

# Comprehensive Colorimetric Study of Anthocyanic Copigmentation in Model Solutions. Effects of pH and Molar Ratio

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**ABSTRACT:** New colorimetric variables have been defined in the uniform CIELAB color space to assess the quantitative and qualitative color changes induced by copigmentation and their incidence on visual perception. The copigmentation process was assayed in model solutions between malvidin 3-glucoside and three phenolic compounds (catechin, epicatechin, and caffeic acid) as a function of the pH and the pigment/copigment molar ratio. Along the pH variation, the greatest magnitude of copigmentation was obtained at pH 3.0, being significantly higher with epicatechin and caffeic acid. At high acidic pH, the main contribution of copigmentation to the total color was qualitative, whereas between pH 2.0 and 4.0, the main colorimetric contribution was quantitative. The contribution of epicatechin and caffeic acid to the color changes was more marked for the quantitative characteristics. On contrast, particularly at higher pH values, the qualitative contribution was more important in catechin copigmented solutions. Increasing copigment concentration induced perceptible color changes at molar ratios higher than 1:2, consisting in a bluish and darkening effect of the anthocyanin solutions. Among the different CIELAB attributes, hue difference was the best correlated parameter with the increase of copigment concentration, proving the relevance of this physicochemical phenomenon on the qualitative changes of anthocyanin color.

**KEYWORDS:** anthocyanin, color, copigmentation, Tristimulus Colorimetry

## INTRODUCTION

Anthocyanins are the pigments accounting for the brilliant red, purple, and blue colors in many fruits, vegetables, and derived food products such as fruits juices, jams, and red wines.<sup>1</sup>

It is well-known that the stability of anthocyanic pigments is greatly limited because their resonating structure confers intrinsic instability against several physical–chemical conditions. Evaluation of the factors affecting the stability of anthocyanins indicates that pH is the most important extrinsic factor of anthocyanin degradation. Depending on the pH of the medium, the red-colored flavylium cation coexists as an equilibrium mixture with other forms of anthocyanins: the blue-purple quinonoidal bases, the colorless hemiacetal B, and the pale yellow chalcones. Therefore, the same anthocyanin solution may show different colors.<sup>2</sup>

However, the chemical and colorimetric stability of anthocyanins can be improved by associations with other substances. The copigmentation phenomenon, among others, represents one of the most complex and efficient mechanisms of anthocyanin chromophore stabilization in nature and food systems.<sup>3</sup> In food science, this phenomenon is considered to be a relevant interaction because obtaining stable and attractive colors is a major focus for quality control purposes.<sup>4</sup> Especially, in winemaking, it is assumed that it plays a key role in the color evolution and stability of young red wines.<sup>5</sup>

Copigmentation reactions consist of noncovalent interactions between anthocyanins among themselves (self-association), between the central anthocyanin chromophore and aromatic acyl residues covalently linked to their glycosyl moieties (intramolecular copigmentation), or through intermolecular interaction with a wide variety of colorless organic

compounds named copigments or copigmentation cofactors (basically other phenolic compounds, but also amino acids, sugars, organic acids, polysaccharides, etc.).<sup>4–7</sup>

From a molecular point of view, the anthocyanin–copigment complexes adopt a typical sandwich configuration ( $\pi$ – $\pi$  stacking) via hydrophobic interaction. This structural conformation protects the red-colored flavylium cation against the nucleophilic attack of water, peroxide and sulfur dioxide bleaching, and pH changes, reducing the formation of the other colorless species in the anthocyanin equilibrium (hemiacetal and chalcone).<sup>6</sup>

In addition, copigmentation not only confers greater stability to anthocyanins but also induces color variations. This kind of molecular association is responsible for the typical changes in the spectral properties of the chromophore group, that is, an increase of the absorptivity and frequently a shift of the visible  $\lambda_{\max}$  toward greater wavelengths. Consequently, copigmentation produces both quantitative and qualitative color changes in anthocyanin solutions. In this sense, the measurement and evaluation of these colorimetric changes is of great interest to the food industry because color is one of the main sensory parameters for the quality of foods influencing customer selection.<sup>7</sup>

The contribution of the copigmentation phenomenon in color has been widely studied using spectrophotometric methods, in both model and food systems that contain

**Received:** November 11, 2011

**Revised:** February 29, 2012

**Accepted:** February 29, 2012

**Published:** February 29, 2012

individual anthocyanins and added copigments or in red wines in which anthocyanins naturally co-occur with other phenolic compounds.<sup>8–15</sup> The evaluation of the changes in the visible spectrum of anthocyanins, or specifically in the  $\lambda_{\text{max}}$  (520 nm), has permitted the determination of the influence of numerous factors on the effectiveness of the copigmentation including the concentrations of pigments and cofactors, their chemical structures, the cofactor/pigment molar ratio, the pH of the medium, etc. In the same way, several studies in red wines have shown that the magnitude of copigmentation and its evolution during winemaking is extremely variable according to viticultural, agronomical, or enological practices, accounting for approximately 25–50% of the total color of young red wines.<sup>5,16–20</sup>

In any case, although spectral methods have been demonstrated to be valid, simple, and quick tools to quantitative estimations, it is generally accepted that they provide limited precision and accuracy for color specifications. The lower precision and accuracy achievable can be explained because an adequate description of the color variations requires (i) that spectral variations considered should be those affecting the entire spectral curve, not only its visible  $\lambda_{\text{max}}$  and (ii) the use of at least three colorimetric attributes: hue, saturation, and lightness.<sup>21</sup> Moreover, the specific changes at the  $\lambda_{\text{max}}$  are also often interpreted incorrectly in colorimetric terms. It has been described that the hyperchromic and bathochromic effects make anthocyanin solutions appear bluer and with more intense color.<sup>3,22,23</sup> However, the variation in a single wavelength does not explain the complete behavior of the color due to this phenomenon.

For these reasons, to advance the knowledge of the global colorimetric role of copigmentation it is necessary to consider both quantitative and qualitative color changes. In this context, Tristimulus Colorimetry, which is based on transmittance values of the whole spectra, represents a useful methodology that widely improves the objective analysis of color.

Thus, through Tristimulus Colorimetry, in this study is performed a precise colorimetric interpretation of the copigmentation phenomenon using the uniform 1976- $(L^*a^*b^*)$  color space (CIELAB), which has been recommended by the Commission Internationale de l'Éclairage (CIE) as a more appropriate tool for color specification in most industrial applications. For this purpose, diverse colorimetric variables based on both the rectangular ( $L^*$ ,  $a^*$ ,  $b^*$ ) and cylindrical ( $L^*$ ,  $C^*_{ab}$ ,  $h_{ab}$ ) color coordinates have been defined to assess the quantitative and qualitative color implications associated to copigmentation and their incidence on visual perception. These colorimetric variables have been applied and compared with the most used simplified method in the evaluation of the copigmentation effects in model solutions.

For the model solution assay, the pigment chosen was malvidin 3-glucoside, one of the six most common anthocyanins in nature, fruits, and vegetables. The flavanols catechin, epicatechin, and caffeic acid were also selected because they are some of the main polyphenolic copigments described, especially in red wines.

## MATERIALS AND METHODS

**Standards and Copigmented Models Solutions.** The pigment malvidin 3-glucoside (Mv 3-gl) was isolated in the laboratory from skins of *Vitis vinifera* red grapes of the Tempranillo variety. Extraction was made with acidic methanol (methanol/HCl 1 N; 95:5 v/v), and the extracts were purified by semipressure liquid chromatography

using a reversed-phase column, as described by Heredia et al.<sup>24</sup> The copigments (+)-catechin, (–)-epicatechin, and caffeic acid were purchased from Sigma Chemical Co. (St. Louis, MO).

All of the model solutions were prepared in a wine-like medium containing 5 g/L tartaric acid in 12% ethanol with ionic strength adjusted to 0.2 M by the addition of sodium chloride.

To evaluate the effect of the pH on the copigmentation phenomenon, three copigmented solutions of Mv 3-gl/(+)-catechin (MC), Mv 3-gl/(–)-epicatechin (ME), and Mv 3-gl/caffeic acid (MF), as well as a reference solution (Mv 3-gl), were prepared in wine-like medium at different pH values: 1.0, 2.0, 3.0, 4.0, and 5.0. The reference solution contained 200 mg/L (0.41 mM) Mv 3-gl. Copigmented solutions contained the same anthocyanin concentration and the corresponding copigment using a pigment/copigment molar ratio of 1:5.

The effect of the copigment concentration was also assessed. Two copigmented solutions of Mv 3-gl/(+)-catechin (MC) and Mv 3-gl/(–)-epicatechin (ME) and a reference solution were prepared in the same wine-like medium adjusted to pH 3.60. Copigmented solutions contained the same anthocyanin concentration (0.41 mM) and the corresponding copigments to give the required pigment/copigment molar ratios: 1:1, 1:2, 1:5, and 1:7.

All of the solutions were prepared in triplicate and equilibrated to reach the equilibrium for 2 h and stored closed in darkness at 25 °C, after which their absorption spectra were recorded.

**Color Analysis.** The absorption spectra (380–770 nm) of all the model solutions were recorded at constant intervals ( $\Delta\lambda = 2$  nm) with a Hewlett-Packard UV-vis HP8452 spectrophotometer (Palo Alto, CA), using 2 mm path length glass cells and distilled water as a reference. The CIELAB parameters ( $L^*$ ,  $a^*$ ,  $b^*$ ,  $C^*_{ab}$ , and  $h_{ab}$ ) were determined by using CromaLab software,<sup>25</sup> following the recommendations of the Commission Internationale de l'Éclairage:<sup>26</sup> the 10° Standard Observer and Standard Illuminant D65.

The  $L^*$  value is the vertical axis and defines the lightness, the property according to which each color can be considered as equivalent to a member of the gray scale, between black and white, taking values within the range of 0–100, respectively. The  $a^*$  and  $b^*$  values represent the chromaticity scalar coordinates, which in turn represent opponent red–green and blue–yellow scales.

From  $L^*$ ,  $a^*$ , and  $b^*$ , other parameters are defined, such as hue ( $h_{ab}$ ) and chroma ( $C^*_{ab}$ ). Hue angle ( $h_{ab}$ ) is the attribute according to which colors have been traditionally defined as red, green, etc. On the other hand, chroma ( $C^*_{ab}$ ) is the attribute that allows each hue to be determined by its degree of difference in comparison to a gray color with the same lightness. Moreover, these colorimetric parameters can be distinguished as quantitative or qualitative color attributes as they indicate quantitative ( $L^*$  and  $C^*_{ab}$ ), or qualitative ( $h_{ab}$ ) contributions to color.

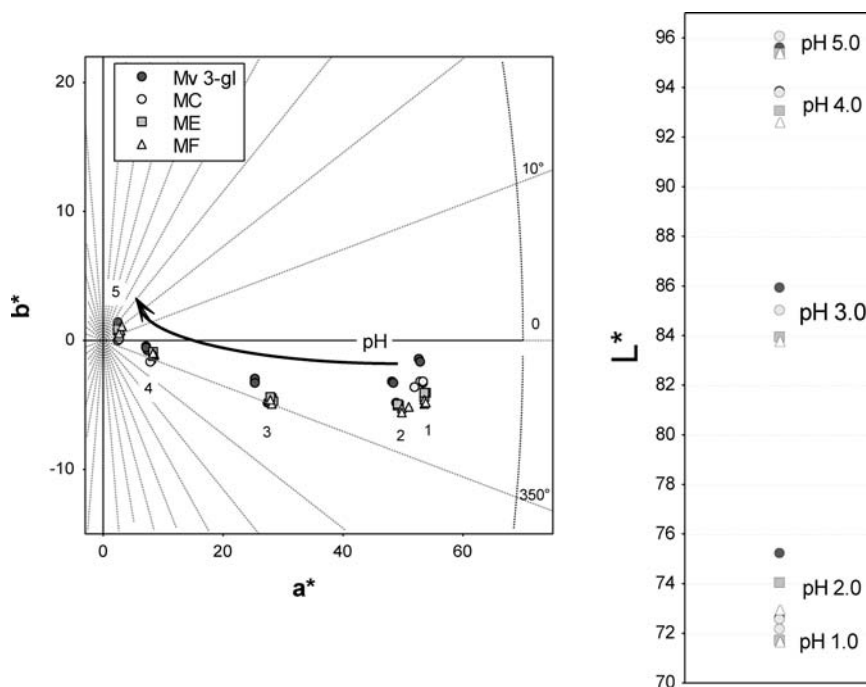
Color difference, which is very important to evaluate relationships between visual and numerical analyses,<sup>27</sup> was determined by means of the CIE76 color difference parameter ( $\Delta E^*_{ab}$ ). It was calculated as the Euclidean distance between two points in three-dimensional space defined by  $L^*$ ,  $a^*$ , and  $b^*$ :  $\Delta E^*_{ab} = ((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2}$

**Colorimetric Analysis of Copigmentation in the CIELAB Color Space.** The colorimetric effect of copigmentation was evaluated by comparing the color of the pure anthocyanin solutions and the color of the same solutions containing different copigment concentrations.

We consider a new variable  $E$ , which expresses the “total color” of a solution as the color difference between the corresponding  $L^*$ ,  $a^*$ , and  $b^*$  values with respect to distilled water ( $L^* = 100$ ,  $a^* = 0$ ,  $b^* = 0$ ). Therefore, the total color of the copigmented and noncopigmented solutions was expressed as  $E_C$  and  $E_0$ , respectively. From  $E_C$  and  $E_0$ , the percentage of the anthocyanin color solutions that is due to copigmentation was calculated as the following equation:

$$CCI = ((E_C - E_0)/E_0) \times 100$$

The absolute color variation induced by copigmentation was assessed as the CIELAB color difference formula ( $\Delta E^*_{ab}$ ) applied



**Figure 1.** Change in the location of the pure Mv 3-gl and copigmented solutions (MC, ME, and MF) within (left panel) the ( $a^*b^*$ ) diagram and (right panel) lightness values ( $L^*$ ) as a function of the pH value.

between the color of the copigmented and noncopigmented anthocyanin solutions, as follows:

$$\Delta E_{ab(C-0)}^* = ((L_C^* - L_0^*)^2 + (a_C^* - a_0^*)^2 + (b_C^* - b_0^*)^2)^{1/2}$$

In the same way, the absolute lightness, chroma, and hue differences ( $\Delta L^*$ ,  $\Delta C_{ab}^*$ , and  $\Delta h_{ab}$ ) were used to assess the trend of the color changes induced by copigmentation. Specifically,  $\Delta h_{ab}$  is the difference between two hues, in sexagesimal degrees.

In addition, the relative contribution of the three color attributes that make up the total CIELAB color difference was also calculated to compare the copigmentation effect according to different factors. Thus, the weight of each color attribute was calculated as

$$\% \Delta L = ((\Delta L)^2 / (\Delta E_{ab}^*)^2) \times 100$$

$$\% \Delta C = ((\Delta C)^2 / (\Delta E_{ab}^*)^2) \times 100$$

$$\% \Delta H = ((\Delta H)^2 / (\Delta E_{ab}^*)^2) \times 100$$

$\Delta H$  being deduced from  $\Delta E_{ab}^*$ ,  $\Delta L$ , and  $\Delta C$  values as follows:

$$\Delta H = ((\Delta E_{ab}^*)^2 - ((\Delta L)^2 + (\Delta C)^2))^{1/2}$$

Therefore,  $\Delta H$ ,  $\Delta L$ , and  $\Delta C$  are scalar magnitudes in CIELAB units.

**Statistical Analysis.** For the statistical treatment of the data, Statistica v. 8.0 software<sup>28</sup> was used.

## RESULTS AND DISCUSSION

**Effect of pH and the Chemical Structure of Copigment on Copigmentation.** A color analysis of the Mv 3-gl copigmented with different phenolic compounds was performed in the CIELAB space at different pH values (from 1 to 5 units) to evaluate the influence of the copigmentation reaction on the anthocyanin equilibria. Figure 1 depicts the location of the pure Mv 3-gl solution and its corresponding copigmentation with catechin (MC), epicatechin (ME), and

caffeic acid (MF) within the ( $a^*b^*$ ) diagram and lightness values ( $L^*$ ) according to pH value.

As previously reported by Heredia,<sup>24</sup> pH variations provoke important changes in both quantitative and qualitative psychophysical components of the anthocyanin color. As the pH increases, the total color ( $E$ ) of the pure Mv 3-gl solution strongly decreased from 59.51 to 5.12 units, the progressive color degradation being especially remarkable at pH values  $>3.0$  (Table 1). However, the addition of the different copigments increased significantly the total color of the pure Mv 3-gl solution, confirming the protective effect of the copigmentation phenomenon against color degradation.

As can be seen in Figure 1, between pH 1.0 and 3.0, the color of the pure Mv 3-gl solution underwent a slight decrease to blue hues and became less vivid and lighter. This color evolution reflects the kinetic and thermodynamic competition between the flavylium cation and the other colorless species in the anthocyanin equilibria, that is, hemiacetals, chalcones, and the blue anionic quinonoidal bases.<sup>2</sup> However, at this pH range, a notable displacement of all copigmented solutions with respect to the pure Mv 3-gl solution was produced in the CIELAB space, which tended progressively toward 350° color area ( $-10^\circ$ ). Therefore, whereas the pH effect diminishes the values of  $a^*$  and increases the values of  $L^*$ , which agrees with the increase of the discoloration (evolution to achromatic colors), copigmentation mainly diminishes the values of  $L^*$  and  $b^*$ . As a consequence, pure Mv 3-gl solutions exhibited the darkest and more vivid bluish color when they were in the presence of the copigments, revealing the positive influence of the copigmentation phenomenon on the anthocyanin equilibrium and, thus, on its color. Specifically, at pH 3.0, the total color ( $E$ ) increased significantly from 29.1 (Mv 3-gl) to 31.8, 32.7, and 32.8 units, respectively.

On the contrary, according to their location in the ( $a^*b^*$ ) colorimetric diagram, at pH values  $>3$  units, both copigmented and noncopigmented solutions appear to be more grouped in

**Table 1. Mean Values of the  $A_{520\text{nm}}$  and the Total Color ( $E$ ) Obtained for the Pure Anthocyanic Solution and Its Respective Copigmentation at Each pH Value (MV 3-gl: 200 mg/L, Molar Ratio 1:5) as well as the CIELAB Differences ( $\Delta E^*_{ab}$ ,  $\Delta L^*$ ,  $\Delta C^*_{ab}$ ,  $\Delta h_{ab}$ ) between Mv 3-gl Solutions with and without Copigments**

|                    | pH | pigment        |                 | pigment/copigment |                 |
|--------------------|----|----------------|-----------------|-------------------|-----------------|
|                    |    | Mv 3-gl        | MC              | ME                | MF              |
| $A_{520\text{nm}}$ | 1  | 0.86 ± 0.001 a | 0.84 ± 0.001 b  | 0.84 ± 0.008 b    | 0.83 ± 0.004 b  |
|                    | 2  | 0.73 ± 0.002 a | 0.78 ± 0.03 a   | 0.73 ± 0.002 a    | 0.77 ± 0.03 a   |
|                    | 3  | 0.32 ± 0.001 a | 0.34 ± 0.001 b  | 0.36 ± 0.004 c    | 0.37 ± 0.003 c  |
|                    | 4  | 0.10 ± 0.003a  | 0.10 ± 0.003 a  | 0.11 ± 0.001 b    | 0.12 ± 0.005b   |
|                    | 5  | 0.06 ± 0.001 a | 0.05 ± 0.005a   | 0.06 ± 0.001 a    | 0.05 ± 0.002 a  |
| $E$                | 1  | 59.51 ± 0.98 a | 60.10 ± 0.23 b  | 60.77 ± 0.26 c    | 60.79 ± 0.16 c  |
|                    | 2  | 54.37 ± 1.67 a | 57.51 ± 1.77 b  | 55.83 ± 0.17 b    | 57.88 ± 1.42 b  |
|                    | 3  | 29.13 ± 0.98 a | 31.85 ± 0.17 b  | 32.68 ± 0.34 c    | 32.79 ± 0.20 c  |
|                    | 4  | 9.47 ± 1.67 a  | 10.22 ± 0.27 b  | 10.82 ± 0.01 c    | 11.06 ± 0.38c   |
|                    | 5  | 5.12 ± 0.60 a  | 4.93 ± 0.38 a   | 5.40 ± 0.12 a     | 5.52 ± 0.47 a   |
| $\Delta E^*_{ab}$  | 1  |                | 1.73 ± 0.06 a   | 2.88 ± 0.04 b     | 3.39 ± 0.13 c   |
|                    | 2  |                | 2.92 ± 0.48 a   | 2.29 ± 0.12 a     | 3.78 ± 1.06 a   |
|                    | 3  |                | 3.06 ± 0.17 a   | 3.69 ± 0.37 b     | 3.87 ± 0.24 b   |
|                    | 4  |                | 1.29 ± 0.08 a   | 1.38 ± 0.02 a     | 1.59 ± 0.27 a   |
|                    | 5  |                | 1.01 ± 0.14 a   | 0.54 ± 0.09 a     | 0.71 ± 0.14 a   |
| $\Delta L^*$       | 1  |                | -0.46 ± 0.11 a  | -0.91 ± 0.29 ab   | -0.95 ± 0.10 b  |
|                    | 2  |                | -2.64 ± 0.23 a  | -1.16 ± 0.02 a    | -2.27 ± 0.82 a  |
|                    | 3  |                | -0.89 ± 0.01 a  | -1.97 ± 0.20 b    | -2.14 ± 0.13 b  |
|                    | 4  |                | -0.03 ± 0.20 a  | -0.77 ± 0.21 b    | -1.03 ± 0.40 b  |
|                    | 5  |                | +0.23 ± 0.28 a  | -0.29 ± 0.10 a    | -0.20 ± 0.40 a  |
| $\Delta C^*_{ab}$  | 1  |                | +0.41 ± 0.21 a  | +0.95 ± 0.15 b    | +0.94 ± 0.14 b  |
|                    | 2  |                | +0.47 ± 0.20 a  | +1.03 ± 0.19 a    | +2.77 ± 1.17 b  |
|                    | 3  |                | +2.59 ± 0.18 a  | +2.95 ± 0.27 a    | +2.98 ± 0.15 a  |
|                    | 4  |                | +0.91 ± 0.19 a  | +1.09 ± 0.01 a    | +1.17 ± 0.16 a  |
|                    | 5  |                | +0.02 ± 0.06 a  | +0.05 ± 0.01 a    | +0.44 ± 0.24 b  |
| $\Delta h_{ab}$    | 1  |                | -1.74 ± 0.07 a  | -2.74 ± 0.20 b    | -3.35 ± 0.15 c  |
|                    | 2  |                | -1.30 ± 0.75 a  | -1.93 ± 0.07 a    | -1.99 ± 0.53 a  |
|                    | 3  |                | -2.91 ± 0.08 a  | -2.12 ± 0.29 b    | -2.61 ± 0.35 ab |
|                    | 4  |                | -6.65 ± 0.65 a  | -2.75 ± 0.84 b    | -2.20 ± 0.17 b  |
|                    | 5  |                | -17.28 ± 2.30 a | -5.56 ± 0.92 b    | -6.41 ± 3.07 b  |

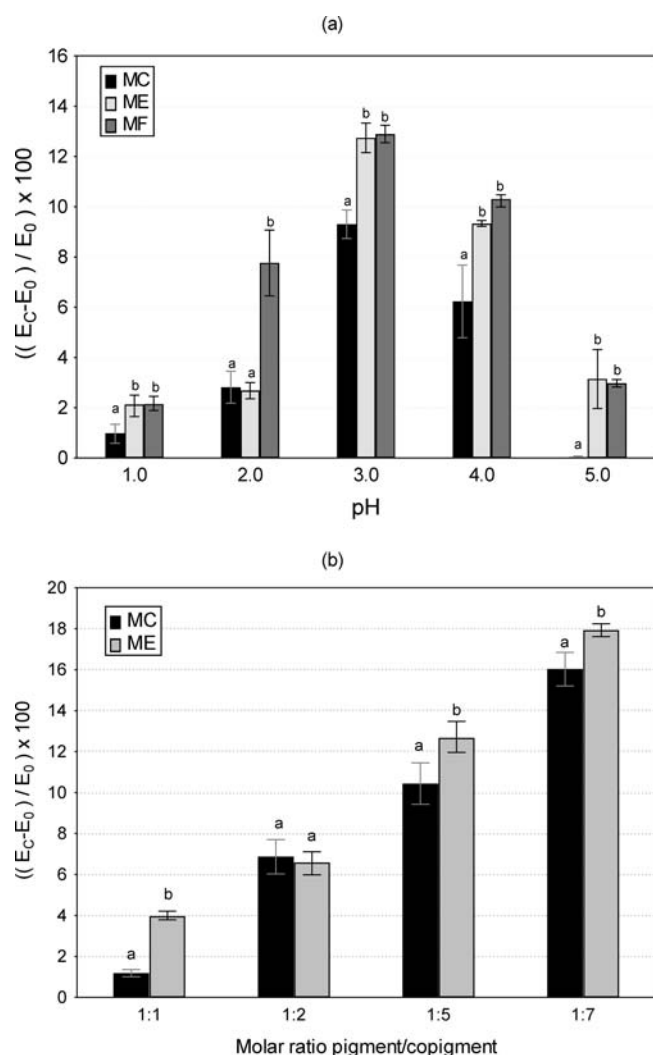
the same color area nearer the coordinates origin, reflecting that the effect of the copigmentation was less relevant, being almost imperceptible at pH 5.0. At this pH range, all of the anthocyanin solutions became more and more achromatic ( $a^*$  and  $b^*$  tended toward zero) and clearer ( $L^*$  increases) and had a lower color intensity, which means a progressive loss of color.

Figure 2 shows the change in the magnitude of copigmentation for the copigmented solutions evaluated by Tristimulus Colorimetry as the ratio  $((E_C - E_0)/E_0) \times 100$ . The results showed that copigmentation occurred over the entire pH range we have studied for all of the phenolics used as copigments. However, the magnitude of the copigmentation and its colorimetric effect was strongly pH-dependent and influenced by the nature of the copigment used. The greatest magnitude of copigmentation was obtained at pH 3.0, being significantly higher with epicatechin and caffeic acid. They both increased respectively the total color of Mv 3-gl by 13.7 and 13.8%, whereas catechin, which was the less effective copigment, reached an increase of only 9.3%. This agrees with other studies that have shown that among the different

flavan-3-ol copigments, the more planar molecules such as (-)-epicatechin or with electron-donor substituents such as cinnamic acids can better stack with anthocyanins, resulting in higher copigmentation effect.<sup>11,29-31</sup> The slightly lower values of the copigmentation magnitude obtained in this study with respect to those reported in the literature could be due to both the lower pigment/copigment molar ratio used and the chemical nature of the tested copigments. It has been established that the differences in the number, size, or spatial location of the substituents make monomeric flavan-3-ols, such as catechin, epicatechin, or simple phenolic acids, exhibit weaker copigmentation than more planar phenolic compounds such as flavonols.<sup>32</sup>

As shown in Table 1, the CIELAB differences ( $\Delta E^*_{ab}$ ,  $\Delta L^*$ ,  $\Delta C^*_{ab}$ ,  $\Delta h_{ab}$ ) between Mv 3-gl solutions with and without copigments were also calculated. Along the pH variation, the highest color differences ( $\Delta E^*_{ab}$ ) were produced from pH 1.0 to 3.0, confirming the colorimetric stabilization of the flavylium ion by copigmentation at lower rather than higher pH values. Specifically, the highest color differences were reached at pH 3.0, being 3.1, 3.7, and from 3.4 units in MC, ME, and MF

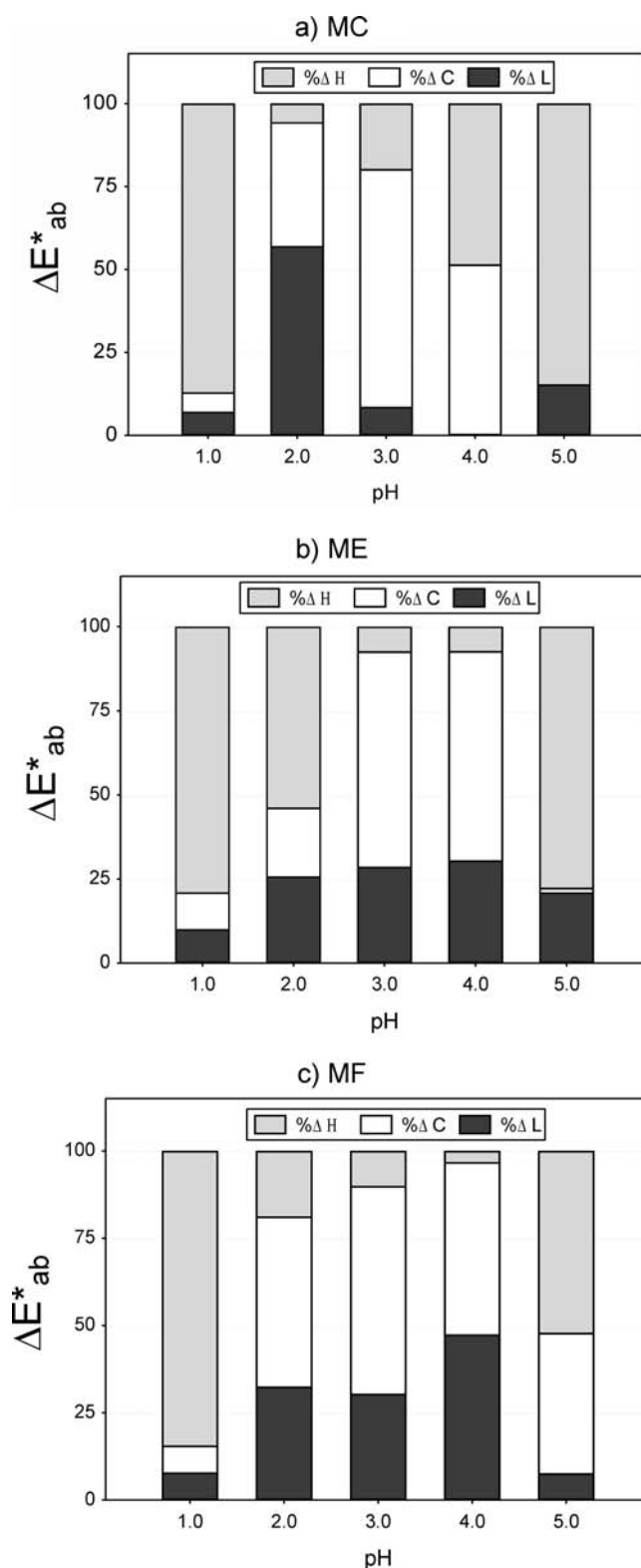




**Figure 2.** Changes in the magnitude of copigmentation for the Mv 3-gl solutions copigmented with catechin, epicatechin, and caffeic acid (MC, ME, and MF) (a) as a function of the pH and (b) as a function of the molar ratio, evaluated by Tristimulus Colorimetry as the ratio  $((E_C - E_0) / E_0) \times 100$ .

solutions, respectively. Therefore, although flavan-3-ols have been usually considered to be poorer anthocyanin copigments, at most acidic pH values, the color changes induced were always perceptible to the human eye.<sup>33</sup> However, at pH values  $>3.0$  units, there was no significant difference between the color effect induced by the flavanols tested, all of the color differences being  $<2$  units, that is, not visually distinguished. At higher pH values it could be expected a more marked effect of copigmentation due to the higher proportion of colorless anthocyanin. However, this is not always the case since each copigment has a particular optimum conditions and effectiveness of copigmentation which is determined by several factors (stereochemistry, pK value, etc.), as reported by Mazza et al.<sup>9</sup>

In general, caffeic acid produced the largest color effects, which was coherent with the results obtained about the magnitude of copigmentation when it was assessed by Tristimulus Colorimetry. On the contrary, as can be checked in Table 1, at some pH values, whereas color changes were detected in the CIELAB color space, no changes at  $A_{520nm}$  were observed. This divergence between the two analytical methods has been previously reported by González-Manzano<sup>14</sup> and



**Figure 3.** Relative contribution of lightness (% $\Delta L$ ), chroma (% $\Delta C$ ), and hue (% $\Delta H$ ) to the total color difference for each pigment/copigment as a function of the pH value.

confirms that the most simplified methods not always can achieve a reliable evaluation of the copigmentation process.

For a more comprehensive analysis of the colorimetric implication of copigmentation process as a function of the pH, the relative contributions of lightness (% $\Delta L$ ), chroma (% $\Delta C$ ),

**Table 2.** Mean Values of the  $A_{520\text{nm}}$  and the Total Color ( $E$ ) Obtained for the Pure Anthocyanic Solution and Its Respective Copigmented Solution at Each Molar Ratio (Mv 3-gl: 200 mg/L, pH 3.6); as well as the CIELAB Differences ( $\Delta E^*_{ab}$ ,  $\Delta L^*$ ,  $\Delta C^*_{ab}$ ,  $\Delta h_{ab}$ ) between Mv 3-gl Solutions with and without Copigments

| colorimetric parameter |                    | molar ratio     |                |                 |                |                |
|------------------------|--------------------|-----------------|----------------|-----------------|----------------|----------------|
|                        |                    | 0               | 1              | 2               | 5              | 7              |
| MC                     | $A_{520\text{nm}}$ | 0.66 ± 0.001 a  | 0.68 ± 0.001 a | 0.77 ± 0.008 b  | 0.77 ± 0.008 b | 0.84 ± 0.004 c |
|                        | $E$                | 49.26 ± 0.45 a  | 49.84 ± 0.12 a | 52.64 ± 0.58 b  | 54.40 ± 0.50 b | 57.32 ± 0.90 c |
|                        | $\Delta E^*_{ab}$  |                 | 1.01 ± 0.33 a  | 4.89 ± 0.37 b   | 6.36 ± 0.19 b  | 9.76 ± 1.27 c  |
|                        | $\Delta L^*$       |                 | -0.16 ± 0.54 a | -4.51 ± 0.33 b  | -3.95 ± 0.66 b | -8.56 ± 0.99 c |
|                        | $\Delta C^*_{ab}$  |                 | +0.60 ± 0.26 a | +0.65 ± 1.06 a  | +3.40 ± 1.17 a | +2.59 ± 3.60 a |
|                        | $\Delta h_{ab}$    |                 |                | -1.21 ± 0.60 a  | -2.56 ± 0.99 a | -5.05 ± 0.13 b |
| ME                     | $A_{520\text{nm}}$ | 0.67 ± 0.004 a  | 0.72 ± 0.01 b  | 0.76 ± 0.004 c  | 0.78 ± 0.008 d | 0.82 ± 0.002 e |
|                        | $E$                | 49.26 ± 0.001 a | 51.21 ± 0.10 b | 52.51 ± 0.28 c  | 55.50 ± 0.38 d | 57.90 ± 0.36 e |
|                        | $\Delta E^*_{ab}$  |                 | 2.27 ± 0.20 a  | 4.53 ± 0.91 b   | 6.65 ± 0.33 c  | 10.69 ± 0.44 d |
|                        | $\Delta L^*$       |                 | -1.77 ± 0.23 a | -3.99 ± 1.12 b  | -4.16 ± 0.37 b | -5.53 ± 0.24 b |
|                        | $\Delta C^*_{ab}$  |                 | +1.08 ± 0.30 a | +0.90 ± 0.95 a  | +4.67 ± 0.34 b | +6.64 ± 0.63 c |
|                        | $\Delta h_{ab}$    |                 | -1.02 ± 0.54 a | -2.39 ± 0.83 ab | -3.11 ± 0.47 b | -3.38 ± 0.50 b |

and hue ( $\% \Delta H$ ) to the total color difference for each pigment/copigment solution were also calculated. As can be seen in Figure 3, the main contribution to the measured color differences  $\Delta E^*_{ab}$  at pH 1.0 was qualitative, which was evidenced by the significantly higher contribution of hue  $\% \Delta H$  (80–88%) with respect to lightness  $\% \Delta L$  or chroma  $\% \Delta C$  (7–10 and 6–10%, respectively). These results are coherent because at pH < 2, the anthocyanin exists primarily in the form of the red cation flavylium and the amount of the other colorless species is almost nonexistent. Therefore, at these pH values, the copigmented complexes do not provide important displacement of the anthocyanin equilibrium toward the red-colored flavylium cation, which is also reflected by the lower variations obtained for  $E$  and  $\Delta E^*_{ab}$ . However, an interesting observation is that between pH 1.0 and 2.0, for which the distribution between anthocyanin forms should be very similar, the colorimetric changes induced by copigmentation were different. At pH 2.0, quantitative color changes ( $\% \Delta C$  and  $\% \Delta L$ ) became more pronounced, especially for MC and MF copigmented solutions. As reported by some authors, at a given copigment-to-pigment molar ratio and a given pH, the color will be characteristic, not only of malvin but also of the type of associated copigment.<sup>8,22</sup> In this way, an infinite variety of colors may be produced by only one anthocyanin associated with various copigments. In this context, another interesting observation is that these different colorimetric changes could be revealed by Tristimulus Colorimetry instead of other simplified methods, confirming that it is a useful and objective technique for assessing the integral copigmentation effect on color.

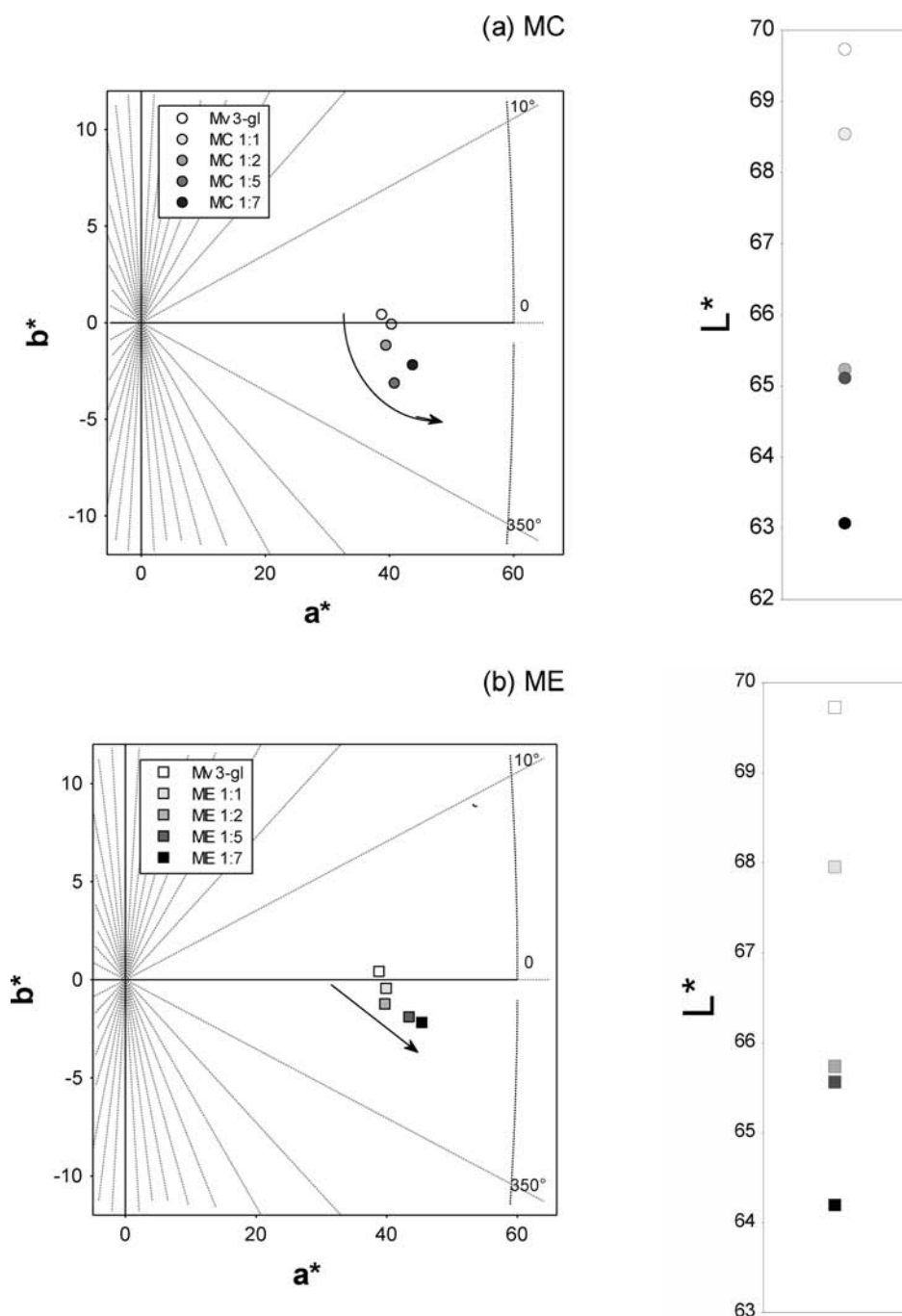
On the other hand, between pH 2.0 and 4.0, the main colorimetric contribution was quantitative, the chroma modifications  $\% \Delta C$  being particularly more marked than lightness  $\% \Delta L$  (35–65 and 22–38%, respectively). In this pH range, the proportion of the other colorless species increases, coexisting with the red-colored flavylium cation in solution, so copigmentation complexes can provide at the same time color stabilization and variation, which coincides with the greatest changes in the  $E$  and  $\Delta E^*_{ab}$  values. At pH 5.0, the most abundant colored species present is the quinoidal form, and thus Mv 3-gl itself does not confer much color to a solution; so again, the contribution of the copigmentation to color is mainly qualitative, that is, significantly due to hue changes.

Moreover, the relative contributions of lightness, chroma, and hue permitted us to compare the colorimetric effect induced by the different flavanols tested. In general terms, epicatechin and caffeic acid produced similar effects on the total color of the Mv 3-gl across the pH changes, their contributions being more marked with quantitative changes than qualitative ones ( $\% \Delta L + \% \Delta C = 55\text{--}63\%$  and  $\% \Delta H = 45\text{--}37\%$ ). On the contrary, particularly at the higher pH values, the qualitative contribution was more important in catechin copigmented solutions.

**Effect of the Molar Ratio on Copigmentation.** The influence of the pigment/copigment molar ratio was also assayed at pH 3.60. For this purpose, two copigmented solutions of Mv 3-gl/catechin (MC) and Mv 3-gl/epicatechin (ME) were prepared in the same wine-like medium at molar ratios 1:1, 1:2, 1:5, and 1:7.

For both assays, the positive effect of the copigmentation on the Mv 3-gl total color was increased with increasing copigment concentration (Table 2), which is in agreement with previous studies reported in the bibliography despite the range of the pigment/copigment molar ratios used being lower.<sup>34</sup> The maximum displacement of hydration/dehydration equilibrium toward the colored species was achieved using the highest molar ratio (1:7) because it caused the greatest total color increases.

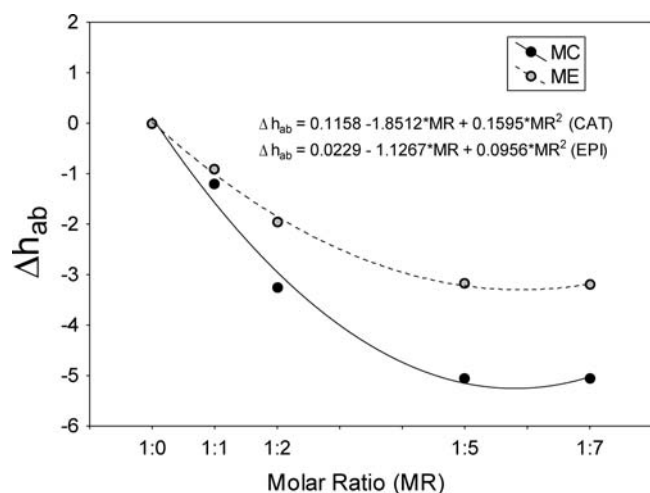
Moreover, significant differences in  $L^*$ ,  $a^*$ , and  $b^*$  values among the pure Mv 3-gl solution and its corresponding copigmented solution were found, especially at molar ratios higher than 1:2, resulting in a different location of the samples in the ( $a^*b^*$ ) colorimetric diagram (Figure 4). In the absence of any copigments, the original anthocyanin color took positive  $a^*$  and  $b^*$  values and appeared located near  $0^\circ$ , that is, in a well-defined reddish color area ( $L^* = 69.71$ ,  $a^* = 38.84$ , and  $b^* = 0.39$  CIELAB). However, with the increasing addition of the flavanol copigments, the samples appeared located progressively farther from the coordinates origin and experienced a remarkable evolution from the first toward the fourth quadrant, that is, from the reddish to the purple or red-bluish color region. The behavior of the color parameters as a function of the concentration was similar for both copigments used. At the highest molar ratio (1:7), copigmented samples reached the lowest  $L^*$  and  $b^*$  values but the highest  $a^*$  values, and, thus, they exhibited the darkest and most vivid bluish color.



**Figure 4.** Change in the location of the pure Mv 3-gl and copigmented solutions (MC, ME) within (a) the  $(a^*b^*)$  diagram and (b) lightness values ( $L^*$ ) as a function of the molar ratio.

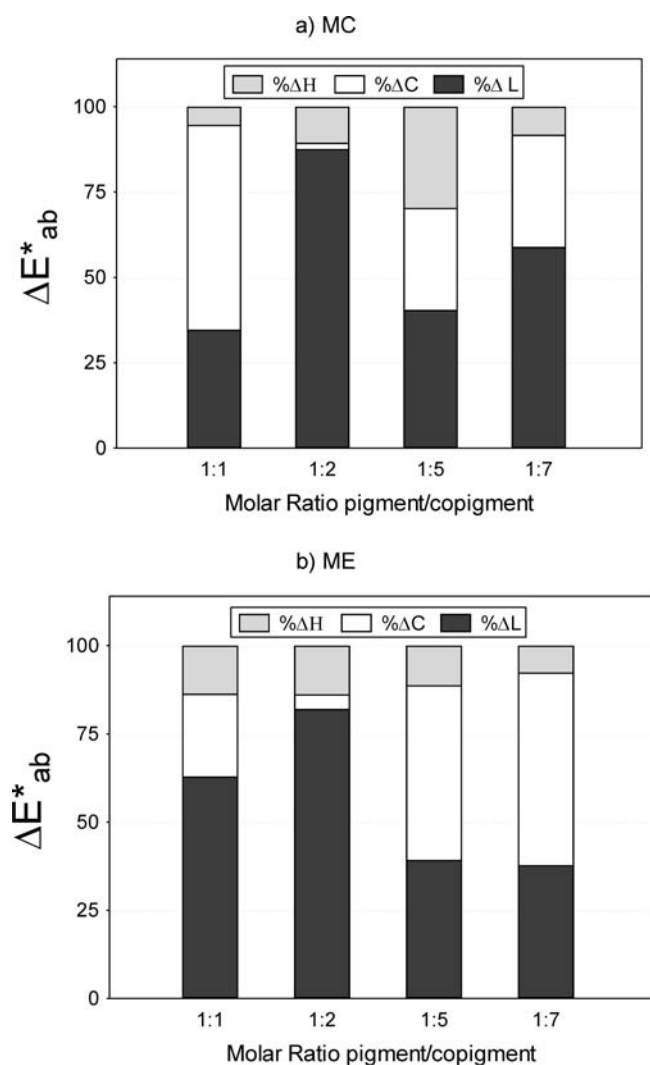
With regard to the changes in the contribution of the copigmentation to the total anthocyanin color, the added copigments caused significant concentration-dependent increases in the magnitude of the effect, as can be seen in Figure 2. From 1:1 to 1:7 molar ratios, catechin induced an increase of the Mv 3-gl total color from 1.2 to 16.0%, whereas with epicatechin, the increase was from 3.9 to 17.9%. Notwithstanding, although the pattern evolution was similar between both flavanol copigments, once again, the ability of the epicatechin to act as Mv-3gl copigment was stronger than that of catechin because significantly higher values for copigmentation were achieved for most of the molar ratios tested.

As noted in the previous section of this study, the dependence between the amplitude of the color effect induced by copigmentation was evaluated by means of the CIELAB color differences ( $\Delta E^*_{ab}$ ) (Table 2). As expected, the progressive formation of copigmentation complexes was confirmed by a successive increase in the color differences between anthocyanin and flavanol solutions when copigment concentrations were increasingly added. For all of the concentration levels tested, epicatechin caused always significant increases ( $p < 0.05$ ) in the color differences. In contrast, higher increases in the molar ratio were necessary to induce significant changes with catechin copigmented solutions.



**Figure 5.** CIELAB hue differences ( $\Delta h_{ab}$ ) as a function of molar ratio for Mv 3-gl/catechin (MC) and Mv 3-gl/epicatechin (ME) solutions.

In any case, calculation of the simple regression coefficients between total color differences and the copigment concen-



**Figure 6.** Relative contribution of lightness (% $\Delta L$ ), chroma (% $\Delta C$ ), and hue (% $\Delta H$ ) to the total color difference for each pigment/copigment as a function of the molar ratio.

tration for each copigmented solution confirmed that the relationship was significantly high with regard to the copigment used ( $r^*$  values ranged from 0.95 to 0.98 units,  $p < 0.05$ ). The lowest color differences were found between pure Mv 3-gl solution and the copigmented ones at the lowest molar ratio used (1:1), taking values of 1.0 and 2.3 units with catechin and epicatechin, respectively. However, the color differences obtained at all molar ratios higher than 1:1 were  $>3$  units and, hence, visually relevant. The largest color changes were induced with the highest copigment concentration (molar ratio 1:7), being slightly more marked with epicatechin (10.79 vs 9.76 units, in MC solutions). This was consistent with the higher values also obtained regarding the total color ( $E$ ) and the magnitude of copigmentation, although the difference was not significant.

In relation to the quantitative and qualitative color changes that the original Mv 3-gl solution underwent with increasing molar ratio, the tendency was always toward higher decreases of  $\Delta L^*$  and  $\Delta h_{ab}$ , which means an increasing bluish and darkening effect. Simultaneously, chroma differences  $\Delta C^*_{ab}$  tended toward higher increases resulting in an increasing vivid effect, although this tendency was less marked than lightness and hue trends.

Univariate correlations between individual color attributes differences ( $\Delta L^*$ ,  $\Delta C^*_{ab}$ ,  $\Delta h_{ab}$ ) and copigment concentration were explored to determine the significance of these changes. Across the different copigmented solutions, the best relationships were obtained with simple regression for quantitative attributes ( $\Delta L^*$ ,  $\Delta C^*_{ab}$ ) and with second-degree polynomial regression for the qualitative ( $\Delta h_{ab}$ ). Calculation of the coefficient regressions revealed that all of the relationships were strong and significant ( $p < 0.05$ ), except for those corresponding to  $\Delta C^*_{ab}$  for MC solutions. Regression coefficients took negative signs for lightness and hue differences, ranging as mean values between 0.71 and 0.95 and between 0.93 and 0.99, respectively; these values were positive for chroma differences. Among the different CIELAB attributes, hue differences ( $\Delta h_{ab}$ ) were the best correlated parameter because  $>95\%$  of cases showed high quadratic regression coefficients ( $R^2 = 0.99$ ), proving the relevance of this physicochemical phenomenon in the qualitative changes of anthocyanin color. A schematic representation of the hue differences ( $\Delta h_{ab}$ ) evolution as a function of molar ratio is shown in Figure 5.

Again, the higher precision and accuracy of the colorimetric method to better evaluate the global color changes associated with copigmentation were manifested by comparing the  $A_{520nm}$ ,  $E$ , and  $\Delta E^*_{ab}$  values. As can be observed in Table 2, no effect of copigmentation was detected when the molar ratio was increased from 1:2 to 1:5 in MC solution, because no changes on the  $A_{520nm}$  were obtained. However, the increase in the catechin concentration resulted in increases of the total color and of the total color difference.

Finally, by comparing the relative contributions of lightness (% $\Delta L$ ), chroma (% $\Delta C$ ), and hue (% $\Delta H$ ) obtained at each molar ratio (Figure 6), it was observed that for all of the molar ratios tested, the absolute color differences induced by copigmentation were due mainly to quantitative changes (% $\Delta L$  + % $\Delta C$  = 87.5%, as mean values) and to a lesser extent to the qualitative ones (% $\Delta H$  = 12.5%), the weight of the lightness modifications being, in general, more marked than in chroma. However, increasing concentration for both copigments influenced especially the pattern evolution of the



lightness contribution, which notably decreased from low to high molar ratios (% $\Delta L$  = 67 vs 44%, respectively).

In contrast, chroma and hue contributions were in general more variable and did not show a clear tendency. Interestingly, at a given molar ratio value, both chroma and hue changes were influenced in different ways by the type of copigment. For example, at a molar ratio of 1:5, both copigments induced similar absolute color differences (Table 2), but catechin induced higher changes in hue and epicatechin in chroma (% $\Delta H$  = 30 vs 11% and % $\Delta C$  = 30 vs 50%, respectively).

As a summary, the colorimetric interpretation of copigmentation based on the CIELAB color space has demonstrated to be of practical interest because both quantitative and qualitative color changes can be better understood. It has been demonstrated that pH, copigment structure, and concentration have significant influences on the copigmentation process, which induced different absolute and relative color changes in anthocyanin solutions. The greatest magnitude of copigmentation and color effect was obtained at pH 3.0, being significantly higher with epicatechin and caffeic acid. At high acidic pH values (1.0) the main contribution to color differences was qualitative, whereas between pH 2.0 and 4.0, the main colorimetric contribution was quantitative, the chroma modifications % $\Delta C$  being particularly more marked than lightness % $\Delta L$ . At pH 3.60, the greatest color effects were induced by epicatechin at a molar ratio of 1:7. Increasing molar ratio produced always an increasing bluish, vivid, and darkening effect on anthocyanin solutions, affecting especially the lightness contribution % $\Delta L$ . On the other hand, epicatechin and caffeic acid produced similar effects on the total color of the Mv 3-gl, contributing more marked quantitatively changes than qualitative changes. In contrast, particularly at the higher pH values, the qualitative contribution was more important in catechin copigmented solutions.

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### Funding

We are indebted to the Ministry of Science and Innovation of Spain (Project AGL2008-05569-C02-02 and Fellowship BES-2009-025429) and the Consejería de Economía, Innovación y Ciencia, Junta de Andalucía (Project P07-AGR-02893) for financial support.

### Notes

The authors declare no competing financial interest.

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