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The influence of phenolic acyl groups on the color of purple sweet potato anthocyanins and their metal complexes

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24 **Abstract**

25 Anthocyanins from purple sweet potato (PSP) are peonidin and cyanidin glycosides acylated
26 by *p*-hydroxycinnamic and *p*-hydroxybenzoic acids. For six individual PSP pigments, the
27 thermodynamic constants of proton transfer and water addition were determined, from which
28 the speciation diagrams for the colored and colorless forms and the UV-visible spectra of
29 individual colored forms could be constructed. The data confirm that acylation by phenolic
30 acids protects the chromophore against water addition (a consequence of acyl – anthocyanidin
31 π -stacking interactions) and that this protection depends on the type and number of acyl
32 residues, diacylation being much more efficient than monoacylation, and *p*-hydroxycinnamoyl
33 more efficient than *p*-hydroxybenzoyl. Most PSP anthocyanins can bind metal ions (Fe^{2+} ,
34 Al^{3+}) through their cyanidin chromophore and/or their caffeoyl residue(s). At pH 7, a cyanidin
35 glycoside bearing a caffeoyl residue can bind a single metal ion by simultaneous involvement
36 of its two binding units. With Fe^{2+} a strong bluing effect was observed. Although the caffeoyl
37 residues efficiently slow down color loss, their redox activity actually accelerates the thermal
38 degradation of anthocyanins. Consistently, two-electron autoxidation of anthocyanins bearing
39 caffeoyl residues could be evidenced by UPLC-MS/DAD analysis. These new pigments
40 possibly stem from intramolecular coupling between the chromophore and *o*-quinones derived
41 from the caffeoyl residues.

42

43 1. Introduction

44 Anthocyanins are plant pigments that typically exhibit bright red, purple and blue colors
45 as the pH is changed from 1 to 9 [1,2]. This property makes them potential natural food
46 colorants. However, color instability in the pH range 4 - 7 greatly limits the industrial
47 applications of anthocyanins. Color loss results from a combination of reversible (water
48 addition) and irreversible (autoxidation, hydrolysis) mechanisms [3]. Polyacylation by
49 phenolic acids, *i.e.* hydroxycinnamic acids (HCAs) or hydroxybenzoic acids (HBAs),
50 efficiently increases the color stability owing to π -stacking interactions between the
51 anthocyanidin chromophore and the acyl residues. Within the compact conformations
52 (intramolecular copigmentation) and/or aggregates (self-association) thus formed, the
53 anthocyanidin is less available to attacks by bleaching agents, such as water, bisulfite and
54 hydrogen peroxide [4]. Polyacylated anthocyanins, which are common in flower petals, can
55 also be found in edible sources, especially in intensely colored vegetables, such as red
56 cabbage (RC) and purple sweet potato (PSP), and the corresponding extracts bear great
57 potential for application as food colorants.

58 RC and PSP anthocyanins are both 3-O-sophorosyl-5-O-glucosylcyanidin and peonidin
59 derivatives bearing a variety of acyl groups on the sophorosyl moiety. In RC, acylation occurs
60 at C6-OH of the first D-glucose unit (Glc-1) and/or at C2-OH of the second D-glucose unit
61 (Glc-2). By contrast, acylation in PSP anthocyanins only occurs at the C6-OH positions of
62 both Glc units (Scheme 1). Diacylation of RC and PSP anthocyanins provides a remarkable
63 protection against water addition to the C2 position (C-ring) of the flavylium ion (AH^+),
64 which leads to a colorless hemiketal (B) in equilibrium with minor concentrations of *cis*- and
65 *trans*-chalcones (Cc and Ct) (Scheme 1-SI) [5,6]. Sandwich-type conformations with the
66 chromophore intercalated between the two acyl residues are assumed to be involved in this
67 gain in color stability [7].

68 While PSP anthocyanins are most commonly peonidin (major) and cyanidin (minor)
69 derivatives, RC are essentially cyanidin derivatives, which were shown to bind metal ions
70 above pH 5 with concomitant intense bathochromic shifts in the visible band and color
71 stabilization [8]. Unlike their RC cabbage homologs, some major PSP pigments have a caffeic
72 acid residue, which itself can bind metal ions. Hence, depending on the PSP anthocyanin
73 selected, metal binding can occur via the chromophore (RC anthocyanins) or acyl group (P9a,
74 P10, P11, P12) or both (P9b) (Scheme 1).

75 In this work, from a selection of individual PSP anthocyanins differing by the number
76 and type (HCA vs. HBA) of acyl residues, the influence of acylation on the color loss by
77 reversible water addition was quantitatively investigated. The superiority of anthocyanins
78 bearing two HCA residues is clearly demonstrated. The relative affinity of the cyanidin and
79 caffeoyl binding sites for metal ions and its consequences on the color expressed was
80 investigated at pH 7 with Fe^{2+} and Al^{3+} . Finally, the impact of the caffeoyl residues, free or
81 coordinated to iron, on the irreversible color loss (autoxidation) was also assessed. Evidence
82 for the formation of new pigments derived from intramolecular caffeoyl – anthocyanidin
83 oxidative coupling is provided.

84

85 **2. Materials and methods**

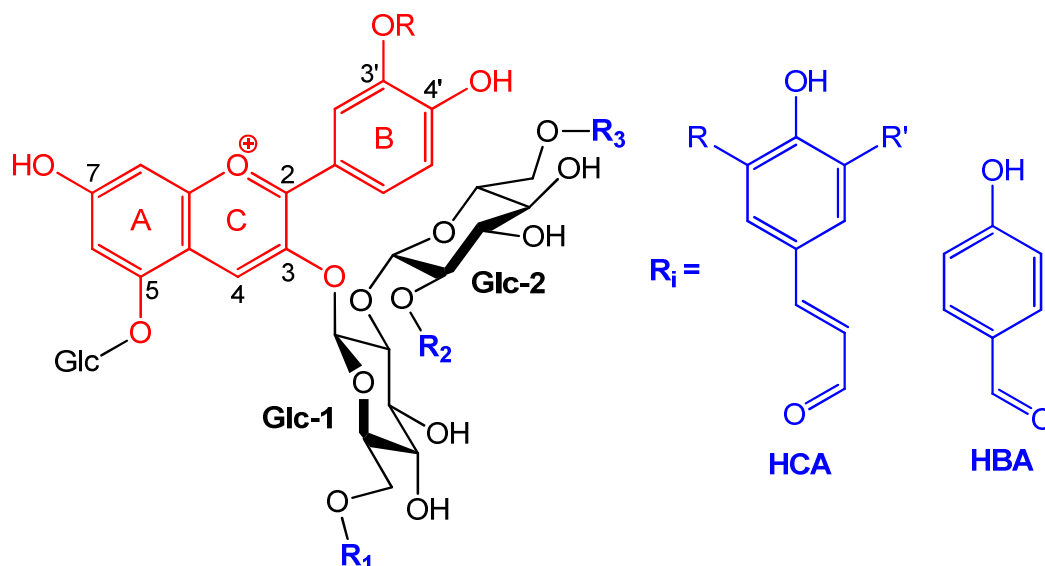
86 *2.1. Chemicals*

87 Anthocyanin extracts and isolated anthocyanins from purple sweet potato and red
88 cabbage (Scheme 1) were isolated by semi-preparative reverse phase HPLC [9]. They are
89 acylated derivatives of cyanidin- or peonidin-3-O-sophorosyl-5-O-glucoside. HPLC-grade
90 water was used for all aqueous solutions. Caffeic acid, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, KCl,
91 $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ were all purchased from Sigma-Aldrich. Acetic acid
92 (VWR), trace metal grade HCl (Fisher Scientific) and NaOH (Alpha Aesar) were also used.
93 Concentrated stock solutions (5 mM) of pigment were prepared in 0.05 M HCl.

94 *2.2. Structural transformations of anthocyanins*

95 For six major PSP anthocyanins, the following parameters were determined according
96 to a method recently described with details [5]: the first and second acidity constants of the
97 flavylium ion $\text{p}K_{\text{a}1}$ and $\text{p}K_{\text{a}2}$ (successively connecting the AH^+ and the neutral and anionic
98 bases A and A^-), the overall acidity constant of the flavylium ion $\text{p}K'_a$ (defined as $K'_a = K'_h +$
99 $K_{\text{a}1}$), the apparent hydration constant of the flavylium ion $\text{p}K'_h$ (connecting AH^+ and the set of
100 hydrated colorless forms, B, Cc and Ct) and the corresponding rate constants of hydration k_h
101 (s^{-1}) and dehydration k_{-h} ($\text{M}^{-1} \text{s}^{-1}$). The latter is actually an apparent rate constant for the
102 dehydration of B and Cc in fast tautomeric equilibrium (Scheme 1-SI).

103



104

	R	R ₁	R ₂	R ₃	
PA'	Me	H	H	H	<i>p</i> -Coumaroyl (pC): R = R' = H
P9a ^a	Me	Caffeoyl	H	H	Caffeoyl (Cf): R = OH, R' = H
P9b ^b	H	Caffeoyl	H	Feruloyl	Feruloyl (Fl): R = OMe, R = H
P10 ^b	Me	Caffeoyl	H	Caffeoyl	
P11 ^b	Me	Caffeoyl	H	<i>p</i> -Hydroxybenzoyl	Sinapoyl (Sp): R = R' = OMe
P12 ^b	Me	Caffeoyl	H	Feruloyl	
PA ^c	H	H	H	H	
P4 ^c	H	<i>p</i> -Coumaroyl	Sinapoyl	H	

105 ^a Structure inferred from those of the PSP diacylated pigments. ^b Structures from [10].

106 ^c From red cabbage [5].

107

108 **Scheme 1.** Structure of the purple sweet potato anthocyanins studied

109

110 Briefly, 10⁻² M acetate (pH 3.0 – 6.0) and phosphate (pH 6.0 – 8.5) buffers were used
 111 and the ionic strength fixed with 0.1 M KCl. Absorption spectra were recorded on a Agilent
 112 8453 diode-array spectrometer in thermostated and magnetically stirred quartz cuvettes
 113 (pathlength = 1 cm). For each pigment, the kinetics of the hydration reaction was monitored
 114 over the pH range 2 - 6. The apparent first-order rate constant of hydration (*k*_{obs}) was
 115 calculated for each final pH value. The first acidity constant *K*_{a1} (AH⁺/A couple) and rate
 116 constants *k*_h and *k*_{-h} were deduced from the pH dependence of *k*_{obs} and the ratio of the initial to
 117 final visible absorbance [5]. These solutions were left for 1 - 2 h to reach the hydration

118 equilibrium and their UV-visible spectra were recorded. From the plot of A_{eq} (at the
119 flavylum's λ_{max}) as a function of pH in the range 1 - 6, the apparent acidity constant pK'_a was
120 estimated (example provided in Fig. 1-SI). The apparent hydration constant K'_h was deduced
121 from the relationship: $K'_a = K'_h + K_{a1}$. Finally, the second acidity constant K_{a2} (A/A^- couple)
122 was estimated from the pH dependence of the absorbance at the anionic base's λ_{max}
123 immediately after addition of pigment to near neutral solutions (pH 6.0 - 8.5). Under such
124 conditions, the slow hydration can be neglected. By expanding the pH range to 9.2 and by
125 monitoring in the UV range (375 nm), the first acidity constant of P9a's caffeoyl moiety could
126 also be estimated.

127 Speciation diagrams showing the pH-dependence of the individual forms were
128 constructed from the pK_{a1} , pK_{a2} and pK'_a values (Fig. 2-SI). They were determined at $t = 0$
129 (hydration excluded) and at equilibrium.

130 2.3. Colorimetric data

131 In the color industry, it is common to express color characteristics by the $L^*a^*b^*$
132 coordinates. L^* corresponds to the light intensity, expressed from 0 (no light) to 100.
133 Parameters a^* and b^* quantify the contribution of four colors: green (-a), red (+a), blue (-b)
134 and yellow (+b). A program was developed that converts the UV-visible spectra (from 380 to
135 780 nm with 5 nm intervals) to the XYZ tri-stimulus values, then to the $L^*a^*b^*$ coordinates,
136 using the CIE (Commission Internationale de l'Eclairage) equations [11] for the standard D_{65}
137 illuminant and an observer at 10° (see Table 1-SI). From these coordinates, an open-access
138 online digital color calculator (<http://colorizer.org/>) was used to create color patches that
139 provide a reliable picture of the color actually expressed by the pigments in solution at a
140 concentration of 50 μM .

141 2.4. Metal binding experiments

142 Fresh 5 mM solutions of Fe^{2+} and Al^{3+} were prepared from $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{AlCl}_3 \cdot$
143 $6\text{H}_2\text{O}$ in 1 mM HCl. In the quartz cuvette of the UV-visible spectrometer, the following
144 solutions were added in this order: pH 7 phosphate buffer, 20 μL of anthocyanin stock
145 solution and, after a few seconds, a small volume of the 5 mM Fe^{2+} solution (final
146 iron/anthocyanin molar ratio = 1 or 2). The full UV-visible spectra were recorded in kinetic
147 mode. The duration of acquisition varied between 1 and 2 min. For an optimal sensitivity, the
148 detection in the visible range was set at 550 or 610 nm with Al^{3+} (close to the complex's λ_{max})
149 and at 670 nm with Fe^{2+} (charge transfer contribution of the Fe^{3+} complexes). For free and

150 bound caffeic acid, the absorbance was recorded at 350 nm and 370 nm, respectively. When
151 applicable, the hyperchromic and bathochromic shifts were calculated from the initial (free
152 ligand) and final (metal complex) spectra as $(A_{\max,f} - A_{\max,0})/A_{\max,0}$ and $\lambda_{\max,f} - \lambda_{\max,0}$,
153 respectively.

154 2.5. Kinetic modeling

155 The kinetic curves were analyzed with the Scientist[®] software (Micromath, St Louis,
156 USA). A two-step process was usually observed, which is interpreted as the successive
157 formation of complex C1 (second-order rate constant k_1 , molar absorption coefficient ϵ_1)
158 evolving into complex C2 (first-order rate constant k_2 , molar absorption coefficient ϵ_2).
159 Optimized values for the rate constants and molar absorption coefficients are reported.

160 2.6. Thermal degradation

161 Thermal degradation was performed at pH 7 and 50°C in a thermostated water bath
162 according to a method previously reported [4]. Briefly, the pigments were diluted to 50 μ M in
163 the phosphate buffer at 50°C and UV-vis spectra were recorded over 8h. The residual fraction
164 of color species at pH 7 (a mixture of neutral and anionic bases) was determined at λ_{\max} as %
165 Color = $100 \times A_{\lambda_{\max}}(t) / A_{\lambda_{\max}}(t = 0)$. Aliquots of 1.5 mL were taken up at time zero, at regular
166 time intervals over 8h, and finally at $t = 24$ and 48h. They were cooled down, acidified to pH
167 1 and stabilized at room temperature for 15h (nonacylated anthocyanins) to 48h (diacylated
168 anthocyanins) (to ensure complete regeneration of the flavylium ion from the colorless
169 forms). The absorption spectra were then recorded and the residual fraction of flavylium ion
170 was calculated as % AH⁺ = $100 \times A_{\lambda_{\max}}(t) / A_{\lambda_{\max}}(t = 0)$ and plotted as a function of time.
171 Finally, the percentage of degradation products was simply deduced from % D = $100 - \%$
172 AH⁺.

173 2.7. Product identification

174 The acidified and stabilized samples were analyzed with an Acquity UPLC (Waters
175 Corporation, Milford, USA) equipped with a diode array detector (DAD) and a ESI-Q-trap
176 HCT Ultra mass spectrometer (Bruker Daltonics, Bremen, Germany) in ultrascan mode.
177 Samples (5 μ L) were injected onto an Acquity UPLC BEH C18 reversed phase column
178 (50x2.1 mm, 1.7 μ m) at 30°C. Phase A (1% HCO₂H in H₂O) and B (1% HCO₂H in MeCN)
179 were used for elution at 0.4 mL/min. Gradient for P12 was %B: 0 min: 6%, 3 min: 12%, 9
180 min: 18%, 11 min: 24%, 14 min: 80%, 15-18 min: 6%. Gradient for P4 was %B: 0 min: 6%, 5
181 min: 12%, 10 min: 24%, 12-13 min: 80%, 15-18 min: 6%. The capillary voltage was -1.8 kV

182 (positive mode) or 2.2 kV (negative mode) with a 120-2200 m/z scanning interval at a speed
183 of $26 \times 10^3 m/z s^{-1}$. Desolvation was conducted with N_2 at 365°C, 40 psi, 540 L/h. The cone
184 voltage and the fragmentation amplitude were 40 V and 1.2 V, respectively.

185

186 **3. Results and discussion**

187 *3.1. Structural transformations of PSP anthocyanins*

188 Non-, mono- and diacylated anthocyanins were compared through the thermodynamic
189 and kinetic parameters characteristic of their structural transformations in acidic to mildly
190 alkaline solution (Scheme 1-SI). Whereas the first and second acidity constants are weakly
191 impacted by the acylation with no clear trend emerging, the overall acidity constant, which
192 includes the hydration component, is strongly affected: the pK'_a value increases from
193 nonacylated to diacylated anthocyanins, with a gap more marked when a second acyl residue
194 is introduced. As expected, this trend is translated in the pK'_h value, the global
195 thermodynamic constant of water addition to the flavylium ion. The diacylated anthocyanins
196 of purple sweet potato appear much more resistant to water addition than their non- and
197 monoacylated analogs, meaning that the second acyl residue triggers a better protection of the
198 flavylium ion against water addition than the first one, as observed with the red cabbage
199 anthocyanins [5]. This is consistent with the hypothesis of diacylated anthocyanins adopting
200 sandwich conformations with the anthocyanidin intercalated between the 2 acyl residues for
201 optimal protection. The second acylation also occurs on the external sugar of the sophorose,
202 and thus displays a higher flexibility. This may result in more efficient π -stacking
203 interactions.

204 The speciation diagrams express the calculated fractions of flavylium, neutral base,
205 anionic base and the mixture of colorless forms, plotted for each pigment over the pH range 1
206 – 10. They show that the fraction of colored forms in mildly acidic solution (pH 5 - 7) ranges
207 from *ca.* 1% for PA' to *ca.* 60% for P9b. As for the red cabbage anthocyanins [5], the
208 coloring potential of the diacylated anthocyanins far outreaches that of the non- and
209 monoacylated homologs at all pHs. This protection against hydration is mostly rooted in
210 smaller hydration rate constants (a factor *ca.* 30 between the k_h values of PA' and P12). As
211 diacylated anthocyanins make a large contribution to PSP anthocyanin extracts, representing

212 48% to 75% or more according to the cultivar [12,13], these pigments are mostly responsible
 213 for the color of the extract at the typical food pHs.

214

215 **Table 1.** Thermodynamic and rate constants for the structural transformations of the PSP
 216 anthocyanins (25°C).

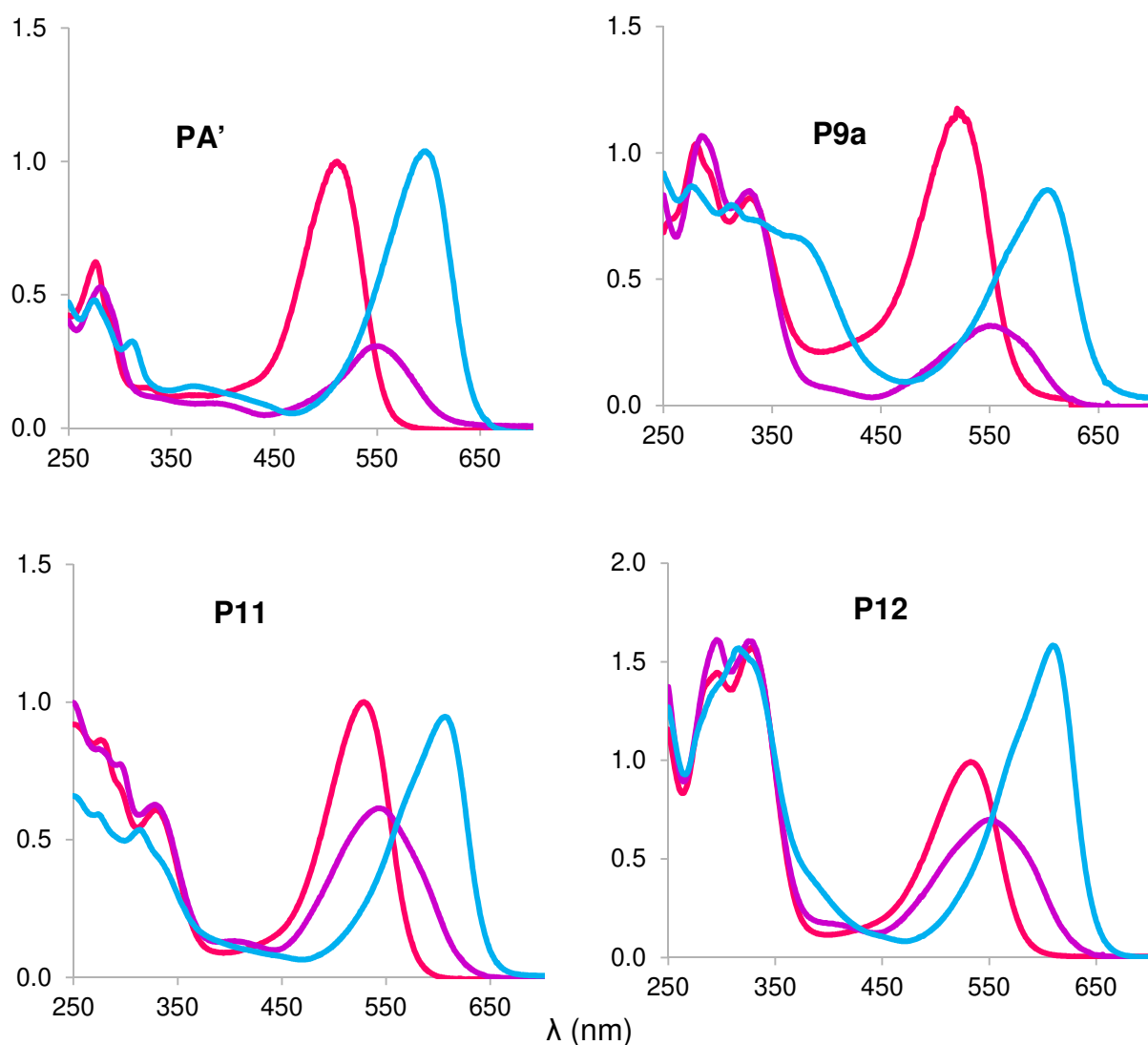
	Pigment	pK'_a	pK_{a1}	pK_{a2}	pK'_h ^a	pK_h ^b	k_h (s ⁻¹)	k_{-h} (M ⁻¹ s ⁻¹)
PA'	Peo	2.04 (± 0.04)	4.21 (± 0.08)	7.08 (± 0.04)	2.04	2.55	0.33 (± 0.01)	116 (± 7)
P9a	Peo(Cf)	2.43 (± 0.02)	4.06 (± 0.12)	7.11 (± 0.02)	2.44	2.82	0.132 (± 0.011)	87.8 (± 6.6)
P9b	Cya(Cf,Fl)	3.71 (± 0.07)	3.93 (± 0.04)	7.15 (± 0.06)	4.11	3.91	0.049 (± 0.004)	394 (± 24)
P10	Peo(Cf,Cf)	3.53 (± 0.03)	4.11 (± 0.06)	7.16 (± 0.05)	3.66	3.95	0.028 (± 0.004)	251 (± 25)
P11	Peo(Cf,HB)	3.25 (± 0.03) ^c	3.99 (± 0.06) ^c	7.29 (± 0.02) ^c	3.34	3.74	0.030 (± 0.004)	162 (± 16)
P12	Peo(Cf,Fl)	3.85 (± 0.04)	4.34 (± 0.07)	7.49 (± 0.05)	4.02	4.25	0.010 (± 0.002)	176 (± 27)

217 ^a $K'_h = K'_a - K_{a1}$ (Ct included), ^b $K_h = k_h / k_{-h}$ (Ct excluded). ^c From [6] (phosphate / citrate /
 218 borate buffer): $pK'_a = 3.15$, $pK_{a1} = 4.2$, $pK_{a2} = 7.8$. Peo(HB): $pK'_a = 2.69$, $pK_{a1} = 4.1$, $pK_{a2} =$
 219 7.5.

220

221 Among the diacylated anthocyanins of PSP, P11 bears a *p*-hydroxybenzoyl residue,
 222 which is much less common than the hydroxycinnamoyl residues. This peculiarity makes P11
 223 more vulnerable to water addition than the other diacylated pigments (P9b, P10 and P12),
 224 which display 2 HCA residues. For instance, the percentage of colored forms at equilibrium at
 225 pH 7 is *ca.* 40% for P12, *vs.* only 10% for P11. In addition, the formation of the colorless
 226 species (hydration) is 3 times as fast with P11 as for P12. This is consistent with HBAs being
 227 less potent copigments than HCAs [7] and suggests that the HBA residue of P11 develops

228 weaker π -stacking interactions with the anthocyanidin than the wider more polarizable HCA
 229 residues.



λ_{\max} (nm)	Flavylium ion	Neutral base	Anionic base
PA' (no acyl)	511	548	597
P9a (acyl = Cf)	522	542	603
P11 (acyl = Cf, HB)	528	543	607
P12 (acyl = Cf, Fl)	528	553	610

230 **Fig. 1.** UV-visible spectra of pure colored forms for peonidin derivatives PA', P9a, P11 and
 231 P12. —: flavylium ion, —: neutral base, —: anionic base; color patches (from the
 232 L*a*b*coordinates).

233

234 The spectra of the pure neutral and anionic bases can be calculated from the
235 experimental spectra at pH 1 (pure flavylium), 5.5 and 7.5 (recorded before significant
236 hydration) and the pK_{a1} and pK_{a2} values. For instance, from the total pigment concentration
237 and the pK_{a1} value, the concentration of the flavylium ion is determined at pH 5.5 and the
238 corresponding spectrum is generated, then subtracted from the experimental one, thus yielding
239 the spectrum of the pure base. This spectrum can then be used for similar corrections at pH
240 7.5 to unveil the spectrum of the pure anionic base. Such analyses permit to rigorously
241 compare the coloring properties for a selection of peonidin derivatives from PSP as a function
242 of their acylation pattern (Fig. 1). As usual, acylation results in a shifting of the visible band
243 to higher wavelengths but this phenomenon, typically associated with acyl – anthocyanidin π -
244 stacking interactions, is more significant with the flavylium ion and the anionic base.
245 Acylation by a *p*-hydroxybenzoyl vs. *p*-hydroxycinnamoyl residue has no consequence on the
246 flavylium spectrum (same λ_{max} for P11 and P12) but results in slightly lower λ_{max} values for
247 the neutral and anionic bases (Fig. 1).

248 The spectra of the anionic bases of P9a, P9b and P12 all show a narrow asymmetric
249 absorption band associated with a high chromaticity (Table 1-SI), which is an advantage in
250 terms of color expression. Based on our previous work [5], purple sweet potato and red
251 cabbage diacylated anthocyanins (except for P11) cannot be discriminated by their sensitivity
252 to water addition. Thus, the position on Glc-2 of the second HCA residue (C6-OH in PSP vs.
253 C2-OH in RC) has little influence on its capacity to hinder water addition to the flavylium ion.

254 From the spectral changes in the UV range at higher pH, the pK_a of the P9a's caffeoyl
255 residue was estimated at 8.27 ± 0.05 . This residue is more acidic than free caffeic acid ($pK_a =$
256 8.48) [14], 5-caffeoylquinic acid ($pK_{a2} = 8.42$) [15] and the caffeoyl moiety of the heavenly
257 blue anthocyanin ($pK_{a3} = 9.02$) [16]. The dissociation of the HCA residues is thus largely
258 negligible at food pHs.

259 3.2. Metal binding

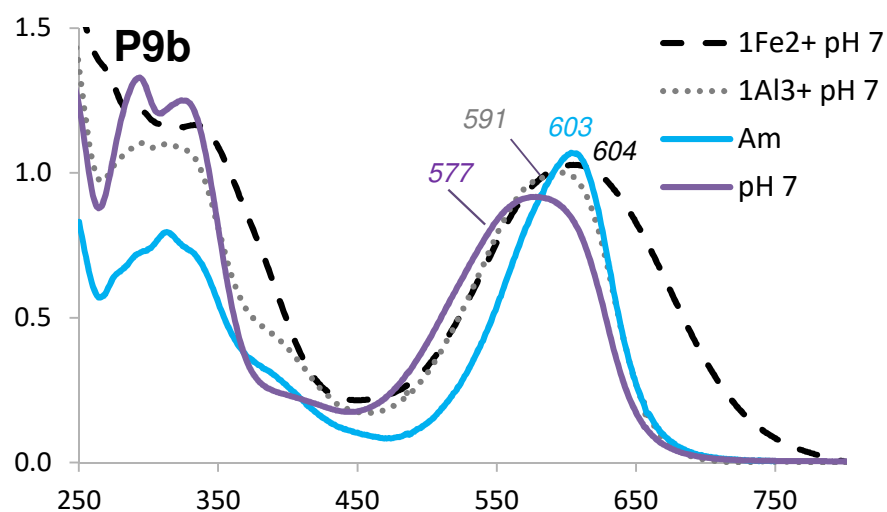
260 In our study of metal – anthocyanin binding, a neutral moderately concentrated (10
261 mM) phosphate buffer was used to set the pH constant and also simply mimic the competition
262 polyphenols may encounter in natural media with other common oxygenated ligands (organic
263 acids, phosphate and phosphatidyl groups) for metal ions. Aluminum and iron binding is an
264 important mechanism of color variation in plants, especially for the expression of blue colors

265 in flowers [17]. In PSP anthocyanins, the cyanidin nucleus and/or the caffeoyl residues can
266 bind metal ions owing to their catechol rings. For comparison, nonacylated pigment PA and
267 free caffeic acid were also studied. The spectral modifications of the PSP pigments (Fig. 2,
268 Table 2-SI) are highly dependent on pH, the metal ion, and the presence, number and position
269 of acyl residues.

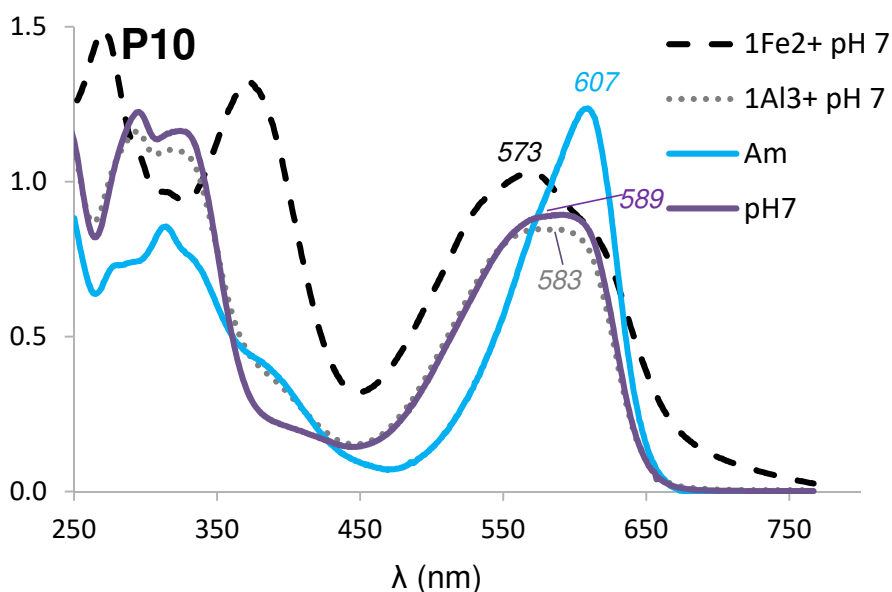
270 3.2.1. Aluminum binding

271 Caffeic acid does not bind to 1 molar equiv. of Al^{3+} at pH 7, whether free or as the
272 single acyl group of peonidin derivative P9a (Glc-1). However, for P10, the presence of the
273 second caffeoyl residue (Glc-2) allows Al^{3+} binding (Fig. 2, Table 2-SI).

274



275



276 **Fig. 2.** UV-visible spectra of pigment P9b (Cya, acyl = Cf, HB) and P10 (Peo, acyl = Cf, Cf),
277 at pH 7, its pure anionic base (Am, calculated) and its Fe^{2+} and Al^{3+} complexes (1 equiv.).

278

279 The binding of 1 or 2 equiv. Al^{3+} is primarily manifested by weak modifications in the UV
280 band of the acyl residues (the appearance of a shoulder at *ca.* 400 nm), while the visible band
281 remains mostly unaffected. Besides, reaching saturation in the spectral modifications requires
282 an excess Al^{3+} , which suggests that Al^{3+} - P10 binding is reversible under our conditions.

283 P9b combines two potential binding units, the cyanidin nucleus and the caffeoyl residue
284 on Glc-1 (inert to Al^{3+} as observed with P9a). The spectral changes in the UV range are the
285 same as for P10 but the visible band is now shifted to higher wavelengths and broadened (Fig.
286 2). Moreover, the final spectra with 1 and 2 equiv. Al^{3+} are almost identical. Overall, these
287 observations suggest a simultaneous binding of 1 equiv. Al^{3+} for the cyanidin and caffeoyl
288 moieties. The absorption spectrum of the P9b - Al^{3+} complex is close to the calculated
289 spectrum of the pure anionic base (Fig. 2). From the anionic base (proton loss from the acidic
290 C7-OH and C4'-OH groups), the coordination of Al^{3+} induces an additional proton loss from
291 C3'-OH, which apparently has only a weak spectral impact.

292 3.2.2. Iron binding

293 In a neutral dilute phosphate buffer, natural catechols typically bind Fe^{3+} much more
294 slowly than Fe^{2+} because of the strong competition between phosphate and phenol for Fe^{3+}
295 [18]. However, fast Fe^{2+} binding is typically followed by fast autoxidation of Fe^{2+} within the
296 complexes, which was confirmed by Fe^{2+} titration (ferrozine test). Independent experiments
297 with RC anthocyanins confirmed the much faster binding to Fe^{2+} in a 10^{-2} M phosphate
298 buffer. Moreover, the final spectra were actually the same, whether Fe^{2+} or Fe^{3+} was added, in
299 agreement with iron autoxidation during binding (Fig. 3-SI). Direct high-resolution mass
300 analysis of the solutions also confirmed that the final complexes involve Fe^{3+} (unpublished
301 data). Although free Fe^{2+} is already quite prone to autoxidation in neutral solution, its
302 conversion to Fe^{3+} is expected to be accelerated by binding to catechols, given the much
303 higher intrinsic affinity of these ligands for Fe^{3+} ($\log K_b = 20$ for Fe^{3+} , vs. 8 for Fe^{2+} [19]).

304 Fe^{2+} binding to caffeic acid or P9a results in the formation of a characteristic shoulder
305 (between 350 and 370 nm) from the UV band. By contrast, full deprotonation of caffeic acid
306 (pH *ca.* 10) shifts the absorption band to 344 nm (Fig. 4-SI). With caffeic acid, a new
307 absorption band typical of ligand-to-iron charge transfer is also observed at $\lambda_{\text{max}} \approx 610$ nm
308 (Fig. 4-SI) with a weak molar absorption coefficient ($\epsilon \approx 900 \text{ M}^{-1} \text{ cm}^{-1}$). Fe^{3+} being a much
309 stronger electron acceptor than Fe^{2+} , the development of the charge transfer band is another

310 evidence of Fe²⁺ autoxidation within the complex [18,19]. With P10, the spectral changes in
311 the UV range are much more intense and a true new absorption band at $\lambda_{\text{max}} = 374$ nm
312 emerges (Fig. 2). Its extension into the visible range adds a yellow component to the P10's
313 color. This new band points to cooperation between the two Cf residues in iron binding.
314 Although the peonidin nucleus has no metal binding ability, iron shifts the visible band to
315 shorter wavelengths (Fig. 2, Table 2-SI). This hypsochromic shift could reflect the
316 perturbation of the peonidin – acyl π -stacking interactions (a consequence of the iron-induced
317 perturbation of the electron density on the caffeoyl residues) and is also another evidence of
318 the compact folded conformations adopted by diacylated anthocyanins.

319 Caffeic acid - Fe²⁺ binding results in a weak shoulder at 370 nm (Fig. 4-SI), which is
320 not further increased by higher Fe²⁺ concentrations. In the presence of iron, the visible
321 spectrum of PA exhibits a weak bathochromic shift of 9 nm (Fig. 4-SI). Both bindings occur
322 with similar kinetics (Table 2). In comparison with PA and caffeic acid, the spectral
323 modifications induced by Fe²⁺ - P9b binding are much more spectacular (a bathochromic shift
324 of 27 nm and a more intense shoulder at 370 nm) and indicate that both binding sites
325 participate (Figs. 2 & 5-SI).

326 3.2.3. *Binding stoichiometry*

327 In the presence of increasing Fe²⁺ concentrations, the bathochromic shift of P9b's
328 visible band reaches saturation at 1 equivalent (Fig. 6-SI). This is evidence of a dominant 1:1
329 binding, which is the stoichiometry typically evidenced with other iron – flavonoid complexes
330 [19–21]. However, 1:2 binding was also reported with quercetin and kaempferol [22]. In
331 agreement with a 1:1 iron – anthocyanin binding, the same final UV-visible spectra were
332 recorded with P9b, whether 1 or 2 equiv. Fe²⁺ were added (data not shown). The same
333 observation holds for P10 and Fe²⁺. By contrast, with caffeic acid, saturation was reached at
334 lower iron concentrations, 1/3 to 2/3 equiv. (Fig. 6-SI), suggesting its possible involvement in
335 1:2 and 1:3 coordination complexes.

336 3.2.4. *Quantitative kinetic analysis*

337 Simple binding models were used to simultaneously analyze the spectral changes in the
338 visible (anthocyanidin) and UV (acyl) domains (Table 2, Fig. 3).

339 When metal binding is observed, this is often through a two-step kinetic process (Fig.
340 3). The 2 kinetic steps can be evidenced at the same monitoring wavelength, either by an

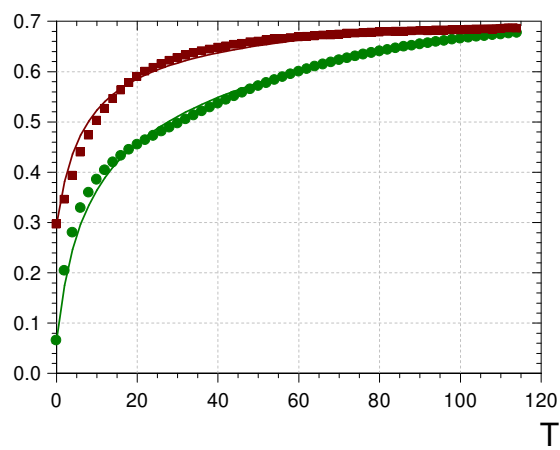
341 increase in absorbance followed by a decay (*e.g.*, caffeic acid + Fe²⁺, P9a + Fe²⁺, P9b + Al³⁺),
342 or a clearly biphasic (fast, then slow) increase in absorbance (*e.g.*, P9b + Fe²⁺, P10 + Al³⁺).
343 With ligands having a single binding site (caffeic acid, P9a), the second step (following a
344 second-order step of metal binding) is assumed to reflect a rearrangement in the coordination
345 sphere (possibly involving the phosphate ions) to a more stable complex. With P9b, the two
346 steps could in principle be ascribed to sequential metal binding to the two binding sites.
347 However, as both the UV (Cf) and the visible (Cya) bands are concomitantly intensified (Fig.
348 3), it is proposed that P9b binds a single Fe²⁺ equivalent simultaneously through its two
349 binding units and that the second (first-order) step most likely reflects a rearrangement in the
350 coordination sphere. This double coordination should occur at a minimal reorganization cost
351 as the two moieties are already in π -stacking interaction within the free pigment.

352 Similarly, as the free caffeic acid and pigments PA and P9a do not bind Al³⁺ under our
353 conditions, Al³⁺-P9b binding is probably driven by the joined coordination of Al³⁺ to the
354 cyanidin and caffeoyl moieties stacked onto each other by intramolecular copigmentation.
355 The fast coordination of P10 (2 caffeoyl residues) to Al³⁺ (at least as fast as with the cyanidin
356 derivative P9b) emphasizes the specific affinity of the external caffeoyl residue for Al³⁺. As
357 the same residue is critical to providing protection against water addition to the peonidin
358 nucleus (Table 1), it can be proposed that the strong π -stacking interactions developed by
359 these two moieties are key to the affinity of P10 for Al³⁺.

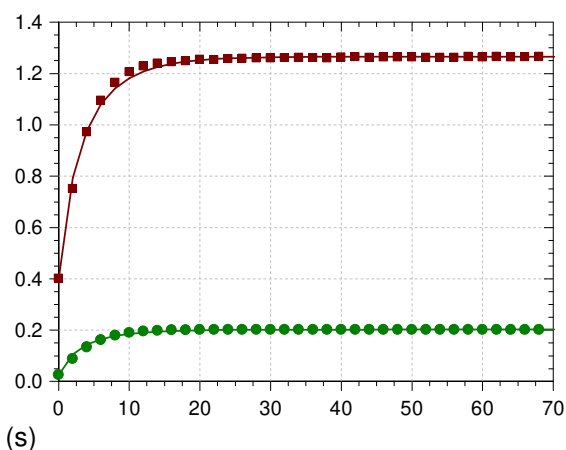
360 The spectral changes observed in iron - cyanidin binding combine the bathochromic
361 shift featuring the complete conversion of the ligand to the anionic base and the underlying
362 ligand-to-metal charge transfer. As the latter effect is absent with aluminum, the overall
363 bathochromic shift is much weaker (for P9b, 8 nm, *vs.* 36 nm with iron) (Table 2-SI). With
364 Al³⁺, a small fraction of unbound pigment may also remain in solution (reversible binding).
365 The influence of the acyl residues is critical and, for instance, the iron-induced bathochromic
366 shift drops to 9 nm for nonacylated PA. It is thus proposed that the simultaneous binding of
367 Fe²⁺ by cyanidin and the caffeoyl residue of P9b is the driving force in the intense bluing
368 effect observed with this pigment. By comparison, the highest bathochromic shift achieved by
369 adding Fe³⁺ (1 equiv.) to a neutral solution of red cabbage anthocyanins (non-coordinating
370 HCA residues) is *ca.* 20 nm (8 nm with Al³⁺) [8].

371

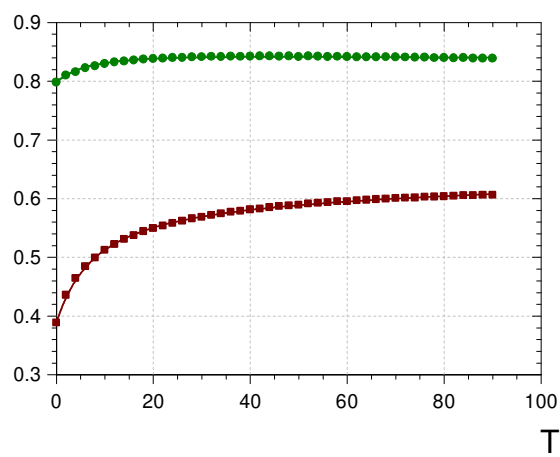
P9b + Fe²⁺



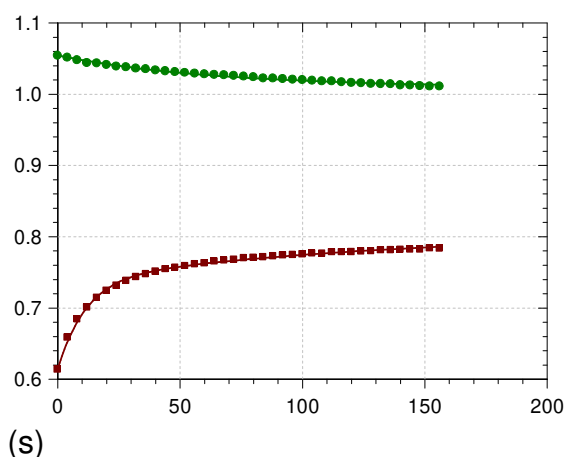
P10 + Fe²⁺



P9b + Al³⁺



P10 + Al³⁺



372 **Fig. 3.** The kinetics of metal binding to P9b and P10 (pH 7, 2 equiv. metal ion). ■: Monitoring
373 in the UV range (370 nm), ●: Monitoring in the visible range (Fe²⁺: 670 nm, Al³⁺: 550 nm).
374

375 Overall, iron binding appears faster than aluminum binding (when observable), a likely
376 consequence of a stronger competition between phosphate and the anthocyanin's binding sites
377 for Al³⁺.

378

380 **Table 2.** Kinetic analysis of metal – ligand binding (pH 7, 0.01 M phosphate buffer, 25°C).

Metal, Pigment	M equiv.	$10^3 k_1$ ($M^{-1} s^{-1}$) ^a	k_2 (s^{-1}) ^b	λ (nm) ^c $10^3 \varepsilon_1$ ($M^{-1} cm^{-1}$)	λ (nm) ^c $10^3 \varepsilon_2$ ($M^{-1} cm^{-1}$)
Fe, P9a	1	10.6 (\pm 0.7)	0.17 (\pm 0.01)	370: 29.9 (\pm 0.7) 670: 5.9 (\pm 0.2)	370: 20.5 (\pm 0.1) 670: 3.7 (\pm 0.1)
Fe, P9b	1	2.4 (\pm 0.1) 7.2 (\pm 0.2)	- 16.9 (\pm 0.5) $\times 10^{-3}$	370: 17.5 (\pm 0.1) 670: 10.8 (\pm 0.1)	- 670: 18.1 (\pm 0.1)
^d		3.5 (\pm 0.1)	16.9 $\times 10^{-3}$	370: 16.2 (\pm 0.1) 670: 13.2 (\pm 0.2)	370: 17.5 (\pm 0.1) 670: 18.1 (\pm 0.1)
Fe, P9b	2	10.0 (\pm 0.2)	23.8 (\pm 0.4) $\times 10^{-3}$	370: 17.2 (\pm 0.1) 670: 12.3 (\pm 0.1)	370: 18.6 (\pm 0.1) 670: 17.7 (\pm 0.1)
Fe, P10	1	3.7 (\pm 0.1)	-	370: 29.6 (\pm 0.1) 670: 4.8 (\pm 0.1)	
Fe, P10	2	4.3 (\pm 0.1)	-	370: 31.6 (\pm 0.1) 670: 5.1 (\pm 0.1)	
Fe, Cf	1	13.0 (\pm 0.5)	39 (\pm 1) $\times 10^{-3}$	370: 4.7 (\pm 0.1) 670: 0.92 (\pm 0.01)	370: 1.9 (\pm 0.1) 670: 0.32 (\pm 0.01)
Fe, PA	1	5.10 (\pm 0.6)	199 (\pm 14) $\times 10^{-3}$	670: 26.3 (\pm 0.22)	670: 5.19 (\pm 0.05)
Al, P9a	1	No binding			
Al, P9b	1	1.3 (\pm 0.1)	-	370: 12.6 (\pm 0.1) 610: 21.4 (\pm 0.1)	-
Al, P9b	2	1.9 (\pm 0.1)	19 (\pm 1) $\times 10^{-3}$	370: 13.3 (\pm 0.1) 550: 20.3 (\pm 0.1)	370: 14.9 (\pm 0.1) 550: 20.2 (\pm 0.1)
Al, P10	1	2.2 (\pm 0.1)	-	370: 13.7 (\pm 0.1)	-
Al, Cf	1	No binding			
Al, PA'	1	No binding			

381 ^a k_1 : bimolecular rate constant of metal binding leading to complex 1. ^b k_2 : first-order rate
382 constant for the possible evolution of complex 1 to complex 2. ^c ε_1 , ε_2 : molar absorption
383 coefficients of complex 1 and complex 2 at the specified wavelengths. ^d Final (refined) curve-
384 fitting at both wavelengths. M equiv: metal to pigment molar ratio.

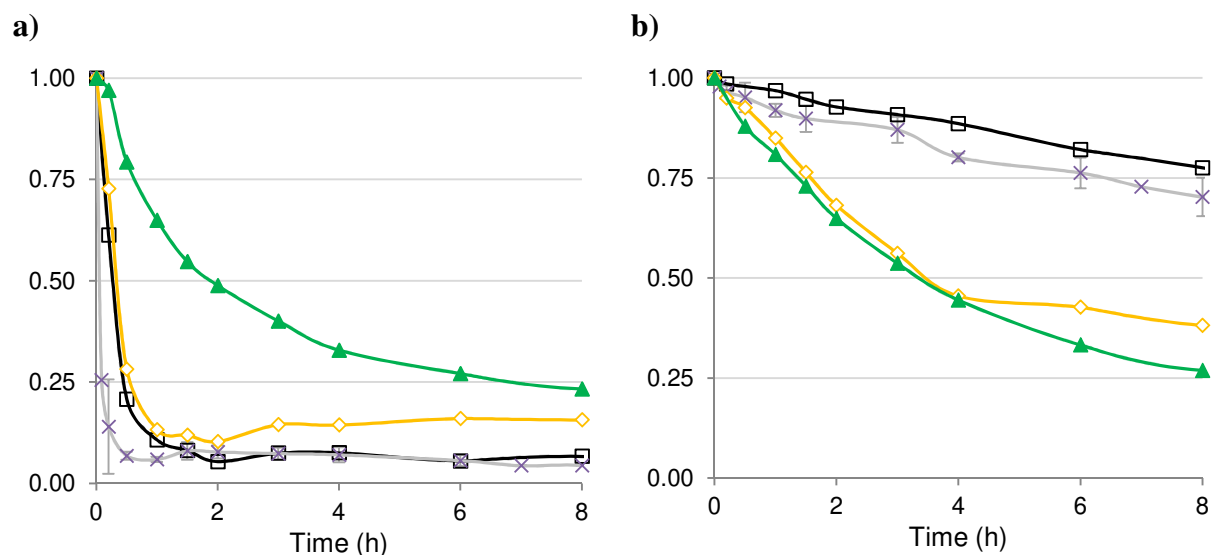
386 3.3. Thermal stability

387 3.3.1. Rate of degradation

388 The stability of individual anthocyanins was investigated at pH 7, 50°C (Fig. 4). In the
389 peonidin series, P10 (Peo-Cf,Cf) is more resistant to color loss than PA', a protection afforded
390 by the acyl - peonidin π -stacking interactions (Fig. 4a). In the presence of caffeic acid (2
391 equiv.), the rate of color loss for PA' is unchanged, thus suggesting that intermolecular
392 copigmentation is ineffective under such conditions. Unexpectedly, total pigment
393 quantification (after acidification) shows that P10 is much less resistant to true (irreversible)
394 degradation than its nonacylated counterpart PA' (Fig. 4b). P11 and P12, which also display
395 caffeoyl residues, have degradation rates close to that of P10 (Fig. 7-SI). Moreover, the
396 addition of caffeic acid (2 equiv.) also accelerates the degradation of PA' (Fig. 4b). By
397 contrast, the irreversible degradation of the red cabbage anthocyanins (acyl = pC, Fl, Sp) is
398 barely impacted by the acylation pattern [4]. Thus, it seems that the redox active caffeoyl
399 residue [23] favors the oxidative degradation of PSP anthocyanins.

400 Iron – anthocyanin binding is a major way of producing stable blue colors [8,17].
401 However, even moderate Fe^{2+} concentrations were shown to accelerate the degradation of red
402 cabbage anthocyanins, specifically the non- and monoacylated ones [4]. Nonacylated PA'
403 from PSP (Peo-3-O-Soph-5-O-Glc) and PA, its homolog from RC (Cya-3-O-Soph-5-O-Glc),
404 undergo degradation at similar rates (Fig. 4b). However, PA is much more destabilized by
405 Fe^{2+} addition than PA' ($t_{50} = 2\text{h}$ vs. 17h). This is consistent with a degradation initiated by iron
406 binding followed by a two-electron transfer to O_2 . On the other hand, Fe^{2+} (1.5 equiv.) has no
407 impact on the rate of P10 degradation (Fig. 7-SI). In this case, tight iron – caffeoyl binding
408 cancels the pro-oxidant effect of Fe^{2+} , as observed with the diacylated anthocyanins of red
409 cabbage [4].

410



411 **Fig. 4.** Kinetics of **a)** color loss and **b)** pigment degradation (pH 7, 50°C). PA (Cya, no acyl,
 412 x), PA' (Peo, no acyl, □), P10 (Peo, Cf, Cf, ▲), PA' + 2 equiv. caffeic acid (◇).

413

414 In summary, caffeic acid, either free or bound to the glycosyl moieties, accelerates the
 415 degradation of PSP anthocyanins at pH 7 but this effect can be suppressed by iron - caffeoyl
 416 binding. More generally, the presence of redox-active catechols, such as catechins and caffeic
 417 acid esters, may contribute to the overall chemical instability of anthocyanin-rich extracts
 418 [24]. Indeed, in spite of its higher percentage of diacylated anthocyanins, the PSP extract is
 419 less stable than the RC extract at pH 7, 50°C (Fig. 8-SI).

420 3.3.1. Degradation products

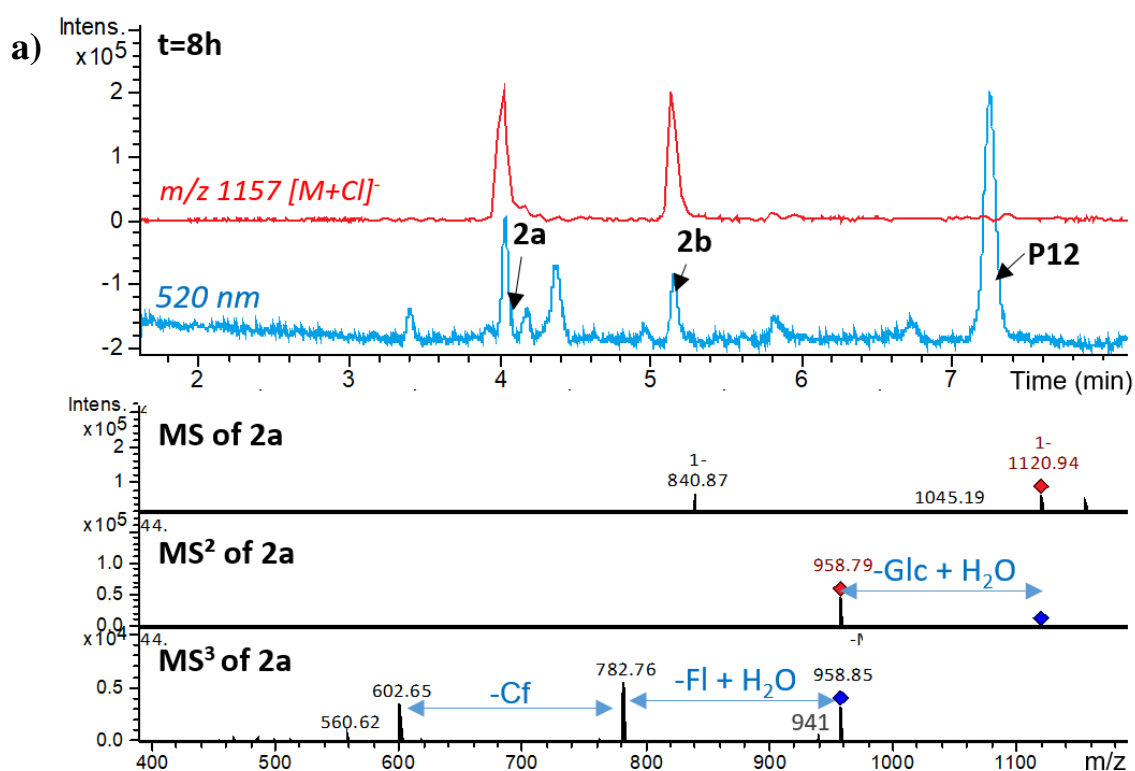
421 The degradation products of P11 and P12 (diacylated peonidin derivatives having one
 422 caffeoyl residue) and of P4 (diacylated cyanidin derivative without caffeoyl residue)
 423 supplemented with caffeic acid (1 equiv.) were analyzed by UPLC-MS/DAD. Pigments
 424 having lost the caffeoyl residue (m/z 905 from P11, m/z 961 from P12) were detected as well
 425 as diacylsophorose moieties (m/z 623 from P11, m/z 679 from P12), feruloylsophorose (2.8
 426 μM in ferulic acid equiv. after 24h) and caffeoylsophorose (1.3 μM in caffeic acid equiv. after
 427 24h) in low concentration. Under similar degradation conditions, *p*-coumaroylsophorose was
 428 detected as a major degradation product of red cabbage anthocyanins at pH 7 [3].

429 A group of new pigments also was detected, corresponding to P11 – 2H and P12 – 2H.
 430 Similar two-electron oxidized products were not detected with red cabbage anthocyanins
 431 under the same conditions [3]. For instance, with P12, 2 isomers of **2** having a m/z of 1121

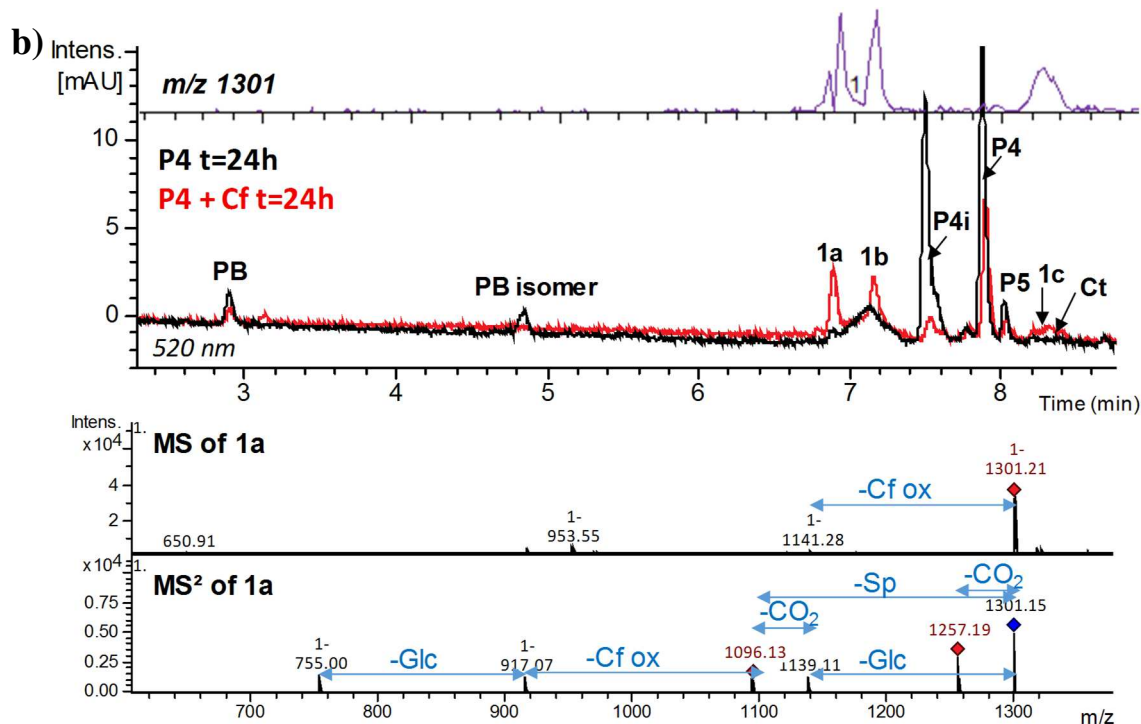
432 were observed at $R_t = 4.0$ and 5.2 min (Fig. 5a). Their λ_{\max} of 536 nm corresponds to a shift of
 433 *ca.* $+4$ nm compared to P12 (Fig. 9-SI). Products **2** are probably formed by autoxidation of the
 434 caffeoyl residue (initiated by metal traces) with concomitant formation of a *o*-quinone and
 435 H_2O_2 [18]. The *o*-quinone could then evolve by intramolecular nucleophilic addition of the
 436 peonidin nucleus (under its nucleophilic anionic base or hemiketal form), as already observed
 437 in an intermolecular version [25,26]. As the *o*-quinone of a caffeoyl residue has several
 438 electrophilic centers and the peonidin nucleus (anionic base and/or hemiketal) has 2
 439 nucleophilic centers (C6 and C8), the formation of several isomers is actually possible.

440 For comparison, a solution of red cabbage anthocyanin P4 (m/z 1123) supplemented
 441 with caffeic acid was heated under the same conditions. A new pigment noted **1** was detected
 442 with a λ_{\max} of 525 nm (*vs.* 537 nm for P4) and a m/z of 1301 consistent with an oxidative
 443 coupling to caffeic acid (Figs 5b & 10-SI). This compound has 3 isomers ($R_t = 6.92, 7.16$ and
 444 8.30 min) and yields a m/z 1141 fragment, corresponding to the P4 hemiketal. Besides, **1** also
 445 losses CO_2 to yield a m/z 1257 ion. Product **1** is thus proposed to result from the nucleophilic
 446 addition of P4 to the caffeic acid *o*-quinone. Similar products have already been observed
 447 when nonacylated anthocyanins are treated by the *o*-quinone of caffeic or caffeoyltartaric acid
 448 (generated by enzymatic oxidation) [25,26].

449



450



451
 452 **Fig. 5.** UPLC-MS/DAD monitoring of thermal degradation at pH 7, 50°C (detection at 520
 453 nm + ion current). **a)** P12 after 8h ($[M-2H]^-$ ion: m/z 1123). Detection of two-electron
 454 oxidized isomeric pigments 2a and 2b (m/z 1121). **b)** P4 after 24h ($[M-2H]^-$ ion: m/z 1123).
 455 Detection of pigments 1a and 1b (m/z 1301) resulting from oxidative coupling between P4
 456 and caffeic acid (1 equiv.). P5: initial contamination. Ct: P4 *trans*-chalcone.

457
 458 Pigment **1** concentration after 24h at pH 7, 50°C was estimated at 5.2 μM (in cyanin
 459 equivalent), *i.e.* roughly equal to the residual P4 concentration (4.9 μM , *i.e.* ca. 10% of the
 460 initial concentration). Interestingly, while P4 alone is very prone to isomerization (up to 53%)
 461 *via* intramolecular migration of its sinapoyl residue (at C2-OH of Glc-2) [3], addition of
 462 caffeic acid inhibits this phenomenon (only 19% under the same conditions).

463 In brief, acylation by caffeic acid or supplementation by free caffeic acid both concur to
 464 making anthocyanins more prone to autoxidation at neutral pH. The anthocyanin derivatives
 465 thus formed still absorb in the visible range (Figs 9-SI & 10-SI). Their contribution to the
 466 global color and its stability would deserve additional investigation.

467

468 **4. Conclusion**

469 Diacylated PSP anthocyanins express more intense purple and blue colors in near
470 neutral solution than non- and monoacylated ones. Their color is also more stable, thanks to
471 efficient π -stacking interactions between the acyl residues and the anthocyanidin nucleus.
472 However, a vulnerable point of the PSP anthocyanins evidenced in this work is the presence
473 of redox-active caffeoyl residues that accelerate their oxidative degradation, thus making
474 purple sweet potato extracts less stable than red cabbage extracts, despite the higher content in
475 diacylated anthocyanins of the former. Thus, under moderate heating at pH 7, caffeoyl
476 residues undergo autoxidation to electrophilic/oxidizing *o*-quinones produced by autoxidation
477 of the caffeoyl residues, a reaction probably initiated by iron traces. Metal - caffeoyl binding
478 only weakly modifies the color expressed through a modulation of the acyl - peonidin π -
479 stacking interactions. Through a tight iron coordination, anthocyanins bearing two caffeoyl
480 residues appear resistant to the pro-oxidant effect of moderate Fe^{2+} concentrations (10 to 100
481 times the trace concentrations in tap water). Al^{3+} binding could be an alternative to erase the
482 redox activity of the caffeoyl residues. On the other hand, a minor PSP pigment combining a
483 cyanidin nucleus and a caffeoyl residue can strongly bind iron through its two interacting
484 catechol nuclei with concomitant strong bathochromism and blue color development.

485 In summary, diacylated PSP anthocyanins have a high potential for development as
486 natural blue colors, provided that the reactivity of their caffeoyl residues be kept under
487 control. To this purpose, food-grade nucleophiles and antioxidants (thiols, ascorbate) could be
488 worth testing. In crude extracts, a purification step aimed at eliminating caffeoylquinic acids
489 from the PSP extracts could help limit the oxidative degradation of anthocyanins.

490

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494

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496

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