ELSEVIER

Food Chemistry 64 (1999) 21–26

Food Chemistry

Influence of structure on colour stability of anthocyanins and flavylium salts with ascorbic acid

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Received 27 February 1998; accepted 2 April 1998

Abstract

Ascorbic acid (330 mg/l) was added to buffered solutions (pH 2.35) of malvidin 3-glucoside, malvidin 3,5-diglucoside and flavylium salts with differing 4-substituents, viz.: 5,7-dihydroxy-4′-methoxyflavylium chloride, 5,7-dihydroxy-4-methyl-4′-methoxyflavylium chloride and 5,7-dihydroxy-4-phenyl-4′-methoxyflavylium chloride. Malvidin 3,5-diglucoside lost colour slower than malvidin 3-glucoside and flavylium colour stability was in the order 4-phenyl > 4-methyl > 4-H. The results demonstrate that anthocyanin reactivity and the status of the flavylium 4-substituent play an important role in colour stability under these conditions.

1. Introduction

It has long been known that anthocyanins and ascorbic acid (vitamin C) are mutually destructive in the presence of oxygen (Sondheimer and Kertesz, 1953). Starr and Francis (1968) showed that cranberry juice pigments degraded most rapidly when the greatest amounts of ascorbic acid and oxygen were present. Under aerobic conditions, the addition of transition metals, e.g. copper ions, accelerates the destruction of ascorbic acid and anthocyanins in their mutual presence; hydrogen peroxide produced by copper-catalysed break-down of ascorbic acid (Timberlake, 1960a,b) is believed to be the cause of pigment degradation under these conditions, and the possible nature of the activated oxygen species responsible has been discussed (King et al., 1980). That oxygen is an essential requirement in the decolorisation of anthocyanidins by ascorbic acid was confirmed by these authors, when rigorously deoxygenated systems were colour-stable for 10 days, but lost colour within a few hours after oxygen was admitted. Earlier work (Shrikhande and Francis, 1974) demonstrated the protective anti-oxidative property of some flavonols (e.g. quercetin) which retarded the degradation process.

The presence of anthocyanin breakdown products in a decolorised system showed, according to Iacobucci and Sweeny (1983), that colour-bleaching of anthocyanins by ascorbic acid occurs by oxidative cleavage of the pyrilium ring. This result diverged from the earlier work of Jurd (1972) who proposed a direct condensation mechanism, albeit without any experimental evidence, but later reinforced by Poei-Langston and Wrolstad (1981). These latter authors noted that anthocyanin colour decreased more rapidly under oxygen free conditions (nitrogen sparging) than under oxygenated conditions (oxygen sparging), favouring a predominant condensation mechanism to explain the observed colour loss.

The effect of anthocyanin structure on this reaction was observed by Hrazdina and Franzese (1974), when, in acidic solution, malvidin 3,5-diglucoside was oxidised more rapidly than the acylated malvidin analogue—an effect ascribed to decreased activity of the C2 position and/or steric hindrance. Similarly, Timberlake and Bridle (1968), highlighted possible steric effects on colour stability in studies with flavylium salts, when the nature of the substituent at position 4 was a key factor influencing resistance to colour-bleaching by sulphur dioxide. Jurd (1972) concluded that ascorbic acid could condense with the 4-position of anthocyanins in a manner analogous to the bisulphite ion reaction.

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0308-8146/98/$19.00
PII: S0308-8146(98)00107-1

To extend this area of research, we studied the effect of structure of some flavylium and anthocyanin compounds on reactivity with ascorbic acid, to provide further evidence for a mode of action of the degradation process. This work was conducted at the Institute of Food Research, Reading Laboratory.

2. Materials and methods

Experiments were carried out at pH 2.35, a pH value which permitted a direct comparison of anthocyanin behaviour with that of the less soluble synthetic flavylium derivatives. This is in accordance with previously reported data (Jurd, 1972) that show that these compounds are stable only in strongly acidic solutions

2.1. Material

Malvidin 3,5 diglucoside was obtained commercially (Aldrich Chem, USA). Malvidin 3-glucoside was isolated and purified in the laboratory by semi-preparative high performance liquid chromatography (HPLC) of a concentrated methanol-1%HCl *Vitis vinifera* grape skin extract. 5,7-Dihydroxy-4′-methoxyflavylium chloride salts with different 4-substituents (H-, methyl- and phenyl-) were prepared by analogy with the method of Robinson and Walker (1934).

Anthocyanin solutions with an absorbance value ca. 1.5 (10 mm path length, 520 nm) were prepared in 1% citric acid (pH 2.35) with 0.1% sodium benzoate, using malvidin 3-glucoside (1.19 mg/l) and malvidin 3,5-diglucoside (1.85 mg/l) with initial absorbance values ca. 1.45. To a portion of each solution, ascorbic acid (solid) was added to give a final concentration of 330 mg/l.

Flavylium, salts were dissolved in 1% citric acid buffer, as above, to achieve an initial absorbance of ca. 1.0 (10 mm path length; 4-H λ_{max} 468 nm; 4-CH_3 λ_{max} 449 nm and 4-phenyl λ_{max} 470 nm). Concentrations of these solutions are not defined since small additions of solid were made until the absorbances were stable for 24 h in the dark. Ascorbic acid was added to a portion of each solution, to give a final concentration of 330 mg/l.

Reaction mixtures (5 ml) were kept in screw-top vials and stored in the dark at 20°C. Duplicate solutions were prepared for each experiment and all analytical measurements were done in duplicate and the mean values reported in the data. Anthocyanin concentrations were calculated by spectrophotometry as described earlier (Picinelli et al., 1994)

2.2. HPLC analysis

Ascorbic acid concentration was determined by HPLC (Castillo and Greppin, 1988) on a Hewlett-Packard 1090M Series II chromatograph with a ODS-Hypersil reversed-phase column (250×4.6 mm, particle size 5 μm), flow rate 1 ml/min at 40°C, injection volume (20 μl) and diode array detector (245 nm). The elution solvent was aqueous 2% (NH_4) H_2PO_4 adjusted with phosphoric acid to pH 2.8.

2.3. Colour measurements

Solutions were measured in glass cells of 10 mm path length using a Philips PU8740 spectrophotometer. L^* a^* b^* values were calculated using illuminant D65 and a 10° observer according to the CIELAB 76 convention (McLaren, 1980). Hue angle (H) was calculated from arctan b^*/a^* and chroma from $(a^{*2}+b^{*2})^{1/2}$. Data were analysed using UNICAM, ASDS Windows 3.1 and Excel 5.0 PC based system software.

3. Results and discussion

3.1. Changes in anthocyanin and ascorbic acid (AA)

3.1.1. Changes in concentration

The rate of decrease of anthocyanin concentration is faster in samples containing AA, at the beginning of the experiment (greatest difference day 9), but after 10 days, is independent of the presence of ascorbic acid, for both anthocyanins tested (Fig. 1(a)). The influence of ascorbic acid on anthocyanin degradation during the first six days is slightly greater for the monoglucoside solution than the diglucoside solution. Also, the percentage loss of malvidin 3-glucoside (>90% with or without AA, after 13 days) is much higher than the malvidin 3,5-diglucoside (ca. 57% with or without AA, after 13 days).

The AA control was completely degraded after nine days (Fig. 1(b)). It is remarkable that the presence of anthocyanins and the nature of these compounds influences the rate of degradation of ascorbic acid in this pH buffer. Thus, although 100% of ascorbic acid control disappeared after nine days, 15% remained in the presence of malvidin 3-glucoside and 23% in the presence of malvidin 3,5-diglucoside after the same period. In summary, total loss of AA occurs after 15 days in the presence of malvidin 3-glucoside while 5% remains after 17 days in the presence of the diglucoside. showing that anthocyanins offer some degree of stabilisation towards AA at this pH.

Furthermore, AA degradation is slower according to the degree of substitution of the anthocyanin, presumably due to a higher stability of 3,5-diglucosides compared to 3-glucosides. This effect could possibly be due to the antioxidant properties recently described for anthocyanins (Wang et al., 1997). Also, at the low pH used here, the predominant anthocyanin form is the flavylium cation, known to be more active as a free radical scavenger.

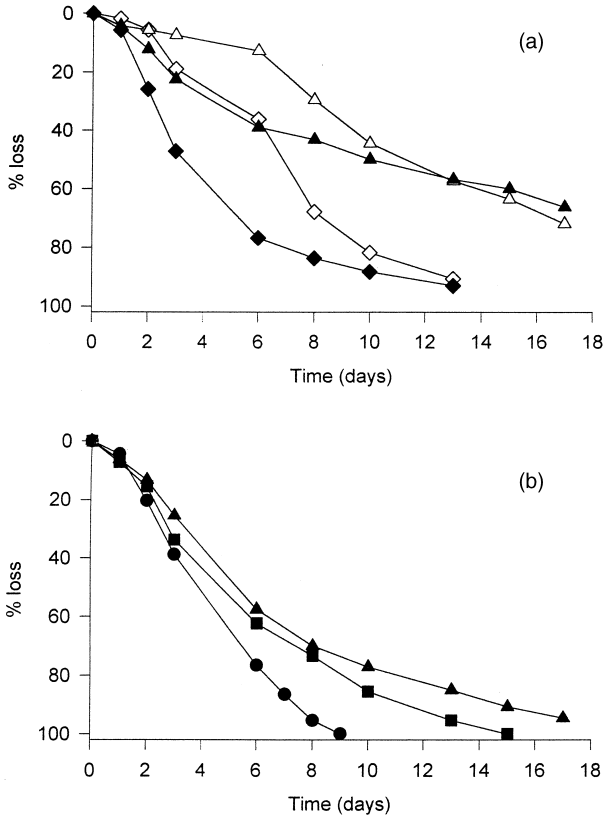

Fig. 1. (a) Percentage loss of anthocyanins dissolved in CA (citric acid) buffer. ◇, Malvidin 3-glucoside control; ◆, malvidin 3-glucoside with AA; △, malvidin 3,5-diglucoside control; ▲, malvidin 3,5 diglucoside with AA. (b) Percentage loss of AA in CA buffer, with anthocyanins. ●, AA control; ■, AA with malvidin 3-glucoside; ▲, AA with malvidin 3,5-diglucoside.

3.1.2. Changes in anthocyanins colour

During the previously reported reactions, some changes in visible colour were also observed (Table 1). These were more dependent on the type of anthocyanin than on the presence of AA. The samples became lighter (L^* increasing), more pronounced for malvidin 3-glucoside than for malvidin 3,5-diglucoside. However, the greatest changes were observed in a^*, indicating loss of red colour in solutions with malvidin 3-glucoside but almost independent of the content of AA. Nevertheless, the presence of AA appears to promote browning, as hue angle increases (ca. 18° over 13 days) in monoglucoside anthocyanin solution, with no similar effect on the diglucoside solution. Chroma also showed a gradual decrease, indicating a minor contribution of the a^* value to the colour observed (less red), again more appreciable with malvidin 3-glucoside than with 3,5-diglucoside. All these results are in accordance with the behaviour shown in absorbance values (represented in Fig. 1(a) by %loss of anthocyanin concentration). It is remarkable that no differences in the λ_{max} were measured in any of the experiments.

Table 1
Photocolorimetric measurement of anthocyanins in CA buffer

Time (days)	L^*	a^*	b^*	Chroma	H
M3G C					
0	64.40	60.19	9.66	60.96	9.12
1	64.40	60.19	9.66	60.96	9.12
2	65.96	60.44	9.04	61.11	8.50
3	66.32	59.84	8.80	60.48	8.37
6	72.21	50.87	2.91	50.95	3.27
8	81.94	30.75	0.33	30.75	0.61
10	87.02	16.19	1.43	16.26	5.05
13	92.59	7.81	2.46	8.19	17.49
M3G AA					
0	64.40	60.19	9.66	60.96	9.12
1	65.49	59.92	8.99	60.59	8.53
2	69.77	54.60	5.19	54.84	5.43
3	74.87	44.48	2.59	44.56	3.33
6	85.14	21.83	1.65	21.89	4.33
8	88.84	15.43	1.98	15.55	7.33
10	90.76	9.44	2.33	9.73	13.85
13	93.87	4.72	2.50	5.34	27.89
M3,5dG C					
0	67.17	62.87	−1.97	62.90	358.21
1	67.17	622.87	−1.97	621.90	358.21
2	67.65	62.97	−2.47	63.02	357.76
3	67.74	62.23	−2.37	62.28	357.82
6	68.97	61.65	−3.51	61.75	356.74
8	71.71	55.10	−3.96	55.24	355.89
10	75.40	48.91	−5.39	49.20	353.71
13	78.97	40.70	−4.73	40.98	353.38
15	79.89	34.23	−3.36	34.40	354.40
17	84.01	29.41	−3.00	29.56	354.17
M3,5dG AA					
0	67.17	62.87	−1.97	62.90	358.21
1	66.94	62.72	−2.23	62.76	357.96
2	68.59	61.39	−3.40	61.48	356.84
3	70.29	58.00	−4.05	58.15	356.00
6	74.07	52.01	−5.66	52.32	353.79
8	73.70	47.52	−3.50	47.65	355.79
10	76.48	45.41	−5.16	45.71	353.51
13	78.49	41.25	−5.00	41.55	353.09
15	79.32	37.05	−4.04	37.23	353.78
17	81.48	33.90	−4.27	34.16	352.83

M3G C: malvidin 3-glucoside control; M3G AA: malvidin 3-glucoside with AA: M3, 5dG C: malvidin 3,5-diglucoside control: M3,5dG AA: malvidin 3,5-diglucoside with AA.

3.2. Changes in flavylium salts and AA behaviour

3.2.1. Changes in concentration

In contrast to the results obtained for ascorbic acid degradation in the presence of anthocyanins, the type of flavylium salt had no significant influence on the rate of loss of ascorbic acid (Fig. 2(b)) (less than 3% left after 10 days for 4-phenyl and 0% for the others). However, this is not in correlation with the results obtained when loss of flavylium salt concentrations is analysed. Fig. 2(a) shows the changes in concentration in flavylium salts in all model solutions with time. Concentration decreased ca. 10% in all control samples after 33 days whereas, in the samples containing ascorbic acid, a

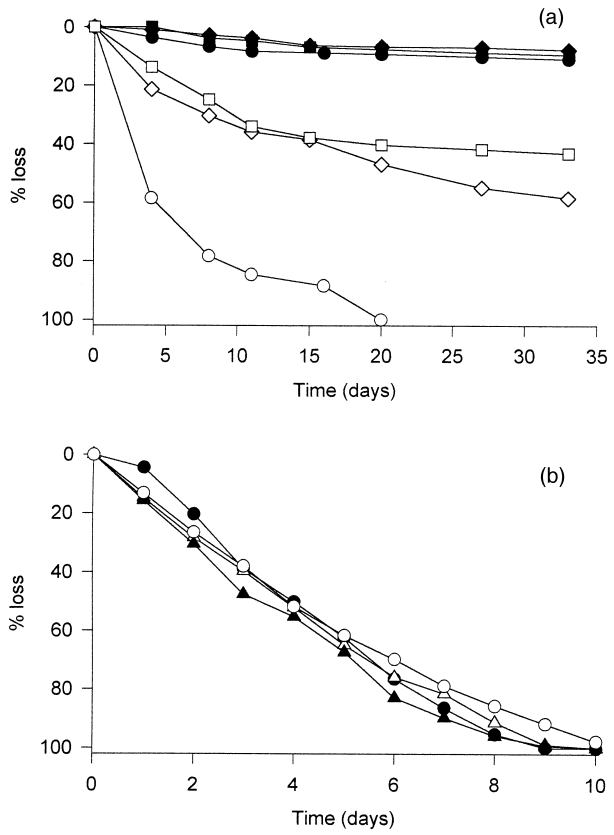

Fig. 2. (a) Percentage loss of flavylium salts in CA buffer. ●, 4H control; ○, 4H with AA; ◆, 4 methyl control; ◇, 4 methyl with AA; ■, 4 phenyl control; □, 4 phenyl with AA. (b) Percentage loss of AA with flavylium salts. ●, AA control; △, AA with 4H flavylium salt; ▲, AA with 4 methyl flavylium salt; ○, AA with 4 phenyl flavylium salt.

more marked decrease occurred, more pronounced with the least substituted salt. Thus, the 5,7 dihydroxy-4'methoxy flavylium chloride salt was not detectable after 20 days, while 60% and 40% of the 4 methyl and 4 phenyl salts, respectively, was lost after 33 days. The fact that the 4H flavylium salt did not stop the degradation, even when no AA was detectable, may be due to the reaction of this salt with the AA degradation product (DHA) which has been shown to react with these compounds (Francis, 1989; Starr and Francis, 1968).

3.2.2. Changes in flavylium salts colour

Only small changes in visible colour were observed during these reactions, especially in the control solutions (Table 2). Intensity assessment did not show any significant changes in any of the analysed models, L^* being constant during the whole experiment, as was a^*. Significant changes were noticeable only in b^* value, due to the predominant yellowish colour of these compounds, especially when AA was present, and more pronounced if position 4 was free. This was correlated with changes in chroma with time. Chroma did not present significant changes in control samples whereas, in the samples containing AA, a decrease occurred, most notably during the first 20 days, in solutions with position 4 unsubstituted. However, the samples did not brown, even though b^* decreased significantly (70.41 to 2.06; 63.11 to 28.98 and 52.33 to 32.01 in 4H, 4 methyl and 4 phenyl flavylium salts, respectively), as can be seen when analysing hue angle values (H). As with anthocyanins, it is noteworthy that no differences in the λ_{max} were measured in any of the experiments.

3.3. Some discussion about type of reaction that may take place

The anthocyanin/flavylium nucleus is electron-deficient and consequently highly reactive and unstable and susceptible to attack by many nucleophilic reagents, including water, peroxides, sulphur dioxide etc. Charge distributions of the flavylium cation have been calculated by several authors (e.g. Bendz et al., 1967), showing that electrophilic attack could occur at positions 6, 8 and 3′ (or 5′). But, because of their positive charge, flavylium salts (cations) are most susceptible to nucleophilic attack, principally at carbons 2 and 4. From our results, position 4 seems to be the more susceptible to the above mentioned attack since, when this position is substituted, the degradation of the flavylium salt is slower (and the degradation rate depends on the type of substitution).

On the other hand, it has also been demonstrated that flavylium salts condense easily with amino acids (Shriner and Sutton, 1963), phloroglucinol (Jurd and Waiss Jr, 1965) and catechin (Jurd, 1967) to yield colourless 4-substituted flav-2-enes. The result of these condensation reactions is the loss of the flavylium pigmentation. The β-diketone dimedone condenses very readily with flavylium salts and, assuming that ascorbic acid is structurally similar to dimedone, Jurd (1972) postulated that a similar condensation may account for the observed effect of this substance.

Nevertheless, from the results obtained in our experiment, this sort of reaction is unlikely to take place between anthocyanins and AA, for several reasons: (1) colour loss is slow, rather than instantaneous as seen with SO_2 (Timberlake and Bridle, 1968) and colour does not return immediately on acidification. (2) No new UV absorbing compounds were seen in HPLC analyses (data not shown); nor were there any changes in λ_{max} that could indicate new compounds formed. (3) AA has a degradation effect on flavylium salts even when position 4 is substituted. Thus it seems that degradation is more likely to be produced by the free radical mechanism (Iacobucci and Sweeny, 1983) rather than by direct condensation at position 4 (Jurd, 1972).

Moreover, considering the oxygen radical absorbing capacity of anthocyanins, which confers potent antioxidant properties on these compounds, as recently

Table 2
Photocolorimetric measurements of flavilium salts in CA buffer

Time (days)	L^*	a^*	b^*	Chroma	H
F14H C					
0	89.93	2.16	70.38	70.41	88.24
1	88.68	2.73	71.24	71.29	87.81
4	89.07	2.66	69.69	69.74	87.81
6	89.49	1.43	68.59	68.64	87.97
8	89.50	2.53	68.24	68.29	87.87
11	89.65	2.58	68.06	68.11	87.82
16	89.70	2.94	67.38	67.44	87.51
20	89.72	3.15	67.09	67.16	87.31
27	89.78	3.25	66.85	66.93	87.22
33	89.83	3.42	66.80	66.89	87.07
F14H AA					
0	89.93	2.16	70.38	70.41	88.24
1	89.64	1.66	62.54	62.56	88.47
4	93.03	−0.18	34.49	34.49	90.29
6	93.37	−0.44	25.91	25.91	90.98
8	94.75	−0.57	17.86	17.87	91.82
11	95.58	−0.40	14.52	14.53	92.01
16	96.04	−0.34	8.82	8.83	92.18
20	96.51	−0.30	2.06	2.08	92.30
F14Met C					
0	93.76	−11.25	63.11	64.09	100.11
1	93.79	−11.43	62.82	63.86	100.32
4	93.33	−11.29	62.15	63.17	100.29
6	93.29	−11.24	61.86	62.88	100.29
7	93.94	−11.10	62.45	63.43	100.08
8	92.83	−10.91	61.31	62.28	100.09
11	93.56	−10.93	61.14	62.11	100.14
13	93.25	−10.92	60.20	61.18	100.28
15	93.73	−10.89	60.03	61.01	100.29
20	93.73	−10.13	59.87	60.72	99.61
27	93.85	−10.46	59.84	60.75	99.91
33	94.00	−10.56	59.60	60.53	100.05
F14Met AA					
0	93.76	−11.25	63.11	64.09	100.11
1	93.71	−11.38	61.74	62.78	100.45
4	93.41	−10.14	51.30	52.29	101.18
6	93.77	−9.86	48.97	49.95	101.39
7	94.52	−9.51	48.39	49.32	101.12
8	93.48	−9.22	46.21	47.12	101.28
11	94.39	−8.81	44.16	45.03	101.28
13	93.64	−8.51	42.52	43.36	101.32
15	93.73	−7.86	41.75	42.48	100.66
20	93.42	−5.47	36.02	36.43	98.63
27	94.21	−4.83	31.44	31.81	98.73
33	94.35	−4.60	28.98	29.34	99.01
F14Ph C					
0	90.52	1.57	52.33	52.36	88.28
1	90.64	1.44	52.95	52.93	88.44
4	90.34	1.39	52.62	52.64	88.49
6	90.46	1.34	52.28	52.30	88.53
7	91.31	1.34	52.27	52.59	88.54
8	90.21	1.32	51.75	51.77	88.53
11	91.24	1.27	51.67	51.68	88.59
13	90.54	1.08	50.68	50.69	88.78
15	91.17	1.01	50.42	50.43	88.85
20	91.51	1.01	50.45	50.46	88.85
27	91.71	0.92	50.13	50.14	88.95
33	91.58	0.86	49.56	49.57	89.00
F14Ph AA					
0	90.52	1.57	52.33	52.36	88.28
1	90.59	1.42	52.77	52.79	88.46
4	91.06	0.88	45.87	45.88	88.89
6	91.19	0.67	43.32	43.32	89.11
7	92.35	0.58	42.27	42.27	89.21
8	91.39	0.55	40.19	40.20	89.21
11	92.56	0.05	36.65	36.65	89.60
13	92.16	0.25	34.85	34.85	89.92
15	92.72	0.02	34.37	34.37	89.97
20	93.17	0.15	33.70	33.70	89.75
27	93.24	0.10	32.80	32.80	89.82
33	93.23	0.10	32.01	32.01	89.83

Fl: flavylium salt; C: control; AA: flavylium salt with AA; Met: methyl; Ph: phenyl.

shown by Wang et al. (1997), this lends support to the theory of oxidation reaction in which the AA acts as an activator of molecular oxygen producing free-radicals which cleave the pyrilium ring, as postulated by Iacobucci and Sweeny (1983).

Furthermore, diglucoside anthocyanins stack vertically in a helical manner when self-association occurs at low pH (Hoshino et al., 1981) and further condensation reactions may not be possible due to steric hindrance. Thereby, it could be assumed that interaction with AA would be similarly inhibited. Nevertheless, when observing the degradation rate of malvidin 3,5-diglucoside, during the first nine days of the experiment, the reaction is faster if AA is present indicating that AA is reacting with the anthocyanin.

The greater stability of the diglucoside may be explained by the presence of a 3-glycoside on the flavylium molecule (anthocyanins) which lends colour stability compared to the more acidic 3-hydroxylated flavylia (anthocyanidins) (Timberlake and Bridle, 1967), which readily undergo equilibrium rearrangement in aqueous solution to the colourless carbinol base forms, with subsequent irreversible oxidative degradation. The reaction of flavylium salts with nucleophiles occurs readily when the C5 position is unsubstituted (Timberlake and Bridle, 1967; Iacobucci and Sweeny, 1983). The addition of a further glycosidic group (3,5-diglycosides) produces a more acidic anthocyanin than the corresponding 3-glucoside. Glycosyl substitution at C5 reduces the nucleophilic character of the C6 and C8 positions, thus anthocyanin 3,5-diglycosides are less prone to electrophilic attack than 3-glycosides (Timberlake and Bridle, 1977).

Acknowledgements

C.G.V. wishes to thank the Spanish CICYT (AL195-0001) for financial support and CSIC for a contract. P.B. is funded by the Office of Science and Technology via the Biotechnology and Biological Sciences Research Council

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