SPECIALIA

Structural transformation reactions of anthocyanins

L.-J. Chen[1] and G. Hrazdina[2]

Department of Food Science and Technology, Cornell University, Geneva (New York 14456, USA), 18 September 1981

Summary. Spectroscopic evidence shows that the flavylium cation (**1**) undergoes hydroxylation to form the carbinol base (**2**) and can also deprotonate to form the anhydro (quinonemethane) bases (**3a** and **3b**). The anhydro bases hydrate to the carbinol base. The carbinol base above pH 6, or the ionized carbinol base (**2a**), undergoes ring fission to produce the cis-chalcone (**4**).

Investigations with synthetic flavylium salts[3-8] showed that these compounds in aqueous media undergo structural transformation reactions. The mechanism in these reactions was suggested to be the deprotonation of the flavylium cation (**1**) to the anhydro (quinonemethane) base(s) (**3a** and **3b**) and hydration of the anhydro base(s) in the 2-or 4-position to form the carbinol base. The formation of yellow chalcones (**4**) from the synthetic flavylium salts was reported to occur even at low pH values. Naturally occurring anthocyanins were not included in these experiments. Recent kinetic investigations by Brouillard et al.[9-14] with malvidin 3-glucoside and 3,5-diglucoside suggested a similar behavior. A revised structural transformation mechanism proposed by the above authors excluded the hydration of the anhydro bases (**3a** and **3b**) to form the colorless carbinol base (**2**) and claimed the formation of (**2**) by hydroxylation of the flavylium cation (**1**) exclusively. In this paper we present evidence for the existence of 8 structural transformation forms of naturally occurring anthocyanins in the pH range 1-7, and for the direct hydration of the anhydro bases. Hydroxylation of the flavylium cations from naturally occurring anthocyanins in the 4-position was not considered due to the deactivating effect of the 3-hydroxyl or glycosyl group. Previous studies showed that the major site for nucleophylic attack on the flavylium cation is at C-2[15].

Malvidin 3,5-diglucoside was isolated as described earlier[16,17]. H^1NMR (CD_3OD) δ:2.05 (s, 6H, 3′,5′-OMe), 3.50-3.90 (m, 12H, glucose protons), 5.15-5.50 (m, 2H, glucose C-1 protons), 7.14 (d, J=2.2 Hz, H-6), 7.20 (d, J=2.2 Hz, H-8), 8.06 (s, H-2′, H-6′), 9.20 ppm (s, H-4).

Changes in the spectrum of malvidin 3,5-diglucoside solutions were investigated in the pH range 1-12.5. At pH 1 and below the pigment was present quantitatively in the red colored flavylium cation (**1**) form, exhibiting an absorption maximum at 517 nm. As the pH increased to 4.5, the

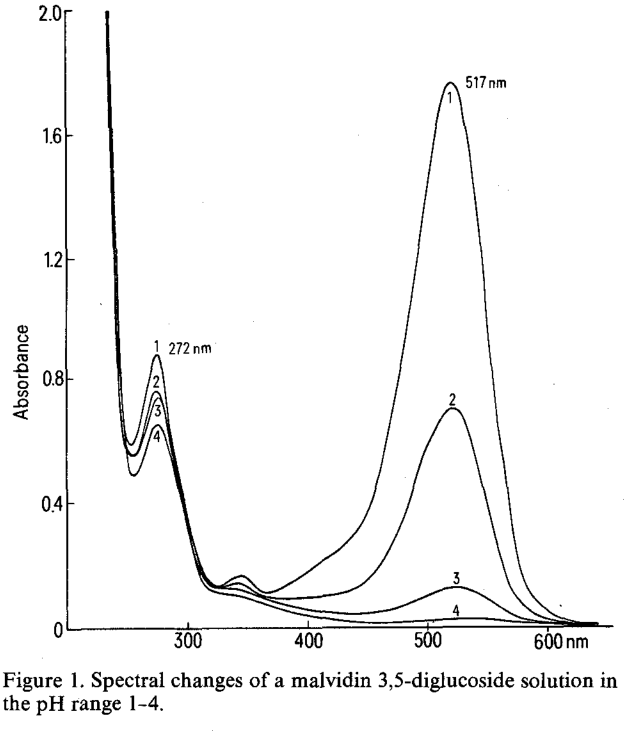


Figure 1. Spectral changes of a malvidin 3,5-diglucoside solution in the pH range 1-4.

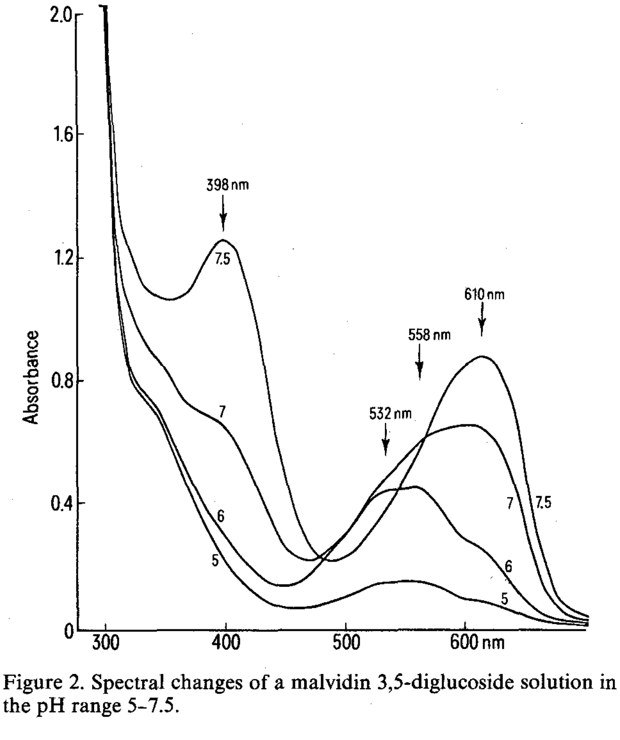


Figure 2. Spectral changes of a malvidin 3,5-diglucoside solution in the pH range 5-7.5.

intensity of the red colored anthocyanin solution progressively decreased to nearly colorless, as the flavylium cation underwent nucleophilic attack by water to form the carbinol base (**2**) (fig. 1). Above pH 4.5 a purple color appeared and increased in intensity up to pH 6.5 (fig. 2). The visible spectrum of this solution exhibited absorption maxima at 532 and 558 nm, indicating the presence of 2 different chromophoric species. These are the 7-keto anhydro base (**3a**) (λ_{max} 532 nm) and the 4′-keto anhydro base (**3b**) (λ_{max} 558 nm) present in equilibrium in the solution (λ_{max} of the anhydro base from the 7-hydroxy-4′-methoxy flavylium chloride is at 484 nm, that of the 4′-hydroxy-7-methoxy flavylium chloride at 506 nm[5]). Naturally occurring anthocyanins containing a 3-hydroxyl or glycosyl group apparently behave differently from synthetic flavylium salts because it was reported that 4′,7-dihydroxy flavylium chloride produced the 7-keto anhydro base only[5].

In addition to the 2 above absorption maxima a new λ_{max} at 610 nm appeared when the pH of the solution was increased to 6 and above, as shown in figure 2. This band is caused by the presence of the ionized anhydro base (**5**). Since there is only 1 absorption maximum, it is obvious that the same ionized anhydro base is obtained from either of the 2 anhydro base structures (**3a** and **3b**).

At neutral pH the anhydro bases were quantitatively ionized and the color of the anthocyanin solution appeared blue. In addition to the absorption maximum at 610 nm, a new peak appeared at 398 nm, caused by the formation of the yellow chalcone (**4**). In solutions with increasing alkalinity the absorbance at 610 nm progressively decreased as the chalcone was formed. The chalcone is also formed when the anthocyanin solution is stored at alkaline pH's, as seen in figure 3.

At pH 12 the color of the anthocyanin solution is yellow (λ_{max} 398 nm) and the presence of the ionized anhydro base (**5**) could not be detected. In addition to the absorption peak at 398 nm a shoulder at 430 nm was also observed in the spectrum, deriving from the ionized chalcone (**4a**).

Malvidin 3-glucoside[18] behaved similarly. Due to the higher pK values of anthocyanin 3-glycosides[7], the relative amount of the structural transformation forms was slightly different from those of malvidin 3,5-diglucoside. The color of the malvidin 3-glucoside solution was more intense at pH values of 3–4.5 than that of the 3,5-diglucoside, caused by the presence of higher amounts of the flavylium ion in the solution. The formation of the ionized anhydro bases occurred at higher pH's. Complete ionization of the anhydro bases occurred at pH 8.5. At pH 12 the color of the malvidin 3-glucoside solution appeared green, due to the presence of both the ionized anhydro bases and the yellow chalcone. At this pH no ionization of the chalcone could be detected.

When a solution of malvidin 3,5-diglucoside with the pH of 1.0 was placed in a buffer solution with the pH of 4.0, the initial red color of the resulting solution slowly faded to colorless. This indicated the conversion of the flavylium cation (**1**) to the carbinol base (**2**). When a pigment solution with the pH of 6.0, containing the anhydro bases (**3a** and **3b**) was added to the same buffer solution with the pH of 4.0, the original purple color also faded slowly, producing the same carbinol base (**2**) by hydration. During the course of the reaction the formation of the flavylium cation (**1**) could not be detected in the spectrum.

When a malvidin 3,5-diglucoside solution with the pH of 1.0 was added to a buffer solution with the pH of 6.5, the original deep red solution immediately turned purple and than slowly faded to colorless. This indicated a deprotonation of the flavylium cation (**1**) to the anhydro bases (**3a** and **3b**) in a fast reaction and hydration of the anhydro bases (**3a** and **3b**) to the colorless carbinol base (**2**) in a 2nd, slow reaction. In this reaction a small peak at 398 nm also appeared in the spectrum, indicating the formation of the chalcone (**4**). The chalcone does not seem to appear below this pH in detectable amounts (fig. 3). Addition of malvidin 3,5-diglucoside solution with the pH of 1.0 to a pH 7.5 buffer solution resulted in an immediate appearance of blue color, caused by the formation of the ionized anhydro base (**5**). The blue color of the solution slowly changed to greenish-yellow (λ_{max} 398 nm) as the chalcone was formed.

These above experiments indicate an equilibrium between the flavylium cation (**1**) and carbinol base (**2**) and also between the anhydro bases (**3a** and **3b**) and the carbinol base.

From our data and observations the structural transformation of anthocyanins appears to take place as follows:

Structural transformation forms from naturally occurring anthocyanins other than the flavylium cation (**1**) eluded isolation and chemical characterization. However, investigations with simple flavylium salts such as 3-methyl 4′-hydroxy and 3-ethyl 4′-hydroxy flavylium chloride[19] support the above mechanism. Both these compounds form readily the carbinol bases in aqueous solutions and the corresponding 2-methoxyflav-3-enes in aqueous methanolic solutions and permit their isolation and characterization. The equilibrium between anhydro base and the carbinol

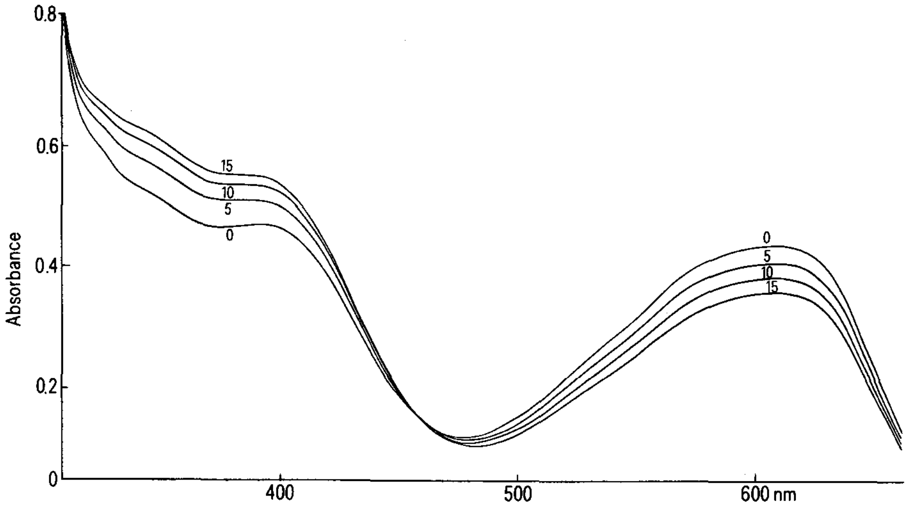
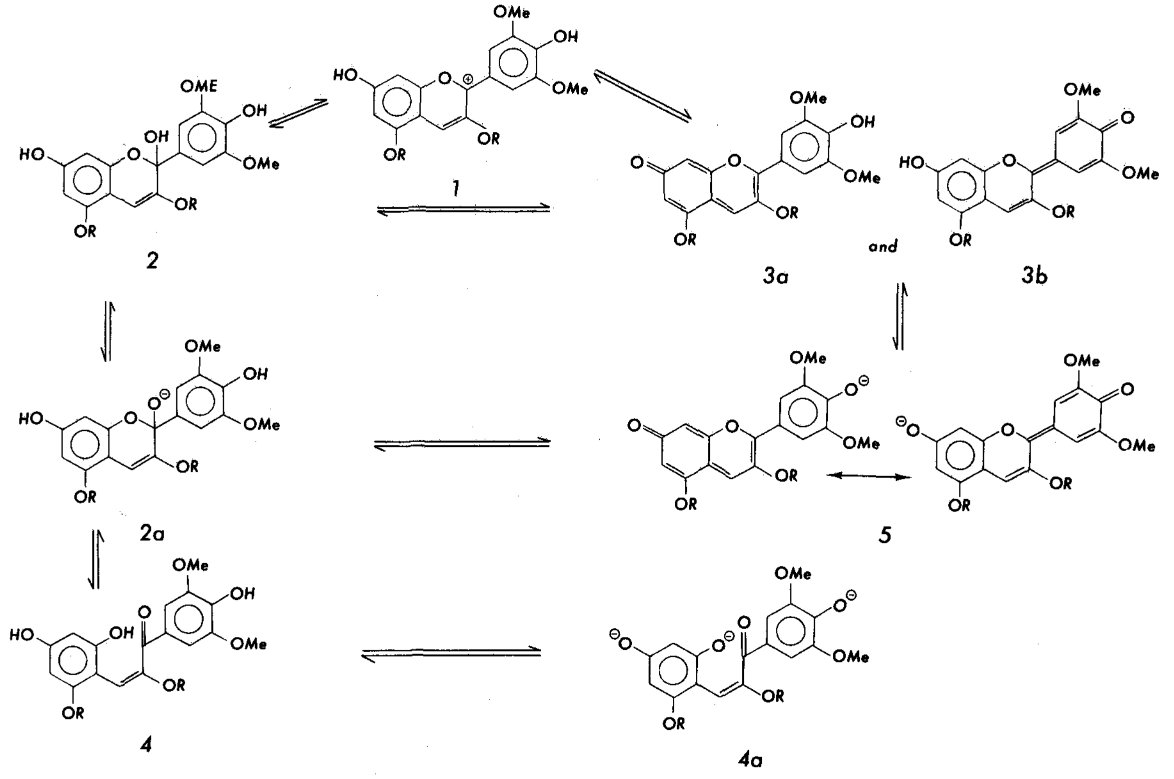


Figure 3. Spectral changes of a malvidin 3,5-diglucoside solution at pH 7.5 as a function of time.



base of the above flavylium salts in acetone solution was established by H^{1}NMR spectrometry. When the pure, colorless carbinol base of 3-methyl 2,4′-dihydroxyflav-3-ene was dissolved in acetone-d_6 and its H^{1}NMR spectrum recorded (δ:1.66) (s, 3-Me), 6.49 (s, H-4), 6.70–7.20 (m), 7.50 (d, J = 9 Hz, assigned to anhydrobase H-2′, H-6′), 7.82 (d, J = 9 Hz, assigned to carbinol base H-2′, H-6′), it showed that the 2 structural transformation forms, e.g. carbinol base and the anhydro base, were present at the ratio of 2:5 (ratio of δ:7.82 to 7.50 was 2:5). The direct formation of the anhydro base from 3-ethyl-2-methoxy-4′-hydroxyflav-3-ene in acetone d_6 (δ:1.00 and 1.10 (triplet), 1.95 and 2.50 (quartets), 3.25 (s, 2-OMe), 6.50 (s, H-4), 6.60–7.30 (m, H-5,6,7,8), 7.50 and 7.83 (d, J = 8 Hz, H-2′, H-6′) was also observed, the 2 structures reaching an equilibrium at the ratio of 1:4. (The H^{1}NMR spectrum indicated the presence of 2 compounds in the solution by the presence of duplicate signals. The signals at δ:7.83 and 7.50 were present at a ratio of 1:4). The carbinol base of the above flavylium salts has also been observed to convert to the corresponding anhydro base upon exposure to elevated temperatures, under development of an intense cerise color.

Under electron impact fragmentation the carbinol base of the 3-methyl-4′-hydroxy flavylium salt (m/e 268) loses a hydroxy group to form the flavylium cation at m/e 251. Also detected is a major signal m/e 250, caused by the formation of the anhydro base either directly by dehydration of the carbinol base or by deprotonation of the flavylium cation.

These above data are in agreement with the proposed structural transformation reaction mechanism, and do not support the claims by Brouillard et al.[9-14] for the presence of the chalcone form in aqueous acidic solutions under pH 6, or the formation of the carbinol base from the flavylium cation exclusively. The first compound produced by the fission of the heterocyclic ring of an anthocyanin molecule is the cis-ortho-hydroxy chalcone (**4**), having a λ_{max} at 398 nm. This structure is most likely stabilized by hydrogen bond formation between the 2-hydroxyl and the carbonyl groups[20]. Therefore, in a cis-trans equilibrium, the cis form is expected to be prevailing. The absorption band at ca. 340 nm used by the above authors as evidence for the presence of the chalcone is caused by a hitherto undetermined structural transformation form present in both aqueous and alcoholic acidic solvents.

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1 Present address: The Upjohn Co., P.O. Box 685, La Porte, Texas 77571/USA.
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