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Anthocyanins with 4′-glucosidation from red onion, *Allium cepa*

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Abstract

The anthocyanins, cyanidin 3-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-malonyl-β-glucopyranoside)-4′-*O*-β-glucopyranoside, cyanidin 7-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-malonyl-β-glucopyranoside)-4′-*O*-β-glucopyranoside, cyanidin 3,4′-di-*O*-β-glucopyranoside, cyanidin 4′-*O*-β-glucoside, peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside)-5-*O*-β-glucopyranoside and peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) have been isolated in minor amounts from pigmented scales of red onion, *Allium cepa*, in addition to six known anthocyanins. The structures were established mainly by extensive use of 2D NMR spectroscopy and electrospray LC–MS. With exception of cyanidin 4′-glucoside and cyanidin 3,4′-diglucoside reported from *Hibiscus esculentus* with inadequate documentation, this is the first identification of anthocyanins with 4′-glycosidation. Compared to cyanidin 3-glycosides the cyanidin 4′-glucoside derivatives showed hypsochromic shifts of visible λ_{max} and hyperchromic effects on wavelengths around 440 nm, similar to pelargonidin 3-glycosides.

Keywords: Red onion; *Allium cepa*; Alliaceae; Pigmented scales; Anthocyanins; 4′-Glucosidation; Spectral properties; 2D NMR

1. Introduction

The genus *Allium* (Alliaceae) contains important vegetables like onions and chive. The main anthocyanin in the red onion, *Allium cepa* L., cultivars 'Ruby', 'Southport Red Globe' and the Japanese variety 'Kurenai' was first identified as cyanidin 3-glucoside (Robinson and Robinson, 1932; Foussain, 1956; Brandwein, 1965; Fuleki, 1969, 1971; Du et al., 1974; Kenmochi and Katayama, 1975). Cyanidin 3-(3″-glucosylglucoside = 3-laminariobioside) and some uncharacterised cyanidin derivatives were also reported. The identification of peonidin 3-arabinoside as the main pigment of the cultivar 'Southport Red Globe' (Brandwein, 1965) was not confirmed by Fuleki (1971), who instead identified minor amounts of peonidin 3-glucoside in pigmented scales of the same cultivar. Later Moore et al. (1982a,b) indicated that the main anthocyanins of red onion were acylated, however, without determination of the acyl group nor the substitution pattern. Then the main anthocyanins of the red onion cultivars 'Kurenai', 'Red Baron', 'Comred', 'Tropea', 'Mambo', 'Red Jumbo', 'Red Bone' and 'Red Granex' were identified as the 3-(6″-malonylglucoside), 3-(3″-glucosyl-6″-malonylglucoside), 3-(3″-glucosylglucoside) and 3-glucoside of cyanidin (Terahara et al., 1994; Fossen et al., 1996; Donner et al., 1997). Fossen et al. (1996) also identified some of the minor anthocyanins to be the 3-(3″,6″-dimalonylglucoside) and 3-(3″-malonylglucoside) of cyanidin as well as the 3,5-diglucosides of cyanidin and peonidin. Additionally, Donner et al. (1997) identified cyanidin 3-(3″-malonylglucoside), peonidin 3-glucoside and peonidin 3-malonylglucoside, however without determination of the linkage between the acyl group and the sugar of the latter pigment. They also indicated minor occurrence of a cyanidin 3-laminariobioside derivative with both glucose units substituted with malonic acid. In contrast to previous report of Du et al. (1974), who identified cyanidin 3-glucoside and cyanidin 3-laminariobioside as main anthocyanins in Spanish red onion, Ferreres et al. (1996) have without support from NMR or MS data identified the 3-arabinoside and 3-malonylarabinoside of cyanidin among the

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main anthocyanins of Spanish red onion (cultivar 'Morada de Amposta'). Gennaro et al. (2002) have recently reported the presence of minor amounts of delphinidin and petunidin derivatives in pigmented scales of the red onion cultivar 'Tropea'. Recently we have reported the presence of several C-4-substituted anthocyanins isolated in minor amounts from pigmented scales of red onion (Fossen et al., 2003).

In this paper we present isolation and characterisation of six minor anthocyanins (**1–4**, **10**, **11**) from pigmented scales of red onion. Their structures were established by extensive use of NMR spectroscopy and electrospray MS. The four cyanidin derivatives (**1–4**) share one glucose unit located in the 4′-position. This 4′-substitution, which has not been identified adequately for any anthocyanin previously, lead to UV–Vis spectral properties of these cyanidin derivatives which are similar to those of pelargonidin derivatives.

2. Results and discussion

The HPLC chromatogram of the methanolic extract of pigmented scales of red onion, *A. cepa*, detected in the visible spectral region, showed four major and several minor anthocyanins. The anthocyanins were purified by partition against ethyl acetate followed by Amberlite XAD-7 column chromatography. The pigments were fractionated by Sephadex LH-20 column chromatography and isolated by preparative HPLC. The pure anthocyanins, **1–11**, were checked for homogeneity by analytical HPLC (Table 1). Based on NMR spectroscopy and MS, the four major (**6–9**) and the minor anthocyanin **5** have previously been identified as the 3-(3″-glucosyl-6″-malonylglucoside), 3-(6″-malonylglucoside), 3-(3″-glucosylglucoside), 3-glucoside and 3,5-diglucoside of cyanidin, respectively (Terahara et al., 1994; Fossen et al., 1996).

The UV–Vis spectrum of pigment **2** taken on-line during HPLC showed a visible maximum at 508 nm with A_{440}/A_{508} of 45%. The downfield part of the 1D ^{1}H NMR spectrum of **2** showed a 3H ABX system at δ 9.26 (*d*, 0.9 Hz; H-4), δ 7.04 (*dd*, 1.9 Hz, 0.9 Hz; H-8) and δ 6.78 (*d*, 1.9 Hz; H-6), a 3H AMX system at δ 8.35 (*dd*, 8.7 Hz, 2.2 Hz; H-6′), δ 8.16 (*d*, 2.2 Hz; H-2′) and δ 7.50 (*d*, 8.7 Hz; H-5′) in accordance with a cyanidin derivative. The sugar region showed the presence of two monosaccharides. All the ^{1}H sugar resonances (Table 2) were assigned by the DQF-COSY and

Table 1
Chromatographic (HPLC) and spectral (UV-Vis and MS) data recorded for cyanidin 4′-*O*-β-glucopyranoside (**1**), cyanidin 3,4′-di-*O*-β-glucopyranoside (**2**), cyanidin 3-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-malonyl-β-glucopyranoside)-4′-*O*-β-glucopyranoside (**3**), cyanidin 7-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-malonyl-β-glucopyranoside)-4′-*O*-β-glucopyranoside (**4**), cyanidin 3,5-di-*O*-β-glucopyranoside (**5**), cyanidin 3-*O*-β-glucopyranoside (**6**), cyanidin 3-*O*-(3″-*O*-β-glucopyranosyl-β-glucopyranoside) (**7**), cyanidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) (**8**), cyanidin 3-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-malonyl-β-glucopyranoside) (**9**), cyanidin 3-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-methylmalonyl-β-glucopyranoside) (**9b**), peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside)-5-*O*-β-glucopyranoside (**10**) and peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) (**11**)

Compd	On-line HPLC	ES-MS			
	UV–Vis-max (nm)	$A_{440}/A_{\text{Vis-max}}$ (%)	t_R (min)	$[M]^+$ *m/z*	$[A]^+$ *m/z*
1	508	50	11.13	449	287
2	508	45	12.53	611	287
3	508	50	14.37	859	287
4	508	50	13.96	859	287
5	516	18	13.30	611	287
6	520	30	14.25	449	287
7	520	30	14.72	612 $[MH]^+$	
8	522	29	18.09	536 $[MH]^+$	
9	522	29	18.67	698 $[MH]^+$	
9b	523	28	20.96		
10	519	18	17.30	750 $[MK]^+$	301
11	523	28	20.56		

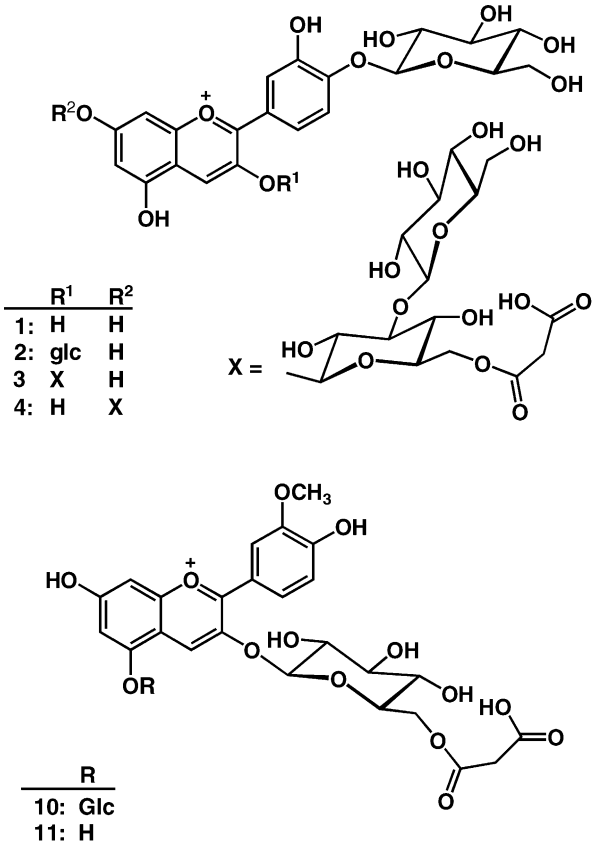

Fig. 1. The structures of cyanidin 4′-*O*-β-glucopyranoside (**1**), cyanidin 3,4′-di-*O*-β-glucopyranoside (**2**), cyanidin 3-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-malonyl-β-glucopyranoside)-4′-*O*-β-glucopyranoside (**3**), cyanidin 7-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-malonyl-β-glucopyranoside)-4′-*O*-β-glucopyranoside (**4**), peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside)-5-*O*-β-glucopyranoside (**10**), and peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) (**11**) isolated from red onion, *Allium cepa*.

Table 2
^{1}H-NMR spectral data for cyanidin 3,4′-di-*O*-β-glucopyranoside (**2**), cyanidin 3-*O*-(3″-*O*-glucosyl-6″-*O*-malonyl-β-glucopyranoside)-4′-*O*-β-glucopyranoside (**3**), cyanidin 7-*O*-(3″-*O*-glucosyl-6″-*O*-malonyl-β-glucopyranoside)-4′-*O*-β-glucopyranoside (**4**), cyanidin 3,5-di-*O*-β-glucopyranoside (**5**), cyanidin 3-*O*-(3″-*O*-glucosyl-6″-*O*-methylmalonyl-β-glucopyranoside) (**9b**), peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside)-5-*O*-β-glucopyranoside (**10**), and peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) (**11**) in CF_3COOD–CD_3OD (5:95; v/v) at 25 °C

	2	**3**	**4**	**5**	**9b**	**10**	**11**
Aglycone							
4	9.26 *d*, 0.9 Hz	9.19 *s (b)*	9.19 *s*	9.24 *d*, 0.9 Hz	9.02 *s*	9.08 *d*, 0.9 Hz	9.06 *s (b)*
6	6.78 *d*, 1.9 Hz	6.80 *d*, 2.1 Hz	6.29 *d*, 2.1 Hz	7.16 *d*, 1.9 Hz	6.76 *d*, 1.8 Hz	7.13 *d*, 2.0 Hz	6.77 *d*, 1.8 Hz
8	7.04 *dd*, 0.9 Hz, 1.9 Hz	7.04 *dd*, 2.1 Hz, 0.9 Hz	6.37 *d*, 2.1 Hz	7.18 *dd*, 1.9 Hz, 0.9 Hz	6.98 *d*, 1.8 Hz	7.23 *dd*, 2.0 Hz, 0.9 Hz	7.01 *dd*, 1.8 Hz, 0.9 Hz
2′	8.16 *d*, 2.2 Hz	8.16 *d*, 2.2 Hz	7.70 *d*, 2.2 Hz	8.17 *d*, 2.3 Hz	8.09 *d*, 2.3 Hz	8.34 *d*, 2.3 Hz	8.29 *d*, 2.2 Hz
5′	7.50 *d*, 8.7 Hz	7.50 *d*, 8.8 Hz	7.37 *d*, 8.8 Hz	7.13 *d*, 8.8 Hz	7.09 *d*, 8.8 Hz	7.18 *d*, 8.8 Hz	7.14 *d*, 8.8 Hz
6′	8.35 *dd*, 8.7 Hz, 2.2 Hz	8.32 *dd*, (8.8 Hz, 2.2 Hz	7.76 *dd*, 8.8 Hz, 2.2 Hz	8.44 *dd*, 8.8 Hz, 2.3 Hz	8.35 *dd*, 8.8 Hz, 2.3 Hz	8.41 *dd*, 8.8 Hz, 2.3 Hz	8.34 *dd*, 8.8 Hz, 2.2 Hz
OMe						4.12 *s*	4.11 *s*
3-*O*-β-Glucopyranoside			7-*O*-β-Glucopyranoside	3-*O*-β-Glucopyranoside			
1″	5.40 *d*, 7.8 Hz	5.44 *d*, 7.7 Hz	5.53 *d*, 7.7 Hz	5.38 *d*, 7.9 Hz	5.43 *d*, 7.7 Hz	5.53 *d*, 7.7 Hz	5.39 *d*, 7.7 Hz
2″	3.75 *dd*, 7.8 Hz, 9.2 Hz	3.98 *m*	3.55 *m*	3.78 *dd*, 7.9 Hz, 9.3 Hz	3.98 *dd*, 7.7 Hz, 9.0 Hz	3.78 *m*	3.75 *m*
3″	3.61 *t*, 9.2 Hz	3.84 *m*	3.69 *m*	3.62 *t*, 9.3 Hz	3.84 *t*, 9.0 Hz	3.65 *m*	3.64 *t*, 9.0 Hz
4″	3.51 *m*	3.65 *m*	3.52 *m*	3.49 *t*, 9.3 Hz	3.63 *dd*, 9.3 Hz, 9.0 Hz	3.53 *m*	3.51 *m*
5″	3.64 *m*	3.96 *m*	3.67 *m*	3.71 *m*	3.95 *ddd*, 1.8 Hz, 7.0 Hz, 9.3 Hz	3.98 *m*	3.91 *ddd*, 2.0 Hz, 7.0 Hz, 9.5 Hz
6A″	4.00 *dd*, 12.0 Hz, 2.0 Hz	4.68 *dd*, 12.1 Hz, 1.8 Hz	4.49 *dd*, 12.1 Hz, 1.8 Hz	4.06 *dd*, 12.0 Hz, 2.2 Hz	4.67 *dd*, 11.8 Hz, 1.8 Hz	4.53 *dd*, 11.9 Hz, 2.3 Hz	4.63 *dd*, 11.8 Hz, 2.0 Hz
6B″	3.79 *dd*, 12.0 Hz, 6.1 Hz	4.39 *dd*, 12.1 Hz, 7.3 Hz	4.35 *dd*, 12.1 Hz, 5.9 Hz	3.81 *dd*, 12.0 Hz, 6.0 Hz	4.40 *dd*, 11.8 Hz, 7.0 Hz	4.45 *dd*, 11.9 Hz, 7.2 Hz	4.39 *dd*, 11.8 Hz, 7.0 Hz
5-*O*-β-Glucopyranoside							
1‴				5.25 *d*, 7.9 Hz		5.29 *d*, 7.7 Hz	
2‴				3.76 *dd*, 7.9 Hz, 9.3 Hz		3.79 *m*	
3‴				3.64 *m*		3.65 *m*	
4‴				3.54 *t*, 9.3 Hz		3.55 *m*	
5‴				3.65 *m*		3.69 *m*	
6A‴				4.04 *dd*, 12.0 Hz, 2.2 Hz		4.05 *dd*, 12.1 Hz, 2.3 Hz	
6B‴				3.83 *dd*, 12.0 Hz, 5.8 Hz		3.84 *dd*, 12.1 Hz, 6.5 Hz	
4′-*O*-β-Glucopyranoside							
1‴	5.17 *d*, 7.8 Hz	5.17 *d*, 7.7 Hz	5.07 *d*, 7.7 Hz				
2‴	3.69 *dd*, 7.8 Hz, 9.2 Hz	3.69 *m*	3.66 *m*				
3‴	3.62 *t*, 9.2 Hz	3.62 *m*	3.62 *m*				

(continued on next page)

Table 2 (*continued*)

	2	3	4	5	9b	10	11
4‴	3.53 *t*, 9.2 Hz	3.54 *m*	3.54 *m*				
5‴	3.64 *m*	3.64 *m*	3.65 *m*				
6A‴	4.04 *dd*, 12.0 Hz, 2.1 Hz	4.04 *dd*, 12.1 Hz, 1.8 Hz	4.03 *m*				
6B‴	3.84 *dd*, 12.0 Hz, 5.7 Hz	3.84 *dd*, 12.1 Hz, 3.0 Hz	3.84 *m*				
3″-*O*-β-Glucopyranosyl							
1‴		4.71 *d*, 7.7 Hz	4.65 *d*, 7.7 Hz		4.71 *d*, 7.7 Hz		
2‴		3.44 *m*	3.38 *m*		3.43 *m*		
3‴		3.39 *m*	3.49 *m*		3.51 *t*, 9.1 Hz		
4‴		3.51 *m*	3.39 *m*		3.39 *m*		
5‴		3.47 *m*	3.43 *m*		3.47 *ddd*, 2.0 Hz, 6.8 Hz, 9.5 Hz		
6A‴		4.01 *dd*, 12.1 Hz, 2.2 Hz	3.98 *m*		4.01 *dd*, 11.8 Hz, 2.0 Hz		
6B‴		3.75 *m*	3.74 *m*		3.75 *dd*, 11.8 Hz, 6.8 Hz		
6‴-*O*-Malonyl							
2		3.44 *s*	3.44 *s*		3.54 *s*	3.44 *s*	3.44 *s*
OMe					3.69 *s*		

TOCSY experiments, and the corresponding ^{13}C resonances were thereafter assigned by the ^{1}H–^{13}C HSQC experiment (Table 3). The anomeric coupling constants (7.8 Hz) and the 12 ^{13}C resonances in the sugar region were in accordance with two β-glucopyranose units. The downfield shift of H-5′ (δ 7.50) compared to the analogous signal of cyanidin 3,5-diglucoside, **5** (δ 7.13), and the crosspeak at δ 5.40/9.26 (H-1″/H-4) in the ^{1}H–^{1}H NOESY spectrum of **2** confirmed the linkages between the sugar units and the aglycone 4′- and 3-positions, respectively. A molecular ion at *m*/*z* 611 in the ES-MS spectrum of **2** corresponding to cyanidin diglycoside, in addition to a fragment ion at *m*/*z* 287 corresponding to cyanidin, were in accordance with cyanidin 3,4′-di-*O*-β-glucopyranoside (Fig. 1).

The UV–Vis spectrum and the downfield part of the ^{1}H NMR spectrum of **3** were very similar to analogous spectra of **2** (Tables 1–3). The sugar region in the ^{1}H NMR spectrum indicated the presence of malonyl and three monoglycosyl moieties (Table 2). All the ^{1}H sugar resonances were assigned by the 2D DQF-COSY and TOCSY experiments, and the corresponding ^{13}C resonances (Table 3) were then assigned by the ^{1}H–^{13}C HSQC experiment. The anomeric coupling constants (7.7 Hz) and the 18 ^{13}C resonances in the sugar region were in accordance with three β-glucopyranose units. The downfield shift of H-5′ (δ 7.50), the crosspeaks at δ 5.40/9.26 (H-1″/H-4) and δ 5.17/7.50 (H-1‴/H-5′) in the NOESY spectrum of **3** confirmed the linkages between two of the glucose units to the aglycone 3- and 4′-positions, respectively. The 3″-linkage of the third glucosyl unit was revealed by the downfield shifts of C-3″ (δ 87.5) (Table 3) and H-3″ (δ 3.84) (Table 2). Thus, the sugar attached to the aglycone 3-position was identified as the rare disaccharide laminariobioside. The downfield shifts of H-6A″ (δ 4.68), H-6B″ (δ 4.39) and C-6″ (δ 65.5) confirmed the linkage of the acyl moiety. A molecular ion in the ES-MS spectrum at *m*/*z* 859 corresponding to cyanidin malonyltriglucoside, in addition to a fragment ion at *m*/*z* 287 corresponding to cyanidin, confirmed the identity of **3**, cyanidin 3-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-malonyl-β-glucopyranoside)-4′-*O*-β-glucopyranoside (Fig. 1).

The UV–Vis, ES-MS and NMR spectra of **4** showed many similarities with the analogous spectra of **3** (Tables 1–3). However, the upfield shifts of H-6 (δ 6.29) and H-8 (δ 6.37) compared to the analogous signals of **3** (δ 6.80 and 7.04, respectively) indicated a different substitution pattern. Indeed the downfield shift of C-8 (δ 101.2) showed that one of the glucosyl units was linked to the aglycone 7-position. The downfield shifts of H-5′ (δ 7.37) and C-3″ (δ 87.3), revealed the linkages of the two other glucosyl units, while the downfield shifts of H-6A″ (δ 4.49), H-6B″ (δ 4.35) and C-6″ (δ 65.5) confirmed the 6″-position of the malonyl moiety in accordance with cyanidin 7-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-malonyl-β-

Table 3
^{13}C-NMR spectral data for cyanidin 3,4′-di-*O*-β-glucopyranoside (**2**), cyanidin 3-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-malonyl-β-glucopyranoside)-4′-*O*-β-glucopyranoside (**3**), cyanidin 7-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-malonyl-β-glucopyranoside)-4′-*O*-β-glucopyranoside (**4**), cyanidin 3,5-di-*O*-β-glucopyranoside (**5**), cyanidin 3-*O*-β-glucopyranoside (**6**), cyanidin 3-*O*-(3″-*O*-β-glucopyranosyl-β-glucopyranoside) (**7**), cyanidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) (**8**), cyanidin 3-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-malonyl-β-glucopyranoside) (**9**), cyanidin 3-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-methylmalonyl-β-glucopyranoside) (**9b**), peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside)-5-*O*-β-glucopyranoside (**10**) and peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) (**11**) in CF_3COOD–CD_3OD (5:95; v/v) at 25 °C

	2	**3**	**4**	**5**	**6**	**8**	**9**	**9b**	**10**	**11**
Aglycone										
2	*	163.3	*	165.0	163.91	164.30	164.58	164.61	*	164.32
3	*	145.3	*	146.6	145.57	145.55	145.55	145.50	*	145.44
4	138.5	141.6	139.1	136.0	136.68	136.69	137.00	136.93	*	137.34
5	*	158.5	*	156.8	159.25	159.70	*	159.10	*	159.20
6	*	*	101.7	105.8	103.45	103.43	103.40	103.48	105.4	103.40
7	*	*	*	169.5	170.55	170.49	173.54	170.50	*	170.54
8	*	*	101.2	97.4	95.17	95.26	*	95.31	97.5	95.50
9	*	*	*	157.2	157.58	157.71	*	157.77	*	157.91
10	*	*	*	113.4	113.32	113.23	113.20	113.24	*	113.52
1′	*	123.9	*	121.2	121.16	121.18	121.23	121.19	*	121.09
2′	118.7	119.2	118.5	118.6	118.39	118.37	118.50	118.48	115.3	115.28
3′	*	148.6	*	147.7	147.34	147.44	147.46	147.57	*	149.59
4′	*	152.8	*	156.7	155.77	155.86	155.94	155.95	*	156.62
5′	117.0	117.2	117.0	117.6	117.46	117.39	117.33	117.32	117.8	117.65
6′	126.4	127.8	124.3	129.1	128.25	128.47	128.48	128.51	*	129.04
OMe									57.1	56.91
3-*O*-β-Glucopyranoside										
1″	103.7	103.3		104.6	103.72	103.58	103.34	103.32	102.6	103.81
2″	74.6	74.2		74.7	74.79	74.64	73.91	73.90	74.7	74.81
3″	77.6	87.5		78.3	78.14	77.90	87.56	87.55	78.1	78.00
4″	71.0	70.0		71.4	71.08	71.31	69.98	69.92	71.5	71.33
5″	78.3	75.6		79.0	78.77	75.93	75.60	75.58	75.7	76.01
6″	62.4	65.5		62.7	62.36	65.47	65.44	65.45	65.6	65.45
5-*O*-β-Glucopyranoside										
1‴				102.7					102.8	
2‴				74.6					74.7	
3‴				78.1					78.1	
4‴				71.2					71.3	
5‴				78.6					78.5	
6‴				62.6					62.8	
4′-*O*-β-Glucopyranoside										
1‴	102.3	102.4	102.7							
2‴	74.5	74.9	75.0							
3‴	77.8	77.9	77.7							
4‴	71.1	71.2	71.2							
5‴	78.3	78.1	78.1							
6‴	62.4	62.5	62.5							
3″-*O*-β-Glucopyranosyl										
1⁗		104.8	104.9				105.14	105.13		
2⁗		75.5	75.5				75.46	75.46		
3⁗		78.0	78.0				77.89	77.88		
4⁗		71.2	71.6				71.61	71.61		
5⁗		78.2	78.2				78.29	78.28		
6⁗		62.7	62.7				62.64	62.64		
7-*O*-β-Glucopyranoside										
1‴			95.3							
2‴			74.9							
3‴			87.3							
4‴			71.2							
5‴			75.0							
6‴			65.4							
6″-*O*-Malonyl										
1						166.65	168.59	168.15		168.59
2								41.70		*
3						170.19	170.88	168.76		168.76
OMe								52.86		

*Not detected adequately.

glucopyranoside)-4′-*O*-β-glucopyranoside (Fig. 1). A molecular ion at *m*/*z* 859 in the ES-MS spectrum of **4** confirmed this identity. The location of the (3″-*O*-glucosyl-6″-*O*-malonyl-β-glucopyranoside) moiety in the aglycone 7-position seems to have a considerable upfield effect on all aglycone protons but H-4 (Table 2). This effect is expected for H-6 and H-8, however, the upfield effect on the B-ring protons may indicate intramolecular interaction between the glycosyl unit in the 7-position and the aglycone B-ring.

The UV–Vis spectrum of **1** showed the same characteristics as the other cyanidin derivatives with 4′-glucosidation (Table 1). A fragment ion at *m*/*z* 287 in the ES-MS spectrum of **1** corresponding to cyanidin, and a molecular ion at *m*/*z* 449 corresponding to cyanidin connected to one monoglycosyl unit, showed the identity of **1** to be cyanidin 4′-*O*-β-glycoside. Since glucose is the only monosaccharide identified in the other anthocyanins (**2**–**11**) isolated from this plant, pigment **1** is most probably cyanidin 4′-*O*-β-glucoside (Fig. 1).

The UV-Vis spectrum of pigment **10** taken on-line during HPLC showed a visible maximum at 519 nm with A_{440}/A_{519} of 18% in accordance with a 3,5-disubstituted cyanidin or peonidin derivative (Harborne, 1958). The downfield part of the 1D ^{1}H NMR spectrum of **10** showed two 3H ABX systems with many similarities to that of **5** (Table 2). However, a 3H singlet at *δ* 4.12 (OCH_3) indicated the aglycone peonidin. The sugar region of the ^{1}H NMR spectrum indicated the presence of two monosaccharides and one malonyl moiety. The anomeric coupling constants (7.7 Hz), the crosspeaks in the DQF-COSY and TOCSY spectra, and the 12 ^{13}C resonances in the sugar region of the ^{1}H/^{13}C HSQC spectrum were in accordance with two β-glucopyranosyl units. The crosspeaks at *δ* 5.53/9.08 (H-1″/H-4) and *δ* 5.29/7.13 (H-1‴/H-6) in the NOESY spectrum confirmed peonidin 3- and 5-glucosidation, respectively. The downfield shift of H-6A″ (*δ* 4.53), H-6B″ (*δ* 4.45) and C-6″ (*δ* 65.6) showed the linkage of the acyl moiety. A molecular ion $[MK]^+$ at *m*/*z* 750 in the ES-MS spectrum of **10** corresponding to peonidin malonyldiglucoside, in addition to a fragment ion at *m*/*z* 301 corresponding to peonidin, confirmed the identity of **10** to be peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside)-5-*O*-β-glucopyranoside (Fig. 1).

The UV–Vis spectrum of **11** showed a visible maximum at 523 nm with A_{440}/A_{523} of 28% in accordance with a 3-substituted cyanidin or peonidin derivative (Harborne, 1958). The NMR spectra of **11** showed many similarities with the analogous spectra of **10** (Tables 2 and 3). However, the sugar regions in the NMR spectra showed the presence of one malonyl unit and only one sugar. The crosspeak at δ 5.39/145.4 (H-1″/C-3) in the HMBC spectrum of **11** confirmed that the sugar was attached to the aglycone 3-position. The downfield shifts of H-6A″ (*δ* 4.63), H-6B″ (*δ* 4.39) and C-6″ (*δ* 65.4) (Tables 2 and 3), and the crosspeaks at δ 4.63/168.6 (H-6A″/C-1‴) and 4.39/168.6 (H-6B″/C-1‴) in the HMBC spectrum, showed that the malonyl moiety was linked to the sugar 6″-position. Thus, the identity of **11** was confirmed to be peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) (Fig. 1).

In this paper we report identification of four cyanidin 4′-glucosides, **1**–**4**. With exception of cyanidin 4′-glucoside, **1**, and cyanidin 3,4′-diglucoside, **2**, previously reported to occur in flowers of *Hibiscus esculentus* (Malvaceae) by Hedin et al. (1968), this is the first identification of anthocyanins with 4′-glycosidation. However, the identification of the 4′-glucoside linkages of **1** and **2** from *H. esculentus,* were mainly based on lack of bathochromic shifts by the addition of $AlCl_3$ to a solution containing the anthocyanins. This proof, which leans on lack of the typical complexation between Al^{3+} and the 3′- and 4′-hydroxyl groups on the B-ring of cyanidin derivatives without B-ring substitution, is inadequate for the absolute identification of the 4′-linkages published by Hedin et al. (1968). Kuwada (1961) has indeed identified cyanidin 3-xylosylglucoside and cyanidin 3-glucoside from flowers of *H. esculentus*, and all other papers on anthocyanins from species in the genus *Hibiscus* have reported similar anthocyanins with their glycosyl moieties in the 3- and 5-positions. Among the deoxyanthocyanins, Nair and Mohandoss (1985) have indicated the occurrence of luteolinidin 4′-glucuronide from flowers *Holmskioldia sanguinea* (Verbenaceae). With exception of the 3′-(2″-galloylgalactoside) and 3′-(2″-galloyl-6″-acetylgalactoside) of delphinidin isolated from flowers of *Nymphaea caerulea* (Fossen and Andersen, 1999), pigments **4** and **1** are the only anthocyanins with B-ring sugar substitution and no glycosyl moiety in the anthocyanidin 3-position. Peonidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside)-5-*O*-β-glucopyranoside, **10**, has been reported to occur in *Lamium amplexicaule* and *Prunella* sp. (Labiatae) (Saito and Harborne, 1992).

During the isolation process the free carboxyl terminus of the malonyl group may be esterified by the solvent, a reaction which is catalysed by acid (TFA) in the solvent. Thus, the methylmalonyl derivatives of **3**, **4** and **9** (**9b**) from the acidified methanolic extract of red onion were isolated and identified. We have observed that methylation of the free carboxyl group of the malonyl restrict the exchange of the malonyl methylene protons with deuterium, which allow detection of the corresponding methylene ^{13}C signal. The methylene ^{13}C signal of malonyl units with free carboxyl group, is normally difficult to detect. Chromatographic and spectroscopic data on cyanidin 3-*O*-(3″-*O*-β-glucopyranosyl-6″-*O*-methylmalonyl-β-glucopyranoside), **9b**, have been included in Tables 1–3.

The four cyanidin derivatives, **1**–**4**, which have a glucose unit located in the 4′-position, showed UV–Vis spectral properties similar to those of pelargonidin

derivatives (Table 1). Compared to cyanidin glycosides without substitution on the B-ring, for instance pigments **5**–**9**, the cyanidin 4′-glucoside derivatives showed hypsochromic shifts (8–12 nm) of their visible λ_{max} values. 4′-Glucosidation of these cyanidin derivatives resulted also in hyperchromic effects on wavelengths around 440 nm as revealed by their $A_{440}/A_{Vis\text{-}max}$ ratio in the range of 45–50%. Both the visible λ_{max} values and these $A_{440}/A_{Vis\text{-}max}$ ratios have previously been used to characterize pelargonidin derivatives (Andersen, 1988).

3. Experimental

3.1. Plant material

Red onion, *A. cepa* was bought on a local food market. A voucher specimen has been deposited at the University of Bergen.

3.2. Isolation of pigments

The pigmented scales of 2.87 kg red onion were cut into pieces and extracted 20 h (2 times) with 0.5% TFA in MeOH at 4 °C. The filtered extract was concd under red. pres., purified by partition against EtOAc (equal volume, four times) and applied to an Amberlite XAD-7 column (Andersen, 1988). The anthocyanins were further fractionated by Sephadex LH-20 column chromatography using MeOH–H_2O–TFA (39.6:60:0.4; v/v) as eluent. Anthocyanins were isolated by preparative HPLC (ODS-Hypersil column (25×2.2 cm, 5 μm) and Gilson 305/306 pump equipped with an HP-1040A detector) using the solvents HCOOH–H_2O (1:19, v/v) (A) and HCOOH–H_2O–MeOH (1:9:10, v/v) (B). The elution profile consisted of a linear gradient from 10% B to 100% B for 45 min, isocratic elution (100% B) for the next 13 min, followed by linear gradient from 100% B to 10% B for 1 min. The flow rate was 14 ml min^{-1}, and aliquots of 300 μl were injected.

Analytical HPLC was performed with an ODS-Hypersil column (25×0.4 cm, 5 μm) using the solvents HCOOH–H_2O (1:19) (A) and HCOOH–H_2O–MeOH (1:9:10) (B). The elution profile consisted of a linear gradient from 10% B to 100% B for 23 min, isocratic elution, 100% B, for the next 5 min, followed by linear gradient from 100% B to 10% B for 1 min. The flow rate was 0.75 ml min^{-1}, and aliquots of 10 μl were injected.

3.3. Spectroscopy

UV–Vis absorption spectra were recorded on-line during HPLC analysis. Spectral measurements were made over the wavelength range 240–600 nm in steps of 2 nm.

The NMR experiments were obtained at 600.13 MHz 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 SEFT experiment which was performed at 100.61 MHz on a Bruker DMX-400 instrument equipped with an inverse probe. Sample temperatures were stabilised at 25 °C. The deuteriomethyl ^{13}C signal and the residual ^1H signal of the solvent (CF_3CO_2D–CD_3OD; 5:95, v/v) were used as secondary references (δ 49.0 and δ 3.40 from TMS, respectively). The Spin-Echo Fourier Transform (SEFT) experiment was performed with 1600 transients. The spectral width was 20 161 Hz. The one-bond proton-carbon shift correlations were established using phase-sensitive gradient-selected Heteronuclear Single Quantum Coherence, HSQC (Braun et al., 1996). The experiment was optimised for a one-bond proton–carbon coupling constant of 145 Hz. 140 FIDs were recorded in t_1 and 2K data points in t_2, and 705 transients were collected for each t_1 increment. The spectral widths were 21 128 Hz in f_1 and 5000 Hz in f_2. The proton–carbon shift correlations by long-range coupling were established using the heteronuclear multiple bond correlations (HMBC) experiment (Braun et al., 1996). 256 FIDs were recorded in t_1 and 2K data points in t_2, and 200 transients were collected for each t_1 increment. The spectral widths were 22 638 Hz in f_1 and 3720 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 (Braun et al., 1996). The experiment was optimised for a one-bond proton-proton coupling constant of 7.5 Hz. 300 FIDs were recorded in t_1 and 4K data points in t_2, and 70 transients were collected for each t_1 increment. The spectral width was 1395 Hz. The total correlations between the protons belonging to each sugar unit were established by the TOtal Correlation SpectroscopY experiments (TOCSY) (Bax and Davis, 1985). 140 FIDs were recorded in t_1 and 4K data points in t_2, and 80 transients were collected for each t_1 increment. The spectral width was 1532 Hz.

Mass spectral data were achieved by a LC–MS system (Waters 2690 HPLC-system connected to Micromass LCZ mass spectrometer) with electrospray ionization in positive mode (ESP+). The following ion optics were used: Capillary 3 kV, cone 30 and 60 V, and extractor 7 V. The source block temperature was 120 °C and the desolvation temperature was 150 °C. The electrospray probe-flow was adjusted to 100 μl/min. Continuous mass spectra were recorded over the range m/z 150–1200 with scan time 1 s and interscan delay 0.1 s.

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