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Influence of Yeasts in Wine Colour

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Abstract

Colour is the first impression that the consumer receives from wine and it influences the taste. Colour gives an idea about wine quality, age, oxidation and structure, so it has an important repercussion on the consumer perception of wine. Yeasts promote the formation of stable pigments by the production and release of fermentative metabolites affecting the formation of vitisin A and B type pyranoanthocyanins. The hydrox- and ycinnamate decarboxylase activity showed by some yeast strains produces highly reactive vinylphenols stimulating the formation of vinylphenolic pyranoanthocyanins from grape anthocyanin precursors during fermentation. Some yeasts also influence the formation of polymeric pigments by unclear mechanisms that can include the production of linking molecules such as acetaldehyde. Grape anthocyanins adsorbed in yeast cell walls during fermentation are removed from wine after racking processes affecting final pigment content. Moreover, the intensive use of non-*Saccharomyces* yeasts in current oenology makes it interesting to assess the effect of new species in the improvement of wine colour.

Keywords: *Saccharomyces*, non-*Saccharomyces*, anthocyanins, pyranoanthocyanins, polymeric pigments, wines

1. Introduction

In the past, the formation and evolution of wine colour was conditioned by the anthocyanin composition of the grape variety, the degree of extraction during winemaking and the physicochemical evolution of the pigments during tank, barrel and bottle ageing. In these last processes, the influence of grape proanthocyanins, other flavonoids and oxygen affects the formation of stable polymeric pigments [1].

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Grape pigments are monomeric anthocyanins glycosylated in position 3. The colour of anthocyanins is strongly dependent on pH, SO_2 levels and hydration. According to these factors, several anthocyanin derivatives can be found (**Figure 1**). Moreover, the solubility of anthocyanins is affected by the polarity of the medium. During fermentation, alcohol production reduces must polarity and the concentration of anthocyanins decreases.
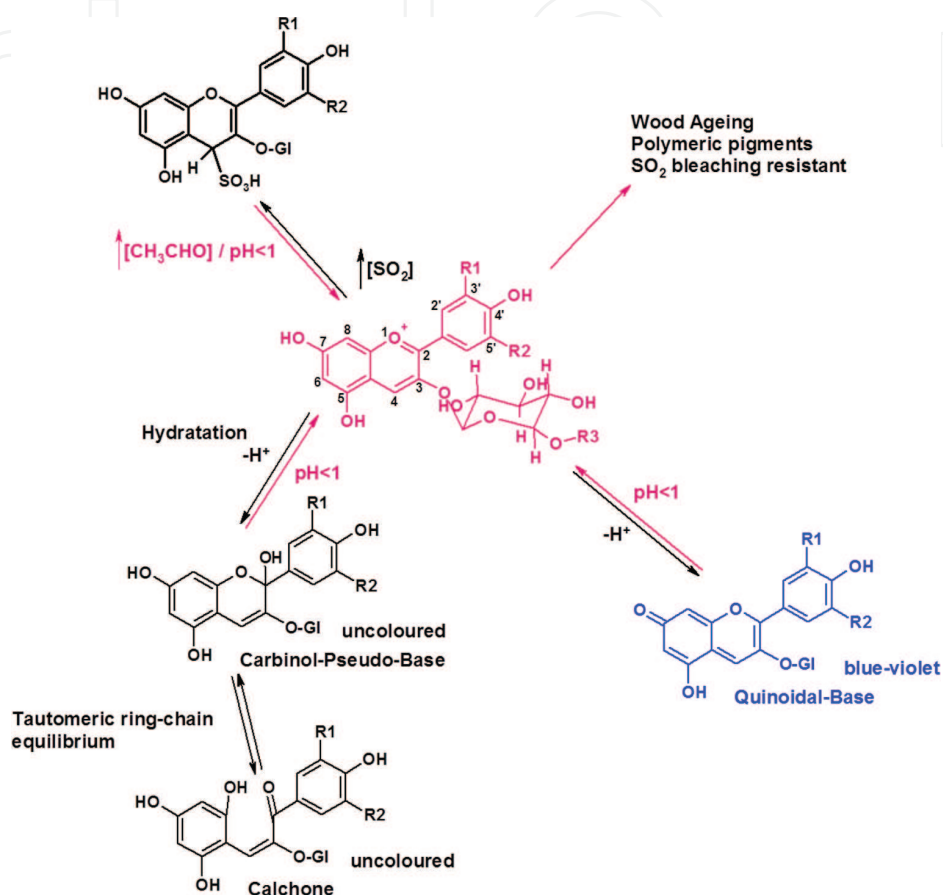

Figure 1. Reactions that can affect anthocyanins during winemaking and the effects on their structure.

Since 1990s the role of yeast in colour stability has been studied deeply. In fact, anthocyanin insolubilisation during fermentation is a consequence of yeast metabolism. However, that is not the only contribution of yeasts to wine colour, the production of derived pigments during fermentation using yeast metabolites as precursors or by means of yeast enzymatic activities are also major concerns that have been analysed in detail in the last decades [2, 3].

Pigment adsorption by yeast cell walls reduces the concentration of anthocyanins affecting wine colour, especially in low colour varieties. This property has been used traditionally to reduce pigment contents in the production of white sparkling wines from red varieties (*blanc de noirs*).

The formation of polymeric pigments has been considered as a way of colour stabilization during ageing. Grape anthocyanins condense with other flavonoids forming polymeric structures (n × [C6-C3-C6]). Sometimes, flavonoids can be linked by some intermediate

molecules like acetaldehyde. The polymerization reactions occur during ageing and are affected by precursor levels, oxygen levels and pH. Currently, some evidences point up that yeast can also promote the formation of polymeric pigments during fermentation.

The strong effect of pH in anthocyanins colour intensity can cause slight modifications in wine colour by some yeast species able to either degrade or produce organic acids during fermentation.

Some of these processes with effect on colour can also be enhanced by the use of non-*Saccharomyces* yeasts during fermentation.

2. Formation of pyranoanthocyanins with yeast metabolites

Some yeast metabolites can react with grape anthocyanins during fermentation forming derived pigments with enhanced stability in oenological conditions and slightly different chromatic features. The formation of vitisin A, a pyranoanthocyanin pigment formed by chemical reaction between malvidin-3-*O*-glucoside (malvidin or m3g), a major grape anthocyanin, and pyruvic acid released by yeast, is produced during fermentation. It can be also produced during ageing. In a similar way, vitisin B is formed during fermentation by the reaction between acetaldehyde and m3g (**Figure 2**).
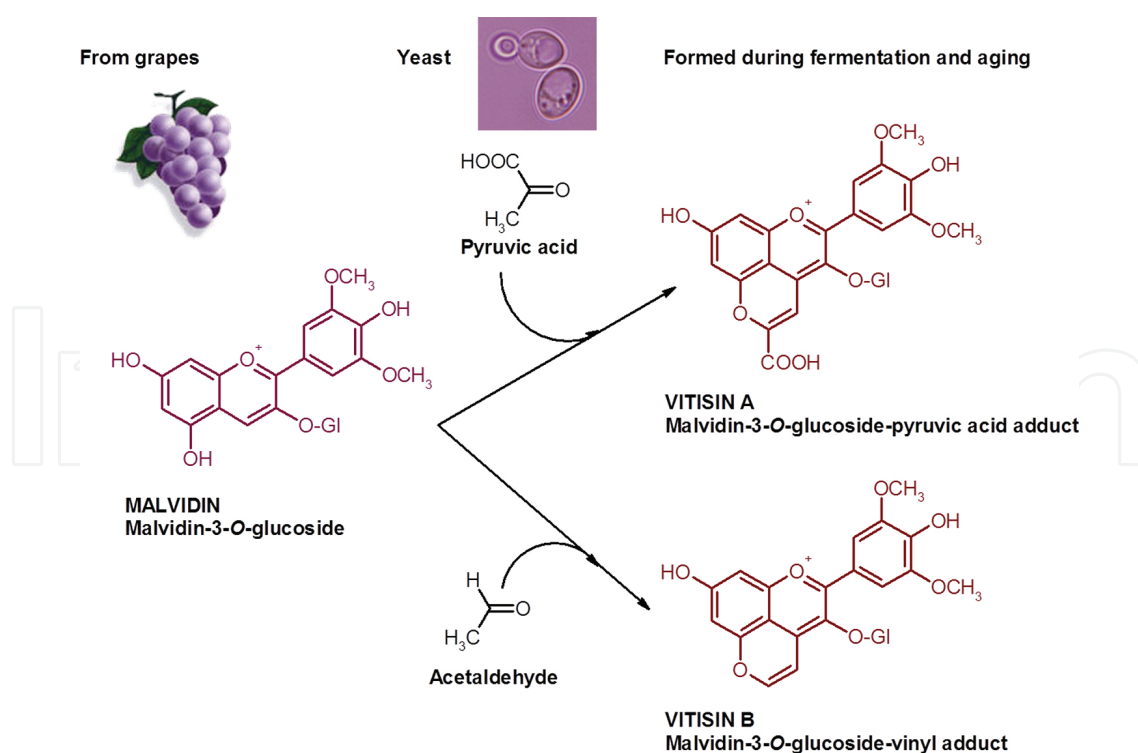

Figure 2. Formation of vitisins A and B by chemical reaction between malvidin and pyruvic acid or acetaldehyde, respectively.

Vitisins A and B are stable pyranoanthocyanin in oenological conditions with stable colour intensity under variable pH [4]. They are also more resistant to oxidative damage probably because of the higher number of resonant forms they have due to the double pyranose ring structure. Moreover, they are not sensitive to sulphur dioxide bleaching because position C4 is fully saturated being unable to react with the bisulphite ion (**Figure 3**).
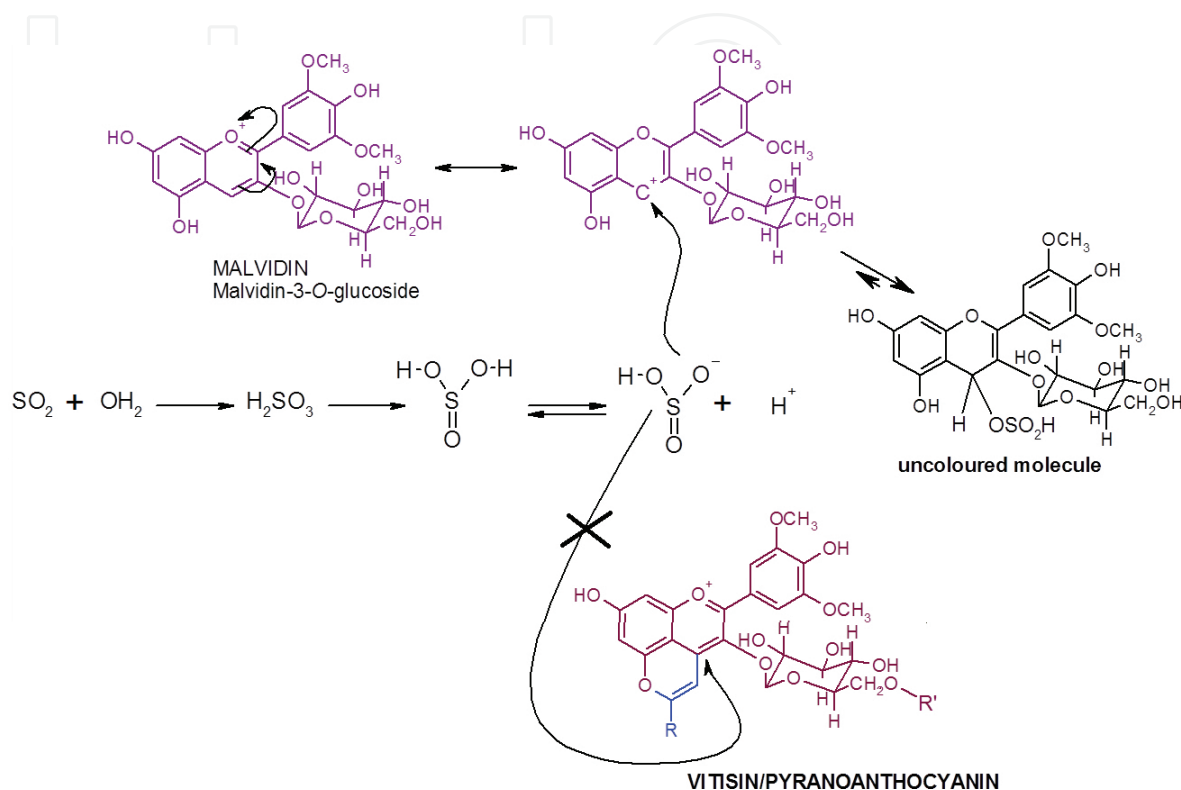

Figure 3. Dis-colouration reaction in anthocyanins by bisulphite attack. This reaction is not possible in pyranoanthocyanins because C4 is saturated.

Chromatic properties of vitisins are slightly different from grape anthocyanins. The maximum of absorption in visible spectra for malvidin is approximately 528 nm, but vitisin A shows a maximum of 515 nm and vitisin B of 495 nm (**Figure 4**). That means that vitisins are red-orange pigments and consumers currently prefer red-bluish colours in wines. However, the normal evolution in wine colour during ageing is from purple to red-orange, and, in this situation, colour of vitisins can be better integrated in wine appearance, indeed, they can even improve it. The stability of vitisins that makes them more persistent during ageing must be also considered.

Vitisins are usually analysed by HPLC-DAD separation and quantitation, using an external standard of malvidin-3-*O*-glucoside. Traditional LC separations are done in C18 reverse phase columns and the elution is produced with a gradient of water and methanol or water and acetonitrile. To keep anthocyanins in the cationic form (pyrilium form), the use of formic acid as the pH regulator is very convenient. Also, it is important to ensure a good ionization in electrospray when ESI-MS analysers are used. In the past, 30 cm columns with 5 μm particles were used, thus obtaining good separation times near or higher than one hour. The newer low

size particle columns (1.8 or 2.6 μm) can be used to perform the wine anthocyanins separations in a few minutes. In some chromatographic conditions, vitisins can co-elute with acetylated delphinidin. Co-elution of vitisins A and B is also frequent. The separation of these compounds can be assured by managing both solvent gradient and formic acid concentration. Anthocyanins and vitisins are identified by UV-visible spectra. The quantification is done by interpolation in a calibration curve of an external standard of malvidin-3-O-glucoside.
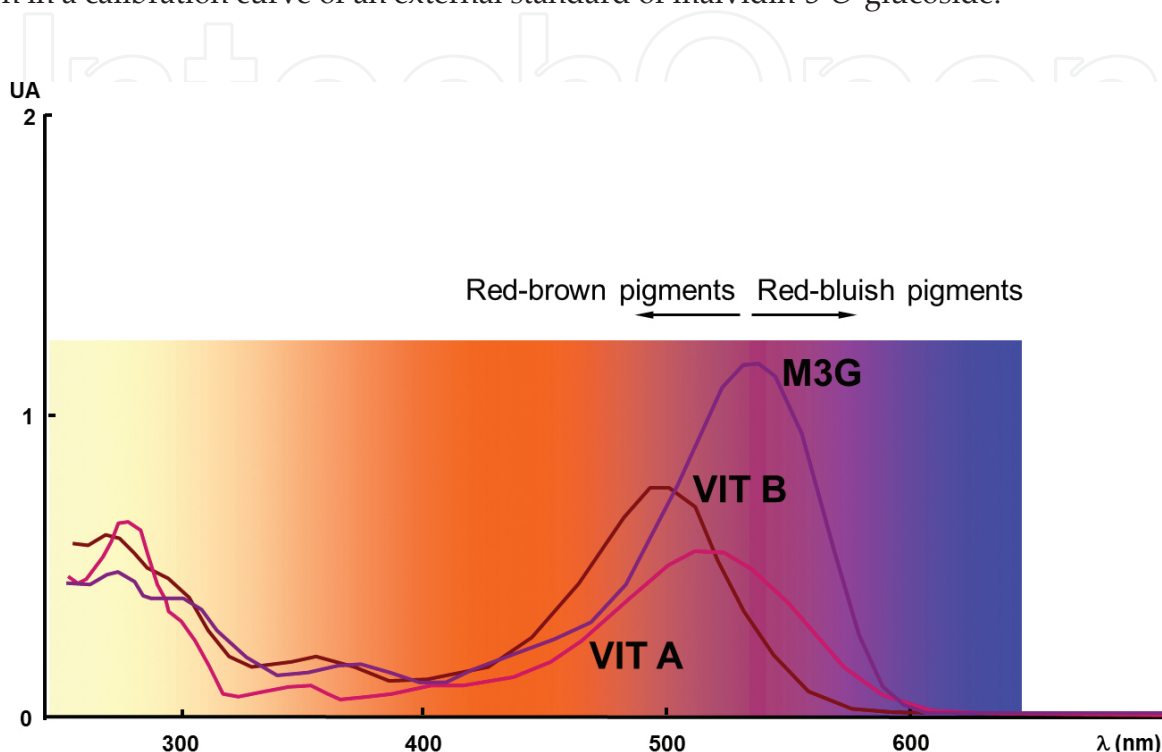

Figure 4. UV-visible spectra of malvidin, and vitisins A and B.

Vitisins A and B can be also identified by mass spectrometry (MS) by the specific fragmentation patterns; in fact, MS facilitates the full identification of these pigments after LC separation. The m/z for molecular ion (M^+) of vitisin A is 561 and the aglycon fragment is 399 mass units. The fragment is also common for acetyl and coumaroyl vitisins A. M^+ of Vitisin B is 517 and the corresponding aglycon fragment is 355. This last mass moiety also appears in MS spectra of acetyl and coumaroyl vitisins B.

Concentration of vitisins in wines could range from traces to a few mg/l. The amount formed during fermentation can be improved by using selected *Saccharomyces cerevisiae* strains with suitable production of pyruvic acid and acetaldehyde [5]. We observed that the release of pyruvate and acetaldehyde by yeasts during fermentation can be correlated with the formation of vitisin A and B, respectively ($R^2 > 0.8$) [5]. Oenological parameters such as pH, temperature or SO_2 concentration also affect the formation of vitisins during fermentation [6].

The production of pyruvate and acetaldehyde behaves differently in *Saccharomyces*. Usually, the maximum concentration of pyruvic acid is reached on the fourth/fifth day of fermentation and, then, this value decreases to reach a stable concentration towards the end of fermentation. Since pyruvate is an intermediate compound in many metabolic routes, a possible explanation

to this behaviour is that at the beginning of fermentation, when enough nutrients are available, the yeast produces and releases it in big amounts. However, at the end of alcoholic fermentation, when nutrients become scarce, the yeast may take the pyruvate previously released into the fermentation medium for use in metabolic processes. The utility of acetaldehyde is less and is basically released progressively from the beginning to the end of fermentation. The patterns of excretion of pyruvate and acetaldehyde influence the formation of the respective vitisins A and B. Maximum concentrations of vitisin A are found after the maximum production of pyruvate in the middle of fermentation, and the higher concentration of vitisin B is produced at the end of fermentation [5, 6].

Selected yeast strains of *S. cerevisiae* with higher production of pyruvate and acetaldehyde increase the formation of vitisins A and B because of the higher concentration of precursors (**Figure 5**). The use of specific strains with high release rates of precursors, along with an optimization of the oenological conditions (pH, SO_2 and temperature) to favour the chemical reactions of condensation between malvidin and either pyruvate or acetaldehyde, is a way to modulate the vitisin formation during fermentation.
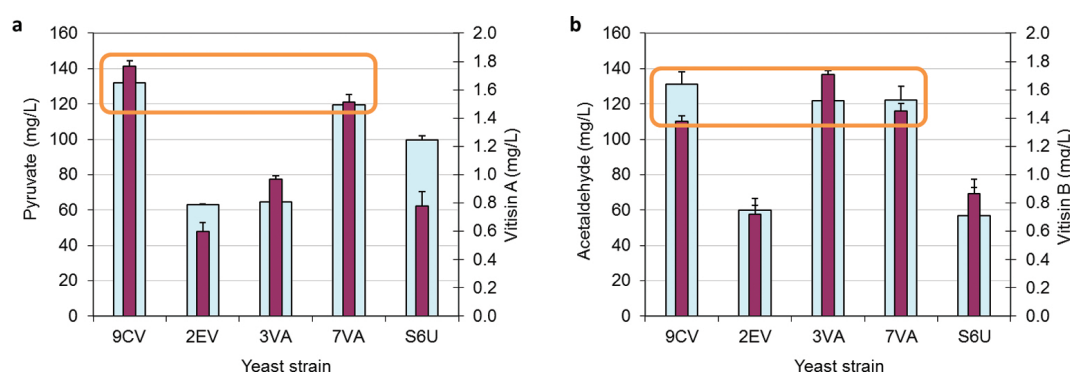

Figure 5. Production of pyruvate (a) and acetaldehyde (b) by several strains of *S. cerevisiae* and the corresponding formation of vitisins A and B respectively. In blue columns precursor (pyruvate or acetaldehyde) in red ones (vitisin A or B).

Recently, we have observed that some non-*Saccharomyces* are able to develop some specific metabolic processes affecting the global excretion of pyruvate. *Schizosaccharomyces pombe* is a fission yeast with asexual reproduction by bipartition instead of budding, and also able to develop singular catabolic pathways like maloalcoholic fermentation. *S. pombe* yeast is able to ferment sugar concentrations representing more than 13% (v/v) of potential ethanol, so it is useful to perform complete wine fermentations and it can also be used in mixed or sequential fermentations. The slower speed of fermentation and the production of excessive amounts of acetic acid, when *S. cerevisiae* is used as the sole fermentative yeast, could present some drawbacks. We have observed a release of pyruvate during the fermentation with *S. pombe* in average higher than with *S. cerevisiae* strains, probably due to its metabolic peculiarities. Thereby, when used to ferment red grapes, the formation of vitisin A and its derivatives is greater than with *S. cerevisiae* [7]. In some cases, the levels can overcome 10 mg/L. Fermentation

with *S. pombe* can be used to enhance the formation of vitisin A-type pigments (**Figure 6**) using either pure or sequential cultures with *S. cerevisiae*.
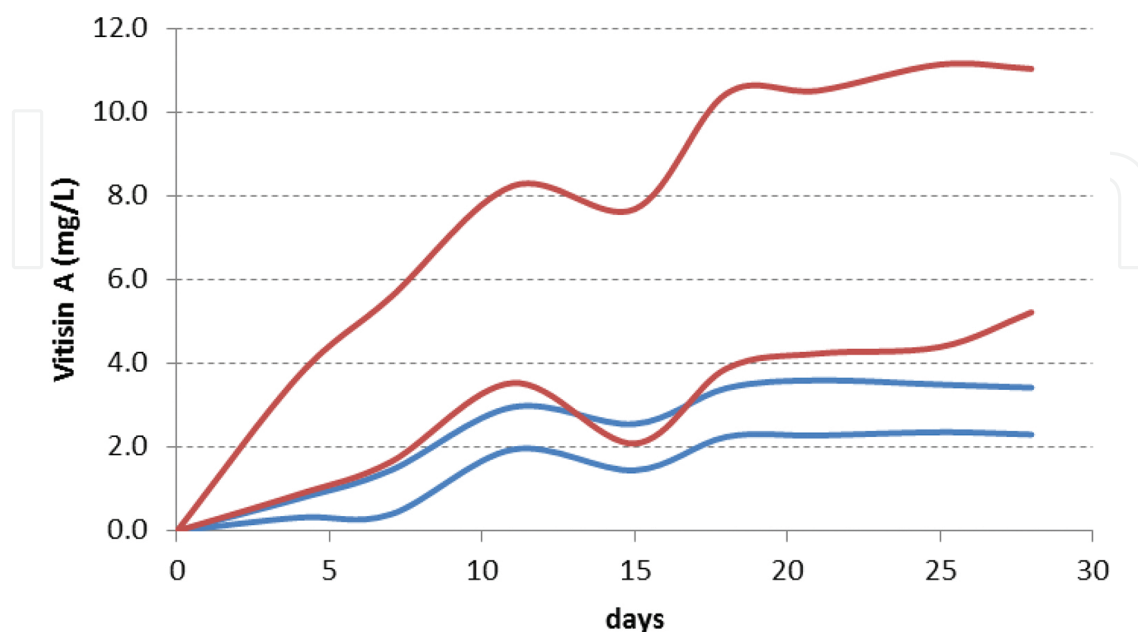

Figure 6. Formation of vitisin A during the fermentation with *S. pombe*. Red lines minimum and maximum values formed during fermentation by *S. pombe* (four strains). Blue line minimum and maximum values formed by *S. cerevisiae* (two strains).

Vitisins are formed by chemical condensation of malvidin with pyruvate and acetaldehyde so the addition of these precursors in wines enhances their formation. The addition of pyruvate is especially effective [8]. However, pyruvate and acetaldehyde are not allowed as oenological additives. Moreover, the use of acetaldehyde has many other effects on reactions between phenolic compounds promoting the condensation between tannins and the precipitation of phenols and pigments.

3. Hydroxycinnamate decarboxylase activity influence on the formation of vinylphenolic pyranoanthocyanins

Vinylphenolic pyranoanthocyanins (VPAs) are also stable pigments with similar properties to vitisins. They were discovered initially in pinotage (*Vitis vinifera* L.) wines and the derived pigment from malvidin and caffeic acid was called pinotin A (malvidin-3-*O*-glucoside-4-vinylcatechol) [9]. A mechanism of formation by chemical reaction between caffeic acid and malvidin with an uncoloured intermediate that recovers colour by slow oxidation under the typical conditions of wine barrel ageing was proposed (**Figure 7**) [10]. Due to their slow formation, these pigments were suggested as age markers in barrel ageing.

Later, the formation of these pigments derived from malvidin and hydroxycinnamic acids (HCAs) (either caffeic, ferulic or *p*-coumaric) was observed during fermentation when yeasts

with hydroxycinnamate decarboxylase (HCDC) activity were used [6, 11]. HCDC+ strains are able to decarboxylate HCAs in must during fermentation producing highly reactive vinyl-phenols (VPh) that spontaneously condense with malvidin and other grape anthocyanins forming VPAs (**Figure 8**) [11]. These compounds can be separated and analysed by LC-ESI/MS in wines. In typical LC conditions to separate anthocyanins, VPAs appear at the end of the chromatogram because of their high apolar structures with five aromatic rings. UV and MS key parameters of VPAs are detailed in **Table 1**.
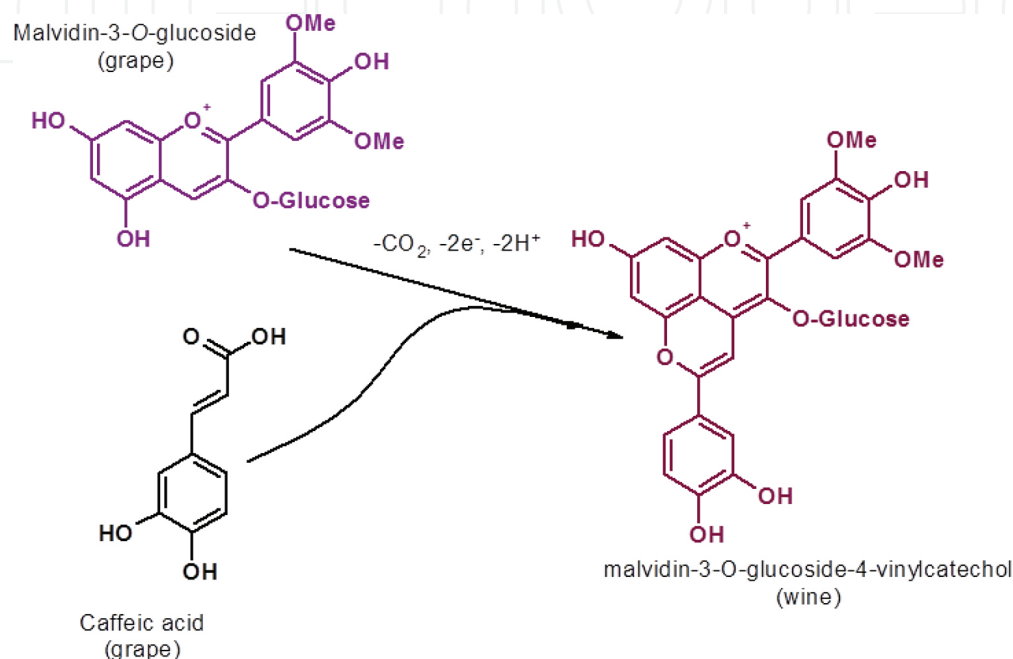

Figure 7. Formation of pinotin A from both malvidin and caffeic acid during wine ageing by a physicochemical process.

The use of HCDC+ strains of *Saccharomyces* and non-*Saccharomyces* during fermentation is a way to promote the formation of stable VPAs during fermentation. Some non-*Saccharomyces* strains (*P. guillermondii, S. pombe*) have been described as HCDC+ and they can favour the formation of VPAs at a higher concentration than *S. cerevisiae* [7, 12]. When low fermentative non-*Saccharomyces* are used to ferment must, they should be used in mixed or sequential cultures with *Saccharomyces* to ensure complete sugar depletion [13].

The selection of HCDC+ and determination of the intensity of enzymatic activity can be tested by using a fermentative medium with hydroxycinnamic acids. The use of *p*-coumaric acid is a good option. The medium can be prepared using grape must from fresh grape or a dilution of concentrated must with 220 g/l of sugars, the pH can be adjusted at 3.5 and a suitable concentration of *p*-coumaric acid is 50 mg/l. The evolution in the degradation of *p*-coumaric acid to 4-vinylphenol can be checked by LC-DAD [14]. The yeast strain can be considered HCDC+ when more than 10% of *p*-coumaric acid is transformed into 4-vinylphenol. The greater the degradation of *p*-coumaric acid and, subsequently, the formation of 4-vinylphenol, the higher the HCDC+ activity of the yeast. It is convenient to use HCDC negative and positive

controls during the experiment. The test can be also performed with other grape HCAs such as either caffeic or ferulic.
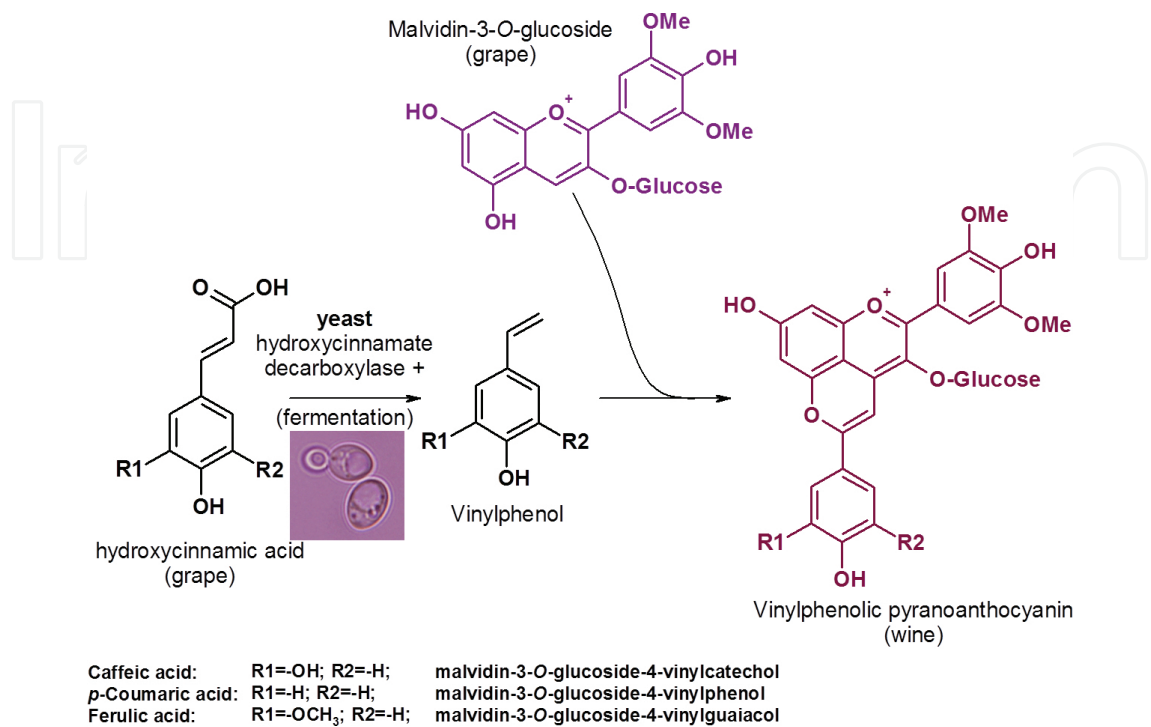

Caffeic acid:	R1=-OH; R2=-H;	malvidin-3-O-glucoside-4-vinylcatechol
p-Coumaric acid:	R1=-H; R2=-H;	malvidin-3-O-glucoside-4-vinylphenol
Ferulic acid:	R1=-OCH₃; R2=-H;	malvidin-3-O-glucoside-4-vinylguaiacol

Figure 8. Enzymatic decarboxylation of grape hydroxycinnamic acids by HCDC+ yeast strains and subsequent chemical condensation with malvidin.

Compound	Molecular ion [M]$^+$ (*m/z*)	Aglycone fragment (*m/z*)
Malvidin-3-*O*-glucoside-4-vinylcatechol	625	463
Malvidin-3-*O*-glucoside-4-vinylguaiacol	639	477
Petunidin-3-*O*-glucoside-4-vinylphenol	595	433
Malvidin-3-*O*-glucoside-4-vinylcatechol	625	463
Malvidin-3-*O*-glucoside-4-vinylphenol	609	447
Malvidin-3-*O*-(6-acetyl)-glucoside-4-vinylphenol	651	447
Malvidin-3-*O*-(6-p-coumaroyl)-glucoside-4-vinylphenol	755	447
Malvidin-3-*O*-glucoside-4-vinylcatechol	625	463
Malvidin-3-*O*-glucoside-4-vinylphenol	609	447
Malvidin-3-*O*-glucoside-4-vinylguaiacol	639	477
Malvidin-3-*O*-(6-acetyl)-glucoside-4-vinylguaiacol	681	477
Malvidin-3-*O*-(6-p-coumaroyl)-glucoside-4-vinylguaiacol	785	477

Table 1. Vinylphenolic pyranoanthocyanin pigments identified by HPLC/ESI-MS in musts containing extra hydroxycinnamic acids fermented by selected yeast strains.

HCAs are precursors of ethyl phenols (EPs) in wines, controversial off-smells that highly degrade the wine quality. During barrel ageing, some spoilage yeasts are able to transform

grape HCAs into ethyl phenols by means of two enzymatic steps. First, an HCDC activity transform HCAs into VPhs and later a vinylphenol reductase enzyme induce the reduction of VPhs in EPs. Sensory threshold of EPs is very low, about 400 ppb of 4-ethylphenol can be perceptible in wines, although it also depends on wine polyphenolic structure. Higher concentrations can strongly depreciate the wine quality. Formation of VPAs by yeasts during fermentation is a natural way to block VPhs and, consequently, to reduce EP precursors of wines (**Figure 9**).
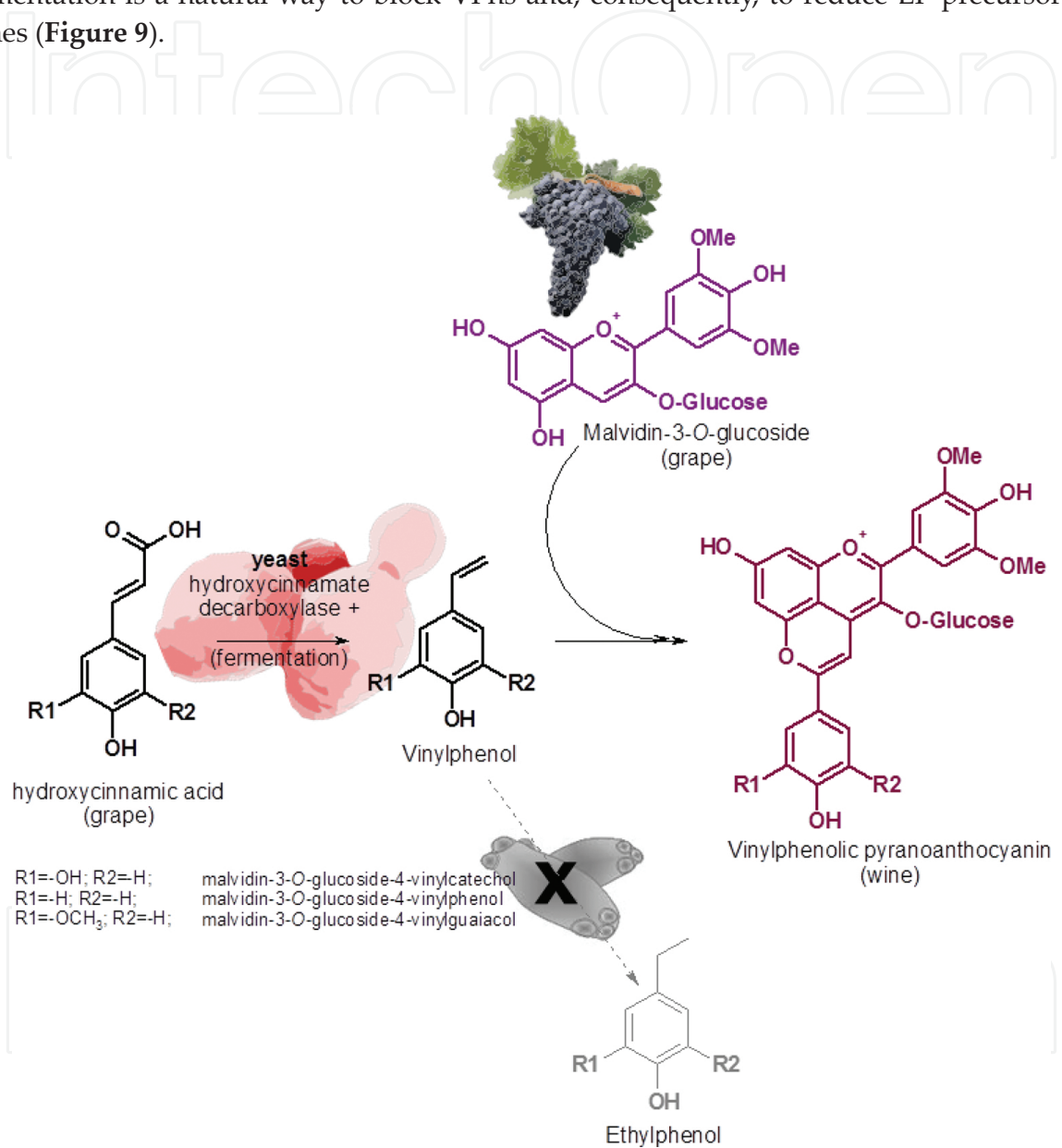

Figure 9. Formation of VPAs by a mix mechanism of enzymatic decarboxylation of HCAs by yeast and chemical condensation with wine anthocyanins.

A reduction in the amount of HCAs, correlated with the amount of VPAs that were formed, has been observed when the effect of ferment with HCDC+ *S. cerevisiae* yeast has been studied. After massive contamination with *Dekkera bruxellensis* (10^6 cfu/ml), the fermentations performed with HCDC+ *Saccharomyces* finished with 470 ppb of 4-ethylphenol and quite closed

to the sensory threshold. However, the fermentations by HCDC- *Saccharomyces* reached 1100 ppb meaning near three folds the sensory threshold [15].

Other problem in the formation of ethylphenols in wines is that the levels of tartaric esters of HCAs (TE-HCAs) are frequently higher than free HCAs. These esters can release free HCAs during storage and barrel ageing increasing the amount of ethylphenol precursors. The use of cinnamyl esterases enzymes (CEs) during maceration is a way to release free HCAs. If, at the same time, fermentation is done with HCDC+ yeasts, the HCAs can be used to form VPAs reducing the precursors of EPs. This is a natural enzymatic-biologic-chemical way to decrease the precursors of ethylphenols protecting wines against ulterior contaminations by *Brettanomyces/Dekkera* yeasts (**Figure 10**) [14].
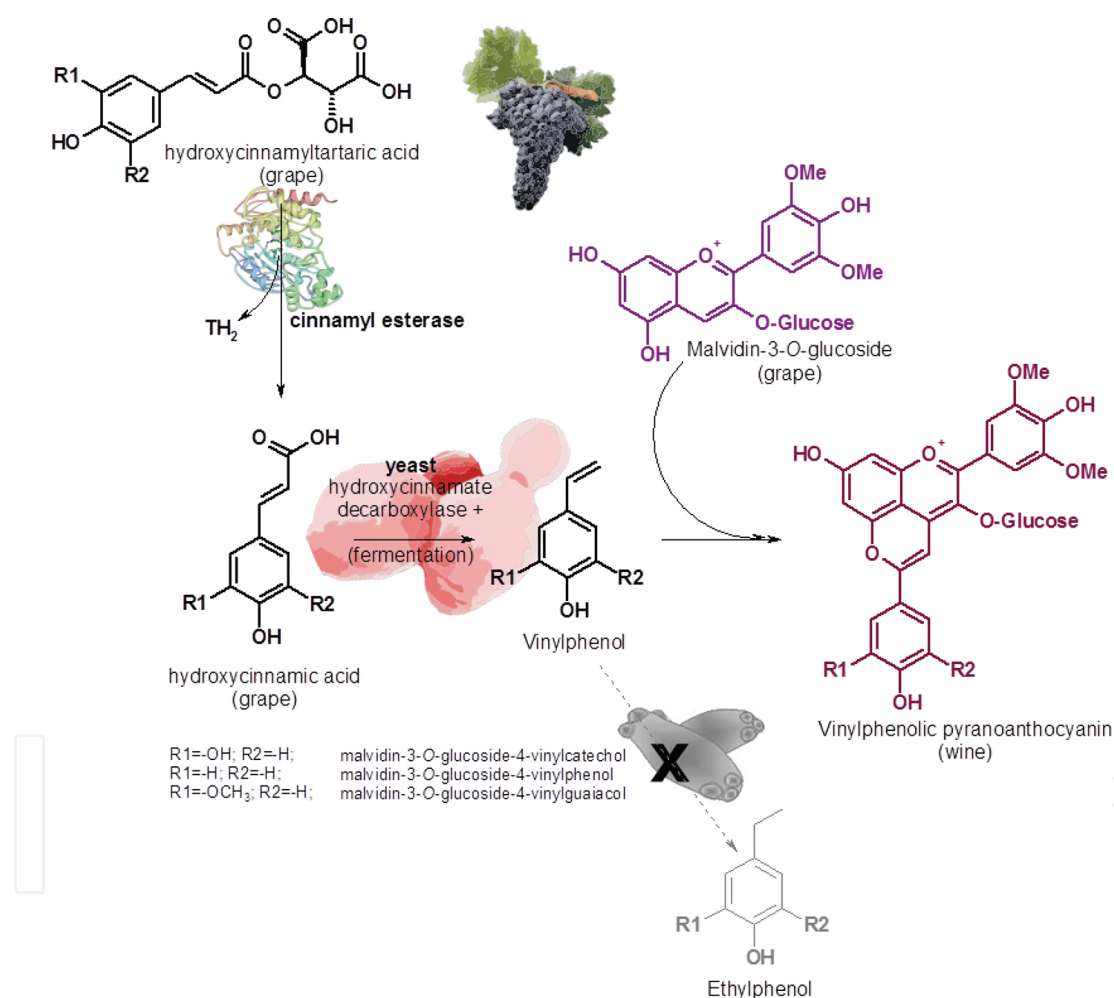

Figure 10. Mobilization of TE-HCAs to form stable VPAs decreasing the amount of 4-ethylphenol precursors during fermentation.

The simultaneous use of CEs and HCDC+ yeasts promotes the formation of stable pigments decreasing at the same time the amount of EPs precursors. We can observe the effect on scale fermentations when HCDC+ (*S. cerevisiae* strains 7VA and TP2A16) and HCDC- (*S. uvarum* strain S6U) yeasts are used in fermentations with or without CEs (**Figure 11**).
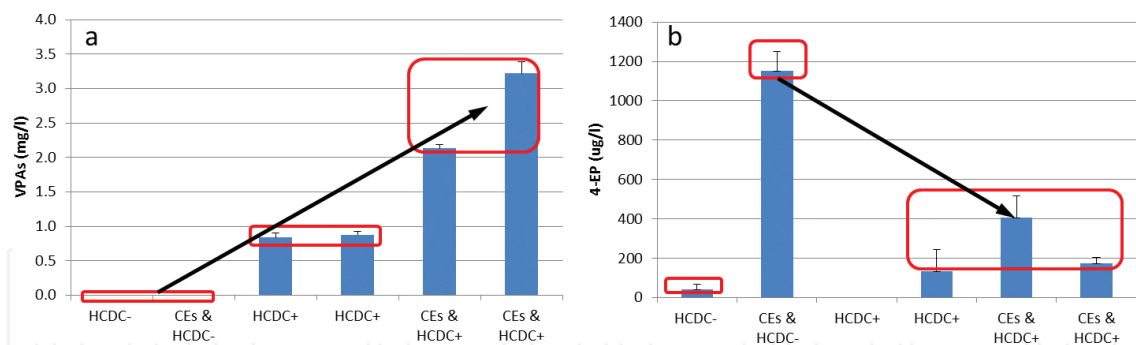
Figure 11. (a) Formation of stable pigments from grape anthocyanins (VPAs) during fermentation with and without CEs. HCDC+ yeast with hydroxycinnamate decarboxylase activity. Two *S. cerevisiae* yeasts were used 7VA and TP2A16. HCDC- yeast without hydroxycinnamate decarboxylase activity. (b) Formation of 4-EP after *Dekkera* contamination (10^6 cfu/ml) in wines from musts treated with and without CEs and fermented by yeasts HCDC- and HCDC+.

4. Formation of polymeric pigments

It is known that the wine colour evolves from red-bluish to red-orange during the ageing and this phenomenon is affected by oxygen levels and temperature (**Figure 12**). During barrel ageing, microoxygenation through the porous surface of wood promotes the browning of the wine pigments, and, at the same time, helps to modulate the aromatic profile and causes tannins smoothing. It is also known that long reductive ageing, as happens in vintage Porto wines, helps to keep red-bluish pigments and to preserve initial colour. During ageing, the colour of wine, initially due to grape anthocyanins, is being substituted by polymeric pigments; these pigments could be responsible of 50% of the colour density after the first year [1].
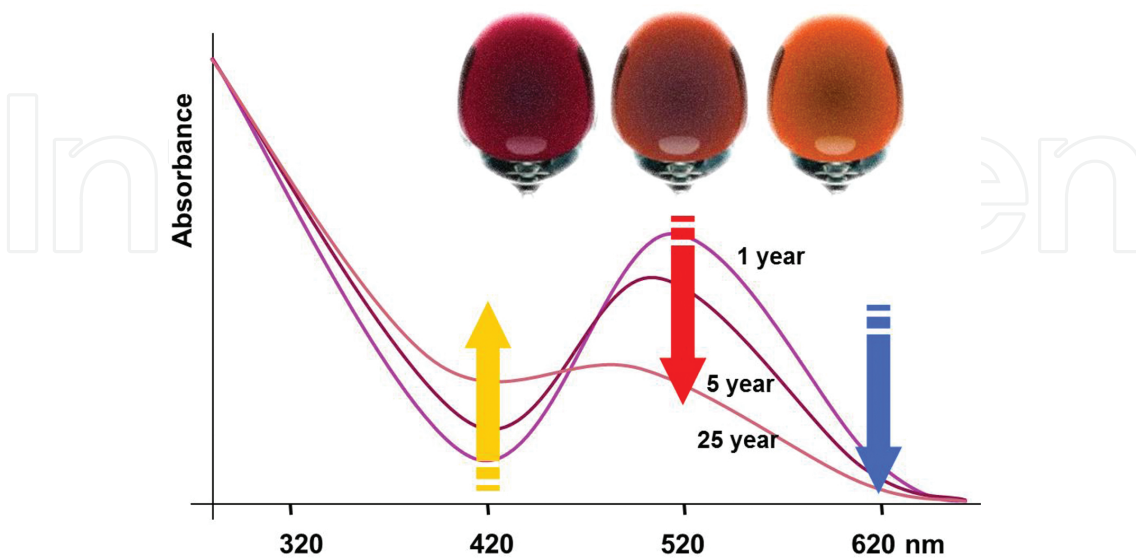

Figure 12. Typical wine colour evolution during barrel ageing.

Polymeric pigments are formed by more than one flavonoid unit, which means compounds with structure $n \times$ [C6-C3-C6]. In this polymer, the colour is due to the anthocyanin moiety but the non-anthocyanin fraction is affecting the colour tonality. Several structures have been proposed for oligomeric pigments found in wines in which anthocyanin can be either the beginning or the end of the chain with structures that can reach up to six flavonoid monomers, the non-pigment moiety is (epi) catechin [16]. Also oligomers formed by 2 or 3 anthocyanin units can be found in grapes [17].

Polymeric pigments show red-orange colours and higher stability against both oxidative damage and SO_2 bleaching, so they are really important in the colour of aged red wines. The analysis of these pigments can be done by LC-MS, capillary electrophoresis and gel electrophoresis. When wine anthocyanins are separated by mass/charge ratio in gel electrophoresis, the monomeric grape anthocyanins run faster being easily separated in the gel front and polymeric pigments are delayed at the end, because all wine anthocyanins have a positive charge in the pyrilium ring, but the mass increase strongly in the oligomers depending on the number of flavonoid units. A red-bluish or red colour in anthocyanin monomers and a red-orange colour in oligomeric pigments can be observed (**Figure 13**).
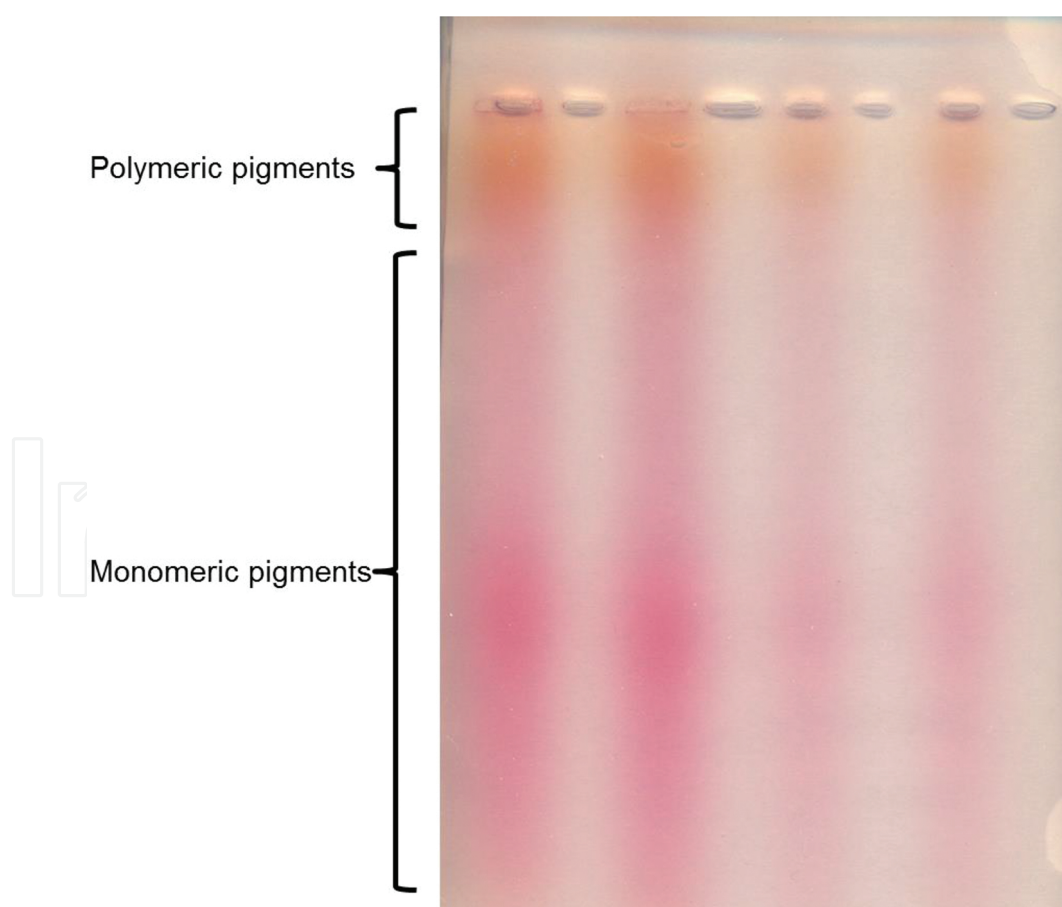

Figure 13. Gel electrophoresis of wine anthocyanins.

Capillary electrophoresis (CE) has also been used to separate polymeric anthocyanins. This technique is quite similar to gel electrophoresis, however, it improves the resolution that is possible to get in traditional gel technique. Although LC separations are usually preferred for separation of monomeric anthocyanins, its performance is worst to identify and separate polymeric pigments. However, CE is good for charged compounds such as anthocyanins that can be easily separated according to the charge/mass ratio.

Formation of polymeric pigments has been traditionally considered as a natural chemical process produced during ageing and promoted in acidic media and under oxidative conditions of barrel ageing. However, recently, the role of yeast in the formation of polymeric pigments during must fermentation has been considered. Moreover, some polymeric pigments can be formed faster because of the connection between anthocyanin and catechins or procyanidins by acetaldehyde bridges. When musts supplemented with catechin and procyanidin B2 were fermented by several selected *Saccharomyces* yeast strains, it was possible to find four dimeric pigments derived from catechin (CA1, CA2, CA3, C4) and also 1 oligomeric compound derived from Procyanidin B2 [18]. Probably, the amount and rate of acetaldehyde produced by the yeasts can affect the formation of these compounds during fermentation. These pigments show a shoulder at 460 nm characteristic of the ethyl linkage in indirect anthocyanin-flavanol condensations [19]. Pigments CA1 and CA2 were identified by their molecular ion *m/z* 809, so both are enantiomers of a dimer formed by malvidin-3-*O*-glucoside and catechin linked by an acetaldehyde bridge [20]. In the same way, CA3 and CA4 were enantiomers of petunidin-3-*O*-glucoside and catechin linked by acetaldehyde [21], and their molecular ion was *m/z* 795. Oligomeric pigment P1 was formed in fermentations that were added with procyanidin B2, its molecular ion had an *m/z* ratio of 1097 corresponding to the malvidin-3-*O*-glucoside-acetaldehyde-procyanidin B2 adduct [22]. The concentration of catechin dimers was higher than procyanidin B2 oligomers, ranging between 0.4 and 1.6 mg/l depending on the yeast strain used to ferment the must [18]. The use of specific strains of *S. cerevisiae* could be an interesting tool to promote the formation of polymeric pigment during fermentation to improve colour stability.

We are also studying the role of non-*Saccharomyces* in the formation of polymeric pigments during fermentation and, so far, it is possible to observe that some species can promote the formation of polymers better than *S. cerevisiae* [23]. Maybe, in the future, the use of mixed or sequential fermentations involving non-*Saccharomyces* yeasts and also the use of polymers precursors could be a way to increase stable pigments in wines.

5. Pigment adsorption in yeast cell walls

During fermentation, yeasts are able to adsorb the molecules in external cell wall surface. The adsorption of anthocyanins [24, 25], phenols [26, 27], aromatic compounds [28] and toxic molecules [29, 30], have been previously reported. In the exponential fermentation, yeast population range 10^8–10^9 cfu/ml, and considering the typical elliptic geometry and size of *S. cerevisiae*, it provides a specific surface of around 10 m^2/l of must [24]. So, yeast cells are able to adsorb big amounts of pigments during must fermentation.

Moreover, cell adsorption is a strain-dependant phenomena being possible to select yeasts with lower anthocyanin adsorption than others [24]. The adsorption of anthocyanins in cell walls is not yet well understood, but probably depends on cell wall surface structure and composition. It has been observed that the amount of each anthocyanin type molecule adsorbed on cell walls is affected by the polarity of the anthocyanin. Polarity of grape anthocyanins is affected by B ring substitution pattern (methoxylation makes anthocyanin more apolar, hydroxylation makes it more polar), and the type of acylation: none, acetylation, coumaroylation or caffeoylation in decreasing order of polarity. It has been observed that apolar anthocyanins can be more strongly adsorbed than polar ones.
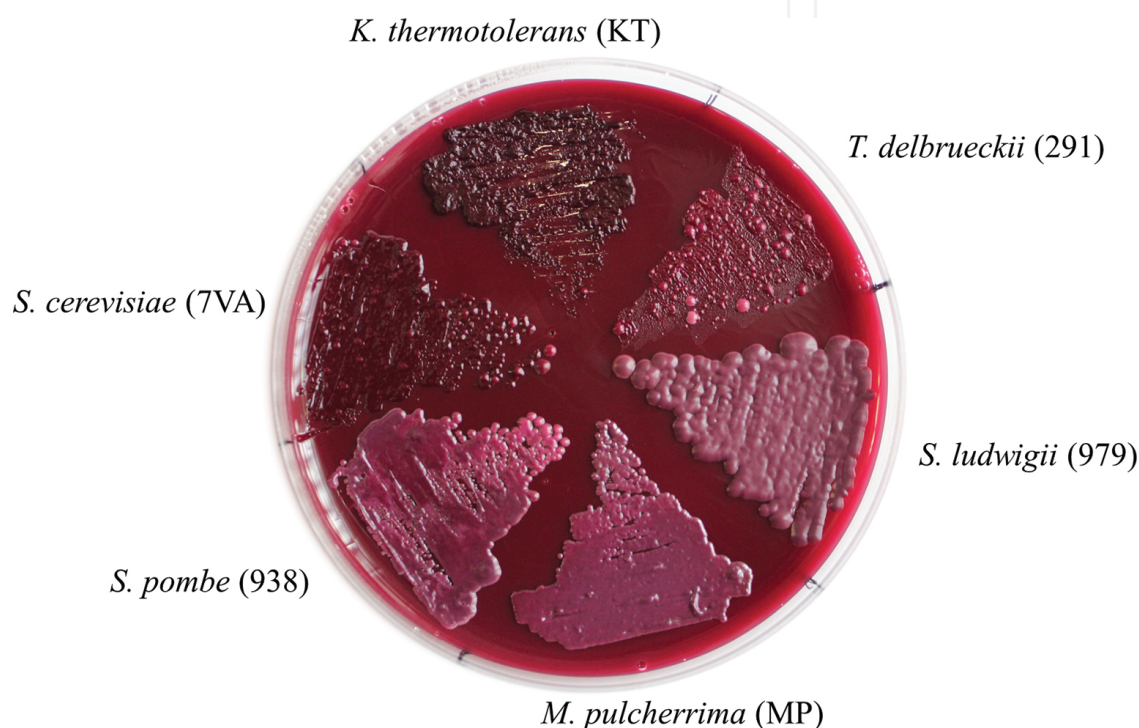

Figure 14. Several yeast species growing in YEPD-agar medium supplemented with anthocyanins.

The selection of yeasts with low anthocyanin adsorption helps to keep more anthocyanins in solution, what means wines with higher amount of anthocyanins. Of course, this will be especially interesting for those grape varieties in which production of anthocyanins is low (Pinot noir, Grenache) or in regions where the synthesis of anthocyanins is inhibited by unsuitable climatic conditions. Although global adsorption in *S. cerevisiae* strains have been evaluated in the range 1.6–5.8% of total anthocyanins (average 3.5%) [25], the range for coumaroylated anthocyanins, that are more strongly adsorbed, is 8–28%, moreover, these pigments show the red-blue colours to improving the wine tonality.

To evaluate the ability to adsorb anthocyanins by yeast, two kinds of procedures have been used. The first one, that is fast and easy to apply, is the plating in agar medium enriched in grape anthocyanins. The medium is a YEPD-agar but supplemented with a high concentration of anthocyanins extracted from grape skins [18, 31]. When yeasts colonies grow and develop

in plate surface adsorb anthocyanins from the surrounding medium, and this adsorption is proportional to the affinity of their cell walls to link anthocyanins. So, more pigmented colonies are coming from strains with strong anthocyanin adsorption (**Figure 14**). This technique allows to perform a fast screening for selecting either yeast strains or species with low anthocyanin adsorption.

The second procedure to evaluate anthocyanins adsorption by yeasts, that is more precise but more difficult and tedious to apply, is the recovery of anthocyanins adsorbed from cell walls and the characterization and quantitation of them by LC-DAD or LC-ESI/MS [24, 25]. The procedure requires separating all the lees from fermentation and it is possible to do it when red wines are fermented without skins maceration. If winemaking is done with skin maceration, what is the usual industrial process, which is very difficult to separate the yeast lees? But, it is easy to produce a red must with enough colour and tannins to make fermentation in absence of skins by using accelerated maceration, for example: heating and pressing the grapes, freezing or using ultrasounds. Thus, the lees can be separated from the wine by centrifugation at the end of fermentation.

Later, the lees can be washed with water or water-ethanol (88/12, v/v) solutions to remove anthocyanins that are not strongly adsorbed in cell wall but only in suspension in the surrounding medium among cells. It is not easy to evaluate what is the degree of extraction in this preliminary clean up and maybe some of the removed anthocyanins can be partially/weakly retained on cell walls. The following step, that is particularly delicate, is the separation of anthocyanins from cell walls. Yeast cell wall is a thick layer that externally covers the cell, and is involved in relationship function and giving resistance to osmotic pressure. It is formed by globular mannoproteins sustained by a net of fibrillar polysaccharides mainly formed by β-glucans and chitin [32, 33]. Anthocyanins might be forming links by polar interactions with these cell wall components, but the nature of this process is not yet well described.

Separation of anthocyanins from cell walls must be done by using solvents. The use of formic acid-methanol mixtures has been described [25]. Several washings are necessary to remove most of the adsorbed compounds and some anthocyanins can still remain linked after extraction. The detachment process can be facilitated by applying energy in form of shaking, ultrasounds or temperature. After each washing, supernatant is recovered by centrifugation at $3000 \times g$. Supernatants are collected together, concentrated under vacuum and analysed by LC-DAD to identify and quantify anthocyanins.

6. Biological ageing: ageing on lees

Yeasts are used not only to ferment musts but also in the ageing process of wines. Many traditional wines as Sherry-flor wines, natural sparkling wines and barrel fermented and aged Chardonnays improve their quality after a long period together with the yeast lees produced during fermentation. Along the biological ageing, many cell metabolites and structural components are released into the wine improving sensory quality. Also, the ageing on lees (AOL) technique can be used during barrel maturation of red wines [34]. AOL has a reductive

role because yeast lees are oxygen consumers and, moreover, some cell wall constituents such as glutathione (GSH) are antioxidant compounds with a protective effect on aromatic compounds and anthocyanins.

The simultaneous use of barrel ageing and AOL reduce the oxidative degree partially preserving anthocyanins from oxidative degradations [34]. Yeast selection is also a tool to get better strains to improve the wine quality and to protect pigments during AOL [35]. The use of non-*Saccharomyces* yeasts can also be a tool to improve the performance of AOL regarding the release of yeast polysaccharides and colour stabilization [36]. Osmophilic yeasts, able to grow in media with high sugar concentration, frequently show a thick cell wall that releases polysaccharides faster during ageing on lees. Some species like *S. pombe* have a cell wall configured in a double-layer structure with higher thickness than *S. cerevisiae* and some specific polymers.

7. Future trends

Probably, the future of red winemaking will be the separation of maceration and fermentation by means of fast macerations (minutes-hours) using new technologies such as high hydrostatic pressures [37], pulsed electric fields [38], irradiation [39] and ultrasounds, among others, to ensure enough amounts of anthocyanins and tannins in the must. In this situation, the fermentation will be produced in absence of skins and seeds and at low temperature to preserve sensory quality. The use of selected strains of *Saccharomyces* and, especially, new non-*Saccharomyces* will be a complementary biotechnology to improve wine quality and wine colour by promoting the formation of both pyranoanthocyanins and polymeric pigments, and also reducing the adsorption of anthocyanins in cell walls.

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