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Formation of Hydroxyphenyl-pyranoanthocyanins in Grenache Wines: Precursor Levels and Evolution during Aging

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Grenache red wines were produced following three different winemaking techniques, that is, small-scale standard experimental wines and industrial-scale wines by both double-mash fermentation and fermentation in Ganimede vats. Wines were analyzed for their color properties, as well as the anthocyanin, flavonol, hydroxycinnamic acid, and pyranoanthocyanin profiles following alcoholic and malolactic fermentation. The evolution of pyranoanthocyanins and their corresponding precursors in the experimental wines was monitored at 6 and 10 months of aging. Wines produced by double-mash fermentation exhibited superior color properties compared to Ganimede wines and the experimental red wines, due to better extraction of flavonols and anthocyanins as well as a lower degree of polymerization. Pyranoanthocyanin formation varied within the different classes of pigments. Vitisins A and B were formed only during alcoholic fermentation. Pinotin A (i.e., the reaction product from malvidin 3-glucoside and caffeic acid) formation took place only during the aging process, whereas formation of hydroxyphenyl-pyranoanthocyanins derived from *p*-coumaric and ferulic acid followed two different pathways, that is, an enzymatically assisted production during fermentation and a pure chemical formation during aging.

KEYWORDS: Pyranoanthocyanin; pinotin A; vitisin A; caffeic acid; hydroxycinnamic acids; Grenache, red wine color; aging

INTRODUCTION

The red grape cultivar Grenache is one of the most widespread *Vitis vinifera* varieties in the world (*1*). It is very popular in Spain (called Garnacha) and other winemaking regions with a hot and dry climate, such as California or Australia. Because of its thin skin and high ripeness level, Grenache grapes are usually used to produce rosé or fortified wines. Grenache is typically blended with other varieties to speed maturation, being a key ingredient in Rioja wines (blending with Tempranillo creates a robust, hearty red wine) or in Rhône region wines (the so-called GSM or Rhône red blends include Grenache, Syrah, and Mourvèdre). Because Grenache is particularly susceptible to oxidation, the wines are considered to have only a short aging potential (*2*). One likely reason for the inclination of Grenache wines to oxidation is their high concentration of hydroxycinnamic acid derivatives, reaching levels as high as 270−460 mg/L, quantified as caftaric acid (*3*). The mean value reported for other wine varieties is within the range of 50−60 mg/L (*4*). Caftaric acid is known to hydrolyze during wine aging, and the released caffeic acid is involved in the oxidative browning of wine (*5*).

To improve the color of Grenache red wines, it is a general practice to make use of the pomace remaining from rosé wine production. By adding a second batch of freshly mashed grapes, the so-called "doble pasta" (double-mash), Grenache red wines are obtained that are characterized by a deeper color and enhanced astringency and bitterness. Recently, some new winemaking practices have emerged that enable an overextraction of the red wine pigments. The new treatment follows the strategy of increased phenolic extraction by damaging the cell and vacuole membranes, such as in the flash release process (*6*). Recently, a new type of fermenting vat, referred to as Ganimede vat, has been designed for avoiding cap formation during alcoholic fermentation. The Ganimede fermenting vats capture the CO_2 formed during fermentation in a special conical funnel unit mounted in the tank approximately halfway up (*7*, *8*). The accumulated CO_2 gas bubbles up through the neck of the funnel section of the CO_2 collector and agitates the grape skin cap, preventing it from drying and forming a crust. The agitation by bubbling is relatively gentle, and the gas movement in the wine keeps the wine circulating in the vat, the effect being similar as described for délestage.

The color of red wine strongly depends on the levels of the anthocyanins malvidin and peonidin 3-*O*-β-D-glucoside and their

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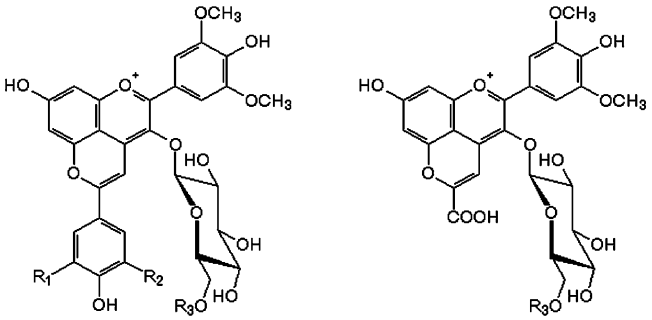

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Figure 1. General structures of hydroxyphenyl-pyranoanthocyanins (**1**) and 5-carboxy-pyranoanthocyanins (**2**). R_1, R_2 = H, OH, OCH_3. R_3 = H, acetyl, *p*-coumaroyl.

6″-acetylated and 6″-*p*-coumaroylated derivatives. As minor compounds, derivates of delphinidin, petunidin, and cyanidin are also involved. During wine fermentation and maturation the anthocyanins and other wine constituents, for example, flavonols and proanthocyanins, can interact and create numerous novel monomeric, oligomeric, and polymeric compounds. Besides the color enhancement through copigmentation effect that is of great importance in young red wines, the contribution of polymeric pigments is crucial to red wine color, especially in the case of aged red wines. Among the newly formed pigments, the class of pyranoanthocyanins plays an important role (**Figure 1**).

The first red wine-derived pyranoanthocyanin was isolated and completely structurally characterized by a French group (*9*, *10*). It was found to be the reaction product of malvidin 3-glucoside and 4-vinylphenol. The authors postulated the formation of 4-vinylphenol via the decarboxylation of *p*-coumaric acid by an enzymatic side activity of the wine yeast. Additional studies however, revealed that the formation of pyranoanthocyanins with a hydroxyphenyl moiety (**Figure 1**, **1**) cannot be explained only by the reaction of the decarboxylated hydroxycinnamic acid (*11*, *12*). Direct reactions of hydroxycinnamic acids and anthocyanins also occur and seem to be responsible for the slow formation of pyranoanthocyanins during the maturation of red wines. In the case of pinotin A, formation can be explained only by a direct reaction of caffeic acid and malvidin 3-*O*-glucoside because so far there is no evidence for an enzymatic decarboxylation of caffeic acid in wine (*13*).

This paper focuses on the formation of hydroxyphenyl-pyranoanthocyanins (**1**) in wines made from the *V. vinifera* cultivar Grenache grown in the Spanish region of Méntrida. Grenache wines were selected for this study because of their high levels of hydroxycinnamic acid derivatives. Experimental wines made by the traditional way of periodically punching down the cap and industrial wines produced by the overextracting techniques of double mash as well as Ganimede vats were investigated. The wines were analyzed with regard to their contents of anthocyanins and hydroxycinnamic acid derivatives, the precursors of hydroxyphenyl-pyranoanthocyanins. In the case of the experimental red wines, the evolution of pyranoanthocyanins during aging was monitored.

MATERIALS AND METHODS

Chemicals. All solvents were of HPLC quality and all chemicals of analytical grade (>99%). Water was of NANOpure quality. The HPLC commercial standards used for quantification were myricetin and quercetin (Sigma, St. Louis, MO); malvidin-3-glucoside, 3-glucosides of quercetin, kaempferol, and isorhamnetin, and quercetin 3-galactoside (Extrasynthese, Genay, France); quercetin-3-rutinoside (rutin), kaempferol, and isorhamnetin (Fluka, Buchs, Switzerland); and caffeic, *p*-coumaric, and ferulic acids (Merck, Darmstadt, Germany).

Wines. Experimental wines were made from healthy Grenache grapes grown in Fuensalida (Méntrida Appellation of Origin, central western Spain). The grapes were supplied by the Cooperativa Condes de Fuensalida cellar (Fuensalida, Spain) at the degree of ripeness that is common for this winemaking region: 13.5% of potential ethanol content and total acidity of 4.75 g/L (as tartaric acid). The grapes (75 kg) were destemmed and crushed and subsequently divided into three vats of 25 L after the addition of 100 mg/L of SO_2 (as $K_2S_2O_7$). Alcoholic fermentation conditions were as follows: inoculation with *Saccharomyces cerevisiae* yeast (UCLM S325, Fould-Springer); fermentation temperature, 24 °C; manual punching down every 12 h; wine separation after 7 days, when the relative density had reached a constant value. After the alcoholic fermentation, malolactic fermentation was induced by inoculation with *Oenococcus oeni* lactic acid bacteria (Lactobacter SP1, Laffort). The end of the malolactic fermentation was confirmed by TLC (Vinikit, Panreac), and then the wines were racked. Finally, after 100 days of storage at 18 °C, the wines were filtered through 1.2 μm filters (Millipore), bottled, and stored at 18 °C. Wine samples were analyzed after alcoholic fermentation, after malolactic fermentation, and after 6 and 10 months of storage, respectively.

The industrial wines were supplied, after malolactic fermentation, by the same cellar that supplied the grapes. The ripeness of the Grenache grapes used was within the ranges of 13.0−13.5% for the potential alcohol content and 4.75−5.00 g/L for the total acidity. The Ganimede wines were elaborated in vats of 50000 L filled with 50000 kg of grapes. The double-mash wines were produced in vats of 50000 L equipped with a system of pumping over for facilitating the skin extraction. From a first batch of 50000 kg of Grenache grapes the free-run must was removed after 24 h for rosé wine production and a second batch of fresh Grenache grapes was back-added (the double mash). Alcoholic fermentation at 24 °C was performed with the help of yeast inoculation (Actiflore C; Laffort), and malolactic fermentation developed spontaneously. Wine samples were taken from the storage vats and kept at −18 °C until analysis.

Analysis of Phenolic Compounds. Total polyphenols and anthocyanins were determined using a modification of the method proposed by Glories (*14*, *15*) as described by Mazza et al. (*16*). Tannin concentration was measured according to the method of Ribéreau-Gayon and Stonestreet (*17*). Astringency of tannins was measured following the methodology described by Glories (*18*) for the determination of the gelatin index. In the present case, the results were expressed as "intensity of astringency", that is, the tannins that precipitated after a treatment with cold soluble gelatin (Atoclar M, Esseco, Italy). Tannin concentration was calculated using the corrected formula adapted to young red wines and obtained by comparison of a standard solution of oligomeric procyanidin from grape seeds: C (g/L) = $16.16D_2 - 24.24D_1 + 1.71$[Al], where D_2 and D_1 were the optical density at 520 nm after heating and without heating, respectively, on a 10 mm optical path, and [Al] was the free anthocyanin concentration in g/L that was estimated from the results of HPLC for monomeric anthocyanins.

Individual phenolic compounds (anthocyanins, flavonols, and hydroxycinnamic acid derivatives) were determined by HPLC analysis. After centrifugation (2500*g*) and filtration (0.45 μm nylon membranes, Millipore), the wine samples were directly injected (10 μL) into a Varian ProStar HPLC apparatus (Varian Inc., Walnut Creek, CA) comprising a ProStar 240 ternary pump, a ProStar 410 autosampler, and a ProStar 330 photodiode array detector. The column was thermostated at 20 °C in an MFE-01 oven (Análisis Vínicos, Tomelloso, Spain). Separation was performed in an Ace 5 C18 250 × 4.6 mm column (Advanced Chromatography Technologies, Aberdeen, Scotland). 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 nm (hydroxycinnamic acid derivatives), 360 nm (flavonols), and 520 nm

(anthocyanins). For quantification, calibration curves were obtained by injecting solutions of the corresponding standards (malvidin 3-glucoside; the flavonol aglycones myricetin, quercetin, kaempferol, and isorhamnetin; the 3-glucosides of quercetin, kaempferol, and isorhamnetin; quercetin 3-rutinoside; quercetin 3-galactoside; caffeic acid; *p*-coumaric acid; ferulic acid), whereas for the other compounds the concentrations were expressed as the most similar standard, but the differing molecular weights were taken into account: caffeic acid for caftaric acid and *S*-glutathionyl-caftaric acid; *p*-coumaric acid for coutaric acid; ferulic acid for fertaric acid; malvidin 3-glucoside for monomeric anthocyanins; and quercetin 3-glucoside for myricetin 3-glucoside and quercetin 3-glucuronide.

Color Properties. Wine color properties were determined after centrifugation (2500*g*, 15 min) and adjustment of 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 (percent copigmentation) and the degree of anthocyanin polymerization (percent polymerization) were determined following the method developed by Boulton (*19*) as described by Hermosín-Gutiérrez (*20*). 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 (*21, 22*).

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, an LG-980-02 ternary gradient unit, and an 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 for solvent B was as follows: 6% (minute 0); 20% (minute 20); 40% (minute 35); 60% (minute 40); 90% (minute 45); 90% (minute 50); and 6% (minute 55).

Pinotin A was quantified using a previously obtained calibration curve at 510 nm (*23*). 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, 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 were as follows: 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, *m*/*z* 50−1200; chromatographic conditions as above.

Statistical Analysis. The data corresponding to the different Grenache wines (experimental, Ganimede, and double mash) were analyzed by Student−Newman−Keuls test (SPSS version 10.0, SPSS Inc.).

RESULTS AND DISCUSSION

Color Properties and Anthocyanin Content. After the malolactic fermentation, the color properties of the three different young wines were analyzed, by determination of the chromatic CIELAB characteristics (**Table 1**). Here, the double-mash Grenache wines exhibited the most intense color. This is not unexpected because of the higher skin-to-juice ratio. The experimental wines exhibited the lightest (highest values of L^*) and less pure red color (lowest values of C^*), having little purple hue (highest values of h^*). The chromatic characteristics of the Ganimede and double-mash wines were very similar, but the double-mash wines had the purest color (highest values of C^*). These results suggested that the double-mash Grenache wines contained not only a higher amount of red pigments but also more of other phenolic compounds (**Table 2**), which would contribute to the stabilization of the color through copigmentation (*24*). No differences were found regarding the content of tannins and their astringency within the three types of Grenache wines produced. However, the amount of total anthocyanins was significantly higher in the double-mash wines as compared to the Ganimede (29%) and experimental (160%) wines. It is noteworthy that the increase in the amount of monomeric anthocyanins was even much higher, 56% as compared to Ganimede wines and 278% as compared to experimental wines. The double-mash technology increases the skin-to-juice ratio, facilitating a higher extraction of anthocyanins. However, the observed increase in the extraction of anthocyanins in double-mash wines was much higher than expected as compared to experimental wines, and one likely explanation is the higher amount of flavonols present in the double-mash wines. This was 2-fold the concentration found in the Ganimede wines and 10-fold the concentration found in the experimental wines. The higher transfer of flavonols from the skins to the wine helped to extract more anthocyanins, most likely by means of copigmentation complexes as has been recently demonstrated for wines elaborated with the addition of the flavonol rutin before the fermentation (*24*). With regard to the contribution of copigmented and polymerized anthocyanins to their total wine color (**Table 1**) the differences observed were also in agreement with the above-mentioned results.

Anthocyanin, Flavonol, and Hydroxycinnamic Acid Profile. The increase in monomeric anthocyanins for double-mash Grenache wines did not yield any changes in the characteristic anthocyanin profile of Grenache wines (data not shown), which is dominated by malvidin 3-glucoside (*25*). The corresponding increase in the flavonol concentration, however, introduced some

Table 1. Average Value for CIELAB Chromatic Parameters, Measured at pH 3.6, for Experimental ($n = 3$), Ganimede ($n = 10$), and "Doble Pasta" (Double Mash; $n = 6$) Grenache Wines after Malolactic Fermentation

chromatic parameter	experimental	Ganimede	double mash
L^*	78.30 a[a]	64.63 b	59.09 b
C^*	24.32 a	35.27 b	44.66 c
h^*	10.34 a	–0.42 b	–2.01 b

[a] Different letters in the same row indicate significant differences according to the Student–Newman–Keuls test ($\alpha = 0.05$).

Table 2. Average Phenolic Composition of Experimental ($n = 3$), Ganimede ($n = 10$), and "Doble Pasta" (Double Mash; $n = 6$) Grenache Wines after Malolactic Fermentation

phenolic compound	experimental	Ganimede	double mash
total polyphenols[a] (mg/L)	647.9 a[d]	833.4 b	1049.3 c
tannins (g/L)	1.292	1.265	1.472
intensity of astringency (g/L)	1.084	1.020	1.108
total anthocyanins[b] (mg/L)	98.3 a	198.0 b	254.9 c
total monomeric anthocyanins[b,c] (mg/L)	49.0 a	119.1 b	185.3 c
malvidin 3-glucoside (mg/L)	41.9 a	91.9 b	140.7 c
% polymerization	39.2 a	36.4 a	31.6 b
% copigmentation	15.6 a	29.2 b	33.2 b
total flavonols[c] (μmol/L)	13.3 a	65.0 b	135.2 c
total hydroxycinnamic acid derivatives[c] (μmol/L)	414.7 a	720.7 b	901.3 c

[a] As gallic acid. [b] As malvidin 3-glucoside. [c] Summation of the individual compounds quantified by HPLC. [d] Different letters in the same row indicate significant differences according to the Student–Newman–Keuls test ($\alpha = 0.05$).

Table 3. Average Flavonol Profile (Molar Percentage[a] of Each Individual Flavonol) of Experimental ($n = 3$), Ganimede ($n = 10$), and "Doble Pasta" (Double Mash; $n = 6$) Grenache Wines after Malolactic Fermentation

flavonol	experimental	Ganimede	double mash
myricetin 3-glucoside	17.0 a	20.7 ab	24.4 b
quercetin 3-rutinoside	2.9 a	2.2 ab	0.9 b
quercetin 3-galactoside	17.0 a	4.5 b	4.3 b
quercetin 3-glucoside	ND[b]	6.3 a	12.7 b
quercetin 3-glucuronide	32.7 a	49.7 b	41.1 c
kaempferol 3-glucoside	ND	ND	1.5
isorhamnetin 3-glucoside	29.1 a	13.6 b	12.5 b
myricetin	0.4	1.4	1.4
quercetin	0.9	1.5	1.3

[a] Calculated from the quantification data, expressed in μmol/L, obtained by HPLC. Different letters in the same row mean significant differences according to the Student–Newman–Keuls ($\alpha = 0.05$). [b] ND, not detected.

Table 4. Average Concentrations[a] (Milligrams per Liter) of Hydroxycinnamic Acid Derivatives for Experimental ($n = 3$), Ganimede ($n = 10$), and "Doble Pasta" (Double Mash; $n = 6$) Grenache Wines after Malolactic Fermentation

hydroxycinnamic acid derivative	experimental	Ganimede	double mash
S-glutathionyl-caftaric acid	12.98 a	54.50 b	49.94 c
cis-caftaric acid	2.42 a	4.47 b	5.93 b
trans-caftaric acid	49.91 a	136.21 b	183.44 c
caffeic acid	20.23	ND[b]	ND
cis-coutaric acid	5.40 a	11.01 b	11.26 b
trans-coutaric acid	9.72 a	33.03 b	42.13 c
p-coumaric acid	2.72	ND	ND
trans-fertaric acid	5.42 a	9.80 b	10.79 b
ferulic acid	1.30	ND	ND

[a] Quantification by HPLC. Different letters in the same row mean significant differences according to the Student–Newman–Keuls test ($\alpha = 0.05$). [b] ND, not detected.

changes in their profile (**Table 3**). The main flavonol was always quercetin 3-glucuronide, although its concentration was significantly higher for Ganimede wines and lower for experimental wines. The second important flavonol was myricetin 3-glucoside, having a higher concentration for double-mash wines and a lower concentration for experimental wines, whereas Ganimede wines showed an intermediate value. Isorhamnetin 3-glucoside and quercetin 3-galactoside in contrast were important flavonols in the experimental wines. Quercetin 3-glucoside was not detected in experimental wines, and the molar percentage found in double-mash wines was 2-fold the value found in Ganimede wines. It is possible that quercetin 3-glucoside was partially hydrolyzed in wines, although in all wines the observed concentration of free flavonols (hydrolyzed flavonol glycosides) was low.

The double-mash Grenache wines also had the highest concentrations of hydroxycinnamic acid derivatives (**Table 2**). Mainly hydroxycinnamoyltartaric acids were found in the analyzed Grenache wines (**Table 4**) after completion of malolactic fermentation. Only experimental wines showed appreciable amounts of the corresponding free hydroxycinnamic acids released by hydrolysis of the former esters. The caffeic acid and its derivatives (the ester formed with tartaric acid, known as caftaric acid, and the reaction product between oxidized caftaric acid and the tripeptide glutathione, known as *S*-glutathionyl-caftaric acid) were the dominant ones (three-fourths of the total molar concentration), and caftaric acid was present in similar amounts as reported for Pinotage wines (*23*).

Table 5. Average Concentrations[a] (Milligrams per Liter) of Pyranoanthocyanins for Experimental ($n = 3$), Ganimede ($n = 10$), and "Doble Pasta" (Double Mash; $n = 6$) Grenache Wines after Malolactic Fermentation

pyranoanthocyanin	experimental	Ganimede	double mash
vitisin A	0.77 a	2.74 b	4.05 b
vitisin B	0.76 a	1.62 b	1.22 b
pinotin A	ND[b]	ND	ND
malvidin 3-glc-4-VP[c]	0.22	0.30	0.36
malvidin 3-glc-4-VG	0.16	0.20	0.29

[a] Quantification by HPLC. Different letters in the same row mean significant differences according to the Student–Newman–Keuls test ($\alpha = 0.05$) [b] ND, not detected. [c] glc, glucoside; VP, vinylphenol; VG, vinylguaiacol.

It has been outlined that the pyranoanthocyanin pinotin A derives from caffeic acid (*26*) and its formation can be explained by direct reaction between caffeic acid and malvidin 3-glucoside (*27*). As caffeic acid is present in wines only after hydrolysis of caftaric acid, Grenache wines were expected to show a high potential for the formation of the pyranoanthocyanin pinotin A.

Formation of Pyranoanthocyanins. Pinotin A has been obtained in model solutions by direct reaction between caffeic acid and malvidin 3-glucoside (*27*). Pyranoanthocyanins derived from other hydroxycinnamic acids, that is, those derived from *p*-coumaric and ferulic acids, have been postulated to be formed in wines after a previous enzymatic decarboxylation of the hydroxycinnamic acids giving rise to the corresponding 4-vinylphenols (*10*). Only recently was formation of pinotin A reported to be due to the release of caffeic acid from caftaric acid (*24*). Our findings are in line with this hypothesis, because pinotin A was not detected in any of the analyzed Grenache wines (**Table 5**). Importantly, additional pyranoanthocyanins, that is, malvidin 3-glucoside-4-vinylphenol and malvidin 3-glucoside-4-vinylguaiacol, were present in concentrations that were in accordance with the concentrations of their corresponding precursors. This suggests a formation already during alcoholic fermentation, most likely due to enzymatic hydrolysis of their corresponding hydroxycinnamoyltartaric acids and subsequent enzymatic decarboxylation of the resulting *p*-coumaric and ferulic acids. Moreover, vitisin-like pyranoanthocyanins (**Figure 1**, **2**) (*28*, *29*) were also found in all of the analyzed Grenache wines (**Table 5**). Together with the expected vitisin A and vitisin B, the 6″-acetyl and the 6″-*p*-coumaroyl derivatives of vitisin B were detected in trace amounts using LC/MS analysis (*30*). In addition, the aglycon of vitisin B, having a molecular ion at *m*/*z* 355, was detected. The concentrations of both vitisins A and B were always higher in double-mash wines. One explanation can be seen in the addition of the second batch of fresh mash when the alcoholic fermentation was in progress and some of the secondary products of yeast activity, that is, pyruvic acid, were already available for the reaction with malvidin 3-glucoside. The levels of vitisin A in the double-mash Grenache wines were in the range of the values found for young Chilean Cabernet Sauvignon wines reported by Schwarz et al. (*31*).

Evolution of Hydroxyphenyl-pyranoanthocyanins and Their Precursors during Aging. There was no pinotin A detectable in Grenache wines at the end of malolactic fermentation even when some caffeic acid was present. The experimental wines were therefore analyzed over an aging period of 10 months (18−20 °C in the dark). The results confirmed the progress of the hydrolysis of the hydroxycinnamoyltartaric acids and the subsequent reaction of free hydroxycinnamic acids with the major anthocyanin present in Grenache wines, that is,

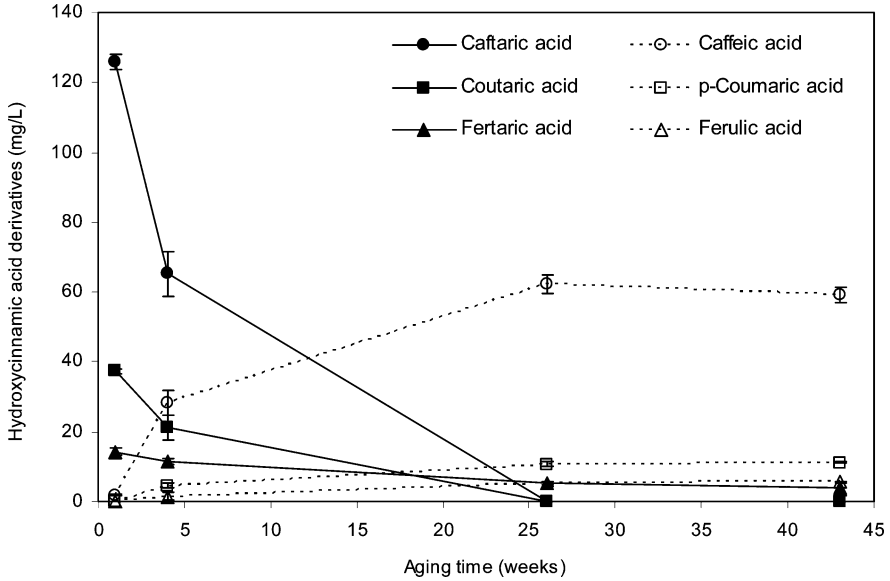

Figure 2. Evolution of hydroxycinnamic acid derivatives in experimental Grenache wines during aging. Time starts at the beginning of the alcoholic fermentation.

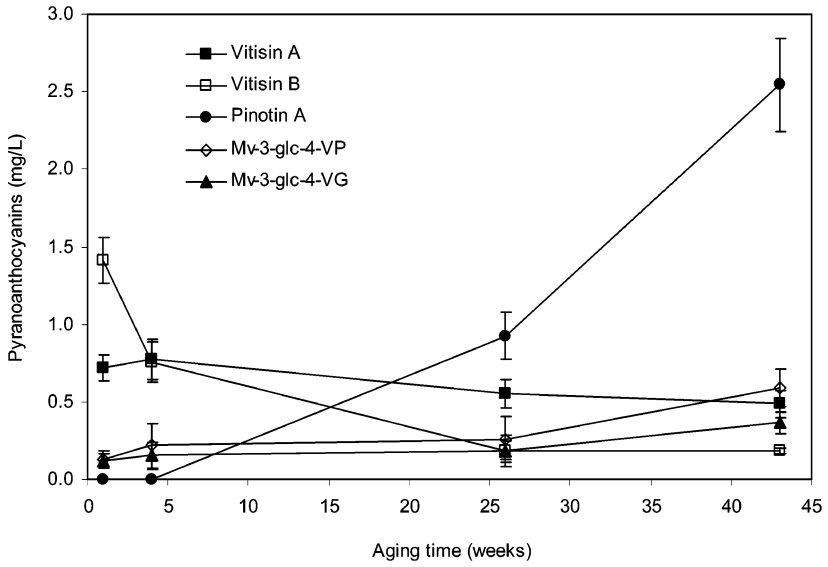

Figure 3. Evolution of carboxy- and hydroxyphenyl-pyranoanthocyanins in experimental Grenache wines during aging. Mv, malvidin; glc, glucoside; VP, vinylphenol; VG, vinylguaiacol. Time starts at the beginning of the alcoholic fermentation.

malvidin 3-glucoside. The hydrolysis of both caftaric and coutaric acid was completed after 6 months of aging, whereas fertaric acid hydrolyzed more slowly. The latter compound was still detectable after an aging period of 10 months (**Figure 2**). The concentrations of free hydroxycinnamic acids reached their maximum values after 6 months of aging, and a decrease in the concentration of caffeic acid was observed only after 10 months of aging. At the same time, formation of pinotin A was observed. Pinotin A formation started at the end of the malolactic fermentation, and this substance became the most important pyranoanthocyanin after a period of 6 months of aging (**Figure 3**). Even after 10 months of aging the concentration of pinotin A continued to increase, reaching a concentration of 2.54 mg/L. For Pinotage wines a nonlinear relationship between the molar concentration of pinotin A and both the aging time and the molar concentration of caffeic acid was reported: $\log[\text{pinotin A}] = -4.07 + 0.54 \times \text{age} + 0.84 \times \log[\text{caffeic acid}]$ (*23*). Application of this equation to the Grenache wines predicted very low values for the concentrations of pinotin A, thus suggesting that individual grape cultivars could exert some influence on the kinetics of pinotin A formation.

Formation of the other two hydroxyphenyl-pyranoanthocyanins, that is, malvidin 3-glucoside-4-vinylphenol and malvidin 3-glucoside-4-guaiacol, occurred in two separate periods (**Figure 3**). They were initially produced during the alcoholic and malolactic fermentations following most likely the enzymatic pathway, that is, the production of vinylphenols. Then their concentrations remained almost constant until the fourth month of aging. From then on the concentrations of both *p*-coumaric and ferulic acid reached their maximum values and, in a parallel way, the synthesis of malvidin 3-glucoside-4-vinylphenol and malvidin 3-glucoside-4-vinylguaiacol was reactivated. This suggests a direct reaction of released hydroxycinnamic acids with malvidin 3-glucoside. The vitisin-like pyranoanthocyanins were produced only during the alcoholic fermentation, and vitisin A proved to be more stable than vitisin B (**Figure 3**).

In summary, the two overextraction winemaking techniques (Ganimede vats or double-mash fermentation) proved to be

superior to the traditional way of manually punching down the cap with respect to the color properties of the resulting red wines. Especially the double-mash wines were characterized by a higher concentration of anthocyanins and a lower degree of polymerization products as compared to the experimental red wines and also the wines produced in Ganimede vats. This effect might be explained by the protective properties of coextracted flavonols. Agitation of the mash also resulted in increased concentrations of the higher water-soluble hydroxycinnamic acid derivatives. The profile of pyranoanthocyanin formation supports previous findings that pinotin A is solely formed through a chemical reaction between caffeic acid and malvidin 3-glucoside during aging. In contrast, hydroxyphenyl-pyranoanthocyanins derived from *p*-coumaric and ferulic acid are likely to be initially formed by enzymatic decarboxylation of the hydroxycinnamic acid precursors during alcoholic and malolactic fermentation followed by reaction of the resulting vinylphenols with anthocyanins and later during the aging period again through the direct reaction of released hydroxycinnamic acid as described for pinotin A.

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