Evolution and Stability of Anthocyanin-Derived Pigments during Port Wine Aging

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For three years, the evolution of the three major anthocyanidin monoglucosides (malvidin 3-glucoside, malvidin 3-acetylglucoside, and malvidin 3-coumaroylglucoside) and their anthocyanin−pyruvic acid adducts was monitored in Port wines stored in oak barrels. The degradation reactions of all pigments followed first-order kinetics in all the wines studied. The degradation rate constants of the anthocyanin−pyruvic acid adducts were much lower than those of the anthocyanidin monoglucosides. The results of both anthocyanins and pyruvic acid adducts show that acylation on the sugar moiety of all the pigments decreased their stability in wine. The levels of malvidin 3-glucoside−pyruvic acid adduct and its acylated forms increased right after wine fortification with wine spirit before starting to decrease around 100 days. The initial formation of anthocyanin−pyruvic acid adducts was concurrent with the degradation of anthocyanidin monoglucosides.

Keywords: *Port wine; pigments; pyruvic acid; anthocyanin; aging*

INTRODUCTION

It has been stated that the most rapid change in wine color composition occurs during the first year of maturation when the wine is normally in bulk storage (*1*). This phase is considered to be quite distinct from the latter "aging phase", when the wine is in-bottle and well protected from any further contact with air (*2, 3*). The color changes during wine maturation are usually attributed to anthocyanin polymerization reactions and formation of new pigments resulting from interactions between anthocyanins and other phenolic compounds, such as catechins. These newly formed pigments are thought to arise from direct condensation between anthocyanins and catechins (*4*−*7*) or to involve ethyl bridging (*8*−*13*). All these events result in the formation of more stable pigments that stabilize wine color changing it to a more brick red hue. Besides these newly formed pigments, other anthocyanin-derived compounds have been reported in red wines over the last years (*14*−*16*). Dallas and Laureano have signalized (by HPLC) unknown anthocyanins in wine samples, especially a major one eluting soon after malvidin 3-glucoside. This pigment, which was present in wines in the range of concentration between 1 and 12 mgL^{-1}, was isolated and showed resistance to sulfur bleaching (*17*). Furthermore, a new malvidin 3-glucoside-derived pigment named vitisin A, together with its acylated esters, was found in fortified wines and revealed a visible maximum absorption at 511 nm, resistance to bleaching by sulfur dioxide, and a greater color expression at higher pH values than original anthocyanins (*15*). The structure of these malvidin−pyruvic acid adducts results from the cyclization between carbon 4 and the hydroxyl group at carbon 5 of the original flavylium moiety yielding a fourth ring, which has been referred to be responsible for their higher stability compared to that of the original anthocyanins (*15, 18*) (Figure 1). The color properties of these pigments are expected to influence the Port wine color shift to their characteristic orange "tawny" hue. Vitisin A was shown to have a higher color expression at wine pH than the original anthocyanidin monoglucosides (*15*). Other compounds with similar characteristics were found in grape pomace, but a slightly different structure was attributed (*18*). These compounds were recently isolated from Port wines (*19*), and their structures were found to be similar to the ones proposed by Fulcrand et al. (*17*). Concerning their formation mechanism, malvidin−pyruvic acid adducts (mv−py) were obtained from the reaction of malvidin 3-glucoside with pyruvic acid (*18, 20*). Similarly, its acetyl and coumaroyl forms were obtained from the reaction of pyruvic acid with malvidin 3-acetylglucoside and malvidin 3-coumaroylglucoside, respectively. Studies performed in model solutions have shown that their concentrations depend on several factors including pyruvic acid, acetaldehyde, anthocyanin levels, pH, and temperature (*18*−*22*). These studies have shown that acetaldehyde competes with pyruvic acid in the reaction with the original anthocyanins (*21*). The aim of the present work is to monitor the evolution of these anthocyanin-derived pigments, namely mv−py, mv-ac−py, and mv-coum−py in Port wines along with the other major anthocyanins, with particular regard to their stability. Kinetic parameters such as reaction order and rate constants were determined to characterize these reactions.

MATERIALS AND METHODS

Wine Samples. Two monovarietal Touriga Nacional wines and two monovarietal Touriga Francesa wines (both red *Vitis vinifera* Portuguese varieties) were made from grapes grown in different Douro vineyards in Northern Portugal (1997 vintage). Microvinifications were performed according to the usual Port winemaking procedure. The grapes (25 kg) were de-stemmed and crushed into stainless steel wine vats. When

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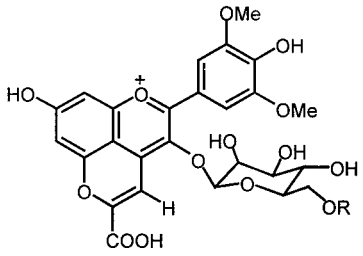
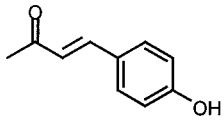


R = H , malvidin 3-glucoside pyruvic acid adduct

R =  , malvidin 3-coumaroylglucoside pyruvic acid adduct

R =  , malvidin 3-acetylglucoside pyruvic acid adduct

Figure 1. Structures of the anthocyanin−pyruvic acid adducts.

Table 1. Enological Parameters of Touriga Nacional (TN) and Touriga Francesa (TF) Varietal Port Wines (Vintage 1997)

	ρ (g·cm^{-3})	alcohol (% v/v)	pH	total acidity (g·L^{-1})	total SO_2 (mg·L^{-1})
TN 1	1.019	18.4	3.48	5.16	20
TN 2	1.022	20.2	3.56	7.43	24
TF 1	1.019	18.1	3.51	5.61	19
TF 2	1.018	18.1	3.66	4.01	15

about half of the original sugar content was converted to alcohol (2−3 days), the must fermentation was stopped by addition of wine spirit (ratio wine/wine spirit, ≈5) in order to maintain its natural sweetness. Every wine was separated from pomace by filtration and pumped to other vats. The final alcohol content was set to 18−20% alcohol (v/v) and the wines were stored in oak barrels in a dark room with an average temperature of 15 °C. The microvinifications were performed in triplicate. The wines were analyzed before storage, and samples were taken from each barrel after 8, 12, 21, 30, and 38 months of aging. Some wine enological parameters are shown in Table 1.

A monovarietal Touriga Nacional Port wine made according to the procedure described above was used to follow the evolution of anthocyanins and anthocyanin−pyruvic adducts during the first 200 days of wine bottle aging.

The 10 commercial Port wines were made from grapes of Touriga Nacional and Touriga Francesa from the 1997 vintage.

HPLC Analysis. The wines were directly analyzed by HPLC using a Merck ODS (C18) (250 × 4.6 mm i.d.) column with diode array detection (20 μL). The solvents were A, H_2O/HCOOH (9:1), and B, CH_3CN/H_2O/HCOOH (3:6:1). The gradient consisted of 20 to 85% B for 70 min, 85 to 100% B for 5 min, and then isocratic for 10 min at a flow rate of 1 mL/min. The anthocyanidin monoglucosides and pyruvic adducts were identified on the basis of their UV−vis spectra and retention time of standards (Figure 2). The malvidin−pyruvic acid adduct standards were isolated from one-year-old Port wines prepurified by low-pressure Toyopearl gel chromatography. The wine fraction was concentrated, and the pigments were isolated by semipreparative HPLC using a 500-μL injector and using the same chromatographic conditions described above. The pigments structures were elucidated by mass spectrometry and NMR (*19*).

Pigment Quantification. Calibration curves were obtained by injecting different concentrations of malvidin 3-glucoside and malvidin 3-glucoside−pyruvic acid derivative (mv−py) standards. Linear calibration curves (r^2 > 0.98) were obtained in the range of the concentrations studied with a detection limit of 1.0 mg/L. Unknown concentrations were determined from the linear regression equations.

Statistical Analysis. All measurements were performed in triplicate. Linear regression, analysis of standard deviation, and T-test experiments were performed for every mean using the SPSS computer package (p < 0.05). Where error bars are not visible in the graphs, determinations are within the size of the symbols.

Extinction Coefficients (ϵ). The ϵ values (Lmol^{-1}cm^{-1}) of mv and its pyruvic acid adduct (mv−py) were determined in methanol/HCl 0.01% and performed at λ_{max} of both pigments (538.0 and 531.8 nm for mv and mv−py, respectively). Absorbency was measured in several standard solutions of mv and mv−py in a UV-265 Shimadzu spectrophotometer using 10-mm cells at 20 °C.

RESULTS AND DISCUSSION

The pigments detected from HPLC analysis were expressed on malvidin 3-glucoside (mv) for anthocyanidin monoglucosides and malvidin 3-glucoside−pyruvic acid adduct (mv−py) for anthocyanin−pyruvic adducts. Expression of concentrations on mv or mv−py is important because these pyruvic adducts present spectroscopic characteristics in the visible spectrum that are different from those of the original anthocyanins. In our experimental conditions, the molar extinction coefficients values (ϵ) found for mv and mv−py measured at 538.0 nm (λ_{max} of mv) are 1.60 × 10^4 and 1.29 × 10^4 Lmol^{-1}cm^{-1}, respectively. Additionally, the ϵ values were also measured at 531.8 nm (λ_{max} of mv−py) which gave 1.56 × 10^4 and 1.30 × 10^4 Lmol^{-1}cm^{-1} for mv and mv−py, respectively. The differences found between the ϵ values of both pigments were not as significant as we expected them to be, attending to the unique spectroscopic features of mv−py described in the literature. The molar extinction coefficient found for mv seems to be consistent with values found in the literature (*23*). To our knowledge, no data are available concerning the molar extinction coefficient of mv−py.

Evolution of Anthocyanins and Pyruvic Adducts. The evolution of the levels of the three major anthocyanidin 3-monoglucosides (malvidin 3-glucoside, mv; malvidin 3-acetylglucoside, mv-ac; and malvidin 3-coumaroylglucoside, mv-coum) and the three detected malvidin−pyruvic acid adducts (mv−pyruvic acid adduct, mv−py; mv-ac−pyruvic acid adduct, mv-ac−py;

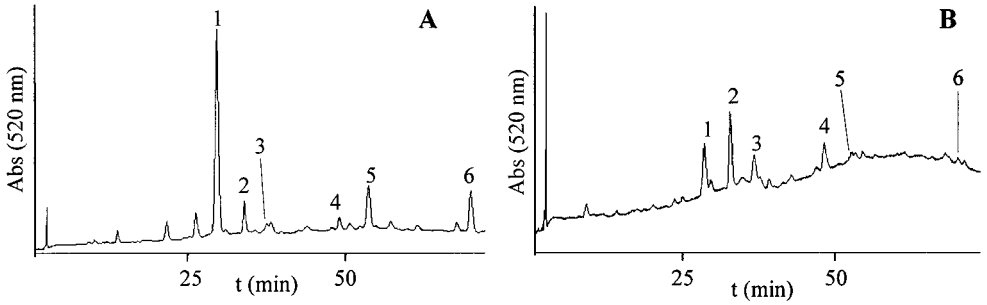


Figure 2. HPLC profile recorded at 520 nm of young (A) and one-year-old (B) Port wine. (1) Malvidin 3-glucoside, (2) malvidin 3-glucoside−pyruvic adduct, (3) malvidin 3-acetylglucoside−pyruvic adduct, (4) malvidin 3-coumaroylglucoside−pyruvic adduct, (5) malvidin 3-acetylglucoside, and (6) malvidin 3-coumaroylglucoside.

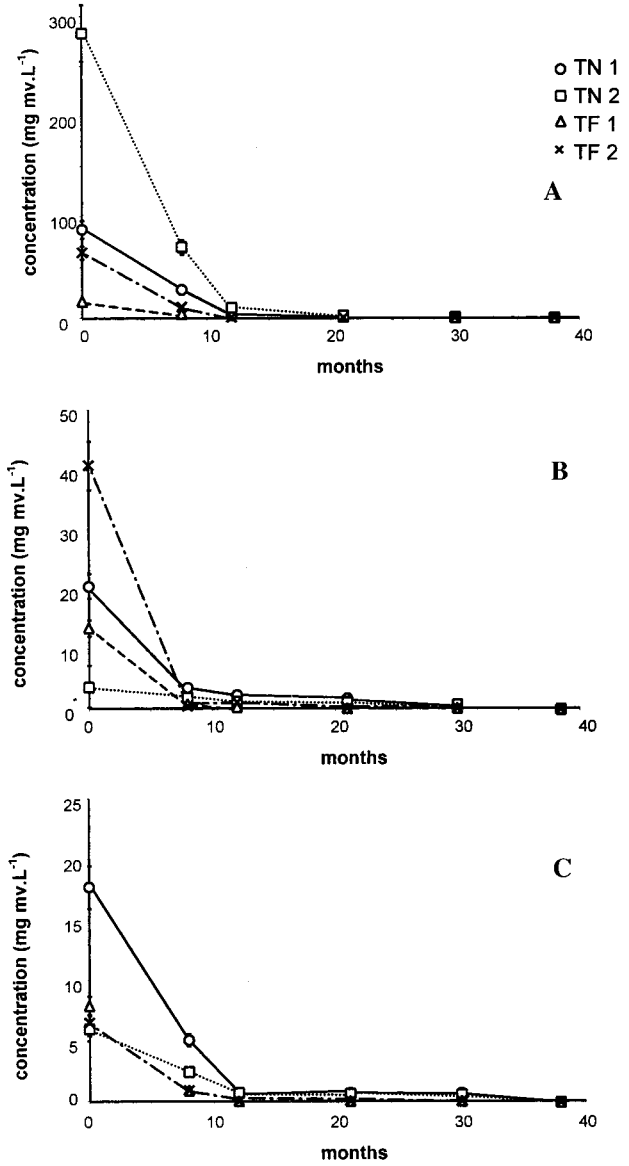


Figure 3. Evolution of malvidin 3-glucoside (A), malvidin 3-acetylglucoside (B), and malvidin 3-coumaroylglucoside (C) in Touriga Nacional (TN 1 and TN 2) and Touriga Francesa (TF 1 and TF 2) Port wines during 38 months storage in oak barrels.

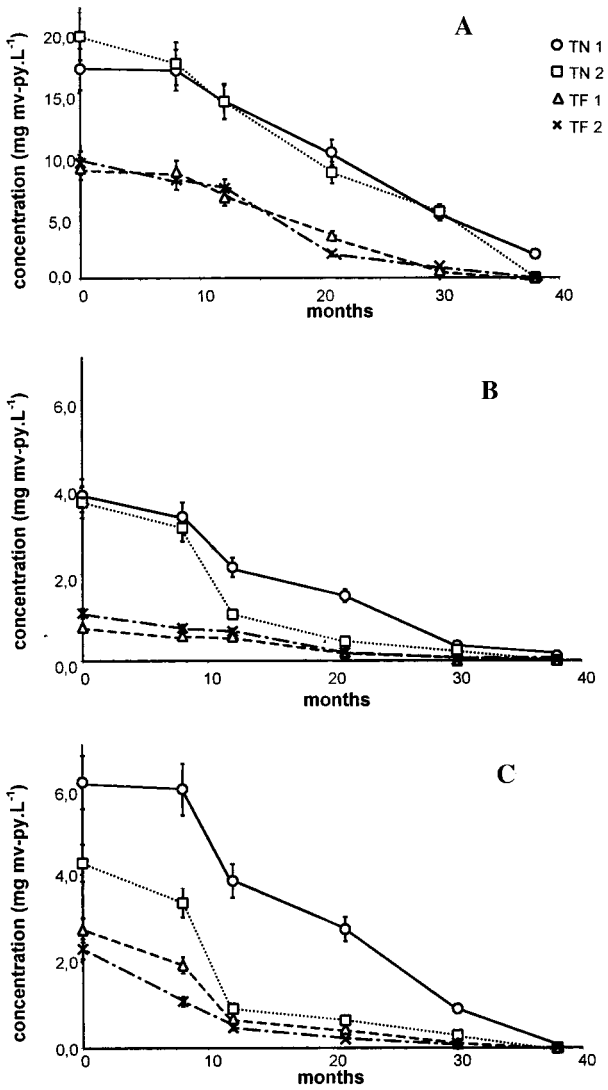


Figure 4. Evolution of malvidin−pyruvic acid (A), malvidin 3-acetylglucoside−pyruvic acid (B), and malvidin 3-coumaroylglucoside−pyruvic acid (C) in Touriga Nacional (TN 1 and TN 2) and Touriga Francesa (TF 1 and TF 2) Port wines during 38 months storage in oak barrels.

mv-coum−pyruvic acid adduct, mv-coum−py) were followed during 38 months in varietal Port wines stored in oak barrels (Figures 3 and 4). The newly formed pigments were found to be much more stable than the original anthocyanidin monoglucosides, as their concentration in Port wines appear to be less affected during aging. Indeed, after one year of aging, the major grape anthocyanidin monoglucosides underwent a decrease between 80 and 90% in amount, while mv−py decreased only 15−25% during the same period. The other two pigments reported, mv-ac−py and mv-coum−py, decreased 15−30% and 30−45%, respectively. After 30 months of aging, practically no anthocyanidin monoglucosides were detected in all the Port wines studied, but 20−35% of the original content of anthocyanin−

Table 2. Degradation Rate Constants (k; month^{-1}) of Malvidin 3-Glucoside (mv), Malvidin 3-Acetylglucoside (mv-ac), Malvidin 3-Coumaroylglucoside (mv-coum), Malvidin−Pyruvic Acid (mv−py), Malvidin 3-Acetylglucoside−Pyruvic Acid (mv-ac−py), and Malvidin 3-Coumaroylglucoside−Pyruvic Acid (mv-coum−py) in Touriga Nacional (TN) and Touriga Francesa (TF) Port Wines Stored in Oak Barrels[a]

	mv		mv-ac		mv-coum		mv−py		mv-ac−py		mv-coum−py	
wine	k month^{-1}	r^2	k month^{-1}	r^2	k month^{-1}	r^2	k month^{-1}	r^2	k month^{-1}	r^2	k month^{-1}	r^2
TN 1	−0.142	0.925	−0.192	0.901	−0.282	0.890	−0.057	0.883	−0.097	0.920	−0.064	0.894
TN 2	−0.175	0.971	−0.193	0.931	−0.182	0.918	−0.046	0.963	−0.099	0.943	−0.092	0.914
TF 1	−0.179	0.819	−0.365	1.000	−0.272	0.986	−0.047	0.852	−0.067	0.863	−0.109	0.951
TF 2	−0.239	0.883	−0.350	0.842	−0.249	0.979	−0.083	0.907	−0.085	0.888	−0.112	0.805

[a] Values are the average of triplicate assays ($p < 0.05$).

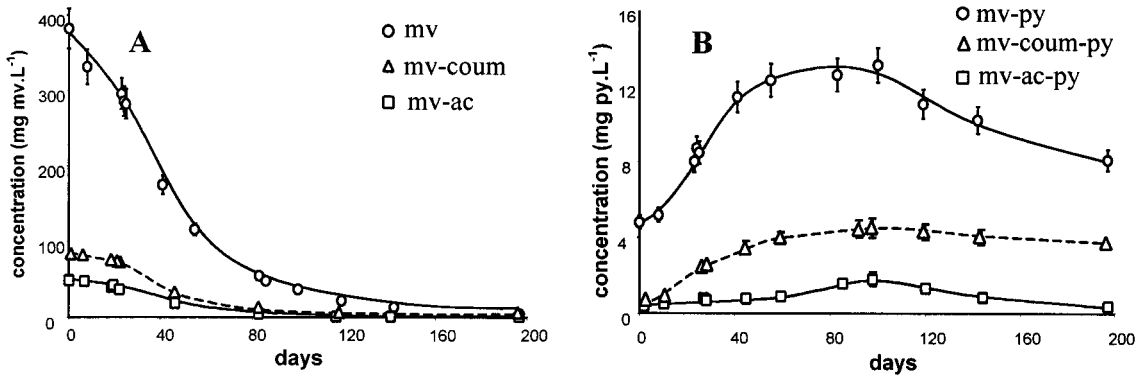


Figure 5. Evolution of the level of malvidin 3-glucoside, malvidin 3-acetylglucoside, and malvidin 3-coumaroylglucoside (A), and malvidin−pyruvic acid adduct, malvidin−acetylglucoside−pyruvic acid adduct (mv-ac−py), and malvidin−coumaroylglucoside−pyruvic acid adduct (mv-coum−py) (B) during the first 200 days after fortification of TN 1 with wine spirit.

pyruvic adducts was still present in the wines. After 38 months of aging, mv−py was the only pigment detected in both TN wines, but no pigments were found in the TF wines (Figure 4), which is in strong agreement with recent reports (*24*).

Pigment Degradation Kinetics. The results obtained showed that the degradation reactions of the major anthocyanidin monoglucosides and malvidin−pyruvic acid adducts followed first-order kinetics. This agrees with previous works (*9*, *20*, *24*−*26*). The reaction rate constants (k) were determined by calculating the slopes after linear regressions of the graphs $ln\ C = f(t)$ where C is the concentration of the pigment and t is the period (month) of aging (Table 2). For the same pigment the differences observed in the k values of the different wines studied results from differences in their chemical composition. These intrinsic chemical differences could prevent or accelerate the pigment degradation process. This degradation seems to be less important in TN wines than in TF wines. Furthermore, the degradation rate constants of mv-ac and mv-coum were much higher than for mv. In general, mv-ac disappears faster than mv-coum (except for TN 1) which agrees with some works performed in model solutions (*20*) or in wine (*27*), but contrasts with the findings of McCloskey and Yengoyan (*28*).

The anthocyanin−pyruvic acid adducts were found to have much lower degradation rate constants than the original anthocyanins (Table 2). Similarly to the data obtained for the three major anthocyanidin monoglucosides, the pyruvic acid adducts acylated derivatives (mv-ac−py and mv-coum−py) showed higher rate loss constants than mv−py (Table 2). Nevertheless, no conclusion could be made regarding the effect of the type of acylation (with acetic or coumaric acid) on their stability as their relative degradation changed between the different wines studied. Effectively, the wine chemical composition seems to play an important role in the relative degradation of these pigments.

Pigment Evolution after Wine Fortification. The absence of anthocyanin−pyruvic acid adducts in fresh grape skins led us to assume that they are formed during vinification and through the period of wine maturation. The formation mechanism is thought to occur through the reaction of original grape anthocyanins with pyruvic acid mainly resulting from yeast metabolism during fermentation (*18*). Therefore, it is expected that the levels of these pigments increase immediately after grape crushing. This is not perceptible from Figure 4 because large periods of time elapsed between the measurements.

To study the initial evolution of anthocyanins and pyruvic acid adducts, all pigment concentration was followed immediately after red wine fortification (after approximately 2 days of fermentation) (Figure 5A). At this point the grape pomace was separated from the wine and the fermentation was stopped by adding wine spirit (ratio wine/wine spirit ≈5). In this experiment this point was considered to be day zero regarding pigment evolution. The initial concentration of pyruvic acid adducts probably results from their formation during the period between grape crushing and fortification. The anthocyanin−pyruvic acid adducts mv−py and mv-coum−py started to increase, reaching their maximum concentration value around 60 days, then started to decrease after 100 days, although more slowly for mv-coum−py than mv−py (Figure 5B). For mv-ac−py the maximum level was reached near 100 days and started thereafter to decrease (Figure 5B). Despite the great difference between their concentrations, the initial formation of anthocyanin−pyruvic acid adducts followed the degradation of anthocyanidin monoglucosides. As expected, the three major anthocyanidin 3-glucosides started to decrease following first order kinetics. The levels of anthocyanins were not significant after 200

Table 3. Concentration of Malvidin 3-Glucoside (mv), Malvidin 3-Acetylglucoside (mv-ac), Malvidin 3-Coumaroylglucoside (mv-coum), Malvidin−Pyruvic Acid (mv−py), Malvidin 3-Acetylglucoside−Pyruvic Acid (mv-ac−py), and Malvidin 3-Coumaroylglucoside−Pyruvic Acid (mv-coum−py) in 10 Commercial Port Wines (Young and after 2 years of Bottle Aging)[a]

	mv		mv-ac		mv-coum		mv−py		mv-ac−py		mv-coum−py	
wine	6 months	2 years	6 months	2 years	6 months	2 years	6 months	2 years	6 months	2 years	6 months	2 years
1	30.9	1.5	3.4	nd	4.8	nd	14.2	12.9	7.0	1.6	10.5	6.5
2	30.0	0.7	3.5	nd	4.3	nd	15.4	13.1	7.5	0.2	10.8	8.1
3	29.6	0.5	3.6	nd	4.6	nd	14.9	12.2	7.1	1.3	10.1	6.4
4	28.3	0.6	3.7	nd	4.6	nd	14.9	12.7	6.8	2.5	9.9	6.3
5	32.3	0.6	3.5	nd	4.6	nd	14.3	13.7	7.0	0.9	8.9	6.4
6	32.6	0.4	3.4	nd	4.7	nd	14.1	11.1	7.2	1.5	10.0	5.1
7	32.8	0.2	3.5	nd	4.8	nd	14.9	11.3	7.3	0.9	10.6	6.8
8	31.0	0.3	3.3	nd	4.8	nd	14.3	12.2	7.2	0.7	10.2	7.1
9	30.4	2.8	3.2	nd	3.9	nd	14.6	14.1	7.4	1.0	9.8	6.3
10	10.5	0.7	2.2	nd	0.9	nd	9.5	7.0	2.1	1.5	5.2	4.8

[a] Values are the average of triplicate assays ($p < 0.05$). Concentrations of anthocyanins and pyruvic acid adducts are expressed in mg of mv per L and mg of mv−py per L, respectively; nd: not detected.

days for mv and after 120 days for its acylated forms. Besides the reaction with pyruvic acid, others probably more important involving anthocyanin polymerization are likely to occur in wine and are the main factor responsible for their decrease. Nevertheless, very little is known about these degradation mechanisms. Reactions involving anthocyanins and catechins are particularly relevant and may occur directly or involve small molecules such as acetaldehyde and glyoxylic acid (*5*, *11*, *29*).

Pigment Evolution During In-Bottle Aging. Several commercial Port wines were analyzed after 6 months (when bottled) and after two years of aging regarding their pigment content (Table 3). As expected, only traces of malvidin 3-glucoside were detected but all the other anthocyanidin monoglucosides had disappeared. Surprisingly, after two years of bottle aging the levels of mv−py decreased only by 9−18%. Comparing the evolution between the pyruvic adducts in-bottle, the losses of mv-ac−py were higher than the losses of mv−py and mv-coum−py. The losses of mv−py were much lower than that observed in the wines stored in oak barrels, which decreased around 70% during the same period of time. This behavior was also observed for mv-coum−py and mv-ac. The results obtained point to the much higher stability of anthocyanin-derived pigments when well protected from any air contact comparatively to the stability of the original anthocyanidin monoglucosides. During bottle aging, wines develop in a reducing environment and the oxidation−reduction potential decreases regularly until it reaches a minimum value that prevents oxidation reactions.

CONCLUSION

The decrease in concentration of the three major anthocyanidin monoglucosides (mv, mv-ac, and mv-coum) and the anthocyanin−pyruvic acid adducts (mv−py, mv-ac−py and mv-coum−py) during three years of aging followed first order kinetics in all the wines studied. The pyruvic acid adducts were found to be very resistant to wine aging when compared to the original grape anthocyanins. The results of both anthocyanins and pyruvic acid adducts show that acylation on the sugar moiety of all the pigments decreased their stability in wine. After wine spirit addition, it was shown that the levels of mv−py and its acylated forms increased importantly before starting to decrease around 100 days. The three major anthocyanins (which are precursors of the newly formed pigments) decreased almost totally during the same period. An initial increase of anthocyanin−pyruvic acid adduct concentrations was concurrent with the degradation of anthocyanidin monoglucosides, although in a different range of concentrations.

These studies performed in wines are very complex due to their high chemical complexity, and the assessment of all factors involved in the formation of new pigments is very difficult. Anthocyanin−pyruvic acid adducts are more abundant in Port wines than in red table wines, as seen from previous analysis in our laboratories (data not shown) and as reported by other authors (*20*). The characteristic chemical and physical properties of Port wine could be at the origin of their higher levels of newly formed pigments. First, when wine spirit is added in order to stop fermentation, the pyruvic acid concentration is expected to be higher than when the fermentation is allowed to go to dryness. Effectively, the pyruvic acid excreted by the yeast at the beginning of the fermentation is further used in the yeast metabolism (*30*). Beside this, Port wines present slightly higher pH values than red table wines, which is an important factor that influences anthocyanin−pyruvic acid adduct formation. Additionally, the higher content of ethanol, which is known to be a good solvent for polyphenols, increases the pigment solubility and can probably favor the formation of new pigments. Finally, another important aspect is the chemical composition of the wine spirit used, which represents about 20% of the final Port wine volume. It is known that wine spirit has a high level of aldehyde compounds, especially acetaldehyde, which favors the reaction between anthocyanins and tannins. Nevertheless, its real contribution is practically unknown.

Further studies are thus needed regarding the influence of some of these factors in the evolution and stabilization of Port wine color.

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