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Study of the components of quality in SO_2-free wines obtained by innovative vinification protocols Evaluation of the pre-fermentative addition of lysozyme and oenological tannins

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Table of Contents

INTRODUCTION

In recent years, the increasing interest demonstrated by consumers in the selection of food products has stimulated considerable research efforts towards the identification of innovative technologies for assuring safer and healthier food.

In oenology, a major growing concern is the use of sulphur dioxide during the technological processes involved in winemaking. Sulphur dioxide (SO_2) is commonly used as a preservative because of its well established technological properties. These include its function as an antioxidant, protecting wine phenols from oxidation and as an inhibitor for must endogenous oxidases; it's bacteriostatic properties, which prevent the onset of undesirable fermentations (such as acetic or malolactic fermentation) and its usefulness for the extraction of skin pigments.

However, there is a general trend towards the reduced use of SO_2 in wine processing because, over certain ingestion doses, this compound could have toxic effects on human health. Moreover, SO_2 is commonly known to trigger adverse reactions in certain people who can be sensitive to its presence, and can be a major factor in wine intolerance. It is also important to reduce the amount of SO_2 in wine, since this compound is also found in many food products as an additive, and the amount consumed is accumulative in an organism.

Although it can be technologically challenging and appear often difficult to make wines of high quality without the addition of SO_2, considerable research efforts have been undertaken to develop oenological protocols in which alternative additives, to substitute the presence of sulphites in the above-mentioned functions, can be used for the production of high quality wines, with low SO_2 levels, stable and well characterized.

Since the early 1990's, the use of lysozyme has been proposed to control malolactic fermentation in winemaking, supporting or even replacing the use of sulphur dioxide. Lysozyme (E.C. 3.2.1.17) is an enzyme present in the hen egg white which possesses a lytic activity on the cell wall of Gram-positive bacteria, such as lactic acid bacteria. Its use in musts and wines was authorised by the Organisation International de la Vigne et du Vin (OIV) in 1997. However, even if there is only scant information available concerning the risk of its presence in wines and some contradictory experimental results exist, in 2005 the European Commission decided to permanently include lysozyme on the list of ingredients that must be indicated on the wine labels.

Since lysozyme lacks any antioxidant properties, in order to control the browning phenomena of sulphite-free wines, it is suggested its use can be associated with others additives or adjuvants

which could prevent the oxidation of volatile and polyphenolic compounds, which are considered the most important quality parameters of musts and wines.
In particular, oenological tannin has already proved to be highly reactive against oxidative intermediates and could well be proposed as a suitable additive.

The aims of the following PhD research study were to evaluate the quality of white wines, as a function of the reduction in SO_2 use during the first steps of the winemaking process.
In order to investigate the mechanism and intensity of interactions occurring between lysozyme and the principal macro-components of musts and wines, a series of experiments on model wine solutions were undertaken, focusing attention on the polyphenols, SO_2, oenological tannins, pectines, ethanol, and sugar components.
In the second part of this research program, a series of conventional sulphite added vinifications were compared to vinifications in which sulphur dioxide was replaced by lysozyme and consequently define potential winemaking protocols suitable for the production of SO_2-free wines.
To reach the final goal, the technological performance of two selected yeast strains with a low aptitude to produce SO_2 during fermentation were also evaluated.
Together with the study of the oenological parameters in the resulting wines (eg. density, pH, alcohol content, total and volatile acidity, total polyphenol index, etc) and the amount of lysozyme present at the end of the alcoholic fermentation, analyses were focused on the evaluation of major wine quality parameters such as the phenolic amount, the concentration of volatile compounds and amino acids by using High Performance Liquid Chromatography/ Diode Array Detection/ Fluorescence Detection (HPLC/DAD/FLD) and Gas Chromatography/Mass Spectroscopy (GC/MS) techniques. Furthermore, to more completely understand the mechanisms linked to the oxidative phenomena of white SO_2-free wines, analysis of the volatile and polyphenolic compounds over a one year period of wine ageing in bottles was also undertaken.
The last part of the research program was focused on the fundamental chemistry relevant to the oxidative phenolic spoilage of white wines. More specifically, the impact of glutathione as a potential inhibitor for preventing the oxidative ageing of white wines (via the formation of xanthilium cation pigments from (+)-catechin and glyoxylic acid) was studied. This training program was developed at the National Wine and Grape Industry Centre in Wagga Wagga (NSW, Australia) and at the University of Melbourne (VIC, Australia).

Chapter 1

Interactions between hen egg white lysozyme and some of the principal macro-components of musts and wines

ABSTRACT: *The lytic activity on the cell wall of Gram-positive bacteria showed by lysozyme, an enzyme present in hen egg white, has promoted its successful use in the pharmaceutical and food industry as an antimicrobial agent for prolonging the shelf-life.*
Since the 1990's, the use of egg white lysozyme to control malolactic fermentation in winemaking, supporting or even replacing SO_2, has been proposed in several studies, demonstrating the efficacy of lysozyme addition in winemaking under several conditions. However, the lack of further information concerning the mechanism and intensity of interactions occurring between lysozyme and the principal macro-components of musts and wines stimulated the following work.
In this study, the ability of lysozyme to interact with several must and wine macro-components was investigated in model wine solutions, in order to simplify the interpretation of results. Six different must and wine macro-components (sugars, ethanol, SO_2, oenological tannins, pectines and polyphenol extract) were added individually to wine-like media containing 250 mg L^{-1} of lysozyme, and the amount of residual protein together with enzyme activity 2, 24 and 48 hours after addition were monitored.
The results show that glucose and fructose appear to not significantly decrease the bioactivity and amount of residual protein, whereas for ethanol, tannins, pectines, SO_2 and polyphenols, the data highlighted an increased interaction. In particular, the interaction between polyphenols and lysozyme was found to be very strong and completely inhibited the protein's enzymatic activity from the beginning of contact until the end of the experiment. The same trend was found for the residual protein percentage in solution, which decreased quickly after only 2 hours of contact, confirming that the interaction between lysozyme and polyphenols causes precipitation of the enzyme from the medium.

Sections of this work have been published in:

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1. INTRODUCTION

1.1 Activity of lysozyme

Lysozyme, E.C. 3.2.1.17, is an enzyme first discovered by Fleming in 1922, which is found widely distributed in the natural environment. It is present in most physiological liquids (eg. milk, blood, tears, urine, cervical mucus, etc.) and also in different plants.

Several lysozyme types that share common properties, including antibacterial activity, have been discovered (Ibrahim, 1996a; Wang et al., 1990).

The most common type is the c-type, which is the lysozyme present in hen egg-white. This lysozyme has a lytic activity on the cell wall of Gram-positive bacteria, and has been successfully used in the pharmaceutical and food industry as an antimicrobial agent for prolonging product shelf-life since the 1950's. It has been used to preserve fresh fruits and vegetables, seafood, meats and a great deal of cheese varieties, to prevent late blowing caused by butyric acid bacterial contamination. (Ghitti et al., 1983; Proctor et al, 1988; Cunningham et al., 1991; Croguennec et al, 1999). Lysozyme has also been added to infant feeding formulae in order to make it more closely resemble human milk and increase the amount of *Bifidus bacillus* bacteria in the intestines, contributing to a healthy flora and aiding digestion. (Nishihava et al., 1967).

Lysozyme's lytic activity is based on the hydrolysis of the *β*-(1-4) linkage between *N*-acetylmuramic acid (NAM) and *N*-acetylglucoseamine (NAG), which constitutes the peptoglycan layer of the bacteria cell wall (Fig. 1).

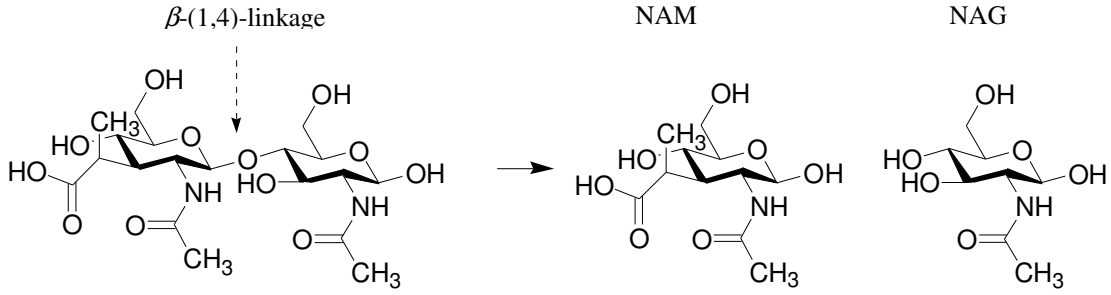

Figure 1. The enzyme hydrolyzes the 1-4 glycosidic linkage between alternating N -acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues.

This chitinolytic activity changes the cell's solidity and permeability, causing degradation of the bacterial cell wall and thus accelerating cell lysis (Cunningham et al., 1991).

Lysozyme is a small monomeric globular protein, consisting of 129 amino acids as shown in Table 1. It contains six tryptophan (Trp) residues, three of which are located at the substrate binding sites, two in the hydrophobic matrix box, and one which is separate from the others. Among them Trp 62 and Trp 108 are the most dominant fluorophores, both being located at the

substrate binding site. These fluorescent properties allowed for the detection and quantification of lysozyme via fluorimetric detection (FDL), as highlighted by Riponi et al., 2007. The molecular mass of egg white lysozyme is 14307 Da, and its isoelectric point (pI), the pH at which it carries no net electrical charge, is 10.5-11.0, due to the high proportion of lysine and arginine residues (Canfield, 1963; Ghosh et al., 2008).

Aminoacids	*Hen egg-white lysozyme*
Alanine	12
Valine	6
Leucine	8
Isoleucine	6
Proline	2
Phenylalanine	3
Tryptophane	6
Methionine	2
Glicine	12
Aspartic acid + Asparagine	21
Glutamic acid + Glutamine	5
Lysine	6
Arginine	11
Serine	10
Threonine	7
Cysteine	8
Tyrosine	3
Histidine	1
n. total residues	*129*

Table 1. Hen egg-white lysozyme amino acid composition (Innovazione tecnologica nell'industria di lavorazione delle uova. M.Pizzichini, P.Marcolini, P.Erbisti, A.Serse. ENEA, Dipartimento Innovazione, Settore Biotecnologie e Agricoltura, Roma).

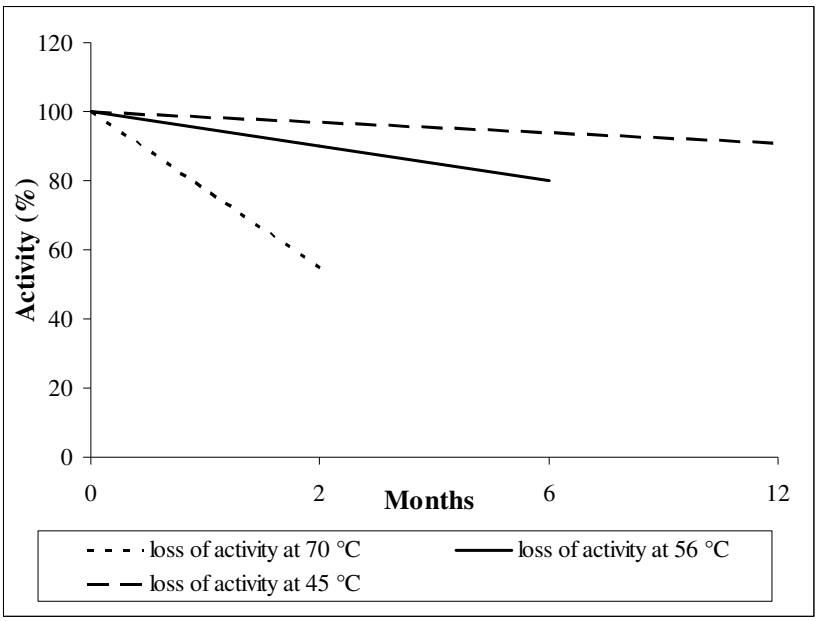

Figure 2. Lysozyme activity loss as a function of different storage temperatures (www.fordras.com).

The maximum stability and activity for lysozyme is found at pH values lower than 7.0, namely, in the range of 2.8-4.2, which is coincidentally the pH range of most wines (Pitotti et al. 1991). There are many factors which can affect the activity of lysozyme (*eg*. temperature, certain chemicals, processing and complexation). Generally, lysozyme is very stable toward temperature fluctuation. At temperatures higher than room temperature, lysozyme maintains an appreciable stability with a loss of activity of only 9% when stored at 45°C for 12 months and a loss of 20% at 56°C for six months (Fig. 2) (www.fordras.com).

This heat resistance of lysozyme at high temperature is quite remarkable, but its stability, as measured by the "residual activity", is more properly a function of two key parameters, namely the temperature and pH.

In acidic solutions, lysozyme is very heat stable and has been reported to withstand 100°C with little loss of activity (Bordet, 1928; Meyer et al., 1936; Linz, 1937; Smolelis et al., 1952). Matsuoka et al. (1966) also found lysozyme to be stable in acidic solutions at pH 4.5, 100°C for 3 min and at pH 5.29, 100°C for 30 min. Using loss of activity as a criterion, Beychok et al (1959) showed that stability in the temperature range 85-95°C was maximum about pH 5.5. This stability decreased rapidly at about pH 6, although low solubility in more alkaline solutions prevented further study.

Gorini et al. (1953) reported a 25% inactivation of lysozyme at 70°C in borate buffer at pH 7.9 after 30 min and Sandow (1926) reported that lysozyme in egg-white at pH 8.0 was destroyed in

15 min, whereas at pH 5 and 60°C, no loss of activity occurred in 60 min. Lysozyme seems to be over 50 times more heat stable in phosphate buffer (pH 6.2) than in egg-white (pH 9) at 62.5°C. Its inactivation in egg-white varied from about 10% at pH 7 to over 95% at pH 9. Neither Al^{3+} nor Fe^{3+} (10^{-3} M) had any influence on the stability of lysozyme in egg-white. No inactivation of lysozyme occurred at 62.5°C in phosphate buffer, even at pH 9, while at 65°C in egg-white, inactivation occurred in 10 min at pH 9. The loss of lysozyme activity is enhanced in egg-white at lower temperatures than in buffer due to the presence of sulfydryl groups in ovalbumin, the main protein found in egg white, which can reduce at least one disulphide bond of the lysozyme (Cunningham et al., 1991).

The activity of 1 mg mL^{-1} lysozyme in bovine skim milk was found to decrease at temperatures greater than 60°C by Weaver et al. (1978). Lysozyme polymerized through disulphide linkages and at 180°C both polymerisation and degradation occurred. When the temperature was raised to 200°C many changes occurred, including cleavage and recombination of peptide bonds. Above 200°C, polymerization and degradation occurred more violently (Hayase et al., 1975).

The effect of fluctuating temperature on lysozyme activity was studied by Wu et al. (1975). In contrast to the effects on simple chemical reactions, fluctuating temperatures were found to affect the activation, deactivation and reactivation of enzymatic reactions in complex ways. The reaction rate seems influenced by certain patterns of temperature adaptation unique to particular types of enzyme molecules. Overshoot and undershoot phenomena occurred when temperatures were changed and, as a result, inverse compensation was shown. The final product yield after subjecting an enzyme to temperature fluctuation depended on the compensation between the overall magnitude of overshoot from theoretical values. The cycle-down mode and a lower frequency of fluctuation had a greater influence on ratio changes. Faster rates and higher yields resulted when lysozyme was not heat-inactivated (20°C region). When heat inactivation was apparent (*eg*. 50°C region for lysozyme), the lower frequency and the cycle-down mode resulted in greater inactivation as well as greater activation. However, the former effect overshadowed the latter and resulted in a lower yield.

Lysozyme is extracted from edible hen egg white by a procedure of separating ion-exchange resin. The microbiological purity guarantees the security for its usage in food. The egg white used in the preparation of enzymes are compatible with parameters established by inspection agencies and is treated in compliance with hygienic manufacturing procedures.

Lysozyme is commonly sold as a white, odourless microcrystalline powder, because of its lower stability in aqueous solution (22%). Soluble in water, but insoluble in the more common organic

solvents or concentrated saline solutions, the powder form is stable for five years when maintained free from humidity and at room temperature, whereas the aqueous solution has a shelf life of 9 months (www.fordras.com).

1.2 Effect of Lysozyme on some bacterial strains

As an enzyme, lysozyme shows a specific action toward both species and strain. Its efficacy toward Gram-negative bacteria (i.e., acetic bacteria) is much less than Gram-positive bacteria (i.e., lactic acid bacteria) and could be defined as bacteriostatic rather than bactericidal (Cunningham et al., 1991; Hughey et al., 1987), presumably because the outer membrane acts as a barrier. It is inactive against the eukaryotic cell wall (Mc Kenzie et al., 1991). Some researchers found that a partial unfolding of lysozyme with proper acquisition of the hydrophobic pocket to the surface can increase its antimicrobial activity to include Gram-negative bacteria, without a detrimental effect on the inherent bactericidal effect against Gram-positive ones (Ibrahim et al. 1996).

To comprehend how lysozyme functions as a preservative in foods, extensive studies have been undertaken over the years to understand the effect of lysozyme on certain strains and types of bacteria.

Salton et al. (1960) used various Gram-positive bacteria to study the susceptibility of their cell walls toward degradation. Strains of *Bacillus*, *Corynebacterium*, *Lactobacillus*, *Micrococcus*, *Sarcina*, *Sporosarcina*, *Staphylococcus* and Gram-negative *Streptococcus* were studied, and the isolated cell walls from all the organisms were shown to be sensitive to lysozyme.

Since lysozyme has also been isolated from human and bovine milk, researchers have sought to determine how the enzyme affects bacteria in the milk. Vakil et al. (1969) used eight Gram-positive strains including two acid lactic bacteria (*Micrococcus lysodeikticus*, *Lactobacillus casei*, *Staphylococcus aureus*, *Sarcina lutea*, *Streptococcus lactis*, *Streptococcus faecalis, Bacillus subtilis* and *Bacillus cereus*) and five Gram-negative organisms (*Escherichia coli*, *Serratia marcescens*, *Proteus vulgaris*, *Pseudomonas fluorescens* and *aeruginosa*) that were either live resting cells or UV-killed cells. They found that all were susceptible to bovine milk muramidase and all except the lactic acid bacteria (*Lactobacillus casei* and *Streptococcus lactis*) were susceptible to human milk muramidase. This result suggested that lysozyme played a significant role in the inherent antibacterial activity of milk.

Vedmina et al. (1979) tested the sensitivity of lysozyme against 476 strains of Gram-negative bacteria. They found high resistance to lysozyme in *Eltor* and *Pseudomonas*. Cultures of various sensitivity included *Aeromonas*, enteropathogenic *Escherichia coli* and NAG-vibrios.

Bottazzi et al. (1978) studied the effect of lysozyme on thermophilic lactic acid bacteria. The natural culture of lactic acid bacteria grown in whey was very sensitive to lysozyme. They found that concentrations up to 10 mg $L^{-1}$of lysozyme were proportional to the lityc effect.

Lysozyme has also been known to be more detrimental to spoilage and phatogenic bacteria in milk than to lactic acid-producing bacteria. In fact, lysozyme in small concentration was found to activate the growth of two *Streptococcus cromoris* strains that are used in cheese processing (Akashi, 1972). Because of this, lysozyme has been suggested as a preservative in dairy fermented products such as cheese.

Lysozyme can inhibit many types of spoilage organisms that are able to shorten the shelf-life of foods. In a Japanese study on the inhibitory effect of egg-white lysozyme on growth of lactobacilli from mirin liquor, *Lactobacillus heterohiochii*, *L. fermenti*, *L. plantarum*, and *L. casei* (all of which spoil wine) were isolated from mirin liquor and used as test organisms. Growth of these organisms was completely inhibited by 20 mg L^{-1} of lysozyme. After one year of storage at room temperature, more than 95% of the original activity of lysozyme remained (Uchida et al., 1972). Another study of sake putrefying lactic acid bacteria found that *Lactobacillus acidophilus* was resistant to lysozyme, while all the others were inhibited in sake containing more than 15% ethanol (Yajima et al., 1971).

Using direct microscopic counts, lower total microbial numbers than expected in some egg products after bacterial decomposition were found (Hall et al., 1971). The data obtained suggested that the bactericidal effectiveness of lysozyme in reducing bacterial populations should not to be overlooked when producing low bacterial count, pasteurized products. These results were confirmed by Ashton et al. (1975), finding lysozyme in commercial crystalline egg albumen. The data suggested that destruction of the thermophilic aerobes by lysozyme should not be considered when performing counts on egg products. The addition of lysozyme to pasteurised milk has been shown to substantially reduce the direct microscopic count.

1.3 Lysozyme as a preservative in food

In the 1970's, considerable interest was stimulated in the use of lysozyme as a food preservative, mostly in Japan, where the majority of work using lysozyme in food systems has been performed. Akashi (1972) found that egg-white suspensions had a lityc action on Escherichia coli suspensions. Food product such as sausages, fish cakes and bacon could be preserved with lysozyme. There are also many Japanese patents on the use of lysozyme; as a coating for the surface of fresh vegetables, fish, meat and fruit (Japanese patent 4831-905), as a process to preserve tofu bean curd by adding lysozyme to soya milk during processing (Japanese patent 46-

336/72), for the incorporation of egg-white lysozyme with ovalbumin and ovomucin to preserve dried milk compositions for pediatric use (Japanese patent, 16-780/70), for treatment with aqueous solution of lysozyme and NaCl on oysters, shrimps and other seafood preserved in refrigerated storage (Japanese patent 5710/72), for soaking fresh marine products in aqueous solution containing a lysozyme salt, amino acids and NaCl (Japanese patent 19576/71), for the addition of lysozyme or its salts together with *p*-hydroxybenzoic esters to wines (Japanese patent 3-115/71) and together with β-glycopyranose dehydrogenase to sake (Japanese examinated patent 5535105).

1.3.1 Meat

The use of lysozyme with meat products has also been successfully applied.

Some studies about the preservative effect of lysozyme added to cooked sausage, salami sausage and Vienna sausage showed that meat was more effectively preserved when lysozyme was used in combination with NaCl and $NaNO_2$ than by either lysozyme or salt alone. Omitting the effect of heat, lysozyme retarded microbial growth to a greater extent than did the standard preservative mixture of NaCl and $NaNO_2$. The number of species of bacteria found initially on sausages after lysozyme treatment (Streptococcus, Pseudomonas, Achromobacter and Flavobacterium) toward the middle to last stages of storage time decreased (Akashi, 1969, 1970, 1971).

Also, the preservative effects of egg-white lysozyme on non-packaged Kamaboko (a type of cured surimi, a Japanese processed seafood product, in which various white fish are pureed, combined with additives, formed into distinctive loaves, and then steamed until fully cooked and firm) was studied by Akashi et al. (1972). In this experiment, the viable count, slime changes, binding capacity and brown colour changes were monitored. Kamaboko preserved with lysozyme in the meat performed better than the mixture with more traditional preservatives such as 2-[2furyl-3-(5-nitro-2-furyl)acrilamide] (AF2) or ascorbic acid. Egg-white lysozyme exhibited binding capacity and brown colour changes similar to those of AF2 or sorbic acid.

1.3.2 Cheese

Undoubtedly, cheese is the food for which most of the research using lysozyme as a preservative has been performed.

Because of the lytic activity on the cell wall of bacteria like *Clostridium tyrobutyricum*, hen egg white lysozyme has been successful used in cheesemaking to prevent late blowing of semi-hard and hard cheeses, like Edam, Gouda and some Italian varieties (IDF, 1987; Cunningham et al., 1991). Late blowing is a defect in high-pH cheeses caused by *Clostridium tyrobutyricum* spores

present in raw milk ferments lactate. This defect is characterized by eyes, slits, and cracks caused by the production of gas bubbles, and can create considerable loss of product.

Some studies tried to make Edam cheese and incorporated lysozyme in the form of concentrated suspensions just before the addition of rennet. The minimum amount of lysozyme required to prevent late blowing of cheese made from low quality milk was about 500 units mL^{-1}, corresponding to approximately 0.6% egg-white. Purified lysozyme and egg-white were both effective inhibitors but dried ovalbumin was not completely effective. No significant differences were found organoleptically between cheese made with lysozyme or with nitrate, and lysozyme did not have any effect on the cheesemaking process (Wasserfall et al., 1976). Another research group showed that only 0.2% egg-white on four different batches of Edam cheese, containing progressively increasing amounts of butyric acid spores, improved the cheese organoleptically and microbiologically (Koterska et al., 1972).

For *Clostridium tyrobutyricum*, the butyric acid bacteria that can cause late blowing or late gassing in Italian cheeses, Edam and Gouda, Wasserfall and Teuber (1979) have used egg-white lysozyme at a concentration of 500 units mL^{-1} to kill 99% of 5×10^5 resting vegetative cells of the bacteria within 24 hours of incubation at 25°C. Spores were resistant to lysozyme, however proliferating vegetative cells were severely inhibited. Though lysozyme-resistant cells developed in growing cultures in the presence of lysozyme, the overall outgrowth of spores to vegetative cells was delayed one day in the presence of 550 units of lysozyme mL^{-1}. These results suggested this inhibition of the lactate-fermenting bacteria was the basis for observation that lysozyme could substitute for nitrate in preventing the late gas defect. The same research group prepared Edam cheese without lysozyme and with 500 units mL^{-1} of lysozyme from 25 litres of low-count milk containing approximately 1000 bacteria spores L^{-1}. After five weeks of ripening, cheese made with lysozyme had resisted late blowing, while the other was no longer suitable for consumption. The same results occurred in the presence of lysozyme-resistant vegetative cells. A patent process in the UK in which lysozyme or its non-toxic salts were added to butter or cheese to prevent the development of undesirable microorganisms was developed (patent application 2014032A).

The use of preservatives, like lysozyme, is limited or not permitted in most cheeses having protected designation of origin (PDO), because its presence in food could compromise the image of quality perceived by consumers (Tirelli et al., 2007).

However, the manufacturers of Grana Padano, an Italian PDO long-ripened hard cheese produced with raw milk, have started to employ lysozyme extracted from egg white to counter late blowing caused by the out-growth of clostridia spores (*C. butyricum* and *C. tyrobutyricum*).

Some applications involved the addition of 50 mg L^{-1} lysozyme to rennet milk in Grana Padano cheese making. The results showed that after 24 months of ripening the physical and organoleptic properties were excellent, whereas control cheese was blown because of *C. tyrobutyricum* (Wasserfall et al. 1979). Today the maximum amount allowed in Grana Padano PDO production is 2.5 g 100 kg^{-1} of milk used.

For its antibacterial properties, lysozyme from hen egg-white was declared a cheese preservative (E1105), according to both the current EU legislation (Council Directive 95/2/CE) and the Codex Alimentarius (Codex Stan A-6-1978, Rev. 1-1999). They approved the utilisation of lysozyme as a cheese additive with a tolerability of 10-35 g 100 L^{-1} of milk used in cheese production, corresponding to 10-35 g 100 kg^{-1} of finished product.

Since the presence of hen egg-white lysozyme in cheese could be potentially hazardous for subjects with allergies toward egg products, in 2005 the European Community included lysozyme on the allergenic additive list (Council Directive 2000/13/CE), because of its extraction from hen-egg, and it must be declared on the label in accordance (Iaconelli et al., 2008).

1.3.3 Fermented beverages

The preservative effect of lysozyme has also been shown in fermented beverages, like sake, mirin liquor, beer and wine.

Sake is a Japanese alcoholic beverage made from rice, through a brewing process similar to that of beer, containing 18-20% alcohol.

A study on the effect of lysozyme on *Lactobacillus homohiochii* and *heterohiochii* in sake reported that the growth-inhibitory action of lysozyme on these bacteria was more potent in sake pretreated with activate carbon than in sake without pre-treatment. The minimum inhibitory concentration of lysozyme in sake of approximately 20% alcohol content was 5 mg L^{-1}. A commercial sample of sake with added lysozyme maintained the enzyme activity even in presence of residual kaki-shibu, a type of tannin prepared from Japanese persimmon. In the same study, it was also found that sake degradation caused from *Lactobacillum* was prevented by adding lysozyme or its salts together with *p*-hydrobenzoic esters (Yajima et al., 1971).

Lysozyme was effectively used to inhibit spoilage lactic acid bacteria (hiochi bacteria) in mirin liquor, which is a sweetened sake containing less than 20% alcohol, used for cooking Japanese food. A lysozyme amount of 20 mg L^{-1} added to mirin liquor was found to completely inhibit the growth of the organism tested. More than 95% of the original activity of lysozyme added to mirin liquor remained after one year of storage at room temperature and no loss of lysozyme

activity was observed after pasteurization at 65°C for 5 hours. The clarification treatment of mirin liquor with wheat gluten and persimmon tannin gave a 2.5% loss of lysozyme activity (Uchida et al., 1972; Yashitake et al., 1977).

The relatively high thermal stability of lysozyme has also made it attractive for use in heat-sterilised and in pasteurized food products, such as beer, possibly allowing reduced thermal processes, and therefore, minimising nutritional and sensory quality loss. Makki et al. (1996) evaluated the thermal inactivation kinetics of lysozyme in beer at four different temperatures (75, 82, 91 and 95°C) and investigated lysozyme's potential to prevent or delay growth of lactic acid bacteria, responsible for spoilage in beer. The results of thermal inactivation of lysozyme showed a linear relationship between temperature increase and lysozyme activity reduction. Concerning the effect on lactic acid bacteria, lysozyme at 10 and 50 mg L^{-1} delayed growth of the spoilage bacteria *Lactobacillus brevis* and *Pediococcus damnosus*, but did not prevent growth. Similar findings were reported by Daeschel et al. (1999), who also noted that lysozyme does not appear to contribute any sensory properties to beer either after immediate addition or upon extended storage (3 months) in beer held refrigerated or at room temperature.

1.4 Lysozyme activity in winemaking

1.4.1 Effect on musts and wine bacterial strain

The winemaking process involves the interaction of many different microbial species, including yeast, fungi, lactic acid bacteria and acetic acid bacteria. The alcoholic conversion of grape sugars by yeast is not the only pivotal microbial involvement in modern oenology. The secondary fermentation, such as malolactic fermentation (MLF) caused by lactic acid bacteria also plays a critical role in the final quality of the wine (Amati, 1988).

Most lactic acid bacteria associated with wine are capable of conducting malolactic fermentation, while certain species are also reportedly responsible for wine spoilage (i.e. *Pediococcus spp*., *Lactobacillus spp*., *Oenococcus oeni*). (Gao et al., 2002). Some spoilage lactic acid bacteria can produce high levels of acetic acid with a consequent detrimental impact on wine quality (Du Toit et al., 2000; Edwards et al, 1998). In addition to this sensory defect, high concentrations of acetic acid have often been implicated in stuck and sluggish fermentations (Rasmussen et al. 1995; Edwards et al., 1999).

To avoid the onset of undesirable fermentations (such as acetic or malolactic fermentation), sulphur dioxide is traditionally used as a preservative in winemaking (Ribéreau-Gayon et al., 2007). However, there is a general trend towards reduced use of SO_2 in wine processing, because it can elicit an allergic response in sensitive people and has potential toxic effects on human

health over certain ingestion doses, suggesting the necessity to develop oenological protocols in which alternative additives can be used to substitute sulphites in the mentioned functions (Taylor SL et al, 1986; Romano et al, 1993; Sonni et al., 2008).

Since the early 1990's, the use of egg white lysozyme has been proposed to control malolactic fermentation in winemaking, supporting or even replacing sulphur dioxide (Amati et al.,1994; Amati et al., 1996; Gerbaux et al., 1997; Chinnici et al. 1996).

The antimicrobial activity of lysozyme toward lactic bacteria was reviewed by Cunningham et al. (1991) and has been since shown to depend on both the cell physiological state and the lysozyme structure in the medium (H^+ concentration, reacting compounds) (Ibrahim et al., 1996a; Ibrahim et al., 1996b).

Bacterial sensitivity to lysozyme depends on the peptidoglycan structure in the cell wall and the lysozyme efficacy toward Gram-negative bacteria (i.e. acetic bacteria) has been shown to be much less compared to the Gram-positive bacteria and more bacteriostatic than bactericidal, presumably because the outer membrane acts as a barrier (Cunningham et al., 1991; Hughey et al., 1987).

The antimicrobial action of lysozyme has been modified to include Gram-negative bacteria by altering its surface hydrophobicity through genetic and chemical modifications or by interactions between phenolic aldehyde with the molecule (Ibrahim et al., 1993; Ibrahim et al., 1994a; Ibrahim et al., 1994b).

Research conducted by Ibrahim et al. (1996b) has found that a partial unfolding of lysozyme to allow proper acquisition of the hydrophobic pocket to the surface can switch its antimicrobial activity to include Gram-negative bacteria without a detrimental effect on the inherent bactericidal effect against Gram-positive bacteria.

1.4.2 Effect of some must and wine components in model solution

A number of studies have reported possible interactions between the protein with some components of musts and wines. The primary factor that has been observed to affect the bacteriolytic action of lysozyme in wines is believed to be polyphenolic components, present at higher concentration in musts and red wines, which can quickly bind proteins (Gerland et al., 2006; Chinnici et al., 1997). There are also some other macro-components that can influence the lytic activity of lysozyme, exposing wines to potential lactic alterations and causing problems especially in the case of winemaking using low levels of sulphur dioxide addition (Sonni et al., 2009; Bellachioma et al., 2008; Amati et al., 1996).

The stability of lysozyme in the presence of some microbial substances used in foods, such as benzoic esthers, sodium nitrite, calcium propionate, potassium sorbate, and butylated hydroanisole (BHA), has been determined in phosphate buffered solutions, although these studies neglected some other important wine conditions, such as low pH (3.0-4.0) and high ethanol concentration (10-14%) (Cunningham et al., 1991; Yang et al., 1993). An interesting study of model solutions compared with white and red musts and wines was carried out by Amati et al. (1996) in order to understand the influence of some oenological operations on lysozyme activity. For the model solutions, this study showed that physical treatments, such as centrifugation, filtration at 0.45 µm and cooling at -5°C for 120 hours, did not cause any variation in enzymatic activity.

With regard to the effect of free SO_2 in the model solution, a concentration of 15 mg L^{-1} of free SO_2 decreased the enzyme activity mainly during the first hours of contact between the enzyme and SO_2, likely due to the reduction of disulphide bonds in the enzyme, consequently decreasing the protein's stability. A significant difference in residual lysozyme activity between SO_2 free white wine and white wine containing 80 mg L^{-1} of SO_2 was found, although in that case, the difference was ascribed to differences in the polyphenolic content relating to partial solubilisation of the suspended solids in the presence of SO_2 during fermentation.

1.4.3 Effect of fining agents and adjuvants on lysozyme activity

The addition of fining agents used in the winemaking industry (*eg*. fish, gelatine, silica sol, bentonite, active carbon polyvinylpolypyrrolidone, cellulose, fossil flour, pectolytic enzymes, yeast walls, hydrated potassium ferrocyanide) was also evaluated. Bentonite showed a relevant effect of decreasing enzymatic activity in model solutions, even at lower levels than those normally used in enology. According to other authors, bentonite exerts an adsorbing action on proteins (Saywell, 1935; Ribereau-Gayon et al., 1988). In musts and wines, high doses of bentonite nullified lysozyme activity, while low doses caused a decrease in the enzymatic activity, especially in musts and wines rich in polyphenols. The same trend of results was found in the case of active carbon addition, for which a marked decrease in enzyme activity was observed both in model solutions and in white must and wines.

For gelatine powder and potassium caseinate, the negative effect on enzyme activity in the model solution could not be confirmed in must and white wines, even at high dosages. This might be due to fast precipitation of both agents by polymerisation with other substances present in the matrix, without including lysozyme in the floccules formation.

The efficacy of lysozyme addition for both red and white wine production has been demonstrated in musts and wines under several conditions. Delfini et al. (2004) have highlighted higher precipitation for the protein added to wines instead of musts, while Amati et al. (1996) have shown a stronger enzymatic inhibition in red wine compared to white wine.

The comparison between wine with lysozyme added at the end of alcoholic fermentation, and must with lysozyme added after clarification highlighted that the latter addition can control the start of malolactic fermentation. Pitotti et al. (1991) obtained positive results by adding lysozyme at concentrations over 100 mg L^{-1} immediately after crushing and cold juice clarification, while Castino et al. (2001) prevented the malolactic activity in a fermenting Moscato juice contaminated with commercial malolactic bacteria using a concentration of 500 mg L^{-1}. Gerbeaux et al. (1999) and Gerland (2002) found lysozyme advantageous, at concentration of 250-500 mg L^{-1}, in musts with sluggish fermentation due to the growth by spoilage lactic acid bacteria. *Sangiovese* grape musts were early oxygenated and treated with lysozyme in order to obtain wines without SO_2 addition by Chinnici et al. (1996). The addition of 250 mg L^{-1} lysozyme could ensure the inhibition of malolactic fermentation for a period of 9 months and wine obtained showed a suitable high level of acidity.

Studies on musts and wines with different origins and compositions showed that the major decrease of lysozyme lytic activity occurred in a matrix rich in polyphenols, as a consequence of interactions between protein and tannins. Bartowsky et al. (2004) have studied the impact of lysozyme on the chemical and sensorial properties of commercially vinified red (Cabernet and Shiraz) and white (Riesling) wines. They found that lysozyme retained 75-80% activity in the white wine after six months; however no detectable activity remained in red wines after two days. Upon addition of lysozyme to both the red wines, a rapid decrease in colour density and phenolic content occurred in combination with the formation of a light precipitate, due to the formation of complexes between lysozyme and wine pigment phenolic compounds (Green et al., 1994).

Lysozyme addition on red grape must was evaluated by Chinnici et al. (1997) taking into consideration both phenolic compounds and colour density. In the SO_2 free and added samples, they found a relevant increase in the brown polymeric fraction amount which was detrimental for the purple red monomer fraction that usually represents the most important part of the wine colour. The hue of these wines was lower when compared to the SO_2 added wines, due to their high acidity.

Concerning white wine, the lysozyme added induced cloudiness/haze (heat instability), suggesting that white wines may require protein stabilization following treatment with lysozyme.

This phenomenon was also highlighted in further studies (Chinnici et al., 1996; Bartowsky et al., 2004).

1.4.4 Legal framework

In 1992, a F.A.O/W.H.O. committee, formed for the revision of food labelling, authorized the use of the lysozyme in food processing.

In 1997, the Organisation International de la Vigne et du Vin (OIV) authorized the use of lysozyme in musts and wines and this enzyme was added to the additives list that can be used during winemaking.

Because both the frequency and severity of food allergies has increased considerably in recent years, attention has been focused on certain food proteins, used as additives, which were hypothesized may act as hidden allergens. Since 31% of children and 8% of adults with food allergies result from an allergy to eggs, together with ovalbumin, ovomucoid and ovotransferrin, researchers have started to study potential lysozyme allergic effects. Some publications have shown that lysozyme was indeed allergenic, but some other studies highlighted an absence of allergic reaction to lysozyme in egg allergic subjects (Langeland, 1983; Anet et al., 1985; Frèmont et al., 1997; Iaconelli et al., 2008).

Because differences between the laws, regulations and administrative provisions of the member states of European Community on the labelling of foodstuffs has impeded the free circulation of these products and can lead to unequal conditions of competition, in 2000 the European Parliament regarded indication of the ingredients presents in foodstuffs to achieve a high level of health protection for consumers and to guarantee their information, and amended the Directive 2000/13/EC fixing a list of ingredients that had to compare on labels (Directive 2000/13/EC).

In 2003, the European Parliament added a list of allergenic substances (Annex *IIIa*) that must be included on labels, like eggs and products thereof (Directive 2003/89/EC).

Furthermore, in 2004, a Commission Regulation referred that any ingredient included in Annex *IIIa* must be indicated on the labelling of any beverages containing more than 1.2% by volume of alcohol, including wine (Commission Regulation No 1991/2204).

In 2005, the European Commission excluded certain ingredients or products of those ingredients from Annex *IIIa* (including egg-lysozyme used in wine as an additive and egg-albumin used for wine and beer clarification) and submitted them to the European Food Safety Authority (EFSA) for a scientific opinion. The Scientific Panel on Diet Products, Nutrition and Allergies of EFSA, taking into account the information provided with clinical studies, considered that wines fined with egg products might trigger adverse allergic reactions in susceptible individuals under the

conditions of use stated by the applicant. For this reason, some ingredients like lysozyme were permanently re-entered into Annex *IIIa* and must be indicated on wine labelling.

The efficacy of lysozyme addition in winemaking has been demonstrated in musts and wines under several conditions. However, a lack of further information concerning the mechanism and intensity of interactions occurring between lysozyme and the principal macro-components of musts and wines stimulated the following work.
A series of experiments were carried out on model wine solutions in order to simplify the interpretation of results.

2. EXPERIMENTAL

2.1 Wine model solutions

Wine model solutions were prepared with tartaric acid 3 g L^{-1} in water and adjusted with HCl to pH 3.2. To these were added six different must and wine macro-components, individually, as shown in Table 2. The same table also shows the amounts and some characteristics of the components used.

Components	Amount added	Characteristics
Sugars	200 g L^{-1}	Added Glucose and Fructose 1:1
SO_2	100 mg L^{-1}	Added in potassium salt form
Ethanol	12% v/v	
Apple pectins	2 g L^{-1}	Average degree of methylation: 70%
Tannins	100 mg L^{-1}	Excellent Gold White (Oliver Ogar)
Polyphenol extract	g L^{-1} of PFT (expressed as gallic acid equivalent)	Extracted from red wine according to Tomàs-Barberàn et al. (1992), after vacuum dealcoholation

Table 2. Amounts of must and wine components added to wine model solutions, and their characteristics.

To each prepared wine model solution was added 250 mg L^{-1} of lysozyme. The polyphenolic extract was obtained from *cv Sangiovese* grapes, fermented in our laboratories and extracted by the method of Tomás–Barberán (1992). A blank solution was prepared from the wine model

solution with addition of lysozyme, but lacking addition of any other components. After 2, 24 and 48 hours, the amount and the activity of the residual enzyme were calculated, using two procedures that will be explained in the following paragraphs. The resulting data show the percentage of each trial with respect to the blank, evaluated after the same number of hours.

2.2 Chemicals and Standards

Tartaric acid, glucose, fructose and pectines were purchased from Aldrich (Milan, Italy). Sulphur dioxide was used as potassium salt (Carlo Erba, Milan, Italy), liquid gallic tannin (Excellent Gold White) was purchased from Oliver Ogar Italia (Verona, Italy) and ethanol from Merk (Darmstadt, Germany). Reagents and solvents of analytical or HPLC grade were purchased from Merck (Darmstadt, Germany). HPLC-quality water was purified in a Simplicity system (Millipore, Bedford, MA). Pure lysozyme was provided by Fordras S.A. (Lugano, Switzerland). Stock solutions of lysozyme were prepared, at various concentrations, in a model matrix (12% ethanol, 3 g L^{-1} tartaric acid, adjusted to pH 3.2 with potassium tartrate), and stirred for 15 hours to permit complete dissolution of the powder. For the turbidimetric method of analysis of lysozyme biological activity, *Micrococcus lysodeikticus* was purchased from Aldrich (Milan, Italy). Phosphate buffer (0.15 M, pH 6.6) with disodium phosphate dodecahydrate and monosodium phosphate monohydrate was similarly purchased from Aldrich (Milan, Italy).

2.3 Quantification of the residual protein

The quantity of lysozyme present in the medium after reaction with the tested macro-components was conducted following the HPLC-FLD method developed by Riponi et al. 2007, using a Jasco HPLC apparatus (Tokyo, Japan) equipped with a binary pump (PU 1580), a 20 µL loop, a Rheodyne valve (Cotati, CA, USA), a photodiode detector (PU MD 910; Tokyo, Japan), a fluorimetric detector (FP 2020; Tokyo, Japan) and a column oven (Hengoed Mid Glamorgan, UK). The column was a Tosoh Bioscience (Stuttgart, Germany) TSK gel Phenyl 5PW RP (7.5 cm x 4.6 mm i.d.), protected with a guard column filled with the same resin. All runs were acquired and processed using Borwin 5.0 software (JMBS Developments, Grenoble, France). UV detection was performed at 280 and 225 nm. The fluorometric detector was set at λ_{ex} 276 nm and λ_{em} 345 nm (gain 10, spectrum bandwidth 18 nm). The elution solvents were 1% acetonitrile (CH_3CN), 0.2% trifluoroacetic acid (TFA), 98.8% H_2O (solvent A), and 70% CH_3CN, 0.2% TFA, 29.8% H_2O (solvent B). Gradient elution was as follows: 100% A for 3 min, then to 65.0% A in 7 min, maintained for 5 min, then to 40.5% A in 12 min, then to 0% A in 2 min, maintained 5 min, then to 100% A in 2 min, followed by 10 min of re-equilibration to the initial conditions.

The column operating conditions were at 30°C and with a flow of 1 mL min^{-1}. Identification of lysozyme in actual samples was carried out by comparing its retention time and UV-spectra to those of standard solutions.

The samples were acidified with HCl (10 M), diluted 1 in 10, then filtered using a nylon filter with 0.22 µm diameter pores, 5 minutes after the addition. The chromatographic analysis was carried out immediately after filtering. Quantification was performed using an external standard; peak areas of standard lysozyme solutions at five different concentrations within the chosen range were determined, in triplicate.

2.4 Quantification of the enzyme activity

For the determination of lysozyme bioactivity, a validated turbidimetric method authorised by the Organisation International de la Vigne et du Vin was used (Resolution Oeno 15/2001, OIV, Paris). It is based on measuring the degree of lysis induced by the enzyme on cells of bacteria such as *Micrococcus lysodeykticus* by means of turbidimetry. This technique provides an estimate of the decrease in turbidity of a cell wall suspension via spectrophotometrical measurement and can be easily used to quantify the enzyme concentration in a sample by comparing the calculated activity to authentic lysozyme (as mg L^{-1}) from a standard calibration curve, previously prepared with a lysozyme standard solution. The standard solution was prepared dissolving 50 mg of lysozyme chloride in water and diluting to 100 mL in a graduated flask. 5 mL of this solution was further diluted with water up to 50 mL, while 2 mL of the same solution was diluted with a 0.15 M phosphate buffer up to 100 mL to obtain a 1 mg L^{-1} lysozyme (standard solution). The phosphate buffer (0.15 M, pH 6.6) was prepared with disodium phosphate dodecahydrate 18.9 g L^{-1} and monosodium phosphate monohydrate 13.41 g L^{-1}.

For the samples, two test tubes with 5 mL of buffer as a suspension control sample for *Micrococcus luteus* were prepared (the first control sample was used in the beginning and the second one at the end of the trial). After exactly 30 seconds, 5 mL of *Micrococcus luteus* suspension were added, mixing manually to avoid spilling. The tube were subsequently mixed with a Vortex and kept at 37°C (± 5°C) in a water bath for exactly 12 minutes. The final quantity of lysozyme in the tubes was 0.2 – 0.28 – 0.4 mg L^{-1}. After incubation, the tubes were removed in the same order they were placed, with an interval of 30 seconds. Each sample was mixed and the absorbance was read against the control buffer. The apparatus used for the spectrophotometric measurements was a Uvidec 610 spectrophotometer (Jasco, Tokyo, Japan) and the measurements were performed at 540 nm. The value obtained depends not only on the

protein quantity present in the medium, but also on the biological activity of the enzyme and can change as a function of the inactivated level of the enzyme.

3. RESULTS AND DISCUSSION

Table 3 shows the percentage of residual protein and enzyme bioactivity on model solutions with the macro-components of musts and wines added. Almost all of the macro-components studied seemed to interact with lysozyme. Only glucose and fructose, both added at the average concentration usually found in musts, appeared to not decrease the bioactivity and the amount of residual protein.

		Bioactivity (%)	Residual protein (%)
Glucose/Fructose	2 h	82.30	97.00
	24 h	91.97	98.30
	48 h	96.81	95.79
Ethanol	2 h	108.70	82.12
	24 h	93.20	81.89
	48 h	79.09	81.25
SO_2	2 h	57.81	85.60
	24 h	19.55	67.24
	48 h	0.00	65.23
Gallic tannins	2 h	79.87	88.20
	24 h	75.22	94.13
	48 h	62.49	88.15
Pectins	2 h	91.57	87.04
	24 h	85.47	94.51
	48 h	58.14	75.63
Poliphenolic extract	2 h	0.00	2.13
	24 h	0.00	3.97
	48 h	0.00	1.72

Table 3. Percentage of residual protein and enzyme bioactivity on model solutions as a function of the micro-components of musts and wines added.

3.1 Ethanol

During the alcoholic fermentation, the progressive accumulation of alcohol can cause a reduction in enzyme activity. Table 3 shows that after 48 hours from the addition of lysozyme to a model solution with 12% (v/v) ethanol, the residual enzyme activity has a 20% reduction.

Concerning the effect of increment of lytic activity after 2 hours from the addition, a previous study, using 9.5% (v/v) ethanol for 200 minutes, highlighted that at this value of ethanol concentration, there is probably a slightly non-specific interaction between ethanol and the three-dimensional structure of the protein that could promote enzyme-substrate interactions. (Brecher et al., 1995; Millar et al., 1982).

By contrast, higher concentrations of alcohol and longer contact periods change the structure of the protein causing a reduction in enzyme activity. This has been shown also for other enzymatic products used in winemaking (Zinnai et al., 2007).

For the residual protein, the amount of lysozyme in solution decreases very quickly during the first 2 hours (20%) due to its insolubilisation in the medium.

3.2 Sulphur dioxide

Sulphur dioxide is commonly used as a preservative in winemaking because of its technological functions (e.g. as an antioxidant, extractive solvent, antimicrobial agent). In terms of its antimicrobial function, SO_2 inhibits must endogenous oxidases and stops the onset of undesirable fermentations (such as acetic or malolactic fermentation). Upon interaction with lysozyme, a reduction in activity was found at concentrations of free SO_2 exceeding 10 $mg\ L^{-1}$ (Amati et al., 1994), probably due to interaction with the disulphide bonds of the enzyme molecule (Cecil et al., 1962). Nevertheless, strong denaturating conditions and high SO_2 concentrations are needed to cleave the disulphide bonds of lysozyme (Cecil et al., 1962). Tirelli et al. (2007) showed that SO_2 can interact with one of the four disulphide bonds of lysozyme resulting in the formation of a mono-thiosulphonate linkage which subsequently decreases the amount of the active residual protein. This reaction was favoured by increasing pH values and sulphur dioxide concentrations.

The data in Table 3, in agreement with the results obtained from Amati et al. (1996), show that 100 $mg\ L^{-1}$ of free SO_2 can strongly reduce the lytic activity of lysozyme in the first hours of addition. The enzyme activity inhibition increases over time and after 48 hours the decay of the lysozyme muramidase activity is complete.

The strong decrease in percentage of enzyme activity does not correspond to a strong decrease in the percentage content of lysozyme as residual protein in the medium. After 48 hours, the contact between lysozyme and SO_2 reduced the residual protein to 65% of the initial amount.

The results obtained highlight that, under these conditions, the residual protein in the medium, even if quantifiable with chromatographic analysis, is not able to carry out an antibacterial function, probably due to a change in the structure as a result of the presence of HSO_3^-. Therefore, to monitor both the residual protein together and the enzyme activity appear fundamental in order to understand the mechanism of interactions and develop oenological protocols in which lysozyme is associated with the use of SO_2.

3.3 Tannins

Tannins can be described as phenolic compounds (molecular weights between 500 and 3000 $g \cdot mol^{-1}$) that are usually classified in two groups, hydrolysable tannins and condensed tannins according to their structural type. Hydrolysable tannins (or gallotannins and ellagitannins) consist of a carbohydrate core, the hydroxyl groups of which are esterified by gallic acid or one of its derivatives (digallic, trigallic and ellagic acids). Condensed tannins (or proanthocyanidins) are formed by the condensation of hydroxyflavans which can release anthocyianidins by acid hydrolysis (Vivas, 2001).

Tannins can combine with proteins to form soluble complexes, which can grow to colloidal size, at which point they scatter light, and larger still, which can lead to sediment formation. Complexation can take place mediated by hydrogen bonds between tannins and peptide links, and also via hydrophobic interactions and/or polar interactions (Spencer et al. 1988, Siebert et al. 1996, Serafini et al., 1997). For this reason, in oenology, tannins are traditionally used to facilitate the clarification of wines and musts (protein stabilisation).

In recent years, a better understanding about the physical-chemical properties of tannins has permitted their successful addition at different steps of the winemaking process, not only for their protein stabilisation function.

In addition to their bitter taste perception, their effect of varying redox potential under low oxygen conditions and their capacity to form complexes with metal cations (chelates), tannins can link with sulphur compounds, such as thiols, promoting their exclusion from the medium, particularly in the case of malodorous sulphur compounds. In particular, this latter property which occurs only under oxidative conditions can be favourably used to limit the accumulation of thiols in wines during the ageing process in large barrels (Vivas, 2001; Bosso et al., 2001).

A study conducted on red wines with added ellagitannins and catechin showed ellagitannin is an impressive oxidation regulator, quickly absorbing the dissolved oxygen and facilitating the hydroperoxidation of wine constituents. This reaction induced tannin/anthocyanin condensation via acetaldehyde, favouring stabilisation and deepening the crimson colour; while the limited

oxidation of wine phenolic compounds prevented the development of brick-yellow colour. In addition, the structure of the wine tannins was modified, reducing their astringency due to more advanced polymerisation (Vivas et al., 1996).

An Italian study conducted on wines with added ellagitannins and gallotannins compared to wine without any additive found that tannins limited oxidation reactions in wines and promoted their slower evolution over time. Wine with added gallotannins, particularly, appeared less oxidised than the same wine without additives (Bosso et al., 2001).

Gallic tannins are hydrolysable tannins consisting of oligomers of gallic and digallic acids, and can be a valid additive to prevent oxidative phenomena during winemaking. Rawel et al.(2001) have found that these chemical compounds can link via covalent bonds to free amine functions, reducing protein activity and solubility. As already determined in our research group, the pre-fermentative addition of oenological tannins can effectively inhibit the oxidative phenomena of musts and wines made with low amounts of SO_2 and increase the concentrations of some ethyl-esters, probably as a result of a dual mechanism involving enzyme inhibition and radical scavenging activity (Bellachioma et al. 2008; Sonni et al., 2009).

Table 3 shows a progressive decrease in the enzymatic activity during the 48 hours of contact between lysozyme and tannin. This result suggests that the use of tannins in winemaking associated with lysozyme must be rationalised, to avoid an excessive decrease in the enzymatic activity of lysozyme.

Therefore, it could be useful to add lysozyme only 12-24 hours after the addition of gallic tannins, to permit the tannins to activate their antiradical and antioxidant function, as well as to inactivate the oxidases. In this trial, the trend of the residual protein (around 88% of the initial amount) do not decrease as fast as the reduction of the lytic activity of the lysozyme in the medium. The same behaviour was previously highlighted in the ethanol and SO_2 trials.

3.4 Pectins

The cell wall of a grape cell is made of hemicellulose, pectins and structural proteins interwoven within a network of cellulose microfibrils. They are a source of wine pectic polysaccharides, which may create turbidity during wine processing and hinder filtration. The cell wall pectins composition changes as a function of the cultivar, the ripening and healthy stage of grapes (Ortega-Regules et al., 2008). At the beginning of winemaking, in which the degree of methylation is higher than 70%, the amount of this glucosidic polymer can range between 1 and 2 g L^{-1}. In the presence of low pH or cations in the medium, pectin is organised into a colloidal structure composed of a double helix (hydrogel) that can retain water and demonstrate

mechanical resistance (Thom et al., 1982). In our trial, 2 g L^{-1} of apple pectins were added to the model wine solution and the development of a precipitate phase was observed.

An increase in the precipitate phase over time was associated to a decrease in the enzymatic activity and the quantity of the residual protein in the medium (40% and 25% decrease in 48 hours respectively). After 48 hours, the residual enzyme activity for evaluating the interaction between lysozyme and pectins was analysed. For this purpose, the wine model solution after addition of pectin was centrifuged to separate the supernatant from the precipitated phase, then the latter was diluted with the wine model solution 1:1 (v/v).

A strong lytic activity of the precipitated phase is shown in Fig.3, which is due to its self association and the majority of the concentration of the enzyme that was previously in solution. Therefore, the precipitate phase seems to have preserved the initial enzymatic characteristics.

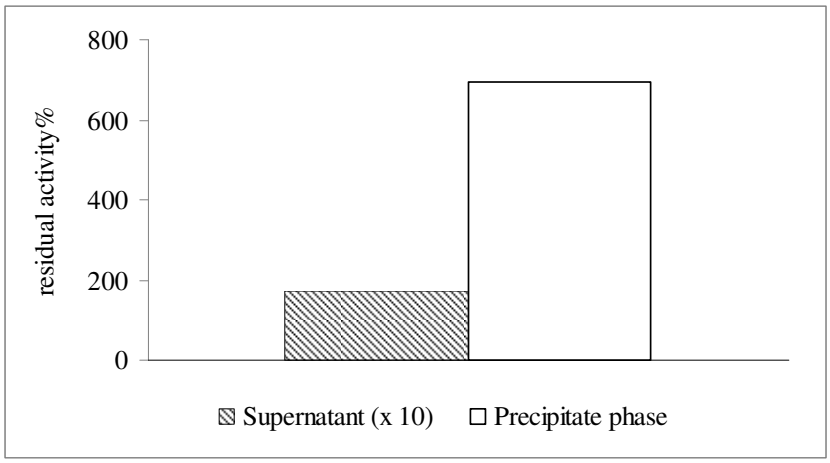

Figure 3. Lysozyme interaction with pectins: percentage of lysozyme residual activity in the supernatant and in precipitate phase (the supernatant data are multiply by 10, to enhance clarity).

These results could be explained with a trapping phenomenon by the colloidal polysaccharide structure of the pectins on the lysozyme, which does not inactivate the antimicrobial ability of the enzyme.

With regard to real production conditions, the data obtained suggest that the movement of the juice during winemaking can increase the lytic activity of lysozyme present in the medium. In addition, the use of pectolitic enzymes could significantly contribute to improve the lysozyme activity by hydrolysing the pectic colloidal structure.

The supernatant showed 17.4% of residual enzyme activity, confirming that processes that promote precipitation phenomena and polysaccharides deposits can reduce the presence of lysozyme in musts and wine.

3.5 Polyphenolic extract

The polyphenol extract was obtained following the Tomàs-Barberan et al. (1992) method. After addition of polyphenolic compounds the model solution showed these values:

a) Total Polyphenols Amount: 1.50 g L^{-1} (expressed as gallic acid equivalent); b) Total Anthocyans Amount: 260 mg L^{-1} (expressed as malvidine equivalent) c) Hydroxycinnamic acids: 165 mg L^{-1} (expressed as caffeic acid equivalent).

Many authors highlighted a strong interaction of lysozyme with the phenolic component of musts and wines. The primary factor that has been observed to inactivate the bacteriolytic action of lysozyme in wines is believed to be the polyphenolic components, which can quickly bind proteins (Chinnici et al., 1996; Chinnici et al., 1997). The different concentration levels of phenolic compounds that occur in must before settling, at the beginning of the alcoholic fermentation or at the end, can influence the solubilisation level of lysozyme. Delfini at al. (2004) highlighted higher precipitation amounts of protein added to musts after clarification compared to wines at the end of alcoholic fermentation, while Amati et al. (1996) showed a stronger enzymatic inhibition on red wine compared to white wines.

The interaction mechanisms between monomeric phenolic components and lysozyme were studied by Rawel et al. (2001), who suggested that, in the presence of oxygen, the phenolic compounds are subjected to enzymatic oxidation involving two reaction steps: the first step consisting of the hydroxylation of monophenols into *o*-diphenols and the oxidation of the *o*-diphenols into *o*-quinones. The quinones represent a reactive electrophilic intermediate that can be attacked by nucleophiles such as lysine, methionine, cysteine and tryptophan residues in a protein chain, such as lysozyme. The lysozyme derivatives appear less soluble, more hydrophobic and with a lower lytic activity. The major interaction between lysozyme and *o*-quinones seems to be with quinones generated from oxidation of *o*-diphenols (for example caffeic acid) and gallic acid, while ferulic acid and coumaric acid react to a lesser extent.

Concerning flavonoids (catechins, anthocyans and flavonols), another important group of wine polyphenolic substrates, some studies revealed that for several phenolic compounds the phenomena of binding/quenching to proteins is affected by the structure of the polyphenols. The binding affinity and consequently the precipitation percentage increased with the molecular weight of the polyphenol compound, and in the presence of galloyl groups (Soares et al, 2007).

Moreover, in this case the interactions between flavonoids and lysozyme, Van der Waals forces or hydrogen bonds, are weaker than the interactions between quinones and lysozyme (Ghosh et al., 2008).

Table 3 shows a strong interaction between lysozyme and polyphenols already 2 hours of after addition. The enzymatic activity is completely inhibited at the beginning of the contact time until the end of the experiment. The same trend is found for the residual protein percentage in solution, which decreases quickly after 2 hours of contact, confirming that interactions between lysozyme and polyphenols cause a precipitation of the enzyme from the medium.

In this experiment, the lysozyme-polyphenols interaction was stronger than the one found in red/white musts and wines experiments reported from our research group in the past, in which the precipitation percentage was between 20 and 50% (Amati et al., 1996a; Amati et al., 1996b). Moreover, in the past studies, the free lysozyme decreased in the medium very gradually (until 50-60%), permitting its enzymatic activity during fermentation and also during the following stabilisation and storage stage.

The contradictory results obtained in this work, could be due to a high percentage of tannin present in the medium or to a polyphenol extraction procedure that could have generated a large amount of oxidised species with a higher interaction level with lysozyme.

The precipitate fraction analysis, obtained in the same way as for the pectins trial, showed that the interaction between polyphenols and lysozyme was very strong and strongly inhibited the protein's enzymatic activity.

4. CONCLUSIONS

The data obtained showed that glucose and fructose appear to not significantly affect the bioactivity and the amount of residual protein, whereas for ethanol, tannins, pectins, SO_2 and polyphenols, the results highlighted an increased interaction.

The progressive decrease in the enzymatic activity during the 48 hours of contact between lysozyme and tannins, due to their capacity for combination to lead to sediment formation, suggests that the use of tannins in winemaking associated with lysozyme must be rationalised, to avoid an excessive decrease in enzymatic activity of lysozyme. Therefore, it could be useful to add lysozyme only 12-24 hours after the addition of gallic tannins, to permit the tannins to activate their antiradical and antioxidant function, as well as to inactivate the oxidases.

Concerning the SO_2, the results obtained highlight that a strong decrease in the lytic activity of lysozyme in the first hours of addition doesn't correspond to a strong decrease in the percentage

content of lysozyme as residual protein in the medium, suggesting that, under these conditions, the residual protein in the medium is not able to carry out an antibacterial function, probably due to a change in the structure as a result of the presence of HSO_3^-.

By contrast, the interaction between polyphenols and lysozyme was found to be very strong and completely inhibit the protein's enzymatic activity at the beginning of contact until the end of the experiment together with the residual protein percentage in solution, which decreased quickly already after 2 hours of contact. These results confirm that interaction between lysozyme and polyphenols cause a precipitation of the enzyme from the medium.

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Chapter 2

Replacement of sulphur dioxide by lysozyme and oenological tannins during fermentation: influence on volatile composition of white wines

***ABSTRACT**: In recent years the use of sulphur dioxide, a commonly used additive in winemaking, has been questioned because of its toxic effects on human health. Studies have been conducted to find alternative auxiliaries which can effectively substitute this additive in all its several technological functions. In previous works, lysozyme and enological tannins were found as possible substitutes in controlling undesirable bacterial fermentations and phenolic oxidation. However, data on volatile composition of wines obtained by those protocols are lacking. In this work, the effects on volatile composition of white wines with the substitution of SO_2 during fermentation by means of lysozyme and tannin were studied. At the same time, the technological performance of two low SO_2 producing selected strains of yeasts were evaluated.*

The results showed that both SO_2 and lysozyme prevented the development of undesirable bacterial fermentations. The study of volatile compounds show differences in contents of alcohols, acids and esters among final products: wines fermented with strain 1042 and lysozyme had higher total alcohols concentration, while SO_2 addition promoted a higher production of 3-methyl-1-butanol, 3-methyltio-1-propanol, phenylethyl alcohol and 4-hydroxy-benzenethanol. Esters, as a total, were influenced by the different strain and tannins added, while medium-chain fatty acids ethyl esters amounts and their correspondent fatty acids were found at higher amounts in wines coming from fermentations with lysozyme. The sensory analysis revealed a preference for wines with added lysozyme and tannins. The data suggest that the addition of lysozyme and enological tannins during alcoholic fermentation could represent a promising alternative to the use of sulphur dioxide and for the production of wines with reduced content of SO_2. Volatile composition of the final wines was affected by the different vinification protocols (mainly concerning alcohols and ethyl esters).

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1. INTRODUCTION

During the last few years, a number of researchers aimed to identify innovative technologies for assuring safer and healthy food. In oenology, one major concern was the use of sulphur dioxide during the technological process. Sulphur dioxide is commonly used as a preservative in winemaking because of its several technological functions. In fact, it acts as an antioxidant to protect wine phenols from oxidation. Furthermore, SO_2 inhibits must endogenous oxidases and contrasts the onset of undesirable fermentations (such as acetic or malolactic fermentation)

(Ribéreau-Gayon et al., 2007). However, the general trend toward reduced use of SO_2 in wine processing, because it can elicit an allergic response in sensitive people and has potential toxic effects on human health over certain ingestion doses, suggests the necessity to develop oenological protocols in which alternative additives can be used to substitute sulphites in the mentioned functions (Taylor et al., 1986; Romano et al., 1993).

Since this compound is found in many foods as an additive and the amount taken is accumulative in the organism, the Organisation International de la Vigne et du Vin (OIV) have set down maximum limits for winemaking.

Several studies have been undertaken to develop oenological protocols in which alternative auxiliaries substitute sulphites in the above mentioned functions, sometimes with the declared final goal to produce sulphite-free wines. In particular, since the early 1990s, the use of egg white lysozyme has been proposed to control malolactic fermentation in winemaking (Amati et al., 1994; Amati et al., 1996; Gerbaux et al., 1997), supporting or even replacing sulphur dioxide (Chinnici et al., 1996). Lysozyme, E.C. 3.2.1.17, an enzyme present in the hen egg white, has a lytic activity on cell wall of Gram-positive bacteria, and has been used successfully as antimicrobial agent in the food industry, particularly in cheesemaking (Cunningham et al., 1991; Proctor et al., 1988; Ghitti et al., 1983). Its lytic activity is based on the hydrolysis of the 1-4 linkage between *N*-acetylmuramic acid and *N*-acetylglucoseamine, which constitute the peptoglycan layer of the bacteria cell wall. Its efficacy for both red and white wine production was demonstrated on musts and wines under several conditions (Amati et al., 1996; Delfini et al., 2004).

The antimicrobial activity of lysozyme toward lactic bacteria was reviewed by Cunningham et al. (1991) and has been since shown to depend on both the cell physiological state and the lysozyme structure in the medium (H+ concentration, reacting compounds) (Ibrahim et al., 1996a; Ibrahim et al., 1996b). Bacterial sensitivity to lysozyme depends on the peptidoglycan structure in the cell wall and the lysozyme efficacy toward Gram-negative bacteria (i.e. acetic bacteria) has been shown to be much less compared to the Gram-positive bacteria and more bacteriostatic than bactericidal, presumably because the outer membrane acts as a barrier (Cunningham et al., 1991; Hughey et al., 1987).

A number of studies have reported possible interactions between lysozyme and some components of musts and wines. The primary factor that has been observed to affect the bacteriolytic action of lysozyme in wines is believed to be polyphenolic components, present at higher concentration in musts and red wines, which can quickly bind proteins (Gerland et al., 2006; Chinnici et al., 1997). There are also some other macro-components that can influence the

lytic activity of lysozyme, like ethanol, pectines, SO_2 and tannins exposing wines to potential lactic alterations and causing problems especially in the case of winemaking using low levels of sulphur dioxide addition (Bellachioma et al., 2008; Amati et al., 1996). Oenological tannins, in particular, represent an interesting group of adjuvants that could effectively prevent the oxidative phenomena of musts and wines, probably as a consequence of their radical scavenging activity, when added in pre-fermentation (Bellachioma et al., 2008).

Tannins are phenolic compounds usually classified in two groups, hydrolysable tannins and condensed tannins according to their structural type. Hydrolysable tannins (or gallotannins and ellagitannins) consist of a carbohydrate core, the hydroxyl groups of which are esterified by gallic acid or one of its derivatives (digallic, trigallic and ellagic acids). Condensed tannins (or proanthocyanidins) are formed by the condensation of hydroxyflavans which can release anthocyianidins by acid hydrolisis (Vivas, 2001).

Industry uses various plant materials (leaves, fruit, galls, bark and wood) to produce numerous kind of commercial tannin extracts (Tang et al.,1992). These products are largely used in leather tannins, in metallurgy, for modifying the taste of foodstuffs, in the preparation of adhesives, as a source of pharmacological drugs and in the treatment and ageing of wine and spirits (Vivas et al., 1996). Industrial oenological tannins are hydrolizable tannins extracted from some parts (wood, fruits, pathogenic protuberances) of several vegetal species, like chestnut trees (*Castanea sativa*), oak (*Quercus robur* and *petraea*), dry fruits (*Terminalia chebula, Caesalpina spinosa*), grape seeds (*Vitis vinifera*), ipertrophic excrescences due patogens attacks, exotic wood (Vivas, 2001; Res. Oenol., 12/2002).

Figure 1 shows some of the most important and used oenological tannins, with information on the plant origin (species and tissue) and extraction solvent used.

In 2002, the Organisation International de la Vigne et du Vin (OIV) established the analytical methods with which the botanical origins of tannins can be recognized and in 2008 amended and supplemented the resolution published with a new one: in fact, it is possible to characterise the botanical origin of tannins with the aid of criteria like ultraviolet absorption spectrum, flavanol content, proanthocyanidines, digallic acid and scopoletine (Res. Oenol., 12/2002; Res. Oenol., 6/2008).

Tannins can combine with proteins to form soluble complexes, which can grow to colloidal size, at which point they scatter light, and larger still, which can lead to sediment formation. (Spencer et al. 1988, Siebert et al. 1996, Serafini et al., 1997). For this reason, in oenology, tannins are traditionally used to facilitate the clarification of wines and musts (protein stabilisation).

In recent years, a better understanding about physical-chemical properties of tannins has permitted their successful addition at different steps of the winemaking process, not only for their protein stabilisation function. An Italian study conducted on wines with added ellagitannins and gallotannins compared to wine without any additive found that tannins limited oxidation reactions in wines and promoted their slower evolution over time. Wine with added gallotannins, particularly, appeared less oxidised than the same wine without additives (Bosso et al., 2001).

Family	Species	Common name of commercial tannin extract	Tissue	Tannin contents (%)	Tannins nature
Anacardiaceae	Rhus semialata	Chinese tannins	Galls	80	gallotannins
Combretaceae	Terminalia chebula	Myrabolans	Fruits	40	gallo/ellagitannins
Fagaceae	Castanea sativa	Sweet chestnut tannins	Wood	10	ellagitannins
	Quercus robur	Peduncolata oak tannins	Wood	10	ellagitannins
	Quercus petraea	Sessile oak tannins	Wood	10	ellagitannins
	Quercus infectoria	Turkish tannins	Galls	45	gallotannins
Acaceae	Robinia speudoaccacia	Officinal tannins	Galls	40	gallotannins
Leguminosae	Caesalpina spinosa	Tara	Fruits, pods	40	gallotannins
Vitaceae	Vitis Vinifera	Grapes tannins	Fruits, seeds	15	procyanidines

Figure 1. Sources of commercial tannin extracts (Tang et al., 1992; Vivas, 2001).

When used on white musts, lysozyme efficiently controls the malolactic spoilage, hence suggesting the possibility to carry out an alcoholic fermentation without the use of sulphur dioxide. However, information on volatile composition of SO_2 free wines obtained by using lysozyme is scarce. The aim of this research was, hence, to study the effects on wine volatile composition after pre-fermentative addition of lysozyme and/or oenological tannin to SO_2 free musts, and to compare the volatile profiles of such wines with respect to "conventional" sulphites added wines.

2. EXPERIMENTAL

2.1 Fermentations

Forty litres of blended fresh must (50% cv *Trebbiano* and 50% cv *Sauvignon Blanc*) were fermented in two litre laboratory glass fermentors that were saturated with N_2 before the filling. A glass trap (filled with 4 N H_2SO_4) prevented microbial contamination and oxygen entrance. Two low SO_2 producing selected strains of *Saccharomyces cerevisiae* (strains 333 and 1042 from University of Bologna - ESAVE collection), were used to carry out fermentations and were

inoculated at an initial cell concentration of 1.5 x 10^6 CFU ml^{-1}. Four trials for each strain were defined with the aim to study the effect of the following variables: 1) strain, 2) lysozyme/SO_2, 3) tannin (Table 1).

Factor \ Trials	*Strain 333*				*Strain 1042*			
	A1	*B1*	*C1*	*D1*	*A2*	*B2*	*C2*	*D2*
Lysozyme (g L^{-1})	0.25	0.25	-	-	0.25	0.25	-	-
$K_2S_2O_5$ (mg L^{-1})	-	-	160	160	-	-	160	160
Tannin (g L^{-1})	-	0.1	-	0.1	-	0.1	-	0.1

Table 1. Scheme of fermentation trials

Fermentations were performed in triplicate. Must were stirred daily to ensure a homogenous fermentation. Fermentations were monitored by daily weighting the fermentors and the samples were taken at the end of fermentations, when the loss of weight stopped.

2.2 Oenological parameters

Determination of density, total and volatile acidity, dry extract, total SO_2 were made according to OIV (1990). The pH was determined by using a pH-meter (Mettler Toledo, Spain). The alcoholic strenght of wines was determined by using an oenochemical distilling unit (Gibertini, Italy). The total polyphenol index (PFT) was determined by a direct lecture (after filtration at 0.45nm with PTFE filters) at 280 nm using an Uvidec 610 spectrophotometer (Jasco, Japan) and results were expressed as mg L^{-1} of gallic acid equivalent. All the analysis were made in duplicate.

2.3 Chemicals and Standards

Dichloromethane (Suprasolv) was purchased from Merck KGaA (Darmstadt, Germany). Standard compounds were supplied by Aldrich (Milano, Italy), Sigma Chemicals (St. Louis, Missouri, USA), Fluka Chimie AG (Buchs, Switzerland). Hydromatrix resin was from Varian Inc. (Palo Alto, California, USA). Water was of HPLC grade. Lysozyme Chloride was furnished by Fordras S.A. (Lugano, Switzerland), while liquid gallic tannin (Excellent Gold White) was purchased from Oliver Ogar Italia (Verona, Italy). Sulphur dioxide has been used as potassium salt (Carlo Erba, Italy).

2.4 HPLC Analysis

Organic acids and lysozyme quantifications were conducted following the procedure described by Castellari et al. (2000) and Riponi et al. (2007), respectively. The HPLC used was a Jasco apparatus (Tokyo, Japan) equipped with a binary pump (PU 1580), a 20 µl loop, a Rheodyne valve (Cotati, CA), a photodiode detector (PU MD 910), a fluorimetric detector (FP 2020), and a column oven. The column was a Bio-Rad Aminex HPX 87H (300 mm x 7,8 mm) for the analysis of organic acids and a Tosoh Bioscience (Stuttgart, Germany) TSK gel Phenyl 5PW RP (7.5 cm x 4.6 mm i.d.), protected with a guard column filled with the same resin, for lysozyme.

2.5 GC Analysis

Compounds with high volatility and high concentration (acetaldehyde, ethylacetate, *n*-propanol, *i*-butanol, isoamyl alcohol) were analyzed according to the method outlined by A.O.A.C. (2000). A gas-chromatograph 8000 series (Fisons) equipped with a flame ionisation detector and a packed column 23% Carbowax 1500 (w/w) on Chromosorb W (60-80 mesh) were used. The working condition were: GC grade nitrogen as carrier gas at flow rate (constant flow) of 3.0 mL min^{-1}, column temperature of 70 °C (isothermal), detector and inlet temperature was 150 °C.

2.6 GC-MS Analysis

For the analysis of all the other volatiles, the procedure of sample preparation proposed by Gerbi et al. (1992) was used. The analysis of the extracts was carried out in a GC-MS Thermo Finnigan Trace GC ultra gas chromatograph (San Jose, CA), equipped with a Thermo Finnigan Trace DSQ mass selective detector and a fused silica capillary column Stabilwax (Restek, Bellefonte, PA; 30 m, 0.25 mm i.d., and 0.25 µm film thickness), under the following working conditions: GC grade helium as carrier gas at a flow rate (constant flow) of 1.0 mL min^{-1}; column temperature program, 40 °C heated at 3 °C min^{-1} to 100 °C and then heated at 5 °C min^{-1} to 240 °C (held for 10 min). The injection temperature was 250 °C. Samples (1 µl) were injected in the splitless mode. Detection was carried out by positive ion electron impact (EI) mass spectrometry in the full scan mode, using an ionization energy of 70 eV and a transfer line temperature of 280 °C. The mass acquisition range was *m/z* 30-400 and the scanning rate 1 scan s^{-1}. Chromatographic peaks were identified by comparing their mass spectra with those of standards and/or those reported in the literature and in commercial libraries NIST 2.0 and Wiley 7. Quantification was carried out from total ion current peak areas according to the internal standard method (100 µL of a 514 mg L^{-1} solution of 2-octanol were added to 20 mL of each sample); the response factor of standard volatile compounds to the internal standard was

experimentally obtained and applied to correct the peak area of each analyte. For compounds lacking reference standards, the response factors of standards with similar chemical structures were used.

2.7 Sensorial Analysis

White wines at the end of fermentation were tasted by a group of 10 assessors ranging from 22 to 35 years of age with previous experience in sensory analysis. They were recruited from the staff of University of Bologna.

Assessors were training in Descriptive Sensory Analysis using fresh wines during three sessions. Then judges generated sensory terms individually. Over the course of these three sessions, 6 attributes (citrus, floral, fruity, tropical fruit, banana notes and astringency) were selected by consensus in order to describe the aroma of fresh wines samples. Another four formal sessions were employed to evaluate the intensity of the 6 attributes selected using an unstructured 10 cm straight line. For each trial, all the replicates were tested.

2.8 Statistical Analysis

For each final wine, significant differences in mean concentrations of volatile compounds were tested by means of ANOVA analysis followed by a Post Hoc comparison (Tuckey's test at $p>0.01$). To evaluate the influence of each tested factor (yeast strain, lysozyme/SO_2, tannins) on volatiles produced during fermentations, the data were subjected to multiple regression analysis after a graphical exploration to exclude outliers. Furthermore a Principal Component Analysis (PCA) with a Varimax rotation of data was carried out with the aim to highlight the main contributors to the variance among samples. All the analyses were conducted using "Statistica 6" package (StatSoft Italia Srl, Italy).

3. RESULTS AND DISCUSSION

3.1 General parameters of final wines

Table 2 show the oenological parameters of the wines obtained from the different fermentations: all wines had similar pH, density, DO 420 nm, total polyphenol index, alcoholic strength and dry extract. Total SO_2 in samples without sulphites addition (0.4-1.3 mg L^{-1}), confirmed that both the strains were low SO_2 producers.

It has been shown that the pre-fermentative use of sulphur dioxide can result in an accumulation of acetaldehyde in the final wines (Romano et al., 1993). In our SO_2 added wines, acetaldehyde

amounts were from 3 to 5 times higher if compared to samples obtained without SO_2 addition and this fact could well contribute to the sensory attributes of the wines.

The low values for volatile acidity (0.2 g L^{-1}) confirmed the lack of acetic fermentation, that could be responsible of wines quality decline. Concerning malic acid, data obtained by HPLC analysis were very similar whatever the samples and, taking into account the initial amount in the must, they highlight the absence of malolactic spoilage in final wines.

At the end of fermentation, the residual lysozyme was about 80% and 50% in wines obtained from yeast strain 333 and 1042 respectively. The reduction of free lysozyme in wines due to its interaction with must constituents (mainly phenolics) has been already reported (Amati et al., 1996; Delfini et al., 2004; Bellachioma et al., 2008). However our data suggest that yeast strain could play a further role in the amount of residual lysozyme in wines after the fermentation process.

3.2 Volatile characterisation of final wines

The volatile compounds identified in each final wine were grouped into chemical classes and are reported in tables 3, 4 and 5 together with the Tuckey's test results. On the right hand side, the tables also show the significant ($p>0.01$) standardized beta coefficients coming from the multiple regression analysis, carried out with the aim of highlighting significant correlations between each factor and the production of volatiles during fermentation. The higher the regression coefficient (beta), the stronger the impact of the factor on that specific compound. Furthermore the sign of the beta values indicate the direct (positive sign) or reversed (negative sign) correlation. Hence, positive signs refer to a direct relationship between tannins, lysozyme and strain 1042 on the level of single compounds while, for negative signs, a reversed correlation should be considered.

	Strain 333				
	must	SO_2	lysozyme	SO_2+tannin	lysozyme+tannin
DO 420 nm	0.181	0.037 ± 0.001	0.046 ± 0.001	0.042 ± 0.002	0.052 ± 0.002
PFT (mg L^{-1})	101	109 ± 0.94	112 ± 10.9	122 ± 4.09	113 ± 5.21
density	1.0706	0.9921 ± 0.0002	0.9926 ± 0.0002	0.9921 ± 0.0002	0.9924 ± 0.0001
pH	3.08	3.01 ± 0.02	3.05 ± 0.00	3.03 ± 0.02	3.04 ± 0.02
Total acidity (g L^{-1} tartaric acid)	5.66	6.09 ± 0.19	5.81 ± 0.05	6.20 ± 0.09	6.00 ± 0.08
Volatile acidity (g L^{-1} acetic acid)	-	0.24 ± 0.00	0.23 ± 0.02	0.21 ± 0.03	0.21 ± 0.10
Degree alcoholic (%)	-	10.5 ± 0.08	10.5 ± 0.06	10.7 ± 0.12	10.6 ± 0.08
Dry extract (g L^{-1})	-	15.9 ± 0.29	17.5 ± 0.58	16.7 ± 0.17	17.3 ± 0.46
Total SO_2 (mg L^{-1})	-	48.0 ± 1.60	0.43 ± 0.18	46.4 ± 1.39	0.75 ± 0.18
Acetaldehyde (mg L^{-1})	-	29.8 ± 0.91	6.49 ± 0.17	28.8 ± 1.24	4.99 ± 0.58
Lysozyme (mg L^{-1})	-	-	214 ± 20.8	-	200 ± 14.8
Malic acid (g L^{-1})	2.57	2.21 ± 0.18	2.19 ± 0.03	2.32 ± 0.05	2.04 ± 0.22

	Strain 1042			
	SO_2	lysozyme	SO_2+tannin	lysozyme+tannin
DO 420 nm	0.022 ± 0.003	0.066 ± 0.004	0.027 ± 0.002	0.071 ± 0.003
PFT (mg L^{-1})	114 ± 0.55	111 ± 2.48	118 ± 2.71	120 ± 2.50
density	0.9922 ± 0.0001	0.9922 ± 0.0002	0.9927 ± 0.0002	0.9922 ± 0.0002
pH	2.99 ± 0.00	3.00 ± 0.01	3.00 ± 0.00	3.00 ± 0.00
Total acidity (g L^{-1} tartaric acid)	7,83 ± 0.04	6.93 ± 0.04	7.85 ± 0.10	7.14 ± 0.22
Volatile acidity (g L^{-1} acetic acid)	0.22 ± 0.02	0.16 ± 0.02	0.23 ± 0.02	0.16 ± 0.02
Degree alcoholic (%)	11.1 ± 0.28	10.3 ± 0.32	11.1 ± 0.10	10.5 ± 0.48
Dry extract (g L^{-1})	18.2 ± 0.60	15.8 ± 0.57	19.6 ± 0.29	16.7 ± 1.01
Total SO_2 (mg L^{-1})	39.0 ± 1.22	0.75 ± 0.18	40.0 ± 0.46	1.39 ± 0.18
Acetaldehyde (mg L^{-1})	15.6 ± 2.28	5.04 ± 0.77	23.5 ± 5.08	5.22 ± 0.50
Lysozyme (mg L^{-1})	-	132 ± 3.97	-	113 ± 1.59
Malic acid (g L^{-1})	2.64 ± 0.33	2.48 ± 0.11	2.87 ± 0.18	2.61 ± 0.15

Table 2 General oenological parameters of final wines fermented by strain 333 and 1042

3.2.1 Alcohols

Table 3 show the mean concentration in alcohols of the eight wines.

As expected, the yeast strain had a stronger relationship with the sum of alcohols in final wines (beta = 0.763). Strain 1042 tends to furnish higher amounts of such volatiles, if compared with strain 333. Among single compounds, considerable correlations were found between yeast strain and 1-butanol, 2-methyl-3-pentanol and 2-furanmethanol. This could be likely due to differences in metabolic pathways between the two strains (Bell et al., 2005). Sulphur dioxide as well, had a significant influence on alcohols production: total alcohols amount in wines obtained from fermentation with SO_2 was higher than counterparts obtained with lysozyme and without SO_2 addition.

At concentrations below 300 mg L^{-1}, higher alcohols certainly contribute to a desirable level of complexity in wines flavour, whereas concentration over 400 mg L^{-1} have a detrimental effect on wines quality (Rapp et al., 1991). In our samples, total alcohols spanned between 256 to 397 mg L^{-1} hence positively contributing to wine aroma.

For 3-methyl-1-butanol, 3-methyltio-1-propanol, phenylethyl alcohol and 4-hydroxy-benzenethanol, they were overall found at higher concentrations in wines fermented with SO_2 (even if with different responses as a function of the strain used) likely as a consequence of the increased consumption of must amino acids, promoted by sulphites, during fermentation (Herraiz et al., 1989; Garde-Cerdan et al., 2007; Margheri et al., 1986).

Wine alcohols, in fact, can be formed during fermentation by two different ways: a catabolic process starting from amino acid-derivatives α-ketoacid (the Ehrlich pathway) and an anabolic process starting from α-ketoacids acting as intermediates in cell glucose metabolism (Bell et al., 2005; Hernandez-Orte et al., 2006; Nykanen, 1986).

In particular, the above cited alcohols are synthesized from leucine, methionine, phenylalanine and tyrosine respectively, and the increased degradation of these amino acids by yeasts could well drive to higher contents their corresponding alcohols (Nykanen, 1986). Similar results on the role of SO_2 during alcohols fermentation have been already reported by other authors (Herraiz et al., 1989; Garde-Cerdan et al., 2007; Margheri et al., 1986).

On the other side, the amount of other alcohols, such as *n*-propanol and 3-ethoxy-1-propanol, was higher in wines fermented without SO_2, as already reported by Herraiz et al. (1988) and Margheri et al. (1986). For this class of compounds, tannins did not show any appreciable effect.

3.2.2 Esters

The concentration of esters as a sum (Table 4) tended to be higher in samples fermented with strain 1042. This result, however, greatly depends on the relevant production of ethyl hydrogen succinate that affects the total amount of these compounds. Individually, in fact, a number of esters, (hexanoic, octanoic, decanoic, dodecanoic ethyl esters) were significantly correlated with the strain 333. The influence of yeast strain on esters production has been already reported by Vila et al. (1998) and Lema et al. (1996). SO_2 had no significant relationship on total ester amounts, diversely from alcohols. However, if single compounds are considered, higher concentrations of medium-chain fatty acid ethyl esters (MCFA ethyl esters), such as ethyl hexanoate, ethyl octanoate and ethyl decanoate, were found in SO_2 free wines obtained with lysozyme addition (Table 4), at values above their threshold level, hence contributing to the fruity aroma of the wines (Peinado et al., 2004).

These results were in contrast with some works reported in the bibliography. For example, Herraiz et al. (1989), evaluating the differences between wines fermented with or without sulphur dioxide, found that, if compared with SO_2 free fermentations, wines fermented with SO_2 were characterized by higher levels of ethyl octanoate, although there weren't differences in ethyl hexanoate amounts. Similar findings were reported by Margheri et al. (1986), for whom also ethyl decanoate was produced at higher concentration in SO_2 added musts. Other authors (Shinohara et al., 1981), however, showed that esters concentration started to increase only after an SO_2 addition > 100 mg L^{-1}.

In fact, the effect of SO_2 addition on esters production does not seem to be systematic and may depend on several factors. Nykanen (1986) showed that reduced oxygen concentration increased the production of MCFA ethyl esters; Moio et al. (2004) associated an increase in esters concentration to combined action of higher SO_2 amounts and low O_2 availability, regardless of the type of yeast strain employed for fermentation. Furthermore Herraiz Tomico (1990), according to our results, found higher concentration of ethyl hexanoate in wines fermented without SO_2. Bardi et al. (1998) postulated that during alcoholic fermentation, unsaturated fatty acids can be synthesized by oxidation of free saturated fatty acids, with a process that involves the presence of free oxygen. Lacking this element, the synthesis stops, with the corresponding accumulation of acyl-CoA. Under these conditions, in order to recover free coenzyme A, the yeasts promote ester formation and the wine obtained in these conditions is richer in esters containing the corresponding acyl group. (Moio et al., 2004; Bardi et al., 1998). In our wines, the added SO_2 amount (80 mg L^{-1}) had probably been unable to reduce in a significant way the availability of free oxygen during the alcoholic fermentation and further studies may be

necessary to consider other factors influencing the MCFA ethyl esters formation in SO_2 free wines. On the other side, our data show that tannins, especially in the presence of SO_2, increased the concentration of C_{12}-C_{16} ethyl esters (Table 4), likely due to the fast drop of oxygen availability as a result of the tannins oxygen scavenging activity (Bosso et al., 2001).

For the acetate esters, a positive influence of strain 1042 on ethyl acetate was found, while 2-phenylethyl acetate was higher in wines obtained with strain 333. Our data are in accordance with results obtained by Daudt et al. (1973) about the role of different type yeast strains on acetate esters production.

3.2.3 Acids

Acids amounts (Table 5) followed the trend of the corresponding fatty acid ethyl esters as a consequence of their common biosynthetic pathway, which leads to the production of long chain unsaturated fatty acids (Soumalainen et al., 1979): in particular, the MCFA (octanoic, decanoic, dodecanoic and tetradecanoic acids) were directly correlated to strain 333. Furthermore, hexanoic, octanoic and decanoic acids were also positively influenced by the presence of lysozyme. On the other hand, strain 1042 is linked to higher amounts of acetic, butanoic and isovaleric acids. For this group of compounds, tannins had no significant influence.

Fatty acids contribute to fresh flavour of wine, or for an unpleasant flavour if they are in excess, and they also help to modify the perception of other taste sensations (Ribéreau-Gayon et al., 2007). The total fatty acid concentration in wines samples was found to be around 15-25 mg L^{-1}, a value that didn't impair wine aroma (Miranda-Lopez et al., 1992).

Table 3. Final wines: Alcohols concentrations (mg L^{-1}) and contribution of the tested factors on their production as assessed by multiple regression analysis

	Strain 333				Strain 1042				Regr. Coefficient[(1)]		
	Lyso	Lyso+ tan	SO_2	SO_2 + tan	Lyso	Lyso+tan	SO_2	SO_2 + tan	Yeast	Lyso	Tannin
metanol	35.0 ± 2.40 [a]	35.4 ± 4.70 [a]	30.5 ± 1.39 [a]	30.7 ± 0.51 [a]	50.8 ± 2.54 [bc]	45.1 ± 1.84 [b]	40.0 ± 1.73 [b]	59.0 ± 18.8 [c]	0.713	-	-
n-propanol	11.6 ± 0.19 [bc]	11.5 ± 0.42 [bc]	6.30 ± 0.19 [a]	7.55 ± 1.96 [ab]	12.1 ± 0.63 [c]	12.5 ± 1.00 [c]	9.40 ± 0.69 [b]	12.5 ± 3.73 [c]	0.502	0.44	-
i-butanol	30.7 ± 1.51 [bc]	34.9 ± 1.95 [c]	14.7 ± 2.60 [a]	12.7 ± 1.03 [a]	23.8 ± 1.55 [b]	26.7 ± 1.50 [b]	21.6 ± 1.77 [ab]	29.8 ± 6.31 [bc]	-	0.613	-
3-methyl-1-butanol	144 ± 6.03 [a]	170 ± 5.64 [a]	184 ± 18.0 [ab]	170 ± 7.62 [a]	172 ± 12.1 [a]	179 ± 8.89 [ab]	208 ± 11.5 [b]	248 ± 51.2 [bc]	0.527	-0.4	-
1-butanol	0.78 ± 0.03 [a]	0.55 ± 0.10 [a]	0.51 ± 0.06 [a]	0.46 ± 0.11 [a]	7.23 ± 0.98 [bc]	7.79 ± 0.88 [c]	4.84 ± 2.21 [b]	4.95 ± 0.96 [b]	0.896	0.267	-
4-methyl-1-pentanol	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00			-
3-methyl-1-pentanol	0.06 ± 0.00 [a]	0.07 ± 0.00 [a]	0.08 ± 0.01 [ab]	0.08 ± 0.02 [ab]	0.07 ± 0.05 [a]	0.14 ± 0.01 [c]	0.13 ± 0.00 [bc]	0.13 ± 0.01 [bc]	0.684	-	-
2-methyl-3-pentanol	0.10 ± 0.00 [b]	0.06 ± 0.01 [ab]	0.10 ± 0.06 [b]	0.12 ± 0.03 [b]	0.00 ± 0.00 [a]	0.01 ± 0.00 [a]	0.01 ± 0.00 [a]	0.01 ± 0.00 [a]	-0.864	-	-
1-hexanol	1.22 ± 0.19 [ab]	0.85 ± 0.06 [ab]	1.59 ± 0.88 [b]	0.67 ± 0.06 [a]	0.80 ± 0.04 [ab]	0.82 ± 0.04 [ab]	0.71 ± 0.09 [ab]	0.65 ± 0.06 [a]	-0.384	-	-
3-ethoxy-1-propanol	1.07 ± 0.13 [d]	0.74 ± 0.27 [c]	0.01 ± 0.01 [a]	0.02 ± 0.01 [a]	0.05 ± 0.01 [b]	0.04 ± 0.01 [ab]	0.03 ± 0.00 [a]	0.03 ± 0.00 [a]	-0.534	0.568	-
cis 3-hexen-1-ol	0.05 ± 0.01	0.05 ± 0.00	0.13 ± 0.12	0.08 ± 0.00	0.07 ± 0.01	0.06 ± 0.00	0.07 ± 0.01	0.06 ± 0.00	-	-	-
2-furanmethanol	0.01 ± 0.00 [ab]	0.02 ± 0.01 [ab]	0.01 ± 0.00 [a]	0.03 ± 0.01 [b]	0.07 ± 0.00 [c]	0.05 ± 0.01 [c]	0.13 ± 0.01 [d]	0.12 ± 0.00 [d]	0.831	-0.36	-
3-methylthio-1-propanol	0.62 ± 0.01 [a]	0.66 ± 0.03 [a]	1.21 ± 0.2 [b]	1.20 ± 0.18 [b]	0.63 ± 0.07 [a]	0.59 ± 0.06 [A]	0.91 ± 0.17 [ab]	1.04 ± 0.03 [b]	0.219	-0.895	-
benzyl alcohol	0.04 ± 0.01 [ab]	0.03 ± 0.01 [ab]	0.05 ± 0.01 [b]	0.04 ± 0.00 [ab]	0.04 ± 0.01 [ab]	0.03 ± 0.00 [ab]	0.02 ± 0.00 [a]	0.02 ± 0.00 [a]	-0.482	-	-
phenylethyl alcohol	18.1 ± 0.96 [b]	19.0 ± 2.31 [b]	35.0 ± 8.07 [c]	35.1 ± 7.52 [c]	14.5 ± 1.10 [a]	15.9 ± 1.21 [a]	17.0 ± 3.09 [b]	19.1 ± 1.72 [b]	-0.573	-0.594	-
4-OH-benzenethanol	12.5 ± 0.90 [a]	17.8 ± 3.62 [a]	41.0 ± 11.0 [c]	49.0 ± 8.18 [c]	13.2 ± 4.77 [a]	21.6 ± 2.06 [ab]	17.3 ± 13.4 [a]	21.0 ± 4.58 [ab]	-0.433	-0.558	-
Sum	*256 ± 12.4* [a]	*292 ± 19.1* [b]	*315 ± 22.5* [bc]	*307 ± 17.2* [bc]	*296 ± 18.9* [b]	*330 ± 17.5* [c]	*320 ± 24.6* [c]	*397 ± 27.5* [d]	*0.763*	*-0.628*	-

In the same row, different letters denote significant differences at $p < 0.01$

[(1)] only standardized regression coefficients (beta values) with $p < 0.01$, are reported

Table 4 Final wines: Esters concentrations (mg L^{-1}) and influence of the tested factors on their production as assessed by multiple regression analysis

	Strain 333				Strain 1042				Regr. Coefficient[(1)]		
	Lyso	Lyso+ tan	SO_2	SO_2 + tan	Lyso	Lyso+tan	SO_2	SO_2 + tan	Yeast	Lyso	Tannin
ethyl-acetate	36.1 ± 3.44 ^{ab}	31.6 ± 3.61 ^{a}	25.0 ± 3.23 ^{a}	29.5 ± 5.13 ^{a}	33.1 ± 4.10 ^{a}	43.3 ± 5.78 ^{ab}	42.2 ± 11.9 ^{ab}	55.0 ± 11.3 ^{b}	0.608	-	-
ethyl hexanoate	0.84 ± 0.06 ^{d}	0.76 ± 0.04 ^{cd}	0.70 ± 0.07 ^{bc}	0.63 ± 0.08 ^{bc}	0.75 ± 0.05 ^{c}	0.70 ± 0.03 ^{bc}	0.54 ± 0.10 ^{ab}	0.51 ± 0.03 ^{a}	-0.502	0.725	-
hexyl acetate	0.15 ± 0.04 ^{a}	0.11 ± 0.01 ^{a}	0.23 ± 0.04 ^{b}	0.27 ± 0.03 ^{b}	0.24 ± 0.02 ^{b}	0.24 ± 0.02 ^{bc}	0.13 ± 0.02 ^{a}	0.14 ± 0.01 ^{a}	-	-	-
ethyl lactate	1.69 ± 0.20 ^{ab}	4.10 ± 3.04 ^{b}	1.04 ± 0.32 ^{ab}	0.96 ± 0.04 ^{a}	1.43 ± 0.14 ^{ab}	1.77 ± 0.15 ^{ab}	2.31 ± 0.24 ^{ab}	2.18 ± 0.03 ^{ab}	-	0.277	-
ethyl octanoate	1.15 ± 0.14 ^{c}	1.11 ± 0.01 ^{c}	0.79 ± 0.16 ^{b}	0.68 ± 0.08 ^{ab}	0.69 ± 0.02 ^{ab}	0.64 ± 0.11 ^{ab}	0.50 ± 0.06 ^{a}	0.54 ± 0.04 ^{ab}	-0.702	0.561	-
ethyl-3-hydroxybutanoate	0.25 ± 0.01 ^{d}	0.23 ± 0.01 ^{d}	0.14 ± 0.01 ^{ab}	0.14 ± 0.01 ^{ab}	0.19 ± 0.01 ^{c}	0.17 ± 0.01 ^{bc}	0.12 ± 0.01 ^{a}	0.13 ± 0.01 ^{a}	-0.422	0.809	-
ethyl decanoate	0.06 ± 0.00 ^{c}	0.04 ± 0.00 ^{b}	0.04 ± 0.01 ^{b}	0.03 ± 0.00 ^{ab}	0.02 ± 0.00 ^{a}	0.03 ± 0.00 ^{ab}	0.01 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	-0.855	0.231	-
ethyl4-hydroxybutanoate	1.04 ± 0.07 ^{a}	1.28 ± 0.17 ^{a}	0.65 ± 0.11 ^{a}	2.08 ± 0.30 ^{b}	0.77 ± 0.32 ^{a}	0.77 ± 0.11 ^{a}	2.00 ± 0.50 ^{b}	0.63 ± 0.03 ^{a}	-	-	-
2-phenylethyl acetate	1.65 ± 0.28 ^{d}	1.53 ± 0.11 ^{cd}	1.03 ± 0.36 ^{bc}	2.54 ± 0.17 ^{e}	0.48 ± 0.14 ^{a}	0.45 ± 0.04 ^{a}	0.61 ± 0.12 ^{ab}	0.80 ± 0.02 ^{bc}	-0.826	-	0.201
ethyl dodecanoate	0.10 ± 0.02 ^{c}	0.12 ± 0.04 ^{c}	0.08 ± 0.01 ^{bc}	0.11 ± 0.04 ^{c}	0.00 ± 0.01 ^{a}	0.01 ± 0.00 ^{a}	0.02 ± 0.01 ^{ab}	0.03 ± 0.01 ^{ab}	-0.87	-	0.206
S-(3-hydroxypropyl) thioacetate	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	32.1 ± 2.98 ^{c}	37.0 ± 3.07 ^{c}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	12.6 ± 1.01 ^{b}	14.4 ± 0.99 ^{B}	-0.34	-0.847	-
diethyl malate	0.07 ± 0.02 ^{a}	0.07 ± 0.03 ^{a}	0.05 ± 0.01 ^{a}	0.06 ± 0.03 ^{a}	0.06 ± 0.02 ^{a}	0.07 ± 0.01 ^{a}	0.12 ± 0.05 ^{ab}	0.19 ± 0.03 ^{c}	0.486	-0.383	-
ethyl hexadecanoate	0.01 ± 0.01 ^{a}	0.01 ± 0.01 ^{a}	0.01 ± 0.01 ^{a}	0.09 ± 0.06 ^{c}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.03 ± 0.00 ^{ab}	-	-0.488	0.377
ethyl hydrogen succinate	8.80 ± 1.20 ^{a}	9.73 ± 2.29 ^{a}	8.81 ± 3.18 ^{a}	13.5 ± 0.85 ^{b}	13.4 ± 6.55 ^{ab}	16.00 ± 2.50 ^{c}	9.80 ± 5.56 ^{a}	16.60 ± 4.05 ^{c}	0.488	-	0.426
Sum (excluding ethylacetate)	*15.8 ± 2.05* ^{a}	*19.1 ± 5.76* ^{b}	*13.6 ± 4.31* ^{a}	*21.1 ± 1.71* ^{C}	*18.0 ± 7.31* ^{b}	*20.9 ± 3.01* ^{c}	*16.2 ± 6.70* ^{ab}	*21.5 ± 4.30* ^{c}	*0.376*	-	*0.439*

In the same row, different letters denote significant differences at $p< 0.01$

[(1)] only standardized regression coefficients (beta values) with $p < 0.01$, are reported

Table 5. Final wines: Acids concentrations (mg L^{-1}) and influence of the tested factors on their production as assessed by multiple regression analysis

	Strain 333				Strain 1042				Regr. Coefficient[(1)]		
	Lyso	Lyso+ tan	SO_2	SO_2 + tan	Lyso	Lyso+tan	SO_2	SO_2 + tan	Yeast	Lyso	Tannin
acetic acid	3.07 ± 0.40 [ab]	3.99 ± 0.18 [ab]	1.85 ± 0.91 [a]	1.97 ± 0.12 [a]	3.92 ± 0.72 [ab]	6.06 ± 2.91 [b]	10.6 ± 1.30 [c]	10.5 ± 0.83 [c]	0.668	-	-
propanoic acid	0.64 ± 0.09 [ab]	0.98 ± 0.59 [bc]	0.31 ± 0.06 [a]	0.63 ± 0.26 [ab]	2.20 ± 0.77 [c]	0.49 ± 0.13 [a]	0.61 ± 0.22 [ab]	1.76 ± 0.67 [bc]	-	-	-
isobutyric acid	1.71 ± 0.17 [c]	2.30 ± 0.31 [d]	0.80 ± 0.25 [ab]	0.32 ± 0.20 [a]	1.05 ± 0.22 [bc]	1.35 ± 0.07 [bc]	1.57 ± 0.09 [bc]	1.27 ± 0.07 [bc]	-	0.544	-
butanoic acid	0.38 ± 0.04 [ab]	0.61 ± 0.04 [b]	0.32 ± 0.08 [a]	0.54 ± 0.11 [ab]	0.58 ± 0.03 [ab]	0.62 ± 0.09 [b]	0.63 ± 0.10 [b]	0.53 ± 0.20 [ab]	0.398	-	-
isovaleric acid	0.33 ± 0.05 [a]	0.51 ± 0.19 [a]	0.82 ± 0.39 [ab]	0.75 ± 0.04 [ab]	0.77 ± 0.08 [ab]	1.20 ± 0.10 [b]	1.05 ± 0.10 [b]	1.10 ± 0.18 [b]	0.652	-0.328	-0.268
hexanoic acid	4.52 ± 0.37 [c]	4.11 ± 0.41 [bc]	2.89 ± 0.34 [a]	2.75 ± 0.34 [a]	4.61 ± 1.27 [c]	3.80 ± 0.27 [bc]	2.07 ± 0.42 [a]	2.30 ± 0.15 [a]	-	0.870	-
octanoic acid	8.44 ± 0.60 [c]	8.57 ± 0.92 [c]	5.52 ± 0.60 [ab]	5.96 ± 0.26 [ab]	7.27 ± 3.25 [b]	6.06 ± 0.24 [ab]	2.97 ± 1.14 [a]	4.26 ± 0.31 [ab]	-0.506	0.691	-
n-decanoic acid	3.25 ± 0.33 [b]	3.67 ± 0.61 [b]	2.09 ± 0.75 [ab]	2.52 ± 0.42 [ab]	2.06 ± 1.06 [ab]	1.67 ± 0.16 [ab]	0.90 ± 0.45 [a]	1.35 ± 0.19 [a]	-0.661	0.396	-
dodecanoic acid	0.23 ± 0.06 [ab]	0.29 ± 0.10 [b]	0.18 ± 0.07 [ab]	0.20 ± 0.06 [ab]	0.13 ± 0.04 [ab]	0.11 ± 0.04 [ab]	0.10 ± 0.08 [a]	0.14 ± 0.05 [a]	-0.480	-	-
tetradecanoic acid	0.03 ± 0.01	0.04 ± 0.03	0.02 ± 0.02	0.03 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	-0.518	-	-
n-hexadecanoic acid	1.07 ± 0.89 [c]	0.96 ± 0.89 [c]	0.77 ± 0.66 [b]	0.63 ± 0.34 [ab]	0.35 ± 0.07 [a]	0.47 ± 0.12 [a]	0.36 ± 0.30 [a]	0.63 ± 0.29 [ab]	-	-	-
Sum	*23.7 ± 3.01* [b]	*26.1 ± 4.26* [b]	*15.6 ± 4.15* [a]	*16.3 ± 2.17* [a]	*23.0 ± 7.50* [b]	*21.9 ± 4.14* [b]	*21.0 ± 4.22* [b]	*22.6 ± 3.0* [ab]	-	*0.358*	-

In the same row, different letters denote significant differences at $p < 0.01$

[(1)] only standardized regression coefficients (beta values) with $p < 0.01$, are reported

3.2.4 Principal component analysis

To try to deepen the understanding of which volatile compound mainly contribute to characterize each final wine, a PCA analysis was carried out on the whole set of data (Fig. 2).

Three groups were clearly discriminated in the plane formed by the first two components (53% of total variance as a sum). In the lower right side of the graph, all the twelve SO_2 free/lysozyme added wines were grouped, independently from the presence or the absence of tannins. For these samples, discriminating variables were hexanoic and octanoic acids together with their ethyl esters (variables 8, 12, 1 and 2, respectively, in the plot) and ethyl-3-hydroxybutanoate (variable 4).

A second group included the six SO_2 added wines fermented with strain 1042 (lower left side of the graph) and was characterized by 2-furanmethanol, diethyl malate and acetic acid (compounds 5, 11 and 3 respectively). Finally, for the SO_2 added samples fermented by strain 333, the major discriminating variables were the sulphur alcohols 3-methylthio-1-propanol and 3-ethylthio-1-propanol (variables 6 and 7), together with phenylethyl alcohol and 3-hydroxypropyl thioacetate (variables 9 and 10 respectively).

The different trials (flagged with rhombus) are identified as in table 1. Triangles refer to significant variables as follows: 1: ethyl hexanoate; 2: ethyl octanoate; 3: acetic acid; 4: ethyl-3-hydroxybutanoate; 5: 2-furanmethanol; 6: 3-methylthio-1-propanol; 7: 3-ethylthio-1-propanol; 8: hexanoic acid; 9: phenylethyl alcohol; 10: 3-hydroxypropyl thioacetate; 11: diethyl malate; 12: octanoic acid.

3.2.5 Sensory evaluation

Sensory descriptive analysis showed that the two strains of yeast used in fermentation contributed in a different way on sensory profile of wines obtained.

In particular, higher values for fruity, banana and tropical fruit attributes, were found in samples fermented by strain 1042 (Fig. 3a).

In strain 333, the highest scores for floral, fruity, tropical and banana attributes were obtained by samples fermented with lysozyme (Fig. 3b).

For the citrus fruits attribute, the two strain showed a different behaviour as a function of oenological protocol carried out: in samples fermented by 1042 strain this attribute was increased by lysozyme addition, while in samples fermented by 333 strain the same attribute was decreased by use of lysozyme.

Concerning the use of oenological tannins, they influenced in a positive way citrus attributes for both strains. Nevertheless, it affected negatively the fruity and banana attributes in the case of

1042 strain, while the opposite was found in wines from 333 yeast strain. Tropical fruit attribute was increased by the contemporary addition of tannins and lysozyme, a fact that was not observed in case of the SO_2 added samples, for both yeast strains.

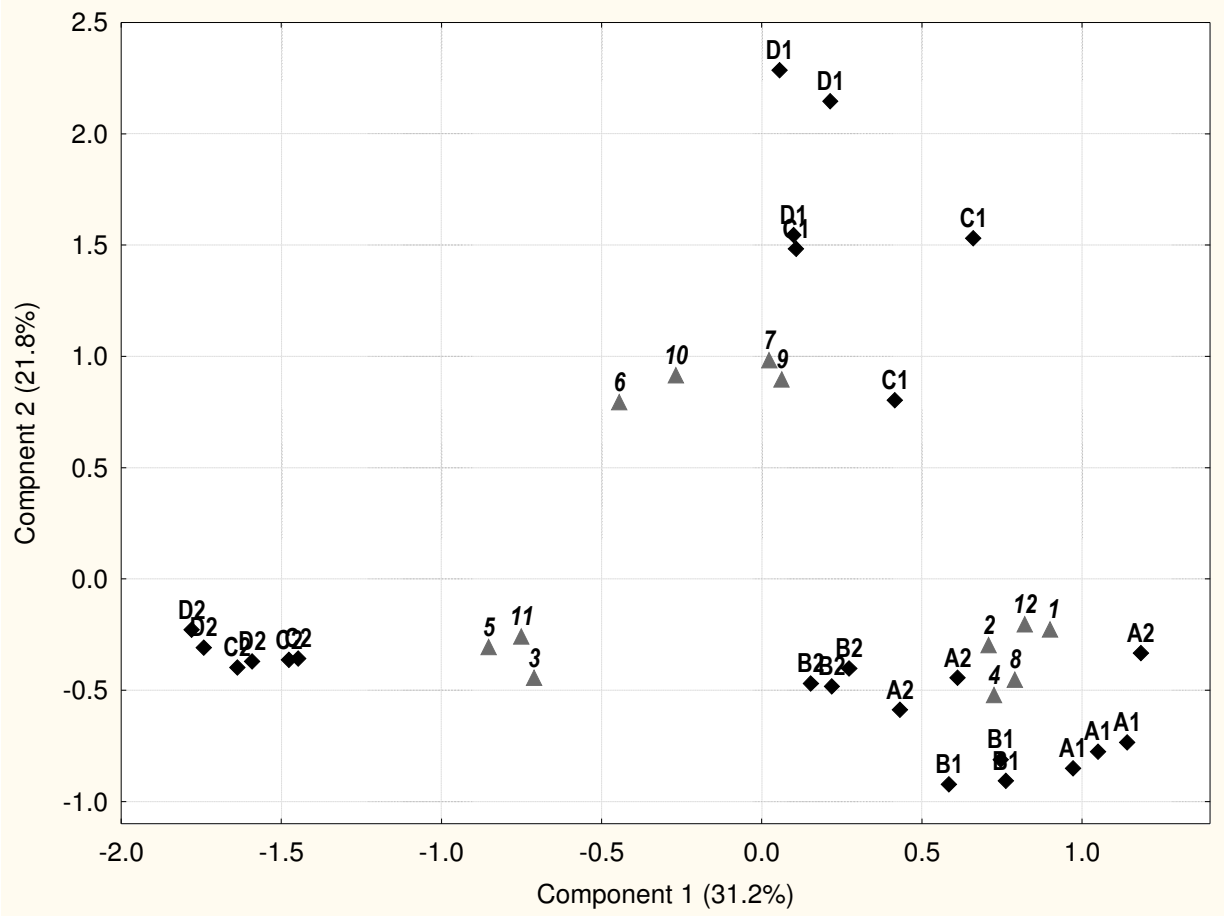

Figure 2. Biplot of the first two principal components.

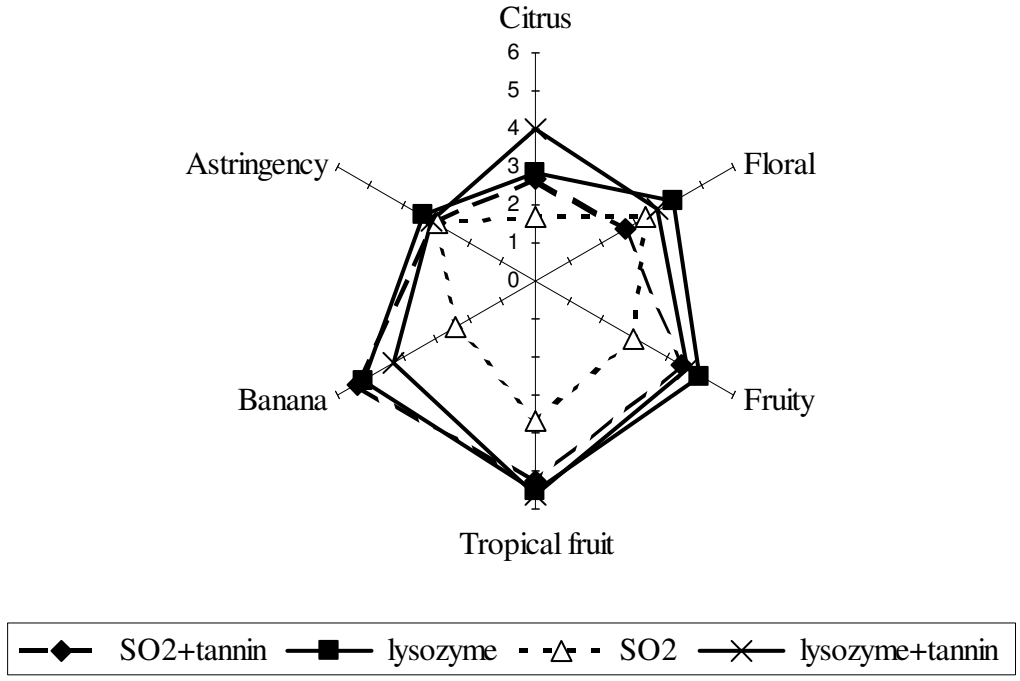

Figure 3a. Results of descriptive sensory analysis of final wines from strain 1042

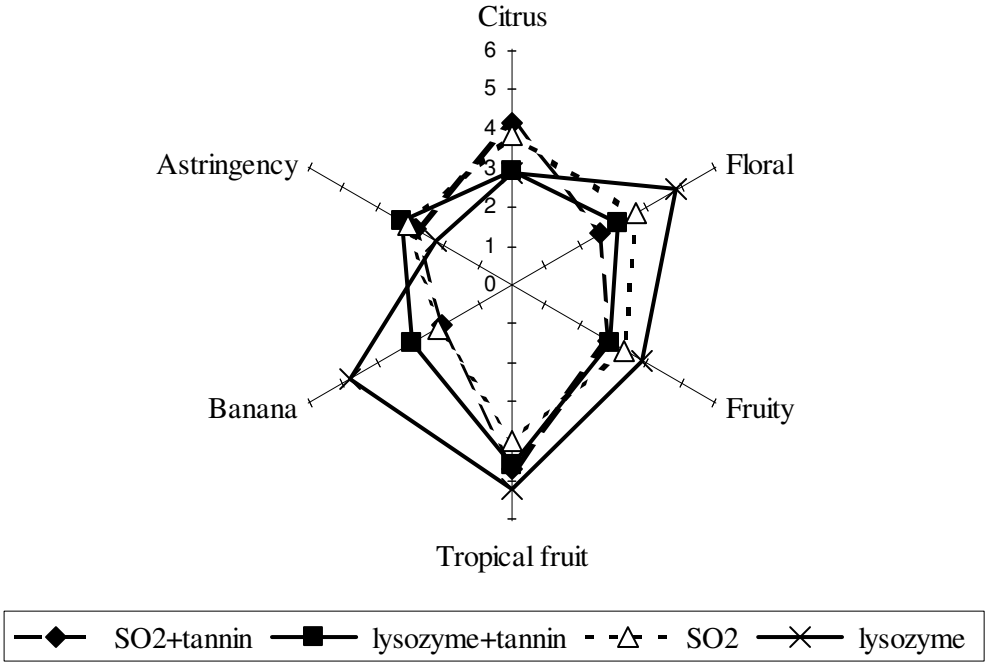

Figure 3b. Results of descriptive sensory analysis of final wines from strain 333

4. CONCLUSIONS

Overall, the obtained data suggest that the addition of lysozyme and enological tannins during alcoholic fermentation could represent a promising alternative to the use of sulphur dioxide and a reliable starting point for the production of SO_2-free wines. Volatile composition of the final wines was affected by the different vinification protocols (mainly concerning alcohols and ethyl esters) also as a consequence of yeast responses to the absence of sulphites during fermentation. The use of lysozyme and tannins on musts furnished wines with distinct sensory impact, if compared with "conventional" musts fermented with sulphites. Further studies will be set down to investigate the evolution of such wines during the storage and to deepen possible correlations between must amino acid consumption and presence/absence of SO_2 during the alcoholic fermentation.

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Chapter 3

Fermentation of sulphite-free white musts with added lysozyme and oenological tannins: nitrogen consumption and biogenic amines composition of final wines

ABSTRACT: *The influence on amino acid consumption and biogenic amines composition of white wines obtained by replacing SO_2 during fermentation with lysozyme and tannins was studied. At the same time, the fermentative performance of two low SO_2 producing selected yeasts strains was evaluated. For this purpose, a series of laboratory scale fermentations of fresh white must and a HPLC-DAD method for the analysis of amino acids, biogenic amines and ammonium ion were undertaken. The presence of SO_2 or lysozyme affected the consumption of nitrogen as a function of the yeast strain, while oenological tannin had no substantial influence. Strain 1042 increased the consumption of total YAN in the presence of SO_2, as a consequence of the enhanced utilization of ammonium ion and a number of amino acids. By contrast, strain 333 tended not to change the total YAN uptake, whatever the juice treatment, and reduced the consumption of aspartic and glutamic acids, GABA and other compounds in the case of samples added with SO_2. When compared with lysozyme addition, for both strains, SO_2 increased the consumption of alanine and glutamine, the latter being a major contributor to the assimilable nitrogen of the must. No influence of must treatments was found on the content of biogenic amines in the final wines.*

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1. INTRODUCTION

The nitrogen composition of grape must mainly depends on the grape cultivar and maturity, vineyard fertilization, climatic conditions, oenological practices and ripeness (Huang & Ough, 1991; Huang & Hough, 1989; Soufleros, Bouloumpasi, Tsarchopoulos & Biliaderis, 2003). Nitrogen composition of must affects both the rate of alcoholic fermentation due to the rapid increase of biomass production, and the aroma of final wines since amino acids are precursors for volatile compounds (Äyräpää, 1971; Bell & Henscke, 2005). In addition, the consumption rate and metabolic fate of amino acids depends on the yeast strain and the physicochemical properties of the must or wine (Soufleros & Bertrand, 1979; Valero, Millán, Ortega & Mauricio, 2003). Deficiencies in the amount of yeast assimilable nitrogen (YAN) in grape musts can cause

fermentation faults (Jiranek, Langridge & Henschke, 1995; Bely, Sablayrolles & Barre, 1990; Ingledew & Kunkee, 1985; Bell & Henscke, 2005; Filipe-Ribeiro & Mendes-Faia, 2007).

Among the several nitrogen compounds, biogenic amines are endowed with biological activity. They are frequently found in fermented foods and beverages and are produced mainly through the decarboxylation of amino acids (Romero, Sánchez-Viñas, Gázquez & Bagur, 2002). High concentrations of biogenic amines are associated with deficient sanitary conditions and may cause toxicological effects (Silla-Santos, 1996). Moreover, secondary amines can form nitrosamines, compounds of known carcinogenic action (Torrea & Ancín, 2002). Due to these issues, upper limits for histamine in wine have been defined in different European countries, varying from 2 to 10 mg L^{-1} (Landete, Ferrer, Polo & Pardo, 2005).

In oenology, the use of SO_2 is very widespread in production processes, because of its antioxidant and antimicrobial activities. However, excess SO_2 has been reported to have toxic effects on human health (Romano & Suzzi, 1993; Gao, Zhang, Krentz, Darius, Power & Lagarde, 2002). During the last few years, lysozyme has been proposed to support or even replace SO_2 in wines (Chinnici, Piva, Arfelli & Amati, 1996; Bartowsky, Costello, Villa & Henschke, 2004). Lysozyme, which is already used as an antimicrobial agent in the food industry (Cunningham, Proctor & Goetsch, 1991; Proctor & Cunningham, 1988; Ghitti, Mosca, Lavezzari & Bianchi Salvatori, 1983), has no adverse effect on the growth of yeast and can be used during alcoholic fermentation to prevent the growth of spoiling lactic acid bacteria and to reduce the occurrence of stuck/sluggish alcoholic fermentations (Gao et al., 2002). Also, this substance does not enhance the browning of white wines during their storage (Bartowsky et al., 2004) and its activity is not significantly influenced by the most common technological operations (Amati, Chinnici, Piva, Arfelli & Riponi, 1996). Furthermore, the pre-fermentative addition of oenological tannins can help to avoid the oxidative phenomena of musts and wines, to contribute to wine structure, to stabilize the colouring material and improve the sensory impact (Bautista-Ortín, Martínez-Cutillas, Ros-García, López-Roca & Gómez-Plaza, 2005; Bellachioma, Riponi, Sonni & Chinnici, 2008; Sanz, Martínez-Castro & Moreno-Arribas, 2008).

There is evidence that the presence of sulphites in musts may influence the consumption of must amino acids during alcoholic fermentation by interfering with the glycolysis and respiratory chain phosphorylation (Maier, Hinze & Leuschel, 1986). As a consequence, differences on volatile composition and sensory features of sulphite added wine over sulphite-free wines have been found (Frivik & Ebeler, 2003; Garde-Cerdán & Ancín-Azpilicueta, 2007; Sonni, Cejudo-Bastante, Chinnici, Natali & Riponi, 2009).

The aim of this research is to study the amino acid consumption and biogenic amine composition of sulfite-free wines obtained with lysozyme and/or oenological tannin addition during alcoholic fermentation compared with conventional fermentation in the presence of SO_2. In order to investigate the influence of each single factor, a series of laboratory-scale fermentation were carried and an existing HPLC methodology was improved to simultaneously analyse for 23 amino acids, the ammonium ion and 6 biogenic amines using UV detection at 280 nm.

2. EXPERIMENTAL

2.1 Chemicals and Standards

HPLC grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Water was of MilliQ quality. Boric acid was obtained from Sigma-Aldrich and NaOH 1M and acetic acid from Merck. L-2-aminoadipic acid and diethylethoxymethylenmalonate (DEEMM) were supplied from Sigma-Aldrich (Steinheim, Germany). Solutions of amino acids and biogenic amines (Sigma-Aldrich) were prepared with HCl 0.1N.

2.2. Vinification

Alcoholic fermentation was studied in a must obtained at the experimental winery of the University of Bologna by blending grapes from Trebbiano and Sauvignon Blanc cv (60 Kg each). The grapes were destemmed, crushed at 0.9 bars in a bladder press, cold-settled at 4°C for 24 h and racked. The racked must was filtered through a 0.45 μm Seitz-Supra EK filter from Seitz (Bad Kreuznach, Germany) and placed in two litre laboratory glass fermentors, previously saturated with N_2, to start the fermentation, which was conducted with no temperature adjustment. A glass trap (filled with 37% H_2SO_4) prevented microbial contamination and oxygen entrance. Sample extraction was achieved through a sealed septum during the fermentation. All recipients and materials, which were in contact with the samples, were previously sterilized. Two *Saccharomyces cerevisiae* strains, 333 and 1042 (University of Bologna - ESAVE collection), previously characterized as low SO_2 producing yeasts (Sonni et al., 2009), were used to carry out fermentations and were inoculated after the rehydration of about 1.5 x 10^6 CFU mL^{-1} into 25 mL of sterilized must in 250-mL Erlenmeyer flasks plugged with cotton wool, incubated for 24 hr. These cultures were then used to inoculate fermentation trials to a cell density of 5 x 10^6 CFU ml^{-1}. Four different trials were defined with the aim to study the effect of the following three variables: 1) strain, 2) lysozyme/SO_2, 3) tannin (Table 1).

Factor \ Trial	*Strain (333 or 1042)*			
	S	*ST*	*L*	*LT*
Lysozyme (g L^{-1})	-	-	0.25	0.25
$K_2S_2O_5$ (mg L^{-1})	160	160	-	-
Tannin (g L^{-1})	-	0.1	-	0.1

Table 1. Scheme of fermentation trials. Legend for samples: S: Sulphur dioxide addition, ST: Sulphur dioxide and tannin addition, L: Lysozyme addition, LT: Lysozyme and tannin addition.

The fermentors were placed over magnetic stirrers to ensure homogenous fermentation and were monitored daily by weighing. Samples were taken immediately at the end of fermentation, when the loss of weight stopped, and all fermentations were performed in triplicate.

2.3. Assimilable nitrogen and ratio of nitrogen compounds

Yeast assimilable nitrogen (YAN) was expressed as the sum of the assimilable amino acid nitrogen (not including proline, hydroxyproline and ornithine which are not suitable nitrogen sources for yeasts) plus ammoniacal nitrogen, both quantified by HPLC, and taking into account the nitrogen percent of each amino acid.

The consumption of individual amino acids was estimated from the loss of concentration in wines in relation to the initial quantity in the grape juice.

Formaldehyde titration of free amino acids was also performed following the procedure proposed by Aerny (1996) which is based on the reaction of formaldehyde with α-amino acids and ammonium.

2.4. Analysis of free amino acids and biogenic amines.

2.4.1. Reaction of Derivatization

The method used to determine aminoenone derivates is a modification of the methodology described by Gómez-Alonso, Hermosín-Gutiérrez and García-Romero (2007). Briefly, 1.75 mL of borate buffer 1M (pH = 9), 750 µL of methanol, 1 mL of sample without any pre-treatment, 20 µL of internal standard (L-2-aminoadipic acid, 1 g L^{-1}), and 20 µL of DEEMM were left to react in a screw-cap test tube over 30 min in an ultrasound bath. Warming the solution at 70 °C for 30 min was beneficial in avoiding the presence of derivatizing artefacts emerging in the chromatogram between 20 and 22 min.

2.4.2. HPLC Analysis

A modification of the HPLC method proposed by Gómez-Alonso et al. (2007) for the identification and quantification of 23 amino acids, the ammonium ion and six biogenic amines was used (Fig. 1). The partial overlapping of asparagine, serine and hydroxyproline reported in the original method, was resolved by changing eluents and their pH. Detection and quantification was carried out at 280 nm. Diagnostic maximum absorption at 292 and 267 nm were displayed by hydroxyproline, proline and ammonium respectively which were used for identification purposes. The present procedure required a shorter run time compared with the original method (70 min compared to 85 min).

The studied twenty-three amino acids were: (L-aspartic acid, L-glutamic acid, *trans*-4-hydroxy-L-proline, L-serine, L-asparagine, L-glutamine, glycine, L-histidine, L-threonine, L-arginine, L-*α*-alanine, proline, GABA (γ-aminobutyric acid), L-tyrosine, L-valine, L-methionine, L-cysteine, L-tryptophan, L-isoleucine, L-leucine, L-phenylalanine, L-ornithine and L-lysine) plus 2-aminoadipic acid (internal standard), ammonium ion, and six biogenic amines (tyramine, histamine, spermidine, putrescine, cadaverine and tryptamine). HPLC separation was performed on a Jasco apparatus (Tokyo, Japan), equipped with a binary gradient pump (PU 1580), a 20 µl loop, a Reodyne valve (Cotati, USA), a photodiode detector (PU MD 910) and a column oven. The derivatized samples, after filtration (0.45 µm, nylon membrane, Teknokroma), were injected on a Waters (Milford, MA, USA) reversed-phase column Nova-Pak® C18 (3.9 x 300 mm; 4µm particle), thermostated at 40ºC. The Borwin 5.0 (JMBS Developments, Grenoble, France) software package was employed for chromatographic control. The composition of the mobile phases were as follows: phase A, 25mM acetate buffer pH = 5,65 and phase B, 80:20 mixture of acetonitrile and methanol. The mobile phases used were filtered through a 0.45 µm nylon Millipore filter. The flow rate was 1.1 mL/min.

The linear gradient for solvent A was as follows: 0 min, 100%; 7 min, 96%; 18 min, 94%; 23 min, 92%; 25 min, 92%; 28 min, 85%; 50 min, 77%; 60 min, 55%; 65 min, 40%; 67 min, 20%; 70 min, 100%. Detection was performed at 280 nm while quantification was based upon the internal standard method.

2.5. Statistical Analysis

To evaluate the influence and the possible interactions of the investigated variables on the nitrogen consumption, the data corresponding to the wines coming from must fermented in different conditions were organized in two groups (amino acids and biogenic amines) and

subjected to multivariate statistical analysis (factorial ANOVA) by using the Statistica 6 (StatSoft Italia srl, Italy) software package.

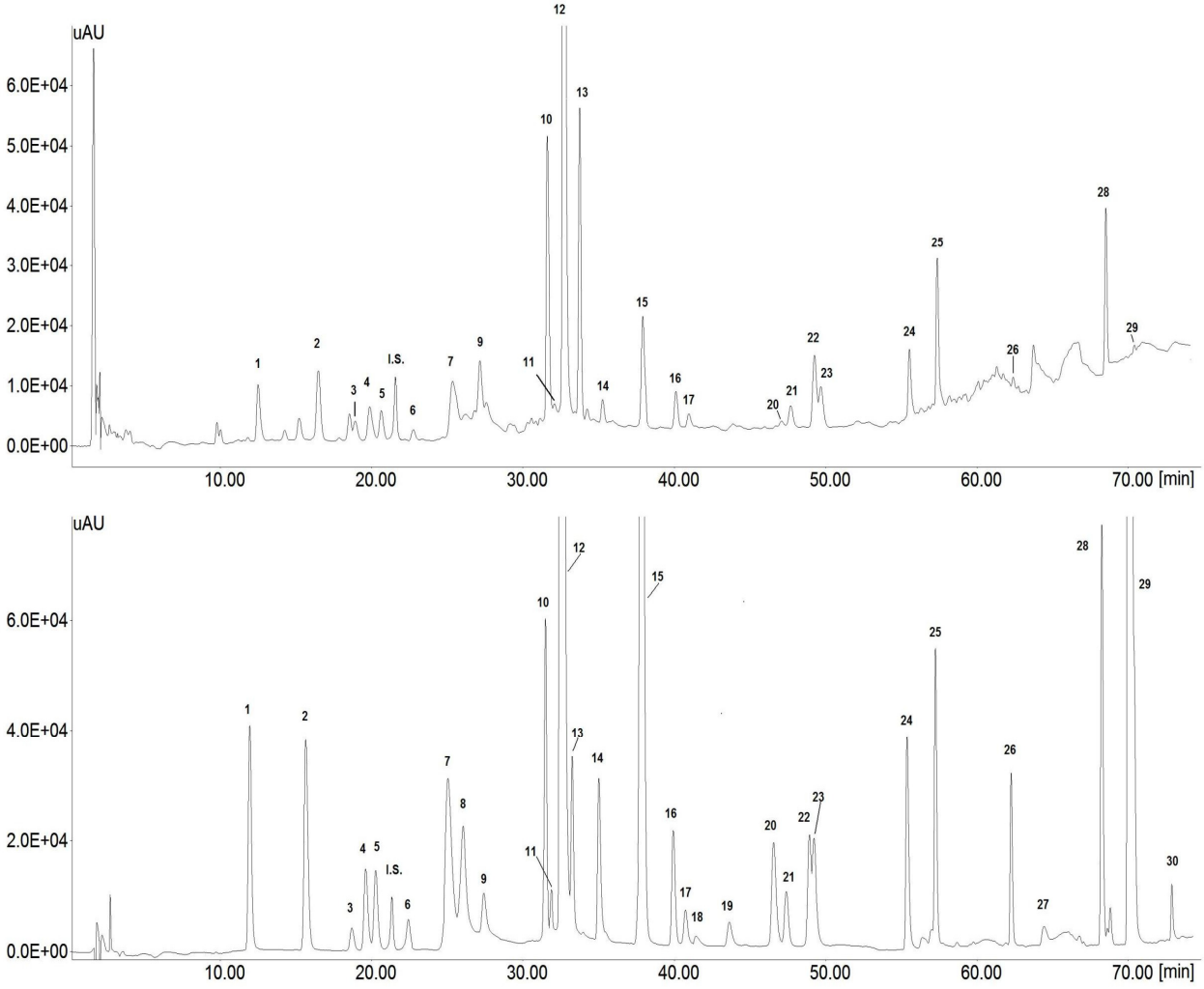

Figure 1: HPLC profile of the amino acids, ammonium ion and biogenic amines at 280nm of a wine (upper panel) and a standard solution (lower panel). Legend: 1: aspartic acid; 2: glutamic acid; 3: OH-proline; 4: serine; 5: asparagine; 6: glutamine; 7: glycine; 8: histidine; 9: threonine; 10: arginine; 11: α-alanine; 12: proline; 13: GABA; 14: tyrosine; 15: ammonium ion; 16: valine; 17: methionine; 18: histamine 19: cysteine; 20: tryptophan; 21: isoleucine; 22: leucine; 23: phenylalanine; 24: ornithine; 25: lysine; 26: tyramine; 27: spermidine; 28: putrescine; 29: cadaverine; 30: tryptamine. I.S.: 2-aminoadipic acid

3. RESULTS AND DISCUSSION

3.1 Amino acid composition of must

Arginine, glutamine and GABA, together with proline, were the most abundant amino acids (Table 2), representing 78% of the total amino acid content of must, followed by ammonium ion, glutamic acid, alanine and serine (15% as a sum). According to Huang et al., (1991), Dizy et al. (1996) and Garde-Cerdán, Marsellés-Fontanet, Arias-Gil, Martín-Belloso and Ancín-Azpilicueta (2007), these amino acids constitute the major nitrogen sources in musts. Minor amino acid compounds were methionine, glycine and tryptophan, in agreement with Valero et al. (2003) and Huang et al. (1991). Spermidine and putrescine were the only biogenic amines present in must. These amines are thought to be indispensable components of living cells for regulating nucleic acid function and protein synthesis and probably also in the stabilization of membranes (Silla-Santos, 1996; Halász, Baráth, Simon-Sarkadi & Holzaptfel, 1994). According to Ancín-azpilicueta, González-marco and Jiménez-moreno (2008), spermidine and putrescine are the major amines in grapes and musts before their transformation into wine.

3.2 Yeast Assimilable Nitrogen (YAN) of must

In Table 3, the overall must nitrogen sources for yeasts are reported.

Ammonium and amino nitrogen (the both derived from HPLC values) represented about 19% (56.5 mg N/L) and 81% (237 mg N/L) of the total assimilable nitrogen respectively while, for the contribution to YAN of the single amino acids, following the mean concentration in musts, arginine was largely the major contributor (109 mg N/L), followed by glutamine and GABA (59.4 and 38.8 mg N/L respectively) (Table 2).

The total YAN of juice was 294 mg N/L (Table 3), which is more than double the minimum nitrogen amount thought to be needed to efficiently complete the alcoholic fermentation (estimated at around 140 mg N/L) (Bely, Sablayrolles & Barre, 1991). It's worthy of mention that to calculate such an HPLC-derived YAN value, the three assimilable nitrogens provided by arginine were taken into account (Martin, Brandriss, Schneider & Bakalinsky, 2003), obtaining a value considerably higher than the YAN estimated by means of the commonly used formol titration (Table 3).

This may be due to the fact that in this latter method, formaldehyde titrates only one of the four arginine nitrogens, driving an underestimation of the YAN values in arginine-rich musts.

In fact, as a confirmation of this hypothesis, calculating the HPLC-derived YAN by considering only one single nitrogen to be provided by arginine, the final value would be 221 mg N/L which is consistent with the value furnished by the formol method (Table 3).

Other drawbacks of the formol titration, such as the underestimation of primary amino acids and ammonium (85-90 % mean recovery) are compensated by the partial reaction with proline (about 17 % is quantified), (Gump, Zoecklin, Fugelsang & Whiton, 2002; Felipe-Ribeiro & Mendez-Faia, 2007), usually not considered as a contributor to YAN because it is an unsuitable source of nitrogen for yeast (Castor, 1952).

	Amino acids (mg L^{-1})			*mg N/L*
Aspartic acid	14.0	±	1.12	1.47
Glutamic acid	74.0	±	3.91	7.09
Hydroxyproline		tr		-
Serine	60.0	±	3.13	8.00
Asparagine	3.00	±	0.31	0.67
Glutamine	310	±	12.5	59.4
Glycine		tr		-
Histidine	10.5	±	1.58	0.95
Threonine	11.0	±	0.64	1.29
Arginine	453	±	14.5	109
Alanine	41.7	±	3.45	6.56
Proline	206	±	25.6	-
GABA	286	±	31.0	38.8
Tyrosine	2.21	±	0.32	0.17
Valine	14.3	±	1.33	1.69
Methionine	0.33	±	0.31	0.03
Cysteine		tr		-
Tryptophan	1.16	±	0.29	0.08
Isoleucine	8.48	±	0.56	0.90
Leucine	3.77	±	0.64	0.40
Phenylalanine	5.22	±	1.23	0.44
Ornithine		tr		-
Lysine	1.04	±	0.19	0.10
Sum	*1505*	±	*85.6*	*237*

	Biogenic amines (mg L^{-1})		
Histamine		n.d	
Tyramine		n.d	
Spermidine	3.78	±	0.21
Putrescine	2.06	±	0.18
Cadaverine		Tr	
Tryptamine		Tr	

Table 2. Amino acids and biogenic amines (mg L^{-1}±SD) composition of the initial grape juice. The right hand column shows the contribution to the YAN of each single amino acid.

3.3 Nitrogen consumption during fermentation

The course of fermentation was monitored by following the weight loss of fermentors. Fermentations were completed in 12- 16 days, depending on the yeast strain and the must treatment, when all the samples had < 2.5 g L^{-1} of sugars (Figure 2). In general, strain 333 accomplished shorter fermentation times even if the presence of sulphur dioxide resulted in a delayed onset of the tumultuous sugar consumption phase (upper diagram of Figure 2, samples S and ST), likely as a result of the prolonged lag-phase due to cells adaptation. However, starting from the 4^{th} day, the weight loss of these samples was faster and permitted the completion of fermentation within 12 days, eg. 2 days before the lysozyme-added samples (L and LT) which, on the other hand, showed quicker initial sugar consumption.

The fermentative behaviour of strain 1042 appeared less affected by sulphur dioxide in terms of initiation and duration (Figure 2, lower diagram) except for the higher sugars consumption rate in the central part of the fermentation demonstrated by samples S and ST.

At the end of fermentation, the total consumed YAN varied from 260 to 283 mg N/L (Table 3) which corresponded to the consumption of 89 and 96% available YAN, respectively. These values are lower than the 400 mg N/L reported for seven yeast strains cultivated in a synthetic medium of unrestricted nitrogen availability (Jiranek et al., 1995) but agree very well with the estimate of another study carried out on grape juice (300 mg N/L) (Bely et al., 1990). The presence of SO_2 appeared to influence the nitrogen metabolism of strain 1042, increasing the consumption of both the aminic (in samples S and ST) and the ammonium fraction (samples S) driving higher total nitrogen consumption (Table 3). It is known that ammonium is the preferred nitrogen source for yeasts (Magasanik, 2003). During the fermentation, the transport of other non-preferred amino acids across the plasma membrane is regulated by GAP1, the general amino acid permease, which is repressed when ammonium is present in grape juice (Salmon & Barre, 1998). Hence the fast and thorough consumption of ammonium may promote the uptake of the other amino acids in the juice. The figures we found are in accordance with the findings of Garde-Cerdán et al. (2007) who, by using even lower amounts of sulphites with respect to our trials (20 mg L^{-1} in place of 80 mg L^{-1}), reported very similar differences in nitrogen consumption between fermentations conducted with (328 mg N/L) and without (315 mg N/L) SO_2 addition.

Nitrogen utilization of strain 333 was shown to be less affected by sulphites addition and only sample S consumed a significantly higher amount of ammonium which, however, was not reflected in an enhanced YAN uptake.

In Table 4, the consumption of each single amino acid during the fermentation is separately reported for the two yeast strains. The data were obtained by differences between the initial amount in juice and the residual content of amino acids in final wines. With the aim to minimize the aminic nitrogen release from yeast cells after the completion of fermentation, final wines were withdrawn the same day the fermentation stopped.

The main sources of nitrogen for yeasts were the most abundant amino acids arginine, GABA, glutamine and serine which were consumed to an extent varying from 95 to 100%. All these compounds (except GABA), are considered good nitrogen sources, characterized by a fast consumption even in the presence of ammonium in the medium (Jiranek et al., 1995). On the contrary, GABA is usually consumed only in the second half of the fermentation to extents varying from 50 to 100% (Monteiro & Bisson, 1991; Garde-Cerdán et al., 2007).

Hydroxyproline, glycine and lysine were not utilized by the yeast since they are not good nitrogen sources (Castor, 1952). Furthermore, their amounts in final wines may increase due to the ethanol-driven change in permeability of the plasmatic cell membrane at the end of fermentation (Ferreras, Iglesias & Girbes, 1989; Bidan, Feuillat & Moulin, 1986) which causes a passive process of desorption.

Our results also revealed a consistent excretion of proline in wines. On the other hand, it is known that proline is not consumed under anaerobic fermentations and that its accumulation in wines is due to the metabolism of arginine, of which it is a metabolite (Martin et al., 2003).

Table 4 also shows the main effects and interactions of the tested factors on the amino acid consumption. The presence of SO_2 or lysozyme was largely the most influent factor on amino nitrogen metabolism, while tannin had virtually no influence. For both the strains, the addition of sulphites to musts before the fermentation resulted in a statistically significant increased uptake of glutamine and alanine. For the latter amino acid, this is in accordance with the findings of Garde-Cerdán et al. (2007) who, quite surprisingly, did not mention glutamine in their work. Glutamine, together with glutamate and ammonium, is the main source of cellular nitrogen and their presence may repress the utilization of other non-preferred nitrogen sources (Magasanik & Kaiser, 2002). With respect to the other compounds, the behaviour of the two strains was not consistent since they were characterized by the almost generally increased consumption of amino acids in SO_2 added samples fermented with strain 1042, while for strain 333, the opposite was true. Differences in amino acid metabolism among different yeast strains as a function of the presence or absence of sulphites during fermentation have been already reported by Dizy et al. (1996) and may be somehow linked to the general strain-dependent resistance of yeasts to sulphites.

In fact, despite the well established influence of sulphite on microbial growth and metabolism, little is known on its possible impact on amino acids assimilation during fermentation.

Sulphite acts both on glycolysis and respiratory chain phosphorylation in yeasts causing ATP depletion, as a consequence of the inhibition of a number of enzymes, such as glyceraldehyde-3-phosphate dehydrogenase and glutamate dehydrogenase (inhibited by 98 and 60% respectively) (Maier et al., 1986). In such conditions, cells verify an energy deficiency status which may require the modification of some metabolic pathways to gain a more efficient utilization of the energy. It can be shown, for example, that to incorporate ammonia for the synthesis of other amino acids, the use of glutamine in place of glutamate represents a suitable way to reduce the energy needs of the cell (Magasanik, 2003). This could justify the increased consumption of glutamine in the fermentations carried out with sulphite. In addition, the decline of the intracellular pH which follows the entry of SO_2 into the cell lowers the transmembrane pH gradient, dissipating the proton-motive force across the membrane (Pilkington & Rose, 1988). This may result in a diminished efficiency of processes such as the active transport of solutes and amino acids, which requires the proton-motive force, leading to modifications of the metabolic behaviour of the yeast. In the already cited studies of Garde-Cerdán et al. (2007) and Polo et al. (1996), the effects of SO_2 on nitrogen consumption have been investigated in Spanish musts. A number of their findings substantially agree with ours, although no tentative explanation for the phenomena involved was given.

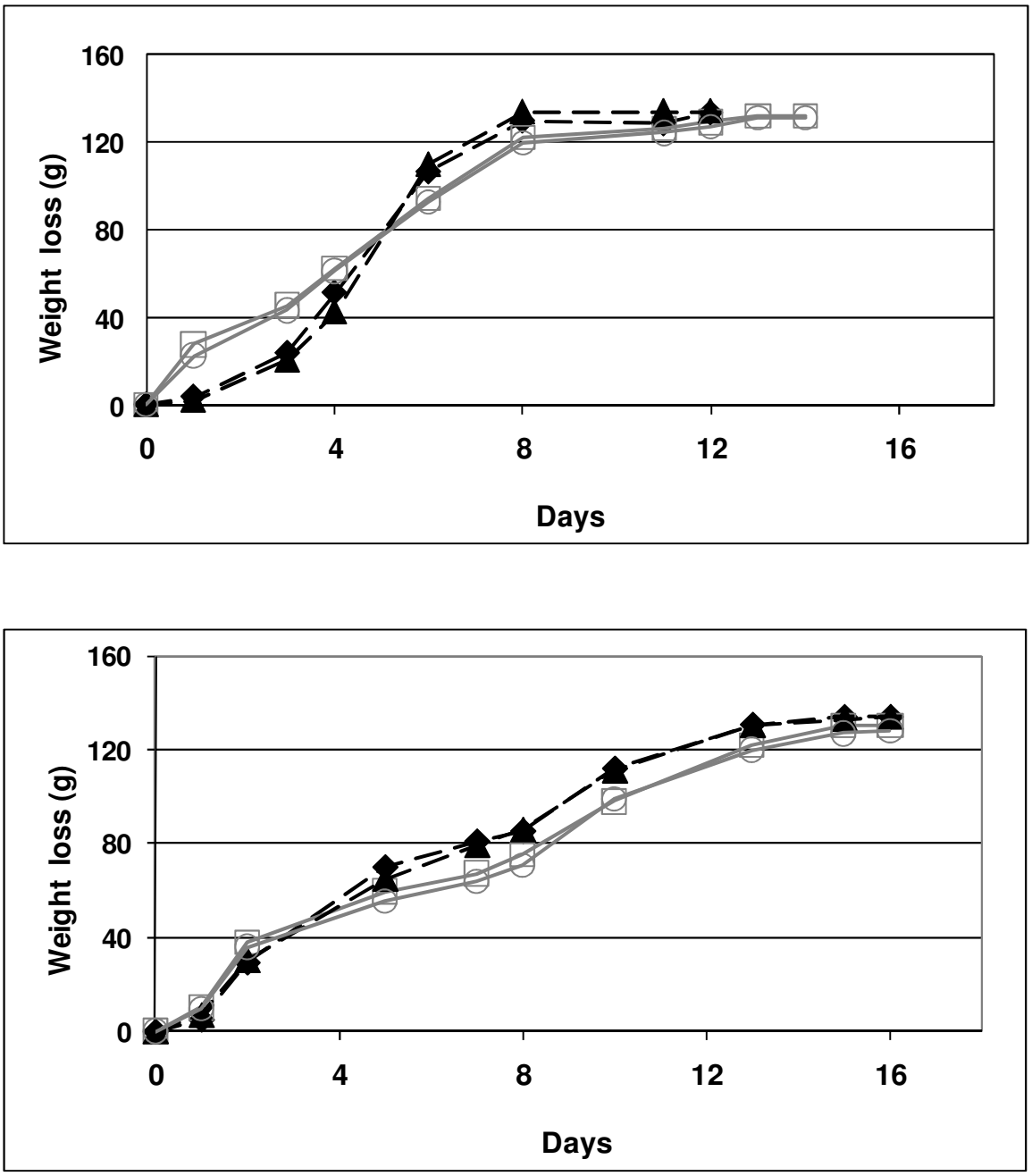

Figure 2: Weight loss of the grape musts during fermentation. Upper and lower panel refer to yeast strain 333 and 1042 respectively.

Table 3. Nitrogen (mg N/L ± SD) amount in the initial must and its uptake by the two yeast strains after the completion of the alcoholic fermentation.
In the same column, different letters flag significantly differences for $p < 0.01$

		Ammonium Nitrogen (mg N/L ± SD)	Amino Nitrogen (mg N/L ± SD)	YAN (mg N/L ± SD)
		Initial nitrogen amount		
Must	HPLC*	56.5 ± 4.4	237 ± 12.1	294 ± 17.4
	Formol*	-	-	224 ± 25.6
		Nitrogen uptake		
Strain 333	S	47.9 ± 1.2^{c}	215 ± 4.60^{a}	264 ± 5.59^{a}
	ST	41.7 ± 1.6^{a}	217 ± 1.84^{a}	260 ± 3.24^{a}
	L	41.3 ± 0.5^{a}	221 ± 1.21^{a}	262 ± 1.32^{a}
	LT	42.7 ± 1.2^{ab}	221 ± 0.66^{a}	264 ± 0.70^{a}
Strain 1042	S	48.5 ± 1.4^{c}	234 ± 0.46^{b}	283 ± 1.20^{b}
	ST	46.3 ± 0.1^{b}	231 ± 4.79^{b}	278 ± 4.70^{b}
	L	45.5 ± 0.8^{b}	220 ± 0.91^{a}	266 ± 0.92^{a}
	LT	45.6 ± 2.0^{b}	220 ± 2.83^{a}	266 ± 4.70^{a}

* Method used to estimate the nitrogen fraction

Table 4: Mean amino acid consumption (mg L^{-1} ± SD) during the fermentation of each single trials (n=6) and influence of the tested factors as assessed by factorial Anova. Excretion of compounds is flagged with negative values.

	S	ST	L	LT	Main Effects[a]	
					Lysozyme/SO_2	Tannin
			Strain 333			
Aspartic acid	9.67 ± 1.80	9.15 ± 1.04	13.7 ± 0.22	13.8 ± 0.06	*	
Glutamic acid	47.6 ± 1.52	46.9 ± 2.34	51.5 ± 1.30	52.1 ± 2.00	*	
Hydroxyproline	-6.49 ± 1.16	-0.86 ± 0.22	-5.16 ± 0.43	-5.78 ± 0.61		
Serine	60.1 ± 0.00	60.1 ± 0.00	60.1 ± 0.00	60.1 ± 0.00		
Asparagine	-5.18 ± 0.54	-1.34 ± 4.63	1.00 ± 2.23	1.47 ± 0.08	*	
Glutamine	297 ± 2.29	294 ± 2.12	283 ± 2.65	278 ± 4.72	*	
Glycine	-4.88 ± 0.06	-3.21 ± 0.44	-3.23 ± 0.11	-2.97 ± 0.08	*	*
Histidine	10.32 ± 0.34	7.14 ± 0.30	10.1 ± 0.05	9.84 ± 0.61		*
Threonine	7.86 ± 0.81	9.23 ± 0.63	10.1 ± 0.25	10.1 ± 0.39		
Arginine	431 ± 3.14	437 ± 2.52	433 ± 0.43	436 ± 0.65		
Alanine	29.6 ± 0.70	28.1 ± 1.39	26.2 ± 0.23	25.4 ± 0.92	*	
Proline	-577 ± 149	-528 ± 22.3	-425 ± 45.7	-485 ± 144		
GABA	267 ± 15.1	258 ± 4.89	286 ± 0.00	286 ± 0.00	*	
Tyrosine	2.61 ± 3.38	1.01 ± 0.21	1.51 ± 0.22	1.79 ± 0.05		
Valine	12.8 ± 0.75	14.1 ± 0.00	14.1 ± 0.11	14.1 ± 0.00		
Methionine	-2.93 ± 0.92	0.33 ± 0.00	-0.63 ± 0.23	-0.99 ± 0.14		*
Cysteine	n.d.	n.d.	n.d.	n.d.		
Tryptophan	1.13 ± 0.06	0.95 ± 0.07	0.92 ± 0.09	0.97 ± 0.01	*	
Isoleucine	4.61 ± 1.55	4.91 ± 0.43	7.25 ± 0.11	7.40 ± 0.28	*	
Leucine	0.17 ± 0.85	-0.05 ± 0.93	2.50 ± 0.22	2.87 ± 0.38	*	
Phenylalanine	2.61 ± 0.46	1.95 ± 0.16	3.91 ± 0.11	4.93 ± 0.13	*	
Ornithine	n.d.	n.d.	n.d.	n.d.		
Lysine	-10.1 ± 1.18	-11.6 ± 1.85	-6.51 ± 0.06	-4.80 ± 0.21	*	
			Strain 1042			
Aspartic acid	14.0 ± 0.00	14.0 ± 0.00	13.7 ± 0.05	13.1 ± 0.74		
Glutamic acid	72.2 ± 0.86	71.8 ± 0.54	44.9 ± 1.00	41.1 ± 4.64	*	
Hydroxyproline	0.00 ± 0.00	0.00 ± 0.00	-3.65 ± 0.80	0.00 ± 0.00		
Serine	60.1 ± 0.00	60.1 ± 0.00	60.1 ± 0.00	60.1 ± 0.00		
Asparagine	3.14 ± 0.00	3.08 ± 0.06	1.07 ± 0.10	1.91 ± 0.11	*	
Glutamine	307 ± 0.57	305 ± 1.64	287 ± 3.08	295 ± 11.8	*	
Glycine	0.00 ± 0.00	0.00 ± 0.00	-0.46 ± 0.08	-0.73 ± 0.08	*	
Histidine	10.5 ± 0.04	10.5 ± 0.03	9.47 ± 0.47	5.86 ± 0.26	*	
Threonine	11.0 ± 0.00	9.26 ± 1.51	9.84 ± 0.06	10.1 ± 0.79		
Arginine	448 ± 0.13	452 ± 2.45	426 ± 0.84	427 ± 1.97	*	
Alanine	36.6 ± 0.27	36.6 ± 0.30	27.8 ± 0.45	26.0 ± 0.55	*	
Proline	-93.6 ± 25.2	-113 ± 191	-203 ± 60.6	-289 ± 84.0		
GABA	285 ± 2.41	282 ± 3.62	286 ± 0.00	286 ± 0.00		
Tyrosine	2.21 ± 0.00	2.21 ± 0.00	2.05 ± 0.08	1.79 ± 0.20	*	
Valine	14.1 ± 0.00	14.1 ± 0.00	13.9 ± 0.08	14.1 ± 0.00		
Methionine	0.33 ± 0.00	0.33 ± 0.00	-0.30 ± 0.01	-1.04 ± 0.20	*	
Cysteine	n.d.	n.d.	n.d.	n.d.		
Tryptophan	1.15 ± 0.01	1.16 ± 0.00	1.01 ± 0.05	0.99 ± 0.10	*	
Isoleucine	8.13 ± 0.08	8.48 ± 0.00	7.09 ± 0.09	6.43 ± 0.13	*	
Leucine	3.77 ± 0.00	3.77 ± 0.00	2.79 ± 0.05	1.79 ± 0.18	*	
Phenylalanine	5.22 ± 0.00	5.22 ± 0.00	4.34 ± 0.05	3.27 ± 0.23	*	
Ornithine	n.d.	n.d.	n.d.	n.d.		
Lysine	1.05 ± 0.00	1.05 ± 0.00	-5.89 ± 0.46	-7.35 ± 1.40	*	

[a] asterisks denote significant effects at $p \leq 0.01$

3.4 Biogenic amines content of final wines

Table 5 shows the mean concentration of biogenic amines in wines. Their amounts spanned between about 3.0 mg L^{-1} and 7.5 mg $L^{-}1$ as a sum. Putrescine, tryptamine and histamine were the compounds most widely detected in the samples. By contrast, tyramine and cadaverine were found in few samples only at trace levels. According to Gónzalez-Marco, Jimenez-Moreno and Ancín-Azpilicueta (2006), both the strains we used have been demonstrated to produce putrescine, tryptamine and histamine during fermentation even though, for this latter compound, *Saccharomyces cerevisiae* has been previously classified as a very low producer (Caruso, Fiore, Contursi, Salzano, Paparella & Romano, 2002). Spermidine decreased during alcoholic fermentation in spite of the relevant presence of arginine, its precursor, in the must, confirming the lack of correlation between the presence of precursor amino acids in the juice and the amount of derived amines after fermentation (Gónzalez-Marco et al., 2006).

Overall, and regardless of the strain, the data shown in Table 5 indicate that the pre-fermentative addition to grape must of lysozyme or tannin had no specific influence on the final amount of biogenic amines in the wines. Sulphites, also, appeared not to influence their presence in white wines, confirming the findings of Vidal-Carou, Codony-Salcedo and Mariné-Font (1990). ANOVA analysis carried out on this data set did not reveal a significant effect for the variables investigated.

	S	ST	L	LT
		Strain 333		
Histamine	n.d.	2.27 ± 1.74	2.62 ± 0.52	n.d.
Tyramine	tr	n.d.	n.d.	tr
Spermidine	n.d.	n.d.	n.d.	1.84 ± 0.18
Putrescine	4.20 ± 0.43	4.20 ± 0.14	2.43 ± 0.01	tr
Cadaverine	tr	tr	n.d.	tr
Tryptamine	tr	1.04 ± 0.08	n.d.	1.08 ± 0.14
Sum	***4.20 ± 0.43***	***7.51 ± 2.83***	***5.15 ± 0.53***	***2.94 ± 0.45***
		Strain 1042		
Histamine	tr	1.53 ± 0.15	1.91 ± 0.50	tr
Tyramine	tr	tr	tr	tr
Spermidine	tr	tr	1.96 ± 0.12	tr
Putrescine	3.01 ± 0.89	3.73 ± 0.11	2.97 ± 0.16	3.12 ± 0.60
Cadaverine	tr	tr	tr	tr
Tryptamine	1.02 ± 0.08	tr	0.70 ± 0.08	0.90 ± 0.04
Sum	***3.24 ± 0.96***	***5.28 ± 0.27***	***7.56 ± 0.65***	***4.01 ± 0.65***

Table 5: Concentration of biogenic amines (mg L^{-1} ± SD) in final wines (n=6).

4. CONCLUSIONS

This study demonstrated that, under our experimental conditions, the consumption of amino acids and ammonium during fermentation was influenced by the presence of SO_2, and also as a function of the yeast strain we used. Only strain 1042 consumed a higher amount of ammonium and total YAN in the presence of SO_2, while for strain 333, no significant difference in total nitrogen uptake was found between the samples. Both the strains demonstrated an increased utilization of glutamine and alanine in samples with SO_2 addition. For the other amino acids, the two strains behaved in an opposite manner, with enhanced uptake in lysozyme-added samples for strain 333 whereas strain 1042 lowered its consumption. Lastly, the presence of biogenic amines in final wines was not influenced by the must treatments. In order to confirm these findings on other grape cultivars and yeast strains, further studies may be necessary and are under consideration by our research group.

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Chapter 4

Replacement of sulphur dioxide by lysozyme and oenological tannins during fermentation: influence of bottle storage on the evolution of volatile compounds of white wines

***ABSTRACT**: The aim of this work was to study the effects on volatile compounds in white wines by substituting SO_2 during fermentation with lysozyme and oenological tannins.*
For this purpose, a series of laboratory fermented Sauvignon Blanc musts with and without added SO_2, which were stored in bottles at room temperature for 1 year, were analysed by gas-chromatography mass spectrometry (GC-MS) to determine the content of major alcohols, esters and acids at the end of the alcoholic fermentation and during their later evolution. At the same time, the technological performances of two strains of yeast that produce low amounts of SO_2 were evaluated.
The results showed that the addition of lysozyme and oenological tannins during alcoholic fermentation could positively replace the use of sulphur dioxide. The different vinification protocols influenced the volatile composition of final wines, also as a consequence of the yeast's response to the presence or absence of sulphites during fermentation.
The volatile compound evolution of such wines during bottle storage showed a strong influence in the presence of SO_2 on the evolution of alcohols and esters. Also the presence of oenological tannins displayed a positive role in scavenging oxygen and maintaining the amounts of esters over certain levels in wine stored for 1 year. By contrast, acids were virtually unaffected by SO_2, lysozyme or tannins during the storage time.

1. INTRODUCTION

Wine is a complex mixture of hundreds of compounds, many of which contribute substantially to the colour, mouthfeel and aromatic properties of this beverage. As a result of their pronounced effect on our sensory organs, aroma compounds play a definitive role in the quality evaluation of foodstuffs. Various factors can influence the quality of grapes and wine, including the cultivar, soil quality, water management, vine canopy management and the ripeness of the grapes. Technological aspects and vinification methods (like methodologies used for grape crushing, must treatment and skin contact time), fermentation conditions (such as pH, temperature, yeast flora) and aging of wine (such as bottle or wood maturation) also have a significant influence on the final aroma (Swiegers et al., 2005; Ribéreau-Gayon et al., 2000a,b; Rapp, 1990; Boulton et al., 1998).

When dealing with wine aroma, a distinction is made among:

- *primary* or *grape aroma*: aroma compounds as they occur in the undamaged plant cells of the grape;

- *secondary grape aroma*: aroma compounds formed during the processing of the grapes (crushing, pressing, skin contact) and by chemical, enzymatic-chemical, and thermal reactions in grape must;
- *fermentation bouquet*: aroma compounds formed during the alcoholic fermentation;
- *maturation bouquet*: caused by chemical reactions during maturation of the wine.

1.1 Primary and Secondary Aroma

The monoterpenes are a class of natural compounds (terpene ethers, monoterpene alcohols, monoterpene diols) that contribute important floral and citrus character to wines. They are produced by higher plants, algae, fungi and even some yeasts, from a common precursor, geranyl pyrophosphate (GPP). In particular, one of the plant species that produce monoterpenoids is *V. vinifera* (grapes). These monoterpenes are largely localized in the skin, their overall levels increase during grape maturation and they are extracted into the wines from the grapes during the alcoholic fermentation (Ebeler et al., 2009; Swiegers et al., 2005).

The terpene composition of grapes can be influenced by climate and viticultural conditions. By contrast, numerous studies have shown that monoterpenes compounds are not changed by yeast metabolism during fermentation (except for geraniol and nerol), and therefore they can be used analytically for varietal characterisation (Ebeler et al., 2009; Rapp et al., 1996; Rapp, 1990).

Recently, work conducted by Carrau et al., (2005) suggested that some strains of *Saccharomyces cerevisiae* can contribute to the floral aroma of wine by *de novo* biosynthesis of some monoterpenes (sterols and terpenes), and this contribution could be augmented by certain fermentation conditions such as musts with higher concentrations of assimilable nitrogen, for example from the ammonium ion, in combination with microaerobic fermentation.

Another group of compounds that belong to the grape aroma are the methoxypyrazines. In the late 1960's, Buttery et al (1969) identified 3-isobutyl-2-methoxypyrazine (IBMP) as the main impact compound responsible for the aroma of bell peppers and associated this aroma character with the aroma of Cabernet Sauvignon and Sauvignon Blanc grape varieties. In addition to IBMP, 2-sec-butyl-3-methoxypyrazine (SBMP) and 3-isopropyl- 2-methoxypyrazine (IPMP) have also been identified in grapes and wines. IBMP levels range from 4 to 30 ng L^{-1}, depending on variety, maturity and growing conditions. High levels (>15 ng L^{-1} in white wines, >25 ng L^{-1} in red wines) contribute to an "undesirable" herbaceous aroma in wines and each of the pyrazines have slightly different aroma qualities. While IBMP is described as having a bell pepper/green gooseberry aroma, IPMP is described as asparagus/green bean and SBMP as

pea/bell pepper. It is important to note, however, that not all vegetal aromas can be related to methoxypyrazines (Buttery et al., 1969; Ebeler et al., 2009).

Heymann et al. (1986) reported that IBMP was readily degraded with light exposure and its level seemed to be influenced by grape maturity (levels decrease with maturation), temperature of the grape cluster, microclimate (cooler climates have higher levels), pruning (i.e, manipulation of buds per vine), and vine water potential (Heymann et al., 1986; Ebeler et al., 2009).

1.2 Aroma compounds formed during fermentation

While some aroma compounds arise directly from chemical components of the grapes, many grape-derived compounds are released and/or modified by the action of flavour-active yeast and bacteria, and a further substantial portion of wine flavour substances result from the metabolic activities of these wine microbes (Swiegers et al., 2005; Schreirer 1979). It is for this reason that wine has more flavour than the grape juice from which it was produced.

Apart from ethanol and glycerol, as well as diols and higher alcohols (2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol) numerous other wine constituents are formed by yeast metabolism (especially acids, esters, aldehydes, ketones and sulphur compounds), as is shown in Fig.1 (Rapp, 1990).

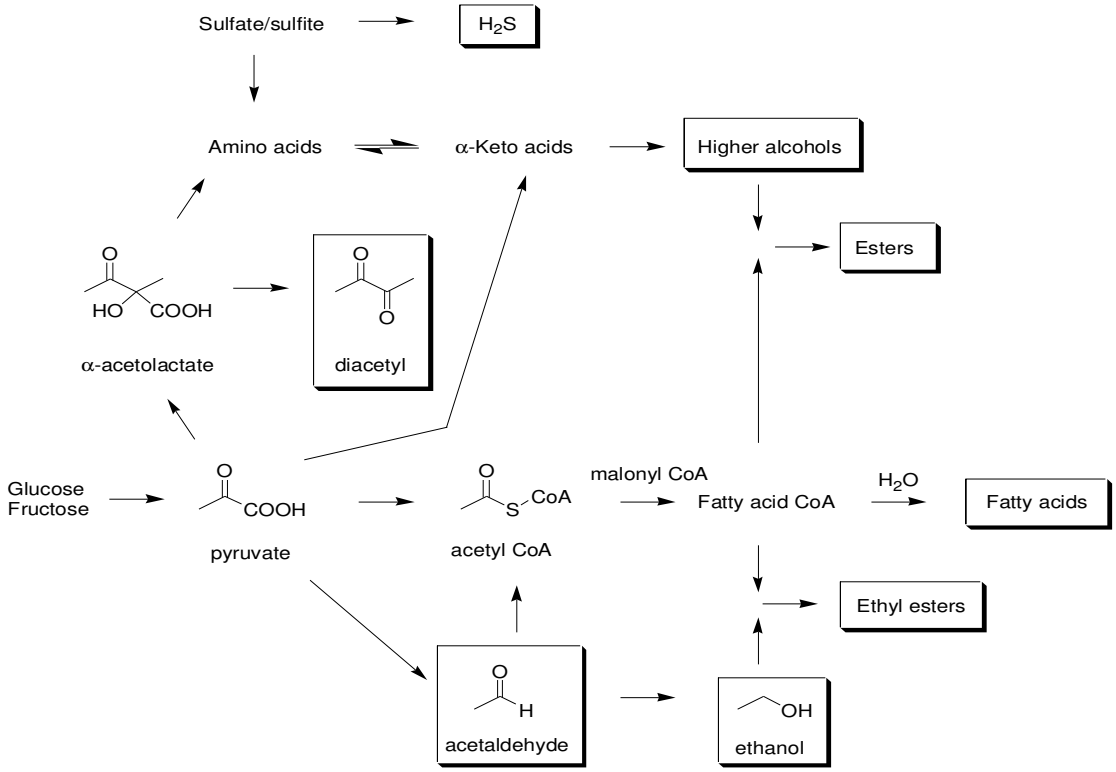


Fig 1. Representation of derivation and synthesis of flavour-active compounds from sugar, amino acids and sulphur metabolism by wine yeast

1.2.1 Volatile Acids

Little is known concerning volatile carboxylic acids (e.g. fatty acids) in grapes. Older papers report their presence and composition only in grape seed oil, the main fraction being unsaturated fatty acids, e.g. linoleic acid (Kliewer et al., 1967; Schreier, 1979). Fatty-acids, a group of volatile organic acids with an aliphatic chain, are formed earlier during the alcoholic fermentation and in higher concentration than their corresponding fatty acid ethyl esters. In particular, the contents of almost all fatty acids (from C-4 to C-10) increase during fermentation, whereas the long-chain acids (from C-16 to C-18) decrease (Herraiz et al., 1990). The presence or absence of sulphur dioxide in fermentation also seems to influence their evolution, showing an increase of hexanoic, octanoic and decanoic acids in wines produced without SO_2 addition compared to others fermented in the presence of SO_2 (Sonni et al., 2009).

Fatty acids contribute to a fresh flavour in wine, or for an unpleasant flavour if they are in excess, and they also help to modify the perception of other taste sensations (Ribéreau-Gayon et al., 2007). The total fatty acid concentration in wines samples was found to be around 15 - 25 mg L^{-1} (Miranda-Lopez et al., 1992). They represent only the 10-15% of the total acid content of wines, the rest being constituted by acetic acid (Fowles 1992, Henschke and Jiranek 1993, Radler 1993).

Acetic acid is of particular importance, because at elevated concentrations it imparts a vinegar-like character to wine. Acetic acid becomes objectionable at concentrations of 0.7–1.1 g L^{-1}, depending on the style of wine; the optimal concentration is 0.2–0.7 g L^{-1} (Corison et al. 1979, Dubois 1994). Acetic acid production by the strains of *Saccharomyces cerevisiae* used in winemaking has been reported to vary widely and, during fermentation, as little as 100 mg L^{-1} and up to 2 g L^{-1} are produced (Radler 1993). Strains in current use tend to produce acetic acid concentrations at the lower end of the range for dry wines but tend to higher values for sweet wines (Monk and Cowley 1984, Henschke and Dixon 1990, Millan et al. 1991, Bely et al. 2003, Erasmus et al. 2004).

Although *Saccharomyces spp.* can produce acetic acid, excessive concentrations in wine are largely the result of metabolism of ethanol by aerobic acetic acid bacteria, like *Gluconobacter oxydans*, *Acetobacter aceti*, *Acetobacter pasteurianus*, *Gluconacetobacter liquefaciens* and *Gluconacetobacter hansenii*. Injudicious aeration during and/or after the winemaking process can result in the growth and activity of acetic acid bacteria, high volatile acidity and a vinegary taint in wine.

The oxidation of ethanol to acetic acid is the best-known characteristic of these wine-associated acetic acid bacteria. In this reaction, a membrane-bound alcohol dehydrogenase oxidises ethanol

to acetaldehyde, which is further oxidised to acetate by a membrane-bound aldehyde dehydrogenase. The concentration of oxygen required for metabolic activity and survival in wine is much lower than previously thought; acetic acid bacteria can survive in wine barrels for long periods of low oxygen tension and, somewhat unexpectedly, spoilage of bottled red wine by acetic acid bacteria has been reported (Drysdale and Fleet 1988, Bartowsky et al. 2003).

A small increase in volatile acids is often observed after the completion of malolactic fermentation conducted by malolactic bacteria. Two pathways can be involved. Acetic acid can be produced from residual sugar through heterolactic metabolism (phosphoketolase pathway) (Henick-Kling 1993, Ribéreau-Gayon et al. 2000a) and the first step in citric acid metabolism produces acetic acid (Cogan 1987, Ramos et al. 1995, Ramos and Santos 1996).

1.2.2 Alcohols

Higher alcohols

Higher alcohols (also known as fusel alcohols) are secondary yeast metabolites, and can have both positive and negative impacts on the aroma and flavour of wine. Excessive concentrations of higher alcohols can result in a strong, pungent smell and taste, whereas optimal levels impart fruity characters (Nykänen et al. 1977, Lambrechts et al., 2000, Swiegers et al., 2005).

Some of the most important alcohols that contribute to wine flavour are shown in Table 1. Higher alcohols are divided into two categories, aliphatic and aromatic alcohols, and are also extremely important in wine and distillates (Nykänen et al. 1977). The aliphatic alcohols include propanol, isoamyl alcohol, isobutanol and active amyl alcohol. The aromatic alcohols consist of 2-phenylethyl alcohol and tyrosol. It has been reported that concentrations below 300 mg L^{-1} add a desirable level of complexity to wine, whereas concentrations that exceed 400 mg L^{-1} can have a detrimental effect (Rapp et al., 1996). The use of different yeast strains during fermentation contributes considerably to variations in higher alcohol profiles and concentrations in wine (Rankine 1968b, Giudici et al. 1990). The concentration of amino acids (the precursors for higher alcohols) in the must also influence higher alcohol production, where the total production of higher alcohols increases as concentrations of the corresponding amino acids increase (Schulthess and Ettlinger 1978). In the majority of studies nitrogen applied in the vineyard decreased the higher alcohol concentration in wine compared to wine prepared from vines that received no nitrogen (Bell et al., 2005). Furthermore, ethanol concentration, fermentation temperature, the pH and composition of grape must, aeration, level of solids, grape variety, maturity and skin contact time also affect the concentration of higher alcohols in the final product (Fleet and Heard 1993). In case of SO_2 addition (80 mg L^{-1}) in fermentation, some

alcohols (like 3-methyl-1-butanol, 3-methyltio-1-propanol, phenylethyl alcohol and 4-hydroxy-benzenethanol) were found at higher concentrations in wines fermented with SO_2 compared to wines fermented without SO_2, likely as a consequence of the increased consumption of musts amino acids, promoted by sulphites, during fermentation (Sonni et al., 2009).

Wine alcohols, in fact, can be formed during fermentation by two different ways: a catabolic process starting from amino acid-derivatives *α*-ketoacid (the Ehrlich pathway) and an anabolic process starting from *α*-ketoacids acting as intermediates in cell glucose metabolism (Bell et al., 2005; Hernandez-Orte et al., 2006; Nykanen, 1986).

Concerning the Ehrlich pathway, the first step in the catabolism of branched-chain amino acids is transamination to form the respective α-keto acids (e.g. α-ketobutyric acid from threonine, α-ketoisocaproic acid from leucine, α-ketoisovaleric acid from valine, and α-keto-β-methylvaleric acid from isoleucine). A pyruvate decarboxylase converts the resulting α-keto acid to the corresponding branched-chain aldehyde with one less carbon atom, and alcohol dehydrogenase catalyses the NADH-dependent reduction of this aldehyde to the corresponding fusel alcohol (1-propanol, isoamyl alcohol, isobutanol, active amyl alcohol) (Bell et al., 2005; Dickinson et al., 1993) (Fig. 2).

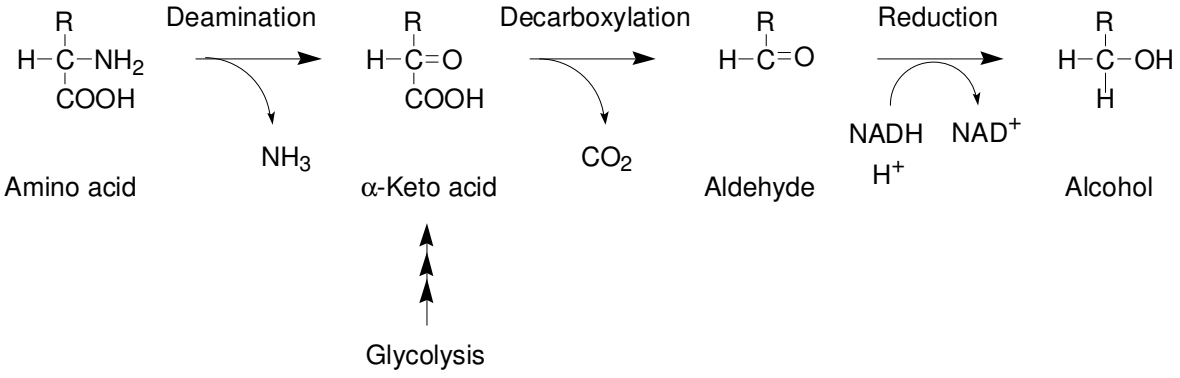
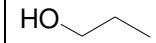
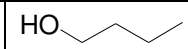
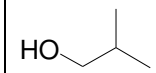
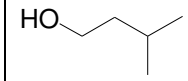
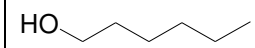
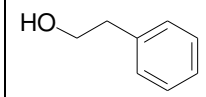
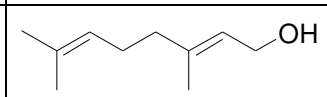
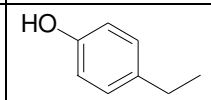
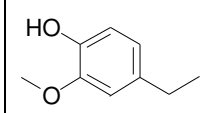
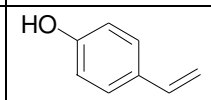
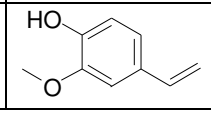


Figure 2. The Ehrlich pathway for the formation of higher alcohols from amino acids and sugar (Bell et al., 2005).

Table 1. A summary of the major alcohols reported in wine: their structure, aroma characteristics, concentration in wine and aroma thresholds (Sweigers et al., 2005).

Compound Name	**Structure**	**Reported Aroma charcteristics**	**Concentration in wine (mg L^{-1})**	**Aroma threshold (mg L^{-1})**
1-propanol		Pungent, harsh	9.0–68	500**
1-butanol		Fusel, spiritous	0.5–8.5	150*
Isobutanol		Fusel, spiritous	9.0–174	40*
Isoamyl alcohol		Harsh, nail polish	6.0–490	30*
Hexanol		Green, grass	0.3–12.0	4**
2-Phenylethyl alcohol		Floral, rose	4.0–197	10*
trans-3,7-Dimethyl-2,6-octadien-1-ol (Geraniol)		Rose-like	0.001–0.044	5******/30*
4-ethylphenol		Medicinal, barnyard	0.012–6.5	0.14*/0.6***
4-ethyl guaiacol		Phenolic, sweet	0.001–0.44	0.033*/0.11***
4-vinyl phenol		phamaceutical	0.04–0.45	0.02******
4-vinyl guaiacol		Clove-like, phenolic	0.0014–0.71	10******

* 10% ethanol, ** wine, *** red wine, **** beer, ***** synthetic wine, ****** water

Ethanol

Ethanol is an alcohol produced during alcoholic fermentation starting from sugar grapes and its concentration affects the sensory perception of wine flavour-active compounds.

The presence of ethanol is essential to enhance the sensory attributes of other wine components, while an excessive amount can produce a perceived 'hotness' and mask the overall aroma and flavour of wine (Guth et al., 2002; Bell et al., 2005).

This, along with heightened health consciousness, stricter drinking and driving laws, and increased tax rates associated with high ethanol wines, have increased the demand for wines with reduced alcohol concentrations, putting pressure on wine producers, particularly those in warm climates where grape sugar levels can become high (Day et al. 2002).

The removal or reduction of alcohol in wine can be achieved by various physical processes, including reverse osmosis, adsorption, distillation, centrifugation, evaporation, extraction, freeze concentration, membrane, and partial fermentation. There are restrictions on the use of some of these techniques in some countries because they can cause a detrimental loss or modifications to aroma and flavour compounds during the process (Swiegers et al., 2005).

1.2.3 Esters

Esters, a large group of flavour compounds, are considered the next major constituents in wine after water, ethanol and fusel alcohols and the primary source of fruity aromas. As such, esters are extremely important for the flavour profile of fermented beverages as wine, with the presence of different esters often having a synergistic effect, impacting on the individual flavours well below their individual threshold concentrations. The fact that most esters are present in concentrations around their threshold value implies that modest concentration changes might have a dramatic effect on wine flavour (Sumby et al., 2009).

Esters are formed when alcohol and carboxylic acid functional groups react, and a water molecule is eliminated. In wine, esters can be classified into two groups: those formed enzymatically by esterase, lipases and alcohol acetyltransferases enzymes, in which are included esters like ethyl acetate, ethyl butanoate, ethyl hexanoate and ethyl octanoate; and the other formed during wine ageing, by chemical esterification between alcohol and acids at low pH (Margalit, 1997). Enzymatic accumulation of esters in wines during fermentation is known to be the result of a balance of the enzymatic synthesis and hydrolysis reactions involving esterase and lipase, and synthesis reactions involving alcohol acetyltransferases. Substrates for these enzymes are alcohols or thiols and fatty acids (or their acyl CoA-activated forms) produced during the lipid, sugar and amino acid metabolism (Sumby et al., 2009).

With a large number of different acids and alcohols in wine there is considerable potential for the formation of a wide range of esters, many of which are in fact found in wine (Table 2). The C4–C10 ethyl esters of organic acids, ethyl esters of straight chain fatty acids (ethyl esters of branched chain fatty acids to a lesser degree) and acetates of higher alcohols are largely responsible for the fruity aroma of wine and are particularly pronounced in young wines (Ebeler, 2001). The ethyl esters are comprised of an alcohol group (ethanol) and an acid group (medium-chain fatty acid) and include ethyl hexanoate, ethyl octanoate and ethyl decanoate. The acetate esters are comprised of an acid group (acetate) and an alcohol group which is either ethanol or a complex alcohol derived from amino acid metabolism, and includes esters such as ethyl acetate and isoamyl acetate. In particular, ethyl acetate is qualitatively the most common ester in wine, due to its ready formation from the predominant ethanol and acetic acid. It is often an important contributor to wine aroma; at low concentrations (around 20 mg L^{-1}) it gives a desirable and fruity character to the wine, while at higher concentrations (around 50 mg L^{-1}) imparting a solvent/nail varnish- like aroma (Saerens et al., 2008; Swiegers et al., 2005; Ribereau-Gayon et al., 2000b).

The formation of esters during fermentation is a dynamic process with numerous variables interacting, like the quantity of esters or their precursors originally present in the grape, the temperature of fermentation, the yeast strain that predominates and the nutrients present, especially the concentration of nitrogen compounds and must solids (Sumby et al., 2009).

The average esters production and their relative proportions are highly dependent on the yeast strain and the influence of other parameters, such as temperature, oxygen and nitrogen (Vilanova et al., 2007; Vila et al., 1998; Lema et al., 1996).

The effect of different variables on wine composition is well documented. For example, a recent study using a commercial wine yeast strain reported that there were higher concentrations of fresh and fruity aromas after fermentation at 15 °C as opposed to a 28 °C fermentation, which produced higher concentrations of compounds with flowery aroma (Molina et al., 2007).

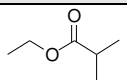
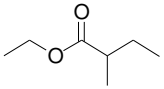
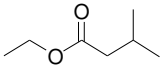
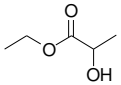
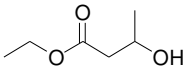
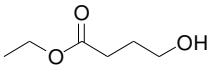
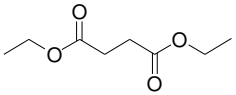
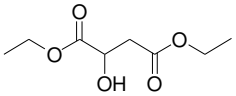
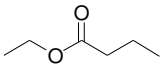
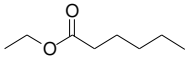
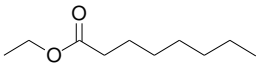
Concerning the role of SO_2 addition in winemaking on ester production, Sonni et al., (2009) reported that higher concentrations of medium-chain fatty acid ethyl esters (MCFA ethyl esters) were found in SO_2-free wine obtained with lysozyme addition at values above their threshold level, hence contributing to the fruity aroma of the wines. On the contrary, other authors noted that some MCFA ethyl esters increased in wine fermented with SO_2, suggesting that the effect of SO_2 addition on ester production does not seem to be systematic and may depend on several factors, such as O_2 availability (Nikanen, 1986; Bardi et al., 1998; Mioi et al., 2004).

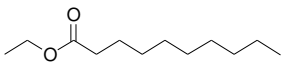
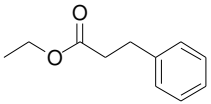
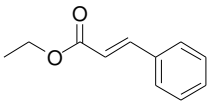
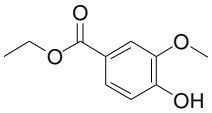
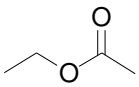
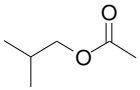
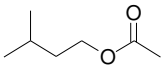
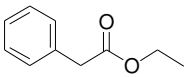
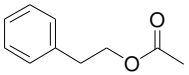
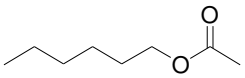
The overall volatile composition of most grape varieties is similar despite clear differences in their aromas. Most varietal differences occur from changes in relative ratios of volatile compounds. There is considerable variability in ester content amongst different grape cultivars. Ferreira et al. (2000) reported that the yeast-derived esters are strongly linked to the variety of grape. Gurbuz et al. (2006) have also reported that Australian Merlot had a higher proportion of esters (83%) amongst the identified volatiles, compared to a Californian Merlot (60%) and Cabernet Sauvignon from the same sources. This study used commercially available finished wines from the same region (Barossa Valley in Australia and Napa Valley in the USA), however differences in winemaking practice were not taken into account.

Malolactic fermentation (MLF), involving the bioconversion of malic acid to lactic acid and carbon dioxide, can also impact on the ester profile of the final wine product in particular with the ester ethyl 2-hydroxypropanoate (ethyl lactate). Its production is coupled to lactic acid formation and its synthesis can be correlated with the percentage degradation of malic acid (Sumby et al., 2009).

After significant modifications in composition during fermentation, chemical constituents generally react slowly during ageing to move to their equilibrium position, resulting in gradual changes in flavour. A wide variability in the development of esters during wine maturation was found, which will be discussed further in section 1.4 (Moreno et al., 2006; Sivertsen et al., 2001; Ramey et al., 1980).

Table 2. A summary of the major esters reported in wine: their structure, aroma characteristics, concentration in wine and aroma thresholds (Sumby et al., 2009; Sweigers et al., 2005).

Compound Name	**Structure**	**Reported Aroma charcteristics**	**Reported concentration in wine (mg L^{-1})**	**Aroma threshold (mg L^{-1})**
Ethyl 2-methylpropanoate (ethyl isobutyrate)		Fruity, strawberry, lemon	0.01–0.48	0.001, 0.015a, s, 5.0b
Ethyl 2-methylbutanoate		Apple, strawberry, berry, cider, anise	Trace-0.03	0.0001, 0.001a, 0.018s
Ethyl 3-methylbutanoate (ethyl isovalerate)		Sweet fruit, pineapple, lemon, anise, floral	Trace-0.07	0.0001, 0.003a, s, 1.3b
Ethyl 2-hydroxypropanoate (ethyl lactate)		Milk, soapy, buttery, fruity	3.05–297.5	0.05–0.2, 150w
Ethyl 3-hydroxybutanoate		Fruity (winey), green, marshmallow	0.05–0.58	20
Ethyl 4-hydroxybutanoate		Caramel	6.61	NR
Diethyl butanedioate (diethyl succinate)		Fruity, fermented, floral	1.21–61.11	NR
Diethyl hydroxybutandioate (diethyl malate)		Brown sugar, sweet	0.81	NR
Ethyl butanoate		Floral, fruity, strawberry, sweet	0.07–0.53	0.001, 0.02a, 0.4b
Ethyl hexanoate		Fruity, strawberry, green apple, anise	0.15–1.64	0.005a, s, 0.08w, 0.85w
Ethyl octanoate		Sweet, fruity, ripe fruit, burned, beer	0.14–2.61	0.002s, 0.005a, 0.012, 0.58w

Ethyl decanoate		Oily, fruity (grape), floral	0.01–0.70	0.2s, 0.012, 0.51w
Ethyl 3-phenylpropanoate (ethyl dihydrocinnamate)		Flower	Trace-0.003	0.002s
Ethyl 3-phenylprop-2-enoate (Ethyl cinnamate)		Honey, cinnamon	Trace-0.01	0.001a, s, 0.048w
Ethyl 4-hydroxy-3-methoxybenzoate (Ethyl vanillate)		Flower, fruit, sweet, vanilla	0.46	NR
Ethyl acetate		Fruity, solvent, balsamic	22.0–63.5	7.5a, 60, 12.27w
2-Methylpropyl acetate (isobutyl acetate)		Fruity, apple	Trace-0.17	1.6b
3-Methylbutyl acetate (isoamyl acetate)		Banana, fruity	0.03–5.52	0.03a, 0.16w
Ethyl 2-phenylacetate		Rose, floral	0.03–0.39	NR
2-Phenylethyl acetate		Flowery, rose	Trace-0.26	0.25a, 0.65, 1.80w
Hexyl acetate		Green, herbaceous, fruit, grape	Trace-3.90	0.002–0.48, 0.67/2.4w

Aroma thresholds values determined in water except where specified (a, 10% (v/v) aqueous ethanol; b, beer; s, synthetic wine [11% (v/v) ethanol, 7 g L^{-1} glycerol, 5 g L^{-1} tartaric acid, pH 3.4]; w, wine).

1.2.4 Carbonyl compounds

Acetaldehyde is the major carbonyl compound found in wine with concentrations ranging from 10 mg L^{-1} to 75 mg L^{-1} and a sensory threshold value of 100 mg L^{-1} (Schreier 1979). Aldehydes contribute to flavour with aroma descriptors such as 'bruised apple' and 'nutty' but can also be a marker of wine oxidation (Table 3).

As the last precursor before ethanol is formed, acetaldehyde is one of the major metabolic intermediates in yeast fermentation. Pyruvate, the end-product of glycolysis, is converted to acetaldehyde via the pyruvate decarboxylase enzymes, and then converted to ethanol via the alcohol dehydrogenase enzyme. This step is crucial for maintaining a redox balance in the cell, as it reoxidises NADH to NAD+, which is required for glycolysis (Pronk et al. 1996). During fermentation, the most rapid accumulation of acetaldehyde occurs when the rate of carbon dissimilation is at its maximum, after which it falls to a low level at the end of fermentation and then slowly increases over time. Fermentation conditions such as medium composition, nature of insoluble material used to clarify the must, and extreme aerobic growth conditions greatly affect acetaldehyde concentrations (Delfini et al., 1993).

In wine, the amount of acetaldehyde can increase over time due to oxidation of ethanol, activity of film yeast and aeration (Fleet et al., 1993). It has also been shown that the pre-fermentative use of high concentrations of sulphur dioxide can result in an accumulation of acetaldehyde in the final wine (Romano et al., 1993). A study comparing wine produced with and without SO_2 addition showed the amount of acetaldehyde significantly higher in wine to which SO_2 had been added, and this could well contribute to the sensory attributes of the wines (Sonni et al., 2009). Acetaldehyde concentrations have been recently shown to increase with increasing fermentation temperature: e.g. a fermentation carried out at 30ºC resulted in a significantly higher concentration of acetaldehyde (Romano et al. 1994), whereas some earlier studies found that temperature did not affect aldehyde concentrations (Amerine et al., 1980). Acetaldehyde concentration can also vary considerably (from 6 to 190 mg L^{-1}) depending on the yeast strain (Then et al., 1971; Sonni et al., 2009).

The presence of acetaldehyde in white wines is an indication of wine oxidation. The process of converting ethanol to acetaldehyde in the presence of oxygen is also referred to as 'madeirisation' and this produces a slightly almondy flavour that resembles the fortified sweet wine, Madeira. It is usually facilitated by prolonged storage in a barrel at high temperatures and the resulting wine lacks freshness and has a musty taste known as *rancio* (Robinson 1999). Acetaldehyde in red wines can contribute to aroma complexity as long as the concentration does not exceed 100 mg L^{-1}. It also enhances the colour development of red wine by promoting

condensation reactions between anthocyanins and catechins to tannins, forming stable polymeric pigments resistant to sulphur dioxide bleaching (Somers et al., 1987).

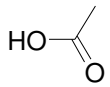
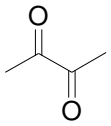
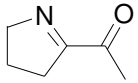
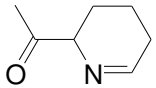
It is, therefore, inevitable that any bacterial activity that affects the concentration of acetaldehyde in wine potentially can affect its colour and flavour. Some strains of *Oenococcus oeni* and *Lactobacillus* (but not *Pediococcus*) can metabolise acetaldehyde to acetic acid and ethanol. The ability to metabolise acetaldehyde bound to sulphur dioxide can inhibit the growth of bacteria by releasing sulphur dioxide, which accumulates to form an inhibitory concentration. The chemical and sensory impact of the ethanol and acetic acid formed by the metabolism of acetaldehyde by lactic acid bacteria is believed to be limited, but the reduction in the acetaldehyde pool in wine is believed to influence final wine colour (Swiegers et al., 2005).

Another important carbonyl compound in wine is diacetyl (or 2,3-butanedione), which, at around 1-4 mg L^{-1}, depending on the style and the type of wine, contributes to a buttery or 'butterscotch' aroma (Table 3). Although yeasts biosynthesise some diacetyl (0.2–0.3 mg L^{-1}) in wine, most of it originates from the metabolic activities of lactic acid bacteria (Swiegers et al., 2005).

A variety of factors, including some that the winemaker can control, affect the concentration of diacetyl in wine, including oxygen exposure, fermentation temperature, sulphur dioxide levels and duration of malolactic fermentation (Bartowsky et al., 2004).

In the presence of sulphur dioxide, the concentration of free diacetyl in wine is lowered, however as the sulphur dioxide content decreases, for example during ageing, the ratio of free diacetyl will increase again, thus increasing its sensory impact (Nielsen et al., 1999).

Table 3. A summary of the some other aroma and flavour compounds reported in wine: their structure, aroma characteristics, concentration in wine and aroma thresholds (Sumby et al., 2009; Sweigers et al., 2005).

Compound Name	**Structure**	**Aroma charcteristics**	**Concentration in wine** (mg L^{-1})	**Aroma threshold** (mg L^{-1})
Acetic acid		VA, vinegar	100–1150	280*
Acetaldehyde		Sherry, nutty, bruised apple	10–75	100**
Diacetyl		Buttery	<5	0.2** / 2.8***
2-acetyl-1-pyrroline (ACPY)		Mousy	Trace	0.0001******
2-acetyltetrahydropyridine (ACPTY)		Mousy	0.0048–0.1	0.0016******

* 10% ethanol, ** wine, *** red wine, **** beer, ***** synthetic wine, ****** water

1.2.5 Sulphur compounds

Sulphur-containing flavour compounds typically occur in wine at very low concentrations, have very low detection thresholds and generally confer a negative sensory contribution to wine (Table 4). On the basis of their chemical structure, sulphur compounds in wine fall into five different categories, namely sulfides, polysulfides, heterocyclic compounds, thioesters and thiols. These compounds vary widely in their sensory properties, Many of them are associated with negative descriptors, which include cabbage, rotten egg, sulphurous, garlic, onion and rubber, whereas some can contribute positive aromas to wine, such as strawberry, passionfruit and grapefruit (Mestres et al. 2000, Vermeulen et al. 2005; Tominaga et al. 1996, 1998a,b). A variety of biochemical as well as chemical mechanisms are involved in the formation of sulphur compounds in wine and foods, however many of these mechanisms are still poorly defined. The development of these sulphur compounds by yeasts include the degradation of sulphur-containing amino acids, the degradation of sulphur-containing pesticides, and the release and/or the metabolism of grape-derived sulphur-containing precursors (Swiegers et al., 2005).

Sulfides (hydrogen sulfide, polysulfides, mercaptans)

Probably the best known sulphur compound in wine is hydrogen sulfide, a highly volatile thiol with a very low odour threshold (50–80 μg L^{-1}) which imparts a 'rotten egg' aroma conferring a negative sensory contribution to wine. However, this problem is relatively easily dealt with through the use of copper (which results in the formation of copper sulfide) or aeration (resulting in oxidation of the sulfide) (Monk 1986). Nevertheless, eliminating the use of copper salts by wineries is a desirable food processing goal and the presence of oxidised sulphur compounds in young wine could be related to the reductive character in bottled wine.

Hydrogen sulfide can be formed metabolically by yeast from either inorganic sulphur compounds, such as sulfate and sulfite, or organic sulphur compounds, such as cysteine and glutathione. When these organic compounds are absent, the cell must synthesise them from inorganic sulphur compounds accumulated from must. Under certain conditions, sulfide is liberated during the reduction of inorganic sulphur to become detectable by the winemaker. The concentration of hydrogen sulfide produced in fermentation varies with the availability of sulphur compounds, yeast strain and fermentation conditions, and the nutritional status of the environment, especially the availability of diammonium phosphate (DAP) (Sweigers et al., 2005).

The mechanism(s) for hydrogen sulfide formation during the final stages of fermentation are not clear. In white wine ferments, hydrogen sulfide formation is inversely correlated with initial total

nitrogen, and glutathione measured after fermentation (Park et al. 2000). During the final stages of fermentation in red wine ferments, however, hydrogen sulfide production appears to be unresponsive to DAP addition, but, at least in several cases, some evidence suggests that aeration and vitamin addition can moderate hydrogen sulfide production (Henschke 1996).

During fermentation, in association with hydrogen sulfide, the formation of mercaptanes also take place, including methyl mercaptan and ethyl marcaptan, highly reactive compounds with low aroma thresholds (around 1.1 $\mu g\ L^{-1}$).

Disulfides can be reduced to mercaptans by the action of sulfite ions, which can then be removed by copper or silver fining (not permitted in Europe and some other countries). However, the disulfides left as by-products of the reaction cannot be removed by copper ions, and not all of the off-flavours can, therefore, be removed in this way (Swiegers et al., 2005).

Thiols

The volatile thiols are one of the most potent groups of aroma compounds found in wine, some imparting negative aromas, others contributing positively (Table 4). Furfurylthiol is a potent aroma compound identified in Bordeaux red wines, white Petite Manseng, and also in toasted barrel staves (Tominaga et al. 2000b). Its presence in wine has been shown to be the result of yeast transformation of furfural released from toasted oak staves during fermentation (Blanchard et al. 2001). Fermentations that have an added nitrogen source, such as asparagine, do not produce as much furfurylthiol. Therefore, production of furfurylthiol is linked to the production of the HS^- anion, which is not produced when ammonium sulfate is added in sufficient quantities in a fermentation (Blanchard et al. 2001).

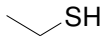
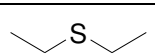
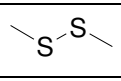
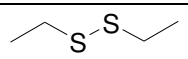
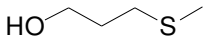
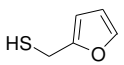
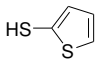
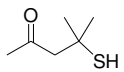
The volatile thiols 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) are of particular importance to wine aroma. These sulphur-containing compounds (thiol referring to the SH functional group) have extremely low perception thresholds: 3 ng L^{-1} (4MMP), 60 ng L^{-1} (3MH) and 4 ng L^{-1} (3MHA) (Table 4). In Sauvignon Blanc wine, these compounds are of particular importance to the varietal character as it imparts box tree (4MMP), passionfruit, grapefruit, gooseberry and guava aromas (3MH and 3MHA) (Dubourdieu et al. 2000). However, 4MMP, 3MH and 3MHA have also been identified in wines made from Colombard, Riesling, Semillon, Merlot and Cabernet Sauvignon in varying concentrations and can, therefore, potentially impact the aroma (Tominaga et al. 2000a, Murat et al. 2001b).

The volatile thiols are almost non-existent in the grape juice and only develop during fermentation. However, Darriet et al. (1995) clarified that 4MMP and 3MH do exist in the grapes

but in the form of non-volatile, cysteine bound conjugates and that yeast are responsible for cleaving the thiol from this precursor.

A mechanism of thiol release was proposed on the basis of experiments showing that a cell-free enzyme extract of the bacteria *Eubacterium limosum* containing carbon-sulphur lyase enzymes can release 4MMP from its precursor S-4-(4- methylpentan-2-one)-L-cysteine (Cys-4MMP) (Tominaga et al. 1995). Therefore, it was suggested that the amplification of Sauvignon Blanc varietal aromas during fermentation occurs through the action of yeast carbon-sulphur lyases (Tominaga et al. 1998a,b). The laboratory of Prof. Dubourdieu has shown in model ferments that when the chemically synthesised precursor, S-3-(hexan-1-ol)-L-cysteine (Cys-3MH) decreases in concentration, 3MH increases. However, only a small fraction (1.6% at day 6 of fermentation) of the cysteine-bound precursor was released as 3MH (Dubourdieu et al. 2000). In Cabernet Sauvignon and Merlot musts, it was shown that the amount of 3MH released was proportional to the Cys-3MH concentration. Therefore, the higher the concentration of the cysteine conjugate thiol precursors in the must, the higher the volatile thiol concentration in the resulting wine (Murat et al. 2001a). However, on average, only 3.2% of the precursor was released during fermentation. It is, therefore, clear that there is a huge, untapped flavour potential remaining in the wine after fermentation but that this source of flavour is not fully utilised due to the metabolic limitations of the yeast cell. The amount of 4MMP released in wine ferments is dependent on which yeast strain is used to conduct the fermentation (Dubourdieu et al. 2000). Therefore, the genetic and physiological characteristics of the yeast strain have a large effect on its ability to release thiols. Work at the Australian Wine Research Institute (AWRI) has confirmed these findings, showing that different commercial wine strains have variable abilities to release 4MMP from the Cys-4MMP precursor in model ferments (Swiegers et al., 2005).

Table 4. A summary of sulphur compounds, including thiols, reported in wine: their structure, aroma characteristics, concentration in wine and aroma thresholds (Sweigers et al., 2005).

Compound Name	Structure	Aroma descriptor	Concentration in wine (µg L^{-1})	Aroma threshold (mg L^{-1})
Hydrogen sulfide		rotten egg	Trace–>80	10–80
Methanethiol (methyl mercaptan)		cooked cabbage, onion, putrefaction, rubber	5.1, 2.1	0.3
Ethanethiol (ethyl mercaptan)		onion, rubber, natural gas	1.9–18.7	1.1
Dimethyl sulfide		asparagus, corn, molasses	1.4–61.9	25
Diethyl sulfide		cooked vegetables, onion, garlic	4.1–31.8	0.93
Dimethyl disulfide		cooked cabbage, intense onion	2	15, 29
Diethyl disulfide		garlic, burnt rubber	Trace–85	4.3
3-(Methylthio)-1-propanol (methionol)		cauliflower, cabbage, potato	140–5000	500
Benzothiazole		rubber	11	50
Thiazole		popcorn, peanut	0–34	38
4-Methylthiazole		green hazelnut	0–11	55
2-Furanmethanethiol		roasted coffee, burnt rubber	0–350 ng L^{-1}	1 ng L^{-1}
Thiophene-2-thiol		burned, burned rubber, roasted coffee	0–11	0.8
4-Mercapto-4-methylpentan-2-one (4MMP)		cat urine, box tree/ blackcurrant, broom	0–30 ng L^{-1}	3 ng L^{-1}

1.4 Maturation bouquet

The composition of wine changes continuously during storage as a function of parameters such as temperature, illumination, position of bottles, oxygen content and storage time. These changes are varied and intricate and can affect its aroma and colour, as well as its phenolic and metal composition. Aside from changes involving phenolics, oxidative processes lead to the loss of some characteristic volatiles and the appearance of new and distinctive aromas of older wines and/or atypical ones associated with wine deterioration (Hernanz et al., 2009).

Most work on the development of aroma compounds during wine maturation, especially alcohols and esters, was done during storage in white wine bottles and a wide variability in the results was shown.

In young white wines, the loss of freshness and fruity traits and browning reactions take place in a broad period that can vary from one month to several years, depending on both the type of the wine and the storage conditions. In this sense, there are studies on white wines that have revealed changes in the concentration of volatiles after two years, with an evolution from a characteristic fruity odour to a vegetative aroma resembling either asparagus or straw (Ghisholm et al., 1995), and losses of their fruity attributes after 18 months of storage under common commercial conditions, associated with an increase in diethyl succinate and acetaldehyde content and a decrease in other esters like isopentyl acetate and 2-phenylethyl acetate (Gónzalez-Viñas et al., 1996). Concerning acetaldehyde, also in wines made without SO_2 addition, the high level of acetaldehyde after 6 months of storage suggested a higher chemical oxidation of ethanol to acetaldehyde in wine aged without SO_2 than in wines stored with addition of SO_2 (Garde-Cerdan et al., 2007). Ramey & Ough (1980) found that the acetate esters of higher alcohols were generally degraded more rapidly than the ethyl esters of fatty acids in both wine and model solutions. They also observed that the rate of hydrolysis of fatty acid esters varied in proportion to their molecular weight and consequently a rapid degradation of heavy esters was highlighted.

Escudero et al. (2002) studied the aroma of young wines altered by oxygen (stored under oxygen for 1 week), reporting that wine aroma degradation was primarily caused by the appearance of a cooked-vegetable odour nuance given from t-2-nonenal, eugenol, benzaldehyde and furfural. The acetaldehyde content of wines, however, did not vary significantly during the oxidation process.

Also, elevated temperatures during storage caused significant differences in the aroma of white wines after only five days (De la Presa-Owens et al., 1997). A recent study conducted by Hernanz et al. (2009) on wines stored for one year in bottles under different conditions of temperature (seasonal temperature variations, 4°C and 15°C) showed a progressive decrease in the total volatile concentration. This was mainly due to the loss of alcohols, especially for wines

stored a 4°C. Additionally, the total acids and total esters increased during the storage of both wines, while the carbonyl compound amounts like acetaldehyde decreased.

Due to their gradual hydrolysis over time, volatile esters are sometimes considered unimportant in the favourable effects of wine. In fact, depending on the acid–ester equilibrium, branched fatty acid ethyl esters can increase during wine ageing (Díaz-Maroto et al., 2005). The branched fatty acid ethyl esters are less volatile than their straight-chain analogues, however, they are also important odourants of wine (Table 2). The ethyl esters of diprotic acids (e.g. diethyl butanedioate; Table 2) have also been shown to increase significantly with time, due to chemical esterification during wine ageing (Câmara et al., 2006). Because of a tendency of esters to return to their equilibrium levels, any effect of microorganisms on the ester profile of wine will mostly be advantageous for young 'fruity' wines. A recent study by Roussis et al. (2007) tested the inhibition of volatile ester degradation during storage of wine using caffeic acid or glutathione. Addition of caffeic acid protected several important esters involved in wine aroma during storage, including isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate, suggesting that this could be a useful method to extend a wine's fruity character. A decrease in fatty acids ethyl ester hydrolysis during storage was also shown in fermentations carried out with SO_2 by Garde-Cerdán et al. (2007). Furthermore, the presence or absence of SO_2 has greater influence on the evolution of alcohols during the storage of wine in bottles than in their formation during the alcoholic fermentation. At 3 months of aging, the stored wine with SO_2 showed a higher concentration of total alcohols than the wine aged in bottles without SO_2 and at 6 months the differences in their concentration become very significant, highlighting a higher concentration in the bottled wine with SO_2 than in the wine aged without SO_2. Similar results were not found for acids, probably due to a less significant effect of SO_2 on their evolution (Garde-Cerdán et al., 2007).

Concerning red wines, an interesting study on the development of esters during maturation of Merlot red wines in oak barrels over a period of 18 months showed that not all the esters have the same pattern of development during ageing. The development of isoamyl acetate, ethyl butyrate and ethyl hexanoate were all different depending on the turbidity grade of wine and, unlike data reported by other authors working with white wine aged in bottles, the fatty acid and ethanol esters were depleted more than the isoamyl acetate (Moreno et al., 2006).

In previous work conducted by our research group, the effects on volatile compositions of white wines by the substitution of SO_2 during the alcoholic fermentation with lysozyme and oenological tannins was investigated (Sonni et al., 2009). At the same time, the technological

performance of two strains of yeast that produce low amounts of SO_2 were evaluated. The data suggested that the composition of the volatiles in the final wines was affected by the different vinification protocols, especially regarding alcohol and ethyl ester contents, concluding that the addition of lysozyme and oenological tannins during alcoholic fermentation could represent a promising alternative to the use of SO_2 for the production of wines with reduced content of SO_2.
The aim of the following research was to confirm the effects on wine volatile composition of pre-fermentative addition of lysozyme and/or oenological tannin to SO_2 free musts, and to compare the volatile profiles of such wines with respect to "conventional" sulphite added wines. In the same study, the evolution of the aroma composition of wines during the bottle storage for one year was also analyzed, following the potential oxidation phenomena of volatile compounds.

2. EXPERIMENTAL

2.1 Fermentations

Eighty three litres of fresh must from cv *Sauvignon Blanc* were fermented in two litre laboratory glass fermentors that were saturated with N_2 before filling. A glass trap (filled with 4 N H_2SO_4) prevented microbial contamination and oxygen entrance. Two low SO_2 producing selected strains of *Saccharomyces cerevisiae* (strains 333 and 1042 from University of Bologna - ESAVE collection), were used to carry out fermentations and were inoculated at an initial cell concentration of 1.5 x 10^6 CFU ml^{-1}. Six samples for 333 strain and for 1042 strain respectively were defined with the aim of studying the effects of the following variables: 1) strain, 2) lysozyme, 3) SO_2, 4) tannin (Table 5).

Factor \ Trials	Strain (333 or 1042)						
	T	*L*	*LT*	*S*	*ST*	*SL*	*SLT*
Lysozyme (g L^{-1})	-	0.25	0.25	-	-	0.25	0.25
$K_2S_2O_5$ (mg L^{-1})	-	-	-	120	120	120	120
Tannin (g L^{-1})	-	-	0.15	-	0.15	-	0.15

Table 5. Scheme of fermentation trials. (Legend for samples: S: Sulphur dioxide addition, ST: Sulphur dioxide and tannin addition, L: Lysozyme addition, LT: Lysozyme and tannin addition).

Fermentations were performed in triplicate. Must were stirred daily to ensure a homogenous fermentation. Fermentations were monitored by daily weighing of the fermentors and samples were taken at the end of fermentations, when the loss of weight stopped. The final wines were bottled, under a nitrogen flux, in 125 mL bottles, and stored in the dark, at 15°C for 1 year. Analysis of the volatile composition of the wines after 3 and 12 months of storage were performed in duplicate.

2.2 Oenological parameters

Determination of density, total and volatile acidity, dry extract and total SO_2 were made according to OIV (1990). The pH was determined using a pH-meter (Mettler Toledo, Spain). The alcoholic strength of wines was determined by using an oenochemical distilling unit (Gibertini, Italy). The total polyphenol index (PFT) was determined by a direct lecture (after filtration at 0,45nm with PTFE filters) at 280 nm using an Uvidec 610 spectrophotometer (Jasco, Japan) and results were expressed as mg L^{-1} of gallic acid equivalent. All the analyses were made in duplicate.

2.3 Chemicals and Standards

Dichloromethane (Suprasolv) was purchased from Merck KGaA (Darmstadt, Germany). Standard compounds were supplied by Aldrich (Milano, Italy), Sigma Chemicals (St. Louis, Missouri, USA), Fluka Chimie AG (Buchs, Switzerland). Hydromatrix resin was from Varian Inc. (Palo Alto, California, USA). Water was of HPLC grade. Lysozyme Chloride was furnished by Fordras S.A. (Lugano, Switzerland), while liquid gallic tannin (Excellent Gold White) was purchased from Oliver Ogar Italia (Verona, Italy). Sulphur dioxide has been used as the potassium salt (Carlo Erba, Italy).

2.4 HPLC Analysis

The method used to determine organic acids is a modification of the methodology previously described by Castellari et al. (2000), which permits an improved identification of succinic, shikimic and pyruvic acids. Lysozyme quantification was conducted following the procedure described by Riponi et al. (2007). Both the HPLC analysis were performed using a Jasco apparatus (Tokyo, Japan) equipped with a binary pump (PU 2089), a 20 µl loop, a Rheodyne valve (Cotati, CA), a photodiode detector (PU MD 910), a fluorimetric detector (FP 2020), and a column oven. The column was a Bio-Rad Aminex HPX 87H (300 mm x 7,8 mm) for the analysis

of organic acids and a Tosoh Bioscience (Stuttgart, Germany) TSK gel Phenyl 5PW RP (7.5 cm x 4.6 mm i.d.), protected with a guard column filled with the same resin, for lysozyme.

2.5 GC Analysis

Compounds with high volatility and high concentration (acetaldehyde, ethylacetate, *n*-propanol, *i*-butanol, isoamyl alcohol) were analyzed according to the method outlined by A.O.A.C. (2000). A gas-chromatograph 8000 series (Fisons) equipped with a flame ionisation detector and a packed column 23% Carbowax 1500 (w/w) on Chromosorb W (60-80 mesh) were used. The working conditions were: GC grade nitrogen as carrier gas at flow rate (constant flow) of 3.0 mL min^{-1}, column temperature of 70°C (isothermal), detector and inlet temperature was 150°C.

2.6 GC-MS Analysis

For the analysis of all the other volatiles, the sample preparation procedure proposed by Gerbi et al. (1992) was used. The analysis of the extracts was carried out in a GC-MS Thermo Finnigan Trace GC ultra gas chromatograph (San Jose, CA), equipped with a Thermo Finnigan Trace DSQ mass selective detector and a fused silica capillary column Stabilwax (Restek, Bellefonte, PA; 30 m, 0.25 mm i.d., and 0.25 μm film thickness), under the following working conditions: GC grade helium as carrier gas at a flow rate (constant flow) of 1.0 mL min^{-1}; column temperature program, 40°C heated at 3°C min^{-1} to 100°C and then heated at 5°C min^{-1} to 240°C (held for 10 min). The injection temperature was 250°C. Samples (1 μl) were injected in the splitless mode. Detection was carried out by positive ion electron impact (EI) mass spectrometry in the full scan mode, using an ionization energy of 70 eV and a transfer line temperature of 280°C. The mass acquisition range was *m/z* 30-400 and the scanning rate 1 scan s^{-1}. Chromatographic peaks were identified by comparing their mass spectra with those of standards and/or those reported in the literature and in commercial libraries NIST 2.0 and Wiley 7. Quantification was carried out from total ion current peak areas according to the internal standard method (100 μL of a 514 mg L^{-1} solution of 2-octanol were added to 20 mL of each sample); the response factor of standard volatile compounds to the internal standard was experimentally obtained and applied to correct the peak area of each analyte. For compounds lacking reference standards, the response factors of standards with similar chemical structures were used.

2.7 Statistical Analysis

For each final wine, significant differences in mean concentrations of volatile compounds were tested by means of ANOVA analysis followed by a Post Hoc comparison (Tuckey's test at $p>0.01$). To evaluate the influence of each tested factor (yeast strain, lysozyme, SO_2 and tannins) on volatiles produced during fermentations, the data were subjected to multiple regression analysis after a graphical exploration to exclude outliers. All analyses were conducted using "Statistica 6" package (StatSoft Italia Srl, Italy).

3. RESULTS AND DISCUSSION

3.1 General parameters of final wines

The oenological parameters of the wines obtained from the different fermentations are shown in Tables 6a, 6b and Tables 7a, 7b.

The pH of the wines (with strain 333, 2.9-3.0; with strain 1042, 3.2-3.5) was within the range considered normal for this product.

All wines presented similar density, total polyphenol index, alcoholic strength and low values for volatile acidity (0.3-0.5 g L^{-1}), confirming the lack of acetic fermentation that could otherwise be responsible for the decline in quality.

By contrast, wines fermented with strain 1042 showed a higher dry extract compared to the samples fermented with strain 333, due to the higher sugar residue level.

Total SO_2 in all samples without sulphites addition (0.3-2.1 mg L^{-1}), confirmed that both strains 333 and 1042 were low SO_2 producers, as was highlighted in the preview study conducted by Sonni et al. (2009).

It has been already shown that the pre-fermentative use of sulphur dioxide can result in an accumulation of acetaldehyde in the final wines (Romano et al., 1993; Sonni et al., 2009). In our SO_2 added wines, acetaldehyde amounts were around 4-5 times higher when compared to samples obtained without SO_2 addition and this fact could well contribute to the sensory attributes of the wines.

For the organic acid data obtained by HPLC analysis (Tables 8a and 8b), all were very similar irrespective of the sample and, taking into account the initial amount in the must, they highlight the absence of malolactic spoilage in final wines, confirming that both SO_2 and lysozyme can efficiently control malolactic fermentation, as was shown in our preview study (Sonni et al., 2009).

By contrast with results obtained by Sonni et al. (2009) and Garde-Cerdan et al. (2007), there were no differences in the total acidity amount (expressed in g L^{-1} of tartaric acid) in wines fermented with SO_2 compared to the SO_2-free wines. However, slightly higher values were observed for the samples fermented with strain 1042 to those obtained from 333 strain inoculation.

Table 6a. General parameters of wines fermented with strain 333 at the end of alcoholic fermentation

	Strain 333							
	Degree alcoholic (% v/v)	*Density*	*Dry extract (g L^{-1})*	*Total SO_2 (mg L^{-1})*	*pH*	*Total acidity (g L^{-1} tartaric acid)*	*Volatile acidity (g L^{-1} acetic acid)*	*Acetaldehyde (mg L^{-1})*
Wine without addition	11,6 ± 3,51	1,001 ± 0,012	17,2 ± 2,19	0.64 ± 0,74	3,00 ± 0,03	5,138 ± 2,16	0,490 ± 0,00	10,7 ± 6,44
Lysozyme	11,8 ± 1,00	0,993 ± 0,001	18,0 ± 1,78	0.32 ± 0,00	3,01 ± 0,03	7,210 ± 0,73	0,498 ± 0,02	12,9 ± 7,84
Lysozyme+Tannins	12,2 ± 0,35	0,972 ± 0,012	17,0 ± 1,72	1.60 ± 0,55	3,04 ± 0,01	7,475 ± 0,23	0,450 ± 0,06	13,2 ± 4,53
SO_2	11,3 ± 0,75	0,991 ± 0,000	16,3 ± 1,05	46.1 ± 0,46	3,01 ± 0,02	7,025 ± 0,30	0,410 ± 0,05	47,5 ± 14,7
SO_2+Tannins	11,6 ± 0,54	0,993 ± 0,002	17,3 ± 2,47	46.1 ± 0,46	2,97 ± 0,03	7,575 ± 0,27	0,490 ± 0,06	33,9 ± 15,8
SO_2+Lysozyme	11,8 ± 0,16	1,007 ± 0,009	17,9 ± 0,14	45.6 ± 2,83	2,91 ± 0,01	8,250 ± 0,11	0,465 ± 0,02	33,2 ± 10,5
SO_2+Lysozyme+Tannins	12,0 ± 0,19	0,991 ± 0,001	18,2 ± 0,79	46.9 ± 0,46	2,94 ± 0,00	7,425 ± 0,20	0,480 ± 0,00	61,5 ± 7,01

Table 6b. General parameters of wines fermented with strain 1042 at the end of alcoholic fermentation

	Strain 1042							
	Degree alcoholic (% v/v)	*Density*	*Dry extract (g L^{-1})*	*Total SO_2 (mg L^{-1})*	*pH*	*Total acidity (g L^{-1} tartaric acid)*	*Volatile acidity*	*Acetaldehyde (% v/v)*
Wine without addition	12.4 ± 0.12	0.992 ± 0.000	21.6 ± 1.10	1.60 ± 0.00	3.50 ± 0.30	8.050 ± 0.28	0.290 ± 0.03	11.6 ± 2.36
Lysozyme	12.4 ± 0.15	0.992 ± 0.000	22.3 ± 0.57	2.13 ± 0.92	3.34 ± 0.00	8.075 ± 0.04	0.250 ± 0.02	11.2 ± 1.34
Lysozyme+Tannins	12.3 ± 0.24	0.993 ± 0.000	24.7 ± 0.42	5.33 ± 2.44	3.34 ± 0.01	7.925 ± 0.11	0.350 ± 0.02	14.3 ± 2.14
SO_2	12.3 ± 0.42	0.992 ± 0.001	22.4 ± 2.06	58.1 ± 9.38	3.25 ± 0.02	7.800 ± 0.07	0.370 ± 0.09	58.5 ± 7.31
SO_2+Tannins	11.1 ± 0.87	0.997 ± 0.003	26.1 ± 0.35	60.8 ± 6.97	3.33 ± 0.02	7.775 ± 0.11	0.370 ± 0.09	68.0 ± 4.21
SO_2+Lysozyme	12.0 ± 0.35	0.994 ± 0.002	26.1 ± 4.74	56.0 ± 2.26	3.32 ± 0.00	8.175 ± 0.11	0.420 ± 0.04	60.5 ± 2.04
SO_2+Lysozyme+Tannins	12.0 ± 0.20	0.994 ± 0.001	25.5 ± 1.16	43.7 ± 2.44	3.32 ± 0.00	8.000 ± 0.04	0.390 ± 0.06	56.0 ± 1.98

Table 7a. DO 420 nm, accelerate ageing test and Total polyphenols index of wines fermented with strain 333 at the end of alcoholic fermentation.

	Strain 333		
	D.O.420 nm	*Accelerate ageing Test* *Esp2-Esp1*	*PFT 280nm* *mg L^{-1}*
Wine without addition	0,053 ± 0,006	0,039 ± 0,01	99 ± 11,5
Lysozyme	0,060 ± 0,006	0,008 ± 0,01	103 ± 7,22
Lysozyme+Tannins	0,079 ± 0,001	0,013 ± 0,00	150 ± 2,05
SO_2	0,068 ± 0,011	0,011 ± 0,03	117 ± 9,14
SO_2+Tannins	0,086 ± 0,007	0,021 ± 0,01	146 ± 4,41
SO_2+Lysozyme	0,096 ± 0,007	0,014 ± 0,00	149 ± 8,81
SO_2+Lysozyme+Tannins	0,073 ± 0,007	0,009 ± 0,00	147 ± 4,38

Table 7b. DO 420 nm, accelerate ageing test and Total polyphenols index of wines fermented with strain 1042 at the end of alcoholic fermentation.

	Strain 1042		
	D.O.420 nm	*Accelerate ageing Test* *Esp2-Esp1*	*PFT 280nm* *mg L^{-1}*
Wine without addition	0.153 ± 0.008	0.012 ± 0.09	235 ± 3.69
Lysozyme	0.137 ± 0.001	0.015 ± 0.00	302 ± 5.74
Lysozyme+Tannins	0.138 ± 0.002	0.018 ± 0.00	278 ± 1.43
SO_2	0.126 ± 0.010	0.006 ± 0.08	274 ± 9.55
SO_2+Tannins	0.145 ± 0.010	0.010 ± 0.00	262 ± 8.44
SO_2+Lysozyme	0.125 ± 0.004	0.009 ± 0.00	284 ± 12.7
SO_2+Lysozyme+Tannins	0.121 ± 0.001	0.015 ± 0.01	291 ± 34.3

Table 8a. Organic acids content (in g L^{-1}) of wines fermented with strain 333 at the end of alcoholic fermentation.

	Strain 333						
	Tartaric a. $g\ L^{-1}$	*Pyruvic a.* $g\ L^{-1}$	*Malic a.* $g\ L^{-1}$	*Shikimic a.* $g\ L^{-1}$	*Lactic a.* $g\ L^{-1}$	*Citric a.* $g\ L^{-1}$	*Succinic a.* $g\ L^{-1}$
must	3.330	0,020	3.434	0,014	0,028	0,104	n.d.
Wine without addition	2,588 ± 0,02	0,109 ± 0,00	2,750 ± 0,09	0,020 ± 0,00	0,329 ± 0,03	0,296 ± 0,01	0,698 ± 0,05
Lysozyme	3,480 ± 0,48	0,089 ± 0,01	2,850 ± 0,45	0,018 ± 0,00	0,205 ± 0,17	0,293 ± 0,01	0,485 ± 0,04
Lysozyme+Tannins	3,877 ± 0,29	0,083 ± 0,01	3,037 ± 0,28	0,023 ± 0,00	0,200 ± 0,01	0,159 ± 0,01	0,548 ± 0,02
SO_2	2,509 ± 0,25	0,192 ± 0,02	2,747 ± 0,19	0,030 ± 0,00	0,210 ± 0,02	0,033 ± 0,01	1,224 ± 0,09
SO_2+Tannins	3,577 ± 0,18	0,169 ± 0,02	3,148 ± 0,16	0,028 ± 0,00	0,175 ± 0,03	0,029 ± 0,01	1,220 ± 0,10
SO_2+Lysozyme	2,659 ± 0,26	0,198 ± 0,01	3,715 ± 0,38	0,027 ± 0,00	0,213 ± 0,03	0,039 ± 0,00	1,159 ± 0,00
SO_2+Lysozyme+Tannins	3,067 ± 0,85	0,157 ± 0,01	3,157 ± 0,07	0,027 ± 0,00	0,190 ± 0,02	0,038 ± 0,01	1,106 ± 0,02

Table 8b. Organic acids content (in g L^{-1}) of wines fermented with strain 1042 at the end of alcoholic fermentation

	Strain 1042						
	Tartaric a. $g\ L^{-1}$	*Pyruvic a.* $g\ L^{-1}$	*Malic a.* $g\ L^{-1}$	*Shikimic a.* $g\ L^{-1}$	*Lactic a.* $g\ L^{-1}$	*Citric a.* $g\ L^{-1}$	*Succinic a.* $g\ L^{-1}$
Wine without addition	3.061 ± 0.17	0.097 ± 0.02	2.733 ± 0.13	0.016 ± 0.00	0.386 ± 0.11	0.276 ± 0.04	1.324 ± 0.03
Lysozyme	3.856 ± 0.19	0.060 ± 0.01	2.795 ± 0.05	0.007 ± 0.07	0.275 ± 0.01	0.302 ± 0.00	1.079 ± 0.02
Lysozyme+Tannins	3.439 ± 0.10	0.322 ± 0.42	2.713 ± 0.08	0.015 ± 0.00	0.262 ± 0.02	0.317 ± 0.02	0.869 ± 0.03
SO_2	3.026 ± 0.45	0.095 ± 0.00	2.711 ± 0.11	0.014 ± 0.00	0.369 ± 0.04	0.282 ± 0.01	1.319 ± 0.09
SO_2+Tannins	3.099 ± 0.51	0.109 ± 0.01	2.607 ± 0.05	0.015 ± 0.00	0.270 ± 0.03	0.270 ± 0.01	0.991 ± 0.08
SO_2+Lysozyme	3.198 ± 0.37	0.092 ± 0.01	2.777 ± 0.02	0.014 ± 0.00	0.310 ± 0.01	0.276 ± 0.00	0.973 ± 0.00
SO_2+Lysozyme+Tannins	3.545 ± 0.66	0.144 ± 0.10	2.759 ± 0.17	0.017 ± 0.00	0.267 ± 0.02	0.288 ± 0.02	0.983 ± 0.04

Table 9 shows the lysozyme residual values in samples at the end of fermentation, highlighting only a low reduction in wines obtained from yeast strain 333 and 1042 respectively, compared to the initial added amount that was 250 mg L^{-1}, in accordance with a preview study by Sonni et al. (2009). The reduction of free lysozyme in wines due to its interaction with must constituents (mainly phenolics) has been already reported (Chinnici et al., 2009; Bellachioma et al., 2008; Amati et al., 1996). However, our data suggest that also the utilised yeast strain could play a further role in the amount of residual lysozyme in wines after the fermentation process.

	Lysozyme (mg L^{-1})					
	Strain 333			*Strain 1042*		
Lysozyme	188	±	17.7	140	±	1.73
Lysozyme+Tannins	167	±	27.2	145	±	64.8
SO_2+Lysozyme	152	±	27.6	160	±	24.7
SO_2+Lysozyme+Tannins	155	±	19.3	164	±	35.0

Table 9. Lysozyme residual content of wines fermented with strain 333 and 1042 at the end of alcoholic fermentation.

3.2 Volatile characterisation at the end of alcoholic fermentation of wines inoculated with strain 333 and 1042

The volatile compounds identified in each final wine were grouped into chemical classes and are reported in tables 10a, 10b, 11a, 11b, 12a and 12b, together with the Tuckey's test results. On the right hand side, the tables also show the significant ($p>0.01$) standardized beta coefficients from the multiple regression analysis, carried out with the aim of highlighting significant correlations between each factor (SO_2, lysozyme and tannins) and the production of volatiles during fermentation for each strain. The higher the regression coefficient (beta), the stronger the impact of the factor on that specific compound. Furthermore, the sign of the beta values indicate the direct (positive sign) or reversed (negative sign) correlation. Hence, positive signs refer to a direct relationship between tannins, SO_2, lysozyme and the interaction among them on the level of single compounds while, for negative signs, a reversed correlation should be considered.

3.2.1 Alcohols

Tables 10a and 10b show the concentrations of alcohols for both wines fermented with strain 333 and strain 1042.

For wines fermented with strain 333, the lowest amount of alcohols as a sum was present in samples fermented with lysozyme addition, whereas for samples fermented with strain 1042, the lowest alcohol content was present in samples which had both lysozyme and tannins added. Furthermore, the regression analysis showed a negative influence of lysozyme on total alcohol production for wines fermented with strain 1042.

Strain 1042 tends to furnish higher amounts of alcohols as a sum compared with strain 333, which was also highlighted in our preview study (Sonni et al., 2009). These results suggest the presence of a strong relationship between yeast strain and the sum of alcohols in final wines, as was previously shown by Gonzalez-Vinas et al (1995). Sulphur dioxide addition showed a variable influence on alcohol production depending on the strain. The amount of total alcohols in wine fermented with strain 333 with SO_2 addition was higher (204 mg L^{-1}) than in the counterparts obtained with lysozyme (161 mg L^{-1}). By contrast, in samples fermented with strain 1042, sulphur dioxide and lysozyme addition showed almost the same value of total alcohol production (200 mg L^{-1} for SO_2 addition; 210 mg L^{-1} for lysozyme addition).

Among single compounds, for strain 333 samples, sulphur dioxide had a significant influence on certain alcohol production compared to lysozyme addition. Positive influences were observed for 2-methyl-2-butanol and 3-ethylthio-1-propanol production, while a negative influence for 3-ethoxy-1-propanol was apparent. Concerning the latter alcohol, the higher production in wines fermented with strain 333 and lysozyme addition instead of SO_2 has already been determined in our own previous results (Sonni et al. (2009) and Herraiz et al. (1990)). Furthermore, the production of 3-ethoxy-1-propanol increased when lysozyme and tannins were both added, as was the case for 2-methyl-2-butanol.

The multiple regression analysis highlighted that alcohols production was more negatively influenced by the correlation between SO_2 and tannins, except for 2-methyl-2-butanol; however the impact of each single factor on these alcohols production was positive.

Lysozyme addition alone did not influence strongly the alcohols production.

For samples fermented with strain 1042, the only significant influence of SO_2 addition on alcohol production was a positive one for 1-butanol and a negative one for iso-butanol. In this case, the multiple regression analysis showed a great influence on alcohols production for SO_2 and tannins as single factors, whereas no influences for SO_2 and tannins correlated factor was shown. Concerning lysozyme addition, table 11b showed a negative influence only on *n*-propanol and 3-ethoxy-1-propanol production. As for wines fermented with strain 333 data previously discussed, lysozyme seemed not to show any appreciable effect on alcohols production for fermentations carried out with strain 1042.

In contrast with other preview studies, concerning sulfite promoted musts amino acid consumption during the fermentation, our experiments did not reveal a role of SO_2 on the Ehrlich pathway, a catabolic process of alcohol formation starting from amino acid derivatives and α-ketoacids (Garde-Cerdan et al., 2007; Sonni et al., 2009). For some alcohols involved in the Ehrlich pathway, wines fermented with both strain 333 and 1042 showed no significant influence on 3-methylthio-1-propanol and phenylethyl alcohol either in the presence or absence of SO_2. Furthermore, for fermentations with strain 1042, the SO_2 addition showed a negative influence on isoamyl alcohols, *i*-butanol and 1-propanol, even though no significant differences in their production amount between wines fermented with or without SO_2 have been found.

Alcohols have intense odours that play a role in wine aromas. At concentrations less than 300 mg L^{-1}, they contribute to the wines aromatic complexity, while at higher levels they can mask the wine's aromatic finesse. The concentration of total alcohols in our samples did not exceed this threshold at anytime, so these compounds would have contributed in a positive way to the wine aroma.

3.2.2 Esters

As preview studies have shown, the concentration of esters as a sum (Tables 11a and 11b) tended to be higher for wines fermented with strain 1042, confirming that ester production is strain-dependent (Vilanova et al., 2007; Vila et al., 1998; Lema et al., 1996).

This result, however, greatly depends on the relevant production of ethyl hydrogen succinate, which affects the total amount of these compounds. Tannins showed a positive influence on the total esters amount, whereas SO_2 and lysozyme appeared to not have a significant influence.

For wines fermented with strain 333, tannins result in the most positive influencing factor on esters production, particularly for isoamyl acetate, ethyl octanoate, ethyl-4-hydroxybutanoate, phenylethyl acetate and ethyl hydrogen succinate.

By contrast, for wines fermented with strain 1042, the most positive influencing factors were lysozyme and the interaction between SO_2 and tannins, especially for isoamyl acetate, ethyl hexanoate, hexyl acetate and ethyl octanoate, while tannins showed a negative contribution on ester production, especially for isoamyl acetate, hexyl acetate and ethyl hydrogen succinate.

For the concentrations of medium-chain fatty acid ethyl esters (MCFA ethyl esters), such as ethyl hexanoate, ethyl octanoate and ethyl decanoate, they were found at higher concentration in SO_2-free wines with lysozyme addition fermented with strain 1042, compared to wines with SO_2 addition, while in SO_2-free wines obtained with strain 333, only ethyl decanoate followed the same trend. These results were in accordance with our preview study (Sonni et al., 2009) and in

contrast with others studies in the literature reporting an increase in MCFA ethyl ester production in wines fermented with SO_2, especially in the case of additions larger than 100 mg L^{-1} (Herraiz et al., 1989; Margheri et al., 1986; Shinoara et al., 1981).

It is well known that the effect of SO_2 addition on ester production does not seem to be systematic and may depend on several factors. Nykanen (1986) showed that reduced oxygen concentration increased the production of MCFA ethyl esters; Moio et al. (2004) associated an increase in esters concentrations to the combined action of higher SO_2 amounts and low O_2 availability, regardless of the type of yeast strain employed for fermentation. Furthermore Herraiz Tomico (1990), in accord with our results for wines fermented with strain 1042, found higher concentration of ethyl hexanoate in wines fermented without SO_2. Bardi et al. (1998) postulated that during alcoholic fermentation, unsaturated fatty acids can be synthesized by oxidation of free saturated fatty acids, in a process that involves the presence of free oxygen. Lacking this element, the synthesis stops, with the corresponding accumulation of acyl-CoA. Under these conditions, in order to recover free coenzyme A, the yeasts promotes ester formation and the wine obtained in these conditions is richer in esters containing the corresponding acyl group (Moio et al., 2004; Bardi et al., 1998). In our wines, the added SO_2 amount in pre-fermentation (60 mg L^{-1}) was probably unable to significantly reduce the availability of free oxygen during the alcoholic fermentation. On the other hand, our data show that tannins, especially in the presence of SO_2, increased the concentration of C_{12}-C_{16} ethyl esters (Table 11a and 11b), confirming the hypothesis postulated in our previous study about the oxygen scavenging activity of tannins that causes a fast drop in oxygen availability (Bosso et al., 2001).

However, an important result to highlight is that the statistical analysis showed a significant decrease on ethyl ester compounds only for ethyl-octanoate and ethyl-3-hydroxybutyrate in wines fermented with strain 1042 and for ethyl lactate in wines fermented with strain 333.

With regard to the acetates, a positive influence of lysozyme addition in wines fermented with strain 1042 was found, especially for isoamyl acetate, hexyl acetate and phenylethyl acetate, whereas in samples obtained with strain 333, a significant increase due to lysosyme addition was shown only for ethylacetate, and isoamyl acetate. The differences found on acetate ester concentration values between the two groups of fermentation highlight an existence of the role different types of yeast strain on acetate production may have, in accord with the results obtained by Sonni et al. (2009) and Daudt et al. (1973).

Acetate ester of higher alcohols and ethyl esters of fatty acids are considered important contributors to young wine aromas and exhibit floral and fruity odours, as reported in Table 2.

3.2.3 Acids

The amounts of acid as a sum (Table 12a and 12b) follow the trends in the concentration of esters as a sum. In particular, the concentration of acids as a sum was higher for wines fermented with strain 1042, as compared to wines fermented with strain 333.

Among single compounds, the amount of some acids like hexanoic acid, octanoic acid, decanoic acid and tetradecanoic acid is higher for wines fermented with strain 1042, as was the trend for the corresponding fatty acid ethyl esters. These results confirm their common biosynthetic pathway, which leads to the production of long chain unsaturated fatty acids (Soumalainen et al., 1979).

Concerning the fatty acid contribution to wine flavour, the total fatty acid concentration in wines samples was found to be around 20 - 40 mg L^{-1}, a value that is known to not impair wine aroma (Miranda-Lopez et al., 1992).

Table 10a. Final wines fermented with strain 333: alcohol concentrations (mg L^{-1}) and influence of the tested factors on their production, as assessed by multiple regression analysis

	Strain 333							Regression coefficient*					
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO_2+Lyso+tan	Lyso	SO_2	Tannin	LysoxSO_2	LysoxTan	SO_2xTan
Methanol	10.5 ± 3.61 [a]	18.1 ± 2.67 [ab]	28.3 ± 7.26 [bc]	24.1 ± 6.37 [abc]	29.9 ± 6.50 [bc]	41.3 ± 2.26 [c]	32.6 ± 4.08 [bc]	-	0.663	1.200	-	-	-0.840
n-Propanol	6.36 ± 3.13 [a]	9.87 ± 1.21 [ab]	17.2 ± 1.83 [cd]	10.0 ± 2.15 [abc]	1.37 ± 0.18 [abcd]	18.4 ± 1.03 [d]	15.1 ± 1.14 [bcd]	-	-	1.590	-	-	-1.090
i-Butanol	17.1 ± 11.6 [a]	25.0 ± 6.34 [ab]	35.8 ± 3.07 [ab]	35.1 ± 7.51 [ab]	6.06 ± 0.23 [ab]	43.0 ± 1.07 [b]	43.9 ± 2.09 [b]	-	0.869	-	-	-	-
Isoamhyl alcohols	39.1 ± 17.2	29.6 ± 1.13	36.6 ± 3.93	45.1 ± 2.95	39.1 ± 2.20	42.1 ± 0.88	42.9 ± 0.75	-	-	-	-	-	-
2-Methyl-2-butanol	0.04 ± 0.01 [a]	0.05 ± 0.00 [a]	0.37 ± 0.00 [b]	0.58 ± 0.03 [c]	0.17 ± 0.03 [a]	0.12 ± 0.03 [a]	0.75 ± 0.10 [d]	-	1.027	-1.351	-0.815	1.793	0.537
1-Propanol	0.66 ± 0.07 [a]	1.07 ± 0.29 [ab]	2.14 ± 0.18 [c]	1.20 ± 0.41 [ab]	1.73 ± 0.08 [bc]	1.81 ± 0.30 [bc]	1.37 ± 0.10 [abc]	-	-	2.006	-	-0.875	-1.352
1-butanol	0.17 ± 0.02 [a]	0.30 ± 0.10 [a]	0.39 ± 0.03 [a]	0.36 ± 0.12 [a]	1.06 ± 0.07 [c]	1.24 ± 0.04 [c]	0.69 ± 0.09 [b]	-	0.242	1.727	0.888	-1.470	-0.757
2 hexanol	0.06 ± 0.00	0.07 ± 0.01	0.09 ± 0.02	0.09 ± 0.00	0.07 ± 0.01	0.07 ± 0.00	0.10 ± 0.02	-	-	-	-	1.335	-
4-Methyl-1-pentanol	0.01 ± 0.00 [ab]	0.00 ± 0.00 [a]	0.01 ± 0.00 [ab]	0.01 ± 0.00 [ab]	0.01 ± 0.00 [ab]	0.02 ± 0.00 [b]	0.01 ± 0.00 [ab]	-	-	-	-	-	-
3-Methyl-1-pentanol	0.09 ± 0.03 [ab]	0.07 ± 0.01 [a]	0.11 ± 0.01 [ab]	0.10 ± 0.02 [ab]	0.11 ± 0.01 [ab]	0.12 ± 0.00 [ab]	0.13 ± 0.01 [b]	-	-	-	-	-	-
2-Methyl-3-pentanol	0.14 ± 0.05 [ab]	0.15 ± 0.03 [ab]	0.17 ± 0.02 [ab]	0.11 ± 0.00 [a]	0.13 ± 0.01 [ab]	0.19 ± 0.03 [b]	0.13 ± 0.01 [ab]	-	-	1.741	-	-1.267	-1.216
1-Hexanol	0.93 ± 0.31	0.95 ± 0.04	0.91 ± 0.04	0.77 ± 0.05	1.09 ± 0.23	0.95 ± 0.06	0.91 ± 0.05	-	-	-	-	-	-
trans-3-Hexen-1-ol	0.01 ± 0.01 [ab]	0.01 ± 0.00 [a]	0.01 ± 0.00 [ab]	0.01 ± 0.00 [ab]	0.02 ± 0.00 [ab]	0.02 ± 0.00 [b]	0.02 ± 0.00 [ab]	-	-	-	1.091	-	-
3-Ethoxy-1-propanol	0.36 ± 0.07 [c]	0.20 ± 0.02 [b]	0.45 ± 0.02 [c]	0.06 ± 0.02 [a]	0.10 ± 0.02 [ab]	0.21 ± 0.05 [b]	0.11 ± 0.01 [ab]	-0.577	-1.081	1.469	1.047	-0.497	-1.189
cis-3-Hexen-1-ol	0.07 ± 0.03 [ab]	0.05 ± 0.00 [a]	0.07 ± 0.00 [ab]	0.08 ± 0.01 [ab]	0.07 ± 0.01 [ab]	0.09 ± 0.00 [b]	0.08 ± 0.00 [ab]	-	-	-	-	-	-
2-Methylthio-ethanol (µg/L)	15.4 ± 5.76 [b]	4.41 ± 2.33 [a]	18.2 ± 2.91 [b]	12.7 ± 1.69 [ab]	15.6 ± 2.57 [b]	13.7 ± 1.00 [ab]	17.8 ± 2.47 [b]	-1.099	-	-	1.092	-	-
3-Methythio-1-propanol	0.59 ± 0.07	0.56 ± 0.04	0.60 ± 0.05	0.93 ± 0.22	0.79 ± 0.24	0.70 ± 0.17	0.92 ± 0.13	-	-	-	-	-	-
3-Ethylthio-1-propanol (µg/L)	n.d ± n.d [a]	n.d. ± n.d. [a]	n.d. ± n.d. [a]	5.43 ± 1.13 [b]	6.45 ± 2.14 [b]	7.44 ± 1.10 [b]	5.93 ± 1.19 [b]	-	0.814	-	-	-	-
2-(2-butoxyethoxy)ethanol	0.04 ± 0.01 [b]	0.02 ± 0.00 [ab]	0.02 ± 0.00 [ab]	0.01 ± 0.00 [a]	0.01 ± 0.01 [a]	0.02 ± 0.01 [ab]	0.03 ± 0.01 [ab]	-0.934	-1.170	-	1.302	-	-
4-Methylthio-1-butanol (µg/L)	1.51 ± 1.21	1.74 ± 1.24	n.d. ± n.d.	0.40 ± 0.14	0.44 ± 0.18	0.15 ± 0.15	n.d. ± n.d.	-	-	-	-	-	-
Benzyl alcohol	0.09 ± 0.01 [ab]	0.09 ± 0.02 [ab]	0.14 ± 0.01 [b]	0.10 ± 0.03 [ab]	0.08 ± 0.02 [a]	0.09 ± 0.01 [a]	0.11 ± 0.02 [ab]	-	-	-	-	-	-
Phenylethyl alcohol	32.3 ± 5.74	25.9 ± 1.17	27.7 ± 1.21	27.9 ± 8.13	40.0 ± 0.10	27.5 ± 7.39	33.7 ± 2.63	-	-	-	-	-	-
1-Octadecanol (stenol)	0.01 ± 0.00 [ab]	0.02 ± 0.01 [bc]	0.02 ± 0.00 [bc]	0.01 ± 0.00 [a]	0.00 ± 0.00 [a]	0.03 ± 0.00 [c]	0.01 ± 0.00 [ab]	0.621	-	-	-	-	-0.800
Sum	150 ± 35.7 [a]	161 ± 6.14 [a]	211 ± 17.5 [ab]	204 ± 24.7 [ab]	198 ± 15.50 [ab]	241 ± 8.2 [c]	243 ± 5.53 [c]	-	0.731	-	-	-	-

In the same row, different letters denote significant differences at $p < 0.01$

(1) only standardized regression coefficients (beta values) with $p < 0.01$, are reported

Table 10b. Final wines fermented with strain 1042: alcohol concentrations (mg L^{-1}) and influence of the tested factors on their production, as assessed by multiple regression analysis

	Strain 1042							Regression coefficient*					
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO2 + Lyso	SO2+Lyso+tan	Lyso	SO_2	Tannin	LysoxSO$_2$	LysoxTan	SO$_2$xTan
Methanol	36.4 ± 1.27 ^{bc}	34.5 ± 1.19 ^{abc}	37.6 ± 1.25 ^{c}	29.9 ± 1.03 ^{ab}	34.9 ± 4.59 ^{abc}	38.1 ± 0.95 ^{c}	34.1 ± 0.71 ^{abc}	-	-	-	-	-	-
n-Propanol	14.9 ± 0.12 ^{b}	12.0 ± 0.74 ^{a}	10.3 ± 0.34 ^{a}	11.6 ± 0.43 ^{a}	11.0 ± 0.14 ^{a}	10.5 ± 0.03 ^{a}	11.0 ± 0.57 ^{a}	-0.742	-0.807	0.564	0.793	-	-
i-Butanol	50.8 ± 0.74 ^{c}	50.9 ± 0.53 ^{c}	34.2 ± 0.81 ^{ab}	38.2 ± 3.01 ^{b}	23.9 ± 6.28 ^{a}	38.7 ± 5.37 ^{b}	28.4 ± 1.44 ^{ab}	-	-0.890	-	0.916	-	-
1-Butanol	0.40 ± 0.12 ^{ab}	0.31 ± 0.05 ^{a}	0.42 ± 0.05 ^{abc}	0.61 ± 0.04 ^{bcd}	0.65 ± 0.11 ^{dce}	0.80 ± 0.00 ^{ce}	0.88 ± 0.01 ^{e}	-	0.473	0.403	0.747	-	-
Isoamylalcohols	260 ± 21.3 ^{cd}	272 ± 3.27 ^{d}	212 ± 18.5 ^{abcd}	218 ± 5.15 ^{abcd}	162 ± 26.1 ^{a}	238 ± 35.7 ^{bcd}	185 ± 6.19 ^{ab}	-	-0.574	-0.781	-	-	-
2-Hexanol (µg/L)	35.3 ± 14.5 ^{a}	25.7 ± 3.76 ^{a}	33.2 ± 6.99 ^{a}	30.0 ± 3.34 ^{a}	29.7 ± 1.87 ^{a}	24.2 ± 5.38 ^{a}	35.2 ± 2.05 ^{a}	-	-	-	-	-	-
3-Methyl-1-pentanol	0.13 ± 0.03 ^{ab}	0.14 ± 0.01 ^{ab}	0.08 ± 0.00 ^{a}	0.19 ± 0.01 ^{b}	0.13 ± 0.00 ^{ab}	0.19 ± 0.08 ^{b}	0.14 ± 0.01 ^{ab}	-	-	-0.644	-	-	-
1-Hexanol	0.75 ± 0.17 ^{a}	0.61 ± 0.02 ^{a}	0.69 ± 0.01 ^{a}	0.71 ± 0.06 ^{a}	0.72 ± 0.00 ^{a}	0.57 ± 0.13 ^{a}	0.80 ± 0.01 ^{a}	-	-	-	-	0.978	-
trans-3-Hexen-1-ol (µg/L)	50.6 ± 31.1 ^{a}	30.7 ± 10.8 ^{a}	78.0 ± 10.6 ^{a}	351 ± 200 ^{a}	753 ± 0.19 ^{a}	234 ± 133 ^{a}	73.1 ± 42.3 ^{a}	-	-0.721	-0.814	-	0.83	-
3-Ehoxy-1-propanol (µg/L)	46.3 ± 4.34 ^{a}	35.8 ± 4.48 ^{a}	15.9 ± 6.93 ^{a}	9.60 ± 4.27 ^{a}	13.6 ± 6.25 ^{a}	6.50 ± 1.42 ^{a}	7.20 ± 1.41 ^{a}	-0.450	-0.759	0.783	0.640	-0.599	
cis-3-Hexen-1-ol	0.06 ± 0.02 ^{a}	0.06 ± 0.01 ^{a}	0.06 ± 0.00 ^{a}	0.06 ± 0.00 ^{a}	0.06 ± 0.00 ^{a}	0.05 ± 0.01 ^{a}	0.06 ± 0.01 ^{a}	-	-	-	-	-	-
1-Octanol (µg/L)	43.9 ± 23.2 ^{a}	34.1 ± 22.7 ^{a}	63.2 ± 2.22 ^{a}	49.0 ± 1.84 ^{a}	56.5 ± 1.41 ^{a}	40.1 ± 8.71 ^{a}	57.3 ± 1.43 ^{a}	-	-	-	-	-	-
3-(Methythio)-1-propanol	0.81 ± 0.17 ^{a}	0.88 ± 0.10 ^{a}	0.91 ± 0.09 ^{a}	1.02 ± 0.11 ^{a}	0.97 ± 0.02 ^{a}	0.96 ± 0.25 ^{a}	0.98 ± 0.08 ^{a}	-	-	-	-	-	-
3-(Ethylthio)-1-propanol (µg/L)	27.1 ± 10.3 ^{a}	21.0 ± 5.26 ^{a}	35.1 ± 1.13 ^{a}	28.5 ± 13.1 ^{a}	44.5 ± 16.6 ^{a}	27.2 ± 6.24 ^{a}	33.5 ± 2.12 ^{a}	-	0.686	-	-	-	-
Benzyl alcohol	0.42 + 0.04 ^{a}	0.40 + 0.11 ^{a}	0.42 + 0.03 ^{a}	0.39 + 0.01 ^{a}	0.45 + 0.03 ^{a}	0.39 + 0.08 ^{a}	0.37 + 0.01 ^{a}	-	-	-	-	-	-
Phenylethyl alcohol	35.7 ± 2.97 ^{a}	33.7 ± 7.06 ^{a}	34.9 ± 2.09 ^{a}	33.0 ± 2.63 ^{a}	34.8 ± 2.37 ^{a}	37.2 ± 8.09 ^{a}	34.7 ± 4.02 ^{a}	-	-	-	-	-	-
1-Octadecanol (stenol)	0.26 ± 0.00 ^{b}	0.14 ± 0.06 ^{ab}	0.15 ± 0.03 ^{ab}	0.14 ± 0.04 ^{ab}	0.21 ± 0.01 ^{ab}	0.12 ± 0.03 ^{ab}	0.11 ± 0.07 ^{a}	-	-	-	-	-	-
4-Hydroxy-benzeneethanol	93.5 ± 5.93 ^{b}	51.8 ± 32.7 ^{ab}	34.2 ± 7.46 ^{a}	56.2 ± 16.1 ^{ab}	56.1 ± 1.60 ^{ab}	58.4 ± 14.5 ^{ab}	34.7 ± 8.22 ^{a}	-	-	-1.073	-	-	-
Sum	269 ± 21.9 ^{b}	210 ± 36.2 ^{ab}	184 ± 18.0 ^{a}	200 ± 14.7 ^{ab}	190 ± 16.6 ^{ab}	233 ± 23.6 ^{ab}	186 ± 14.4 ^{a}	-0.779	-	-	-	-	-

In the same row, different letters denote significant differences at $p< 0.01$

(1) only standardized regression coefficients (beta values) with $p < 0.01$, are reported

Table 11a. Final wines fermented with strain 333: ester concentrations (mg L^{-1}) and influence of the tested factors on their production, as assessed by multiple regression analysis

	Strain 333							Regression coefficient*					
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO_2+Lyso+tan	Lyso	SO_2	Tannin	LysoxSO$_2$	LysoxTan	SO_2xTan
Ethylacetate	39.2 ± 28.2 ^{a}	120 ± 36.4 ^{b}	89.1 ± 12.3 ^{ab}	49.6 ± 6.06 ^{ab}	64.1 ± 22.4 ^{ab}	90.2 ± 1.94 ^{ab}	94.8 ± 10.5 ^{ab}	1.286	-	-	-	-	-
Isoamyl acetate	0.49 ± 0.02 ^{a}	0.81 ± 0.02 ^{b}	0.95 ± 0.14 ^{bc}	0.89 ± 0.12 ^{bc}	1.06 ± 0.07 ^{bc}	1.12 ± 0.01 ^{c}	0.86 ± 0.11 ^{bc}	0.782	0.971	1.399	-	-0.970	-0.885
Ethyl hexanoate	0.71 ± 0.32	0.67 ± 0.17	1.02 ± 0.16	0.74 ± 0.18	1.03 ± 0.19	1.07 ± 0.05	1.18 ± 0.12	-	-	-	-	-	-
Hexyl acetate	0.17 ± 0.16	0.09 ± 0.01	0.14 ± 0.03	0.10 ± 0.02	0.16 ± 0.04	0.22 ± 0.02	0.19 ± 0.03	-	-	-	-	-	-
Ethyl lactate	3.73 ± 0.50 ^{a}	7.28 ± 1.31 ^{b}	2.94 ± 0.19 ^{a}	1.84 ± 0.10 ^{a}	2.51 ± 0.19 ^{a}	2.80 ± 0.06 ^{a}	2.41 ± 0.08 ^{a}	1.007	-0.535	-0.930	-0.671	-	1.022
Ethyl octanoate	0.12 ± 0.03	0.12 ± 0.03	0.20 ± 0.02	0.14 ± 0.03	0.24 ± 0.13	0.09 ± 0.09	0.22 ± 0.05	-	-	1.300	-	-1.056	-
Ethyl 3 idroxybutyrate	0.16 ± 0.10	0.28 ± 0.06	0.26 ± 0.00	0.15 ± 0.02	0.16 ± 0.04	0.17 ± 0.01	0.17 ± 0.01	0.952	-	-	-	-	-
Ethyl decanoate (µg/L)	165 ± 96.0 ^{a}	283 ± 64.2 ^{a}	256 ± 4.55 ^{ab}	155 ± 22.3 ^{a}	164 ± 38.6 ^{a}	172 ± 12.4 ^{a}	170 ± 8.99 ^{b}	-	-	-	-	-	-
Diethyl succinate	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.00	0.01 ± 0.00	0.03 ± 0.02	0.03 ± 0.00	0.02 ± 0.00	-	-	-	-	-	-
Methyl-4-hydroxybutanoate	0.06 ± 0.04	0.02 ± 0.00	0.05 ± 0.01	0.06 ± 0.01	0.04 ± 0.02	0.08 ± 0.03	0.07 ± 0.02	-	-	-	-	-	-
Ethyl-4-hydroxybutanoate	2.05 ± 1.53	1.02 ± 0.30	3.04 ± 0.52	1.63 ± 0.27	3.28 ± 0.13	2.61 ± 0.88	3.19 ± 0.42	-	-	1.534	-		-
Phenylethyl acetate	0.29 ± 0.14	0.26 ± 0.01	0.34 ± 0.02	0.35 ± 0.08	0.61 ± 0.44	0.36 ± 0.13	0.47 ± 0.05	-	-	1.321	-	-1.203	-
i-P ropyl dodecanoate	0.03 ± 0.00 ^{ab}	0.02 ± 0.00 ^{ab}	0.03 ± 0.00 ^{ab}	0.01 ± 0.00 ^{a}	0.02 ± 0.01 ^{ab}	0.02 ± 0.02 ^{ab}	0.04 ± 0.01 ^{b}	-	-	-	-	-	-
Ethyl dodecanoate	3.55 ± 0.71	2.85 ± 0.43	3.42 ± 0.22	2.43 ± 0.27	2.55 ± 0.96	2.93 ± 0.63	3.26 ± 0.40	-	-	-	-	-	-
S-(3-hydroxypropyl) thioacetate (µg/L)	4.33 ± 2.53 ^{a}	3.37 ± 1.13 ^{a}	n.d. ± n.d ^{a}	515 ± 88.9 ^{b}	12.6 ± 7.55 ^{a}	8.25 ± 3.34 ^{a}	9.82 ± 4.56 ^{a}	-	1.403	-1.394	-1.268	1.264	-
1-Methylbutyl acetate	0.14 ± 0.08	0.08 ± 0.01	0.18 ± 0.02	0.11 ± 0.01	0.16 ± 0.03	0.13 ± 0.07	0.18 ± 0.04	-	-	-	-	-	-
Acetylglycine ethyl ester	0.07 ± 0.03	0.06 ± 0.02	0.07 ± 0.00	0.09 ± 0.01	0.02 ± 0.01	0.05 ± 0.04	0.09 ± 0.03	-	-	-	-	1.661	-
Ethyl 5-oxotetrahydro-2-furancarboxylate	0.05 ± 0.02	0.03 ± 0.01	0.06 ± 0.01	0.04 ± 0.00	0.04 ± 0.02	0.07 ± 0.02	0.06 ± 0.02	-	-	-	-	-	-
Ethyl hydrogen succinate	23.3 ± 0.47 ^{b}	15.2 ± 0.09 ^{ab}	24.7 ± 1.63 ^{b}	10.3 ± 3.01 ^{a}	19.9 ± 5.01 ^{ab}	26.0 ± 3.97 ^{b}	21.6 ± 5.32 ^{ab}	-	-1.090	1.980	1.823	-1.081	-1.068
Sum (excluding ethylacetate)	34.9 ± 3.52 ^{b}	28.9 ± 0.26 ^{ab}	37.4 ± 1.32 ^{b}	19.5 ± 10.9 ^{a}	31.9 ± 4.24 ^{ab}	37.8 ± 5.99 ^{b}	34.0 ± 6.00 ^{b}	-	-1,131	1,815	1,632	-1,081	

In the same row, different letters denote significant differences at $p< 0.01$

(1) only standardized regression coefficients (beta values) with $p < 0.01$, are reported

Table 11b. Final wines fermented with strain 1042: ester concentrations (mg L^{-1}) and influence of the tested factors on their production, as assessed by multiple regression analysis.

	Strain 1042							Regression coefficient*					
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO_2+Lyso+Tan	Lyso	SO_2	Tannin	LysoxSO$_2$	LysoxTan	SO$_2$xTan
Ethylacctatc	79.0 ± 2.05 ^{a}	78.2 ± 1.77 ^{a}	71.4 ± 2.15 ^{a}	75.2 ± 11.0 ^{a}	59.3 ± 6.13 ^{a}	70.7 ± 4.59 ^{a}	75.6 ± 4.05 ^{a}	-	-	-	-	-	-
Isoamyl acetate	1.08 ± 0.50 ^{bc}	1.52 ± 0.01 ^{c}	0.95 ± 0.07 ^{bc}	0.68 ± 0.10 ^{ab}	0.62 ± 0.13 ^{ab}	1.24 ± 0.00 ^{bc}	0.88 ± 0.02 ^{bc}	0.718	-	-0.911	-	-	0.552
Ethyl hexanoate	0.50 ± 0.19 ^{ab}	0.75 ± 0.04 ^{b}	0.67 ± 0.06 ^{b}	0.40 ± 0.12 ^{ab}	0.61 ± 0.13 ^{b}	0.52 ± 0.11 ^{ab}	0.67 ± 0.04 ^{b}	0.875	-	-	-	-	0.851
Hexyl acetate	0.16 ± 0.03 ^{b}	0.21 ± 0.01 ^{b}	0.20 ± 0.01 ^{b}	0.14 ± 0.02 ^{b}	0.19 ± 0.02 ^{b}	0.16 ± 0.05 ^{b}	0.22 ± 0.01 ^{b}	0.742	-	-0.735	-0.607	-	0.856
Ethyl lactate	5.34 ± 1.62 ^{a}	3.56 ± 0.19 ^{a}	3.32 ± 0.26 ^{a}	4.61 ± 0.39 ^{a}	3.17 ± 0.46 ^{a}	3.26 ± 0.72 ^{a}	3.66 ± 0.06 ^{a}	-1.129	-	-	-	-	-
Ethyl octanoate	0.64 ± 0.01 ^{abc}	1.02 ± 0.06 ^{d}	1.00 ± 0.14 ^{cd}	0.46 ± 0.08 ^{ab}	0.77 ± 0.18 ^{bcd}	0.66 ± 0.15 ^{abcd}	0.75 ± 0.04 ^{bcd}	1.056	-	-	-0.791	-	0.662
Ethyl-3 -hidroxybutyrate	1.90 ± 0.21 ^{ab}	2.47 ± 0.11 ^{b}	1.38 ± 0.05 ^{a}	1.69 ± 0.38 ^{a}	1.46 ± 0.03 ^{a}	1.74 ± 0.38 ^{ab}	1.36 ± 0.08 ^{a}	-	-	-	-	-0.709	-
Ethyl decanoate	0.26 ± 0.05 ^{ab}	0.31 ± 0.06 ^{ab}	0.38 ± 0.11 ^{b}	0.16 ± 0.04 ^{ab}	0.39 ± 0.10 ^{b}	0.16 ± 0.05 ^{ab}	0.18 ± 0.02 ^{ab}	-	-	-	-1.004	-	-
Diethyl succinate	0.37 ± 0.10 ^{bc}	0.31 ± 0.03 ^{ab}	0.26 ± 0.02 ^{ab}	0.36 ± 0.06 ^{abc}	0.20 ± 0.02 ^{a}	0.25 ± 0.06 ^{ab}	0.19 ± 0.01 ^{a}	-0.647	-	-	-	-	-0.617
Methyl-4-hydroxybutanoate	0.03 ± 0.01 ^{a}	0.02 ± 0.01 ^{a}	0.04 ± 0.00 ^{a}	0.03 ± 0.01 ^{a}	0.04 ± 0.02 ^{a}	0.03 ± 0.01 ^{a}	0.03 ± 0.00 ^{a}	-	-	-	-	-	-
Ethyl-4-hydroxybutanoate	8.74 ± 1.04 ^{a}	7.88 ± 0.48 ^{a}	10.4 ± 1.34 ^{a}	10.1 ± 1.07 ^{a}	11.7 ± 3.45 ^{a}	9.85 ± 2.36 ^{a}	11.7 ± 0.98 ^{a}	-	-	-	-	-	-
Phenylethyl acetate	0.55 ± 0.05 ^{ab}	1.20 ± 0.01 ^{c}	0.72 ± 0.04 ^{ab}	0.52 ± 0.08 ^{ab}	0.64 ± 0.05 ^{ab}	0.84 ± 0.29 ^{bc}	0.54 ± 0.02 ^{ab}	1.320	-	-	-0.677	-0.577	-
Isopropyl dodecanoate	0.02 ± 0.01 ^{a}	0.01 ± 0.01 ^{a}	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.02 ± 0.01 ^{a}	-	-	-	-	-	-
Diethyl hydroxybutanedioate	0.34 ± 0.08 ^{a}	0.28 ± 0.12 ^{a}	0.34 ± 0.07 ^{a}	0.32 ± 0.05 ^{a}	0.35 ± 0.11 ^{a}	0.32 ± 0.09 ^{a}	0.32 ± 0.03 ^{a}	-	-	-	-	-	-
Acetylglycine ethyl ester	0.20 ± 0.03 ^{b}	0.07 ± 0.03 ^{a}	0.06 ± 0.01 ^{a}	0.13 ± 0.05 ^{ab}	0.10 ± 0.01 ^{a}	0.07 ± 0.02 ^{a}	0.07 ± 0.00 ^{a}	-1.126	-	-0.963	-	-	-
Ethyl 5-oxotetrahydro-2-furancarboxylate	0.31 ± 0.06 ^{a}	0.20 ± 0.09 ^{a}	0.20 ± 0.05 ^{a}	0.19 ± 0.02 ^{a}	0.22 ± 0.06 ^{a}	0.21 ± 0.06 ^{a}	0.19 ± 0.02 ^{a}	-	-	-	-	-	-
Ethyl hydrogen succinate	114 ± 15.0 ^{b}	71.0 ± 30.8 ^{ab}	61.7 ± 12.0 ^{ab}	73.1 ± 6.53 ^{ab}	67.0 ± 13.1 ^{ab}	57.0 ± 13.7 ^{a}	57.7 ± 3.75 ^{a}	-0.889	-0.838	-0.741	-	-	-
Sum (excluding ethylacetate)	135 ± 17.5 ^{a}	90.8 ± 31.4 ^{a}	81.6 ± 14.0 ^{a}	92.9 ± 6.50 ^{a}	87.4 ± 17.0 ^{a}	76.4 ± 17.9 ^{a}	78.5 ± 4.26 ^{a}	-	-	0.860	-	-	-

In the same row, different letters denote significant differences at $p< 0.01$

(1) only standardized regression coefficients (beta values) with $p < 0.01$, are reported

Table 12a. Final wines fermented with strain 333: acid concentrations (mg L^{-1}) and influence of the tested factors on their production, as assessed by multiple regression analysis

	Strain 333							Regression coefficient*					
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO2+Lyso+tan	Lyso	SO_2	Tannin	LysoxSO$_2$	LysoxTan	SO$_2$xTan
Acetic acid	10.7 ± 5.00 ^{a}	11.1 ± 2.26 ^{a}	19.3 ± 4.57 ^{ab}	17.6 ± 3.28 ^{ab}	23.7 ± 0.54 ^{bc}	32.1 ± 0.95 ^{c}	18.5 ± 2.52 ^{ab}	-	-	1.887	0.876	-1.216	-1.350
Isobutyric acid	1.46 ± 0.19 ^{b}	1.55 ± 0.15 ^{b}	0.12 ± 0.01 ^{a}	2.20 ± 0.29 ^{b}	1.87 ± 0.23 ^{b}	2.00 ± 0.29 ^{b}	2.13 ± 0.20 ^{b}	-	0.533	-1.360	-	-	1.026
Butanoic acid	0.35 ± 0.06 ^{ab}	0.35 ± 0.03 ^{a}	0.33 ± 0.02 ^{a}	0.60 ± 0.09 ^{b}	0.46 ± 0.15 ^{ab}	0.37 ± 0.00 ^{ab}	0.38 ± 0.05 ^{ab}	-	1.147	-	-	-	-
Octanoic acid	8.61 ± 1.93	6.48 ± 1.25	8.88 ± 0.79	5.44 ± 1.02	8.36 ± 0.16	6.91 ± 3.11	8.96 ± 1.22	-	-	-	-	-	-
n-Decanoic acid	2.31 ± 0.94	1.40 ± 0.42	2.77 ± 0.19	1.32 ± 0.33	1.74 ± 1.01	3.39 ± 1.03	3.07 ± 0.50	-	-	-	1.402	-	-
Dodecanoic acid	0.15 ± 0.06	0.10 ± 0.03	0.19 ± 0.01	0.17 ± 0.12	0.13 ± 0.03	0.24 ± 0.03	0.27 ± 0.07	-	-	-	-	-	-
Tetradecanoic acid	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	-	-	-	-	-	-
n-Hexadecanoic acid	0.53 ± 0.20 ^{ab}	0.60 ± 0.02 ^{ab}	0.61 ± 0.11 ^{ab}	0.80 ± 0.12 ^{b}	0.21 ± 0.12 ^{a}	0.81 ± 0.07 ^{b}	0.57 ± 0.09 ^{ab}	-	-	-	-	-	-
Sum (excluding acetic acid)	13.4 ± 2.81 ^{a}	10.5 ± 1.51 ^{a}	12.9 ± 0.91 ^{a}	10.5 ± 1.75 ^{a}	12.8 ± 1.15 ^{a}	13.7 ± 4.40 ^{a}	15.4 ± 2.04 ^{a}	-	-	-	-	-	-

Table 12b. Final wines fermented with strain 1042: acid concentrations (mg L^{-1}) and influence of the tested factors on their production, as assessed by multiple regression analysis

	Strain 1042							Regression coefficient*					
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO2 + Lyso	SO2+Lyso+tan	Lyso	SO_2	Tannin	LysoxSO$_2$	LysoxTan	SO$_2$xTan
Acetic acid	11.5 ± 3.02 ^{a}	7.53 ± 1.11 ^{a}	10.9 ± 1.27 ^{a}	11.8 ± 0.19 ^{a}	8.93 ± 3.05 ^{a}	8.80 ± 3.46 ^{a}	11.6 ± 3.56 ^{a}	-0.733	-	0.809	-	-	-
Propionic acid	0.04 ± 0.01 ^{a}	0.32 ± 0.12 ^{b}	0.10 ± 0.06 ^{a}	0.04 ± 0.01 ^{a}	0.03 ± 0.01 ^{a}	0.03 ± 0.01 ^{a}	0.03 ± 0.01 ^{a}	1.076	-	-	-0.720	-	-
Isobutyric acid	3.34 ± 0.47 ^{c}	2.99 ± 0.15 ^{bc}	2.06 ± 0.11 ^{ab}	2.09 ± 0.36 ^{ab}	1.20 ± 0.26 ^{a}	1.75 ± 0.64 ^{a}	1.46 ± 0.28 ^{a}	-	-0.750	0.605	0.626	-	-
Butanoic acid	0.18 ± 0.01 ^{a}	0.20 ± 0.05 ^{a}	0.20 ± 0.02 ^{a}	0.18 ± 0.02 ^{a}	0.17 ± 0.00 ^{a}	0.18 ± 0.04 ^{a}	0.18 ± 0.02 ^{a}	-	-	-	-	-	-
Pentanoic acid	0.78 ± 0.11 ^{ab}	0.58 ± 0.42 ^{a}	0.68 ± 0.04 ^{a}	0.74 ± 0.05 ^{ab}	0.61 ± 0.07 ^{a}	0.75 ± 0.22 ^{ab}	0.61 ± 0.02 ^{a}	-	-	0.808	-	-	-0.735
Hexanoic acid	0.91 ± 0.19 ^{ab}	1.57 ± 0.09 ^{c}	1.66 ± 0.04 ^{c}	0.72 ± 0.05 ^{a}	1.54 ± 0.25 ^{c}	1.25 ± 0.27 ^{ab}	1.45 ± 0.12 ^{bc}	1.164	-	-	-0.561	-	0.531
Octanoic acid	6.21 ± 1.85 ^{a}	7.37 ± 3.93 ^{a}	9.84 ± 1.98 ^{a}	5.15 ± 1.39 ^{a}	9.83 ± 1.52 ^{a}	6.53 ± 1.52 ^{a}	7.23 ± 0.29 ^{a}	-	-	-	-	-	-
Decanoic acid	6.09 ± 1.71 ^{ab}	4.55 ± 2.91 ^{ab}	6.36 ± 0.31 ^{b}	3.56 ± 1.33 ^{ab}	6.61 ± 1.10 ^{b}	3.28 ± 0.72 ^{ab}	4.73 ± 0.81 ^{ab}	-	-	-	-	-	-
Dodecanoic acid	1.18 ± 0.43 ^{b}	0.52 ± 0.53 ^{ab}	0.64 ± 0.22 ^{ab}	0.58 ± 0.28 ^{ab}	0.97 ± 0.19 ^{ab}	0.40 ± 0.10 ^{ab}	0.15 ± 0.16 ^{a}	-	-	-	-	-	-
Tetradecanoic acid	0.63 ± 0.00 ^{c}	0.38 ± 0.33 ^{abc}	0.26 ± 0.00 ^{abc}	0.21 ± 0.03 ^{abc}	0.45 ± 0.08 ^{bc}	0.16 ± 0.04 ^{ab}	0.00 ± 0.00 ^{a}	-	-	-	-	-	-
Hexadecanoic acid	8.21 ± 0.17 ^{a}	2.94 ± 2.55 ^{a}	2.92 ± 0.13 ^{a}	4.37 ± 2.27 ^{a}	7.50 ± 2.60 ^{a}	4.11 ± 1.28 ^{a}	3.74 ± 4.56 ^{a}	-	-	-	-	-	-
Sum	27.6 ± 4.06 ^{a}	21.4 ± 10.2 ^{a}	24.7 ± 2.29 ^{a}	17.6 ± 4.59 ^{a}	28.9 ± 0.06 ^{a}	18.4 ± 4.10 ^{a}	19.6 ± 5.38 ^{a}	-	-	-	-	-	-

In the same row, different letters denote significant differences at $p< 0.01$

(1) only standardized regression coefficients (beta values) with $p < 0.01$, are reported.

3.3 Evolution of volatile compounds in wines fermented with strain 333 during 1 year of bottle ageing

In order to better understand the evolution of volatile compounds of wine during storage, the wine samples fermented with strain 333 were aged for 1 year in bottles stored in the dark and at cellar temperature. The analysis of volatile compounds was carried out after 3 months and after 1 year of storage.

3.3.1 Alcohols

Concerning the alcohols amount as a sum (Tables 10a, 13 and 14), the data showed a greater influence of SO_2 as compared to lysozyme addition on the evolution of alcohols during the storage, as was the case for the samples at the end of the alcoholic fermentation.

Among the single compounds, the amount of phenylethyl alcohol and benzyl alcohol increased during the storage time. Furthermore, they were found in higher concentration in wine fermented with SO_2 and SO_2 together with added tannins, compared to wines with lysozyme and lysozyme together with added tannins, suggesting an influence of SO_2 on the evolution of alcohols during the storage, previously noted by Garde-Cerdan (2007).

For 3-ethoxy-1-propanol, the data showed a negative influence of SO_2 during all of the storage time, confirming the results obtained for the samples at the end of the alcoholic fermentation. The amount of this alcohol is constant during all storage times for samples with SO_2 and SO_2 together with added tannins, whereas its concentration showed a decrease after 3 months of storage followed by a constant trend after 1 year of storage in the lysozyme and lysozyme together with added tannin samples.

For 3-ethylthio-1-propanol production, the data suggested a positive influence of SO_2 addition starting at the end of the fermentation and during all the storage period. Furthermore, the SO_2 addition showed a positive influence that continues upon ageing. The data highlight a strong increment in the value after 3 months of storage that is maintained after 1 year of bottle storage.

An increment in the amount of isoamyl alcohol was also found after 3 months of storage and it continued after 1 year for the samples fermented with SO_2 and with added lysozyme. In this case, the addition of tannins had a detrimental effect, causing a decrease in isoamyl alcohol values after 1 year of storage. However, for this compound the multiple regression analysis did not show any significant influence due to the factors studied.

3.3.2 Esters

The production of esters during the storage period increased widely, varying from less than 50 mg L^{-1} at the end of the alcoholic fermentation to about 200-300 mg L^{-1} after 1 year of ageing (Tables 11a, 15 and 16). However this trend is almost completely due to the large augmentation of ethyl lactate and ethyl hydrogen succinate.

Multiple regression analysis showed a positive influence of tannins on total ester amounts during the storage, whereas the addition of SO_2 showed a negative influence at the end of the fermentation, and a positive influence after 3 months of bottle aging. In fact, at 3 months of bottle storage, SO_2 resulted in the most positive influencing parameter, whereas after 1 year, tannins resulted in the most positive influence, as was the case at the end of the alcoholic fermentation.

Among the single compounds, hexyl acetate, ethyl lactate and diethyl succinate showed the same trend of evolution, increasing during the storage time up to one 1 year for all the samples developed (Fig. 3a,b,c). Furthermore, hexyl acetate and ethyl lactate production and evolution during the storage time are significantly influenced by the presence of SO_2. The statistical data showed a negative regression coefficient for SO_2 and tannin factors, and a positive coefficient for lysozyme addition for ethyl lactate, whereas a positive regression coefficient was found for the SO_2 factor for hexyl acetate.

Concerning the MCFA ethyl esters, the higher value of the sample with tannins compared to others without added tannins confirmed the hypothesis that tannins have an oxygen scavenging activity, which maintains the amount of these esters over certain levels (Sonni et al, 2009; Bosso et al., 2001).

The same MCFA ethyl esters, however, showed a variable trend during the bottle storage time. For example, ethyl hexanoate decreased during the storage in all samples, except the SO_2 sample (that showed an increase after 3 month of storage), while ethyl octanoate decreased during the storage for wines with added SO_2 and wines with SO_2 together with added tannins, but increased after 3 months of storage for samples with lysozyme and lysozyme together with added tannins, and decreased after 1 year of aging, such as the ethyl decanoate trend for all the samples. Furthermore, a positive influence for the tannins parameter was highlighted for all the analysis steps, at the end of fermentation, and after 3 months and 1 year of bottle storage.

With regard to the acetates, the data showed a decrease during the storage ageing reaching a low amount for isoamylacetate (20-30 mg L^{-1}) whereas an increase after 3 month followed by a decrease after 1 year of bottle storage, coming back to the same values shown at the end of

alcoholic fermentation was found for phenylethyl acetate. For hexyl acetate, the increasing amount trend during the ageing time was previously discussed.

3.3.3 Acids

Tables 17a and 17b shows the acid concentrations after 3 months and 1 year of bottle storage.
Lysozyme addition samples showed a lower total concentration amount during the aging time.
In addition, the effect of SO_2, lysozyme or added tannins on the evolution of acids during wine storage in bottles was less than in the case of esters and alcohols.
Fatty acids contribute to either the fresh flavour of wine if they are present in the correct amount, or to an unpleasant flavour if they are in excess, and they also help to modify the perception of other taste sensations. The total fatty acid concentrations at the end of the fermentation and during the storage time were found to be around 10-25 mg L^{-1}, a value that did not impair wine aroma (Sonni et al., 2009).

4. CONCLUSIONS

The data obtained confirmed that the addition of lysozyme and oenological tannins during alcoholic fermentation can positively replace the use of sulphur dioxide, as was shown in our previous study (Sonni et al., 2009). The different oenological protocols influenced the volatile composition of final wines, also as a consequence of the responses to yeast in the presence/absence of sulphites during fermentation.
The volatile compound evolution of such wines during storage showed the strong influence of the presence of SO_2 on the evolution of alcohols and esters. Also the presence of gallotannins displayed a positive role in scavenging oxygen and maintaining the amounts of esters over certain levels in wine stored for 12 months. By contrast, acids were virtually unaffected by the investigated variables, (SO_2, lysozyme and tannins) during the storage time.

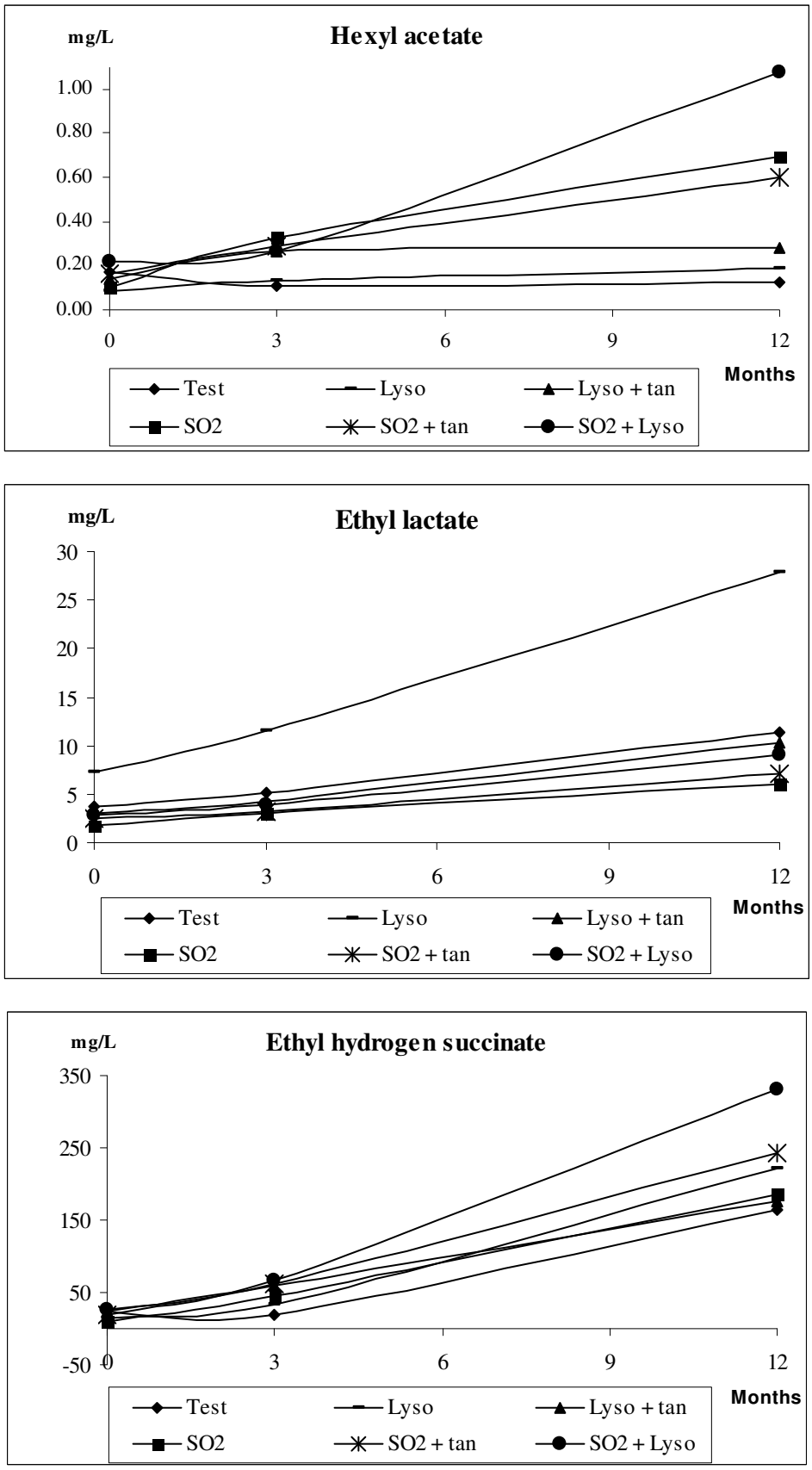


Figure 3. Evolution of hexyl acetate, ethyl lactate and ethyl hydrogen succinate during the storage of wine. Standard deviation of data have been omitted to enhance clarity.

Table 13. Wines fermented with strain 333 after 3 months of storage: alcohol concentrations (mg L^{-1}) and influence of the tested factors on their production, as assessed by multiple regression analysis

	Strain 333							Regression coefficient*					
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO_2+Lyso+tan	Lyso	SO_2	Tannin	LysoxSO$_2$	LysoxTan	SO$_2$xTan
Isobutyl alcohol	4.10 ± 0.01 ^{ab}	4.85 ± 0.51 ^{ab}	8.38 ± 1.95 ^{b}	6.34 ± 0.56 ^{ab}	6.20 ± 2.12 ^{ab}	5.24 ± 1.04 ^{ab}	3.15 ± 1.15 ^{a}	-	-	-	-	-	-0.74
1-Butanol	0.25 ± 0.00 ^{a}	0.27 ± 0.06 ^{a}	0.54 ± 0.05 ^{a}	0.58 ± 0.21 ^{a}	0.59 ± 0.12 ^{a}	0.58 ± 0.06 ^{a}	0.33 ± 0.09 ^{a}	-	-	-	-	-	-0.77
Isoamylalcohol	43.1 ± 2.93 ^{a}	50.6 ± 7.34 ^{a}	67.9 ± 10.7 ^{a}	55.1 ± 4.52 ^{a}	57.4 ± 16.4 ^{a}	51.2 ± 6.77 ^{a}	37.2 ± 7.23 ^{a}	-	-	-	-	-	-0.66
2-Hexanol	0.07 ± 0.02 ^{bc}	0.07 ± 0.01 ^{bc}	0.08 ± 0.01 ^{bc}	0.08 ± 0.00 ^{c}	0.04 ± 0.02 ^{abc}	0.02 ± 0.00 ^{a}	0.03 ± 0.01 ^{ab}	-	-	-	-0.53	0.50	
3-Methyl-1-pentanol	0.07 ± 0.01 ^{ab}	0.06 ± 0.01 ^{a}	0.09 ± 0.01 ^{abc}	0.11 ± 0.02 ^{c}	0.10 ± 0.00 ^{bc}	0.11 ± 0.00 ^{c}	0.09 ± 0.01 ^{abc}	-	0.52	-	-	-	-0.58
1-Hexanol	0.79 ± 0.11 ^{a}	0.86 ± 0.13 ^{ab}	1.11 ± 0.05 ^{b}	0.82 ± 0.07 ^{a}	0.78 ± 0.04 ^{a}	0.80 ± 0.02 ^{a}	0.68 ± 0.01 ^{a}	-	-0.72	-	-	-	-0.67
3-Ehoxy-1-propanol	0.38 ± 0.23 ^{b}	0.13 ± 0.02 ^{ab}	0.37 ± 0.04 ^{ab}	0.06 ± 0.03 ^{a}	0.09 ± 0.02 ^{ab}	0.13 ± 0.01 ^{ab}	0.08 ± 0.01 ^{ab}	-	-1.02	-	0.56	-	-
cis-3-Hexen-1-ol	0.05 ± 0.01 ^{ab}	0.04 ± 0.00 ^{a}	0.07 ± 0.01 ^{abc}	0.08 ± 0.01 ^{bc}	0.07 ± 0.00 ^{abc}	0.09 ± 0.01 ^{c}	0.07 ± 0.01 ^{abc}	-		-	-	-	-0.74
1-Octanol	0.04 ± 0.00 ^{a}	0.05 ± 0.00 ^{a}	0.05 ± 0.00 ^{a}	0.05 ± 0.01 ^{a}	0.04 ± 0.01 ^{a}	0.04 ± 0.00 ^{a}	0.05 ± 0.00 ^{a}	-	0.52	-	-	-	-0.58
3-(Methythio)-1-propanol	0.56 ± 0.13 ^{ab}	0.35 ± 0.06 ^{a}	0.29 ± 0.16 ^{a}	0.98 ± 0.11 ^{c}	0.96 ± 0.05 ^{c}	0.82 ± 0.01 ^{bc}	1.14 ± 0.04 ^{c}	-	0.82	-	-	-	-
3-(Ethylthio)-1-propanol (µg/L)	10.2 ± 4.58 ^{a}	10.7 ± 4.01 ^{a}	11.9 ± 1.16 ^{a}	13.0 ± 7.45 ^{a}	6.12 ± 2.24 ^{a}	4.58 ± 0.66 ^{a}	34.7 ± 1.58 ^{b}	0.40	0.61	0.49	-	-	-
Benzyl alcohol	0.43 ± 0.13 ^{a}	0.37 ± 0.04 ^{a}	0.49 ± 0.04 ^{a}	0.37 ± 0.06 ^{a}	0.46 ± 0.02 ^{a}	0.49 ± 0.04 ^{a}	0.78 ± 0.05 ^{b}	0.50	0.75	-	-	-	-
Phenylethyl alcohol	28.0 ± 0.41 ^{a}	32.5 ± 2.49 ^{a}	37.9 ± 8.16 ^{ab}	38.1 ± 2.56 ^{ab}	42.5 ± 11.1 ^{ab}	39.7 ± 3.95 ^{ab}	58.3 ± 4.83 ^{b}	-0.71	-0.71	-	0.71	-	-
1-Octadecanol (stenol)	0.08 ± 0.02 ^{a}	0.09 ± 0.01 ^{ab}	0.09 ± 0.04 ^{ab}	0.09 ± 0.08 ^{ab}	0.05 ± 0.01 ^{a}	0.08 ± 0.02 ^{ab}	0.29 ± 0.13 ^{b}	0.37	0.77	-	-	0.46	0.44
4-Hydroxy-benzeneethanol	40.6 ± 11.0 ^{a}	37.2 ± 6.85 ^{a}	40.1 ± 7.54 ^{a}	51.1 ± 11.4 ^{a}	61.0 ± 9.35 ^{a}	58.7 ± 10.3 ^{a}	150 ± 30.3 ^{b}	-	-	-	-	-	-
Sum	124 ± 12.0 ^{a}	132 ± 1.42 ^{a}	163 ± 19.1 ^{a}	158 ± 13.5 ^{a}	176 ± 37.7 ^{ab}	164 ± 21.8 ^{a}	258 ± 41.6 ^{b}	0.49	0.70	-	-	-	-

In the same row, different letters denote significant differences at $p < 0.01$

(1) only standardized regression coefficients (beta values) with $p < 0.01$, are reported

Table 14. Wines fermented with strain 333 after 1 year of storage: alcohol concentrations (mg L^{-1}) and influence of the tested factors on their production, as assessed by multiple regression analysis

	Strain 333							Regression coefficient*					
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO_2+Lyso+tan	Lyso	SO_2	Tannin	LysoxSO$_2$	LysoxTan	SO$_2$xTan
Isobutyl alcohol	5.01 ± 2.84 ^{a}	6.35 ± 1.67 ^{a}	8.53 ± 1.54 ^{a}	7.31 ± 1.85 ^{a}	7.29 ± 2.88 ^{a}	7.38 ± 0.35 ^{a}	9.80 ± 2.93 ^{a}	-	-	-	-	-	-
1-Butanol	0.42 ± 0.31 ^{a}	0.45 ± 0.15 ^{a}	0.51 ± 0.04 ^{a}	0.51 ± 0.13 ^{a}	0.56 ± 0.21 ^{a}	0.71 ± 0.03 ^{a}	0.43 ± 0.17 ^{a}	-	-	-	-	-	-
Isoamylalcohol	42.9 ± 8.66 ^{a}	52.8 ± 11.4 ^{a}	64.2 ± 0.92 ^{a}	58.0 ± 4.51 ^{a}	52.9 ± 13.4 ^{a}	56.9 ± 2.71 ^{a}	63.0 ± 20.9 ^{a}	-	-	-	-	-	-
2-Hexanol	0.03 ± 0.01 ^{a}	0.10 ± 0.01 ^{b}	0.09 ± 0.01 ^{ab}	0.08 ± 0.01 ^{ab}	0.08 ± 0.02 ^{ab}	0.07 ± 0.00 ^{ab}	0.09 ± 0.03 ^{ab}	0.82	-	-	-0.77	-	-
3-Methyl-1-pentanol	0.07 ± 0.03 ^{a}	0.08 ± 0.02 ^{a}	0.11 ± 0.01 ^{a}	0.11 ± 0.01 ^{a}	0.11 ± 0.02 ^{a}	0.12 ± 0.01 ^{a}	0.13 ± 0.03 ^{a}	-	-	-	-	-	-
1-Hexanol	0.72 ± 0.25 ^{a}	0.75 ± 0.10 ^{a}	0.88 ± 0.04 ^{a}	0.77 ± 0.06 ^{a}	0.73 ± 0.06 ^{a}	0.88 ± 0.04 ^{a}	0.84 ± 0.14 ^{a}	-	-	-	-	-	-
trans -3-Hexen-1-ol (µg/L)	8.62 ± 5.65 ^{ab}	9.07 ± 0.68 ^{ab}	10.4 ± 2.49 ^{ab}	10.3 ± 5.38 ^{ab}	13.6 ± 5.86 ^{b}	17.6 ± 0.84 ^{b}	0.00 ± 0.00 ^{a}	-	-	-	-	-0.88	-0.80
3-Ehoxy-1-propanol	0.23 ± 0.00 ^{b}	0.14 ± 0.03 ^{ab}	0.37 ± 0.05 ^{c}	0.05 ± 0.01 ^{a}	0.08 ± 0.03 ^{a}	0.12 ± 0.01 ^{a}	0.09 ± 0.02 ^{a}	-	-1.08	0.61	0.36	-	-0.61
cis-3-Hexen-1-ol	0.05 ± 0.03 ^{a}	0.05 ± 0.01 ^{a}	0.06 ± 0.01 ^{a}	0.07 ± 0.01 ^{a}	0.06 ± 0.01 ^{a}	0.08 ± 0.00 ^{a}	0.07 ± 0.02 ^{a}	-	-	-	-	-	-
1-Octanol	0.02 ± 0.00 ^{a}	0.06 ± 0.00 ^{ab}	0.06 ± 0.02 ^{b}	0.06 ± 0.00 ^{b}	0.05 ± 0.01 ^{ab}	0.04 ± 0.00 ^{ab}	0.06 ± 0.01 ^{b}	0.64	0.60	-	-0.88	-	-
2-(Methylthio)-ethanol	1.60 ± 0.14 ^{a}	2.32 ± 0.50 ^{a}	5.50 ± 0.46 ^{b}	3.64 ± 1.20 ^{ab}	4.38 ± 0.35 ^{ab}	5.70 ± 0.27 ^{b}	5.92 ± 1.56 ^{b}	-	-	0.57	-	-	-0.43
3-(Methythio)-1-propanol	0.50 ± 0.11 ^{a}	0.60 ± 0.02 ^{a}	0.56 ± 0.09 ^{a}	1.01 ± 0.12 ^{b}	0.85 ± 0.16 ^{ab}	0.80 ± 0.04 ^{ab}	0.80 ± 0.14 ^{ab}	-	0.97	-	-	-	-
3-(Ethylthio)-1-propanol (µg/L)	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	4.04 ± 0.44 ^{bc}	5.27 ± 0.42 ^{c}	7.10 ± 0.34 ^{d}	2.72 ± 0.92 ^{b}	-0.23	0.62	-	0.28	-0.52	-0.40
Benzyl alcohol	0.72 ± 0.29 ^{a}	1.04 ± 0.22 ^{a}	1.15 ± 0.18 ^{a}	1.04 ± 0.01 ^{a}	0.75 ± 0.18 ^{a}	0.58 ± 0.03 ^{a}	0.97 ± 0.26 ^{a}	-	-	-	-0.77	0.67	-
Phenylethyl alcohol	38.4 ± 4.99 ^{a}	40.9 ± 11.6 ^{a}	32.9 ± 2.30 ^{a}	47.6 ± 5.80 ^{a}	52.1 ± 6.15 ^{a}	49.8 ± 2.37 ^{a}	54.3 ± 13.9 ^{a}	-	0.78	-	-	-	-
1-Octadecanol (stenol)	0.06 ± 0.03 ^{a}	0.10 ± 0.03 ^{a}	0.07 ± 0.02 ^{a}	0.08 ± 0.01 ^{a}	0.04 ± 0.02 ^{a}	0.08 ± 0.00 ^{a}	0.05 ± 0.03 ^{a}	-	-	-	-	-	-
4-Hydroxy-benzeneethanol	32.8 ± 9.35 ^{b}	45.1 ± 6.62 ^{ab}	41.4 ± 9.08 ^{ab}	52.5 ± 12.6 ^{ab}	36.8 ± 10.9 ^{a}	70.9 ± 3.38 ^{b}	33.6 ± 12.2 ^{a}	-	-	-	-	-	-0.56
Sum	126 ± 22.0 ^{a}	158 ± 29.2 ^{ab}	159 ± 11.3 ^{ab}	176 ± 13.8 ^{ab}	159 ± 26.0 ^{ab}	197 ± 9.25 ^{b}	173 ± 15.1 ^{ab}	-	-	-	-	-	-

In the same row, different letters denote significant differences at $p< 0.01$

(1) only standardized regression coefficients (beta values) with $p < 0.01$, are reported

Table 15. Wines fermented with strain 333 after 3 months of storage: ester concentrations (mg L^{-1}) and influence of the tested factors on their production, as assessed by multiple regression analysis

	Strain 333							Regression coefficient*					
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO_2+Lyso+tan	Lyso	SO_2	Tannin	LysoxSO$_2$	LysoxTan	SO$_2$xTan
Isoamyl acetate	0.54 ± 0.03 ^{a}	0.64 ± 0.07 ^{a}	0.91 ± 0.07 ^{a}	0.79 ± 0.28 ^{a}	0.78 ± 0.11 ^{a}	0.66 ± 0.02 ^{a}	0.57 ± 0.09 ^{a}	-	-	-	-	-	-
Ethyl hexanoate	0.66 ± 0.20 ^{a}	0.64 ± 0.07 ^{a}	1.15 ± 0.04 ^{a}	1.03 ± 0.26 ^{a}	1.04 ± 0.06 ^{a}	0.97 ± 0.01 ^{a}	1.07 ± 0.19 ^{a}	-	-	0.58	-	-	-
Hexyl acetate	0.11 ± 0.00 ^{a}	0.13 ± 0.03 ^{a}	0.26 ± 0.02 ^{b}	0.33 ± 0.03 ^{b}	0.29 ± 0.06 ^{b}	0.26 ± 0.01 ^{b}	0.30 ± 0.05 ^{b}	-	0.74	-	-	-	-
Ethyl lactate	5.17 ± 0.73 ^{a}	11.5 ± 1.75 ^{b}	4.22 ± 0.57 ^{a}	3.07 ± 0.47 ^{a}	3.24 ± 0.36 ^{a}	3.83 ± 0.21 ^{a}	2.65 ± 0.22 ^{a}	0.49	-0.31	-0.61	-0.47	-	0.52
Ethyl octanoate	0.74 ± 0.20 ^{a}	0.75 ± 0.14 ^{a}	1.42 ± 0.14 ^{c}	0.92 ± 0.08 ^{ab}	1.37 ± 0.22 ^{bc}	1.59 ± 0.05 ^{c}	1.80 ± 0.04 ^{c}	-	-	0.57	-	-0.60	-0.92
Ethyl-3 -hydroxybutyrate	1.72 ± 0.98 ^{a}	1.67 ± 0.54 ^{a}	3.09 ± 0.29 ^{a}	2.15 ± 0.42 ^{a}	2.27 ± 0.29 ^{a}	2.36 ± 0.04 ^{a}	2.13 ± 0.02 ^{a}	0.68	0.72	-	-	-	-
Ethyl decanoate (µg/L)	213 ± 128 ^{a}	157 ± 26.6 ^{a}	332 ± 80.9 ^{abc}	253 ± 127 ^{ab}	357 ± 157 ^{abc}	584 ± 5.59 ^{bc}	647 ± 12.0 ^{c}	-	0.84	-	-	-	-
Diethyl succinate	0.19 ± 0.00 ^{a}	0.14 ± 0.04 ^{a}	0.33 ± 0.02 ^{ab}	0.23 ± 0.11 ^{a}	0.34 ± 0.08 ^{ab}	0.47 ± 0.00 ^{b}	0.45 ± 0.06 ^{b}	-	0.99	-	-	0.27	0.29
Methyl-4-hydroxybutanoate	0.02 ± 0.01 ^{ab}	0.01 ± 0.00 ^{a}	0.03 ± 0.00 ^{ab}	0.03 ± 0.02 ^{ab}	0.04 ± 0.01 ^{ab}	0.03 ± 0.00 ^{ab}	0.04 ± 0.00 ^{b}	0.74	0.65	-	-	0.94	0.73
Ethyl-4-hydroxybutanoate	2.17 ± 0.12 ^{a}	3.01 ± 0.81 ^{a}	9.01 ± 0.81 ^{bc}	6.34 ± 2.75 ^{ab}	9.70 ± 0.82 ^{bc}	11.2 ± 0.20 ^{c}	16.4 ± 1.53 ^{d}	0.41	0.94	-	-	-	-
Phenylethyl acetate	0.51 ± 0.04 ^{a}	0.72 ± 0.14 ^{ab}	0.86 ± 0.07 ^{ab}	1.19 ± 0.43 ^{b}	1.34 ± 0.08 ^{bc}	1.20 ± 0.00 ^{b}	1.88 ± 0.16 ^{c}	-	-	-	-	0.71	-
Isopropyl dodecanoate	0.02 ± 0.01 ^{a}	0.02 ± 0.01 ^{a}	0.02 ± 0.00 ^{a}	0.02 ± 0.01 ^{a}	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.03 ± 0.01 ^{a}	0.54	0.53	-	-	-	-
Diethyl hydroxybutanedioate	0.34 ± 0.41 ^{ab}	0.15 ± 0.00 ^{a}	0.40 ± 0.03 ^{ab}	0.36 ± 0.18 ^{ab}	0.45 ± 0.03 ^{ab}	0.65 ± 0.04 ^{ab}	0.92 ± 0.03 ^{b}	0.61	0.71	-	-	0.47	0.35
Acetylglycine ethyl ester	0.05 ± 0.01 ^{a}	0.05 ± 0.02 ^{a}	0.07 ± 0.01 ^{a}	0.09 ± 0.01 ^{a}	0.10 ± 0.01 ^{a}	0.10 ± 0.00 ^{a}	0.24 ± 0.05 ^{b}	-	0.78	-	-	-	-
Ethyl 5-oxotetrahydro-2-furancarboxylate	0.20 ± 0.13 ^{a}	0.11 ± 0.03 ^{a}	0.23 ± 0.01 ^{a}	0.35 ± 0.17 ^{ab}	0.28 ± 0.04 ^{a}	0.41 ± 0.02 ^{ab}	0.65 ± 0.10 ^{b}	0.49	0.66	-	0.40	0.25	0.24
Ethyl hydrogen succinate	18.5 ± 3.01 ^{a}	33.4 ± 0.67 ^{ab}	58.4 ± 6.28 ^{c}	44.5 ± 9.71 ^{bc}	61.6 ± 11.5 ^{c}	66.9 ± 6.86 ^{c}	119 ± 1.83 ^{d}	0.82	0.77	-	-	0.38	0.57
Sum	31.2 ± 4.31 ^{a}	53.1 ± 1.29 ^{ab}	80.8 ± 5.81 ^{cd}	61.6 ± 13.6 ^{bc}	83.2 ± 10.9	91.2 ± 7.29 ^{d}	148 ± 2.23 ^{e}	0.62	0.70	0.35	-	0.25	0.21

In the same row, different letters denote significant differences at $p < 0.01$

(1) only standardized regression coefficients (beta values) with $p < 0.01$, are reported

Table 16. Wines fermented with strain 333 after 1 year of storage: ester concentrations (mg L^{-1}) and influence of the tested factors on their production, as assessed by multiple regression analysis

	Strain 333							Regression coefficient*					
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO_2+Lyso+tan	Lyso	SO_2	Tannin	LysoxSO$_2$	LysoxTan	SO$_2$xTan
Isoamyl acetate	0.21 ± 0.15 ^{a}	0.20 ± 0.06 ^{a}	0.24 ± 0.02 ^{a}	0.20 ± 0.05 ^{a}	0.21 ± 0.04 ^{a}	0.30 ± 0.01 ^{a}	0.26 ± 0.12 ^{a}	-	-	-	-	-	-
Ethyl hexanoate	0.54 ± 0.26 ^{a}	0.48 ± 0.20 ^{a}	0.78 ± 0.09 ^{a}	0.58 ± 0.10 ^{a}	0.68 ± 0.14 ^{a}	0.91 ± 0.04 ^{a}	0.83 ± 0.26 ^{a}	-	-	-	-	-	-
Hexyl acetate	0.13 ± 0.01 ^{a}	0.19 ± 0.02 ^{a}	0.28 ± 0.04 ^{ab}	0.69 ± 0.09 ^{c}	0.60 ± 0.13 ^{bc}	1.08 ± 0.05 ^{d}	0.59 ± 0.21 ^{bc}	-	0.68	-	-	-0.30	-0.44
Ethyl lactate	11.4 ± 1.39 ^{b}	27.9 ± 1.50 ^{c}	10.2 ± 0.16 ^{ab}	6.00 ± 1.35 ^{a}	7.01 ± 1.75 ^{ab}	9.07 ± 0.43 ^{ab}	7.95 ± 2.15 ^{ab}	0.61	-0.27	-0.58	-0.47	-	0.58
Ethyl octanoate	0.51 ± 0.17 ^{ab}	0.42 ± 0.18 ^{a}	0.85 ± 0.02 ^{bc}	0.64 ± 0.12 ^{ab}	0.81 ± 0.07 ^{abc}	1.07 ± 0.05 ^{c}	0.85 ± 0.08 ^{bc}	-	-	0.65	0.57	-0.43	-0.71
Ethyl-3 -hydroxybutyrate	1.65 ± 0.95 ^{a}	30.8 ± 5.92 ^{b}	3.05 ± 0.20 ^{a}	2.07 ± 0.16 ^{a}	1.70 ± 0.10 ^{a}	2.09 ± 0.10 ^{a}	1.87 ± 0.22 ^{a}	0.71	-	-0.68	-0.70	-	0.67
Ethyl decanoate (µg/L)	159 ± 136 ^{ab}	28.5 ± 14.6 ^{a}	83.3 ± 5.73 ^{a}	43.1 ± 18.5 ^{a}	142 ± 81.3 ^{a}	393 ± 18.7 ^{b}	111 ± 77.1 ^{a}	-	-	-	0.94	-0.75	-0.66
Diethyl succinate	1.80 ± 0.40 ^{a}	2.28 ± 0.77 ^{ab}	4.35 ± 0.62 ^{bc}	3.60 ± 0.68 ^{abc}	4.69 ± 0.39 ^{c}	5.49 ± 0.26 ^{c}	3.97 ± 1.15 ^{abc}	-	-	0.59	-	-0.49	-0.67
Ethyl-4-hydroxybutanoate	0.25 ± 0.08 ^{a}	0.45 ± 0.19 ^{ab}	1.28 ± 0.30 ^{bc}	0.76 ± 0.26 ^{ab}	1.29 ± 0.15 ^{bc}	1.71 ± 0.08 ^{c}	1.18 ± 0.43 ^{bc}	-	-	0.65	-	-0.50	-0.65
Phenylethyl acetate	0.16 ± 0.08 ^{a}	0.24 ± 0.06 ^{ab}	0.29 ± 0.06 ^{ab}	0.38 ± 0.02 ^{b}	0.39 ± 0.03 ^{b}	0.44 ± 0.02 ^{b}	0.37 ± 0.10 ^{ab}	-	0.70	-	-	-	-
Isopropyl dodecanoate	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{ab}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{ab}	0.00 ± 0.00 ^{a}	-	-	-0.72	-	-	-
Diethyl hydroxybutanedioate	1.32 ± 0.69 ^{a}	2.55 ± 1.58 ^{ab}	5.83 ± 2.01 ^{abc}	5.38 ± 1.81 ^{abc}	6.94 ± 1.82 ^{bc}	10.1 ± 0.48 ^{c}	5.79 ± 0.96 ^{abc}	-	-	-	-	-0.50	-0.65
Acetylglycine ethyl ester	0.05 ± 0.02 ^{a}	0.08 ± 0.04 ^{a}	0.07 ± 0.03 ^{a}	0.09 ± 0.02 ^{a}	0.07 ± 0.02 ^{a}	0.12 ± 0.01 ^{a}	0.05 ± 0.01 ^{a}	-	-	-	-	-	-
Ethyl 5-oxotetrahydro-2-furancarboxylate	0.42 ± 0.36 ^{ab}	0.36 ± 0.18 ^{ab}	0.57 ± 0.17 ^{ab}	0.66 ± 0.14 ^{b}	0.60 ± 0.11 ^{ab}	0.00 ± 0.00 ^{a}	0.51 ± 0.10 ^{ab}	-	-	-	-	-	-
Ethyl hydrogen succinate	165 ± 107 ^{a}	222 ± 49.9 ^{a}	177 ± 19.4 ^{a}	186 ± 55.3 ^{a}	243 ± 47.8 ^{a}	330 ± 15.7 ^{a}	219 ± 51.1 ^{a}	-	-	-	-	-	-
Sum	183 ± 108 ^{a}	288 ± 52.4 ^{a}	205 ± 20.1 ^{a}	207 ± 58.6 ^{a}	268 ± 52.5 ^{a}	363 ± 17.3 ^{a}	243 ± 51.7 ^{a}	-	-	-	-	-	-

In the same row, different letters denote significant differences at $p< 0.01$

(1) only standardized regression coefficients (beta values) with $p < 0.01$, are reported

Table 17a. Wines fermented with strain 333 after 3 months of storage: acid concentrations (mg L^{-1}) and influence of the tested factors on their production, as assessed by multiple regression analysis

	Strain 333							Regression coefficient*					
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO_2+Lyso+tan	Lyso	SO_2	Tannin	LysoxSO$_2$	LysoxTan	SO$_2$xTan
Acetic acid	2.90 ± 0.16	4.46 ± 1.78	11.8 ± 0.73	8.72 ± 3.00	10.8 ± 0.61	15.0 ± 0.19	7.04 ± 1.31	-	-	-	-	-	-0.70
Propionic acid	0.03 ± 0.02 ^{a}	0.03 ± 0.00 ^{a}	0.03 ± 0.00 ^{a}	0.03 ± 0.00 ^{a}	0.03 ± 0.00 ^{a}	0.04 ± 0.01 ^{a}	0.03 ± 0.00 ^{a}	-	-	-	-	0.85	-
Isobutyric acid	0.97 ± 0.30 ^{a}	1.22 ± 0.05 ^{ab}	1.41 ± 0.13 ^{abc}	1.68 ± 0.19 ^{bc}	1.55 ± 0.07 ^{bc}	1.81 ± 0.10 ^{c}	1.32 ± 0.03 ^{abc}	-	-	0.85	0.43	-0.48	-0.58
Butanoic acid	0.25 ± 0.03 ^{a}	0.26 ± 0.04 ^{a}	0.30 ± 0.00 ^{a}	0.17 ± 0.15 ^{a}	0.28 ± 0.01 ^{a}	0.28 ± 0.00 ^{a}	0.29 ± 0.02 ^{a}	-	0.43	0.51	-	-	-
Pentanoic acid	0.72 ± 0.16 ^{ab}	0.57 ± 0.09 ^{a}	0.68 ± 0.07 ^{ab}	1.00 ± 0.08 ^{b}	0.87 ± 0.11 ^{ab}	0.85 ± 0.01 ^{ab}	0.95 ± 0.07 ^{b}	-	-	0.60	0.57	-	-0.42
Hexanoic acid	1.92 ± 0.39 ^{a}	1.86 ± 0.23 ^{a}	2.24 ± 0.18 ^{a}	1.99 ± 0.44 ^{a}	2.08 ± 0.05 ^{a}	2.46 ± 0.12 ^{ab}	3.22 ± 0.30 ^{b}	0.45	0.41	-	-	-	-
Octanoic acid	6.40 ± 1.64 ^{a}	6.45 ± 1.06 ^{a}	8.43 ± 0.66 ^{a}	8.06 ± 2.33 ^{a}	8.27 ± 0.83 ^{a}	10.0 ± 0.36 ^{a}	17.1 ± 1.89 ^{b}	0.57	0.84	-	-	0.54	0.49
Decanoic acid	2.10 ± 0.82 ^{a}	1.49 ± 0.25 ^{a}	2.11 ± 0.23 ^{a}	2.17 ± 0.78 ^{a}	2.74 ± 0.43 ^{a}	4.59 ± 0.02 ^{b}	7.04 ± 0.40 ^{c}	0.61		-	-	0.74	
Dodecanoic acid	0.18 ± 0.00 ^{a}	0.47 ± 0.32 ^{a}	0.29 ± 0.06 ^{a}	0.32 ± 0.08 ^{a}	0.38 ± 0.09 ^{a}	0.60 ± 0.03 ^{a}	1.16 ± 0.08 ^{b}	0.71	0.62	-	-	0.70	0.60
Tetradecanoic acid	0.11 ± 0.03 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.12 ± 0.03 ^{a}	0.11 ± 0.02 ^{a}	0.18 ± 0.02 ^{a}	0.43 ± 0.15 ^{b}	0.61	0.63	-	-	-	-
Hexadecanoic acid	3.22 ± 1.99 ^{a}	3.09 ± 0.68 ^{a}	1.96 ± 0.36 ^{a}	2.81 ± 0.79 ^{a}	2.52 ± 0.19 ^{a}	5.19 ± 0.24 ^{ab}	12.0 ± 6.02 ^{b}	0.55	0.77	-	-	0.52	0.57
Sum (excluding acetic acid)	15.9 ± 4.65 ^{ab}	15.4 ± 1.99 ^{a}	17.5 ± 0.74 ^{ab}	18.4 ± 4.14 ^{ab}	18.8 ± 0.87 ^{ab}	26.0 ± 0.30 ^{b}	43.5 ± 4.03 ^{c}	0.62	0.74	-	-	0.44	0.40

Table 17b. Wines fermented with strain 333 after 1 year of storage: acid concentrations (mg L^{-1}) and influence of the tested factors on their production, as assessed by multiple regression analysis

	Strain 333							Regression coefficient*					
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO_2+Lyso+tan	Lyso	SO_2	Tannin	LysoxSO$_2$	LysoxTan	SO$_2$xTan
Propionic acid	0.03 ± 0.02 ^{a}	0.03 ± 0.00 ^{a}	0.04 ± 0.00 ^{a}	0.03 ± 0.00 ^{a}	0.04 ± 0.01 ^{a}	0.04 ± 0.00 ^{a}	0.04 ± 0.01 ^{a}	-	-	-	-	-	-
Isobutyric acid	0.93 ± 0.15 ^{a}	1.08 ± 0.01 ^{ab}	1.27 ± 0.13 ^{abc}	1.60 ± 0.18 ^{c}	1.24 ± 0.17 ^{abc}	1.46 ± 0.07 ^{bc}	1.48 ± 0.20 ^{bc}	-	0.87	-	-	-	-
Butanoic acid	0.24 ± 0.01 ^{ab}	0.24 ± 0.01 ^{ab}	0.26 ± 0.01 ^{b}	0.22 ± 0.03 ^{ab}	0.20 ± 0.01 ^{a}	0.27 ± 0.01 ^{b}	0.25 ± 0.01 ^{ab}	-	-	-	-	-	-
Pentanoic acid	0.73 ± 0.27 ^{a}	0.62 ± 0.04 ^{a}	0.69 ± 0.04 ^{a}	1.04 ± 0.15 ^{a}	0.84 ± 0.15 ^{a}	0.85 ± 0.04 ^{a}	0.83 ± 0.02 ^{a}	-	-	-	-	-	-
Hexanoic acid	1.97 ± 0.41 ^{a}	2.00 ± 0.45 ^{a}	2.23 ± 0.30 ^{a}	1.88 ± 0.16 ^{a}	2.02 ± 0.17 ^{a}	2.26 ± 0.11 ^{a}	1.84 ± 0.42 ^{a}	-	-	-	-	-	-
Octanoic acid	5.50 ± 1.63 ^{a}	5.36 ± 2.32 ^{a}	6.32 ± 1.85 ^{a}	6.06 ± 0.97 ^{a}	7.57 ± 1.24 ^{a}	9.30 ± 0.44 ^{a}	6.56 ± 1.95 ^{a}	-	-	-	-	-	-
Decanoic acid	1.21 ± 0.48 ^{a}	0.59 ± 0.30 ^{a}	0.91 ± 0.27 ^{a}	0.67 ± 0.17 ^{a}	1.57 ± 1.11 ^{a}	3.91 ± 0.19 ^{b}	1.20 ± 0.72 ^{a}	-	-	-	0.83	-0.77	-0.65
Dodecanoic acid	0.49 ± 0.47 ^{a}	0.84 ± 0.12 ^{a}	0.42 ± 0.13 ^{a}	0.17 ± 0.04 ^{a}	0.31 ± 0.16 ^{a}	0.64 ± 0.03 ^{a}	0.46 ± 0.22 ^{a}	-	-	-	-	-	-
Tetradecanoic acid	0.11 ± 0.10 ^{a}	0.22 ± 0.06 ^{a}	0.12 ± 0.05 ^{a}	0.00 ± 0.00 ^{a}	0.14 ± 0.08 ^{a}	0.00 ± 0.00 ^{a}	0.15 ± 0.10 ^{a}	-	-	-	-	-	0.65
Hexadecanoic acid	1.55 ± 0.15 ^{a}	2.74 ± 0.21 ^{a}	2.27 ± 0.95 ^{a}	3.49 ± 1.09 ^{a}	2.13 ± 1.23 ^{a}	4.26 ± 0.20 ^{a}	3.54 ± 2.02 ^{a}	-	-	-	-	-	-
Sum (excluding acetic acid)	12.8 ± 2.87 ^{a}	13.7 ± 3.03 ^{ab}	14.5 ± 2.76 ^{abc}	15.2 ± 1.12 ^{ab}	16.1 ± 3.25 ^{abc}	23.0 ± 1.09 ^{b}	16.3 ± 4.17 ^{ab}	-	-	-	-	-	-

In the same row, different letters denote significant differences at $p < 0.01$

(1) only standardized regression coefficients (beta values) with $p < 0.01$, are reported

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Chapter 5

Replacement of sulphur dioxide by lysozyme and oenological tannins during fermentation: evolution of phenolic compounds in white wines stored in bottles

ABSTRACT: *In this work, the effects on phenolic compound profiles for white wines substituting SO_2 during fermentation with lysozyme and tannin were studied. The proposed analytical HPLC-FLD method was used to establish the phenolic composition of Sauvignon at the end of the alcoholic fermentation, and its evolution during bottle storage for one year. The profile of phenolic compounds for all wines analysed were found to decrease during the bottle storage period, due to oxidation effects caused by the presence of oxygen dissolved in the medium.*
The results showed a positive influence of SO_2 in the total amount of phenolic compounds at the end of the fermentation and, together with tannins, a protecting effect against degradation of the phenolic content during the storage period. By contrast, in this work, lysozyme did not show a significant influence on phenolic compound values.

1. INTRODUCTION

Phenolic compounds constitute one of the most important quality parameters of wines, since they contribute to wine organoleptic characteristics such as colour, astringency, bitterness, and aroma. Due to their antioxidant and anti-inflamatory properties, phenolic compounds are associated with several beneficial physiological effects, that are derived from moderate wine consumption such as a decrease in the incidence of cardiovascular diseases, a phenomenon that was initially known as the "French paradox" (Renaud et al., 1992; Teissedre et al., 1996). Likewise, the anticarcinogenic activity of wine phenolic compounds has also been demonstrated (Clifford et al., 1996). The phenolic composition of wine is conditioned by the grape variety and by other factors that affect the berry development, such as soil, geographical location, and weather conditions. On the other hand, winemaking techniques play an important role in the extraction of polyphenols from the grape and in their further stability in wine. The time of maceration and fermentation in contact with grape skins and seeds, pressing, maturation, fining, and bottle aging are all factors that affect the phenolic composition of wines (Monagas et al., 2005). In recent years, considerable effort has been devoted to the study of grape and wine polyphenols, an area that is essential in order to evaluate the potential of different grape varieties, optimize enological processes, obtain products with peculiar and improved characteristics, and achieve a better understanding of wine's physiological properties.

The term "phenolic" or "polyphenolic" describes compounds that possess a benzene ring

substituted by one or more hydroxyl groups (-OH). Their reactivity is due to the acidic character of the phenolic function and to the nucleophilic character of the benzene ring.

Based on their carbon skeleton, polyphenols can be classified as non-flavonoid and flavonoid compounds. Non-flavonoid compounds are mainly contained in the grape's pulp, while flavonoid compounds are located in the grape's skins, seeds, and stems.

1.1 Non-flavonoid phenolic compounds

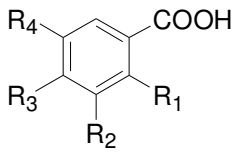
The non-flavonoids, which comprise hydroxybenzoic acids, hydroxycinnamic acids and stilbenes, originate from the grape juice, and are normally the principal phenolic molecules in white wines, with concentrations ranging from 50-250 mg L^{-1}, depending on the cultivar, winemaking techniques, etc.

For the hydroxybenzoic acids (Fig.1), gallic acid is the only form present in the grapes, particularly in the solid parts of the berry, either in its 'free' form or in the form of a flavanol ester (i.e. epicatechin-3-*O*-gallate). However, other hydroxybenzoic acids, including *p*-hydroxybenzoic, protocatechic, vanillic, syringic and gentistic acids have been identified in wines (Monagas et al., 2005).

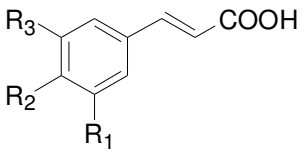
The hydroxycinnamic acids are located in the vacuoles of the skin and pulp cells (Ribéreau-Gayon et al., 2000b). They are the major phenolics in white wine and the main non-flavonoids in red wines. Wine contains free acids, namely caffeic acid, *p*-coumaric acid and ferulic acid, which are usually esterified with tartaric acid to form respectively caffeoyltartaric, *p*-coumaroyltartaric and feruloyltartaric acids (Fig. 1), present in their *trans* form, although small quantities of the *cis* isomers also exist (Singleton et al., 1978; Monagas et al., 2005). The presence of the glucose esters of *trans p*-coumaric and ferulic acids have also been reported in grapes (Reschke et al, 1981).

1.1.1 Stilbenes

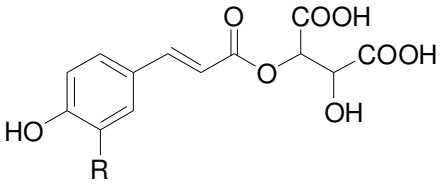
The hydroxylated stilbenes are phytoalexins synthesized by the plant, especially in the skins, leaves, and roots, in response to fungal infections and ultraviolet (UV) light (Korhammer et al., 1995). In fact, grapes and their derived products are considered the most important dietary sources of stilbenes. *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 (Waterhouse et al., 1994; Mattivi et al., 1995) (Fig.2).



Hydroxybenzoic Acids	CAS RN	R1	R2	R3	R4
p-Hydroxybenzoic	99-96-7	-H	-H	-OH	-H
Protocatechuic	99-50-3	-H	-OH	-OH	-H
Vanillic	121-34-6	-H	$-OCH_3$	-OH	-H
Gallic	149-91-7	-H	-OH	-OH	-OH
Syringic	530-57-4	-H	$-OCH_3$	-OH	$-OCH_3$
Salicilic	99-10-5	-OH	-H	-H	-H
Gentisic	530-57-4	-OH	-H	-H	-OH

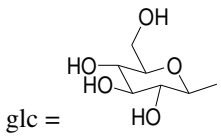


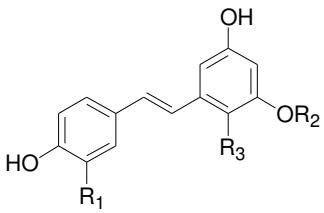
Hydroxycinnamic Acids	CAS RN	R1	R2	R3
p-Coumaric	501-98-4	-H	-OH	-H
Caffeic	501-16-6	-OH	-OH	-H
Ferulic	537-98-4	$-OCH_3$	-OH	-H
Sinapic	7362-37-0	$-OCH_3$	-OH	$-OCH_3$



Hydroxycinnamic Esters	CAS RN	R
Trans-caffeoyltartaric acid (Caftaric acid)	67879-58-7	-OH
Trans-p-coumaroyltartaric acid (Coutaric acid)	27174-07-8	-H
Trans-feruloytartaric acid (Fertaric acid)	74282-22-7	$-OCH_3$

Figure 1. A summary of phenolic acids and their derivatives (Monagas et al., 2005)





Stilbenes	CAS RN	R1	R2	R3
Trans-resveratrol	501-36-0	-H	-H	-H
Trans-resveratrol-3-O-glucoside (piceid)	27208-80-6	-H	-glc	-H
Trans-resveratrol-2-C-glucoside	285562-17-6	-H	-H	-glc
Trans-astringin	29884-49-9	-OH	-glc	-H

Figure 2. A summary of stilbenes (Monagas et al., 2005)

1.2 Flavonoid phenolic compounds: flavanols, anthocyanins and flavonols

The second main group of grape-derived phenolics are the flavonoids (Fig. 3). They have a more complex structure than the non-flavonoids, consisting of a phenolic 'A' ring fused with a pyran containing 'C' ring, and a third single linked phenolic 'B' ring.

In a young wine they are normally present in a less polymerised state, but as the wine matures, they undergo different polymerisation reactions in which O_2 plays an important role. The most important flavonoids in wine are the flavan-3-ols, anthocyanins and flavonols, and to a smaller degree, the flavanonols and flavones. Within each group, compounds differ by the number and the location of the hydroxyl- (-OH) and methoxy- ($-OCH_3$) groups located on the B ring.

1.2.1 Flavan-3-ols

Flavan-3-ols or 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.

The monomeric units consist of (+)-catechin and (-)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin, where the different stereo-isomers are due to the different -H and -OH group substitutions on the C and B rings.

For the dihydroxylated forms, (-)-epicatechin can be esterified by gallic acid at the C-3 position, resulting in (-)-epicathechin-3-*O*-gallate.

These molecules can associate through C4/C6 and C4/C8 bonds to form dimers, trimers and oligomers, and thus form the procyanidins (or condensed tannins).

Proanthocyanidins are a class of compounds that have been variously described as anthocyanogens, leucoanthocyanidins, flavan-3,4-diols, condensed tannins and tannins. They posses the property of liberating anthocyanidins with heating in acidic conditions, as a result of interflavanic bond cleavage (Kennedy et al.,2006).

In grapes, two groups of proanthocyanidins depending on the nature of the liberated anthocyanidin (cyaniding or delphinidin) are distinguished: procyanidins, which are proantochyanidins 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. Dimeric procyanidins can be divided into types A and B, where type A have interflavan C4/C6 and C4/C8 bonds, with ether bonds between the C5 or C7 carbon units of the terminal unit and the C2 carbon of the upper unit, while type B dimeric procyanidins are

characterised by C4/C6 and C4/C8 interflavan bonds. Trimeric procyanidins are divided into Types C and D, where type C has two type B interflavan bonds, and type D have a type A and a type B bond. These molecules can polymerise further to form so-called grape tannins or condensed tannins, which can be classified according to the mean degree of polymerisation (mDP). These molecules are considered oligomers when the mDP is 5 to 10, and polymers when the mDP is greater than 10. The mDP for stems and pips is about 10, but about 30 for skins, indicating that the flavanoid molecules of skins are more polymerised than those of the pips and stems (Ribéreau-Gayon *et al*., 2000b; Herderich et al., 2005). Flavan-3,4-diols can also polymerise in a similar fashion (Monagas *et al*., 2005). These condensed tannins normally exist at 1-3 g L^{-1} in red wine and their concentration depends on the cultivar and winemaking techniques, including factors such as skin maceration time and ageing procedures, etc.

1.2.2 Anthocyanins

Anthocyanins occur mainly in the skins of red grape cultivars and are responsible for the colour of red wine. In young red wines their concentrations can range from 250 mg L^{-1} to more than 1000 mg L^{-1}. Different types occur in wine, depending on the hydroxyl- (-OH) and methoxy- (-OCH_3) constituents of the B-ring of the molecule, and they can be esterified with glucose at the C3 position. This leads to the occurrence in wines of cyanidin, peonidin, delphinidin, petunidin and malvidin-3-monoglucoside, which can also be acylated with both an acetate and a cinnamic acid derivate (Ribéreau-Gayon *et al*., 2000b; Monagas *et al*., 2005).

1.2.3 Flavonols

Flavonols are yellow pigments which exist at lower concentrations in grapes and wines as 3-O-glycosides of the six main aglicones: myricetin, quercitin, kaempherol, isorhamnetin, laricitrin and syringetin. They normally occur in white wine at 1-3 mg L^{-1} and in red wine at about 100 mg L^{-1} (Ribéreau-Gayon *et al*., 2000b).

1.2.4 Flavanonols

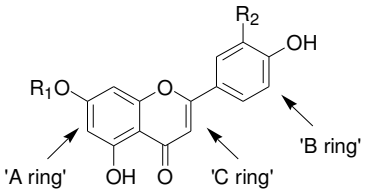
The flavanonols are not usually present in plants used for food. Among the flavanonols found in the grapes of *Vitis vinifera*, astilbin (dihydroquercetin-3-*O*-rhamnoside) and engeletin (dihydrokaempferol-3-*O*-rhamnoside) were first identified in the skin and wine from white grapes by Trousdale et al. (1983). Other flavanonols recently identified are dihydromyricetin-3-*O*-rhamnoside, which has been reported in red wines by Vitrac et al. (2001), dihydrokaempferol, dihydroquercetin (taxifolin), dihydrokaempferol-3-*O*-glucoside, dihydroquercetin-3-*O*-

glucoside, dihydroquercetin-3_-*O*-glucoside, and dihydroquercertin-3-*O*-xyloside, identified for the first time in Riesling wines by Baderschneider and Winterhalter (2001).

1.2.5 Flavones

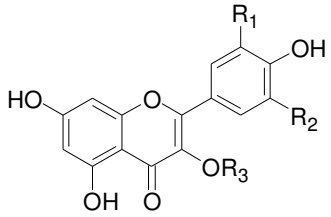
Although more than 100 flavones have been identified in plants, these compounds are not very common or abundant in fruits (Macheix et al., 1990). In the leaves of *Vitis vinifera*, apigenin-8-*C*-glucoside, luteolin as well as the 7-*O*-glucosides of apigenin and luteolin have been identified (Monagas et al., 2005).

Together with glutathione, phenolic compounds are considered "native" antioxidants in must and wine, exhibiting a great capacity to consume oxygen, due to the presence of several hydroxyl groups (Vivas et al., 1996b). The quantity and rate of oxygen consumption is always higher in red wines than in white wines. Considering the amount of oxygen a wine can take up (ranging from about 60 mL L^{-1} to over 600 mL L^{-1} from light white to heavy red wine), there are no other autoxidisable compounds evident in sufficient amounts to react with that much oxygen (Singleton, 1987).



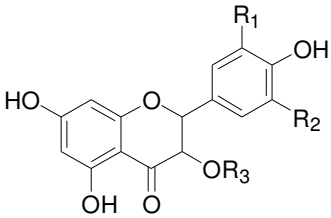
Flavone	CAS-RN	R1	R2
Apigenin	520-36-5	H	H
Apigenin-7-O-glucoside	578-74-5	glc	H
Luteolin	491-70-3	H	OH
Luteolin-7-O-glucoside	5373-11-5	glc	OH

glc = glucose



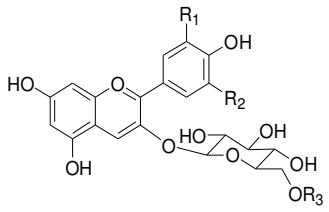
Flavonol	CAS-RN	R1	R2	R3
Kaempferol	520-18-3	H	H	H
Kaempferol-3-O-glucoside	480-10-4	H	H	glc
Kaempferol-3-O-galactoside	23627-87-4	H	H	gal
Kaempferol-3-O-glucuronide	22688-78-4	H	H	gluc
Quercetin	117-39-5	OH	H	H
Quercetin-3-O-glucoside	482-35-9	OH	H	glc
Quercetin-3-O-glucuronide	22688-79-5	OH	H	gluc
Myricetin	529-44-2	OH	OH	H
Myricetin-3-O-glucoside	19833-12-6	OH	OH	glc
Myricetin-3-O-glucuronide	77363-65-6	OH	OH	gluc
Isorhamnetin	480-19-3	OCH_3	H	H
Isorhamnetin-3-O-glucoside	5041-82-7	OCH_3	H	glc

glc = glucose, gal = galactose, gluc = glucuronamide

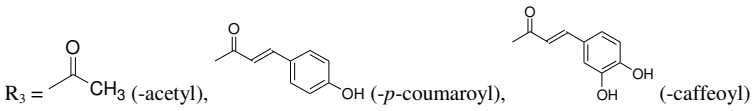


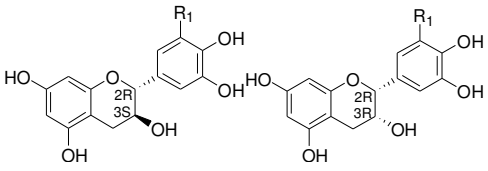
Flavanonol	CAS RN	R1	R2	R3
2,3-dihydrokaempferol	480-20-6	H	H	H
2,3-dihydrokaempferol-3-O-rhamnose	572-31-6	H	H	rha
2,3-dihydrokaempferol-3-O-glucoside	31049-08-8	H	H	glc
2,3-dihydroquercetin	480-18-2	OH	H	H
2,3-dihydroquercetin-3-O-rhamnose	29838-67-3	OH	H	rha
2,3-dihydroquercetin-3-O-glucoside	27297-45-6	OH	H	glc
2,3-dihydroquercetin-3'-O-glucoside	31106-05-5	O-glc	H	H
2,3-dihydroquercetin-3-O-xyloside	549-32-6	OH	H	xyl
2,3-dihydromyricetin-3-O-rhamnose	80443-12-5	OH	OH	rha

glc = glucose, rha = rhamnose, xyl = xylose



Anthocyanidin	CAS-RN	R1	R2
Cyanidin	7084-24-4	OH	H
Delphinidin	6906-38-3	OH	OH
Peonidin	6906-39-4	OCH3	H
Petunidin	6988-81-4	OCH3	OH
Malvidin	7228-78-6	OCH3	OCH3





Flavan-3-ol	CAS-RN	R1	C2	C3
(+)-Catechin	154-23-4	H	R	S
(+)-Gallocatechin	970-73-0	OH	R	S
(-)-Epicatechin	490-46-0	H	R	S
(-)-Epigallocatechin	970-74-1	OH	R	R

Figure 3. Flavones, flavonols, flavanonols, anthocyanins and flavanols (Monaga et al., 2005)

2. THE ROLE OF OXYGEN IN MUST AND WINE

Many compounds are susceptible to oxidation, including some metal ions, tartaric acid, ascorbic acid, ethanol, SO_2, volatile and phenolic compounds. Among these, the phenolic compounds are the main substrates for oxidation in must and wine.

Oxygen can play an important role during the winemaking process, influencing the composition and quality of the must and wine. The oxidation process involves the transfer of an electron between reductive and oxidative partners, and in wine, O_2 is predominantly responsible as the oxidant, itself being reduced to certain intermediates including hydrogen peroxide and then water. Molecular O_2 exists as a diradical in a triplet ground state, which limits its reactivity. However, the addition of a single electron, originating from reduced transition metal ions, can overcome this limitation, leading to the negatively-charged superoxide radical, with a second electron transfer resulting in a peroxide anion (Miller et al., 1990; Danilewicz, 2003). This phenomenon results in O_2 being involved in various reactions in wine.

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, and the addition of sulphur dioxide. For example, 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 et al., 1974). White et al. (1973) showed that increasing 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. The general use of sulphur dioxide as an anti-oxidant dates back to the early 18th century and the protection of wine from unwanted oxidative spoilage has been long recognised. Added sulphur dioxide slows oxygen consumption and SO_2 concentrations of 25 mg L^{-1} to 100 mg L^{-1} inhibits phenol oxidation (White et al., 1973; Schneider, 1998), while in the absence of sulphites, the depletion of oxygen is very rapid and is complete within minutes (4 to 20 on average) (Ribéreau-Gayon et al., 2000a).

However, the presence of oxygen during alcoholic fermentation is essential to correct yeast growth. In fact, when sluggish fermentation is suspected, oxygen is generally added to improve the biomass synthesis, which increases the fermentation rate. The correct alcoholic fermentation procedure can influence the production of flavour metabolites and consequently the sensory characteristics of wine.

From the end of the alcoholic fermentation, wine is exposed to many oenological operations that can increase the concentration of dissolved oxygen, such as transfer from tank to tank, the type of filtration, the bottling, and the type of closure. Once the wines is stored in bottles, the

reactions that occur during the ageing are strongly influenced by the dissolved oxygen concentration and the lack of oxidative enzymes is replaced by metal ion catalysed activity. The final result is a strong influence on the wine organoleptic characters (such as colour, flavour and astringency) and antioxidant potential.

2.1 Enzymatic oxidation

Enzymatic and chemical oxidation processes can take place mainly in musts, with both involving molecular oxygen as a substrate. The enzymatic oxidation of phenolic compounds is performed by two types of enzyme groups, namely the polyphenoloxidase's (PPO) (catecholoxidase, tyrosinase, phenolase, cresolase, and *o*-diphenoloxidase) and the laccase's, which can be found in grapes infected with *Botrytis cinerea* (Ribereau-Gayon et al., 2000a).

Enzymatic oxidation, also known as enzymatic browning, is especially important in white wine making and occurs since the grape is pressed. Although anthocyanins and condensed flavanols (oligomers and polymers) are not good substrates for PPO, possibly due to steric hindrance, the effects of this enzyme can also be evidenced in red wine making, production and red wine discoloration (Cheynier et al., 1991).

Caftaric and cutaric acids are the major phenols and substrates for enzymatic oxidation in white musts (in grape juice protected by oxygen, their amount is about 145 mg L^{-1} and 15 mg L^{-1}, respectively) and they are oxidised to caffeoyltartaric acid *o*-quinone (GRP) and *p*-coumaroyltartaric acid *o*-quinone, respectively (Singleton et al., 1985).

Once formed, the *o*-quinones are very reactive species which may polymerise and/or rapidly oxidise other components of must (coupled oxidation), particularly amino acids and proteins (like lysozyme), ascorbic acid (Cheynier et al., 1990), sulphur dioxide (Cheynier et al., 1989a) and other *o*-diphenols (not PPO substrates), that act as reductants (Cheynier et al., 1988), in this way protecting the anthocyanins, especially if the oxygen supply is limited (Fig. 4). Concerning the reaction between *o*-quinones and lysozyme, Rawel et al. (2001) have shown that the reactivity of phenolic compounds on the free amino groups of lysozyme is influenced by the position and the number of hydroxyl groups present in the respective phenols and this can influence the lytic activity of the lysozyme. In particular, the most reactive phenolic compounds are gallic acid, caffeic acid and cinnamic acid (Bernkop- Schnurch et al., 1998; Cheynier et al., 1989a; Rawel et al., 2001).

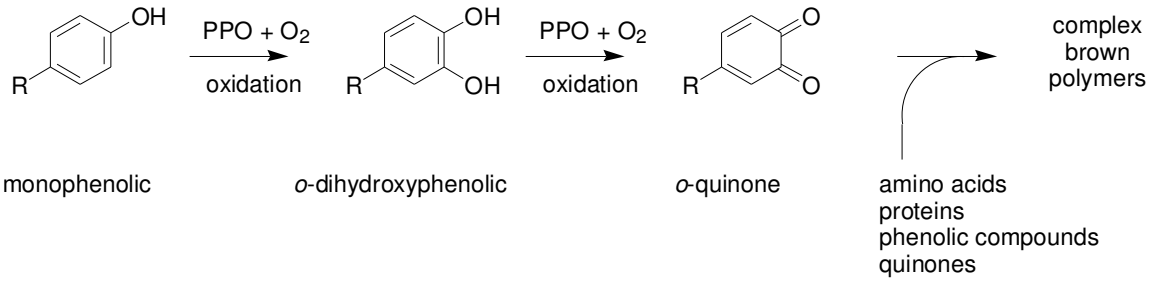


Figure 4. Enzymatic oxidation of phenolic compounds (Scollary, 2002)

In Fig. 5, the first stage of caftaric acid oxidation to a quinone is shown. Owing to its high concentration and reactivity, this primary oxidation product is the origin of a further cascade of non-enzymatic reactions.

Most sensitivity toward oxidative browning has been shown to be largely dependent on the hydroxycinnamate to glutathione ratio (and possibly other grape constituents able to trap caffeoyltartaric acid *o*-quinones) (Cheynier et al., 1990). Caftaric acid *o*-quinone formed by enzymatic oxidation taking place immediately when the grapes are crushed, reacts readily with the available glutathione, a compound present in grapes in rather large concentrations. This reaction forms 2-S-glutathionylcaffeoyltartaric acid, a colourless compound also referred to as grape reaction product (GRP) (Cheynier et al., 1986; Singleton et al., 1985). This reaction has several important consequences. Firstly, it regenerates a phenolic species from the *o*-quinone, which then has the capacity to absorb another equivalent of oxygen, and it produces a colourless catechol that is not a substrate for further enzymatic oxidations. Hence, the formation of GRP competes with reactions that lead to must degradation and it is believed to limit must browning, maintaining high concentration of unaltered phenolic compounds in wines (Sarni-Manchado et al., 1995).

After glutathione depletion, the excess caftaric acid *o*-quinone can oxidize other must constituents, including GRP, catechin, procyanidin dimers Bl-B4, and epicatechin 3-O-gallate, to the corresponding *o*-quinones and be simultaneously reduced back to caftaric acid. Additionally, mixed catechin-caffeoyltartaric acid condensation products form more easily than caffeoyltartaric acid oligomers (Cheynier et al., 1989a). The partial regeneration of caftaric acid enables its re-oxidation by PPO and further oxygen consumption.

In the absence of reducing or trapping agents, the *o*-quinones may react with one another or condense with hydroquinones, either by a mechanism analogous to a Michael 1,4 addition (Gramshaw, 1970; McDonald and Hamilton, 1973; Singleton, 1987) or through two semiquinone radical intermediates (Cilliers and Singleton, 1991; Singleton, 1987).

All these reactions are interdependent. In particular, the mentioned trapping of caftaric acid *o*-quinone by glutathione or reduction with sulfite limits the other reactions.

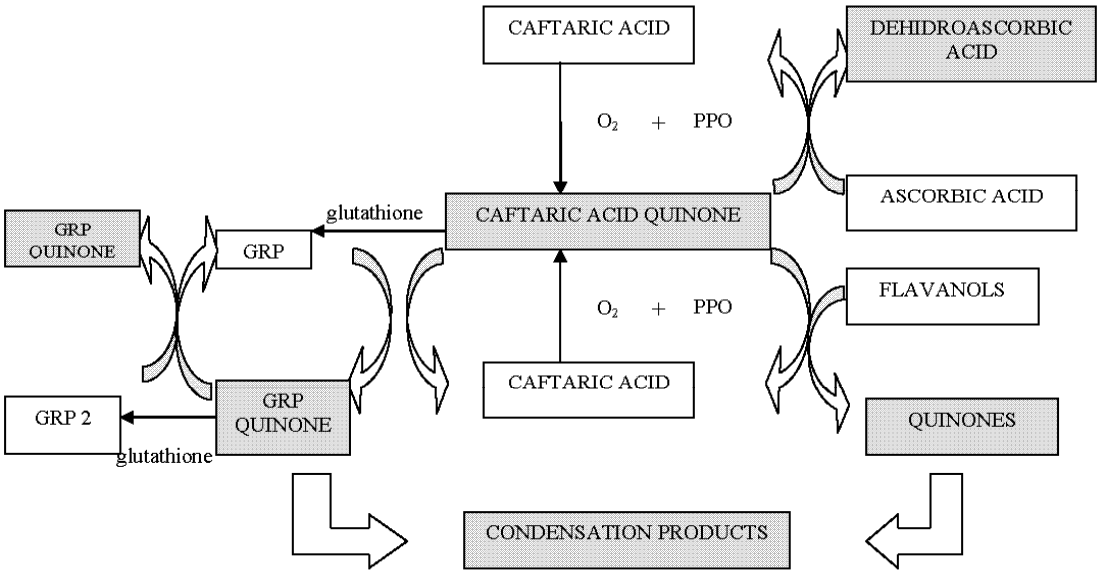


Figure 5. Reaction mechanism of *trans*-caftaric acid and GRP oxidation (Silva et al., 1999)

2,5-di-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, cathechin, epicatechin, procyanidin B2 and B3 have a browning potential about 10-fold higher than hydroxycinnamic acid derivates. Cheynier et al. (1991) have found that polyphenoloxidase did not degrade proanthocyanidins alone, but in the presence of caftaric acid, the oxidative condensation of non-galloylated procyanidins proceed more quickly than the non-oxidative condensation of non-galloylated procyanidins.

2.2 Non enzymatic oxidation

Non-enzymatic oxidation is a reaction that occurs in wines after fermentation, when the oxidative enzymes activity decreases. The oxidation reactions occurring in wines during the storage are technological and have nutritional significance due to their influence on wine organoleptic characters and antioxidant status.

The main difference between enzymatic and non-enzymatic oxidation is the way in which *o*-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 an organic molecule is a "spin forbidden" process due to the triplet ground state arrangement of electrons in the former. As such, 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 *o*-quinone, only one atom of oxygen is needed and the second appears as hydrogen peroxide. Under acidic conditions, this hydrogen peroxide oxidizes additional substances, including ethanol in wines, which would otherwise not readily autoxidize. By contrast, under enzymatic oxidation, hydrogen peroxide is not produced (Singleton et al., 1995).

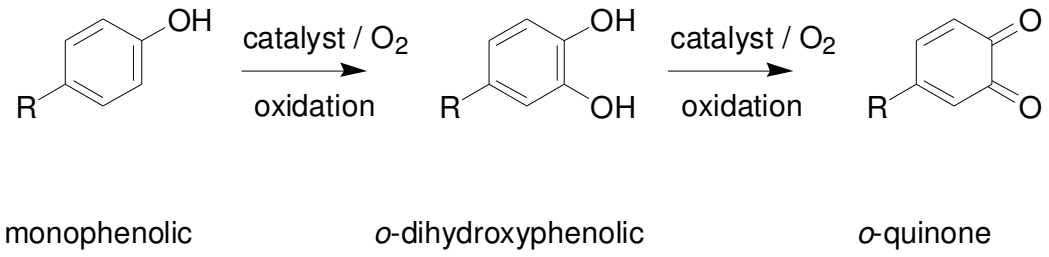


Figure 6. Non-enzymatic oxidation of a phenolic compound (Scollary 2002).

Due to the aforementioned poor reactivity of molecular 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 ($OOH^{\bullet}$) (Fig. 7). This step requires a catalyst, presumably a transition state metal such as iron (Waterhouse et al., 2006). The transfer of a second electron then produces 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 Fenton reaction between hydrogen peroxide and ferrous iron salts (Fig. 8). The last reaction produces water, the final product of oxygen reduction (Danilewicz, 2003).

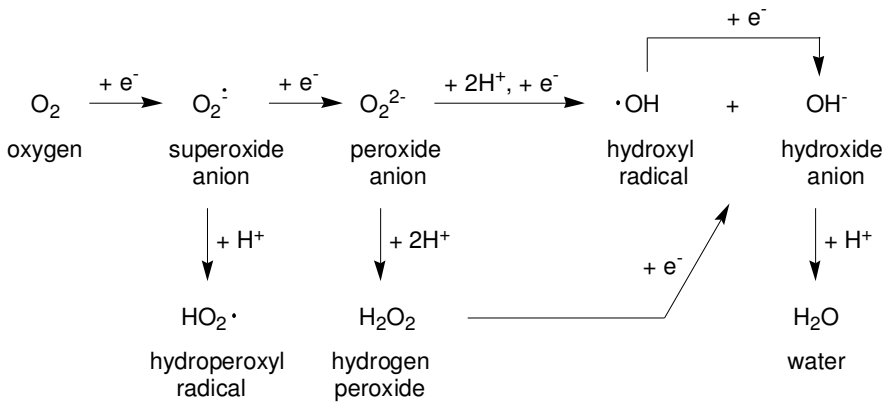


Figure 7. Ladder of oxygen reduction.

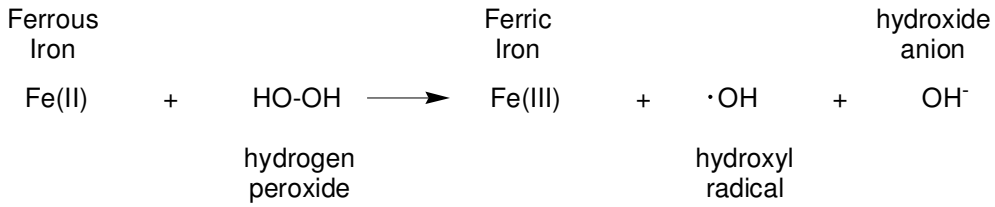


Figure 8. Fenton reaction.

The aim of this work was to study the influence on phenolic composition and oxidation of must and wines of two different vinification technologies: namely a conventional one (with sulphites addition in pre-fermentation) compared to a vinification in which sulphur dioxide was replaced by oenological tannins, which are phenolic polymers with high antioxidant and antiradical activities, useful in controlling oxygen's influence and effect on the composition and quality of must and wine. Tannins were used in association with lysozyme, an enzyme extracted from hen egg white which possesses a lytic activity that showed an inhibition effect on the growth of Gram-positive bacteria, such as lactic acid bacteria.

In the same work, the evolution of phenolics composition of wines during bottle storage for one year was also evaluated, following the potential oxidation phenomena of phenolic compounds. At the same time, the evaluation of the changes in the colour during the bottle storage period was carried out.

3. EXPERIMENTAL

3.1 Fermentations

Forty two litres of fresh must from cv *Sauvignon Blanc* were fermented in two litre laboratory glass fermentors that were saturated with N_2 before the filling. A glass trap (filled with 4 N H_2SO_4) prevented microbial contamination and oxygen entrance. A low SO_2 producing selected strains of *Saccharomyces cerevisiae* (strains 333 from University of Bologna - ESAVE collection), was used to carry out fermentations and was inoculated at an initial cell concentration of 1.5 x 10^6 CFU mL^{-1}. Six samples were evaluated with the overall aim of studying the effect of the following variables: 1) strain, 2) lysozyme, 3) SO_2, 4) tannin (Table 1).

Trials / Factor	Strain (333)						
	T	*L*	*LT*	*S*	*ST*	*SL*	*SLT*
Lysozyme (g L^{-1})	-	0.25	0.25	-	-	0.25	0.25
$K_2S_2O_5$ (mg L^{-1})	-	-	-	120	120	120	120
Tannin (g L^{-1})	-	-	0.15	-	0.15	-	0.15

Table 1. Scheme of fermentation trials (Legend for samples: T: control, S: Sulphur dioxide addition, ST: Sulphur dioxide and tannin addition, L: Lysozyme addition, LT: Lysozyme and tannin addition).

Fermentations were performed in triplicate. Must were stirred daily to ensure a homogenous fermentation. Fermentations were monitored by daily weighing of the fermentors, and samples were taken at the end of fermentation, when the loss of weight stopped.

The final wines were bottled, under a nitrogen flux in 125 mL bottles, and stored in the dark, at 15°C for 1 year. Analysis of the phenolic compounds of these wines was carried out after 3 and 12 months of storage.

3.2 Chemicals and standards

Acetic acid and HPLC grade acetonitrile were purchased from Merck (Darmstadt, Germany). Standard compounds were supplied by Aldrich (Milano, Italy), Sigma Chemicals (St. Louis, Missouri, USA), Fluka Chimie AG (Buchs, Switzerland). Water was of HPLC grade.

3.3 Accelerated browing test

The model used to asses browning development was described by Singleton et al., (1976).

Wines lots of 20 mL were filtered through 0.45 nm PTFE filters and placed in 50 mL screw-cap glass vials. Samples were subjected to heating at a constant temperature of 50°C for 48 hours in an oven. Optical density of wine samples before and after treatment was determined from absorbance measurement at 420 nm by a direct lecture on an Uvidec 610 spectrophotometer (Jasco, Japan) with a 1 cm quartz cuvette.

Wine samples were analysed at the end of the fermentation, after 3 month and after 1 year of bottle storage.

3.4 HPLC Analysis

Phenolic compound determinations were performed by HPLC analysis using a Jasco apparatus (Tokyo, Japan) equipped with a binary pump (PU 2089), a 20 µl loop, a Rheodyne valve (Cotati, CA), a photodiode detector (PU MD 910), a fluorometric detector (FP 2020), and a column oven. The column was a C18 Synergy 4µ Hydro RP 8021 (250 mm x 3,00 mm). Chromatographic data were acquired and processed using Chromnav 1.11.02 software (Jasco Corporation, Tokyo, Japan). The method used to determine phenolic compounds was devised and validated by the research group of Food Science Department of University of Bologna. Samples were filtered with syringe filters (0.45 µm; cellulose membrane) and injected without any-pre-treatment.

Table 2 lists the optimum instrumental parameter values for the chromatographic determinations of gallic acid, ethylgallate, (+)-catechin, (-)-epicatechin, *trans*-caffeic and *trans*-caftaric acid, GRP, ethyl caffeoate, *p*-coumaric, *cis* and *trans*-coutaric acid, ethyl cumarate, ferulic acid, tirosol, myricetin, myricetin glucuronide, myricetin glucoside, quercitin, quercitin glucuronide, quercitin glucoside, isorhamnetin, kaempherol, procyanidin B1 and B2, *cis* and *trans*-resveratrol.

Identification of phenolic compounds in wines was carried out by UV-Vis spectroscopy and, wherever possible, by comparing the retention times to those of standards solutions. (+)-Catechin, (-)-epicatechin, *cis* and *trans*-resveratrol, tyrosol, procyanidin B1 and B2 were identified via fluorometric detection. Quantification was performed using an external standard and calibration curves generated using standard solutions. Caftaric acid, GRP and ethylcaffeoate

were quantified using the caffeic acid calibration curve. Cutaric acid and ethyl cumarate were quantified using the *p*-cumaric acid calibration curve.

Chromatographic conditions	
Injected volume	20 μL
Analytical column	C 18 Synergi 4μ Hydro RP 80A (250 mm x 3.00 mm) (Phenomenex)
Mobile phase	A (H_2O 98% - 2% acetic acid) B (acetonitrile 98% - 2% acetic acid)

Time (min.)	Mobile phase A (%)	Mobile phase B (%)
0	98	2
15	95	5
25	90	10
32	82	18
40	80	20
45	70	30
50	50	50
54	20	80
55	0	100
57	98	2

Flow rate	0.5 mL min^{-1}
Oven Temperature	35°C
Detection conditions	
DAD	
Scanning	220-450 nm
Detection wavelenght	280 nm: gallic acid, ethylgallate, catechin, epicathechin; 320 nm: caffeic acid, caftaric acid, GRP, ethylcaffeoate, coumaric acid, cutaric acid, ethylcumarate, ferulic acid; 350 nm: myricetin, myricetin glucoside and glucuronide, quercitin, quercitin glucoside and glucuronide, isorhamnetina, kaempherol;
Bandwidth	4 nm
FLUOROMETER	

Time (min)	λ Exc	λ Em	Gain
0	280	315	10
40	324	370	100
48.5	260	370	100
60	280	315	10

Detection wavelength (λ Exc)	280 nm: cetechin, epicathechin, tyrosol, procyanidin B1 and B2 324 nm: *trans*-resveratrol 260 nm: *cis*-resveratrol

Table 2. Chromatographic conditions for the determination of phenolic compounds in wines.

3.5 Statistical Analysis

For each final wine, significant differences in mean concentrations of phenolic compounds were tested by means of ANOVA analysis followed by a Post Hoc comparison (Tuckey's test at $p>0.01$). To evaluate the influence of each tested factor (yeast strain, lysozyme, SO_2 and tannins) on phenolic compound profile during the fermentation and storage period, the data were

subjected to multiple regression analysis after a graphical exploration to exclude outliers. All the analyses were conducted using the "Statistica 6" package (StatSoft Italia Srl, Italy).

4. RESULTS AND DISCUSSION

4.1 Evolution of colour intensity during the accelerated ageing

Table 3 shows the influence of the accelerated browning test on wine samples at the end of the alcoholic fermentation and during the bottle storage, together with the Tuckey's test results.

At the end of the fermentation the accelerated browning test did not reveal significant differences among the wine samples, whereas some differences were observed between the SO_2-free sample and samples with SO_2 addition after 3 months of bottle storage. In particular, lysozyme associated with tannin addition showed a significantly higher value of optical density that can be explained by the oxygen-scavenging activity of tannins that may preserve the phenolic content during the storage period, but at the same time oxidise its own chemical constituents (gallic acid and its derivatives) increasing the DO 420 nm value.

Thereafter, until the end of the study after one year, the tendency of browning remained constant for the SO_2-free samples, whereas a sharp increase was observed for samples with SO_2 addition.

Table 3. Accelerated browning on wine samples data.

	Accelerated browning Test						
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO_2+Lyso+tan
end of fermentation	0.006 ± 0.001	0.004 ± 0.002	0.008 ± 0.001	0.006 ± 0.001	0.002 ± 0.005	0.001 ± 0.004	0.001 ± 0.003
3 months	0.015 ± 0.001 ^{ab}	0.011 ± 0.004 ^{ab}	0.020 ± 0.010 ^{b}	0.002 ± 0.003 ^{a}	0.000 ± 0.000 ^{a}	0.008 ± 0.006 ^{ab}	0.005 ± 0.003 ^{ab}
1 year	0.017 ± 0.006 ^{ab}	0.015 ± 0.002 ^{ab}	0.019 ± 0.005 ^{b}	0.019 ± 0.003 ^{b}	0.019 ± 0.005 ^{b}	0.025 ± 0.003 ^{b}	0.021 ± 0.003 ^{b}

In the same row, different letters denote significant differences at $p< 0.01$

(1) only standardized regression coefficients (beta values) with $p < 0.01$, are reported

4.2 Phenolic compound characterisations at the end of alcoholic fermentation of wines inoculated with strain 333 and their evolution during 1 year of bottle ageing

The phenolic compounds identified in each wine at the end of the alcoholic fermentation and during the bottle storage are reported in Tables 4 and 5, together with the Tuckey's test results. The right side of the tables show the significant ($p>0.01$) standardised beta coefficients obtained from the multiple regression analysis, carried out with the aim of highlighting significant correlations between each factor (SO_2, lysozyme and tannins) and the production and evolution

of phenolic compounds. The higher the regression coefficient (beta), the stronger the impact of the factor on that specific compound. Furthermore, the sign of the beta values indicate a direct (positive sign) or reversed (negative sign) correlation. Hence, positive signs refer to a direct relationship between tannins, SO_2, lysozyme and the single compounds while, for negative signs, a reversed correlation should be considered.

From a general consideration of the results, the amount of phenolic compound found in wines suggest that the samples were not particularly rich in phenolic compounds (Table 4 and 5) and during the bottle storage, a further decrease in profile (starting from about 70 mg L^{-1} total amounts at the end of the fermentation, until about 11 mg L^{-1} after 1 year of bottle storage) that affected all the samples studied was shown (Tables 5).

Concerning the sum of phenolic compounds at the end of the alcoholic fermentation (Table 4), the lowest amounts were found for wines fermented with lysozyme addition (39.4 mg L^{-1}) and the highest values for wines fermented with SO_2 associated with tannins (69.9 mg L^{-1}) (Fig. 9). The multiple regression analysis confirmed this trend, highlighting a positive relationship between phenolic compounds as a sum and the SO_2 factor.

During the bottle storage period, the lowest amount of total phenolic compounds is associated to wines fermented using lysozyme (10.2 mg L^{-1} and 11.5 mg L^{-1}, after 3 months and 1 year of storage, respectively) while a positive correlation for SO_2 and tannins factors was found. In particular, tannins showed a positive influence on the total amount of phenolics during the entire storage period, not only due to the augmented concentration of gallic acid.

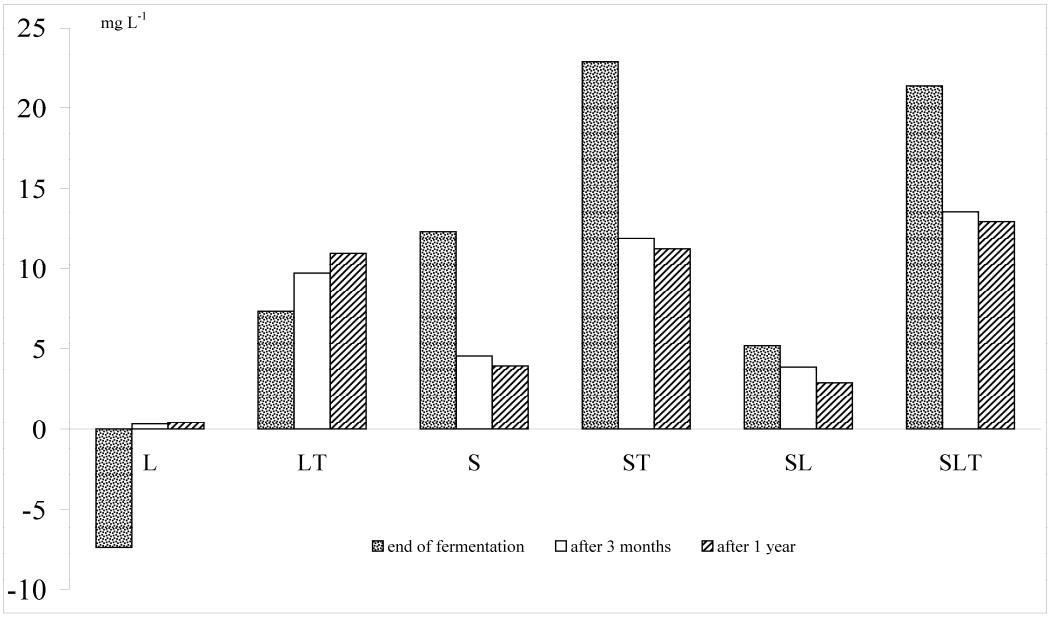


Figure 9. Variation in total amount of phenolic compounds found in wines at the end of the fermentation, and after 3 months and 1 year of storage. The phenolic amount of the control samples represent the "0" line (L = lysozyme; LT = lysozyme+tannin; S = SO_2; ST = SO_2+tannin).

Among individual compounds, tannin addition showed a positive influence on gallic acid concentration at the end of the fermentation and during the storage time, as was expected because its chemical composition consists of a carbohydrate core with hydroxyl groups esterified with gallic acid or one of its derivatives (digallic, trigallic) (Vivas, 2001). Furthermore, data suggest that at our conditions the addition of 100 mg L^{-1} of gallotannins before the start of the fermentation, would increase the amount of gallic acid in final wines up to 8 mg L^{-1}. The constant value in gallic acid concentration during the storage period for samples with added tannins is likely due to an equilibrium between its loss due to oxidative processes, and the release promoted by the hydrolysis of native tannins, which yield gallic acid and glucose as a final products. Lysozyme did not have any influence on gallic acid amount, in contrast with results obtained by Rawel et al. (2001), that showed a high reactivity between gallic acid and lysozyme free amino groups.

As expected, ethylgallate showed the same trend of gallic acid: a significantly higher amount for samples fermented with SO_2 and lysozyme associated with tannin addition, compared to the samples without tannins and a positive regression coefficient for the tannin factor at the end of the fermentation and during the storage period.

Concerning tyrosol, the phenol present in highest concentration for all samples, a relevant decrease was shown during the first 3 months of storage after fermentation for all samples, after

which its amount was maintained constant until 1 year of storage. The statistical analysis showed a positive influence of SO_2 presence on tyrosol values. As with other wine alcohols, tyrosol is formed during alcoholic fermentation by a catabolic process starting from the amino acid derived α–ketoacids (the Erlich pathway) (Nykanen, 1986; Bell et al., 2005; Hernandez-Orte et al., 2006). In particular, tyrosol is synthesised from tyrosine and the higher concentrations found in wines fermented with SO_2 could well be a consequence of increased consumption of that amino acid by yeast, promoted by sulphites during the alcoholic fermentation (Sonni et al., 2009; Garde-Cerdan et al., 2007).

At the end of alcoholic fermentation, lysozyme showed a negative influence on *t*-caffeic acid and ethylcaffeoate content, probably due to the rapid interaction between the free amino groups of lysozyme and the hydroxyl groups of caffeic acid, as reported by Rawel et al. (2001), which inhibit the production of ethylcaffeoate.

Concerning *t*-caftaric and *c*-coutaric acids, which are considered the major phenols and substrate for enzymatic oxidation in white musts, they were found in higher concentration in samples with SO_2 addition and SO_2 with tannins added for the whole experimental period, compared to samples with added lysozyme, confirming the antioxidant activity that SO_2 and tannins are able to perform, acting as reductants and preserving the phenolic compounds profile of wines (Cheynier et al., 1989a) (Fig. 10). A decrease in concentration of the esters of tartaric acid during the storage time found for all the samples studied is in agreement with the results obtained by other researchers (Kallithraka et al., 2009; Recamales et al., 2006; Zafrilla et al., 2003).

Furthermore, a positive influence due to tannins addition appeared for GRP, which was quantified in low concentrations at the end of the fermentation before disappearing after 1 year of storage. During the fermentation and for the first 3 months of bottle storage, the presence of SO_2 associated with tannin addition probably demonstrated antioxidant activity, contributing to preserve the GRP oxidation. In fact, data at the end of fermentation (Table 4) showed a higher amount of GRP in wine samples fermented with SO_2 and with SO_2 associated with tannins compared to the other samples, and the regression analysis of interactions between the factors (data not reported on Table 4) highlighted a negative influence of lysozyme associated with tannins on wine GRP amounts, probably due to the phenomenon of tannins binding with and/or quenching lysozyme and their subsequent precipitation (Soares et al., 2007).

In this experimentation no values for (+)-catechin and (-)-epicatechin were detected.

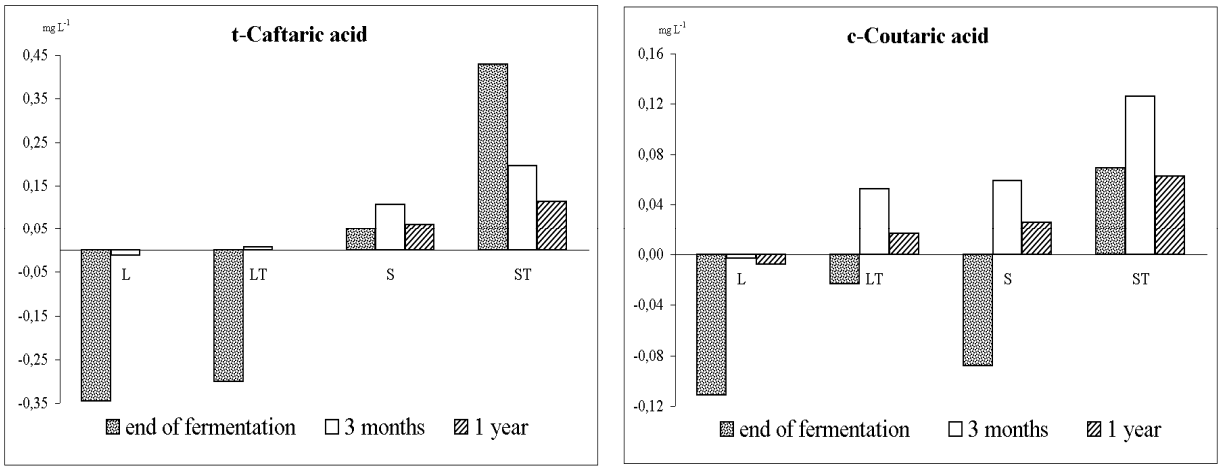


Figure 10. Variation in *t*-caftaric and *c*-coutaric acids amount found in wines at the end of the fermentation, and after 3 months and 1 year of storage. The phenolic amount of the control samples represent the "0" line (L = lysozyme; LT = lysozyme+tannin; S = SO_2; ST = SO_2+tannin).

Table 4. Phenolic concentrations (mg L^{-1}) of final wines fermented with strain 333 and influence of the tested factors on their production, as assessed by multiple regression analysis

	Strain 333							Regression coefficient*					
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO_2+Lyso+tan	Lyso	SO_2	Tannin	LysoxSO$_2$	LysoxTan	SO$_2$xTan
Gallic acid	0.74 ± 0.09 ^{a}	0.34 ± 0.06 ^{a}	8.13 ± 0.19 ^{b}	0.38 ± 0.06 ^{a}	7.86 ± 1.01 ^{b}	0.50 ± 0.13 ^{a}	8.25 ± 0.27 ^{b}			0.986			
Tyrosol	41.8 ± 0.41 ^{ab}	37.0 ± 1.86 ^{a}	42.3 ± 2.18 ^{ab}	56.3 ± 4.68 ^{c}	55.1 ± 1.09 ^{c}	47.7 ± 3.58 ^{bc}	53.0 ± 0.32 ^{c}		0.994				
Ethylgallate	1.09 ± 0.45 ^{b}	0.64 ± 0.11 ^{ab}	1.73 ± 0.31 ^{c}	0.46 ± 0.07 ^{a}	1.97 ± 0.16 ^{c}	0.90 ± 0.02 ^{ab}	2.08 ± 0.01 ^{c}		-0.490	1.116	0.634		
t-Caftaric acid	0.44 ± 0.34 ^{ab}	0.10 ± 0.03 ^{a}	0.14 ± 0.17 ^{a}	0.49 ± 0.12 ^{abc}	0.87 ± 0.40 ^{bc}	0.49 ± 0.05 ^{abc}	1.02 ± 0.06 ^{c}						
GRP	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.13 ± 0.11 ^{a}	0.09 ± 0.09 ^{a}	1.14 ± 0.02 ^{c}	0.00 ± 0.00 ^{a}	0.75 ± 0.10 ^{b}			0.495		-0.318	0.660
c-Coutaric acid	0.27 ± 0.10 ^{a}	0.16 ± 0.02 ^{a}	0.24 ± 0.02 ^{a}	0.18 ± 0.02 ^{a}	0.34 ± 0.03 ^{a}	0.28 ± 0.03 ^{a}	0.23 ± 0.17 ^{a}						
t-Caffeic acid	1.02 ± 0.67 ^{bc}	0.31 ± 0.11 ^{a}	0.28 ± 0.02 ^{a}	0.50 ± 0.03 ^{ab}	1.08 ± 0.33 ^{bc}	0.85 ± 0.08 ^{abc}	1.26 ± 0.12 ^{c}	-0.814			1.104		
p-Coumaric acid	0.15 ± 0.06 ^{a}	0.10 ± 0.03 ^{a}	0.11 ± 0.01 ^{a}	0.10 ± 0.00 ^{a}	0.25 ± 0.06 ^{b}	0.16 ± 0.02 ^{a}	0.29 ± 0.01 ^{b}						
Ferulic acid	0.81 ± 0.40 ^{a}	0.62 ± 0.07 ^{a}	0.93 ± 0.12 ^{a}	0.37 ± 0.09 ^{a}	0.61 ± 0.04 ^{a}	0.67 ± 0.10 ^{a}	0.76 ± 0.07 ^{a}						
Ethylcaffeoate	0.33 ± 0.24 ^{a}	0.05 ± 0.08 ^{a}	0.06 ± 0.01 ^{a}	0.12 ± 0.02 ^{a}	0.34 ± 0.11 ^{a}	0.24 ± 0.00 ^{a}	0.37 ± 0.04 ^{a}	-0.896			1.173		
Ethylcoumarate	0.03 ± 0.02 ^{a}	0.01 ± 0.02 ^{a}	0.02 ± 0.00 ^{a}	0.02 ± 0.01 ^{a}	0.05 ± 0.01 ^{a}	0.04 ± 0.00 ^{a}	0.06 ± 0.01 ^{a}						
Sum	46.7 ± 3.93 ^{ab}	39.4 ± 1.58 ^{a}	54.1 ± 2.14 ^{b}	59.0 ± 7.00 ^{bc}	69.6 ± 4.59 ^{c}	51.9 ± 4.59 ^{ab}	68.1 ± 0.65 ^{c}		0.568				

In the same row, different letters denote significant differences at $p< 0.01$

(1) only standardized regression coefficients (beta values) with $p < 0.01$, are reported

Table 5. Phenolic concentrations (mg L^{-1}) of wines fermented with strain 333 after 3 months and 1 year of storage and influence of the tested factors on their production, as assessed by multiple regression analysis

	3 months							Regression coefficient*		
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO_2+Lyso+tan	Lyso	SO_2	Tannin
Gallic acid	0.01 ± 0.01 [a]	0.02 ± 0.02 [a]	6.80 ± 0.21 [b]	0.09 ± 0.06 [a]	5.96 ± 0.90 [b]	0.05 ± 0.01 [a]	6.70 ± 0.33 [b]			0.923
Tyrosol	8.63 ± 0.29 [a]	8.68 ± 0.36 [a]	9.61 ± 0.11 [a]	12.1 ± 1.23 [b]	12.0 ± 0.67 [b]	10.2 ± 0.19 [ab]	11.7 ± 0.35 [b]		1.158	
Ethylgallate	n.d. [a]	0.29 ± 0.01 [b]	1.03 ± 0.01 [e]	< 0.25 [a]	0.71 ± 0.04 [c]	< 0.25 [a]	0.85 ± 0.05 [d]	0.344		0.735
t -Caftaric acid	0.04 ± 0.01	0.02 ± 0.01	0.04 ± 0.03	0.14 ± 0.04	0.23 ± 0.16	0.18 ± 0.02	0.23 ± 0.04			
GRP	trace	trace	trace	trace	trace	trace	trace			
c -Coutaric acid	0.06 ± 0.00 [a]	0.06 ± 0.01 [a]	0.11 ± 0.01 [ab]	0.12 ± 0.03 [ab]	0.19 ± 0.04 [c]	0.20 ± 0.01 [c]	0.18 ± 0.01 [bc]		0.515	1.254
t- Caffeic acid	0.26 ± 0.02 [a]	0.24 ± 0.07 [a]	0.20 ± 0.01 [a]	0.51 ± 0.08 [ab]	0.68 ± 0.52 [ab]	0.70 ± 0.07 [ab]	1.17 ± 0.09 [b]			
p -Coumaric acid	0.17 ± 0.01	0.24 ± 0.03	0.25 ± 0.04	0.23 ± 0.04	0.24 ± 0.12	0.18 ± 0.01	0.35 ± 0.01			
Ferulic acid	0.35 ± 0.05	0.40 ± 0.01	0.62 ± 0.21	0.25 ± 0.14	0.39 ± 0.11	0.50 ± 0.14	0.51 ± 0.10			
Ethylcaffeoate	0.07 ± 0.01 [a]	0.07 ± 0.02 [a]	0.07 ± 0.01 [a]	0.19 ± 0.04 [ab]	0.29 ± 0.20 [ab]	0.27 ± 0.04 [ab]	0.46 ± 0.06 [b]			
Ethylcoumarate	0.03 ± 0.02	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.05 ± 0.03	0.03 ± 0.01			
Protocatechic acid	0.27 ± 0.03 [a]	0.29 ± 0.06 [a]	0.44 ± 0.04 [a]	0.64 ± 0.19 [ab]	0.71 ± 0.23 [ab]	1.01 ± 0.13 [b]	0.67 ± 0.12 [ab]		0.684	
Sum	9.83 ± 0.35 [a]	10.2 ± 0.46 [a]	19.5 ± 0.43 [c]	14.4 ± 1.36 [b]	21.7 ± 2.52 [c]	13.7 ± 0.54 [ab]	23.4 ± 0.46 [c]		0.435	0.674

	1 year							Regression coefficient*		
	Test	Lysozyme	Lyso + tan	SO_2	SO_2 + tan	SO_2 + Lyso	SO_2+Lyso+tan	Lyso	SO_2	Tannin
Gallic acid	trace [a]	trace [a]	8.15 ± 0.11 [c]	trace [a]	6.53 ± 1.02 [b]	trace [a]	7.81 ± 0.52 [bc]			0.904
Tyrosol	9.13 ± 0.30 [a]	9.36 ± 0.68 [ab]	10.6 ± 0.22 [bcd]	11.8 ± 0.56 [d]	11.9 ± 0.21 [d]	10.3 ± 0.07 [abc]	11.5 ± 0.10 [cd]		1.221	0.022
Ethylgallate	0.26 0.02 [a]	0.30 ± 0.05 [ab]	0.45 ± 0.01 [bc]	0.42 0.07 [abc]	0.50 ± 0.06 [c]	0.58 0.08 [c]	0.50 ± 0.05 [c]		0.716	1.295
t -Caftaric acid	n.d [a]	n.d [a]	n.d [a]	0.06 ± 0.02 [b]	0.11 ± 0.02 [c]	0.12 ± 0.00 [c]	0.12 ± 0.00 [c]		0.535	0.461
GRP	n.d	n.d	n.d	n.d	n.d	n.d	n.d			
c -Coutaric acid	0.03 ± 0.00 [ab]	0.02 ± 0.00 [a]	0.05 ± 0.00 [abc]	0.06 ± 0.01 [abc]	0.09 ± 0.04 [c]	0.08 ± 0.00 [bc]	0.07 ± 0.01 [bc]			1.168
t- Caffeic acid	0.32 ± 0.00 [a]	0.27 ± 0.07 [a]	0.27 ± 0.00 [a]	0.61 ± 0.04 [ab]	0.78 ± 0.52 [ab]	0.84 ± 0.10 [ab]	1.22 ± 0.19 [b]			
p -Coumaric acid	0.23 ± 0.02 [a]	0.26 ± 0.06 [ab]	0.36 ± 0.02 [abc]	0.35 ± 0.02 [abc]	0.40 ± 0.10 [bc]	0.41 ± 0.00 [bc]	0.51 ± 0.02 [c]		0.622	
Ferulic acid	0.79 ± 0.10 [a]	0.89 ± 0.15 [a]	1.42 ± 0.11 [b]	0.80 ± 0.08 [a]	0.93 ± 0.05 [a]	0.85 ± 0.07 [a]	1.06 ± 0.01 [a]			1.023
Ethylcaffeoate	n.d [a]	0.05 ± 0.01 [a]	0.08 ± 0.00 [ab]	0.23 ± 0.03 [b]	0.42 ± 0.11 [c]	n.d. [a]	0.48 ± 0.04 [c]		0.603	-0.675
Ethylcoumarate	n.d	n.d	n.d	n.d	n.d	n.d	n.d			
Protocatechic acid	0.33 ± 0.03 [a]	0.35 ± 0.05 [a]	0.64 ± 0.08 [ab]	0.66 ± 0.16 [ab]	0.65 ± 0.12 [ab]	0.77 ± 0.21 [b]	0.67 ± 0.08 [ab]		0.877	
Sum	11.1 ± 0.48 [a]	11.5 ± 0.73 [a]	22.0 ± 0.31 [c]	15.0 ± 0.62 [b]	22.3 ± 2.07 [c]	14.0 ± 0.24 [ab]	24.0 ± 0.89 [c]		0.380	0.747

In the same row, different letters denote significant differences at $p< 0.01$

[(1)] only standardized regression coefficients (beta values) with $p < 0.01$, are reported

5. CONCLUSIONS

The profile of phenolic compound for all wines analysed was found to decrease during the bottle storage period, due to oxidation effects caused by the presence of oxygen dissolved in the medium.

The presence of SO_2 influenced in a positive way the total amount of phenolic compounds at the end of the fermentation and together with tannins contributed to preserve the phenolic content during the storage period.

Tannins showed a central role in protecting the total amount of phenolics during the entire storage period, and particularly against degradation of gallic acid, ethylgallate and GRP. The last one was also influenced by the presence of SO_2, together with tyrosol, *t*-caftaric and *c*-coutaric acids.

In this work, lysozyme showed a significant negative influence only for *t*-caffeic acid and ethylcaffeoate at the end of the fermentation.

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Chapter 6

The impact of glutathione on the formation of xanthylium cations from (+)-catechin and glyoxylic acid in a model wine system

ABSTRACT: *The ability of glutathione to prevent the production of xanthylium cations in a wine-like media, containing catechin and an aldehydic degradation product of tartaric acid, was investigated. Catechin (0.50 mM) and glyoxylic acid (0.25 mM) were added to a model wine system with 0, 0.25 and 1.25 mM of glutathione. At 1.25 mM glutathione the production of yellow xanthylium cations and the corresponding 440 nm absorbance of samples were significantly decreased while no protective effect was observed at 0.25 mM glutathione. The ability of glutathione to inhibit xanthylium cation production was due to the ability of glutathione to bind to the aldehyde-portion of glyoxylic acid. The equilibrium constants for the binding of glutathione with glyoxylic acid, at pH 3.2, was found to be 0.00129 M as compared to 0.00038 M for the binding of sulphur dioxide and glyoxylic acid. Alternatively, glutathione was not found to bind acetaldehyde under identical conditions. The results of this study suggest that at the concentrations that glutathione exists in wine, glutathione has the potential to inhibit the production of yellow xanthylium cation pigments generated from flavanols and glyoxylic acid.*

1. INTRODUCTION

Tartaric acid is one of the strongest naturally occurring acids in fruit and is the strongest acid in grapes and wine (pK_{a1}=2.90) (Azab et al., 1997; Ough et al., 1988). It is used in the production of jams, sweets, jelly, tinned fruit and vegetables, coca powder and frozen dairy produce, mainly to adjust the acidity but also as an emulsifier. In the wine industry, it is well known that tartaric acid, present in grapes and musts at concentrations between 3 and 15 g L^{-1}, is relatively stable microbiologically compared to the other naturally occurring organic acids, such as malic and citric acids. The use of tartaric acid to acidify musts and wines is permitted in the EU by Reg. 1493/99 (1.5 mg L^{-1} for musts and 2.5 g L^{-1} for wines are the maximum value permitted). It is traditionally used in the form of potassium hydrogen tartrate (KHT), associated with cooling, to speed up the precipitation and settling of KHT naturally present in wines that, otherwise, may accumulate during bottle storage, causing a not well accepted deposit. Another application of tartaric acid is in tartrate stabilisaton, which is the addition of metatartaric acid, a compound produced by heating tartaric acid, which retards the growth of KHT crystals and inhibits their precipitation (Rybéreau-Gayon et al., 2000).

During the 1990's, the oxidative degradation of tartaric acid has been linked to the production of pigments in model wine media and several studies using model wine systems to understand colour changes have demonstrated that tartaric acid can undergo degradation to form glyoxlic

acid, as shown in Fig. 1a. The glyoxylic acid formed by tartaric acid oxidation can then react at the aldehyde functional group with (+)-catechin to form a carboxymethine-linked (+)-catechin dimer (colourless compound in which the two flavanol units are linked by a carboxymethine bridge between the C-8 or C-6 positions, either symetrically [eg. C-6,C-6 and C-8,C-8] or asymmetrically [eg. C-6,C-8]), which undergoes cyclisation to form a xanthene, before a final oxidation step which generates yellow xanthylium cation pigments (Fig. 1a, 1b) (Fulcrand et al., 1997; Es-Safi et al., 1999; Es-Safi et al., 2000). The production of such pigments constitutes a new xanthylium formation pathway, since up until this point, only anthocyanin-flavanol reactions were known to contribute to the 'oxidative browning' spoilage phenomenon of the wine.

The isolation and further incubation of xanthylium cation pigments was found to yield two types of yellowish pigments showing visible absorption maxima at 440 nm and 460 nm, respectively. Mass spectroscopy (MS) spectral analysis showed that the first pigment type were xanthylium cation salts (coming from the 6-6, 6-8 and 8-6 carboxymethine-linked (+)-catechin dimer isomers), while the second type were shown to be ester derivatives of the former (coming from 8-8 carboxymethine-linked (+)-catechin dimer isomer) (Es-Safi et al., 2000).

A study on the spectral characteristics and colour intensity of xanthylium pigments in aqueous solutions at different pH values showed that in acidic aqueous medium, as is the case in wine, the pigments occurred with an intense yellow colour, typical of the xanthylium form. From pH 5.6, the pigments colour intensity increased, while there was a gradual bathochromic shift to a more pinkish red colour (Es-Safi, 2004).

The presence of either iron(II) or copper(II) in the model wine media is also known to accelerate the production of the xanthylium cation pigments (Oszmianski et al., 1996; Clark et al, 2002). The postulated role of these cations is to enhance the oxidative degradation of tartaric acid, while copper(II) is also known to accelerate the reaction between (+)-catechin and glyoxylic acid. Kinetics for formation of the carboxymethine-linked (+)-catechin dimer and xanthylium cation pigment suggested that copper(II) accelerates the bridging of the two (+)-catechin units via glyoxylic acid (Fulcrand et al., 1997; Clark et al., 2003). Subsequent studies showed that iron was more efficient than copper in both colouration and production of xanthylium cation pigments in wine-like solutions of tartaric acid and (+)-catechin (George et al., 2006).

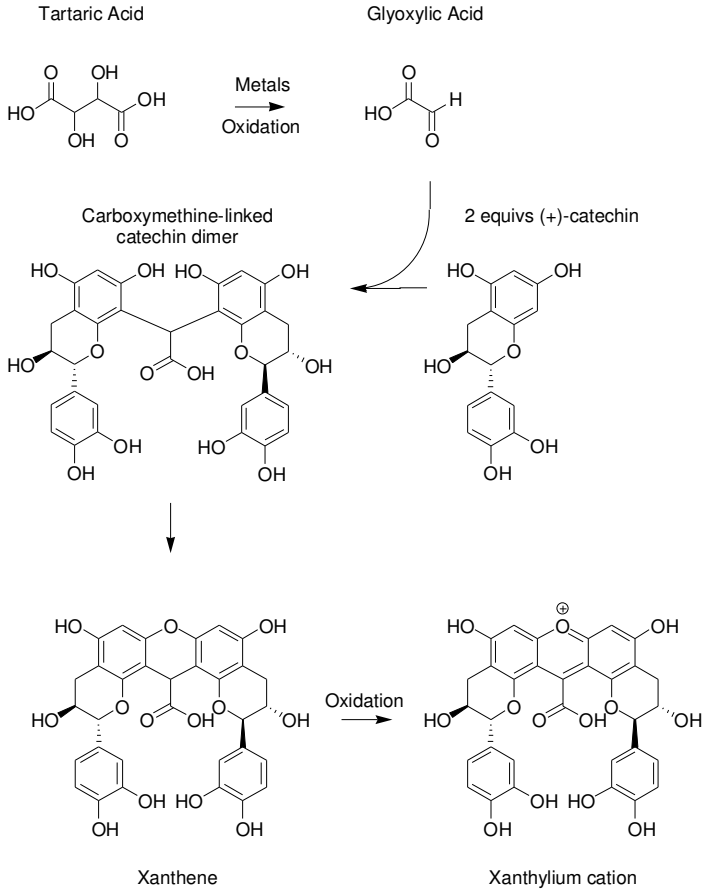

Figure 1a. The formation of xanthylium cations from (+)-catechin and glyoxylic acid (George et al., 2006).

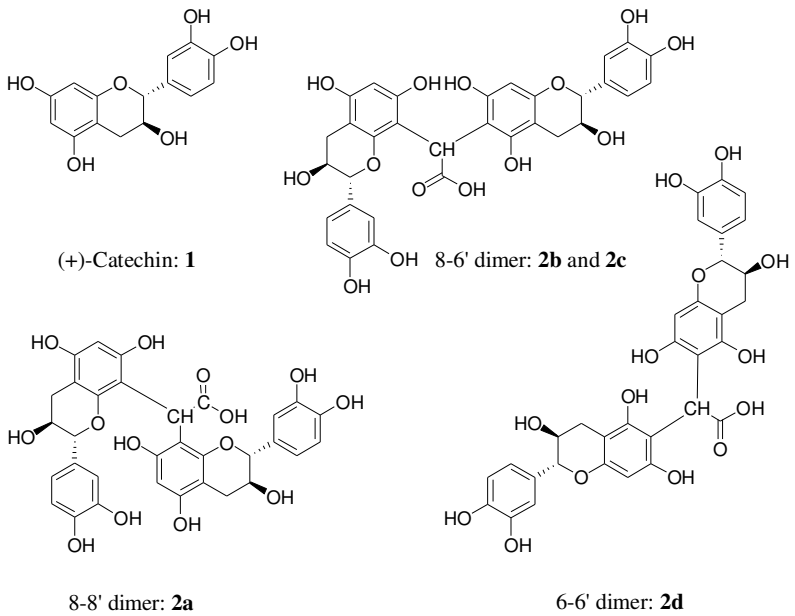

Figure 1b. Structure of (+)-catechin the formed colourless dimers 2a, 2b, 2c, 2d (Es-Safi et al, 2000)

Outdoor storage of tartaric acid solutions is known to generate glyoxylic acid and other oxidation products (Clark et al., 2003). Recently, experiments that followed the production and the stability of glyoxylic acid during the storage of tartaric acid solutions under various conditions of temperature, light exposure and in the presence of ethanol confirmed that glyoxylic acid was only detected in tartaric acid samples that had been stored outdoors and sunlight was identified as the critical component of outdoor storage that allowed its formation (Clark et al., 2007).

Notably, xanthylium cations have been identified in red wines (Es-Safi et al., 2000) but have not yet been reported in white wines, presumably due to the low concentration of flavanols in the latter. Also, these pigments are known to degrade to other pigments in the presence of non-flavonoids compounds such as caffeic acid in the medium. This instability in a model wine system occurred with caffeic acid, and highlighted that xanthylium cations are a transitory species during white wine oxidation (George et al., 2006).

One of the factors that can influence the production and the stability of these pigments is sulphur dioxide. Sulphur dioxide is successfully utilised in winemaking to limit the impact of any oxygen ingress into the wine and its main function in this role is to efficiently scavenge hydrogen peroxide and bind to aldehyde compounds, particularly acetaldehyde, and *o*-quinone compounds formed as a result of wine oxidation (Burroughs et al., 1964; Burroughs et al., 1973; Danilewicz et al., 2008).

However, as sulphur dioxide can induce allergic reactions in certain consumers there are obvious incentives to lower the concentrations of this preservative in wine. Indeed, a recent study has investigated the ability of glutathione, in combination with certain non-flavonoids such as caffeic acid and/or sulphur dioxide at lower levels than normally adopted, to inhibit the loss of desirable aroma compounds in white wines and model wines. That study showed the ability of combined non-flavonoid and glutathione additions to offer increased protection for desirable aroma compounds at various sulphur dioxide levels (Roussis et al., 2007).

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 et al., 2006) This cysteine-containing tripeptide is composed of glutamic acid, cysteine and glycine, and exists either in reduced (GSH) or oxidized (GSSG) form, participating in redox reactions via the reversible oxidation of its active thiol. In grape juice, it plays a specific role in enzymatic oxidation and browning of white juice (Singleton et al., 1985; Friedman, 1994). Glutathione is thus considered to be a powerful, versatile and important self-defence molecule. The glutathione content varies from 17 to 114 mg kg^{-1} in grapes and from 14 to 102 mg L^{-1} in musts (Cheynier et

al., 1989a), depending on the grape variety, the viticultural practises (i.e. water management, nitrogen fertilisation) and the oenological processes (including methodologies used for grape crushing, must treatment, skin contact time, and the increasing amount of soluble solids) (Okuda et al. 1999; Adams et al., 1993; Dubourdieu, 2006). However, the significant part of glutathione found in wines is due to its release from yeast solid parts at the end of the alcoholic fermentation (Park et al., 2000).

It has been well established for some time that glutathione is not only able to increase protection for desirable aroma compounds, but can also undergo addition reactions with *o*-quinone compounds and convert them back to less reactive phenolic compounds, albeit with a substituted unit attached. The oxidation of caftaric acid and coutaric acid (its coumaric analogue), catalysed by the grape polyphenoloxidase (PPO), leads to the formation of 2-S-glutathionylcaffeoyltartaric acid (GRP), referred to as grape reaction product. The reaction involves enzymatic oxidation of caftaric acid, followed by spontaneous reaction of the generated *o*-quinone with glutathione (Cheynier et al., 1988). Conversion of caftaric acid into GRP, which is known to occur when grapes are first crushed (Cheynier et al.,1995, 1990, 1989), and also during the non-enzymatic oxidations which prevail in the later stages of winemaking (Singleton, 1987; Cilliers et al., 1998), is therefore believed to be a way of limiting must-browning by trapping the *o*-quinones generated from caftaric acid in the form of the a stable glutathione-substitute product which prevents them from proceeding to brown polymers. Indeed, it is in wines whose musts have been protected from oxygen during processing that have the highest levels of glutathione in their corresponding wines. Du toit et al. (2007) studied the effect of oxygen on the levels of glutathione in must and wine. The storage of grape juice at high SO_2 and ascorbic acid levels at -20°C was found not to lead to a decrease in reduced glutathione levels. Alcoholic fermentation and oxygen additions to the must led to lower reduced glutathione levels in the wine. Reduced glutathione levels were only significantly higher in the wine made from reductive juice that had the highest initial reduced glutathione levels in the grapes.

Less certain is the ability of glutathione to fulfil the remaining antioxidant roles of sulphur dioxide in wine conditions, such as binding to aldehyde compounds and to scavenge hydrogen peroxide. Acetaldehyde, glyoxylic acid and glutathione are all actually present in the liver after the consumption of wine and in such physiological environments it is known that acetaldehyde and glutathione do not react (Kera et al., 1985; Pivetta et al., 2006), while glyoxylic acid and glutathione can do form an addition product (Gunshore et al., 1985).

It is well known that the main anti-oxidative activity of sulphur dioxide in wine is the generation of the bisulphite ion, which reacts with H_2O_2 to produce sulphuric acid, thereby limiting further oxidation of phenolic molecules or ethanol (Du Toit et al., 2006). For the activity of glutathione, it is known that it can act as an antioxidant to prevent cellular damage by scavenging hydrogen peroxide, as illustrated by the follow equation;

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$ (Fahey et al., 1991; Field et al., 1996),

although the same activity in wine conditions is less certain.

This study was undertaken in order to investigate the potential of glutathione to prevent the production of yellow xanthylium cation pigments from glyoxylic acid and (+)-catechin. The impact of glutathione concentration on the oxidative colouration, degradation kinetics, and products were followed by UV/Visible spectra, UPLC-PDA and LCMS.

2. EXPERIMENTAL

2.1 Reagents and apparatus

All glassware and plasticware were soaked for at least 16 hours in 10 % nitric acid (BDH, AnalaR) and then rinsed with copious amounts of Grade 1 water (ISO 3696). Solutions and dilutions were prepared using Grade 1 water. Potassium hydrogen tartrate (> 99 %), L(+)-tartaric acid (> 99.5 %) and L-glutathione (> 98 %) were obtained from Sigma (St. Louis, MO, USA). Phosphoric acid (98%) was from Merck (Darmstadt, Germany). (+)-Catechin monohydrate (Sigma, 98 %) was used without further purification. Glyoxylic acid, sodium metabisulfite and acetaldehyde were purchased from Sigma (St. Louis, MO, USA).

Absorbance measurements and spectra were recorded on a µQuant Universal Microplate Spectrophotometer (Biotek Instruments) with the KC4 v3.0 (Biotek Instruments) software package. Absorbance measurements were monitored at 440 nm and spectra were recorded from 200 to 600 nm.

CIELab measurements were conducted on a Shimadzu UV-1700 UV-Visible spectrophotomer with UVPC Colour Analysis software (version 3.00). The L*, a* and b* CIELab values were calculated at the daylight illuminant D65 and with a 10 degree observer angle. The transmission was scanned over the range 380 to 780nm with samples in 10 mm quartz cuvettes. The wine-like solution was used as the blank solution.

Liquid chromatography for samples with (+)-catechin were conducted with an Ultra Performance Liquid Chromatography (UPLC) system consisting of a Waters Acquity binary solvent manager connected to a sample manager and a PDA detector all run by Empower^2 chromatography manager software. The column was a Waters Acquity BEH C18 (2.1 × 50 mm) with 1.7 µm particle diameter. Injection volume was 7.5 µL and the elution gradient consisted of solvent A: 0.5% acetic acid (CH_3COOH) in water and B: 0.5% (CH_3COOH) in methanol (MeOH), as follows (expressed in solvent A): from 100 to 85% over 5 minutes, down to 71% in 30 minutes and to 0% in 5 minutes; after 10 minutes at 0%, up to 100% in 5 minutes followed by 5 minutes at 100% to equilibrate the column. The flow rate was 0.45 mL/min. Chromatograms and UV-visible spectra were recorded over the range 200-700 nm.

Liquid chromatography for equilibrium constant determination was carried out with the equipment as described by Labrouche et al. (2005): the LC-DAD-RI experiment was conducted on a Waters 2690 separation module run by Millenium32 software and connected to a Waters 2996 photodiode array detector and to a Waters 2414 refractive Index detector. The two Bio-Rad Aminex-HPX 87H (300 x 7.8 mm) ion exclusion columns in series were used and the column operating conditions were at 40°C. The elution solvent was phosphoric acid 0.065% in water, brought to pH 3.2 with potassium hydroxide (10% (w/v)).

2.2 Reactions

The wine-like solution was prepared by adding 0.011 M potassium hydrogen tartrate and 0.008 M tartaric acid to aqueous ethanol (12 % v/v, 2L) and stirring overnight at room temperature. The pH of the wine-like solution was 3.2 ± 0.1. (+)-catechin (0.50 mM), and glyoxylic acid (0.25 mM) were added to this solution and stirred until dissolved. The ratio of 2:1 for (+)-catechin to glyoxylic acid was chosen as it is consistent with the stoichiometry of the reagents in the final xanthylium cation product (Figure 1). This solution, containing 0.50 mM (+)-catechin and 0.25 mM glyoxylic acid, was then divided into 9 x 150 mL aliquots in 250 mL Schott bottles, as shown in Fig. 2. Glutathione at 0.25 mM and 1.25 mM was added to three different sets of the bottles thereby creating triplicate samples at 0, 0.25 and 1.25 mM glutathione. The samples were held in darkness at 45 °C for 12 days and the sample bottles were only opened on measurement days. The plotted data is the mean of the replicates with the error bars representing the 95% confidence limits.

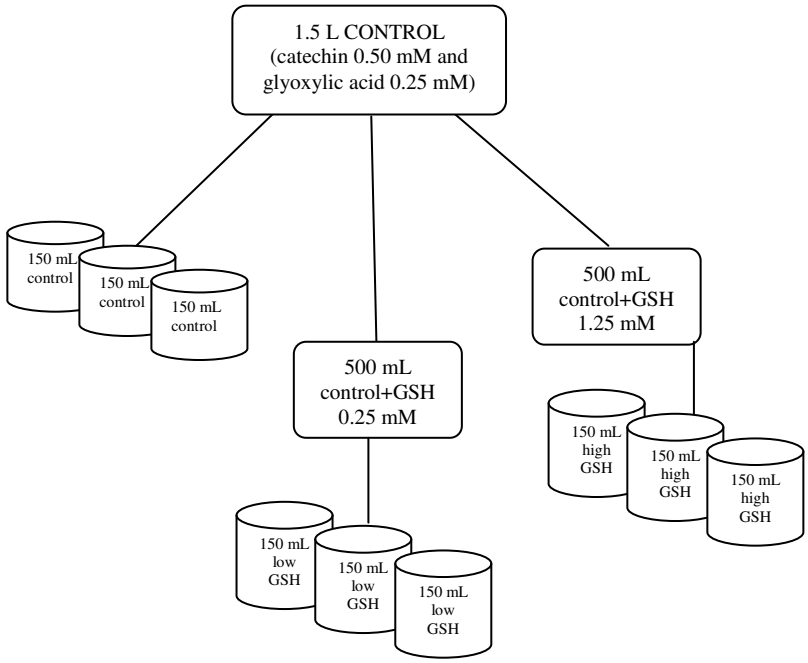

Figure 2. The wine-like solution trials scheme.

2.3 Determination of equilibrium constants for the dissociation of aldehyde addition products

For the determination of dissociation constants between glutathione and SO_2 with acetaldehyde or glyoxylic acid, solutions were prepared by adding these compounds at different concentrations to aqueous H_3PO_4 (0.065%) brought to pH 3.2 with potassium hydroxide (10% (w/v)), following the scheme showed in Table 1: This specific buffer was required as it is transparent at wavelengths around 210 nm and has a refractive index similar to water.

All the samples were prepared in triplicate, in 2.0 mL capped vials with no head space and held in darkness at 30°C for 2 hours before equilibrating to room temperature (1 hour) and being injected into the HPLC.

Quantification of free glyoxylic acid and acetaldehyde were performed using calibration curves generated with three standard solutions (at 5, 10 and 15 mM respectively) injected in triplicate and analysed via the refractive index detector for glyoxylic acid and at 275 nm for acetaldehyde. For the quantification of glutathione and SO_2, the concentration of glutathione and SO_2 added to the solution as shown in Table 1 were used.

	GSH	**Glyoxylic acid**	**Acetaldehyde**
	mM	mM	mM
$\mathbf{a_1}$	0	20	20
$\mathbf{b_1}$	10	20	20
$\mathbf{c_1}$	20	20	20
$\mathbf{d_1}$	100	20	20

	SO_2	**Glyoxylic acid**	**Acetaldehyde**
	mM	mM	mM
$\mathbf{a_2}$	0	20	20
$\mathbf{b_2}$	5	20	20
$\mathbf{c_2}$	10	20	20
$\mathbf{d_2}$	15	20	20

Table 1. Scheme of solution trials.

The apparent equilibrium constants for dissociation were calculated according to the Law of Mass Action:

$$\text{A-B} \rightleftharpoons \text{A} + \text{B} \qquad K_d = \frac{[A]\,[B]}{[A\text{-}B]}$$

Where [A] = concentration (in M) of free glyoxylic acid or acetaldehyde, as quantified by HPLC, [B] = concentration (in M) of free glutathione or SO_2 (equivalent to $[B]_{added}$-[A-B]), quantified as a difference between the value added to the starting solutions and the concentration of bound

glutathione/SO_2, and [A-B] = concentration (in M) of bound glutathione or SO_2, ($[A]_{initial}$-[A]), quantified as the difference between the glyoxylic acid or acetaldehyde concentration added to the starting solutions and the value of the remaining free glyoxylic acid or acetaldehyde quantified by HPLC. Such calculations are similar to those used by Burroughs et al. (1973), and this equation represents a simplification of the true situation since both the addition product and free sulphur dioxide exist in different structures according to pH, and the free aldehydes exist in hydrated or non-hydrated forms. Therefore the equilibrium constants calculated are 'apparent' equilibrium constants using the total concentration of reactants irrespective of their standard forms.

3. RESULTS AND DISCUSSION

The reaction of (+)-catechin and glyoxylic acid was conducted at glyoxylic acid to glutathione ratios of 1:0, 1:1 and 1:5. These ratios were chosen as the production of glyoxylic acid in oxidising wine systems is known to be low, and compared to the typical concentrations of glutathione in wine (10-30 mg L^{-1}), the glutathione concentration should be well in excess of the glyoxylic acid concentration. The ratios adopted would provide some insight into the efficiency of glutathione for the prevention of the yellow xanthylium cation products.

3.1 Oxidative colouration of model wine system

Once prepared, all samples were of a similar faint yellow colour. During the experiment the samples at 0 and 0.25 mM glutathione increased in colour intensity (Fig. 3) and their corresponding spectra (data not shown) showed a broad band with a maximum around 440 nm.

The broad absorbance band meant significant absorbance of wavelengths corresponding to both yellow (440 nm) and red (500 nm) colouration, which was consistent with the appearance of the samples. By contrast, the sample with 1.25 mM glutathione remained clear during the reaction period (Fig. 3).

Intriguingly, despite their identical treatment to the other samples, the samples with 0.25 mM glutathione had poor agreement in terms of their increase in 440 nm absorbance during the 12-day experiment. Although the confidence limits for the 0.25 mM glutathione sample are not shown in Figure 3 (for clarity), the average 440 nm absorbance on day-12 was 0.4 ± 0.3 absorbance units (n=3, 95% confidence limit) for these particular samples. Indeed, some of the 0.25 mM glutathione samples had greater 440 nm absorbance than the 0 mM glutathione samples by day-12, while other 0.25 mM samples had lesser absorbance. Therefore, the average 440 nm

absorbance for the 0.25 mM glutathione samples at day-12 was not significantly different (P=0.05) from the samples without glutathione, but was significantly higher (P = 0.05) than the samples containing 1.25 mM glutathione. The exact cause for the large confidence limits in the 0.25 mM sample was not certain but evident in all subsequent LC data.

A CIELab analysis of samples was performed which allows a both quantitative and descriptive analysis of samples that is comparable with their visual assessment. The L* parameter gives an indication of the intensity of colour from essentially none (100) to maximum (0), a* gives an indication of green (-a*) to red (+a*) colouration, and b* gives an indication of yellow (+b*) to blue (-b*).

	Day 12			
	L*	a*	b*	deltaE*ab
(+)-Catechin/ glyoxylic acid	93.4 ^{a}	-1.4 ^{a}	51.0 ^{b}	106.5 ^{b}
(+)-Catechin/ glyoxylic acid/ glutathione 0.25 mM	91.9 ^{a}	1.5 ^{a}	50.3 ^{b}	104.4 ^{ab}
(+)-Catechin/ glyoxylic acid/ glutathione 1.25 mM	99.0 ^{b}	-0.1 ^{a}	3.3 ^{a}	99.1 ^{a}

Table 2. CIELab values for the model wine samples. The values quoted are the average CIELab values (n=3) for samples at day 12 of the experiment. In the same row, different letters denote significant differences at p< 0.01

The CIELab data at day 12 (Table 2) showed that the samples at 0 and 0.25 mM glutathione had higher colour intensity (L*) than the 1.25 mM glutathione sample. The a* parameter was negative for the 0 and 1.25 mM glutathione samples indicating a green colour, which for the 0 mM glutathione sample, was pronounced (Fig. 4). For the b* parameter, the 1.25 mM glutathione sample appeared the least yellow, confirming the results obtained with the absorbance measurements at 440 nm (Fig. 9).

These results highlighted the efficiency of glutathione over a certain ratio to prevent the formation of yellow xanthylium cation products.

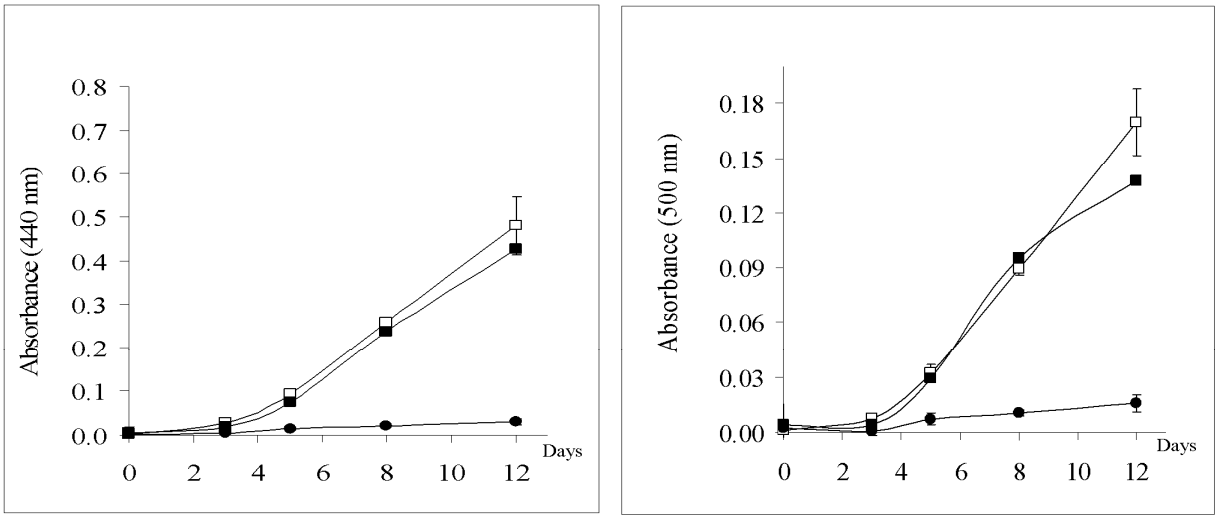

Figure 3. The change in 440 nm and 500 nm absorbance during the storage of samples at 45 °C in darkness. The samples are: (□) control, (■) control + 0.25 mM glutathione, and (●) control + 1.25 mM glutathione. Error bars indicate the 95 % confidence limits, and have been omitted for ■ to enhance clarity.

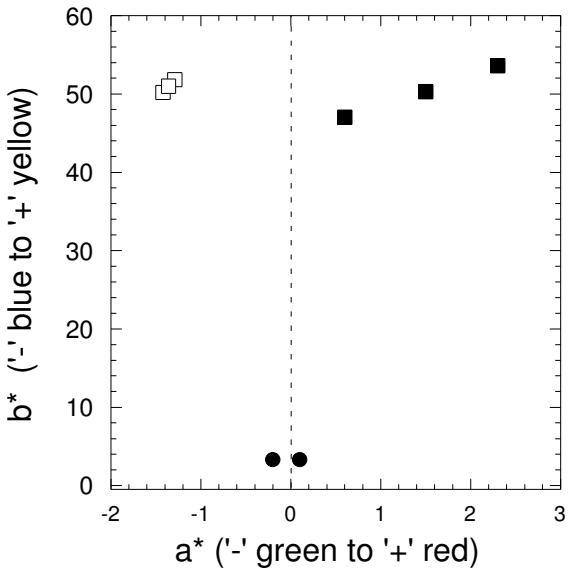

Figure 4. The CIELab analysis results after the 12 day of storage at 45 °C in darkness. The samples are: (□) control, (■) control + 0.25 mM glutathione, and (●) control + 1.25 mM glutathione.

3.2 Composition of the model wine during oxidation

During the experiment, the composition of the model wine systems was monitored by UPLC-DAD in order to determine the decay rates for (+)-catechin and the production rates of the xanthylium cation pigments as well as their precursors. The xanthylium cation pigments formed from (+)-catechin and glyoxylic acid are well characterised (Es-Safi et al., 2000; Maury et al., 2010) and consequently were identified based on their retention times, UV/Vis spectra and LC-MS analysis. The carboxymethine-linked (+)-catechin dimers, which are known intermediates in the formation of the xanthylium cations from glyoxylic acid and (+)-catechin, were similarly identified by their retention times, UV/Vis spectra and LC-MS analysis.

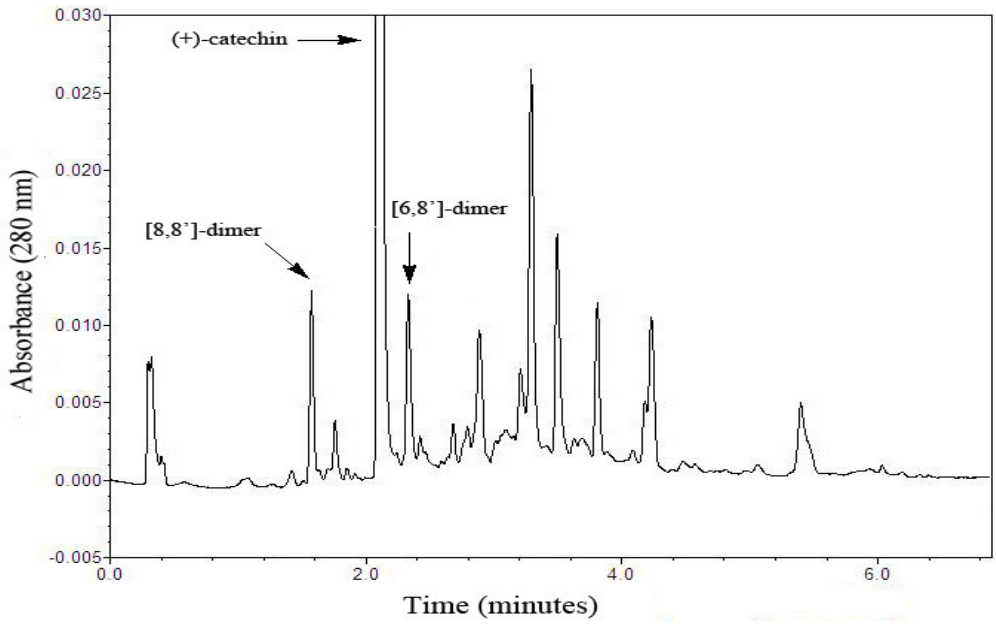

Figure 5. UPLC chromatographic profile monitored at 280 nm of (+)-catechin and glyoxylic acid mixture for a control sample at day 8 with no GSH added, showing residual (+)-catechin and newly formed 8-8 and 6-8 dimers (xanthylium intermediates). The 8-6 and 6-6 dimers were not resolved.

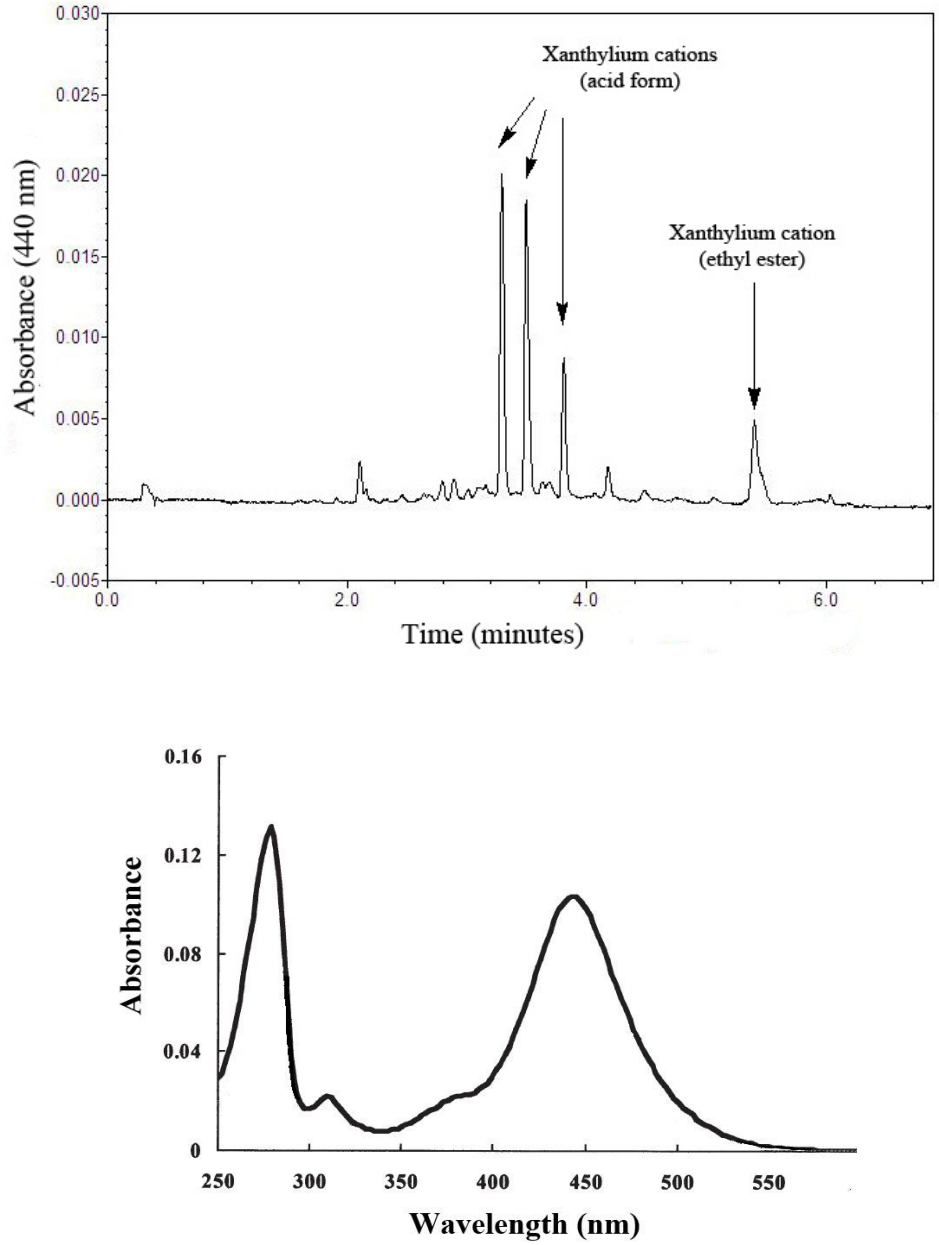

Figure 6. UPLC chromatographic profile registered at 440 nm of (+)-catechin and glyoxylic acid mixture for a control sample at day 8 with no GSH added, showing the four yellow xanthylium cation pigments and UV-visible spectra of two yellow pigments formed from the 6-8 dimer xanthylium intermediate.

After 12-days the (+)-catechin levels in the 0, 0.25 and 1.25 mM glutathione samples had reached 84 ± 4%, 70 ± 30% and 101 ± 4% of the original total level (0.5 mM) respectively, as shown in Fig. 7. Consequently, not only was it apparent that the ratio of 1:5 for glyoxylic acid to glutathione decreased the 440 nm absorbance in samples, but it also inhibited the loss of (+)-catechin. This latter outcome suggests that glutathione prevents the initial addition reaction between glyoxylic acid and (+)-catechin (Fig. 1).

For this to occur, the most likely mechanism is via the competitive binding of glutathione to glyoxylic acid, and such binding has been reported previously (Gunshore et al., 1985) albeit under physiological conditions far removed from the pH and composition of wine. Consistent with the 440 nm absorbance results was also the poor confidence limits for the loss of (+)-catechin for the samples with 0.25 mM glutathione.

The production of the pigment precursors (Fig. 8) known as the carboxymethine-linked (+)-catechin dimers (Fig. 1), and the final xanthylium cation pigments (Fig. 9) were consistent with the 440 nm absorbance data. The high glutathione levels of 1.25 mM prevented the formation of both compounds compared to the samples with 0 and 0.25 mM glutathione. The samples with 0.25 mM had high variability in the production of both the carboxymethine-linked (+)-catechin dimers and xanthylium cation pigments, but in both cases were significantly higher than for the 1.25 mM glutathione sample.

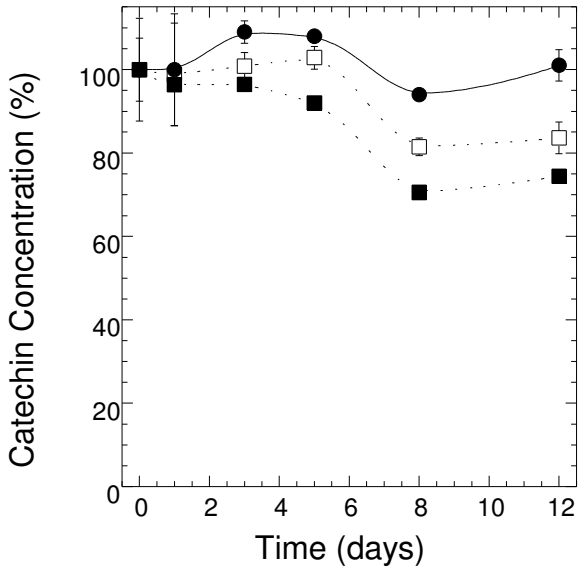

Figure 7. The decay of (+)-catechin concentration (%) at 280 nm of absorbance during the storage of samples at 45 °C in darkness. The samples are: (□) control, (■) control + 0.25 mM glutathione, and (●) control + 1.25 mM glutathione. Error bars indicate the 95 % confidence limits, and have been omitted for ■ to enhance clarity.

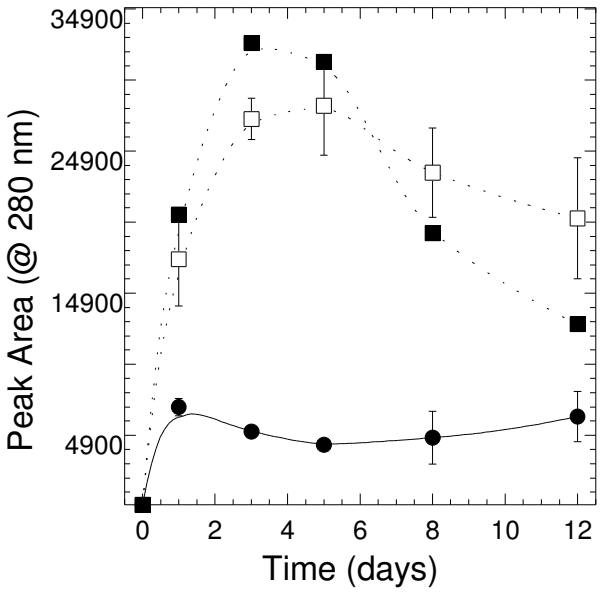

Figure 8. The production of one of the three xanthylium cation pigment precursors at 280 nm during the storage of samples at 45 °C. The samples are: (□) control, (■) control + 0.25 mM glutathione, and (●) control + 1.25 mM glutathione. Error bars indicate the 95 % confidence limits.

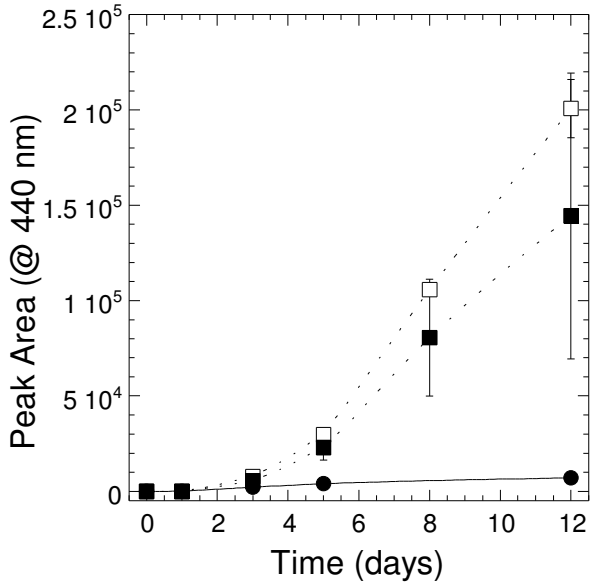

Figure 9. The production of the xanthylium cation pigments during the storage of samples at 45 °C. The samples are: (□) control, (■) control + 0.25 mM glutathione, and (●) control + 1.25 mM glutathione. Error bars indicate the 95 % confidence limits.

3.3 Determination of glutathione-aldehyde dissociation constants

To establish the extent of glutathione binding to glyoxylic acid (Fig 10) and provide an explanation of the results in Section 3.2, the equilibrium constant for such a reaction was determined utilising LC-DAD-RI with an ion exchange column. The technique had the advantage of allowing quantification of several components of the equilibrium (i.e. [A] and [B]) in a single analysis. However, the perceived limitation of the technique was the chromatographic separation of the equilibrium species that could lead to adduct dissociation during the measurement. In this sense, the technique was providing an 'operationally defined' measure of the equilibrium constant but would allow insight into whether binding was occurring at wine pH.

For comparison, the equilibrium constants for the binding of glutathione to acetaldehyde were also calculated, along with the binding of sulphur dioxide to both glyoxylic acid and acetaldehyde (Fig.10). Both these aldehydes have the ability to participate in the chemical evolution of phenolic compounds in wine.

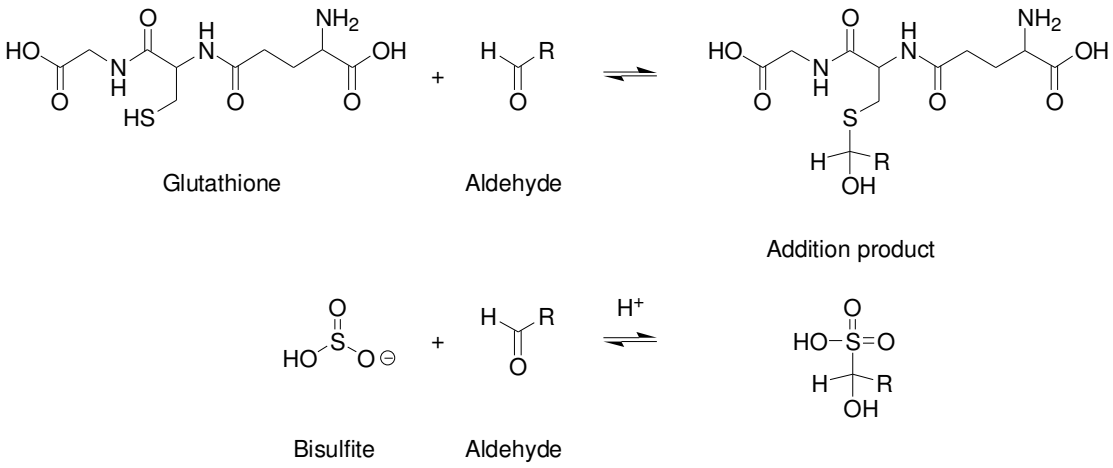

Figure 10. The addition reactions between glutathione or bisulfite with either acetaldehyde or glyoxylic acid, where R = CH_3 for acetaldehyde and R = COOH for glyoxylic acid.

Glutathione		Sulphur Dioxide	
Compound	*Dissociation Constant K_d (M)*	*Compound*	*Dissociation Constant K_d (M)*
Glyoxylic acid	0.00129	Glyoxylic acid	0.00038
Acetaldehyde	0.34407	Acetaldehyde	0.00061

Table 3. Dissociation constants K_d for the reaction between glutathione or SO_2 with glyoxylic acid or acetaldehyde.

From the data in Table 3, it is evident that glutathione showed an appreciable affinity with glyoxylic acid (resulting in a relatively low value for the equilibrium constant) at wine pH conditions (about pH 3) compared to acetaldehyde, confirming the ability of glutathione to bind to the aldehyde portion of glyoxylic acid, and thus the potential for it to prevent the formation of yellow xanthylium pigments. The data also suggest that, compared to glutathione, sulphur dioxide is a more reactive molecule toward the two aldehyde compounds we investigated. Furthermore, glyoxylic acid seems to be more reactive than acetaldehyde to the binding of SO_2 or glutathione. For the sulphur dioxide binding, this order of aldehyde reactivity is consistent with the dissociation constant data summarised within Bradshaw et al. (2010).

The values obtained in Table 3 compares well to the apparent equilibrium constant value obtained by Gunshore et al. (1985) for glyoxylic acid and glutathione at pH 7.4 (i.e. 0.0015 M). Whilst the equilibrium constants in Table 3 appear larger for acetaldehyde than those found in the literature. Burroughs et al. (1973) reported 'apparent' equilibrium constants for the sulphur dioxide /acetaldehyde system of 1.5 x 10^{-6} M (pH 3) and Kanchuger and Byers (1979) reported 0.083 M (pH 4-5) for the acetaldehyde and glutathione system. Therefore, it would appear based on the discrepancies between the measured and literature values, the acetaldehyde system seems less suited to the HPLC-DAD-RI determination of the apparent equilibrium constants. This may have been a consequence of the kinetics of dissociation of the adduct during the chromatographic separation at 40^{o}C and therefore a limitation of the technique. Future work could be conducted on the determination of the dissociation constants using non-invasive techniques such as proton nuclear magnetic resonance (^{1}H-NMR).

4. CONCLUSIONS

The results of this study suggest that glutathione is a promising candidate for inhibiting the formation of yellow xanthylium cation pigments production generated from (+)-catechin and glyoxylic acid at the concentrations that glutathione typically exists in wine. It must be noted that this is not the only pathway for the production of xanthylium cation pigments as these compounds may also be generated from ascorbic acid and (+)-catechin via a mechanism that does not involve glyoxylic acid (Barril et al. 2009). The ability of glutathione to bind glyoxylic acid rather than acetaldehyde may enable glutathione to be used as a 'switch' for glyoxylic acid-induced polymerisation mechanisms, as opposed to the equivalent acetaldehyde polymerisation, in processes such as microoxidation. Further research is required to assess the ability of glutathione to prevent xanthylium cation production during the in-situ production of glyoxylic acid and in the presence of sulphur dioxide.

Acknowledgements

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CONCLUSIONS

The data obtained from this research study confirm the capacity of lysozyme to control the bacterial activity and the consequent malolactic fermentation. The addition of lysozyme associated with oenological tannins in pre-fermentation of white musts could represent a promising alternative to the use of sulphur dioxide and serves as a reliable starting point for the production of SO_2-free wines.

The different vinification protocols studied influenced the composition of the volatile profile in wines at the end of the alcoholic fermentation, especially with regards to alcohols and ethyl esters also a consequence of the yeast's response to the presence or absence of sulphites during fermentation, contributing in different ways to the sensory profiles of wines. In fact, the aminoacids analysis showed that lysozyme can affect the consumption of nitrogen as a function of the yeast strain used in fermentation.

During the bottle storage, the evolution of volatile compounds is affected by the presence of SO_2 and oenological tannins, confirming their positive role in scaveging oxygen and maintaining the amounts of esters over certain levels, avoiding a decline in the wine's quality.

Even though a natural decrease was found on phenolic profiles due to oxidation effects caused by the presence of oxygen dissolved in the medium during the storage period, the presence of SO_2 together with tannins contrasted the decay of phenolic content at the end of the fermentation.

Tannins also showed a central role in preserving the polyphenolic profile of wines during the storage period, confirming their antioxidant property, acting as reductants.

To define a good winemaking protocol that includes lysozyme addition, it was fundamental to understand the interaction between lysozyme and the macro-components of musts and wines. In fact, the inhibition of the protein's enzymatic activity caused by the strong interaction between polyphenols and lysozyme should be taken into account.

The decrease in the enzymatic activity due to the interaction between lysozyme and tannins suggests that the use of tannins in winemaking associated with lysozyme must be rationalised. Therefore, it could be useful to add lysozyme only 12-24 hours after the addition of gallic tannins, to permit the tannins to activate their antiradical and antioxidant function, as well as to inactivate oxidases.

Concerning SO_2, the strong decrease in the lytic activity of lysozyme in the first hours of addition does not correspond to a strong decrease in the percentage content of lysozyme as

residual protein in the medium, suggesting that, under these conditions, the residual protein in the medium is not able to carry out an antibacterial function, probably due to a change in the structure as a result of the linkage with HSO_3^-.

Our study focused on the fundamental chemistry relevant to the oxidative phenolic spoilage of white wines has demonstrated the suitability of glutathione to inhibit the production of yellow xanthylium cation pigments generated from flavanols and glyoxylic acid at the concentration that it typically exists in wine. The ability of glutathione to bind glyoxylic acid rather than acetaldehyde may enable glutathione to be used as a 'switch' for glyoxylic acid-induced polymerisation mechanisms, as opposed to the equivalent acetaldehyde polymerisation, in processes such as microoxidation. Further research is required to assess the ability of glutathione to prevent xanthylium cation production during the in-situ production of glyoxylic acid and in the presence of sulphur dioxide.