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Grape anthocyanin oligomerization: A putative mechanism for red color stabilization?

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ABSTRACT

The equilibrium forms of malvidin-3-*O*-glucoside trimer present in grape skins were studied in aqueous solution at different pH values through UV–Visible spectroscopy. It was observed that the reactivity of this compound is strongly dominated by acid–base chemistry (pK_{a1} = 3.61 ± 0.03; pK_{a2} = 6.83 ± 0.06), with the reaction sequence hydration–tautomerization–isomerization accounting less than 10% of the overall reactivity. The decrease of hydration of this flavylium cation derivative when compared to the original anthocyanins results from the formation of a cluster of the pigment with a high-energy of solvation that inhibits the access of water molecules to the flavylium cation core preventing by this way the hydration reactions. Overall of these results raise the hypothesis that polymerization may be a natural stabilization mechanism for the red color of anthocyanins.

1. Introduction

Anthocyanins are polyphenolic compounds present in many flowers and fruits being responsible for the great diversity of colors (red, violet and blue) found in Nature. Their color depend on their structure and is strongly affected by pH when extracted into solution (Brouillard and Lang, 1990). At very acidic pH (pH ~1) anthocyanins present a very intense red color and are present in their flavylium cation form. With the increase of pH for values up to 3–4 anthocyanins are hydrated at 2- or 4-position of the anthocyanidin moiety leading to the formation of the non-colored hemiketal form (Brouillard, 1982; Goto and Kondo, 1991; Kondo et al., 1992). This form is also in equilibrium with the *cis* and *trans*-chalcone forms presenting a yellowish color. For pH values up to 6 the violet neutral quinoidal form is formed from the deprotonation of the flavylium cation form and for higher pH values a second deprotonation equilibrium occurs originating the equilibrium between the neutral and the anionic quinoidal forms (Brouillard and Dubois, 1977; Brouillard and Lang, 1990; Pina, 1998; Santos et al., 1993). Nevertheless, it seems that nature has found many mechanisms to stabilize the colored forms of anthocyanins. These mechanisms include auto-association (self-assembling) (Gonzalez-Manzano et al., 2008; Mistry et al., 1991), intra and inter-molecular co-pigmentation (Brouillard, 1983; Dangles et al., 1993a,b; Goto and Kondo, 1991; Mistry et al., 1991; Yoshida et al., 2009), metal complexation (Dangles et al., 1994; Goto and Kondo, 1991; Yoshida et al., 2009) and reaction with other molecules leading to the formation of more stable anthocyanin-derived compounds (Cruz et al., 2010; Oliveira et al., 2013b, 2014, 2009, 2011). The mechanism and extend of stabilization is greatly affected by the structure of anthocyanin but also by the compounds present in the nearby environment.

For instance, the blue color presented by some flowers is the most intriguing question and has been the subject of numerous studies over the years (Goto and Kondo, 1991; Katsumoto et al., 2007; Kondo et al., 1992). It is well-known that anthocyanins present a blue color in basic pH conditions, however it is also known that the color is very unstable and fades with time (Goto and Kondo, 1991). Hayashi and colleagues have dedicated their work to the isolation of the blue pigments (named commelinin) directly from the petals of blue dayflower *Commelina communis* (Hayashi, 1958). The structure of commelinin was reported to be a supramolecular metal complex composed by malonylawobanin, flavocommelin and Mg^{2+} ions in a 6:6:2 ratio (Tamura et al., 1986). Similar results were reported for the protocyanin pigment of blue cornflower *Centaurea cyanus* but in this case with Fe^{3+} and Mg^{2+} ions (Goto and Kondo, 1991; Goto et al., 1986). Over the years

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other metalloanthocyanins have been described to be responsible for the blue color of some flowers (Ishikawa et al., 1999; Mori et al., 2008; Takeda et al., 1994). In other cases, the blue color presented by flowers was claimed to be due to the intramolecular co-pigmentation observed between polyacylated anthocyanins molecules. Polyacylated anthocyanins which contain two or more aromatic acyl residues (caffeoyl, *p*-coumaroyl, feruloyl and *p*-hydroxybenzoyl) linked to the sugar moiety can interact in a intramolecular sandwich-type stacking between the aromatic acyl residues and the anthocyanidin chromophore being responsible for their higher stability and the blue color (Goto et al., 1982; Saitô et al., 1971; Yoshida et al., 1992). In other matrices, such as wines and especially young red wines the red color of the flavylium cation of anthocyanins is stabilized by auto-association (Asen et al., 1972; Gonzalez-Manzano et al., 2008) and intermolecular co-pigmentation mechanisms (Asen et al., 1972; Brouillard and Dangles, 1994; Davies and Mazza, 1993; Gonzalez-Manzano et al., 2009; Liao et al., 1992; Osawa, 1982). Both mechanisms rely on hydrophobic and hydrogen-bond interactions between the anthocyanin molecules in self-assembling (Asen et al., 1972; Gonzalez-Manzano et al., 2008) and between anthocyanins with other non-colored compounds such as flavones, flavonols and flavanols in intermolecular copigmentation (Asen et al., 1972; Brouillard and Dangles, 1994; Davies and Mazza, 1993; Gonzalez-Manzano et al., 2009; Liao et al., 1992). However, during wine ageing and maturation the concentration of anthocyanins starts to decrease leading to the formation of several anthocyanin-derivatives such as A and B-type vitisins and other pyranoanthocyanins that have been described in the literature over the years (Bakker and Timberlake, 1997; Fulcrand et al., 1996; He et al., 2006; Oliveira et al., 2010; Schwarz et al., 2003a,b). Those compounds formed present a more stable color than their precursors (anthocyanins) (Bras et al., 2011; Cruz et al., 2010; Oliveira et al., 2013b, 2014, 2009, 2011). Recently, it was detected and isolated from a 2 years-old Port wine a trimeric malvidin-3-*O*-glucoside compound which is extracted from grape skins during winemaking (Oliveira et al., 2013a). The aim of this work is to study the equilibrium forms of this trimeric anthocyanin (Fig. 1) in aqueous solutions at different pH values in order to evaluate the influence of anthocyanin polymerization in the proton transfer and hydration constants of this kind of compounds.

2. Results and discussion

2.1. pH dependent behaviour in aqueous solution; color stabilisation

Anthocyanins, and flavylium compounds in general, undergo in aqueous solution a series of reactions responsible for the known pH dependence of their colors (Brouillard and Dubois, 1977; Brouillard and Lang, 1990; Melo et al., 2009; Pina et al., 2012) (Fig. 2). In contrast with the strong orange to pink hues of the flavylium cation ($\mathbf{AH^+}$) and the red to blue hues of the quinoidal base (**A**) and anionic quinoidal base ($\mathbf{A^-}$) deprotonated forms, the hemiketal (**B**) is colorless and the chalcones (**Cc** and **Ct**) are usually yellowish. These species are connected through a network of reactions (Fig. 2) that take place in different time scales. The proton transfer reactions to form **A** and $\mathbf{A^-}$ occur in microseconds; the hydration of $\mathbf{AH^+}$ to form **B** occurs in the subsecond to minutes time range, depending on pH; the cycle-chain tautomerization leading to **Cc** occurs in seconds and finally, **Cc** isomerizes to **Ct** in minutes to hours, depending on the substituents in the 2-phenyl-1-benzopyrylium core. After reaching the final thermodynamic equilibrium, a global apparent equilibrium constant, K'_a, between $\mathbf{AH^+}$ and all the other species (**CB**), Eqs. (1)–(3), can be determined (Leydet et al., 2012; Melo et al., 2009). The value pK'_a corresponds to the pH where upon thermal equilibration 50% of the flavylium cation has been transformed into the other species; it is a measure of the color stability of the flavylium species with pH: the higher the pK'_a the more stable is the flavylium towards deprotonation (K_a) and hydration (K_h), Fig. 2.

$$\mathbf{AH^+} + H_2O \leftrightharpoons \mathbf{CB} + H_3O^+ \quad (1)$$

$$[\mathbf{CB}] = [\mathbf{A}] + [\mathbf{B}] + [\mathbf{Cc}] + [\mathbf{Ct}] \quad (2)$$

$$K'_a = K_a + K_h + K_hK_t + K_hK_tK_i \quad (3)$$

In order to see the initial distribution of species in trimeric malvidin-3-*O*-glucoside compound **1** (Fig. 1), a batch UV–Vis spectrophotometric pH titration was carried out through a series of pH jumps from a mother solution in 0.1 M HCl to higher pH values and the spectra run in ca. 1 min (Fig. 3). The spectra red-shift with increasing pH showing one set of isosbestic points up to pH ~5 that evolves to a second set of isosbestic points for pH > 5, suggesting the existence of three species and two pK_a's (Fig. 3A). Simultaneous fitting of the absorbances at three wavelengths allows to obtain $pK_{a1} = 3.61 \pm 0.03$ and $pK_{a2} = 6.83 \pm 0.06$ (Fig. 3B). These pK_a values correspond to the deprotonation of one of the phenol groups in the malvidin-3-glucoside core of **1** to form a zwitterion in resonance with the quinoidal base **A** (Fig. 2) and to the deprotonation of the second phenol group to yield $\mathbf{A^-}$. The deprotonation order proposed in Fig. 2, where the OH group in position 5 of the flavylium moiety is the first to deprotonate followed by the OH group in position 4′, is based on ^{1}H NMR data (see Supplementary content). The other five phenol groups of **1** are located in the two neutral flavan moieties and are expected to deprotonate at higher pH values (pH > 8) with concomitant changes in absorption spectra closer to the UV region. Deconvoluting the data in Fig. 3A further allows to obtain the individual spectra of $\mathbf{AH^+}$, **A** and $\mathbf{A^-}$ (Fig. 3C) with maxima at 546, 568 and 632 nm, respectively.

The absorption maximum of compound **1** (546 nm) is significantly red-shifted in comparison with that of the parent compound malvidin-3-glucoside (oenin) that has $\lambda_{max} = 519$ nm in water (Houbiers et al., 1998; Leydet et al., 2012; Lima et al., 2002). This is indicative of an environment less polar than water, since the flavylium chromophore, characterized by π–π^* transitions with strong charge-transfer character, presents negative solvatochromism, i.e., the electric dipole moment decreases upon going from the ground to the lowest excited state and the transition energy decreases with decreasing polarity (Mataga and Kubota, 1970). The absorption maximum is indeed closer to that of oenin when stabilized into SDS micelles (536 nm) (Lima et al., 2002). The observed red-shift is thus compatible with a structure where the flavylium core of **1** is wrapped around by the two flavan moieties, as also suggested by the computational studies reported in the literature (Oliveira et al., 2013a). The presence of an A-type linkage in the malvidin-3-glucoside moiety of compound **1** increases the electron donor ability of ring A relatively to the parent malvidin-3-glucosidefurther contributing to the observed red-shift of the lowest energy transition.

After the fast proton transfer reactions that transform $\mathbf{AH^+}$ into **A** and $\mathbf{A^-}$ upon adequate pH jumps, the other reactions in Fig. 2 enter into play until the final thermodynamic equilibrium is reached. To assess the kinetics of these latter processes, the time spectral evolution after each pH jump was carried out (Fig. 4). Except for the more acidic pH's values where the flavylium form is stable (pH $\lesssim$ 2), partial conversion of the acid–base forms ($\mathbf{AH^+}$, **A** and $\mathbf{A^-}$) into the other species of the network is observed. The extents of these conversions range from ca. 2% at pH ~3 (Fig. 4A) to ca. 9% at pH ~6 (Fig. 4C) and are strikingly small in comparison with common anthocyanins (Leydet et al., 2012; Pina, 1998) and flavylium salts in general (Pina et al., 2012). In

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