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Klemen LISJAK

# THE ROLE OF OXYGEN IN NEW VINIFICATION TECHNOLOGIES OF WHITE AND RED WINES

DOCTORAL DISSERTATION

# VLOGA KISIKA V NOVIH TEHNOLOGIJAH VINIFIKACIJE BELIH IN RDEČIH VIN

DOKTORSKA DISERTACIJA

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Doctoral Dissertation was carried out at the Oenological Department, Central Laboratory of Agricultural Institute of Slovenia, Ljubljana, at the Istituto Agrario San Michele All'Adige in Italy and Deparment of Viticulture and Oenology, University of Stellenbosch, Stellenbosch, South Africa.
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Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehnioške fakultete in sklepu Senata Univerze z dne, 14.02.2006 je za mentorico doktorata znanosti s področja živilstva imenovana doc. dr. Urška Vrhovšek ter dne 04.07.2007 somentor Dr. Wessel Johannes Du Toit.

Supervisor (mentorica): Assist. Prof. Urška Vrhovšek, PhD
Co-advisor (somentor): Dr Wessel Johannes Du Toit

Committee for Evaluation and Defence (Komisija za oceno in zagovor):

| | |
|---|---|
| Chairman (predsednik): | Prof. dr. Veronika Abram, PhD<br>University of Ljubljana, Biotechnical Faculty |
| Member (član): | Assist. Prof. Urška Vrhovšek, PhD<br>University of Ljubljana, Biotechnical Faculty<br>IASMA Research Center, Italy |
| Member (član): | Dr. Wessel Johannes Du Toit<br>University of Stellenbosch, Department of Viticulture and Oenology |
| Member (član): | Assist. Prof. Tatjana Košmerl<br>University of Ljubljana, Biotechnical Faculty |
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AB The aim of the study was to provide new data about oxygen's effect on the chemical composition of must and wine. Furthermore, the rationale of our work was to study two different technologies in controlling oxygen's influence and its effect on the composition and quality of must and wine. These two technologies included 'hyperreduction', an innovative grape pressing of white varieties, and 'microoxygenation', a controlled and regulated oxygen addition into red wines. In the hyperreduction experiment we studied the influence of hyperreductive pressing of white grapes on the content of glutathione, hydroxycinnamic acids and their esters and flavan-3-ols. A new, innovative wine press was used, in which the oxygen concentration in the press atmosphere was below 1%. The results showed that hyperreductive pressing prevents the loss of glutathione in grape must and increases the level of hydroxycinnamic acids and their tartaric esters. It was also found that fermentation and aging decrease the glutathione concentration while hydroxycinnamic acids and their esters do not change significantly during fermentation and wine aging. In the microoxygenation experiments the influence of different oxygen addition in combination with oak segments addition on wine phenolics changes was studied. The colour intensity increased in microoxygenation treated wines in comparison to control wines. Aging with oak segments additionally increased the colour intensity. On the other hand, free anthocyanins, analyzed by HPLC showed greater decrease in microoxygenated wines compared with control wines. Low molecular weight and high molecular weight flavan-3-ols showed no significant differences between microoxygenated and control wines. Microoxygenation can also increase *Brettanomyces* and acetic acid bacteria survival. Both new technologies may be also applied for the production of Slovenian wines; by their use it is possible to improve the quality of wines.

## KLJUČNA DOKUMENTACIJSKA INFORMACIJA

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JI en/sl
AI Raziskava preverja nove tehnologije in odkriva nova spoznanja o vplivu kisika na kemijsko sestavo mošta in vina. Preučili smo dve različni tehnologiji, pri kateri ima kontrola kisika pomembno vlogo ne samo na sestavo, ampak tudi na kakovost mošta ter vina. Ena tehnologija vključuje hiperredukcijo kisika z inovativnim stiskanjem grozdja belih sort, druga pa mikrooksigenacijo, to je kontrolirano in regulirano dodajanje kisika v rdeča vina. Pri poskusih s hiperredukcijo smo preučevali njen vpliv na vsebnost glutationa, hidroksicimetnih kislin in flavan-3-olov. Pri tem smo uporabili stiskalnico, ki omogoča stiskanje grozdja v atmosferi z manj kot 1% kisika. Rezultati kažejo, da hiperreduktivno stiskanje preprečuje izgubo glutationa v moštu in poveča vsebnost hidroksicimetnih kislin in njihovih estrov. Ugotovili smo tudi, da fermentacija in zorenje zmanjšata koncentracijo glutationa, medtem ko se vsebnost hidroksicimetnih kislin in njihovih estrov med fermentacijo in zorenjem ne spreminja bistveno. V mikrooksigenacijskih poskusih smo preučevali vpliv različnih dodatkov kisika na sestavo fenolnih spojin in namenili pozornost tudi kombinacijam zorenja z dodanimi hrastovimi segmenti. Intenziteta barve se bolj poveča v mikrooksigeniranih vinih v primerjavi s kontrolnim vinom. Zorenje z dodanimi hrastovimi segmenti še dodatno poveča intenziteto barve. Ugotovili smo tudi, da prosti antociani, ki smo jih analizirali s HPLC, kažejo večji padec pri mikrooksigenaciji kot pri kontrolnih vinih. Nizkomolekularni flavan-3-oli in visokomolekularni flavan-3-oli ne kažejo značilnih razlik med mikrooksigeniranimi in kontrolnimi vini. Mikrooksigenacija lahko pospeši rast kvasovk *Brettanomyces* in ocetnokislinskih bakterij. Obe novi tehnologiji sta primerni tudi v pridelavi slovenskih vin, saj je z njihovo aplikacijo mogoče izboljšati končno kakovost vin.

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## ABBREVATIONS AND ACRONYMS

| | |
|---|---|
| Caffeic acid | *trans*-3,4-dihydroxycinnamic acid |
| Caftaric acid | trivial name for caffeoyltartaric acid |
| CTA | caftaric acid |
| CoTA | coutaric acid |
| Coutaric acid | trivial name for coumaroyltartaric acid |
| DAD | diode-array detector |
| Ferulic acid | *trans*-3-methoxy-4-hydroxycinnamic acid |
| Fertaric acid | trivial name for feruloyltartaric acid |
| FTA | fertaric acid |
| GSH | reduced glutathione |
| GSSG | oxidised glutathione |
| GRP | grape reaction product (2-S-glutathionylcaftaric acid) |
| GRP 2 | 2,5-di-glutathionyl-caffeoyltartaric acid |
| HCA | Free and tartaric esters of hydroxycinnamic acids |
| HPLC | high pressure liquid chromatography |
| Hyperreduction | technology of grape pressing, where oxygen concentration in the press atmosphere is modified and decreased below 1% |
| Hydroxycinnamates | term used to refer to all compounds containing a hydroxylated cinnamic structure, thus including: free cinnamic acids (e.g., caffeic, *p*-coumaric and ferulic); their esters (e.g., with tartaric acid in grap and wine: caftaric, *p*-coumaric and all glutathione derivates) |
| IP | index of polymerization |
| LC-MS-MS | liquid chromatography mass spectrometry |
| MOX | microoxygenation |
| Microoxygenation | the term refers to winemaking technology, where oxygen is introduced into red wine with the aim of improving wine quality |
| *p*-coumaric acid | *trans*-4-hydroxycinnamic acid |
| PPO | polyphenoloxidase |
| SPE | solid phase extraction |
| UV | ultraviolet light |
| VIS | visible light |

# 1 INTRODUCTION

Oxygen ($O_2$) constitutes 20.9 % of air and it is indispensable for living organisms on the earth. Living systems have developed mechanisms how to use oxygen for their metabolism and how to resist excessive oxidative damage. Must and wine, both have 'systems' how to deal with oxygen; however, these 'systems' are not infinite. Therefore, new technologies in wine production pay great attention to the way of managing the role of oxygen in wine making.

When white grape juice is processed without sulphur dioxide, enzymatically induced oxidation occurs and leads to the precipitation of phenolic compounds as insoluble brown pigments. The consequences of these reactions are losses of wine varietal characteristics and lowering of its quality. In order to improve the wine quality, a new technology called hyperreduction has been developed. The aim of this technology is the protection of grape must already during grape pressing. Grapes are pressed in a special wine press, where oxygen concentration is maintained below 1%. With $SO_2$ and ascorbic acid addition complete reductive conditions are obtained and oxidation is prevented already at an early stage of the vinification chain. This can increase the polyphenol and glutathione content, an important wine antioxidants which can protect the aromatic qualities of white wines and increase the varietal characteristics of them.

On the other hand, red wines benefit if low level of oxygen is added into the wine. In the early 1990s, in order to mimic the effect of aging in old barrels, the technique of microoxygenation was developed. The technique involves the continuous bubbling of small amounts of oxygen into the wine. This technique has now been spread throughout the winemaking world on a commercial basis and is now systematically used in the whole winemaking process of some wineries. Microoxygenation allows control over the amount of oxygen, which can be regulated over time. Like in other oxidation, wine antioxidants play a pivotal role in the microoxygenation process. The aim of the microoxygenation process is therefore to have a positive influence on the structural changes of wine polyphenols and consequently wine colour, aroma and mouth-feel.

## 1.1 SCOPE AND RATIONALE FOR THE THESIS

Phenolic compounds are primary reactants that are oxidized in the presence of oxygen, a process which initiates a cascade of chemical transformations that result in the deterioration of foods and wine. The management of these transformations is critical to the production of wine and its quality. Therefore the rationale of our work was to study two different technologies in controlling the oxygen influence and its effect on must and wine composition and quality. These two technologies include 'hyperreduction', an innovative grape pressing of white varieties, and 'microoxygenation', a controlled and regulated oxygen addition into red wines.

### 1.1.1 Hyperreduction

An innovative grape press was tested for the pressing of different white varieties in oxygen free atmosphere. With the absence of oxygen in the atmosphere during grape pressing we hypothesized that oxidation reaction stops due to the absence of oxygen – the most important substrate of this reaction. To confirm this technique small and industrial scale experiments were made and different analytical markers, which include glutathione and hydroxycinnamates, were followed. A novel LC-MS-MS method for glutathione was developed to analyze its content in different pressing fractions during fermentation and wine aging. Low molecular weight phenolic compounds were also analyzed to confirm the effect of hyperreductive technology.

### 1.1.2 Microoxygenation

The effect of microoxygenation treatments on the phenolic composition of different red wines was evaluated. Four different large scale experiments were conducted on different red wines varieties. The influence of oak segment addition in combination with different oxygen additions was also investigated. Spectrophotometric and HPLC analyses of phenolic compounds were followed during and after treatments. We hypothesized that microoxygenation increased the mean degree of proanthocyanidin polymerization (mDP); therefore we analyzed mDP on LC-MS at the end of microoxygenation. As oxygen also influences the microbial population, certain wine was also tasted and the survival of certain aerobic spoilage microorganims monitored.

# 2 LITERATURE REVIEW

## 2.1 INTRODUCTION

As long ago as 1873, Pasteur stated: ”L'Oxygène est le pire ennemi du vin”, (oxygen is the worst enemy of wine) but also, “C'est l'oxygène qui fait le vin, c'est par son influence qu'il vieillit” (Oxygen makes the wine, which ages under its influence).

Since that time, various researchers have studied the relationship between oxygen and wine. It is commonly admitted that extensive oxidation is unfavorable to wine quality, but slow and continuous oxygen dissolution may play a positive role in wine aging (Cheynier et al., 2002). To promote the beneficial effects of oxygen exposure while avoiding spoilage risks it is essential to understand the mechanisms governing oxygen dissolution and consumption in wine.

## 2.2 OXYGEN

### 2.2.1 Measuring of dissolved oxygen

Various methods have been developed to measure oxygen or the oxidation level of beverages after bottling. These include measurement of the oxidation-reduction potential, measurement of dissolved oxygen by polarographic probe, measurement of total oxygen in bottled beer using an oximeter based on Henry’s law and measurement of gas composition in the headspace of a bottle by gas chromatography (Lopes et al., 2005).

The first assays for oxygen content determination used a chemical method based on the oxidation of sodium hydrosulfite into bisulfite by free oxygen, with carmine indigo as the colour indicator (Ribéreau-Gayon et al., 2000b). The currently preferred method is the polarographic analysis developed by Clark (Ribereau-Gayon et al., 2000b). The apparatus consists of two electrodes, a silver anode and a gold cathode, linked by potassium chloride gel. They are separated from the medium by a membrane selectively permeable to oxygen. The difference in potential established between the two electrodes (on the order of 0.6 to 0.8 V) is modified by circulating oxygen through the membrane. The following reactions take place:

- at the cathode: $O_2 + 2H_2 + 4e^- \rightarrow 4OH^-$
- at the anode: $Ag^+ + Cl^- \rightarrow AgCl + e^-$

The intensity of the electrical current caused by the movement of electrons is directly proportional to the quantity of dissolved oxygen expressed in mg/L (Ribereau-Gayon et al., 2000b). Recently, Lopes et al. (2005) developed a nondestructive colourimetric method to measure oxygen diffusion from 1 to 9.8 mg/L during the post bottling period. The method is based on the reduction of indigo carmine by sodium dithionite and reoxidation of the reduced indigo carmine by atmospheric oxygen followed by tristimulus measurements (L*, a*, b*).

### 2.2.2 Oxidation reduction potential

Substances are oxidized when they fix oxygen or lose either hydrogen or one or more electrons. Reduction is the reverse of these reactions. In organic molecules, oxidation produces compounds with a higher oxygen or lower hydrogen content. In fact, there is always balance between the two phenomena. When an oxidation reaction occurs there is always a parallel reduction reaction:

$$Red_1 + Ox_2 \leftrightarrow Ox_1 + Red_2$$

Many chemical reactions in wine are characterized by electron transfer leading to oxidation and reduction phenomena. These reactions occur simultaneously and continue until oxidation-reduction equilibrium is reached. The oxidation-reduction potential of wine is a function of the oxidation and reduction levels of the medium at certain equilibrium. Therefore, redox phenomena are responsible for profound modifications leading to alterations principally in chemical wine color and aroma.

The wine's resistance to oxidation is a function of three main parameters: redox potential, the total concentration of native or added antioxidants, and the amount of dissolved oxygen (Oliveira et al., 2002).

The oxidation-reduction potential ($E_H$) of wine can be measured with an electrode similar to dissolved oxygen concentration measurement. Oliviera et al. (2002) described a new potentiometric method to evaluate the resistance to oxidation of white wines. The method uses the addition of trichlorotitanum (TCT) as a reductant following the titration with the oxidizing agent dichlorophenolindophenol (PIP) to obtain redox titration curves with a single potentiometric end-point.

### 2.2.3 Oxygen solubility

In a gas mixture, a gas exerts a partial pressure that corresponds to the pressure it would have if it occupied the entire volume alone. The proportion of oxygen in dry air is 20.9%, so its partial pressure ($pO_2$) is $1.013 \times 10^3 \times 20.9 = 21.2 \times 10^3$ Pascal (Pa), at 20°C and atmospheric pressure. In air saturated with water, the partial pressure of oxygen is $18.8 \times 10^3$ Pa. At equilibrium, the partial pressure of a gas dissolved in a liquid is identical to the partial pressure of the gas in the gas phase. Thus, at equilibrium, the partial pressure of oxygen in air saturated wine or water at 20 °C and atmospheric pressure is 18.8 kPa. If the liquid phase were saturated with oxygen instead of air, the partial pressure would be around five times more. Methods that measure dissolved oxygen using electrolytic cells give access to partial pressure and can be calibrated in saturation percentage. The liquid medium is saturated with oxygen when the measured value (100% saturation) is the same in the liquid phase as in the vapor phase.

The dissolved oxygen concentration can be calculated by using a solubility coefficient, using Henry's law: $pO_2 = H \times C^*$, where H is the oxygen solubility coefficient (H) and C* is the gaseous oxygen concentration at equilibrium. The oxygen solubility coefficient depends on temperature, pressure and the liquid composition.

A liter of air contains approximately 300 mg/L of oxygen. The oxygen concentration decreases as the temperature increases: in water, the solubility is 14.67 mg/L at 0°C, 9.2 mg/L at 20°C and only 5.6 mg/L at 50°C (Cheynier et al., 2002). The oxygen solubility decreases as the ethanol content increases up to 30%, but beyond that ethanol content the oxygen solubility strongly increases. The presence of solutes (sugar) in the liquid phase also decreases the oxygen solubility (Cheynier et al., 2002). Thus, the oxygen concentration in wine that is saturated with air is 6 mL/L or 8.4 mg/L whereas, in water, 100% saturation corresponds to 9.2 mg/L oxygen at 20°C and atmospheric pressure (Cheynier et al., 2002). Berta et al. (1999) report that wine is saturated with oxygen at 7.7 mg/L and 20°C. Moutounet and Mazauric (2001) report that the consumption of oxygen in red wine saturated with oxygen takes 25 days at 13°C, 18 days at 17°C, 4 days at 20°C, 3 days at 30°C and just few minutes at 70°C. An increase in pH and phenolic compounds enhances the consumption of oxygen. In general, the kinetics of oxygen dissolution in wine is much higher than its consumption.

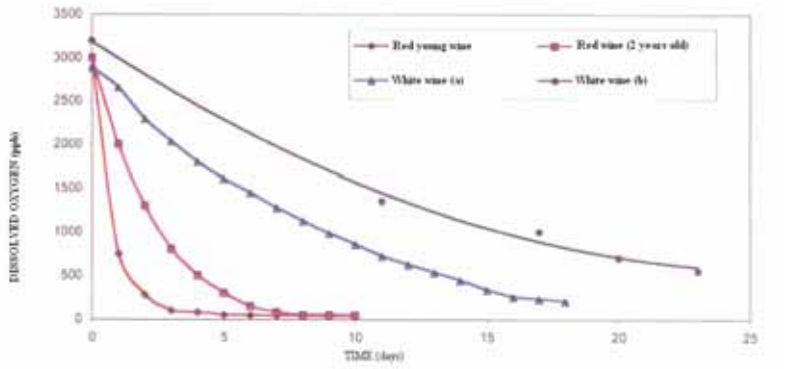



Figure 1: Oxygen consumption during wine storage in a hermetical vessel (Ferrarini and D'Andrea., 2000: 26)

## 2.3 ANTIOXIDANTS IN MUST AND WINE

An antioxidant is any substance that when present in low concentrations compared to those of an oxidizable substrate significantly delays or prevents the oxidation of substrates. Beside native antioxidants (phenolic compounds and glutathione), additional antioxidants are used in wine-making, namely ascorbic acid and sulphur dioxide. Polyphenols exhibit a great capacity to consume oxygen, which is due to the presence of several hydroxyl groups (Vivas and Glories, 1996b). Therefore, the quantity and rate of oxygen consumption are always higher in red wines than in white wines. Considering the amount of oxygen a wine can take up (ranging from about 60 to over 600 mL/L from light white to heavy red) there are no other autoxidizable substances evident in sufficient amounts to react with that much oxygen (Singleton, 1987). The detailed mechanisms of wine polyphenols reacting with oxygen are described in the following chapters.

### 2.3.1 Polyphenols

Oxygen consumption is much faster in red wines than in white wines, indicating that it is largely due to the oxidation of polyphenols, which are one of the most important antioxidants of wine. They are also responsible for wine colour, astringency, bitterness and partially its taste.

Due to structural similarity, the major groups of phenolics which are related to wine and winemaking can be divided into two classes: **non-flavonoid** ($C_6 – C_1$ the *p*-hydroxybenzoic group, $C_6 – C_3$ the cinnamic group and $C_6 – C_2 – C_6$ stilbenes) and **flavonoid phenolics** ($C_6 – C_3 – C_6$). These are the major phenolics in wine and in the fruit of other plants as well (Margalit, 2004).

#### 2.3.1.1 Non Flavonoids

The non-flavonoids present in grape and wine are hydroxycinnamates (HCA), hydroxybenzoic acids and stilbenes.

Hydroxybenzoic acid

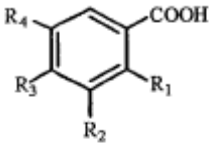
Gallic acid is the only hydroxybenzoic acid that has been formally identified in a native state in grapes, found in the solid parts of the berry, either in free form or in the form of flavanol ester (i.e. epicatehin-3-O-gallate). However, other hydroxybenzoic acids (HCA) can also be found in wines, including *p*-hydroxybenzoic, protocatechuic, vanillic, syringic and gentistic acids (Monagas et al., 2005).

Hydroxycinnamic acids

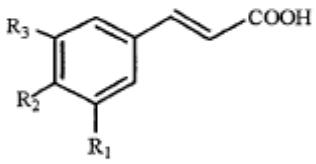
The hydroxycinnamic acids are located in the vacuoles of the skin and pulp cells (Ribéreau-Gayon et al., 2000b). They are the major phenolics in white wine and the main non-flavonoids in red wines. There are 3 free acids found in wines: caffeic acid, *p*-coumaric acid and ferulic acid (Sommers et al., 1987), which are usually esterified with tartaric acid and form caffeoyltartaric (caftaric), *p*-coumaroyltartaric (coutaric) and feruloyltartaric (fertaric) acids (Figure 2). They are present in their *trans*-form, although small quantities of the *cis* isomers also exist (Singleton et al., 1978). The presence of the glucose esters of *trans*-*p*-coumaric and ferulic acids has also been reported in grapes (Reschke and Herrmann, 1981)

Stilbenes

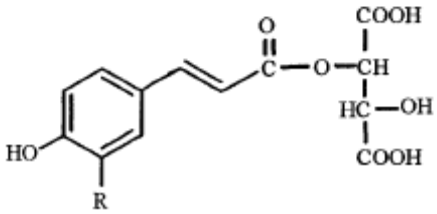
The hydroxylated stilbenes ($C_6 - C_2 - C_6$) are phytoalexins synthesized by the plant, especially in the skins, leaves and roots in response to fungal infections and ultraviolet (UV) light (Korhammer et al., 1995). *Trans*- and *cis*- resveratrol (3,5,4'-trihydroxystilbene) as well as their glucose derivatives (*trans*- and *cis*- piceid) have been identified in grapes and wines (Siemman and Creasy, 1992).




| Benzoic acid | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| *p*-Hydroxybenzoic | H | H | OH | H |
| Protocatechuic | H | OH | OH | H |
| Vanillic | H | $OCH_3$ | OH | H |
| Gallic | H | OH | OH | OH |
| Syringic | H | $OCH_3$ | OH | $OCH_3$ |
| Salicilic | OH | H | H | H |
| Gentisic | OH | H | H | OH |




| Hydroxycinnamic acid | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| *p*-Coumaric | H | OH | H |
| Caffeic | OH | OH | H |
| Ferulic | $OCH_3$ | OH | H |
| Sinapic | $OCH_3$ | OH | $OCH_3$ |




| Hydroxycinnamic ester | R |
|---|---|
| *Trans*- caffeoyltartaric acid (caftaric acid) | OH |
| *Trans*-*p*-coumaroyltartaric acid (cutaric acid) | H |
| *Trans*- feruloyltartaric acid (fertaric acid) | $OCH_3$ |

Figure 2: Phenolic acids and their derivatives (Monagas et al., 2005: 86)

#### 2.3.1.2 Flavonoids

Flavonoid compounds (flavone = 'yellow') include the whole class of plant phenolics which have the $C_6 - C_3 - C_6$ frame. The main groups of flavonoid compounds present in grapes and in wine from *Vitis vinifera* are flavonols, flavan-3-ols and anthocyanins and, in a smaller degree, flavanonols and flavones. Within each group, compounds differ by the number and by the localization of the hydroxyl and methoxyl groups located in the B rings.

Flavones, Flavonols and Flavanonols

Although more than 100 flavones have been identified in plants, these compounds are not yet common or abundant in fruits (Macheix et al., 1990). They were found mostly in the leaves of *Vitis vinifera*.

Flavonols are yellow pigments mainly located in the vacuoles of the epidermal tissues. In *Vitis vinifera* grapes, they exist as 3-*O*-glycosides of the four main aglycones: myricetin, quercetin, kaempherol, isorhamnetin, laricitrin, syringetin (Mattivi et al., 2006a). Eight flavonol monoglycosides and three diglycosides were characterized in the skin of *Vitis vinifera* grapes (Cheynier and Rigaud, 1986). The flavonol profile of wine is distinguished from that of grapes by the additional presence of the aglycone forms, which originated most likely from the hydrolysis of the glycosilated forms during vinification, maturation and/or aging of wine (Monagas et al., 2005). Price et al. (1995) report that quercetin level in Pinot Noir is highly correlated with clusters sun exposure.

The flavanonols are not usually present in plants used for food; they are normally found in wood in the form of free aglycones. Astilbin (dihydroquercetin-3-*O*-rhamnoside) and engeletin (dihydrokaempferol-3-*O*-rhamnoside) were first identified in the skin and in the wine from white grapes by Trousdale and Singleton (1983). Both astilbin and engeletin have been recently reported in white wines (Baderschneider and Winterhalder, 2001; Chamkha et al., 2003)

Anthocyanins

Anthocyanins constitute a large family of differently coloured compounds and occur in countless mixtures in practically all parts of higher plants. They are mainly located in the skins of grape beries. The anthocyanins identified in grape skins and in wine from *Vitis vinifera* are the 3-*O*-monoglucosides and 3-*O*-acetyl monoglucosides of five main anthocyanidins: delphinidin, cyanidin, petunidin, peonidin and malvidin, which differ from each other by the number and position of the hydroxyl and methoxyl groups located in the B-ring of the molecule (Figure 3) (Mazza and Miniati, 1993). The presence of anthocyanins diglucoside in large quantities is specific to certain species like *Vitis riparia* and *Vitis rupestris* (Ribéreau-Gayon et al, 2000b).

It has been demonstrated that, in acidic or neutral medium, four different anthocyanin structures exist in equilibrium: the flavylium cation (red), the quinoidal base (blue), the carbinol pseudo-base (colourless), and the chalcone (colourless or yellow) (Brouillard and Dubois, 1977). The amount of the anthocyanins present in red grape varies greatly, depending highly on the variety of grape and also on other factors such as: clone, maturity, seasonal conditions, production area, yield, etc. During the winemaking process, anthocyanins are involved in oxidation, hydrolysis and condensation reactions that are responsible for important wine colour changes.

Flavan-3-ols and Proanthocyanidins

Flavan-3-ols of flavanols are found in the solid parts of the berry (seed, skin and stem) in monomeric, oligomeric or polymeric forms; the latter two forms are also known as proanthocyanidins or condensed tannins.

*Monomeric Units*

The flavan-3-ol monomeric units found in *Vitis vinifera* grapes are (+)-catehin, (–)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin (Su and Singelton, 1969). In relation to the dihydroxylated forms, (-)-epicatechin can be esterified by gallic acid at C-3 position, resulting in (-)-epicatehin-3-*O*-gallate. They are responsible for bitterness in wine and may also have some associated astringency (Kennedy et al., 2006).

*Proanthocyanidins or Condensed Tannins*

Proanthocyanidins are a class of compounds that has been variously described as anthocyanogens, leucoanthocyanidins, flavan-3,4-diols, condensed tannins and tannins

(Kennedy et al., 2006). They possess the property of liberating anthocyanidins under heated acidic conditions as a result of the interflavanic bond cleavage (Porter et al., 1986). In *Vitis vinifera* grapes, two groups of proanthocyanidins depending on the nature of the liberated anthocyanidin (cyanidin or delphinidin) are distinguished: procyanidins, which are proanthocyanidins composed of (+)-catechin and (-)-epicatechin and prodelphinidins, proanthocyanidins composed of (+)-gallocatechin and (-)-epigallocatechin. Grape seeds have only procyanidins whereas skins posses both procyanidins and prodelphinidins.

Proanthocyanidins are also distinguished by their chain length and by the nature of the interflavanic bond. In relation to the chain length, the term 'oligomer' refers to the molecule corresponding to proanthocyanidins with a mean degree of polymerization (mDP) between 2 and 5 units. The term 'polymer' refers to those molecules with mDP > 5 units that can not be resolved or separated due to the high number of possible isomers (Waterhouse et al., 2000).

Numerous B-type oligomeric procyanidins, including dimmers (B1: epicatechin (4β→8)-catechin; B2: epicatechin (4β→8)-epicatechin; B3: catechin (4α→8)-catechin; B4: catechin (4α→8)-epicatechin;…), trimers, and tetramers consisting of (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-*O*-gallate units, have been identified in the *Vitis vinifera* seeds.

Polymeric proanthocyanidins represent the largest proportion of the total flavan-3-ol content in the different parts of the grape. The quantity, structure, and degree of polymerization of grape polymeric proanthocyanidins differ depending on their localization in the different plant tissues (Monagas et al., 2005).

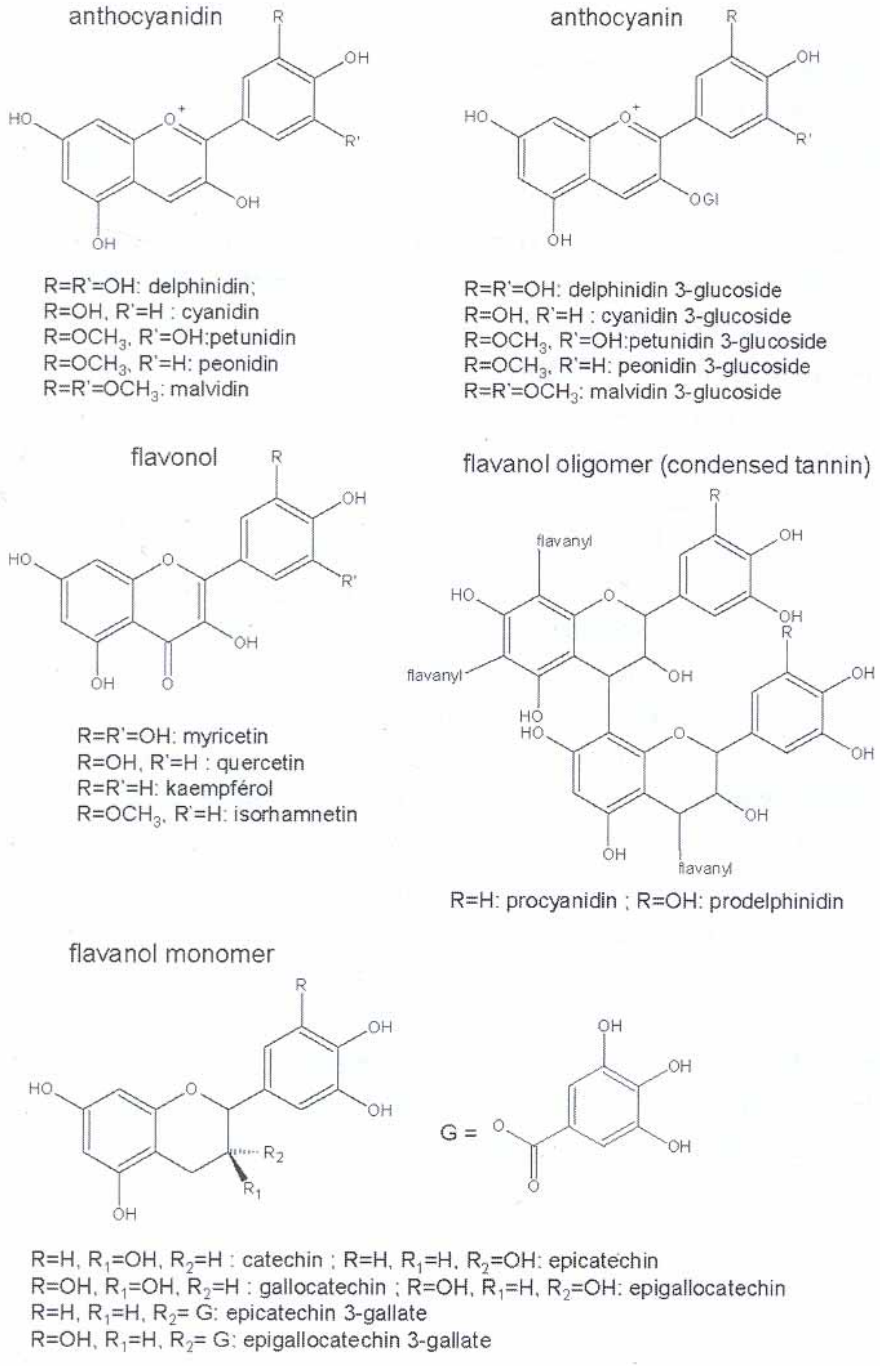



Figure 3: Structure of important flavonoids in grapes and wines (Cheynier et al., 2006: 299)

### 2.3.2 Ascorbic acid

Ascorbic acid content in grapes is low compared with other fruits, ranging from 5 to 150 mg/kg of fruit (Zoecklein et al., 1999). It is monobasic acid with lactone ring formation occurring between carbons 1 and 4. Due to asymmetrism at C4 and C5, four stereoisomers may occur: D- and L-ascorbic, D-isoascorbic (erythorbic acid), and D-erythro-3-keto-hexuronic acids. Among these, D- and L-ascorbic and erythorbic acids are of interest to winemakers.

Ascorbic acid, unless added in large amount or in later stages, is usually oxidized by the end of must preparation (Singleton, 1987). This does not seem to be due to ascorbic acid oxidase or non-enzymatic catechin oxidation, but results rather from enzymatic oxidation of phenols followed immediately by the reduction by ascorbic acid (with its consequent oxidation) of the quinones back to the phenols. The reaction of ascorbic acid and oxygen generates hydrogen peroxide, which can, through coupled oxidation with ethanol, produce acetaldehyde. The latter binds with the free $SO_2$, making it unavailable as an antioxidant. Bradshaw et al. (2003) showed that hydrogen peroxide did not induce (+)-catechin browning to the extent observed for ascorbic acid in conditions similar to that of white wine. Although dehydroascorbic acid is generally referred to as the major oxidation product derived from ascorbic acid, other additional products reported under wine-like conditions include acetaldehyde, 2,3-diketo-L-gulonic acid, L-threonic acid, oxalic acid, L-threo-2-pentulosonic acid, 4,5,5,6-tetrahydroxy-2,3-diketohexanoic acid and furfural (Bradshaw et al., 2003). However, it is difficult to see how these compounds can be directly involved in the browning reaction of (+)-catechin. Niemela (1987) has also demonstrated that the aerobic oxidation of ascorbic acid, in alkaline conditions, can lead to the formation of up to 7 major products, with more than 50 compounds being observed in total. Dehydroascorbic acid is just one of the initial oxidation products. Although there is a lack of certainty as to the nature of the ascorbic acid degradation products, it does appear that most are smaller than ascorbic acid and that those containing an aldehyde functional group could be involved in bridging phenolic compounds, similar to the established acetaldehyde bridging process (Scollary, 2002). Bradshaw et al. (2003) showed that after ascorbic acid addition to model wine solutions containing (+)-catechin the absorbance at 420 nm first decreased and than increased. This period prior to the absorbance increase

was named as the 'lag period'. When adding pre-oxidized ascorbic acid, browning commences immediately; this shows that oxidation products of ascorbic acid can increase the browning. There was also no lag period observed compared to the fresh ascorbic acid addition. It also confirmed the critical role of molecular oxygen concentration in the initiation of the browning process. Peng et al. (1998) also showed that oxidative browning under accelerated conditions was enhanced by ascorbic acid, but reduced by $SO_2$. They also showed that wine with ascorbic acid added at bottling showed a slightly faster browning than the wine without that addition. The $SO_2$ consumption in the wine with ascorbic acid was about twice as fast as in the wine without ascorbic acid. $SO_2$ is consumed rapidly at the beginning of the reaction process and protects the ascorbic acid from breaking down itself until all sulphur dioxide is consumed. Following the loss of sulphur dioxide, ascorbic acid degraded and (+)-catechin oxidation ensued (Scollary, 2002). In model wine matrix the 'cross-over' of ascorbic acid from antioxidant to pro-oxidant occurs when all ascorbic acid has been oxidized (Bradshaw et al., 2003). Flanzy (1959) indicated that wines with added ascorbic acid had lighter colour at the beginning of storage and darker colour some time later. Skouroumounis et al. (2005) also showed that wines were less oxidized after storage for two to five years when ascorbic acid was added at bottling. According to Scollary (2002) the concentration of ascorbic acid must be sufficiently high to ensure that it does not break down over the life of the wine. This concentration must take into consideration all factors including random oxygen ingress that might occur when the wine is in the bottle.

Oliveira et al. (2002) found that 1 mmol of oxygen in wine oxidize 0.84 mmol of ascorbic acid or 1 mg/L of oxygen will oxidize approximately 11 mg/L of ascorbic acid. They also found the antioxidant power of ascorbic acid to be much higher than that of $SO_2$.

### 2.3.3 Sulphur dioxide

In its several commercially available forms, $SO_2$ is used in wine as a chemical antioxidant and inhibitor of microbial activity. In wine it occurs in two forms, bound (or fixed) and free, their sum equalling total $SO_2$. At the pH of wine, the dominant free forms of $SO_2$ are the molecular and the bisulphite. In grape must, added sulphur dioxide acts in different ways to inactivate the mechanism of flavonoid precipitation in must. It inhibits and

destroys tyrosinase; the total activity decrease is 75% to 90% when 50 mg/L $SO_2$ is added (Dubernet and Ribéreau-Gayon, 1974). In addition, sulphur dioxide can suppress nonenzymatic oxidative reactions. This may result from sulphites acting reductively, by converting oxidation products back to their reduced forms, for example, caftaric acid quinone, primary oxidation product of PPO and directly responsible for flavonoid oxidation is reduced back by $SO_2$ (Jackson, 1994). At wine pH, the reaction of oxygen with $SO_2$, as its sulphite ion, is very slow and essentially irrelevant; however, one of the most important effects of $SO_2$ in wine is to react with hydrogen peroxide, a by-product of the antioxidant action of ascorbic acid (Waterhouse and Laurie, 2006). In white wine, sulphur dioxide bleaches brown pigments, causing the wine to develop a pale colour (Jackson, 1994). However, the same action can result in undesirable colour loss in red wines. By destroying the hydrogen peroxide produced during the autooxidation of phenols, the $SO_2$ limits the formation of acetaldehyde and the generation of colour stabilizing anthocyanin-tannin polymers.

### 2.3.4 Glutathione

Glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine) is the most abundant non-protein thiol compound widely present in living organisms, from prokaryotes to eukaryotes (Rollini and Manzoni, 2006) This cysteine-containing tripeptide, composed of glutamic acid, cysteine and glycine, exists either in reduced - GSH (Figure 4) or oxidized - GSSG form and participates in redox reactions by the reversible oxidation of its active thiol. It is a critical factor in protecting organisms against toxicity and diseases and its depletion is linked to a number of disease states including cancer, neurodegenerative and cardiovascular diseases (Masella et al., 2005).

In plants it is involved in a redox state, maintained in a reduced form by the ascorbate-glutathione cycle in which ascorbate, glutathione, dehydroascorbate reductase (DHARD), glutathione reductase (GSR) and ascorbate peroxidase participate (Okuda and Yokotsuka, 1999). Glutathione is also involved in maintaining ascorbic acid in its reduced form (Anderson, 1998).

It can be also very important in the interaction of plants with their environment, where it serves as the precursor for phytochelatins, binding toxic heavy metals such as cadmium

(Grill et al., 1989). It plays a big role also in the detoxification of organic xenobiotics, through its role as a substrate for glutathione-S-transferase. In grape juice it plays a specific role in enzymatic oxidation and browning of white juice (Singleton et al., 1985) discussed in details in subsequent chapters. Glutathione is thus considered to be a powerful, versatile and important self-defence molecule.

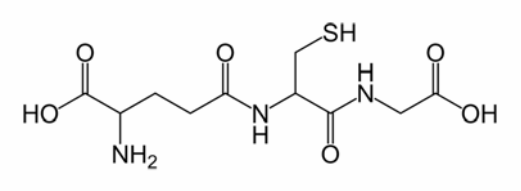



Figure 4: Structural formula of glutathione (Camera and Picardo, 2002:185)

In addition, GSH is of interest in the food additive industry and sports nutrition (Rollini and Manzoni, 2006). It is also of increasing interest in medical treatment and health care.

The glutathione content varies from 17 to 114 mg/kg in grapes and from 14 to 102 mg/L in musts (Cheynier et al., 1989a). Okuda and Yokotsuka (1999) reports that the reduced glutathione concentration in six varieties of grapes harvested between 18° and 20°Brix (commercial maturity) ranged from 51.2 to 83.8 nmol/g fresh weight, which is 15.7 to 25.7 mg/kg.

During ripening, the glutathione content increased with increasing amounts of soluble solids in both Koshu and Cabernet Sauvignon cultivars (Okuda and Yokotsuka, 1999). The correlation between °Brix and GSH content was high ($r = 0.914$, $p = 0.001$) for Koshu, but lower ($r = 0.814$, $p<0.05$) for Cabernet Sauvignon. Adams and Liyanage (1993) showed that close correlation between GSH and soluble solids only persists until berries reach about

16 °Brix. Thereafter little change in the GSH content occurs whereas the soluble solids continue to accumulate.

In red varieties the GSH level increases along with skin coloration, which is coincident with the onset of soluble solids accumulation (Adams and Liyanage, 1993). Dubourdieu (2006) also showed that water supply and nitrogen based fertilization to the vine seems to affect the accumulation of glutathione in the grapes. A moderate water deficit is more favorable to glutathione accumulation than severe stress water.

Peyrot et al. (2002) identified *S*-3-(hexan-1-ol)-glutathione in Sauvignon Blanc as a possible precursor for *S*-3-(hexan-1-ol)-L-cysteine and further 3-mercaptohexan-1-ol, a varietal aroma compound of Sauvignon Blanc.

Glutathione plays an important role in yeast metabolism (Penninckx, 2000). A GSH cycle in the yeast plays a leading role in the regulation of the sulphur fluxes and is closely integrated into the yeast sulphur metabolism (Penninckx, 2002). It might be also a precursor of $H_2S$ or mercaptans, because it is abundant in dry yeast and it is also formed during fermentation. One of its components, cysteine, can be converted to $H_2S$ and further it is possible that the strong reducing agent GSH could reduce elementary sulphur to $H_2S$ (Park et al., 2000b). Park et al. (2000a) also showed that glutathione steadily increased towards the end of fermentation and the final wine concentration ranging from 0.1 to 5.1 mg/L was correlated with both total nitrogen ($p<0.01$) and assimilable amino acid content in juice.

Park et al. (2000a) showed that glutathione concentration increased during the fermentation in two studied musts. Its concentration was found to be 5.1 mg/L in Palomino wine and 2.1 mg/L in Sauvignon Blanc wine.

## 2.4 OXYGEN IN MUST

### 2.4.1 Oxygen uptake in wine musts

During the crushing, pressing and other processing steps, $O_2$ comes into contact with the grape must. Oxygen uptake in musts is related to many factors, including polyphenol content, polyphenoloxidase enzyme content and its activity, pH, temperature, etc. White and Ough (1973) showed that rising the temperature from 25 °C to 32 °C increased the oxygen uptake rate more than 10 fold and cooling the must from 25 °C to 0 °C nearly halved the rate. Added sulphur dioxide slows oxygen consumption and the concentration of 25 mg/L to 100 mg/L $SO_2$ inhibits the polyphenoloxidase (White and Ough, 1973). On the other hand, other authors report that approximately 500 mg/L of free $SO_2$ was able to completely inhibit phenol oxidation when oxygen was absorbed (Schneider, 1998). Various other must treatments also affect the rate of oxygen uptake. Two minutes at 80 °C denatures the enzymes and completely inhibits oxygen uptake (White and Ough, 1973).

Dubernet and Ribéreau-Gayon (1974) report that the rate of oxygen consumption in must is very variable and in 35 musts it varied from 0.5 to 4.6 mg $O_2$/L/min, with the average value 2 mg $O_2$/L/min. In such case, one saturation with oxygen at 25°C or 8 mg/L can be consumed in 4 minutes. The rate of oxygen consumption in must is three times faster at 30°C than at 10°C and decreases at temperature above 40°C because of polyphenoloxidase enzyme inactivation (Dubernet and Ribéreau-Gayon, 1974). The consumption of $O_2$ by tyrosynase is very fast, ranging from 30 to 200 mg/L, with 10-15 mg/L being taken up during whole bunch crushing (Cheynier et al., 1993). The uptake is also faster initially, but decreases as the phenolic substrate is depleted. Laccase, an enzyme present in grapes infected with *Botrytis*, increases the total oxygen uptake (Cheynier et al., 1993). In the absence of sulfiting, the depletion of oxygen is very rapid and is complete within minutes (4 to 20 on average) (Ribéreau-Gayon et al., 2000a). Addition of $SO_2$ in the must stops the oxygen consumption (Dubernet and Ribéreau-Gayon et al., 1974).

### 2.4.2 Enzymatic oxidation

The oxidation of must is an enzymatically catalyzed reaction (Schneider, 1998). An important role in the oxidation reactions is performed by two types of enzyme group: (1) polyphenoloxidases (PPO) (cateholoxidases, tyrosinase, phenolase, cresolase, and *o*-diphenoloxidase) and (2) laccase, which can be found in grapes infected with *Botrytis cinerea*. *Botrytis cinerea* laccase attacks a wider range of substrates than PPO, which also includes Grape reaction product (GRP) (Salgues et al., 1986). In the presence of air oxygen, hydroxycinnamates are both oxidized by grape polyphenoloxidase to caftaric acid *o*-quinone. The later can undergo coupled oxidations in which other phenolic compounds are oxidized to the corresponding quinones, while the caftaric acid quinones are reduced back to caftaric acid, which is again oxidizable by the enzyme. The quinones generated by enzymatic and coupled oxidations polymerize to brown pigments (Sapis et al., 1983).

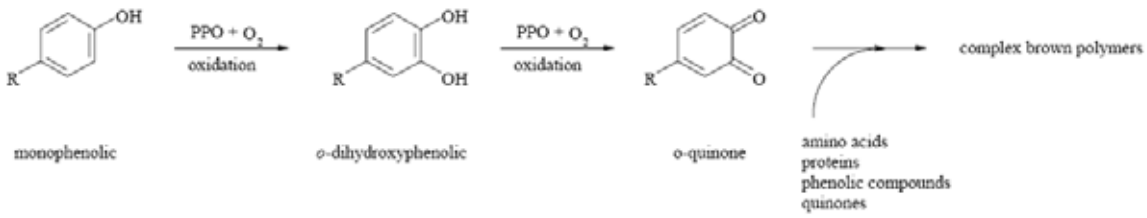



Figure 5: Enzymatic oxidation of phenolic compound (Scollary, 2002: 7)

Caffeoyltartaric (caftaric) acid and *p*-coumaroyltartaric (coutaric) acid are the major phenols and substrates for enzymatic oxidation in white musts (Singleton et al., 1985). The typical *Vitis vinifera* wine grape has about 145 mg/L of *trans*-caftaric acid in the juice protected by oxygen and about 15 mg/L of *trans*-coutaric acid (Singleton, 1987).

Glutathione interferes with oxidation mechanism by trapping the caftaric acid quinones in the form of 2-*S*-glutathionyl caftaric acid (Figure 6) also referred to as grape reaction product (Singleton et al., 1985). This reaction has several important consequences. First, it regenerates a phenolic species from the quinone, which then has the capacity to absorb another equivalent of oxidation. Second, the colourless catechol product of this reaction is not a substrate for enzymatic oxidation, thus this reaction captured oxidation in a product that had no browning potential. Thus the formation of GRP is thus believed to limit must browning (Cheynier et al., 1989b). Singleton et al. (1985) studied the reaction of this

quinone with several other thiols. Nearly all reacted, and the reaction was not reversible under wine conditions. Another reaction of thiols with quinones was reported by Blanchard et al. (2004), who showed that when catechin is oxidized, it can react with 3-mercaptohexanol, an important factor in the fruity aroma of Cabernet Sauvignon, Merlot and Cabernet Franc. The result was a loss of fruity varietal character.

2,5-di-*S*-glutathionylcaftaric acid (GRP2) can also be formed in the presence of glutathione. If sufficient glutathione is available, the formation of GRP2 seems to be an efficient way of limiting the browning (Salgues et al., 1986). Browning is highly correlated to flavanols. Based on enzymatic oxidation of individual phenolic compounds at equal molar concentrations, catechin, epicatechin, procyanidin B2, and procyanidin B3 have a browning potential about 10-fold higher than hydroxycinnamic acid derivates (Lee and Jaworski, 1988). Cheynier and Ricardo da Silva (1991) found that polyphenoloxidase did not degrade proanthocyanidins alone, but in the presence of caftaric acid, the oxidative condensation of non-galloylated procyanidins preceded more quickly than the non-oxidative condensation of non-galloylated procyanidins.

In the first stage of oxidation, caftaric acid is converted to quinone. This primary oxidation product is, owing to its high concentration and reactivity, at the origin of three further nonenzymatic reactions (Cheynier et al., 1990).

- It combines with glutathione to a colourless compound initially called Grape Reaction Product - GRP.
- After glutathione depletion, the excess caftaric acid quinone can oxidize other must constituents, including GRP and flavanols, and be simultaneously reduced back to caftaric acid. The partial regeneration of caftaric acid enables its re-oxidation by PPO and further oxygen consumption.
- It polymerizes with its own precursor, caftaric acid, regenerating the original phenol form, which is able to be re-oxidized.

All three reactions are interdependent. Particularly, trapping of caftaric acid quinone by glutathione or reduction with sulfite limits the other reactions (Figure 7).

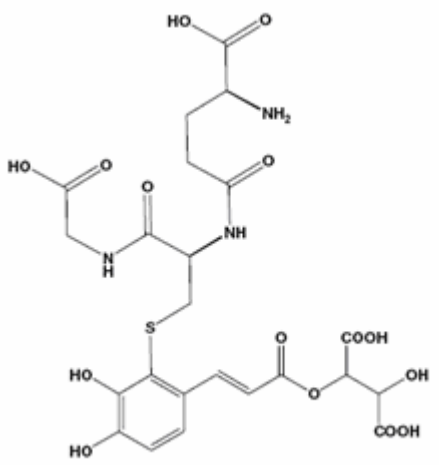



Figure 6: Chemical structure of 2-*S*-glutathionylcaftaric acid – GRP (Cheynier et al., 1986: 219)

The ratio between hydroxycinnamates (HCA) and glutathione in grape juice can determine the amount and fate of GRP, which can therefore influence the degree of browning (Cheynier et al., 1989a). The must samples can be separated into three classes (A, B, C), namely light coloured oxidized musts with low hydroxycinnamic acid concentration, intermediate musts and dark oxidized musts with high hydroxycinnamic acid concentration (Cheynier et al., 1989a). When the HCA/glutathione molar ratio is below 1 (class A), all the CTA quinones formed are expected to be trapped as GRP, and therefore prevented from proceeding to brown polymers in must containing excess glutathione. On the other hand when, hydroxycinnamic acid to glutathione ratios is between 1.1 and 3.6 (class B). GRP concentration reached a maximum after a few minutes and then decreased steadily. Following glutathione depletion, caftaric acid- and GRP-*o*-quinones first accumulated and then reacted further to condensation products. Small amounts of GRP2 are formed but rapidly disappear from the solution, presumably by coupled oxidation.

In the class C the maximum levels of GRP and GRP-*o*-quinone were much lower and that of caftaric *o*-quinone higher. No GRP2 could be detected, meaning that glutathione was depleted before GRP oxidation started as would be expected given the large hydroxycinnamic acid to glutathione ratios (3.8 to 5.9) found in these musts.

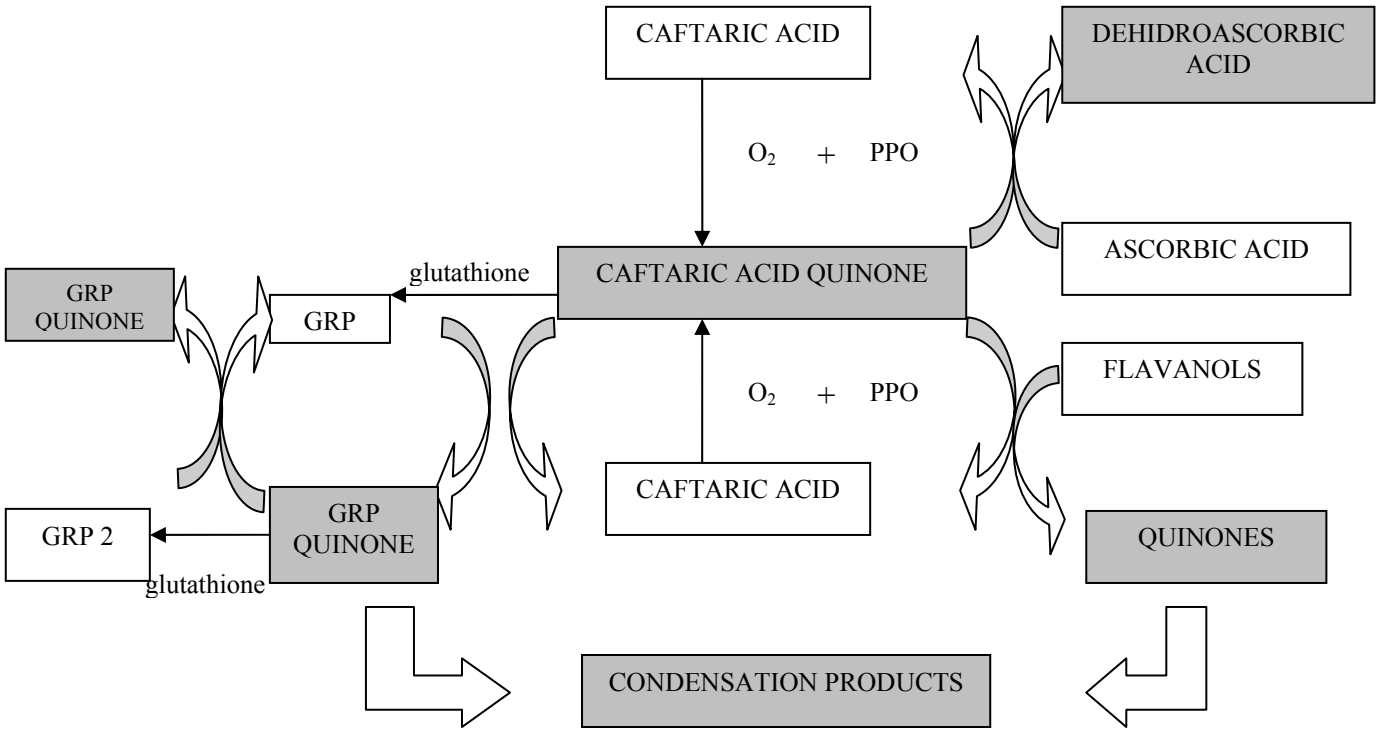



Figure 7: Proposed reaction mechanism for the coupled oxidation of *trans*-caftaric acid and GRP (Silva et al., 1999: 6)

## 2.5 OXYGEN IN ALCOHOLIC FERMENTATION

During alcoholic fermentations, air or oxygen diffusion is an accepted and legal practice. When sluggish fermentation is suspected, oxygen is generally added to improve the biomass synthesis, which increases the fermentation rate (Salmon, 2005). Under anaerobic conditions, yeast growth normally requires added oxygen to synthesize lipids (sterols and unsaturated fatty acids). These lipids are incorporated into cell wall, enhancing ethanol tolerance of the yeast. A recent study showed that lipid synthesis and optimal growth of *Saccharomyces cerevisiae* during alcoholic fermentation required about 5-7.5 mg/L of oxygen (Rosenfeld et al., 2003). Valero et al. (2002) showed that must oxygenation (up to saturation) increased total esters and higher alcohol concentration, but the ratio between Σ esters/Σ higher alcohols is lower in the fermentation with oxygenation, which does not lead to improved sensory profile for the wines. Mauricio et al. (1997) report that short aeration after 2 days of fermentation led to an immediate (6 h) increase in the concentrations of acetates in the wine, probably as a result of partial stimulation of cellular metabolism and growth. Aerating the must also stimulated the production of ethanol, 1-propanol, isoamyl alcohol and isobutyl alcohol. Blateyron et al. (2003) found that the addition of 5 mg/L $O_2$ to fermenting must did not affect the sensory characteristics of the wine compared to the control, but the addition of an excess (50 mg/L) did decrease the quality, with an increase in brown color. Furthermore, proline, not assimilable under anaerobic conditions, can be used as a supplementary nitrogen source in the presence of molecular oxygen (Ribéreau-Gayon et al., 2000a; Ingledew and Kunkee, 1985).

Sablayrolles (1990) reports that during fermentation yeast can consume between 15 and 20 mg/L of oxygen. Oxygen has higher effective on yeast growth, if the addition is made at the end of growing phase or at ¼ of fermentation; however the addition is effective until ½ of fermentation. Yeast lees can consume oxygen during the ageing of wine. Yeast cells and yeast lees (Rosenfeld et al., 2003) both have much higher potential oxygen consumption rates and affinities for oxygen than wine polyphenols.

Oxygen during malolactic fermentation can also influence the sensory characteristics of wine. Oxygen enhances the conversion of α-acetoacetate to diacetyl, a major flavour metabolite produced by lactic acid bacteria (Bartowsky and Henschke, 2004).

## 2.6 OXYGEN AND WINE

Oxygen has a major effect on the oxidation-reduction potential ($E_H$). Differences of 150-250 mV have been observed at oxygen levels ranging from 1 to 6 mg/L.

Table 1: Effect of oxygen content on the oxidation-reduction potential of a red wine (Vivas and Glories, 1996a: 12)

| $O_2$ (mg/l) | $E_H$ (mV) | $\Delta E_H$ (mV) |
|---|---|---|
| 0.1 | 263 | |
| 0.8 | 280 | 17 |
| 2.5 | 340 | 77 |
| 4.8 | 424 | 161 |
| 5.0 | 434 | 171 |

Varying compounds has a different influence on the oxidation-reduction potential. Ethanol increases the instantaneous oxidation rate and slightly reduces the potential, on the other hand, tartaric, malic and lactic acids produce only a few minor modifications. Glycerol has no effect on oxidation mechanisms (Ribéreau-Gayon et al., 2000b). Phenols, on the other hand, inhibit variations in potential. Anthocyanins, in particular, consume oxygen rapidly, leading to a rapid drop in potential (Ribereau-Gayon et al., 2000b). Catechins and oligomeric procyanidins are more active than polymers. Furthermore, temperature causes wide variations in the oxidation-reduction potential (100 mV between 0 and 30 °C) in proportion to the quantity of dissolved oxygen. Additionally, ellagitannins increase the redox potential (Ribéreau-Gayon et al., 2000b). Finally, the types of containers used for ageing and storing have an influence on the oxidative process, depending on their permeability to air (Ribéreau-Gayon et al., 2000b). Racking of wine increases the redox potential from 30 to 150 mV; however after 8-10 days the level of redox potential decreased to its initial values (Vivas and Glories, 1996a).

### 2.6.1 Factors affecting oxygen dissolution in wine

Wine that is stirred with air becomes saturated with $O_2$ in approximately 30 seconds (Ribéreau-Gayon et al., 2000b). So any operation involving air contact, such as wine transfer or stirring, rapidly induces oxygen dissolution. In storage tanks or barrels, the level of dissolved oxygen is extremely low and usually between 0.02 and 0.05 mg/L. Oxygen in

wine is distributed in layers, reaching levels close to saturation at the interface with the tank headspace and down to almost zero oxygen below the first 10 to 20 cm of wine surface (Waterhouse and Laurie, 2006; Cheynier et al., 2002). This distribution depends on several factors, including the shape of the tank and the wine's variation in temperature.

Table 2: Dissolved oxygen concentration in red wine stored in a 2700 L tank (Cheynier et al., 2002: 23)

| Distance to wine surface (cm) | Dissolved oxygen, concentration (mg/L) |
|---|---|
| 0 | 8.4 |
| 2 | 1.0 |
| 5 | 0.1 |
| 10 | 0.02 |

From the end of the alcoholic fermentation, wine is exposed to many enological operations that can increase the concentration of dissolved oxygen. The effect of single enological processes on the level of dissolved oxygen in wines can be classified as 'high enrichment' and 'low enrichment' treatments (Figures 8 and 9) (Castellari et al. 2004). According to Castellari et al. (2004), racking increases the level of oxygen in wines with a median value of 0.370 – 0.375 mg/L. When wine temperature was bellow 10 °C, even if care was exerted in transferring the wines from tank to tank (bottom to bottom), the dissolved oxygen increases by a 3-fold factor, with a median value 1.290 mg/L (Figure 8, number 3).
Vidal et al. (2001) reports that wine transfer with different pumping systems has not a big influence on oxygen dissolution in red wines. Its concentration depends mostly on operating condition (T, air contact...) during wine transferring.

Kieselguhr filtration

Filtration is not a critical operation in comparison with wine aeration, since the median increase in oxygen was always less than 300 µg/L (Figure 9, numbers 7-10). Like other operations, the highest oxygen absorption occurs at the beginning of kieselguhr filtration. According to Vidal et al. (2001), the concentration of dissolved oxygen at the beginning of kieselguhr filtration (in the first 15 minutes) can be between 2 and 4 mg/L, which could be due to microporous structure of kieselguhr that contains a lot of air.

Tangential microfiltration and centrifugation

Vidal et al. (2001) report that oxygen enrichment during tangential microfiltration can vary from 0.6 mg/L to 2.2 mg/L. The use of centrifuges (Figure 8, number 11), by contrast, may cause significant wine oxidation (median value of 1.200 mg/L) (Castellari et al. 2004). These values coincide with those obtained by Vidal et al. (2001) who found the average oxygen absorption for centrifugation to be 0.95 mg/L. It is commonly recognized that tartaric stabilization can produce great oxygen absorption due to very low temperature. Castellari et al. (2004) reports median increase of dissolved oxygen 1.265 mg/L during static tartrate stabilization. Continuous systems speeded up the process, but in many cases produced complete saturation of wine with oxygen (Figure 8, number 14). This data correlates with Vidal et al. (2001) who found an average oxygen absorption 2.38 mg/L during continuous tartrate stabilization. The critical point of tartaric stabilization is filtration, because lower temperature can increase oxygen absorption. This value can be reduced with nitrogen sparging at the exit of the filter unit. Therefore, the working conditions (inertage, temperature) can play a significant role in the oxygen uptake during wine stabilization.

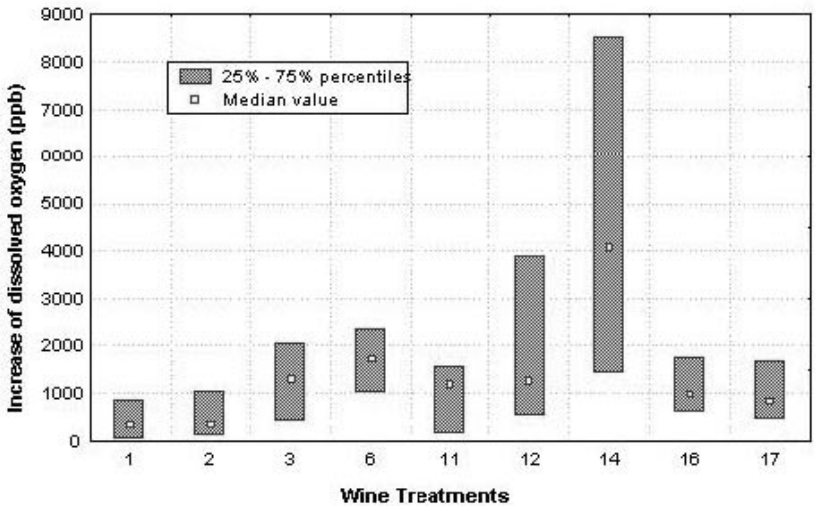



Figure 8: Increase of dissolved oxygen in wines after 'High Enrichment' treatments. 1-Racking (bottom-bottom, 15 - 20 °C); 2-Racking (bottom-up, 15 - 20 °C); 3-Racking (bottom-bottom 5 - 10 °C); 'barrique' assembling; 11-Centrifugation; 12-Refrigeration; 14-Continuous Tartaric Stabilization; 16-17 Filing glass bottles (Castellari et al., 2004: 391).

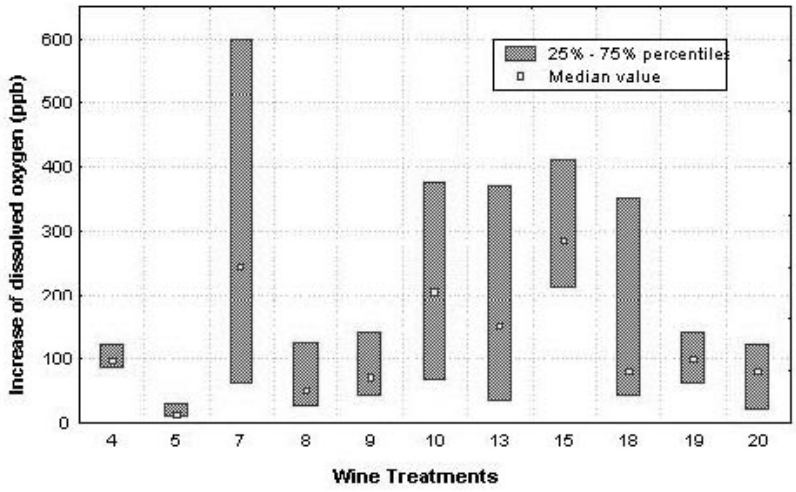



Figure 9: Increase of dissolved oxygen in wines after 'Low Enrichment' treatments. 4-Pumping; 5-'Barrique batonnage'; 7-10 Filtration; 13- Heat Exchange; 15- Electrodyalisis; 18-20 Filling (Castellari et al. 2004: 392).

Wine bottling

Filling of glass bottles remains a problem in the winemaking process. Different filling procedures produce a variable increase of dissolved oxygen in wines. Castellari et al. (2004) reports a median enrichment of dissolved oxygen 0.83 mg/L during this step. Nevertheless, this value can vary significantly, depending on the filling system and conditions during the wine bottling. Vidal et al. (2003b) report that the average global oxygen absorption measured during 19 static bottling lines was 1.6 mg/L, but varying from 0.23 to 3.87 mg/L (Table 3). In fact, there are many parameters that can influence oxygen uptake during wine bottling: the use of inert gas during wine transfer; the length and diameter of tubes; the number of pumps; the topography of filling machine; the type of filling machine and corking system; the use of vacuum before filling, etc.

Beside this, the working conditions during bottling can also significantly contribute to dissolved oxygen. Wines with low oxygen concentration (< 0.5 mg/L) and low $CO_2$ concentration have higher affinity to oxygen dissolution due to the Fick's law (Vidal et al., 2003b). Wine temperature, number of machine arrests, level of wine in the tank and its protection with inert gas also contribute to the oxygen level in a bottle.

Table 3: Oxygen absorption during wine bottling (Vidal et al, 2003b: 36).

| **Operation** | **Volume of wine (hL)** | **Number of studied bottling** | **Oxygen absorption (mg/L)** | | |
|---|---|---|---|---|---|
| | | | Minimum | Average | Maximum |
| Filtration and stabilization | 2073 | 9 | 0.05 | 0.1-0.2 | 0.50 |
| Filling at fixed bottling machine | 2463 | 19 | 0.23 | 1.60 | 3.87 |
| Filling at mobile bottling machine | 479 | 6 | 0.63 | 1.43 | 2.00 |
| Bag-in-box (conditioning) | 432 | 2 | 0.09 | 0.42 | 0.74 |

In particular, the amount of oxygen dissolved during bottle filling depends on the technology used. It is useful that the first and the last 500 bottles (the first and the last palette) of bottled wine are separated as this wine contains higher values of oxygen.

It is critical to consider the headspace volume between wine and cork and system of bottling to reduce the oxygen content in headspace. Usually there is 8 mL of volume, which at normal conditions (T = 0 °C, p = 1,013 bar, R ($O_2$) = 0,0446 mol/L) brings: 8 mL x 1.43 mg/mL $O_2$ x 0.209 = 2.4 mg/750 mL wine or 3.2 mg/L oxygen disposable for consumption.

Oxygen and bottle closures

The type of closure can play a critical role during the ageing of wine. Many reports of oxidation of commercial bottled white wines under conditions of cellar storage have been reported (Waters et al., 1996). Waters et al. (1996) examined the permeability of two different cork types and storage in the atmosphere of either nitrogen, oxygen or air by comparing the level of browning, concentration of free and total sulphur dioxide and ascorbic and erythorbic acids in wines. Corks stored for 8 months under oxygen and air had a higher on the browning level and showed a greater loss of the antioxidants, indicating that corks were permeable to oxygen. There was no significant difference in the loss of ascorbic and erythorbic acid or the level of browning between bottled wines stored under different inert gasses.

It is generally presumed that corks are permeable to oxygen and it has been estimated that, over one year, several millilitres of oxygen could enter a wine via this route (Ribéreau-Gayon et al., 2000b). However, the oxygen diffusion kinetics depends on the type of closures. Lopes et al. (2005) studied the kinetics of oxygen diffusion through different

closure types available on the market including natural cork stoppers, technical cork stoppers, synthetic stoppers and a glass stopper as control. They found that technical cork closures (agglomerate, twin top) exhibited low oxygen permeability (below 3 mg/L after 365 days), synthetic closures showed substantial oxygen permeability and reached the limit of oxygen quantification (9.8 mg/L) within 140 days of storage. The natural cork closures displayed lots of variation with regard to oxygen diffusion and ranged from 8.3 mg/L to 5.9 mg/L over 365 days of storage. Their results also showed no significant correlation between the amount of oxygen and dimensions, the coefficient of porosity or moisture content of the natural cork. The rates of oxygen diffusion through different closures were greater in the first month of storage. The majority of closures displayed an oxygen diffusion rate between 2 and 4 mg/L/month during this period of time. However, in the following months (between 2 and 12 months) diffusion was extremely variable ranging from 0.24 mg/L/month to 0.50 mg/L/month for natural corks and 1 mg/L/month for synthetic closures.

Godden et al. (2001) showed that the $SO_2$ loss in white wines was most evident for synthetic closures. The $SO_2$ losses were intermediate for the natural cork stoppers the least evident for the technical cork closures. Francis et al. (2003) showed that wine bottled mostly with synthetic closures exhibited undesirably high oxidized aroma in comparison to other closures after a period of 24 months. This was also reflected in the colour of the wines, with wines closed with synthetic corks generally sooner becoming browner. These wines also had an oxidised aroma and lost a high percentage of fruity character.

The exact method of $O_2$ diffusion into the bottles is unknown, but it could mean that $O_2$ inside the corks initially permeates into wine, with atmospheric $O_2$ permeating later. This may explain the high initial rate of $O_2$ diffusion in the first month (Lopes et al., 2005). However, oxygen is not the only factor affecting oxidative spoilage in bottled wines. The causative agent of oxidation in the corks could have been hydrogene peroxide or chloride residues, since corks are washed with solutions containing these oxidants during processing (Waters, 1996). Buiatti et al. (1997) studied the preservation of red and white wines in containers other than glass (PET, wine boxes, multilayer cartons) in comparison to the glass bottle ageing. After 24 months of ageing they found a significantly higher concentration of dissolved oxygen in white wines packed in a bag-in-box compared to glass bottles. Sensory preference test found no significant differences between containers.

Ough (1987) suggests that wine stored in PET bottles was as effective in the wine preservation as glass up to 10 to 12 month, but the concentration of $SO_2$ was lower compared to glass bottles.

Oxygen exchange of wines stored in barrels

Wines stored in wooden barrels of small volume had twice as much of dissolved oxygen as those matured in stainless steel tanks, indicating that storage in wood barrels induced a moderate oxygenation during wine maturation. The oxygen transfer in barrique barrel is estimated between 2 and 5 mL/L/year in the situation of permanent topping up whereas in barrels with an airtight bung it is 15 to 50 mL/L/year (Moutounet et al., 1998). This data can be explained by the existence of negative pressure inside the barrel which favours gas entry. The amount of dissolved oxygen in wines matured in new barrels is higher than in used barrels, because a prolonged use causes a progressive colmatation of wood pores and a consequent decrease in the wine oxygen content. Moutounet and Mazauric (2001) report that dissolved oxygen in wines, matured in barriques, ranged between 20 and 50 μg/L. After the barrel has been used three to five times, the quantity of dissolved oxygen in wines will be very close to that of wines stored in tanks, so different wine colour characteristics will be obtained (Vivas and Glories, 1997). Barrel volume may also influence the oxidative process. The four kinds of maturation effects which are attributed to barrels (evaporation, extraction, oxidation and component reaction) would all be intensified by a greater wood surface in contact with a unit of beverage (Perez-Coello et al., 1999). Oxygen consumption by yeast lees has to be considered during white wine ageing for the following reasons: (i) barrel filling and simple opening of filled barrels are technological steps that strongly favour oxygen dissolution in the wine (up to 0.2 mg of $O_2$/L) (Moutounet et al., 1998); (ii) there are many examples of periodic stirring of the lees during wine ageing on lees, including repetitive additions of small amounts of oxygen (up to oxygen saturation).

## 2.7 OXYGEN IN WINE AGEING

### 2.7.1 Non-enzymatic oxidation

The main difference between enzymatic and non-enzymatic oxidation is in the way in which quinone is formed. It is similar to enzymatic oxidation, except that a metal ion is required in place of the enzyme (Scollary, 2002; Danilewicz, 2003). The direct interaction between molecular oxygen and organic molecules is 'spin forbidden' due to the arrangement of electrons in the oxygen molecule. Conversion of molecular oxygen from its lowest energy (ground) state to a higher energy (excited) state is required before a reaction can occur. As an *o*-dihydroxyphenol reacts with $O_2$ to produce its quinone, only one atom of oxygen is needed and the second appears as hydrogen peroxide (Singleton and Cilliers, 1995). Under acidic conditions, this hydrogen peroxide oxidizes additional substances, including ethanol in wines, which would otherwise not readily autooxidize. By contrast, under enzymatic oxidation, hydrogen peroxide is not produced (Singleton and Cilliers, 1995).

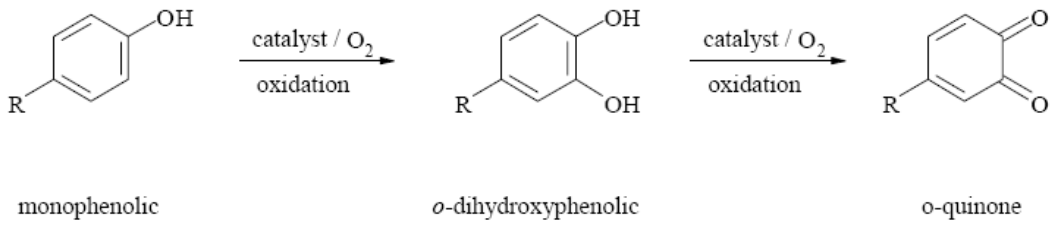



Figure 10: Non-enzymatic oxidation of a phenolic compound (Scollary, 2002: 8)

Due to the poor direct reactivity of oxygen with organic molecules, the oxidising potential of molecular oxygen is harnessed by the generation of reactive oxygen species (ROS) that constitute a reductive ladder of oxidation. The initial transfer of an electron leads to the formation of superoxide ion, $O_2^{\bullet -}$, which at wine pH exists as the hydroperoxide radical ($OOH^{\bullet}$). This step requires a catalyst, presumably a transition state metal such as iron (Waterhouse and Laurie, 2006). The transfer of a second electron would then produce a

peroxide, hydrogen peroxide ($H_2O_2$) being the specific form generated in wine. The next reduction creates an oxidative agent even more reactive than the previous one, namely the hydroxyl radical ($OH^{\bullet}$), via the Fenton reaction between hydrogen peroxide and ferrous iron salts (Figure 12). The last reaction produces water, the final product of oxygen reduction (Danilewicz, 2003).

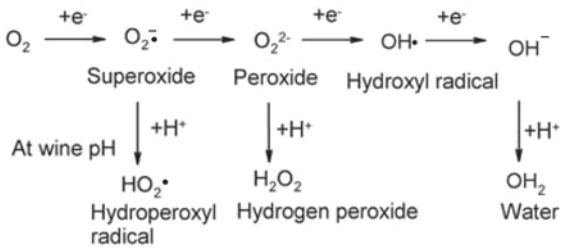



Figure 11: Ladder of oxygen reduction (Waterhouse and Laurie, 2006: 308)

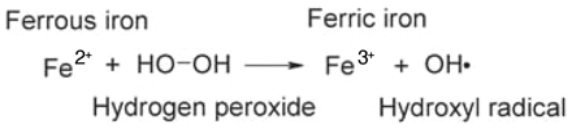



Figure 12: Fenton reaction (Waterhouse and Laurie, 2006: 308)

### 2.7.2 White wine

2.7.2.1. The influence of oxygen on white wine colour and aroma

The colour of white wine is one of the important quality parameters. The brown colour is normally unwanted, because it indicates the oxidation or spoilage of white wine.

In must the enzymatic oxidations are more significant than chemical oxidations because they are more rapid. In wine, however, chemical oxidations play an unquestionable role, since oxidative enzymes no longer exist (Ribéreau-Gayon et al., 2000b).

Simpson (1982), in his studies on 'browning' reactions (30 weeks at 15°C) and 'accelerated browning' reactions (3 weeks at 50°C), found a significant positive correlation between the total phenolic content and browning, but also reported that monomeric catechin-type compounds and dimeric procyanidin compounds are important indicators of browning susceptibility. Among the monomeric catechins, (–)-epicatechin was more positively correlated ($P < 0.001$) with both browning and accelerated browning than catechin ($P < 0.01$) (Simpson, 1982). The rate of autoxidation of phenolic compounds is

also pH dependent, and for some compounds it is nine times higher at pH 4 than at pH 3 (Singleton, 1987). The browning in white wine is a chemical process and can be due to three mechanisms. The first one is the oxidation of phenolic molecules to their corresponding quinones in varying degrees of polymerization which is also influenced by the copper and iron concentration (Lopez-Toledano et al., 2002). It produces a yellow-brown colouration (Es-Safi et al., 1999). The second mechanism is the oxidation of tartaric acid to glyoxylic acid, which leads to the condensation of phenolic molecules (Monagas et al., 2005). In white wine the model solutions containing (+)-catechin, tartaric acid is oxidized to glyoxylic acid (OHC-COOH) in the presence of iron traces, leading to the formation of both colourless and yellow compounds that exhibit a maximum absorption between 440 and 460 nm (Oszmianski et al., 1996). Similarly, the reaction can also be catalyzed by copper ions (Clark and Scollary, 2002; Es-Safi et al., 2003). The major colourless product has been identified in model solutions as a catechin dimer linked by a carboxy-methine bridge, resulting from the reaction between (+)-catechin and glyoxylic acid through a mechanism similar to that of the acetaldehyde-mediated condensation reaction (Fulcrand et al., 1997). The formation of yellow xanthylium compounds is associated with the disappearance of the colourless carboxy-methine-linked catechin dimers.

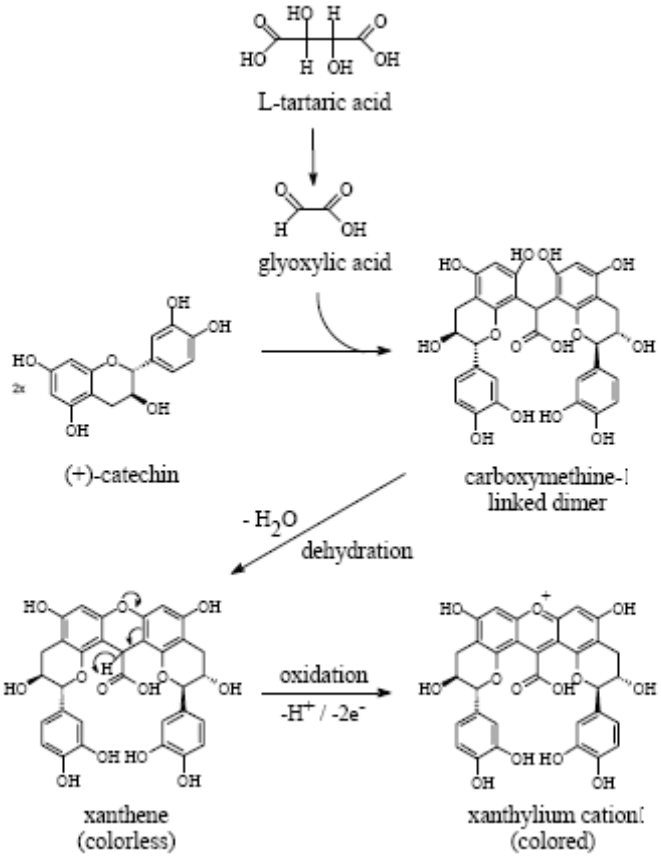



Figure 13: Four step reaction sequence leading to the formation of a xanthylium pigment from (+) catechin (Scollary, 2004: 14)

Cacho et al. (1995) studied the influence of iron, copper and manganese on wine oxidation. They found that manganese favours the increase of acetaldehyde and iron catalyzes the acetaldehyde combination with phenolic compounds. Copper does not seem to influence the acetaldehyde formation during the oxidation (Cacho et al. 1995).

Acetaldehyde, produced during the coupled oxidation or fermentation, is well-known to bridge catechin-type phenolic compounds to give a colourless ethyl-bridged dimer (Saucier et al., 1997a) similar to the carboxymethine-linked dimer formed as an intermediate compound in the production of xanthylium cations (Fulcrand et al., 1997).

Fernandez-Zurbano et al. (1995) found that the content of hydroxycinnamic acids in the wines poorly correlated with browning. They also found that the content of hydroxycinnamic acids and esters decreased significantly during the process; however, the decrease was seemingly unrelated to the extent of browning of the wine (Fernandez-

Zurbano et al., 1998). On the other hand, the flavanol contents of the wines were found to be correlated with the degree of browning at the end of the oxidation process. Cilliers and Singleton (1990) report that caffeic acid and derivatives are shown to be the major sources of brown products, because there was a significant correlation between the brown product and caffeic acid consumed in both model systems and wine containing other compounds that could potentially interfere.

Some white wines undergo a subtle colour change called 'pinking' after processing that makes the wine appear slightly or noticeably pink (Lamuela-Raventos et al., 2001). This problem, considered serious by many winemakers, appears after a wine has been stored and then exposed to small amounts of air or oxygen. The compound responsible for pinking has not been identified, but is thought to be a phenolic chromophore (Margalit, 2004). According to Jones (1989) the pink phenomenon can be caused by at least ten different compounds plus polymeric material. However, several of these possibilities appear to be unfeasible. There are, however, strong indications that the pink compound could be a derivative of GRP, the reaction product of glutathione with caftaric acid-*o*-quinone, which is generated by enzymatic oxidation of caffeoyl- and *p*-coumaroyl tartaric acids (Singleton et al., 1985). The grape reaction product survives intact after fermentation and is thus present at the time of pinking. It can form a chromophore following oxidation via peroxidase (Margalit, 2004).
Simpson (1977) used hydrogen peroxide to evaluate the pinking susceptibility in white wines and concluded that 75 mg/L is the best concentration to induce pinking. He found that PVPP could remove the pink materials and their precursors. An assay where $H_2O_2$ is added to wine has been developed to test for potential pinking (Lamuela-Raventos et al., 2001). Ascorbic acid at the level of 30 mg/L decreased pinking and at 45 mg/L prevented it completely. Higher temperature and light also increase the pinking rate (Lamuela-Raventos et al., 2001).

There are several phenomena related to the oxidative degradation of wine. One is colour degradation expressed through the browning and pinking process, another is flavour degradation which, however, has received less attention. The literature on this topic focuses mainly on acetaldehyde generation from the oxidation of ethanol (Wildenradt and

Singleton, 1974), but although acetaldehyde plays a role in wine oxidation, it does not appear to be responsible for the taste and flavour of spoiled wines (Marais and Pool, 1980, Escudero et al., 2002). Oxidation-spoiled white wines are related with descriptors such as 'honey-like', 'farm-feed', 'hay' and 'woody-like'. Comparison of the aroma by gas chromatography-olfactometry showed that these aromas were attributed to 3-(methylthio)propionaldehyde (methional), phenylacetaldehyde, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), and 4,5-dimethyl-3-hydroxy-2(5H)-furanone (sotolon) (Ferreira et al., 2003). Dissolved oxygen plays a fundamental role in the formation of methional, due to both the direct oxidation of methionol and the formation of $\alpha$-di-carbonyl compounds, which are reactants in the Strecker reaction (Ferreira et al., 2002). Escudero et al. (2002) analyzed 27 young white wines stored under oxygen for 1 week and found that *trans*-2-nonenal, eugenol, benzaldehyde and furfural are the main compounds responsible for cooked-vegetable odour nuance.

### 2.7.3 Red wines

#### 2.7.3.1 Polyphenolic reactions in red wine and their effect on the red wine colour and aroma

The polyphenolic reactions involved in red wine aging are basically described as anthocyanin/tannin combinations that can either be direct, generating xanthylium salts, or involve acetaldehyde, leading to purple pigments (Somers, 1971; Timberlake and Bridle, 1976). Relative amounts of anthocyanins and tannins, dissolved oxygen, pH and the presence of yeast metabolites influence these reactions. The pH controls reactivity because at low pH the electrophilic flavylium ion is the most abundant form of anthocyanins whereas at higher pH the nucleophilic hydrated ion is the most abundant form.

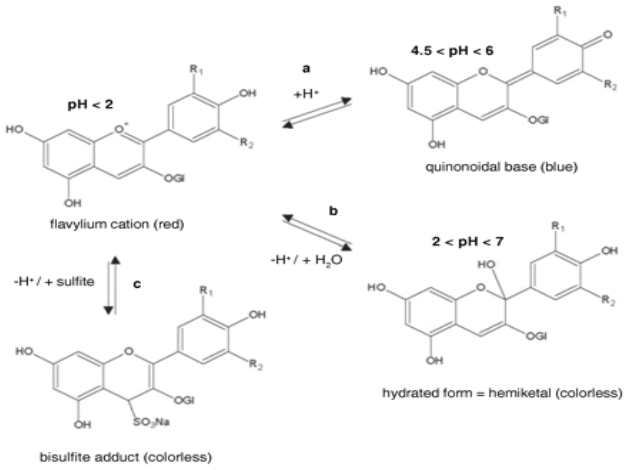



Figure 14: Reactions of anthocyanins in aqueous solutions and wine ($R_1$, $R_2$ = H, OH, $OCH_3$): proton transfer (a), hydration (b), and sulphite bleaching (c) (Cheynier et al., 2006)

Understanding the chemistry of free anthocyanins is an essential step towards understanding the chemistry of condensation and the behaviour of anthocyanin containing pigments in wine solution. Different types of pigments were identified in the last decade. They can be classified into three groups with respect to their formation pathways (Fulcrand et al., 2004):

1. Reactions involved in the conversion of grape anthocyanins to more stable pigments are classically described as anthocyanin-flavanol reactions. They can be direct, leading to the first group of products, including flavanol-anthocyanin ($F\text{-}A^+$)

or anthocyanin- flavanol ($A^+$-F) pigments as well as colourless A(-O-)F compounds that contain an additional ether linkage and a structure analogous to that of A-type proanthocyanins.

2. The second type of reactions involved in conversion of grape anthocyanins to wine pigments is a polymerization process involving an aldehyde component. Acetaldehyde-induced reactions involve protonation of the aldehyde followed by nucleophilic addition of the flavonoid onto the resulting carbocation (Fulcrand et al., 1996). Ethyl-linked products, including ethyl-linked flavanols (F-Et-F) and ethyl-linked anthocyanin-flavanol pigments ($A^+$-Et-F) have been detected in wines (Saucier et al., 1997b). Other aldehydes including glyoxylic acid and furfural were shown to react in the same way (Fulcrand et al., 1997), however, products resulting from flavanol reactions with these aldehydes undergo further rearrangement to xanthylium pigments.

3. The third group of wine pigments corresponds to pyranoanthocyanins formed by the reaction of genuine anthocyanins with yeast metabolites released during fermentation (Fulcrand et al., 2004). Egliton et al. (2004) showed that yeast metabolites were actively involved in the formation of pigmented polymers. Pyruvic acid leads to the major pyranoanthocyanins currently found in wines, i.e. carboxy-pyranoanthocyanins (R=COOH), sometimes referred to as vitisin A (Baker et al., 1997; Singleton and Trousdale, 1992). In aged Pinotage wines, the pigment pinotin A has been discovered. It is formed between the anthocyanin and a hydroxycinnamic acid moiety, especially the caffeic acid in Pinotage (Schwarz and Winterhalter, 2004). Pyranoanthocyanins are exceptionally stable pigments towards sulphite bleaching and pH variations, due to substitution of the flavylium C-4. Lee et al. (2004) showed that most of the vitisin A formation occurred during fermentation when yeast activity was the highest and oxygen concentration was low. It is suggested that more than oxygen itself, reactive oxygen species are responsible for the formation of vitisin A. Therefore, appropriate cap management techniques and/or the presence of components in the wine which promote the

production of oxidation substrates, such as ellagitannin, may be used to enhance the formation of stable wine pigments such as vitisin A.

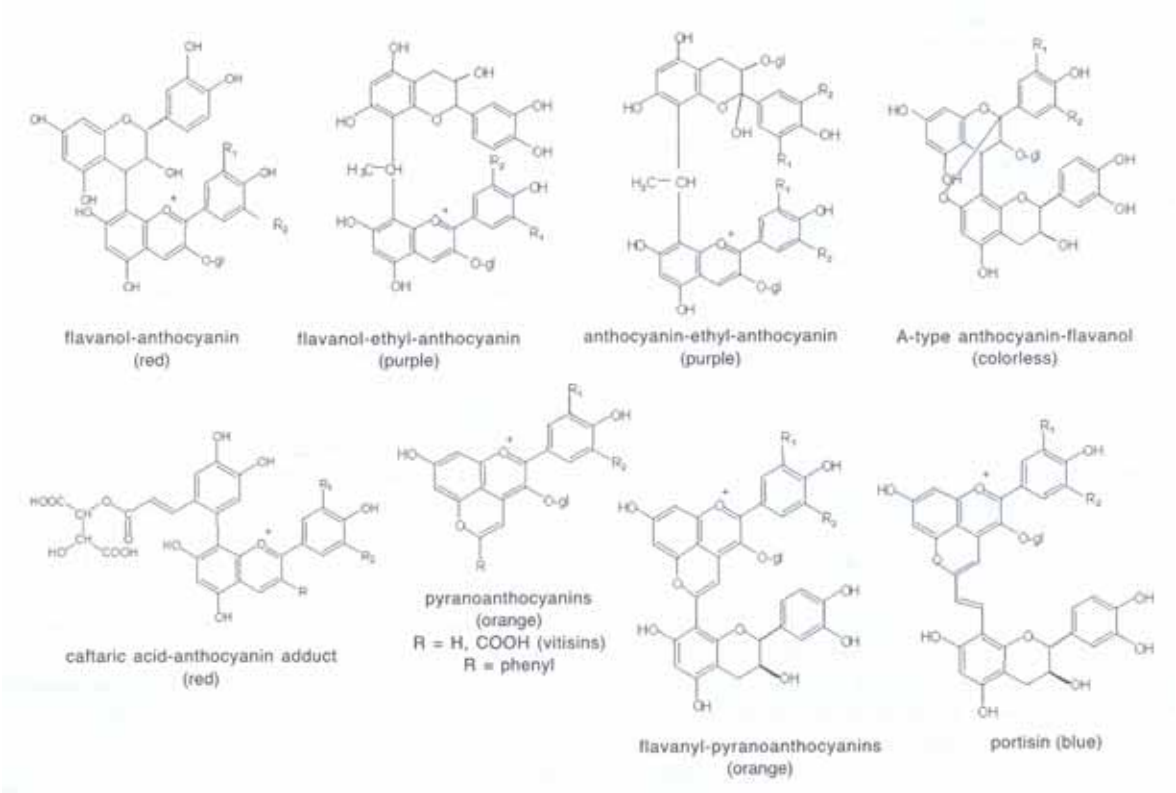



Figure 15: Anthocyanin derivates detected in wine ($R_1$, $R_2$ = H, OH, $OCH_3$) (Cheynier et al., 2006)

Colour is one of the main organoleptic properties of red wine and it is of crucial importance for the consumer since it is the first characteristic to be perceived in the glass. Anthocyanins are largely responsible for the colour of young red wine and the concentration of individual anthocyanins can reflect grape composition. After less than twelve months of ageing, the concentration of free anthocyanins declines substantially and pigments that have been formed after crushing, during fermentation and during ageing are essential to maintain the red colour of wine. Based on spectrophotometric measurements, pigmented polymers are regarded to contribute as much as 90% to the colour of red wine after 2 years of storage (Somers, 1971).

The use of oak barrels in winemaking is thought to improve the wine quality. Many constituents can be extracted from staves during ageing in barrels, such as ellagitannins (vascalagin and castalagin), tannins, ferulic, vanillic, syringic and ellagic acids, cumarins and volatile compounds (Canas et al., 1999; Viriot et al., 1993). Ellagitannins have an

important role in the red wine ageing and may be involved in both red and white wine oxidation mechanisms. Vivas and Glories (1996b) found that when tannins oxidize, due to their greater oxidising capacity; they produce larger amounts of acetaldehyde than condensed tannins. This leads to higher levels of polymerization, which induces tannin-anthocyanin polymerization, leading to higher colour density. These tannins also seem to buffer catechins from oxidation by being oxidized themselves and thus preventing formation of a brick-yellow colour (Vivas and Glories, 1996b). High storage temperature in combination with high $O_2$ concentration can lead to anthocyanins and tannin breakdown reactions, which can increase the yellow hue of the wine.

The effect of oxygen on red wine aroma has not been investigated in detail. According to Blanchard et al. (2004) the quinones generated can easily react with thiols, generating $H_2O_2$, which can further oxidize 3-mercapthohexanol (3MH), a fermentation product, responsible for fruity aromas of red and rose wines made from Merlot, Cabernet Sauvignon and Grenache grapes. Higher dissolved oxygen concentration resulted in higher decrease in 3MH, but the decrease was not directly linked to dissolved oxygen and was probably due to other molecules present in red wines that react more readily with oxygen than thiols. The addition of $SO_2$ (30 mg/L) and anthocyanins attenuated the decrease in 3MH. Anthocyanins and $SO_2$ thus seem to play a synergetic role in reducing this thiol. The oxidative environment inside an oak barrel also stimulates the formation of sotolon, which forms due to the oxidation of threonine. Phenylacetaldehyde, a compound responsible for old-wood-oxidation character of red wines, is also formed in wines stored in oak casks. This compound, with odour thresholds 2 μg/L, is related to the oxidative degradation of phenylalanine or direct oxidation of β-phenylethanol (Jarauta et al., 2005). On the other hand, oxidation, taking place in oak cask, leads to disappearing of some important wine alcohols, such as hexanol, β-phenylethanol and methionol. It is suggested that these three alcohols are oxidized to their corresponding aldehydes. This can change the sensory effect of wine, as aldehydes have smaller odor thresholds than alcohols (Jarauta et al., 2005). However, the effect of $O_2$ on the aroma composition of red wine should be investigated further.

#### 2.7.3.2 Influence of phenolic structure on taste

Phenolics may be bitter or astringent, and some of them are also volatile and contribute specific odours such as vanillin, ethyl phenols and vinyl phenols. Formation of anthocyanin-proanthocyanidin adducts was commonly proposed to explain the loss of astringency during wine maturation, and wine quality was speculated to have something to do with anthocyanin to proantocyanidin ratio due to interaction between anthocyanins and proanthocyanidins during wine maturation (Cheynier et al. 1998). Brossaud et al. (2001) observed that anthocyanin fraction reduced grape proanthocyanidin astringency and did not contribute to bitterness. In the study of Vidal et al. (2004) anthocyanin fraction only slightly increased the astringency of the model wine solution. However, isolated anthocyanin fraction studied in these experiments contained up to 22% of non-monomeric anthocyanin material. The level of astringency of this anthocyanin fraction was lower than tannins fraction with medium degree of proanthocyanidin polymerization 3.

Procyanidins become gradually less bitter and more astringent as the molecular weight increases up to about 10 units (Cheynier et al. 1998). Beyond this limit, they are believed to be insoluble and thus no longer astringent. According to these authors, astringency, defined as an extreme drying or puckering sensation within the mouth resulting from interactions between tannins and mouth proteins, is essentially due to procyanidins, especially galloylated proanthocyanidins. Their experiments suggest that formation of anthocyanin-tannin adducts is the major mechanism involved in the conversion of astringent proanthocyanidins (‘hard’ tannins) to ‘soft’ tannins during wine maturation. In contrast, colourless catechin oxidation products were shown to interact with proteins as did procyanidin dimers, and should therefore be similarly astringent. Besides, catechin quinones generated by oxidation are likely to proceed to different – and possibly ‘softer’ – products in the presence of anthocyanins (Sarni-Manchado et al., 1997). According to Cheynier et al. (2006) flavanol polymerization reactions, regardless of the polymers formed (proanthocyanidins, oxidation products or ethyl-flavanols), enhance rather than reduce astringency. However, wine tannins also undergo cleavage reactions generating low molecular weight species that contribute to the loss of astringency during wine ageing. Other wine components such as polysaccharides and proteins interfere with astringency perception, presumably through competition with salivary proteins in the formation of

tannin complexes (Cheynier et al., 2006). Catechin and epicatechin are by far the main constituents of seed flavanols, since epicatechin gallate occurs only at a lower concentration (Mattivi et al., 2006b). In addition, small differences in flavonoid configurations can produce significant differences in sensory properties. Epicatechin is more bitter and astringent than its chiral isomer catechin (Lesschaeve and Noble, 2005) and dimer B6 (catechin-4,6-catechin) was more bitter and astringent than dimer B3 (catechin-4,8-catechin) and dimer B4 (catechin-4,8-epicatechin). Vidal et al. (2003a) studied the effects of grape tannin structure on bitterness and astringency. They showed that modifying the molecular structure by introducing an ethyl bridge between anthocyanins and proanthocyanidins, decreased astringency but increased bitterness. In contrast, for grape seed tannin, reducing the level of esterification with gallic acid decreased astringency (Vidal et al., 2003a). Mattivi et al. (2006b) showed that epicatechin gallate is highly variable in seed proanthocyanidin extracts. The lowest values were observed in the Syrah and Marzemino grape seed extract. Hard tannin perception is associated with high tannin concentrations, especially of large polymers and galloylated tannins from seeds that aggregate easily. It is uncertain whether soft tannin perception is due to low concentrations of such tannins, to the presence of specific compounds such as anthocyanin-tannin adducts, or to the incorporation of tannins into protein or polysaccharide complexes. Finally, the role of psychological factors should not be overlooked. In particular, green tannin perception appeared linked with the lack of red colour associated with poor ripeness. ‘Green’ versus ‘soft’ tannin perception was changed when the same series of wines was tested in white or black glasses by an expert wine panel (Brossaud, 1999), indicating mental associations between colour and taste perception.

## 2.8 OXYGEN AND WINE SPOILAGE MICROORGANISMS

*Brettanomyces/Dekkera* have been recognised as wine spoilage yeast, imparting undesirable odours and flavours and high producer of acetic acid. They are capable of producing at least 10 different aromatic components which destroy the fruity characteristics of the wine (Licker et al. 1998). The principal spoiler compounds associated with *Brettanomyces* are two volatile phenols, 4-ethylphenol and 4-ethylguaiacol. The relationship between *Brettanomyces* and oxygen is rather complex. Ciani and Ferraro

(1997) showed that the oxygen concentration exerted a strong influence on both growth and acetic acid production by *Brettanomyces* yeast in winemaking. Full aerobiosis leads to a large production of acetic acid. Semi-aerobiosis resulted in the best condition for alcoholic fermentation (Custers effect) combined with acetic acid production. In anaerobic condition *Brettanomyces* yeast growth did not result in high acetic acid production and a pure, even if slow, alcoholic fermentation occurred. Uscanga et al. (2003) studied the influence of the oxygen supply on the growth, acetic acid and ethanol production by *Brettanomyces bruxellensis* in glucose medium. They observed greater acetic acid production and lower ethanol production at higher oxygen supply.

According to Gills et al. (2002), a supply of 10 mL/L of oxygen per month is a 'limit' value, below which *Brettanomyces* growth is equivalent to that obtained in anaerobiosis. Above this level, *Brettanomyces* growth is encouraged and the cellular concentration obtained is 30% higher. Racking the wine during *Brettanomyces* growth encourages their growth, as much as excessive continuous oxygen supply would be.

High volatile acidity and a vinegary taint in wine are often associated with the activity of acetic acid bacteria such as *Acetobacter aceti, Acetobacter pasteurians and Gluconobacter oxydans* (Du Toit and Lambrechts, 2002). Acetic acid bacteria can produce acetaldehyde at concentrations of up to 250 mg/L and high concentrations of acetic acid ranging from 0.7 to 1.2 g/L. Another product of the metabolism of acetic acid bacteria that could affect wine quality is ethyl acetate.

Oxygen is considered necessary for the growth of acetic acid bacteria, as they are classified as strictly aerobic microorganisms. Wine surfaces left in contact with air quickly develop a surface flora of these bacteria. Moreover, momentary aeration of wine during pumping and transfer operations is sufficient to encourage a significant growth of the low populations of these organisms (Drysdale and Fleet, 1988). Observations that acetic acid bacteria can use substances such as quinones and reducible dyes as electron acceptors in substitute for oxygen support the possibility of these bacteria surviving and growing in an anaerobic to semi-anaerobic environment.

It has been reported that levels of molecular $SO_2$ needed to prevent the growth of *Acetobacter* were dependent on the strain and ranged from 0.05 to 0.6 mg/L whereas 0.8 mg/L molecular $SO_2$ was needed to prevent the growth of *Gluconobacter hansenii* (Du Toit and Pretorius, 2002). A strain of *Brettanomyces bruxellensis* was found to be more

sensitive than a strain od *A. pasteurianus* to molecular $SO_2$, with 0.25 mg/L molecular $SO_2$ preventing it from growing on media as well as drastically reducing its viability (Du Toit et al., 2005). However, strains differences probably exist regarding these aspects and should be investigated further.

## 2.9 THE ROLE OF OXYGEN IN VINIFICATION TECHNOLOGIES

### 2.9.1 Microoxygenation

Microoxygenation (MOX) is a process during which measured amounts of oxygen are introduced to wines with the aim of bringing about desirable changes. Some of these include enhanced colour stability and intensity, softening of astringent tannins, and decreased reductive and vegetative aromas, and greater intensity of aroma attributes regarded as grape-derived (the so called 'varietal aroma') (Parish et al., 2000, Paul, 2002). Microoxygenation can be started at any point during the winemaking process, but usually it is stopped during malolactic fermentation, because some lactic acid bacteria are capable of metabolizing acetaldehyde, even the acetaldehyde bound to sulfur dioxide (Osborne et al., 2000).

During microoxygenation $O_2$ is supplied in the form of compressed gas via a micron-size diffuser positioned close to the bottom of the tank, which should be at least 2.5 meters high to guarantee that the oxygen bubbles would have dissolved (Oenodev, 2001). The amount of oxygen introduced to the wine varies from 2-30 mg/L of wine per month. The clarity of wine is also important, as the yeast lees are able to absorb large quantities of oxygen that might otherwise go into the desirable reactions. Microoxygenation has also been suggested as a means of replacing expensive oak barrels by combining the process with alternative oak products in a stainless steel tank. Cano-Lopez et al. (2006a) compared oak barrel maturation vs. microoxygenation (3 mL/L/month) during 3 and 6 month maturation. They found higher colour intensity in wine matured during three months in oak barrel; however, microoxygenated wines showed high concentration of pyranoanthocyanins. During microoxygenation, $O_2$ should be supplied at a slower rate than is its rate of consumption by the wine to prevent unwanted accumulation in the headspace of the tank (Nikfardjam and Dykes, 2003). Waterhouse and Laurie (2006) determined dissolved oxygen levels to be between 200 and 250 μg/L in microoxygenation treatment of Cabernet Sauvignon at 5 mg/L/month oxygenation rate. A controlled flow of oxygen to the wine must be sufficient to produce a high enough acetaldehyde concentration for inducing the polymerization and combination reactions (Es-Safi et al., 1999). The acetaldehyde can in turn react with flavanols to induce the formation of a very reactive carbonation that quickly reacts either with another flavanol molecule or with an anthocyanin, producing ethyl-bridged flavanol-

flavanol and flavanol-anthocyanin oligomers stable to $SO_2$ and pH change (Silva et al., 2002). A general trend in analytical results of MOX-treated wines is a decrease in the concentration of anthocyanins, concomitant with an increase in total colour intensity (Moutounet et al. 1995, McCord, 2002). Too great oxygen flow may be unsuitable, however, as this can lead to the oxidation of aromas, the precipitation of high-molecular-weight polymers and browning. Precipitation of polymeric material can cause the reduction of colour intensity. Many of these oxidative reactions are irreversible and may detrimentally affect the development and existence of many phenolic compounds. There is also the possibility of adverse microbial activity.

Cano-Lopez et al. (2006b) showed that after 10 weeks the microoxygenation of wine made from the Monastrell grape variety, most of the vitisin A-like compounds (petunidin 3-glucoside pyruvate, vitisin A, coumaryl vitisin A) showed lower concentrations in the control wine and increased concentrations in the MOX wines, the greatest increases in the MOX wines receiving the highest oxygen dose. They found no accumulation of acetaldehyde; however, Carbi et al. (2002) found that higher doses of oxygen generate higher concentrations in acetaldehyde and higher colour intensity. The temperature has a direct influence on the speed of the reactions, leading to the increased structuring effect between polyphenols, but it also has an influence on the oxygen solubility, which increases as the temperature decreases. MOX seems not to have a direct effect on $SO_2$ concentration, and vice versa, but if an accumulation of dissolved oxygen occurs, the free $SO_2$ concentration drops. However, the rate of decrease of free $SO_2$ can be indicative of how the wine is responding to microoxygenation. Too rapid rate of decrease indicates too high rate of oxygenation. McCord (2002) showed that microoxygenation decreased methyl and ethyl mercaptan concentration; however there was no increase in the dimethyl sulphide in his study. Dimethyl sulphide is typically formed from oxidation of sulphide or mercaptan precursors and their sensory threshold is much higher than mercaptan threshold.

Llaudy et al. (2006) observed that applying microoxygenation before oak ageing produces wines with a slightly (though significantly) higher mean degree of proanthocyanidin polymerization and a drastically lower astringency. McCord (2002) also showed that microoxygenation led to a decrease in monomeric proanthocyanidins, epicatechin and an increase in polymeric phenolics. These wines, complementary with oak ageing, also present more integrated wood aromas.

MOX has been shown to impact herbaceousness. The effect of MOX on methoxypyrazine, compounds responsible for green and vegetal aromas, is not well understood. It appears that the reduction in the herbal character may not be the result of changes in methoxypyrazines, but changes in thiols or sulphur-containing compounds, that help to reinforce the herbal or vegetative sensory perception. Some thiol compounds complement the odor of methoxypyrazines. Sulphur-containing compounds, unlike methoxypyrazines, are not stable. During MOX, it is the oxidation of some sulphur-containing compounds that may result in the muting of the vegetal character of treated wines (Zoecklein, 2006).

Du Toit et al. (2006) found that microoxygenation can be used to enhance the quality of a younger red wine, but should be used with care in an older red wine because over-oxidation can lead to a faulty/spoiled character. $SO_2$ levels of the wine should be checked regularly because oxygen can stimulate growth of *Brettanomyces* and acetic acid bacteria.

### 2.9.2 Hyperoxygenation

Flavonoids undergo regenerative polymerization when oxygen is consumed and their flavour thresholds are lowered as polymerization progresses. Thus, hyperoxidation must be seen as a technique to remove flavonoid phenols using the natural enzymatic constitution of grapes and to contribute to sensory stability of white wine (Schneider, 1998).

When flavanols are oxidized by caftaric acid quinone, the respective quinones polymerize rapidly and precipitate as brown pigments. The polymerization reaction in must, is essentially the same as in wine, but the rate of quinone production is much faster in must. Furthermore, while the pigments are more or less soluble in an alcoholic medium, they are insoluble in must.

One miligrame per liter of oxygen could precipitate from 0 to 8.6 mg/L of flavonoids (as catechin). In order to precipitate the contents of must flavonoid lower than 100 mg/L (as catechin), one saturation concentration (9 mg/L) of oxygen may be sufficient. Higher contents of flavonoids as might be obtained by pomace contact, require an oxygen consumption of about 30 mg/L, corresponding to approximately three saturation concentrations (Schneider, 1998). According to Nicolini et al. (1991) hyperoxygenation of must caused an average reduction of 87% in the total hydroxycinnamate concentration as determined by HPLC and up to 40% in the total phenol concentration.

Wines made from oxidized musts were less susceptible to oxidative browning (Nagel and Graber, 1988). The more oxygen a must absorbs, the less oxygen is consumed by the corisponding wine, and a lower percentage of oxygen consumed by wine reacts with phenols. As must oxidation kinetics require the presence of PPO, all technical means of lowering this enzymatic activity have to be avoided before the hyperoxidation procedure is accomplished. Press juice has more tyrosinase activity than free-run juice. Oxidation products inactivate and precipitate PPO and other proteins. Bentonite-fining by 100 g/hL leads to a loss of 30% of the initial PPO activity, eliminating soluble enzyme (Schneider, 1998). The sulphur dioxide effect on PPO inactivation is also already been mentioned. The combined effect of $SO_2$, bentonite and clarification on PPO explains why must hyperoxidation has to be carried out directly after pressing and before any further juice processing. Zironi et al. (1997) proposed a ratio $A_{320nm}$ : $A_{280\ nm}$ as a marker of the hyperoxygenation technique. It was found that wines with a value below 0.5 were probably made following the hyperoxygenation of the must. During fermentation oxidised quinines probably associate with yeast and thus precipitate out, or are reduced due to the reductive environment created by fermentation.

Hyperoxidation can reduce bitterness and astringency (Schneider, 1998), but there are other reports stressing no difference in sensory preference (Cheynier et al., 1990). Nagel and Graber (1988) report that wines produced from the oxidized juices were significantly more bitter. Oxidation of the hydroxycinnamic acids can result in an increase in harshness and bitterness. White wines made from oxidized musts were comparable in colour to the control wines and were judged as inferior in flavour. Verette et al. (1988) report that the non-flavonoid phenol fraction does not contribute to bitterness at the concentration found in wine. However, reports on olfactory changes generated by hyperoxidation are conflicting and needs further investigation.

### 2.9.3 Hyperreduction

The oxidation of juice is much more complex than the simple elimination of readily oxidizable phenols. There are conflicting views on the effect of reductive as opposed to oxidative treatment of juice prior to fermentation on wine quality. However, quite a number of authors also reported increases in fruity aroma intensity as well as wine quality of wines produced from reductive juices as opposed to those made from oxidized juices

(Singleton et al., 1980; Nicolini et al., 1991; Van Wyk et al., 1996). Reductive treatment of juice is commonly used to produce fresh and fruity Sauvignon Blanc wines of high quality (Marais, 1998). Dubourdieu et al. (1993) found that must oxidation decreased levels of the non-volatile precursor of 4-mercapto-4-methyl-pentane-2-one which is formed during alcoholic fermentation and which is believed to be particularly contributing to the fruity varietal aroma of Sauvignon Blanc wines.

The exposure of white musts or wines to oxygen will lower its quality and slightly increase its bitterness. A decrease of fruitiness and varietal character can also be noticed (Margalit, 2004).

Therefore, to increase the varietal wine aroma different reductive technologies have already been applied, especially in the vinification of Sauvignon Blanc and other varieties which contain the sulphur aroma compounds and which oxidize easily in the presence of a low amount of oxygen (Mattivi et al., 2005). As important oxidation occurs already inside the presses, where oxygen uptake during pressing of whole clusters was estimated at 10 to 15 mg/L (Cheynier et al., 1993), vinification with hyperreduction using inert gases such as nitrogen, argon and others is of particularly interesting. This technique can improve the juice from different pressings fractions, which represents must samples at differnet pressure, because they are very important for the extraction of aromatic compounds which can be found mostly in grape skins.

Hydroxycinnamic acids and their esters are the most important polyphenolic antioxidants of white wines and good indicators for the control of hyperreductive technology, as they are the first to enter in the oxidation reaction. The contents of hydroxycinnamic acids and their esters in grape berry and must are much higher in the must completely protected from oxygen than the values usually found in white wines (Vrhovšek, 1998). An important issue is also the decrease of $SO_2$ addition in wines, as it has not been used up in enzymatic oxidative reactions in the prefermentation phase (Mattivi et al., 2005).

# 3 MATERIAL AND METHODS

## 3.1.1. General chemicals

### 3.1.1.1 Chemicals and reagents used in wine cellar

Chemicals and reagents used in wine cellars during technical experiment include $K_2S_2O_5$ (Anchor Yeast Biotechnologies, Cape Town, South Africa; AEB, Brescia, Italy), ascorbic acid (Sigma-Aldrich, Steinheim, Germany), bentonite (Bentogran, AEB, Brescia, Italy), yeast cultures *Saccharomyces cerevisiaes* (VL3, Laffort, Bordeaux, France; BCS 103 Springer Oenologie, Maison-Alfort, France; VIN13, Anchor Yeast Biotechnologies, Cape Town, South Africa; Fermol Rouge, AEB, Brescia, Italy), yeast nutrient (DAP, Anchor Yeast Biotechnologies, Cape Town, South Africa; Biostimol, Polo Enologia, Oderzo, Italy), Fermotan oak tannin (AEB, Brescia, Italy), malolactic starter culture bacteria (Biolact, Pascal-Biotech, Paris, France), NT116 yeast (Anchor Yeast Biotechnologies, Cape Town, South Africa) and diammonium hydrogenphosphate (Anchor Yeast Biotechnologies, Cape Town, South Africa).

### 3.1.1.2 Solvents

The solvents included: formic acid (Riedel-de Haen, Seelze, Germany) and acetonitril (Chromasolve, Riedel-de Haen, Seelze, Germany), *ortho*-phosphoric acid (Riedel-de Haën, Seelze, Germany). Other chemicals, methanol (HPLC grade), sulphuric acid, hydrochloric acid, perchloric acid, formic acid, Folin Ciocalteau, $Na_2CO_3$, ethanol, $FeSO_4*7H_2O$, acetaldehyde and vanillin were obtained from Merck (Darmstadt, Germany). Aqueous solutions were made with Milli-Q water (Milipore, Bedford, USA).

### 3.1.1.3 Chemical Standards

L-Glutathione reduced (Fluka 49750) and L-Glutathione oxidized (Fluka 49740) were purchased from Sigma-Aldrich (Steinheim, Germany).

Some hydroxycinnamic acids and their esters were isolated from grapes (Istituto Agrario San Michele all'Adige, Italy) while some (caffeic acid, *p*-coumaric, ferulic acid) were purchased from Sigma-Aldrich (Steinheim, Germany). Other standards include: (+)-catechin hydrate (Fluka, Buchs, Switzerland), gallic acid (Fluka, Buchs, Switzerland), vanillic acid (Fluka, Buchs, Switzerland), *p*-coumaric acid (Sigma, Buchs, Switzerland),

malvidin-3-glucoside (Fluka, Buchs, Switzerland), ellagic acid (Fluka, Buchs, Switzerland), quercetin-3-glucoside (Fluka, Buchs, Switzerland) and quercetin (Extrasynthèse, Lyon, France).

#### 3.1.1.4 Microbiology chemicals

The microbiology experiment included Man Rogosa Sharp – MRS (Merck, Darmstadt, Germany), penicillin (Sigma-Aldrich, Steinheim, Germany), pimaricin (Actistab, Gistbrocades, Heerlen, Nederlands), yeast extract, peptone, glucose (Merck, Darmstadt, Germany), chloramphenicol (Sigma-Aldrich, Steinheim, Germany) and cyclohexamide (Sigma-Aldrich, Steinheim, Germany).

### 3.1.2. General equipment and materials

#### 3.1.2.1 Materials

Other materials included: solid phase extraction (SPE) cartridge (Sep Pak C18, 0.35 g; Waters, Milford, USA), guard hypersil column 2.1 x 20 mm (5µm) (Agilent Technologies, Palo Alto, USA), Chromolith Peformance RP-18e column and pre-column (Merck, Darmstadt, Germany), 0.45 µm syringe filters (Sartorius, Goettingen, Germany)

#### 3.1.2.2 Equipment

Technical material included Bűchi rotavapor (Flawil, Switzerland), two microoxygenation units (SAEn and SAEn 4000), supplied by Parsec (Firenze, Italy), oak barrels, oak staves (Radoux, Stellenbosch, South Africa) and oak blocks (AEB, Sežana, Slovenia), Micro III G202 oxygenmeter (Gesellschaft für Gerätebau, Dortmund, Germany), Oxi 330i oxygenmeter cell ox 325 probe (Wissenschaftlich-Technische Werkstätten – WTW, Weilheim, Germany), oxygenmeter Oxymet, Isolcell (Laives, Italy), GrapeScan FT 120 instrument (Foss Electric, Hillerod, Denmark), Metrohm titration unit (Metrohm Ltd., Switzerland), Waters Quattro micro API triple quadropole mass spectrometer with a 2690 Alliance (Waters, Milford, USA), Agilent 1100 HPLC with DAD connected to an Agilent NDS ChemStation (Agilent Technologies, Palo Alto, USA), Gymar Selector System (Occimiano, Italy).

## 3.2 HYPERREDUCTION OF WHITE WINE

### 3.2.1. Grapes

Different varieties of grapes were used for the hyperreduction experiment. In the small scale hyperreductive pressing three different grapes were used, two Sauvignon Blanc and Colombar grapes from Stellenbosch region (South Africa), all picked at full maturity. The industrial hyperreductive experiment performed in Italy includes Muller Thurgau (Trento, Italy), Chardonnay (Trento, Italy) and Greco di Tufo (Campagnia region, Italy). All grapes except Chardonnay, which was used for sparkling base wine, were harvested at full maturity.

### 3.2.2 Technological experiments

#### 3.2.2.1 Small scale hyperreduction pressing

A custom built small scale press was constructed at Stellenbosch University and used to extract the juice with minimum oxygen pick up, which ensures an oxygen level below 1% in the press atmosphere (Figure 16).

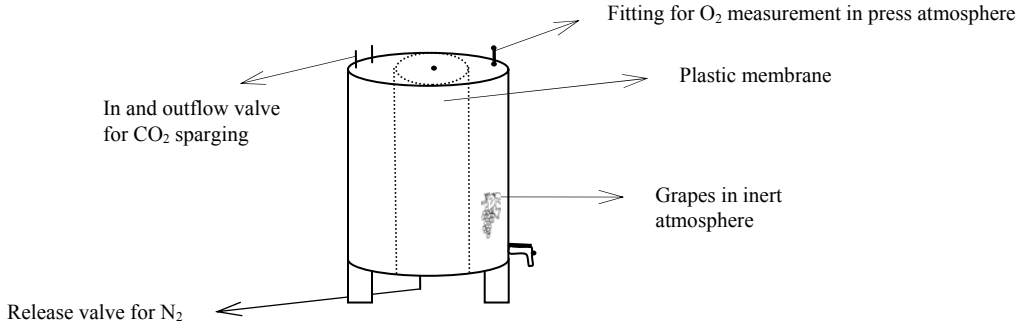



Figure 16: Microvinification wine press used for hyperreductive pressing
Slika 16: Mikrovinifikacijska stiskalnica za hiperreduktivno stiskanje grozdja

This was achieved by placing 9 kg of whole grape bunches (not destemmed and crushed) around a central plastic membrane in the press. The press was filled with grapes, sealed and filled with water. The water was displaced with $CO_2$ (Afrox SA, Stellenbosch, South Africa) to achieve inert atmosphere around the grapes before pressing. The plastic membrane was subsequently filled with nitrogen gas (Afrox SA, Stellenbosch, South

Africa). The pressure applied in the plastic membrane was slowly increased to 2 bars. The nitrogen gas was released through a pressure release valve and the grapes pressed again to 2 bars. An extra in and outflow valve on top of the press allowed continuous flow of $CO_2$ through the press during the procedure. Around 3.5 liters of the juice generated by this pressing action was accumulated in 4.5 L glass bottles that had previously been filled with water and displaced with $CO_2$. Sulfur dioxide (60 mg/L) and ascorbic acid (50 mg/L) were added in the 4.5 L glass bottles just before filling with juice from the press. In spite of double pressing up to 2 bars, quite low percentages (40%) of juice were obtained in all treatments. Oxygen ($O_2$) concentrations were measured in the headspace of the press with the Micro III G202 of Gesellschaft für Gerätebau (Dortmund, Germany) according to the supplier's recommendations. Oxygen concentrations in the juice in 4.5L glass containers were measured with an Oxi 330i oxygen meter with a cell ox 325 probe (Wissenschaftlich-Technische Werkstätten – WTW, Weilheim, Germany). Oxygen levels during pressing were kept below 1% inside the headspace of the press and less than 0.3 mg/L in the juice during the hyperreductive treatment. These treatments were considered the reductive juice treatments. The control juice treatment was conducted in the same manner, but the press and 4.5 L bottles were not filled with water and displaced by $CO_2$ prior to the pressing of the juice. Sulfur dioxide (60 mg/L) and ascorbic acid (50 mg/L) were also added into the bottle immediately after pressing. The oxygen pick-up in the control juices was between 1.0-1.5 mg/L. The hyperoxidative juice treatments were also pressed without water and $CO_2$ in the press and 4.5 L glass bottles. Neither $SO_2$ nor ascorbic acid was added to these juices. After pressing, the juices were racked four times into a plastic 20 L bucket to encourage $O_2$ pick up. This resulted in 3.5-4 mg/L dissolved $O_2$ in total, measured immediately after the fourth air racking. However, the exact concentration was difficult to measure, because of rapid decrease of oxygen concentration in the conditions without $SO_2$ and ascorbic acid. All treatments in all three grape types were performed in triplicate.

After settling over the night (12 h, 15 °C), the juice was racked from the grape lees with $CO_2$. It was then inoculated with the yeast strain Vin13 (Anchor Yeast Biotechnologies, Cape Town, South Africa) at 30 g/hL according to the supplier's recommendations and fermented at 15 ºC. Two days after the start of fermentation diammonium phosphate (Anchor Yeast Biotechnologies, Cape Town, South Africa) was added at 50 g/hL. At the end of fermentation, an additional 50 mg/L $SO_2$ was added to all treatments and the wine

was racked with $CO_2$ from the yeast lees and bottled in green 750 mL wine bottles under $CO_2$ gas and sealed with screw caps.

**Sampling procedure from different juice treatments.** Samples destined for LC-MSMS glutathione analyses and HPLC analyses (hydroxycinnamic acids and their esters, catechin, epicatechin) were taken from the juice in the reductive treatments just after the juice was collected in the 4.5 L glass bottles. This was done by transferring 80 mL of juice, with $CO_2$ pressure, from the 4.5 L glass bottles into 100 mL glass vials containing 1000 mg/L $SO_2$ and 500 mg/L ascorbic acid. The 5% $SO_2$ solution was made from $K_2S_2O_5$ (Anchor Yeast Biotechnologies, Cape Town, South Africa) and 1.6 mL was added to 80 mL of juice to obtain a desired concentration. 5% ascorbic acid (Sigma-Aldrich, Steinheim, Germany) was also used and prepared daily before starting the experiments. Carbon dioxide was also blown inside the 100 mL glass vials before and after the juice was transferred into the glass vials. This was done to completely inhibit any residual oxidation enzyme activity. Additional $CO_2$ was blown on the headspace and the vials sealed hermetically. The vials were then frozen at –20 ºC until analyzed after 4 weeks. Samples from the oxidative treatments were stored in the same manner, but the juice was collected for analysis after 8 hours to allow the enzymatic oxidative reactions to take place.

3.2.2.2 Industrial hyperreductive pressing

The industrial scale vinification experiment was carried out at Pojer & Sandri winery in Faedo, Trentino (Northern Italy). Special wine press invented by Mr. Pojer was used for hyperreductive pressing. The press consisted of a hermetical system, which enables pressing of all fractions in the press atmosphere bellow 1% oxygen. The hyperreductive pressing system is also available by Bucher-Vaslin Company as Intertys patented system (Ardilouze, 2006). The oxygen concentration was measured with oxygen meter Oxymet, Isolcell (Laives, Italy). There are some important aspects (Figure 17) which should be noticed when using hyperreductive press operating with overpressure:

- the press (1) consisted of a closed system, which is hermetically connected with buffer tank (3),
- the buffer tank (3) is connected with a membrane – ‘baloon’(2), where inert gas ($N_2$) is stored,

- the vacuum pump (4) enables the extraction of most oxygen present from the system (press, buffer tank and tubes) before filling with inert gas from membrane storage,
- the atmosphere of all three objects (1,2,3) is in equilibrium with the same gas during operation of the press, which enables the total absence of oxygen,
- protected grape and juice is pressed to a buffer tank and then transferred by pump to a sedimentation tank, where a controlled atmosphere should also be achieved,
- the system with gas membrane storage enables the recycling of the same gas through all grape pressings, which minimizes the operation costs during the vintage,
- sampling of juice was carried out from the tube connecting the wine press and the buffer tank.

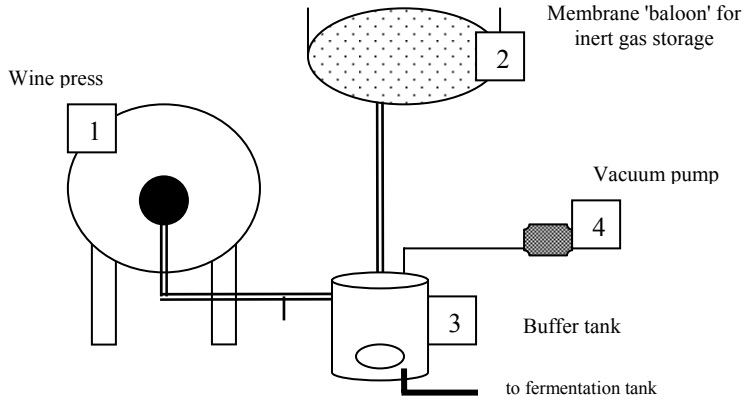



Figure 17: Schematic presentation of an industrial hyperreductive wine press
Slika 17: Shematski prikaz industrijske hiperreduktivne stiskalnice

The hyperreductive technology was performed on different white varieties which included Muller Thurgau, Chardonnay and Greco di Tufo (Figure 18).

After picking, grapes were stored overnight in the cold store at 2°C. The grapes were then divided into two equal parts (cca. 1000 kg each) and pressed by two different technologies:

1.) normal ($O_2$) – in contact with air and

2.) hyperreductive pressing ($N_2$) - in nitrogen atmosphere.

Vinification process started with the pressing of whole grape clusters in the absence of oxygen – hyperreduction and in air atmosphere – normal pressing. 30 mg/L of $SO_2$ and 20 mg/L of ascorbic acid were added to the grapes at the moment of pumping the grape

into the press. Different pressing fractions were analysed for the following parameters: glutathione, hydroxycinnamic acids and their esters and low molecular weight flavan-3-ols – vanillin index. The same compounds were also investigated during fermentation and before and after each treatment that was made in the vinification chain and during wine ageing. Before juice sedimentation, 20 g/hL bentonite (Bentogran, AEB, Brescia, Italy) was added to the juice and after 24 h the clear juice was racked from the grape lees. Wine fermentation was carried out at 18-20°C with two commercial yeast strains (10 g/hL VL3, Laffort Oenologie Bordeaux, France; and 10 g/hL BCS 103 Springer Oenologie, Maison-Alfort, France) and yeast nutrient (Biostimol, Polo Enologia, Oderzo, Italy) was added before fermentation. During fermentation, oxygen (single addition 5 mg/L) was added to stimulate yeast growth and prevent $H_2S$ formation. After fermentation, wines were racked in the protected atmosphere from gross lees, sulphited and aged in contact with lees. Stirring of the yeast lees was performed twice per month.

The $O_2$ concentration in the press was measured in the buffer tank with oxygenmeter, Oxymet (Isolcell, Laives, Italy). Pressing cycles for Muller Thurgau are indicated in Figure 19. Chardonnay and Greco di Tufo variety had a slightly different pressing management, but with no remarkable differences.

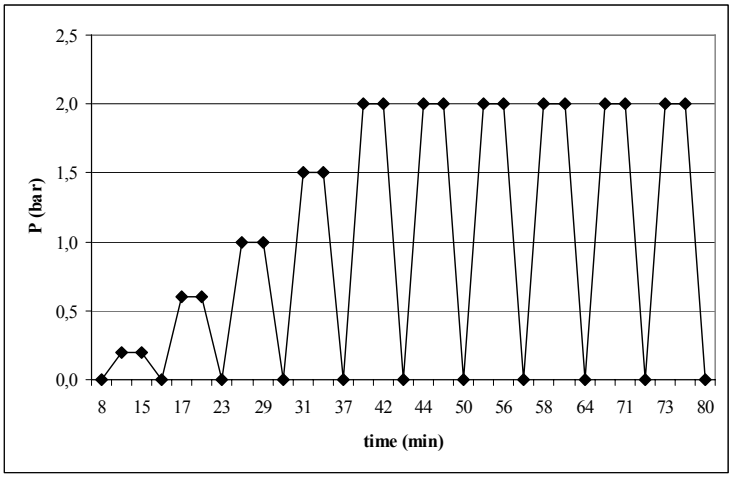



Figure 19: Diagram of pressing cycles of Muller Thurgau grapes

Slika 19: Diagram ciklusa stiskanja grozdja sorte Muller Thurgau

**Sampling**

Samples of each pressing fraction were collected from the buffer tank and from the tube connecting press and buffer tank. Before sampling 1 g/L of $K_2S_2O_5$ and 0.5 g/L of ascorbic was added into the 0.75 L bottle. Bottles were also sparged with inert gas before sampling. Samples were analyzed in the same day or were stored at 4 °C during the night. Samples of wine during wine ageing were taken without addition of $SO_2$ or ascorbic acid into the sampling bottles.

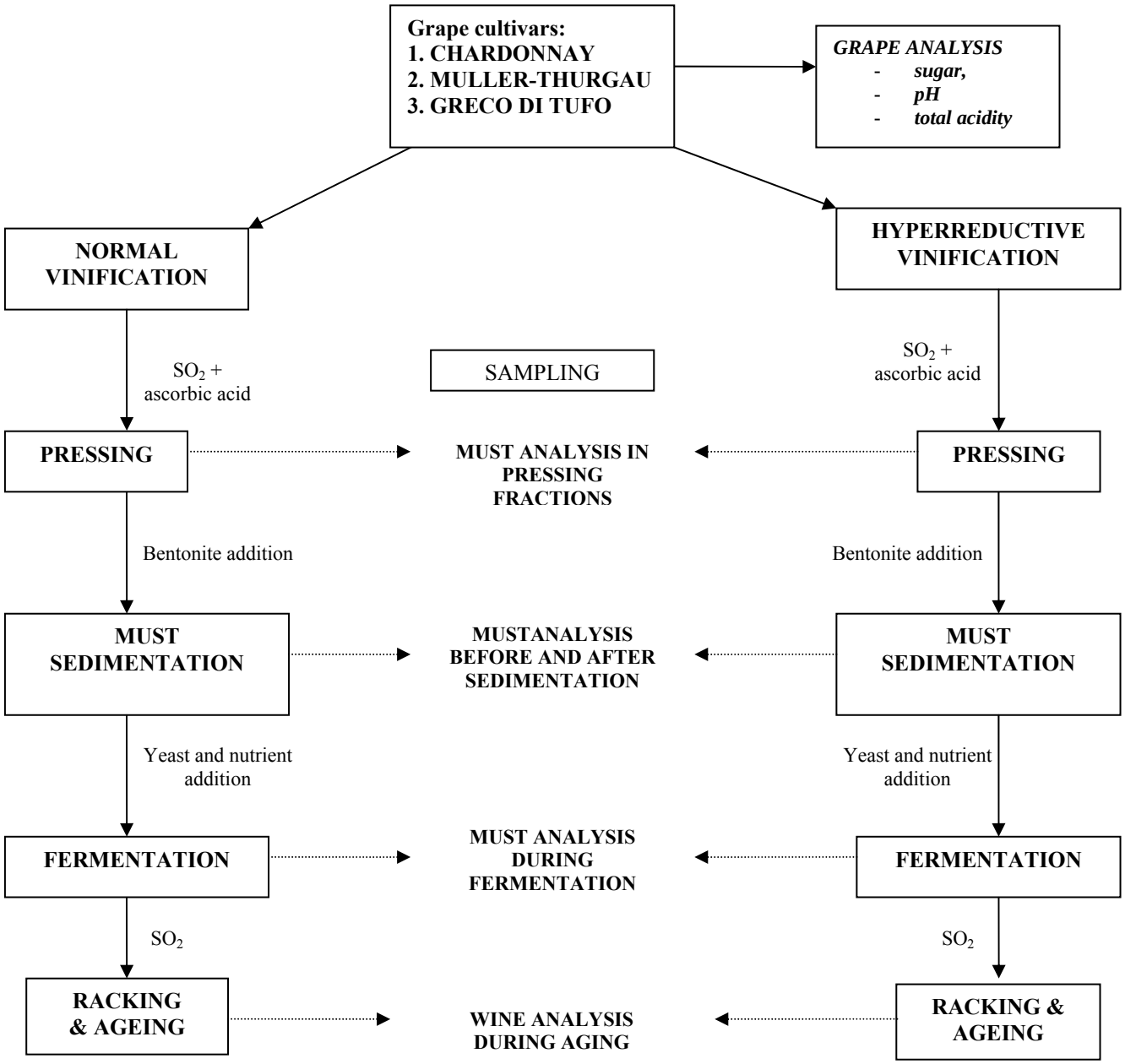



Figure 18: Flow sheet of industrial trial

Slika 18: Shematski potek industrijskega poskusa

### 3.2.3 Analytical methods

#### 3.2.3.1 Standard analytical methods

Analyses included pH, total acidity, alcohol, residual sugar, volatile acidity, extract, free and total $SO_2$. Different methods were used in different laboratories. Standard analysis of wine produced in Slovenia was made according to the Official Journal of European Communities, Commission Regulation ECC Methods for Wine established and accredited at Agricultural Institute of Slovenia (Table 4).

Table 4: Review of analytical methods and apparatus applied for must and wine analyses
Preglednica 4: Pregled analitičnih metod in aparatur uporabljenih pri analizah mošta in vina

| Parameter | Principe of the method | Apparatus | Reference |
|---|---|---|---|
| Relative density | Measurement of density at 20°C | Resonant U-tube | EEC, No. 355/2005 |
| Alcohol | Distillation and density measurement | Resonant U-tube | EEC, No. 355/2005 |
| Sulphur dioxide | Direct iodometric titration | / | EEC, No. 2676/90 |
| Reducing sugars | Iodometrically | / | EEC, No. 2676/90 |
| pH | Potentiometric | Mettler Toledo DL 53 | ECC, No. 2676/90 |
| Titritable acidity | Potentiometric | Mettler Toledo DL 53 | ECC, No. 2676/90 |
| Volatile acidity | Distillation and titration | Oenoextracteur Chenard (APA 107) | ECC, No. 2676/90 |
| Total dry extract | Calculation from density | / | ECC, No. 2676/90 |
| Dry matter | Refractometric | Refractometer | ECC, No. 2676/90 |

Standard analyses (pH, sugar, titritable acidity, volatile acidity) of wines treated in South Africa were made with the GrapeScan FT 120 instrument (Foss Electric, Hillerod, Denmark). The instrument utilizes Fourier transform infrared spectroscopy (FT-IR). Sulfur dioxide analyses were done potentiometrically with the Metrohm titration unit (Metrohm Ltd., Switzerland). Standard analyses (pH, titratable acidity, dry matter) of juice from Italy were made at Pojer & Sandri winery using refractometer and pH meter.

3.2.3.2 LC-MS-MS analysis of glutathione

A Waters Quattro micro API triple quadropole mass spectrometer with a 2690 Alliance HPLC was used for LCMSMS analysis. Separation was performed on a Waters Atlantis C18, 3 μm, 2.1x150 mm column using a 0.1% formic acid (solvent A) to acetonitrile (solvent B) gradient. The solvent composition was kept at 100% solvent A for the first 0.5 min, followed by a linear gradient over 6.5 minutes to 80% solvent B and re-equilibration to 100% A for 7 min. The reduced glutathione eluted at 3.5 minutes and oxidized at 5.2 minutes.

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

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

**Stability of reduced glutathione in must during the storage.**

The stability of reduced glutathione over time was also assessed to see the effect of storage on glutathione. Hanepoot table grapes were pressed in the hyperreductive manner with the small scale custom build press and the juice collected into vials as other sample preparation procedure. Three of these vials contained juice with no $SO_2$ and ascorbic acid added while three more vials contained the same juice with the addition of ascorbic acid and $SO_2$ (at 500 mg/L and 1000 mg/L, respectively). The reduced glutathione concentrations of these six vials were analyzed by the LC-MS-MS system on the same day. Additional vials containing $SO_2$ and ascorbic acid were also stored at 4 ºC or –20 ºC and analyzed after four weeks of storage. In addition, three vials, stored at –20 ºC with $SO_2$ and ascorbic acid were thawed after two weeks, refrozen and thawed again for analyses after four weeks of

storage. This was done to asses the effect of repeated thawing on reduced glutathione levels.

3.2.3.3 HPLC analysis of hydroxycinnamic acids and their esters in grape juice and wine

An Agilent 1100 HPLC with DAD connected to an Agilent NDS ChemStation (Agilent Technologies, Palo Alto, USA) was used for HCA detection and quantification. Separation was performed using an ODS Hypersil C18 column 2.1x250 mm (5 μm) with an ODS Hypersil guard column 2.1x20 mm (5 μm) (Agilent Technologies, Palo Alto, USA). The mobile phase consisted of A: 0.5% formic acid in water and B: 2% formic acid in methanol. Separation was carried out at 40 °C for 33 min. The gradient conditions were linear starting at 16% B, to 25% B in 15 min, to 43% B in 13 min, to 100% B in 0.1 min, 100% B for 4.9 min and back to 16% B in 0.1 min. The column was equilibrated for 10 min prior to each analysis. The flow rate was 0.4 mL/min and the injection volume was 10 μl. The UV-VIS spectra were recorded from 220 to 700 nm, with detection at 320 nm.

3.2.3.4 HPLC analysis of other phenolic compounds in white and red wine

Reverse Phase High Performance Liquid Chromatography was performed on an Agilent 1100 series HPLC system equipped with a diode array detector (Agilent Technologies, Palo Alto, CA, USA). Data processing was done with Chemstation software (Hewlett-Packard, Waldbronn, Germany). A 100 mm x 4.6 mm Chromolith Performance RP-18e column and pre-column was used (Merck, Darmstadt, Germany). The mobile phases used were: Solvent A containing de-ionized water adjusted to a pH of 2.04 with Ortho-phosphoric acid (Riedel-de Haën, Seelze, Germany), and Solvent B, consisting of Acetonitrile (Chromasolve, Riedel-de Haën, Seelze, Germany) with 20% of Solvent A. A flow rate of 2 mL/min was used and column temperature was maintained at 35 ºC. The gradient profile used can be seen in Table 5.

Table 5: Gradient profile of the HPLC analysis of phenolic compounds

Preglednica 5: Gradientni profil pri HPLC analizi fenolnih komponent

| Time (min) | % Solvent A | % Solvent B |
|---|---|---|
| 0 | 99 | 1 |
| 2 | 99 | 1 |
| 17 | 96 | 4 |
| 31 | 90 | 10 |
| 55 | 84 | 16 |
| 75 | 75 | 25 |
| 80 | 20 | 80 |
| 84 | 20 | 80 |
| 85 | 99 | 1 |

Quantification was done using external standards: (+)-catechin hydrate, gallic acid, vanillic acid, *p*-coumaric acid, malvidin-3-glucoside, ellagic acid, quercetin-3-glucoside and quercetin.

Flavan-3-ols were quantified at 280 nm as mg/L catechin, benzoic acids at 280 nm as mg/L vanillic acid, cinnamic acids at 320 nm as mg/L *p*-coumaric acid, anthocyanins at 520 nm as mg/L malvidin-3-glucoside, flavonol-glucoside at 360 nm as mg/L quercetin-3-glucoside and flavonol aglycones at 360 nm as mg/L quercetin.

### 3.2.3.5 Vanilin Index

The catechins and proanthocyanidins reactive to vanillin were analyzed according to the optimized and controlled vanillin – HCl method following the conditions described by Di Stefano et al. (1989b). White wine was diluted (2-5 times, to obtain a final reading between 0.2 and 0.4 AU) with 0.5 M $H_2SO_4$, and 2 mL of the solution was loaded on a conditioned Sep-Pak. The column was washed with 2 mL of 5 mM $H_2SO_4$ and the flavanols were eluted with 5 mL of MeOH into test tube. One milliliter of the methanolic solution containing the flavanols was placed in a test tube (shielded from light) together with 6 mL of vanillin (4% in MeOH) and immersed in a water bath at 20°C. A 3 mL of concentrated HCl was carefully added. After exactly 15 min, the absorbance of pink complex was read at 500 nm in a 10 mm cell against a blank prepared in the same conditions, containing MeOH instead of vanillin. Concentrations were calculated as (+)-catechin (mg/L) by means of a calibration curve (Rigo et al., 2000).

#### 3.2.3.6 Colour

Brown colour was determined by direct colorimetric reading of undiluted sample at 420 nm (1 mm).

## 3.3 MICROOXYGENATION OF RED WINE

### 3.3.1. Grapes

Different varieties of grapes were used for the microoxygenation experiment. Merlot 2004 and Cabernet Sauvignon 2005 grapes came from the Vipava Valley in Slovenia while Pinotage 2004 and Cabernet Sauvignon 2006 was from the Stellenbosch region in South Africa.

### 3.3.2 Technological experiments

Various microoxygenation treatments were made at different commercial cellars which participated with different red wines, with the aim of seeing the effect of different oxygen levels and oak segments on the phenolic profile of wine. They are listed in Table 6 with the dosage of $O_2$ added to each tank. In all the wines, a control tank of similar size was kept which received no microoxygenation. All wines were treated with microoxygenation equipment supplied by Parsec from Italy. This equipment doses the required $O_2$ dosage in mg/L/month.

Table 6: Microoxygenation treatments applied in experiments

Preglednica 6: Uporabljeni pogoji mikrooksigenacije pri poskusih mikrooksigenacije

| Experiment | Cultivar and vintage | Origin of wine | Treatment |
|---|---|---|---|
| 1 | Merlot 2004 | Vipava Valley Slovenia | 6 tanks were microoxygenated in combination with oak blocks and different oxygen concentration (Table 7) |
| 2 | Cabernet Sauvignon 2005 | Vipava Valley Slovenia | 6 tanks were microoxygenated in combination with oak blocks and different oxygen concentration. 5 and 3 mg/L/month oxygen was added during the wine aging. The same wine was also matured in a new oak barrel. |
| 3 | Pinotage 2004 | Stellenbosch, South Africa | 0, 1.5 and 3 mg $O_2$/L/month with oak staves starting seven months after the completion of malolactic fermentation. The same wine was also matured in an oak barrel of the same wood as the staves used (USA MT+). |
| 4 | Cabernet Sauvignon 2006 | Stellenbosch South Africa | Oxygen addition starts just after the finished malolactic fermentation. Oak staves made of the same wood as barrels were added in two different internal surface areas of 225 L oak barrel. |

#### 3.3.2.1 Experiment 1: Merlot 2004

60.000 kg of Merlot (vintage 2004, Biljenski griči, Slovenia) grape was destemmed, crushed, sulphited (50 mg/L) and crushed into Gymar Selector System (Occimiano, Italy) vinificator, where maceration and fermentation was performed. Rehydrated yeast (20 g/hL) (Fermol Rouge, AEB, Brescia, Italy) and yeast nutrient (20 g/hL Enovit, AEB, Brescia, Italy) was added just after crushing. 20 g/hL tannins (Fermotan, AEB, Brescia, Italy) was also added at the beginning. Total polyphenols and anthocyanins were analyzed during maceration which lasted for 7 days. After the maceration, malolactic bacteria 1 g/hL (Biolact, Pascal-Biotech, Paris, France) was added and wine temperature was maintained at 22°C. The malolactic fermentation lasted for 3 weeks and the wine was sulphited and racked into 6 tanks of 7000 L each. The tanks were 5 m high. Microoxygenation at two different oxygen concentrations was performed, starting with 10 and 30 mg/L/month and slowly decreased to lower concentration (Table 7). The decision for oxygen reduction was made on the basis of sensory trials. Medium-toast French oak blocks at 1 g/L concentration were added into three tanks to simulate barrel aging. Oxygen additions were the same as in tanks without oak.

Table 7: Oxygen addition (mg/L/month) management in the experiment 1
Preglednica 7: Dinamika dodajanja kisika (mg/L/mesec) pri poskusu 1

| Date | Weeks | Control | Low | High |
|---|---|---|---|---|
| 19th November 2004 | 0 | 0 | 10 | 30 |
| 20th December 2004 | 5 | 0 | 10 | 30 |
| 3rd January 2005 | 7 | 0 | 10 | 10 |
| 17th January 2005 | 9 | 0 | 5 | 5 |
| 24th January 2005 | 10 | 0 | 3 | 3 |
| 26th January 2005 | 10 | 0 | 1 | 3 |
| 7th April 2005 | 20 | 0 | 0 | 0 |

During and after the microoxygenation different analyses followed: colour intensity and colour hue, colour distribution, total polyphenols, total anthocyanins, proanthocyanidins, vanillin index (low molecular weight flavan-3-ols). HPLC analysis of anthocyanins profile and flavanols were also performed at the end of microoxygenation. mDP was analyzed with LC-MS-MS.

#### 3.3.2.2 Experiment 2: Cabernet Sauvignon 2005

The second microoxygenation experiment (Cabernet Sauvignon 2005) started 5 months after the fermentation in the same tanks and by the same procedure as in the previous year. Grapes were desteemed, crushed, sulphited (50 mg/L) and filled into Gymar Selector System (Occimiano, Italy) vinificator. Rehydrated dry yeast (20 g/hL) Wineferm Rouge Cru (AEB, Bresica, Italy) and 20 g/hL yeast nutrient (Enovit, AEB, Brescia, Italy) was added at the beginning of fermentation. After maceration malolactic bacteria (1 g/hL Biolact, Pascal-Biotech, Paris, France) were added and malolactic fermentation was performed within 3 weeks. After malolactic fermentation the wine was racked into another tank and sulphited. 5 months after fermentation the wine was transferred into 6 tanks of 7000 L each and the microoxygenation was performed. However, lower concentrations of oxygen were used than the concentrations in the experiment 1, starting with 5 and 3 mg/L/month and reduced to 3 and 1 mg/L/month after 8 weeks. Medium-toast French oak blocks at 1 g/L concentration were added into three tanks. Parallel aging in new oak barrels (USA, MT) was also applied.

During and after the treatment, colour intensity, colour hue and total polyphenols (OD 280) were followed. Phenolic profile and anthocyanin profile was made at the end of microoxygenation.

#### 3.3.2.3 Experiment 3: Pinotage 2004

In this experiment, Pinotage grapes of the Stellenbosch origin were used. The grapes were destemmed, crushed, sulphited and filled into a static vinificator. Fermentation was conducted with NT116 (Anchor Yeast Biotechnologies, Cape Town, South Africa) at 20 g/hL and diammonium phosphate (Anchor Yeast Biotechnologies, Cape Town, South Africa) at 40 g/hL was added during the fermentation. After 6 days of fermentation and maceration, spontaneous malolactic fermentation followed. The wine was sulphited and after seven months transferred into three 1.5 m high tanks of 1100 L. American oak staves (medium toasted plus, Radoux, Stellenbosch, South Africa) were added to 70% of the internal surface of a 300 L oak barrel, which followed the supplier's (Radoux, Stellenbosch, South Africa) recommendations to simulate an oak barrel. The same

Pinotage wine was also matured in a 300 L American oak barrel (medium toasted plus, Radoux, Stellenbosch, South Africa).

The oxygen rate of microoxygenated wine was 1.5 and 3 mg/L and was constant during the treatment. One tank received no oxygen and was used as a control.

During and after the treatment colour density, colour hue and total phenolics (OD 280) were followed. At the end of microoxygenation treatment detailed phenolics profile was analyzed by HPLC. Microbiology analysis was also performed during the treatment.

#### 3.3.2.4 Experiment 4: Cabernet Sauvignon 2006

The aim of the experiment 4 (Cabernet Sauvignon 2006) was also to compare the influence of barrel ageing and microoxygenation with the addition of oak staves. Cabernet Sauvignon from the Stellenbosch region was vinificated in the same manner as wine in the experiment 3. After destemming and crushing the must was inoculated with NT116 (Anchor Yeast Biotechnologies, Cape Town, South Africa) at 20 g/hL and diammonium phosphate (Anchor Yeast Biotechnologies, Cape Town, South Africa) at 30 g/hL was added during the fermentation. The fermentation and maceration lasted for 7 days in static fermentors. After fermentation, spontaneous malolactic fermentation was conducted. Microoxygenation started just after malolactic fermentation. Four (1100 L) tanks were used in the microoxygenation treatment. Two of them received 5 mg/L/month while the other two were used as a control and did not receive any oxygen. Medium toasted American oak staves (Radeaux, Stellenbosch, South Africa) were added into the tanks at 40% and 100% internal surface area of 225 L barrel oak.

Colour intensity, colour hue and total phenolics (OD 280) were analyzed during and after the treatment. After 6 months the phenolics spectrum was analyzed.

### 3.3.3 Analytical methods

#### 3.3.3.1 Spectrophotometric analyses

Colour intensity and hue were measured with 0.1 cm path length glass cells as described by Glories (1984). Total polyphenols (OD 280) were determined following the methods described by Somers et al. (1978). One millilitre of wine is deluteted 100 times and absorbance at 280 nm is measured in 1.0 cm length glass cells. The result is multiplied with delution factor and expressd without units.

The fractions of co-pigmented anthocyanin, free anthocyanins and polymeric colour pigment content in the red wines were also determined according to the method of Levengood and Boulton (2004).

**Total polyphenols**

The total phenols were assessed by the reduction of phosphotungitic and phosphomolybdic acids (Folin Ciocalteau reagent) to blue pigments by phenols in alkaline solutions (Di Stefano and Guidoni, 1989a). The red wine was diluted (usually 10-20 times) with 0.5 M $H_2SO_4$ and the dilution factor was adjusted to obtain a final reading between 0.3 to 0.6 AU. One milliliter of diluted wine was then slowly loaded on the conditioned Sep-Pak, and the polar substances were removed with 2 mL of 5 mM $H_2SO_4$. The phenolic compounds were eluted into a 20 mL calibrated flask, with 2 mL of MeOH followed by 5 mL of distilled water. One milliliter of Folin Ciocalteau reagent and after 3-4 min, 4 mL of 10% $Na_2CO_3$ was added and the solution was brought to 20 mL with distilled water. After 90 min at 20°C, the absorbance of the sample (filtered through 0.45 μm) was read at 700 nm in a 10 mm cell, against a blank test prepared by using distilled water in place of the wine. Concentrations were determined by means of a calibration curve as (+)-catechin in mg/L (Rigo et al., 2000).

**Total anthocyanins**

An aliquot of 5 mL of red wine diluted (5-20 times, to obtain a final reading in the range of 0.3-0.6 AU) with 0,5 M $H_2SO_4$ was loaded on a conditioned Sep-Pak. The column was washed with 2 mL of 5 mM $H_2SO_4$ and the red pigments were eluted with 3 mL of methanol into 20 mL calibrated flask. A volume of 0.1 mL of concentrated HCl was added,

and the volume was brought to 20 mL with etanol/water/HCl (70:30:1). The total anthocyanins were directly quantified on the basis of the maximal absorbance in the visible range (536-540 nm) against a blank (ethanol/water/HCl 70:30:1). The wine pigment content was calculated in milligrams per liter by assuming the average absorbance of the mixture of anthocyanins (average MW = 500) extracted from Cabernet Sauvignon grape to equal 18800 M (Glories, 1984, Di Stefano et al., 1989b). The conventional numerical value thus obtained can be easily converted in other common units by means of the appropriate absorbance of available standard compounds (Rigo et al., 2000).

**Proanthocyanidins**

The proanthocyanidins were evaluated according to Di Stefano et al. (1989b) by transformation into cyanidin. This method was the original acid butanol assay of Swan and Hills optimized by adding adequate amounts of iron salts as catalyst to increase the reproducibility of yield of cyanidin and by replacing the toxic solvent n-butanol with the optimal percentage of ethanol (Rigo et al., 2000).

An aliquot of 2 mL of red wine diluted (10-20 times) with 0.05 M $H_2SO_4$ was loaded in a conditioned Sep-Pak. The column was washed with 2 mL of 5 mM $H_2SO_4$ and proanthocyanidins were eluted with 3 mL of methanol, collected into a 50 mL flask shielded from light (aluminum foil) and containing 9.5 mL absolute ethanol. An amount of 12.5 mL of $FeSO_4*7H_2O$ (300 mg/L) in concentrated HCl was added, and the flask was then placed in a boiling water bath and refluxed for 50 min, after which time it was rapidly cooled by immersion in cold water (20°C). The spectrum from 380 to 700 nm was recorded in a 10 mm cell after 10 min against a blank (water). The tangent from the minimum (approx. 450 nm) was drawn, and the absorbance between the maximum (approx. 550 nm) and a slope of tangent was calculated. To subtract natural anthocyanins present in the sample, the corresponding value of the wine prepared under the same conditions and placed in ice instead of warming was subtracted to obtain the net value of absorbance. Under such conditions the average yield has been estimated from the author to be 20% (Di Stefano et al., 1989b) and the proanthocyanidin concentration (mg/L) can be conventionally expressed as 5 times the amount of cyanidin formed by means of a calibration curve with cyanidin chloride ($\varepsilon$ = 34700 $nmLcm^{-1}mg^{-1}$, according to Di Stefano et al., 1989b).

**Vanilin Index**

The method by Rigo et al. (2000) was used and is described in chapter 3.2.3.5

3.3.3.2 HPLC analysis of anthocyanins in red wines

Clean-up of red wine samples using a solid phase extraction

Red wine samples were purified using a solid phase extraction (SPE) before being injected into HPLC. The SPE cartridge was previously conditioned with 4 mL methanol followed by 10 ml water. The wine sample was diluted 4-5 times in water (it should remain less than 3% ethanol in a sample) and quantitatively loaded on the cartridge. The cartridge was washed with 6 ml of 0.3% perchloric acid in water, dried with nitrogen and the sample was eluted with 5-10 ml methanol in 50 mL flask. The methanol fraction was evaporated at 38 °C to dryness under the reduced pressure using a rotavapor. The residue was redissolved in 0.5-1.0 ml of a mixture suitable for HPLC (0.3% perchloric acid in water (70:30, v/v) and 30% of methanol (HPLC grade). Samples were filtered through a 0.22 μm PVDF syringe driven filter and analyzed by HPLC within 2 hours.

**Analysis of anthocyanins in red wines**

An Agilent 1100 HPLC with DAD detector coupled to an Agilent NDS ChemStation (Agilent Technologies, Palo Alto, USA) was used for anthocyanin detection and quantification. Separation was performed using a column Purospher C18 250x4.6 mm (5 μm) with a guard column Purospher C18 4x4 mm (5 μm) (Merck, Darmstadt, Germany). The mobile phase consisted of A: 0.3% perchloric acid in water and B: 100% methanol. Separation was carried out at 35 °C for 50 min. The gradient conditions were linear starting at 28.5% B, to 51% B in 42 min, to 68.5% B in 3 min, to 100% B in 2 min, 100% B for 3 min. The column was equilibrated for 6 min prior to the next analysis. The flow rate was 0.55 mL/min and the injection volume was 10 μL. The UV-VIS spectra were recorded from 200 to 700 nm, with the detection at 520 nm.

3.3.3.3 HPLC analysis of other phenolic compounds in red wine

The method used is described in chapter 3.2.3.4

3.3.3.4 Analysis of colour fractions

The fraction of copigmented anthocyanin, free anthocyanins and polymeric colour pigment content in the red wines were determined according to the method of Boulton et al. (1996). The method measures the absorbance of a sample altered by dilution together with those resulting from additions of acetaldehyde or sulphur dioxide. After pH adjustment to 3.6 and filtering the wine 0.45µm pore size filter, the following absorbance readings at 520 nm are measured:

$A^{acet}$: 20 µL 10% acetaldehyde solution is added to 2 mL of wine sample in a 10 mm cuvette. After 45 min the wine sample is measured at 520 nm

$A^{20}$: the wine sample is diluted 1/20 by placing 100 µL wine and 1900 µL buffer in a cuvette with 10 mm pathlength. The absorbance is measured at 520 nm after 10 minutes. The reading is corrected for dilution by multiplying by 20.

$A^{SO2}$: 160 µL 5% SO2 solution is added to 2 mL of wine sample in a cuvette with 10 mm pathlength. The absorbance is measured at 520 nm after 10 minutes.

The following equitations are used to calculate the colour contributions: $SO_2$

Colour due to Copigmentation (AU): $[C] = (A^{acet} – A^{20})$

Colour due to Anthocyanins (AU): $[C] = (A^{20} – A^{SO2})$

Colour due to Polymeric Pigment (AU): $[C] = A^{SO2}$

3.3.3.5 LC - MS analysis of mean degree of proanthocyanidin polymerization (mDP)

The proanthocyanidin mDP and the percentage of monomers were measured according to the method published by Mattivi et al. (2006b). Analysis of the flavanols and of their benzyl-thioethers were carried on a Waters 2690 HPLC system equipped with a Waters 996 DAD, Micromass ZQ electrospray ionization-mass spectrometer (ESI-MS) in negative mode. Separation was performed using a column Xterra MS C18, 3.5 µm, 2.1 x 150 mm (Waters). The flow rate was 0.25 mL/min and the injection volume was 5 µL. The mobile phases were 2.5% acetic acid in $H_2O$ (A) and acetonitrile (B). The separation of flavanol monomers was carried out at 40 °C over 30 min under the following conditions: linear gradients ranging from 0% B to 20% B over 30 min. The column was then washed with 95% B for 1 min and afterwards equilibrated for 7 min prior to each analysis. The gradient

for the analysis after thioacidolysis was as described above, up to 30 min, whereafter the run was prolonged for 15 min using up to 60% B in order to also elute the benzyl-thioethers. After each run the column was washed for 4 min with 95% B and equilibrated for 7 min. The UV-VIS spectra were recorded from 210 nm to 400 nm, with detection at 280 nm. The MS detector operated at capillary voltage 3000 V, extractor voltage -6V, source temperature 105 °C, desolvation gas flow (N2) at 450 L/h. The outlet of the HPLC system was split (9:1) to the ESI interface of the mass analyzer. ESI-mass spectra ranging from m/z 100 to 1500 were recorded in negative mode with a dwell time of 0.1 s. Quantification of the flavanols was performed by MS, by means of the external standard method, on their molecular ions $(M-H)^-$.

#### 3.3.3.6 Microbiology analysis

Acetic acid bacteria and *Brettanomyces* yeasts in experiment 3 were enumerated by plating out the wine on selective media at the beginning, after 4, 14 and 20 weeks of microoxygenation. For the enumeration of acetic acid bacteria, the culture medium consisted of 57 g/L Man Rogosa Sharp (MRS) medium (pH to 5 with HCl, 20 g/L agar) to which 20% of sterile red wine was added after sterilization. This medium was supplemented with 7 mg/L penicillin (Sigma-Aldrich) and 50 mg/L pimaricin (Actistab, Gistbrocades, Anchor Yeast Biotechnologies) to eliminate lactic acid bacteria and yeast, respectively. *Brettanomyces* strains were isolated on a YPD medium [containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose (pH 4.8 adjusted with HCl, 20 g/L agar)], supplemented with 50 mg/L chloramphenicol (Sigma) and 300 mg/L cyclohexamide (Sigma) to eliminate bacteria and non-*Brettanomyces* yeasts, respectively. Plates were incubated at 30 °C for 10 days before counting the colonies.

### 3.4 SENSORY EVALUATION

Various sensory evaluations were used for different wine treatments. In the hyperreduction and microoxygenation trials, triangle test were used. All samples were presented for assessment in 30-mL aliquots in three-digit-coded, covered ISO standard tasting glasses under red lights at room temperature.

## 3.5. STATISTICAL ANALYSIS

The results from hyperreductive and microoxygenation treatments were statistically analyzed with Statgraph program. Triangle tests were tested at the 0.05 and 0.01 probability level using the significance in triangle test (p=1/3).

# 4. RESULTS

## 4.1 HYPERREDUCTION

### 4.1.1 Validation of LC-MS-MS method for glutathione in juice and wine

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

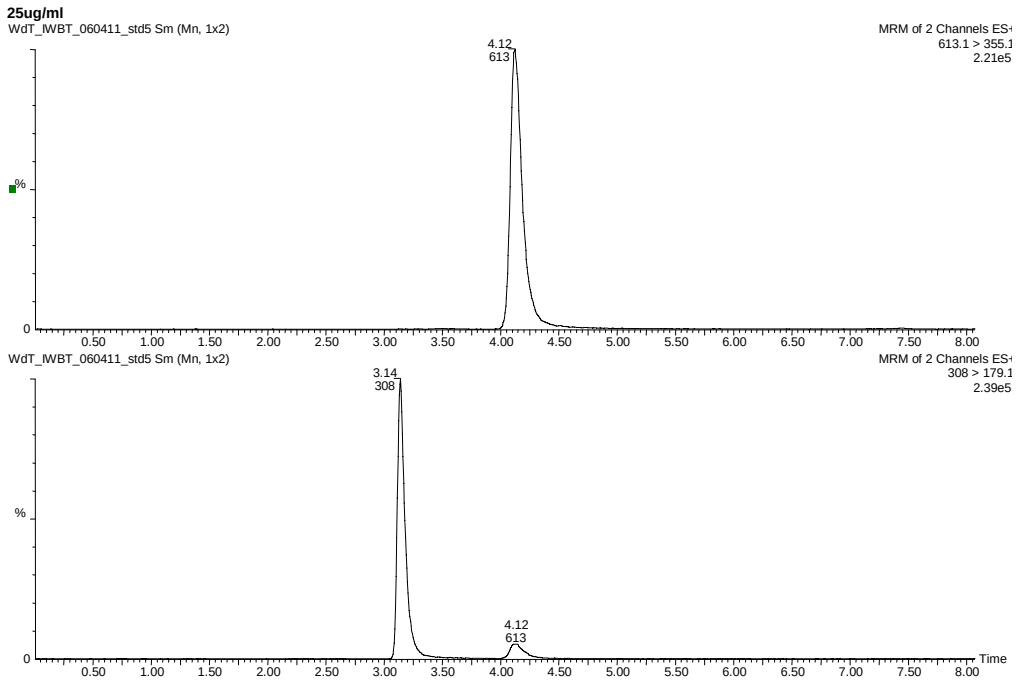



Figure 20: LC-MS-MS chromatograms of oxidized (4.12 min) and reduced (3.14 min) glutathione standards.
Slika 20: LC-MS-MS kromatogram oksidirane (4.12 min) in reducirane (3.14 min) oblike glutationa (standard)

The method conditions are described in chapter 3.2.3.2

The limit of detection for the method (signal to noise of 3:1) was 0.2 mg/L for oxidized and 0.4 mg/L for reduced glutathione at an injection volume of 5 µL with the upper limit of the linear range 200 mg/L. Relative standard deviation (RSD) for 6 injections was 0.4% in the 40 mg/L range and the limit of quantitation for both compounds was 0.8 mg/L. The limit of quantitation was taken as the lowest concentration where the % RSD of 6 injections was better than 20%.

The reduced glutathione concentrations in grape juice during the stability test can be seen in Figure 21. It is clear that the samples in the absence of $SO_2$ and ascorbic acid oxidized rapidly, with reduced glutathione levels dropping significantly compared to the same samples that received $SO_2$ and ascorbic acid. After 4 weeks reduced glutathione concentrations in the samples stored at 4 ºC decreased slightly (thought not significantly), but remained constant in the samples frozen at -20 ºC to which high concentrations of $SO_2$ and ascorbic acid were added. Even when a sample was thawed twice before analyses, the reduced glutathione levels stayed relatively constant over the 4 weeks period (Du Toit et al., 2007).

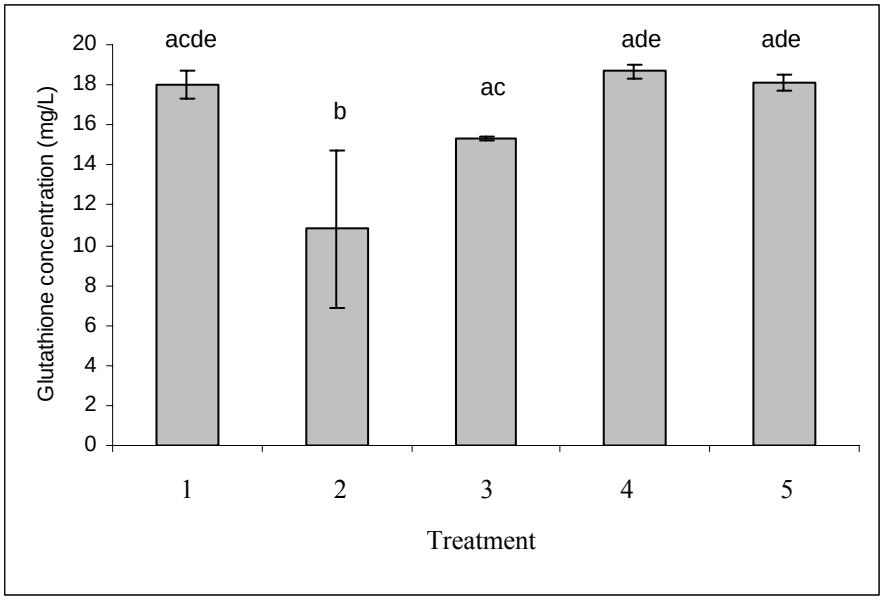



Figure 21: Reduced glutathione levels in grape juice stored under different conditions

Legend:

1 = samples analyzed immediately after pressing, with 1000 mg/L $SO_2$ and 500 mg/L ascorbic acid added;

2 = samples analyzed immediately after pressing, with no $SO_2$ or ascorbic acid added;

3 = samples stored at 4 ºC for 4 weeks;

4 = samples stored at -20 ºC for 4 weeks. Sample thawed only once;

5 = samples stored at -20 ºC for 4 weeks. Sampled thawed twice. All the samples analyzed at week 4 had 1000 mg/L $SO_2$ and 500 mg/L ascorbic acid added initially.

Error bars indicate the standard deviation from the mean. Different letters indicate significant differences ($p \leq 0.05$).

Slika 21: Koncentracija reduciranega glutationa v moštu, shranjenega v različnih pogojih.

Legenda:

1 = vzorec analiziran takoj po stiskanju z dodanima 1000 mg/L $SO_2$ in 500 mg/L askorbinske kisline;

2 = vzorec analiziran takoj po stiskanju brez dodatka $SO_2$ in askorbinske kisline

3 = vzorec hranjen 4 tedne pri 4 °C;

4 = vzorec hranjen 4 tedne pri -20 ºC. Vzorec odmrznjen samo enkrat;

5= vzorec hranjen 4 tedne pri -20 ºC. Vzorec dvakrat odtajen. Vsi vzorci analizirani pri 4 tednih so imeli v začetku dodano 1000 mg/L $SO_2$ in 500 mg/L askorbinske kisline.

Odstopanja označujejo standardno deviacijo od povprečja. Različne črke označujejo značilne razlike ($p \leq 0,05$).

### 4.1.2 Small scale hyperreduction pressing

Grapes used for experiments were picked at optimal maturity and baseic chemical parameters of must are described in Table 8.

Table 8: Chemical parameters of grapes used for hyperreductive pressing experiment

Preglednica 8: Kemijski parametri grozdja uporabljenega pri hiperreduktivnem poskusu

| **Experiment** | **Variety and Origin** | **°Brix** | **Titratable acid (g/L as tartaric acid)** | **pH** |
|---|---|---|---|---|
| Sauvignon 1 | Sauvignon Blanc, Elgin | 20.0 | 4.98 | 3.13 |
| Colombar | Colombar, Robertson | 20.9 | 5.19 | 3.12 |
| Sauvignon 2 | Sauvignon Blanc, Stellenbosch | 19.2 | 4.20 | 3.21 |

#### 4.1.2.1 Glutathione (reduced and oxidised)

Glutathione (reduced and oxidised) was analysed after pressing and after fermentation. The results are indicated in Tables 9 and 10. The results represent average values of three pressings for each treatment.

Table 9: Reduced glutathione (mg/L) in juice and wine in different pressing treatments

Preglednica 9: Reducirani glutation (mg/L) v moštu in vinu v različnih pogojih stiskanja

| **Treatment** | **Sauvignon Blanc (Elgin)** | | **Colombar** | | **Sauvignon Blanc (Stellenbosch)** | |
|---|---|---|---|---|---|---|
| | Juice | Wine | Juice | Wine | Juice | Wine |
| Hyperreductive | 32.6 a* | 8.7 a | 52.8 a | 13.0 a | 71.2 a | 34.2 a |
| Normal | 27.1 b | 6.3 b | 48.2 a | 11.4 a | 63.5 b | 16.5 b |
| Oxidative | 3.5 c | 1.9 c | 7.9 b | 6.6 b | 12.2 c | 1.2 c |

* Means in columns followed by the same letter are not significantly different by Duncan's multiple range test p=0.05

Table 10: Oxidised glutathione (mg/L) in juice and wine pressed with different treatments

Preglednica 10: Oksidiran glutation (mg/L) v moštu in vinu v različnih pogojih stiskanja

| **Treatment** | **Sauvignon Blanc (Elgin)** | | **Colombar** | | **Sauvignon Blanc (Stellenbosch)** | |
|---|---|---|---|---|---|---|
| | Juice | Wine | Juice | Wine | Juice | Wine |
| Hyperreductive | 0.46 a* | nd | 1.7 a | nd | 1.7 a | nd |
| Normal | 0.47 a | nd | 1.7 a | nd | 1.8 a | nd |
| Oxidative | 0.80 b | nd | 2.9 b | nd | 2.3 b | nd |

nd - not detectable

* Means in columns followed by the same letter are not significantly different by Duncan's multiple range test p=0.05

It is clearly seen that pressing with differnet $O_2$ concentrations present greatly affects the reduced glutathione level. Pressing in very reductive conditions prevents glutathione degradation and higher concentrations were observed in all three grape varieties. The concentration of the reduced glutathione in hyperreductive pressings ranged from 33-71 mg/L. The highest concentration of the reduced glutathione was found in Sauvignon Blanc from the Stellenbosch region (71.2 mg/L). The glutathione profile during the fermentation was not analyzed, however glutathione concentration decreased after fermentation in all treatments.

The level of oxidised glutathione ranged from 0.46-2.3 mg/L, however its concentration is significantly higher only in oxidative treatment, while there is no difference between the hyperreductive and normal treatment. The oxidised glutathione concentration in wine was under the limit of detection.

### 4.1.2.2 Hydroxycinnamic acids, their tartaric esters, (+)-catechin and (–)-epicatechin

HPLC analyses of juices and wines were performed to asses the contents of hydroxycinnamic acid, their tartaric esters, (+)-catechin and (–)-epicatechin. The results are indicated in Table 11.

Table 11: Phenolic content in juices and wines at different pressing treatment

Preglednica 11: Vsebnost fenolnih spojin v moštu in vinu pri različnih načinih stiskanja

| | (+)-catech. | (–)-epicat. | *trans*-CTA | *trans*-CoTA | GRP | *trans*-FTA | caffeic | *p*-coumaric | ferulic |
|---|---|---|---|---|---|---|---|---|---|
| **Sauvignon Blanc** (Elgin) | | | | | | | | | |
| **JUICE** | | | | | | | | | |
| hyperreductive | 5.41 b* | 11.15 b | 37.69 c | 0.47 b | 0.76 a | 3.83 b | 0.00 a | 0.20 b | 0.00 a |
| normal | 4.15 a | 7.43 a | 22.90 b | 0.33 a | 2.99 b | 2.28 a | 0.00 a | 0.20 b | 0.00 a |
| oxidised | 7.79 c | 10.47 b | 12.82 a | 0.42 b | 11.64 c | 1.63 a | 0.00 a | 0.00 a | 0.00 a |
| **WINE** | | | | | | | | | |
| hyperreductive | 3.98 c | 11.20 b | 35.01 c | 0.43 b | 0.40 a | 3.32 c | 0.46 c | 0.16 a | 0.30 b |
| normal | 3.54 b | 9.91 ab | 26.98 b | 0.41 ab | 2.93 b | 2.82 b | 0.39 b | 0.15 a | 0.29 ab |
| oxidised | 2.86 a | 9.33 a | 7.46 a | 0.36 a | 6.75 c | 1.26 a | 0.27 a | 0.13 a | 0.27 a |
| **Colombar** | | | | | | | | | |
| **JUICE** | | | | | | | | | |
| hyperreductive | 9.50 c | 19.05 b | 30.29 c | 0.60 c | 0.60 a | 3.38 c | 0.00 a | 0.09 b | 0.00 a |
| normal | 8.27 b | 17.31 b | 22.47 b | 0.49 b | 2.17 b | 2.48 b | 0.00 a | 0.10 b | 0.00 a |
| oxidised | 3.62 a | 13.22 a | 3.99 a | 0.37 a | 10.75 c | 1.39 a | 0.38 b | 0.00 a | 0.00 a |
| **WINE** | | | | | | | | | |
| hyperreductive | 3.91 b | 19.49 b | 22.81 c | 0.47 b | 0.13 a | 2.54 c | 0.50 b | 0.19 a | 0.29 a |
| normal | 3.50 ab | 18.37 b | 19.25 b | 0.45 b | 1.78 b | 2.15 b | 0.52 b | 0.35 a | 0.34 ab |
| oxidised | 2.92 a | 15.65 a | 0.83 a | 0.31 a | 8.98 c | 0.41 a | 0.26 a | 0.32 a | 0.40 b |
| **Sauvignon Blanc** (Stellenbosch) | | | | | | | | | |
| **JUICE** | | | | | | | | | |
| hyperreductive | 3.52 a | 13.21 b | 51.06 c | 0.45 b | 0.80 a | 4.31 b | 0.00 a | 0.10 a | 0.00 a |
| normal | 2.94 a | 10.89 a | 35.82 b | 0.34 a | 2.48 b | 2.67 a | 0.00 a | 0.11 a | 0.00 a |
| oxidised | 3.12 a | 11.67 a | 28.45 a | 0.33 a | 10.81 c | 2.60 a | 0.49 b | 0.09 a | 0.00 a |
| **WINE** | | | | | | | | | |
| hyperreductive | 4.95 ab | 13.72 b | 41.42 b | 0.35 b | 0.27 a | 3.29 b | 0.57 a | 0.26 a | 0.47 a |
| normal | 4.69 a | 12.84 a | 37.50 b | 0.38 b | 1.19 b | 2.77 ab | 0.61 a | 0.27 a | 0.50 a |
| oxidised | 5.27 b | 13.35 ab | 22.39 a | 0.29 a | 7.77 c | 2.48 a | 0.54 a | 0.27 a | 0.48 a |

* Means in columns followed by the same letter are not significantly different by Duncan's multiple range test p=0.05

Legend: *trans*-CTA: *trans*-caftaric acid; *trans*-CoTA: *trans*-coutaric acid; GRP: grape reaction product; *trans*-FTA: *trans*-fertaric acid

The results in Table 11 show that *trans*-caftaric acid plays a significant role in wine oxidation. Its value ranged from 30 to 51 mg/L in hyperreductive pressed grape juice while the values in the oxidised treatment are much lower and ranged between 4 and 28 mg/L. The values of *trans*-coutaric acid were very low, ranging from 0.28 to 0.60 mg/L. Free hydroxycinnamic acids (caffeic, *p*-coumaric and ferulic) were found mostly in wine, but at low concentrations, too. (+)-Catechin and (–)-epicatechin concentration between pressings with increased concentration of $O_2$ differed only in Colombar grape juice while there were no differences in the Sauvignon Blanc variety. However, concentrations were very low, which could be due to gentle pressing and low must yields (40%) after grape pressing.

Oxygen and sulphur dioxide play crucial roles in the oxidation process of grape juice. In Figure 22 the consumption kinetics of oxygen was measured in relation to different concentrations of $SO_2$ and ascorbic acid addition. The amount of dissolved oxygen was analyzed in fresh grape juice, pressed under hyperreductive conditions and aerated.

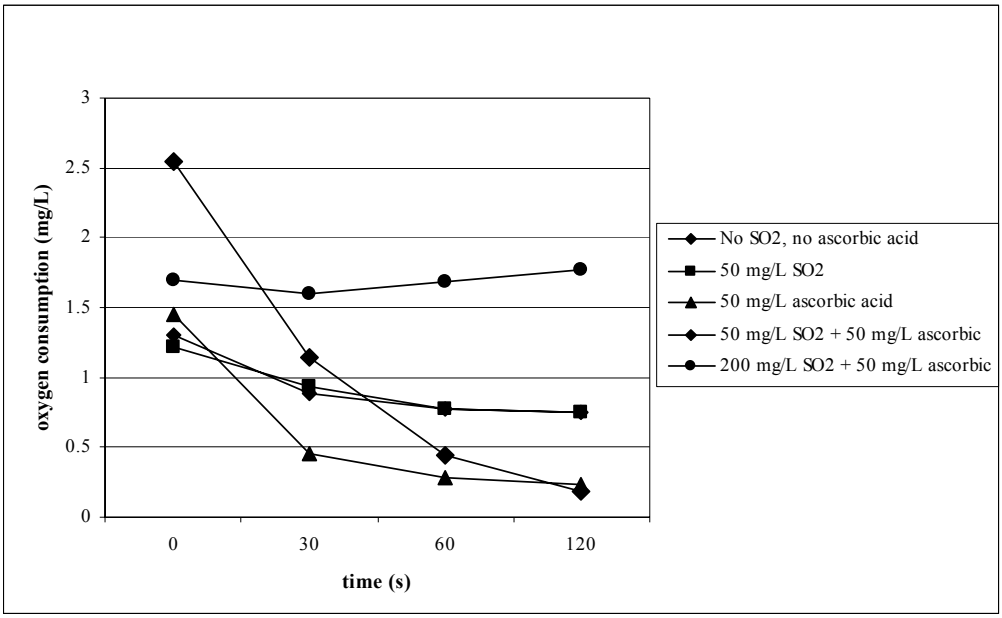



Figure 22: Oxygen consumption in grape juice in relation to different concentrations of sulphur dioxide and ascorbic acid additions in Sauvignon Blanc (Stellenbosch)

Slika 22: Poraba kisika v moštu glede na dodane različne koncentracije žveplovega dioksida in askorbinske kisline pri sorti Sauvignon Blanc (Stellenbosch)

The Figure 22 shows the speed of oxygen consumption in Sauvignon Blanc grape juice originated from Stellenbosch. If no $SO_2$ is added, the oxygen concentration dropped rapidly

to initial values (before aeration). However, addition of 50 mg/L of $SO_2$ retards the oxygen consumption while addition of ascorbic acid alone increased the consumption of $O_2$ due to its antioxidant effect. Addition of 200 mg/L $SO_2$ and 50 mg/L ascorbic acid completely inhibited oxygen consumption and its concentration stayed stable.

#### 4.1.2.3 Sensory evaluation

Triangle tasting of all three wines was performed one month after the fermentation. The panel consisted of 7 experienced tasters. The results can be found in Table 12.

Table 12: Influence of pressing treatment on statistically significant ($p<0.05$) sensory differences in three different wines (Hr: hyperreductive pressing, N: normal, Ox: oxidative)
Preglednica 12: Vpliv načina stiskanja na statistično značilne ($p<0,05$) senzorične razlike v treh različnih vinih (Hr: hiperreduktivno stiskanje, N: normalno, Ox: oksidativno)

| Treatment | Sauvignon Blanc (Elgin) | Colombar | Sauvignon Blanc (Stellenbosch) |
|---|---|---|---|
| **Hr:N** | Yes | Yes | No |
| **Hr:Ox** | Yes | Yes | Yes |
| **N:Ox** | No | No | Yes |

In Sauvignon Blanc wine (Elgin) and Colombar wine there were statistically significant differences ($p<0.05$) in the sensory perception between hyperreductive and normally pressed grapes. Significant differences were also observed between the hyperreductive and oxidative treatment while there was no difference between normal and oxidative pressing. In Sauvignon Blanc from Stellenbosch the panel could not distinguish between hyperreductive and normal pressing treatment. However, there was a significant difference in hyperreductive and oxidative pressing and between normal and oxidative treatment.

### 4.1.3 Industrial hyperreductive pressing

Special wine press made by Mr. Mario Pojer, winemaker from the Trentino region in Italy was used for hyperreductive experiment. Three grape varieties were pressed in hyperreductive ($N_2$) and normal atmosphere ($O_2$).

Table 13: Chemical parameters of crushed grapes (vintage 2005)

Preglednica 13: Kemijski parametri grozdja, vključenega v poskus (trgatev 2005)

| Variety | °Brix | Titrable acid (g/L as tartaric acid) | pH |
|---|---|---|---|
| Muller Thurgau | 18.1 | 6.0 | 3.17 |
| Chardonnay (Sparkling base) | 18.2 | 10.0 | 3.03 |
| Greco di Tufo | 20.6 | 10.1 | 2.98 |

Before crushing the random sample of grapes was taken and analyzed for glutathione and esterified and free hydroxycinnamic acid content (HCA). The results are shown in Table 14. Three varieties (Chardonnay, Muller Thurgau and Greco di Tufo) were used for hyperreductive experiments; however the analysis of two other varieties (Sauvignon Blanc and Malvasia) were also performed to see the content of glutathione and HCA in their flesh and skins.

### 4.1.3.1 The contents of hydroxycinnamic acids, their tartaric esters and glutathione

Table 14: Glutathione and hydroxycinnamic acids and their tartaric esters content in grapes

Preglednica 14: Vsebnost glutationa in hidroksicimetnih kislin ter njihovih estrov v grozdju

| **Variety** | | **reduced glutath.** | **oxydised glutath.** | *cis* - **CTA** | *trans* - **CTA** | *cis* - **CoTA** | **GRP** | *trans* - **CoTA** | **FTA** | *trans* - **caffeic** | *trans* -p- **coumaric** | *trans* - **ferulic** | sum **(HCA)** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | mg/kg | mg/kg | mg/kg | mg/kg | mg/kg | mg/kg | mg/kg | mg/kg | mg/kg | mg/kg | mg/kg | mg/kg |
| Chardonnay | | | | | | | | | | | | | |
| | Flesh | 22.73 | nd | nd | 39.73 | 0.44 | 0.45 | 2.53 | 1.25 | nd | nd | nd | 44.40 |
| | Skins | 13.39 | nd | 2.74 | 65.96 | 6.96 | n.d. | 23.13 | 0.87 | nd | nd | nd | 99.66 |
| | | | | | | | | | | nd | nd | nd | |
| Muller Thurgau | Flesh | 11.22 | nd | nd | 23.83 | 0.40 | 0.23 | 1.74 | 1.11 | nd | nd | nd | 27.31 |
| | Skins | 5.77 | nd | 1.54 | 32.11 | 7.38 | n.d. | 16.98 | 0.57 | nd | nd | nd | 58.58 |
| Greco di Tufo | Flesh | 15.07 | 0.61 | 1.25 | 92.07 | 1.98 | n.d. | 6.97 | 2.67 | nd | nd | nd | 104.94 |
| | Skins | 17.97 | 0.54 | 2.84 | 81.85 | 11.69 | n.d. | 44.23 | 1.90 | nd | nd | nd | 142.51 |
| Sauvignon | | | | | | | | | | | | | |
| | Flesh | 30.42 | nd | nd | 38.09 | 0.43 | 0.52 | 2.18 | 0.69 | nd | nd | nd | 41.91 |
| | Skins | 11.18 | nd | 1.45 | 33.31 | 7.41 | n.d. | 21.64 | n.d. | nd | nd | nd | 63.81 |
| | | | | | | | | | | nd | nd | nd | |
| Malvasia (healthy berries) | Flesh | 8.97 | nd | 0.74 | 43.90 | 0.27 | 0.27 | 1.14 | 1.13 | nd | nd | nd | 47.45 |
| | Skins | 10.02 | nd | 1.50 | 34.64 | 1.00 | n.d. | 2.75 | 0.45 | nd | nd | nd | 40.34 |
| Malvasia (botrytizied berries) | | | | | | | | | | | | | |
| | Flesh | 1.59 | nd | 0.62 | 72.11 | 0.52 | 0.34 | 2.41 | 1.80 | nd | nd | nd | 77.80 |
| | Skins | 3.14 | nd | 2.26 | 72.95 | 1.88 | n.d. | 6.25 | 1.07 | nd | nd | nd | 84.41 |

nd - not detectable

Legend: cis-CTA: cis-caftaric acid*;* *trans*-CTA: *trans*-caftaric acid; *cis*-CoTA: *cis*-coutaric acid; *trans*-CoTA: *trans*-coutaric acid*;* GRP: grape reaction product; FTA: *trans*-fertaric acid; HCA: esterified and free hydroxycinnamic acids

The concentration of glutathione in the grape flesh ranged from 11 to 22 mg/kg while the concentration in berry skin was from 6 to 18 mg/kg. This indicates that in some varieties half of glutathione can be found in grape skins. Oxidized glutathione was not found in Chardonnay and Muller Thurgau while in Greco di Tufo its concentration was very low. Malvasia grape originated from Slovenia (Biljenski griči) was also analyzed. The glutathione level was lower than other four varieties, but it is clearly seen that botrytized berries contained much lower reduced glutathione content comparing to the sound grapes. Healthy berries of Malvasia grapes contained 8.97 mg/kg of reduced glutathione while botrytized berries contained 1.59 mg/kg glutathione in the must. The sum of hydroxycinnamic acids and their tartaric esters in the flesh ranged from 27 to 105 mg/kg, while the berry skin showed a slightly higher concentration (59-142 mg/kg). *trans* – caftaric acid was the major hydroxycinnamic ester, with the concentration ranging from

23.8 mg/kg to 92.1 mg/kg in the grape juice. The concentration of *trans*-coutaric acid was much lower in grape juice, ranging from 1.7 to 7.0 mg/kg, however its concentration in berry skin was much higher. Free hydroxycinnamic acids (caffeic, coumaric and ferulic) were not found in grape juices or skins.

Glutathione (reduced and oxidized), hydroxycinamic acids, their tartaric esters and low molecular weight phenolics (vanillin index) were monitored during normal and hyperreductive pressing, fermentation and aging. The effect of bentonite addition and microoxygenation was also assessed in one treatment. Data are shown in Annexes A1, A2 and A3.

### 4.1.3.2 Influence of pressing management on reduced glutathione content

There were evident differences in reduced glutathione concentration between the hyperreductive ($N_2$) and normal ($O_2$) pressing (Figure 23). Reduced glutathione concentration in tank after pressing in an inert atmosphere ranged from 16.4 mg/L (Chardonnay) to 16.7 (Muller Thurgau) and 22.5 mg/L in Greco di Tufo variety while the concentration in normally pressed grape juice was much lower, ranging from 0.07 mg/L (Chardonnay) to 8.7 mg/L (Muller Thurgau) and 8.1 mg/L in Greco di Tufo. Bentonite fining (20 g/hL) was used in musts after pressing. However, bentonite addition, sedimentation and racking of must did not show a marked decrease in reduced glutathione content (Annexes A1, A2, A3). $CO_2$ inert gas was used to sparge the empty tank before racking, which may additionally protect the juice while racking into empty tank. Filling the tank from the bottom was used during racking.

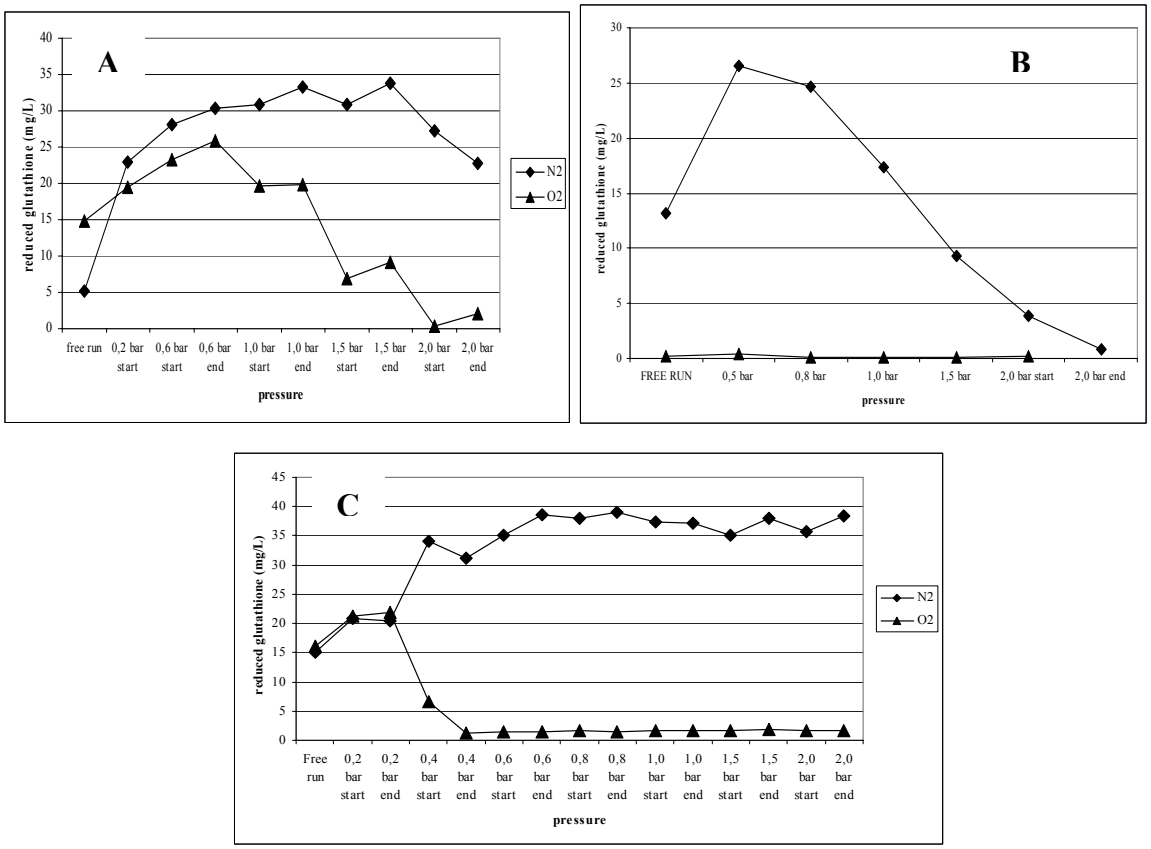



Figure 23: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on reduced glutathione content in Muller Thurgau (A), Chardonnay (B) and Greco di Tufo grape juice (C) at different pressing fractions

Slika 23: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na vsebnost reduciranega glutationa v moštu sorte Muller Thurgau (A), Chardonnay (B) in Greco di Tufo (C) v različnih frakcijah stiskanja

During fermentation reduced glutathione decreased in all hyperreductive pressed musts (Figure 24).

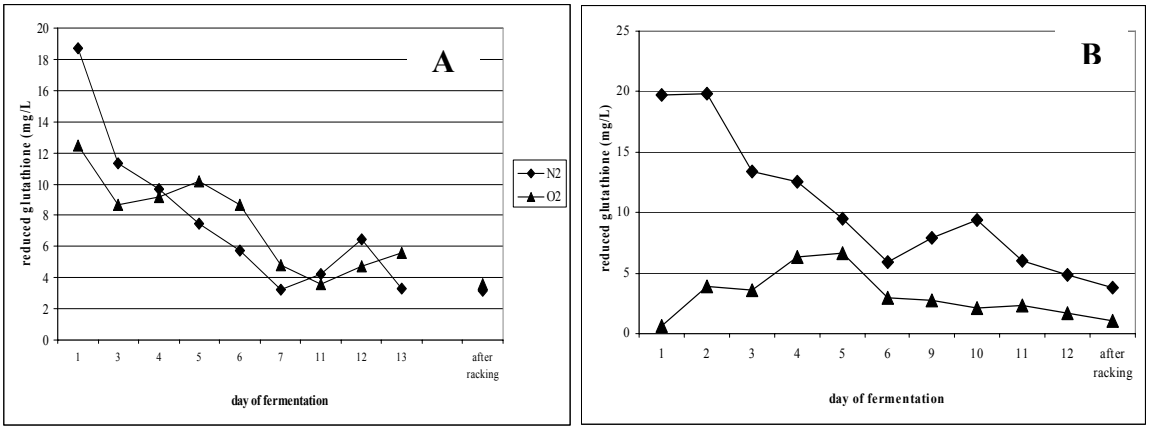



Figure 24: Influence of fermentation on reduced glutathione content in Muller Thurgau must (A) pressed in hyperreductive ($N_2$) and normal ($O_2$) conditions, and Chardonnay must (B) pressed in hyperreductive ($N_2$) and normal ($O_2$) conditions

Slika 24: Vpliv fermentacije na vsebnost reduciranega glutationa v moštu sorte Muller Thurgau (A), stiskanem pri hiperreduktivnih ($N_2$) in normalnih ($O_2$) pogojih in moštu sorte Chardonnay (B), stiskanem pri hiperreduktivnih ($N_2$) in normalnih ($O_2$) pogojih

Oxygen addition (5 mg/L) was applied during the fermentation of Muller Thurgau pressed in normal ($O_2$) conditions. The data are listed in Table 15.

Table 15: Influence of oxygen addition on reduced glutathione and hydroxycinnamic acid content (mg/L) of Muller Thurgau grape variety during fermentation

Preglednica 15: Vpliv dodatka kisika med fermentacijo na vsebnost reduciranega glutationa in hidroksicimetnih kislin (mg/L) v moštu sorte Muller Thurgau (mg/L)

| | glutathione reduced | glutathione oxidised | *cis*-CTA | *trans* -CTA | *cis* -CoTA | GRP (*) | *trans* -CoTA | FTA | *trans* caffeic | *trans p-coumaric* | *trans* -ferulic | Vanillin index (+) catechin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Before | 8,47 | 0,92 | 1,24 | 31,72 | 2,54 | 3,28 | 4,26 | 1,46 | 0,57 | n.d. | n.d. | 75,6 |
| After | 8,68 | 1,17 | 1,11 | 31,69 | 2,53 | 3,38 | 4,30 | 1,46 | 0,57 | n.d. | n.d. | 75,6 |

*quantified as caffeic acid
n.d. – not detectable

### 4.1.3.3 Influence of pressing management on oxidized glutathione content

The concentration of oxidized glutathione was much lower compared to the reduced form (Annexe A1, A2, A3). Its concentration also increased during pressing. It was higher in the normal ($O_2$) pressing, but only in Muller Thurgau grapes. Chardonnay and Greco di Tufo juices showed higher concentration of oxidized glutathione in hyperreductive pressing

compared with the control. During fermentation its concentration felt to very low values and after 9 months its concentration fell to 0 mg/L.

4.1.3.4 Influence of pressing management on hydroxycinnamic acid and their tartaric esters content

There were evident differences in hydroxycinnamic acids and their esters content between hyperreductive and normal pressing.

***Caftaric acid***

*trans*-Caftaric acid was the major esterified hydroxycinnamic acid. It is seen from Figures 25, 29, 33 that its concentration increases with the increasing pressure and absence of oxygen. Final pressing at 2 bar had 95% (Greco di Tufo) to 133% (Muller Thurgau) more *trans*-caftaric acid compared to free run. Its concentration in tank after hyperreductive pressing ranged from 49 mg/L (77% of total HCA without GRP) for Muller Thurgau to 73 mg/L (82%) for Chardonnay and 216 mg/L (82%) for Greco di Tufo variety. During fermentation the *trans*-caftaric acid concentration slightly decreased from 3% to 16.6%. However, its concentration during ageing was stable in the Chardonnay and Muller Thurgau wine while it decreased in the Greco di Tufo wine. The concentration of *cis*-caftaric acid was much lower than *trans*-caftaric, but it followed the same trend as the *trans*-caftaric acid.

***Grape Reaction Product (GRP)***

The content of GRP increases with the increasing pressure and after pressing its concentration in tank was 1.9 (Muller Thurgau, Chardonnay) to 3.5 times (Greco di Tufo) higher in normal pressing compared to the hyperreductive pressing (Figures 26, 30, 34). During fermentation GRP decreased with 11-15% in Chardonnay and Muller Thurgau juice.

***Coutaric acid***

There was from 37% (Muller Thurgau) to 51% (Chardonnay) less *trans*-coutaric acid in the control pressing compared to the hyperreductive pressing (Figure 27, 31, 35). During fermentation *trans*-coutaric acid decreased for 10 to 18% in Chardonnay and Muller Thurgau juice respectively while the concentration in Greco di Tufo variety stayed stable. Its concentration during aging was also stable and after 9 month did not change significantly.

### *Fertaric acid*

Its concentration was quite low compared with other hydroxycinnamic acids, ranging from 1.84 mg/L to 5.62 mg/L (Figure 28, 32, 36). During fermentation it slightly decreased and was more or less stable during ageing.

### Free hydroxycinnamic acids

Free forms of hydroxycinnamic acids (caffeic, *p*-coumaric and ferulic) were not found in grape juice. *trans*-Caffeic acid appeared only during fermentation, which could be due to enzymatic activity of wine yeast. *p-C*oumaric and *trans*-ferulic acid were not found in wine. During wine ageing *trans*-caffeic acid increased, but its concentration was very low, ranging from 0.20 to 2.23 mg/L.

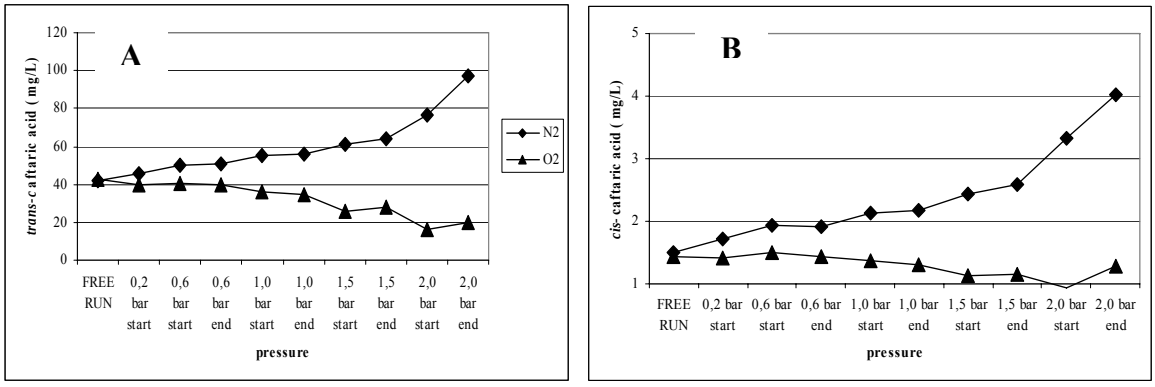



Figure 25: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on *trans*-caftaric acid (A) and *cis*-caftaric acid (B) in Muller Thurgau grape juice

Slika 25: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na vsebnost *trans*-kaftarne (A) and *cis*-kaftarne kisline (B) v moštu sorte Muller Thurgau

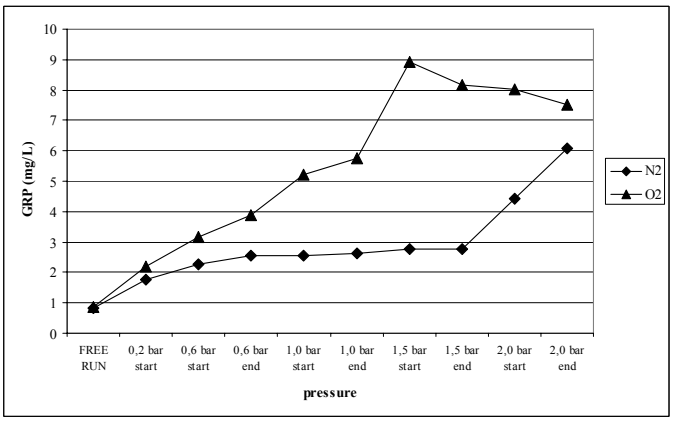



Figure 26: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on GRP in Muller Thurgau grape juice

Slika 26: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na vsebnost GRP v moštu sorte Muller Thurgau

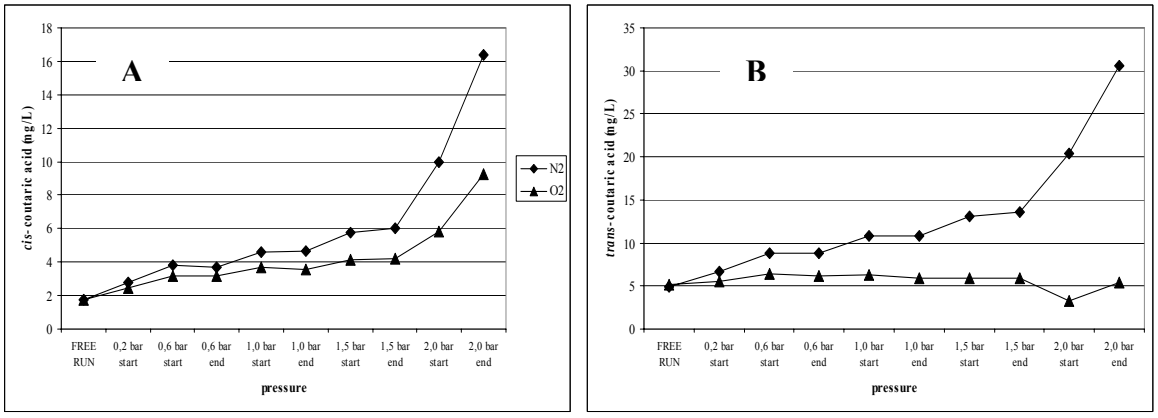



Figure 27: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on *cis*-coutaric acid (A) and *trans*-coutaric acid (B) in Muller Thurgau grape juice

Slika 27: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na vsebnost *cis*-kutarne (A) and *trans*-kutarne kisline (B) v moštu sorte Muller Thurgau

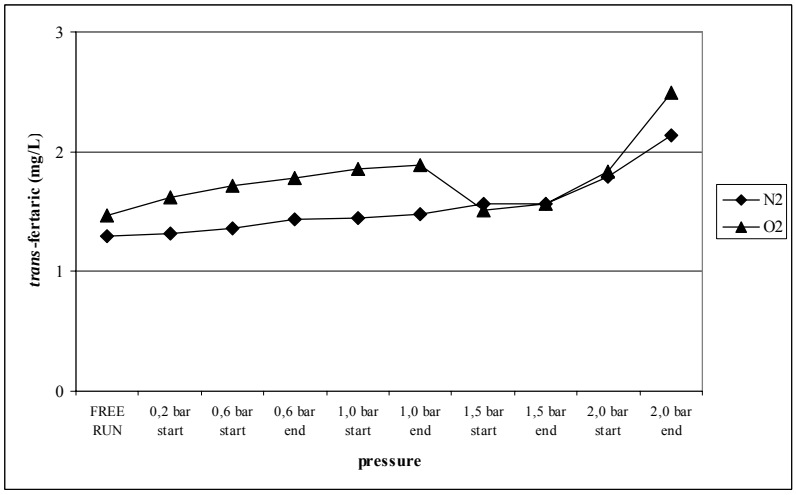



Figure 28: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on *trans*-fertaric acid in Muller Thurgau grape juice

Slika 28: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na vsebnost *trans*-fertarne kisline v moštu sorte Muller Thurgau

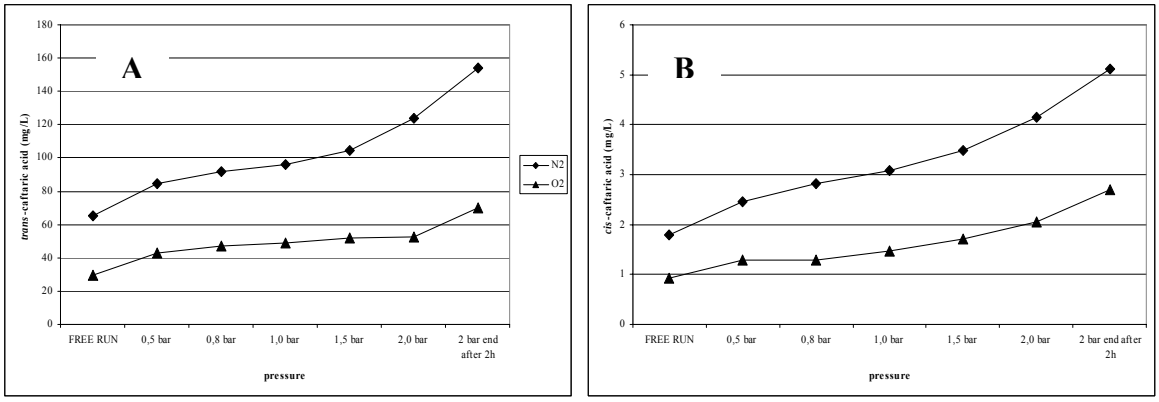



Figure 29: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on *trans*-caftaric acid (A) and *cis*-caftaric acid (B) in Chardonnay grape juice

Slika 29: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na vsebnost *trans*-kaftarne (A) and *cis*-kaftarne kisline (B) v moštu sorte Chardonnay

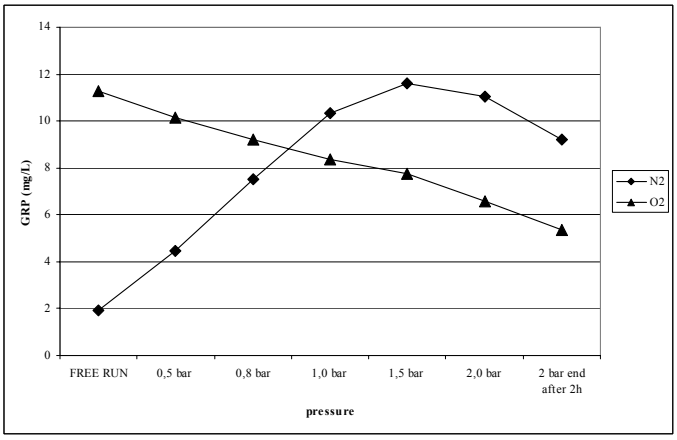



Figure 30: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on GRP in Chardonnay grape juice

Slika 30: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na vsebnost GRP v moštu sorte Chardonnay

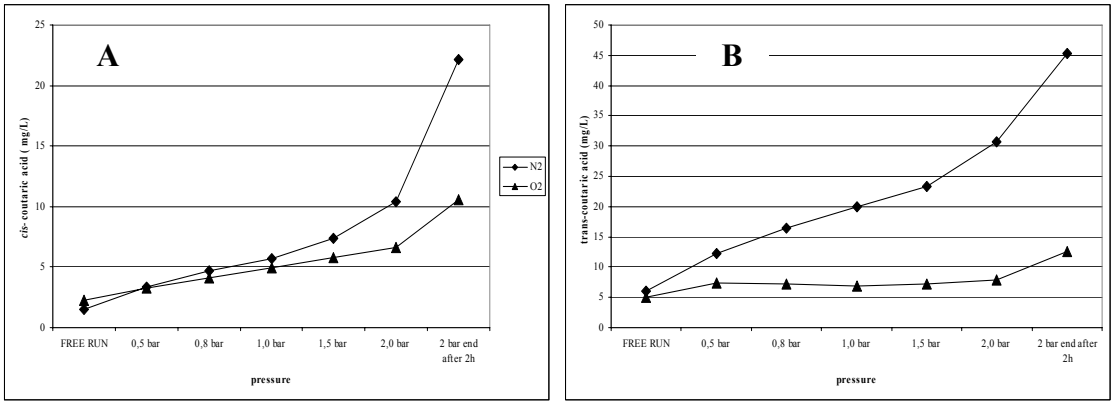



Figure 31: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on *cis*-coutaric acid (A) and *trans*-coutaric acid (B) in Chardonnay grape juice

Slika 31: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na vsebnost *cis*-kutarne (A) and *trans*-kutarne kisline (B) v moštu sorte Chardonnay

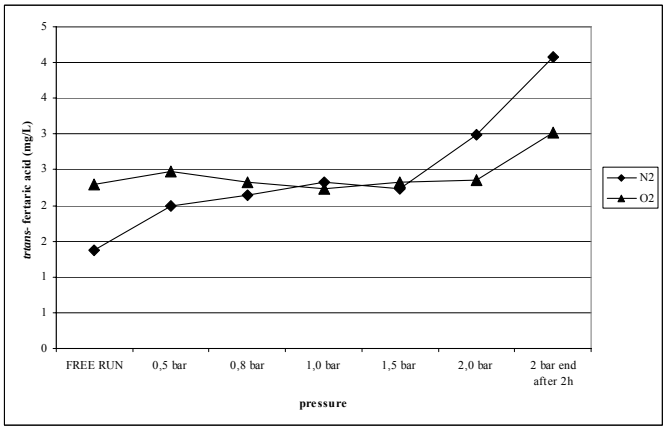



Figure 32: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on *trans*-fertaric acid in Chardonnay grape juice

Slika 32: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na vsebnost *trans*-fertarne kisline v moštu sorte Chardonnay

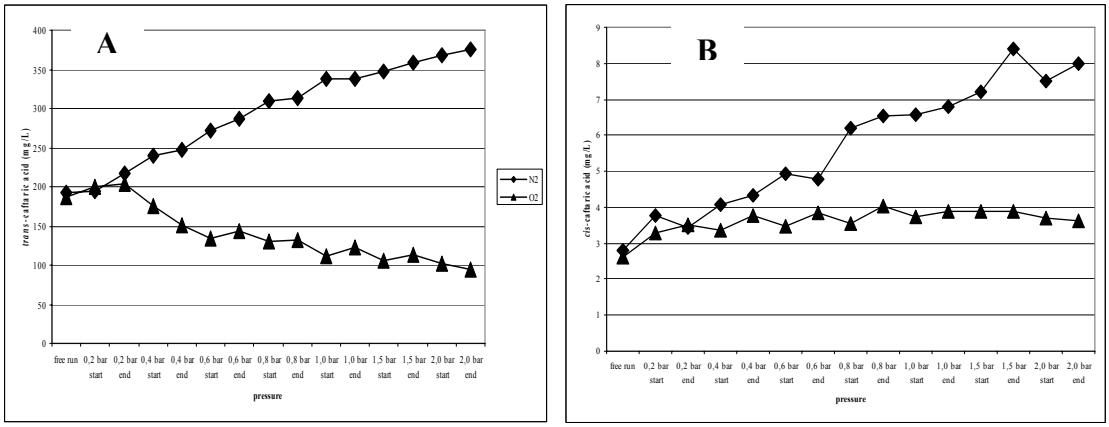



Figure 33: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on *trans*-caftaric acid (A) and *cis*-caftaric acid (B) in Greco di Tufo grape juice

Slika 33: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na vsebnost *trans*-kaftarne (A) and *cis*-kaftarne kisline (B) v moštu sorte Greco di Tufo

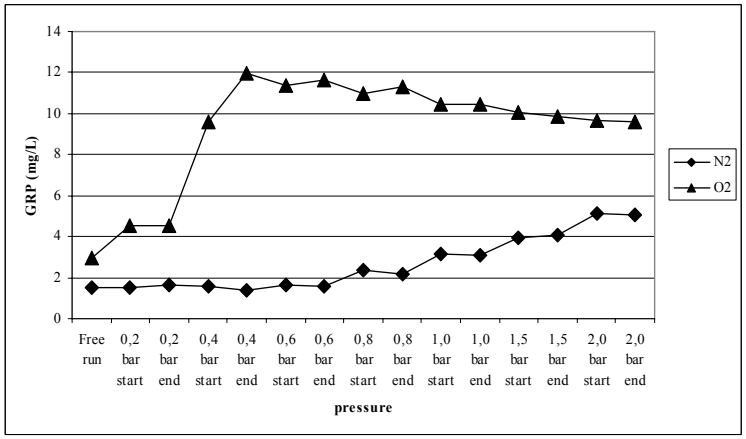



Figure 34: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on GRP in Greco di Tufo grape juice

Slika 34: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na vsebnost GRP v moštu sorte Greco di Tufo

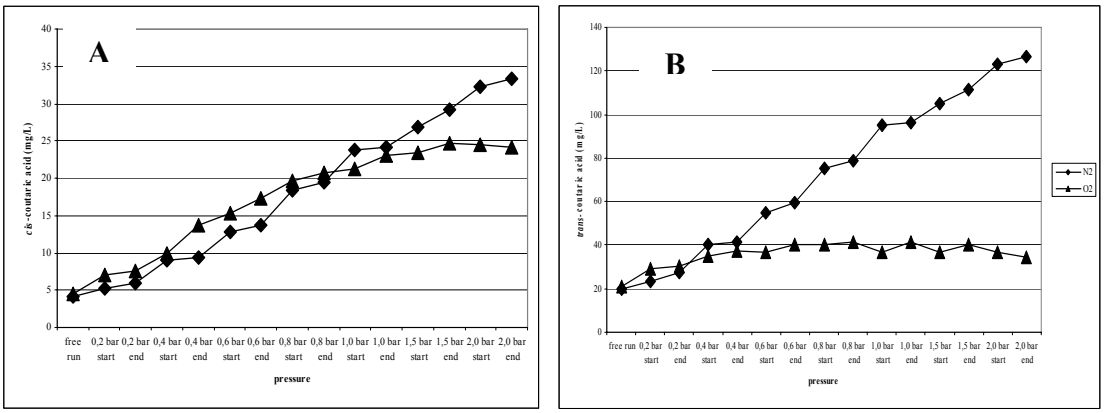



Figure 35: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on *cis*-coutaric acid (A) and *trans*-coutaric acid (B) in Greco di Tufo grape juice

Slika 35: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na vsebnost *cis*-kutarne (A) and *trans*-kutarne kisline (B) v moštu sorte Greco di Tufo

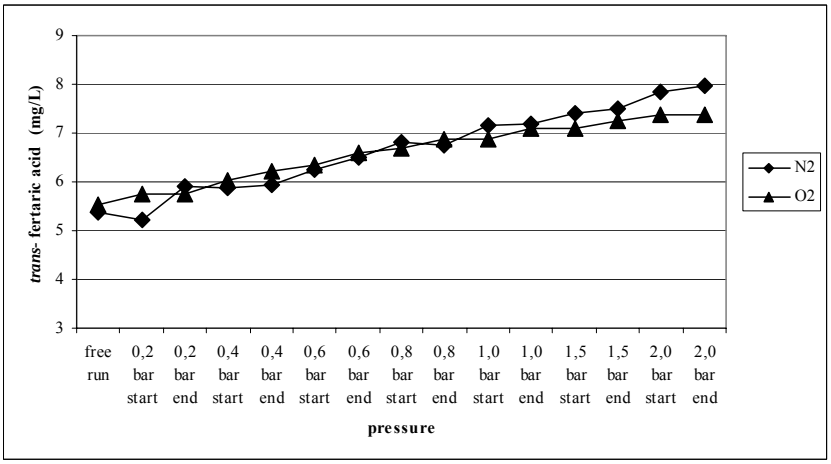



Figure 36: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on *trans*-fertaric acid in Greco di Tufo grape juice

Slika 32: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na vsebnost *trans*-fertarne kisline v moštu sorte Greco di Tufo

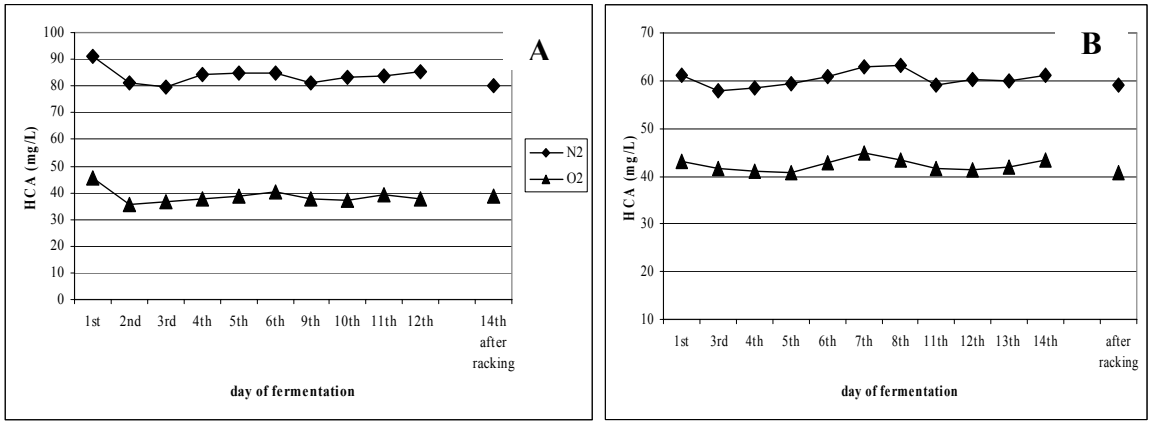



Figure 37: Influence of fermentation on hydroxycinamic acids and their esters (sum value, without GRP) in Chardonnay (A) and Muller Thurgau (B) wine pressed in hyperreductive ($N_2$) and normal ($O_2$) conditions

Slika 37: Vpliv fermentacije na vsebnost hidroksicimetnih kislin in njihovih estrov (skupna vrednost, brez GRP) v vinu sorte Chardonnay (A) in Muller Thurgau (B), stiskanem v hiperreduktivnih ($N_2$) in normalnih ($O_2$) pogojih

### 4.1.3.5 Low molecular weight flavan-3-ols (vanillin Index)

Low molecular weight procyanidins was measured by vanillin – a cyclic aldehyde that reacts preferably with monomer phenols like catechin and epicatechin. The content of flavan-3-ols also increased with the increasing pressure. Pressings at 2 bars in hyperreductive atmosphere showed 3-4 times more flavan-3-ols compared to free run juice (Figure 38).

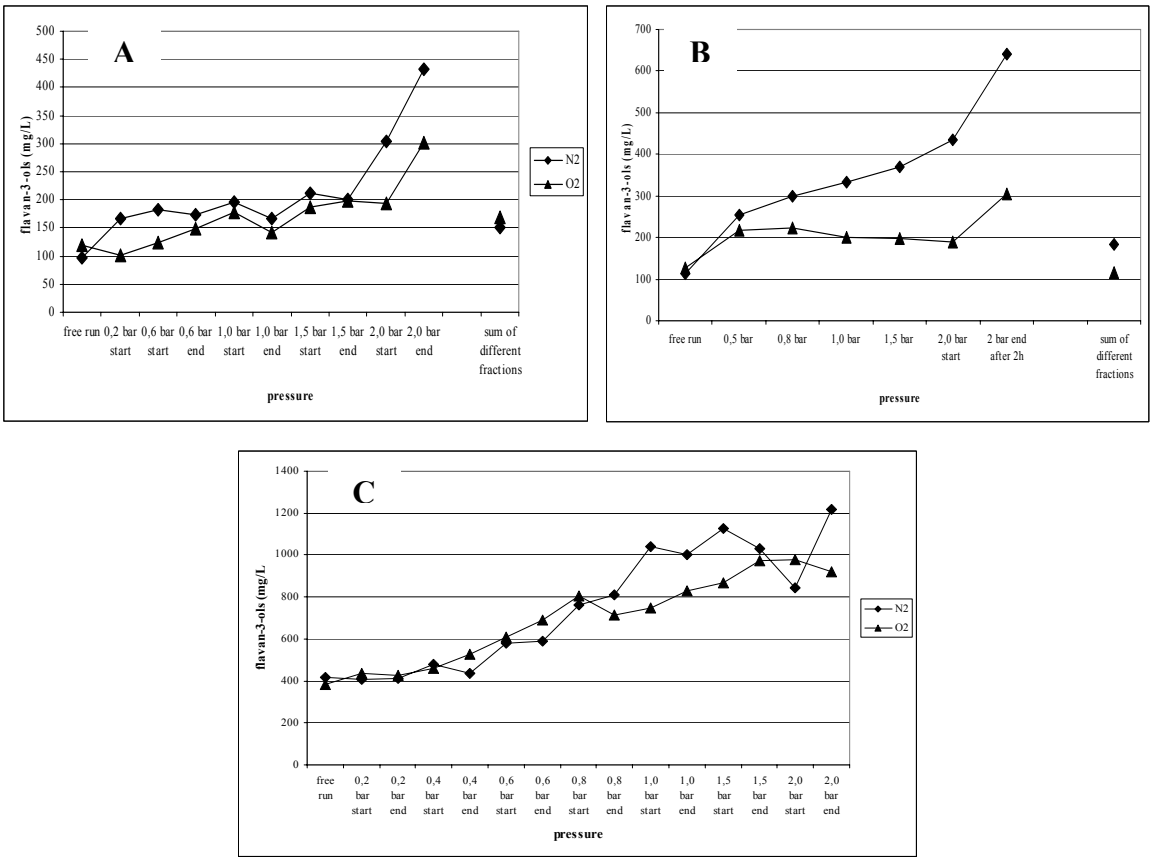



Figure 38: Influence of hyperreductive ($N_2$) and normal ($O_2$) pressing on flavan-3-ols in Muller Thurgau (A), Chardonnay (B) and Greco di Tufo variety (C) in differnet must fractions

Slika 38: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja na flavan-3-ole v moštu sorte Muller Thurgau (A), Chardonnay (B) in Greco di Tufo (C) v različnih frakcijah

During fermentation low molecular weight flavan-3-ols decreased significantly in both, normal and hyperreductive pressed grape juice. After fermentation, the hyperreductive pressed Muller Thurgau wine had 18% more flavan-3-ols while Chardonnay wine had 59% more flavan-3-ols (Figure 39).

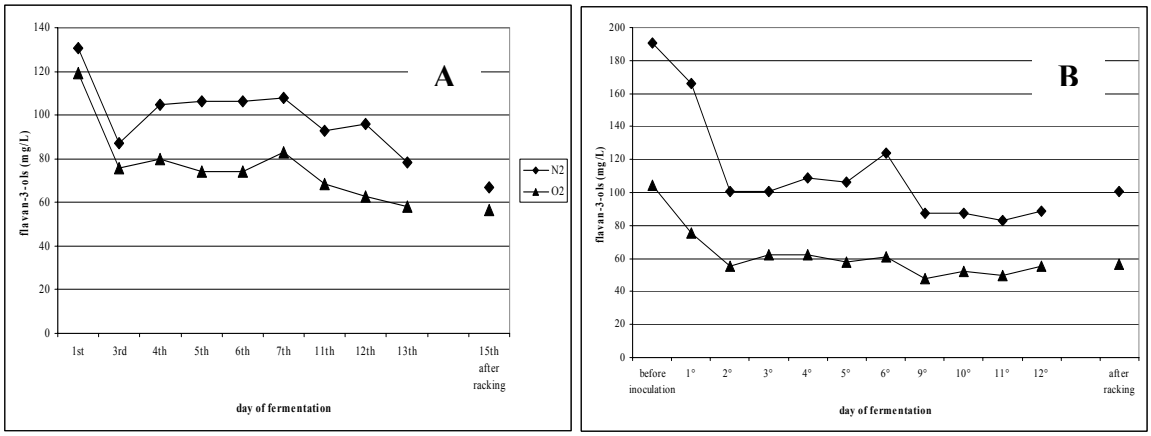



Figure 39: Influence of fermentation on vanillin index in hyperreductive ($N_2$) and normal ($O_2$) pressed Muller Thurgau juice (A) and Chardonnay juice (B)

Slika 39: Vpliv fermentacije na vanilin index v hiperreduktivno ($N_2$) in normalno ($O_2$) stiskanem moštu sorte Muller Thurgau (A) in Chardonnay (B)

### 4.1.3.6 Total polyphenols content and absorbance at $A_{420}$

Table 16 shows the influence of hyperreductive treatment on total polyphenols content and absorbance at 420 nm, which is a good indicator of wine browning. Hyperreductive pressing of Chardonnay showed slightly higher absorbance at 420 nm while other two wines showed no differences.

Table 16: Influence of hyperreductive and normal treatment on total phenolic content (Folin Ciocalteau) and $A_{420}$ after 9 months of storage.

Preglednica 16: Vpliv hiperreduktivne in normalne predelave na vsebnost skupnih polifenolov (Folin Ciocalteau) in $A_{420}$ po 9 mesecih zorenja.

| | **Total polyphenols** mg/L (+)-catechin | **$A_{420}$** (1cm) |
|---|---|---|
| Chardonnay | | |
| normal | 166 | 0,099 |
| hyperreductive | 241 | 0,110 |
| Muller Thurgau | | |
| normal | 163 | 0,062 |
| hyperreductive | 186 | 0,062 |
| Greco di Tufo | | |
| normal | 403 | 0,121 |
| hyperreductive | 396 | 0,121 |

### 4.1.3.7 Sensory evaluation of experimental wines

Triangle tasting of the wines of all three experiments was carried out 10 months after the completition of fermentation. The panel consisted of 10 tasters.

Table 17: Number of positive response and statistical differences between hyperreductive and normal technology.

Preglednica 17: Število pozitivnih odgovorov in statistične razlike med hiperreduktivno in normalno tehnologijo

| Treatment | Muller Thurgau | Chardonnay | Greco di Tufo |
|---|---|---|---|
| **No. of positive response (out of 10)** | 8 | 6 | 6 |
| **Statistical difference at (p < )** | 0.01 | 0.1 | 0.1 |

Eight out of ten tasters could distinguish between hyperreductive and normally produced Muller Thurgau wine, which corresponds to 0.01 level of significance. However, significant difference in Chardonnay and Greco di Tufo wine was much lower and 6 out of 10 tasters could distinguish the differences. In Greco di Tufo wine, different fractions were included in wines, that is why a low level of statistically significant differences was achieved. In normally pressed Greco di Tufo wine, pressings up to 0.4 bar were used while in hyperreductive pressed wine, pressings up to 2 bar were used.

## 4.2. MICROOXYGENATION

Microoxygenation experiments were conducted only in large scale tanks. This is due to microoxygenation requirements, which needs at least 2 m high tanks to perform the dissolution of oxygen successfully.

### 4.2.1. Colour intensity and colour hue

Colour intensity and colour hue were followed during the treatment in all four experiments. Experiment 1 represents Merlot wine 2004 from the Vipava Valley, experiment 2 Cabernet Sauvignon, vintage 2005, from the Vipava Valley, Experiment 3 was a microoxygenation treatment of Pinotage wine, vintage 2004, from the Stellenbosch region, and experiment 4 Cabernet Sauvignon, vintage 2006 also from the Stellenbosch region in South Africa.

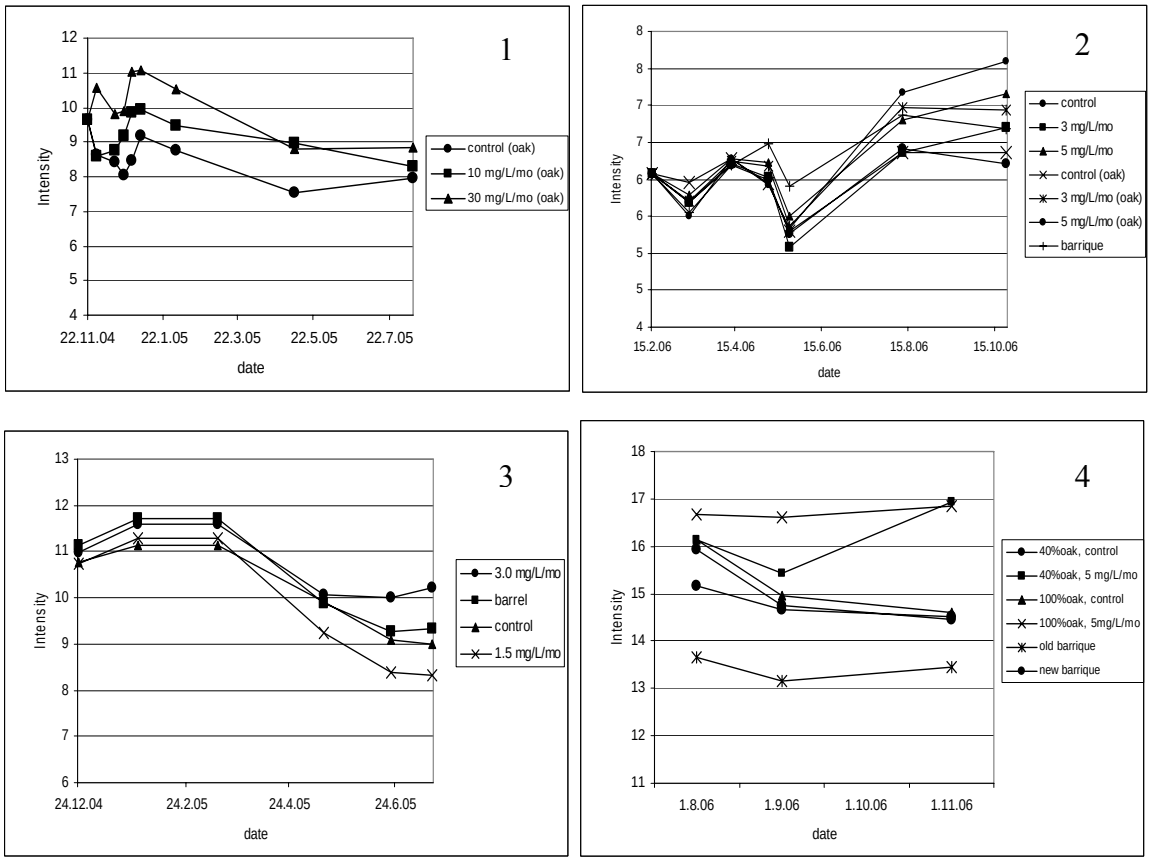



Figure 40: Colour intensity kinetics in experiment 1 (wine aged with oak segments), experiment 2 (wine aged with oak segments and in barrel), and experiment 3 and 4

Slika 40: Kinetika intenzitete barve v vinu poskusa 1 (vino zorjeno z dodanimi hrastovimi segmenti), vinu poskusa 2 (s hrastovimi segmenti in barrique sodu) ter vinu poskusa 3 in 4

As shown in figure 40, colour intensity in the experiment 1 (Merlot 2004) increased at the beginning and then slowly decreased. Wines which received higher dose of oxygen showed higher color intensity. At the end of microoxygenation treatment, the wine with higher oxygen addition had 11% higher color intensity while the wine with lower addition had 4.5% higher color intensity compared to the control.

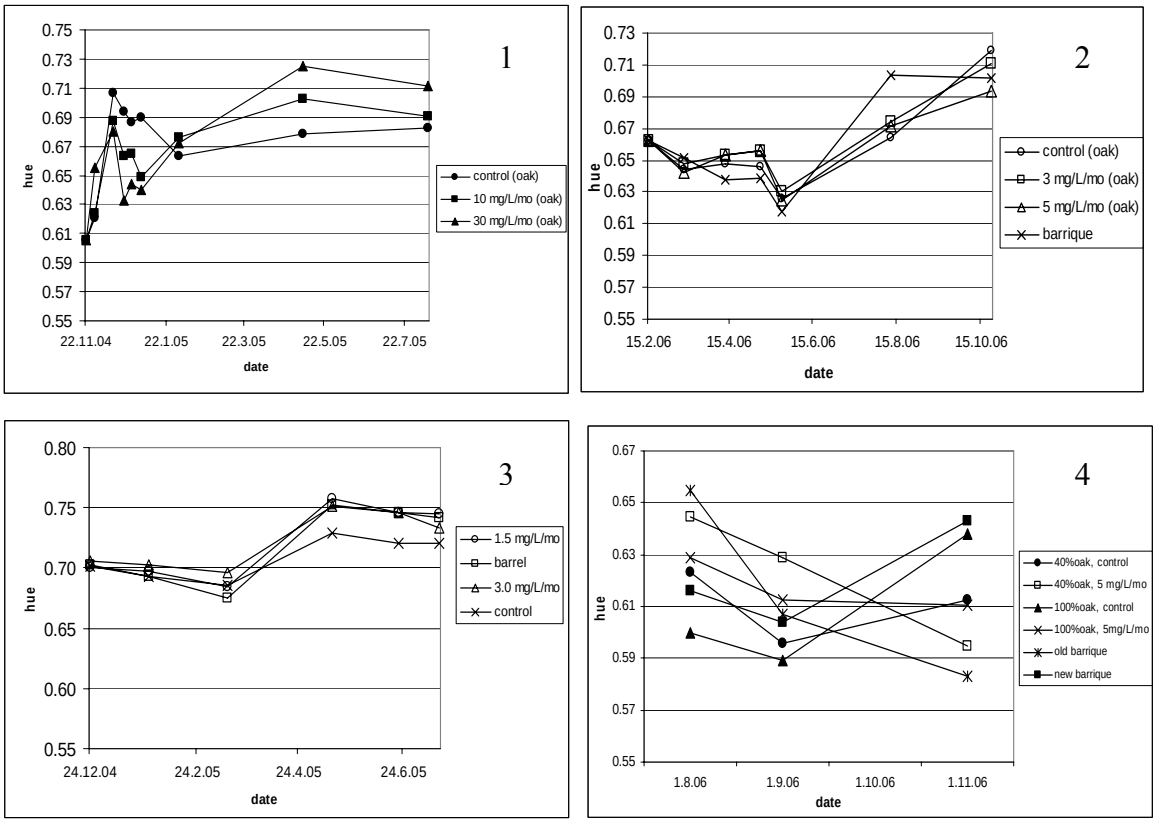



Figure 41: Color hue kinetics in experiment 1 (wine aged with oak segments), experiment 2 (wine aged with oak segments and in barrel), experiment 3 and 4.

Slika 41: Kinetika tona barve v poskusu 1 (vino zoreno z dodanimi hrastovimi segmenti), poskusu 2 (vino s s hrastovimi segmenti in barrique sodu) ter vinu poskusa 3 in 4.

In the experiment 2, where 3 and 5 mg/L/month of oxygen was added to Cabernet Sauvignon, the colour intensity was 8 to 19% higher in the treated wines. Differences in colour intensity in the wines of experiment 3 (Pinotage 2004), which received 1,5 and 3 mg/L/month, can be seen after 6 months of treatment. The highest color intensity had wine treated with the highest amount of oxygen addition (3 mg/L/month). However, the lowest colour intensity had wine treated with 1.5 mg/L/month. Wine aged in a barrel had slightly higher colour intensity compared with wine aged in a tank with no oxygen added. In the wine of experiment 4, the differences in colour intensity were perceived between

microoxygenated and control wines. The highest color intensity showed the microoxygenated wine (5 mg/L/month) with 100% internal surface area of 225 L oak barrel. Colour hue ($A_{420}/A_{520}$) in wines of experiment 1 was 4% higher in wine treated with higher oxygen concentration. However, in wines of second experiment treated with 3 and 5 mg $O_2$/L/month there was no difference in colour hue between treatments. It can be seen that colour hue fell during the first 20 weeks and then started to increase. In wines of experiment 3 the same tendency can be observed during the first 10 weeks. After that, colour hue increased and after 28 weeks the microoxygenated wines has higher colour hue compared to the control. Wine aged in a barrel also had higher value than wine aged in microoxygenated tank. In wines of the fourth experiment, a decrease tendency was observed in colour hue in all wines except in the wine aged in an old barrel and the wine aged in a tank with 100% internal surface area of 225 L oak barrel.

### 4.2.2 Total anthocyanins

Total anthocyanins were measured spectrophotometrically as described in Chapter 3.3.3.1. In the experiment 1 kinetics of anthocyanin changes were followed during the microoxygenation. It is clearly seen that the total anthocyanins decrease is proportional to oxygen addition. Wine with higher $O_2$ addition showed higher decrease in total anthocyanins (Figure 42).

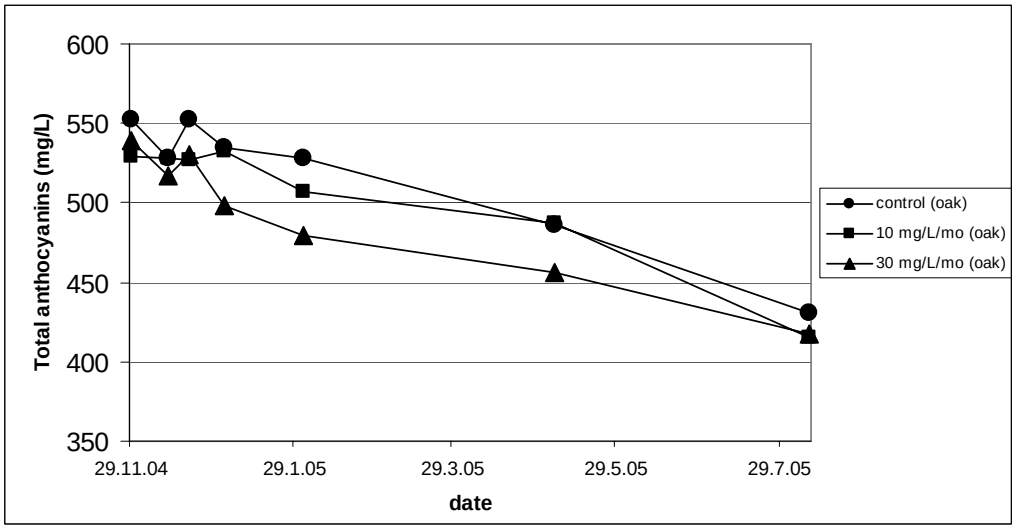



Figure 42: Total anthocyanins changes during microoxygenation process in wines of experiment 1, aged with oak segments

Slika 42: Spremembe skupnih antocianov med procesom mikrooksigenacije v vinih poskusa 1, zorenih s hrastovimi segmenti

HPLC profiles of free anthocyanins were also determined at the end of microoxygenation (Figure 43). It can be seen (Annexe B22 - B27) that malvidin-3-glucosid was the most abundant free anthocyanin in red wines. In wines aged with oak segments of experiment 1, malvidin-3-glucoside was lowered by 5.1% in lower oxygen addition and 7.2% in higher oxygen addition (Annexe B22). Peonidin-3-glucoside was lower for 17 and 16% and petunidin-3-glucoside for 4 and 9% compared to the control (Annexe B22). However, their concentration was 6 to 8 times lower than malvidin-3-glucoside concentration. The concentration of anthocyanins (Figure 43, 1) was however significantly lower in the micro-oxygenated and oak treated wines compared to the control which received no oak and microoxygenation treatments. The concentration of other anthocyanins did not show a significant change. In wines of experiment 2, malvidin-3-glucoside was lowered from 8 to 17% compared to the control wine (Annexes B24).

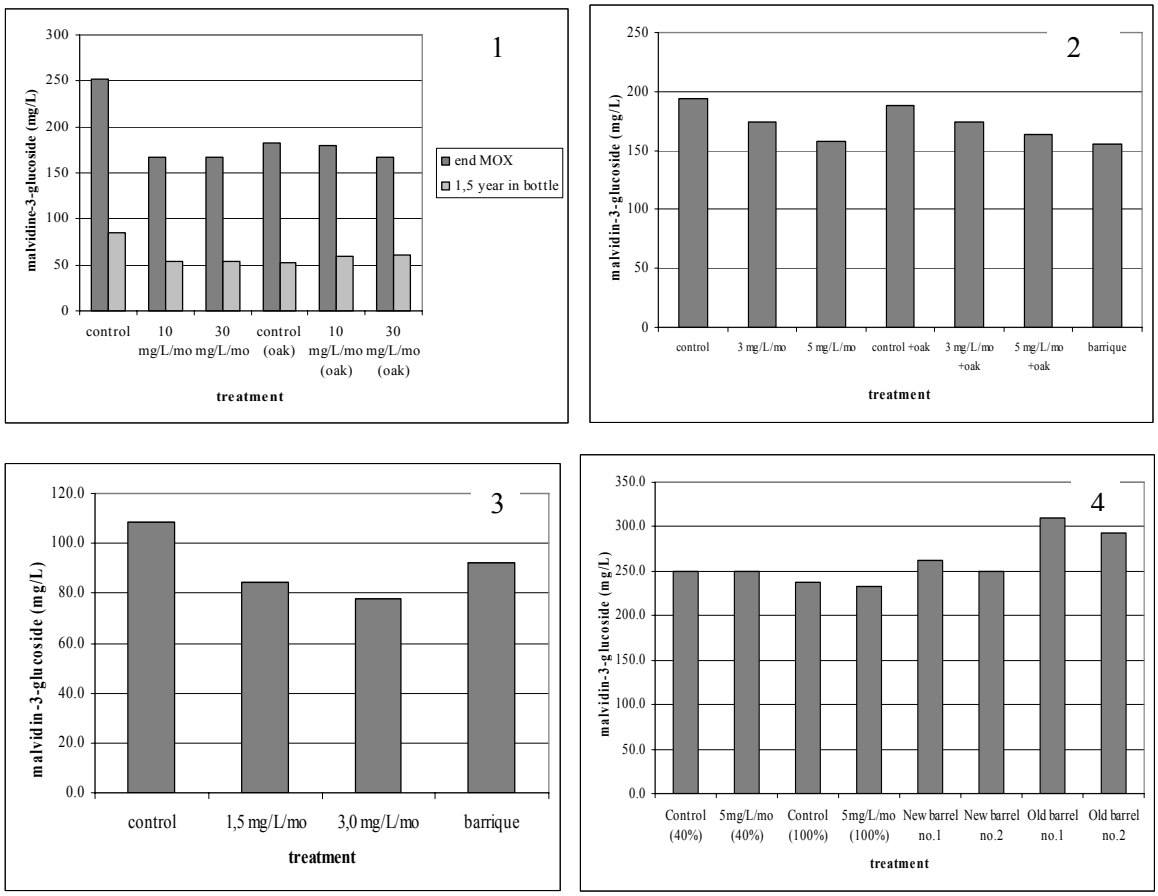



Figure 43: Sum of free anthocyanins measured by HPLC at the end of microoxygenation treatment in wines of experiments 1, 2, 3 and 4.

Slika 43: Vsota prostih antocianov, analiziranih s HPLC, na koncu procesa mikrooksigenacije v vinih poskusov 1, 2, 3 in 4.

### 4.2.3 Total polyphenols

Different analyses of phenolic compounds were made during treatments. Total polyphenols analyzed by Folin Ciocalteau showed no correlation within oxygen additions. Optical density at 280 nm was also measured during different treatments and it is presented in the following graphs (Figure 44).

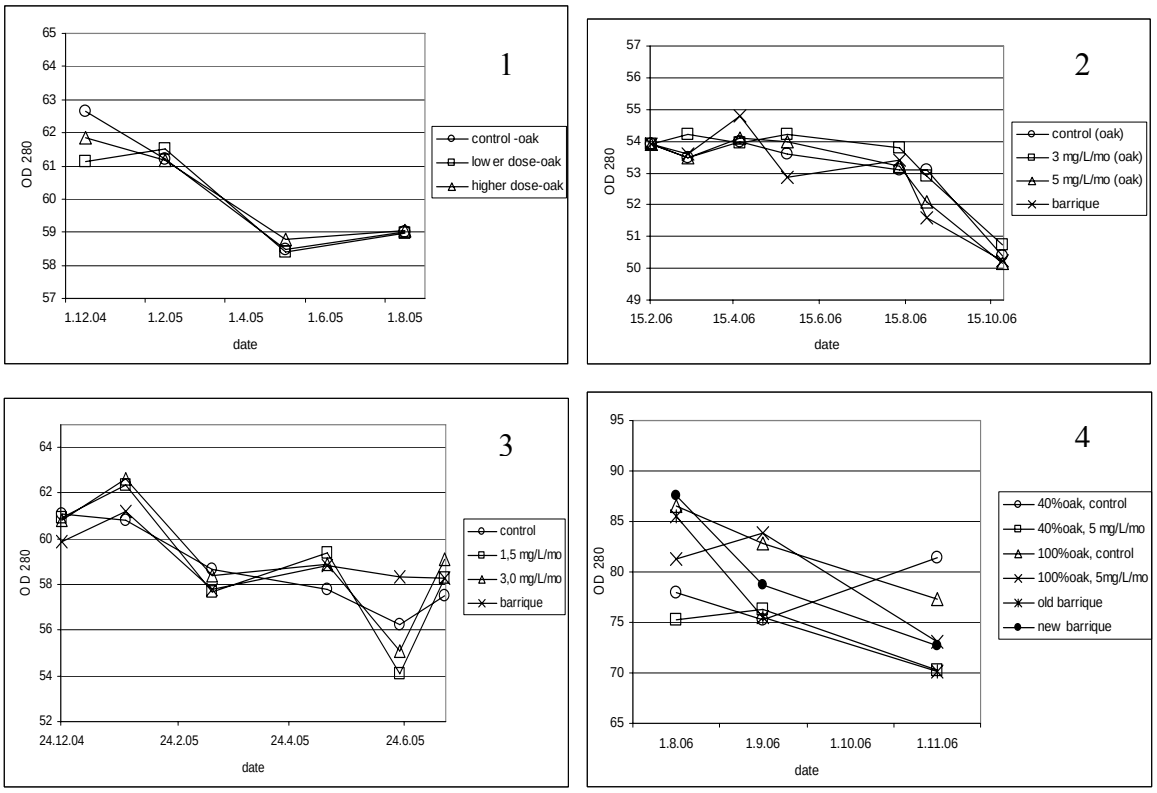



Figure 44: Total phenolics (OD 280) in experiment 1 (wines aged with oak segments), experiment 2 (wine aged with oak segments and in an oak barrel) and wines of experiments 3 and 4

Slika 44: Skupni fenoli (OD280) pri poskusu 1 (vino zoreno s hrastovimi segmenti), poskusu 2 (vinu, zorjeno s hrastovimi segmenti in bariku ter vinih poskusov 3 in 4

It can be seen that total phenols (OD 280) decreased during wine ageing, but a correlation between oxygenated and non-oxygenated wines is hardly seen.

High molecular weight proanthocyanidins in wines of experiment 1, measured by modified Bate-Smith method (Di Stefano et al., 1989b) increased at the beginning during the first two months, but after that they started to decrease. The wine which received higher oxygen dose showed higher proanthocyanidin concentration during ageing (Figure 45).

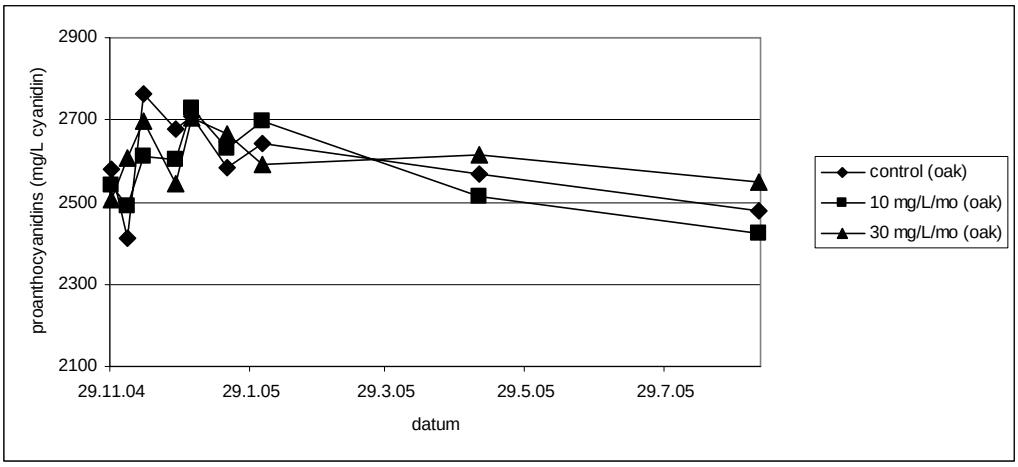



Figure 45: Proanthocyanidin kinetics during the microoxygenation of wines with oak segments in experiment 1

Slika 45: Kinetika proantocianidinov med procesom mikrooksigenacije v vinih poskusa 1, z dodanimi hrastovimi segmenti

Low molecular weight flavan-3-ols (vanillin index) decreased during wine ageing but no correlations between oxygenation and control wine were found. The average drop of low molecular weight flavan-3-ols during 9 months ageing of wines in experiment 1 was 17.5% (Figure 46). In wine of the second experiment the average drop of 18% was observed during the microoxygenation treatment.

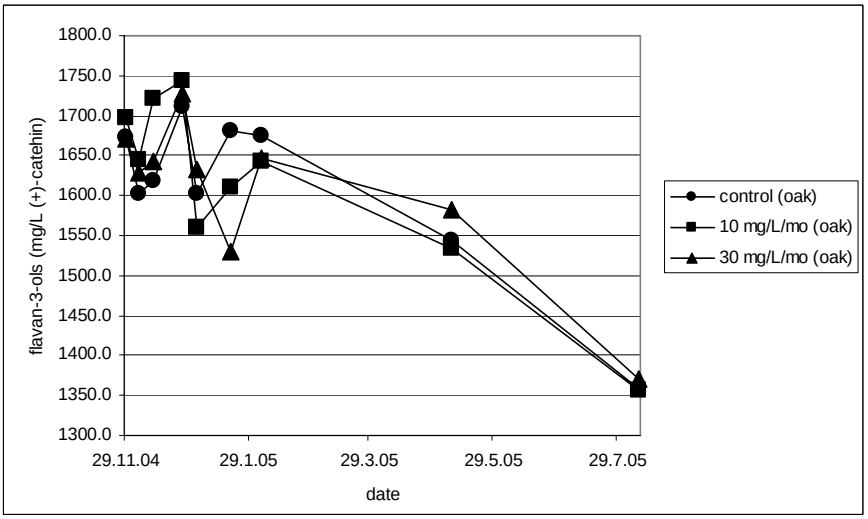



Figure 46: Vanillin index kinetics during the microoxygenation process of wines of experiment 1 with oak segments

Slika 46: Kinetika vanilin indeksa med procesom mikrooksigenacije v vinih s hrastovimi segmenti v poskusu 1

Polymerization index calculated as the ratio between low and high molecular weight proanthocyanidins was followed during microoxygenation. Figure 47 shows its development during the microoxygenation of wines of experiment 1.

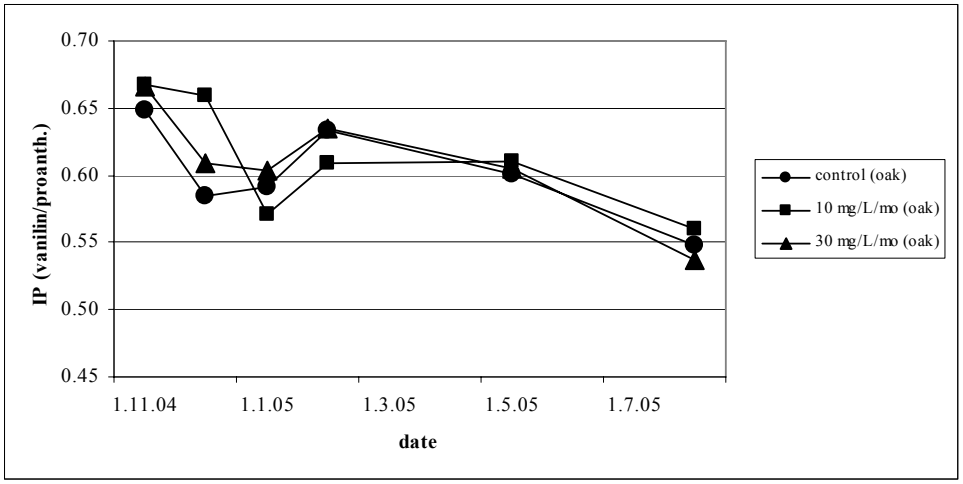



Figure 47: Polymerization index (vanillin/proanthocyanidin) development during the microoxygenation of wines of experiment 1

Slika 47: Indeks polimerizacije (vanilin/proantocianidini) med mikrooksigenacijo vin v poskusu 1

### 4.2.4 Colour proportion

Figure 48 shows the proportion of colour fraction in wine as proposed by Boulton et al. (1996). It can be seen that microoxygenation increased the proportion of polymeric pigments in wines of experiment 1 from 36% in the control to 39% in the microoxygenated wine at higher oxygen dose. Consequently free anthocyanins dropped from 47 to 45%. An increase of polymeric pigments can be seen also in microoxygenated wines of experiment 2, aged with oak segments and microoxagented wines of experiment 4. Microoxygenated wines of experiment 3 showed no differnece in polymeric pigments comparing with control wine.

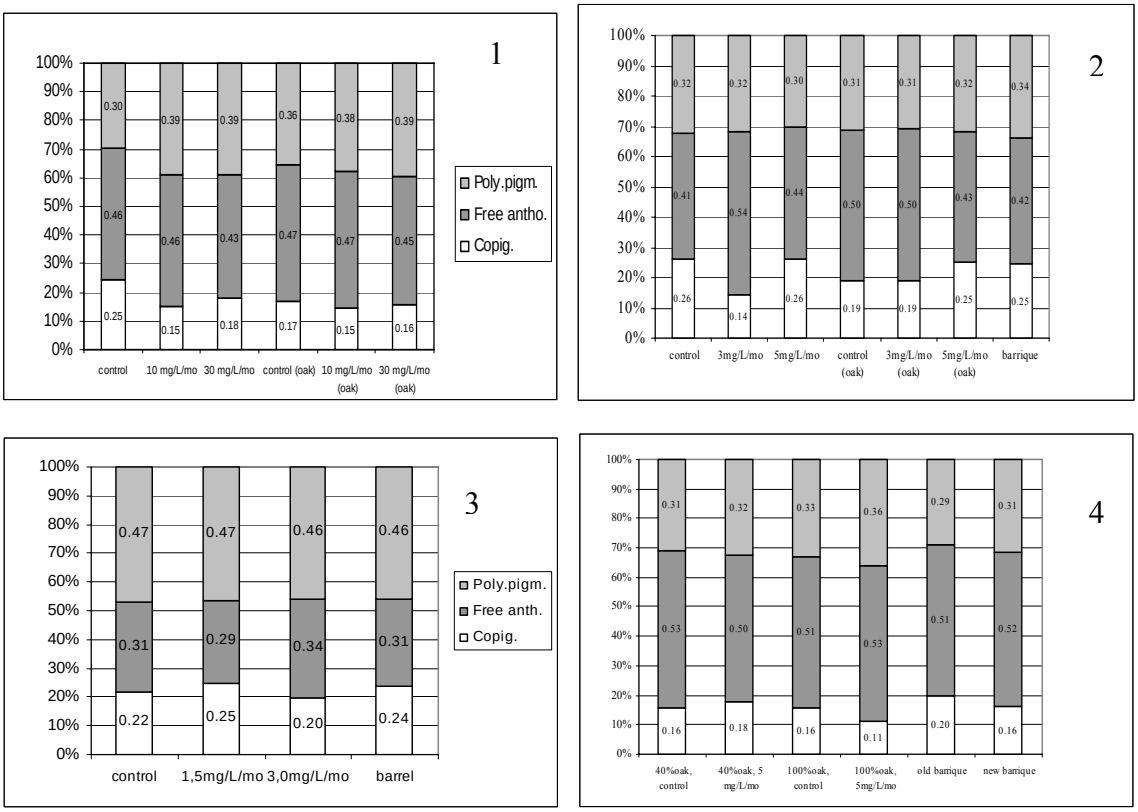



Figure 48: Proportion of colour fraction of the wines in experiment 1, 2, 3 and 4 analyzed at the end of microoxygenation treatment. Copig.: fraction of color due to co-pigmentation, Free anth.: fraction of color due to free anthocyanins, Poly. pigm.: color due to polymeric fraction.

Slika 48: Delež barvne frakcije v vinih poskusa 1, 2, 3 in 4, analiziranih na koncu mikrooksigenacijskega poskusa. Copig.: delež kopigmentirane barve, Free anth.: delež prostih antocianov, Poli. pigm.: delež polimerne barve

### 4.2.5 Microbiology

Acetic acid bacteria and *Brettanomyces* yeast in wine C were enumerated by plating out the wine on selective media at the beginning of microoxygenation and after 4, 14 and 20 weeks, which can be seen in Table 18. Acetic acid bacteria counts increased in microoxygenated wines and wines aged in barrel. *Brettanomyces* yeast counts also increased in microoxygenated wines.

Table 18: Acetic acid bacteria growth in cfu/mL (left) and *Brettanomyces* yeast cfu/mL (right) in wines of experiment 3

Preglednica 18: Rast ocetnokislinskih bakterij v cfu/mL (levo) in *Brettanomyces* kvasovk (desno) v vinih poskusa 3

| | **Acetic acid bacteria** | | | | ***Brettanomyces* yeast** | | | |
|---|---|---|---|---|---|---|---|---|
| **weeks** | **0** | **4** | **14** | **20** | **0** | **4** | **14** | **20** |
| Control | 80000 | 1 | 1 | 34 | 30 | 30 | 40 | 8 |
| 1.5mg/L/month | 80000 | 95000 | 150 | 10 | 30 | 31 | 1400 | 1 |
| 3mg/L/month | 81000 | 69000 | 360 | 34 | 28 | 30 | 40 | 370 |
| Barrel | 82000 | 74000 | 10000 | 16000 | 28 | 28 | 50 | 1 |

### 4.2.6 Sensory evaluation

Sensory evaluation was also performed in all treatments. In the first experiment the wines were evaluated at two different stages of microoxygenation, in the middle of microoxygenation process and three months after its completion (Figure 49).

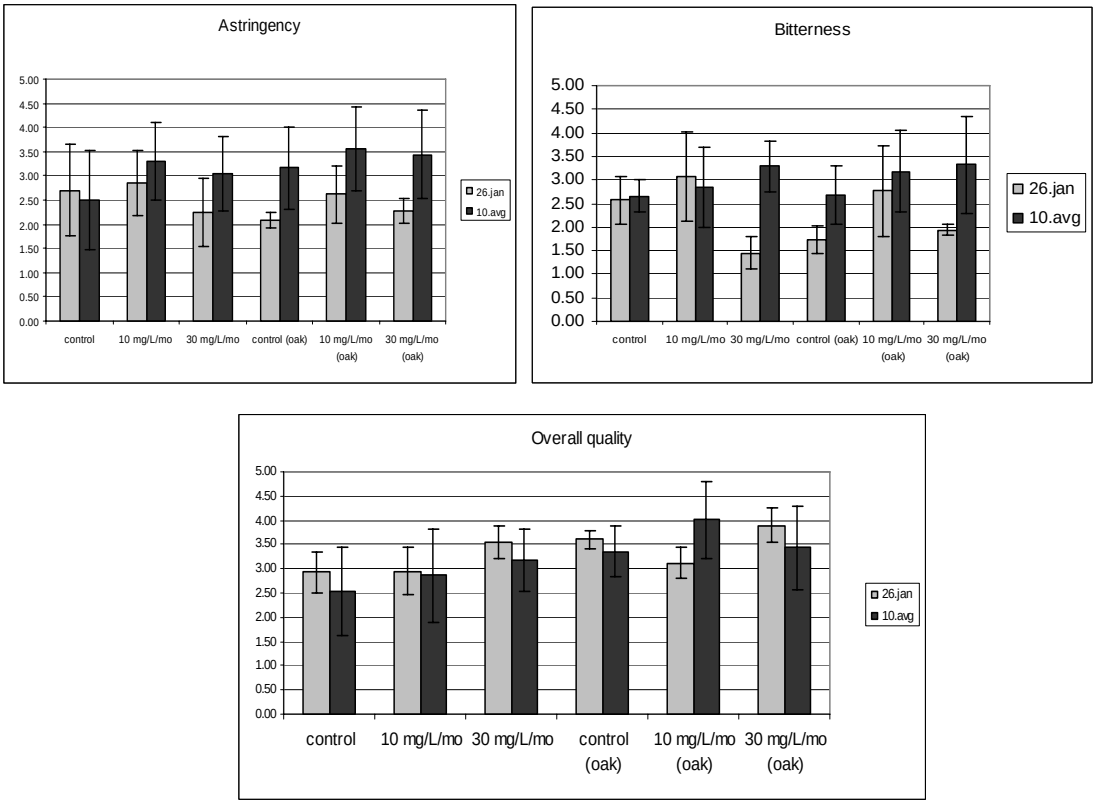



Figure 49: Comparison of astringency, bitterness and overall quality at two different stages of microoxygenation of wines of experiment 1 (Merlot, 2004). Error bars represent standard deviation of 10 degustators

Slika 49: Primerjava trpkosti, grenkobe in celokupne kakovosti v dveh časovnih obdobjih mikrooksigenacije vina poskusa 1 (Merlot, 2004). Odstopanja predstavljajo standardno deviacijo desetih degustatorjev

Table 19 shows the statistically significant differences between different treatments in wines of experiment 2 (Cabernet Sauvignon) 3 months after the end of microoxygenation treatment. The results show that all treatments differ, both in wines without added oak segments and in wines with added oak segments.

Table 19: Influence of microoxygenation on statistically significant ($p<0.05$) sensory differences in wines of experiment 2

Preglednica 19: Vpliv mikrooksigenacije na statistično različne ($p<0,05$) senzorične spremembe v vinih poskusa 2

| **Wine B (Cabernet Sauvignon 2005, Vipava Valley)** | **Treatment** | **Statistically difference at 0,05 level of significance** |
|---|---|---|
| Wine without oak segments | control vs. 3 mg/L/mo | Yes |
| | control vs. 5 mg/L/mo | Yes |
| | 3 mg/L/mo vs. 5 mg/L/mo | Yes |
| Wine with added oak segments | control vs. 3 mg/L/mo | Yes |
| | control vs. 5 mg/L/mo | Yes |
| | 3 mg/L/mo vs. 5 mg/L/mo | Yes |

Table 20 shows statistical differences between treatments. Medium toasted American oak staves (Radoux, Stellenbosch, South Africa) were added into the tanks at 40% and 100% of internal surface area of 225 L barrel oak.

Table 20: Influence of microoxygenation and different internal surface of oak addition on statistically significant ($p<0.05$) sensory differences in wines of experiment 4

Preglednica 20: Vpliv mikrooksigenacije in različnih deležev površin hrastovine na statistično različne ($p < 0,05$) senzorične spremembe v vinih poskusa 4

| **Treatment** | **Statistically difference at 0.05 level of significance** |
|---|---|
| 40% control vs. 40% MOX | No |
| 40% control vs. 100% control | Yes |
| 40% control vs. 100% MOX | Yes |
| 40% control vs. barrique | No |
| 40% MOX vs. 100% control | No |
| 40% MOX vs. 100% MOX | Yes |
| 40% MOX vs. barrique | No |
| 100% control vs. 100% MOX | Yes |
| 100% control vs. barrique | Yes |
| 100% MOX vs. barrique | Yes |

# 5. DISCUSSION AND CONCLUSION

## 5.1 DISCUSSION

The influence of oxygen and its effect on must and wine composition was studied. Since phenolics play a pivotal role in oxidation reactions, the main objective of this study was to investigate changes in two innovative technologies, hyperreduction and microoxygenation.

### 5.1.1. Hyperreduction of white wines

#### 5.1.1.1 Glutathione analysis

Glutathione has received much attention in the past due to its important role in many detoxification processes in living cells. It has a strong anti-oxydative effect and prevents cellular damage by maintaining certain thiols in their reduced state, scavenges hydrogen peroxide and hydroxyl radicals, and maintains the redox potential in cells (Field et al., 1996). It has been recently found, that glutathione can play positive role also in wine technology. Beside its influence in enzymatic oxidation, as already described in literature review, it can also protect aromatic volatiles in white wines during their storage (Roussis et al., 2007; Herbst et al., 2006; Dubourdieu, 2006).

A novel method for reduced and oxidised glutathione was set up on LC-MS-MS. The limit of detection was 0.2 mg/L for oxidized and 0.4 mg/L for reduced glutathione. Okuda and Yokotsuka (1999) reports that over 90% of the original glutathione concentration in the grape juice was lost within five minutes when extracted without $HClO_4$, compared with the level in its presence. Therefore, to prevent glutathione losses, a high dose (1000 mg/L) of $SO_2$ and 500 mg/L of ascorbic acid was added into the bottles at the moment of sampling. Stability test on glutathione concentration was also performed to see the effect of storage condition on glutathione losses before analyzing. It was found that samples stored for 4 weeks at -20°C, with $SO_2$ and ascorbic acid did not change the glutathione concentration (Figure 21).

The concentration of reduced glutathione was analyzed in different grape varieties in grape juice and grape skins. It can be seen that roughly half of the glutathione can be found in

grape flash and the other half in grape skins (Table 14). Glutathione concentration in must ranged from 11 to 30 mg/L (Table 14), which coincides with Cheynier et al. (1989a) who found 14 to 102 mg/L glutathione in grape musts. Among the varieties included into this study, the highest reduced glutathione level was contained by Sauvignon Blanc must, followed by Chardonnay and Greco di Tufo (Table 14). In this study glutathione content was not analyzed during grape ripening; however, Okuda and Yokotsuka (1999) found that glutathione content increased during ripening, with increasing amounts of soluble solids in both Koshu and Cabernet Sauvignon cultivars. They found that correlation between °Brix and glutathione content was high ($r = 0.914$, $p = 0.001$) for Koshu variety, but lower ($r = 0.814$, $p < 0.05$) for Cabernet Sauvignon.

*Botrytis* infection can greatly influence the glutathione level. Healthy Malvasia berries contained more than 5 fold less reduced glutathione compared to botrytized berries. However, this is only a preliminary result, since only one variety was analyzed (Table 14).

#### 5.1.1.2 Small scale hyperreduction pressing

Two different Sauvignon Blanc and Colombar grapes were used and pressed as whole bunches with a custom built press. Oxygen levels during pressing were kept below 1% inside the headspace of the press and less than 0.3 mg/L in the must during hyperreductive pressing and racking. These treatments were considered as hyperreductive must treatments. The oxygen pick-up in the control juices was between 1.0-1.5 mg/L. The oxidative grape pressing was made without $SO_2$ or ascorbic acid and four air rackings were made to encourage $O_2$ pick-up. This resulted in 3.5-4 mg/L dissolved $O_2$ in total measured just after four air rackings. However, the exact concentration was difficult to measure because of rapid decrease of oxygen concentration in conditions without $SO_2$. This was evident from measuring the oxygen consumption after racking and additions of different concentration of $SO_2$ and ascorbic acid (Figure 22). If no $SO_2$ was added, the oxygen concentration dropped rapidly while addition of 50 mg/L of $SO_2$ retards oxygen consumption, which could be due to inhibition of polyphenol oxidase activity. Boulton et al. (1996) reported that addition of 75 mg/L $SO_2$ to must led to 90% inhibition of polyphenol oxidase activity. Our data showed that addition of 200 mg/L $SO_2$ and 50 mg/L ascorbic acid completely inhibited the decrease of oxygen concentration while addition of only ascorbic acid

increased the consumption of oxygen in the most. This could be due to its role in enzymatic reactions of caftaric acid. Ascorbic acid, however, regenerate caftaric acid from its quinone, thus enabling higher oxygen consumption. Bradsow et al. (2003) found that ascorbic acid reacts 1000 times faster with $O_2$ than $SO_2$.

According to Rankine (1989), the freshly crushed must is normally saturated with oxygen because of the aerating action of crushing. From the viewpoint of definition, aeration is the physical process of dissolving oxygen in must or wine while oxidation is the subsequent chemical process of oxidising the juice or wine constituents (Rankine, 1998).

Our experiments included hyperreductive, normal and hyperoxydative treatment of grape juice prior to fermentation. Since phenolic compounds and glutathione are the first compounds that enter oxidation reactions, they are good indicators of oxidation treatments and the oxidative status of grape must. Glutathione (reduced and oxidised), hydroxycinnamic acids and their esters and flavanols were therefore measured during the trials (Annexes A1, A2, A3).

Levels of glutathione content in three different grapes ranged from 33-71 mg/L, which is similar to previously published data by Cheynier et al. (1989a). Park et al. (2000a and 2000b), however, found glutathione level in Sauvignon Blanc juice to be between 0.01 mg/L to 1.28 mg/L, which is much lower than what we found. The authors were not clear on how the grapes were crushed and whether it was done under very reductive or more oxidative conditions, with the latter possibly leading to degradation of the reduced glutathione before the start of fermentation. Our reductive treatments led to the highest levels of reduced glutathione in the juice, with control treatments having slightly lower values. In two experiments with Sauvignon Blanc grapes the content in reduced glutathione significantly differed ($p=0.05$) between all three treatments. However, there was no statistically significant difference between hyperreductive and normal treatment in Colombar grape pressing. Low concentrations of $O_2$ contact as in the control (about 1.0-1.5 mg/L) led to a reduction in the reduced glutathione levels. This is low in terms of commercial winemaking, where $O_2$ levels of a few mg/L often come in contact with must, which is observed with standard commercial crushers and presses (Schneider, 1998).

The relatively small differences in the reduced glutathione concentration between hyperreduced and normal pressing are due to sulphuring, which was performed very quickly after pressing. $SO_2$ can inhibit enzymatic oxidation in must and thus the

consumption of reduced glutathione (Boulton et al., 1996). Hyperoxidative treatment of juice without added sulphur dioxide greatly decreased glutathione levels. This confirms an oxidation reaction of caftaric acid by grape polyphenoloxidase (*ortho*-diphenol oxidase) to the corresponding *o*-quinone and subsequent reaction of available glutathione to form 2-*S*-glutathionyl caftaric acid – Grape Reaction Product (GRP).

At high $O_2$ concentrations, such as in the oxidative treatment, reduced glutathione levels dropped drastically in the juice, which is due to the absence of $SO_2$ and higher concentration of dissolved $O_2$. Glutathione concentration in this treatment was significantly lower compared to the control or hyperreductive pressing.

Reduced glutathione levels in wines were constantly lower than in the corresponding juices in all the treatments. However, our findings show that higher glutathione concentration in must also results in higher glutathione concentration in wine. Park et al., (2000a and 2000b) found glutathione levels to increase during alcoholic fermentation, which is contradictory to our findings. They reported that glutathione production is directly correlated with both nitrogen and assimilable amino acid concentration. The differences in initial amino acid composition as well as the large difference in initial glutathione levels could explain the difference between our results and that of other researchers. Yeast strains could also differ in their glutathione metabolism. Only in Sauvignon Blanc from Stellenbosch region, which had the highest initial value of the reduced glutathione, did the reductive treatment lead to relatively high reduced glutathione levels in wine. It thus seems that the reductive treatment of Sauvignon Blanc as currently being conducted in several wine producing countries should be re-assessed. Marais (1998) investigated the reductive and oxidative treatments of Sauvignon Blanc juice and its effect on the aroma and composition of the resulting wine. They found that wines made reductively gave the best quality wine, but the oxygen levels in the headspace of the press and in the juice were not, according to our knowledge, monitored in this work. However, 2-methoxy-3-isobutylpyrazine levels, which give the typical green pepper aroma to Sauvignon Blanc, were not influenced by the addition of hydrogen peroxide, a strong oxidizing agent, to wine.

*trans*-Caftaric acid plays a pivotal role in enzymatic oxidation (Figure 7). Its concentration dropped with increasing oxygen concentration and oxidative treatment. Hyperreductive

pressed grapes showed the highest concentration while hyperoxidised treatment showed the lowest (Table 11). In all three grapes and treatments there were significant differences in *trans*-caftaric acid concentrations, both in must and in wines after fermentation. On the other hand, the concentrations of GRP were statistically significant higher in normal and hyperoxidated treatments. The decrease of caftaric acid in oxidative treatment causes an increase in GRP concentration, which is the consequence of the enzymatic oxidation in the grape juice in the presence of oxygen (Cheynier et al., 1989b). There were no differences in *trans*-coutaric acid concentrations between treatments. This could be due to very low initial concentration, which could be the consequence of gentle pressing and low must yields. There were no significant differences in free hydroxycinnamic acids (caffeic, *p*-coumaric and ferulic). Their concentration increased in wine, which could be due to enzymatic activities of wine yeast.

Flavan-3-ols, (+)-catechin and (–)-epicatechin were also analyzed, but the differences between treatments were hardly seen. Significant difference was only observed in Colombar juice, where hyperreductive pressed grapes had more (+)-catechin. However, all musts had low levels of both (+)-catechin and (–)-epicatechin, which is again due to soft pressing used in the experiment.

Triangle sensory tasting of all three wines was also performed. The panel could distinguish statistically ($p<0.05$) all three hyperreduced wines from hyperoxidised treated wines and two hyperreduced treatments from normally pressed grapes. Normal and hyperoxidised treatments were difficult to distinguish. The reason could be due to the fast oxidation reactions that occur during pressing, before $SO_2$ addition, and therefore influence the taste of wine. This coincides with Cheynier et al., (1993) who found that important oxidation occurs already inside the press, where oxygen uptake during pressing of whole clusters was estimated at 10 to 15 mg/L.

#### 5.1.1.3 Industrial hyperreductive experiment

Hyperreductive and control pressings were performed in a specially designed wine press. Oxygen concentration in the press atmosphere was measured and concentration below 1% was achieved during the hyperreductive pressing. The reduced oxygen in the atmosphere

retards enzymatic oxidative reactions and influences the level of phenolics and glutathione in grape juice.

**Influence of grape pressing on glutathione, hydroxycinnamic acids and their tartaric esters and low molecular weight flavan-3-ols**

Glutathione concentration was significantly higher in hyperreductive pressed juice when compared to the normally pressed. During pressing glutathione content increased with increasing pressure, which confirms that there is a significant content of glutathione present in skin berries. Our data show that pressing between 0.8 and 1.0 bar led to the highest glutathione concentration.

It can be seen from Figure 23 that glutathione in Muller Thurgau and Greco di Tufo musts pressed in inert atmosphere is not consumed while in normal conditions it oxidizes very fast. This can also depend on the pressing management. If more cycles are used, more oxidation can occur inside the press. Normal pressings above 1.5 bar led to just a small glutathione content which could be due to the absence of $SO_2$ and higher tyrosinase activity in the press juice compared to free-run juice (Schneider, 1998). The concentration of oxidised glutathione was much lower compared to the reduced glutathione. Its concentration also increased with increasing pressure.

The content of hydroxycinnamic acids and their esters in the grape juices ranged from 27 to 105 mg/L (Table 14). *trans*-Caftaric acid was the main esterified hydroxycinnamic acid, representing 87 – 92% of all hydroxycinnamic acid and their esters in the must with the concentration ranging from 24 to 92 mg/L. Singleton et al. (1984) found the average hydroxycinnamic acids and their esters concentration to be 106 mg/L in the juice.

In hyperreductive pressings, esterified hydroxycinnamic acids increased with increasing pressure, because they are mostly located in skin berries. This is most evident for coutaric acid confirming its location in grape skins. This is in agreement with other authors (Vrhovšek, 1997), who found *cis*- and *trans*- coutaric acid located mostly in grape skins.

After pressing there was from 28% (in Muller Thurgau) to 52% (in Chardonnay) more total hydroxycinnamic acids and their esters (without GRP) in hyperreductive compared to normally ($O_2$) pressed grape must. In Greco di Tufo variety there was about 20% more hydroxycinnamic acids and their esters in hyperreductive pressed grapes compared to

normal pressing. This is because in normally pressed grapes only pressings up to 0.4 bar were used, which coincides with the traditional pressing of this variety.

The reason for lower hydroxycinnamic acids and their esters content in normal pressing could be due to their oxidation by grape polyphenoloxidase, so their concentration decreases even if the pressure increases. This is most evident for caftaric and coutaric acid, because they first enter to oxidation reaction to form *o*-quinone. Singleton et al. (1985) observed that caftaric acid was more easily oxidised than coutaric acid.

The concentration of *trans*-caftaric acid in the must after pressing was from 28% (Muller Thurgau) to 55% (Chardonnay) lower in normally pressed grape juice if compared with the hyperreductive pressing. This is in agreement with Singleton et al. (1985) who found a loss of 35% to 100% of the original caftaric acid with regards to crushing conditions in a winery. c*is*-Caftaric acid was also higher in hyperreductive pressing compared to the normal one, but its concentration was much lower regarding the *trans*-isomer.

As it was already found in grape analysis, coutaric acid was mostly located in grape skins. The concentration of *trans*-coutaric acid was 5 to 6 times higher at 2 bar compared with the free run (Figures 27, 31, 35).

The concentration of GRP increased in normal pressing if compared to the hyperreductive treatment, which is the consequence of the reaction between caftaric acid quinone and glutathione in the presence of PPO and oxygen. Cheynier et al. (1988) showed that 0.4 mol of molecular oxygen was consumed per mol of caftaric acid. Conversion of caftaric acid into GRP is therefore believed to be a way of limiting must browning by trapping the caftaric acid quinones in the form of stable glutathione-substituted product and preventing them from proceeding to brown polymers.

Fertaric acid did not show any decrease in normal $O_2$ pressing, which could indicate that it was not involved in oxidation reactions (Figures 28, 32, 36). This is in agreement with the results of other authors (Vrhovšek, 1997; Cheynier et al., 1993), who have also found that fertaric acid was not oxidizable during the pressing. Free forms of hydroxycinnamic acids (caffeic, *p*-coumaric and ferulic) were not found in grape juice (Table 14).

Low molecular weight polyphenols also increased with the increasing pressure, indicating their presence in grape skins. Pressings at 2 bars in hyperreductive atmosphere showed 3-4 times more polyphenols compared to free run juice (Figure 38). In normal pressing, polyphenols also increased with the increasing pressure but their concentration was lower

in comparison with hyperreductive pressing because of their oxidation. Schneider (1998) found that one oxygen saturation (9 mg/L) may be sufficient to precipitate must flavonoid contents to lower than 100 mg/L (expressed as catechin).

**Influence of fermentation on glutathione, hydroxycinnamic acids and their tartaric esters and low molecular weight polyphenols**

The decrease in glutathione during fermentation was 71% (Chardonnay), 81% (Muller Thurgau) and 82% in Greco di Tufo fermenting juice (Figure 24). This could be due to the metabolism of glutathione by yeast. Some of the factors influencing the glutathione level in must during fermentation were already mentioned in the previous chapter. Lavigne et al. (2007) showed that yeast strain also plays an important role in glutathione metabolism. In Chardonnay must, however, an initial increase and later decrease of glutathione was observed during the fermentation of normally ($O_2$) pressed Chardonnay grapes. Hyperreduced wines showed higher concentration of glutathione, but the difference was not significant compared with juice before fermentation. Bentonite addition and must sedimentation had no effect on glutathione content. Single addition of oxygen during fermentation (5 mg/L) also showed no effect on glutathione content.

Hydroxycinnamic tartaric esters (*trans*-caftaric, *trans*-coutaric and fertaric acid) and GRP slightly decreased during fermentation (Figure 37). The decrease was from 3 – 18%. This could be due to the interactions between hydroxycinnamic acids and their esters and yeast as was also found by Somers et al. (1987). They found higher losses of hydroxycinnamic acids and their esters during fermentation, however our data show that fermentation plays a minor role in the modification of hydroxycinnamic acids and their estes compared to the pre-fermentation processing. On the other hand, *trans*-caffeic acid appeared in fermenting must which could be due to hydrolysis of caftaric acid as a consequence of yeast enzymatic activities.

Low molecular weight polyphenols also decreased during fermentation (Figure 39). The decrease was 33% to 53%, which could be due to the interaction with yeast and proteins.

Microoxygenation of the fermenting must was also applied in one treatment. However, no changes were observed in phenolic compounds before and after must microoxygenation. We assume that oxygen was consumed for yeast metabolism before oxydising the phenolic compounds. This coincides with Rosenfeld et al. (2003) and Fornairon-Bonnefond and

Salmon (2003) who found that yeast cells and yeast lees have much higher consumption rates and affinities for oxygen than wine polyphenols. Additional ethanol formed during fermentation can also inhibit the PPO activity and thus retards the oxidation of hydroxycinnamic tartaric esters. Bentonite addition after pressing and must sedimentation decreased the content of low molecular weight polyphenols by an average 8.5 %.

**Influence of aging on glutathione, hydroxycinnamic acids and their tartaric esters and low molecular weight polyphenols**

Dubourdieu (2006) found that the best ageing conditions for preserving the aromatic qualities of dry white wines were also those which minimize the decrease in their glutathione content. Therefore the knowledge of aging conditions on glutathione is important to wine technology. Our data showed that the average glutathione decrease was 37% within three months of wine aging on lees (battonage). However its concentration in wine was very low, ranging from 1.8 to 5.7 mg/L with no difference between hyperreductive and normally grape pressing (Annexe A1, A2, A3). Chardonnay wine was aged in old barrels, but the decrease was not enhanced compared to other two wines aged in tanks. Glutathione concentration fell below the limit of detection after 9 months of wine storage and after the wine had been racked off the lees. Lavigne et al. (2007) found that glutathione content decreased rapidly and significantly when wine was separated from its lees. This effect was accentuated in new barrels, where oxidative phenomena are more prevalent. Herbst et al. (2006) showed that addition of sulphur dioxide and glutathione lowered the decrease in 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate, an important aroma coumpounds in Sauvignon Blanc, over a four week period. Dubourdieu (2006) reports that control wine cv. Sauvignon Blanc had absorbance at 420 nm of 0.203 after three years, while wine with added 10 mg/L glutathione had an absorbance value of 0.136. 3-mercaptohexanol was also higher in wines with glutathione addition while sotolon and 2-aminoacetophenone were lower. Glutathione therefore plays an important role in preventing oxidative spoilage of white wines and can increase the maturation potential of white wines, particularly Sauvignon Blanc.

Hydroxycinnamic tartrate esters did not change significantly during wine aging. There was a slight decrease of *trans*-caftaric acid in Greco di Tufo. *trans*-Coutaric and fertaric acid also showed no significant decrease during wine aging. This is in agreement with other

authors, who found small losses during wine conservation (Gunata et al., 1986, Somers et al., 1987). The GRP concentration decreased during aging by 27% in the average. This is also in agreement with the results which have been obtained by other researchers and their experiments (Gunata et al., 1986; Singleton and Cilliers, 1995; Vrhovšek, 1997). *trans*-Caffeic acid slightly increased during aging and *trans*-coumaric acid appeared in wine after 9 months of aging. This could be due to hydrolysis of tartaric esters during storage and coincides with results obtained by many authors (Archier et al., 1993; Somers et al., 1987; Vrhovšek, 1997).

Low molecular weight flavan-3-ols decreased by 22 to 62% after 9 months of aging but there was no correlation between the normal or hyperreductive technology. The decrease of flavan-3-ols could be due to oxidation and interactions with proteins and yeast. The content of total polyphenols measured with Folin-Ciocalteau after 9 months of aging was higher in hyperreductive Muller Thurgau (14%) and Chardonnay (45%) wine while there were no differences in Greco di Tufo wine (Table 16). The absorbance at 420 nm was higher only in Chardonnay hyperreductive pressed grapes, while there was no influence of hyperreductive pressing in other two varieties. Vinification in hyperreduction can increase brown colour, but more research is needed to confirm this.

**Sensory evaluation**

Hyperreductive technology greatly affects the organoleptic characteristics of wine. Hyperreductively produced Muller Thurgau differed significantly ($p<0.05$) from the normally pressed grapes, since 8 out of 10 tasters found the difference (Table 17). However, none of the tasters found that wine more bitter or astringent; in hyperreductive produced wine even higher values of hydroxycinnamic acids and their esters and phenolic compound were found. This is not in agreement with other authors (Okamura and Watanabe, 1981; Ong and Nagel, 1978) who found hydroxycinnamic acids and their esters in wine to be bitter and astringent. Verrete et al. (1988) reports that added caffeoyl tartaric acid did not produce any detectable difference in the taste of wine, even at 150 mg/L, but it was easily detected in 1% aqueous ethanol at 100 mg/L ($p<0.001$). No significant difference in taste was produced when caffeic acid and *p*-coumaric acid were added at 120 mg/L and 30 mg/L respectively. GRP was not detectable at 50 mg/L (Verrette et al., 1988). Hyperreductive produced Muller Thurgau wine was judged as more fresh and fruity with

softer and harmonious taste. Some tasters also found hyperreductive wine more closed and reductive on the nose. On the other hand, wines produced by normal pressing were described as more bitter and phenolic, less harmonious with less varietal and aromatic characteristics, but more clean and open on the nose.
Sensory difference in Chardonnay and Greco di Tufo wines was lower and 6 out of 10 tasters found the differences. The reason could due to different winemaking technique, because the Chardonnay wine was aging 10 months in 225 L used barrels, therefore the effect of hyperreductive pressing can be masked by oak compounds. The taster, who found the difference between wines, described the hyperreductive Chardonnay as more fruity with sweaty aroma and softer, less astringent taste.

Greco di Tufo wine produced in normal ($O_2$) conditions contained pressings just up to 0.4 bar while hyperreductive pressed grapes and wine contained pressings up to 2.0 bar. The pressing management changes the structure of the wine and modifies the perception of wine flavour; therefore a fewer number of tasters found the difference between both wines. Like in other two varieties, the main descriptive difference for hyperreductive produced Greco di Tufo was enhanced freshness and fruitiness, better roundness with less bitterness and astringency aftertaste. However, Greco di Tufo produced by normal technology had more complex and more persistent aftertaste.

Our results show that hyperreduction can increase the freshness and fruitiness of white wines, with better roundness and softness on the taste. The varietal aroma also seems to increase when grape are vinificated with hyperreduction. However, care must be taken, because these wines can be more reductive and closed on the nose. The complexiness also seems to decrease when working in hyperreduction. We can assume that the oxidative state of wine phenolics greatly influences the taste of wine, probably more than their absolute concentration.

### 5.1.2. Microoxygenation of red wines

The effect of microoxygenation on phenolic compounds was evaluated. Due to microoxygenation technique requirements, where appropriate tank height is needed, only large scale experiments were investigated. Due to industrial incapability to perform experiments in triplicate, all microoxygenation trials were made only in one model. Thus we should not consider the absolute values reported, but rather the tendencies observed.

Microoxygenation is usually applied to red wines to improve and stabilize their colour, therefore great attention was paid to colour development during wine aging and anthocyanins structure. In general the treated wines colour intensity increased in microoxygenation wines in comparison to the control (Figure 40). The increase was proportional to oxygen addition, therefore, the higher oxygen dose was added, the higher colour intensity the wine obtained. This is in agreement with Perez-Magarino et al. (2007) who found the same tendency in their experiments. Higher colour intensities in microoxygenated wines could be due to combination of anthocyanins with proanthocyanidins. Oxygen addition leads to oxidation of phenolic compounds and formation of $H_2O_2$. The latter can form a bridge between an anthocyanin molecule and a proantocyanidin moiety (Es-Safi et al., 1999). The resulting molecule is also coloured, subsequently leading to the increase in colour intensity during aging. Our results also showed that higher proportion of polymeric pigments was obtained in microoxygenated wines at the end of the treatment. Additionally, HPLC analysis of free anthocyanins also showed lower values of free anthocyanins in all microoxygenation treatment (Figure 43). This could be again due to augmented combination of anthocyanins with proanthocyanidins. Bosso et al. (2000) and Castel et al. (2001) also found an increase in polymeric anthocyanins, concluding that the addition of oxygen activated the reactions among free anthocyanins and flavanols, forming new coloured compounds stable to changes in $SO_2$ concentration and pH.

The increase in colour intensity was mostly due to the increase of $A_{420}$ while $A_{520}$ decreased during aging. The proportion of $A_{620}$ also slightly increased during aging and was found to be higher in microoxygenated wines compared to the control. The higher colour intensity could have been due to the contribution of ethyl-linked pigments; since the absorbance of these molecules at 620 nm is higher than that of genuine anthocyanins (the

percentage of blue colour in MOX wines is higher than in control wines). Pyranoanthocyanins may also participate in the higher colour intensity, as they show higher absorbance at 420 nm and contribute to the redness of wines. These anthocyanin-derived pigments are present at very low concentrations in wine, but their true importance can not be judged by their concentration as assessed by HPLC. At wine pH, they may contribute greatly to wine colour. At pH 3.6, the concentration of malvidin-3-glucoside to give an absorbance value of 1 at 520 nm is 260 mg/L, compared with a concentration of 29 mg/L for vitisin A (Lee et al., 2004). Anthocyanins at wine pH are mainly in the hemiketal form and therefore do not contribute to wine colour.

One of the alleged uses of microoxygenation is to simulate an oak barrel, leading to the observed colour changes (Santos-Buelga et al., 1999). Our results showed that the addition of oak segments in combination with oxygen addition increased colour intensity. This could be due to anthocyanin combination with oak tannins in the presence of oxygen. The same can be also seen in wine aged in old and new barrels. Wines aged in new barrels show significantly higher colour intensity compared with wine aged in old barrels, which could be due to higher oxygen exchange in new barrels. Our results showed that colour intensity was higher in microoxygenated wines compared with wines aged in barrique. This is, however, not in accordance with Cano-Lopez et al. (2006a) who found higher colour intensity in wines matured for 6 months in oak barrels than microoxygenated wines. This could be due to lower oxygen addition in microoxygenated wines compared to oxygen transfer through oak barrel or a difference in the constitution of the oak used for the barrels and staves.

Colour hue increased during wine aging, however there were no significant differences between microoxygenated and control wine (Figure 41). This is also in agreement with other authors (Perez-Magarino et al., 2007). An increase in colour hue can be seen only in Merlot wine (Vipava Valley) oxygenated at higher rate and in microoxygenated Pinotage (Stellenbosch region) wine. During 1.5 year bottle aging of wine A, colour hue increased by 12.4% in average while colour intensity decreased by 16.6% in average. The reason could be the oxygen left in bottle headspace and gas exchanges through the cork, which influence oxidative reactions between anthocyanins and tannins also during bottle aging.

Total anthocyanins were also measured in our experimental wines. Our results showed that total anthocyanins were lower in most of the microoxygenated wines compared with the control wines (Figure 42). The same trend was also confirmed with HPLC determination of free anthocyanins, where concentration of free anthocyanins decreased with the increased oxygen concentration. These losses are in agreement with experimental studies carried out by Amati et al. (2000) and Ferrarini et al. (2001) who found lower concentrations of anthocyanins, catechins and proanthocyanidins in microoxygenated wines than in the control one. Perez-Magarino et al. (2007) also showed higher monomeric anthocyanin glucosides, acetylated and cinnamylated in control wine, compared with microoxygenated wine. On the other hand, Llaudy et al. (2006) found significantly higher levels of anthocyanidin-3-glycosides, acetylated and *p*-coumaroyl anthocyanins in microoxygenated wines compared with the control wines. The reason for anthocyanin decrease is their combination with flavanols and formation of ethyl bridges, as also found by Atanasova et al. (2002). Malvidin-3-glucoside, which represented 44-59% of all free anthocyanins in our treated wines, showed the highest decrease in absolute value. However, in terms of relative value, petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside showed a similar decrease, from 7 - 18% in average compared with control tank. Wines with a higher proportion of added oak staves had lower concentration of malvidin-3-glucoside. This may indicate that anthocyanins react with oak components or they more effectively link to proanthocyanidins in the presence of toasted oak extracts. Gomez-Plaza (2004) also found that monomeric anthocyanins decreased during the wood aging period. The age of the barrel, rather than the volume of the barrels or the origin of the wood were the main cause of differences in monomeric anthocyanin concentration among wines. Revilla and Gonzales-San Jose (2001) found that the decrease in the monomeric anthocyanin content in wines stored in used barrels was also lower and the evolution of these phenolic compounds in all the wines was slower in the bottle than in the barrel. It is clear that monomeric anthocyanins are not the main factor responsible for colour density in aged wines but, rather, the formation of new compounds, mainly condensation products between anthocyanins and tannins.

It can also be seen that Cabernet Sauvignon from South Africa contained 10% higher proportion of malvidin-3-glucoside in comparison with Cabernet Sauvignon from Slovenia. If this is a coincidence or a general fact it is not possible to state from these

results. However, this could be the consequence of different terroir, clones or other climate differences between countries. In any case, free anthocyanins decreased during wine aging (Ribéreau-Gayon et al., 2000b), an average decrease during 1.5 years bottle aging of wines in experiment 1 was 67% (Figure 43).

Total polyphenols also decreased during wine aging, but the correlation between control and microoxygenated wines was not significant. Low molecular weight flavan-3-ols (vanillin index) and high molecular weigh flavan-3-ols (proanthocyanidins) were measured during microoxygenation of wines in experiment 1 (Merlot, 2004) and experiment 2 (Cabernet Sauvignon, 2005). Low molecular weight flavan-3-ols decreased during wine aging by 18% in average (Figure 46). However, there was no difference between control and microoxygenated wines. Proanthocyanidins increased at the beginning of microoxygenation of wines in experiment 1, but then started to decrease (Figure 45). The ratio between vanillin index and proanthocyanidin analyzed by Bath Smith was proposed as apparent polymerization index. Lower ratio indicates that wine contains less low molecular flavan-3-ols and more proanthocyanidins. The ratio decreased during wine aging, but there was no difference between control and microoxygenated wines (Figure 47). Nevertheless, two wines from South Africa show a much lower ratio between low and high molecular weight polyphenols, indicating that these South African grape tannins could have had higher molecular weight in comparison to Slovenian wines.

The mean degree of polymerization (mDP) was also analyzed in experiment 1 (Merlot, 2004, Vipava Valley) after 9 months of the microoxygenation treatment. Though the difference between microoxygenated and control wine is small, the microoxygenated wines showed lower mean degree of polymerization. This is not in accordance with other authors who showed higher mDP in microoxygenated wines compared with the control. However, in an acidic-medium such as wine, tannins also undergo cleavage reactions (Vidal et al., 2002). Some of the cleavage products serve as intermediates in polymerization reactions, and some bonds in the new polymers are also labile. In particular, ethyl bridges formed through acetaldehyde-induced polymerization are unstable, and through cleavage and addition reactions are gradually converted to more stable pyranoanthocyanin derivatives (Cheynier et al., 1999). As a consequence, phenolic changes during wine aging are a dynamic process leading to increasing structural diversity but not necessarily to larger polymers. However, an estimation of tannin mDP becomes

increasingly inaccurate in older wines as some modified constitutive units (e.g., through oxidation) and other units incorporated into the polymers cannot be analyzed. McCord (2002) showed no significant difference in the quantity of oligomers and polymers between oxygenated and non-oxygenated wines.

HPLC phenolic profile was also made in wines of experiment 3 (Pinotage, 2004, Stellenbosch) at the end of the microoxygenation experiment. Vanillic acid was much higher in the barrel-treated wine than in the other treatments, probably due to the higher oak contact. Concentration of (+)-catechin decreased with 10% in oxygenated wine compared with the control wine and with 16% in barrel. Polymeric pigment and polymeric phenol increased during microoxygenation, with barrel having the highest value.
Polymeric pigment increased in the 3 mg/L/month and barrel treatments and was also higher with regard to polymeric phenols after 6 months of treatment. This is probably due to more acetaldehyde being produced in these treatments as a result of the higher $O_2$ additions, which led to enhanced acetaldehyde polymerization (Ribéreau-Gayon et al., 2000b). (–)-Epicatechin was lower at the end of microoxygenation compared with the control. This is in agreement with McCord (2002) and Vidal et al. (2002) who have shown in wine-like model solution that flavanol monomers such as epicatechin, react with products of the breakdown of polymeric proanthocyanidins. This leads to a reduction in the average size of the proanthocyanidin polymers and this change of polymer size may influence the perceived astringency.

Acetic acid bacteria and *Brettanomyces* are both well known spoilage micro-organisms of wine. Acetic acid bacteria can form unwanted elevated levels of acetic acid through an oxidative metabolism of ethanol. *Brettanomyces* can cause medical, barnyard characters in wine due to the production of volatile phenols. Both these organisms have been proven to grow in wine when $O_2$ has been added to it (Du Toit and Pretorius, 2002 and Du Toit et al., 2005). There has been no report, according to our knowledge, on the effect of microoxygenation on acetic acid and *Brettanomyces* counts in wine. Acetic acid bacterial numbers decreased in the control tank after four weeks, with counts staying constant in the tanks receiving $O_2$ and in the barrel. Acetic acid bacteria can go into a viable, but non-culturable state in wine, which can be negated by the addition of $O_2$ (Du Toit et al., 2005).

*Brettanomyces* counts increased in the 1.5 mg/L/month treatment from 10 to $10^3$ cfu/mL after 14 weeks (Figure 49). At this time the free $SO_2$ concentration decreased in this tank to 18 mg/L and was increased to 35 mg/L. This led to a decrease in *Brettanomyces* cell counts in this tank, but the counts subsequently increased in the wine, microoxygenated with 3 mg/L/month, with a correlating decrease in the free $SO_2$ (17 mg/L). The $SO_2$ at this stage was then adjusted in all the treatments to 35 mg/L/month. Microoxygenation thus does not seem to support the growth of acetic acid bacteria, but possibly their survival. With the permeation of $O_2$ through oak staves (Vivas et al., 2003) small amounts of $O_2$ coming into contact with wine could hence support the survival and growth of acetic acid bacteria and *Brettanomyces*. The judicial use and monitoring of $SO_2$ in combination with microoxygenation is important to prevent this, especially in the case of *Brettanomyces*. Too high $SO_2$ concentrations could, however, lead to inhibition of favourable phenolic polymerization reactions (Ribéreau-Gayon et al., 2000b).

Sensory analyses were also performed in all treatments. In the first experiment descriptive analyses were made in the middle of microoxygenation treatment (after 10 weeks) and 3 months after it's completion (Figure 50). Astringency and bitterness were lower in microoxygenated wines and overall quality was higher after 10 weeks of treatment (Figure 50). However, when microoxygenation was applied too long, both astringency and bitterness increased (Du Toit et al., 2006). Microoxygenated wines also showed higher overall quality and wines with oak segment addition were preferred to wines without oak segments.

There were high variations in the descriptive evaluation of wines; therefore triangle test was applied in other three experiments. In this way a statistically significant difference can be obtained. In wines of experiment 2 (Cabernet Sauvignon, Vipava Valley), tasters could distinguish at 0.05 level of significance all different oxygen additions (Table 19). The tasting was performed 3 months after microoxygenation had been completed. In wines of experiment 3 the panel could not statistically distinguish (after three months) between the control, 1.5- and 3 mg/L/month treatment and barrel aging. In wines from the forth experiment the panel could distinguish between different treatments and oak additions; however they could not distinguish between control and microoxygenated wines with 40% internal surface of oak addition from each other and the barrel. Microoxygenation used in

combination with oak staves could thus be an alternative to using oak barrels for red wine maturation and development. However, it is important that the correct quality oak staves are used. Further research on this is also required.

## 5.2 CONCLUSIONS

- the reduced oxygen concentration in the must during the processing of grapes retards enzymatic oxidative reactions and influences the level of phenolics and glutathione in grape juice
- glutathione, caftaric acid and GRP concentration can be appropriate markers to follow the oxidative status of must
- oxygen addition into the must can decrease the glutathione content; on the other hand, hyperreductive pressing of grapes prevents the loss of glutathione
- hyperreduction pressing can increase the content of esterified hydroxycinnamic acids, especially caftaric and coutaric acid, the main esterified hydroxycinnamic acid in must; the content of fertaric acid is not influenced by hyperreduction technology
- fermentation decreases the glutathione content in must while esterified hydroxycinnamic acids decreased just slightly during fermentation
- during wine aging glutathione decreases while hydroxycinnamic acids and their esters do not change significantly
- hyperreductive technology greatly affects the organoleptic characteristics of wine and can be an appropriate technique to increase the freshness and fruitiness of white wines, with better roundness and softness of the taste
- microoxygenation can be applied into red wine maturation with the aim of improving colour intensity
- colour hue is not greatly affected when using microoxygenation
- addition of oak segments in combination with microoxygenation can greater increase the colour intensity than using microoxygenation alone
- the decrease of free anthocyanins was often greater when higher oxygen additions were used
- total phenolics (OD 280), flavan-3-ols (vanillin index) and proanthocyanidins (Bate-Smith reaction) decrease during wine aging, but there is no difference between control and microoxygenated wines
- there was no difference in mDP between control and microoxygenated wines
- microoxygenation can increase the growth of *Brettanomyces* yeast and the survival of acetic acid bacteria

- sensory characteristics can be greatly influenced when microoxygenation is used
- both technologies are suitable for production of Slovenian wines.

## 6 SUMMARY

**Background:** oxygen plays an important role in winemaking. It is commonly admitted that extensive oxidation is unfavorable to wine quality, but slow and continuous oxygen dissolution may play a positive role in wine aging (Cheynier, 2002). To promote the beneficial effects of oxygen exposure while avoiding spoilage risks, it is essential to understand the mechanisms governing oxygen dissolution and consumption in wine. Therefore, new technologies in wine production – hyperreduction and microoxygenation; focus a great intention how to manage the role of oxgen in wine making.

**Objective of the work:** to study two different technologies in controlling oxygen's influence and its effect on must and wine composition and quality. These two technologies include 'hyperreduction', an innovative grape pressing of white varieties and 'microoxygenation' a controlled and regulated oxygen addition into red wines.

**Experimental design**: thesis work was divided into two experimental parts: hyperreduction trials and microoxygenation experiments.

In hyperreduction an innovative grape press was tested for pressing of different white varieties in oxygen free atmosphere. With the total absence of oxygen in the atmosphere during grape pressing we hypothesized that oxidation reactions stops due to absence of oxygen – the most important substrate of these reactions. To confirm this technique small and real scale experiments were made and different analytical markers were followed, which include glutathione and esterified hidroxycinnamic acids. A novel LC-MS-MS method for reduced and oxydised glutathione was developed to analyze its content in different pressing fraction, during fermentation and wine aging. Low molecular weight flavan-3-ols were also analyzed to confirm the effect of hyperreductive technology. All treated wines were also submitted into sensory evaluation.

In microoxygention treatment the effect of oxygen addition on the phenolic composition of different red wines was evaluated. Four different large scale experiments were conducted on different red wine varieties. The influence of oak segments addition in combination with different oxygen additions was also investigated. Spectrophotometric and HPLC DAD and MS analysis of phenolic compound were followed during and after treatments.

**Results:**

**Hyperreduction**: a novel method for reduced and oxidised glutathione was set up on LC-MS-MS. The limit of detection was 0.2 mg/L for oxidized and 0.4 mg/L for reduced glutathione. Stability test on glutathione concentration was also performed to see the effect of storage condition on glutathione losses before analyzing. It was found that storage for 4 weeks at -20°C, with 1000 mg/L $SO_2$ and 500 mg/L ascorbic acid, did not change glutathione concentration. The concentration of reduced glutathione was analyzed in Muller Thurgau, Chardonnay, Greco di Tufo, Sauvignon Blanc, and Malvasia grape varieties in grape flesh and grape skins. Glutathione concentration in must ranged from 9 to 30 mg/L. Out of the varieties included in vinification experiments, the highest reduced glutathione level contained were in Sauvignon Blanc must, followed by Colombar and Chardonnay.

a) Microvinification trial: Our reductive treatments led to the highest levels of reduced glutathione in the juice, with control treatments having slightly lower values. At high $O_2$ concentrations, such as in the oxydative treatment, reduced glutathione levels dropped drastically in the juice, which is due to the absence of $SO_2$ and higher concentration of dissolved $O_2$. Reduced glutathione levels in the wines were constantly lower than in the corresponding juice in all the treatments. However, our findings show that higher glutathione concentration in must results also in higher glutathione concentration in wine.

b) Industrial experiments: in industrial experiments, glutathione concentration was significantly higher in hyperreductive pressed juice comparing to normal pressed. During pressing glutathione content increased with increasing pressure, which confirms that there is significant content of glutathione present in skin berries. Esterified hydroxycinnamic acids were also higher in hyperreductive pressing comparing with normal processing. *trans*-Caftaric acid, which ranged from 27 - 105 mg/L and represented from 87 - 92% of all hydroxycinnamic acids dropped significantly during normal ($O_2$) pressing of grapes. On the other hand GRP increased in normal, oxidative grape processing. Fertaric acid showed no chages between normal and hyperreductive pressing, which indicates that it is not included in oxidative, enzymatic reactions. Glutathione showed a huge decrease during fermentation, while esterified hydroxycinnamic acids decreased just slightly during fermentation. During wine aging glutathione decreased, while esterified hydroxycinnamic

acids did not change significantly. Hyperreductive technology increased the freshness and fruitiness of white wines, with better roundness and softness on the taste.

**Microoxygenation:** due to industrial incapability to perform experiments in triplicate, all microoxygenation trials were made only in one repetition. In all treated wines colour intensity increased in microoxygenation wines in comparison with control. The increase was proportional to the oxygen addition, therefore the higher oxygen dose was added, the higher colour intensity of wine was obtained. Colour hue increased during wine aging, however there were no significant differences between microoxygenated and control wine. Our results also showed that addition of oak segments in combination with oxygen addition increased colour intensity. Total anthocyanins were lower in the microoxygenated wines comparing with the control wines. The same trend was confirmed also with HPLC determination of free anthocyanins, where concentrations of free anthocyanins decreased with increased oxygen concentration, which could be due to augmented combination of anthocyanins with proanthocyanidins. Total polyphenols decreased during wine aging, but correlations between control and microoxygenated wines was not significant. Low molecular weight flavan-3-ols and high molecular weight proanthocyanidins also showed no difference between control and microoxygenated wines. The mean degree of polymerization (mDP) was also analyzed in experiment A (Merlot, 2004, Vipava Valley) after 9 months of microoxygenation treatment. Though the difference between microoxygenated and control wine is small, the microoxygenated wines show lower mean degree of polymerization. Microbiological analyses were also performed in one experiment. Acetic acid counts decreased in the control tank after four weeks, with counts staying constant in the tanks receiving $O_2$ and in the barrel.

*Brettanomyces* counts increased in microoxygenated wines – in the 1.5 mg/L/month treatment from 10 to $10^3$ cfu/mL after 14 weeks. However, after $SO_2$ addition cell counts decreased. The judicial use and monitoring of $SO_2$ in combination with microoxygenation is important to prevent microbiological spoilage. Sensory evaluations showed lower astringency and bitterness of microoxygenated wines. However, when microoxygenation was applied for too long, both astringency and bitterness increased. Microoxygenated wines also showed higher overall quality and wines with oak segments addition were preferred with regard to wines without oak segments.

**Conclusions**: grape pressing is crucial factors in must oxidation, because oxidative enzymatic reactions are very fast. The hyperreduction seems to be an effective technology to prevent must oxidation and increase glutathione and hydroxycinnamic acids and their esters content in must and wines. However, great care must be taken to perform a correct hyperreduction pressing and maintain oxygen concentration in press atmosphere below 1%. Using this technology the varietal characteristics of certain wines can be improved, increase freshness and fruitines of wines and decrease its bitterness and astringency. Microoxygenation of red wines was approved to increase colour intensity of red wine. In this investigation tendencies were observed, but further trials investigating the effect of microoxygenation on the evolution of the taste of red wine should be carried out. This study did show that many complex analytical methods are not adequate alone to monitor microoxygenation, because the chemical reactions in red wines are very complex and still not enough controlled. On the other hand, microbiology can also be considered. However, microoxygenation can be a good tool to improve sensory quality of red wines.

## 6.1 SUMMARY IN SLOVENE LANGUAGE (POVZETEK V SLOVENSKEM JEZIKU)

**Uvod**

Kisik ima pri predelavi vin zelo pomembno vlogo, tako pozitivno kot negativno. Znano je, da izpostavljanje mošta ali vina kisiku zmanjšuje kakovost in sortnost samega vina zaradi oksidacije, izgube sadnosti, karamelizacije in sprememb drugih lastnosti. V zadnjih letih sta se v predelavi vin razvili dve novi metodi pridelave vin, ki smo ju eksperimentalno proučili, da bi ugotovili njune prednosti, slabosti im možnosti za njihovo aplikacijo pri pridelavi slovenskih vin.

Prva metoda je hiperredukcija – tehnologija vinifikacije belih vin s popolno odsotnostjo kisika. V zadnjem času se vse bolj uveljavlja pri predelavi in zorenju belih sortnih vin. Grozdje se predela in stiska v posebej prirejeni stiskalnici, kjer je koncentracija kisika pod 1% med celotno fazo stiskanja. Prednosti take tehnologije naj bi bile večplastne, saj naj bi odsotnost kisika preprečevala tako encimske kot neencimske reakcije oksidacije, kar vodi do vin z bolj izraženimi sortnimi značilnostmi, bogatejšo polifenolno sestavo in manjšo uporabo žveplovega dioksida.

Temu nasprotna mikrooksigenacija pa se vse bolj uveljavlja v tehnologijah pridelave rdečih vin. Z dodatkom mikro količin kisika optimiziramo in pospešimo zorenje rdečega vina. Ta tehnika se je razvila v zgodnjih 90-tih kot simulacija zorenja vina v lesenih sodih barrique, saj je znano, da les prepušča majhne količine kisika. S kontroliranim dodajanjem kisika vplivamo na strukturne spremembe polifenolov rdečega vina in posledično na barvo, aromo in okus.

Polifenolne spojine so primarne komponente, ki se oksidirajo s kisikom, oksidacijski produkti pa posledično spremenijo sestavo in kakovost vina. Raziskava želi pridobiti nova spoznanja o posledicah dveh različnih tehnologij, pri kateri ima kisik ključno vlogo. Delo je potekalo v dveh raziskovalnih sklopih: eksperimentalno smo proučevali tako hiperreduktivno kot tudi tehnologijo mikrooksigenacije. Tako smo pri belih vinih raziskovali vpliv hiperreduktivne tehnologije na sestavo in kakovost mošta in vina, pri rdečih vinih pa vpliv mikrooksigenacije na strukturne spremembe njihovih polifenolov. Z laboratorijskimi in industrijskimi poskusi smo ugotavljali vpliv različnih tehnik stiskanja belega grozdja v modificirani atmosferi na vsebnost hidroksicimetnih kislin in glutationa v

moštu ter vinu. Pri rdečih vinih pa smo z različnimi poskusi spremljali vpliv dodajanja različnih koncentracij kisika na polifenolno sestavo vina.

## Materiali in metode

### Hiperredukcija

Hiperreduktivno tehnologijo smo proučili na laboratorijski in industrijski ravni. Na laboratorijski ravni smo za stiskanje grozdja v modificirani atmosferi (vsebovala je manj kot 1% kisika) uporabili posebno stiskalnico. Vanjo smo dali 9 kg celega grozdja, jo pokrili s pokrovom in napolnili z vodo. Vodo smo nato izpodrinili s $CO_2$ plinom (Afrox SA, Stellenbosch, JAR) in tako dosegli inertno atmosfero v notranjosti stiskalnice. Z dušikom (Afrox SA, Stellenbosch, JAR) smo napolnili notranjost membrane in grozdje dvakrat stisnili do nadtlaka 2 bar. Notranjost stiskalnice smo kontinuirano prepihovali s $CO_2$. Okrog 3,5 L mošta smo zbrali v 4,5 L steklenih posodah, ki smo jih predhodno napolnili z vodo in jo nato izpodrinili s $CO_2$. Za tem smo v steklene posode dodali 60 mg/L žveplovega dioksida in 50 mg/L askorbinske kisline. Kljub dvojnemu stiskanju do 2 barov nadtlaka, smo dobili dokaj majhne izkoristke (40%). Kisik v plinasti fazi stiskalnice smo kontinuirano merili z oksimetrom Micro III G202 Gesellschaft für Gerätebau (Dortmund, Nemčija), kisik v moštu v 4,5 L steklenih posodah pa z oksimetrom Oxi 330i oxygen meter with a cell ox 325 probe (Wissenschaftlich-Technische Werkstätten – WTW, Weilheim, Nemčija). Vsebnost kisika v atmosferi stiskalnice je bila pri hiperreduktivnem stiskanju vedno pod 1%, v moštu pa pod 0,3 mg/L.

Kontrolno (normalno) stiskanje grozdja je bilo opravljeno na podoben način, le brez dodajanja $CO_2$ v stiskalnico in stekleno posodo. Koncentraciji žveplovega dioksida in askorbinske kisline sta bili enaki kot pri hiperreduktivnem stiskanju. Prevzem kisika v moštu je bil 1,0 - 1,5 mg/L. Hiperoksidativna predelava je bila opravljena brez dodatka žveplovega dioksida in askorbinske kisline. Po stiskanju smo mošt štirikrat zračno pretočili v plastični posodi. Na ta način smo pospešili raztapljanje kisika v moštu. Izmerjena koncentracija je bila med 3,5 - 4,0 mg/L. Natančno merjenje kisika v moštu brez dodatka $SO_2$ in askorbinske kisline je zelo težko, saj njegova koncentracija hitro pade zaradi porabe v reakcijah encimskega delovanja. Po sedimentaciji smo mošt pretočili s pomočjo $CO_2$, inokulirali s 30 g/hL rehidriranim kvasnim sevom Vin 13 (Anchor Yeast Biotechnologies,

Cape Town, JAR) in dodatkom 50 g/hL diamonijevim fosfatom (Anchor Yeast Biotechnologies, Cape Town, JAR). Po fermentaciji smo mlado vino zažveplali (50 mg/L) in pretočili v steklenice z navojnim pokrovčkom. Za te poskuse smo uporabili grozdje treh različnih sort (dva Sauvignona in Colombar), vsako obdelavo pa trikrat ponovili.
Vzorčenje za HPLC in LC-MS-MS analize je potekalo tik po opravljenem stiskanju ter po fermentaciji. 80 mL vzorca smo shranili v 100 mL steklenih vialah, ki smo jih predhodno napolnili s $CO_2$, dodali 1000 mg/L $SO_2$ in 500 mg/L askorbinske kisline. S tem smo popolnoma inhibirali encimsko delovanje in preprečili izgubo merjenih substanc. Viale smo do analiz zamrznili na -20° C.

Industrijski poskus hiperreduktivne predelave je potekal v vinski kleti Pojer&Sandri v Faedu (Trentino, Italija). Poseben sistem stiskanja je omogočal stiskanje grozdja v modificirani atmosferi s koncentracijo kisika pod 1 odstotkom. Prednost omenjene stiskalnice je sistem za recikliranje inertnega plina, ki se shranjuje v posebnem balonu. Inertni plin tako kroži v zaprtem sistemu stiskalnice, zbirni posodi in balonu. Za poskus smo uporabili tri različne sorte grozdja: Chardonnay, Muller Thurgau in Greco di Tufo. Obrano grozdje smo najprej 12 ur hranili v hladilnici na temperaturi 2 °C, ga razdelili v dva enaka dela po cca. 1000 kg in predelali po dveh različnih tehnologijah:

- s kontrolnim stiskanjem v normalni atmosferi,
- s hiperreduktivnim stiskanjem v atmosferi dušika.

Stiskali smo celo grozdje, ki smo mu predhodno dodali 30 mg/L $SO_2$ in 20 mg/L askorbinske kisline. Pri različnih nadtlakih smo prešance vzorčili v 0,75 L steklenice, v katere smo predhodno dodali 1 g/L $K_2S_2O_5$ in 0,5 g/L askorbinske kisline. Steklenice smo predhodno prepihali z inertnim plinom (argonom). V vseh vzorcih smo opravili analize glutationa, hidroksicimetnih kislin in njihovih estrov ter nizkomolekularnih flavan-3-olov.
Koncentracijo kisika v atmosferi stiskalnice smo merili z oksimetrom Oxymet (Isolcell, Laives, Italija). Po stiskanju je sledil dodatek bentonita 20 g/hL (Bentogran, AEB, Brescia, Italija) in sedimentacija. Po 24 urah je sledil pretok in dodatek dveh različnih sevov kvasovk: 10 g/hL VL 3 (Laffort Oenologie Bordeaux, Francija) in 10 g/hL BCS 103 (Springer Oenologie, Maison-Alfort, Francija) ter 20g/hL hranila za kvasovke Biostimol (Polo Enologia, Oderzo, Italija). Po fermentaciji, ki je trajala 12 – 15 dni, smo vina oddvojili od grobih droži in žveplali. Vino je nato zorelo na finih drožeh.

V vseh frakcijah prešancev smo določali vsebnost glutationa, hidroksicimetnih kislin in njihovih estrov ter nizkomolekularnih flavan-3-olov. Omenjene spojine smo zasledovali tudi med fermentacijo in zorenjem vina.

Za analizo glutationa smo razvili metodo na LC-MS-MS s pomočjo Waters Quattro micro API triple quadroupole masnim spektrofotometrom z 2690 Alliance HPLC. Separacija je potekala na Waters Atlantis C18, 3 μm, 2,1x150 mm koloni z 0,1 % mravljinčno kislino (topilo A) in acetonitrilom (topilo B). Sestava topila je bila prve 0,5 min 100% topila A, kateremu je sledil linearni gradient 6,5 min topila B do koncentracije 80% in re-ekvilibracija na 100% s topilom A, naslednjih 7 minut. Retenzijski čas za reducirani glutation je bil 3,5 min, za oksidirani glutation pa 5,2 min.

Preverili smo tudi stabilnost glutationa med hranjenjem vzorca pri različnih temperaturah in vsebnosti $SO_2$. Grozdje smo hipereduktivno stisnili in vzorce isti dan analizirali z in brez dodatka $SO_2$ ter askorbinske kisline. To smo naredili v treh ponovitvah. Vzorce smo nato 4 tedne hranili pri temperaturi 4 °C in -20 °C. Po dveh tednih smo del vzorcev odmrznili, analizirali in ponovno zamrznili. S tem smo hoteli proučiti vpliv tajanja na koncentracijo glutationa.

Hidroksicimetne kisline smo analizirali z Agilent 1100 HPLC z DAD detektorjem, povezanim z Agilent NDS ChemStation (Agilent Technologies, Palo Alto, USA). Separacijo smo izvedli s Hypersil C18 kolono 2,1x250 mm (5 μm) in z ODS Hypersil predkolono 2,1 x 20 mm (5 μm) (Agilent Technologies, Palo Alto, USA). Mobilno fazo je sestavljala:

A) 0,5% mravljinčna kislina v vodi,
B) 2% mravljinčna kislina v metanolu.

Separacija je potekala pri 40 °C. Pretok je bil 0,4 mL/min, injiciran volumen pa 10 μL. Beležili smo UV-VIS spekter od 220 do 700 nm, detekcija pa se je vršila pri 320 nm. Nizkomolekularne flavan-3-ole, reaktivne na vanilin smo analizirali po metodi opisani v Di Stefano in Guidoni (1989a) ter Rigo in sod. (2000).

Razliko senzoričnih sprememb smo ugotavljali s pomočjo triangel testov, s katerimi smo želeli ugotoviti, ali omenjene tehnologije statistično pomembno vplivajo na senzorične spremembe vin.

**Mikrooksigenacija**

V okviru mikrooksigenacijskih analiz smo opravili 4 različne poskuse na treh različnih sortah (poskusi št. 1 do 4). Vsi so potekali na industrijskem nivoju v večjih tankih, saj je eden izmed pogojev tehnike mikrooksigeniranja sama višina tankov, ki naj znaša vsaj 2 m. V večini poskusov smo poleg kisika dodali različne koncentracije in oblike hrastovih dodatkov (ploščice, deske), saj smo želeli ugotoviti vpliv kisika na kakovost vina v kombinaciji s hrastom. Vse poskuse smo tretirali z mikrooksigenacijsko napravo Parsec (Firenze, Italija), ki dodaja kisik v mg/L/mesec.

Preglednica 1: Opis mikrooksigenacijskih poskusov

| POSKUS | SORTA IN LETNIK | IZVOR VINA | OBDELAVA |
|---|---|---|---|
| 1 | Merlot 2004 | Vipavska dolina Slovenija | 6 tankov smo mikrooksigenirali v kombinaciji z dodanimi hrastovimi ploščicami in različnimi koncentracijami kisika. Začetek mikrooksigeniranja po biološkem razkisu. Vino smo tretirali s 30 in 10 mg/L/mesec, koncentracijo pa smo postopno zmanjševali. |
| 2 | Cabernet sauvignon 2005 | Vipavska dolina Slovenija | 6 tankov smo mikrooksigenirali v kombinaciji z dodanimi hrastovimi ploščicami in različnimi koncentracijami kisika. Vino smo tretirali s 5 in 3 mg/L/mesec kisika. Isto vino je zorelo v novih hrastovih sodih. |
| 3 | Pinotage 2004 | Stellenbosch, Južno Afriška Republika | 0, 1,5 and 3 mg $O_2$/L/mesec z dodanimi hrastovimi dogami in začetkom mikrooksigeniranja 7 mesecev po mlečnokislinski fermentaciji. Isto vino je zorelo tudi v hrastovem sodu iz istega lesa kot doge (USA MT+). |
| 4 | Cabernet sauvignon 2006 | Stellenbosch Južno Afriška Republika | Začetek takoj po končanem biološkem razkisu. Hrastove doge istega lesa in ožganosti so bile dodane v mikrooksigenacijski tank v dveh različnih koncentracijah |

Med mikrooksigeniranjem smo spremljali spremembe fenolne sestave vina. Predvsem nas je zanimala dinamika barvnih sprememb (intenzitete in tona barve) ter spremembe v sami

koncentraciji skupnih in prostih antocianov. Skupne polifenole, antociane, visokomolekularne flavan-3-ole (proantocianidine) in nizkomolekularne flavan-3-ole (vanilin index) smo določali spektrofotometrično po metodi Di Stefano in Guidoni (1989a) ter Rigo in sod. (2000).

Proste antociane smo določali s pomočjo metode HPLC-DAD pri 520 nm in detekcijo vseh 15 antocianov. Vzorce smo pred injiciranjem na HPLC prečistili s pomočjo ekstrakcije na trdni fazi (SPE) ter jih filtrirali skozi 0,22 µm PVDF filter. Posamezne antociane smo identificirali s pomočjo spektrov pripadajočih standardov v območju valovnih dolžin med 300 in 700 nm ter retenzijskih časov. Antociane smo ovrednotili kot ekvivalente eksternega standarda malvidin-3-glukozida, glede na površino pod vrhom, s pomočjo umeritvene krivulje za malvidin-3-glukozid.

Povprečno stopnjo polimerizacije proantocianidinov smo določali po metodi Mattivi et al. (2006b). V poskusu št. 3 (Pinotage, 2004) smo opravili tudi mikrobiološko analizo, s katero smo med mikrooksigeniranjem na selektivnih gojiščih spremljali rast kvarljivcev vina in sicer ocetnokislinskih bakterij in kvasovk rodu *Brettanomyces.*

Na obravnavanih vinih smo s pomočjo triangelnih testov opravili senzorične analize vin in tako preverili, ali obstajajo razlike med različnimi obdelavami.

## REZULTATI IN RAZPRAVA

### Hiperredukcija

Vpliv kisika na sestavo mošta in vina smo spremljali v laboratorijskem in industrijskem merilu. Glede na to, da imajo polifenoli najpomembnejšo vlogo, je bil glavni namen poskusov spremljanje sprememb v koncentraciji teh spojin, ki so pomembni antioksidanti.

Pri poskusih hiperreduktivne predelave na laboratorijski in industrijski ravni smo spremljali vpliv omenjene tehnologije na koncentracijo glutationa in hidroksicimetnih kislin, saj so to najboljši markerji za spremljanje te tehnologije.

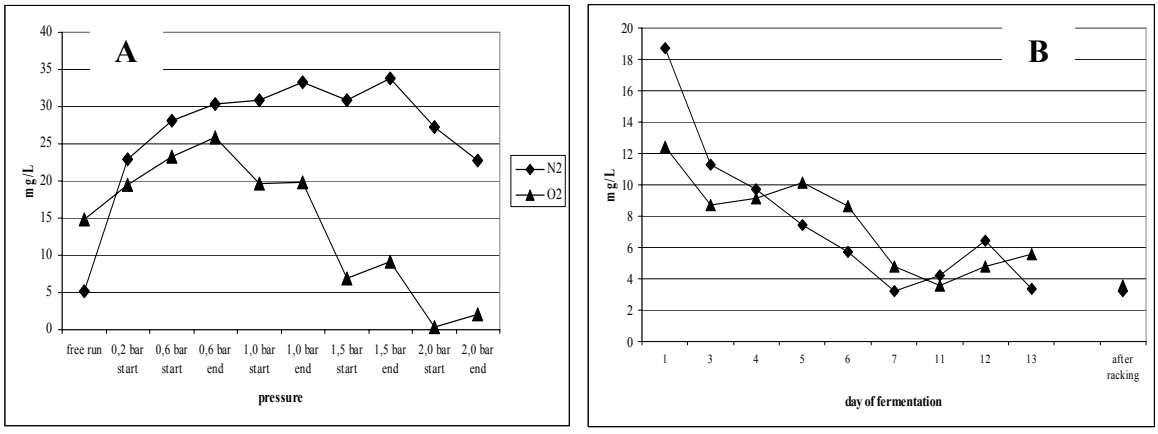
Meja detekcije za reducirani glutation z metodo LC-MS-MS je bila 0,2 mg/L, za oksidirani glutation pa 0,4 mg/L. Test stabilnosti je pokazal, da štiritedensko hranjenje vzorca z dodatkom $SO_2$ in askorbinske kisline pri -20 °C, ne vpliva na zmanjšanje koncentracije glutationa. Koncentracija glutationa v moštih je bila v vseh opravljenih poskusih med 11 in 72 mg/L, kar sovpada s podatki Cheynier s sod. (1989a), ki so našli od 14 do 102 mg/L

glutationa v grozdnem moštu. Največ glutationa je vseboval mošt Sauvignona, ki sta mu sledila Colombar in Chardonnay.

Laboratorijski poskusi so vsebovali tri različne pred-fermentacijske tehnologije predelave grozdja, in sicer hiperreduktivno, normalno in oksidativno stiskanje. Vsebnost glutationa pri različnih načinih stiskanja je podana v preglednicah št. 9 in 10.

Iz rezultatov je razvidno, da vsebnost reduciranega glutationa pade med predelavami z različno vsebnost kisika. V hiperreduktivno predelanem moštu je vsebnost reduciranega glutationa največja, medtem ko je vsebnost pri normalno predelanih manjša, pri hiperoksidiranih moštih pa znatno manjša. Vsebnost reduciranega glutationa se po fermentaciji znatno zmanjša. Poleg reduciranega glutationa se je s stopnjo oksidacije zmanjšala tudi kaftarna kislina, vsebnost grozdnega reakcijkega produkta (GRP, grape reaction product) pa se je statistično značilno povečala. Padec reduciranega glutationa in kaftarne kisline ter naraščanje GRP-ja je posledica encimskih reakcij v grozdnem moštu (Cheynier s sod., 1989b).

Industrijski poskus je prav tako pokazal razlike v koncentraciji glutationa in hidroksicimetnih kislin. Mošt hiperreduktivno predelanega grozdja je vseboval večjo vsebnost reduciranega glutationa v primerjavi z normalno predelanim. Vsebnost reduciranega glutationa je naraščala z naraščanjem tlaka in dosegla maksimalno vrednost v prešancih med 0,8 in 1,0 bara nadtlaka v inertni atmosferi, kar pomeni, da se nahaja tudi v jagodni kožici.




Slika 1: Vpliv hiperreduktivnega ($N_2$) in normalnega ($O_2$) stiskanja (A) in fermentacije (B) na vsebnost reduciranega glutationa v sorti Muller Thurgau

Vsebnost esterificiranih hidroksicimetnih kislin je večja tudi v hiperreduktivno predelanem grozdju, kjer *trans*-kaftarna kislina predstavlja od 87 do 92% vseh hidroksicimetnih kislin in njihovih estrov v moštu, njena koncentracija pa se giblje med 24 in 92 mg/L. Koncentracija hidroksicimetnih kislin in njihovih estrov narašča z naraščajočim nadtlakom, kar potrjuje dejstvo, da se večinoma nahajajo v jagodni kožici. To velja predvsem za kutarno kislino, kar se ujema tudi z ugotovitvami drugih avtorjev (Vrhovšek, 1997). Z uporabo hiperreduktivnega stiskanja na ta način znatno prispevamo k večji vsebnosti kutarne kisline v moštu. Dodatek bentonita v mošt pred sedimentacijo in dodatek kisika med fermentacijo (5 mg/L) nista vplivala na vsebnost esterificiranih hidroksicimetnih kislin. Vsebnost GRP-ja se pri normalnem stiskanju grozdja poveča v primerjavi s hiperreduktivnem, kar nakazuje pomen encimskih reakcij med estri hidroksicimetnih kislin in glutationom. Vsebnost fertarne kisline je ostala ista ne glede na način stiskanja, kar dodatno potrjuje dejstvo, da ne vstopa v oksidacijske reakcije (Vrhovšek, 1997; Cheynier et al., 1993). To nakazuje na dejstvo, da fertarna kislina ni primeren marker spremljanja hiperreduktivne predelave. Z našimi raziskavami smo zato odprli pot za povečanje predvsem kaftarne in kutarne kisline v moštu. Prostih hidroksicimetnih kislin (kumarne, ferulne in kavne kisline) v moštu nismo našli, kar pomeni, da so v grozdju predvsem v vezani obliki.

Nizkomolekularni polifenoli naraščajo tudi z večanjem nadtlaka. V naših poskusih je bila vsebnost polifenolov v prešancih pri 2 barih 3 do 4- krat večja kot v samotoku.

Med fermentacijo se je vsebnost glutationa zmanjšala v povprečju za 71 do 82 odstotkov, kar je verjetno posledica metabolizma glutationa v kvasovkah. Vina hiperreduktivno predelanega grozdja so pokazala večjo vsebnost glutationa, vendar razlika ni bistvena, iz česar lahko sklepamo, da fermentacija bistveno vpliva na vsebnost glutationa. Koncentracija esterificiranih hidroksicimetnih kislin se je med fermentacijo tudi rahlo zmanjšala, kar je lahko posledica interakcij s kvasovkami. Encimska aktivnost med fermentacijo povzroči razpad zaestrenih hidroksicimetnih kislin (kaftarne kisline) v proste hidroksicimetne kisline (kavna kislina). Koncentracija nizkomolekularnih polifenolov se je med fermentacijo prav tako precej zmanjšala, kar je lahko posledica interakcij s kvasovkami ali proteini. Med zorenjem je vsebnost glutationa padla v povprečju za 37 odstotkov v prvem trimesečju in je znašala med 1,8 in 5,7 mg/L, med hiperreduktivnim in normalnim stiskanjem pa ni bilo večje razlike. Po 9 mesecih zorenja je vsebnost glutationa

padla pod mejo detekcije. Koncentracija hidroksicimetnih kislin in njihovih estrov je bila med zorenjem konstantna, kar je v skladu tudi z drugimi raziskovalci (Gunata in sod., 1986; Somers in sod., 1987). Vsebnost GRP-ja se je med zorenjem zmanjšala, vsebnost kavne in kumarne kisline pa povečala, kar je posledica hidrolize zaestrenih hidroksicimetnih kislin (Archier in sod., 1993; Somers in sod., 1987; Vrhovšek, 1997). Naše raziskave pa so poleg tega pokazale, da se je vsebnost nizkomolekularnih flavan-3-olov med zorenjem vina zmanjšala od 22 pa vse do 62 odstotkov v prvih devetih mesecih zorenja, kar je lahko posledica interakcij z odmrlimi kvasovkami in proteini.

Raziskave so nas privedle do spoznanja, da s hipereduktivno predelavo vplivamo tudi na senzorične spremembe vina. Vse rezultate poskusov smo preverjali s triangel senzoričnim testiranjem. Laboratorijski poskus je pokazal, da so se vsa vina hiperreduktivno predelanega grozdja statistično pomembno razlikovala od oksidativne predelave grozdja ($p<0,05$). Prav tako sta se dve hiperreduktivno pridelani vini statistično pomembno razlikovali od tradicionalne, kontrolne predelave. Industrijski poskusi so prav tako pokazali statistično pomembne razlike med hiperreduktivnim in normalnim stiskanjem grozdja. Pri sorti Muller Thurgau je bila stopnja tveganja 0,05, pri sortah Chardonnay in Greco di Tufo pa 0,1. Degustatorji so hiperreduktivno predelana vina ocenili kot bolj sortno izražena, sadna in sveža s poudarjeno mehkobo in polnostjo okusa. Na podlagi navedenih rezultatov lahko potrdimo, da uporaba hiperreduktivne tehnologije pozitivno vpliva na senzorične spremembe belih vin.

**Mikrooksigenacija**

Vpliv mikrooksigenacije na polifenolne spremembe smo spremljali z industrijskimi poskusi št. 1 (Merlot, 2004), št. 2 (Cabernet Sauvignon, 2005), št. 3 (Pinotage, 2004) in št. 4 (Cabernet Sauvignon, 2006). Ker se mikrooksigenacija uporablja za stabilizacijo rdečih vin, smo spremljali razvoj intenzitete in tona barve. Pri vseh mikrooksigeniranih vinih se je intenziteta barve povečala. Vedno je bila sorazmerna z dodanim kisikom, kar pomeni, da z večanjem koncentracije kisika vplivamo na večjo intenziteto barve. Večja intenziteta barve je posledica reakcij med antociani in tanini. Kisik namreč oksidira fenolne spojine, pri tem pa se tvori $H_2O_2$. Slednji deluje kot prekurzor med antociani in proantocianidini in tako se tvorijo stabilna barvila (Es-Safi in sod., 1999). Naši rezultati so pokazali, da se je vsebnost

polimernih pigmentov povečala, skupnih antocianov pa zmanjšala. Tako smo pojasnili vpliv kisika na reakcije polifenolov v rdečem vinu, kar omogoča tvorbo stabilnejših barvil. Poleg tega smo s HPLC analizami prostih antocianov potrdili, da se vsebnost prostih antocianov zmanjša z večjim dodatkom kisika, kar pomeni, da monomerni antociani hitreje polimerizirajo. Povečanje intenzitete barve je v naših primerih nastala predvsem zaradi povečanja absorbance pri 420 nm in 620 nm. Povečanje absorbance pri 420 nm je lahko posledica večje vsebnosti piranoantocianidinov, ki izboljšajo rdečo barvo vina.

Ena izmed pomembnih možnosti uporabe mikrooksigenacije je tudi simulacija barrique sodov. Naši rezultati kažejo, da uporaba kisika v kombinaciji z dodanimi hrastovimi segmenti poveča intenziteto barve. Prav tako so vina, ki so zorela v novih barrique sodih pokazala večjo intenziteto barve, kar je lahko posledica dejstva, da kisik v novih barrique sodih hitreje prehaja skozi lesene doge kot v rabljenih sodih. Simulacija barikiranja ima tako velike finančne učinke na pridelavo rdečih sort vina, kar pomeni dodatno prednost pri uporabi mikrooksigenacije.

Ton barve se je med zorenjem vina povečal, vendar med kontrolnimi in mikrooksigeniranimi vini ni bilo večjih razlik, kar se sklada z ugotovitvami drugih raziskovalcev (Perez-Magarino in sod., 2007). Povečanje tona barve je bilo opazno samo pri vinih poskusa št. 1 (Merlot, 2004) , ki smo jih tretirali z večjo koncentracijo kisika.

Vsebnost prostih antocianov se je zmanjšala v vseh obravnavanih vinih predvsem zaradi zmanjšanja vsebnosti malvidin-3-glukozida, ki predstavlja od 44 do 59 odstotkov vseh prostih antocianov. Vina z dodanimi hrastovimi segmenti so imela večje zmanjšanje malvidin-3-glukozida, kar je lahko posledica reakcij s hrastovimi komponentami.

Vsebnost skupnih polifenolov se je med zorenjem vina zmanjšala, vendar korelacija med tretiranimi in kontrolnimi vini ni statistično značilna. Vsebnost nizkomolekularnih flavan-3-olov (vanilin index) se je zmanjšala med zorenjem, vendar večjih razlik med različnimi načini obdelave ni bilo. Vsebnost proantocianidinov, merjenjih po metodi Bate-Smith, tudi ni dalo bistveno različnih rezultatov kljub različnim obdelavam. Analiza povprečne stopnje polimerizacije procianidinov v vinih poskusa št. 1 (Merlot, 2004) je pokazala nižje stopnje polimerizacije v primerjavi s kontrolnim vinom, kar pa ni v skladu z ugotovitvami drugih raziskovalcev. Ponovno se je potrdilo, da je zorenje vina dinamičen proces, ki vodi v povečanje strukturnih sprememb polifenolov, vendar ne vedno v večje polimere (Cheynier in sod., 1999).

HPLC analize polifenolov v vinih poskusa 3 (Pinotage, 2004) so pokazale manjše vsebnosti katehina in epikatehina v mikrooksigeniranih vinih v primerjavi s kontrolnim vinom.

V okviru raziskav na področju mikrooksigenacije smo v poskusu št. 3 preverili tudi vpliv kisika na rast nezaželenih mikroorganizmov v vinu, predvsem ocetnokislinskih bakterij in kvasovk rodu *Brettanomyces*, saj je znano, da se obe vrsti organizmov razvijata v prisotnosti kisika (Du Toit in Pretorius, 2002, Du Toit in sod., 2005). Rast kvasovk *Brettanomyces* se je povečala z 10 na $10^3$ cfu/mL po 14 tednih obravnave. Istočasno je vsebnost prostega $SO_2$ padla na 18 mg/L. Po korekciji prostega $SO_2$ na 35 mg/L se je vsebnost kvasovk *Brettanomyces* občutno zmanjšala. Na drugi strani pa dodatek kisika ni stimuliral rasti ocetnokislinskih bakterij, ampak pripomogel k njihovi preživelosti. Ugotovitve nas vodijo do spoznanja, da mikrooksigenacija lahko poveča rast *Brettanomyces* kvasovk in pripomore k preživetju ocetnokislinskih bakterij, zato je kontrola prostega $SO_2$ med tretiranjem zelo pomembna.

Senzorične analize so pokazale statistične razlike v različnih obdelavah. V vinih, zajetih v poskus št. 1 (Merlot, 2004), smo opravili deskriptivne senzorične analize, v ostalih pa primerjalne triangel analize. Trpkost in grenkoba sta se v mikrooksigeniranih vinih (poskus št. 1) zmanjšala po 10 tednih obdelave, medtem ko je nadaljnjo mikrooksigeniranje povečalo trpkost in grenkobo. Skupna kakovost obdelanih vin se je v primerjavi s kontrolnim vinom povečala. Zaradi velikih odstopanj v deskriptivni senzorični analizi smo v ostalih vinih opravili le triangel primerjalne analize. V vinih, zajetih v poskus št. 2 (Cabernet Sauvignon, 2005), so ocenjevalci tri mesece po končani mikrooksigenaciji lahko statistično relevantno razlikovali vse tri dodatke kisika ($p < 0,05$). V vinih, zajetih v poskus št. 3 (Pinotage, 2004), degustatorji po treh mesecih mikrooksigenacije niso ugotovili razlike med tretiranimi vini in kontrolo, vendar so bila vina, vključena v poskus št. 4 (Cabernet Sauvignon, 2006), statistično pomembno različna. Iz tega sledi, da z uporabo mikrooksigenacije lahko spremenimo senzorični profil vina in pozitivno vplivamo na izboljšanje senzorične kakovosti rdečih vin.

SKLEPI

- zmanjšana koncentracija kisika v moštu med predelavo grozdja upočasni encimske oksidacijske reakcije in vpliva na vsebnost fenolov in glutationa v grozdnem moštu,
- glutation, kaftarna kislina in GRP so lahko primerni markerji za sledenje oksidativnega stanja mošta,
- dodatek kisika v mošt zmanjša vsebnost glutationa, na drugi strani pa hiperreduktivno stiskanje grozdja ohrani njegovo koncentracijo,
- hiperreduktivno stiskanje lahko poveča vsebnost zaestrenih hidroksicimetnih kislin, predvsem kaftarne in kutarne kisline, dveh glavnih zaestrenih hidroksicimetnih kislin v moštu,
- fermentacija zmanjša vsebnost glutationa v moštu, medtem ko se koncentracija hidroksicimetnih kislin le malo zmanjša,
- vsebnost glutationa se zmanjša med zorenjem vina, medtem ko je koncentracija hidroksicimetnih kislin konstantna,
- hiperreduktivna tehnologija pozitivno vpliva na organoleptične karakteristike vina in je primerna tehnologija za izboljšanje sadnosti in svežine belih vin, izboljša njihovo polnost in mehkobo okusa,
- mikrooksigenacija se uporablja pri rdečih vinih za izboljšanje intenzitete barve,
- mikrooksigenacija ne vpliva pomembno na ton barve,
- dodatek hrastovih segmentov v kombinaciji z mikrooksigenacijo bolj poveča intenzteto barve kot zorenje brez dodanih hrastovih segmentov,
- zmanjšanje prostih antocianov je večje pri tretiranju z večjo koncentracijo kisika,
- skupni polifenoli (OD 280), flavan-3-oli (vanilin index) in proantocianidini (Bate-Smith) se zmanjšajo med zorenjem vina, vendar razlike med mikrooksigeniranimi in kontrolnimi vini niso značilne,
- v povprečni stopnji polimerizacije proantocianidinov (mDP) ni razlik med kontrolnim in mikrooksigeniranimi vini,
- mikrooksigenacija lahko poveča število kvasovk rodu *Brettanomyces* in ocetnokislinskih bakterij,
- senzorične spremembe se lahko zelo spremenijo z uporabo mikrooksigenacije,
- obe novi tehnologiji sta primerni tudi za pridelavo slovenskih vin.

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# ANNEXES

Annexe A1: Comparison in glutathione, hydroxycinnamic acids and their esters and vanillin index content in normal (O2) and hiperreductive (N2) pressing Chardonnay grape (22nd September 2005)
Priloga A1: Primerjava vsebnosti glutationa, hidroksicimetnih kislin in njihovih estrov in vanillin indeksa pri normalnem ($O_2$) in hiperreduktivnem ($N_2$) stiskanju grozdja Chardonnay (22. september 2005)

| | glutathione reduced | | glutathione oxidised | | *cis* -caftaric | | *trans* - caftaric | | *cis* - coutaric | | GRP (*) | | *trans* - coutaric | | fertaric | | *trans* - caffeic | | *trans* p-coumaric | | *trans* - ferulic | | Vanillin index | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) |
| | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L (+) | |
| FREE RUN | 0,23 | 13,14 | 2,95 | 1,9 | 0,93 | 1,79 | 29,54 | 64,97 | 2,29 | 1,47 | 11,29 | 1,92 | 5,09 | 6,08 | 2,3 | 1,37 | nd | nd | nd | nd | nd | nd | 128 | 113,4 |
| 0.5 bar | 0,41 | 26,51 | 2,53 | 4,4 | 1,29 | 2,45 | 42,67 | 84,74 | 3,29 | 3,39 | 10,14 | 4,47 | 7,46 | 12,27 | 2,48 | 1,99 | nd | nd | nd | nd | nd | nd | 216,6 | 253 |
| 0.8 bar | 0,14 | 24,72 | 3,08 | 4,56 | 1,29 | 2,82 | 47,15 | 91,61 | 4,07 | 4,69 | 9,22 | 7,51 | 7,23 | 16,48 | 2,33 | 2,14 | nd | nd | nd | nd | nd | nd | 222,5 | 299,5 |
| 1.0 bar | 0,14 | 17,35 | 3,05 | 4,59 | 1,46 | 3,08 | 49,14 | 95,82 | 4,98 | 5,74 | 8,38 | 10,33 | 6,96 | 19,94 | 2,24 | 2,32 | nd | nd | nd | nd | nd | nd | 199,2 | 333 |
| 1.5 bar | 0,11 | 9,32 | 2,81 | 4,5 | 1,71 | 3,49 | 52,07 | 104,32 | 5,82 | 7,39 | 7,76 | 11,59 | 7,25 | 23,35 | 2,32 | 2,23 | nd | nd | nd | nd | nd | nd | 197,7 | 370,8 |
| 2.0 bar | 0,16 | 3,88 | 2,64 | 3,88 | 2,05 | 4,15 | 52,76 | 123,53 | 6,66 | 10,43 | 6,59 | 11,03 | 7,94 | 30,65 | 2,36 | 2,99 | nd | nd | nd | nd | nd | nd | 189 | 433,3 |
| tank after press (before bentonite) | 0,07 | 16,45 | 1,52 | 7,46 | 0,96 | 2,08 | 33,22 | 73,51 | 2,46 | 2,72 | 9,55 | 4,89 | 4,76 | 9,68 | 1,91 | 1,84 | nd | nd | nd | nd | nd | nd | 116,3 | 184,7 |
| after sedimentation (before racking) | 0,07 | 15,6 | 1,43 | 6,98 | 0,84 | 1,88 | 34,76 | 77,97 | 2,44 | 2,83 | 9,53 | 5,22 | 4,97 | 10,21 | 2,07 | 2,01 | nd | nd | nd | nd | nd | nd | 112 | 170,1 |
| before yeast inoculation | 0,03 | 12,98 | 1,69 | 3,31 | 0,82 | 1,67 | 34,36 | 76,56 | 2,5 | 2,66 | 9,61 | 5 | 4,89 | 10,32 | 2,04 | 2,18 | nd | nd | nd | nd | nd | nd | 104,7 | 190,5 |
| 1° day fermentation | 0,6 | 19,74 | n.d. | 5,5 | 1,28 | 1,93 | 33,91 | 74,26 | 2,49 | 2,64 | 9,68 | 4,69 | 5,16 | 9,96 | 2,38 | 1,91 | 0,35 | 0,34 | nd | nd | nd | nd | 75,6 | 165,8 |
| 2° day fermentation | 3,95 | 19,78 | n.d. | 2,36 | 0,81 | 1,96 | 26,78 | 65,68 | 1,76 | 2,48 | 8,22 | 4,33 | 3,88 | 8,79 | 1,86 | 1,69 | 0,32 | 0,31 | nd | nd | nd | nd | 55,3 | 100,3 |
| 3° day fermentation | 3,61 | 13,36 | n.d. | 2,65 | 0,86 | 1,79 | 27,57 | 64,94 | 1,85 | 2,43 | 8,21 | 4,16 | 4,02 | 8,6 | 1,87 | 1,67 | 0,32 | 0,31 | nd | nd | nd | nd | 62,5 | 100,3 |
| 4° day fermentation | 6,28 | 12,52 | n.d. | 3,58 | 0,79 | 2,02 | 28,64 | 68,64 | 2,06 | 2,58 | 8,63 | 4,85 | 4,07 | 9,18 | 1,92 | 1,8 | 0,29 | 0,31 | nd | nd | nd | nd | 62,5 | 109,1 |
| 5° day fermentation | 6,62 | 9,49 | n.d. | 2,83 | 1,05 | 2,23 | 28,9 | 68,48 | 2,25 | 2,64 | 8,67 | 4,78 | 4,11 | 9,18 | 1,97 | 1,78 | 0,32 | 0,32 | nd | nd | nd | nd | 58,2 | 106,1 |
| 6° day fermentation | 2,93 | 5,88 | n.d. | 1,19 | 1,14 | 2,28 | 30,05 | 68,78 | 2,29 | 2,6 | 8,71 | 4,59 | 4,24 | 9,23 | 2,05 | 1,74 | 0,32 | 0,32 | nd | nd | nd | nd | 61,1 | 123,6 |
| 9° day fermentation | 2,78 | 7,87 | 0,43 | 4,72 | 0,94 | 2,13 | 28,29 | 65,44 | 2,34 | 2,67 | 6,97 | 3,6 | 4,01 | 8,71 | 1,51 | 1,7 | 0,41 | 0,44 | nd | nd | nd | nd | 48 | 87,2 |
| 10° day fermentation | 2,06 | 9,36 | 0,48 | 5,85 | 0,9 | 2,25 | 28,06 | 67,19 | 2,29 | 2,7 | 6,68 | 3,87 | 3,92 | 8,91 | 1,76 | 1,72 | 0,39 | 0,45 | nd | nd | nd | nd | 52,3 | 87,2 |
| 11° day fermentation | 2,31 | 6,05 | 0,65 | 6,28 | 0,99 | 2,23 | 29,51 | 67,78 | 2,42 | 2,63 | 8,14 | 4,27 | 4,2 | 8,93 | 1,54 | 1,72 | 0,43 | 0,46 | nd | nd | nd | nd | 49,4 | 82,9 |
| 12° day fermentation | 1,68 | 4,83 | 0,58 | 5,5 | 0,97 | 2,3 | 28,64 | 69,07 | 2,36 | 2,74 | 8,24 | 4,5 | 4 | 9,19 | 1,47 | 1,75 | 0,28 | 0,34 | nd | nd | nd | nd | 55,3 | 88,7 |
| 14° day – after *trans* fer into barrique (6.10.2005) | 1,08 | 3,78 | n.d. | 3,56 | 1,07 | 2,15 | 28,9 | 65,2 | 2,18 | 2,42 | 8,42 | 4,52 | 4,16 | 8,61 | 1,99 | 1,73 | 0,34 | 0,24 | nd | nd | nd | nd | 56,7 | 100,3 |
| 20.10.2005 | 3,37 | 7,6 | 0,82 | 5,3 | 0,83 | 2,05 | 28,09 | 65,83 | 2,67 | 2,97 | 4,41 | 2,67 | 3,94 | 8,18 | 1,8 | 2,15 | 0,1 | 0,1 | nd | nd | nd | nd | 50,9 | 96 |
| 17.11.2005 | 1,12 | 4,75 | 0,17 | 2,66 | 0 | 2,71 | 30,66 | 70,67 | 3,47 | 0,48 | 0,49 | 0 | 4,11 | 8,73 | 1,61 | 0,32 | 0,46 | 0,71 | nd | nd | nd | nd | 48 | 82,9 |
| 6.2.2006 | 1,18 | 5,7 | 0,59 | 2,48 | 1,19 | 2,61 | 30,78 | 66,53 | 3 | 3,44 | 4,49 | 2,95 | 3,99 | 8,1 | 1,54 | 1,86 | 0,85 | 0,92 | nd | nd | nd | nd | | |
| 25.7.2006 | 0 | 0 | 0 | 0,74 | 0,82 | 2,02 | 30,72 | 65,22 | 3,18 | 3,3 | 3,47 | 2,33 | 3,96 | 7,87 | 2,47 | 2,52 | 1,02 | 1,36 | 0,01 | 0,13 | nd | nd | 29,08 | 63,98 |

*quantified as caffeic acid
nd - not detectable

Annexe A2: Comparison in glutathione, hydroxycinnamic acids and their esters and vanillin index content in normal ($O_2$) and hiperreductive ($N_2$) pressing of Muller Thurgau grapes (20th September 2005)
Priloga A2: Primerjava vsebnosti glutationa, hidroksicimetnih kislin in njihovih estrov in vanillin indeksa pri normalnem ($O_2$) in hiperreduktivnem ($N_2$) stiskanju grozdja Muller Thurgau (20. september 2005)

| | glutathione reduced | | glutathione oxidised | | *cis* -caftaric | | *trans* - caftaric | | *cis* - coutaric | | GRP' (*) | | *trans* - coutaric | | fertaric | | *trans* - caffeic | | *trans* p-coumaric | | *trans* - ferulic | | Vanillin index | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | normal ($O_2$) | | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) |
| | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L (+) catehin | |
| FREE RUN | 14,86 | 5,16 | 2,46 | 1,07 | 1,43 | 1,51 | 42,53 | 41,66 | 1,78 | 1,72 | 0,88 | 0,84 | 5,1 | 5 | 1,47 | 1,29 | nd | nd | nd | nd | nd | nd | 119,2 | 97,4 |
| 0.2 bar start | 19,56 | 22,91 | 4,26 | 2,09 | 1,42 | 1,71 | 39,98 | 45,46 | 2,43 | 2,8 | 2,21 | 1,77 | 5,6 | 6,7 | 1,62 | 1,32 | nd | nd | nd | nd | nd | nd | 100,3 | 165,8 |
| 0.6 bar start | 23,25 | 28,14 | 4,79 | 1,76 | 1,49 | 1,93 | 40,49 | 49,94 | 3,19 | 3,82 | 3,18 | 2,27 | 6,4 | 8,8 | 1,72 | 1,36 | nd | nd | nd | nd | nd | nd | 123,6 | 181,8 |
| 0.6 bar end | 25,92 | 30,3 | 3,97 | 1,56 | 1,43 | 1,92 | 39,58 | 50,51 | 3,17 | 3,71 | 3,89 | 2,56 | 6,2 | 8,8 | 1,78 | 1,44 | nd | nd | nd | nd | nd | nd | 148,3 | 173 |
| 1.0 bar start | 19,68 | 30,92 | 4,29 | 1,72 | 1,37 | 2,13 | 36,17 | 55,08 | 3,72 | 4,59 | 5,23 | 2,56 | 6,3 | 11 | 1,86 | 1,45 | nd | nd | nd | nd | nd | nd | 178,8 | 194,8 |
| 1.0 bar end | 19,79 | 33,33 | 4,2 | 1,53 | 1,3 | 2,17 | 34,82 | 56,24 | 3,59 | 4,63 | 5,74 | 2,63 | 6 | 11 | 1,89 | 1,48 | nd | nd | nd | nd | nd | nd | 142,5 | 165,8 |
| 1.5 bar start | 6,89 | 30,86 | 3,86 | 1,62 | 1,13 | 2,43 | 25,72 | 61,26 | 4,14 | 5,74 | 8,93 | 2,77 | 5,9 | 13 | 1,51 | 1,56 | nd | nd | nd | nd | nd | nd | 186,1 | 210,8 |
| 1.5 bar end | 9,11 | 33,76 | 3,16 | 1,82 | 1,15 | 2,58 | 28,19 | 64,22 | 4,21 | 6,01 | 8,18 | 2,77 | 5,9 | 14 | 1,57 | 1,56 | nd | nd | nd | nd | nd | nd | 197,7 | 200,7 |
| 2.0 bar start | 0,34 | 27,16 | 3,33 | 1,49 | 0,94 | 3,32 | 16,45 | 76,31 | 5,82 | 10 | 8,02 | 4,44 | 3,3 | 20 | 1,83 | 1,79 | nd | nd | nd | nd | nd | nd | 193,4 | 303,9 |
| 2.0 bar end | 2,02 | 22,75 | 1,72 | 0,89 | 1,29 | 4,03 | 19,93 | 97,01 | 9,26 | 16,4 | 7,52 | 6,07 | 5,4 | 31 | 2,49 | 2,14 | nd | nd | nd | nd | nd | nd | 301 | 433,3 |
| | | | | | | | | | | | | | | | | | | | | | | | | |
| tank after pressing (before bentonite) | 8,67 | 16,67 | 7,11 | 4,7 | 1,37 | 1,89 | 35,41 | 49,18 | 2,85 | 3,46 | 3,47 | 1,81 | 5,1 | 8,1 | 1,43 | 1,37 | nd | nd | nd | nd | nd | nd | 168,7 | 151,2 |
| tank after sedimentation | 6,76 | 17,47 | 9,25 | 5,95 | 1,3 | 1,7 | 35,56 | 49,88 | 2,86 | 3,45 | 3,55 | 1,79 | 5 | 8,2 | 1,81 | 1,46 | nd | nd | nd | nd | nd | nd | 128 | 171,6 |
| | | | | | | | | | | | | | | | | | nd | nd | nd | nd | nd | nd | | |
| must sediment | 9,76 | 20,06 | 9,54 | 5,48 | 1,16 | 1,74 | 34,21 | 49,26 | 2,78 | 3,41 | 3,79 | 1,89 | 4,5 | 7,9 | 1,53 | 1,44 | nd | nd | nd | nd | nd | nd | 128 | 311,2 |
| before yeast inoculation | 6,57 | 17,05 | 10,03 | 5,51 | 1,28 | 1,69 | 35,7 | 50,17 | 2,81 | 3,47 | 3,45 | 1,81 | 5,2 | 8,2 | 1,79 | 1,46 | nd | nd | nd | nd | nd | nd | 130,9 | 161,4 |
| 1° day fermentation | 12,46 | 18,7 | 8,35 | 3,22 | 1,06 | 1,4 | 33,28 | 47,26 | 2,66 | 3,31 | 3,55 | 1,74 | 4,7 | 7,8 | 1,35 | 1,39 | nd | nd | nd | nd | nd | nd | 119,2 | 130,9 |
| 3° day fermentation | 8,47 | 11,32 | 0,92 | nd | 1,24 | 1,66 | 31,72 | 44,47 | 2,54 | 3,16 | 3,28 | 1,65 | 4,3 | 7,2 | 1,46 | 1,41 | 0,57 | 0,63 | nd | nd | nd | nd | 75,6 | 87,2 |
| 4° day fermentation | 9,16 | 9,7 | 2,05 | nd | 1,21 | 1,65 | 31,1 | 45,25 | 2,52 | 3,19 | 3,13 | 1,68 | 4,2 | 7,3 | 1,34 | 1,38 | 0,61 | 0,61 | nd | nd | nd | nd | 80 | 104,7 |
| 5° day fermentation | 10,15 | 7,43 | 2,74 | nd | 1,25 | 1,73 | 30,83 | 46,35 | 2,55 | 3,26 | 3,22 | 1,76 | 4,2 | 7,5 | 1,36 | 1,46 | 0,56 | 0,63 | nd | nd | nd | nd | 74,2 | 106,1 |
| 6° day fermentation | 8,64 | 5,74 | 2,92 | nd | 1,31 | 1,97 | 32,64 | 47,8 | 2,55 | 3,38 | 3,41 | 1,83 | 4,4 | 7,8 | 1,4 | 1,47 | 0,59 | 0,61 | nd | nd | nd | nd | 74,2 | 106,1 |
| 7° day fermentation | 4,8 | 3,25 | 1,07 | nd | 1,32 | 1,98 | 33,91 | 48,08 | 2,81 | 3,38 | 3,47 | 1,75 | 4,7 | 7,7 | 1,45 | 1,44 | 0,57 | 0,61 | nd | nd | nd | nd | 82,9 | 107,6 |
| 11° day fermentation | 3,55 | 4,25 | 2,38 | 1,28 | 1,33 | 1,86 | 31,32 | 45,38 | 2,59 | 3,28 | 2,55 | 1,38 | 4,2 | 7,4 | 1,42 | 1,51 | 0,67 | 0,76 | nd | nd | nd | nd | 68,3 | 93,1 |
| 12° day fermentation | 4,76 | 6,45 | 3,1 | 1,62 | 1,36 | 1,87 | 31,22 | 45,22 | 2,64 | 3,3 | 2,63 | 1,5 | 4,2 | 7,4 | 1,36 | 1,4 | 0,67 | 0,75 | nd | nd | nd | nd | 62,5 | 96 |
| 13° day fermentation | 5,59 | 3,33 | 2,93 | 1,66 | 1,33 | 1,93 | 31,54 | 46,3 | 2,64 | 3,33 | 2,92 | 1,54 | 4,3 | 7,4 | 1,41 | 1,48 | 0,66 | 0,58 | nd | nd | nd | nd | 58,2 | 78,5 |
| 15° day fermentation, after rack from lees (15.10.2005) | 3,55 | 3,18 | 1,88 | n.d. | 1,29 | 1,8 | 30,91 | 44,81 | 2,56 | 3,24 | 3,05 | 1,55 | 4,2 | 7,3 | 1,35 | 1,4 | 0,54 | 0,64 | nd | nd | nd | nd | 56,7 | 66,9 |
| 20.10.2005 | 7,1 | 7,2 | 2,67 | 1,77 | 1,22 | 1,84 | 33,29 | 44,83 | 2,96 | 3,6 | 2,29 | 1,06 | 4,4 | 7,1 | 0,21 | 1,85 | 1,77 | 0,22 | nd | nd | nd | nd | 59,6 | 78,5 |
| 17.11.2005 | 5,31 | 5,38 | 0,62 | 0,38 | 1,46 | 2,03 | 32,89 | 46,7 | 3,15 | 3,81 | 2,29 | 1,22 | 4,4 | 7,3 | 1,5 | 1,75 | 0,14 | 0,17 | nd | nd | nd | nd | 55,25 | 66,88 |
| 6.2.2006 | 5,71 | 5,61 | 1,16 | 0,85 | 1,39 | 1,88 | 32,42 | 42,16 | 3,07 | 3,48 | 1,96 | 1,05 | 4,4 | 6,7 | 1,51 | 1,58 | 0,29 | 0,36 | nd | nd | nd | nd | | |
| 25.7.2006 | 0 | 0 | 0 | 0 | 0,85 | 1,5 | 34,29 | 46,42 | 3,21 | 3,87 | 1,03 | 0,53 | 4,8 | 7,4 | 1,42 | 1,94 | 0,2 | 0,79 | nd | nd | nd | nd | 46,53 | 55,25 |

*quantified as caffeic acid
nd - not detectable

Annexe A3: Comparison of glutathione, hydroxycinnamic acids and their esters and vanillin index content in normal ($O_2$) and hiperreductive ($N_2$) pressing of Greco di Tufo variety (18th October 2005)
Priloga A3: Primerjava vsebnosti glutationa, hidroksicimetnih kislin in njihovih estrov in vanillin indeksa pri normalnem ($O_2$) in hiperreduktivnem ($N_2$) stiskanju grozdja Greco di Tufo (18. oktober 2005)

| | glutathione reduced | | glutathione oxidised | | *cis* -caftaric | | *trans* - caftaric | | *cis* - coutaric | | GRP' (*) | | *trans* - coutaric | | fertaric | | *trans* - caffeic | | *trans* p-coumaric | | *trans* - ferulic | | Vanillin index | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) | normal ($O_2$) | hiprereduction ($N_2$) |
| | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | | mg/L | |
| FREE RUN | 16,2 | 15,07 | 4,04 | 4,76 | 2,62 | 2,8 | 186,83 | 192,43 | 4,53 | 4,12 | 2,95 | 1,5 | 20,86 | 20,05 | 5,52 | 5,37 | nd | nd | nd | nd | nd | nd | 384 | 415,8 |
| FRE RUN in buffer tank | 14,68 | 15,62 | 4,49 | 5,23 | 2,9 | 3,56 | 184,69 | 190,52 | 4,58 | 4,15 | 3,13 | 1,52 | 20,64 | 19,58 | 5,54 | 5,32 | nd | nd | nd | nd | nd | nd | 394 | 392,6 |
| 0.2 bar start | 21,2 | 20,76 | 4,29 | 5,21 | 3,27 | 3,78 | 199,73 | 194,27 | 7,1 | 5,17 | 4,56 | 1,5 | 29,45 | 23,28 | 5,75 | 5,22 | nd | nd | nd | nd | nd | nd | 436 | 407,1 |
| 0.2 bar end | 21,92 | 20,51 | 4,95 | 4,64 | 3,5 | 3,45 | 202,89 | 217,66 | 7,51 | 5,89 | 4,54 | 1,67 | 30,37 | 27,41 | 5,74 | 5,9 | nd | nd | nd | nd | nd | nd | 426 | 412,9 |
| 0.4 bar start | 6,66 | 33,96 | 4,09 | 4,31 | 3,35 | 4,06 | 176,31 | 240,48 | 10 | 9,02 | 9,62 | 1,56 | 34,76 | 40 | 6,03 | 5,89 | nd | nd | nd | nd | nd | nd | 458 | 479,8 |
| 0.4 bar end 2°cicle | 2,17 | *** | 3,98 | *** | 3,64 | *** | 156,55 | *** | 12,5 | *** | 11,16 | *** | 36,34 | *** | 6,22 | *** | nd | *** | nd | *** | nd | *** | 486 | *** |
| 0.4 bar end | 1,27 | 31,25 | 4,39 | 4,1 | 3,78 | 4,34 | 151 | 247 | 13,64 | 9,4 | 11,93 | 1,41 | 37,24 | 41,16 | 6,23 | 5,93 | nd | nd | nd | nd | nd | nd | 528 | 436,2 |
| 0.6 bar start | 1,41 | 35,03 | 3,54 | 3,87 | 3,49 | 4,94 | 134,71 | 271,72 | 15,27 | 12,83 | 11,37 | 1,63 | 36,51 | 54,94 | 6,34 | 6,24 | nd | nd | nd | nd | nd | nd | 611 | 581,6 |
| 0.6 bar end | 1,49 | 38,54 | 3,59 | 4,15 | 3,86 | 4,77 | 142,51 | 286,24 | 17,3 | 13,73 | 11,61 | 1,58 | 40,27 | 59,31 | 6,59 | 6,51 | nd | nd | nd | nd | nd | nd | 692 | 590,3 |
| 0.8 bar start | 1,55 | 37,9 | 2,85 | 4,58 | 3,53 | 6,2 | 129,4 | 310,35 | 19,58 | 18,43 | 10,97 | 2,37 | 40,52 | 75,52 | 6,69 | 6,82 | nd | nd | nd | nd | nd | nd | 806 | 761,9 |
| 0.8 bar end | 1,52 | 39,09 | 3,27 | 4,42 | 4,02 | 6,53 | 131,61 | 314 | 20,65 | 19,43 | 11,29 | 2,17 | 41,18 | 78,95 | 6,87 | 6,74 | nd | nd | nd | nd | nd | nd | 715 | 808,4 |
| 1.0 bar start | 1,61 | 37,27 | 3,23 | 3,45 | 3,73 | 6,57 | 111,75 | 337,62 | 21,33 | 23,81 | 10,44 | 3,18 | 36,8 | 95,25 | 6,89 | 7,15 | nd | nd | nd | nd | nd | nd | 747 | 1041,1 |
| 1.0 bar end | 1,73 | 37,06 | 2,58 | 3,67 | 3,87 | 6,78 | 123,56 | 337,75 | 23,13 | 24,14 | 10,43 | 3,11 | 41,6 | 96,05 | 7,09 | 7,18 | nd | nd | nd | nd | nd | nd | 832 | 1003,3 |
| 1.5 bar start | 1,63 | 35,13 | 3,03 | 4,03 | 3,9 | 7,19 | 105,14 | 347,34 | 23,43 | 26,92 | 10,08 | 3,94 | 36,68 | 105 | 7,09 | 7,41 | nd | nd | nd | nd | nd | nd | 867 | 1125,4 |
| 1.5 bar end | 1,77 | 37,95 | 2,33 | 4,29 | 3,87 | 8,39 | 113,18 | 358,83 | 24,63 | 29,12 | 9,85 | 4,06 | 40,37 | 111,67 | 7,24 | 7,5 | nd | nd | nd | nd | nd | nd | 971 | 1029,4 |
| 2.0 bar start | 1,72 | 35,76 | 2,02 | 3,05 | 3,71 | 7,49 | 101,18 | 367,04 | 24,52 | 32,27 | 9,69 | 5,14 | 36,91 | 123,11 | 7,37 | 7,85 | nd | nd | nd | nd | nd | nd | 980 | 846,2 |
| 2.0 bar end | 1,59 | 38,32 | 2,1 | 3,48 | 3,62 | 7,99 | 93,53 | 375,02 | 24,19 | 33,42 | 9,6 | 5,09 | 34,57 | 126,82 | 7,36 | 7,97 | nd | nd | nd | nd | nd | nd | 922 | 1215,5 |
| tank after pressing** (before bentonite) | 8,15 | 22,5 | 4,8 | 5,54 | 2,85 | 3,39 | 174,31 | 216,04 | 5,85 | 7,07 | 6 | 1,73 | 22,69 | 31,29 | 5,48 | 5,62 | nd | nd | nd | nd | nd | nd | 364 | 430,4 |
| tank after sedimentation (after racking) | 5,16 | 22,49 | 7,99 | 4,4 | 2,88 | 2,92 | 168,02 | 213,3 | 5,7 | 6,78 | 7,66 | 1,93 | 21,89 | 30,24 | 5,42 | 5,61 | nd | nd | nd | nd | nd | nd | 329 | 405,7 |
| 1st day ferment. | 8,08 | 24,99 | 8,7 | 6,22 | 3,75 | 4,17 | 143,86 | 187,6 | 5,03 | 6,28 | 5,78 | 1,41 | 19,11 | 28,64 | 4,81 | 5,13 | 0,22 | 0,29 | nd | nd | nd | nd | 329 | 405,7 |
| 5th day ferment | 0,41 | 4,06 | 0,32 | 1,58 | 5 | 5,68 | 150,42 | 196,8 | 5,43 | 6,67 | 6,62 | 1,65 | 20,11 | 28,64 | 5 | 5,33 | 0,17 | 0,19 | nd | nd | nd | nd | 336 | 365 |
| 13th day after rack (31.10.2005) | 0,11 | 4,07 | n.d. | 1,28 | 5,13 | 5,96 | 163,09 | 207 | 5,72 | 6,98 | 5,43 | 1,27 | 22,38 | 30,16 | 5,53 | 5,74 | 0,26 | 0,39 | nd | nd | nd | nd | | |
| 17.11.2005 | 2,09 | 3,98 | n.d. | 0,88 | 4,65 | 5,35 | 148,77 | 190,28 | 0,87 | 6,69 | nd | nd | 19,47 | 27,32 | 4,59 | 4,98 | 0,46 | 0,59 | nd | nd | nd | nd | 195 | 271,9 |
| 6.2.2006 | 1,24 | 2 | 0,79 | 2,05 | 4,56 | 5,11 | 114,38 | 179 | 5,97 | 6,35 | 5,06 | 1,43 | 18,64 | 25,25 | 4,38 | 4,58 | 1,25 | 1,47 | nd | nd | nd | nd | | |
| 25.7.2006 | 0 | 0 | 0,02 | 0,45 | 1,86 | 2,81 | 146,32 | 162,79 | 5,32 | 5,92 | 3,98 | 0,87 | 19,39 | 23,18 | 5,78 | 1,93 | 2,23 | 0,79 | 0,2 | 0,22 | nd | nd | 145 | 101,8 |

**quantified as caffeic acid*
** in normal ($O_2$) pressed only pressing up to 0.4 bar were used
*** data not available
nd – not detectable

Annexe A4: Oxygen consumption kinetics in Sauvignon Blanc (Stellenbosh) grape juice in relation to differnet concentration of $SO_2$ and asorbic acid addition

Priloga A4: Kinetika porabe kisika v grozdnem moštu sorte Sauvignon Blanc (Stellenboch) glede na različne koncentracije $SO_2$ in askorbinske kisline

| **Addition / time (s)** | **0** | **30** | **60** | **120** |
|---|---|---|---|---|
| No $SO_2$. no ascorbic | 2.54 | 1.14 | 0.44 | 0.18 |
| 50 mg/L $SO_2$ | 1.22 | 0.94 | 0.78 | 0.75 |
| 50 mg/L ascorbic | 1.45 | 0.45 | 0.28 | 0.23 |
| 50 mg/L $SO_2$ + 50mg/L ascorbic | 1.3 | 0.89 | 0.78 | 0.75 |
| 200 mg/L $SO_2$ + 50mg/L ascorbic | 1.7 | 1.6 | 1.68 | 1.77 |

Annexe B1: Colour intensity kinetics during microoxygenation treatment of experiment 1 in 2004/2005

Priloga B1: Kinetika intenzitete barve med procesom mikrooksigenacije vin poskusa 1 v letu 2004/2005

| **Treatment / Date** | **22 Nov** | **29 Nov** | **13 Dec** | **21 Dec** | **27 Dec** | **3 Jan** | **1 Feb** | **6 Jun** | **9 Aug** |
|---|---|---|---|---|---|---|---|---|---|
| control | 9.65 | 8.81 | 7.82 | 7.30 | 7.38 | 7.77 | 7.11 | 6.51 | 6.78 |
| 10 mg/L/mo | 9.65 | 9.21 | 8.47 | 9.07 | 9.79 | 10.12 | 9.86 | 8.65 | 8.70 |
| 30 mg/L/mo | 9.65 | 8.54 | 9.40 | 10.20 | 11.00 | 11.16 | 10.93 | 8.90 | 8.69 |
| control (oak) | 9.65 | 8.63 | 8.41 | 8.04 | 8.48 | 9.19 | 8.76 | 7.55 | 7.95 |
| 10 mg/L/mo (oak) | 9.65 | 8.58 | 8.76 | 9.20 | 9.86 | 9.95 | 9.48 | 8.98 | 8.31 |
| 30 mg/L/mo (oak) | 9.65 | 10.55 | 9.81 | 9.91 | 11.02 | 11.07 | 10.51 | 8.82 | 8.84 |

Annexe B2: Colour intensity kinetics during microoxygenation treatment in experiment 2 in 2005/2006

Priloga B2: Kinetika intenzitete barve med mikrooksigenacijo vin poskusa 2 v letu 2005/2006

| **Treatment / Date** | **15 Feb** | **13 Mar** | **12 Apr** | **8 May** | **23 May** | **11 Aug** | **23 Oct** |
|---|---|---|---|---|---|---|---|
| control | 6.07 | 5.69 | 6.19 | 5.99 | 5.26 | 6.41 | 6.20 |
| 3 mg/L/mo | 6.07 | 5.68 | 6.20 | 6.02 | 5.08 | 6.37 | 6.70 |
| 5 mg/L/mo | 6.07 | 5.79 | 6.27 | 6.22 | 5.50 | 6.80 | 7.16 |
| control (oak) | 6.07 | 5.95 | 6.28 | 5.94 | 5.29 | 6.37 | 6.36 |
| 3 mg/L/mo (oak) | 6.07 | 5.71 | 6.24 | 6.17 | 5.36 | 6.97 | 6.93 |
| 5 mg/L/mo (oak) | 6.07 | 5.49 | 6.28 | 5.92 | 5.34 | 7.17 | 7.59 |
| barrique | 6.07 | 5.55 | 6.19 | 6.48 | 5.91 | 6.87 | 6.68 |

Annexe B3: Colour intensity kinetics during microoxygenation treatment in experiment 3 in 2004/2005

Priloga B3: Kinetika intenzitete barve med mikrooksigenacijo vin poskusa 3 v letu in 2004/2005

| **Treatment / Date** | **24 Dec** | **27 Jan** | **14 Mar** | **14 May** | **21 Jun** | **15 Jul** |
|---|---|---|---|---|---|---|
| control | 10.78 | 11.14 | 11.14 | 9.92 | 9.08 | 8.99 |
| 1.5 mg/L/mo | 10.75 | 11.29 | 11.29 | 9.25 | 8.38 | 8.34 |
| 3.0 mg/L/mo | 10.98 | 11.60 | 11.60 | 10.07 | 9.99 | 10.22 |
| barrel | 11.15 | 11.72 | 11.72 | 9.87 | 9.28 | 9.33 |

Annexe B4: Colour intensity kinetics during microoxygenation of wines in experiment 4 in 2006

Priloga B4: Kinetika intenzitete barve med mikrooksigenacijo vin v poskusu 4 v letu 2006

| Treatment / Date | 25 Aug | 28 Sept | 29 Nov |
|---|---|---|---|
| 40% oak. control | 15.17 | 14.67 | 14.52 |
| 40% oak. 5 mg/L/mo | 16.14 | 15.42 | 16.94 |
| 100% oak. control | 16.13 | 14.97 | 14.61 |
| 100% oak. 5mg/L/mo | 16.68 | 16.61 | 16.86 |
| old barrique 1 | 13.37 | 13.06 | 13.33 |
| old barrique 2 | 13.97 | 13.23 | 13.55 |
| new barrique 1 | 16.02 | 14.85 | 14.57 |
| new barrique 2 | 15.83 | 14.64 | 14.36 |

Annexe B5: Colour hue kinetics during microoxygenation treatment of experiment 1 in 2004/2005

Priloga B5: Kinetika tona barve med mikrooksigenacijo vin poskusa 1 v letu 2004/2005

| Treatment / Date | 22 Nov | 29 Nov | 13 Dec | 21 Dec | 27 Dec | 3 Jan | 1 Feb | 6 Jun | 9 Aug |
|---|---|---|---|---|---|---|---|---|---|
| control | 0.605 | 0.595 | 0.700 | 0.686 | 0.681 | 0.717 | 0.675 | 0.687 | 0.691 |
| 10 mg/L/mo | 0.605 | 0.633 | 0.687 | 0.669 | 0.661 | 0.654 | 0.654 | 0.687 | 0.680 |
| 30 mg/L/mo | 0.605 | 0.631 | 0.662 | 0.634 | 0.638 | 0.636 | 0.667 | 0.730 | 0.709 |
| control (oak) | 0.605 | 0.621 | 0.707 | 0.694 | 0.687 | 0.690 | 0.663 | 0.678 | 0.682 |
| 10 mg/L/mo (oak) | 0.605 | 0.624 | 0.687 | 0.663 | 0.665 | 0.649 | 0.676 | 0.702 | 0.691 |
| 30 mg/L/mo (oak) | 0.605 | 0.655 | 0.680 | 0.633 | 0.644 | 0.640 | 0.672 | 0.725 | 0.711 |

Annexe B6: Colour hue kinetics during microoxygenation treatment of in experiment 2 in 2005/2006

Priloga B6: Kinetika tona barve med mikrooksigenacijo vin poskusa 2 v letu 2005/2006

| Treatment / Date | 15 Feb | 13 Mar | 12 Apr | 8 May | 23 May | 11 Aug | 23 Oct |
|---|---|---|---|---|---|---|---|
| control | 0.662 | 0.636 | 0.657 | 0.652 | 0.632 | 0.660 | 0.725 |
| 3 mg/L/mo | 0.662 | 0.650 | 0.654 | 0.656 | 0.622 | 0.679 | 0.716 |
| 5 mg/L/mo | 0.662 | 0.652 | 0.647 | 0.645 | 0.624 | 0.654 | 0.700 |
| control (oak) | 0.662 | 0.644 | 0.648 | 0.646 | 0.626 | 0.665 | 0.719 |
| 3 mg/L/mo (oak) | 0.662 | 0.648 | 0.653 | 0.656 | 0.631 | 0.674 | 0.710 |
| 5 mg/L/mo (oak) | 0.662 | 0.642 | 0.653 | 0.656 | 0.625 | 0.672 | 0.694 |
| barrique | 0.662 | 0.651 | 0.638 | 0.639 | 0.617 | 0.703 | 0.702 |

Annexe B7: Colour hue kinetics during microxygenation treatment in experiment 3 in 2004/2005

Priloga B7: Kinetika tona barve med mikrooksigenacijo vin poskusa 3 v letu in 2004/2005

| Treatment / Date | 24 Dec | 27 Jan | 14 Mar | 14 May | 21 Jun | 15 Jul |
|---|---|---|---|---|---|---|
| control | 0.701 | 0.693 | 0.686 | 0.729 | 0.721 | 0.721 |
| 1.5 mg/L/mo | 0.701 | 0.698 | 0.685 | 0.757 | 0.746 | 0.744 |
| 3.0 mg/L/mo | 0.705 | 0.703 | 0.696 | 0.751 | 0.745 | 0.733 |
| barrique | 0.703 | 0.694 | 0.675 | 0.752 | 0.746 | 0.742 |

Annexe B8: Colour hue kinetics during microoxygenation of wines in experiment 4 in 2006

Priloga B8: Kinetika tona barve med mikrooksigenacijo vin v poskusu 4 v letu 2006

| Treatment / Date | 25 Aug | 28 Sept | 29 Nov |
|---|---|---|---|
| 40% oak. control | 0.623 | 0.596 | 0.613 |
| 40% oak. 5 mg/L/mo | 0.644 | 0.629 | 0.595 |
| 100% oak. control | 0.600 | 0.589 | 0.638 |
| 100% oak. 5mg/L/mo | 0.629 | 0.612 | 0.611 |
| old barrique 1 | 0.665 | 0.609 | 0.640 |
| old barrique 2 | 0.645 | 0.605 | 0.526 |
| new barrique 1 | 0.615 | 0.602 | 0.644 |
| new barrique 2 | 0.616 | 0.606 | 0.641 |

Annexe B9: Polyphenol index (OD 280) kinetics during microoxygenation of wines in experiment 1 in years 2004/2005

Priloga B9: Kinetika indeksa polifenolov (OD 280) med mikrooksigenacijo vin poskusa 1 v letu 2004/2005

| Treatment / Date | 17 Dec | 1 Feb | 6 May | 9 Aug |
|---|---|---|---|---|
| control | 54.4 | 53.9 | 54.9 | 52.5 |
| 10 mg/L/mo | 60.6 | 61.5 | 59.5 | 58.5 |
| 30 mg/L/mo | 61.1 | 61.8 | 59.8 | 59.2 |
| control (oak) | 62.7 | 61.2 | 58.5 | 59.0 |
| 10 mg/L/mo (oak) | 61.1 | 61.5 | 58.4 | 59.0 |
| 30 mg/L/mo (oak) | 61.9 | 61.2 | 58.8 | 59.1 |

Annexe B10: Polyphenol index (OD 280) kinetics during microoxygenation of wines in experiment 2 in years 2005/2006

Priloga B10: Kinetika indeksa polifenolov (OD2 80) med mikrooksigenacijo vin poskusa 2 v letu 2005/2006

| Treatment / Date | 15 Feb | 13 Mar | 19 Apr | 23 May | 11 Aug | 30 Aug | 23 Oct |
|---|---|---|---|---|---|---|---|
| control | 53.9 | 53.8 | 54.0 | 53.9 | 53.9 | 51.7 | 50.1 |
| 3 mg/L/mo | 53.9 | 53.3 | 54.2 | 53.6 | 51.5 | 51.1 | 49.6 |
| 5 mg/L/mo | 53.9 | 53.0 | 54.4 | 53.5 | 53.2 | 52.4 | 49.0 |
| controlo (oak) | 53.9 | 53.5 | 54.0 | 53.6 | 53.1 | 53.1 | 50.4 |
| 3 mg/L/mo (oak) | 53.9 | 54.2 | 54.0 | 54.2 | 53.8 | 52.9 | 50.7 |
| 5 mg/L/mo (oak) | 53.9 | 53.5 | 54.1 | 54.0 | 53.2 | 52.1 | 50.2 |
| barrique | 53.9 | 53.6 | 54.8 | 52.9 | 53.4 | 51.6 | 50.2 |

Annexe B11: Polyphenol index (OD 280) kinetics during microoxygenation of wines in experiment 3 in years 2004/2005

Priloga B11: Kinetika indeksa polifenolov (OD 280) med mikrooksigenacijo vin poskusa 3 v letu 2004/2005

| Treatment / Date | 24 Dec | 27 Jan | 14 Mar | 14 May | 21 Jun | 15 Jul |
|---|---|---|---|---|---|---|
| control | 61.1 | 60.8 | 58.6 | 57.8 | 56.3 | 57.5 |
| 1.5 mg/L/mo | 60.9 | 62.4 | 57.7 | 59.4 | 54.1 | 58.3 |
| 3.0 mg/L/mo | 60.8 | 62.6 | 58.4 | 58.9 | 55.1 | 59.1 |
| barrique | 59.9 | 61.2 | 57.8 | 58.8 | 58.3 | 58.3 |

Annexe B12: Polyphenol index (OD 280) kinetics during microoxygenation of wines in experiment 4 in year 2006

Priloga B12: Kinetika indeksa polifenolov (OD 280) med mikrooksigenacijo vin v poskusu 4 v letu 2006

| Treatment / Date | 25 Aug | 28 Sept | 29 Nov |
|---|---|---|---|
| 40% oak. control | 77.9 | 75.3 | 81.4 |
| 40% oak. 5 mg/L/mo | 75.2 | 76.2 | 70.2 |
| 100% oak. control | 86.5 | 82.8 | 77.3 |
| 100% oak. 5mg/L/mo | 81.2 | 83.8 | 73.1 |
| old barrique 1 | 83.0 | 75.7 | 71.5 |
| old barrique 2 | 88.1 | 75.2 | 68.8 |
| new barrique 1 | 89.8 | 73.4 | 71.2 |
| new barrique 2 | 85.4 | 84.1 | 74.1 |

Annexe B13: Total anthocyanins (mg/L) kinetics during the microoxygenation of wines in experiment 1 in years 2004/2005

Priloga B13: Kinetika skupnih antocianov (mg/L) med mikrooksigenacijo vin poskusa 1 v letu 2004/2005

| Treatment / Date | 29 Nov | 13 Dec | 21 Dec | 3 Jan | 1 Feb | 6 May | 9 Aug |
|---|---|---|---|---|---|---|---|
| control | 675 | 645 | 659 | 657 | 617 | 598 | 522 |
| 10 mg/L/mo | 543 | 565 | 540 | 534 | 506 | 478 | 410 |
| 30 mg/L/mo | 541 | 544 | 516 | 521 | 479 | 459 | 396 |
| control (oak) | 552 | 528 | 552 | 535 | 528 | 486 | 431 |
| 10 mg/L/mo (oak) | 529 | 528 | 527 | 533 | 507 | 487 | 416 |
| 30 mg/L/mo (oak) | 539 | 517 | 531 | 498 | 479 | 456 | 417 |

Annexe B14: Total polyphenols (Folin Ciocalteau; mg/L) kinetics during the microoxygenation of wines in experiment 1 in years 2004/2005

Priloga B14: Kinetika skupnih polifenolov (Folin Ciocalteau; mg/L) med mikrooksigenacijo vin poskusa 1 v letu 2004/2005

| Treatment / Date | 29 Nov | 17 Dec | 21 Dec | 3 Jan | 1 Feb | 6 May | 9 Aug |
|---|---|---|---|---|---|---|---|
| control | 1917 | 2179 | 1964 | 2102 | 2176 | 1953 | 1956 |
| 10 mg/L/mo | 2015 | 2317 | 2128 | 2193 | 2208 | 2240 | 2211 |
| 30 mg/L/mo | 2278 | 2020 | 2345 | 2134 | 2476 | 2341 | 2248 |
| control (oak) | 2292 | 2283 | 2401 | 2257 | 2311 | 2300 | 2457 |
| 10 mg/L/mo (oak) | 2165 | 2410 | 2286 | 2313 | 2518 | 2309 | 2152 |
| 30 mg/L/mo (oak) | 2180 | 2433 | 2344 | 2097 | 2228 | 2356 | 2183 |

Annexe B15: Proantocyanidins (mg/L) kinetics during the microoxygenation treatment of wines in experiment 1 in years 2004/2005

Priloga B15: Kinetika proatocianidinov (mg/L) med mikrooksigenacijo vin v poskusu 1 v letu 2004/2005

| Treatment / Date | 29 Nov | 6 Dec | 13 Dec | 27 Dec | 3 Jan | 19 Jan | 3 Feb | 9 May | 8 Sep |
|---|---|---|---|---|---|---|---|---|---|
| control | 2361 | 2306 | 2348 | 2381 | 2305 | 2358 | 2305 | 2317 | 2279 |
| 10 mg/L/mo | 2555 | 2696 | 2624 | 2619 | 2656 | 2573 | 2469 | 2576 | 2449 |
| 30 mg/L/mo | 2622 | 2570 | 2666 | 2642 | 2666 | 2644 | 2620 | 2690 | 2508 |
| control (oak) | 2581 | 2413 | 2765 | 2678 | 2707 | 2585 | 2641 | 2570 | 2480 |
| 10 mg/L/mo (oak) | 2540 | 2491 | 2612 | 2603 | 2730 | 2632 | 2698 | 2514 | 2425 |
| 30 mg/L/mo (oak) | 2507 | 2608 | 2699 | 2543 | 2705 | 2665 | 2593 | 2614 | 2548 |

Annexe B16: Vanillin index (mg (+)-catechin/L) kinetics during the microoxygenation treatment of wines in experiment 1 in years 2004/2005

Priloga B16: Kinetika vanilin indeksa (mg (+)-katehin/L) med mikrooksigenacijo vin poskusa 1 v letu 2004/2005

| Treatment / Date | 29 Nov | 6 Dec | 13 Dec | 27 Dec | 3 Jan | 20 Jan | 4 Feb | 9 May | 9 Aug |
|---|---|---|---|---|---|---|---|---|---|
| control | 1359 | 1466 | 1472 | 1527 | 1457 | 1575 | 1442 | 1315 | 1115 |
| 10 mg/L/mo | 1647 | 1657 | 1677 | 1782 | 1689 | 1753 | 1698 | 1397 | 1374 |
| 30 mg/L/mo | 1524 | 1678 | 1642 | 1815 | 1696 | 1592 | 1601 | 1447 | 1292 |
| control (oak) | 1673 | 1601 | 1617 | 1712 | 1602 | 1681 | 1674 | 1543 | 1358 |
| 10 mg/L/mo (oak) | 1696 | 1644 | 1721 | 1743 | 1559 | 1610 | 1643 | 1534 | 1357 |
| 30 mg/L/mo (oak) | 1670 | 1629 | 1642 | 1727 | 1632 | 1529 | 1647 | 1582 | 1369 |

Annexe B17: Polymerization index (vanillin/proanthocyanidin) kinetics of wines in experiment 1 in years 2004/2005

Priloga B17: Kinetika indeksa polimerizacije (vanillin/proantocianidini) v vinih poskusa 1 v letu 2004/2005

| Treatment / Date | 29 Nov | 13 Dec | 3 Jan | 4 Feb | 9 May | 9 Aug |
|---|---|---|---|---|---|---|
| control | 0.58 | 0.63 | 0.63 | 0.63 | 0.57 | 0.49 |
| 10 mg/L/mo | 0.64 | 0.64 | 0.64 | 0.69 | 0.54 | 0.56 |
| 30 mg/L/mo | 0.58 | 0.62 | 0.64 | 0.61 | 0.54 | 0.52 |
| control (oak) | 0.65 | 0.59 | 0.59 | 0.63 | 0.60 | 0.55 |
| 10 mg/L/mo (oak) | 0.67 | 0.66 | 0.57 | 0.61 | 0.61 | 0.56 |
| 30 mg/L/mo (oak) | 0.67 | 0.61 | 0.60 | 0.64 | 0.61 | 0.54 |

Annexe 18: Proportion (%) of colour fraction of wines of experiment 1 as proposed by Boulton: copigmentation: fraction of colour due to co-pigmentation. Free anthocyanins: fraction of colour due to free anthocyanins. Polymeric pigments: colour due to polymeric fraction. Analyzed at the end of the experiment (August 2005)

Priloga 18: Delež barvnih frakcij v vinih poskusa 1 (po Boultonu) : copigmentation : frakcija barve glede na kopigmentacijo. Free anthocyanins : frakcija barve glede na proste antociane. Polymeric pigments: delež barve polimernih pigmentov. Analizirano na koncu poskusa (avgust 2005)

| Treatment / Proportion | Copigmentation | Free anthocyanins | Polymeric pigments |
|---|---|---|---|
| control | 25 | 46 | 30 |
| 10 mg/L/mo | 15 | 46 | 39 |
| 30 mg/L/mo | 18 | 43 | 39 |
| control (oak) | 17 | 47 | 36 |
| 10 mg/L/mo (oak) | 15 | 47 | 38 |
| 30 mg/L/mo (oak) | 16 | 45 | 39 |

Annexe B19: Proportion (%) of colour fraction of wines of experiment 2 as proposed by Boulton: copigmentation: fraction of colour due to co-pigmentation. Free anthocyanins: fraction of colour due to free anthocyanins. Polymeric pigments: colour due to polymeric fraction. Analyzed at the end of the microoxygenation (October 2006)

Priloga B19: Delež barvnih frakcij v vinu B (po Boultonu): copigmentation : frakcija barve glede na kopigmentacijo. Free anthocyanins : frakcija barve glede na proste antociane. Polymeric pigments: delež barve polimernih pigmentov. Analizirano na koncu poskusa (oktober 2006)

| Treatment / Proportion | Copigmentation | Free anthocyanins | Polymeric pigments |
|---|---|---|---|
| control | 26 | 41 | 32 |
| 3 mg/L/mo | 14 | 54 | 32 |
| 5 mg/L/mo | 26 | 44 | 30 |
| control (oak) | 19 | 50 | 31 |
| 3 mg/L/mo (oak) | 19 | 50 | 31 |
| 5 mg/L/mo (oak) | 25 | 43 | 32 |
| barrique | 25 | 42 | 34 |

Annexe B20: Proportion (%) of colour fraction of wines of experiment 3 as proposed by Boulton: copigmentation: fraction of colour due to co-pigmentation. Free anthocyanins: fraction of colour due to free anthocyanins. Polymeric pigments: colour due to polymeric fraction. Analyzed at the end of microoxygenation (July 2005)

Priloga B20: Delež barvnih frakcij v vinih poskusa 3 (po Boultonu): copigmentation : frakcija barve glede na kopigmentacijo. Free anthocyanins : frakcija barve glede na proste antociane. Polymeric pigments: delež barve polimernih pigmentov. Analizirano na koncu poskusa (julij 2005)

| Treatment / Proportion | Copigmentation | Free anthocyanins | Polymeric pigments |
|---|---|---|---|
| control | 22 | 31 | 47 |
| 1.5 mg/L/mo | 25 | 29 | 47 |
| 3.0 mg/L/mo | 20 | 34 | 46 |
| barrel | 24 | 31 | 46 |

Annexe B21: Proportion (%) of colour fraction of wines of experiment 4 as proposed by Boulton: copigmentation: fraction of colour due to co-pigmentation. Free anthocyanins: fraction of colour due to free anthocyanins. Polymeric pigments: colour due to polymeric fraction. Analyzed at the end of the microoxygenation (November 2006)

Priloga B21: Delež barvnih frakcij v vinih poskusa 4 (po Boultonu): copigmentation : frakcija barve glede na kopigmentacijo. Free anthocyanins: frakcija barve glede na proste antociane. Polymeric pigments: delež barve polimernih pigmentov. Analizirano na koncu poskusa (November. 2006)

| Treatment / Proportion | Copigmentation | Free anthocyanins | Polymeric pigments |
|---|---|---|---|
| 40 % oak. control | 16 | 53 | 31 |
| 40 % oak. 5 mg/L/mo | 18 | 50 | 32 |
| 100 % oak. control | 16 | 51 | 33 |
| 100 % oak. 5 mg/L/mo | 11 | 53 | 36 |
| old barrique 1 | 20 | 51 | 29 |
| old barrique 2 | 19 | 51 | 29 |
| new barrique 1 | 16 | 52 | 31 |
| new barrique 2 | 17 | 52 | 31 |

Annexe B22: Polyphenolic analysis of wines of experiment 1, analyzed three months after the microoxygenation treatment

Priloga B22: Polifenolne analize vin poskusa 1, analizirane tri mesece po mikrooksigencijskem poskusu

| | Control | 10 mg/L/mo | 30 mg/L/mo | Control (oak) | 10 mg/L/mo (oak) | 30 mg/L/mo (oak) |
|---|---|---|---|---|---|---|
| Total anthocyanins (spectroph.) | 522.1 | 410.3 | 395.6 | 430.9 | 415.5 | 417.2 |
| Total polyphenols (Folin Ciocalteau) | 1956 | 2211 | 2248 | 2457 | 2152 | 2183 |
| OD 280 | 52.5 | 58.5 | 59.2 | 59.0 | 59.0 | 59.1 |
| Colour Intensity | 6.78 | 8.70 | 8.69 | 7.95 | 8.31 | 8.84 |
| % A420 | 36.0 | 35.4 | 36.3 | 35.7 | 35.9 | 36.0 |
| % A520 | 52.1 | 52.1 | 51.2 | 52.3 | 51.9 | 50.6 |
| % A620 | 11.9 | 12.5 | 12.5 | 12.0 | 12.2 | 13.4 |
| Color Hue | 0.691 | 0.680 | 0.709 | 0.682 | 0.691 | 0.711 |
| Vanilin Index | 1116 | 1374 | 1293 | 1359 | 1357 | 1370 |
| Proantocyanidnis | 2279 | 2449 | 2508 | 2480 | 2425 | 2548 |
| IP (Vanilin/Proantocyanidins) | 0.49 | 0.56 | 0.52 | 0.55 | 0.56 | 0.54 |
| | | | | | | |
| Delphinidin-3-glucoside | 15.1 | 10.2 | 8.8 | 9.7 | 11.7 | 10.0 |
| Cyanidin-3-glucoside | 1.9 | 1.7 | 3.1 | 2.4 | 3.1 | 2.3 |
| Petunidin-3-glucoside | 17.9 | 12.2 | 11.4 | 13.1 | 12.6 | 11.9 |
| Peonidin-3-glucoside | 11.7 | 8.5 | 8.7 | 10.1 | 8.6 | 8.5 |
| Malvidin-3-glucoside | 116.2 | 76.0 | 70.2 | 81.3 | 77.1 | 75.4 |
| Delphinidin-3-acetylglucoside | 6.4 | 3.5 | 3.1 | 4.2 | 3.8 | 3.6 |
| Cyanidin-3-acetylglucoside | 1.9 | 1.9 | 1.8 | 2.2 | 1.8 | 1.9 |
| Petunidin-3-acetylglucoside | 6.7 | 4.1 | 4.2 | 5.4 | 4.4 | 4.1 |
| Peonidin-3-acetylglucoside | 9.0 | 8.6 | 9.7 | 9.2 | 10.2 | 8.8 |
| Malvidin-3-acetylglucoside | 46.5 | 25.0 | 25.8 | 27.4 | 28.0 | 24.9 |
| Delphinidin-3-coumaroylglucoside | 1.7 | 2.0 | 2.9 | 1.9 | 2.7 | 2.1 |
| Malvidin-3-caffeoylglucoiside | 0.9 | 0.7 | 1.3 | 0.8 | 1.2 | 0.6 |
| Cyanidin-3-coumaroylglucoside | 0.5 | 0.6 | 1.1 | 0.5 | 0.8 | 0.6 |
| Petunidin-3-coumaroylglucoside | 1.3 | 1.1 | 2.2 | 1.1 | 1.4 | 1.2 |
| Peonidin-3-coumaroylglucoside | 2.5 | 2.2 | 3.7 | 2.4 | 2.3 | 2.3 |
| Malvidin-3-coumaroylglucoside | 11.7 | 8.7 | 9.2 | 10.2 | 10.1 | 8.6 |
| Sum (anthocyanins) | 251.9 | 166.9 | 167.2 | 181.9 | 179.8 | 166.8 |
| | | | | | | |
| B1 | 113.4 | 119.1 | 108.5 | 127.4 | 113.5 | 129.3 |
| B2 | 94.3 | 111.4 | 115.0 | 120.5 | 108.7 | 125.1 |
| (+)-Catechin | 98.4 | 129.3 | 127.4 | 121.2 | 116.5 | 112.8 |
| (–)-Epicatechin | 166.9 | 192.2 | 189.1 | 202.3 | 190.3 | 205.6 |
| Epicatechingallate | 46.1 | 21.7 | 19.4 | 22.9 | 24.6 | 54.3 |
| Quercitin | 8.5 | 8.5 | 8.3 | 8.3 | 7.9 | 6.9 |
| Polymeric phenols | 1316 | 933 | 950 | 852 | 919 | 914 |
| Polymeric pigment | 18 | 24 | 23 | 22 | 22 | 24 |
| Gallic acid | 69.2 | 82.6 | 80.5 | 81.9 | 81.7 | 80.5 |
| Ellagic acid | 1.8 | 2.6 | 2.5 | 3.1 | 3.2 | 2.9 |
| | | | | | | |
| mDP | 3.15 | 3.08 | 3.11 | 3.16 | 2.87 | 2.73 |

Annexe B23: Polyphenolic analysis of wines of experiment 1, analyzed 1.5 years after microoxygenation treatment

Priloga B23: Polifenolne analize vin poskusa 1, analizirane 1,5 leta po končanem poskusu mikrooksigenacije

| | Control | 10 mg/L/mo | 30 mg/L/mo | Control (oak) | 10 mg/L/mo (oak) | 30 mg/L/mo (oak) |
|---|---|---|---|---|---|---|
| Total anthocyanins (spectroph.) | 324 | 257 | 272 | 260 | 279 | 270 |
| OD 280 | 52.1 | 58.0 | 58.3 | 56.9 | 57.5 | 58.2 |
| Color Intensity | 6.46 | 7.08 | 7.03 | 6.70 | 6.72 | 6.85 |
| % A420 | 39.0 | 38.6 | 38.9 | 39.4 | 39.3 | 39.1 |
| % A520 | 49.5 | 50.4 | 50.5 | 49.9 | 50.0 | 50.4 |
| % A620 | 11.5 | 10.9 | 10.6 | 10.7 | 10.7 | 10.6 |
| Color Hue | 0.788 | 0.767 | 0.771 | 0.790 | 0.787 | 0.776 |
| Vanilin Index | 1229 | 1371 | 1400 | 1266 | 1254 | 1260 |
| Proantocyanidnis | 2321 | 2477 | 2690 | 2466 | 2696 | 2526 |
| IP (Vanilin/Proantocyanidins) | 0.53 | 0.55 | 0.52 | 0.51 | 0.47 | 0.50 |
| | | | | | | |
| Delphinidin-3-glucoside | 8.00 | 3.17 | 3.00 | 3.48 | 3.77 | 3.78 |
| Cyanidin-3-glucoside | 0.92 | 0.64 | 0.64 | 0.68 | 0.80 | 0.77 |
| Petunidin-3-glucoside | 6.55 | 3.79 | 3.73 | 3.83 | 4.45 | 4.53 |
| Peonidin-3-glucoside | 3.93 | 2.96 | 2.65 | 2.85 | 3.06 | 3.09 |
| Malvidin-3-glucoside | 38.64 | 24.26 | 24.75 | 23.46 | 27.21 | 28.28 |
| Delphinidin-3-acetylglucoside | 1.96 | 0.91 | 0.89 | 0.96 | 1.14 | 1.16 |
| Cyanidin-3-acetylglucoside | 0.22 | 0.38 | 0.36 | 0.36 | 0.37 | 0.63 |
| Petunidin-3-acetylglucoside | 2.18 | 1.44 | 1.63 | 1.46 | 1.68 | 1.59 |
| Peonidin-3-acetylglucoside | 3.52 | 4.84 | 4.76 | 4.36 | 4.67 | 4.73 |
| Malvidin-3-acetylglucoside | 13.17 | 6.62 | 6.71 | 6.38 | 7.53 | 7.60 |
| Malvidin-3-caffeoylglucoiside | 1.24 | 1.38 | 1.27 | 1.37 | 1.30 | 1.30 |
| Delphinidin-3-coumaroylglucoside | 0.41 | 0.19 | 0.25 | 0.16 | 0.32 | 0.31 |
| Cyanidin-3-coumaroylglucoside | 0.13 | 0.10 | 0.19 | 0.18 | 0.10 | 0.10 |
| Petunidin-3-coumaroylglucoside | 0.38 | 0.39 | 0.40 | 0.32 | 0.42 | 0.12 |
| Peonidin-3-coumaroylglucoside | 0.47 | 0.32 | 0.37 | 0.33 | 0.44 | 0.46 |
| Malvidin-3-coumaroylglucoside | 2.75 | 2.29 | 2.55 | 1.98 | 2.75 | 2.90 |
| | | | | | | |
| Sum (anthocyanins) | 84.48 | 53.68 | 54.13 | 52.17 | 60.01 | 61.34 |

Annexe B24: Polyphenolic analysis of wines of experiment 2, analyzed three months after the microoxygenation treatment

Priloga B24: polifenolne analize vin poskusa 2, analizirane tri mesece po mikrooksigenacijskem poskusu

| | Control | 3 mg/L/mo | 5 mg/L/mo | Control +oak | 3 mg/L/mo +oak | 5 mg/L/mo +oak | Barrique |
|---|---|---|---|---|---|---|---|
| Total anthocyanins (spectroph.) | 527 | 508 | 505 | 528 | 504 | 498 | 484 |
| OD 280 | 50.1 | 49.6 | 49.0 | 50.4 | 50.7 | 50.2 | 50.2 |
| Color Intensity | 6.20 | 6.70 | 7.16 | 6.36 | 6.93 | 7.59 | 6.68 |
| % A420 | 36.9 | 36.7 | 36.1 | 36.7 | 36.4 | 35.9 | 36.5 |
| % A520 | 50.9 | 51.3 | 51.6 | 51.0 | 51.2 | 51.7 | 52.0 |
| % A620 | 12.2 | 12.0 | 12.3 | 12.3 | 12.4 | 12.4 | 11.5 |
| Color Hue | 0.725 | 0.716 | 0.700 | 0.719 | 0.710 | 0.694 | 0.702 |
| Vanilin Index | 1124 | 1134 | 1079 | 1151 | 1145 | 1095 | 1077 |
| Proantocyanidnis | 1994 | 1977 | 1994 | 1979 | 1932 | 1977 | 1983 |
| IP (Vanilin/Proantocyanidins) | 0.56 | 0.57 | 0.54 | 0.58 | 0.59 | 0.55 | 0.54 |
| | | | | | | | |
| Delphinidin-3-glucoside | 12.31 | 11.59 | 10.65 | 11.34 | 12.85 | 12.85 | 10.30 |
| Cyanidin-3-glucoside | 1.36 | 1.21 | 1.14 | 1.34 | 1.26 | 1.19 | 1.13 |
| Petunidin-3-glucoside | 12.27 | 11.36 | 10.26 | 11.99 | 11.63 | 10.71 | 9.97 |
| Peonidin-3-glucoside | 9.85 | 8.86 | 8.06 | 9.63 | 8.77 | 8.04 | 7.93 |
| Malvidin-3-glucoside | 93.54 | 84.44 | 76.76 | 91.16 | 83.68 | 75.82 | 75.91 |
| Delphinidin-3-acetylglucoside | 4.30 | 3.98 | 3.63 | 4.27 | 4.04 | 3.62 | 3.48 |
| Cyanidin-3-acetylglucoside | 1.17 | 1.19 | 1.11 | 1.16 | 1.16 | 1.09 | 1.01 |
| Petunidin-3-acetylglucoside | 4.39 | 3.87 | 3.57 | 4.25 | 3.89 | 3.46 | 3.48 |
| Peonidin-3-acetylglucoside | 4.39 | 3.90 | 3.50 | 4.25 | 3.85 | 3.37 | 3.38 |
| Malvidin-3-acetylglucoside | 38.37 | 33.98 | 30.57 | 37.17 | 33.36 | 29.57 | 29.84 |
| Delphinidin-3-coumaroylglucoside | 0.64 | 0.53 | 0.46 | 0.61 | 0.54 | 0.34 | 0.40 |
| Malvidin-3-caffeoylglucoiside | 0.26 | 0.25 | 0.24 | 0.25 | 0.24 | 0.21 | 0.19 |
| Cyanidin-3-coumaroylglucoside | 0.17 | 0.22 | 0.31 | 0.15 | 0.22 | 2.57 | 0.13 |
| Petunidin-3-coumaroylglucoside | 0.53 | 0.45 | 0.46 | 0.50 | 0.49 | 4.13 | 0.41 |
| Peonidin-3-coumaroylglucoside | 1.52 | 1.30 | 1.04 | 1.44 | 1.21 | 1.00 | 1.04 |
| Malvidin-3-coumaroylglucoside | 8.48 | 6.88 | 6.33 | 8.12 | 7.09 | 6.07 | 6.23 |
| | | | | | | | |
| Sum (anthocyanins) | 193.55 | 174.00 | 158.10 | 187.64 | 174.28 | 164.03 | 154.84 |

Annexe B25: Concentrations of different polyphenolic compounds in wines of experiment 3 initially and after a 24 weeks treatment

Priloga B25: Koncentracija različnih polifenolnih component v vinih poskusa 3 v začetku in po 24 tednih tretiranja

| Compound | Concentration (mg/L) for each treatment | | | | |
|---|---|---|---|---|---|
| | Initially | Control | 1.5 mg $O_2$/L/month | 3 mg $O_2$/L/month | Barrel |
| Gallic acid | 46.4 | 56.1 | 50.3 | 57.2 | 47.3 |
| Gentisic acid | 1.5 | 2.6 | nd | 1.5 | nd |
| Caftaric acid | 17.3 | 16.3 | 17.5 | 16.8 | 17.3 |
| Vanillic acid | 2.6 | 4.1 | 5.1 | 3.3 | 43.7 |
| Catechin | 790.2 | 784.0 | 704.4 | 698.6 | 659.5 |
| Caffeic acid | 64.2 | 59.0 | 56.8 | 57.0 | 52.6 |
| Procyanidin B1 | 60.0 | 91.0 | 93.5 | 55.6 | 78.5 |
| p-Coumaric acid | 3.6 | 3.8 | 4.2 | 6.4 | 12.2 |
| Procyanidin B2 | 49.1 | 40.8 | 40.4 | 40.0 | 39.8 |
| Epicatechin | 90.1 | 73.6 | 76.2 | 68.8 | 80.5 |
| Delphinidin-3-glucoside | 11.2 | 7.7 | 7.4 | 9.6 | 7.2 |
| Petunidin-3- glucoside | 13.8 | 7.6 | 9.3 | 8.5 | 8.7 |
| Peonidin-3- glucoside | 5.5 | 2.9 | 4.1 | 3.4 | 4.1 |
| Malvidin-3- glucoside | 117.9 | 63.4 | 83.8 | 72.0 | 79.6 |
| Ellagic acid | 3.7 | 4.8 | 4.5 | 5.4 | 3.3 |
| Quercetin-3- glucoside | 15.8 | 12.3 | 13.6 | 11.1 | 14.7 |
| Myricetin | 4.3 | 3.2 | 5.1 | 5.1 | 4.3 |
| Quercetin-3-rhamnoside | 5.0 | 4.2 | 3.4 | 4.7 | 4.0 |
| Malvidin-3-Acetate | 40.1 | 18.4 | 23.8 | 24.2 | 22.4 |
| Quercitin | 7.8 | 4.8 | 6.4 | 5.8 | 5.2 |
| Malvidin-3-*p*-coumaric acid | 19.6 | 6.8 | 11.1 | 9.6 | 10.0 |
| Polymeric pigment | 28.8 | 29.0 | 33.9 | 34.8 | 35.3 |
| Polymeric phenols | 791.6 | 782.9 | 947.1 | 1021.5 | 1132.0 |

Annexe B26: Polyphenolic analysis of wines of experiment 3, analyzed one year after the microoxygenation treatment

Priloga B26: Polifenolne analize v vinih poskusa 3, analizirane eno leto po mikrooksigenacijskem poskusu

| | Control | 1.5 mg/L/mo | 3.0 mg/L/mo | Barrique |
|---|---|---|---|---|
| Total anthocyanins (spectroph.) | 449 | 415 | 381 | 404 |
| OD 280 | 54.3 | 52.6 | 51.5 | 52.4 |
| Color Intensity | 8.78 | 8.09 | 8.58 | 8.47 |
| % A420 | 38.3 | 38.5 | 38.0 | 37.9 |
| % A520 | 49.5 | 49.8 | 50.3 | 50.3 |
| % A 620 | 12.2 | 11.8 | 11.7 | 11.9 |
| Color Hue | 0.775 | 0.773 | 0.756 | 0.753 |
| Vanilin Index | 752 | 810 | 704 | 605 |
| Proantocyanidnis | 2444 | 2474 | 2572 | 2654 |
| IP (Vanilin/Proantocyanidins) | 0.31 | 0.33 | 0.27 | 0.23 |
| | | | | |
| Delphinidin-3-glucoside | 3.34 | 2.56 | 2.73 | 3.30 |
| Cyanidin-3-glucoside | 0.36 | 0.15 | 0.30 | 0.35 |
| Petunidin-3-glucoside | 5.92 | 4.85 | 4.60 | 5.65 |
| Peonidin-3-glucoside | 3.18 | 2.61 | 2.41 | 2.88 |
| Malvidin-3-glucoside | 63.62 | 50.23 | 45.99 | 53.95 |
| Delphinidin-3-acetylglucoside | 0.91 | 0.80 | 0.79 | 0.96 |
| Cyanidin-3-acetylglucoside | 0.58 | 0.35 | 0.42 | 0.09 |
| Petunidin-3-acetylglucoside | 1.48 | 1.11 | 0.93 | 1.15 |
| Peonidin-3-acetylglucoside | 1.27 | 0.98 | 1.38 | 1.27 |
| Malvidin-3-acetylglucoside | 18.16 | 14.04 | 12.34 | 15.01 |
| Delphinidin-3-coumaroylglucoside | 0.15 | 0.22 | 0.03 | 0.26 |
| Malvidin-3-caffeoylglucoiside | nd | nd* | 0.17 | 0.21 |
| Cyanidin-3-coumaroylglucoside | nd | nd | 0.16 | 0.02 |
| Petunidin-3-coumaroylglucoside | 0.63 | 0.43 | 0.33 | 0.52 |
| Peonidin-3-coumaroylglucoside | 0.76 | 0.53 | 0.44 | 0.59 |
| Malvidin-3-coumaroylglucoside | 8.08 | 5.58 | 4.63 | 5.89 |
| | | | | |
| Sum (anthocyanins) | 108.45 | 84.47 | 77.66 | 92.11 |

Annexe B27: Poliphenolic analysis of wines of experiment 4, analyzed three months after the microoxygenation treatment

Priloga B27: Polifenolne analize vin poskusa 4, analizirane tri mesece po mikrooksigenacijskem poskusu

| | Control (40%) | 5mg/L/mo (40%) | Control (100%) | 5mg/L/mo (100%) | New barrel no.1 | New barrel no.2 | Old barrel no.1 | Old barrel no.2 |
|---|---|---|---|---|---|---|---|---|
| Total anthocyanins (spectroph.) | 768 | 744 | 734 | 721 | 729 | 758 | 796 | 778 |
| OD 280 | 69.0 | 69.4 | 71.1 | 70.1 | 69.0 | 68.6 | 67.2 | 69.0 |
| Color Intensity | 14.33 | 15.21 | 15.36 | 16.30 | 15.31 | 14.86 | 13.41 | 14.38 |
| % A420 | 34.6 | 34.6 | 34.4 | 34.3 | 34.4 | 34.3 | 34.8 | 34.5 |
| % A520 | 53.0 | 53.1 | 52.9 | 53.1 | 53.0 | 53.0 | 52.9 | 53.1 |
| % A 620 | 12.4 | 12.3 | 12.6 | 12.5 | 12.6 | 12.7 | 12.4 | 12.3 |
| Color Hue | 0.652 | 0.651 | 0.651 | 0.647 | 0.649 | 0.647 | 0.657 | 0.650 |
| Vanilin Index | 1266 | 1368 | 1212 | 1221 | 1294 | 1223 | 922 | 1114 |
| Proantocyanidnis | 3241 | 3051 | 3221 | 3277 | 3577 | 3151 | 3119 | 2958 |
| IP (Vanilin/Proantocyanidins) | 0.39 | 0.45 | 0.38 | 0.37 | 0.36 | 0.39 | 0.30 | 0.38 |
| | | | | | | | | |
| Delphinidin-3-glucoside | 19.78 | 20.79 | 17.47 | 17.77 | 19.52 | 18.04 | 23.40 | 19.90 |
| Cyanidin-3-glucoside | 1.82 | 1.84 | 1.68 | 1.57 | 1.88 | 1.74 | 2.28 | 2.00 |
| Petunidin-3-glucoside | 19.00 | 19.33 | 17.47 | 16.48 | 19.54 | 18.29 | 22.94 | 21.00 |
| Peonidin-3-glucoside | 11.31 | 11.40 | 10.76 | 9.88 | 12.06 | 11.13 | 13.61 | 13.34 |
| Malvidin-3-glucoside | 149.41 | 149.10 | 140.95 | 129.48 | 155.73 | 148.29 | 181.31 | 170.65 |
| Delphinidin-3-acetylglucoside | 0.77 | 0.62 | 0.86 | 4.11 | 1.00 | 0.94 | 1.29 | 1.31 |
| Cyanidin-3-acetylglucoside | 0.55 | 0.82 | 0.80 | 9.52 | 0.78 | 0.57 | 0.59 | 0.84 |
| Petunidin-3-acetylglucoside | 1.26 | 1.51 | 2.16 | 7.92 | 1.39 | 1.51 | 1.91 | 3.03 |
| Peonidin-3-acetylglucoside | 2.70 | 3.07 | 3.59 | 3.38 | 2.88 | 2.82 | 2.74 | 3.17 |
| Malvidin-3-acetylglucoside | 26.19 | 23.83 | 24.43 | 18.23 | 28.52 | 28.38 | 37.45 | 35.39 |
| Delphinidin-3-coumaroylglucoside | 0.70 | 0.62 | 0.70 | 0.54 | 0.71 | 0.69 | 0.90 | 0.91 |
| Malvidin-3-caffeoylglucoiside | 0.37 | 0.36 | 0.54 | 0.34 | 0.41 | 0.42 | 0.57 | 0.57 |
| Cyanidin-3-coumaroylglucoside | 0.22 | 0.25 | 0.36 | 0.31 | 0.26 | 0.22 | 0.33 | 0.34 |
| Petunidin-3-coumaroylglucoside | 1.01 | 1.03 | 0.99 | 0.91 | 1.08 | 1.00 | 1.16 | 1.19 |
| Peonidin-3-coumaroylglucoside | 1.42 | 1.48 | 1.32 | 1.26 | 1.58 | 1.46 | 1.81 | 1.90 |
| Malvidin-3-coumaroylglucoside | 13.70 | 13.67 | 12.64 | 11.47 | 14.50 | 13.98 | 17.62 | 16.88 |
| | | | | | | | | |
| Sum (anthocyanins) | 250.21 | 249.73 | 236.72 | 233.17 | 261.84 | 249.47 | 309.49 | 292.41 |