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# Effect of water content on the acid–base equilibrium of cyanidin-3-glucoside

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#### ABSTRACT

Laser Flash Photolysis was employed to measure the deprotonation and reprotonation rate constants of cyanidin 3-monoglucoside (kuromanin) in water/methanol mixtures. It was found that the deprotonation rate constant  $k_d$  decreases with decreasing water content, reflecting the lack of free water molecules around kuromanin, which may accommodate and stabilize the outgoing protons. On the other hand, the reprotonation rate constant,  $k_p$ , increases with the decrease in water concentration from a value of  $k_p = 2 \times 10^{10} \, \mathrm{I \, mol^{-1} \, s^{-1}}$  in water up to  $k_p = 6 \times 10^{10} \, \mathrm{I \, mol^{-1} \, s^{-1}}$  at 5.6 M water concentration in the mixture. The higher value of  $k_p$  at lower water concentrations reflects the fact that the proton is not freely escaping the solvation shell of the molecule. The deprotonation rate constant decreases with decreasing water content, reflecting the lack of free water molecules around kuromanin that can accommodate the outgoing protons. Overall, the acidity constant of the flavylium cation decreases with the decrease in water concentration from  $pK_a$  values of 3.8 in water to approximately 4.8 in water-depleted media, thus shifting the equilibrium towards the red-coloured form, **AH**<sup>+</sup>, at low water contents.

The presence, or lack, of water, will affect the colour shade (red to blue) of kuromanin. This is relevant for its role as an intrinsic food component and as a food pigment additive (E163).

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## 1. Introduction

Cyanidin 3-monoglucoside (kuromanin) belongs to the anthocyanin family of natural polyphenols and is the major pigment present in passion fruit rind (Kidoey et al., 1997). Like all anthocyanins, kuromanin undergoes several equilibria in aqueous media, which affect the colour displayed by the pigment as shown in Scheme 1 (Pina, Melo, Laia, Parola, & Lima, 2012).

The equilibrium between the two coloured forms (flavylium cation,  $AH^+$ , and quinoidal bases, A, see Scheme 1), is responsible for the shading between red (flavylium cation,  $AH^+$ ) and blue (quinoidal base, A) observed in the fruit. Upon light absorption by the pigment, the excited state acid–base reaction provides a pathway for energy dissipation which aids in pigment photostability and photoprotection of the plant cells (Ferreira da Silva et al., 2012).

The effect of the solvent medium on the multiequilibria of anthocyanins is of relevance, since in nature the pigment accumulates in vacuoles, where the composition is different from dilute aqueous solutions, and the presence of other organic components constant than pure water. Several works focusing on the effect of non-aqueous media on the hydration reaction leading to colour loss exist in the literature (Gomes, Parola, Lima, & Pina, 2006; Lima et al., 2002; Pina et al., 2012; Vautier-Giongo et al., 2002). The major purpose of these works has been to find ways to displace the hemiketal (**B**)-flavylium equilibrium towards **AH**<sup>+</sup>, either by stabilization of **AH**<sup>+</sup> in anionic micelles, or through co-pigmentation, thus promoting the existence of the coloured species at higher pH values. However, the effect of the medium (low water concentration, low dielectric constant) on the shading of the colour, i.e., in the equilibrium between the coloured forms **AH**<sup>+</sup> and **A**, has not been tackled to date. It was shown previously that ultrafast excited state conversion of flowilum extings into their respective bases provides an efficient

provides microheterogeneous environments of lower dielectric

of flavylium cations into their respective bases provides an efficient tool to probe the ground-state interconversion between these species (Maçanita et al., 2002). Because deprotonation of  $AH^{**}$  (where \* stands for excited state) occurs with 99% efficiency (in less than 10 ps) and  $A^*$  decays to the ground-state in less than 300 ps, absorption of a ns-laser pulse by  $AH^+$  generates a transient ground-state concentration of A which is superior to its ground state equilibrium concentrations of  $AH^+$  and A can be conveniently







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followed by transient absorption spectroscopy and follows a first order law with an observed rate constant  $k_{obs}$ , which is the sum of the direct and reverse rate constants (Eq. (1)), (Moreira et al., 2003).

$$k_{\rm obs} = k_{\rm d} + k_{\rm p} [\rm H^+] \tag{1}$$

#### 2. Materials and methods

Kuromanin (cyanidin-3-monoglucoside, PM 484.84 for the chloride salt,  $\varepsilon_{510} = 26,900 \text{ M}^{-1} \text{ cm}^{-1}$  in 0.1 M HCl) was isolated from the rind of purple passion fruit *Passiflora edulis* using standard separation procedures. Purity was assessed *via* reversed phase HPLC (Hypersil ODS C18) with detection at 520 nm (>99% peak area) and at 280 nm (>95% peak area) and by mass spectrometry.

Samples used in the various experiments were prepared by adding HCl to a kuromanin solution in distilled water. An aliquot of the aqueous solution of known pH was then mixed with methanol (CHROMASOLV<sup>®</sup>, Sigma–Aldrich) to obtain a final volume of 5 ml with the correct H<sub>2</sub>O:MeOH proportions [volume MeOH:H<sub>2</sub>O ratios of 0:100 (55.5 M H<sub>2</sub>O), 50:50 (27.7 M H<sub>2</sub>O), 70:30 (16.6 M H<sub>2</sub>O) and 90:10 (5.55 M H<sub>2</sub>O)]; final anthocyanin concentrations within the sample varied from  $1 \times 10^{-5}$  M to  $4 \times 10^{-5}$  M. pH values were determined for the samples prepared in water, before the addition of methanol, using a calibrated pH metre (MeterLab pHM240 pH metre from Radiometer Copenhagen or using a Crison BasiC 20 pH metre combined with a Mettler Toledo InLab Micro Ag/AgCl microelectrode).

The [H<sup>+</sup>] values used in the correlations are calculated from the dilution factor and the measured pH in the aqueous phase  $[H^+] = (V_{H_2O}/V_{mix})[H^+]_{aq}$ . For the measurements of the  $pK_a$  of the kuromanin in the water:methanol mixtures at 20 °C, the determination of the <sup>s</sup><sub>s</sub>pH (i.e., the –log  $a_{H^+}$  in solvent mixture) was carried out as described previously (Castells, Ràfols, Rosés, & Bosch, 2003).

Flash photolysis experiments were carried out at room temperature using the LK60 Laser Flash Photolysis equipment from Applied Photophysics (Pinheiro, Parola, Baptista, & Lima, 2010), with laser excitation at 532 nm (SHG), and monitoring the recovery of the ground-state protonated flavylium absorption band at 510 nm and the decay of the quinoidal base at 570 nm. The observed rate constants ( $k_{obs}$ ) were obtained through the global fitting of the kinetic traces with single-exponential recovery or decay functions, respectively. The values of  $k_{obs}$  used in the linear representations against [H<sup>+</sup>] are averages of 3 independent measurements, the error in the individual values is always less than 5%.

#### 3. Results and discussion

#### 3.1. Multiequilibria of kuromanin in water

Absorption spectra of kuromanin aqueous solutions equilibrated overnight at moderately acidic conditions are shown in Fig. 1.

At pH 2 the absorption spectrum of the flavylium cation ( $AH^*$ ) dominates, with an absorption maximum at 512 nm. At pH 5.07, the equilibrium is completely shifted towards the base (A), hemiketal (B) and chalcones ( $C_E$  and  $C_Z$ ), with absorption maxima at 550, 270 and 330 nm, respectively. The equilibrium concentration of the quinonoidal base is very low, since the overall equilibrium is shifted mainly towards the colourless forms (hemiketal, B and chalcones,  $C_E$  and  $C_Z$ , Scheme 1), which absorb below 350 nm.

Laser Flash Photolysis of aqueous solutions of kuromanin at pH values below 4 ( $\lambda_{exc}$  = 532 nm) results in bleaching of the flavylium cation absorption at 512 nm and the appearance of a positive  $\Delta$ OD assigned to the S<sub>1</sub>  $\leftarrow$  S<sub>0</sub> absorption of **A**, formed during the flash (Fig. 2a).

The positive absorption bands formed after the laser flash are due to the formation of the quinoidal base in the ground state with a concentration that is in excess with respect to the ground state equilibrium. The base form thus reacts to yield the flavylium cation, re-establishing the ground state equilibrium concentrations for that particular pH. Both the recovery of the flavylium and



Scheme 1. Multiequilibiria of cyanidin-3-glucoside in moderately acidic aqueous solutions.

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