Color and Stability of Pigments Derived from the Acetaldehyde-Mediated Condensation between Malvidin 3-*O*-Glucoside and (+)-Catechin

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A pigment derived from the acetaldehyde-mediated condensation between (+)-catechin and malvidin 3-*O*-glucoside has been prepared and isolated by semipreparative HPLC, and its characteristics of color and stability have been studied and compared with that of malvidin glucoside in aqueous solutions. When the pH was increased from 2.2 to 5.5, the solution of the pigment became progressively more violet (λ_{max} = 560 nm at pH 5.5), whereas similar solutions of the anthocyanin were almost colorless at pH 4.0. This behavior indicated that the anthocyanin moiety of the pigment was more protected against water attack, and thus the formation of its quinonoidal forms was favored. The color of the pigment also showed more stability with regard to bleaching by SO_2 than that of malvidin glucoside. Nevertheless, the pigment was more sensitive to degradation in aqueous solution than the anthocyanin. The cleavage of the ethyl bridge that links the anthocyanin and the catechin constituted the first step in its degradation, as demonstrated by the formation of malvidin glucoside as a major product.

INTRODUCTION

In the presence of acetaldehyde, flavanols condense with one another (*1*, *2*) and with anthocyanins (*3*–*7*) through ethyl bridges, which are established between nucleophilic positions of their phloroglucinol rings. As a result of the condensation between malvidin 3-*O*-glucoside and (+)-catechin, two major pigments are formed that have been proposed to correspond to two diastereomers of a flavanol—anthocyanin dimer linked by an ethyl bridge through their C-8 positions (Figure 1) (*5*, *6*). When sufficient acetaldehyde is available, these pigments rapidly progress to larger structures by the incorporation of new ethyl—flavanol units until a critical mass is reached, after which they precipitate (*5*, *8*). It has been reported that solutions containing this type of condensed pigments have a violet hue and a more stable color against changes in pH and SO_2 bleaching than anthocyanins (*3*, *4*, *9*–*11*). However, this apparently increased color stability of the pigments has not been studied in detail for the compounds isolated.

In wine, acetaldehyde is formed as an intermediate compound during the fermentation process and may also be produced in further stages as a result of the ethanol oxidation coupled to the autoxidation of phenolic compounds containing ortho hydroxy groups (*12*, *13*). The importance of the acetaldehyde-mediated condensation for the color of red wines is debatable. Some authors
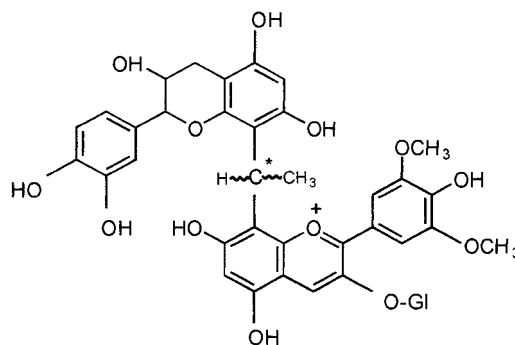

Figure 1. Basic structure of the pigments derived from the acetaldehyde-mediated condensation between anthocyanins and catechins. C* is an asymmetric carbon, which makes possible the existence of diastereomer pigments.

have given it an important role (*2*, *10*, *14*), whereas others have suggested that it would not be very relevant, taking into account the reduced availability of acetaldehyde due to the habitual presence of SO_2 (*15*, *16*). Only recently has development of the LC-MS technique allowed the direct detection in wine of products formed in these reactions, both colorless, the formation of which involves only flavanols (*17*), and colored ones, derived from the reaction between anthocyanins and flavanols (*18*, *19*).

In the present work we studied the color characteristics and stability in relation to pH, discoloration by SO_2, and storage in aqueous solution of a pigment formed in the acetaldehyde-mediated condensation between malvidin glucoside and catechin.

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MATERIALS AND METHODS

Isolation of Malvidin Glucoside and Catechin. Malvidin glucoside and catechin were extracted and purified from the skins (*20*) and seeds (*21*) of *Vitis vinifera* grapes, respectively. The identity of the isolated compounds was established by comparison of their chromatographic and spectral characteristics with commercial standards of (+)-catechin and malvidin 3-*O*-glucoside (Sigma). Their purity was checked by HPLC-DAD.

Preparation of the Condensed Pigment. Equimolar amounts of malvidin glucoside and catechin were dissolved in 20% acetic acid (pH 1.3) in the presence of acetaldehyde (2.5 times the amount of catechin) and allowed to react for 48 h at 25 °C. The formation of two pigments, I and II, was observed. Pigment II (majority pigment) was isolated from the solution by semipreparative HPLC using Waters 600 equipment and a PrepNova-Pak HR C18 6 μm (100 × 25 mm) radial compression cartridge. The solvents were methanol (A) and 5% acetic acid (B). A gradient was established from 10 to 15% A over 30 min, from 15 to 20% A over 15 min, from 20 to 30% A over 15 min, and from 30 to 40% A over 10 min, at a flow rate of 8 mL/min. Detection was carried out at 280 nm, and the peaks were collected in a fraction collector. Solvents were eliminated under vacuum, and the compound was transferred to water and freeze-dried. The purity and identity of the pigments isolated were checked by HPLC-DAS and MS.

Mass spectrometry analysis was performed in a Finnigan LCQ equipped with an API source using an electrospray ionization (ESI) interface. The compound was directly injected into the spectrometer with a pump at a flow rate of 10 μL/min. Both the auxiliary and sheath gas were a mixture of nitrogen and helium. The capillary temperature was 180 °C and the voltage 3 V. A full scan from 150 to 1500 uma and an MS-MS using a relative collision energy of 20% were obtained. Spectra were recorded in positive ion mode.

HPLC Analysis. HPLC analyses were carried out in Hewlett-Packard 1050 equipment using a 3 μm Spherisorb ODS2 column (150 × 4.6 mm). The solvents were 4.5% formic acid (A) and acetonitrile (B). For elution, a linear gradient was established from 10 to 20% B over 20 min, from 20 to 25% B over 10 min, from 25 to 35% B over 10 min, and 35% B isocratic 10 min, at a flow rate of 1.5 mL/min. Detection was performed at 280 and 520 nm in a Shimadzu SPD-M10AVP photodiode array detector.

Influence of pH and SO₂ on the Color. For the pH assay, solutions of pigment II (8×10^{-5} M) were prepared in a citric acid buffer at different pH values ranging from 2.2 to 5.5 and allowed to stand for 2 h at 20 °C prior to the spectrophotometric measurement. To study the bleaching by SO₂, solutions of pigment II (8×10^{-5} M) were prepared in a citric acid buffer at three pH values: 2.5, 4.0, and 5.0. Aqueous solutions of sodium bisulfite (5 mg/mL) were added to each solution to achieve SO₂ concentrations in the range 0–200 μg/mL. In both assays, UV–vis spectra (350–700 nm) were obtained in a Lambda 3 Perkin-Elmer spectrophotometer using a quartz cell of optical path length 1 cm and further submitted to chromatic analysis.

Stability of the Pigment in Aqueous Solution at 32 °C. Solutions of the pigment II (8×10^{-5}M) were prepared in a citric acid buffer at two pH values, 2.5 and 5, and maintained at 32 °C in closed vials in the dark. Changes were monitored by UV–vis spectrophotometry and HPLC-DAD.

In all cases, similar assays were carried out with solutions of malvidin glucoside.

Tristimulus Colorimetry. From the visible spectra obtained, chromatic parameters were obtained by the weighted-ordinate method ($\Delta\lambda = 1$ nm), using the CIE 1964 standard observer (10° visual field) and the CIE standard illuminant D_{65} as references. Chromatic analyses were performed according to CIE recommendations (CIE, 1986, 1995) in the CIE 1976 ($L^*a^*b^*$) space (CIELAB). Calculations were made using PCROM original PC software (*22*).
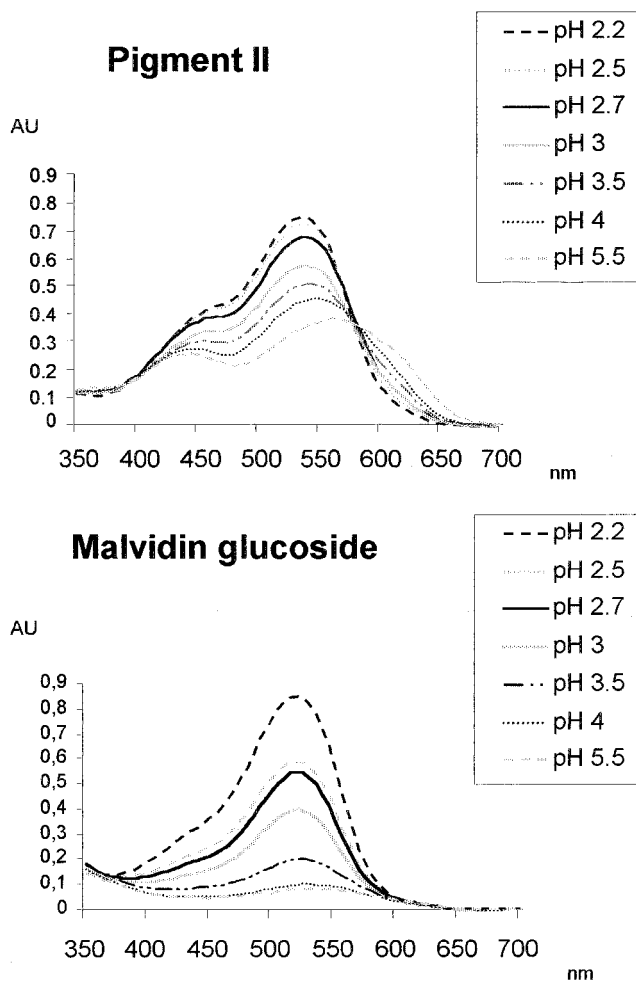

Figure 2. Changes in the visible spectra of pigment II and malvidin glucoside in the range of pH values from 2.2 to 5.5.

RESULTS AND DISCUSSION

Pigment Preparation and Molar Extinction. Previous assays were carried out to adjust the reaction conditions between malvidin glucoside and catechin in order to facilitate the best formation of pigment, minimizing that of polymers. The conditions finally employed are described under Materials and Methods. As expected, the rapid formation of two pigments was observed, one of which was minor (pigment I) with respect to the other (pigment II), the peak of which reached a maximum area after 48 h, at which time it was isolated. The identity of the isolated pigment was established by comparison of its chromatographic and UV–vis characteristics and mass spectrum (positive molecular ion at m/z 809) with those of an identical pigment previously identified in our laboratory (*5*). Small amounts of pigment I were also obtained in the isolation process. However, this pigment proved to be much more unstable and, in aqueous solution, it was rapidly partially transformed into pigment II; for this reason, no assays with pigment I could be performed.

The extinction of pigment II at its maximum wavelength of absorption ($\lambda_{max} = 535$ nm) was established in 10% ethanol solutions adjusted to pH 0.5 with HCl. The value obtained was 17100 M^{-1}, slightly lower than that previously determined for malvidin glucoside (20200 M^{-1}) (*20*).

Influence of the pH Value on the Color of Pigment II in Aqueous Solution. Figure 2 shows the

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Table 1. L^*, h_{ab}, and C^*_{ab} Values in CIE 1976 ($L^*a^*b^*$) Color Space (CIELAB) of Malvidin Glucoside and Pigment II Solutions in the Range of pH Values from 2.2 to 5.5

	malvidin glucoside			pigment II		
pH	L^*	h_{ab}	C^*_{ab}	L^*	h_{ab}	C^*_{ab}
2.2	73.25	5.22	52.63	70.23	−5.54	44.62
3	84.17	−4.16	31.01	73.46	−10.92	32.81
4	94.21	−14.45	7.95	75.67	−24.77	20.80
5.5	94.48	−15.81	4.73	77.80	−46.34	10.79

Figure 3. Effects of the addition of increasing concentrations of SO_2 on the visible spectra of pH 2.5 solutions of pigment II and malvidin glucoside.

variations occurring in the visible region of the spectrum in aqueous solutions of pigment II with pH values in the 2.2–5.5 range. Comparison with the changes occurring in solutions of malvidin glucoside under identical assay conditions revealed that the pigment had a considerably more stable color against the increase of pH than the anthocyanin, the discoloration of which was almost complete at pH 4. Additionally, the solutions of pigment II showed hues different from those of malvidin glucoside. At the more acidic pH values the solutions of pigment II were already more violet (λ_{max} = 535 nm) than those of malvidin glucoside (λ_{max} = 520 nm). As pH increased, the solutions of pigment II acquired an increasingly more violet color and their λ_{max} values shifted to 560 nm. The color changes of the solutions had effects on the chromatic parameters (Table 1). A spectacular decrease occurred in the chroma values (C^*_{ab}) for both compounds as pH increased. The variation in the lightness (L^*) was of up to 20 CIELAB units in the case of malvidin glucoside when the pH was increased from 2.2 to 4.0, after which it stabilized, whereas the variation in the lightness of pigment II did not surpass 8 units throughout the range of pH values assayed. The greater increase in lightness observed in the case of malvidin glucoside should therefore be interpreted in terms of a greater discoloration of its solutions with respect to those of pigment II. The stabilization in the values of both parameters that occurred in malvidin glucoside solutions from pH 4.0 could thus be explained because the anthocyanin had reached its degree of maximum discoloration. With regard to hue (h_{ab}), the solutions of malvidin glucoside presented behavior similar to the quantitative color parameters, stabilization being observed from pH 4.0. In contrast, in the solutions of pigment II the hue dropped to lower values (bluer hues) at this pH value.

The explanation for these observations can be found in the equilibria between the different structural forms of anthocyanins. The increase in pH leads to a progressive loss of the red color typical of the flavylium form, accompanied by a discoloration of the solutions due to the formation of the hydrated pseudobase, which, according to the results obtained, would reach a maximum at pH 4.0. The higher resistance to discoloration in the solutions of pigment II and the shift of color to bluer hues must therefore be due to a greater protection of its anthocyanin moiety against the nucleophilic attack of water. Thus, when the pH rises, equilibria are displaced toward the formation of blue quinonoidal structures. The presence of an isosbestic point at ∼580 nm in the visible spectra of pigment II obtained at different pH values (Figure 2) is proof of the shift of the equilibria in that direction.

Bleaching by SO_2. The effect of SO_2 on the color of the solutions of pigment II was studied at pH 2.5, 4, and 5. However, comparison with the effect exerted by this additive on malvidin glucoside was made only at
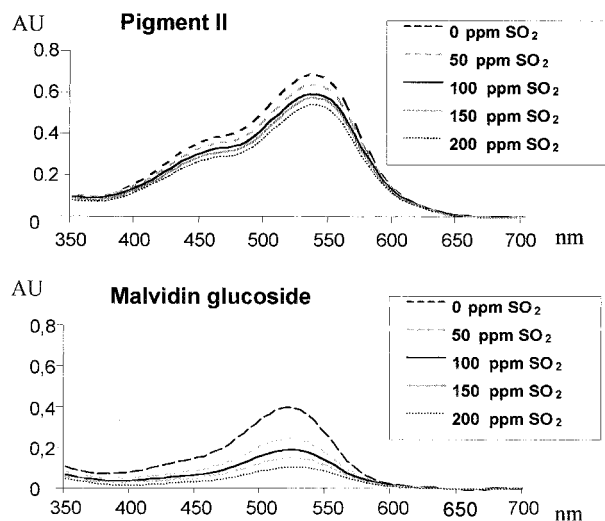
pH 2.5, because at pH 4 and 5 the solutions of the anthocyanin were already discolored by the effect of the low acidity. At pH 2.5, the solution of pigment II showed greater resistance to discoloration by the addition of increasing amounts of SO_2 (0–200 μg/mL) than did malvidin glucoside (Figure 3). This indicates the existence of a higher protection of the pigment against the nucleophilic attack of bisulfite, as described above for water. In the solutions of pigment II at pH 4 and 5 more discoloration occurred than at pH 2.5, which may be explained by the different reactivity of SO_2 as a function of the pH. The pK of the equilibrium between sulfurous acid and the bisulfite anion is 1.8; thus, at a pH value of 2.5 both forms must still exist in the solutions, whereas at pH 4–5 only the reactive bisulfite ion will be present (*23*).

The effects of the addition of SO_2 on the chromatic parameters of the solutions are shown in Table 2. A considerable increase in lightness (L^*) and a very important decrease in the amount of color, represented by the chroma (C_{ab}) were observed as a result of the SO_2 addition to the solutions of malvidin glucoside (pH 2.5). The SO_2 effect was already appreciable at low concentrations. However, only small changes were observed in the solutions of pigment II at pH 2.5, indicating that its color is more resistant to the discoloring action of SO_2 than malvidin glucoside.

Stability in Aqueous Solution. The stabilities of pigment II and malvidin glucoside were studied at pH 2.5 and 5 in aqueous solutions kept at 32 °C for 70 days, sufficient time for the complete disappearance of pigment II. The rate of degradation of both pigment II and malvidin glucoside was greater at the more acidic pH (Figure 4). This can be explained by an easier hydrolysis of the glucose moiety, with the production of unstable aglycons. In the case of pigment II, the more acidic pH could also favor its cleavage at the level of the ethyl bridge between anthocyanin and catechin. Interestingly, at both pH values the decrease of pigment II in the solutions was faster than that of malvidin glucoside, indicating a lower stability of the pigment with respect to the anthocyanin.

The process of degradation of malvidin glucoside in aqueous solution occurs through cleavage of the heterocycle, with the formation of products corresponding

Table 2. *L**, *h*$_{ab}$, and *C**$_{ab}$ Values in CIE 1976 (*L**a*b*) Color Space (CIELAB) of Solutions of Malvidin Glucoside at pH 2.5 and Pigment II at pH 2.5, 4, and 5 with Addition of Increasing Concentrations of SO$_2$ (0–200 ppm)

SO$_2$ (ppm)	malvidin glucoside, pH 2.5			pigment II, pH 2.5			pigment II, pH 4			pigment II, pH 5		
	L*	*h*$_{ab}$	*C**$_{ab}$	*L**	*h*$_{ab}$	*C**$_{ab}$	L*	*h*$_{ab}$	*C*$_{ab}$	*L**	*h*$_{ab}$	*C**$_{ab}$
0	73.25	5.22	52.63	70.23	−5.54	44.62	71.18	−33.88	22.02	75.50	−36.51	15.63
50	89.95	−7.96	21.55	71.75	−7.00	42.00	80.96	−21.38	20.18	83.48	−36.83	12.05
100	93.37	−11.64	14.27	73.21	−7.69	39.81	84.74	−22.90	15.94	87.40	−37.72	9.11
150	95.16	−15.49	10.58	74.12	−8.43	39.02	87.20	−24.43	12.97	89.80	−38.24	7.23
200	96.19	−18.78	8.30	75.47	−9.14	37.50	88.96	−25.36	11.15	91.20	−39.55	6.14
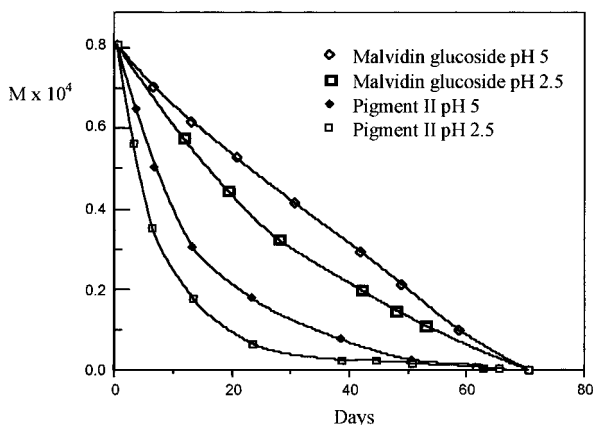
Figure 4. Changes in the concentrations of malvidin monoglucoside and pigment II in the solutions of pH 2.5 and 5.
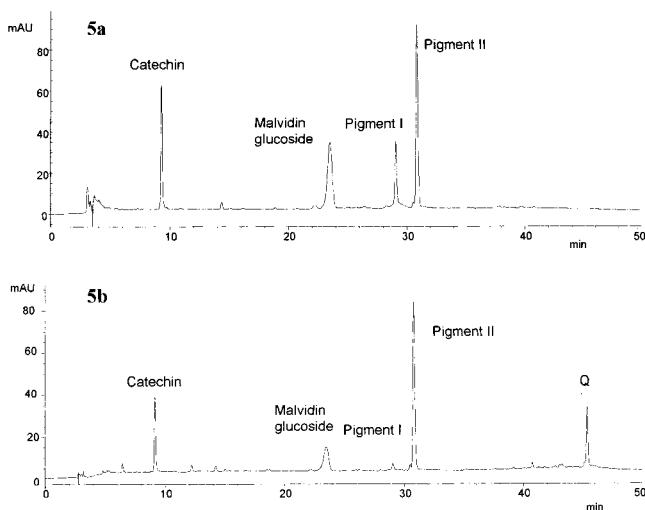
Figure 5. Chromatograms recorded at 280 nm of a solution of pigment II after 6 days of reaction at 32 °C (a) at pH 2.5 and (b) at pH 5. Peak Q is suggested to be a quinone form of pigment II.

to the B (syringic acid) and A (trihydroxybenzaldehyde and phloroglucinol) rings (*24*, *25*). This process is confirmed in our assays, in which the formation of syringic acid, identified by comparison with a standard (Sigma), was observed in the chromatograms.

The decrease of pigment II in the solutions was accompanied by the formation of pigment I, malvidin glucoside, and catechin (Figure 5a). The cleavage of the pigment at the level of the ethyl bridge must thus occur, which is accompanied by a process of structural reorganization, surely as a result of the further reaction of the products of degradation. Pigment II had completely disappeared by day 55 in the pH 2.5 solution, although this solution continued to display red color due to the presence of malvidin glucoside formed in its degradation.
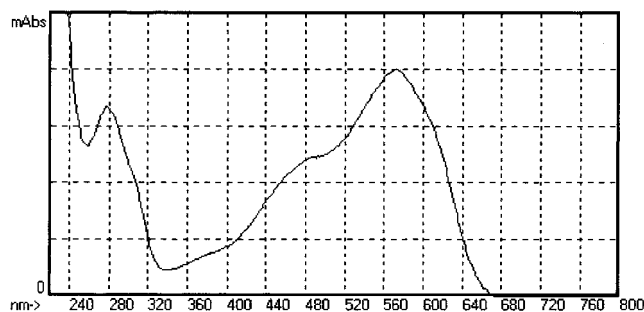
Figure 6. Spectrum of peak Q of the chromatogram of Figure 5b.

As mentioned above, the decrease of pigment II was slightly faster at pH 2.5 than at pH 5 (Figure 4). In this latter solution lower amounts of pigment I were formed (Figure 5b), possibly due not only to a lower rate of pigment II cleavage but also to a more difficult formation of the ethyl–catechin cation necessary for further reaction, which would be facilitated at more acidic pH (*5*). In the chromatograms of the solution of pigment II at pH 5, a peak was observed that did not appear at pH 2.5 (peak Q in Figure 5b). The presence of this peak was observed from the first day of the assay, and its area decreased simultaneously to that of the pigment II. Taking into account its absorption spectrum (Figure 6), which shows λ_{max} in the visible at 574 nm, we suggest that it could correspond to the quinone form of pigment II. It should be noted that the anthocyanin pigments are usually detected in the HPLC chromatograms as their flavylium cations, due to the low pH of the mobile phase. Peaks corresponding to quinonoidal forms are not observed in the chromatograms because these forms are very scarce in aqueous solutions and rapidly revert to the flavylium form in the acidic medium provided by the HPLC solvents. The fact that a quinonoidal form is seen in the chromatograms of the pigment II solution at pH 5 could be explained by an incomplete reversion to the flavylium cation during the HPLC analysis, which would be favored by the greater importance of its protonation equilibrium compared to anthocyanins and the low acidity of the solution.

From the observations made, it can be reasonably inferred that the absence or very low amounts of these kinds of condensed pigments which are normally present in red wines could be a consequence of their low stability in aqueous solutions.

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