# A New Procedure to Calculate All Equilibrium Constants in Flavylium Compounds: Application to the Co-Pigmentation of Anthocyanins.

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## Appendix 1A

*The complex multistate simplified as a triprotic acid.*

Let us consider the extension of the anthocyanins and related compounds equilibrium to the mono and di-ionized species. The extension to more acid base equilibria is straightforward

First global acid base equilibrium

$\mathbf{AH^+} + H_2O \rightleftharpoons \mathbf{A} + H_3O^+$ $K_a$ proton transfer (1)

$\mathbf{AH^+} + H_2O \rightleftharpoons \mathbf{B} + H_3O^+$ $K_h$ hydration (2)

$\mathbf{B} \rightleftharpoons \mathbf{Cc}$ $K_t$ tautomerization (3)

$\mathbf{Cc} \rightleftharpoons \mathbf{Ct}$ $K_i$ isomerization (4)

Second global acid base equilibrium

$\mathbf{A} + H_2O \rightleftharpoons \mathbf{A^-} + H_3O^+$ $K_{A/A^-}$ proton transfer (5)

$\mathbf{B} + H_2O \rightleftharpoons \mathbf{B^-} + H_3O^+$ $K_{B/B^-}$ proton transfer (6)

$\mathbf{Cc} + H_2O \rightleftharpoons \mathbf{Cc^-} + H_3O^+$ $K_{Cc/Cc^-}$ proton transfer (7)

$\mathbf{Ct} + H_2O \rightleftharpoons \mathbf{Ct^-} + H_3O^+$ $K_{Ct/Ct^-}$ proton transfer (8)

Third global acid base equilibrium

$\mathbf{A^-} + H_2O \rightleftharpoons \mathbf{A^{2-}} + H_3O^+$ $K_{A^-/A^{2-}}$ proton transfer (9)

$\mathbf{B^-} + H_2O \rightleftharpoons \mathbf{B^{2-}} + H_3O^+$ $K_{B^-/B^{2-}}$ proton transfer (10)

$\mathbf{Cc^-} + H_2O \rightleftharpoons \mathbf{Cc^{2-}} + H_3O^+$ $K_{Cc^-/Cc^{2-}}$ proton transfer (11)

$\mathbf{Ct^-} + H_2O \rightleftharpoons \mathbf{Ct^{2-}} + H_3O^+$ $K_{Ct^-/Ct^{2-}}$ proton transfer (12)

Inspection of the multistate of reactions shows that except for the first global acid base equilibrium that generates the species A (also obtained by proton transfer), B, Cc and Ct, the following global equilibria correspond to the generation of the deprotonated forms of the former.

The mole fractions of all the species of the multistate are straightforwardly calculated by a simple mass balance in function of the species AH$^+$, by using the equilibrium constants of eq.(1) to eq.(12)

The mass balance gives

$$C_0 = [AH^+]+[A]+[B]+[Cc]+[Ct]+[A^-]+[B^-]+[Cc^-]+[Ct^-]+[A^{2-}]+[B^{2-}]+[Cc^{2-}]+[Ct^{2-}] \tag{13}$$

$$C_0 = [AH^+](1+\frac{K_a}{[H^+]}+\frac{K_h}{[H^+]}+\frac{K_hK_t}{[H^+]}+\frac{K_hK_tK_i}{[H^+]}+\frac{K_{A/A^-}K_a}{[H^+]^2}+\frac{K_{B/B^-}K_h}{[H^+]^2}+\frac{K_{Cc/Cc^-}K_hK_t}{[H^+]^2}+\frac{K_{Ct/Ct^-}K_hK_tK_i}{[H^+]^2}+ \\ +\frac{K_{A^-/A^{2-}}K_{A/A^-}K_a}{[H^+]^3}+\frac{K_{B^-/B^{2-}}K_{B/B^-}K_h}{[H^+]^3}+\frac{K_{Cc^-/Cc^{2-}}K_{Cc/Cc^-}K_hK_t}{[H^+]^3}+\frac{K_{Ct^-/Ct^{2-}}K_{Ct/Ct^-}K_hK_tK_i}{[H^+]^3} \tag{14}$$

From eq.(14) the mole fraction distribution of AH$^+$ can be calculated, eq.(15)

$$X_{AH^+} = \frac{[AH^+]}{C_0} = \frac{[H^+]^3}{D} \tag{15}$$

where

$$D = [H^+]^3 + (K_a + K_h + K_hK_t + K_hK_tK_i)[H^+]^2 + (K_{A/A^-}K_a + K_{B/B^-}K_h + K_{Cc/Cc^-}K_hK_t + K_{Ct/Ct^-}K_hK_tK_i)[H^+] + \\ + K_{A^-/A^{2-}}K_{A/A^-}K_a + K_{B^-/B^{2-}}K_{B/B^-}K_h + K_{Cc^-/Cc^{2-}}K_{Cc/Cc^-}K_hK_t + K_{Ct^-/Ct^{2-}}K_{Ct/Ct^-}K_hK_tK_i$$

By analogy with a tri-protic acid where $K'_a$, $K''_a$ and $K'''_a$ are the first second and third acidity constants the mole fraction of the fully protonated form is given by eq.(16)

$$X_{AH^+} = \frac{[H^+]^3}{[H^+]^3 + K'_a[H^+]^2 + K'_aK''_a[H^+] + K'_aK''_aK'''_a} \tag{16}$$

With

$$K'_a = K_a + K_h + K_hK_t + K_hK_tK_i \tag{17}$$

$$K'_aK''_a = K_{A/A^-}K_a + K_{B/B^-}K_h + K_{Cc/Cc^-}K_hK_t + K_{Ct/Ct^-}K_hK_tK_i \tag{18}$$

$$K'_aK''_aK'''_a = K_{A^-/A^{2-}}K_{A/A^-}K_a + K_{B^-/B^{2-}}K_{B/B^-}K_h + K_{Cc^-/Cc^{2-}}K_{Cc/Cc^-}K_hK_t + \\ + K_{Ct^-/Ct^{2-}}K_{Ct/Ct^-}K_hK_tK \tag{19}$$

The complex equilibria from eq.(1) to eq.(12) can thus be summarized in eq.(20) to eq.(22).

**AH**$^+$ + $H_2O \rightleftharpoons$ **CB** + $H_3O^+$ $K'_a$ (20)

**CB** + $H_2O \rightleftharpoons$ **CB**$^-$ + $H_3O^+$ $K''_a$ (21)

**CB**$^-$ + $H_2O \rightleftharpoons$ **CB**$^{2-}$ + $H_3O^+$ $K'''_a$ (22)

where

[**CB**]=[**A**]+ [**B**]+ [**Cc**]+ [**Ct**] (23)

[**CB**$^-$]=[**A**$^-$]+ [**B**$^-$]+ [**Cc**$^-$]+[**Ct**$^-$] (24)

[**CB**$^{2-}$]=[**A**$^{2-}$]+ [**B**$^{2-}$]+ [**Cc**$^{2-}$]+[**Ct**$^{2-}$] (25)

with $K'_a$ defined above by eq.(17) and $K''_a$ and $K'''_a$ by eq.(26) and eq.(27)

$$K''_a = \frac{K_{A/A^-}K_a + K_{B/B^-}K_h + K_{Cc/Cc^-}K_hK_t + K_{Ct/Ct^-}K_hK_tK_i}{K'_a} \tag{26}$$

$$K'''_a = \frac{K_{A^-/A^{2-}}K_{A/A^-}K_a + K_{B^-/B^{2-}}K_{B/B^-}K_h + K_{Cc^-/Cc^{2-}}K_{Cc/Cc^-}K_hK_t + K_{Ct^-/Ct^{2-}}K_{Ct/Ct^-}K_hK_tK}{K'_aK''_a} \tag{27}$$

The mole fraction of the remaining species is now easy to obtain form eq.(28) to eq.(39)

$$X_A = \frac{K_a[H^+]^2}{[H^+]^3 + K'_a[H^+]^2 + K'_aK''_a[H^+] + K'_aK''_aK'''_a} \tag{28}$$

$$X_B = \frac{K_h[H^+]^2}{[H^+]^3 + K'_a[H^+]^2 + K'_aK''_a[H^+] + K'_aK''_aK'''_a} \tag{29}$$

$$X_{Cc} = \frac{K_hK_t[H^+]^2}{[H^+]^3 + K'_a[H^+]^2 + K'_aK''_a[H^+] + K'_aK''_aK'''_a} \tag{30}$$

$$X_{Ct} = \frac{K_hK_tK_i[H^+]^2}{[H^+]^3 + K'_a[H^+]^2 + K'_aK''_a[H^+] + K'_aK''_aK'''_a} \tag{31}$$

$$X_{A^-} = \frac{K_aK_{A/A^-}[H^+]}{[H^+]^3 + K'_a[H^+]^2 + K'_aK''_a[H^+] + K'_aK''_aK'''_a} \tag{32}$$

$$X_{B^-} = \frac{K_h K_{B/B^-}[H^+]}{[H^+]^3 + K'_a[H^+]^2 + K'_a K''_a[H^+] + K'_a K''_a K'''_a} \tag{33}$$

$$X_{Cc^-} = \frac{K_h K_t K_{Cc/Cc^-}[H^+]}{[H^+]^3 + K'_a[H^+]^2 + K'_a K''_a[H^+] + K'_a K''_a K'''_a} \tag{34}$$

$$X_{Ct^-} = \frac{K_h K_t K_i K_{Ct/Ct^-}[H^+]}{[H^+]^3 + K'_a[H^+]^2 + K'_a K''_a[H^+] + K'_a K''_a K'''_a} \tag{35}$$

$$X_{A^{2-}} = \frac{K_a K_{A/A^-} K_{A^-/A^{2-}}}{[H^+]^3 + K'_a[H^+]^2 + K'_a K''_a[H^+] + K'_a K''_a K'''_a} \tag{36}$$

$$X_{B^{2-}} = \frac{K_h K_{B/B^-} K_{B^-/B^{2-}}}{[H^+]^3 + K'_a[H^+]^2 + K'_a K''_a[H^+] + K'_a K''_a K'''_a} \tag{37}$$

$$X_{Cc^{2-}} = \frac{K_h K_t K_{Cc/Cc^-} K_{Cc^-/Cc^{2-}}}{[H^+]^3 + K'_a[H^+]^2 + K'_a K''_a[H^+] + K'_a K''_a K'''_a} \tag{38}$$

$$X_{Ct^{2-}} = \frac{K_h K_t K_i K_{Ct/Ct^-} K_{Cc^-/Cc^{2-}}}{[H^+]^3 + K'_a[H^+]^2 + K'_a K''_a[H^+] + K'_a K''_a K'''_a} \tag{39}$$

On the other hand, eq.(20) to eq.(22) show that the system behaves as a triprotic acid with constants $K'_a$, $K''_a$ and $K'''_a$. The respective mole fractions are given by eq.(40) to eq.(43)

$$X_{AH+} = \frac{[H^+]^3}{D} \tag{40}$$

$$D = [H^+]^3 + K_a^{'}[H^+]^2 + K_a^{'}K_a^{''}[H^+] + K_a^{'}K_a^{''}K_a^{'''}$$

$$X_{CB} = \frac{K_a^{'}[H^+]^2}{D} \tag{41}$$

$$X_{CB-} = \frac{K_a^{'}K_a^{''}[H^+]}{D} \tag{42}$$

$$X_{CB2-} = \frac{K_a^{'}K_a^{''}K_a^{'''}}{D} \tag{43}$$

Pseudo equilibrium

All equations above described can be applied at the pseudo-equilibrium. The pseudo equilibrium is reached when the isomerization constants is by far the slowest step of the kinetics towards the equilibrium.

**AH**$^+$ + $H_2O \rightleftharpoons$ **CB**$^\wedge$ + $H_3O^+$ $K^\wedge{}_a$ (44)

**CB**$^\wedge$ + $H_2O \rightleftharpoons$ **CB**$^{\wedge-}$ + $H_3O^+$ $K^{\wedge\wedge}{}_a$ (45)

**CB**$^{\wedge-}$ + $H_2O \rightleftharpoons$ **CB**$^{\wedge 2-}$ + $H_3O^+$ $K^{\wedge\wedge\wedge}{}_a$ (46)

Where

[**CB**$^\wedge$]=[**A**]+ [**B**]+ [**Cc**]+ [**Ct**] (47)

[**CB**$^{\wedge-}$]=[**A**$^-$]+ [**B**$^-$]+ [**Cc**$^-$]+[**Ct**$^-$] (48)

[**CB**$^{\wedge 2-}$]=[**A**$^{2-}$]+ [**B**$^{2-}$]+ [**Cc**$^{2-}$]+[**Ct**$^{2-}$] (49)

The mole fractions of the pseudo equilibrium are given by the same expression of the equilibrium making $K_i=0$

With

$$K_a^\wedge = K_a + K_h + K_h K_t \tag{50}$$

$$K^\wedge{}_a K^{\wedge\wedge}{}_a = K_{A/A^-} K_a + K_{B/B^-} K_h + K_{Cc/Cc^-} K_h K_t \tag{51}$$

$$K^\wedge{}_a K^{\wedge\wedge}{}_a K^{\wedge\wedge\wedge}{}_a = K_{A^-/A^{2-}} K_{A/A^-} K_a + K_{B^-/B^{2-}} K_{B/B^-} K_h + K_{Cc^-/Cc^{2-}} K_{Cc/Cc^-} K_h K_t \tag{52}$$

and by consequence

$$K_a^{\wedge\wedge} = \frac{K_{A/A^-} K_a + K_{B/B^-} K_h + K_{Cc/Cc^-} K_h K_t}{K_a^\wedge} \tag{53}$$

$$K_a^{\wedge\wedge\wedge} = \frac{K_{A^-/A^{2-}} K_{A/A^-} K_a + K_{B^-/B^{2-}} K_{B/B^-} K_h + K_{Cc^-/Cc^{2-}} K_{Cc/Cc^-} K_h K_t}{K_a^\wedge K^\wedge{}_a} \tag{54}$$

The mole fractions of the global triprotic acid at the pseudo-equilibrium is given by

$$X_{AH+} = \frac{[H^+]^3}{D} \tag{55}$$

$$D = [H^+]^3 + K_a^\wedge [H^+]^2 + K_a^\wedge K_a^{\wedge\wedge} [H^+] + K_a^\wedge K_a^{\wedge\wedge} K_a^{\wedge\wedge\wedge}$$

$$X_{CB^\wedge} = \frac{K_a^\wedge [H^+]^2}{D} \tag{56}$$

$$X_{CB^{\wedge}-} = \frac{K_a^{\wedge} K_a^{\wedge\wedge} [H^+]}{D} \tag{57}$$

$$X_{CB^{\wedge}2-} = \frac{K_a^{\wedge} K_a^{\wedge\wedge} K_a^{\wedge\wedge\wedge}}{D} \tag{58}$$

**Appendix 1B**

In appendix 1A it was shown that the system behaves at the equilibrium or pseudo equilibrium as a triprotic acid.

$$\mathbf{AH^+} + H_2O \rightleftharpoons \mathbf{CB} + H_3O^+ \qquad K'_a \tag{1}$$

$$\mathbf{CB} + H_2O \rightleftharpoons \mathbf{CB^-} + H_3O^+ \qquad K''_a \tag{2}$$

$$\mathbf{CB^-} + H_2O \rightleftharpoons \mathbf{CB^{2-}} + H_3O^+ \qquad K'''_a \tag{3}$$

$$X_{AH+} = \frac{[H^+]^3}{D} \tag{4}$$

$$D = [H^+]^3 + K_a^{'}[H^+]^2 + K_a^{'}K_a^{''}[H^+] + K_a^{'}K_a^{''}K_a^{'''}$$

$$X_{CB} = \frac{K_a^{'}[H^+]^2}{D} \tag{5}$$

$$X_{CB-} = \frac{K_a^{'}K_a^{''}[H^+]}{D} \tag{6}$$

$$X_{CB2-} = \frac{K_a^{'}K_a^{''}K_a^{'''}}{D} \tag{7}$$

The same expressions can be used for the pseudo equilibrium making $K_i$=0

As shown in the main text at the pseudo equilibrium (*mutatis mutandis* for the equilibrium) the normalized mole fraction distribution of the species A, B and Cc can be calculated trough the reverse pH jumps. Fig. S1 shows a simulation of these mole fractions for several pH values. In other words, CB$^{\wedge}$, CB$^{\wedge-}$ and CB$^{\wedge 2-}$, can be decomposed in their components, Fig. S.1. This means that for example the mole fraction of CB, eq.(5) can be written as follows

$$X_{CB} = \frac{K_a^{\wedge}[H^+]^2}{D} = a_0\frac{K_a^{\wedge}[H^+]^2}{D} + b_0\frac{K_a^{\wedge}[H^+]^2}{D} + c_0\frac{K_a^{\wedge}[H^+]^2}{D} \tag{8}$$

where $a_0$, $b_0$ and $c_0$ are the normalized mole fractions respectively of A, B and Cc

$$a_0 + b_0 + c_0 = 1 \tag{9}$$

The same for CB$^{\wedge-}$ and CB$^{\wedge 2-}$.

$$X_{CB^{\wedge}-} = \frac{K_a^{\wedge}K_a^{\wedge\wedge}[H^+]}{D} = a_1\frac{K_a^{\wedge}K_a^{\wedge\wedge}[H^+]}{D} + b_1\frac{K_a^{\wedge}K_a^{\wedge\wedge}[H^+]}{D} + c_1\frac{K_a^{\wedge}K_a^{\wedge\wedge}[H^+]}{D} \tag{10}$$

$$a_1 + b_1 + c_1 = 1 \tag{11}$$

$$X_{CB^\wedge 2-} = \frac{K^\wedge_a K^{\wedge\wedge}_a K^{\wedge\wedge\wedge}_a}{D} = a_1 \frac{K^\wedge_a K^{\wedge\wedge}_a K^{\wedge\wedge\wedge}_a}{D} + b_1 \frac{K^\wedge_a K^{\wedge\wedge}_a K^{\wedge\wedge\wedge}_a}{D} + c_1 \frac{K^\wedge_a K^{\wedge\wedge}_a K^{\wedge\wedge\wedge}_a}{D} \tag{12}$$

$$a_2 + b_2 + c_2 = 1 \tag{13}$$

The normalized mole fractions of each species and the respective ionized forms can be written

$$X_A + X_{A-} + X_{A2-} = a_0 \frac{K^\wedge_a [H^+]^2}{D} + a_1 \frac{K^\wedge_a K^{\wedge\wedge}_a [H^+]}{D} + a_2 \frac{K^\wedge_a K^{\wedge\wedge}_a K^{\wedge\wedge\wedge}_a}{D} \tag{14}$$

$$X_B + X_{B-} + X_{B2-} = b_0 \frac{K^\wedge_a [H^+]^2}{D} + b_1 \frac{K^\wedge_a K^{\wedge\wedge}_a [H^+]}{D} + b_2 \frac{K^\wedge_a K^{\wedge\wedge}_a K^{\wedge\wedge\wedge}_a}{D} \tag{15}$$

$$X_{Cc} + X_{Cc-} + X_{Cc2-} = c_0 \frac{K^\wedge_a [H^+]^2}{D} + c_1 \frac{K^\wedge_a K^{\wedge\wedge}_a [H^+]}{D} + c_2 \frac{K^\wedge_a K^{\wedge\wedge}_a K^{\wedge\wedge\wedge}_a}{D} \tag{16}$$

The mole fractions calculated in Appendix 1can be re-written as follows

$$X_A + X_{A-} + X_{A2-} = \frac{K_a[H^+]^2 + K_{A/A-}K_a[H^+] + K_{A-/A2-}K_{A/A-}K_a}{[H^+]^3 + K'_a[H^+]^2 + K'_a K''_a [H^+] + K'_a K''_a K'''_a} \tag{17}$$

$$X_B + X_{B-} + X_{B2-} = \frac{K_h[H^+]^2 + K_{B/B-}K_h[H^+] + K_{B-/B2-}K_{B/B-}K_h}{[H^+]^3 + K'_a[H^+]^2 + K'_a K''_a [H^+] + K'_a K''_a K'''_a} \tag{18}$$

$$X_{Cc} + X_{Cc-} + X_{Cc2-} = \frac{K_h K_t[H^+]^2 + K_{Cc/Cc-}K_h K_t[H^+] + K_{Cc-/Cc2-}K_{Cc/Cc-}K_h K_t}{[H^+]^3 + K'_a[H^+]^2 + K'_a K''_a [H^+] + K'_a K''_a K'''_a} \tag{19}$$

Comparing eq.(14) to eq.(16) with eq.(17) to eq.(19)

$$K_a = a_0 K^\wedge_a; \quad K_{A/A-}K_a = a_1 K^\wedge_a K^{\wedge\wedge}_a; \quad K_{A-/A2-}K_{A/A-}K_a = a_2 K^\wedge_a K^{\wedge\wedge}_a K^{\wedge\wedge\wedge}_a$$

$$K_h = b_0 K^\wedge_a; \quad K_{B/B-}K_h = b_1 K^\wedge_a K^{\wedge\wedge}_a; \quad K_{B-/B2-}K_{B/B-}K_h = b_2 K^\wedge_a K^{\wedge\wedge}_a K^{\wedge\wedge\wedge}_a$$

$$K_h K_t = c_0 K^\wedge_a; \quad K_{Cc/CC-}K_h K_t = c_1 K^\wedge_a K^{\wedge\wedge}_a; \quad K_{Cc-/Cc2-}K_{Cc/Cc-}K_h K_t = c_2 K^\wedge_a K^{\wedge\wedge}_a K^{\wedge\wedge\wedge}_a$$

This permits to calculate all equilibrium constants, since $K^\wedge_a$, $K^{\wedge\wedge}_a$ and $K^{\wedge\wedge\wedge}_a$ are calculated from the pseudo-equilibrated absorption spectra. At any wavelength the absorption as a function of pH exhibits 3 inflection points corresponding to the three p$K_a$s.

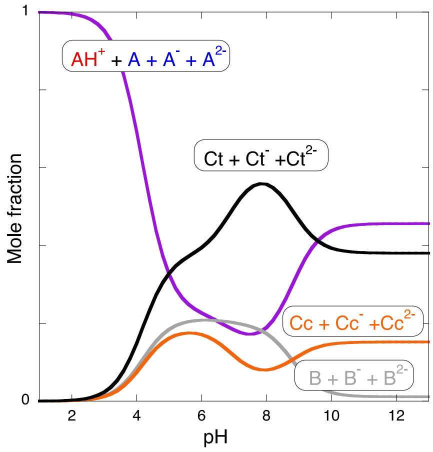

**Figure S1.** Simulation of the mole fraction distribution of the species A, B, Cc, Ct and their respective ionized forms obtained by reverse pH jumps. $pK_a$=4.8; $K_h$=1.4x10$^{-5}$; $K_t$=0.88; $K_i$=2; $pK_{A/A-}$=7.1; $pK_{B/B-}$=6.9; $pK_{Cc/Cc-}$= 7.4; $pK_{Ct/Ct-}$=6.7 ; $pK_{A-/A2-}$=8.3; $pK_{B-/B2-}$=10.0; $pK_{Cc-/Cc2-}$=8.4; $pK_{Ct-/Ct2-}$=9.0.

**Appendix 2**

Considering the flavylium cation and the neutral species

$\mathbf{AH^+} + H_2O \rightleftharpoons \mathbf{A} + H_3O^+$ $K_a$ proton transfer (1)

$\mathbf{AH^+} + H_2O \rightleftharpoons \mathbf{B} + H_3O^+$ $K_h$ hydration (2)

$\mathbf{B} \rightleftharpoons \mathbf{Cc}$ $K_t$ tautomerization (3)

This equation is reduced to

$\mathbf{AH^+} + H_2O \rightleftharpoons \mathbf{CB^{\wedge}} + H_3O^+$ $K^{\wedge}_a$ proton transfer (4)

$[CB^{\wedge}]=[A]+[B]+[Cc]$

$$K^{\wedge}_a = K_a + K_h + K_h K_t \tag{5}$$

Considering the 1:1 complexation with neutral species

$\mathbf{AH^+} + CP \rightleftharpoons \mathbf{AH^+CP}$ $K_{AH+CP}$ complexation (6)

$\mathbf{A} + CP \rightleftharpoons \mathbf{ACP}$ $K_{ACP}$ complexation (7)

$\mathbf{B} + CP \rightleftharpoons \mathbf{BCP}$ $K_{BCP}$ complexation (8)

$\mathbf{Cc} + CP \rightleftharpoons \mathbf{CcCP}$ $K_{CcCP}$ complexation (9)

This complex set of equations can be reduced to eq.(4) and eq.(10)

$\mathbf{CB^{\wedge}} + CP \rightleftharpoons \mathbf{CB^{\wedge}CP}$ $K_{CBCP}$ complexation (10)

$[CB^{\wedge}CP]=[ACP]+[BCP]+[CcCP]$

$$K_{CB^{\wedge}CP} = \frac{[CB^{\wedge}CP]}{[CB][CP]} = \frac{[ACP]+[BCP]+[CcCP]}{[A][CP]+[B][CP]+[Cc][CP]} \tag{11}$$

Simplifying

$$K_{CB^{\wedge}CP} = \frac{[CB^{\wedge}CP]}{[CB][CP]} = \frac{(K_{ACP}K_a + K_{BCP}K_h + K_{CcCP}K_hK_t)}{K^{\wedge}_a} \tag{12}$$

Considering the mono-ionized species, those that still have some importance in anthocyanins (the higher ionized species are not stable)

$\mathbf{A} + H_2O \rightleftharpoons \mathbf{A^-} + H_3O^+$ $K_{A/A-}$ proton transfer (13)

$\mathbf{B} + H_2O \rightleftharpoons \mathbf{B^-} + H_3O^+$ $K_{B/B-}$ proton transfer (14)

$\mathbf{Cc} + H_2O \rightleftharpoons \mathbf{Cc^-} + H_3O^+$ $K_{Cc/cc-}$ proton transfer (15)

Eq.(13) to eq.(15) are reduced to eq.(16)

$\mathbf{CB^{\wedge}} + H_2O \rightleftharpoons \mathbf{CB^{\wedge -}} + H_3O^+$ $K_{CB^{\wedge}/CB^{\wedge}-}$ proton transfer (16)

This constant can be written as in eq.(17)

$$K_{CB^\wedge/CB^\wedge-} = \frac{([A^-]+[B^-]+[Cc^-])[H^+]}{[CB]} = \frac{K_{A/A-}K_a + K_{B/B-}K_h + K_{Cc/CC-}K_hK_t}{K_a^\wedge} \qquad (17)$$

Regarding the complexation of the ionized species

$\mathbf{A^-} + \mathrm{CP} \rightleftharpoons \mathbf{A^-CP}$ $K_{\mathrm{ACP}}$ complexation (18)

$\mathbf{B^-} + \mathrm{CP} \rightleftharpoons \mathbf{B^-CP}$ $K_{\mathrm{BCP}}$ complexation (19)

$\mathbf{Cc^-} + \mathrm{CP} \rightleftharpoons \mathbf{Cc^-CP}$ $K_{\mathrm{CcCP}}$ complexation (20)

Can be reduced to

$\mathbf{CB^{-\wedge}} + \mathrm{CP} \rightleftharpoons \mathbf{CB^{\wedge-}CP}$ $K_{(\mathrm{CB^\wedge\text{-}\ CP})}$ (21)

$$K_{(CB^\wedge-CP)} = \frac{[CB^{\wedge-}CP]}{[CB^{\wedge-}][CP]} = \frac{[A^-CP]+[B^-CP]+[Cc^-CP]}{[A^-][CP]+[B^-][CP]+[Cc^-][CP]} \qquad (22)$$

giving

$$K_{(CB^{\wedge-}CP)} = \frac{K_{ACP/ACP-}K_{A/A-}K_a + K_{BCP/BCP-}K_{B/B-}K_h + K_{CcCP/CcCP-}K_{Cc/Cc-}K_hK_t}{K_{CB^\wedge/CB^{\wedge-}}K_a^\wedge} \qquad (23)$$

Summarizing, the complex set of equations can be dramatically simplified to a diprotic acid where the three species can be involved in the complexation. The system is defined by two acid base constants given by eq.(5) and eq.(17) respectively $AH^+/CB^\wedge$ and $CB^\wedge/CB^{\wedge-}$ and three complexation constants eq.(6), eq.(12) and eq.(23) respectively with flavylium cation, $CB^\wedge$ and $CB^{\wedge-}$.

In order to get the mole fractions of all species a balance mass must be done

$$C_0 = [AH^+]+[A]+[B]+[Cc]+[A^-]+[B^-]+[Cc^-]+[AH^+CP]+[ACP]+[BCP]+[CcCP]+[A^-CP]+[B^-CP]+[Cc^-CP] \qquad (24)$$

From the set of equations above all species can be written in terms of $AH^+$.

$$C_0=[AH^+](1+\frac{K_a}{[H^+]}+\frac{K_h}{[H^+]}+\frac{K_hK_t}{[H^+]}+\frac{K_{A/A-}K_a}{[H^+]^2}+\frac{K_{B/B-}K_h}{[H^+]^2}+\frac{K_{Cc/CC-}K_hK_t}{[H^+]^2}+$$

$$+K_{AH+CP}[CP]+K_{ACP}\frac{K_a}{[H^+]}[CP]+K_{BCP}\frac{K_h}{[H^+]}[CP]+K_{CcCP}\frac{K_hK_t}{[H^+]}[CP]+$$

$$+\frac{K_{ACP/ACP-}K_{ACP}K_a}{[H^+]^2}[CP]+\frac{K_{BCP/BCP-}K_{BCP}K_h}{[H^+]^2}[CP]+\frac{K_{CcCP/CcCP-}K_{CcCP}K_hK_t}{[H^+]^2}[CP]) \tag{25}$$

$$C_0=[AH^+](1+K_{AH+CP}[CP]+\frac{K_a+K_h+K_hK_t+K_{ACP}K_a[CP]+K_{BCP}K_h[CP]+K_{CcCP}K_hK_t[CP]}{[H^+]}+$$

$$\frac{K_{A/A-}K_a+K_{B/B-}K_h+K_{Cc/CC-}K_hK_t+(K_{ACP/ACP-}K_{ACP}K_a+K_{BCP/BCP-}K_{BCP}K_h+K_{CcCP/CcCP-}K_{CcCP}K_hK_t)[CP]}{[H^+]^2}) \tag{26}$$

or simplifying

$$C_0=[AH^+](1+K_{AH+CP}[CP]+\frac{K_a^{\wedge}+K_1[CP]}{[H^+]}+\frac{K_2+K_3[CP]}{[H^+]^2}) \tag{27}$$

with

$$\begin{aligned}
K_a^{\wedge}&=K_a+K_h+K_hK_t\\
K_1&=K_{ACP}K_a+K_{BCP}K_h+K_{CcCP}K_hK_t\\
K_2&=K_{A/A-}K_a+K_{B/B-}K_h+K_{Cc/CC-}K_hK_t\\
K_3&=K_{ACP/ACP-}K_{ACP}K_a+K_{BCP/BCP-}K_{BCP}K_h+K_{CcCP/CcCP-}K_{CcCP}K_hK_t
\end{aligned} \tag{28}$$

The mole fraction of AH$^+$ can now be written

$$X_{AH+}=\frac{[AH^+]}{C_0}=\frac{1}{(1+K_{AH+CP}[CP])+\frac{K_a^{\wedge}+K_1[CP]}{[H^+]}+\frac{K_2+K_3[CP]}{[H^+]^2}}=\frac{\frac{1}{(1+K_{AH+CP}[CP])}[H^+]^2}{[H^+]^2+\frac{K_a^{\wedge}+K_1[CP]}{(1+K_{AH+CP}[CP])}[H^+]+\frac{K_2+K_3[CP]}{(1+K_{AH+CP}[CP])}} \tag{29}$$

$$X_{AH+CP}=\frac{[AH^+CP]}{C_0}=\frac{\frac{K_{AH+CP}[CP]}{(1+K_{AH+CP}[CP])}[H^+]^2}{[H^+]^2+\frac{K_a^{\wedge}+K_1[CP]}{(1+K_{AH+CP}[CP])}[H^+]+\frac{K_2+K_3[CP]}{(1+K_{AH+CP}[CP])}} \tag{30}$$

The sum of both mole fractions

$$X_{AH+}+X_{AH+CP}=\frac{[H^+]^2}{[H^+]^2+K_{a(CP)}^{\wedge}[H^+]+K_{a(CP)}^{\wedge}K_{a(CP)}^{\wedge\wedge}} \tag{31}$$

The values $K^{\wedge}_{\mathrm{a(CP)}}$ and $K^{\wedge\wedge}_{\mathrm{a(CP)}}$ are obtained experimentally. Correspond to the inflection points when the absorbance is represented as a function of pH, as well as in the experiments of the reverse pH jumps. The constant $K_{\mathrm{AH+CP}}$ is calculated by representing the absorption of the flavylium cation at pH=1 as a function of the co-pigment concentration.

The mole fraction of A and ACP and their ionized species is obtained from the contributions

$$X_{A}+X_{ACP}+X_{A-}+X_{ACP-}=\frac{\frac{K_{a}+A_{ACP}K_{a}[CP]}{(1+K_{AH+CP}[CP])}[H^{+}]+\frac{K_{A/A-}K_{a}+K_{ACP/ACP-}K_{ACP}K_{a}[CP]}{1+K_{AH+CP}[CP])}}{[H^{+}]^{2}+K^{\wedge}_{a(CP)}[H^{+}]+K^{\wedge}_{a(CP)}K^{\wedge\wedge}_{a(CP)}} \tag{32}$$

$$\begin{aligned}
K^{\wedge}_{a} &= K_{a}\\
K_{1} &= K_{ACP}K_{a}\\
K_{2} &= K_{A/A-}K_{a}\\
K_{3} &= K_{ACP/ACP-}K_{ACP}K_{a}
\end{aligned} \tag{33}$$

Identically for the hemiketal and *cis*-chalcones

$$X_{B}+X_{BCP}+X_{B^{-}}+X_{B^{-}CP}=\frac{\frac{(K_{h}+K_{BCP}K_{h}[CP])[H^{+}]+K_{B/B^{-}}K_{h}+K_{BCP/BCP^{-}}K_{BCP}K_{h}[CP]}{(1+K_{AH^{+}CP}[CP])}}{[H^{+}]^{2}+K^{\wedge}_{a(CP)}[H^{+}]+K^{\wedge}_{a(CP)}K^{\wedge\wedge}_{a(CP)}} \tag{34}$$

$$X_{Cc}+X_{CcCP}+X_{Cc^{-}}+X_{Cc^{-}CP}=\frac{\frac{(K_{h}K_{t}+K_{CcCP}K_{h}K_{t}[CP])[H^{+}]+K_{Cc/Cc^{-}}K_{h}K_{t}+K_{CcCP/CcCP^{-}}K_{CcCP}K_{h}K_{t}[CP]}{(1+K_{AH^{+}CP}[CP])}}{[H^{+}]^{2}+K^{\wedge}_{a(CP)}[H^{+}]+K^{\wedge}_{a(CP)}K^{\wedge\wedge}_{a(CP)}} \tag{35}$$

On the other hand the reverse pH jumps in the presence of co-pigment can be written as follows for AH$^{+}$, their co-pigments together with A its co-pigment and the respective ionized species (in reverse pH jumps these species appear as the initial absorption).

$$X_{AH+}+X_{AH+CP}+X_{A}+X_{ACP}+X_{A-}+X_{A-CP}=\frac{[H^{+}]^{2}+a_{0(CP)}K^{\wedge}_{a(CP)}[H^{+}]+a_{1(CP)}K^{\wedge}_{a(CP)}K^{\wedge\wedge}_{a(CP)}}{[H^{+}]^{2}+K^{\wedge}_{a(CP)}[H^{+}]+K^{\wedge}_{a(CP)}K^{\wedge\wedge}_{a(CP)}} \tag{36}$$

where $a_{0(\mathrm{CP})}$ and $a_{1(\mathrm{CP})}$ are respectively the mole fraction of A plus ACP and A$^{-}$ plus ACP$^{-}$.

Consequently

$$a_{0(CP)}K^{\wedge}_{a(CP)}=\frac{K_{a}+A_{ACP}K_{a}[CP]}{(1+K_{AH+CP}[CP])} \tag{37}$$

$$a_{1(CP)}K^{\wedge}_{a(CP)}K^{\wedge\wedge}_{a(CP)}=\frac{K_{ACP/ACP-}K_{ACP}K_{a}}{1+K_{AH+CP}[CP]} \tag{38}$$

Proceeding identically for the other species

$$X_{B}+X_{BCP}+X_{B-}+X_{B-CP}=\frac{b_{0(CP)}K^{\wedge}_{a(CP)}[H^{+}]+b_{1(CP)}K^{\wedge}_{a(CP)}K^{\wedge\wedge}_{a(CP)}}{[H^{+}]^{2}+K^{\wedge}_{a(CP)}[H^{+}]+K^{\wedge}_{a(CP)}K^{\wedge\wedge}_{a(CP)}} \tag{39}$$

$$X_{Cc}+X_{CcCP}+X_{Cc-}+X_{Cc-CP}=\frac{c_{0(CP)}K^{\wedge}_{a(CP)}[H^{+}]+c_{1(CP)}K^{\wedge}_{a(CP)}K^{\wedge\wedge}_{a(CP)}}{[H^{+}]^{2}+K^{\wedge}_{a(CP)}[H^{+}]+K^{\wedge}_{a(CP)}K^{\wedge\wedge}_{a(CP)}} \tag{40}$$

$$b_{0(CP)}K^{\wedge}_{a(CP)}=\frac{K_{h}+A_{BCP}K_{h}[CP]}{(1+K_{AH+CP}[CP])} \tag{41}$$

$$b_{1(CP)}K^{\wedge}_{a(CP)}K^{\wedge\wedge}_{a(CP)}=\frac{K_{BCP/BCP-}K_{BCP}K_{h}}{1+K_{AH+CP}[CP]} \tag{42}$$

$$c_{0(CP)}K^{\wedge}_{a(CP)}=\frac{K_{h}K_{t}+K_{CcCP}K_{h}K_{t}[CP]}{(1+K_{AH+CP}[CP])} \tag{43}$$

$$c_{1(CP)}K^{\wedge}_{a(CP)}K^{\wedge\wedge}_{a(CP)}=\frac{K_{CcCP/CCCP-}K_{CcCP}K_{h}K_{t}}{1+K_{AH+CP}[CP]} \tag{44}$$

Summarizing eq.(37) eq.(39) and eq.(41) give respectively $K_{\mathrm{ACP}}$, $K_{\mathrm{BCP}}$ and $K_{\mathrm{CcCP}}$

On the other hand eq.(38) eq.(40) and eq.(42) give respectively the ionization constant of the complexes $K_{\mathrm{ACP/ACP-}}$, $K_{\mathrm{ABCP/BCP-}}$, and $K_{\mathrm{CcCP/CcCP-}}$.

It is easy to prove that

$$K_{ACP/ACP-}=\frac{K_{ACP-}}{K_{ACP}}K_{A/A-}; \qquad K_{BCP/BCP-}=\frac{K_{BCP-}}{K_{BCP}}K_{B/B-} \qquad K_{CcCP/CcCP-}=\frac{K_{CcCP-}}{K_{CcCP}}K_{Cc/Cc-} \tag{45}$$

When the equilibrium is considered the contribution of *trans*-chalcones should be added

$$X_{Ct}+X_{CtCP}+X_{Ct^{-}}+X_{Ct^{-}CP}=\frac{\dfrac{(K_{h}K_{t}K_{i}+K_{CtCP}K_{h}K_{t}K_{i}[CP])[H^{+}]+K_{Ct/Ct^{-}}K_{h}K_{t}K_{i}+K_{CtCP/CtCP^{-}}K_{CtCP}K_{h}K_{t}K_{i}[CP]}{(1+K_{AH^{+}CP}[CP])}}{[H^{+}]^{2}+K'_{a(CP)}[H^{+}]+K'_{a(CP)}K''_{a(CP)}} \tag{46}$$

$$X_{Ct} + X_{CtCP} + X_{Ct-} + X_{Ct-CP} = \frac{d_{0(CP)}K^{'}_{a(CP)}[H^{+}] + d_{1(CP)}K^{'}_{a(CP)}K^{''}_{a(CP)}}{[H^{+}]^{2} + K^{'}_{a(CP)}[H^{+}] + K^{'}_{a(CP)}K^{''}_{a(CP)}} \tag{47}$$

$$d_{0(CP)}K^{'}_{a(CP)} = \frac{K_{h}K_{t}K_{i} + K_{CtCP}K_{h}K_{t}K_{i}[CP]}{(1 + K_{AH+CP}[CP])} \tag{48}$$

$$d_{1(CP)}K^{'}_{a(CP)}K^{''}_{a(CP)} = \frac{K_{CtCP/CtCP-}K_{CcCP}K_{h}K_{t}K_{i}}{1 + K_{AH+CP}[CP]} \tag{49}$$

Comparing eq.(46) with eq.(48)

$$d_{0(CP)}K^{'}_{a(CP)} = \frac{K_{h}K_{t}K_{i} + K_{CtCP}K_{h}K_{t}K_{i}[CP]}{(1 + K_{AH+CP}[CP])} \tag{50}$$

$$d_{1(CP)}K^{'}_{a(CP)}K^{''}_{a(CP)} = \frac{K_{CtCP/CtCP-}K_{CtCP}K_{h}K_{t}K_{i}}{1 + K_{AH+CP}[CP]} \tag{51}$$

and

$$K_{CtCP/CtCP-} = \frac{K_{CtCP-}}{K_{CtCP}}K_{Ct/Ct-} \tag{52}$$