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ACYLATED FLAVONOL GLYCOSIDES FROM SPINACH LEAVES (*SPINACIA OLERACEA*)

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; flavonoids; acylated and feruloylated flavonol glycosides.

Abstract—From spinach leaves (cv Viroflay), five new naturally occurring flavonoids have been isolated and identified by ^{13}C NMR, ^{1}H NMR, FAB-MS, UV and hydrolytic and enzymatic procedures. The new compounds were identified as spinacetin 3-*O*-*β*-D-glucopyranosyl(1 → 6)-[*β*-D-apiofuranosyl(1 → 2)]-*β*-D-glucopyranoside, patuletin 3-*O*-*β*-D-(2″feruloylglucopyranosyl)(1 → 6)-[*β*-D-apiofuranosyl(1 → 2)]-*β*-D-glucopyranoside, spinacetin 3-*O*-*β*-D-(2″-*p*-coumaroylglucopyranosyl)(1 → 6)-[*β*-D-apiofuranosyl(1 → 2)]-*β*-D-glucopyranoside, spinacetin 3-*O*-*β*-D-(2″feruloylglucopyranosyl)(1 → 6)-[*β*-D-apiofuranosyl(1 → 2)]-*β*-D-glucopyranoside and spinacetin 3-*O*-*β*-D-(2″feruloylglucopyranosyl)(1 → 6)-*β*-D-glucopyranoside. The known compounds jaceidin 4′-glucuronide, 5,3′,4′-trihydroxy-3-methoxy-6:7-methylenedioxyflavone 4′-glucuronide, 5,4′-dihydroxy-3,3′-dimethoxy-6:7-methylenedioxyflavone 4′-glucuronide, patuletin 3-glucosyl(1 → 6)-[apiosyl(1 → 2)] glucoside and patuletin and spinacetin 3-gentiobiosides, were also detected. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

As part of our studies on phenolic compounds from vegetables, we reinvestigated the flavonoids present in the leaves of spinach, since this is a major vegetable consumed in most developed countries after decoction of either fresh or frozen leaves.

In a previous study, the flavonoids jaceidin 4′-glucuronide; 5,3′,4′-trihydroxy-3-methoxy-6:7-methylenedioxyflavone 4′-glucuronide and 5,4′-dihydroxy-3,3′-dimethoxy-6:7-methylenedioxyflavone 4′-glucuronide were identified from *Spinacia oleracea* L. leaves [1]. The same authors later described some additional constituents including spinatoside (5,7,3′,4′-tetrahydroxy-3,6-dimethoxyflavone 4′-*β*-D-glucuronide), patuletin 3-glucosyl-(1 → 6)[apiosyl (1 → 2)]-glucoside and patuletin and spinacetin 3-gentiobiosides [2]. The purpose of the present work was the isolation and identification of some acylated flavonoid glycosides which had not been previously reported.

RESULTS AND DISCUSSION

The HPLC analyses of spinach leaf extracts revealed the presence of 10 flavonoids (**1**–**10**). The diode-array detector showed that four of them (**3**–**5** and **7**) had UV spectra which suggested acylation with hydroxycinnamic acid derivatives [3, 4]. The HPLC retention times of **1**–**10** are shown in Table 1. Compounds **1**, **6**, **8**, **9** and **10**, coincided with authentic markers previously isolated and identified from spinach leaves [1, 2]. The remaining compounds, which had not been previously reported, were isolated by a combination of Sephadex LH-20 chromatography, reversed-phase LPLC and semipreparative HPLC. The purity of the isolated compounds was checked by analytical HPLC.

Compound **2**, from its UV spectrum appeared to be a non-acylated flavonol with similar characteristics to **1**. The main difference revealed by the UV-VIS study was that **2** did not have an *ortho*-dihydroxy substitution on ring B. UV spectral analysis of **2**, revealed free hydroxyls at the 5, 7 and 4′ positions [5], and acid hydrolysis gave spinacetin, glucose and apiose. Its FAB-MS was consistent with a tetrahydroxy-dimethoxyflavone (A+H = 346) glycosylated with two hexoses and one pentose (M = 802). The ^{1}H NMR data confirmed the structure of the flavonoid nucleus (spinacetin), and the configuration and nature of the three sugars. The spectrum was very similar to that of **1** (Table 2), the only difference being an additional methoxyl signal for the methyl ether at the 3′ position. ^{13}C NMR analysis confirmed the structure

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Table 1. Spinach leaf flavonoids

No.	Structure	R_t (HPLC)* min
1	Patuletin 3-*O*-*β*-D-glucopyranosyl(1 → 6)-[*β*-D-apiofuranosyl(1 → 2)]-*β*-D-glucopyranoside	8.5
2	Spinacetin 3-*O*-*β*-D-glucopyranosyl(1 → 6)-[*β*-D-apiofuranosyl(1 → 2)]-*β*-D-glucopyranoside	11.3
3	Patuletin 3-*O*-*β*-D-(2″feruloylglucopyranosyl)(1 → 6)-[*β*-D-apiofuranosyl(1 → 2)]-*β*-D-glucopyranoside	11.8
4	Spinacetin 3-*O*-*β*-D-(2″-*p*-coumaroylglucopyranosyl)(1 → 6)-[*β*-D-apiofuranosyl(1 → 2)]-*β*-D-glucopyranoside	13.4
5	Spinacetin 3-*O*-*β*-D-(2″feruloylglucopyranosyl)(1 → 6)-[*β*-D-apiofuranosyl(1 → 2)]-*β*-D-glucopyranoside	14.0
6	Spinacetin 3-*O*-*β*-D-glucopyranosyl(1 → 6)-*β*-D-glucopyranoside	15.6
7	Spinacetin 3-*O*-*β*-D-(2″feruloylglucopyransoyl)(1 → 6)-*β*-D-glucopyranoside	17.1
8	Jaceidin 4′-glucuronide	17.5
9	5,3′,4′-Trihydroxy-3-methoxy-6:7-methylenedioxyflavone 4′-glucuronide	19.5
10	5,4′-Dihydroxy-3,3′-dimethoxy-6:7-methylenedioxyflavone 4′-glucuronide	21.5

Patuletin = 3,5,7,3′,4′-pentahydroxy-6-methoxyflavone.
Spinacetin = 3,5,7,4′-tetrahydroxy-6,3′-dimethoxyflavone.
Jaceidin = 5,7,4′-trihydroxy-3,6,3′-trimethoxyflavone.
* For HPLC conditions see Experimental.

Table 2. ^{1}H NMR spectra of **1**-**7**

H	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Flavonoid							
H-8	6.45 *s*	6.52 *s*	6.47 *s*	6.50 *s*	6.51 *s*	6.54 *s*	6. 52 *s*
H-2′	7.52 *d*	7.90 *d*	7.58 *d*	7.87 *d*	7.86 *d*	7.93 *d*	7.86 *d*
	$J_{2',6'}$ = 1 Hz	$J_{2',6'}$ = 1 Hz	$J_{2',6'}$ = 1 Hz	$J_{2',6'}$ = 1 Hz	$J_{2',6'}$ = 1 Hz	$J_{2',6'}$ = 1 Hz	$J_{2',6'}$ = 1 Hz
H-5′	6.82 *d*	6.91 *d*	6.82 *d*	6.68 *d*	6.88 *d*	6.91 *d*	6.88 *d*
	$J_{5',6'}$ = 8 Hz	$J_{5',6'}$ = 8 Hz	$J_{5',6'}$ = 8 Hz	$J_{5',6'}$ = 8 Hz	$J_{5',6'}$ = 8 Hz	$J_{5',6'}$ = 8 Hz	$J_{5',6'}$ = 8 Hz
H-6′	7.63 *dd*	7.54 *dd*	7.62 *dd*	7.58 *dd*	7.56 *dd*	7.49 *dd*	7.55 *dd*
OMe 6	3.74 *s*	3.75 *s*	3.69 *s*	3.70 *s*	3.68 *s*	3.75 *s*	3.68 *s*
OMe 3′	—	3.85 *s*		3.85 *s*	3.78 *s*	3.84 *s*	3.85 *s*
Acid							
H-α			6.21 *d*	6.20 *d*	6.16 *d*		6.16 *d*
			$J_{\alpha,\beta}$ = 17 Hz	$J_{\alpha,\beta}$ = 17 Hz	$J_{\alpha,\beta}$ = 17 Hz		$K_{\alpha,\beta}$ = 17 Hz
H-*β*			7.54 *d*	7.46 *d*	7.46 *d*		7.46 *d*
H-2			7.38 *d*	7.53 *d*	7.37 *d*		7.37 *d*
			$J_{2,6}$ = 0.5 Hz	$J_{2,3}$ = 3 Hz	$J_{2,6}$ = 0.5 Hz		$J_{2,6}$ = 0.5 Hz
H-3				6.87 *d*			
H-5			6.85 *d*	6.87 *d*	6.86 *d*		6.85 *d*
			$J_{5,6}$ = 8 Hz		$J_{5,6}$ = 8 Hz		$J_{5,6}$ = 8 Hz
H-6			7.12 *dd*	7.53 *d*	7.12 *dd*		7.12 *dd*
OMe 3			3.94 *s*		3.94 *s*		3.95 *s*
Inner glucose							
H-1	5.52 *d*	5.58 *d*	5.59 *d*	5.57 *d*	5.63 *d*	5.49 *d*	5.63 *d*
	J = 7 Hz	*J* = 7 Hz	*J* = 7 Hz	*J* = 7 Hz	*J* = 7 Hz	*J* = 7 Hz	*J* = 7 Hz
Apiose							
H-1	5.34 *s*	5.32 *s*	5.33 *s*	5.30 *s*	5.31 *s*	—	—
Terminal glucose							
H-1	4.0 *d*	4.0 *d*	4.28 *d*	4.28 *d*	4.30 *d*	4.07 *d*	4.30 *d*
	J = 7 Hz	*J* = 7 Hz	*J* = 7 Hz	*J* = 7 Hz	*J* = 7 Hz	*J* = 7 Hz	*J* = 7 Hz
H-2	—		4.39 *t*	4.36 *t*	4.38 *t*	—	4.39 *t*
			J = 7 Hz	*J* = 7 Hz	*J* = 7 Hz		*J* = 7 Hz

Measurements at 300 MHz in DMSO-d_6 with TMS as internal reference.

Table 3. ^{13}C NMR data of flavonoids **1**, **2**, **5** and **6**

C	1	2	5	6
Flavonoid				
2	156.1	156.1	156.1	156.5
3	132.8	132.5	132.6	132.7
4	177.5	177.4	177.7	177.7
5	152.4	152.3	152.4	152.4
6	131.3	131.2	131.6	131.3
7	151.6	151.6	151.7	151.7
8	93.9	94.0	94.6	94.1
9	157.8	157.6	158.2	157.5
10	104.3	104.4	104.4	104.5
1′	122.0	122.2	122.4	122.2
2′	116.1	115.2	115.7	115.3
3′	144.9	146.9	147.0	147.0
4′	148.5	149.4	149.4	149.5
5′	115.2	113.2	115.4	113.4
6′	121.2	121.1	121.2	121.1
OMe 6	60.0	60.0	60.0	60.1
OMe 3′		55.8	55.9	55.9
Acid				
CO			165.9	
C-α			114.3	
C-β			145.2	
1			125.8	
2			110.7	
3			149.7	
4			148.2	
5			113.0	
6			123.4	
OMe 3			55.7	
Inner glucose				
1	98.7	98.8	98.8	101.0
2	77.0[a]	76.8[a]	76.9[a]	76.8[a]
3	76.9[a]	76.6[a]	76.4[a]	76.6[a]
4	69.7[b]	70.1[b]	70.4[b]	69.7[b]
5	76.6[a]	76.5[a]	76.1[a]	76.6[a]
6	68.1	67.8	66.9	67.8
Apiose				
1	108.7	108.6	108.7	
2	76.6[a]	76.8[a]	76.4[a]	
3	79.3	79.3	79.4	
4	74.0	74.1	74.3	
5	64.4	64.5	64.7	
Terminal glucose				
1	103.2	103.1	100.2	103.2
2	73.4	73.4	73.8	73.5
3	76.3[a]	76.3[a]	73.5	76.5[a]
4	70.1[b]	69.7[b]	69.7[b]	69.7[b]
5	76.2[a]	76.0[a]	78.5	76.3[a]
6	60.8	60.8	60.3	60.8

Measured at 75 MHz in DMSO-d_6 with TMS as internal reference. Values with the same superscript may be interchangeable in the vertical column.

of **2** and the interglycosidic linkages (Table 3). Thus **2** was identified as spinacetin (3-*O*-β-D-glucopyranosyl(1 → 6)-[β-D-apiofuranosyl(1 → 2)]-β-D-glucopyranoside.

Compounds **3**, **5** and **7**, all produced ferulic acid after alkaline hydrolysis. Acylation with ferulic acid was also envisaged from their UV spectra with maxima for BI at 336–337 nm [4]. Alkaline hydrolysis, of

3 rendered **1**, **5** rendered **2**, and of **7** rendered **6** as shown by the HPLC analyses of the hydrolysis products and indicated that both acylated and deacylated flavonoids were present in spinach leaves. Acid hydrolysis of **3** gave ferulic acid, patuletin, glucose and apiose, of **5** gave spinacetin, glucose and apiose and of **7** gave spinacetin and glucose. Enzyme hydrolysis with β-D-glucosidase had no effect on these compounds, while glucose was readily released from **2**, showing that the ferulic acid residue was located on the terminal glucose in **3**, **5** and **7**. The ^{1}H NMR study confirmed the nature of the aglycones and sugars, as well as the presence of one feruloyl residue per molecule (Table 2). These results were consistent with the data obtained from the FAB-MS analyses. The feruloyl residues are shown to be attached to the 2-hydroxyl of the terminal glucoses by the ^{1}H NMR spectra, in which both the terminal glucose H-1 doublet at δ 4.28–4.30 and the coupled (^{1}H–^{1}H-COSY) H-2 triplets at δ 4.38–4.39 appeared markedly downfield from those in the deacylated derivatives **1**, **2** and **6** (Table 2) (H-1 terminal glucose at δ 4.0–4.07 and H-2 at δ 3.5–3.7) [6]. The ^{13}C NMR spectrum of **5** (Table 3), is also consistent with acylation at this position since both C-1 and C-3 signals of the terminal glucose appear upfield by about 3 ppm, from signals of the corresponding deacylated derivatives [7]. Thus, **3** was identified as patuletin 3-*O*-β-D-(2″feruloylglucopyranosyl)(1 → 6)-[β-D-apiofuranosyl(1 → 2)]-β-D-glucopyranoside, **5** as spinacetin 3-*O*-β-D-(2″feruloylglucopyranosyl)(1 → 6)-[β-D-apiofuranosyl(1 → 2)]-β-D-glucopyranoside and **7** as spinacetin 3-*O*-β-D-(2″feruloylglucopyranosyl)(1 → 6)-β-D-glucopyranoside.

Compound **4** also showed a UV spectrum typical of an acylated derivative, but in this case the maximum for BI was at 316 nm. Alkaline hydrolysis of **4** produced *p*-coumaric acid and **2** while acid hydrolysis gave spinacetin, glucose and apiose in addition to *p*-coumaric acid. Enzyme hydrolysis with β-glucosidase, did not release glucose, suggesting that an acyl group was attached to the terminal glucose. FAB-MS analysis confirmed the nature of the aglycone and the occurrence of a single *p*-coumaric residue in the molecule. The ^{1}H NMR data confirmed the aglycone substitution pattern, the presence of two glucose residues and one apiose, and acylation with *p*-coumaric acid at the 2-hydroxyl of the terminal glucose, since the H-1 doublet of the terminal glucose at δ 4.28 and the coupled H-2 triplet at δ 4.36 appear markedly downfield from those in the deacylated compound **2** (Table 2). Thus **4** was identified as spinacetin 3-*O*-β-D-(2″-*p*-coumaroylglucopyranosyl)(1 → 6)-[β-D-apiofuranosyl (1 → 2)]-β-D-glucopyranoside.

Compounds **2**–**5** and **7** are new naturally occurring flavonoids.

EXPERIMENTAL

Plant material and flavonoid extraction. Spinach (cv Viroflay) leaves (*ca* 1.5 kg) were frozen at −70° and freeze dried. The dried plant material was ground and extracted in a Soxhlet first with Et_2O and then with MeOH. The filtered concd MeOH extract was dissolved in H_2O (100 ml) and re-extracted with Et_2O (20 ml × 3) and then concd and redissolved in MeOH.

Flavonoid isolation and purification. The MeOH extract was then chromatographed on a Sephadex LH-20 column (Pharmacia, Uppsala) (45 × 3 cm column) with MeOH, and the composition of the different fractions visualized under UV light (360 nm), was tested by HPLC (column LiCrochart RP-18, 12.5 × 0.4 cm, 5 μm particle size, Merck-Darmstadt) using as mobile phase H_2O–HCOOH (19:1) (A), and MeOH (B). Elution was performed at a solvent flow-rate of 1 ml/min using a gradient starting with 15% B to reach 35% B at 15 min and 40% B at 25 min. Detection was achieved with a photo-diode array detector in order to register the UV spectra of the phenolic constituents. The collected fractions were rechromatographed on a LPLC (Lobar Column (Merck-Darmstadt) RP-18, 40–63 μm particle size, 44 × 3.7 cm) and the resulting fractions visualized under UV light (360 nm). Elution was isocratic in all cases starting with MeOH–H_2O (1:3) followed by 30, 35, 40 and 45% MeOH in H_2O. The composition of the fractions obtained was checked again by HPLC, and the compounds purified by semiprep. HPLC on a Spherisorb ODS-2 column (25 × 0.7 cm, 5 μm particle size), using as solvent isocratic MeOH–H_2O mixts (25–35% MeOH) depending on the polarity of the different flavonoids.

Acid hydrolysis. The isolated flavonoids (*ca* 1 mg) in MeOH (0.5 ml) were heated with 1 ml of 2 N HCl at 90° for 40 min. The resultant aglycones and released cinnamic acids were extracted into EtOAc and analysed by HPLC (see conditions above), and the sugars in the water layer by PC in PhOH–H_2O (4:1).

Alkaline hydrolysis. The isolated flavonoids (*ca* 1 mg) were hydrolysed with 2 N NaOH (1 ml) in the presence of N_2 in the dark for 24 hr at room temp. The product was directly injected, after filtration through 0.45 μm, into the HPLC for cinnamic acid and flavonoid glycoside analysis.

Enzyme hydrolysis. Enzyme hydrolysis was carried out using β-D-glucosidase in a pH 5, 0.1 M acetate buffer for 5 hr at 35°. The products were recovered by filtration though a solid-phase extraction cartridge (RP-18, Whatman), and eluted with MeOH. The products were analysed by HPLC.

Flavonoid identification. The isolated flavonoids were subjected to UV–VIS spectral analysis using classical shift reagents [5], ^{1}H NMR, ^{13}C NMR, H–H COSY and FAB-MS.

2. UV λ_{max}^{MeOH}: 257, 272sh, 349; +NaOMe: 272, 337, 405; +$AlCl_3$: 274, 283sh, 307sh, 364, 410sh; +$AlCl_3$HCl: 275, 283sh, 310sh, 367, 411sh; +NaOAc: 273, 322, 385; +NaOAc/H_3BO_3: 258, 272sh, 354 nm. FAB-MS (positive mode in nitrobenzylalcohol): 800 (M-2); 346 (A + H).

3. UV λ_{max}^{MeOH}: 255, 274, 295sh, 337, 371sh;

+ NaOMe: 269, 312sh, 389; + $AlCl_3$: 277, 282sh, 323, 433; + $AlCl_3$/HCl: 276, 283sh, 323, 364; + NaOAc: 274, 336, 394; + NaOAc/H_3BO_3: 266, 275sh, 334, 380 nm. FAB-MS (positive mode in nitrobenzylalcohol): 964 M+; 332 (A + H).

4. UV λ_{max}^{MeOH}: 258, 275, 300sh, 319, 356sh; + NaOMe: 271, 313sh, 376, 415sh; + $AlCl_3$: 276, 282sh, 316, 361; + $AlCl_3$/HCl: 277, 283sh, 311, 366; + NaOAc: 277, 319, 380; + NaOAc/H_3BO_3: 260sh, 276, 320, 360sh nm. FAB-MS (positive mode in nitrobenzylalcohol): 948 (M+), 346 (A + H).

5. UV λ_{max}^{MeOH}: 255, 275, 300sh, 336, 373sh; + NaOMe: 268, 278sh, 314sh, 340sh, 392; + $AlCl_3$: 275, 283sh, 331, 363sh; + $AlCl_3$/HCl: 276, 283sh, 322, 358; + NaOAc: 273, 335, 391; + NaOAc/H_3BO_3: 275, 301sh, 335, 379sh nm. FAB-MS (positive mode in nitrobenzylalcohol): 978 (M+), 346 (A + H).

7. UV λ_{max}^{MeOH}: 255, 275, 298sh, 336, 370sh; + NaOMe: 269, 277sh, 348sh, 392, 432sh; + $AlCl_3$: 275, 282sh, 323, 365; + $AlCl_3$/HCl: 275, 282sh, 318, 362; + NaOAc: 275, 329, 396; + NaOAc/H_3BO_3: 276, 299sh, 332, 375sh nm. FAB-MS (positive mode in nitrobenzylalcohol): 844 (M-2); 346 (A + H).

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