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Nikolaos Kontoudakis

Grape phenolic maturity; Determination methods and consequences on wine phenolic composition

Ph Doctoral Thesis

Directed by Dr. Fernando Zamora and Dr. Joan Miquel Canals

Departament de Bioquímica i Biotecnologia

UNIVERSITAT ROVIRA I VIRGILI

Tarragona
2010

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Fernando Zamora Marín i Joan Miquel Canals, professors del Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili,

CERTIFIQUEM:

Que aquest treball, titulat “Grape phenolic maturity; Determination methods and consequences on wine phenolic composition”, que presenta Nikolaos Kontoudakis per a l’obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia d’aquesta universitat i que acompleix els requeriments per poder optar a Menció Europea.

Tarragona, 6 de Maig de 2010

Fernando Zamora Marín Joan Miquel Canals

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Acknowledgements

Original Greek	English Translation
Σα βγείς στον πηγαιμό για την Ιθάκη,	When you set out on your journey to Ithaca,
να εύχεσαι νάναι μακρύς ο δρόμος,	pray that the road is long,
γεμάτος περιπέτειες, γεμάτος γνώσεις.	full of adventure, full of knowledge.
....	...
Η Ιθάκη σ' έδωσε το ωραίο ταξίδι.	Ithaca has given you the beautiful voyage.
Χωρίς αυτήν δεν θά βγαινες στον δρόμο.	Without her you would have never set out on the road.
Αλλο δεν έχει να σε δώσει πια.	She has nothing more to give you.
Κι αν πτωχική την βρεις, η Ιθάκη δεν σε γέλασε.	And if you find her poor, Ithaca has not deceived you.
Ετσι σοφός που έγινες, με τόση πείρα,	Wise as you have become, with so much experience,
ήδη θα το κατάλαβες η Ιθάκες τι σημαίνουν.	you must already have understood what Ithacas mean.
Κ. Καβάφης, 1911.	**C. Cavafis, 1911.**

As Odysseys everyone has its 'Ithaca'; put its targets and want to make them true. One of mine 'Ithaca's' was to realize a doctorate in Oenology. This target now is almost realized. It wasn't an easy 'travel', however, it offers my many experiences and it was full of emotions. At this long trip, I wasn't alone. Many persons help me all this time to get here, where I am now. The contact with all those people, from the beginning until now, filled me with strength and courage and I am grateful to them.

First of all, I would like to thank me first contacts in Spain, when I was still in Greece and I was looking over for a PhD program. I contacted first with the university of Salamanca and then with the university of Rovila I Virgili. They really tried to understand me necessities and give me the possible alternatives to satisfy them. After those contacts I decided to go to Tarragona and begun to find a group to work with them. I came in touch with my actual supervisors expressing my interest in wine technology. They trust me from the beginning and they encourage me to every step of me work. Of course it was little bit difficult at least the first months, as I didn't speak Spanish, but

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finally we could communicate with any problem, with the build of a relation of respect and understanding. For that reason I would like to express my gratitude to make me part of the group.

From the first days in the laboratory the technicians of our department as also the workers of the experimental winery of the university were a great support in every moment. Actually, they solved every problem that appears and without their help it would have been difficult to make any work. For that reason I would like to thank them, as also the actual secretariat of biotechnology department for their support. Moreover, I would like to thank the professors of the department that they were there to give me advises at every question that I had.

My instants in Port, permits me to know the other part of Iberian Peninsula. I become in contact with the group of professors and students of the department of chemistry that works with polyphenols. They make a really good work and permit me to have a better knowledge about this group of compounds. They accept me and made my stay there comfortable and I am glad that I visit their town and laboratory.

These lines serve to show my appreciation to all the friends and colleagues that I made in Tarragona. With my arrival in the town, I began to know people, form Catalonia, Spain, Europe, Latin America, Africa…The most of them doctorates like me, some of them colleagues of the laboratory, but all good friends. We shared good and bad moments, in the work, out of the work and we developed relationships that I hope their will withstand through the time.

With one of these relationships we share more than a friendship. She became my partner, 'mi pequeñita', who stand by me every moment. She is a follower traveler for the next 'Ithacas'.

Finally, I would like to thanks my family, especially my parents, my brother and my grandfathers, for their support all these years. Great part of this endeavor belongs to them and it would have been impossible without their encouragement and perseverance.

Now let the 'muse' to narrate us the records…

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Dedicated to

my parents, my brother and mi pequeñita.

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I.INTRODUCTION

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INTRODUCTION

Wine has a long recorded history that goes back more than 7.5 thousand years. The earliest suspected wine residues come from the early to mid-fifth millennium b.c. – Hajji Firuz Tepe, in the northern Zagros Mountains of Iran (Duque et al. 2005). In ancient times, wine was considered to be a magical, spontaneous gift of nature and has been highly regarded by high society, witnessing essential in any important event.

Nowadays, wine is an integral component of the culture of many countries and is expanding in new ones. Consumers in the traditional producer countries as also in the 'new world', demand for high quality wines. Especially, for the red wines consumers are looking to find wines with deep red color, full body, soft tannins and fruit scents. To obtain these characteristics one of the essential parameters is that grapes have achieved a high grape phenolic maturity.

This introduction focuses on the importance of grape phenolic compounds in wine quality, the importance of predicting phenolic maturity and the probable consequences that a high phenolic maturity can produce in wine characteristics.

1. Phenolic composition of grapes and wines

Phenolic compounds represent a large group of molecules which are present in grape and wine with an essential role in enology. The phenolic compounds of the grapes contribute to wine organoleptic characteristics such as color, taste, astringency and bitterness and their capacity for aging (Ribéreau-Gayon et al. 2006). The antioxidant properties of phenolic compounds have also been associated the moderate consume of red wine with health-promoting effects (Pitsavos et al. 2005). This phenomena was initially known as ''French paradox'' (Renaud and de Lorgeril 1992). More recently, a special interest has been devoted to phenolic compounds for their anticarcenogenic ability (Block 1992) and the neuroprotective effect (Ma et al. 2010).

From a chemical standpoint, the phenolic compounds dispose a benzenic ring directly attached by one or more hydroxyl groups. Their reactivity is due to the acid character of the phenolic function and to the nucleophilic character of the benzene ring (Monagas et al. 2005).

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Phenolic compounds can be classified in various ways. The most common classification is based on the distinction in flavonoid compounds with a C3-C6-C3 skeleton and non-flavonoid compounds. Each group is further divided into several families (Figure 1), with share structural features that confer specific properties such as color, aroma and taste (Cheynier et al. 2006).

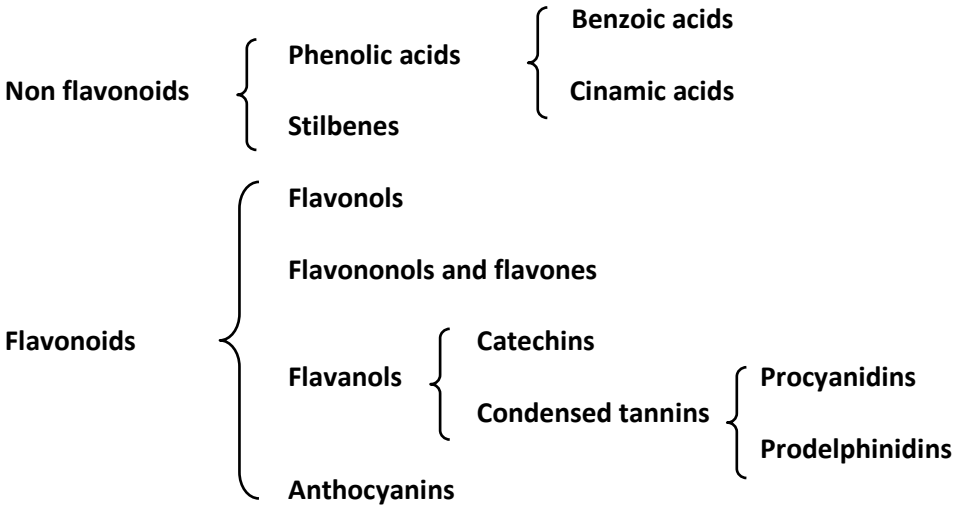

Figure 1. Classification of phenolic compounds (adapted from Zamora 2003).

Grapes have flavonoid compounds mainly in the skin, seeds and stem and non-flavonoid mainly in pulp but also in the other parts of the grapes (Zamora 2003).

1.1. Non- flavonoid

The non-flavonoid compounds present in grapes and wine are phenolic acids and stilbenes.

1.1.1. Phenolic acids

Phenolic acid compounds are divided into hydroxybenzoic and hydroxicinnamic acids. Hydroxybenzoic acids are derived from benzoic acid and possess a C6-C1 skeleton. Grapes

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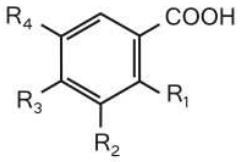
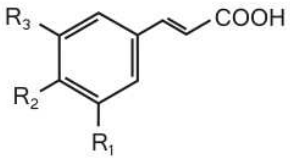
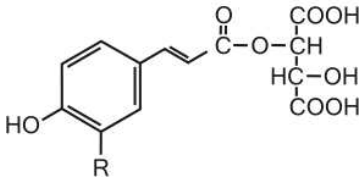
contain gallic acid in the pulp (Lu and Foo 1999), and ester-linked form with flavan-3-ols in seeds (Su and Singleton 1969). However, other hydroxibenzoic acids (Figure 2) and their derivatives can be found in wine (Monagas et al. 2005).



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



Hydroxycinnamic acid	R_1	R_2	R_3
p-Cumaric	H	OH	H
Caffeic	OH	OH	H
Ferulic	OCH_3	OH	H
Sinapic	OCH_3	OH	OCH_3



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

Figure 2. Phenolic acid and derivates (adapted from Monegas et al. 2005).

On the other hand, hydroxicinnamic acids are composed of a benzene ring with substitutions at carbons C6-C3. There are located in the vacuoles of the skin and pulp cells in the form of tartaric esters (Ribéreau-Gayon 1965). The principal hydroxicinnamic acids present at grapes are the caftaric, cutaric and fertaric acids in *trans* form, although small quantities of the *cis* isomers can also be detected (Singleton et al. 1978). These tartaric esters are present in wine as well as their hydrolyzed form that gives rise to free hydroxicinnamic acids (Sommers et al. 1987). Finally, a number of hydroxicinnamic acids derivates have been observed in wine (Rentzsch et al. 2009). Figure 2 present the most important compounds of this group in grapes and wine.

1.1.2. Stilbenes

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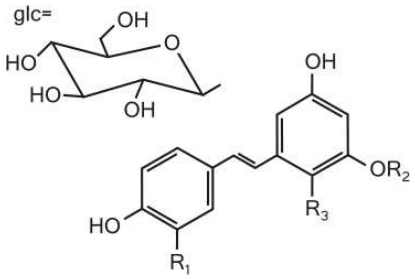
Stilbenes are formed by an ethene double bond substituted with a benzene ring on both carbon atoms of the double bond. The most abundant is the *trans*-resveratrol. Its chemical structure and its glucoside derivatives are shown in Figure 3.

Stilbenes can also occur in oligomeric and polymeric form and have been identified in grapevine and wine (Rentzsch et al. 2009).

It appears that the plant synthesize these compounds as a defense respond to fungal infection and ultraviolet light irradiation, in leaves, roots and berry skin (Langcake and Pryce 1976, Jeandet et al. 1991), although there have been detected in other parts of berry especially in seeds of muscadine grapes (*Vitis rotundifolia*) (Ector et al. 1996).



Stilbene	R_1	R_2	R_3
Trans-resveratrol	H	H	H
Trans-resveratrol-3-*O*-glucoside (piceid)	H	glc	H
Trans-resveratrol-2-*C*-glucoside	H	H	glc
Trans-astringin	OH	glc	H

Figure 3. Stilbenes (adapted from Monegas et al. 2005).

Stilbenes have no significance in the organoleptic characteristics of wine, however, due to their potential antioxidative, anticarcinogenic and neuroprotective effects are considered to play a relevant role in human health (Pérez-López et al. 2009).

1.2. Flavonoids

Flavonoids are characterized by a basic skeleton of 15 carbon atoms (C6-C3-C6) of 2-phenyl-benzopyrene type and are divided in four major subclasses on the base of the oxidation state of the pyran ring: the flavonols, the flavanonols and flavones, the flavanols and the anthocyanins

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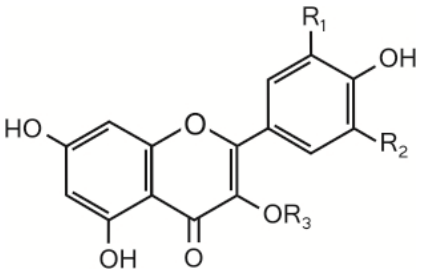
(Cheynier et al. 2000). Among them, flavanols and anthocyanins are the most abundant in grape and wine and are particularly important to the quality of red wine.

1.2.1. Flavonols

Flavonols are yellow pigments that act as protectors against the ultraviolet light irradiation (Flint et al. 1985). They are mainly found at grape skin (Cheynier and Rigaud 1986), although they have also been detected in the pulp (Pereira et al., 2006). In grapes they are present mainly in the glycosilated form of the four main aglycones (Figure 4). In wine the aglycone form is also observed, but it is probably originated by the hydrolysis of the glycosilated forms during the winemaking process (Cheynier et al. 2000).



Flavonol	R_1	R_2	R_3
Kaempferol	H	H	H
Kaempferol-3-*O* -glucoside	H	H	glc
Kaempferol-3-*O* -galactoside	H	H	gal
Kaempferol-3-*O* -glucuronide	H	H	gluc
Quercetin	OH	H	H
Quercetin-3-*O* -glucoside	OH	H	glc
Quercetin-3-*O* -glucoronide	OH	H	gluc
Myricetin	OH	OH	H
Myricetin-3-*O* -glucoside	OH	OH	glc
Myricetin-3-*O* -glucuronide	OH	OH	gluc
Isorhamnetin	OCH_3	H	H
Isorhamnetin-3-*O* -glucoside	OCH_3	H	glc

gal=galactose; glc=glucose; gluc=glucuronide acid

Figure 4. Flavonol (adapted from Monegas et al. 2005).

1.2.2. Flavanonols and flavones

Flavanonols astilbin and engeletin are the most common compounds of this group (Figure 5). They have been identified in the skin and wine of white grapes (Trousdale and Singleton, 1983), in grape pomace (Lu and Foo, 1999), in grape stems (Souquet et al., 2000) and also in red wine (Vitrac et al., 2001).

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Flavones present a similar structure with flavonols (Figure 5). The compounds that are detected in wine are apigenin, baicalein and luteolin (Wang and Huang, 2004).

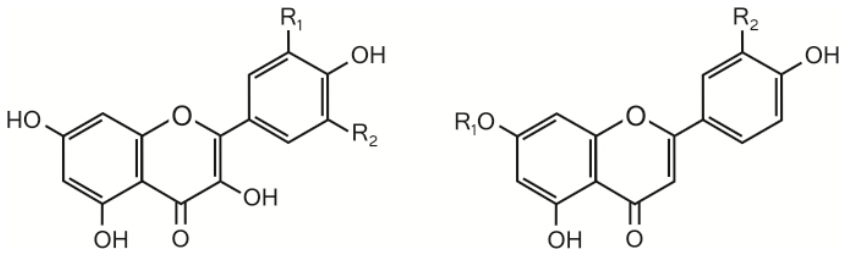

Figure 5. Structure of flavononols and flavones.

1.2.3. Flavanols

Flavanols or flavan-3-ols are found in high concentrations in grapes seed, skin and stem (Escribano-Bailón et al. 1995, Prieur et al. 1994, Souquet et al. 1996, Souquet et al, 2000, Su and Singleton 1969). They play an important role in wine organoleptic characteristics and they are found on monomeric, oligomeric and polymeric form.

Flavan-3-ols monomers are often referred as "catechins" and are distinguished by the degree of hydroxylation of the B ring (Figure 6). In their structure they have two asymmetric carbons (C2 and C3), which makes possible the existence of four optical isomers for each catechin, the (+)/(-) catechin and (+)/(-) epicatechin. Moreover, the present of a third OH group at the aromatic B ring leads to the corresponding (+)/(-) gallocatechin and (+)/(-) epigallocatechin. In nature, these monomers are found as such, although the methylated, glycosylated or acylated derivatives of them can also be found, mainly the esterified derivatives by gallic acid at C3 position.

In grapes the major flavan-3-ols monomers are (+)-catechin, its isomer (-)-epicatechin and in a relative high concentration (–)-epicatechin-3-gallate (Cheynier et al. 2000). Also, to a lesser concentration, (+)-gallocatechin (Piretti et al., 1976), (-)-epigallocatechin (Escribano-Bailón et al. 1995), (+)-catechin-3-gallate and (+)-gallocatechin-3-gallate (Lee and Jaworski et al. 1990)

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are detected. Nevertheless, flavan-3-ols monomers represent only a small proportion inasmuch as the major part of flavan-3-ols is in oligomeric and polymeric form (Zamora 2003).

Flavan-3-ols oligomers and polymers are also known as proanthocyanidins or condensed tannins. The term tannin refers to the capacity to 'tan' animal hide, which is done by reacting with the proteins of the animal's skin. When condensed tannins heated under mineral acid solution they are hydrolyzed to red anthocyanidin pigments, as result of the acid-catalyze cleavage of the interflavanic bond, hence the term proanthocyanidins. Proanthocyanidins that are hydrolyzed to cyanidins are known as procyanidins and are composed from (+)-catechin and (-)-epicatechin. Nevertheless, in the event that the polymers consist of (+)-gallocatechin and/or (-)-epigallocatechin units, the acid hydrolysis results in delphinidins and therefore is defined as prodelphinidins (Porter et al. 1986).

In function of the interflavanic bond nature, proanthocyanidins are distinguished to B-type and A-type (Figure 6). B-type are those in which links are established between carbon 4 of a flavan-

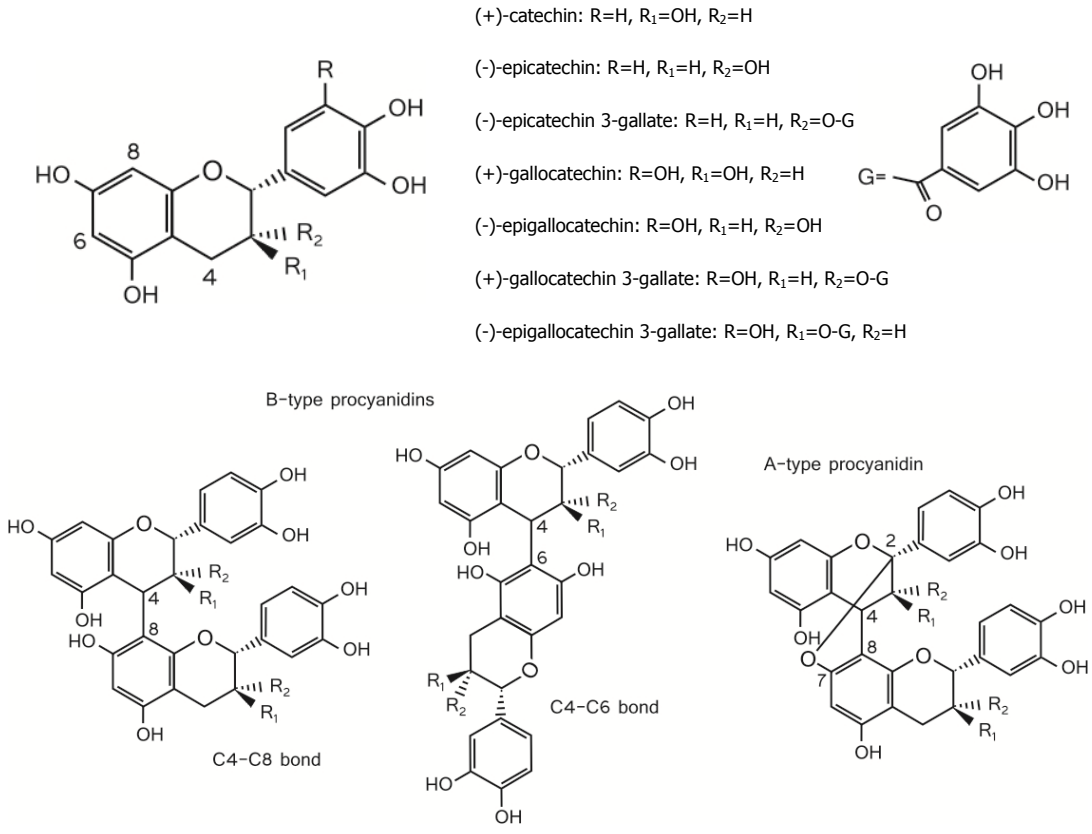

Figure 6. Flavan-3-ol monomers and dimers of type-A and type-B (adapted from Terrier et al. 2009).

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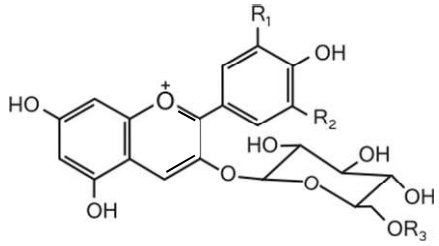
3-ol unit (considered ‘‘upper subunit’’ or ‘‘extension’’) and carbons 8 or 6 of another monomer (called ‘‘lower subunit’’). From the B-type proanthocyanins identified in grapes, B1, Ec-(4β→8)-Cat, has been reported to be the main oligomer in skins, whereas all C4→C8 proanthocyanidin dimers are usually found in seeds, with the B2, Cat-(4β→8)-Ec, as the most abundant one (González-Manzano et al. 2004).

Proanthocyanidins of type-A, that are compounds with an C4→C8 or C6 bond and an additional ether type C2→C7 or C5 bond, have been mention to be present in grapes and wine (Vivas de Gaulejaac et al. 2001).

1.2.4. Anthocyanins

Anthocyanins (from the Greek anthos-*ανθός* flower and kyanos-*κυανός* purple) are mainly located in the grape skin tissue of red grapes and are responsible for red wine color (Zamora 2003). Also there are some few *teinturier* varieties that also contain anthocyanins in the pulp (Castillo-Muñoz et al. 2009).



Anthocyanidin	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

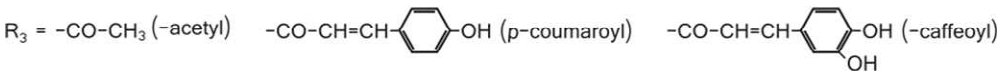


Figure 7. Anthocyanins (adapted from Monagas et al. 2005).

The anthocyanins are water-soluble pigments, consisting of an aglycon (or anthocyanidin), derived from the ion flavylium or phenyl-2-benzopyrilium variously substituted, that is attached to one or more sugar molecules, which in turn can be esterified with different organic acids. The

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anthocyanins of grapes and wine from *Vitis vinifera* are the 3-*O*-monoglucosides and the 3-*O*-acylated monoglucosides of the anthocyanidins, delphinidin, cyanidin, petunidin, peonidin and malvidin. They differ from each other by the number and position of the hydroxyl and methoxyl groups located in the B-ring of the molecule as shown in Figure 7 (Monagas. and Bartolomé 2009). Moreover, pelargonidin-based anthocyanins have also been detected at red grapes and wines (Castillo-Muñoz et al. 2009).

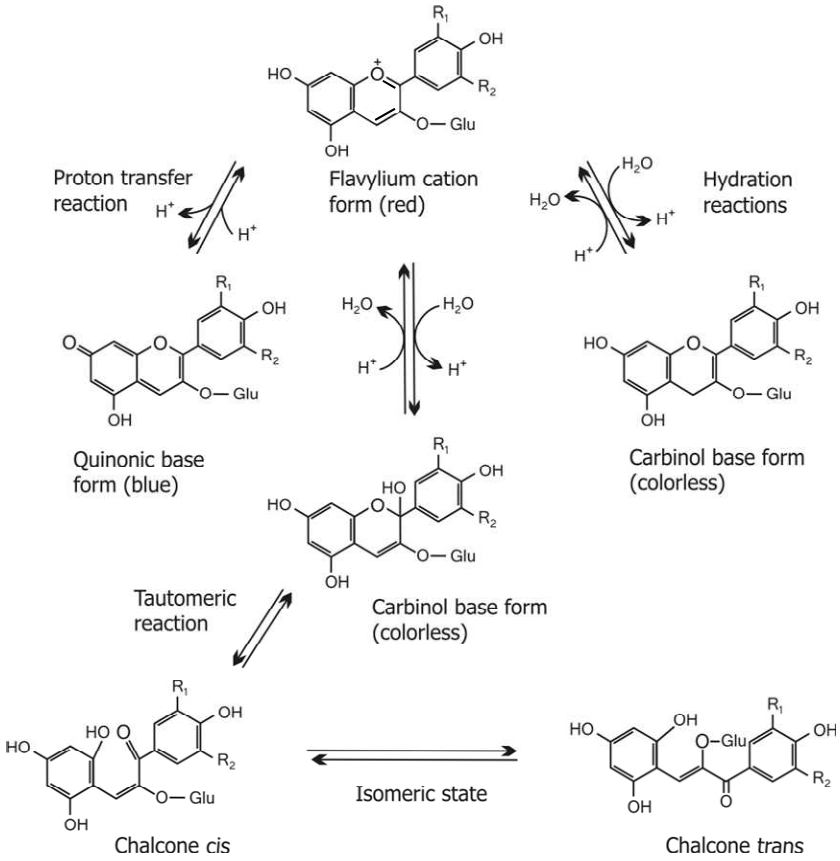

Figure 8. Anthocyanin equilibrium depends on the pH (adapted from Ribéreau-Gayon et al. 2006).

Acylation occurs at the C-6 position of the glucose molecule by esterification with acetic, *p*-coumaric or caffeic acids (Mazza and Miniati 1993) and lately reported with lactic acid (Alcalde-Eon et al. 2006). In grapes and wine predominates the 3-*O*-acetylmonoglucosides and 3-*O*-coumaroylmonoglucosides of the five main anthocyanidins. The malvidin- and peonidin-3-*O*-caffeoylmonoglucosides are found in low concentrations (Ribéreau-Gayon 1968, Baldi et al. 1995). Also the presence of the 3-*O*-caffeoylmonoglucosides of cyanidin, delphinidin and petunidin has been reported in few cases (Vidal et al. 2004b). Additionally, it has been recently

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confirmed the existence of 3,5-diglucosides in grapes and wine (Vidal et al. 2004b, Castillo-Muñoz et al. 2009), 3,7-diglucosides in wine (Alcalde-Eon et al. 2006) and also anthocyanin oligomers up to trimers in grape skin extract.

Anthocyanins in aqueous solution exist in the form of four basic structures, the flavylium cation, the quinoidal anhydrous base, the hemiketal o carbinol pseudo-base and the chalcone base. There are in equilibrium, depending on the pH (Figure 8). The relative amounts of each of the structural forms that coexist in equilibrium are a function of pH of the medium and its substituents. At very acidic pH (pH <2) anthocyanins are mainly in the form of flavylium cation. The flavylium cation is highly colored and appears red. As pH increases, the flavylium cation quickly disappears to give rise by deprotonation at several quinoidal base forms which have bluish color. Another reaction that occurs is the flavylium cation hydration leading to the formation of colorless carbinol pseudobases. Both reactions occurred simultaneously in accordance with the equilibrium constants. On the other hand, in weakly acidic medium, when the temperature of the solutions is high, pseudobase carbinol can be converted to a *cis* or *trans* chalcone by opening the pyrilium ring. The chalcones can be neutrals and colorless or can be ionized and take a slight yellow color. Reversion to flavylium form by decreasing the pH occurs much more slowly in the case of the *trans* than in the case of the *cis* form, indicating that *trans* form could be irreversible.

At the pH of the wine we can consider that there is a balance among the different forms. The red flavylium form is present only in a small proportion while the major proportion of anthocyanins is found in colorless or weakly colored forms.

2. Anthocyanins and proantocyanidin evolution during berry ripening

Grape berries development consist of two successive sigmoid growth cycles, which are separated by a short lag-phase (Coombe 1992). The first period-berry formation-begins after anthesis, with the fruit set, the cell division-enlargement and with the seeds approaching their full size. At this phase proanthocyanidins and their respective monomers are accumulated in seed (epidermis, inner cells of the soft seed coat, and inner cell layer of the inner integument) (Cadot et al. 2006) and the vacuoles of the grape skin cells (Amrani Joutei and Glories 1995).

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The lag-phase characterized by little change in berry size, the seed embryo development and the hardening of the seed coat. The third period-berry ripening- begins at veraison with the onset of sugar accumulation, the berry softening and an increase in berry size. Parallel, anthocyanins accumulation in the vacuoles of the skin begins in veraison and rich their maximum concentration around harvest period (Kennedy et al. 2002, Canals et al. 2005). A decrease in the total amount of antocyanins just before harvest and/or during over-ripening has been observed in some works (Mateus et al. 2002, Ryan et al. 2003, Fournand et al. 2006). On the other hand, proanthocyanidins and flavan-3-ol monomers were maximal in skin and seed before veraison (Downey et al. 2003). After veraison their behavior is not very clear. Some authors describe a decrease in skin proanthocyanidins, flavan-3-ol monomers and in the mean degree of polymerization but others an increase (de Freitas et al. 2000, Kennedy et al. 2001, Habertson et al. 2002, Kennedy at al. 2002). The flavan-3-ol concentration of seeds diminishes after veraison and until the harvest, which could be explained by oxidation (Kennedy et al. 2000, Downey et al. 2003).

In addition, except the quantitative changes that occur during grape development also qualitative modification has been observed. It is noted a change in the proportional flavan-3-ol composition. More specific it seems that the proportion of (-)-epicatechin-3-gallate diminish, the (-)-epicatechin increase and the (+)-catechin has small variation during berry ripening (Kennedy et al. 2000, Kennedy et al. 2002, Pastor del Rio and Kennedy 2006). The average degree of polymerization increases with maturity (Downey et al. 2003) although there are studies that present a decline (Kennedy et al. 2000, Kennedy et al. 2002). Finally, the total amount of berry proanthocyanidins throughout ripening seems to be decreased (de Freitas et al. 2000, Harbertson et al. 2002) even though an increase has also been reported (Delgado 2004, Canals et al. 2005).

2.1. Factors influencing phenolic synthesis and accumulation during berry ripening

Phenolic biosynthesis and accumulation through berry ripening is affected by different factors as genotype, environment and cultural practices.

It is obvious that *Vitis vinifera* cultivars determine the biosynthesis and accumulation of phenolic compounds of grapes and therefore wine. They determine the quantitative as also the

qualitative phenolic composition. It is characteristic that anthocyanin profile can be used as chemical markers with the aim of classifying red-grape varieties and wines. Besides the variety, intravarietal heterogeneity is a factor that induces a very different behavior among the different clones. It should be noted that most researches in the development of clones have been directed towards obtaining high yielding clones, resulting in a decrease of color and phenolic compounds. Therefore, the choice of clone is almost as important as variety.

In additional, rootstock genotypes is related with water and gas exchange status (Candolfi-Vasconcelos et al. 1994), canopy growth (Koundouras et al. 2008), and yield (Ezzahouani et al. 2005). For that reason, rootstock is possibly affecting phenolic composition (Doazan 1996, Koundardas et al. 2009). Moreover, the rootstock can also influence the harvest date. Therefore the choice of rootstock should also take into account their overall influence on the quality of the raw material.

Environmental factors (topographical, agro-pedological, climatic), usually described by the French term "*terroir*", have been acknowledged to influence grape and wine quality. Sunlight exposure and temperature are two factors with certain impact on phenolic composition of grapes (Spayd et al. 2002). Low diurnal and night temperatures have been reported to favor anthocyanin accumulation whereas high temperatures reduce their concentration. On the contrary, anthocyanin accumulation seems to be increased linearly with increasing sunlight exposure. Apart from the concentration, ultraviolet light exposure and temperature are related with alterations in anthocyanin composition (Downey et al. 2006).

Water conditions have been recognized as one of the most important factors that determine wine quality. Vine water status has been mentioned to influence accumulation of phenolic compounds in grapes with a clear positive effect of water deficit on berry phenolic composition (Esteban et al. 2001). For that reason, rainfall distribution and efficiency are of great importance as also the soil water storage capacity. Moreover, in relation with the soil characteristics, soil depth, structure, texture and mainly fertility affect phenolic composition (Downey et al. 2006).

Furthermore, macroclimatic characteristics like topography, latitude and altitude may affect the grape composition. It was observed an increase in anthocyanin concentration with increasing altitude from 100-150 meters to above 250 meters. Altitude also affected flavan-3-ol composition (Mateus et al. 2001, 2002).

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Agricultural practices modulate the expression of phenolic composition of grapes throughout the grape ripening process. Cultural aspects such as training system (Zoecklein et al. 2008), row vine spacing, pruning, bunch thinning, bud and leaf removal as well as the water and fertilize management (Esteban et al. 2001, González-Neves et al. 2002, Delgado et al. 2004, Poni et al. 2009) noted to have an influence on phenolic biosynthesis and accumulation. Agronomic practices like conventional, organic or biodynamic systems are also shown to have a relation with berry phenolic composition (Reeve et al. 2005, Vian et al. 2006).

Finally, other factors that may have an impact in phenolic composition of grapes are the vine age (Reynolds et al. 2007) and pathogenesis (Amati et al. 1996).

2.2. Influence of phenolic composition of grapes on wine composition and its sensoriality; the phenolic maturity concept

For the production of high quality wines is crucial, without any doubt, the degree of ripeness of the grapes. It should be noted that the maturation process affects not only the concentration of sugars and acids in berries, but also exerts, as it was already mentioned, a large effect on the phenolic composition of the grapes.

Anthocyanins and flavan-3-ols composition of the grapes at the harvest time have a great influence on wine color and color stability during wine aging. Consequently, more ripen grapes, would permit the elaboration of wines with deep red color. The ripen grapes, besides having a higher concentration of anthocyanins, present also a greater extractability of phenolic compounds from grape skin. Differences of polysaccharides, based on galactose and arabinose, together with the cellulose content and the degree of methylation of the pectins could be responsible for different anthocyanin extractability, keeping in mind that differences on the thickness or density of the skin cell-wall could also influence the extractability (Ortega-Regules et al. 2006).

Additionally, flavan-3-ols composition of grapes, especially originated from seeds, affects the astringency and the tannic intensity of the grapes and wines. The tendency is to diminish as the maturity degree increases (Llaudy et al. 2008). This fact is related not only with the total quantity of flavan-3-ols since sometimes samples containing higher contents in flavano-3-ols can exhibit less astringency and tannic intensity than others with lower ones (Ferrer-Gallego et

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al. 2010). It has been suggested that the qualitative profile of grapes can affect these sensorial parameters. In fact, the degree of galloylation correlates well with astringency (Vidal et al. 2003). For that reason a decrease of galloylation during grape maturity may diminish astringency of grapes and wines. Moreover, the greatest the degree of polymerization the greatest the astringency (Vidal et al. 2003), so the degree of polymerization of the grape proanthocyanidins at the harvest time will have an effect on wine astringency.

It is clear, therefore, that phenolic composition of grapes at the harvest time will determine the wine quality. For this reason and also because consumers demand wines with deep red color, full body, soft tannins and fruit scents, all vineyard work has been oriented during last years in the search of greater maturity.

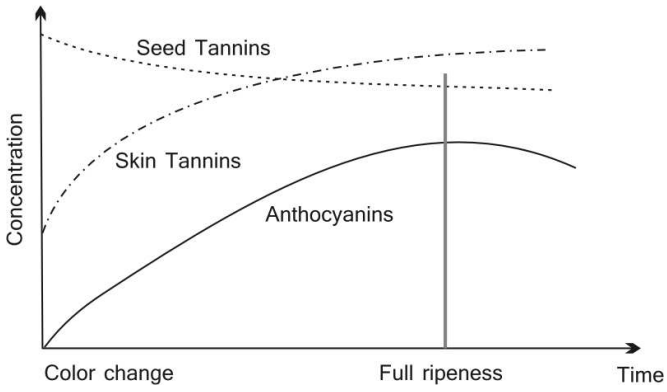

Figure 9. Evolution of grape phenolic compounds throughout grape maturity (adapted from Ribéreau-Gayon et al. 2006).

Commonly, the maturity of the grapes, and therefore the date of harvest, is determined only by analyzing the concentration of sugars, titratable acidity and pH of the grape juice (Ribéreau-Gayon et al. 2006). However, these parameters only give information about the ripeness of the pulp of the grapes and overlook the maturity of the skins and seeds.

Figure 9 shows that there is no necessarily a direct relation between the pulp maturation and the phenolic concentration of the red grapes. This makes essential the introduction of the phenolic maturity concept; which measures the potential of proanthocyanidins and anthocyanins present in grapes, as also their capacity to be extracted from grapes during winemaking.

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2.3. Methods for measuring phenolic maturity

The determination of phenolic maturity is very useful for deciding the optimum harvest date. Furthermore, the knowledge of phenolic ripeness of the grapes can be very useful for the classification of grapes at the winery in accordance with their level of quality and even to influence, by the adequate winemaking method, on the level of phenolic compounds extraction (Zamora 2003).

In recent years, several methods have been proposed to determine the phenolic maturity. Among the different techniques that have been development, the most applied methods are based on obtaining extracts from grapes with maceration in different solvents. Probably the most used method is that described by Glories (Glories and Agustin 1993., Saint-Cricq de Gaulejac et al. 1998). Nowadays, other methods are also used like the ITV method (Dupuch, 1993, Lamadon 1995, Cayla et al. 2002) developed by the *Institut Français de la Vigne et du Vin* and the method proclaimed by Iland (2004). However, all these methods are slow, laborious and require the participation of well-trained technicians.

Recently, the group of Celotti has proposed an extraction using microwaves that permits accelerate the extraction time (Celotti et al. 2007a). Besides, another method, Cromoenos, has begun to be popular, especially in Spain, as allows very rapid and easy determination (Cromoenos 2010). In addition, it should be noted that the techniques FTIR (Fourier Transform Infrared) are already being applied in the analysis of the extracts, which should allow more complex analytical results achieved at present (Dubernet et al. 2000).

The principle of these methods consists on rapidly extraction of the anthocyanins and proanthocyanidins from the skins and partial extraction of seeds proanthocyanidins, with roughly crushing of grapes and maceration in the proposed solvent and subsequent analysis of the parameters that are considered more appropriate. In that way it can be assessed the phenolic maturity of the grapes and the phenolic content of the future wine.

Moreover, the last years have been presented techniques that consist on the determination of the physical characteristics of the grapes, directly at the vineyard. Such methods are the skin texture parameters, like the grape skin hardness and thickness (Segade et al. 2008), the direct measurement of the color absorption of the grape skins, based on the screening of fruit

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chlorophyll fluorescence, that allows both flavan-3-ol and anthocyanin contents of intact berry skin to be measured (Celotti et al. 2007b, Cerovic et al. 2008) and the most advanced method, the analysis of multispectral airborne high resolution images (Lamb et al. 2004). These techniques reduce drastically the analysis time, but need more experimentation to demonstrate their reliability and repeatability.

Finally, the methods based on tasting grape berries and seeds have been introduced in recent years (Le Moigne et al. 2008). These methods are really very useful for determining grape maturity. However, tasting methods require experience, a laborious sampling and their parameterization is very difficult. For all those reasons, tasting methods are applied mainly to small and highly controlled vineyards.

3. Anthocyanins and proantocyanidin evolution during winemaking and aging

Immediately after the harvest the winemaking process begins. During this process the diffusion and extraction of the grape phenolic compounds take place and a perpetual evolution of the phenolic composition of the must at beginning and of the wine later occurs with the participation of biochemical and chemical phenomena.

3.1. Extraction kinetics of anthocyanins and flavan-3-ols during red winemaking

The winemaking of red wines is a complex process in which two phenomena take place simultaneously: alcoholic fermentation and maceration. It is the superposition of both phenomena which affects the kinetics of solubilization of most of the molecules of wine organoleptic impact (Zamora 2003). Figure 10 shows the kinetics of phenolic compounds extraction during fermentation / maceration (Ribéreau-Gayon et al. 2006).

According to this figure, the maceration is composed of three stages. The first one is the prefermentative maceration, which is the period before the beginning of the alcoholic fermentation. Therefore, takes place in an aqueous medium at generally moderate temperatures.

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The length of this stage depends on the latency phase of yeast and it is dependent on SO_2 dose and temperature. The second maceration stage is during the alcoholic fermentation. In this phase the medium change gradually from an aqueous to a hydroalcoholic medium with a simultaneously rise of the temperature. The duration of this phase will depend on multiple factors, including the initial concentration of sugars, the yeast strain, the nutrients present in the medium, the level of aeration and temperature. Finally, the third phase corresponds to the postfermentative maceration and it takes place after the achievement of alcoholic fermentation. This postfermentative maceration is usually only applied for obtaining wines with high aging potential and its length depends on the winemaker decision.

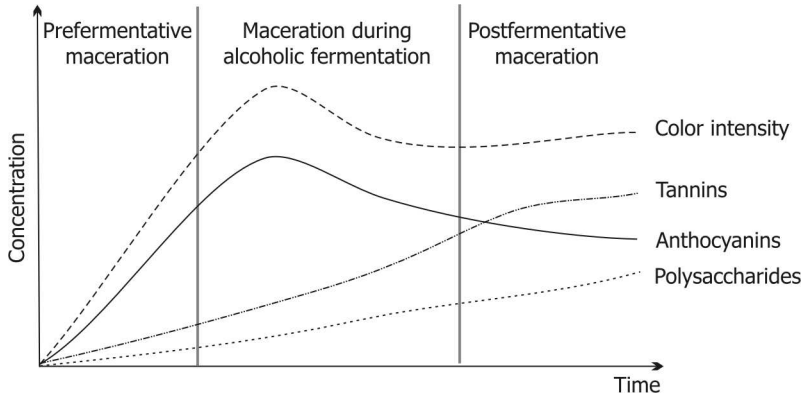

Figure 10. Kinetics of phenolic compounds extraction during fermentation/maceration (adapted from Ribéreau-Gayon et al. 2006).

The phenolic compounds extraction is related with the level of the grape maturity (see 1.2.2). Moreover it is affected by the temperature. Higher temperatures reduce the time required to reach maximum concentration and also increase the maximal amount (Sacchi et al. 2005). Since ethanol concentration increases during winemaking process, the phenolic extractability is also facilitated (Canals et al. 2005).

As the Figure 10 shows, anthocyanins are extracted relatively quickly, during the first few days of maceration. Once anthocyanins reach their maximum levels, no more extraction is usually observed and a downward trend occurs, mainly due to oxidation, precipitation, modifications in their structure and adsorption in yeast cell walls (Zamora 2003).

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Flavan-3-ols are extracted more slowly. At the beginning of the maceration when the temperature is still moderate and alcohol concentration is still low, the extraction is very slow. However, as the alcoholic fermentation progresses, alcohol and temperature increases which favor flavan-3-ols extraction (Canals et al. 2005, Sacchi et al. 2005).

It is also necessary to distinguish between the flavan-3-ols released by skins and seeds because their extraction kinetic is different. The flavan-3-ols of the skins starts to be dissolved at the same time that anthocyanins, although their release is slower and longer. In contrast, the flavan-3-ols of the seeds do not begin to dissolve until the middle of fermentation.

Flavan-3-ols extraction increases throughout fermentation and maceration. At the beginning of the maceration flavan-3-ols composition is characterized by an important presence of skins flavan-3-ols. The extension of maceration process leads to a progressively increase of seeds flavan-3-ols, which become predominant with a main influence on the qualitative composition of wine flavan-3-ols (González-Manzano et al. 2004, Llaudy et al. 2008). For that reason, depending on the wine style, it is necessary to adapt the extraction of flavan-3-ols. It is also possible to select the proportion of skin and seed flavan-3-ols using one or more winemaking techniques that affect differently phenolic extraction (Zamora 2003, Sacchi et al. 2005).

3.2. Evolution of phenolic compounds during wine aging

The changes in phenolic composition which take place during winemaking and aging involve two processes. The first process involves enzymatic reactions and occurs mostly at the early stages of winemaking while the second one involves chemical reactivity of the phenolic compounds and begins during winemaking process and continues during aging.

Enzymatic reactions

Enzymatic oxidation and degradation involves mainly the polyphenoloxidases (PPO). These reactions are more important for white wines while in red wines are not so important. Generally, catechins are poor substrates for grape PPO and also proanthocyanidins and anthocyanins cannot be oxidized directly by grape PPO due to steric hindrance (Cheynier et al. 2000). However, they can react with the enzymatically generated caffeoyltartaric acid quinine through

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coupled oxidation and nucleophilic condensation reactions (Cheynier and Ricardo da Silva 1991, Sanri-Manchado et al. 1995, 1997).

Also the *β*-glucosidase activity is responsible for the breakdown of the glucosidic bond of the anthocyanidin-3-glucosides. The released anthocyanidins are more unstable and are rapidly degraded leading to losses in wine color (Monagas and Bartolomé 2009).

Chemical reactions

Several chemical reactions taking place during the winemaking and aging, with an important influence on the color, the stability of the color, the body and the astringency of the wine.

As it has already mentioned (see 1.2.4.) in a slight acidic medium, as is the wine, anthocyanins are present in four structural forms that are in equilibrium depending on the pH, with the predomination of the less colored and colorless forms. Moreover, anthocyanins react with the bisulfite ions (HSO3-) at the C-4 position of the flavylium cation resulting in the formation of a colorless adduct which is stable at the wine pH (Berke et al. 1998). The proportion of the anthocyanins that are trapped as colorless bisulfate adducts is according to pH and the amount of sulfite added in must or wine (Ribéreau et al. 2006).

More complex reaction with the participation of anthocyanins and other phenolic compounds has led to propose the existence of copigmentation phenomenon. Copigmentation is a solution phenomenon in which the planar polarisable nuclei of the colored forms of the anthocyanins and other non-colored organic components form molecular associations or complexes (Boulton 2001). They form vertical stacking complexes held by low energy bonds (Van der Waals, hydrophobic interactions) that are stabilized by the disposal of the sugar molecules on the outside, which among them there are established hydrogen bonds. Water molecules cannot enter in the interior of these complexes and thus copigmentation prevents hydration of anthocyanins and fading, making the equilibrium to shift to colored forms of anthocyanins. This normally results in increased absorbance intensity (hyperchromism) and a positive shift in the visible wavelength (bathochromism), with the color becoming purple (Asen et al. 1972).

Several compounds are studied to be as potential anthocyanin copigments. Copigments may be phenolic compounds, alkaloids, amino acids, organic acids, nucleotides, polysaccharides (Robinson and Robinson 1931, Asen et al. 1972, Mazza and Brouillard 1990). In additional, intramolecular copigmentation has also been mentioned as a possible mechanism occurring with

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the association of the anthocyanins positively charged pyrylium nucleus and their aromatic residues of their own molecule, as well as the self-association of them and the interaction with metal cations (metal complexation) (Asen et al. 1972, Brouillard 1981).

Among red wine compounds, flavonoids, hydroxycinnamic acids appears to be the most promising compounds to act as anthocyanins copigments. Flavan-3-ols present small strength of copigmentation in comparison with the other compounds, due to their non-planar structure (Gómez-Míguez et al. 2006). However, as there are present in great amounts in red wines their copigmentation with anthocyanins can contribute significantly in wine color of red young wines (González-Manzano et al. 2009). However, important differences can be present in their ability to interact with anthocyanins through the different structure and stereochemistry of flavan-3-ol (Berké and De Freitas, 2005).

Generally, the factors that influence the copigmentation are pH, ethanol, temperature, molecular structure of the copigments and concentration ratio between anthocyanins and copigments (Boulton 2001).

The copigmentation is of great interest as it seems to contribute significantly to the color of young red wines. It is also possible that copigmentation would be the first step in the formation of new pigments that determine the color of aged red wines (Brouillard and Dangles 1994).

The formation of pigments with direct condensation between anthocyanins and flavan-3-ols has been suggested (Jurd and Somers 1970, Somers 1971). There have been proposed two different mechanisms for this type of reactions, the anthocyanin-flavan-3-ol (A-F) and the flavan-3-ol-anthocyanin (F-A) direct condensation reaction (Figure 11).

The anthocyanin-flavan-3-ol direct condensation reaction starts with the nucleophilic attack of the C-8 or C-6 position of the flavan-3-ol to the electrophilic C-4 position of the anthocyanin in the form of flavylium cation, giving rise to either a flavene which can later undergo an oxidative reorganization to give rise to the formation of yellowish pigments with a possible xanthylium structure (Jurd and Somers 1970, Somers 1971, Santos-Buelga et al. 1995), although this proposal has been questioned (Santos-Buelga et al. 1999), or to a colorless bicyclic condensation product (Bishop and Nagel 1984, Remy-Tanneau et al. 2003).

In the case of the F-A direct condensation reaction, the proposal mechanism suggest that carbocations generated from the acid-catalyzed interflavanic bond cleavage of the

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proanthocyanidins act as electrophilic agents and react with the nucleophilic C-6 or C-8 position of the anthocyanin in its hydrated hemiketal form, giving rise to a colorless pigment (Halsam 1980, Cheynier et al. 2000). These carbocations may also react with flavan-3-ols (flavan-3-ol-flavan-3-ol condensation reaction) generating new proanthocyanidin molecules that can increase their degree of polymerization or can decrease it if an excess of monomeric flavan-3-ols is present in the medium (Halsam 1980).

Direct condensation reactions between anthocyanins and flavanol monomers are strongly related to pH, due to electrophilic and nucleophilic characters of the anthocyanins and flavan-3-ols. F-A

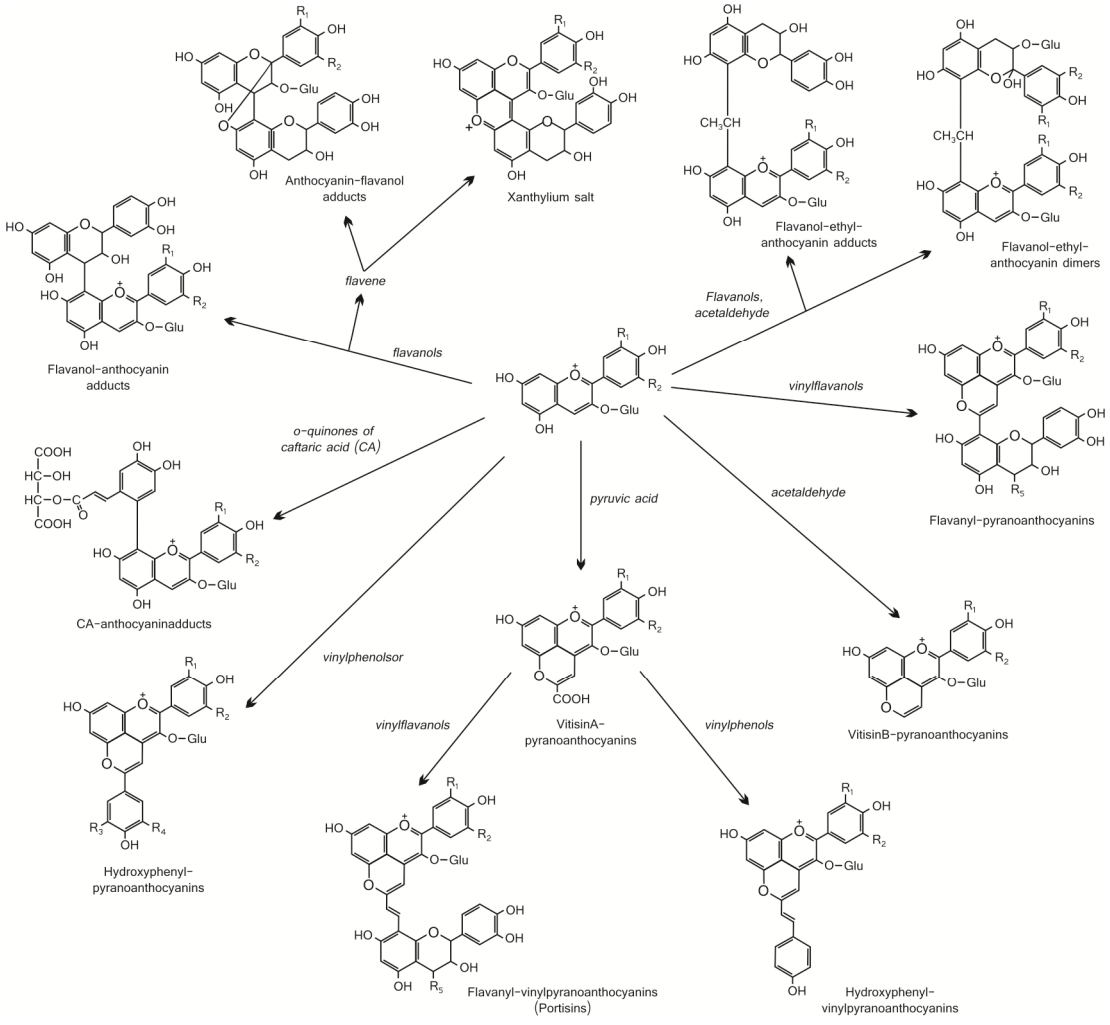

Figure 11. Main chemical reactions of anthocyanins during winemaking and aging (adapted from Monagas and Bartolomé 2009).

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adducts are formed at low pH values and A-F adducts above pH 3.8 (Salas et al. 2003). Temperature seems to favor formation of the latter as well as flavan-3-ol size (Malien-Aubert et al. 2002). These pigments appear to have an increased resistance to sulfite bleaching and to the pH effect (Salas et al. 2003). The products of both mechanisms have been detected in wine fractions (Remy et al. 2000, Vivar-Quintana et al. 2002).

Other possible mechanisms (Figure 11 and 12) that have been suggested are the condensation reactions mediated by aldehydes (anthocyanin-anthocyanin, flavan-3-ol-flavan-3-ol and anthocyanin-flavan-3-ol). The main aldehyde that undergoes this type of condensation reactions is acetaldehyde, which is present in wine is originated from the yeast metabolism (Romano et al. 1994) during alcoholic fermentation and from the oxidation of ethanol in the presence of polyphenols (Wildenradt and Singleton 1974). Other aldehydes like propionaldehyde, isovaleraldehyde, isobutyraldehyde and benzaldehyde are present in wine in fewer concentrations, with the exception of the Port wines (Pissarra et al. 2005). Moreover, other substances such as glyoxylic acid, which result from oxidation of tartaric acid (Fulcrand et al. 1997), furfural, 5-hydroxymethylfurfural and vainillin that can be extracted in wine from barrels (Es-Safi et al. 2000, Sousa et al. 2007) are also aldehydes which may participate in this type of reactions.

Timberlake and Bridle (1976) proposed that the aldehyde, in the form of carbocation, reacts with the flavan-3-ol at position C-6 or C-8 of the phloroglucinol ring, and after dehydratation, the flavan-3-ol aldehyde adduct can react with anthocyanins or flavan-3-ols giving rise to new structures that are linked by an ethyl bridge (Figure 11 and 12).

Flavan-3-ol-anthocyanin acetaldehyde-mediated pigments have a purplish color and greater color intensity than anthocyanins. Their color is more stable against pH changes and there are only partially bisulfate bleached. Nevertheless, these pigments are more sensitive to degradation in aqueous solution than anthocyanin. Moreover, the cleavage of the ethyl bridges may cause the formation of new precursor compounds which can react with other polyphenols and be responsible of reorganizations and new condensation reactions (Escribano-Bailón et al. 2001).

The formation of pigments between proanthocyanidin oligomers and anthocyanins has been observed (Francia-Aricha et al. 1997, Vivar-Quintana et al. 1999). In the presence of enough acetaldehyde concentration, condensed pigments are progressing rapidly towards larger

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structures by incorporating new ethyl-flavanol units, reaching a critical mass in which pigments precipitate, leading to loss of color (Rivas-Gonzalo et al. 1995).

Flavan-3-ols also undergo condensation reactions with each other mediated by acetaldehyde, which give rise in colorless pigments. The polymers that can be formed through these reactions are unstable and undergo other reactions. These ethyl linkages are very labile and can cleave into vinylflavanol oligomers which can then react with anthocyanins to produce flavanyl-pyranoanthocyanins (Fulcrand et al. 2006). Other aldehydes such as glyoxilic acid or furfural, as it was quoted above, undergo condensation reactions and form xanthylium salts (Figure 12).

The formation of pigments resulting from the acetaldehyde-mediated condensation between anthocyanins has been proved in synthetic medium and these pigments were later found in red wine (Atanasova et al. 2002b).

It is necessary to keep in mind that in general the availability of acetaldehyde in wine is limited by the presence of sulfite. Nevertheless, acetaldehyde presents an important role in the evolution of wine color depending on the wine physicochemical characteristics and the conditions that is stored.

The factors that mainly affect the acetaldehyde production are the oxygen, the pH and the temperature. The reaction rate is higher in the present of oxygen and at acidic pH, since the formation of acetaldehyde and its protonated form are favored under these conditions (Rivas-Gonzalo et al. 1995, Atanasova et al. 2002a). On the other hand, the formation of ethyl-bridged pigments is slower in low temperatures and the formed compounds are more stable in relation to their degradation and precipitation (Rivas-Gonzalo et al. 1995).

Over the last decades, several studies showed that a number of cycloaddition reactions occurs between anthocyanins and other small molecules in red wine, such as acetaldehyde (vitisin B) (Bakker and Timberlake 1997, Vivar-Quintana et al. 1999), acetoacetic acid (He et al. 2006), pyruvic acid (vitisin A) (Fulcrand et al. 1998, Mateus et al. 2001), vinylphenol (Fulcrand et al. 1996, Schwarz et al. 2003b), vinylguaiacol (Hayasaka and Asenstorfer 2002), vinylcatechol (pinotin A) (Schwarz et al. 2003a), and vinylcatechin (Cruz et al. 2008) giving rise to new families of anthocyanin-derived pigments called pyranoanthocyanins (Figure 11).

This type of pigments present more stability at color changes of pH and at SO_2 bleaching in comparison with natural anthocyanins from grapes (Sarni-Manchado et al. 1996, Bakker and

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Timberlake 1997, France-Aricha et al. 1997). This fact together with theirs red-orange hues similar to that acquired during red wine aging, has suggested that they may play a crucial role in color changes that occur in wine red during aging.

Recently, a new class of anthocyanin-derivate pigments, named portisins (Figure 11), was detected and isolated directly from Port red wines (Mateus et al. 2003). This type of pigments appears to display a bluish color under acidic conditions. Its formation mechanism appears to occur between a pyranoanthocyanin moiety and a flavan-3-ol molecule through a vinyl bridge.

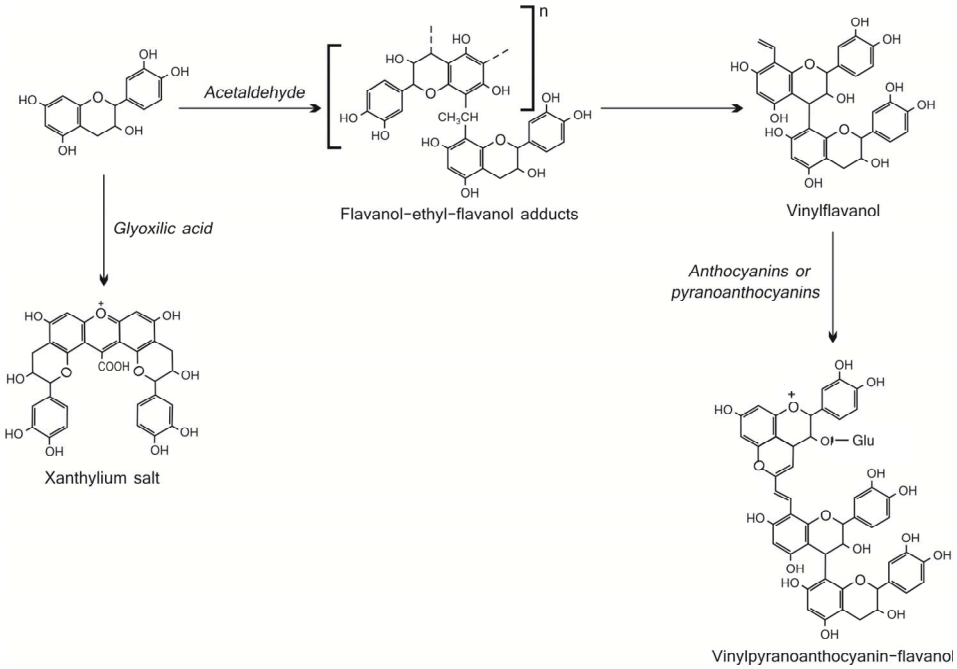

Figure 12. Reaction of flavanols with glyoxilic acid and condensation reactions mediated by acetaldehyde during winemaking and aging (adapted from Monagas and Bartolomé 2009).

Moreover, other compounds newly displaying a structure similar to that of the other reported portisins were detected. These new pigments were shown to possibly arise from the reaction between carboxypyranoanthocyanins and hydroxycinnamic acids (caffeic, coumaric, sinapic, and ferulic acids) (Oliveira et al. 2007).

Finally, during the wine-maturing processes in oak barrels or with the addition of oak chips, hydrolysable tannins are dossolved in the wine. It is suggested that condensation reactions

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among anthocyanins and the C-glycosidic ellagitannins can take place with a possible bathochromic effect in wine color, however more research is necessary (Chassaing et al. 2010).

3.3. Sensory implications of phenolic compounds

The phenolic composition of grapes and wine has a significant importance on wine quality. Specifically, the most abundant compounds, anthocyanins and flavan-3-ols, and their reactivity during winemaking and aging define the wine color, astringency, bitterness and the evolution of all these sensory attributes.

Anthocyanins are structurally dependent on the conditions and the composition of the wine medium. The pH of the wine and the presence of sulfites provoke discoloration of the anthocyanins. However, red young wines appear to have intense red color as product of different mechanism. Copigmentation is one of the main mechanisms that provoke the color stabilization (Boulton 2001). Also it is possible that copigmentation is the first stage in the formation of new more stable pigments thus affecting wine color stability (Brouillard and Dangles 1994). As it has shown above, depending on the type of anthocyanins and copigments and also of their concentrations, there would be variations in wine color and intensity. It is believed that the contributions of copigmentation in young wines color is quite high and gradually diminish during aging (Hermosín Gutiérrez et al. 2005).

The anthocyanin-flavan-3-ol direct condensation through the two different mechanisms, produce colorless compounds or give rise to the formation of yellowish xanthylium pigments (see 1.3.2.). The contribution of these pigments pigments, that present violet hues, seem to contribute more indirectly than directly in wine color. These compounds are found in very small amounts in wine, most probable as result of their instability that provokes their cleavage. Their contribution in wine color it seems to be negligible as there are present in low concentrations.

In addition, ethyl-linked moieties after the cleavage can react again and give ethyl-bridge derivatives or flavan-pyranoanthocyanins (see 1.3.2.). Besides, in the presence of enough acetaldehyde concentration, ethyl-linked pigments polymerized giving rise progressively to large polymers that after a critical size will precipitate (Rivas-Gonzalo et al. 1995), which indirectly leads to the gradual change of the color.

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The formation of the more stable pyranoanthocyanins seems to promote the change of the initial purple-red color in hues more oranges. Although they appear in very small concentrations, as they present stability under pH value and sulfite bleaching, it is possible that their contribution in color is significant. On the other hand, portisins appears a blue color and they are found in small amounts mainly in Port wines. However, it is believed that these pigments contribute in wine color change (Mateus et al. 2003).

Other pigments that have been mentioned (see 1.3.2), like caftaric acid-anthocyanin adducts, anthocyanin-ellagitannins or anthocyanin monomers are found in very low concentrations in wine and more studies need to define their importance on wine color.

All these progressive structural changes appear to result in gradually shift of the initial purple-red color to reddish-brown hues. Furthermore, phenolic compounds are responsible for essential organoleptic features of red wine, like astringency and bitterness.

Bitterness is one of the basic tastes and is mainly induced by flavan-3-ols. It has been demonstrated that bitterness decreases from monomer to trimer flavan-3-ols (Peleg et al. 1999). Bitterness intensity is higher when ethanol level increases. Additionally, since bitterness is a taste sensation resulting from an interaction between bitter compounds and specific tongue receptors, the bitterness increase could be directly related to the increase of the bitter compound concentration (Fontoin et al. 2008). Finally, it seems that ethyl-bridged flavan-3-ols formed during wine aging could increase bitterness (Vidal et al. 2004a).

As opposed to bitterness, astringency is a tactile sensation most commonly described as drying, roughing and puckering of the epithelium of the oral cavity. American Society for the Testing of Materials define astringency as the complex sensation due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins. (ASTM, 1989). Although the basic mechanism of astringency is not yet well understood, it suggested that oral astringency results primarily from the precipitation of salivary proteins, which impairs the natural lubrication of oral surfaces (Green et al. 1993). Wine proanthocyanidins can interact and precipitate salivary proteins.

Astringency sensation depends on flavan-3-ols concentration and chemical structure, although concentration appears to be less important than chemical structure (see 1.2.2.). From monomer to trimer astringency increases (Peleg et al. 1999). Also at larger proanthocyanidin polymers the perception of the astringency increases with the molecular weight as well as with the degree of

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galloylation (Vidal et al. 2003). However, there are different opinions about the influence of high molecular weight proanthocyanidins in astringency. Some authors have reported that proanthocyanidins with mean molecular size bigger than 7 are insoluble and unable to contribute in astringency (Lee 1990). However, more recent studies have shown that high polymerized proanthocyanidins are soluble in wine-like water-alcoholic solution and are very astringent (Vidal et al. 2003).

The astringency in general is considered to decrease during wine aging. Some of the condensation reactions that take place between anthocyanins and flavan-3-ols may contribute to the declination of wine astringency (Vidal et al. 2004a). One other theory is that proanthocyanidins undergo cleavage reactions by acid catalysis, thereby becoming smaller and reducing astringency (Cheynier et al. 2006).

The flavan-3-ols astringency is related not only with their chemical structure and concentration. Ethanol content and pH of wine also affects astringency of proanthocyanidins. It is observed that the increase of ethanol content and pH diminish astringency (Fontoin et al. 2008).

4. Impact of climate change on grape maturity; implications on wine composition and quality

4.1. The Climate Change

Climate change is certainly one of the most studied scientific issues of the last years. Although there is a big debate about the impact of human activity on the climate change and especially on the impact of the anthropogenic greenhouse gas emissions (Crowley 2000, Barnett et al. 2005), insurance is that the climate is changing. An increasing number of observations give a more complete picture of global warming (IPCC 2007, Barnett et al. 2005, Mears and Wentz 2005) during the past century and the early of the last one. The Intergovernmental Panel on Climate Change 2007 (IPCC Forth Assessment Report) mentioned that the tendency of global warming at the 20th century was 0.6 ± 0.2°C and the decade of 90s was the warmest of the century (IPCC 2007). Eleven of the twelve years between 1995 and 2006 were in the rank among the 12 warmest years of global surface temperature since 1850. NASA mention that January 2000 to December 2009 was the warmest decade on record and the 2009 was the second warmest year

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since recordkeeping began (NASA 2010).

The IPCC (2007) emphasized that the majority of the warming over the last 50 years has likely been attributed by the increase of the emissions of carbon dioxide (CO_2) and other greenhouse gases, like methane (CH_4), nitrous oxide (N_2O), hydrofluorocarbons (HFCs), perfluorocarbons (PFCs) and sulphurhexafluoride (SF_6), produced mainly by burning fossil fuels and contributed by land-use change and deforestation. Levels of carbon dioxide in January 2010 appears to be 389 parts per million (ppm), much higher than any other time in the past (NOAA 2010). Greenhouse gases trap incoming heat near the surface of the Earth and are the key factors causing the rise in temperatures. There are also other key factors such as volcanic eruptions, sun's irradiance, oscillations of sea surface temperature in the tropics, and changes in aerosol levels. Overall, the evidence suggests that these effects are not enough to account for the global warming observed since 1880. Especially, volcanic eruptions in 2009 have not had a significant impact and solar irradiance decreased although the global surface temperature was continued increasing (NASA 2010).

If the emission of greenhouse gases continues with the same intensity, the estimation of mean global surface air temperature, while recognizing deep uncertainties, will probably be between 2-6°C until the end of the century (IPCC 2007).

The most obvious effects of the climate change will be on one hand the sea level rise. A higher temperature means more speed in the thawing of large masses of continental ice, Arctic and Antarctic (Church 2001, Mitrovica et al. 2001, Huybrechts and Joughin 2005). On the other hand, will bring increased risks associated with extreme weather events. There will be more heat waves and floods, the incidence of hurricanes, typhoons and cyclones will increase in certain areas of the planet, while in others the drought and desertification are inevitable (IPCC 2007).

This probable rise of mean global temperatures will have an important impact on natural and human system, like asymmetry ecological responses (Walther et al. 2002), extinction risk for both flora and fauna spans (Thomas et al. 2004), risk for water resources (Arnell et al. 1999), decrease of global food production (Parrya et al. 2004) and risk for human health (McMichael et al. 2006).

4.2. Impact of climate change on grape maturity; implications on wine quality

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Certainly, the consequences of climate change on agriculture in general (Rosenzweigb et al. 2004, IPCC 2007) and viticulture in particular (Jones et al. 2005) are of great importance. In this sense, the research of Jones et al. (2005) is very illustrative about the influence that will have for the wine quality. Jones et al. (2005) have studied the change of temperature at the growing season in 27 of the most prominent wine growing regions worldwide during the period 1950-1999. They found that average growing season temperatures warmed 1.26 ºC and dormant season temperatures 1.38 ºC. Although this warming was not uniform, greatest in western U.S and Europe, (Iberian Peninsula, Southern France, and parts of Washington and California reached an increase more than 2.5 ºC), less in Chile, Australia and South Africa, has promoted the quality of wines in all areas, with the sole exception of the Rhine Valley. This phenomenon happened because the average temperature was close to the optimum growing season temperature for the cultivars adapted well to the climate of each region, thus encouraging the correct maturation of the grapes.

Using climate models warming, recent research on the projected climate changes in wine regions worldwide predict continued warming for the period 2000-49. The model output used in this analysis, Hadley Centre climate model-HadCM3, (Pope et al. 2000) comes from the SRES A2 scenario (Special Report on Emissions Scenarios) and represents mid-range predictions compared to the other climate models (Houghton et al. 2001). This model projected an increase of 2.04 ºC on average for growing season temperatures and parallel predict a warming during dormant period. The temperature increase predicted to be greater in the Northern Hemisphere-NH- (2.11 ◦C) than in the Southern Hemisphere-SH- (1.71 ◦C). This warming will divert most of the actual vineyards of their optimal temperatures such as the vineyards of California (Nemani et al. 2001, Jones 2005, Jones et al. 2005, White et al. 2006), Australia (Webb 2006), South Africa (Carter 2006) and Europe with more negative influences in Iberian Peninsula (Kenny and Harrison 1993, Schultz 2002, Jones 2005).

Grapevine regions can be categorized (Figure 13) taking in account temperature-based parameters (e.g. degree days, mean temperature of the warmest month, average growing-season temperatures, etc.) for the production of high quality wines in regions with cool, intermediate, warm, and hot climates (Jones 2007). A possible warming of 2 ºC that is predicted for the period 2000-2049 can provoke, for example, the transformation of one region with cool climate to one with intermediate and push a region outside the ability to ripen correctly existing varieties with obvious consequence in wine quality. This occurs because each cultivar has an optimum average

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growing temperature that favor the grape maturity needed for obtaining high quality wines. A change in temperature can therefore affect negatively grape maturity.

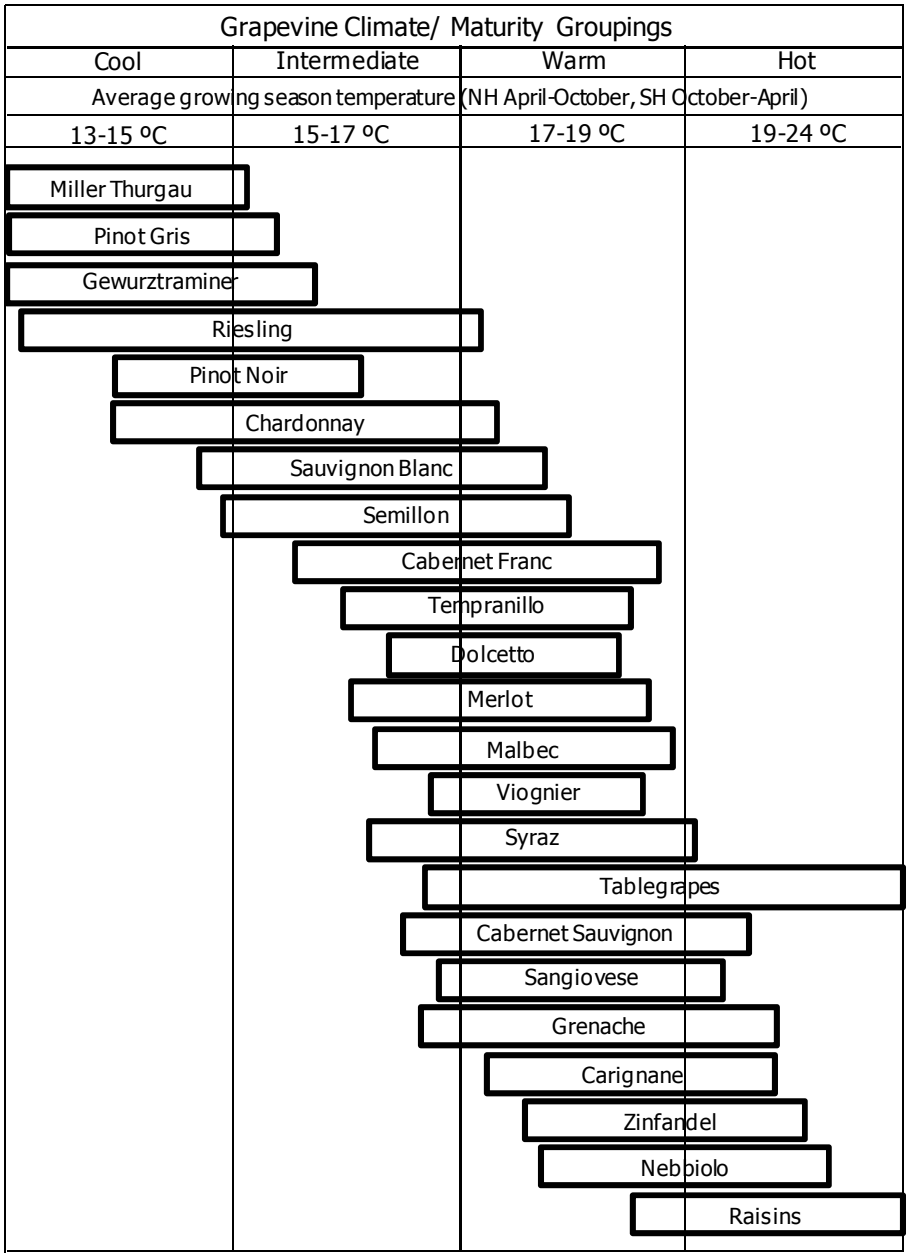

Figure 13. The climate-maturity groupings based on relationships between phonologicalrequirements and climate for high to premium quality wine production in the world's benchmark regions for each variety (adapted from Jones 2007).

Moreover it is observed that global warming is shifting areas of vine cultivation. It has been estimated that the northern limits of its cultivation in Europe is moving at a rate between 10 and 30 km per decade and that the speed of travel is expected to double between 2020 and 2050 (Kenny and Harrison 1993). This means that some regions that are adequate for cultivars

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adapted well to the climate of the region conditions, could not be suitable at the future, without the adoption of appropriate adaptive measures, and other regions that until now appeared as unsuitable wine grape production regions under warmer temperature scenarios may become more suitable for the production of high quality wines.

In the light of these previsions there is no doubt that climate change will affect the quality of grape and wines well adapted in the viticulture regions that are now growing. The increase of temperature and the evolution of rainfall distribution and efficiency (IPCC 2007) as also point potential evaporation change (Webb 2006) will probable affect the vine and grape physiology and biochemistry. Although rise in CO2 concentration in the atmosphere may increase grapevine photosynthesis and grape production without causing negative impacts on the quality of grapes and red wine (Gonçalves et al. 2009), water availability and increase of temperatures will probable affect negatively. Schultz (2000) illustrates the probable effects of the possible increase in surface level ultraviolet UV-B radiation due to depletion of stratospheric ozone on grape physiology and production (Table 1).

UV-B effects	Possible relevance for grape production
• activation of genes of the phytopropanoid pathway	➢ accumulation of flavonoids and anthocyanins (color formation and wine composition)
• inactivation (damage) of photosystem II and of photosynthetic enzymes	➢ decreased photosynthesis
• reduced chlorophyll and carotenoid concentration	➢ decreased photosynthesis ➢ altered aroma compounds ? ➢ xanthophylls, leaf and berry energy balance?
• effects on nitrogen metabolism (via carbon supply or direct effects on key enzymes)	➢ decreased amino acid concentration (yeast metabolism, fermentation kinetics, higher alcoh formation, secondary aromatic compounds)
• thicker leaves, wax composition • photo-oxidation of indole acetic acid (IAA, auxin), UV-B absorption by tryptophan	➢ more disease resistance ➢ possible formation of *o*-aminoacetophenone (off-flavor in white wine)
• increase in ascorbic acid and glutathione content through the formation of free radicals	➢ photoprotection, sulfur metabolism, induction of enzyme activities?
• flowering and phenology	➢ may be affected in some varieties
• alterations in soil microflora and fauna	➢ nutrient availability

Table 1. Effects of the possible increase in surface level ultraviolet UV-B radiation due to depletion of stratospheric ozone on grape physiology and production (adapted from Schultz 2000).

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The most enlightened effects from climate change would be: inhibition of photosynthesis, partial or total inhibition of berry development and biosynthesis, inhibition of ripening, loss of fruit volume with consequences on yield and the increase of sugar content at the harvest time.

It is clear that if at the maturity period the temperature is higher than the optimum, the pulp of the grapes mature more rapidly, reaching high concentrations of sugar, low acid concentrations and thereby the period between veraison and industrial maturity decline, making it more difficult to reach the proper aromatic and phenolic grapes maturity (Zamora 2005), resulting in unbalanced wines with high pH and alcoholic content.

High pH values in wines implies some problems such as less color, less antiseptic effectiveness of sulfur dioxide and higher risk of microbiological spoilage (Beech et al. 1979). On the other hand, high alcohol concentration is very likely to provoke stuck and sluggish alcoholic fermentation and to inhibit malolactic fermentation. Moreover, an excess of alcohol is unpleasant. Moreover, wines with high alcohol content are imposed with higher tax rates in some countries.

It is a verifiable fact that most of the wines have gradually increased their alcohol content in recent years and winemakers are really concerned about the problem. The tendency of higher alcohol levels is studied in many viticulture areas with more characteristic the case of Alsace region which potential alcoholic levels of Riesling at harvest moment have increased 2.5% (v/v) between 1972-2003 (Duchêne and Schneider 2005).

4.3. Winemaking techniques for reducing impact of climate change on wine quality

Several techniques have been proposed to mitigate climate impacts. Firstly, it is necessary to study varieties, clones of vines and rootstocks (Main et al. 2002) that may be able to retard pulp sugar accumulation and acid consumption at a certain regional conditions. Additionally, the translation of the vineyards to other production regions can be a positive move (Schultz 2000). These solutions maybe are the most suitable but require considerable time and inversions to study and replace the existing vineyards. Moreover, other possible available options for managing excessive alcohols in the vineyard can be the correct election of training system, row

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vine spacing, pruning, bunch thinning, bud and leaf removal as also the adequate water management (Reynolds et al. 1996, Bruce et al. 2008, Mandelli et al. 2008, Poli et al. 2009).

The harvest of grapes at early stage of ripening can be adapted but with negative effects in wine quality since the phenolic and aromatic maturity would not achieve the levels for the production of high quality wines. Other solution is the addition of water and mineral acids to the grape juice before the beginning of the fermentation but except the fact that it is strictly forbidden in the most of the winemaking countries, there is the possible of alternating the wine quality. Finally, the blending of wines with high alcoholic concentration with other with low concentration is an alternative solution when the wine with low alcoholic concentration do not present the disadvantages described above.

The use of Glucose oxidase (EC 1.1.3.4) has been also proposed to obtain less alcoholic wines (Pickering et al. 1998). This enzyme break down glucose into gluconic acid, but is deactivated in the presence of alcohol in concentrations greater than about 1% by volume. They therefore need to be added to must before it has begun fermenting.

However, the high gluconic acid concentration in wine is undesirable. Moreover, this enzyme use oxygen as substrate and consequently it is necessary to aerate excessively the grape juice to operate adequately which may involve an oxidation of other wine components (Pickering et al. 1999).

It is proposed the selection of *Saccharomyce cerevisiae* strains with a lower yield transformation of sugar into ethanol. However, the possibility of selecting a strain that produce less ethanol is limited by the fact that oenological strains present similar yields. Alcoholic fermentation carried out with non-*Saccharomyces* yeasts (Ciani and Ferraro 1996) can be a possible alternative solution but there is the possibility of produce unbalanced wines. The combination of non-*Saccharomyces* with *Saccharomyce cerevisiae* yeasts can be a promising answer.

The investigations about yeast and alcohol products are focused mainly on the genetically modified organisms. Several metabolic engineering strategies have been proposed (Dequin and Barre 1994, De Barros Lopes et al. 2000, Remize et al. 2000, Malherbe et al. 2003, Heux et al. 2006). These strategies are based on metabolic redirection of sugars in the must by reducing ethanol production and increasing the cell biomass or fermentation end products such as glycerol or lactic acid. But genetically modified products, and especially genetically modified wine, are not accepted from the most of the consumers. So, more studies are necessary about the

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possible environmental and human health risks of these yeasts and it is also necessary to study how they affect the wine characteristics.

Several other technological innovations during winemaking process have been proposed to remove alcohol and decrease pH. Vacuum distillation methods using either evaporators or distillation column are widely applied in wine industry for alcohol reduction, being the spinning cone column probably the more utilized (Pickering 2000). In addition, membrane processes have been applied during the last years to reduce sugar content in must or alcohol in wine. Among diverse methods (dialysis (Regan 1990), membrane contactors (Diban et al. 2008), pervaporation (Takács et al. 2007) and reverse osmosis (Bui et al. 1986), the reverse osmosis is the most popular. Other techniques that maybe can be applied for reducing alcoholic content of wines are the use of supercritical fluid extraction (Ruiz-Rodrigueza et al. 2010) and the adsorption (on resins or on silica gels) (Pickering 2000).

The pH decrease can be achieved by adding organic acids however when the pH is too high the result is questionable. The reduction treatments are based basically at cationic exchange columns and electrodyalisis techniques (Walker et al. 2004). These physical methods are effective but involve the use of large and expensive equipment and many winemakers have concerns about their effects on wine quality.

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II.HYPOTHESIS AND OBJECTIVES

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HYPOTHESIS AND OBJECTIVES

During the last years, consumers demand wines with deep red color, full body, soft tannins and fruit scents. Those characteristics can be only achieved with grapes that have reached a high phenolic maturity. Very well-ripe grapes have a high concentration of anthocyanins that proportionate wines with deep red color. Additionally, full ripen grapes present low astringency and bitterness probably due to the diminution of the proportion of seed proanthocyanidins which are very galloylated. On the contrary, unripe grapes can lead to wines with poor color as also high levels of bitterness and astringency.

At this point, it is essential to introduce the phenolic maturity notion. This concept makes reference to the potential of proanthocyanidins and anthocyanins presents in grapes, as also to their structure and capacity to be extracted from grapes during winemaking. Consequently, oenologists are very interested in phenolic maturity measurement in order to know which will be the color intensity and the final polyphenol composition of their wines. The aim is to facilitate the decision of the harvest day and/or even to separate grapes in function of their real quality. In recent years, several methods have been proposed to measure phenolic maturity. Among the different techniques that have been developed, the most applied of them are based on obtaining extracts from grapes with maceration in different solvents. Although these methods are commonly employed by some wineries there is a lack of scientific information about their exactitude, precision and especially their real predictive capacity. Indeed, only few studies correlate the phenolic composition of the grapes with the color and phenolic composition of the corresponding wines.

On the other hand, all these methods only provide the average value of a representative sample from the whole grape vineyard and do not consider any possible heterogeneity in their degree of maturity. Winemakers know that the presence of a non-negligible proportion of unripe berries at the harvest time can considerably alter wine characteristics and that the only solution is the manual elimination of unripe berries with a grape sorting table for minimizing its negative impact.

In view of the above, the main objectives of the first part of this thesis were:

- To compare three of the most used methods for measuring the phenolic maturity of grapes at the harvest time - Glories, ITV and Cromoenos - in order to determine their

predictive effectiveness on the color and phenolic composition of the corresponding wines.

- To study the heterogeneity of the grapes, according to their density, during the grape ripening and determine its real influence on wine characteristics.

Once we achieved those objectives, we focused on the probably implications that well-ripe grapes could have at wine quality. Grapes with very high phenolic maturity frequently have high sugar and low acid concentration; thereby the obtained wines have the inconvenience of presenting very high pH and alcohol content. During recent years this tendency seems to be increasing especially as result of the climate change.

To mitigate the excess of pH and alcoholic concentration of wines we proposed the following objective:

- To study the use of unripe grapes harvested during cluster thinning as a new winemaking procedure for partially reducing the wine pH and alcoholic content.

During last years, micro-oxygenation has begun to be a common application technique in several wineries. Most authors have suggested that the reactions induced by oxygen produce wine color stabilization and astringency decrease. In fact, all winemakers know that micro-oxygenation is very useful for stabilizing color, for improving structure and body, and for decreasing the astringency, bitterness and herbaceous characters of wine. Therefore, its employment in wines obtained with insufficiently-ripe grapes is highly recommended.

As it is already mentioned, well-ripe grapes probably lead to wines with high pH and insufficiently-ripe grapes generally produce wines with low pH. The pH conditions the equilibrium among the different anthocyanin forms and has an unquestionable impact on wine color. On the other hand, anthocyanins can act, depending on pH, as electrophiles in the flavylium form or as nucleophiles in the hemiketal form. Therefore, it is quite logical that pH can influence the reactivity of anthocyanins. Moreover, the first step on the reactions induced by oxygen implies the formation of ethanal from ethanol. Subsequently, ethanal must capture a proton and become into a carbocation which will react with flavanols to start the process of formation of ethyl bridges. Therefore it is also logical that pH exert a non-negligible influence on these mechanisms.

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In this context, it is logical to consider that the proton concentration, and consequently the pH, affects the process of this reaction. For that reason it would be interesting to study how wines with different pH evolve with the application of micro-oxygenation.

Bearing in mind this observation we proposed the following objective:

- To study the influence of wine pH on the effectiveness of micro-oxygenation.

This research is part of a more general project developed by the Oenological Technology Research Group of the Department of Biochemistry and Biotechnology of the Rovira i Virgili University in Tarragona, Spain (project: AGL2007-66338 and CDTI (Project CENIT Demeter). An international stage has been done completed at the Chemistry Department of the Porto University down the supervision of the professor Dr. Victor de Freitas.

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III.RESULTS

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i. Comparison of methods for estimating phenolic maturity in grapes: Correlation between predicted and obtained parameters

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Nikolaos Kontoudakis
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Abstract

With the aim of determining the real predictive ability of three methods for measuring phenolic maturity (Glories, ITV and Cromoenos), representative grapes of Merlot, Cabernet sauvignon, Grenache and Tempranillo were harvested at three different ripening levels. The grapes were vinified by triplicate and were also used for phenolic maturity estimation. After that, colour intensity, CIELAB coordinates, anthocyanins by spectrometry and HPLC and total phenolics were analysed in wines and also in the different extracts from the three extraction methods. Statistical analysis of data was carried out to determine the real performance of prediction of the different methods. Glories method predicted reasonably the color intensity, CIELAB coordinates and the concentration of anthocyanins and total phenolics in wine although it needs a lot of time and it requires working carefully. On the other hand, Cromoenos method predicted similarly or even better the color and phenolic composition of wine. This method also presents the advantage of being much faster and easier to apply. In contrast, although the ITV method provided reasonable results for anthocyanins and total phenolic compounds its colour prediction was not adequate.

Keywords: *Phenolic maturity, Glories, Institut Français de la Vigne et du Vin, method, Cromoenos, Predictive ability*

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1. Introduction

One of the most important parameters for obtaining high quality red wines is probably the ripeness of the grapes at the harvest time. Commonly, the grape maturity, and therefore the decision of harvesting, is to be taken only after analyzing sugar concentration, titratable acidity and the pH of the grape juice [1]. However, these parameters only provide information about the pulp ripeness and overlook the real degree of skins and seeds maturities [2].

The maturity of skins and seeds is considered as a key factor for red winemaking because they are the major source of phenolic compounds which are dissolved into the wine during maceration process [1,3]. Anthocyanins, the molecules responsible of red wine color, accumulate gradually in the skins during ripening [4-6]. However, anthocyanins are not always easily extracted from skins, and low extraction levels can lead to poorly colored wines, even though if the anthocyanins concentration in the original grapes is sufficient [7]. Therefore, the extractability of anthocyanins is also one of the main factors affecting their future concentration in wine [2,7]. Moreover, the extractability of anthocyanins increases throughout grape ripening [1]. In fact, it has been verified that the maceration of skins from well-ripened grapes in a model wine solution originated a greater anthocyanin concentration and higher color intensity [8].

Grape skins and seeds also contain many other phenolic compounds that are incorporated into the wine during the maceration process. Among these, proanthocyanidins, also known as condensed tannins, have a major role in wine quality [1]. Unlike proanthocyanidins from seeds, proanthocyanidins from skins contain prodelphinidins and have a higher degree of polymerization and a lower proportion of galloylated subunits [8]. Proanthocyanidins contribute to long-term color stability by combining with anthocyanins [1]. Besides, proanthocyanidins are also associated with such texture sensations as body and astringency [8,9].

Nowadays, deeply colored and full-bodied wines are highly valued by the market. However, these kind of wines require fully ripen grapes or on the contrary there is a risk of obtaining very bitter and astringent wines if the maceration technique applied provokes an overextraction of tannins. Consequently, winemakers are very interested in phenolic maturity measurement in order to know which will be the color intensity and the final polyphenol composition of the wine. The aim is to facilitate the decision of the day of harvest or even to separate grapes in function of their real quality. Several methods for measuring phenolic maturity have been

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proposed using different techniques, such as the skin texture measurement [10], the direct measurement of the color absorption of the grape skins [11] and even the analysis of multispectral airborne high resolution images [12]. Nevertheless, these methods still remain experimental and are not usually employed by wineries. Nowadays, the most applied methods are based on obtaining extracts from grapes by means of maceration in different solvents [7, 13-17]. Among them, Glories method [7, 18] and ITV method [16,19] are probably the most employed. However, all these methods are slow, laborious and require the participation of well-trained technicians. Recently, another method, Cromoenos, has been proposed [20]. This method uses two commercial reagents and specific equipment for extracting phenolic compounds, enabling results easily in just 10 minutes.

Although these methods are commonly employed by some wineries there is not a clear criterion for selecting which one of these methods is the most appropriate. This is probably due to the lack of scientific information about their exactitude, precision and especially their real predictive capacity. In fact, to our knowledge there are only few studies which have tried to correlate the phenolic composition of the grapes with the color and phenolic composition of the corresponding wines [21-23]. The aim of this study was the comparison of three methods of measuring phenolic maturity, Glories [7], ITV [13] and Cromoenos [20] in order to determine their predictive performance on the color and phenolic composition of the corresponding wines.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Methanol, acetonitrile and formic acid were HPLC grade were purchased from Panreac (Barcelona, Spain). Malvidin-3-O-glucoside chloride was purchased from Extrasynthès (Genay, France). The rest of the chemicals were of high purity and were purchased from Panreac (Barcelona, Spain).

2.1.2. Grape samples

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This study was carried out with grapes of the cultivars *Vitis vinifera* cv. Merlot, Cabernet sauvignon, Grenache and Tempranillo. Grapes were harvested from the experimental vineyard belonging to the Enology Faculty in Tarragona (Rovira i Virgili University) at Constantí (Tarragona) in 2007 at three levels of ripeness.

For each cultivar, the grapes were only collected from the two central rows of each plot. For obtaining random samples and for avoiding picking grapes from the same vine, every third vine was marked.

The first harvest was collected three weeks after veraison and only from the marked vines. The second harvest was carried out five weeks after veraison collecting only grapes from the vines right next to the marked ones. Finally, the third harvest was done 7 weeks after veraison picking only grapes from the remaining vines.

All bunches were manually destemed and the grapes were randomly grouped and used immediately for standard and phenolic maturity measurements and also for winemaking.

2.2. Methods

All the maturity analysis and vinifications were carried out by triplicate.

2.2.1. Standard maturity analysis.

One hundred grape berries were weighted and used for determining the sugar content, the titratable acidity and the pH according with the analytical methods recommended by the OIV [25]. Sugar concentration was measured using a refractometer (Fabre réfractométres, Sarl Germain, France). The titratable acidity was measured by titrimetry using NaOH 0.1 N and Bromothymol blue as indicator. The pH was measured using a pHmeter (micropH 2002, Crison, Barcelona, Spain). Table 1 shows the sugar content, the probable alcoholic degree, the titratable acidity, pH and the weight of 100 berries of the four cultivars at the three different maturity levels in which they were harvested.

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Table 1. Evolution of standard parameters of grapes throughout ripening.

Cultivars	Parameters	Weeks after veraison		
		3	5	7
Merlot	S (g/L)	201.1 ± 0.1a	233.9 ± 0.1b	242.4 ± 0.1c
	PAD (% v/v)	12.0 ± 0.1a	13.9 ± 0.1b	14.4 ± 0.1c
	TA (g/l)	6.65 ± 0.16a	5.42 ± 0.03b	4.67 ± 0.23c
	pH	3.23 ± 0.01a	3.39 ± 0.01b	3.45 ± 0.01c
	W (g)	147.7 ± 2.4a	150.1 ± 1.7a	169.4 ± 6.1b
Cabernet Sauvignon	S (g/L)	185.1 ± 0.1a	207.6 ± 1.9b	222.2 ± 0.1c
	PAD (% v/v)	11.0 ± 0.1a	12.3 ± 0.2b	13.2 ± 0.1c
	TA (g/l)	8.95 ± 0.23a	6.90 ± 0.40b	5.65 ± 0.09c
	pH	3.07 ± 0.01a	3.14 ± 0.02b	3.28 ± 0.01c
	W (g)	135.1 ± 0.7a	137.6 ± 3.5a	153.6 ± 5.0b
Grenache	S (g/L)	221.0 ± 1.9a	233.9 ± 0.1b	233.4 ± 0.9b
	PAD (% v/v)	12.0 ± 0.3a	13.9 ± 0.1b	13.9 ± 0.2b
	TA (g/l)	5.58 ± 0.17a	4.03 ± 0.03b	3.90 ± 0.01c
	pH	3.07 ± 0.01a	3.30 ± 0.01b	3.30 ± 0.03b
	W (g)	200.9 ± 5.06a	203.9 ± 5.8a	222.2 ± 5.7b
Tempranillo	S (g/L)	194.1 ± 1.0a	203.6 ± 0.1b	233.9 ± 1.7c
	PAD (% v/v)	11.5 ± 0.2a	12.1 ± 0.1b	13.9 ± 0.3c
	TA (g/l)	5.85 ± 0.15a	4.46 ± 0.05b	4.20 ± 0.01c
	pH	3.15 ± 0.01a	3.23 ± 0.03b	3.28 ± 0.01c
	W (g)	209.2 ± 5.9a	216.9 ± 15.2ab	223.9 ± 8.46b

All data expressed the arithmetic average of three replicates ± standard deviation. S: sugar concentration, PDA: probable alcoholic degree, TA: titratable acidity, W: weight of 100 berries. Different letter indicates the existence of statistically significant differences.

2.2.2. Phenolic maturity analysis.

Six hundred grape berries from the four grape varieties and from the different ripening stages were milled by triplicate for 1 minute using a blender (Krups GMBH, F575, Solingen, Germany). This extract was used for the measurement of phenolic maturity using the Glories method, the ITV method and the Cromoenos method.

2.2.2.1. Glories method

It has been applied a modification of the methodology previously described by Professor Glories [7,18]. The modification consisted in the use of two buffers to guarantee the conservation of the pH throughout the maceration period. Samples of 50 g of the extract were macerated for 4 hours in two different buffers. First buffer was oxalic acid 0.3 M (pH 1.00) and the second buffer was phosphoric acid 0.3 M (pH 3.2). The pH of both buffers was adjusted using NaOH solution (0.2 N). The samples were manually shacked every half hour. After 4 h, the macerated samples were

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centrifuged (12,000 rpm, 10 min) in a Biofuge primo centrifuge (Heraus, Osterode, Germany) and used for colour measurement and for anthocyanins and total phenolic compounds content.

2.2.2.2. ITV method

It has been applied the methodology previously described by Dupuch [13]. Samples of 50 g of the extract were macerated for 1 h after the addition of 15 mL of ethanol (96 %) and 85 mL of HCl (0.1 %). The samples were manually shacked every 15 minutes. After 1 h, the macerated samples were centrifuged (12,000 rpm, 10 min) in a Biofuge primo centrifuge (Heraus, Osterode, Germany) and used for colour measurement and for anthocyanins and total phenolic compounds content.

2.2.2.3. Cromoenos method

This method use specific equipment and reagents provided by the manufacturer (Bioenos, Cariñena, Spain) [20]. Samples of 40 mL of the extract were introduced in the thermoextractor after the addition of 1 mL of reagent A, 4 mL of reagent B and 40 mL of deionised water. Once upon the temperature arrives till 80 ºC (environ 2 min) 1 mL of the sample was centrifuged (13,400 rpm; 2 min) in a Hermle Z233 MK2 centrifuge (Wehingen, Germany).

60 µL of the the supernatant were diluted till 4 mL with HCl 2 % (v/v). This solution was used for the measurement of the absorbance at 520 and 280 nm. The predicted colour intensity of the corresponding wines were obtained by means of the software supplied by the manufacturer after introducing the absorbances at 520 and 280 nm as well as the cultivar name, the probable alcoholic degree, titratable acidity and pH. Simultaneously, this solution was also used for the determination of anthocyanins and total phenolic compounds content.

2.2.2.4. Modified Cromoenos method

A modification of the Cromoenos was also applied to the measurement of the color. 500 µL of the supernatant were diluted with 1 mL of a synthetic solution containing 4 g of tartaric acid L^{-1} and 13 % (v/v) ethanol adjusted at a pH of 3.80. This solution was selected after some previous

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assays (data not shown) because the mixture with the Cromoenos extract presented a pH similar than that of most of the wines of the region (pH 3.50). This mixture was directly used for colour measurements.

2.2.3. Winemaking procedure.

For each variety and ripening stage, the grapes were harvested and manually destemed. After that, the berries were randomly distributed in three groups of eight kilos each one, crushed with a semi-automatic crusher machine (Gual, Villafranca del Penedès, Spain), sulphited (100 mg of $K_2S_2O_5$ L^{-1}) and introduced in 10 L tanks. All tanks were immediately inoculated with 200 mg L^{-1} of selected yeast (DV10, Martin Vialatte, Epernay, France) and maintained at a room temperature of 25 ± 1 ºC. All these microvinifications were controlled diary by measuring the temperature and the density of the must. Each day a punch down was carried out to favour phenolic compounds dissolution. After 14 days of maceration, the wines were racked, sulphited (100 mg of $K_2S_2O_5$ L^{-1}) and refrigerated for three weeks at 4 ºC. Hence, malolactic fermentation was inhibited to avoid possible variations in the rhythm of this transformation that could affect differently each wine. After that, wines were decanted and bottled. All the samples were stocked at 15 ± 1 ºC until the moment of the analysis.

2.2.4. Wine and phenolic extract analysis

With the aim of making directly comparable extracts and wines, the results of grape extracts were corrected considering the applied dilution factors and a theoretical yield of 80 % between the volume of wine and the weight of the grapes.

2.2.4.1. Colour parameters.

The color intensity (*CI*) was estimated using the method described by Glories [26]. The CIELAB parameters, lightness (*L**), chroma (*C**), hue (*H**), redness (*a**) and yellowness (*b**) were determined according to Ayala et al. [27].

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2.2.4.2. Phenolic compounds.

The total anthocyanins concentration was determined spectrophotometrically using the method described by Niketic-Aleksic and Hrazdrina [28]. HPLC analyses of anthocyanins were carried out with an Agilent (1100 series) liquid chromathograph and Waters Spherisorb column (ODS2) in accordance with the method described by González-SanJosé [14]. The anthocyanin standard curves were made using malvidin-3-glucoside (Extrasynthase, Lyon, France). The total phenolic index (TPI) was determined by measuring absorbance at 280 nm [2] and expressed as absorbance units [1].

2.2.5. Statistics.

All of the data are expressed as the arithmetic average ± standard deviation from three replicates. Linear regressions as well as Fisher's correlation analysis were carried out using Statview (software for Macintosh). Statistical comparisons between values were established with a one-factor ANOVA and Schffee test using SPSS software.

3. Results and discussion

Table 1 shows the sugar content, probable alcoholic degree, titratable acidity, pH and the weight of 100 berries of the four cultivars at the three selected harvest maturity levels. The four cultivars presented the expected evolution in all parameter. The first harvest presented an insufficient maturity inasmuch as the probable alcoholic degree and pH were relatively low and the titratable acidity relatively high in relation to the potentiality of each one of these cultivars. In contrast, the grapes of the third harvest were very ripe because their probable alcoholic degree and pH were relatively high and their titratable acidity comparatively low. The grapes of the second harvest of the four cultivars presented intermediate values.

Figure 1 shows the linear regression analysis between the color intensity of the different wines and their corresponding predicted values obtained by applying the different phenolic maturity methods. These results indicate that Glories and Cromoenos methods originated reasonably linear regression coefficients (r^2 = 0.8489 and 0.8869 respectively), whereas the ITV method did

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not (r^2 = 0.3879). On the other hand, the modification of Cromoenos method enhanced the quality of the measure to the point that the linear regression coefficient (r^2 = 0.9517) increased notably in respect to the original method.

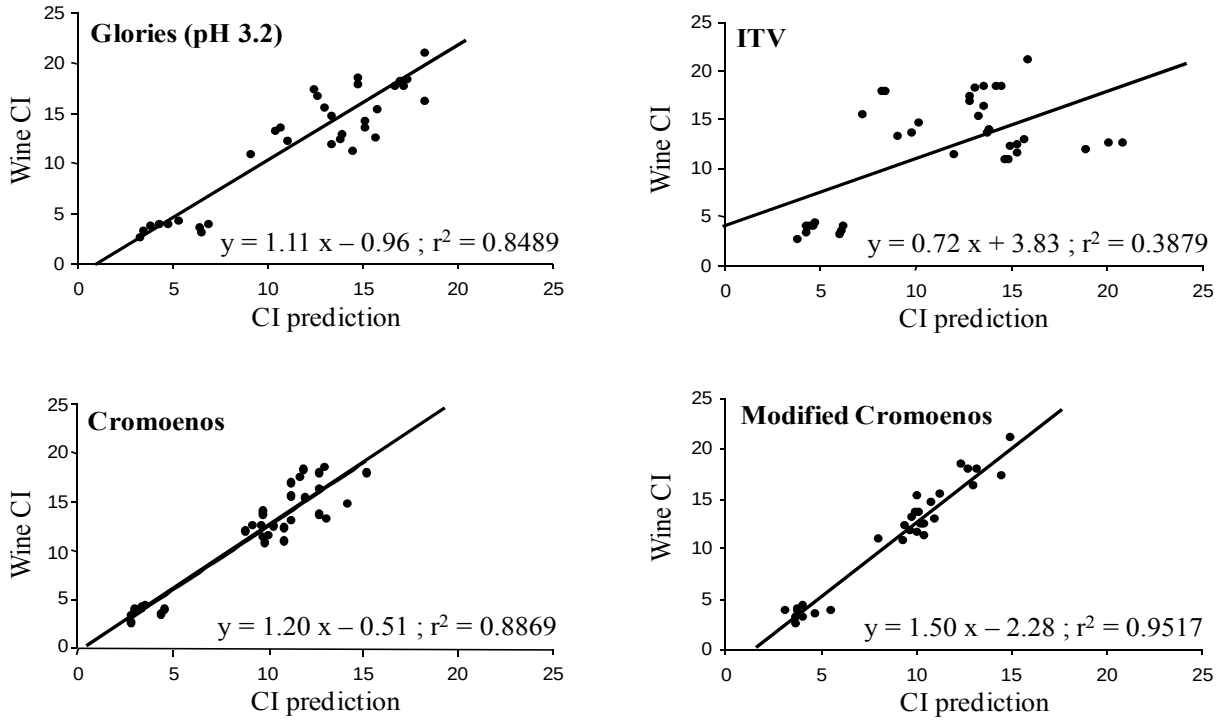

Fig. 1. Linear regression analysis between the obtained and the predicted color intensity.

Figure 2 present the linear regression analysis between the CIELAB coordinates of the different wines and their corresponding predicted values obtained with Glories, ITV and modified Cromoenos method. In that case, the measurement of CIELAB coordinates for Cromoenos method was not possible because of the high absorbance of the solutions. In contrast, the dilution used in the modified Cromoenos method allowed its determination. Glories, ITV and specially modified Cromoenos originated satisfactory linear regression coefficients for Chroma (r^2 = 0.8474, 0.7514 and 0.9114 respectively) and Luminosity (r^2 = 0.7900, 0.8284 and 0.8803 respectively). However, none of these methods was able to provide acceptable linear regression coefficients for the Hue (r^2 = 0.3951, 0.1427 and 0.5895 respectively). Even, in the case of Glories method the slope was negative indicating an inverse tendency between the extract and the wine.

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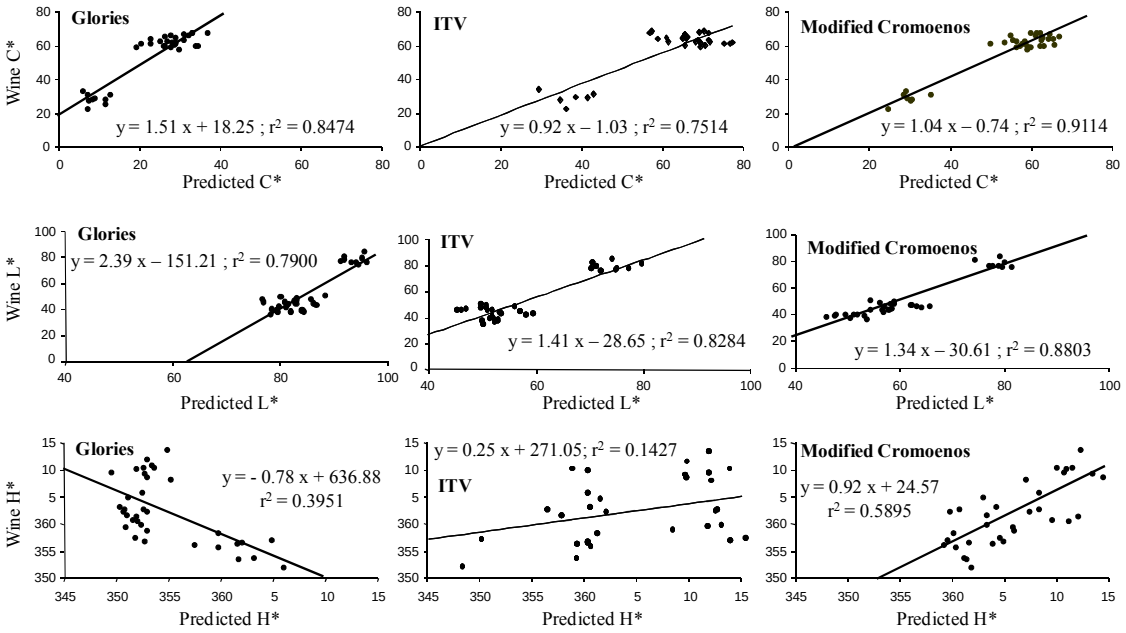

Fig. 2. Linear regression analysis between the obtained and the predicted CIELAB coordinates.

Table 2 summarizes the predictive capabilities for color intensity and CIELAB coordinates of the different phenolic maturity methods. All this data indicates that the modified Cromoenos method originated in all the parameters the higher linear regression and Fisher's correlation coefficients, existing in all the cases, even in H* and b*, a statistical significant correlation ($p < 0.05$). On the other hand, Glories method also led to reasonably good linear regression and Fisher's correlation coefficients for CI, C*, L* and a* but not for H* or b*. Finally, ITV method only correlated adequately C*, L* and a* whereas no significant correlations were found in CI, H* and b*. The ensemble of these results indicates that Glories and specially modified Cromoenos method can be used for wine color prediction with an adequate degree of precision, whereas ITV method is not suitable for this purpose.

As it was quoted in the introduction, only few studies have tried to verify the real predictive performance of some methods for measuring grapes phenolic maturity. Specifically, Jensen et al. (2008) [21], working with microvinifications of only 250 g, have obtained a linear regression coefficient (r^2) of 0.9178 when compare the color intensity prediction with the one in wines. This value is slightly higher than our results corresponding to Glories and Cromoenos methods but somewhat lower than those of modified Cromoenos method.

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Table 2. Comparison between the predictive capabilities for color intensity and CIELAB coordinates of the different methods.

Predicted parameter	Method	Slope (m)	Y Intercept (b)	r^2	Fisher's correlation coefficient	Significance
Color intensity	Glories (pH 3.2)	1.11	-0.96	0.8489	0.9214	***
	ITV	0.21	-3.83	0.3879	0.5624	ns
	Cromoenos	1.20	-0.51	0.8869	0.9418	***
	Modified Cromoenos	1.50	-2.28	0.9517	0.9756	***
C*	Glories (pH 3.2)	1.51	18,25	0.8474	0.9205	***
	ITV	0.92	-1.03	0.7514	0.8668	***
	Modified Cromoenos	1.05	0.74	0.9114	0.9547	***
L*	Glories (pH 3.2)	2.39	-151.21	0.7900	0.8888	***
	ITV	1.41	-28.65	0.8284	0.9102	***
	Modified Cromoenos	1.34	-30.61	0.8803	0.9382	***
H*	Glories (pH 3.2)	-0.78	636.88	0.3951	0.5756	ns
	ITV	0.25	271.05	0.1427	0.3778	ns
	Modified Cromoenos	0.92	24.57	0.5895	0.7678	*
a*	Glories (pH 3.2)	1.54	18.45	0.8283	0.9101	***
	ITV	1.11	-14.15	0.8145	0.9025	***
	Modified Cromoenos	1.14	-6.38	0.9015	0.9495	***
b*	Glories (pH 3.2)	-1.36	-0.17	0.1625	0.4031	ns
	ITV	0.14	2.44	0.0604	0.2458	ns
	Modified Cromoenos	0.96	-14.82	0.6456	0.8035	**

All data represent the statistical comparison of 36 wines and their corresponding phenolic maturity extracts. ns: non significant.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.005$.

Other studies have tried to correlate grape phenolics with the final wine composition [23,24] but none of them have directly compared the grape extract color with the wine color. Finally, González-Neves et al. (2002) [29] have directly compared the color and the concentration of anthocyanin and other phenolic compounds of wines with the parameters obtained by the Glories method. In this experiment, authors obtained good statistical correlations when they compare the total polyphenols in wine with the anthocyanin extracted at pH 3.2 and between the color intensity and the anthocyanin extracted at pH 1.0. Other parameters presented significant correlation coefficient but not at the same level.

Figure 3 shows the linear regression analysis between the anthocyanins concentration, measured by spectrophotometry, of the different wines and their corresponding predicted values obtained by applying the different phenolic maturity methods. Glories method originated reasonably good linear regression coefficients at both pH. Specifically, r^2 was 0.7897 at pH 1.0 and 0.8809 at pH 3.2. According to Glories method, the anthocyanin concentration obtained at pH 1.0 represent

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all the anthocyanins present in the skins whereas anthocyanin concentration obtained at pH 3.2 represent all the anthocyanin extractable in a standard winemaking. These results are in agreement with this postulate since the slope at pH 3.2 is close to 1 (0.96) suggesting that extraction at this pH was very similar to that obtained in the wine. Moreover, the slope at pH 1.0 is close to 0.5 (0.52), suggesting that during winemaking were extracted about 50% of all anthocyanins present.

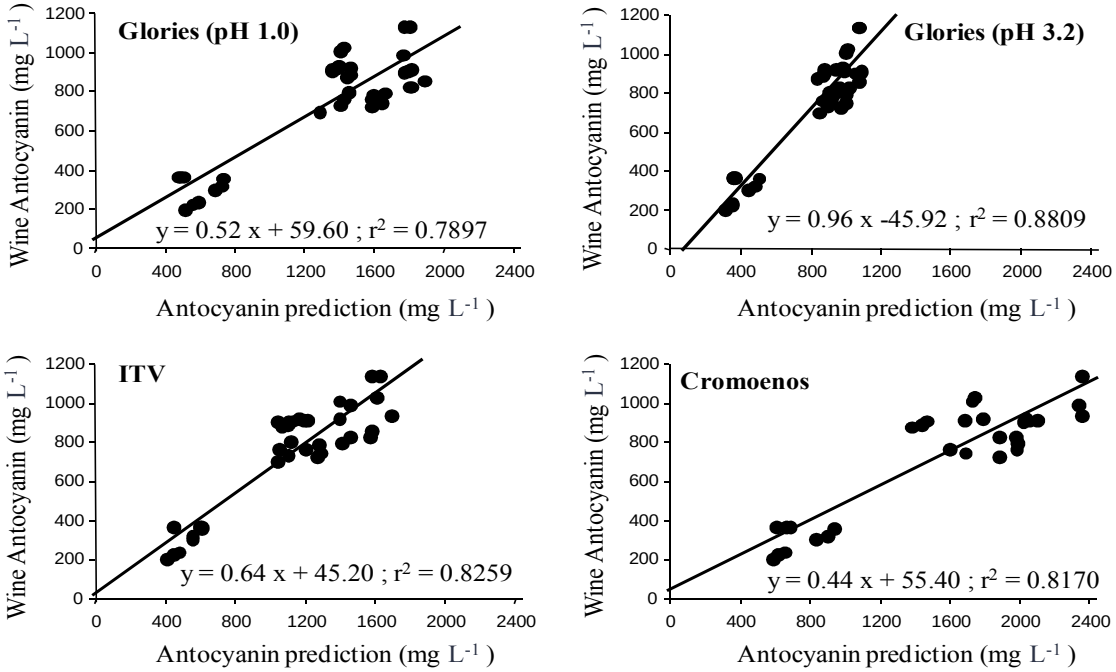

Fig. 3. Linear regression analysis between the obtained and the predicted anthocyanin concentration.

The other two methods, ITV and Cromoenos, also provided reasonably linear regression coefficients for anthocyanin concentration (r^2 = 0.8259 and r^2 = 0.8170 respectively). However, the obtained slopes are in both cases quite lower than 1.0 (0.64 and 0.44) indicating that both methods overextracted anthocyanins in relation to what happens during winemaking.

Other authors have tried to correlate the spectrophotometric anthocyanin concentration of the extracts and the final wine anthocyanin concentration. González-Neves et al. (2004) [23] and Romero-Cascales et al. (2005) [24], using the Glories method have obtained linear regression coefficients of 0.7225 and 0.8742 respectively, whereas Jensen et al. (2008) [21], using other methodology [15] obtained better results (0.9025). Our results are comparable and confirm that the three methods predict reasonably the future wine anthocyanin concentration.

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Figure 4 shows the linear regression analysis between the total phenolic index (TPI) of the different wines and their corresponding predicted values obtained by applying the different phenolic maturity methods. For this parameter, the three methods provided reasonably good linear regression coefficients (between 0.8028 and 0.8839) and comparables to those previously described in the bibliography [21,23,24]. However, the slopes were in all the cases lower than 1.0 suggesting that all three methods produce an overextraction of phenolic compounds. Specifically, Glories method presented a slope of 0.75 which indicates an overextraction of around 33 %, which can be considered not very high. However, ITV and Cromoenos presented slopes considerably lower, 0.36 and 0.19, thus implying very high overextraction (around three and five folds more than in wine). This overextraction is probably because the conditions of extraction of all methods are very drastic and involve the complete crushing of seeds.

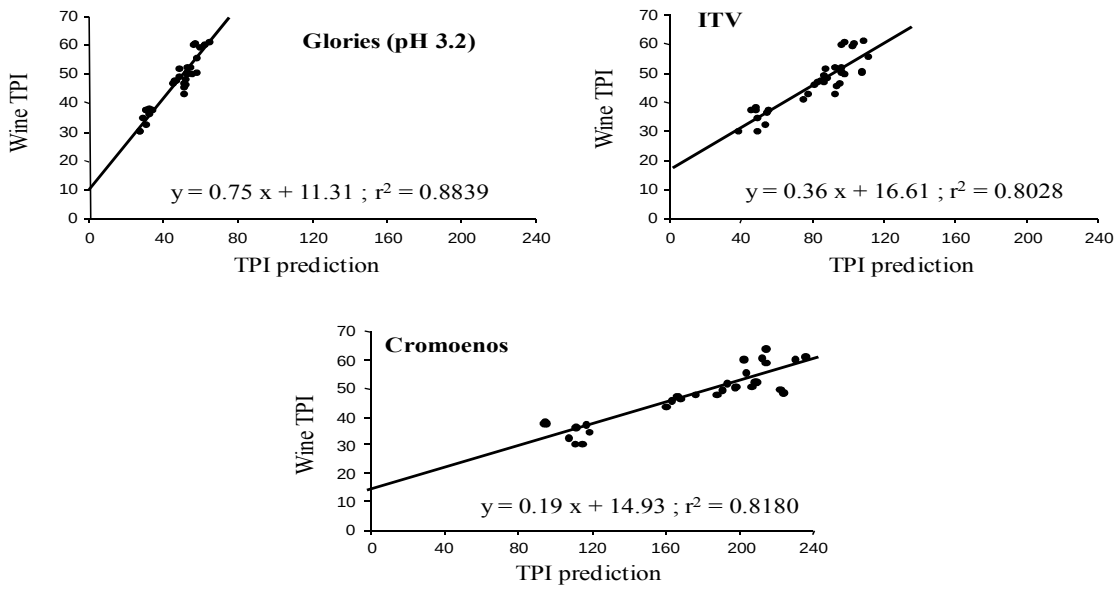

Fig. 4. Linear regression analysis between the obtained and the predicted TPI.

Table 3 summarizes the predictive capabilities for anthocyanins, measured by spectrophotometry, and TPI of the different phenolic maturity methods. This table also include the statistical comparison between the anthocyanin analysis by HPLC of the wines and the extracts obtained by the different methods. The three methods provide reasonably linear regression and Fisher's correlation coefficients in anthocyanins (by spectrophotometry) and TPI, existing in all the cases a statistical significant correlation ($p < 0.001$). When the statistical

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analysis was applied to the total anthocyanins measured by HPLC the linear regression and Fisher's correlation coefficients of all the methods decreased considerably although the existence of a statistical significance in the correlation was maintained in all the cases. It is necessary to remark that the slopes were in the three methods quite lower than 1.0 confirming that an overextraction of anthocyanins has taken place especially in the ITV and Cromoenos methods. These results are in agreement to the few data previously described [21,23,24] and can be considered as quite satisfactory.

Table 3. Comparison between the predictive capabilities for phenolic compounds of the different methods.

Predicted parameter	Method	Slope (m)	Y Intercept (b)	r^2	Fisher's correlation coefficient	Significance
Total anthocyanins by spectrophotometry	Glories (pH 3.2)	0.96	- 46	0.8809	0.9386	***
	ITV	0.74	45	0.8259	0.9088	***
	Cromoenos	0.44	55	0.8170	0.9039	***
Total anthocyanins by HPLC	Glories (pH 3.2)	0.48	101	0.4884	0.6988	*
	ITV	0.21	137	0.4515	0.6719	*
	Cromoenos	0.19	117	0.5680	0.7536	**
Total anthocyanins monoglucosides	Glories (pH 3.2)	0.31	122	0.4172	0.6459	*
	ITV	0.16	128	0.4808	0.6934	*
	Cromoenos	0.17	100	0.6152	0.7843	**
Total acylated anthocyanins	Glories (pH 3.2)	0.97	1.0	0.9017	0.9499	***
	ITV	0.49	-19.1	0.8864	0.9441	***
	Cromoenos	0.33	11.4	0.8908	0.9438	***
Total acetilated anthocyanins	Glories (pH 3.2)	0.88	3.4	0.9531	0.9763	***
	ITV	0.47	0.7	0.9698	0.9848	***
	Cromoenos	0.36	5.1	0.9180	0.9581	***
Total coumarylated anthocyanins	Glories (pH 3.2)	0.47	18.4	0.2989	0.5468	ns
	ITV	0.11	22.1	0.2814	0.5302	ns
	Cromoenos	0.12	17.5	0.4143	0.6431	*
TPI	Glories (pH 3.2)	0.75	11.3	0.8839	0.9402	***
	ITV	0.36	16.6	0.8028	0.8961	***
	Cromoenos	0.19	14.9	0.8180	0.9044	***

All data represent the statistical comparison of 36 wines and their corresponding phenolic maturity extracts. ns: non significant.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.005$.

When this statistical analysis was applied to the monoglucosides, the acetylated and the coumarylated anthocyanins, the results varied in function of the anthocyanins group. Specifically, the monoglucosides presented a very similar behaviour than total anthocyanins as it was expected due to the great proportion of this anthocyanins type. However, acetylated and coumarylated anthocyanins behaves in a different manner. The three methods provide very good linear regression and Fisher's correlation coefficients in acetylated anthocyanins. However, in the case of coumarylated anthocyanins the results were quite worst, being only significant in the case of Cromoenos method (Table 3).

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3. Conclusions

It can be concluded that the three studied methods for measuring phenolic maturity can be used for predicting some of the characteristics of the future wines. Specifically, the three methods provide very good linear regression and correlation coefficients for anthocyanins by spectrophotometry and TPI. However, only Glories and Cromoenos offer good results for color parameters. Moreover, in the case of Cromoenos, a simple modification allows to considerably enhance its performances. Nevertheless, all three methods provide somewhat worse results when were applied to compare the anthocyanins measured by HPLC.

Another consideration to bear in mind are the cost and the easiness of the manipulation process of the three methods. Glories and ITV method do not use any specific equipment or specific reactive. However, both methods are slow, laborious and require the participation of well-trained technicians which represents a not negligible cost for the winery. On the other hand, Cromoenos needs specific equipment and reactives, which represent a cost of around 7.5 € $sample^{-1}$. Nevertheless, this method enables results easily in just 10 minutes thereby reducing the cost of labor necessary. Consequently, Cromoenos method presents the best balance between its predictive ability and its simplicity of use, making it a very good analytical procedure for wineries.

Acknowledgments

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ii. Influence of the heterogeneity of grape phenolic maturity on wine composition and quality

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Abstract

Nowadays, consumers demand red wines with deep color, soft tannins and fruit scents, but these wines can only be obtained from grapes with complete phenolic maturity. Diverse methods have been proposed for measuring phenolic maturity. However, all these methods only provide the average value and do not consider any possible heterogeneity. Throughout ripening, grapes were separated according to their density, which revealed the existence of a large heterogeneity. Grapes at harvest were also separated by density in three groups. The higher the density of the grapes the higher ethanol content, pH, color intensity, total phenolic index and anthocyanin and proanthocyanidin concentrations, and the lower the titratable acidity and bitterness of the wines. When the grapes were denser the wines were also better balanced in flavor and mouthfeel sensation. These results suggest that grape heterogeneity may influence the final wine composition and quality and therefore it should be consider at harvest.

Keywords: *Phenolic maturity, Heterogeneity, Red wine color, Phenolic compounds, Anthocyanin*

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1. Introduction

One of the major factors affecting red wine quality is the real degree of phenolic maturity in the grapes at harvest time (Ribereau-Gayon, Glories, Maujean & Dubourdieu, 2006). Most of wine's sensory attributes such as color, body, and tactile and taste sensations like as, astringency and bitterness, are directly associated with the composition of anthocyanins and proanthocyanidins (Arnold & Noble, 1978; Gawel, 1998; Noble, 1990; Vidal et al., 2003) and this composition is strongly affected by ripeness (Canals et al., 2005; Llaudy, Canals, Canals, & Zamora, 2008).

Anthocyanins are only present in grape skins whereas proanthocyanidins are present in skins and seeds (Ribereau-Gayon et al., 2006). Seed proanthocyanidins are made up of (+)-catechin, (–)-epicatechin and (–)-epicatechin-3-gallate (Kennedy, Matthewa, & Waterhouse, 2002; Prieur, Rigaud, Cheynier, & Moutounet, 1994), whereas skin proanthocyanidins have a much lower proportion of (–)-epicatechin-3-gallate and also contain (–)-epigallocatechin (Gonzalez-Manzano, Rivas-Gonzalo, & Santos-Buelga, 2004; Souquet, Cheynier, Brossaud, & Moutounet, 1996).

Molecular sizes and the monomeric composition of proanthocyanidins in particular have a large influence on the sensation of astringency. More specifically, the greater degree of polymerization and the greater percentage of galloylation, will cause a greater sensation of astringency (Herderich & Smith, 2005; Vidal et al., 2003; Vivas & Glories, 1996).

It is generally considered that ripeness strongly influences the phenolic composition of red wines (O´-Marques, Reguinga, Laureano, & Ricardo-da-Silva, 2005; Ryan & Revilla, 2003). It has been reported, for example, that insufficiently ripened grapes have a lower extractability of anthocyanins and proanthocyanidins from skins and a higher extractability of proanthocyanidins from seeds (Canals et al., 2005; Peyrot des Gachons & Kennedy, 2003). For this reason, it is generally thought that insufficiently ripened grapes may produce more astringent and bitter wines because their seeds can release a higher amount of proanthocyanidins, which are highly galloylated (Romeyer, Macheix, & Sapis, 1986).

Consequently, for the last two decades winemakers have been very interested in the concept of "Phenolic Maturity". The reason for this is very simple. Consumers demand wines with deep red

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color, full body, soft tannins and fruit scents, and this kind of wine can only be obtained from grapes that have reached complete phenolic maturity. If this is not the case, wines may present bitter and astringent sensations and a poor color.

This interest has led to several investigations to try and find effective methodologies for determining the real level of phenolic ripeness in grapes and thus provide a better criterion for deciding the optimum time of harvest. In fact, various methods for measuring phenolic maturity have been proposed (Celotti, Della Vedova, Ferrarini, & Martinand, 2007; Dupuch, 1993; Glories, & Agustin, 1993).

Some of these methods can predict reasonably well the phenolic maturity and especially the color intensity of a wine (Kontoudakis et al., 2010). However, all these methods only provide the average value of a representative sample from the whole grape vineyard and do not consider any possible heterogeneity in their degree of maturity.

In a vineyard, grapes do not ripen homogeneously. Each cluster and even each berry matures at different rates through the influence of multiple factors. The location of vines in the vineyard (exposure, altitude, soil composition, temperature, humidity, vine density, etc.), the position of the cluster on the vine and even the position of the berries in the cluster can produce some differences in the ripening rate (Haselgrove et al., 2000; Smart, Robinson, Due, & Brien, 1985). Furthermore, an uneven grape ripeness can affect the quality of the final product. The presence of a non-negligible percentage proportion of unripe berries can considerably increase the appearance of bitter and astringent characters in wine.

Several studies are focused on the influence of grapes harvested at different stages of maturity on wine phenolic composition and quality (Canals et al., 2005; Gambuti, Strollo, Lecce, & Moio, 2007; Llaudy et al., 2008; Pérez-Magariño, & González-San José, 2006). However, to our knowledge, none of them study the extent and the impact of grape heterogeneity. Since this aspect has not been investigated in detail to date, the aim of this study was to determine the real influence of the heterogeneity in the degree of grape ripeness on wine composition and quality.

2. Materials and methods

2.1. *Chemicals*

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Methanol, acetonitrile, formic acid and acetic acid were HPLC-grade and were purchased from Panreac (Barcelona, Spain). Malvidin-3-O-glucoside chloride, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin-3-O-gallate were purchased from Extrasynthès (Genay, France). TSK Toyopearl gel HW-40(S) was purchased from Tosoh (Japan). Ovoalbumin, phloroglucinol and L-ascorbic were purchased from Sigma (Madrid, Spain). The rest of the chemicals were of high purity and were purchased from Panreac (Barcelona, Spain).

2.2. *Grapes*

This study was carried out with grapes of the cultivar *Vitis vinifera* cv. Cabernet sauvignon from the experimental vineyard at Constantí (AOC Tarragona) which belongs to the Enology Faculty in Tarragona of the Rovira i Virgili University. The grapes were collected in 2007 and 2008 and were only taken from the two central rows of the plot.

2.3. *Maturity controls*

Six hundred grapes were randomly collected at one, three, five and seven weeks after veraison. One hundred berries were used for measuring the sugar content, titratable acidity, pH and weight. Solutions of NaCl of 100 to190 g/l were prepared and to analyze ripening heterogeneity according to the methodology previously described (Fournand et al., 2006). These solutions had densities comprised between 1020 and 1120 mg/ml. One hundred berries were introduced to the less dense solution (1020 mg/ml). The floating berries were considered that have the same density than the solution. There were separated from sank berries and were counted. After that, sank berries were removed and introduced to the following denser solution (1031.1 mg/ml). The same process was repeated with all NaCl solutions. All these measurements were done in triplicate.

2.4. *Winemaking procedure*

Seven weeks after veraison, 70 kg of grapes were harvested and manually destemmed. Grapes were separated into three groups by flotation in two solutions of sucrose of different concentrations. Sucrose solutions of 232 g/l and 244 g/l were used for the 2007 vintage and of 244 g/L and 256 g/l for the 2008 vintage. These solutions presented the following densities at

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20ºC: 1090 mg/l and 1095 mg/l for the 2007 vintage and 1095 mg/l and 1100 mg/l for the 2008 vintage. These solutions were chosen each year to adapt it to the maturity conditions of the vintage.

Table 1. Evolution of standard parameters of grapes throughout ripening.

Parameters	Vintage	Weeks after veraison			
		1	3	5	7
S (g/L)	2007	154.3 ± 3.9a	185.1 ± 0.1b	207.6 ± 1.9c	222.2 ± 1.3d
	2008	176.7 ± 1.7a	190.8 ± 2.3b	197.8 ± 5.6b	206.5 ± 1.0c
PAD (% v/v)	2007	9.17 ± 0.23a	11.0 ± 0.3b	12.3 ± 0.2c	13.2 ± 1.9d
	2008	10.50 ± 0.10a	10.9 ± 0.1b	11.6 ± 0.2c	12.3 ± 0.1d
TA (g/l)	2007	13.34 ± 0.10a	8.95 ± 0.23b	6.90 ± 0.40c	5.65 ± 0.09d
	2008	14.10 ± 0.09a	9.45 ± 0.05b	7.03 ± 0.49c	6.55 ± 0.35c
pH	2007	2.82 ± 0.01a	3.07 ± 0.02b	3.14 ± 0.02c	3.28 ± 0.01d
	2008	2.90 ± 0.02a	3.05 ± 0.02b	3.16 ± 0.02c	3.21 ± 0.01c
W (g)	2007	109.1 ± 1.a	120.4 ± 0.7b	137.6 ± 3.5b	153.6 ± 5.1c
	2008	115.1 ± 3.8a	117.6 ± 3.5ab	124.2 ± 5.8b	127.1 ± 0.5c

All data are expressed as the arithmetic average of three replicates ± standard deviation (n=3). S: sugar concentration, PAD: prabable alcoholic degree, TA: titratable acidity, W: weight of 100 berries. Statistical analysis: one-factor ANOVA and Scheffe' s test (both p=0.05). Different letters indicates the existence of statistically significant differences between values of the same vintage.

After that, the berries of each lot were randomly distributed into three groups of four kilograms, crushed with a semi-automatic crusher machine (Gual, Villafranca del Penedès, Spain), sulphited (100 mg of $K_2S_2O_5$/l) and introduced in six liter tanks. All tanks were immediately inoculated with 200 mg/l of selected yeast (DV10, Martin Vialatte, Epernay, France) and maintained at a room temperature of 25 ± 1 ºC. All these microvinifications were controlled daily by measuring the temperature and the density of the must. Each day a punch down was carried out to encourage the extraction of phenolic compounds. After 14 days of maceration, the wines were racked, sulphited (100 mg of $K_2S_2O_5$/l) and refrigerated for three weeks at 4 ºC. After that, wines were decanted and bottled. All the samples were stored at 15 ± 1 ºC until the moment of the analysis.

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2.5. *Standard grape and wine analysis*

The analytical methods recommended by the OIV (O.I.V., 2005) were used to determine sugar content, probable alcoholic degree, titratable acidity and the pH of the grapes and the ethanol content, titratable acidity and pH of the wines.

2.6. *Color parameters*

The color intensity (*CI*) was estimated using the method described by Glories (1984). The CIELAB coordinates, lightness (*L**), chroma (*C**), hue (*h**), red-greenness (a*) and yellow-blueness (b*) were determined according to Ayala, Echávarri and Negueruela (1997) and the data were processed with the MSCV® software (Ayala et al., 2001). The total colour difference (ΔEab*) between two samples was obtained using the expression: $\Delta Eab^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ (Pérez-Magariño & González-Sanjose, 2003). All absorbance measurements were taken with a Helios Alpha (Thermo Fisher Scientific Inc.,Waltman, MA) UV–vis spectrophotometer using quartz cells of 1 mm path length.

2.7. *Anthocyanin Analysis*

The total anthocyanin content was determined by spectrophotometry using the method described by Niketic-Alksic and Hrazdina (1972). Free and combined anthocyanins were calculated using the PVPP index (Glories, 1984). Reversed-phase HPLC analyses of anthocyanins and the anthocyanin-derived pigments Vitisin A and Vitisin B were carried out with an Agilent 1200 series liquid chromathograph (HPLC-DAD) and an Agilent Zorbax Eclipse XDB-C18, 4.6 x 250mm 5µm column (Agilent Technologies, Santa Clara, USA) in accordance with the method described by González-San José, Diez, Santa María, & Garrido. (1988). Anthocyanins and anthocyanin-derived compounds were quantified at 520 nm as malvidin-3-glucoside, using malvidin-3-glucoside chloride as an external standard.

2.8. *Analysis of Flavanols*

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2.8.1. *HPLC and HPLC-ESI-MS analysis of Catechins and Oligomeric Proanthocyanidins*

20 ml of wine was extracted thrice with 20 ml of ethyl acetate to analyze the catechins and oligomeric proanthocyanidins. The wine extract was evaporated under vacuum (Buchi rotavoporate, Flawil, Switzerland) and then redissolved with methanol. This wine extract was fractionated through a TSK Toyopearl HW-40(s) gel column (250mm x 16mm) using purified methanol as described previously (De Freitas, & Glories, 1999). Flow rate was regulated at 0.8 ml/min using a peristaltic pump (Gilson, Middleton, USA). This gave us three fractions of 120 ml each one. The first fraction contained procyanidin monomers, (+)-catechin and (-)-epicatechin, the second fraction contained procyanidin dimers and trimers and the third fraction contained procyanidin trimers and tetramers (De Freitas, Glories, Bourgeios, & Vitry, 1998). All fractions were evaporated and redissolved on 1 ml synthetic solution (12% ethanol, 4 g/l tartaric acid and pH 3.2).

Quantitative analysis was carried out by reverse-phase HPLC (Merck-Hitachi L6200, Darmstadt, Germany) using two Beckman Ultrasphere C18 ODS columns (250 × 4.6 mm, 5µm) (Beckman Coulter, Fullerton, USA) connected in series and protected with a guard column packed with the same packing. The chromatograms were monitored at 280nm using a UV detector and in accordance with the method of De Freitas and Glories (1999). The procyanidin monomers and dimers were identified by comparing its retention time with that of the pure compound and by ESI-MS analysis. Trimers and tetramers were identified by ESI-MS analysis.

Mass spectrometry analyses were performed using a Finnigan Surveyor series liquid chromatography equipped with a Thermo Finnigan (Hypersil Gold) reversed-phase column (150 mm × 4.6 mm, 5 μm, C18) thermostated at 25 °C. The samples were analyzed using the same solvents, gradients, injection volume, and flow rate for HPLC analysis. Double-online detection was done by a photodiode spectrophotometer and mass spectrometry. The mass detector was a Finnigan LCQ DECA XP MAX quadrupole ion trap (Finnigan Corp., San Jose, CA) equipped with an atmospheric pressure ionization (API) source and using an electrospray ionization (ESI) interface. The vaporizer and the capillary voltages were 5 kV and 4 V, respectively. The capillary temperature was set at 325 °C. Nitrogen was used as both sheath and auxiliary gas at flow rates of 90 and 25, respectively (in arbitrary units). Spectra were recorded in positive ion mode between m/z 250 and 1500.

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2.8.2. *Analysis of Proanthocyanidins following acid catalysis with phloroglucinol*

Acid-catalysis cleavage in the presence of excess phloroglucinol (Kennedy & Jones, 2001) was used to analyze monomeric proanthocyanidin composition and its mean degree of polymerization (mDP). 10ml of wine was evaporated under a low pressure vacuum (Univapo 100 ECH, Uni Equip, Martinsried, Germany). After that it was resuspended in 6 ml distilled water and then applied to Set Pak Plus tC18 Environmental cartridges (Waters, Milford, USA) that had been previously activated with 10 ml methanol and 15 ml water. The sample was washed with 15 ml distilled water and then the proanthocyanidins were eluted with 12 ml methanol, immediately evaporated under vacuum and later eluted in 2ml methanol. Finally, 100 µl of this sample were reacted with 100 µl phloroglucinol solution (0.2N HCl in methanol, containing 100g/l phloroglucinol and 20 g/l ascorbic acid) at 50 ºC for 20 min. The reaction was stopped by adding 1000 µL of 40 mM aqueous sodium acetate (Kennedy & Jones, 2001). Reversed-phase HPLC analysis (Agilent serie 1200 HPLC-DAD) was carried out according to the method of Kennedy and Jones (2001). The monomers (+)-catechin, (-)-epicatechin, (-)-epicaechin-3-O-gallate were identified by comparing its retention time with that of the pure compounds. The phoroglucinol adducts of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin-3-O-gallate were identified by HPLC-TOF analysis. Analyses were performed with Agilent 1200 series HPLC using an Agilent 6210 time of flight (TOF) mass spectrometer equipped with an electrospray ionization (ESI) system. Elution was carried out under the same HPLC analysis conditions as described by Kennedy and Jones (2001). The capillary voltage was 3.5 kV. Nitrogen was used as both dry gas at a flow rate of 12 l/min at 350 ºC and nebulizer gas at 60 psi. Spectra were recorded in positive ion mode between m/z 50 and 2400. This assay was also carried out without addition of phloroglucinol in order to measure the quantity of proanthocyanidin monomers naturally present in the wines.

The number of terminal subunits were considered as the difference between total monomers without phoroglucinol and thus obtained in the analysis performed without phloroglucinol addition. The addition of all phloroglucinol adducts was consider as the extension subunits of the proanthocyanidins. The mean degree of polymerization (mDP) was calculated by adding terminal and extension subunits (in moles) and dividing by terminal subunits. The total proanthocyanidin concentration was considered as the addition of all terminal and extension subunits. Because acid catalysis with phloroglucinol is not completely efficient, the real yield of

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the reaction was measured using a pure B2 proanthocyanidin dimer [(-)-epicatechin-(4→8)-(-)-epicatechin]. This yield was used to calculate the total proanthocyanidin concentration.

2.9. *Other Phenolic Compounds*

The total phenolic index (TPI) was determined by measuring the absorbance at 280 nm (Ribereau-Gayon et al., 2006). Proanthocyanidin concentration was also estimated by precipitation with methyl cellulose (Sarneckis et al., 2006).

2.10. *Astringency Index*

Astringency index was estimated using ovoalbumin as a precipitation agent and tannic acid solutions as standards in accordance with in accordance with Llaudy et al.'s method (2004).

2.11. *Sensory Analysis*

All the wines were tasted by a group of 10 expert enologists from the Rovira i Virgili University 6 weeks after bottling. Dark glasses were used to prevent the influence of color intensity. Three sensory triangle tests were conducted to compare the three wines in pairs. In all the cases, the first objective was to recognize the different wines and after then indicate which was their favorite and for what reason.

2.12. *Statistics*

All the physical and chemical data are expressed as the arithmetic average ± of the standard deviation from three replicates. One-factor ANOVA and Scheffe`s test were carried out with SPSS software. The level of significance of sensory triangle tests was determined following Jackson's method (2002).

3. Results and discussion

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Table 1 shows the evolution of standard parameters of grapes throughout ripening. In both vintages the sugar content, the probable alcoholic degree, the pH and the weight of 100 berries increased and the titratable acidity decreased during ripening as expected. According to these parameters, vintage 2007 reached a greater level of technological maturity than vintage 2008 although both vintages can be considered normal. However, these parameters were measured using an ensemble of 100 berries randomly collected; therefore, only the average value of each parameter is reflected and the berries' heterogeneity is not considered.

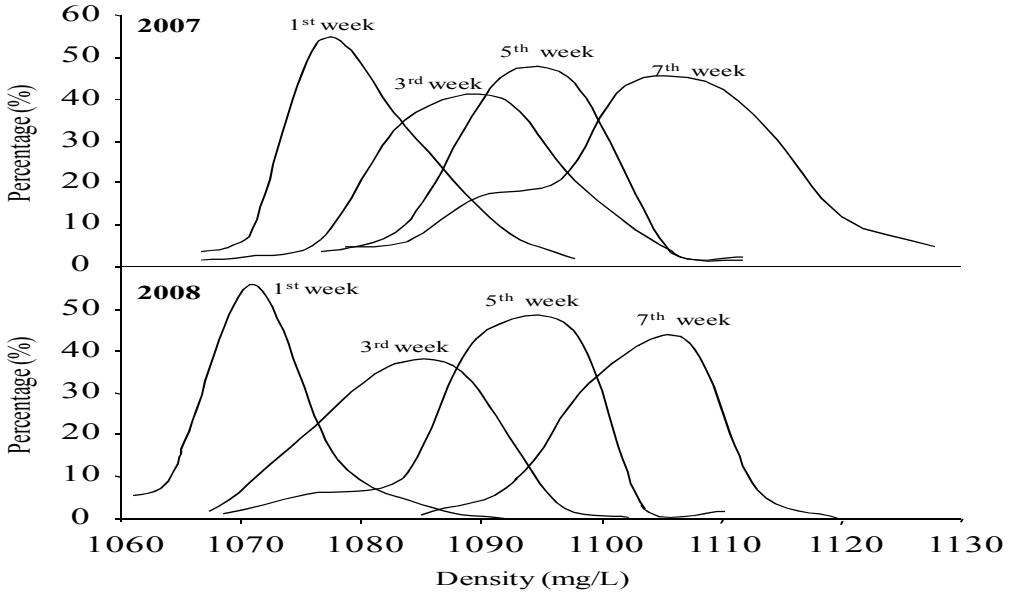

Figure 1. Distribution of grapes densities throughout ripening.

To determine the real dispersion of these parameters, several assays were performed using solutions of NaCl of increasing concentration. Separating the grapes that floated from those that sank and counting them made it possible to determine the density distribution of berries throughout the maturation process (Figure 1). A quick glance at the figure shows a Gaussian bell-shaped distribution in both vintages and at all sampling points. These data confirm that a non-negligible heterogeneity is present from the beginning of the maturation process until the moment of harvest. Consequently, this heterogeneity implies that a considerable percentage of unripe grapes are harvested and introduced into the vinification tanks. Since unripe grapes provide a lower sugar content, higher acidity, fewer anthocyanins and in particular more seed

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tannins (Llaudy et al., 2008), their presence can increase bitterness and astringency and therefore adversely affect the final quality of wine.

Table 2. Wine Parameters.

Parameters	Vintage	Low density	Medium density	High density
Ethanol (% v/v)	2007	12.2±0.1a	13.2±0.1b	14.2±0.1c
	2008	11.0±0.1 a	12.7±0.2 b	13.7±0.2 c
TA (g of tartaric acid/L)	2007	5.90±0.35a	5.85±0.30a	5.35±0.09b
	2008	6.17±0.15a	6.00±0.10ab	5.93±0.06b
pH	2007	3.56±0.02a	3.61±0.03b	3.66±0.03c
	2008	3.18±0.01a	3.30±0.02b	3.35±0.01a

All data are expressed as the arithmetic average of three replicates ± standard deviation (*n*=3). TA: titratable acidity. Statistical analysis: one-factor ANOVA and Scheffe' s test (both p=0.05). Different letters indicates the existence of statistically significant differences between values of the same vintage.

Table 2 shows the standard parameters of the wines. As was expected, the higher the density of grapes the higher ethanol content and pH of wines. In contrast, the titratable acidity showed the opposite tendency in both vintages. These data confirm that the existing heterogeneity in grape densities also affects the wine composition, at least where the standard parameters are concerned.

Table 3 shows the color parameters of the wines of both vintages. In general, the wines from the 2007 vintage that have a higher level of technological maturity indicators also presented higher values of Color Intensity (CI) and Chroma (C *), and lower Luminosity (L*) values (more dark color) than those of 2008.

An overall view of these data indicates that the density of the grapes significantly influences the wine color. Specifically, the higher the density of grapes the higher the CI, C* and red-greenness (a*). On the other hand, L* had the opposite tendency for both years. The other CIELAB coordinates, hue (h*) and yellow-blueness (b*) did not show uniform behavior. On one hand, h* tended to increase with the density of the grapes in the 2007 wines whereas it tended to decrease in the 2008 wines. On the other hand, b * also tended to increase with the density of the grapes in the 2007 wines whereas it was erratic in the 2008 wines.

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Table 3. Color Parameters.

Parameters	Vintage	Low density	Medium density	High density
CI	2007	17.13±0.40a	19.62±1.01b	21.80±0.73c
	2008	9.17±0.49a	12.05±1.13b	14.80±1.29c
C*	2007	67.71±0.22a	68.88±0.21b	69.60±0.25c
	2008	48.13±2.70a	54.43±2.13b	59.02±1.39c
L*	2007	40.90±0.95a	38.53±1.14b	36.30±0.56c
	2008	57.35±1.63a	48.83±3.05b	42.83±2.29c
h*	2007	11.87±0.71a	15.22±1.96b	19.27±1.23c
	2008	14.51±1.31a	9.38±1.32b	8.61±1.60b
a*	2007	65.26±0.38a	66.44±0.46b	66.59±0.13b
	2008	46.57±2.33a	53.69±2.30b	58.34±1.21c
b*	2007	13.93±0.78a	18.08±2.32b	22.94±1.56c
	2008	11.09±0.05a	8.84±0.89b	9.84±0.58c

All data are expressed as the arithmetic average of three replicates ± standard deviation (*n*=3). CI: color intensity, C*: chroma, L*: luminosity, h*= hue, a*= red-grenness and b*= yellow-blueness. Statistical analysis: one-factor ANOVA and Scheffe' s test (both p=0.05). Different letters indicates the existence of statistically significant differences between values of the same vintage.

Table 4 presents the total color differences (ΔEab*) among wines of the same vintage. The human eye can generally distinguish two colors when ΔEab* ≥ 1 (Pérez-Magariño & González-Sanjose, 2003). However, it is also generally accepted that tasters can only distinguish the color of two wines through the glass when ΔEab* ≥ 5 units (Pérez-Magariño & González-Sanjose, 2003). In fact, the differences that can be distinguished by the human eye also depend on the color intensity, because the discriminating capacity becomes less accurate when color perception reaches the saturation level. In our experimental conditions, the three wines obtained for both vintages all had ΔEab* differences greater than 5 units. Therefore, the existing color differences among the wines obtained from grapes of different densities are great enough to be detected by the human eye even when the wines come from grapes of relatively close densities. These data confirm that the existing heterogeneity in the maturity of the grapes has a real impact on wine color because the presence of less dense grapes may affect the visual quality of wine.

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Table 4. Total color differences (Δeab*) among wines.

	Low	Medium	High
Low	-	4.9±1.7	10.1±1.1
Medium	10.1±1.2	-	5.5±0.9
High	17.5±0.5	7.76±1.2	-

All data are expressed as the average of three replicates ± standard deviation (*n*=3). Values in dark grey correspond to 2007 vintage. Values en clear grey correspond to 2008 vintage.

Table 5 shows the anthocyanin concentration, measured by spectrophotometry, of the wines of both vintages. Parallel to what was observed in the color, the total anthocyanin concentration of wines from the 2007 vintage was also higher than that of 2008, reconfirming the greater maturity of this vintage. In both years, the density of the grapes also played a key role in the anthocyanin concentration of the wines. The higher the grape density, the higher the anthocyanin concentration in the wine. This behavior was similar for free anthocyanins and also for anthocyanins combined to flavanols. The higher anthocyanin concentration found in denser grapes can mainly due to the higher concentration of anthocyanins in the skins and also maybe to the higher ethanol concentration of these grapes which exerts a significant effect on anthocyanin extraction during winemaking (Canals et al., 2005).

Table 5. Spectrophotometric Analysis of Antocyanins.

Parameters	Vintage	Low density	Medium density	High density
Free anthocyanins (mg/L)	2007	559±26a	801±10b	806±23b
	2008	283±15a	474±58b	666±54c
Combined anthocyanins (mg/L)	2007	165±9a	221±21b	302±34c
	2008	133±11a	177±3b	212±17c
Total anthocyanin (mg/L)	2007	725±35a	1021±11b	1108±49c
	2008	415±25a	651±61b	878±38c

All data are expressed as the arithmetic average of three replicates ± standard deviation (*n*=3). Statistical analysis: one-factor ANOVA and Scheffe' s test (both p=0.05). Different letters indicates the existence of statistically significant differences between values of the same vintage.

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Anthocyanins were also analyzed by HPLC (Table 6) and the overall results were similar to those obtained by spectrophotometry, although the values were significantly lower. This is because spectrophotometric analysis overestimates the total anthocyanin concentration because it also detects other pigments (Rivas-Gonzalo, Gutierrez, Hebrero, & Santos-Buelga, 1992), whereas HPLC only detects free anthocyanins (Rivas-Gonzalo, 2003). Furthermore, conversion of spectrophotometric data to anthocyanin concentration is necessarily imprecise, because many different pigments showing different extinction coefficients contribute to the absorbance.

Table 6. HPLC Analysis of Anthocyanins and Anthocyanin-Derived Pigments.

Parameters	Vintage	Low density	Medium density	High density
Anthocyanidin-3-monoglycosides (mg/L)	2007	194.3±3.7a	229.4±15.1b	242.1±13.9b
	2008	72.4±4.1a	173.2±34.9b	228.2±18.6c
Acetylated anthocyanins (mg/L)	2007	61.7±1.3a	68.0±3.9b	70.2±4.1b
	2008	27.5±0.9a	68.1±14.9b	91.5±6.1c
p-Coumaroyl anthocyanins (mg/L)	2007	12.0±3.8a	16.9±0.4b	20.7±0.7b
	2008	4.2±0.1a	12.5±3.6b	19.0±1.7c
Total free anthocyanins (mg/L)	2007	275.4±1.8a	313.4±19.5b	351.0±18.7b
	2008	104.2±4.2a	253.8±53.4b	338.7±26.1c
Vitisin A (mg/L)	2007	0.10±0.01a	0.15±0.01b	0.21±0.04c
	2008	10.20±0.65a	11.53±0.56ab	13.97±1.56b
Vitisin B (mg/L)	2007	3.17±1.50a	5.14±0.73ab	5.49±0.68b
	2008	2.04±0.41a	3.28±0.37b	4.36±0.84b

All data are expressed as the arithmetic average of three replicates ± standard deviation (n=3). Statistical analysis: one-factor ANOVA and Scheffe' s test (both p=0.05). Different letters indicates the existence of statistically significant differences between values of the same vintage.

The total anthocyanin concentration measured by HPLC was also higher in wines from 2007 than in those from 2008 when wines of same grape density were compared, confirming again the greater maturity of this vintage. As with spectrophotometry, HPLC also showed that the density of the grapes had a large influence on wine anthocyanin concentration. The higher the density of the grapes, the higher the anthocyanin concentration of the wines. A similar tendency was observed in anthocyanidin-3-monoglucosides and in acetylated and coumarylated anthocyanins as well as in derived pigments such as vitisin A and vitisin B. Together these results indicate that the density of the grapes greatly affects the concentration of the principal molecules responsible for wine color. In particular, the presence of a high proportion of less dense grapes in the whole harvest can lower the anthocyanin concentration below expected

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levels, which corroborate again that the grape heterogeneity may be more important than is commonly thought.

Table 7 shows the results for total phenolic compounds, proanthocyanidins, measured by means of a precipitation with methyl-cellulose, and astringency. The total phenolic indexes (TPI) and proanthocyanidin concentration of 2007 wines were once more greater than those of 2008 when similar densities were compared. Again it was confirmed that the maturity of the 2007 vintage was higher. In this case the trends were also very clear and indicated that the density of the grapes had a strong influence on the final quality of wine. Both the IPT and proanthocyanidin concentration of the wine increased significantly with the density of the grapes. As with the anthocyanins, there are two causes for this. First, denser grapes have a higher proanthocyanin accumulation and second, more these compounds are extracted due to the higher ethanol concentration in the wines obtained from these grapes (Canals et al., 2005). The astringency of the wines was also measured and presented behavior that was parallel to that of the proanthocyanidins.

Table 7. Total Phenolic Compounds, Proanthocyanidins and Related Parameters.

Parameters	Vintage	Low density	Medium density	High density
TPI	2007	39.9±2.0a	49.1±1.6b	51.8±1.3b
	2008	35.4±1.8a	40.8±2.7b	49.9±3.4c
Proanthocyanidins (mg epicatechin/L)	2007	992.3±129.9a	1112.7±240.5a	1570.5±350.1a
	2008	886.5±65.8a	836.4±50.1a	1321.8±+215.5b
Astringency Index (g tannic acid/L)	2007	0.159±0.009a	0.202±0.005b	0.231±0.004c
	2008	0.086±0.003a	0.096±0.013a	0.142±0.030b

All data are expressed as the arithmetic average of three replicates ± standard deviation (n=3). Statistical analysis: one-factor ANOVA and Scheffe' s test (both p=0.05). Different letters indicates the existence of statistically significant differences between values of the same vintage.

Table 8 shows the results of the analysis of wine proanthocyanidins obtained by acid-catalysis in the presence of excess phloroglucinol (2008 vintage only). The results of the proanthocyanidin concentration measured by this method do not exactly match those

obtained by precipitation with methyl-cellulose. However, the tendencies observed by both methods are very similar, the proanthocyanidin concentration of the wines being higher when the density of the grapes was greater. Moreover, when the density of the berries increased, the

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proportion of (+)-catechin significantly decreased and the proportion of (-)-epigallocatechin significantly increased. Meanwhile, the proportions of the other two monomers, (-)-epicatechin and (-)-epicatechin-3-O-gallate remained constant. Since epigallocatechin is only present in skin proanthocyanidins (Souquet et al., 1996) these data indicate that the contribution of skins to the wine proanthocyainidin concentration increases when the grapes are denser and therefore riper. It seems therefore that denser grapes provide more proanthocyanidins which would produce more tannic wines with a greater capacity for aging. In contrast, less dense grapes would produce slight wines without capacity for aging. Hence, the grape heterogeneity can affect these attributes.

Table 8. Analysis of Total Proanthocyanidins and Related Parameters Following Acid-Catalysis in the Present of Excess Phloroglucinol for the wines of 2008.

Parameters	Low density	Medium density	High density
Total proanthocyanidins (mg/l)	639.1±92.5a	715.4±168.6ab	1006.2±191.9b
mDP	3.70±0.22a	4.71±0.48b	4.91±0.25b
(+)-Catechin (%)	16.8±0.4a	13.3±1.7b	12.1±0.4b
(-)-Epicatechin (%)	62.0±0.2a	61.8±1.6a	60.6±0.9a
(-)-Epigallocatechin (%)	18.2±0.8a	21.7±1.0b	24.1±0.9c
(-)-Epicatechin Gallate(%)	2.9±0.1a	3.2±0.2a	3.2±0.2a
Total proanthocyanidins (µmol/L)	585±115a	572±59a	683±101a
Molecular weight average (Da)	1100±66a	1403±145b	1466±76b

All data are expressed as the arithmetic average of three replicates ± standard deviation (*n*=3). mDP, mean Degree of Polymerization. Statistical analysis: one-factor ANOVA and Scheffe' s test (both p=0.05). Different letters indicates the existence of statistically significant differences between values of the same vintage.

The mean degree of polymerization (mDP) of proanthocyanidins of the wines obtained with the grapes of medium and high density present similar values. However, the mDP of the wine obtained with berries of low density was significantly lower. This data confirms that the degree of polymerization of proanthocyanidins is higher when the grapes are riper. Also it would suggest that the lower density grapes mainly provide seed proanthocyanidins since the mDP of skin proanthocyanidins are higher than the mDP of seed proanthocyanidins (Prieur et al.,1994; Souquet et al., 1996). These results agree with those of Kennedy et al. (2002) who have found that the degree of proanthocyanidin polymerization increases with maturity.

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Acid-catalysis in the presence of excess phloroglucinol also allows the molar concentration and consequently the molecular weight average of proanthocyanidins to be obtained. As was expected, the molecular weight average followed a similar trend to the mDP. However, the proanthocyanidin molar concentration surprisingly did not vary significantly among the wines made from grapes of different densities. This fact indicates that all the grapes, regardless of their density, release a similar number of proanthocyanidin molecules during winemaking. Therefore, the increase observed in the proanthocyanidin concentration, expressed in mg/l, is only to the result of an increase in the degree of proanthocyanidin polymerization and not to an increase in the number of molecules. These data also justify why the wines from denser grapes presented higher astringency. According to Vidal et al. (2003) astringency augments when the degree of proanthocyanidin polymerization increases. Given that wines made with high density grapes presented higher proanthocyanin concentration and that their proanthocyanins also presented a higher mDP, it is logical that their astringency was greater too.

Table 9. HPLC Analysis of Flavanols (Catechins and Proanthocyanidins Oligomers) for the wines of 2008.

Parameters	Low density	Medium density	High density
(+)-Catechin (mg/L)	377.8±24.5a	522.4±54.4b	671.2±2.5c
(-)-Epicatechin (mg/L)	415.0±42.7a	444.9±23.4a	533.0±91.1a
Catechins (mg/L)	792.8±66.6a	967.3±77.8b	1152.8±24.1c
B1 (mg/L)	11.4±2.9a	32.2±3.2b	58.9±3.4c
B3 (mg/L)	67.7±11.1a	103.6±16.0b	104.9±25.7c
B4 (mg/L)	13.8±0.9a	12.1±1.9a	13.0±2.0a
B5 (mg/L)	3.4±0.1a	3.3±0.5a	3.0±0.4a
B6 (mg/L)	48.6±1.6a	49.0±1.9a	50.3±2.0a
Dimers (mg/L)	132.1±9.7a	196.1±16.6b	230.5±27.4b
Trimers (mg/L)	39.2±1.3a	41.7±16.0ab	67.1±10.2b
Tetramers (mg/L)	8.6±0.7a	11.5±1.1b	15.0±0.1c
Oligomers (mg/L)	179.9±10.1a	252.7±3.3b	312.7±35.3c
Catechins + Oligomers (mg/L)	1005.2±20.1a	1220.0±74.5b	1468.3±73.5c

All data are expressed as the arithmetic average of three replicates ± standard deviation (*n*=3). Statistical analysis: one-factor ANOVA and Scheffe' s test (both p=0.05). Different letters indicates the existence of statistically significant differences between values of the same vintage.

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Table 9 presents the HPLC analysis of flavanols (catechins and oligomers). Also in this case, the density of the berries significantly affected the concentration of proanthocyanidin monomers and oligomers. The total monomer concentration increased when the density of the grapes was higher. This augmentation mainly occurs due to the increase of (+)-catechin concentration while (-)-epicatechin concentration remained at similar levels. On the other hand, the wine concentration of proanthocyanidin dimers also increased when the grapes were denser. However, this increased was only due to dimers B1 and in particular B3 whereas other dimers such as B4, B5 and B6 present similar values in all wines. The trimers and tetramers followed a similar profile to the dimers. The denser the grapes, the higher their concentration. This increase in oligomers agrees with the previously mentioned results concerning the mDP.

The sensory triangular tests were carried out by comparing in pairs the three wines obtained from the different density grapes. Only 5 of 10 tasters were able to differentiate between the high density wines and the medium density wines. This result was not statistically significant ($p > 0.05$). In contrast, all the tasters distinguished the low density wine when it was compared with the medium or high density wine, being the results statistically significant ($p < 0.05$). All these sensory analyses were done in dark glasses to prevent the tasters from being influenced by the color. Therefore, these differences must be attributed to flavors and/or mouth sensation and not wine color. In all cases, the tasters preferred wines from denser grapes. The reason given was that these wines had a higher fruit and floral scents. Also there were better balanced as there were sweeter, less acidic and less bitter than the low density wines.

4. Conclusions

It can be concluded that the heterogeneity of the grapes at the moment of the harvest is more important that it is usually thought. The ensemble of all the grapes always has a proportion of very well ripened grapes, another proportion of sufficiently ripened grapes and finally a proportion of less ripened grapes. This fact must be taken into account because the presence of less ripened grapes can affect seriously the final composition and consequently the quality of the wine. These less ripened grapes diminish the final ethanol content, pH, anthocyanin concentration, color intensity, total phenolic index and proanthocyanidin concentration and increase titratable acidity. Moreover, these lower density grapes contribute less polymerized

proanthocyanidins, lower proportions of (-)-epigallocatechin and higher proportions of (+)-catechin. These data suggest that lower density grapes release more seed proanthocyanidins than skin proanthocyanidins. Finally, the sensory comparison of wines leaves no doubt because as the wine obtained from lower density grapes was always recognized in all the triangle trials and was considered as less balanced and bitterer. Further research is needed to determine how this heterogeneity can be measured easily and quickly. Only in this way will winemakers have the tools which enable them to take the heterogeneity of the grapes into account in their maturity controls and even to consider the possibility of applying a sorting table for eliminating the unripe grapes.

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iii. Use of unripe grapes harvested during cluster thinning as a method for reducing alcohol content and pH of wine

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Abstract

Background and Aims: Deep red full-bodied wines can only be obtained from grapes with complete phenolic maturity which frequently produce wines with high pH and alcohol content. The present study focuses on a new procedure for simultaneously reducing pH and ethanol content.

Methods and Results: Grapes from cluster thinning were used to produce a very acidic low-alcohol wine. The wine was treated with high doses of charcoal and bentonite. This odorless and colorless wine was used to reduce pH and ethanol content when the grapes reached complete phenolic maturity. The anthocyanin and proanthocyanidin concentrations, the mean degree of polymerization and the monomeric composition of proanthocyanidin of reduced-alcohol wines were similar than those of their corresponding controls. Since the pH was lower, the color of the reduced-alcohol wines was more intense. No significant differences were found between reduced-alcohol wines and their controls by triangle sensory tests using dark glasses for two of the three studied cultivars.

Conclusion: The proposed procedure may be useful for reducing simultaneously the alcohol content and the pH of wines.

Significance of the Study: The proposed procedure allows decreasing simultaneously alcohol and pH. Moreover, it is very easy to apply and does not require specific equipment.

Keywords: *Reduced-Alcohol Wine, pH Reduction, Color, Phenolic Compounds*

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Introduction

Nowadays, strongly colored full-bodied red wines are highly appreciated by the market. However, producing this kind of wine requires an intense extraction of phenolic compounds from the cap during winemaking and this can cause bitterness, excess of astringency and herbaceous smells if the grapes are not ripe enough. In fact, grape maturity strongly influences the phenolic composition of red wines (O´-Marques et al. 2005, Ryan and Revilla 2003). Insufficiently ripened grapes have a lower extractability of anthocyanins and proanthocyanidins from skins and a higher extractability of proanthocyanidins from seeds (Canals et al. 2005, Peyrot des Gachons and Kennedy 2003). Consequently, it is generally thought that insufficiently ripened grapes can release a higher amount of seed proanthocyanidins, which are highly galloylated (Romeyer et al. 1986). This is probably the reason why poorly matured grapes often produce astringent bitter wines.

For this reason, winemakers are very interested in measuring phenolic maturity so that they can harvest grapes that are ripe as possible. Nevertheless, grapes with very high phenolic maturity frequently present high sugar and low acid concentrations. Consequently, the resulting wines have the drawback of very high pH and alcohol content. High pH values in wines cause such problems as less color and less antiseptic effectiveness of sulfur dioxide (Beech et al. 1979). On the other hand, excess ethanol may cause stuck and sluggish fermentation (Bisson 1999), and can also alter the sensory balance of the wine (Williams 1972, Fischer and Noble 1994). Moreover, high-alcohol wines have higher tax rates in some countries and are sometimes not well accepted by consumers.

It is a verifiable fact that most wines have gradually increased their alcohol content and pH in recent years and winemakers are concerned about the problem. Some authors even consider that climate change may increase this tendency (Tate 2001, Duchêne and Schneider 2005, Jones et al. 2005). The increase in temperature and the changes in rainfall distribution will probable affect vine and grape physiology, and impact on wine composition and quality. If the temperature during ripening is higher than the optimal, the grape pulp matures faster, and the pH and sugar concentration are too high. Thereby the period between veraison and industrial maturity decline, which makes it more difficult to reach the proper aromatic and phenolic maturity, and leads to unbalanced wines (Zamora 2003, 2005). Moreover, it is observed that

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global warming is shifting areas of vine cultivation. It has been estimated that the northern limits of its cultivation in Europe is moving at a rate between 10 and 30 km per decade and that the speed of travel is expected to double between 2020 and 2050 (Kenny and Harrison 1993). This means that some regions that are nowadays adequate for grape growing could not be suitable at the future, without the adoption of appropriate adaptive measures. In contrast, other regions that until now appeared as unsuitable for wine grape production because of its warmer temperature may become more suitable in the future for wine production.

Several techniques have been proposed to mitigate climate impacts. One is to harvest grapes at an early stage of ripening. However, this is not a good solution because grapes have not reached an adequate phenolic and aromatic maturity, which would certainly produce bitter and herbaceous wines. Another is to add water and mineral acids to the grape juice before the fermentation begins. This reduces the sugar concentration and pH but has a general negative effect on wine quality because it dilutes all the other compounds, and although this practice is authorized in some countries, it is strictly forbidden in others. It is also possible to reduce this problem by blending high-alcohol wines with low-alcohol wines. However, a sufficient volume of low-alcohol wine, which does not present the disadvantages described above, is required.

Other possibilities are to introduce new cultivars, modify culture techniques and even move vineyards to other production areas to delay pulp sugar accumulation and acid consumption (Schultz 2000). It should be taken into account that all the work on clonal selection in recent years has aimed to obtain grapevines that can quickly produce high sugar content. Now we need to retrace our steps and search for the old vines that are best adapted to the new climate conditions. This solution is probably the best one but it requires long and laborious studies and a considerable economic investment to replant most of the vineyards.

Glucose oxidase (EC 1.1.3.4) has also been proposed as one way of obtaining less alcoholic wines (Pickering et al. 1998). However, it has two drawbacks. It oxidize glucose by generating a very high concentration of gluconic acid in wine. Moreover, it uses oxygen as a substrate so the grape juice needs to be excessively aerated which may oxidize other wine components (Pickering et al. 1999).

The use of yeast with a lower ethanol yield production has also been proposed. However, it seems that natural *Saccharomyce cerevisiae* strains present similar yields, which leads to an impasse unless genetically modified organisms (Dequin and Barre 1994, Malherbe et al. 2003) or non-*Saccharomyces* yeasts are used (Ciani and Ferraro 1996).

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However, nowadays the most commonly used methods for modifying alcohol content and pH are probably physical ones. In fact, several physical techniques have been applied to partially reduce the sugar concentration in grape juice or the alcohol in wine. Some of the proposed methods are supercritical fluid extraction, vacuum distillation and membrane techniques (Bui et al. 1986, Sykes et al. 1992, Pickering 2000, Takács et al. 2007, Diban et al. 2008, Ruiz-Rodrigueza et al. 2010). Of these, the most frequently used in the wine industry are the spinning cone column and reverse osmosis system (Pickering 2000). To decrease wine pH, on the other hand, physical methods such as cationic exchange columns and electrodyalisis are the most used (Bonorden et al.1986, Walker et al. 2004). These physical methods are effective but involve bulky and expensive equipment and many winemakers have concerns about their effects on wine quality.

The present study focuses on a new winemaking process for simultaneously reducing the pH and alcohol content of wine. It involves using the grape berries collected during cluster thinning. Grape growers often remove some of the grape clusters at the beginning of the veraison in order to improve the development ones remaining. These clusters, which are usually left on the ground of the vineyard, can be harvested and used to obtain a highly acidic wine with a very low degree of alcohol. Once alcoholic fermentation has ended, this wine can be treated with a very high dose of charcoal and bentonite in order to completely eliminate phenolic compounds and herbaceous smells. This odorless and colorless wine can be used later, when the rest of the grapes have reached the complete phenolic maturity to reduce pH and ethanol content.

Materials and Methods

Chemicals and reagents

Methanol, acetonitrile, formic acid and acetic acid were HPLC-grade and purchased from Panreac (Barcelona, Spain). Malvidin-3-O-glucoside chloride, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin 3-O-gallate were purchased from Extrasynthès (Genay, France). Ovoalbumin, phloroglucinol and L-ascorbic were purchased from Sigma (Madrid, Spain). Charcoal and bentonite were purchased by Martin Vialatte (Epernay, France). The rest of the chemicals were of high purity and were purchased from Panreac (Barcelona, Spain).

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Winemaking procedure

The yield of the vineyards of the cultivar *Vitis vinifera* cv. Grenache can sometimes be reduced by applying cluster thinning in order to improve the phenolic maturity. During the vintage 2008 just at the beginning of the veraison, the clusters of this cultivar were thinned in the experimental vineyard belonging to the Enology Faculty in Tarragona (Rovira i Virgili University) at Constantí (AOC Tarragona). Five hundred kg of the grapes were not rejected but taken to the experimental winery instead.

The grapes were crushed (Delta E2, Bucher Vaslin, Chalonnes sur Loire-France) and lightly pressed in a pneumatic press (M-15c, Marzola, Logroño, Spain) until 250 L of grape juice was obtained. The must was immediately sulphited with 100 mg of $K_2S_2O_5$/L and placed in a 100 L stainless steel tank. The grape juice was allowed to settle for 20 hours, racked to another tank and inoculated with selected yeast (EC 1118, Lallemand, Montreal, Canada). Alcoholic fermentation was carried out at 18 ± 1 ºC. When it has finished, the tank was sulphited with 100 mg of $K_2S_2O_5$/L and treated with 5 g/L of charcoal and 1 g/L of bentonite to obtain the absolute discoloration and deodorization. This low-ethanol wine presented the following analytical parameters: 5 % (v/v) of ethanol content, 17.8 g of tartaric acid/l of titratable acidity and pH 2.64. Then, it was conserved at 4 ºC.

Subsequently, grapes of the cultivar *Vitis vinifera* cv. Cabernet Sauvignon and Merlot from the vineyards of Juvé & Camps in Mediona (AOC Penedes) and Bobal from the vineyards of Dominio de la Vega in San Antonio (AOC Requena) were harvested at two different ripening stages. The first harvest (H1) was carried out when the probable degree of alcohol was between 13.0 and 14.0 %. The second harvest (H2) was carried out when the grapes reached optimum phenolic maturity.

For each cultivar, 60 Kg (H1) or 120 Kg (H2) of grapes were collected and manually destemmed. After that, berries were randomly distributed into three lots (H1) or six lots (H2) of eight kilograms each. Then the grapes were crushed with a semi-automatic crusher machine (Gual, Villafranca del Penedès, Spain), sulphited (100 mg of $K_2S_2O_5$/L) and placed in ten-liter tanks. Three tanks from the first harvest (H1) and also three tanks from the second harvest (CH2) were used without any of the low-ethanol wine being added. The other three tanks from the second harvest (RAH2) were used for the reduction alcohol experiment. Specifically, a part of the total volume of the grape juice was extracted and replaced with the same volume of low-

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alcohol wine. This volume (V) was calculated for each sample with the aim of reproducing the probable alcoholic degree of the corresponding wine from the first harvest. The following equation was used to calculate the exact volume (V) to be replaced in each sample:

$$V\ (liters) = 6.4\ (G2-G1)/(G2-5)$$

where 6.4 is the yield of grape juice (in liters) from 8 kg of grapes, G2 is the probable degree of alcohol of the grape juice, G1 is the degree of alcohol of the wine from the first harvest, and 5% is the degree of alcohol of the low-alcohol wine. Specifically, the volume of grape juice replaced by the low-alcohol wine was 0.85 L for Cabernet Sauvignon, 1.50 L for Merlot and 2.00 L for Bobal.

All tanks were inoculated with 200 mg/L of selected yeast (EC1118, Lallemand, Montreal, Canada) and maintained at a room temperature of 25 ± 1 ºC until the end of the maceration/fermentation process. All these microvinifications were controlled daily by measuring the temperature and the density of the must. Every day, a manual punch down was carried out to improve the extraction of phenolic compounds. After 14 days of maceration, the wines were decanted, bottled and inoculated with 100 mg/L of the lactic bacteria, *Oenococcus oeni* (Vitilactic F, Martin Vialatte, Epernay, France). Once malolactic fermentation had finished, all the wines were racked, sulphited (100 mg of $K_2S_2O_5$/L) and refrigerated for three weeks at 4 ºC. Finally, all samples were stored at 15 ± 1 ºC until analysis.

Standard grape and wine analysis

The analytical methods recommended by the OIV (O.I.V. 2005) were used to determine sugar content, probable alcohol content, titratable acidity, the pH of the grapes and their ethanol content, the titratable acidity and the pH of the wines.

Color parameters

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The color intensity (CI) was estimated using the method described by Glories (1984b). The CIELab coordinates, lightness (L*), chroma (C*), hue (h*), red-greenness (a*) and yellow-blueness (b*) were determined according to Ayala et al. (1997) and the data were processed with the MSCV software (Ayala et al. 2001). The total colour difference (ΔEab*) between two samples was obtained using the expression: $\Delta Eab^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ (Pérez-Magariño and González-Sanjose 2003). All absorbance measurements were taken with a Helios Alpha (Thermo Fisher Scientific Inc.,Waltman, MA) UV–vis spectrophotometer using quartz cells with a path length of 1 mm.

Anthocyanin analysis

Reversed-phase HPLC analyses of anthocyanins and the anthocyanin-derived pigments Vitisin A and Vitisin B were carried out with an Agilent 1200 series liquid chromatograph (HPLC-DAD) and an Agilent Zorbax Eclipse XDB-C18, 4.6 x 250mm 5µm column (Agilent Technologies, Santa Clara, USA) in accordance with the method described by González-San José et al. (1988). Anthocyanin standard curves were made using malvidin-3-glucoside.

Analysis of proanthocyanidins following acid catalysis with phloroglucinol

Acid-catalysis cleavage in the presence of excess phloroglucinol was used to analyze monomeric proanthocyanidin composition and its mean degree of polymerization (mDP). The samples were prepared and analyzed by reversed-phase HPLC according to the method described by Kennedy and Jones (2001). The monomers (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-O-gallate were identified by comparing their retention times with those of the pure compounds. The phoroglucinol adducts of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin-3-O-gallate were identified by HPLC-TOF analysis. Analyses were performed with Agilent 1200 series HPLC using an Agilent 6210 time of flight (TOF) mass spectrometer equipped with an electrospray ionization (ESI) system. Elution was carried out under the same HPLC analysis conditions as described by Kennedy and Jones (2001). The capillary voltage was 3.5 kV. Nitrogen was used as both dry gas at a flow rate of 12 l/min at 350 ºC and nebulizer gas at 60 psi. Spectra were recorded in positive ion mode between

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m/z 50 and 2400. This assay was also carried out without addition of phloroglucinol so that the quantity of proanthocyanidin monomers naturally present in the wines could be measured.

The number of terminal subunits were considered as the difference between total monomers without phoroglucinol and thus obtained in the analysis performed without phloroglucinol addition. The number of extension subunits was considered as the addition of all the phloroglucinol adduct. The mean degree of polymerization (mDP) was calculated by adding the number of terminal and extension subunits (in moles) and dividing by the number of terminal subunits. The total proanthocyanidin concentration was considered as the addition of all terminal and extension subunits. Because acid catalysis with phloroglucinol is not completely efficient, the real yield of the reaction was measured using a pure B2 proanthocyanidin dimer [(-)-epicatechin-(4→8)-(-)-epicatechin]. This yield was used to calculate the total proanthocyanidin concentration.

Other phenolic compounds

The total phenolic index (TPI) was determined by measuring the absorbance at 280 nm (Ribereau-Gayon et al. 2006). The proanthocyanidin concentration was also estimated by precipitation with methyl cellulose (Sarneckis et al. 2006).

Astringency index

The astringency index was estimated using ovoalbumin as a precipitation agent and tannic acid solutions as standards in accordance with Llaudy et al.'s method (2004).

Sensory analysis

All the wines were tasted by a group of 10 expert enologists from the Rovira i Virgili University 6 weeks after bottling. Two previous training sessions of tasting were carried out to standardize criteria among the panelists. Dark glasses were used to prevent them from being influenced by color intensity (Norton and Johnson 1987, Stillman 1993). Three sensory triangle tests were conducted for each cultivar to compare the three wines in pairs. In all the cases, the main

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objective was to recognize the different wine and after then indicate which of them was preferred for its olfactory and/or tasting sensations.

Statistics

All the physical and chemical data are expressed as the arithmetic average ± the standard deviation from three replicates. One-factor ANOVA and Scheffe's test s test were carried out with SPSS software. The level of significance of sensory triangle tests was determined following Jackson's method (2002).

Results and discussion

The fermentation times were, in general, closely related with the sugar concentration of the different grape juices. The higher sugar concentration the slower alcoholic fermentation. More specifically, H1 and RAH2 fermentations were finished in a range of 7-8 days, while Merlot and Cabernet Sauvignon CH2 fermentations were completed in a range of 8-10 days. In the case of CH2 Bobal, the fermentation took longer to be ended (12-14 days) probably because of their higher sugar content.

Standard wine parameters are shown in Table 1. Naturally, wines from the first harvest (H1) had higher titratable acidity, lower ethanol content and lower pH (Table 1) than the corresponding control wines from the second harvest (CH2), which indicated that the three grape varieties had really ripened during the period between the first and the second harvest. Moreover, the wines from the second harvest reached very high degrees of alcohol, more than 15 % in the three cultivars, and also high pH values (more than 3.75 for Merlot and Bobal).

As expected, all the wines to which part of their juice had been replaced by the low-alcohol wine (RAH2) had a lower ethanol content and pH than their corresponding controls (CH2). In fact, the ethanol content, the pH and the titratable acidity of the RAH2 wines were closer to those of the corresponding H1 wines than to those of their corresponding CH2 wines for the three cultivars. Specifically, RAH2 wines had 0.9 % (Cabernet Sauvignon), 1.7 % (Merlot) and 3.0 % (Bobal) less alcohol content than their corresponding CH2 wines. These results are quite

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conclusive and show that replacement of some of the grape juice by the low-alcohol wine can be useful for obtaining reduced-alcohol wines with a lower pH.

Table 1. Standard wine parameters.

Cultivar	Parameters	H1	H2	
			CH2	RAH2
Cabernet Sauvignon	Ethanol (% v/v)	14.0± 0.1[a]	15.4 ± 0.1[b]	14.5 ± 0.1c
	TA (g/L)	6.50 ± 0.13[a]	5.90 ± 0.04[b]	6.72 ± 0.19[a]
	pH	3.48 ± 0.02[a]	3.55 ± 0.02[b]	3.46 ± 0.03[a]
Merlot	Ethanol (% v/v)	13.4 ± 0.1[a]	15.9 ± 0.1[b]	14.2 ± 0.1[c]
	TA (g/L)	7.00 ± 0.17[a]	6.35 ± 0.24[b]	7.15 ± 0.09[c]
	pH	3.45 ± 0.01[a]	3.76 ± 0.03[b]	3.55 ± 0.07[c]
Bobal	Ethanol (% v/v)	13.2 ± 0.1[a]	16.9 ± 0.1[b]	13.9 ± 0.1[c]
	TA (g/L)	7.45 ± 0.09[a]	6.70 ± 0.05[b]	8.94 ± 0.06[c]
	pH	3.46 ± 0.01[a]	3.80 ± 0.01[b]	3.34 ± 0.08[c]

TA: titratable acidity, H1: first harvest, H2: second harvest, CH2: control wines of H2, RAH2: reduced alcohol wines from H2. All data are expressed as the average of the three replicates ± standard deviation (n = 3). Statistical analysis: one-factor ANOVA and Scheffe´s test (both $p < 0.05$). Different letters indicate the existence of statistically significant differences.

The results corresponding to anthocyanin concentration (Table 2) indicates that the wines from the second harvest (H2) presented higher total anthocyanin concentrations than their corresponding H1 wines and no statistically significant differences were found between the CH2 and the RAH2 wines in the three cultivars. These results confirm that the riper grapes produced wines with a higher anthocyanin concentration in the three studied cultivars (Bautista-Ortin et al. 2006, Fournand et al. 2006). The increase of total anthocyanins throughout ripening was mainly due to anthocyanidin-3-monoglucosides although the same trend was observed in acetyl and p-coumaryl anthocyanidins.

Contrary to what was expected, the anthocyanin concentrations of RAH2 wines of the three cultivars were similar to those of their controls (CH2). Theoretically, the extraction of some of the grape juice and the replacement by the low-alcohol wine should remove some of the anthocyanins, specifically those extracted during the crushing process and also the anthocyanins solved during the short time of skin contact. It has also been reported that higher ethanol content favors anthocyanin extraction throughout skin contact (Canals et al. 2005).

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One possible explanation for these paradoxical results may be due to the lower pH values of RAH2 wines. A lower pH may favor anthocyanin extraction during winemaking compensating the positive effect of ethanol. A lower pH may also protect anthocyanins against oxidation by means of two mechanisms: it shifts the equilibrium of anthocyanins from carbinol towards to the formation of flavylium cation (Glories, 1984a) and it inhibits the polyphenol oxidase activity of the grape juice (Rapeanu et al. 2006).

Table 2. Concentration of anthocyanins (mg/L) determined by HPLC.

Cultivar	Parameters	H1	H2	
			CH2	RAH2
Cabernet Sauvignon	Anthocyanidin-3-monoglucosides	131.9 ± 11.9^{a}	184.7 ± 12.5^{b}	191.5 ± 21.0^{b}
	Acetylated anthocyanidins	46.7 ± 6.4^{a}	66.5 ± 4.5^{b}	69.1 ± 8.1^{b}
	p-Coumaroyl anthocyanidins	6.5 ± 1.7^{a}	11.0 ± 0.6^{b}	11.0 ± 1.1^{b}
	Total anthocyanins	185.1 ± 32.5^{a}	262.2 ± 17.8^{b}	271.7 ± 30.5^{b}
Merlot	Anthocyanidin-3-monoglucosides	135.7 ± 13.9^{a}	187.0 ± 20.2^{b}	201.9 ± 4.2^{b}
	Acetylated anthocyanidins	42.3 ± 4.5^{a}	47.9 ± 3.2^{ab}	49.2 ± 1.2^{b}
	p-Coumaroyl anthocyanidins	13.4 ± 2.0^{a}	17.5 ± 1.1^{b}	19.3 ± 0.4^{c}
	Total anthocyanins	191.4 ± 20.2^{a}	252.4 ± 24.7^{b}	270.5 ± 4.8^{b}
Bobal	Anthocyanidin-3-monoglucosides	241.7 ± 21.1^{a}	293.1 ± 15.2^{b}	320.6 ± 45.6^{b}
	Acetylated anthocyanidins	18.5 ± 1.0^{a}	18.9 ± 0.7^{a}	19.7 ± 2.1^{a}
	p-Coumaroyl anthocyanidins	18.4 ± 2.2^{a}	22.5 ± 1.5^{b}	24.1 ± 3.0^{b}
	Total anthocyanins	278.6 ± 24.4^{a}	334.5 ± 17.5^{b}	364.4 ± 51.0^{b}

H1: first harvest, H2: second harvest, CH2: control wines of H2, RAH2: reduced alcohol wines from H2. All data are expressed as the average of the three replicates ± standard deviation (n = 3). Statistical analysis: one-factor ANOVA and Scheffe´s test (both $p < 0.05$). Different letters indicate the existence of statistically significant differences.

Color intensity and CIELab coordinates are shown in Table 3. In the three cultivars the color intensity, and C* of the CH2 wines were higher and the L* lower than the equivalent values of the corresponding H1 wines. Other authors have found similar results (Canals et al 2005, Gambuti et al. 2007), which confirms that the riper grapes generally produce more intensely colored wines.

On the other hand, the Merlot and Bobal RAH2 wines showed higher color intensity, and C* and lower L* than their corresponding controls (H2) although the anthocyanin concentrations of Cabernet Sauvignon and Bobal were similar. The same trend was observed with Cabernet Sauvignon wines although the results were not statistically significant. Therefore, the more intense color of all RAH2 wines was probably because the lower pH of RAH2 wines increased

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the proportion of the anthocyanins' flavylium cation (Glories 1984a). Indeed, the changes in color parameters were greater in Merlot and even more so in Bobal because the alcohol decrease was greater, which led to a considerable decrease in the pH. Another possible cause is that a lower concentration of ethanol in the RAH2 wines favors copigmentation (Canals et al. 2005).

Table 3. Color Intensity and ClELab coordinates.

Cultivar	Parameters	H1	H2	
			CH2	RAH2
Cabernet Sauvignon	CI	9.3 ± 0.8^{a}	12.6 ± 0.5^{b}	13.9 ± 2.3^{b}
	C*	44.54 ± 1.13^{a}	58.54 ± 0.66^{b}	60.63 ± 2.47^{b}
	L*	42.19 ± 2.62^{a}	48.50 ± 3.12^{b}	47.37 ± 5.72^{b}
	h*	1.97 ± 0.67^{a}	2.78 ± 0.98^{a}	2.73 ± 1.36^{a}
	a*	36.21±1.45^{a}	58.44 ± 0.63^{b}	60.26 ± 2.13^{b}
	b*	0.69 ±0.47^{a}	3.39 ± 0.52^{b}	2.79 ± 1.42^{b}
Merlot	CI	8.7 ± 0.9^{a}	12.6 ± 1.5^{b}	17.0 ± 1.9^{c}
	C*	55.57 ± 2.64^{a}	59.06 ± 1.65^{b}	66.57 ± 1.42^{c}
	L*	60.27 ± 2.80^{a}	50.70 ± 5.00^{b}	42.17 ± 2.57^{c}
	h*	2.83 ± 0.75^{a}	7.47 ± 1.66^{b}	15.04 ± 2.73^{c}
	a*	55.50 ± 2.60^{a}	58.44 ± 1.41^{a}	63.89 ± 0.64^{b}
	b*	2.76 ± 0.84^{a}	7.48 ± 2.08^{b}	17.30 ± 3.41^{c}
Bobal	CI	10.6 ± 1.4^{a}	15.8 ± 0.8^{b}	22.9 ± 1.7^{c}
	C*	57.08 ± 1.56^{a}	59.28 ± 0.20^{b}	67.38 ± 1.23^{c}
	L*	52.33 ± 4.06^{a}	36.63 ± 1.42^{b}	34.05 ± 1.06^{c}
	h*	1.67 ± 0.08^{a}	9.92 ± 0.86^{b}	17.88 ± 1.39^{c}
	a*	56.19 ± 0.57^{a}	58.39 ± 0.05^{b}	63.62 ± 0.66^{c}
	b*	1.60 ± 0.14^{a}	10.22 ± 0.91^{b}	24.76 ± 3.05^{c}

H1: first harvest, H2: second harvest, CH2: control wines of H2, RAH2: reduced alcohol wines from H2. All data are expressed as the average of the three replicates ± standard deviation (n = 3). Statistical analysis: one-factor ANOVA and Scheffe´s test (both $p < 0.05$). Different letters indicate the existence of statistically significant differences.

However, for winemakers it is much more important to know whether the human eye can really distinguish between the colors of two wines, than whether there are statistically significant differences between these parameters. Consequently, the total color differences (ΔEab*) were calculated (Table 4) in order to determine if the human eye is really capable of distinguishing between the different wines. The human eye can generally distinguish two colors when $\Delta Eab^* \geq 1$ (Pérez-Magariño and González-Sanjose 2003). However, it is also generally accepted that

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tasters can only distinguish the color of two wines through the glass when ΔEab* ≥ 5 units (Pérez-Magariño and González-Sanjose 2003). In fact, the differences that can be distinguished by the human eye also depend on the color intensity, because discriminating capacity is less accurate when color perception reaches saturation level. In our experimental conditions, the differences in ΔEab* between H1 wines and all their corresponding H2 wines (CH2 and RAH2) were greater than 5 units. Therefore, it can be conclude that all the H2 wines could be clearly distinguished from their corresponding H1 wines with the naked eye. The differences in ΔEab* between RAH2 and CH2 wines of Merlot and Bobal were also greater than 5 units, indicating that the differences in color were sufficiently large to be distinguished by the human eye. In the case of Cabernet Sauvignon, the difference in ΔEab* between RAH2 and CH2 wines was only 3.9, probably because the alcohol reduction and consequently the decrease in its pH were lower than in the other cultivars.

Table 4. Total color differences (ΔEab*) among wines.

Cultivar	H1 vs CH2	H1 vs RAH2	H2 vs RAH2
Cabernet Sauvignon	22.6 ± 0.8	24.7 ± 1.9	3.9 ± 1.4
Merlot	11.8 ± 3.0	25.3 ± 2.1	16.3 ± 1.4
Bobal	14.9 ± 1.9	25.6 ± 3.8	13.6 ± 3.9

H1: first harvest, H2: second harvest, CH2: control wines of H2, RAH2: reduced alcohol wines from H2. All data are expressed as the average of the three replicates ± standard deviation (n = 3).

The total phenolic compounds, proanthocyanidin concentration, measured by precipitation with methyl-cellulose, and the astringency index are shown in Table 5. The total phenolic indexes (TPI), proanthocyanidin concentrations and the astringency indexes of all the H2 wines were significantly higher than their corresponding H1 wines. This data confirms that the riper grapes produced the more tannic wines. Other authors have found similar results (Llaudy et al., 2008, Kennedy et al. 2002). On the other hand, the proanthocyanidin concentrations of the RAH2 wines from the three cultivars were similar to those of their corresponding CH2 wines, indicating that replacing part of their grape juice by low-alcohol wine had not affected their tannicity. However some small differences were detected in the TPI and astringency index.

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Specifically, the TPI of the CH2 Merlot was significant lower and the astringency index of the Bobal CH2 was significant higher than in their respective reduced-alcohol wines (RAH2). These differences may be due to changes in the wine matrix other than the proanthocyanidin concentration.

Table 5. Total phenolic compounds, proanthocyanidins (mg epicatechin/L) and astrigency index (mg tannic acid/L).

Cultivar	Parameters	H1	H2	
			CH2	RAH2
Cabernet Sauvignon	TPI	40.3 ± 1.5^{a}	44.6 ± 1.8^{b}	45.3 ± 5.2^{ab}
	Proanthocyanidins	740 ± 100^{a}	1010 ± 310^{a}	1140 ± 250^{a}
	Astringency Index	146 ± 21^{a}	171 ± 10^{b}	183 ± 13^{b}
Merlot	TPI	24.2 ± 2.6^{a}	37.8 ± 5.2^{b}	49.3 ± 1.3^{c}
	Proanthocyanidins	330 ± 60^{a}	1010 ± 120^{b}	1140 ± 60^{b}
	Astringency Index	56 ± 6^{a}	118 ± 42^{b}	145 ± 39^{b}
Bobal	TPI	35.4 ± 3.6^{a}	68.1 ± 2.6^{b}	63.0 ± 3.0^{b}
	Proanthocyanidins	740 ± 200^{a}	1650 ± 220^{b}	1950 ± 400^{b}
	Astringency Index	96 ± 27^{a}	342 ± 19^{b}	204 ± 1^{c}

H1: first harvest, H2: second harvest, CH2: control wines of H2, RAH2: reduced alcohol wines from H2. All data are expressed as the average of the three replicates ± standard deviation (n = 3). Statistical analysis: one-factor ANOVA and Scheffe´s test (both $p < 0.05$). Different letters indicate the existence of statistically significant differences.

Proanthocyanidins were also measured by acid-catalysis in the presence of excess phloroglucinol (Table 6) and the results were generally very similar to those obtained by methyl-cellulose precipitation. This data confirms that H2 wines had a higher proanthocyanidin concentration than H1 wines and that the proposed method for alcohol reduction did not affect the proanthocyanidin concentration (see above). This method also allows the mean degree of polymerization (mDP) and the percentage of the different monomers of proanthocyanidins to be measured. These results indicate that the mDP was significantly higher in all the H2 wines than in the H1 wines. The higher mDP observed in the wines from riper grapes may be due to the fact that the polymerization of proanthocyanidins increased with grape maturity and/or that the higher ethanol content favored the extraction of larger molecules. If we compare only H1 with CH2, both causes are possible. However, the mDP of Cabernet Sauvignon and Merlot RAH2 was similar to that of their respective controls although their ethanol content is closer to that of H1 than CH2 wines. This should indicate that polymerization during grape ripening is the main reason for the higher proanthocyanidin mDP observed in the H2 wines. Nevertheless, it has been

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reported than ethanol plays a non-negligible role in the proanthocyanidin extraction (Canals et al. 2005) and the results obtained seem to confirm this. In this regard, the proanthocyanidin concentration, measured by phoroglucinolysis, of all RAH2 wines tended to be lower than that of their respective controls although it was only significant in the case of Merlot. Moreover, the mDP of RAH2 Bobal wine was slightly but significantly lower than its respective CH2. RAH2 Bobal wine was precisely the sample in which alcohol was most drastically reduced (3%) and it is quite logical that this difference hinders the extraction of larger molecules. In the other cultivars, the difference in ethanol content was not so important and therefore the differences in mDP were not significant.

Table 6. HPLC Analysis of Total Proanthocyanidins and Related Parameters Following Acid –Catalysis in the Present of Excess Phloroglucinol.

Cultivar	Parameters	H1	H2	
			CH2	RAH2
Cabernet Sauvignon	Proanthocyanidins (mg/L)	703.6 ± 31.3^{a}	1104 ± 183.4^{b}	940.1 ± 197.8^{b}
	mDP	4.35 ± 0.09^{a}	6.54 ± 0.20^{b}	6.34 ± 0.14^{b}
	(+)-Catechin (%)	18.6 ± 0.4^{a}	12.4 ± 0.5^{b}	13.1 ± 0.4^{b}
	(-)-Epicatechin (%)	56.2 ± 1.5^{a}	54.6 ± 1.2^{a}	55.0 ± 0.7^{a}
	(-)-Epicatechin-3-O-gallate (%)	4.5 ± 0.1^{a}	4.8 ± 0.4^{a}	4.3 ± 0.2^{a}
	(-)-Epigallocatechin (%)	20.5 ± 0.9^{a}	28.2 ± 0.1^{b}	28.4 ± 0.7^{b}
Merlot	Proanthocyanidins (mg/L)	427.0 ± 115.5^{a}	1070.1 ± 17.2^{b}	969.9 ± 41.4^{c}
	mDP	2.72 ± 0.13^{a}	4.80 ± 1.84^{b}	4.43 ± 0.36^{b}
	(+)-Catechin (%)	26.2 ± 2.2^{a}	21.8 ± 1.4^{b}	19.0 ± 2.1^{b}
	(-)-Epicatechin (%)	57.8 ± 0.7^{a}	57.6 ± 0.1^{a}	57.6 ± 1.9^{a}
	(-)-Epicatechin-3-O-gallate (%)	4.4 ± 0.4^{a}	4.9 ± 0.2^{b}	5.7 ± 0.3^{c}
	(-)-Epigallocatechin (%)	11.6 ± 1.3^{a}	16.5 ± 0.6^{b}	17.7 ± 0.7^{b}
Bobal	Proanthocyanidins (mg/L)	761.4 ± 54.0^{a}	1648.3 ± 38.0^{b}	1573.5 ± 78.6^{b}
	mDP	6.60 ± 0.14^{a}	9.54 ± 0.30^{b}	8.77 ± 0.34^{c}
	(+)-Catechin (%)	18.9 ± 0.3^{a}	11.3 ± 0.6^{b}	13.2 ± 1.1^{c}
	(-)-Epicatechin (%)	54.5 ± 0.8^{a}	60.4 ± 0.3^{b}	57.5 ± 1.0^{c}
	(-)-Epicatechin-3-O-gallate (%)	3.2 ± 0.1^{a}	3.6 ± 0.3^{a}	3.6 ± 0.3^{a}
	(-)-Epigallocatechin (%)	23.3 ± 1.0^{a}	24.7 ± 0.5^{b}	25.6 ± 0.2^{b}

H1: first harvest, H2: second harvest, CH2: control wines of H2, RAH2: reduced alcohol wines from H2. All data are expressed as the average of the three replicates ± standard deviation (n = 3). Statistical analysis: one-factor ANOVA and Scheffe´s test (both $p < 0.05$). Different letters indicate the existence of statistically significant differences.

On the other hand, grape ripening also affected the proportion of the different proanthocyanidin monomers. Specifically, the proportion of (+)-catechin significantly decreased and the proportion of (-)-epigallocatechin significantly increased in the three cultivars throughout

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maturation. In contrast, the proportions of (-)-epicatechin and (-)-epicatechin-3-O-gallate remained almost unchanged. Only slight but significant fluctuations were detected. In particular, the proportion of (-)-epicatechin-3-O-gallate in Merlot and the proportion of (-)-epicatechin in Bobal tended to increase with maturation.

Since epigallocatechin is only present in skin proanthocyanidins (Souquet et al. 1996) the changes in the proportion of this monomer in the three cultivars seems to indicate that the contribution of skins to the wine proanthocyainidin concentration increases when the grapes are riper.

Only slight differences were found in the proanthocyanidin monomer proportion between the reduced-alcohol wines (RAH2) and their respective controls (CH2). In fact their compositions were very much closer to CH2 wines than to H1 wines, which indicate that grape ripening has a greater influence on wine proanthocyanin composition than other factors such as ethanol content or pH.

Table 7. Sensory analysis (triangular test)

Cultivars	Triangular test	Positive identifications	p	Preferences	
				Flavor	Taste
Cabernet Sauvignon	H1 vs CH2	7/10	0.05	2/5	1/6
	H1 vs RAH2	5/10	ns	1/4	2/3
	CH2 vs RAH2	4/10	ns	2/2	3/1
Merlot	H1 vs CH2	7/10	0.05	1/6	2/5
	H1 vs RAH2	7/10	0.05	3/4	3/4
	CH2 vs RAH2	3/10	ns	2/1	1/2
Bobal	H1 vs CH2	8/10	0.005	2/6	7/1
	H1 vs RAH2	9/10	0.001	4/5	8/1
	CH2 vs RAH2	9/10	0.001	4/5	9/0

H1: first harvest, H2: second harvest, CH2: control wines of H2, RAH2: Reduced Alcohol wines from H2. n.s: non significant.

The sensory triangular tests were carried out by comparing the three wines of each cultivar by pairs (Table 7). As it was quoted above, the color differences among wines were in some cases so evident that all sensory analyses were done in dark glasses. It is well known that color has a non-negligible effect on the sensory appreciation of flavor and texture (Norton and Johnson,

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1987; Stillman, 1993). Therefore, using dark glasses, the highlighted differences could be attributed to flavors and/or mouth sensation, without color interfering. Tasters were able to significantly distinguish the wines of the first harvest from the controls of the second one. As was expected due that H1 grapes were in all the cases less ripe than CH2 grapes. Consequently, the differences in ethanol content, acidity, and polyphenols composition between these wines were noticeable. In the case of Cabernet Sauvignon and Merlot, tasters preferred CH2 wines than H1, by both nose and mouth sensations. The results from Bobal evaluation were less clear. In fact, tasters appreciated the riper flavor of CH2 wines but they preferred H1 in mouth, probably by the excessive alcohol content in CH2 samples which made them too aggressive in the palate.

Tasters were also able to distinguish between H1 and RAH2 wines in the case of Merlot and Bobal but they were unable to differentiate between Cabernet Sauvignon H1 and RAH2 wines. That is also logical because the differences in maturity of Cabernet Sauvignon grapes from the first and the second harvest were lower than in the case of the other two cultivars. In fact, analytically RAH2 and H1 wines of Cabernet are more similar than the other equivalent pairs (Tables 1 to 6). On the other hand, Cabernet Sauvignon RAH2 was the sample in which the addition of low-alcohol wine was the lowest. The preferences of the tasters have not sense in the case of Cabernet sauvignon inasmuch as they did not differentiate the wines. However, they preferred by slight majority the CH2 than H1 wines, by their aroma complexity, of both Merlot and Bobal cultivar; as well in the case of CH2 Merlot wines tasting sensations were appreciated. In apparently contradiction, tasters' preference by H1 than RAH2 Bobal wine could be justified by the excessive acidity provided by the addition of the low alcohol wine into RAH2 wine.

Finally, tasters were able to distinguish between CH2 and RAH2 wines only for Bobal cultivar; unexpectedly, the panel was not able to differentiate these wines for the other two cultivars. It is clear that RAH2 and CH2 of Bobal were very different because it was the experiment in which the addition of low-alcohol wine was higher. In fact, RAH2 wine was actually very acidic and almost all panelists can distinguish it. However, in the case of Cabernet Sauvignon and Merlot, although the differences in the ethanol content were quite high, the other analytical parameters were not so different and in most of the cases minor than the differences between H1 and CH2. Since tasters were unable to distinguish between CH2 and RH2 wines of Cabernet and Merlot, the preferences are meaningless. In the case of Bobal, tasters preferred by slight majority the aroma of RAH2 wine, but in contrast all of them preferred the CH2 by palate sensations. This clear preference was very likely due to the excessive acidity of the RAH2 wine. That is a main

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consideration to take into account in future experiences because it should be prevented that the reduction of alcohol content do not produces an excessive acidification of wine.

It can be concluded then that the proposed procedure may be useful for partially reduction of alcohol content and simultaneously decreasing pH of wines. The color of the reduced-alcohol wines obtained was better than their corresponding controls and their phenolic composition was similar. Moreover, the procedure proposed in this paper does not require specific equipments and is very easy to apply in standard wineries. Further experimentation is needed to better adapt the process to obtain more balanced wines without the problems of excess alcohol and high pH.

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iv. Influence of wine pH in the changes of color and polyphenol composition induced by micro-oxygenation

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Abstract

The presence of oxygen in red wine leads to the transformation of ethanol into ethanal which after capturing a proton will react with flavanols to start the process of formation of ethyl bridges. Wine pH also conditions the equilibrium among the different anthocyanin structures and may consequently affect anthocyanins reactivity. Consequently, the aim of this paper was to study how the pH can affect the changes induced by micro-oxygenation in two wines with different phenolic composition. The differences observed in micro-oxygenation wines in comparison to their controls were, in general, greater when the pH was more acidic. Specifically, the differences found in color intensity, anthocyanin concentration, PVPP Index, ethyl-linked pigments, type B vitisins, polymeric pigments and ethylidene-bridged flavanols between micro-oxygenated wines and their corresponding controls were higher at lower pH. On the contrary, the effects of micro-oxygenation when the pH was less acidic were much smaller and sometimes practically nonexistent.

Keywords: *Wine, pH, phenolic compounds, color, micro-oxygenation*

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Introduction

Phenolic compounds are one of the main determinants of the quality of red wines. Anthocyanins, which are the main responsible of the color of red wine, are extracted from grape skins during the maceration and fermentation processes. Other phenolic compounds, present in skins and seeds, are also extracted. Among these compounds, proanthocyanidins, also known as condensed tannins, are the main determinant of texture sensations such as body, bitterness and astringency (*1-3*). The molecular size and monomeric composition of proanthocyanidins seem to be related to the sensation of astringency: the greater the degree of polymerization and the greater the percentage of galloylation, the greater is the sensation of astringency (*3,4*).

During winemaking and aging, phenolic compounds undergo progressive structural changes with an undoubted influence in wine organoleptic characteristics. In particular, anthocyanins, which are unstable, present a high chemical reactivity giving rise to new more stable pigments. Different mechanisms have been proposed to explain the formation of new pigments. Some of them imply the direct condensation of anthocyanins and flavanols without the participation of oxygen (*5-7*). But probably, the reactions in which oxygen is involved have utmost importance. During winemaking and aging, the presence of small quantities of oxygen leads to the formation of ethanal from ethanol. The ethanal can in turn react with flavanols to induce the formation of a very reactive carbocation that quickly reacts either with another flavanol molecule or with an anthocyanin, producing ethyl-bridged flavanol-flavanol or flavanol-anthocyanin oligomers (*8*). However, it has recently been shown that the compounds formed by ethyl bridges are unstable and that its cleavage can originate new compounds (*9*).

On the other hand, cycloaddition reactions between anthocyanins and other small molecules can produce a new family of anthocyanin-derived pigments called pyranoanthocyanins. Specifically, the reaction with pyruvic acid or ethanal generates vitisin A or B respectively (*10-13*). In addition, the reaction with vinylphenol can generate vinylphenol adducts (*14,15*), and finally the previously formed ethylidene-bridged compounds can dissociate and generate vinylphenol adducts (*16,17*).

All those reactions result in gradually shift of the initial purple-red color to reddish-brown hues. Additionally, astringency diminishes but the mechanism because it happens it is not so clear. Theoretically, the formation of ethyl bridges should increase the degree of polymerization of the

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flavanoles which should increase the astringency (*3*). However, what happens is exactly the opposite. One possible explanation may be the condensation reactions that take place between anthocyanins and flavanols which can diminish the astringency (*4*). Even some authors have suggested that the cleavage reactions of proanthocyanidins by acid catalysis may be the cause of the observed reduction of the astringency (*18*).

The reactivity of phenolic compounds is affected by several factors such as temperature, pH, and free SO_2 concentration (*19,20*) but probably the oxygen exposure is the main determinant (*21-23*). In fact, wines are traditionally aged in oak barrels because the porosity of the wood, the interstices between staves, and the bunghole allow the entry of small amounts of oxygen which can induce all the above quoted reactions (*24*).

Nevertheless, oak aging is an expensive and laborious process that cannot be used for all wines. Micro-oxygenation (MOX) has therefore been proposed for reproducing, and even accelerating, the transformations of color and phenolic compounds that take place during oak aging (*25,26*). MOX technique consists on providing a controlled flow of oxygen into the wine in the form of microbubbles through the injection of gaseous oxygen using a microdiffuser (*27*).

Since its appearance, MOX has become a common application technique in wineries worldwide. It is believed that MOX stabilize the color and decrease the astringency, bitterness and herbaceous characters of wine (*28*). The influence of MOX on the wine quality depends on several parameters with the most important of them to be the moment and the duration of the application, the dose of the oxygen and the composition of the initial wine.

Several publications in the literature have studied the influence of MOX on wine color, composition, and sensory attributes (*21, 26, 28-30*). To our knowledge, however, none of these have studied the influence of wine pH on the effectiveness of MOX. The pH condition the equilibrium among four different anthocyanin structures. The flavylium cation (red) is the main form at very acid pH but its concentration decrease progressively when pH augments, which originate the appearance of the quinoidal base (blue) and the hemiketal form (colorless) and the chalcone (slightly yellow) (*31*). Depending on pH, anthocyanins can act as electrophiles in the flavylium form or as nucleophiles in the hemiketal form (*32*). Therefore, it is quite logical that pH can influence the reactivity of anthocyanins with oxygen. Moreover, the first step on the reactions induced by oxygen implies the formation of ethanal from ethanol. Subsequently, ethanal must capture a proton and become into a carbocation which will react with flavanols to

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start the process of formation of ethyl bridges. Therefore it is also logical that the proton concentration exert a non-negligible influence on this mechanism. In this paper, therefore, we study how the pH can affect the changes induced by MOX in two wines with different phenolic composition.

Materials and methods

Chemicals. Methanol, acetonitrile, formic acid and acetic acid were HPLC-grade and were purchased from Panreac (Barcelona, Spain). Malvidin-3-O-glucoside chloride, (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-O-gallate were purchased from Extrasynthès (Genay, France). Phloroglucinol, L-ascorbic, 4-methylcatechol and 4-dimethylaminocinnamaldehyde (DMACH) were purchased from Sigma (Madrid, Spain). The rest of the chemicals were of high purity and were purchased from Panreac (Barcelona, Spain).

Wines. This study was carried out with two Cabernet Sauvignon wines from the 2008 vintage of the AOC Penedes. These wines were selected because of its different phenolic composition. The wine A had a very low phenolic content, whereas the wine B had a very high phenolic content. Specifically the standard parameters of the wine A at the start of the experiment were: ethanol content, 12.5%; titratable acidity, 5.2 g of tartaric acid/L; volatile acidity, 0.42 g of acetic acid/L; pH, 3.5 free SO2, 20 mg/L. The standard parameters of the wine B at the start of the experiment were: ethanol content, 12.8%; titratable acidity, 5.9 g of tartaric acid/L; volatile acidity, 0.49 g of acetic acid/L; pH, 3.5 free SO2, 20 mg/L.

All racking of the wines were made with previous drainage with argon to ensure that only received the dose of oxygen corresponding to the micro-oxygenation. 4,500 L of both wines were divided in three stainless steel tanks of 1,500 L. The first tanks of each wine was added with NaOH 2M to increase the pH up to 3.9. The second tank of each wine was added with H_2SO_4 2M to diminish pH down to 3.1 and it was also added a complementary volume of distilled water to equal the total volume added to the first tanks. Finally, a similar total volume of distilled water was added to the third tank to minimize the dilution effect without altering the pH. After that, wines were pumped without aeration with the aim to homogenize them and were

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left to stand for 24 h. Twenty four glass bottles (750 mL), previously drained with argon, were filled with each wine and sealed with 49 mm natural cork. The bottled wines were considered as controls. Simultaneously, each one of the 6 different wines (wine A and B at three different pH) was racked to three MOX tanks previously drained with argon for carrying out the experiment in triplicate.

Micro-Oxygenation Equipment. The multiple diffuser microoxygenator (VISIO 2/6-Oenodev, France) was connected to each one of MOX stainless steel tanks of 165 L. These tanks were 2.5 m in height, had a diameter of 0.30 m, and were equipped with a ceramic diffuser placed at 10 cm above the bottom of the tank. These dimensions were necessary so that the oxygen bubbles produced during micro-oxygenation would have a sufficient height of displacement to guarantee their complete dissolution.

Experimental Conditions. All wines, controls and micro-oxygenated, were kept at a temperature of 16 ± 2 °C. MOX were carried out for 3 months with an oxygen flow of 15 mg/L per month. After that, 24 glass bottles (750 mL), previously drained with argon, were filled with each tank and sealed with 49 mm natural cork. Analyses were done immediately after bottling and also 8 months later.

Color parameters. The color intensity (CI) was estimated using the method described by Glories (*33*). The CIELAB coordinates, lightness (L*), chroma (C*), hue (H*), were determined according to Ayala et al. (*34*) and the data were processed with the MSCV® software (*35*). All absorbance measurements were taken with a Helios Alpha (Thermo Fisher Scientific Inc., Waltman, MA) UV–vis spectrophotometer using quartz cells of 1 mm path length.

Anthocyanin Analysis. The total anthocyanin content was determined by spectrophotometry using the method described by Niketic-Alksic and Hrazdina (*36*). The PVPP index was calculated according to Glories (*33*).

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HPLC Analyses of Anthocyanins and Derived Pigments. Reversed-phase HPLC analyses of anthocyanins and the anthocyanin-derived pigments were carried out with an Agilent 1200 series liquid chromathograph (HPLC-DAD) and an Agilent Zorbax Eclipse XDB-C18, 4.6 x 250mm 5µm column (Agilent Technologies, Santa Clara, USA) according to the method described by Cano-López et al. (*29*). As solvents were used 4.5% aqueous formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.8 mL/min. Elution was performed with a gradient starting with 10% B to reach 15% B at 30 min, 15.2% B at 45 min, 18% B at 60 min, 25% B at 100 min and 25 to 100% B in 20 min. Chromatograms were recorded at 520 nm and anthocyanin standard curves were made using malvidin-3-O-glucoside chloride.

Compounds were identified by comparing their UV spectra recorded with the diode array detector and those reported in the literature (*37*). In addition, to confirm each peak identity analyses were performed with Agilent 1200 series HPLC using an Agilent 6210 time of flight (TOF) mass spectrometer equipped with an electrospray ionization (ESI) system. Elution was carried out under the same HPLC analysis conditions as described by Cano-López (*29*). The capillary voltage was 3.5 kV. Nitrogen was used as both dry gas at a flow rate of 12 L/min at 350 ºC and nebulizer gas at 60 psi. Spectra were recorded in positive ion mode between m/z 50 and 2400.

Analysis of Proanthocyanidins following acid catalysis with phloroglucinol. Acid-catalysis cleavage in the presence of excess phloroglucinol (*38*) was used to analyze monomeric proanthocyanidin composition and its mean degree of polymerization (mDP). 10 mL of wine was evaporated under a low pressure vacuum (Univapo 100 ECH, Uni Equip, Martinsried, Germany). After that it was resuspended in 6 ml distilled water and then applied to Set Pak Plus tC18 Enviromental cartridges (Waters, Milford, USA) that had been previously activated with 10 mL methanol and 15 mL water. The sample was washed with 15 mL distilled water and then the proanthocyanidins were eluted with 12 mL methanol, immediately evaporated under vacuum and later redissolved in 2 mL methanol. Finally, 100 µL of this sample were reacted with 100 µL phloroglucinol solution (0.2N HCl in methanol, containing 100g/L phloroglucinol and 20 g/L ascorbic acid) at 50 ºC for 20 min. The reaction was stopped by adding 1000 µL of 40 mM aqueous sodium acetate (*38*). Reversed-phase HPLC analysis (Agilent serie 1200 HPLC-DAD) was carried out according to the method of Kennedy and Jones (*38*). As solvents were used 1% aqueous acetic acid (solvent A) and methanol (solvent B) at a flow rate of 1 mL/min. The

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elution conditions were 1.0 mL/min. Elution was performed with a gradient starting with 5% B for 10 min, a linear gradient from 5 to 20% B in 20 min, a linear gradient from 20 to 40% B in 25 min. The column was then washed with 90% B for 10 min and reequilibrated with 5% B for 5 min before the next injection. The monomers (+)-catechin, (-)-epicatechin, (-)-epicaechin-3-O-gallate were identified by comparing its retention time with that of the pure compounds. The phoroglucinol adducts of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin-3-O-gallate were identified by HPLC-TOF analysis. Analyses were performed with Agilent 1200 series HPLC using an Agilent 6210 time of flight (TOF) mass spectrometer equipped with an electrospray ionization (ESI) system. Elution was carried out under the same HPLC analysis conditions as described by Kennedy and Jones (*38*). The capillary voltage was 3.5 kV. Nitrogen was used as both dry gas at a flow rate of 12 L/min at 350 ºC and nebulizer gas at 60 psi. Spectra were recorded in positive ion mode between m/z 50 and 2400. This assay was also carried out without addition of phloroglucinol in order to measure the quantity of proanthocyanidin monomers naturally present in the wines.

The number of terminal subunits were considered as the difference between total monomers without phoroglucinol and thus obtained in the analysis performed without phloroglucinol addition. The number of extension subunits was considered as the addition of all the phloroglucinol adduct. The mean degree of polymerization (mDP) was calculated by adding terminal and extension subunits (in moles) and dividing by terminal subunits. The total proanthocyanidin concentration was considered as the addition of all terminal and extension subunits. Because acid catalysis with phloroglucinol is not completely efficient, the real yield of the reaction was measured using a pure B2 proanthocyanidin dimer [(-)-epicatechin-(4→8)-(-)-epicatechin]. This yield was used to calculate the total proanthocyanidin concentration.

Wine EDP Phloroglucinolysis Method. The determination of the ethylidene-bridged flavanols in wine samples was done with the use of 2,2´-ethylidenediphloroglucinol (EDP) phloroglucinolysis according to the method described by Drinkine et al. (*39)*. 5 mL of wine was diluted in 10 mL of water. By 10 mL, the sample was applied on the column after conditioning. After that 10 mL of the sample were applied to Set Pak Plus tC18 (5g) Enviromental cartridges (Waters, Milford, USA) that had been previously activated with 50 mL methanol and 50 mL water. The sample was washed with 50 mL distilled water and then the sample was eluted with 50 mL methanol, immediately evaporated under vacuum and later redissolved in 1.5 mL methanol. Finally, 100 µL of this sample were reacted with 100 µL phloroglucinol solution

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(0.2N HCl in methanol, containing 100g/L phloroglucinol and 20 g/L ascorbic acid) at 50 ºC for 20 min. The reaction was stopped by adding 200 µL of 400 mM aqueous sodium acetate (*39*). Aqueous 4-methylcatechol (20 µL of 500 mg/L) was then added as internal standard.

Analyses were performed with Agilent 1200 series HPLC using an Agilent 6210 time of flight (TOF) mass spectrometer equipped with electrospray ionization (ESI) system and an Agilent Zorbax Eclipse XDB-C18, 4.6 x 150mm 5µm column. As solvent were used 5% aqueous acetic acid (solvent A), and acetonitrile as solvent B. The sample loop was 20 µL and the elution gradient was as follows: 10% B for 2 min, from 10 to 50% B in 8 min, from 50 to 100% B in 1 min, 100% B for 4 min, from 100 to 10% B in 1 min, 10% B for 4 min with a 0.3 mL/min flow. The capillary voltage was 3.5 kV. Nitrogen was used as both dry gas at a flow rate of 12 L/min at 350 ºC and nebulizer gas at 60 psi. Spectra were recorded in positive ion mode between m/z 50 and1100. For comparing the wine samples we used the area of the identified EDP.

Other Phenolic Compounds. The total phenolic index (TPI) was determined by measuring the absorbance at 280 nm (*19*). Dimethylaminocinnamaldehyde index (DMACH index) was measured according to Nagel and Glories (*40*).

Statistics. All the data are expressed as the arithmetic average ± of the standard deviation from three replicates. Two and one-factor ANOVA and Scheffe`s test were carried out with SPSS software.

Results and discussion

Tables 1 (Wine A) and 2 (Wine B) show the wines color characteristics. In regard to the original wines (pH 3.5), it is clear that they have very different color characteristics. Wine A have lower Color Intensity (CI), Chroma (C*) and Hue (H*) and higher Luninosity (L*) than wine B. This data confirms that wine A is a light wine while wine B is a highly concentrated wine, at least in terms of color.

As expected, CI, C* and L* of both wines were drastically influenced by the pH. Specifically, CI and C* were higher and L* lower as the pH is lower in both wines. H* was also higher when

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the pH was more acid in wine B, but this effect was not so clear in the case of wine A. These changes generated by the pH in the chromatic characteristics observed in both wines can be easily explained. The lower the pH the higher the proportion of flavylium cation, and consequently, the higher the contribution of anthocyanins to red color (*31*). In addition, the higher the pH the higher the proportion of quinonoidal base. Since this form of anthocyanins is blue, at lower pH, H* should also be lower.

CI tended to decrease over time in both wines at any pH. However, all the MOX wines had higher CI than their corresponding controls with the only exception of the wine A at pH 3.9. These differences were observed immediately after bottling and also 8 months later. Moreover, a clear trend was observed after 8 months of bottle storage inasmuch as the differences in CI appear to be higher when pH is lower. Specifically, in wine A the differences in absorbance units were 1.3 at pH 3.1, 0.4 at pH 3.5 and 0.2 at pH 3.9, and in wine B were 2.4 at pH 3.1, 1.5 at pH 3.5 and 1.1 at pH 3.9.

In regard to Cielab coordinates, C* tend to decrease in all the wines over time but no clear tend is observed. On the other hand, L* tend to increase in all the control wines over time and have not a clear behavior in both micro-oxygenated wines at any pH during the three first months. However, 8 months later, nearly all the microoxygenated wines have an L* value lower than their corresponding controls, and this differences are higher when the pH is lower. Specifically, in wine A the differences in L* units were 4.3 at pH 3.1, 1.0 at pH 3.5 and 0.1 at pH 3.9, and in wine B were 3.3 at pH 3.1, 2.2 at pH 3.5 and 0.8 at pH 3.9. Finally, changes in H* are even more complicated. In the slight wine (wine A) H* tend to increase in the MOX wines at any pH. However, in the high phenolic content wine (wine B) the changes on H* of the MOX wines vary in function of the pH. Thus, H* decreases at pH 3.1, it is stable at pH 3.5 and it increases at pH 3.9. It seems, therefore, pH and polyphenols concentration exert an effect on the color changes induced by MOX.

Tables 3 (Wine A) and 4 (Wine B) show the results from the spectrophotometric analysis of anthocyanins and the PVPP Index. This data confirms that wine A had an anthocyanin concentration much lower than wine B and therefore they were completely different at this level.

As expected, total anthocyanin decreased over time in all the conditions. However, some different pattern can be observed between controls and MOX wines. In general, the decrease in anthocyanin concentration after three months tends to be higher in the MOX wines than in their

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corresponding controls. Eight months later, these differences remained in wine A, but wine B behaved differently in function of pH. In this high phenolic content wine, the anthocyanin concentration of MOX wine was higher at pH 3.1, equal at pH 3.5 and lower at pH 3.9 than their corresponding controls.

The PVPP Index tended to increase over time in all wines and did it faster when MOX was applied, which would confirm that oxygen favors the combination of anthocyanins with flavanols (*21*). On the other hand, after three months of MOX, the difference of PVPP index of MOX wines in respect to their controls was higher when the pH was lower. Specifically, in wine A the differences in PVVP Index were 9.9 at pH 3.1, 2.2 at pH 3.5 and 2.2 at pH 3.9, and in wine B were 14.0 at pH 3.1, 7.4 at pH 3.5 and 3.4 at pH 3.9. Although these differences are minimized eight months later, this data suggests that when the pH is lower, MOX is more effective, at least in terms of its effect on the combination of anthocyanins and flavanols.

Anthocyanins and anthocyanin-derived pigments were also analyzed by HPLC. In both wines, A (Table 5) and B (Table 6), the five anthocyanin monoglucosides (malvidin, petunidin, delphinidin, peonidin, and cyanidin) and their respective acetyl and coumaryl derivatives were detected. Overall, total monomeric anthocyanin concentration decreased over time and did it faster when MOX was applied and when pH was lower. Broadly, this trend, which was observed in the five monoglucosides and also in their respective acyl-derivatives, does not coincide exactly with that observed for the anthocyanins measured by spectrophotometry. This is because spectrophotometric analysis overestimates the total anthocyanin concentration since it includes the contribution from other pigments, whereas only free anthocyanins are detected by the HPLC method (*41*).

Malvidin 3-glucoside-(epi)catechin was the only detected direct adduct of anthocyanins and flavanols and its concentration tends to increase over time in all wines. However, nor pH nor microoxygenation seem to exert any influence on its evolution.

Malvidin-3-glucoside-ethyl-catechin, malvidin-3-acetylglucoside-ethyl-catechin and malvidin-3-p-coumaroyl-glucoside-ethyl-catechin were detected in wine samples and all of them are grouped in Tables 5 and 6 as ethyl-bridged linked pigments. After three months, none of the control wines differ significantly with respect to their original wines. Similar results were obtained with MOX wines at pH 3.9 and 3.5. However, both MOX wines had significantly higher levels ethyl-bridged linked pigments when the pH was more acidic (3.1). Eight months

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later, all the MOX wines had small but significant higher concentrations of these pigments than their corresponding controls. These results confirm that MOX favor the combination of anthocyanins with flavanols by means of ethyl bridges although the differences found are minor than expected.

Several pyranoanthocyanin compounds were found in our wines. Specifically, type-A vitisins which include: vitisin A and A-type vitisins of peonidin-3-glucoside and malvidin 3-acetylglucoside; type-B-vitisins which include vitisin B and B-type vitisin of malvidin 3-acetylglucoside; vinyl-adducts which include malvidin-3-glucoside-4-vinyl-catechin adduct and malvidin-3-glucoside-4-vinylphenol adduct.

After three months, all the MOX wines had small but significant higher concentration of type-A vitisins than their respective controls without observing any effect of pH. These differences remained 8 months later only in the high phenolic content wine (B) but not in the slight wine (A).

A different trend was found in type B vitisins because three months later all the wines, control, and MOX, had a significant increase in the concentration of these pigments. It is interesting to note that both MOX wines at pH 3.1 had significant higher concentration of type B vitisins than their corresponding controls and that these differences were not found at the other pH. Therefore, the very acidic pH seems to favor the cycloaddition of ethanal with anthocyanins.

After three months, the vinyl adducts concentration of all MOX wines was significant higher than their corresponding controls but 8 months later this differences are not so clear. In fact some control wines after 8 months had higher concentrations of these pigments than their corresponding MOX wines. On the other hand, the pH did not appear to exert any effect on these compounds.

Some authors have suggested that a broad peak at the end of the HPLC chromatogram of anthocyanins and derived-pigments correspond to the polymeric pigments (*29,30*). This polymeric peak tended to increase its surface over time in all wines which suggest that the formation of polymeric structures of pigments took place both in the control and MOX wines. This polymeric peak tend to increased faster when MOX was applied on the wine B at any pH but only at pH 3.1 in the wine A.

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At three months, this polymeric peak was significant higher when MOX was applied on the wine B at any pH but only at pH 3.1 in the wine A. Eight months later the differences between MOX and control wines were more clearly influenced by the pH. Specifically, at pH 3.1 the surface of the polymeric peak was around 50 % higher in both MOX wines than in their controls whereas at pH 3.5 were around 15 % and practically inexistent at pH 3.9. These data suggest, therefore, that the formation of polymeric pigments in the presence of oxygen is more favorable when the pH of the wine is very acidic.

Tables 7 and 8 show the total phenolic compounds and related parameters. These data confirm again that both wines are very different in their phenolic composition inasmuch as the wine A have a TPI and a proanthocyanidin concentration of about half of the wine B.

As expected, no differences were found in the TPI, DMACH Index, proanthocyanidin concentration and mDP of both initial wines in function of the pH. Nevertheless, the TPI and the proanthocyanidin concentration tended to decrease over time in all wines probably caused by the precipitation of great polymers. In general, these decreases were similar in control and MOX wines at any pH. The DMACH index also decreased in all wines over time but in that case some general trends were observed. Specifically, DMACH Index decreased faster in MOX wines than in their controls. Moreover, this decrease seemed to be more drastic when the pH was lower especially in the less concentrated wine (A). Since DMACH reacts only with the extremes of proanthocyanidin polymers, its diminution must be due to a decrease in the number of molecules (Citas). Therefore, the observed decrease of DMACH Index may be caused either by precipitation of some of the proanthocyanidin molecules or by increasing their degree of polymerization. However, the mDP measured by phloroglucionolisis do not follow a similar pattern than DMACH Index, remaining always in very similar values in all wines. These results indicate that MOX have not induced changes in the degree of polymerization and suggest that the decrease in DMACH is more concerned with precipitation of some of the proanthocyaninidins molecules. These results can be considered as surprising because it was expected that the MOX induced the polymerization of proanthocyaninidins but other author have also reported similar results (*21*).

Table 9 show the evaluation of ethyl-bridged flavanols by means of EDP phloroglucionolysis. In that case, only wines at 8 months were analyzed because at the beginning of the experiment the method was not ready. In none of the wines, the application of MOX generated significant differences in the presence of ethylidene-bridged flavanols at pH 3.9. However, when the pH

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were lower significant differences appeared. Specifically, at pH 3.5 higher presence of ethylidene-bridged flavanols was found in microxigenated wine A but not in wine B and at pH 3.1 the differences became very high in both wines. These data indicates that the formation of ethyl-bridged flavanols is highly favored at pH very acidic. Moreover, since no differences were found at pH 3.9 between control and MOX wines, this data also suggest that MOX can be less effective when the pH of wines is too high.

It can be concluded that pH exert a major effect on the evolution of color and phenolic compounds of wine during aging, especially when oxygen is added by MOX. When pH is more acidic, the effects of MOX are clearer. The differences observed in MOX wines in comparison to their controls indicate that they had more intense color and higher PVPP Index, higher concentrations of ethyl-linked pigments, type B vitisins, polymeric pigments and ethylidene-bridged flavanols when the pH is more acidic. On the contrary, the effects of MOX when the pH is less acidic are much smaller and sometimes practically nonexistent.

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Table 1. Color Parameters - wine A -

Parameter	pH	Treatment	Initial Wine	After 3 months of microoxygenation	8 months after bottling
CI	3.1	Control	10.9 ± 0.2A	10.0 ± 0.1B,α	9.0 ± 0.1C,α
		MO		11.1 ± 0.4A,β	10.3 ± 0.3B,β
	3.5	Control	9.1 ± 0.1A	8.7 ± 0.1B,α	8.3 ± 0.1C,α
		MO		9.1 ± 0.1A,β	8.7 ± 0.1B,β
	3.9	Control	7.9 ± 0.1A	8.3 ± 0.2B,α	7.8 ± 0.1A,α
		MO		8.3 ± 0.2B,α	8.0 ± 0.2AB,α
L*	3.1	Control	50.8 ± 0.5A	53.3 ± 0.2B,α	56.1 ± 0.2C,α
		MO		49.9 ± 1.3A,β	51.8 ± 1.0A,β
	3.5	Control	55.1 ± 0.2A	56.7 ± 0.2B,α	58.6 ± 0.1C,α
		MO		56.1 ± 0.4B,α	57.6 ± 0.4C,β
	3.9	Control	58.3 ± 0.1A	58.4 ± 0.7A,α	60.6 ± 0.1 B,α
		MO		58.9 ± 0.7A,α	60.5 ± 0.2B,α
C*	3.1	Control	57.8 ± 0.3A	54.1 ± 0.1B,α	50.1 ± 0.1C,α
		MO		53.9 ± 0.2B,α	51.4 ± 0.4C,β
	3.5	Control	49.3 ± 0.1A	46.8 ± 0.1B,α	44.2 ± 0.2C,α
		MO		46.2 ± 0.2B,β	44.0 ± 0.2C,α
	3.9	Control	40.4 ± 0.2A	40.1 ± 0.4A,α	40.1 ± 0.1A,α
		MO		40.1 ± 0.3AB,α	39.5 ± 0.3B,β
H*	3.1	Control	5.7 ± 0.2A	5.6 ± 0.2A,α	5.8 ± 0.1A,α
		MO		10.0 ± 0.6B,β	9.6 ± 0.3B,β
	3.5	Control	3.8 ± 0.1A	4.2 ± 0.2B,α	6.3 ± 0.2C,α
		MO		8.3 ± 0.6B,β	8.9 ± 0.4B,β
	3.9	Control	3.9 ± 0.1A	6.0 ± 0.3B,α	8.3 ± 0.1C,α
		MO		9.3 ± 0.5B,β	10.4 ± 0.2C,β

All data are expressed as the average values of three replicates ± standard deviation (n=3). Statistical analysis: two-factor ANOVA and Scheffe's test (both, p=0.05). Different letters indicate statistical differences. Latin letters (A, B, C) are used to compare the wines of the same pH throughout the time. Greek letters (α, β) are used to compare control and micro-oxygenation samples of the same pH at the same time.

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Table 2. Color Parameters - wine B -

Parameter	pH	Treatment	Initial Wine	After 3 months of microoxygenation	8 months after bottling
CI	3.1	Control	28.1 ± 0.2A	25.8 ± 0.1B,α	20.2 ± 0.1C,α
		MO		26.9 ± 0.3B,β	22.6 ± 0.1C,β
	3.5	Control	23.4 ± 0.3A	21.5 ± 0.1B,α	18.2 ± 0.1C,α
		MO		23.6 ± 0.3B,β	19.7 ± 0.3C,β
	3.9	Control	20.8 ± 0.2A	19.3 ± 0.1B,α	16.6 ± 0.2C,α
		MO		20.7 ± 0.2B,β	17.7 ± 0.1C,β
L*	3.1	Control	29.6 ± 0.1A	30.0 ± 0.1B,α	33.6 ± 0.1C,α
		MO		26.9 ± 0.3B,β	30.0 ± 0.3C,β
	3.5	Control	29.1 ± 0.3A	29.8 ± 0.2B,α	33.8 ± 0.1C,α
		MO		26.8 ± 0.2B,β	31.6 ± 0.3C,β
	3.9	Control	28.7 ± 0.1A	30.1 ± 0.1B,α	34.8 ± 0.1C,α
		MO		28.3 ± 0.2B,β	34.0 ± 0.2C,β
C*	3.1	Control	68.1 ± 0.3A	66.1 ± 0.1B,α	64.1 ± 0.1C,α
		MO		63.3 ± 0.7B,β	62.3 ± 0.5B,β
	3.5	Control	60.8 ± 0.1A	58.6 ± 0.1B,α	56.9 ± 0.1C,α
		MO		57.1 ± 0.2B,β	57.0 ± 0.2B,α
	3.9	Control	55.2 ± 0.3A	52.5 ± 0.1B,α	48.8 ± 0.1C,α
		MO		52.3 ± 0.3B,α	53.1 ± 0.1C,β
H*	3.1	Control	26.9 ± 0.4A	24.5 ± 0.1B,α	20.8 ± 0.1C,α
		MO		25.4 ± 1.0B,β	22.6 ± 0.7C,β
	3.5	Control	17.2 ± 0.4A	15.9 ± 0.1B,α	15.6 ± 0.1C,α
		MO		18.3 ± 1.0A,β	17.9 ± 0.8A,β
	3.9	Control	9.8 ± 0.4A	10.1 ± 0.1A,α	11.6 ± 0.1B,α
		MO		14.9 ± 0.1B,β	16.1 ± 0.0B,β

All data are expressed as the average values of three replicates ± standard deviation (n=3). Statistical analysis: two-factor ANOVA and Scheffe's test (both, p=0.05). Different letters indicate statistical differences. Latin letters (A, B, C) are used to compare the wines of the same pH throughout the time. Greek letters (α, β) are used to compare control and micro-oxygenation samples of the same pH at the same time.

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Table 3. Spectrophotometric Analysis of Anthocyanins and PVPP Index - wine A -

parameter	pH	Treatment	Initial Wine	After 3 months of microoxygenation	8 months after bottling
Total Anthocyanins (mg/L)	3.1	Control	417.1 ± 3.3A	362.3 ± 13.1B,α	264.8 ± 1.0C,α
		MO		316.2 ± 4.1B,β	246.2 ± 0.5C,β
	3.5	Control	415.9 ± 14.0A	377.4 ± 6.0B,α	282.3 ± 2.7C,α
		MO		343.9 ± 2.5B,β	265.2 ± 0.5C,β
	3.9	Control	424.7 ± 7.1A	394.9 ± 4.5B,α	314.4 ± 2.2C,α
		MO		376.5 ± 1.0B,β	289.0 ± 2.2C,β
PVPP Index	3.1	Control	34.7 ± 1.3A	48.6 ± 2.3B,α	52.5 ± 1.1C,α
		MO		58.5 ± 6.8B,β	60.9 ± 0.8B,β
	3.5	Control	34.5 ± 2.6A	44.0 ± 3.4B,α	45.3 ± 2.5B,α
		MO		46.2 ± 0.5B,α	53.1 ± 1.2C,β
	3.9	Control	35.9 ± 4.3A	40.0 ± 1.1A,α	43.8 ± 1.1B,α
		MO		42.2 ± 2.9A,α	53.1 ± 5.8B,β

All data are expressed as the average values of three replicates ± standard deviation (n=3). Statistical analysis: two-factor ANOVA and Scheffe's test (both, p=0.05). Different letters indicate statistical differences. Latin letters (A, B, C) are used to compare the wines of the same pH throughout the time. Greek letters (α, β) are used to compare control and micro-oxygenation samples of the same pH at the same time.

Table 4. Spectrophotometric Analysis of Anthocyanins and PVPP Index - wine B -

parameter	pH	Treatment	Initial Wine	After 3 months of microoxygenation	8 months after bottling
Total Anthocyanins (mg/L)	3.1	Control	1009.8 ± 10.1A	798.6 ± 4.1B,α	481.3 ± 3.7C,α
		MO		748.1 ± 6.1B,β	511.0 ± 6.6C,β
	3.5	Control	981.8 ± 13.7A	796.3 ± 1.5B,α	521.5 ± 2.5C,α
		MO		741.7 ± 10.4B,β	517.4 ± 3.9C,α
	3.9	Control	994.3 ± 11.3A	806.8 ± 8.6B,α	544.3 ± 6.2C,α
		MO		782.8 ± 18.7B,α	517.4 ± 3.9C,β
PVPP Index	3.1	Control	32.7 ± 2.1A	44.6 ± 0.3B,α	67.7 ± 0.1C,α
		MO		58.6 ± 1.6B,β	71.9 ± 2.7C,β
	3.5	Control	32.0 ± 2.1A	51.5 ± 0.9B,α	62.4 ± 0.3C,α
		MO		58.9 ± 2.3B,β	66.4 ± 1.8C,β
	3.9	Control	33.6 ± 1.1A	49.8 ± 1.3B,α	57.3 ± 1.5C,α
		MO		53.2 ± 3.3B,α	64.0 ± 1.4C,β

All data are expressed as the average values of three replicates ± standard deviation (n=3). Statistical analysis: two-factor ANOVA and Scheffe's test (both, p=0.05). Different letters indicate statistical differences. Latin letters (A, B, C) are used to compare the wines of the same pH throughout the time. Greek letters (α, β) are used to compare control and micro-oxygenation samples of the same pH at the same time.

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Table 5. HPLC Analysis of Anthocyanins and Anthocyanin-Derived Pigments - wine A -

Parameter	pH	Treatment	Initial Wine	After 3 months of microoxygenation	8 months after bottling
Monomeric anthocyanins (mg/L)	3.1	Control	158.5 ± 0.4A	106.3 ± 1.7B,α	58.9 ± 1.1C,α
		MO		65.6 ± 4.6B,β	32.7 ± 0.6C,β
	3.5	Control	155.5 ± 0.3A	118.3 ± 1.2B,α	69.0 ± 2.0C,α
		MO		96.6 ± 3.4B,β	54.3 ± 1.3C,β
	3.9	Control	152.9 ± 0.5A	121.8 ± 1.0B,β	85.9 ± 1.2C,α
		MO		108.7 ± 0.5B,β	74.4 ± 0.3C,β
Direct adducts (mg/L)	3.1	Control	1.36 ± 0.04A	1.62 ± 0.21B,α	1.69 ± 0.13B,α
		MO		1.68 ± 0.07B,α	1.45 ± 0.03C,β
	3.5	Control	1.30 ± 0.01A	1.54 ± 0.04B,α	1.48 ± 0.12B,α
		MO		1.60 ± 0.03B,α	1.38 ± 0.03C,α
	3.9	Control	1.23 ± 0.01A	1.37 ± 0.01B,α	1.35 ± 0.07B,α
		MO		1.36 ± 0.03B,α	1.38 ± 0.02B,α
Ethyl-linked pigments (mg/L)	3.1	Control	0.45 ± 0.07A	0.43 ± 0.02A,α	0.38 ± 0.01A,α
		MO		0.76 ± 0.06B,β	0.76 ± 0.08B,β
	3.5	Control	0.45 ± 0.07A	0.44 ± 0.03A,α	0.36 ± 0.04B,α
		MO		0.42 ± 0.05A,α	0.56 ± 0.10A,β
	3.9	Control	0.45 ± 0.05A	0.50 ± 0.05A,α	0.51 ± 0.01A,α
		MO		0.47 ± 0.04A,α	0.74 ± 0.07B,β
A type Vitisins (mg/L)	3.1	Control	2.49 ± 0.04A	2.03 ± 0.06B,α	2.12 ± 0.03B,α
		MO		2.60 ± 0.06B,β	2.16 ± 0.02C,α
	3.5	Control	2.47 ± 0.02A	2.06 ± 0.05B,α	2.05 ± 0.05B,α
		MO		2.62 ± 0.05B,β	2.12 ± 0.09C,α
	3.9	Control	2.43 ± 0.04A	1.91 ± 0.05B,α	1.95 ± 0.04B,α
		MO		2.95 ± 0.04B,β	2.03 ± 0.12C,α
B type Vitisins (mg/L)	3.1	Control	0.30 ± 0.02A	0.84 ± 0.03B,α	0.37 ± 0.04A,α
		MO		1.97 ± 0.25B,β	1.31 ± 0.20C,β
	3.5	Control	0.34 ± 0.01A	0.90 ± 0.02B,α	0.27 ± 0.04C,α
		MO		0.65 ± 0.04B,β	0.46 ± 0.05C,β
	3.9	Control	0.34 ± 0.06A	0.91 ± 0.09B,α	0.21 ± 0.01C,α
		MO		0.92 ± 0.02B,α	0.42 ± 0.04C,β
Vinyl adducts (mg/L)	3.1	Control	1.19 ± 0.01A	1.28 ± 0.03B,α	1.18 ± 0.19AB,α
		MO		1.69 ± 0.24B,β	1.58 ± 0.22B,α
	3.5	Control	1.19 ± 0.04A	1.41 ± 0.05B,α	1.42 ± 0.05B,α
		MO		1.71 ± 0.08B,β	1.73 ± 0.07B,β
	3.9	Control	1.18 ± 0.01A	1.65 ± 0.03B,α	1.92 ± 0.09C,α
		MO		1.96 ± 0.06B,β	2.07 ± 0.15B,α
Polymeric Peak (mg/L)	3.1	Control	5.18 ± 0.29A	5.46 ± 0.31A,α	6.21 ± 0.06B,α
		MO		9.20 ± 0.85B,β	9.50 ± 1.00B,β
	3.5	Control	5.05 ± 0.53A	6.22 ± 0.22B,α	6.23 ± 0.19B,α
		MO		6.36 ± 0.26B,α	6.83 ± 0.24B,α
	3.9	Control	5.15 ± 0.19A	5.94 ± 0.15B,α	6.16 ± 0.58B,α
		MO		6.23 ± 0.24B,α	6.56 ± 0.06B,α

All data are expressed as the average values of three replicates ± standard deviation (n=3). Statistical analysis: two-factor ANOVA and Scheffe's test (both, p=0.05). Different letters indicate statistical differences. Latin letters (A, B, C) are used to compare the wines of the same pH throughout the time. Greek letters (α, β) are used to compare control and micro-oxygenation samples of the same pH at the same time.

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Table 6. HPLC Analysis of Anthocyanins and Anthocyanin-Derived Pigments - wine B -

Parameter	pH	Treatment	Initial Wine	After 3 months of microoxygenation	8 months after bottling
Monomeric anthocyanins (mg/L)	3.1	Control	270.4 ± 10.4A	205.1 ± 27.8B,α	61.41 ± 0.19C,α
		MO		143.0 ± 6.2B,β	42.46 ± 2.16C,β
	3.5	Control	270.3 ± 4.8A	195.6 ± 4.9B,α	79.32 ± 1.9C,α
		MO		156.3 ± 6.3B,β	61.85 ± 3.7C,β
	3.9	Control	245.5 ± 6.1A	204.4 ± 2.4B,α	96.8 ± 0.1C,α
		MO		195.1 ± 2.2B,β	72.9 ± 0.1C,β
Direct adducts (mg/L)	3.1	Control	1.52 ± 0.05A	2.45 ± 0.12B,α	2.10 ± 0.10C,α
		MO		2.02 ± 0.06B,β	2.75 ± 0.04C,β
	3.5	Control	1.66 ± 0.03A	2.03 ± 0.05B,α	2.41 ± 0.09C,α
		MO		1.96 ± 0.05B,α	2.87 ± 0.01C,β
	3.9	Control	1.60 ± 0.07A	1.79 ± 0.05B,α	2.39 ± 0.02C,α
		MO		1.88 ± 0.06B,α	2.34 ± 0.08C,α
Ethyl-linked pigments (mg/L)	3.1	Control	1.19 ± 0.10A	1.24 ± 0.05A,α	1.62 ± 0.18B,α
		MO		2.23 ± 0.06B,β	1.91 ± 0.06C,β
	3.5	Control	1.23 ± 0.10A	1.23 ± 0.06A,α	1.45 ± 0.05B,α
		MO		1.37 ± 0.15A,α	1.68 ± 0.12B,β
	3.9	Control	1.22 ± 0.05A	1.11 ± 0.05A,α	1.42 ± 0.02 B,α
		MO		1.04 ± 0.14B,α	1.54 ± 0.04B,β
A type Vitisins (mg/L)	3.1	Control	7.68 ± 0.01A	7.48 ± 0.05B,α	7.40 ± 0.01C,α
		MO		8.09 ± 0.04B,β	7.85 ± 0.05C,β
	3.5	Control	7.60 ± 0.10A	7.42 ± 0.02B,α	7.30 ± 0.01C,α
		MO		9.91 ± 0.03B,β	9.38 ± 0.05C,β
	3.9	Control	7.61 ± 0.05A	6.66 ± 0.06B,α	6.53 ± 0.01C,α
		MO		7.42 ± 0.01B,β	6.93 ± 0.01C,β
B type Vitisins (mg/L)	3.1	Control	0.40 ± 0.05A	0.85 ± 0.02B,α	0.48 ± 0.01A,α
		MO		1.31 ± 0.05B,β	0.83 ± 0.02B,β
	3.5	Control	0.39 ± 0.03A	0.68 ± 0.02B,α	0.28 ± 0.01C,α
		MO		0.63 ± 0.06B,α	0.33 ± 0.01C,β
	3.9	Control	0.43 ± 0.05A	0.67 ± 0.01B,α	0.27 ± 0.01C,α
		MO		0.65 ± 0.02B,α	0.25 ± 0.01C,α
Vinyl adducts (mg/L)	3.1	Control	1.01 ± 0.07A	1.21 ± 0.03B,α	2.12 ± 0.08C,α
		MO		1.57 ± 0.01B,β	2.97 ± 0.08C,β
	3.5	Control	1.11 ± 0.05A	1.29 ± 0.07B,α	2.05 ± 0.04C,α
		MO		1.51 ± 0.03B,β	1.62 ± 0.14B,β
	3.9	Control	1.15 ± 0.06A	1.18 ± 0.05A,α	1.88 ± 0.09C,α
		MO		1.32 ± 0.07B,β	1.47 ± 0.09B,β
Polymeric Peak (mg/L)	3.1	Control	5.99 ± 0.21A	7.38 ± 0.34B,α	7.57 ± 0.09B,α
		MO		8.39 ± 0.35B,β	11.23 ± 0.43C,β
	3.5	Control	5.89 ± 0.08A	6.30 ± 0.16B,α	8.83 ± 0.01C,α
		MO		7.47 ± 0.32B,β	10.07 ± 0.03C,β
	3.9	Control	5.43 ± 0.13A	6.63 ± 0.08B,α	8.58 ± 0.08C,α
		MO		8.02 ± 0.08B,β	9.22 ± 0.16C,β

All data are expressed as the average values of three replicates ± standard deviation (n=3). Statistical analysis: two-factor ANOVA and Scheffe's test (both, p=0.05). Different letters indicate statistical differences. Latin letters (A, B, C) are used to compare the wines of the same pH throughout the time. Greek letters (α, β) are used to compare control and micro-oxygenation samples of the same pH at the same time.

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Table 7. Total Phenolic Compounds, Proanthocyanidins, and Related Parameters - wine A -

parameter	pH	Treatment	Initial Wine	After 3 months of microoxygenation	8 months after bottling
TPI	3.1	Control	49.3 ± 0.2A	49.0 ± 0.1A,α	45.7 ± 0.6B,α
		MO		48.5 ± 0.9A,α	45.2 ± 0.3B,α
	3.5	Control	49.1 ± 0.2A	48.8 ± 0.2A,α	45.4 ± 0.1B,α
		MO		48.4 ± 0.8A,α	45.1 ± 0.3B,α
	3.9	Control	49.5 ± 0.1A	46.7 ± 0.8B,α	43.5 ± 0.2C,α
		MO		46.1 ± 0.3B,α	43.4 ± 1.1C,α
Proanthocyanidins (mg/L)	3.1	Control	1473 ± 21A	1423 ± 42A,α	1286 ± 27B,α
		MO		1377 ± 21B,α	1215 ± 81C,α
	3.5	Control	1475 ± 25A	1429 ± 46A,α	1352 ± 21B,α
		MO		1420 ± 41A,α	1258 ± 27B,β
	3.9	Control	1476 ± 37A	1423 ± 16A,α	1397 ± 36A,α
		MO		1483 ± 70A,α	1312 ± 26B,β
DMACH Index	3.1	Control	38.50 ± 0.35A	35.75 ± 0.43B,α	31.83 ± 0.52C,α
		MO		29.92 ± 1.38B,β	27.83 ± 1.38B,β
	3.5	Control	39.50 ± 0.10A	35.50 ± 0.66B,α	32.33 ± 0.58C,α
		MO		32.83 ± 1.81B,β	29.08 ± 0.29C,β
	3.9	Control	38.13 ± 0.88A	36.42 ± 1.13A,α	33.08 ± 0.72B,α
		MO		35.58 ± 1.61B,α	32.50 ± 0.43C,α
mDP	3.1	Control	4.65 ± 0.04A	4.58 ± 0.07A,α	4.52 ± 0.20A,α
		MO		4.45 ± 0.08A,β	4.58 ± 0.09A,α
	3.5	Control	4.72 ± 0.13A	4.58 ± 0.07A,α	4.85 ± 0.32A,α
		MO		4.67 ± 0.03A,α	4.71 ± 0.12A,α
	3.9	Control	4.61 ± 0.01A	4.80 ± 0.06B,α	5.08 ± 0.03C,α
		MO		4.80 ± 0.13B,α	5.28 ± 0.08C,β

All data are expressed as the average values of three replicates ± standard deviation (n=3). Statistical analysis: two-factor ANOVA and Scheffe's test (both, p=0.05). Different letters indicate statistical differences. Latin letters (A, B, C) are used to compare the wines of the same pH throughout the time. Greek letters (α, β) are used to compare control and micro-oxygenation samples of the same pH at the same time.

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Table 8. Total Phenolic Compounds, Proanthocyanidins, and Related Parameters - wine B -

parameter	pH	Treatment	Initial Wine	After 3 months of microoxygenation	8 months after bottling
TPI	3.1	Control	95.7 ± 1.8A	92.7 ± 0.2B,α	92.8 ± 0.7B,α
		MO		91.8 ± 0.7B,α	92.9 ± 0.8B,α
	3.5	Control	95.6 ± 2.5A	91.9 ± 0.9B,α	92.7 ± 0.5B,α
		MO		91.1 ± 1.1B,α	92.5 ± 0.6B,α
	3.9	Control	95.6 ± 0.5A	91.3 ± 1.2B,α	92.2 ± 0.5B,α
		MO		90.5 ± 1.2B,α	91.1 ± 0.6B,α
Proanthocyanidins (mg/L)	3.1	Control	2753 ± 30A	2435 ± 49B,α	2243 ± 170C,α
		MO		2560 ± 26B,β	2017 ± 152C,β
	3.5	Control	2781 ± 59A	2489 ± 43B,α	2371 ± 6C,α
		MO		2579 ± 61B,α	2327 ± 5C,β
	3.9	Control	2742 ± 17A	2512 ± 71B,α	2373 ± 44C,α
		MO		2634 ± 31B,β	2223 ± 234C,α
DMACH Index	3.1	Control	65.67 ± 1.42A	65.00 ± 1.50A,α	56.25 ± 0.71B,α
		MO		57.50 ± 0.25B,β	51.83 ± 1.18C,β
	3.5	Control	67.25 ± 2.18A	65.17 ± 1.15A,α	58.00 ± 1.12B,α
		MO		60.25 ± 1.75B,β	54.92 ± 1.27C,β
	3.9	Control	65.92 ± 1.13A	64.42 ± 1.04A,α	58.88 ± 1.24B,α
		MO		60.75 ± 1.64B,β	55.75 ± 1.30C,β
mDP	3.1	Control	4.47 ± 0.04A	4.63 ± 0.05B,α	4.91 ± 0.01C,α
		MO		4.61 ± 0.21B,α	4.74 ± 0.10B,β
	3.5	Control	4.58 ± 0.10A	4.85 ± 0.17B,α	4.91 ± 0.04B,α
		MO		4.79 ± 0.09B,α	4.75 ± 0.20B,α
	3.9	Control	4.52 ± 0.07A	4.65 ± 0.10A,α	5.24 ± 0.02B,α
		MO		4.64 ± 0.05A,α	4.90 ± 0.01B,β

All data are expressed as the average values of three replicates ± standard deviation (n=3). Statistical analysis: two-factor ANOVA and Scheffe's test (both, p=0.05). Different letters indicate statistical differences. Latin letters (A, B, C) are used to compare the wines of the same pH throughout the time. Greek letters (α, β) are used to compare control and micro-oxygenation samples of the same pH at the same time.

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Table 9. Evaluation of Ethylidene-Bridged Flavan-3-ols

Parameter	pH	Treatment	8 months after bottling (Wine A)	8 months after bottling (Wine B)
Ethyl bridges (Area x10^4)	3.1	Control	31.7 ± 2.2α	100.3 ± 3.7α
		MO	100.2 ± 29.7β	170.45 ± 18.5β
	3.5	Control	34.1 ± 2.4α	55.8 ± 2.7α
		MO	52.5 ± 4.9β	61.0 ± 7.1α
	3.9	Control	25.1 ± 0.7α	55.6 ± 7.9α
		MO	24.9 ± 2.6α	46.2 ± 2.0α

All data are expressed as the average values of three replicates ± standard deviation (n=3). Statistical analysis: one-factor ANOVA and Scheffe's test (both, p=0.05). Different letters indicate statistical differences.Greek letters (α, β) are used to compare control and micro-oxygenation samples of the same pH at the same time.

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IV. DISCUSSION AND PERSPECTIVES

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DISCUSSION AND PERSPECTIVES

The aim of this thesis was to study the phenolic maturity of the grapes, their influence on wine phenolic composition and their sensory implications, as well as the application of some techniques to compensate the lack or excess of grape maturity.

Grape phenolic maturity has a key role on wine quality. The actual market demands wines with deep red color, full body, soft tannins and fruit scents which can only be achieved if the grapes have reached a complete phenolic maturity. As it was already mentioned, very well ripened grapes proportionate wines with a high concentration of anthocyanins and consequently a deep red color (Kennedy et al. 2002, Canals et al. 2005). Additionally, full ripen grapes present low astringency and bitterness as result of the diminution of the seed proanthocyanidins proportion which are very galloylated (Kennedy et al. 2000, Kennedy et al. 2002). On the contrary, unripe grapes can lead to wines with poor color as also high levels of bitterness and astringency (Canals et al. 2005).

The importance of grapes phenolic maturity, on wine characteristics, increases the interest of winemakers to have adequate tools that permit its measurement. This gave a special importance on the first part of this thesis as there are only a few studies that correlate the predictive effectiveness of the methods for measuring phenolic maturity and the color and phenolic composition of the corresponding wines (Gonzáles-Neves et al 2004, Romero-Cascales et al. 2005, Jensen et al. 2008). Moreover, these studies have used only one the existent prediction methods. In our study we compare three of the most used methods (Glories, ITV, Cromoenos), at least in Spain.

Several micro-vinifications were carried out to achieve this object at three different levels of grape maturity with grapes of the *Vitis vinifera* cultivars Tempranillo, Grenache, Merlot and Cabernet Sauvignon. The first two cultivars are among the most cultivated in Spain whereas the other two are probable the most cultivated worldwide. There are several types of winemakings and for that reason we decided to elaborate according to standard conditions. Temperature was maintained at 25 ºC, the cap was punched down once by day to favor phenolic compounds dissolution, and the wines were racked after of 14 days of maceration.

The results of this experiment indicated that there is a quite good linear regression between the color intensity of the different wines and their corresponding predicted values for the Glories

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and Cromoenos methods. The linear regression coefficients were r^2 = 0.8489 and r^2 = 0.8869 respectively. In contrast, the regression coefficient for the ITV method was very low (r^2 = 0.3879). Moreover, a small modification of Cromoenos method improved the quality of the measure. Specifically, the linear regression coefficient (r^2 = 0.9517) increased notably in respect to the original method. These results shows that using Glories, Cromoenos or the modification of this method it is possible to predict reasonably well the wine color of the obtained wines, but not with ITV.

Additionally, Glories, ITV and specially modified Cromoenos originated satisfactory linear regression coefficients for Chroma (r^2 = 0.8474, 0.7514 and 0.9114 respectively) and Luminosity (r^2 = 0.7900, 0.8284 and 0.8803 respectively). It was not the same for the Hue, as the linear regression coefficients were very low in all the cases (r^2 = 0.3951, 0.1427 and 0.5895 respectively). Even, the obtained slope of Glories method was negative indicating an inverse tendency between the extract and the wine. This lead us to conclude that the Chroma and the Luminosity can be predicted quite well using anyone of the three methods, but the Hue prediction is not possible.

The linear regression coefficients obtained for anthocyanins concentration using the three methods provide quite good results (r^2 = 0.7897 at pH 1.0 and 0.8809 at pH 3.2. for Glories and r^2 = 0.8259 and r^2 =0.8170 for ITV and Cromoenos respectively). Moreover, Glories at pH 3.2 had a slope close to one but the other two methods had slopes quite lower than one, indicating that the two last methods provoked an overextraction of anthocyanins.

Finally, the linear regression analysis between the total phenolic index of the different wines and their corresponding predicted values obtained by applying the different phenolic maturity methods was quite good (between 0.8028 and 0.8839). However, the slopes indicate that all three methods produce an overextraction of phenolic compounds.

In our opinion, this study was necessary due to the lack of information about the prediction effectiveness of the different methods for phenolic maturity (Gonzáles-Neves et al 2004, Romero-Cascales et al. 2005, Jensen et al. 2008).

In our study, we conclude that the three studied methods provide good linear regression and correlation coefficients for anthocyanins by spectrophotometry and TPI. However, only Glories and Cromoenos offer good results for color parameters, especially the Cromoenos modified method. Glories is probable the most used method and it seems that can be a useful method. On

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the other hand, Cromoenos has good prediction effectiveness for color intensity and it is also quite rapid, that give it an advantage. At the harvest, wineries need a technique that can give results as quickly as possible, and Cromoenos can do it. However, new methods appears, like the direct measurement techniques FTIR (Fourier Transform Infrared) that permit achieve more complex analytical results (Dubernet et al., 2000). The direct measurement of grape maturity in the vineyard using multispectral airborne high resolution image analysis seems to be an interesting possibility (Lamb et al. 2004). Nevertheless, all these methods need to be studied more deeply to demonstrate their effectiveness. On the other hand, these techniques are quite expensive and a small winery cannot afford. Therefore, the methods using extracts such as Glories and Cromoenos could still have future. However, further studies are necessary to develop more efficient and quicker methods. Only this way, wineries could improve their controls for deciding the optimal harvest date and even for classifying grapes in function of their quality (or prize).

In the case of our experiment, we realized nine micro-vinifications for each cultivar. This number is quite small to study separately each variety. A greater number of micro-vinifications would permit the individual study of each one, and this is a proposal for future studies. Moreover, we decided to realize micro-vinifications because it was the only possibility for working with enough replicates to have statistically validity. As a next step it would be interesting to make vinifications in tanks of industrial scale which would permit to draw conclusions on real winemaking conditions.

The second part of this thesis is focused on the influence of heterogeneity of grape phenolic maturity on the final quality of wine. It is well known that grapes do not ripe homogeneously in the vineyard. Several parameters can affect the maturation process of clusters and berries. The main factors that can be underlined are the location of vines in the vineyard (exposure, altitude, soil composition, temperature, humidity, vine density, etc.), the position of the cluster on the vine and even the position of the berries in the cluster can cause differences in the ripening rate (Haselgrove et al. 2000, Smart et al. 1985).

Our results demonstrate that grapes do not mature at the same rate. During two consecutive years the berries of Cabernet Sauvignon had a huge density distribution throughout the maturation process. This heterogeneity continues until the harvest date. For that reason, we decided to separate the berries according to their density at three groups with the use of two solutions of sucrose of different concentrations. The berries were vinificated separately.

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As expected, the grapes of different densities originated wine with different characteristics. The higher the density of grapes the higher alcohol content and pH of wines and on the contrary, the titratable acidity showed the opposite tendency in both vintages. Moreover, wine color intensity and Cielab parameters, Chroma (C*) and red-greenness (a*), were higher in wines obtained from grapes with high density. These have an influence in total color differences (ΔEab*) among wines of the same vintage, which indicates that wines from grapes of different density can be distinguished by the human eye.

Grape density also affected the anthocyanin and proantocyanidin composition. Data shows that the total concentration of these compounds was greater in wines obtained from grapes with higher density. More specifically, about the proanthocyanidin composition, when the density of the berries increased the proportion of (+)-catechin significantly decreased and the proportion of (-)-epigallocatechin significantly increased. Meanwhile, the proportions of the other two monomers, (-)-epicatechin and (-)-epicatechin-3-O-gallate remained constant. Since epigallocatechin is only present in skin proanthocyanidins (Souquet et al., 1996) these data indicate that the contribution of skins to the wine proanthocyainidin concentration increases when the grapes are denser and therefore riper. The same tendency had the mean degree of polymerization (mDP) of proanthocyanidins. More ripen grapes had higher mDP. According to Vidal et al. (2003) astringency augments when the degree of proanthocyanidin polymerization increases. The wines made with high density grapes presented higher proanthocyanin concentration and their proanthocyanins also presented a higher mDP. Subsequently, their astringency was also greater. In the same way, anthocyans concentration tends to be higher in wines obtained from grapes with higher density. These results suggest that wines from denser grapes would have a more intense color and also would have a better capacity for aging.

Finally, the sensorial analyses present differences between the wines of the low density and the wines of medium or high density, but not between the wines of medium and high density. The tasters preferred the wines of medium and high density as there appeared to be more balanced, with more floral and fruit scents and with less bitterness.

This experiment confirms our hypothesis, that grape heterogeneity can affect wine quality. The problem is how to separate the grapes depends on the density. In small amount of grapes it is easy, but in greater amounts, maybe it has same practical inconvenience. The separation of the grapes according to their density by the use of aqueous solutions with different density maybe can be automated, but this is work of other studies related with engineering. A technique that is

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utilized widely, in small wineries that want to improve the quality of their wines, is the manual elimination of unripe and defective berries with a grape sorting table.

As was already shown, grapes with high phenolic maturity permit the production of high concentrated wines, with full body, high color appearance and smooth tannins. But nowadays, especially after the changes that cause global warming (IPCC 2007), in several winemaking zones, to achieve a high phenolic ripeness, grapes needs to reach high sugar and low acid concentrations. The final wines would have high alcoholic concentration and high pH (Jones 2005), characteristics that are not desirable (Zamora 2005).

The problem described above leads to the appearance of several solutions for diminishing the negative effects. Our proposal is described on the third part of this thesis and consists in using unripe grapes harvested in a cluster thinning to reduce the alcohol content of wines from very ripe grapes. This study was carried out with grapes of the cultivar Cabernet Sauvignon and Merlot from the vineyards of the AOC Penedes and Bobal from the AOC Requena. The Bobal from Requena was chosen as it uses to have very high sugar content when it achieves the optimum phenolic maturity. On the other hand, Cabernet Sauvignon and Merlot, were chosen as there are cultivated worldwide.

The wine obtained from unripe grapes was discolored and deodorized with charcoal and later used for substituting a proportion of the grape juice of very ripe grapes. The wines obtained with the application of the proposal method had a lower alcoholic content and also a lower pH. This data confirms that the proposed method can be useful for reducing simultaneously alcohol and pH.

Furthermore, the obtained reduced-alcohol wines had similar phenolic composition than wines obtained from the same grapes without applying this procedure. These wines had similar anthocyanin and proanthocyanidin concentration, and also similar mean degree of polymerization and momoneric composition. However, as its pH is lower, their color intensity is significant higher.

In additional, we can confirm that the wines produced from riper grapes, with or without the application of the proposal technique, had higher phenolic content and higher color intensity than wines from less ripe grapes. The results are in agreement with those described in the second part of this Thesis. Specifically, riper grapes provide wines with higher anthocyanin and proanthocyanidin concentration. Moreover, the tendency was that the proportion of (+)-catechin

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significantly decreased and the proportion of (-)-epigallocatechin significantly increased throughout maturation. In contrast, the proportions of (-)-epicatechin and (-)-epicatechin-3-O-gallate remained almost unchanged. Finally, the mDP increased during the maturity.

Although it is clear that anthocyanins concentration increases during maturity (Kennedy et al. 2002, Canals et al. 2005) and may suffer a decline just before harvest and/or during over-ripening (Mateus et al. 2002, Fournand et al. 2006), it is not clear what happen with proanthocyanidins. Some authors have described that the berry proanthocyanidins concentration decrease throughout ripening (De Freitas et al. 2000, Harbertson et al. 2002) whereas others have reported an increase (Delgado 2004, Canals et al. 2005). In the same way there are discrepancies about what happens with the mDP. Some authors affirm that decreases (Kennedy et al. 2000, Kennedy et al. 2002) while others say exactly the opposite (Downey et al. 2003). Our results (second and third parts) indicate that both, proanthocyanidin concentration and mDP, tend to increase throughout ripening.

It can be concluded that the applied method for obtaining wines with decreased pH and alcoholic content do not alter the wine characteristics, at least at phenolic level. Besides, this procedure is a cheap method that does not need expensive equipments. Nevertheless, it is necessary to explore more about this technique. For that reason, our group is now realizing a new study using the odorless and colorless low-alcohol wine but after applying a total dealcoholization. In the present experiment this wine had 5 % (v/v) of alcohol. A dealcolization will permit to use lower proportion of low-alcohol wine for obtaining the same alcohol reduction which would minimize the effect on acidity and pH. In the case of Bobal, as it was already mentioned, the needed decrease of the alcoholic degree has provoked an excessive acidification. Using this modification it is possible to minimize the negative effect that the low alcoholic wine can produce. Additionally, it is necessary to develop experiments of this technique at industrial scale to verify the viability of this procedure. In this study, micro-vinifications did not permit a better study of the sensorial impact of this technique. Finally, a comparative study with other methods for reducing alcohol content will be interesting.

The last part is related with the pH influence on the effectiveness of micro-oxygenation on wine phenolic composition and color. Brouillard (1982) demonstrated that pH has a great influence on the anthocyanin reactivity. Moreover, when the oxygen is consumed by the red wine during winemaking and aging, ethanol is transformed in ethanal according to the mechanism described by Singleton (1987). Subsequently, ethanal must capture a proton becoming into a carbocation

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which will react with flavanols to begin the process of formation of ethyl bridges. Therefore it is also logical that the pH has an influence on these mechanisms. Moreover, it must be taken into account that well-ripe grapes usually lead to high pH wines whereas unripe grapes use to produce low pH wines. All these arguments, together with that maturity influences the pH of wine, prompted us to undertake this study.

The obtained data are quite interesting. It is demonstrated that pH affect certainly the evolution of wine color and phenolic compounds when micro-oxygenation was applied. As it was expected, the micro-oxygenated wines had more color in comparison with their controls at any pH and the effect was greater when the pH was more acidic. In the same way, the anthocyanins reactivity and the formation of new anthocyanin derived-pigments were higher at lower pH. Specifically, the increase on the concentration of ethyl-linked pigments, type B vitisins, polymeric pigments and ethylidene-bridged linked flavanols originated by micro-oxygenation were higher at low pH. On the contrary, when the pH was less acidic the effectiveness of micro-oxygenation was much smaller and sometimes practically nonexistent.

Surprisingly, the mDP vary very few between controls and micro-oxygenated wines and also throughout the time. It was expected that the mDP reach greater values in the micro-oxygenated wines than in their corresponding controls but it was not. Similar results were reported by Atanasova et al. (2002), although in this work a greater increase during the time was observed.

As final observation of this work, it can be extracted that the oenologist should take in account the pH of the wine before applying a micro-oxygenation treatment. It would be interesting to develop further studies about how the pH influences the transformation induced by micro-oxygenation using different wines as also applying different oxygen doses.

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V. GENERAL CONCLUSIONS

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GENERAL CONCLUSIONS

- The three studied methods for measuring phenolic maturity, Glories, ITV and Cromoenos, can be useful for predicting some of the characteristics of the future wines. Specifically, the three methods provide very good linear regression and correlation coefficients for anthocyanins by spectrophotometry and TPI. However, only Glories and Cromoenos offer good results for color parameters.

- Cromoenos method enables results easily in just 10 min thereby reducing the cost of labor necessary. Consequently, Cromoenos method presents the best balance between its predictive ability and its simplicity of use, making it a very good analytical procedure for wineries. Moreover, a simple modification of Cromoenos method is proposed. This modification allows to considerably enhancing its performances.

- The heterogeneity of the grapes at the moment of the harvest was greater than expected. The ensemble of all the grapes has always a proportion of very well-ripe grapes, another proportion of sufficiently-ripe grapes and finally a proportion of less-ripe grapes. This fact must be taken into account because the presence of less-ripe grapes can affect the final composition and consequently the quality of the wine.

- The presence of a proportion of less-ripe grapes diminish the final ethanol content, pH, anthocyanin concentration, color intensity, total phenolic index and proanthocyanidin concentration and increase titratable acidity. Moreover, these lower density grapes contribute with less polymerized proanthocyanidins, lower proportions of (-)-epigallocatechin and higher proportions of (+)-catechin. These data suggest that lower density grapes release more seed proanthocyanidins than skin proanthocyanidins. Finally, the sensory comparison of wines leaves no doubt because the wine obtained from lower density grapes was always recognized in all the triangle trials and it was considered as the worst balanced and the bitterest.

- The color, the concentration of anthocyanin and proanthocyanidin as well as their mean degree of polymerization increased throughout ripening in the wines of the three studied cultivars. Moreover, the monomeric composition of the wine proanthocyanidins changed with

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maturity. Specifically, (+)-catechin decreased and (-)-epigallocatechin increased significantly. Since, (-)-epigallocatechin is only present in the skin prodelphinidins, this results suggest that the proportion of skin tannins of wine are greater in the riper grapes.

- The use of unripe grapes is proposed as a method for obtaining wines with lower pH and ethanol content. The method consists in the substitution of a proportion of the grape juice of well-ripe grapes by a colorless and odorless low-alcohol wine obtained from cluster thinning. This procedure is cheap, very easy to apply in wineries and does not require specific equipment.

- Applying the proposed method for decreasing ethanol and pH it is possible to obtain wines with a similar phenolic composition than the wines obtained with the same grapes without applying it. These wines had similar anthocyanin and proanthocyanidin concentration, and also similar mean degree of polymerization and momoneric composition than their controls. However, as its pH is lower, their color intensities are significant higher.

- The differences observed in micro-oxygenation wines in comparison to their controls were, in general, greater when the pH was more acidic. Specifically, the differences found in color intensity, anthocyanin concentration, PVPP Index, ethyl-linked pigments, type B vitisins and polymeric pigments between micro-oxygenated wines and their corresponding controls were higher at lower pH. On the contrary, the effects of micro-oxygenation when the pH was less acidic were much smaller and sometimes practically nonexistent.

- The micro-oxygenated wines in comparison to their controls do not show great differences in the proanthocyanidin concentrations or in their mean degree of polymerization. The pH does not appear to exert effects on these parameters. However, the analysis by EDP-phloglucinolysis shows that there are greater amounts of ethyl-bridged flavanols in the micro-oxygenated wines at pH very acidic.

- Micro-oxygenation is much more effective when the pH is very acidic and therefore pH must be taken into account as an important factor for considering in wineries when this technique is applied.

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