UV–Visible Spectroscopic Investigation of the 8,8-Methylmethine Catechin-malvidin 3-Glucoside Pigments in Aqueous Solution: Structural Transformations and Molecular Complexation with Chlorogenic Acid

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The physicochemical properties of 8,8-methylmethine catechin-malvidin 3-*O*-glucoside isomers, commonly referred to as catechin-ethyl-malvidin 3-*O*-glucoside, have been studied in aqueous solutions and compared with those of the parent anthocyanin (malvidin 3-*O*-glucoside). The hydration and acidity constants (pK_h and pK_a) of the catechin-ethyl-malvidin 3-*O*-glucoside pigments and malvidin 3-*O*-glucoside were determined by UV–visible spectroscopic measurements. The ethyl-linked catechin-malvidin 3-*O*-glucoside pigments present higher stability toward hydration than the parent anthocyanin. The high resistance of these ethyl-linked pigments toward the hydration is related to the self-association that offers optimal protection from the nucleophilic attack of water. Moreover, the ethyl link may confer to the molecule enough flexibility to undergo intramolecular interaction, further protecting it from hydration and bisulfite discoloration. In the wine pH range (3.2–4.0), due to the low pK_a and high pK_h values, the ethyl-linked pigments are present as colored forms (flavylium cation and quinonoid bases).

KEYWORDS: Malvidin 3-*O*-glucoside; ethyl-linked pigments; hydration and transfer proton constants; intramolecular interaction and self-association

INTRODUCTION

Anthocyanins extracted from grape skins during winemaking are responsible for the color of red wines. The red color of anthocyanins is contributed by their cationic flavylium form (AH^+), which is predominant only in acidic media (pH <2). The flavylium cation is in equilibrium with various other forms through proton transfer, hydration, and tautomerization reactions. At wine pH, the nucleophilic attack of water at position 2 of the pyrylium nucleus leads to the colorless hemiketal form (AOH), which is itself partly converted into chalcones (C). AOH and C correspond to the thermodynamic products of anthocyanin structural transformations in aqueous solutions (*1*). Concurrently, fast deprotonation of the flavylium cation leads to the kinetic products, the neutral quinonoid bases (A) in mildly acidic solutions, which further deprotonate into the anionic quinonoid base (A^-) in neutral to mildly alkaline solutions (**Figure 1**). The overall hydration equilibrium relating the flavylium cation and the mixture of hemiketal and chalcone forms can be characterized by the thermodynamic constant K_h. Similarly, the overall proton-transfer equilibrium relating the flavylium ion and the mixture of tautomeric neutral quinonoid bases is characterized by the thermodynamic constant K_a. The pK_h and pK_a values allow one to specify the distribution of a given anthocyanin into its different forms in acidic to neutral aqueous solutions. Native anthocyanins have pK_h values in the range of 2–3. Hence, in red wine (pH 3.2–4.0), anthocyanins are expected to be present largely as colorless hydrated forms (>70%). Thus, some color-stabilizing mechanisms must take place.

Such mechanisms may initially occur in grapes. Due to the planarity and polarizability of their chromophores, the colored forms of anthocyanins are prone to developing π-stacking interactions (a combination of van der Waals interactions and the hydrophobic effect) with themselves (self-association), with their own aromatic acyl residues (intramolecular copigmentation), or with other phenols (intermolecular copigmentation). Being largely selective of the colored forms, copigmentation tends to shift the hydration equilibrium toward the flavylium cation, thus resulting in color enhancement (hyperchromic effect). Metal complexation, especially with Al^{3+}, can also produce a similar effect (*2–4*).

Another color-stabilizing mechanism, especially important in wine, is the conversion of native anthocyanins into more stable

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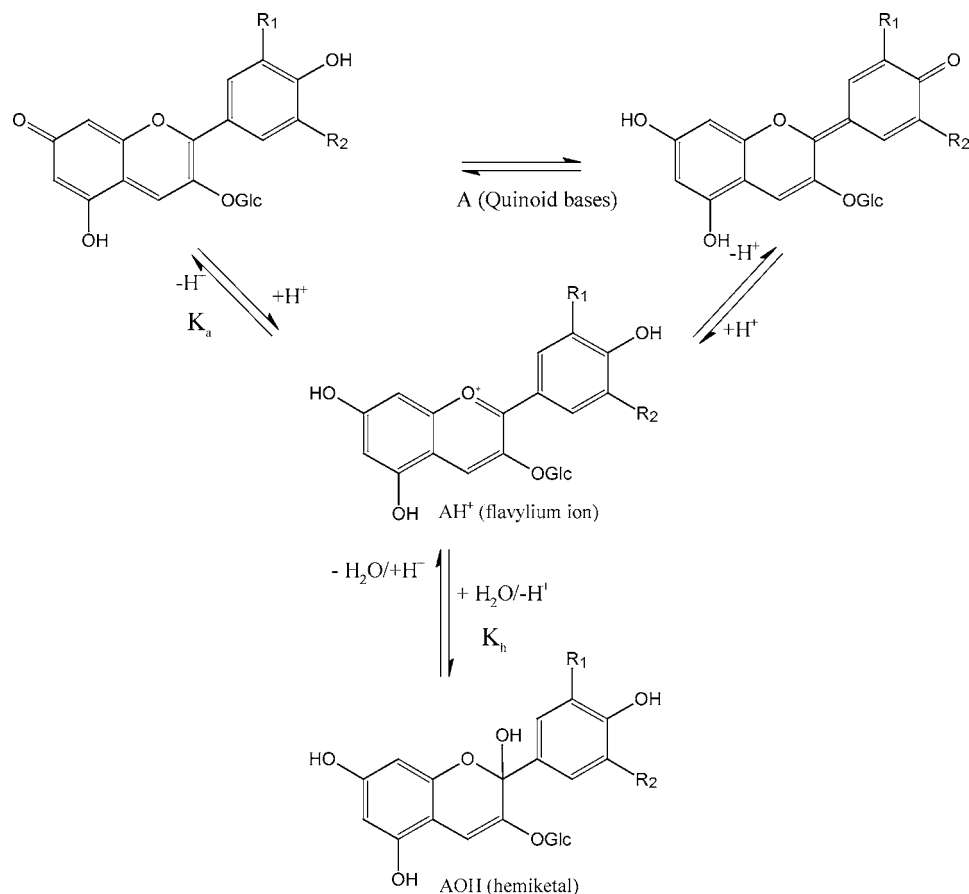§ UMR A 408 INRA-Université d'Avignon.

Figure 1. Structural transformations of anthocyanin in slightly acidic aqueous solutions.

pigments through various reactions involving other wine components such as tannins and yeast metabolites. Several pathways have been proposed to explain the formation of more stable pigments in wines. The first mechanism is a direct reaction between anthocyanins and flavan-3-ols (F), leading to tannin–anthocyanin (F–A$^+$) and anthocyanin–flavanol (A$^+$–F) adducts (5–8).

Another mechanism consists of the acetaldehyde-mediated coupling between anthocyanins and flavanols. In the case of malvidin 3-*O*-glucoside (oenin, the main anthocyanin in grape) and (epi)catechin, two major violet pigments are formed that have been proposed to be two epimers both having an 8,8-methylmethine bridge (usually referred to as an ethyl bridge) (**Figure 2**). Recently, the corresponding structures have been fully identified by NMR (9). These pigments are characterized by a higher wavelength of maximal visible absorption (540 nm) with respect to the native anthocyanin (10–12). The two epimeric pigments have been also characterized by thiolysis and HPLC/ESI-MS (13).

The methylmethine flavanol–anthocyanin adducts, commonly referred to as F-ethyl-A, were found in model solutions as well as in red wine (14–16). Moreover, a methyl-methine dimalvidin 3-*O*-glucoside pigment formed in a winelike model solution has been also recently characterized by NMR (17) and finally detected in red wine by HPLC-MS analysis. Pigments of the F-ethyl-A type have been reported to be more resistant to hydration and sulfite bleaching than their precursor anthocyanins (10, 18, 19).

The purpose of this work was to study the structural transformations of the 8,8-methylmethine catechin-Mv3glc epimers in aqueous solutions and to determine their hydration and acidity constants. Copigmentation was also investigated
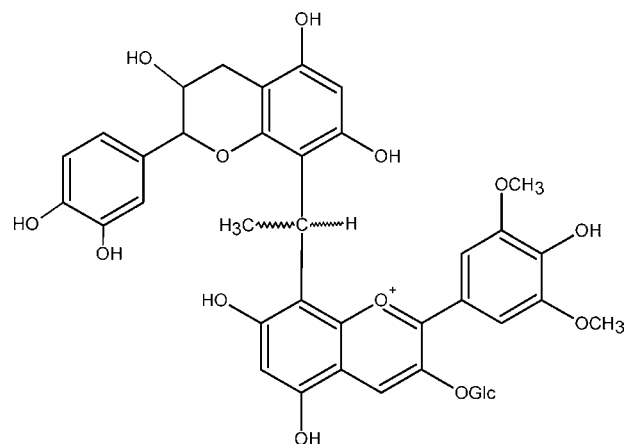

Figure 2. Structure of 8,8-methylmethine catechin-malvidin 3-glucoside pigments.

using chlorogenic acid as copigment. The results were compared with those of malvidin 3-glucoside to evaluate their relative contributions to wine color.

MATERIALS AND METHODS

Materials. Malvidin 3-*O*-glucoside (Mv3glc or oenin) was either isolated from a grape anthocyanin extract as described by Vidal et al. (20) for the preparation of the two methylmethine catechin-malvidin 3-*O*-glucoside epimers or purchased from Extrasynthèse (Genay, France) for the physicochemical studies. (+)-Catechin was purchased from Sigma. Acetaldehyde and trifluoroacetic acid were purchased from Sigma (St. Louis, MO). Acetonitrile and methanol were from Merck (Darmstadt, Germany). Formic and acetic acids were purchased from Prolabo (Fontenay s/Bois, France), and chlorogenic acid was from Sigma.

Synthesis of the 8,8-Methylmethine Catechin-malvidin 3-O-Glucoside Pigments. Mv3glc (3.3 g/L, 6.8 mM) and (+)-catechin (2.1 g/L, 7.3 mM) were dissolved in a 5% acetic acid solution (pH 2.4) in the presence of acetaldehyde. The sample was kept in darkness at 30 °C for 48 h (*10*). The formation of two pigments, referred to as I (minor pigment) and II (major pigment), was observed, and both isomers were isolated by semipreparative HPLC as described below.

Isolation and Purification of the Two Isomeric Pigments, I and II. The two isomers (I and II) were isolated and purified by semipreparative HPLC. Semipreparative HPLC was performed using a Gilson system including a 305 master and a 306 slave pump, an 807 manometric module, an 811B dynamic mixer, a 7725 Rheodyne valve injector, and an 875 UV−visible Jasco detector at 280 nm. The column was a reversed-phase Lichrospher 100 RP18 (5 µm packing, 125 × 22 mm i.d.). Elution conditions were as follows: 10 mL/min flow rate; room temperature; solvent A, water/trifluoroacetic acid (100:0.1, v/v); solvent B, acetonitrile/water/trifluoroacetic acid (80:20:0.1, v/v/v); elution from 30 to 34% B in 16 min, from 34 to 90% in 2 min, and then an isocratic step for 2 min with 90% B followed by washing and re-equilibration of the column. The isolated compounds were concentrated to dryness under a vacuum and freeze-dried prior to use for further experiments. The chemical nature and purity of the isomers were checked by HPLC-DAD and MS.

HPLC-DAD. HPLC-DAD analyses were performed using a Waters 2690 system equipped with an autosampler system, a Waters 996 photodiode array detector, and Millenium 32 chromatography manager software (Milford, MA). Separation was achieved on a Lichrospher 100-RP18 column (5 µm packing, 250 × 22 mm i.d.) (Merck) protected with a guard column of the same material (Merck). The elution conditions were as follows: 0.250 mL/min flow rate; oven temperature, 30 °C; solvent A, water/formic acid (95:5, v/v); solvent B, acetonitrile/solvent A (80:20 v/v). Elution began isocratically with 2% B for 7 min and was continued with linear gradients from 2 to 20% B in 15 min, from 20 to 30% B in 8 min, from 30 to 40% B in 10 min, from 40 to 50% B in 5 min, and from 50 to 80% B in 5 min, followed by washing and re-equilibration of the column.

HPLC−Electrospray Ionization (ESI)/MS. MS experiments and fragmentation analyses were performed on a ThermoFinnigan LCQ Advantage (San Jose, CA) mass spectrometer (MS) equipped with an ESI source and an ion trap mass analyzer, which were controlled by the LCA navigator software. The mass spectrometer was operated in the positive mode in the range of m/z 250−2000 and under the following conditions: source voltage, 4.5 kV; capillary voltage, 23.5 V; capillary temperature, 250 °C; collision energy for fragmentation, 25% for MS^2 and 30% for MS^3. Chromatographic separation was achieved using a Waters 2690 system equipped with an autosampler system, a Waters 996 photodiode array detector, and Millenium 32 chromatography manager software. The column was a Lichrospher 100-RP18 (5 µm packing, 250 × 22 mm i.d.) protected with a guard column of the same material (Merck). The elution conditions were as follows: 0.250 mL/min flow rate; oven temperature, 30 °C; solvent A, water/formic acid (98:2, v/v); solvent B, acetonitrile/solvent A (80:20 v/v). Elution began isocratically with 2% B for 7 min and continued with linear gradients from 2 to 20% B in 15 min, from 20 to 30% B in 8 min, from 30 to 40% B in 10 min, from 40 to 50% B in 5 min, and from 50 to 80% B in 5 min, followed by washing and re-equilibration of the column.

Absorption Spectra. Spectra were recorded with a Hewlett-Packard diode array spectrophotometer fitted with a quartz cell (optical path length = 1 cm) equipped with a stirring magnet. A constant temperature in the cell was obtained by use of a water-thermostated bath. Temperature was measured with a Comark thermocouple and was kept at 25 °C.

Kinetic Measurements. Pigment solutions were prepared in a pH 2.5 buffer (0.1 M citric acid; 0.1 M $Na_2PO_4 \cdot 12H_2O$; ionic strength adjusted to 1 M by NaCl). The solutions were kept for 2 h to reach complete hydration equilibrium. One milliliter of the equilibrated pigment solution was magnetically stirred in the spectrometer cell. One milliliter of $Na_2PO_4 \cdot 12H_2O$ solutions of concentrations ranging from 0.01 to 0.1 M was quickly added to the pigment solution. The final concentration of the two F-ethyl-A isomers and Mv3glc was 8×10^{-5} M. The molecular mass of the F-ethyl-A pigment taken into account

for concentration calculations was 922 g mol^{-1}, assuming that the pigments were isolated as flavylium trifluoroacetates.

The visible absorbance at the wavelength of visible absorption maximum was immediately recorded every 0.5 s until hydration equilibrium (1−2 min; the possible formation of low concentrations of *trans*-chalcone via slow *cis−trans* isomerization was not considered). The final pH value at equilibrium was carefully measured and ranged from 2.7 to 4.7. The spectrometer software automatically calculated the first-order apparent rate constant of the hydration reaction (k).

Thermodynamic Measurements. After the kinetic measurements, the pigment solutions at different pH values were kept for 2 h at 25 °C to ensure complete hydration equilibrium including the *cis−trans* isomerization of the chalcone and their UV−visible spectra recorded. The values of the $K_h + K_a$ parameter were deduced from the plot of the absorbance at 535 nm as a function of pH.

Copigmentation Experiments. The set of F-ethyl-A solutions was collected within the pH range of 2−5 and mixed for each isomer. The pH of the combined solutions was then adjusted to 3.5 with a more acidic pigment solution at the same concentration (8×10^{-5} M). For each pigment, the resulting solution was divided into two parts: chlorogenic acid was added to one part so as to obtain a final copigment concentration of 8×10^{-3} M. The pH was again adjusted to 3.5 by adding a few drops of 1 M NaOH (without significant dilution). The two solutions (with and without chlorogenic acid) were mixed in different proportions so as to obtain a range of solutions with copigment to pigment molar ratios ranging from 0 to 100. The UV−visible spectrum of each equilibrated pigment solutions was recorded.

Sulfite Bleaching Experiments. Pigment solutions (I, II, catechin-Mv3glc, and Mv3Glc) were prepared in HCl solutions, and the pH was adjusted to 2.5, 3.5, and 4.5, respectively, by the addition of various amounts of 0.01 M NaOH. The final concentration of pigment in these solutions was 8×10^{-5} M. To 1 mL of each pigment solution was added 15 µL of aqueous sodium bisulfite (0.2 and 200 g/L). The solutions were held for 20 min. Then, the UV−vis spectra were recorded.

Mathematical Data Treatments. These were carried out on a Pentium 120 PC using Scientist software (Micro Math, Salt Lake City, UT).

RESULTS AND DISCUSSION

The pigments studied were synthesized according to a procedure described under Materials and Methods. The minor pigment is referred to as pigment I and the major pigment as pigment II. In accordance with reported literature (*9, 19*), pigment II corresponds to the *S* configuration and pigment I corresponds to the *R* configuration.

The molar absorption coefficients of pigments I and II (at $\lambda_{max} = 535$ nm) were determined in 0.1 M citric acid adjusted to pH 1 with concentrated HCl: $\epsilon_{pigmentI} = 12120$ M^{-1} cm^{-1}, $\epsilon_{pigmentII} = 10870$ M^{-1} cm^{-1}. These values were much lower than that of Mv3glc at 515 nm ($\epsilon_{Mv3glc} = 23050$ M^{-1} cm^{-1}) under the same conditions. Escribano-Bailon and co-workers (*10*) obtained similar results using different conditions.

Thermodynamic Measurements. Figure 3 shows the pH dependence of the UV−visible spectrum of Mv3glc. In the pH range of 1−4, an almost complete disappearance of the visible band (93% absorbance loss at the wavelength of maximum absorption) without bathochromic effect was observed, which indicates the occurrence of a pure hydration process. By contrast, in the pH range of 1−3, only a slight decrease of the visible band (10−20% absorbance loss at the wavelength of maximum absorption) without bathochromic effect was observed for pigments I and II. At pH >3.7 for pigment I and >3.3 for pigment II, the decay of the visible band is associated with a bathochromic effect, which reveals that additional deprotonation of the residual flavylium ion begins to take place. At pH >5.9, an increase in visible absorption around 610 nm becomes
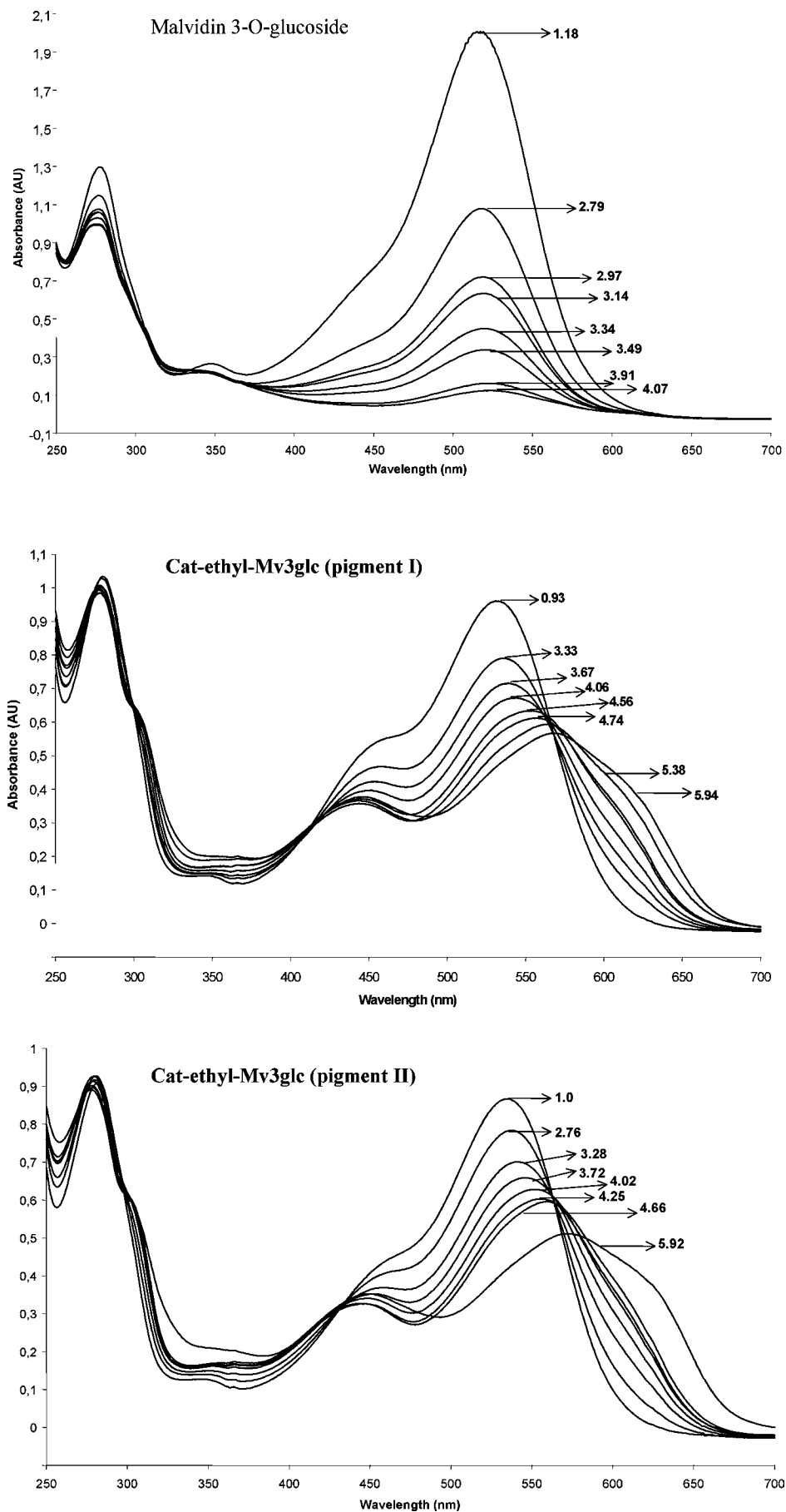
Figure 3. UV–visible spectra of malvidin 3-*O*-glucoside and pigments I and II at different pH values.

Table 1. Hydration and Proton Transfer Constants of Pigments I and II

	pK	pK_h	pK_a[a]	pK_{a2}
malvidin 3-O-glucoside	2.78	2.83	3.78	
pigment I	3.44	4.17	3.53 (3.39)	5.04
pigment II	3.24		3.29 (3.23)	5.38

[a] Values in parentheses were determined from the curve fittings of the whole *A* versus pH curves (eq 3), hydration being neglected (see text).

Table 2. Thermodynamic and Kinetic Parameters for Mv3glc

	pK	pK_h	pK_a	k_1 (min^{-1})	$k_2 \times 10^3$ (M^{-1} min^{-1})
this work	2.78	2.83	3.78	3.3	2.4
ref *1*		2.60	4.25	5.1	2.0

detectable with both pigments, which points to the formation of the anionic quinonoid bases. Moreover, at pH >4.4, the appearance of a shoulder around 310 nm suggests the formation of chalcones in low concentrations.

The value of $K = K_h + K_a$ was deduced from a plot of the visible absorbance (at the flavylium λ_{max}) against pH according to eq 1 (*21*):

$$\frac{A_0}{A_0 - A} = \frac{K_h + K_a}{K_h + K_a(1 - r_A)} + \frac{10^{-pH}}{K_h + K_a(1 - r_A)} \quad (1)$$

In eq 1, A_0 is the absorbance of a strongly acidic solution (pH <1) in which the anthocyanin is under pure flavylium form and *A* is the absorbance at a given slightly acidic pH. Parameter r_A is the ratio of molar absorption coefficients of the same pigment under its quinonoidal form (A) and under its flavylium form (AH$^+$), $r_A = \epsilon_A/\epsilon_{AH+}$.

Plotting $A_0/(A_0 - A)$ versus 10^{-pH} results in a straight line. The K value was deduced from the intercept-to-slope ratio. The corresponding pK value for Mv3glc is 2.78, being significantly lower than those of pigments I and II (3.44 and 3.24, respectively) (**Table 1**). Because malvidin 3-glucoside undergoes pure hydration in the pH range investigated, its pK value must be essentially equal to pK_h. However, for pigments I and II, some contribution of K_a is expected. Hence, the pK_h values must be slightly higher than the pK values. Taken together, these results confirm that pigments I and II are both more resistant to hydration than the parent pigment oenin.

Kinetic Measurements. To calculate the proton transfer thermodynamic constant (K_a), a kinetic study was carried out from pH jump experiments. In the hydration−deprotonation competition, the hemiketal form (AOH) is the thermodynamic product, whereas the quinonoid bases (A) are the kinetic products. When the pH of a fairly acidic equilibrated solution of pigment (flavylium ion as the major form) is quickly increased (pH jump), the mixture relaxes toward equilibrium, corresponding to the new pH value according to a kinetics that is governed by water addition on the flavylium ion. However, the quinonoid base−flavylium ratio is instantaneously raised (fast proton transfer), and the lower flavylium concentration results in an apparently slower hydration process (rate constant *k*). Plotting the *k* value as a function of pH allows one to determine the pK_a value. For native anthocyanins, this method is much more accurate than a direct pK_a determination from equilibrated solutions because the quinonoid bases, in those conditions, are only very minor products as a consequence of the successful competition of hydration. Using the $K_h + K_a$ value determined from the experiments on equilibrated solutions, a plot of $(K_a + K_h + 10^{-pH})/k$ versus 10^{pH} allows one to calculate K_a and k_2 (rate constant of hemiketal dehydration) according to eq 2 (*21*):

$$(K_a + K_h + 10^{-pH})/k = 1/k_2 + K_a 10^{pH}/k_2 \quad (2)$$

From K and K_a, K_h can be calculated as well as the hydration rate constant $k_1 = k_2 K_h$.

In the case of Mv3glc, the plot gave a satisfying straight line in the pH range of 2.8−4.1. The different constants calculated for Mv3glc are given in **Table 2**. The pK_h and pK_a values are 2.83 and 3.78, respectively. Both are slightly different from values reported in the literature (p$K_h = 2.60$, p$K_a = 4.25$) in different conditions (25 °C, ionic strength adjusted to 0.2 M KNO$_3$) (*1*). Unexpectedly, the pK_a values of pigments I and II could not be determined according to this procedure. Deviations from linearity were observed at the limits of the pH range, especially for the least acidic values. A possible reason is that the absorbance amplitudes in the pH jump experiments are typically very small (<0.1, i.e., ~10 times smaller than that of Mv3glc), especially for pigment II, thus preventing the accurate determination of the apparent rate constant of hydration. These observations are in agreement with the high pK_h values estimated for pigments I and II from investigations on equilibrated solutions. This resistance to hydration may reflect self-association, as suggested earlier by Escribano-Bailon et al. (*18*) for a pigment formed by coupling catechin and a synthetic flavylium ion by a methylmethine linkage. To a lesser degree, self-association of Mv3glc could also explain the above-mentioned discrepancies between our results and literature data since a higher pigment concentration was used in our experiments. Moreover, the ionic strength and buffer, which are different from those used by Brouillard et al. (*1*), might also enhance aggregation as found for other polyphenols (*22*). Anyway, the lower pK_a value estimated for oenin in this work is consistent with self-association because electrostatic repulsions between stacked flavylium cations is expected to favor deprotonation.

An alternative approach was attempted to determine the acidity constants of pigments I and II. The kinetics experiments were simply used to evaluate $r_A = \epsilon_A/\epsilon_{AH+}$ by comparing the visible spectrum of the pigments in strongly acidic solutions and the spectrum obtained immediately after pH jump (no significant hydration) to a pH value high enough to ensure complete conversion of the flavylium ion into the quinonoid bases based on a maximal bathochromic shift. These pH values were 4.74 for pigment I and 4.25 for pigment II. The method gave $r_A = 0.74$ for pigment I and $r_A = 0.73$ for pigment II. Using these r_A values, the pK_a values of pigments I and II were calculated from the slope (*a*) and intercept (*b*) of the linear plot using eq 1: $K_a r_A = (b - 1)/a$ (**Table 1**). From the K_a value, $K_h = K - K_a$ was calculated.

The hydration constant (pK_h) is the most relevant measure of the stability of a given anthocyanin in water. The value estimated at 4.17 for pigment I is much higher than that of the parent Mv3glc. The hydration constant for pigment II is too small to be estimated ($K \approx K_a$). Hence, the spectral changes recorded on equilibrated solutions essentially reflect deprotonation of the flavylium ion to give the quinonoid bases. The higher stability of pigment II toward hydration could be due to its adopting a conformation that allows stronger self-association with simultaneous efficient protection of the flavylium nucleus from the surrounding water molecules. Alternatively, intramolecular copigmentation could operate as in acylated anthocyanins (*21, 23*), although it is not clear whether the short methylmethine

bridge can provide sufficient flexibility for the catechin residue to stack onto the pyrylium ring of the anthocyanin residue.

For native anthocyanins, the hydration constant K_h is higher than the proton-transfer constant K_a ($pK_h < pK_a$), so that the colorless forms largely prevail in mildly acidic equilibrated aqueous solutions. The inverse behavior observed with pigments I and II may be due not only to remarkably high pK_h values (thermodynamically unfavorable hydration because of self-aggregation) but also rather low pK_a values (3.53 and 3.29 for pigments I and II, respectively). Once more, these low pK_a values are consistent with self-association because deprotonation is expected to alleviate electrostatic repulsion between flavylium units within aggregates.

Using the pK_h and pK_a values (**Table 1**), it is straightforward to plot distribution diagrams of anthocyanin forms as a function of pH (**Figure 4**). The differences in species distribution between the parent anthocyanin Mv3glc and pigments I and II are particularly striking in the pH range of wine (3.2−4.0). The majority of pigments I and II are actually present as colored forms (flavylium cation + quinonoid bases), whereas the Mv3glc is present mostly as the colorless hemiketal form (65−85%).

As mentioned above, the visible spectra of the two methyl-methine Cat-Mv3glc pigments undergo additional modifications when the pH is raised above 4−5. Theoretical curves assuming two successive proton transfers from the non-covalent dimer $(AH^+)_2$ (eq 3) could actually be fitted to the whole experimental absorbance versus pH curves. Escribano-Bailon et al. (*18*) applied the same data treatment to their methylmethine Cat-flavylium pigment.

$$A = A_0 \frac{1 + B_1 10^{pH} + B_2 10^{2pH}}{1 + 10^{pH-pKa1} + 10^{2pH-pKa1-pKa2}} \qquad (3)$$

In eq 3, parameters B_1 and B_2 are defined as $B_1 = r_A K_{a1}$ and $B_2 = r_A K_{a2}$ with $r_A = \epsilon_A/\epsilon_{AH+}$.

K_{a1} and K_{a2} represent the thermodynamic constants for the conversion of $(AH^+)_2$ into (AH^+,A) and for the conversion of (AH^+,A) into $(A)_2$, respectively. Hydration is neglected.

The curve-fitting procedure yielded pK_{a1} values (3.39 for pigment I and 3.23 for pigment II) in good agreement with those previously determined (**Table 1**), especially for pigment II for which hydration is actually negligible.

The pK_{a2} values were estimated at 5.04 for pigment I and at 5.38 for pigment II. Both values are higher than those estimated for model pigments (devoid of glucose residue on the flavylium nucleus) investigated by Escribano-Bailon et al. (*18*). This suggests that non-covalent dimerization is probably weaker for pigments I and II, maybe due to the steric hindrance and hydrophilic character brought about by the glucose residues.

In contrast to pigments I and II, the Cat-Mv3glc pigment (direct linkage between the flavanol and anthocyanin units) investigated by Salas et al. (*8*) has a pK_h value of 2.6, that is, close to that of the parent anthocyanin Mv3glc. This result shows that the methylmethine bridge is crucial for the development of the color-stabilizing mechanism. On the other hand, the second apparent hydration constant for the methylmethine di-Mv3glc ($pK_{h2} = 4.6$) (*24*) is close to that of pigment II ($pK_h = 4.17$), as expected for two closely related pigments in which the oenin flavylium nucleus is linked via a methylmethine bridge either to a catechin unit or to an oenin hemiketal unit.

Copigmentation. Interactions of anthocyanins with colorless polyphenols (copigmentation) is an important process for color stabilization in flowers, fruits, and wine. Anthocyanin copig-
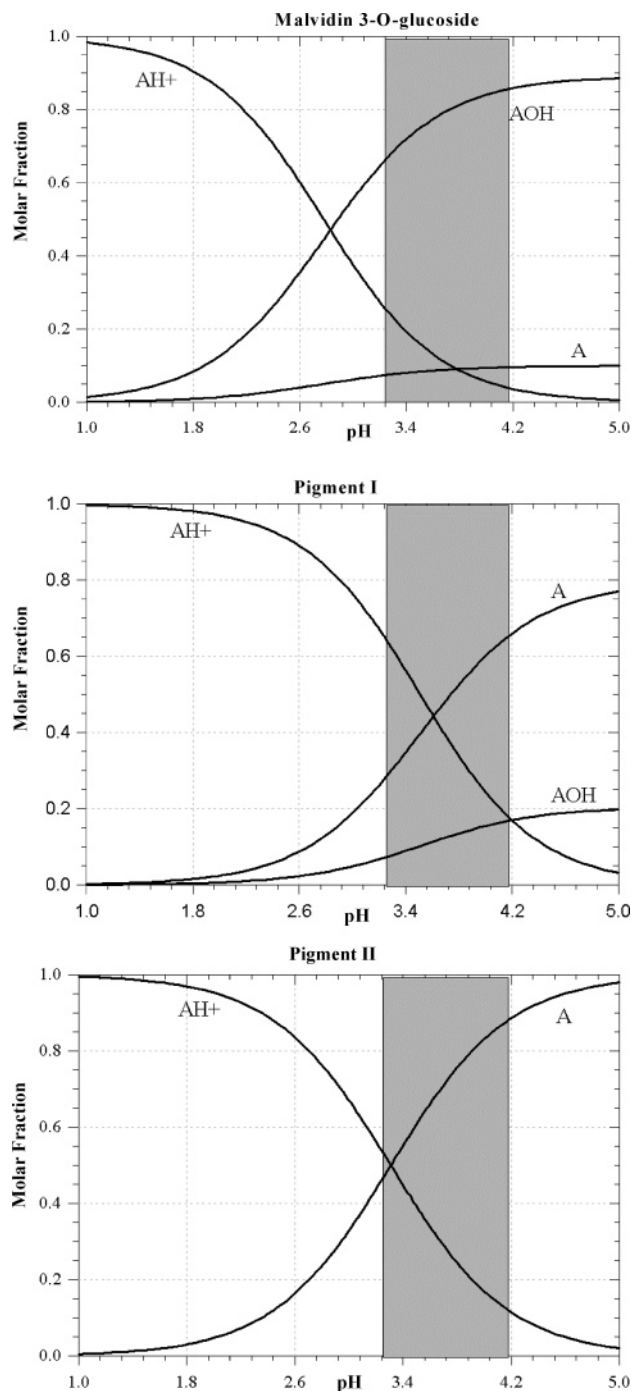

Figure 4. Distribution diagrams for equilibrated solutions of malvidin 3-*O*-glucoside and pigments I and II as a function of pH.

mentation gives more intense and more stable colors than those expressed by free anthocyanin (*3, 25−28*). The hyperchromic effect typical of copigmentation results from a displacement of the hydration equilibrium toward the flavylium form due to its selective interaction with the copigment. On the other hand, the bathochromic shift usually observed could reflect more favorable electrostatic interactions between the pigment and copigment once the former has reached the electronic excited state corresponding to the visible absorption band (*29*).

In this work, copigmentation experiments were carried out with chlorogenic acid (5-caffeoylquinic acid) as copigment at the pH of wine (pH 3.5). **Figure 5** shows the changes in the UV−vis spectrum of each pigment as a function of the chlorogenic acid concentration. The visible spectra of pigments

Physicochemical Properties of Catechin-ethyl-malvidin 3-O-Glucoside Pigments

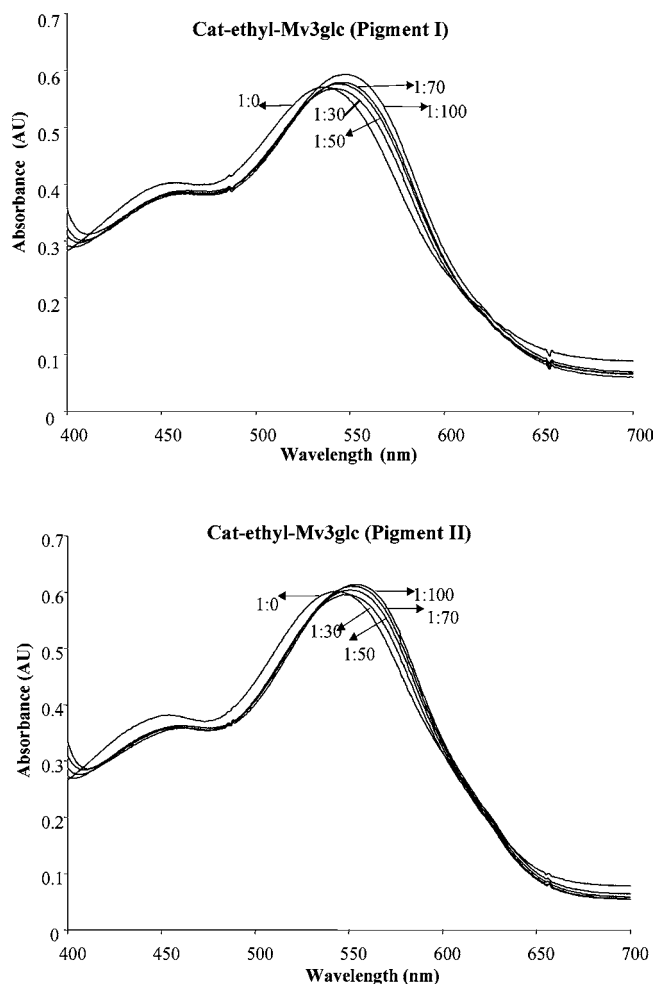*J. Agric. Food Chem.*, Vol. 54, No. 1, 2006 **195**

Figure 5. Visible spectra of pigments I and II after addition of chlorogenic acid for different pigment/copigment molar ratios.

I and II showed weak bathochromic shifts (11 and 10 nm, respectively) that point to an interaction taking place. This interaction may be weak because of unfavorable competition with self-aggregation. Moreover, no hyperchromic effect was observed in agreement with our proposal that hydration is negligible for these pigments. Hence, only very low concentrations of colorless forms are available for conversion into the flavylium ion upon interaction with the copigment. By contrast, the Cat-Mv3glc pigment (direct linkage between the flavanol and anthocyanin units) was recently shown (8) to display more spectacular copigmentation effects in the presence of chlorogenic acid (hyperchromic effect = 171%, bathochromic shift = 14 nm for a pigment/copigment molar ratio of 1/100), as also observed with the parent anthocyanin Mv3glc. The different behaviors between the methylmethine Cat-Mv3glc pigments and the Cat-Mv3glc pigment suggest that the methylmethine bridge provides the proper flexibility to promote intramolecular catechin−flavylium interactions and/or (more probably) self-association, thereby severely limiting the access of water and copigment molecules to the flavylium nucleus.

Bleaching of Pigments. Bleaching is caused by the addition of bisulfite at C-4 of the pyrylium nucleus, thus generating a colorless adduct (30). The effect of bisulfite on the color of the cat-ethyl-Mv3glc solutions was studied at pH 2.5, 3.5, and 4.5 using two concentrations of sodium bisulfite. The more diluted bisulfite solution was used to mimic enological concentration, and the more concentrated one was used to be sure of bisulfite saturation and bleaching completion of the C-4 unsubstituted
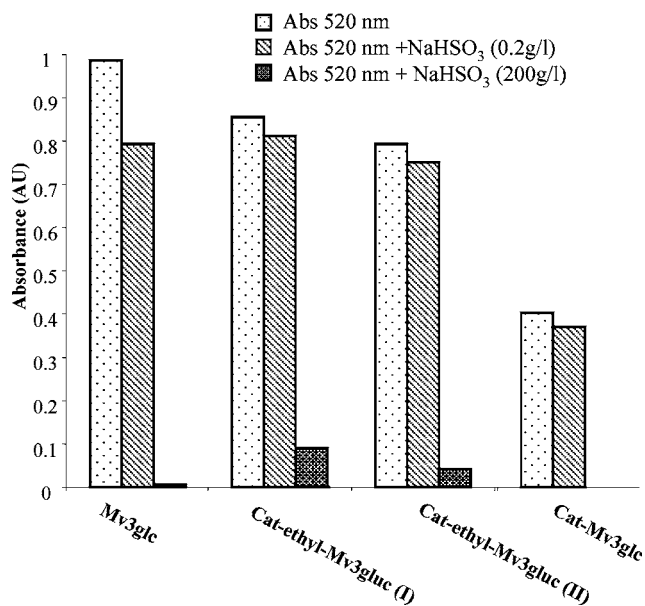

Figure 6. Absorbance values at pH 2.5 for malvidin 3-glucoside, catechin-malvidin 3-glucoside pigment, and pigments I and II in the presence or absence of sodium bisulfite.

pigments. In the case of Mv3glc and catechin-Mv3glc, the bleaching experiment was performed only at pH 2.5, because at higher pH, both catechin-Mv3glc and Mv3glc solutions are already almost colorless. In the same way as copigmentation studies, the effect of bisulfite bleaching was compared between the directly linked catechin-Mv3glc dimer and the methyl-methine-bridged dimers. At pH 2.5, pigments I and II are more resistant to bisulfite-induced discoloration than Mv3glc and catechin-Mv3glc. Unlike catechin-Mv3glc and its parent anthocyanin Mv3glc, the two cat-ethyl-Mv3glc epimers are not totally discolored (5−10% of the initial color is preserved) when treated by the concentrated bisulfite solution (**Figure 6**). At pH 3.5 and 4.5, solutions of pigments I and II were more bleached than at pH 2.5, in agreement with the formation of the more reactive sulfite ion (30).

Overall, the catechin-ethyl group provides more efficient protection to the flavylium chromophore when the attacking nucleophile is water than when it is bisulfite. This difference could reflect the higher nucleophilicity of bisulfite as well as the larger accessibility of the flavylium C-4 center (compared with C-2) within the aggregates.

In conclusion, the proton transfer and hydration constants of the methylmethine catechin-Mv3glc pigments have been determined. Pigment II is totally resistant to water addition, whereas pigment I undergoes little hydration ($pK_h = 4.17$) in comparison to the parent anthocyanin. The color-stabilizing mechanism seems to be linked to the propensity of such pigments for self-aggregation. Because of their low pK_a and high pK_h, ∼50% of these pigments must be present in wine as quinonoid bases, thus contributing to the red-purple hue of aged wine.

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