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Formation of new stable pigments from condensation reaction between malvidin 3-glucoside and (−)-epicatechin mediated by acetaldehyde: Effect of tartaric acid concentration

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Abstract

The objective of this work was to study the effect of tartaric acid concentration on the condensation reaction between malvidin 3-glucoside (Mv-glc) and flavanols mediated by acetaldehyde in the model solution. The model wine solutions were prepared by 12% ethanol in water (v/v) with two different L-tartaric acid concentrations (5 g/l and 25 g/l, respectively) and at two different pH values (3.2 and 1.7, respectively). Four new pigments were detected in model wine solutions containing Mv-glc, (−)-epicatechin and acetaldehyde. By reverse-phase HPLC-DAD, ESI-MS and MS^n fragmentation analysis, the four new pigments were tentatively identified as four isomers of hydroxyethyl malvidin-3-glucoside-ethyl-flavanol. The decrease in the concentration of Mv-glc and (−)-epicatechin and the increase in the concentration of the new identified pigments were more pronounced at higher tartaric acid concentration. At pH 1.7, although the two well-recognized ethyl-linked Mv-glc-flavanol isomers were quantitatively the major pigmented products in the reaction solution throughout the assay period, they appeared less stable than the four new pigments. At pH 3.2, the rate of formation of ethyl-linked Mv-glc-flavanol pigments was much slower than at pH 1.7, whereas the four new pigments were quantitatively the predominant pigmented products at the latter stage of the reaction.

Keywords: Malvidin 3-glucoside; (−)-Epicatechin; Acetaldehyde; Tartaric acid; Condensation reaction

1. Introduction

It is well known that the reaction of anthocyanins and flavanols is one of the most important reactions during ageing and storage of red wine. The newly formed pigments are more stable than their anthocyanins precursors, and present distinct sensory properties. Two major ways of anthocyanins and flavanols reactions have been reported: direct reaction and indirect reaction (Timberlake & Bridle, 1976). Both of these two types of reactions have been intensively studied during the last years (Cheynier, 2002; Dueñas, Fulcrand & Cheynier, 2006; Es-Safi, Cheynier & Moutounet, 2000; Fulcrand, Atanasova, Salas & Cheynier, 2004, chap. 6; Pissarra, Mateus, Rivas-Gonzalo, Santos-Buelga & De Freitas, 2003; Rivas-Gonzalo, Bravo-Haro & Santos-Buelga, 1995; Salas, Fulcrand, Meudec & Cheynier, 2003).

For direct reaction between anthocyanins and flavanols, two mechanisms have been proposed. One is the formation of anthocyanin–flavanol (A^+–T) adduct, and another one is the formation of flavanol–anthocyanin (T–A^+) adduct. The mechanisms of formation of T–A^+ adduct and A^+–T adduct are presented in Fig. 1.

In the formation of A^+–T adduct, anthocyanin is in the flavylium form and acts as an electrophile. Nucleophilic addition of the flavanol onto the flavylium cation produces the colourless flavene, which can be either oxidized to the red flavylium, or proceed to a colourless cyclic condensation product.

In the formation of T–A^+ adduct, procyanidins release the intermediate carbocation by acid-catalyzed cleavage of their interflavanic bond, which acts as an electrophile,

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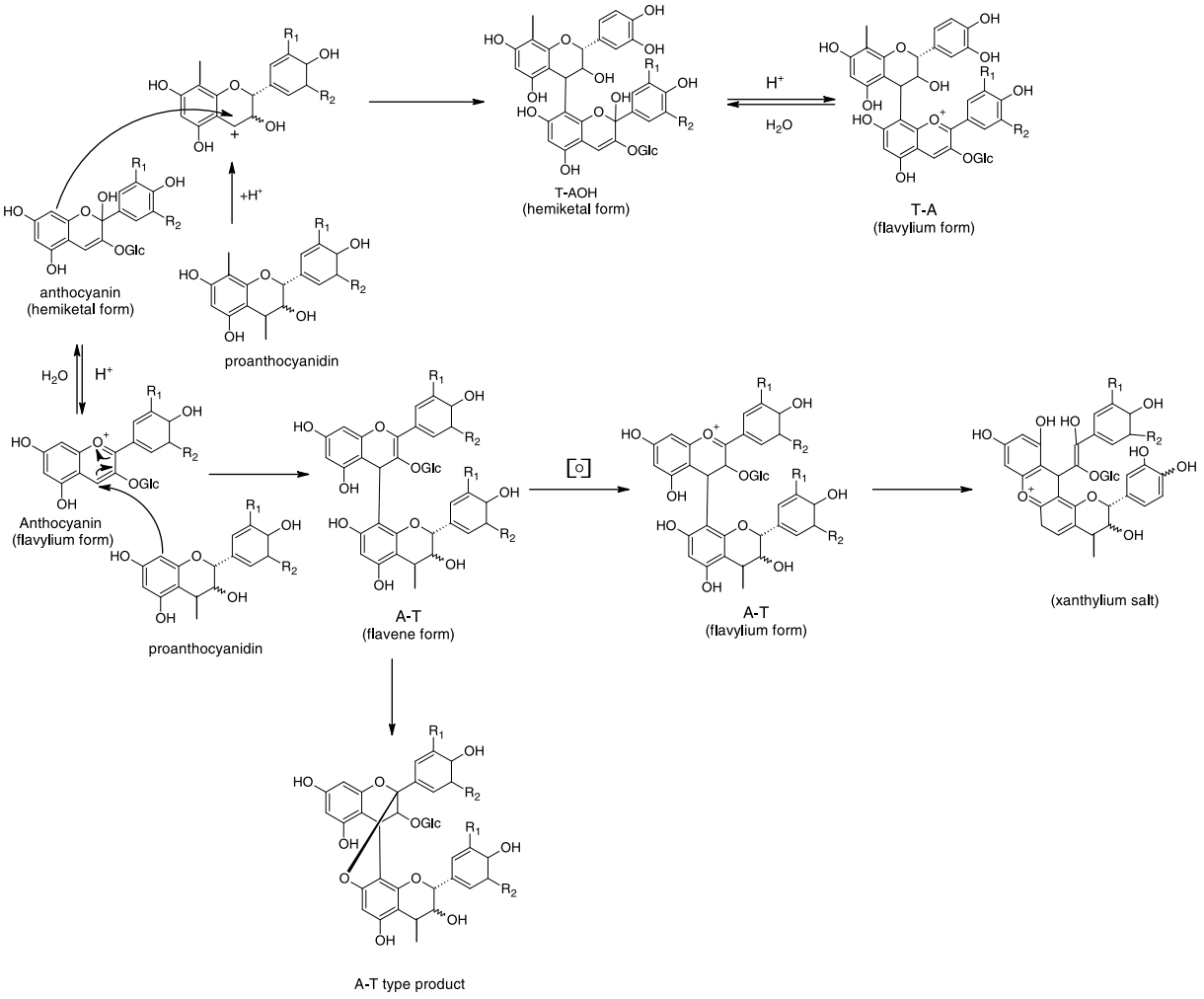

Fig. 1. Hypothetical mechanisms of direct reaction between anthocyanins and flavanols in wine (Cheynier, 2002).

while the anthocyanin, in its hemiketal form, acts as a nucleophile. The produced colourless dimer in the hemiketal form will further dehydrate to the red flavylium form.

For indirect reaction between anthocyanins and flavanols mediated by acetaldehyde, the reaction begins with nucleophilic addition of the flavanol on protonated acetaldehyde. The product thus formed loses a water molecule to give a new carbocation intermediate which, proceeds nucleophilic addition by anthocyanins in the hemiketal form, to produce ethyl linked T–A adducts. The nucleophilic addition may also occur by another flavanol, to ethyl-linked T–T adduct. Moreover, cycloaddition of anthocyanins with acetaldehyde can also occur, producing orange pigment. Fig. 2 presents the hypothetical mechanisms of indirect reaction between anthocyanins and flavanols mediated by acetaldehyde.

It has been well documented that the ratio of anthocyanin to flavanol, pH and temperature, oxygen availability and other co-factors like metal ions can affect anthocyanin–flavanol interactions (Dueñas et al., 2006; Es-Safi, Fulcrand, Cheynier & Moutounet, 1999; Guerra, 1997; Remy, Fulcrand, Labarbe, Cheynier & Moutounet, 2000; Salas et al., 2003). However, it was not clear if the different tartaric acid concentration can also affect such reactions. The motive that we verified the effect of tartaric acid concentration on this reaction is because some red wines, such as one of the characteristic wines in Portugal – "Vinho Verde" wines – often contain very high amounts of tartaric acid.

2. Materials and methods

2.1. Chemicals

Mv-glc was isolated from Pinot Noir grape skins as described previously (Sun, Santos, Leandro, De Freitas, & Spranger, 2007). (−)-Epicatechin was purchased from Fluka A. G. (Buchs, Switzerland). Acetaldehyde (analytical grade) was obtained from Merck (Darmstadt, Germany). All organic solvents were of HPLC grade.

2.2. Preparation of model solution

Model solution used for the reaction was prepared as described previously (Sun et al., 2007). Furthermore, 12%

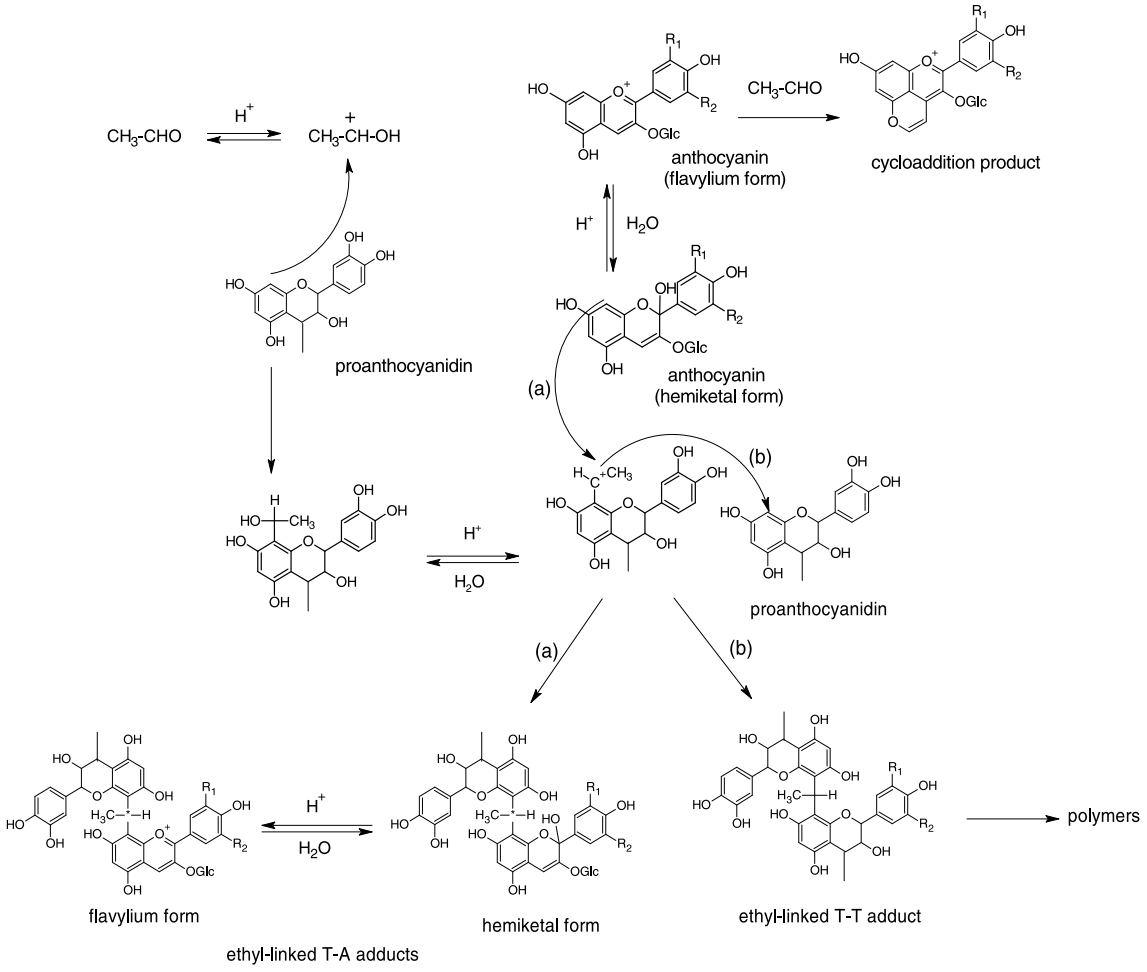

Fig. 2. Hypothetical mechanisms of indirect reaction between anthocyanins and flavanols mediated by acetaldehyde in wine (Cheynier, 2002).

ethanol in water (v/v) was acidified with L-tartaric acid at concentrations of 5 g/l and 25 g/l, respectively. Two pH values, 3.2 and 1.7, of the model solution were chosen, which were adjusted by addition of 1 N of HCl or 1 N of NaOH. The two pH values 3.2 and 1.7 represent, respectively, the pH of red wine and the pH at which anthocyanins are present essentially in the flavylium form. For each pH value, the reaction medium was composed of Mv-glc, (−)-epicatechin and acetaldehyde in molar ratio 1:5:11. The molar ratio of anthocyanins/flavanols (1:5) used in this study represents a red wine rich in these compounds (Guerra, 1997). The reaction solution was kept at 30 °C in brown glass vials. No special attention to oxygen exposure was paid during the reaction. The reaction products were monitored periodically by reverse-phase HPLC-DAD and ESI-MS under the conditions as described below.

2.3. Reverse-phase HPLC with photodiode array detection

The Reverse-phase HPLC-DAD analysis used for monitoring the reaction products was a Waters system, equipped with a quaternary pump (Waters 600), a Controller (Waters 600), a thermostat controlling the column temperature and an autosampler (Waters 717 plus) and a Photodiode Array detector (Waters 996) coupled to a data processing computer – Millennium 32. The column (250 × 4 mm) was a cartridge of 4 μm Superspher 100 RP18 (Merck). The mobile phase flow-rate was fixed at 0.7 ml/min. throughout this study. The detection was ranged from 200 to 780 nm, being 525 nm for detection of anthocyanins and their derivatives and 280 nm for detection of all types of polyphenols. The column temperature was set at 30 °C. Two elution solvents A (formic acid/water; 10/90; v/v) and B (acetonitrile/water/formic acid; 30/60/10; v/v/v) were used. Elution program was as follows: gradient elution from 18% to 47% of B for 42 min, isocratic elution with 47% of B for 5 min, gradient elution from 47% to 100% of B for 30 min, followed by isocratic elution with 100% B during 33 min. The column was washed by acetonitrile/water/formic acid (70/20/10) for 10 min, followed by re-equilibration to the initial conditions. Detection wavelength ranged from 250 to 600 nm. Injection volume was 30 μl.

2.4. ESI-MS analysis

Identification of newly-formed coloured and non-coloured products in the reaction solution was performed by

ESI-MS and MS^n fragmentation analyses. Mass spectra were recorded from $m/z = 150$ to $m/z = 3000$ in a positive and negative ionization mode, respectively. Auto MS (4) with the fragmentation amplitude 0.85 and enhanced scan resolution (5.500 m/z/s) were selected. Voltages for the skimmer and the capillary were −40 and +4000 V for negative ion mode or 40 to −4000 V for positive ion mode, respectively. Other MS conditions were as follows: nebulizer gas (N_2), 30 psi; drying gas (N_2), 10 l/min; dry temperature 300 °C.

3. Results and discussion

Table 1 presents the effect of tartaric acid concentration and pH value of the reaction medium on the reactivity of Mv-glc towards (−)-epicatechin in the presence of acetaldehyde. There is no surprise to note in Table 1, as already confirmed previously by various authors (Dueñas et al., 2006; García-Viguera, Bridle & Bakker, 1995; Pissarra et al., 2003), that the reactions between flavanols and malvidins 3-glucoside are highly related to pH values: the lower the pH value of reaction medium is, the higher rate constant of any of these reactions. However, this work also showed that such reactions were also dependent on tartaric acid concentration of the reaction medium. The decrease in the concentration of Mv-glc and flavanols and the formation of new coloured products are more pronounced at the higher tartaric acid concentration.

Fig. 3 shows the typical HPLC chromatograms of the reaction solutions of Mv-glc with epicatechin mediated by acetaldehyde at pH 1.7. It can be noted that the profile of HPLC chromatogram at the initial stage of the reaction (Fig. 3Y) is similar to those of already published works (Es-Safi & Cheynier, 2004; Rivas-Gonzalo et al., 1995). The two major peaks A and B are well known compounds, namely the two isomers of ethyl-linked flavanol-Mv-glc condensation products, which were identified several years ago by various authors (Bakker, Picinelli & Bridle, 1993; Escribano-Bailón, Dangles & Brouillard, 1996; Mateus, Pascual-Teresa, Rivas-Gonzalo, Santos- Buelga & De Freitas, 2002; Rivas-Gonzalo et al., 1995). In this work, we confirm the identity of Peaks A and B. The UV–vis spectra of peaks A and B can be distinguished from that of Mv-glc; the wavelengths of their maximum absorption (λ_{max}) in the visible region were 537 nm and 542 nm, respectively, shifted to higher wavelength. Moreover, both spectra feature a shoulder (landing) at about $\lambda = 460$ nm, a characteristic of the ethyl linkage of indirect anthocyanidins–flavanols condensation products. MS analysis of Peaks A and B in a positive ion mode gave an ion at m/z 809, corresponding to the molecules of ethyl-linked flavanol-Mv-glc condensation products. Furthermore, MS^n fragmentation of the ion at m/z 809 gave the confirmation of the identity of such compounds: MS^2 fragmentation of the ion at m/z 809 produced major ion at m/z 519, corresponding to the loss of one molecule of (−)-epicatechin (−290 amu). MS^3 fragmentation of the ion at m/z 519 gave ion at m/z 357, corresponding to the loss of a glucose moiety (−162 amu).

Table 1
Rate constant of reaction of Mv-glc and (−)-epicatechin mediated by acetaldehyde at different pH values and different tartaric acid concentrations

Tartaric acid concentration (g/l)	pH 1.7			pH 3.2		
	Rate constant K (h^{-1})	R^2	SD	Rate constant K (h^{-1})	R^2	SD
5	2.31	0.9952	0.0801	0.0950	0.9952	0.0025
25	2.37	0.9922	0.1048	0.1440	0.9914	0.0051

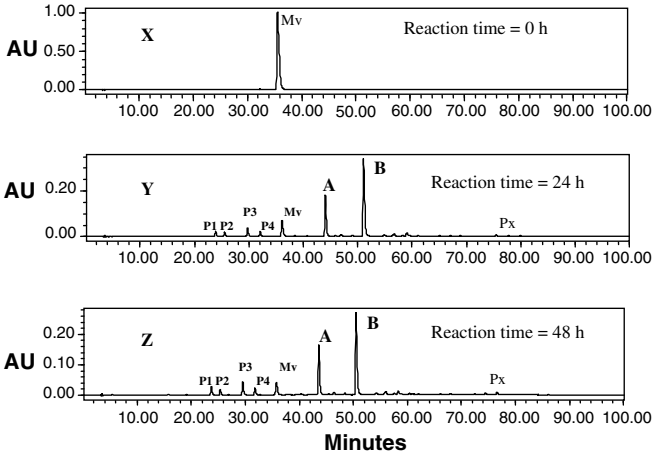

Fig. 3. HPLC chromatograms of the reaction solutions of Mv-glc with (−)-epicatechin mediated by acetaldehyde at pH 1.7.

Also, it is no surprise to find, as reaction time increases (Fig. 3Z), various small peaks appear after the major pigments A and B. As reported by other authors, these peaks are attributed to higher polymeric pigments (Es-Safi & Cheynier, 2004), such as ethyl-linked trimer, tetramer condensation products, containing two flavanol units and one

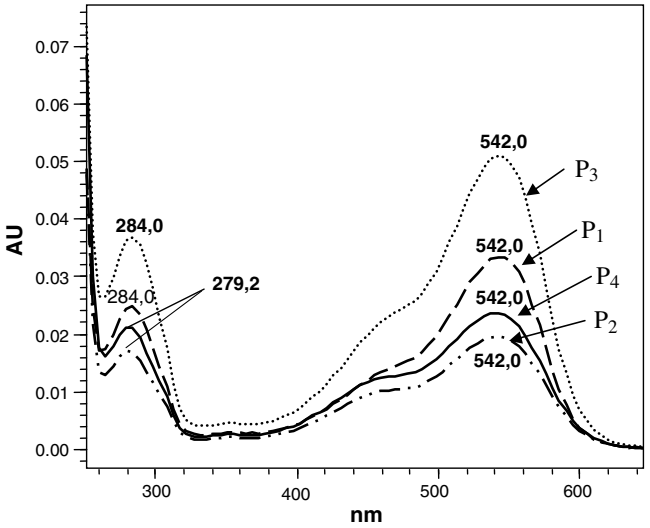

Fig. 4. UV–vis spectra of peaks P_1, P_2, P_3 and P_4 of HPLC chromatogram in Fig. 3.

Mv-glc unit (*m*/*z*: 1125 amu), one flavanol unit and two Mv-glc units (*m*/*z*: 664 amu), three flavanol units and one Mv-glc unit (*m*/*z*: 1441 amu), two flavanol units and two Mv-glc units (*m*/*z*: 822 amu). Moreover, pyranoanthocyanins are eluted much later than anthocyanin–procyanidin complexes (Sun et al., 2007).

However, what is worth noting is that as reaction time increases, four additional peaks appear before the peak of Mv-glc, i.e., peaks P_1, P_2, P_3 and P_4, which correspond to new pigments not identified previously. The good separation of these four peaks corresponding to new pigments, in this work, which appeared before the peak of Mv-glc, is probably due to the very gradual elution program we used (see Section 2). The UV–vis spectra of these four peaks are similar, with the maximum absorption at 542 nm, shifted to higher wavelength than that of Mv-glc (Fig. 4).

It is interesting to note that the spectra of these four peaks are similar to those of A and B. All these four peaks gave the same maximum absorption in the visible region (542 nm, exactly identical to that of peak B), shifted to higher than that of Mv-glc. These data would suggest that these four peaks were also condensation products from the interaction between epicatechin and Mv-glc mediated by acetaldehyde, with structures similar to A and B.

Furthermore, all these four peaks present, in their mass spectrum in positive mode, an ion at *m*/*z* 853. This ion suggests that they are four isomers of a compound already identified in our previous work (Sun et al., 2007), i.e., hydroxyethyl Mv-glc-ethyl-flavanol. We confirmed its identity by multiple MS^n fragmentation analysis. Fig. 5 presents the hypothetical structure of this compound.

It is worth mentioning that in our previous work, the obtained HPLC chromatogram of the same reaction medium did not show any peak before Mv-glc, but only one, not well-resolved peak corresponding to hydroxyethyl Mv-glc-ethyl-flavanol which appeared after Mv-glc peak. In this work, by using very gradual HPLC elution

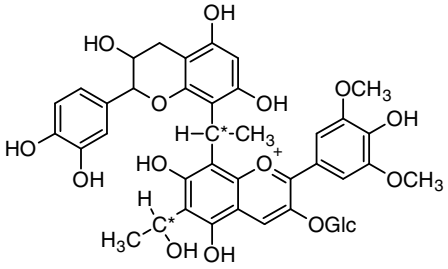

Fig. 5. Hypothetical structure of P_1 to P_4.

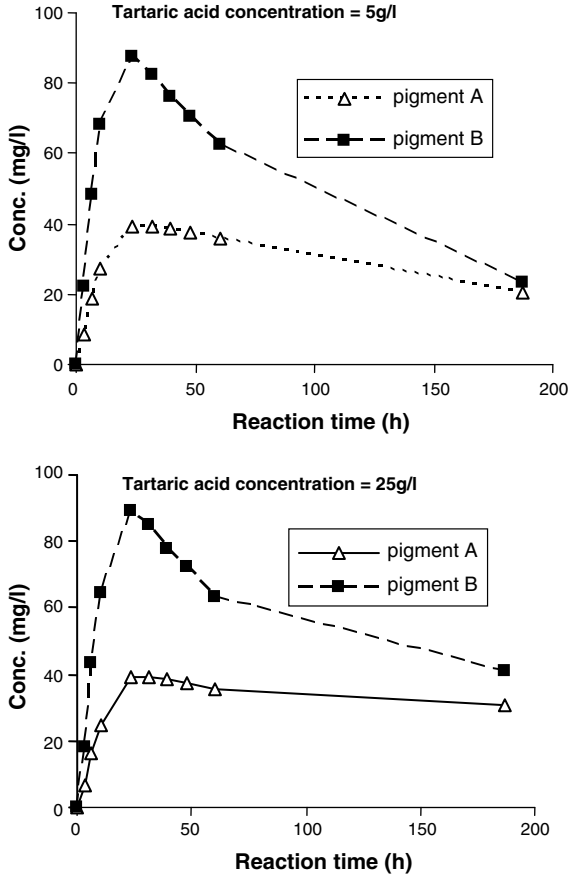

Fig. 6. Evolution of pigments A and B during the reaction between Mv-glc and (−)-epicatechin in the presence of acetaldehyde at pH 1.7 and at different tartaric acid concentration.

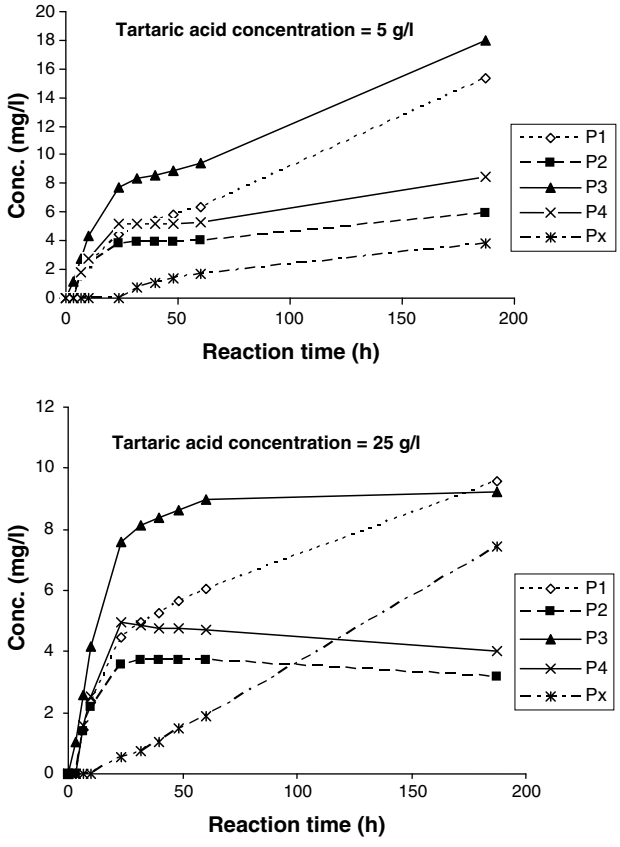

Fig. 7. Evolution of pigments P_1–P_4 and P_x during the reaction between Mv-glc and (−)-epicatechin in the presence of acetaldehyde at pH 1.7 and at different tartaric acid concentration.

program, we have succeeded in good separation of the four isomers of such compound and all of them appeared before the peak of Mv-glc. Consequently, the baseline of HPLC chromatogram was much improved, thus permitting their more precise identification and quantification.

Because the hypothetical structure has two asymmetric carbons (C^*), it is reasonable to suggest that these four peaks are two pairs of enantiomers. According to the profile of HPLC chromatogram (Fig. 3) and their specific spectra (Fig. 4), we may suggest that one pair of enantiomers are P_1 (*trans*) and P_2 (*cis*) and another pair of enantiomers are P_3 (*trans*) and P_4 (*cis*). In fact, Fig. 4 showed that in the UV region the maximum absorption of P_1 and P_3 are the same (284.0 nm), while P_2 and P_4 present the same maximum absorption in this region (279.2 nm). However, for full identification of these four peaks, NMR analysis is needed, which permit to confirm the position of ethyl and hydroxyethyl group.

The peak P_x is another pigment with much higher retention time than Mv-glc. The maximum absorption in its UV–vis spectrum in the visible region is 508 nm. The hypsochromic shift of the maximum absorption let us suggest that this compound belongs to the pyranoanthocyanin group. The MS analysis of this peak, in positive ion mode, gave an ion at m/z 805 in its mass spectrum, which corresponds to a compound of Mv-glc-vinyl-flavanol. Furthermore, its identity was confirmed by MS^n fragmentation analysis. MS^2 fragmentation of the ion at m/z 805 gave a major ion at m/z 643, corresponding to the loss of one glucose moiety (−162 amu). MS^3 fragmentation of the ion at m/z 643 gave the ion at m/z 491, corresponding to the loss of the fragment released by the retro Diels–Alder decomposition (−152 amu). The presence of such compounds in red wine, grape pomace and model wine solution has been proposed previously by other authors (Asenstorfer, Hayasaka & Jones, 2001).

Fig. 6 presents the evolution of pigments A and B during the reaction between Mv-glc and epicatechin in the presence of acetaldehyde at pH 1.7 and different tartaric acid concentration.

From this figure, we may see clearly that at pH 1.7, the major pigments A and B reach their maximum concentration at 24 h and then decrease continuously, both at 5 g/l and 25 g/l of tartaric acid. However, this decrease appears less pronounced at higher tartaric acid concentration. In other words, pigment A and B appeared more stable at higher tartaric acid concentration.

For newly-identified compounds (Fig. 7), the concentrations of P_1 and P_3 increase continuously throughout the reaction period, while P_2 and P_4 reach their maximum concentration at earlier stage of the reaction and then appeared more stable. Moreover, more decrease in

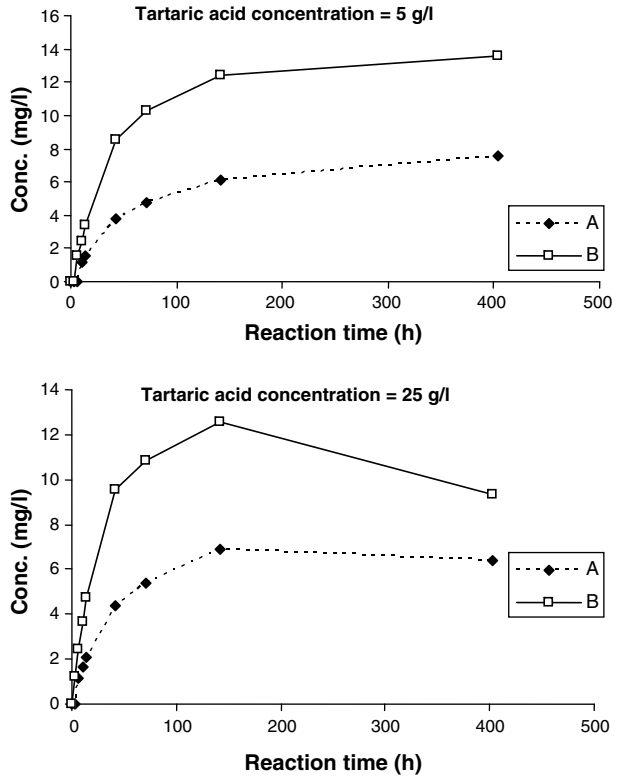

Fig. 8. Evolution of pigments A and B during the reaction between Mv-glc and (−)-epicatechin in the presence of acetaldehyde at pH 3.2 and at different tartaric acid concentration.

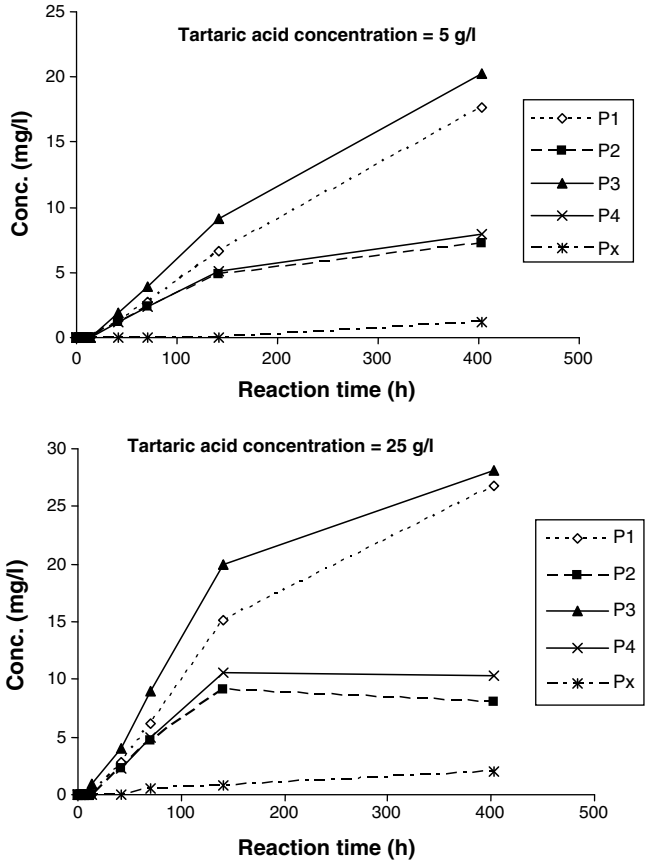

Fig. 9. Evolution of pigments P_1–P_4 and P_x during the reaction between Mv-glc and (−)-epicatechin in the presence of acetaldehyde at pH 3.2 and at different tartaric acid concentration.

pigments A and B, led to higher amount of formation of the newly-identified pigments, particularly P_1 and P_3.

At pH 3.2, that is to say at wine pH value, the formation of pigments A and B are much slower than at pH 1.7 (Fig. 8). It can be also observed from Fig. 8 that at the initial stage of the reaction, the rates of formation of pigments A and B were identical at two different tartaric acid concentrations, while at the later stage of reactions, the pigments A and B appeared more degraded at higher tartaric acid concentration.

As compared with those at pH 1.7, the pigments P_1–P_4 and P_x newly-formed in the reaction conducted at 3.2 can reach relatively high concentration, particularly P_1 and P_3 (Fig. 9). Quantitatively the suggested four isomers, i.e., pigments P_1–P_4, are predominant pigments in the latter stage of the reaction because the total amounts of these four pigments were much higher than total amounts of other major pigments, i.e., pigments A and B and Mv-glc. Moreover, tartaric acid concentration can affect significantly the formation of these newly-identified pigments. At tartaric acid concentration of 25 g/l, the concentrations of these newly-identified pigments are nearly twice of those at tartaric acid concentration at 5 g/l. In addition, even at 400 h of reaction, the amounts of P_1 and P_3 were continuously increased. In addition, degradation of pigment A appeared positively correlated with the formation of P_1 and P_3.

From these results, the formation of newly-identified pigments P_1–P_4 might be suggested to be originated from the reaction of compounds A and B with acetaldehyde, namely hemiketal form of A and B with protonated acetaldehyde. Although we have no further chemical evidence for supporting this suggestion, statistical analysis showed that for the reaction conducted at pH 1.7 and at 5 g/l of tartaric acid concentration, the degradation of pigment A was very significantly correlated to the formation of pigment P_1 (correlation coefficient = −0.9988) and to that of the sum of pigments P_1 and P_2 (correlation coefficient = −0.9980), while the degradation of pigment B was significantly correlated to that of formation of pigment P_3 (correlation coefficient = −0.9751) and to that of sum of pigments P_3 and P_4 (correlation coefficient = −0.9702). Thus, the formation pathway of P_1–P_4 may be supposed as shown in Fig. 10.

We may also suggest, in the similar reaction solution containing oligomeric procyanidins, the presence of similar pigments to P_1–P_4, but with two or more flavanol units in their structures. In fact, in the previous work (Sun et al., 2007), we used HPLC-ESI-MS to analyse a reaction solution containing Mv-glc, dimer procyanidins and acedaldehyde and we did detect two peaks in the HPLC chromatogram which gave the same positive ion at m/z 1141, corresponding to the structure identical to P_1–P_4, but with two flavanol units (Sun et al., 2007).

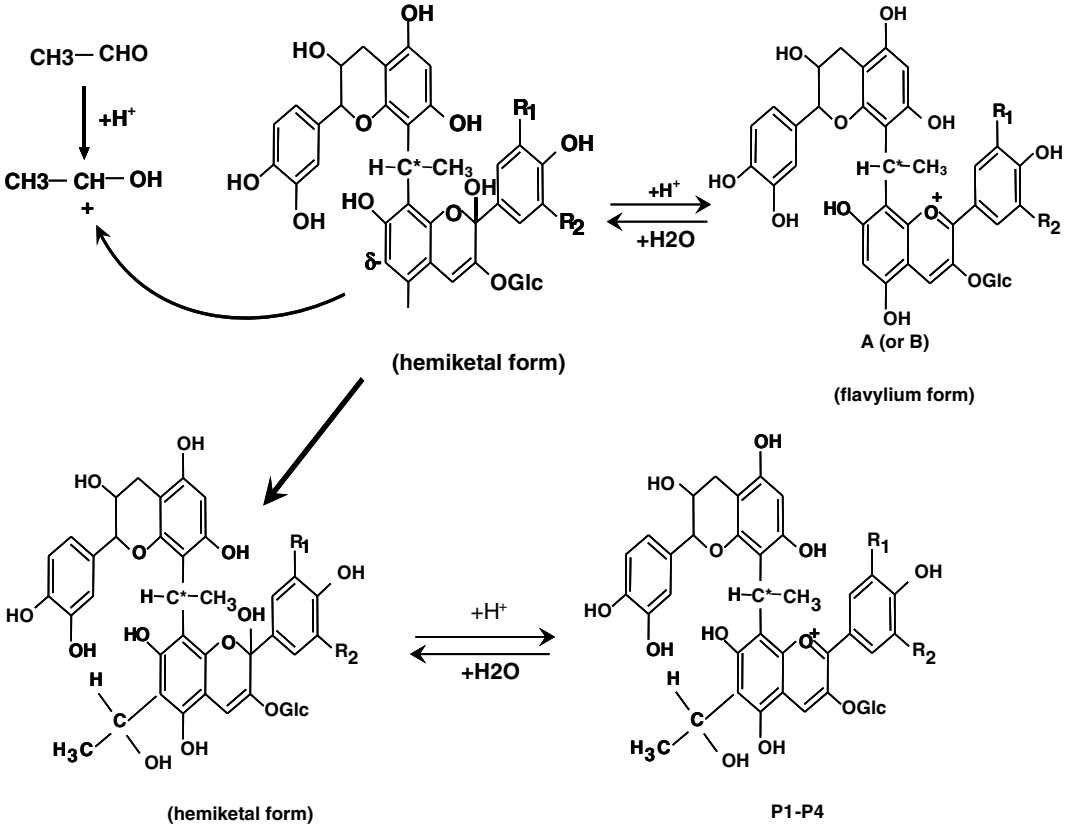

Fig. 10. Proposed formation pathway of P_1–P_4 in Fig. 3.

4. Conclusions

In conclusion, the four new pigments detected in model wine solutions containing Mv-glc, flavanols and acetaldehyde appeared more stable than the two well-recognized isomer pigments A and B, i.e., ethyl-linked Mv-glc-flavanol, throughout the reaction period. At wine pH value (pH 3.2), the four new pigments were quantitatively the predominant pigmented products at the latter stage of the reaction. Thus, from a practical point of view, such pigments may play an important role in sensory properties of red wine during the ageing process. Further works to confirm the structures of the four new pigments by NMR (i.e., the position of ethyl linkage and hydroxyethyl group) and their formation mechanisms, and to identify these or similar compounds in red wine are under way in our laboratory.

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