

## Influence of Ethanol Content on the Extent of Copigmentation in a Cencibel Young Red Wine

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The effect of ethanol content on the copigmentation of a Cencibel young red wine was studied, by means of ethanol elimination and reconstitution of the initial volume with different ethanol proportions. The reference wine (14.0% ethanol, 0.40 g/L volatile acidity) showed a bathochromic shift of 4 nm and a color enhancement ( $\Delta\text{Color}$ ) of 41%, lower than that found for the reconstituted wine with the same ethanol content (53%). This discrepancy could be attributable to the loss of acetic acid during the ethanol elimination step.  $\Delta\text{Color}$  was 95% for the reconstituted wine without ethanol and decreased until 18% for the reconstituted wine with 22% ethanol. Copigmentation was important for reconstituted wines with ethanol contents typical for table red wine, showing  $\Delta\text{Color}$  between 53 and 57%. An increase in ethanol content in reconstituted wines was accompanied by an increase in the pH value. Perceivable changes in color ( $\Delta E^* > 1$ ) followed every increase in ethanol content.

**KEYWORDS:** Anthocyanin; copigmentation; Cencibel; CIELAB; color; ethanol content; polymerization; red wine; Tempranillo

### INTRODUCTION

Copigmentation in young red wines from *Vitis vinifera* grapes seems to lead to an increase in the pigment concentration and an enhancement in the color due to them. Besides, it was suggested that copigmentation could play an important role in the subsequent evolution of young red wine during aging, affecting the speed of pigment polymerization reactions and protecting anthocyanins against oxidation. Moreover, copigmentation could also have sensory implications with respect to perceived astringency and mouthfeel attributable to polyphenols (1).

In model wine solutions, anthocyanins have a tendency to interact with themselves (self-association), with the result of an increase in the expected color intensity. There are experimental proofs, derived from the study of circular dichroism (CD) spectra of such solutions, indicating that self-association occurs by weak  $\pi$ - $\pi$  interactions leading to the formation of planar stacks in spirals between anthocyanin molecules (2–5). The formation of a copigmentation complex between an anthocyanin and a cofactor in model wine solutions, by similar planar stacking in spirals with a stoichiometry of 1:1, has been the proposed mechanism for the observed copigmentation (2). In young red wines, one could expect that color enhancement was attributable both to anthocyanin self-association and to copigmentation between anthocyanins and cofactors. However, the CD spectra registered for young red wines at pH values 3.6 and 1.0 showed that only copigmentation is relevant to color enhancement in these wines (1). Loss in color intensity by easy dissociation of copigmentation complexes is observed in model

wine solutions when nonaqueous solvents, like ethanol, are added (6–8) or when concentration is diminished by dilution (7, 9–11).

Another effect attributable to ethanol in young red wines could be the contribution to the characteristic purple hue of their red color. The majority of anthocyanins shows a bathochromic shift up to 25 nm in acidified ethanol, in relation to their solutions in acidified water (12). Young Porto fortified red wines have ethanol contents between 18 and 21% and show a purple-red color that could be the result of a combination of copigmentation and ethanol effects. It is thought that flavonols, the cofactors with the highest values of equilibrium constants for copigmentation complex formation in model wine solutions, are more soluble in the conditions of these wines; the copigmentation observed is still important.

The measure of the contribution of copigmentation to the total color of a young red wine can be made using the method based upon the dissociating effect of dilution on the copigmentation complex (13). This method considers the possible ethanol effects and the optimum pH value for the observation of copigmentation in red wines. Using this method, the contribution of copigmentation to the total color of young Cabernet Sauvignon wines from California accounted for 25–50% (14). This method has also been applied in recent studies about the effects of prefermentative additions of certain cofactors on the color intensity and stability, as well as in anthocyanin content, of young red wines (15, 16).

Because of the dissociating effect on the copigmentation complex shown by nonaqueous solvents, the importance of copigmentation in red wines containing ethanol is uncertain. In model wine solutions at a pH value of 5.0 and with a molar

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ratio of 12 of cofactor to pigment, the loss of color, with respect to the model solution in water, was 15% with ethanol contents close to those typical of table red wines and 20% when ethanol contents reached the values found in fortified wines (12). In 10% ethanol, with respect to water, model wine solutions at a pH value of 3.5 showed a loss of color between 7 and 20% when only one pigment was present and negligible loss of color when pigments were mixed; some pigment–cofactor pairs were unaffected by ethanol (17). It appears that in model wine solutions with ethanol contents close to those typical of table red wines, ethanol has a poor influence on copigmentation. However, the content in ethanol must be considered when comparing copigmentation measures for real wines and those obtained for model wine aqueous solutions.

The aim of this work was to study the influence of ethanol content on the extent of copigmentation in a model system closer to real wines. After vacuum concentration in a rotary evaporator of a Cencibel (Tempranillo) young red wine, its original volume was reconstituted by adding ethanol and water in different ratios, to get similar reconstituted wines but with different ethanol contents. The contribution of copigmentation to the total color, phenolic composition, and chromatic characteristics was determined in these wines.

## MATERIALS AND METHODS

**Preparation of Young Red Wines with Different Ethanol Content.** A young red wine was elaborated from Cencibel (Tempranillo) grapes in Naranjo Cellars (Ciudad Real, Spain). After alcoholic fermentation, a sample of this wine was centrifuged at 2500g for 15 min at 10 °C (reference wine). A sample of the reference wine was concentrated by a third under vacuum in a rotary evaporator at 40 °C. The concentrated and dealcoholized resulting wine was diluted with water (Milli-Q) up to the 75% of its original volume (concentrated wine). Several samples of the concentrated wine were reconstituted up to the original reference wine volume, adding the necessary amount of absolute ethanol and water to get similar wines with ethanol contents in the range of 0–22% (reconstituted wines). For every wine, the subsequent measures were made together with a duplicate. Conventional analyses were performed according to OIV methods.

**Analysis of Ethanol Content by GC.** Residual ethanol content in concentrated wine was determined by gas chromatography (GC). A Perkin-Elmer 8700 gas chromatograph was used, fitted with a conventional injector (180 °C) and a flame ionization detector (FID) (200 °C). A packed column MFE-Vinicol of 2 m length and 1/8 in. diameter was used, with nitrogen as the carrier gas at 15 mL/min. The temperature program was 40 °C (3 min) at 10 °C/min to 60 °C (0 min) at 3 °C/min to 145 °C (10 min). Chromatograms of reference wine (diluted 1/1000 with water) and concentrated wine (diluted 1/10 with water) were registered, after addition of the same amount of an aqueous solution of 2-pentanol (Sigma-Aldrich) used as an internal standard. The relative area of ethanol peak was measured for both samples, and the residual ethanol content in the concentrated wine was calculated comparing the relative areas in both the concentrated wine and the reference wine.

**Analysis of Monomeric Anthocyanins by HPLC.** Individual monomeric anthocyanins in the wines were analyzed by HPLC using a Waters 2690 liquid chromatograph, coupled to a Millennium 3.2 data station. The column used was 250 mm length and 4.6 mm internal diameter, packed with 5 μm particle diameter of Spherisorb C18 (Waters), held at 30 °C, and protected with a column guard of 10 mm × 4.6 mm. Detection was performed at 520 nm using a photodiode array detector (Waters, model 996), and quantification was made by means of a calibration curve obtained with standard solutions of malvidin 3-monoglucoside chloride (Extrasynthese). Eluents A (formic acid–water, 10:90) and B (formic acid–water–methanol, 10:30:60) were used with the following gradient: 30% B, 40% B (25 min), 100% B (60 min), 30% B (70 min).

**Spectrophotometric Analysis of Phenolics.** The content of several kinds of phenolics was determined using a modification of the Glories's

method (18, 19), as described by Mazza et al. (20). Wines and standards samples were diluted 1:10 with 10% ethanol. To 0.25 mL of wine or standard diluted samples was added 0.25 mL of 0.1% HCl in 96% ethanol, together with 4.55 mL of 2% aqueous HCl. After it was mixed and kept for 15 min, absorbances at 280 (total phenolics), 320 (hydroxycinnamic acid esters), 360 (flavonols), and 520 nm (total anthocyanins) were measured, using water as a blank. Quantification was made using calibration curves obtained with standard solutions of gallic acid (Sigma-Aldrich) for total phenolics, caffeic acid (Sigma-Aldrich) for hydroxycinnamic acid esters, quercetin (Sigma-Aldrich) for flavonols, and malvidin 3-monoglucoside chloride (Extrasynthese) for total anthocyanins.

**Copigmented and Polymerized Anthocyanins.** The contribution of copigmentation to the total wine color at pH 3.6 (% Copigmentation), the color enhancement due to copigmentation ( $\Delta\text{Color}$ ), and the degree of anthocyanin polymerization (% Polymerization) were determined following the method proposed by Boulton (13), with the modification of using the same ethanol content in the dilution wine as in the reconstituted wine. Wine samples were first adjusted to pH 3.6 using 4 N NaOH or HCl and then centrifuged at 2500g for 15 min at 10 °C. Total wine color at a pH value of 3.6 is assumed to be  $A^{\text{acet}}$ , the measure of absorbance at 520 nm, using water as a blank, after addition of 20 μL of 10% acetaldehyde to 2 mL of wine sample, and keeping for 45 min; wine color without copigmentation effects is considered to be  $A^{\text{dil}}$ , the measure of absorbance at 520 nm of the wine sample diluted 1:20 with a synthetic dilution wine made with 5 g/L of tartaric acid in water, adjusted to pH 3.6 and having the same ethanol content as the measured wine sample (for the reference wine, the ethanol content of dilution wine was 12%, as in the original method); finally, polymeric pigment wine color was assumed as  $A^{\text{SO}_2}$ , the measure of absorbance at 520 nm after addition of 160 μL of 5% SO<sub>2</sub> to 2 mL of wine sample. The former measures were corrected for dilutions and used to calculate the following data:

$$\% \text{ Copigmentation} = [(A^{\text{acet}} - A^{\text{dil}})/A^{\text{acet}}] \times 100$$

$$\Delta\text{Color} = [(A^{\text{acet}} - A^{\text{dil}})/(A^{\text{dil}} - A^{\text{SO}_2})] \times 100$$

$$\% \text{ Polymerization} = (A^{\text{SO}_2}/A^{\text{dil}}) \times 100$$

**Chromatic Characteristics.** Chromatic characteristics of the wine samples in the CIELAB space were obtained using the simplified method proposed by Ayala et al. (21), from absorbance measures at 450, 520, 570, and 630 nm for wine samples after adjusting the pH values to 3.6. Necessary calculations to obtain the values for chromatic parameters  $L^*$ ,  $a^*$ ,  $b^*$ ,  $C^*$ , and  $H^*$  were made using the computer application performed by authors (22). Chromatic differences, following an increase in ethanol content of the reconstituted wines, were calculated as

$$\Delta E^* = [\Delta L^{*2} + \Delta C^{*2} + \Delta H^{*2}]^{1/2}$$

**Statistical Analysis.** The data for the phenolic composition and for the monomeric anthocyanins corresponding to the reference wine and to the reconstituted wines were analyzed by Student's *t*-test (SPSS version 10.0, SPSS Inc.), searching for significant differences.

## RESULTS AND DISCUSSION

The reference wine used was representative of actual young red wines from the La Mancha region (middle south of Spain) elaborated with the variety Cencibel (Tempranillo). It was just recently elaborated red wine, in which malolactic fermentation was not performed. Its conventional analyses were 14.0% ethanol, pH = 3.66, total acidity of 5.86 g/L (as tartaric acid), volatile acidity of 0.40 g/L (as acetic acid), 2.41 g/L of reducing sugars, 85 units of DO<sub>280</sub>, 19.2 and 48.0 mg/L of free and total SO<sub>2</sub>, respectively, and a color intensity of 16.7.

The concentration of the reference wine, under vacuum and low temperature, allowed the almost total elimination of the

**Table 1.** Phenolic Composition for the Cencibel Reference Wine and the Average Phenolic Composition for the Reconstituted Wines with Different Ethanol Contents

phenolics	reference wine		reconstituted wines	
	MV ( <i>n</i> = 2)	SD	MV ( <i>n</i> = 12)	SD
total phenolics <sup>a</sup>	1815	38	1819	75
hydroxycinnamic acid esters <sup>b</sup>	368	6	372	16
flavonols <sup>c</sup>	215	4	218	10
total anthocyanins <sup>d</sup>	735	10	720	33

<sup>a</sup> As mg/L of gallic acid. <sup>b</sup> As mg/L of caffeic acid. <sup>c</sup> As mg/L of quercetin. <sup>d</sup> As mg/L of malvidin 3-monoglucoside.

**Table 2.** Concentration of Total Monomeric Anthocyanins (as Malvidin 3-Monoglucoside) and Percentage of Every Individual Monomeric Anthocyanin (Anthocyanin Profile) for a Young Cencibel Reference Wine and for Its Reconstituted Wine Using Only Water (0% Ethanol), as Derived from Their HPLC Chromatograms

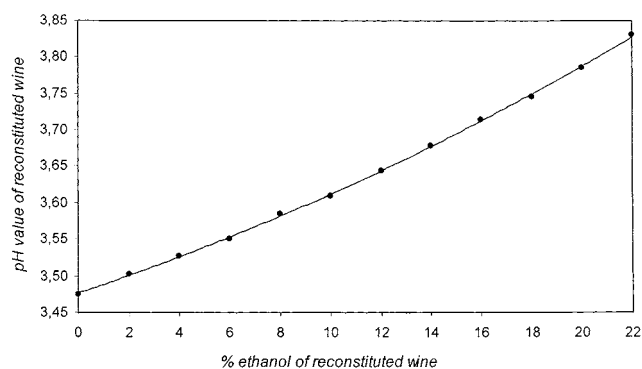
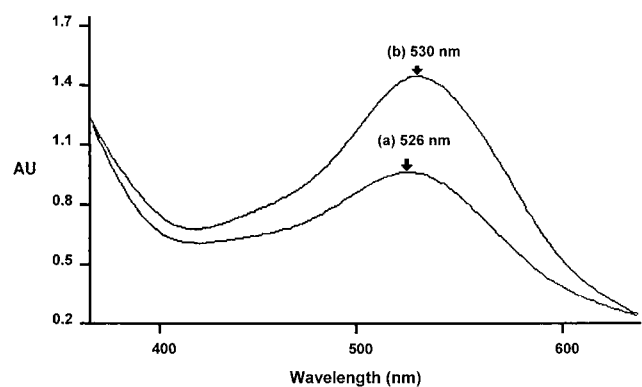
	reference wine		reconstituted wine (0%)	
	MV ( <i>n</i> = 2)	SD	MV ( <i>n</i> = 2)	SD
Σ monomeric anthocyanins (mg/L)	478	8	485	6
anthocyanin profile <sup>a</sup>				
delphinidin 3-monoglucoside	10.93	0.65	10.67	0.69
cyanidin 3-monoglucoside	0.67	0.05	0.70	0.03
petunidin 3-monoglucoside	12.40	0.78	12.24	0.55
peonidin 3-monoglucoside	3.53	0.04	3.48	0.04
malvidin 3-monoglucoside	52.32	1.08	51.82	0.98
Ac-delphinidin 3-monoglucoside	1.05	0.01	1.05	0.01
Ac-cyanidin 3-monoglucoside	0.09	0.00	0.10	0.00
Ac-petunidin 3-monoglucoside	1.15	0.01	1.10	0.01
Ac-peonidin 3-monoglucoside	0.39	0.00	0.39	0.01
Ac-malvidin 3-monoglucoside	4.03	0.02	3.92	0.03
Cm-delphinidin 3-monoglucoside	1.77	0.01	1.82	0.00
Cm-cyanidin 3-monoglucoside	0.36	0.00	0.42	0.00
Cm-petunidin 3-monoglucoside	1.76	0.01	1.87	0.01
Cm-(peonidin + malvidin) 3-monoglucosides	9.52	0.18	10.42	0.23

<sup>a</sup> Ac and Cm, mean acetyl and *p*-coumaryl derivatives, respectively, of the anthocyanidin 3-monoglucosides.

ethanol (only 0.95% of the initial ethanol content was remaining). Concentrated wine maintained almost the total acidity, but volatile acidity (0.24 g/L) decreased in one-third, and free and total SO<sub>2</sub> slightly decreased (11.0 and 34.2 mg/L, respectively). From this concentrated wine, reconstituted wines were built with different ethanol contents. Reconstituted wine with no ethanol added had an ethanol content of 0.13%, belonging to the residual ethanol not eliminated, and was considered "0% ethanol" reconstituted wine.

The concentration process had no effect on the phenolic composition of the reference wine, and no significant differences were found between this wine and the reconstituted wines in attention to the content of total phenolics, hydroxycinnamic acid esters, flavonols, and total anthocyanins (**Table 1**). With respect to the anthocyanin fraction, no significant differences were found also in the content of monomeric anthocyanins (**Table 2**), showing both the reference and the reconstituted wines the characteristic monomeric anthocyanin profile of the Cencibel red wines (23).

The higher the ethanol content, the higher the pH value in the reconstituted wines was (**Figure 1**), in a very well-adjusted quadratic relation ( $r^2 = 0.9996$ ). This could mean that in wines

**Figure 1.** Plot of pH vs ethanol content for reconstituted wines from a concentrated Cencibel young red wine.**Figure 2.** Normalized visible spectra of the Cencibel reference young red wine, recorded at pH 3.6 (a) after dilution (1:20) of wine adjusted at pH 3.6, with 12% ethanol solution containing tartaric acid (5 g/L, adjusted at pH 3.6) and (b) after acetaldehyde addition (20  $\mu$ L of 10% acetaldehyde, to 2 mL of wine adjusted at pH 3.6).

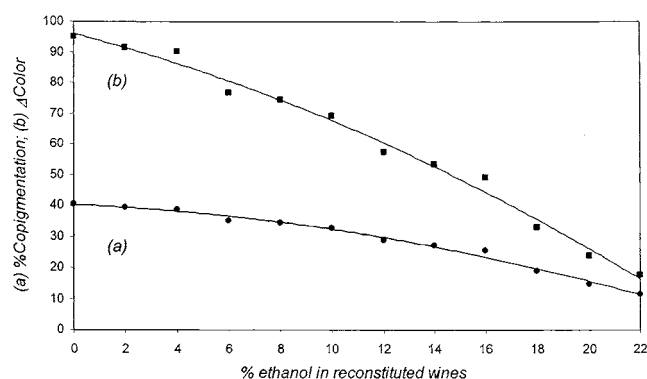
with similar compositions with respect to anthocyanins and total acidity, an increase in the ethanol concentration, for example, when Porto wines are fortified, could lead to a diminution of the fraction of anthocyanins in the form of flavilium cation, due to the increase in the pH value.

Visible spectra of the reference wine were registered for the determination of the degree of polymerization and the contribution of copigmentation to the total color in this wine. The reference wine, diluted to dissociate the copigmentation complexes, showed an absorbance maximum at 526 nm (**Figure 2a**). The reference wine, after addition of acetaldehyde for releasing the bisulfite bleached anthocyanins, showed an enhancement of 22.4% for the absorbance at 520 nm, with respect to the diluted reference wine (%Copigmentation = 22.4%), as well as a bathochromic shift of the absorbance maximum toward 530 nm (**Figure 2b**). The %Copigmentation found for this young Cencibel reference wine was comparable to the lower values described for Cabernet Sauvignon wines (14). Bisulfite addition to the reference wine led to the breakdown of the copigmentation complexes (14), together with the bleaching of a fraction of anthocyanins, being the absorbance at 520 nm only the 28.7% of the absorbance value for diluted reference wine (%Polymerization = 28.7%). Polymeric pigments remaining after bisulfite bleaching had the absorbance maximum at 522 nm. The effect of the copigmentation on the color of the reference wine, attributable to the nonpolymerized anthocyanins, was a color enhancement of 40.6% ( $\Delta$ Color = 40.6%).

**Table 3.** Chromatic Characteristics (Space CIELAB; MVs and SD) of Cencibel Reconstituted Wines at pH 3.6 after Addition of Acetaldehyde, as a Function of Ethanol Concentration<sup>a</sup>

% ethanol	<i>L</i> *		<i>a</i> *		<i>b</i> *		<i>C</i> *		<i>H</i> *		$\Delta E^*$
	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	
0	19.95	1.34	48.65	0.72	4.04	0.68	48.82	0.78	4.74	0.72	
2	24.45	0.64	52.92	0.78	8.11	0.52	53.54	0.86	8.71	0.42	7.63
4	26.55	1.06	54.98	1.11	10.07	0.52	55.89	1.19	10.38	0.33	3.57
6	28.85	0.21	56.76	0.54	10.94	0.02	57.81	0.53	10.91	0.08	3.04
8	30.25	0.49	57.75	0.52	11.24	0.35	58.83	0.45	11.01	0.43	1.74
10	32.10	0.57	58.54	0.46	10.23	0.52	59.43	0.36	9.91	0.57	2.23
12	32.75	0.78	59.08	0.47	10.99	0.69	60.09	0.34	10.53	0.72	1.12
14	34.15	0.64	59.59	0.47	10.50	0.66	60.51	0.34	10.00	0.70	1.56
16	35.90	0.57	59.86	0.32	9.45	0.74	60.60	0.20	8.97	0.75	2.03
18	35.80	0.28	55.32	0.28	7.35	0.04	55.81	0.28	7.57	0.08	5.00
20	35.10	0.00	56.00	0.06	8.46	0.02	56.64	0.05	8.59	0.01	1.49
22	36.30	0.28	55.37	0.08	7.85	0.10	55.92	0.09	8.07	0.08	1.49

<sup>a</sup> The chromatic differences ( $\Delta E^*$ ) are calculated as  $[\Delta L^{*2} + \Delta C^{*2} + \Delta H^{*2}]^{1/2}$ , following an increase in the ethanol content of the reconstituted wine (i.e., from 0 to 2%, from 2 to 4%, etc.).

**Figure 3.** Plot of %Copigmentation (a) and  $\Delta$ Color (b) vs ethanol content for Cencibel reconstituted young wines.

The degree of anthocyanin polymerization can also be derived from the difference between the total anthocyanin content (**Table 1**) and the monomeric anthocyanins content as determined by HPLC (**Table 2**). The reference wine had 735 mg/L of total anthocyanins and 478 mg/L of monomeric anthocyanins, 35.0% being the percentage of nonmonomeric anthocyanins over the total anthocyanins. The nonmonomeric anthocyanins result from the reaction of monomeric anthocyanins with other substances and can be considered as anthocyanic pigments more or less polymerized. The nonmonomeric anthocyanins fraction represents a higher percentage over the total anthocyanins than the fraction of bisulfite nonbleached anthocyanins, because the bisulfite bleaching reactions occur through bisulfite addition to the C-4 of the flavonoid structure of monomeric anthocyanins (24) and in some of the nonmonomeric anthocyanins this position remains free.

The increase of the ethanol content for reconstituted wines led to a progressive diminution in the value of  $A^{\text{acet}}$ , whereas  $A^{\text{dil}}$  and  $A^{\text{SO}_2}$  remained almost constant. The contribution of copigmentation to the total wine color (%Copigmentation) for the 0% ethanol reconstituted wine was two times the value found for the reference wine. As ethanol content increased, the %Copigmentation diminished following a quadratic relation ( $r^2 = 0.9911$ ), until a minimum value of 11.5% was reached for the higher ethanol content reconstituted wine (**Figure 3a**). This result confirms the dissociating role of ethanol on copigmentation complexes and also shows that copigmentation is an important phenomenon that occurs in table young red wine. The reconstituted wines with ethanol contents between 12 and 14% maintained the three quarters of the %Copigmentation observed

when ethanol was not present. This result was the same as the results obtained for model wine solutions containing a single anthocyanin (12, 17). Nevertheless, a real red wine is a complex mixture of anthocyanins, and the result found differs from those obtained when several anthocyanins were mixed in the same model wine solution, in which no effect of ethanol content on copigmentation was observed (17). The color enhancement due to copigmentation ( $\Delta$ Color) observed in Cencibel reconstituted wines at ethanol contents of table young red wine was between 53 and 57%, being 95% for the 0% ethanol reconstituted wine and following a decreasing quadratic pattern ( $r^2 = 0.9893$ ) until a value of 18% for the maximum ethanol content reconstituted wine (**Figure 3b**). The degree of anthocyanin polymerization was unaffected by ethanol content and was the same as that measured for the reference wine (mean value (MV) = 28.7%; standard deviation (SD) = 1.32).

The reference wine had values for %Copigmentation (22.4%) and  $\Delta$ Color (40.6%) lower than the corresponding reconstituted wine with the same ethanol content (27.3 and 49.2%, respectively). It would be possible that some volatile compounds eliminated during wine concentration had some dissociating effect on the copigmentation complexes of young red wine. In particular, during the concentration process, the content in volatile acidity for the wine diminished from 0.40 to 0.24 g/L (as acetic acid), and it is known that acetic acid is a very strong dissociating cosolvent for copigmentation in model solutions (12).

The increase in ethanol content for reconstituted wines had no bathochromic effect on the value of absorbance maximum of the related visible spectra registered after dilution (526 nm), after acetaldehyde addition (530 nm), and after bisulfite bleaching (522 nm). Only a slight shift from 526 to 528 nm was observed in diluted wines when the ethanol content reached the value of 20–22%.

In relation to the chromatic characteristics, the results showed that having the same content of anthocyanins, the higher the ethanol content of the reconstituted wines, the higher the value of luminosity ( $L^*$ ) found, reaching a maximum value of 35.78 (SD = 0.50) at ethanol contents in the range of 16–22% (**Table 3**). A decrease in the value of  $L^*$  means, in the case of a red wine, that there is less absorption at 520 nm (red color), and this is an expected result because pH is increasing (less proportion of anthocyanins in the red flavilium form) and the degree of copigmentation is lower (less hyperchromic effect). At the same time, the red color component ( $a^*$ ) and the color saturation ( $C^*$ ) had increasing values until a maximum at ethanol



content of 12–16% was reached, whereas the yellow-blue color component ( $b^*$ ) and the hue angle ( $H^*$ ) reached their maximum values at the range of ethanol content between 6 and 14%. Both color components values decreased at 18% and higher ethanol contents, possibly due to the diminution in the fraction of anthocyanins in the form of flavilium cation following pH increase (lower values of  $a^*$ ; less red color wine), as well as the slight bathochromic shift observed at high ethanol contents (lower values of  $b^*$ ; less yellow or more blue color wine). Chromatic differences ( $\Delta E^*$ ) between reconstituted wines were calculated following an increase in the ethanol content of the reconstituted wine (i.e., from 0 to 2%, from 2 to 4%, etc.). The  $\Delta E^*$  values were always higher than 1.1, showing that every increase in 2% of the ethanol content led to a perceivable color change ( $\Delta E^* > 1$ ). The highest value found for  $\Delta E^*$  corresponded to the increase from 0 to 2% in the ethanol content of the reconstituted wine.

#### ACKNOWLEDGMENT

I acknowledge Naranjo Cellars (Ciudad Real, Spain) for a supply of young red wine samples.

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Received for review October 9, 2002. Revised manuscript received April 9, 2003. Accepted April 10, 2003. I acknowledge the Ministerio de Ciencia y Tecnología for financial support (Project AGL 2000-0280).

JF021029K