

## Influence of oenological tannins on malvidin-3-*O*-monoglucoside copigmentation in a model wine solution

Jordi Gombau<sup>1</sup>, Adeline Vignault<sup>1,2</sup>, Olga Pascual<sup>1</sup>, Sergio Gomez-Alonso<sup>3</sup>, Esteban García-Romero<sup>4</sup>, Isidro Herminos<sup>3</sup>, Joan Miquel Canals<sup>1</sup>, Pierre-Louis Teissedre<sup>2</sup> and Fernando Zamora<sup>1</sup>

<sup>1</sup>Departament de Bioquímica i Biotecnologia, Facultat d'Enologia de Tarragona, Universitat Rovira i Virgili, Carrer de Marcel·lí Domingo 1, 43007 Tarragona (Tarragona), Spain

<sup>2</sup>Institut des Sciences de la Vigne et du Vin, Université de Bordeaux, EA 4577 Œnologie, 210 Chemin de Leysotte, 33140 Villenave d'Ornon, France

<sup>3</sup>Instituto Regional de Investigación Científica Aplicada, Universidad de Castilla-La Mancha, Campus Universitario s/n, 13071 Ciudad Real (Castilla-La Mancha), Spain

<sup>4</sup>Instituto de la Vid y el Vino de Castilla-La Mancha, Carretera Toledo-Albacete s/n, 13700 Tomelloso (Ciudad Real), Spain

Corresponding author: fernando.zamora@urv.cat

### ABSTRACT

**Aim:** The aim of the study was to evaluate the effects of five different oenological tannins on the color of a malvidin-3-*O*-monoglucoside solution, and thereby assess the effectiveness of these tannins as copigments.

**Methods and Results:** Solutions containing malvidin-3-*O*-glucoside and different doses of copigments were prepared. The malvidin-3-*O*-glucoside concentration of the solutions, and color parameters, were measured after 1 and 7 days.

**Conclusions:** On days 1 and 7, a decrease in lightness and hue, and an increase in chroma, absorption at 520 nm and wavelength of maximal absorption were observed in solutions containing the oenological tannins. A decrease in malvidin-3-*O*-glucoside concentration in the tannin-containing solutions compared with the control solution was detected on day 7. The extent of this decrease depended on the specific tannin. Some changes in color parameters on day 7 compared with day 1 were observed, which were probably due to the formation of new pigments. The total color difference between the different malvidin-3-*O*-glucoside solutions and a pure white solution was calculated to estimate the effectiveness of the different tannins as copigments.

**Significance and impact of the study:** These results prove that supplementation with oenological tannins is a viable option for improving the color of red wines. A copigmentation index is proposed for measurement of the effectiveness of copigments.

### KEYWORDS

color improvement, copigmentation, malvidin-3-*O*-glucoside, oenological tannins, pigments

## INTRODUCTION

The use of oenological tannins is common practice in winemaking. Although the use of tannins is authorized by the International Organisation of Vine and Wine (OIV) only for facilitating the clarification of wines and musts (OIV, 2017), it is unquestionable that they are also used for many other purposes. Indeed, several other properties have been attributed to oenological tannins, such as antioxidant activity (for protection of wines against oxidation) (Hagerman *et al.*, 1998), direct consumption of dissolved oxygen (Pascual *et al.*, 2017), the ability to scavenge peroxy radicals (Magalhaes *et al.*, 2014), the ability to chelate iron(II), prevention of oxidative damage mediated by Fenton-based reactions (Pérez *et al.*, 2009), antioxidasic activity (antilaccase activity) (Obradovic *et al.*, 2005), improvement of wine structure and mouthfeel (Vivas, 2001), color improvement and stabilization of red wines (Canuti *et al.*, 2012), copigmentation effect (Neves *et al.*, 2010), direct formation of new pigments (Versari *et al.*, 1998), elimination of reduction odors (Vivas, 2001) and even bacteriostatic effects (Lempereur *et al.*, 2002a).

Many commercial tannins of different botanical origins and chemical compositions are currently available on the market (Obreque-Sliver *et al.*, 2009; Malacarne *et al.*, 2016). The so-called oenological tannins include ellagitannins from oak or chestnut, gallotannins from gall nuts, and condensed tannins from grape seeds and skins or from other plant sources, such as tara, quebracho and mimosa (Versari *et al.*, 2013).

The chemical structure of ellagitannins consists of an open-chain glucose esterified at positions 4 and 6 by a hexahydroxydiphenoyl unit and a nonahydroxyterphenoyl unit esterified at positions 2, 3 and 5 with a C-glycosidic bond between the carbon of the glucose and position 2 of the trihydroxyphenoyl unit (Quideau *et al.*, 2004; Okuda *et al.*, 2009). The structure of various ellagitannins have been described, but castalagin and vescalagin are the most abundant in oak wood, accounting for between 40% and 60% of the total by weight (Fernández de Simón *et al.*, 1999).

Gallotannins are formed by the esterification of gallic acid with the hydroxyl group of a polyol carbohydrate such as glucose (Hagerman, 1998). Gallotannin extracts, which are also

commercially known as tannic acid, are mixtures of polygalloyl glucose or polygalloylquinic acid esters with a number of galloyl moieties per molecule ranging from 2 to 12, depending on the plant source used to extract the tannic acid (Sylla *et al.*, 2015).

Condensed tannins are polymers of flavan-3-ol units; they are also called proanthocyanidins, because they release anthocyanins by acidic cleavage in accordance with the Bate-Smith reaction (Bate-Smith, 1973). Their composition, which depends on their botanical origin, varies mainly as regards the monomer released after acidic cleavage, the mean degree of polymerization, and their levels of galloylation and ramification (Versari *et al.*, 2013).

Grape-skin tannins are composed of procyanidins and prodelfinidins, because their acidic cleavage produces cyanidin and delphinidin. In contrast, grape-seed tannins comprise only procyanidins, because they release only cyanidin (Bate-Smith, 1973). Additionally, grape-skin proanthocyanidins have a high mean degree of polymerization and a low level of galloylation, whereas grape-seed proanthocyanidins have a lower mean degree of polymerization and a high level of galloylation (Souquet *et al.*, 1996). Regarding tannins from non-grape sources, quebracho tannins have a high level of ramification and are profisetinidins, because their acidic cleavage produces fisetinidin, whereas mimosa tannins are prorobinetidins, because they release robinetidin (Celzard *et al.*, 2015).

Of the various properties attributed to oenological tannins, their ability to improve the color intensity and stability of red wines is probably one of the main reasons why they are widely used in winemaking. The color of red wines is without doubt one of their main quality parameters, because it is the first sensory attribute experienced by consumers (Parpinello *et al.*, 2009). Indeed, poor color can be an indicator of possible wine deficiencies, such as insufficient grape phenolic maturity, infection of grapes by grey rot, and inadequate winemaking and stabilization processes. Because it evolves over time, the color of a wine also gives information about its age, its conservation status and its aging ability.

Anthocyanins are the main compounds that determine red wine color, the visible expression

of which depends, among other factors, on pH. They are mainly present in the form of the flavylium cation (which is red) at very acidic pH, but its proportion decreases progressively as pH increases, and this in turn generates the appearance of the quinoidal base (which is blue) and the hemiketal form (which is colorless) (Brouillard and Dubois, 1977). The hemiketal form can in turn give rise, via the opening of its heterocyclic ring, to the chalcone form (which is slightly yellow), and this may later be degraded in the presence of oxygen. This degradation is greatly favored by light and high temperature (Furtado *et al.*, 1993).

Wine color is also strongly determined by copigmentation, a phenomenon based on interactions between anthocyanins and either other anthocyanins or other molecules acting as copigments (Boulton, 2001). This comes about through the formation of vertical  $\pi$ - $\pi$  stacking non-covalent complexes (van der Waals interactions) between the planar polarizable nuclei of anthocyanins and the colorless copigment (de Freitas *et al.*, 2017). The resulting complexes adopt a sandwich-like structure that protects the flavylium cation against nucleophilic attack by water, thus avoiding formation of the colorless hemiketal (Santos-Buelga and de Freitas, 2009). Copigmentation therefore increases wine color intensity (i.e. it has a hyperchromic effect), but it can also change the color hue through bathochromic shift (Brouillard and Dangles, 1994).

Red wine contains several substances that can play a role as copigments, such as some amino acids, phenolic acids, flavonols, flavan-3-ol monomers, proanthocyanidins and even anthocyanins themselves (He *et al.*, 2012). It has also been suggested that oenological tannins may contribute as copigments to improve the color of red wines (Lempereur *et al.*, 2002b), although to our knowledge no direct scientific evidence for this effect has been reported.

Anthocyanins can also react with other molecules, especially flavanols (flavan-3-ol monomers and proanthocyanidins), to produce new pigments (Francia-Aricha *et al.*, 1997; He *et al.*, 2012). Different mechanisms have been proposed to explain the formation of these new pigments. Some of these involve the direct condensation of anthocyanins and flavanols without the participation of oxygen (Santos-Buelga *et al.*, 1995), but the most important

reactions are probably those involving oxygen (Timberlake and Bridle, 1976). 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 with either another flavanol molecule or an anthocyanin, thus producing ethyl-bridged flavanol-flavanol or flavanol-anthocyanin oligomers (Es-Safi *et al.*, 1999).

Additionally, 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 (Bakker and Timberlake, 1997). The reaction between anthocyanins and vinylphenol can also generate vinylphenol adducts (Schwarz *et al.*, 2003), and the previously formed ethylidene-bridged compounds can dissociate and generate vinylflavanol adducts (Cheynier *et al.*, 2006). More recently, it has been reported that anthocyanins can also react with ellagitannins to form new pigments (Chassaing *et al.*, 2010).

It is generally accepted that copigmentation is responsible for a substantial proportion (32–43%) of the color of young red wines (Hermosín-Gutiérrez *et al.*, 2005). It has also been suggested that copigmentation may be the first step in the formation of covalent bonds between anthocyanins and flavanols (flavan-3-ol monomers and proanthocyanidins), thus creating new anthocyanin-derived pigments either directly or through the participation of ethanal (Francia-Aricha *et al.*, 1997; de Freitas *et al.*, 2017). Indeed, during storage or aging, copigmentation decreases and the concentration of polymerized structures increases, with consequent changes in the wine (Brouillard and Dangles, 1994; Gutiérrez *et al.*, 2005; Liao *et al.*, 1992).

In previous studies, some researchers have shown the effectiveness as copigments of different pure phenolic compounds in model wine solutions. However, to our knowledge very little scientific information exists about the effectiveness as copigments of these commercial tannins, which are evidently not pure substances. As previously mentioned, the use of commercial oenological tannins is a common practice in modern winemaking, and for this reason the OIV

is now reconsidering the properties of oenological tannins in order to rewrite the OIV *International Oenological Codex* and authorize their use for other purposes.

Considering all the above, the aim of the present research was to study the effect of diverse oenological tannins on the color of the anthocyanin malvidin-3-*O*-monoglucoside in a model wine solution, and to propose a new index to measure the actual effectiveness as copigments of the different commercial products.

## MATERIALS AND METHODS

### 1. Chemicals

Methanol and formic acid were high-performance liquid chromatography (HPLC) grade and purchased from Panreac (Barcelona, Spain). Tartaric acid and sodium hydroxide were also purchased from Panreac. Absolute ethanol was purchased from MerckKGaA (Darmstadt, Germany). Malvidin-3-glucoside chloride and (-)-epicatechin were purchased from Extrasynthese (Genay, France).

### 2. Oenological tannins

Five different oenological tannins were used in this study: seed tannin, skin tannin, quebracho tannin, gallotannin and ellagitannin. They were provided by AEB Ibérica S.A. (Castellbisbal, Barcelona, Spain), Agrovin (Alcazar de San Juan, Ciudad Real, Spain), Institut Oenologique de Champagne (Épernay, France) and Laffort (Bordeaux, France), all members of Oenoppia, an internationally minded association of producers of oenological products. Each of the tannins has been analyzed using the OIV official method (OIV, 2017) to determine its richness in phenolic compounds. The results (expressed as % w/w) were seed tannin,  $73.1 \pm 0.7\%$ ; skin tannin,  $61.1 \pm 2.4\%$ ; quebracho tannin,  $79.5 \pm 6.9\%$ ; gallotannin,  $84.2 \pm 2.4\%$ ; and ellagitannin,  $66.7 \pm 4.3\%$ .

### 3. Sample preparation

All the samples were prepared in a model wine solution (ethanol 12% v/v, with tartaric acid 4 g/L, adjusted to pH 3.5 with sodium hydroxide) previously purged with nitrogen to remove dissolved oxygen. A solution containing only 50 mg/L of malvidin-3-*O*-glucoside (i.e. the pigment) was prepared; samples of this solution, with no added tannin, served as positive

controls. Simultaneously, solutions containing 50 mg/L of malvidin-3-*O*-glucoside and 100, 200 or 400 mg/L of (-)-epicatechin or each of the various commercial oenological tannins (i.e. the copigments) were also prepared to give a copigment-to-pigment weight ratio of 2, 4 and 8, respectively. Finally, solutions containing only 100, 200 or 400 mg/L of (-)-epicatechin or each tannin were prepared as negative controls.

Aliquots of 1.5 mL of each of these solutions were introduced into Eppendorf tubes previously saturated with nitrogen. Solutions were kept in the dark at 20°C for 7 days. All samples were prepared in triplicate. All the subsequent analyses were carried out 1 and 7 days after the beginning of the experiment.

### 4. Color analysis

One and seven days after sample preparation, the full absorption spectrum in the visible range (400–800 nm) of all the samples was measured with a spectrophotometer (Helios Alpha UV-vis, Thermo Fisher Scientific, Waltman, MA, USA) using a quartz cell with a path length of 10 mm). The visible spectra of the solutions containing only the copigments (i.e. (-)-epicatechin and the oenological tannins) were subtracted from the spectra of the samples containing the pigment (i.e. malvidin-3-*O*-glucoside) and the corresponding concentration of copigment (i.e. the oenological tannins or (-)-epicatechin) to eliminate the color contribution of each of the copigments. CIELab coordinates were determined following Ayala *et al.* (1997), and the data were processed using MSCV software (Ayala *et al.*, 2001).

The effectiveness of the hyperchromic and bathochromic effects of the different copigments was evaluated by comparing the absorption at 520 nm and the CIELab coordinates (chroma, C\*; lightness, L\*; and hue, h\*) of the samples containing only malvidin-3-*O*-glucoside (i.e. the positive controls) with those of the samples also containing the copigments, after subtracting the values for the negative controls. The total color difference ( $\Delta E_{ab}^*$ ) between each solution and a pure white solution, and the wavelength of maximal absorption ( $\lambda_{max}$ ) of the different spectra, were also determined to evaluate the effectiveness as copigments of the different oenological tannins.

## 5. Malvidin-3-O-glucoside quantification

Malvidin-3-O-monoglucoside concentration was determined 1 and 7 days after sample preparation by reverse-phase HPLC analyses with an Agilent 1200 series liquid chromatograph (HPLC–diode array detection) using an Agilent Zorbax Eclipse XDB-C18, 4.6 × 250 mm 5-mm column (Agilent Technologies, Santa Clara, CA, USA), in accordance with the method described by Gil *et al.* (2012). Malvidin-3-O-glucoside was used to produce anthocyanin standard curves. The aim of this quantification was to estimate the possible losses of malvidin-3-O-glucoside due to oxidation or the possible formation of polymeric pigments between malvidin and the oenological tannins.

## 6. Copigmentation effectiveness index

The following equation is proposed to provide an index of effectiveness as copigments, for use when comparing different commercial tannins.

$$\text{copigmentation index (\%)} = 100 \times \frac{\Delta E_{ab} * TS - \Delta E_{ab} * CS}{\Delta E_{ab} * CS}$$

In the equation,  $\Delta E_{ab} * CS$  is the total color difference between the positive control solution (containing malvidin-3-O-monoglucoside without copigment) and a pure white color solution, and  $\Delta E_{ab} * TS$  is the total color difference between the solution of malvidin-3-O-monoglucoside containing 400 mg/L of tannin and a pure white solution. The CIELab coordinates of a pure white color solution are  $L^* = 100.00$ ,  $a^* = 0.00$  and  $b^* = 0.00$ .

## 7. Statistics

All data are expressed as the arithmetic mean  $\pm$  the standard deviation from three replicates. One-factor ANOVA was carried out using XLSTAT software (Addinsoft, Long Island City, NY, USA). Comparison of the slopes of the regression lines was carried out using ULC 2.0.1 Univariate Linear Calibration software (University Rovira i Virgili, Tarragona, Spain).

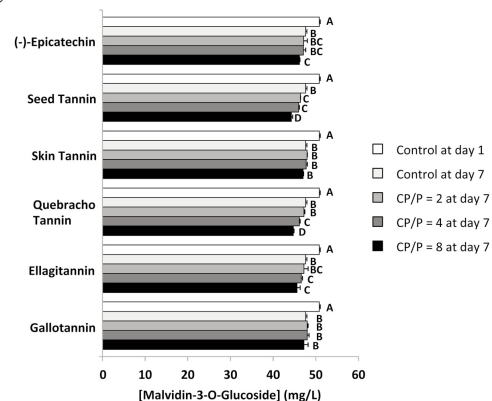
# RESULTS AND DISCUSSION

## 1. Effects on malvidin-3-O-glucoside concentration

Figure 1 shows the concentration of malvidin-3-O-monoglucoside on days 1 and 7 in the positive control solution and the solutions containing

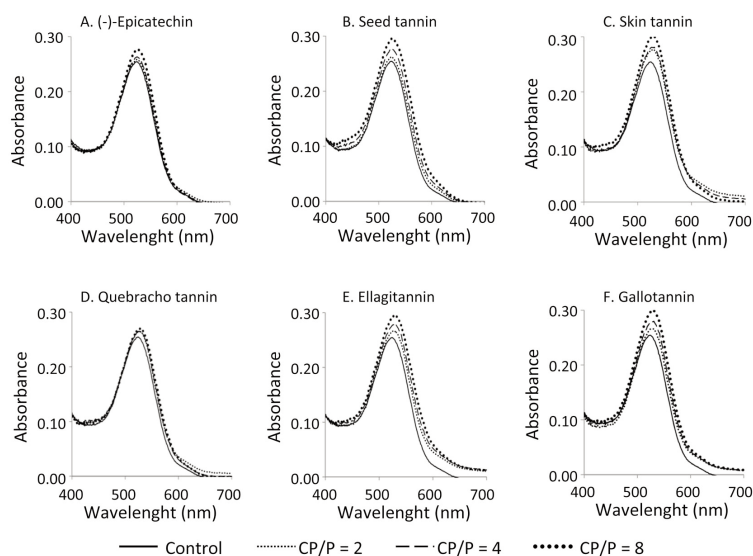
malvidin-3-O-glucoside and different doses of (–)-epicatechin and the various oenological tannins. On day 1, the concentration of malvidin-3-O-glucoside was  $50.8 \pm 0.2$  mg/L in all samples. After 7 days of stockage, the concentration of malvidin-3-O-monoglucoside of the control solution containing only this anthocyanin decreased significantly to  $47.6 \pm 0.3$  mg/L. This reduction (about 6.3 %) was probably due to oxidation (Furtado *et al.*, 1993). Similar results were obtained by Jordao *et al.* (2008) with malvidin-3-O-glucoside in a model wine solution without the presence of any other phenolic compounds.

Figure 1 also shows how the solutions of malvidin-3-O-glucoside were affected by the presence in the media of the copigments, (–)-epicatechin and the oenological tannins, at different concentrations after 7 days. The presence of all copigments except skin tannin and gallotannin brought about a slight but significant decrease in the concentration of malvidin-3-O-glucoside, and this reduction tended to be greater at higher concentrations of copigment. However, the decrease in malvidin-3-O-glucoside concentration was small and may be related to its oxidation or transformation; it has been reported that proanthocyanidins, flavan-3-ol monomers (Francia-Aricha *et al.*, 1997) and even ellagitannins (Jordão *et al.*, 2008; Chassaing *et al.*, 2010) can react with malvidin-3-O-glucoside to produce new pigments.



**Figure 1.** Malvidin-3-O-glucoside concentration of the model wine solution supplemented with increasing concentrations of (–)-epicatechin and diverse oenological tannins.

All data are the mean  $\pm$  SD of three replicates. Different letters indicate the existence of statistically significant differences ( $p < 0.05$ ) between different doses of each copigment. CP/P ratio correspond to the quotient between the copigment and pigment concentrations expressed as g/L.



**FIGURE 2.** Visible spectra of a model wine solution of malvidin-3-*O*-glucoside (50 mg/L) supplemented with increasing concentrations of (-)-epicatechin and diverse oenological tannins at seventh day of experimentation.

CP/P ratio correspond to the quotient between the copigment and pigment concentrations expressed as g/L.

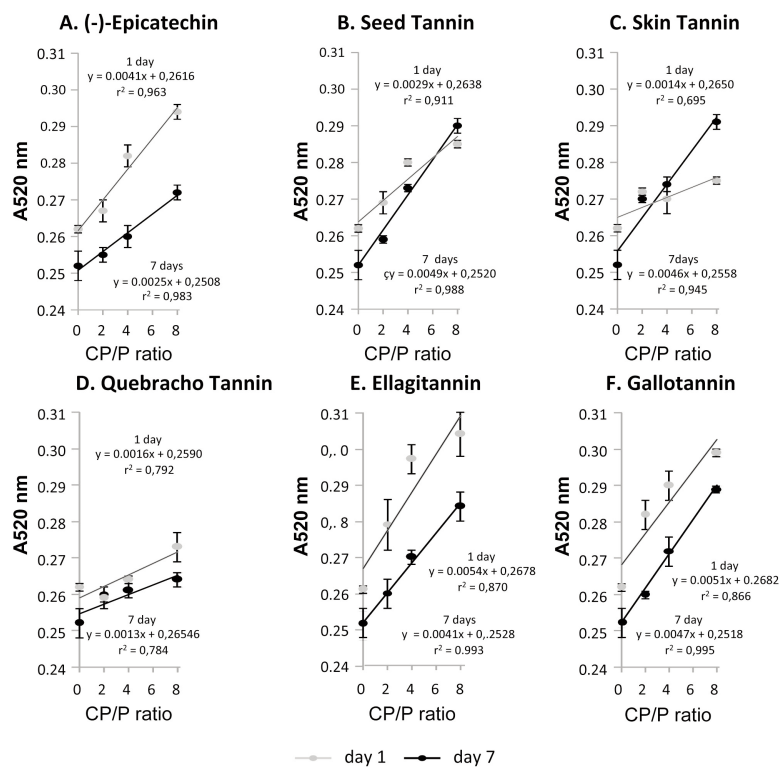
The absorption spectra of these new pigments differ from those of malvidin-3-*O*-glucoside, and the degree of difference depends greatly on the nature of the new pigment. Pigments created by direct unions between malvidin-3-*O*-glucoside and flavanols have a red hue, for example, whereas those created by unions between malvidin-3-*O*-glucoside and flavanols via an ethyl bridge have a purple hue and the different products of cycloaddition (pyranoanthocyanins) have an orange hue (Francia-Aricha *et al.*, 1997; de Freitas *et al.*, 2017). Consequently, the formation of these new pigments can change the absorption spectrum of the solution. However, the variations in malvidin-3-*O*-glucoside concentration brought about by the presence of the different copigments were not very important, with values ranging from 0 (low doses of skin tannin or gallotannin) to 6.6% (in the case of the highest dose of seed tannin), and a global mean value below 2%. For this reason, changes in the absorption spectrum of the different samples are attributable mainly to copigmentation phenomena, although in some cases it could be hypothesized that a small and unknown proportion may be due to the presence of very low amounts of new pigments derived from malvidin-3-*O*-glucoside.

## 2. Effects on absorption spectra and CIELab color parameters

### 2.1 Effects on absorption spectra

Figure 2 shows the full spectrum in the visible range (400–800 nm) on day 7 of the malvidin-3-*O*-glucoside control sample and solutions containing also different doses of (-)-epicatechin and the various oenological tannins. In all cases, the presence of copigments increased the height and the surface of the absorption spectrum in the visible range, and this effect was dose-dependent. These results confirm that all the oenological tannins investigated can improve the color of a malvidin-3-*O*-glucoside solution and probably also the color of red wines. Similar results have been reported previously (Lempereur *et al.*, 2002b; Vignault *et al.*, 2019).

Because the absorption spectrum results give only a general idea of the effects of (-)-epicatechin and the various oenological tannins on color, the effects on the red color specifically (absorption at 520 nm,  $A_{520\text{nm}}$ ) and the CIELab coordinates were measured to better understand the copigmentation phenomenon:  $A_{520\text{nm}}$ , lightness ( $L^*$ ) and chroma ( $C^*$ ) describe quantitative attributes of the color, whereas hue ( $h^*$ ) describes qualitative attributes. The evaluation of these parameters makes it possible to determine the hyperchromic and bathochromic effects produced by the presence of the various copigments.



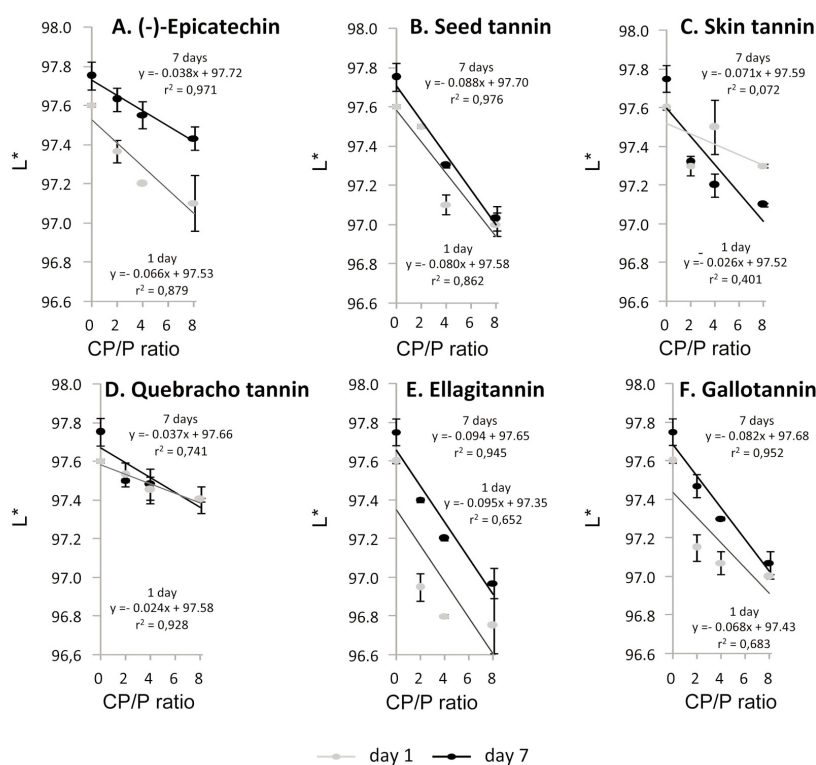
**FIGURE 3.** Influence of the copigment/pigment ratio on the absorbance at 520 nm of a model wine solution of malvidin-3-O-glucoside (50 mg/L) supplemented with (-)-epicatechin and diverse oenological tannins. All data are the mean  $\pm$  SD of three replicates. CP/P ratio correspond to the quotient between the copigment and pigment concentrations expressed as g/L.

Figure 3 shows the  $A_{520\text{nm}}$  on days 1 and 7 of the solutions of malvidin-3-O-glucoside containing the different doses of (-)-epicatechin and the various oenological tannins. As shown in each graph, the  $A_{520\text{nm}}$  of the solution clearly decreased between day 1 and day 7, when no copigments were present. This decrease was undoubtedly related to the reduction in malvidin-3-O-glucoside concentration shown in Figure 1.

The  $A_{520\text{nm}}$  of the malvidin-3-O-glucoside solutions increased with the presence of (-)-epicatechin and each of the oenological tannins, showing a reasonably linear correlation with copigment-to-pigment ratio (CP:P) (see Figure 3). The slopes of the regression lines obtained, along with their statistical significance, are shown in Table 1. The values of the slopes indicate the effectiveness of (-)-epicatechin and the various oenological tannins in improving the red color of the solution; the steeper the slope, the greater their effectiveness. Berké and de Freitas (2005) have reported a similar slope for (-)-epicatechin, although under different experimental conditions. As shown by the slopes

obtained, ellagitannin produced the greatest hyperchromic effect on day 1, followed in decreasing order by gallotannin and (-)-epicatechin (the slopes for these two tannins were statistically similar). In contrast, the smallest hyperchromic effects were produced by seed tannin, skin tannin and quebracho tannin.

No significant differences were observed between the slopes of the regression lines for day 1 and day 7 in the case of quebracho tannin, ellagitannin and gallotannin; the lines were nearly parallel (see Figure 3D–F). However, some differences were observed in the case of (-)-epicatechin, seed tannin and skin tannin (see Figure 3A–C). Specifically, the slopes for the seed and skin tannins became significantly steeper, whereas the slope of (-)-epicatechin did the opposite. The steeper slopes of the regression lines for the seed and skin tannins on day 7, crossing and passing the regression line for day 1 at the highest CP:P ratio, suggest that the copigmentation phenomenon needs time to reach its maximal effect, or that new pigments with a greater contribution to the red color were being



**FIGURE 4.** Influence of the copigment/pigment ratio on the Lightness ( $L^*$ ) of a model wine solution of malvidin-3-O-glucoside (50 mg/L) supplemented with (-)-epicatechin and diverse oenological tannins. All data are the mean  $\pm$  SD of three replicates. CP/P ratio correspond to the quotient between the copigment and pigment concentrations expressed as g/L.

formed. Oligomeric and polymeric proanthocyanidins have been reported to react with malvidin-3-O-glucoside to form new red pigments (Malien-Auberg *et al.*, 2002).

The significantly less steep slope of the regression line for (-)-epicatechin on day 7 compared to that on day 1 suggests that the maximal copigmentation effect had already been reached on the first day. There are a number of possible explanations for the decrease in the incline of the slope. On the one hand, it may be due to the formation of new pigments with a lesser effect on the intensity of the red color. According to Dueñas *et al.* (2006), (-)-epicatechin and malvidin-3-O-glucoside can react to form an adduct anthocyanin-flavanol, which in turn is quickly transformed into a xanthylium cation with a yellow color. On the other hand, (-)-epicatechin can also form other compounds. Indeed, it has been reported that (-)-epicatechin can form adducts in the presence of glyoxylic acid produced by oxidation of tartaric acid and later form xanthylium pigments (Gómez-Míguez *et al.*, 2006). These new compounds are probably less effective

copigments than (-)-epicatechin; however, further studies are needed to verify this.

## 2.2 Effects on lightness

Figure 4 shows the relation between  $L^*$  and CP:P for (-)-epicatechin and each of the oenological tannins. A decrease in  $L^*$  was brought about by the presence in the media of each of the copigments. This indicates that their presence increased the color intensity of the resultant solution. It should be highlighted that a decrease in  $L^*$  better indicates the existence of a hyperchromic effect than an increase in  $A_{520\text{nm}}$ , because  $L^*$  refers to all the visible range of the spectrum and not just to a single wavelength.

The value of  $L^*$  on day 7 was significantly higher than on day 1, when no copigments were present. This significant increase in  $L^*$  is to be expected, because the malvidin-3-O-glucoside concentration and the  $A_{520\text{nm}}$  on day 7 were significantly lower than on day 1.

The decrease in  $L^*$  showed a linear relation with CP:P. However, in this case the regression coefficients were not as clear as in the case of

**TABLE 1.** Slopes of the regression lines corresponding to the red color (A520), Lightness (L\*), Chroma (C\*) and  $\Delta E_{ab}^*$  in function of the copigment/pigment ratio after 1 or 7 days of the supplementation with (-)-epicatechin and diverse oenological tannins.

Copigment	Time (days)	A520	Lightness (L*)	Chroma (C*)	$\Delta E_{ab}^*$
(-)-Epicatechin	1	0.0041 ± 0.0004 <b>C</b> $\beta$	-0.066 ± 0.011 <b>B</b> $\beta$	0.108 ± 0.007 <b>C</b> $\beta$	0.118 ± 0.009 <b>C</b> $\beta$
	7	0.0025 ± 0.0003 <b>b</b> $\alpha$	-0.038 ± 0.005 <b>a</b> $\alpha$	0.058 ± 0.005 <b>b</b> $\alpha$	0.073 ± 0.006 <b>b</b> $\alpha$
Seed tannins	1	0.0029 ± 0.0003 <b>B</b> $\alpha$	-0.080 ± 0.010 <b>B</b> $\alpha$	0.042 ± 0.003 <b>A</b> $\alpha$	0.069 ± 0.006 <b>B</b> $\alpha$
	7	0.0049 ± 0.0002 <b>c</b> $\beta$	-0.088 ± 0.006 <b>b</b> $\alpha$	0.067 ± 0.005 <b>b</b> $\beta$	0.102 ± 0.004 <b>c</b> $\beta$
Skin tannins	1	0.0014 ± 0.0006 <b>A</b> $\alpha$	-0.026 ± 0.011 <b>A</b> $\alpha$	0.044 ± 0.005 <b>A</b> $\alpha$	0.043 ± 0.010 <b>A</b> $\alpha$
	7	0.0046 ± 0.0004 <b>c</b> $\beta$	-0.071 ± 0.013 <b>b</b> $\beta$	0.086 ± 0.004 <b>c</b> $\beta$	0.111 ± 0.008 <b>cd</b> $\beta$
Quebracho tannins	1	0.0016 ± 0.0005 <b>A</b> $\alpha$	-0.021 ± 0.007 <b>A</b> $\alpha$	0.049 ± 0.002 <b>A</b> $\beta$	0.057 ± 0.005 <b>AB</b> $\beta$
	7	0.0013 ± 0.0004 <b>a</b> $\alpha$	-0.037 ± 0.011 <b>a</b> $\alpha$	0.014 ± 0.004 <b>a</b> $\alpha$	0.034 ± 0.005 <b>a</b> $\alpha$
Ellagitannins	1	0.0054 ± 0.0010 <b>C</b> $\alpha$	-0.095 ± 0.022 <b>B</b> $\alpha$	0.073 ± 0.005 <b>B</b> $\beta$	0.100 ± 0.018 <b>C</b> $\alpha$
	7	0.0041 ± 0.0004 <b>c</b> $\alpha$	-0.094 ± 0.009 <b>b</b> $\alpha$	0.054 ± 0.008 <b>b</b> $\alpha$	0.096 ± 0.009 <b>c</b> $\alpha$
Gallotannins	1	0.0051 ± 0.0010 <b>C</b> $\alpha$	-0.068 ± 0.015 <b>B</b> $\alpha$	0.081 ± 0.004 <b>B</b> $\alpha$	0.098 ± 0.014 <b>C</b> $\alpha$
	7	0.0047 ± 0.0002 <b>c</b> $\alpha$	-0.082 ± 0.007 <b>b</b> $\alpha$	0.081 ± 0.005 <b>c</b> $\alpha$	0.115 ± 0.006 <b>d</b> $\beta$

All data are the mean ± SD of three replicates. Different capital letters indicate the existence of statistically significant differences ( $p < 0.05$ ) between the different tannins at day 1. Lowercase letters indicate the existence of statistically significant differences ( $p < 0.05$ ) between the different tannins at day 7. Different Greek letters indicate the existence of statistically significant differences ( $p < 0.05$ ) between times of treatment.

$A_{520\text{nm}}$ , probably because  $L^*$  relates to the whole absorption spectrum, not just absorption at a single wavelength. The slopes for each copigment were negative (see Table 1), therefore the higher the absolute value, the greater the hyperchromic effect.

On day 1, the highest absolute slope value and consequently the greatest hyperchromic effect was produced by ellagitannin (see Figure 4E), followed in decreasing order by seed tannin, gallotannin, (-)-epicatechin, skin tannin and quebracho tannin (see Figures 4A–D,F). The only significant difference was found between quebracho tannin and skin tannins with all the other copigments.

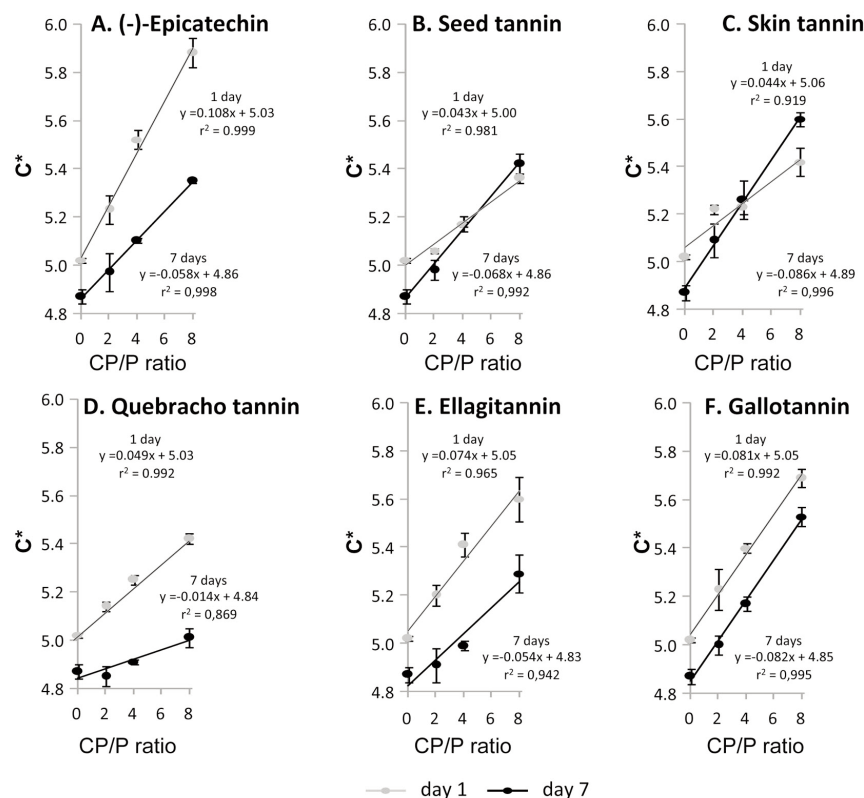
The slope for the  $L^*$  of the solutions containing (-)-epicatechin was significantly less steep on day 7 than on day 1 (see Figure 4A), which suggests, as mentioned earlier, that the copigmentation effect of this compound tends to decrease over time, probably because of the formation of xanthylium cations (Dueñas *et al.*, 2006; Gómez-Míguez *et al.*, 2006). In contrast, the  $L^*$  slope for skin tannin increased significantly in absolute value (see Figure 4C), showing a similar pattern to that observed for  $A_{520\text{nm}}$ . No significant differences were found between the slopes for day 1 and day 7 for any of the other oenological tannins. Consequently, on day 7 the greatest hyperchromic effect was produced by ellagitannin, followed by seed

tannin, skin tannin and gallotannin, which exert a similar effect. The least effective copigments in terms of hyperchromic effect were (-)-epicatechin and quebracho tannin.

### 2.3. Effects on chroma

Figure 5 shows the relation between  $C^*$  and CP:P for (-)-epicatechin and each of the oenological tannins. As expected,  $C^*$  values were significantly lower on day 7 than on day 1, when no copigments were present. Again, the decrease in malvidin-3-*O*-glucoside concentration explains this result. Generally, the graphs show a similar pattern to that obtained for  $A_{520\text{nm}}$ . The results confirm that (-)-epicatechin and each of the tannins exert a hyperchromic effect, because the higher the CP:P, the higher the  $C^*$ . The  $C^*$  values were more in line with CP:P, showing reasonably clear regression coefficients and confirming that the color of the malvidin-3-*O*-glucoside solution was more vivid in the presence of these copigments.

On day 1, the steepest slope was obtained with (-)-epicatechin (see Figure 5A), followed in decreasing order by gallotannin, ellagitannin, quebracho tannin, skin tannin and seed tannin (see Figure 5B–F). In contrast, on day 7 some interesting changes in the slopes were observed. Specifically, the slopes were significantly steeper in the case of seed tannin and skin tannin, and significantly less steep in the case of



**FIGURE 5.** Influence of the copigment/pigment ratio on the Chroma ( $C^*$ ) of a model wine solution of malvidin-3-*O*-glucoside (50 mg/L) supplemented with (-)-epicatechin and diverse oenological tannins. All data are the mean  $\pm$  SD of three replicates. CP/P ratio correspond to the quotient between the copigment and pigment concentrations expressed as g/L.

(-)-epicatechin, ellagitannin and quebracho tannin. No differences were observed in the case of gallotannin.

An increase in the steepness of the  $C^*$  slope between days 1 and 7 suggests that the copigmentation effect had increased or that new red pigments had been formed (Malien-Auberg *et al.*, 2002). In the case of skin tannin, the steeper slope on day 7 was probably due to an increase in the copigmentation effect, because there was no significant decrease in malvidin-3-*O*-glucoside concentration compared with the control solution for the same day. In contrast, the increase in incline of the  $C^*$  slope on day 7 for the solutions containing seed tannin was probably due to both an increase in copigmentation and the formation of new pigments, because the malvidin-3-*O*-glucoside concentration was significant lower than that of the control solution on day 7.

In the case of (-)-epicatechin, ellagitannin and quebracho tannin, the decrease in the incline of the  $C^*$  slope between days 1 and 7 suggests that

their copigmentation effect is lower or that malvidin-3-*O*-glucoside had reacted to form new compounds with a different hue. Such a hypothesis seems probable, because a decrease in the malvidin-3-*O*-glucoside concentration was observed in the solutions containing (-)-epicatechin, ellagitannin and quebracho tannin compared with the concentration in the control solutions without these compounds. It has been reported that colorless molecules are formed in solutions containing malvidin-3-*O*-glucoside and oak extract, and that the ellagitannin concentration decreases over time (Jordão *et al.*, 2008).

#### 2.4 Effects on wavelength of maximal absorption

The wavelength of maximal absorption ( $\lambda_{\max}$ ) of the absorption spectra of the malvidin-3-*O*-glucoside solutions was determined (Table 2), with the aim of evaluating the bathochromic effect produced by the presence of the different copigments. No significant differences in  $\lambda_{\max}$  (around 524 nm) between days 1 and 7 were detected in the absence of copigment.

**TABLE 2.** Wavelength (nm) of maximum absorbance of a model wine solution of malvidin-3-*O*-glucoside (50 mg/L) supplemented with increasing concentrations of (-)-epicatechin and diverse oenological tannins at 1 and 7 days of treatment.

Copigment	(g/L)	1 day			7 days		
Control	0.0	524.0 ± 1.4	<b>A</b>	$\alpha$	523.3 ± 0.6	<b>A</b>	$\alpha$
	0.1	526.7 ± 1.2	<b>AB</b>	$\beta$	524.3 ± 0.6	<b>A</b>	$\alpha$
	0.2	528.3 ± 2.5	<b>B</b>	$\beta$	524.5 ± 0.6	<b>A</b>	$\alpha$
EC	0.4	529.3 ± 2.9	<b>B</b>	$\beta$	524.3 ± 0.6	<b>A</b>	$\alpha$
	0.1	525.7 ± 1.2	<b>AB</b>	$\alpha$	524.5 ± 0.5	<b>A</b>	$\alpha$
SDT	0.2	527.0 ± 1.0	<b>B</b>	$\beta$	524.7 ± 0.6	<b>A</b>	$\alpha$
	0.4	528.7 ± 2.1	<b>B</b>	$\beta$	524.7 ± 0.6	<b>A</b>	$\alpha$
	0.1	528.0 ± 0.0	<b>B</b>	$\alpha$	527.0 ± 1.0	<b>B</b>	$\alpha$
SKT	0.2	528.7 ± 1.2	<b>B</b>	$\alpha$	526.7 ± 0.8	<b>B</b>	$\alpha$
	0.4	528.7 ± 1.2	<b>B</b>	$\alpha$	531.0 ± 0.1	<b>C</b>	$\beta$
	0.1	531.0 ± 0.1	<b>C</b>	$\beta$	526.0 ± 0.1	<b>B</b>	$\alpha$
QT	0.2	531.0 ± 0.1	<b>C</b>	$\beta$	526.3 ± 0.6	<b>B</b>	$\alpha$
	0.4	527.3 ± 1.2	<b>B</b>	$\alpha$	527.7 ± 1.2	<b>B</b>	$\alpha$
	0.1	528.0 ± 0.1	<b>B</b>	$\beta$	526.3 ± 0.6	<b>B</b>	$\alpha$
ET	0.2	530.0 ± 1.7	<b>C</b>	$\alpha$	531.0 ± 0.1	<b>C</b>	$\alpha$
	0.4	531.0 ± 0.1	<b>C</b>	$\alpha$	531.0 ± 0.1	<b>C</b>	$\alpha$
	0.1	530.0 ± 1.7	<b>B</b>	$\alpha$	526.8 ± 0.3	<b>B</b>	$\alpha$
GT	0.2	528.0 ± 0.1	<b>B</b>	$\alpha$	530.3 ± 0.6	<b>C</b>	$\beta$
	0.4	529.3 ± 1.5	<b>B</b>	$\alpha$	531.0 ± 0.1	<b>C</b>	$\beta$

All data are the mean ± SD of three replicates. EC: (-)-epicatechin; SDT: seed tannin; SKT: skin tannin; QT: quebracho tannin; ET: ellagitannin; GT: gallotannin. Different capital letters indicate the existence of statistically significant differences ( $p < 0.05$ ) between different doses of each copigment. Different Greek letters indicate the existence of statistically significant differences ( $p < 0.05$ ) between times of treatment.

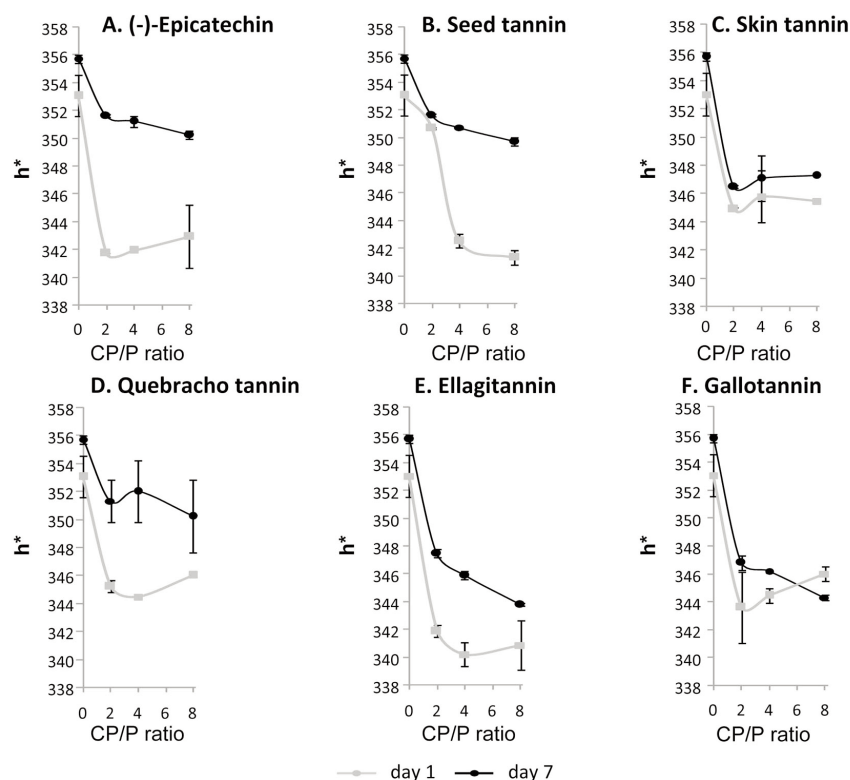
Consequently,  $\lambda_{\max}$  was not displaced by the slight but significant degradation of malvidin-3-*O*-glucoside observed under such conditions.

On day 1, the presence of (-)-epicatechin and each of the oenological tannins produced a significant bathochromic displacement. This effect seems to be dose-dependent, because generally the higher the concentration of copigment, the higher the  $\lambda_{\max}$  with the exception of solutions containing skin tannin and quebracho tannin, which reached  $\lambda_{\max}$  at lower concentrations. The bathochromic displacement oscillated between 1.7 and 7 nm of  $\lambda_{\max}$ , being particularly high in the case of ellagitannin and quebracho tannin.

On day 7, the solutions containing skin tannin, quebracho tannin, gallotannin and ellagitannin had maintained the bathochromic displacement, although in the case of quebracho tannin the  $\lambda_{\max}$  was significantly lower than on day 1. In

contrast, the bathochromic effect of the solutions containing (-)-epicatechin and seed tannin had completely disappeared, because the  $\lambda_{\max}$  was similar to that of the control solution of malvidin-3-*O*-glucoside.

The decrease in the bathochromic effect observed for (-)-epicatechin, seed tannin and, to a lesser extent, quebracho tannin may have come about for two reasons. It could have been due to a reduction in copigmentation effectiveness, or alternatively the formation of new pigments with a more red-orange hue (Malien-Auberg *et al.*, 2002; Dueñas *et al.*, 2006). The result may have been due to the simultaneous occurrence of both. In contrast, the maintenance of bathochromic displacement in the solutions containing skin tannin and gallotannin, together with the fact that the malvidin-3-*O*-glucoside concentration was similar to that in the control solution, point to the permanence of the copigmentation effect.



**FIGURE 6.** Influence of the copigment/pigment ratio on the Hue ( $h^*$ ) of a model wine solution of malvidin-3-O-glucoside (50 mg/L) supplemented with (-)-epicatechin and diverse oenological tannins. All data are the mean  $\pm$  SD of three replicates. CP/P ratio correspond to the quotient between the copigment and pigment concentrations expressed as g/L.

The particular case of solutions containing ellagitannins is more complicated, because a significant decrease in malvidin-3-O-glucoside concentration was detected and it has been reported that new pigments can be formed from these compounds, which have a bluish hue (Jordão *et al.*, 2008; Chassaing *et al.*, 2010). In such case, maintenance of the higher  $\lambda_{\max}$  could be associated with the formation of these new pigments and also to maintenance of the bathochromic effect.

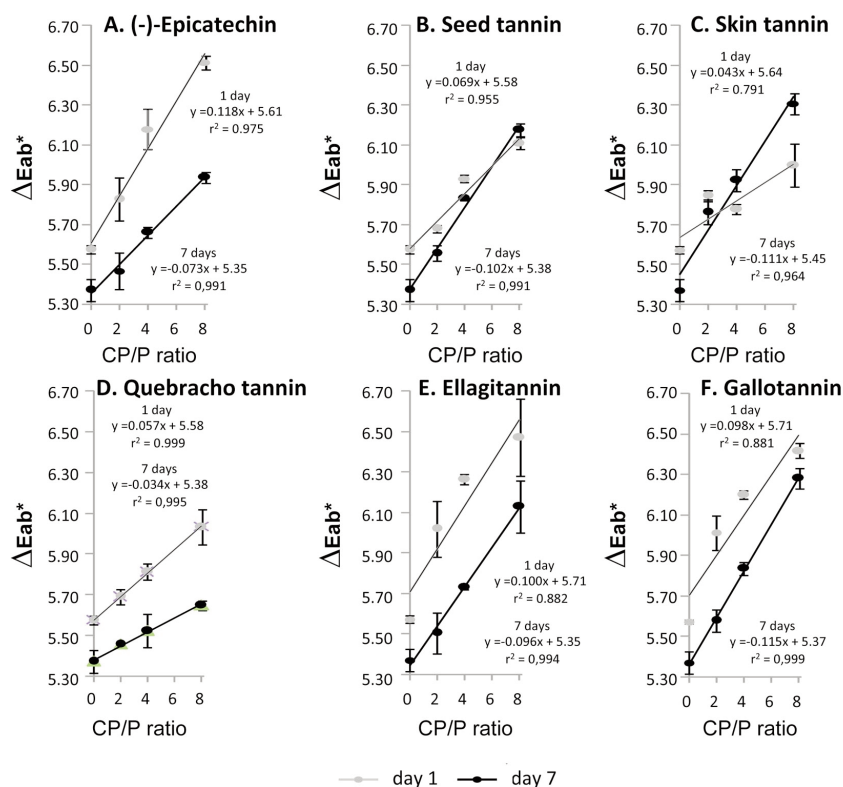
## 2.5 Effects on hue

Figure 6 shows the relation between  $h^*$  and CP:P for (-)-epicatechin and each of the oenological tannins. An increase in  $h^*$  angle for the control solutions of malvidin-3-O-glucoside had come about by day 7 compared with day 1. This again confirms the degradation of malvidin-3-O-glucoside, which results in a more red-orange color. Supplementation with (-)-epicatechin and each of the tannins brought about a significant decrease in  $h^*$  on day 1, therefore the color of these solutions was clearly

more bluish than that of the corresponding control solution. This effect was not dose-dependent, because the decrease in  $h^*$  was generally reached at the lowest CP:P. The decrease in  $h^*$  was clearly maintained on day 7 in the case of solutions containing skin tannin and gallotannin (see Figure 6C,F). The other copigments also caused a decrease in  $h^*$  on day 7, but to a lower extent than on day 1 (see Figure 6A,B,D,E).

The solutions containing skin tannin and gallotannin were the only solutions in which no significant decrease in malvidin-3-O-glucoside concentration was detected, therefore it seems that the copigmentation effect is maintained between days 1 and 7. In contrast, the clear increase in  $h^*$  values observed in the presence of the other copigments on day 7 compared with day 1 can be explained by the formation of new pigments and/or a decrease in the copigmentation effect, as discussed above.

The changes in  $h^*$  values between days 1 and 7 suggest not only that skin tannin, gallotannin



**FIGURE 7.** Influence of the copigment/pigment ratio on the total color differences ( $\Delta E_{ab}^*$ ) of a model wine solution of malvidin-3-O-glucoside (50 mg/L) supplemented with (-)-epicatechin and diverse oenological tannins with a pure white color solution. All data are the mean  $\pm$  SD of three replicates. CP/P ratio correspond to the quotient between the copigment and pigment concentrations expressed as g/L.

and, to a lesser degree, ellagitannin increase the bluish hue of the solution, but also that this effect is maintained probably because the copigmentation effect lasts longer, and in the case of ellagitannins, because new pigments with a bluish hue may be formed (Jordão *et al.*, 2008; Chassaing *et al.*, 2010). In contrast, (-)-epicatechin and the other oenological tannins exert a bathochromic effect initially, but this decreases over time, probably because new pigments with a red-orange hue are formed (Malien-Auberg *et al.*, 2002; Dueñas *et al.*, 2006) and/or because these compounds are transformed into others with a lower copigmentation effectiveness (Gómez-Míguez *et al.*, 2006).

## 2.6 Effects on total color differences

To evaluate the effect of the different copigments on the intensity of color, the total color differences (the  $\Delta E_{ab}^*$  values) between the solutions of malvidin-3-O-glucoside with or without the different copigments and a pure white color solution were calculated. Generally,

$\Delta E_{ab}^*$  showed a similar trend to that of  $C^*$ , although with some minor differences (Figure 7).

## 3. Copigmentation index values

Table 3 summarizes the results for copigmentation index values of (-)-epicatechin and the different commercial tannins investigated. The copigmentation index illustrates simply the effectiveness of the different commercial tannins to improve wine color, which, as discussed, is mainly due to the copigmentation phenomenon. According to the index, on day 1 the best copigment is (-)-epicatechin and ellagitannin, followed in decreasing order by gallotannin, seed tannin, quebracho tannin and skin tannin. However, on day 7 some interesting differences can be observed. First, the copigmentation index of (-)-epicatechin and, to a lesser extent, quebracho tannin and ellagitannin, decrease significantly, suggesting that the copigmentation effect decreases or that malvidin-3-O-glucoside reacts to form new compounds with a lesser contribution to the color. In contrast, the

**TABLE 3.** Copigmentation Index of the different copigments (commercial tannins and (-)-epicatechin)

Copigment	Time					
	1 day			7 days		
(-)-Epicatechin	16.82 ± 0.36	<b>D</b>	$\beta$	10.5 ± 0.16	<b>B</b>	$\alpha$
Seed tannin	9.53 ± 0.08	<b>B</b>	$\alpha$	14.96 ± 0.5	<b>C</b>	$\beta$
Skin tannin	7.81 ± 0.17	<b>A</b>	$\alpha$	17.4 ± 0.26	<b>D</b>	$\beta$
Quebracho tannin	8.22 ± 0.24	<b>A</b>	$\beta$	5.14 ± 0.07	<b>A</b>	$\alpha$
Ellagitannin	16.11 ± 0.53	<b>D</b>	$\beta$	14.11 ± 0.44	<b>C</b>	$\alpha$
Gallotannin	15.13 ± 0.13	<b>C</b>	$\alpha$	16.95 ± 0.39	<b>D</b>	$\beta$

All data are the mean ± SD of three replicates. Different capital letters indicate the existence of statistically significant differences ( $p < 0.05$ ) between the different tannins. Different Greek letters indicate the existence of statistically significant differences ( $p < 0.05$ ) between times of treatment.

copigmentation index of seed tannin, skin tannin and gallotannin increases significantly, probably because the staking structures responsible for the copigmentation effect need time to form completely or because new pigments with a greater contribution to the color are formed (at least in the case of the case of seed tannin).

## CONCLUSION

It can be concluded that all the oenological tannins investigated are good copigments, because they improve the color of a malvidin-3-*O*-glucoside solution. The improvement in color was dose-dependent for each of the tannins, because it was greater when the copigment concentration was higher. Vignault *et al.* (2019) obtained similar results and have shown that pH and ethanol content also have an important influence on the copigmentation effectiveness of oenological tannins. The effect on color varied depending on the botanical origin of the tannins and their chemical structure, probably because some of the tannins can form new pigments with malvidin-3-*O*-glucoside. Specifically, the changes in malvidin-3-*O*-glucoside concentration and color parameters between days 1 and 7 suggest that seed tannin, quebracho tannin and ellagitannin can form new pigments, whereas skin tannin and gallotannin do not seem to do.

Further studies, using not only synthetic media but also real wine, are required to expand our knowledge of the mechanisms by which oenological tannins can improve wine color. It seems that the improvement in color exerted by each of the oenological tannins investigated in the present study was mainly due to the copigmentation effect, because the decrease in malvidin-3-*O*-glucoside concentration was

always very low (6.6% in the case of the maximal decrease, with a mean value below 2%). The results prove that supplementation with oenological tannins is an interesting tool for improvement of the color of red wines, and therefore these findings point to the need to include this functionality of oenological tannins in the OIV *International Oenological Codex*.

Finally, this study proposes a copigmentation index (see the equation) to measure the actual effectiveness of commercial tannins, which could be useful for tannin manufacturers and potential users of oenological tannins. The index is based on the total color difference ( $\Delta E_{ab}^*$ ) between each copigment solution and a pure white color solution; it is the best parameter for determining true differences between the color of two solutions, because it reflects the Euclidian distance between two points in the CIELab space. The index also accounts for the percentage increase in  $\Delta E_{ab}^*$  in response to supplementation with the highest dose of tannins (400 mg/L), thereby facilitating its quantification.

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