Detection of Compounds Formed through the Reaction of Malvidin 3-Monoglucoside and Catechin in the Presence of Acetaldehyde

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The interaction between catechin and malvidin 3-monoglucoside in the presence of acetaldehyde was studied in model solutions. The initial formation of two pigments (I and II), which later evolved to more condensed structures, was observed. Evidence was found that for these pigments to be formed, catechin should first react with acetaldehyde and the resulting adduct later condenses with anthocyanin; it is proposed that pigments I and II are two enantiomeric structures of the catechin-(8→)-acetyl-(→8)-anthocyanin dimer. For their formation, a relatively acidic pH is necessary; this is attributed to the demand that the acetaldehyde should be in cationic form for condensation to occur. Condensation among catechin units involving acetaldehyde was also seen. Minor amounts of substances from the degradation of anthocyanin were detected, indicating that in the presence of acetaldehyde it is basically condensation and not degradation of the anthocyanin that occurs.

Keywords: *Anthocyanin; catechin; condensation; color*

INTRODUCTION

During the conservation and aging of red wines a change in color occurs. This can be explained in terms of the progressive formation of condensed pigments resulting from the interaction between anthocyanins and other phenolic products extracted from the grape. Although this process has been described in earlier works (Jurd, 1969; Singleton and Esau, 1969; Somers, 1971), the structure of the pigments formed and the mechanisms through which they are produced are still to be established. Two main processes have been suggested: a direct reaction between anthocyanins and flavanols giving rise to compounds with a yellowish brown hue (Jurd, 1969; Liao et al., 1992) and the reaction between the above compounds involving acetaldehyde with the formation of violet pigments (Timberlake and Bridle, 1976).

The increase in color intensity occurring in red wines due to the effect of acetaldehyde is well-known (Ribéreau-Gayon et al., 1983; Sims and Morris, 1986) and has been studied by several authors in model solutions (Baranowski and Nagel, 1983; Bakker et al., 1993; Bravo-Haro et al., 1992; Roggero et al., 1987; Timberlake and Bridle, 1976, 1977). Using HPLC, Roggero et al. (1987) observed that, in the presence of acetaldehyde, malvidin 3-monoglucoside reacts with catechin to yield two major compounds. The use of diode array detectors associated with HPLC afforded the spectra of these substances, showing them to be reddish blue pigments with spectra similar to those of anthocyanins, although with their maximum wavelength in the visible range bathochromically shifted with respect to these (Bakker et al., 1993; Bravo-Haro et al., 1992).

It has been suggested that these substances have a dimeric structure, in which the anthocyanin is bound to the catechin through an acetyl bridge (Bakker et al., 1993), in concordance with the mechanism previously proposed by Timberlake and Bridle (1976). These sub-

stances are transient and they later evolve to form substances with a greater degree of condensation which finally precipitate (Bakker et al., 1993; García-Viguera et al., 1994).

The present work studies the interaction in model solutions between catechin and malvidin monoglucoside in the presence of acetaldehyde and offers new information about the analysis and characterization of the compounds formed in these reactions using the chromatographic and spectrophotometric data obtained.

MATERIALS AND METHODS

Standards. Syringic acid and (+)-catechin were obtained from Sigma Chemical Co. (St. Louis, MO); 2,4,6-trihydroxybenzoic acid was from Aldrich-Chemie (Steinheim, Germany) and phloroglucinolcarboxaldehyde (2,4,6-trihydroxybenzaldehyde) from Fluka Chemie AG (Buchs, Switzerland). Malvidin 3-monoglucoside was isolated in our laboratory from grape skins, as described below.

Isolation of Malvidin 3-Monoglucoside. Skins of red grapes (*Vitis vinifera*) were subjected to extraction with methanol plus 1 N HCl (95 + 5); the sugars and other polar substances were removed from the extracts in a mixed stationary phase column of silica gel 60/Polyclar AT, as described by Hebrero et al. (1988). Malvidin 3-monoglucoside (Mv3mg) was isolated from the purified extracts of anthocyanins by medium-pressure chromatography using a Büchi chromatograph equipped with a model 681 pump, a Model 687 gradient former, and a UV–vis Model 683 detector. A 46 × 2.6 cm column was used, packed at our laboratory with Lichoprep RP-18 of 24–40 μm (Merck). Elution was performed through linear gradients between 2.5% acetic acid and methanol at a flow rate of approximately 28 mL/ min. Detection was made at 520 nm, and the peaks were collected in a Frac-100 (Pharmacia) fraction collector. The fractions containing malvidin 3-monoglucoside were transferred to an aqueous solution and then lyophilized. The purity of the Mv3mg (93 ± 1%) in the lyophilisate was determined by HPLC/DAS.

Preparation of Model Solutions. Solutions containing 250 mg/L (0.51 mM) Mv3mg, 700 mg/L (2.6 mM) (+)-catechin, and 250 mg/L (5.7 mM) acetaldehyde were prepared in 0.5% (w/v) tartaric acid containing 10% ethanol and adjusted to two different pH values (3.2 and 4.0) with 0.1 N NaOH. The preparations (25 mL) were placed in reaction vials of 50 mL and sealed hermetically with septa of Teflon and silicone and

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stored in darkness at different temperatures (32 and 15 °C). Samples were taken periodically during 1 month by extracting a sufficient volume for analysis through the septum by puncture. The first sample (day 0) was collected 1 h after preparation of the solution.

HPLC Analysis. A Hewlett-Packard Series 1050 pump was used. The column (25 × 0.4 cm i.d.) was a 5 μm Lichrospher 100 RP-18 (Merck), thermostatically controlled at 30 °C. Five percent formic acid (A) and gradient grade acetonitrile (B) were used as solvents, establishing a linear gradient between 10% B and 35% B over 50 min at a flow of 1.5 mL/min. Detection was made at 520 and 280 nm with an HP1040M Series II diode array detector coupled to an HP79994A data treatment station. Mv3mg was quantified from the area of its peak recorded at 520 nm and catechin from its peak area recorded at 280 nm, both by comparison with external standards.

Spectral Measurements. Spectra of the solutions in the visible light region were obtained in a 0.2 cm cuvette using a Lambda 3β UV—vis (Perkin-Elmer) spectrophotometer.

Preparation of Substances Resulting from Acetaldehyde—Catechin Condensation. Solutions containing (+)-catechin and acetaldehyde (1:1 molar ratio) were prepared in 0.5% (w/v) tartaric acid with 10% ethanol at pH 3.2 and allowed to react at room temperature for 24 h. The substances formed were separated by semipreparative HPLC on a Waters 600E pump using a 5 μm Ultracarb ODS 20 column (25 × 1 cm) and establishing a linear gradient between 2.5% acetic acid and methanol at a flow rate of 3.5 mL/min. The fractions collected were evaporated under vacuum at 30 °C, and the residues were redissolved in an identical solution at pH 3.2.

RESULTS AND DISCUSSION

Figure 1 shows three chromatograms recorded at 520 nm of the pH 3.2 solution kept at 32 °C after 1 h and 3 and 10 days of reaction. The progressive appearance of new peaks corresponding to colored substances was noted. Apart from the peak corresponding to Mv3mg (M), another two major peaks (I and II) may also be seen in the chromatograms, corresponding to two condensed pigments previously reported (Bakker et al., 1993; Roggero et al., 1987). The spectra of these compounds (Figure 2) are similar to those of the anthocyanins, but their maxima in the visible region (around 540 nm) are bathochromically shifted with respect to that of Mv3mg (525 nm). With longer retention times other peaks appear, which are attributed to polymeric pigments (García-Viguera et al., 1994). The good resolution obtained for these peaks in our chromatograms should be noted, since under the conditions employed by other authors (Roggero et al., 1987; Singleton and Trousdale, 1992) polymeric pigments usually elute in the form of humps. This good separation allows the individual obtention of peak spectra and permits the following of their evolution through time. It offers the possibility of isolating compounds and further characterizing their structure. Most of these peaks have spectra similar to those of pigments I and II, although in one of them (marked with an asterisk in Figure 1C) the maximum is shifted toward lower wavelengths (505 nm, see spectrum in Figure 2).

At 32 °C pigments I and II appeared in a few hours after preparation of pH 3.2 solutions (Figure 1A). Bakker et al. (1993) by FAB MS determined a molecular ion at m/z 809 for pigments I and II; this is coherent with a dimeric structure in which the anthocyanin and the catechin are bound by an acetyl bridge. These authors do not propose a concrete structure for the pigments, but they suggest that their formation could occur through the mechanism proposed by Timberlake and Bridle (1976), according to which the acetaldehyde in cationic form reacts with catechin and, in an acid
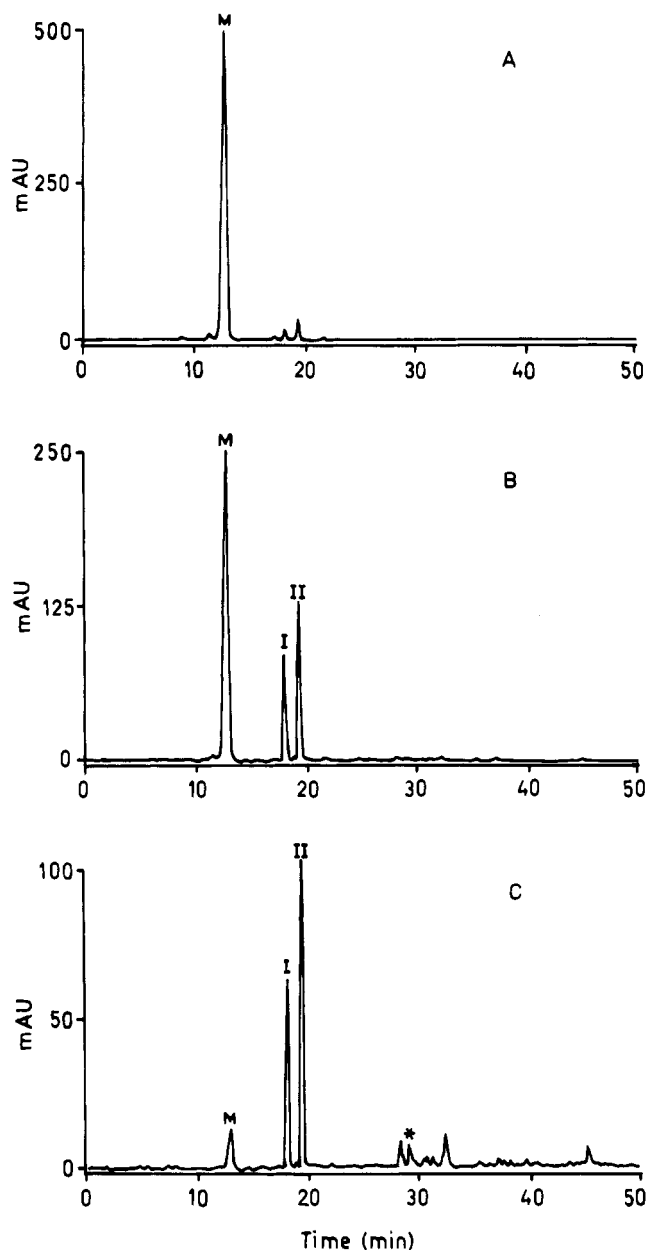

Figure 1. HPLC chromatograms recorded at 520 nm of a model solution [malvidin 3-monoglucoside + (+)-catechin + acetaldehyde] at pH 3.2 after 1 h (A), 3 days (B), and 10 days (C) of reaction at 32 °C. M, malvidin 3-monoglucoside; I, II, and *, selected condensed pigments (spectra shown in Figure 2).

medium, the adduct formed leads to a new carbocation that reacts with the anthocyanin. The positions involved in this reaction would be C-8 and/or C-6 of both anthocyanin and catechin. Another mechanism was proposed by Dournel (1985), according to which acetaldehyde would react at position 4 of the anthocyanin in flavylium form and the new resulting cation would substitute the catechin at its position 8 or 6, giving rise to colorless dimers that form red pigments after being oxidized.

Evidence that this latter mechanism cannot be responsible for the formation of substances I and II was obtained in our experiments. In solutions of Mv3mg alone in the presence of acetaldehyde at pH 3.2 there was hardly any reaction, and after 30 days at room temperature, essentially only colorless substances from the degradation of anthocyanin were detected. In preparations containing catechin and acetaldehyde in
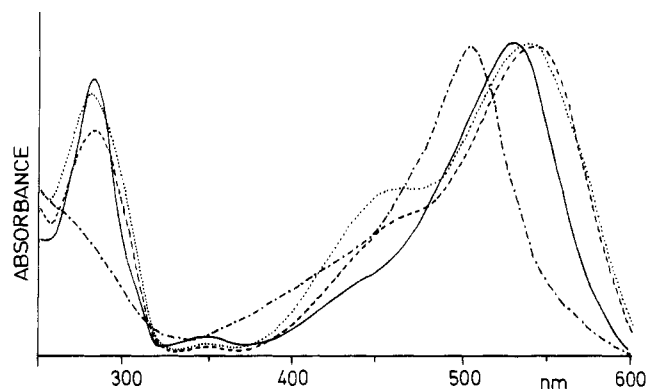
1446 J. Agric. Food Chem., Vol. 43, No. 6, 1995

Rivas-Gonzalo et al.

Figure 2. Spectra of malvidin 3-monoglucoside (—) and pigments: I (···); II (- - -); and * (— · —).
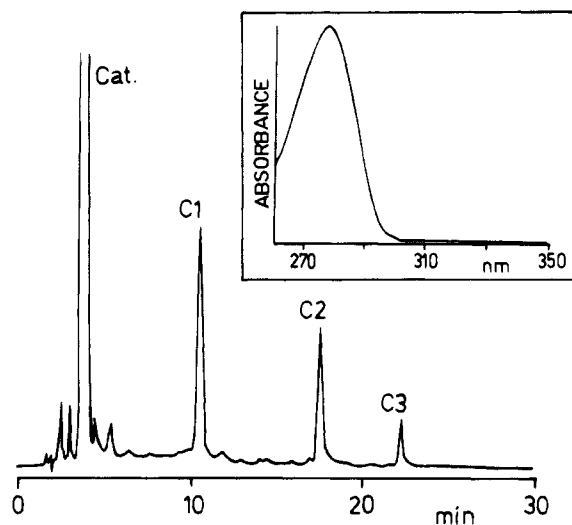

Figure 3. HPLC chromatogram recorded at 280 nm corresponding to a semipreparative separation of the compounds formed in (+)-catechin/acetaldehyde reaction. (Inset) Spectrum corresponding to catechin and peaks C1, C2, and C3.

Figure 4. Proposed structure for pigments I and II.

the same conditions, during a few hours the rapid formation of three colorless compounds with spectra identical to that of catechin was observed (Figure 3). To isolate these three compounds, solutions containing catechin and acetaldehyde were made up for preparative purposes and the substances formed were isolated by semipreparative HPLC, as indicated under Materials and Methods. Each of these compounds was introduced separately at pH 3.2 in the presence of malvidin 3-monoglucoside. Only compound C1 showed an immediate reaction with the anthocyanin, giving rise to the formation of both pigments I and II; the other two compounds did not react with anthocyanin. The results obtained in this assay suggest that the formation of pigments I and II must occur through a mechanism similar to that proposed by Timberlake and Bridle (1976). That is, as a cation, acetaldehyde reacts with catechin and forms an adduct that, in sufficiently acid medium, gives rise to a carbocation that binds to anthocyanin.

García-Viguera et al. (1994) proposed that pigments I and II could result from substitution of the anthocyanin A ring at positions 8 and 6, respectively. We believe that substitution at carbon 6 is unlikely owing to the hindrance due to the presence of hydroxyl groups at positions 5 and 7. On the other hand, if these pigments arise from substitution of anthocyanin at positions 8 and 6, it seems logical to suppose that the catechin could also be substituted at these positions and, therefore, the
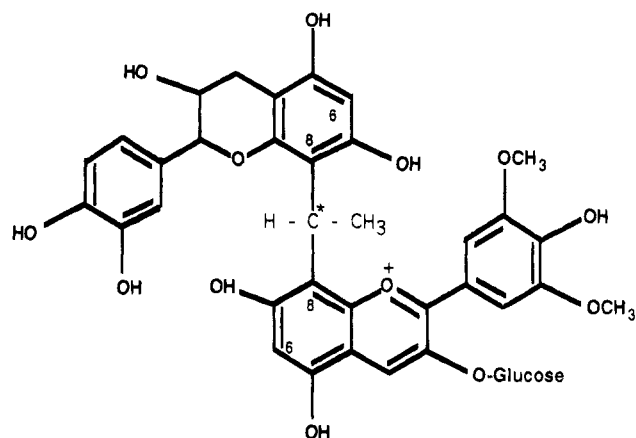
formation of two catechin–acetaldehyde adducts and four different pigments would be possible. Additionally, position 2′ in the anthocyanin B ring, also with nucleophilic characteristics, could be considered for substitution, thus increasing the number of possible alternative pigments.

The fact that in the reaction between catechin and acetaldehyde only one compound able to react with anthocyanin is formed indicates the existence of a clearly preferential substitution site, which must be position 8 of the A ring, considered to be the most nucleophilic. This position must also be the preferential site of substitution in the anthocyanin. We suggest that pigments I and II are enantiomers of the dimer catechin-(8→)-acetyl-(→8)-anthocyanin (Figure 4), given the existence of an asymmetric carbon (*) in the acetyl bridge. This proposal does not exclude the possibility that other minor pigments formed in the solutions might come from alternative substitutions of other nucleophilic positions.

Under our assay conditions, pigments I and II accumulate very rapidly at pH 3.2, but their formation is notably reduced at pH 4, as can be observed in Figure 5, where the changes in the area of peak II (peak I shows similar behavior) in the different conditions of pH and temperature tested are presented. The decrease in Mv3mg concentration is faster at the more acidic pH and, after 10 days at 32 °C, the anthocyanin levels were close to 3% (pH 3.2) and 30% (pH 4) of the initial values (Figure 6). At the end of the assay period, the loss of Mv3mg is nearly complete at both pH values, since the lower condensation rate existing at pH 4 favors a greater anthocyanin degradation.

The effect of pH on the formation of pigments I and II has been studied by García-Viguera et al. (1994), who found that in the pH 2–5 range both the formation rate and the amounts of these pigments accumulated rose with the decrease in pH. This finding was linked by these authors to the availability of the flavylium cation existing as a function of the pH. However, if condensation occurs according to the above proposed mechanism, it cannot be very strongly affected by the form in which the anthocyanin is present, since it would essentially depend on the nucleophilic nature of carbon 8 (Baranowski and Nagel, 1983). It would be more logical to assume that it is the availability of acetaldehyde that limits the reaction rate, since in insufficiently acidic hydroalcoholic media acetaldehyde would be prevented from forming a cation and would therefore react with catechin to a lesser extent, thus explaining the low condensation rate observed at pH 4.
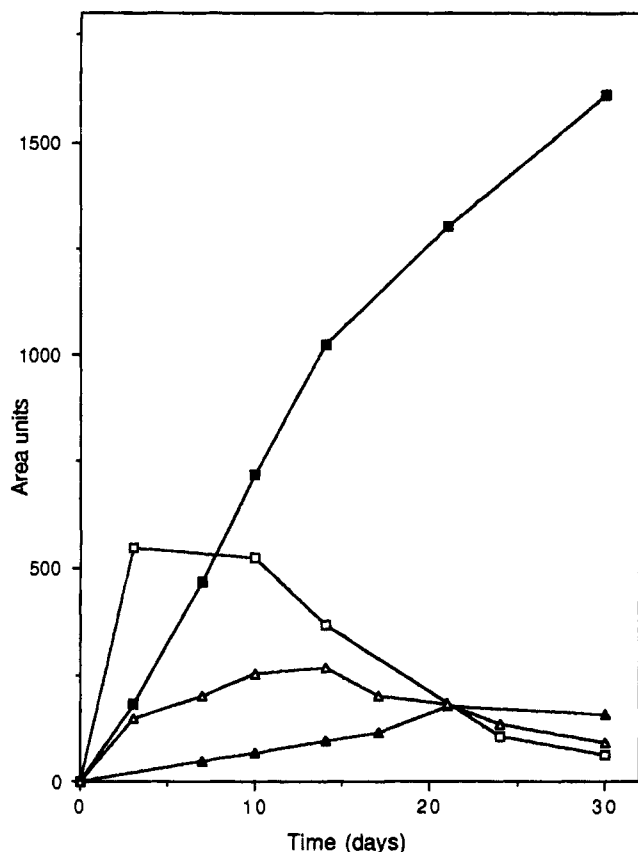
Figure 5. Changes in peak II chromatographic area recorded at 520 nm in solutions kept at 32 °C, pH 3.2 (□); 32°C, pH 4 (△); 15 °C, pH 3.2 (■); and 15 °C, pH 4 (▲).

Figure 6. Percentages of the retention of the concentration of malvidin 3-monoglucoside in solutions kept at 32 °C, pH 3.2 (□); 32 °C, pH 4 (△); 15 °C, pH 3.2 (■); and 15 °C, pH 4 (▲) over 30 days.

Pigments **I** and **II** reach a maximum (day 3) rapidly, at pH 3.2 and 32 °C (Figure 5), and then decrease, while formation of new pigments with longer retention times is simultaneously observed. The existence of two differentiated groups of substances should be noted: one characterized by peaks with retention times between 25 and 35 min, which were first formed, and another giving rise to peaks with retention times longer than 35 min, which appeared later as a purple precipitate was formed. These new pigments must possess a higher degree of polymerization and their formation must occur through successive incorporation of catechin units, since they appear when the availability of free anthocyanin is very low or has even been exhausted. It has been suggested that the incorporation of new catechin units to condensed pigments could occur with or without the involvement of acetaldehyde (García-Viguera et al., 1994). In our experiments, the speed at which solutions at pH 3.2 evolve toward the formation of these polymers suggests that the progression of the polymerization in these conditions must essentially take place involving acetaldehyde.

Temperature is a determining factor in the evolution and accumulation of new pigments. In the same assay period, solutions of pH 3.2 reached greater color intensities at 15 °C than at 32 °C (Figure 7). This is explained by the rapid loss of Mv3mg observed at 32 °C and the progression of pigments **I** and **II** toward more polymerized structures, which eventually precipitated, as could be seen from the appearance of a purple precipitate in the reaction vials. However, at 15 °C the evolution of pigments **I** and **II** toward more polymerized structures was slower, thus allowing them to be accumulated in greater amounts. A similar observation was made by Baranowski and Nagel (1983), who re-
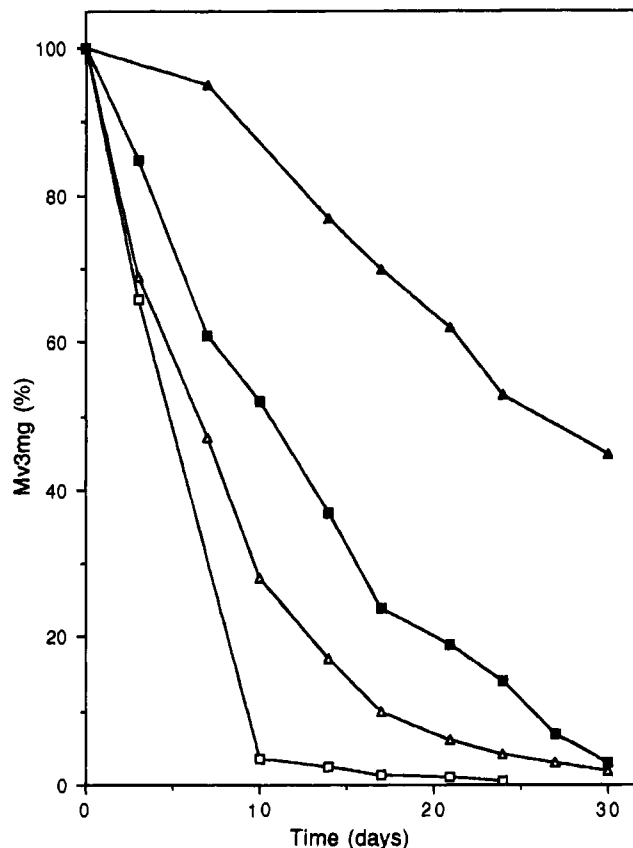
ported that higher temperatures produce more polymeric material in less time but that the polymers are more stable with regard to degradation and precipitation at lower temperatures.

In solutions of Mv3mg and catechin to which acetaldehyde was not added, we observed that direct condensation between anthocyanin and catechin occurs; this type of condensation takes place very slowly and finally leads to the formation of yellowish pigments whose spectra have maxima at wavelengths ranging between 425 and 450 nm (Santos-Buelga et al., 1995). In the presence of acetaldehyde, as studied here, very low amounts of a pigment from direct condensation were only detected at pH 4 (peak x in Figure 8A), suggesting that condensation involving acetaldehyde is what principally occurs.

In Figure 8B the same chromatogram as in Figure 1C is shown but registered at 280 nm. By comparison of these two chromatograms, the presence of some peaks showing only absorbance in the UV region can be observed. Spectra of peaks c, e, and f are similar to that of (+)-catechin (peak a). They correspond to compounds resulting from the catechin—acetaldehyde condensation, identical to those present in the chromatogram of Figure 3, as confirmed by injection of that preparation in the chromatographic conditions used for model solutions. Peak c (corresponding to C1 in Figure 3) was tentatively ascribed to a catechin—acetaldehyde adduct; in the chromatograms in Figure 8, this peak shows a small area, because of its rapid reaction with Mv3mg to yield pigments **I** and **II**. Peaks e and f (C2 and C3 in Figure 3) could correspond to catechin dimers involving acetaldehyde, similar to pigments **I** and **II**. The dimeric nature of these compounds could also explain why they
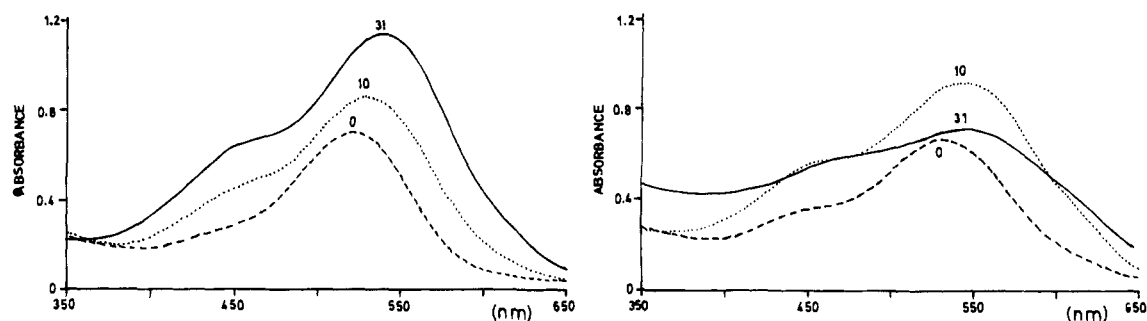
Figure 7. Evolution of the spectrum of a model solution [malvidin 3-monoglucoside + (+)-catechin + acetaldehyde] at pH 3.2 at 15 °C (left) and at 32 °C (right).
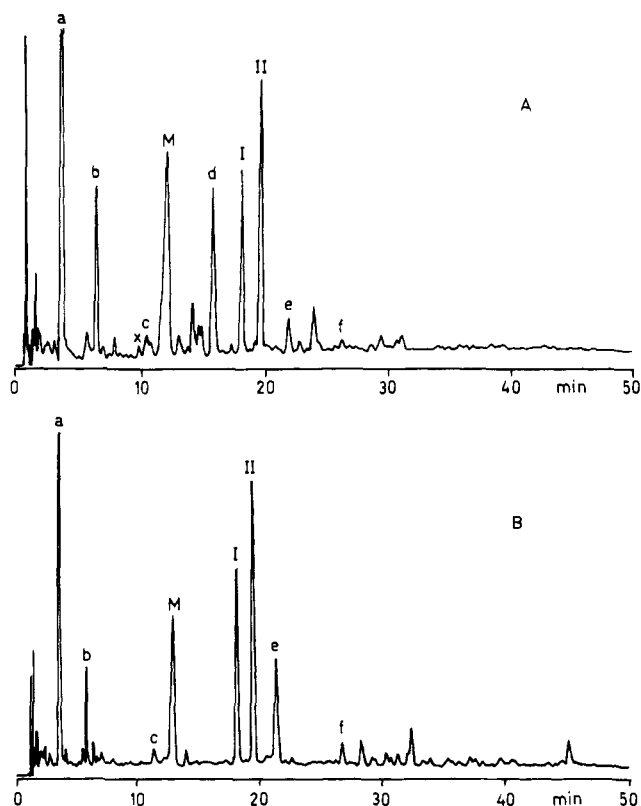

Figure 8. HPLC chromatograms recorded at 280 nm of the pH 4 (A) and pH 3.2 (B) solutions after 10 days of reaction at 32 °C. See text for peak identification.

Figure 9. Spectra corresponding to peaks b (—) and d (···) from Figure 8.

do not react with Mv3mg, as mentioned above; thus, the ratio between their areas is similar to that of C2 and C3. The accumulation of these peaks is much lower at pH 4 (Figure 8A), which can be explained by the lower availability of acetadehyde in reactive form, as previously suggested for the formation of pigments I and II.

Peaks b and d correspond to the two main compounds formed in the degradation of Mv3mg, as confirmed by the results obtained in solutions to which anthocyanin was introduced alone. Peak b (spectrum in Figure 9) was identified as syringic acid, arising from ring B of Mv3mg (Furtado et al., 1993; Piffaut et al., 1994). Peak d must be related to the anthocyanin ring A and, according to these authors, the release of 2,4,6-trihy-droxybenzaldehyde should be expected from this moiety. However, neither the spectrum (Figure 9) nor HPLC retention time of peak d coincides with that of a standard of this substance. Similarly, this peak does not correspond to 2,4,6-trihydroxybenzoic acid, which could be formed after 2,4,6-trihydroxybenzaldehyde oxidation, as was also shown by comparison with a standard. No further information could be obtained about peak d, which remains unidentified.
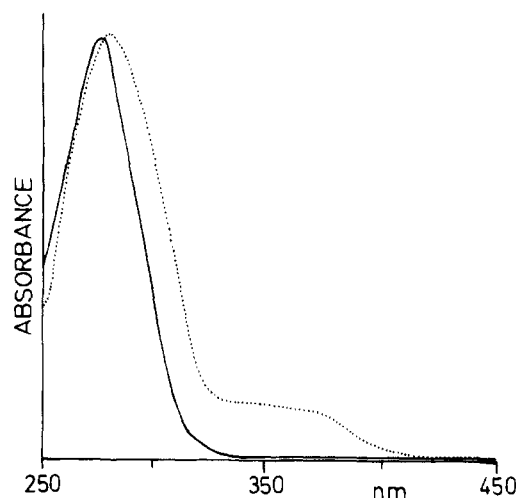
At 3.2 and 32 °C, peak b area is rather weak, when compared with those observed in similar solutions of Mv3mg to which no acetaldehyde or catechin was added (Santos-Buelga et al., 1995). This fact shows that the degradation rate was rather low, since anthocyanin was rapidly lost through condensation reactions, hence confirming previous reports (Baranowski and Nagel, 1983). Nevertheless, at pH 4, at which the rate of condensation is lower than at pH 3.2, higher amounts of peaks b and d are accumulated, suggesting that, at this pH, the structural degradation is an important factor in the explanation of the loss of Mv3mg.

At 15 °C almost negligible amounts of compounds from anthocyanin degradation are observed at both pH values over a period of 30 days; nevertheless, an almost complete loss of Mv3mg at pH 3.2 (final retention of 3%) and a decrease of 55% at pH 4 were observed. Under these conditions, the anthocyanin decrease can only be explained by its involvement in condensation reactions. Low temperature, on the other hand, causes the anthocyanin to be lost according to a linear and not an exponential model, as occurred at 32 °C. In these circumstances, at 15 °C, there is more Mv3mg available for the formation of more and more stable condensed pigments.

The observations made are relevant, since they suggest that in red wines with suitable levels of anthocyanins and flavanols the maintenance of pH and temperatures as low as those compatible with enological practice should confer greater and more stable coloring.

Anthocyanin Condensation in the Presence of Acetaldehyde

J. Agric. Food Chem., Vol. 43, No. 6, 1995 **1449**

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