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Spectral Features and Stability of Oligomeric Pyranoanthocyanin-flavanol Pigments Isolated from Red Wines

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The spectral characteristics and stability of three red wine pigments (pyranoMv-catechin, pyranoMv-epicatechin, and pyranoMv-dimer B3) toward pH variation and bisulfite bleaching, as well as their color stability and degradation during storage, have been studied. Unlike the absorbance spectra of most wine pigments, the intensity of which is more increased in more acidic conditions, oligomeric pyranoMv-flavanols have maximum absorbance at wine pH around 3.6, up to 30−50% higher than that in pH 1.0. This particular hyperchromic effect shown in mildly acidic solutions suggests the presence of intramolecular copigmentation in the molecule of pyranoanthocyanin-flavanols, giving rise to higher molar extinction coefficients around wine pH and contributing to the overall wine color. The most probable conformations were determined by computer-assisted model building and molecular mechanics. Besides exceptional stability toward pH variations, pyranoanthocyanin-flavanols were also shown to be entirely resistant to bleaching by sulfur dioxide. During a 6 month storage period, pyranoanthocyanin-flavanols were much more stable against degradation than the anthocyanin with the following order: pyranoMv-catechin > pyranoMv-dimer B3 > pyranoMv-epicatechin > carboxy-pyranoMv ≫ Mv. Kinetic decomposition monitored by high-performance liquid chromatography−diode array detection−mass spectrometry revealed the formation of a new pigment (pyranone-Mv structure) and the cleavage of the interflavanic linkage of procyanidin dimer in the solutions containing carboxy-pyranoMv and pyranoMv-dimer B3, respectively. Despite some degree of decline of these oligomeric pyranoanthocyanins, their color intensity was surprisingly enhanced, and their color stability greatly improved throughout the entire storage period, thus contributing significantly as long-lived orange-red pigments to the maintenance of aged wine color.

KEYWORDS: Pyranoanthocyanin-flavanols; oligomeric pigments; spectral characteristics; intramolecular copigmentation; conformation; storage stability; red wine

INTRODUCTION

The color evolution of red wines during aging is usually attributed to the progressive formation of new pigments resulting from interactions between anthocyanins and other compounds, especially flavan-3-ols such as catechins and procyanidins (condensed tannins) (*1*). In aged red wine, only small amounts of original anthocyanins that are extracted from grape skins during vinification and fermentation can be detected, even though the wine color is largely maintained (*2*, *3*). Together with the anthocyanins, pigmented polymers and pyranoanthocyanins are two types of pigments believed to contribute to the color of red wines. The former is a very heterogeneous group of biomacromolecules involved in the condensation reactions of anthocyanins with other grape-derived polyphenols such as tannins through the formation of an interflavan bond or an ethyl bridge (*1*, *4*, *5*). On the other hand, pyranoanthocyanins result from the cycloaddition reaction of anthocyanins with another molecule giving rise to an additional pyran ring between C-4 and the hydroxyl group at C-5 of the anthocyanidin moiety. Many compounds present in wines corresponding to secondary metabolites of fermentation such as pyruvic acid (*6*), vinyl-phenol (*7*), acetaldehyde (*8*), acetoacetate (*9*), and cinnamic acid derivatives (*10*) can react with anthocyanins, giving rise to different pyranoanthocyanins.

The importance of the formation of pyranoanthocyanins to the brick-red color of the more aged wines is well established. All of these pigments have been shown to have different chromatic characteristics than original anthocyanins, representing a change toward yellow-orange, exactly what happens during the maturation of wine, and constitute one important family of new pigments

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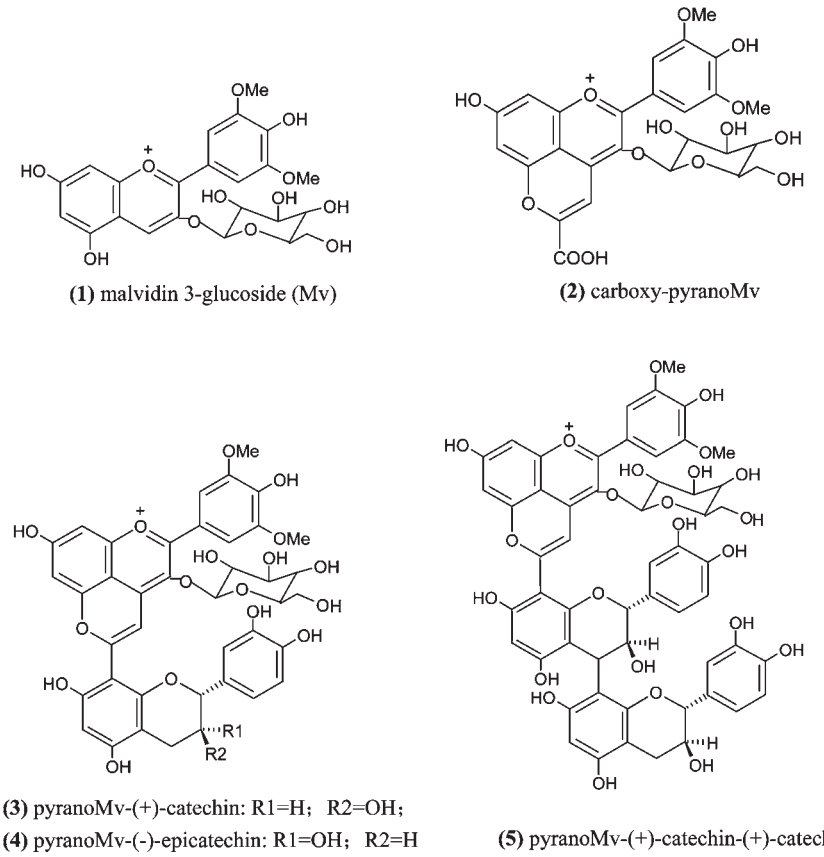

Figure 1. Chemical structures of Mv, carboxy-pyranoMv, and oligomeric pyranoMv-flavanols [(+)-catechin, (−)-epicatechin, and (+)-catechin-(+)-catechin (dimer B3)].

that stabilize and, thus, contribute significantly to the overall expression of the color of wine. The color of pyranoanthocyanins such as vitisin A is more stable toward the increase of pH and bleaching by bisulfite than that of the anthocyanins because of structural features. Wine solutions containing pyranoanthocyanin-flavanol pigments were shown to have a more stable color against changes in pH and SO_2 bleaching than anthocyanins and ethyl-linked anthocyanin-flavanol adducts (*11*, *12*). However, this apparently increased color stability of the pigments has not been studied in detail for the isolated oligomeric compounds. More recently, these oligomeric pyranoanthocyanins bearing different flavanols (catechin, epicatechin, or procyanidin dimers) were identified in Port wines and fully characterized and quantified (*13*−*15*) (**Figure 1**).

Interactions between anthocyanins and flavanols (tannins) are believed to first result in the formation of anthocyanin-ethyl-flavanol adducts during winemaking, which have been shown to be generated rapidly and early in fermentation and likely to consume much of the anthocyanins extracted from the grapes. Although these pigments are more stable toward pH-dependent hydration and bisulfite bleaching than the parent anthocyanins (*16*), phenomena explained by steric hindrance preventing the attack by nucleophiles, they are temporally short-lived and degrade quickly with time to low concentrations in red wines (*17*−*19*). However, the loss of unstable ethyl-linked anthocyanin-flavanol pigments is linked to the formation of reactive intermediates that may go on to form vinyl-linked anthocyanin-flavanol pigments (pyranoanthocyanin-flavanols), although detected only in trace quantities (*18*).

The present work deals with the spectral characteristics and color stability with respect to pH and bisulfite bleaching of isolated oligomeric pyranoanthocyanins bearing different flavanol monomers and dimers in comparison with the parent anthocyanins and major low molecular pyranoanthocyanins (carboxy-pyranoanthocyanins) as well as their storage stability in winelike model solutions.

MATERIALS AND METHODS

Isolation of Oligomeric Pyranoanthocyanin-flavanol Pigments. Oligomeric pyranomalvidin 3-glucoside compounds (**3**−**5** in **Figure 1**) were prepared and purified from a 3 year old Port red wine by combination of column chromatography techniques using polyamide resin, Toyopearl gel, and reversed-phase C18 as described previously (*13*, *15*).

Isolation of Malvidin 3-Glucoside (Mv). Grape skins (*Vitis vinifera*) were subjected to extraction with a solution of 50% aqueous ethanol (pH 1.5) for 1 day at room temperature. The grape skin anthocyanin extract was isolated by Toyopearl gel column chromatography, and individual malvidin 3-*O*-glucoside was purified by semipreparative high-performance liquid chromatography (HPLC) according to the procedure described elsewhere (*20*).

Preparation and Isolation of Carboxy-pyranoMv. The formation of carboxy-pyranoMv was achieved through reaction of Mv with pyruvic acid (molar ratio pyruvic acid/anthocyanin of 50:1) in water (pH 2.6, 35 °C) during 5 days. The extract obtained was purified by Toyopearl gel column chromatography and the carboxy-pyranoMv fraction eluted with water/ethanol 20% (v/v) and further purified by semipreparative HPLC (*21*).

Influence of pH on the Color. The spectral changes of all pigments isolated and purified were characterized. For the pH assay, solutions of each pigment (in the concentrations between 0.01 and 0.02 mM) were prepared in a winelike model solution in 18% aqueous ethanol at different pH values in a range between 1.0 and 11.0 adjusted with HCl or NaOH. Spectral absorbance curves were recorded for all of these solutions from 360 to 750 nm with a 1 nm sampling interval, using a 10 mm × 10 mm cell in a UV-3101 Shimadzu spectrophotometer.

The pH values were measured using a WTW pH 320 pH meter (Weilheim, Germany) with a CRISON 5209 combined glass electrode of 3 mm diameter (Barcelona, Spain). The pH meter was standardized with

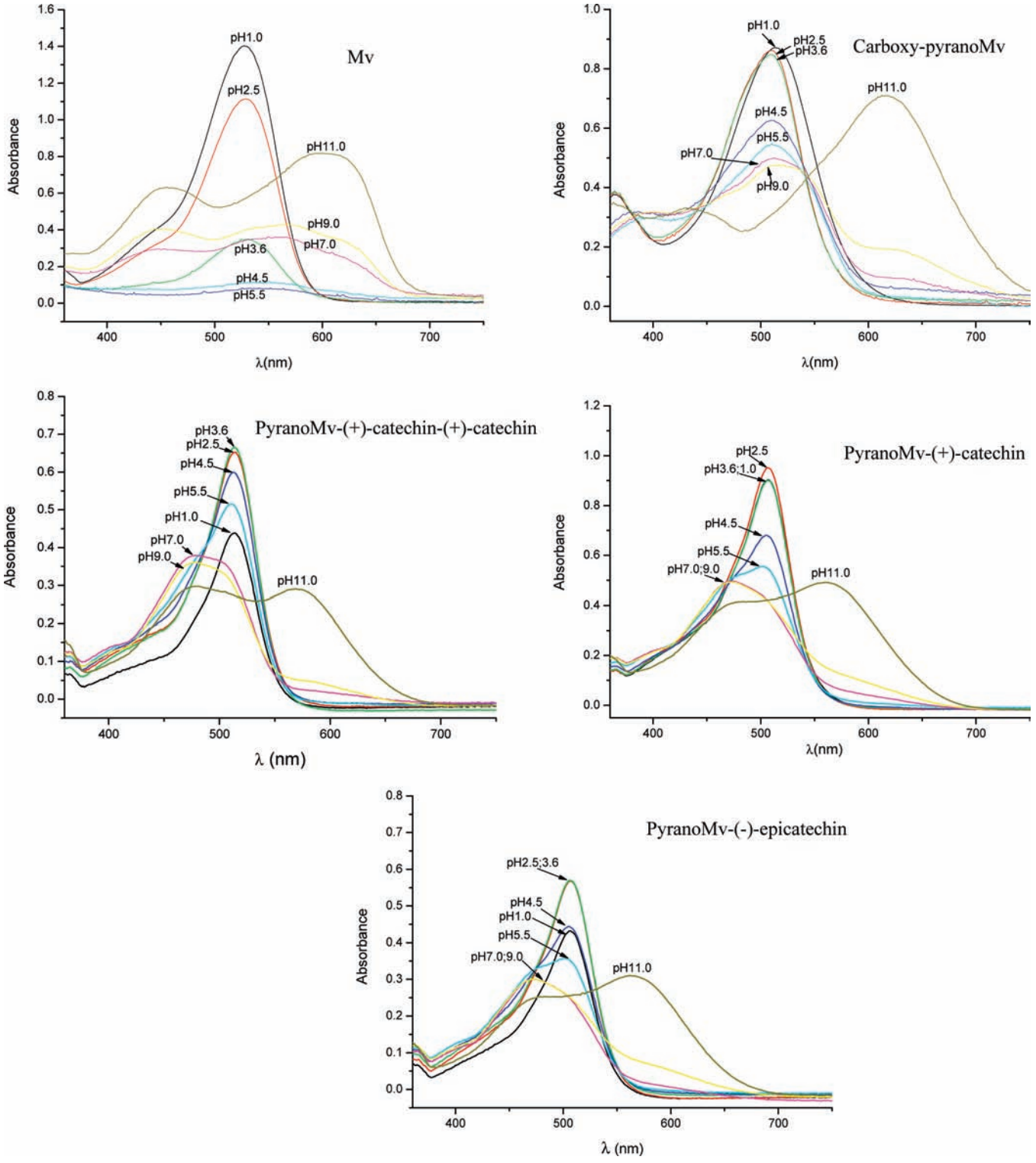

Figure 2. Absorption spectra of Mv, carboxy-pyranoMv, and oligomeric pyranoMv-flavanols at different pH values (1.0−11.0).

pH 1 and pH 4 buffer solutions for pH values below 2.5 and pH 4 and pH 7 buffer solutions for pH values above 2.5.

Conformational Analysis. Conformational analysis was performed with the MM3 (*22*, *23*) force field considering the bond stretch, bond angle, dihedral angle, improper torsion, torsion stretch, bend−bend interactions, van der Waals, and hydrogen bond and electrostatics bond dipoles energy terms. All flexible dihedrals were labeled with convenient step sizes in Cache (*24*), and the conformational space was explored with conflex (*25*, *26*) using edge flip, corner flap, and dihedral rotation.

Influence of Bisulfite Bleaching on the Color. To study the bleaching by SO_2, solutions of each pigment at pH 3.6 were prepared (except for the solution of Mv, which was prepared at pH 2.5), and different aliquots of an aqueous solution of sodium bisulfite (5 mg mL^{-1}) were added to this solution to achieve SO_2 concentrations in the range between 0 and 250 ppm. Spectral absorbance curves were recorded for all of these solutions from 360 to 750 nm with a 1 nm sampling interval, using a 10 mm × 10 mm cell in a UV-3101 Shimadzu spectrophotometer. The bleaching constant (K_{SO_2}) was calculated from the slope of the graphic of color intensity (at the λ_{max} of each pigment) as a function of the concentration of SO_2.

Molar Extinction Coefficient (ε). The molar extinction coefficient (ε) was determined using solutions of all pigments with different concentrations in a range between 0.0025 and 0.02 mM in a Port winelike medium

in 18% aqueous ethanol at pH 1.0 and 3.6, respectively. The coefficient was calculated from the slope of the graphic of color intensity (at the λ_{max} of each pigment) as a function of the concentration of the pigment. Spectral absorbance curves were recorded for all of these solutions from 360 to 750 nm with a 1 nm sampling interval, using a 10 mm × 10 mm cell in a UV-3101 Shimadzu spectrophotometer.

Stability of the Pigments in Aqueous Solution. Solutions of all of the pigments were prepared in a winelike model solution in 18% aqueous ethanol at pH 3.6 and kept at room temperature in closed vials in the dark for 6 months. Changes were monitored by UV−vis spectrophotometry, HPLC−diode array detection (DAD), and LC-DAD/MS analysis.

HPLC Analysis Conditions. All of the pigments were analyzed by HPLC (model 96 Knauer, Berlin, Germany) on a 250 mm × 4.6 mm i.d. reversed-phase C18 column (Merck, Darmstadt, Germany), and detection was carried out using a DAD (Knauer, K-2800). An HPLC pump Knauer K-1001 was used together with a Kauer K-3800 autosampler. The solvents were A, H_2O/HCOOH (9:1), and B, CH_3CN/H_2O/HCOOH (8:1:1). The gradient consisted of 15−35% B over 70 min, 35−80% B over 5 min, and then isocratic for 10 min at a flow rate of 1.0 mL/min.

LC-MS Conditions. A Hewlett-Packard 1100 Series liquid chromatography, equipped with an AQUA (Phenomenex, Torrance, CA) reversed-phase column (150 mm × 4.6 mm, 5 μm, C18) thermostatted at 35 °C was used. Solvents were (A) aqueous 0.1% trifluoroacetic acid and (B) acetonitrile, using the gradient previously reported (*15*). The mass detector was a Finnigan LCQ (Finnigan Corp., San Jose, CA), equipped with an atmospheric pressure ionization source, using an electrospray ionization (ESI) interface. The capillary voltage was 3 V, and the capillary temperature was 190 °C. Spectra were recorded in positive ion mode between m/z 120 and 1500. MS-MS spectra were registered using relative collision energies of 30 and 60 V.

RESULTS AND DISCUSSION

The spectral properties of oligomeric pyranoanthocyanin-flavanol pigments, namely, the color intensity and variation in relation to pH and bisulfite discoloration, were characterized, and their color stability on storage as well as their degradative stability and possible chemical changes during storage at wine pH were also studied. In all cases, similar assays were carried out with solutions of Mv and that of structurally simple pyranoanthocyanin, carboxy-pyranoMv.

Table 1. Maximal Wavelength and Molar Extinction Coefficients (ε)[a] of Mv, Carboxy-pyranoMv, and PyranoMv-flavanols Determined in Model Wine Solutions at pH 3.6 and pH 1.0

		ε (mol^{-1} dm^3 cm^{-1})		ratio of ε
pigment	λ_{max}	pH 1.0	pH 3.6	$100 \times \varepsilon_{pH3.6}/\varepsilon_{pH1.0}$
Mv (**1**)	528	14519	3613	24.88
carboxy-pyranoMv (**2**)	514	8987	8918	99.23
pyranoMv-(+)-catechin (**3**)	506	14823	14855	100.22
pyranoMv-(−)-epicatechin (**4**)	506	6085	8025	131.88
pyranoMv-(+)-catechin-(+)-catechin (**5**)	514	4511	6845	151.73

[a] In model wine solution at the λ_{max} of each pigment.

Spectral Characteristics. The chemical structure of the pigment itself is one of the main factors affecting the color of anthocyanins bearing the same chromophore moiety. **Figure 2** shows the visible spectrum variation in aqueous solutions of malvidin-based pyranoanthocyanins at different pH values in the range 1.0−11.0. The λ_{max} obtained at a mildly acidic model wine solution for the pyranoanthocyanin molecules (**2**−**5**) is similar and presents a hypsochromic shift when compared to the parent compound Mv, as shown in **Table 1**. Moreover, all of the pyranoMv-flavanols also had a considerably stable color against the increase of pH in mildly acidic aqueous solution (∼2−6), as compared with Mv, which is almost colorless due to the displacement of the hydration equilibrium toward the hemiacetal and chalcone forms (**Figure 2**). The higher resistance to discoloration of pyranoMv-flavanol pigments (as well as carboxy-pyranoMv) should be attributed to their structural properties of pyranoanthocyanins, characterized by the substitution at C-4 of the anthocyanin molecule, thus protecting the colored forms against the nucleophilic water attack, which is known to occur at positions 2 and 4 of the chromophore. With the increase of pH up to the alkaline region between 7.0 and 9.0, all of the pyranoMv-flavanols showed a similar hypsochromic shift in λ_{max} toward 470 nm. Further pH changes until 11.0 yielded a maximum of absorption around 570 nm, which may be explained by the equilibrium displacement toward the formation of the quinonoidal base forms (**Figure 2**).

Additionally, the pH variation affects appreciably and differently the color intensities of the individual pyranoMv-flavanols in mildly acidic aqueous solution and especially at wine pH (**Figure 3**). Contrary to what is observed for Mv **1** and its pyruvic adduct **2** in **Figure 3** and other anthocyanins and derivatives reported in the literature (*16*, *27*), the absorptivities of which are highest in more acidic solution (pH 1.0) and decrease toward neutrality, pyranoMv-falvanols (**3**−**5**) showed a significant hyperchromic effect at pH values above 1.0, until maxima were achieved at pH 2.5−3.6. An increase in the relative absorptivity to pH 1.0 (i.e., existence of a hyperchromic effect) was found to occur in all solutions of the three pyranoMv-flavanols, especially for pyranoMv-dimer B3, which presented the highest hyperchromic effect (over 50%) in a wider range of slightly acidic to neutral solution media (**Figure 3**).

The particular behavior of the pyranoanthocyanin bearing different flavanols around wine pH indicates the existence of the

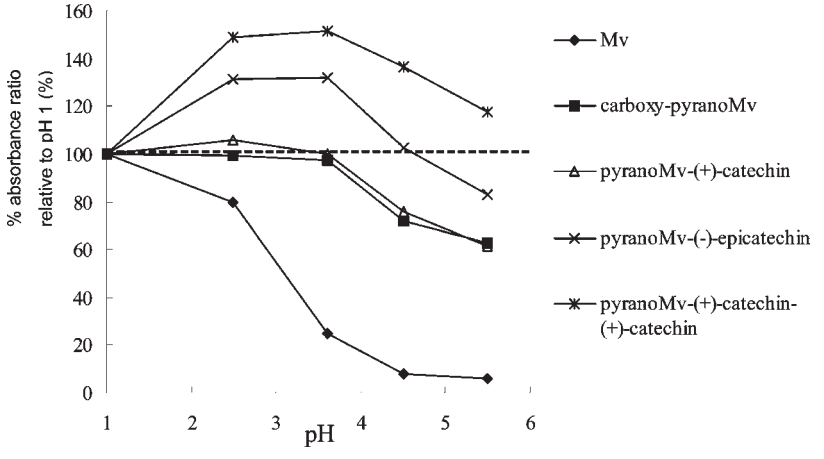

Figure 3. Absorptivity changes at the λ_{max} of each pigment in mildly acidic aqueous solutions (pH ∼ 2.5−5.5) relative to pH 1.

formation of either inter- or intramolecular associations of pyranoMv-flavanols. This fact might be related to the particular structure presented by these molecules. In the present case, where pigments **2**−**5** studied share the same chromophore (pyranoMv), a clear effect of color change results from the different substitution pattern at position 10 of these molecules. The three pyranoMv-flavanols possess chemical structures constituted by the planar aromatic moieties (pyranic or catechol rings of different flavanol residues), which can tightly stack onto and interact with the π-system of the planar pyrylium core of pyranoanthocyanins, a phenomenon of intramolecular copigmentation (*28*, *29*).

Conformational Analysis. To attempt an interpretation of these results, a study of molecular aspects was performed using computer-assisted model building and molecular mechanics (MM3) (*22*−*26*) for all three pyranoanthocyanins bearing different flavanol monomers and dimers (**Figure 4**). Indeed, it is interesting to find that the preferred conformations corresponding to the least energy conformer (the most stable one) have demonstrated a relatively closed "cage" structure, thus favoring the overlap and interactions between the planar pyrylium chromophore of pyranoanthocyanins and the pyranic or catechol rings of the flavanol groups. The most probable conformer structure of pyranoMv-dimer B3 shows an intramolecular π−π stacking arrangement between the central chromophore and the second catechin moiety, the distance between the two aromatic planes being 3.249 Å (**Figure 4**). Moreover, the thermodynamic and kinetic data of natural anthocyanins, especially those with planar aromatic substituents such as aromatic acyl or flavonoid group, already confirmed the existence of strong intramolecular copigmentation effects that confer stability to the colored forms of the molecules by blocking the formation of hydrated species (*29*, *30*). The pyranoMv-epicatechin showed a much higher hyperchromicity (about 32%) between pH 2.5 and 3.6 than its isomeric pyranoMv-catechin (**Figure 3**), indicating stronger intramolecular associations between the coplanar moieties of the former molecule. In fact, the conformational analysis shows that pyranoMv-catechin presents a less closed structure than pyranoMv-epicatechin. Similarly, the highest hyperchromic effect found in the solution of pyranoMv-dimer B3 throughout all of the acidic to neutral pH range should therefore be attributed to the facile and strong stacking interactions between both planes of the procyanidin dimer B3 and the central pyranoflavylium chromophore, as shown in **Figure 4**. Also, this intramolecular association was pronounced at pH 3.6, as clearly shown in **Figure 3**.

The explanation for these observations can also be found in the copigmentation reaction of anthocyanins with polyphenols. It has been quantitatively demonstrated that the intermolecular copigment effect reaches its highest point within the pH range of 3−5, depending on the pigment−copigment pair (*31*, *32*). In the case of Mv copigmented by polyphenols, maximal color hyperchromicity was found close to pH 3.6, in agreement with the results obtained herein for intramolecular copigmentation involving a flavylium moiety and different flavanol residues. On the other hand, the different capacity of hyperchromicity among the three pyranoMv molecules bearing different flavanols showed that the type and structure of the flavanol moieties, covalently bound to the pyranoanthocyanin chromophore, had a great effect on the color expression of pyranoanthocyanin pigments of oligomeric nature, as shown in **Table 1**, in both color variation (maximum visible wavelength, λ_{max}) and color intensity (molar extinction coefficient, ε). The λ_{max} obtained for the most complex pigment pyranoMv-dimer B3 presents a significant bathochromic shift (8 nm) when compared to the pyranoMv-flavanol monomer, also suggesting the existence of intramolecular copigmentation

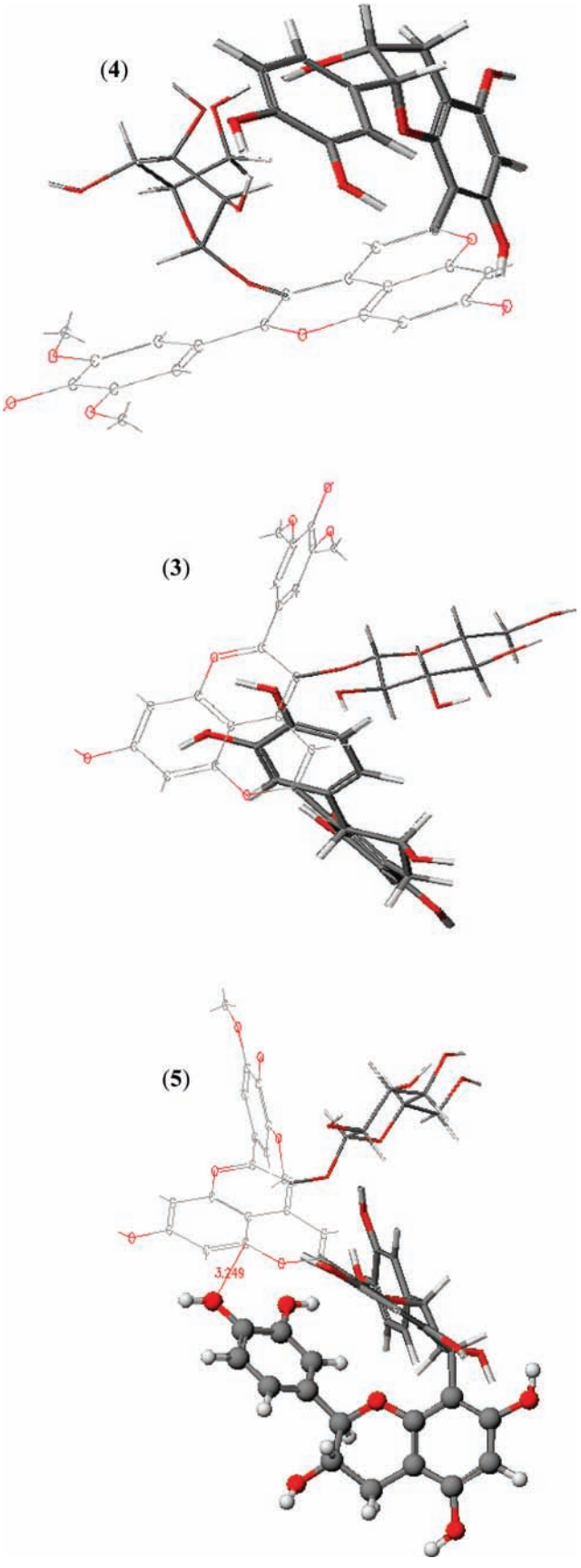

Figure 4. Preferred conformations of pyranoMv-(−)-epicatechin (**4**), pyranoMv-(+)-catechin (**3**), and pyranoMv-(+)-catechin-(+)-catechin (**5**), determined by computer-assisted model building (MacroModel) and molecular mechanics (MM3).

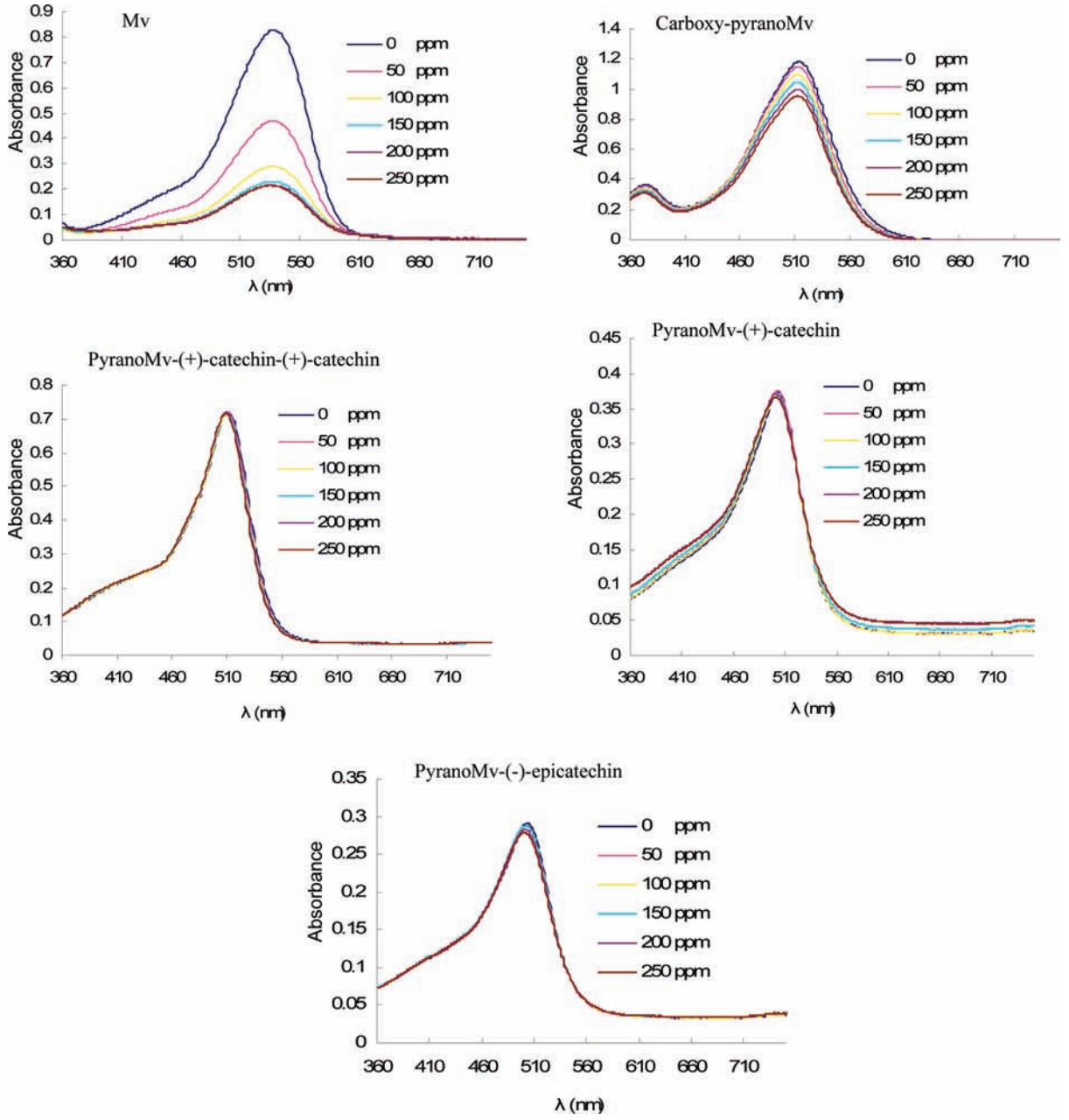

Figure 5. Effects of the addition of increasing concentrations of bisulfite on the visible spectra of pH 3.6 model wine solutions of oligomeric pyranoMv-flavanols and carboxy-pyranoMv, in comparison with Mv.

between the procyanidin dimer B3 residue and the flavylium chromophore. At very acidic pH 1, the ε values of all pyranoanthocyanins were inferior to the one of Mv, except pyranoMv-cat, which has the same magnitude as Mv. However, because of the noticeable hyperchromism around wine pH (3.6), there is a pronounced increase in the ε values for the other two pyranoMv-flavanol pigments going beyond the one of Mv and close to the one of carboxy-pyranoMv (**Table 1**). Therefore, it is important to note that these oligomeric pyranoanthocyanin-flavanol pigments can contribute greatly at wine pH to maintain the color of aged red wines.

Bisulfite Bleaching Stability. The effect of SO_2 on the color stability of the wine model solutions of pyranoMv-flavanols pigments was also studied (**Figure 5**). The three oligomeric pigments, as well as carboxy-pyranoMv, showed a greater resistance to bleaching by addition of bisulfite in the range of 0−250 ppm, when compared with Mv. This improved stability against bisulfite bleaching should be attributed to the C-4 substituted structure of pyranoanthocyanin pigments, which prevent the nucleophilic attack of bisulfite in that position, as described above for water. The bleaching constant determined by plotting the color intensity at the λ_{max} of each pigment as the function of SO_2 concentration (**Table 2**) showed that oligomeric pyranoanthocyanins are entirely resistant to bleaching by sulfur dioxide. The maximum absorbance of the model solutions of pyranoMv-flavanols at pH 3.6 remained unchanged even 2 days after the addition of up to 250 ppm bisulfite. The carboxy-pyranoMv was partly bleached by sulfur dioxide, as compared with pyranoMv-flavanols, but was much more resistant than Mv, which was almost completely bleached under similar conditions (**Figure 5**).

Stability in Aqueous Solution during Storage. The degradative stabilities of all pyranoanthocyanin pigments and malvidin glucoside were studied at the pH value more typical for red wines (pH 3.6). **Figure 6** shows the relative changes in the concentration

of each pigment monitored along the 6 months of storage. All of the pyranoanthocyanins were found to be much more stable than the original malvidin glucoside. Indeed, the differences between the degradation of pigments were already very pronounced during the first 2 months of storage, when only 20% of the original content of Mv was detected in the solution, in agreement with the previous result reported (*16*), while 50−65% of the remaining pyranoanthocyanins was still present after the same period of storage. Along storage, pyranoMv-catechin and pyranoMv-catechin-catechin presented a higher stability after half a year of storage, especially the former, remaining 30% of the original amount, while only 10% carboxy-pyranoMv is still present. Overall, along the storage period, the oligomeric pyranoanthocyanins exhibited some degree of stability higher than structurally simple pyranoanthocyanins (carboxy-pyranoMv), as shown by the degradation rate constants of each pigment (**Table 3**).

The disappearance of pigments was modeled through first-order kinetics for each solution. The degradation reaction rate constants (k) were determined by calculating the slopes after linear regression from the plot of ln[C] against time as well as the half-life time ($t_{1/2}$) that corresponds to the time required for a 50% reduction of the initial concentration of each pigment (**Table 3**). The good correlation coefficients calculated from the fitting of kinetics suggested that the pigments degradation in model wine solution followed apparent first-order kinetics satisfactorily. The degradation rate constant of Mv (k), which is in strong agreement with recent reports (*33*), was much higher than that of all pyranoMv derivatives, especially oligomeric pyranoMv-flavanol pigments, indicating a higher stability of these latter pigments. Interestingly, this is quite different for ethyl-linked oligomeric Mv-flavanol pigments, which showed a lower stability than the anthocyanins itself (*16*). Thus, from a structural point of view, it could be concluded that the C-4 substitution of the flavylium ring (pyranoanthocyanin nature) seems to first confer superior stability of pyranoMv-flavanols, which is further enhanced by the presence of additional flavanol groups (oligomeric nature). Nevertheless, the increased stability did not parallel with the increased complexity of the molecular structure, as observed for the k values of pyranoMv-flavanol dimer and pyranoMv-flavanol monomer.

In the model wine solutions, the time required for a 50% ($t_{1/2}$) reduction of the initial content of oligomeric pyranoanthocyanin pigments is approximately three or four times longer than that for Mv and, in general, also longer than that for carboxy-pyranoMv up to 66% (except for pyranoMv-epicatechin, which has a slightly higher $t_{1/2}$) under the same conditions (**Table 3**). The longevities of these pyranoMv-flavanols are in agreement with their stability described above. Obviously, many protective cofactors existing in wines could prevent or limit the degradation process of these pigments, whose decline, therefore, would take place much slower than it does in model solutions, thus occurring as long-lived orange-red pigments in practical grape wines.

The degradation process and possible chemical changes of each pigment in model solutions was monitored by HPLC-DAD-MS. The degradation reactions of malvidin glucoside in the solution are known to occur through cleavage of the heterocycle, with the formation of products corresponding to different moieties of the anthocyanin rings. Among those, the formation of syringic acid, identified on the basis of its UV spectrum (λ_{max} 276 nm), retention time, and mass spectrum (molecular ion at m/z 199), was confirmed in the chromatograms observed (data no shown). As demonstrated in the literature (*16*, *33*, *34*), it resulted from the B ring released after degradation of malvidin glucoside.

The decrease of carboxy-pyranoMv along the storage gave rise to the appearance of a new compound in the chromatogram showing a λ_{max} at 373 nm (peak O in **Figure 7**). The presence of this peak was observed at day 15 of the assay, and its area increased simultaneously with the decrease of carboxy-pyranoMv

Table 2. Bleaching Constants by SO_2 (K_{SO_2}) of Mv, Carboxy-pyranoMv, and Oligomeric PyranoMv-flavanols in Model Wine Solutions

pigment	K_{SO_2} ($\times 10^3$)
Mv (**1**)	2.9
carboxy-pyranoMv (**2**)	0.94
pyranoMv-(+)-catechin (**3**)	0.038
pyranoMv-(−)-epicatechin (**4**)	0.046
pyranoMv-(+)-catechin-(+)-catechin (**5**)	0.032

Table 3. Degradation Rate and Half-Life Time ($t_{1/2}$) of Mv, Carboxy-pyranoMv, and Oligomeric PyranoMv-flavanols in Wine Model Solutions of pH 3.6

pigment	$k \times 10^{-3}$ (days^{-1})	R^2	$t_{1/2}$ (days)
Mv (**1**)	48.6	0.947	26.3
carboxy-pyranoMv (**2**)	12.7	0.992	61.4
pyranoMv-(+)-catechin (**3**)	6.6	0.993	102.2
pyranoMv-(−)-epicatechin (**4**)	10.1	0.996	66.7
pyranoMv-(+)-catechin-(+)-catechin (**5**)	8.0	0.980	84.8

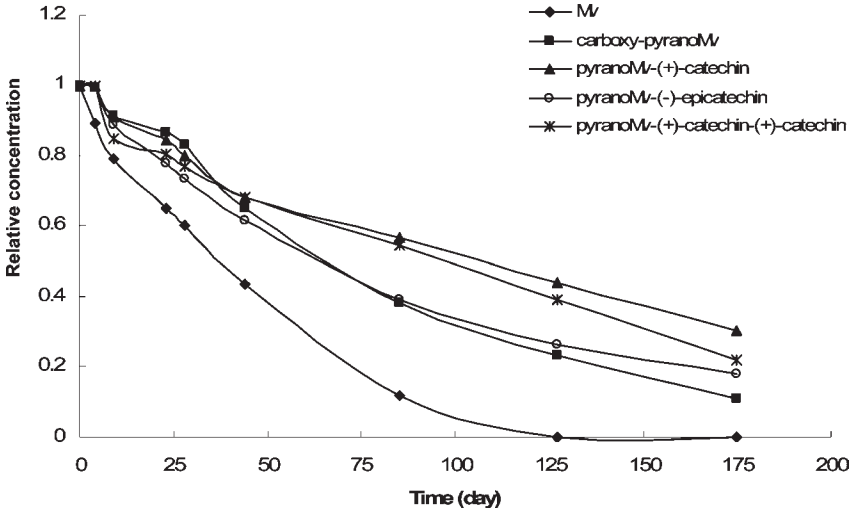

Figure 6. Relative changes in the concentrations of Mv, carboxy-pyranoMv, and oligomeric pyranoMv-flavanols in model wine solutions at pH 3.6 during the storage period.

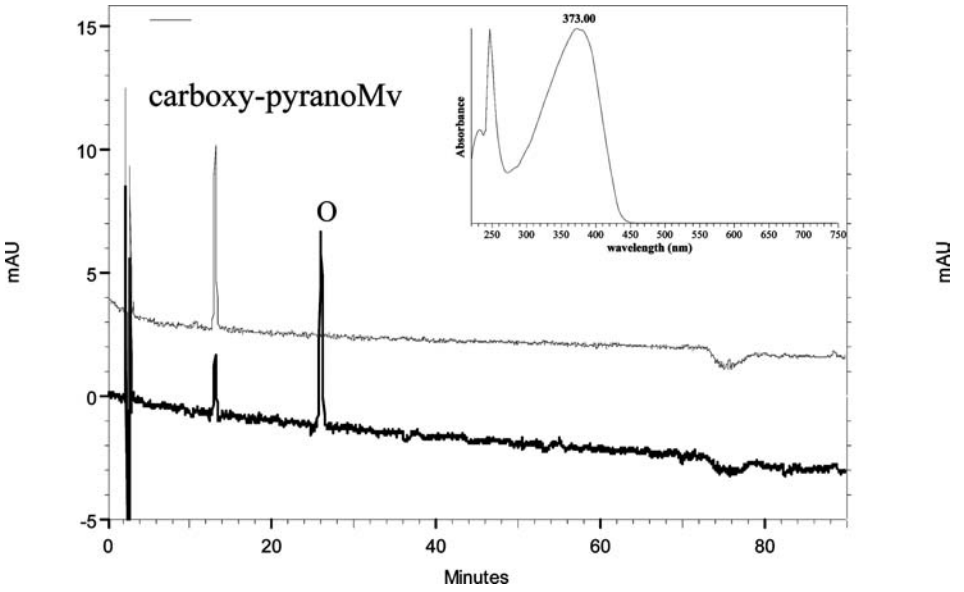

Figure 7. HPLC chromatogram recorded at 510 (above) and 373 nm (below) of the aqueous solution of carboxy-pyranoMv at the end of storage period. Peak O is a newly formed pigment with its UV−vis spectrum (insert).

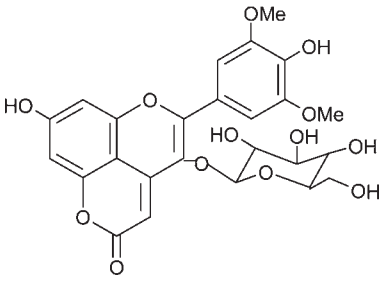

Figure 8. Suggested structure of peak O in **Figure 7**.

until the maximum was achieved at the end of storage, when carboxy-pyranoMv had almost disappeared (**Figure 7**). The mass spectrum of peak O showed a quasimolecular ion signal at m/z 533 (positive ion mode), a MS^2 fragment at m/z 371 ($[M - 162]^+$), corresponding to the loss of a glucose moiety, and MS^3 fragments at m/z 343, 311, and 283. The UV−vis spectra, mass data, and retention time are in agreement with the new family of neutral pyranone-anthocyanin derivatives, named oxovitisins, recently reported in old aged Port wines (*35*). This new compound (**Figure 8**) could be generated gradually through the oxidative degradation reactions of carboxy-pyranoMv and accumulated increasingly along storage.

As compared to the solutions of carboxy-pyranoMv, hardly any formation of major compounds was observed in the solutions of pyranoanthocyanin-flavanols, whose degradative stability was higher than that of the former throughout the storage period, as summarized before in **Figure 6** and **Table 3**. Among these oligomeric pigments, the degradation of pyranoMv-dimer B3 was accompanied by the formation of pigment pyranoMv-catechin (peak 3 in **Figure 9**), indicating that a cleavage of the pigment must thus occur at the interflavanic linkage of the procyanidin dimer unit, which proceeded quickly at the first 3 weeks of storage as shown in **Figure 6**. Along with the breakdown of the pigment during storage, the resulting compound pyranoMv-catechin was already the dominating one in the solution of pyranoMv-dimer B3 in the last 3 months of the assay (**Figure 9**). While in aqueous solutions of pyranoMv-flavanol monomers (catechin or epicatechin), besides syringic acid derived from the degradation of the pyranoanthocyanin moiety of the pigment, only some very tiny amounts of colored products showing λ_{max} at 499 or 514 nm as well as colorless products showing λ_{max} around 310 nm were detected with the time of storage,. However, no further discussion can be made about the identities of the tiny products derived from the degradation of these pyranoMv-flavanol pigments, since no good mass spectra could be obtained in the conditions of ionization used. It is also predictable that the oxidative degradation of these compounds yields other compounds and polymerized structures that are not eluted in the HPLC chromatograms.

Despite the decline of these oligomeric pyranoanthocyanins in model wine solution, the color of their solutions is better maintained during the 6 month storage period (**Figure 10**). All three pyranoMv-flavanols of oligomeric nature studied had much higher color stability than small molecular carboxy-pyranoMv and parent anthocyanin Mv, which lost its color quickly, being imperceptible after 55 days. Carboxy-pyranoMv retained 65% of its color at the end of storage. The general increased color stability of pyranoanthocyanins during storage is in agreement with the results of other studies (*11*, *36*). Surprisingly, the oligomeric pyranoanthocyanins bearing different flavanols showed greatly increased color throughout the entire storage period. The color intensity at the end of 2 months was already 135 and 115% of the original intensity for pyranoMv-dimer B3 (**5**) and pyranoMv-flavanol monomer (**3** and **4**), respectively, and maintained well with a slight increase until the end of storage. This enhancement in color intensity and improvement in color stability may be explained by the appearance of other compounds that, on one hand, may present a similar chromophore groups but that have a slightly higher molar absorptivity, and on other hand, these new compounds may act as copigments and complex with the pyranoanthocyanins promoting a hyperchromic effect. The intermolecular copigmentation between the pyranoanthocyanic chromophore and the flavanols or phenolic acids in wine conditions is well-known to give rise to an increase of absorptivity (hyperchromic effect) and the subsequent protection against hydration (*34*, *36*), thus leading to the increased color intensity and color stability of oligomeric pyranoanthocyanins throughout the storage period.

To our knowledge, there are no previous reports on the color characteristics of oligomeric pyranoanthocyanins bearing different flavanols and their stability during storage. One of the most

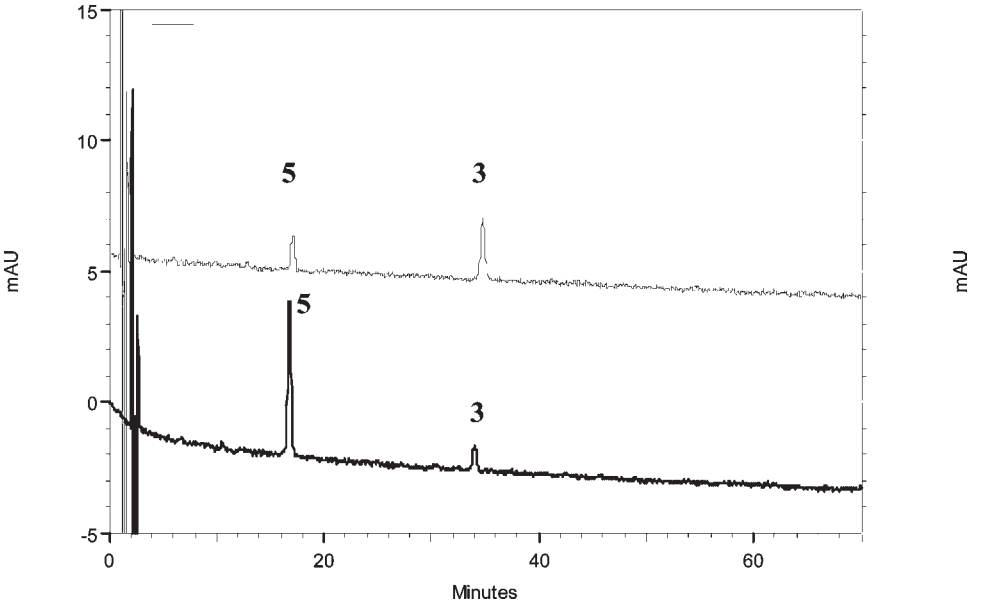

Figure 9. HPLC chromatogram recorded at 510 nm of the aqueous solution of pyranoMv-procyanidin dimer B3 3 months before (below) and at (above) the end of the storage period; **5**, pyranoMv-dimer B3; and **3**, pyranoMv-catechin.

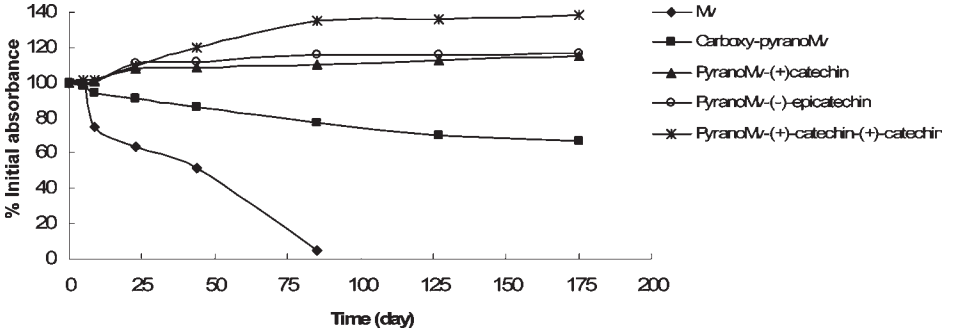

Figure 10. Relative changes in the absorbances (at the λ_{max} of each pigment) of the aqueous solutions containing Mv, carboxy-pyranoMv, and oligomeric pyranoMv-flavanols in model wine solutions at pH 3.6 during the storage period.

interesting findings in the present study was their particular spectral behavior around wine pH, which showed a dramatic hyperchromic effect (30−50%) at mildly acidic aqueous solution, indicating intramolecular associations between the different coplanar moieties of the pyranoMv-flavanols. Besides their higher stability (longevities) against decomposition than parent anthocyanin and carboxy-pyranoMv, as determined by kinetic rate constants, the aqueous solutions of oligomeric pyranoanthocyanins showed surprisingly enhanced color intensity and greatly improved color stability throughout the entire storage period. Altogether, from a structural point of view at the studied pigments, which possess both the C-4 substitution of the flavylium ring (pyranoanthocyanin nature) and the presence of additional flavanol groups (oligomeric nature), it can be suggested that these long-lived orange-red pigments could contribute greatly at wine pH to the maintenance of aged wine color.

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