PERGAMON

Phytochemistry 52 (1999) 1687–1692

PHYTOCHEMISTRY

Anthocyanin pigments of tulips

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Received 24 March 1999; received in revised form 3 June 1999; accepted 10 June 1999

Abstract

The complete structures of the four major anthocyanins in orange-red *Tulipa* 'Queen Wilhelmina' were found to be the novel pigments pelargonidin 3-*O*-[6″-*O*-(2‴-*O*-acetyl-α-rhamnopyranosyl)-β-glucopyranoside] (34%) and cyanidin 3-*O*-[6″-*O*-(2‴-*O*-acetyl-α-rhamnopyranosyl)-β-glucopyranoside] (11%) in addition to pelargonidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (29%) and cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (15%). These structures were elucidated on the basis of chromatography, one- and two-dimensional nuclear magnetic resonance spectroscopy and electrospray mass spectrometry. This is the first report of anthocyanins with acyl group linked to axial sugar position.

Keywords: *Tulipa* 'Queen Wilhelmina'; Liliaceae; Flowers; Anthocyanins; Pelargonidin 3-*O*-[6″-*O*-(2‴-*O*-acetyl-α-rhamnopyranosyl)-β-glucopyranoside]; Cyanidin 3-*O*-[6″-*O*-(2‴-*O*-acetyl-α-rhamnopyranosyl)-β-glucopyranoside; Chemotaxonomy

1. Introduction

The anthocyanins of cultivated tulip flowers were first investigated by Willstätter and Bolton (1916), who found that the scarlet-red colour of some varieties of *Tulipa gesneriana* was due to a mixture of cyanidin diglucoside (cyanin) and carotenoids. Robinson and Robinson (1931) found that garden tulips contained either a mixture of cyanidin and pelargonidin biosides or cyanidin bioside and delphinidin diglucoside. They state that 'the examination of the anthocyanins of the tulip is much more difficult than that of most other plants.' Then Beal, Price and Sturgess (1941) found that a pelargonidin 3-pentoseglycoside was restricted to species in the sub-genus *Leiostemones* (26 species examined) and a delphinidin 3-pentoseglycoside was restricted to species in the sub-genus *Eriostemones* (six species examined). Other delphinidin as well as cyanidin derivatives were uniformly present throughout the genus. Although the anthocyanins in tulips in most studies are not fully characterised and often limited to a description of the anthocyanidins (aglycones), altogether seven pigments have been identified in *Tulipa*: the 3-glucosides and 3-rhamnoglucosides of pelargonidin, cyanidin and delphinidin in addition to delphinidin 3,5-diglucoside (Halevy & Asen, 1959; Shibata, 1956; Shibata & Sakai, 1958; Shibata & Ishikura, 1960). More recently, Nieuwhof, Van Raamsdonk and Van Eijk (1990) revealed by principal component analysis on the basis of carotenoids and the anthocyanidins pelargonidin, cyanidin and delphinidin, that the two sections *Leiostemones* (17 species examined) and *Eriostemones* (nine species examined) could be separated almost completely. A cosmetic remedy containing anthocyanins extracted from tulip flowers has been patented (Kawai, Ando & Ibata, 1989), and cotton and silk have been dyed medium reddish purple by *T. gesneriana* petals (Urabe & Yanagisawa, 1988).

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0031-9422/99/$ - see front matter
PII: S0031-9422(99)00328-3

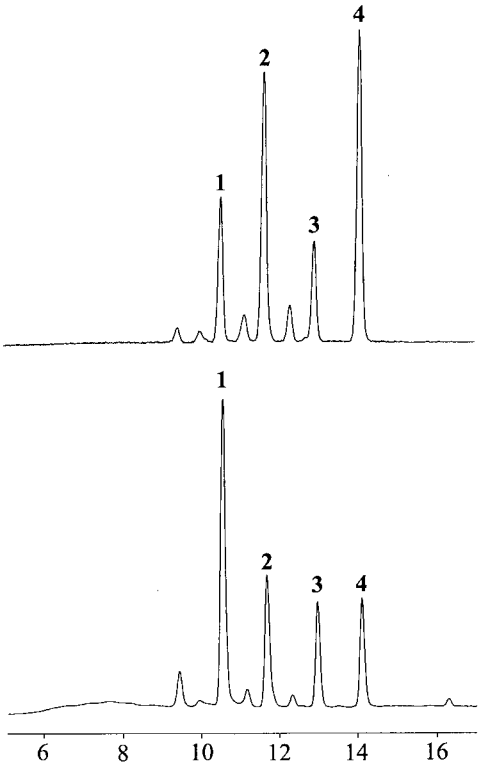
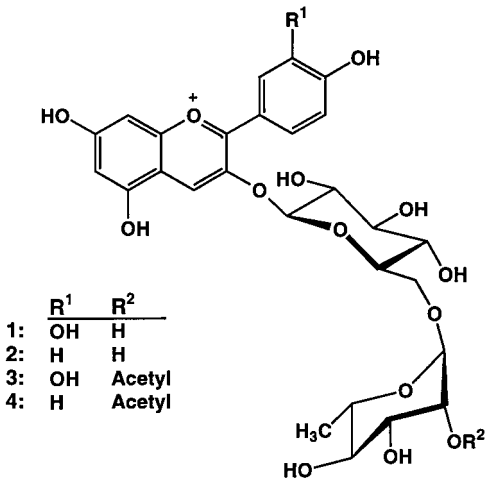


Fig. 1. HPLC separation of the anthocyanins in flowers of *Tulipa* 'Queen Wilhelmina' (top) and a deep red cultivar (bottom). **1** = cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside), **2** = pelargonidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside), **3** = cyanidin 3-*O*-[6″-*O*-(2‴-*O*-acetyl-α-rhamnopyranosyl)-β-glucopyranoside], **4** = pelargonidin 3-*O*-[6″-*O*-(2‴-*O*-acetyl-α-rhamnopyranosyl)-β-glucopyranoside].

During our survey of the anthocyanin content of two *Tulipa* cultivars with different colours, we discovered that two of the major anthocyanins were different from the anthocyanins previously reported to occur in the genus *Tulipa*. The aim of this paper is to present their qualitative and relative quantitative anthocyanin content, including two novel compounds.



2. Results and discussion

The HPLC chromatogram of the crude extract of *Tulipa* 'Queen Wilhelmina' showed four major anthocyanins, **1**–**4** (Fig. 1). The pigments in the extract were purified by partition against ethyl acetate followed by Amberlite XAD-7 column chromatography, and isolated by Sephadex LH-20 chromatography and preparative HPLC.

The UV–Vis spectrum and $A_{440}/A_{\text{vis.max}}$ ratio of **4** (Table 1) indicated a pelargonidin-glycoside without aromatic acylation (Andersen, 1988). The orange-red colour of the pigment (TLC) and the relative long retention time (HPLC) and high mobility (TLC) (Table 1) indicated a pelargonidin glycoside with aliphatic acylation. The two doublets in the ^{1}H NMR-spectrum of **4** at δ 8.68 and 7.14 integrating each for two protons, had the same coupling constant (J = 9.0 Hz). The DQF–COSY NMR spectrum showed a cross-peak between these two signals. The

Table 1
Chromatographic and spectral data and relative amounts of the anthocyanins in *Tulipa* 'Queen Wilhelmina' (QW), and a deep red cultivar (RP)

Compound	R_f (TLC) (FHW)	R_f (TLC) (BAW)	t_R (HPLC) (min)	$A_{440}/A_{\text{vis.max}}$ (%)	$\lambda_{\max}$ (nm)	ES–MS[a] m/z	Relative amounts (%)	
							QW	RP
1	0.62	0.23	10.50	25.4	530	597	15	40
2	0.82	0.59	11.61	40.0	510	581	29	17
3	0.72	0.53	12.89	29.8	527	639	11	13
4	0.87	0.64	14.04	45.2	510	623	34	14
Minor							11	16

[a] The MS spectra were taken after pigment storage in deuterated solvent. Due to considerable exchange of aglycone H-6 and H-8 with deuterium, the $[M\text{-}2H + 2D]^+$ are recorded.

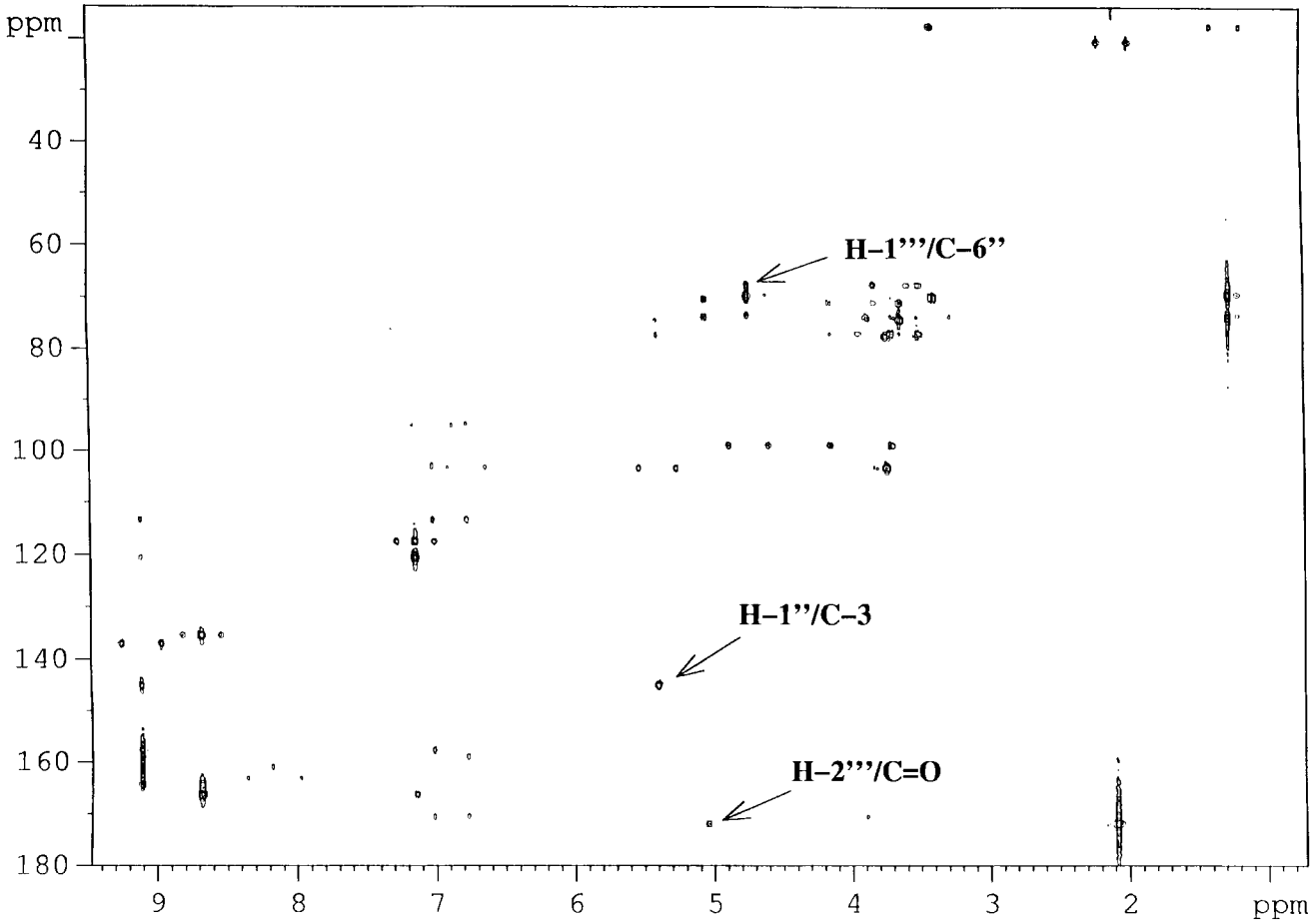


Fig. 2. Heteronuclear multiple bond correlation NMR spectrum (HMBC) of pelargonidin 3-*O*-[6″-*O*-(2‴-*O*-acetyl-α-rhamnopyranosyl)-β-glucopyranoside], **4**, dissolved in CD_3OD:CF_3COOD (19:1). The most important cross-peaks for determination of linkage points are labelled.

other aglycone signals were a 1H singlet at δ 9.11 (H-4), and a 2H AX-system at δ 7.01 (H-8) and δ 6.77 (H-6) (Pedersen, Andersen, Aksnes & Nerdal, 1993). The corresponding aglycone carbons assigned in the HSQC spectrum, and the quarternary carbons assigned in the HMBC spectrum (Fig. 2) were in accordance with a pelargonidin derivative.

The two-dimensional (2-D) TOCSY NMR spectrum of **4** is in agreement with two sugar units. Starting from the anomeric proton at δ 5.39, seven protons could be assigned through their cross-peaks in the DQF–COSY spectrum. The corresponding sugar carbons (Table 3) were assigned by the cross-peaks in the HSQC spectrum. The five non-anomeric carbons had chemical shift values from δ 68.0 to 78.0 (Table 3), indicating a hexose with a pyranose form (Markham & Chari, 1982). The chemical shifts and the coupling constants (Tables 2 and 3) showed a β-glucopyranoside. A cross-peak at δ 5.39/145.5 in the HMBC spectrum (Fig. 2) showed that this sugar unit was connected to the 3-position of the aglycone.

From the TOCSY spectrum it was observed that one of the protons of the second sugar unit was a high-field (δ 1.26). This 3H-doublet (J = 6.5 Hz) is typical for a rhamnose moiety. By using the DQF–COSY and HSQC spectra, it was possible to assign all the chemical shifts for the rhamnopyranosyl moiety (Tables 2 and 3). For anthocyanin sugars it is known that a $^1J_{CH}$-coupling with the proton in the axial position is less than the corresponding coupling for the proton in the equatorial position (Pedersen, Andersen, Aksnes & Nerdal, 1995). A 175 Hz $^1J_{CH}$-coupling for the anomeric rhamnose signal is thus in accordance with an α-rhamnopyranosyl. Confirmed by the downfield shift of C-6″ and the cross-peak δ 68.0/4.73 between C-6″ and H-1‴ in the HMBC-spectrum (Fig. 2), the rhamnosyl moiety was found to be connected to the glucosyl 6-position.

The acyl group was identified as acetic acid by the signals at δ 20.77 and 172.24 in the ^{13}C NMR spectrum, the 3H singlet at δ 2.08 in the ^1H NMR spectrum and the cross-peak at δ 2.08/172.2 in the HMBC-

Table 2
^{1}H NMR spectral data for the four major anthocyanins isolated from flowers of *Tulipa* 'Queen Wilhelmina' dissolved in CD_3OD:CF_3COOD (19:1) at 25°C[a]

	1 δ (ppm), *J* (Hz)	**2** δ (ppm), *J* (Hz)	**3** δ (ppm), *J* (Hz)	**4** δ (ppm), *J* (Hz)
Aglycone				
4	9.02, *s*	9.09 *s*	9.11 *s*	9.44 *s*
6	6.76 *d* 1.9	6.77 *d* 1.8	6.76 *d* 1.9	6.77 *d* 2.0
8	6.98 *s br*	7.01 *s br*	6.99 *s br*	7.01 *s br*
2′	8.12 *d* 2.2	8.69 *d* 9.0	8.13 *d* 2.3	8.68 *d* 9.0
3′		7.14 *d* 9.0		7.14 *d* 9.0
5′	7.11 *d* 8.8	7.14 *d* 9.0	7.12 *d* 8.7	7.14 *d* 9.0
6′	8.36 *dd* 8.7, 2.2	8.69 *d* 9.0	8.36 *dd* 8.7, 2.3	8.68 *d* 9.0
3-*O*-glucopyranoside				
1″	5.39 *d* 7.9	5.36 *d* 7.7	5.40 *d* 7.8	5.39 *d* 7.7
2″	3.76 *dd* 9.1, 7.9	3.72[b] *m*	3.77 *dd* 9.1, 7.8	3.73 *dd* 9.0, 7.7
3″	3.64[b] *m*	3.61[b] *m*	3.64 *t* 9.1	3.63 *t* 9.0
4″	3.51 *t* 9.5	3.49[b] *m*	3.50 *t* 9.4	3.49 *t* 9.5
5″	3.83 *m*	3.79[b] *m*	3.82 *m*	3.81 *m*
$6''_A$	4.16 *d br* 11.1	4.14 *d br* 11.4	4.14 *d br* 11.3	4.13 *dd* 11.4, 1.5
$6''_B$	3.68[b] *m*	3.68[b] *m*	3.69[b] *m*	3.70[b] *m*
6″-*O*-α-rhamnopyranosyl				
1‴	4.74 *s br*	4.74 *d* 1.4	4.73 *s br*	4.73 *s br*
2‴	3.90 *dd* 3.5, 1.5	3.88 *s br*	5.04 *dd* 3.2, 1.3	5.04 *dd* 3.6, 1.5
3‴	3.73 *dd* 9.8, 3.5	3.71[b] *m*	3.88 *dd* 9.7, 3.6	3.88 *dd* 9.7, 3.6
4‴	3.42[b] *m*	3.41[b] *m*	3.39[b] *m*	3.38[b] *m*
5‴	3.66[b] *m*	3.64[b] *m*	3.67[b] *m*	3.68[b] *m*
6‴	1.26 *d* 6.3	1.25 *d* 6.0	1.26 *d* 6.2	1.26 *d* 6.5
2‴-*O*-acetyl				
2‴′			2.08 *s*	2.08 *s*

[a] See Fig. 1 for pigment identification.
[b] Assigned from HSQC/HMBC.

spectrum (Fig. 2). The downfield chemical shift of the rhamnosyl H-2‴ (δ 5.04), and the cross-peak at δ 5.04/172.2 in the HMBC spectrum between H-2‴ and the carboxylic carbon (Fig. 2) confirmed the linkage position between the acyl group and the rhamnosyl. Thus, **4** was identified as pelargonidin 3-*O*-[6″-*O*-(2‴-*O*-acetyl-α-rhamnopyranosyl)-β-glucopyranoside].

The UV–Vis spectrum of **3** indicated an anthocyanin with two oxygen-functions on the B-ring (Table 1) (Andersen, 1988). This pattern was confirmed by the 3H AMX system at δ 8.36 (*dd*, 8.7, 2.3 Hz, H-6′), 8.13 (*d*, 2.3 Hz, H-2′) and 7.12 (*d*, 8.7 Hz, H-5′). In the downfield region additional signals were observed at δ 9.06 (H-4), and a 2H AX-system at δ 6.99 (H-8) and δ 6.76 (H-6) (Table 2). The chemical shifts for the corresponding aglycone carbons and the quarternary carbons were assigned by the HSQC and HMBC NMR spectra, respectively, in accordance with the anthocyanidin cyanidin (Table 3).

The different NMR-spectra of **3** showed many similarities with respect to the aliphatic regions of the corresponding spectra of **4**. After assignments of the chemical shifts (Tables 2 and 3), the cross-peak in the HMBC spectrum of δ 5.40/145.7 between H-1″ and C-3 showed that a β-glucopyranosyl unit was linked to the aglycone 3-position. In a similar way a cross-peak at δ 68.0/4.73 between C-6″ and H-1‴ in the HMBC-spectrum, showed that an α-rhamnopyranosyl unit was connected to the glucosyl 6-position. The relative large $^1J_{CH}$-coupling (174.2 Hz) for the anomeric rhamnose signal was in agreement with an equatorial position for this anomeric proton. After identification of the acetyl moiety (Tables 2 and 3), the identity of **3** was found to be cyanidin 3-*O*-[6″-*O*-(2‴-*O*-acetyl-α-rhamnopyranosyl)-β-glucopyranoside].

The UV–Vis spectra of **1** and **2** were in accordance with cyanidin-/peonidin- and pelargonidin 3-glycosides, respectively (Andersen, 1988). Pigment **1** co-chromatographed (HPLC and TLC) with cyanidin-3-rutinoside from blackcurrant. Their structures were further confirmed by one- and two-dimensional NMR results (Tables 2 and 3) to be cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) (Nygård, Aksnes, Andersen & Bakken, 1997) and pelargonidin 3-*O*-(6″-

Table 3
^{13}C NMR spectral data for the four major anthocyanins isolated from flowers of *Tulipa* 'Queen Wilhelmina' dissolved in CD_3OD:CF_3COOD (19:1) at 25°C[a]

	1 δ (ppm)	**2** δ (ppm)	**3** δ (ppm)	**4** δ (ppm)
Aglycone				
2	164.28	164.68	164.37	164.59
3	145.64	145.48	145.68	145.46
4	136.27	137.22	136.49	137.29
5	159.13	159.26	159.1[b]	159.17
6	103.5[b]	103.54	103.3[b]	103.1[b]
7	170.52	170.72	170.93	170.70
8	95.22	95.33	95.0[b]	94.9[b]
9	157.72	157.92	157.9[b]	157.92
10	113.27	113.53	113.6[b]	113.69
1′	121.25	120.86	121.32	120.89
2′	118.47	135.79	118.46	135.68
3′	147.46	117.89	147.47	117.86
4′	155.89	166.68	155.81	166.57
5′	117.7[b]	117.89	117.43	117.86
6′	128.41	135.79	128.31	135.68
3-*O*-β-glucopyranoside				
1″	103.52	103.79	103.57	103.77
2″	74.70	74.74	74.69	74.71
3″	78.03	78.06	78.00	78.01
4″	71.22	71.23	71.33	71.32
5″	77.43	77.46	77.43	77.44
6″	67.80	67.84	67.97	67.99
6″-*O*-α-rhamnopyranosyl				
1‴	102.18	102.19	99.38	99.35
2‴	71.88	71.88	73.80	73.81
3‴	72.45	72.46	70.59	70.58
4‴	73.94	73.92	74.18	74.16
5‴	69.78	69.79	69.87	69.86
6‴	17.88	17.89	17.89	17.91
2‴-*O*-acetyl				
1⁗ (C═O)			172.25	172.24
2⁗			20.77	20.77

[a] See Fig. 1 for pigment identification.
[b] Assigned from HSQC/HMBC.

O-α-rhamnopyranosyl-β-glucopyranoside), respectively. The structures of **1**–**4** were confirmed by electrospray MS data (Table 1).

The novel pigments, pelargonidin 3-*O*-[6″-*O*-(2‴-*O*-acetyl-α-rhamnopyranosyl)-β-glucopyranoside] and cyanidin 3-*O*-[6″-*O*-(2‴-*O*-acetyl-α-rhamnopyranosyl)-β-glucopyranoside], constitute together 45 and 27% of the total anthocyanin content of the orange-red *Tulipa* 'Queen Wilhelmina' and deep-red unknown cultivar, respectively (Fig. 1). Most probably these pigments have a much wider distribution in this genus. They are easily degraded by mineral acids like hydrochloric acid, which may be a reason for their absence in previous studies of this well-known genus. The acetyl moiety of these pigments are linked to the rhamnose 2-position through the hydroxyl group in the axial position. This is the first report of anthocyanins with an acyl group linked to any axial sugar position, and may thus have chemotaxonomic importance.

3. Experimental

3.1. Isolation of pigments

Flowers of *Tulipa* 'Queen Wilhelmina' and a deep red unknown cultivar were collected in June 1998 in Bergen and extracted twice with MeOH containing 1% TFA. The concd and combined extracts based on 380 g 'Queen Wilhelmina' were purified by partition against ethyl acetate before application on an Amberlite XAD-7 column (Andersen, 1988). The anthocyanins were further purified on a Sephadex LH-20 column (100 × 5.0 cm, Pharmacia) using H_2O–MeOH–TFA (80:19.6:0.4, v/v) as eluent. Further purification and separation was done by preparative HPLC (Gilson 305/306 pump system with Hewlett-Packard 1040A diode array detection) using an ODS Econosil column (25 × 1.0 cm, 10 μm). Two solvents were used for elution: H_2O–HCO_2H (18:1, v/v) (A) and MeOH–H_2O–HCO_2H (10:8:1, v/v) (B). The elution profile consisted of a linear gradient from 10 to 100% B during the first 25 min, isocratic elution using 100% B for the next 5 min followed by a new linear gradient back to 10% B during the next 5 min. The flow rate was 6 ml min^{-1}.

Analytical HPLC was performed on an HP-1050 module system (Hewlett-Packard) using an ODS Hypersil column (20 × 0.5 cm, 5 μm). The elution consisted of a linear gradient from 10 to 100% B during the first 17 min, isocratic elution using 100% B for the next 4 min followed by a linear gradient back to 10% B during 1 min. The flow rate was 1.2 ml min^{-1}, and aliquots of 15 μl were injected. TLC was carried out on microcrystalline cellulose (F1440, Schleicher and Schüll) using two different solvent systems, BAW (1-butanol–HOAc–H_2O; 4:1:5 v/v, upper phase) and FHW (HCO_2H-concd HCl–H_2O; 1:1:2 v/v).

3.2. Spectroscopy

UV–Vis absorption spectra were recorded in 0.01% concd HCl in MeOH. Spectral measurements were made over the wavelength range 240–600 nm in steps of 2 nm. Relative amounts of each anthocyanin are reported as percentages of total peak area in HPLC chromatograms based on absorptions recorded for every second nm between 500 and 540 nm.

The 1-D ^{1}H and ^{13}C SEFT experiments of **1**, and the ^{1}H and TOCSY experiments of **4** were obtained at 400.13 and 100.61 MHz for ^{1}H and ^{13}C, respectively, on a Bruker DMX 400 MHz instrument at 25°C. The

1-D ^{1}H and the 1-D SEFT experiments of **1** were performed on a 5 mm ^{1}H–^{13}C dual-probe, and the 1-D ^{1}H and the 2-D spin-lock (TOCSY) experiments of **4** were performed on a 5 mm inverse-probe. The other NMR experiments were obtained at 600.13 MHz and 150.92 MHz for ^{1}H and ^{13}C, respectively, on a Bruker DRX 600 MHz instrument at 25°C. The deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvent (CF_3CO_2D–CD_3OD; 5:95) were used as secondary references (δ 49.0 and 3.4 from TMS, respectively). The 1-D ^{1}H experiments, except for **1** and **4**, the 2-D homonuclear correlation experiment (DQF–COSY) and the 2-D heteronuclear experiments (HMBC and HSQC) were performed on a 5 mm TBI-probe. The 1-D SEFT-experiments of **3** and **4** and the 1-D CAPT-experiment of **2** were performed on a 5 mm BBO-probe.

The electrospray MS analyses were performed on a Quattro II MS/MS (Micromass, UK) with API source and flow injection. The instrument was operated in the positive ion mode with cone voltage 35 V and capillary voltage 3 kV. The mobile phase carrier was a methanol–water (50:50) mixture. The carrier was pumped into the source at a flow rate of 100 μl min^{-1}. The samples were dissolved in methanol containing 3% formic acid (v/v) prior to analysis.

Acknowledgements

The authors thank Marianne Pile and Elisabeth Salbu (Bergen Kommune) for identification of the cultivars and provision of the plant material, and Åsmund Larsen (Nycomed) for running the MS spectra. K.T. gratefully acknowledges The Norwegian Research Council for a fellowship.

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