Reducing SO₂ content in wine by combining High Pressure and glutathione addition

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ABSTRACT

The aim of this work was to examine the potential of High Pressure (HP) technology as an alternative technique to SO₂ addition for red wine preservation. It focused on producing wines with reduced added SO₂ and the simultaneous addition of glutathione (GSH) as a natural antioxidant. Selected quality parameters of red wine samples from Mouchtaro grapes treated by HP using various pressure parameters were tested. HP processing studied was applied at 200, 400 and 600 MPa for 0, 5 and 15 min. The application of HP for a long duration resulted in a significant reduction in phenolic compound concentrations (TP) due to both extended polymerisation and reduced volatile acidity (VA) and acetic acid concentrations (AAC), which in turn was mainly due to better antimicrobial protection. Based on the changes to the contents of all TP, VA and AAC groups, processing for 5 min at 400 MPa was selected as the optimum HP condition. Red wine samples from Mouchtaro grapes containing 0, 20, 40, 60, 80 and 100 mg/L of SO₂ and 10 mg/L of GSH were HP-treated under the selected pressure/time conditions. Untreated samples containing the same concentrations of SO₂ and GSH were used as control samples. Indices such as AAC, Antioxidant activity (AOA), total anthocyanins, TP, mean Degree of Tannin Polymerisation (mDP) and the composition of volatiles was determined over a period of 12 months. Sensory analysis of the samples took place during the 12th month of storage. After the 12-month period, the pressurised samples with GSH showed higher content of total aldehyde/ketone and higher-alcohols, consistently lower concentrations of acetic acid, ethyl acetate and total esters, and lower VA values. Finally, based on the results obtained from the sensory analysis, untreated samples were characterised by «Red fruits» odours, whereas treated samples were distinguished by their «chocolate» aroma. These results suggest that HP could be used for the production of more «mature» wines. A reduced SO₂ concentration of up to 40 or 60 mg/L may be sufficient for wine stabilisation when combining HP treatment and GSH additions, depending on grape variety.

KEYWORDS

high pressure, sulphur dioxide (SO₂), glutathione, wine stability, quality deterioration
INTRODUCTION

The quality and safety of food products are among the most important parameters that influence consumer choices. There is an increasing demand for high quality and healthier wine that is free from additives and microbiologically safe, with excellent taste and extended shelf life. The wine industry has been dependent on sulphur dioxide (SO₂) for centuries, mainly due to its antioxidant and antimicrobial effects (Santos et al., 2013a; Santos et al., 2013b; Ribereau-Gayon et al., 2006a) and because it prevents browning and colour loss, (Bakker et al., 1998; Oliveira et al., 2011; Ribereau-Gayon et al., 2006b); however, SO₂ has been related to allergic reactions in many consumers (Vally and Thompson, 2001; Vally et al., 2003; Vally et al., 2009). Consequently, the maximum concentration of SO₂ allowed by legislation in wines has been gradually reduced (Regulation (EC) No 607/2009), and research efforts are focusing on other preservatives with healthier profiles, as well as on innovative technologies aiming to partially or totally replace SO₂ in winemaking. Methodologies involving the addition to wines of natural or synthetic molecules or plant extracts (e.g., dimethyl dicarbonate, bacteriocins, phenolic compounds, glutathione, lysozyme, black radish extract) (Divol et al., 2005; Gerbaux et al., 1997; Threlfall and Morris, 2002; Bauernfeind and Pinkert, 1970; Chung and Hancock, 2000; Delfini et al., 2004) and physical techniques (pulsed electric fields, ultrasound irradiation and ultraviolet treatments) (Delsart et al., 2016; Davaux et al., 2011) have already been studied as ways of substituting or reducing the use of SO₂ in winemaking. However, they have limitations (only antimicrobial or antioxidant activity), are costly and have negative effects on the organoleptic properties of wine; therefore, they are not sufficiently effective as stand-alone approaches to fully substituting SO₂ in winemaking.

High Pressure (HP) is a physical, non-thermal technique involving antimicrobial activity due to the resulting inactivation of certain microorganisms and enzymes (Katsaros et al., 2010; Katsaros et al., 2017). Moreover, products subjected to HP retain their sensorial and nutritional properties since the covalent bonds are unaffected (Cao et al., 2011; Alexandrakis et al., 2014). Based on these facts, the use of HP has already been tested in winemaking for the preservation of grape juice, must and wine (Santos et al., 2016), and it could thus be an alternative process to SO₂ addition. Initial studies were related to the sustainability of wine in terms of microbial contamination; a diminution in the initial microbial wine population (indigenous or after inoculation) has been observed, without any changes to physicochemical and organoleptic characteristics and the activity of polyphenoloxidase in wine (Delfini et al., 1995; Puig et al., 2003; Puig et al., 2008). A correlation between the microbial inactivation and the pressure treatment and holding time was found (Mok et al., 2006; Morata et al., 2012). Aerobic bacteria, yeasts and lactic acid bacteria were found to differ in sensitivity to pressurisation (Morata et al., 2012; Morata et al., 2014). In addition, HP treatment effectively reduced wild microorganisms in grapes, especially yeasts, thus facilitating the growth and multiplication of non-Saccharomyces yeasts as starter cultures and making it possible to reduce the SO₂ doses required for maintaining wine quality (Bañuelos et al., 2016).

Research later focused on the effects of HP treatment on the physiochemical and sensorial properties of wines. Numerous studies came to similar prevailing conclusions, regardless of treatments with different pressure and pressure holding time. A comparable behaviour between HP-treated wines and wines naturally aged in oak barrels was revealed (Morata et al., 2014; Santos et al., 2013a, Santos et al., 2016, Tao et al., 2012, Tao et al., 2013). Recent studies have focused on the modification of protein structures, concluding that HP treatment can lead in protein thermal stabilisation (Tabilo-Munizaga et al., 2014). Furthermore, Sun et al. (2015) found that HP-treatments had no effects on the region and phenolic characteristics (Sun et al., 2015), and Tao et al. (2015) managed to produce wines with better colour intensity, higher concentrations of methanol and ethanol, and higher aromatic quality via HP processing in parallel with oak chip maceration (Tao et al., 2015).

Nevertheless, in the absence of SO₂, HP can only protect wine from undesirable microorganisms. A promising alternative for complementing SO₂ and HP in terms of protecting wine from oxidation is glutathione (GSH). GSH is a natural antioxidant contained in grapes that plays key roles in winemaking, from preserving important varietal aroma compounds, to limiting browning and the development of typical ageing off-flavours (Badea and Antoce, 2015). GSH is a tripeptide of glutamate, cysteine and glycine, which has two characteristic structural features:
a γ-peptide bond between glutamate and cysteine that offers prevention against hydrolysis, and a sulphhydryl group (SH) on which GSH’s antioxidant effect is based (Anderson, 1998; Pastore et al., 2003). In 2015, OIV approved the use of GSH in concentrations of up to 20 mg/L (OIV-OENO 445-2015, 2015; OIV-OENO 446-2015, 2015).

The first quantitative analysis of GSH in grapes was established in 1989 by Cheynier, Souquet and Moutoumet (Cheynier et al., 1989). In this study, variations in GSH content in berries and must were attributed to differentiations in varieties, vintage, location and technological practices (e.g., harvesting method (i.e., mechanical vs handpicked), oxygen exposure, grape skin maceration during the prefermentation period, pressing and oxidative or reductive treatments) (Du Toit et al., 2007; Maggu et al., 2007; Kritzinger, 2012). Ripening status can also affect GSH content, with high sugar levels increasing GSH concentrations (Adams and Liyanage, 1993; Okuda and Yokotsuka, 1999; Šuklje et al., 2012). Further increases in GSH content have been observed with a parallel increment in yeast assimilable nitrogen (YAN) in must (Chone et al., 2006; Lacroux et al., 2008). Apart from the raw materials (grapes), it is possible for diversifications in measured GSH to result from the yeast strains used during alcoholic fermentation (Lavigne et al., 2007; Park et al., 2000; Andujar-Ortiz et al., 2012) and the aging process (Lavigne et al., 2007; Penna et al., 2001). During aging, GSH levels generally decline due to the greater oxidation effect, especially in the absence of yeast lees and when new barrels are used (Kritzinger, 2012; Kritzinger et al., 2012).

GSH offers adequate defence against chemical oxidation by delaying the polymerisation of susceptible phenolic compounds and generating xanthilium ions and further transition of wine colour tint and intensity (Sonni et al., 2011a; Sonni et al., 2011b). Besides the protective effect in colour parameters, it has been proved that GSH inhibits the reduction of compounds from a range of aromatic groups, such as esters of higher alcohols, ethyl esters, terpenes and varietal thiols in a dose-dependent manner (Papadopoulou and Roussis, 2001; Papadopoulou and Roussis, 2008; Ugliano et al., 2011; Dubourdieu and Lavigne-Cruege, 2004; Roussis et al., 2007). It is believed that the action of sulphhydryl moiety preserves esters and terpenes, while competitive behaviour between GSH and varietal thiols in the addition reaction with o-quinones (responsible for the oxidation of thiols) has been found to preserve this group of compounds (Roussis et al., 2009; Tirelli et al., 2010). GSH can impact wine aromas negatively as it is a source of hydrogen sulphite (H₂S) (Tokuyama et al., 1973); however, H₂S concentration has been found to significantly decrease in a medium containing a specific inhibitor of GSH synthesis (Hallinan et al., 1999). In addition, GSH suppresses the formation of compounds responsible for aged-like aroma characteristics during bottling (Dubourdieu and Lavigne-Cruege, 2004).

Our previous research (Christofi et al., 2017; Christofi et al., 2020) focused on the examination of the combined effect of High Pressure (HP) treatment and the addition of different amounts of sulphur dioxide on wine stability. The results obtained suggested that HP could only offer antimicrobial protection to wine (reduced volatile acidity and acetic acid contents of treated wines), and that HP-treated samples tend to have analogous characteristics as aged wines due to increased oxidation rates (increased acetaldehyde content of treated wines). So far, HP treatment has not been tested in combination with SO₂ and the simultaneous presence of an antioxidant such as GSH. Since SO₂ is a cheap and effective wine preservative, its complete replacement does not seem feasible at present. Therefore, the primary aim of the present study was to determine the minimum/optimal amounts of sulphur dioxide required, alongside glutathione and HP, to protect wine without deteriorating its quality. The optimal HP parameters for maintaining selected wine quality parameters were also determined.

MATERIALS & METHODS

1. Grapes & Fermentation

For this study, a red grape variety from Vitis Vinifera cv. Mouchtaro (“Ktima Mouson”, 2018 vintage) was used. Mouchtaro is an increasingly popular, indigenous variety from Central Greece, which is mostly cultivated in the region of Biotia. It is believed that Mouchtaro is a clone of Mandilaria (Stavrakaki and Biniari, 2017) and that it is closely related to “Mavri Koundoura” (Merkouropolous et al., 2015). Even though it is largely unexploited, a few experimental fermentations have indicated high acidity, colour intensity and concentration of phenolic compounds, with red fruit, spicy and herbal aromas. Red wine was produced according to methods reported by Christofi et al. (2020).
2. Determination of optimal HP parameters

After the completion of the alcoholic and malolactic fermentations, the wine was racked and 40 mg/L SO₂ was added to it. The wine was then split into four groups of samples and transferred to 350 ml polyethylene bags suitable for HP treatment. The samples from the first group were pressurised at 20 C and 200 MPa in a HP Unit (NC Hyperbaric wave 6000/300) with pressure holding times of 0, 5 and 15 min respectively. Pressure holding time was defined as the time required by the hydrostatic press to reach the selected pressure values. In more detail, pressurisation was terminated as soon as the hydrostatic press reached 200 MPa for the ‘0 min’ samples. The second and third groups of samples were pressurised at 400 MPa and 600 MPa respectively for 0, 5 and 15 min. The last group of samples was not subjected to HP treatment and was used as a control. Each treatment was performed twice.

3. Determination of optimal/minimum SO₂ contents

After the alcoholic and malolactic fermentations, in order to select the lowest possible concentration of SO₂, the wine was separated into two groups of samples and transferred to 350 ml polyethylene bags suitable for HP treatment. In the first group, samples containing 10 mg/L GSH and 0, 20, 40, 60, 80 and 100 mg/L of SO₂ respectively were pressurised under the optimum selected conditions (for 5 min at 20 C at 400 MPa). The second group consisted of samples with identical concentrations of SO₂ and GSH, but without any HP treatment. Each treatment was performed twice. After high pressure treatment, all samples were bottled and stored in the absence of light at a temperature ranging between 15 and 17 °C. All chemical analyses were performed in duplicate after 6 and 12 months of bottle storage.

4. Wine chemical analysis

In the produced wines, several classical analytical parameters (free and total SO₂ contents, % vol., pH, titratable and volatile acidity) and colour parameters (hue and colour intensity) were determined according to the OIV (2009) methods. The results were as follows: titratable acidity = 6.9 g/L (expressed as tartaric acid), volatile acidity = 0.48 g/L (expressed as acetic acid), % vol = 14.0 % (v/v), pH = 4.1, colour intensity = 1.8 and total phenolic content = 2.1 g/L. Antioxidant activity was determined according to Brand-Williams et al. (1995), and total flavanol (catechin) content according to Sun et al. (1998). Acetaldehyde content was determined by spectrophotometry according to the OIV (2009) method. Concentrations of anthocyanins and acetic acid and were measured according to Cristofi et al. (2017), and total phenolic content was determined by Folin-Ciocalteu method (Folin and Ciocalteu, 1927). The % Red (% R) was calculated using the following formula: [(A₅₂₀nm/Colour Intensity)*100].

The tannin mean degree of polymerisation (mDP) and percentage of galloylation (% G) were determined according to the method described by Chira, Jourdes, and Teissedre (Chira et al., 2012).

### TABLE 1. Sample coding and corresponding treatment.

| Sample | Pressure (Mpa) | Pressure holding time (min) |
|--------|----------------|-----------------------------|
| control | 0 | 0 |
| 0/200 | 0 | 0 |
| 5/200 | 200 | 5 |
| 10/200 | 200 | 15 |
| 0/400 | 400 | 0 |
| 5/400 | 400 | 5 |
| 10/400 | 400 | 15 |
| 0/600 | 600 | 0 |
| 5/600 | 600 | 5 |
| 10/600 | 600 | 15 |

### TABLE 2. Sample coding and corresponding treatment. The letter “X” indicates untreated samples. The HP treated samples are coded without ‘X’.

| Sample | SO₂ (mg/L) | GSH (mg/L) |
|--------|------------|------------|
| 0 | 0 | 10 |
| X0 | 0 | 10 |
| 20 | 20 | 10 |
| X20 | 20 | 10 |
| 40 | 40 | 10 |
| X40 | 40 | 10 |
| 60 | 60 | 10 |
| X60 | 60 | 10 |
| 80 | 80 | 10 |
| X80 | 80 | 10 |
| 100 | 100 | 10 |
| X100 | 100 | 10 |
The proanthocyanidins were isolated using a C18 (Lichrolut® C18, 5 g octadecyl bonded endcapped silica, 25 mL vol) SPE cartridge (Merck®, Darmstadt, Germany) according to the method described by Chira et al. (2012). Ten (10) mL of wine was evaporated in a vacuum in order to remove the ethanol. The remaining wine (approximately 4 mL) was resuspended in water to reach a final volume of 20 mL. The cartridge was activated by sequentially adding 25 mL methanol, 25 mL distilled water and the wine sample. The cartridge was then washed with 50 mL distilled water and left to dry for 15 min. Elution of the proanthocyanidins was performed with 50 mL methanol, and the tannin extracts were evaporated under reduced pressure at 30 °C and lyophilised to obtain a dry powder. The final tannin extracts were weighed and dissolved in methanol (to reach a final content of 5 g/L). Acid-catalyzed depolymerisation took place in the presence of phloroglucinol (50 g/L) and (-)-epicatechin gallate (ECG), as well as their phloroglucinol adducts, were analysed by LC/MS on a Shimadzu 2010A (Shimatzu® Corporation) coupled to a single quadrupole mass spectrometer equipped with an electrospray ion source, according to the method described by Kyraleou et al. (2017). All analyses were performed in triplicate.

The monomeric anthocyanins were determined by HPLC according to Kyraleou et al. (2015). The equipment used consisted of a JASCO AS-1555 Intelligent Sampler, a JASCO PU 2089 Plus Quaternary Gradient Pump, a JASCO MD-910 Diode Array Detector and a JASCO LC-Net II / ADC. The column was a Restek Pinnacle II C18 (250 x 4.6 mm, 5 μm). Eluent A comprised 10 % aqueous formic acid solution and Eluent B comprised methanol, and the flow rate 1 mL/min. The volatile composition of the red wines was determined using liquid-liquid extraction combined with GC-MS after 6 and 12 months of storage. For the liquid-liquid extraction of volatile components, 20 mL of sample was placed in 50 mL tubes and 8 mL of dichloromethane. After vortexing for 1 min, the samples were centrifuged at 6000 rpm for 15 min for phase separation. The organic phase was collected and concentrated under a nitrogen flow. After the extraction/preconcentration step, the extracts were injected into a GCMS-QP2010 Ultra (Shimadzu® Inc., Japan) system at 240 °C in split mode (split ratio 1/20). The compounds were separated in a DB-Wax capillary column (30 m X 0.25 mm i.d., 0.25 μm film thickness, Agilent, USA) with Helium as a carrier gas at a constant linear velocity of 36 cm/s. The oven temperature was programmed at 40 °C for 5 min, increased by 5 °C/min to 180 °C, and then by 30 °C/min to 240 °C (and held for 5 min). The mass spectrometer was operated in the electron ionisation mode with the electron energy set at 70 eV and 40-300 m/z scan mass range. Source and interface temperatures were set at 200 °C and 240 °C respectively. During the elution of ethanol (3.4 - 4.0 min), the filament was programmed to turn off to extend its life. The identification of the volatile compounds was performed using AMDIS software (v. 2.65 build 116,66) based on retention time and mass spectra, with a parallel use of NIST library as confirmation. A semi-quantitative analysis of the volatile compounds was performed based on the absolute values of the peak area of each compound, and they were expressed as a percentage of the total peak area [(compound peak area/sum of peak areas)*100].

5. Sensory analysis

A sensory assessment of all the wines was carried out by a group of 12 trained panellists after 12 months of storage. The training and the sensory evaluation took place according to the method described by Kallithraka et al. (2015). The samples were presented in a completely randomised order to each panellist. The attributes selected were grouped into two categories: olfactive descriptors (fresh fruits, dry fruits, chocolate, tobacco, odour of oxidation) and gustative descriptors (astringency, bitterness, sourness and body). The judges also performed an overall quality assessment. All samples were evaluated in triplicate. The intensity of the sensory attributes examined was rated on a 5-point scale (0: null, 5: very strong).

6. Statistical analysis

All chemical determinations were run in triplicate and values averaged. The percentage changes were calculated as follows: % change = (measured parameter value of the sample-measured parameter value of the control) x 100. All data, including those from the sensory analysis, were subjected to one-way analysis.
of variance (ANOVA), with STATISTICA V.7 Software (Statsoft® InC., Tulsa, OK). Mean values were compared using Tukey’s HSD test when the samples were significantly different (p < 0.05).

RESULTS-DISCUSSION

In general, no significant differences were observed for the majority of cases in terms of the analytical parameters of the HP-treated and untreated wines studied immediately after pressurisation and up to a period of 6 months (data not shown). This indicates that pressure treatment did not significantly affect the chemical composition of the samples at the beginning of storage, which is in agreement with previous studies (Santos et al., 2013a; Santos et al., 2016). Since the physicochemical parameters of the pressurised red wines started showing significant differences after 6 months of storage, only the results obtained after 12 months of storage are given.

1. Effect of different HP treatments on chemical composition

To select the optimum applied pressure and pressure holding time, different physiochemical parameters were determined in the samples treated at various pressures and times.

1.1. Polyphenols, Proanthocyanidins and Antioxidant Activity (AOA)

Total phenolic content (TP) was generally reduced after HP treatment (Figure 1). Although the wine samples did not show statistical differences (p < 0.05) at the beginning of storage, the HP treated wines were characterised by lower TP contents after 6 months of storage (data not shown). Due to the positive correlation of TP content and AOA, a similar pattern was observed for AOA as well. This is in agreement with previous studies, which mention the reduction of TP content and AOA independently from the treatment parameters (Santos et al., 2013a; Santos et al., 2016; Tao et al., 2015; Tao et al., 2012; Sun et al., 2015). After 12 months of storage the reduction of total phenolics was statistically higher for samples treated for 15 min (50.8 %) compared to samples treated for holding time t = 0 (after pressure build up, the pressure was immediately released) (45.4 %) and 5 min (44.8 %), while the absolute values of TP ranging from 0.99 to 1.13 g/L. Samples treated at 600 MPa also showed higher TP reduction (48.8 %) (absolute value = 1.03 g/L) compared to the other treatments in terms of pressure factor, but no statistically significant differences were observed (Figure 1).

A similar trend was also observed for proanthocyanidin content (Figure 2). Samples treated at 600 MPa were the most affected, recording the highest decrease (26.5 %; 99.2 mg/L absolute value after 12 months of storage). Statistically significant differences were observed between treated samples. In terms of pressure holding times, 15 min engendered the greatest loss of proanthocyanidins (25.8 %; 100.2 mg/L absolute value after 12 months of storage), with no statistically significant differences between samples (Figure 2).

1.2. Anthocyanins and % Red color (% R)

As a result of HP treatment, the concentration of total anthocyanins (expressed as the sum of malvidin-3-O-glucoside, delphinidin-3-O-glucoside, cyanidin-3-O-glucoside, peonidin-3-O-glucoside, petunidin-3-O-glucoside) decreased in all samples, regardless of pressure and pressure holding time (initial concentration of anthocyanins 490 mg/L). Subsequently, the % R decreased as well. Samples treated at 600 MPa revealed the highest reduction in anthocyanin content (63.9 %) and the highest decrease in % R (4.0 %) after 12 months of storage. Statistically significant differences were observed between samples treated under different pressure parameters. Moreover, 15 min of pressure holding time had the greatest reducing effect on anthocyanin content, followed by 5 min and 0 min. However, these differences were not statistically significant (Figure 3).

These results are in agreement with previous studies, underlining the significant (p < 0.05) changes induced by HP (Santos et al., 2013a; Santos et al., 2016; Tao et al., 2015; Tao et al., 2012; Sun et al., 2015; Christofi et al., 2020). Apart from the natural ageing procedure that takes place (i.e., the decrease in anthocyanins in control samples), the further reduction in content of all groups of flavonoids in the treated samples may be a result of the enhancement of polymerisation reactions due, in turn, to enhanced oxidation or even to the reduction of volume induced by HP (Bolumar et al., 2012). The formation of polymers due to reactions between anthocyanins and other phenolic compounds decreases absorbance to 520 nm (red colour), resulting in a shift from a red-purple colour to the orange-brown hues similar to those of aged wines (He et al., 2006). The acceleration of the formation of anthocyanin
FIGURE 1. Percentage decrease in TP after 12 months of storage based on a) pressure holding time (samples treated for 0, 5 and 15 min) and b) pressure (samples treated at 200, 400 and 600 MPa). Different letters in each column indicate significant differences (p < 0.05) between treatments.

FIGURE 2. Percentage decrease in proanthocyanidins after 12 months of storage for all samples treated under different pressure and pressure holding times. Different letters in each column indicate significant differences (p < 0.05) between treatments.

FIGURE 3. Percentage reduction in the concentration of anthocyanins based on a) pressure (samples treated at 200, 400 and 600 MPa) and b) pressure holding time (samples treated for 0, 5 and 15 min), after 12 months of storage. Different letters in each column indicate significant differences (p < 0.05) between treatments.
pyruvic complexes such as vitisin A-type derivatives could be another explanation for the colour change (Corrales et al., 2008). To a lesser extent, the reduction in monomeric anthocyanin content could be attributed to hydrolytic and degradation reactions (Santos et al., 2016; Christofi et al., 2020).

1.3. Volatile acidity (VA) and Acetic Acid

A longer storage time resulted in increased VA and acetic acid content in both the control and HP-treated samples, regardless of pressure and pressure holding time. Samples with lower applied pressure or pressure holding time showed higher VA values after 12 months of storage. In terms of pressure, the control and the 200 MPa samples were characterised by the highest percentage increase in VA during storage. The highest absolute VA values were also obtained for these samples (1.40 and 1.18 g/L respectively), with the control sample reaching the legal limit for VA in red wines after 12 months of storage. The absolute VA values for samples treated at 400 and 600 MPa were lower (0.56 and 0.62 g/L respectively) after 12 months of storage, indicating that these wines are microbiologically safer. Statistically significant differences (p < 0.05) were observed as from the 3rd month (data not shown). Based on pressure holding time, the control sample and samples treated for 0 min attained the highest increment and absolute VA values (1.40 and 1.04 g/L respectively) with statistically significant differences in comparison with the samples treated for 5 and 15 min (Figure 4) (0.72 and 0.61 g/L respectively). These results are in line with previous studies and can be attributed to the higher inactivation of acetic acid bacteria at higher pressure and longer pressure holding time (Bolumar et al., 2012). It is worth noting that even after 12 months of storage none of the samples exceeded the legal limits for VA levels in red wines (1.4 g/L); however, the control samples and samples treated at 200 MPa for 0 min contained the highest content of volatile acids. Further research would be necessary to determine the impact of HP treatment solely on acetic acid and acetic acid bacteria.

1.4. Selection of the optimum HP parameters

By taking into account all of the above results, it was possible to determine the optimum HP conditions. The reduction in phenolic compounds being considered one of the most important aspects of red wine quality, pressure at 600 MPa and pressure holding time for 15 min were excluded. When focusing on volatile acidity and acetic acid content, the control and samples treated at 200 MPa inevitably showed the worst results; therefore, they were excluded from further studies. Finally, pressure at 400 MPa for 5 min was selected as the optimum parameter due to the lower decrease in anthocyanins, proanthocyanidins and polyphenols, and the lower increase in VA and acetic acid content compared to the corresponding values of all the other samples.

2. Effect of different dosage of SO2 on chemical composition

After selecting the optimum HP conditions, the aim of this work was to select the lowest possible concentration of SO2 required to adequately protect wines when combined with HP treatment in the presence of an antioxidant compound such as GSH.

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**FIGURE 4.** Percentage increase in VA depending on a) pressure holding time (samples treated for 0, 5 and 15 min) and b) pressure (samples treated at 200, 400 and 600 MPa), after 12 months of storage. Different letters in each column indicate significant differences (p < 0.05) between treatments.
For this purpose, we determined the evolution of the chemical composition and the sensory characteristics of wine samples treated with HP and containing various amounts of SO₂ and glutathione.

2.1. Anthocyanins, polyphenols & colour intensity (CI)

The HP-treated samples were characterised by reduced anthocyanin and total polyphenolic content and lower Colour Intensity (CI) compared to the untreated samples.

It was possible to divide the samples into two groups, depending on the reduction of both TP and anthocyanins. The first group was characterised by lower absolute values (302-330 mg/L of anthocyanins and 1.14-1.15 g/L of TP) and a greater reduction in polyphenolic compounds. The second group was defined as having higher absolute values (350-395 mg/L of anthocyanins and 1.17-1.21 g/L of TP) and a more limited reduction in anthocyanins and polyphenols. The samples containing 0, 20 and 40 mg/L SO₂ were assigned to the first group, and the samples with 60, 80 and 100 mg/L SO₂ were assigned to the second. No statistically important differences were observed between samples in terms of TP. In contrast, % reduction in anthocyanin was lowest in the samples containing 80 and 100 mg/L SO₂ due to higher protection against oxidation (Figure 5).

These results are in agreement with our previous study (Christofi et al., 2020) in which colour intensity was also found to be lower in treated samples due to the enhancement of polymerisation reactions caused by HP treatment, independently of the obstructive effect of GSH on the formation of dimers. The rate of the condensation reactions should be dependent on the SO₂ and GSH content of the wines, explaining the differentiation of the samples (Christofi et al., 2020; Santos et al., 2013a). Moreover, the GSH antioxidant mechanism may have offered antioxidant protection to all the phenolic groups leading to a more limited reduction in their concentrations after 12 months of storage, compared to results in our previous study (Christofi et al., 2020). In contrast to our previous study, significant differences in anthocyanin content between samples with 60 and 100 mg/L GSH were observed, indicating that GSH probably has a greater impact than expected on the polymerisation reactions.

2.2. Mean Degree of tannin polymerisation (mDP) & % Galloylation (% G)

The mDP of proanthocyanidins and the % G are two parameters of tannin chemical structure that influence wine astringency and bitterness (Monagas et al., 2003; Prieur et al., 1994). HP treatment played a key role in the changes to these parameters for all samples regardless of SO₂ concentration. All treated wines showed higher mDP and % G values compared to the untreated ones, probably due to the enhancement of polymerisation reactions after pressure. Statistically important differences between treated and untreated samples were noted for mDP, but not for % G. After 12 months of storage, the greatest increase in mDP was observed for the HP-treated samples containing medium concentrations of SO₂ (40 mg/L and 60 mg/L) (Figure 6). This could potentially be attributed to the oxidation of samples with 0 and 20 mg/L of SO₂, as well as the partial obstruction to polymerisation in the 80 and 100 mg/L samples due to occupation of electrophilic centres from SO₂. Further research is necessary to confirm this assumption.

![FIGURE 5. Percentage decrease in a) anthocyanins and b) TP after 12 months of storage of treated samples with different concentrations of SO₂. Different letters in each column indicate significant differences (p < 0.05) between treatments.](image-url)
Higher mDP and %G values for HP-treated samples compared to untreated ones are in line with previous studies, which found that pressure treatments enhance condensation reactions and result in a possible rearrangement of tannin complexes; this favours the incorporation of galloyl over EGC moieties into their structure (Santos et al., 2013a; Christofi et al., 2020). The significant differences between mDP values contrasts with results of previous studies (Christofi et al., 2020; Santos et al., 2016). The addition of GSH may interfere in the simultaneous polymerisation, degradation and precipitation of tannins with higher molecular weight, thus leading to significant differences between samples. In addition, since increased mDP is a general characteristic of natural wine aging, it may be possible to use HP treatment with medium concentrations of SO2 for the production of red wines with ‘ageing-like’ sensory characteristics.

2.3. Volatile composition

More than 100 volatile compounds were identified as belonging to the 5 chemical groups of acids, alcohols, aldehydes and ketones, esters and aging esters (esters of succinic and lactic acid; e.g., ethyl lactate, butyl lactate, isovaleryl lactate, diethyl succinate, ethyl-2-hexyl succinate, 2-hydroxy-3-methyl diethyl succinate). Acetic acid and ethyl acetate were calculated separately from the acids and esters group. The esters group contained the highest number of identified compounds (average of 43), followed by alcohols (average of 36) and aldehydes and ketones (average of 24).

After 12 months of storage, a slightly lower content of total esters was observed in the pressurised samples compared to that in unpressurised wines (Figure 7a). Samples treated with lower concentrations of SO2 were characterised by lower ester content, which is mainly due to the lower concentration of the most abundant esters (ethyl lactate, ethyl isovalerate, etc.), which are products of fermentation with fruity odours (Figure 7b). The same samples also showed higher values for aging esters mainly due to the higher content of diethyl succinate, which has a mild fruity cooked apple flavour (Figure 7c). No statistically important differences were observed between pressurised and unpressurised samples, probably.
FIGURE 7. Percentage content of volatile compounds. a) total esters in pressurised and non-pressurised samples; b) esters in pressurised samples with different SO$_2$ content; c) aging esters in pressurised samples with different SO$_2$ content; d) acids in pressurised and non-pressurised samples; e) aldehydes and ketones in pressurised and non-pressurised samples; f) aldehydes and ketones of pressurised samples with different SO$_2$ content; g) higher alcohols in pressurised and non-pressurised samples; and h) higher alcohols in pressurised samples with different SO$_2$ content after 12 months of storage. Values followed by different letters in each column indicate significant differences (p < 0.05) among different treatments.
due to the role of GSH in protection from oxidation; it has been shown that GSH can inhibit the decline of several volatiles, such as esters and alcohols (Kritzinger et al., 2012). This is in agreement with previous studies supporting accelerated aging with HP treatment (Santos et al., 2015).

After 12 months of storage, higher values were obtained for acid content in non-pressurised samples due to the higher percentage of acetic acid, but no statistically important differences were noted (Figure 7d). In contrast, ethyl acetate content was 9.6 and 8.4 % and acetic acid content was 4.8 and 3.5 % in non-pressurised and pressurised samples respectively. Acetic acid was one of the most dominant acids, negatively affecting the wine bouquet. These results are in agreement with the results of VA (data not shown). Moreover, it was observed that the lower the SO₂ content, the higher the acetic acid and ethyl acetate content. Generally, these results are in agreement with observations in previous studies of an increased inactivation of acetic bacteria after HP treatment (Jeon et al., 2014).

Aldehyde and ketone content was found to be higher in HP-treated samples during storage, but without statistically important differences (Figure 7e). These differences were mainly due to the increased content of acetoin and 2,3-butanedione with “buttery”, “fatty” and “chocolate” odours. The absence of statistically important differences between treated and untreated samples could be explained by the addition of GSH limiting the formation of atypical aging off-odours (Kritzinger et al., 2012). Unexpectedly, samples with higher contents of SO₂ were richer in total aldehydes and ketones (Figure 7f). This is possibly due to the role of SO₂ in preventing the oxidation of carbonyl compounds to their corresponding acids. Tao et al. (2012) suggest that the equilibrium of the chemical forms of SO₂ are altered by HP, thus affecting the reaction of SO₂ with phenolic compounds and aldehydes.

Finally, untreated samples had lower alcohol content during the whole storage period, with statistically important differences (Figure 7g). Furthermore, a positive correlation between SO₂ content and the content of alcohols was observed (Figure 7h). This is an indication of the protective effect of SO₂ in this group of volatile compounds, especially in terms of monoterpane alcohols, such as geraniol which is responsible for the floral aroma in wines.

The results from the GS-MS analysis are in line with previous studies in which higher concentrations of aldehydes and ketones were found in pressurised samples, as well as lower amounts of higher alcohols and fatty acids; this is due to an acceleration in Maillard reactions, which are responsible for the oxidation of alcohols and acids (Santos et al., 2015).

2.4. Sensory Analysis

The results of the sensory analysis of both treated and untreated samples after 12 months of storage in relation to their SO₂ content are shown in Figure 8. The ‘spider web’ diagrams were obtained from the average scores of the olfactory attributes.

The treated and untreated wines without SO₂ addition were significantly different (p < 0.05) in terms of the following attributes: aroma intensity, spicy odour, chocolate odour and astringency intensity (Figure 8a). The pressurised wines were perceived as being more balanced and spicier, with more intense sun-dried fruit, fruit jam and chocolate odours. The non-pressurised wines were perceived as being more astringent with higher overall aroma intensity, in particular that of red fruits.

Regarding the wines that contained 20 mg/L SO₂, the panellists found the following attributes to be significantly different: hue, astringency intensity and bitterness, red fruits, spicy, chocolate and fruit jam odours and balance, and overall quality (Figure 8b). The untreated wines received the highest score in terms of red fruit odour and astringency, while the treated ones were perceived as being more balanced and having an oxidation odour. The odours that were characteristic of the HP-treated samples were those of spices, chocolate and fruit jam.

The pressurised and non-pressurised wines that contained 40 mg/L SO₂ were different in the following sensory parameters: Astringency intensity, bitterness, aroma intensity, and sun-dried fruit and spice odours (Figure 8c). The treated samples were again perceived as being spicier and more intense in sundried fruit odour; nonetheless, they were considered to be more balanced and higher in overall quality. The non-pressurised samples scored higher in aroma intensity, red fruit odour, bitterness and astringency. However, it is worth mentioning that the overall quality and oxidation odour did not differ significantly between the two groups of samples.
As regards the wines with 60 mg/L SO$_2$, the only attributes, which differed significantly were colour intensity and hue, bitterness, intensity of the spicy odour, body and balance (Figure 8d). The HP wines scored higher for fruit jam, chocolate, spicy and sundried fruit odours, while being more balanced. Untreated samples were still characterised by higher aroma intensity, especially in terms of red fruit odour, and they were more astringent.

Significant differences between the samples containing 80 mg/L SO$_2$ were found for balance, overall quality and red fruit attributes only (Figure 8e). HP-treated samples were perceived as being spicier, with higher intensity of sundried fruit odours, and better balance and overall quality, while untreated samples were distinguished by their higher red fruit odours and astringency intensity.

Finally, significant differences were found for wines containing 100 mg/L SO$_2$ in terms of spiciness, sundried fruit and red fruit odours and astringency intensity descriptors (Figure 8f). Pressurised samples still scored higher in fruit jam, chocolate, spice and sundried odours, with higher balance and overall quality. Untreated samples were perceived as more bitter with higher astringency and aroma intensity, particularly that of red fruit.

In general, all treated samples, irrespective of their SO$_2$ content, were perceived as being spicier than untreated samples, with a higher intensity of sundried fruit, fruit jam and chocolate odours, indicating higher oxidation and an aged-like wine character (Santos et al., 2016). These results are in agreement with those of the GC-MS analysis, which showed that the pressurised samples had higher aldehyde and ketone content. In contrast, non-pressurised samples scored higher in astringency and overall aroma intensity, particularly in terms of red fruits, independently of the SO$_2$ content (Figure 8). These results are in line with those of the chemical analysis for total esters, since the non-pressurized samples received a better score. They are also in agreement with sensory data in our previous study (Christofi et al., 2020).

It has been reported that pressurised samples were perceived as being less fruity and floral with higher spicy and sundried fruit odours, possibly due to the higher acetal content (Santos et al., 2016). Higher furan, aldehyde and ketone content has also been attributed to pressurised wines, implying the acceleration of Mallaird reactions and the oxidation of fatty acids and higher alcohols by HP treatment, resulting in odours of aged wines (Santos et al., 2015).

![FIGURE 8](image-url) Sensory analysis of treated and untreated samples after 12 months of storage containing a) 0, b) 20, c) 40, d) 60, e) 80 and f) 100 mg/L of SO$_2$. 
Furthermore, treatment conditions comparable to our previous study also resulted in significant differences between the two groups of samples in ‘astringency’ attributes. The sensory data regarding astringency are in agreement with the results of the chemical analysis which showed that treated samples were characterized by higher mDP and % G content and significantly lower amounts of flavonols, indicating that the HP-treated samples aged faster (Christofi et al., 2020). The sample treated with 60 mg/L SO₂ stood out as being the most astringent, with the highest mDP value.

Finally, pressurised samples scored more for balance and overall quality than the untreated samples, which is in agreement with previous studies (Christofi et al., 2020; Santos et al., 2013a). Out of the treated samples, the sample with 40 mg/L SO₂ was distinguished as having the best “body” and “balance and overall quality”. These results are in accordance with previous observations that HP treatment may result in wines with more mature aging sensory characteristics.

**CONCLUSIONS**

Based on the selected quality parameters of the samples treated using different HP parameters, pressurisation for 5 minutes at 400 MPa was selected as the optimum treatment; this is due to the lower observed decrease in anthocyanin, proanthocyanidin and polyphenol concentrations, followed by the lower increase in their VA and acetic acid contents. Pressure at 600 MPa and pressure holding time for 15 minutes were not considered suitable due to the resulting higher reduction in wine total phenolic content. Non-pressed samples and samples pressed at 200 MPa were also excluded due to their higher volatile acidity and acetic acid contents, indicating limited inactivation of acetic bacteria. The results obtained after 12 months of bottle storage suggest that HP treatment results in wines with more “mature” sensory characteristics which contain lower amounts of phenolic compounds and esters, and higher amount of aldehydes and ketones. In addition, when treating wine samples for 5 min at 400 MPa in the presence of GSH, a lower amount of SO₂ might be adequate to offer the required antioxidant and antimicrobial protection. Pressurised samples containing 40 and 60 mg/L SO₂ were perceived as being less astringent and bitter and more balanced, as well as having better body and overall quality. This indicates that HP has potential for reducing the required SO₂ doses in wine. This observation was further supported by the results of the chemical analyses, which showed that the samples with intermediate SO₂ content had lower acetic acid and ethyl acetate content, and medium aldehyde, ketone and higher alcohol concentrations.

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