JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of Copigments and Grape Cultivar on the Color of Red Wines Fermented after the Addition of Copigments

MICHAEL SCHWARZ,† JOSÉ JOAQUÍN PICAZO-BACETE,‡
PETER WINTERHALTER,† AND ISIDRO HERMOSÍN-GUTIÉRREZ*,‡

Institute of Food Chemistry, Technical University of Braunschweig, Schleinitzstrasse 20, 38106 Braunschweig, Germany, and Área de Tecnología de Alimentos, Escuela Universitaria de Ingeniería Técnica Agrícola, Ronda de Calatrava 7, 13071 Ciudad Real, Spain

The prefermentation addition of copigments led to significantly different red wines according to the copigment structure (flavonol or hydroxycinnamic acid) and the grape cultivar [Tempranillo (= Cencibel) or Cabernet Sauvignon]. The flavonol rutin enhanced copigmentation and anthocyanin extraction, improving the red color, but the hydroxycinnamic acids (especially caffeic acid) had converse results. The above effects were higher in Cabernet Sauvignon wines, particularly if rutin or *p*-coumaric acid was used. These wines showed the highest copigmentation as they contained more anthocyanins and flavonols, whereas the coumaroylated anthocyanins of Tempranillo wines could have prevented the action of the added copigments. After 21 months, the main pyranoanthocyanins found were the malvidin-3-glucoside 4-vinylphenol and the malvidin-3-glucoside 4-vinylcatechol (pinotin A) adducts. The results suggested that the former adduct was primarily generated following enzymatic decarboxylation of *p*-coumaric acid during fermentation, whereas pinotin A was formed through a pure chemical reaction, which depended on the concentration of free caffeic acid during aging.

KEYWORDS: ***Vitis vinifera*; red wine; Cabernet Sauvignon; tempranillo; cencibel; anthocyanins; pyranoanthocyanins; aging products; copigmentation; rutin; caffeic acid; coumaric acid; pinotin A; vinylphenol**

INTRODUCTION

Red wine color is affected by several factors, one of them being the copigmentation phenomenon. Copigmentation is defined as a loose association between anthocyanins and colorless phenolic copigments (*1*). The formation of copigment complexes between colorless phenolic compounds and anthocyanins in their red form, i.e., the flavylium cation, enhances the red color intensity of the solution by shifting the equilibrium distribution of the anthocyanins toward the flavylium ion (hyperchromic effect); another consequence associated with copigmentation is a bathochromic effect, leading to a bluish hue of the wine's red color (*2*). Ethanol can disrupt the copigmentation complexes, but the actual effect of copigmentation in young red wines is still important and accounts for as much as 30−50% of the total red wine color (*1*).

In the last years, many researchers have focused on the most abundant copigments occurring in red wines, i.e., the monomeric (catechin and epicatechin), oligomeric (dimeric and trimeric B type proanthocyanidins), and polymeric (tannins) flavan-3-ols, and evaluated their effects on copigmentation and the formation of new anthocyanin-derived red wine pigments (*3*). However, recent research assigns more relevance with regard to copigmentation to hydroxycinnamic acid derivatives than flavan-3-ols. Caffeic acid has been shown to exhibit a strong copigmentation effect, thus contributing to a higher degree of anthocyanin extraction during winemaking if added prior to the fermentation, together with a stabilization of the red wine color over aging time (*4*, *5*). The latter result suggests that the levels of copigments in red wine are at least as important as the levels of anthocyanins in determining the color of red wine. The stabilizing effect of the acylation of the sugar moiety is also well-established, especially when the acyl group is a cinnamoyl residue (*6*−*9*). This additional stabilization has been attributed to the formation of intramolecular copigmentation complexes between the cinnamoyl residue and the anthocyanidin moiety, that is thermodynamically favored over the intermolecular copigmentation between anthocyanins and external copigments (*10*). Thus, the prefermentation addition of *p*-coumaric acid had a remarkable effect on the color of Pinot Noir red wines (a variety that does not contain any acylated anthocyanins), whereas its effect was very limited in Cabernet Sauvignon wines (*11*). In addition to the hydroxycinnamic acids, the flavonols found in red wine have been demonstrated to be the most powerful copigments in model systems (*12*−*14*). However, to our knowledge, the effect on red wine color of a prefermentation addition of flavonols has not yet been investigated. A comparison of the studies published so far is sometimes difficult because often they refer solely to the copigmentation effects on the

* To whom correspondence should be addressed. Tel: ++34-926-295300. Fax: ++34-926-295351. E-mail: Isidro.Hermosin@uclm.es.
† Technical University of Braunschweig.
‡ Escuela Universitaria de Ingeniería Técnica Agrícola.

10.1021/jf051005o CCC: $30.25
Published on Web 09/23/2005

absorbance measured at 520 nm and at wine pH. Red wine pH values, however, were not necessarily identical in all of these experiments but may have varied due to the influence of the grape cultivar, the degree of grape ripeness, or the addition of acidic copigments. The wine pH value strongly influences the anthocyanin equilibrium in solution and the formation of copigmentation complexes, thus having a dramatic effect on the absorbance value at 520 nm. It has been suggested that copigmentation is the first step in the formation of stable pigments in aged red wines (*15*), and recently, it was shown that hydroxycinnamic acids themselves (coumaric, caffeic, ferulic, or sinapic acid) or their decarboxylation products (4-vinylphenols) can covalently react with anthocyanins, giving rise to the formation of pyranoanthocyanins (*16*−*19*). Pyranoanthocyanins were initially reported to be produced from anthocyanins and certain secondary yeast metabolites such as acetaldehyde or pyruvic acid (*20*, *21*). More generally speaking, the pyranoanthocyanin structure can be formed through the reaction of a compound containing a polarizable double-bound with an anthocyanin.

The aim of this work was to evaluate the possible color improvement of red wines by the prefermentation addition of naturally occurring copigments: a flavonol (rutin) and two hydroxycinnamic acids (caffeic and *p*-coumaric acid). The two selected hydroxycinnamic acids have already been employed in studies on wine copigmentation (*4*, *5*, *11*), but rutin was only used in model solutions (*12*, *13*). Although rutin is a minor quercetin type flavonol found in wine, it was selected due to being representative of the main type of wine flavonols and for its greater solubility in aqueous and hydro alcoholic media than quercetin itself. The grape cultivars assayed were Tempranillo, a Spanish cultivar widely grown in the vast winemaking region of La Mancha (middle-southern Spain) where this cultivar is also known as Cencibel, and Cabernet Sauvignon, a worldwide grown grape variety. Besides analyzing the red color intensity, the chromatic characteristics, and the contribution of copigmented and polymerized anthocyanins to the total wine color, we also determined the detailed composition of the red pigments, and colorless copigments. The results were compared at the end of the alcoholic fermentation and during an aging period of 9 months. After 21 months, the effect of the added copigments on the formation of pyranoanthocyanins was investigated.

MATERIALS AND METHODS

Chemicals. All solvents were of high-performance liquid chromatography (HPLC) quality, and all chemicals were of analytical grade (>99%). Water was of nanopure quality.

Winemaking. Healthy red wine grapes from the cultivars Tempranillo (also known as Cencibel) and Cabernet Sauvignon grown in Ciudad Real (La Mancha, middle-southern Spain) were collected at optimum ripeness: 13.8% of potential ethanol content and total acidity of 5.96 g/L (as tartaric acid) for Tempranillo and 13.9% of potential ethanol content and total acidity of 5.14 g/L (as tartaric acid) for Cabernet Sauvignon. The grapes (60 kg of each cultivar) were destemmed and crushed, and 100 mg/L of SO_2 (as $K_2S_2O_7$) was added to the resulting mash to avoid oxidation. After soft pressing, the musts and solids were equally distributed into 12 vats. For each cultivar assayed, three vats corresponded to the so-called control wines, whereas the others were divided into three treatments, the prefermentation addition of the copigments rutin, caffeic acid, and *p*-coumaric acid, respectively. The copigments were added in the same molar amount (1 mM in relation to the expected must yield, 0.7 L per kg of grape).

Alcoholic fermentation conditions were as follows: inoculation with *Saccharomyces cerevisiae* yeast (UCLM S325, Fould-Springer); temperature kept at 24 °C; manual punching down every 12 h; wine separation after 7 days, when the relative density had reached a constant value. After completion of the alcoholic fermentation, malolactic fermentation was induced by inoculation with *Oenococcus oeni* lactic acid bacteria (Lactobacter SP1; Laffort). The completion of the malolactic fermentation was confirmed by thin-layer chromatography (TLC) (Vinikit, Panreac), and then, the wines were racked. Finally, after 100 days of storage at 18−20 °C, the wines were filtered through 1.2 μm filters (Millipore), bottled (125 mL volume bottles), and stored at 8 °C. Wine samples were analyzed after alcoholic fermentation, after malolactic fermentation, 4 and 9 months of storage.

Analysis of Phenolic Compounds. Total anthocyanins were determined using a modification of the Glories's method (*22*, *23*) as described by Mazza et al. (*24*). Individual phenolic compounds (anthocyanins, flavonols, and hydroxycinnamic acid derivatives) were determined by HPLC (*25*): The wine samples, after centrifugation (2500*g*) and filtration (0.45 μm nylon membranes, Millipore), were directly injected (10 μL) into a Waters 2690 liquid chromatograph (Waters, Milford, MA) equipped with a Waters 996 photodiode array detector. Separations were carried out on a Spherisorb C18, 5 μm, 250 mm × 4.6 mm i.d. column at 40 °C. The solvents were A ($NH_4H_2PO_4$, 50 mM, pH 2.6), B (20% A + 80% acetonitrile), and C (H_3PO_4, 200 mM, pH 1.5). The elution gradient was as follows: 100% A (min 0); 100% A (min 2); 92% A + 8% B (min 5); 14% B + 86% C (min 17); 18% B + 82% C (min 22); 21.5% B + 78.5% C (min 32); 43% B + 57% C (min 62); 100% A (min 70); and 100% A (min 75). Individual chromatograms were extracted at 320 (hydroxycinnamic acid derivatives), 360 (flavonols), and 520 nm (anthocyanins). For quantification, calibration curves were obtained by injecting solutions of the corresponding standards if commercially available (malvidin 3-glucoside; the flavonol aglycones myricetin, quercetin, kaempferol, and isorhamnetin; the 3-glucosides of quercetin, kaempferol, and isorhamnetin; rutin; kaempferol 3-rutinoside; caffeic acid; and *p*-coumaric acid), whereas for the other compounds the concentrations were expressed in mg/L of the most similar standard: caffeic acid for caftaric acid; *p*-coumaric acid for coutaric acid; malvidin 3-glucoside for monomeric anthocyanins; quercetin 3-glucoside for myricetin 3-glucoside and quercetin 3-glucuronide; and rutin for quercetin 3-glucosylgalactoside and 3-glucosylxyloside.

Color Properties. Absorbances of the centrifuged wines (2500*g*, 15 min) at 520 nm were determined directly and after adjusting the pH value to 3.6, using a Unicam UV 540 spectrophotometer. The contribution of copigmented anthocyanins to the total wine color at pH 3.6 (% copigmentation) and the degree of anthocyanin polymerization (% polymerization) were determined following the method developed by Boulton (*26*) as described by Hermosín Gutiérrez (*27*). Chromatic CIELAB characteristics (L^*, C^*, and h^*) were calculated from the absorbances at 450, 520, 570, and 630 nm of the centrifuged and pH-adjusted wines, according to a proposed simplified method (*28*, *29*).

Statistical Analysis. The data corresponding to the control and copigment-added wines for each grape cultivar were analyzed by Student−Newman−Keuls test (SPSS version 10.0, SPSS Inc.).

Quantitative Analysis of Pyranoanthocyanins. Wines were analyzed by HPLC with diode array detection. A PU-980 Intelligent HPLC pump equipped with a DG-980-50 three-line degasser, LG-980-02 ternary gradient unit, and MD-1510 multiwavelength detector were used (Jasco, Germany). Samples were injected via a Rheodyne 7175 injection valve (Techlab, Germany) equipped with a 20 μL loop, and separations were carried out on a Synergi MaxRP-12, 4 μm, 250 × 4.6 mm i.d. column (Phenomenex, Germany). Solvents were water/acetonitrile/formic acid (87:3:10, v/v/v, solvent A; 40:50:10, v/v/v, solvent B), and the flow rate was 0.5 mL/min. The linear gradient was from 6 to 20% B for 0−20 min, from 20 to 40% B for 20−35 min, from 40 to 60% B for 35−40 min, from 60 to 90% B for 40−45 min, and held at 90% B for 45−50 min.

Pinotin A was quantified using a previously obtained calibration curve at 510 nm (*30*). The concentration of all other pyranoanthocyanins was also expressed as Pinotin A, but the differing molecular weights were taken into account.

Identification of Pyranoanthocyanins by HPLC with Electrospray Ionization Multiple Mass Spectrometry (HPLC-ESI-MS^n). A Bruker Esquire ion trap LC-MS system was used (Bruker Daltonik,

Table 1. Mean ($n = 3$) Phenolic Composition and Chromatic Characteristics for Control and Copigment-Added Wines after Completion of Alcoholic Fermentation[a]

phenolic compound or chromatic parameter	Tempranillo				Cabernet Sauvignon			
	control	+ caffeic acid	+ *p*-coumaric acid	+ rutin	control	+ caffeic acid	+ *p*-coumaric acid	+ rutin
AU at 520 nm (wine pH)	1.0430 a	0.9437 b	1.0104 a	1.1134 c	1.1921 a	1.0924 b	1.3990 c	1.5217 d
AU at 520 nm (pH 3.6)	1.0890 a	1.0121 a	1.0487 a	1.1894 b	1.1177 a	1.0569 b	1.3641 c	1.5031 d
total anthocyanins[b]	360 a	320 b	334 b	396 c	318 a	288 b	292 b	383 c
% polymerization	28.5 a	30.6 a	39.5 b	28.5 a	33.8 a	34.5 a	46.4 b	33.4 a
% copigmentation	23.9 a	19.7 b	19.7 b	25.7 a	26.9 a	24.5 b	25.1 b	28.8 c
malvidin 3-glucoside[b]	234 a	170 b	189 b	218 c	156 a	145 b	143 b	181 c
malvidin 3-(6″-acetylglucoside)[b]	19.6 a	15.1 b	15.6 b	18.9 a	81.2 a	71.0 b	70.3 b	91.4 c
malvidin 3-(6″-coumaroylglucoside)[b]	40.8 a	25.5 b	27.0 c	37.1 d	14.1 a	12.8 a	12.5 a	21.5 b
total flavonols[c]	34.1 a	30.6 a	31.0 a	97.8 b	94.0 a	88.7 a	95.3 a	178.7 b
total flavonols (without rutin)[c]	32.5 a,b	28.9 b	29.5 b	35.3 a	87.6 a	82.6 a	88.9 a	102.5 b
L^* (pH 3.6)	53.7 a	56.2 b	56.7 b	50.9 c	51.3 a	53.9 b	54.2 b	43.9 c
C^* (pH 3.6)	49.8 a	46.6 b	46.9 b	51.3 a	50.5 a	47.6 b	46.8 b	55.0 c
h^* (pH 3.6)	1.19 a	3.16 b	5.26 c	0.00 a	1.36 a	3.16 a	6.90 b	2.83 a

[a] Different letters in the same row, for wines of the same cultivar, indicate significant differences according to the test of Student–Newman–Keuls ($\alpha = 0.05$). [b] mg/L as malvidin 3-glucoside. [c] mg/L.

Germany). The HPLC system consisted of a System 1100 Binary Pump G1312A (Agilent, Germany) and a Rheodyne 7725i injection valve with a 20 μL loop (Techlab, Germany). MS parameters: positive ion mode; dry gas, N_2; 11 L/min; drying temperature, 325 °C; nebulizer, 60 psi; capillary, −2500 V; capillary exit offset, 70 V; end plate offset, −500 V; skimmer 1, 20 V; skimmer 2, 10 V; scan range, 50−1200 *m*/*z*; chromatographic conditions as above.

RESULTS AND DISCUSSION

Red Wine Color at the End of Alcoholic Fermentation. At the end of alcoholic fermentation, Tempranillo wines had reached a pH value of 3.75. Statistically significant differences in the absorbances at 520 nm (A_{520}) were found between the control wine and the copigment-added wines, except for the *p*-coumaric acid treatment. However, upon adjustment of the pH to the reference value of pH 3.6, only the rutin addition had a significant effect on the absorbance as compared to the other treatments and the control wine (**Table 1**). For Cabernet Sauvignon wines, the original wine pH of 3.54 was very close to the reference value and only slight decreases were observed in A_{520} measured at pH 3.6. Significant differences were observed between all control and copigment-added wines at both pH values. The prefermentation addition of rutin was accompanied by a significant hyperchromic effect in both varieties, which can be explained by the formation of copigment complexes between rutin and wine anthocyanins. At reference pH, the magnitude of the hyperchromic effect was 9% for Tempranillo and 35% for Cabernet Sauvignon wines. No comparable experiments of a prefermentation addition of a flavonol have been described in the literature, but the hyperchromic effect was to be expected, since flavonols have been demonstrated to be the most powerful copigments in model systems (*12*−*14*).

The prefermentation addition of hydroxycinnamic acids only led to a hyperchromic effect if *p*-coumaric acid was added to Cabernet Sauvignon wines (**Table 1**). With a 22% increase of A_{520} at reference pH, the magnitude of this effect was lower as compared to rutin. The prefermentation addition of caffeic acid always resulted in hypochromic effects, decreasing the A_{520} at reference pH by 7% (Tempranillo) and 5% (Cabernet Sauvignon). These results seem to contradict those previously described for the prefermentation addition of caffeic acid to wines made from the Spanish cultivars Listán Negro and Negramoll (*4*, *5*), although for Negramoll wines the effects of caffeic acid were negligible when it was added in amounts of 120 and 240 mg/L, similar to the 1 mM concentration in our experiments. Moreover, our results are in line with those described for Cabernet Sauvignon wines (*11*), in which the prefermentation addition of 150 mg/L of *p*-coumaric acid acid increased A_{520} by 30%, whereas the addition of the same amount of caffeic acid had the opposite effect, decreasing A_{520} by 4%.

The total anthocyanin content in all of the rutin-added wines was higher as compared to the control wines, 10% in Tempranillo and 20% in Cabernet Sauvignon wines (**Table 1**). These results suggest that rutin favored the extraction of grape anthocyanins during winemaking through the formation of stable copigment complexes with anthocyanins. Conversely, the addition of the hydroxycinnamic acids always resulted in a lower content of total anthocyanins in relation to the control wines: 11% lower for Tempranillo and 9% lower for Cabernet Sauvignon, following the addition of caffeic acid; 7% lower for Tempranillo and 8% lower for Cabernet Sauvignon, following the addition of *p*-coumaric acid. The low total anthocyanin content of the *p*-coumaric acid-added Cabernet Sauvignon wines, as compared to the control wines, seemed not to be correlated to their higher value of A_{520}. This apparent discrepancy can be explained on the basis of the parameters that affect the aqueous equilibrium of anthocyanins: At reference pH, only the 8.6% of monomeric anthocyanins are in their red-colored form (*31*, *32*) and they form copigmentation complexes, whereas the less pH sensitive nonmonomeric anthocyanins predominate in their red-colored form; at the low pH value used for measuring A_{520}, both monomeric and nonmonomeric anthocyanins predominate in their respective red-colored forms, but the monomeric anthocyanins are currently not copigmented due to the dilution required for this measurement (*32*, *33*).

The content of monomeric anthocyanins, as well as the contribution of copigmented (% copigmentation) and polymerized (% polymerization) anthocyanins to the total wine color (**Table 1**), can provide additional proof for the above explanation. Wines with added hydroxycinnamic acids always had lower contents of the predominant malvidin type anthocyanins than the respective control wines. The former wines also had lower values for % copigmentation, especially the Tempranillo wines (18% lower; only 7% lower for Cabernet Sauvignon). In contrast, the values of % polymerization for the *p*-coumaric acid-

added wines were significantly higher than for the control wines, the increase being very similar for both cultivars (39% for Tempranillo and 37% for Cabernet Sauvignon). No significant effect on % polymerization was recorded for the caffeic acid-added wines. A similar increase in % polymerization for both Tempranillo and Cabernet Sauvignon wines, but a lesser decrease in % copigmentation for only Cabernet Sauvignon wines, could be the reason for the converse effects observed in these wines after the addition of *p*-coumaric acid.

The addition of rutin always led to the highest values for % copigmentation as compared to the control wines or hydroxycinnamic acid treatments, although it significantly lowered the content of monomeric anthocyanins in Tempranillo (**Table 1**). A surprising result associated with the addition of rutin was the significant increase in the amount of extracted flavonols (total flavonols without rutin, **Table 1**) in the Cabernet Sauvignon wines. This result suggests a mutual synergism in the extraction of both anthocyanins and flavonols from the grapes into the must: The addition of rutin could favor the formation of copigmentation complexes, resulting in a decrease in the concentration of free anthocyanins in solution and thus promoting a higher degree of anthocyanin extraction from the grapes; subsequently, because of the increase of the anthocyanin concentration in the must, more flavonols would be extracted from the grapes.

The observed chromatic characteristics of the wines were in accordance with their different phenolic compositions (**Table 1**). Addition of rutin gave the darkest wines (lowest values of L^*), due to the hyperchromic effect associated with the enhancement of copigmentation. These wines also showed the most saturated red colors (highest values of C^*), with purple hues (very low h^* values) that suggest an additional bathochromic effect. In contrast, wines made with the addition of hydroxycinnamic acids had the lowest content in total anthocyanins and % copigmentation, and thus, they were also the lightest wines; besides, the extent of polymerization was the greatest in the caffeic acid-added wines, resulting in less saturated colors with weak purple tonalities.

Evolution of Red Wine Color during Aging. After the completion of the malolactic fermentation and during the aging period, the chromatic characteristics and the phenolic composition underwent a similar evolution in both control and copigment-added wines. The positive effects on color by the addition of rutin were maintained over the aging time. It can be concluded that prefermentative addition of rutin enhanced the extraction of anthocyanins during winemaking and also helped to maintain the highest levels of anthocyanins over the aging time. The addition of hydroxycinnamic acids had variable effects depending on structure and the grape cultivar.

The value of A_{520} at reference pH decreased in all wines during the aging time (**Figure 1**). The differences found for this measure between the control and the copigment-added Cabernet Sauvignon wines at the end of the alcoholic fermentation decreased for rutin (from +35 to +15% after 9 months) and *p*-coumaric acid (from +22 to +10% after 9 months), whereas they increased for caffeic acid (from −5 to −12% after 9 months). A correspondent evolution in Tempranillo wines, starting from slighter differences at the end of the alcoholic fermentation, led to very similar wines after 9 months of aging and a reversal of the initial effects of hydroxycinnamic acids was observed (from −4 to +3%, in *p*-coumaric acid-added wines; from −7 to +1%, in caffeic acid-added wines). Thus, the addition of copigments to Tempranillo wines had no significant effect after 9 months in any of the wines.

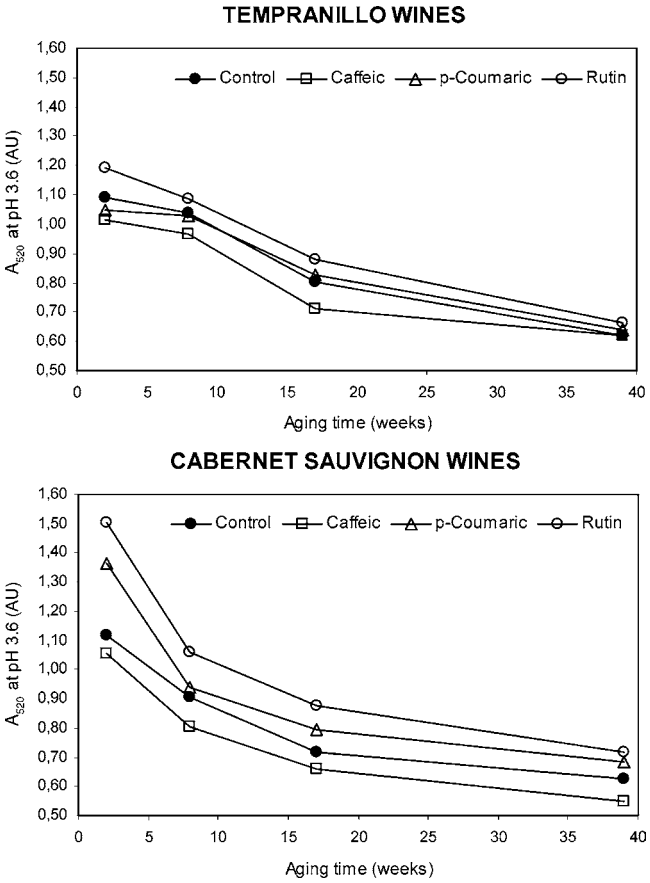

Figure 1. Absorbance at 520 nm (pH 3.6) during the aging period of control and copigment-added Tempranillo and Cabernet Sauvignon wines.

As expected, the content of total anthocyanins decreased with time (**Figure 2**). The rutin-added wines always displayed the highest total anthocyanin content, while the hydroxycinnamic acid-added wines had the lowest; only the *p*-coumaric acid-added Cabernet Sauvignon wine reached a slightly higher content than its control after 9 months. The above differences found for total anthocyanins were also observed for every individual malvidin type anthocyanin and maintained over the aging time.

The decrease in monomeric anthocyanin during aging is mainly attributed to formation of polymeric pigments, as confirmed by the increasing % polymerization (**Figure 3**). However, from the 4th to the 9th month of aging the concentration of polymeric pigments in the wines decreased due to an observed precipitation of coloring matter. The formation of polymeric pigments diminished the concentration of monomeric anthocyanins in the wines, and consequently, % copigmentation also decreased until disappearance (**Figure 4**). This was a constant trend for Cabernet Sauvignon wines, whereas for Tempranillo wines a delay of 4 months was observed. As already shown after alcoholic fermentation, addition of rutin resulted in wines with the highest percentage of copigmented anthocyanins over the aging time, whereas the hydroxycinnamic acid addition led to wines with a lower % copigmentation as compared to the control; for Cabernet Sauvignon wines, the addition of hydroxycinnamic acids only resulted in a significant lower % copigmentation just after the end of alcoholic fermentation.

The evolution of the CIELAB parameters confirmed the disappearance of the copigmentation phenomenon and the decreasing content of monomeric anthocyanins by conversion into anthocyanin-derived pigments with different color properties

Table 2. Mean Value ($n = 3$) for CIELAB Chromatic Parameters of Control and Copigment-Added Wines at Different Aging Times[a]

		Tempranillo				Cabernet Sauvignon			
chromatic parameter	aging time	control	+ caffeic acid	+ *p*-coumaric acid	+ rutin	control	+ caffeic acid	+ *p*-coumaric acid	+ rutin
*L** (pH 3.6)	EAF	53.7 a	56.2 b	56.7 b	50.9 c	51.3 a	53.9 b	54.2 b	43.9 c
	EMLF	53.9 a	56.0 a	54.9 a	51.6 b	56.1 a	60.7 b	58.1 b	52.1 c
	4 months	61.0 a	64.4 b	62.0 a	57.8 c	63.3 a	65.8 a	65.3 a	57.4 b
	9 months	65.7 a	65.4 a	66.6 a	63.5 b	66.0 a	69.9 b	66.1 a	62.4 c
*C** (pH 3.6)	EAF	49.8 a	46.6 b	46.9 b	51.3 a	50.5 a	47.6 b	46.8 b	55.0 c
	EMLF	44.4 a	42.2 b	43.4 c	44.8 a	39.8 a,b	37.3 a	40.9 b	44.9 c
	4 months	39.2 a	36.1 b	40.1 a	41.7 a	34.9 a	33.2 a	35.1 a	40.8 b
	9 months	29.1	29.0	31.2	30.4	30.7 a	29.0 a	34.0 b	33.9 b
*h** (pH 3.6)	EAF	1.19 a	3.16 b	5.26 c	0.00 a	1.36 a	3.16 a	6.90 b	2.83 a
	EMLF	7.16 a	9.85 b	11.68 c	6.40 a	13.63 a	14.39 a	16.41 b	8.71 c
	4 months	7.56 a	10.19 b	11.11 b	5.68 a	17.14 a	19.31 b	20.20 b	9.91 c
	9 months	20.80	22.20	20.50	20.60	26.19 a	30.60 b	28.80 b	22.60 c

[a] Different letters in the same row, for wines of the same cultivar, indicate significant differences according to the test of Student–Newman–Keuls ($\alpha = 0.05$). EAF, end of alcoholic fermentation; EMLF, end of malolactic fermentation.

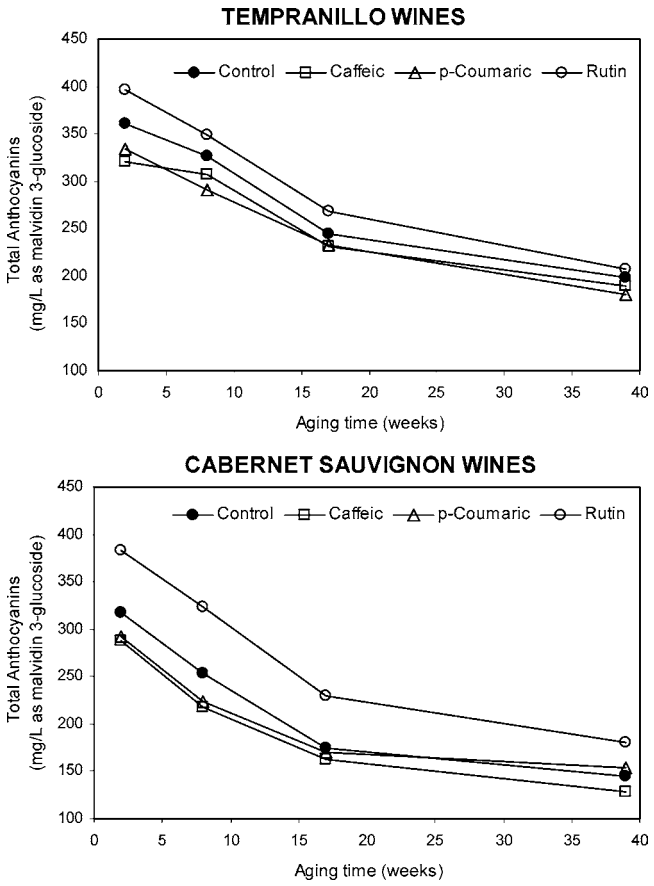

Figure 2. Content of total anthocyanins during the aging period of control and copigment-added Tempranillo and Cabernet Sauvignon wines.

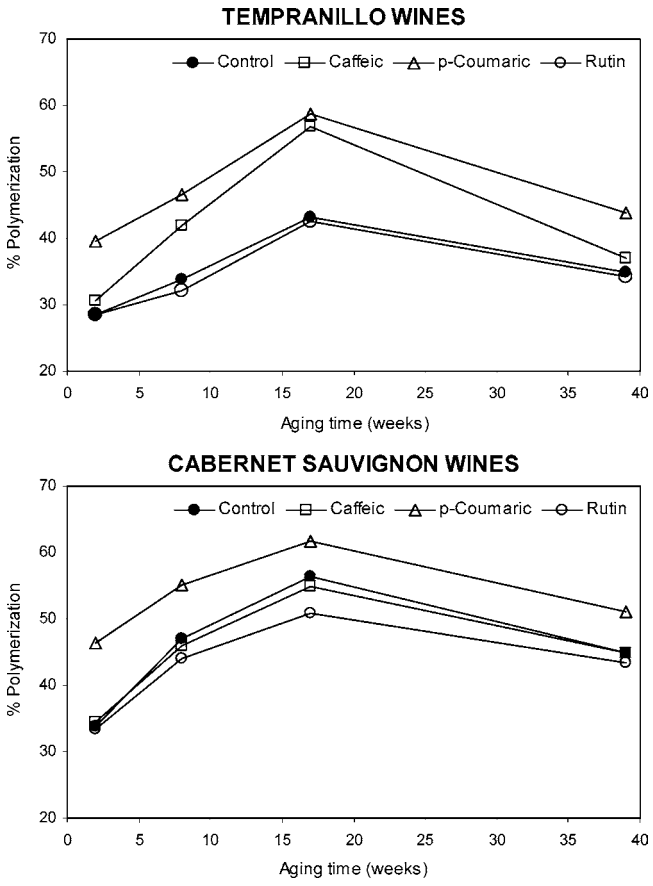

Figure 3. Percent polymerization during the aging period for control and copigment-added Tempranillo and Cabernet Sauvignon wines.

(**Table 2**). All the wines turned lighter during aging (increasing *L** values), and the resulting color was less saturated (decreasing *C** values). The diminishing bathochromic effect associated with copigmentation, together with the increase in % polymerization, led to an increase of the *h** values. The color of the wines was finally described as a pure red, without purple hues. All chromatic characteristics changed more slowly in the rutin-added wines, especially in Cabernet Sauvignon wines.

Effect of the Grape Cultivar on Red Wine Color. The Cabernet Sauvignon wines were more sensitive to the prefermentation addition of copigments than those from the cultivar Tempranillo, especially when rutin or *p*-coumaric acid was added. Some relationships can be established between the cultivar-dependent behavior described above and the characteristic phenolic composition (anthocyanins and copigments) of the grape variety.

The molar ratio copigments/anthocyanins in red wines ranges from 0.05 to 2 and exerts a dramatic influence on copigmentation effects (*1*). In our study, the molar concentration of monomeric anthocyanins ([MA]) at the end of alcoholic fermentation was lower in Cabernet Sauvignon than in Tempranillo control wines (625 vs 728 μmol/L). In contrast, flavonols were found in a higher molar concentration ([Flv]) in Cabernet Sauvignon control wines (191 vs 71 μmol/L in Tempranillo). Therefore, the highest ratio [Flv]/[MA] occurred in the Cabernet Sauvignon control wines (0.306 vs 0.098 for Tempranillo), and

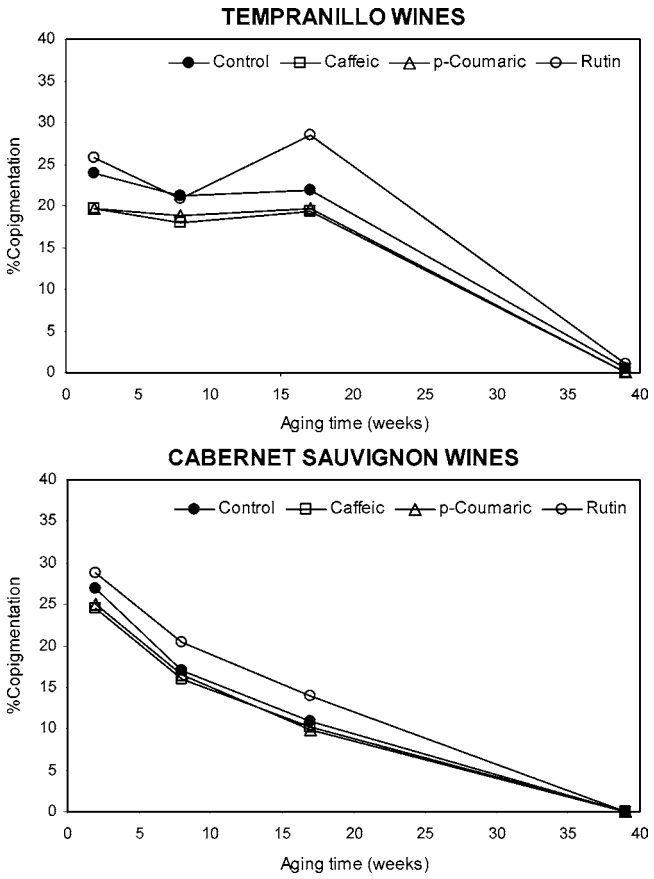

Figure 4. Percent copigmentation during the aging period for control and copigment-added Tempranillo and Cabernet Sauvignon wines.

this can also explain why they had the highest values for % copigmentation (26.9 vs 23.9% for Tempranillo). Addition of rutin led to an increase of both [MA] and [Flv] in Cabernet Sauvignon wines (+19 and +76%, respectively), whereas in Tempranillo wines [Flv] increased by 146% but [MA] decreased by 8%. Consequently, the ratio [Flv]/[MA] increased for Cabernet Sauvignon wines by 47% and for Tempranillo wines by 165%. Thus, a higher percentage of color enhancement was to be expected for the rutin-added Tempranillo wines, but contrary to expectations, the Cabernet Sauvignon wines were more affected. Cabernet Sauvignon wines still had the highest concentrations of flavonols and monomeric anthocyanins after rutin addition, and thus, more copigment complexes could be formed.

The monomeric anthocyanin profile of a certain grape variety is a useful tool in the differentiation of cultivars (*34* and references therein) and may also have had an influence on the results obtained in this study. The two cultivars assayed in our experiment differed in the composition of the acylated anthocyanins. Tempranillo wines contained 76% of nonacylated, 7% of acetylated, and 17% of *p*-coumaroylated anthocyanins, while Cabernet Sauvignon wines contained 60% of nonacylated, 31% of acetylated, and 9% of *p*-coumaroylated anthocyanins. In model systems containing intramolecular copigmented anthocyanins (anthocyanins with two sugar molecules between the anthocyanidin and the cinnamoyl moiety), the formation of intermolecular copigmentation complexes with added copigments was hindered (*10*). It appears likely that the *p*-coumaroyl substituent of wine anthocyanins could be implicated in copigmentation. To our knowledge, there is no evidence that intramolecular copigmentation occurs in wine anthocyanins, although molecular modeling allows this possibility and Brouillard suggested intramolecular copigmentation in winelike anthocyanins, with only one sugar molecule between the anthocyanidin and the caffeoyl moiety (*35*). However, even if the *p*-coumaroyl substituent of wine anthocyanins was only implicated in intermolecular copigmentation, the higher proportion of *p*-coumaroylated anthocyanins in Tempranillo wines could diminish the effect of added copigments in these wines.

Effect of Copigments on the Formation of Pyranoanthocyanins. It has been demonstrated that hydroxycinnamic acids can react with anthocyanins in red wines or fruit juices (*19*, *36*−*39*). The chromatic characteristics of the resulting hydroxyphenyl-substituted pyranoanthocyanins have been found to be only slightly affected by pH changes or bisulfite addition as compared to the respective genuine anthocyanins (*40*−*42*). Thus, pyranoanthocyanins may have a noticeable impact on the perceived color of wines and juices. After an aging time of 21 months, the elaborated wines were analyzed for the presence of pyranoanthocyanins by HPLC-ESI-MS^n. A total of 12 different pyranoanthocyanins were detected and identified by their mass spectrometric characteristics and through comparison with reference compounds that were either previously isolated from Pinotage red wines or synthesized in model solutions (*19*). The concentration of pyranoanthocyanins was determined by HPLC with diode array detection. As only pinotin A (the malvidin 3-glucoside 4-vinylcatechol adduct) was available as a pure reference standard, all other pyranoanthocyanins were quantified as pinotin A taking into account the differing molecular weights. The average concentration of all pigments is given in **Table 3**. Pinotin A and the malvidin 3-glucoside 4-vinylphenol adduct were the major pyranoanthocyanins and could be detected in noteworthy quantities in all control and copigment-added wines. The other 10 pyranoanthocyanins were found in some of the wines only and were usually present just above the detection limit or in trace amounts below 0.2 mg/L. However, the coumaric acid-enriched wines contained exceptionally high concentrations of the 4-vinylphenol adducts of malvidin 3-(6″-acetylglucoside) and malvidin 3-(6″-coumaroylglucoside).

From the concentrations of pinotin A and the malvidin 3-glucoside 4-vinylphenol adduct, together with the content of hydroxycinnamic acids in the wines after alcoholic and malolactic fermentation, and after 4 and 9 months of aging, certain conclusions can be drawn on the most likely pathway of formation for these two compounds. Two alternatives have been suggested (**Figure 5**). After the isolation and identification of the malvidin 3-glucoside 4-vinylphenol adduct, it has been proposed that this compound can be formed in red wines by the reaction of malvidin 3-glucoside with free 4-vinylphenol, that in turn is formed via a yeast-mediated decarboxylation of the precursor coumaric acid during fermentation (*16*). Later, this hypothesis has been extended, and a general formation of red wine pyranoanthocyanins by the reaction of anthocyanins with enzymatically decarboxylated hydroxycinnamic acids was assumed (*17*). Following the isolation and structure elucidation of pinotin A, it was demonstrated that all naturally occurring hydroxycinnamic acids, e.g., coumaric, caffeic, ferulic, and sinapic acid, can directly react with malvidin 3-glucoside in a purely chemical reaction without the need of enzymatic support (*19*).

The percentage content of pinotin A (after 21 months) and the percentage and absolute content of its related precursor caffeic acid (after alcoholic and malolactic fermentation, 4 and 9 months of storage) in all Cabernet Sauvignon and Tempranillo wines is shown in **Table 4**. In Cabernet Sauvignon wines, the

Table 3. Average Concentration in mg/L ($n = 3$) of Pyranoanthocyanins (Expressed as Pinotin A) in the Elaborated Wines after 21 Months of Aging[a]

	Cabernet Sauvignon				Tempranillo			
	control	+ *p*-coumaric acid	+ caffeic acid	+ rutin	control	+ *p*-coumaric acid	+ caffeic acid	+ rutin
pinotin A	0.26 (4.5)	0.56 (8.9)	0.52 (2.6)	0.49 (6.1)	0.71 (27.7)	1.15 (10.6)	1.36 (1.9)	0.82 (4.2)
ac-pinotin A	tr	ND	0.24 (6.9)	ND	ND	ND	0.09 (4.1)	ND
cu-pinotin A	ND	ND	ND	ND	ND	0.22 (38.6)	0.18 (5.2)	ND
mv-3-glc 4-VP	tr	3.51 (2.3)	0.15 (3.2)	0.24 (53.6)	0.34 (14.6)	3.34 (11.8)	0.34 (6.2)	0.42 (10.7)
mv-3-acglc 4-VP	tr	2.32 (5.8)	0.04 (5.9)	0.20 (8.0)	0.10 (43.7)	0.34 (16.2)	tr	0.08 (31.1)
mv-3-cuglc 4-VP	ND	0.68 (10.0)	ND	ND	ND	0.84 (19.6)	ND	0.12 (15.9)
mv-3-glc 4-VG	tr	tr	0.10 (6.2)	0.10 (86.7)	0.17 (8.1)	0.15 (10.0)	0.17 (6.4)	0.17 (9.1)
mv-3-acglc 4-VG	ND	tr	0.06 (4.9)	tr	ND	tr	ND	ND
peo-3-glc 4-VC	ND	0.20 (7.1)	ND	ND	ND	0.30 (10.5)	tr	ND
peo-3-acglc 4-VC	ND	tr	ND	ND	ND	tr	ND	ND
peo-3-cuglc 4-VC	ND	ND	ND	ND	ND	0.09 (46.8)	ND	ND
peo-3-glc 4-VP	ND	tr	ND	ND	ND	tr	ND	ND

[a] The coefficient of variation (%) is given in parentheses. ND, not detected; tr, detected in trace amounts; ac, 6″-acetyl; cu, 6″-coumaroyl; mv, malvidin; peo, peonidin; glc, glucoside; VP, vinylphenol; VG, vinylguaiacol; and VC, vinylcatechol.

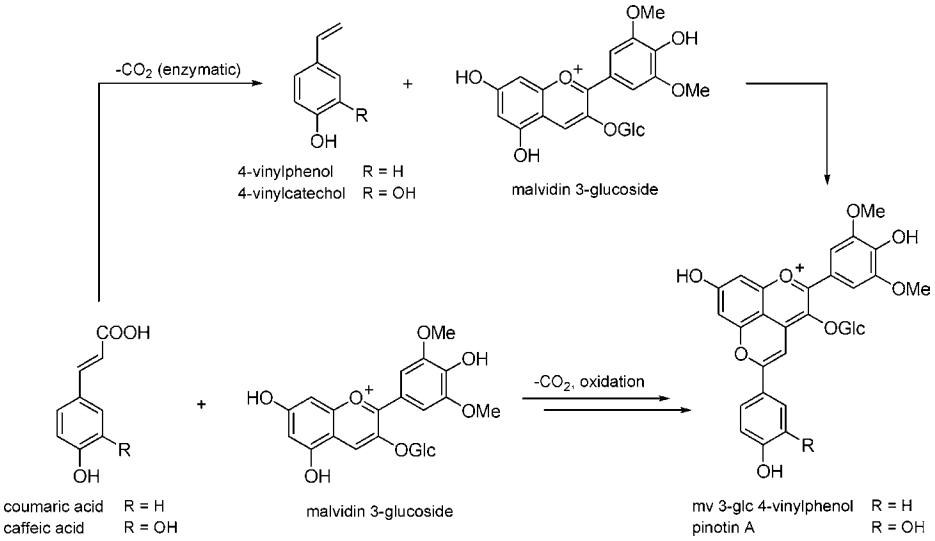

Figure 5. Formation of pyranoanthocyanins via the two postulated pathways (*16*, *19*).

Table 4. Relative Concentration of Pinotin A and Average ($n = 3$) Relative and Absolute (mg/L) Concentration of Its Precursor Caffeic Acid in the Wines at Various Stages of Aging[a]

		caffeic acid % and (mg/L)			
	pinotin A 21 months %	end of alcoholic fermentation	end of malolactic fermentation	4 months	9 months
		Cabernet Sauvignon			
control	46.4	50.9 (5.9)	57.5 (9.2)	60.0 (7.5)	58.8 (9.0)
+ *p*-coumaric acid	100.0	54.3 (6.3)	83.8 (13.4)	78.4 (9.8)	86.2 (13.2)
+ caffeic acid	92.9	100.0 (11.6)	100.0 (16.0)	100.0 (12.5)	100.0 (15.3)
+ rutin	87.5	59.5 (6.9)	88.8 (14.2)	92.0 (11.5)	94.1 (14.4)
		Tempranillo			
control	51.7	38.9 (7.2)	59.1 (13.6)	57.0 (11.8)	62.6 (13.2)
+ *p*-coumaric acid	84.3	37.8 (7.0)	63.5 (14.6)	68.1 (14.1)	64.5 (13.6)
+ caffeic acid	100.0	100.0 (18.5)	100.0 (23.0)	100.0 (20.7)	100.0 (21.1)
+ rutin	60.1	37.3 (6.9)	56.1 (12.9)	58.0 (12.0)	58.3 (12.3)

[a] For abbreviations, cf. **Table 3**.

average concentration of pinotin A in all wines with added copigments was very similar and ranged from 0.49 to 0.56 mg/L (**Table 3**) or 87.5−100%. The control wine contained with 0.26 mg/L pinotin A only 46.4% of the maximum observed content. The percentage distribution of free caffeic acid in all Cabernet Sauvignon wines remained constant following the malolactic fermentation. Just after the end of alcoholic fermentation, the caffeic acid-enriched wine contained ∼12 mg/L, while only around 6 mg/L was found in the others. Because of the hydrolysis of caftaric acid (data not shown), the concentration of free caffeic acid increased during the malolactic fermentation, and the difference in the caffeic acid content in all copigment-added wines vanished (83.8−100% after the malolactic fermentation vs 54.3−100% at the end of alcoholic fermentation).

Table 5. Relative Concentration of the Malvidin 3-Glucoside 4-Vinylphenol Adduct and Average ($n = 3$) Relative and Absolute (mg/L) Concentration of Its Precursor Coumaric Acid in the Wines at Various Stages of Aging[a]

		coumaric acid % and (mg/L)			
	mv 3-glc 4-VP 21 months %	end of alcoholic fermentation	end of malolactic fermentation	4 months	9 months
		Cabernet Sauvignon			
control	tr	12.5 (0.1)	72.2 (2.6)	71.9 (2.3)	76.2 (1.6)
+ *p*-coumaric acid	100.0	100.0 (0.8)	97.2 (3.5)	90.6 (2.9)	100.0 (2.1)
+ caffeic acid	4.3	12.5 (0.1)	77.7 (2.8)	81.3 (2.6)	81.0 (1.7)
+ rutin	6.8	25.0 (0.2)	100.0 (3.6)	100.0 (3.2)	95.2 (2.0)
		Tempranillo			
control	10.1	25.0 (0.3)	94.1 (8.0)	84.6 (6.6)	88.9 (4.0)
+ *p*-coumaric acid	100.0	100.0 (1.2)	100.0 (8.5)	100.0 (7.8)	100.0 (4.5)
+ caffeic acid	10.2	25.0 (0.3)	96.5 (8.2)	89.7 (7.0)	91.1 (4.1)
+ rutin	12.6	16.7 (0.2)	85.9 (7.3)	83.3 (6.5)	84.4 (3.8)

[a] For abbreviations, cf. **Tables 3** and **4**.

Only in the Cabernet Sauvignon control wine the level of free caffeic acid did not increase to more than 60.0% of the maximum content. Thus, the constant concentration of free caffeic acid from malolactic fermentation onward (last data obtained after 9 months of aging) directly reflects the final concentration of pinotin A in the elaborated Cabernet Sauvignon wines. The same observation was made in the Tempranillo wines. The caffeic acid content was highest in the caffeic acid-enriched wine and remained constant around 60% of this value (56.1−68.1%) in the control, coumaric acid-, and rutin-enriched wines from malolactic fermentation onward (approximately 40% at the end of alcoholic fermentation). Consequently, the final relative pinotin A content was 51.7% in the control and 60.1% in the rutin-added wine. Only the coumaric acid-enriched wine showed with 84.3% a concentration for pinotin A, which was somewhat elevated in relation to its caffeic acid content. These results are in line with a previous study on pinotin A in Pinotage red wines of various vintages. Pinotin A was shown to be a suitable aging indicator, as its concentration increased with storage time and depending on the concentration of free caffeic acid that remained largely constant throughout the aging period of several years (*30*).

A completely different behavior was found for the malvidin 3-glucoside 4-vinylphenol adduct and its apparent precursor *p*-coumaric acid (**Table 5**). At the end of alcoholic fermentation, the concentration of coumaric acid in the *p*-coumaric acid-enriched wines was substantially higher as compared to the others (4−6 times in Tempranillo and 4−8 times in Cabernet Sauvignon). The absolute content, however, was with a maximum of 1.2 mg/L well below the caffeic acid concentration. Because of the rapid hydrolysis of coutaric acid, whose concentration at the end of alcoholic fermentation was by an order of magnitude higher as compared to free *p*-coumaric acid (data not shown), the concentration of free coumaric acid was virtually the same in all control and copigment-added wines (including the *p*-coumaric acid-enriched wines) from malolactic fermentation onward (71.9−100% in Cabernet Sauvignon and 83.3−100% in Tempranillo). Despite this almost identical concentration of *p*-coumaric acid in all wines throughout the aging period, the *p*-coumaric acid-added wines contained a vast excess of the malvidin 3-glucoside 4-vinylphenol adduct (3.5 vs ∼0.2−0.3 mg/L in Cabernet Sauvignon; 3.3 vs 0.3−0.4 mg/L in Tempranillo), as well as of the 6″-acetylated and 6″-coumaroylated derivatives. Thus, it can be hypothesized that the fermentation process (alcoholic and/or malolactic fermentation) strongly influences the formation of 4-vinylphenol adducts, as this was the only period in which the addition of coumaric acid had a significant impact on the coumaric acid concentration in the must (although obviously a large extent of the added copigment was subject to precipitation or adsorption to grape solids). Following the pathway suggested by Fulcrand et al. (*16*), 4-vinylphenol could have reacted rapidly with the wine's anthocyanins after enzymatic decarboxylation of *p*-coumaric acid. The following slow chemical reaction between the anthocyanins and the *p*-coumaric acid during the aging period would not be able to adjust the differences between the wines, as the concentration of coumaric acid was the same in all samples. In contrast, caffeic acid does not appear to undergo enzymatic decarboxylation [as already suggested by Chatonnet et al. (*43*)], and formation of pinotin A is controlled only by the slow chemical reaction between malvidin 3-glucoside and caffeic acid. To prove this hypothesis, it would have been necessary to analyze the wines for pyranoanthocyanins not only after 21 months but also directly after alcoholic and malolactic fermentations. If the assumption is correct, high concentrations of the 4-vinylphenol adducts should have been found at this stage in the coumaric acid-enriched wines already, while addition of caffeic acid after such a short period of time should not have resulted in the formation of significant amounts of pinotin A. Unfortunately, these experiments were not performed, as in 2002 information on two possible pathways of formation was not yet available.

By application of the color activity concept (*44*), the visual detection limit of pinotin A at pH 3.6 was determined to be at 0.068 mg/L (*45*), well below its concentration of 0.26−1.36 mg/L in the elaborated wines. Thus, pinotin A contributes with color activity values (CAV) of 4−20 (CAV = concentration/visual detection limit) to the overall wine color. However, this contribution cannot be considered significant, if remaining genuine anthocyanins and newly formed polymeric pigments are taken into account: The visual detection limits of malvidin 3-glucoside, malvidin 3-(6″-acetylglucoside), and polymeric pigments were determined to be at 0.138, 0.182, and 0.939 mg/L, respectively (*45*, *46*). Besides other monomeric anthocyanins that were not individually quantified, the 21 month old wines still contained 11.6−34.4 mg/L of malvidin 3-glucoside (CAV = 84−249) and 4.8−19.7 mg/L of malvidin 3-(6″-acetylglucoside) (CAV = 26−108). Although the exact concentration of polymeric pigments was not determined in this study, their contribution to overall wine color should not be underestimated. It was already demonstrated for vitisin A, a pyranoanthocyanin derived from the reaction between malvidin 3-glucoside and pyruvic acid, which despite its lower visual detection limit (0.034 mg/L) and comparable concentration (1−2 mg/L) it could

not compete against the large excess of polymeric pigments and contributed only ~3−5% to the color of aged red wines (*46*).

ACKNOWLEDGMENT

We thank the Bodegas Naranjo, S.A. (Carrión de Calatrava, Ciudad Real) for supplying grapes.

LITERATURE CITED

(1) Boulton, R. The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *Am. J. Enol. Vitic.* **2001**, *52*, 67−87.

(2) Asen, R.; Stewart, R. N.; Norris, K. H. Co-pigmentation of anthocyanins in plant tissues and its effect on color. *Phytochemistry* **1972**, *11*, 1139−1145.

(3) Mirabel, M.; Saucier, C.; Guerra, C.; Glories, Y. Copigmentation in model wine solutions: occurrence and relation to wine aging. *Am. J. Enol. Vitic.* **1999**, *50*, 211−218.

(4) Darias-Martín, J.; Carrillo, M.; Díaz, E.; Boulton, R. B. Enhancement of red wine colour by pre-fermentation addition of copigments. *Food Chem.* **2001**, *73*, 217−220.

(5) Darias-Martín, J.; Martín-Luis, B.; Carrillo-López, M.; Lamuela-Raventós, R.; Díaz-Romero, C.; Boulton, R. Effect of caffeic acid on the color of red wine. *J. Agric. Food Chem.* **2002**, *50*, 2062−2067.

(6) Dangles, O.; Saito, N.; Brouillard, R. Anthocyanin intramolecular copigment effect. *Phytochemistry* **1993**, *34*, 119−124.

(7) Malien-Aubert, C.; Dangles, O.; Amiot, M. J. Color stability of commercial anthocyanin-based extracts in relation to the phenolic composition. Protective effects by intra- and intermolecular copigmentation. *J. Agric. Food Chem.* **2001**, *49*, 170−176.

(8) Cevallos-Casals, B. A.; Cisneros-Zevallos, L. Stability of anthocyanin-based aqueous extracts of Andean purple corn and red-fleshed sweet potato compared to synthetic and natural colorants. *Food Chem.* **2004**, *86*, 69−77.

(9) Alcalde-Eon, C.; Saavedra, G.; de Pascual-Teresa, S.; Rivas-Gonzalo, J. C. Identification of anthocyanins of pinta boca (*Solanum stenotomum*) tubers. *Food Chem.* **2004**, *86*, 441−448.

(10) Eiro, M. J.; Heinonen, M. Anthocyanin color behavior and stability during storage: Effect of intermolecular copigmentation. *J. Agric. Food Chem.* **2002**, *50*, 7461−7466.

(11) Bloomfield, D. G.; Heatherbell, D. A.; Nikfardjam, M. S. P. Effect of *p*-coumaric acid on the color in red wine. *Mitt. Klosterneuburg* **2003**, *53*, 195−198.

(12) Davies, A. J.; Maza, G. Copigmentation of simple and acylated anthocyanins with colorless phenolic compounds. *J. Agric. Food Chem.* **1993**, *41*, 716−720.

(13) Baranac, J. M.; Petranovic, N. A.; Dimitricmarkovic, J. M. Spectrophotometric study of anthocyan copigmentation reactions. *J. Agric. Food Chem.* **1996**, *44*, 1333−1336.

(14) Baranac, J. M.; Petranovic, N. A.; Dimitricmarkovic, J. M. Spectrophotometric study of anthocyan copigmentation reactions. 2. Malvin and the nonglycosidized flavone quercetin. *J. Agric. Food Chem.* **1997**, *45*, 1694−1697.

(15) Brouillard, R.; Dangles, O. Anthocyanin molecular-interactions: The first step in the formation of new pigments during wine aging? *Food Chem.* **1994**, *51*, 365−371.

(16) Fulcrand, H.; Cameira dos Santos, P.-J.; Sarni-Manchado, P.; Cheynier, V.; Favre-Bonvin, J. Structure of new anthocyanin-derived wine pigments. *J. Chem. Soc., Perkin Trans. 1* **1996**, 735−739.

(17) Hayasaka, Y.; Asenstorfer, R. E. Screening for potential pigments derived from anthocyanins in red wine using nanoelectrospray tandem mass spectrometry. *J. Agric. Food Chem.* **2002**, *50*, 756−761.

(18) Schwarz, M.; Jerz, G.; Winterhalter, P. Isolation and structure of Pinotin A, a new anthocyanin derivative from Pinotage wine. *Vitis* **2003**, *42*, 105−106.

(19) Schwarz, M.; Wabnitz, T. C.; Winterhalter, P. Pathway leading to the formation of anthocyanin-vinylphenol adducts and related pigments in red wines. *J. Agric. Food Chem.* **2003**, *51*, 3682−3687.

(20) Bakker, J.; Bridle, P.; Honda, T.; Kuwano, H.; Saito, N.; Terahara, N.; Timberlake, C. F. Identification of an anthocyanin occurring in some red wines. *Phytochemistry* **1997**, *44*, 1375−1382.

(21) Fulcrand, H.; Benabdeljalil, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. *Phytochemistry* **1998**, *47*, 1401−1407.

(22) Glories, Y. Recherches sur la matière colorante des vins rouges. *Bull. Chim.* **1979**, *9*, 2649−2652.

(23) Miniati, E.; Damiani, P.; Mazza, G. Copigmentation and self-association of anthocyanins in food model systems. *Ital. J. Food Sci.* **1992**, *4*, 109−116.

(24) Mazza, G.; Fukumoto, L.; Delaquis, P.; Girard, B.; Ewert, B. Anthocyanins, phenolics, and color of Cabernet Franc, Merlot, and Pinot Noir wines from British Columbia. *J. Agric. Food Chem.* **1999**, *47*, 4009−4017.

(25) Hermosín Gutiérrez, I.; Sánchez-Palomo Lorenzo, E.; Vicario Espinosa, A. Phenolic composition and magnitude of copigmentation in young and shortly aged red wines made from the cultivars Cabernet Sauvignon, Tempranillo, and Syrah. *Food Chem.* **2005**, *92*, 269−283.

(26) Boulton, R. B. A method for the assessment of copigmentation in red wine. Proceedings of the ASEV 47th Annual Meeting, Reno, NV, 1996.

(27) Hermosín Gutiérrez, I. Influence of ethanol content on the extent of copigmentation in a Tempranillo young red wine. *J. Agric. Food Chem.* **2003**, *51*, 4079−4083.

(28) Ayala, F.; Echávarri, J. F.; Negueruela, A. I. A new simplified method for measuring the color of wines. III. All wines and brandies. *Am. J. Enol. Vitic.* **1999**, *50*, 359−363.

(29) Pérez-Caballero, V.; Ayala, F.; Echávarri, J. F.; Negueruela, A. I. Proposal for a new standard OIV method for determination of chromatic characteristics of wine. *Am. J. Enol. Vitic.* **2003**, *54*, 59−62.

(30) Schwarz, M.; Hofmann, G.; Winterhalter, P. Investigations on anthocyanins in wines from *Vitis vinifera* cv. Pinotage: Factors influencing the formation of pinotin A and its correlation with wine age. *J. Agric. Food Chem.* **2004**, *52*, 498−504.

(31) Brouillard, R.; Delaporte, B. Chemistry of anthocyanin pigments. 2. Kinetic and thermodynamic study of proton-transfer, hydration, and tautomeric reactions of malvidin 3-glucoside. *J. Am. Chem. Soc.* **1977**, *99*, 8461−8468.

(32) Levengood, J. A survey of copigmentation in Cabernet Sauvignon. Master of Science Thesis, University of California, Davis, 1996.

(33) Somers, T. C.; Pocock, K. F. Evolution of red wines. 3. Promotion of the maturation phase. *Vitis* **1990**, *29*, 109−121.

(34) Hermosín Gutiérrez, I.; García-Romero, E. Anthocyanins of red wine grape cultivars grown in the Spanish region of La Mancha: Characteristic cultivar patterns of grapes and single cultivar wines, and evolution during the ripening of the berry. *Alimentaria* **2004**, *41*, 127−139.

(35) Brouillard, R. The *in vivo* expression of anthocyanin colour in plants. *Phytochemistry* **1983**, *22*, 1311−1323.

(36) Schwarz, M.; Wray, V.; Winterhalter, P. Isolation and identification of novel pyranoanthocyanins from black carrot (*Daucus carota* L.) juice. *J. Agric. Food Chem.* **2004**, *52*, 5095−5101.

(37) Hillebrand, S.; Schwarz, M.; Winterhalter, P. Characterization of anthocyanins and pyranoanthocyanins from blood orange (*Citrus sinensis* (L.) Osbeck) juice. *J. Agric. Food Chem.* **2004**, *52*, 7331−7338.

(38) Rein, M. J.; Heinonen, M. Stability and enhancement of berry juice color. *J. Agric. Food Chem.* **2004**, *52*, 3106−3114.

(39) Rein, M. J.; Ollilainen, V.; Vahermo, M.; Yli-Kauhaluoma, J.; Heinonen, M. Identification of novel pyranoanthocyanins in berry juices. *Eur. Food Res. Technol.* **2005**, *220*, 239−244.

(40) Schwarz, M.; Winterhalter, P. A novel synthetic route to substituted pyranoanthocyanins with unique colour properties. *Tetrahedron Lett.* **2003**, *44*, 7583−7587.
(41) Sarni-Manchado, P.; Fulcrand, H.; Souquet, J.-M.; Cheynier, V.; Moutounet, M. Stability and color of unreported wine anthocyanin-derived pigments. *J. Food Sci.* **1996**, *61*, 938−941.
(42) Håkansson, A. E.; Pardon, K.; Hayasaka, Y.; de Sa, M.; Herderich, M. Structures and colour properties of new red wine pigments. *Tetrahedron Lett.* **2003**, *44*, 4887−4891.
(43) Chatonnet, P.; Dubourdieu, D.; Boidron, J.-N.; Lavigne, V. Synthesis of volatile phenols by *Saccharomyces* cerevisiae in wines. *J. Sci. Food Agric.* **1993**, *62*, 191−202.
(44) Hofmann, T. Studies on the influence of the solvent on the contribution of single Maillard reaction products to the total color of browned pentose/alanine solutions−A quantitative correlation using the color activity concept. *J. Agric. Food Chem.* **1998**, *46*, 3912−3917.
(45) Schwarz, M.; Winterhalter, P. Novel aged anthocyanins from Pinotage wines: Isolation, characterization, and pathway of formation. In *Red Wine Color. Revealing the Mysteries*; Waterhouse, A., Kennedy, J. A., Eds.; ACS Symposium Series 886; American Chemical Society: Washington, DC, 2004; pp 179−197.
(46) Schwarz, M.; Quast, P.; von Baer, D.; Winterhalter, P. Vitisin A content in Chilean wines from *Vitis vinifera* cv. Cabernet Sauvignon and contribution to the color of aged red wines. *J. Agric. Food Chem.* **2003**, *51*, 6261−6267.

Received for review May 2, 2005. Revised manuscript received August 8, 2005. Accepted August 18, 2005. We gratefully acknowledge financial support from the Instituto Nacional de Investigaciones Agrarias (INIA, Project VIN 02-007).

JF051005O