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1 **A COMPREHENSIVE COLORIMETRIC STUDY OF ANTHOCYANIC**
2 **COPIGMENTATION IN MODEL SOLUTIONS. EFFECTS OF pH AND MOLAR**
3 **RATIO**

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11 ***Abbreviated running title:***

12 Color and copigmentation of anthocyanins

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26 **Abstract**

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

46

47 **Keywords:** Anthocyanin; color; copigmentation; Tristimulus Colorimetry

48 INTRODUCTION

49 Anthocyanins are the pigments accounting for the brilliant red, purple,
50 and blue colors in many fruits, vegetables, and derived food products such as
51 fruits juices, jams, and red wines (1).

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

61 However, the chemical and colorimetric stability of anthocyanins can be
62 improved by associations with other substances. Copigmentation phenomenon,
63 among others, represents one of the most complex and efficient mechanisms of
64 anthocyanin chromophore stabilization in nature and food systems (3). In food
65 science, this phenomenon is considered a relevant interaction because
66 obtaining stable and attractive colors is a major focus for quality control
67 purposes (4). Especially, in winemaking, is assumed it plays a key role on the
68 color evolution and stability of the young red wines (5).

69 Copigmentation reactions consist of non-covalent interactions between
70 anthocyanins among themselves (self-association) or with a wide variety of
71 colorless organic compounds named copigments or copigmentation cofactors

72 (basically other phenolic compounds, but also amino acids, sugars, organic
73 acids, polysaccharides, etc.).

74 From a molecular point of view, the anthocyanin-copigments complexes
75 adopt a typical sandwich configuration (π - π stacking) via hydrophobic
76 interaction. This structural conformation protects the red colored flavylum cation
77 against the nucleophilic attack of water, peroxide and sulphur dioxide bleaching,
78 and pH changes reducing the formation of the other colorless species in the
79 anthocyanin equilibrium (hemiacetal and chalcone) (6).

80 In addition, copigmentation not only confers greater stability to
81 anthocyanins, but also induces color variations. This kind of molecular
82 association is responsible for the typical changes in the spectral properties of
83 the chromophore group, that is, an increase of the absorptivity and frequently a
84 shift of the visible λ_{\max} toward greater wavelengths. Consequently,
85 copigmentation produces both quantitative and qualitative color changes on the
86 anthocyanins solutions. In this sense, the measurement and evaluation of these
87 colorimetric changes is of great interest in food industry since color is one of the
88 main sensory parameter for the quality of foods influencing customer selection
89 (7).

90 The contribution of the copigmentation phenomenon on color has been
91 widely studied using spectrophotometric methods, both in model or food
92 systems which contain individual anthocyanins and added copigments; or in red
93 wines where anthocyanins naturally co-occurs with other phenolic compounds
94 (8-15). The evaluation of the changes in the VIS spectrum of anthocyanins, or
95 specifically in the λ_{\max} (520 nm), has permitted to know the influence of
96 numerous factors on the effectiveness of the copigmentation including the

97 concentrations of pigments and cofactors, their chemical structures, the
98 cofactor/pigment molar ratio, the pH of the medium, etc. In the same way,
99 several studies in red wines have shown that the magnitude of copigmentation
100 and its evolution during winemaking is extremely variable according to
101 viticultural, agronomical or oenological practices accounting approximately from
102 25 to 50% to the total color of young red wines (5, 16-20).

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

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

120 Thus, through Tristimulus Colorimetry, in this study is performed a
121 precise colorimetric interpretation of the copigmentation phenomenon using the

122 uniform 1976-(L*a*b*) color space (CIELAB), which has been recommended by
123 the CIE (Commission Internationale de l'Éclairage) as a more appropriate tool
124 for color specification in most industrial applications. For this purpose, diverse
125 colorimetric variables based both on the scalar (L*, a*, b*) and angular (L*, C*_{ab},
126 h_{ab}) color coordinates has been defined to assess the quantitative and
127 qualitative color implications associated to copigmentation, and their incidence
128 on visual perception. These colorimetric variables have been applied and
129 compared with the most used simplified method in the evaluation of the
130 copigmentation effects in model solutions.

131

132 **MATERIALS AND METHODS**

133

134 **Standards and copigmented models solutions**

135 The pigment malvidin 3-glucoside (Mv 3-gl) was isolated in the laboratory
136 from skins of *Vitis vinifera* red grapes of Tempranillo variety. Extraction was
137 made with acidic methanol (methanol: HCl 1 N; 95:5 v/v) and the extracts were
138 purified by semipressure liquid chromatography using a reversed-phase
139 column, as described by Heredia, Francia-Aricha, Rivas-Gonzalo, Vicario &
140 Santos-Buelga, 1998. The copigments (+)-catechin (CAT) and (-) -epicatechin
141 (EPI) and caffeic acid (CAF) were purchased from Sigma Chemical Co. (St.
142 Louis, MO).

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

146 In order to evaluate the effect of the pH on the copigmentation
147 phenomenon, three copigmented solutions of malvidin 3-gl:(+)-catechin (MC),

148 malvidin 3-gl:(-)-epicatechin (ME), and malvidin 3-gl: caffeic acid (MF), as well as
149 a reference solution (Mv 3-gl) were prepared in wine-like medium at different pH
150 values: 1.0, 2.0, 3.0, 4.0, and 5.0. The reference solution contained 200 mg/L
151 (0.41 mM) of Mv 3-gl. Copigmented solutions contained the same anthocyanin
152 concentration and the corresponding copigment using a pigment/copigment
153 molar ratio of 1:5.

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

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

163 **Color analysis**

164 The absorption spectra (380- 770 nm) of all the model solutions were
165 recorded at constant intervals ($\Delta\lambda=2$ nm) with a Hewlett- Packard UV-vis
166 HP8452 spectrophotometer (Palo Alto, CA), using 2 mm path length glass cells
167 and distilled water as a reference. The CIELAB parameters (L^* , a^* , b^* , C^*_{ab} , and
168 h_{ab}) were determined by using the CromaLab software (24), following the
169 Commission International de L'Eclairage's recommendations (25): the 10°
170 Standard Observer and the Standard Illuminant D65.

171 The L^* value is the vertical axis and defines the lightness, the property
172 according to which each color can be considered as equivalent to a member of

173 the grey scale, between black and white, taking values within the range 0–100,
174 respectively. The a^* and b^* values represent the chromaticity scalar
175 coordinates, which in turn represent opponent red–green and blue–yellow
176 scales.

177 From L^* , a^* , and b^* , other parameters are defined, such as hue (h_{ab}) and
178 chroma (C^*_{ab}). Hue angle (h_{ab}) is the attribute according to which colors have
179 been traditionally defined as red, green, etc. On the other hand, the chroma
180 (C^*_{ab}) is the attribute that allows each hue to be determined by its degree of
181 difference in comparison to a grey color with the same lightness.

182 Color difference, which are very important to evaluate relationships between
183 visual and numerical analyses (26), was determined by means of the CIE76
184 color difference parameter (ΔE^*_{ab}). It was calculated as the Euclidean distance
185 between two points in the three-dimensional space defined by L^* , a^* and b^* :

$$186 \Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

187 **Colorimetric analysis of copigmentation in the CIELAB color space**

188 The colorimetric effect of copigmentation was evaluated by comparing the
189 color of the pure anthocyanin solutions and the color of the same solutions
190 containing different copigments concentrations.

191 The “total color” (E) of the copigmented and no copigmented solutions (E_C
192 and E_0 , respectively) was expressed as the color difference between the
193 corresponding L^* , a^* , and b^* values with respect to the distilled water ($L^*=100$,
194 $a^*=0$, $b^*=0$). From E_C and E_0 , the percentage of the anthocyanin color that is
195 due to copigmentation was calculated as the following equation:

$$196 CCI = [(E_C - E_0) / E_0] \times 100$$

197 The absolute color variation induced by copigmentation was assessed as
 198 the CIELAB color difference formula (ΔE^*_{ab}) applied between the color of the
 199 copigmented and no copigmented anthocyanin solutions, as follows:

$$200 \quad \Delta E^*_{ab (C-0)} = [(L^*_C - L_0)^2 + (a^*_C - a^*_0)^2 + (b^*_C - b^*_0)^2]^{1/2}$$

201 In the same way, the absolute lightness, chroma and hue differences
 202 (ΔL^* , ΔC^*_{ab} and Δh_{ab}) was used to asses the trend of the color changes induced
 203 by copigmentation. In addition, the relative contribution of the three color
 204 attributes that makes up the total CIELAB color difference was also calculated
 205 in order to compare the copigmentation effect according to different factors.
 206 Thus, the weight of each color attribute was calculated as follow:

$$207 \quad \% \Delta L = [(\Delta L)^2 / (\Delta E^*_{ab})^2] \times 100$$

$$208 \quad \% \Delta C = [(\Delta C)^2 / (\Delta E^*_{ab})^2] \times 100$$

$$209 \quad \% \Delta H = [(\Delta H)^2 / (\Delta E^*_{ab})^2] \times 100$$

210 being ΔH deduced from $\Delta E^*_{ab (C-0)}$, ΔL , and ΔC values, which are scalar
 211 magnitudes:

212 *2.4. Statistical analysis*

213 For the statistical treatment of the data the Statistica v.8.0 software (StatSoft
 214 Inc., 2007) was used.

215

216 **RESULTS AND DISCUSSIONS**

217 **Effect of pH and the chemical structure of copigment on copigmentation**

218 A color analysis of the Mv 3-gl copigmented with different phenolic
 219 compounds was performed in the CIELAB space at different pH values (from 1
 220 to 5 units) to evaluate the influence of the copigmentation reaction on the
 221 anthocyanin equilibrium. Figure 1 depicts the location of the pure Mv 3-gl

222 solution and its corresponding copigmented with (+)-catechin (MC), (-)-
223 epicatechin (ME), and caffeic acid (MF) within (a) the (a^*b^*) diagram and (b)
224 lightness values (L^*) according to the pH value. The changes of the mean
225 values of the total color (E) obtained for each anthocyanin solution are shown in
226 Table 1.

227 As previously reported by Heredia (27), pH variations provokes important
228 changes both on quantitative and qualitative psychophysical components of the
229 anthocyanin color. As the pH increases, the total color (E) of the pure Mv 3-gl
230 solution strongly decreased from 59.51 CIELAB u. at pH 1.0 to 5.12 CIELAB u.
231 at pH 5.0. The progressive color degradation was especially remarkable at pH
232 values up to 3.0, being 51%, 84%, and 91% at pH 3.0, 4.0 and 5.0, respectively.
233 However, the addition of the different copigments increased significantly the
234 total color of the pure Mv 3-gl solution from pH 1.0 to 4.0, confirming the
235 protective effect of the copigmentation phenomenon against color degradation.
236 The prevention of anthocyanin decoloration was more pronounced at pH 2.0
237 and 3.0., avoiding the color loss between 2-7% across the different
238 copigmented solution.

239 As can be seen in Figure 1, between pH 1.0 and 3.0, the color of the
240 pure Mv 3-gl solution underwent a slight decrease to blue hues and become
241 less vivid and lighter ($L^*= 72.0$ to 85.90 , $a^*= 52.81$ to 25.31 and $b^*= -1.58$ to -
242 3.14 CIELAB u., from pH 1.0 to 3.0). This color evolution reflects the kinetic and
243 thermodynamic competition between the flavylium cation and the other
244 colorless species in the anthocyanin equilibrium, that is, hemiacetals, chalcones
245 and the blue anionic quinonoidal bases (2). However, at this pH range, a
246 notable displacement of all the copigmented solutions respecting to the pure Mv

247 3-gl solution was produced, which tended progressively toward 350° color area
248 (-10°). For the three copigments tested, copigmented solution showed the
249 lowest L* and b* values, and simultaneously they had the highest a* values. As
250 a consequence, pure Mv 3-gl solutions exhibited the darkest and more vivid
251 bluish color when they were in the presence of the copigments, revealing the
252 positive influence of the copigmentation phenomenon on the anthocyanin
253 equilibrium and thus, on its color. Particularly, when copigmentation effect was
254 more notable (pH 3.0), L* and b* values changed from 85.9 in the pure Mv 3-gl
255 to 85.0, 83.93 and 83.76; and from -3.12 to -4.86, -4.53 and - 4.77 CIELAB u. in
256 (+)-catequin, (-)-epicatechin and caffeic acid, respectively, while a* values
257 changed from 25.31 to 27.67, 28.09, and 28.10.CIELAB u. Consequently, the
258 total color (E) increased significantly from 29.13 (Mv 3-gl) to 31.85, 32. 68 and
259 32.79 CIELAB u., respectively.

260 On the contrary, according to their location in the (a*b*) colorimetric
261 diagram, at pH values up to 3 units, both copigmented and no copigmented
262 solutions appear more grouped in the same color area nearer to the coordinates
263 origin, reflecting that the effect of the copigmentation was less relevant, being
264 almost imperceptible at pH 5.0. At this pH range, all the anthocyanin solutions
265 become more and more achromatic (a* and b* tended toward zero), clearer (L*
266 increases) and with a lower color intensity, which means a progressive loss of
267 color. At pH 5.0, no significant difference were found in the total color among
268 the different anthocyanin solutions, being 5.12 in pure Mv 3-gl solutions and
269 4.93, 5.40 and 5.52 CIELAB u. with the respective copigments.

270 Figure 2 shows the change on the magnitude of copigmentation for the
271 copigmented solutions evaluated by Tristimulus Colorimetry as the ratio [(E_{C-}

272 $E_0) / E_0] * 100$. The results showed that copigmentation occurred from pH values
273 close to 1 up to neutrality for all the phenolics used as copigments. However,
274 the magnitude of the copigmentation and its colorimetric effect was strongly pH-
275 dependent and influenced by the nature of the copigment used. The greatest
276 magnitude of copigmentation was obtained at pH 3.0, being significantly higher
277 with (-)-epicatechin and caffeic acid. They both increased respectively the total
278 color of Mv 3-gl by 13.70% and 13.80%, while (+)-catechin, which was the less
279 effective copigment, only reached an increase of 9.30%. This agree with other
280 studies that have shown that among the different flavan-3-ols copigments, the
281 more planar molecules as (-)-epicatechin, or with electron-donor substituents as
282 cinnamic acids can better stack with anthocyanins resulting in higher
283 copigmentation effect (11, 28-30). The slightly lower values of the
284 copigmentation magnitude obtained in this study respecting to those reported in
285 the literature could be due both to the lower pigment/copigment molar ratio used
286 and the chemical nature of the tested copigments. It has been established that
287 the differences in the number, the size or the spatial location of the substituents
288 make monomeric flavan-3-ols as catechin, epicatechin or simple phenolic acids,
289 exhibit weaker copigmentation than more planar phenolic compounds as
290 flavonols (31).

291 As shown in Table 1, the CIELAB differences (ΔE^*_{ab} , ΔL^* , ΔC^*_{ab} , Δh_{ab})
292 between Mv 3-gl solutions with and without copigments were also calculated.
293 Along the pH variation, the highest color differences (ΔE^*_{ab}) were produced from
294 pH 1.0 to pH 3.0, confirming the colorimetric stabilization of the flavylium ion at
295 lower than a higher pH values (Yan et al., 2011). Specifically, the color
296 differences increased from 1.73 to 3.06, from 2.88 to 3.69 and from 3.39 to 3.87

297 CIELAB units, in Mv 3-gl solution copigmented with (+)-catechin, (-)-epicatechin
298 and caffeic acid, respectively. Therefore, although flavan-3-ols has been usually
299 considered poorer anthocyanin copigments, at most acidic pH values, the color
300 changes induced were always perceptible by the human eye (32). However, at
301 pH 4.0 and 5.0, there was no significant difference between the color effect
302 induced by the flavanols tested, and all the color differences calculated were
303 smaller than 2 CIELAB units, that is, not visually distinguished.

304 In general, caffeic acid produced the largest color effects, which was
305 coherent with the results obtained about the magnitude of copigmentation when
306 it was assessed by Tristimulus Colorimetry. On the contrary, as can be checked
307 in Table 1, at some pH values, while color changes were detected in the
308 CIELAB color space, no changes at A_{520} nm was observed. This divergence
309 between both analytical methods has been previously reported by González-
310 Manzano (14) and confirms that the most simplified methods not always can
311 achieve a reliable evaluation of the copigmentation process.

312 With respect to the individual color attributes, regardless of pH value and
313 the copigment used, copigmentation induced significant colorimetric changes
314 consisting in a decrease of lightness (L^*) and hue (h_{ab}), as well as an increase
315 of chroma (C^*_{ab}). The lightness and chroma differences (ΔL^* and ΔC^*_{ab}) were
316 more pronounced between pH 2.0 and 3.0, while the largest changes on the
317 hue (Δh_{ab}) were produced at higher pH values. This finding means that in
318 quantitative terms, across the different pH values studied, the original Mv 3-gl
319 control solution change toward darker and more intense color due specifically to
320 the copigmentation phenomenon. However, in qualitative terms, due to the
321 notable influence of the pH on the anthocyanin color, the trend of the hue

322 changes were more variable. From pH 1.0 to 4.0, Mv 3-gl control solution
323 decreased towards more bluish hues while decreased towards more reddish at
324 pH 5.0, as can be observed in Figure 2. The fact that hues differences are
325 almost indiscernible in the (a^*b^*) diagram at pH 4.0 and 5.0 can be explained
326 based on the important degradation of the colored forms in the anthocyanin
327 equilibrium at higher pH values, which made Mv 3-gl solution practically
328 achromatic.

329 For a more comprehensive analysis of the colorimetric implication of
330 copigmentation process as a function of the pH, the relative contribution of
331 lightness ($\% \Delta L$), chroma ($\% \Delta C$), and hue ($\% \Delta H$) to the total color difference for
332 each pigment/copigment solution were also calculated. As can be seen in
333 Figure 3, the main contribution to the measured color differences ΔE^*_{ab} at pH
334 1.0 was qualitative, which was evidenced by the significant higher contribution
335 of hue $\% \Delta H$ (80-88%) with respect to the lightness $\% \Delta L$ or chroma $\% \Delta C$ (7-
336 10% and 6-10%, respectively). These results are coherent since at $\text{pH} < 2$, the
337 anthocyanin exists primarily in the form of the red cation flavylium and the
338 amount of the other colorless species is almost inexistent. Therefore, at this pH
339 value, the copigmented complexes can induce basically color variation but can
340 not provide important displacement of the anthocyanin equilibrium toward the red
341 colored flavylium cation, which is also reflected by the lower variations obtained
342 for E and ΔE^*_{ab} . On the other hand, between pH 1.0 and pH 4.0, the main
343 colorimetric contribution was quantitative; being particularly the chroma
344 modifications $\% \Delta C$ more marked than lightness $\% \Delta L$ (35-65% and 22-38%,
345 respectively). In this pH range, the proportion of the other colorless species
346 increase coexisting with the red colored flavylium cation in solution, so

347 copigmentation complexes can provide at the same time color stabilization and
348 variation, which coincides with the greatest changes on the E and ΔE^*_{ab} values.
349 From pH 5.0 to neutrality, the most abundant colored species present is the
350 quinoidal form and thus Mv 3-gl itself does not confer much color to a solution,
351 so again the contribution of the copigmentation on color is mainly qualitative,
352 that is, significantly due to hue changes.

353 Moreover, the relative contribution of lightness ($\% \Delta L$), chroma ($\% \Delta C$),
354 and hue ($\% \Delta H$) permitted us to compare the colorimetric effect induced by the
355 different flavanols tested. In general terms, (-)-epicatechin and caffeic acid
356 produced similar effects on the total color of the Mv 3-gl across to the pH
357 changes, contributing more marked with quantitative changes than qualitative
358 ones ($\% \Delta L + \% \Delta C = 55-63\%$, and $\% \Delta H = 45-37\%$). On the contrary, particularly
359 at the higher pH values, the qualitative contribution was more important in (+)-
360 catechin copigmented solutions. Thus, it can be said that the copigmentation of
361 (-)-epicatechin and caffeic acid provide notably color stabilization and variation,
362 while (+)-catechin provide more intense color variation than stabilization.

363

364 **Effect of the molar ratio on copigmentation**

365 The influence of the pigment /copigment molar ratio was also assayed at
366 pH 3.60. For this purpose, two copigmented solutions of malvidin 3-gl:(+)-
367 catechin (MC) and malvidin 3-gl:(-)-epicatechin (ME) were prepared in the same
368 wine-like medium at the molar ratios 1:1, 1:2, 1:5, and 1:7.

369 The mean values of the total color (E) of the original Mv 3-gl solution and
370 each copigmented solution as a function of the molar ratio is presented in Table
371 2. For both assays, the positive effect of the copigmentation on the Mv 3-gl total

372 color was increased with increasing copigment concentration, which is in
373 agreement with previous studies reported in bibliography despite being lower
374 the range of the pigment/copigment molar ratio used (33). The maximum
375 displacement of hydration/dehydration equilibrium towards the colored species
376 was achieved using the highest molar ratio (1:7) since it caused the greatest
377 total color increases (from 49.26 to 57.32 and 57.90 CIELAB u. in MC and ME
378 solutions, respectively).

379 Moreover, significant differences on the L^* , a^* , and b^* values among the
380 pure Mv 3-gl solution and its corresponding copigmented were found, especially
381 at molar ratios higher than 1:2, resulting in a different location of the samples in
382 the (a^*b^*) colorimetric diagram (Figure 4). In absence of any copigments, the
383 original anthocyanin color took positive a^* and b^* values and appeared located
384 near 0^0 , that is, in a well defined reddish color area ($L^* = 69.71$, $a^* = 38.84$, and
385 $b^* = 0.39$ CIELAB u.). However, with the increasing addition of the flavanol
386 copigments, the samples appeared located progressively farther to the
387 coordinates origin and experimented a remarkable evolution from the first
388 toward the fourth quadrant, that is, from the reddish to the purple or red-bluish
389 colors region. The behavior of the color parameters as a function of the
390 concentration was similar for both the copigment used. At the highest molar
391 ratio (1:7), copigmented samples reached the lowest L^* and b^* values but the
392 highest a^* values, and thus, they exhibited the darkest and most vivid bluish
393 color ($L^* = 64.18$ and 63.05 , $a^* = 45.44$ and 43.76 , and $b^* = -2.23$ and -2.21
394 CIELAB u. for MC and ME solutions, respectively).

395 Regarding the changes on the contribution of the copigmentation to the
396 total anthocyanin color, the added copigments caused significant concentration-

397 dependent increases on the magnitude of the effect, as can be seen in Figure 2.
398 From 1:1 to 1:7 molar ratio, (+)-catechin induced an increase of the Mv 3-gl total
399 color from 1.2% to 16.0%, while with (-)-epicatechin, the increase was from
400 3.9% to 17.9%. Notwithstanding, although the pattern evolution was similar
401 between both flavanols copigments, once again, the ability of the (-)-epicatechin
402 to act as Mv-3gl copigments was stronger than for (+)-catechin since significant
403 highest values for copigmentation was achieved for most of the molar ratios
404 tested.

405 As was made in the previous section of this study, the dependence
406 between the amplitude of the color effect induced by copigmentation was
407 evaluated by means of the CIELAB color differences (ΔE^*_{ab}) (Table 2). As
408 expected, the progressive formation of copigmentation complexes was
409 confirmed by a successive increase on the color differences between
410 anthocyanins and flavanols solutions when copigment concentrations were
411 increasingly added. For all the concentration level tested, (-)-epicatechin caused
412 always significant increases ($p < 0.05$) on the color differences. In contrast,
413 higher increases on the molar ratio were necessary to induce significative
414 changes with (+)-catechin copigmented solutions.

415 In any case, calculation of the simple regression coefficients between
416 total color differences and the copigment concentration for each copigmented
417 solution confirmed that the relationship was significantly high regarding the
418 copigment used (r^* values ranged from +0.95 to +0.98 units, $p < 0.05$). The
419 lowest color differences were found between pure Mv 3-gl solution and the
420 copigmented ones at the lowest molar ratio used (1:1), taking values of 1.01
421 and 2.27 CIELAB u. with (+)-catechin and (-)-epicatechin, respectively.

422 However, the color differences obtained at all molar ratios higher than 1:1 were
423 higher than 3 units CIELAB, hence visually relevant. The largest color changes
424 were induced with the highest copigment concentration (molar ratio 1:7), being
425 slightly more marked with (-)-epicatechin (10.79 vs. 9.76 CIELAB units, in MC
426 solutions). This was consistent with the higher values also obtained regarding
427 the total color and the magnitude of copigmentation, although the difference
428 was not significant.

429 In relation to the quantitative and qualitative color changes that the
430 original Mv 3-gl solution underwent with increasing molar ratio, the tendency
431 was always toward higher decreases of ΔL^* and Δh_{ab} , which means an
432 increasing bluish and darkening effect. Simultaneously, chroma differences
433 ΔC^*_{ab} tended toward higher increases resulting in an increasing vivid effect,
434 although this tendency was less marked than lightness and hue trends.
435 Specifically, the highest variations in ΔL^* and Δh_{ab} were reached with (+)-
436 catechin at molar ratio 1:7, which decreased in 8.5 and 5.12 CIEALB u.,
437 respectively. On the contrary, the maximum ΔC^*_{ab} occurred with (-) epicatechin
438 at the same molar ratio, increasing in 6.6 CIELAB u.

439 Univariate correlations between individual color attributes differences
440 (ΔL^* , ΔC^*_{ab} , Δh_{ab}) and copigment concentration were explored in order to find
441 out the significance of these changes. Across the different copigmented
442 solutions, the best relationships were obtained with simple regression for
443 quantitative attributes (ΔL^* ΔC^*_{ab}), and with second degree polynomial
444 regression for the qualitative (Δh_{ab}). Calculation of the coefficient regressions
445 revealed that all the relationships were strong and significant ($p < 0.05$), except
446 for those corresponding to ΔC^*_{ab} for MC solutions. Regression coefficients took

447 negative signs for lightness and hue differences, ranging as a mean values
448 between 0.71-0.95 and 0.93-0.99, respectively; while were positive for chroma
449 differences. Among the different CIELAB attributes, hue differences (Δh_{ab}) was
450 the best correlated parameter since more than 95% of cases showed high
451 quadratic regression coefficients ($R^2 = 0.99$), proving the relevance of this
452 physicochemical phenomenon on the qualitative changes of anthocyanin color.
453 A schematic representation of the hue differences (Δh_{ab}) evolution as a function
454 of molar ratio is shown in Figure 5.

455 Again, the higher precision and accuracy of the colorimetric method to
456 better evaluate the global color changes associated to copigmentation was
457 manifested by comparing the A_{520} nm, E, and ΔE^*_{ab} values. As can be observed
458 in Table 2, no effect of copigmentation was detected when the molar ratio was
459 increased from 1:2 to 1:5 in MC solution, since no changes on the A_{520} nm was
460 obtained. However, the increase on the (+)-catechin concentration resulted in
461 increases of the total color (E = 52.54 and 54.40 CIELAB u.) and of the total
462 color difference ($\Delta E^*_{ab} = 4.89$ and 6.36 CIELAB u.).

463 Finally, comparing the relative contribution of lightness ($\% \Delta L$), chroma
464 ($\% \Delta C$), and hue ($\% \Delta H$) obtained at each molar ratio (Figure 6) it was observed
465 that for all the molar ratio tested, the absolute color differences induced by
466 copigmentation were due mainly to quantitative changes ($\% \Delta L + \% \Delta C = 87.5\%$,
467 as mean values) and in lower extend to the qualitative ones ($\% \Delta H = 12.5\%$),
468 being in general the weight of the lightness modifications $\% \Delta L$ more marked
469 than in chroma $\% \Delta C$ (55.47% vs. 32.03%, respectively). However, increasing
470 concentration for both copigment influenced specially the pattern evolution of

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

473 On contrast, chroma and hue contributions were more variable and did
474 not show a clear tendency, which seem to be more influenced by the type of
475 copigment than for the molar ratio used. For example, an interesting
476 observation is that for the same concentration (molar ratio 1:5), both
477 copigments induced similar absolute color differences (Table 2), but the
478 variation on the contribution of each individual attributes was different.
479 Specifically, the increase of the anthocyanin total color consisted in a higher
480 contribution of the hue changes with (+)-catechin and in a higher contribution of
481 the chroma with (-)-epicatechin ($\% \Delta H = 30$ vs. 11% and $\% \Delta C = 30$ vs. 50% ,
482 respectively).

483 As a summary, the colorimetric interpretation of copigmentation based on
484 the CIELAB color space has demonstrated to be of practical interest since both
485 quantitative and qualitative color changes can be better understood. It has been
486 demonstrated that pH, copigment structure and concentration have a significant
487 influence on the copigmentation process, which induced different absolute and
488 relative color changes on anthocyanin solutions. The greatest magnitude of
489 copigmentation and color effect was obtained at pH 3.0, being significantly
490 higher with (-)-epicatechin and caffeic acid. At high acidic pH values (1.0) the
491 main contribution to color differences was qualitative, while between pH 1.0 and
492 pH 4.0, the main colorimetric contribution was quantitative being particularly the
493 chroma modifications $\% \Delta C$ more marked than lightness $\% \Delta L$. At pH 3.65, the
494 greatest color effects were induced by (-)-epicatechin at molar ratio 1:7.
495 Increasing molar ratio produced always an increasing bluish, vivid and

496 darkening effect on anthocyanin solutions, affecting specially the lightness
497 contribution $\% \Delta L$. On the other hand, (-)-epicatechin and caffeic acid produced
498 similar effects on the total color of the Mv 3-gl, contributing more marked with
499 quantitatively changes than qualitatively. On contrast, particularly at the higher
500 pH values, the qualitative contribution was more important in (+)-catechin
501 copigmented solutions.

502

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508

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623
624

625 **Table 1.** Mean values of the A_{520} and the total color (E) obtained for the pure
 626 anthocyanic solution and its respective copigmented at each pH value; as well as, the
 627 CIELAB differences (ΔE^*_{ab} , ΔL^* , ΔC^*_{ab} , Δh_{ab}) between Mv 3-gl solutions with and
 628 without copigments.
 629

	pH	Pigment	Pigment/Copigment		
		Mv 3-gl	MC	ME	MF
A_{520}	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±0.003 _a	0.10±0.003 _a	0.11±0.001 _b	0.12±0.005 _b
	5	0.06±0.001 _a	0.05±0.005 _a	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±0.38 _c
	5	5.12±0.60 _a	4.93±0.38 _a	5.40±0.12 _a	5.52±0.47 _a
ΔE^*_{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
ΔL^*	-1	-	-0.46±0.11 _a	-0.91±0.29 _{ab}	-0.95±0.10 _b
	-	-	-2.64±0.23 _a	-1.16±0.02 _a	-2.27±0.82 _a
	2	-	-0.89±0.01 _a	-1.97±0.20 _b	-2.14±0.13 _b
	3	-	-0.03±0.20 _a	-0.77±0.21 _b	-1.03±0.40 _b
	4	-	+0.23±0.28 _a	-0.29±0.10 _a	-0.20±0.40 _a
ΔC^*_{ab}	5	-	+0.41±0.21 _a	+0.95±0.15 _b	+0.94±0.14 _b
	-1	-	+0.47±0.20 _a	+1.03±0.19 _a	+2.77±1.17 _b
	-	-	+2.59±0.18 _a	+2.95±0.27 _a	+2.98±0.15 _a
	2	-	+0.91±0.19 _a	+1.09±0.01 _a	+1.17±0.16 _a
	3	-	+0.02±0.06 _a	+0.05±0.01 _a	+0.44±0.24 _b
Δh_{ab}	4	-	-1.74±0.07 _a	-2.74±0.20 _b	-3.35±0.15 _c
	5	-	1.30±0.75 _a	-1.93±0.07 _a	1.99±0.53 _a
	-1	-	2.91±0.08 _a	-2.12±0.29 _b	2.61±0.35 _{ab}
	-	-	6.65±0.65 _a	-2.75±0.84 _b	2.20±0.17 _b
	2	-	-17.28±2.30 _a	-5.56±0.92 _b	-6.41±3.07 _b
Δh_{ab}	3	-	-	-	-
	4	-	-	-	-
	5	-	-	-	-

630 **Table 2.** Mean values of the A_{520} and the total color (E) obtained for the pure
 631 anthocyanic solution and its respective copigmented at each molar ratio; as well as, the
 632 CIELAB differences (ΔE^*_{ab} , ΔL^* , ΔC^*_{ab} , Δh_{ab}) between Mv 3-gl solutions with and
 633 without copigments.
 634

Colorimetric parameters	Molar Ratio					
	0	1	2	5	7	
A_{520}	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	
MC	ΔE^*_{ab}	-	1.01±0.33 _a	4.89±0.37 _b	6.36±0.19 _b	9.76±1.27 _c
	ΔL^*	-	-0.16±0.54 _a	-4.51±0.33 _b	-3.95±0.66 _b	-8.56±0.99 _c
	ΔC^*_{ab}	-	+0.60±0.26 _a	+0.65±1.06 _a	+3.40±1.17 _a	+2.59±3.60 _a
	Δh_{ab}	-	-1.21±0.60 _a	-2.56±0.99 _a	-5.05±0.13 _b	-5.12±0.13 _b
A_{520}	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	
ME	ΔE^*_{ab}	-	2.27±0.20 _a	4.53±0.91 _b	6.65±0.33 _c	10.69±0.44 _d
	ΔL^*	-	-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
Δh_{ab}	-	-1.02±0.54 _a	-2.39±0.83 _{ab}	-3.11±0.47 _b	-3.38±0.50 _b	

635 **FIGURE CAPTIONS**

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

639

640 **Figure 2.** (a) Changes on the magnitude of copigmentation for the Mv 3-gl
641 solutions copigmented with (+)-catechin (MC), (-)-epicatechin (ME), and caffeic
642 acid (MF) as a function of the pH, and (b) with (+)-catechin (MC), (-)-epicatechin
643 (ME) as a function of the molar ratio, evaluated by Tristimulus Colorimetry as
644 the ratio $[(E_C - E_0) / E_0] * 100$.

645

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

649

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

653

654 **Figure 5.** CIELAB hue differences (Δh_{ab}) as a function of molar ratio for
655 corresponding to Mv 3-gl: CAT and Mv 3-gl: EPI solutions.

656

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

Figure 1.

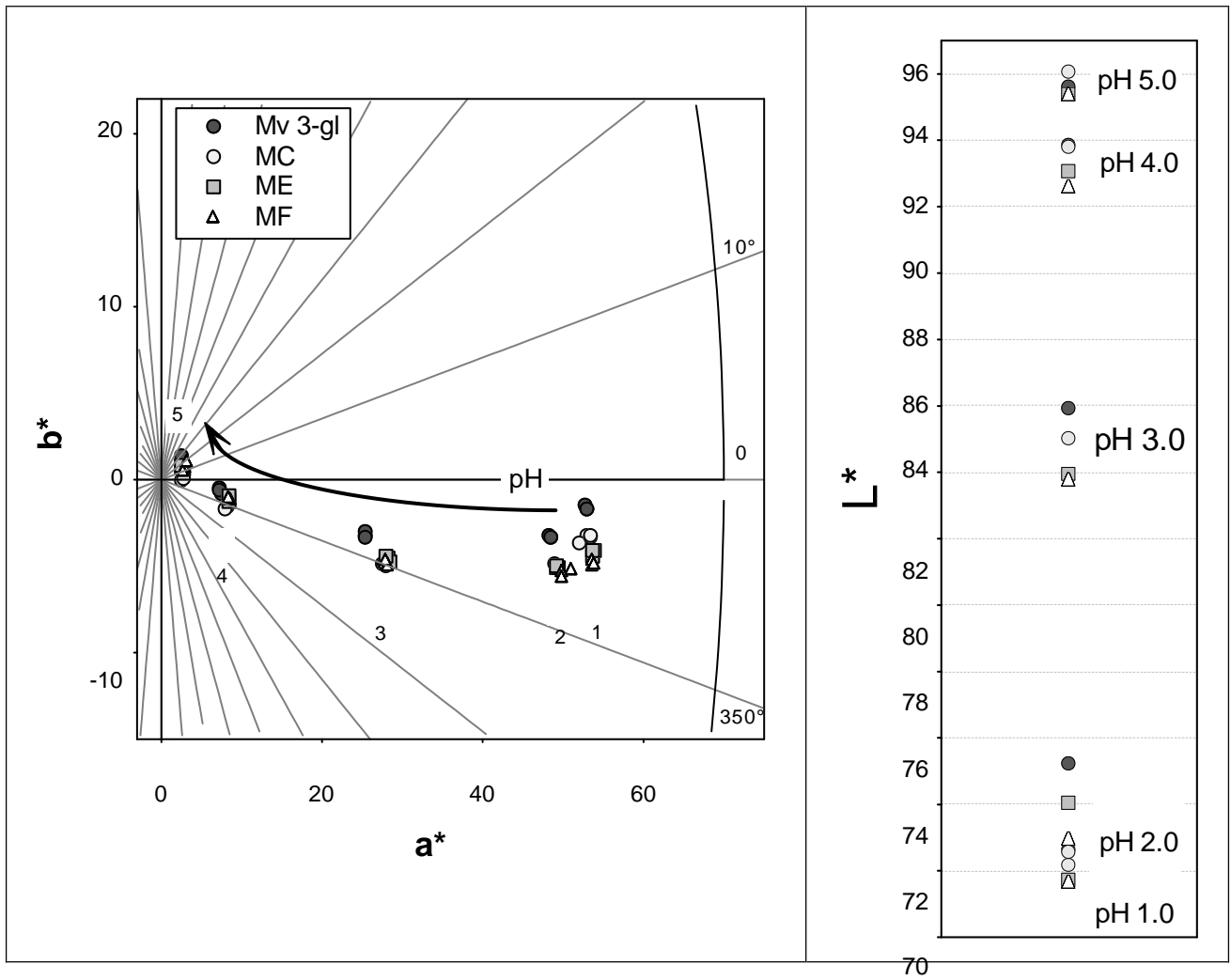


Figure 2

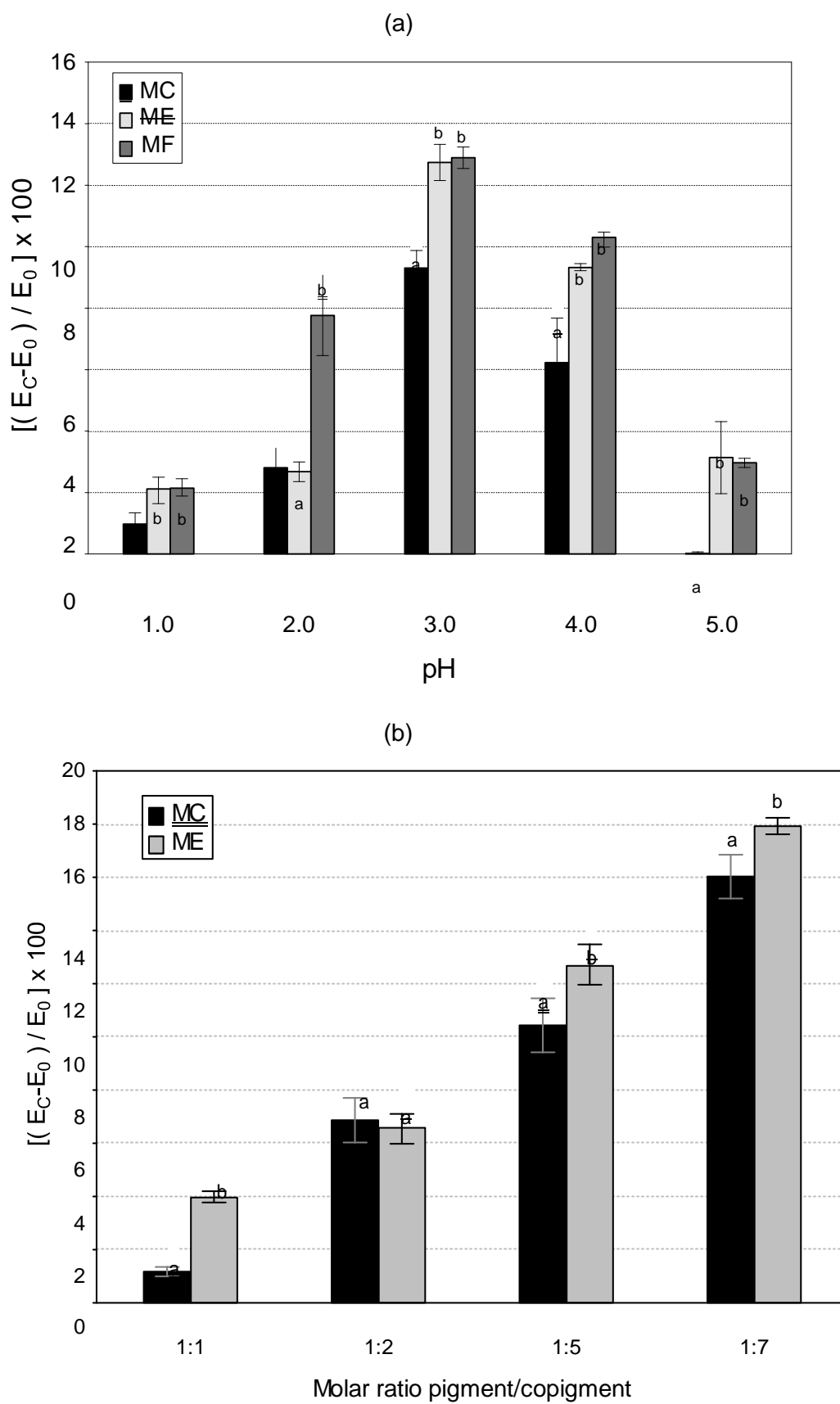


Figure 3.

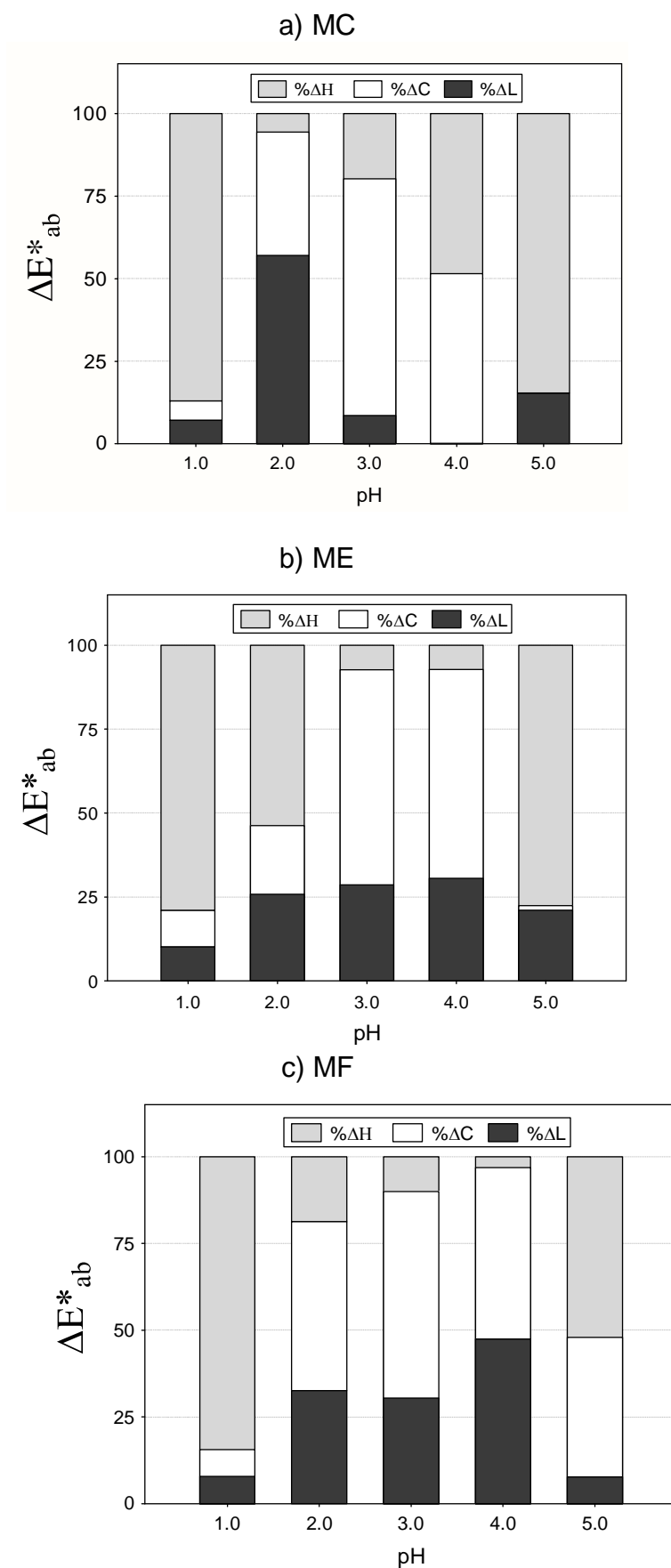
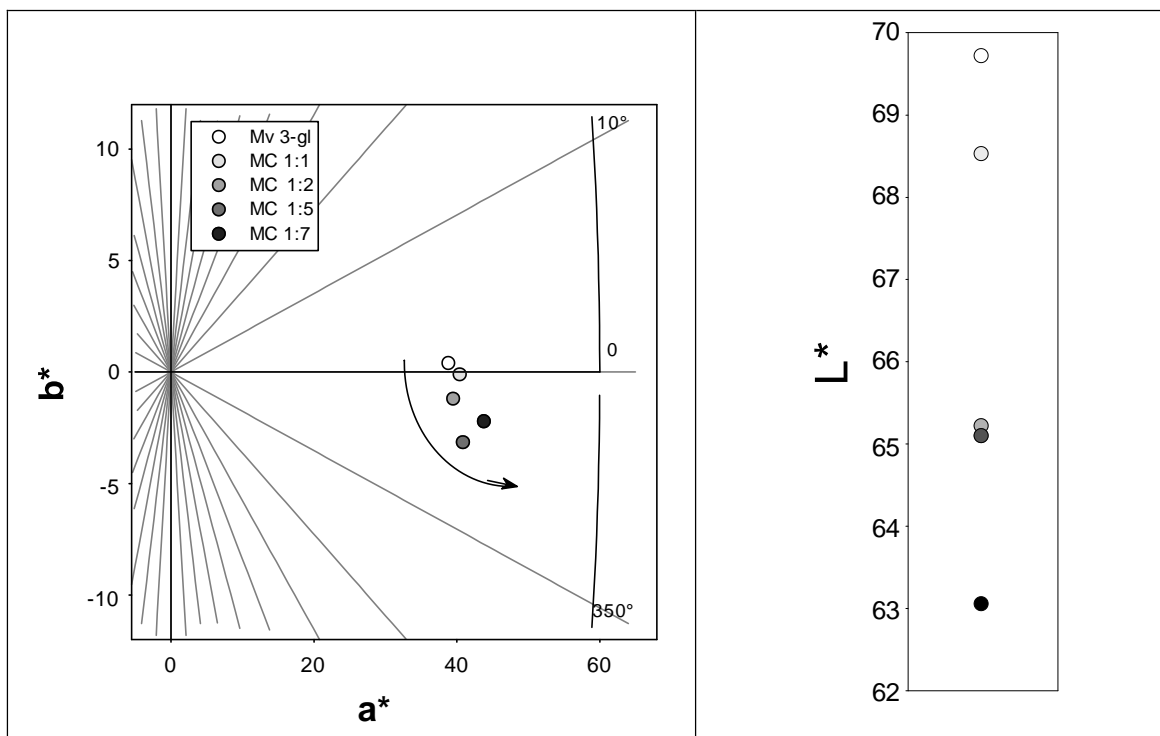


Figure 4.

(a) MC



(b) ME

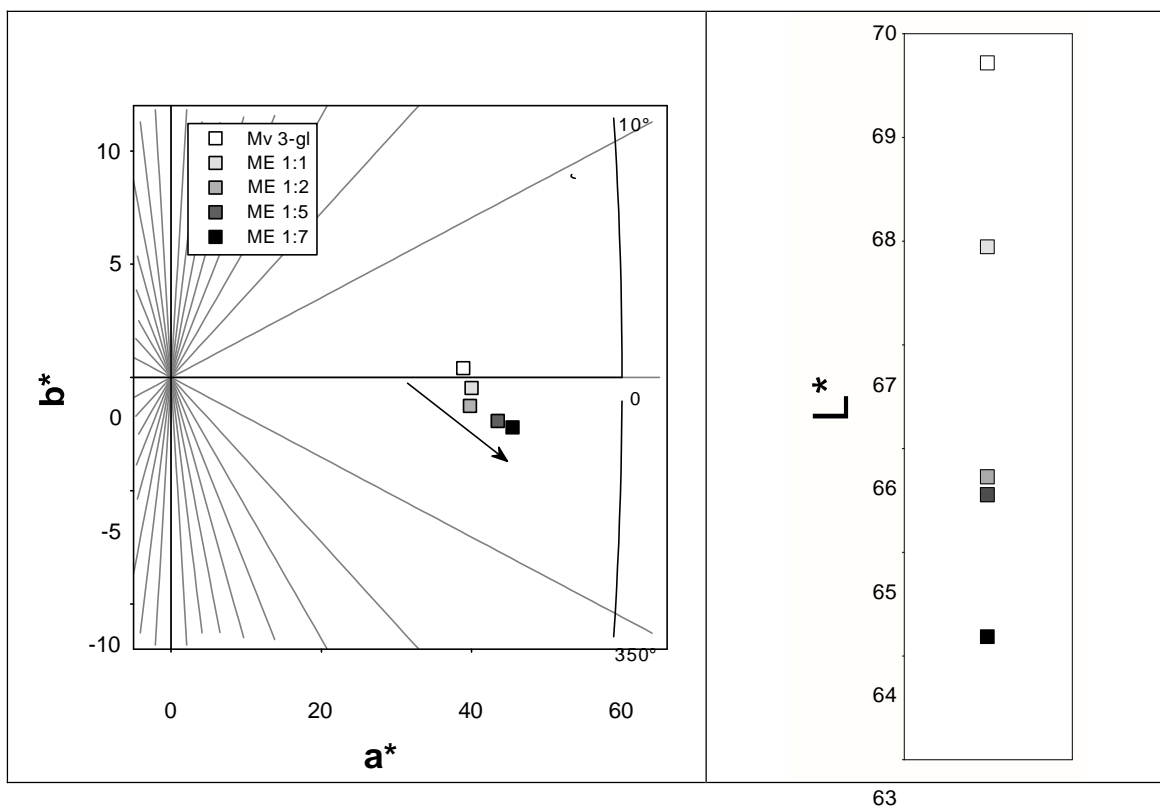


Figure 5.

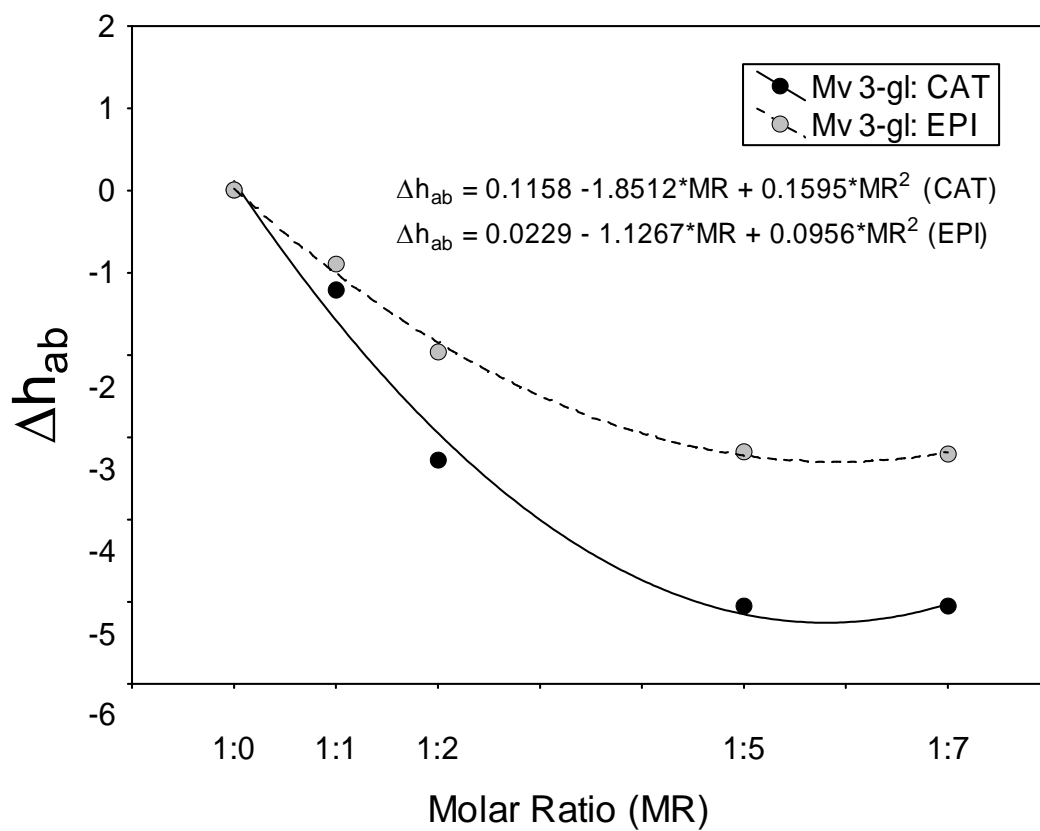


Figure 6.

