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SELECTION AND USE OF YEAST STRAINS ISOLATED FROM AUTOCHTHONOUS SOUTHERN ITALY GRAPEWINE CULTIVARS AND IMPACT ON AROMA PROFILE OF PRODUCED WINES

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RIASSUNTO

L'impiego di lieviti commerciali selezionati all'interno del gruppo *Saccharomyces* "sensu stricto", permette di ridurre i rischi di produzione e di standardizzare il processo di vinificazione, garantendo la qualità del prodotto finale. La diffusione dell'uso di colture starter sta determinando un appiattimento delle differenze sensoriali che contribuiscono a distinguere i vini delle differenti cultivar. La ricerca, è focalizzata allo studio di nuove colture di lieviti che arricchiscano, il vino di quelle componenti aromatiche in grado di esaltarne il carattere di tipicità (varietale). La produzione di molte di queste molecole aromatiche è dovuta ai lieviti non-*Saccharomyces*, presenti sulle uve e nel mosto, che predominano nella prima fase della fermentazione alcolica e che, in questa breve fase, rilasciano metaboliti ed enzimi, che contribuiscono alla formazione di composti che determinano la della complessità aromatica. Infatti, prove sperimentali hanno evidenziato il ruolo positivo dei lieviti non-*Saccharomyces* nella fermentazione vinaria, sia migliorando il comportamento fermentativo dello starter sia aumentando la complessità della composizione del vino, influenzando, in particolare, le proprietà chimiche e sensoriali. La trasformazione biochimica da parte dei lieviti, dei costituenti base del mosto d'uva in composti aromatici rappresenta un meccanismo importante, attraverso il quale può essere influenzato, significativamente, l'aroma ed il flavour del vino ed esprimere meglio il carattere varietale del vitigno. Per cui, accanto all'etanolo, composto primario della fermentazione alcolica, troviamo numerosi composti quali esteri, alcoli superiori, aldeidi, acidi, terpeni, chetoni, che ne determinano la qualità finale del prodotto. I lieviti vinari possiedono la capacità di produrre questi composti, ma è il livello quantitativo che determina la differenza tra le diverse specie e poi, nell'ambito della stessa specie, tra i diversi biotipi.
I lieviti vinari, per lo sviluppo di colture starter, vengono isolati dalle uve, dagli ambienti di lavorazione o cantine antiche e dalle fermentazioni naturali che hanno portato alla produzione di vini con qualità accettabili e peculiari. Essi vengono selezionati in base a caratteristiche quali vigore fermentativo, potere alcoligeno e resistenza agli antisettici, assenza del carattere filmogeno, nonché produrre una bilanciata quantità di metaboliti aromatici, senza eccessi di composti volatili indesiderati e produrre enzimi che trasformano i composti neutri dell'uva in sostanze aromatiche attive. Glicosidasi ed esterasi sono alcuni degli enzimi prodotti dai lieviti che possono dare un contributo significativo all'aroma del vino. In particolare, i lieviti non-*Saccharomyces* sono maggiormente dotati di queste attività enzimatiche rispetto ai lieviti appartenenti alla specie *S. cerevisiae*. Lo scopo di questo lavoro è stata la selezione e l'uso di lieviti isolati da cultivar autoctone dell'Italia meridionale e il loro l'impatto sul profilo aromatico dei vini ottenuti. La tecnica di elezione per l'analisi della componente volatile dei vini ampiamente applicata è la Microestrazione in fase solida (SPME) abbinata alla GasCromatografia accoppiata alla Spettrometria di Massa (GC/MS), che permette la determinazione del profilo volatile senza nessuna manipolazione del campione dando una reale identificazione delle molecole volatili che vanno a caratterizzare il prodotto.
Lo scopo di questo lavoro è stato la selezione e l'uso di lieviti isolati da cultivar autoctone di *"Vitis Vinifera"*dell'Italia meridionale e l'impatto sul profilo aromatico dei vini. Abbiamo testato diverse combinazioni di lieviti autoctoni (*S.cerevisiae* e non-*Saccharomyces*) per migliorare la qualità e le caratteristiche organolettiche del prodotto finale ma anche per enfatizzare il suo legame con il territorio.
Al fine di valutare il profilo aromatico, i vini ottenuti sono stati caratterizzati mediante SolidPhaseMicroExtraction-abbinata alla GasCromatografia accoppiata alla Spettrometria di Massa (SPME-GC/MS).

ABSTRACT

Today, the use of selected commercial yeasts (LSA) from *Saccharomyces* species "*sensu stricto*" is widespread, because it allows to minimize risks in the production process, to standardize the winemaking procedure and ensure the quality of the final product. On the other hand, the widespread use of starter cultures in the wine industry has resulted in a loss of wine sensory characteristics and in a flattening of those differences crucial to distinguish wines of different cultivars.
For this reason, the research is focused on studying new selected yeast cultures that can give a final product enrich of those aromatic compounds which enhance the character of the wine. It is known that the production of many of this aromatic molecules occurs due to the presence of non-*Saccharomyces* yeasts, found on the grapes and must. These non-*Saccharomyces* yeast strains predominate in the first phase of the alcoholic fermentation, are able to release metabolites and enzymes, responsible of the aromatic complexity of the wine. Indeed, experimental tests have shown the positive role of non-*Saccharomyces* yeasts in the fermentation, improving the behavior of the starter and increasing the complexity of the composition of the wine, in particular, the chemical and sensory properties. The biochemical transformation of flavour-inactive grape juice constituents into aromatic components has emerged, as an important, additional mechanism whereby yeasts substantially impact on wine aroma and flavour and facilitate greater expression of grape varietal character. In addition to ethanol, produced of the primary

alcoholic fermentation, there are numerous compounds such as esters, higher alcohols, aldehydes, acids, terpenes, ketones, that determine the final quality of the product. The yeasts have the ability to produce these compounds, but it is the quantitative level that determines the difference between the different species and then, within the same species, between different biotypes. The wine yeasts for starter culture development have been sourced from grapes, winery environment or old cellars and spontaneous fermentations that have given wines of acceptable or unique quality. They are selected based on characteristics such as vigor fermentative, alcoholigenous power and they should be tolerant to the antioxidant and antimicrobial compounds, produce minimal foam, as well as produce a balanced array of flavour metabolites, without undesirable excess of volatile compounds and produce enzymes that transform the neutral compounds in flavor active component. Glycosidases and esterases are some of the enzymes produced by yeasts that can make a significant contribution to the aroma of the wine. In particular, the non-*Saccharomyces* yeasts have more enzymatic activities respect to yeasts *S. cerevisiae*. The aim of this work was the selection and use of autochthonous yeast strains isolated from cultivars of "*Vitis Vinifera*" of southern Italy and their impact on aroma profile of wines. We have tested different combination of autochthonous yeasts (*S. cerevisiae* and non-*Saccharomyces*) to improve the quality and organoleptic characteristics of the final product but also to emphasize its link with the territory and the ancient wine cultivars.
In order to evaluated the aromatic profile, the different wine samples were characterized by Solid Phase Micro Extraction-Gas Crhomatography/Mass Spectrometry (SPME-GC/MS technique).

1 GENERAL INTRODUCTION. Wine: Past and future prospects

Throughout antiquity the conversion of grapes into wine was considered a gift from the gods and the best wines were thus reserved for the elite of society. Wine was one of the first commodities to be bartered by early civilizations engaged in international trade. Today, wine is an integral component of the culture of many countries, a form of entertainment in others, and a libation of choice for advocates of its health benefits. Wine consumers are in over the world, both in developed nations that in poor countries, where it is used as energy source because these people have difficulty in the food supply. Regardless of the geographical area in which wine is produced or the social status of the consumer, all the wines should give pleasant emotions to the consumer. In past generations, the definition of quality was the preserve of the wine producer, and consumers who did not like a particular style of wine were often made to feel uncultured. But globalization and the accompanying rapid worldwide access to information has resulted in a more knowledgeable and empowered consumer with a more sophisticated understanding of product value and a discriminating demand for quality. The control of the definition of quality has thus shifted to the consumer. Success as a wine producer in the twenty-first century requires a thorough appreciation of human behavior and product choice. The intrinsic sensory aspect of wine taste and aroma are only one component in the modern definition of the quality for consumers.

In contrast to other types of crops, grapes can be grown in diverse climates and soils. Although scientifically still unproven, environmental stress is believed to improve the sensory characteristics of grapes and wine, resulting in a better product. The French concept of “terroir” states that the composition of grapes produced in a specific growing region will be influenced by the local environment, which will carry through to the wines of the area (Laville, 1990). This concept also includes as fundamental the minimal intervention in modification of the growing environment so that the terroir may be evident. Thus, in contrast to other agricultural commodities, wine is marketed by the geographical location of production (DOCG), and quality is associated with minimal vineyard inputs or manipulation. Consumers expect that wine from a particular region possess unique qualities that differentiate it from other wines of the same varietal, from other regions. This peculiarity of the industry is a great economic equalizer across the globe. It means that wines perceived to be of high quality can be produced anywhere. Indeed, quality wines are currently being produced on all six arable continents, and affluent as well as emerging nations are active in the international wine trade. The heightened tourism that accompanies the ‘discovery’ of a new wine-producing regions is economically important to many countries. This ‘value added’ economic aspect of wine production is remarkable, and that is the main reason of many efforts that are doing many countries to development and improvement of their wine industries.

On the other hand the consumption declined in the traditional wine producing and consuming countries, while emerged high competition from the ‘New World’ nations as the United States, Australia and Chile, where prosperous consumers chose quality rather than quantity in consumption. In 2012, France, Italy and Spain represent 43% of global wine production (down from 54% in 2010) and 25% of global consumption (down from 35% in 2010) data by Morgan Stanley Research.

Many factors, other than enhanced product quality have fuelled the increase in US consumption of wine, in fact, in 1991, a study by Serge Renaud coined the term ‘French paradox’ to describe the relationship between the high intake of fats in the French diet and the low incidence of coronary heart disease (Renaud & De lorgeril, 1992). This well-turned phrase galvanized the attention of the media, the public and other scientists. Although there are many differences between the French and American diets, attention quickly focused on the disparity in wine consumption between the two countries. This was because of the long recognized benefit of moderate alcohol consumption (Lucia, 1963; Marmot et al., 1981). Renaud had no mechanistic explanation for the paradox, which led many to doubt its validity, but Kinsella and colleagues (Kinsella et al., 1993) proposed that the natural antioxidant phenolic compounds of wine and fruits and vegetables of the Mediterranean diet might protect against heart disease. This conclusion was based partly upon a new theory advanced by Steinberg (Steinberg et al., 1993) that linked oxidation in the blood to disease. The power of the antioxidant hypothesis, coupled to the high visibility of Renaud’s report and strong pressure from the public sector, promoted investigation into the chemical and biological activities of alcohol, dietary phenols and flavonoids. Today there is scientific evidence that moderate alcohol and/or wine consumption protects against the incidence of many diseases of modern society The benefits of antioxidants are more pronounced in red wines as these wines contain a higher phenolic content, but white wines also offer some benefit to the consumer. The impact of the French paradox and the popularization of this study by the media had a pronounced impact on the international wine industry. Consumers were willing to pay more for a product with a perceived health benefit, while still expecting a satisfying sensory experience. This market remains highly competitive, as wines produced anywhere in the world may possess the same health promoting effects. Some growing regions develop the marketing of their wines based on the antioxidants content.

In vino veritas, "there is truth in wine" is the often-quoted saying from the writings of Pliny the Elder, a Roman writer who was killed while observing the cataclysmic eruption of the Vesuvius nearly 2000 years ago.

Grapes, ancient and modern

Ancient vineyards are the key to the revitalized wines of the modern region of Campania in the south of Italy, which is greatly to the winemakers' credit. Rather than pursue an international market by planting Chardonnay, Merlot or Cabernet Sauvignon, the winemakers of Campania have looked to their roots and replanted their own vineyards with locally vigorous vines. Most of these are grapes are unheard of in the rest of the world. However, they are the cultivar of "*Vitis vinifera*" that grow best in this climate and soil. In particular, the Aglianico ("Ellenico" or Hellenic), is a red grape which was brought to the region by the Greeks and then cultivated by the Romans. Piedirosso or Sciascinoso, red grape, is named after the gnarled red bases of the vines, which look like the red feet of a native dove. The white Coda di Volpe grows in a long bunch reminiscent of the tail of a fox. The Falanghina is a white wine grape, the vines being trained on phalanges (stakes) by the Greeks. Fiano, another white, usually referred to as "Fiano di Avellino" is grown in the Avellino district of Campania; the name is derived from the Roman apiana which, legend has it, refers to the fact that bees favoured this grape. The most popular white is Greco di Tufo another reference to a grape brought here by the Greeks. The Campania region has been well-known for its wines since the Greeks and Romans settled there, when it was known as Oenotria, "the land of wine." The most famous wine, Falernum, is currently made by Villa Matilde as Fallerno del Massico DOC. Campanian winemakers may be reviving many of the ancient grape varieties, but they are making their wines with modern vinification techniques.

Both red and white wines are actively being developed by large, and smaller, family-owned wineries. Many of these wines are lively, full of youthful fruit and reasonably priced to compete with other new Southern European and New World wines. Some of the new Campanian wines have already reached retailers in major cities of the UK and US, and you can look forward to many more appearing this coming year.

The red Aglianico, sometimes blended with Piedirosso, is made in a variety of styles, and though it may have begun as a sturdy country wine, it can be vinified with great balance and elegance. These reds can be served with light, as well as hearty dishes.

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2 Yeast

A yeast is a unicellular fungus which reproduces asexually by budding or division, especially the genus *Saccharomyces* which is important in food fermentations (Walker, 1988). Yeasts and yeast-like fungi are widely distributed in nature. They are present in orchards and vineyards, in the air, the soil and the intestinal tract of animals. Like bacteria and moulds, they can have beneficial and non-beneficial effects in foods. The most well known examples of yeast fermentation are in the production of alcoholic drinks and the leavening of bread. For their participation in these two processes, yeasts are of major importance in the food industry. Although there is a large diversity of yeasts and yeast-like fungi, (about 500 species), only a few are commonly associated with the production of fermented foods. Varieties of the *Saccharomyces* genus are the most common yeasts in fermented foods and beverages based on fruit and vegetables. All strains of this genus ferment glucose and many ferment other plant derived carbohydrates such as sucrose, maltose and raffinose.

2.1 Ecology of the grapes and must

The diversity, composition and distribution of yeast in association with grapes and must are influenced by a variety of environmental and technological factors: climate and geographical location (Versavaud et al., 1995; Beltran et al., 2002; Schuller et al., 2005), grape variety (Martini et al., 1996), presence of yeast starters (Heard and Fleet, 1985; Valero et al., 2007), and the fermentation temperature used (Torija et al., 2003a). Early stages of wine fermentation involve many different microorganisms, including bacteria of various kinds as well as molds and yeasts. Specifically, the yeasts belong mainly to the genera *Kloeckera*, *Metschnikowia*, *Torulaspora*, *Candida*, *Kluyveromyces*, *Pichia*, *Brettanomyces*, *Dekkera*, *Zygosaccharomyces*, and *Saccharomyces* (Fleet and Heard, 1993). During the course of the alcoholic fermentation, the *S. cerevisiae* replace the non-*Saccharomyces* species and dominates the final stages of process.

2.2 Winemaking process

Winemaking is a complex chemical and biological process in which different genera of yeast and bacteria are involved. The principal yeast responsible of alcoholic fermentation in must grape belong to the *Saccharomyces* genus. In particular, the *Saccharomyces* "sensu stricto" group, that contains the species *S. cerevisiae*, *S. pastorianus S. paradoxus* and *S. uvarum*, mainly responsible of the grapes juice fermentation (Demuyter et al., 2004; Massoutier et al., 1998; Naumov et al., 2000, 2001; Sipiczki, 2008). Both *S. cerevisiae* and *S. uvarum* are able to grow on substrates characterized by high sugar and ethanol content, low pH, high sulphur dioxide concentrations and remains of fungicides, demonstrating that their genomes are well adapted to the oenological conditions (Sipiczki, 2008). However, *S. cerevisiae* has higher resistance to high temperature stress (up to 37ºC) and higher ethanol resistance (up to 15%) than *S. uvarum* (Belloch et al., 2008). Wine fermentation is a complex microbial process, where the physicochemical conditions and microbial interactions influence the growth and metabolism of the microorganisms involved. Natural or spontaneous alcoholic fermentation involves a multitude of biochemical and ecological interactions between many microbial species (Pretorius, 2000). The *Saccharomyces* yeast rapidly converting the available sugars in ethanol, a toxic compounds for non-*Saccharomyces* yeasts. Ethanol concentration creates strong pressure which limits growth of non-*Saccharomyces*, consequently, they can't compete with the *Saccharomyces*, which generally survive to higher ethanol levels (10-15% v/v) (Fleet and Heard, 1993; Rainieri and Pretorius 2000).
Indeed, yeasts of the genera *Hanseniaspora* and *Kloeckera*, generally present on the bunches skin with high populations, are able to start the fermentation of grape juice, but they tend not to be ethanol- tolerant. During the fermentation process as the ethanol concentrations reach 2-4% (v/v) only ethanol-tolerant yeasts predominate present both within the non-*Saccharomyces* that of *Saccharomyces* species, in particular more belonging to *Saccharomyces cerevisiae*. Moreover, the temperature increase produced by *Saccharomyces* during fermentation provides these species with a clear advantage over non-*Saccharomyces* species (Salvadó et al., 2011). Therefore, due to the non-sterile environment during wine fermentation, different yeast species and/or strains can be involved in several interactions through the production of toxic compounds, or as a result of competition for nutrients. In terms of inhibitory interactions that are mediated by metabolites with toxic effects, the most evident example is the production of ethanol by *S. cerevisiae*. Indeed, the selective pressure exerted by high levels of alcohols has been defined as the main factor responsible for the dominance of *S. cerevisiae* towards other non-*Saccharomyces* yeast (Pretorius 2000). Together with ethanol, other factors can have strong selective pressure in wine fermentation. In particular, the production of medium-chain fatty acids and high amounts of acetic acid can negatively affect the growth of a co-fermenting yeast species. Also cell-to-cell contact appears to be involved in the interactions between *S. cerevisiae* and other non-*Saccharomyces* species, such as *Torulaspora delbrueckii*, *Hanseniaspora uvarum* and *Kluyveromyces thermotolerans* (now reclassified as *Lachanchea thermotolerans*)

(Arneborg et al., 2005). Another mechanism that regulates the presence and dominance of yeast species during wine fermentation is the involvement of oxygen. Reduced oxygen availability under grape juice fermentation might have an important role as a selective factor in mixed cultures starters. Indeed, low tolerance to low available oxygen exhibited by *K. thermotolerans* and *T. delbrueckii* could in part explain their relative competitiveness, and consequently their rapid death in the presence of *S. cerevisiae* (Hansen et al., 2001). Many studies across all winemaking areas have established the yeast succession of *Hanseniaspora* to *Saccharomyces* during spontaneous fermentation of grape juice. However, other yeast species that belong to other genera have occasionally been found, such as those of *Metschnikowia, Candida, Torulaspora, Lachancea/Kluyveromyces and Zygosaccharomyces* (Bell et al., 2013; Jolly et al., 2014; Pretorius 2000).
Today the spontaneous fermentation of the must not be seen as an abandonment of the most to his fate, leaving freely develop low alcohol-tollerant wild yeast, but as an opportunity to drive the fermentation process, trying to encourage the growth of elliptical yeast species Saccharomyces cerevisiae. The spontaneous fermentation, despite the apparent unpredictability of its outcome and the risk of the onset of microbiological problems, allows obtaining products with a strong stylistic distinction, consequence of greater complexity of aroma, flavor and texture, regard products obtained by inoculation of selected strains, which, conversely, would be responsible for a "flattening" of the differences (Pretorius, 2000).The more complexity the wine obtained by spontaneous fermentation would directly correlated with the nature of the process, started and completed by the combined action and/or in sequence of yeasts, among them different at the species level and, within the same species, at the level of strain, each, however comes with its own imprint qualitative transferable to the final product in proportion to the weight of the action took place in fermentation process.
On the other hand the inoculation of the grape must with selected cultures of *Saccharomyces cerevisiae* was introduced with the aim of improving the fermentation rate and controlling the fermentation process, obtaining at the same time wines with desired or more complex oenological characters. But, the main critics of the practice of guided fermentation (using starter cultures) dislike the fact that the commercial yeast-strains, despite being numerous, possess very ordinary characteristics.
Commercial yeast strains produce wines with average qualities and do not enhance the aromatic traits that characterize many yeasts isolated from specific geographical areas. In the last years, studies on the improvement and the selection of wine yeasts to overcome this problem have been carried out. It is generally believed that the inoculation of the fermentation with selected cultures of *S. cerevisiae* will suppress any indigenous non-*Saccharomyces* yeast. (Jolly et al., 2014;Viana et al., 2008). Following numerous studies on the influence of non-*Saccharomyces* yeast in winemaking, there has been a reevaluation of the role of these yeasts. Indeed, some non-*Saccharomyces* yeast can enhance the analytical composition and aroma profile of the wine. In this context, over the last two decades, the use of controlled multistarter fermentation using selected cultures of non-*Saccharomyces* and *S. cerevisiae* yeast strains has been encouraged (Comitini et al., 2011.) Several aspects support the use of multi-starter fermentation in winemaking, the most important of which are: (i) modification of some specific analytical compounds, such as increased glycerol content, enhanced total acidity, or reduced acetic acid content of the wine; (ii) enhancement of the analytical profile of the wine (esters, volatile thyols); (iii) reduction of the ethanol content of the wine; (iv) control of the spoilage microflora in the wine; and (v) improvements to the overall quality and complexity of the wine

2.2.1 Alcoholic fermentation

Alcoholic fermentation is the anaerobic transformation of sugars, mainly glucose and fructose, into ethanol and carbon dioxide. This process, which is carried out by yeast and also by some bacteria such, can be summarised by this overall reaction.

$$C_6H_{12}O_6 \longrightarrow 2\ CH_3CH_2OH + 2\ CO_2$$

However, alcoholic fermentation is a much more complex process. At the same time as this overall reaction proceeds, a lot of other biochemical, chemical and physicochemical processes take place, making it possible to turn the grape juice into wine. Besides ethanol, several other compounds are produced throughout alcoholic fermentation such as higher alcohols, esters, glycerol, organic acids, acetaldehyde. These metabolites are very important in the final quality of wine. The grape juice largely consists of sugars, mainly glucose and fructose. Organic acids are also important in must composition: in particular tartaric and malic acids, citric acid to a lesser extent, and also succinic and keto acids. Finally, phenolic compounds and aromas contribute to wine aroma, although they do not play an essential role in fermentation kinetics.
Yeasts can degrade sugars by two metabolic pathways: fermentative and oxidative. Glycolysis is the common route for both these processes (Figure 2.1). The sugars in grape juice are metabolized to pyruvate by the enzymes of the glycolytic pathway. Afterwards, in the fermentative one, the pyruvate is decarboxylated to acetaldehyde,

which is reduced to ethanol, whereas, in the respirative pathway, the pyruvate that arose from glycolysis undergoes an oxidative decarboxylation in the presence of coenzyme A inside the mitochondria. The respiration can take place at a low sugar concentration and in the presence of oxygen. However, for high glucose concentrations yeasts only metabolize sugars by the fermentative pathway. Even in the presence of oxygen, respiration is blocked. This phenomenon is known as the Crabtree effect, catabolic repression by glucose or the Pasteur contrary effect (Ribéreau-Gayon et al., 2006). The high sugar concentration of the grape must makes the fermentative pathway the main sugar catabolic route. In a standard fermentation, one molecule of sugar (glucose/fructose) yields two molecules of ethanol and carbon dioxide. However, only 90-95% of the sugar is converted into ethanol and carbon dioxide, 1-2% into cellular material and 4-9% into other secondary metabolites such as glycerol, succinic and acetic acids, fusel alcohols and esters (Boulton et al., 1996). Fermentation activity decreases under stressful fermentation conditions, such as nutrient limitation, low pH, lack of oxygen, extreme temperatures, and the presence of toxic substances.

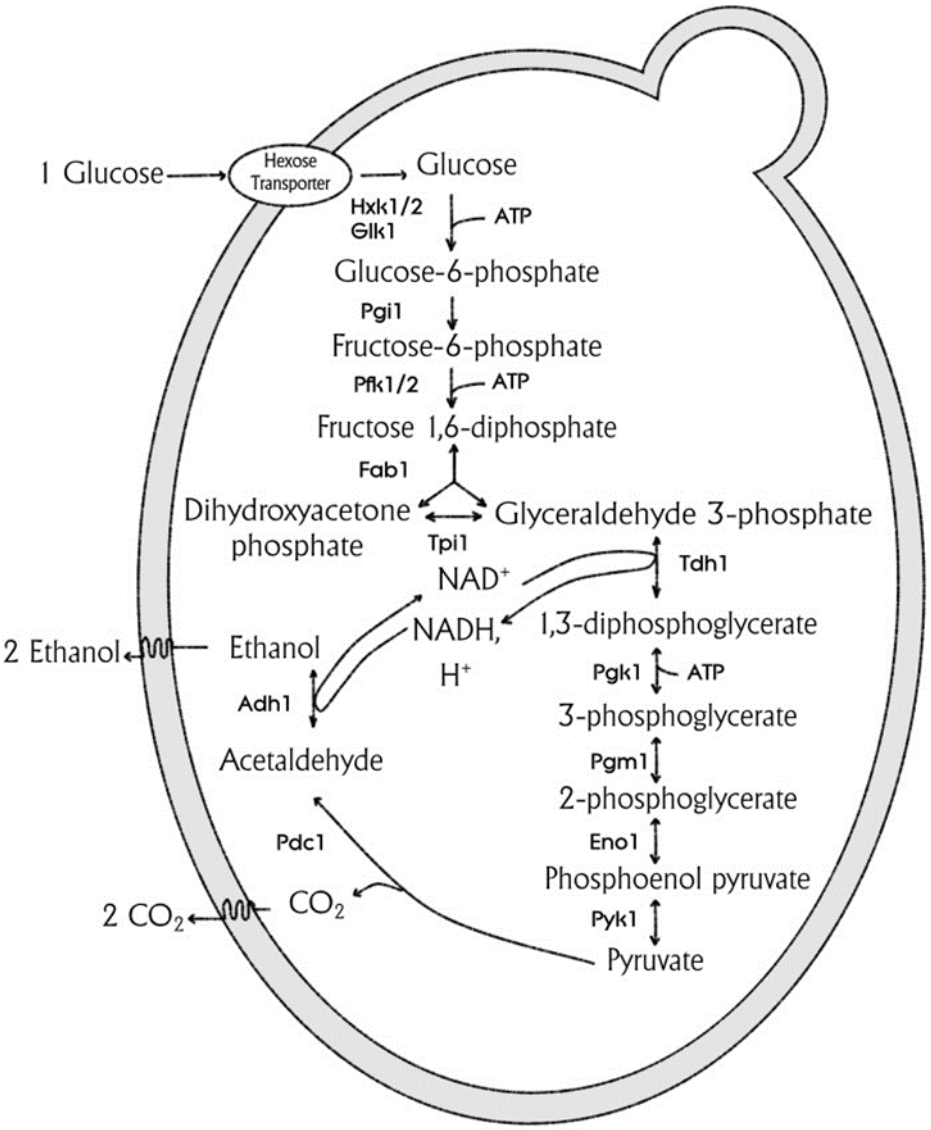

Figure 2.1 Glycolitic pathway in wine yeast

Therefore, the alcoholic fermentation is not only the simple transformation of sugars into ethanol, on the contrary, it is a very complex process that allows us to obtain a very pleasant beverage. It represents the transformation of sugars mainly into ethanol but also in other subproducts, which can contribute positively or negatively to sensory quality.

2.3 Yeasts selection

Criteria for the selection and development of yeasts strain for wine, can be considered under three categories: (1) properties that affect the performance of the fermentation process, (2) fermentation properties that determine wine quality and character and (3) properties associated with the commercial production of wine yeasts (Degre 1993; Rainieri & Pretorius 2000; Mannazzu et al., 2002; Pretorius & Bauer 2002; Bisson 2004; Schuller & Casal 2005). Within each category, there are properties of varying degrees of significance and importance, some being essential and some being desirable.

Fast, vigorous and complete fermentation of grape juice sugars to high ethanol concentrations are essential requirements of wine yeasts. The yeast should be tolerant at the sulphur dioxide concentrations added usually to the juice as an antioxidant and antimicrobial, exhibit uniform dispersion and mixing throughout the fermenting grapes juice, produce minimal foam and to sediment quickly from the wine at the end of fermentation. These processing properties should be well expressed at low temperatures (e.g. 15 °C) for white wine fermentations and at higher temperatures (e.g. 25 °C) for red wine fermentations. It is most important that the yeast does not give slow, sluggish or stuck fermentations (Bisson, 1999). Respect to wine quality and character, it is essential that any yeast produces a balanced array of flavour metabolites, without undesirable excesses of volatiles compounds such as acetic acid, ethyl acetate, hydrogen sulphide and sulphur dioxide. It should not give undesirable autolytic flavours after fermentation. It should not adversely affect wine colour or its tannic characteristics. In essence, the yeast must give a wine with a good clean flavour, free of sensory faults, and allow the grape varietal character to be perceived by the consumer (Lambrechts & Pretorius, 2000; Bisson, 2004; Swiegers et al., 2005) (Table 2.1).

Companies that produce yeasts for the wine industry also have basic needs that must be built into the selection and development process (Degre, 1993). The cost of production needs to be contained so that the final product is affordable to the wine industry. Consequently, the yeast must be amenable to large-scale cultivation on relatively inexpensive substrates such as molasses. Subsequently, it needs to be tolerant of the stresses of drying, packaging, storage and, finally, rehydration and reactivation by the winemaker (Soubeyrand et al., 2006). These requirements need to be achieved without loss of the essential and desirable winemaking properties. Although technologies are well established for the selection, development and production of wine yeasts with good basic oenological criteria, there are increasing environmental pressures for a wine industry that is more efficient and more sustainable, and there are increasing demands from consumers for wines with more distinctive and specific styles, including those with a healthier appeal (e.g. less ethanol, increased antioxidant levels) (Bisson et al., 2002; Bisson, 2004). These directions require a more strategic approach to the development of wine yeasts than in the past. For this purpose, a desired wine attribute is identified, and the property to give that quality is designed into the yeast selection and development process, without compromising the essential oenological criteria already mentioned. Pretorius and colleagues (Pretorius & Bauer, 2002; Pretorius & Hoj, 2005; Verstrepen et al., 2006) and Bisson (2004) broadly describe these developmental targets under five categories. These are, with some examples, as follows:

(1) Improved fermentation performance (e.g. yeasts with greater efficiency in sugar and nitrogen utilization, increased ethanol tolerance, decreased foam production). (2) Improved process efficiency (e.g. yeasts with greater production of extracellular enzymes such as proteases, glucanases and pectinases to facilitate wine clarification; yeasts with altered surface properties to enhance cell sedimentation, floatation and flor formation, as needed; and yeasts that conduct combined alcoholic-malolactic fermentations). (3) Improved control of wine spoilage microorganisms (e.g. yeasts producing lysozyme, bacteriocins and sulphur dioxide that restrict spoilage bacteria). (4) Improved wine wholesomeness (e.g. yeasts that give less ethanol, decreased formation of ethyl carbamate and biogenic amines, increased production of resveratrol and antioxidants). (5) Improved wine sensory quality (e.g. yeasts that give increased release of grape terpenoids and volatile thiols, increased glycerol and desirable esters, increased or decreased acidity and optimized impact on grape phenolics).

2.3.1 Sources of new wine yeasts

During the past 50–75 years, wine production has been transformed into a modern, industrialized process, largely based around the activities of only two yeast species: *S. cerevisiae* and *S bayanus*. Future developments will continue to be based on innovation with these species, but opportunities for innovation using other species of yeasts cannot be overlooked. As mentioned already, various species of *Hanseniaspora*, *Kloeckera*, *Metschnikowia*, *Candida*, *Kluyveromyces* and *Pichia* play significant roles in the early stages of most wine fermentations, and there is increasing interest in more strategic exploitation of these species as novel starter cultures (Ciani & Maccarelli, 1998; Heard, 1999; Ciani et al., 2002; Jolly et al., 2003b).

Essentially, there are two strategies for obtaining new strains of wine yeasts for development as commercial starter cultures: (1) isolation from natural sources and (2) genetic improvement of natural isolates. Once a prospective isolate has been obtained, it is screened in laboratory trials for essential oenological criteria as mentioned already. Isolates meeting acceptable criteria are then used in micro-scale wine fermentations and the resulting wines are

then subjected to sensory evaluation. Strains giving good fermentation criteria and acceptable-quality wines under these conditions are then selected for further development as starter culture preparations.

2.3.2 Natural sources

Generally, wine yeasts for starter culture development have been sourced from two ecological habitats, namely, the vineyard (primarily the grapes) and spontaneous or natural fermentations that have given wines of acceptable or unique quality. The yeasts are part of the natural microbial communities of grapes (Fleet et al., 2002). Understandably, therefore, grapes are always considered a potential source of new wine yeasts. Moreover, there is an attraction that unique strains of yeasts will be associated with particular grape varieties in specific geographical locations and, through this association, they could introduce significant diversity and regional character or 'terroir' into the winemaking process (Vezinhet et al., 1992; Pretorius et al., 1999; Martinez et al., 2007; Raspor et al., 2006; Valero et al., 2007). Thus, in the interests of preserving biodiversity and regional influence on wine character, grapes of the region would represent an important source of yeasts for starter culture development. Yeasts associated with grape berries have been studied for over 100 years (reviewed in Fleet et al., 2002), although more detailed studies are needed to obtain a clearer understanding of their ecology and factors that affect this ecology. The yeast species and populations evolve as the grape berry matures on the vine and are influenced by climatic conditions such as temperature and rainfall, application of agrichemicals and physical damage by wind, hail and attack by insects, birds and animals. The predominant epiphytic flora isolated from grapes at the time of harvest for winemaking display low-fermentative and rarely fermentative characteristics, it composed by mostly species belonging to *Hanseniaspora*, *Kloeckera*, *Candida*, *Metschnikowia*, *Pichia* and *Kluyveromyces* genera, although this data are not always accepted (Martini et al., 1996; Prakitchaiwattana et al., 2004; Renouf et al., 2005, 2007; Raspor et al., 2006; Barata et al., 2008). If the berries are over-ripe, become damaged or are infected with filamentous fungi (mould), the yeast populations tend to be higher and include a greater incidence of fermentative species such as those of *Saccharomyces, Zygosaccharomyces, Saccharomycodes* and *Zygoascus* (Barata et al., 2008; Nisiotou et al., 2007). Consequently, grapes will be a very good source of non-*Saccharomyces* yeasts, should there be a future direction for using such species as novel starters in wine fermentations. It is difficult to isolate *Saccharomyces* species from mature, undamaged grapes by direct culture on agar media, but they are frequently found by enrichment culture methods, suggesting their presence in very low numbers. While, if the grape berries are aseptically harvested from vines, crushed and left to ferment, strains of *S. cerevisiae* and *S. bayanus* would be easily isolated from the fully fermented must (Vezinhet et al., 1992; Khan et al., 2000; van der Westhuizen et al., 2000; Demuyter et al., 2004; Schuller et al., 2005; Mercado et al., 2007; Valero et al., 2007).

However, recovery of *Saccharomyces* species from such ferments is not always consistent and can be determined by many factors that are likely to affect the presence and survival of yeasts on the grape surface, such as amount of rainfall, temperature and agronomic applications.

Wines that have undergone a successful natural fermentation have been a main source of yeasts for development as starter cultures. In the past, the focus has been to isolate suitable strains of *S. cerevisiae* and *S. bayanus*, but these wines will also be a good reservoir of non-*Saccharomyces* species. The precise origin of the indigenous strains of *Saccharomyces* in these wine fermentations has been a question of much debate and controversy (Martini et al., 1996; Mortimer & Polsinelli, 1999). Clearly, the grape itself is a primary source of the yeasts that occur in the grapes juice and it is logical to conclude that any *Saccharomyces* strains from this source would be prominent in the final fermentation. However, processing of the grapes juice and its transfer to fermentation tanks contributes added microbial communities. These microbial communities originate as contamination from the surfaces of winery equipment and are widely considered to be "residential flora" that have built up in the winery over time, through a process of adaptation and selection, despite cleaning and sanitation operations. These flora are dominated by fermenting ethanol-tolerant yeast species such as *S. cerevisiae* and *S. bayanus* because of the selective conditions presented by the properties of fermenting grape juice. Many researchers consider winery flora to be the main source of the diversity in *Saccharomyces* strains, involved in grape juice fermentations and, in recent years, this has been demonstrated using molecular techniques to track strain origin and development. Strains isolated from winery equipment, in addition to any inoculated strain, are found in the fermenting wine, and similar strains can be found in the one winery in consecutive years, suggesting a carry-over from a year to the next (Constanti et al., 1997; Gutierrez et al., 1999; Cocolin et al., 2004; Santamaria et al., 2005; Mercado et al., 2007). Presumably, the *Saccharomyces* flora in the winery originally came from grapes and evolved with time. The source of *Saccharomyces* yeasts on the grapes is still a mystery, but contamination from insects in the vineyard is thought to be a likely possibility (Mortimer & Polsinelli, 1999; Fleet et al., 2002).

Table 2.1 Oenological characteristics to be considered in the selection of wine strains (adapted from Schuller and Casal, 2005)

Oenoligical characteristics	Comment	Desirable
Fermentation vigour	Maximum amount of ethanol (%, v/v) produced at the end of fermentation.	Good ethanol production and tolerance. Complete fermentation of sugars.
Fermentation rate	Grams of CO2 produced during the first 48 h of fermentation.	Prompt fermentation initiation. Minimization of lag phase.
Mode of growth in liquid medium	Dispersed or flocculent growth. Sedimentation speed.	Dispersed yeast growth during, but sedimentation at the end of fermentation.
Foam production	Height of foam produced during fermentation.	Increased foam production.
Optimum fermentation temperature	Thermotolerance and cryotolerance is related to oenological properties.	Optimum fermentation temperature ranges between 18 and 28 ºC. Capacity of fermentation at low temperatures
Volatile acidity	Mainly produced by acetic acid.	Strains should not release more than 100-400 mg/L during fermentation.
Introduction Malic acid degradation or production		Depends on the characteristics of the must.
Glycerol production	Contributing to wine sweetness, body and fullness.	Production between 5-8 g/L.
Esters, higher alcohols and Volatile compounds	Contribute positively to global sensorial characteristics.	Desirable metabolites which influence wine flavour and depend on the presence of precursors.
SO_2 tolerance and production	Antioxidant and antimicrobial agent.	High fermentation capacity in the presence of SO_2.
H2S production	Detrimental to wine quality with very low threshold value.	No production
Cooper resistance	High cooper concentrations may cause stuck fermentations.	High cooper resistance and the ability to reduce the cooper content.
Stress resistance	Several stress during fermentation.	Tolerance to different stress in the same strain

2.4 Controlled fermentation with mixed culture of yeasts

Mixed fermentations using controlled inoculation of *Saccharomyces cerevisiae* starter cultures and non-*Saccharomyces* yeasts represent a feasible way towards improving the complexity and enhancing the particular and specific characteristics of wines. The profusion of selected starter cultures has allowed the more widespread use of inoculated fermentations, with consequent improvements to the control of the fermentation process, and the use of new biotechnological processes in winemaking. Over the last few years, as a consequence of the re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking, there have been several studies that have evaluated the use of controlled mixed fermentations using *Saccharomyces* and different non-*Saccharomyces* yeast species from the wine environment. The combined use of different species often results in unpredictable compounds and/or different levels of fermentation products being produced, which can affect both the chemical and the aromatic composition of wines. Moreover, possible synergistic interactions between different yeasts might provide a tool for the implementation of new fermentation technologies (Ciani et al 2010). At present, it is known that the yeast ecology of the fermentation process is more complex than previously thought, and that non-

Saccharomyces yeast species play relevant roles in the metabolic impact and aroma complexity of the final product. In recent years, there has been a growing demand for new and improved wine-yeast strains that are adapted to different types and styles of wines. In this context, to improve the chemical composition and sensory properties of wine, the inclusion of non-*Saccharomyces* wine yeasts, together with *Saccharomyces* strains as part of mixed and multistarter fermentations, has been proposed as a tool to take advantage of spontaneous fermentation, but avoiding the risks of stuck fermentations (Bisson & Kunkee, 1993; Heard, 1999; Ciani et al., 2006;).

Pure culture fermentations with non-*Saccharomyces* wine yeasts have shown several negative metabolite and fermentation characteristic that generally exclude their use as starter cultures. The most important spoilage metabolites produced by non-*Saccharomyces* wine yeasts are acetic acid, acetaldehyde, acetoin and ethyl acetate, along with off-odours, such as the vinyl and ethyl phenols that are linked to the development of *Brettanomyces/Dekkera* spp. (Chatonnet et al., 1995). Moreover, most of the non-*Saccharomyces* wine-related species show limited fermentation aptitudes, such as low fermentation power and a low SO2 resistance. However, in mixed fermentations such as natural fermentations, some negative enological characteristic of non-*Saccharomyces* yeasts may not be expressed or be modified by *S. cerevisiae* cultures. In this context, following the investigations of the last decades on the quantitative presence and persistence of non-*Saccharomyces* wine yeasts during fermentation, several studies have been carried out to determine their oenological properties and their possible roles in winemaking (Romano et al., 1992, 1997; Ciani & Picciotti, 1995; Lema et al., 1996; Ciani & Maccarelli, 1998; Egli et al., 1998; Fleet, 2003; Jolly et al., 2003; Farkas et al., 2005; Hermle et al., 2005; Domizio et al., 2007; Kim et al., 2008; Viana et al., 2008). Experimental evidence has highlighted the positive role of non-*Saccharomyces* yeasts in the analytical composition of wine (Cabrera et al., 1988; Herraiz et al., 1990; Moreno et al., 1991; Lema et al., 1996). Some non-*Saccharomyces* yeast species can improve the fermentation behavior of yeast starter cultures and the analytical composition of wine, or lead to a more complex aroma.

In this context, the enzymatic activities of non-*Saccharomyces* wine yeasts can influence the wine profile. Investigations on the biocatalytic activity widely associated with non-*Saccharomyces* wine yeasts is β-glucosidase activity. β-Glucosidase hydrolyses terpenyl-glycosides, and can enhance the wine aroma. In contrast to grape glucosidase, β-glucosidase produced by yeast is not inhibited by glucose, and it is involved in the release of terpenols during fermentation. This β-glucosidase activity has been found in several yeast species associated with winemaking, especially among the non-*Saccharomyce*s species (Vasserot et al., 1989; Günata et al., 1990; Manzanares et al., 1999; Ferreira et al., 2001; Rodriguez et al., 2004; Fia et al., 2005; Gonzàlez-Pombo et al., 2008). The diffusion of this activity among non-*Saccharomyces* wine yeasts has confirmed the role of these yeasts in enhancing wine aroma (Manzanares et al., 1999; Ferreira et al., 2001; Gonzàlez-Pombo et al., 2008).

In addition to the enzymatic activities of non-*Saccharomyces* wine yeasts, other specific properties of winemaking interest have been evaluated to improve our knowledge of the metabolic characteristics, and to test the intraspecific variability of these wine yeasts. Non-*Saccharomyces* strains can be selected on the basis of their ability to produce favourable metabolites that contribute to the definition of the final bouquet of a wine. Viana et al. (2008) screened 38 yeast strains belonging to the *Candida*, *Hanseniaspora*, *Pichia*, *Torulaspora* and *Zygosaccharomyces* genera for acetate ester formation. Here, they identified *Hanseniaspora osmophila* as a good candidate for mixed cultures, due to its glucophilic nature, the ability to produce acetaldehyde within a range compatible for wine and acetate ester production, in particular of 2-phenylethyl acetate. Also, Moreira et al. (2008) investigated the role of *H. guilliermondii* and *Hanseniaspora uvarum* in pure and mixed starter cultures with *S. cerevisiae*, for the production of heavy sulphur compounds and esters. Their results highlight that these apiculate yeasts enhance the production of desirable compounds, such as esters, without increasing the undesirable heavy sulphur compounds.

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3 CHEMICAL COMPOSITION OF GRAPE AND WINE

From an enological point of view, the term “wine” is defined as “the drink resulting from the fermentation by the yeast-cells, and also in certain cases by the cells of lactic bacteria, of the juice from the crushing or maceration of grape-cells” (Peynaud 1984). Of the grape genus *Vitis*, the species *V. vinifera* is often cultivated for wine production.

3.1 Chemical composition of grape must

Freshly expressed grape must consists of 70 to 80% water and many dissolved solids. These soluble solids include numerous organic and inorganic compounds. The important groups of compounds, from the winemaking point of view, are the following:

3.1.1 Sugars

In grapes, a large portion of the soluble solids are sugars, mainly glucose and fructose in equal amounts. The sugar content of the must of ripe grapes varies between 150 to 250 g/L. During the course of fermentation, yeasts convert fermentable sugars (glucose and fructose) into alcohol and carbon dioxide. The amount of alcohol produced is related to the amount of sugar initially present in the must; thus, by controlling the amount of sugar in the juice, it is possible to control the amount of alcohol in the resulting wine.

3.1.2 Organic acids

Organic acids are the most abundant solids present in grape juice. They are responsible for the tart taste and have a markedinfluence on wine stability, color, and pH. The principal organic acids found in grapes are tartaric, malic, and to a small extent, citric. Many other organic acids are also found in must and wines, but tartaric and malic acid account for over 90% of the total acids present. During the early period of berry growth, concentration of both acids increases in the fruit. With the onset of ripening, sugars accumulate in the fruit and acids concentration decreases. Generally the reduction in malic acid is greater, and consequently, at maturity, the fruit contains more tartaric acid than malic. Tartaric acid is present as free acid and as a salt, such as potassium bitartrate, which is an important constituent affecting pH and cold stability of wine. Acid composition of grapes is influenced by many factors such as variety, climatic region, and cultural practices. Generally in ripe grapes, the acid levels are lower in a warm climatic region than in a cold region. The acidity is expressed as titratable acidity (TA), which is an important parameter used in quality evaluation of must and wine. Acid content of the juice has importance on must and wine pH. However, the relationship is neither direct nor predictable due to the presence of various kinds of acids and their salts. Understanding the role of pH in winemaking is crucial to make good wines.

3.1.3 Phenolic compounds

Phenolic compounds are important constituents of grapes and wines. They are the most abundant constituents present in grapes after sugars and acids. Phenolic compounds are a group of substances structurally diverse and present in different amounts. They play a vital role in determining the wine's color and flavor. They are involved in browning reactions in grapes and wines and also play a key role in the aging and maturation of wines. Phenolic substances are primarily located in seeds and skins of the berry. As white wines are usually produced from must with low skin and seed contact, their phenolic content is low (100-250 mg/L gallic acid equivalent, GAE). On the contrary, red wines are commonly produced with skin and seed contact. Depending on the contact time, the phenolic content of a red wine generally varies between 1000 to 3500 mg/L GAE. The two main substances included in this group of compounds are anthocyanins and tannins. Anthocyanins are pigments responsible for the red and purple color of the grapes and wines. They exist in both colored and colorless forms. In young red wines, most of the colored anthocyanins are present in free (uncombined) forms. As the red wine ages, the anthocyanins combine with other phenolic compounds. In a combined state, the pigment contributes to color stability. Tannins are very large and complex compounds with a molecular weight over 500. They are yellow, brown, and red colored and give astringency and bitterness. During processing and aging, the tannins polymerize, increasing their molecular size, being astringency more perceived than bitterness. However, increase in molecular size also makes these compounds insoluble, precipitating and causing decrease of wine´s astringency.

3.1.4 Nitrogenous compounds

Grapes contain ammonium cations and organic nitrogenous compounds, such as amino acids, peptides, and proteins. Nitrogen content of the grapes depends on grape variety, climate, soil, fertilization, and other cultural practices. Total nitrogen concentration of the fruit increases during maturation period. Nitrogen containing compounds are important because they serve as the nutrient for yeast and lactic acid bacteria, influencing biomass formation, fermentation rate, and byproducts synthesis, thus affecting sensory wine attributes. Proteins are involved in wine stability. In addition, insufficient nitrogen in must can cause sluggish or stuck fermentations and H_2S formation ("rotten egg" odour). To avoid this problem, must is often supplemented with diammonium phosphate (DAP).

3.1.5 Aroma compounds

Aromatic compounds in grapes are largely present in the skin and the layers of cells immediately beneath it. Their concentration tends to increase during ripening. It is important that the grapes be harvested when the flavor is at its peak. Many factors affect the concentration of aroma compounds in grapes. Manipulation and control of these factors is necessary for attaining the desired flavor level at harvest.

3.1.6 Minerals

Minerals are taken up by the vine from the soil. They usually constitute approximately from 0.2 to 0.6% of the fresh weight of the fruit. The main mineral compounds include: potassium, sodium, iron, phosphates, sulfate, and chloride, being potassium the most important (50-70% of must cations). During ripening, potassium grape content increases. The formation of potassium bitartrate reduces acidity and increases pH of the must, but this salt is involved in wine instability problems.

3.1.7 Pectic substances

Pectin substances are cementing agents present in the cell wall. Chemically, these compounds are complex polysaccharides made of galacturonic acid molecules linked together. During ripening, pectin is hydrolyzed by naturally occurring pectolytic enzymes, softening the berry. In must, pectin causes turbidity by holding the particles of fruit pulp in suspension. To allow the suspended solids to settle and clarify the juice, commercial preparations of pectolytic enzymes are often used.

3.2 Main metabolites in wine

Wine is constituted by chemical compounds whose principal origin is grapes and yeast alcoholic fermentation.The main component of this alcoholic beverage is water, which constitutes about 85%. In addition, several compounds also can be found in wines:

3.2.1 Ethanol

Ethanol is one of the main compounds synthesized in wine fermentation. This compound decreases wine aroma and flavor perception by increasing aromatic compounds solubility in the wine and lowering the volatile fraction (Ferreira, 2007). Furthermore, in the last years, there is an increasing demand to produce wines with lower ethanol contents, due to its negative effects on health (Zhang et al., 2011).

3.2.2 Glycerol

Glycerol is involved in osmoregulation (Ansell et al., 1997; Nevoigt & Stahl, 1997) and in low-temperature tolerance in yeasts (Izawa et al., 2004). The amount of glycerol produced by *S. cerevisiae* in wines is typically between 4 and 9 g/L, with average values approximately of 7 g/L (Scanes et al., 1998). The oenological importance of glycerol lies in its contribution to wine quality by providing slight sweetness, smoothness and fullness, reducing wine astringency (Ishikawa and Noble, 1995; Remize et al., 2000).

3.2.3 Organic acids

Organic acids constitute about 1% of wine. Tartaric acid is the major acid that is derived from grapes. Volatile acids like acetic, succinic, lactic, etc. are produced during fermentation. Acetic acid is the main volatile acid of wine, and its presence at high concentrations gives a vinegar odour and a disagreeable sensation in mouth. Acetic

acid can be produced by yeast, lactic acid bacteria and acetic acid bacteria. *Saccharomyces cerevisiae* only produce small quantities of this compound in fermentation (0.1-0.3 g/L). Conversely, stuck and sluggish fermentations can generate large amounts of this acid due to high production by yeasts or lactic disease development (Zamora, 2009). Besides, there are other organic acids such as tartaric, malic, lactic, succinic and citric acids.

3.2.4 Acetaldehyde

Acetaldehyde, also called ethanal, is an intermediary of alcoholic fermentation obtained by the decarboxylation of pyruvate. Later on, acetaldehyde is reduced to ethanol although a little quantity always remains in the wine. Excessive acetaldehyde amount contributes to perception of oxidation in wine, although in some wines such as Fino and Manzanilla from Jerez high quantity of this compound is desirable (Zamora, 2009).

3.2.5 Sugars

Sweet wines have about 10% sugar content whereas dry wines have only about 0.1%. Sugars are the responsible for the wine sweetness.

3.2.6 Minerals

Minerals constitute from 0.2 to 0.4% of wine. Common mineral salts that are found in wines are sodium, potassium, magnesium, calcium, and iron.

3.2.7 Sulfites

Sulfites are added in winemaking process to achieve sterilization and wine preservation.

3.2.8 Phenolic compounds

Phenols form about 0.01 to 0.5% of wine. The main phenolic compounds are anthocyanins and tannins, which give red color and astringency to wines, respectively.

3.2.9 Aromatic compounds

Alcohols, esters, volatile organic acid and to lesser extent aldehydes, constitute the main compounds of secondary aroma

3.3 Wine Aroma

Differences in wine aroma and flavor are e primary reason that people seek out, enjoy and pay sometimes large amounts for particular wines. The aroma and flavor properties of wine express important regional, varietal and stylistic differences, and create interest and attractiveness of wines produced worldwide. Wine aroma is made up of several hundreds of volatile compounds, in concentrations ranging from several mg/L to a few ng/L, or even less (Schreier, 1979; Nykänen, 1986; Ebeler, 2001). Wine aroma is derived from multiple sources and processes, including: i) grape metabolism, depending on grape variety, climate, soil and vineyard management techniques; ii) biochemical phenomena (oxidation and hydrolysis) occurring prior to fermentation, during extraction of the juice and maceration; iii) metabolic activity of the microorganisms responsible for alcoholic and malolactic fermentations, and iv) chemical or enzymatic reactions that occur after fermentation, particularly during ageing in vat, barrel or bottle (Swiegers et al., 2005; Ribéreau-Gayon et al., 2000a,b; Boulton et al., 1998).

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

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

- secondary or fermentative 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 and formed during the alcoholic fermentation;

- tertiary aroma or bouquet: caused by chemical reactions during maturation of the wine.

3.3.1 Primary Aroma, grape-derived aroma

Primary or varietal aroma is constituted by different chemical compounds whose origin is grapes. These chemical compounds can be in two forms:

- Free aromatic molecules: methoxypyrazines, varietal thiols and monoterpenes (Loscos, 2009)
- Precursors: unsaturated fatty acids, phenolic acids, S-cysteine conjugates, dimethylsulfide precursors, carotenoids and glycoconjugates (Baumes, 2009; Loscos, 2009)

Grape derived aroma compounds are produced in the grape berry during development and generally contribute varietal differences to wine. The factors that influence wine aroma compounds formation for grape-derived compounds include nutrient levels in the soil, water availability, climatic conditions, sunlight exposure and the balance of vegetative and fruit growth (Reynolds & Vanden Heuvel 2009; Marais et al., 1999). The grape-derived components responsible for the primary aroma or varietal character of wines are predominantly localized in the exocarp (skin) tissue. The majority of these compounds are stored as sugar or aminoacid conjugates in the exocarp cell vacuoles, and some are present as free volatiles. The compounds stored as conjugates in the vacuoles of exocarp cells are released through the action of glycosidases and peptidases introduced at the time of crushing and pressing as well as during fermentation, thereby increasing the amounts available for perception in the finished wine (Lund & Bohlmann, 2006). These compounds include terpenes, methoxypyrazine, norisoprenoids and thiols (figure 3.1)

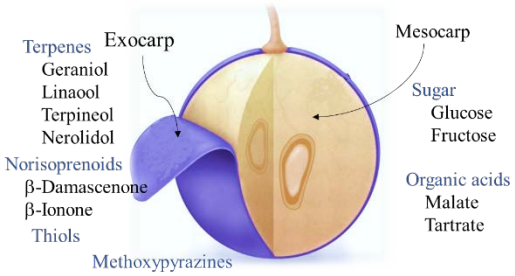


Figura 3.1 Schematic diagram of some grape-derived compounds and their location in the grape berry. Modified from Pretorius (2006)

3.3.1.1 Terpenes

Monoterpenes and sesquiterpenes are biologically synthesized from isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). These precursors are formed either through the cytosolic mevalonic-acid (MVA) pathway from three molecules of acetyl-CoA (Newman & Chappell, 1999) or through the plastidial 2-C-methylerythritol-4-phosphate (MEP) pathway from pyruvate and glyceraldehyde-3-phosphate (Rohmer, 1999). Monoterpenes are subsequently formed from 2E-geranyl diphosphate (GPP) and sesquiterpenes are formed from 6E-farnesyl diphosphate (FPP) through the action of terpene synthases (TPS) (figure 3.2) (Lücker et al., 2004; Martin et al., 2010).

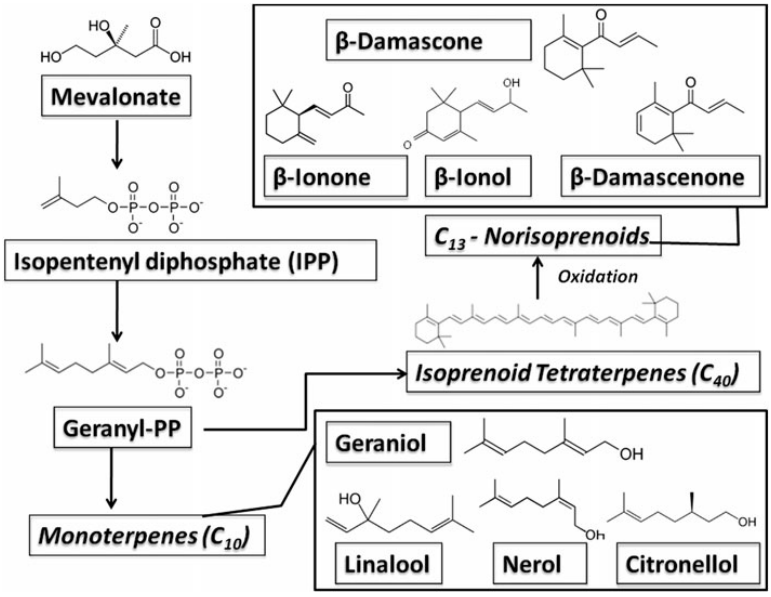


Figure 3.2 Formation of the most important chemical compounds responsible for varietal aroma in wine. Both monoterpens and C13-norisoprenoids are formed from the precursor mevalonate, itself a metabolite of acetyl-CoA. (Figure adapted from Styger et al.,2011)

Floral, rose-like aroma in Muscat varieties (Muscat d'Alexandrie, Muscat de Frontignan)) and aromatic non-Muscaat varietis (Weisser Riesling, Bukettraube, Gewürztraminer, Fernao Pires, and Scheurebe) is due mainly to the presence of monoterpenes, such as linalool, geraniol, nerol, α-terpineol, and hotrienol, occurring in complex combinations (Marais, 1983). Differences in the concentrations depend on the cultivar, climatic conditions, grape maturity, pH, enzymes, storage time, extraction, and wine-making procedures (Marais, 1983).
Sesquiterpenes have gained little attention with repsect to grape and wine analysis, with only three major studies reporting multiple sesquiterpenes in Riesling, Traminer, Ruländer, Müller-Thurgau, Scheurebe, Optima, Rieslaner, Baga, and Shiraz grape varieties (Coelho et al., 2006; Parker et al., 2007). The sesquiterpene α-ylangene was identified as a candidate marker of pepper character in Australian Shiraz wines, but its aroma contribution to wine could not be confirmed (Parker et al., 2007). The sesquiterpene rotundone was subsequently identified as the potent aroma- impact compound responsible for the black pepper aroma in wines produced from Vitis vinifera cv. Shiraz (Siebert et al., 2008; Wood et al., 2008) as well as in a number of other plants including black pepper (Piper nigrum), marjoram (Origanum majorana), oregano (Origanum vulgare), geranium (Pelargonium alchemilloides), nut grass (Cyperus rotundus), rosemary (Rosmarinus officinalis), saltbush (Atriplex cinerea), basil (Ocimum basilicum), and thyme (Thymus vulgaris) (Wood et al., 2008).
Clearly, terpenes and sesquiterpenes play important roles in a number of different winegrape varieties. With continuing discoveries of important new compounds, such as rotundone, it is clear that this group of compounds will continue to be a focus of wine aroma research into the future (Robinson et al., 2014).

3.3.1.2 Norisoprenoids

Norisoprenoids (or apocarotenoids) are derived from carotenoids, are found commonly in nature, and have attracted considerable attention as odorants in many food and fragrance products (Baumes et al., 2002; Winterhalter & Rouseff, 2002; Winterhalter & Ebeler, 2013). They consist of a megastigmane carbon skeleton and differ in the position of the oxygen functional group, being either absent (megastigmanes), attached to carbon 7 (damascones), or attached to carbon 9 (ionones) (Winterhalter & Rouseff, 2002). Norisoprenoids are ubiquitous among grape cultivars, although they are most abundant in aromatic cultivars (Strauss et al., 1987; Winterhalter et al., 1990a; Marais et al., 1992; Schneider et al., 2001), and they are thought to play an important role in the aroma of many wine varieties including Semillon, Sauvignon blanc, Chardonnay, Merlot, Syrah, and Cabernet Sauvignon (Razungles et al., 1993; Sefton et al. 1993, 1994, 1996; Sefton 1998). These compounds are responsible for the typical aroma of some grape varieties. They are also important in both white and red wines because of very low odor thresholds (Aznar et al. 2004; Ferreira et al. 2000; Guth, 1997). Winterhalter et al. (1999) showed that norisoprenoids in Riesling wines originated from several precursors and were stored as glycoconjugates.

Enzymatic oxidation and cleavage of β-carotene (and other carotenoids) during crushing of grapes and in bottle aging are thought to produce a diverse group of norisoprenoids (Ribereau-Gayon et al., 2000). These compounds include β-ionone (aroma of viola), damascenone (aroma of exotic fruits), β-damascone (aroma of rose and fruits), β-ionol (aroma of fruit and flowers), 3-oxo-β-ionone (tobacco smell), and vitispirane (aroma of fresher flowery-fruity and/or exotic flowers and earthy-woody undertone) among others, as reviewed by Mendes-Pinto (2009). Additional important aroma active norisoprenoids in wine include 1,1,6-trimethyl-l,2-dihydronaphthalene (TDN), associated with the kerosene bottle-aged character of Riesling wines (Simpson, 1979; Winterhalter et al., 1990b), and (E)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB), which may be associated with the floral, geranium, and tobacco characters of aged Semillon wines (Janusz et al., 2003; Cox et al., 2005).

3.3.1.3 Methoxypyrazine

The methoxypyrazines have low aroma detection thresholds (in the parts per trillion range) and generally impart "green" characteristics to wine (Allen & Lacey 1999). 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, depending on variety, maturity and growing conditions. High levels (>15 ng/L in white wines, >25 ng/L 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).

3.3.1.4 Volatile thiol compounds

The volatile thiol compounds 4-mercapto-4-methylpentan-2-one (4MMP) and 3-mercaptohexan-1-ol (3MH) exist in the grape berry bound to the aminoacids, L-cysteine and glutathione (Subileau et al., 2008a) and the volatile thiol 3-mercaptohexyl acetate (3MHA) is the product of the 3MH esterification by yeasts (Swiegers at al., 2009). The commons descriptros for these volatile compounds include "box tree", "grapefruit" and "passionfrutis". The descriptors substantially differ, however, depending on the concentration of these compounds in wine. At high concentration, these colatile thiols have been associated with "sweaty", "cat urine" or "sulfur-like" aromas (Dubourdieu et al., 2006; Howell et al., 2004). Therefore, the sensory properties conferred by these compounds may be perceived as either positive or negative, depending upon the concentration in wine and/or the sensitivity and expectations of the individual.

3.3.1.5 Glycosidic precursors

Synthesis of glycosidic precursors during grape ripening is affected by environmental temperature changes (Park et al., 1991) and solar exposition, having less synthesis with less solar exposition (Bureau et al., 2000a, 2000b). In general, aroma compounds and aromatic precursors are more concentrated in grape skins; therefore an increase in the time of contact between grape skins and must means an increase in the transference of these compounds to the must (Sánchez-Palomo et al., 2006; Tamborra et al., 2004; Castro Vázquez et al., 2002). Moreover, an increase in maceration time would conduct to colour enhancement, bitterness and astringency. Glycosidic precursors are a potential source of aromatic compounds, which can be released during winemaking or wine aging by biological or chemical action (Sefton et al., 1993, 1994; Sefton, 1998). Sometimes additional reactions are necessary to form an aromatic molecule (ex. β-damascenone, β-ionone, TDN, TPB, Riesling acetal and vitispirane). Enzymatic hydrolysis is quantitatively more important (Loscos et al., 2009), whereas acid hydrolysis has more qualitative importance (Francis et al., 1998; Sefton et al., 1993; Williams et al., 1989).

In wines, there are several diglycosides (Voirin et al., 1990; Williams et al., 1982):

- β-D-glucopyranose
- α-L-arabinofuranosyl-β-D-glucopyranose
- α-L-rhamnopyranosil-β-D-glucopyranose

- β-D-apiofuranosyl-β-D-glucopyranose

Enzymatic hydrolysis of diglycosides is carried out in two steps. First, the enzymatic activity of α-arabinosidase, α-rhamnosidase or β-apiosidase breaks the bond between the two sugars, and second, β-glucosidases release the aromatic molecule (Günata et al., 1988). However, enzymes able to release the aromatic molecule in only one step have been described (Günata et al., 1998). In winemaking there are several natural sources for glycosidases: grapes, yeasts and bacteria, however exogenous addition of commercial enzymes is also common. Glycosidase activity in yeasts, *Saccharomyces* and non-*Saccharomyces* has been described by several authors (Delcroix et al., 1994; Fleet, 2008; Günata et al., 1993; Mateo et al., 1997; Ugliano et al., 2006). Recent studies have demonstrated yeasts are capable of releasing aromatic molecules from glycosidic precursors in winemaking conditions (Hernández-Orte et al., 2008; Loscos et al., 2007; Ugliano et al., 2006). In addition to hydrolysis of glycosides of monoterpenes and other aromas, *Saccharomyces* yeasts are able to carry out terpenes biotransformation into other terpenes (Gramatica et al., 1982; King & Dickinson, 2000; Zea et al., 1995; Zoecklein et al., 1997).

3.3.2 Secondary or fermentative 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). The fermentation factors that influence wine aroma include solids level, fermentation vessel, type and amount of yeast and bacteria strains used to conduct fermentation, fermentation temperature, nutrient addition and mixing techniques (Swiegers et al., 2005, Ugliano et al., 2009). There are many more volatile compounds in wine than grape juice, as yeasts and bacteria produce hundreds of volatile compounds during fermentation that contribute to wine aroma. These include esters, alcohols, volatile fatty acids, carbonyl and sulphur compounds as is shown in figure 3.3 (Styger et al., 2011).

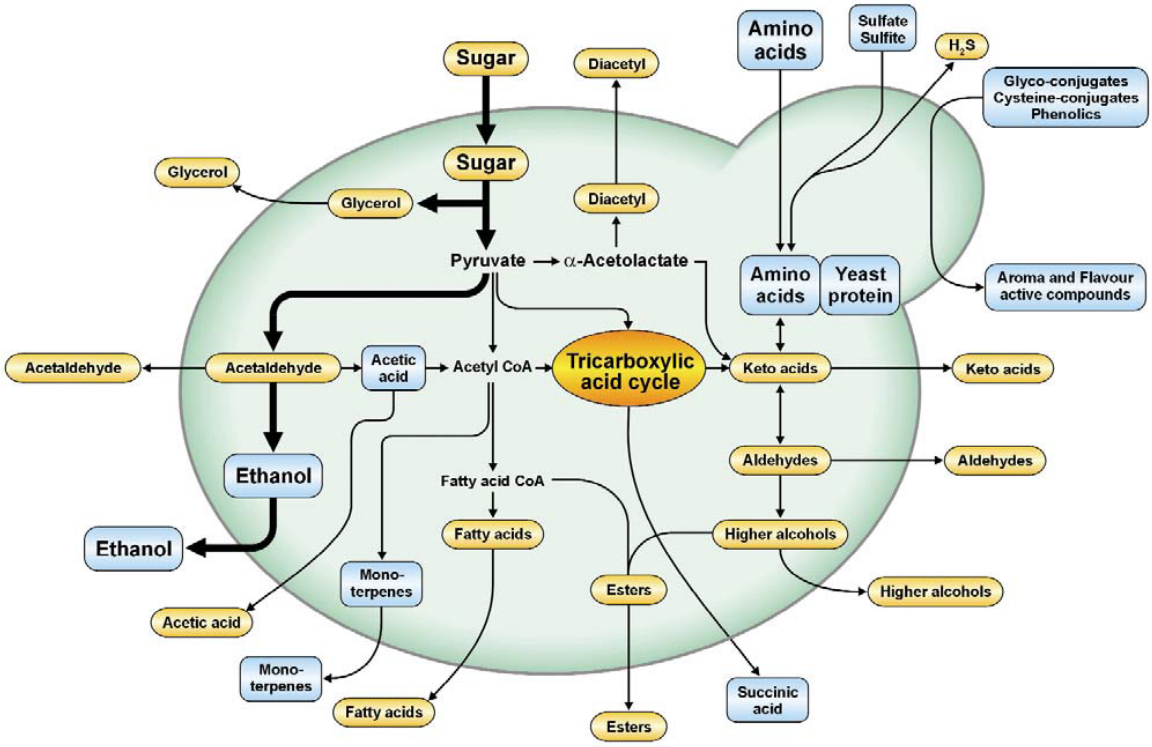


Figure 3.3. Schematic representation of derivation and synthesis of flavour–active compounds from sugar, amino acids and sulgur metabolism by wine yeast (Sweigers et al., 2005)

3.3.2.1 Volatile Acids

Yeasts produce short-(<6 carbons), medium-(6 to 12 carbons), and long chain (16 to 18 carbons) fatty acids a group of volatile organic acids that 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). Short-chain fatty acids that potentially contribute to wine flavor include the branched-chain isobutyric and isovaleric and the straight-chained butyric, and propanoic acids (Francis & Newton, 2005), but the role these compounds play in wine sensory characteristics has not been studied extensively. Isobutyric and isovaleric acids have been noted as markers of *Brettanomyces bruxellensis* spoilage and are thought to be capable of masking the ‘‘Brett character” attributed to 4-ethylphenol and 4-ethylguaiacol, which is somewhat counter intuitive (Romano et al., 2009), since the short-chain fatty acids have sweaty, cheesy-like aromas (Francis & Newton, 2005). The medium-chain fatty acids, hexanoic (C6), octanoic (C8), and decanoic (10), also contribute to wine aroma (Francis & Newton, 2005), and their concentrations are dependent on anaerobic growth conditions, must composition, grape cultivar, yeast strain, fermentation temperature, and winemaking practices (Edwards et al., 1990; Bardi et al., 1999). Medium chain fatty acids are correlated with stuck and sluggish fermentations, as they are inhibitory to *S. cerevisiae* and to some bacteria (Bisson, 1999). The inhibitory effect of medium-chain fatty acids usually occurs under conditions of low pH, low temperature, and high ethanol concentrations (Viegas & Sá Correia, 1995, 1997). However, another study has suggested that cell growth is arrested because fatty acid biosynthesis is prevented by the lack of oxygen and that elevated medium chain fatty acids are not the primary cause of stuck fermentation (Bardi et al., 1999).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 SO2 (Sonni et al., 2009). The total fatty acid concentration in wines samples was found to be around 15 - 25 mg/L (Lopez M 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 & 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, depending on the style of wine; the optimal concentration is 0.2–0.7 g/L (Corison et al., 1979). 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 and up to 2 g/L 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 (Henschke & Dixon, 1990, 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 (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).

3.3.2.2 Alcohols: Ethanol and higher alcohols

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 (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 (De Orduna, 2010). 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).

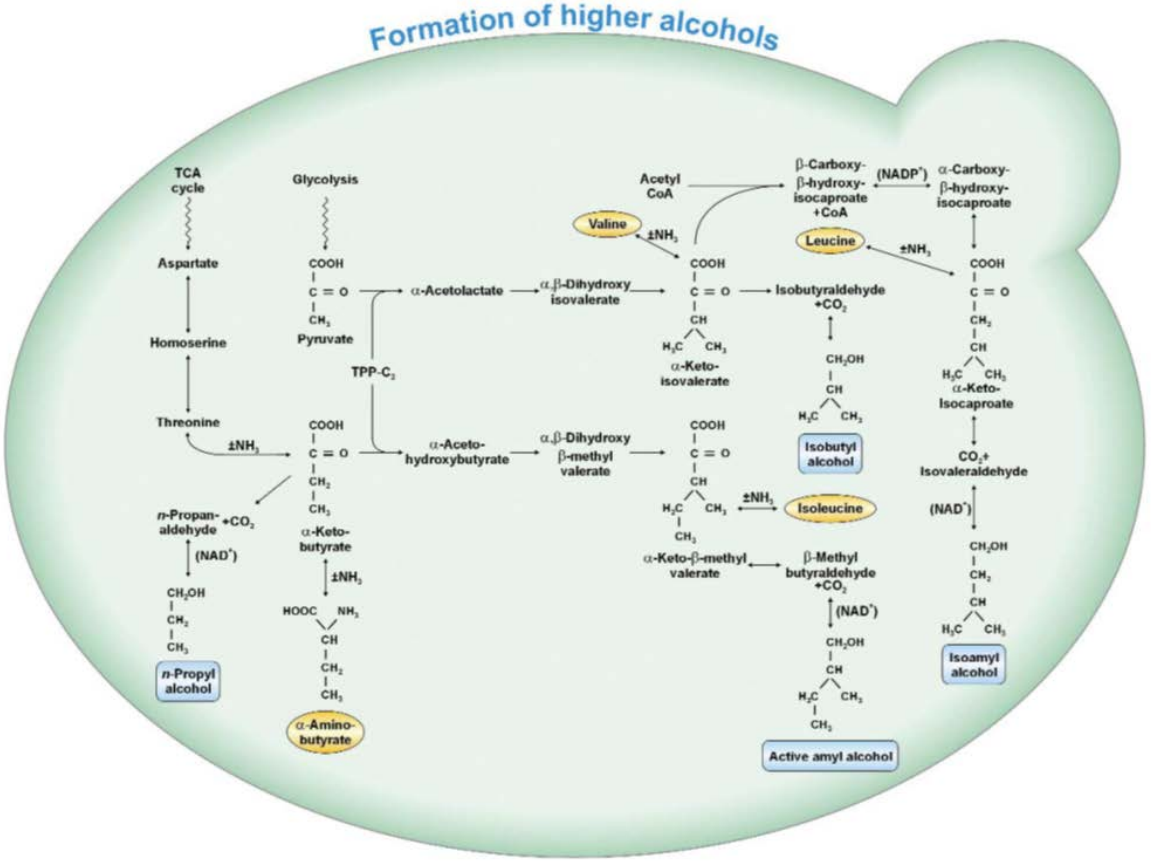


Figura 3.4 A schematic representation of the biosynthesis of higher alcohols in wine yeast (Swiegers et al., 2005).

Higher 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 (Lambrechts et al., 2000; Swiegers et al., 2005). Higher alcohols are divided into two categories, aliphatic and aromatic alcohols, the aliphatic alcohols include propanol, isoamyl alcohol, isobutanol and active amyl alcohol. The aromatic alcohols consist of 2-phenylethyl alcohol and benzyl alcohol. It has been reported that concentrations below 300 mg/L add a desirable level of complexity to wine, whereas concentrations that exceed 400 mg/L can have a detrimental effect (Rapp et al., 1998). The use of different yeast strains during fermentation contributes considerably to variations in higher alcohol profiles and concentrations in wine (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 & Ettlinger, 1978). 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 & Heard, 1993). Non-*Saccharomyce*s yeast can also contribute to the levels of higher alcohols. For example, mixed fermentation with *Pichia* fermentans and S*accharomyces cerevisiae* produced a substantial increase in higher alcohols such as 1-propanol, n-butanol and 1-hexanol compared to fermentation with *Saccharomyces cerevisiae* alone (Clemente-Jimenez et al., 2005).

In case of SO_2 addition (80 mg/L) in fermentation, some alcohols (like 3-methyl-1-butanol, 3-methyltio-1-propanol, phenylethyl alcohol and 4-hydroxybenzenethanol) 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 a ketoacid (the Ehrlich pathway) and an anabolic process starting from a ketoacids acting as intermediates in cell glucose metabolism (Hernandez-Orte et al., 2006).
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.1. 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) (Dickinson et al.,1993) (Fig. 3.5).

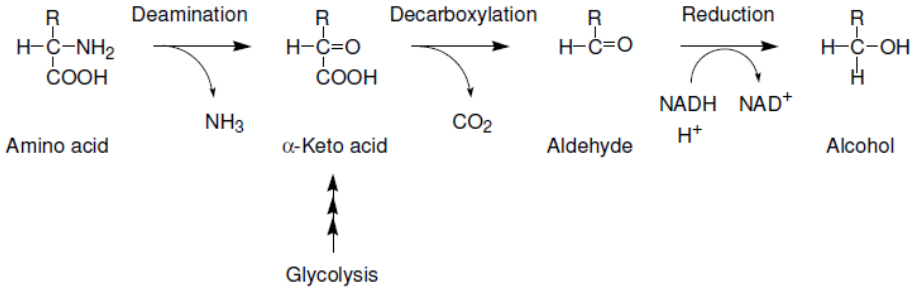
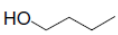
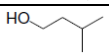
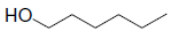
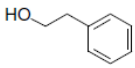
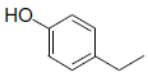
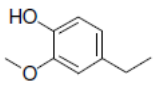
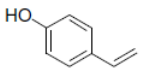
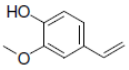


Figure 3.5 The Ehrlich pathway for the formation of higher alcohols from amino acids and sugar.

Table 3.1 A summay of the major alcohols reported in wine: their structure, aroma characteriscs, concentration in wine and aroma thresholds (Swigers et al., 2005).

Compounds Name	**Structure**	**Aroma**	**Concentration in wine (mg/L)**	**Aroma threshold (mg/L)**
1-propanol		Pungent, harsh	9.0-68	500**
1-butanol		Fusel, spirituous	0.5-8.5	150*
Isobutanol		Fusel, spirituous	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*
4-ethylphenol		Medicinal, baynyard	0.012-6.5	0.14*/0.6***
4-ethylguaiacol		Phenolic, sweet	0.001-0.44	0.033*/0.11***
4-vinylphenol		Pharmaceutical	0.04-0.45	0.02******
4-vinylguaicol		Clove-like, phenolic	0.0014-0.71	

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

3.3.2.3 Carbonyl compounds

Acetaldehyde is the major carbonyl compound found in wine with concentrations ranging from 10 mg/L to 75 mg/L and a sensory threshold value of 100 mg/L (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 3.2). 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. 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.i. 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) depending on the yeast strain (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. 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, depending on the style and the type of wine, contributes to a buttery or 'butterscotch' aroma (Table 3.2). Although yeasts biosynthesise some diacetyl (0.2–0.3 mg/L) 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 3.2 Principals carbonyl compounds reported in wine: their structure, aroma characteriscs, concentration in wine and aroma thresholds (Swigers et al., 2005).

Compounds Name	**Structure**	**Aroma**	**Concentration in wine (mg/L)**	**Aroma threshold (mg/L)**
Acetaldehyde		Sherry, nutty, bruised apple	10-75	100**
Diacetyl(2,3-butanedione)		Buttery	<5	0.2**/2.8***

** wine, ***red wine

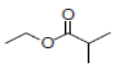
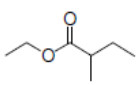
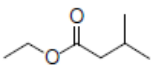
3.3.2.4 Volatile Phenols

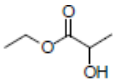
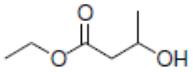
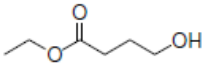
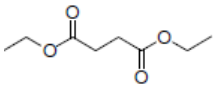
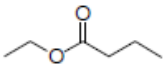
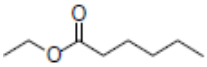
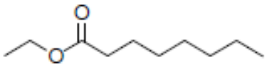
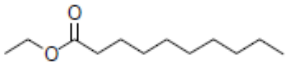
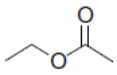
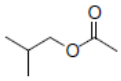
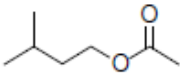
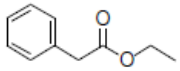
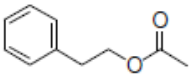
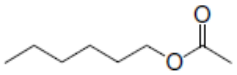
Volatile phenols (formed from the hydroxycinnamic acid precursors in the grape must) have a relatively low detection threshold and are, therefore, easily detected. Although volatile phenols can contribute positively to the aroma of some wines, they are better known for their contribution to off-flavours such 'Band-aid', 'barnyard' or 'stable', which results from high concentrations of ethylphenols (Dubois, 1983). The prominent ethylphenols are are 4-ethylguaiacol and 4-ethylphenol. Vinylphenols, especially 4-vinylguaiacol and 4-vinylphenol, produce a pharmaceutical odour, particularly in white wines (Ribéreau-Gayon et al., 2000b). Trace amounts of volatile phenols are present in grape must, but they are predominantly produced by yeast during fermentation. The nonflavonoid hydroxycinnamic acids, such as p-coumaric acid and ferulic acid, are decarboxylated in a non-oxidative process by *Saccharomyces cerevisiae* to form the volatile phenols 4- vinylguaiacol and 4-vinylphenol, respectively (Chatonnet et al., 1993). The *Brettanomyces/Dekkera* spp. yeasts are well-known for their ability to form volatile phenols in wine (Chatonnet et al., 1992, du Toit and Pretorius 2000). These yeasts are associated with the more unpleasant odourous ethylphenols, and are therefore regarded as spoilage organisms resulting in aromas described as 'Band-aid', 'medicinal', 'pharmaceutical', 'barnyard-like', 'horsey', 'sweaty', 'leathery', 'mouse urine', 'wet dog', 'smoky', 'spicy', 'cheesy', 'rancid' and 'metallic' (Chatonnet et al., 1995). Phenolic acids can also be decarboxylated into volatile phenols, usually first into 4-vinyl derivatives and then reduced to 4-ethyl derivatives through enzymes called phenolic acid decarboxylases. In addition to the metabolic activity of yeast and bacteria, other factors such as oak maturation can also increase the amount of volatile phenols in wine (Pollnitz et al., 2000). In particular, 4-ethylguaiacol and the 4-ethylphenol concentrations showed a marked increase during oak maturation.

3.3.2.5 Esters

The production of esters by the yeast during fermentation can have a significant effect on the fruity flavours in wine (Figure 3.6). The most significant esters are ethyl acetate (fruity, solvent-like), isoamyl acetate (isopentyl acetate, pear-drops aromas), isobutyl acetate (banana aroma), ethyl caproate (ethyl hexanoate, apple aroma) and 2-phenylethyl acetate (honey, fruity, flowery aromas) (Table 3.3) (Thurston et al., 1981). Commercial wine strains produce variable amounts of esters, such as isoamyl acetate, hexyl acetate, ethyl hexanoate and ethyl octanoate, which have a potential impact on the aroma profile (Soles et al., 1982; Lambrechts & Pretorius, 2000). However, there are several non-*Saccharomyces* wine yeasts that can contribute to the ester aromas of wine. For example, mixed culture fermentations by wild yeasts, such as *Hanseniaspora guilliermondii* and *Pichia* anomala, together with *Saccharomyces cerevisiae* showed increased acetate ester concentrations compared to fermentations with *Saccharomyces cerevisiae* alone, without significantly affecting acetaldehyde, acetic acid, glycerol and total higher alcohols (Viana et al., 2008; 2011). Although esters in wine are mainly produced by yeast metabolism (through lipid and acetyl-CoA metabolism), their production can be influenced by the grape variety. In Pinot Noir wines the characteristic fruity flavours of plum, cherry, strawberry, raspberry, blackcurrant and blackberry characters were shown to be influenced by four distinct esters: ethyl anthranilate, ethyl cinnamate, 2,3-dihydrocinnamate, and methyl anthranilate (Moio & Etiévant, 1995). These esters are synthesised by the yeast from grape precursors and have distinct aromas: sweet-fruity and grape-like odour (ethyl anthranilate) and cinnamonlike, sweet-balsamic, sweet-fruity, plum and cherry-like flavour (ethyl cinnamate). The aroma of ethyl 2,3-dihydrocinnamate is very similar to ethyl cinnamate, but its contribution to the overall aroma is smaller (Moio & Etiévant, 1995).

Table 3.3 A summary of the major esters reported in wine: their structure, aroma characteristics, concentration in wine and aroma thresholds (Sweigers et al., 2005).

Compounds Name	**Structure**	**Aroma**	**Concentration in wine (mg/L)**	**Aroma threshold (mg/L)**
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
Ethyl butanoate		Floral,fruity, strawberry, sweet	0.07-0.53	0.001,0.015a,s,5.0b
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 acetate		Fruity, solvent, balsamic	22.0-63.5	7.5a,60,12.27w
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-phenylethylacetate		Flowery, rose	Trace-0.26	0.25a, 0.65, 1.80w
Hexyl acetate		Green, herbaceous, fruit, grape	Trace-3.9	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, syntetic wine; w, wine)

It has been shown that Chardonnay wines characteristically contain ethyl esters such as ethyl-2-methyl propanoate, ethyl-2-butanoate, 3-methyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, and the acetate esters hexyl acetate, 2-methylbutyl acetate and 3-methylbutyl acetate. Although, Riesling wines contained similar esters,

3-methyl butanoate and ethyl hexanoate were found to be unimportant to the final aroma of the wines (Smyth et al., 2005). Ester concentrations differed among wine types, and there appears to be a synergy between the grape and the yeast metabolism in establishing the characteristic ester blueprint of different grape varieties.

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., 2010).

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., 2010).

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 (mediumchain 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) it gives a desirable and fruity character to the wine, while at higher concentrations (around 50 mg/L) 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., 2010). 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; 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 SO2 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 SO2, suggesting that the effect of SO2 addition on ester production does not seem to be systematic and may depend on several factors, such as O2 availability (Bardi et al., 1998; Moio et al., 2004).

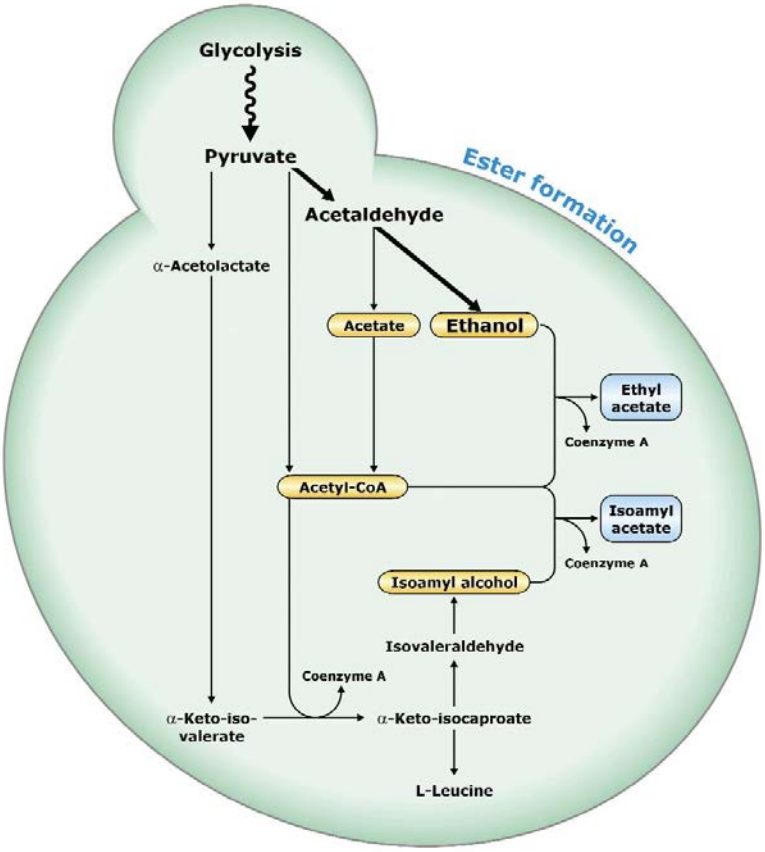


Figura 3.6 A Schematic representation of the formation of ethyl acetate and isoamyl acetate in wine yeast (Sweigers et al., 2005).

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., 2010).
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. Table 2. A summary of the major esters reported in wine: their structure, aroma characteristics, concentration in wine and aroma thresholds (Sumby et al., 2010; Sweigers et al., 2005).

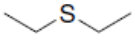
3.3.2.6 Sulphur compounds

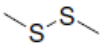
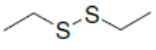
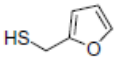
Sulfur-containing volatile compounds were originally associated with malodors mainly due to molecules such as hydrogen sulfide (H_2S), methylmercaptan (methanthiol), ethanethiol, and methionol. However, that is no longer the case with the discovery of a number of volatile thiols that impart pleasant herbaceous, fruity, mineral, smoky, and toasty aromas in wine (Dubourdieu & Tominaga, 2009). The most abundant volatile sulfur compounds in wines are H_2S, methanthiol, dimethylmercaptans (dimethylsulfide, dimethyldisulfide, dimethyltrisulfide), methylthioesters (S-methyl thioacetate, S-methyl thiopropanoate, and S-methyl thiobutanoate), and liberated glutathione and cysteine polyfunctional thiols (4-mercapto-4-methylpentan-2-one, 4MMP; 3-mercaptohexan-1-ol, 3MH; and 3-mercaptohexyl acetate, 3MHA) (Swiegers & Pretorius, 2007, Dubourdieu & Tominaga, 2009; Roland

et al., 2010). Hydrogen sulfide can be generated by *S. cerevisiae* through the degradation of sulfur-containing amino acids (cysteine and glutathione), the reduction of elemental sulfur, or the reduction of sulfite or sulfate (Rauhut & Körbel 1994; Rauhut, 2009). Hydrogen sulfide production varies across yeast strains and with the nitrogen status of the juice (Acree et al., 1972, Rauhut et al., 1996, Bell & Henschke, 2005, Linderholm et al., 2008, Kumar et al., 2010). It is generally understood that the addition of nitrogen, in the form of amino acids, with the exception of cysteine, or ammonium, reduces the production of H2S by yeast. That is because these sources of nitrogen are precursors for O-acetylserine or O-acetylhomoserine synthesis, which are important in the synthesis of cysteine, methionine, and glutathione (Giudici & Kunkee, 1994, Jiranek et al., 1995, Linderholm et al., 2008). The activity of O-acetylserine/ O-acetylhomoserine sulfhydrylase (the enzyme responsible for incorporating reduced sulfur into organiccompounds) is not the only factor important for reducing H2S production, but rather the activity of a complement of enzymes involved in the synthesis of O-acetyl-L-homoserine and homocysteine can help to reduce H_2S production in *S. cerevisiae* (Spiropoulos & Bisson, 2000, Linderholm et al., 2008). Interestingly, a recent study indicates that the nutrients used during rehydration of yeasts for use in grape juice fermentations can influence formation of H_2S (and other volatiles) (Winter et al., 2011). Methionine and cysteine are thought to be regulators of the sulfur reduction pathway. However, it is hypothesized that under the anaerobic conditions experienced during fermentation, cysteine concentrations may play a more important regulatory role in sulfate reduction (Linderholm et al., 2008). It has been shown that yeast respond to the addition of cysteine by increasing the production of H_2S in preference to methionol while the addition of methionine results in an increase in methionol in preference to H_2S (Moreira et al., 2002). This could be partly attributed to cysteine inhibiting serine O-acetyltransferase, which lowers the cellular concentration of O-acetylserine required for induction of the sulfate reduction pathway (Ono et al., 1996, 1999), and/or cysteine repressing the genes which encode cystathionine β-synthase and cystathionine γ-lyase in addition to the genes involved in the sulfate reduction pathway (Hansen & Francke Johannesen, 2000). A number of other volatile sulfur compounds can be formed from reactions of H_2S with other organic compounds; for example, H_2S in combination with ethanol or acetaldehyde forms ethanethiol (Swiegers et al., 2005a). Dimethylsulfide (DMS) is thought to be formed from microbial degradation of methionine and cysteine, although definitive pathways in wine fermentations have not been demonstrated (de Mora et al., 1986). Dimethylsulfide has been noted to increase black olive, truffle, and undergrowth sensory attributes in Syrah wines (Segurel et al., 2004) and can also enhance the fruit aroma of red wines as a result of complex interactions with other volatile compounds, including esters and norisoprenoids (Segurel et al., 2004; Escudero et al., 2007) (Table 3.4). However, DMS is generally not considered to contribute positively to white wine aroma since it enhances asparagus, corn, and molasses characters, although this could be considered as increasing the complexity of the aroma (Goniak & Noble, 1987). DMS, methionol, diethyl sulfide, and diethyl disulfide increase in wine with age and with increased temperature and may contribute to the aroma of aged wines (Marais, 1979, Fedrizzi et al., 2007).

Methionol contributes to the raw potato or cauliflower character of wines, can be found in wines at concentrations up to 5 mg/L, and is produced by either *S. cerevisiae* or *Oenococcus oeni* through the catabolism of methionine (Moreira et al., 2002; Ugliano & Moio, 2005; Vallet et al., 2008, 2009). It contributes to the cooked vegetable aroma of oxidized wines (Escudero et al., 2000). Methional concentrations increase in white wines exposed to elevated temperatures and oxygen. Under these conditions, the compound is produced via a Strecker degradation of methionine to methional in the presence of a dicarbonyl compound or via direct peroxidation of methionol (Escudero et al., 2000, Silva Ferreira et al., 2002).

Table 3.4 A summary of sulphur compounds, including thiols, reported in wine: theire structure, aroma characteristics, concentration in wine and aroma thresholds (Sweigers et al., 2005).

Compounds Name	**Structure**	**Aroma**	**Concentration in wine (μg/L)**	**Aroma threshold (mg/L)**
Hydrogen sulfide	H2S	Rotten egg	Trace->80	10-80
Methanethiol (methyl mercaptan)		Cooked cabbage, onion, putrefaction	5.1,2.1	0.3
Ethanethiol (ethyl mercaptan)		Onion, rubber, natural gas	1.9-18.7	1.1
Dimethyl sulfide		Asparague, 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	0-350ng/l	1ng/L
Thiophene-2-thiol		Burned, burned rubber, roasted coffee	0-11	0.8
4-Mercapto-4-methylpentan-2-one (4MMP)		Cat urine, box tree, broom	0-30ng/L	3ng/L

3.3.3 Tertiary aroma

Tertiary aroma is the consequence of several enzymatic and chemical reactions which take place during wine aging. Depending on the kind of aging it is possible to distinguish between oxidative “bouquet” (in wood barrels) and reductive “bouquet” (in bottles). During these processes some aromatic compounds are synthesized and some are modified, increasing or decreasing their levels (Oliveira et al., 2008; Rapp et al., 1985; Simpson, 1978a). Oxidative “bouquet” consists of acetaldehydes and acetals synthesis and extraction of several wood compounds, such as phenolic compounds from lignin degradation or lactones like 3-methyl-γ-octalactone (Masuda & Nishimura, 1971). Reductive “bouquet” is based on the interaction among wine compounds produced in fermentation. In this wine ageing, reduced sulphurous compounds, like dimethyl sulfide are produced (Marais et al., 1979). Furthermore, most primary aroma compounds show a significant increase and later a steady decrease. Even the levels of some volatile compounds related to wine ageing such as vitispiranes, Riesling acetal, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), and trans-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB) were found to decrease after the initial increase. However, vanillin derivatives, furan linalool oxides, 3-oxo- ionone, actinidiols, 4-ethylphenol and guaiacol showed a continuous increase along the ageing process. Levels of lactones, benzenes, guaiacol, terpenes and volatile phenols after ageing depend on grape variety used to carry out the fermentation process (Loscos et al., 2010).

Monoterpenes, such as linalool, geraniol and citronellol decrease, whereas there is an increase in the levels of nerol oxide, hotrienol, hydroxylinalool, and hydroxycitronellol (Rapp & Mandery, 1986). In addition, higher alcohols remain constant or diminish (Simpson, 1979; Marais & Pool, 1980), whereas acetate esters diminish, except for ethyl acetate, dimethyl succinate, ethyl caproate and ethyl caprylate. Consequently, at the end of the ageing process there is a loss of fresh character and fruitiness (Uber, 2006).

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4 VOLATILE COMPOUNDS: ANALYTIC TECNIQUES

Wine is one of the most complex food products in terms of volatile compounds composition. Several hundred volatiles have been identified in wine, belonging to different chemical classes. Dominating classes of volatile compounds in wines are higher alcohols and esters, also carbonyls are present, acids, terpenes, norisoprenoids, sulfur compounds and pyrazines. Some groups, as terpenes or pyrazines are related to a specific wine/grape types (Mateo & Jimenez, 2000; Sala et al., 2002). Volatile compounds in wines are derived from grapes and transferred from must, formed in the fermentation process and during ageing, as a result of interactions between wine constituents, or extracted from the oak barrels used for ageing (Ebeler, 2001). Volatile compounds in wine are usually present in a concentrations ranging from mg/L down to a few ng/L. In the analysis of key odorants by gas chromatography – mass spectrometry, extraction and preconcentration of these compounds, often present in trace concentrations pose a serious analytical challenge, both for the extraction and preconcentration step and for the detection and quantification process (Guth, 1997; Siebert et al., 2005). For the isolation of wine and grapes volatile compounds different sampling techniques are used, usually liquid/liquid extraction, static headspace (Ortega-Heras et al., 2002), solid phase extraction (SPE) especially for fractionation of free and bound volatile compounds (Piñeiro et al., 2004), stir bar sorptive extraction (SBSE) (Zalacain et al., 2007), and solid phase microextraction (SPME) (Sánchez-Palomo et al., 2005; Fan et al., 2010). Apart from target analysis of selected odorants, volatile compounds in wine are analysed also for the wines of different origin, variety and ageing comparison. For this purpose a fast method of extraction, which enables a full profile of extracted compounds, which would be sensitive, robust and fast is required. Solid phase microextraction (SPME) developed in late eighties by Pawliszyn and coworkers (Pawliszyn, 2009) is a method that proved its suitability in the analysis of volatile compounds in food matrices and can be used for volatiles profiling. SPME is a new sample preparation technique using a fused silica fiber that is coated on the outside with an appropriate stationary phase. The method saves preparation time, solvent purchase and disposal costs, and can improve the detection limitis. It has been used routinely in combination with GC and GC/MS provides high sensitivity. This sample extraction technique is suitable for the extraction and concentration of a high number of volatile and semi-volatile compounds from aqueous solutions (Harmon, 1997; Yu et al., 2012).
For these reasons it has been used to study the volatile profile of many fruit varieties, vegetables, and beverages, including grapes and wine (Vas & Vékey, 2004; Castro et al., 2008; Flamini & Traldi, 2010).

4.1 Headspace Solid Phase Microextraction (HS-SPME)

The fiber SPME device consists of fiber holder and fiber assembly with built-in fiber inside the needle which looks like a modified syringe (Figure 4.1). The fiber holder consists of a spring-loaded plunger, a stainless-steel barrel and an adjustable depth gauge with needle, and is designed to be used with reusable and replaceable fiber assemblies. The fused-silica fiber is coated with a relatively thin film of several polymeric stationary phases. This film acts like a ‘sponge’, concentrating the organic analytes on its surface during absorption or adsorption from the sample matrix. As shown in Table 4.1, seven kinds of fibers are commercially available. Stationary phases are immobilized by non-bonding, bonding, partial crosslinking or high crosslinking. Non-bonded phases are stable with some water-miscible organic but slight swelling may occur when used with non-polar solvents. Bonded phases are stable with all organic solvents except for some non-polar solvents. Partially crosslinked phases are stable in most water miscible organic solvents and some non polar solvents. Highly crosslinked phases are equivalent to partially crosslinked phases, except that some bonding to the core has occurred. Advantages of these phases for SPME applications are similar to the advantages in their use as GC stationary phases. The recovery capabilities of PDMS and PA are based on the phenomena of sorption, whereas the others exploit sorption and adsorption to capture target analytes. PDMS is liquid or highly viscous and is available in different film thickness (7μm, 30μm and 100μm). The PDMS coating supports a temperature of 280ºC, it is non-polar and therefore more suitable for apolar compounds and less for polar ones. The PA coating is solid at room temperature and is used to recover polar compounds, for instance phenols, because of its polar nature. The other fibres consist of two or three different components which extend the range of polarity and efficiency. The characteristic of PDMS/DVB is to be non-polar therefore it absorbs non-polar analytes and uses hydrophobic interactions with lipophilic compounds. The fibre CW/DVB consists of carbowax (polyethylenglycol) and divinylbenzene including insaturations because of the aromatic rings and support π-π interactions with double bonds of analytes, e.g. terpenes. This coating is also very effective to adsorb large compounds, whereas CAR/PDMS, composed of porous carbon, favours recovery of small molecules. The polymer fibre with three phases (1 or 2cm length) supports an operating temperature of 270ºC. The PDMS fibre is particularly suitable for the analysis of esters and acetates (Vianna & Ebeler, 2001) and a greater extraction of norisoprenoids (e.i., damascenone) and terpenes (e.i,linalool) (Canuti et al., 2009)

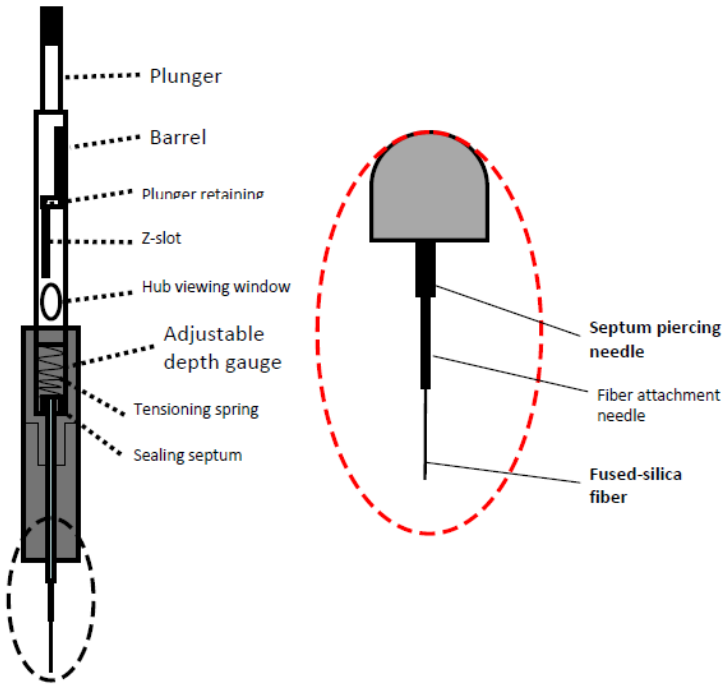

Figure 4.1. commercial SPME device made by Supelco.

Fiber Core (Stationary Phase)	Thickness	Bond Type	pH range	Recommended Operation Temperature °C	Application	Recommended Use
Polydimethylsiloxane (PDMS)	100 µm	Non-bonded	2-10	200-280	Volatile	GC/HPLC
	30µm	Non-bonded	2-11	200-280	Non-polar; semivolatile; moderately polar	GC/HPLC
	7µm	bonded	2-11	220-320	To non-polar; semivolatiles	GC/HPLC
Polydimethylsiloxane/Divinylbenzene (PDMS/DVB)	65µm	Partially crosslinked	2-11	200-270	Polar volatile	GC
Carboxen/ Polydimethylsiloxane (Carboxen/PDMS)	75µm	Partially crosslinked	2-11	250-310	Trace-level volatile	GC
Polyacrylate (PA)	85µm	Partially crosslinked	2-11	220-300	Polar semivolatile	GC/HPLC
Polyethylene glycol (PEG)	60µm	crosslinked	2-9	200-250	alcohols and polar compounds	GC
Divinylbenzene/Carboxen/ Polydimethylsiloxane (DVB/CAR/PDMS)	50/30µm	Highly crosslinked	2-11	230-270	analytes C3-C20 (trace compounds)	GC
Carbowax/Divinylbenzene (Carbowax/DVB)	70µm	Highly crosslinked	2-9	200-240	Polar analytes	GC
	65µm	Partially crosslinked	2-9	200-250	polar analytes, especially for alcohols	GC

Table 4.1: Commercially available SPME fibers (Supelco, Bellefonte, PA)

4.2 Fiber Solid phase Microextraction

The process of fiber SPME is illustrated in Figure 4.2. The sample is placed in a vial, which is sealed with a septum-type cap. The fiber should be cleaned analyzing any sample in order to remove contaminants, which give a high background in the chromatogram. Cleaning can be done by inserting the fiber in the GC injection port (at T>230°C) or a syringe cleaner. When the SPME needle pierces the septum and the fiber is extended through the needle into the sample, the target analytes partition from the sample matrix into the stationary phase. Although SPME has a maximum sensitivity at the partition equilibrium, a proportional relationship is obtained between the amount of analyte adsorbed by the spme fiber and its initial concentration in the sample matrix before reaching partition equilibrium reaching partition equilibrium (Ai, 1997). Therefore, full equilibration is not necessary for quantitative analysis by SPME.

Agitation of the sample is often carried out to increase the rate of for equilibration. After a suitable extraction time, the fiber is withdrawn into the needle, which is removed from the septum and is then inserted directly into the injection port of the GC.

The desorption of analyte from the fiber coating is performed by heating the fiber in the injection port of a GC or GC–MS.

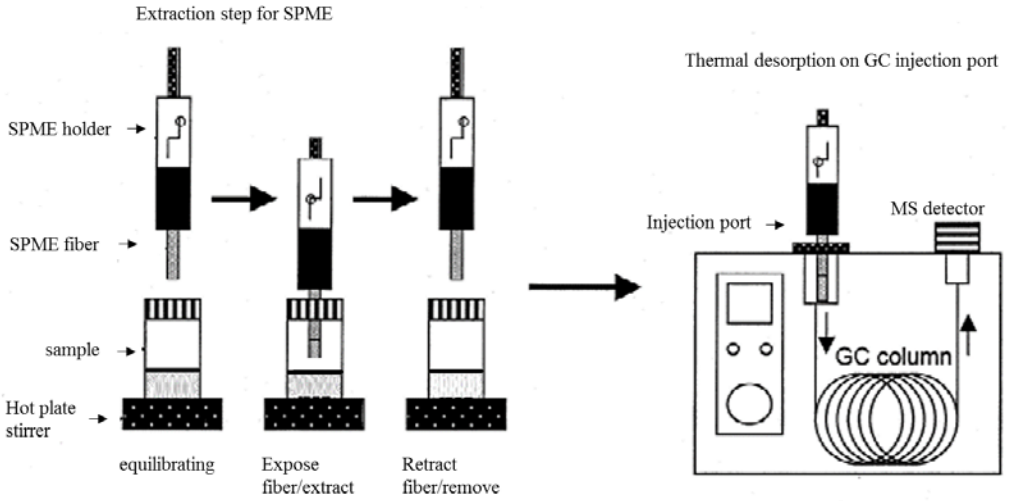

Figure 4.2 process of fiber SPME

4.3 Theoretical aspects of SPME

The principle behind SPME is the partitioning of analytes between the sample matrix and the extraction medium. If a liquid polymeric coating is used, the amount of analyte absorbed by the coating at equilibrium is directly related to its concentrations in the sample

$$n = C_0 V_f V_s K_{fs} / (K_{fs} V_f + V_s) \qquad \text{equation 1}$$

where n is the mass of an analyte absorbed by the coating; V_f and V_s are the volumes of the coating and the sample, respectively; K_{fs}, is the partition coefficient of the analyte between the coating and the sample matrix; and C_0 is the initial concentration of the analyte in the sample. Equation 1 clearly indicates the linear relationship between the amount of analytes absorbed by the fiber coating and the initial concentration of these analytes in a sample. Because the coatings used in SPME have strong affinities for organic compounds, K_{fs} values for targeted analytes are quite large, which means that SPME has a very high concentrating effect and leads to good sensitivity. In many cases, however, K_{fs} values are not large enough to exhaustively extract most analytes in the matrix. Instead, SPME, like static headspace analysis, is an equilibrium sampling method and, through proper calibration, can be used to accurately determine the concentration of target analytes in a sample matrix. As Equation 1 indicates, if *V,* is very large *(V,* >> $K_{fs}V_f$**),** the amount of analyte extracted by the fiber coating

$$n = K_{fs}\, V_f\, C_0$$

is not related to the sample volume. This feature, combined with its simple geometry, makes SPME ideally suited for field sampling and analysis. Because the fiber can be exposed to air or dipped directly into a well, lake, or

river, SPME reduces field analysis time by combining sampling, extraction, concentration, and injection into a single uninterrupted process. The speed of extraction is controlled by the mass transport of the analytes from the sample matrix to the coating. This process involves convective transport in an air or liquid sample, the rate of desorption of analytes from the solid surface when particulate matter is present, and diffusion of analytes in the coating. In direct SPME sampling, the mass transfer rate is determined by the diffusion of analytes in the coating if the sample matrix is perfectly agitated. When the mass transfer rate is determined by the diffusion of the analyte in the coating, for most analytes equilibrium is achieved in <1 min. Rapid extraction is ensured because the coating is very thin, typically between 10 and 100 μm. In practice, this limit can be achieved for gaseous samples because of large diffusion coefficients. For aqueous samples, however, this case is possible only when using very vigorous agitation methods such as sonication. For more practical agitation methods such as magnetic stirring, the equilibration time is much longer and is determined by diffusion through a thin static aqueous layer adjacent to the fiber. This thin layer of water, which surrounds the fiber, is very difficult to remove even when water is stirred rapidly to enhance the mass transfer of analytes. The analytes must diffuse through the water before they can be absorbed by the fiber coating.

Placing a fiber directly into a sample to extract organic compounds works well for gaseous samples and relatively clean water samples. If we want to sample analytes from a solid matrix or from a wastewater sample with grease, oil, and high molecular weight humic acid, however, direct SPME sampling may not work well; sampling analytes from the headspace above the sample matrices is necessary. Thus, SPME can be used to extract organic compounds from virtually any matrix as long as target compounds can be released from the matrix into the headspace. For volatile compounds, the release of analytes into the headspace is relatively easy because analytes tend to vaporize once they are dissociated from their matrix. For semivolatile compounds, the low volatility and relatively large molecular size may slow the mass transfer from the matrix to the headspace and, in some cases, the kinetically controlled desorption or swelling process can also limit the speed of extraction, resulting in a long extraction time. When the matrix adsorbs analytes more strongly than the extracting medium does, the analytes partition poorly into the extraction phase. Because of the limited amount of the extraction phase in SPME (as in SPE), the extraction will have a thermodynamic limitation. In other words, the partition coefficient K_{fs} is too small, resulting in poor sensitivity. If the coating has a stronger ability to adsorb analytes than the matrix does, it is only a matter of time for a substantial amount of analytes to be extracted by the fiber coating, and only kinetics plays an important role during extraction. One of the most efficient ways to overcome the kinetic limitation is to heat the sample to higher temperatures, which increases the vapor pressure of analytes, provides the energy necessary for analytes to be dissociated from the matrix, and at the same time speeds up the mass transport of analytes. In headspace SPME, three phases (coating, headspace, and matrix) are involved, and the chemical potential difference of analytes among the three phases is the driving force that moves analytes from their matrix to the fiber coating. For aqueous samples, the headspace/water partition coefficients K_{hs} are directly related to the analytes' Henry's constants, which are determined by their volatility and hydrophobicity.

In an aqueous matrix, most compounds have quite small K_{hs} values (< 0.25), and the capacity of the headspace for trapping analytes is small. As a result, the sensitivity of headspace SPME is almost the same as that of direct SPME. The loss in sensitivity is important only when the target analytes partition well into the headspace (large K_{hs}), and/or a large headspace volume is used. For VOCs in water, headspace SPME sampling is faster than direct SPME. In headspace SPME, the mass transfer from water to headspace can be speeded up rapidly by constantly stirring the water sample to generate a continuously fresh surface. The mass transfer of volatile compounds from the headspace to the fiber coating is very fast because of large diffusion coefficients of analytes in the gas phase, and volatile compounds transfer more efficiently from water to headspace to coating than from water directly to coating. When the limiting mass transfer step is the mass transport of analytes from matrix to headspace, the extraction time profile curve is characterized by an initial rapid rise (associated with partitioning of analytes originally present in the gaseous headspace) followed by a section with a smaller slope (determined by slower mass transport of analytes from the matrix). This rate is determined by the convective and diffusive transport of analytes in the sample matrix and/or by the slow desorption kinetics. Because partition coefficients are temperature dependent, there is usually an optimum temperature for headspace SPME. As the temperature rises, more analytes are released from the matrix to the headspace, a process that results in high analyte concentrations in the headspace and favors SPME extraction. However, at high temperature, coating headspace partition coefficients decrease, and the matrix-dependent optimum extraction temperature is determined by the interactions among analytes, coating, and matrix. With a good coating, headspace SPME can be used to extract both volatile and semivolatile compounds from their matrices. Although SPME is mainly an equilibrium extraction technique, it has the ability to perform exhaustive extraction. If the coating/matrix partition coefficient, K_{fs} is very large ($K_{fs} V_f >> V_s$), the amount of analyte absorbed by the coating is $n= C_0V_s$ and exhaustive extraction is achieved (Pawliszyn, 2009).

4.4 Optimization of extraction

In fiber SPME, the amount of analyte extracted onto the fiber depends not only on the polarity and thickness of the stationary phase, but also the extraction time and the concentration of analyte in the sample. Extraction of analyte is also typically improved by agitation, addition of salt to the sample, changing the pH and temperature. Extraction time is mainly determined by the agitation rate and the partition coefficient of the analyte between the fiber coating and sample matrix. Although SPME has a maximum sensitivity at the equilibrium point, full equilibration is not necessary for accurate and precise analysis by SPME because of the linearship between the amount of analyte adsorbed by the SPME fiber and its initial concentration in the sample matrix in non-equilibrium conditions (Ai, 1997). However, in such cases, the extraction time and mass transfer conditions have to be carefully matched across runs. Agitation accelerates the transfer of analytes from the sample matrix to the coating fiber. Although the equilibration times progressively decreases with increasing agitation rate, faster agitation tends to be uncontrollable and the rotational speed might cause a change in often observed in the equilibration time and poor measurement precision. The extraction efficiency is also improved by adding soluble salts to the sample. Sodium chloride, sodium hydrogencarbonate, potassium carbonate and ammonium sulphate are generally used for this purpose. In principle, supersaturation of the sample with salts is most effective for the extraction of analytes onto the fiber due to the salting-out effect. The form of analytes present in the sample mainly depends on the pH of the matrix relative to the analyte and influences the extraction efficiency. In general, the sample is acidified for the extraction of acidic analytes and is made alkaline for the extraction of basic analytes. In order to increase the concentration of the analytes in the gaseous phase in HS-SPME, the sample is usually heated. An increase in extraction temperature causes an increase in extraction rate, and simultaneously a decrease in the distribution constant. Therefore, an adequate temperature which provides satisfactory sensitivity and extraction rate should used. For accurate and precise analysis, a consistent extraction time and other SPME parameters are essential. Another critical point is that the vial size and sample volume should be the same during analysis by SPME.

4.5 Headspace Solid Phase Microextraction Coupling Gas Chromatography with Mass Spectrometry (HS-SPME GC/MS)

HS-SPME GC-FID is a method of analysis that combines two sampling techniques, headspace (HS) and solid-phase microextraction (SPME), interfacing with gas chromatography (GC) coupled with Mass Spectrometry (MS).

4.5.1 GasChromatography

GasChromatography is an applied technique for separation, identification and determination of volatile compounds and its applications and improvements have increased since the introduction of the concept of GC by Martin and Synge in 1941. Currently, there are almost a million of gas chromatographs in use throughout the world (Rouessac & Rouessac, 2000).

In gas liquid chromatography, the stationary phase is a liquid, as the name implies, which is immobilised on the capillary inner surface. The mobile phase is a gas which carries out the components of a vaporised sample through the stationary phase. This carrier gas has to be chemically inert. Mostly helium but also nitrogen, argon and hydrogen are used as a mobile gas phase. The flow rates of the carrier gas are controlled either by a two-stage pressure regulator or directly at the column by an electronic flowmeter. A gas chromatograph consists basically of an injector, a column and a detector, as shown in figure 4.3. The analysis starts when an aliquot of a sample (liquid or gas) is inserted by a micro syringe or a SPME fibre into the inlet port (injector). The injector has two functions, first to vaporise the sample analytes and second to mix the analytes with the mobile phase. The mobile phase sweeps the vaporised analytes into the column which is situated in an oven and contains the stationary phase. Further, the mobile phase carries out the mixture of the analytes through the column. Each molecule, depending on its type (volatility, polarity) and on the stationary phase material, is retained for different times. This process is based on adsorption, mass distribution and size exclusion, thus each molecule is separated and comes to the end of the column at different times (retention times). The sample molecules are determined by a detector at the end of the column. The signals of the detector are transmitted either to a recorder or a chromatographic data system which displays the results as a chromatogram. To obtain a high level of efficiency in the column the sample has to be an appropriate size and injected as a “plug” of vapour, otherwise band spreading or poor resolution can occur (Skoog et al., 2007). Therefore, calibrated micro syringes and often fast autosamplers are used to provide reproducibility and time-optimisation. The temperature of the injector is about 50^{o}C above the boiling point of least vaporised analyte. Further, different modes of injection, depending on the column used can be selected, such as split/splitless, direct vaporisation injection and cold on-column injection (Rouessac & Rouessac, 2000).

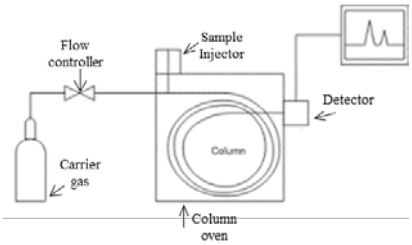

Figure 4.3 Scheme of Gas Chromatograpy.

A split or splitless injector is often needed for capillary columns, because they require very small sample volumes. The carrier gas either sweeps the totality (splitless) in the case of diluted samples or only a part (split) of the sample into the column. Direct vaporisation injection is often used for packed columns. The whole sample is directly vaporised and injected through a septum into the column in a few seconds. Cold on-column injection is an approach for capillary columns where the sample is cold injected as a liquid and is vaporised by a temperature program of the injector or the column. It is useful for thermolabile samples or for separating analytes from the solvent by the thermal effects. Currently, for most applications, the capillary columns are used. These columns are composed of fused silica tubes which are coated with stationary phase. They are formed as coils and placed in an oven at the right temperature by means of an oven program. This program improves the separation of samples with a broad boiling range by increasing the temperature continuously or in steps.

At the end of the column, there is always a detector system, which produces an electronic signal of the eluate. This signal depends on the time since injection (retention time) and is shown as a 2D graph, called chromatogram by means of data system software. There are several detectors available, each with its own characteristics. Some are universal, meaning that they are sensitive to nearly all separated components, however, most detectors are more sensitive only to selected components. In addition, GC can be coupled with spectroscopic instruments, for example infrared spectrometers (IR) or mass spectrometers (MS).
The most common detectors are, the flame ionization detector (FID), the thermal conductivity detector (TCD), the electron capture detector (ECD), the nitrogen phosphorus detector (NPO), the flame photometric detector (FPD), the mass spectrometer (MS) and, to a lesser extent, the Fourier transform infrared spectrometer (FTIR) (Skoog et al., 2007; Rouessac & Rouessac, 2000). This thesis work focuses mainly the evaluation of the response given by MS detectors.

4.5.2 GC-Mass Spectrometry

Mass Spectrometry coupled with GasChromatography is one of the most powerful tools to separate, identify and quantify biochemical, organic or sometimes inorganic compounds in a mixture. The principle is based on the measurement of the ratio of the mass to the charges of ions (m/z) in the gas phase. This ratio is declared in atomic mass units (amu) or in daltons (Da). In general, GC-MS consists of four main parts: GC (Gas Chromatography), Ion source, analyser and detector. The Ion source, analyser and detectron are placed in a high vacuum chamber.

In GC/MS, a capillary column is directly introduced into the ionisation chamber. The separated components from the end of the column are directly fed into the ion source where they are converted into ionised ones by collision with electrons, ions, photons and molecules. After the component fragmentation, ions are focused by several electronic lenses and accelerated as well. The analyser separates the ions according to their mass/charge ratio (m/z). After filtration, the ions conclude their path in a detector where the electrical charges are measured (Skoog et al., 2007; Rouessac & Rouessac, 2000). The scheme of a gas chromatography mass spectrometry is shown in figure 4.4.

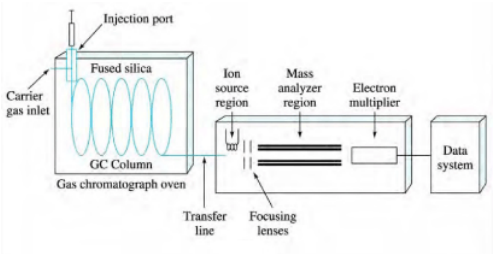

Figure 4.4 Scheme of GC/MS.

The first step of the eluate of GC in a MS-device is ionisation, therefore several methods are available: electron impact (EI), chemical ionisation (CI), fast-atom bombardment (FAB), Matrix-assisted laser desorption ionisation (MALDI), electrospray, atmospheric pressure chemical ionisation (APCI) and thermospray.
The electron impact is the most widely used technique for the analysis of volatile organic compounds. The ionisation process occurs by a beam of electrons which collide on the sample components with an energy of 70eV. The following reaction describes the process:

$$M + e^- \rightarrow M^{+\bullet} + 2e^-$$

where M is the sample molecule, e^- is the electron and $M^{+\bullet}$ is the resulting ion.

After ionisation of each sample component, the ions are separated with an analyser such as a quadrupole, ion trap or time of flight analyser. An ion-trap analyser is based on the same principle as quadrupoles but with a three dimensions electric field. Whereas the time of flight analyser measures the time which each mass needs to travel through a field free fixed pathway. That time is proportional to the square root of the m/z ratio. The most often applied analysers coupled with GC are the quadrupoles. They have been developed because they are more compact, rugged and less expensive than other types of mass spectrometry; furthermore, they achieve very high scan rates. The heart of a quadrupole consists of four parallel hyperbolic or cyclindrical rods. They are arranged in opposite pairs and each pair is connected electrically. A potential, which is a result of a constant component and an alternating component, is applied across these pairs. The ions of the source are transmitted into the space between the rods. Their separation occurs by increasing the voltages applied to the rods while the ratio of continuous voltage to alternating voltage keeps constant. In addition, only ions with a certain value of m/z can reach the ion transducer. For the detection of the separated ions several transducers can be applied, however, the electron multipliers are mostly selected. There are two types of electron multipliers, discrete-dynode electron multiplier and continuous-dynode electron multiplier. Both collect the separated ions and convert them into an electrical signal by the means of an increasing voltage applied to a resistive conductive surface. Other detectors of mass spectrometry are the Faraday cup and array transducers such as microchannel plates.
The signal acquisition of the ion abundances can be achieved in two different modes:

▪ the continuous spectrum which considers an interval of masses or a selected mass. In this spectrum, ions are displayed as peaks of different abundances, depending on the particular instrument. High performance devices can discover the masses with a accuracy about 10 parts in a million (10^{-5} Da);

▪ the fragmentation spectrum. It is generated by summing up the intensities of ion masses which are expressed as a percentage of the base peak (the most intense peak). Therefore, these intensities represent a nominal mass which is closest to the exact mass of the determined ions.

Furthermore, mass spectrometry is a very suitable method for identification of compounds. The structure of molecules can be reconstructed from the ion fragmentation pattern as a fingerprint of the specific compound. Either a library of mass spectra or retention indices can be used for the identification of each compound detected by MS (Skoog et al., 2007; Rouessac & Rouessac, 2000). In summary, although GC/MS requires a high vacuum and purchase and preservation are very expensive, it has been applied for the identification of thousands of components which occur in natural systems. In particular, MS plays an important role for the characterisation of flavour components of food and studies of plants. The reason is its special ability to separate signals from noisy background or signals of coeluated compounds when different specific ions are available.

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5 MATERIALS AND METHODS

The grapes and musts samples of Coda di Volpe, Aglianico and Fiano come from vineyards located in different areas of Campania region (Irpinian and Sannio). Grape sampling was carried out in the period from June to September, in order to obtain a sufficiently representative range of the various states of fruit ripeness, from fruit setting (initial fruit development stage which occurs after flowering) to the stage of fruit ripening during which the colour of the epicarp changes, up until the ripe grapes immediately prior to harvest. Sampling was carried out in order to have a sample that was representative of the plot investigated. The clusters were cut so as to cause no external contamination, were placed in sterile plastic bags and stored in refrigerated containers prior to transportation to the laboratory for analysis.

5.1 Yeast isolation

Once in the laboratory, grape samples were cut into branches and berries, with scissors previously dipped in alcohol, and placed in sterile bags. The Ringer solution (8.5 g/l NaCl and 1 g/l peptone) was then added to each (10g) sample at a ratio of 1:2 and the sample homogenized for 30s at 230 rpm by way of a Stomacker homogenizer (Seward, UK). The sample then underwent various dilution tests in order to reach a concentration suitable for counting and 0.1 mL was spread onto plates with different media (Oxoid, Hampshire, UK):

• **YPD**: a complete medium which allows growth of yeasts, moulds and bacteria (dextrose 20 g/L, peptone 20 g/L, yeast extract 10 g/L, agar 20 g/L).

• **WL nutrient medium**: complete medium allowing growth of the main species of yeast and other microorganisms, but the colonies develop specific colours allowing an initial differentiation (yeast extract 4 g/L, tryptone 5 g/L, glucose 50 g/L; potassium bisphosphate 0.55 g/L, potassium chloride 0.425 g/L, calcium chloride 0.125 g / L, magnesium sulphate 0.1125 g/L, ferric chloride 0.0025 g/L; manganese sulfate 0.0025 g/L; bromocresol green 0.0022 g L, agar 15 g/L)

• **Lysine medium** is a selective medium because it contains nitrogen as the only form of lysine and therefore should not allow growth of the *Saccharomyces* spp. genus (dextrose 44.5 g/L, monoacidic potassium phosphate 1.78 g/L, magnesium sulphate 0.89 g/L, calcium chloride 0.178 g/L, sodium chloride 0.089 g/L; adenine 0.00178 g/L, DL-methionine 0.000891 g/L, L-histidine 0.000891 g/L, DL-tryptophan 0.000891 g/L, boric acid 0.0000089 g/L, zinc sulphate 0.0000356 g/L, ammonium molybdate 0.0000178 g/L, manganese sulphate 0.0000356 g/L, ferrous sulphate 0.0002225 g/L; lysine 1 g/L; inositol 0.02 g/L, calcium pantothenate 0.002 g/L; aneurine 0.0004 g/L, pyridoxine 0.0004 g/L; acid p-aminobenzoic 0.0002 g/L, nicotinic acid, 0.0004 g/L; riboflavin 0.0002 g/L; biotin 0.000002 g/L; Folic acid 0.000001 g/L, agar 17.8 g L).

The plates were incubated at 28°C for 5 days, the colonies counted and the randomly isolated yeasts were picked and stored on YPD slants at 4°C.

5.2 Yeast identification

5.2.1 Biochemical and morphological identification

The strains of yeast isolates were classified according to colony colour and morphology on WL, as well as by their microscopic and biochemical characteristics. The yeast cultures were inoculated on YPD broth and incubated at 28°C for 24 hrs. They were used to prepare the slides for observation at the microscope (Olympus optical BX40, Japan) to determine the shape of the cells. As far as the microscopic characteristics are concerned, the following parameters were analyzed:

Morphological analysis (apiculate, ellipsoid, coccoid)

Multiplication method (division or budding)

Ability to sporulate

Number of spores per ascus

Sporulation capacity was tested on sodium acetate agar medium (sodium acetate 4 g/L, dextrose 1 g/L, agar 15 g/L). An amount of yeast cells was spread on this solid medium and were incubated at 28°C for 15 day.

For the analysis of biochemical characteristics, API 20 C AUX kits supplied by Bio-Merieux were used, in which identification was made based on the ability of the microorganis to metabolize specific substrates represented by the different types of sugars in the table 5.1. The gallery consists of 20 strips containing dehydrated substrates for the execution of 19 assimilation tests. The strips were filled with minimal semi-agarised medium which had been previously inoculated with the microorganism, and the growth achieved was verified on the basis of the substrates that the yeast was able to use. The growth achieved was verified by comparison with the control growth (containing no substrate), while the identification was undertaken using an analytical index. The strips were filled with an inoculum obtained by transferring a small quantity of a colony from the YPD medium plate and resuspended in 3 ml of saline to a vial containing the C API medium to achieve a level of turbidity of 2 on the McFarland scale (0.5 OD at 625 nm). The gallery, placed in a container closed by a lid, was incubated at 28 °C in an incubator for 48-72 hours.

Table 5.1 different substates in the API C AUX kit (Bio-Merieux).

Test	Substrate	Quantity (mg/cup)
0	None	1.2
GLU	D-glucose	1.2
GLY	Glycerol	1.2
2KG	2-cheto calcium gluconate	1.2
ARA	L-arabinose	1.2
XYL	D-xylose	1.2
ADO	Adonitol	1.2
XLT	Xylitol	1.2
GAL	D-galactose	1.2
INO	Inositol	1.2
SOR	D-sorbitol	2.36
MDG	alpha-methyl glucopyranoside	1.2
NAG	N-acetyl glucosamine	1.2
CEL	D-cellobiose	1.2
LAC	D-lactose (of bovine origin)	1.2
MAL	D-maltose	1.2
SAC	D-sucrose	1.2
TRE	D-trehalose	1.2
MLZ	D-melezitose	1.2
RAF	D-raffinose	1.9

After 48 hours of incubation (or 72 hours, if tests, particularly glucose, were inconclusive), growth of yeast compared to a negative control was assessed. A more turbid strip than the control indicated a positive reaction, which was then recorded on a results card. Microorganism identification was achieved by constructing a numerical profile on the results sheet. On the latter, test results were reported which were separated into groups of three, with each test being awarded a value of 1, 2 or 4. By addition of test values corresponding to positive reactions within each group, a 7-digit profile number was obtained. This profiling allowed the genus and species of each yeast species of interest to be categorized according to an analytical index. The following is an example of the card:

Figure 5.1 before incubation

Figure 5.2 after 48/72h of incubation

5.2.2 Molecular identification of yeasts

In order to unambiguously determine the yeasts belonging to a particular species, the D1/D2 region of 26S rDNA gene consisting of a 600 bp region was sequenced. This was achieved by PCR using yeast cells obtained directly from a colony on YPD medium plates. The standard primers used, commonly referred to as NL1 and NL4 in the literature (Arroyo-Lòpez et al., 2006; Esteve-Zarzoso et al., 2000), have the following sequences:

NL1 5'-GCATATCAATAAGCGGAGGAAAAG-3'

NL4 5'-GGTCCGTGTTTCAAGACGG-3'

The PCR reaction was conducted using the EuroTaq polymerase (Euroclone) with a Mastercycler Personal thermocycler (Epphendorf, Hamburg, GE). The reaction mix was as follows:

73.8 µl H2O
5.0 µl dNTP (10 mM)
3.0 µl MgCl2 (50 mM)
2.5 µl primer NL1 (0.5 µM)
2.5 µl primer NL4 (0.5 mM)
10.0 µl 10X PCR buffer
1.2 µl ETaq (unit 5/µl)
Total volume 98.0 µl

The PCR reaction was performed using the following parameters:

Stage	Number of Cycles	Temperature	Time (minutes)
1	1	94°C	15
2	40	94°C 55°C 72°C	1 2 2
3	1	72°C	10

The first extended cycle was included in order to lyse the cells, and the polymerase was added immediately prior to stage 1 completion. Sequencing was carried out using the Sanger method with a 3730 Genetic Analyzer DNA sequencer (Applied Biosystems).

5.3 Selection of yeasts

5.3.1 Selection for resistance to sulfur dioxide

The strains of yeast isolates were tested for resistance to two concentrations of sulfur dioxide, 100 ppm and 250 ppm. The tests were conducted on the 2007 vintage Fiano musts pasteurized for 30' in tubes stoppered with cotton wool. Prior to inoculation, strains were grown in YPD at 28 °C under agitation at 160 rpm in a shaker incubator (New Brunswick Inc., USA) until early exponential phase (A_{600nm} = 0.4-0.6) and the inoculum was added at a concentration of 1% of the total (10 mL). Potassium metabisulfite (MBK) was used as the source of sulphur dioxide (Costanti et al., 1998)

5.3.2 Determination of the yeast fermentative power

One hundred mL of pasteurized must (100°C for 30 min) was inoculated with a 1 % (v/v) microbial biomass suspension (24 h growth). The must fermentation tests were carried out in shake-flasks incuba-ted at 28°C and 200 rpm on an orbital shaker (New Brunswick Scientific Co., Inc., USA). The weight loss caused by CO_2 production was determined every day until a constant weight has been reached and maintained for three days. The fermentative vigor was expressed as g of CO2 per 100 mL of must.

5.4 Analysis of wine samples

5.4.1 Determination of alcohol content

The alcohol content of all the wines was determined by means of a Malligand ebulliometer (Tecnolab, Belpasso, CT, Italy).

5.4.2 Analysis of total acidity

Total acidity was measured using a titrimetric method in which 25 mL of sample was diluted 1:2 with dd H_2O and, under constant stirring, 4N NaOH was added until the pH value reached 7. The number of NaOH milliequivalents required to neutralize the solution, compared to that required for a one litre volume, equates to the titratable acidity in milliequivalents of tartaric acid per litre (mEq/L). Milliequivalents can be converted into grammes, by considering that a tartaric acid equivalent weighs 75 g.

5.4.3 Analysis of total polyphenols

Total polyphenol content of wines was determined using the Folin-Ciocalteu method adapted to a micro scale (Arnous et al., 2002). 0.79 ml distilled water, 0.01 mL appropriately diluted sample, and 0.05 mL Folin-Ciocalteu reagent were combined in a 1.5 mL polypropylene tube and vortexed. After exactly 1 min, 0.15 mL of sodium carbonate (20%) was added, the mixture vortexed and allowed to stand at room temperature in the dark for 120 min. The absorbance was read at 750 nm, and the total polyphenol concentration was calculated from a calibration curve using gallic acid as standard (60-500 mg/L, R2 = 0.9918, y= 0.001x + 0.0253). Results were expressed as mg/L gallic acid equivalents (GAME).

5.4.4 Total flavonoid assay

Total flavonoid content was measured by the aluminium chloride colorimetric assay (Marinova et al., 2005). An aliquot (1 mL) of diluted sample or standard solution of catechin (10, 40, 80, 120, 150 and 200 mg/L in 80 % MeOH) was added to 10 ml volumetric flask containing 4 mL of dd H_2O. 0.3 ml 5% $NaNO_2$ was then added to the flask. After 5 min, 0.3 mL 10 % $AlCl_3$ was added. After 6 minutes, 2 mL 1 M NaOH was added and the total volume was made up to 10 mL with dd H_2O. The solution was mixed thoroughly and the absorbance measured against a blank prepared at 510 nm. The results were corrected for dilution and expressed as mg/L of catechin.

5.4.5 Determination of total and colored anthocyanins

Measurements were performed using a well-established spectrophotometric method (Somers & Evans 1977). The wine sample was placed in a 0.2-cm path length quartz cuvette, and the absorbance measured at 520 nm (A_{520}). Following this, 0.02ml of a 20 % sodium metabisulphite solution was added, the sample was mixed thoroughly, and after 1 min the absorbance read at 520nm (A_{520} SO_2). 12 % ethanol was used as a blank. All measurements were corrected to a 1.0 cm path length. Further, wine (0.02 mL) was mixed with 0.98mL 1 N HCl solution (dilution 1:50) in a 1.5-mL Eppendorf tube, vortexed, and allowed to stand for 180 min at room temperature. The absorbance was read at 520nm (A_{520}HCl) using a 1.0 cm path length cuvette. For the blank, 0.02 mL of 12 % ethanol was used instead of wine. The concentration of total anthocyanins (TA) and coloured (ionized) anthocyanins (AC) was calculated as follows:

$$TA\ (mg/L) = 20 \times [A_{520}HCl - (5 / 3) \times A_{520}\ SO_2]$$

$$CA\ (mg/L) = 20 \times (A_{520} - A_{520}\ SO_2)$$

5.4.6 Analysis of polyphenols by HPLC

To assess the polyphenol content by HPLC, the sample was centrifuged at room temperature for 10 minutes at 13000 rpm. The supernatant was removed and then filtered through a membrane with a 0.45 μm cut-off (Millipore). The sample was then loaded onto C-18 column Hypersill Gold (Thermo) previously equilibrated with eluent A (2 % acetic acid) flow 1 mL/min., and eluted with eluent B (0.5 % acetic acid in water / acetonitrile 50:50) applying the following gradient:

Time	Eluent A (%)	Eluent B (%)
0	90	10
35	45	55
40	0	100
45	0	100
50	90	10

The peak area obtained is compared to that of runs made with standard known concentrations of polyphenols allowing quantitative determination. The HPLC system used is a Finnigan Surveyor LC Pump with SpectraSystem detector RI-150. The wavelengths analyzed are 280 nm, 320 nm and 370 nm.

5.4.7 Determination of Wine Aroma

5.4.7.1 Sample preparation of HS-SPME-GC/MS analysis

The SPME fiber (PDMS-100um, polydimethylsiloxane) was conditioned according to the manufacturer's recommendations prior to its first use. To a 20 mL Headspace vial 5 mL of wine samples was added, along with 3 g of NaCl and octan-3-ol in hydro-alcoholic solution (1/1, v/v) at 100 μg/L as an internal standard. The solution was homogenized with a vortex shaker and then loaded onto a Gerstel autosampling device. The program consisted of swir-ling the vial at 250 rpm for 5 min at 40°C, then inserting the fiber into the headspace for 30 min at 40°C as the solution was swirled again, then transferring the fiber to the injector for desorption at 240°C for 30 min.13

5.4.7.2 Gas Cromatography-Mass Spectrometry

Gas chromatography analyses were carried out using a 7890 Agilent GC system coupled to an Agilent 5975 inert quadrupole mass spectrometer equipped with a Gerstel MPS2 autosampler. The capillary column employed was a HP-Innowax (Agilent technologies) (30 m x 0,25 mm id. 0,50 μm film thickness) and the carrier gas was helium. Splitless injections were used. The initial oven temperature was set to 40°C for 1 min. The temperature was increased in four steps: 40 – 60°C at 2°C/min; 60 – 150°C at 3°C/min, 150 – 200°C at 10°C/min and 200 – 240°C at 25°C/min; the final temperature was maintained for 7 min. The injector, the quadrupole, the source and the transfer line temperature were maintained at 240°C, 150°C, 230°C and 200°C, respectively. Electron ionization mass spectra in full-scan mode were recorded at 70eV electron energy in the range 40 – 300 amu. Peaks were identified using both the NIST 98 and Wiley libraries. Quantification was performed by using the relative concentration in μg/L of the internal standard, calculated as the ratio between each compound area and the internal standard area. The samples were analyzed in triplicate and blank runs were made by using an empty vial every two analysis (Canuti et al., 2009)

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RESULTS AND DISCUSSION

In the Results the Chapter 6, 7, 8, 9 are manuscripts published in different journals resulting from this experimental thesis. As such, the text and figures in these chapters are formatted differently, according to the requirements of the specified journals.

Chapter 6

Sorrentino A., **Boscaino Floriana**, Cozzolino R., Volpe M.G., Ionata E. and La Cara F., 2012. Autochthonous fermentation starters for the production of aglianico wines. Chemical Engineering Transactions 27:211-216. DOI:10.3303/CET1227036, ISSN 1974-9791

Chapter 7

Sorrentino A., **Boscaino Floriana,** Cozzolino R., Volpe M.G., Ionata E., Guerriero S., Picariello T., La Cara F., 2013. Characterization of Free Volatile Compounds in Fiano Wine Produced by Different Selected Autochthonous Yeasts. Chemical Engineering Transactions 32:1837-1842- DOI: 10.3303/CET 1332307, ISBN 978-88-95608-23-5, ISSN 1974-9791

Chapter 8

Boscaino Floriana, Sorrentino A., Ionata E., La Cara F., Volpe M.G., 2014. Evaluation of autochthonous selected yeasts from grapes and cellar in winemaking of aglianico vine. Bioporspect 24th Volume, 3: 66-70, ISSN: 1210-1737

Chapter 9

Boscaino Floriana, Cutri G., Volpe M.G., Blaiotta G., Sorrentino A., Evolution of polyphenols, volatile aroma compounds and natural yeast flora of Coda di Volpe white grape. Chemical Engineering Transactions 43: ISBN 978-88-95608-34-1; **ISSN** 2283-9216 (accepted, in press)

Chapter 6

Sorrentino A., **Boscaino Floriana**, Cozzolino R., Volpe M.G., Ionata E. and La Cara F., 2012. Autochthonous fermentation starters for the production of aglianico wines. Chemical Engineering Transactions 27:211-216. DOI:10.3303/CET1227036, ISSN 1974-9791

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Autochthonous Fermentation Starters for the Production of Aglianico Wines

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Aglianico cultivar is among the most ancient black grape varieties native of Campania, a region of the Southern of Italy. Traditionally wines are produced by natural fermentation carried out by the autochthonous non-*Saccharomyces* and *Saccharomyces* yeasts present on the grapes and in cellar environment respectively. The aim of this work is the selection and the exploitation of new combinations of microorganisms, isolated from the native microflora of Aglianico grapes and musts, to improve the organoleptic and sensory characteristics of the produced wine. The yeasts isolated and identified by morphological, biochemical and molecular methods mainly belonged to the genera *Saccharomyces, Kloeckera, Candida, Metschnikowia, Hanseniaspora* and *Rhodotorula*. Among them two indigenous strains of *Saccharomyces cerevisiae* and *Metschnikowia fructicula* were characterized through laboratory tests and semi-industrial-scale fermentations, and selected as good candidates for autochthonous fermentation starters. The fermentations carried out with the new starters showed some positive differences compared to those carried out with commercial yeasts: in fact the selected strains efficiently completed the fermentations and positively affected the wine quality. In particular, the indigenous yeasts increased the must total acidity, reaching the expected values of pH and alcohol content, without producing excessive levels of acetic acid. They also enhanced the colour and the content of polyphenols, flavonoids and anthocyanins. HPLC analysis showed a significant increase of gallic acid, catechin and resveratrol concentrations. The results demonstrated that the two strains successfully dominated the fermentation process and contributed to improve the wines' organoleptic quality preserving the peculiarities of these typical regional wines.

1. Introduction

Aglianico cultivar is a red grape variety of Greek origin autochthonous of Irpinia, a geographical area of Southern Italy that has an established wine industry. The oldest and simplest way for wine production is the spontaneous fermentation that is carried out by the indigenous yeasts present on the surface of the grape and on the cellar equipments. (Clemente-Jimenez et al., 2004; Fleet, 2008). The grape epiphytic microflora is responsible for the early stages of the must fermentation and is mainly composed by non-*Saccharomyces* yeasts with a low fermentative power. As the fermentation proceeds, the fermentative *Saccharomyces* yeasts, become predominant, replacing the non-*Saccharomyces* ones. The *Saccharomyces* "sensu stricto" strains are "winery" yeasts present on the winemaking equipments. Each winery is characterized by its own fermentative microflora originated by the selection occurred among the species "resident" in the cellar. Recently, to plan and standardize the winemaking process, the utilization of microbial starter composed by selected *S. cerevisiae* yeasts

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have been suggested. However, this procedure inhibits the growth of the non-*Saccharomyces* strains that greatly contribute to the wine quality (Jolly et al., 2003). In fact, they are involved in the determination of properties such as the colour and aromatic complexity of the wine that are important determinants of product typicality. Several researchers promoted the utilization of such selected yeasts strains in order to get mixed fermentation inocula (Ciani et al., 2010) and many studies revealed significant positive differences in the qualitative and quantitative volatile compounds composition of the wines obtained with a guided fermentations compared to those produced with spontaneous yeasts (Calabretti et al. 2011; Comitini et al. 2011). The aim of our work was the selection and the exploitation of new combinations of autochthonous *Saccharomyces* and non-*Saccharomyces* yeast strains, isolated from the native microflora of Irpinian Aglianico grapes and musts, in order to produce an Aglianico wine with improved organoleptic and sensory characteristics, thus preserving the peculiarities of this typical regional wine.

2. Materials and Methods

2.1 Chemicals

The culture media for the isolation, the reactivation and the growth of different yeast strains were purchased from Oxoid (Hampshire, UK). Chemical reagents and solvents are all of analytical grade and were from SIGMA-Aldrich (St.Louis, MO, USA).

2.2 Isolation and molecular identification of yeasts

To isolate the different yeasts populations, serial dilutions of Aglianico grape samples, previously homogenized in sterile Ringer solution, were plated on three different solid agar media: YPD, WL-nutrient and Lysine-agar On WL medium, yeast species have been distinguished by different colony morphologies and colours. The isolation of non-*Saccharomyces* species has been carried out on Lysine medium, in which *Saccharomyces spp* could not grow (Calabretti et al., 2011). The yeast species identification was performed by the analysis of the D1/D2 domain of 26S rDNA sequence. The genetic region was PCR amplified directly from individual yeast colony following the protocol, as described by Arroyo-Lopez et al. (2006). The standard primers utilized were the commonly referred as NL1 and NL4 in the literature (O'Donnell, 1993).

2.3 Determination of the yeast fermentative power

One hundred mL of pasteurized must (100 °C for 30 min) was inoculated with a 1 % (v/v) microbial biomass suspension (24 h growth). The must fermentation tests were carried out in shake-flasks incubated at 28 °C. The flasks weight decrease was measured every day until a constant weight has been reached and maintained for three days.

2.4 Yeasts selection for the resistance to sulphur dioxide

The tests were carried out on Aglianico must, it was pasteurized for 30 min and inoculated with a 1 % of microbial biomass suspension (24 h growth). Potassium metabisulfite (MBK) was used as the source of sulphur dioxide and the yeasts resistance at concentrations of 100 and 250 mg/L was tested. The tests were performed at 28 °C for 7 days (Costanti et al. 1998).

2.5 Growth of yeast strains

The selected yeast strains were inoculated in 100 mL YPD liquid medium. The growth was carried out at 28 °C in shake-flasks at 200 rev min^{-1} (rpm) in an orbital shaker (New Brunswick Scientific Co., Inc., USA) for 24 h. The obtained yeast suspension was used to inoculate, at (1 % v/v) 10 L of YPD liquid medium in a pilot plant fermenter. The obtained biomass was stored at 4 °C.

2.6 Winemaking process

Aglianico grapes were harvested at 18.4 °Brix, destemmed, pressed and then fermented with their skins. MBK was added at a final concentration of 100 mg/L to the juice and, after the addition of 20 mg/L of Trenolin® Rouge DF pectolitic enzyme (Erbslöh Geisenheim AG, Geisenheim, Germany), it was warmed at 18 °C for 18 h. The must was then divided in five 30 L tanks for fermentation heated up to 26 °C. All the tanks with the exception of one that was utilized as control were inoculated with different combination of the selected autochthonous strains *Saccharomyces cerevisiae* AGYP37 and

Metchnikowia fructicola AGYP28*,* with or without the commercial yeast (C.Y.), (about 3 x 10^6 CFU/mL). The control was inoculated with 0.15 g/L (3.2 x10^{10} CFU/g) of dry *S. cerevisiae* Oenoferm® Structure (Erbslöh) previously rehydrated according to manufacturer instructions. At the beginning of the fermentation a mobilisator and a nutrient (0.2 g/L Vita*Drive*®, Vitamon® Combi from Erbslöh) were added. Moreover, in the middle of fermentation, another aliquot of the nutrient, at the same concentration, was added. The fermentation process was completed in 10 days and when the residual sugars concentration reached a value smaller than 2 g/L, 60 mg/L of MBK was added. The wine was decanted with aeration for 3 days followed by 10 days without aeration. Sugars evaluation was carried out by densitometric analysis using a Babo mustimeter.

2.7 Analysis of total polyphenols, flavonoids and anthocianins

Total polyphenol content was determined using the Folin-Ciocalteu reagent (Waterman and Mole, 1994) following a micro scale as described by Arnous et al. (2002). The absorbance at 750 nm was recorded, and the total polyphenol concentration was calculated from a calibration curve using gallic acid as standard and expressed as mg gallic acid equivalents/L (GAME). The total flavonoid content was determined by the aluminium chloride colorimetric method as reported by Marinova et al. (2005). The assay was carried out utilizing aliquots of 1 mL of Aglianico wine diluted with methanol (ratio 1:9) and catechin solutions in 80 % methanol as standards. The absorbance at 510 nm was measured and the total flavonoid concentration was calculated from a calibration curve using catechin as standard and expressed as mg catechin equivalents/L (CE). Total and colored anthocyanins measurements were performed using a well-established spectrophotometric method (Arnous et al. 2002).

2.8 HPLC analysis of phenolic compounds

Phenolic compounds analysis was performed by HPLC/UV with a C18 column Hypersil (Thermo Electron Corporation, Bellefonte, PA, North America). The injected sample volume was 20 µL. Peak identifications were confirmed from retention times and direct comparison to pure standards. The solvent flow rate was 0.9 mL/min and the mobile phase was a four-step linear solvent gradient system (0–5 min, 10 % B; 5-40 min, 45 % B; 40–45 min, 100 % B; 45-50 min, 100 % B; 50-55 min, 10 % B) using 2 % acetic acid in water as solvent A and 0.5 % acetic acid in 50 % acetonitrile as solvent B. UV detection was carried out at 280 nm and 254 nm.

3. Results and Discussion

Microbiological analysis of Aglianico must microflora revealed the presence of species mainly belonging to the *Hanseniaspora, Kloeckera, Metchnikowia, Candida* and *Saccharomyces* genera according to Beltran et al. (2002). In particular *Hanseniaspora, Kloeckera, Metchnikowia* were predominant in the must before the addition of MBK, after the inoculation and in the first days of the fermentative process. These species, except *Metschnikowia* spp.*,* together with *Saccharomyces* spp. were present during the early stages of the alcoholic fermentation, due to their ability to tolerate alcoholic concentrations up to 3 or 4 % alcohol by volume (Hierro et al., 2006). A non-*Saccharomyces* (AGYP28) and a *Saccharomyces* (AGYP37) strain were selected for their resistance to sulphur dioxide and the alcohologenic and fermentative power (data not shown). The experimental winemaking processes carried out with the Aglianico must inoculated with the starters obtained from a combination of the isolated yeasts and the commercial *S. cerevisiae* (C.Y.) showed that the autochtonous yeasts were able to trigger and complete the alcoholic fermentation leaving a sugar residue smaller than 2 g/L (Figure 1).

Moreover content in polyphenols, flavonoids and anthocyanins was increased compared to that obtained with C.Y., as reported in Figure 2 and 3. In particular, the best yield in polyphenols and flavonoids were obtained using a combinations of the two autochthonous yeasts (AGPY37, AGYP28) (Figure 2).

It is well known that polyphenol compounds have positive effects against the reactive oxygen species (ROS), that are involved in many diseases, such as cardiovascular and chronic kidney pathologies, certain types of cancer, neurological disorders, inflammation and hypertensive conditions (Zenebe et al., 2001).

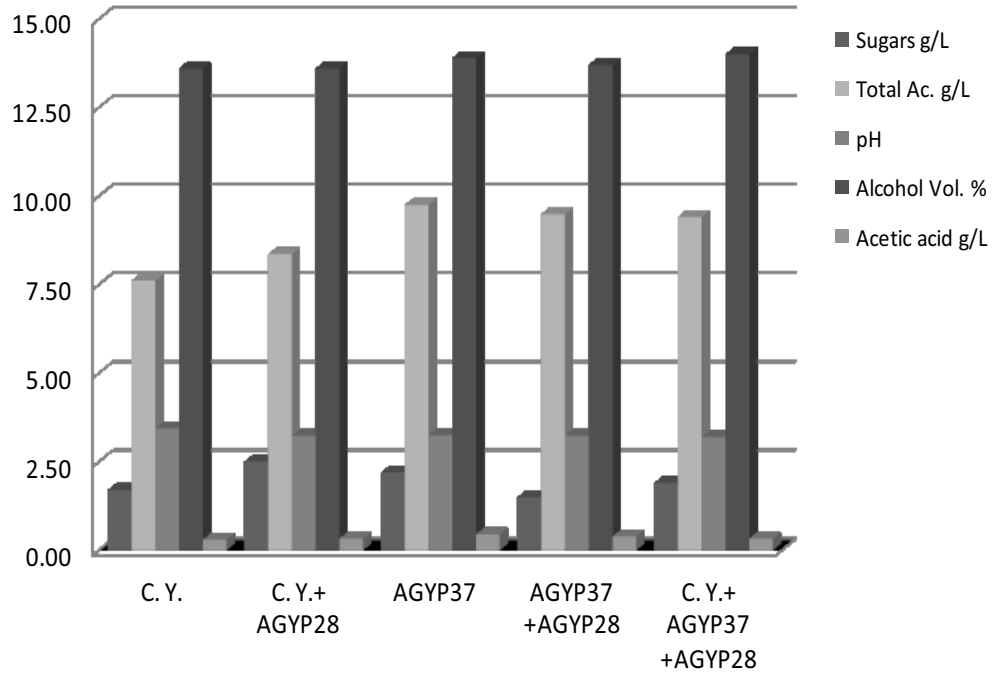

Figure 1: Oenological parameters in Aglianico fermentations

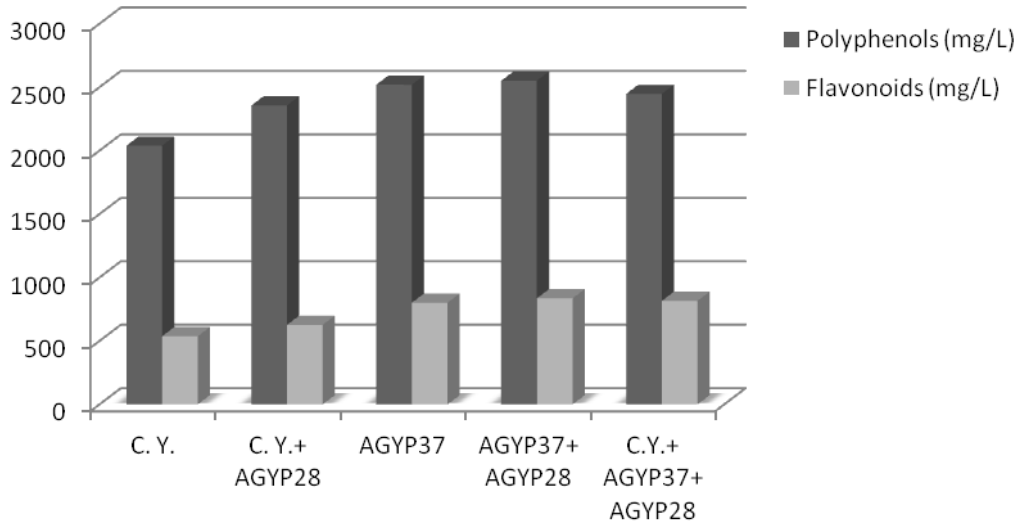

Figure 2: Concentrations of polyphenols and flavonoids in Aglianico fermentations

Therefore, the production of wine with an enhanced content of these molecules could be desirable. The flavonoid content generally increases utilizing the autochthonous yeasts reaching the highest value for the fermentation with *S. cerevisiae* AGPY37 and *M. fructicola* AGYP28. On the other hand, the maximum concentration of total antocyanins was reached with a the combination of C.Y. and AGYP28 (Figure 3).

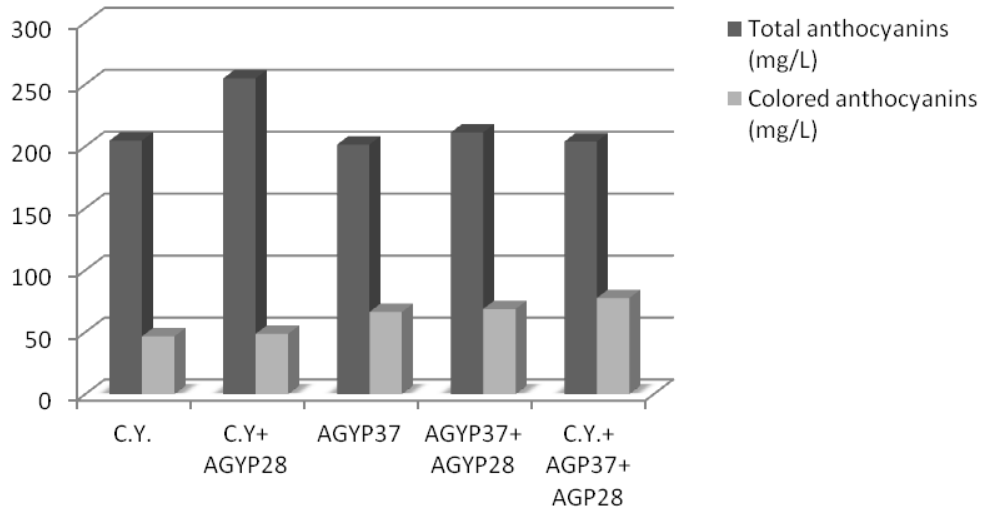

Figure 3: Concentrations of anthocyanins in Aglianico fermentations

The results of HPLC analysis obtained for the Aglianico wine are reported in Table 1. The fermentation experiments with native yeasts showed that the gallic acid content was higher in wines obtained with one or both autochthonous strains. The presence of a larger amount of gallic acid could be a consequence of hydrolysis of gallotannins and esters of gallic acid with glucose. In the fermentations carried out with indigenous strains, the level of catechins increased of about 25 % while the epicatechin content remained unchanged. In the wine produced with the combination of all three yeasts, the level of catechins did not change while the epicatechin content drastically decreased. Coumaric acid and cinnamic acid concentrations always substantially decreased in the presence of native yeasts. The resveratrol content was higher in wines produced with the three strains or with a combination containing the native yeast AGPY37. On the contrary, wine obtained with the combination C.Y./AGYP28 had the lowest content of resveratrol.

Table 1: Final concentrations of phenolic compounds in Aglianico wines obtained after the different fermentations

Compounds mg/L	C.Y.	C.Y. AGPY28	AGPY37	AGPY37 AGPY28	C.Y. AGPY37 AGPY2
Gallic Acid	46.1 ± 2.0	54.2 ± 2.1	61.3 ± 4.2	59.2 ± 3.2	51.1 ± 3.0
Catechin	75.3 ± 4.4	94.2 ± 5.2	97.1 ± 6.1	95.3 ± 5.1	72.2 ± 5.2
Chlorogenic acid	n.d.	n.d.	36.3 ± 2.4	n.d.	n.d.
Epicatechin	57.3 ± 3,4	68.4 ± 4.3	60.2 ± 4.1	57.1 ± 4.2	5.1 ± 0.3
Coumaric acid	18.0 ± 1.0	6.1 ± 0.4	14 ± 0.9	11 ± 0.9	9 ± 0.6
Resveratrol	6.0 ± 0.5	4.3 ± 0.4	7.0 ± 0.5	8.1 ± 0.5	8.4 ± 0.1
Cinnamic acid	11.0 ± 1.1	3.1 ± 0.1	3.0 ± 0.1	7.3 ± 0.5	0.5 ± 0.1

Interestingly, the wine produced with AGYP37 had the highest level of gallic acid and catechin and only in this wine chlorogenic acid was detected. As the absence of this compound, usually present in wine, may be correlated with the presence of an enzymatic activity capable of splitting the ester-linkages between cinnamic and quinic acid, it could be suggested that this activity is not repressed in the fermentation with AGYP37. Finally, the combination of the two native strains led to production of wines with the highest resveratrol level, also in the presence of C.Y.

In conclusion, the results demonstrated that the two strains successfully dominated the fermentation process and contributed to improve the wines' organoleptic quality preserving the peculiarities of these typical regional wines.

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Chapter 7

Sorrentino A., **Boscaino Floriana,** Cozzolino R., Volpe M.G., Ionata E., Guerriero S., Picariello T., La Cara F., 2013. Characterization of Free Volatile Compounds in Fiano Wine Produced by Different Selected Autochthonous Yeasts. Chemical Engineering Transactions 32:1837-1842- DOI: 10.3303/CET 1332307, ISBN 978-88-95608-23-5, ISSN 1974-9791

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Characterization of Free Volatile Compounds in Fiano Wine Produced by Different Selected Autochthonous Yeasts

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Fiano cultivar is widespread in the South of Italy and is the most representative white wine variety in the Campania region designated as DOCG (Denomination of Controlled and Guarenteed Origin). For many years, wines have been produced by natural fermentation carried out by non-*Saccharomyces* and *Saccharomyces* yeasts originate from both the grapes and the cellar. Recently, non-*Saccharomyces* yeasts role in the wine production has been reassessed, consequently to the contribution that they give in the first part of the fermentation process which positively influences the qualitative features of the wine flavor, that is notoriously ''the backbone'' of the wine quality. It is therefore critical to increase and improve such aromas in order to characterize the typicalness of the native vineyards. The aim of this study was the selection of both non-*Saccharomyces* and *Saccharomyces* yeasts, native of Fiano grape and must. Among them, two indigenous strains, *Hanseniaspora guilliermondii* and *Saccharomyces cerevisiae,* were selected to prepare new different combination of autochthonous selected yeast strains to compare with commercial yeast *S. cerevisiae*. These combinations were applied for experimental cellar wine productions and the new starters showed some peculiar aroma production compared to commercial yeast. Moreover, in order to evaluate the aromatic profiles, the different wine samples were characterized by Solid Phase Micro Extraction–Gas Chromatography/Mass Spectrometry (SPME-GC/MS) technique. The results showed that the two native strains successfully dominated the fermentation process and contributed to improve the wines aromatic profiles.

1. Introduction

Many studies have been carried out on the ecology of wine yeasts and established the complexity of alcoholic fermentation, whether spontaneous or inoculated. It is now accepted that wine fermentation involve the growth of non-*Saccharomyces* and *Saccharomyces* species, and that the former play a relevant role in the organoleptic characteristics of a wine (Fleet, 2008). Today, the use of selected commercial yeasts from *Saccharomyces* species "sensu stricto" is widespread, because it allows to minimize risks in the production process, to standardize the winemaking procedure and ensure the quality of the final product. On the other hand, the widespread use of starter cultures in the wine industry has resulted in a loss of wine sensory characteristics and in a flattening of those differences crucial to distinguish wines of different cultivars. For this reason, research in the wine industry is focused on studying new yeast cultures that can enrich the final product, especially with those aromatic compounds which enhance the character of the wine. It is known that the production of many of this aromatic molecules occurs due to the presence of non-*Saccharomyces* yeasts, found on the grapes and the must. These non-*Saccharomyces* strains that predominate in the first phase of the alcoholic fermentation are able to release metabolites and enzymes, responsible of the aromatic complexity of the wine (Romano et al., 2003; Viana et al., 2011). In this regard, several researchers have promoted the utilization of such selected yeasts strains in order to get mixed fermentation inocula (Ciani et al., 2010) and many studies revealed significant positive differences in the qualitative and quantitative volatile compounds profile of the wines obtained with a guided fermentations compared to those produced with spontaneous yeasts (Calabretti et al., 2011). In the literature, a lot examples of analytical methods for studying

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the composition of volatile compounds of the wine are present (Canuti et al., 2009) and some of them have been carried out by SPME-GC/MS (Pawliszyn J. 2009). The aim of our work was the exploitation of new combinations of selected yeasts, obtained from the native microflora of Fiano grapes and musts, not only to improve the quality and organoleptic characteristics of the final product, also to emphasizeing its link with the territory and the autochthonous wine cultivar.

2.Materials and Methods

2.1 Chemicals

The culture media for the isolation, the reactivation and the growth of yeast strains were purchased from Oxoid (Hampshire, UK). All the enzymes and nutrients was obtained from Erbslöh Geisenheim AG, Geisenheim,Germany. Chemical reagents and solvents are all of analytical grade and were from Sigma-Aldrich (St.Louis, MO, USA). The SPME fibers PDMS- 100μm (polydimethylsiloxane), was from Supelco (Bellefonte, PA, USA).

2.2 Isolation and molecular identification of yeasts

To isolate the different yeasts populations, serial dilutions of Fiano grape and must samples, previously homogenized in sterile Ringer solution, were plated on two different solid agar media: WL-nutrient and Lysine-agar. On WL medium, yeast species have been distinguished by different colony morphologies and colours. The isolation of non-*Saccharomyces* species has been carried out on Lysine medium, that does not allow the growth *Saccharomyces spp.* The yeast species identification was performed by the analysis of the D1/D2 domain of 26S rDNA sequence. The genetic region was PCR amplified directly from individual yeast colony, following the protocol described by Arroyo-Lopez et al. (2006). The standard primers utilized were the commonly referred as NL1 and NL4 in the literature (O'Donnell 1993).

2.3 Determination of the yeast fermentative power

The must (100ml) after pasteurization (100 °C for 30 min) was inoculated with a 1 % (v/v) microbial biomass suspension (24h growth). The must fermentation tests were carried out in shake-flasks incubated at 28 °C. The flasks weight decrease was measured every day, until a constant weight has been reached and maintained for three days.

2.4 Yeasts selection for the resistance to sulphur dioxide

The tests were carried out on Fiano must which was pasteurized and inoculated with a 1 % microbial biomass suspension (24h growth). Potassium metabisulfite (MBK) was used as the source of sulphur dioxide and the yeasts resistance at concentrations of 100 and 250 mg/L was checked. The tests were performed at 28 °C for 7 days.

2.5 Growth of yeast strains

The selected yeast strains were inoculated in 100 mL YPD broth (2 % pepton+2 % dextrose+1 % yeast extract + 2 % agar). The growth was carried out at 28 °C in shake-flasks at 200 rev min^{-1} (rpm) in an orbital shaker (New Brunswick Scientific Co., Inc., USA) for 24 h. The obtained yeast suspension was used to inoculate, at (1 % v/v) 10 L of YPD broth, in a pilot plant fermenter. The obtained biomass was stored at 4 °C.

2.6 Winemaking process

Fiano grapes were harvested at 21.1 °Brix in a vineyard located in the area surrounding Avellino a town of the Campania region. The winemaking process was carried out in the cellar of ''Istituto Tecnico Agrario, F. de Sanctis'' in Avellino. The Fiano grapes were crushed, destemmed and separated from the skins by pressing. One hundred milligrams of potassium metabisulfite per liter of obtained juice were added, which was subsequently clarified by cold settling at 10 °C for 18 h, with the addition of 20 mg/L Trenolin® Opti DF pectolitic enzyme. The juice was then racked, divided in five 50 L tanks, warmed up to 16 °C, one of which inoculated with 0.15 g/L ($4.8x10^6$ cells/mL) of dry *S. cerevisiae* (var. *bayanus*) (ST5), Oenoferm® Freddo ($3.2 x 10^{10}$ CFU/g) (Erbslöh Geisenheim AG, Geisenheim, Germany), previously rehydrated according to manufacturer instructions. The other four tanks were inoculated with $2x10^6$ cells/mL of different selected yeast (commercial and autochthonous) combination: FWL66 (*H.guillermondii)*+FYP69 (*S. cerevisiae)* (ST1); FYP69 (ST3); ST5+ FWL66 + FYP69 (ST2); ST5+FWL66 (ST4). A quantity of 0.2 g/L of a biological mobilisator and nutrient (VitaDrive®, Vitamon® Combi), respectively, was added at the beginning of fermentation. Moreover, 0.2 g/L of a nutrient (Vitamon® Combi) were added in the middle of fermentation and the process was completed in 21 days, when the residual sugars concentration was<2 g/L. The wine was racked and cold settled for 4 months at 10 °C after the addition of 60 mg/L of potassium metabisulfite. On the Fiano wine obtained, following OIV methods (www.oiv.int), chemical-physical analyses were performed.

2.7 Monitoring of yeast populations during winemaking process

In order to monitor the alcoholic fermentation, samples of must were taken before the addition of potassium metabisulfite, after the inoculum with the different selected yeast strain (autochthonous and commercial) and at different intervals of times during the process. The samples were withdrawn in sterile containers and maintained at 4 °C, until the laboratory analysis was performed. Serial dilutions of the samples were plated onto WL Nutrient (WL) to evaluate the total yeast populations and to analyze the different colony morphologies and on Lysine Medium to assess the presence of non-*Saccharomyces* yeasts. The plates were incubated at 28 °C for 5 days to allow the development of the colonies and viable counts.

2.8 Sample preparation of HS-SPME-GC analysis

The SPME fiber (PDMS-100μm, polydimethylsiloxane) was conditioned according to the manufacturer's recommendations prior to its first use. To a 20 mL Headspace vial was added 5 mL of wine samples, together to 3g of NaCl and octan-3-ol, in hydro-alcoholic solution (1/1, v/v) at 100 μg/L, as Internal Standard. The solution was homogenized with a vortex shaker and then loaded onto a Gerstel autosampling device. The program consisted of swirling the vial at 250 rpm for 5 min at 40 °C, then inserting the fiber into the headspace for 30 min at 40 °C as the solution was swirled again, then transferring the fiber to the injector for desorption at 240 °C for 30 min.

2.9 GasCromatography-MassSpectrometry

Gas chromatography analysis were carried out using a 7890 Agilent GC system coupled to an Agilent 5975 inert quadrupole mass spectrometer equipped with a Gerstel MPS2 autosampler. The capillary column employed was a HP-Innowax (Agilent technologies) (30 m x 0,25 mm id. 0,50 μm film thickness) and the carrier gas was Helium. Splitless injections were used. The initial oven temperature was set to 40 °C for 1 min. The temperature was increased in four steps: 40–60 °C at 2 °C/min; 60–150 °C at 3 °C/min, 150–200 °C at 10 °C/min and 200-240 °C at 25 °C/min; the final temperature was maintened for 7 min. The injector, the quadrupole, the source and the transfer line temperature were maintained at 240 °C, 150 °C, 230 °C and 200 °C, respectively. Electron ionization mass spectra in full-scan mode were recorded at 70eV electron energy in the range 40–300 amu. Peaks were identified using both the NIST 98 and Wiley libraries. Quantification was performed by using the relative concentration in μg/L of the Internal Standard, calculated as the ratio between each compound area and the internal standard area. The samples were analyzed in triplicate and blank runs were made by using an empty vial every two analysis.

3.Results and discussion

3.2 Chemical-physical analyses

The chemical-physical analyses conducted on the Fiano wine, obtained employing the different winemaking starters selected in this study, are reported in Table 1

Table 1. Chemical-physical analyses of Fiano wine obtained with different winemaking starters(OIV met)

	ST1	ST2	ST3	ST4	ST5
Total acidity	6,53 g/L	6,37 g/L	5,55 g/L	7,58 g/L	7,73 g/L
Volatile acidity	0,30 g/L	0,30 g/L	0,30 g/L	0,42 g/L	0,39 g/L
pH	3,24	3,27	3,40	3,16	3,13
Sulfur dioxide	38,4 mg/L	44,8 mg/L	38,4 mg/L	57,6 mg/L	38,4 mg/L
Density	0,99275	0,99275	0,99195	0,99315	0,99325
% Alcohol	11,76 %vol	11,75 %vol	11,88 %vol	11,80 %vol	11,71 %vol
Total dry matter	21,7 g/L	21,6 g/L	19,5 g/L	22,8 g/L	22,7 g/L

In the Table 1, the quantities of free sulfur dioxide, pH, volatile acidity, density, % alcohol, total dry matter and total acidity in the experimental wines are presented. All values are the average of two duplicate experiments and were within the ranges acceptable for the wine industry. No significant differences in the levels of these items among the wines were observed.

3.1 Monitoring of yeast populations during the winemaking process

Microbiological analysis of Fiano grape and must microflora revealed the presence of species belonging mainly to the *Hanseniaspora, Kloeckera, Candida* and *Saccharomyces* genera, according to Beltran et al. (2002). In particular, *Hanseniaspora* and *Kloeckera* were predominant in the must before the addition of MBK, after the inoculation and in the first days of the fermentative process. These non-*Saccharomyces* yeast species were usually present, together with *Saccharomyces* spp., during the early stages of the alcoholic fermentation, due to their ability to tolerate alcoholic concentrations up to 3 or 4 % alcohol by volume(Fleet 2008). From the results obtained from technological experiments, such as resistance to sulphur dioxide and the alcohologenic and fermentative power (data not shown), two strains of non-*Saccharomyces* (FWL66) and *Saccharomyces* (FYP69) were selected. These two strains, by the analysis of the D1/D2 domain of 26S rDNA

sequence, have been identified as belonging to *H. guilliermondii* and *S. cerevisiae* species. The experimental winemaking processes carried out with the Fiano must inoculated with the starters obtained from a combination of the isolated yeasts and the commercial *S. cerevisiae* showed that the autochtonous yeasts were able to trigger and complete the alcoholic fermentation, leaving a sugar residue smaller than 2 g/L, as reported in Figure 1.

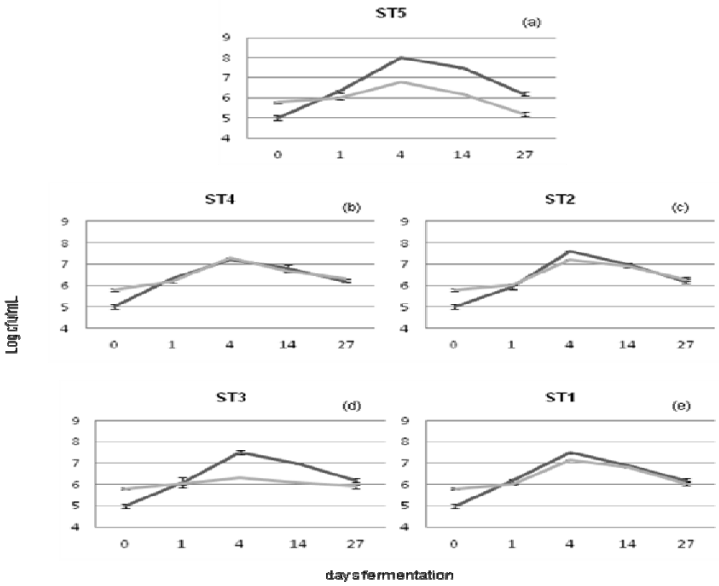

Figure1: Evolution of yeast population during winemaking process of must inoculated with selected yeast mix (□) Saccharomyces; (□) non-Saccharomyces:ST1 (S.cerevisiae FYP69 + H.guilliermondii FWL66);ST2 (Oenoferm® Freddo + S.cerevisiae FYP69 + H.guilliermondii FWL66); ST3 (S.cerevisiae FYP69); ST4(Oenoferm® Freddo + H.guilliermondii FWL66); ST5 (Oenoferm® Freddo)

Figure 1 shows the kinetics of fermentations obtained by employing the 5 starters used in the present trial. From the curve profile of fermentation conducted by commercial yeast (ST5), used as control (Figure 1.a), it is possible to noted that the population of the *Saccharomyces* yeast reaches an order of magnitude of about 10*8 cfu/mL during the fourth day of the process, remaining high for the entire winemaking process. Furthermore, the population of non-*Saccharomyces* yeasts is higher at the beginning of the winemaking process and then decreases during the other phases of the process, as already reported (Ciani et al.,2010; Prakitchaiwattana et al., 2004). The growth curves of native selected cultures, ST3 and ST1 (Figure 1.d and 1.e), show that native *S.cerevisiae* yeast (FYP69) is able to start and complete the winemaking process reaching, after 4 days, a population higher than 10*7 cfu/mL and also to counteract the growth of non-*Saccharomyces* microflora, naturally present in the must (Figure 1.d). On the other side, Figure 1.e, related to the starter ST1, we can note that the yeast *H.guilliermondii* (FWL66) was able to grow and to show a growth trend similar to that of *S.cerevisiae* (FYP69). The Figure 1.b and 1.c show the behavior of the yeast mixtures containing the commercial yeast. In Figure 1.b we can see that both the curves relating to commercial yeast and non-*Saccharomyces* yeasts are very similar, presenting a comparable kinetics of growth. This behavior can be explained by the fact that the selected non-*Saccharomyces* yeast is able to compete both with the commercial yeast and with the autochthonous *S.cerevisiae* yeast (Figure 1.d). This result, strengthening what is observed in Figure 1.a and 1.d, related to cultures in which the yeast FWL66 is absent, allows to conclude that *S. cerevisiae* yeasts (both commercial and autochthonous) are able to predominate and limit the growth of the non-*Saccharomyces* component.

3.3 Volatile compounds detected by SPME-GC/MS analysis

The data about the volatile compounds identified in Fiano wine samples by SPME-GC/MS, reported in Table2, are in general agreement with those reported in literature (Ugliano et al., 2008; Genovese et al.,2007). In particular, according to Romano et al. (2003), it is to underline that the differences in the volatile molecules composition of wine samples, obtained utilizing different yeast species, appear to be quantitative rather than qualitative. Ethyl esters of straight-chain fatty acids and acetates of higher alcohols are the dominating esters in wine samples and they are formed during the alcoholic fermentation process. The aroma compounds isoamyl acetate, ethyl caproate, ethyl caprylate, ethyl caprate and diethyl succinate were determined as the major esters. Also, other types of acetates such as ethyl acetate, hexyl acetate and phenylethyl acetate were

identified. Hexanoic, octanoic and decanoic acids, which also contribute to the aroma of the wine, were detected as the most abundant acids. Acetaldehyde and benzaldehyde were identified in the aldehydes class, while, in the alcohol group, isobutyl alcohol, 1-butanol, isoamyl alcohol, 1-hexanol, 1-pentanol-4-methyl, 1-pentanol-3-methyl, (Z)-3-hexen-1-ol and 2-phenylethanol were detected. Terpenes have been found to play an important role in the aroma composition of wine and have been used to differentiate different grape cultivar (Komes et al., 2006). Specifically, among this class of compounds, in the Fiano wine samples, linalool, 4-terpineol, α-terpineol, geraniol, nerol and β-citronellol were identified, whereas in the class of nor-isoprenoids, α-ionone, β-damascenone, TDN and vitispiranes were determinated. Furthermore, the volatile phenol and 4-vinylguaiacol was identified. Among the esters, ethyl caproate, ethyl caprate and ethyl caprylate, responsible for the fruity, green apple and soap flavor, appear to be present in higher concentration. In particular, the amounts of these compounds seem to be comparable in wines produced by using commercial *S. cerevisiae* (ST5) or autochthonous *S. cerevisiae*, both alone than mixed with other yeast (ST3, ST1) (see also table 3). Even the amount of diethylsuccinate, which mainly contributes to create the body of the wine (Viana et al., 2011); appears to be present in a slightly higher quantity in wines obtained by using ST1 starter, compared to wine samples produced by other starters. Among terpenes, linalol (floral aroma), α-terpineol (lilac aroma), geraniol (floral and orange citrus flavor) and β-citronellol (citrus and grapefruits flavor) (Genovese et al., 2007), are present in higher amount in wines produced by ST3 and ST1 starters, compared to control starter (ST5).

Table 2. Volatile compounds detected on wines obtained by using different starters in winemaking process

	ST3	ST1	ST4	ST2	ST5
	µg/L ± SD	µg/L ± SD	µg/L ± SD	µg/L ± SD	µg/L ± SD
aldehydes					
acetaldehyde	20,16 ±1,81	12,4±0,10	6,7±0,14	9,44±0,08	15,04±0,11
benzaldehyde	57,58±0,83	80,94±0,78	37,36±1,57	76,00±1,09	46,39±1,46
alcohols					
isobutyl alcohol	60,99±1,4	77,27±0,37	85,83±0,03	68,57±0,45	113,65±2,31
1-butanol	1,35±0,04	1,29±0,06	3,55±0,07	1,33±0,04	6,48±0,33
isoamyl alcohol	2017,11±6,79	2409,24±11,65	2109,58±12,73	1906,4±1,39	2672,75±26,52
1-pentanol,4-methyl	3,57±0,11	4,07±0,32	3,56±0,09	3,41±0,21	3,2±0,66
1-pentanol,3-methyl	4,73±0,25	5,95±0,22	5,71±0,29	4,89±0,43	5,01±0,37
1-hexanol	82,29±0,42	94,81±1,00	86,38±4,02	86,34±0,48	91,72±0,67
3-hexen-1-ol cis	4,44±0,27	5,04±0,21	3,68±0,57	3,73±0,25	4,56±0,43
2-phenylethanol	743,54±2,18	1156,66±7,16	638,54±2,76	779,3±0,43	511,16±1,64
esters					
ethyl acetate	865,88±4,08	867,06±9,91	908,26±2,82	728,45±4,88	943,23±1,73
isobutyl acetate	4,55±0,07	4,00±0,81	2,96±0,07	4,2±0,07	7,39±0,20
ethyl butanoate	92,66±1,17	87,66±0,51	85,74±1,42	81,02±0,02	96,81±1,42
ethyl 2methylbutyrate	15,39±0,56	11,66±0,9	7,14±0,19	15,39±0,22	16,43±1,42
ethyl isovalerate	21,35±0,39	22,06±0,56	9,44±0,02	19,59±0,49	7,03±0,04
isoamyl acetate	105,73±2,45	84,28±0,05	79,34±0,80	101,17±1,44	137,08±1,18
ethyl caproate	1645,6±7,91	1128,15±14,35	1504,27±4,62	1334,77±6,75	1435,93±18,28
hexylacetate	3,4±0,15	2,72±0,36	3,92±0,92	3,47±0,31	3,58±0,38
ethyl lactate	66,39±0,55	99,38±0,38	43,74±0,33	56,28±0,23	72,3±1,41
ethyl caprylate	7746,58±9,31	5500,6±1,52	8981,06±12,65	6724,19±17,24	7855,11±7,23
ethyl caprate	3865,54±5,01	2788,33±11,38	5541,41±33,36	4014,02±2,85	3631,37±9,00
diethyl succinate	264,92±4,13	294,12±5,66	205,1±2,83	343,81±2,56	269,99±14,13
phenethyl acetate	16,58±0,53	19,72±0,97	13,88±0,35	83,18±0,25	9,83±0,43
acids					
hexanoic acid	88,43±0,60	114,17±1,39	60,6±2,27	167,43±0,60	81,42±0,27
octanoic acid	1230,83±1,18	1336,51±7,05	995,44±1,59	1198,73±3,85	1017,64±3,74
decanoic acid	883,43±2,02	822,57±9,9	901,58±10,49	844,72±1,01	584,97±4,20
terpenes					
linalol	35,58±1,59	30,99±1,51	36,24±0,84	32,78±3,02	27,57±0,06
4-terpineol	5,99±0,05	5,6±0,23	5,26±0,16	5,97±0,33	5,43±1,15
α-terpineol	21,55±1,27	19,07±1,30	13,28±1,56	18,4±0,85	18,24±0,71
geraniol	6,05±0,31	5,74±0,03	6,37±0,67	3,63±1,70	5,4±0,55
nerol	3,97±0,21	3,95±0,41	5,47±2,5	3,00±0,97	3,67±0,81
β-citronellol	7,45±7,45	6,71±0,01	3,61±0,54	8,27±0,19	4,13±0,03
Nor-isoprenoids					
α-ionone	1,71±0,42	1,2±0,28	0,51±0,04	0,32±0,10	0,18±0,01
β-damascenone	11,7±1,83	15,06±1,14	14,94±3,00	14,38±4,40	9,8±2,00
vitispirane	28,14±0,89	16,05±0,16	16,24±0,30	39,20±0,68	18,53±0,81
TDN	7,64±0,91	3,01±0,28	6,12±0,58	6,78±0,27	6,76±0,33
other					
4-vinylguaiacol	8,71±0,66	6,86±0,09	9,23±0,09	4,87±1,01	12,76±0,57

Vitispirane and β-damascenone are the most abundant norisoprenoids found in the present study, but, it is very reasonably that, between them, the contribution to the flavor is mostly given by β-damascenone (roses and honey aroma), which is present in concentrations higher than the threshold of perception (0.002 μg/L) (Fan et al., 2010). Furthermore, wine samples produced by using ST1 and ST3 contain a higher amount of β-damascenone compared to that produced by ST5 starter. The concentrations of TDN and the 4-vinylguaiacol are similar in all the experimental wines obtained and, being below the threshold of perception, their contribution to the aroma can be considered negligible (Fan et al., 2010). Benzaldehyde, which gives a characteristic sweety and fruity aroma, is more abundant in wines obtained by employing ST1 and ST3 starters compared to ST5. Among the alcohols, 2-phenylethanol (rose fragrance) presents a higher concentration in wines produced by ST3 and ST1 compared to that derived by commercial yeast (ST5). Finally, wines produced using ST2 and ST4 starters have showed a volatile compounds content intermediate, compared to those of the products obtained by ST3, ST1 and ST5 starters. This result can be probably due to the presence in ST2 and ST4 mixtures of commercial yeast, which did not confer a relevant qualitative and quantitative difference to the wine, in comparison with the products obtained utilizing autochthonous starter.

4.Conclusions

In conclusion, many non-*Saccharomyces* yeasts showed interesting oenological properties, in terms of production of ethanol, secondary metabolites and fermentation purity. When used in mixed cultures with *S. cerevisiae*, these non-*Saccharomyces* strains can contribute to increase the production of volatile compounds, enhance the characteristics of the cultivar and then strengthen the concept of "terroir" of a wine.

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Chapter 8

Boscaino Floriana, Sorrentino A., Ionata E., La Cara F., Volpe M.G., 2014. Evaluation of autochthonous selected yeasts from grapes and cellar in winemaking of aglianico vine. Bioporspect 24th Volume, 3: 66-70, ISSN: 1210-1737

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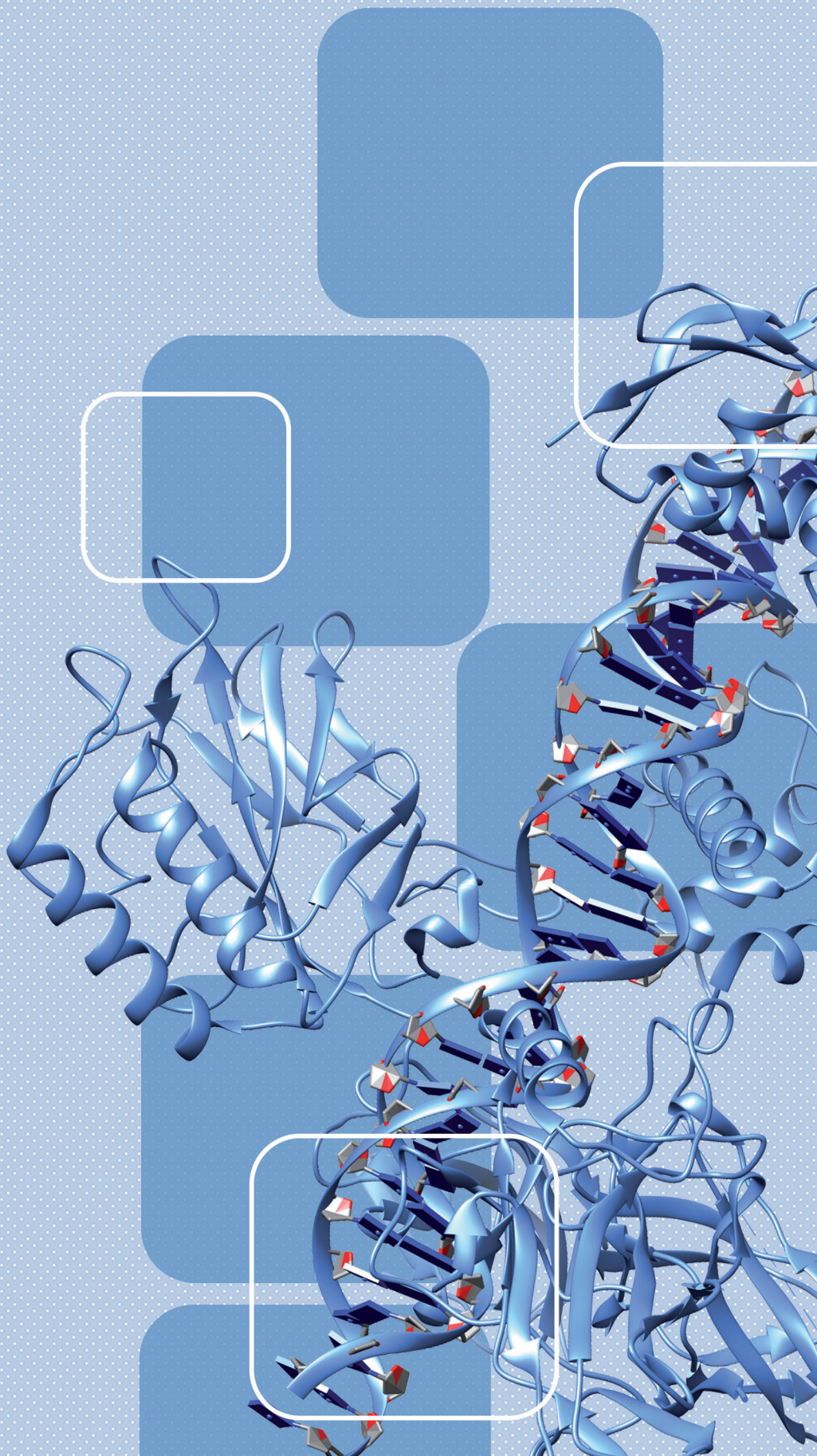

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EVALUATION OF AUTOCHTHONOUS SELECTED YEASTS FROM GRAPES AND CELLAR IN WINEMAKING OF AGLIANICO VINE

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Introduction

Aglianico cultivar is a red grape variety of Greek origin autochthonous of Irpinia, a small district of Campania region in Southern Italy characterized by an established wine industry. Wine production following the oldest way is obtained with the spontaneous fermentation, carried out by the indigenous yeasts present on the surface of the grape and on the cellar equipments.[1,2] The grape native microflora responsible for the early stages of the grape must fermentation are mainly composed by non-*Saccharomyces* yeasts with a low fermentative power. As the alcoholic fermentation proceeds, the fermentative yeasts such as *Saccharomyces cerevisiae* become predominant and replace the non-*Saccharomyces* ones, during the mid-to-final phases of fermentation. The *Saccharomyces* "sensu stricto" strains, named "winery" yeasts are mainly present on the winery equipments surfaces. Each winery is characterized by its own fermentative microflora originated by selection in a very long time among the species "resident" in the cellar. Recently, to plan the winemaking process and standardize the quality of the product, the utilization of microbial starter composed by selected *S. cerevisiae* yeasts has been suggested. However, this procedure inhibits the growth of the non-*Saccharomyces* strains that greatly contribute to the wine quality.[3] In fact, in the first fermentation-phase these species utilize sufficient sugars and amino acids from the grape juice, and generate enough amounts of end-products to have an imprint on wine character. In particular, they are involved in the determination of the colour and aromatic complexity of the wine, which are important determinants of the product typicality. Several researchers have promoted the utilization of such selected native yeasts in order to get mixed fermentation inocula.[4] In addition, several studies revealed that significant positive differences in the qualitative and quantitative volatile compounds composition of the wines have been obtained with a guided fermentations compared to those produced with spontaneous fermentation.[5,6] The aim of this work was the selection of new combinations of yeast strains isolated from the autochthonous microflora of Aglianico grapes and wine cellar, located in Irpinia, and their use in winemaking process to improve the organoleptic and sensory characteristic of the wine thus preserving the peculiarities of this typical regional product.

Materials and Methods

Chemicals

The culture media for the isolation, the reactivation and the growth of different yeast strains were purchased from Oxoid (Hampshire, UK). Chemical reagents and solvents are all of analytical grade and were from SIGMA-Aldrich (St. Louis, MO, USA).

Isolation and molecular identification of yeasts

To isolate different yeasts populations, serial dilutions of Aglianico grape samples, previously homogenized in sterile Ringer solution, were plated on two different solid agar media: WL nutrient agar and Lysine-agar.[7,8] On WL medium, yeast species have been distinguished by different colony morphologies and colours. The isolation of non-*Saccharomyces* species has been carried out on Lysine medium in which *Saccharomyces* spp. cannot grow.[9] The yeast species identification was performed by the analysis of the D1/D2 domain of 26S rDNA sequence. The genetic region was PCR amplified directly from individual yeast colonies, as described by Arroyo-Lopez et al.[10] The standard primers utilized were those commonly referred to as NL1 and NL4 in the literature.[11]

Determination of the yeast fermentative power

One hundred mL of pasteurized must (100°C for 30 min) was inoculated with a 1 % (v/v) microbial biomass suspension (24 h growth). The must fermentation tests were carried out in shake-flasks incubated at 28°C and 200 rpm on an orbital shaker (New Brunswick Scientific Co., Inc., USA). The weight loss caused by CO_2 production was determined every day until a constant weight has been reached and maintained for three days. The fermentative vigor was expressed as g of CO_2 per 100 mL of must.

Yeasts selection for the resistance to sulphur dioxide

The tests were carried out on Aglianico must, pasteurized for 30 min and inoculated with a 1 % of microbial biomass suspension (24 h growth). Potassium metabisulfite (MBK) was used as the source of sulphur dioxide and the yeasts resistance at concentrations of 100 and 250 mg/L was tested. The tests were performed at 28°C for 7 days.[12]

Growth of yeast strains

The selected yeast strains were inoculated in 100 mL YPD liquid medium. The growth was carried out at 28°C in shake-flasks at 200 rpm on an orbital shaker for 24 h. The obtained yeast suspension was used to inoculate (1 % v/v) 10 L of YPD liquid medium

in a pilot plant fermenter. The obtained biomass was stored at 4°C.

Winemaking process

Aglianico grapes were harvested at 18.4°C Brix, destemmed, pressed and then fermented with their skins. MBK was added at a final concentration of 100 mg/L to the juice and, after the addition of 20 mg/L of Trenolin® Rouge DF pectolytic enzyme (Erbslöh Geisenheim AG, Geisenheim, Germany), it was warmed at 18°C for 18 h. The must was then divided into two 30 L tanks for fermentation heated up to 26°C. One tank was inoculated with the starter ST1 containing *S. cerevisiae* FLOSW4 and *H. uvarum* AGSW15 (3 x x 10^6 CFU/mL) and the other tank was inoculated, as control, with 0.15 g/L (about 3.2 x10^{10} CFU/g) of dry *S. cerevisiae 254D-ICV, Lalvin (*Lallemand-Italia), starter ST5, previously rehydrated according to manufacturer's instructions. At the beginning of the fermentation a mobilisator and a nutrient (0.2 g/L Vita*Drive*®, Vitamon® Combi from Erbslöh) were added. Moreover, in the middle of fermentation, another aliquot of the nutrient, at the same concentration, was added. The fermentation process was completed in 15 days and when the residual sugars concentration reached a value smaller than 2 g/L, 60 mg/L of MBK was added. The wine was decanted with aeration for 3 days followed by 10 days without aeration.

Sample preparation of HS-SPME-GC analysis

The SPME fiber (PDMS-100um, polydimethylsiloxane) was conditioned according to the manufacturer's recommendations prior to its first use. To a 20 mL Headspace vial 5 mL of wine samples was added, along with 3 g of NaCl and octan-3-ol in hydro-alcoholic solution (1/1, v/v) at 100 µg/L as an internal standard. The solution was homogenized with a vortex shaker and then loaded onto a Gerstel autosampling device. The program consisted of swirling the vial at 250 rpm for 5 min at 40°C, then inserting the fiber into the headspace for 30 min at 40°C as the solution was swirled again, then transferring the fiber to the injector for desorption at 240°C for 30 min.[13]

Gas Cromatography-Mass Spectrometry

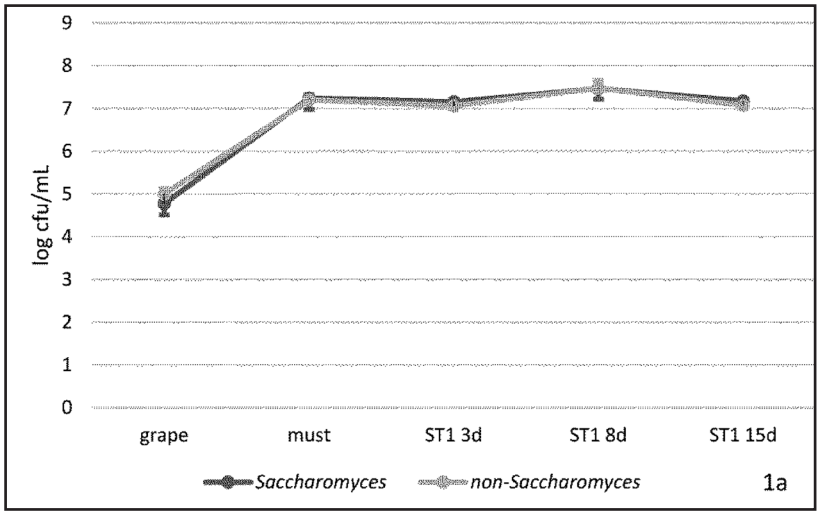
Gas chromatography analyses were carried out using a 7890 Agilent GC system coupled to an Agilent 5975 inert quadrupole mass spectrometer equipped with a Gerstel MPS2 autosampler. The capillary column employed was a HP-Innowax (Agilent technologies) (30 m x 0,25 mm id. 0,50 µm film thickness) and the carrier gas was helium. Splitless injections were used. The initial oven temperature was set to 40°C for 1 min. The temperature was increased in four steps: 40 – 60°C at 2°C/min; 60 – 150°C at 3°C/min, 150 – 200°C at 10°C/min and 200 – 240°C at 25°C/min; the final temperature was maintained for 7 min. The injector, the quadrupole, the source and the transfer line temperature were maintained at 240°C, 150°C, 230°C and 200°C, respectively. Electron ionization mass spectra in full-scan mode were recorded at 70eV electron energy in the range 40 – 300 amu. Peaks were identified using both the NIST 98 and Wiley libraries. Quantification was performed by using the relative concentration in µg/L of the internal standard, calculated as the ratio between each compound area and the internal standard area. The samples were analyzed in triplicate and blank runs were made by using an empty vial every two analysis.[14]

Results and Discussion

Microbiological analysis

We have previously reported[15] that the microbiological analysis of Aglianico grapes revealed the presence of the yeast species mainly belonging to the genera *Hanseniaspora, Kloeckera, Metchnikowia, Candida* and *Saccharomyces* according to Beltran et al.[16]. On the other hand, the yeast strains identified as *Saccharomyces* spp. were the most represented components in the cellar microflora as reported by Ciani et al.[17]. Moreover, some yeast belonging to *Hanseniaspora*, *Kloeckera*, *Metchnikowia* genera were found to be predominant in the must with MBK and in the first days of the fermentative process according to Hierro et al and Fleet. [18,2]

Technological screenings involving the resistance to sulphur dioxide, the alcohologenic power and fermentative power analyses (data not shown), conducted with the isolated yeasts in this study pointed to two strains, FLOSW4 and AGSW15, belonging to *Saccharomyces* and non-*Saccharomyces* spp*.,* respectively, which showed the best performance among the isolates. These two strains have been identified as *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* by the analysis of the D1/D2 domain of 26S rDNA sequence. The experimental winemaking processes carried out with the Aglianico grape must inoculated with the mixed culture of the isolated yeast strains showed that these autochtonous yeasts were able to trigger and complete the alcoholic fermentation leaving a sugar residue smaller than 2 g/L. The Figure 1 shows the kinetics of fermentations obtained by employing of autochtonous starter (ST1) and commercial yeast (ST5), used as control. The growth curves of autochtonous starter, ST1 (Figure 1a), showed that native *S. cerevisiae* yeast (FLOSW4) and the non-*Saccharomyces* yeast (*H. uvarum* AGSW15) are able to start and complete the winemaking process. After 3 days, the load of yeast population was higher than 10*7 cfu/mL and was able to grow over time. Instead, in Figure 1b, with regard to the ST5 commercial starter, we can note that the *S. cerevisiae* strain was able to lead the fermentation process but on the other hand, it has not been able to inhibit the native non-*Saccharomyces* yeasts growth, that remained during all winemaking process, as already reported.[4,19] The data obtained from the trials showed that the autochtonous *S. cerevisiae* yeast has a similar behavior as the commercial *S. cerevisiae* strain to pilot the alcoholic fermentation. Finally, it has been shown to have the ability to modulate the growth of non-*Saccharomyces* yeast as reported previously.[20]



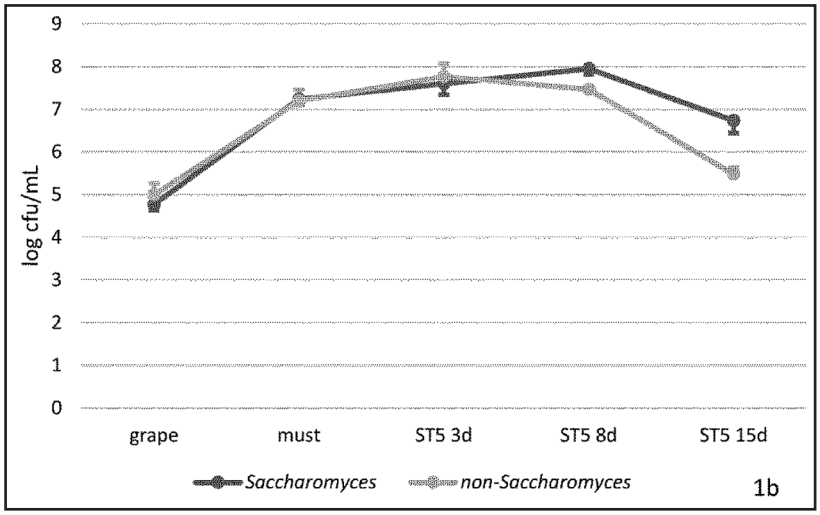


Fig. 1: Kinetics of must Aglianico of trials fermentation with autochtonous starter ST1 (*H. uvarum* + *S. cerevisiae*) and commercial yeast ST5 (*S. cerevisiae* 254D-ICV). The values are the means of three determinations for each sample, the vertical bars represent standard deviation (RSD<5 %)

Volatile compounds detected by SPME-GC/MS analysis

The data about the volatile compounds identified in Aglianico wine samples by SPME-GC/MS, reported in Table 2, are in general agreement with those cited in literature.[21,22] In particular, according to Romano et al.[23], it is to underlined that the differences in the volatile molecules composition of wine samples obtained by utilizing different yeast species appear to be quantitative rather than qualitative. Ethyl esters of straight-chain fatty acids and acetates of higher alcohols are the dominating esters in wine samples and they are formed during the alcoholic fermentation process. The aroma compounds such as isoamyl acetate, ethyl caproate, ethyl caprylate, ethyl caprate and diethyl succinate were detected as the major esters. Also, other types of acetates such as ethyl acetate, hexyl acetate and phenylethyl acetate were identified. Hexanoic, octanoic, decanoic acids and dodecanoic acid, which also contribute to the aroma of the wine, were detected as the most abundant acids. In the alcohols group, in addition to ethanol, isobutyl alcohol, 1-butanol, isoamyl alcohol, 1-hexanol, 1-pentanol-4-methyl, 1-pentanol-3-methyl, (Z)-3-hexen-1-ol, (E)-3-hexen--1-ol and 2-phenylethanol were found. Terpenes that play an important role in the aroma composition of wine have often been used to differentiate different grape cultivar.[24] Specifically, among the compounds of this class, linalool, limonene, alfa terpinene, neryl acetate, α-terpineol, geraniol, nerolidol, β-citronellol, 2,3-dihydrofarnesol and farnesol were identified in the aAglianico wine samples. Furthermore, the volatile phenol, 4-ethylphenol and 4-ethylguaiacol were also revealed. Among the esters, ethyl caproate, ethyl caprate and ethyl caprylate, which are responsible for the fruity, green apple and soap flavor, appeared to be present at higher concentration especially when the autochthonous starter (ST1) was utilized. Also the amount of diethylsuccinate, which mainly contributes to create the body of the wine[25] appeared to be present in more elevated amounts in wines obtained by using the ST1 starter. Among terpenes, in particular the 2,3-dihydrofarnesol was at higher levels in the experimental wine respect control one. This sesquiterpenic compound, known for its antioxidant, anti-inflammatory, antibacterial and anti-cancer properties, may arise directly from grape and/ or rearrangement reactions during the winemaking process and/or aging processes., As this kind of hydrophobic analytes present in several fruits and vegetables has been proposed to promote long and medium-term health beneficial effects it can be hypothesized that also the experimental Aglianico wine could have these healthy properties.[26]

Regarding the total alcohols, their amount was higher in wines produced by ST1 compared to that obtained by commercial yeast (ST5).

On the other hand in the class of phenols the 4-ethylguaiacol and the 4-ethylphenol were more abundant in ST1 wine. These compounds may negatively affect the quality of the wine producing unpleasant odors. Specifically, the presence of ethylphenols (4-ethylphenol and 4-ethylguaiacol) in red wine produces "phenolic" and animal odor. In the wine ST1 the 4-ethylguaiacol was at a higher level respect to the corresponding olfactory threshold. Moreover the experimental Aglianico wine, could have beneficial effect on health, because it contained the 2,3-dihydrofarnesol compound, belonging to sesquiterpenoids class. This class has a known beneficial effect on health.[26]

Conclusions

The fermentation carried out with the new starter showed positive differences compared to commercial yeasts. The results demonstrated that the two native selected strains successfully dominated the winemaking process and the Aglianico wine obtained showed a higher amount of the esters, responsible for the fruity and green apple flavor that enhance the aromatic complexity and strenghth the "terroir".

Table I: Quantification of volatile compounds identified in the wine produced by ST1 *(H. uvarum + S. cerevisiae*) and commercial yeast ST5 (*S. cerevisiae* 254D-ICV)

	ST5 (µg/L ± SD)	ST1(µg/L ± SD)		ST5 (µg / L ± SD)	ST1(µg/L ± SD)
Esters and Acetates			2,3-butandiol	60,98 ± 0,68	28,51 ± 0,39
ethyl acetate	2410,1 ± 26,9	2744,1 ± 38,0	1-octanol	17,2 ± 0,2	26,9 ± 0,4
ethyl propanoate	45,5 ± 0,5	30,8 ± 0,4	1-nonanol	2,3 ± 0,0	26,5 ± 0,4
ethyl isobutyrate	43,2 ± 0,5	27,5 ± 0,4	1-decanol	19,4 ± 0,2	22,0 ± 0,3
isobutyl acetate	25,0 ± 0,3	25,9 ± 0,4	benzyl alcohol	10,1 ± 0,1	11,5 ± 0,2
ethyl butyrate	125,1 ± 1,4	134,3 ± 1,9	2-pheneylethanol	1531,2 ± 17,1	1406,4 ± 19,5
ethyl, 2-methylbutanoate	21,0 ± 0,2	23,7 ± 0,3	methionol	8,9 ± 0,1	6,8 ± 0,1
ethyl isovalerate	20,3 ± 0,2	24,8 ± 0,3	*Total alcohols and thiols*	*31805,9 ± 354,5*	*32397,4 ± 448,7*
isoamylacetate	853,4 ± 9,5	842,7 ± 11,7	**Aldehydes and ketones**		
ethyl caproate	1165,6 ± 13,0	1736,5 ± 24,1	acetaldehyde	6,2 ± 0,1	ND
butylisovalerate	5,5 ± 0,1	5,8 ± 0,1	2,3-butanedione	26,6 ± 0,3	5,2 ± 0,1
hexyl acetate	23,9 ± 0,3	20,2 ± 0,3	*Total aldehydes,ketones*	*32,8 ± 0,4*	*5,2 ± 0,1*
ethyl heptanoate	22,6 ± 0,3	40,9 ± 0,6	**Acids**		
ethyl lactate	234,6 ± 2,6	369,8 ± 5,1	acetic acid	263,5 ± 2,9	293,7 ± 4,1
methyl octanoate	14,3 ± 0,2	14,5 ± 0,2	butanoic acid	2,7 ± 0,0	ND
ethyl caprylate	3664,0 ± 40,8	5915,9 ± 81,9	butanoic acid ,2-methyl	27,4 ± 0,3	20,5 ± 0,3
isoamyl caprylate	35,4 ± 0,4	61,6 ± 0,9	hexanoic acid	47,1 ± 0,5	23,4 ± 0,3
ethyl nonanoate	34,3 ± 0,4	36,7 ± 0,5	octanoic acid	286,2 ± 3,2	377,5 ± 5,2
ethyl 2-hydroxy caproate	15,9 ± 0,2	31,1 ± 0,4	nonanoic acid	6,8 ± 0,1	6,5 ± 0,1
isoamyl lactate	12,4 ± 0,1	7,6 ± 0,1	decanoic acid	14,1 ± 0,2	162,3 ± 2,3
methyl decanoate	10,2 ± 0,1	8,0 ± 0,1	dodecanoic acid	38,8 ± 0,4	13,9 ± 0,2
ethyl caprate	2020,2 ± 22,5	3349,2 ± 46,4	*Total acids*	*686,5 ± 7,7*	*897,7 ± 12,4*
ethyl benzoate	3,7 ± 0,0	ND	**Volatile phenols**		
diethyl succinate	566,4 ± 6,3	625,3 ± 8,7	phenol 4-ethyl-3-methyl	8,4 ± 0,1	ND
ethyl 9-decenoate	20,2 ± 0,2	58,3 ± 0,8	phenol, 2-methoxy-4-methyl	11,4 ± 0,1	3,8 ± 0,1
methyl salicylate	6,6 ± 0,1	5,0 ± 0,1	4-ethyl guaicol	9,3 ± 0,1	57,5 ± 0,8
ethyl phenylacetate	20,5 ± 0,2	27,4 ± 0,4	phenol 4-ethyl	38,5 ± 0,4	155,5 ± 2,2
diisobutylsuccinate	17,2 ± 0,2	13,4 ± 0,2	*Total volatile phenols*	*67,6 ± 0,8*	*216,8 ± 3,0*
ethyl 4-hydroxybutanoate	24,7 ± 0,3	25,6 ± 0,4	**Terpenes**		
phenethyl acetate	70,2 ± 0,8	46,5 ± 0,6	limonene	16,8 ± 0,2	15,3 ± 0,2
ethyl dodecanoate	164,6 ± 1,8	672,1 ± 9,3	α terpinene	10,6 ± 0,1	4,5 ± 0,1
ethyl hydrocinnamate	143,3 ± 1,6	2,6 ± 0,0	neyl acetate	9,5 ± 0,1	ND
Total esters and acetates	*11865,1 ± 132,6*	*16927,6 ± 235,4*	linalolo	40,5 ± 0,5	22,5 ± 0,3
Alcohols and thiols			βcitronellol	29,1 ± 0,3	35,7 ± 0,5
ethanol	19776,6 ± 220,4	20198,1 ± 279,7	α terpineol	9,8 ± 0,1	4,0 ± 0,1
isobutyl alcohol	802,5 ± 9,0	699,3 ± 9,7	geraniol	6,5 ± 0,1	4,1 ± 0,1
1-butanol	20,6 ± 0,2	13,5 ± 0,2	nerolidol	69,7 ± 0,8	10,0 ± 0,1
isoamyl alcohol	9211,3 ± 102,7	9588,3 ± 132,8	2,3-dihydrofarnesol	10,7 ± 0,1	197,0 ± 2,7
1-pentanol,4-methyl	5,7 ± 0,1	7,4 ± 0,1	farnesol	14,1 ± 0,2	19,9 ± 0,3
1-pentanol,3-methyl	16,6 ± 0,2	21,5 ± 0,3	*Total terpenes*	*217,4 ± 2,4*	*313,0 ± 4,3*
1-hexanol	308,8 ± 3,4	330,3 ± 4,6	**Lactones**		
3-hexen-1-ol (E)	6,9 ± 0,1	6,1 ± 0,1	butyrolactone	16,8 ± 0,2	14,2 ± 0,2
3-hexen-1-ol (Z)	2,5 ± 0,0	2,1 ± 0,0	γ n-amylbutyrolactone	1,3 ± 0,0	2,7 ± 0,0
1-octen-3-ol	4,6 ± 0,1	2,3 ± 0,0	*Total lactones*	*18,1 ± 0,2*	*16,9 ± 0,2*

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Summary

Boscaino F., Sorrentino A., Ionata E., La Cara F., Volpe MG.: Avaluation of Autochthonous selected yeasts from grapes and cellar in winemaking of Aglianico vine

Aglianico cultivar is an ancient red grape variety native of Campania region of the Southern of Italy. Until a few decades ago, wine was produced by natural fermentation carried out by autochthonous yeasts present on the grapes and in the cellar. The grapes epiphytic microflora is prevalently composed of apiculate yeasts with a poor fermentative power and by oxidative yeasts, belonging to non-*Saccharomyces* group. On the other hand, most of the yeast strains present in the cellar belong to *Saccharomyces* "sensu stricto". The aim of this work was the selection of new combinations of yeast strains isolated from the autochthonous microflora of Aglianico grapes and wine cellar, located in the Irpinian area, and their use in winemaking process to improve the organoleptic and sensory peculiarities of the wine obtained. The yeasts isolated from grapes and cellar were characterized by morphological, biochemical, and technological analysis. They belonged mainly to the genus *Saccharomyces, Hanseniaspora*, Kloeckera, *Rhodotorula, Metschnikowia* and *Candida*. Among these yeasts the two strains (AGSW15 and FLOSW4) that showed the best winemaking performance were selected and identified as *H. uvarum* and *S. cerevisiae* by 26S rDNA D1/D2 region sequence analysis. These yeasts, when utilized in cellar for semi-industrial-scale fermentations, successfully dominate the alcoholic fermentation and contributed to the improvement of the wine organoleptic qualities as assessed by the flavor profile obtained through the SPME-GC/MS technique. These results clearly suggest the utilization of these selected strains in autochthonous fermentation starter to preserve the peculiarities of this typical regional wine.

Keywords: Aglianico cultivar, autochthonous yeasts, starter, volatile compounds

Souhrn

Boscaino F., Sorrentino A., Ionata E., La Cara F., Volpe MG.: Hodnocení vybraných přirozeně se vyskytujících kvasinek z hroznů a sklepa při výrobě vína Aglianico

Kultivar Aglianico je velmi stará odrůda červeného vína pocházející z regionu Campania v jižní Italii. Do nedávna bylo víno vyráběno fermentací pomocí kvasinek vyskytujících se na hroznech a ve sklepě. Mikroflora ulpívající na hroznech je složena především z kvasinek se slabými fermetačními a oxidačními schopnostmi, které nepatří do skupiny *Saccharomyces*. Naproti tomu většina kmenů kvasinek přítomných ve sklepě patří mezi sacharomycety. Úkolem této práce bylo vybrat nové kombinace kmenů kvasinek izolovaných z přirozeně se vyskytující mikroflory hroznů Aglianico a vinného sklepa lokalizovaného v irpinské oblasti a tyto kombinace kvasinek využít ke zlepšení organoleptických a sensorických vlastností takto připravených vín. Kvasinky izolované z hroznů i sklepa byly charakterizovány po stránce morfologické, biochemické a technické. Tyto kvasinky patří zejména mezi rody *Saccharomyces, Hanseniaspora, Kloeckera, Rhodotorula, Metschnikowia and Candida.* Mezi těmito kvasinkami měly nejlepší vlastnosti při přípravě vína dva kmeny (AGSW15 a FLOSW4), identifikované jako *H. uvarum a S. cerevisiae* na základě sekvenční analýzy úseku D1/D2 26S rDNA. Tyto kvasinky, použité při poloprovozní fermentaci, úspěšně dominovaly v průběhu alkoholické fermentace a ve zlepšení organoleptických vlastností potvrzených technikou SPME-GC/MS. Dosažené výsledky podporují použití vybraných kmenů kvasinek k zahájení fermentace umožňující zachovat zvláštnosti charakteristické pro toto typicky regionální víno.

Klíčová slova: Kultivar Aglianico, autochtonní kvasinky

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Chapter 9

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Evolution of polyphenols fraction and natural yeast flora of Coda di Volpe white grape

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Wine is one of the main product of the agro-food products in Italy especially for its economic importance to local and regional level. The Campania region is excellent for the production of wine especially those of value, thanks to the presence of wide known varieties of native grapes. The aim of this work is been to characterize the chemical, biochemical and microbiological properties of Coda di Volpe grapes, cultivated in two areas, the hills and flat lands of Benevento. The name Coda di Volpe means tail of the fox, which was given in reference to the varieties of long, pendulous bunches of grapes, which resembles a fox's bushy tail.
The polyphenols, pH, total acidity and °Brix were determined at different stages of maturation in order to identify the optimal time for the harvest. In particular, the total polyphenols content was evaluated with Folin-Ciocalteu method, while the specific polyphenols were determined by RP-HPLC. The grapes were harvested in sterile bag, sealed, pressed and incubated at 20°C for 1 month. During the fermentation it was isolated epiphytic microflora and characterized in order to analyse the physiological, oenological and technological properties. Three different yeast strains were assayed in their oenolegical ability, these were used in experimental fermentation with Coda di Volpe must. During alcholic fermentation have been evaluated yeast population, alcoholic grade and the volatile profile by the SPME-GC/MS technique. The data obtained have highlighted their ability to conduct the fermentation process, dominate the indigenous microflora and contribute to the definition of the flavour profile.

Introduction

Coda di Volpe is a golden-yellow grapes variety, used to produce wines in and around the region of Campania in southern Italy. The wine produced from this cultivar is often described as medium to medium-full bodied, fruity (citrus, peach, pineapple) and spicy (nutmeg, cinnamon) and flavors of grapefruit, lemon, and almond.
Since the 1980's, wineries in Campania have begun making single-varietal wines from Coda di Volpe, and now used in the DOC wines of Irpinia and Sannio area.
Industrial wine fermentations are currently conducted by starters of special wine yeast strains of *Saccharomyces cerevisiae* in contrast to traditional spontaneous fermentations conducted by the flora present on the grapes and in the winery. Despite the advantages of using pure cultures of *S. cerevisiae* with regard to the easy control and homogeneity of fermentations, wine produced with pure yeast monocultures lacks the complexity of flavour, stylistic distinction and vintage variability caused by indigenous yeasts (Swiegers et al., 2005). In recent years the inclusion of non-*Saccharomyces* wine yeast species as part of mixed starters together with *S. cerevisiae* to improve wine quality has been suggested as a way of taking advantage of spontaneous fermentations without running the risks of stuck fermentations or wine spoilage (Romano et al., 2003; Ciani et al., 2006). Although non-*Saccharomyces* wine yeast species have traditionally been associated with high volatile acidity, ethyl acetate production, off-flavours and wine spoilage (Sponholz, 1993; Ciani and Maccarelli, 1997), the potential positive role they play in the organoleptic characteristics of wine has been emphasized in numerous studies (Fleet, 2003). Metabolic interactions between non-*Saccharomyces* and *S. cerevisiae* wine yeasts during fermentation could positively or negatively interfere with the growth and fermentation behaviour of yeast species,

particularly *S. cerevisiae*. It has also been noted that the presence/absence of *S. cerevisiae* differs according to each plant and grape cluster (Pretorius et al., 1999). For this reason it is not always possible to obtain the same product from spontaneous wine fermentation. This problem is being solved at present by the use of commercial strains in the fermentation process, to the detriment of the wine's autochthonous character. On the other hand, the widespread use of LSA has led to a leveling of the aromatic characteristics of wines produced with different cultivars. Today is increasing the demand for selected native yeasts starter that could enhance the sensory characteristics peculiar of the wine. The purpose of this work was the identification of cultures of native yeast to be used in starter mix for use on a large scale

2. Materials and Methods

2.1 Chemicals

The culture media for the isolation, the reactivation and the growth of yeast strains were purchased from Oxoid (Hampshire, UK). Chemical reagents and solvents are all of analytical grade and were from Sigma- Aldrich (St.Louis, MO, USA). The SPME fibers PDMS- 100µm (polydimethylsiloxane), was from Supelco (Bellefonte, PA, USA).

2.2 Sampling

The Coda di Volpe grapes were harvested in two different stage of maturation, in the last week of September and in the second week of October. To evaluate the change in total polyphenols and antioxidant activity.

2.3 Analysis of total polyphenols

Total polyphenol content was determined using the Folin-Ciocalteu reagent (Waterman and Mole,1994) following a micro scale as described by Arnous et al. (2002). The absorbance at 750 nm was recorded, and the total polyphenol concentration was calculated from a calibration curve using gallic acid as standard and expressed as mg gallic acid equivalents/L (GAME).

2.4 HPLC analysis of phenolic compounds

Phenolic compounds analysis was performed by HPLC/UV with a C18 column Hypersil (ThermoElectron Corporation, Bellefonte, PA, North America). The injected sample volume was 20 µL. Peak identifications were confirmed from retention times and direct comparison to pure standards. The solvent flow rate was 0.9 mL/min and the mobile phase was a four-step linear solvent gradient system(0–5 min, 10 % B; 5-40 min, 45 % B; 40–45 min, 100 % B; 45-50 min, 100 % B; 50-55 min, 10 % B)using 2 % acetic acid in water as solvent A and 0.5 % acetic acid in 50 % acetonitrile as solvent B. UV detection was carried out at 280 nm (Katalinić et al., 2010).

2.5 Antioxidant Activity of grape skin extracts

Antioxidant properties of grape skin extracts were determined as free radical-scavenging ability (DPPH method), reducing power (FRAP method), Fe2+-chelating ability of plant extracts, and ability to prevent oxidation of linoleic acid (Katalinić et al., 2010).

2.6 Isolation of yeasts

To isolate the different yeasts populations, serial dilutions of Coda di Volpe grape from sealed bag, 1 mL of must was used to prepare Ringer solution serial dilutions and appropriate dilutions were plated on two different solid agar media: WL-nutrient and Lysine agar. On WL medium, yeast species have been distinguished by different colony morphologies and colours. The isolation of non-*Saccharomyces* species has been carried out on Lysine medium, that does not allow the growth *Saccharomyces spp (*Cavazza et al 1992). After 4 days of incubation at 25 °C, the colonies were enumerated to determine the order of population. Five colonies were isolated randomly from the plates and stored at 4°C for fisiological and technological characterization. The culture yeasts were identified according to their morphological aspect (elliptical and lemon shaped) and biochemical test API 20C Aux (bioMerieux, France). The yeasts selected were *S.cerevisae* YTECCVWL7 (a), non-*Saccharomyces* YTECMCVWL4 (b) and *S.cerevisae* YOCMCVWL4 (c), they were used in microfermentations.

2.7 Determination of the yeast fermentative power

The must (100mL) after pasteurization (100 °C for 30 min) was inoculated with a 1 % (v/v) microbial biomass suspension (24h growth). The must fermentation tests were carried out in shake-flasks incubated at 28 °C. The flasks weight decrease was measured every day, until a constant weight has been reached and maintained for three days (Zambonelli, 1998).

2.8 Yeasts selection for the resistance to sulphur dioxide

The tests were carried out on Coda di Volpe must which was pasteurized and inoculated with a 1 % microbial biomass suspension (24h growth). Potassium metabisulfite (MBK) was used as the source of sulphur dioxide

and the yeasts resistance at concentrations of 100 and 250 mg/L was checked. The tests were performed at 28 °C for 7 days (Costanti et al., 1998).

2.9 Inoculated fermentations at laboratory-scale selected yeast isolates

Three yeasts (YOCMCVWL4, YTECMCVWL4, YTECCVWL7), selected on the base of technological traits were inoculated in must in single and different combination. Inoculated fermentation assay was performed in 1L Erlenmeyer flasks filled with 500 mL of Coda di Volpe grape must with 10% MBK. Grape must characteristics were: 21,5 °Brix, pH 3,2. Each strain was inoculated in grape must at a concentration of 10^6 cells/mL. The fermentation was performed at 20°C and the fermentative course was monitored by measuring weight loss, determined by carbon dioxide evolution during the process. At the end of the process, the wine samples, immediately, were analyzed by SPME-GC/MS to evaluate the volatile compounds.

2.10 Analysis of volatile compounds by HS-SPME-GC/MS

The SPME fiber (PDMS-100µm, polydimethylsiloxane) was conditioned according to the manufacturer's recommendations prior to its first use (Pawliszyn 2009). To a 20 mL Headspace vial was added 5 mL of wine samples, together to 3g of NaCl and octan-3-ol, in hydro-alcoholic solution (1/1, v/v) at 100 µg/L, as Internal Standard. The solution was homogenized with a vortex shaker and then loaded onto a Gerstel autosampling device. The program consisted of swirling the vial at 250 rpm for 5 min at 40 °C, then inserting the fiber into the headspace for 30 min at 40 °C as the solution was swirled again, then transferring the fiber to the injector for desorption at 240 °C for 30 min.

2.11 GasCromatography-MassSpectrometry

Gas chromatography analysis were carried out using a 7890 Agilent GC system coupled to an Agilent 5975 inert quadrupole mass spectrometer equipped with a Gerstel MPS2 autosampler. The capillary column employed was a HP-Innowax (Agilent technologies) (30 m x 0,25 mm id. 0,50 µm film thickness) and the carrier gas was Helium. Splitless injections were used. The initial oven temperature was set to 40 °C for 1 min.
The temperature was increased in four steps: 40–60 °C at 2 °C/min; 60–150 °C at 3 °C/min, 150–200 °C at 10 °C/min and 200-240 °C at 25 °C/min; the final temperature was maintened for 7 min. The injector, the quadrupole, the source and the transfer line temperature were maintained at 240 °C, 150 °C, 230 °C and 200 °C, respectively. Electron ionization mass spectra in full-scan mode were recorded at 70eV electron energy in the range 40–300 amu. Peaks were identified using both the NIST 98 and Wiley libraries. Quantification was performed by using the relative concentration in µg/L of the Internal Standard, calculated as the ratio between each compound area and the internal standard area. The samples were analyzed in triplicate and blank runs were made by using an empty vial every two analysis (Sorrentino et al., 2013).

3. Results and Discussion

3.1 Total polyphenols and antioxidant activity

The data reported in Fig.1 show the content of total polyphenols and antioxidant activity in Coda di Volpe berry grapes. They show that the amounts was highest in the skin respect to pulp berry. These results were confirmed in all samples collected to the different times (last week of September and the second week of October). In addition, experimental data have shown that polyphenols were higher in the skin of the grape berry harvested in the first sampling while the antioxidant activity was higher in the berry in second. The HPLC analysis on grape skin (data not shown), confirmed the presence of catechins and epicatechins peaks in both sampling, this data was in according to literature (Katalinić et al., 2010).

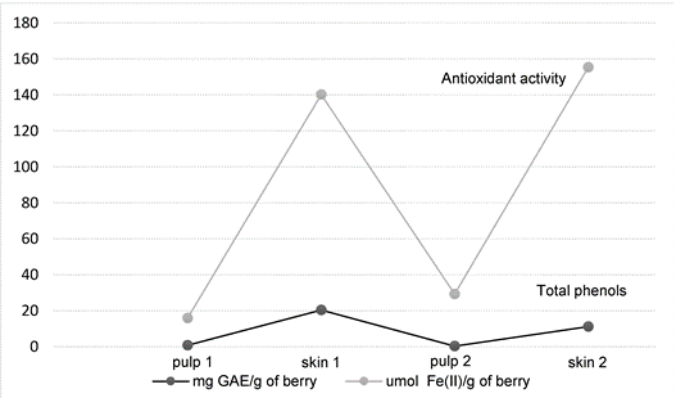

Figure 1: total phenols and antioxidant activity relative to skin and pulp grape berry in two different time sampling

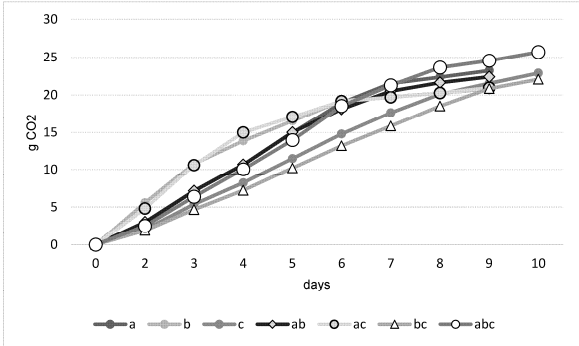

Figure 2: fermentative power of single and mix yeast selected, evaluated by loss in CO_2

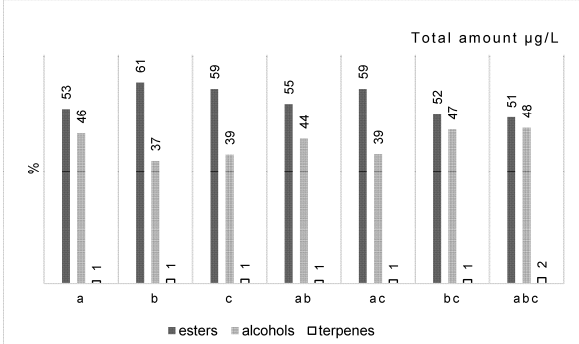

Figure 3: principal VOC classes on Coda di Volpe wine, determined by SPME-GC/MS, reported in percentage.

a: YTECCVWL7; **b**: YTECMCVWL4; **c**: YOCMCVWL4; **ab**: YTECCVWL7+ YTECMCVWL4;**ac:** YTECCVWL7+ YOCMCVWL4; **bc**: YTECMCVWL4+ YOCMCVWL4;**abc**: YTECCVWL7+ YTECMCVWL4+ YOCMCVWL4

3.2 Monitoring of yeast populations on Coda di Volpe grapes

The results of microbiological analyses on the grapes showed that in the first sampling the epiphytic microflora was around $3*10^3$ cfu/g on WL agar and $2*10^4$ cfu/g on Lysine medium, while on the second sampling the load of yeast population was respectively $5*10^5$ cfu/g and $6*10^5$ cfu/g. After enumeration, from the plates contained from 15 to 300 colonies were isolated randomly five colonies. These were stored at 4°C for identification and technological characterization. After fisiological screening, were selected yeasts with elliptical and lemon shaped (Martini et al., 1996). These yeasts were used in technological tests.

3.3 Technological characterization

On yeast collection was carried out in order to ascertain resistance of the indigenous yeasts to sulphur dioxide. Sulphur dioxide is the most widely antiseptic agent used in wine cellars to control the growth of spontaneous microflora during fermentation. For our selection, we checked the resistance at two different concentrations (100 mg/L and 250 mg/L). The results demonstrated that most of the yeasts were resistant to SO_2 concentration 100 mg/L. Our data, revealed that many indigenous yeast belonged to non-*Saccharomyces* genera showed resistance to SO_2. Despite the literature data (Romano and Suzzi, 1993; Henick-Kling et al., 1998) that suggest the lack of such resistance in many yeast non-*Saccharomyces* genera. The resistance to sulphur dioxide could be caused by a "selective pressure" of sulphur levels used on grapevines throughout the years. Moreover, a significant number of indigenous yeasts non-*Saccharomyces* can to grow after the addition of the usual dose of SO_2 until they die of ethanol intoxication after three or four days from the beginning of fermentation (Fleet, 2003). Based on the results, we chosen three yeasts that showed best performance. These yeasts were used to perform fermentative power test along and in mix in Coda di Volpe must. The results (Fig.2) showed that all yeast as along as mix, were able to start and complete the wine fermentation. Moreover, the **abc** mix showed the best fermentative power as demonstrated by higher CO_2 production.

3.4 Volatile compounds

The results obtained from SPME-GC/MS analysis on the product of microvinification in batch, were reported in Table 1. The Esters are considered important to the sensory properties of wines, contributing with positive aroma, essentially with fruity notes (Pereira et al., 2014). The results (Fig.3) showed that esters were one of the most abundant groups (about 55%) as they are secondary aromas. While, the higher alcohols may be present in healthy grapes, but seldom occur in significant amount, they are essentially formed either from sugar catabolism or from amino acid decarboxylation and deamination and they have high perception threshold. They commonly account for about 50% of the aromatic constituents of wine, excluding ethanol. Moreover, the terpenes are varietal compounds present in grapes, especially in skins, o arise from glycosil precursors and they have low perception threshold.

In conclusion, we selected the mix **abc**, to apply in future winemaking process on large scale, because it showed the best power fermentative and a balance in the amount of esters and alcohols products, and a higher content of terpenes (2%), being these volatile compounds classes responsible to modulation of wine aroma and flavor.

Table 1: Volatile compounds identified in microvinification with single and different combination of selected yeasts. Each value is expressed in µg/L and is the mean of three replicates ± sd (standard deviation)

	a		b		c		ab		ac		bc		abc		odor [a]
	µg/L	sd	µg/L	sd	µg/L	sd	µg/L	sd	µg/L	sd	µg/L	sd	µg/L	sd	
Esters (14)															
ethyl acetate	3166,	± 241,	1134,6	± 118,2	871,4	± 20,3	3110,3	± 44,9	1840,5	± 4,1	2066,1	± 153,8	2704,3	± 54,4	fruity
isobutyl acetate	7,7	± 0,6	19,8	± 2,1	nd		19,4	± 0,2	9,6	± 0,02	nd		8,90	± 0,2	fruity
Ethyl butanoate	121,1	± 9,2	90,4	± 9,4	92,2	± 2,1	178,0	± 2,5	117,2	± 0,2	126,5	± 9,4	123,3	± 2,4	floral,
ethyl isovalerate	9,27	± 0,7	10,54	± 1,1	8,26	± 0,1	16,50	± 0,2	11,75	± 0,03	15,76	± 1,1	9,34	± 0,1	sweet fruit
Ethyl hexanoate	1153,	± 87,9	1284,8	± 133,9	760,1	± 17,7	1693,1	± 24,4	1112,4	± 2,5	1100,3	± 81,9	1034,6	± 20,8	fruity
Hexyl acetate	8,5	± 0,6	63,6	± 6,6	6,7	± 0,1	26,1	± 0,3	40,2	± 0,01	7,1	± 0,5	9,2	± 0,1	herbaceou
ethyl lactate	nd		nd		7,8	± 0,2	nd		112,0	± 0,3	54,2	± 4,1	nd		Milk,butter
Ethyl octanoate	6493,	± 495,	9094,5	± 947,9	6364,	± 148,	8058,5	± 116,	7898,7	± 17,8	6422,6	± 478,1	5638,2	± 113,	sweet fruit
isoamyl acetate	783,2	± 59,7	1332,8	± 138,3	539,5	± 12,6	1322,2	± 19,1	1013,8	± 2,3	940,6	± 70,0	803,8	± 16,1	banana
Ethyl decanoate	3612,	± 275,	4112,4	± 301,3	3172,	± 74,1	4270,2	± 61,7	2767,1	± 100,1	3400,6	± 250,1	2560,7	± 51,5	oily, fruity
diethyl succinate	13,7	± 1,1	16,3	± 1,7	18,6	± 0,4	17,6	± 0,2	38,1	± 0,1	45,3	± 3,3	29,8	± 0,6	fruity
ethyl 9-decenoate	848,8	± 64,7	2496,1	± 260,1	2795,	± 65,3	1720,8	± 24,7	3298,1	± 70,4	1267,5	± 94,7	1075,9	± 21,5	-
Phenethylacetate	351,4	± 26,8	306,4	± 10,1	328,8	± 7,6	443,5	± 9,1	491,59	± 20,3	349,7	± 25,5	403,5	± 8,2	flowery
ethyl laurate	345,5	± 26,3	410,6	± 42,8	269,2	± 6,2	482,2	± 6,9	188,4	± 10,2	337,8	± 30,3	271,5	± 5,7	sweet
Alcohols (8)															
Isobutanol	733,7	± 55,9	962,7	± 100,3	303,3	± 7,1	1111,5	± 16,1	790,3	± 1,7	736,4	± 54,8	857,5	± 17,6	fusel
isoamyl alcohol	1062	± 810,	10340,	± 1077,	8088,	± 188,	13202,	± 190,	9897,3	± 22,4	11728,	± 873,1	10553,	± 212,	fusel
4-methyl-1-pentanol	nd		10,5	± 1,1	5,5	± 0,1	nd		nd		6,4	± 0,4	3,9	± 0,1	almond
3-methyl 1-pentanol	nd		15,3	± 1,6	17,6	± 0,4	13,5	± 0,2	11,9	± 0,03	16,9	± 1,6	10,7	± 0,2	vinous
1-hexanol	53,1	± 4,05	6,8	± 0,7	38,7	± 0,9	52,5	± 0,7	9,1	± 0,02	nd		45,8	± 0,2	herbaceou
2-ethyl hexanol	nd		nd		4,3	± 0,1	nd		nd		nd		8,7	± 0,2	mushroom
2,3-butandiol	33,6	± 2,5	nd		nd		18,3	± 0,2	nd		17,6	± 1,3	9,4	± 0,2	butter
phenethyl alcohol	3122,	± 238,	1084,6	± 113,1	1621,	± 37,8	2838,3	± 41,1	1878,3	± 4,2	2147,2	± 159,8	2256,5	± 45,1	rose
Acids (3)															
acetic acid	232,9	± 17,7	31,0	± 3,2	24,8	± 0,6	255,4	± 3,6	69,1	± 0,1	62,9	± 4,6	237,9	± 4,9	vinegar
octanoic acid	391,6	± 29,8	65,3	± 6,8	386,4	± 9,1	12,2	± 0,1	869,5	± 1,9	475,1	± 35,3	512,6	± 10,	fatty
decanoic acid	352,1	± 26,8	69,3	± 7,2	486,6	± 11,4	128,2	± 1,8	894,6	± 2,1	533,3	± 39,7	504,4	± 10,1	fatty
carbonyl comp (2)															
acetoino	29,5	± 2,2	38,9	± 4,1	nd		61,0	± 0,8	42,5	± 0,1	nd		nd		butter
acetaldehyde	76,3	± 5,8	14,0	± 1,4	30,4	± 0,7	61,3	± 0,8	47,2	± 0,1	25,6	± 1,9	93,9	± 1,8	ripe apple
Terpenes (6)															
beta farnesene	11,1	± 0,8	16,3	± 1,7	17,6	± 0,4	16,6	± 0,2	27,5	± 0,1	20,8	± 1,5	22,4	± 0,4	citrus
alfa-farnesene	5,2	± 0,4	12,9	± 1,3	11,4	± 0,2	11,9	± 0,1	17,6	± 0,11	19,7	± 1,4	14,6	± 0,3	wood
nerolidol	93,7	± 7,1	186,6	± 19,3	175,2	± 5,1	180,1	± 2,1	129,6	± 0,3	151,3	± 11,2	173,6	± 3,4	wood
2,3-dihydrofarnesol	60,7	± 4,6	97,7	± 5,6	92,5	± 3,7	80,0	± 0,4	96,9	± 0,3	75,3	± 6,5	143,5	± 4,1	swee
farnesol	85,3	± 9,3	98,2	± 4,6	94,1	± 3,8	80,2	± 0,6	78,1	± 0,3	80,3	± 9,7	112,7	± 3,2	flower
beta citronellol	nd		nd		14,5	± 0,3	nd		9,3	± 0,02	10,6	± 0,7	39,2	± 0,7	citrus

[a] Based on flavournet (www.flavournet.org) and pherobase (www.pherobase.com) online databases

nd: not detected

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Conclusions:

- The yeasts isolated from Aglianico, Fiano and Coda di Volpe grapes, mainly belong to *Metschnikowia*, *Hanseniaspora, Kloeckera*, *Rhodutorula, Pichia* and *Candida* genera; while, in the must and in the cellar, were isolated yeasts belonging to the genera *Saccharomyces*, *Metschnikowia*, and *Hanseniaspora*.

- The experimental mix starters (*Saccharomyces-Metschnikowia* and *Saccharomyces-Hanseniaspora*) were able to trigger and complete the alcoholic fermentation both in the laboratory and in the winery.

- The experimental wines show chemical-physical parameters in the range of acceptability, and similar to those of commercial wines.

- The multiple starters with *S. cerevisiae-H. guilliermondii* and *S. cerevisiae-H. uvarum* are able to produce wines with a greater aromatic complexity compared to commercial yeast strain.

- The use of yeast *S. cerevisiae* and non-*Saccharomyces* isolated from vineyards autochtonous may be a useful strategy to diversify the wines, to increase their aromatic complexity as well as to strengthen the bond with the territory also known as "terroir"