Anthocyanins from Maize (*Zea mays*) and Reed Canarygrass (*Phalaris arundinacea*)

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Flowers of maize, *Zea mays*, and reed canarygrass, *Phalaris arundinacea*, contain the same anthocyanins: cyanidin 3-glucoside, cyanidin 3-(6″-malonylglucoside), cyanidin 3-(3″,6″-dimalonylglucoside), peonidin 3-glucoside, peonidin 3-(6″-malonylglucoside), and peonidin 3-(dimalonylglucoside). The latter pigment has previously not been reported to occur in plants. Structure elucidations were primarily based on homo- and heteronuclear two-dimensional NMR and electrospray MS. During the isolation procedure using various mixtures of H_2O, CF_3CO_2H, and CH_3OH, and during storage in NMR solvent (CF_3CO_2D/CD_3OD; 1:19, v/v) methyl esterification of the free acid function of the malonyl units of the pigments occurs. The acylated anthocyanins constitute more than 80% and 40% of the anthocyanins in *P. arundinacea* and *Z. mays*, respectively. Flowers and leaves of maize, *Zea mays*, contain the same anthocyanins in nearly equal relative proportions.

Keywords: *Maize; Zea mays; reed canarygrass; Phalaris arundinacea; anthocyanins; cyanidin 3-(3″,6″-dimalonylglucoside); peonidin 3-dimalonylglucoside*

INTRODUCTION

The anthocyanin content of maize, *Zea mays* (family Poaceae), has been an important parameter in a number of papers dealing with, for instance, genetic and developmental control of anthocyanin biosynthesis (*1*), effects of N-, P- and K-deficiencies on light-dependent anthocyanin formation (*2*), induction of pink pigmentation by abscisic acid (*3*), high-irradiance responses induced by far-red light (*4*), physiological responses to enhanced UV−B radiation (*5*), DNA protection from induction of damage caused by UV radiation (*6*), and promotion of haustoria formation in the root parasite (*7*). The inheritance of anthocyanin pigmentation in *Z. mays* is well-documented (*8*, *9*).

Several publications have also dealt with the isolation and structure elucidations of anthocyanins in *Z. mays*, however, they present a rather divergent anthocyanin picture. Cyanidin 3-glucoside has been reported to occur in seed coats (*10, 11*), cobs (*12*), leaves (*13*), and throughout the plant (*14*). Lawanson and Osude (*15*) reported cyanidin 3-galactoside, rather than the analogous 3-glucoside, accompanied by pelargonidin 3-glucoside in the shoots of maize seedlings. Cyanidin 3-galactoside has also been suggested to occur in Peruvian dark-seeded corn, where it was reported to be acylated with *p*-coumaric acid (*16*), and in leaves of a *Z. mays* hybrid together with cyanidin-3-glucosides and free aglycon, cyanidin (*17*). Minor amounts of pelargonidin 3-glucoside and peonidin 3-glucoside were also identified in cobs of a Bolivian purple corn variety (*11*). Using a mild extraction procedure, FABMS, and cochromatography (TLC and HPLC), Harborne and Self (*13*) identified cyanidin 3-(6″-malonylglucoside) and cyanidin 3-dimalonylglucoside in reddened maize leaves. This latter pigment, for which determination of exact linkage points of the acyl groups is lacking, seems to accumulate in uninfected epidermal cells that surround restricted lesions on leaves of *Z. mays*, genetically resistant to the fungus *Bipolaris maydis* race O (*18*).

The aim of this work was to determine the individual anthocyanins in flowers and leaves of *Z. mays*. To simplify structure elucidation, flower tops of reed canarygrass, *Phalaris arundinacea*, (Poaceae), which are rich in the same type of anthocyanins as found in *Z. mays*, were also analyzed. Newer varieties of *P. arundinacea* with low levels of alkaloids can be used for pasture and hay. During the analysis a new dimalonylated peonidin 3-glucoside was detected in both *P. arundinacea* and *Z. mays*.

MATERIALS AND METHODS

Plant Material. Flower tops of reed canarygrass, *P. arundinacea*, were collected in Arna, near Bergen, Norway, in August 1998. Leaves and flowers of maize, *Z. mays*, were collected near Exeter, England in July 1998. Voucher specimens have been deposited in Bergen.

Isolation of Pigments. Flower tops (500 g) of *P. arundinacea* were extracted with 1% TFA in MeOH at 5 °C. The filtered extract was concentrated under reduced pressure, purified by partition (several times) against EtOAc, and applied to an Amberlite XAD-7 column (*19*). The anthocyanins were further purified by Sephadex LH-20 column chromatography using gradient elution (MeOH/TFA/H_2O; 19.8:0.2:80.0 (v/v) to MeOH/TFA/H_2O; 59.4:0.6:40.0 v/v). Individual pigments were separated by preparative HPLC according to previously published procedures (*20*).

Analytical Chromatography. Analytical HPLC was performed with an ODS-Hypersil column (20 × 0.5 cm, 5 μm) using the solvents HCOOH/H_2O (1:9) (A) and HCOOH/H_2O/MeOH (1:4:5) (B). The elution protocol consisted of a linear gradient from 10% B to 100% B for 17 min, isocratic elution for the next 4 min (100% B), followed by a linear gradient from

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100% B to 10% B for 1 min. The flow rate was 1.2 mL min^{-1}, and 15-μL aliquots of samples were injected.

Spectroscopy. UV−Vis absorption spectra were recorded in 0.01% concentrated HCl in MeOH. Spectral measurements were made over the wavelength range 240−600 nm in steps of 2 nm. The NMR experiments were obtained at 600.13 and 150.92 MHz for ^1H and ^{13}C respectively, on a Bruker DRX-600 instrument equipped with a multinuclear inverse probe for all but the ^{13}C 1D Spin−Echo Fourier Transform (SEFT) experiments which were performed on a $^1H/^{13}C$ BBO probe. Sample temperatures were stabilized at 25 °C. The deuteriomethyl ^{13}C signal and the residual ^1H signal of the solvent (CF_3CO_2D/CD_3OD; 1:19, v/v) were used as secondary references (δ 49.0 and δ 3.4 from TMS, respectively). The NMR sample concentrations were approximately 2 mM, 10 mM, and 2 mM for compounds **3**, **3b**, and **5**, respectively. The SEFT experiment (*21*) was performed with 12 000 transients. The spectral width was 26 455 Hz. The one-bond proton−carbon shift correlations were established using phase-sensitive gradient-selected heteronuclear single quantum coherence (HSQC) (*21*). The experiment was optimized for a one-bond proton−carbon coupling constant of 145 Hz. 256 FIDs were recorded in t_1 and 2K data points were recorded in t_2, and 187 transients were collected for each t_1 increment. The spectral widths were 16 600 Hz in f_1 and 3687 Hz in f_2. The proton−carbon shift correlations by long-range coupling were established using the heteronuclear multiple bond correlations (HMBC) experiment (*21*). 512 FIDs were recorded in t_1 and 2K data points were recorded in t_2, and 128 transients were collected for each t_1 increment. The spectral widths were 25 063 Hz in f_1 and 3931 Hz in f_2. The one-bond proton−proton shift correlations were established using phase-sensitive gradient-selected double quantum filtered correlation spectroscopy (DQF−COSY) with solvent suppression (*21*). The experiment was optimized for a one-bond proton−proton coupling constant of 7.5 Hz. 256 FIDs were recorded in t_1 and 4K data points were recorded in t_2, and 128 transients were collected for each t_1 increment. The spectral width was 1517 Hz.

The mass spectra were obtained on a Micromass, Platform LCZ, using electrospray operated in the positive ion mode. The sample was extracted by ethanol, and separated by HPLC (Waters 2690) using an ODS-Hypersil column (20 × 0.5 cm, 5 μm) and the solvents $HCOOH/H_2O$ (1:19) (A) and $HCOOH/H_2O$/MeOH (1:9:10) (B). The elution protocol consisted of a linear gradient from 10% B to 100% B for 20 min followed by isocratic elution (100% B) for the next 4 min. The flow rate was 1.2 mL min^{-1}, while the inlet flow into the mass spectrometer was 100 μL min^{-1}. Cone voltages used for detection of $[M]^+$ and $[A]^+$ were 32 V and 70 V, respectively.

Cyanidin 3-(3″,6″-dimalonylglucopyranoside), 3. ^1H NMR: 9.06 (1H, s, H-4), 6.77 (1H, d, J = 1.9 Hz, H-6), 7.00 (1H, d, J = 1.9 Hz, H-8), 8.10 (1H, d, J = 2.3 Hz, H-2′), 7.11 (1H, d, J = 8.7 Hz, H-5′), 8.34 (1H, dd, J = 2.3, 8.7 Hz, H-6′), 5.48 (1H, d, J = 7.7 Hz, H-1″), 3.95 (1H, dd, J = 7.7, 9.5 Hz, H-2″), 5.24 (1H, t, J = 9.5 Hz, H-3″), 3.71 (1H, m, H-4″), 4.00 (1H, m, H-5″), 4.63 (1H, dd, J = 12.0, 2.1 Hz, H-6A″), 4.40 (1H, dd, J = 12.0, 6.9 Hz, H-6B″).

Cyanidin 3-(3″,6″-dimethylmalonylglucopyranoside), 3b. ^1H NMR: 9.06 (1H, s, H-4), 6.76 (1H, d, J = 2.0 Hz, H-6), 6.98 (1H, d, J = 2.0 Hz, H-8), 8.08 (1H, d, J = 2.3 Hz, H-2′), 7.10 (1H, d, J = 8.7 Hz, H-5′), 8.32 (1H, dd, J = 2.3, 8.7 Hz, H-6′), 5.47 (1H, d, J = 7.7 Hz, H-1″), 3.94 (1H, dd, J = 7.7, 9.4 Hz, H-2″), 5.24 (1H, t, J = 9.4 Hz, H-3″), 3.71 (1H, t, J = 9.4 Hz, H-4″), 3.99 (1H, ddd, J = 2.0, 6.7, 9.7 Hz, H-5″), 4.63 (1H, dd, J = 12.0, 2.0 Hz, H-6A″), 4.40 (1H, dd, J = 12.0, 6.7 Hz, H-6B″), 3.68 (2H, s, H-2‴), 3.86 (3H, s, OCH_3‴), 3.54 (2H, s, H-2′^{V}), 3.70 (3H, s, OCH_3′^{V}). ^{13}C NMR: δ 164.69 (C2), 145.35 (C3), 137.15 (C4), 159.3 (C5), 103.49 (C6), 170.62 (C7), 95.29 (C8), 157.84 (C9), 113.27 (C10), 121.17 (C1′), 118.45 (C2′), 147.52 (C3′), 155.96 (C4′), 117.42 (C5′), 128.48 (C6′), 103.40 (C1″), 72.77 (C2″), 79.69 (C3″), 69.36 (C4″), 75.70 (C5″), 65.10 (C6″), 168.12 (C1‴), 41.93 (C2‴), 169.01 (C3‴), 53.01 (OCH_3‴), 168.12 (C1′^{V}), 41.55 (C2′^{V}), 168.78 (C3′^{V}), 52.88 (OCH_3′^{V}).

Peonidin 3-(6″-malonylglucopyranoside), 5. ^1H NMR: 9.08 (1H, s, H-4), 6.78 (1H, d, J = 1.9 Hz, H-6), 7.03 (1H, dd, J = 0.8, 1.9 Hz, H-8), 8.31 (1H, d, J = 2.3 Hz, H-2′), 7.16 (1H, d, J = 8.7 Hz, H-5′), 8.37 (1H, dd, J = 2.3, 8.7 Hz, H-6′), 4.11 (3H, s, OCH_3′), 5.38 (1H, d, J = 7.7 Hz, H-1″), 3.75 (1H, dd, J = 7.7, 9.1 Hz, H-2″), 3.63 (1H, t, J = 9.1 Hz, H-3″), 3.50 (1H, t, J = 9.3 Hz, H-4″), 3.90 (1H, m, H-5″), 4.62 (1H, dd, J = 11.9, 2.1 Hz, H-6A″), 4.38 (1H, dd, J = 11.9, 7.2 Hz, H-6B″), 3.47 (2H, s, H-2′^{V}). ^{13}C NMR (shift values are recorded in the HSQC experiment): δ 137.1 (C4), 103.3 (C6), 95.2 (C8), 115.1 (C2′), 117.4 (C5′), 128.8 (C6′), 56.7 (OCH_3), 103.6 (C1″), 74.6 (C2″), 77.9 (C3″), 71.1 (C4″), 75.8 (C5″), 65.2 (C6″).

Table 1. Chromatographic and Spectral Data of the Anthocyanins Identified in *Phalaris arundinacea* and *Zea mays* (see Figure 1 for peak identification of 1−6[a]).

	HPLC		ESIMS		
compound	vis-max (nm)	$A_{440}/A_{vis-max}$ (%)	t_R (min)	$[M^+]$	$[A^+]$
1	520	29	9.18	449	287
2	520	28	11.90	535	287
3	522	29	13.12	621	287
3b	523	28	15.80	649	287
4	520	30	10.49	463	301
5	521	29	13.46	549	301
6	522	29	14.61	635	301

[a] **3b** = cyanidin 3-(3″,6″-di-methylmalonylglucoside); $[M^+]$ = molecular ion; $[A^+]$ = aglycon moiety ion.

RESULTS AND DISCUSSION

The acidic methanol extract of *P. arundinacea* was purified by partition against ethyl acetate followed by Amberlite XAD-7 column chromatography. The pigments were separated by Sephadex LH-20 column chromatography and semipreparative HPLC. Pure anthocyanins were checked for homogeneity by analytical HPLC (Table 1). The UV−Vis spectra of the anthocyanins of *P. arundinacea* recorded on-line during HPLC analysis showed visible maxima around 520 nm with A_{440}/A_{520} of 28−30% (Table 1), indicating 3-substituted cyanidin or peonidin derivatives. The lack of extended UV absorption and the relatively long retention time for some of the pigments compared to that of standard cyanidin 3-glucoside (Table 1), indicated the presence of some anthocyanins with aliphatic acyl groups.

Pigments **1**, **2**, and **4** co-chromatographed (HPLC) with authentic cyanidin 3-*O*-β-glucopyranoside, cyanidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside), and peonidin 3-*O*-β-glucopyranoside, respectively. Signals in their ESIMS spectra (Table 1) confirmed these structures.

The ^1H NMR spectrum of the main pigment, **3**, showed a one-proton singlet at δ 9.06 (H-4); a three-proton AMX system at δ 8.34 (*dd*, 2.3 Hz, 8.7 Hz, H-6′), δ 8.10 (*d*, 2.3 Hz, H-2′), and δ 7.11 (*d*, 8.7 Hz, H-5′); and a two-proton AX system at δ 7.00 (*d*, 1.9 Hz, H-8) and δ 6.77 (*d*, 1.9 Hz, H-6), which was in accordance with a cyanidin derivative. The number of signals and coupling constants in the sugar region corresponded to one β-glucopyranosyl moiety. After assignment of all the sugar ^1H resonances by the 2D DQF−COSY experiment, the pronounced downfield shift of H-3″ (δ 5.24), H-6A″ (δ 4.63), and H-6B″ (δ 4.40) revealed diacylation at the sugar 3″- and 6″-positions. During the isolation procedure and during storage in the NMR solvent (CF_3CO_2D/CD_3OD; 1:19, v/v), a derivative (**3b**) was formed. This derivative gave similar UV−Vis values as **3**, and **3b** had a retention time which was higher (Table 1). It also showed a very similar ^1H NMR spectrum, with the exception of additional resonances in the region δ 3.5−3.9 (see experimental data). All the ^1H and ^{13}C reso-

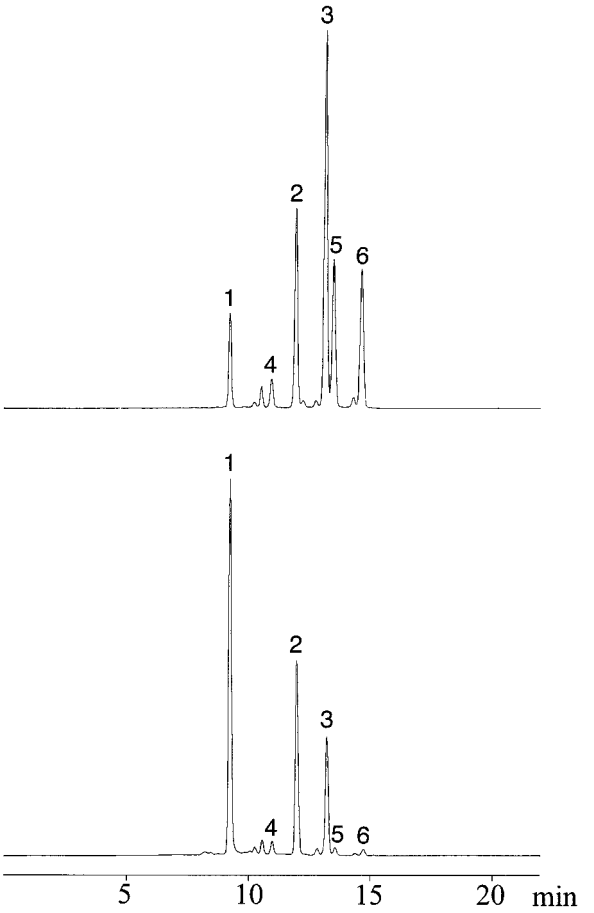
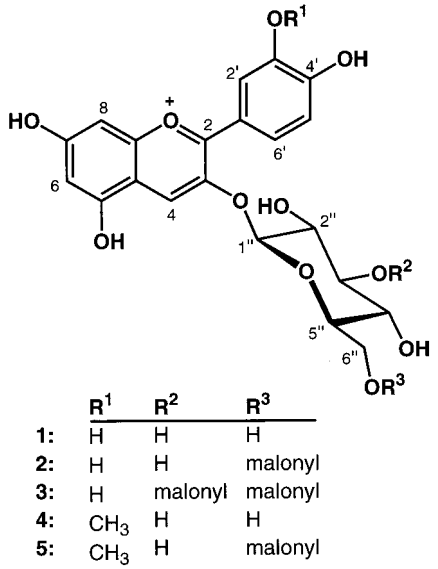


Figure 1. HPLC profiles of the crude anthocyanin extracts of flower tops of *Phalaris arundinacea* (top) and *Zea mays* (bottom) detected at 520 ± 20 nm. See Figure 2 for identification.



	R^1	R^2	R^3
1:	H	H	H
2:	H	H	malonyl
3:	H	malonyl	malonyl
4:	CH_3	H	H
5:	CH_3	H	malonyl

Figure 2. Structures of the anthocyanins identified in *Phalaris arundinacea* and *Zea mays*: Cyanidin 3-glucoside (**1**), cyanidin 3-(6″-malonylglucoside) (**2**), cyanidin 3-(3″,6″-dimalonylglucoside) (**3**), peonidin 3-glucoside (**4**), and peonidin 3-(6″-malonylglucoside) (**5**). Pigment **6** has been identified as peonidin 3-(dimalonylglucoside), without determination of the linkages between the malonyls and the glucose moiety.

Table 2. Relative Quantitative Anthocyanin Content in *Phalaris arundinacea* and *Zea mays* (see Figure 2 for Identification of 1−6)

Latin name	1	2	3	4	5	6	**X**[a]
Phalaris arundinacea	8	18	36	2	15	15	6
Zea mays (flowers)	49	26	17	2	t	1	5
Zea mays (leaves)	48	26	16	2	t	1	7

[a] **X** = unknown.

nances of **3b** were assigned by $^3J_{HH}$ correlations in the 2D COSY spectrum and $^{1-3}J_{CH}$ correlations in the HSQC and HMBC spectra, respectively. Two two-proton singlets at δ 3.54 and 3.68, in addition to two three-proton singlets at δ 3.70 and 3.86, were in accordance with two methylmalonyl acyl groups. The pronounced downfield shift of H-3″ (δ 5.24), H-6A″ (δ 4.63), H-6B″ (δ 4.40), and C-6″ (δ 65.10), indicated the linkage points between the sugar and the acyl groups, and the cross-peaks at δ 4.63/δ 168.1 (H-6A″/C-1′^{V}), δ 4.40/δ 168.1 (H-6B″/C-1′^{V}), and δ 5.24/δ 168.1 (H-3″/C-1‴) in the HMBC spectrum confirmed the ester linkages at the sugar 6″- and 3″-positions, respectively. A molecular ion at *m/z* 649 confirmed two methylmalonyl units, and **3b** was determined to be formed by methyl esterification of the free acid function of the two malonyl units of **3**. Ions at *m/z* 535 ([M-86]$^+$) and 621 ([M]$^+$) in the ESIMS spectrum confirmed **3** to be cyanidin 3-*O*-(3″,6″-di-*O*-malonyl-β-glucopyranoside). The complete structure of this pigment has previously been determined only in the genera *Allium* (*22*) and *Dendrantheum* (*23*).

The ^{1}H NMR spectrum of pigment **5** showed a one-proton singlet at δ 9.08 (H-4); a three-proton AMX system at δ 8.37 (*dd*, 2.3 Hz, 8.7 Hz, H-6′), δ 8.31 (*d*, 2.3 Hz, H-2′), and δ 7.16 (*d*, 8.7 Hz, H-5′); a two-proton AX system at δ 7.03 (*dd*, 0.8 Hz, 1.9 Hz, H-8) and δ 6.78 (*d*, 1.9 Hz, H-6); and a three-proton singlet at δ 4.11 in accordance with a peonidin derivative. After assignments of the sugar ^{1}H resonances by the 2D DQF−COSY experiment, the corresponding ^{13}C resonances were assigned by the 2D heteronuclear HSQC experiment, in agreement with one β-glucopyranosyl moiety. The pronounced downfield shifts of H-6A″ (δ 4.62), H-6B″ (δ 4.38), and C-6″ (δ 65.2) showed a linkage between the sugar 6″-hydroxyl and an acyl group. A molecular ion at *m/z* 549 and a fragment ion at *m/z* 301 in the ESIMS spectrum of **5** corresponding to peonidin malonylglucoside and peonidin, respectively, confirmed the identity of **5** to be peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside).

The relatively long retention time of 6 compared to that of 1 on an ODS−HPLC column (Table 1) indicated the presence of acylation. The ions in the ESIMS spectrum at *m/z* 635 ([M]$^+$), *m/z* 549 ([M-86]$^+$), *m/z* 463 ([M-172]$^+$), and *m/z* 301 ([A]$^+$) were in agreement with the novel pigment peonidin 3-dimalonylglucoside.

The HPLC chromatograms of the acidified methanolic extracts of leaves of *Z. mays* and flower tops of *P. arundinacea* detected in the visible spectral region, showed a very similar qualitative anthocyanin content (Figure 1). The individual anthocyanins in *Z. mays* (Table 2) were identified by co-chromatography (HPLC) with authentic pigments from *P. arundinacea*, and by absorption spectra obtained by diode-array detection during on-line HPLC. Although cyanidin derivatives predominate in *Z. mays* (above 90%), and only small amounts of peonidin derivatives are detected, flowers of *P. arundinacea* contain more than 30% peonidin derivatives (Table 2). The acylated anthocyanins constitute more than 80% and 40% of the anthocyanins in *P. arundinacea* and *Z. mays*, respectively. Flowers and

leaves of maize contain the same anthocyanins in nearly equal relative proportions.

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