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GLUTATHIONE AS A POSSIBLE REPLACEMENT OF SULFUR DIOXIDE IN WINEMAKING TECHNOLOGIES: A REVIEW

Gianina Antonela BADEA, Arina Oana ANTOCE

University of Agronomical Sciences and Veterinary Medicine of Bucharest,
Faculty of Horticulture, Department of Bioengineering of Horti-Viticultural Systems,
59, Mărăşti Ave., Sector 1, 011464 Bucharest, Romania

Corresponding author email: arina.antoce@horticultura-bucuresti.ro

Abstract

The paper presents a literature review regarding the role of glutathione as a natural antioxidant and its usage in various fields, with a special emphasis on aspects relevant to winemaking. Recent studies demonstrated the beneficial influence of the addition of glutathione in the white wine production technology, especially for the preservation of the varietal character of the wines obtained from aromatic grapes. Considering that in all living cells it has a similar role of antioxidant protection, it is a logical assumption that glutathione can contribute to the reduction of the dosage of sulfur dioxide used for wine protection and, in the future, it might be a good candidate for the replacement of sulphur dioxide.

Key words*: Glutathione, sulphur dioxide, white wine oxidation, varietal character preservation.*

1. INTRODUCTION

Glutathione (GSH, γ-L-Glutamyl-L-Cysteinyl Glycine), the most established antioxidant of endogenous origin, produced in both animal and vegetal cells, has as its main role the elimination of free radicals and protection of reactive compounds, which otherwise would rapidly interact with oxygen, ensuring as well the protection against various toxins and detrimental heavy metal actions, which interfere in the processes of cellular aging. Pompella *et al*. (2003) defined glutathione (GSH) as "an important antioxidant in plants, animals, fungi, and some bacteria and archaea, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides". In fact, glutathione (Fig. 1) is a tripeptide with a gamma peptide bond between the carboxyl group of the glutamate side-chain and the amine group of cysteine (which is attached by normal peptide bond to a glycine). Due to its chemical structure, glutathione may protect against oxidation.

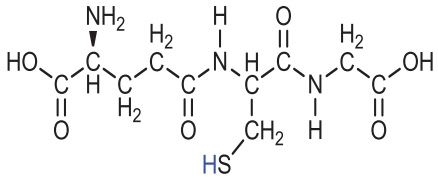

Figure 1. Glutathione (γ-L-Glutamyl-L-Cysteinyl Glycine, GSH)

A simple mechanism of protection is the one by which glutathione in its reduced form (GSH) is oxidized to its dimer form, thereby releasing protons and electrons (H^++e^-) used in coupled reactions for the protection of other molecules against oxidation (Antoce, 2007).

Glutathione exists in both reduced (GSH) and oxidized (GSSG) forms. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent (H^++ e^-) to other unstable molecules, such as reactive oxygen species. By donating an electron, glutathione itself becomes highly reactive, therefore readily reacting with another reactive glutathione to form GSSG (Fig. 2).

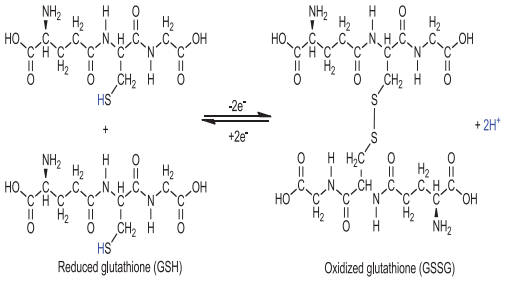

Figure 2. The oxidation reaction of GSH to GSSH

2. GLUTATHIONE AND ITS ROLE IN PREVENTING HUMAN DISEASES

Studies about glutathione appeared as early as the 1980's, but it was only after the year 2000 that an abundance of research results with relevance especially for the medical field was published. Investigations carried out on this tripeptide, which is synthesized by all the cells of the organism, revealed its valuable antioxidant, anti-inflammatory and detoxifying traits. This, in turn, made the researchers to consider it as a possible solution for the prevention and treatment of various immunitary, degenerative and metabolic diseases, including cancer. The fact that this tripeptide can be naturally found in all cells and qualities conferred by its antioxidant capacity, the cellular immune stimulation and detoxification encouraged researchers to consider glutathione a solution to fight incurable diseases such as different cancer types, neurodegenerative diseases such as sclerosis, Parkinson's disease and Alzheimer's disease, autism, Down syndrome, chronic fatigue, heart disease, chronic infections, autoimmune diseases, diabetes, arthritis, asthma, kidney problems, liver disease and more. Glutathione is the most spread intracellular thiolic antioxidant, being essential for redox defense mechanisms activated against oxidative stress. Glutathione metabolism is extremely accurate and is involved in redox signaling and protection against harmful actions of many environmental oxidants. Changes in the ratio of its reduced form and the disulfide form (GSH / GSSG) can destabilize the pathways that cause a wide range of physiological responses of the body, starting from cell proliferation to autophagy and apoptosis, due to the expression of genes involving H_2O_2 as a second messenger (Biswas *et al*., 2009). The signalling mechanism involved is quite simple: reduced glutathione bonds to cysteine of another reduced glutathione, by serving as an electron donor used by the cells to detoxify ROS (reactive oxygen species), the glutathione being in this way converted to its oxidized form. Once oxidized, the dimer GSSG can be reduced back to GSH through a reverse process controlled by glutathione reductase, by using nicotinamide adenine dinucleotide phosphate as an electron donor (Couto *et al.*, 2013). A wealth of recent studies have concluded that ROS cause the development and evolution of tumors by inducing DNA mutations, genomic instability and aberrant pro-tumorigenic signalling. On the other hand, a high level of ROS may be toxic as well to cancer cells and can lead to their death, some treatment schemes relying on this very effect. To equilibrate the state of intracellular oxidative stress, cancer cells intensify their antioxidant protection mechanisms based on glutathione, which leads to the conclusion that high ROS levels can indeed block the tumors development (Glasauer *et al*., 2014). An imbalance in the oxidant/antioxidant intracellular ratio and additional ROS generated by environmental factors have been demonstrated to have an important role in the development of respiratory diseases such as asthma, acute respiratory distress syndrome, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis and so on (Biswas *et al*., 2008). Obvious deterioration of GSH homeostasis has been proven by Fitzpatrick and his team (2009) in the case of children with severe asthma refractory to the usual treatment. Decreased GSH, increased GSSG and higher oxidation levels were measured in their epithelial lining fluid. According to Livingstone and Davis (2007), "Glutathione is a central marker of oxidative stress and the most abundant, predominantly intracellular, antioxidant in the central nervous system". Previous studies (Cruz *et al.*, 2003) have suggested that maintaining glutathione balance is imperative for antioxidant protection mechanisms, synaptic plasticity, memory function and learning processes, the huge importance of glutathione for the proper functioning of the nervous system being

underlined. Recent studies made by Michels and his co-workers (2014) demonstrated that, as compared to the case of non-PTSD participants, in PTSD (posttraumatic stress disorder) patients the levels of γ-amino butyric acid (a primary inhibitory neurotransmitter) and glutathione (a marker for neuronal oxidative stress) are found in significantly higher concentrations, strongly suggesting an abnormal oxidative stress. A deficiency in the glutathione metabolism was also identified in autism, a syndrome of specific mental pathology of children characterized by severe communication difficulties, repetitive behaviours and impairment in the ability to socially interact. Until now it was not clear whether the cause of the GSH deficiency in the cerebellum tissues of autistic subjects lay in the decreased synthesis, increased consumption and/or deficient regeneration mechanisms, but recent researches (Gu *et al.*, 2013) have reported that out of various enzymes involved in GSH metabolism, the glutamate cysteine ligase (GCL), catalyzing both GSH regeneration and synthesis, has an insufficient activity, most likely due to a decreased protein expression of GCL catalytic and modulatory subunits. A protective effect of glutathione was demonstrated in acute poisoning with omethoate, an organophosphorous insecticide, which may lead to hepatocellular edema and fatty degeneration of the liver. Lu and his team (2010) showed that exogenous reduced glutathione (GSH) prevents liver damage, by restoring the activity of acetylcholinesterase (AChE), which is significantly inhibited by the insecticide, and by also mitigating the stress response of the cells, by attenuating the increase in the activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), Tumor Necrosis Factor-alpha (TNF-α) and in nitric oxide (NO) production. As expected, the total concentration of glutathione in the cells and the balance in the GSH/GSSG forms is genetically determined. By studiying mono-and dizygotic twin pairs, Van ‘t Erve and his co-workers (2013) have demonstrated that the concentration of glutathione in human erythrocytes is an inheritable trait. They have also anticipated that this holds true for other types of cells too. As the couple GSH/GSSG is a marker of oxidative status in the cells, future research can focus on determining the contributions of both genes and environment to the antioxidant protection each individual can naturally achieve, so that personalized preventive or treatment strategies be applied. In this sense, up-regulation of glutathione-related genes (Schuliga *et al.*, 2002), modulating glutathione-related defenses, as well as modulating glutathione-related enzyme activities, may be a new approach in various therapeutic strategies. Knowing that GSH cannot be directly administered *per se* with any clinical consequence (Chen *et al*., 1998a), various precursors (Chen *et al.*, 1998b; Olney *et al.* 1990; Ortolani *et al.*, 2000) or similar products with chemically modified formula (Yamamoto *et al.*, 1993; Burg *et al.*, 2002; Hamilton *et al.*, 2004; Kals *et al.*, 2008) were developed in order to simulate the physiological or pharmacological effects of glutathione, some of them used to intensify the anti-oxidant activity (Shibata *et al.*, 1995; Wang *et al.*, 1996; Ehrlich *et al.*, 2007), while others to target the enzymes involved in GSH metabolism (Batist *et al.*, 1986; Kunze *et al.*, 2000; Gate *et al.*, 2001; Ruscoe *et al.*, 2001;Wu *et al.*, 2010). The two approaches have shown significant therapeutic potential and a lot of new molecules are now in clinical tests and development (Green *et al.*, 2012). Modulation of glutathione, both by increasing it or by reducing it, has a huge potential and clinical significance in a wide range of human diseases (Wu and Batist, 2013).

3. GLUTATHIONE AND ITS ROLE IN PLANTS

The glutathione, as in all living cells, plays an indispensable role in plant cells, being involved in the antioxidant system, sulfur metabolism and the detoxification of xenobiotics (Noctor and Foyer, 1998). Glutathione is found in all plant cells, being synthesized either in the cytosol or in the chloroplast. As in human/animal cells, it has an antioxidant role, being associated with plant responses to various stress factors, participating in neutralizing free radicals and hydrogen peroxide produced by large temperature differences, lack of water, pesticides, air pollution (Alscher, 1989). Glutathione has vital importance for plants, because it fulfills certain essential functions in their metabolism. GSH participates in some

biosynthesis processes and cell detoxification. It is involved in photorespiratory and respiratory metabolism control, phytohormones modulation and in redox mechanism against damaging actions of ROS (Noctor *et al.*, 2012). In some plants glutathione is found together with his homologues, molecules which have in their structure other amino acids in C-terminal position than the glycine (Rennenberg, 1980; Klapheck, 1988; Klapheck *et al*., 1992), such as alanine or serine. Homoglutathione (γ-Glutamyl-Cysteinyl-β-Alanine) is found mainly in legumes (MacNicol, 1987; Klapheck 1988), while in cereals, another GSH analogous, hydroxymethyl GSH (γ-Glutamyl-Cysteinyl-Serine), is predominant. Some researchers concluded that these analogues are not formed by specific synthesis, but rather by glutathione molecule modification (Klapheck, *et al*. 1992; Okumura *et al.*, 2003; Skipsey *et al.*, 2005). As in animal cells, disulfide forms of glutathione and its homologues are reduced to GSH through reactions catalyzed by GSH reductases (Klapheck 1988; Klapheck *et al*., 1992). Glutathione fulfills other important functions in plants, being involved in sulfur assimilation and inhibition of sulfate absorption (Herschbach and Rennenberg, 1994; Lappartient *et al*., 1999; Vauclare *et al*., 2002; Buchner *et al*., 2004), pollen germination and pollen tube growth (Zechmann *et al*., 2011) and cell division and meristem development (Vernoux *et al*., 2000; Cairns *et al*., 2006; Reichheld *et al*., 2007; Frottin *et al*., 2009; Bashandy *et al*., 2010). GSH is part of the mechanism to fight against infections caused by molds, such as *Botrytis cinerea* (Chassot *et al*., 2008). Oxidants are produced in plants following the metabolic processes of photosynthesis, photorespiration and respiration (Foyer and Noctor, 2003), but they are also generated by unfavourable weather conditions (cold, heat, drought, heavy rainfall), soil and air pollution and various pathogen agents, all affecting the GSH redox state in plants leaves (Sen Gupta *et al.*, 1991; Vanacker *et al*., 2000; Bick *et al*., 2001; Gomez *et al*., 2004). Interestingly, the content of GSH or its homologues in leaves is not very much influenced by the variation of light intensity during the day/night alternance. The ratio GSH/GSH homologues is constant in plants leaves, for example almost all soybean leaves with the exception of those damaged by age, contain similar total amounts and similar GSH/GSH homologues ratios. However, important gradients were measured between young and aged poplar tree leaves (Arisi *et al*., 1997). Toxic metals and metalloids determine an excessive production of ROS responsible for the oxidative stress, plants being among the most sensitive organisms to metal toxicity. GSH controls directly or indirectly the defense mechanisms against ROS, thus being involved in plants protection against oxidative stress generated by toxic metals and metalloids (Anjum *et al*., 2012). The anti-oxidant mechanism of glutathione observed in living cells is also valid in food products containing reduced-glutathione or their precursors. Their presence contributes to a better protection against the aggression of oxygen and to a longer shelf life of these products. Among foods, one that can benefit the most from anti-oxidant protection is the wine, therefore, from ancient times men was in pursuit of powerful (and healthy) anti-oxidant molecules to be added to protect the aroma and colour. It is therefore no surprise that the naturally-occurring GSH attracted recently a lot of attention, especially for the white wine anti-oxidant protection. Glutathione has crucial roles in winemaking, from the preservation of important varietal aroma compounds originating in grapes, to the limitation of browning and of atypical ageing off-flavours development in wines. The simplest protection mechanism against oxidation is the oxidation of reduced form of glutathione to its disulfide form. Another mechanism is based on the fact that, due to its end cysteine residue, the GSH reacts with quinones and forms colorless complex molecules, more resistant to further oxidation (Antoce, 2007).

4. OXIDATION MECHANISMS OCCURING IN WHITE WINES

Oxygen control, especially during white winemaking process, plays an extremely important role for the quality of wine. The lack of proper management of oxygen can promote oxidation reactions of certain phenolic compounds responsible for maintaining varietal character and typicity of the wine. Oxidative transformations of grape must start during the

harvest process and transportion of grapes to the cellar, due to the exposure to air of the must leaking from the broken berries. Protection against oxidation should be taken into consideration particularly in pre-fermentation phases, the only cases in which positive effects of oxygen are apparent being during the first stages of wine fermentation and during maturation (Antoce, 2007). In the presence of oxygen, the very well known polyphenol oxidation processes are rapidly occurring and are followed by changes in the must/wine chemistry, with negative effects on the white wine quality, mostly expressed by wine browning and fruity aroma loss. However, oxygen supplementation is desired in some cases, going even as far as the hyper-oxygenation of the must. Winemaking by hyper-oxygenation technology is only recommended for non-aromatic varieties, in order to obtain neutral wines to serve as a base for sparkling wines or to obtain wines with enhanced fermentation aroma. Loss of aromatic compounds, browning and phenol precipitate formation are specific incidents associated with oxidation of white wines (Kilmartin, 2010). Oxidation of phenolic compounds in wine takes place by uptaking the oxygen dissolved in wine, either by chemical or by enzymatic pathways, both oxidation mechanisms occurring simultaneously. The oxidation of polyphenols from must occurs very rapidly under the catalysis of various polyphenoloxidases (PPOs). The tyrosinase enzymes catalyze the oxidation of cinnamic acid and its esters with tartaric acid (caftaric acid and cutaric acid) causing the formation of quinones, which are involved in various metabolic reactions of oxidation and oxidative polymerization. Laccases, another polyphenoloxidase found only in musts from grapes attacked by the fungus *Botrytis cinerea*, catalyze the quick oxidation of various substrates and is very hard to control (Antoce, 2007). On the other hand, it is believed that the non-enzymatic absorption of oxygen in the wine leads to similar products and further chemical processes as in the case of the enzymatic oxidation in the presence of PPO, except that the formation of hydrogen peroxide is only linked with the non-enzymatic oxidation (Singleton, 1987). Non-enzymatic oxidation of *ortho*-catechins leads to the formation of *ortho*-diquinones, with elimination in must of a molecule of H_2O_2, which is also very reactive and can oxidise other substances in wine, such as alcohols, producing more volatile aldehydes. One of the common oxidation reactions that occurs immediately following the chemical transformation of *ortho*-diphenols is the transformation of the ethanol into acetaldehyde (Antoce, 2007). The oxidation of *ortho*-diphenolic compounds allows for some further reactions resulting in the formation of quinones, by means of a semi-quinone radical intermediate. In this case oxygen is initially reduced to hydrogen peroxide, by a coupled transformation reaction of iron(II) into iron(III) (Waterhouse and Laurie, 2006). The resulted quinones have electrophilic properties, which prompts them to react with the nearby nucleophilic molecules, so that the catechol radical is converted back to a reduced phenolic form, although with a substituted group attached. The semi-quinones radicals are able to react with other radical species, including hydrogen atoms, regenerating in this way the *ortho*-diphenol structure. The chemical transformation of ethanol into acetaldehyde occurs in a significant proportion only when coupled with the oxidation of readily-oxidisable polyphenols, such as caffeic acid, a hydroxycinnamic acid typical for white wine (Wildenradt and Singleton, 1974). As such, these *ortho*-diphenolic compounds are oxidation propagators. In the absence of polyphenols, considered the main initial substrates for oxidation of wine, ethanol and tartaric acid are very stable against oxidation.

4.1. OXIDATIVE BROWNING OF WHITE WINES

The reaction of browning of white wines normally starts with a lag time, followed by a low speed phase, before reaching an accelerated phase, which leads to the conclusion that this phenomenon is rather an autocatalytic process, in which the easiest oxidizable polyphenol oligomeric products are involved. The browning of the white wines was rather correlated to the total flavonoid content than to the total phenols content or to the concentration of hydroxycinnamic acids (Singleton, 1987). The browning of the white wines is due to the

oxidation of oxidisable polyphenols and is associated with the loss of varietal aroma compounds that give wine varietal typicity. Chemical analyses show that white wines have lower concentrations of total polyphenols (typically 200–500 mg/L), with a predominance of hydroxycinnamic acids. White wines contain low concentrations of flavonoids (quercetin glycosides or catechins), but even so, their presence is important in the context of wine browning. Higher concentrations of these compounds were found in musts with a longer time of contact with the skin and harder pressing (Maggu *et al.*, 2007), which make these particular musts more susceptible to browning. The mechanism by which the polyphenoloxidases (PPO) catalyze the oxidation reactions of the very easily oxidisable compounds in the presence of oxygen and in the absence of any protection with sulfur dioxide is very well known (Singleton, 1987). PPO enzymes determine the formation of reactive quinones, which can react further with other polyphenols, with glutathione or different varietal thiols, such as 3-sulfanylhexanol, in all cases resulting brown coloured products (Cheynier *et al.*, 1989a, 1993; Nikolantonaki *et al.*, 2014). Studies have shown that the preferred substrate of PPO is not the catechin, but the caftaric acid (caffeoyl tartaric acid) and the quinones resulted from the oxidation of caffeic acid have a higher affinity to bond with catechin, rather than with another caffeic acid, leading to catechin quinones, which in turn form condensation compounds (Cheynier *et al.*, 1989a). However, the PPO enzymes are playing this role in the white wine browning processes, only under certain conditions, such as the lack of SO_2 and only for a limited period of time (Traverso-Rueda and Singleton, 1973). Moreover, Lutter *et al.* (2007) showed in a model wine solution that in the presence of an Fe(II) catalyst the oxidation of caffeic acid leads to dihydroxy-benzaldehyde. When the dihydroxy-benzaldehyde reacted with catechin, not brown, but various colourless and yellow/red compounds were produced, including bridged-catechin dimmers (Cheynier *et al.*, 1989b; Schneider, 1998; Ho *et al.*, 2000). For the instability of varietal aromatic compounds (i.e. 3-sulfanylhexan-1-ol and for the wine browning reactions occuring during maturation) again the polyphenols are responsible, especially the flavonoids (i.e. flavan-3-ols and their condensation products, proanthocyanidins), which are readily oxidisable compounds (Blanchard *et al.*, 2004; Fernandez-Zurbano *et al.*, 1995; Nikolantonaki *et al.*, 2012; Rossi and Singleton, 1966). All the compounds responsible for the browning reaction of white wine were difficult to determine, but some studies have indicated some possible other directions and methods. George *et al.* (2006) have demonstrated that the glyoxylic acid, resulted from the oxidation of tartaric acid, binds with two catechin molecules trough a reaction catalysed by metalic ions (iron, copper) and form yellow coloured xanthylium pigments. The xanthylium cation pigments originating formed from epicatechin proved to be two times more intensely colored than those forming from solutions containing catechins (Labrouche *et al.*, 2005). The same pigments were generated in reactions between catechin and dihydroxfumaric acid, resulted from tartaric acid oxidation by hydroxyl radicals (Clark, 2008). The researches made by Merida *et al.* (2006) have demonstrated that in the presence of yeasts, a lower quantity of coloured products have been produced in the reaction between catechin and glyoxylic acid, this phenomen being explained by the fact that yeasts consumed the oxygen and that they are also capable to absorb the brown products. On the other hand, to prevent the browing of the finished wine, the hyperoxidation of musts can be used to eliminate the excessive easily oxidisable phenolic compounds, which are in this way oxidized and precipitated (Cheynier *et al.*, 1989b; Schneider, 1998; Ho *et al.*, 2000). As a consequence, wines with very low concentrations of polyphenols and with lower browning potential are produced. The aroma of these wines may achieve a high intensity if it is enhanced by freeze-concentration or the grapes are late harvested (i.e.: the icewines cases) (Kilmartin *et al.*, 2007)

4.2. OXIDATION OF AROMA COMPOUNDS

Another important general effect of wine oxidation is directly seen upon wine aroma (du Toit *et al.*, 2006), ranging from the benefic

elimination of reductive smells generated by sulfur-containing compounds, to the detrimental aroma losses, through their oxidative degradation and the production of new 'oxidised' aromas. Moreover, many sulfur-containing compounds may produce unwanted aromas in wines (Mestres *et al.*, 2000), which must be removed later through oxygenation operations, racking or copper fining. However, some sulfur-containing compounds have positive effects to wine aromas (i.e: the dimethyl sulfide is enhancing the berry fruit note of the wines and varietal thiols, such as 3 mercaptohexanol (3MH), determine the grapefruit/ passionfruit aromas of Sauvignon blanc and other wines (Tominaga *et al.*, 1998)). Such compounds have to be protected against oxidation (Segurel *et al.*, 2004; Escudero *et al.*, 2007). A lot of studies have been made to understand how aroma compounds oxidation occurs in wines. As we saw previously, the polyphenols oxidation products are the quinones, which are easily reacting with the sulfides. Direct oxidation of thiols (mercaptans) to disulfide forms can also occur and is detrimental for the wine quality as these products are not anymore removable by copper fining (Rauhut *et al.*, 1996; Mestres *et al.*, 2000). Fedrizzi and his team (2007) found that in older wines, higher concentrations of dimethyl disulfide and diethyl disulfide, coupled with lower concentrations of ethyl mercaptan and 2-mercaptoethanol, indicate the occurance of oxidation processes. Marais (1979) reported as well increases in dimethyl sulfide with aging, due to the degradation of S-methyl methionine. In the case of the thiol group-containing amino acid cysteine, a very fast oxidation reaction occurs in the presence of O_2, Fe(II) and Cu(II), as the oxidation of thiols is catalysed by metals (Danilewicz *et al.*, 2008). The disulfides have the capacity to convert back to thiols due to the reducing ability of sulfites in wine, which has been demonstrated in model solution studies by Bobet *et al.* (1990). During wine ageing thiol compounds with lower sensorial thresholds than the disulfides they originate from are being released. During bottle storage mercaptans release from the hydrolysis of thioacetic esters has also been described by Rauhut and co-workers (1996). During wine-ageing, aldehydes are important intermediates in the redox processes occuring, leading to color and flavor changes. If the off-flavors resulted from wine oxidation remain at low concentrations they can contribute to the complexity of a wine, but when their proportion is increasing, the wine quality is affected (Oliveira *et al.*, 2011). Unsolved questions and issues remain regarding the way in which the behaviour of the aroma compounds can be controlled and modulated to obtain a better aromatic profile of the wines. Studies aiming to improve the aromatic profile of wines are continuously performed, a multitude of products with antioxidant properties and antioxidant protection technologies being proposed for various stages of the wine evolution, from grape harvesting and pre-fermentation phases, to wine stabilization, aging in bottles and final consumption.

4.3. ANTIOXIDANT PROPERTIES OF DIFFERENT ADDITIVES IN WHITE WINE

White wines resistance to oxidation is an imperious condition for the preservation of quality and extension of their shelf life. Winemakers can choose among a multitude of available choices for anti-oxygen protection, taking into account the rate and extent of oxygen exposure at various stages in winemaking. Among the most used methods for the management of unwanted sulfur compounds formed by oxidation it is the addition of antioxidant molecules such as SO_2, glutathione (GSH) and ascorbic acid, independently or in combinations. Another very well known technique is to maintain the wine on yeast lees during ageing, in order to harness lees ability for oxygen consumption. The use of various fining agents, especially before bottling, is another way to preserve wine quality, used to remove the substrates prone to oxidation, such as polyphenols and sulfur-containing compounds. As the metals act as catalysts for oxidation reactions, procedures to decrease the wine's metal content (especially iron and copper) or the use of chelating agents to block these metals and reduce their catalytic oxidative effect became available. Another direction is to use different antioxidant agents, in order to consume the dissolved oxygen or to reverse the oxidative processes from the wine, as antioxidants can act

in a multitude of directions in achieving their antioxidant effect. One of the most used antioxidant compounds is sulfur dioxide (SO_2) widely applied in a lot of operations and with a multitude of techniques. Attempts to protect selected aroma compounds in a model wine solution containing isoamyl acetate, ethyl hexanoate and linalool were made by Roussis and Sergianitis (2008) by using as antioxidants SO_2 and mixtures of glutathione with either caffeic acid or gallic acid at concentrations similar to those found in wine. Polyphenolic compounds such as caffeic acid and gallic acid were used to provide protection against the loss of certain aroma compounds, by exploiting their preferential affinity for oxygen, as compared to other oxidisable substrates. In this case, the phenolic compounds proved to have completely different effect from the one shown in browning reactions (Kilmartin, 2010). Preservatives like SO_2, ascorbic acid, glutathione, having the ability to act as quinones reductants and/or scavengers are decisive factors for managing the wine resistance to oxidative aging and varietal thiol stability (Brajkovich *et al.*, 2005; Lavigne Cruège *et al.*, 2003; Ugliano *et al.*, 2011). The use of sulfur dioxide and ascorbic acid combined in different proportions determined the inhibition of polyphenol oxidation in wines to various extents (Oliveira *et al.*, 2002). However, as Barril *et al.* (2009) underlined, ascorbic acid is a highly unpredictable molecule and the usage brings some risks, because in the presence of catechin its degradation products can eventually react farther and lead to yellow coloured xanthylium pigments. Pons *et al.* (2010) emphasized that the ascorbic acid has a big potential to compromise the wine flavour, considering the fact that it stays at the origin of sotolon in dry white wines. As it is known, sulfur dioxide does not have the ability to capture oxidative degradation products of ascorbic acid, which means it is possible that SO_2 could not reduce the degradation of ascorbic acid (Barril *et al.*, 2012). Apart from SO_2 and ascorbic acid, the other native antioxidant compound found in grapes, the tripeptide glutathione (GSH), was studied in the last years and at present it is occasionally employed in winemaking, as the product is under evaluation by the OIV for the addition in must and wine (OIV resolutions OENO-TECHNO 10-445 and OENO-TECHNO 10-446, stage 5 in 2015) up to a concentration of 20 mg/l. Previous researches have demonstrated that the glutathione, combined with small quantities of SO_2, inhibit the loss of desirable aromatic compounds like mono-terpenes and esters, and delay the browning reactions in wines, especially the yellow coloured xanthylium cation pigments formation (Bouzanquet *et al.*, 2012; Roussis *et al.*,2007; Sonni *et al.*, 2011a). Lavigne and Dubourdieu (2002) were the first who have observed that reduced glutathione confers direct protection of the volatile thiols during the oxidative processes or aging in barrels, while Ugliano *et al.* (2011), confirmed the same protective effect by measuring the loss of volatile thiols in samples of Sauvignon Blanc with 20 mg/L of GSH added at bottling, finding that after 6 months of aging in bottles the loss of thiolic compounds was highly reduced. Vaimakis and his co-workers (1996) have measured in the white wine a higher un-oxidised phenol content after the addition of another thiolic compound, the amino acid cysteine. Other researchers (Nikolantonaki *et al.*, 2014) assessed with modern techniques the protective effect of antioxidant agents in wine, including SO_2, GSH, ascorbic acid and tannins. In spite of the intensifying research regarding the glutathione in wines, the present knowledge about the GSH antioxidant role and its complementary activity with the most common wine preservatives such as sulfur dioxide and ascorbic acid need to be further studied, as these combinations seem to be very promising for the wine oxidation control (Kritzinger *et al.*, 2012).

4.4. THE SULFUR DIOXIDE AS ANTIOXIDANT IN WINE

The most common antioxidant and preservative agent used in winemaking is the sulfur dioxide, as it has both oxidation preventing activity and antimicrobial role. An important proportion of SO_2 in wine is bound to carbonyl compounds, such as acetaldehyde and the free SO_2 is found mostly in the bisulfite ion (HSO_3^-) form, only a small proportion being identified as molecular SO_2 (Abramovic˘*et al.*, 2014). This leads us to the conclusion that bigger quantities of free sulfur dioxide in wine, although not welcomed

by the health-concerned consumers, determine higher molecular SO_2 levels and a better wine quality, as the amounts of hydrogen peroxide, o-quinones and carbonyl compounds are smaller (Webber *et al*., 2014). The sulfur dioxide is used in winemaking to limit the detrimental impact of oxygen intake into the wine, as its principal ability is to scavenge the above mentioned hydrogen peroxide, ortho-quinones and carbonyl compounds (Adachi *et al.*, 1979; Danilewicz and Wallbridge, 2010). The sulfur dioxide was seen first as an inhibitor of PPO activity (Singleton *et al.*, 1985). Later, SO_2 has been proven to be a fast scavenging agent of hydrogen peroxide, but not by directly reacting with oxygen. SO_2 can be oxidised by O_2 in model wine solutions only in the presence of catalytic metals (such as Fe and Cu) which are increasing the oxidation of ethanol to acetaldehyde, followed by an acetaldehyde-bound SO_2 accumulation (Danilewicz, 2003, 2007; Danilewicz *et al.*, 2008). Vivas and his co-workers (1997) have proposed an inhibitory role of sulfur dioxide for the polyphenols auto-oxidation reaction, as in their experiments the oxidation of catechol-containing polyphenols occurred much faster, with a bigger SO_2 consumption, but with minimal ethanol oxidation. The researchers have concluded that SO_2, at the concentrations typically found in wine, did not act as a superoxide ion scavenger, while superoxide was actually effectively removed by ascorbic acid and polyphenols (Vivas *et al.*, 1997). Sulfur dioxide has also an important role in the rapid reduction of oxidized polyphenols (Cheynier *et al.*, 1989a, 1993), which has also been demonstrated by Saucier and Waterhouse (1999) in the synergistic activity of SO_2 and catechin. Both tests applied (Folin–Ciocalteu and Randox (ABTS) total antioxidant assays) have indicated a minimal response with SO_2 alone, but a significant increasing response in the case of catechin and SO_2, demonstrating the ability of SO_2 to bring back the catechin quinone oxidation products in the form of reduced catechin, allowing it to start the reaction over again. In accordance to Lambropoulos and Roussis (2006), in model wine studies, the sulfur dioxide increases the ability of caffeic acid and gallic acid to protect from oxidation several esters and terpenes. Although any carcinogenic or genetic mutations caused by sulfur dioxide have not been demonstrated, it is agreed that the sulfur dioxide may adversely affect human health because of its potential allergenicity (Walker, 1985; Garde-Cerdán and Ancín-Azpilicueta, 2007). As a consequence, because SO_2 is actually used not only for wine production and preservation, but also as an additive in a multitude of food products, the amount ingested being cumulative, reducing its utilization had to become a priority for food and beverages industry. Even if at present producing wine without SO_2 addition is not acceptable for many oenologists, in the view of its multiple protection abilities to prevent the enzymatic oxidation of musts and to inhibit the growth of unwanted microorganisms (Garde-Cerdán and Ancín-Azpilicueta, 2007), for the sake of the consumers' health, finding other suitable replacement products or new compounds with similar or better preservative actions, has to be a priority for the wine researchers.

4.5. GLUTATHIONE AND ITS ROLE IN PREVENTING THE MUST AND WINE OXIDATION

One of the most promising molecules with abilities to at least replace the sulfur dioxide in its antioxidants actions is the glutathione. As mentioned before, glutathione (GSH) is a natural antioxidant contained in grapes, that plays key roles in winemaking, from the preservation of important varietal aroma compounds, to the limitation of browning and of atypical ageing off-flavours development in wines. The GSH content found in the various varieties of *Vitis vinifera* is extremely variable, depending on the genetic component, the level of grapes ripening, nutrition or environmental stresses. Grape juices contain different concentrations of GSH, from traces to more than 100 mg/L, the content being influenced by oxygen exposure, polyphenoloxidases activities, crushing operations, grape skin contact period and pressing conditions (Cheynier *et al.*, 1989c; Park *et al.*, 2000a; du Toit *et al.*, 2007; Maggu *et al.*, 2007; Patel *et al.*, 2010). Lower concentrations of GSH were found in a more oxidative must treatment, compared to a reductive one. In the pressed grape juice researchers measured lower concentrations of

GSH in the absence of added SO_2 and ascorbic acid, but the GSH content found during frozen storage, was quite stable (du Toit *et al.*, 2007). To assure the antioxidant protection of musts, high concentrations (50–100 mg/L) of free glutathione in crushed grapes are needed (Singleton *et al.*, 1985). Even during the alcoholic fermentation a possible variation of GSH content can be observed, related to the yeast activity (Mezzetti and de Vero, 2014). In finished wines, the GSH concentration may considerably vary (Kritzinger *et al.*, 2013; Park *et al.*, 2000a) due to several technological conditions. Starting from the grapes, GSH production is directly correlated with both total nitrogen and assimilable amino acid content of grape juice, therefore, even from the beginnig, the raw materials differ significantly. Afterwards, the fermentation yeast may also influence the GSH concentration in the medium, and the lees contact can have an even greater influence. To establish the GSH positive influence to the wine quality, intending specifically to limit the browning reactions and losses of aroma compounds, some researchers analyzed its antioxidant status in the presence of various oenological factors, such as yeast strain choice, extended lees contact and the manipulation of juice assimilable nitrogen content (Kritzinger *et al.*, 2013). The GSH concentration during the alcoholic fermentation was measured too within various experimental studies. Irregular levels of GSH during the alcoholic fermentation were found, some researchers reporting higher levels (Park *et al.* 2000a, b; Fracassetti, 2010; Andújar-Ortiz *et al.*, 2012), other lower levels (du Toit *et al.*, 2007; Patel *et al.*, 2010; Coetzee, 2011) as compared to GSH initial amount. Lavigne and his collaborators (2007) established that the glutathione concentration after alcoholic fermentation completion is directly influenced by the yeast strain, as they measured different GSH concentrations in the same Sauvignon Blanc juice inoculated with different yeast strains. Working with other yeasts, other researchers did not confirmed these results. Results obtained by Fracassetti (2010) lead to the conclusion that the influence of yeast strain on the GSH wine concentration is insignificant. Kritzinger and his team (2013) studied too the influence of some commercial wine yeast strains, lees contact and assimilable nitrogen content on glutathione concentration in wine. Decreased contents of glutathione were reported by many researchers during wine ageing (Penna *et al.*, 2001; Ugliano *et al.*, 2011). However, the GSH concentration remaining in wine is indeed affected by the wine yeasts, as it was observed that the lees prevents the consumption of the glutathione content (Lavigne *et al.*, 2007). Kritzinger *et al.* (2012) demonstrated that the use of GSH-enriched inactive dry yeast preparations also have influence on the GSH concentration in the wine, fact also supported by other researchers (Lavigne *et al.* 2007), who showed that keeping the wines on the lees could contribute to maintaining of a good level of glutathione in the aging wine. Some yeasts are at present specifically selected to impact on the total content of GSH and resolutions are under debate at the OIV, regarding the approval of inactivated yeasts rich in glutathione containing at least 8 mg/g of reduced glutathione (resolutions OENO-TECHNO 13-532 and OENO-TECHNO 13-533, Stage 5 in 2015).

As suggested by Kritzinger (2012), the final concentration of GSH in wine may be influenced by the *Saccharomyces cerevisiae* metabolism during alcoholic fermentation. As GSH is an intracellular compound, it is released during the process of yeast autolysis, and can be absorbed as well from the extracellular environment by the yeast cells. Kritzinger and his co-workers (2013) concluded that the yeast strains could alter the GSH content in wines, by either utilising or secreting glutathione during fermentation, leading in this way to a variable wine GSH content. In supporting this theory, several research teams have identified and described in *S. cerevisiae* transporters for both the absorbtion and secretion of GSH (Miyake *et al.*, 1998; Bourbouloux *et al.*, 2000; Dhaoui *et al.*, 2011). Because GSH can be transported to the vacuoles of yeast cells, Jaspers and his collaborators (1985) have shown that GSH may be degraded by the enzymes form vacuolar membrane, such as c-glutamyltranspeptidase (c-GT) and L-cysteinylglycine dipeptidase. Kumar *et al.* (2003) described an alternative pathway of GSH degradation in *Saccharomyces cerevisiae*, independent of the enzyme c-GT, mediated by a novel protein complex encoded by three new genes (Ganguli *et al.*, 2007). GSH plays a

crucial role during the oxidation of white must and wine, being involved even in some oxidation reactions of phenolic compounds, as it is the case of caftaric acid quinones, with which it forms 2-S-glutathionyl caftaric acid, the so called Grape Reaction Product (GRP) (Singleton *et al.*, 1985; Cheynier *et al.*, 1986; Antoce, 2007; Sonni *et al.*, 2011a,b). These reactions usually occur during the operation of grapes crushing, when most of the phenolic compounds come in contact with oxygen and in the presence of PPO are rapidly oxidized, but these reactions can also occur later, without enzymes, when there only is chemical oxidation (Sonni *et al.*, 2011a, Ugliano *et al.*, 2011). GSH can also operate indirectly as a cofactor for several antioxidant enzymes, such as GSH peroxidase, GSH reductase, glutaredoxins and GSH S-transferases (Grant, 2001). This is the way in which GSH inhibits the browning reactions in wine, by blocking the *ortho*-quinones in uncoloured polymers (Singleton *et al.*, 1985; Antoce, 2007), making the ratio of glutathione to caftaric acid important for the browning susceptibility of a wine (Cheynier and van Hulst, 1988). Singleton suggested in 1987 to calculate an index of the enzymatic oxidation to which a must was exposed, as the ratio of caftaric acid to the GRP. Although it is thought that during alcoholic fermentation and storage of wine no significant changes of caftaric acid or S-glutathionyl caftaric acid (GRP) content occur, in further studies performed on bottled red wines kept for 170 days at 20°C, Giovanelli and Brenna (2007) found an increase of GRP concentration. The increase in GRP may be explained by a reaction of glutathione with caftaric acid quinones resulted from the chemical oxidation that can still occur in wine during aging in bottle. A similar evolution has been proven by Bassil *et al.* (2005), who obtained S-cysteinecaffeic acid by adding only the amino acid cysteine to caffeic acid oxidised by using sodium periodate as oxidant agent. To balance the dual effect of glutathione, which can protect from oxidation and contribute to oxidation as well, Vaimakis and Roussis (1996) have proposed a combination of white must oxidation and glutathione addition. It has been already demonstrated that glutathione is useful for the protection of different varietal aroma compounds during the aging of wines, as it is the case of 3-mercaptohexanol or other polyphenols (Dubourdieu *et al.*, 2000), volatile thiols (Lavigne-Cruège and Dubourdieu, 2002; Dubourdieu and Lavigne, 2004; Ugliano *et al.* 2011), esters and terpenes (Papadopoulou and Roussis, 2001, 2008; Roussis *et al.*, 2009). GSH appears to have an inhibitory effect on the formation of sotolon and 2-aminoacetophenone, which contribute to the development of atypical ageing characters (Dubourdieu and Lavigne, 2004). Other studies were not able to show any significant correlation between browning and GSH content. The statistical analyses of physicochemical parameters of thirteen Lebanese 2 years old Chardonnay dry wines of the same vintage studied by El Hosry and his team (2009) have lead to the conclusion that the main contributors to the wine browning are the pH, total phenols and total SO_2, but not the glutathione. Previously similar results were reported by Fernandez-Zurbano and co-workers (1995). In these cases, rapid oxidation of glutathione might explain the lack of correlation between GSH and browning predisposition of wine. By adding GSH, the aromatic characteristics of wines are primarily improved (du Toit *et al.*, 2006), as the first step of wine oxidation affects the aroma compounds, while browning is a later step (Singleton, 1987). The wines produced from oxygenated musts to which GSH has been added registered a considerable improvement of quality, without signs of specific oxidation flavour (Vaimakis and Roussis, 1996). In order to determine the doses of exogenous GSH which should be added to wine to obtain antioxidant protection and improved wine aromatic profile, different variants were studied. The protection against the loss of α-terpineol and linalool has been ensured by the addition of 20 mg/L of glutathione in Muscat wines kept in contact with the air at 20°C for 3 days (Papadopoulou and Roussis, 2001). The results were also confirmed in 2008 by the same authors during the storage of Debina white wines added with 20 mg/L of GSH. Lavigne-Cruege and Dubourdieu (2002) have showed that the addition of only 10 mg/L of GSH prevented the apparition of yellow shading of wine, ageing defects and the loss of the varietal aroma. Du Toit *et al.* (2006) have found that during 8 days of accelerated oxidation (55°C), a concentration of 10-20 mg/l

added GSH lead to a maximum total phenol content, while in the samples with 30 mg/L a moderate diminution of the total content of phenol occurred. The control samples, without any addition of GSH registered the lowest total phenol content. A clear improvement in total phenol content was initially observed (on day 0) for all wine samples with added GSH in comparison with control sample (with no added GSH). After 8 days of oxidation, irrespective of the glutathione addition, the GSH concentration in all the studied samples, ranged from 4.79 to 5.11 mg/L. Because a similar GSH concentration of 5.1 mg/L was found in white Palomino wine from Davis USA (Park *et al.*, 2000a) some researchers (Du Toit *et al.*, 2006) drew the conclusion that the value of about 5 mg/L of GSH is a steady-state value for the white wines. Webber and his collaborators (2014) have evaluated the effect of glutathione (GSH) addition on secondary aromas and on the phenolic compounds of sparkling wine elaborated by traditional method. When 10-20 mg/l GSH were added to the base must, lower levels of total phenolic compounds, hydroxycinnamic acids, ethyl decanoate, octanoic and decanoic acids were recorded, along with higher levels of 2-phenylethanol, 3-methyl-1-butanol and diethyl succinate. The GSH addition has a better effect for the sparkling wine quality when it is performed in the base must and not in the base wine. The highest level of total glutathione, as reported by Webber and his team (2014), was found in sparkling wine in which GSH was added to the must. GSH addition to base wine determined higher levels of free SO_2, irrespective of the amount of added GSH (Webber *et al*., 2014). The levels of GSH in sparkling wines are similar to those found in still white wines (Marchand and de Revel, 2010; Janes *et al.*, 2010; Fracassetti *et al.*, 2011). The content of GSH in the sparkling wine production diminished during fermentation, as observed also in some previous studies (du Toit *et al.*, 2007; Kritzinger, 2012) and was lower than the quantity of GSH added to the must and/or base wine. In the case of sparkling wines too, GSH may have been consumed by the reaction with *ortho*-quinones to form the GRP or by its interaction with the yeast (du Toit *et al.*, 2007; Kritzinger, 2012). In previous studies Penninckx (2002) underlined that GSH is involved in many stress response mechanisms of *Saccharomyces cerevisiae* and it may also play a role in the maintenance of basic cell functions such as cell structural integrity. GSH is an important metabolite for the yeast multiplication during alcoholic fermentation and it is also a potential source of nitrogen and sulfur (Penninckx, 2002). GSH is the most abundant sulfur-containing organic compound in *Saccharomyces cerevisiae,* which can account for 0.5–1.0% of the cell dry weight (Elskens *et al.*, 1991). As a source of sulfur, glutathione may also generate undesirable sulfur-based odours. Some studies regarding actually pointed out the potential of GSH to be a source of hydrogen sulfide (H_2S), because cysteine, one of the amino acids constituents of GSH, can be degraded by cysteine desulfhydrase to form H_2S (Tokuyama *et al.*, 1973). Hallinan and co-workers (1999) researches reported that glutathione may contribute to up to 40% of the H_2S release, liberated from sulfate by nitrogen starved yeast, incubated in the presence of sulfate. In a more recent study Ugliano *et al.* (2011) reported the formation during aging of a higher concentration of H_2S in the wines treated with GSH prior to bottling than in untreated wines.
GSH influence and functions in must and wine, along with its evolution during the alcoholic fermentation or aging, is thus not completely understood, therefore, further studies are necessary to completely clarify the way in which GSH can contribute to the wine quality improving during all winemaking stages.

5. CONCLUSIONS

Glutathione is found in plants, especially in fruits, among which the grapes are an important source. Its presence in grapes ensures the antioxidant protection of the must and the crushed grape mash, as its chemical structure allows for the oxidation of its thiol group into a disulfuric group, thus protecting other molecules from the attack of reactive oxygen species. With its high affinity for oxygen, glutathione preserves the fruity aromatic notes of young wines and prevents the premature aging of the wine. Glutathione can also block the formation of *ortho*-quinones, which confer brownish colour to the wines, by reducing them

to incolor phenolic compounds. Glutathione from grapes can also be used during must fermentation by the *Saccharomyces* yeasts as a source of sulfur, provided by its cysteinyl residue. Recent studies also demonstrated the beneficial influence of the addition of glutathione in the white wine production technology, especially for the preservation of the varietal character of the wines obtained from aromatic grapes. Many studies have demonstrated a direct relation between the oxidative stability of white wines, the values of pH, total phenol and total SO_2 levels and of GSH added to must or to wine during bottling. GSH can improve the wine stability and prevent the apparition of atypical oxidative flavours during wine ageing. Considering that it has a similar role of antioxidant protection across all living things, it is a logical assumption that glutathione can function similarly in processed foods and that can contribute to the reduction of the dosage of sulfur dioxide used for antioxidant wine protection. Athough, due to sulfur dioxide multiple functions in preservation of foods and wines, a complete replacement of sulfur dioxide cannot be envisaged, in the future, it might become a good candidate for partial replacement of sulfur dioxide. For now, it is important to continue the researches in order to establish the minimum/optimal amounts of free sulfur dioxide and/or ascorbic acid needed besides GSH to assure the wine stability.

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