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Health promoting compounds in Sangiovese wines

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Parole chiave: antociani, flavonoli, flavan-3-oli monomeri, *Saccharomyces cerevisiae*, *Candida zemplinina*, tirosolo, idrossitirosolo, triptofolo, Sangiovese.

Riassunto

Scopo: Lo scopo di questo lavoro è stato quello di indagare sui fattori che influenzano l'accumulo di diversi composti dotati di proprietà benefiche per la salute in vini Sangiovese. Dunque, il lavoro di tesi si è incentrato sullo studio dell'incidenza di diverse variabili capaci di influenzare la composizione delle uve ed il ruolo dei lieviti durante la fermentazione alcolica nel determinare l'accumulo di tali sostanze nei vini. In particolare, i composti presi in considerazione in questa tesi includono molecole derivanti dalle uve (antociani, flavonoli e flavan-3-oli monomeri) e tre alcoli superiori prodotti dai lieviti (tirosolo, idrossitirosolo e triptofolo).

Metodi e Risultati: Per studiare l'incidenza delle singole variabili che influenzano l'accumulo di tali sostanze nei vini**, s**ono state definite ed adottate delle procedure standardizzate che hanno riguardato le operazioni di campionamento delle uve, la conservazione di campioni prima della vinificazione, le operazioni di fermentazione e macerazione e, infine, le analisi dei vini finiti. La composizione dei vini sperimentali Sangiovese ottenuti rifletteva le caratteristiche compositive delle uve di partenza. In questo lavoro, l'effetto di zona viticola risultava profondamente combinato all'effetto dell'annata, sottolineando così la ben nota importanza del *Terroir*, nella sua accezione più ampia, nel determinare la composizione dei vini. Tuttavia, riferendosi agli studi condotti in un singolo vigneto, le caratteristiche dell'annata, descritta dalle temperature dell'aria, il vigore delle piante e le specie di lievito coinvolte durante la fermentazione alcolica risultavano essere variabili capaci di influenzare l'accumulo di composti dotati di proprietà benefiche per la salute nei vini sperimentali. In generale, le uve esposte a maggior numero di giorni caratterizzati da temperature comprese tra 16-22 °C nell'annata (dalla fioritura alla vendemmia), uve appartenenti a piante caratterizzate da un basso vigore, e fermentazioni caratterizzate da basse cinetiche di produzione di etanolo condotte da un unico ceppo di *S. cerevisiae* hanno portato ad un maggiore accumulo di tali sostanze nei vini sperimentali.

Conclusioni: La gestione di pratiche colturali in relazione alle condizioni climatiche dell'annata e il controllo delle popolazioni di lievito coinvolte durante la fermentazione alcolica possono essere cosiderati strumenti utili per ottenere un maggior accumulo nei vini di sostanze dotate di proprietà benefiche per la salute, inoltre coinvolte nella qualità sensoriale e longevità di finale prodotti.

Significato ed impatto dello studio: I risultati riportati implementano la conoscenza sulla variabilità della composizione delle uve e dell'incidenza del comportamento fermentativo dei lieviti nel determinare l'accumulo di diversi composti dotati di proprietà benefiche per la salute nei vini Sangiovese.

Keywords: anthocyanins, flavonols, flavan-3-ol monomers, *Saccharomyces cerevisiae*, *Candida zemplinina*, tyrosol, hydroxytyrosol, tryptophol, Sangiovese.

Summary

Aims: The aim of this work was to investigate on the factors affecting the accumulation of different health promoting compounds in Sangiovese wines. In particular, the attention was focused on the incidence of several variables able to influence grape composition and the role of yeasts during wine fermentation. Health promoting compounds here studied included grape derived molecules (anthocyanins, flavonols and flavan-3-ol monomers) as well as higher alcohol produced by yeasts (tyrosol, hydroxytyrosol and tryptophol).

Methods and Results: Firstly, a method for the evaluation of health promoting compound contents in Sangiovese experimental wines was developed. Standardized procedures were defined starting from the sampling operations and storage of samples, to winemaking and finished wines' analysis. Experimental wine composition deeply reflected the specific conditions experimented by grapes. On the basis of results here obtained, the accumulation of health promoting compounds in Sangiovese wines resulted to be influenced by a series of variables. In this work, the effect of viticultural area was found to be deeply combined to the effect of the vintage, thus remarking the well known importance of *Terroir*, in its widest meaning, in determining wine composition. However, when referred to grapes from a single vineyard, it emerged that air temperature, vigor and yeast species involved during the alcoholic fermentation were variables affecting the accumulation and partitioning of health promoting compounds in experimental wines. In general, grapes exposed during the vintage (from blooming to harvest) to higher number of days characterized by temperatures between 16-22°C, grapes from low vigor vines and fermentations characterized by lower kinetics carried out by a single strain of *S. cerevisiae* led to a higher accumulation of health promoting compounds in the experimental wines.

Conclusions: The management of viticultural practices according to the climatic conditions of vintage and the control of yeast populations involved during the alcoholic fermentation can be considered useful tools in order to obtain wines richer in health promoting compounds which are also potentially involved in sensory quality and longevity of final products.

Significance and Impact of the Study: The results here reported improve the knowledge on variability of grape composition and the incidence of yeast fermentative behavior in determining the accumulation of different health promoting compounds in Sangiovese wines.

Papers related to the thesis

Mangani, S., Buscioni, G., Romboli, Y. and Vincenzini, M.: **Variability of anthocyanin and flavonol profiles in Sangiovese wines**. Paper to be submitted to Italian Journal of Food Science. (In this thesis, Cap. Results 2.2)

Romboli, Y., Mangani, S., Buscioni, G., Granchi, L. and Vincenzini, M.: **Effect of Saccharomyces cerevisiae and Candida zemplinina on quercetin, vitisin A and hydroxytyrosol contents in Sangiovese wines.** Paper to be submitted to World Journal of Microbiology and Biotechnology . (In this thesis, Cap. Results 2.4.2 and 3.1)

Contributions to congress

Poster:

Romboli, Y., Mangani, S., Buscioni, G., and Vincenzini, M.: **Variability of Tyrosol, Hydroxytyrosol and Tryptophol concentrations in wines obtained from Sangiovese grapes for Brunello di Montalcino wine production, vintage 2011.** SIMTREA congress (Società Italiana di Microbiologia Agraria, Alimentare e Ambientale), Bari (Italy) 26-28 June 2012.

Romboli, Y., Mangani, S., Buscioni, G., and Vincenzini, M.: **Tyrosol, Hydroxytyrosol and Tryptophol accumulation in wine as affected by microbial ecology of alcoholic fermentation**. SIMTREA congress (Società Italiana di Microbiologia Agraria, Alimentare e Ambientale), Bari (Italy) 26-28 June 2012.

Romboli, Y., Mangani, S., Buscioni, G., and Vincenzini, M.: **Bioactive compounds of microbial origin: variability of Tyrosol, Hydroxytyrosol and Tryptophol contents in Sangiovese wines. ENOFORUM 2013**, Arezzo (Italy) 7-9 May 2013.

Romboli, Y., Mangani, S., Buscioni, G., and Vincenzini, M.: **Tyrosol, Hydroxytyrosol and Tryptophol contents in wine as affected by fermenting yeast species and must aeration.** WAC 2014 (International Congress of Wine Active Compounds), Beaune (France), 26-28 March 2014.

Romboli, Y., Mangani, S., Buscioni, G., and Vincenzini, M.: **Quercetin contents of Sangiovese wines as affected by fermenting yeast species and must aeration**. X Congresso Italiano di Chimica degli Alimenti, Firenze (Italy), 6-10 Luglio 2014, Firenze

Oral presentation:

Quercetin, vitisin and hydroxytyrosol contents of sangiovese wines as affected by fermenting yeast species and must aeration - Convegno Internazionale Environmental Sustainability and Food Security, Potenza 17-19 Giugno 2014

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Introduction

1. Brief literature review on moderate wine consumption and health aspects

The effects of wine on health is a subject that goes back a long way in the history of human population. The medicinal use of wine has been recorded since the Sumerians wrote recipes for wine-based medicines in 2200 BC. The Greek physician Hippocrates recommended wine as a disinfectant and recognized to it an important role in the healthy diet. Galen, in the ancient Rome, treated the wounds of gladiators. Moreover, the Jewish Talmud described wine as “the foremost of all medicine”(Denning et al., 2004). On the basis of such information, still nowadays, wine is considered the "oldest known medicine" (Robinson, 2006). More in particular, wine has been described as an antiseptic, a painkiller and to treat dermatological conditions and digestive disorders (Robinson, 2006). To date, the Mediterranean diet has become the reference diet for the prevention of cardiovascular diseases. Red wine seems to be an essential component of the diet, since its moderate consumption is associated with lower risk and mortality from cardiovascular diseases (Guerrero et al., 2009).

In the early 1990s the research efforts on health benefits of red wine have been encouraged as a consequence of the media impact of the French Paradox. In 1992, Renaud and de Lorgeril explained the "French paradox" publishing a study on *The Lancet* in which they revealed a lower incidence of coronary heart disease in France despite high levels of saturated fat in the traditional French diet. A moderate daily consumption of red wine has been proposed to contribute to this effect (Renaud de Lorgeril, 1992; Renaud and Gueguen 1998). From then on, various epidemiological studies were carried out on wine and health field contributing to strengthen the knowledge of the effects of wine moderate consuming on a reduction in all-cause mortality, particularly cardiovascular mortality, when compared with individuals who abstain or who drink alcohol to excess (Ruf , 2003; Guilford and Pezzuto, 2011). Critics of these studies argue that the average moderate wine drinker is more likely to exercise, to be health conscious, and to be of a higher educational and socioeconomic class (Lindberg and Amsterdam 2008).

However, as reviewed by Guilford and Pezzuto (2011), it must be emphasized that the benefits associated with red wine are dependent upon regular and moderate consumption. Although general recommendations are one drink

(150 mL) daily for women and two drinks (300 mL) daily for men, individual ideals may vary based on age, gender, genetics, body type, and drug/supplement use. The pattern of wine consumption is also important. Moderate regular drinking gives many health benefits that are lost when drinking is only periodic and heavy, even though the weekly average amount may be the same.

It is important to stress that there is also a large body of evidence that supports the health benefits derived from grape and wine by-products (for recent reviews, Dinicola et al., 2014; Teixeira, et al., 2014) Although the chemical composition of grape and wine differ to some degree, similar effects on human health have been recorded. Some investigators believe that these benefits may actually be enhanced in wine, perhaps due to additive effects with the alcohol component of wine and/or to an increased bioavailability of wine phenols as a result of the fermentation process (Gilford and Pezzuto, 2011).

The mechanisms responsible for the healthful effects of wine are extremely complex. Both the alcohol and the phenolic components have been extensively studied and there is controversy over which component is more important. Indeed, several studies revealed that a moderate intake of alcoholic beverages produces positive effects on antioxidants, lipids, and platelets (for a review, Lindberg and Amsterdam, 2008); whereas, others provide evidence that wine demonstrates beneficial properties that are independent from the presence of alcohol, while they are attributed to the polyphenol content of wine (for a review, Ruf 2003). As reported by Guilford and Pezzuto (2011) the alcohol component increases HDL cholesterol levels, inhibits platelet aggregation, and reduces systemic inflammation. Besides, phenols present in wine independently provide antioxidant protection, decrease platelet aggregation, and increase endothelial function.

Among wine phenols, resveratrol has massively attracted the attention of reserchers from the early 90s. Indeed, it was considered as one of the most important compounds related to the French Paradox. Resveratrol is a key compound implicated in the cardiovascular benefits associated with moderate red wine consumption (Nakata and Inoue 2014). Besides its widely documented biological activities its popularity is also due to the fact that grape, grape juice and wine represent the main western dietary sources of resveratrol (Wenzel and Somoza 2005; Bertelli, 2007). Other sources are peanuts and berries but the amount resulted lower than in wine. Thus,

resveratrol is an important marker in research not only because of its well-known biological activity, which is associated with a broad variety of effects, but also because the calculation of its ingestion in red wine is not confused by the consumption of other beverages or foodstuffs (Bertelli, 2007). Besides resveratrol, wine, and in particular red wine, is a complex mixture of phenolic compounds that possess health enhancing properties and are able to act synergistically to produce health benefits (Guilford and Pezzuto, 2011).

However, wines differ in alcohol and phenols content and composition. Firstly, the peculiar composition and contents of wine can be modulated by a wide range of factors involving both grape and wine chemistry. In particular, variety, geopedological characteristics, climate and agronomical practices are reported to strongly influence the composition of grapes. Moreover, enological practices involved in the whole winemaking may influence the content and composition of wines. As reported by Muller and Fugelsang (1997), yeast type, fermentation temperature, maceration length, the use of clarifying agents, oak-wood aging, and others are practices adopted to increase the acceptability of products, but may change the constituency of the very same compounds purportedly responsible for health attributes of wine. Moreover the Authors affirmed *"...“older” wines. whose phenolic compounds have had the opportunity to extensively polymerize (and entrap other molecules). might not be as good a source of antioxidants as “younger” wines. These polymeric aggregates might not cross the intestinal barrier as easily as do smaller molecules..."*. Therefore, essentially no two bottles of wine have the exact same chemical composition. In addition, the storage procedures and duration of wine aging after it is purchased may also alter its chemical profile (Guilford and Pezzuto 2011). Indeed, several Authors pointed out the necessity to fully characterize wine analytically when carrying out studies on wine moderate consumption and health related benefits (Muller and Fugelsang 1997; Bertelli, 2007; Guilford and Pezzuto, 2011).

2. Flavonoids: health promoting compounds of plant origin

2.1. Classification, chemistry and role in grape and wine

Phenolic compounds can be defined as molecules naturally derived from plants or microbes, consisting of a phenyl ring backbone with a hydroxyl group or other substituents. In grape, phenolic compounds are classified in non-flavonoid, such as hydroxybenzoic and hydroxycinnamic acids, volatile phenols and stilbenes (characterized by a simple C6 backbone), and flavonoid compounds. Despite a not negligible presence of non-flavonoid compounds, flavonoids make up a significant portion of phenols in grape and wines (Teixeira et al., 2013).

Flavonoids are a large class of substances widely diffused in plant kingdom produced by the phenylpropanoid pathway. To date, over 6000 compounds belonging to this large family of secondary metabolites have been identified (Erlund, 2004; Hichiri et al., 2011). Plants synthesize flavonoid as they confer UV-protection, are involved in the mechanism of attraction of pollinators and in seed dispersal strategy, and they act as protectors of tissue in case of oxidative damage and pathogen attack (Harborne and Williams, 2011). In grapes, flavonoid accumulate principally in skins and seed and anthocyanins, flavonols and flavan-3-ols are the most abundant subgroups detected (Downey et al., 2006). The berry growth follows a double sigmoid curve that can be divided into two growth phases (Stage I and III) separated by a lag phase (Stage II). The transition from stage II to stage III, is named veraison and it is considered the onset of ripening, i.e. the moment at which sugars and anthocyanins begin to accumulate. Flavonols and flavan-3-ols are synthesized before veraison (flowering) and their accumulation continues from fruit set to one or two weeks after veraison (Downey et al., 2006; Castellarin et al., 2007)

The increasing interest on these compounds, from the early 1990s until now, resides in the fact that they are ubiquitous in the human diet and their intake has been associated with several health benefits (Georgiev et al., 2014). However, flavonoids are divided into various subgroups and their biological and chemical properties are closely related to the subgroup of belonging (Erlund, 2004). Therefore, since wine is a complex mixture, it is likely that a multitude of chemical constituents, as well as their metabolites, act synergistically on human health (Banc et al., 2014).

From a chemical point of view, flavonoids have a common basic structure consisting by two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle (C6-C3-C6). The oxidation state of the C rings

define several structural classes that include flavonols, flavan-3-ols (monomers, dimers, trimers and polymers) and anthocyanins (Teixeira et al., 2013).

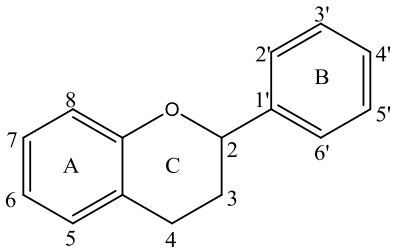


Figure 1: Flavonoid basic structure and numbering

In wines, flavonoid contributes significantly to sensorial quality. Indeed, they are the main responsible of color, flavor, texture and astringency. Moreover, they contribute to antioxidant properties of wines. The antioxidant activity of phenols in wines depends on the easiness of reaction with oxidants in the form of reactive oxygen species (ROS) (Waterhouse, 2002). Oxygen is not very active directly on organic molecules. However, after several reactions involving the transfer of electrons from different compounds, such as iron and phenols, the formation of hydroperoxide radical (HO_2•), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) occur. The hydroperoxyl radical is not reactive enough to abstract a hydrogen from many substrates, but the good hydrogen-donating properties of phenolics make them an exception. When phenolics react with ROS such as the hydroperoxyl radical, the reaction rate of each phenolic depends on its ability to form a stable product radical.

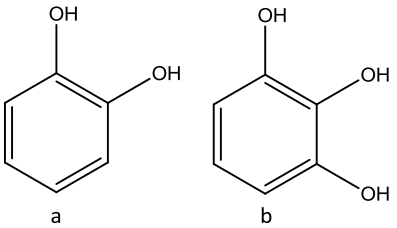


Figure 2: Simple phenols not found in wines but present as functional group in grape and wine phenols. a) pyrocatechol, b) pyrogallol

Compounds containing a 1,2,3-trihydroxyl group (pyrogallol), or a 1,2-dihydroxy aromatic ring (catechol), reacts very readily with oxidants in the form of free radical reactive oxygen species to form a very stable radical anion,

the semi-quinone radical. It is stable enough that it does not abstract hydrogens from other substances and will persist long enough to react with another semi-quinone radical, resulting in a disproportionation reaction which yields a quinone and a phenol, in total quenching two radicals. Nearly all wine phenolics are very reactive towards the hydroperoxyl radical (Waterhouse, 2002; Waterhouse and Laurie, 2005). These reaction of oxidation lead to the formation of polymeric pigments by direct condensation of anthocyanins with other flavonoids or a combination of pigments with acetaldehyde. As a consequence, the production of highly colored compounds, and reduced astringency, occurs, affecting both the wine color and the palatability. In spite of this positive effect, adding oxygen to red wine (e.g. during maturation) reduced the amount of some monomer and oligomeric phenols known to be related to the antioxidant status of wine (Castellari et al., 2000).

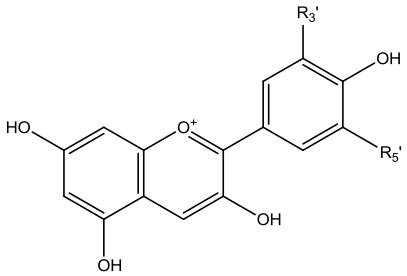
The amount of flavonoids extracted during vinification is influenced by many factors, including temperature, length of skin contact, mixing, type of fermentation vessel, ethanol concentrations, SO_2, yeast strain, pH, and pectolytic enzymes. Extraction is ultimately limited by the amount present in the fruit, and this varies with cultivar, vintage, macro- and micro-climatic conditions, and vinification process (Soleas et al., 1997). Indeed, sensorial quality and health enhancing properties of finished wines depends on many factors involving both grape composition and enological practices.

2.1.1.Anthocyanins

Anthocyanins are the pigments responsible for red, purple and blue color of grape berries and, consequently, of the red wines in which their concentration and composition influence both hue and color stability. They accumulate, generally, in berry skins (hypodermis) in which they play several roles including attraction of predators for seed dispersal, protection against photo-oxidative stress, defense against different pathogens, free radical scavenging (He et al., 2010) and play a roles in DNA protection (Teixeira et al., 2013).

Anthocyanins are defined as the glycosides and acylglycosides of anthocyanidins that differ one to another for the hydroxyl or methoxyl substitutions occurring at the 3' and 5' position of the B ring of flavylium, the core of anthocyanidins. Anthocyanins can also be esterified by acids, such as acetic, *p*-coumaric or caffeic, linked to the 6' position of the glucose bonded to the position 3 of the C ring. Five anthocyanidins are reported in grapevine: delphinidin, cyanidin, petunidin, peonidin and malvidin, despite recently several authors reported the presence of pelargonidin at trace levels (Castillo-

Muñoz et al., 2009; He et al., 2010). In *Vitis vinifera*, only anthocyanidin-3-O-monoglycosides are found due to two mutations in the 5-*O*-glucosyltransferase gene; these mutations implicated the loss of the dominant allele involved in the production of diglucosidic anthocyanins. Generally, malvidin-3-O-glucoside, along with its acylated forms, is the most abundant anthocyanin.



R_3'	R_5'	Name of aglycone
H	H	pelargonidin
OH	H	cyanidin
OCH_3	H	peonidin
OH	OH	delphinidin
OCH_3	OH	petunidin
OCH_3	OCH_3	malvidin

R	Name of derivative
H	Mv-3-*O*-glucoside
-acetyl	Mv-3-*O*-(6-*O*-acetyl)-glucoside
-*p*-coumaroyl	Mv-3-*O*-(6-*O*-*p*-coumaroyl)-glucoside
-caffeoyl	Mv-3-*O*-(6-*O*-caffeoyl)-glucoside

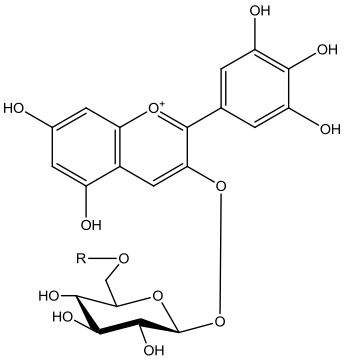


Figure 3: Anthocyanidin structures and malvidin derivatives

The molecular structures of the different anthocyanins influences the color. The substitution of the lateral B ring leads to a bathochromic shift of the maximum adsorption wavelength (towards violet). On the other hand, despite acylation is reported to shift the color tonality to purple (Stintzing et al., 2002), glucose fixation and acylation shift the color towards orange when compared to native anthocyanidin (Ribéreau-Gayon et al., 2000b). In wines, the color of this pigment resulted dependent by the medium conditions such as pH and SO_2 (Ribéreau-Gayon et al., 2000b).

The anthocyanin profile, defined as the partitioning of glucosides and acylated anthocyanins, is reported to be a varietal characteristic. Therefore, it can be used as a chemotaxonomical parameter for classification of red *Vitis vinifera*

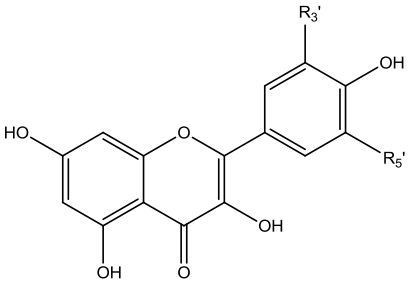
varieties (Von Baer et al., 2005). As an example, Sangiovese, the most widely cultivated red-berried cultivar in Tuscany (Italy), is characterized by a higher percentage of 3-*O*-glucosides of cyanidin, delphinidin and petunidin. However, the concentration of these anthocyanins falls during winemaking with respect to that of methoxylated anthocyanins, such as peonidin 3-*O*-glucoside and malvidin 3-*O*-glucoside (Arapitsas et al., 2012). Moreover, a lower abundance of acylated pigments is also reported (Mangani et al., 2011).

In berry skins, anthocyanins appear at veraison where, initially, only the glucosides of disubstituted anthocyanins as cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside are synthesized, followed by the trisusbstituted anthocyanins delphinidin, petunidin, and malvidin. Their accumulation in epidermal cells continues throughout the ripening process, reaching a maximum concentration at full maturity and then decreases (Downey et al., 2006).

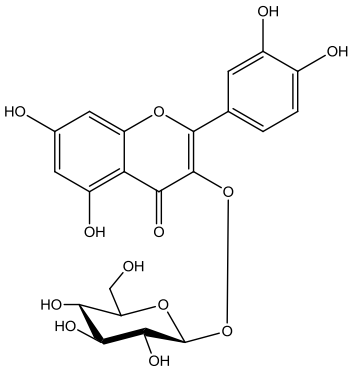
2.1.2.Flavonols

Flavonols are a class of substances widely distributed in several plants organs (e.g. in grape vine: leaves, stems, flowers and fruits) in which they play a fundamental role as UV-A and UV-B protectants localized in the outer epidermis (Keller, 2010; Flamini et al., 2013) and as copigments with the anthocyanins in flowers and fruits (Asen et al., 1972). Moreover, flavonols seems to be involved in plant-pathogen interactions (Macheix et al., 2005).

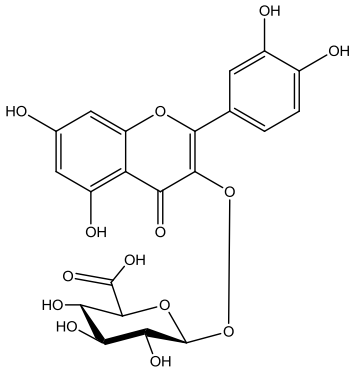
Structurally, flavonols are characterized by a 3-hydroxyflavone backbone. The number and the types of substitutions on the B ring determine the difference of molecules belonging to this class. Conventionally, they accumulate in the vacuoles of epidermal tissues as glycosides (Price et al., 1995). In *Vitis vinifera*, flavonols constitute the third most abundant component of flavonoids in the skin fraction, after anthocyanins and flavan-3-ols. They occur in grapes as glucosides, glucuronides, galactosides and to a lesser extent as rhamnosides and rutinosides (Flamini et al., 2013), with the sugar bond attached to the 3 position of the flavonoid skeleton (Teixeira et al., 2013). In the berry skins kaempferol, quercetin, myrcetin and, in smaller abundance, their methylated forms isoharmnetin, laricitrin and syringetin are found. Generally, the main flavonols reported in grape berries are quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronide (Downey et al., 2003).



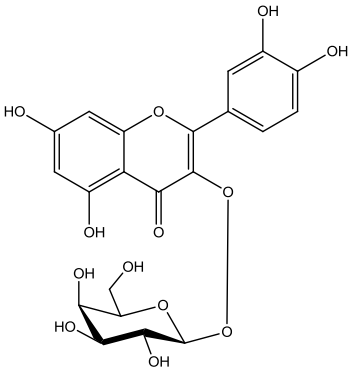
R_3'	R_5'	Name of aglycone
H	H	kaempferol
OH	H	quercetin
OCH_3	H	isorhamnetin
OH	OH	myricetin
OCH_3	OH	laricitin
OCH_3	OCH_3	syringetin



quercetin-3-O-glucoside



quercetin-3-O-glucuronide



quercetin-3-O-galactoside

Figure 4: Flavonol structures and quercetin glycosides

As for anthocyanins, also flavonols are highly variable across genotype and thus, their patterns are consider useful markers in *Vitis vinifera* chemotaxonomy (Downey et al., 2003; Flamini et al., 2013). Moreover, the pattern of distribution of flavonols seems to be related to that of anthocyanins for a given variety. In particular, Mattivi et al. (2006), studying the pattern of

64 different grape varieties, found that a substantial presence of myricetin was found in the majority of red varieties characterized by their purple color, due to the large amount of delphinidin derivatives. Conversely, the red varieties characterized by a dominant amount of cyanidin derivatives in the anthocyanins were also characterized by the substantial prevalence of quercetin among flavonols. Sangiovese is reported to have a high concentration of quercetin, together with other light red varieties such as Nebbiolo and Pinot Noir. Besides, the abundance of myricetin is reported to be inferior or closer to 20%. Varieties characterized by high abundance of myricetin (> 45%) are, among others, Cabernet Sauvignon, Sagrantino and Teroldego (Mattivi et al., 2006).

Highest flavonol concentrations in grapes are detected at flowering. Then, the increasing in size of grape berry coincides with a decrease in flavonol amounts. A subsequently significant level of flavonols biosynthesis was reported during berry development. Finally, the greatest increase in flavonols accumulation can be observed one to two weeks postveraison and continuing throughout ripening (Downey et al., 2006).

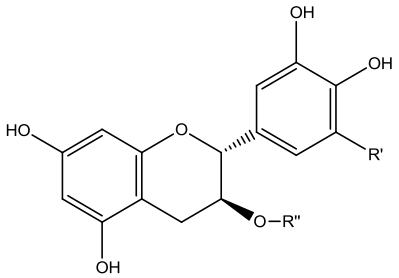
During the winemaking process, the glycosides are hydrolyzed and flavonol aglycones are released (Price et al., 1995). Quercetin and its glycosides participate to wine color by acting as copigmentation cofactors (Boulton, 2001) However, due to its low solubility, free quercetin can be responsible of haze and yellow deposits in wines (Somers and Ziemelis, 1985).

Flavonols are also studied from a nutritional point of view, being highly bioactive compounds widely distributed in dietary plants (Manach et al., 2004). In particular, the antioxidant and anti-inflammatory properties of quercetin and its glycosides, generally the most abundant flavonols in grapes, have been extensively reviewed due to their association in the prevention and therapy of cardiovascular diseases and cancer (Russo et al., 2012).

2.1.3.Flavan-3-ols

Flavan-3-ols are the most abundant class of phenolics in grape berry. They are found as monomers or polymeric structure. Thiese polymers are also referred to as proanthocyanidins (PAs) or condensed tannins (Ribéreau-Gayon et al., 2000b). Principally, flavan-3-ols are located in the seeds and in stems, then in skins, and also are found in the vasculature of the berry (pulp). The basic units of PAs that accumulate in grapes skins and seeds, also referred to as catechins, are (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin-3-O-gallate (ECG), and (-)-epigallocatechin (EGC), the latter being present only in skins (Cheynier et

al., 1998). In the seeds, procyanidins are characterized by a lower degree of polymerization and, generally, by a higher content of flavan-3-ol monomers than in skins (Downey et al., 2003).



R'	R''	
H	H	(+)-catechin
OH	H	(+)-gallocatechin

R'	R''	
H	H	(-)-epicatechin
OH	H	(-)-epigallocatechin
H	-galloyl	(-)-epicatechin-3-*O*-gallate

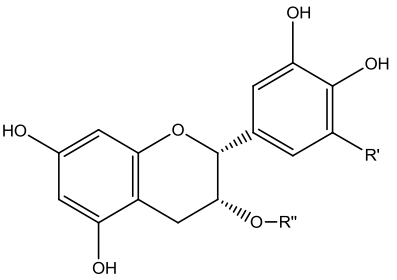


Figure 4: Flavan-3-ol monomer structures

Flavan-3-ol monomers are reported to concur to astringency and principally to bitterness of wines, while astringency increases with the degree of polymerization. However, relatively to their astringency, larger polymers and monomers are more bitter than dimers, trimers or tetramers (Gawel, 1998). Due to their sensorial properties, flavan-3-ols and in particular PAs are thought to act as a feeding deterrent to herbivorous and insects (Teixeira et al., 2013). Moreover, phenolic compounds in the seed coat may act as biochemical barrier to permeability to oxygen. As a consequence, phenolic compounds reduces oxygen supply to the seed embryo, protecting it from oxidation (Werker, 1997).

As for flavonols, flavan-3-ols start to accumulate at flowering. The main period of accumulation of tannins occurs immediately after fruit set with the maximum level observed at veraison. Their accumulation continues until 1 to 2 weeks after veraison (Downey et al., 2006). In seeds, flavan-3-ols accumulate in the soft parenchyma of seed coat between the cuticle and the hard seed coat. Tannins represent the most soluble phenols in seeds, in which are also

present in lesser amounts flavan-3-ol monomers. During ripening, a general decline in seed tannins has been reported. Such decline may reflect their covalent attachment to the insoluble matrix of the seed. In this phase, a color change of seeds has been observed. Kennedy et al. (2000). Seed browning is considered the result of tannins oxidation. Downey et al. (2003) reported that at ripe stage, up to 30% of the seed tannin extension subunit may be covalent bound and thus unavailable for extraction during winemaking. Thus, the contribution of seed flavan-3-ols to wine appear to be more consistent as long the maceration length is (Glories and Saucier, 2000). Moreover, during ripening, a decrease of 90% in flavan-3-ol monomers and 60% of procyanidins has been reported in Cabernet Sauvignon (Kennedy et al., 2000).

In skins, flavan-3-ols are principally found as PAs co-located with anthocyanins in the vacuole of thick-walled hypodermal cells. The concentration of skin tannins declines during ripening in proportion to berry growth. During the period from fruit development and ripening, skin tannins show an increasing degree of polymerization (mDP) from green berries to red berries to maturity. As reported for seed tannins, the level of extractability of skin tannins decrease between veraison and harvest (Downey et al., 2006).

Flavan-3-ols, in particular tannins, are compounds of great importance for wine quality due to their astringent and bitter properties. Indeed, tannins in wines undergo to reaction of polymerization with other tannins thus decreasing the astringency sensation (Ribéreau-Gayon et al., 2000b). Even long polymeric tannins, as long as 70 subunits, remain in solution; tannins precipitate only when they bind to proteins in wine. Polymerization is a dynamic process in wine; polymeric tannins are susceptible to cleavage so that their degree of polymerization can increase or decrease during aging (Keller, 2010). Moreover, by the interaction with anthocyanins, pigmented polymers are produced leading to a higher long-term color stability (Vidal et al., 2003). Finally, the level of catechin monomers in the wines decreased with increasing grape maturity(Downey et al., 2006)

As in the case of flavonols and anthocyanins, the biosynthesis of (+)-catechin and (-)-epicatechin was supposed to be dependent on genetic factors related to each grape cultivar, and the ratio between these compounds was used to differentiate the cultivar (Pinot Noir and Merlot) employed during winemaking (Goldberg et al., 1998).

2.2. Biosynthesis of anthocyanins, flavonols and flavan-3-ols

Flavonoids are biosynthesized in plants through the widely distributed secondary metabolic phenylpropanoid and flavonoid pathways. The major classes of flavonoid compounds found in grape berries include flavonols, flavan-3-ols and anthocyanins, which share a common upstream pathway involving some key enzymes, such as phenylalanine ammonialyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumarmate: CoA ligase (4CL), chalcone synthase (CHS) and chalcone isomerase (CHI) (He et al., 2014).

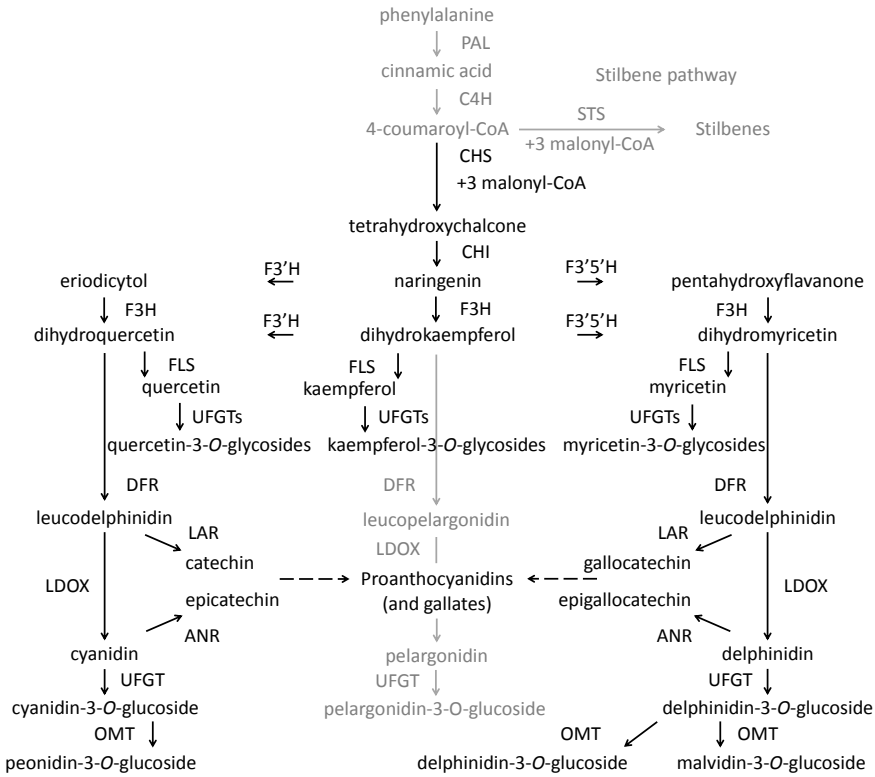


Figure 5: The phenylpropanoid/flavonoid pathway (source: Bogs et al., 2005; Pfeiffer et al., 2006; Castellarin et al., 2007; He et al., 2010b;Azuma et al., 2012; Flamini et al., 2013; Huang et al., 2013;Teixeira et al., 2013; Soubeyrand et al., 2014).

Briefly, PAL catalyzes the first step in the phenylpropanoid pathway by removing the NH_3 radical from L-phenilalanine, and then, catalyzed by C4H and 4CL, p-coumaroyl-COA is produced. It is worth noting that *p*-coumaroyl-CoA is a branch point in the phenylpropanoid pathway at which CHS leads towards

flavonoid synthesis and STS leads towards stilbene synthesis. The starting point of the flavonoid pathway is represented by the formation of naringenin chalcone, as a consequence of condensation between three malonyl-CoA molecules and one p-coumaroyl-CoA molecule catalyzed by the action of chalcone synthase (CHS). Then, naringenin chalcone is quickly converted in its naringenin flavanone after the action of chalcone isomerase (CHI). The B ring of naringenin flavanone can be hydroxilated by the flavonoid 3'-hydroxylase (F3'H) or flavonoid 3'5'-hydroxylase (F3'5'H) to produce eriodictyol or pentahydroxyflavanone, respectively. This three flavanones are then involved by the action of flavanone 3β-hydroxylase (F3H, also known as FHT) to produce the corresponding dihydroflavonols. In particular, dihydrokaempferol, dihydroquercetin and dihydromyricetin are produced by the reaction catalyzed by F3H from naringenin flavanone, eriodictyol or pentahydroxyflavanone. Moreover, dihydrokaempferol is a potential substrate for F3'H and F3'5'H, to produce dihydroquercetin and dihydromyricetin, respectively.

The formation of dihydroflavonols in the flavonoid pathway is the crucial step for flavonol synthesis (Catsellarin et al., 2007). Flavonol synthase (FLS) is the key enzyme in the conversion of dihydroflavonols into flavonols (Spribille and Forkmann, 1984). In particular, quercetin, myricetin and kaempferol derived from diydroquercetin, dihydromyricetin and dihydrokaempferol. This three basic flavonols differ one to another by the number of hydroxyl group in the B ring. Kaempferol is monohydroxylated in position 3'; quercetin is dihydroxylated in positions 3' and 4'; and myricetin is trihydroxylated in positions 3', 4' and 5'. Moreover, despite in lower concentration, also isorhamnetin, laricitrin and syringetin are found in grapes (Flamini et al., 2013). These molecules are produced by methylation of quercetin, the former, and myricetin, the latters by the enzyme *O*-methyltransferase (OMT). However, flavonols are much worse substrates than anthocyanins, with differences of 1 order of magnitude in the activities of OMT on these two classes of flavonoids. Indeed, the hydroxylated forms of flavonols, quercetin and myricetin, resulted dominant in comparison to the corresponding *O*-methylated forms (Mattivi, et al., 2006). In white and light red varieties the lack of expression of the enzyme F3'5'H prevents the synthesis myricetin. Indeed, in these varieties the flavonols composition is characterized by the presence of quercetin, kaempferol and isorhamnetin derivatives (Flamini et al., 2013).

In grapes (*Vitis vinifera* sp.), flavonols are found in form of 3-*O*-glycosides (Markis et al., 2006). As for anthocyanins, glycosylation enhances their water

solubility, allowing their accumulation in plant cells (Ono et al., 2010). Genarally, flavonols occur in grapes as glucosides, glucuronides, galactosides, rhamnosides and rutinosides, mostly represented by the former three (Flamini et al., 2013). Regarding flavonol-3-*O*-glucosides, it has been demonstrated that UDP-glucose:flavonoid 3-*O*-glucosyl transferase (UFGT, GT1) catalyses glucosylation of both anthocyanidins and flavonols, but its efficiency is much higher for the former (Ford et al., 1998). Other two glycosyltransferases that contribute to the structural diversification of flavonol glycosides in grape vine were recently identified (Ono et al., 2010). In particular, GT5 is a UDP-glucuronic acid:flavonol-3-*O*-glucuronosyltransferase.The second, GT6, is a bifunctional UDP-glucose / UDP-galactose : flavonol-3-*O*-glucosyltransferase / galactosyltransferase (Ono et al., 2010).

The three dihydroflavonols, produced in the flavonoid pathway, can be also reduced by dihydroflavonol 4-reductase DFR with consequent production of their corresponding leucoanthocyanidins. Finally, the leucoanthocyanidins can be oxidized to their corresponding colored anthocyanidins by the action of anthocyanidin synthase (ANS), also known as leucoanthocyanidin dioxygenase (LDOX).

After the action of ANS, the unstable anthocyanidins have to be converted in more stable vacuolar anthocyanins. This is the begin of the specific anthocyanin downstream branch (He et al., 2010b). Hence, immediately after the production of anthocyanidins in the cytosol, reaction of glycosilation, methylation and acylation occur. The first reaction that occurs in red grape variety is the glycosilation of anthocyanidins. This reaction is fundamental for the accumulation of stable anthocyanins in berries. Indeed, mutants of red grape varieties lacking the expression of the genes for the initial glycosylation of anthocyanidins usually they do not accumulate anthocyanins in their berries, despite the intact anthocyanidin biosynthetic pathway exists, (Boss et al., 1996). Generally, *Vitis vinifera* red varieties present only 3-O-glucosides derivates, while 3-5-O-diglucosides are found in other grapevine species (Janvary et al., 2009). The glucosylation of anthocyanins is catalized by UDP-glucose:flavonoid 3-*O*-glucosyl transferase (UFGT). In *Vitis vinifera*, anthocyanin can only be *O*-glucosidated. Thus, UFGT used to be called as 3-*O*-glucosiltranferase (3-GT or GT1) (Ford et al., 1998; He et al., 2010b). As for mutants of red grapes, white varieties do not accumulate anthocyanins in the skins because the gene encoding UFGT is not expressed (Boss et al., 1996),

remarking the fundamental role of such enzyme in determining the accumulation of anthocyanins in grapes.

Another reaction that confer stability to anthocyanins is the methylation of the hydroxyl groups at the C_3 positions or at both C_3 and C_5 positions on the B ring. These reactions are mediated by the enzyme *O*-methyltransferase (OMT) which allow the formation of peonidin-3-*O*-glucoside from cyanidin-3-*O*-glucoside and petunidin-3-*O*-glucoside and malvidin-3-*O*-glucoside from delphinidin-3-*O*-glucoside.

Finally, acylation is one of the most common modifications involving plant phenols, including anthocyanins. The acylation confers to the anthocyanins a higher stability on color. In *Vitis vinifera*, the most common acylated anthocyanins are acetyl, *p*-cumaroyl and caffeoyl derivatives. The addition of acetyl, *p*-cumaroyl and caffeoyl constituents linked to the C6'' positions of glucosyl groups is catalyzed by the action of anthocyanin acyltransferases (ACT).

Thus, anthocyanins could be transported into the vacuoles *via* the non-covalent activity of glutathione S-transferase (GST). GST proteins cannot transport anthocyanins themselves, but need to work together with the glutathione *S*-conjugate pump, which is an ATP-binding cassette (ABC) transporter and belongs to the multidrug resistance-associated protein (MRP) family (He et al., 2010b). On the other hand, multidrug and toxic compound extrusion (MATE) transporters are another class of putative transport factors that may be involved in the intracellular transportation of anthocyanins (He et al., 2010b). While the biosynthesis and regulation mechanisms of anthocyanin synthesis have been extensively studied, the knowledge on the mechanisms of their sequestration in the vacuole and on to what extent their color is affected by vacuole storage is still limited (Teixeira et al., 2013).

The key enzymes for the synthesis of flavan-3-ol monomers are leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR). Leucoanthocyanidins are produced by the two lateral branches of the flavonoid pathway. In particular, they are classified as leucocyanidin and leucodelphinidin as a consequence of the hydroxyl substituent in their B ring. LAR is responsible of the reduction of leucoanthocyanidins which lead to the formation of (+)-catechin, from leucocyanidin, and (+)-gallocatechin, from leucodelphinidin. Besides, ANR is the crucial enzyme that catalyzed anthocyanidins in flavan-3-ol monomers. In particular, (-)-epicatechin is formed from cyanidin and (-)-epigallocatechin is formed from delphinidin

(Huang et al., 2014). These four monomeric flavan-3-ols, together with their acylated forms (esters with gallic acid), are the fundamental constituents of PAs in grapes. Indeed, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin-3-O-gallate, with traces of (+)-gallocatechin are the fundamental constituents most commonly found in grape PAs (Souquet et al., 1996). PAs are defined as oligomeric and polymeric flavan-3-ols, in which the monomeric the "extension" subunits are linked by C-4-C-8 or C-4-C-6 interflavonid bond (Souquet et al., 1996; De Freitas and Glories, 1999). PAs are also referred to as condensed tannins. Substantial differences exist in seed and skins flavan-3-ols composition. In fact, the average size of skin tannins, as their mean is much larger than seed tannins (mDP 4 to 6 subunits), and skin tannins contain epigallocatechin subunits whereas seed tannins generally lack epigallocatechin. Indeed, it is well known that in seeds only procyanidins ((+)-catechin, (-)-epicatechin) are detectable; in skins, instead, procyanidins and prodelphinidin ((-)-epigallocatechin and (+)-gallocatechin) are found (González-Manzano et al., 2004). Moreover, the smaller seed tannins usually have a higher proportion of their subunits as epicatechin-3-*O*- gallate which is found only in trace in skins (Souquet et al., 1996). Finally, seed tannins are constituted by (+)-catechin and (-)-epicatechin in a similar amount whereas in skin tannins the latter is prevalent (Downey et al., 2006).

2.3. Factors influencing biosynthesis and accumulation of flavonoids in grapes

Considering the importance of flavonoids, responsible of various aspects related to wine quality and their health benefits, several Authors in the literature discussed and reviewed the effects of factors affecting flavonoid biosynthesis and accumulation in grape berries. In particular, it emerged that phenolic synthesis and accumulation is deeply determined by the interaction between genotype and environment (Teixeira et al., 2013). Moreover, agronomical practices are able to modify the microclimatic condition of grape berries thus altering flavonoid biosynthesis and accumulation. Indeed, many variables have been taken into consideration such as light exposure, temperature, water, nutritional status, altitude, soil type, microbial interaction, pathogenesis, wounding, defoliation, plant growth regulators, and various developmental processes (Downey et al., 2006).

However, many of these factors are closely interrelated and difficult to isolate experimentally, even excluding the diversity of genotype (about 10,000 known Vitaceae cultivars) (Downey et al., 2006). Indeed, variety is the first variable affecting contents and composition of anthocyanins, flavonols and flavan-3-

ols. In particular, anthocyanin and flavonol profiles resulted characteristic for each variety and are considered useful tools for the classification of *Vitis vinifera* red variety (Mattivi et al., 2006). Moreover, it seems that variety determines both the amount and the structure of wine flava-3ols (Mattivi et al., 2009).

Among many environmental factors reported to influence flavonoid content and composition of a given grape cultivar, site and season have been indicated to impact dramatically (Downey et al., 2006).

Indeed, these inputs can be crudely summarized as light/radiation and temperature, as well as water and nutritional status (Teixeira et al., 2013).

Nitrogen

The mineral nutrients are fundamental for plant growth and accumulation of metabolites in various organs. Nitrogen is the most effective of them, influencing vine growth, morphology, fruit production and tissue composition (Keller, 2010). High nitrogen supply favors vegetative growth, inducing also growth of lateral shoots and thus influencing canopy density which has been associated with reduced fruit sugar, high acidity and poor color. As first a consequence of high nitrogen supply is the competition between growing shoots and clusters for supply of assimilates, inducing a delay in fruit ripening. Moreover, a direct influence of nitrogen on fruit composition is also reported, as nitrate uptake leads to a reprogramming of the expression of many genes involved in metabolism. Besides an effect of accumulation of organic acids and aminoacid at the expense of sugar and phenolic compounds, nitrate supply is reported to suppress the expression of the genes involved in phenolic production (Keller, 2010). Both low and excessively high levels of nitrogen have been shown to decrease color of grape berries. In this connection, research on *Terroir* show that a moderate nitrogen deficiency has been correlated with improved grape quality (Chone et al., 2001).

A typical plants response to low nitrogen status is the accumulation of phenolic compounds. In particular, the accumulation of anthocyanins during veraison result maximized at low to moderate nitrogen availability. In this connection, low nitrogen status is also reported to increase the accumulation of flavonols in berries in preveraison, although differences in berries treated at different levels of nitrogen supply tend to decrease towards maturity (Keller and Hrazdina, 1998). In a recent study carried out on Cabernet Sauvignon, an up regulation of several structural genes involved in flavonoid metabolism was

assessed in low nitrogen supply conditions (*PAL*, *CHS*, *F3H*, *F3'5'H*, *DFR*, *LDOX* and *OMT*) from veraison towards maturity. Moreover, despite a not significant difference in the ratio between di- and trisubstituted anthocyanins at mid-ripe and full maturity, an increase between 8 and 10% of cyanidin and delphinidin was observed in these two periods (Soubeyrand, et al., 2014).

Nitrogen availability is one of the most important factors in determining vine vigor. Tannin contents in skins of Pinot Noir were found to be affected by vine vigor. In particular, Cortell et al. (2005) observed an increase in proportion of (-)-epigallocatechin subunits in PAs and a general increase in the average size and contents of polymers with decreasing of vine vigor. However, as suggested by Downey et al. (2006), these differences may be due not only to an effect of vine vigor but also to an indirect effect of changes induced by canopy architecture, resulting in differential bunch exposure effects.

Water status

The major consequence of soil type is related to the capacity of the soil to hold water while remaining well drained to avoid waterlogging. Water status has been recognized as a potent agent modulating secondary metabolism in grapevine during berry development. Several Authors report that large fluxes of water are not essential for the optimal plant performance for agricultural purpose. Indeed, managing a moderate water deficit represents a useful tool in order to guarantee a high quality fruit production. It has been demonstrated that, in grape cell cultures, anthocyanin synthesis is extremely sensitive to osmotic stress. The higher accumulation of anthocyanin under osmotic stress suggest that a deficit of irrigation may be manage in order to favor their accumulation in berries (Downey et al., 2006).

However, the effects of an imposed deficit or water apply (i.e. irrigation) may not be univocal. Indeed, the level and the moment at which water status is modulated results crucial. Moreover, the response to moderate irrigation or water stress might also be cultivar dependent (Teixeira et al., 2013). Water availability is reported to impact on a wide range of plant processes other than flavonoid biosynthesis. For example, stomatal closure in response to water deficit reduces photosynthesis, thus reducing all metabolite accumulation and root and shoot growth. Moreover, the primary effect of water deficit is to decrease berry size thus resulting in a concentration of flavonoids in berries (Downey et al., 2006).

In a study carried out on Cabernet Sauvignon, it was demonstrated that both early and late season water deficit lead to a higher accumulation of anthocyanins in berries after veraison. Moreover, early water deficit accelerates sugar and anthocyanin synthesis. Expression profiling revealed that the increased anthocyanin accumulation resulted from earlier and greater expression of the genes controlling flux through the anthocyanin biosynthetic pathway, including *F3H*, *DFR*, *UFGT* and *GST*. Moreover, a shift towards a predominance of trisubstituted anthocyanins was observed, as a consequence of a differential regulation of *F3'H* and *F3'5'H*. In this study also the expression of genes involved in flavonol and flavan-3-ol accumulation were studied, but limited effects were observed at full maturity (Castellarin et al., 2007). Other studies report an increase of flavonols and skin PAs in grapes under drought stress, but it seemed to be cultivar-dependent (Teixeira et al., 2013). Finally, Keddey et al. (2000) observed that water defcits decreased the amounts of flavan-3-ol monomers and greatly increased their rate of loss during fruit ripening.

Light and temperature

As suggested by Downey et al. (2006), for a given site, in an irrigated vineyard, soil will remain constant, nutrition will be adeguate and viticultural practices will be roughly the same from year to year, so the primary seasonal difference will be climatic, predominantly as regards sunlight and temperature.

As the source of energy for photosynthesis, sunlight is the most important climatic factor affecting grape composition. Often it is referred to in terms of the effect of shade. Shade is responsible of decreasing of both light intensity and temperature during the day, and it is very difficult to separate the effect of each of these variables.

Generally, it is reported that shading leads to a general decrease in carbon fixation in grapes that are characterized by lower sugar and total amino acids (with lower abundance of proline and higher of arginine) and higher malic acid contents (Keller et al., 2010). Moreover, the application of shading treatments has also been shown to alter temperature and humidity of the canopy. As a consequence, two contrasting effects have been reported. By lowering vapor pressure deficit, transpiration and photosynthesis are decreased, reducing growth and flavonoid accumulation. Secondly, high humidity have been associated with increasing risk of pathogenesis which may cause a general wound response inducing flavonoid accumulation (Downey et al., 2006).

Respect to flavonoid accumulation, anthocyanins resulted influenced by visible light, whereas flavonols react predominantly to UV-B. Although light is essential for anthocyanin production, direct sun exposure of fruit is not nearly as important for anthocyanins as for flavonols accumulations. There is evidence that biosynthesis of flavonols is more strongly modulated by light than other berry metabolites, thus flavonols are considered useful markers for light exposure (Keller, 2010). Higher flavonol glycosides are accumulate in exposed fruits when compared to shaded cluster composition (Price et al., 1995). Moreover, it was demonstrated that almost negligible contents of flavonols were detected in leaf and fruit when those tissues had not been exposed to sunlight (Downey et al., 2006). Indeed, light modulates the expression of the key flavonols structural gene, *FLS*, and *MYBF1*, a transcriptional regulator involved in their synthesis (Teixeira et al., 2013). As a consequence, any viticultural practice which favors exposing grapes to direct sunlight is expected to have a positive influence on their concentration in berries (Flamini et al., 2013).

Skin flavan-3-ols and PAs are more sensitive than the seed ones to environmental cues; sunlight has been shown to affect their relative content. Indeed, sunlight exposure consistently increased the relative abundance of the tri-hydroxylated gallocatechins at the expense of the di-hydroxylated catechins and increased the length of tannins polymers (Teixeira et al., 2013). However, shading treatments demonstrated to increase the amount of seed PAs and affected their composition in Pinot Noir (Cortell et al., 2006) while had no effects in Shiraz (Downey et al., 2004) reiterating the importance to discriminate between irradiation and temperature effects. However Downey et al. (2006) reported that the decrease in tannin extractability during ripening seemed to be greater in exposed fruit leading to a substantial equal amount of PAs in both exposed and not exposed clusters at harvest.

Bergqvist et al. (2001) observed that anthocyanins increased linearly as sunlight exposure on the north side of the canopy increased, but declined when cluster exposure on the south exceeded 100µmol/m^2/s. Too much radiation can even inhibit anthocyanin accumulation or induce degradation, perhaps due to the formation of peroxide (H_2O_2) as a result of oxidative stress (Keller, 2010). Downey et al. (2006) reviewed the effect of bunch exposure on anthocyanins composition. It was reported that shaded fruits are characterized by a higher proportion of trisubstituted anthocyanins associated with an increase of proportion of coumaroylglucosides compared to anthocyanidin-3-

O-glucosedes. However, anthocyanins accumulation and composition are reported to be deeply influenced by temperature.

Berries holding different positions in a canopy experience different temperatures. In particular, the surface of berries exposed to sunlight can be 12°C (green berries) or 17°C (dark-colored berries) warmer than surrounding air. Berries exposed to higher temperatures, but not too high to induce a stop in photosynthesis, may be characterized by higher sugar contents, as a consequence of dehydration, lower acidity, as a consequence of an accelerate malate respiration, higher pH and amino acid contents with higher proportion of proline at maturity (Keller, 2010).

The principal flavonoid components affected by air temperature are undoubtedly anthocyanins. Previous researches demonstrated that anthocyanin biosynthesis is generally favored at relatively low temperatures, between 16 and 22°C (Nicholas et al. 2011). On the contrary, at temperatures lower than 10°C and higher than 30°C, anthocyanin accumulation is inhibited (Downey et al. 2006), and, at temperatures higher than 35°C, a certain anthocyanin degradation is reported to occur (He et al. 2010b). Such consideration have been also demonstrated for flavonol contents (Flamini et al., 2013). As concerns anthocyanin partitioning, a study carried out on Merlot grapes showed that high berry temperatures, between 34 and 44°C, alter the relative proportions between acylated and non-acylated forms of anthocyanins, and between di- (Cy, Pn) and tri-substituted (Dp, Pt, Mv) anthocyanins (Tarara et al. 2008). Downey et al. (2004) reported a proportional increase in coumaroyl glucosides in a season characterized by higher temperatures. Moreover, the compression of the diurnal temperature range was found to favor the partitioning of anthocyanins and flavonols towards di-substitution, so that Cy, Pn and quercetin (Q) derivatives were accumulated to a higher extent in grape berries (Cohen et al. 2012).

Concerning flavan-3-ols, their higher stability under diverse growing conditions is reported in both skin and seeds. However, some studies have shown a positive association between temperature and the number of seeds and total proanthocyanidin levels per berry at harvest. When the effect of cluster temperature on proanthocyanidins biosynthesis was studied it was shown that there is no consistent relationship between temperature and total proanthocyanidins accumulation across three seasons. However, cooling berries resulted in a significant increase in the proportion of (–)-epigallocatechin as an extension subunit (Cohen et al., 2012b).

2.4. Health benefits associated with flavonoids

Several studies have shown that moderate wine intake may have many beneficial effects on human health and these are mainly attributed to phenolic compounds, especially flavonoids. The interest in the possible health benefit of flavonoids has increased due to their potent antioxidant and free radical scavenging activities observed *in vitro*. In addition, flavonoids have been reported to exhibit multiple biological effects, such as cardioprotective, anti-carcinogenic, anti-atherogenic, anti-inflamatory, antiviral, antimicrobial, vasodilatatory (Procházková et al., 2011). From epidemiological and clinical studies, it emerges that regular and moderate wine consumption is associated with decreased incidence of cardiovascular disease, hypertension, diabetes, and certain types of cancer, including lung, esophagus, stomach, colon, endometrium, ovarian and prostate cancer (Banc et al., 2014).

Among wine, red wine is considered to have a stronger protective effect due to the high content of antioxidant substances released from skin and seeds of grapes (Paixão et al., 2007). A strong correlation was found between antioxidant capacity in vitro and total phenols content of wines. Indeed, red wines demonstrate a stronger health promoting activity than beer and spirits due to its richer content in phenols (Cordova and Sumpio, 2009). Flavonoids can prevent injury caused by free radicals by several mechanisms, such as direct scavenging of reactive oxygen species (ROS), activation of antioxidant enzymes, metal chelating activity, reduction of α-tocopheryl radicals, inhibition of oxidases, mitigation of oxidative stress caused by nitric oxide, increase in uric acid levels, increase in antioxidant properties of low molecular antioxidants (Procházková et al., 2011).

The bioavailability of grape and wine flavonoids is largely influenced by their chemical structure. Indeed, the degree of glycosilation, type of sugar, acyl esterification and othr funtional groups on the flavan nucleus are reported to largely affect the mechanisms of absorption and their biological activities. It is important to keep in mind that bioavailability of compounds may differ greatly, so that the most abundant phenolic compounds in our diet are not necessarily those leading to the highest concentration of active metabolites in target tissues (Manach et al., 2005). Thus, the composition of wines derived by different grape varieties or different winemaking conditions may alter not only the total content of flavonoids, but also their composition, leading to different health promoting effects on the human body (Georgiev et al., 2014). Moreover, gender and gentic differences, dietary habits, dosage and intestinal

microflora are reported to influence the absorption of flavonoids (Banc et al., 2014).

After ingestion, flavonoid glycosides can be modified in the oral cavity by the hydrolysing activity of saliva, although after passing through the stomach most reach the small intestine and thereafter the colon. In the gastrointestinal tract their absorption is associated with the hydrolysing activity. Several enzymes are involved and then the aglycone can enter the epithelial cells by passive diffusion due to their increased lipophilicity. Once absorbed, flavonoids and related phenolics undergo phase II enzymatic metabolism, which firstly occurs firstly in small intestine. Flavonoids can be conjugated with glucuronic acid, sulphate and methyl groups, in reactions catalysed by UDP-glucuronosyltransferases (UGTs), sulphotransferases (SULTs) and catechol-O-methyltransferases (COMT), respectively. Metabolites pass through the portal vein to the liver where they may be again converted and then enter the systemic circulation and eventually undergo renal excretion. However, a substantial amount of metabolites and their parent compounds reach the large intestine where they are exposed to the resident microflora. By the action of gut microflora, low molecular weight catabolites that can be efficiently absorbed *in situ*; some of them undergo further phase II metabolism locally and/or in the liver before entering the circulation and being excreted in urine (Rodriguez-Mateos et al., 2014).

Anthocyanins are absorbed very rapidly but inefficiently and their major derived metabolites isolated from human urine and serum are glucuronidated and methylated conjugates. In contrast to flavan-3-ol monomers, procyanidins are less permeable through cell walls thus are absorbed less readily, and their polymerization may compromise intestinal absorption. Indeed, low molecular weight oligomers, with a polymerization degree ≤ 3, are absorbed intact in the gastrointestinal tract, differently from polymers, which are degraded into small phenolic acids by the colonic microflora (Banc et al., 2014).

As described above, the health benefits of flavonoids depend on their chemical structure. Flavan-3-ol monomers act as antioxidants, free radical scavengers and anticarcinogenic; they have cardio-preventive, anti-microbial and anti-viral properties and may also play an important role in maintaining neurological health. On the basis of the B ring structure and number of catechol groups, flavan-3-ol monomers show a relative hierarchy of effectiveness as radical scavengers (–)-epicatechingallate > (–)-epigallocatechingallate > (–)-epigallocatechin> (–)-epicatechin > (+)-catechin . Research studies about

health promoting effects of flavan-3-ols revealed that they have beneficial effects on heart and liver diseases, they cause a reduction of plasma oxidation stress, they can slow down aging processes and neurodegenerative processes, enhance weight loss, protect the skin from damage caused by ionising radiation, and they have also been described as preservatives against microbes Moreover, they possess anti-inflammatory, anti-hypertensive, anti-diabetic and anti-mutagenic effects. However, this is still controversial, since several works highlighted also the contrary, namely that flavan-3-ols can act as anti-nutrients, pro-carcinogens, pro-oxidants, hemorrhagic inductors, mutagens or hepatotoxin, according to the their source, type, amount and availability of other dietary factors (Banc et al., 2014). Regarding PAs, increasing the degree of polymerization improves efficiency against a variety of radical species, PAs dimers and trimers being more effective than monomeric flavonoids against the superoxide anion. The consumption of PAs-rich foods, such as red wine, has been shown to increase the plasma antioxidant capacity, to have positive effects on vascular function and to reduce platelet activity in humans. Moreover, they have shown anticarcinogenic properties (Banc et al., 2014).

Flavonols are a class of substances which comprises some of the most prominent dietary antioxidants, among these quercetin can be singled out, for it presents a variety of pharmacological activities that provide protection not only against osteoporosis, certain forms of cancer, pulmonary and cardiovascular diseases but also against aging. Moreover, as described in the literature, one of the reasons for the success of quercetin is probably due to the relatively high bioavailability of the molecule compared to other phytochemicals. In wine, quercetin aglycone is released by the partial hydrolysis of glycosides during the winemaking and thus is present in finished wines, together with its different glycosides. Despite the original belief that only the free form of quercetin could be absorbed at the intestinal level by passive diffusion due to its hydrophobic nature, later studies have surprisingly demonstrated that the adsorption of quercetin glycosides almost doubles that of its corresponding aglycone. (Russo et al., 2012). Quercetin can be considered the prototype of a naturally occurring chemopreventive agent because its described biological activities (antioxidant, anti inflammatory, anti-proliferative, pro-apoptotic and anti-angiogenic) span through all stages of carcinogenesis from initiation to invasion and metastasis and act on different genetic, biochemical and immunological aspects that underpin the development and maintenance of tumors. Furthermore, quercetin plays a key

role in the reduction of blood pressure by reducing oxidative stress in a dose-dependent manner (Banc et al., 2014). Regarding myricetin, it has several therapeutic actions, such as anticarcinogenic, antiviral and antimicrobial, antiplatelet, antioxidant and cytoprotective (Banc et al., 2014). Finally, the meta analysis of epidemiological studies show an inverse association between flavonol intake and coronary heart disease and stroke (Banc et al., 2014).

Anthocyanins have been reported to be strong antioxidants; they inhibit the growth of cancerous cells, inhibit inflammation, act as vasoprotectors, and have anti-obesity effects (Manach et al., 2005). Among wine flavonoids, specifically anthocyanins can prevent neurodegenerative processes, both by inhibition of neuro-inflammation and oxidative stress reduction. In *in vitro* assays, anthocyanins have exhibited multiple antitoxic and anti-carcinogenic effects such as: directly scavenging reactive oxygen species (ROS), increasing the oxygen-radical absorbing capacity of cells, stimulating the expression of Phase II detoxification enzymes, reducing the formation of oxidative adducts in DNA, decreasing lipid peroxidation, inhibiting mutagenesis by environmental toxins and carcinogens, and reducing cellular proliferation by modulating signal transduction pathways. *In vivo* studies have shown that anthocyanins are able to inhibit the development of cancer in carcinogen-treated animals and in animals with a hereditary predisposition to cancer, in contrast to epidemiological studies in humans that have not provided convincing evidence of the anti-cancer effects of anthocyanins. Although epidemiological studies have not shown that anthocyanin intake reduces cancer risk in humans, they suggest that anthocyanin intake may reduce certain parameters of oxidative damage (Banc et al., 2014). The health benefits associated in epidemiologic studies with the consumption of anthocyanin-rich foods contradict the apparent low bioavailability of these compounds. Nevertheless, the biological activity of absorbed parent compounds, their metabolites and microbial catabolites and the potential synergy between them could be the answer to the anthocyanin paradox bioactivity (Fernandes et al., 2014).

3. Yeasts and health promoting compounds in wines

Besides genetics, environmental and agronomical factors, the wine making conditions could affect significantly the transfer of bioactive compounds from grape to wine (Waterhouse, 2002). As reviewed by Sacchi et al. (2005), fermentation temperature, thermovinification, must freezing, *saignée*, addition of pectolitic enzymes and extended maceration have been found to affect significantly the contents of phenolic compounds in wines. Other winemaking techniques like carbonic maceration, management of sulfur dioxide level and cold soak treatments have been shown to have little to no effect in phenolic contents.

The impact of yeasts on the content of bioactive compounds of wine was also reviewed. Even if variable results are reported, mainly depending on grape varieties studied (Sacchi et al., 2005), several Authors affirmed that microorganisms involved in wine-making can contribute to the total amount of bioactive compounds in the final product (Pretorius , 2000; Monagas et al., 2007; Suárez-Lepe and Morata, 2012). The traditional role of wine yeasts, *i.e.*, that of transforming grape sugars into ethanol, has been significantly widened by the advent of modern oenological microbiology. Louis Pasteur (1866) indicated that the types of yeast used in the wineries of a particular region are responsible for its wines having similar organoleptic characteristics. Thus, metabolic peculiarities and physiological properties of a particular yeast may lead to the formation of metabolites and the transformation of grape compounds influencing the organoleptic and health enhancing properties of a wine.

3.1. Interaction with flavonoids

The major metabolite of alcoholic fermentation is ethanol. Its production mainly depends on the sugar content of musts and by the metabolic activity of yeasts species involved in the alcoholic fermentation. Alcohol represents a good solvent for phenol extraction and in the presence of skins and seeds, as in red fermentations, the opportunity for extraction is wide. Modulating the maceration length, different results can be achieved in the extraction of phenols although using the same grapes (Waterhous, 2002; Hernández-Jiménez et al., 2012). In a study conducted on the extraction of flavan-3-ols in wines, González-Manzano et al. (2004) reported that the length of the maceration not only had an influence in the total levels of flavan-3-ols reached in the wines, but also in their qualitative composition. Indeed, maximum release of flavan-3-ols from the skins was reached after 24 h of maceration in

12.5% ethanol, whereas long maceration and higher ethanol percentages were required for extraction from the seeds. In particular, maximum seed flavan-3-ol extraction was observed after 2–3 weeks of maceration, reaching the 90% of total falvan-3ol in model wine tested. Thus, since the ethanol yield is consider a strain dependent trait (Giudici et al., 1995), yeast species and strains involved during the alcoholic fermentation may play a fundamental role in length and efficacy of phenols extraction.

The activity of yeast involved in the winemaking process may be reflected also on flavonoid profile through direct or indirect modification of specific compounds. As an example, it is well known that flavonol glycosides coexist in juice and wine with their relative aglycones, while the latter are not detectable in healthy grapes. The presence of aglycones is consider to be the result of acid hydrolysis during processing and storage (Castillo-Muñoz et al., 2007). However, yeasts involved in the alcoholic fermentation could potentially contribute to the glycoside hydrolysis, as it is known that some strains possess glycosidase activity, including β-glucosidase, which is active on flavonol-3-*O*-glucosides (Terrier et al., 2009; Suárez-Lepe and Morata, 2012). The most abundant flavonol in grapes is quercetin, and the release of the aglycone may produce a precipitation as a consequence of the lower solubility of quercetin respect to its glycosides (Somers and Ziemelis, 1985). Moreover, β-glucosidase was reported to be active also on anthocyanin-3-*O*-glucosides, producing unstable anthocyanidins that are susceptible to degradation phenomena (Terrier et al., 2009).

Several secondary metabolites produced by yeasts may interact with flavonoids thus producing new molecules possessing important feature for sensorial and health enhancing aspects. During fermentation, glucose is turned into pyruvate to obtain energy. Pyruvate is then metabolized into acetaldehyde, which serves as a terminal electron acceptor. However, some of the pyruvate and acetaldehyde produced diffuse out of the yeast cytoplasm, providing precursor molecules for the formation of more stable phenolic compounds. The release of pyruvic acid and acetaldehyde varies according to the yeast strain, a criterion that might be used in yeast selection. Pyruvate and acetaldehyde molecules may condense with grape anthocyanins (principally malvidin-3-*O*-glucoside) to produce highly stable pyranoanthocyanin adducts such as vitisin A and B, respectively. These pigments have been described as resistant to SO_2 bleaching, very stable to color modifications caused by pH and to oxidative damage during aging (Morata et al., 2007; Suárez-Lepe and

Morata 2012). From a salutistic point of view, vitisin A shows a higher resistance to gastrointestinal conditions, presumably leading to enhanced bioavailability compared to its parent anthocyanin (McDougall et al., 2005).Finally, acetaldehyde produced may act as a link for the formation of flavan-3-ol polymers and/or pigmented polymers thus contributing to color stability and flavor of wines (Pretorius, 2000).

Yeasts with hydroxycinnamate decarboxylase (HCDC) activity can also be used to decarboxylate hydroxycinnamic acids and form vinylphenols that condense with grape anthocyanins (again, principally malvidin-3-Oglucoside) to produce color-stable vinylphenolic pyranoanthocyanin adducts. A number of *S. cerevisiae* strains have HCDC activity, although varying in strength. While some show very little activity, others may transform of up to 15% of the hydroxycinnamic acid present into vinylphenols. However, yeast species deeply influence the production of vinylphenolic. Indeed, Strains of *Dekkera bruxellensis* can show conversion rates of 90% (Dias et al., 2003).

Another phenomenon of interaction between flavonoid and yeasts is relative to the adsorption of anthocyanins on the cell walls. Indeed, several Authors reported that the composition and the porosity of the cell walls of yeasts involved during the alcoholic fermentation can cause a loss of color of wines *via* the adsorption of anthocyanins (Vasserot et al., 1997; Morata et al., 2005). The adsorption and the retention of anthocyanins by the cell walls of yeasts have been reported to vary significantly on the basis of anthocyanin structure (as degree of methoxylation and steric hindrance), ethanol concentration (Vasserot et al., 1997) and yeast species and strains involved in the winemaking (Medina et al., 2005). Morata et al. (2003), studied the anthocyanin adsorption profiles of the cell walls of different strains of *Saccharomyces* spp isolated from grapes collected in three Spanish *appellation controlée* regions during the vinification of Cabernet Sauvignon grapes. In the study they highlighted that differential anthocyanin adsorption was seen in the cell walls of different strains of *Saccharomyces* spp, as a consequence of the differences in the structure and composition of the cells walls of the different strains. Acyl derivatives (*p*-coumaryl and acetyl) were more strongly adsorbed than nonacyl derivatives. Also, anthocyanins with a greater degree of methoxylation (malvidin and peonidin) were more adsorbed than those most hydroxylated (delphinidin and petunidin). This suggests that adsorption involves a hydrophobic interaction. Moreover, peonidin and its derivatives

were more adsorbed than malvidin probably due to steric differences of these molecules.
Finally, in a water-ethanol solution (EtOH 15%, v/v) containing Fe^{2+} and tartaric acid, the presence of yeast partially inhibited the degradation of (+)-catechin and (-)-epicatechin, with a higher efficiency in protection of the former. The Authors hypothesized that the presence in the medium of some compounds or fraction released by the yeast cells under conditions of autolysis may exert an inhibitory/protective effect on flavan-3-ols oxidation, thus decreasing the rate of browning (Lopez-Toledano et al., 2002).

3.2. Tyrosol, hydroxytyrosol and tryptophol: health promoting compounds of microbial origin

3.2.1. Production by yeast cell and contents in wines

Grapes are the main source of health enhancing compounds in wines, but are not the only. In fact yeast metabolism is also a source of wine compounds possessing salutistic properties like the phenolic alcohol tyrosol and the non-phenolic alcohol tryptophol.
Tyrosol, tryptophol and hydroxytyrosol are secondary metabolites produced by yeasts during the alcoholic fermentation. In particular, tyrosol and tryptophol are higher alcohols produced, as well as phenyl ethanol, by the catabolism of aromatic amino acids (Mas et al., 2014), whereas hydroxytyrosol is a tyrosol derivative formed by the hydroxylation of tyrosol (Piňeiro et al., 2011). Aromatic amino acids (tryrosine, tryptophane and phenylalanine) are catabolised by the Ehrilch pathway, which starts with the transamination of the amino group and the formation of the α-keto acid. Then, α-keto acids are decarboxylated to the corresponding aldehydes and finally, depending on the redox state of the cell, they can be further metabolised to the corresponding aromatic alcohol, or are oxidised to the corresponding acids (Mas et al., 2014).
The production of higher alcohols by yeast have been associated to different physiological functions. Indeed, the oxidative deamination is reported to be one of the mechanisms for obtaining nitrogen when the yeast pool has been depleted (Vollbrecht and Radler, 1973). Moreover, it was suggested that higher alcohol production might act as a detoxification process for the intracellular medium of α-keto acids and aldehydes, or as a means of regulating the metabolism of amino acids (Ribéreau-Gayon et al., 2000). Finally it was proposed that the production of higher alcohols contributes to the maintenance of the redox balance in the cell, because of the final reduction

steps of the pathways involves the reoxidation of NADH+H$^+$ to NAD$^+$ (Zoecklein et al., 1995). However, it has also been stated that there appears to be enough acetaldehyde to maintain the redox balance, thus the formation of higher alcohols is not considered to be an important means for the reoxidation of NADH (Boulton et al., 1995).

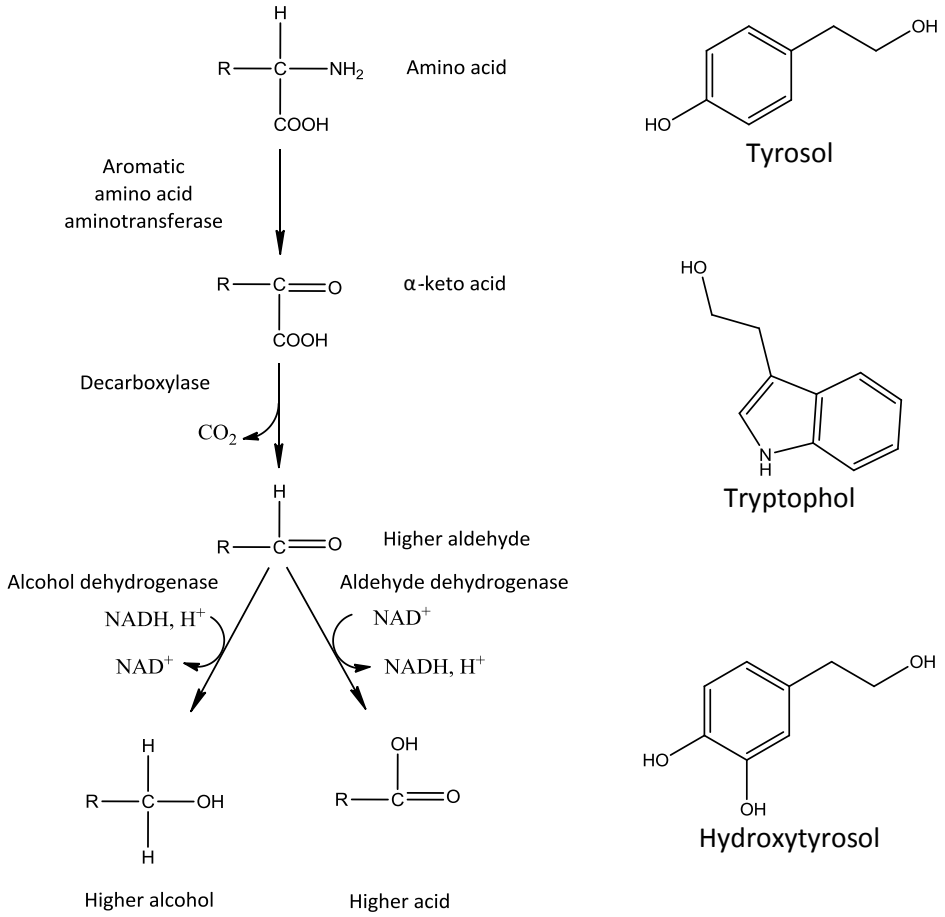


Figure 6: The Ehrilch pathway and structure of tyrosol, tryptophol and hydroxytyrosol (source: Hazelwood et al., 2008).

Tyrosol and tryptophol have been recently described as the molecular modulators of some physiological and morphological processes considered involved in cell signaling. This aspect is also called as quorum sensing in different yeast species and appear to be related to population size, morphological change from yeast to pseudohypha and nutritional state of the

environment (Chen et al., 2004; Chen and Fink, 2006). Moreover, tryptophol was indicated as an autoregulatory signaling molecule inducing the accumulation of aromatic higher alcohols in Saccharomyces cerevisiae when in stationary phase with high cell density (Chen and Fink, 2006).
In wines, tyrosol concentration ranges from 4 to 44 mg/L, hydroxytyrosol from not detectable to 5 mg/L in red wines (Piñeiro et al., 2011; Fernández-Mar et al., 2012) and tryptophol from 0,02 to 10 mg/L (Monagas et al., 2007). Conversely to other higher alcohols which are produced during the initial part of alcoholic fermentation, the contents of tyrosol and tryptophol became higher in last part of fermentation (Garde-Cerdàn and Ancìn-Azpilicueta , 2006). Their origin seems to be related mainly with nitrogen contents of musts. Generally, it appears that low level of amino acids in must provokes an enrichment in higher alcohols (Hernández-Orte et al., 2005). Moreover, it is well know that different yeasts species and strain can differ in the uptake and patterns of utilization of nitrogen sources (Large, 1986). Indeed, the final contents of tyrosol, tryptophol and hydroxytyrosol in wine could be influenced by the microbial ecology of alcoholic fermentation. Garde-Cerdàn and Ancìn-Azpilicueta (2006) carried out a study about the contribution of wild yeasts in terms of tyrosol and tryptophol contents in wine. Tyrosol showed variation in contents as a consequence of the yeasts involved in the alcoholic fermentation, whereas the concentration of tryptophol did not show significant differences. Finally, it seems that tyrosol remains at relatively constant concentration during wine aging (Ribéreau-Gayon et al., 2000b).

3.2.2.Health related aspects

Tyrosol and hydroxytyrosol are phenolic alcohols commonly found in red and white wines. Particularly in white wines, they may represent an important fraction of phenolic compounds, thus contributing to protection of products. In the Mediterranean diet, they are found principally in olive oil and their bioavailability and pharmacokinetic are principally studied in this matrix (; Fernández-Mar et al., 2012). In 2011, the European Food Safety Authority, (EFSA) reviewed the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage, maintenance of normal blood HDL cholesterol concentrations, maintenance of normal blood pressure, “anti-inflammatory properties”, “contributes to the upper respiratory tract health”, “can help to maintain a normal function of gastrointestinal tract” and “contributes to body defences against external agents” attributed to these molecules. Indeed, they concluded that

“polyphenols in olive standardised by their content of hydroxytyrosol and its derivatives (e.g. oleuropein complex) are sufficiently characterized in relation to the claimed effects.” Although studies on humans are still in the initial stages and therefore further studies are needed, tyrosol and hydroxytyrosol are reported to possess several health promoting activities, such as antioxidant and radical scavenging, cardioprotective capacity, anticancer, antimicrobial, antidiabetic and neuroprotective. These specific properties in wines are currently under discussion, as they were settled basically from *in vitro* assays and cannot be directly correlated with *in vivo* studies (Fernández-Mar et al., 2012). However, Schröder et al. (2009) reported that wine is an important source of hydroxytyrosol and they suggested alcohol as an indirect promoter of endogenous hydroxytyrosol generation.

Regarding tryptophol, it is reported to have a sleep-inducing property similar to the effects of serotonin or melatonin. It is present in wine and beer as byproducts of fermentation can also be produced in the human liver after disulfiram treatment for chronic alcoholism (Cornford et al., 1979). Moreover, tryptophol showed antimicrobial activity against a food-borne pathogen which causes gastrointestinal conditions (Gañan et al., 2009) and inhibitory effects on the growth of enological lactic acid bacteria (García-Ruiz et al., 2011)

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Aim of the thesis

Aims and structure of the thesis

The aim of this thesis was to investigate on the effects of several variables affecting Sangiovese grape composition and the incidence of yeast fermentative behavior in determining the accumulation of different health promoting compounds in Sangiovese wines. In particular, the study was focused on grape derived compounds possessing health enhancing properties and also involved in quality related aspects, such as anthocyanins, flavonols and flavan-3-ol monomers. Moreover, three higher alcohols produced by yeasts, tyrosol, hydroxytyrosol and tryptophol, were included in this study as their health enhancing properties have been reported in the literature.

Before showing the general structure of this thesis, it is important to remark that all the experimental Sangiovese wines were characterized in terms of health promoting compounds at the end of a standardized maceration length. It is well known that stabilization phenomena involving most of the health enhancing compounds here taken into consideration occur in wines after its separation from skin and seeds. These phenomena lead to the formation of more stable polymeric compounds, but a general loss of monomeric compounds related to the total antioxidant activity of wines is associated. Indeed, the stage immediately after the separation of wine from its skin and seed is to be considered the moment at which the highest concentration of health promoting compounds in wine is detected.

To fulfill the aim, a method for the evaluation of health promoting compound contents in wines was described in the first part of the result section. The method consisted in the definition of standardized procedures necessary for the Sangiovese grape vinification, starting from the sampling operations and storage of samples, to winemaking and finished wines.

The following sections are constituted by the results of various experiments. All the results are organized in two macro topics on the basis of the origin of health promoting compounds here considered:

i) Effect of grape composition and yeasts species involved in the alcoholic fermentation on anthocyanin, flavonol and flavan-3-ol monomer contents and profiles.

ii) Effect of grape composition and yeasts species involved in the alcoholic fermentation on tyrosol, hydroxytyrosol and tryptophol contents.

The first section was articulated starting from the study of variability in terms of health promoting compound composition of experimental Sangiovese wines obtained by grape samples collected different enological areas of Tuscany during three consecutive vintages (2011, 2012, 2013). In this first study, it was possible to investigate the effect of vintage and origin of grapes on the experimental wines composition. Then, grapes from a single vineyard harvested in four consecutive vintages (2011-2014) were vinified. The four vintages were characterized by a climatic point of view and the variability of wines composition was related to climatic conditions experienced by grapes.
Thirdly, in a single vintage, parcels of a specific vineyard were chosen on the basis of vine vigor. Then, the grape samples were harvested in these specific parcels and experimental wines were compared in order to evaluate the impact of vine vigor on grape and wine compositions. Lastly, laboratory fermentations of the same Sangiovese grape must were carried out by *Saccharomyces cerevisiae* alone or by sequential inoculum of *Candida zemplinina* and *S. cerevisiae*. This study was aimed at highlighing the impact of yeast species involved during the alcoholic fermentation on flavonoids in wine, paying particular attention on quercetin and its glycosides and vitisin A concentration.

Regarding the second macro topic, the study was focused on health promoting compounds of microbial origin (tyrosol, hydroxytyrosol and tryptophol). At first, the effect of grape must composition was investigated as a variable capable of modifying the production of the three higher alcohols by a single strain of *Saccharomyces cerevisiae*.
Then, the accumulation of these molecules was assessed in Sangiovese wines produced by both pure inoculum of *S. cerevisiae* alone or by sequential inoculum of *C. zemplinina* and *S. cerevisiae*, in aerated and non-aerated conditions. The effect of yeast species and aeration was highlighted.

Results

1. Development of a methodological approach for laboratory microvinifications

Abstract

The huge variability in health promoting compounds composition, assessed among clusters in the same vineyard, among cluster of the same vine and berries in the same cluster constitutes a tangible problem involving both enologists and researchers. In this connection, a preliminary study was focused on the definition of a method capable to reduce such real existing variability allowing to guarantee the repeatability of measurements and, as a consequence, to obtain an acceptable robustness of results.

In the first part of this work, frozen storage was tested in order to define the maximum period applicable to grape samples without compromising their phenolic composition, with particular attention to anthocyanin, flavonol and flavan-3-ol monomer contents, classes of molecules discussed in this thesis. No significant differences (p<0.01) in phenolic parameters of grapes were assessed if grape samples underwent to a frozen storage up to 2 months. For this, frozen storage period of grape samples used in this thesis is specified in each materials and methods section of the works here presented. When applied, this period did not exceed 1 month.

Then, it was possible to define a methodological approach that include some preliminary steps for row material preparation prior vinification. In particular, grape clusters were sampled from different vines in the same vineyard. Once in laboratory, destemming operation were carried out while clusters were still frozen and then well mixed in order to obtain homogeneous sub-samples used for grape analysis and vinifications replicates. By following this procedure, an acceptable reproducibility of the data on grape samples characterization and consequently in experimental wines composition was achieved.

Introduction

Wine composition is deeply influenced by chemical and physical characteristics of grapes, also as regards the contents of compounds possessing biological activities. Grapes are the major source of phenolic compounds (Waterhouse, 2002), a class of molecules deeply studied for their health enhancing properties (Fernández-Mar et al., 2012). In addition, grape must composition is responsible of metabolic behavior of microrganisms involved during alcoholic fermentation which may represent another source of bioactives in wines (Ribéreau-Gayon et al., 2000[b]; Morata et al., 2003).

However, as reported by Ribéreau-Gayon et al. (2000[a]), the chemical composition of grapes of a specific variety in the same vineyard at a given moment is deeply heterogeneous. In the literature, several Authors discussed about the difference in grapes composition during the ripening phases regarding both technological parameters (Wolpert et al., 1983) and phenolic contents (Kontoudakis et al., 2011). Such variability in grape composition is an issue involving vineyard managers and enologists to set the most appropriate harvest date and to define the proper winemaking conditions. Furthermore, grape heterogeneity must be taken into consideration by researchers in the definition of experimental plans for their studies. By knowing the magnitude of such variability it is possible to plan the appropriate sampling operations capable to minimize errors and to provide best estimate for vineyard parameters (Wolpert et al., 1983).

Also laboratory procedures are fundamental for a correct estimation of grape characteristics. In fact, all the operations on grape samples must meet some basic requirements such as the preservation of physical and chemical characteristics of row material and, especially in research field, the possibility of constitution of stable and homogeneous sub-samples with the aim to guarantee the repeatability of quality measurements (Cynkar et al., 2004).

The aim of this work was to assess the differences in composition of wines obtained from clusters harvested in a single vineyard, as a consequence of grape biological variability. Then, it was possible to develop and to perform an experimental method capable to contain the intrinsic variability of row materials and to reduce the differences in chemical composition of experimental wines produced from grapes belonging to the same vineyard.

Materials and methods

Grape samples. Grape samples used in this study were harvested and transferred in laboratory at low temperature and then analyzed and vinified.

Experimental vinifications. The experimental red wine vinifications were carried out using 450 g grape samples which were manually crushed, placed in 500 mL Erlenmeyer flasks provided with Müller valves, inoculated with Saccharomyces cerevisiae Sc1 strain and then incubated at 28°C for 14 days. Flasks were gentle mixed (1 minute) once a day to allow the diffusion of phenolic compounds from skin and seeds into wine. Then, flasks were weighted in order to monitor the fermentation progress (CO_2 evolution).

Yeast inoculum. Yeast inoculum was originated from one-day-old culture, maintained at 30°C in yeast extract-peptone-dextrose (YEPD). Inoculum size was calculated to give an initial cell density of 10^6 cells/mL. The yeast strain *Saccharomyces cerevisiae* Sc1 was previously isolated from commercial Sangiovese wine fermentations and it is included in the yeast culture collection of the Department of Management of Agricultural, Food and Forestry Systems (GESAAF, University of Florence, Italy).

Chemicals and analytical determinations. All solvents were of HPLC quality, chemicals of analytical grade (>99%) and water was of MilliQ® quality (Millipore, Billerica, USA).
Phenolic maturity of grapes was evaluated in accordance to Saint-Cricq De Gaulejac et al. (1998), determining total anthocyanin contents (Tot A, mg/L), Extractable anthocyanins (Extr A, mg/L), and total phenolic richness (RPT, OD_{280}). The complementary indexes were calculated as follow:
EA% = [(Tot A - Extr A) / Tot A] x 100 = Cellular maturity index;
dTpep = RPT - [(Extr A x 40) / 1000] = Seeds tannins levels;
Mp% = (dTpep / RPT) x 100 = Relative proportion of seed tannins, seeds maturity.
For the determination of anthocyanins, hydroxybenzoic acids, hydroxycinnamic acids, flavonols, flavan-3-ols, stilbenes on Sangiovese grape samples, 40 berries, in triplicates, were randomly picked. At first, the berries were weighed. Then, seeds were removed from berries paying attention to avoid pulp loss. Seeds were weighed. Skins and pulp were extracted together. Hence, skins+pulp and seeds were extracted in 150 mL of solvent constituted by methanol/water/formic acid (500:485:15). The maceration length was carried out overnight at 20°C. Quantification of phenolic were expressed in

mg/g for seeds and in mg/Kg of grapes for skins+pulp considering the volume increase produced as a consequence of extraction of pulp.

Anthocyanins quantification of grapes and wines was performed by HPLC separation and identification of individual anthocyanins according to the Office International de la Vigne et du Vin method for analysis of anthocyanins in red wines (OIV 2009) as previous described (Mangani et al. 2011). Anthocyanins were quantified using malvidin-3-*O*-glucoside as a standard. Grape extracts and wines were injected in HPLC after filtration through 0.45 micron. In table 1, the anthocyanins identified in Sangiovese wines are reported.

For the determination of flavonols, flavan-3-ol monomers, and the three higher alcohols hydroxytyrosol, tyrosol and tryptophol the analytical conditions were those described by Hernández et al. (2005). Grape extracts and wines were extracted with diethyl ether and ethyl acetate and then injected in a HPLC (Varian Pro-star 210), equipped with a Diode Array Detector and a reversed phase column Chromsep Omnispher (5µm particle, 250 x 4.6 mm; Varian Inc.), thermostated at 25 °C. The mobile phase was composed of (A) acetonitrile and (B) 2% (v/v) acetic acid; the gradient profile was 0-55 minutes, 100%-80% B; 55-70 minutes, 80-50% B; 70-80 minutes, 50-5% B, followed by washing with acetonitrile and re-equilibration of the column from 110 to 125 minutes; the flow rate was 1 mL/min from the beginning to 60 minutes and 1.2 ml/min from this point to the end. Detection was performed by scanning from 210 to 400 nm. Identification of various peaks was performed on the basis of retention time and specific absorption spectra of each single peak. Quantification was performed using specific compounds as standards. In table 2, the compounds identified in Sangiovese wines and the standards used for their quantification are reported.

Glucose, fructose, ethanol, glycerol, acetate, lactate, succinate, pyruvate and 2-3-butandiols concentrations in wine were determined by HPLC, according to Granchi et al. (1998), utilizing a MetaCarb H Plus Column (8µm particle, 300 x 7.8 mm; Varian Inc.) and a Pro-star 210 chromatograph equipped with a Diode Array Detector at 210 nm and a Refractive Index Detector, in series (Varian Inc.); α-aminocid N content was determined by the NOPA procedure (Dukes and Butzke, 1998); malate was determined by enzymatic assay (Steroglass S.r.l., Perugia, Italy); total acidity and pH analysis were carried out according to O.I.V. (2007).

Table 1: Anthocyanins determined with HPLC-DAD method as described by OIV 2009; standard compound and wavelenght used for quantification

Detected compounds	Standard	Quantification wavelength (nm)
Glucosides †, §		
delphinidin-3-*O*-glucoside	Mv-3-*O*-glu	520
cyanidin-3-*O*-glucoside	Mv-3-*O*-glu	
petunidin-3-*O*-glucoside	Mv-3-*O*-glu	
peonidin-3-*O*-glucoside	Mv-3-*O*-glu	
malvidin-3-*O*-glucoside	Mv-3-*O*-glu	
Acilated anthocyanins †, §		
peonidin-3-*O*-(6″-acetyl)-glucoside	Mv-3-*O*-glu	520
malvidin-3-*O*-(6″-acetyl)-glucoside	Mv-3-*O*-glu	
peonidin-3-*O*-(6″-p-coumaroyl)-glucoside	Mv-3-*O*-glu	
malvidin-3-*O*-(6″-p-coumaroyl)-glucoside	Mv-3-*O*-glu	
Pyranoanthocyanins §		
malvidin-3-*O*-glucoside-pyruvic acid (vitisin A)	Mv-3-*O*-glu	520
malvidin-3-*O*-glucoside-vinil adduct (vitisin B)	Mv-3-*O*-glu	
Pigmented polymers		
Pigmented polymers	Mv-3-*O*-glu	520

Mv-3-*O*-glu = malvidin-3-*O*-glucoside

†: Free Anthocyanins

§: Non polymeric Anthocyanins

Table 2: Monomeric phenolic compounds and derivatives determined with HPLC-DAD method as described by Hernández et al. (2005); standard compounds and wavelenght used for quantification

Detected compounds	Standards	Quantification wavelength (nm)
Flavan-3-ol monomers		
Catechin	Catechin	
Epicatechin	Epicatechin	280 nm
Epicatechin-3-*O*-gallate	Epicatechin	
Flavonols		
Myricetin-3-*O*-glucoside	Myricetin	
Myricetin-3-*O*-galactoside	Myricetin	
Myricetin-3-*O*-glucuronide	Myricetin	
Myricetin	Myricetin	280 – 340 nm
Quercetin-3-*O*-galactoside	Quercetin	
Quercetin-3-*O*-glucuronide	Quercetin	
Quercetin-3-*O*-glucoside	Quercetin	
Quercetin	Quercetin	
Kaempferol	Kaempferol	
Kaempferol-3-*O*-glucoside	Kaempferol	
Higher alcohols		
Tyrosol	Tyrosol	
Hydroxytyrosol	Tyrosol	280 nm
Tryptophol	Tryptophol	

Results and discussion

Effect of frozen storage on grapes composition. Frozen storage is a compromise widely used in laboratories to preserve the condition of samples before analysis by standard chemical and physical methods if many samples are awaiting analysis (Cynkar et al., 2004). However, it is well known that freezing and thawing can alter grape composition. In fact, the formation of crystals, dehydration of tissues, chemical reactions and component degradations are possible phenomenon that may occur during freezing/thawing operations (Garcia et al., 2011). Hence, the first part of this section was aimed to assess the maintenance of chemical and physical grape samples characteristics after different period of storage at -20°C.

This preliminary experiment, aimed to determine a maximum frozen storage period for grape samples, was carried out by using Sangiovese grapes harvested in 4 different vineyards (A, B, C, D). Berries were randomly picked taking them alternately from each side of the row at different heights on the vine, for a total of 2.5 – 3 kg of berries sampled. Grape composition was evaluate at harvest time for all the samples. Then, the chemical and physical characteristics of grapes were evaluate after 1, 2, 3 and 4 months of storage at -20°C. In particular, sample A was analyzed after 1 and 2 months, while sample B, C and D were analyzed after 2, 3 and 4 months of frozen storage respectively. In table 3, the results of chemical and physical characterization of fresh grape samples (A, B, C, D), the same samples after different period of storage (1, 2, 3, 4 months after harvest) and the ratio between the analytical data are reported (Frozen / fresh ratio).

Table 3: Effect of storage time on grape composition

		Tot S	pH	TA	Malic acid	α-AN	Tot A	Extr A	EA%	RPT
Fresh										
A		231	3.39	7.4	2.44	113	1189	708	40.5	43.1
B		221	3.41	5.4	0.62	130	987	508	48.5	39.5
C		253	3.55	6.0	2.33	234	1145	722	36.9	54.5
D		283	3.53	6.0	1.9	230	891	623	30.1	45.0
Frozen	Mo									
A	1	229	3.41	5.3	2.23	110	1164	695	40.3	44.5
A	2	226	3.40	5.4	2.23	110	1187	686	41.6	42.1
B	2	226	3.58	3.80	0.69	125	984	498	49.4	40.1
C	3	256	3.66	4.70	2.42	196	1148	744	35.2	50.5
D	4	290	3.7	3.40	2.17	132	881	495	43.8	41.0
Frozen:Fresh										
A	1	0.99	1.01	0.72	0.91	0.98	0.98	0.98	1.00	1.03
A	2	0.98	1.00	0.72	0.91	0.98	1.00	0.97	1.03	0.99
B	2	1.02	1.05	0.70	1.11	0.96	1.00	0.98	1.02	1.01
C	3	1.01	1.03	0.78	1.04	0.84	1.00	1.03	0.95	0.93
D	4	1.02	1.05	0.57	1.16	0.57	0.99	0.80	1.45	0.91

Tot S = Total sugars (g/L); TA = Titratable acidity (gTartaric acid/L); α-AN = α-aminoacidic nitrogen content (mgN/L); Tot A = Total anthocyanins (mg/L); Extr A = Extractable anthocyanins (mg/L); EA% =Cellular maturity index; RPT = Total phenolic richness (OD_{280}). A, B, C, D = Sangiovese grape samples. Mo = Month(s) after harvest

By evaluating the results and, in particular, by comparing the evolution of the ratio between data of frozen and fresh grape samples, it is possible to confirm an impact of freezing on grapes composition. In particular, all the parameters linked to the acidic component of grapes, such as pH, total acidity (TA) and malic acid contents resulted deeply influenced by the storage at -20°C. Moreover, the ratio between frozen samples and fresh samples indicated that the α-aminoacidic nitrogen content seems to decrease during freezing time, a result not entirely negligible given the importance of this parameter on the alcoholic fermentation progress. This results are in accordance with studies carried out by other Author on different grape varieties (Cynkar et al., 2004; Garcia et al., 2011).

However, focusing the attention on phenolic maturity parameters (Tot A, Extr A, EA%, RPT), it can be affirmed that storage time up to 2 months does not produce a significant effect on phenolic composition of grape samples. In addition, total sugar contents of grape samples seems to be stable at an acceptable level during all the frozen storage time here taken into consideration. This resulted in agreement with results of works carried out by other Author affirming no statistical differences in total anthocyanins contents, total phenolic richness and total sugar contents in grape samples stored at -18°C for a period up to 3 months (Cynkar et al., 2004).

Grape sample A were also characterized in terms of anthocyanin, flavonol and flavan-3-ol monomer contents in order to evaluate the impact of 1 and 2 months frozen storage on grape composition. Indeed, the anthocyanin composition of fresh grapes are compared with those of the same grapes after 1 month of frozen storage. Table 4 reported the result of grape extract carried out with methanol/water/formic acid solvent (500:485:15), and with aqueous solution (pH 3.2) used for the determination of extractable anthocyanins according to phenolic maturity procedure (Saint-Cricq De Gaulejac et al., 1998).

Table 4: Effect of storage time on grape composition: Anthocyanin contents of fresh grapes (Fresh) and after 1 month of frozen storage at -20°C; related statistical analysis (ANOVA, $p<0.01$). Values are reported as mean of triplicates ± coefficient of variation as percentage.

	Solvent extraction			ANOVA	Extraction pH 3,2			ANOVA
	Fresh	Frozen 1 Month	Frozen 2 Month	$p<0.01$	Fresh	Frozen 1 Month	Frozen 2 Month	$p<0.01$
Dp-3-*O*-G	66.6 ± 6.8	88.5 ± 0.1	82.8 ± 3.6	ns	45.7 ± 10.0	55.3 ± 4.6	47.5 ± 0.7	ns
Cy-3-*O*-G	145.4 ± 6.4	161.9 ± 0.7	151.9 ± 4.1	ns	117.5 ± 5.6	127.0 ± 2.7	114.5 ± 0.3	ns
Pt-3-*O*-G	84.2 ± 5.9	101.1 ± 1.6	94.1 ± 3.9	ns	56.0 ± 8.2	64.4 ± 4.0	57.7 ± 0.9	ns
Pn-3-*O*-G	139.4 ± 8.4	143.5 ± 4.5	124.9 ± 1.7	ns	97.6 ± 4.7	107.2 ± 2.4	106.7 ± 0.2	ns
Mv-3-*O*-G	233.1 ± 8.2	249.1 ± 2.3	224.1 ± 2.2	ns	173.0 ± 6.1	188.3 ± 3.5	185.6 ± 0.1	ns
Acylated A	5.8 ± 10.2	6.9 ± 20.0	7.0 ± 1.5	ns	3.0 ± 17.0	3.6 ± 0.0	3.5 ± 4.9	ns
Tot A	674.5 ± 7.5	750.9 ± 1.8	684.7 ± 2.9	ns	492.7 ± 6.3	545.8 ± 3.2	515.5 ± 0.1	ns

Dp = delphinidin-3-*O*-glucoside, Cy = cyanidin-3-*O*-glucoside, Pt = petunidin-3-*O*-glucoside, Pn = peonidin-3-*O*-glucoside, Mv = malvidin-3-*O*-glucoside, Ac = acetilated anthocyanins, Cou = coumarilated anthocyanins, Tot = Total anthocyanins.

Firstly, no detrimental effect of 1 and 2 months of frozen storage were highlighted for grape samples analyzed. According to the results, despite a slight trend towards an increased cellular maturity index after 1 month of frozen storage, as also reported by Garcia et al. (2011), no significant differences were found on anthocyanin contents of grapes. Finally, it is noteworthy to highlight a reduction of variability of anthocyanin contents in grape samples after 1 month of frozen storage.Freezing berry resulted an applicable approach allowing to reach a better reproducibility of subsamples regarding anthocyanin composition of grapes. Indeed, the subsample preparation was obtained by mixing the berries. During mixing operation the attention to avoid rupture of the berries in fresh samples led to a less efficient homogenization, whereas for frozen samples the efficiency of the mixing was higher because of the berries' lower susceptibility to rupture.
In table 5, the comparison of flavonols and flavan-3-ol monomers, contents in fresh and after1 month of frozen storage grapes is reported.

Table 4: Effect of storage time on grape composition: flavonols, flavan-3-ol monomers, contents of fresh grapes (Fresh) and after 1 and 2 month of frozen storage at -20°C; related statistical analysis (Student's t-Test). Values are reported as mean of triplicates ± coefficient of variation as percentage.

	Fresh	Frozen 1 Month	Frozen 2 Month	ANOVA p<0.01
mg/Kg grapes				
M-3-*O*-glc	0,9 ± 37.7	1,4 ± 15.2	1,9 ± 10.9	ns
M-3-*O*-glu	3,9 ± 16.8	3,6 ± 2.2	3,5 ± 21.0	ns
M-3-*O*- gal	1,5 ± 42.6	1,3 ± 34.5	1,2 ± 5.3	ns
Q-3-*O*-gal	4,0 ± 1.0	5.0 ± 0.5	5.0 ± 13.6	ns
Q-3-*O*-glc	7,6 ± 24.1	9,8 ± 3.4	9,2 ± 9.5	ns
Q-3-glu	31,1 ± 0.0	33,4 ± 13.3	33,8 ± 13.4	ns
K-3-*O*-glu	2,1 ± 6.5	2,9 ± 3.2	2,9 ± 14.2	ns
mg/g seed				
C	1,6 ± 22.3	1,4 ± 15.9	1,2 ± 11.6	ns
EC	2,7 ± 18.6	2,2 ± 20.6	1,9 ± 10.0	ns
ECG	0,2 ± 30.7	0,2 ± 13.3	0,2 ± 2.2	ns

M-3-*O*-glc = myricetin-3-*O*-glucuronide, M-3-*O*-glu = myricetin-3-*O*-glucoside, M-3-*O*-gal = myrictin-3-*O*-galactoside. Q-3-*O*-gal = quercetin-3-*O*-galactoside, Q-3-*O*-glc = quercetin-3-*O*-glucuronide, Q-3-*O*-glu = quercetin-3-*O*-glucoside. C = (+)-catechin, EC = (-)-epicatechin, ECG = (-)-epicatechin gallate

No detrimental effect of 1 and 2 months frozen storage emerged in composition of flavonol and flavan-3-ol monomer contents in grapes and seeds. Once again, contents of determined phenolic compounds found in grape sample after 1 and 2 months of frozen storage were characterized by a generally lower variability, remarking a better constitution of homogenous subsamples.
By the results obtained on grape anthocyanins, flavonols and flavan-3-ol monomers, frozen storage was chosen as a preliminary step for grape samples preparation prior vinification.

Variability of grapes and wines composition. Sangiovese grape samples were collected at the same time in a nine-years old vineyard located in Montalcino (SI, Italy). In the vineyard were individuated 4 parcels in which 5 clusters were sampled from 4 distinct vines in each parcel. The 5 clusters belonging to each vine were placed together in a codified plastic bag. A total of 80 clusters were sampled. Samples were finally transferred into laboratory at low temperature.
Once at laboratory, 32 clusters, 2 for each bag, were joint together. Indeed, 17 lots were obtained: 16 constituted by 3 clusters harvested from 16 vines and 1 lot constituted by 32 clusters randomly picked from 16 vines. Finally, the lots were stored at -20°C. The storage period at -20°C did not exceed 2 months, on the basis of the results obtained in the first part of the study.
While still frozen, all 32 clusters picked randomly from the 16 vines were hand plucked one day before their use and replaced in the bags. After destemming, berries were well mixed in order to create homogeneous triplicates which were used for chemical and physical analysis of grapes and for vinification. This crucial step was carried out while berries were still frozen in order to avoid their rupture and consequently the formation of must.
Then, berry thawing was carried out overnight at 4°C for all lots. A small amount of grapes from each lot were used for chemical and physical determinations. In figure 1, the schemes of operations carried out prior the fermentation of the 16 lots constituted by 3 cluster each (a) and for the lot constituted by 32 clusters (b) are reported.
Indeed, the heterogeneity of grapes and correspondent wines composition was studied by (i) assessing the differences in grapes and wines from 16 different vines of the same vineyard (fig. 1a), (ii) developing a method capable to contain such variability by joint together clusters from the same vineyard

belonging to different vines, evaluating differences in grape and wines triplicates composition (fig.1b).

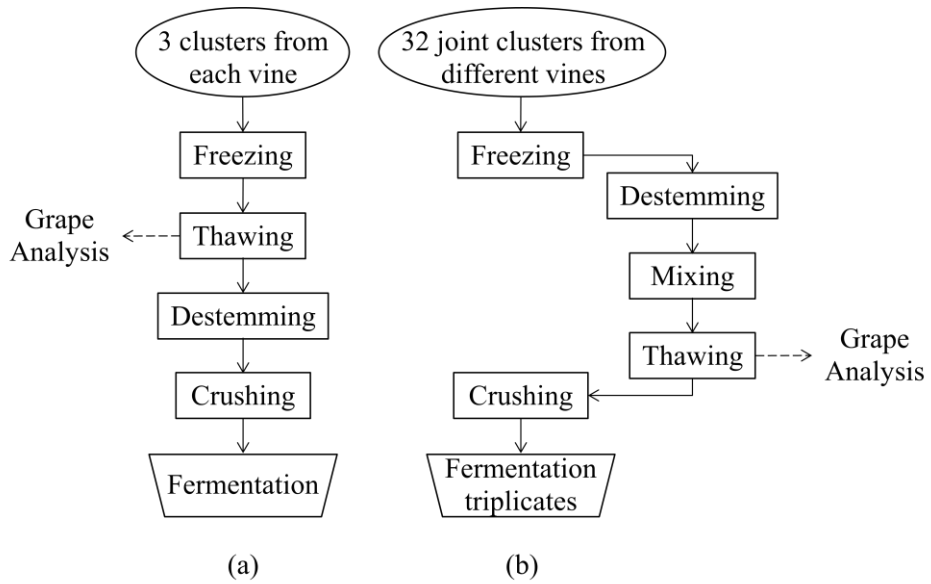

Figure 1: Preliminary steps for grape samples preparation prior vinification: (a) clusters from the same vine, (b) joint clusters from different vines.

In table 4, the characterization of technologic and phenolic maturity of grape samples is reported. In the left side of the table 1 the frequency distribution and the coefficient of variation of chemical and physical parameters measured on grape samples belonging to a single vine is shown; in the right side, the table 1 shows the results obtained in the case of the lot formed by 32 joint clusters from different vines.

From the table 4 is possible to assess the impact of intrinsic variability of grape samples belonging to a single vine. In fact, by considering the values reported on the left side of the table, the values of all parameters measured were characterized by an high coefficient of variation. On the other hand, joint grape samples, reported in the right side of table 1, showed a marked reduction of variability between replicates. This was considered the first step in the validation of a method for grape treatment before the alcoholic fermentation.

Table 4: Chemical and physical characterization of grape samples. Results are reported as frequency distribution and coefficient of variation for grape samples belonging to specific vine, on the left side, and as mean and coefficient of variation as percentage for joint grape samples.

	Clusters from single vines						Joint clusters	
	Min	25th Perc	Median	75th Perc	Max	%CV	Mean	%CV
pH	3.46	3.65	3.79	3.90	4.12	-	3.66*	-
Tot S(g/L)	208.0	224.0	246.5	268.8	280.0	*9.7*	255.7	*1.3*
Malic acid (g/L)	1.1	1.3	1.7	2.2	2.9	*31.4*	2.4	*1.1*
α-AN (mgN/L)	100.4	156.7	198.7	394.3	560.0	*57.2*	196.0	*1.3*
Tot A (mg/L)	786	881	1040	1222	1648	*22*	1148	*3.4*
Extr A (mg/L)	574	661	692	760	947	*13*	744	*1.7*
EA%	17.3	26.1	33.7	40.0	43.1	*23.9*	42.7	*6.7*
RPT	37.3	40.1	42.1	46.3	50.1	*9.0*	39.2	*4.1*
dTpep	10.4	12.5	14.2	17.4	21.3	*20.8*	17.0	*8.4*
Mp%	21.6	32.4	33.1	39.4	43.1	*17.3*	36.3	*7.5*

Tot S = Total sugars; α-AN = α-aminoacidic nitrogen content; Tot A = Total anthocyanins; Extr A = Extractable anthocyanins; EA% =Cellular maturity index; RPT = Total phenolic richness; dTpep = seeds tannins levels; Mp% = seeds maturity

*The variability of pH values in grape samples constituted by 32 joint clusters: 3.64, 3.66 and 3.68.

After 14 days of alcoholic fermentation/maceration, the residual sugar concentration in all wines was under 4 g/L. Similarly to grape samples, chemical parameters measured on the experimental wines are reported in table 5.

As expected, the variability found in grape composition among clusters belonging to a single vine was reflected in the correspondent wines. Indeed, the experimental wines were characterized by a strong heterogeneity in the various class of phenols. Moreover, the different composition in grape must deeply affects the contents of all the metabolites produced by the yeast strain used in this work in the experimental wines, underlining its fundamental role in determining the metabolic behavior of yeast. On the other hand, the experimental wines obtained by joint clusters showed a strong reduction of composition variability, confirming a good acceptance of the method here proposed.

Table 5: Chemical and physical characterization of experimental wines. Results are reported as frequency distribution and coefficient of variation for grape samples belonging to specific vine, on the left side, and as mean and coefficient of variation for joint grape samples.

	Wine from single vine clusters						Wine from joint clusters	
	Min	25th Perc	Med	75th Perc	Max	%CV	Mean	%CV
pH	3.49	3.56	3.68	4.01	4.10	-	3.60*	-
TA (g /L)	6.0	6.7	7.8	8.4	11.9	*19.4*	8.7	*1.4*
Glyc (g/L)	7.9	9.3	10.1	10.9	11.9	*10.7*	9.3	*2.2*
EtOH(%v/v)	11.3	12.7	14.5	15.1	16.0	*10.7*	14.5	*1.2*
Lact (g/L)	0.08	0.18	0.21	0.27	0.39	*31.8*	0.16	*7.4*
Acet (g/L)	0.17	0.23	0.25	0.28	0.49	*26.9*	0.23	*6.2*
Succ (g/L)	0.92	1.28	1.46	1.83	1.98	*21.2*	1.01	*7.6*
2,3-buts	0.60	0.73	0.92	1.09	1.27	*21.7*	1.07	*6.6*
mg/L								
Tot A	103.6	148.8	187.7	226.6	309.8	*29.8*	213.2	*4.8*
F-3-ols	59.4	82.2	103.5	113.8	152.2	*24.2*	143.8	*4.6*
F	34.4	45.7	59.4	76.5	86.7	*26.5*	75.1	*1.5*
Tyr	3.6	10.1	17.4	26.5	34.4	*52.2*	25.2	*7.5*
Hyt	3.5	8.2	11.3	15.1	28.0	*48.4*	11.3	*8.0*
Trp	2.3	3.3	3.9	5.3	13.8	*58.3*	3.9	*3.8*

*The variability of pH values in experimental wines obtained by 32 joint clusters: 3.57, 3.61 and 3.63.

TA = titratable acidity ($g_{tartaric\ acid}$/L), Glyc = glycerol, EtOH = ethanol, Lact = lactate, Acet = acetate, Succ = succinate, 2,3-buts = 2,3-butanediols. Tot A = sum of non polymeric anthocyanins and pigmented polymers, F-3-ols = flavan-3-ol monomers, F = flavonols, Tyr = tyrosol, Hyt = hydroxytyrosol, Trp =tryptophol.

Finally, the experimental wines were characterized in terms of relative abundances of anthocyanins, flavonols and flavan-3-ol monomers (tab. 6).

Table 6: Anthocyanin, flavonol and flavan-3-ol monomer profiles of the experimental wines. Relative abundance of glucosilated, vitisins and acylated anthocyanins to non polymeric anthocyanins. Relative abundances of myricetin and its glycosides, quercetin and its glycosides, kaempferol and kampferol glucoside to total flavonols. Relatrive abundance of catechin, epicatechin and epicatechin-3-O-gallate to total flavan-3-ol monomers.

	Wine from single vine clusters						Wine from joint clusters	
	Min	25th Perc	Med	75th Perc	Max	%CV	Mean	%CV
A profile (%)								
Dp-3-*O*-G	1,6	3,9	9,1	10,1	13,8	*48,2*	10,8	*1,5*
Cy-3-*O*-G	0,7	1,4	3,3	5,4	8,3	*64,5*	7,1	*1,6*
Pt-3-*O*-G	7,9	12,9	17,3	18,4	19,8	*23,7*	17,8	*0,5*
Pn-3-*O*-G	3,7	7,3	10,5	13,6	15,9	*35,4*	13,5	*1,3*
Mv-3-*O*-G	41,9	51,6	56,8	72,9	85,1	*20,8*	49,6	*1,0*
Vitisins	0,2	0,4	0,5	0,6	0,8	*31,9*	0,3	*5,7*
Acylated A	0,4	0,7	0,9	1,1	1,3	*27,3*	0,9	*5,7*
F profile (%)								
Ms	12,0	14,8	16,0	16,7	21,4	*14,6*	18,2	*7,1*
Qs	74,2	79,4	80,5	82,2	83,1	*3,0*	79,0	*1,5*
Ks	1,8	2,9	3,7	4,3	4,9	*22,5*	2,8	*9,9*
F3ol profile (%)								
C	27,8	35,3	39,9	43,1	49,6	*14,1*	39,3	*9,1*
EC	45,1	52,1	54,8	59,2	66,6	*11,1*	59,8	*6,5*
ECG	3,0	4,5	5,3	5,6	8,5	*24,8*	0,8	*37,2*

A profile (%) = anthocyanins profile, Dp-3-*O*-G = delphinidin-3-*O*-glucoside, Cy-3-*O*-G = cyanidin-3-*O*-glucoside, Pt-3-*O*-G = petunidin-3-*O*-glucoside , Pn-3-*O*-G = peonidin-3-*O*-glucoside, Mv-3-*O*-G = malvidin-3-*O*-glucoside, Vitisins = sum of vitisin A and vitisin B, Acylated A = sum of acetilated and coumarilated anthocyanins to non polymeric anthocyanins. F profile (%) = flavonols profile, Ms = sum of quercetin and its glycosides, Qs = sum of quercetin and its glycosides, Ks = sum of kaempferol and kaempferol-3-*O*-glucoside to total flavonols. F3ol profile(%) = Flavan-3-ol monomers profile C = (+)-catechin, EC = (-)-epicatechin, ECG = (-)-epicatechin gallate

Once again, the experimental wine obtained by 32 joint clusters showed a marked reduction of the variability, with the sole exception for epicatechin-3-*O*-gallate, between replicates when compared with the extremely different

results reported for the experimental wines produced by clusters belonging to a single vine. Indeed, the latter experimental wines reflected the different pattern of distribution of single anthocyanins flavonols and flavan-3-ol monomers of native grapes due to specific microclimatic conditions characterizing each vines. In fact, as it is known that such compounds are strongly affected by several factors such as vine vigor, temperature, exposition and water status (Downey et al., 2002), the peculiar position of vine in the vineyard and clusters on the vine resulted determinant factors for the biosynthesis and their accumulation in grape berries.

Conclusion

In this work, the noticeable heterogeneity in grape composition was investigated by determining the variability in chemical and physical characteristics of grapes harvested in the same vineyard and their corresponding wines; a methodological approach capable to keep it under control was, hence, proposed.

The first part of the work was carried out with the aim to understand the possibility of frozen storage on grape samples prior their fermentation. This crucial step was essential to ensure a good reproducibility of biological triplicates in order to obtain experimental wines whose chemical composition was comparable. By the result obtained, supported also by other studies in the literature (Cynkar et al., 2004; Santesteban et al., 2013), it was possible to verify that frozen storage modify grape composition when the acidic components are taken into consideration. However, it was also possible to confirm results of other author in the literature regarding phenolic composition of grapes (Cynkar et al., 2004; García et al., 2011). In fact, no significant differences (p<0.01) in phenolic parameters of grapes were assessed if grape samples underwent to a frozen storage up to 2 months. For this, frozen storage period of grape samples used in this thesis is specified in each materials and methods section of the works here presented. When applied, this period did not exceed 1 month.

Secondly, by understanding the magnitude of such variability in grape samples composition, it was possible to define a methodological approach that include some preliminary steps for row material preparation prior vinification, from sampling operations in vineyard to laboratory procedures, aimed to guarantee a reliable reproducibility of results. In particular, grape clusters were sampled from different vines in the same vineyard. Once in laboratory, destemming operation were carried out while clusters were still frozen and then well mixed

in order to obtain homogeneous sub-samples used as replicates for grape analysis and vinifications. By following this procedure, as reported in figure 1b, an acceptable reproducibility of the data on grape samples characterization and consequently in experimental wines composition was achieved. Hence, this methodology was adopted in all the works presented in this thesis.

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2. Health promoting compounds of grape origin in Sangiovese wines.

2.1. Vinifications of Sangiovese grapes cultivated in different viticultural areas: a three consecutive vintages study

Abstract

In this work the variability in contents and quality of several health promoting compounds of plant origin in Sangiovese wines as affected by native grapes composition was investigated. In particular, the work was focused on contents and partitioning of anthocyanins, flavonols and flavan-3-ol monomers, as they are widely recognized for their importance in determining wine quality and their health enhancing properties.

In the three vintages 2011,2012 and 2013, grapes from 7 Tuscany estates located in three distinct areas of high quality wines production (Brunello di Montalcino, Chianti Classico, Chianti colli Aretini) were harvested and fermented under laboratory conditions by a single strain of *Saccharomyces cerevisiae* in order to exclude any incidence of technological and microbiological factors during the winemaking.

Firstly, the characterization of grape samples on the basis of their phenolic maturity was analyzed with a multivariate approach. By applying the principal components analysis (PCA) a differentiation of grape samples on the basis of vintage year was assessed. Indeed, grape samples and correspondent experimental wines underwent ANOVA analysis on the basis of vintage year.

At first, vintage year seemed to be the variable able to discriminate grape samples on regards to their phenolic contents. The contents of falvan-3-ols monomers in the experimental wines resulted to be strongly affected by vintage year, with higher amounts found in 2013 wines. Despite significant correlation found between anthocyanin contents and total phenols of grapes and related experimental wines, no significant effect of vintage year was detected for total anthocyanin and flavonol contents in the experimental wines. Indeed, the contents of anthocyanins and flavonols showed peculiar fluctuations in the three vintages for wines obtained by grape samples from each single estate.

However, a general effect of vintage year involving all the experimental wines was reported when anthocyanins profiles were considered. In fact, the ratio between trisubstituted to disubstituted anthocyanins resulted to be

characteristic on the basis of vantage year. On the contrary, regarding flavonol partitioning, the contribution of quercetin and its glycosides (Qs) to total flavonols occurring in Sangiovese experimental wines was relatively stable, so that it might be seen as a characteristic of the grape variety. Finally, no significant differences emerged in the partitioning of flavan-3-ol monomers as a consequence of vintage year.

Introduction

Flavonoids are a class of compounds found in wines possessing well known antioxidant properties and probably involved in the prevention of various disease associated to oxidative stress, such as cardiovascular diseases and cancer (Manach et al., 2004). Moreover, they are well known to contribute to the wine quality by influencing color, astringency, bitterness and structure of the final product (Sacchi et al., 2005). Wine composition depends largely by grapes used and also by winemaking practices, including technological and microbiological factors, adopted for its production (Garrido and Borges 2013). However, as reported by Revilla et al. (1997) grape phenolic composition resulted to be deeply influenced by diverse agroecological factors such as the cultivar, the vintage climatic conditions, the site of production (geopedological properties and agronomical practices), and the degree of maturation. Among flavonoids, anthocyanins, flavonols and flavan-3-ols are three class of molecules deeply studied because of their implication in various aspects. Anthocyanins are the pigments of red grapes (He et al., 2010), flavonols contribute to wine color by acting as anthocyanins copigments (Boulton, 2001) and seems to concur to bitterness of wines (Rodríguez Montealegre, 2006) and falvan-3-ol monomers induce astringent and bitter mouth sensation (Gawel, 1998) other than taking part in polymerization reaction with anthocyanins, increasing and stabilizing wine color (Timberlake and Bridle, 1977). Moreover, the health enhancing properties of flavonols, in particular quercetin and its derivatives, anthocyanins and flavan-3-ols have been extensively reviewed (Erlund, 2004; Yilmaz, and Toledo, 2004; McGhie and Walton, 2007; Pascual-Teresa et al., 2010). Finally, the relative proportion of single anthocyanins, flavonols and flavan-3-ol monomers resulted to be haracteristic for each variety playing a role of chemotaxonomic tools for discrimination of red *Vitis vinifera* varieties (Goldberg et al., 1998; Von Baer et al., 2005; Mattivi et al., 2006;).

The aim of this work was to assess the variability of health promoting compounds contents and composition of wines, as affected by row material by

using a method capable to exclude the incidence of technological and microbiological factors during the transformation of grape musts into wines. Indeed, in the three vintages 2011,2012 and 2013, grapes from 7 Tuscany estates, were harvested and fermented under laboratory conditions by a single strain of *Saccharomyces cerevisiae*.

Materials and methods

Grape samples. Grape samples used in this work were sampled during three vintage (2011, 2012, 2013) in 7 different vineyards located in 3 enological areas of Tuscany. In particular, 2 vineyards were located in the Chianti Classico DOCG area (Firenze - Siena), 4 vineyards in the Brunello di Montalcino DOCG area (Siena) and 1 vineyards in Chianti colli Aretini DOCG area (Arezzo).
For each vintage, 30 clusters were sampled from 30 vines. The harvesting date was set according to quality standards of grape ripen as decided by the owners of the vineyards. In table 1, the sample codes, the characteristics of vineyards and the harvesting dates in the three vintages are reported.

Table 1: Samples codifications, vineyard characteristics and harvesting dates of grape samples in vintages 2011, 2012, 2013.

	Vineyard characteristics				Harvesting date (DOY)		
Vineyard code	Planting year	Training system	Plant spacing (m x m)	Farming practices	2011	2012	2013
BdM1	1972	Spur cordon	3.0 x 1.2	Conventional	255	262	271
BdM2	2007	Spur cordon	2.5 x 1.0	Biological	278	275	283
BdM3	1998	Spur cordon	2.5 x 0.7	Biological	258	261	277
BdM4	2002	Spur cordon	2.3 x 1.2	Organic	269	262	275
CC1	2003	Spur cordon	2.0 x 1.0	Conventional	258	263	271
CC2	2004	Spur cordon	2.5 x 1.3	Integrate	273	276	276
CA1	2000	Spur cordon	2.5 x 1.0	Integrate	251	269	268

CC = Chianti Classico, BdM = Montalcino, CA = Chianti Colli Aretini
DOY = Day-of-Year

Grape samples were transferred to laboratory at low temperature and stored at -20°C for a period of time not exceeding 1 months. Grape samples preparation methodologies prior analysis and vinifications are reported in Chapter 3.1 (3.1.3 Results, figure 1b).

Experimental vinifications. The laboratory vinifications were carried out using 450g of grapes each, in triplicates for each grape samples. Technological condition of alcoholic fermentation/maceration are reported in Chapter 3.1 (3.1.2 Materials and methods).

Yeast Inoculum. The alcoholic fermentation was induced by inoculating *Saccharomyces cerevisiae* Sc1 strain. Yeast inoculum preparation is reported in Chapter 3.1 (3.1.2 Materials and methods).

Chemical and analytical determination. Chemical and analytical determination on grapes, musts and wines are reported in Chapter 3.1 (3.1.2 Materials and methods).

Statistical analysis. All statistical calculations were performed by using Statistica software (version 7, StatSoft, Tulsa, OK, USA) and GraphPad Prism 5 (GraphPad Software, Inc., CA,USA).

Results and discussion

At first, the analytical results from all the grape samples (21 samples, that is 7 samples from each vintage) were taken into consideration (tab. 2)

Table 2: Analytical and chemical parameters characterizing the 21 grape samples. Results are reported as frequency distribution and coefficient of variation (CV%)

Grapes	Min	25th Perc	Median	75th Perc	Max	CV%
Tot S (g/L)	199	227	237	253	293	10.5
Malic acid (g/L)	0.44	0.92	1.34	1.85	2.42	40.9
Tartaric acid (g/L)	3.54	4.19	4.77	5.22	7.02	16.2
Citric acid (g/L)	0.09	0.185	0.52	0.65	0.95	55.9
pH	3.42	3.58	3.66	3.72	4.03	-
α-AN (mgN/L)	75	121	132	181	297	36.3
Tot A (mg/L)	668	1019	1162	1410	1911	22.6
Extr A (mg/L)	436	534.5	622	745	1148	24.8
EA%	34.7	40.0	45.5	49.4	54.2	13.3
RPT (OD_{280})	32.1	37.6	41.6	55.2	73.1	24.6
dTpep	5.5	14.6	17.2	24.2	46.3	48.3
Mp%	14.2	35.2	42.7	49.7	72.7	29.9

Tot S = Total Sugars, sum of glucose and fructose concentrations; α-AN = α-aminoacidic nitrogen content; Tot A = Total anthocyanins; Extr A = Extractable anthocyanins; EA% = Cellular maturity index; RPT = Total phenolic richness; dTpep = seeds tannins levels; Mp% = seeds maturity

Grape samples showed a noticeable variability in all the parameters here taken into consideration for their chemical and analytical characterization. In particular, malic acid, citric acid and α-aminoacidic nitrogen content where the non-phenolic parameters characterized by an high coefficient of variation. Regarding the evaluation of phenolic maturity indexes, the highest range of values was found in terms of seed tannins levels (dTpep) in grape samples.

Principal component analysis (PCA) was performed on phenolic maturity parameters determined for each grape samples (fig. 1)

The first two components considered explained the 91.16% of the total variance. The first component, which explained the 51.10% of total variance, resulted to be negatively related with seeds tannins levels (dTpep), seeds maturity (MP%) and total phenolic richness (RPT). The second component, which explained the 40.06% of total variance, was positively related with

extractable (Extr A) and total anthocyanins (Tot A). Finally, samples distributed in the lower right corner of the score plot were characterized by an higher values of cellular maturity index (EA%).

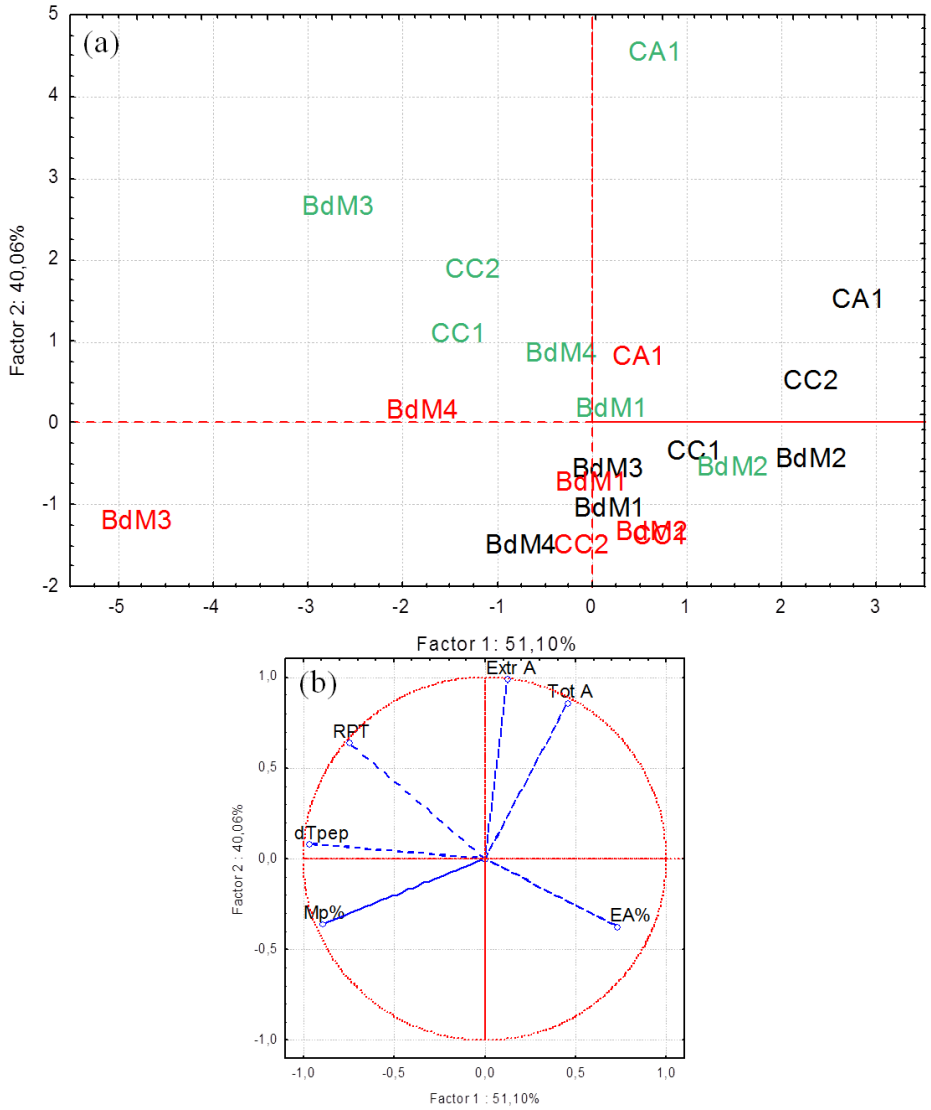

Figure 1: Principal component analysis of phenolic maturity data from Sangiovese grape samples: **(a)**; factor 1 vs factor2 score plot. Different colors of scores are referred to different vintages: 2011 (black), 2012 (red), 2013 (green); **(b)** projection of variables total anthocyanins (Tot A), extractable anthocyanins (Extr A), cellular maturity index (EA%), total phenolic richness (RPT), seeds tannins levels (dTpep), seeds maturity (Mp%)

Considering the score plot, the grape samples were generally distributed on the basis of vintage year. The effect of origins of grapes did not seem to play a crucial role in samples distribution. In particular, grape samples harvested in 2013 (green scores in fig. 1) tended to distribute in the higher left corner of the score plot. On the other hand, grape samples 2011 (black scores in fig. 1) were distributed on the right while grape samples 2012 (red scores in fig. 1) resulted to be mostly distributed in the lower part of the plot.

However, the samples derived from each estate follow a characteristic pattern of distribution among the three vintages. Indeed, the variability in grape composition during the three vintage resulted characteristic for samples from the same estate. For example, the samples codified as BdM3 showed a high variability in composition between the three vintages, while the three grape samples BdM1 resulted more similar. This could be due to peculiar fluctuations of microclimatic conditions characterizing each vineyard as well as to different agronomical management of vineyards performed by the 7 estates.

The multivariate approach adopted (PCA) seemed to highlight an effect in determining differences in analytical and chemical features of Sangiovese grape samples. Hence, the analysis of variance (one-way ANOVA) was performed on the basis of vintage, in order to assess the effect of vintage year on analytical characteristics of Sangiovese grapes. In table 3 results of one-way ANOVA of technologic and phenolic parameters of grape samples is reported.

Significant differences were assessed for grape samples harvested in three different vintages. In particular, grapes from 2013 vintage were characterized by an higher level of malic acid and by lower citric acid contents and pH values. Indeed, the 2013 vintage were cooler than 2011 and 2012 and these seemed to be related to acidity parameters of grapes, in particular for malic acid contents as it is known that its concentration in grapes is related to air temperature (Ruffner et al., 1976). Moreover, higher contents of extractable anthocyanins and higher values of total phenolic richness were assessed on 2013 grape samples when compared with grapes from the other vintages. On the contrary, 2012 grapes were characterized by lower level of malic acid, citric acid, extractable anthocyanins contents and higher seed maturity index when compared with grapes belonging to the other vintages studied.

Table 3: One-way ANOVA of technologic and phenolic parameters determined on grape samples in the three vintages. Results are reported as mean ± coefficient of variation, except for pH values were the results are reported as mean ± standard deviation †. Means in the same line followed by different letters are significantly different (Tukey test at $p < 0.05$).

	2011	2012	2013
Tot S (g/L)	254 ± 0.08	234± 0.13	234 ± 0.09
Malic acid (g/L)	1.35 ± 0.42 ab	0.93 ± 0.34 b	1.86 ± 0.19 a
Tartaric acid (g/L)	4.07 ± 0.08	5.46 ± 0.15	4.95 ± 0.06
Citric acid (g/L)	0.69 ± 0.19 a	0.51 ± 0.17 b	0.14 ± 0.38 b
pH	3.76 ± 0.13† a	3.63 ± 0.09† ab	3.56 ± 0.05† b
α-AN (mgN/L)	155 ± 0.25	164 ± 0.43	122 ± 0.35
Tot A (mg/L)	1198 ± 0.21	1045 ± 0.21	1357 ± 0.21
Extr A (mg/L)	628 ± 0.17 ab	560 ± 0.18 b	709 ± 0.24 a
EA%	47.0 ± 0.08	45.6 ± 0.16	41.1 ± 0.13
RPT (OD_{280})	38.1 ± 0.08 b	46.1 ± 0.21 ab	54.8 ± 0.23 a
dTpep	13.1 ± 0.43	23.7 ± 0.45	22.6 ± 0.40
Mp%	33.8 ± 0.38 b	49.8 ± 0.22 a	40.4 ± 0.22 ab

Tot S = Total Sugars, sum of glucose and fructose concentrations; α-AN = α-aminoacidic nitrogen content; Tot A = Total anthocyanins; Extr A = Extractable anthocyanins; EA% = Cellular maturity index; RPT = Total phenolic richness; dTpep = seeds tannins levels; Mp% = seeds maturity

After 14 days of alcoholic fermentation/maceration, the experimental wines were chemically analyzed. In table 4, standard parameters, total anthocyanin and flavonol contents and total phenols index are reported (tab. 4).

As expected, the variability assessed in grape composition (tab. 2) was reflected into experimental wines composition. In particular, the class of phenolic compounds determined showed a high level of variability, with coefficient of variation (CV%) of 33.6 for total anthocyanin contents and 36.8 for total flavonol contents.

Table 4: Analytical and chemical parameters characterizing the 21 experimental wines. Results are reported as frequency distribution and coefficient of variation (CV%)

Wines	Min	25th Perc	Median	75th Perc	Max	CV%
pH	3.38	3.51	3.58	3.68	3.84	-
Ethanol (%v/v)	11.4	12.9	13.6	14.3	16.3	9.3
Acetic acid (g/L)	0.07	0.11	0.17	0.24	0.36	46.6
Total anthocyanins (mg/L)	98.3	166.7	213.2	257.3	420.1	34.2
Total flavonols (mg/L)	27.6	65.1	71.7	81.1	140.5	36.8
Total flavan-3-ols (mg/L)	16.9	57.6	81.6	124.2	204.5	52.7
TP (OD_{280})	29.0	46.9	53.3	66.9	82.8	23.3

TP = Total phenols.

Moreover, correlation analysis showed significant correlation between some grape and experimental wine parameters. In particular, highly significant correlation were assessed between total anthocyanins (Tot A) and extractable anthocyanins (Extr A) of grapes and total anthocyanins contents of related experimental wines (Person's r = 0.78 and 0.72, $p < 0.001$, respectively) and between total phenolic richness (RPT) of grapes and total phenols (TP) of related experimental wines (Person's r = 0.81, $p < 0.001$). This results confirmed that the origin of variability in composition of wines is strongly related with grapes composition when the vinification is carried out in standardized conditions.

As in the case of grape samples, the experimental wines obtained in the three vintages were analyzed by performing the one-way ANOVA (tab. 5). No significant difference were assessed in terms of pH, ethanol and acetic acid contents of wines. Similarly, total anthocyanins, total flavonols and total phenols were not significantly affected by vintage years. Conversely, total contents of flavan-3-ol monomers were related to vintage year, with the experimental wines produced in 2013 characterized by higher contents. This result is consistent with findings of other Authors, which reported a strong relation between these compounds and vintage year, principally attributable to grape maturity (Downey et al., 2006).

Table 5: One-way ANOVA of pH, ethanol, acetic acid and phenolic compounds determined on the experimental wines obtained in the three vintages. Results are reported as mean ± coefficient of variation, except for pH values were the results are reported as mean ± standard deviation †. Means in the same line followed by different letters are significantly different (Tukey test at $p < 0.05$).

	2011	2012	2013
pH	3.64 ± 0.11†	3.59 ± 0.12†	3.55 ± 0.12†
Ethanol (%v/v)	14.0 ± 0.08	13.2 ± 0.10	13.7 ± 0.06
Acetic acid (g/L)	0.21 ± 0.38	0.14 ± 0.60	0.23 ± 0.33
Anthocyanins (mg/L)	247.5 ± 0.31	203.6 ± 0.28	219.9 ± 0.42
Flavonols (mg/L)	75.1 ± 0.37	63.8 ± 0.19	89.1 ± 0.38
Flavan-3-ols (mg/L)	58.5 ± 0.59 b	87.7 ± 0.39 ab	129.4 ± 0.28 a
TP (OD_{280})	55.2 ± 0.16	52.8 ± 0.29	61.8 ± 0.25

TP = Total phenols.

As reported by figure 2, a generalized effect toward higher contents of flavan-3-ols in all the 2013 experimental wines was reported, with the sole exception of BdM2 which reached higher concentrations in 2012. Despite this result, it was possible to assess that vintage played a strong effect in determining the concentration of flavan-3-ol monomers in wines. Conversely, the variability in total anthocyanin and flavonol contents in the experimental wines, did not reproduce specific pattern on the basis of vintage year. The anthocyanin and flavonol contents of the experimental wines BdM1 resulted relatively stable throughout the vintages studied while BdM3 wines reported appreciable differences. Moreover, even considering experimental wines obtained by grapes cultivated in a specific area, the effect of vintage in their concentration seemed to be not the same. As an example the higher contents of anthocyanins and flavonols in samples BdM2 were detected in 2012 experimental wines while in the same vintage BdM3 was characterized by the lowest values of anthocyanins and flavonols recorded for BdM3 samples. Finally, the experimental wines CA1 resulted generally more concentrated in anthocyanins in when compared with the other samples of the same vintage.
Indeed the vintage year seemed to produce an effect on total contents of anthocyanins, flavonols and flavan-3-ol monomers but the magnitude of its impact resulted specific for each experimental wines (tab.5 and fig. 2).

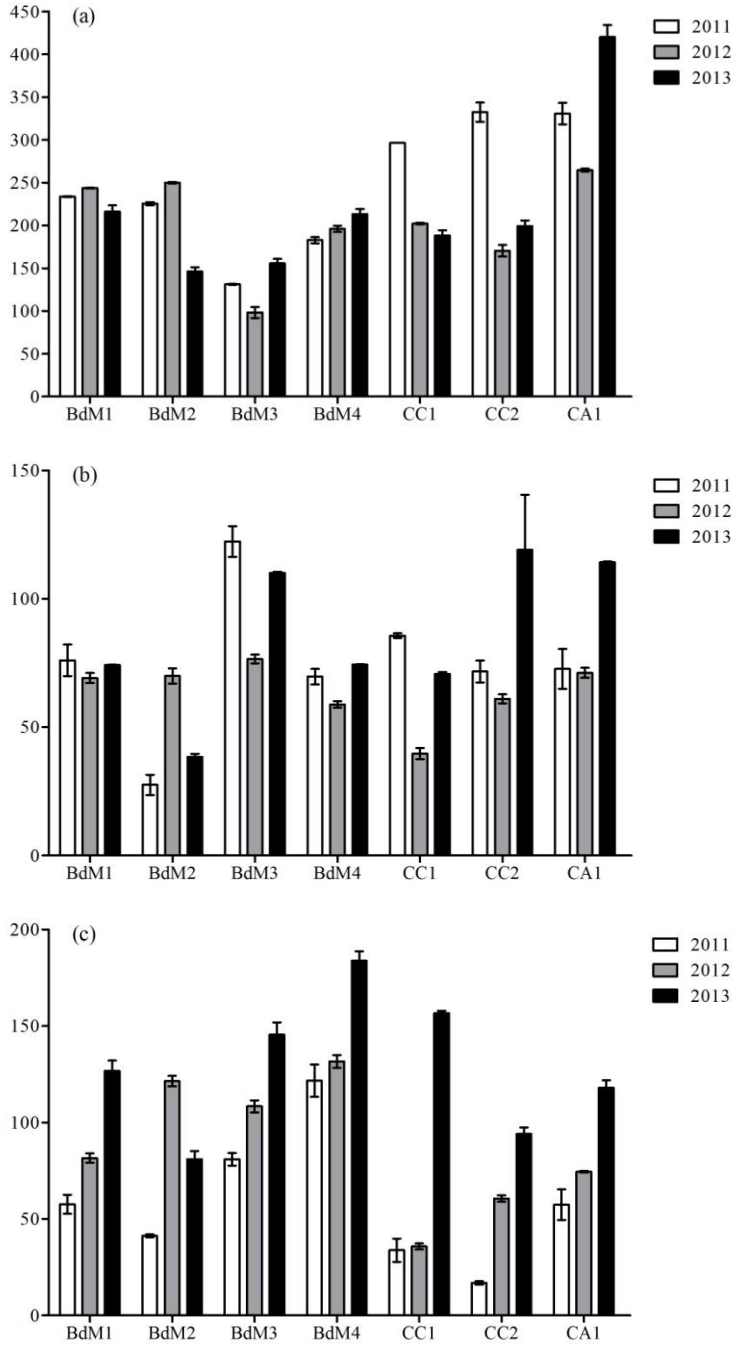

Figure 2: Total anthocyanins (a) total flavonols (b) and total flavan-3-ol monomers (c) contents of Sangiovese experimental wines. Values are reported as mean, error bars are SEM.

This seemed to suggest that the effect of vintage is reflected on wine composition as a consequence of microclimatic conditions, geopedological features and agronomical managements of specific micro area, identified in this study as each estate, strengthen the considerations on the influence of vintage year on phenolic maturity parameter of grape samples. Moreover, is noteworthy to highlight that although the technological and microbiological factors characterizing the method of vinification were taken under standardized conditions, the process acts as a new source of variability. Indeed, the fermentation kinetics, intended as ethanol rate of formation during fermentation, probably may play a significant role in hiding the differences reported for grape samples. As a consequence, despite a significant correlation between RPT of grape samples and TP of corresponding experimental wines, the significant differences characteristic of single vintage recorded on grapes did not emerged in wines.

However, it is well known that vintage conditions, as the sum of climatic factors, geopedological features and vineyard managements, can affect grapes composition also in qualitative distribution of single flavonoinds (Brossaud et al., 1999). Although anthocyanins composition is a feature relatively stable for a given grape variety (Arapitsas et al., 2012), it is well known that vintage year could effects relative proportion of the different anthocyanins (He et al., 2010). Moreover, the characteristic condition of a single year can also affect the composition of flavonols in grape (Brossaud et al., 1999). As it is known that wine qualitative composition inherit the grape qualitative composition, even if a larger variability occurs (Arapitsas et al., 2012), the experimental wines were characterized on the basis of vintage year in order to evaluate difference in anthocyanins and flavonols profiles due to characteristics of single vintage (tab. 6).

Table 6: Anthocyanins and flavonols profiles of experimental wines obtained in the 3 vintage, mean of 7 experimental wines for each vintage. Values are expressed as mean ± coefficient of variation (CV = standard deviation/mean). Means in the same line followed by different letters are significantly different (Tukey test at $p < 0.05$).

	2011	2012	2013
Anthocyanins profile (%)			
Dp-3-*O*-G	8.0 ± 0.26	8.5 ± 0.21	8.4 ± 0.36
Cy-3-*O*-G	2.5 ± 0.31 b	4.1 ± 0.46 b	7.0 ± 0.33 a
Pt-3-*O*-G	17.0 ± 0.10	15.9 ± 0.09	15.6 ± 0.18
Pn-3-*O*-G	8.3 ± 0.14 b	10.6 ± 0.25 b	15.2 ± 0.28 a
Mv-3-*O*-G	62.6 ± 0.08 a	59.4 ± 0.12 a	52.2 ± 0.18 b
Vitisins	0.3 ± 0.41 b	0.7 ± 0.37 a	0.4 ± 0.30 b
Acylated A	1.3 ± 0.21	0.8 ± 0.46	1.2 ± 0.82
A ratio	8.3 ± 0.19 a	6.2 ± 0.34 b	3.8 ± 0.38 c
Flavonols profile (%)			
Ms	16.8 ± 0.33	17.7 ± 0.24	16.9 ± 0.39
Qs	77.7 ± 0.06	77.6 ± 0.05	79.5 ± 0.08
Ks	5.5 ± 0.38 a	4.8 ± 0.20 ab	3.7 ± 0.19 b
F ratio	0.22 ± 0.40	0.23 ± 0.28	0.22 ± 0.49
Flavan-3-ols profile%			
C	51.5 ± 0.11	46.5 ± 0.16	49.0 ±0.13
EC	44.2 ± 0.11 b	50.7 ± 0.12 a	48.1 ± 0.16 ab
ECG	4.3 ± 0.52	2.8 ± 0.96	2.9 ± 0.92
F3ols ratio	1.2 ± 0.20	0.9 ± 0.27	1.1 ± 0.30

Dp-3-*O*-G = delphinidin-3-*O*-glucoside, Cy-3-*O*-G = cyanidin-3-*O*-glucoside, Pt-3-*O*-G = petunidin-3-*O*-glucoside , Pn-3-*O*-G = peonidin-3-*O*-glucoside, Mv-3-*O*-G = malvidin-3-*O*-glucoside, Vitisins = sum of vitisin A and vitisin B, Acylated A = sum of acetilated and coumarilated anthocyanins to non-polymeric anthocyanins contents; A ratio = trisubstituted (Dp-3-O-G, Pt-3-*O*-G and Mv-3-*O*-G) to disubstituted (Cy-3-*O*-G and Pn-3-*O*-G) ratio. Ms = sum of myricetin and its glycosides, Qs = sum of quercetin and its glycosides, Ks = sum of kaempferol and kaempferol-3-*O*-glucoside to total flavonols; F ratio = Ms contents (mg/L) / Qs contents (mg/L). C = (+)-catechin, EC = (-)-epicatechin, ECG = (-)-epicatechin-3-*O*-gallate; F3ols ratio = C contents (mg/L) / EC contents (mg/L).

The experimental wines were characterized by an high variability in relative abundance of anthocyanins profile, while the flavonols profiles resulted more similar. Regarding anthocyanins profiles, as reported by other Authors (Mangani et al., 2011; Arapitsas et al., 2012),Sangiovese experimental wines were characterized by a high abundance of malvidin-3-*O*-glucoside (Mv-3-*O*-G) and by a traceable presence of acylated anthocyanins and vitisins. Moreover, Mv-3-*O*-G relative abundances, despite other anthocyanins with the exception of petunidin-3-*O*-glucoside (Pt-3-*O*-G) were characterized by a relative stability in the three vintages, as reported by the low value of coefficient of variation. On the basis of the three vintages, significant differences emerged in the content of Mv-3-*O*-G assessing the lower value in 2013 experimental wines. On the other hand, the 2013 experimental wines were also characterized by a higher relative abundance of cyanidin-3-*O*-glucoside (Cy-3-*O*-G) and peonidin-3-*O*-glucoside (Pn-3-*O*-G) when compared with the other experimental wines. As a consequence, the ratio between trisustituted and disubtituted anthocyanins resulted significantly different among the experimental wines of the three vintage. In particular, a lower value of A ratio was assessed in 2013 experimental wines while the higher value was assessed in 2011 experimental wines.

Regarding Flavonols profile, significant differences were found only in the relative abundance of kaempferol and its glycosides (Ks) of experimental wines, with the lowest value found in 2013 experimental wines. On the other hand, the relative abundance of myricetin and its glycosides (Ms) and quercetin and its glycosides (Qs) showed no significant difference in the experimental wines of the three vintages. Moreover, the relative abundance of quercetin and its glycosides respect to total flavonols in all the experimental wines exhibited a low coefficient of variation, with values quite comparable to those found by other Authors in Sangiovese wines (Ghiselli et al., 1998; McDonald et al., 1998). Such results suggested that the contribution of Qs to total flavonols occurring in Sangiovese experimental wines was relatively stable, so that it might be seen as a characteristic of the grape variety.

Finally, flavan-3-ol monomers partitioning was considered. In literature is reported that with the increasing of the degree of maturation catechin as a consequence of polymerization reaction decrease while epicatechin and proanthocyanidin dimers and trimers increased (Downey et al., 2006). By the results obtained no significant differences in the partitioning was assessed with the exception of epicatechin (EC). However, the F3ols ratio, proposed as a

characteristic features of *Vitis vinifera* varieties (Goldberg et al., 1998) did not differ significantly among the experimental wines of the three vintage studied.

Conclusion

In this work the variability in contents of plant derived health promoting compounds of experimental wines obtained in three consecutive vintages and produced from Sangiovese grapes cultivated in different enological areas of Tuscany was investigated. By excluding the influences of technological and microbiological variables during the transformation of grape musts into wines, it was possible to evaluate the incidence of grape composition on plant derived health promoting compounds of experimental wines. In particular, the results here reported showed an influence of vintage year on grapes phenolic maturity involving all grape samples independently by their geographical origin. However, despite a general variability induced by vintage year on grapes composition, grapes from each estate showed a peculiar fluctuation in composition among the three vintages maybe due to microclimatic or specific agronomical factors identifying each single vineyard. Such findings were then transferred into experimental wines, although an increased variability in phenolic parameters analyzed induced by the winemaking was detected. Indeed, the impact of vintage year was not reflected as univocal effect on anthocyanin and flavonol contents of experimental wines. Conversely, a significant effect of vintage year was assessed in flavan-3-ol monomers contents of the experimental wines.

Moreover, the effect of vintage year was also reported in flavan-3-ols concentrations and anthocyanins profiles of experimental wines were considered. In fact, by the results obtained, the ratio between trisubstituted to disubstituted of experimental wines resulted characteristic for all the experimental wines on the basis of vantage year.

In conclusion, the variability in Sangiovese grape compositions, which is reflected in correspondent experimental wines, is the result of specific climatic conditions characterizing each vintage year in a given micro area, identified as single vineyard. Climatic factors, geopedological features and vineyard managements interact together in determining the specific condition of grapes growth in a specific vineyard in a given year, influencing and inducing peculiarity in grapes composition. As a consequence, a noticeable variability in grapes and wines composition was found. Given that, further works reported in this thesis were aimed to investigate on this variability trying to characterize deeply the factors related to vintage year able to affect the contents of health

promoting molecules of plant origin, such as anthocyanins, flavonols and flavan-3-ol monomers found in wines.

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2.2. Variability of anthocyanin and flavonol profiles in Sangiovese wines

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Abstract.
To investigate on the origin of the anthocyanin profile variability of Sangiovese wines, grapes of four consecutive years harvested from the same vineyard, managed from year to year with the same viticultural practices, were vinified under laboratory conditions, ruling out any possible incidence of microbial ecology and winemaking technology. This study also included flavonols, and flavan-3-ols, biosynthetically related to anthocyanins.
Wines showed significantly different anthocyanin profiles. The relative proportions between tri- and di-substituted anthocyanins were strongly dependent on air temperature: elevated air temperatures and high values of heat accumulation favored tri-substituted anthocyanins with respect to di-substituted ones.
Key words: Climate; flavonols; Sangiovese; traceability; wine anthocyanins.

Introduction

A recent paper demonstrated that the anthocyanin profile of Sangiovese wines is characterized by a noticeable prevalence of malvidin-3-glucoside (Mv) and a negligible relative abundance of acylated anthocyanins. Hence, Sangiovese wines are well distinguishable from wine made with grapes containing significant proportions of acylated anthocyanins, such as Merlot or Cabernet Sauvignon. However the analysis of several wines, produced in years 2005-2010 by different wineries in Tuscany, showed a wide variability in the relative abundance of cyanidin-3-glucoside (Cy), peonidin-3-glucoside (Pn), and Mv, the latter anthocyanin ranging from 33.2 % to 66.9 % (MANGANI et al., 2011). As a consequence, the anthocyanin profile significantly differed among Sangiovese wines. In the same paper, it was demonstrated that this variability was unaffected by the microbial ecology of the vinification process, suggesting that other factors should be taken into consideration to understand and to justify the observed variability. In this regard, it is known that the phenolic content of a wine is roughly dependent on the vinification process, but the primary determinant is the composition of the grapes at harvest. On the other hand, phenolic composition of grapes is affected by numerous factors, such as *cultivar*, climatic and geo-pedological conditions, viticultural practices and maturation degree (DOWNEY et al., 2006; GINJOM et al., 2011; NICHOLAS et al., 2011). However, it is reasonable that phenolic composition of grapes harvested in the same vineyard, managed from year to year with the same viticultural practices, is mainly dependent on the climatic conditions of the vintage, which can be crudely summarized as air temperature and sunlight. Indeed, among climatic conditions, temperature is considered the most important factor in affecting anthocyanins biosynthesis (DOWNEY et al., 2006; KLIEWER and TORRES, 1972; SPAYD et al., 2002; TARARA et al., 2008), because it can significantly modify both the content and the composition of grape berry anthocyanins by affecting the expression of both the structural and the regulatory genes (DAL SANTO et al., 2013; HE et al., 2010). Previous researches demonstrated that anthocyanin biosynthesis is generally favored at relatively low temperatures, between 16 and 22°C (NICHOLAS et al., 2011). On the contrary, at temperatures lower than 10°C and higher than 30°C, anthocyanin accumulation is inhibited (DOWNEY et al., 2006; KLIEWER and TORRES, 1972), and, at temperatures higher than 35°C, a certain anthocyanin degradation is reported to occur (HE et al., 2010). As concerns anthocyanin partitioning, a study carried out on Merlot grapes showed that high berry temperatures,

between 34 and 44°C, alter the relative proportions between acylated and non-acylated forms of anthocyanins, and between di- (Cy, Pn) and tri-substituted (Dp, Pt, Mv) anthocyanins (TARARA et al., 2008). Moreover, the compression of the diurnal temperature range was found to favor the partitioning of anthocyanins and flavonols toward di-substitution, so that Cy, Pn and quercetin (Q) derivatives were accumulated to a higher extent in grape berries (COHEN et al., 2012). In spite of these findings, it is to be underlined that most studies so far carried out concerned the influence of climatic factors on the phenolic composition of wine grapes, without furnishing useful information on phenolics in resulting wines (COHEN et al., 2012; DOWNEY et al., 2004; LORRAIN et al., 2011; MORI et al., 2007; NICHOLAS et al., 2011; TARARA et al., 2008). However, it is known that the data obtained from grape analysis are not directly transferable to wine, owing to the influence of the winemaking process on both the content and the composition of phenolic compounds (GINJOM et al., 2011).

The aim of this work was to investigate on the origin of the anthocyanin profile variability of Sangiovese wines. To this purpose, Sangiovese grapes of four consecutive years (2011-2014) were harvested from the same vineyard managed from year to year with the same viticultural practices, and transformed into wines under strictly controlled laboratory conditions, in order to rule out any possible variability due to both microbial ecology and winemaking technology. This study also included flavonols and flavan-3-ol monomers, linked to anthocyanins under a biosynthetically point of view.

Materials and methods.

Grape sampling.

Sangiovese grape clusters, harvested during 2011, 2012, 2013 and 2014 vintages were sampled in a 11 years old Sangiovese vineyard. The vineyard covered approximately 0.38ha with NE-SW oriented rows and was located in Montalcino (Italy). Vines were trained to cordons, spur-pruned and spaced 2.5x0.9m (approximately 4500 vines/ha); shoots were maintained in a vertical position by two couples of movable wires.

Agronomical practices were the same in the four vintages: same fertilization practices, green pruning during growth season, leaf removal and cluster thinning during ripening of grapes, no irrigation.

A total of approximately 15kg grapes were harvested from the same vines at each vintage.

In order to reduce the burden of berry composition variability within the clusters, the vine and between vines, consequence of the considerable phenotypic plasticity of grapevine (DAL SANTO et al., 2013), clusters were destemmed, berries being suddenly transferred in a plastic bag and hence gently mixed.

Experimental microvinifications.

Three aliquots of mixed berries (weight 460g) were transferred in separated plastic bags and crushed under aseptic conditions; each juice with its skins and seeds was transferred into a sterilized 500mL Erlenmeyer flask provided with Müller valves filled with concentrated sulfuric acid. Microvinifications were induced by inoculating the musts with a *S. cerevisiae* strain previously isolated from a spontaneous commercial wine fermentation and included in the yeast culture collection of the Dipartimento di Gestione dei Sistemi Agrari, Alimentari e Forestali (GESAAF, University of Florence, Italy). Yeast inocula, originated from a one-day-old culture grown at 30°C in yeast extract-peptone-dextrose agar (YEPD), were made to give an initial cell concentration of 10^6 cells/mL. Must fermentations were carried out at 28°C and their time course was monitored by determining the weight loss caused by CO_2 evolution. Once CO_2 production ceased, fermentation was considered to be finished. After 15 days, experimental wines were separated from grape solids and immediately analyzed. Samples were taken aseptically at the middle of alcoholic fermentation to verify the effective dominance of the inoculated *S. cerevisiae* strain.

Chemicals and analytical determinations.

All solvents were of HPLC quality and all chemicals of analytical grade (>99%); water was of MilliQ® quality (Millipore, Billerica, USA).

The dominance of the inoculated yeast strain was verified by characterizing, at strain level, 25 *S. cerevisiae* isolates from the middle phase of the alcoholic fermentation (RFLP-mtDNA analysis, according to GRANCHI et al., 2003).

The phenolic maturity of grapes was calculated according to the method described by RIBÉREAU-GAYON et al. (2006), the total phenolic content (TP_{grape}) was calculated by measuring the absorbance at 280nm of the solution at pH3.2.

Free α-amino nitrogen content (FAN) was determined by the NOPA procedure (DUKES and BUTZKE, 1998). Total acidity was determined according to the official method for wine analysis (EEC 2676/90), color intensity (CI) was determined according to GLORIES (1984), and total phenolic content of wines

(TP_{wines}) was estimated by measuring the absorbance at 280 nm of the wine according to RIBÉREAU-GAYON et al. (2006).

HPLC separation and identification of individual anthocyanins were performed according to the *Office International de la Vigne et du Vin* method for analysis of anthocyanins in red wines (OIV 2009) as previous described (MANGANI et al., 2011). Anthocyanins were quantified using malvidin-3-glucoside as a standard. Wines were injected in HPLC after filtration through 0.45 micron.

For flavonol and flavan-3-ol monomers quantification the analytical conditions were those described by HERNÁNDEZ et al. (2005). Wine samples extracted with diethyl ether and ethyl acetate were injected in a HPLC (Varian Pro-star 210), equipped with a Diode Array Detector at 360nm and at 280nm and a reversed phase column Chromsep Omnispher (5µm particle, 250 x 4.6mm; Varian Inc.), thermostated at 25°C. Flavonols were quantified using as standards Q, myricetin (M) and kaempferol (K). Glycoside concentrations were expressed in equivalents of the correspondent aglycone.

Anthocyanin and flavonol profiles were determined by relative proportions of compound concentration.

Glucose, fructose, ethanol and glycerol concentrations were determined by HPLC, according to GRANCHI et al. (1998), utilizing a MetaCarb H Plus Column (8µm particle, 300 x 7.8mm; Varian Inc.) and a Pro-star 210 chromatograph equipped with a Diode Array Detector at 210nm and a Refractive Index Detector, in series (Varian Inc.).

All analytical data are the mean of two separate determinations.

Climate data collection and analysis.

One datalogger A753 GPRS RTU, a sensor for air temperature SEN-R Combisensor Temp/RH TR1, and a rain gauge RG1, 200cm^2, 0.2mm, unheated (Adcon Telemetry, Austria) were installed in the vineyard. The original data were used to calculate daily minimum, average, and maximum temperatures, daily temperature range, and growing degree days base 18°C (KELLER, 2010).

All statistical calculations were performed by using Statistica software (version 7, StatSoft, Tulsa, OK, USA).

Results and discussion

Anthocyanin and flavonol profiles of experimental wines.

Chemical composition of both grapes and experimental wines are summarized in tables 1 and 2. The 2014 wines, obtained from the grapes showing the highest values of anthocyanin content (A pH 1 and A pH 3.2) as well as the

lowest values of pH (Table 1), really exhibited statistically significantly higher values of total free anthocyanins and color intensity (CI, table 2). The 2012 wines showed significantly higher TP values (Table 1), according to the highest TP values of grapes (table 2). As concerns flavonol concentration, the highest value was detected in 2014 wines, 2012 wines showing the lowest one. The flavan-3-ol monomer contents, on the contrary were higher in 2013 wines, whereas the lowest contents were detected in 2014 wines.

Anthocyanin profiles of all Sangiovese wines, reported in table 3, were characterized by the prevalence of Mv with low levels of acylated anthocyanins, in agreement with previous findings (MANGANI et al., 2011). The anthocyanin profile of 2014 wines were characterized by significant higher percentages of Dp, Cy, Pn and significant lower percentages of Mv, whereas 2012 wines on the contrary were characterized by significant lower percentages of Dp, Cy, Pt and Pn and significant higher percentages of Mv. However, a wide variability in the anthocyanin profiles was observed, the variation coefficient of anthocyanin percentages ranging between 9 and 34% (table 3). The highest variation was recorded for Cy; this was not surprising because Cy possesses an *o*-diphenol structure, that is known to be more susceptible to degradation (HE et al. 2010).

As concerns flavonols (table 4), the profiles (calculated as the sum of all glycosilated and aglycone forms, ΣM, ΣQ, ΣK) were always characterized by the prevalence of ΣQ. This was in agreement with previous findings, reporting that Q derivatives typically dominate the flavonol profile of Sangiovese grapes (MATTIVI et al., 2006). The flavonol profile of 2011 wines showed significantly higher ΣQ and lower ΣM, whereas 2014 wines were characterized by the lowest ΣQ and the highest ΣM values. However, it should be stressed that the ΣQ percentage of the wines was characterized by a low variation coefficient (CV=5%), so that ΣQ could be considered as a typical feature of Sangiovese wines.

Considering the biosynthetic pathway, anthocyanins and flavonols are closely related because both classes of flavonoids derive from the same or parallel enzymatic activities. Actually, Flavonoid-3'-hydroxylase (F 3'OH) and Flavonoid-3',5'-hydroxylase (F3',5'OH) are the enzymes involved in the biosynthetic pathway of both anthocyanins (tri-substituted and di-substituted ones) and flavonols (Q, M and K). In grapes, the ratios of both tri-substituted to di-substituted anthocyanins (Dp, Pt, Mv / Cy and Pn) and flavonol (M / Q) derivatives are related to the enzymatic activities of F 3'OH and F3',5'OH, and

are characteristics of each variety (FIGUEIREDO-GONZÁLEZ et al., 2012; MATTIVI et al., 2006). These characteristic ratios are inherited by the wine from the grapes, although the winemaking process may introduce further sources of variability (ARAPITSAS et al., 2012). The calculated values (tables 3, and 4) showed a wide variability among the wines, the variation coefficients varying between 22 and 25%.

To investigate on the origin of the observed variability on both anthocyanin and flavonol profiles, a correlation study was performed among the profile indexes of wines (table 3, 4) and both grape and wine characteristics (tables 1, and 2). The tri-substituted/di-substituted anthocyanin ratios were inversely correlated with phenolic maturity indices of grapes, ApH1 and ApH3.2 ($r=-0.98$ and $=-0.87$, respectively, $p<0.01$). In particular, these indices were positively correlated with the relative abundances of both the di-substituted anthocyanins ($r=0.80$ to 0.96, $p<0.01$). Furthermore, a direct and highly significant correlation was found among tri-substituted/di-substituted anthocyanin ratios and total phenolics, ethanol content and pH of wines ($r=0.84$ to$=0.89$, respectively, $p<0.01$). A significant negative correlation was found among tri-substituted/di-substituted anthocyanin ratios and free anthocyanin contents in wines ($r=-0.78$, $p<0.01$). Concerning flavonols, no significant correlations ($p<0.01$) were found among flavonol profiles and both grape and wine characteristics.

In conclusion, anthocyanin profile indexes of experimental wines were closely related to some grape maturity indices, that in turn depends on weather conditions during the phenological periods of flavonoid biosynthesis. As a consequence, weather conditions of the four years were also evaluated.

Climate variables from blooming to harvest

Seven climate variables, listed in table 5, were identified to describe the climatic time courses of the four years 2011-2014 throughout the phenological periods of flavonoid biosynthesis. Focusing the attention on the most relevant parameters known to affect anthocyanin biosynthesis, in the pre-veraison time period, 2012 was characterized by the highest value of heat accumulation (expressed as growing degree days base 18°C), whereas 2014 showed the lowest value. High values of heat accumulation in the pre-veraison time period could have favored an accumulation of flavonoid precursors thus justifying the highest values of total phenolics recorded in both 2012 grapes and wines. Moreover, in the post-veraison time period, 2011 and 2012 were both characterized by the highest number of days with maximum temperatures in

the range of inhibition of anthocyanin accumulation in the skin (i.e.>30°C; according to CHORTI et al., 2010), by the highest number of days with maximum temperatures exceeding 35°C, temperatures generally associated with anthocyanin degradation (HE et al., 2010), and by the highest total heat accumulation values. A significant inverse correlation was found among free anthocyanin contents in wines and heat accumulation in the post-veraison time period ($r=-0.91$, $p<0.01$) and with both the number of days with maximum temperatures in the range of inhibition of anthocyanin accumulation in the skin (i.e.≥30°C; $r=-0.89$, $p<0.01$), the number of days with maximum temperatures exceeding 35°C ($r=-0.83$, $p<0.01$), confirming the hypothesis of a detrimental effect of elevated temperatures on anthocyanin biosynthesis. Besides, considering that the formation of Mv-derivatives seems to be less sensitive to temperature than the other anthocyanins (KELLER, 2010), the higher value of heat accumulation in 2011 and 2012 could have caused a higher relative abundance of Mv in the corresponding wines, as it really occurred (table 3).

In order to investigate if the tri-substituted/di-substituted ratios were linked to climatic conditions, a correlation study was carried out among the A and F ratios (tables 3, and 4) and the climatic parameters in table 5. A direct and significant correlation was found among the tri-substituted/di-substituted anthocyanin ratios and heat accumulation in both pre and post-veraison time periods ($r=0.94$ and 0.95, respectively, $p<0.01$), and with both the number of days with maximum temperatures in the range of inhibition of anthocyanin accumulation in the skin (i.e.≥30°C; $r=0.96$, $p<0.01$), the number of days with maximum temperatures exceeding 35°C ($r=0.94$, $p<0.01$) and daily temperature range ($r=0.97$, $p<0.01$) in the post-veraison time period. Thus, elevated values of heat accumulation seems to affect the anthocyanin profile of the wines, characterized by higher percentages of Mv, and lower percentages of Cy and Pn.

Concerning flavonols, a significant inverse correlation was found between tri-substituted/di-substituted flavonol ratio and the number of days with average temperatures between 16 and 22°C ($r=-0.79$, $p<0.01$) in the pre-veraison time period.

Conclusions.

The anthocyanin profile of Sangiovese wines possesses a typical feature, linked to the genetic characteristics of the grapes and evidenced by both a negligible relative abundance of acylated derivatives and a prevalence of Mv. However,

the profiles of wines produced with grapes harvested in consecutive years in the same vineyard, managed with the same viticultural practices, showed significantly different Mv percentages. This variability of Mv percentage was found to be strongly dependent on air temperature time course in the post-veraison time period. Consequently, also the relative proportions between tri- and di-substituted anthocyanins was strongly dependent on air temperature. As a matter of fact, elevated air temperatures favored tri-substituted anthocyanins (Mv, Pt, Dp) with respect to di-substituted ones (Cy, Pn). Furthermore, high values of heat accumulation favored tri-substituted forms. In this connection, a proper canopy management, aimed to avoid excessive grape heating, could be an useful tool to limit the effect of air temperature on grape composition. However, it cannot be ruled out that the light exposition of the clusters, which is known to increase Mv percentage in wines (RUSTIONI et al., 2011; STERNAD LEMUT et al., 2013), could have had a synergistic effect with heat accumulation.

The tri-substituted to di-substituted flavonol ratio, on the contrary, in spite of the high variability (CV 22%), was not significantly correlated with air temperatures, highlighting that flavonol biosynthesis (tri-substituted / di-substituted flavonols) is modulated differently from anthocyanin biosynthesis (tri-substituted / di-substituted anthocyanins), although the metabolic pathway leading to anthocyanins and flavonols is generally considered the same for both classes of phenolics.

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Table 1.

Phenolic and technological maturities of Sangiovese grapes.

	2011	2012	2013	2014	CV%
Phenolic and technological maturity indices [a]					
A pH 1 (mg/L)	881	1009	1147	1236	15
A pH 3.2 (mg/L)	495	647	744	717	17
EA (%)	44	36	35	42	11
TP_{grape}	41.0	52.2	46.7	42.5	11
Mp (%)	51.7	50.4	36.3	32.6	23
S (g/L)	293 ± 4.2	296 ± 4.2	256 ± 3.2	240 ± 3.2	10
pH	3.7 ± 0.1	3.8 ± 0.1	3.7 ± 0.0	3.4 ± 0.0	5
TA	6.0	5.7	4.8	5.3	10
FAN (mg/L)	131 ± 6.4	297 ± 6.0	196 ± 2.9	91 ± 4.0	46

[a]Abbreviations: Anthocyanins extracted at pH 1 (A pH 1), anthocyanins extracted at pH 3.2 (A pH 3.2), anthocyanin extractability index (EA), total phenolics (TP), seed maturity index (Mp), glucose and fructose concentrations (S), titratable acidity (TA), free α-amino nitrogen content (FAN), coefficient of variation (CV %).

Table 2.

Chemical composition (mean ± SD) of Sangiovese experimental wines analysed after alcoholic fermentation.

Experimental wines	2011	2012	2013	2014	CV%
Parameters [a]					
Ethanol (%)	16.3 ± 0.1 a	14.8 ± 0.1 b	14.5 ± 0.2 b	13.6 ± 0.1 c	7
Glycerol (g/L)	10.5 ± 0.1 ab	10.8 ± 0.0 a	9.4 ± 0.1 ab	9.1 ± 0.5 b	8
Free A (mg/L)	171 ± 3.8 b	179 ± 4.7 b	200 ± 11.0 b	283 ± 14 a	23
Pigmented polymers (mg/L)	11.5 ± 1.2 a	16.4 ± 0.6 a	13.1 ± 0.7 a	13.4 ± 1.5 a	14
Flavonols (mg/L)	69.7 ± 4.3 ab	58.9 ± 1.8 b	75.1 ± 1.1 a	78.0 ± 3.8 a	11
Flavan-3-ols (mg/L)	122 ± 4.3 b	132 ± 4.6 b	184 ± 8.4 a	112 ± 4.5 b	23
CI ($_{420,520,620}$)	10.9 ± 0.4 b	12.2 ± 0.1 ab	10.3 ± 0.3 b	14.0 ± 0.9 a	14
TP_{wine}	68.0 ± 0.5 a	70.3 ± 0.2 a	54.7 ± 0.9 b	57.9 ± 1.7 b	11
pH	3.7 ± 0.0 a	3.7 ± 0.0 a	3.6 ± 0.1 a	3.5 ± 0.0 b	3
TA	6.8 ± 0.1 b	6.1 ± 0.0 c	8.7 ± 0.1 a	8.6 ± 0.1 a	15

[a]Abbreviations: anthocyanins (A), color intensity (CI), total phenolics (TP), titratable acidity (TA), coefficient of variation (CV %).

[b] Means in the same row followed by different letters are significantly different (Tukey test at $p<0.01$).

Table 3. Anthocyanin profile (mean ± SD) of Sangiovese experimental wines analysed after alcoholic fermentation.

Experimental wines	2011	2012	2013	2014	CV %
Anthocyanins [a] (%)					
Dp	9,7 ± 0.1 c	7,5 ± 0.0 d	10,8 ± 0.2 b	11,8 ± 0.2 a	16
Cy	3,8 ± 0.0 c	4,3 ± 0.0 c	7,1 ± 0.1 b	8,8 ± 0.7 a	34
Pt	18,3 ± 0.1 a	14,3 ± 0.0 c	17,8 ± 0.1 a	16,9 ± 0.3 b	9
Pn	10,6 ± 0.1 b	11,3 ± 0.1 b	13,5 ± 0.2 a	14,3 ± 0.7 a	13
Mv	56,0 ± 0.3 b	61,3 ± 0.1 a	49,6 ± 0.5c	46,4 ± 0.9 d	11
Vitisin A	0,4 ±0.0 a	0,3 ± 0.0 a	0,3 ± 0.0 a	0,4 ± 0.1 a	16
Acyl. A	1,3 ± 0.0 a	1,0 ± 0.0 b	0,9 ± 0.0 b	1,4 ± 0.1 a	20
A (ratio)	5.8 ± 0.0 a	5.3 ± 0.0 a	3.8 ± 0.1 b	3,3 ± 0.2 b	25

[a]Abbreviations: Delphinidin-3-glucoside (Dp), cyanidin-3-glucoside (Cy), petunidin-3-glucoside (Pt), peonidin-3-glucoside (Pn), malvidin-3-glucoside (Mv), acylated anthocyanins (Acyl. A), sum of delphinidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside percentages over sum of cyanidin-3-glucoside, and peonidin-3-glucoside percentages (A ratio), coefficient of variation (CV %).
[b] Means in the same row followed by different letters are significantly different (Tukey test at $p<0.01$).

Table 4. Flavonol profile (mean ± SD) of Sangiovese experimental wines analysed after alcoholic fermentation.

Experimental wines	2011	2012	2013	2014	CV %
Flavonols [a] (%)					
ΣM	13,9 ± 0.6 b	22,6 ± 2.5 a	18,2 ± 1.3 ab	21,8 ± 0.4 a	18
ΣQ	80,8 ± 0.6 a	73,6 ± 2.7 bc	79,0 ± 1.2 ab	73,3 ± 0.5 c	5
ΣK	5,3 ± 0.1 a	3,8 ± 0.3 ab	2,8 ± 0.3 b	4,9 ± 0.7 a	28
F (ratio)	0.17 ± 0.01 b	0.31 ± 0.04 a	0.23 ± 0.02 ab	0,30 ± 0.01 a	22
M-glycosides	11,4 ± 0.6 a	15,4 ± 2.9 a	14,6 ± 0.8 a	14,5 ± 0.5 a	13
Q-glycosides	68,5 ± 3.2 a	37,0 ± 0.3 b	58,4 ± 2.4 a	43,7 ± 2.9 b	24
K-glycosides	4,7 ± 0.2 a	1,5 ± 0.4 bc	1,2 ± 0.4 c	3,4 ± 0.6 ab	59
M	2,5 ± 0.1 b	7,3 ± 0.5 a	3,6 ±1.0 b	7,3 ± 0.4 a	43
Q	12,3 ± 3.8 c	36,6 ± 2.4 a	20,7 ± 1.7 bc	29,7 ± 2.9 ab	37
K	0,6 ± 0.3 c	2,3 ± 0.1 a	1,6 ± 0.2ab	1,5 ± 0.1 b	40

[a]Abbreviations: myricetin (M), quercetin (Q), kaempferol (K); sum of the glycosilated derivatives (-glycosides); sum of glycoside and aglycone forms (Σ) sum of percentages of glycosilated and aglycone forms of myricetin over sum of percentages of glycosilated and aglycone forms of quercetin (F ratio), coefficient of variation (CV %).
[b] Means in the same row followed by different letters are significantly different (Tukey test at $p<0.01$).

Table 5.

Climate variables of 2011, 2012, 2013 and 2014.

	From blooming to veraison				From veraison to harvest			
Years[a]	**2011**	**2012**	**2013**	**2014**	**2011**	**2012**	**2013**	**2014**
DOY	135-205	140-198	145-209	142-208	206-269	199-261	210-275	209-260
Climate variables								
days T°C min < 10	4	3	10	1	2	0	0	0
days 22 ≥ T°C avg ≥ 16	38	20	27	11	17	19	32	31
T°C max ≥ 30	24	36	24	13	60	57	30	7
days T°C max > 35	2	3	-	-	13	16	5	-
DTR (°C, mean±SD)	13.1 ± 2.3 ab	14.0 ± 3.0 b	13.1 ± 2.5 ab	12.4 ± 2.5 a	15.5 ± 2.6 c	14.6 ± 3.9 c	13.3 ± 2.4 b	11.8 ± 2.2a
GDD (base 18°C)	258	267	223	190	384	381	272	167
Rainfall (mm)	190.8	36.4	211	156	74	63.8	49	121

[a]Abbreviations: day of year (DOY), daily temperature range (DTR, difference between maximum and minimum), cumulative growing degree-days (GDD, °C).

2.3. Influence of vine vigor on the composition of flavonoids in Sangiovese grapes and wines

Abstract

The relationships between variation in vine vigor and Sangiovese grapes and wines compositions were investigated. In particular, the study was focused on anthocyanin, flavonol and flavan-3-ol monomer concentrations and profiles of Sangiovese grapes and wines as affected by vine vigor.

The study was carried out in a commercial vineyard located in the production area of Brunello di Montalcino (SI, Italy) during the vintage 2013. Spatial variations of vine vigor in the vineyard studied (northeast-southwest oriented) was defined by the measurements of Normalized Difference Vegetation Index (NDVI) which is reported as a suitable tool for the discrimination of grapevine vigor. Two different vigor zones were then identified. Microclimatic analysis were carried out from DOY 201 to DOY 275 (commercial maturity), monitoring daily temperatures in the fruiting zone of vines and hourly temperatures of clusters located in north and south side of the canopies in high and low vine vigor zones.

Higher temperature were reached in the fruiting zone of low vigor vines and clusters heating dynamics resulted faster in the first part of the day respect to those recorded for high vigor clusters, as a consequence of a higher exposition to sunlight of low vigor grapes.

Vine vigor affects flavonoids monomer composition of clusters. In particular, higher prevalence of trisubstituted and acylated anthocyanins, higher contents of total flavonols and lower contents of flavan-3-ol monomers were found in low vigor clusters. Moreover, the exposition of clusters in the south side of the canopy influence negatively the relative abundance of Dp-3-*O*-and Pt-3-*O*-G, and positively total flavonols contents.

Finally, low vigor experimental wines showed higher contents of polymeric and non polymeric anthocyanins, total flavonols and quercetin glycosides and lower flavan-3-ol monomer contents. Moreover, reduction of vine vigor seemed to affect anthocyanins profiles of grapes and wines, increasing the values of trisubstituted to disubstituted anthocyanins ratio.

Introduction

Anthocyanins, flavonols and flavan-3-ols are three classes of flavonoids deeply studied for their implications in wine quality and their health enhancing properties (Mattivi et al., 2006). Anthocyanins are the pigments of red grapes and consequently of red wines in which their concentration and composition influence both hue and color stability (He et al., 2010). In most varieties of *Vitis vinifera*, with exception for the so called "teinturiers", they accumulate in berry skins in which they play several roles including attraction of predators for seed dispersal, protection against solar exposure and UV radiation, defense against different pathogens, free radical scavenging and antioxidant activities (He et al., 2010). Moreover, therapeutic properties of anthocyanins are reported (Kong et al., 2003). In berries, anthocyanins appear at veraison where, initially, only the glucosides of disubstituted anthocyanins as cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside are synthesized, followed by the trisubstituted anthocyanins based on delphinidin, petunidin, and malvidin. Their accumulation continues throughout the ripening process, reaching a maximum concentration at full maturity and then decreases (Downey et al., 2006).

Flavonols are a class of substances widely distributed in dietary plants and also found in wines that have been receiving much attention for their highly bioactive properties (Manach et al., 2004). In particular, the antioxidant and anti-inflammatory properties of quercetin and its glycosides, the most abundant flavonols in grapes, have been extensively reviewed due to their association in the prevention and therapy of cardiovascular diseases and cancer (Russo et al., 2012). Moreover, flavonols are involved in determining wine color acting as anthocyanins copigments (Downey et al., 2006). As anthocyanins and flavan-3-ols, flavonols are products of the phenylpropanoid pathway and accumulate in berry skins as UV protectants and free radical scavengers (Downey et al., 2006). Flavonols are synthesized in two distinct periods, the first around flowering and the second in post-veraison and continuing throughout ripening (Downey et al., 2006).

Flavan-3-ol monomers are the fundamental constituent of the most abundant class of flavonoids found in grapes, condensed tannins (Keller, 2010). This polymer are also referred as procyanidins (Ribéreau-Gayon et al., 2000). The basic units of procyanidins accumulate in grapes skins and seeds are (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin-3-O-gallate (ECG), and (-)-epigallocatechin (EGC), the latter being present only in skins (Cheynier et al.,

1998). Seed procyanidins are characterized by lower degree of polymerization and, generally, by a higher contents of flavan-3-ol monomers when compared to skins. (Downey et al., 2003). They accumulate in grapes immediately after fruit-set with maximum levels observed around veraison (Downey et al., 2003). Flavan-3-ol monomers are reported to concur to astringency and principally to bitterness of wines (Gawel, 1998). As in the case of anthocyanins and flavonols, several Author focused their studies on health enhancing properties (Scott et al., 1993; Bors et al., 1999).

Several factors are reputed to influence flavonoids accumulation in grape berries. Such factors include, among others, light, temperature, water, soil type, nutritional status (Downey et al., 2006). Specific studies were found in literature in which the essential components (as the above cited) have been individually manipulated in order to understand the effect on grape composition (Price et al., 1995; Chorti et al., 2010; Cohen et al. 2012). However, given the complex nature of plant growth, it can be difficult to evaluate the effect of a single factors in a vineyard where multiple influences exist (Cortell et al., 2005). Indeed, the existing variability in vine growth in a given vineyard is traduced in different microclimatic conditions affecting the fruiting zone and, as a consequence, grape composition. The effect of vine vigor, intended as canopy structure and density, on chemical characteristics of grapes have been extensively studied. In vineyard, several factors have been reputed to induce spatial variation in vine vigor as physical and chemical characteristics of the soil, nutrient availability, water, pests and diseases, in turn influencing crop yield, clusters morphology and composition (Jackson and Lombard, 1993; Bramley and Hamilton, 2004).

Remote sensing analysis is considered an useful monitoring tool because of its ability to provide a synoptic view of grapevine shape, size and vigor in a given vineyard by using vegetation indices (Fiorillo et al., 2012). Moreover, a relation between vegetation indices like Normalized Difference Vegetation Index (NDVI) and grape phenols and color was proposed for Cabernet (Lamb et al., 2004). On the basis of such considerations, the authors individuate in the output of their monitoring a powerful tool for viticulturist in the management of canopy architecture and, as a consequence, grape quality.

The aim of this work was to assess the effect of vine vigor, defined on the basis of NDVI, on Sangiovese grapes composition. The vigor zones were also characterized in terms of microclimatic conditions of fruiting zone. Moreover, temperatures of clusters positioned in different zone of the canopy were

monitored as it is known that low vigor zones tend to be characterized by a higher bunch exposure. Such information were used to relate differences found in anthocyanins, flavonols and flavan-3-ol monomers composition of Sangiovese grapes. Morphological and technological parameter of grape maturity are reported in order to evaluate the influence of vine vigor on such characteristics. Moreover, grapes from zones identified as high and low vigor were vinified in standardized laboratory conditions allowing to evaluate the differences in grape compositions on flavonoid monomer contents and profiles in experimental wines.

Materials and methods

Site description and vigor zones determination. The study was performed in a 0.38 ha vineyard located in the Brunello di Montalcino domain (SI, Italy) during the vintage 2013. The vineyard was planted in 2002 to Sangiovese in a Northeast-Southwest direction with vine and row spacing of 1.3 and 2.3 respectively. Vines were trained to spur cordon, not irrigated and located at 320 m above sea level.

An Unmanned Aerial Vehicle (UAV) platform was used to assess the heterogeneity of the vineyard in terms of vine vigor and two parcels of high and low vigor were identified. In the vintages 2013, three flights were carried in the period between June, and October to monitor fruit set, veraison and ripening of grapes. The UAV platform used in this work was provided by Ibimet-CNR and its technological characteristics, the camera used and image processing for the determination of Normalized Difference Vegetation Index (NDVI) in the two zones were described by Primicerio et al. (2012).

Microclimatic conditions. Microclimatic parameters were monitored in each parcel with hourly frequency. In particular, temperature of grape clusters was monitored by using a microprobe located inside the bunches. A total of 4 sample clusters per parcel were studied. Sensor provided clusters were characterized by different location within the canopy: 2 in the north and 2 in south side of the canopy. Moreover, microclimatic parameters of fruiting zone were monitored by two control units, one for each vigor zones, located on vine cordon which provided the measurement of temperature and humidity. Acquisition of microclimatic parameters of grape clusters and fruiting zones in the two high and low vigor zones were provided by a data logger WatchDog 1400 Microstation (Spectrum Technologies, 3600 Thayer Court, Aurora, IL 60504, United States).

As an example, the Normalized difference vegetation index (NDVI) of the vineyard studied is reported in figure 1. Moreover, the position of microprobes and control units for the determinations of microclimatic parameters related to sensor provided clusters and fruiting zones are also showed. The colors reported in the map are related to NDVI values: red and deep green indicate lower and higher vigor zones, respectively.

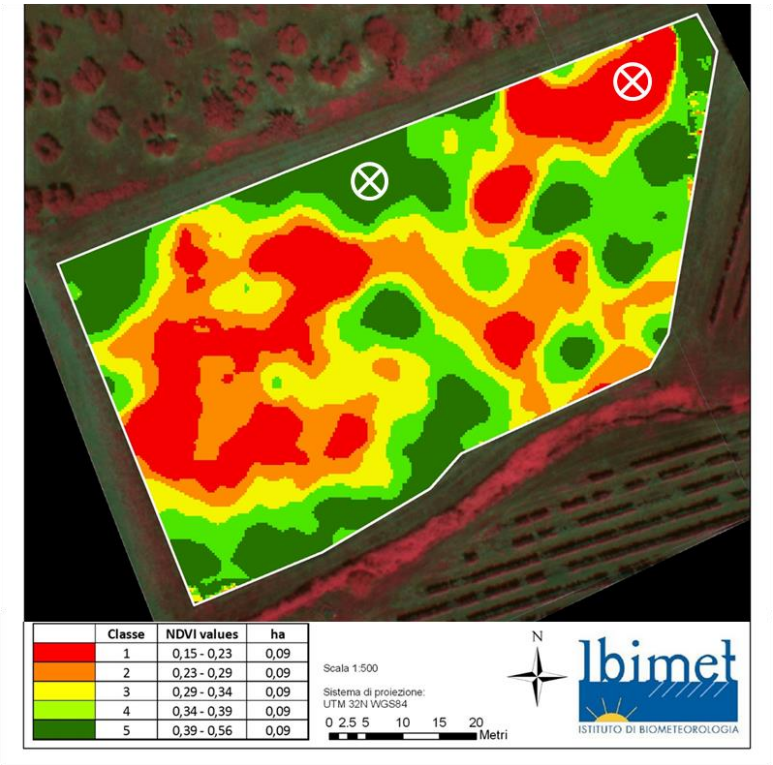

Figure 1: Normalized difference vegetation index (NDVI) of the vineyard studied, vintage 2013. Symbols refer to the position of sensor provided clusters and control units for the determination of microclimatic parameters in high and low vigor zones.

Grape samples. For the characterization of grapes composition and morphology, the 4 clusters from each zone were used. In particular, sensor provided clusters were: 2 in the north side of the canopy from high vigor (HV North), 2 in the south side of the canopy from high vigor (HV South), 2 in the north side of the canopy from low vigor (LV North), 2 in the south side of the canopy from low vigor (LV South).

For the experimental vinifications, a total of 30 clusters from 30 distinct vines in each zone were sampled. Grape samples were transferred to laboratory at low temperature and stored at -20°C for a period of time not exceeding 1 months.

Experimental vinifications. The laboratory vinifications were carried out using 450g of grapes each, in triplicates. Technological condition of alcoholic fermentation/maceration are reported in Chapter 3.1 (3.1.2 Materials and methods).

Yeast Inoculum. The alcoholic fermentation was induced by inoculating *Saccharomyces cerevisiae* Sc1 strain. Yeast inoculum preparation is reported in Chapter 3.1 (3.1.2 Materials and methods).

Chemical and analytical determination. After being weighed, sensor provided clusters were characterized in terms of anthocyanins and flavonols contents. Berries from each clusters were divided on the basis of their dimension assuming a spherical form of single berry. In particular, 6 categories were constituted on the basis of berry diameter: <6 mm, 7-9 mm, 10-12 mm, 13-15 mm, 16-18 mm, >19 mm. Berries from each categories were weighted. For the determination of anthocyanins and flavonols contents, 40 berries per cluster were randomly picked respecting the relative proportions of berry dimensions in the cluster. Then, the 40 berries were handily peeled and skins, seeds and pulp were divided. Skins and seeds were dried with paper and weighed. The extraction of berry skins were carried according to Rodríguez Montealegre et al. (2006). Skins were extracted in 150 mL of solvent constituted by methanol/water/formic acid (500:485:15). The maceration length was carried out overnight at 20°C. Anthocyanins determination was performed by direct injection of the extract in HPLC. For flavonols determination, the extract underwent to a second extraction as described in Chapter 3.1 (3.1.2 Materials and methods) before the HPLC analysis.
Other Chemical determination on grapes and wines are reported in Chapter 3.1 (3.1.2 Materials and methods).

Statistical analysis. All statistical calculations were performed by using Statistica software (version 7, StatSoft, Tulsa, OK, USA) and GraphPad Prism 5 (GraphPad Software, Inc., CA,USA).

Results and discussion

Canopy microclimatic conditions in high and low vigor zones. Identification of high and low vigor zones in the vineyard studied was performed by evaluating the Normalized difference vegetation index (NDVI) in the vintage 2013 (tab. 1).

Table 1: Normalized difference vegetation index (NDVI) of high and low vigor zone in the vineyard studied, vintage 2013.

	NDVI		
	June	August	September
HV	0,649	0,656	0,623
LV	0,636	0,603	0,563

HV = high vigor; LV = low vigor

During the period from June to September, three data were collected from each vigor zone. By evaluating the results, it was possible to assess that despite a substantial uniformity of the two zone in June, the results obtained in August and September showed an increasing discrepancy in NDVI of high vigor compared to low vigor zones.

As vigor is one of the factor influencing the grape composition (Downey et al., 2006), the first part of the work was aimed to describe the microclimatic conditions of the fruiting zone in high and low vigor vines. In figure 2, the daily minimum, average and maximum temperatures recorded by the two control units located on cordon of vines of the two vigor zones during the period from 201 to 268 DOY (20th of July - 25th of September) are reported. The beginning of veraison was assessed at 210 DOY (29th of July, vertical dotted line in the graph), while harvesting date was established at 275 DOY (2nd of October)

Differences in temperatures between the two zones were assessed in the first part of the period monitored. In particular, maximum temperatures and, to a lesser extent, average temperatures resulted higher in low vigor zone in the period from DOY 201 to 219, as a consequence of a higher light exposure and temperature in the fruiting zone (Jackson and Lombard, 1993)

For better understand the impact of air temperatures and light exposure on clusters from different vigor zone and position within the canopy, figure 3 reported the hourly temperatures of sensor provided clusters in the period from DOY 201 to 219. As previously described, low vigor clusters temperatures resulted higher than high vigor clusters even comparing south exposed clusters of vine characterized by higher vigor with north exposed clusters from low vigor vines. By observing the slope of temperature increase during the first

part of the day, it is possible to affirm that clusters from low vigor zone were characterized by faster heating dynamics, reaching temperatures up to almost 8°C higher than clusters from high vigor zone. In particular, clusters temperature in low vigor zone frequently reached temperatures above 35°C in the second part of the day. No differences were assessed regarding the cooling dynamics of clusters during the period from dusk to dawn (8.00 pm - 06.00 am).

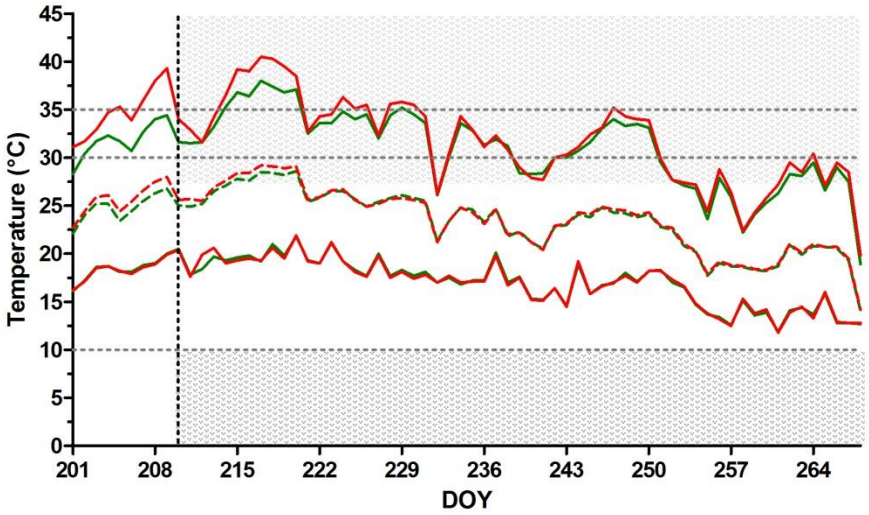

Figure 2: average (dotted lines), minimum and maximum (continuous lines) temperatures time course during the period from 201 to 268 DOY recorded by the control units located on vine cordons. Vertical dotted line represent the beginning of veraison (DOY 210). Dotted areas in graph represent crucial range of temperature reported to influence the anthocyanins biosynthesis: optimum range (from 16 to 22°C, Nicholas et al. 2011), inhibition of biosynthesis (higher than 30°C, Downey et al. 2006, Kliewer and Torres 1972), degradation phenomenon (higher than 35°C, He et al. 2010).

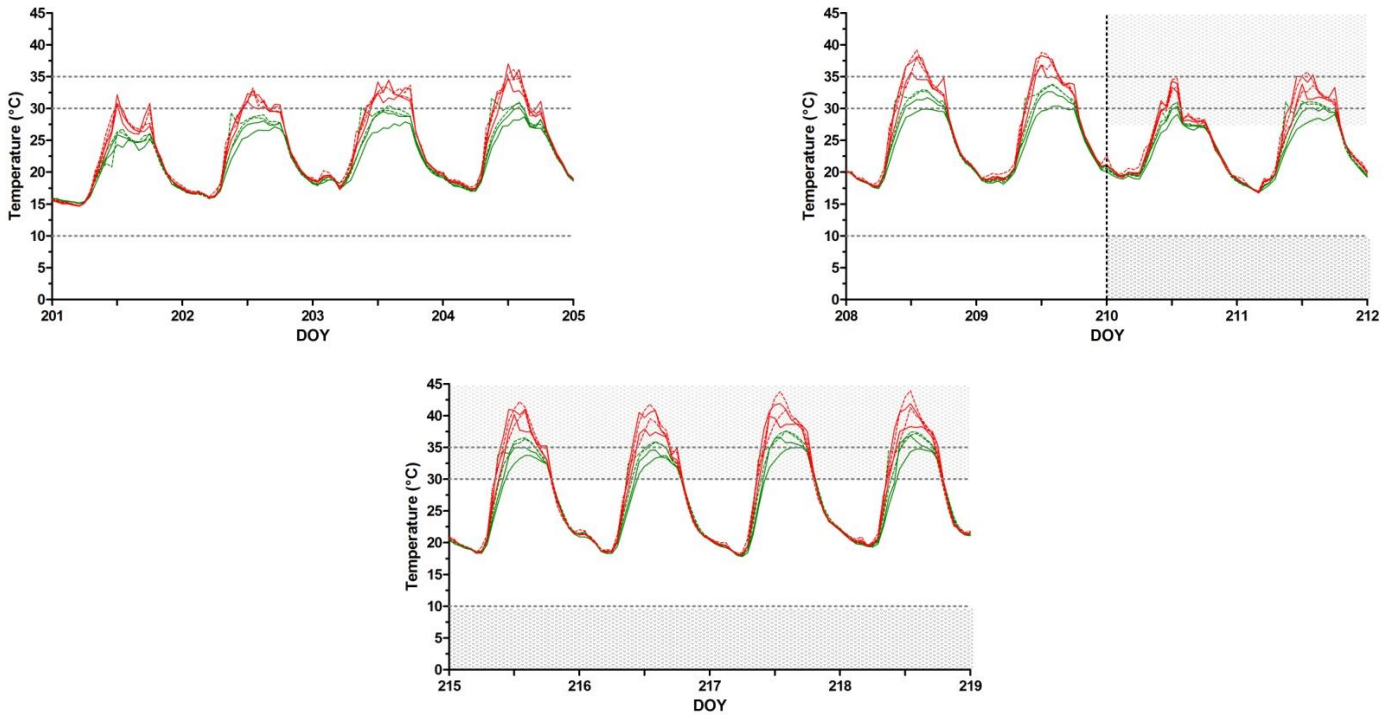

Figure 3: Hourly temperatures recorded during the period from DOY 201 to 219 in north exposed (continuous lines) and south exposed (dotted lines) clusters of vines from low (red lines) and high vigor (green) zones. Vertical dotted line represent the veraison (DOY 210). Dotted areas in graph represent crucial range of temperature reported to influence the anthocyanins accumulation: inhibition of biosynthesis (lower than 10°C and higher than 30°C, Downey et al. 2006, Kliewer and Torres 1972), degradation phenomenon (higher than 35°C, He et al. 2010).

Sensor provided clusters characterization. At harvest, sensor provided clusters were transferred into laboratory in order to evaluate their morphological and chemical features. Data underwent to two-way ANOVA in order to evaluate the influence of vigor and position of clusters within vine canopy (tab. 2).

Table 2: Morphological and chemical features of sensor provided clusters and related statistical analysis (two-way ANOVA). Results are reported as mean ± coefficient of variation except for pH values were the results are reported as mean ± standard deviation[†].

	Sensor provided clusters				Source of variation		
	HV North	HV South	LV North	LV South	V	P	V x P
Cluster weight (g)	366±0.21	400±0.05	314±0.14	130±0.19	**	ns	*
Berry weight (g)	2,16±0.11	1,94±0.17	1,31±0.10	1,27±0.16	*	ns	ns
Skin weight (g)	0,41±0.07	0,40±0.15	0,23±0.12	0,25±0.30	*	ns	ns
Skin:Berry ratio (g/g, %)	19,2±0.04	20,5±0.02	17,8±0.02	19,3±0.14	ns	ns	ns
n seeds per berry	2,00±0.02	1,61±0.39	2,21±0.24	1,50±0.12	ns	ns	ns
Seed weight (mg)	41.4±0.03	39.9±0.11	34.2±0.12	39.0±0.07	ns	ns	ns
Tot S (g/L)	238±0.00	245±0.00	194±0.07	229±0.08	*	ns	ns
pH[†]	3,85±0.07	3,90±0.00	3,95±0.07	4,10±0.00	*	*	ns
Malic acid (g/L)	3,24±0.13	2,47±0.11	2,38±0.04	2,25±0.01	*	ns	ns
α-AN (mgN/L)	362±0.11	348±0.03	439±0.20	652±0.22	*	ns	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant. V = Vigor; P = Position of clusters on vine; V x P = Interaction V x P; HV = high vigor; LV = low vigor; North = clusters from north side of the canopy; South = clusters from south side of the canopy. Tot S = Total Sugars; α-AN = α-amino acid nitrogen content.

Clusters from high vigor zone were characterized by a higher weight, heavier berries and skins when compared with sample clusters from low vigor zone. No significant differences were found as concern skin-berry weight ratio and number of seeds per berry.

Regarding chemical features, α-amino acid nitrogen content and pH resulted lower in the clusters from high vigor zone. Moreover, the position of clusters seemed to affect pH values. On the other hand, total sugar and malic acid contents were lower in sample clusters from high vigor zone.

Concerning the α-amino acid nitrogen and total sugar contents, the data reported in table 1 seems to be in contrast with the findings of other Author in the literature. Indeed, several Authors found that nitrogen content in must is related to nitrogen content in the soil (Conradie and Saayman, 1989) and higher values of nitrogen availability in the soil lead to a higher vigor of vines and to a lower accumulation of sugar in grape berries (Delas and Pouget, 1984). On the other hand, several studies on vine vigor on the accumulation of sugar in grape berries highlight that clusters from low vigor zones were characterized by a higher amount of total soluble solids when compared with high vigor zones clusters (Cortell et al., 2007).

Nitrogen contents of soil seems to be one of the most important variables affecting vine vigor (Downey et al., 2006). However, knowing a positive relation between soil and grapes nitrogen contents (Conradie and Saayman, 1989), it seemed that clusters from low vigor vines in this study were characterized by a higher content of α-aminoacidic nitrogen as a consequence of concentration effect, hence the differences in vine vigor in the vineyard studied may be determined by factors other than nitrogen content in the soil. Indeed, it is well known that climatic conditions, physical and chemical characteristics of the soil, pests, diseases and water status may affect vine vigor (Jackson and Lombard, 1993; Bramley and Hamilton, 2004).

As described above, the accumulation of sugars in clusters could be affected by vine vigor. In this work, low vigor vines in the vineyard studied were characterized by lower NDVI values that means a lower density of vine canopy respect to high vigor vines. These evaluations were also confirmed by several surveys on the vineyard. Such lower density of the canopy may have influenced the ratio leaf area/weight of fruit, a characteristic known to affect the sugar accumulation in berries (Kliewer and Dokoozlian, 2005).

Moreover, carbohydrate reserves are known to be responsible of sugar accumulation in berries at veraison (Fregoni, 2005) and their storage in perennial and annual organs can be reduced by water stress (Smith and Holzapfel, 2009). In the previous vintage respect to that reported in this work, the same pattern of distribution of vigor zones was assessed in the vineyard studied utilizing the same monitoring procedures as reported in this work for the description of vigor zones in 2013. Indeed, the 2012 was considered a drought vintage, thus, similarly to 2013, low vigor vines were characterized by a lower canopy density which probably led to a lower accumulation of carbohydrate reserves. However, carbohydrates content in cane, trunk and

roots would have to be monitored in order to validate this hypothesis on the cause of sugar contents in grapes from different vigor zones in the vineyard.
Despite a noticeable variability recorded in contents and partitioning of various anthocyanins, vigor and, to a lesser extent, position of clusters on the vine showed an effect in determining differences between clusters belonging to different vigor vines (tab. 3). Although not significant differences were found in anthocyanins contents, clusters from low vigor vines showed a trend towards a higher concentrations in total anthocyanins.

Table 3: Contents and partitioning of anthocyanins in sample clusters and related statistical analysis (two-way ANOVA). Results are reported as mean ± coefficient of variation.

	Sensor provided clusters				Source of variation		
	HV North	HV South	LV North	LV South	V	P	V x P
mg/Kg fresh berries							
Tot A	796.4±0.27	816.8±0.38	1005.7±0.12	957.9±0.04	ns	ns	ns
A prof %							
Dp-3-*O*-G	8.8±0.05	11.9±0.13	8.1±0.14	10.7±0.12	ns	*	ns
Cy-3-*O*-G	21.3±0.11	23.0±0.09	11.2±0.22	13.2±0.03	**	ns	ns
Pt-3-*O*-G	10.9±0.01	13.5±0.11	11.5±0.11	13.9±0.06	ns	*	ns
Pn-3-*O*-G	27.9±0.03	19.7±0.11	17.3±0.02	17.0±0.19	**	*	*
Mv-3-*O*-G	30.0±0.07	30.5±0.05	50.1±0.09	43.7±0.02	***	ns	ns
Acylated A	1.1±0.11	1.2±0.21	1.9±0.02	1.5±0.02	**	ns	ns
A ratio	1.0±0.06	1.3±0.18	2.5±0.10	2.3±0.13	**	ns	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant. V = Vigor; P = Position of clusters on vine; V x P = Interaction V x P; HV = high vigor; LV = low vigor; North = clusters from north side of the canopy; South = clusters from south side of the canopy.
Tot S = Total Sugars; α-AN = α-aminoacidic nitrogen content; Tot A = ∑ glucosides and acylated anthocyanins;
A profile (%) = anthocyanins profile Dp-3-*O*-G = delphinidin-3-*O*-glucoside, Cy-3-*O*-G = cyanidin-3-*O*-glucoside, Pt-3-*O*-G = petunidin-3-*O*-glucoside , Pn-3-*O*-G = peonidin-3-*O*-glucoside, Mv-3-*O*-G = malvidin-3-*O*-glucoside, Acylated A = sum of acetilated and coumarilated anthocyanins to non-polymeric anthocyanins contents of grapes; A ratio = trisubstituted (Dp-3-O-G, Pt-3-*O*-G and Mv-3-*O*-G) to disobstituted (Cy-3-*O*-G and Pn-3-*O*-G) ratio.

As regard anthocyanins partitioning (tab. 3), clusters from low vigor vines were characterized by a lower percentage of disubstituted anthocyanins (Cy-3-*O*-G and Pn-3-*O*-G) and higher relative abundance of Mv-3-*O*-G. This findings may be related to higher temperature monitored in low vigor clusters especially at the beginning of veraison when Cy-3-*O*-G and Pn-3-*O*-G are the prevalent anthocyanins synthesized in grape berries (Downey et al., 2006). In this connection, a higher sensitivity to temperature of the enzyme catalyzing the disubstituted anthocyanins was suggested by Guidoni et al. (2008). Moreover, the compression of the diurnal temperature range was found to favor the partitioning of anthocyanins and flavonols toward dihydroxylation, so that Cy and Pn derivatives were accumulated to a higher extent in grape berries (Cohen et al. 2012). The temperature time course reported in figure 1, showed that, at the beginning of veraison, sample clusters from low vigor zone reached higher temperature (30-35°C), so that inhibition and degradation phenomena of existing anthocyanins, as reported for Merlot grapes (Spayd et al., 2002), may have occurred, in particular involving Cy-3-*O*-G and Pn-3-O-G. Moreover, low vigor zone were characterized by a higher range between minimum and maximum temperatures. As a consequence, the trisubstituted to disubstituted anthocyanins ratio was found higher in sample grapes from low vigor zone.

The position of clusters within the vine canopy resulted significative in determining difference in Dp-3-*O*-G and Pt-3-*O*-G proportion. In particular, south exposed clusters were characterized by a higher percentage of Dp-3-*O*-G and Pt-3-*O*-G, possibly due to higher light exposure of sample clusters since it is known a light responsive behaviour of these anthocyanins (Downey et al., 2004; Cortell et al., 2007).

Furthermore, clusters from low vigor vines showed a higher proportion of acylated anthocyanins. Indeed it is well known that higher temperatures can influence gene expression and enzyme activity involved for the acylation reaction of anthocyanins (Chorti et al., 2010) although their low incidence in the anthocyanins profiles of Sangiovese grapes and wines (Mangani et al., 2011).

Total flavonols resulted noticeably influenced by both vine vigor and position of grape cluster within the vine canopy (tab. 4). In particular, clusters from low vigor zone resulted highly concentrated in flavonols. Moreover, north exposed clusters from low vigor vine showed a higher content of flavonols when compared with south exposed clusters from high vigor vine. Indeed, since flavonols biosynthesis is stimulated by UV-light (Spayd et al., 2002), the

accumulation of total flavonols seemed to be related to sun exposure: higher in clusters from low vigor zone and, when vine vigor is excluded, higher in south exposed clusters.

Table 4: Contents and partitioning of flavonols in sample clusters and related statistical analysis (two-way ANOVA). Results are reported as mean ± coefficient of variation.

	Sensor provided clusters				Source of variation		
	HV North	HV South	LV North	LV South	V	P	V x P
mg/Kg fresh berries							
Tot F	87,4±0.24	123,0±0.09	157,1±0.05	194,9±0.08	**	*	ns
F prof %							
Ms	15,8±0.21	15,9±0.16	19,0±0.34	18,7±0.03	ns	ns	ns
Qs	80,9±0.04	80,5±0.03	76,1±0.08	76,1±0.01	ns	ns	ns
K-3-*O*-G	3,4±0.01	3,7±0.00	4,8±0.12	5,2±0.10	**	ns	ns
F ratio	5,3±0.25	5,2±0.20	4,3±0.41	4,1±0.05	ns	ns	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant. V = Vigor; P = Position of clusters on vine; V x P = Interaction V x P; HV = high vigor; LV = low vigor; North = clusters from north side of the canopy; South = clusters from south side of the canopy.
F profile (%) = flavonols profile, Ms = sum of myricetin and its glycosides, Qs = sum of quercetin and its glycosides, K-3-*O*-G = kaempferol-3-*O*-glucoside to total flavonols; F ratio = Ms contents (mg/L) / Qs contents (mg/L).

Regarding flavonols profile of Sangiovese grapes (tab. 4), Qs represent the most abundant flavonols followed by Ms and K-3-*O*-G, in agreement with the literature for (Mattivi et al., 2006). No significant differences were found in myricetin glycosides and quercetin glycosides relative abundances among the clusters. Low vigor clusters were characterized by a higher proportion of K-3-*O*-G, although its contribute to total flavonols represent a minor percentage when compared with the other flavonols.
Finally, table 5 show the total contents of flavan-3-ol monomers and their relative abundances in grape seeds. As reported for anthocyanins and flavonols, vine vigor was the variable able to affect significantly the accumulation of flavan-3-ol monomers in grape seed. In particular, an increase in total contents of monomer was reached in grapes from high vigor zone. These results are in agreement with the findings of other Authors (Cortell et

al., 2005). Conversly to data reported by Cortell et al. (2005), grapes from low vigor zone were characterized by higher proportion of (+)-catechin when compared to high vigor clusters. As a consequence, the F3ols resulted higher in clusters from low vigor. Indeed, as it is known that the percentage of (+)-catechin decrease during the maturation process, grapes from low vigor were characterized by a lower degree of seed maturation. Although significant, the effect of position of grape cluster within the vine canopy on flavan-3-ol monomers partitioning should be considered negligible (tab. 5).

Table 5: Contents and partitioning of flavonols in sample clusters and related statistical analysis (two-way ANOVA). Results are reported as mean ± coefficient of variation.

	Sensor provided clusters				Source of variation		
	HV North	HV South	LV North	LV South	V	P	V x P
mg/g seed							
Tot F3ols	3.36±0.21	3.17±0.13	1.54±0.02	2.03±0.11	**	ns	ns
F3ols prof %							
C	27.7±0.11	28.2±0.05	35.4±0.09	36.4±0.01	**	*	ns
EC	68.7±0.03	67.5±0.03	58.6±0.00	58.5±0.00	***	*	*
ECG	3.6±0.32	4.3±0.14	6.0±0.55	5.1±0.07	ns	ns	ns
F3ol ratio	0,40±0.14	0,42±0.08	0,60±0.08	0,62±0.00	***	*	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant. V = Vigor; P = Position of clusters on vine; V x P = Interaction V x P; HV = high vigor; LV = low vigor; North = clusters from north side of the canopy; South = clusters from south side of the canopy.
F profile (%) = flavonols profile, C = (+)-catechin, EC = (-)-epicatechin, ECG = (-)-epicatechin-3-*O*-gallate; F3ols ratio = C contents (mg/L) / EC contents (mg/L).

Effect of vine vigor on experimental wines composition. From the two vigor zones identified, 30 grape clusters were harvested at commercial maturity and then vinified under laboratory conditions. After 14 days of alcoholic fermentation/maceration, the residual sugar concentration in all wines was under 4 g/L. Significant differences were found in ethanol and pH and total acidity. In particular, low vigor wines were characterized by 13.5 ± 0.13% (v/v) of ethanol and a pH value of 3.93 ± 0.04 and 7.95 ± 0.13 $g_{tartaric\ acid}$/L of total acidity while in high vigor wines ethanol reached 14.5 ± 0.17% (v/v) and pH values of 3.63 ± 0.04 and 8.72 ± 0.13 $g_{tartaric\ acid}$/L of total acidity (results were reported as mean ± standard deviation).

The anthocyanins contents of experimental wines resulted statistically different (tab. 6). In particular, both non-polymeric anthocyanins and pigmented polymers contents resulted higher in wines obtained by the fermentation of low vigor grapes. These results are probably a consequence of a higher exposition of clusters belonging to low vigor vine. Indeed, Price et al. (1995) reported an increasing in non-polymeric anthocyanins and pigmented polymers in wines from highly exposed clusters.

Table 6: Contents and partitioning of anthocyanins in experimental wines and related statistical analysis (Student's t-Test). Results are reported as mean ± coefficient of variation.

	Experimental wines		
	HV	LV	Student's t-Test
mg/L Anthocyanins in wines			
Non-polymeric anthocyanins	200,1 ± 0.06	234,8 ± 0.05	*
Pigmented polymers	13,1 ± 0.06	16,6 ± 0.05	*
A profile %			
Dp-3-*O*-G	10,8 ± 0.01	6,0 ± 0.00	***
Cy-3-*O*-G	7,1 ± 0.02	2,3 ± 0.01	***
Pt-3-*O*-G	17,8 ± 0.01	14,3 ± 0.00	***
Pn-3-*O*-G	13,5 ± 0.01	8,4 ± 0.01	***
Mv-3-*O*-G	49,6 ± 0.01	66,1 ± 0.00	***
Vitisins	0,3 ± 0.06	0,3 ± 0.00	ns
Acylated A	0,9 ± 0.06	2,6 ± 0.01	***
A ratio	3,8 ± 0.02	8,0 ± 0.01	***

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant.

HV = high vigor; LV = low vigor;

A profile (%) = anthocyanins profile, Dp-3-*O*-G = delphinidin-3-*O*-glucoside, Cy-3-*O*-G = cyanidin-3-*O*-glucoside, Pt-3-*O*-G = petunidin-3-*O*-glucoside , Pn-3-*O*-G = peonidin-3-*O*-glucoside, Mv-3-*O*-G = malvidin-3-*O*-glucoside, Acylated A = sum of acetilated and coumarilated anthocyanins to non-polymeric anthocyanins contents of wines; A ratio = trisubstituted (Dp-3-O-G, Pt-3-*O*-G and Mv-3-*O*-G) to disobstituted (Cy-3-*O*-G and Pn-3-*O*-G) ratio.

Regarding the anthocyanins profile (tab. 6), it is known that during the alcoholic fermentation the anthocyanins composition of native grapes undergo to modifications involving in particular an impoverishment in cyanin and an enrichment in malvin (Di Stefano et al., 1994). Indeed, Mv-3-*O*-G resulted the most abundant anthocyanin in the experimental wines and with a higher proportion in wines obtained from low vigor grapes. In general, glucosides and acylated anthocyanins presented the same pattern of distribution according to sample cluster composition. As a consequence, despite a general increase of A ratio, the values reported in low vigor wines resulted higher than wines obtained by high vigor grapes, reflecting native grapes composition. Vitisins showed the same abundance in both low and high vigor wines, due to the same yeast strain used for the fermentations as it is known that vitisins are strongly related to metabolic production of pyruvate and acetaldehyde (Morata et al., 2003).

Table 7: Contents and partitioning of flavonols and contents of quercetin glycosides and aglycone in experimental wines and related statistical analysis (Student's t-Test). Results are reported as mean ± coefficient of variation.

	Experimental wines		
	HV	LV	Student's t-Test
mg/L Flavonols in wines			
Tot F	75,1 ± 0.01	100,5 ± 0.05	**
F profile %			
Ms	18,2 ± 0.07	18,9 ± 0.03	ns
Qs	79,0 ± 0.01	76,8 ± 0.01	ns
Ks	2,8 ± 0.10	4,2 ± 0.04	**
F ratio	0,2 ± 0.09	0,2 ± 0.04	ns
mg/L Qs in wines			
Q-3-*O*-Gly	43.8 ± 0.04	62.2 ± 0.05	**
Free Q	15.5 ± 0.10	15.0 ± 0.02	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant.

HV = high vigor; LV = low vigor;

F profile (%) = flavonols profile, Ms = sum of myricetin and its glycosides, Qs = sum of quercetin and its glycosides, K-3-*O*-G = kaempferol-3-*O*-glucoside to total flavonols; F ratio = Ms contents (mg/L) / Qs contents (mg/L); Qs in wines = quercetin and its glycosides contents in wines, Q-3-*O*-Gly = ∑ quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoronide and quercetin-3-*O*-glucoside; Free Q = quercetin aglycone.

The experimental wines were also characterized in terms of flavonols composition. Moreover, table 7 shows the contents of quercetin and its glycosides as they are the most abundant flavonols in Sangiovese (Mattivi et al., 2006) and because of their extensively studied healthy properties (Russo et al., 2012). Confirming the results of sample clusters, flavonols contents resulted significantly higher in the experimental wines obtained by low vigor clusters. Once again, flavonols profile of sample clusters harvested in different vigor zones were transferred into the experimental wines maintaining the pattern of flavonols distribution between previously reported for grapes.

Total contents of quercetin glycosides were higher in the experimental wines obtained by low vigor grapes. These results are in agreement with a previous study on Pinot Noir in which quercetin glycosides concentrations found in wines were related to cluster exposure, with wines from sun exposed clusters having the highest concentrations when compared with those from shaded clusters (Price et al., 1995).

Finally, the experimental wines obtained by low vigor clusters were characterized by a lower contents of flavan-3-ol monomers (tab. 8). This is consistent with the data reported for sensor provided clusters and reported by other Authors in literature (Cortell et al., 2005)

Table 8: Contents and partitioning of flavan-3-ol monomers in experimental wines and related statistical analysis (Student's t-Test). Results are reported as mean ± coefficient of variation.

	Experimental wines		
	HV	LV	Student's t-test
mg/L Flavan-3-ols monomer in wines			
Tot F3ols	183,8 ± 0.05	90,5 ± 0.02	***
F3ol profile %			
C	39,3 ± 0.09	41,9 ± 0.05	ns
EC	59,8 ± 0.06	50,8 ± 0.05	ns
ECG	0,8 ± 0.37	7,3 ± 0.06	***
F3ol ratio	0,7 ± 0.16	0,8 ± 0.10	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant. V = Vigor; P = Position of clusters on vine; V x P = Interaction V x P; HV = high vigor; LV = low vigor; North = clusters from north side of the canopy; South = clusters from south side of the canopy.

F3ol profile (%) = flavan-3-ol monomer profile, C = (+)-catechin, EC = (-)-epicatechin, ECG = (-)-epicatechin-3-*O*-gallate; F3ols ratio = C contents (mg/L) / EC contents (mg/L).

Regarding Flavan-3-ol monomer partitioning, significant differences not reported for sensor provided clusters emerged on (-)-epicatechin-3-*O*-gallate relative abundance. Despite not significant, (-)-epicatechin relative abundance resulted tendencially higher in high vigor wines.

Conclusion

In this work, the effect of vine vigor on chemical characteristics of Sangiovese grapes and wines was studied. In particular, anthocyanins, flavonols and flavan-3-ol monomers were taken into consideration because of their implications in wine quality and their health enhancing properties.

The first part of the work was focused on the definition of microclimatic condition of the fruiting zone and temperature of clusters located in two different vigor zones of the vineyard and characterized by different position within the canopy. Higher temperatures were reached in low vigor clusters respect to high vigor clusters, probably as a consequence of a higher exposition to sunlight of clusters belonging to low vigor vines.

Vine vigor also affected clusters morphology and composition. In particular, grapes from low vigor vines in this work were characterized by lower clusters weight, sugar and malic acid contents and higher pH value and α-aminoacidic nitrogen content.

Regarding flavonoid monomer composition of clusters several differences resulted related to vine vigor and cluster position within the canopy. In particular, anthocyanins profile in grapes from low vigor vines resulted characterized by a higher prevalence of trisubstituted anthocyanins while significant differences were found in the contents of flavonols in grapes, with those from high vigor vines showing lower concentration of flavonols respect to low vigor clusters. Moreover, the position of clusters within the canopy showed an effect on partitioning of some anthocyanins (Dp-3-*O*-and Pt-3-*O*-G), total flavonol and total flavan-3-ol monomer contents. By evaluating the microclimatic condition of fruiting zones and temperatures of different exposed clusters, it seemed that differences in anthocyanins and flavonols composition of clusters probably were related to the effects of temperature and light exposure of grapes.

Finally, the experimental wines obtained by fermenting grapes from high and low vigor zones reflected the differences assessed in grape clusters composition. Indeed, low vigor wines showed higher contents of polymeric and non polymeric anthocyanins, total flavonols, quercetin glycosides and lower contents of flavan-3-ol monomers when compared to the experimental

wines obtained from high vigor clusters. Moreover, reduction of vine vigor seemed to affect anthocyanins profiles of grapes and wines, increasing the values of trisubstituted to disubstituted anthocyanins ratio.
By understanding the effect of vine vigor on clusters and, consequently, wines characteristics, viticulturists have the opportunity to modulate practices for the management of spatial variation of vine vigor in vineyards in order to limit the crop productions, to stimulate grape berries synthesis of phenolic compounds of quality and healthy interests and, as a consequence, to enrich the resulting wines.

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2.4. Effect of yeast species involved in the alcoholic fermentation on accumulation and partitioning of flavonoids in Sangiovese wines

2.4.1. Alcoholic fermentation carried out by different yeast species in two condition of aeration: effect on flavonoid accumulation in Sangiovese wines

Abstract

In this work, the effect of different yeast species involved in the alcoholic fermentation and aeration of must on the concentrations and composition of anthocyanins, flavonols and flavan-3-ol monomers of Sangiovese wines produced from the same grape must with its skins and seeds was investigated. Besides, standard characterization and secondary metabolites produced by yeasts were monitored on the experimental wines. At first, the experimental wines obtained by sequential inoculum of *Candida zemplinina* (*Starmerella bacillaris* according to Duarte et al., 2012) strain Cz1 and *Saccharomyces cerevisiae* Sc1 were characterized by higher contents of glycerol, acetic acid and pyruvic acid. Regarding phenolic contents of the experimental wines, yeast different species characterizing the alcoholic fermentations seemed to affect total phenolic index and total flavan-3-ol monomers contents of the experimental wines. Such results were probably linked to the ethanol rate of production which lead to significant differences in wines when maceration length is standardized. Besides, a strong effect of aeration was reported on the concentration of non polymeric anthocyanins, flavonols and, to a lower extent, flavan-3-ols, probably as a consequence of their susceptibly to oxidation reaction.

Despite a non significant effect on flavonol and flavan-3-ol monomer profile, anthocyanin profiles resulted influenced by aeration and, more deeply, by yeast involved during the alcoholic fermentation. In particular, in the experimental wines produced by sequential inoculum were characterized by higher abundance of disubstituted anthocyanins (cyanidin-3-O-glucoside and peonidin-3-O-glucoside) and vitisin A and lower relative abundance of malvidin-3-O-glucoside.

Introduction

The metabolic behavior of different yeast strains may affect significantly the organoleptic properties of a wine (Suárez-Lepe and Morata, 2012). Indeed, the organoleptic properties of a wine may be affected to such a point that, currently, yeast selection criteria include the capability to improve color, aroma and structure of wine in addition to other technological properties. In this connection, although *Saccharomyces cerevisiae* has been the main species selected for winemaking, several Authors consider some non-*Saccharomyces* yeasts potentially able to enrich wines from a sensorial point of view, due to their metabolic and physiological characteristics (Pretorius, 2000; Suárez-Lepe and Morata, 2012). Among non-*Saccharomyces*, *Candida zemplinina* is a yeast species frequently isolated during the early stages of must fermentations in different enological Countries. It is worth mentioning that the name *Candida zemplinina* have to be considered an obligate synonym of *Starmerella bacillaris* (Duarte et al., 2012; Pfliegler et al., 2014). *Candida zemplinina* is known to possess interesting properties from an enological point of view: highly fructophilic character, tolerance to high glucose concentrations, medium ethanol tolerance, high glycerol and low acetic acid production.

In view of these properties, the use of controlled mixed cultures of selected non-*Saccharomyces* and *Saccharomyces cerevisiae* strains are proposed for modulating wine quality (Tofalo et al, 2012). In this work, the effect of different yeast species involved in the alcoholic fermentation and aeration of must on the concentrations and composition of anthocyanins, flavonols and flavan-3-ol monomers of Sangiovese wines produced from the same grape must with its skins and seeds was investigated.

Materials and methods

Grape samples.

A single lot of Sangiovese grapes constituted by 30 clusters was used. Grape samples were transferred to laboratory at low temperature and stored at -20°C for a period of time not exceeding 1 months. Grape samples preparation methodologies prior analysis and vinifications are reported in Chapter 3.1 (3.1.3 Results, figure 1b). Chemical composition of the grape must is here reported: Total Anthocyanin contents (Tot grape A), 883 ± 2 mg/L; Total phenols (TP), 45.7 ± 0.9 ($O.D._{280}$); Glucose 121.4 ± 1.4 g/L, Fructose 128.4 ± 1.7 g/L, pH 3.81 ± 0.01, α-aminoacidic nitrogen content (α-AN) 127.8 ± 2.8 mgN/L.

Experimental vinifications. The laboratory vinifications were carried out using 450g of grapes each, in triplicates. Micro-vinification were carried out in 500 mL flasks at 28°C for 14 days, using as inoculum *S. cerevisiae* Sc1 strain alone (pure inoculated fermentation) and *C. zemplinina* Cz1 strain followed by *S. cerevisiae* Sc1 after 5 days (sequential inoculated fermentation). Daily mixing was carried out under two aeration conditions: (i) open flask (aerated), (ii) close flask (non-aerated). The fermentation progress was monitored by weight loss, as above described.

Yeast inocula. Yeast inoculua of *S. cerevisiae* Sc 1 and *C. zemplinina* Cz1 preparation is reported in Chapter 3.1 (3.1.2 Materials and methods).

Chemicals and analytical determinations. Chemical and analytical determination on grapes, musts and wines are reported in Chapter 3.1 (3.1.2 Materials and methods).

Statistical analysis. All statistical calculations were performed by using Statistica software (version 7, StatSoft, Tulsa, OK, USA) and GraphPad Prism 5 (GraphPad Software, Inc., CA,USA).

Results and discussion

After 14 days of fermentation under different condition of aeration, residual sugars were below 2 g/L in all experimental wines, but fermentation kinetics were quite different, depending on the yeast species dominating the transformation process.

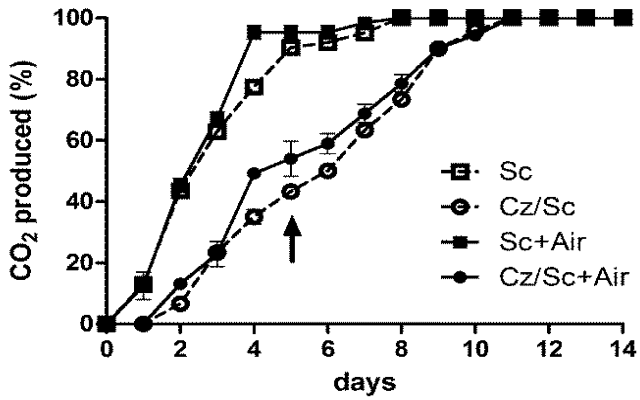

Figure 2: Alcoholic fermentation time courses in Sangiovese grapes fermented under laboratory conditions using S. cerevisiae alone (Sc) or sequential inoculum of C zemplinina and S. cerevisiae (Cz/Sc) and in combination with (+Air) or without aeration of grape must. (The black arrow indicates the inoculum of S. cerevisiae at the fifth day of must fermentation started by C. zemplinina). Values are reported as mean ± standard deviation.

Indeed, as shown in figure 2, alcoholic fermentations (AF) carried out by *S. cerevisiae* Sc1 alone were characterized by a higher fermentative activity. The presence of *C. zemplinina* Cz 1 caused a negative effect on fermentation kinetics in both aerated and non-aerated conditions. Moreover, the rate of CO_2 production resulted faster in fermentation carried out in aerated conditions in both pure and sequential inoculated fermentations.

Analytical characteristics of the experimental wines are reported in table 1.

Table 1. Analytical characteristics of the experimental wines obtained by using *S. cerevisiae* Sc1 alone (Sc) or sequential inoculum of *C. zemplinina* Cz1 and *S. cerevisiae* Sc1 (Cz/Sc) with or without aeration (+Air) of grape must and related statistical analysis (two-way ANOVA). Values are expressed as mean ± standard deviation.

	Experimental wines				Source of variation		
	Sc	Cz/Sc	Sc+Air	Cz/Sc+Air	YI	Air	YI xAir
Ethanol (%, v/v)	13.7 ± 0.3	13.4 ± 0.1	13.8 ± 0.0	13.3 ± 0.3	ns	ns	ns
Glycerol (g/L)	10.4 ± 0.0	16.2 ± 0.1	10.1 ± 0.5	15.7 ± 0.1	***	ns	ns
Acetic acid (g/L)	0.11 ± 0.01	0.65 ± 0.01	0.13 ± 0.01	0.52 ± 0.02	***	**	**
Pyruvic acid (mg/L)	10.6 ± 2.1	87.1 ± 10.7	11.6 ± 0.7	100.6 ± 1.4	***	ns	ns
TA ($g_{tart\ ac}$/L)	6.8 ± 0.1	6.5 ± 0.1	6.7 ± 0.1	6.6 ± 0.0	ns	ns	ns
TP (OD_{280})	44.5 ± 0.1	40.4 ± 1.8	40.5 ± 2.4	34.6 ± 3.2	*	*	ns
pH	3.70 ± 0.01	3.67 ± 0.00	3.71 ± 0.01	3.69 ± 0.02	ns	ns	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant

TA = Total acidity; TP = Total phenols; YI = effect of yeast inoculums; Air = effect of aeration; YI x Air = interaction.

The experimental wines showed significant differences in the content of some fermentative metabolites, which resulted strongly affected by the yeast species. In particular, the experimental wines from sequential inoculum of *C. zemplinina* Cz1 and *S. cerevisiae* Sc1 were characterized by high contents of glycerol and pyruvic acid. According to the two-way ANOVA analysis, acetic acid was significantly affected by both fermenting yeast species and aeration conditions, the lowest concentration being produced in fermentation carried out by *S. cerevisiae* Sc1 alone under non-aerated conditions (0.11 ± 0.01 g/L). Regarding total phenols (TP) of the experimental wines, the presence of *C. zemplinina* Cz1 and aeration resulted detrimental. In particular, lower value of TP were found in experimental wines obtained by sequential inoculated fermentations, probably as a consequence of a lower rate of accumulation of ethanol in the medium. Indeed, a lower production of ethanol in the first part of fermentation characterized by the presence of *C. zemplinina* Cz1, lead to

significant differences in the wines produced by standardizing the maceration length. Finally, the aeration of musts may be responsible of the oxidation of wine phenols, thus affecting the TP. This consideration is in agreement with what reported by Castellari et al. (1998) effect

Regarding anthocyanin, flavonol and flavan-3-ol contents in the experimental wines, results are reported in table 2.

Table 2: Phenolic compounds in the experimental Sangiovese wines obtained by using *S. cerevisiae* Sc1 alone (Sc) or sequential inoculum of *C. zemplinina* Cz1 and *S. cerevisiae* Sc1 (Cz/Sc) with (+Air) or without aeration of grape must and related statistical analysis (two-way ANOVA). Values are expressed as mean ± standard deviation.

	Experimental wines				Source of variation		
	Sc	Cz/Sc	Sc+Air	Cz/Sc+Air	YI	Air	YI xAir
non-Pol A	103.2 ± 1.2	99.0 ± 0.7	83.6 ± 2.8	83.7 ± 1.8	ns	***	ns
Pigm Polym	11.0 ± 2.4	11.9 ± 1.2	11.1 ± 0.2	10.4 ± 0.8	ns	ns	ns
Tot F	51.6 ± 2.9	50.7 ± 1.5	40.7 ± 1.4	39.6 ± 2.2	ns	**	ns
Tot F3ols	77.1 ± 7.4	55.9 ±10.8	58.4 ± 2.5	43.3 ± 5.0	*	*	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant

YI = effect of yeast inoculums; Air = effect of aeration; YI x Air = interaction

The two-way ANOVA analysis suggested that the aeration of must resulted the variable capable to affect the contents of flavonoids in experimental wines. In particular, the loss of non polymeric anthocyanins in experimental wines processed in aerated conditions reached about the 20% when compared with experimental wines produced without aeration. As reported by Castellari et al. (1998), oxygen in must is capable to provoke both direct oxidation of phenolic compounds or favor reaction of polymerization. Hence, the loss of non polymeric anthocyanins may be due to their participation on the formation of pigmented polymer. However, in the present experiment, pigmented polymer were not statistically higher in the experimental wine treated in aerated conditions. Indeed, the loss of non polymeric anthocyanins is imputable to a detrimental effect of air on this compounds.

Similarly, aeration of must provoked a considerable loss of both total flavonols and total flavan-3-ol monomers. However, the latter class of compounds resulted significantly affected also by yeast species involved in the fermentation process. Indeed, the experimental wines produced with sequential inoculum were characterized by about 26% less of total flavan-3-ol

monomers in both aerated and non-aerated conditions. Monomers of flavan-3-ols detected in experimental wines, are principally derived from grape seeds as it is known their higher abundance in seeds rather than in skins (de Freitas and Glories, 1999). The extraction of phenolic compounds from seeds into wine resulted faster and intense in function of ethanol concentration which probably allows the disorganization of the outer lipidic layer that protect seeds (Hernández-Jiménez et al., 2012). Indeed, the lower concentration of flavan-3-ol monomers in experimental wines obtained by sequential inoculum of *C. zemplinina* Cz1 followed by *S. cerevisiae* Sc1 may probably be linked to the rate of ethanol production during the alcoholic fermentation.

Finally, the study focused on anthocyanin, flavonol and flava-3ol monomer profiles in order to evaluate the possible effect of yeast involved during the alcoholic fermentation and aeration conditions of grape must (tabs. 3, 4, 5)

Conversely to what reported non polymeric anthocyanin contents, yeast species involved in the alcoholic fermentation showed a marked influence on anthocyanin profile of the experimental wines respect to aeration conditions (tab.3). With the exception of acylated, all anthocyanin glucosides and vitisin A resulted deeply influenced by the presence of *C. zemplinina* Cz1 under equal conditions of must aeration. In particular, in presence of *C. zemplinina* Cz1, the disubstituted anthocyanins resulted more abundant respect in pure inoculated fermentation. This led to a decreasing in A ratio which has reached values of approximately 50% less than those recorded in wines produced by using *S. cerevisiae* Sc1 alone. Regarding vitisin A, the product of the condensation between malvidin-3-*O*-glucoside and pyruvate, contents and its relative abundance on total non polymeric anthocyanins, a strong relation with the capacity of pyruvate production by yeasts during the alcoholic fermentation emerged. This partially justify a reduction of relative abundance of malvidin-3-*O*-glucoside in the experimental wines obtained with sequential inoculum(results are extensively reported in chapter 5.3 of this thesis).

Table 3: Anthocyanin profile of the experimental Sangiovese wines obtained by using *S. cerevisiae* Sc1 alone (Sc) or sequential inoculum of *C. zemplinina* Cz1 and *S. cerevisiae* Sc1 (Cz/Sc) with (+Air) or without aeration of grape must and related statistical analysis (two-way ANOVA). Values are expressed as mean ± standard deviation.

	Experimental wines				Source of variation		
	Sc	Cz/Sc	Sc+Air	Cz/Sc+Air	YI	Air	YI xAir
Dp-3-*O*-G	6.2 ± 0.2	6.9 ± 0.2	4.7 ± 0.2	5.4 ± 0.4	*	**	ns
Cy-3-*O*-G	2.0 ± 0.2	5.2 ± 0.1	1.9 ± 0.0	4.7 ± 0.5	***	ns	ns
Pt-3-*O*-G	15.1 ± 0.1	13.8 ± 0.3	13.1 ± 0.3	12.4 ± 0.4	*	**	ns
Pn-3-*O*-G	7.2 ± 0.3	11.9 ± 0.2	7.3 ± 0.2	12.2 ± 0.3	***	ns	ns
Mv-3-*O*-G	68.0 ± 0.2	58.3 ± 0.7	71.0 ± 0.3	61.0 ± 1.6	***	*	ns
Vit A	0.6 ± 0.1	2.9 ± 0.3	1.0 ± 0.1	3.2 ± 0.1	***	*	ns
Acylated A	0.8 ± 0.0	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	ns	ns	ns
A ratio	9.7 ± 0.5	4.6 ± 0.0	9.6 ± 0.2	4.7 ± 0.3	***	ns	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant. YI = effect of yeast inoculums; Air = effect of aeration; YI x Air = interaction;

A profile (%) = anthocyanins profile Dp-3-*O*-G = delphinidin-3-*O*-glucoside, Cy-3-*O*-G = cyanidin-3-*O*-glucoside, Pt-3-*O*-G = petunidin-3-*O*-glucoside , Pn-3-*O*-G = peonidin-3-*O*-glucoside, Mv-3-*O*-G = malvidin-3-*O*-glucoside, Acylated A = sum of acetylated and coumarilated anthocyanins to non-polymeric anthocyanins contents of grapes; A ratio = trisubstituted (Dp-3-O-G, Pt-3-*O*-G and Mv-3-*O*-G) to drisubstituted (Cy-3-*O*-G and Pn-3-*O*-G) ratio.

The differences reported in anthocyanin glucosides partitioning may be due to several different phenomenon interactions involving yeast and these pigment flavonoids. Indeed, several Authors reported that the composition and the porosity of the cell walls of yeasts involved during the alcoholic fermentation can cause a loss of color of wines via the adsorption of anthocyanins (Vasserot et al., 1997; Morata et al., 2005). The adsorption and the retention of anthocyanin of the cell walls of yeasts have been reported to vary significantly on the basis of anthocyanin structure, as degree of methoxylation and steric hindrance, ethanol concentration (Vasserot et al., 1997) and yeast species and strains involved in the winemaking (Medina et al., 2005). Moreover, another phenomenon of interaction between yeasts and anthocyanins well documented is related to the production of β-D-glucosidase produced by yeasts and capable to release the correspondent anthocyanidin which spontaneously converts to brown or colorless compounds (Blom et al., 1983;

Manzanares et al., 2000). Once again, the activity of β-D-glucosidase resulted strain specific and, between species, non-*Saccharomyces* seemed to be characterized by a higher level of β-D-glucosidase activity. Indeed, this activity has been investigated in *S. cerevisiae*, but it resulted rare or generally lower than these non-*Saccharomyces* studied (Rosi et al., 1994; Charoenchai et al., 1997). Finally, despite a equal quantity of pigmented polymer assessed in the experimental wine, the composition of these polymer may be differ on the basis of anthocyanins involved. Indeed, in the literature is reported a different kinetics of reaction rate of anthocyanins in the formation of stable pigmented compounds (Dallas et al., 1996). Once again, this could be influenced by a different kinetics of extraction from solid parts of grapes attributable to a diverse rate ethanol production.

Conversely to anthocyanin, no significant effect of yeast species and aeration of must during alcoholic fermentation on flavonol and falvan-3ol monomer profiles of the experimental wines were assessed (tabs. 4 and 5).

Table 4: Flavonol profile of the experimental Sangiovese wines obtained by using *S. cerevisiae* Sc1 alone (Sc) or sequential inoculum of *C. zemplinina* Cz1 and *S. cerevisiae* Sc1 (Cz/Sc) with (+Air) or without aeration of grape must and related statistical analysis (two-way ANOVA). Values are expressed as mean ± standard deviation.

	Experimental wines				Source of variation		
	Sc	Cz/Sc	Sc+Air	Cz/Sc+Air	YI	Air	YI xAir
Ms	13.8 ± 1.1	13.9 ± 2.3	14.6 ± 0.1	13.5 ± 2.3	ns	ns	ns
Qs	81.1 ± 0.0	81.1 ± 1.2	81.5 ± 0.7	81.4 ± 1.3	ns	ns	ns
Ks	5.1 ± 1.1	5.0 ± 1.1	3.9 ± 0.6	5.0 ± 1.0	ns	ns	ns
F ratio	0.17 ± 0.01	0.17 ± 0.03	0.18 ± 0.00	0.17 ± 0.03	ns	ns	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant. YI = effect of yeast inoculums; Air = effect of aeration; YI x Air = interaction.

F profile (%) = flavonols profile, Ms = sum of myricetin and its glycosides, Qs = sum of quercetin and its glycosides, K-3-*O*-G = kaempferol-3-*O*-glucoside to total flavonols; F ratio = Ms contents (mg/L) / Qs contents (mg/L).

Table 5: Flavan-3-ol monomer profile of the experimental Sangiovese wines obtained by using *S. cerevisiae* Sc1 alone (Sc) or sequential inoculum of *C. zemplinina* Cz1 and *S. cerevisiae* Sc1 (Cz/Sc) with (+Air) or without aeration of grape must and related statistical analysis (two-way ANOVA). Values are expressed as mean ± standard deviation.

	Experimental wines				Source of variation		
	Sc	Cz/Sc	Sc+Air	Cz/Sc+Air	YI	Air	YI xAir
C	48.9 ± 1.3	51.2 ± 4.3	52.3 ± 0.6	51.3 ± 1.3	ns	ns	ns
EC	48.9 ± 1.7	46.9 ± 3.8	45.9 ± 1.0	47.0 ± 1.5	ns	ns	ns
ECG	2.2 ± 0.3	1.87 ± 0.6	1.84 ± 0.4	1.70 ± 0.2	ns	ns	ns
F3ols ratio	1.00 ± 0.06	1.10 ± 0.18	1.14 ± 0.04	1.09 ± 0.06	ns	ns	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant. YI = effect of yeast inoculums; Air = effect of aeration; YI x Air = interaction;

F3ols profile (%) = flavan-3-ol monomer profile, C = (+)-catechin, EC = (-)-epicatechin, ECG = (-)-epicatechin gallate; F3ols ratio = C contents (mg/L) / EC contents (mg/L).

Conclusion

In this work, the effect of different yeast species involved in the alcoholic fermentation and aeration of must on the concentrations and composition of anthocyanins, flavonols and flavan-3-ol monomers of Sangiovese wines produced from the same grape must with its skins and seeds was investigated. Yeast species involved in the alcoholic fermentation are not only responsible of the transformation of sugar into ethanol, but also play a significant role in determining the condition of extraction of flavonoids from solid part of grapes into wines. In particular, besides a strong effect on several secondary metabolites found in wines fermented by different yeast species, the rate of production of ethanol resulted fundamental for the dissolution of flavan-3-ol monomers from skins and seeds. Moreover, anthocyanin partitioning resulted deeply influenced by the presence of *C. zemplinina* Cz1 leading to wine with lower A ratio respect to those produced by *S. cerevisiae* Sc1 alone. This results seemed to be attributable to several phenomena of interaction between yeast species and anthocyanins. Further study in this field are needed.

Finally, aeration was applied in order to evaluate the difference in flavonoids extraction by varying the rate of production of ethanol by yeasts. However, in the experiment presented, aeration was able to increase the metabolic activity of yeasts toward a higher rate of ethanol production, but a direct detrimental effect was assessed on the contents of flavonoids in the experimental wines.

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2.4.2.Quercetin and vitisin A contents of Sangiovese wines as affected by fermenting yeast species and must aeration

Abstract

Quercetin and vitisin A are phenolic compounds found in wines covering relevant roles in determining some sensorial properties and showing several positive properties on human health. This work refers on the possible effects of yeast species and must aeration on quercetins and vitisin A accumulations in experimental Sangiovese wines. Indeed, 56 experimental wines were obtained by varying both grape Sangiovese musts, yeast species involved in alcoholic fermentation (*S. cerevisiae* alone or by sequential inoculum of *Candida zemplinina* and *S. cerevisiae*) and must aeration.

The variability in composition of quercetin and its glycosides in wines was principally due to row material, although both yeast species involved in alcoholic fermentation and must aeration could influence the relative abundance of the different forms of quercetin. Regarding Vitisin A, higher contents were found in wines produced in the presence of *C. zemplinina*, as a consequence of its higher production of pyruvic acid than *S. cerevisiae*.

Indeed, the yeast species occurring in the fermentative process or must aeration affected both the relative abundances among the different forms of quercetin as well as the amounts of vitisin A. Finally, this work underlines the role of both yeast species and must aeration in determining phenolic composition of Sangiovese wine.

Introduction

The principal source of phenols in wines is the grape berry, in which the amount of these molecules depends on the cultivar and is influenced by environmental and viticultural factors (Jackson and Lombard, 1993). However, during the winemaking process, native phenols undergo to modifications and other phenolic compounds are formed by either enzymatic reactions or metabolic activities of yeasts (Ribéreau-Gayon et al., 2000; Morata et al., 2003). Therefore, phenols found in wines may originate from different sources. Indeed, this work is focused on some phenolic compounds of high relevance for red wine quality whose in wines may depend by yeast metabolic activity.

Quercetin is the most abundant flavonol found in Sangiovese grapes (Mattivi et al., 2006; Storchi et al., 2008), the most widely cultivated red-berried cultivar in Tuscany (Italy). In *Vitis vinifera*, quercetin usually exists as 3-*O*-glycosides, synthesized in the berry skin in response to UV-B exposure (Keller,

2010). During the winemaking process, the glycosides are hydrolyzed and quercetin is released (Price et al., 1995). Yeasts involved in the alcoholic fermentation could potentially contribute to the glycoside hydrolysis, as it is known that some strains possess glycosidase activity, including β-glucosidase, which is active on flavonol and anthocyanin-3-*O*-glucosides (Terrier et al., 2009; Suárez-Lepe and Morata, 2012). Quercetin and its glycosides participate to wine color by acting as copigmentation cofactors (Boulton, 2001) and seem to contribute to bitterness of wines (Rodríguez Montealegre, 2006). However, due to its low solubility, free quercetin can be responsible of haze and yellow deposits in wines (Somers and Ziemelis, 1985). Moreover, quercetin is deeply studied because it is the most common flavonol in the human diet (Erlund, 2004), its antioxidant and anti-inflammatory properties being associated with the prevention and therapy of cardiovascular diseases and cancer (Russo et al., 2012).

Vitisin A is one of the first oligomeric pigments identified in red wines (Asenstorfer et al., 2003). It is formed through condensation of malvidin-3-*O*-glucoside, that is the most common anthocyanin found in *Vitis vinifera* (Morata et al., 2006), and pyruvate, a secondary metabolite produced by yeasts. The new molecule shows a more stable structure and results more resistant to discoloration by SO_2, less sensitive to color modification by pH compared to malvidin-3-*O*-glucoside (Morata et al., 2003). Moreover, vitisin A shows a higher resistance to gastrointestinal conditions, presumably leading to enhanced bioavailability respect its parent anthocyanin (McDougall et al., 2005).

From the above, the metabolic behavior of different yeast strains may affect significantly the organoleptic properties of a wine (Suárez-Lepe and Morata, 2012). Indeed, the organoleptic properties of a wine may be affected to such a point that, currently, yeast selection criteria include the capability to improve color, aroma and structure of wine in addition to other technological properties. In this connection, although *Saccharomyces cerevisiae* has been the main species selected for winemaking, several Authors consider some non-*Saccharomyces* yeasts potentially able to enrich wines from a sensorial point of view, due to their metabolic and physiological characteristics (Pretorius, 2000; Suárez-Lepe and Morata, 2012). Among non-*Saccharomyces*, *Candida zemplinina* is a yeast species frequently isolated during the early stages of must fermentations in different enological Countries and it is known to possess interesting properties: highly fructophilic character, tolerance to high glucose

concentrations, medium ethanol tolerance, high glycerol and low acetic acid production. In view of these properties, the use of controlled mixed cultures of selected non-*Saccharomyces* and *Saccharomyces cerevisiae* strains are proposed for modulating wine quality (Tofalo et al, 2012).

Therefore, this work was aimed to assess the possible effect of *S. cerevisiae* alone or sequential inoculum of *C zemplinina* and *S. cerevisiae* on the concentrations of quercetin and its glycosides and vitisin A. At first, a specific strain of *Saccharomyces cerevisiae* (Sc1) was used in standardized laboratory scale fermentations of different Sangiovese grape musts with their skins and seeds in order to evaluate the influence of row material on phenolic molecules here taken into consideration. Besides, a sequential inoculum of *Candida zemplinina* Cz1 strain followed by *Saccharomyces cerevisiae* Sc1 was compared to a single inoculated fermentation of *Saccharomyces cerevisiae* Sc1 using the same grape must with its skins and seeds. Moreover, the alcoholic fermentations were carried out in two different conditions of aeration, factor known to directly interact with both phenolic compounds of grapes and fermentative behavior of yeasts (Moreno et al., 1988; Mauricio et al., 1998).

Materials and methods

Grape samples. During three consecutive vintages (2011-2013), from different vineyards located in three Tuscan viticultural areas producing high quality wines (Brunello di Montalcino, Chianti Classico and Chianti Colli Aretini), lots of grape clusters of Sangiovese variety were harvested, paying much attention on cluster integrity. Each lot was constituted by 30 grape clusters for a total of 52 lots. Grape samples were transferred to laboratory at low temperature and stored at -20°C for a period of time not exceeding 1 months. Grape samples preparation methodologies prior analysis and vinifications are reported in Chapter 3.1 (3.1.3 Results, figure 1b).

Experimental vinifications. The laboratory vinifications were carried out using 450g of grapes each, in triplicates for each grape samples, inoculated with *Saccharomyces cerevisiae* Sc1 strain Technological condition of alcoholic fermentation/maceration are reported in Chapter 3.1 (3.1.2 Materials and methods).

For the experiments involving different yeast species, a lot of Sangiovese grapes harvested in 2011 was used. Chemical composition of the grape must is here reported: Total Anthocyanin contents (Tot grape A), 883 ± 2 mg/L; Total phenols (TP), 45.7 ± 0.9 ($O.D._{280}$); Glucose 121.4 ± 1.4 g/L, Fructose 128.4 ± 1.7

g/L, pH 3.81 ± 0.01, α-aminoacidic nitrogen conten (α-AN) 127.8 ± 2.8 mgN/L. Also in this case, triplicates micro-vinification were carried out in 500 mL flasks at 28°C for 14 days, using as inoculum *S. cerevisiae* Sc1 strain alone (pure inoculated fermentation) and *C. zemplinina* Cz1 strain followed by *S. cerevisiae* Sc1 after 5 days (sequential inoculated fermentation). Daily mixing was carried out under two aeration conditions: (i) open flask (aerated), (ii) close flask (non-aerated). The fermentation progress was monitored by weight loss, as above described.

Yeast inocula. Yeast inoculua of *S. cerevisiae* and *C. zemplinina* preparation is reported in Chapter 3.1 (3.1.2 Materials and methods).

Chemicals and analytical determinations. Chemical and analytical determination on grapes, musts and wines are reported in Chapter 3.1 (3.1.2 Materials and methods).

Statistical analysis. All statistical calculations were performed by using Statistica software (version 7, StatSoft, Tulsa, OK, USA) and GraphPad Prism 5 (GraphPad Software, Inc., CA,USA).

Results

Single strain fermentation of different grape musts. The analytical characterization of the 52 grape samples, musts and wines is reported in table 1.

Table 1: Physicochemical characteristics of the 52 Sangiovese grape musts and experimental wines from fermentation by *S. cerevisiae* strain Sc1. Frequency distribution, CV% = coefficient of variation

	Min	Median	Max	Mean	CV%
Grapes					
Total anthocyanins (mg/L)	668	1130	1911	1146	22.9
Total phenols ($O.D._{280}$)	32.1	42.1	73.1	45.1	19.7
Musts					
Total sugar (g/L)	199.0	246.0	296.0	246.1	9.0
Tartaric acid (g/L)	3.48	4.82	7.02	4.79	14.5
α-AN (mgN/L)	75.0	151.9	560.0	189.8	60.0
pH	3.42	3.69	4.12	-	-
Wines					
Residual sugar (g/L)	0.0	0.7	5.0	1.0	99.2
Ethanol (v/v, %)	11.3	13.8	16.3	13.9	8.8
Glycerol (g/L)	7.9	9.5	11.9	9.6	9.3
Acetic acid (g/L)	0.07	0.22	0.35	0.21	32.3
TA ($g_{\text{tartaric acid}}$/L)	5.7	7.3	11.9	7.5	16.4
TP ($O.D._{280}$)	28.95	53.68	83.78	54.93	20.8
CI ($O.D._{420+520+620}$)	5.2	10.5	19.5	11.2	26.2
pH	3.38	3.61	4.10	-	-

α-AN = α-aminoacidic nitrogen content, TA = Total acidity; TP = Total phenols, CI = Color Intensity.

The 52 grape samples showed a remarkable variability in terms of total anthocyanins, total phenols, tartaric acid and α-aminoacidic nitrogen content (α-AN), the latter showing the highest coefficient of variation among the parameters here taken into consideration.

At the end of the alcoholic fermentation, the residual sugar concentration in all wines was under 4 g/L, except one sample showing 5 g/L, the other chemical parameters showing a variability quite consistent with the variability of the

grape composition, with the highest value of acetic acid concentration occurring in the wine originated from the must with the lowest content of α-AN.
Regarding the bioactive molecules studied, the concentrations and the relative abundaces of quercetin and its glycosides and vitisin A are reported (tab. 2).

Table 2: Phenolic compounds in the 52 experimental Sangiovese wines from fermentation by *S. cerevisiae* strain Sc1. Frequency distribution, CV% = coefficient of variation

	Min	Median	Max	Mean	CV%
Qs (mg/L)	15.9	56.1	124.5	56.4	36.1
Qs/Tot F (%)	65.7	79.6	88.6	78.7	5.5
Qs profile (%)					
q-3-*O*-gal	4.6	9.2	21.3	9.5	31.1
q-3-*O*-glc	7.4	13.9	32.1	15.0	34.0
q-3-*O*-glu	5.6	53.3	72.8	50.5	34.3
free q	7.0	23.2	63.4	24.9	55.0
Vit A (mg/L)	0.3	0.7	1.4	0.8	37.6
Vit A/Tot A (%)	0.1	0.4	0.9	0.4	43.2

Qs = sum of quercetin and its glycosides contents; Qs/Tot F = sum of quercetin and its glycosides contents to total flavonols ratio; q-3-*O*-gal = quercetin-3-*O*-galactoside, q-3-*O*-glc = quercetin-3-*O*-glucuronide, q-3-*O*-glu = quercetin-3-*O*-glucoside, free q = quercetin aglycone; Vit. A/Tot A = contents of Vitisin A to total non polymeric anthocyanins ratio; YI = effect of yeast inoculums; Air = effect of aeration; YI x Air = interaction.

In all the experimental wines, the concentration of free quercetin and its glycosides (Qs) showed a wide variability even though the Qs to total flavonols ratio (Qs/Tot F) exhibited a low coefficient of variation (Table 2). Such results suggested that the contribution of Qs to total flavonols occurring in wines from Sangiovese grape must fermentation was relatively stable, so that it might be seen as a characteristic of the grape variety. On the other hand, the Qs-profile, as determined by the relative abundance of free quercetin and its conjugated forms with respect to total Qs, was characterized by a noticeable variability. The analysis of correlation revealed that the relative abundance of free quercetin was highly correlated with the relative abundance of quercetin-3-*O*-

glucoside (Pearson r = -0.96, $p < 0.01$), suggesting that this conjugated form could be the preferential substrate leading to the formation of free quercetin. Finally, the contents of vitisin A ranged from 0.3 to 1.4 mg/L and represented less than 1% of the total non polymeric anthocyanins in the experimental wines.

Alcoholic fermentation carried out by different yeast species. After 14 days of fermentation under different condition of aeration, residual sugars were below 2 g/L in all experimental wines. The analytical characterization of the experimental wines is reported in table 3.

Table 3. Fermentation kinetics and physicochemical characteristics of the experimental wines obtained by using *S. cerevisiae* alone (Sc) or sequential inoculum of *C. zemplinina* and *S. cerevisiae* (Cz/Sc) with or without aeration (+Air) of grape must and related statistical analysis (two-way ANOVA). Values are expressed as mean ± standard deviation.

	Experimental wines				Source of variation		
	Sc	Cz/Sc	Sc+Air	Cz/Sc+Air	YI	Air	YI xAir
Ethanol (%, v/v)	13.7 ± 0.3	13.4 ± 0.1	13.8 ± 0.0	13.3 ± 0.3	ns	ns	ns
Glycerol (g/L)	10.4 ± 0.0	16.2 ± 0.1	10.1 ± 0.5	15.7 ± 0.1	***	ns	ns
Acetic acid (g/L)	0.11 ± 0.01	0.65 ± 0.01	0.13 ± 0.01	0.52 ± 0.02	***	**	**
Pyruvic acid (mg/L)	10.6 ± 2.1	87.1 ± 10.7	11.6 ± 0.7	100.6 ± 1.4	***	ns	ns
TA ($g_{tart\ ac}$/L)	6.8 ± 0.1	6.5 ± 0.1	6.7 ± 0.1	6.6 ± 0.0	ns	ns	ns
TP (OD_{280})	44.5 ± 0.1	40.4 ± 1.8	40.5 ± 2.4	34.6 ± 3.2	*	*	ns
pH	3.70 ± 0.01	3.67 ± 0.00	3.71 ± 0.01	3.69 ± 0.02	ns	ns	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant

TA = Total acidity; TP = Total phenols; YI = effect of yeast inoculums; Air = effect of aeration; YI x Air = interaction.

The experimental wines showed significant differences in the content of some fermentative metabolites, which resulted strongly affected by the yeast species. In particular, the experimental wines from sequential inoculum of *C.*

zemplinina and *S. cerevisiae* were characterized by high contents of glycerol and pyruvic acid. According to the two-way ANOVA analysis, acetic acid was significantly affected by both fermenting yeast species and aeration conditions, the lowest concentration being produced in fermentation carried out by *S. cerevisiae* alone under non-aerated conditions (0.11 ± 0.01 g/L).
As concerns the bioactive molecules taken into consideration in this study, their concentrations in the experimental wines are reported in Table 4.

Table 4: Phenolic compounds in the experimental Sangiovese wines obtained by using *S. cerevisiae* alone (Sc) or sequential inoculum of *C. zemplinina* and *S. cerevisiae* (Cz/Sc) with (+Air) or without aeration of grape must and related statistical analysis (two-way ANOVA). Values are expressed as mean ± standard deviation.

	Experimental wines				Souce of variation		
	Sc	Cz/Sc	Sc+Air	Cz/Sc+Air	YI	air	YIxAir
Qs (mg/L)	41.9 ± 2.4	41.1 ± 1.8	33.2 ± 0.9	32.2 ± 1.3	ns	**	ns
Qs/Tot F (%)	81.1 ± 0.0	81.0 ± 1.1	81.5 ± 0.7	81.3 ± 1.4	ns	ns	ns
Qs profile (%)							
q-3-*O*-gal	9.9 ± 1.4	7.7 ± 0.5	10.3 ± 1.4	6.7 ± 1.4	*	ns	ns
q-3-*O*-glc	11.1 ± 0.1	9.1 ± 0.4	10.3 ± 0.5	8.6 ± 0.8	**	ns	ns
q-3-*O*-glu	68.4 ± 1.2	69.3 ± 1.2	72.9 ± 0.1	74.1 ± 2.0	ns	**	ns
free q	10.7 ± 0.3	13.9 ± 1.1	6.5 ± 1.0	10.5 ± 1.4	**	**	ns
Vit A (mg/L)	0.7 ± 0.1	2.9 ± 0.3	0.9 ± 0.1	2.7 ± 0.0	***	ns	ns
Vit A/Tot A (%)	0.7 ± 0.1	2.9 ± 0.3	1.1± 0.1	3.2 ± 0.1	***	*	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant
Qs = sum of quercetin and its glycosides contents; Qs/Tot F = sum of quercetin and its glycosides contents to total flavonols ratio; q-3-*O*-gal = quercetin-3-*O*-galactoside, q-3-*O*-glc = quercetin-3-*O*-glucuronide, q-3-*O*-glu = quercetin-3-*O*-glucoside, free q = quercetin aglycone; Vit. A/Tot A = contents of Vitisin A to total non polymeric anthocyanins ratio; YI = effect of yeast inoculums; Air = effect of aeration; YI x Air = interaction.

The two-way ANOVA analysis suggested that Qs amount was unaffected by yeast species involved in the fermentation process, but it was negatively influenced by must aeration. In any case, the percentage of Qs with respect to

total flavonols was about 81%, independently of yeast species involved and aeration conditions. Regarding the Qs profile, the relative abundances of free and conjugated forms of quercetin were influenced by both yeast species and aeration, the abundance of quercetin-3-*O*-glucuronide and, to a lower extent, quercetin-3-*O*-galactoside being significantly higher in wines produced by *S. cerevisiae* alone. On the contrary, quercetin-3-*O*-glucoside percentage resulted positively related to the must aeration. Finally, free quercetin abundance resulted higher in wines obtained with sequential inoculum of *C. zemplinina* and *S. cerevisiae*. Moreover, aeration influence negatively its relative abundance in the experimental wines.

Yeast species involved in must fermentation strongly affected also the concentration of vitisin A in the experimental wines. In particular, higher concentration of vitisin A were found in wines produced by sequential inoculum of *C. zemplinina* and *S. cerevisiae,* in both aerated and non-aerated conditions. Moreover, a highly significant correlation was found between Vitisin A and pyruvic acid content in wines (Pearson r = 0.98, $p < 0.01$).

Discussion

The experimental wines showed a noticeable variability in terms of total contents of quercetin and its glycosides (Qs), due to different grape composition and to different technological winemaking conditions, as also reported by other Authors (Price et al., 1995). Moreover, quercetin and its glycosides represented an almost stable percentage of the total flavonols (Qs/Tot F) in all the experimental wines, with values quite comparable to those found by other Authors in Sangiovese wines (Ghiselli et al., 1998; McDonald et al., 1998), strongly suggesting that the Qs to total flavonols ratio in Sangiovese wines may be an almost constant feature.

In terms of Qs-profile (that is the percentages of quercetin and its glycosides on total Qs), if it is widely known that it is strongly affected by sun exposure (Price et al., 1995), this work demonstrates that it is also affected by yeast species involved in grape must fermentation. In particular, the relative abundance and the quantity of quercetin-3-*O*-glucoside was unaffected by yeast species involved in the alcoholic fermentation (*C. zemplinina* Cz1 or *S. cerevisiae* Sc1), whereas noticeable differences have been assessed for the other forms, suggesting that the yeast ecology of wine fermentation could significantly modify the Qs profile.

Regarding the concentration of vitisin A and its relative abundance with respect to total anthocyanin content in the experimental wines, the results

pointed out a strong effect of yeast species involved in the alcoholic fermentation. In particular, *C. zemplinina* Cz1 produced more pyruvic acid than *S. cerevisiae* Sc1, so that a higher concentration of the pigment was produced in the fermentations carried out by the sequential inoculum of the two species, with a positive effect on wine color stability.

Conclusion

In conclusion, the health enhancing molecules here taken into consideration showed a very large variability in concentration. As expected, the main source of this variability is to be ascribed to the grape composition, both directly, as in the case of quercetins, and indirectly, as in the case of available nitrogen which affects fermentation kinetics. However, some fermentation variables, such as yeast species occurring in the process or must aeration, demonstrated to play an important role in determining both the relative abundances among the different forms of quercetin as well as vitisin A contents.

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2.4.3.Contribution to congresses

PO 84 Quercetin contents of Sangiovese wines as affected by fermenting yeast species and must aeration

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Quercetin and its glycosylated derivatives are the main dietary flavonols, known to possess strong antioxidant properties and, thus, playing an important role in the protection against degenerative diseases (Boots 2008). Quercetin can be absorbed by humans from the diet both as aglycone and glycosides, their absorption being promoted by red wine (Dragoni et al 2006). In grapes, quercetin exists only as 3-glycosides, whereas the aglycone can be found in wines, together with the 3-glycosides, as a result of acid hydrolisis that occurs during winemaking and aging. Moreover, quercetin aglycone might also originate from glycosidase activity of the yeasts strains participating in the winemaking process (Villena et al. 2007).

The aim of this work was to compare two styles of fermentations in terms of both aglycone and 3-glycosides quercetin contents. To this purpose, the concentrations of quercetins, aglyconic and glycosylated forms, were determined in wines obtained under laboratory conditions by varying both yeast species (*Saccharomyces cerevisiae* alone or sequential inoculum of *Candida zemplinina* and *S. cerevisiae*) and aeration conditions of grape must during fermentation.

Total flavonol content of wines was not significantly affected by the type of inoculum however significant differences between the contents of quercetins were detected. In particular, wines obtained with sequential fermentation were characterized by significant higher aglycone contents than wines obtained with pure culture of *S. cerevisiae*, whereas glycosilated quercetin contents were significantly lower. Among the glycosilated quercetins, quercetin-3-glucoside was not significantly affected by the type of inoculum, whereas the 3-glucuronide and 3-galattoside were both significantly lower in wines obtained by sequential fermentation. Concerning the effect of must aeration, both flavonols and quercetins, were found at significantly higher concentration in wines obtained without aeration.

In conclusion, concentration of all quercetin forms is affected by must aeration during alcoholic fermentation. Moreover, the ratio between the aglyconic and glycosylated forms seems to be affected by the yeast species participating in the winemaking process.

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Quercetin contents of Sangiovese wines as affected by fermenting yeast species and must aeration

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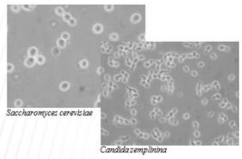

INTRODUCTION

Quercetin and its glycosylated derivatives are the main dietary flavonols, known to possess strong antioxidant and anti-inflammatory properties [1]. Quercetin can be absorbed by humans from the diet both as aglycone and glycosides, their absorption being promoted by red wine [2]. In grapes, quercetin exists only as 3-glycosides, whereas the aglycone can be found in wines, together with the 3-glycosides, as a result of acid hydrolisis that occurs during winemaking and aging. Moreover, quercetin aglycone might also originate from glycosidase activity of the yeast strains participating in the winemaking process [3]. Among non-*Saccharomyces* yeasts, *Candida zemplinina* is a yeast species frequently isolated during the early stages of must fermentations and it is known to possess interesting oenological properties, including medium ethanol tolerance [4]. Moreover, some enological practice as aeration of must during fermentation could influence the content of phenolic compounds in wines [5].

AIM

The aim of this work was to compare two styles of fermentations in terms of both aglycone and 3-glycosides quercetin contents. To this purpose, the concentrations of quercetins, both aglyconic and glycosylated forms, were determined in wines obtained under laboratory conditions by varying both **yeast species** (*Saccharomyces cerevisiae* alone or sequential inoculum of *Candida zemplinina* and *S. cerevisiae*) and **aeration conditions** of grape must during fermentation.

Material and Methods

Laboratory microvinifications (tab.1): 450 g of Sangiovese grape juice with its skins and seeds, triplicates in 500 mL flasks (glucose + fructose, 248 ± 4 g/L; pH, 3.81 ± 0.15; α-aminoacidic N, 128 ± 3 mgN/L), inoculated with specific yeast cultures:
(i) pure inoculated fermentation, *S. cerevisiae* – Sc (10^6 cell/mL),
(ii) sequential inoculated fermentation, *C. zemplinina* – Cz (10^6 cell/mL) followed by *S. cerevisiae* – Sc (10^6 cell/mL) after 5 days.
Yeast strains were previously isolated from Brunello di Montalcino wine fermentations and are included in the yeast culture collection of GESAAF, University of Florence, Italy.
The inoculated musts were incubated at 28°C for 14 days. Fermentation progress was evaluated by CO_2 loss, weighting flasks after mixing by gentle rolling one time/day (1 minute) in two conditions:
(i) open flask (aerated), (ii) close flask (non-aerated).
After 14 days of fermentation under different condition of aeration, residual sugars were below 2 g/L in all the experimental wines.

Table 1. Experimental plan and sample denomination

	Pure inoculated fermentation	Sequential inoculated fermentation
Non-Aerated	Sc	Cz/Sc
Aerated	Sc + Air	Cz/Sc + Air

Chemical analysis: glucose, fructose and ethanol - HPLC [6]; Flavonols - HPLC-UV-DAD detector scanning from 210 to 400 nm [7]. Flavonols were quantified using quercetin, myricetin and kaempferol as standards. Glycoside concentrations were expressed in equivalents of the correspondent aglycone at 280 and 340nm.
Statistical analysis: two-way ANOVA by GraphPad Prism 5 software.

RESULTS

Table 2. Flavonols and quercetins content of experimental Sangiovese wines obtained by using *S. cerevisiae* alone (Sc) or sequential inoculum of *C. zemplinina* and *S. cerevisiae* (Cz/Sc) with (+Air) or without aeration of grape must and related statistical analysis (two-way ANOVA). YI = effect of yeast inoculum, Air = effect of aeration, YI x Air = interaction. Values are expressed as mean ± standard deviation.

	Experimental wines				Source of variation		
	Sc	Cz/Sc	Sc + Air	Cz/Sc + Air	YI	Air	YI x Air
Tot Flavonols (mg/L)	51.6 ± 2.9	50.8 ± 1.6	40.7 ± 1.4	39.6 ± 2.2	ns	**	ns
Qs (mg/L)	41.9 ± 2.4	41.1 ± 1.8	33.2 ± 0.9	32.2 ± 1.3	ns	**	ns
Qs/Tot F (%)	81.1 ± 0.0	81.0 ± 1.1	81.5 ± 0.7	81.3 ± 1.4	ns	ns	ns

ns = non-significant; *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$
Notes: Tot Flavonols = total flavonols contents; Qs = sum of quercetin and its glycosides contents; Qs/Tot F = Qs to total flavonols

Flavonol and quercetin contents (tab.2)

•Both Tot Flavonols and Qs were unaffected by **yeast species** involved in the fermentation process, but they were both negatively affected by **must aeration**.

• The percentage of Qs with respect to total flavonols was about 81%, independently of **yeast species** involved and **aeration conditions**.

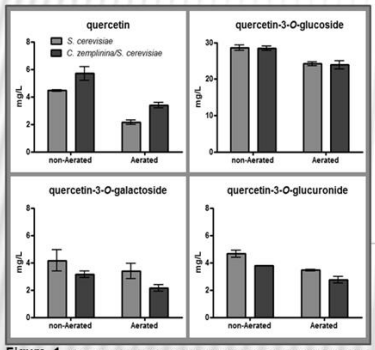

Figure 1. Contents of quercetin and its glycosides in the experimental wines. Values are expressed as mean ± SEM.

Quercetin and its glycosides contents (fig. 1)

•**Must aeration** during alcoholic fermentation negatively affected the contents of quercetin, quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronide (two-way ANOVA).

• Wines obtained with **sequential inoculum** were characterized by significant higher aglycone contents than wines obtained with pure cultures of *S. cerevisiae*.

•Among the glycosilated quercetins, quercetin-3-glucoside was not significantly affected by the type of inoculum, whereas the 3-glucuronide and 3-galattoside were both significantly lower in wines obtained by **sequential inoculum** (two-way ANOVA).

CONCLUSIONS

The concentrations of all quercetin forms in the experimental wines are strongly affected by **must aeration** during alcoholic fermentation. Moreover, the ratio between the aglyconic and glycosylated forms seems to be affected by the **yeast species** participating in the winemaking process.

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3. Health promoting compounds produced by yeasts in Sangiovese wines.

3.1. Tyrosol and hydroxytyrosol contents of Sangiovese wines as affected by fermenting yeast species and must aeration

Abstract

tyrosol and hydroxytyrosol are higher alcohols produced by yeast during the alcoholic fermentation known to possess several positive properties on human health. In this work, 56 experimental wines were obtained by varying both grape Sangiovese musts, yeast species involved in alcoholic fermentation (*S. cerevisiae* alone or by sequential inoculum of *Candida zemplinina* and *S. cerevisiae*) and must aeration.

By the results obtained was possible to assess that the production of tyrosol and hydroxytyrosol resulted influenced by both fermentation kinetics and by the presence of different species during the alcoholic fermentation. The contents of hydroxytyrosol and tyrosol in the experimental wines ranged from 7.2 to 66.5 mg/L. In particular, the higher concentration were found in experimental wines obtained by single strain of *S. cerevisiae* Sc 1 fermentation characterized by slow fermentation kinetics. In this work, both the α-aminoacidic nitrogen content and aeration of must during the alcoholic fermentation influenced the fermentation rates and negatively affected the contents of hydroxytyrosol and tyrosol in experimental wines, pointing out the key role of fermentation kinetics on the accumulation of these two molecules in wine.

Introduction

Tyrosol and hydroxytyrosol are higher alcohols known to possess several beneficial health properties, deriving from their free radical scavenging, anticarcinogenic and cardioprotective properties. In particular, antioxidant, cardioprotective, anticancer, antimicrobial, antidiabetic, neuroprotective activities were claimed (Fernández-Mar, 2012). The main source of hydroxytyrosol in the Mediterranean diet is olive oil in which it is found as a consequence of enzymatic hydrolysis of oleuropein (Bisignano et al., 1999).

Wine seems to be another important source of hydroxytyrosol and tyrosol in the diet (Fernández-Mar, 2012). Their origin in wines is strictly related to microrganisms involved in the wine making, in particular yeasts found during the alcoholic fermentation of musts (Ribéreau-Gayon et al., 2000).

Indeed, tyrosol is formed in wine by yeasts during alcoholic fermentation via the Ehrlich pathway from tyrosine (Sentheshanmuganathan and Elsden, 1958; Hazelwood et al., 2008; Monagas et al., 2005), whereas hydroxytyrosol is a tyrosol derivative formed by hydroxylation of the aromatic ring (Piñeiro et al., 2011). Indeed, their presence in wines is related by both grapevine variety vinified and vintage (Gris et al., 2011). In particular, chemical characteristics of grapes as the nitrogen content of musts may influence the accumulation of tyrosol and hydroxytyrosol in wines (Chen and Fink, 2006).

Moreover, it is well know that different yeasts species and strain can differ in the uptake and patterns of utilization of nitrogen sources (Large, 1986 Hence, the final contents of hydroxytyrosol and tyrosol in wine resulted influenced by the microbial ecology of alcoholic fermentation (Garde-Cerdán and Ancín-Azpilicueta, 2006). Some authors carried out few studies about the contribution of wild yeasts in terms of tyrosol and tryptophol contents in wine (Garde-Cerdán and Ancín-Azpilicueta, 2006). Indeed, tyrosol showed greater concentration in the mixed inoculated fermentation, so it would seem that the wild flora contributed to its synthesis. These Authors also found that, compared to other higher alcohol which are produced during the initial part of alcoholic fermentation, the contents of tyrosol became higher in final part of fermenting process.

Although *Saccharomyces cerevisiae* has been the main species selected for winemaking, several Authors consider some non-*Saccharomyces* yeasts potentially able to enrich wines from a sensorial point of view, due to their metabolic and physiological characteristics (Pretorius, 2000; Suárez-Lepe and Morata, 2012). Among non-*Saccharomyces*, *Candida zemplinina* is a yeast

species frequently isolated during the early stages of must fermentations in different enological Countries and it is known to possess interesting properties: highly fructophilic character, tolerance to high glucose concentrations, medium ethanol tolerance, high glycerol and low acetic acid production. In view of these properties, the use of controlled mixed cultures of selected non-*Saccharomyces* and *Saccharomyces cerevisiae* strains are proposed for modulating wine quality (Tofalo et al, 2012).

Therefore, this work was aimed to assess the possible effect of *S. cerevisiae* alone or sequential inoculum of *C zemplinina* and *S. cerevisiae* on the concentrations of hydroxytyrosol and tyrosol in experimental wines. At first, a specific strain of *Saccharomyces cerevisiae* (Sc1) was used in standardized laboratory scale fermentations of different Sangiovese grape musts with their skins and seeds in order to evaluate the influence of row material on the two higher alcohols. Besides, a sequential inoculum of *Candida zemplinina* Cz1 strain followed by *Saccharomyces cerevisiae* Sc1 was compared to a single inoculated fermentation of *Saccharomyces cerevisiae* Sc1 using the same grape must with its skins and seeds. Moreover, the alcoholic fermentations were carried out in two different conditions of aeration, factor known to directly interact with fermentative behavior of yeasts (Moreno et al., 1988; Mauricio et al., 1998).

Materials and methods

Grape samples. During three consecutive vintages (2011-2013), from different vineyards located in three Tuscan viticultural areas producing high quality wines (Brunello di Montalcino, Chianti Classico and Chianti Colli Aretini), lots of grape clusters of Sangiovese variety were harvested, paying much attention on cluster integrity. Each lot was constituted by 30 grape clusters for a total of 52 lots. Grape samples were transferred to laboratory at low temperature and stored at -20°C for a period of time not exceeding 1 months. Grape samples preparation methodologies prior analysis and vinifications are reported in Chapter 3.1 (3.1.3 Results, figure 1b).

Experimental vinifications. The laboratory vinifications were carried out using 450g of grapes each, in triplicates for each grape samples, inoculated with *Saccharomyces cerevisiae* Sc1 strain Technological condition of alcoholic fermentation/maceration are reported in Chapter 3.1 (3.1.2 Materials and methods).

For the experiments involving different yeast species, a lot of Sangiovese grapes harvested in 2011 was used. Chemical composition of the grape must is here reported: Total Anthocyanin contents (Tot grape A), 883 ± 2 mg/L; Total phenols (TP), 45.7 ± 0.9 ($O.D._{280}$); Glucose 121.4 ± 1.4 g/L, Fructose 128.4 ± 1.7 g/L, pH 3.81 ± 0.01, α-aminoacidic nitrogen conten (α-AN) 127.8 ± 2.8 mgN/L. Also in this case, triplicates micro-vinification were carried out in 500 mL flasks at 28°C for 14 days, using as inoculum *S. cerevisiae* Sc1 strain alone (pure inoculated fermentation) and *C. zemplinina* Cz1 strain followed by *S. cerevisiae* Sc1 after 5 days (sequential inoculated fermentation). Daily mixing was carried out under two aeration conditions: (i) open flask (aerated), (ii) close flask (non-aerated). The fermentation progress was monitored by weight loss, as above described.

Yeast inocula. Yeast inoculua of both *S. cerevisiae* and *C. zemplinina* preparation is reported in Chapter 3.1 (3.1.2 Materials and methods).

Kinetic parameters of alcoholic fermentation. By determining the CO2 loss during the 14 days of fermentation, it was possible to calculate the Fermentation vigor (FV, reported as grams of CO2 produced in the first 48 hours of fermentation by 100 mL of grape must) and the maximum rate of specific CO_2 production (Vmax, determined through non linear fit of CO_2 production kinetics to the Gompertz equation).

Chemicals and analytical determinations. Chemical and analytical determination on musts and wines are reported in Chapter 3.1 (3.1.2 Materials and methods).

Statistical analysis. All statistical calculations were performed by using Statistica software (version 7, StatSoft, Tulsa, OK, USA) and GraphPad Prism 5 (GraphPad Software, Inc., CA,USA).

Results and discussion

Single strain fermentation of different grape musts. The analytical characterization of the 52 Sangiovese musts is reported in table 1.

Table 1: Analytical characterization of the 52 Sangiovese musts from fermentation by *S. cerevisiae* strain Sc1. Frequency distribution, CV% = coefficient of variation

	Min	Median	Max	Mean	CV%
Musts					
Total sugar (g/L)	199.0	246.0	296.0	246.1	9.0
Tartaric acid (g/L)	3.48	4.82	7.02	4.79	14.5
α-AN (mgN/L)	75.0	151.9	560.0	189.8	60.0
pH	3.42	3.69	4.12	-	-

α-AN = α-amino acid nitrogen content

A remarkable variability in terms of α-amino acid nitrogen content (α-AN) was found, showing the highest coefficient of variation among the parameters here taken into consideration. The high variability in α-AN concentration was expected to affect fermentation kinetics, both in terms of specific maximum rate of CO_2 production (Vmax) and fermentation vigor (FV). Indeed, by fermenting the must showing the lowest and the highest α-AN, the fermentations kinetics were characterized by a FV from 2.0 to 7.5 gCO_2/100 mL) and values of Vmax from 16.8 to 82.7, respectively.

At the end of the alcoholic fermentation, the residual sugar concentration in all wines was under 4 g/L, except one sample showing 5 g/L, the other chemical parameters showing a variability quite consistent with the variability of the grape composition, with the highest value of acetic acid concentration occurring in the wine originated from the must with the lowest content of α-AN (tab. 2).

Table 2: Analytical characterization of the 52 Sangiovese experimental wines from fermentation by *S. cerevisiae* strain Sc1. Frequency distribution, CV% = coefficient of variation

	Min	Median	Max	Mean	CV%
Experimental Wines					
Residual sugar (g/L)	0.0	0.7	5.0	1.0	99.2
Ethanol (v/v, %)	11.3	13.8	16.3	13.9	8.8
Glycerol (g/L)	7.9	9.5	11.9	9.6	9.3
Acetic acid (g/L)	0.07	0.22	0.35	0.21	32.3
TA (g tartaric acid/L)	5.7	7.3	11.9	7.5	16.4
TP ($O.D._{280}$)	28.95	53.68	83.78	54.93	20.8
pH	3.38	3.61	4.10	-	-
Hyt+Tyr (mg/L)	7.2	30.3	66.5	30.2	46.9

TA = Total acidity; TP = Total phenols; Hyt+Tyr = Sum of hydroxytyrosol and tyrosol contents.

In all the experimental wines, a high variability was found in the concentrations of hydroxytyrosol and tyrosol, molecules known to be mainly originated by the yeast metabolism, (Table 2), although a unique strain of *S. cerevisiae* was used. Correlation analysis, performed in order to evaluate the influence of chemical parameters of the musts or kinetic parameters of alcoholic fermentation on the production of the two higher alcohols by the *S. cerevisiae* Sc1 strain used (tab. 3).

Table 3: Correlation analysis between the sum of hydroxytyrosol and tyrosol contents and some physicochemical parameters of Sangiovese grape musts and kinetic parameters of fermentations carried out by *S. cerevisiae* strain Sc1.

Coefficient	Total Sugars (g/L)	pH	α-AN (mgN/L)	Vmax	FV
Spearman r	0.25	-0.41**	-0.73***	-0.71***	-0.62***
Pearson r	0.25	-0.23	-0.57***	-0.63***	-0.60***

*p<0,05; **p<0,01; ***p<0,001

α-AN = α-aminoacidic nitrogen content of must; Vmax = specific maximum rate of CO_2 production; FV = fermented vigor expressed as gCO_2/100mL in first 48 hours.

The correlation analysis pointed out that the sum of hydroxytyrosol and tyrosol contents in wines showed significant inverse correlation with the initial α-AN content in grape musts, as well as with the maximum rate of specific CO_2

production and the fermentation vigor (tab. 3). A significant correlation was also found between the sum of hydroxytyrosol and tyrosol and pH of the grape musts. The figure 1, report graphically the correlation analysis between hydroxytyrosol and tyrosol (Hyt+Tyr) contents in the experimental wines and both α-AN and V max.

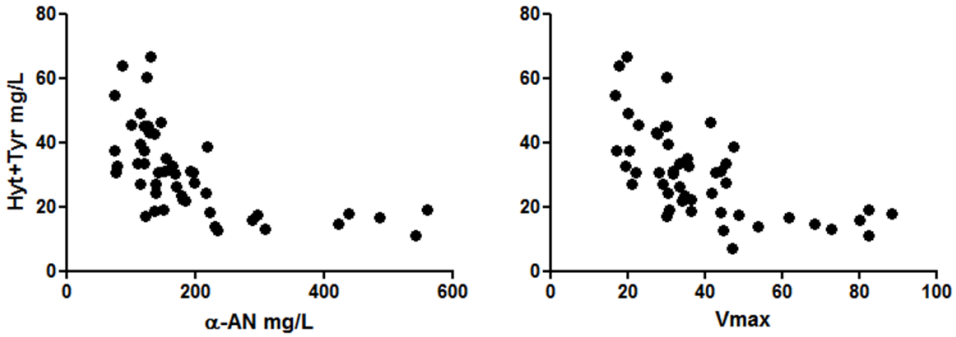

Figure1: Correlation between hydroxytyrosol and tyrosol (Hyt+Tyr) contents in the experimental wines and α-aminoacidic nitrogen content of must(α-AN) and specific maximum rate of CO_2 production (Vmax).

Alcoholic fermentation carried out by different yeast species. After 14 days of fermentation under different condition of aeration, residual sugars were below 2 g/L in all experimental wines, but fermentation kinetics were quite different, depending on the yeast species dominating the transformation process. Indeed, as shown in figure 2, alcoholic fermentations (AF) carried out by *S. cerevisiae* Sc1 alone were characterized by a higher fermentative activity. The presence of *C. zemplinina* Cz 1 caused a negative effect on fermentation kinetics in both aerated and non-aerated conditions.

As expected, aeration conditions improved significantly the values of fermentation vigor and Vmax (Table 4) in both pure and sequential inoculated fermentations.

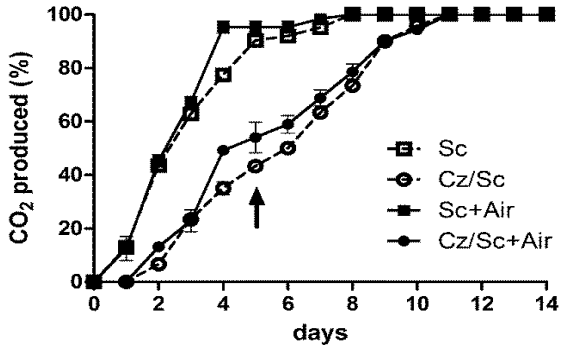

Figure 2: Alcoholic fermentation time courses in Sangiovese grapes fermented under laboratory conditions using S. cerevisiae alone (Sc) or sequential inoculum of C zemplinina and S. cerevisiae (Cz/Sc) and in combination with (+Air) or without aeration of grape must. (The black arrow indicates the inoculum of S. cerevisiae at the fifth day of must fermentation started by C. zemplinina). Values are reported as mean ± standard deviation.

As reported in Table 4, the experimental wines showed significant differences in the content of some fermentative metabolites, which resulted strongly affected by the yeast species. In particular, the wines from sequential inoculum of *C. zemplinina* and *S. cerevisiae* were characterized by high contents of glycerol and pyruvic acid. According to the two-way ANOVA analysis, acetic acid was significantly affected by both fermenting yeast species and aeration conditions, the lowest concentration being produced in fermentation carried out by *S. cerevisiae* alone under non-aerated conditions (0.11 ± 0.01 g/L).

Table 4. Fermentation kinetics and physicochemical characteristics of the experimental wines obtained by using *S. cerevisiae* alone (Sc) or sequential inoculum of *C. zemplinina* and *S. cerevisiae* (Cz/Sc) with or without aeration (+Air) of grape must and related statistical analysis (two-way ANOVA). Values are expressed as mean ± standard deviation.

	Experimental fermentations and wines				Source of variation		
	Sc	Cz/Sc	Sc+Air	Cz/Sc+Air	YI	Air	YI xAir
AF kinetics							
FV	4.5 ± 0.2	0.7 ± 0.0	4.8 ± 0.2	1.3 ± 0.0	***	*	ns
Vmax	25.6 ± 0.2	11.9 ± 0.3	34.8 ± 4.5	12.6 ± 1.2	***	*	ns
Exp Wines							
Ethanol (%, v/v)	13.7 ± 0.3	13.4 ± 0.1	13.8 ± 0.0	13.3 ± 0.3	ns	ns	ns
Glycerol (g/L)	10.4 ± 0.0	16.2 ± 0.1	10.1 ± 0.5	15.7 ± 0.1	***	ns	ns
Acetic acid (g/L)	0.11 ± 0.01	0.65 ± 0.01	0.13 ± 0.01	0.52 ± 0.02	***	**	**
TA ($g_{tart\ ac}$/L)	6.8 ± 0.1	6.5 ± 0.1	6.7 ± 0.1	6.6 ± 0.0	ns	ns	ns
TP (OD_{280})	44.5 ± 0.1	40.4 ± 1.8	40.5 ± 2.4	34.6 ± 3.2	*	*	ns
pH	3.70 ± 0.01	3.67 ± 0.00	3.71 ± 0.01	3.69 ± 0.02	ns	ns	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant

FV = fermented vigor expressed as gCO_2/100mL in first 48 hours; Vmax = specific maximum rate of CO_2 production; YI = effect of yeast inoculums; Air = effect of aeration; YI x Air = interaction.

Regarding the sum of hydroxytyrosol and tyrosol (Hyt+Tyr), the two-way ANOVA analysis highlighted significant interactions between yeast species and aeration conditions (tab. 5).

Table 5: Tyrosol and hydroxytyrosol in the experimental Sangiovese wines obtained by using *S. cerevisiae* alone (Sc) or sequential inoculum of *C. zemplinina* and *S. cerevisiae* (Cz/Sc) with (+Air) or without aeration of grape must and related statistical analysis (two-way ANOVA). Values are expressed as mean ± standard deviation.

	Experimental wines				Souce of variation		
	Sc	Cz/Sc	Sc+Air	Cz/Sc+ Air	YI	air	YIxAir
Hyt+Tyr (mg/L)	18.4 ± 3.3	5.8 ± 0.5	5.6 ± 0.6	3.0 ± 0.1	**	**	*
Tyr/Hyt ratio	3.4 ± 0.5	3.5 ± 0.1	2.8 ± 0.1	2.3 ± 0.2	ns	**	ns

*p< 0.05, **p<0.01; ***p<0.001; ns: non-significant

Hyt+Tyr = Sum of hydroxytyrosol and tyrosol contents; Tyr/Hyt ratio = tyrosol/hydroxytyrosol ratio; YI = effect of yeast inoculums; Air = effect of aeration; YI x Air = interaction.

In particular, the highest content of Hyt+Tyr was found in the wine produced by *S. cerevisiae* alone under non-aerated conditions, whereas the lowest amount was detected in sequentially inoculated fermentation under aerated conditions. Finally, it is worth mentioning that the tyrosol to hydroxytyrosol ratio was higher in non-aerated fermentations. This might suggest that aeration could have an effect on the enzymatic conversion of tyrosol into hydroxytyrosol.

Conclusion

The concentration of hydroxytyrosol and tyrosol (Hyt+Tyr) in the experimental wines was deeply influenced by yeast fermentation kinetics, that, in turn, is affected by grape must composition. In particular, higher amounts of Hyt+Tyr were produced when fermentations were slower, due to a lower concentration of amino acids in grape musts. Hence, this results strengthen the findings of other Authors, who observed that supplementing grape must with any kind of nitrogen lead to a reduction in higher alcohol levels in wines (Hernández-Orte et al., 2005). Moreover, by aerating must during alcoholic fermentation, *S. cerevisiae* Sc1 enhanced significantly its specific maximum rate of CO_2 production (Vmax) and fermentation vigor (FV), leading to lower levels of Hyt+Tyr and, once again, pointing out the key role of fermentation kinetics on the accumulation of these two molecules in wine.

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3.2. Contribution to congresses

AL51
VARIABILITY OF TYROSOL, HYDROXYTYROSOL AND TRYPTOPHOL CONCENTRATIONS IN WINES OBTAINED FROM SANGIOVESE GRAPES FOR *BRUNELLO DI MONTALCINO* WINE PRODUCTION, VINTAGE 2011

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Tyrosol and tryptophol are yeast secondary metabolites, formed in wine during alcoholic fermentation via the Ehrlich pathway from tyrosine and tryptophan, respectively (Monagas *et al.*, 2007), whereas hydroxytyrosol is a tyrosol derivative formed by hydroxylation of the aromatic ring (Piñeiro *et al.*, 2011). Tyrosol and hydroxytyrosol are reported to possess several health-enhancing activities, deriving from their free radical scavenging, anticarcinogenic, cardioprotective and antimicrobial properties. These specific properties are currently under discussion, as they were settled basically from in vitro assays and cannot be directly correlated with in vivo studies. Another source of discussion are the low concentrations of tyrosol, hydroxytyrosol found in wines (tyrosol ranging from 4 to 44 mg/L, hydroxytyrosol from not detectable to 5 mg/L in red wines) (Piñeiro *et al.*, 2011, Fernández-Mar *et al.*, 2012) although their beneficial effects will probably be also related to the synergistic effect with the polyphenols present in wine. Moreover, ingestion of red wine can promote endogenous hydroxytyrosol generation (Fernández-Mar *et al.*, 2012).
As concerns tryptophol, it induces sleep in human, besides it has been reported to have antimicrobial activity against a food-borne pathogen which causes gastrointestinal conditions (Gañan *et al.*, 2009) and inhibitory effects on the growth of enological lactic acid bacteria (García-Ruiz *et al.*, 2011). The levels detected in wine ranged from 0,02 to 10 mg/L (Monagas *et al.*, 2007). The wine content of these fusel alcohols seemed to be variable because it is affected by the must composition and pH (Monagas *et al.*, 2007). Moreover, since climatic parameter are known to influence the grape composition and, consequently, its amino acid content, the concentration of these bioactive compounds could be indirectly determined by the climate.
The aim of this study was to assess the variability of tyrosol, hydroxytyrosol and tryptophol concentrations of wines obtained fermenting Sangiovese grapes harvested in 2011 in the same oenological area. Grapes for *Brunello di Montalcino* wine production, cultivated in 5 different vineyards in the *Brunello di Montalcino* DOCG areal, were fermented under laboratory conditions utilizing a *Saccharomyces cerevisiae* strain isolated in a winery of the same areal. Fusel alcohol contents of the resulting wines were quite variable and not correlated with pH and free amino acid concentration in musts. Fusel alcohol concentrations were consistent with those reported by other Authors, tyrosol ranging from 6 to 37 mg/L, hydroxytyrosol from 6.8 to 26 mg/L and tryptophol from 6 to 16 mg/L. The two wines with significantly higher tyrosol concentrations showed also higher tryptophol contents than the other wines.

Keywords: wine, tyrosol, hydroxytyrosol, tryptophol, *Brunello di Montalcino*

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Variability of tyrosol, hydroxytyrosol and tryptophol concentrations in wines obtained from Sangiovese grapes for Brunello di Montalcino wine production, vintage 2011

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Introduction

Tyrosol, hydroxytyrosol and tryptophol are reported to possess several health-enhancing activities, deriving from their free radical scavenging, anticarcinogenic, cardioprotective and antimicrobial properties (Piñeiro et al., 2011; García-Ruiz et al., 2011) .

The wine content of these fusel alcohols is variable because it is affected by the must composition (Monagas et al., 2007) and indirectly by the climate (Gris et al., 2011).

Aim

To assess the variability of tyrosol, hydroxytyrosol and tryptophol concentrations of wines obtained fermenting Sangiovese grapes harvested in 2011 in the *Brunello di Montalcino* oenological area, during the 2011 vintage.

Materials and methods

Laboratory micro-vinifications were carried out in duplicate using grape juice with its skins, obtained by manual stemming and crushing of Sangiovese grape clusters harvested in 2011 and stored at -20°C. Grapes for *Brunello di Montalcino* wine production, cultivated in 5 different vineyards in the *Brunello di Montalcino* DOCG areal, were fermented utilizing a *Saccharomyces cerevisiae* strain from Department of Agricultural Biotechnology (University of Florence, Italy) culture collection.

Chemical analysis: fusel alcohols and aminoacids were both determined by high performance liquid chromatography (HPLC). Samples for aminoacid analysis were previously derivatised with *orto*-phtaldialdehyde. Free a-amino nitrogen content (FAN) was determined by the NOPA procedure (Dukes et al., 1998).

Results

✓Fusel alcohol concentrations were quite variable, tyrosol ranging from **6** to **37** mg/L, hydroxytyrosol from **6.8** to **26** mg/L and tryptophol from **6** to **16** mg/L.

✓The two wines with significantly higher tyrosol concentrations showed also higher tryptophol contents than the other wines.

✓Hydroxytyrosol content of the resulting wines was positively correlated with must pH.

✓Fusel alcohols contents were not correlated with precursor amino acid concentrations in musts.

✓Tyrosol and hydroxytyrosol contents were negatively correlated with FAN contents of the musts.

Correlation coefficients

	pH	AA Precursor	FAN
Tyrosol	0,50	- 0,49	- 0,64*
Hydroxytyrosol	0,71*	- 0,29	- 0,64*
Tryptophol	0,30	- 0,10	- 0,16

*significant at p<0.05

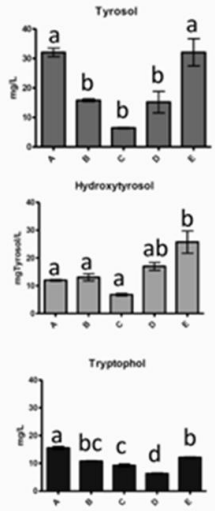

Fig.1: Fusel alcohols concentrations in wines (ANOVA, Tukey's test at p<0.05)

Conclusions

Experimental Sangiovese wines showed high hydroxytyrosol levels.

The production of tyrosol and hydroxytyrosol seems to be affected by nitrogen content of the musts.

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Variability of Tyrosol, Hydroxytyrosol and Tryptophol contents in Sangiovese wines produced by a single strain of Saccharomyces cerevisiae

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Tyrosol, hydroxytyrosol and tryptophol are reported to possess several health-enhancing activities, deriving from their free radical scavenging, anticarcinogenic, cardioprotective and antimicrobial properties.

Tyrosol and tryptophol are produced by yeasts during alcoholic fermentation from the catabolism of amino acids tyrosine and tryptophan, respectively, whereas hydroxytyrosol is produced by hydroxylation of its precursor tyrosol.

Tyrosol and tryptophol are described as quorum sensing molecules in different yeast species. These aromatic alcohols allow S.cerevisiae to respond to both cell density and nutritional state of the environment. The aim of this work was to investigate on the factors, other than yeast strain, affecting the accumulation of tyrosol, hydroxytyrosol and tryptophol in wine

Poster presented at Enoforum 2013, 7-9 May, Arezzo (Italy)

Attached Documents

Open and read the poster (pdf, 907 Kb)

Variability of Tyrosol, Hydroxytyrosol and Tryptophol contents in *Sangiovese* wines produced by a single strain of *Saccharomyces cerevisiae*

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Tyrosol, hydroxytyrosol and tryptophol are reported to possess several health-enhancing activities, deriving from their **free radical scavenging**, **anticarcinogenic**, **cardioprotective** and **antimicrobial properties** [3; 5].

Tyrosol and tryptophol are **produced by yeasts** during alcoholic fermentation from the catabolism of amino acids tyrosine and tryptophan, respectively [4], whereas hydroxytyrosol is produced by hydroxylation of its precursor tyrosol [5].

Tyrosol and tryptophol are described as **quorum sensing molecules** in different yeast species. These aromatic alcohols allow *S.cerevisiae* to respond to both **cell density** and **nutritional state of the environment** [1].

Aim

The aim of this work was to investigate on the factors, other than yeast strain, affecting the accumulation of tyrosol, hydroxytyrosol and tryptophol in wine

Materials and methods

Laboratory micro-vinifications were carried out in triplicate using grape juice with its skins and seeds, obtained by manual stemming and crushing of Sangiovese grape clusters harvested in 8 different vineyards (A-H) in three different viticultural areals of Tuscany (Brunello di Montalcino, Chianti Classico and Chianti colli Aretini) in two different vintages (2011 and 2012) and stored at -20°C. The 16 microvinifications were carried out by inoculating the musts with a single strain of *Saccharomyces cerevisiae*. All fermentations were completed in 15 days.

The yeast strain above mentioned was previously isolated from a commercial alcoholic fermentation of Sangiovese must (Brunello di Montalcino) and is included in the yeast culture collection of GESAAF, University of Florence, Italy.

Chemical analysis: fusel alcohols were determined by high performance liquid chromatography (HPLC) equipped with an UV-DAD detector at 280nm. N-α-aminocid content was determined by the NOPA procedure [2].

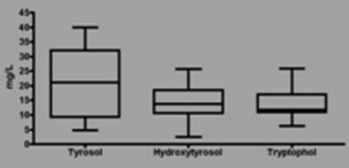
Results

The Sangiovese musts were characterized in terms of **pH**, **sugar** and total **N-α-amino acid** content. Fermentation progress was monitored daily through CO_2 loss to determine **fermentation vigor** (VF48) and **maximum rate of sugar consumption** (Vmax). Highest and lowest detected values of the parameters are reported in table 1.

Tab.1

	Chemical and kinetic parameters	
	min	max
pH	3.47 ± 0.00	4.03 ± 0.00
N-α-amino acid (mg/L)	69 ± 11	297 ± 14
VF 48 (gCO_2)	11.0 ± 0.0	52.0 ± 0.0
V max (gCO_2/day)	4.2 ± 0.0	22.6 ± 0.0

Values reported are expressed as mean ± standard deviation



High variability in terms of tyrosol, hydroxytyrosol and tryptophol contents were assessed in the wines as reported in fig.1.

In particular:

- Tyrosol content ranging from **4.7** to **40.0** mg/L.
- Hydroxytyrosol content ranging from **2.5** to **25.7** mg/L.
- Tryptophol content ranging from **6.3** to **25.9** mg/L.

As reported in Table 2:

Tab.2

	Correlations (r)						
	pH	N-α-amino acid (mg/L)	VF48 (gCO_2)	Vmax (gCO_2/day)	Tyrosol (mg/L)	Hydroxytyrosol (mg/L)	Tryptophol (mg/L)
Tyrosol (mg/L)	-0.11	**-0.59****	-0.19	**-0.54****	-	**0.82****	**0.43***
Hydroxytyrosol (mg/L)	0.08	**-0.65****	**-0.44***	**-0.70****	**0.82****	-	0.23
Tryptophol (mg/L)	-0.27	0.06	0.33	0.04	**0.43***	0.23	-

*p<0.05; **p<0.01

- **N-α-amino acid content** of the musts and **Vmax** of alcoholic fermentations are significantly and inversely correlated (p<0.01) with **tyrosol** and **hydroxytyrosol** contents of the wines.
- **VF48** is significantly and inversely correlated (p<0.05) with **hydroxytyrosol** contents of the obtained wines.
- No correlation was found between tryptophol concentration and chemical and kinetic parameters.
- **Tyrosol** is significantly and directly correlated with **hydroxytyrosol** (p<0.01) and **tryptophol** (p<0.05) content of the obtained wines.

Conclusions

The contents of tyrosol and hydroxytyrosol in wines fermented by a single strain of *Saccharomyces cerevisiae* are higher as the fermentation rate is slower.

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AL50
TYROSOL, HYDROXYTYROSOL AND TRYPTOPHOL ACCUMULATION IN WINE AS AFFECTED BY MICROBIAL ECOLOGY OF ALCOHOLIC FERMENTATION

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Yeasts metabolism is a source of bioactive compounds, such as tyrosol, hydroxytyrosol and tryptophol, possessing potential beneficial effects for human health such as some higher alcohols. Fusel alcohols, or fusel oils, derive from amino acid catabolism via a pathway that was first proposed by Felix Ehrlich. *Saccharomyces cerevisiae* can use tryptophan or tyrosine as the only source of cellular nitrogen, with the main products of their catabolism being tryptophol or tyrosol, respectively (Bordiga *et al.*, 2010). Their origin seems to be related mainly with nitrogen contents of musts. In this connection, it's well know that different yeasts species and strain can differ in the uptake and patterns of utilization of nitrogen sources (Large, 1986). For this reason the final contents of tyrosol, tryptophol and hydroxytyrosol in wine could be influenced by the microbial ecology of alcoholic fermentation. Some authors carried out few studies about the contribution of wild yeasts in terms of tyrosol and tryptophol contents in wine (Garde-Cerdàn *et al.*, 2006). Indeed, tyrosol showed greater concentration in the mixed inoculated fermentation, so it would seem that the wild flora contributed to its synthesis, whereas the concentration of tryptophol did not show significant differences. These Authors also found that, compared to other higher alcohol which are produced during the initial part of alcoholic fermentation, the contents of tyrosol and tryptophol became higher in final part of fermenting process.
The aim of this work was to compare two styles of fermentation in terms of tyrosol, hydroxytyrosol and tryptophol formation during alcoholic fermentation of Sangiovese grapes cultivated in *Brunello di Montalcino* enological areal. Two laboratory micro-vinifications were carried out by inoculating grape must with specific yeasts cultures: 1) *S. cerevisiae*, 2) *Candida zemplinina* followed by *S. cerevisiae*.
C. zemplinina is a yeast species frequently isolated during the early stages of must fermentations in different enological countries and is known to possess interesting properties: highly fructophilic, tolerance to high glucose concentrations, medium ethanol tolerance, high glycerol and low acetic acid production (Tofalo *et al.*, 2012). In view of these properties, this non-*Saccharomyces* yeast was proposed for enological use. Moreover in winemaking, the use of controlled mixed cultures of selected non-*Saccharomyces* and *Saccharomyces* strains, could have advantages over fermentations inoculated with pure cultures of *S. cerevisiae* and could thus lead to the production of wines with more desirable characteristics (Tofalo *et al.*, 2012).
In the present experimentation, HPLC analyses showed that the formation of tyrosol, hydroxytyrosol and tryptophol increased during progress of both fermentative processes. However, final amount of fusel alcohols was significantly lower in wine obtained with sequential inoculation than in wine obtained with pure culture of *S. cerevisiae*. In the former fermentation, tyrosol concentration reached 3 mg/L, tryptophol 1 mg/L and hydroxytyrosol 1,5 mg/L while in the other fermentation tyrosol, tryptophol, and hydroxytyrosol reached concentrations of 5 mg/L, 5,5 mg/L, 3 mg/L, respectively.

Keywords: wine, tyrosol, hydroxytyrosol, tryptophol, *Candida zemplinina*, *Brunello di Montalcino*

References:

Tyrosol, hydroxytyrosol and tryptophol accumulation in wine as affected by microbial ecology of alcoholic fermentation

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Introduction

Tyrosol, hydroxytyrosol and tryptophol are fusel alcohols, formed in wine during alcoholic fermentation by yeast amino acid catabolism (Fig. 1). Yeasts species and strain can differ in the uptake and patterns of utilization of nitrogen sources (Large, 1986). For this reason we can assume that the final contents of tyrosol, tryptophol and hydroxytyrosol in wine could be influenced by the microbial ecology of alcoholic fermentation.

Candida zemplinina is a yeast species frequently isolated during the early stages of must fermentations in different enological countries and is known to possess interesting enological properties. Moreover in winemaking, the use of controlled mixed cultures of selected non-*Saccharomyces* and *Saccharomyces* strains can have advantages over fermentations inoculated with pure cultures of *S. cerevisiae* and can thus lead to the production of wines with more desirable characteristics (Tofalo et al., 2012).

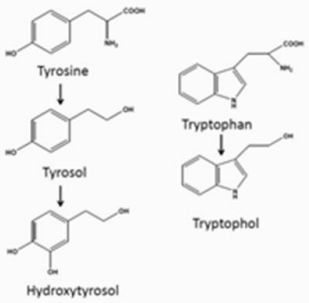

Fig.1: Patways of tyrosol, hydroxytyrosol and tryptophol formation.

Aim

To compare tyrosol, hydroxytyrosol and tryptophol formation during alcoholic fermentation of Sangiovese grapes cultivated in Brunello di Montalcino enological areal carried out by :
1) *Saccharomyces cerevisiae*, 2) *Candida zemplinina* followed by *Saccharomyces cerevisiae*.

Materials and methods

Laboratory micro-vinifications were carried out in triplicate using grape juice with its skins, obtained by manual stemming and crushing of Sangiovese grape clusters harvested in 2010 and stored at -20°C. Microvinification were carried out by inoculating the musts with specific yeast cultures: 1) *Saccharomyces cerevisiae*, 2) *Candida zemplinina* followed by *Saccharomyces cerevisiae* after 5 days.

Yeast strains, one of each above mentioned species, were previously isolated from Brunello di Montalcino wine fermentations and are included in the yeast culture collection of the Department of Agricultural Biotechnology, University of Florence, Italy.

Chemical analysis: fusel alcohols were determined by high performance liquid chromatography (HPLC) equipped with an UV-DAD detector at 280nm.

Results

✓HPLC analyses showed that the formation of tyrosol, hydroxytyrosol and tryptophol increased during progress of both fermentative processes (Fig. 2).

✓Final amount of fusel alcohols was significantly lower in wine obtained with sequential inoculation than in wine obtained with pure culture of *S. cerevisiae*.

Conclusions

Fusel alcohols accumulation seems to be negatively affected by the presence and the activity of *C. zemplinina* in sequential fermentations.

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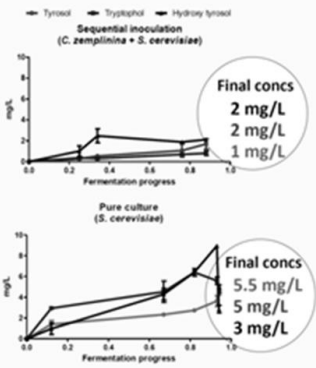

Fig.2: Fusel alcohols formation during progress of fermentative processes.

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TYROSOL, HYDROXYTYROSOL AND TRYPTOPHOL CONTENTS IN WINE AS AFFECTED BY FERMENTING YEAST SPECIES AND MUST AERATION

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Keywords: *Saccharomyces cerevisiae*; *Candida zemplinina*; bioactive compounds; higher alcohols.

Tyrosol, hydroxytyrosol and tryptophol, three higher alcohols produced by yeast during alcoholic fermentation, are reported to possess several health-enhancing activities, deriving from their free radical scavenging, anticarcinogenic, cardioprotective and antimicrobial properties [1; 2]. Some Authors reported that the final contents of tyrosol, tryptophol and hydroxytyrosol in wine could be influenced by the microbial ecology of the alcoholic fermentation [3]. However, also aeration of musts during alcoholic fermentation could influence the production of higher alcohols in wines [4]. In the present work, the concentration of the three bioactive molecules were determined in wines obtained under laboratory conditions by varying both yeast species (*Saccharomyces cerevisiae* alone or sequential inoculum of *Candida zemplinina* and *S. cerevisiae*) and aeration conditions of grape must during fermentation. Since several Authors, in literature, discussed about fermentative activity and production of higher alcohols by wine yeasts [5], the first part of the study was aimed to describe the kinetics of the experimental fermentations. The values of two kinetic parameters, fermentative vigor (FV48h) and maximum fermentation rate (Vmax), demonstrate that the presence of *C. zemplinina* had a negative effect on fermentation kinetics in both aerated and non-aerated conditions. Moreover, aeration improves significantly the values of FV48h and Vmax.

In the experimental wines, the highest concentrations of tyrosol, hydroxytyrosol and tryptophol were found when grape musts were fermented by *S. cerevisiae* alone, under non-aerated conditions. Two-way ANOVA demonstrated that the type of inoculum and aeration affected significantly tyrosol and hydroxytyrosol concentrations in wine, whereas the type of inoculum resulted the sole variable affecting tryptophol concentration

In conclusion, the concentrations of tyrosol, hydroxytyrosol and tryptophol in wine depend markedly on yeast species and to a lower extent on aeration conditions of must during alcoholic fermentation. Indeed, the dominance of *C. zemplinina* in the first stage of alcoholic fermentation strongly affects the contents of all the bioactive molecules here taken into consideration.

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Tyrosol, Hydroxytyrosol and Tryptophol contents in wine as affected by fermenting yeast species and must aeration

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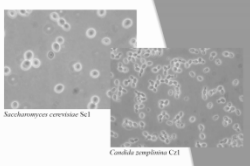
INTRODUCTION

Tyrosol (Tyr), hydroxytyrosol (Hyt) and tryptophol (Trp) are higher alcohols produced by yeasts during alcoholic fermentation. They are reported to possess several health-enhancing activities deriving from their free radical scavenging, anticarcinogenic, cardioprotective and antimicrobial properties [1; 2]. In wines, Tyr and Trp are produced from the catabolism of amino acids tyrosine and tryptophan, respectively [3], whereas Hyt is produced by hydroxylation of its precursor Tyr [2]. Some Authors reported that the final contents of Tyr, Hyt and Trp in wine could be influenced by the microbial ecology of alcoholic fermentation [4]. Moreover also aeration of musts could influence the production of higher alcohols in wines [5], as well as increase the fermentative activity of yeasts.

Between non-*Saccharomyces* yeasts, *Candida zemplinina* is a yeast species frequently isolated during the early stages of must fermentations and it is known to possess interesting oenological properties including medium ethanol tolerance [6].



Saccharomyces cerevisiae Sc1

Candida zemplinina Cz1

AIM

The concentrations of tyrosol, hydroxytyrosol and tryptophol were determined in wines obtained under laboratory conditions by varying both yeast species (*Saccharomyces cerevisiae* alone or sequential inoculum of *Candida zemplinina* and *S. cerevisiae*) and aeration conditions of grape must during fermentation.

Material and Methods

Laboratory microvinifications: triplicates of 450 g of Sangiovese grape juice with its skins and seeds (glucose + fructose, 248 ± 4 g/L; pH, 3.81 ± 0.15; α-aminoacidic N, 128 ± 3 mgN/L) inoculated with specific yeast cultures:

- pure inoculated fermentation, *S. cerevisiae* – Sc1 (106 cell/mL),
- sequential inoculated fermentation, *C. zemplinina* – Cz1 (106 cell/mL) followed by *S. cerevisiae* – Sc1 (106 cell/mL) after 5 days.

Yeast strains, one of each above mentioned species, were previously isolated from spontaneous Brunello di Montalcino wine fermentations and are included in the yeast culture collection of GESAAF, University of Florence, Italy.

The inoculated musts were incubated at 28°C for 14 days. The fermentation progress was evaluated by CO_2 loss, weighting flasks after mixing by gentle rolling one time/day (1 minute) in two conditions:

- open flask (aerated),
- close flask (non-aerated).

Experimental plan and **sample denominations** are reported in table 1.

Table 1. Experimental plan and sample denominations

	Pure inoculated fermentation	Sequential inoculated fermentation
Non-Aerated	Sc	Cz/Sc
Aerated	Sc + Air	Cz/Sc + Air

Kinetic parameters:

- Fermentation vigor (FV48h) - grams of CO_2 produced in the first 48 hours of fermentation.
- Maximum fermentation rates (Vmax) - non linear fit of CO_2 production kinetics to the Gompertz equation.

Chemical analysis: glucose, fructose and ethanol - HPLC [7]; α-aminocid N - NOPA procedure [8]; Tyr, Hyt and Trp - HPLC-UV-DAD detector at 280nm [9].

Statistical analysis: two-way ANOVA by GraphPad Prism 5 software.

RESULTS

1. Kinetic parameters

• All the fermentations were **completed** (residual sugar < 2 g/L) within the maceration period, but with **different kinetics**.

• Alcoholic fermentations carried out by ***S. cerevisiae*** **alone** were characterized by a **higher fermentative activity** (fig. 1).

Table 2. Values of **Fermentation Vigor, FV48h** (gCO_2) and **Maximum fermentation rates, Vmax** ($\%CO_2$/days) (mean ± standard deviation). Effect of type of inoculum (I), Aeration (Air) and I x Air interaction, ns: non-significant (two-way ANOVA).

	Kinetic parameters of AF				Source of variation		
	Sc	Cz/Sc	Sc + Air	Cz/Sc + Air	I	Air	I x Air
FV48h	13.5 ± 0.7	2.0 ± 0.0	14.5 ± 0.7	4.0 ± 0.0	***	*	ns
Vmax	25.6 ± 0.2	11.9 ± 0.3	34.8 ± 4.5	12.6 ± 1.2	**	*	ns

*$p<0.05$, **$p<0.01$, ***$p<0.001$

• The presence of ***C. zemplinina*** had a **negative effect** on **FV48h** and **Vmax** in both aerated and non-aerated conditions (Tab. 2).

• The **Aeration** of must **improves** significantly the values of **FV48h** and **Vmax** (Tab. 2).

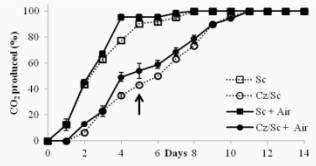


Figure 1. Alcoholic fermentations kinetics. Sc (□), pure inoculated fermentation in non-aerated conditions; Cz/Sc (○), sequential inoculated fermentations in non-aerated conditions; Sc+Air (■), pure inoculated fermentation in aerated conditions; Cz/Sc+Air (●), sequential inoculated fermentations in aerated conditions.
The **black arrow** indicates the inoculum of *S. cerevisiae* at the fifth day of must fermentation initiated by *C. zemplinina*.

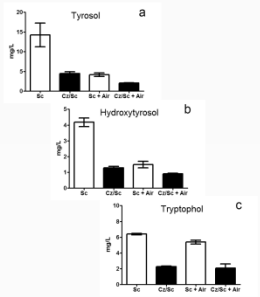


Figure 2. Contents of tyrosol (a), hydroxytyrosol (b) and tryptophol (c) in the experimental wines

2. Tyrosol, hydroxytyrosol and tryptophol concentrations

• The **highest concentrations** of Tyr, Hyt and Trp were found in wines from grape must fermentations inoculated with ***S. cerevisiae*** **alone**, under **non-aerated conditions** (fig. 2; Tab. 3).

Table 3. Tyrosol, hydroxytyrosol, tryptophol contents (mg/L) and tyrosol/hydroxytyrosol ratio (Tyr/Hyt) in wines (mean ± standard deviation). Effect of type of inoculum (I), Aeration (Air) and I x Air interaction, ns: non-significant (two-way ANOVA).

	Contents in wines (mg/L)				Source of variation		
	Sc	Cz/Sc	Sc + Air	Cz/Sc + Air	I	Air	I x Air
Tyrosol	14.3 ± 3.0	4.5 ± 0.4	4.2 ± 0.4	2.1 ± 0.1	**	**	*
Hydroxytyrosol	4.2 ± 0.3	1.3 ± 0.1	1.5 ± 0.2	0.9 ± 0.0	***	***	***
Tyr/Hyt	3.4 ± 0.5	3.5 ± 0.1	2.8 ± 0.1	2.3 ± 0.2	ns	**	ns
Tryptophol	6.4 ± 0.1	2.3 ± 0.1	5.4 ± 0.3	2.1 ± 0.5	***	ns	ns

*$p<0.05$, **$p<0.01$, ***$p<0.001$

• Significant **interactions** between the type of inoculum and the aeration exist for **Tyr** and **Hyt** concentrations in wines (Tab. 3).

• The **ratio** Tyr/Hyt was **higher** in **non-aerated fermentations**. This could indicates that **aeration** affect the enzymatic **conversion** of **Tyr** into **Hyt** (Tab. 3).

• The **type of inoculum** seems to be the sole variable affecting **Trp** concentrations. **Higher concentrations** were found in **pure inoculated fermentations** in both aerated and non-aerated conditions (Tab. 3).

CONCLUSION

The concentrations of **tyrosol**, **hydroxytyrosol** and **tryptophol** in wine **depend markedly on yeast species** and, to a lower extent, on aeration conditions of must during alcoholic fermentation. Indeed, the **dominance of *C. zemplinina*** in the first stage of alcoholic fermentation **strongly affects the contents** of all the bioactive molecules here taken into consideration.

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General conclusions

General conclusions and significance of the study

This thesis pointed out the variability in contents of health promoting compounds, derived from grape and produced by yeasts during the alcoholic fermentation, in Sangiovese experimental wines. It is worth to stress that in order to evaluate the impact of certain variables on the accumulation of such molecules in wines, it was necessary to set up a method consisting in standardized procedures, starting from sampling operations and storage of grape samples, to winemaking practices and finished wines' analysis. By standardizing such operations, it was possible to separate the effects of different variables in order to understand more in deep their contribution to wine composition. Conversely, at commercial level, many other factors may affect their concentration in wines, thus hiding or enhancing the effects of the variables here studied.

In particular, the thesis was articulated in two macro topics on the basis of origin of the health promoting compounds studied. Thus, the first macro topic was focused on grape derived classes of molecules: anthocyanins, flavonols and flavan-3-ol monomers which are classes of molecules biosynthetically related and are also responsible of sensory qualities of wines. The second macro topic was focused on three higher alcohols produced by yeasts during the alcoholic fermentation: tyrosol, hydroxytyrosol and tryptophol.

The first experimental study reported in this thesis considered the variability in contents and in partitioning of anthocyanins, flavonols and flavan-3-ol monomers in Sangiovese wines produced by grapes cultivated in different viticultural areas of Tuscany in three consecutive vintages (2011, 2012, 2013). At first, vintage year seemed to be the variable able to discriminate grape samples on regards to their phenolic contents. Despite experimental wines composition reflected those of native grapes, no univocal effect of vintage was found on anthocyanin and flavonol contents in wines. This seemed to suggest an interactive effect between viticultural area and vintage in determining grape and consequently wine composition. However, the contents of falvan-3-ol monomers in the experimental wines resulted generally affected by vintage year, with higher amounts found in 2013 experimental wines. Moreover, lower values of trisubstituted anthocyanins was reported for 2013 experimental wines.

In the second study, the effect of air temperature on the accumulation of grape derived health promoting compounds in experimental wines was studied by vinification of Sangiovese grapes harvested in the same vineyard in four consecutive vintages. The air temperature and the heat accumulation characterizing each vintage deeply affected the biosynthesis of anthocyanins in grapes, thus influencing anthocyanin partitioning in the corresponding experimental wines. In particular, the higher air temperature in post veraison experimented by grapes of 2011 and 2012 vintages was traduced in higher relative abundance of trisubstituted anthocyanins in experimental wines.
In the third study, vine vigor resulted a variable capable of determining the composition of grapes and experimental wines by affecting contents and partitioning of anthocyanins, flavonols and falvan-3-ol monomers. This was probably related to different temperatures and sun exposition experimented by grapes as a consequence of vine vigor. Sangiovese experimental wines obtained from low vigor grapes showed higher contents of polymeric and non polymeric anthocyanins, total flavonols and quercetin glycosides and lower flavan-3-ol monomer contents. Moreover, reduction of vine vigor seemed to affect anthocyanins profiles of grapes and wines, increasing the values of trisubstituted to disubstituted anthocyanins ratio.
Finally, the effect of different yeast species involved in the alcoholic fermentation was studied. Yeasts were able to affect the contents of flavan-3-ol monomers in Sangiovese experimental wines as a consequence of their specific rate of ethanol production. Moreover, alcoholic fermentation carried out by *Saccharomyces cerevisiae* alone or by a sequential inoculum of *Candida zemplinina* followed by *S. cerevisiae* lead to a different partitioning of anthocyanins and quercetin and its glycosides in the experimental Sangiovese wines.

In the second macro topic, the effects of grape composition, winemaking practices and yeast species on tyrosol, hydroxytyrosol and tryptophol was studied. The accumulation of the three higher alcohols in experimental wines ranged widely and their amounts were mainly influenced by the presence of different species during the alcoholic fermentation. Moreover, tyrosol and hydroxytyrosol contents were deeply influenced by the fermentation kinetics. Higher amounts of tyrosol and hydroxytyrosol were found in experimental wines deriving from the fermentation carried out by a single strain of *S. cerevisiae* and characterized by slow fermentation kinetics. In particular, both

the α-amino acid nitrogen content of must and aeration during the alcoholic fermentation influenced the fermentation rates and negatively affected the contents of tyrosol and hydroxytyrosol in experimental wines. The sequential inoculated fermentation carried out by *C. zemplinina* followed by *S. cerevisiae* lead to lower contents of tyrosol and hydroxytyrosol.

On the basis of the results here obtained, the accumulation of health promoting compounds in Sangiovese wines resulted to be influenced by a series of variables affecting grape composition and yeast species involved in the alcoholic fermentation. In this work, the effect of viticultural area was found to be deeply combined to the effect of the vintage, thus remarking the well known importance of *Terroir*, in its widest meaning, in determining wine composition. However, when referred to grapes from a single vineyard, it emerged that air temperature, vigor and yeast species involved during the alcoholic fermentation were variables affecting the accumulation and partitioning of health promoting compounds in experimental wines. In general, grapes exposed to higher number of days characterized by temperatures between 16-22°C during the vintage (from blooming to harvest), grapes from low vigor vines and fermentation characterized by lower kinetics carried out by a single strain of *S. cerevisiae* led to a higher accumulation of health promoting compounds in the experimental wines.

In conclusion, the results here reported improve the knowledge on variability of grape composition and the incidence of yeast fermentative behavior in determining the accumulation of different health promoting compounds in Sangiovese wines. Indeed, in a determinate site, the management of viticultural practices according to the climatic conditions of vintage and the control of yeast populations involved during the alcoholic fermentation can be considered useful tools in order to obtain wines richer in health promoting compounds which are also potentially involved in quality and longevity of the final product.

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