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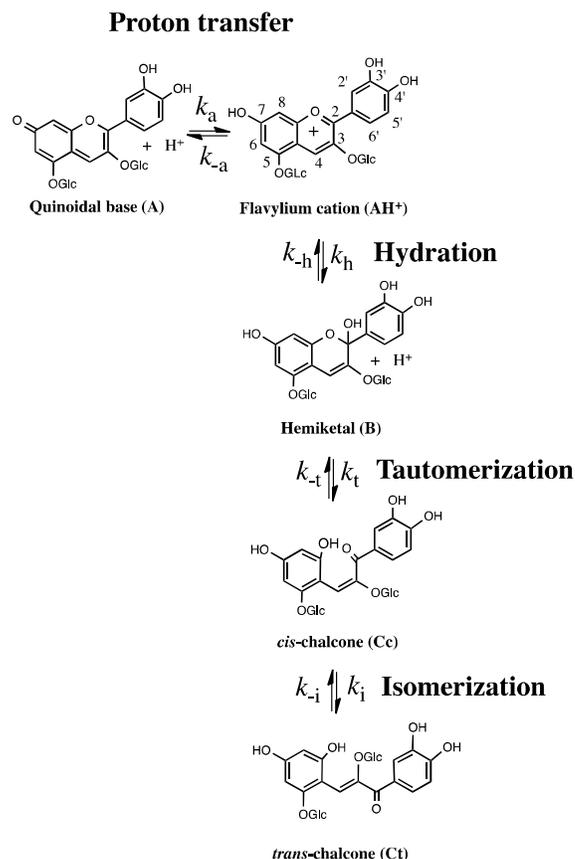Luis Cabrita,[a] Vesselin Petrov [b] and Fernando Pina [a]

Cyanidin was studied by direct pH jumps (from equilibrated solutions at very low pH values to higher pH values) and reverse pH jumps (from equilibrated or not equilibrated solutions at higher pH values to very low ones). The kinetic steps of the direct and reverse pH jumps were followed by stopped flow, absorption spectroscopy and HPLC, at different timescales. The pH dependent rate constant of the slower kinetic process to reach the equilibrium follows a bell shaped curve as described for many synthetic flavylium compounds. Unlike anthocyanins, it was proved that there is no pH dependent reversibility in the system, since the chalcone suffers an irreversible degradation process. The mathematical expression to describe the bell shaped behaviour was deduced. These results contribute to explain why in plants glycosylation is crucial for the stabilization of the anthocyanins.

Introduction

Anthocyanidins are 2-phenyl-1-benzopyrylium derivatives possessing the same structure of anthocyanins but lacking any sugar substituents, see Scheme 1 for cyanidin. Differently from anthocyanidins, the network of chemical reactions involving anthocyanins (those possessing a sugar in position 3 or two sugars in position 3 and 5) is now firmly established. The flavylium cation, AH^+, is the dominant species at low pH values. Raising the pH two parallel reactions, deprotonation to give the quinoidal base (**A**) and hydration leading to the hemiketal (**B**), take place. These two reactions are in competition but occur in different timescales: proton transfer in the micro-second and hydration in minutes to sub-seconds, depending on pH. By consequence upon a pH jump from very acid solutions (where AH^+ is the dominant species) here on designated direct pH jump, **A** appears as a kinetic product that usually fades with time, because it is not the most stable species at the equilibrium. This is due to the crucial achievement on these systems made by Brouillard and Dubois,[1] who discovered that the species **A** is not reactive in acidic to moderately acid medium.

The hemiketal **B** (chromene) is involved in a tautomeric process that leads to the ring opening with formation of a *cis*-chalcone form. This reaction usually occurs in the sub-second timescale. Finally the *cis*-chalcone isomerizes and gives the *trans*-chalcone in the timescale of seconds or many days depending on the flavylium substitution pattern. The coloured species of the network are the flavylium cation and the quinoidal base, the last one red shifted in comparison with the former.

The analysis of the thermodynamic equilibria of the flavylium compounds becomes easier if eq.(1) is considered.[2] Eq.(1) is equivalent to a single acid-base equilibrium between flavylium cation and a conjugate base, **CB**, defined as the sum of the concentrations of the other species in the network

Proton transfer

Quinoidal base (A)   **Flavylium cation (AH+)**

k_{-h} || k_h **Hydration**

Hemiketal (B)

k_{-t} || k_t **Tautomerization**

cis-**chalcone (Cc)**

k_{-i} || k_i **Isomerization**

trans-**chalcone (Ct)**

Scheme 1. Network of chemical reactions of anthocyanins and related compounds. $K_n = k_n/k_{-n}$ (n=a,h,t,i)

The analysis of the thermodynamic equilibria of the flavylium compounds becomes easier if eq.(1) is considered.[2] Eq.(1) is equivalent to a single acid-base equilibrium between flavylium cation and a conjugate base, **CB**, defined as the sum of the concentrations of the other species in the network,
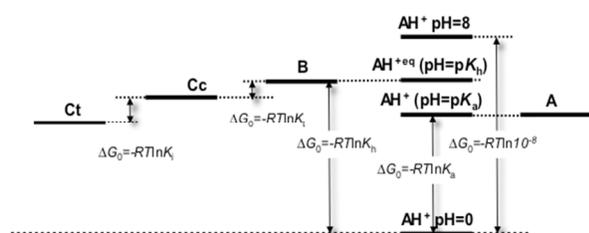
$$[CB]=[A]+[B]+[Cc]+[Ct]$$

$$AH^+ + H_2O \rightleftharpoons CB + H_3O^+$$
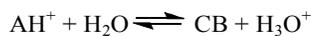
$$K'_a=K_a + K_h+ K_hK_t + K_hK_tK_i \tag{1}$$

Thermodynamics

A convenient way to account for the thermodynamic of the network of chemical reactions involving flavylium derivatives is presented in Scheme 2.[3]

Scheme 2. Energy level diagram for the flavylium network of chemical reactions at the equilibrium.

Once the equilibrium constants are calculated all the network species are positioned from the relationship, ΔG_0=-RT lnK where ΔG_0 is the standard Gibbs energy, R the gas constant, T the absolute temperature and K the equilibrium constant for each process of the network,[4] see Scheme 2. The relative position of the several species is very dependent on the substituents of the flavylium core. For example in the case of anthocyanins, B is the most stable species at higher pH values, while in 4',7-dihydroxyflavylium it is Ct.[5]

Kinetics

Regarding the kinetic process taking place in the network of chemical reactions involving flavylium derivatives two situations according to the existence or not of a thermal barrier for the *cis-trans* isomerization have been described: i) the former followed by anthocyanins, ii) the last by some synthetic flavylium compounds.

Slow formation of *trans*-chalcone

In anthocyanins three kinetic processes can be identified upon a pH jump from equilibrated solutions at pH=1.0 to higher pH values (direct pH jumps).[6] Immediately after the direct pH jump the proton transfer takes place, to form **A**, within micro-seconds, faster than the mixing time of a stopped flow apparatus, eq.(2). In this step **AH**[+] and **A** are in fast equilibrium, the respective mole fraction distribution depending on pH. Moreover, they behave as a single species in the subsequent kinetic steps.

$$k_1 = k_a + k_{-a}[H^+] \tag{2}$$

The second kinetic process observed in anthocyanins is controlled by the hydration. The tautomerization is also much faster than hydration (unless for very low pH values) and thus **B** and **Cc** behave as a single species in equilibrium during this step. At the end of this process all the species except **Ct** are in (pseudo)equilibrium.

$$k_2 = \frac{[H^+]}{[H^+]+K_a}k_h+\frac{1}{1+K_t}k_{-h}[H^+] \tag{3}$$

The last kinetic process corresponds to the formation of **Ct** and is given by eq.(4)

$$k_3 = \frac{K_hK_t}{[H^+]+K_a+K_h(1+K_t)}k_i+k_{-i} \tag{4}$$

Fast formation of trans-chalcone

This is the case of many synthetic and some natural flavylium derivatives.[6] In this case the pseudo-equilibrium is not formed and the steps 2 and 3 above described are transformed in one. Assuming that the equilibrium between **AH**[+] and **A** as well **B** and **Cc** is reached during the kinetic process, the following equation can be deduced,[7] see Scheme 3.
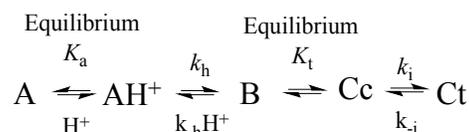
Scheme 3. Network of chemical reactions when the *cis-trans* isomerization barrier is small.

This is equivalent to the following equilibrium, eq.(5) permitting to apply the steady state hypothesis to Y (**B** and **Cc**) leading to eq.(6)

$$X \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Y \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Z \tag{5}$$
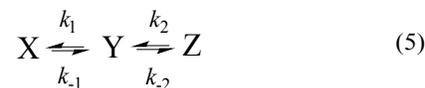
$$k_{obs} = \frac{\frac{[H^+]}{[H^+]+K_a}K_hK_tk_i+k_{-i}[H^+]}{[H^+]+\frac{K_tk_i}{k_{-h}}} \tag{6}$$

Representation of eq.(6) as a function of pH leads to a bell shaped curve similar to the one reported in Fig. 2B, see below.

In the case of anthocyanidins the situation is different since these compounds are highly unstable at moderately acidic pH values. In previous work it was reported the instability of anthocyanidins and some suggestions about the respective degradation mechanism were made.[8] In this work strong evidences for the degradation mechanism of cyanidin are given together with a detailed study of the respective kinetics.

Experimental

Chemicals.

Cyanidin chloride was purchased from Extrasynthèse (Genay, France), 2,4,6-trihydroxybenzaldehyde was purchased from Aldrich, 2,4,6-trihydroxybenzoic acid and 3,4-dihydroxybenzoic acid were purchased from Fluka.

All aqueous solutions were prepared with type I water. Methanol and ethanol were of HPLC grade. A universal buffer of Theorell Stenhagen was made by dissolving 2.25 mL of phosphoric acid (85% w/w), 7.00 g of monohydrated citric acid, 3.54g of boric acid and 343 ml of 1 M NaOH solution in water, completed until 1 L.[9]

Instrumentation and procedures.

A stock solution of cyanidin chloride 1.50×10^{-4} M was prepared by dissolving the appropriate amount of cyanidin in EtOH acidified with 1% concentrated HCl. The use of an ethanolic instead of an aqueous solution conferred an increased stability to the stock solution and avoided precipitation during the experiments.

All pH measurements were made in a Crison BASIC 20+ pH-meter fitted with a Crison electrode. The calibration was made with standard buffers at pH 4.00, 7.00 and 9.00 purchased from CRISON. The UV-Vis measurements were made in a CARY 5000 spectrophotometer (VARIAN) using quartz cuvettes (1 cm path) at 20°C, in the 220-800 nm range and at a scan rate of 1010 nm/min.

The stopped flow experiments were conducted in an Applied Photophysics SX20 stopped flow spectrometer provided with a PDA.1/UV photodiode array detector with a minimum scan time of 0.65 ms and a wavelength range of 200–700 nm.

All thermodynamic and kinetic constants of the cyanidin chloride solution were determined by a spectrophotometric method, as described elsewhere.[10] A stock solution of cyanidin chloride (1.50×10^{-4} M) in acidified ethanol was prepared as described above.

Direct pH jumps (from pH 1 to higher pH values) were carried out by pippeting 0.250 mL of cyanidin stock solution into a quartz cuvette, then rapidly adding 2.25 mL of a universal buffer at the target pH value, and start collecting UV-Vis spectra immediately. This corresponds to a 10-fold dilution and the analyzed solutions were therefore 10% in EtOH (v/v). Minor pH adjustments were made by adding a few microliters of aqueous HCl 10 M or NaOH 1M solutions. The ionic strength was kept constant. Reverse pH jumps (from higher pH values to pH 1) were performed by adding 0.100 mL HCl (10 M) to cyanidin solutions that previously underwent direct pH jumps. Throughout the whole experiment the temperature was kept at 20 °C.

The HPLC system was a Merck-Hitachi comprising a L-6200A pump, a L-4500 Diode Array Detector, a L-5025 column oven, a D-6000 interface controlled with the DSM software, and a Rheodyne 7125 manual injection valve with a 20 μL injection loop. The analysis was performed in a Phenomenex Gemini column (C18, 150 x 4.6 mm, 3 μm). The solvents used were A (Water: Formic Acid, 9:1, v/v) and B (Water:Formic Acid: Methanol, 4:1:5, v/v). The gradient employed started with 10% B, then increasing linearly to 100% B at 30 min. Flow rate was 0.6 ml/min and oven temperature was 40°C. All samples were filtered with 0.45 μm GHP syringe filters (Acrodisc).

Results and discussion

Fig.1 shows the absorption spectra taken 20 ms after a direct pH jump followed by stopped flow. The spectral variations are compatible with an acid base equilibrium between AH^+ and A.

Representation of the absorbance as a function of pH (inset of Fig. 1) gives a pK_a=4.8.
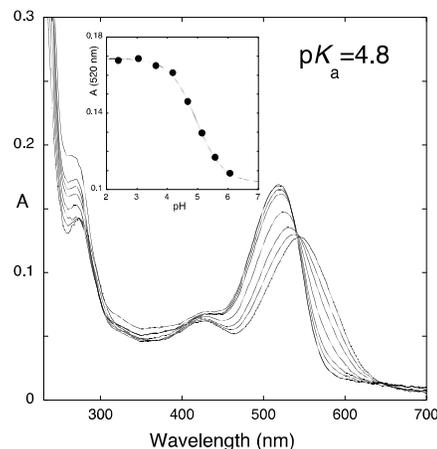

Figure 1. pH dependent absorption spectra obtained 20 ms after a pH jump followed by stopped flow.

The spectra shown in Fig. 1 evolve according to Fig. 2A. The AH^+/A species disappear to give other species absorbing preferentially in the UV. Representation of the rate constant of this process as a function of pH is a bell shaped curve as shown in Fig. 2B.
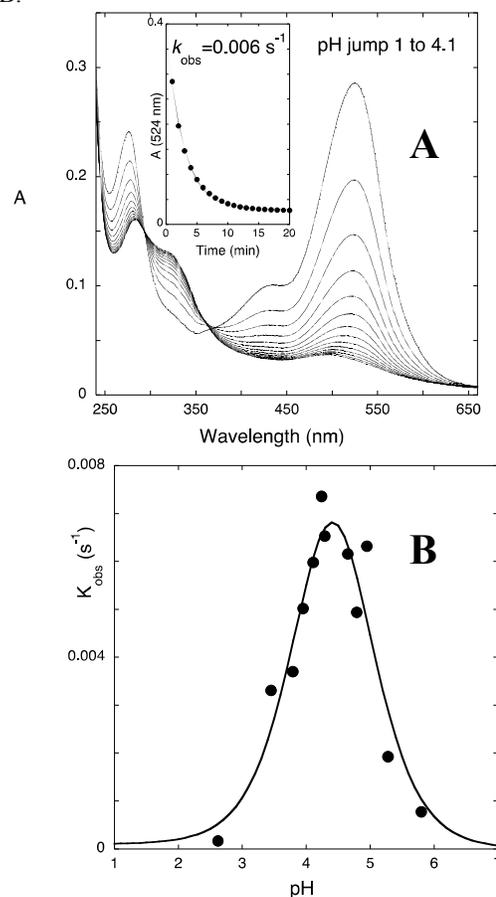

Figure 2. A- Spectral modifications taking place after a direct pH jump; B- representation of the rate constants as in Fig. 2A as a function of pH.

The data shown in Fig. 2B would suggest a behaviour of cyanidin similar to compounds lacking *cis-trans* isomerization barrier, like many synthetic flavylium compounds and also some natural.[6] However, a deeper analysis of the system proves that it is not the case. The question is that in eq.(5) and eq.(6) the complete reversibility of the system is a requirement, which is followed in the systems already reported.[6] The data shown in Fig. 3 clearly shows that this system is not reversible: after a direct pH jump and upon a certain delay, if a reverse pH jump is applied back to pH=1 the absorption of the flavylium is not completely recovered, and increasing the delay decreases the extent of absorption recovery.
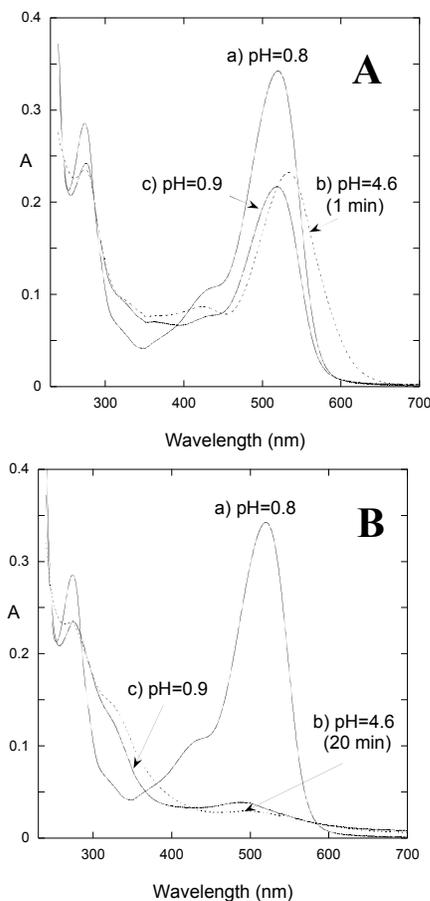

Figure 3. A- The sequence of pH jumps: initial solution of cyanidin at pH 0.8 (a), followed by a direct pH jump to pH 4.6 (b); after a delay of 1 min at this pH, a reverse jump back to pH=0.9 (c). Only 63% of the flavylium absorption was recovered; B- the same sequence but using a delay of 20 min at pH=4.6 before the reverse pH jump. In this case only a small fraction of flavylium absorption was recovered.

Nevertheless, if the reverse pH jump is carried out within 1 min of delay after the direct jump, the re-appearance of (some) flavylium cation can be monitored by stopped flow, Fig. 4. As reported previously, the reverse pH jump converts all **A** into **AH**[+] within a few miliseconds (mixing time of the stopped flow), accounting for the absorbance of flavylium/quinoidal base after the delay(75%).[6] The first observable kinetic process corresponds to the recovery of more **AH**[+] (16%) from **B**, since

at low pH values hydration step is faster than tautomerization (change of regime). The second and last process is the further formation **AH**[+] (9%) now from **Cc** via **B**. The ratio of the amplitudes of the last kinetic step and the first is equal to the equilibrium constant K_t. Some kinetic information is also obtained from Fig. 4. The first and faster process corresponds to the hydration reaction and is given by eq.(3), while the second one and slower is assigned to k-t (there is no reversibility from B to Cc since once B is formed is immediately transformed into AH+). [footnote 1]. At this pH value the acid and basic catalysis was neglected, since it is not expected to be relevant.
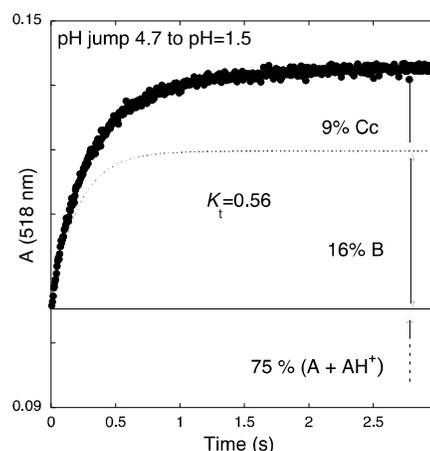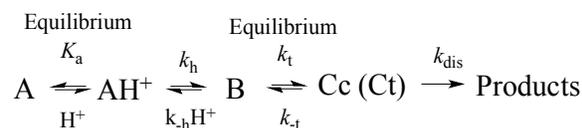

Figure 4. Reverse pH jump to pH=1.5 after a direct pH jump from 1.0 to 4.7 (delay 1 minute) monitored by stopped flow.

As shown in Fig. 3 the bell shaped curve cannot be attributed to a system as shown in eq.(5) and eq.(6). However, using a slight modification to take into account the experimental details of this system a bell shaped curve is also obtained, see Scheme 4.

Scheme 4. Network of chemical reactions in the case of anthocyanidins.

This modification accounts for the existence of irreversible process(es) resulting in the formation of degradation products. From a kinetic point of view, the irreversible step could take place from either **Cc** or **Ct**. Comparing eq.(6) with eq.(5) the systems are kinetically the same if k_i is substituted by k_{dis} and k_{-i} is neglected. [footnote 2] On this basis, the observed rate constant should be given by eq.(7).

$$k_{obs} = \frac{\dfrac{[H^+]}{[H^+]+K_a}K_h K_t k_{diss}}{[H^+]+\dfrac{K_t k_{diss}}{k_{-h}}} \quad (7)$$

A global fitting using pKa from Fig.1, K_t, k_{-t} and eq.(3) in Fig.4 as well as eq.(7) permits to calculate all the parameters see Table 1.

Table 1. Equilibrium and rate constants of cyanidin at pH 4.8 in water (10%EtOH).

pK_a	pK_h	K_t	k_h/s^{-1}	$k_{-h}/M^{-1}s^{-1}$	k_t/s^{-1}	k_{-t}/s^{-1}	k_{diss}
4.8	4.6	0.56	0.009	400	0.9	1.6	0.1

Degradation of cyanidin.

The transformations occurring in solutions of cyanidin upon different delays after direct pH jumps were also followed by HPLC with DAD detection, Fig. 5A. The peaks of some of the network species and degradation products of cyanidin were monitored and identified based on retention time, absorption spectra and co-chromatography, and peak areas were plotted against time, Fig. 5B.

It is clear the disappearance of cyanidin ($\bullet$) in accordance to the results reported in Fig. 4. The variation of peak area as a function of time suggests the initial formation of 2,4,6-trihydroxybenzaldehyde and 3,4-dihydroxybenzoic acid, and a chalcone, which we were not able to distinguish if it is *cis* or *trans*. The chalcone(s) build up to 120 min and then decrease with time, suggesting they are being consumed in subsequent degradation process. The 2,4,6-trihydroxybenzoic acid seems to be a secondary degradation product resulting from the oxidation of the corresponding benzaldehyde. These results are in good agreement with previous work.[8]

Inspection of the bell shaped curve suggests that there is not reversibility from **Ct** ($k_{-i}\approx0$). Otherwise the curve would not be zero at very low pH values, because the limit of eq.(6) when the proton concentration is very high is k_{-i}, as observed previously in the case of synthetic flavylium compounds.[6] In order to account for the experimental data, two possibilities can be considered: i) **Cc** leads to **Ct** and this last one decomposes much faster than the backward reaction **Ct** to **Cc**, k_{-i}, and in this case k_{diss} should be assigned to k_i, ii) **Cc** decomposes without giving **Ct**, and in this case k_{diss} represents the decomposition rate of the former. We were not able to distinguish which of these two assumptions take place.

A possible explanation for the anthocyanidins degradation is shown in Scheme 5. The first step regards a Michael addition of water to C2, resulting in an hydroxylated intermediate which could be easily oxidized into the corresponding products.

In the case of the anthocyanins the first step would be impeded explaining their higher stability.
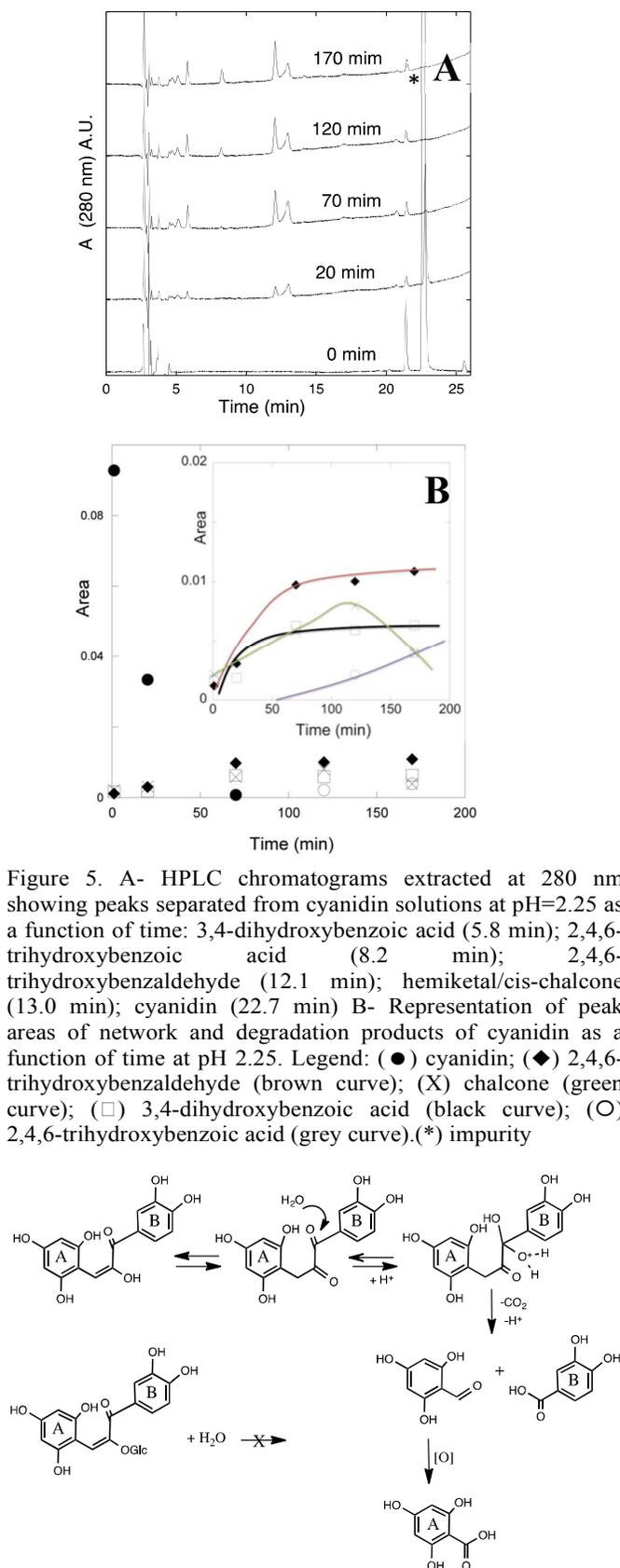

Figure 5. A- HPLC chromatograms extracted at 280 nm showing peaks separated from cyanidin solutions at pH=2.25 as a function of time: 3,4-dihydroxybenzoic acid (5.8 min); 2,4,6-trihydroxybenzoic acid (8.2 min); 2,4,6-trihydroxybenzaldehyde (12.1 min); hemiketal/cis-chalcone (13.0 min); cyanidin (22.7 min) B- Representation of peak areas of network and degradation products of cyanidin as a function of time at pH 2.25. Legend: ($\bullet$) cyanidin; ($\blacklozenge$) 2,4,6-trihydroxybenzaldehyde (brown curve); (X) chalcone (green curve); ($\square$) 3,4-dihydroxybenzoic acid (black curve); (O) 2,4,6-trihydroxybenzoic acid (grey curve).(*) impurity
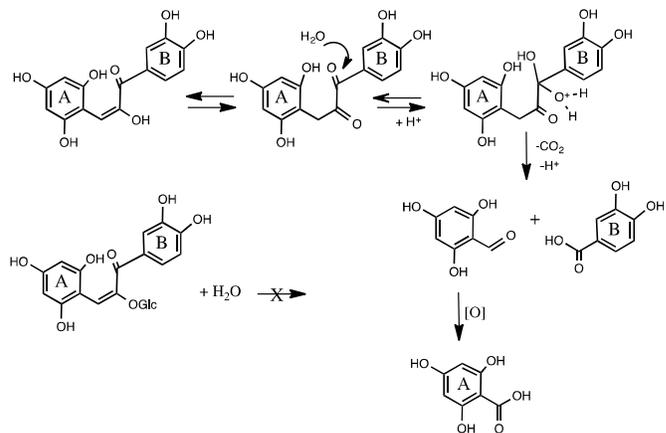
Scheme 5. Proposed mechanism of cyanidin degradation.

Conclusions

The rate of cyanidin disappearance as a function of pH follows a bell shaped curve apparently similar to those previously reported for other flavylium derivatives. However, the latter show reversibility, recovering flavylium cation when equilibrated solutions at higher pH values are acidified. The former is not reversible giving rise to degradation products. The growing of the rate at lower pH values is due to the increasing of the mole fraction of the chalcones; once formed, these species degrade irreversibly. Kinetically this is equivalent to a kinetic control by the *cis-trans* isomerization as observed in other flavylium compounds. The descending branch of the bell shaped curve can be attributed to the hydration control; by increasing the pH the rate of chalcone formation decreases via decreasing of the hemiketal formation.

The kinetic and HPLC results strongly suggest that the chalcones are responsible for the formation of degradation products. The degradation leads to 2,4,6-trihydroxybenzaldehyde and 3,4-dihydroxybenzoic acid. The compound 2,4,6-trihydroxybenzoic acid was also found in a subsequent kinetic process most probably from the oxidation of 2,4,6-trihydroxybenzaldehyde. This result contrasts with the behavior of cyanin and other anthocyanins, which present reversibility in acidic medium. The existence of the sugar in position 3 is thus a crucial requirement to the expression of colour in plants.

Acknowledgements

Notes and references

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† Footnotes

1) At this pH value the acid and basic catalysis was neglected, since it is not expected to be relevant.

2) If there is decomposition the constant k_i lacks of significance.

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