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Colour effects of co-pigmentation of anthocyanin revisited—3. A further description using CIELAB differences and assessment of matched colours using the CMC model

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Abstract

Extensive colour difference calculations (CIELAB and CMC models for CIE $D_{65}/10^{\circ}$, Ill./Obs.) were applied to the colorimetric coordinates previously gathered on 224 pure or co-pigmented (by rutin) solutions of cyanin at pH between 2.5 and 5.5. CIELAB coordinates additionally recorded on acidic (0.1N HCl) methanolic and aqueous cyanin solutions (10^{-5} M-2.5×10^{-3} M) showed the considerable hue gamut covered by each series of solutions (1/6 and 1/8 of the colour circle). A markedly yellowing effect of water vs. methanol was observable and it represented the most influential parameter in the impressive CIELAB differences (ΔE^* up to 38 units) caused by the solvent. On aqueous cyanin solutions, successive one unit pH elevations resulted in huge colour gaps (up to $\Delta E^* > 60$ units, at pH 3.5 vs. 2.5). The corresponding 'loss of colour' was mainly attributable to a decreasing chroma (ΔC^*) while the coupled hue differences (ΔH^*) corresponded to more or less deep blueing effects at pH elevations up to 4.5 and more unexpectedly to yellowing effects at pH increased to 5.5. Using CMC difference calculations, the colours of 0.1 N HCl aqueous solutions at low concentrations were best matched by those of cyanin solutions at pH 2.5 and about 2–2.5 fold higher concentrations; at high concentrations, their colours were preferentially simulated by solutions at each corresponding—or lower—cyanin concentration but co-pigmented by rutin (up to 4:1 ratio). Similar, but more complex features, emerged in the constitution of many best matched couples of colours from the database of model solutions at pHs 2.5–5.5. Close—or even identical—colour stimuli were also shown to be based on either closely matched or quite distinctly shaped spectral curves. The complete 'colour by numbers' approach presented in this series of papers may find applications in many fields in which anthocyanic pigments are employed, as natural food colorants for instance. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Anthocyanins; Co-pigmentation; Colour; CIELAB; CMC colour differences; Colour matching

1. Introduction

In aqueous medium, anthocyanin pigments are in the stable and coloured form of the flavylium cation, observable in very acidic solutions only. In flowers, these molecules are accumulated in the vacuolar compartment of epidermal cells of petals, the pH of which is currently ranging from 2.5 to 7.5 (Stewart, Norris, & Asen, 1975). Because of its high reactivity to environmental conditions, the anthocyanin nucleus has its structure strongly modified as the pH is increased, originating bathochromic shifts of the λ_{max} in the visible area of the pigment spectrum along with a hypochromic effect (Brouillard, 1983). By means of colour measurement of more than 200 model solutions (pH 2.5–5.5), it was shown that the colour effects resulting from the bathochromic shift corresponded to either blueing or yellowing changes of hue, while the currently reported 'loss of colour' coupled with the hypochromic effect corresponded to simultaneous variations of two attributes of the colour perception by the human visual system, increasing lightness and decreasing saturation; in addition, some measurable spectral variations recorded at the highest pH tested corresponded to colorimetric values of visually colourless solutions (Gonnet, 1998). By contrast, at the corresponding pHs in natural conditions (flower petals), anthocyanin pigments display 'intense' colours, resulting from their co-pigmentation with other phenolic molecules, especially flavonoids

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(Mazza & Brouillard, 1987, 1990). The colour effects of this phenomenon according to the pH, the pigment and co-pigment concentrations was extensively described using the CIELAB colorimetric scale and reference light/observer conditions (Gonnet, 1999).

The present paper is focused on the amplitude of colour differences resulting from spectral variations in anthocyanin solutions, especially those resulting from co-pigmentation, with respect to the most influential factors on this phenomenon. It is based on the previously published colorimetric CIELAB data of 224 pure pigment (cyanidin-3,5 di-glucoside or cyanin) and co-pigmented (by flavonol rutin or quercetin-3 rhamnoglucoside) solutions (Gonnet, 1998, 1999). In addition, the visible spectra and CIELAB coordinates of new sets of cyanin solutions in acidic media were recorded, representing the spectral and colorimetric references for this anthocyanin under the most coloured form of the flavylium cation. Finally, the search of couples of solutions with the closest colours in this complete database was performed, to show how perceptually identical or close colour(s) can be obtained by several solutions representing different combinations of pH, pigment and co-pigment concentrations and displaying different spectral features.

Achieving this last purpose required introducing additional colour difference calculations. The CIELAB scale having the properties on an Euclidean space, the distance between any two colour points (a reference and a sample) can be calculated according to the Pythagoras' theorem (Fig. 1). However, perceptually, this total colorimetric difference, (ΔE^*) suffers some shortcomings:

1. It was designed for 'small' differences only ($\Delta E^* < 10$), although adjustments of the formula for larger ones were recently introduced, but presently tested in the field of surface colours only (Guan & Luo, 1999).
2. The CIELAB system is not really uniform in terms of *visual* colour differences i.e. equally spaced colours in the CIELAB solid do not correspond to equal visual colour differences.

More precisely, all the colour points with a constant *colorimetric* difference ΔE^* from a colour standard (reference) are graphically distributed on the surface of a sphere, the radius of which is ΔE^*. Consequently each point on this surface represents, vs. the standard, a unique combination of ΔL^*, ΔC^* and ΔH^* differences, each one being originally considered as equally influential in the perception of the total colour differences. In reality, the results of extensive visual matching experiences have revealed that the perceptibility of colour differences by the human visual system continuously changes with the direction (i.e. it is with lightness, chroma or hue) and the amplitude of the difference. Finally, it resulted that all the colours with a constant *visual* difference from the standard appeared distributed in the CIELAB space on the surface of an ellipsoid—not a sphere—the size and shape (relative lengths of the short and long axes) of which are considerably variable with the position of the standard in the colour solid. Therefore, different calculation models have been introduced as substitutes of the original CIELAB formula, and taking in account all these particularities, gaining a better correlation between the assessment of *visual* and *colorimetric* differences. All these systems were empirically developed from the results of visual matching experiences and are introducing variable correction terms (weighting factors, applying to the ΔL^*, ΔC^* and ΔH^* CIELAB differences) the value of which is depending on the position of the standard in the colorimetric space. The different available models differ by the calculation mode of these coefficients, because the original colorimetric data employed for their derivation were measured on different types of industrial material (textiles paints, ...) and the conditions for performing the correlative visual and colorimetric assessments were specific too. Among the most widely accepted models in the colour industry are the CMC (Colour Measurement Committee; Clarke, MacDonald, & Rigg, 1984) and the recently introduced CIE_{94} (CIE, 1995) colour difference equations. Both models were developed for surface (reflective or object) colours but the CMC was also found to provide good agreement with visual assessment for transparent media (cut sheets transparencies examined over a white background; Luo et al., 1993) and consequently can be adequately considered for the final comparison of colours of anthocyanin solutions.

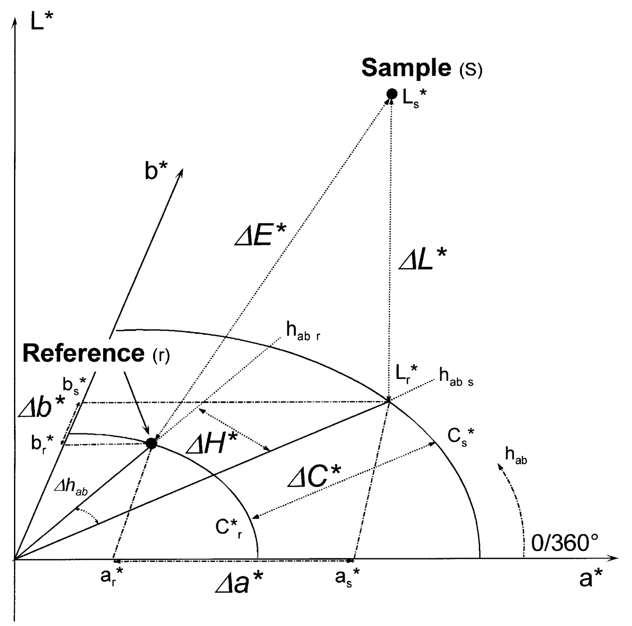

Fig. 1. CIELAB colour differences (ΔE^*) and its components: $\Delta E^* = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{0.5}$ (rectangular notation), or $\Delta E^* = (\Delta L^{*2} + \Delta C^{*2} + \Delta H^{*2})^{0.5}$ (polar notation).

In the total CMC difference (ΔE_{CMC}), the ΔL^*, ΔC^* and ΔH^* differences are each weighted by chroma dependent factors (S_L, S_C and S_H, respectively). In addition, the weighted relative tolerances of $\Delta L^*/S_L$ and $\Delta C^*/S_C$ can be further adjusted using two additional parametric factors (l and c, respectively) according to the following $\Delta E_{(CMC)}$ equation:

$$\Delta E_{(\mathrm{CMC}:l,c)} = \left[\left(\frac{\Delta L^*}{l \times S_L}\right)^2 + \left(\frac{\Delta C^*}{c \times S_C}\right)^2 + \left(\frac{\Delta H^*}{S_H}\right)^2\right]^{0.5}$$

These parametric factors are mainly employed for the adjustment of tolerances of colour control of production in the industry and to adjust the calculation to the specific attributes of the colour appearance of some materials. When the perceptibility is the prevailing factor in the colour difference evaluation, as it will be the case of colour matching of solutions, those coefficients are set to $l = c = 1$ and then the CMC colour difference becomes:

$$\Delta E_{(\mathrm{CMC}:l,l)} = \left[\left(\frac{\Delta L^*}{S_L}\right)^2 + \left(\frac{\Delta C^*}{S_C}\right)^2 + \left(\frac{\Delta H^*}{S_H}\right)^2\right]^{0.5}$$

Originally, both the CMC and CIELAB equations were found to really apply to 'small differences' only (ΔE^* below 10 units). However, further works also revealed that a good agreement with visual judgement was obtained for larger colour differences with the CMC formula for ΔE^* up to 13 units (Addac-Badu, 1986). In this survey, the calculation of colour differences between anthocyanin solutions frequently retrieved values far over this limit. Since no equation for large colour differences really matching the visual assessment was available, the CIELAB equation will be here employed first for *the description* of colour differences between solutions occurring with the variations of pigment/co-pigment and pH parameters, as it was used in the previous paper (Gonnet, 1999), even if some very huge differences perceptually appear meaningless. In the search of colour vicinity among all the solutions in the complete batch of model solutions, the CMC model was introduced.

2. Materials and methods

Recording the spectral data and calculation of the CIELAB coordinates of the new acidic cyanin solutions (0.1N HCl in H_2O and MeOH) were performed according to the procedure described previously for the ones measured at pH 2.5–5.5 (Gonnet, 1998, 1999). From the spectral transmittance curves (380–780 nm, regular transmission, optical path 1 cm), the CIELAB coordinates expressed in terms of lightness (L^*), chroma (C^*) and hue angle (h_{ab}) were calculated for the CIE $D_{65}/10^\circ$ illuminant/observer reference condition (CIE, 1986). This corresponds to the numerical specification of the colour stimulus perceived by an human observer viewing (10° observation field) a perfectly white background illuminated by a daylight source (CIE D_{65} type) through a pigment solution of 1 cm thickness. CIELAB colour differences were calculated using the formulas presented in Fig. 1.

The search of closest visual colours was performed by a specially developed computer program based on the Quattro-Pro® (Corel) and Paradox® (Inprise) softwares. The CIELAB coordinates of each sample solution were successively introduced as the standard (reference) ones in a database containing the colorimetric coordinates of all the model solutions: the solutions with the closest colours to each standard were automatically retrieved from the database and sorted, on the basis of the smallest $\Delta E_{(\mathrm{CMC}:1,1)}$s. This calculation method was applied to 236 pigment and co-pigmented model solutions but in this paper, only the data of solutions displaying really visually perceptible colours (i.e. having a chroma over approximately 20 C^* units, under the conditions of measurement employed) will be considered for discussion. All the colorimetric calculations in this survey were performed on a NEC Dimension 350 PC computer.

3. Results and discussion

3.1. The colours of cyanin in acidic (0.1 N HCl) solutions: influence of the solvent

Most of the available spectral data of pure anthocyanins in the literature are reported for solutions at pH 1 or containing 0.1–3% HCl (Giusti, Rodríguez-Saona, & Wrolstrad, 1999; Harborne, 1958), generally in methanol. However, the spectral properties of these pigments—and consequently their colours—are also intensely affected by the solvent. This study represented an opportunity to record the variations of the spectral and colorimetric properties of cyanin, one of the most common anthocyanins, in different solvents. In addition to the data of acidic aqueous solutions—serving as the reference for discussing the colour variations of this pigment at different concentrations, pH and co-pigment to pigment ratios—the spectra of a batch of corresponding solutions were recorded in methanolic solutions in the presence of 0.1N HCl and their CIELAB coordinates calculated (Table 1). When their absorbances were measurable (i.e. concentrations below 10^{-4} M), all the aqueous solutions shared the same λ_{max} at 510 nm while it was shifted up to 526 nm for all the corresponding methanolic ones, which also displayed higher absorbances at this λ_{max}. The Fig. 2 presents the colorimetric differences (total CIELAB difference ΔE^* and its components, ΔL^*, ΔC^* and ΔH^*) calculated in each couple of methanolic and aqueous solutions at different concentrations, the first ones serving as the reference.

When compared to the currently accepted threshold of perceptibility of CIELAB colorimetric differences (approximately $\Delta E^*=0.8$–1), the total differences recorded between methanolic and aqueous cyanin solutions reached impressive values, from 6.4 to 38.6, the maximum being obtained for a pigment concentration (10^{-4} M) in the middle of the range studied. Hue variations were the most overall influential parameter of the total difference (ΔE^*), specially for the solutions between 2.5×10^{-5} M and 5×10^{-4} M, and represented a strong yellowing effect of water on chromatic tonalities, with a maximum ($\Delta H^*=36.4$) observable at 10^{-4} M. Their amplitude are resulting from the combination of the extent of each hue gamut—the methanolic solutions displaying the largest one covering a 61° portion of the colour circle vs. 46° only for the aqueous ones—with the specific evolution of this attribute with the cyanin concentration in each batch of solutions. For instance, the colour of both the aqueous and methanolic solutions at 10^{-3} M shared the same basic orange tonality (h_{ab} 44.4° for H_2O pH 1 vs. h_{ab} 44.7° for methanol, $\Delta H^*=-0.53$) but when their concentration was decreased to 5×10^{-4} M, the hue of the aqueous solution turned yellower (h_{ab} 50.4°) while the one of its methanolic counterpart moved slightly bluer (h_{ab} 42.7°). Then from there, at lower pigment concentrations the tonality of colour in both series of solutions continuously shifted bluer, but with a specific amplitude in each one, originating the evolution of the ΔH^* curve in Fig. 2. In particular, the final hue in each gamut measured on the solution at 10^{-5} M, was typically magenta (h_{ab} 4.0°) for the aqueous solution vs. purple (h_{ab} 343°, i.e. strongly bluer) for the methanolic one.

Table 1
CIELAB colorimetric coordinates (D65/10°, transmission, 1 cm optical pathlength) of acidic (0.1 N HCl) aqueous and methanolic cyanin solutions (10^{-5}–10^{-3}M)

Cyanin concentration (M)	Water (0.1 N HCl)			MeOH (0.1 N HCl)		
	L^*	C^*	h_{ab}	L^*	C^*	h_{ab}
10^{-3}	40.75	100.23	44.38	43.40	106.00	44.67
5×10^{-4}	52.49	112.79	50.43	49.15	104.10	42.69
2.5×10^{-4}	61.88	96.86	46.17	54.65	86.61	26.07
10^{-4}	71.79	69.29	29.99	62.67	78.39	1.39
5×10^{-5}	78.81	53.13	16.72	70.36	70.45	350.75
2.5×10^{-5}	85.82	36.78	8.64	79.53	52.38	346.02
10^{-5}	92.93	18.71	4.05	89.46	27.63	343.54

Chroma (ΔC^*) was the secondly influential parameter accounting for the total difference, displaying the most complex variations observed with pigment concentration. In the range of the lowest concentrations ($\leqslant10^{-4}$ M), the most saturated solutions were the methanolic ones (maximum effect at 5×10^{-5} M, ΔC^* −17.3) while at the highest concentrations, the highest chroma values were displayed by the aqueous solutions (maximum effect at 2.5×10^{-4} M, ΔC^* 10.2) with the exception of 10^{-3} M (ΔC^* −5.9).

Increasing lightness was the weakest contributor to ΔE^* (maximum ΔL^* 9.1, at 10^{-4} M); all but one of the aqueous solutions appearing lighter than the corresponding methanolic ones, the evolution of this attribute with the cyanin concentration following those observed for ΔE^* and ΔH^* but with a considerably reduced amplitude. Again the colorimetric difference observed in the couple of solutions at 10^{-3} M was quite specific, being the only one in which the colour of the aqueous solution appeared darker (ΔL^* −2.6) than its methanolic reference.

The unexpected differences markedly observed in the range of the highest concentrations originate in the particular evolution of colour parameters with concentration in each series of solutions. For the methanolic one, the upper edge of the chroma and hue gamuts was observed at the highest concentration tested (10^{-3} M) while for aqueous solutions it occurred at a lower concentration (5×10^{-4} M) from which the value of both parameters decreased (C^*) or moved counter-clockwise (h_{ab}) as examined above when the pigment concentration in acidic water was increased (to 10^{-3} M) or decreased (to 2.5×10^{-4} M).

These results emphasize the previously reported complexity of the relationships between the spectral data

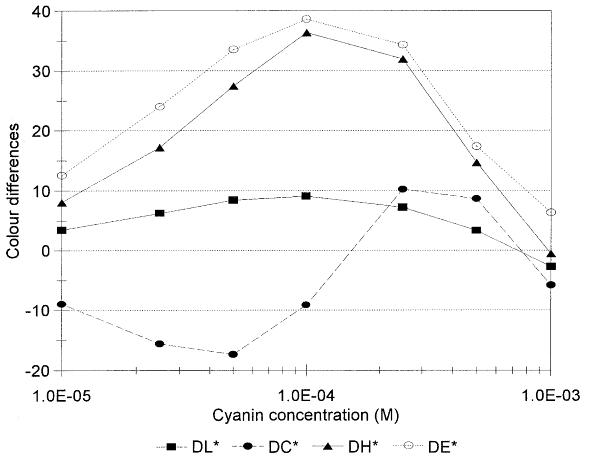

Fig. 2. CIELAB colour differences (D65/10° CIE illuminant/observer condition, transmission, 1 cm optical pathlength) between 0.1N HCl aqueous and methanolic cyanin solutions (10^{-5}–10^{-3}M).

(generally considered at the λ_{max}) and the resulting colours of solutions (Gonnet, 1998). Here again, solutions exhibiting a fixed λ_{max} displayed considerable hue variations (h_{ab} 4–50.4°, for aqueous solutions with the same λ_{max} at 510 nm; h_{ab} 343.5–44.7°, for methanolic ones with λ_{max} at 526 nm); by contrast, two solutions at the same concentration (10^{-3} M) but having shifted spectra (λ_{max} theoretically centred on 510 and 526 nm) share the same basic tonality (h_{ab} 44.4–44.7°). Regarding the hyperchromic effect—here observed for methanolic solutions vs. aqueous ones (their absorbance at the λ_{max} being about 20% higher)—this resulted in decreasing lightness (excepted for the highest concentration tested) coupled with 'erratic' effects on chroma: either increasing or decreasing, with a considerable amplitude of variation. In this regard, the colour differences between the two solutions at 5×10^{-4} M are quite illustrating: spectrally, the aqueous solution was the one having the lowest absorbance in the area of the λ_{max} but colorimetrically, the combination of its (higher) chroma and lightness corresponds to a visually more 'intensely' coloured solution than its methanolic counterpart; this is resulting from spectral effects of solvent affecting some other portions of the visible spectrum (mainly between 410–470 nm).

3.2. Colour differences between pure cyanin solutions with the pH and the pigment concentration (Fig. 3 a–d)

At the highest two pHs tested, only the solutions at concentrations 2.5×10^{-5} M (pH 4.5) or 2.5×10^{-4} M (pH 5.5) and up displayed visually perceptible colours; consequently, the colorimetric differences between solutions at lower concentrations will be ignored. With respect to their reference colours observed at pH 1, the cyanin solutions, almost in totality, suffered a continuous colour fading as the pH was increased, by combined effects on chroma and lightness. The total colorimetric differences between the colours displayed by solutions of the flavylium coloured forms (at pH 1) and by the corresponding ones at pH 5.5, reached impressively huge values (ΔE^* from 20 to 111 for 10^{-5} and 5×10^{-4} M, respectively). Rare exceptions were the solutions at 2.5×10^{-3} and 10^{-3} M which appeared at pH 2.5 (very) slightly more coloured (mainly because of a higher chroma) than their respective counterparts at pH 1.

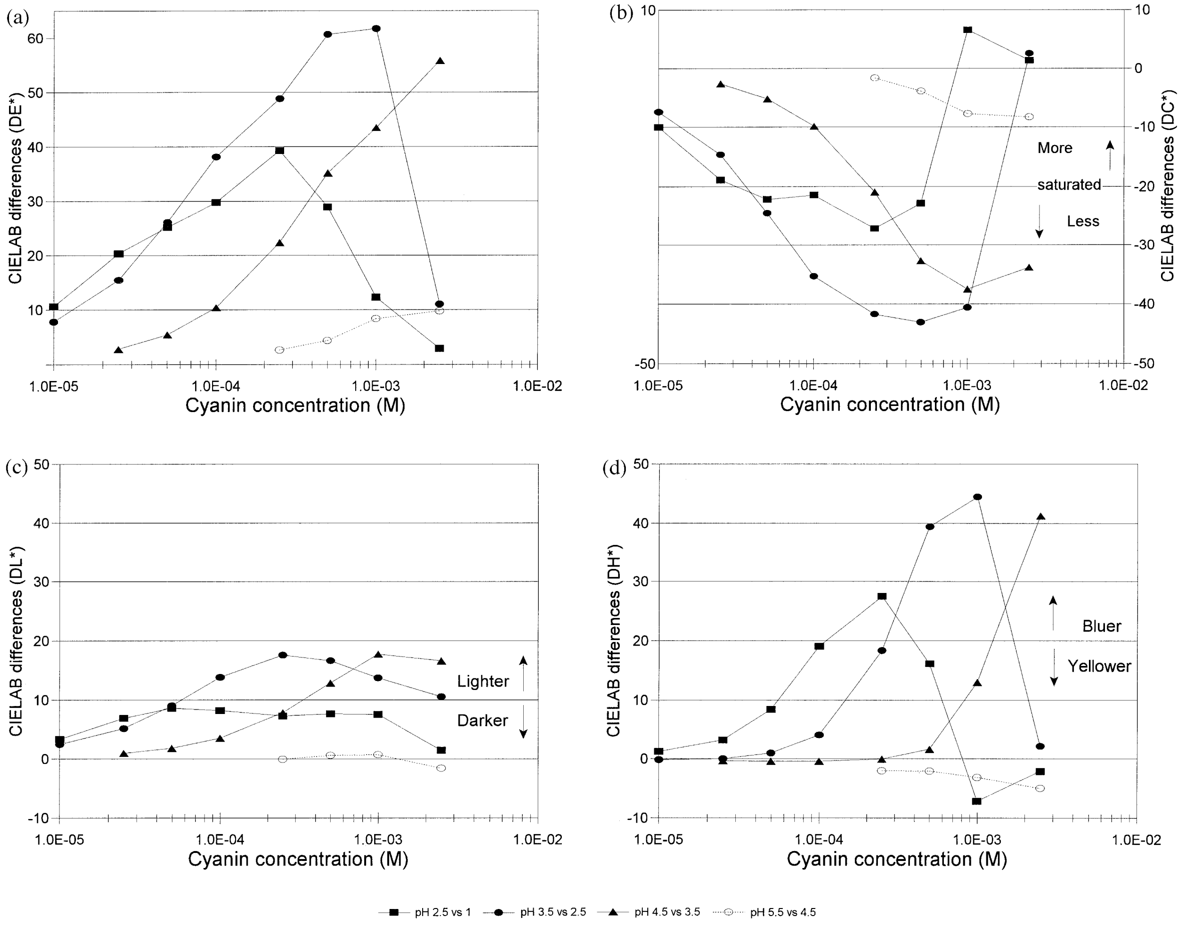

Fig. 3. CIELAB colour differences (D65/10°, transmission, 1 cm optical pathlength) between pure cyanin solutions (10^{-5}–2.5×10^{-3}M) at each pH increase step (between 1 and 5.5). (a) total differences (ΔE^*), (b) chroma differences (ΔC^*), (c) lightness differences (ΔL^*), (d) hue differences (ΔH^*).

The highest total colorimetric difference ($\Delta E^*_{\max}$) coupled with a one unit pH variation in a series was recorded for the step 2.5–3.5 ($\Delta E^*_{\max}$ over 60 units, Fig. 3a), representing a considerable colour change. Some colour variations measured in each couple of solutions at just the higher or lower pH steps remained very impressive too ($\Delta E^*_{\max}$ 56 and 39, respectively) but over 4.5 the pH effects on colour strongly weakened ($\Delta E^*_{\max} < 10$). Simultaneously, the pigment concentration between pairs of solutions with the $\Delta E^*_{\max}$ in each batch shifted to higher values with increasing pHs: from 2.5×10^{-4} M for the first pH step, it moved to 5×10^{-4} M–10^{-3} M ($\Delta E^*_{\max}$ 60.7–61.8) for the next pH step and finally to 2.5×10^{-3} M for the two final ones, 4.5–3.5 and 5.5–4.5.

In each series of different pH steps, the colour variations were mostly determined by chroma (Fig. 3b), the strong decrease of which (ΔC^* up to −43) is representing the main cause of the classically reported 'loss of colour' of anthocyanins with increasing pHs. Exceptionally increasing chroma (three exceptions only) occurred, at the highest two cyanin concentrations and first pH steps. Accordingly, and except for the batch at pH 4.5–3.5, the pigment concentrations for the maximum effect of pH on the chroma of solutions—and the relative amplitude of ΔC^*—were comparable to the ones reported for the total difference.

Lightness was the attribute displaying the smallest variations (maximum ΔL^*17.6, figure 3c), all but one solutions (2.5×10^{-3} M, pH 4.5–5.5) turning lighter (L^* increased) when the pH augmented, with a simultaneous shift of the concentration of the maximum ΔL^* at each pH step from 5×10^{-5} M to 2.5×10^{-3} M. ΔL^*s were virtually negligible between solutions at the highest pHs (ΔL^* below the threshold of perceptibility for all the solutions, excepted 2.5×10^{-3} M); it also remained the least influential parameter of the colour difference between most of solutions at the first pH step and those at the highest concentrations at pH 3.5–2.5, except for the pair at 2.5×10^{-3} M for which ΔL^* accounted for almost the complete total difference (ΔL^*10.6 in ΔE^* 11.1).

Comparably to the other two colour attributes, the cyanin concentration for the strongest hue variation in each series (ΔH^*, Fig. 3d) progressively moved from medium (2.5×10^{-4} M at the first pH step) to high values (2.5×10^{-3} M) at the highest pHs. However, excepted in the series at both ends of the pH gamut studied, the ΔH^* were more sudden here: only small changes occurred with the pH variation at the lowest concentrations while at higher ones (over 10^{-4} and 5×10^{-4} M at pH 4.5–3.5 and 5.5–4.5, respectively) the hue hardly shifted and represented the main factor of the colour variation measured: ΔH^*−44.5 (10^{-3}M, pH 3.5 vs. 2.5: orange to magenta-red hues) or −41.3 (2.5×10^{-3}M, pH 4.5 vs. 3.5: orange-red to magenta hues). Regarding the perceived colour, most of these variations corresponded to a blueing effect of the pH elevation on hues but there were also some solutions where the hue became yellower, namely those at the both ends of the gamut surveyed: 10^{-3} and 2.5×10^{-3} M at pH 2.5 vs. pH 1 and the complete batch of solutions at pH 5.5 vs. the corresponding ones at pH 4.5.

3.3. Colour differences between model co-pigmented solutions

CIELAB colorimetric differences between the corresponding co-pigmented solutions at the successive one unit incremented pHs between 2.5 and 5.5 are listed in Table 2. No data are supplied for colourless or poorly coloured solutions, the differences measured either remaining largely under the threshold of perceptibility or being predominantly based on hue differences (huge yellowing effect) caused by the massive additions of co-pigment. This applies to solutions at pigment concentrations $\leqslant 2.5\times10^{-5}$ M, 5×10^{-5} M and 10^{-4} M at the 3.5–2.5, 4.5–3.5 and 5.5–4.5 pH change steps, respectively.

Most of the greatest total differences (ΔE^*) were measured between solutions at the lowest pHs and between those in the series at pH 4.5–3.5 at the highest cyanin concentrations too, i.e. the most coloured ones, in accordance with the previous reports on the pH effects on the efficiency of co-pigmentation. Comparable to pure cyanin solutions—and with the same exception (2.5×10^{-3} M, pH 3.5)—increasing the pH originated a considerable fading of the colour of co-pigmented solutions, coupled with blueing ($\Delta H^* < 0$) or more unexpectedly yellowing effects ($\Delta H^* > 0$) on tonalities, some of them corresponding to spectacular hue shifts.

The greatest differences—$\Delta E^*_{\max}$ 35 to over 50 units—between corresponding solutions were recorded at pH steps 3.5–2.5 and then 4.5–3.5, the concentrations for which they were measured being intermediate (10^{-4}–10^{-3} M) or the highest ones (5×10^{-4}–2.5×10^{-3} M), respectively. Increasing the pH a step further to 5.5 resulted in more reduced effects ($\Delta E^*_{\max} < 18$) the highest amplitude of which was recorded for the solution at 10^{-3} M.

In addition, when the pH of the solutions at the lowest pigment concentration (5×10^{-5} M) was changed to 3.5 from 2.5, the most important effects on colour occurred in highly co-pigmented ones (co-pigment to pigment ratio 64:1). Then, with increasing cyanin concentration, the maximal effect progressively shifted to solutions at the lowest ratio (down to 0.5:1). In the cyanin concentration range up to 2.5×10^{-4} M, the colour variation mostly depended on the loss of chroma, coupled with a less important lightness relative increase. A growing influence of hue variation (ΔH^*) with increasing co-pigmentation level was observable in the first batch of solutions (5×10^{-5}M). Perceptually, the hue differences recorded here mean a yellowing effect of pH on tonalities, i.e. highly co-pigmented solutions ($\geqslant$4:1 ratio) at pH 3.5 are clearly yellower than their counterparts at pH 2.5. At the 10^{-4} M pigment concentration, most of the hue variations represented a minor parameter of the colour difference and corresponded to a very slight blueing effect of the pH on tonalities ($\Delta H^* < 0$) of weakly co-pigmented solutions which progressively turned to a marked yellowing effect ($\Delta H^* > 0$) as the co-pigmentation level was increased. In the batches of solutions at 2.5×10^{-4} M-10^{-3} M, the hue difference (general intense blueing effect of increasing pH) gained more and more influence and by a large margin originated most of the deep colour variation perceived in the last two series. Finally, in the solutions at the highest pigment concentration tested (2.5×10^{-3} M), only relatively moderate colour variations—and corresponding to a yellowing effect on hues—were caused by increased pH.

A very comparable variation outline in the colour changes was observable at the further two pH step elevations but with regard to the pigment concentration scale, it appeared globally shifted to higher steps (by a 2–4-fold factor). In particular, this evolution resulted in the basic chromatic tonalities of the colours of solutions at pH 5.5 vs. 4.5 all turning yellower, a blueing effect of the pH affecting a unique solution— the one at the highest pigment concentration and 1:1 co-pigment to pigment ratio.

As reported before (Gonnet, 1999), most of the yellowing effects on hues of solutions systematically

Table 2
CIELAB colour differences (D65/10°, transmission, 1 cm optical pathlength) between co-pigmented cyanin solutions (5×10^{-5}–2.5×10^{-3}M) at each pH increase step (pH 2.5–5.5)

Cyanin concentration (M)	Rutin: cyanin ratio	pH 3.5 vs. 2.5				pH 4.5 vs. 3.5				pH 5.5 vs. 4.5			
		ΔE^*	ΔL^*	ΔC^*	ΔH^*	ΔE^*	ΔL^*	ΔC^*	ΔH^*	ΔE^*	ΔL^*	ΔC^*	ΔH^*
5×10^{-5}	0.5:1	**26.96**	9.59	−25.19	−0.68	–	–	–	–	–	–	–	–
	1:1	**27.58**	9.88	−25.75	−0.32	–	–	–	–	–	–	–	–
	2:1	**28.03**	9.60	−26.33	0.47	–	–	–	–	–	–	–	–
	4:1	**29.13**	10.02	−27.22	2.61	–	–	–	–	–	–	–	–
	8:1	**30.92**	11.21	−28.43	4.71	–	–	–	–	–	–	–	–
	16:1	**34.63**	13.40	−31.30	6.30	–	–	–	–	–	–	–	–
	32:1	**33.80**	12.82	−29.33	10.85	–	–	–	–	–	–	–	–
	64:1	**35.55**	14.81	−26.63	18.32	–	–	–	–	–	–	–	–
10^{-4}	0.5:1	**38.55**	14.21	−35.65	−3.64	**11.53**	4.13	−10.71	1.13	–	–	–	–
	1:1	**39.41**	14.68	−36.45	−3.02	**12.25**	4.48	−11.25	1.85	–	–	–	–
	2:1	**40.82**	15.28	−37.81	−1.81	**13.91**	4.97	−12.26	4.30	–	–	–	–
	4:1	**42.89**	16.88	−39.42	−0.27	**15.50**	5.69	−12.98	6.28	–	–	–	–
	8:1	**45.78**	19.24	−41.54	0.49	**20.03**	7.27	−15.14	10.92	–	–	–	–
	16:1	**42.32**	18.00	−38.17	3.21	**26.66**	10.91	−18.39	15.93	–	–	–	–
	32:1	**37.87**	16.74	−33.57	5.19	**31.20**	13.73	−18.15	21.35	–	–	–	–
2.5×10^{-4}	0.5:1	**47.29**	17.73	−40.08	−17.76	**25.54**	9.53	−23.68	0.88	**3.25**	0.77	−2.51	1.91
	1:1	**46.48**	18.27	−38.54	−18.47	**28.57**	10.82	−26.38	1.71	**4.06**	1.14	−3.02	2.46
	2:1	**44.01**	18.74	−35.29	−18.45	**32.90**	12.37	−30.19	4.20	**5.45**	2.20	−4.06	2.90
	4:1	**41.26**	19.40	−30.76	−19.48	**38.48**	15.20	−34.84	6.01	**6.38**	1.65	−3.53	5.05
	8:1	**33.58**	15.69	−23.56	−18.07	**44.12**	19.18	−39.33	5.64	**10.50**	2.66	−5.45	8.57
	16:1	**33.59**	15.70	−23.61	−18.01	**42.34**	19.48	−36.93	7.00	**14.52**	1.11	−0.05	14.48
5×10^{-4}	0.5:1	**54.57**	14.47	−32.66	−41.26	**43.39**	17.49	−39.59	−3.07	**6.63**	1.61	−5.79	2.80
	1:1	**52.27**	13.38	−27.69	−42.27	**45.87**	19.03	−41.56	−3.81	**8.82**	2.74	−7.76	3.18
	2:1	**50.12**	12.52	−22.72	−42.88	**44.76**	20.17	−39.74	−4.12	**12.15**	2.68	−11.40	3.23
	4:1	**45.70**	12.22	−20.16	−39.15	**42.15**	19.68	−36.40	−8.05	**13.71**	2.47	−11.87	6.40
	8:1	**48.14**	13.11	−16.07	−43.44	**37.10**	14.34	−32.94	−9.29	**14.13**	2.14	−11.55	7.85
10^{-3}	0.5:1	**51.99**	10.85	−26.48	−43.41	**44.62**	18.70	−35.20	−20.04	**14.12**	2.99	−12.83	5.09
	1:1	**43.75**	8.92	−20.12	−37.81	**43.90**	18.12	−30.09	−26.34	**14.13**	2.32	−13.64	2.87
	2:1	**39.27**	8.86	−14.42	−35.43	**38.17**	12.99	−22.49	−27.98	**16.29**	3.38	−15.82	1.95
	4:1	**35.93**	9.80	−11.26	−32.68	**34.75**	5.12	−15.58	−30.64	**17.81**	5.13	−16.84	2.75
2.5×10^{-3}	0.5:1	**5.44**	3.31	3.93	1.80	**52.20**	10.57	−16.38	−48.42	**8.70**	0.87	−8.66	0.12
	1:1	**4.58**	2.44	3.33	1.97	**42.07**	6.02	−10.93	−40.18	**8.10**	2.53	−7.31	−2.39

displaying bathochromic shifts at their λ_{max}—instead of expectable blueing ones—originate in spectral variations occurring simultaneously in the shortest visible wavelengths. These are caused by the cumulative effects of the co-pigment absorption (between 380 and 450 nm) when massively added and of the increased absorptivity of cyanin itself in the 400–480 nm area at higher pHs.

Regarding the specific effects of the co-pigment to pigment ratio, it was previously shown (Gonnet, 1999) that in a restricted range of model solutions at a fixed pH (2.5):

1. The intensity of the colour effects of gradual co-pigment addition continuously increased at each co-pigment to pigment ratio with the pigment concentration between 5×10^{-5} M and 10^{-3} M and then decreased (2.5×10^{-3} M).
2. Co-pigmentation acted the most efficiently on colour variation at its lowest level (0.5:1) in the solutions at high cyanin concentrations (5×10^{-4} M and up) and by contrast this was at its highest levels in the solutions at low pigment concentrations (below 10^{-4} M).

When considering the total (i.e. vs. the reference pure pigment solutions) and the stepped (i.e. between each successive co-pigmented solutions in a batch) colorimetric differences in the full line of model solutions at different pH and co-pigment to pigment ratios tested (data not supplied here), very comparable global trends are emerging, with the difference that generally the pigment concentrations for the above described colour effects are progressively shifted to higher values when the pH is increased: for instance the pigment concentration for the maximum co-pigmentation effect observable at the 0.5:1 ratio moved from 5×10^{-4} M to 2.5×10^{-3}M when the pH increased from 2.5 to 5.5.

3.4. *Retrieving the pigment solutions with the closest colours in the database*

3.4.1. *Model solutions vs. reference 0.1N HCl aqueous cyanin solutions*

Table 3 presents the results of the search performed into the colorimetric database to retrieve the model solutions displaying the closest colours to the ones of aqueous cyanin solutions at pH 1 (including some additional ones not considered in the first part of the report). Some model solutions were found to display colours really matching those of reference solutions, at the lower and higher ends of the concentration gamut surveyed, 10^{-5} M and 7.5×10^{-4}–4×10^{-3}M, respectively ($\Delta E_{CMC} \leqslant 1$, i.e. down to the threshold of just visually noticeable differences). In the interval, the colour gap in every other couple of solutions with the closest colours was larger and represents largely perceptible (ΔE_{CMC} 2.5–3.8) or even very important (ΔE_{CMC} 5–6.2) differences.

Versus each acidic reference most of the model solutions with the closest colour were at the nearest higher pH (2.5), but generally at a higher concentration or/and additionally co-pigmented by rutin. The general trend in the range of reference solutions at the lowest concentrations ($\leqslant 7.5\times10^{-4}$M, with one exception 2.5×10^{-5} M), was that their best colour match was the pure cyanin solution at pH 2.5 and at about a 2–2.5 fold higher concentration. Simulating the colour of the four at the highest pigment concentrations ($\geqslant 10^{-3}$M) was preferentially obtained by the effects of co-pigmentation: here, really matched colours were observed, those of model solutions at higher pH (2.5 or even 3.5) at an identical- or even a lower—concentration, but in which rutin co-pigment was present (this also applies to the solution at 2.5×10^{-5}M). In this regard, the reference solutions at 2 and 2.5×10^{-3} M deserve a special interest:

Table 3

CMC $_{(1:1)}$ colour differences (D65/10°, transmission, 1 cm optical pathlength) between each reference pure cyanin solution (0.1 N aqueous HCl) and the retrieved model solution with the closest colour

Reference cyanin solution at pH = 1:	Model solution with the closest colour to the reference:						
Concentration (M)	Cyanin concentration (M)	pH	Rutin:cyanin ratio	**Δ E (CMC:$_{1:1}$) Colour differences:**			
				Δ E	$\Delta L^*/S_L$	$\Delta C^*/S_C$	$\Delta H^*/S_H$
10^{-5}	2.5×10^{-5}	2.5	0.5:1	**0.70**	−0.31	−0.49	0.39
2.5×10^{-5}	2.5×10^{-5}	2.5	2:1	**2.54**	0.00	−1.50	2.05
5×10^{-5}	10^{-4}	2.5	0	**3.84**	0.90	−1.99	3.16
10^{-4}	2.5×10^{-4}	2.5	0	**3.12**	−2.00	0.13	2.39
2×10^{-4}	5×10^{-4}	2.5	0	**5.85**	−4.49	−0.14	3.75
2.5×10^{-4}	5×10^{-4}	2.5	0	**6.21**	−1.44	−2.04	5.69
5×10^{-4}	10^{-3}	2.5	0	**5.00**	−3.72	−1.68	2.90
7.5×10^{-4}	10^{-3}	2.5	0	**0.99**	0.26	−0.91	0.27
10^{-3}	10^{-3}	2.5	0.5:1	**1.07**	1.04	0.07	0.25
2×10^{-3}	10^{-3}	2.5	4:1	**0.85**	−0.39	0.02	0.76
2.5×10^{-3}	2.5×10^{-3}	3.5	0.5:1	**0.84**	−0.01	−0.63	0.56
4×10^{-3}	2.5×10^{-3}	2.5	1:1	**0.51**	−0.42	−0.09	0.27

the colour of the first one was very closely matched by a solution at pH 2.5 at half its own concentration but in return strongly co-pigmented (4:1 ratio) while for the second one, the best match was the solution at the same concentration, a markedly higher pH (3.5) but more moderately co-pigmented (0.5:1 ratio). In the range of intermediate concentrations, the importance of some ΔE_{CMC} retrieved (>3 or 5) signified that no model solution in the database displayed very close colours: in most cases, the mismatch was caused by the amplitude of the hue differences ($\Delta H^*/S_H$).

3.4.2. Closest colours in model solutions at different pH and co-pigment ratios

The retrieved couples of model solutions displaying the closest colours are listed in Table 4. In the batches of solutions at pH 2.5 between 5×10^{-5} and 2.5×10^{-4} M, the chemical features of the pairs of solutions with the closest colours fall into two categories. For each solution at the lowest co-pigment to pigment ratios (0 to 4:1, or 1:1 only for 2.5×10^{-4} M), the best colour matching solution was one at the same pH, concentration and at the closest co-pigment concentration (higher or lower), while the colours of all the solutions at the highest co-pigment ratios (>8:1, or 2:1 for 2.5×10^{-4} M) were most closely matched by those of solutions at higher pH (3.5), concentrations (5 or 4-fold higher) but at a much lower (8 or 16-fold) co-pigment to pigment ratio than their respective reference. For all but one of the solutions at 5×10^{-4} M and 10^{-3}M, the couples with the closest colours were only formed of solutions at the same pH (2.5), identical concentration and generally at the nearest (higher or lower) co-pigment to pigment ratio. Here, the colour differences are far more important than in the batches of solutions at the lowest concentrations (most ΔE_{CMC} were <3). By contrast, each closest colour retrieved for the three solutions at the highest concentration tested (2.5×10^{-3} M) represented a very good match ($\Delta E_{\text{CMC}}<1.5$), based on a specific combination of chemical parameters: solution at the same pH, a lower pigment concentration but a higher co-pigment to pigment ratio for the pure pigment solution—or for the two co-pigmented ones, solutions at higher pH (3.5) at either the same cyanin concentration coupled with a higher co-pigmentation level or at higher cyanin concentration (510^{-3} m) without co-pigment added.

With many solutions at pH 3.5 at the lowest co-pigmentation ranges (up to 8:1, shrinking to 1:1 only with increasing pigment concentrations), being retrieved as the best colour matches for highly co-pigmented solutions at pH 2.5, the reciprocity frequently applies here, i.e. their closest colours were those of solutions at this lower pH, a lower concentration (4 or 5-fold) and a markedly higher co-pigment concentration (4 to 16-fold). For each highly co-pigmented solution at pH 3.5, the closest colour was displayed by the solution at the nearest co-pigment to pigment ratio in the same concentration series. Finally, the best matches for the two most co-pigmented solutions at 10^{-4} M (16 and 32:1; those at ratios <4:1 displaying very pale colours are ignored here) were found in the series of solutions at higher pHs (4.5 or even 5.5), not, or weakly co-pigmented, but in return having a much higher concentration (10^{-3} M).

At pH 4.5 and 5.5, only the solutions at pigment concentration higher than 5×10^{-4} M displayed colours in the range considered in this report (i.e. $C^*>20$).

At pH 4.5, when the relative co-pigment concentration varied from 0 to 8:1 in the solutions at 5×10^{-4}M, the best matching solution was first at lower pH (3.5) and pigment concentration (5-fold) coupled with a higher co-pigment to pigment ratio, then at the same pH and concentration and the closest co-pigment to pigment ratio, and finally, at the same pH (or one step higher), higher pigment concentration (2-fold) and lower co-pigment to pigment ratio. Parallel trends were observed for the solutions at 10^{-3} M: at the lowest co-pigmentation levels (0–1:1), solutions at the same pH, lower pigment concentration (2-fold) and higher co-pigment to pigment ratio were the best respective colour matches while solutions at higher concentration (2.5-fold), higher pH (5.5) but a lower co-pigment to pigment ratio displayed the closest colours to the ones of solutions at the highest co-pigmentation level. Finally, the closest colour of the pure cyanin solution at 2.5×10^{-3} M was displayed by a solution the far lower concentration (10-fold) of which was balanced by the coupled effects of lower pH (3.5) and very strong co-pigmentation level (16:1); for the two co-pigmented solutions (0.5 and 1:1), their best colour matches were the corresponding ones at pH 5.5.

In the series at pH 5.5, the closest colours for all solutions at the highest pigment concentration (2.5×10^{-3}M) were displayed by their counterparts at pH 4.5. As for the only two solutions at 5×10^{-4} M considered, their best colour matches were solutions at the same pH but combining, vs. their respective reference, adverse effects of higher concentration (2-fold) and lower co-pigment to pigment ratio. Finally, the closest colours for all but one of the solutions at 10^{-3} M were found among those at pH 4.5 which were featuring, as the co-pigmentation level of the reference increased, a lower pigmentation concentration (2-fold) and stronger co-pigmentation (2–4-fold) and then the same concentration with the closest lower co-pigmentation ratio. Finally, the best match for the pure pigment solution was again a solution at a much lower concentration (4-fold) the weaker colour of which is reinforced by the effects of massive co-pigment addition (16:1 ratio).

According to the current literature on the co-pigmentation effects one could expect that different solutions

Table 4
CMC $_{(1:1)}$ colour differences (D65/10°, transmission, 1 cm optical pathlength) between each reference co-pigmented cyanin solution and the retrieved model solution with the closest colour[a]

Reference model solution:			Model solution with the closest colour to the reference:						
pH	Cyanin concentration (M)	Rutin: cyanin ratio	PH	Cyanin concentration (M)	Rutin: cyanin ratio	**ΔE (CMC:$_{1:1}$) Colour differences:**			
						ΔE	$\Delta L^*/S_L$	$\Delta C^*/S_C$	$\Delta H^*/S_H$
2.5	5×10^{-5}	0	2.5	5×10^{-5}	0.5:1	**0.54**	−0.41	0.33	0.10
		0.5:1	2.5	5×10^{-5}	0	**0.54**	0.41	−0.33	0.10
		1:1	2.5	5×10^{-5}	0.5:1	**0.54**	0.34	−0.37	0.20
		2:1	2.5	5×10^{-5}	1:1	**0.70**	0.43	−0.48	0.29
		4:1	2.5	5×10^{-5}	2:1	**1.52**	0.68	−0.89	1.03
		8:1	3.5	2.5×10^{-4}	1:1	**1.13**	−0.90	−0.69	0.06
		16:1	3.5	2.5×10^{-4}	2:1	**1.02**	−0.81	−0.47	0.41
		32:1	3.5	2.5×10^{-4}	2:1	**1.72**	1.39	−1.02	0.12
		64:1	3.5	2.5×10^{-4}	4:1	**3.45**	0.64	1.21	3.17
	10^{-4}	0	2.5	10^{-4}	0.5:1	**1.09**	−0.82	0.52	0.50
		0.5:1	2.5	10^{-4}	0	**1.09**	0.82	−0.52	0.50
		1:1	2.5	10^{-4}	0.5:1	**1.13**	0.83	−0.59	0.49
		2:1	2.5	10^{-4}	1:1	**2.16**	1.23	−1.05	1.44
		4:1	2.5	10^{-4}	2:1	**2.66**	2.20	−1.11	1.02
		8:1	3.5	5×10^{-4}	0.5:1	**2.30**	0.44	−1.66	1.53
		16:1	3.5	5×10^{-4}	1:1	**2.03**	0.22	−0.72	1.88
		32:1	3.5	5×10^{-4}	2:1	**1.69**	−1.29	1.09	0.09
	2.5×10^{-4}	0	2.5	2.5×10^{-4}	0.5:1	**3.74**	−2.27	0.74	2.88
		0.5:1	2.5	2.5×10^{-4}	1:1	**2.83**	−2.30	0.70	1.49
		1:1	2.5	2.5×10^{-4}	0.5:1	**2.83**	2.30	−0.70	1.49
		2:1	3.5	10^{-3}	0	**4.08**	2.46	−3.24	0.37
		4:1	3.5	10^{-3}	0.5:1	**2.10**	−0.87	−1.17	1.52
		8:1	3.5	10^{-3}	1:1	**2.82**	−1.61	−0.98	2.10
		16:1	3.5	10^{-3}	1:1	**1.43**	1.18	−0.68	0.46
	5×10^{-4}	0	2.5	5×10^{-4}	0.5:1	**6.38**	−4.91	0.15	4.08
		0.5:1	2.5	5×10^{-4}	1:1	**3.48**	−3.11	0.04	1.57
		1:1	2.5	5×10^{-4}	0.5:1	**3.48**	3.11	−0.04	1.57
		2:1	2.5	5×10^{-4}	1:1	**4.42**	4.31	0.13	0.98
		4:1	2.5	5×10^{-4}	8:1	**2.58**	−2.52	−0.28	0.49
		8:1	2.5	5×10^{-4}	4:1	**2.58**	2.52	0.28	0.49
	10^{-3}	0	2.5	10^{-3}	0.5:1	**8.35**	−6.16	−1.84	5.32
		0.5:1	2.5	10^{-3}	1:1	**4.48**	−3.63	−1.26	2.29
		1:1	2.5	10^{-3}	0.5:1	**4.48**	3.63	1.26	2.29
		2:1	2.5	10^{-3}	4:1	**2.86**	−2.43	−0.97	1.17
		4:1	2.5	2.5×10^{-3}	0	**1.20**	−0.56	−0.71	0.79
	2.5×10^{-3}	0	2.5	10^{-3}	4:1	**1.20**	0.56	0.71	0.79
		0.5:1	3.5	2.5×10^{-3}	1:1	**0.95**	−0.65	−0.60	0.35
		1:1	3.5	5×10^{-3}	0	**1.43**	0.80	−0.28	1.15
3.5	10^{-4}	8:1	2.5	2.5×10^{-5}	32:1	**1.35**	0.30	1.30	0.24
		16:1	4.5	10^{-3}	0	**2.27**	−1.41	1.00	1.48
		32:1	5.5	10^{-3}	0.5:1	**4.05**	−2.06	−2.38	2.54
	2.5×10^{-4}	0	2.5	5×10^{-5}	0	**1.77**	0.46	1.54	0.74
		0.5:1	2.5	5×10^{-5}	2:1	**1.73**	1.28	0.80	0.84
		1:1	2.5	5×10^{-5}	8:1	**1.13**	0.90	0.69	0.06
		2:1	2.5	5×10^{-5}	16:1	**1.03**	0.81	0.47	0.41
		4:1	2.5	5×10^{-5}	32:1	**2.80**	2.39	−1.41	0.41
		8:1	3.5	2.5×10^{-4}	16:1	**2.66**	−2.38	−0.38	1.12
		16:1	3.5	2.5×10^{-4}	8:1	**2.66**	2.38	0.38	1.12

(*continued on next page*)

Table 4 (*continued*)

Reference model solution:			Model solution with the closest colour to the reference:						
pH	Cyanin concentration (M)	Rutin: cyanin ratio	PH	Cyanin concentration (M)	Rutin: cyanin ratio	**Δ*E* (CMC:$_{1:1}$) Colour differences:**			
						Δ*E*	$\Delta L^*/S_L$	$\Delta C^*/S_C$	$\Delta H^*/S_H$
	5×10^{-4}	0	2.5	10^{-4}	1:1	**2.34**	0.76	1.51	1.61
		0.5:1	2.5	10^{-4}	8:1	**2.30**	−0.44	1.66	1.53
		1:1	2.5	10^{-4}	16:1	**2.03**	−0.22	0.73	1.88
		2:1	2.5	10^{-4}	32:1	**1.69**	1.29	−1.09	0.09
		4:1	3.5	5×10^{-4}	8:1	**2.19**	−1.42	1.06	1.30
		8:1	3.5	5×10^{-4}	4:1	**2.19**	1.42	−1.06	1.30
	10^{-3}	0	2.5	2.5×10^{-4}	1:1	**3.75**	1.07	2.67	2.41
		0.5:1	2.5	2.5×10^{-4}	4:1	**2.10**	0.87	1.17	1.52
		1:1	2.5	2.5×10^{-4}	16:1	**1.43**	−1.18	0.68	0.46
		2:1	3.5	10^{-3}	4:1	**1.21**	−1.18	−0.01	0.26
		4:1	3.5	10^{-3}	2:1	**1.21**	1.18	0.01	0.26
	2.5×10^{-3}	0	2.5	5×10^{-4}	4:1	**5.35**	−1.28	0.10	5.19
		0.5:1	2.5	2.5×10^{-3}	0	**2.88**	1.82	1.08	1.95
		1:1	2.5	2.5×10^{-3}	0.5:1	**0.95**	0.65	0.60	0.35
		2:1	3.5	5×10^{-3}	0	**1.26**	−0.56	−0.79	0.80
4.5	5×10^{-4}	0	3.5	10^{-4}	2:1	**1.57**	1.21	0.99	0.11
		0.5:1	3.5	10^{-4}	8:1	**2.21**	0.85	1.75	1.05
		1:1	4.5	5×10^{-4}	0.5:1	**2.92**	2.23	−1.84	0.42
		2:1	4.5	10^{-3}	0	**1.41**	0.72	0.50	1.11
		4:1	4.5	10^{-3}	0.5:1	**3.35**	−1.20	3.12	0.14
		8:1	4.5	10^{-3}	1:1	**3.26**	1.28	−2.64	1.43
	10^{-3}	0	4.5	5×10^{-4}	2:1	**1.41**	−0.72	−0.50	1.11
		0.5:1	4.5	5×10^{-4}	4:1	**3.35**	1.20	−3.12	0.14
		1:1	4.5	5×10^{-4}	8:1	**3.16**	0.58	−3.10	0.20
		2:1	5.5	10^{-3}	4:1	**5.20**	−3.41	−3.76	1.16
		4:1	5.5	2.5×10^{-3}	0.5:1	**7.12**	−5.14	−1.39	4.73
	2.5×10^{-3}	0	3.5	2.5×10^{-4}	16:1	**2.90**	2.33	0.16	1.73
		0.5:1	5.5	2.5×10^{-3}	0.5:1	**3.13**	0.90	−3.00	0.06
		1:1	5.5	2.5×10^{-3}	1:1	**4.11**	3.00	−2.54	1.23
5.5	5×10^{-4}	0	4.5	2.5×10^{-4}	2:1	**0.39**	0.00	0.25	0.31
		0.5:1	4.5	2.5×10^{-4}	4:1	**0.98**	0.24	−0.34	0.89
		1:1	5.5	5×10^{-4}	0.5:1	**1.67**	1.39	−0.85	0.36
		2:1	4.5	2.5×10^{-4}	8:1	**1.70**	1.53	−0.09	0.74
		4:1	5.5	10^{-3}	0	**4.07**	4.00	0.72	0.20
		8:1	5.5	10^{-3}	0.5:1	**5.02**	4.90	−0.42	1.03
	10^{-3}	0	4.5	2.5×10^{-4}	16:1	**2.57**	0.32	−2.00	1.58
		0.5:1	5.5	5×10^{-4}	4:1	**3.84**	0.79	−3.29	1.83
		1:1	4.5	5×10^{-4}	8:1	**3.51**	−1.26	2.87	1.57
		2:1	4.5	5×10^{-4}	8:1	**5.47**	5.44	0.13	0.54
		4:1	4.5	10^{-3}	2:1	**5.20**	3.41	3.76	1.16
	2.5×10^{-3}	0	4.5	2.5×10^{-3}	0	**4.80**	1.37	3.37	3.13
		0.5:1	4.5	2.5×10^{-3}	0.5:1	**3.13**	−0.90	3.00	0.06
		1:1	4.5	2.5×10^{-3}	1:1	**4.11**	−3.00	2.54	1.23

[a] S_L, S_C and S_H weighting factors in the CMC difference formula being Chroma dependent, the ΔE_{CMC} between the colours in most couples of solutions slightly changes according to which one served as the reference in the calculation. For instance, differences between solutions **1** (5×10^{-4}M, pH 2.5 and 8:1 co-pigment to pigment ratio) and **2** (5×10^{-4}M, pH 2.5 and 4:1 co-pigment to pigment ratio) are 2.68 for ΔE_{CMC} **2** vs. **1** and ΔE_{CMC} 2.58 for **1** vs. **2**. For convenience, in the table, the ΔE_{CMC} values for all the retrieved symmetrical couples of colours were harmonized with only the smallest one supplied.

with visually close colours (ΔE_{CMC} < about 2 for instance) should share very similar spectral characteristics resulting from the pH/co-pigmentation combined effects. In fact, the comparison of the spectral data of solutions in some retrieved matching colour couples once again outlines that colours are not in direct connection to spectral features at the λ_{max}.

Very close colours ($\Delta E_{CMC} = 1.13$) and identical spectral maxima (both λ_{max} at 515 nm and A = 0.53 and 0.54, respectively) were effectively shared by two solutions differing by their pH, concentration and co-pigmentation ratio: 2.5×10^{-4} M, 1:1 and pH 3.5 vs. 5×10^{-5} M, 8:1 and pH 2.5. By contrast, within the same colour difference bracket, there were also other colour couples of solutions featuring noticeable spectral differences, especially regarding the position of their λ_{max}:

> 5×10^{-5} M = 32:1, pH 2.5 vs. 2.5×10^{-4} M, 2:1, pH 3.5 ($\Delta E_{CMC} = 1.72 - \lambda_{max}526$ nm/$A = 0.66$ vs. 518 nm/$A = 0.63$, respectively);
>
> 10^{-4} M, 32:1, pH 2.5 vs. 5×10^{-4} M, 2:1, pH 3.5 ($\Delta E_{CMC} = 1.69$, $\lambda_{max}531$ nm/$A = 1.41$ vs. 522 nm/$A = 1.64$, respectively);
>
> 2.5×10^{-4} M, 16:1, pH 2.5 vs. 10^{-3} M, 1:1, pH 3.5 ($\Delta E_{CMC} = 1.43$, $\lambda_{max}532$ nm/$A = 3.47$ vs. 521 nm/$A = 3.34$, respectively) or
>
> 5×10^{-4} M, 2:1, pH 4.5 vs. 10^{-3} M, pH 4.5 ($\Delta E_{CMC} = 1.41$, $\lambda_{max}528$ nm/$A = 0.44$ vs. 520 nm/$A = 0.44$, respectively).

It is noteworthy to emphasize that in the first three couples of solutions the hue difference ($\Delta H^*/S_H$) represents the weakest (or even a negligible) contributor to the ΔE_{CMC} although the λ_{max} are shifted apart. Spectral differences of the same kind are also noted in many other couples of solutions displaying greater shortest colour differences.

4. Conclusion

This ‘colour by numbers’ approach has conclusively proven the real complexity of the colour variations resulting from the spectral changes caused by the pH and/or co-pigmentation of anthocyanins and disclosed the origin of the subsequent misinterpretations in the literature. The major issues of the hue variations coupled with the bathochromic shift of the λ_{max} and the differences in colour ‘intensity’ with absorbance changes (hypo- or hyperchromic effects) were especially clarified:

Bathochromic shifts did not systematically originate bluer hues as shown when the pH of all the cyanin solutions was increased from 4.5 to 5.5 (generalized yellowing effect) or in many co-pigmented ones at low or medium pigment concentrations at lower pH values. Conversely, pure cyanin solutions with a fixed λ_{max} in both the aqueous and methanolic series displayed a considerable gamut of basic tonalities. Absorbance increases were shown to cause lowering effect on lightness (i.e. the colour of the solution became darker) coupled either with increasing effects on chroma (the colour became more vivid), or more rarely, with a chroma remaining stable or even decreasing in the series at the lowest pH and highest cyanin concentrations.

Then, the CIELAB ΔE^* amplitudes first confirmed the pH value for maximal co-pigmentation effects (about 3.5). Increasing the co-pigment to pigment ratio always resulted in stronger cumulative colour effects, but for the poorly coloured solutions (lowest pigment concentrations or/and highest pH values), the maximum colour effect at each stepped co-pigment addition occurred at the highest co-pigment to pigment ratios while it was recorded as the lowest one (0.5:1) for the strongly coloured ones (lowest pH and/or highest cyanin concentrations). Contrary to previous studies, the maximum effects of co-pigmentation (at each co-pigment to pigment ratio) occurred at the highest pigment concentrations only for the solutions at the highest pHs tested (4.5 and 5.5) and it was shifted down to intermediate values (5×10^{-4}M) at lower pH values (2.5 and 3.5).

Colour difference calculations have also revealed that acidic (HCl 0.1 N) aqueous and methanolic cyanin solutions displayed relatively close colours (and shared the same basic orange tonality, although their λ_{max} was shifted apart) at the highest pigment concentration tested and that the solvent effects became very impressive when decreasing the cyanin concentration: these were based on hue—and then chroma—variations, corresponding to a yellowing effect of water on chromatic tonalities.

Finally, using ‘visual’ colour differences calculations (CMC model) it was disclosed that the reference colour of some anthocyanin solutions at pH 1 (stable and coloured form of the flavylium nucleus) could be simulated by the ones of model solutions at higher pHs (2.5 exceptionally 3.5) either by a simple ‘pigment concentration effect’ (pure pigment at a concentration about 2–2.5-fold the reference) or by a ‘co-pigmentation effect’ of solutions at the same—or even lower—pigment concentration co-pigmented by rutin (relative ratio up to 4:1). At the successive higher pH tested (2.5–5.5) similar global conclusions emerged but with more considerable differences in the pigment concentrations and co-pigment to pigment ratios of solutions in each couple retrieved.

In some retrieved couples of solutions with matched colours, it was found that the combined effects of different pH, pigment and co-pigment concentrations resulted in very close spectral features (λ_{max}, absorbance and even shape between 380 and 780 nm). Conversely, the matched colours of some other couples were based on spectral curves featuring strikingly different λ_{max} and absorbance values, balanced by simultaneous spectral differences occurring in the shortest wavelengths area of the visible light.

Many areas concerning the colour effects of the co-pigmentation of anthocyanins have been clarified and developed by the colorimetric analysis presented in the three papers in this series, along with providing an objective, appropriate and accurate definition of this phenomenon. Most of the issues discussed here on model solutions of cyanin co-pigmented by rutin can also find extended applications to any field in which anthocyanic colours are involved, especially the industrial use of these pigments as natural colorants for instance. A further example will come soon with the example of the unusual colours resulting from the co-pigmentation of the macrocyclic anthocyanins recently isolated from carnation flowers (Gonnet & Fenet, 2000).

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