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ORIGINAL PAPER

Changes in the color components and phenolic content of red wines from *Vitis vinifera* L. Cv. "Tempranillo" during vinification and aging

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Abstract Changes in phenolics and color components during vinification and aging of Tempranillo wines were studied. Different phenolics display different diffusion into the must, and with the exception of proanthocyanidins and acetyl-glucoside anthocyanins, with maximum concentration at the end of postmaceration, the rest of the unacylated and coumarated anthocyanins, monomeric flavanols, and hydroxycinnamic acids reached their maximum at the end of alcoholic fermentation. This resulted in a significant increase in both wine color and stable color, mainly due to the formation of copigmentation complexes, although polymeric pigment formation was also important. Malolactic fermentation produced a significant decrease in flavonoid content while nonflavonoid concentrations were maintained, prompting a considerable loss in wine color, despite the dramatic increase in bisulfite-stable color. Wine oak aging did not produce any significant change in the studied parameters, while bottle aging reduced the level of monomeric anthocyanins and flavanols, and hydroxycinnamic acids. However, wine color remained stable due to a significant increase in stable color. Polymerization reactions of anthocyanins prevailed over pigment degradation reactions, and copigmentation was still relevant after 2 years of bottle aging.

Keywords Color components · Anthocyanins · Proanthocyanidins · Monomeric flavanols · Hydroxycinnamic acids · Winemaking · Aging · Evolution

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Introduction

Red grape phenolics play a number of important roles in viticulture, including UV protection, disease resistance, pollination, color and defense against predation in plants [1]. They are also essential for wine quality as they are involved in haze formation and hue, and are responsible for the color, taste and antioxidant capacity of wines [2].

Grape and wine phenolics belong to two main groups: flavonoid and nonflavonoid compounds. Flavonoids, located in grape skins, seeds and stems, include anthocyanins, flavanol monomers, oligomeric and polymeric proanthocyanidins, flavonols, flavanonols and flavones. Nonflavonoids, which derive primarily from the pulp and skins of grape berries, include phenolic acids and resveratrol and its derivatives. Flavonoids are the most important family among grape polyphenols, both in quantity and quality. Anthocyanins and derived pigments are directly responsible for the color of red grapes and wines, while monomeric flavanols and proanthocyanidins are mainly responsible for the bitterness, astringency and structure of wines [3]. Flavonols also contribute to bitterness and affect red wine color [4, 5], and certain phenolic acids participate in copigmentation [5].

Wine phenolic composition is conditioned by the grape used and by the winemaking processes that determine their extraction into the must and their further stability in wine. Grape phenolics depend on the variety and other factors that affect berry development such as soil, geographical location and weather conditions [6]. Fruit ripeness, ethanol content [7], perhaps berry size [8], and maceration conditions influence the extraction of phenolic compounds from the grape, and in particular the relative proportion of anthocyanins and flavanols diffusing into the wine [9]. Numerous reactions of phenolic compounds occur in the course of

winemaking and aging, producing a huge variety of colorless products and pigments.

Changes in polyphenolic composition are mainly due to the participation of grape phenolics in haze formation [10] and in numerous copigmentation, cycloaddition, polymerization, and oxidation reactions [11]. These enzymatic and non-enzymatic reactions start just after grape crushing and continue throughout fermentation and aging [12], contributing to important changes in wine sensory properties [13]. Thus, the decrease in astringency as wine ages is ascribed to reactions of proanthocyanidins. Similarly, the color change from the red–blue of young red wines to brick-red in aged wines results from reactions of grape anthocyanins. In particular, the stable color in red wines is associated with anthocyanin copigmentation and polymerization, and other derived anthocyanin reactions resulting in more stable pigments.

The occurrence of such reactions in wine will depend on many factors but mainly on polyphenolic concentrations during the different stages of winemaking. The extraction of anthocyanins and proanthocyanidins during alcoholic fermentation is generally acknowledged [14–18], and in-depth research into changes in anthocyanin composition during maturity and bottle aging has also been carried out [19–22]. However, few studies describe a global evolution of color and polyphenolic composition from the obtainment of must to aging in bottles, after passing through maturation in barrels. This research aims to study the evolution of anthocyanins, hydroxycinnamic acids and flavanols during maceration–fermentation, malolactic fermentation, maturity and aging in bottles, and their relation with color composition and stability.

Materials and methods

Reagents and samples

All chemicals used were of analytical reagent grade. All chromatographic solvents were of HPLC grade. Malvidin-3-glucoside, peonidin-3-glucoside, caffeic acid, *trans-p*-coumaric acid, (+)catechin and (−)epicatechin, were purchased from Extrasynthèse (Lyon, France), and vanillin from Sigma (St Louis, MO, USA).

Formic acid, acetonitrile and trifluoroacetic acid supplied by Sigma and MilliQ ultrapure water (Millipore, Molsheim, France) were used. Acetone was obtained from Riedel-deHäen (Sigma), and pure methanol and acetaldehyde were purchased from Merck (Darmstadt, Germany). Ethanol 96% v/v, sulfuric acid, sodium metabisulphite and tartaric acid were supplied by Scharlab (Barcelona, Spain), and sodium hydroxide and hydrochloric acid 37% were obtained from Carlo Erba (Rodano, MI, Italy).

Wine samples were produced from *Vitis vinifera* Tempranillo grapes from the Qualified Origin Denomination Rioja (D.O Ca. Rioja). *Saccharomyces cerevisiae* var. *cerevisiae* RC212 was a commercial yeast from Lallemand (Lallemand Inc., Montreal, Canada). The bacterial strain *Oenococcus oeni* was also purchased from Lallemand.

Vinification and sample collection

Tempranillo grapes were sourced from Autol, La Rioja, Spain, and harvested at 21.9 Brix, pH 3.56 and 6.02 g tartaric acid/l. Experimental vinifications were carried out at the Universidad de La Rioja winery and wines were prepared using traditional wine technology. Grapes were destemmed and crushed and distributed into three 100 l stainless steel tanks. The prefermentation process lasted for 6 h at 18 ± 2 C and the experimental scaled fermentations were performed with a yeast inoculum of 25 g/hl *S. cerevisiae* RC212. The fermentation–maceration process was carried out at a maximum temperature of 28 ± 2 C for 10 days. Post-fermentative maceration lasted for 4 days at 24 ± 2 C and wines were run off. The cap was punched down twice a day until it remained submerged during the entire maceration period. During post-maceration, the cap was protected against oxidation by injecting CO_2(g) into the headspace of the tank. Wines were inoculated with a commercial preparation of *Oenococcus oeni* (1 g/hl) to induce malolactic fermentation, carried out at 18.5 ± 1 C. After 20 days of malolactic fermentation, all the wines were racked and clarified by settling for 25 days at 10 C. Wine aging was performed at 13 C and 80–85% relative humidity in new 13-l American oak barrels, which have a larger area/volume than the traditional 225-l barrels. For this reason, and based on the organoleptic analysis, the oak aging process was only performed for 45 days. After this, the wines were filtered through SEITZ K250 filters (2.5–3.0 μm) (Sert Schenk Filter System GmB, Bad Krevnach, Germany) and finally bottled and stored for 24 months at 14 C and 80–85% relative humidity.

Samples were taken during alcoholic maceration–fermentation (beginning, 25–30, 55–60, and 99% of sugars consumed: namely 0AF, 30AF, 60AF, and 99AF, respectively), and at the beginning and end of malolactic fermentation (BMF, EMF). Samples were also taken at the beginning and end of wine oak aging (BOA, EOA), and at the beginning and end of wine bottle aging (BBA, EBA).

Determination of usual enological parameters

Conventional enological parameters (color intensity, ethanol concentration, pH values, and titratable and volatile acidities) were determined at wine pH according to official OIV practices [23].

Color components and total polyphenol index values

Spectrophotometric measurements were performed on a Cary 300 Scan UV-vis spectrophotometer (Varian Inc., Madrid, Spain) using 2- and 10-mm path length quartz cells. All the samples were analyzed in triplicate and all absorbance values corrected to 10-mm path length.

Wine color (WC), monomeric anthocyanins color (MAC), copigmentation color (CC), and bisulfite-stable color (BSC) were determined using the method proposed by Levengood and Boulton [24]. The total polyphenol index (TPI) was determined by absorbance at 280 nm of diluted wine with synthetic wine (12% alcohol, 5 g/l of tartaric acid in water, pH 3.6).

Fractionation of phenolics by GPC

Must and wine samples were directly fractionated by GPC on a Toyopearl gel HP-50F column (Tosohaas, Montgomery-Ville, PA, USA) using the method described by Guadalupe et al. [25]. A first fraction (F1) was eluted with ethanol/water/trifluoroacetic acid (55:45:0.05, v/v/v), and a second fraction (F2) was recovered by elution with acetone/water (60:40, v/v). The two fractions collected per sample were taken to dryness under vacuum, and then the first fraction was analyzed by HPLC-DAD-MS, and the second one was used to determine proanthocyanidin content using the vanillin assay. All samples were fractionated in duplicate.

HPLC-DAD determination of monomeric phenolic compounds in fraction F1

F1 fractions were subjected to HPLC-DAD on an Agilent modular 1100 liquid chromatograph (Waldbronn, Germany) equipped with a G1313A injector, a G1311A HPLC quaternary pump, an on-line G1379A degasser, a G1316A oven, a G1315B photodiode array detector, and Agilent Chemstation software. A Kromasil 100-C18 reverse phase column (5 μm packing, 200 × 4.6 mm i.d.), protected with a guard column of the same material (Teknokroma, Barcelona, Spain) and thermoregulated at 30 C was used.

The HPLC-DAD conditions had been previously used with satisfactory results in our laboratory to analyze fraction F1 [25]. The solvents used were: (A) formic acid/water (2:98, v/v), and (B) acetonitrile/water/formic acid (80:18:2, v/v/v), establishing the following gradient: isocratic 2% B in 3 min, from 2 to 10% B in 2 min, from 10 to 15% B in 10 min, from 15 to 30% B in 10 min, from 30 to 50% B in 10 min, from 50 to 60% B in 5 min, from 60 to 90% B in 5 min, at a flow rate of 1 ml/min. Spectra were recorded from 250 nm to 600 nm.

Quantification was carried out by peak area measurements at 515 nm for anthocyanins, 310 nm for hydroxycinnamic acids and 280 nm for flavanols. Since most of the individual phenolic compounds are not commercially available as reference standards, anthocyanin content was expressed as malvidin-3-glucoside, hydroxycinnamic acid content as caffeic acid and flavanol content as (+)catechin by an external standard calibration curve. Individual phenolic compounds were identified by HPLC-MS [25]. Each measurement was run in triplicate.

The content of non-acylated anthocyanins (A-Glu) was calculated as the sum of delphinidin (Df), cyanidin (Cy), petunidin (Pt), peonidin (Pn) and malvidin-3-glucosides (Mv); the content of acetyl-glucoside anthocyanins (A-Ac) as the sum of delphinidin (Dfac), cyanidin (Cyac), petunidin (Ptac) and malvidin-3-(6-acetyl)-glucosides (Mvac); the content of coumaryl-glucoside anthocyanins (A-Cm) included delphinidin (Dfcm), petunidin (Ptcm), and malvidin-3-(6-*p*-coumaryl)-glucosides (Mvcm). The sum of A-Glu, A-Ac and A-Cm was referred to as total monomeric anthocyanins (TMA). Total hydroxycinnamic acids (TCin) were calculated as the sum of *trans*-caftaric (*trans*-caffeoyl-tartaric acid), *cis*-caftaric (*cis*-caffeoyl-tartaric acid), *trans*-coutaric (*trans*-*p*-coumaryl-tartaric acid), *cis*-coutaric (*cis*-*p*-coumaryl-tartaric acid), caffeic and *trans*-*p*-coumaric acid. Monomeric flavanols (M-Flava) included (+)catechin, (−)epicatechin and (−)epigallocatechin.

Determination of total proanthocyanidin content by the vanillin assay in fraction F2

Total proanthocyanidins (PA) were quantified in F2 fractions by means of the vanillin assay using the method described by Sun et al. [26], but with slight modifications described by Guadalupe et al. [25]. Total flavanols (T-Flava) corresponded to the sum of flavanols monomers (M-Flava) and proanthocyanidins (PA).

Statistical procedures

Phenolic composition and color measurement data corresponding to the samples taken in the different vinification stages were analyzed using Student's t test (SPSS version 12.0, SPSS Inc) to identify significant differences. In this paper, differences between samples always refer to significant differences with at least $P < 0.05$.

Results and discussion

Conventional enological analysis in wines

The pH, titratable acidity, ethanol concentration and volatile acidity values were in the ranges usually found in Tempranillo variety wines (Table 1). The pH, titratable

Table 1 Conventional enological parameters of wines

Wine stage[a]	% v/v[b]	pH	TA[c]	VA[d]	TPI[e]	CI[f]	Hue[g]
BMF	12.6 ± 0.2	3.77 ± 0.01	5.2 ± 0.09	0.23 ± 0.05	40.0 ± 0.7	6.79 ± 0.03	0.53
EMF	12.5 ± 0.2	3.96 ± 0.03	3.69 ± 0.04	0.28 ± 0.06	35.0 ± 0.5	5.80 ± 0.02	0.60
BOA	12.5 ± 0.2	3.97 ± 0.02	3.71 ± 0.05	0.29 ± 0.05	35.5 ± 0.6	5.82 ± 0.01	0.59
EOA	12.6 ± 0.2	3.98 ± 0.01	3.98 ± 0.05	0.33 ± 0.02	36.3 ± 0.4	5.00 ± 0.01	0.62
BBA	12.6 ± 0.2	3.98 ± 0.01	3.98 ± 0.05	0.33 ± 0.05	36.0 ± 0.4	4.89 ± 0.05	0.59
EBA	12.5 ± 0.2	3.98 ± 0.01	3.95 ± 0.05	0.36 ± 0.05	37.0 ± 0.5	4.90 ± 0.01	0.75

[a] Wine stage: *BMF* beginning of malolactic fermentation, *EMF* end of malolactic fermentation, *BOA* beginning of oak aging, *EOA* end of oak aging, *BBA* beginnig of bottle aging, *EBA* end of bottle aging

[b] mL of ethanol for 100 ml of wines at 20 C

[c] Titratable acidity as g of tartaric acid per liter

[d] Volatile acidity as g of acetic acid per liter

[e] Total polyphenol index as absorbance at 280 nm ($A_{280\ nm}$)

[f] Color intensity as sum of absorbances at 420, 520, and 620 nm

[g] $A_{420\ nm}/A_{520\ nm}$

Mean ± SD ($n = 3$)

acidity, and volatile acidity values obtained confirmed the absence of microbial alterations. Wine pH increased 0.2 units at the end of malolactic fermentation and titratable acidity decreased by 29%. Furthermore, malolactic fermentation prompted a decrease in both the total polyphenol index (TPI) and color intensity, while hue increased 0.1 units. The analyses performed on the wine indicated proper storage of wines during oak and bottle aging. Although wood polyphenols are extracted during wine aging, TPI values obtained before and after wine aging were not significantly different ($P < 0.05$), suggesting that polyphenol extraction was minimal during short aging. As expected wine aging was accompanied by a decrease in color intensity and an increase in hue, while the TPI value of the wine increased significantly ($P < 0.05$).

Evolution of must and wine color composition during vinification and aging

The evolution of wine red color (WC), monomeric anthocyanins color (MAC), copigmentation color (CC), and bisulfite-stable color (BSC) was analyzed during maceration–fermentation, post-maceration, malolactic fermentation, oak aging and bottle aging (Fig. 1). Bisulfite-stable compounds are usually termed polymeric pigments, which is incorrect since it includes compounds that are not polymeric in nature (i.e., pyranoanthocyanins) and not all anthocyanin-derived polymers are colored or bisulfite-stable pigments (i.e., so-called T-A polymeric pigments). However, the practice of measuring bisulfite-stable color continues to be very useful [27].

Red winemaking increased the value of WC, MAC, CC and BSC, and maceration was the main process affecting

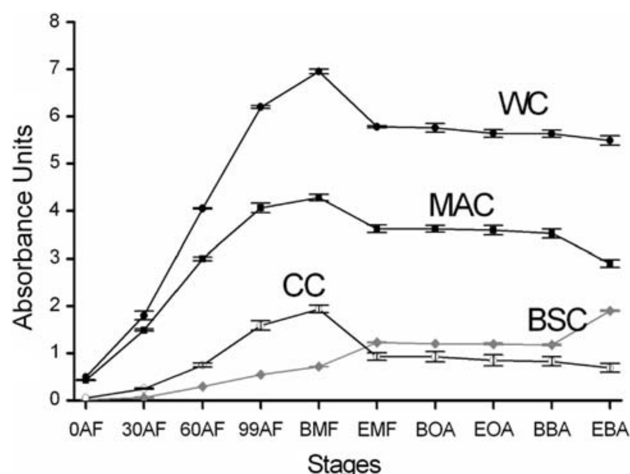

Fig. 1 Wine color (*WC*), monomeric anthocyanins color (*MAC*), copigmentation color (*CC*), and bisulfite-stable color (*BSC*) during vinification and aging. See text for conditions and calculations. Vinification stage: 0AF, 30AF, 60AF, 99AF, alcoholic fermentation (0, 30, 60, 99% of sugars consumed, respectively); BMF and EMF, the beginning and end of malolactic fermentation; BOA and EOA, the beginning and end of wine oak aging; BBA and EBA, the end of wine bottle aging

these values. Throughout the period studied, the value of monomeric anthocyanin color (MAC) was considerably higher than CC and BSC, contributing more than 50% to wine color. However, the ratio of stable bisulfite and copigmentation color (BSC + CC) to unstable anthocyanin color (MAC) changed with the winemaking process (Fig. 2).

The light red color of must samples was due to monomeric anthocyanins color, since the color values due to their polymeric and copigmented forms were negligible. MAC, BSC, and CC values increased significantly in the samples during alcoholic fermentation, indicating a high

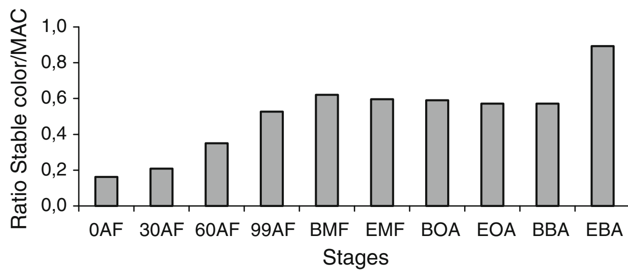

Fig. 2 Ratio of stable color (bisulfite-stable color + copigmentation color) to monomeric anthocyanins color (*MAC*). Vinification stage: 0AF, 30AF, 60AF, 99AF, alcoholic fermentation (0, 30, 60, 99% of sugars consumed, respectively); BMF and EM, the beginning and end of malolactic fermentation; BOA and EOA, the beginning and end of wine oak aging; BBA and EBA, the beginning and end of wine bottle aging

extraction of grape anthocyanins as well as an important transformation of these compounds into stable copigmentation complexes and polymeric pigments. These reactions were seen to be greater with higher ethanol concentrations, and CC increased more than sixfold between 30AF and 99AF, while BSC increased ninefold. As expected, the post-maceration carried out to enhance wine color increased the WC value, and it was seen to be due to an increase in the color-stabilizing components while the MAC remained stable. At the end of this period, the contribution of copigmentation color to wine color was almost 30%, coinciding with the results reported by Boulton [4], who reported that the contribution of copigmented anthocyanins to young red wine color ranged from 30 to 50%. Copigments in young wines are very important for wine color since they increase color intensity and transform the color of wine red to purple. They may also be seen as a storage form of anthocyanin, allowing more anthocyanin to be removed from the skins and stabilized in solution until the polymeric pigments are formed. In this regard, and although it has been reported that polymeric pigments form during wine maturity, we found that the amount of anthocyanin-derived polymeric pigments started to become significant at the end of maceration-fermentation, contributing in around 10% to young wine red color.

Malolactic fermentation resulted in a significant decrease in the content of wine color (16.7%), due to a decrease in both CC and MAC. However, bisulfite-stable color increased dramatically by 71% in the wine, coinciding with previous observations reported by our group [28]. It is widely known that malolactic fermentation produces important losses of color due to the precipitation of unstable colloidal material [29], and it also produced the dissociation of the copigmentation complexes, probably due to ionic shift. However, new and stable pigments were also formed during this period. These new pigments are the source of stable color in aged red wines because they are more resistant to bisulfite bleaching and their color is not pH-dependent like free anthocyanins.

During short-term barrel aging, the MAC, BSC and CC values did not change significantly ($P < 0.05$). Bottle aging induced an important loss of MAC (0.69 absorbance units) and did not prompt a significant change in the value of CC ($P < 0.05$), but this was offset by the significant increase in bisulfite-stable color (0.7 absorbance units), in such a way that wine color remained stable. This fact indicated that reactions of anthocyanins with proanthocyanidins and other phenolic compounds prevailed over pigment degradation reactions, and thus the contribution of bisulfite-stable color to WC was higher than 30%. Although copigmentation phenomena have been described as negligible during wine maturity, some authors describe that copigments disappear completely after 9 months of bottle aging [5, 30], our results confirmed the presence of copigmented anthocyanins in the bottle-aged wine, contributing more than 10% to wine color.

All the changes described above resulted in an increase in the stable/instable color ratio, from less than 0.2 in the must samples to almost 1 at the end of bottle aging, with two main stages of stable color formation: maceration–fermentation and bottle aging (Fig. 2).

Evolution of the content of total and individual phenolic families during vinification and aging

Figure 3 shows the content of total phenolic families (TP), total monomeric anthocyanins (TMA), total hydroxycinnamic acids (TCin), and total flavanols (T-Flava) during vinification and aging. The content of total phenolic families was calculated by adding the amounts TMA, TCin and T-Flava. Monomeric anthocyanins were analyzed because they are directly responsible for red wine color, although both hydroxycinnamic acids and flavanols are associated with the formation of stable color. Hydroxycinnamic acids and monomeric flavanols are involved in copigmentation reactions, the former having been described as highly effective copigments [31, 32]. The so-called polymeric pigments are formed by the reaction between anthocyanins and polymeric flavanols.

Winemaking increased total phenolic families (TP) content, and maceration–fermentation was again the main process affecting this content. TP increased ninefold between day 0 (0AF) and day 14 (end of post-fermentative maceration), the moment when the wine achieved maximum TP concentration, since phenolic extraction continued while pomace contact existed. As in the case of wine color, total polyphenol index (TPI) and color intensity, malolactic fermentation resulted in a substantial decrease in TP (29%). During short-term barrel aging, the total phenolic families content of wine remained stable but the TP value decreased during the 2 years of bottle aging (10%).

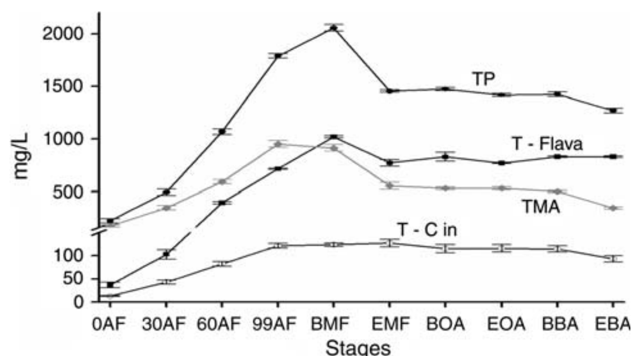

Fig. 3 Concentration (mg/l) of total phenolic families (*TP*), total monomeric anthocyanins (*TMA*), total flavanols (*T-Flava*) and total hydroxycinnamic acids (*TCin*) during vinification and aging. See text for conditions and calculations. Vinification stage: 0AF, 30AF, 60AF, 99AF, alcoholic fermentation (0, 30, 60, 99% of sugars consumed, respectively); BMF and EMF, the beginning and end of malolactic fermentation; BOA and EOA, the beginning and end of wine oak aging; BBA and EBA, the beginning and end of wine bottle aging

As regards the results for individual phenolic families, important differences were observed between the must and wine samples (Fig. 3). Monomeric anthocyanins were the predominant polyphenols in the must aqueous phase, representing more than 77% of total phenolic families (TP) content, followed some way behind by flavanols (16.5%) and hydroxycinnamic acids (5.7%). Alcoholic fermentation produced a fivefold increase in the concentration of monomeric anthocyanins (TMA), while the colorless phenolics TCin and T-Flava increased by more than 9-fold and 19-fold, respectively, the T-Flava/TMA ratio increased from 0.2 in the musts to 0.8 at the end of fermentation. Both anthocyanins and hydroxycinnamic acids reached their maximum concentration at the end of this stage, while flavanol concentration continued to increase during post-maceration favored by the high temperature and ethanol content. As a result, the T-Flava/TMA ratio increased to 1.1, T-Flava representing more than 49% of total phenolic content, followed by monomeric anthocyanins (44%) and hydroxycinnamic acids. It has been widely described that the variation in anthocyanin concentration during maceration depends not only on the degree of extraction or gain but also on the speed of loss, mainly attributed to their conversion to other molecular species either through chemical reactions or colloidal associations and their precipitation or absorption phenomena. As a result, a gradual increase in stable color, both in copigmented and polymeric anthocyanins, was observed during maceration (Fig. 1).

The decrease in monomeric anthocyanin content during malolactic fermentation was 1.6 times greater than that of total flavanols (39.4 vs. 24.2%), while hydroxycinnamic acid concentration was maintained. The considerable reduction in monomeric anthocyanins increased the T-Flava/TMA ratio to 1.4 and coincided with the important loss in wine color (Fig. 1).

During bottle aging, the content of T-Flava was maintained while anthocyanin and hydroxycinnamic acid content was considerably reduced, 31 and 19%, respectively, with the subsequent increase in the T-Flava/TMA ratio (>50%). The loss of monomeric anthocyanins may be due to either reactions of formation of new stable pigments or degradation reactions. The stable value of WC during bottle aging and the increase in BSC (Fig. 1) indicated that the decrease in TMA was due to its transformation into more stable pigments in terms of color and not due to their degradation. The fact that total flavanols remained nearly constant during this stage does not mean that the monomeric flavanols and proanthocyanidins did not change quantitatively and qualitatively (see "Evolution of individual phenolics during vinification and aging").

Evolution of individual phenolics during vinification and aging

The evolution of the mean concentration of individual anthocyanins, individual monomeric flavanols and proanthocyanidins, and individual hydroxycinnamic acids during the stages of the vinification process is shown in Figs. 4, 5, and 6, respectively.

The extraction of grape skin anthocyanins and monomeric flavanols and skin-pulp hydroxycinnamic acids rapidly increased due to the diffusion of non-acylated anthocyanins (Fig. 4), (−)epigallocatechin (Fig. 5), and *trans*-hydroxycinnamate derivatives (Fig. 6) into the must. However, the polymeric flavanols displayed limited solubility into the aqueous medium (Fig. 5). As a result, anthocyanins were the predominant molecules in 0AF must samples; non-acylated anthocyanin glucosides (A-Glu) accounted for 89% of total monomeric anthocyanins while coumaroylated forms (A-Cm) and acetylated forms (A-Ac) accounted for 8.2 and 2.8%, respectively (Table 2). As expected in a *Vitis vinifera* variety, malvidin-3-glucoside was the major anthocyanin, accounting for 72% of total anthocyanins, and its derivates were also the main anthocyanins in the acetylated and coumaroylated forms (Fig. 4). Monomeric flavanols represented 73% of total flavanols (Table 2), and (−)epigallocatechin, present only in skin [33], represented 34% of total monomeric flavanols in must samples (Fig. 5). *Trans*-caftaric acid was found to be the main hydroxycinnamic acid, accounting for 42% of the latter (Fig. 6).

The relative proportions of the different anthocyanins, hydroxycinnamic acids and flavanols significantly changed with the progression of maceration-fermentation (Table 2). Thus, the proportion of non-acylated anthocyanins and *trans*-caftaric acid decreased while the proportion of

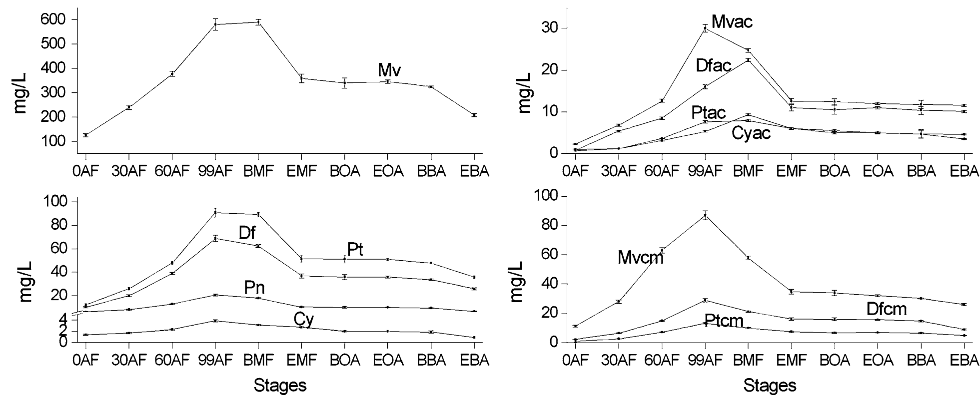

Fig. 4 Concentration (mg/l) of monomeric anthocyanins during vinification and aging: non-acylated anthocyanins, acetyl-glucoside anthocyanins, and coumaryl-glucoside anthocyanins. See text for abbreviations, conditions and calculations. Vinification stage: 0AF, 30AF, 60AF, 99AF, alcoholic fermentation (0, 30, 60, 99% of sugars consumed, respectively); BMF and EMF, the beginning and end of malolactic fermentation; BOA and EOA, the beginning and end of wine oak aging; BBA and EBA, the beginning and end of wine bottle aging

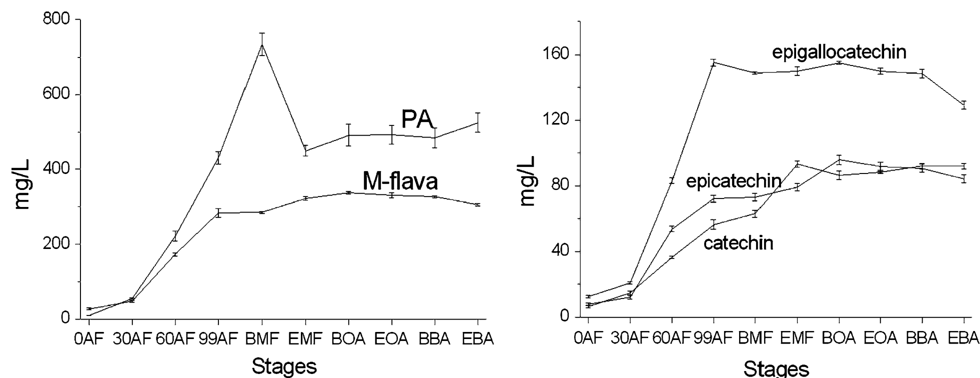

Fig. 5 Concentration (mg/l) of proanthocyanidins (*PA*), total monomeric flavanols (*M-Flava*), and monomeric epigallocatechin, epicatechin and catechin during vinification and aging. See text for conditions and calculations. Vinification stage: 0AF, 30AF, 60AF, 99AF, alcoholic fermentation (0, 30, 60, 99% of sugars consumed, respectively); BMF and EMF, the beginning and end of malolactic fermentation; BOA and EOA, the beginning and end of wine oak aging; BBA and EBA, the beginning and end of wine bottle aging

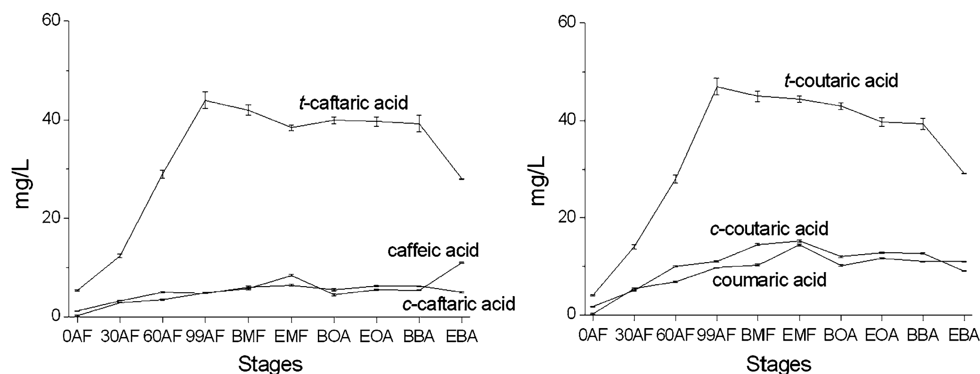

Fig. 6 Concentration of hydroxycinnamic acids during vinification and aging. See text for conditions and calculations. Vinification stage: 0AF, 30AF, 60AF, 99AF: alcoholic fermentation (0, 30, 60, 99% of sugars consumed respectively); BMF and EMF: the beginning and end of malolactic fermentation; BOA and EOA: the beginning and end of wine oak aging; BBA and EBA: the beginning and end of wine bottle aging

acylated anthocyanins and *trans*-coutaric acid increased. This different behavior was attributed not only to the different diffusion of the anthocyanins and hydroxycinnamate derivatives but also to the characteristic profile of the Tempranillo variety. The results obtained at the end of the fermentation stage showed that the content of malvidin-

Table 2 Evolution of relative contents (%) of different anthocyanic forms (non-acylated: *A-Glu*, acetylated: *A-Ac* and coumaroylated forms: *A-Cm*), hydroxycinnamic acid forms, and monomeric (*M-Flava*) and polymeric flavanols (*PA*) in maceration-fermentation and post-maceration (mean ± standard deviations)

Stage[a]	Anthocyanic forms								Hydroxycinnamic acid forms						Flavanols	
	Mv[b]	Pt[b]	Df[b]	Pn[b]	Cy[b]	A-Glu	A-Ac	A-Cm	*t*-Caft[c]	*c*-Caft[d]	Caffeic	*t*-Cout[e]	*c*-Cout[f]	Coumaric	M-Flava	PA
0AF	80.8 ± 1	7.9 ± 0.4	6.5 ± 0.3	3.9 ± 0.3	0.9 ± 0.03	89.1 ± 0.6	2.8 ± 0.2	8.2 ± 0.4	42.5 ± 1	9.4 ± 0.1	1.6 ± 0.3	31.5 ± 0.5	13.4 ± 0.5	1.6 ± 0.0	72.8 ± 0.5	27.2 ± 0.2
30AF	81.2 ± 0.2	8.8 ± 0.2	6.8 ± 0.4	2.7 ± 0.2	0.6 ± 0.1	85.1 ± 0.4	4.2 ± 0.4	10.7 ± 0.5	28.8 ± 0.9	7.7 ± 0.3	6.7 ± 0.2	32.6 ± 0.4	11.6 ± 0.3	12.6 ± 0.3	46.5 ± 0.4	53.5 ± 0.4
60AF	78.7 ± 0.4	10.0 ± 0.3	8.1 ± 0.1	2.7 ± 0.3	0.5 ± 0.06	80.9 ± 0.3	4.7 ± 0.3	14.4 ± 0.5	35.2 ± 0.4	6.1 ± 0.2	4.3 ± 0.1	34.0 ± 0.6	12.2 ± 0.4	8.3 ± 0.2	43.8 ± 0.3	56.2 ± 0.1
99AF	75.9 ± 0.5	11.9 ± 0.2	9.0 ± 0.2	2.7 ± 0.3	0.5 ± 0.04	80.3 ± 0.3	6.2 ± 0.1	13.6 ± 0.6	36.2 ± 0.5	4.0 ± 0.3	4.0 ± 0.3	38.7 ± 0.5	9.1 ± 0.3	8.0 ± 0.5	39.7 ± 0.2	60.3 ± 0.2
BMF	77.3 ± 0.3	11.7 ± 0.1	8.2 ± 0.3	2.3 ± 0.1	0.4 ± 0.06	83.2 ± 0.4	7.0 ± 0.2	9.7 ± 0.6	34.0 ± 0.3	4.9 ± 0.1	4.6 ± 0.2	36.4 ± 0.3	11.7 ± 0.2	8.3 ± 0.2	28.0 ± 0.2	72.0 ± 0.4

[a] Vinification stage: 0AF, 30AF, 60AF, 99AF: alcoholic fermentation (0, 30, 60, 99% of sugars consumed respectively); *BMF* beginning of malolactic fermentation

[b] Relative content of different non-acylated anthocyanins with respect to total A-Glu

[c] *Trans*-caftaric acid

[d] *Cis*-caftaric acid

[e] *Trans*-coutaric acid

[f] *Cis*-coutaric

See text for conditions, calculations, and abbreviations for anthocyanic forms

3-(6-coumaroyl)-glucoside was threefold higher than the content of malvidin-3-(6-acetyl)-glucosides, and that the amounts of *trans*-caftaric acid and *trans*-coutaric acid were comparable (Figs. 4, 6), in accordance with the Tempranillo variety, characterized by a higher proportion of anthocyanin coumarate forms than acetylated forms, and similar quantities of *trans*-caftaric and *trans*-coutaric acid [34].

As regards the relative proportions of different non-acylated anthocyanic forms, we found that the proportion of peonidin (Pn) and cyanidin (Cy) decreased from 0AF to 30AF while the proportion of delphinidin (Df), petunidin (Pt) and malvidin (Mv) increased (Table 2). Although it has been widely described that the initial diffusion of the bi-substituted anthocyanins (Pn and Cy) is higher than that of the tri-substituted anthocyanins (Df, Pt and Mv), our results coincided with those reported by other authors for other grape varieties [35]. As maceration–fermentation progressed, the relative proportions of the non-acylated bi-substituted anthocyanins and malvidin glucoside decreased, and the proportions of Df and Pt increased (Table 2). Thus, the quantity of Df and Pt increased more than tenfold at the end of fermentation although malvidin glucoside was still the prevalent anthocyanin (Fig. 4). As regards the flavanols, both monomeric and polymeric forms significantly increased during maceration–fermentation with a greater increase in the polymeric forms (Fig. 5); as a result, the proportion of polymeric proanthocyanidins doubled more than that of the monomeric flavanols at the end of this period (Table 2). The gradual extraction of seed tannins, which needs higher ethanol content to be solubilized than the skin tannins, probably contributed to the increase in flavanols.

The prolongation of alcoholic maceration favored the extraction and solubility of seed tannins and minority acetylated anthocyanins [36] but not that of majority anthocyanins. The extraction of major anthocyanins (A-Glu and A-Cm) was probably not sufficient to compensate for their loss due to the formation of new polymeric and copigmented anthocyanins (Fig. 1). Post-maceration also produced a decrease in the concentration of *trans*-caftaric and *trans*-coutaric acid, which did not coincide with the increase observed in the corresponding free forms (Fig. 6), and it could be explained by their role as copigments. An additional source of caffeic acid and *trans*-*p*-coumaric acid may also be the hydrolysis of cinnamoyl-glucoside anthocyanins [19].

Proanthocyanidins and non-acylated anthocyanins were the main phenolics in young wines after post-maceration, followed by monomeric flavanols, coumaryl-glucoside anthocyanins, hydroxycinnamic acids and acetyl-glucoside anthocyanins. These compounds represented about 42.7, 24.1, 15.9, 7.2, 6.8 and 3.3% of the content of total phenolic compounds, respectively. Malvidin-3-glucoside was again

Table 3 The percentage decrease (%) of different anthocyanic forms during malolactic fermentation (BMF-EMF) and wine oak aging (BOA-EOA) and wine bottle aging (EBA-EBA)

Stage	Anthocyanic forms											
	Mv	Pt	Df	Pn	Cy	Dfac	Cyac	Ptac	Mvac	Dfcm	Ptcm	Mvcm
BMF-EMF	39.0	42.4	40.9	42.1	12.9	50.9	35.5	24.05	49.0	23.7	25.0	39.9
BOA-EOA	a	b	b	b	b	a	9.1	a	b	b	a	b
EOA-EBA	35.8	25.3	23.5	30.9	52.1	b	b	25.5	b	38.6	26.2	13.6

[a] Percentage decrease not detected

[b] Percentage decrease lower than 5%

See text for abbreviations

the main anthocyanin in wines and its derivates were also the main anthocyanins in the acetylated and coumaroylated forms after post-maceration.

Malolactic fermentation prompted a significant decrease in the content of the different anthocyanins, proanthocyanidins and *trans*-hydroxycinnamate derivatives, 39.6% for A-Glu, 44.6% for A-Ac, 34.4% for A-Cm, 38.7% for PA, and 4.8% for *trans*-hydroxycinnamates. At the same time, monomeric flavanol content increased significantly ($P > 0.05$) while free acid content did not change significantly ($P < 0.05$) (Figs. 4, 5, 6). Although the percentage decrease in minority anthocyanins, that is, A-Ac, was greater than that of majority anthocyanins, that is, A-Cm and A-Glu, the loss in total monomeric anthocyanin content (TMA) could not be related exclusively to the decrease in A-Ac, since the contribution of A-Cm and A-Glu to the decrease in TMA was substantial. The concentration at the beginning of malolactic fermentation of the different anthocyanin structures (Fig. 4) and their corresponding percentage decreases at the end of this stage (Table 3) showed that the rate of loss of each compound did not depend on their initial concentration. The lower values observed at the end of malolactic fermentation for monomeric anthocyanins, color intensity (Table 1) and wine color (Fig. 1) were in good agreement with the bibliography [28, 37]. However, it is important to bear in mind that the stable/instable color ratio remained almost constant (Fig. 2). The tannin–anthocyanin combination and the precipitation of unstable colloids tannin–tannin, tannin–polysaccharides and tannin–proteins would explain the reduction in proanthocyanidins and monomeric anthocyanins. At the same time, the partial disappearance of tartaric esters with caffeic acid and *trans*-*p*-coumaric acid was detected, with an increase in the corresponding free acids. Other authors have reported the complete disappearance of *trans*-caftaric and *trans*-*p*-coutaric acid in Tempranillo wine during this phase [38]. However, the amount of caffeic acid obtained (8.4 mg/l) was similar to that obtained in most varietal red wines [39].

In contrast to what occurred with short-term barrel aging, long-term aging in bottles caused a considerable decrease of anthocyanins and hydroxycinnamic acids (Figs. 4, 6). The anthocyanins most resistant to disappearance were the minority acylated anthocyanins, showing a 5.5% decrease, followed some way behind by majority anthocyanins forms, coumarate derivatives (22%) and non-acylated anthocyanins (34%). This might indicate that the loss in total monomeric anthocyanin content (TMA) could be closely related to the decrease of non-acylated and coumarate anthocyanins. However, the rate of loss of the different anthocyanin structures during aging in bottles did not depend on their initial concentration (Fig. 4; Table 3), this fact confirmed the previous observations in malolactic fermentation. The decrease in the concentration of the different anthocyanin structures did not entail a change in WC but an increase in BSC (Fig. 1). This fact suggests that the decrease in the concentrations of the different anthocyanin structures could be closely related to their participation in the formation reaction of new pigments. The hydroxycinnamic compounds underwent dramatic changes; the forms of combined *trans*-*p*-coumaric and caffeic acid decreased by around 27% after 2 years of bottle aging while free acids increased. It was also observed that the amounts of *trans*-caftaric and *trans*-coutaric acid were comparable, which together with the higher proportion of coumarated anthocyanin forms detected than acetate forms, confirmed that the varietal characteristics of the Tempranillo wine were maintained after 2 years of aging.

Total flavanols remained stable during aging, but in-depth analysis revealed an increase in proanthocyanidins (8.5%) at the same time as a decrease in monomeric flavanols (8%). Epigallocatechin content was reduced during aging and, as previously reported by other authors [17, 21, 40], epicatechin was also reduced (Fig. 5). These changes were probably the result of complex mutual polymerization–depolymerization processes accompanied by combination with the different anthocyanins to give more stable pigments.

Contrary to what was observed in the young wine after postmaceration, the polymeric flavanols were the most

prevalent compounds in the aged wines (41.3%), followed some way behind by monomeric flavanols (24%) and non-acylated anthocyanins (21.8%), and lastly hydroxycinnamates (7.3%), coumaryl-glucoside anthocyanins (3.1%), and acetyl-glucoside anthocyanins (2.3%).

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