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Reactivity of Anthocyanins and Pyranoanthocyanins. Studies on Aromatic Hydrogen–Deuterium Exchange Reactions in Methanol

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Reactivity studies involving anthocyanin structures and their equilibrium forms will lead to better understanding of the properties of these antioxidants. Hydrogen–deuterium (H → D) exchange reactions at various sites of the 3-glucosides of delphinidin (**1**), petunidin (**2**), malvidin (**3**), and the corresponding 3-glucosides of carboxypyranodelphinidin (**4**), carboxypyranopetunidin (**5**), carboxypyranomalvidin (**6**), and the flavonol quercetin 3-*O*-(6-α-rhamnopyranosyl-β-glucopyranoside) (**7**) have been examined at room temperature in pure CD_3OD and in CD_3OD acidified with CF_3CO_2D. The H → D exchange rate constants of H-6 and H-8 of **2** determined from ^{1}H NMR integration data were found to be independent upon pigment concentration (up to 4×10^{-2} M) and trifluoroactic acid concentration (0–15%, v/v), respectively. This suggest that these reactions follow first-order kinetics and unexpectedly to be independent of the acid concentration. H-6 and H-8 of the flavylium cation A-rings of **1**–**3**, and in the corresponding hydrogens of the hemiketal forms, exchanged with half-lives of ∼100 h (**1**) and ∼50 h (**2** and **3**), respectively. The pyranoanthocyanins (**4**–**6**) experienced no H → D exchange for the analogous hydrogens, but H → D exchange of H-β (H-4) ($t_{1/2} \approx 25$ h) for these compounds was observed. Only H-8 underwent significant H → D exchange in **7**. It is concluded that a stabilization of the σ-complexes, assumed to be the intermediates in the reactions, takes place for the common anthocyanins (**1**–**3**) contrary to the pyranoanthocyanins (**4**–**6**).

KEYWORDS: Hydrogen–deuterium exchange; anthocyanidin 3-glucosides; flavylium cation; hemiketal; pyranoanthocyanidin 3-glucosides; rutin; reactivity; ^{1}H NMR

INTRODUCTION

The anthocyanins are known to be responsible for most of the attractive red, purple, and blue colors in plants and belong to the large and widespread group of plant constituents known as flavonoids (*1*). These water-soluble compounds may possess a wide range of biological activities (*2*), and their consumption has been assumed to provide beneficial effects on human health to prevent stroke and other cardiovascular disorders, age-related degenerative diseases, cancer, etc. (*3–7*). Many of their effects have been attributed to their antioxidative potential by contributing to, or enhancing by induction, the endogenous antioxidant properties of living cells or organisms. Interest in the potential health-promoting properties of anthocyanins has therefore intensified during the past decade; the scientific results, however, have primarily been based on in vitro characteristics. Mechanisms associated with absorption and pharmacokinetics of anthocyanins have only recently been examined (*8*). The anthocyanin forms found circulating in blood or tissues after oral ingestion may be metabolites or breakdown products, being different species from the original forms occurring in the diet. These generated compounds may induce different biological effects as compared to their precursors.

Some 575 different anthocyanins have so far been reported (*1*, *9*). They are considered to be present in several equilibrium forms formed by elementary reactions including proton transfer, isomerization, and tautomerization, and these reactions are among the important factors responsible for variations in color and stability of the anthocyanins (*10*, *11*). Anthocyanins containing an extra pyrano ring, the pyranoanthocyanins, have in recent years been found to be present in small amounts in wines, juices, and other processed foodstuffs (*12–17*) and in fresh plant material (*18–21*). It has been reported that pyranoanthocyanins have some different properties from the analogous anthocyanins, including restricted formation of colorless equilibrium forms under weakly acidic–neutral conditions (*21–24*).

The aromatic rings of flavonoids have some electronic density at specific sites due to mesomeric effects of electron-donating substituents such as hydroxyl groups (**Figure 1**). The substitution pattern of the A-ring of flavonoids may therefore facilitate electrophilic aromatic substitution reactions involving exchange

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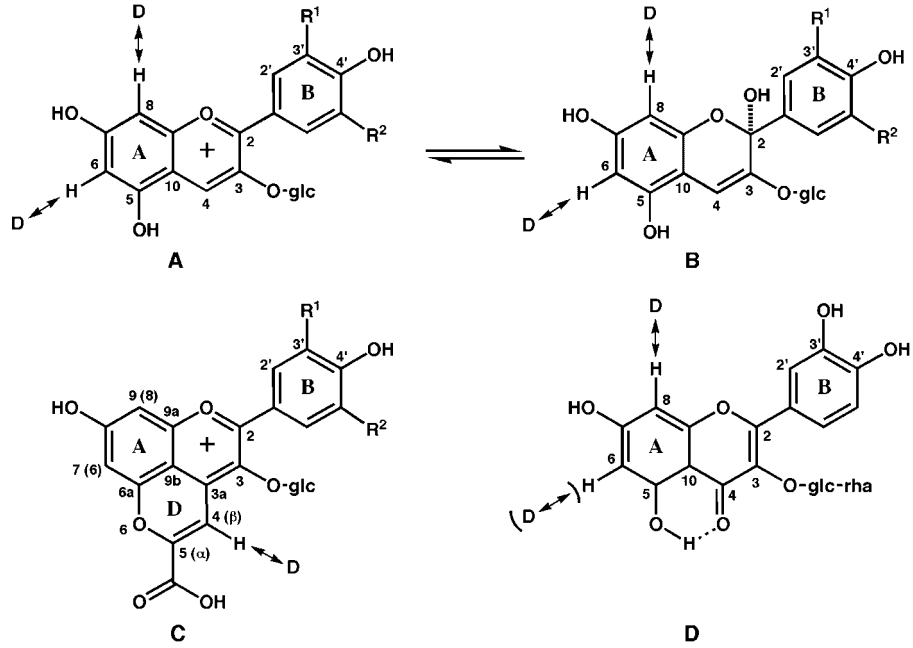

Figure 1. **A**: flavylium cation form of anthocyanins (**1**–**3**); **B**: two hemiketal forms, a (major) and b (minor), of anthocyanins (**1**–**3**); **C**: carboxypyranoanthocyanins (**4**–**6**); **D**: rutin (**7**). **1** = delphinidin 3-*O*-β-glucopyranoside (R^1, R^2 = OH); **2** = petunidin 3-*O*-β-glucopyranoside (R^1 = OCH_3, R^2 = OH); **3** = malvidin 3-*O*-β-glucopyranoside (R^1, R^2 = OCH_3); **4** = 5-carboxypyranodelphinidin 3-*O*-β-glucopyranoside (R^1, R^2 = OH); **5** = 5-carboxypyranopetunidin 3-*O*-β-glucopyranoside (R^1 = OCH_3, R^2 = OH); **6** = 5-carboxypyranomalvidin 3-*O*-β-glucopyranoside (R^1, R^2 = OCH_3); **7** = quercetin 3-*O*-(6-α-rhamnopyranosyl-β-glucopyranoside). Positions experiencing nuclear hydrogen–deuterium (H → D) exchange are labeled with arrows. The H → D exchange at the 6-position in (**7**) is very slow. The numbers of the A-ring in brackets in **C** shows the normal nomenclature for these positions in anthocyanins.

of hydrogens with deuterium at particular carbon atoms. Exchange in hydroxyl groups (OH → OD) of various flavonoids in the gas phase has been used to probe the conformations, gas-phase acidities, and deprotonation sites (*25*, *26*). Using mass spectrometry applied to various flavonoids under chemical ionization conditions, the aromatic hydrogens of flavonoids may undergo exchange with deuterium (*25*). This type of H → D exchange has also been observed in catechin/epicatechin 3′,4′,5,7-tetramethyl ethers, formed after cleavage of a methylated condensed procyanidin in D_2O–dioxane (*27*), in 3′,4′,5,7-tetramethoxyflavan when a solution of this compound in 3:1 D_2O–dioxane is heated at 95 °C for 16 h in Pyrex glass (*27*), and during labeling of isoflavone phytoestrogens (*28*).

Anthocyanins are good candidates for studies of H → D exchange because of the presence of the hydroxyl groups, the various resonance structures arising through three conjugated ring systems, and the occurrence of various equilibrium forms. It has previously been reported that H-6 and H-8 of the flavylium cationic form of pelargonidin (*29*) and malvidin (*30*) in anthocyanins can be exchanged with deuterium in acidified D_2O and acidified CD_3OD. As far as we know, no systematic study of possible exchange of the various aromatic hydrogens with deuterium in anthocyanins, nor H → D exchange reactions influencing anthocyanin in their hemiketal forms, have previously been addressed.

The major aim of the present work was to examine possible H → D exchange reactions at various sites of the anthocyanidin moieties of delphinidin 3-glucoside, petunidin 3-glucoside, and malvidin 3-glucoside (**1**–**3**) in solvents where they exist as flavylium cation forms as well as hemiketal forms (**A** and **B** in **Figure 1**). Similar studies were carried out on corresponding pyranoanthocyanins (**4**–**6**) and the flavonol rutin (**7**) (**C** and **D** in **Figure 1**), dissolved in the same solvents. Comparable studies with focus on specific sites of anthocyanidin structures and their equilibrium forms were carried out to improve understanding of differentiated properties and functions of different anthocyanins found in berries, fruits, vegetables, and derived products.

MATERIALS AND METHODS

Flavonoids (1–7). Dried black beans (*Phaseolus vulgaris* L.) were purchased from a local food shop (Helios, Bergen, Norway) and used as source for the anthocyanins, delphinidin 3-glucoside (**1**), petunidin 3-glucoside (**2**), and malvidin 3-glucoside (**3**) (*31*). The dried black beans (3 kg) were soaked in ∼3.5 L of water containing 0.5% trifluoroacetic acid (TFA) (Merck, Darmstadt, Germany) at 4 °C for 24 h. This extraction procedure was repeated four times. The combined extracts were purified by Amberlite XAD-7 column chromatography as previously described (*32*, *33*). The carboxypyranoanthocyanidins (**4**–**6**) were prepared by mixing the Amberlite XAD-7 purified anthocyanins (**1**–**3**) (10 g) dissolved in ethanol (100 mL) containing 2 mL of TFA and 2-oxopropanoic acid (pyruvic acid) (100 g) (Fluka, Germany) dissolved in distilled water (900 mL). The hemisynthesis of pigments (**4**–**6**) and the isolation of pigments (**1**–**6**) on a Sephadex LH-20 column were performed as previously described (*32*). Quercetin 3-*O*-(6-*O*-α-rhamnopyranosyl-β-glucopyranoside) (**7**) (rutin hydrate, minimum 95% HPLC purity) was obtained from Sigma-Aldrich (Germany).

NMR Spectroscopy. One-dimensional ^1H spectra of pigments (**1**–**7**) were obtained at 600.13 MHz on a Bruker 600 MHz instrument equipped with a cryogenic probe. Sample temperatures were stabilized at 298 K. The residual ^1H signals of the solvents CF_3CO_2D:CD_3OD (5:95, v/v), CF_3CO_2D:CD_3OD (15:85, v/v), and CD_3OD were used as secondary references (δ 3.40 from TMS). The NMR tubes used for the analyses were 528-PP Wilmad NMR tubes (Sigma-Aldrich, Germany) and 506-P-7 Standard Series NMR tubes (Norell). The Norell NMR tubes were soaked in a 0.2 M NaOH solution for several days, washed with distilled water, and dried before use.

1D ^1H NMR Exchange Experiments. Approximately 5 mg of each pigment (**1**–**7**) (**Figure 1**) was dissolved in CF_3CO_2D/CD_3OD (5:95, v/v) (∼10–12 mM). The ^1H spectra were initially recorded after 0.5, 1, 2, 4, 8, 16, and 24 h (**Table 1**). After 24 h the samples were stored

Table 1. Integration Data for H-6 and H-8 from the ^{1}H NMR (600 MHz) Spectra of the 3-*O*-β-Glucopyranosides of Delphinidin (**1**), Petunidin (**2**), Malvidin (**3**), and Quercetin 3-*O*-(6-α-Rhamnopyranosyl-β-glucopyranoside) (**7**) in CF_3CO_2D/CD_3OD (5:95, v/v) Recorded at 298 K[a]

	1		2		3		7	
time (h)	H-6	H-8	H-6	H-8	H-6	H-8	H-6	H-8
0.5	1.00	1.00	0.94	0.96	0.99	0.98	1.00	1.00
1	0.99	0.99	0.92	0.94	0.96	0.95	1.00	1.00
2	0.95	0.98	0.89	0.91	0.94	0.94	0.99	0.99
4	0.93	0.97	0.87	0.89	0.91	0.89	1.00	0.98
8	0.91	0.93	0.82	0.82	0.86	0.84	0.99	0.96
16	0.9	0.91	0.75	0.75	*m*	*m*	0.99	0.92
24	0.76	0.77	0.71	0.69	0.64	0.62	0.98	0.88

[a] Integration data are presented relative to the integrated area (r.i.a.) obtained for H-2′ (**2**) and H-2′ and H-6′ (**1** and **3**). *m* = integration data is missing. Additional integration data after 24 hours in darkness at room temperature. (1), 11 d: H-6 (0.09), H-8 (0.10); 22 d: H-6 (0.05), H-8 (0.07); 145 d: H-6 (0.01), H-8 (0.01). (2), 8 d: H-6 (0.09), H-8 (0.09); 30 d: H-6 (0.05), H-8 (0.05); 37 d: H-6 (0.04), H-8 (0.05) 152 d: H-6 (0.01), H-8 (0.01). (3), 7 d: H-6 (0.09), H-8 (0.09); 17 d: H-6 (0.07), H-8 (0.05); 30 d: H-6 (0.04), H-8 (0.06); 145 d: H-6 (0.01), H-8 (0.01). (7), 8 d: H-6 (0.96), H-8 (0.70). d = days.

in darkness at room temperature whereupon the 1D ^{1}H spectra were recorded after various time intervals.

Exchange experiments were also performed with 5 mg of pigment **2** in pure CD_3OD, in CF_3CO_2D/CD_3OD (15:85, v/v), and with 10 mg (~20 mM) and 20 mg (~40 mM) in CF_3CO_2D/CD_3OD (5:95, v/v) (**Table 2**). Relative integrated area (r.i.a.) for the exchange of H-6 and H-8 with deuterium in the flavylium cationic form (f) and hemiketal (hemiacetal) forms (major (a) and minor (b)) of pigments **1**–**3** in CD_3OD from the ^{1}H NMR spectra recorded at 25 °C are shown in **Table 3** (*33*). The amounts of the flavylium cation (f) and hemiketal forms (a and b) were derived from the ^{1}H NMR spectra of pigments **1**–**3** (*34*). Minor differences between comparable ^{1}H NMR integration data due to intrinsic experimental errors within the integration method were not taken into account.

RESULTS

Deuterium Exchange of Aromatic Hydrogens in Flavylium Cations. The H → D exchange of the aromatic hydrogens at various sites of the anthocyanidin moieties of **1**–**3** in their flavylium cationic forms in CF_3CO_2D/CD_3OD (5:95, v/v) at room temperature were based on integrated ^{1}H NMR data (**Table 1**). No exchange was found for H-4 or any of the B-ring protons, even during storage for more than 4 months. However, after only 24 h the integrated area of H-6 and H-8 on the A-rings of **1**–**3** were reduced by 23–36% compared to the signals measured 30 min after sample preparation (**Figure 2**). After 7–11 days the three pigments had experienced around 90% H → D exchange; after around 5 months the signals representing H-6 and H-8 were barely detectable (**Table 1**). The H → D exchange reactions of H-6 and H-8 of **1**–**3** (**Figure 3**) seemed to follow first-order kinetics according to logarithmic transformations. No distinct differences between the exchange rates of H-6 and H-8 in any of the three pigments could be observed. ^{1}H NMR experiments of these pigments dissolved in pure CD_3OD gave comparable results for the flavylium cations (**Table 3**); in CD_3OD pigments **1**–**3** were present both as flavylium cations and as hemiketals (*34*). However, the exchange rates of both H-6 and H-8 of delphinidin 3-*O*-β-glucopyranoside (**1**) (k = 0.007 s^{-1}) were slightly slower than the rates recorded for their methoxy-substituted analogues (**2** and **3**) (k = 0.014 s^{-1}) (**Figure 3**, **Table 3**).

Experiments performed with pigment **2** in pure CD_3OD and in CF_3CO_2D:CD_3OD (15:85, v/v) showed similar H → D exchange rates for H-6 and H-8 of the flavylium cationic form (**Table 2**) as observed for **2** dissolved in CF_3CO_2D:CD_3OD (5:95, v/v) (**Table 1**). The H → D exchange rates for H-6 and H-8 were thus not affected significantly by a 15% content of the fairly strong acid CF_3CO_2D. Apparently, the exchange rates are independent of the concentration of CF_3CO_2D in the applied solvent.

Deuterium Exchange of Aromatic Hydrogens in Anthocyanin Hemiketals. Anthocyanins are generally assumed to be present in several equilibrium forms depending on several factors (*10*). When anthocyanidin 3-*O*-β-glycopyranosides such as **1**–**3** are dissolved in pure deuterated methanol, both the flavylium cation and two epimeric hemiketals of each pigment have been shown to be present (*34*). The molar proportions of the flavylium cation and the two hemiketals were most similar for all the examined pigments, even after storage for weeks (*34*).

When **1**–**3** were dissolved in CD_3OD at room temperature, no H → D exchange was observed for H-4 or any of the B-ring protons of the hemiketal forms. However, after 24 h the integrated area of the ^{1}H NMR signals of H-6 and H-8 of the hemiketals of **1**–**3** were reduced with 16–33%. The H → D exchange rates of H-6 and H-8 of **1** were slightly slower than those of **2** and **3** (**Table 3**). The H → D exchange rates of H-6 and H-8 in the hemiketal forms of **1**–**3** were thus the same as the exchange rates of these hydrogens in their corresponding flavylium cationic forms. This similarity was caused by the fast equilibrium of the flavylium cation with the hemiketal forms, which was confirmed by observations of strong exchange peaks between analogous protons of these forms in the NOESY spectra of **1**–**3**, when dissolved in pure CD_3OD (*34*). Because this equilibrium is much faster (seconds) than the H → D exchange reaction, which was monitored in hours and days, individual contributions of the flavylium and hemiketal forms to the overall H → D exchange observed cannot be distinguished.

Deuterium Exchange of Hydrogens in Pyranoanthocyanins. The pyranoanthocyanins (**4**–**6**) dissolved in CF_3CO_2D:CD_3OD (5:95, v/v) were subjected to similar H → D exchange experiments as described for the analogous anthocyanins (**1**–**3**). No H → D exchange was observed for any of the B-ring protons of **4**–**6**, in agreement with the results obtained for **1**–**3**. However, contrary to the H → D exchanges observed for the nuclear protons of the A-rings of both the flavylium cation and the hemiketal forms of **1**–**3**, none of the pyranoanthocyanins (**4**–**6**) experienced H → D exchange for any hydrogen of the A-ring, even after 10 days. On the other hand, relatively fast exchange (k = 0.028 s^{-1}) was observed for H-β (H-4) of the D-ring. A comparison of the exchange rate of H-β of carboxypyranopetunidin 3-*O*-β-glucopyranoside (**5**) with the exchange rates of H-8 of petunidin 3-*O*-β-glucopyranoside (~10 mM) (**2**) and H-8 of rutin (~10 mM) (**7**) is shown in **Figure 5**.

Deuterium Exchange of Aromatic Hydrogens of the Flavonol Quercetin 3-Rutinoside. The H → D exchange of the aromatic protons of quercetin as based upon the change in the ^{1}H NMR spectra of rutin (**7**) dissolved in CF_3CO_2D/CD_3OD (5:95, v/v) is presented in **Table 1**. No H → D exchange was observed for any of the B-ring protons (H-2′, H-5′, and H-6′) of this flavonol. After 24 h the integrated area of H-8 (on the A-ring), however, was reduced with 12% (k = 0.002 s^{-1}). A possible decrease (2%) was also observed for H-6. After 8 days the integrated areas due to H-8 and H-6 were reduced with 30 and 4%, respectively.

DISCUSSION

Experiments performed with petunidin 3-*O*-β-glucopyranoside (**2**) dissolved in pure CD_3OD, in CF_3CO_2D/CD_3OD (5:95,

Table 2. Integration Data for H-6 and H-8 from the ^{1}H NMR (600 MHz) Spectra of Petunidin 3-*O*-β-Glucopyranoside (**2**) recorded at 298 K: (**A**) 5 mg in CF_3CO_2D/CD_3OD (5:95, v/v) (~10 mM); (**B**) 10 mg in CF_3CO_2D/CD_3OD (5:95, v/v) (~20 mM); (**C**) 20 mg in CF_3CO_2D/CD_3OD (5:95, v/v) (~40 mM); (**D**) 5 mg in CF_3CO_2D/CD_3OD (15:85, v/v) (~10 mM); (**E**) 5 mg in CD_3OD, Flavylium Form (f) (~8 mM), Hemiketal Forms (a and b) (~2 mM)[a]

	A		B		C		D			E		
time (h)	H-6f	H-8f	H-6f	H-8f	H-6f	H-8f	H-6f	H-8f	H-6f	H-8f	H-6a,b	H-8a,b
0.5	0.94	0.96	1.00	1.00	0.99	0.99	0.95	0.98	0.95	0.96	1.64	1.67
1	0.92	0.94	0.98	0.98	0.97	0.97	0.94	0.96	0.94	0.95	1.48	1.57
2	0.89	0.91	0.96	0.94	0.96	0.95	0.93	0.93	0.93	0.95	1.43	1.45
4	0.87	0.89	0.94	0.93	0.93	0.93	0.91	0.91	0.93	0.93	1.41	1.49
8	0.82	0.82	0.91	0.88	0.91	0.89	0.86	0.86	0.89	0.88	1.37	1.46
16	0.75	0.75	0.83	0.82	0.84	0.81	0.77	0.78	0.80	0.80	1.31	1.26
24	0.71	0.69	0.73	0.71	0.76	0.74	0.69	0.69	0.75	0.73	1.21	1.21

[a] Integration data for the flavylium form are presented relative to the integrated area (r.i.a.) obtained for H-2′ and for the hemiketal forms (a and b) relative to H-2′ of the major (a) hemiketal form. Additional integration data recorded for (**2**) during storage in darkness at room temperature. **A**, 132 d: H-6 (0.01), H-8 (0.01); **B,** 132 d: H-6 (0.01), H-8 (0.01), **C**, 164 d: H-6 (0.01), H-8 (0.01). The H-6 and H-8 signals of the two hemiketal forms (a,b) are integrated together. d = days.

Table 3. Integration Data for H-6 and H-8 from the ^{1}H NMR (600 MHz) Spectra of the Flavylium Cation Form (f) and Hemiketal Forms (a and b) of Delphinidin 3-*O*-β-Glucopyranoside (**1**), Petunidin 3-*O*-β-Glucopyranoside (**2**), and Malvidin 3-*O*-β-Glucopyranoside (**3**) in CD_3OD Recorded at 298 K[a]

	1				2				3			
time (h.min)	H-6f	H-8f	H-6a,b	H-8a,b	H-6f	H-8f	H-6a,b	H-8a,b	H-6f	H-8f	H-6a,b	H-8a,b
0.20									0.97	0.99	1.98	1.96
0.30	1.05	1.09	2.11	2.15	0.91	0.93	1.79	1.69	0.93	0.97	1.95	1.93
0.47	0.99	0.98	1.93	1.87								
24.00	0.82	0.81	1.74	1.67	0.71	0.69	1.50	1.37	0.73	0.69	1.45	1.31
27.19					0.68	0.69	1.38	1.34				
44.10	0.73	0.72	1.66	1.63								
51.23									0.42	0.42	1.04	1.01
62.30	0.64	0.62	1.56	1.46								
85.20	0.53	0.50	1.29	1.15								

[a] Integration data for the flavylium forms (f) are presented relative to the integrated area (r.i.a.) obtained for H-2′ (2) and H-2′ and H-6′ (**1** and **3**). Integration data for the hemiketal forms are presented as r.i.a. of H-2′ (**2**) and H-2′ and H-6′ (**1** and **3**) of the major (a) hemiketal form. The H-6 and H-8 signals of the two hemiketal forms (a, b) are integrated together.

v/v), and in CF_3CO_2D/CD_3OD (15:85, v/v) showed similar H → D exchange rates for both H-6 and H-8 (**Tables 1** and **2**). Kolar (*27*) examined H → D exchange reactions for H-6 and H-8 of some methylated flavanols in D_2O/dioxane (3:1) solutions after heating the samples for 16 h at 95 °C in Pyrex glasses. He concluded that the observed exchange reactions, following first-order kinetics, were typical electrophilic aromatic substitution reactions being catalyzed by acid. When no acid was present, the observed exchange reactions were suggested to be promoted by the Pyrex glass of the applied NMR tube assisting in the exchange process. Convincing evidence for this particular type of catalysis was the finding that both the H → D exchange reaction and cleavage of methylated procyanidins failed when examined in soda glass NMR tubes. The present H → D exchange studies on **2** dissolved in pure CD_3OD using both Wilmad Pyrex glass and Norell N-51A glass NMR tubes, the latter carefully cleansed with 0.2 M NaOH prior to use to remove possible surface acidity, led however to the same rates for the exchange reactions of H-6 and H-8 during the first 24 h (**Figure 4**). Possible D^+ formation from CD_3OD, as H^+ formation from CH_3OH, has previously been shown to take place only at elevated pressure and temperature (*35*). Acidic impurities in solvents and NMR tubes may thus not be the cause for the relatively rapid H → D exchange reactions in **1**–**3** observed in pure CD_3OD.

Studies of the potential influence of pigment concentration on the H → D exchange rates of H-6 and H-8 were performed with 10, 20, and 40 mM samples of petunidin 3-*O*-β-glucopyranoside (**2**) in CF_3CO_2D/CD_3OD (5:95, v/v) (**Table 2**). Similar H → D exchange rates were observed for both H-6 and H-8, showing the exchange mechanism not to be significantly affected by the anthocyanin concentration.

No H → D exchange involving the aromatic B-ring hydrogens of the three pyranoanthocyanins (**4**–**6**) or the flavylium cation and hemiketal forms of the three anthocyanidin 3-monoglucosides (**1**–**3**) was observed during our experiments. When considering the anthocyanidin A-ring, it has previously been reported that H-6 and H-8 of the flavylium cationic forms of pelargonidin 3-glucoside (*29*) and malvidin 3,5-diglucoside (*30*) may exchange with deuterium when dissolved in acidified D_2O or acidified CD_3OD. The 3-glucosides of delphinidin, petunidin, and malvidin (**1**–**3**) in their flavylium cationic forms experienced hydrogen–deuterium exchange at C-6 and C-8 in different solvents (**Tables 2** and **3**). No distinct difference between the exchange rates in these positions could be detected, although C-8 has been considered to be more negatively charged than C-6 (*36*). The exchange rates of H-6 and H-8 in the anthocyanidin hemiketal forms were found to be nearly identical to that of their flavylium cationic forms. These results are in agreement with the existence of a fast equilibrium between the flavylium cation and the hemiketal forms in anthocyanins like **1**–**3** (*34*). However, we cannot exclude that the A-ring hydrogens of the hemiketal forms have the same exchange rates as the corresponding hydrogens of the flavylium cation form. The structures of anthocyanins **1**–**3** are typical representatives for most of the anthocyanidin 3-monoglycosides found in fruits and vegetables (*37*). These pigments have been proposed to be present mainly as hemiketals in slightly acidic to neutral solvents (*10*, *34*, *38*), a relevant pH range in plants, and in the human gastrointestinal tract.

With due allowance for experimental uncertainties the obtained results are consistent with that the anthocyanins (**1**–**3**) exchange hydrogen with deuterium quite readily at position C-6 and C-8 in the A-ring with approximately equal rates. The

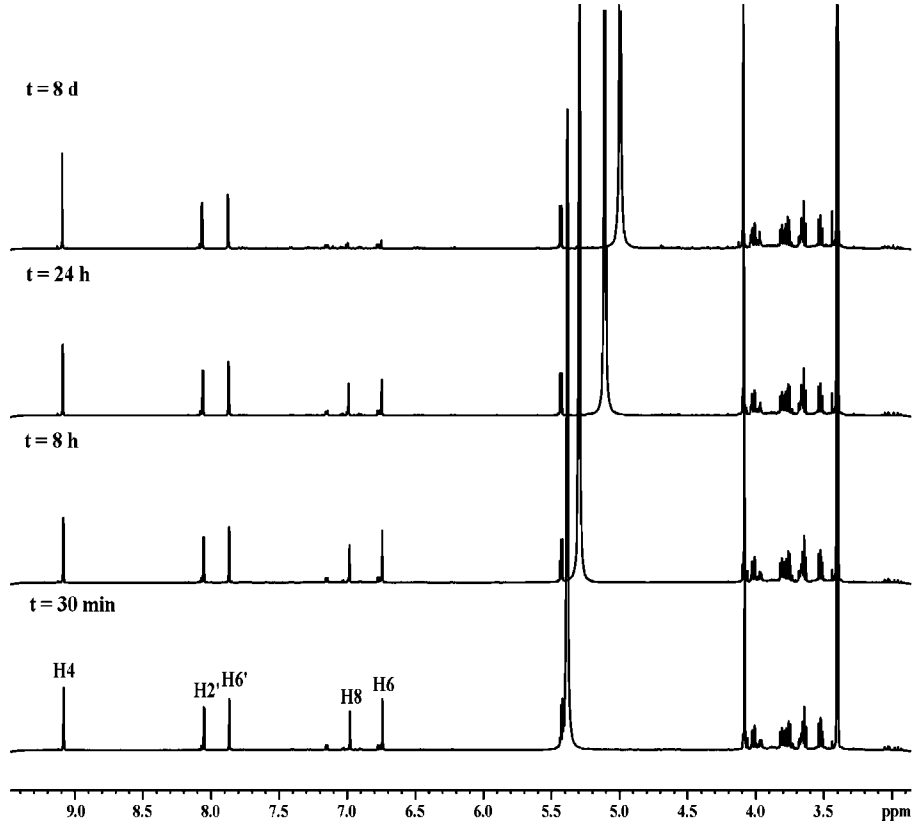

Figure 2. ^{1}H NMR (600 MHz) spectra of petunidin 3-*O*-β-glucopyranoside (**2**) (~10 mM) in CF_3CO_2D/CD_3OD (5:95, v/v) recorded after 30 min, 8 h, 24 h, and 8 days in darkness at room temperature. The protons of the petunidin moiety are labeled.

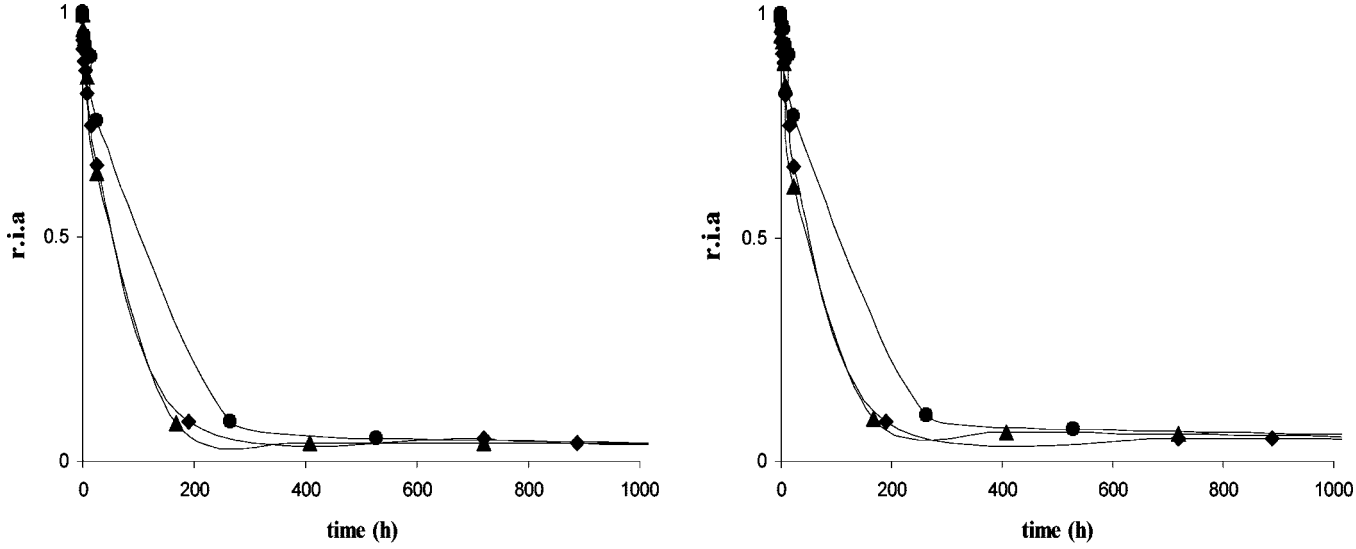

Figure 3. Relative integrated area (r.i.a.) of H-6 (left) and H-8 (right) in the ^{1}H NMR spectra of the flavylium forms of delphinidin 3-*O*-β-glucopyranoside (•) (**1**), petunidin 3-*O*-β-glucopyranoside (♦) (**2**), and malvidin 3-*O*-β-glucopyranoside (▲) (**3**) plotted against time (hours). The first 1000 h (~42 days) after sample preparation are shown in the figure.

reactions, as viewed by the flow of the relative integrated area (r.i.a.) vs time plots (**Figure 3**), appear to follow first-order kinetics. Since attempts to apply second-order rate equations failed, the observed reactions may not appear to be initiated by any form of stacking of the compounds when dissolved in the applied solvent mixtures. This conclusion is substantiated by the fact that the observed H → D exchange rates are independent upon the concentration of the substrates. Previously, it has been shown that C-4 substituted anthocyanins similar to the pyranoanthocyanins (**4**–**6**) exhibit higher resistance to bleaching by sulfur dioxide, higher color intensities, and restricted formation of hemiketals under weakly acidic to neutral solvent conditions as compared to analogous anthocyanidin 3-glucosides such as **1**–**3** (*21*, *24*, *39*). Contrary to the H → D exchange observed in the A-rings of **1**–**3**, pigments **4**–**6** in their flavylium cationic forms showed in the present study no aromatic H → D exchange for any of their A-ring protons (H-6 and H-8). The generalized scheme for H → D exchange reactions of the aromatic A-ring hydrogens of **1**–**3** are shown in **Figure 6**. Apparently, the oxygen atom (6-O) in the D-ring of **4**–**6** does not have the same electron donating/mesomeric effects on C-6 and C-8 as the corresponding 5-OH group in **1**–**3**.

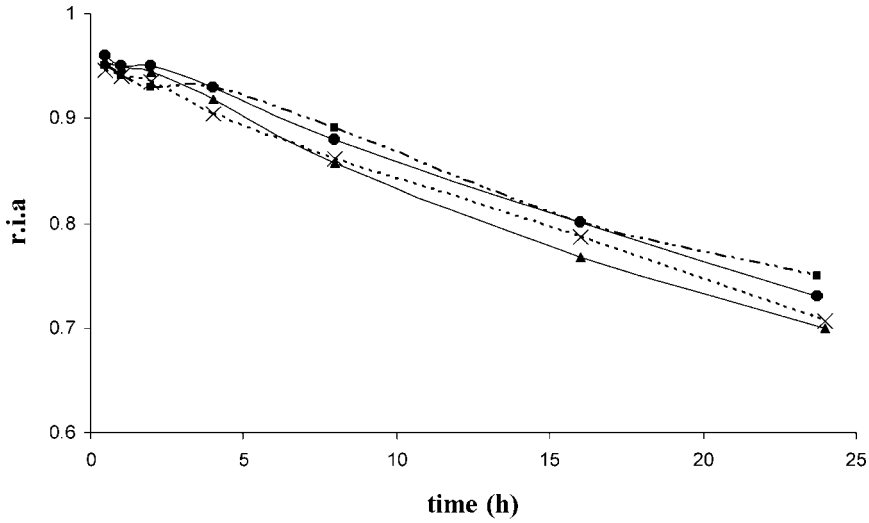

Figure 4. Relative integrated area (r.i.a.) of the H-6 and H-8 resonances in the ^{1}H NMR spectra of the flavylium cation form of petunidin 3-*O*-β-glucopyranoside (**2**) in pure CD_3OD at 298 K: H-8 (•) and H-6 (■) in Wilmad Pyrex glass, H-8 (▲) and H-6 (×) in Norell N-51A glass.

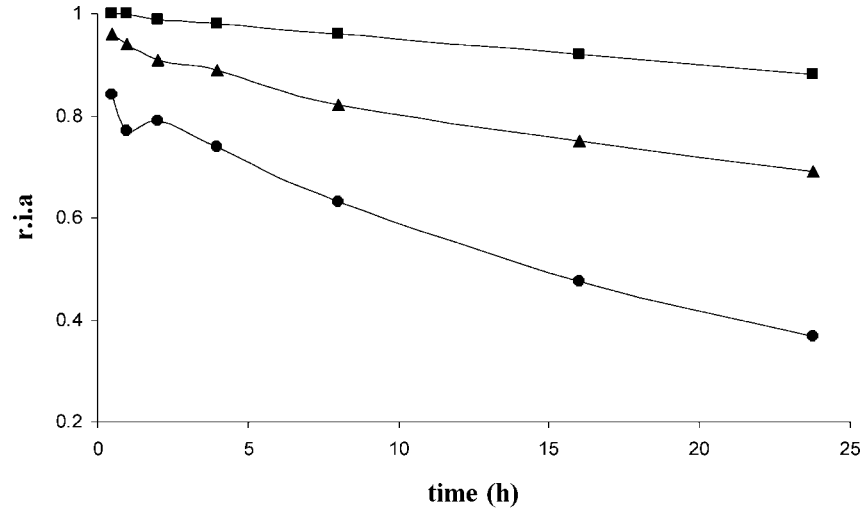

Figure 5. Comparison of the relative integrated area (r.i.a.) of proton resonances in the ^{1}H NMR spectra of H-β in carboxypyranopetunidin 3-*O*-β-glucopyranoside (~10 mM) (**5**) (•), H-8 in petunidin 3-*O*-β-glucopyranoside (~10 mM) (**2**) (▲), and H-8 in rutin (~10 mM) (**7**) (■) as a function of time in hours.

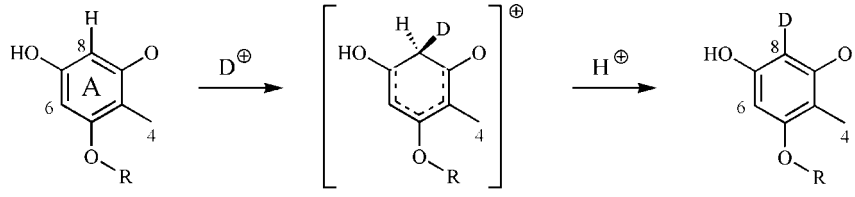

Figure 6. Generalized scheme showing the proposed mechanism for the deuterium exchange of H-8 of the A-ring of anthocyanins (**1**–**3**) and the flavonol rutin (**7**). A similar scheme may represent the corresponding exchange of H-6 in **1**–**3**.

Similar exchange studies were performed with quercetin 3-rutinoside, **7**. When this flavonol, with similar A-ring as **1**–**6**, was dissolved in CF_3CO_2D/CD_3OD (5:95, v/v), the H → D exchange rates of the aromatic A-ring protons were reduced compared to the rates of the corresponding hydrogens of anthocyanins (**1**–**3**) (**Table 1**). However, the H → D exchange of H-8 in rutin was significantly higher than the corresponding rate of H-6; 12% and 2% reduction of signal responses in the NMR spectra after 24 h, respectively. This preference for an exchange reaction at C-8 (**Figure 6**) suggests that this position may have a more positive charge than C-6 in the postulated intermediate, the σ-complex, leading to higher stability of the σ-complex involved in the exchange of H-8. No similar difference between the H → D exchange rates of H-8 and H-6 was observed for the anthocyanins (**1**–**3**).

One notable result in the present study is the observation that the H → D exchange rates at the C-6 and C-8 positions in **2** (**Tables 1** and **2**) are essentially the same in pure CD_3OD and in CD_3OD containing 5% or 15% CF_3CO_2D (v/v), corresponding to CF_3CO_2D being 0.7 or 2.1 M, respectively. Although $[D^+]$ will be significantly less than 0.7 and 2.1 M in these mixtures, particularly since acids are known to be less dissociated in methanol than in water (pK_a of CF_3COOH in H_2O is ~0.0), the present data seem to indicate that the H → D exchange reactions of this type of anthocyanins are independent upon the concentration of D^+. H → D exchange in aromatic

compounds, in deuterated water and alcohols, even when substituted with donor substituents, as in 1,3,5-trimethoxybenzene, are known to proceed only extremely slowly at room temperature when no acids are present (*40*–*42*). Generally, these reactions (when measurable) take place with an early transition state forming a Wheland (Pfeiffer) intermediate (*43*), presumably through a nonplanar aromatic ring with some sp^3- hybridization of the *ipso*-carbon atom. One may speculate whether such intermediates may be possible, allowing for H → D exchange of A-ring hydrogens in anthocyanins, without being influenced by the presence of acid.

Numerous anthocyanins have so far been identified (*1*, *9*), but the impact of their structure on reactivity, metabolism, and functions of the various anthocyanins is only rudimentarily known. The electron density on the aromatic rings, enhanced by the electron-donating oxygen functions, generates differences in the electron density at particular sites in the various anthocyanidins. Specific positions will thus be more susceptible to react with either basic or acidic compounds. As outlined above, structural differences between anthocyanins and pyranoanthocyanins may lead to different reactivity in electrophilic aromatic substitution reactions as revealed by the observed deuterium exchange rates of only some of the aromatic hydrogens.

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