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Products Formed in Model Wine Solutions Involving Anthocyanins, Procyanidin B_2, and Acetaldehyde

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Interaction between anthocyanins (3-glucosides of cyanidin, peonidin and malvidin), procyanidin B_2 (B_2), and acetaldehyde and the formation of new colored compounds was studied in model wine solutions. The anthocyanin−procyanidin B_2 condensation reaction showed first-order kinetics with or without the presence of acetaldehyde, and the reaction rates were calculated. In all the model solutions containing anthocyanins, procyanidin B_2, and acetaldehyde, rapid color augmentation with shifts toward violet were observed. Two principal new compounds (thought to be trimers, linked at different positions) were detectable by HPLC, and their spectral properties were studied by diode-array spectroscopy. Malvidin 3-glucoside reacts more slowly than cyanidin 3-glucoside and peonidin-3 glucoside. Similar color changes were observed in model solutions containing anthocyanins alone with acetaldehyde. Finally, we observed that the degradation constant rates for each anthocyanin increased in the following order: anthocyanins alone < anthocyanins + B_2 < anthocyanins + acetaldehyde < anthocyanins + B_2 + acetaldehyde. Similar increasing order of the reaction rates of procyanidin B_2 was observed. In both cases, the presence of acetaldehyde was of major importance in accelerating the chemical transformation of phenolic compounds, inducing the formation of new products.

Keywords: *Anthocyanins; procyanidin B_2; acetaldehyde; condensation reaction*

INTRODUCTION

During the winemaking processes (maceration, storage, aging, bottling) of red wines, the *Vitis vinifera* anthocyanin glycosides are converted through several reactions into both colorless oxidation products and polymeric pigments (Somers, 1966; Haslam, 1977, 1980; Hrazdina and Franzese, 1974; Wulf and Nagel, 1978; Somers and Verette, 1988). Several explanations for the mechanisms and possible structures for these polymeric pigments have been proposed (Somers, 1966, 1971; Jurd 1967, 1969). One of these condensation mechanisms, which may be very important in wine aging, involves the intervention of acetaldehyde. The addition of acetaldehyde to red wines induced large color changes by reactions with sulfites and by polymerization of the anthocyanins with other phenolic compounds, leading to their partial precipitation (Somers, 1971; Timberlake and Bridle, 1976b; Ribéreau-Gayon *et al.*, 1983; Somers and Wescombe, 1987). The polymerization of anthocyanins has never been unequivocally confirmed by spectroscopic analyses (^{13}C NMR) and remains an active area of research.

In experiments with model solutions, using known pure anthocyanins and other phenolic compounds, similar effects were observed and it was concluded that acetaldehyde has little action on anthocyanins alone but a pronounced effect when other phenolic compounds are also present. It was previously suggested (Timberlake and Bridle, 1976a; Baranowski and Nagel, 1983; Roggero *et al.*, 1987; Bakker *et al.*, 1993) that the color increase was due to the formation of highly colored new compounds, involving anthocyanin and phenolic compounds linked by a $CH(CH_3)$ bridge. The reaction mechanism and the initial position of the linkage have been proposed. The increased color and violet shift can be attributed to the formation of an anhydro base.

The anthocyanin studies were limited to those with malvidin 3-glucoside—the predominant anthocyanin in wine—because of current interest in the relationship between color and quality in red wines. However, interactions between anthocyanins and phenolic compounds of several types were studied in model solution by Timberlake and Bridle (1976a, 1977) using TLC and spectral measurements.

The aim of the present work is to extend the investigation of these condensation reactions, using several types of anthocyanins and procyanidin B_2 [(−)-epicatechin-(4β→8)-(−)-epicatechin]. We studied interactions between cyanidin 3-glucoside, peonidinin 3-glucoside, malvidin 3-glucoside, and procyanidin dimer B_2, with or without the presence of acetaldehyde. High-performance liquid chromatography (HPLC) was used to measure the degradation of anthocyanins and procyanidin concentration and diode-array spectroscopy (DAS) to characterize the new polymeric pigments.

MATERIALS AND METHODS

Samples. Cyanidin 3-glucoside, peonidin 3-glucoside, and malvidin 3-glucoside chlorides and (−)-epicatechin were commercial samples obtained from Extrasynthese (Lyon, France). Procyanidins B_2 and C_1 (homogeneous trimer of epicatechin) were separated from a mixture of grape seed oligomeric procyanidins by gel chromatography and purified by preparative HPLC (Ricardo-da-Silva *et al.*, 1991). Purification levels of all the anthocyanins and procyanidins B_2 and C_1 were tested by analytical reverse-phase HPLC.

Gel Chromatography. Fractionation of the grape seed extract in order to isolate procyanidins B_2 and C_1 was performed on a column (350 × 25 mm i.d.) filled with Fractogel TSK HW-40(s), particle size 0.025−0.040 (Merck, Darmstadt, Germany). Elution was carried out with methanol, using a Pharmacia system (Bromma, Sweden) including a pump of medium pressure (P-500), a UV−vis detector (LKB 2151) and

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a fraction collector (LKB Frac-100). The absorbance of the eluate was measured at 280 nm and the flow rate was 1.2 mL/min. Seven fractions containing various oligomeric procyanidins were collected (Ricardo-da-Silva *et al*., 1991), concentrated under vacuum, and dissolved in CH_3OH/H_2O (50/50 v/v).

Preparative HPLC. A Merck Model L-6200A pump (Merck-Hitachi, Darmstadt, Germany) equipped with a manual Rheodyne injector was used. Detection was made at 280 nm with a Konik UV−visible detector (UV/VIS 200) (Konik Instruments SA, Miami, FL) and the column (250 × 10 mm i.d.) was a reverse-phase Spherisorb (Hichrom) C_{18} (5 μm). The solvents were (A) 2.5% acetic acid, and (B) acetonitrile/2.5% acetic acid (80/20 v/v), and the flow rate was 2 mL/min. A linear gradient was run from 7% B to 30% B during 45 min, and then to pure B for 10 min. The peaks were collected manually at the time of their elution, and each fraction was evaporated under vacuum and dissolved in a model wine solution. Identification of procyanidins B_2 and C_1 was performed by complete acid hydrolysis and partial acid-catalyzed degradation with phloroglucinol according to the method described by Ricardo-da-Silva *et al*. (1991).

Analysis of Anthocyanins. The apparatus was a Perkin-Elmer (Norwalk, CT) system, equipped with a 410-LC pump, a solvent programmer Model 420, and a manual injector (Rheodyne 7125-A) fitted with a 20 μL loop. The column (250 × 4 mm, particle size 5 μm) was a reverse-phase Superspher 100 (Merck), C_{18} protected by a guard column of the same material. The solvents were (A) 40% formic acid, (B) pure acetonitrile and (C) bidistilled water. The initial conditions were 25% A, 10% B, and 65% C, followed by a linear gradient from 10 to 30% B, and 65 to 45% C for 40 min, with a flow rate of 1 mL/mn, and the detection was made at 520 nm.

A Konik diode-array detector (wavelength range 366−800 nm) coupled to a Konichrom data treatment station (Konik Instruments SA) was used to obtain visible spectra of the different compounds present in model solutions.

Analysis of Procyanidins. Analysis of samples were performed with the same chromatographic and detection system (280 nm) employed for preparative HPLC. The column (250 × 4 mm, particle size 5 μm) was a reverse-phase C_{18}, Superspher 100 (Merck) protected by a guard column of the same material, and the flow rate was 0.9 mL/min.

Model Solutions. Anthocyanins and procyanidin B_2 were dissolved in a solution containing 5g/L of tartaric acid, 12% (v/v) ethanol and adjusted at pH= 3.2. The reactants were used in amounts calculated to give the following final concentrations: anthocyanins 0.3 mM (134.7 mg/L, 138.9 mg/L, 147.9 mg/L respectively for cyanidin-3 glucoside, peonidin-3 glucoside, and malvidin-3 glucoside), procyanidin B_2 1.01 mM (584 mg/L), and acetaldehyde (0.2% v/v, 35.8 mM). The solutions were prepared under nitrogen and then put into tubes (3 mL of each sample in 5 mL vials), flushed with nitrogen, and sealed. Six different experimental mixtures were prepared for each anthocyanin, filtered (0.45 μm), and dispensed into six sets of test tubes as follows: (1) anthocyanin alone; (2) anthocyanin + acetaldehyde; (3) anthocyanin + procyanidin B_2; (4) anthocyanin + procyanidin B_2 + acetaldehyde; (5) procyanidin B_2 alone; (6) procyanidin B_2 + acetaldehyde.

All the samples were kept in the dark for 4 months at controlled temperature (22 °C) and analyzed in duplicate (daily for the solutions containing acetaldehyde).

Standardization. Standard solutions containing increasing concentrations of cyanidin 3-glucoside chloride, peonidin 3-glucoside chloride, malvidin 3-glucoside chloride, and procyanidin B_2 were directly injected (in triplicate) into the HPLC system. The calibration lines were determined by least-squares regression analysis.

RESULTS AND DISCUSSION

Several reactions were expected to occur in the model wine solutions studied, containing pure anthocyanins (cyanidin 3-glucoside chloride, peonidin 3-glucoside chloride, malvidin 3-glucoside chloride), procyanidin B_2 (B_2), and acetaldehyde. The reactions to be considered are mainly the condensations between anthocyanins and B_2 with or without an acetaldehyde bridge, but at least four other reactions can occur simultaneously: (i) degradation of anthocyanins, (ii) transformation of B_2, (iii) reaction between anthocyanins and acetaldehyde, and (iv) reaction between B_2 and acetaldehyde.

Table 1. First-Order Apparent Reaction Rates for Disappearance of Anthocyanins in Model Wine Solutions

model solutions[a]	$k = \Delta \ln C/\Delta T$ (day^{-1})	R^2	probability of the model (P)
cyanidin 3-gluc alone	0.005	92	<0.001
cyanidin 3-gluc + acet	0.081	96	<0.001
cyanidin 3-gluc + B_2	0.009	98	<0.001
cyanidin 3-gluc +B_2 + acet	0.420	99	<0.001
peonidin 3-gluc alone	0.005	99	<0.001
peonidin 3-gluc + acet	0.071	96	<0.001
peonidin 3-gluc + B_2	0.009	95	<0.001
peonidin 3-gluc + B_2 + acet	0.526	99	<0.001
malvidin 3-gluc alone	0.006	95	<0.01
malvidin 3-gluc + acet	0.077	96	<0.001
malvidin 3-gluc + B_2	0.009	99	<0.001
malvidin 3-gluc + B_2 + acet	0.340	94	<0.001

[a] gluc, glucoside; acet, acetaldehyde.

Degradation of Phenolic Compounds Alone. Anthocyanins monoglucosides are highly reactive flavonoids. Reactivity is due to anthocyanins formed by several well-known reactions for which equilibria are published (Brouillard and Delaporte, 1977). These involve the heterocyclic ring, and the most important anthocyanin forms are the red flavylium salt, the purple anhydro base, the colorless carbinol base, and the chalcone. During storage a decline of the anthocyanin color is often observed, and hydrolytic or oxidative reactions can be responsible for these alterations.

Anthocyanins (3-glucosides of cyanidin, peonidin, and malvidin) react in model wine solution (without procyanidins) very slowly. We observed a slight decrease in anthocyanin concentrations, and these losses were found to be logarithmic with time. The reaction rates with respect to the disappearance of anthocyanins presented in Table 1 were apparently equal ($k = 0.005-0.006$ day^{-1}).

Procyanidin B_2, alone in a model wine solution, was partly transformed into epicatechin and trimer C_1 (both identified by co-injection) (Figure 1) and other compounds (probably more complex procyanidins), showing that C−C bond breaking and C−C bond forming of procyanidins can occur under such mild conditions, as it was suggested by Timberlake and Bridle (1976a) and Haslam (1977).

Reaction between Anthocyanins and Acetaldehyde. The addition of acetaldehyde to model solutions containing cyanidin 3-glucoside, peonidin 3-glucoside, and malvidin 3-glucoside, separately, increased the color intensity accompanied by a shift toward violet. One new compound was detected by HPLC in each model solution (Figure 2).

Acetaldehyde probably reacts as an electrophile at the 8 or 6 position of the anthocyanin A-ring, with the formation of polymers consisting of monoglucoside units linked by a $CH(CH_3)$ bridge. It is evident that many types of polymers are possible since both anthocyanins and procyanidins contain two reactive positions (6 and 8). Timberlake and Bridle (1976a) in previous studies found that the addition of acetaldehyde to solutions containing anthocyanins alone gave a reaction when the latter was malvidin 3-glucoside, and it was largely

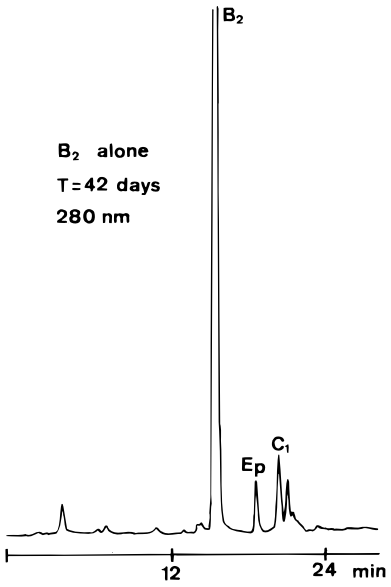

Figure 1. HPLC chromatogram of a model wine solution containing procyanidin B_2 alone, recorded at 280 nm: B_2, procyanidin dimer; Ep, (−)-epicatechin, C_1, procyanidin trimer.

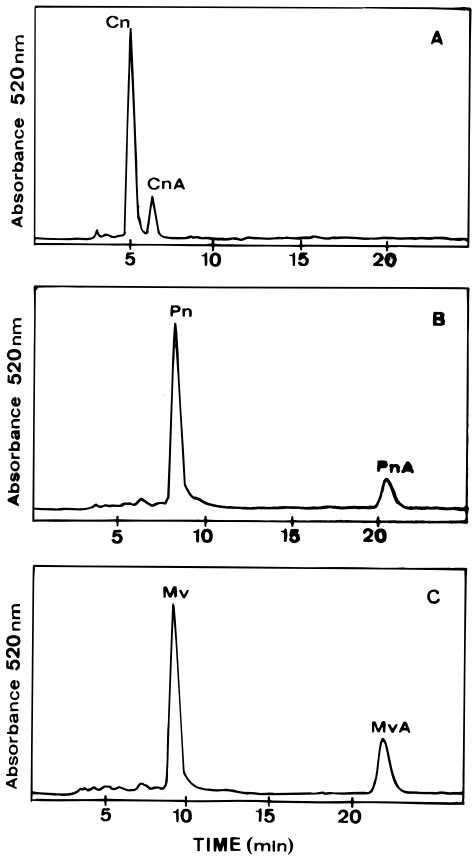

Figure 2. HPLC chromatograms of the three model wine solutions containing anthocyanins alone and acetaldehyde recorded at 520 nm: (A) Cn, cyanidin 3-glucoside, CnA, new product; (B) Pn, peonidin 3-glucoside, PnA new product; (C) Mv, malvidin 3-glucoside, MvA, new product.

without effect on malvidin 3,5-diglucoside and malvidin 3-*p*-coumarylglucoside-5-glucoside.

From our experiments, a logarithmic loss of the anthocyanin concentration was observed in each model solution, and the reaction rates are presented in Table 1.

Cyanidin 3-glucoside decreases faster (k = 0.081 day^{-1}) than the 3-glucosides of peonidin and malvidin

Table 2. First-Order Apparent Reaction Rates for Disappearance of Procyanidin B_2 in Model Wine Solutions

model solutions[a]	$k = \Delta \ln C/\Delta T$ (day^{-1})	R^2	probability of the model (P)
B_2 alone	0.0033	97	<0.05
B_2 + acetaldehyde	0.2980	99	<0.001
B_2 + cyanidin 3-gluc	0.0039	93	<0.001
B_2 + peonidin 3-gluc	0.0035	94	<0.001
B_2 + malvidin 3-gluc	0.0044	99	<0.006
B_2 + cyanidin 3-gluc + acet	0.3100	99	<0.003
B_2 + peonidin 3-gluc + acet	0.3690	99	<0.001
B_2 + malvidin 3-gluc + acet	0.3500	98	<0.001

[a] acet, acetaldehyde; gluc, glucoside.

Table 3. Visible Absorption Maxima of the New Condensation Compounds As Recorded by Diode-Array Spectroscopy

model solutions[a]	symbol	retention time (min)	λ_{max} exptl (nm)
cyanidin 3-gluc alone	Cn	4.9	517
cyanidin 3-gluc + acet	CnA	6.1	523
cyanidin 3-gluc + B_2 + acet	$CnBA_1$	3.5	529
	$CnBA_2$	6.6	521
peonidin 3-gluc alone	Pn	7.4	518
peonidin 3-gluc + acet	PnA	21.5	525
peonidin 3-gluc + B_2 + acet	$PnBA_1$	5.1	530
	$PnBA_2$	8.7	535
malvidin 3-gluc alone	Mv	9.8	528
malvidin 3-gluc + acet	MvA	23.7	534
malvidin 3-gluc + B_2 + acet	$MvBA_1$	11.9	541
	$MvBA_2$	13.3	544

[a] gluc, glucoside; acet, acetaldehyde.

(k = 0.071 and 0.077 day^{-1}, respectively), and in each model solution a precipitation was observed after several days. The new pigments, labeled CnA, PnA, and MvA (Table 3) were monitored by HPLC and their maximum absorption wavelengths were measured by diode-array spectroscopy. CnA showed a maximum at 523 nm while PnA and MvA showed absorption maxima at 525 and 534 nm, respectively (Figure 2).

A model solution, containing B_2 and acetaldehyde, showed that B_2 was not detectable in model solution after 15 days. Although no new compound was detectable by HPLC at 280 nm (in our analysis condition), B_2 may have formed a polidisperse group of procyanidin reaction products below the limits of detection.

Reaction of Anthocyanins with B_2. It is well-known that anthocyanins formed co-pigment complexes with flavonoids and other compounds at pH ranging from 2 to 5 (Asen *et al.*, 1972).

Model solutions containing B_2 and various anthocyanins were examined over a 120 day period. A gradual loss of color in the red region (520 nm) was accompanied by an increase of color in the 420−450 nm region. The eventual formation of xanthylium salts by direct condensation of phenolic compounds and anthocyanins had been proposed by Timberlake and Bridle (1976a) and Ribereau-Gayon (1973). Linkage must be between position 4 of the anthocyanins and position 8 or 6 of the phenolic compounds. Evidence of the formation of xanthylium salts has been reported by Jurd and Somers (1970). A model solution containing a leucocyanidin−phloroglucinol condensation product yielded a yellow pigment (λ_{max} 453 nm) after acid treatment.

During 4 months of storage no new compound was detected by HPLC at 520 nm under our analysis conditions and with cyanidin, peonidin, or malvidin 3-glucoside, anthocyanins concentration decreased very slowly. HPLC chromatograms showed that B_2 has been

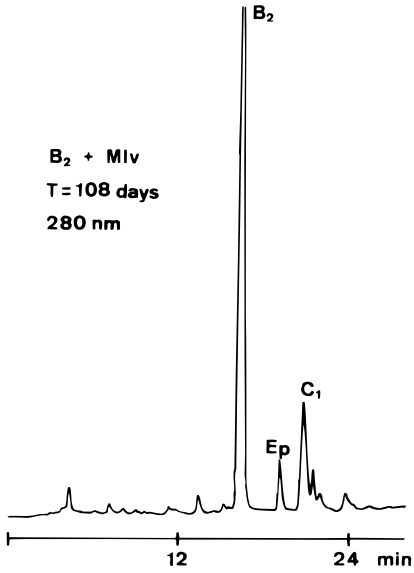

Figure 3. HPLC chromatogram of the model solution containing malvidin-3 glucoside and procyanidin B_2 recorded at 280 nm: B_2, procyanidin dimer; Ep, (−)-epicatechin, C_1, procyanidin trimer.

partly transformed into epicatechin and other procyanidins (Figure 3), but that occurred also in the control solution containing B_2 alone (Figure 1).

The reaction rates calculated for the disappearance of B_2 both in the control solution and in the presence of anthocyanins are presented in Table 2. The rate of loss of B_2 alone ($k = 0.0033$) was always slightly lower than in mixtures containing, separately, the three anthocyanins. On the other hand, reaction rates calculated for the disappearance of the various anthocyanins both in the control solution (anthocyanins alone) and in mixtures with procyanidin B_2 (Table 1) showed some differences between them. In fact, reaction rates for all three anthocyanins in model solutions containing procyanidin B_2 were always equal to 0.009 day^{-1}, while these reactions rates were lower in solutions containing anthocyanins alone ($k = 0.005$ for cyanidin 3-glucoside and peonidin 3-glucoside and $k = 0.006$ for malvidin 3-glucoside). So, this probably suggests that some interaction between the various anthocyanins and procyanidin B_2 had occurred but were not detected under the analytical conditions used in this work.

Reaction of Anthocyanins with B_2 and Acetaldehyde. Interactions between the anthocyanins and procyanidin B_2 were monitored by HPLC, and a general decrease in the concentration of the anthocyanins and B_2 were observed. The losses of the anthocyanins and B_2 were found to be logarithmic with time. The reaction rates k (day^{-1}), calculated following a first-order reaction, are shown in Tables 1 and 2.

Significant differences in the reaction rates ($P < 0.05$) between peonidin 3-glucoside and cyanidin 3-glucoside were observed (0.53 and 0.42 day^{-1}, respectively), the compounds differing only by the nature of the substituent group in the 3′ position (OH for cyanidin 3-glucoside, OCH_3 for peonidin 3-glucoside).

Also significant differences in the reaction rates ($P < 0.05$) between peonidin 3-glucoside and malvidin 3-glucoside ($k = 0.53$ and 0.34 day^{-1}, respectively) were observed. These compounds differing only by the degree of methylation on the B-ring (two methoxyl groups at the 3′ and 5′ positions for malvidin 3-glucoside and only one methoxyl group at the 3′ position for peonidin 3-glucoside).

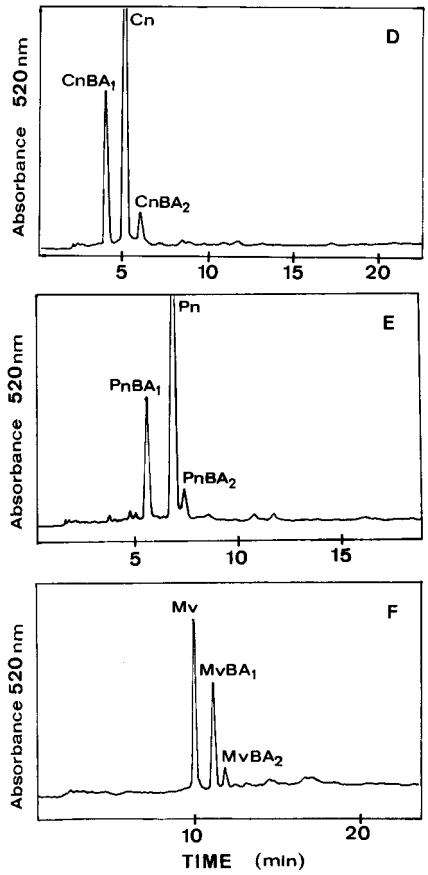

Figure 4. HPLC chromatograms of the three model wine solutions containing anthocyanins, procyanidin B_2, and acetaldehyde recorded at 520 nm: (D) Cn cyanidin 3-glucoside, $CnBA_1$ and $CnBA_2$ new products; (E) Pn, peonidin 3-glucoside, $PnBA_1$ and $PnBA_2$ new products; (F) Mv, malvidin 3-glucoside, $MvBA_1$ and $MvBA_2$ new products.

In the case of cyanidin 3-glucoside, the color of the solution increased rapidly and continuously and became more violet in the presence of acetaldehyde. Two new polymeric pigments distinguishable from cyanidin 3-glucoside were formed and labeled $CnBA_1$ and $CnBA_2$ (Figure 4D). The formation of the new compounds began after several hours, reaching a maximum after 2 days (9.2 and 3.2 mg/L expressed respectively as cyanidin 3-glucoside), and disappeared after 12 days. These two new compounds could be the result of the condensation reaction of B_2 with cyanidin 3-glucoside linked by acetaldehyde, following a mechanism described before by Timberlake and Bridle (1976a). Acetaldehyde reacts probably with procyanidin B_2 at position 8 rather than 6. The resulting compound reacts more favorable to position 8 of the anthocyanins than position 6, by analogy with simple flavylium salt behavior (Timberlake and Bridle, 1977) and also because of the higher negative ground state charge of position 8 (Bendz *et al.*, 1967). Hence, it is possible that $CnBA_1$, always present at the highest concentration, is a trimer linked via position 8 of malvidin 3-glucoside and the corresponding flavanol−acetaldehyde product, while $CnBA_2$ is a trimer corresponding to one of the other possibilities.

The spectra of the new compounds were obtained by diode-array detector. The spectrum of cyanidin 3-glucoside showed an absorption maximum at 517 nm in accordance with previous studies (Dallas and Laureano, 1994), while compound $CnBA_1$ showed a λ_{max} at 529 nm and $CnBA_2$ a λ_{max} at 521 nm (Table 3).

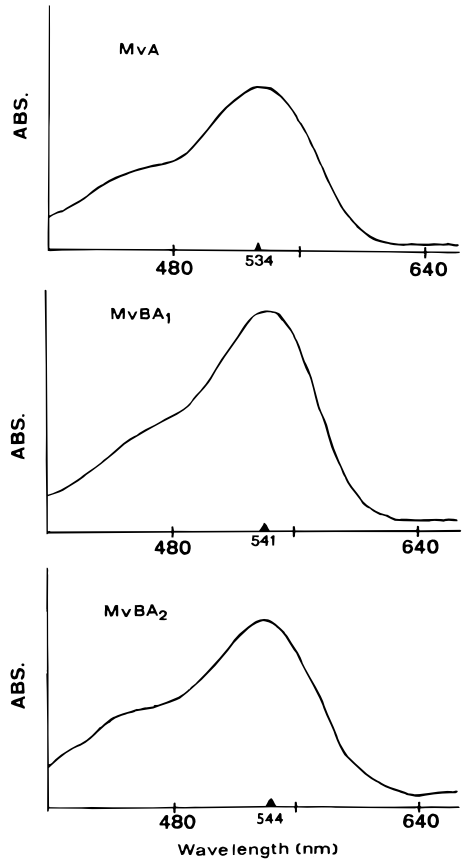

Figure 5. Visible absorption spectra of different condensation compounds, recorded by diode array spectrophotometric detection, formed in model wine solutions: (1) MvA product formed in a model wine solution with malvidin 3-glucoside and acetaldehyde; (2) $MvBA_1$and $MvBA_2$ products formed in a model wine solution containing malvidin 3-glucoside, B_2, and acetaldehyde.

The peonidin 3-glucoside concentration decreased faster than the other two anthocyanins and it was not detectable after 12 days. The formation of two new colored compounds, $PnBA_1$ and $PNBA_2$ (Figure 4E), began simultaneously after 1 h, and the largest amounts of these compounds were present at 2 days (12 and 3.3 mg/L expressed respectively as peonidin 3-glucoside). $PnBA_1$ and $PnBA_2$ then began to decrease and were not detectable after 10 days. The bathochromic shifts in the visible region of these two products (530 and 535 nm for $PnBA_1$ and $PnBA_2$, respectively) were higher than that observed for the products $CnBA_1$ and $CnBA_2$. Considerations about the chemical structure of $PnBA_1$ and $PnBA_2$ are similar to those mentioned before for cyanidin derivatives.

Different behavior was shown in the last model solution, where malvidin 3-glucoside decreased much more slowly ($k = 0.34$ day^{-1}) than the other two anthocyanins, and disappeared after 15 days. Moreover, only one product ($MvBA_1$) was detectable by HPLC at the beginning, in contrast to the other model solutions where the two polymers were formed at the same time. $MvBA_1$ first accumulated to reach a maximum concentration after 2 days (19.4 mg/L expressed as malvidin 3-glucoside) and disappeared after 12 days. The other compound ($MvBA_2$) was detectable (Figure 4F) only after 5 days and reached its maximum levels on the day 6 (1.7 mg/L expressed as malvidin 3-glucoside).

The greatest shift in λ_{max} occurred for product $MvBA_2$ (544 nm), while the maximum absorption wavelength for product $MvBA_1$ was 541 nm (Figure 5).

To establish the unequivocal chemical structure of compounds $CnBA_1$, $CnBA_2$, $PnBA_1$, $PnBA_2$, $MvBA_1$, and $MvBA_2$ identification by spectroscopic measurements (NMR ^{13}C, ^1H) is absolutely necessary.

At the end of our experiments, the three solutions were still colored, although there were no discrete compounds detectable by HPLC. This effect may be explained by the fact that the compounds ($CnBA_1$, $CnBA_2$, $PnBA_1$, $PnBA_2$, $MvBA_1$, $MvBA_2$) polymerize to higher molecular weight colored compounds in the presence of acetaldehyde (Haslam and Lilley, 1988). When this condensation became too great, the pigments became large enough to be insoluble and a precipitate was observed. In all our model solutions some precipitations were observed over 10−12 days.

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