

Analytical, Nutritional and Clinical Methods Section

Colour effects of co-pigmentation of anthocyanins revisited—2. A colorimetric look at the solutions of cyanin co-pigmented by rutin using the CIELAB scale

Jean-François Gonnet*

Laboratoire BMP, Université Lyon I, 43, boulevard du 11 Novembre, F-69622 Villeurbanne, France

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Abstract

Spectral measurements (380–780 nm) were performed on 224 model solutions of cyanin co-pigmented by the flavonol rutin at pH between 2.5 and 5.5. The colour of the solutions was expressed as colorimetric co-ordinates in the CIELAB scale using the L^* (lightness) C^* (chroma) and h (hue angle) notation (for the D65/10° CIE Illuminant/Observer condition). Contrary to previous reports, all the most intensely co-pigmented solutions, although exhibiting the strongest bathochromic of their λ_{\max} , did not displayed the bluest hues; an inverse, i.e. yellowing, effect was observed in the co-pigmented solutions at the lowest cyanin concentrations. The hyperchromic effect of co-pigmentation always caused a decreasing lightness of the solutions coupled with either an increasing or a decreasing chroma for those at low or high pigment concentrations respectively. Based on the amplitude of total colour CIELAB differences (ΔE^*) at each co-pigment to pigment ratio, the most intense effects of co-pigmentation were observed at pH 3.5, except for the solutions at the highest pigment content (pH 4.5); the evolution of colour differences at increasing co-pigment to pigment ratios at pH between 2.5 and 5.5 are discussed. Colour differences calculations also showed that the maximum colour steps at successive increasing co-pigment to pigment ratios were observed at low values (0.5 or 1:1) in the most coloured solutions and at the highest values (up to 8–16:1) in the less coloured ones. In describing the colour effect of co-pigmentation on anthocyanins, the discrepancies found between the results of the present colorimetric approach and those previously reported based on spectral data at the λ_{\max} only are discussed. Only the first one—by considering the spectral variations over the complete range of visible wavelengths and using the psychometric correlates of the three attributes of colour specified in the CIELAB system—correctly describes the colour variations as these are perceived by the human eye. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Co-pigmentation plays a major role in the colour expression of anthocyanin pigments in aqueous media. Besides the stabilization process of the anthocyanin nucleus as coloured forms, this phenomenon is also the main factor originating the extraordinary colour palette that each single anthocyanic chromophore may generate (for instance, from red in many rose flowers to blue in those of cornflower, both containing cyanin). The origin of this colour diversity is in the spectral changes caused by co-pigmentation: vs those of the corresponding basic anthocyanins, the spectra

of co-pigmented structures display a shift of the λ_{\max} to longer wavelengths (bathochromic effect) coupled with a strongly increased absorptivity (hyperchromic effect) (Asen, Stewart, & Norris, 1972; Chen & Hrazdina, 1981; Mazza & Brouillard, 1990). The amplitude of these variations was reported to be dependent on many co-acting factors:

- pH: the value for maximal effects was found to be close to 3.5 (Mazza & Brouillard, 1990; Williams & Hrazdina, 1979).
- Temperature: heating the co-pigment–pigment mixture causes a diminution of both the spectral effects (Baranac, Petranovic, & Dimitric-Markovic, 1996; Mazza & Brouillard, 1987, 1990).
- The nature of the anthocyanic chromophore (Baranac et al., 1996; Chen & Hrazdina, 1981;

* Tel.: +33-472-44-8000; fax: +33-478-84-4847.
e-mail: gonnet@cismsun.univ-lyon1.fr

Williams & Hrazdina, 1979) and of the co-pigment. In particular, among the molecules consistently present in the vacuoles, flavonol glycosides were found to be among the most efficient co-pigments (Asen et al., 1972; Baranac et al.).

Very influential parameters are the co-pigment to pigment molar ratio and the pigment concentration. Both spectral effects are intensified at higher co-pigment to pigment ratios; at constant pH, temperature and co-pigment-pigment ratio, strongly increased absorptivity is observed at higher anthocyanin concentrations (Asen, 1976; Asen et al., 1972; Mazza & Brouillard, 1990).

The previous colorimetric analysis of pure cyanin—and some co-pigmented—solutions at different pH and concentrations using the CIELAB scale (Gonnet, 1998) revealed that the *colour effects* resulting from the above described spectral variations were usually reported incorrectly in the literature. In particular it was shown that a bathochromic shift of the λ_{\max} of solutions did not cause a general blueing effect on their colour: huge variations of hue were observable in different solutions having exactly the same visible λ_{\max} while others exhibiting a strong $\Delta\lambda_{\max}$ in their spectra had either bluer or yellower tonalities than those of their respective reference solutions. Colour measurement also revealed that enhanced colour “intensity”—consistently employed for the description of increased absorptivity colour effects—is a quite ambiguous term, which indistinctly covers variations of the other two attributes of colour perception, lightness and saturation. This study also remembered that communicating efficiently on colour requires that its description is referred to precise standard light and observer conditions.

This work presents the application of the previously described colorimetric method to the revision of the colour effects of co-pigmentation of cyanin (cyanidin 3,5-diglucoside) by the flavonol rutin (quercetin 3-rhamno-glucoside) at different pH, pigment concentrations and co-pigment to pigment molar ratios.

2. Materials and methods

2.1. Pigments

Cyanin (approx. 10 g) was obtained from repeated preparative column chromatographies (Macherey-Nagel SC6 polyamide and Sephadex LH20) of a crude methanolic extract of approx. 2 kg of fresh rose petals (*Polyantha* cultivar). Final purification was achieved after multiple precipitations by diethyl-oxyde from a concentrated methanolic solution. Purity was checked by CCM, HPLC and UV-visible spectrum. Rutin was commercially available from ExtraSynthèse (Genay, France) and recrystallized twice from methanol.

Stock solutions of pigment and co-pigment were prepared in MacIlvaine citrate-phosphate buffers (pH 2.5, 3.5, 4.5 and 5.5) at twice the required normality (ranging from 10^{-5} M to 2.5×10^{-3} M). Each co-pigment-pigment solution was prepared by mixing equal volumes of cyanin and rutin at adequate concentrations (ratios ranging from 0.5:1 to 64:1), then vigorously stirred and left (in darkness) for 10 min at 22°C (thermostatic water bath). Due to solubility limitations of rutin in aqueous solutions, recording the spectra of the complete line of co-pigment to pigment ratios was only possible for the cyanin solutions at 5×10^{-5} M and lower. For the cyanin solutions at higher concentrations, this gamut had to be progressively shrunk, up to 32:1 (10^{-4} M) to 1:1 (2.5×10^{-3} M).

2.2. Spectrophotometric measurements

Spectral curves were recorded (regular transmission, from 380 to 780 nm with a 2 nm bandwidth) in 10 mm optical path quartz cuvettes using a Kontron Uvikon 943 spectrophotometer.

2.3. Colorimetric calculations

From the spectral curves of the solutions, their colorimetric co-ordinates were computerized in the CIE-LAB scale for the CIE D65/10° Illuminant/Observer condition, using a specially developed program run on a Zeos PC computer. In this specification of colours, the CIELCH notation of CIELAB was preferred over the $L^*a^*b^*$ as it uses the direct psychometric equivalents of the three attributes of the human perception, lightness, saturation and hue (or chromatic tonality). In this scale, each colour is numerically specified by a unique set of three cylindrical coordinates:

- L^* for lightness,
- C^* , for metric chroma, the correlate of saturation, and
- h_{ab} , for hue angle, the correlate of chromatic tonality. Hue angles are stepped counterclockwise on a chromatic circle the remarkable values of which are 0/360° (magenta), 90° (yellow), 180° (bluish-green) and 270° (blue). Intermediate basic tonalities specified in the text are named according to their calculated hue angles and can be viewed in the N2S™ Colour Atlas (Mecanorma, 1990), based on the CIELAB scale using the CIE D65/10° Illuminant/Observer condition.

The CIELAB system having the properties of an euclidean space, the distance between any two (or more) colour points (the solutions here) represents their colorimetric difference (ΔE^*) and is calculated from the differences of its components, ΔL^* , ΔC^* and ΔH^* : $\Delta E^* = (\Delta L^{*2} + \Delta C^{*2} + \Delta H^{*2})^{0.5}$. In this work a mean

threshold value of $\Delta E^* = 1$ will be presently assumed as a valuable basis for just noticeable colour difference between solutions. The above colorimetric difference equation was originally designed for the calculation of “small” differences ($\Delta E^* < 10$); higher values—having lesser perceptual signification—will be however considered here for some comparative purposes of the efficiency of co-pigmentation process. Further information on the arrangement of the CIELAB system and the subsequent management of colour data was already presented (Gonnet, 1993, 1995, 1998).

3. Results and discussion

3.1. Spectral variations in cyanin–rutin solutions

Most of the spectral variations observed in the curves recorded for 224 model solutions perfectly matched those published in the literature for the corresponding co-pigment to pigment combinations (at different pH, pigment concentrations and co-pigment to pigment molar ratios) as summarized and discussed previously (Gonnet, 1998). Consequently, the CIELAB co-ordinates based on all the curves recorded can be considered adequately for a subsequent discussion of colour variations caused by the co-pigmentation phenomenon. Additional spectral data will be supplied here, in connection with specific colour variations.

3.2. The revised colour description of co-pigmented cyanin solutions

Table 1 presents the $L^*C^*h_{ab}$ colour co-ordinates (D65/10° Illuminant/Observer condition) of pure cyanin and model cyanin/rutin solutions from 10^{-5} M to 2.5×10^{-3} M at pH 2.5, 3.5, 4.5 and 5.5 and co-pigment to pigment molar ratios ranging from 0.5:1 to 64:1.

3.2.1. Colorimetric variations of reference (non co-pigmented) cyanin solutions with pH

Unsurprisingly, the most acidic solutions (pH 2.5) were the most coloured at each concentration. As the pH was increased up to 5.5, the general trend was a colour fading caused by an important loss of saturation and an increased lightness, coupled with hue shifts (to bluer or yellower tonalities).

No perceptible colours [CIELAB co-ordinates combining a very high L^* (about 96 or more) with very low C^* (5 or less)] were observable for all the solutions at pH 4.5 and 5.5 in the 10^{-5} – 10^{-4} M concentration bracket and those at 10^{-5} and 2.5×10^{-5} M as soon as the pH reached 3.5.

At higher cyanin concentrations (2.5×10^{-4} M and up), the pH step originating the largest colorimetric differences was from 2.5 to 3.5, except for the solution at

2.5×10^{-3} M (pH 3.5 to 4.5). Saturation was the most influential parameter in these variations, the loss of chroma caused by increasing the pH representing for some solutions a huge colour gap: more than 80 chroma units lost for both the solutions at 5×10^{-4} M and 10^{-3} M when the pH augmented of three units. Lightness, the secondly ranked parameter of the colorimetric variations, systematically decreased with increasing pH from 2.5 to 4.5 and remained roughly constant when the pH further raised to 5.5. The solution at the highest concentration tested (2.5×10^{-3} M) exhibited specific variations of these two attributes when the pH moved from 2.5 to 3.5: its chroma augmented, and a decreasing lightness here was the prevailing factor of colour changes.

Hue followed a similar global evolution with the pH value for all the visually coloured solutions. From its initial position on the colour circle—recorded at pH 2.5—the hue angle first moved clockwise (to lower values) when pH was elevated up to 4.5, corresponding to a blueing effect on chromatic tonalities. A further augmentation of pH (to 5.5) caused an important counterclockwise shift of hue angle, meaning that the hues now moved back to yellower tonalities, these ones nevertheless remaining finally bluer than those of the corresponding solutions at pH 2.5. This “unexpected” (vs previous records) colour change—hue moving yellower coupled with the λ_{\max} of the solutions shifted to longer wavelengths (526–528 nm vs 519–520 nm, for the solutions at pH 5.5 vs 4.5, respectively)—originated in a relative hyperchromic effect of the pH on the spectrum of cyanin in the visible range between 400 and 480 nm (Fig. 1).

3.2.2. Colorimetric and spectral variations in co-pigmented cyanin solutions

Whatever were the pH and the pigment concentrations, the addition of co-pigment rutin always resulted in darker colours (lower L^*) and in an enhanced saturation level (higher C^*) in most solutions, or a nearly stable or even decreasing one in the others. As compared to the corresponding reference solutions of pure pigment, the co-pigmented ones displayed a spectacularly extended palette of basic tints, from magenta (h_{ab} 358°) to purple (h_{ab} 348°) at one of its ends and from orange (h_{ab} 48°) to yellowish-green (h_{ab} 112°) at the other one.

3.2.2.1. Influence of co-pigment concentration. In cyanin solutions between 10^{-5} and 10^{-4} M, adding rutin at increasing molar ratios up to 64:1 caused a continuous positive $\Delta\lambda_{\max}$ at pH 5.5 (λ_{\max} shifted from 523 to 535–539 nm), 4.5 (λ_{\max} from 519–522 to 531–536 nm) and 3.5 (λ_{\max} from 510–513 to 520–536 nm), coupled with an intense hyperchromic effect (increased absorptivity up to 380%). Coherent variations of the three attributes of colour resulted throughout—lower L^* , higher C^*

Table 1
 CIELAB coordinates (D65/10° Illuminant/Observer) of pure and co-pigmented cyanin solutions at pH 2.5–5.5

Cyanin concentration (M)	Rutin/cyanin ratio	pH 2.5			pH 3.5			pH 4.5			pH 5.5		
		<i>L</i> *	<i>C</i> *	<i>h_{ab}</i>	<i>L</i> *	<i>C</i> *	<i>h_{ab}</i>	<i>L</i> *	<i>C</i> *	<i>h_{ab}</i>	<i>L</i> *	<i>C</i> *	<i>h_{ab}</i>
10 ⁻⁵	0	96.28	8.67	358.38	98.79	1.23	1.43	99.05	0.33	16.78	99.35	0.19	26.78
	0.5:1	96.55	7.45	1.38	98.68	1.35	5.32	99.03	0.34	52.95	99.43	0.20	53.58
	1:1	96.51	7.50	1.56	98.74	1.22	6.19	99.00	0.37	48.28	99.43	0.21	64.96
	2:1	96.39	7.70	1.74	98.64	1.33	11.45	99.00	0.40	71.31	99.40	0.33	80.77
	4:1	96.84	7.67	357.53	98.37	1.23	26.74	98.99	0.55	80.73	99.32	0.62	94.18
	8:1	96.61	7.74	358.35	98.45	1.49	46.02	98.89	0.90	93.56	99.34	1.02	103.72
	16:1	96.31	7.85	2.24	97.80	2.92	71.27	98.82	1.77	104.75	99.29	2.00	109.07
	32:1	95.19	8.91	8.41	97.62	3.73	83.73	98.45	3.54	108.42	99.07	3.61	110.59
	64:1	95.16	8.50	26.84	97.48	4.41	88.86	98.14	6.17	109.87	98.79	7.74	112.07
2.5×10 ⁻⁵	0	92.73	17.89	1.42	97.90	3.25	1.35	98.8	80.65	14.73	99.1	10.45	22.89
	0.5:1	92.48	17.92	2.74	97.92	3.16	2.90	98.7	10.77	30.16	99.1	10.58	43.48
	1:1	92.33	18.11	2.51	97.82	3.22	5.25	98.71	0.76	39.35	99.1	20.62	55.23
	2:1	92.13	18.49	2.36	97.72	3.31	8.07	98.44	0.90	54.07	99.1	30.73	72.82
	4:1	91.81	18.85	2.15	97.35	3.57	16.60	98.5	91.15	72.88	99.07	1.21	91.03
	8:1	91.18	19.52	1.69	96.91	4.00	27.85	98.1	42.53	94.72	98.88	2.26	100.48
	16:1	89.66	21.54	0.88	96.30	4.48	43.67	97.83	4.23	99.24	98.76	4.40	107.75
	32:1	87.85	23.19	2.90	95.50	5.82	48.41	97.44	6.78	103.42	97.89	8.84	107.37
	64:1	85.98	23.05	10.23	93.93	8.84	62.73	96.53	11.57	104.76	97.25	15.10	108.54
5×10 ⁻⁵	0	87.47	30.99	4.78	96.53	6.49	1.01	98.36	1.33	9.44	98.65	0.98	23.14
	0.5:1	86.89	31.67	5.04	96.48	6.48	2.31	98.19	1.41	20.37	98.66	1.11	35.54
	1:1	86.41	32.44	4.53	96.29	6.69	3.29	98.01	1.48	28.50	98.63	1.16	46.26
	2:1	85.81	33.45	3.80	95.41	7.11	5.54	97.80	1.76	43.51	98.51	1.45	66.50
	4:1	84.87	35.39	1.25	94.89	8.16	10.05	97.54	2.48	70.88	98.31	2.31	84.41
	8:1	82.93	36.99	0.19	94.14	8.55	15.40	96.98	4.06	83.60	98.10	4.13	98.39
	16:1	79.04	42.02	358.28	92.44	10.72	15.34	96.33	6.04	90.19	97.17	8.32	101.07
	32:1	76.10	43.34	359.47	88.92	14.01	24.92	95.39	9.98	96.23	96.47	13.15	105.07
	64:1	72.15	43.93	5.02	86.96	17.30	43.83	92.83	16.68	92.93	94.78	21.29	103.45
10 ⁻⁴	0	80.02	47.87	10.96	93.90	12.54	1.47	97.43	2.70	5.87	97.64	2.11	24.10
	0.5:1	78.92	49.20	9.99	93.13	13.55	1.91	97.26	2.84	12.35	97.69	2.14	28.14
	1:1	77.81	50.72	9.04	92.49	14.26	2.61	96.97	3.01	18.86	97.55	2.31	39.63
	2:1	76.18	53.48	6.28	91.45	15.67	2.69	96.42	3.41	36.87	97.23	2.80	56.03
	4:1	73.30	56.48	4.35	90.18	17.06	3.85	95.87	4.09	48.00	96.79	3.96	75.31
	8:1	68.19	62.54	1.41	87.43	21.00	2.18	94.70	5.86	61.16	95.59	7.35	87.76
	16:1	63.85	65.00	359.96	81.85	26.83	4.38	92.76	8.45	68.26	92.66	13.18	90.63
	32:1	60.31	64.37	2.75	77.05	30.80	9.43	90.78	12.65	74.91	89.93	22.90	92.98
	64:1	56.81	63.85	10.23	72.15	35.93	15.34	88.92	18.92	83.60	88.92	28.15	92.98
2.5×10 ⁻⁴	0	69.20	69.69	26.91	86.82	27.99	2.88	94.69	7.03	3.08	94.63	5.39	22.46
	0.5:1	66.30	71.88	23.13	84.03	31.80	1.71	93.56	8.12	4.86	94.33	5.61	21.13
	1:1	63.43	73.99	21.12	81.69	35.45	0.34	92.52	9.06	5.82	93.66	6.04	24.96
	2:1	59.21	76.19	18.23	77.95	40.90	359.20	90.31	10.71	10.72	92.51	6.65	30.52
	4:1	53.59	77.60	17.16	72.98	46.84	358.57	88.19	12.00	13.12	89.84	8.47	42.13
	8:1	48.84	79.10	13.63	64.53	55.54	357.96	83.71	16.21	8.74	86.37	10.76	46.60
	16:1	45.88	78.14	15.99	61.58	54.53	0.13	81.06	17.59	13.11	82.17	17.54	61.79
	32:1	42.81	77.60	17.16	58.48	53.45	1.41	79.70	19.41	10.72	82.17	17.54	61.79
	64:1	40.31	77.60	17.16	56.81	53.45	1.41	79.70	19.41	10.72	82.17	17.54	61.79
5×10 ⁻⁴	0	60.14	90.00	41.23	76.79	46.95	5.91	89.73	14.28	2.28	90.32	10.41	12.06
	0.5:1	54.27	90.49	37.37	68.75	57.83	4.24	86.23	18.24	358.81	87.85	12.45	9.49
	1:1	50.74	90.62	35.81	64.12	62.93	3.31	83.16	21.37	357.35	85.90	13.62	8.06
	2:1	46.24	90.20	34.80	58.77	67.48	2.90	78.94	27.74	357.44	81.62	16.34	6.14
	4:1	40.96	88.22	33.02	53.18	68.06	3.76	72.86	31.67	353.81	75.33	19.79	8.50
	8:1	38.50	87.32	33.56	51.61	71.25	1.59	65.95	38.31	351.38	68.09	26.76	5.47
	16:1	36.81	87.32	33.56	51.61	71.25	1.59	65.95	38.31	351.38	68.09	26.76	5.47
	32:1	35.14	87.32	33.56	51.61	71.25	1.59	65.95	38.31	351.38	68.09	26.76	5.47
	64:1	33.47	87.32	33.56	51.61	71.25	1.59	65.95	38.31	351.38	68.09	26.76	5.47
10 ⁻³	0	48.35	106.86	48.33	62.13	66.25	17.68	79.92	28.73	0.49	80.63	20.97	7.90
	0.5:1	41.76	100.47	44.17	52.61	73.98	15.01	71.31	38.78	353.44	74.29	25.95	2.64
	1:1	38.18	96.17	42.19	47.10	76.04	16.64	65.22	45.96	350.90	67.54	32.32	355.16
	2:1	34.25	90.75	40.01	43.11	76.33	15.43	56.10	53.84	350.22	59.48	38.03	352.69
	4:1	32.12	87.56	38.86	41.92	76.30	15.80	47.04	60.72	349.78	52.16	43.88	352.83
2.5×10 ⁻³	0	31.65	85.26	39.66	42.23	87.89	38.24	58.83	54.10	3.40	57.24	45.79	9.15
	0.5:1	26.87	77.92	36.34	30.18	81.86	37.64	40.75	65.48	359.01	41.63	56.82	359.13
	1:1	23.95	72.75	34.43	26.39	76.08	35.95	32.41	65.14	2.79	34.94	57.83	0.55

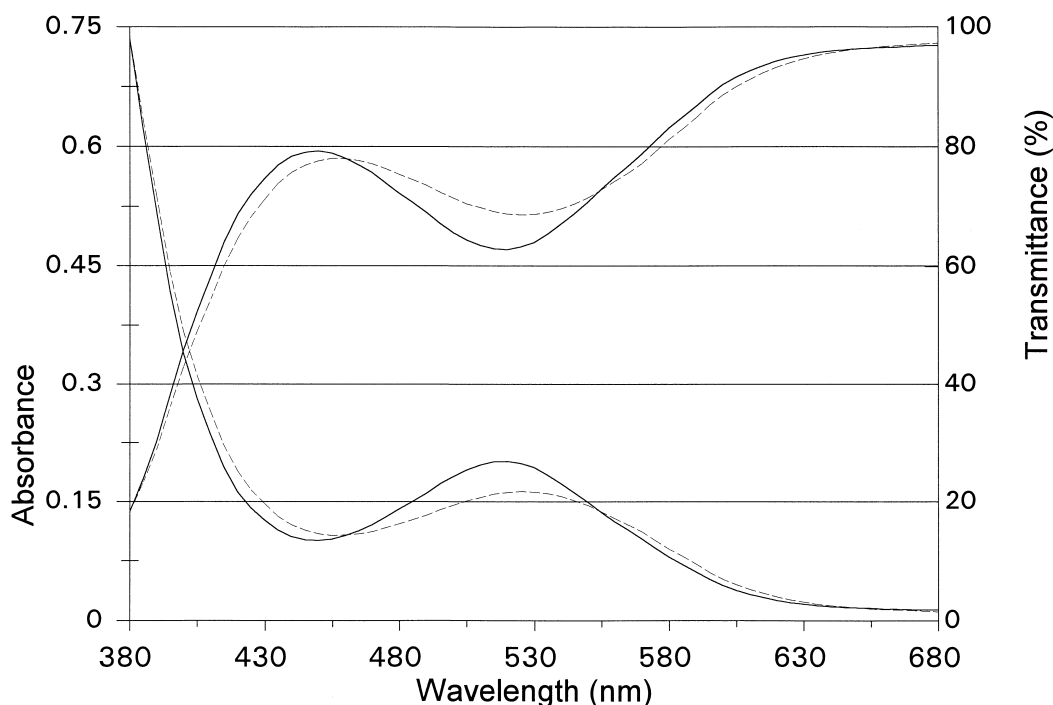


Fig. 1. Spectral absorbance (bottom) and transmittance (top) curves of cyanin solutions at 5×10^{-4} M and pH 4.5 (full lines) or 5.5 (dotted lines).

and h_{ab} shifts—but most did not generate visually perceptible colours: in this batch, only the solutions having a chroma over 4–5 appeared—slightly—coloured. These corresponded to the most intensely co-pigmented solutions (64:1 to 32–8:1 molar ratios), all but two displaying basic hues in the range of yellow tonalities: greenish-yellow (h_{ab} 112°) to yellow (h_{ab} 93°) for the solutions at pH 5.5, greenish-yellow (h_{ab} 109.9°) to orange-yellow (h_{ab} 74.9°) at pH 4.5 and yellow to yellow-orange (h_{ab} 88.9 and 62.7°) at pH 3.5 (10^{-5} and 2.5×10^{-5} M). At 5×10^{-5} and 10^{-4} M and pH 3.5, the initial magenta based colour (h_{ab} 1–1.5°) of pure cyanin solutions also turned yellower, up to orange (h_{ab} 43.8°) or to red–magenta (h_{ab} 9.4°), respectively.

The λ_{max} of cyanin solutions at 2.5×10^{-4} M also suffered important bathochromic shifts on increased co-pigment addition: from 523 (pure pigment) to 543 nm (highly co-pigmented ones), 520 to 540 nm and 511 to 535 nm at pH 5.5, 4.5 and 3.5, respectively. Here again, a yellowing effect on tonalities resulted in the colour of the solutions at the highest two pH values: from pure red (h_{ab} 22.5°) their hues shifted to orange–yellow (h_{ab} 61.8°) at pH 5.5 or from magenta (h_{ab} 3°) to magenta–red (h_{ab} 13°) at pH 4.5. By contrast, at pH 3.5, the tonalities of colour of co-pigmented solutions turned—very slightly—bluer and remained in a very restricted gamut of magenta tints (h_{ab} moving from 2.9° to 357.9–1.7°).

At higher pigment concentrations (5×10^{-4} M and up), a general blueing effect of co-pigmentation was observed: the hue of solutions turned from magenta–red (h_{ab} 12–8°) to magenta (h_{ab} 5.5°) or purple–magenta

(h_{ab} 353°) at pH 5.5, or up to 350° from originally magenta tonalities (h_{ab} 0–3.4°) at pH 4.5. At pH 3.5, the hue variations were far more limited and stepped on the colour circle: magenta (h_{ab} 1.6–4.2°, 5×10^{-4} M), magenta–red (h_{ab} 15–16.6°, 10^{-3} M) or orange–red (h_{ab} 37.6–36°, 2.5×10^{-3} M).

Similar variations of the other two attributes of colour, a higher chroma coupled with a lower lightness consistently resulted for all but two solutions— 2.5×10^{-3} M, pH 3.5 and 4.5—at pH 5.5–3.5 with increasing co-pigment to pigment molar ratios.

All the solutions at pH 2.5 displayed perceptible colours. A yellowing effect on hue coupled with increased concentrations of rutin (λ_{max} shifted from 510 to 526 nm) was observed in the solutions at the lowest two pigment concentrations only. For the cyanin solutions at 5×10^{-5} – 5×10^{-4} M (λ_{max} shifted up to 532 nm), a double-step variation occurred: hue angles shifted first to lower values (blueing effect on colour) from which this movement was reversed (hue turning back to yellower tones); these changes were based on specific initial tonalities, according to the pigment concentration: magenta (h_{ab} 4.8°, 5×10^{-5} M), or orange–red (h_{ab} 41.2°, 5×10^{-4} M). Co-pigmented solutions at the highest cyanin concentrations (5×10^{-4} M and up) also displayed bluer tonalities here coupled with their chroma remaining stable or decreasing when more co-pigment was added.

3.2.2.2. Influence of pH and cyanin (pigment) concentration. Some effects of both of these parameters being already perceptible and examined in the previous

section, only two more examples will be considered, using colour differences calculations. Table 2 presents the CIELAB colorimetric differences between cyanin solutions at 5×10^{-4} M co-pigmented by rutin (ratios 0.5:1 to 8:1) at pH varying from 2.5 to 5.5. Table 3 lists the colorimetric differences between co-pigmented solutions (ratios 0.5:1 to 8:1) at pH 2.5 when the cyanin concentration varied from 5×10^{-5} to 2.5×10^{-3} M. In both tables, the colorimetric differences (ΔE^*) are supplied into two forms: ΔE_s^* (and its components ΔL^* , ΔC^* and ΔH^*) representing the “step by step” colorimetric difference, calculated between the CIELAB coordinates of each successive couple of solutions in a series. ΔE_t^* symbolizes the “total” difference between any co-pigmented solution and the pure cyanin solution in the same series, obtained by using the CIELAB coordinates of the pure pigment solution as the reference for this calculation.

Compared to the threshold for just perceptible differences ($\Delta E^* = 1$), the total differences ΔE_t^* (Table 2) between the pure pigment solutions at 5×10^{-4} M and the co-pigmented ones at the maximum ratio considered (8:1) represented very spectacular colour changes, the strongest ones occurring at pH 3.5 and 4.5 (ΔE_t^* 34–35 vs 24.8 or 27.7 only at pH 2.5 or 5.5). The amplitude of the colour effects of co-pigmentation with increasing co-pigment to pigment ratios also differed with the pH: at the lowest pH (2.5 and 3.5), this phenomenon

was the most efficient (measured by successive ΔE_s^*) at the lowest ratio (0.5:1— ΔE_s^* 8.5 or 13.6 at pH 2.5 or 3.5); when the ratio augmented, the overall trend was the co-pigment efficiency progressively decreased (ΔE_s^* finally falling to 2.7 or 4.4). At higher pH (4.5 and 5.5), an opposite global evolution was noted, the highest ΔE_s^* —ie the strongest colour effect—occurring in the most co-pigmented solutions (at pH 5.5 ΔE_s^* 10.1 for the 8:1 ratio vs 3.2 for 0.5:1).

The pH also influenced which were the major parameter(s) accounting for these colorimetric differences. At pH 2.5, the colour effects of higher co-pigment amounts mainly concerned the lightness and the chromatic tonalities of the solutions (ΔE_s^* mainly depended on ΔL^* and ΔH^*), their chroma displaying relatively minor changes. At pH 3.5 and 4.5, the colour variations were based on chroma and then lightness differences (ΔC^* and ΔL^*); comparably, hue variations remained less influent while remaining perceptible for the most co-pigmented ones. Finally, in the solutions at pH 5.5, the colour changes resulting from co-pigmentation mainly affected lightness, and secondly chroma while the hue variations still remained of small influence.

Table 2
CIELAB colour differences (D65/10°) between co-pigmented (ratio: 0.5:1–8:1) solutions of cyanin (5×10^{-4} M) at pH 2.5–5.5

Rutin:cyanin ratio	ΔE_s^*	ΔL^*	ΔC^*	ΔH^*	ΔE_t^*
pH 2.5					
0.5:1	8.46	-5.87	0.50	-6.07	8.46
1:1	4.31	-3.53	0.13	-2.47	12.71
2:1	4.79	-4.50	-0.42	-1.59	17.18
4:1	6.29	-5.28	-1.98	-2.77	23.10
8:1	2.74	-2.46	-0.91	0.82	24.82
pH 3.5					
0.5:1	13.61	-8.04	10.88	-1.52	13.61
1:1	6.95	-4.62	5.10	-0.98	20.54
2:1	7.05	-5.36	4.55	-0.46	27.48
4:1	5.71	-5.59	0.58	1.01	31.74
8:1	4.42	-1.57	3.18	-2.64	35.26
pH 4.5					
0.5:1	5.38	-3.50	3.96	-0.98	5.38
1:1	4.42	-3.07	3.13	-0.50	9.79
2:1	7.64	-4.22	6.37	0.04	17.34
4:1	7.48	-6.08	3.92	-1.88	24.43
8:1	9.70	-6.91	6.64	-1.48	34.10
pH 5.5					
0.5:1	3.25	-2.47	2.04	-0.51	3.25
1:1	2.29	-1.95	1.17	-0.33	5.53
2:1	5.10	-4.28	2.73	-0.50	10.62
4:1	7.21	-6.29	3.45	0.74	17.71
8:1	10.12	-7.25	6.96	-1.22	27.66

Table 3
CIELAB Colour differences (D65/10°) between co-pigmented solutions of cyanin (5×10^{-5} M– 2.5×10^{-3} M) at pH 2.5

Rutin:cyanin ratio	ΔE_s^*	ΔL^*	ΔC^*	ΔH^*	ΔE_t^*
5×10^{-5} M					
0.5:1	0.91	-0.58	0.68	0.14	0.91
1:1	0.94	-0.48	0.76	-0.29	1.80
2:1	1.25	-0.60	1.01	-0.42	3.01
4:1	2.65	-0.94	1.94	-1.53	5.50
8:1	2.60	-1.94	1.60	-0.67	7.99
10^{-4} M					
0.5:1	1.92	-1.10	1.33	-0.83	1.92
1:1	2.05	-1.11	1.52	-0.83	3.97
2:1	4.08	-1.63	2.77	-2.51	7.96
4:1	4.55	-2.88	3.00	-1.85	12.46
8:1	8.49	-5.11	6.05	-3.05	20.93
2.5×10^{-4} M					
0.5:1	5.91	-2.90	2.18	-4.67	5.91
1:1	4.39	-2.88	2.11	-2.55	10.22
2:1	6.09	-4.22	2.20	-3.79	16.24
4:1	5.97	-5.62	1.41	-1.43	21.51
8:1	6.94	-4.75	1.50	-4.83	28.25
5×10^{-4} M					
See Table 2					
10^{-3} M					
0.5:1	11.87	-6.59	-6.39	-7.53	11.87
1:1	6.54	-3.58	-4.30	-3.40	18.32
2:1	7.58	-3.93	-5.42	-3.55	25.74
4:1	4.23	-2.12	-3.20	-1.78	29.84
2.5×10^{-3} M					
0.5:1	9.95	-4.78	-7.34	-4.72	9.95
1:1	6.46	-2.93	-5.17	-2.52	16.36

At the same pH value (2.5, Table 3) and each co-pigment to pigment ratio, more and more intense colour effects were produced when the pigment concentration increased (except for the highest one, 2.5×10^{-3} M) as shown by the amplifying total differences (ΔE_t^* from 5.5 to 29.8 for the solutions at 5×10^{-5} to 10^{-3} M and the 4:1 ratio for instance). However, identical gradual additions of rutin resulted in differently stepped changes, according to the pigment concentration: in the batches of solutions at the lowest two cyanin concentrations (5×10^{-5} and 10^{-4} M), the amplitude of ΔE_s^* increased with the co-pigment addition, the maximum effect occurring at the highest ratio. In those at higher pigment concentrations, this trend was reversed, the strongest effects being progressively shifted to the lowest ratio (0.5:1) and the amplitude of ΔE_s^* continually shrank with further additions of co-pigment. At 5×10^{-5} and 10^{-4} M cyanin, the colour changes caused by increasing co-pigmentation mainly affected chroma and lightness (ΔC^* and ΔL^* were the main contributors to ΔE_s^*) of the solutions, with an additional raising importance of hue (ΔH^*) in the second one. At higher cyanin concentrations, besides a still important loss of lightness, the effects on colour shifted from chroma—which was a weakly influential parameter in the batch of solutions at 5×10^{-4} M—to hue variations (ΔH^* progressively superseded ΔC^* in the ΔE_s^* values). At the highest two concentrations, the three colour attributes were simultaneously affected by changes of a comparably high amplitude but here the chroma variations corresponded to an important loss of saturation.

Colorimetric results obtained here on model cyanin/rutin mixtures, differing by molecular and environmental factors, are consistent with those previously reported for the pure cyanin solutions (Gonnet, 1998). Unmatched results observed between the present colorimetric approach of the co-pigmentation process and the previous ones—only considering spectral data at the λ_{\max} of the anthocyanin—were confirmed, specially those concerning the so-called *general* “blueing effect” (hue variations) coupled with the “colour enhancement” (“intensity” variations).

Firstly, no colour variations perceptible by the human visual system resulted from the consistent spectral changes occurring in the co-pigmented solutions at the lowest pigment concentrations and higher pH values. In those batches, only the solutions at the highest co-pigment to pigment ratios—and consequently exhibiting the strongest spectral and colorimetric variations—displayed perceptible tonalities, which were in the yellow range. Comparable yellowing effects of co-pigmentation on tonalities were also observed in more intensely coloured solutions, at low pigment concentrations generally, and occasionally at some in the highest ones. In poorly coloured solutions, these “unexpected” chromatic variations result from the simultaneous absorption of

visible wavelengths by both the pigment and the co-pigment. The co-pigment absorption of the shortest radiations (380–450 nm approximately) originates a yellow sensation which is amplifying with its concentration while orange to purplish-red sensations (colours in the “cyanic” range) are resulting from the absorption in the central portion of the spectrum (480–600 nm) by cyanin pigment. The final colour of each solution, being based on the rules of subtractive mixtures of colorants (as a painter does with pigments on his palette), depends on the contribution of each constituent to the shape of the spectral curve. For instance, in the series of solutions at pH 5.5 and 4.5 at the four lowest concentrations, the co-pigment was virtually the sole contributor to their colour, causing the yellow tonalities displayed by the highly co-pigmented ones, although the pigment itself presented here its highest λ_{\max} in this survey.

Moreover, in the solutions at pH 5.5, the increased absorptivity between 400 and 480 nm—itsself causing a yellowing effect—due to specific effects of pH on the cyanin spectrum, was surimposed. Then, when the relative absorption of cyanin increased—by the pH and co-pigmentation combined effects—the yellowing strength of rutin was progressively balanced and finally exceeded by the cyanic colour component. Accordingly, in the batches of solutions at progressively increased pigment content, the hue variations caused by co-pigmentation gradually changed from yellowing to blueing effects although the λ_{\max} of the most co-pigmented solutions always appeared at the longest wavelengths. Such observations meet the results of a micro-spectrocolorimetric survey performed on the vacuolar sap of cells of carnation petals (containing anthocyanins, co-pigments...) and revealing that the colours of the vacuolar solutions having the highest λ_{\max} (resulting from the cumulative actions of the chemical factors affecting the structure of the anthocyanin nucleus *in vivo*) were not systematically those displaying the bluest hues (Gonnet & Hieu, 1992).

The present study confirmed that the enhanced “intensity” of colour—the word currently employed in the literature for describing the effect of co-pigmentation caused by increased absorptivity—corresponded to variations affecting specifically *two* attributes of colour, chroma and lightness. In short, weakly and moderately coloured solutions—i.e. those at the lowest pigment concentrations and/or at highest pH values—generally became more saturated and darker with increasing co-pigment concentration. By contrast, chroma remained stable, or even decreased for the most co-pigmented solutions at higher pigment concentrations or/and lower pH while their lightness was still decreasing.

Concerning the influence of environmental and molecular factors on the efficiency of co-pigmentation, the literature cited in introduction reports that both the recognized effects of this phenomenon on anthocyanin

colours—on their spectra in reality—were intensified at the highest pigment concentrations and pigment to pigment ratios and found maximal at pH 3.6.

In this study, the largest total colour differences between each pure cyanin solution and the co-pigmented ones were effectively measured in the series of solutions at pH 3.5, except for the most concentrated—and visually the most coloured—ones (10^{-3} and 2.5×10^{-3} M) in which the maximal differences were observable in the batches of solutions at pH 4.5.

At each pH and co-pigment to pigment ratio, the amplitude of the total colour difference generally increased with the cyanin concentration, except for the series of solutions at the two highest concentrations and lowest pH value.

With no exception this survey confirmed that in the range of the ratios tested, the more the co-pigment was added the more intense were the total colour effects produced. However, the colorimetric analysis also revealed more subtle differences in the amplitude of colour differences caused by successive additions of co-pigment according to the pH and the pigment concentration. The trend was that the strongest effects of co-pigmentation were observable at the lowest co-pigment to pigment ratio for the most coloured solutions (at the highest cyanin concentration and /or the lowest pH). On contrary, in the solutions at lower pigment concentrations and /or higher pH, only little colour variations resulted from co-pigment addition at low relative concentrations, the maximal colour effects occurring at the highest co-pigment to pigment ratios.

Regarding the influence of chemical parameters on the colour effects of co-pigmentation of anthocyanins, the discrepancies between this study and the previous reports definitively emphasize that the λ_{\max} of solutions is not the only wavelength to be considered. When the spectral variations caused by the co-pigmentation are considered according to the perceptual mechanism of the colour vision system—ie over the entire visible range of wavelengths and by means of a colorimetric approach—dramatic differences are revealed. In particular, the huge interval of hue angles covered by pure cyanin solutions with concentration show that at a fixed wavelength, absorbance changes, besides expectable lightness/chroma variations, also cause important shifts of tonalities. Similarly, variations of the λ_{\max} at comparable absorbances may also originate lightness/chroma variations. These combined features explain why some of the most intense spectral effects—at high cyanin concentrations and co-pigment to pigment ratios particularly—did not systematically produce the most intense effects on colour or why the maximum efficiency of co-pigmentation was observed at the either lowest or the highest ratio, according to the pigment concentration.

4. Conclusion

Whatever the chemical and environmental parameters considered, all the results in the present study definitively show that in describing the colour effects of the co-pigmentation of cyanin by rutin, only a colorimetric analysis—using the psychometric correlates of the three attributes of colour specified in the CIELAB system—really reports on the variations as these are perceived by the human eye. In addition, the CIELAB co-ordinates system also opens to possibilities of computation of visual colour differences allowing retrieving the closest colours in a database, containing the data of the model solutions realised for this survey for instance. Visually, close or even identical colours can be exhibited by solutions differing by pH, pigment concentration and/or co-pigment to pigment ratios. Some even can display colours simulating those of anthocyanin solutions on the most coloured form of the flavylium nucleus (at pH close to 1): this topic will be examined in the third paper in this series.

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