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Characterization of Hemiacetal Forms of Anthocyanidin 3-*O*-β-Glycopyranosides

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The 3-*O*-β-glucopyranosides of delphinidin, petunidin, and malvidin (**1**−**3**) and cyanidin 3-*O*-β-galactopyranoside (**4**) dissolved in deuterated methanolic solutions without and with acid (5%, CF_3-COOD) were identified by homo- and heteronuclear NMR techniques. The hemiacetal forms of all the four anthocyanins were characterized as two epimeric 2-hydroxy-hemiacetals on the basis of assignments of both proton and carbon NMR signals together with chemical shift considerations. This is the first report of ^{13}C NMR assignments of two epimeric anthocyanin hemiacetal forms. No 4-hydroxy-hemiacetal form was detected for any of the pigments. For each anthocyanin dissolved in deuterated methanol, the equilibrium between each of the two epimeric hemiacetals and the corresponding flavylium cation was confirmed by the observed positive exchange cross-peaks in the 2D ^1H NOESY spectra. The molar proportions of the flavylium cation and the two hemiacetals of **1**−**4** in deuterated methanol were very similar for all pigments, even during storage for weeks. The majority of the anthocyanins reported to occur in fruits have the same or similar structures as **1**−**4**. These pigments have been proposed to exist predominantly as hemiacetals in slightly acidic to neutral solvents, which is a relevant pH range in plants and in the human gastrointestinal tract.

KEYWORDS: Anthocyanidin 3-*O*-β-monoglycopyranosides; hemiacetal forms; equilibrium proportions; ^1H and ^{13}C NMR assignments

INTRODUCTION

Anthocyanins are responsible for most red to blue colors of plants (*1*). These water-soluble pigments are considered to occur in several equilibrium forms. Thermodynamic and kinetic studies have led to a generally accepted scheme with respect to the different transformations of the flavylium cation of simple anthocyanins under various pH conditions (*2*−*9*). The flavylium cation is the predominant equilibrium form in strongly acidic solutions. In addition, the existence of tautomeric quinonoidal bases derived from the flavylium cation by deprotonation, and hemiacetal and chalcone forms related to the flavylium form by nucleophilic reaction with water, have been described. The colorless OH adducts known as carbinol bases, pseudobases, or hemiacetals have previously been examined using pH-jump methods, UV−visible and fluorescence spectroscopy (e.g., ref *9*), and in a few cases NMR spectroscopy (*10*−*14*). However, accurate experimental proofs for the anthocyanin hemiacetal structures are very limited. By observing changes in the ^1H NMR spectra of malvidin 3-glucoside dissolved in a CD_3CN/D_2O mixture (1:4, v/v) upon variation in temperature and acidity of the solvent, it has been possible to tentatively assign ^1H NMR chemical shifts considered to be 2-OH and 4-OH hemiacetal forms (*10*). No discrimination between the various NMR signals related to the two 2-OH hemiacetal forms was presented. Santos et al. (*12*) studied various forms of malvin which coexist in slightly acidic aqueous conditions. Two-dimensional NOESY exchange correlation NMR spectra provided valuable information about the structural transformations, including evidence for the relationship between various equilibrium forms; however, there is insufficient information in this paper to determine which proton resonance arises from which hemiacetal form (*12*). The diacylated anthocyanin, malvidin 3-(*p*-coumarylglucoside)-5-acetylxyloside, has been found to exist as a mixture of the flavylium form and one pseudobase form in DMSO/d_6-CF_3-COOD (9:1) (*13*). This is the only anthocyanin hemiacetal form reported with ^{13}C NMR assignments; however, no indication of the existence of two epimeric hemiacetals was presented.

Recent technological advances in development of high-field magnets and cryoprobe technology have improved the resolution and sensitivity of NMR techniques, which have increased the possibilities of accurate assignments of proton and carbon atoms of various anthocyanin equilibrium forms occurring in mixtures using a combination of 1D and 2D NMR techniques. Thus, in this work NMR assignments of all proton and carbon atoms of individual equilibrium forms of the 3-glucosides of delphinidin, petunidin, and malvidin, **1**−**3**, and cyanidin 3-galactoside, **4**, in CD_3OD have been used to characterize the structures of the hemiacetal forms of **1**−**4**. The structures of anthocyanin **1**−**4** are representative for the majority of anthocyanidin 3-monoglycosides found in fruits and vegetables (*15*). These pigments have been proposed to occur mainly as hemiacetals in slightly acidic

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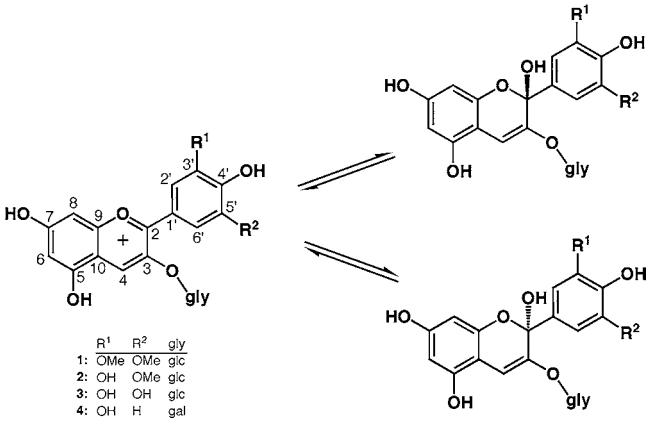

Figure 1. Hemiacetal structures of **1**, malvidin 3-*O*-β-glucopyranoside; **2**, petunidin 3-*O*-β-glucopyranoside; **3**, delphinidin 3-*O*-β-glucopyranoside, and **4**, cyanidin 3-*O*-β-galactopyranoside. For each anthocyanin, **1**–**4**, the equilibrium between the two hemiacetal forms and the corresponding flavylium form was observed by exchange cross-peaks in the 2D ^{1}H NOESY spectra.

to neutral solvents (*9*, *16*). For each anthocyanin, **1**−**4**, the equilibrium between the hemiacetal forms and the corresponding flavylium form (**Figure 1**), was observed by exchange cross-peaks in the 2D ^{1}H NOESY spectra (**Figure 4**). The molar proportions of the various equilibrium forms were determined by integration of signals in the ^{1}H NMR spectra (**Figure 2**).

MATERIALS AND METHODS

Plant Material. Dried black beans (*Phaseolus vulgaris* L.) were purchased from a local food shop, Helios (Bergen, Norway). On-line HPLC analysis of the crude extract showed three major anthocyanins, corresponding to malvidin 3-glucoside (**1**), petunidin 3-glucoside (**2**), and delphinidin 3-glucoside (**3**) (*17*). Black chokeberry (*Aronia melanocarpa* Michx. Ell.) was collected in August 2004 in Bergen, Norway, and immediately stored at −20 °C. Pre-analysis of the crude chokeberry extract by HPLC revealed the 3-galactoside (**4**) (major pigment), 3-arabinoside, 3-xyloside, and 3-glucoside of cyanidin, which are in accordance with the literature (*18*, *19*).

Chromatography. The dried black beans (3 kg) were soaked in approximately 3.5 L of water containing 0.5% trifluoroacetic acid (TFA) (Merck, Darmstadt, Germany) at 4 °C for 24 h. Then the beans were extracted four times with 3.5 L of methanol containing 0.5% TFA for 24 h. After concentration under reduced pressure with a rotary evaporator, the combined concentrates were diluted with water to a total volume of 0.5 L before partition against ethyl acetate (Fisons, Loughborough, UK) (4 × 0.5 L). The water layer containing the anthocyanins was concentrated to 100 mL, and then it was loaded on a 70 × 5 cm Amberlite XAD-7 chromatography column. The column was first washed with 2.0 L of distilled water; then at neutral pH, the anthocyanins were eluted using methanol containing 0.5% TFA (1 L), dried under reduced pressure, and then freeze-dried. Approximately 10 g of XAD-7 purified bean material were applied on a 70 × 9.5-cm-i.d. preparative column, packed with Sephadex LH-20 (Amersham Bioscience, Uppsala, Sweden), using methanol (20%, v/v) and TFA (0.5%, v/v) in water. The flow rate was adjusted to 1000 mL/h. A total of thirty fractions (300−1000 mL) were collected on the basis of band separation, and the anthocyanins were eluted in the following order: malvidin 3-glucoside (**1**) (88% purity), petunidin 3-glucoside (**2**) (94%

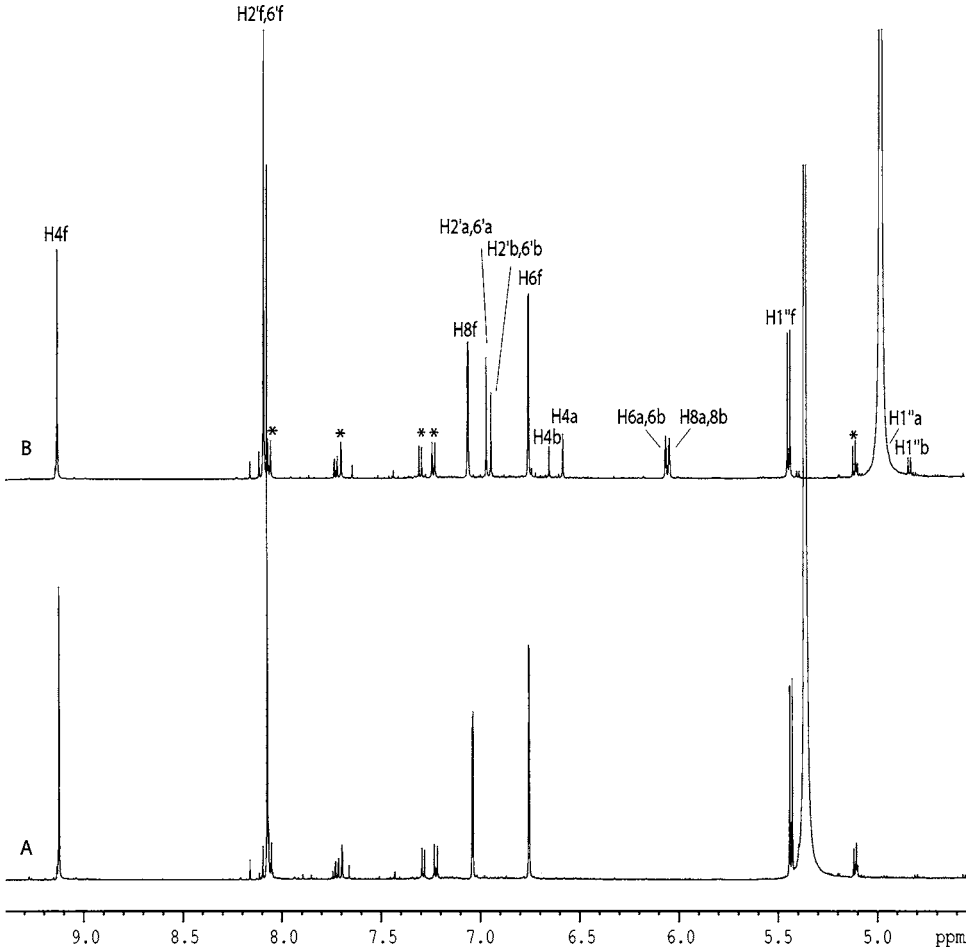

Figure 2. ^{1}H NMR spectra (600.13 MHz) of malvidin 3-*O*-β-glucopyranoside, **1** (concn about 11 mM), at 25 °C in (**A**) CF_3CO_2D/CD_3OD (5:95, v/v) and (**B**) CD_3OD. f = assignment of flavylium cation; a = assignment of hemiacetal **a** (major); b = assignment of hemiacetal **b** (minor); * = impurities.

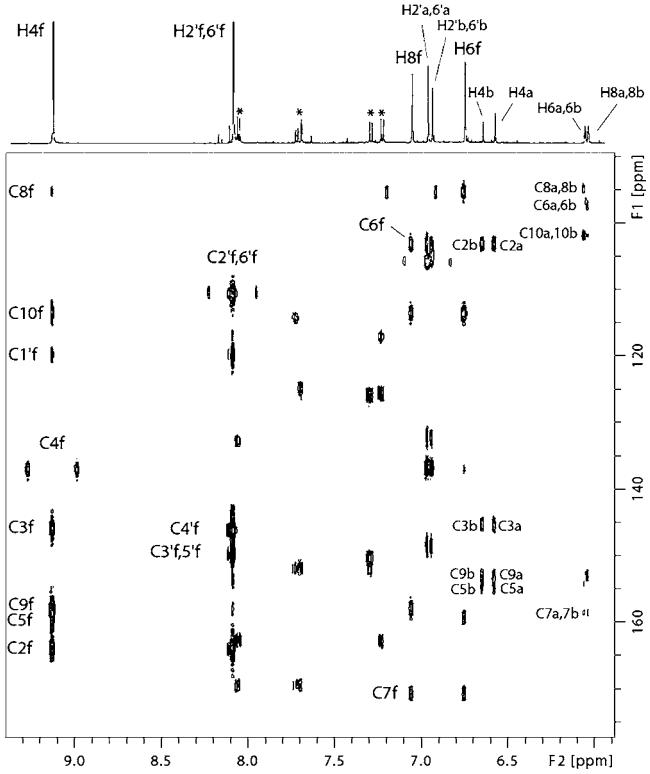

Figure 3. Expanded region of a HMBC spectrum (600.13 MHz) of malvidin 3-*O*-β-glucopyranoside (**1**) of about 11 mM at 25 °C in CD_3OD. f = assignment for the flavylium; a = assignment for the hemiacetal **a** (major); b = assignment for the hemiacetal **b** (minor); * = impurities. Top; ^{1}H NMR spectrum of malvidin 3-*O*-β-glucopyranoside (**1**).

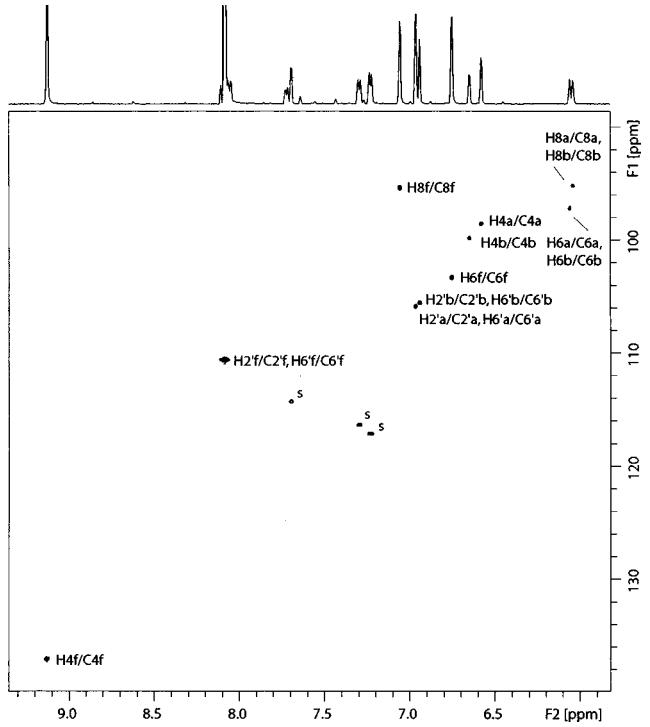

Figure 4. Expanded region of a HSQC spectrum (600.13 MHz) of malvidin 3-*O*-β-glucopyranoside (**1**) of about 11 mM at 25 °C in CD_3OD. f = assignment for the flavylium; a = assignment for the hemiacetal **a** (major); b = assignment for the hemiacetal **b** (minor); *s* = impurities. Top; ^{1}H NMR spectrum of malvidin 3-*O*-β-glucopyranoside (**1**).

purity), and delphinidin 3-glucoside (**3**) (70−80% purity). The purities were determined by HPLC.

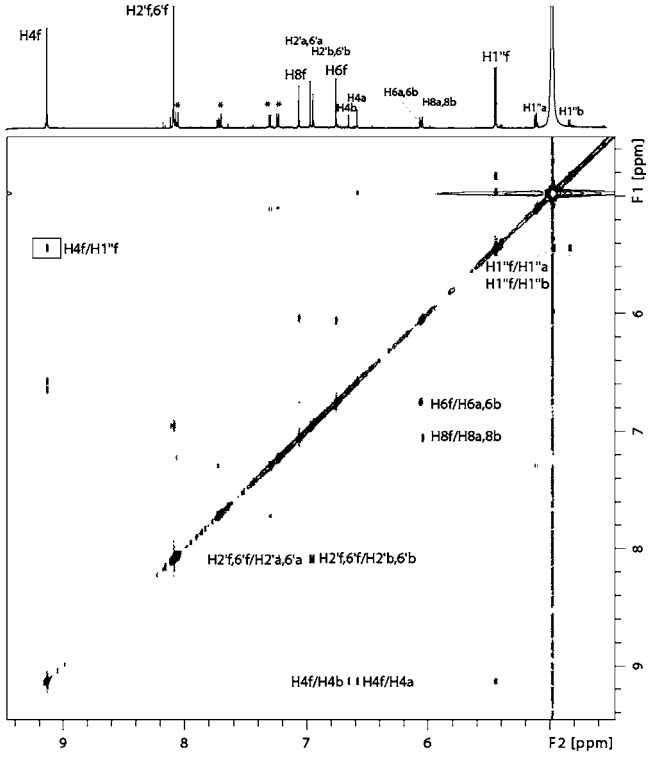

Figure 5. Expanded region of a NOESY spectrum (600.13 MHz) of malvidin 3-*O*-β-glucopyranoside (**1**) of about 11 mM at 25 °C in CD_3OD. A negative cross-peak due to NOE correlation between H-4 and H-1″ of the flavylium cation, locating the position of the monosaccharide to the aglycone, is enclosed in a box. Other labeled cross-peaks are positive and are caused by chemical exchange. f = assignment for the flavylium; a = assignment for the hemiacetal **a** (major); b = assignment for the hemiacetal **b** (minor); * = impurities. Top; ^{1}H NMR spectrum of malvidin 3-*O*-β-glucopyranoside (**1**).

Frozen black chokeberries were extracted four times with 2 L of methanol containing 0.5% TFA for 24 h. The extracts where concentrated under reduced pressure and purified with ethyl acetate and XAD-7. Cyanidin 3-galactoside (**4**) (77−97% purity) was isolated by Sephadex LH-20 column chromatography.

Analytical HPLC. The purity and identity of the fractions were checked with HPLC. The analytical HPLC system (Agilent 1100 Series, Waldbronn, Germany) was equipped with a HP 1050 diode-array detector (Hewlett-Packard), a 20-μL loop, and a 200 × 4.6-mm-i.d., 5-μm ODS Hypersil column (Supelco, Bellefonte, PA). Two solvents, A, water (0.5% TFA) and B, acetonitrile (0.5% TFA) were used for elution. The elution profile for HPLC consisted of initial conditions with 90% A and 10% B followed by gradient elution for 10 min (14% B), isocratic elution 10−14 min, and the subsequent gradient conditions: 18 min (16% B), 22 min (18% B), 26 min (23% B), 31 min (28% B), and 32 min (40% B), isocratic elution 32−40 min, gradient elution 40−43 min (10% B), and final isocratic elution 43−46 min (10% B). The flow-rate was 1.0 mL/min, and aliquots of 15 μL were injected with a Micro Autosampler (Agilent 1100 Series). The UV−vis absorption spectra were recorded on-line during HPLC analysis over the wavelength range 240−600 nm in steps of 2 nm. The purity of the fractions was based on integration data obtained from HPLC profiles monitored at 280 ± 10 nm, without taking into account the different molar absorption coefficients of the compounds.

NMR Spectroscopy. One-dimensional ^{1}H, ^{13}C compensated attached proton test experiment (CAPT), two-dimensional heteronuclear single quantum coherence (^{1}H−^{13}C HSQC), heteronuclear multiple bond correlation (^{1}H−^{13}C HMBC), double quantum filtered correlation (^{1}H−^{1}H DQF-COSY), total correlation (^{1}H−^{1}H TOCSY), and nuclear Overhauser effect (^{1}H−^{1}H NOESY) spectroscopy for pigments **1**−**4** were obtained at 600.13 and 150.90 MHz for ^{1}H and ^{13}C, respectively, on a Bruker 600 MHz instrument (Fällanden Switzerland) equipped

Table 1. ^{1}H (600.13 MHz) and ^{13}C (150.90 MHz) NMR Data for the Flavylium Cation Form of Malvidin 3-*O*-β-Glucopyranoside (**1**), Petunidin 3-*O*-β-Glucopyranoside (**2**), Delphinidin 3-*O*-β-Glucopyranoside (**3**), and Cyanidin 3-*O*-β-Galactopyranoside (**4**) in CD_3OD at 25 °C[a]

	1 ^{1}H δ (ppm), *J* (Hz)	**2** ^{1}H δ (ppm), *J* (Hz)	**3** ^{1}H δ (ppm), *J* (Hz)	**4** ^{1}H δ (ppm), *J* (Hz)	**1** ^{13}C δ (ppm)	**2** ^{13}C δ (ppm)	**3** ^{13}C δ (ppm)	**4** ^{13}C δ (ppm)
2					163.99	164.09	164.22	164.42
3					145.81	145.81	145.87	145.72
4	9.132 *d* 0.8	9.090 *d* 0.8	9.056 *d* 0.8	9.113 *d* 1.0	137.08	136.63	136.12	136.98
5					159.35	159.22	159.16	159.21
6	6.756 *d* 2.1	6.739 *d* 2.0	6.732 *d* 2.0	6.738 *d* 2.0	103.87	103.25	103.19	103.29
7					170.69	170.44	170.24	170.42
8	7.060 *dd* 0.7, 2.1	6.994 *dd* 0.7, 2.0	6.949 *dd* 0.9, 2.0	6.982 *dd* 1.0, 2.0	95.37	95.13	94.96	95.09
9					157.96	157.74	157.63	157.69
10					113.06	113.42	113.20	113.39
1′					119.87	119.96	120.01	121.27
2′	8.091 *s*	8.075 *d* 2.2	7.861 *s*	8.158 *d* 2.3	110.63	109.32	112.55	118.46
3′					149.78	149.77	144.78	147.41
4′					146.25	145.21	147.55	155.78
5′				7.105 *d* 8.8	149.78	147.51	144.78	117.41
6′	8.091 *s*	7.867 *dd* 0.6, 2.2	7.861 *s*	8.358 *dd* 8.8, 2.3	110.63	113.69	112.55	128.23
OMe	4.100 *s*	4.088 *s*			57.17	57.15		
1″	5.44 *d* 7.8	5.43 *d* 7.8	5.41 *d* 7.8	5.35 *d* 7.7	103.76	103.73	103.63	104.45
2″	3.73 *dd* 7.7, 9.2	3.76 *dd* 7.8, 9.0	3.79 *dd* 7.8, 9.1	4.08 *dd* 7.7, 9.6	75.04	74.94	74.76	72.07
3″	3.63 *t* 9.2	3.64 *t* 9.0	3.65 *t* 9.1	3.76 *dd* 9.6, 3.4	78.25	78.18	78.05	74.92
4″	3.49 *dd* 9.2, 9.9	3.51 *dd* 9.0, 9.9	3.54 *dd* 9.1, 9.8	4.04 *dd* 0.5, 3.4	71.22	71.15	71.04	70.10
5″	3.66 *m*	3.66 *m*	3.65 *m*	3.89 *m*	78.97	78.89	78.81	77.79
6A″	4.01 *dd* 2.2, 12.2	4.01 *dd* 2.3, 12.1	4.00 *dd* 2.3, 12.2	3.89 *m*	62.42	62.38	62.32	62.33
6B″	3.78 *dd* 6.3, 12.2	3.79 *dd* 6.3, 12.1	3.82 *dd* 6.3, 12.2	3.86 *m*	62.42	62.38	62.32	62.33

[a] *s*, singlet; *d*, doublet; *dd*, double doublet; *t*, triplet; *m*, multiplet. ^{13}C NMR data are obtained from the ^{13}C CAPT NMR spectra.

Table 2. ^{1}H (600.13 MHz) and ^{13}C (150.90 MHz) NMR Data for the Hemiacetal **a** (Major) Form of Malvidin 3-*O*-β-Glucopyranoside (**1**), Petunidin 3-*O*-β-Glucopyranoside (**2**), Delphinidin 3-*O*-β-Glucopyranoside (**3**), and Cyanidin 3-*O*-β-Galactopyranoside (**4**) in CD_3OD at 25 °C[a]

	1a ^{1}H δ (ppm), *J* (Hz)	**2a** ^{1}H δ (ppm), *J* (Hz)	**3a** ^{1}H δ (ppm), *J* (Hz)	**4a** ^{1}H δ (ppm), *J* (Hz)	**1a** ^{13}C δ (ppm)	**2a** ^{13}C δ (ppm)	**3a** ^{13}C δ (ppm)	**4a** ^{13}C δ (ppm)
2					103.11	103.82	103.19	103.10
3					145.39	145.47	145.55	145.48
4	6.582 *d* 0.8	6.579 *d* 0.7	6.581 *d* 0.6	6.610 *d* 0.7	98.57	98.64	98.78	98.63
5					154.57	153.26	154.31	154.59
6	6.065 *d* 2.2	6.051 *d* 2.0	6.038 *d* 2.2	6.044 *d* 2.2	97.19	97.07	96.98	97.05
7					158.4	158.4	158.4	158.4
8	6.046 *dd* 0.7, 2.2	6.022 *dd* 0.6, 2.2	5.999 *dd* 0.7, 2.2	5.999 *dd* 0.7, 2.2	95.10	95.13	95.14	95.09
9					152.95	153.17	153.17	153.19
10					101.75	101.69	101.76	101.77
1′					132.15	132.56	132.41	133.27
2′	6.968 *s*	6.857 *d* 1.9	6.700 *s*	7.105 *d* 2.2	105.90	103.81	107.37	115.59
3′					148.52	148.98	146.12	146.64
4′					136.81	135.22	134.47	146.56
5′				6.792 *d* 8.3	148.52	145.69	146.12	115.36
6′	6.968 *s*	6.795 *dd* 0.6, 2.0	6.700 *s*	7.011 *dd* 8.3, 2.2	105.90	109.81	107.37	119.77
OMe	3.908 *s*	3.909 *s*			56.69	56.67		
1″	4.97 *d* 7.8	4.95 *d* 7.8	4.93 *d* 7.8	4.90 *d* 7.7	102.11	102.98	102.22	102.73
2″	3.36 *dd* 7.7, 9.1	3.37 *dd* 7.8, 9.1	3.38 *d* 7.8, 9.1	3.69 *dd* 7.7, 9.8	74.78	74.57	74.53	71.89
3″	3.52 *t* 9.1	3.52 *t* 9.1	3.52 *m*	3.64 *dd* 9.8, 3.4	77.88	78.02	77.94	74.84
4″	3.44 *dd* 9.1, 9.9	3.45 *m*	3.47 *m*	3.97 *dd* 1.1, 3.4	71.25	71.09	70.88	70.05
5″	3.52 *m*	3.52 *m*	3.54 *m*	3.78 *m*	78.29	78.04	78.25	76.84
6A″	3.78 *m*	3.95 *m*	3.96 *m*	3.84 *m*	62.45	62.25	62.22	62.16
6B″	3.94 *m*	3.79 *m*	3.81 *m*	3.80 *m*	62.45	62.25	62.22	62.16

[a] s, singlet; *d*, doublet; *dd*, double doublet; *t*, triplet; *m*, multiplet. ^{13}C NMR data are obtained from the ^{13}C CAPT NMR spectra.

with a cryogenic probe. Sample temperatures were stabilized at 298 K. The deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvent (CF_3COOD/CD_3OD; 5:95, v/v) were used as secondary references (δ 49.0 and δ 3.40 from TMS, respectively).

RESULTS AND DISCUSSION

The 3-*O*-β-glucopyranosides of malvidin, petunidin, and delphinidin, **1**−**3**, were isolated from black beans (*Phaseolus vulgaris* L.), while cyanidin 3-*O*-β-galactopyranoside, **4**, was isolated from black chokeberry (*Aronia melanocarpa* Michx.). The anthocyanins, **1**−**4**, were dissolved in CD_3OD without and with CF_3COOD (5%, v/v). The structures of the various forms of each anthocyanin in the two solvents were determined on the basis of proton and carbon values assigned by a combination of 1D (^{1}H, ^{13}C-CAPT) and 2D (^{1}H−^{1}H COSY, ^{1}H−^{1}H TOCSY, ^{1}H−^{1}H NOESY, ^{1}H−^{13}C HSQC, ^{1}H−^{13}C HMBC) NMR spectroscopic techniques. In the acidified solvent only the flavylium cation forms of **1**−**4** were present, as expected.

Table 3. ^{1}H (600.13 MHz) and ^{13}C (150.90 MHz) NMR Data for the Hemiacetal **b** (Minor) Form of Malvidin 3-*O*-β-Glucopyranoside (**1**), Petunidin 3-*O*-β-Glucopyranoside (**2**), Delphinidin 3-*O*-β-Glucopyranoside (**3**), and Cyanidin 3-*O*-β-Galactopyranoside (**4**) in CD_3OD at 25 °C[a]

	1b ^{1}H δ (ppm), *J* (Hz)	**2b** ^{1}H δ (ppm), *J* (Hz)	**3b** ^{1}H δ (ppm), *J* (Hz)	**4b** ^{1}H δ (ppm), *J* (Hz)	**1b** ^{13}C δ (ppm)	**2b** ^{13}C δ (ppm)	**3b** ^{13}C δ (ppm)	**4b** ^{13}C δ (ppm)
2					103.60	102.75	103.11	102.96
3					145.27	145.47	145.39	145.53
4	6.651 *d* 0.8	6.646 *d* 0.7	6.636 *d* 0.6	6.658 *d* 0.7	99.79	99.72	98.57	99.59
5					153.17	154.40	154.57	154.44
6	6.059 *d* 2.2	6.055 *d* 2.0	6.047 *d* 2.2	6.049 *d* 2.2	97.13	97.30	97.19	97.10
7					158.4	158.4	158.4	158.4
8	6.043 *dd* 0.7, 2.2	6.028 *dd* 0.6, 2.3	6.016 *dd* 0.7, 2.2	6.017 *dd* 0.7, 2.2	95.25	95.29	95.29	95.25
9					154.11	152.52	152.95	153.19
10					101.89	101.78	101.75	101.92
1′					132.56	132.32	132.15	133.32
2′	6.944 *s*	6.814 *d* 1.9	6.678 *s*	7.106 *d* 2.2	103.60	107.24	105.90	115.48
3′					148.77	146.28	148.52	145.64
4′					135.48	134.56	136.81	146.66
5′				6.826 *d* 8.3	145.62	1476.28	148.52	115.45
6′	6.944 *s*	6.792 *dd* 0.6, 2.0	6.678 *s*	6.991 *dd* 8.3, 2.2	103.60	107.24	105.90	119.67
OMe	3.911 *s*	3.967 *s*			56.81		56.69	
1″	4.83 *d* 7.8	4.79 *d* 7.8	4.758 *d* 7.8	4.72 *d* 7.7	102.63	103.55	102.11	103.35
2″	3.34 *dd* 7.8, 9.2	3.34 *dd* 7.8, 9.3	3.34 *dd* 7.8, 9.4	3.66 *dd* 7.7, 9.8	74.64	74.66	74.78	71.89
3″	3.46 *t* 9.2	3.45 *t* 9.3	3.45 *t* 9.4	3.56 *dd* 9.8, 3.4	77.67	77.81	77.88	74.46
4″	*	3.49 *m*	*	3.95 *dd* 1.1, 3.4	70.93	70.95	71.25	69.99
5″	3.58 *m*	3.48 *m*	3.48 *m*	3.74 *m*	78.24	78.18	78.29	77.00
6A″	3.82 *m*	3.99 *m*	3.82 *m*	3.89 *m*	62.45	62.44	62.45	62.26
6B″	3.99 *m*	3.83 *m*	3.99 *m*	3.86 *m*	62.45	62.44	62.45	62.26

[a] s, singlet; *d*, doublet; *dd*, double doublet; *t*, triplet; *m*, multiplet. *Overlapped with another signal. ^{13}C NMR data are obtained from the ^{13}C CAPT NMR spectra.

However, in pure CD_3OD, several forms were found to represent each of the four anthocyanins.

Hemiacetal Forms of Malvidin 3-*O*-β-Glucopyranoside (1). In the downfield region of the ^{1}H NMR spectrum of pigment **1** dissolved in pure CD_3OD, more than twelve aromatic proton signals were present. Four of these had similar chemical shift values and coupling constants to the four aromatic proton signals representing the flavylium cation form of **1** in CF_3COOD/CD_3OD (5:95, v/v) (**Figure 2**). These signals, including a singlet at δ 9.132 (H-f4), two 2H *meta*-coupled protons at δ 6.756 (*d*, 2.1 Hz; H-f6) and δ 7.060 (*dd*, 0.7, 2.1 Hz; H-f8), and a singlet at δ 8.091 (H-6′f/H-2′f), showed relatively higher intensities than the other signals present in this region of the spectrum. A methoxy singlet at δ 4.100 integrating for six protons and the observed cross-peaks in the HMBC and HSQC spectra of **1** (**Figures 3** and **4**) were particularly useful for complete assignments of the protons and carbons of the flavylium cation form of malvidin.

The relationship between the proton resonances of the flavylium cation and other signals in the downfield region were revealed by exchange cross-peaks in the 2D ^{1}H−^{1}H NOESY NMR spectrum of **1** dissolved in pure CD_3OD (**Figure 5**). Two more anthocyanidin forms (**a** and **b**) in addition to the flavylium cation form were thus recorded. Starting with the H-4f/H-4a exchange cross-peak at δ 9.13/6.58, which is used to assign H-4a, the proton and carbon chemical shifts of anthocyanidin **a** (the major form) were thereafter assigned. The cross-peaks at δ 6.58/103.1 (H-4a/C-2a), δ 6.58/154.6 (H-4a/C-5a), δ 6.58/152.9 (H-4a/C-9a), and δ 6.58/145.4 (H-4a/C-3a) in the HMBC spectrum of **1** (**Figure 3**) were used to assign C-2a, C-5a, C-9a, and C-3a, respectively. Similarly, the carbons belonging to the B-ring of this anthocyanidin form were assigned from the cross-peaks at δ 6.97/ 103.1 (H-2′a,6′a/C-2a), δ 6.97/136.8 (H-2′a,6′a/C-4′a), δ 6.97/148.5 (H-2′a,6′a/C-3′a,5′a), δ 6.97/132.2 (H-2′a,6′a/C-1′a), δ 6.97/105.9 (H-2′a,6′a/C-2′a,6′a), and δ 3.91/148.5 (−OCH_3-a/C-3′a,5′a), while the rest of the A-ring carbons were identified from the cross-peaks at δ 6.046/158.4 (H-8a/C-7a), δ 6.046/101.8 (H-8a/C-10a), δ 6.046/97.2 (H-8a/C-6a), δ 6.065/158.4 (H-6a/C-7a), δ 6.065/154.6 (H-6a/C-5a), δ 6.065/101.8 (H-6a/C-10a), and δ 6.065/95.1 (H-6a/C-8a). The chemical shift of C-4a was assigned by the cross-peak at δ 6.58/98.6 (H-4a/C-4a) in the HSQC spectrum.

The remaining problem was to address the structural differences between the flavylium cation and anthocyanidin **a**. The most apparent difference between the ^{1}H NMR spectra of these two forms consisted of the 2.55 ppm upfield shift of H-4 of anthocyanidin **a** (**Figure 2**), which was the major structural dissimilarity between the two forms to the C-ring. This upfield shift of H-4 indicated a lower conjugation of anthocyanidin **a** compared to the flavylium cation, in accordance with a hemiacetal. Likewise the outstanding 60.9 and 38.5 ppm upfield shifts of C-2 and C-4 of anthocyanidin **a** compared to the flavylium cation (**Figure 3**) was consistent with anthocyanidin **a** as the 2-hydroxy hemiacetal form. The assignment of the 2-hydroxy hemiacetal instead of the potential 4-hydroxy analogue was also supported by theoretical predictions (using the program ChemDraw Ultra, v.9.0) of chemical shifts of C-2 and C-4, when both the 2-OH hemiacetal and 4-OH hemiacetal were considered.

In an analogous manner the ^{1}H and ^{13}C chemical shifts of anthocyanidin **b** (minor form) were assigned, starting from the H-4f/H-4b exchange cross-peak at δ 9.13/6.65 (**Figure 5**). The small differences between the chemical shifts of hemiacetal **a** and hemiacetal **b** (**Tables 2** and **3**) were in accordance with two epimeric 2-OH hemiacetal forms. This is the first report of ^{13}C NMR assignments of two epimeric anthocyanin hemiacetal forms.

The sugar region of the ^{1}H NMR spectrum of **1** revealed three sugar signals (δ 5.45, δ 4.97, and δ 4.83) with similar anomeric coupling constants (7.8 Hz). The most downfield anomeric signal showed highest intensity and was assigned to the flavylium cation (**f**) by the HMBC cross-peak at δ 5.44/145.3 (H-f1″/C-f3). The NOESY spectrum of **1** revealed exchange cross-peaks at δ 4.97/5.44 (H-1″a/H-f1″) and δ 4.83/5.44 (H-1″b/H-f1″) between the anomeric signal of the flavylium cation

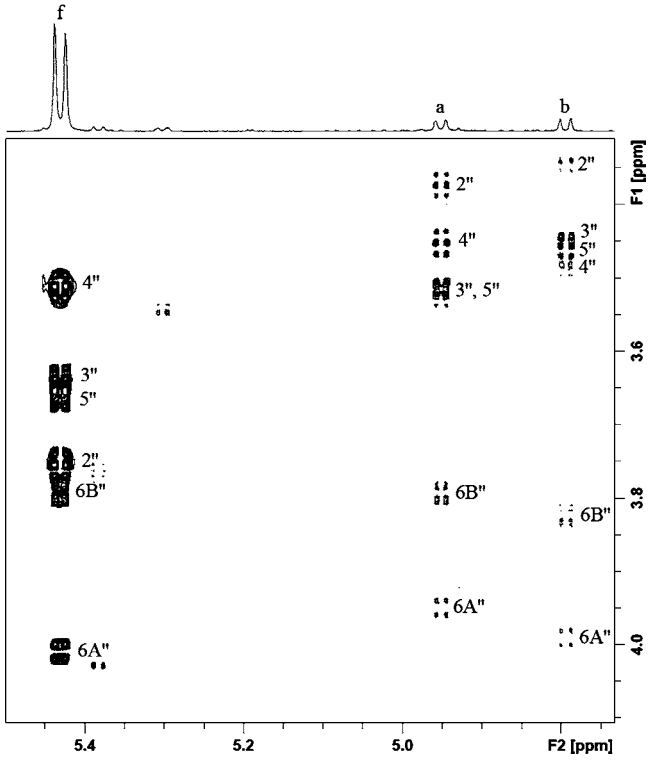

Figure 6. ^{1}H–^{1}H TOCSY NMR spectra (600.13 MHz) of the sugar region of petunidin 3-*O*-β-glucopyranoside, **2** (concn about 11 mM), at 25 °C in CD_3OD. f = flavylium cation; a = hemiacetal **a** (major); b = hemiacetal **b** (minor).

and the two other anomeric signals. The HMBC spectrum of **1** confirmed that the sugars units of the two 2-OH hemiacetals were connected to the respective aglycone 3-positions. The ^{1}H and ^{13}C NMR shift assignments and coupling constants (**Tables 2** and **3**) of the three monosaccharides were all in accordance with β-glucopyranose. Thus, anthocyanidins **a** and **b** were characterized as two epimeric 2-OH hemiacetal forms of malvidin 3-*O*-β-glucopyranoside.

Hemiacetal Forms of Anthocyanins 2−4. Analogous to the resonances in the downfield region of the ^{1}H NMR spectrum of **1**, pigments **2**−**4** dissolved in pure CD_3OD were represented by aromatic proton signals assigned to the flavylium cation forms of petunidin, delphinidin, and cyanidin, respectively. For each of these pigments, analogous to the NMR spectra of **1**, the relationship between the resonances of the flavylium cation forms and resonances belonging to two other forms were revealed by exchange cross-peaks in the respective 2D ^{1}H NOESY spectrum. Thereafter, the elucidation of the flavylium cation and the major and minor hemiacetal forms of petunidin 3-*O*-β-glucopyranoside (**2**), delphinidin 3-*O*-β-glucopyranoside (**3**), and cyanidin 3-*O*-β-galactopyranoside (**4**) were based on similar assignments (**Tables 1**−**3**) and reasoning as done for pigment **1**. **Figure 6** shows a ^{1}H-^{1}H TOCSY spectrum of the sugar signals for the flavylium form and the two hemiacetal forms (**a**, **b**) for pigment **2**. The differences between the chemical shifts of the protons and carbons of the flavylium cation and the hemiacetal forms, were remarkably similar for all four anthocyanins, **1**−**4**, regardless of the nature of the anthocyanidin (**Figure 7**).

Regular consumption of fruits, vegetables, jams, preserves, soft drinks, and wines ensure intake of anthocyanins in our diet, which is associated with probable reduced risks for various diseases (*20*). Interests in these pigments are also related to their role as food colorants (*1*, *16*). The current studied pigments,

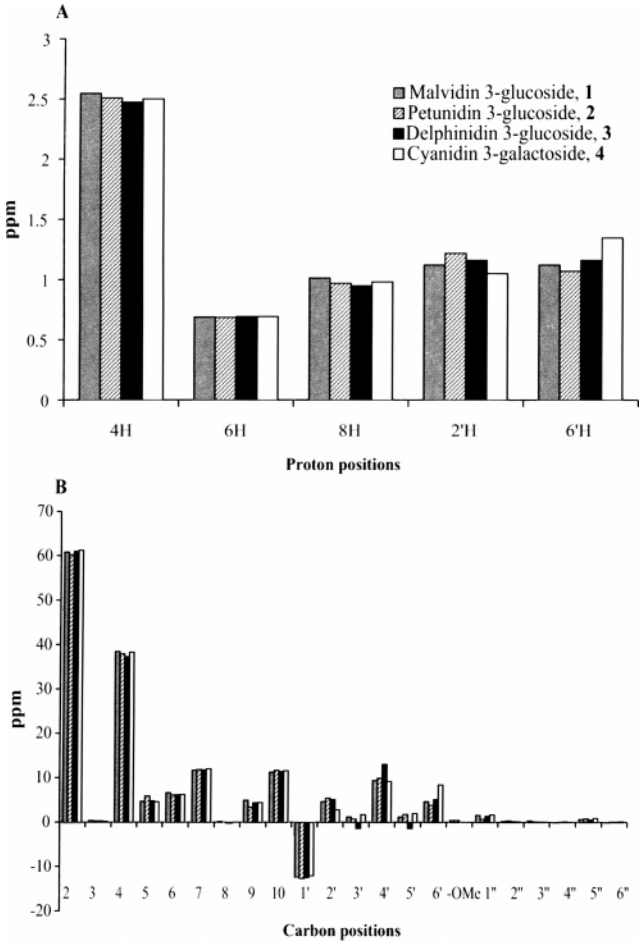

Figure 7. A. ^{1}H NMR chemical shift differences (ppm) between the flavylium form and hemiacetal **a** (major) form of pigments **1**–**4** for selected positions of the anthocyanin aglycones. **B.** ^{13}C NMR chemical shift differences (ppm) between the flavylium form and hemiacetal **a** (major) form of pigments **1**–**4** for all of the carbons in the anthocyanins.

1−**4**, include four of the six most common anthocyanidins (delphinidin, petunidin, malvidin, and cyanidin) reported to be found in plants, and glucose (in pigment **1**−**3**) and galactose (pigment **4**) are the most-frequent and third-most-frequent monosaccharide identified in anthocyanins (*1*). The structures of **1**−**4** are representative for the major intake of anthocyanins in our diet (*15*). Under slightly acidic to neutral conditions, which is a relevant pH range for in-vivo conditions in plants and in the human gastrointestinal tract, this type of anthocyanins has previously been considered to occur predominantly as hemiacetals (*9*, *16*).

There exists only limited information about elucidation of anthocyanin hemiacetal structures in the literature (*10*−*14*). Under in-vivo conditions, simple anthocyanins like **1**−**4** occur on several equilibrium forms, of which some are unstable even during a short time of storage (*21*). Our observation of reduced solubility of anthocyanins in aqueous solutions compared to alcoholic solutions may be another limiting factor. In the present studies, the anthocyanins (**1**−**4**) have been dissolved in deuterated methanol, which has facilitated full assignments of chemical shifts of both the protons and carbons of two epimeric 2-hydroxy hemiacetals of each of these four anthocyanins (**Figure 1**). In the NOESY NMR spectra we have observed negative exchange cross-peaks between the hemiacetals and the flavylium cation form, indicating that the two epimeric hemiacetal forms are in equilibrium with the same flavylium cation

Table 4. Proportions (%) of the Flavylium Cation/Hemiacetal **a**/Hemiacetal **b** Recorded by Integration of ^{1}H NMR Spectra of Malvidin 3-*O*-β-Glucopyranoside (**1**), Petunidin 3-*O*-β-Glucopyranoside (**2**), Delphinidin 3-*O*-β-Glucopyranoside (**3**), and Cyanidin 3-*O*-β-Galactopyranoside (**4**) Stored in CD_3OD for 24 h at 25 °C

	flavylium cation	hemiacetal **a**	hemiacetal **b**
1	75	15	10
2	79	12	9
3	82	10	8
4	67	20	13

form. It was not possible to detect similar exchange cross-peaks between the two epimeric hemiacetal forms for any of the four pigments. It is also interesting to note that the molar ratio between the flavylium cation, hemiacetal **a,** and hemiacetal **b** were nearly similar for all the four examined pigments after 24-h storage (**Table 4**). These ratios were constant for weeks. The equilibrium proportions between the flavylium cation form and the two hemiacetal forms is hereby proposed to be similar for anthocyanidin 3-monoglycosides, regardless of the nature of the anthocyanidin or monosaccharide, at least in deuterated methanol.

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