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Short communication

High-performance liquid chromatographic determination of flavonoids in *Betula pendula* and *Betula pubescens* leaves

Markku Keinänen*, Riitta Julkunen-Tiitto

Department of Biology, University of Joensuu, P.O. Box 111, FIN-80101 Joensuu, Finland

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Abstract

A high-performance liquid chromatography (HPLC) procedure based on a water–methanol gradient with tetrahydrofuran for simultaneous analysis of flavonoids and other phenolics of differing polarities in birch leaves was developed. Mobile phases with and without tetrahydrofuran or orthophosphoric acid provided additional information for tentative identification of the compounds. Forty-five compounds were determined from *Betula pendula* and *B. pubescens* leaves. Five of the isolated flavonol glycosides have not been reported earlier from *B. pendula*. *B. pendula* leaves have a range of myricetin and quercetin glycosides with the same sugar moieties in roughly the same proportions. Flavonoid aglycones were deposited on leaf surfaces. © 1998 Elsevier Science B.V.

Keywords: *Betula pendula*; *Betula pubescens*; Flavonoids; Phenolic compounds; Glycosides; Aglycones

1. Introduction

Flavonoid glycosides of *Betula pendula* Roth and *Betula pubescens* Ehrh. have been investigated in several studies relating to pharmacology, chemotaxonomy or chemical ecology [1–16]. Quercetin-3-galactoside, quercetin-3-rhamnoside and myricetin-3-digalactoside were identified in early studies which treated the species collectively as *B. alba* L. [1–3]. In an early chemotaxonomic survey of the genus *Betula*, quercetin-3-arabinoside, kaempferol-3-glucoside, isorhamnetin-3-galactoside and hesperetin-7-rutinoside were reported from both species [4–7] and quercetin-3-glucoside, luteolin-4′-glucoside and acacetin-7-glucoside from *B. pubescens* [5–7].

More sophisticated techniques were used to identify six flavonoid glycosides from *B. pendula* and *B. pubescens* [10]: quercetin-3-galactoside, quercetin-3-rhamnoside, quercetin-3-glucuronide, quercetin-3-arabinopyranoside, quercetin-3-arabinofuranoside and myricetin-3-galactoside. Four new flavonoid glycosides have recently been isolated from *B. pubescens*: myricetin-3-glucuronide, myricetin-3-O-α-L-(acetyl)-rhamnopyranoside, quercetin-3-O-α-L-(4″-O-acetyl)-rhamnopyranoside and kaempferol-3-rhamnoside [15].

Various flavonoid aglycones have been identified from leaf buds of birches [17–19]. A few aglycones have also been detected in birch leaves [4,5,12,15,16,20]. However, it has not been shown, whether these are artifacts resulting from unintended hydrolysis of glycosides during sample preparation and extraction, or, are deposited externally on epicuticular wax layers of leaves [21].

The purpose of this study was to develop a high-performance liquid chromatography (HPLC) proce-

*Corresponding author.

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dure for simultaneous determination of phenolic compounds of differing polarities, such as phenolic acids, flavonoid glycosides and aglycones, and to identify the phenolics present in birch leaves, at least tentatively. Furthermore, the effect of mobile phase composition on retention and selectivity and as an aid in partial identification of phenolic compounds was studied. Because recent flavonoid studies of *B. pendula* and *B. pubescens* have been partly inconsistent [9–16], the phenolic composition of these two species was compared.

2. Experimental

2.1. Sample preparation

A composite sample of *B. pendula* long-shoot leaves was collected in August from stands on a clear felling near Joensuu, Finland [12]. After air-drying, full-grown leaves were separated from the shoots and milled. The powdered material was stored at −20°C until used. A separate sample of whole air-dried leaves (50 g) from the same trees was used for the analysis of epicuticular components. In addition, samples of *B. pendula* and *B. pubescens* short-shoot leaves were collected in August from full-grown birches in Joensuu.

For the isolation of flavonoid conjugates, powdered leaves (10 g) were extracted twice with 250 ml of 80% ethanol using an Ultra-Turrax homogenizer for 3 min. The extract was filtered and the residue was washed with 100 ml of 96% ethanol. The combined extract was evaporated to dryness, redissolved in 15 ml of 30% ethanol, and fractionated on a polyamide column (40×1.6 cm I.D., Polyamid SC-6, 0.05–0.16 mm, Macherey–Nagel, Düren, Germany) by consecutive elution with 10%, 50%, 80% and 100% ethanol. UV-absorbing fractions were detected at 280 nm (LKB 8300A Uvicord, Bromma, Sweden). After freeze–drying, the fractions containing flavonoid glycosides (50% and 80% ethanol) were taken up in water–methanol (1:1), and combined, because they contained the same flavonoid glycosides. HPLC analysis indicated that three flavonoids tentatively identified as glucuronides were retained in the polyamide column.

For analytical HPLC, two dried leaves (120–350 mg) were cut with scissors and crushed with a glass rod into 5–20 mm^2 pieces and left to steep for 10 min in 25 ml of 80% ethanol. The samples were extracted twice with 25 ml of 80% ethanol using an Ultra-Turrax homogenizer for 3 min. The extract was filtered and the residue was washed with 20 ml of 96% ethanol. The combined extract was evaporated to dryness. Before analysis, extracts were dissolved in methanol–water (1:1).

Epicuticular compounds were extracted by washing air–dried long-shoot leaves (50 g) for 30 s in methanol. The extract was evaporated to dryness and dissolved in 50 ml of warm methanol (50°C). Waxes were precipitated by cooling for 18 h at −20°C [22]. Before analysis, the extract was dissolved in methanol–water (4:1). For comparison, a sample of three washed leaves was analysed by HPLC.

2.2. Standards and solvents

Apigenin, acacetin, luteolin, kaempferol, myricetin, quercetin, naringenin, myricetin-3-O-rhamnoside, quercetin-3-O-galactoside, quercetin-3-O-arabinopyranoside and quercetin-3-O-rhamnoside were from Roth (Karlsruhe, Germany), quercetin-3-O-glucoside, kaempferol-3-O-glucoside and picein were from Extrasynthese (Genay, France) and kaempferol-3-O-rhamnoside was from Apin Chemicals (Abingdon, Oxon, UK). Chlorogenic acid, caffeic acid, 4-hydroxycinnamic acid and (+)-catechin were from Aldrich (Steinheim, Germany). Methanol, acetonitrile, and tetrahydrofuran (THF) were of HPLC grade (Lab-Scan, Dublin, Ireland).

2.3. Chromatographic system

The HPLC system was a Hewlett-Packard (Avondale, PA, USA) instrument with a quaternary pump (HP 1050), an autosampler (HP 1050) and a photodiode array detector (HP 1040A) combined with HP ChemStation. A 3-μm HP Hypersil ODS column (60×4.6 mm I.D.) was used. The solvents were A (aqueous 2.4% THF+0.25% orthophosphoric acid) and B (methanol). The elution system was: 0–4 min, 2–12% of B in A; 4–30 min, 12–35% of B in A; 30–45 min, 35–60% of B in A. The flow-rate was 2 ml/min and the injection volume 20 μl. The analysis was monitored at 220, 280, 320 and 360 nm. In order

to study the effect of pH and THF on elution of birch phenolics, leaf samples of *B. pendula* and *B. pubescens* were analysed on mobile phases containing no orthophosphoric acid or THF.

Individual glycosides were isolated by repeated injection of combined flavonoid glycoside fractions of the polyamide eluate of leaf extracts onto the analytical column. The solvents were A (aqueous 2.4% THF) and B (methanol). The elution system was 0–30 min, 10–30% of B in A. The flow-rate was 2 ml/min and the injection volume 80 μl. For most of the compounds, the separation was repeated by using acetonitrile as solvent B.

2.4. Identification and quantification of compounds

The retention times and UV–Vis spectra of the peaks were compared with authentic reference compounds. Isolated flavonoid glycosides and raw extracts of *B. pendula* and *B. pubescens* leaves were hydrolysed for 10, 30 and 60 min in 1 ml of 2 *M* HCl and methanol (1:1) at 90°C. Spectral analyses of the isolated compounds were performed using standard shift reagents [23]. After acid hydrolyses, the aglycones were determined with HPLC and the sugar residues with gas chromatography–mass spectrometry (GC–MS) by comparison with reference compounds. The GC and GC–MS conditions were as in [24]. Additional information was obtained by HPLC analysis of samples of both species without acid or THF in the mobile phase. Flavonoid glycosides were quantified as equivalents of quercetin-3-galactoside. Flavonoid aglycones were quantified as apigenin, luteolin or kaempferol and flavanones as naringenin. Hydroxycinnamic acid derivatives were quantified as chlorogenic acid, and compound 1 as picein.

3. Results and discussion

3.1. HPLC conditions

Several solvent systems based on acetonitrile, methanol and THF were tested to improve the separation of flavonoids and other phenolic compounds in *B. pendula* leaves. Better results were achieved with acetonitrile and methanol than with THF. Moreover, mobile phases based on methanol or acetonitrile gave comparable results, whereas the use of THF changed the elution order of several peaks altering the chromatogram considerably.

The use of THF in solvent systems based on water–methanol or water–acetonitrile has been reported to give good separations of various phenolic compounds, such as cinnamic acid derivatives [25,26], phenolic glycosides [27], flavonoid glycosides and aglycones [22] and fully methoxylated flavones [28]. Therefore, the addition of THF in solvent systems based on acetonitrile and methanol gradients was tested. In both solvent systems, addition of 1.0–3.0% THF in water acidified with orthophosphoric acid (0.25%) improved the separation of flavonoid glycoside peaks. The exact concentration of THF depends on the initial composition of the mobile phase and the steepness of the gradient. Although acetonitrile gave slightly better separation of flavonoid glycosides, non-flavonoid phenolics separated better with methanol. We preferred methanol-based mobile phase for routine analysis, because it is a good solvent for various kinds of phenolics [e.g., [12,23–25,27]], and has been reported to have excellent properties as a mobile phase for identification of unknown flavonoids by their retention data [29].

Retention parameters of the analytical method (Fig. 1) based on a water–methanol gradient with 2.4% THF in water are given in Table 1. Selectivity factors [30] for the analytical method and mobile phases containing no orthophosphoric acid or THF reveal several changes in elution of the compounds depending on the solvent system. The use of THF affected particularly the elution of compound 1 by reducing considerably its retention. In addition, it increased the retention of glucuronides and rhamnosides compared with other flavonol glycosides. Although this results in better separation of flavonol glycosides than achieved by previously published methods for birch phenolics [8,9,11,15,16], several myricetin peaks were not well separated (Fig. 1, Table 1).

Analysis without orthophosphoric acid in mobile phase provides a simple means of detecting compounds with acidic substituents, such as flavonoid glucuronides or acylated flavonoids, because these eluted much faster without acid (Table 1). The ratio

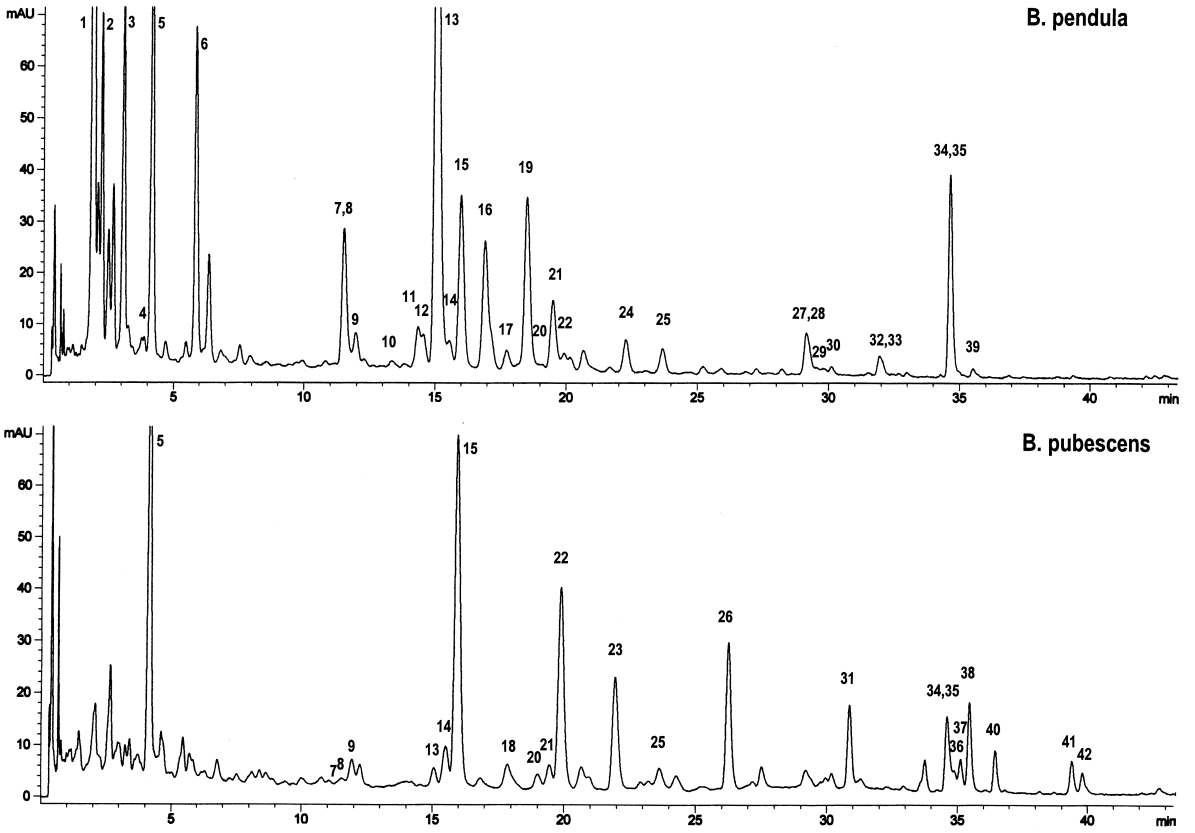

Fig. 1. Chromatograms of *B. pendula* and *B. pubescens* short-shoot leaves at 280 nm. For identification of peaks see Table 1.

of capacity factors of the compounds in these two solvent systems ($k'_{\text{without acid}}/k'_{\text{analytical method}}$) shows that this decrease in retention was especially pronounced for glucuronides (0.30, 0.34 and 0.36 for glucuronides of myricetin, quercetin and kaempferol, respectively; 0.48, 0.42 and 0.53 for compounds 18, 23 and 26, respectively). Although the use of non-acidified mobile phase resulted in poor peak shapes, especially for acidic compounds, the peaks could be identified by their UV–Vis spectra and comparison with quantitative results obtained by analytical method.

3.2. Identification of phenolic compounds

3.2.1. Internal compounds

Compound 1 is invariably present as a major peak in HPLC analyses of *B. pendula* leaves (Fig. 1). Its UV–Vis spectrum resembles that of picein. The compound was tentatively identified as 3,4′-dihydroxypropiophenone-3-β-D-glucopyranoside that has been isolated from leaves of *B. alba* [31], *B. platyphylla* var. *japonica* [32] and *B. pendula* [16]. In *B. pubescens* leaves, chlorogenic acid is the most abundant non-flavonoid phenolic (Table 1). Compounds 2, 3 and 6 were tentatively identified as cinnamic acid derivatives. After acid hydrolysis of leaf extracts of *B. pendula* caffeic and *p*-coumaric acids were detected. On the basis of their UV–Vis spectra and elution order, compounds 2, 3 and 6 could be neochlorogenic acid, *trans*-5-*p*-coumaroylquinic acid and *trans*-3-*p*-coumaroylquinic acid, respectively [16].

A range of compounds were identified as conjugates of myricetin (compounds 7–12), quercetin (13–16, 18, 19, 21, 23) and kaempferol (17, 20, 22, 24–26) (Fig. 1, Table 1), because these peaks disappeared and their corresponding aglycones were

Table 1
Analysis of phenolic compounds in leaves of *B. pendula* and *B. pubescens*

Peak	Compound	Analytical method		No acid,	No THF,	Identification	Content (mg/g dw)	
		t_R (min)	α	α	α	a,b,c,d,e,f,g	*B. pendula*	*B. pubescens*
1	3,4′-Dihydroxypropiophenone-3-glucoside	1.9	0.11	0.12	0.27	a	4.7	−
2	Neochlorogenic acid	2.2	0.13	–	0.19	a,c	0.6	−
3	*p*-Coumaric acid derivative	3.1	0.19	0.05	0.25	a,c	1.1	−
4	(+)-Catechin	3.9	0.24	0.25	–	a,b	0.6	+
5	Chlorogenic acid	4.2	0.26	0.09	0.48	a,b,c	2.7	4.2
6	*p*-Coumaric acid derivative	5.9	0.38	0.13	0.55	a,c	2.1	−
7	Myricetin-3-galactoside	11.5	0.76	0.76	0.81	a,c,d,e	2.6	0.1
8	Myricetin-3-glucoside	11.8	0.78	0.78	0.83	a,c,d	+	0.4
9	Myricetin-3-glucuronide	12.0	0.79	0.24	0.81	a,c,f,g	0.5	0.6
10	Myricetin-3-arabinopyranoside	13.3	0.88	0.88	0.89	a,c,d,e	0.1	0.1
11	Myricetin-3-arabinofuranoside	14.3	0.95	0.95	0.93	a,c,d	0.6	−
12	Myricetin-3-rhamnoside	14.5	0.97	0.96	0.93	a,b,c,d,e	0.2	−
13	Quercetin-3-galactoside	15.0	1.00	1.00	1.00	a,b,c,d,e	8.0	1.2
14	Quercetin-3-glucoside	15.6	1.04	1.03	1.03	a,b,c,d,e	0.3	2.2
15	Quercetin-3-glucuronide	16.0	1.07	0.36	1.03	a,c,f,g	2.2	5.1
16	Quercetin-3-arabinopyranoside	16.9	1.13	1.12	1.11	a,b,c,d,e	1.6	0.2
17	Kaempferol-3-glycoside	17.7	1.18	1.18	1.15	a,c	0.1	+
18	Acylated quercetin conjugate	17.9	1.19	0.57	1.13	a,c,g	−	0.9
19	Quercetin-3-arabinofuranoside	18.5	1.23	1.23	1.16	a,b,c,d,e	2.2	−
20	Kaempferol-3-glucoside	19.0	1.27	1.26	1.21	a,b,c	+	0.9
21	Quercetin-3-rhamnoside	19.5	1.30	1.29	1.20	a,b,c,d,e	0.8	0.3
22	Kaempferol-3-glucuronide	19.9	1.33	0.48	1.23	a,c,f,g	0.2	1.6
23	Acylated quercetin conjugate	21.9	1.47	0.62	1.33	a,c,g	−	3.1
24	Kaempferol glycoside	22.3	1.49	1.48	1.36	a,c	0.3	−
25	Kaempferol-3-rhamnoside	23.7	1.59	1.57	1.39	a,b,c	0.2	0.3
26	Acylated kaempferol conjugate	26.2	1.76	0.93	1.50	a,c,g	−	1.5
27	Apigenin derivative	29.1	1.96	1.96	1.64	a	0.2	−
28	Apigenin	29.2	1.97	1.97	1.61	a,b	+	−
29	Luteolin derivative	9.5	1.98	1.99	1.65	a	+	−
30	Luteolin derivative	30.1	2.02	2.03	1.70	a	+	−
31	Flavanone	30.8	2.07	2.06	1.68	a	−	0.6
32	Luteolin derivative	31.9	2.15	2.16	1.79	a	0.1	−
33	Luteolin derivative	32.0	2.15	2.16	1.80	a	+	−
34	Acacetin	34.6	2.33	2.33	1.89	a,b	+	+
35	Apigenin derivative	34.6	2.33	2.33	1.90	a	0.6	
36	Kaempferol derivative	34.7	2.33	2.33	1.90	a	−	0.4
37	Kaempferol derivative	35.1	2.36	2.35	1.87	a	−	1.1
38	Kaempferol derivative	35.4	2.38	2.37	1.92	a	−	1.6
39	Apigenin derivative	35.5	2.39	2.40	1.94	a	+	−
40	Flavanone	36.4	2.45	2.44	1.97	a	−	0.3
41	Apigenin derivative	39.4	2.65	2.64	2.13	a	−	0.4
42	Kaempferol derivative	39.8	2.68	2.67	2.13	a	−	0.7

Selectivity factors (α) for analytical method with and without orthophosphoric acid or tetrahydrofuran are calculated in relation to quercetin-3-galactoside. The values are averages of 3–6 runs.
Identification: (a) UV–Vis spectrum, (b) co-elution with reference compound, (c) hydrolysis of leaf raw extract and identification of aglycones, (d) isolation from *B. pendula*, acid hydrolysis, and subsequent analysis of aglycones and sugar residues, (e) UV spectra of isolated compounds with standard shift reagents, (f) slow hydrolysis, (g) elution pH-dependent.
Concentrations are averages of short-shoot leaf samples from three individuals.

produced after acid hydrolysis. After 10 min the hydrolysis was nearly complete for all other flavonoid glycosides except for compounds 9, 15 and 22, which were not totally hydrolysed even after 60 min. Furthermore, the elution of these compounds in HPLC analysis was strongly dependent on the pH of the eluent (Table 1). Compounds 9, 15 and 22 were tentatively identified as 3-glucuronides of myricetin [15,16], quercetin [9,10,15,16] and kaempferol, respectively, as the UV–Vis spectra of the compounds were identical to 3-glycosides of corresponding flavonols.

In addition to glucuronides, three other flavonoid peaks (18, 23, and 26) in *B. pubescens* eluted well before other flavonoid glycosides without acid in mobile phase (Table 1). These compounds are probably acylated flavonoid conjugates, as they were easily hydrolysed. Two acylated flavonoids have recently been isolated from *B. pubescens* leaves, and identified with ^{13}C and ^{1}H nuclear magnetic resonance (NMR) as quercetin-3-O-α-L-(4″-O-acetyl)-rhamnopyranoside and myricetin-3-O-α-L-(acetyl)-rhamnopyranoside [15]. Since acyl groups are labile [33], it is possible that they have not been detected in earlier studies of birch flavonoids. In this study, the assumed acylated flavonoids were tentatively identified as derivatives of quercetin (18, 23) and kaempferol (26). Moreover, the UV spectra of these compounds resembled other 3-glycosides of corresponding flavonols rather than 3-rhamnosides with a characteristic shift of band I [34].

After fractionation of *B. pendula* extract on a polyamide column, compounds 7–8, 10–14, 16, 19 and 21 were isolated by repeated injection onto the analytical column. The positions of the sugars were determined by spectral analysis [23]. After acid hydrolysis, the aglycones were determined by HPLC and sugars by GC–MS using reference compounds. The sugar moieties of compounds 10, 11, 16 and 19 were determined by GC–MS to be arabinoses. According to the ratio of TMS–ether peaks m/z 204 and 217, the sugars were assigned as pyranoid (compounds 10 and 16) or furanose forms (compounds 11 and 19) [35]. Myricetin-3-glucoside, myricetin-3-arabinopyranoside, myricetin-3-arabinofuranoside, myricetin-3-rhamnoside and quercetin-3-glucoside have not been isolated before from *B. pendula*.

3.2.2. External compounds

A sample of leaves was washed with methanol in order to extract phenolic compounds present on leaf surfaces. HPLC analyses of these leaf extracts (Fig. 2) and washed leaves indicated a fractionation of foliar phenolics into lipophilic external phenolics (methylated derivatives of apigenin and luteolin) and hydrophilic internal phenolics, such as flavonol glycosides (Fig. 1). In HPLC analysis of washed leaves, only a trace of one flavone aglycone (compound 35) was detected.

Compounds 28 (apigenin) and 34 (acacetin), and a small peak assigned as luteolin (Fig. 2) were co-

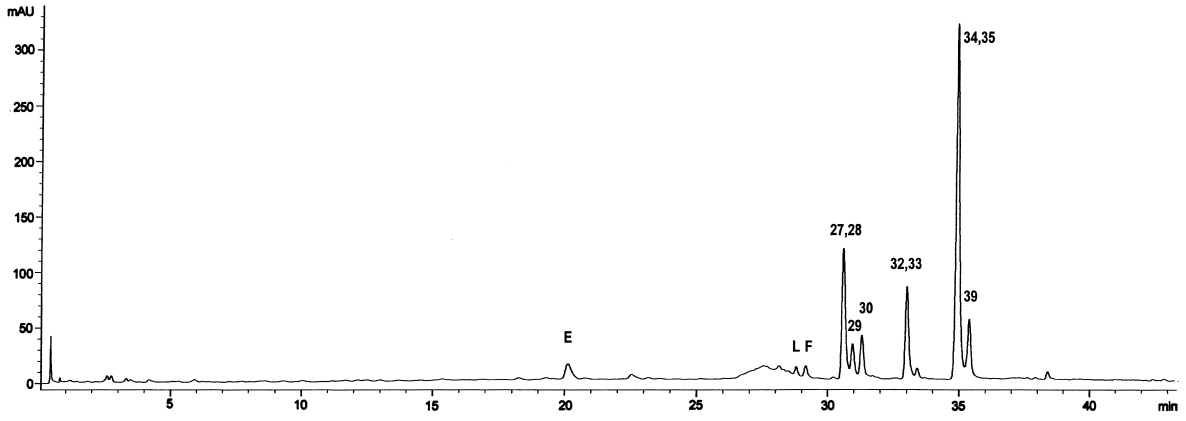

Fig. 2. Chromatogram of the compounds washed from the surfaces of *B. pendula* long-shoot leaves at 280 nm. For identification of peaks, see Table 1 (E=ellagic acid derivative, L=luteolin, F=flavanone).

chromatographed with authentic standards. Based on their UV–Vis spectra, one peak was assigned as an ellagic acid derivative, one as a flavanone, peaks 29, 30, 32 and 33 as derivatives of luteolin, and compounds 35 and 39 as derivatives of apigenin. Compound 35 co-eluted with a small amount of acacetin.

A methanol wash of *B. pubescens* leaves indicated that the compounds 31, 34–38 and 40–42 are deposited on leaf surfaces. The compounds identified as flavanones (31 and 40) eluted later than naringenin, which has been reported from *B. pubescens* [15,16]. Peaks 34–38, 41 and 42 were tentatively assigned as derivatives of apigenin and kaempferol by their UV–Vis spectra (Table 1). Peak 34 (Fig. 1) consisted of at least two flavonoid compounds, one of which was identified as acacetin.

3.3. Flavonoid composition of B. pendula and B. pubescens

Although the total content of low-molecular-mass phenolics (the sum of analysed compounds, Table 1) in short-shoot leaves of adult trees was about the same in both species (33.0 and 29.0 mg/g dry mass, for *B. pendula* and *B. pubescens*, respectively), there were differences in relative proportions of groups of phenolics. External flavonoid aglycones formed 6.2% of the total content of analysed phenolics in *B. pubescens*, but only 1.2% in *B. pendula*. In addition to qualitative differences in flavonoid glycosides, the species differ quantitatively in the ratio of galactosides to glucosides and glucuronides.

In early studies [1–7], the main flavonoids of *B. pendula* and *B. pubescens* were reported to be quercetin-3-galactoside, quercetin-3-rhamnoside and myricetin-3-digalactoside. In this study (Table 1), as well as in other recent studies [9,10,15,16], myricetin-3-digalactoside has not been found, and the main myricetin glycoside was identified as myricetin-3-galactoside. Originally, myricetin-3-digalactoside was identified as a diglycoside after heating in 2% sulfuric acid for 1 h and subsequent quantification of the aglycone and sugar residue [3]. Considering the rather labile structure of myricetin [33], a significant proportion of the aglycone might have degraded during the hydrolysis and thus lead to possibly erroneous identification of the compound as a digalactoside.

Recently, myricetin-3-O-α-L-(acetyl)-rhamnopyranoside was reported to occur in high concentrations in *B. pendula* leaves [16]. We have not found acylated flavonoids in fresh or dried samples of *B. pendula* in this study or during earlier work [12–14]. However, the non-acylated form of this compound, myricetin-3-rhamnoside, is among the main flavonoids in seedlings of *B. pendula* and long-shoot leaves of adult trees [13,36], although it occurs only in low concentration in short-shoot leaves of adult trees (Table 1).

Our results show that *B. pendula* has a characteristic glycosidic pattern of flavonoids, a range of myricetin and quercetin monoglycosides having the same sugar moieties in roughly the same proportions. The same pattern may apply also for kaempferol glycosides (Table 1). However, arabinofuranosides and glucuronides are not observed in all samples of *B. pendula* [14,36]. *B. pubescens* leaves may have an analogous range of flavonol glycosides, but having additional, possibly acylated glycosides.

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References

[1] P. Casparis, P. Sprecher, H.J. Müller, Pharm. Acta Helv. 21 (1946) 341.
[2] R. Hänsel, L. Hörhammer, Arch. Pharm. 287 (1954) 117.
[3] L. Hörhammer, H. Wagner, R. Luck, Arch. Pharm. 290 (1957) 338.
[4] L. Pawlowska, Acta Soc. Bot. Pol. 49 (1980) 281.
[5] L. Pawlowska, Acta Soc. Bot. Pol. 51 (1982) 403.
[6] L. Pawlowska, Acta Soc. Bot. Pol. 51 (1982) 413.
[7] L. Pawlowska, Acta Soc. Bot. Pol. 52 (1983) 301.
[8] H. Wagner, G. Tittel, S. Bladt, Dtsch. Apoth. Ztg. 123 (1983) 515.
[9] K. Dallenbach-Tölke, Sz. Nyiredy, B. Meier, O. Sticher, Planta Med. 53 (1987) 189.
[10] K. Dallenbach-Tölke, Sz. Nyiredy, G.A. Gross, O. Sticher, J. Nat. Prod. 49 (1986) 1155.
[11] P.G. Pietta, P.L. Mauri, E. Manera, P.L. Ceva, Chromatographia 28 (1989) 311.

[12] M. Keinänen, J. Agric. Food Chem. 41 (1993) 1986.
[13] A. Lavola, R. Julkunen-Tiitto, Oecologia 99 (1994) 315.
[14] M. Keinänen, R. Julkunen-Tiitto, J. Agric. Food Chem. 44 (1996) 2724.
[15] V. Ossipov, K. Nurmi, J. Loponen, N. Prokopiev, E. Haukioja, K. Pihlaja, Biochem. Syst. Ecol. 23 (1995) 213.
[16] V. Ossipov, K. Nurmi, J. Loponen, E. Haukioja, K. Pihlaja, J. Chromatogr. A 721 (1996) 59.
[17] K.H. Bauer, H. Dietrich, Chem. Ber. 66 (1933) 1053.
[18] E. Wollenweber, Biochem. Physiol. Pflanzen 166 (1974) 425.
[19] E. Wollenweber, Biochem. Syst. Ecol. 3 (1975) 47.
[20] K. Krawiarz, Arboretum Kornickie 17 (1972) 201.
[21] E. Wollenweber, V.H. Dietz, Phytochemistry 20 (1981) 869.
[22] T. Vogt, P.-G. Gülz, J. Chromatogr. 537 (1991) 453.
[23] K.R. Markham, Techniques of Flavonoid Identification, Academic Press, London, 1982.
[24] R. Julkunen-Tiitto, M. Rousi, J. Bryant, S. Sorsa, M. Keinänen, H. Sikanen, Trees 11 (1996) 16.
[25] E. Roggendorf, R. Spatz, J. Chromatogr. 204 (1981) 263.
[26] P. Mouly, E.M. Gaydou, J. Estienne, J. Chromatogr. 634 (1993) 129.
[27] B. Meier, R. Julkunen-Tiitto, J. Tahvanainen, O. Sticher, J. Chromatogr. 442 (1988) 175.
[28] J.M. Sendra, J.L. Navarro, L. Izquierdo, J. Chromatogr. Sci. 26 (1988) 443.
[29] Y.D. Kahie, C. Pietrogrande, M.I. Medina Mendez, P. Reschiglian, F. Dondi, Chromatographia 30 (1990) 447.
[30] L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley, New York, 1979.
[31] R. Tschesche, A. Hartz, G. Wulff, Phytochemistry 13 (1974) 518.
[32] K. Mori, Z.-H. Qian, S. Watanabe, Liebigs Ann. Chem. (1992) 485.
[33] J.B. Harborne and C.A. Williams, in J.B. Harborne, T.J. Mabry and H. Mabry (Editors), The Flavonoids, Academic Press, New York, 1975, p. 376.
[34] J.B. Harborne, in J.B. Pridham (Editor), Methods in Polyphenol Chemistry, Pergamon Press, Oxford, 1964, p. 13.
[35] M.F. Chaplin and J.F. Kennedy (Editors), Carbohydrate Analysis—A Practical Approach, IRL Press, Oxford, 1986.
[36] M. Keinänen, unpublished results.