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High-performance liquid chromatographic separation and identification of phenolic compounds from leaves of *Betula pubescens* and *Betula pendula*

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Abstract

The following major phenolics with non-flavonoid structure were identified from leaves of *Betula pubescens* (white birch) and *Betula pendula* (silver birch): 1-*O*-galloyl-β-d-(2-*O*-acetyl)-glucopyranose, 1-(4″-hydroxyphenyl)-3′-oxopropyl-β-d-glucopyranose, gallic, chlorogenic, neo-chlorogenic, *cis*- and *trans*-forms of 3- and 5-*p*-coumaroylquinic acids. Chromatographic (analytical and preparative HPLC), chemical (hydrolysis) and spectroscopic (UV, ^{1}H and ^{13}C NMR, MS) techniques were applied for separation, isolation, purification and identification of these phenolics. Moreover, 33 low-molecular-mass phenolics were detected and quantitated and their occurrence was compared in leaves of white and silver birches.

Keywords: *Betula pubescens*; *Betula pendula*; Phenolic compounds

1. Introduction

Phenolic compounds are widely distributed plant constituents. They have often been assumed to play a crucial role in plant–herbivore interactions (e.g. [1], see also [2,3]). *Betula pubescens* has been studied particularly for wound-induced reductions in leaf palatability to insect herbivores [4]. Some studies tried to correlate chemical reactions with the decreased palatability of birch leaves after damage to foliage by demonstrating increased phenol contents after leaf damage [5–7].

Most plant–herbivore studies have quantitated only the total content of phenolic compounds, although it is known that the total consists of a complex set of different types of phenols. It is well known that a diversity of phenolic structures are synthesized and accumulated in cells: hydroxybenzoic and hydroxycinnamic acids, acetophenones, stilbenes, lignans, neolignans, flavonoids, lignin, hydrolyzable and condensed tannins. The ecological activity and mechanisms of action of these compounds on herbivores may be very different. Consequently, to evaluate the true role of phenolics in plant–herbivore rela-

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tionships, it is necessary to be able to quantitate individual phenolic compounds in host plant tissues.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is the most effective and reliable method for quantitative analysis of natural mixtures of phenolic compounds and for their preparative isolation from plant materials [8–10]. Recently, we have used this method for isolation, identification and quantitation of flavonoids from *B. pubescens* leaves [11]. In addition to flavonoids, birch leaves contain a number of non-flavonoid constituents. Identification of these phenolics has proved to be difficult because of scanty information about their composition in birch leaves.

Phenolics of different Betulaceae species, including *B. pubescens* and *B. pendula*, have been studied by Pawlowska [12,13]. Paper chromatography, UV spectroscopy and some chemical methods were used for their separation and identification. In addition to different flavonoids, gallic, protocatechuic and chlorogenic acids were found in foliage of these birches. Up to now, modern methods of analysis (HPLC, MS and ^{1}H NMR) have been applied for the identification of betuloside, platyfylloside, rhododendrin, rhododendrol, salidroside and dehydrosalidroside from the stems of seedlings and from twigs of *B. pendula* [14–16]. We are not aware of any thorough study of non-flavonoid phenolics in the leaves of *B. pendula* or *B. pubescens*.

In this paper, we report results of the isolation and identification of some non-flavonoid phenolics from leaves of white and silver birch. Analytical and preparative RP-HPLC, MS, ^{1}H and ^{13}C NMR were applied to these purposes. Moreover, the main low-molecular-mass phenolics in the leaves of two birch species were quantitated and compared.

2. Experimental

2.1. Plant material

White birch (*Betula pubescens*) and silver birch (*B. pendula*) leaves were sampled in July from trees in the Botanical Garden of University of Turku (Finland). Leaves were collected on ice and transported immediately to the laboratory.

2.2. Sample preparation

Analytical

Fresh birch leaves (1.0–1.5 g) were transferred to 15 ml of methanol, ground for 3 min with an Ultra-turrax T25 and allowed to stand for 60 min with continuous stirring. The homogenate was centrifuged and the pellet was re-extracted twice with 15 ml of 80% methanol. The combined extracts were evaporated to dryness using a rotary evaporator. The dry residue was then dissolved in 20 ml of water and centrifuged.

Preparative

Birch leaves were air-dried for 36 h at the room temperature and for 12 h at 50°C in a heating cupboard. Then they were ground to fine powder and used for preparative isolation of individual phenolics.

Fine powder of birch leaves (40 g dry mass) was suspended in 300 ml 70% ethanol and allowed to stand for 5 h with continuous stirring. After filtration, the insoluble residue was re-extracted twice with the same solvent. The extracts were combined (about 650 ml) and tannins were precipitated by our modification of the Marigo method [17] (NaCl was not added to the extract). The precipitate was separated from the supernatant by centrifuging for 15 min at 2000 *g*. After purification, the extract was evaporated under low pressure, the residue redissolved in 25 ml of water and centrifuged.

2.3. Equipment

Analytical chromatographic analysis was performed with an HPLC system consisting of a Kontron HPLC pump 420 with HPLC gradient Former 425 (Kontron, Zürich, Switzerland), a Perkin-Elmer LC-235 diode-array detector (DAD) with Graphic Printer GP-100 and C-R6A Chromatopac Integrator (Shimadzu, Kyoto, Japan). Injections were made via a Rheodyne rotary valve (Cotati, CA, USA) with a 20-μl

loop. The column used was Spherisorb ODS-2 (250 × 4.6 mm I.D., 5 μm; Phase Step, UK).

A two-step system was used for preparative HPLC. For the first step an LC-pump MMC for gradient elution (Mikrotechna, Czech Republic) and a UV detector Milichrom-4 (Nauchpribor, Orel, Russia) were used. The Rheodyne injector valve was combined with a 1-ml sample loop. The column was Diasorb-130-C16T (250 × 15 mm I.D., 6 μm, BioChimMac, Russia–Austria–Germany). For the second step, a system for analytical HPLC with preparative column μBondapak™ C_{18} (300 × 19 mm I.D., 125 Å, 10 μm, Waters, Millipore Corporation) was used for purification of some individual phenolics. The sample was applied to the column with a 500-μl loop valve.

2.4. Chromatographic conditions

Analytical HPLC

Two solvents were used: (A) 5% formic acid; (B) acetonitrile. The elution profile was: 0–5 min, 100% A (isocratic); 5–60 min, 0–30% B in A (linear gradient); 60–70 min, 30–60% B in A (linear gradient); 70–80 min, 60% B in A (isocratic). Flow-rate, 1 ml min^{-1}; column pressure, 70–134 bar; detection, 280 nm. The acquisition of UV spectra (210–370 nm) was automatic at the apex.

Preparative HPLC

Two solvents were used for the first step: (A) 2.5% acetic acid; (B) 96% ethanol. The elution profile was: 0–60 min, 100% A (isocratic); 60–300 min, 0–90% B in A (linear gradient). Flow-rate, 5 ml min^{-1}. The second step: elution was carried out with solvents for analytical HPLC. The elution profiles for individual phenolics were the next. Compound 21: 0–80 min, 10% B in A (isocratic); 80–140 min, 10–20% B in A (linear gradient); 140–160 min, 20–40% B in A (linear gradient); 160–190 min, 40% B in A (isocratic). Compound 32: 0–80 min, 15% B in A (isocratic); 80–140 min, 15–25% B in A (linear gradient); 140–160 min, 25–40% B in A (linear gradient); 160–190 min, 40% B in A (isocratic). Flow-rate, 5 ml min^{-1}; column pressure, 35 bar.

2.5. Acid hydrolysis and product identification

A 100-μl aliquot of a methanol solution of the respective glycoside or ester was mixed with 100 μl 4 *M* HCl and kept for 60 min at 90°C. The hydrolysate was diluted to 2 ml with water and the aglycone was separated from the liberated sugar or quinic acid by adsorption on a C_{16} Diapac cartridge (BioChimMac, Russia–Austria–Germany). The aglycones were identified by the analytical HPLC procedure. Carbohydrates were analyzed by TLC on silica-gel plates (Kieselgel 60 F_{254}, Merck) impregnated with 0.5 *M* NaH_2PO_4 in H_2O–MeOH (3:1) with *iso*-PrOH–Me_2CO–0.1 *M* lactic acid (2:2:1) according to Hansen [18]. TLC was also applied for separation and identification of quinic acid. The plate was developed with *n*-butanol–acetic acid–water (4:1:1), air-dried and then sprayed with specific reagent to visualize the spots of hydroaromatic acids [19].

2.6. NMR and MS measurements

The ^{1}H and ^{13}C NMR spectra of compounds 1, 13, 19 and 32 were recorded in DMSO-d_6 at room temperature on a JEOL JNM-GX 400 (^{1}H: 400 MHz, ^{13}C: 100 MHz) FT NMR spectrometer. The ^{1}H-^{13}C NMR-COSY and ^{1}H off-resonance decoupling spectra of compound 13 were obtained with a JEOL JNM-A500 (^{1}H: 500 MHz, ^{13}C: 125.65 MHz) FT NMR spectrometer with tetramethylsilane as an internal standard. Chemical shifts are recorded in δ values. DMSO-d_6 was chosen as solvent because of its good ability to dissolve a range of phenolics. Electron-impact (EI^+) mass spectra were recorded at 70 eV on a VG 7070E mass spectrometer equipped with a 11-250 data system. The samples were introduced through the direct inlet (probe temperature ambient).

2.7. Isomerization of *p*-coumaroylquinic and chlorogenic acids

Compounds were dissolved in a saturated solution of $NaHCO_3$ and the temperature was

raised to 90°C [20]. Heating was stopped after 30 min and H_2SO_4 was added to pH 3. The obtained isomers of *p*-coumaroylquinic or caffeoylquinic acids were purified by adsorption on a C_{16} Diapac cartridge and separated by analytical HPLC. For isomerization of *trans-p*-coumaric acid, we have used exposure to UV light as alternative technique: 3 h in methanol solution, UV lamp with broad band (Philips E/73/2, 125 W).

2.8. Quantitation

Quantitation of hydroxybenzoic (compounds 1 and 2) and hydroxycinnamic acid derivatives (compounds 6, 8, 9, 13, 16, 19 and 21), flavanol (10) and flavanons (40 and 41) was performed at 280 nm by using compound 13, which was isolated and purified by preparative HPLC, or (+)-catechin, naringenin, gallic, *p*-coumaric and chlorogenic acids as standards. For flavonoid glycosides (compounds 25–39), quantitation was performed at 360 nm by using quercetin, myricetin or kaempferol as standards, assuming a similar molecular extinction coefficient for aglycones and glycosides [21]. The measurements were done in triplicate. Mean values are expressed in milligrams per gram of dry matter. The standard error in the quantitation of phenolics depends on the area, shape and separability of the peaks. In our case, it ranged from 1.5 to 3.7% for *B. pubescens* and from 1.2 to 5.2% for *B. pendula*.

2.9. Chemicals

The following authentic compounds were used as external standards for identification: gallic, protocatechuic, 2,4,6-trihydroxybenzoic, 2,3,4-trihydroxybenzoic, *p*-hydroxybenzoic, vanillic, salicylic, syringic, caffeic, cinnamic, ferulic, *m*-coumaric, *o*-coumaric, *p*-coumaric, quinic and chlorogenic acids, (+)-catechin, kaempferol, quercetin, naringenin and galactose (Sigma); *p*-acetophenone, sinapic acid, myricetin, xylose and arabinose (Fluka); rhamnose and glucose (Merck).

3. Results and discussion

3.1. HPLC separation of birch phenolics

Characteristic HPLC traces of phenolics from *B. pubescens* and *B. pendula* leaves are shown in Fig. 1. We have tested different combinations of isocratic and gradient techniques and good resolution of individual low-molecular-mass phenolics was achieved. The UV spectra of the phenolics were monitored with a diode-array detector and on the basis of these data, they were divided into flavonoid (nos. 4, 5, 11, 12, 15, 22–41) and non-flavonoid (nos. 1–3, 6, 8–10, 13, 19 and 21) compounds (Table 1).

UV spectral data of phenolic derivatives are important for determination of the nature of the aglycone. Comparison of the spectra of non-flavonoid phenolics (Table 1) with those of the standard aglycones (different hydroxybenzoic and hydroxycinnamic acids) revealed that compound 1 corresponds to derivatives of hydroxybenzoic acids; compound 6 to caffeic acid derivatives; compounds 8, 9, 19 and 21 to *p*-coumaric acid derivatives.

The flavonoids of *B. pubescens* leaves have been identified already [11]. Here, their composition in the leaves of silver birch was determined also. This was done by comparison of retention times and UV spectra of the main individual peaks with those of white birch and co-chromatography of extracts of these two birch species (Table 1). As a result, only one new flavonoid glycoside (32) was found in the *B. pendula* leaves in comparison with *B. pubescens* [11]. On the basis of its UV spectrum, this flavonoid was preliminary identified as quercetin-glycoside. For further identification of birch phenolics, some of them were isolated and purified from crude extracts of leaves by one- or two-step preparative HPLC.

3.2. Isolation and purification of phenolics

Extract from *B. pubescens* leaves was used for isolation of compound 1 and extract from *B. pendula* for isolation of phenolics 13, 19, 21 and 32. A Diasorb-130-C16-T column and a gradient

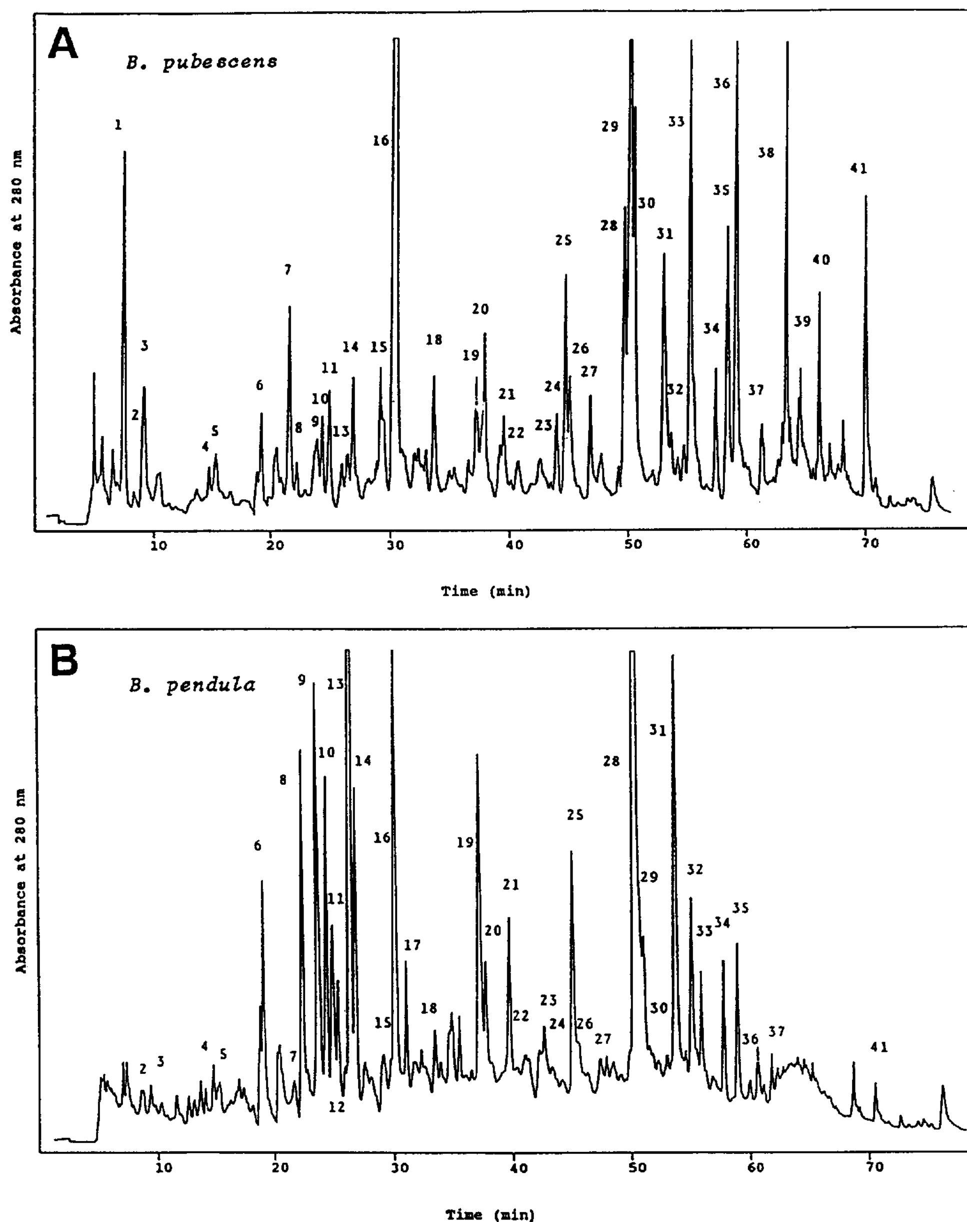

Fig. 1. Traces of HPLC analysis of soluble low molecular mass phenolics (1–41) from *B. pubescens* (A) and *B. pendula* leaves (B). Spherisorb ODS-2 column (250 × 4.6 mm I.D., 5 μm; Phase Step, UK) at flow-rate of 1 ml min^{-1} with gradient of acetonitrile in 5% formic acid; detection wevelength of 280 nm at 0.1 AUFS. Peaks: 1 = 1-*O*-galloyl-D-(2-*O*-acetyl)-glucopyranose; 2 = gallic acid; 4,5 = flavanols; 6 = neochlorogenic acid; 8 = *cis*-5-*p*-coumaroylquinic acid; 9 = *trans*-5-*p*-coumaroylquinic acid; 11 = (+)-catechin; 12 = flavanol; 13 = 1-(4″-hydroxyphenyl)-3′-oxopropyl-β-D-glucopyranose; 16 = chlorogenic acid; 19 = *trans*-3-*p*-coumaroylquinic acid; 21 = *cis*-3-*p*-coumaroylquinic acid; 22–24 = flavanols; 25 = myricetin-3-*O*-β-D-glucuronopyranoside; 26 = myricetin-3-*O*-β-D-galactopyranoside; 27 = flavanol; 28 = myricetin-3-*O*-α-L-(acetyl)-rhamnopyranoside; 29 = quercetin-3-*O*-β-D-glucuronopyranoside; 30 = quercetin-3-*O*-β-D-galactopyranoside; 31 = quercetin-3-*O*-α-L-arabinofuranoside; 32 = quercetin-*O*-β-D-rhamnopyranoside; 33 = kaempferol-3-*O*-β-D-glucopyranoside; 34 = quercetin-glycoside; 35 = kaempferol-3-*O*-α-L-rhamnopyranoside; 36 = quercetin-3-*O*-α-L-(4″-*O*-acetyl)-rhamnopyranoside; 37–39 = kaempferol-glycosides; 40 = naringenin; 41 = flavanone; 3, 7, 10, 12, 13, 14, 17, 18 and 20 = non-identified.

Table 1
Retention time (t_R) and UV maxima (λ_{max}) of some phenolics from leaves of *B. pubescens* and *B. pendula* determined by HPLC–photodiode array detection on Spherisorb ODS-2 using acetonitrile–5% aq. formic acid as eluent

Number of peak[a]	t_R (min)	λ^b_{max} (nm)
1	7.24	281
2	8.21	276
6	19.36	250, 302 sh, 328
8	22.55	308
9	23.97	314
10	24.51	297
13	26.06	280
16	30.30	249, 302 sh, 328
19	37.33	315
21	39.69	307
32	54.27	260, 303 sh, 353

[a] Refer to peak number in Fig. 1.
[b] sh = shoulder.

of ethanol in 2.5% acetic acid as solvent were used in the first step of the isolation and purification of birch phenolics. Individual peaks from multiple injections were collected as they eluted from the column and concentrated by evaporation.

The high efficiency of this method allows the isolation of these phenolics in quantities of 7–30 mg. The purity of the compounds (as % of all peak areas) was as follows: 1, 93%; 13, 98%; 19, 95%; 21, 73%; and 32, 82%. Compounds 21 and 32 were repurified on the μBondapak^{TM} C_{18} column with a gradient of acetonitrile in 5% formic acid as solvent system whereafter the purities of these individual phenolics were within the limits of 96–98%.

3.3. Identification of phenolics

Compounds 2, 6 and 16

The structures of only two phenolic compounds, gallic (2) and chlorogenic (16) acids, were identified by direct chromatographic comparison with reference samples. Their identities were verified by both spectral data and retention time (co-chromatography) (Fig. 1, Table 1).

The UV spectrum of compound 6 was identical to that of chlorogenic acid (Table 1) and it was assumed that this is one of the isomers of caffeoylquinic acid. By heating of chlorogenic acid in a saturated solution of $NaHCO_3$, a mixture of neo-chlorogenic (3-caffeoylquinic), crypto-chlorogenic (4-caffeoylquinic) and chlorogenic (5-caffeoylquinic) acids was obtained [20]. HPLC analysis of these isomers and comparison of their UV spectra and t_R with data for phenolic acid (6), allowed to identify this compound as a neo-chlorogenic acid (Fig. 1, Table 1). For identification of other phenolics, NMR and MS methods were mainly used.

Compound 1

In general the ^1H NMR spectrum of this compound was similar to that of β-glucogallin (1-*O*-galloyl-β-D-glucopyranose) [22]. The ^{13}C NMR spectrum of β-glucogallin is not known but ^{13}C NMR spectra of structurally closely related derivatives [22,23] support our conclusion that the main structure of compound 1 corresponds to an ester of gallic acid and glucose. Hydrolysis of this phenolic, followed by HPLC and spectral analysis of the aglycon, gave gallic acid. The sugar obtained on hydrolysis of this compound was glucose. However, in the ^1H NMR and ^{13}C NMR spectra of compound 1, an additional signal characteristic for protons of acetyl group at 1.82 ppm and two additional shifts for the acetate carbons at 22.5 ppm (CH_3COO^-) and 173.6 ppm (CH3*C*OO$^-$) were established. Acylation of the glucose moiety at C-2 is evidenced by a downfield shift in this signal and an upfield shift of the C-3 resonance [24]. On the basis of these data, compound 1 was identified as 1-*O*-galloyl-β-D-(2-*O*-acetyl)-glucopyranose. β-Glucogallin is considered the primary metabolite in the biosynthesis of hydrolyzable tannins and wide-spread in higher plants [25] but the acetylated form of this compound was now found for the first time.

*NMR and MS data for 1-O-*galloyl-β-D-(2-*O*-acetyl)-glucopyranase

^1H NMR: δ 6.97 (2H, s, H-2′, H-6′), 5.49 (1H, d, $J = 7.8$ Hz, H-1), 3.10–3.66 (sugar protons), 1.82 (3H, s, OAc); ^{13}C NMR: δ 173.6

(C-2, CH3CCOO$^-$), 164.8 (C-7′), 146.0 (C-3′, 5′), 140.2 (C-4′), 118.0 (C-1′), 109.1 (C-2′, C-6′), 101.7 (C-1), 77.8 (C-3), 76.6 (C-5), 72.6 (C-2), 69.5 (C-4), 60.5 (C-6), 22.5 (C-2, CH_3COO^-). EI–MS: *m/z* (relative intensity) 126 $[C_6H_60_3]^+$ (83), 108 $[C_6H_4O_2]^+$ (20), 44 $[C_2H_4O]^+$ (100).

Compound 13

The UV spectrum of this compound was very similar to that of acetophenone but the t_R was not the same (acetophenone, 33.61 min; compound 13, 26.06 min). Acid hydrolysis gave glucose and unknown aglycone with $t_R = 42.02$ min and $\lambda_{max} = 280$ nm. ^{1}H NMR spectrum of compound 13 showed one *p*-substituted aromatic ring and two types of aliphatic protons in addition to signals from glucose protons. Analysis of ^{1}H, ^{13}C NMR, ^{1}H-^{13}C COSY and MS data allowed to identify compound 13 as 1-(4″-hydroxyphenyl)-3′-oxopropyl-β-D-glucopyranose. This compound was reported 20 years ago in the *B. alba* leaves [26] but the authors did not give NMR and MS data of the glucoside in their paper.

NMR and MS data for 1-(4″-hydroxyphenyl)-3′-oxopropyl-β-D-glucopyranose

^{1}H NMR: δ 7.87 (2H, d, $J = 8.6$ Hz, H-2″, H-6″), 6.87 (2H, d, $J = 8.5$ Hz, H-3″, H-5″), 4.21 (1H, d, $J = 7.9$ Hz, H-1), 4.10 (1H, dt, $J = 9.8$ Hz, 6.7 Hz, H-1′a), 3.87 (1H, dt, $J = 10.0$ Hz, 6.7 Hz, H-1′b), 3.68 (1H, dd, $J = 11.6$ Hz, 1.5 Hz, H-6a), 3.45 (1H, dd, $J = 11.9$ Hz, 6.1 Hz, H-6b), 3.23 (2H, m, H-2′), 3.13-3.20 (2H, m, H-3, H-5), 3.07 (1H, t, $J = 9.2$ Hz, H-4), 2.96 (1H, t, $J = 8.2$ Hz, H-2); ^{13}C NMR: δ 197.0 (s, C-3′), 162.3 (s, C-4″), 131.0 (d, C-2″. C-6″), 128.7 (s, C-1″), 115.5 (d, C-3″, C-5″), 103.3 (d, C-1), 77.0 (d, C-3), 76.8 (d, C-5), 73.6 (d, C-2), 70.3 (d, C-4), 65,0 (t, C-1′), 61.3 (t, C-6), 38.4 (t, C-2′). EI-MS: *m/z* (relative intensity) 148 $[C_9H_8O_2]^+$ (45), 121 $[C_7H_5O]^+$ (100), 93 $[C_6H_5O]^+$ (20).

Compound 19

The UV spectrum of this compound shows that it is a derivative of *p*-coumaric acid (Table 1), which is present in higher plants mainly as ester or glycoside. The characteristic ^{1}H NMR spectrum of compound 19 is known [10] and it was identified as *trans*-3-*p*-coumaroylquinic acid. Analysis of hydrolysis products by analytical HPLC, UV and TLC revealed quinic and *trans*-*p*-coumaric acids. Accuracy of identification was confirmed by ^{13}C NMR and MS data.

NMR and MS data for trans-3-p-coumaroylquinic acid

^{1}H NMR: δ 7.53 (2H, d, $J = 8.3$ Hz, H-2′, H-6′), 7.51 (^{1}H, d, $J = 15.9$ Hz, H-7′), 6.80 (2H, d, $J = 8.3$ Hz, H-3′, H-5′), 6.29 (^{1}H, d, $J = 16.0$ Hz, H8′), 5.09 (1H, m, H-3), 3.93 (^{1}H, m, H-5), 3.54 (^{1}H, m, H-4), 1.91-2.02 (3H, m, H-2 a,e, H-6 e), 1.77-1.80 (1H, m, H-6 a); ^{13}C NIMR: δ 177.0 (C-7, COOH), 165.9 (C-9′), 159.8 (C-4′) 144.5 (C-7′), 130.3 (C-2′, C-6′), 125.2 (C-1′), 115.8 (C-3′, C-5′), 77.0 (C-1), 73.0 (C-4), 70.8 (C-3, C-5), 38.9 (C-2), 37.4 (C-6). EI-MS: *m/z* (relative intensity) 338 $[M]^+$ (10), 164 $[A]^+$ (33), 147 $[A-17]^+$ (100).

Compounds 8, 9 and 21

The shape and adsorption maximum of the UV spectrum of two pairs of compounds–8/21 and 9/19, *trans*-3-*p*-coumaroylquinic acid–were similar (Table 1). A small amount of compound 21 was isolated and some of its properties were studied. It was established that the mass spectrum of this compound was completely identical to that of *trans*-3-*p*-coumaroylquinic acid (19, data not shown). Moreover, hydrolysis of compound 21, followed by HPLC and TLC analysis of products, gave quinic acid and *trans*- and *cis*-isomers of *p*-coumaric acid. The *cis*-form was identified by comparison of UV spectra and t_R with those of the reference isomer which was produced from *trans*-form by UV illumination. The shapes of the UV spectra of compounds 8 and 21 were identical to that of *cis*-*p*-coumaric acid. Hence, the aglycone of compounds 8 and 21 is the *cis*-*p*-coumaric acid and that of compound 9 the *trans*-*p*-coumaric acid.

It is well known that *p*-coumaric and quinic acids can appear as several isomers [27,28]. For determination of relationships between struc-

tures of birch phenolics 8, 9, 21 and *trans*-3-*p*-coumaroylquinic acid (19), isomers of the last were produced by a method described in Ref. [20]. Three new derivatives of *trans*-3-*p*-coumaroylquinic acid were found in the reaction mixture by analytical HPLC. They had the UV spectra and t_R characteristic for compounds 8, 9 and 21 (Table 1). HPLC conditions and retention times of esters of 24 hydroxycinnamic acids are known [28]. On the basis of these data, the birch phenolics were identified as *cis*-5-*p*-coumaroylquinic (8), *trans*-5-*p*-coumaroylquinic (9) and *cis*-3-*p*-coumaroylquinic (21) acids (Fig. 1, Table 2).

Table 2
Content of phenolic compounds in the leaves of *B. pubescens* and *B. pendula*

Number of peak[a]	Phenolic compound	Content[b] (mg g^{-1} dry mass)	
		B. pubescens	*B. pendula*
1	1-*O*-Galloyl-β-D-(2-*O*-acetyl)-glucopyranose	1.11	+
2	Gallic acid	0.14	+
4	Flavanol	+	+
5	Flavanol	+	+
6	Neo-chlorogenic acid	0.44	0.54
8	*cis*-5-*p*-Coumaroylquinic acid	0.17	0.33
9	*trans*-5-*p*-Coumaroylquinic acid	0.31	0.67
11	(+)-Catechin	2.01	1.62
12	Flavanol	1.39	+
13	1-(4″-Hydroxyphenyl)-3′-oxopropyl-β-D-glucopyranose	0.80	8.50
16	Chlorogenic acid	20.42	1.28
19	*trans*-3-*p*-Coumaroylquinic acid	0.14	0.30
21	*cis*-3-*p*-Coumaroylquinic acid	0.09	0.18
22	Flavanol	+	+
23	Flavanol	+	+
24	Flavanol	+	+
25	Myricetin-3-*O*-β-D-glucuronopyranoside	1.16	1.45
26	Myricetin-3-*O*-β-D-galactopyranoside	0.80	0.21
27	Flavanol	0.60	+
28	Myricetin-3-*O*-α-L-(acetyl)-rhamnopyranoside	1.65	8.66
29	Quercetin-3-*O*-β-D-glucuronopyranoside	4.92	0.77
30	Quercetin-3-*O*-β-D-galactopyranoside	1.41	0.07
31	Quercetin-3-*O*-α-L-arabinofuranoside	1.88	1.95
32	Quercetin-3-*O*-α-L-rhamnopyranoside	+	0.33
33	Kaempferol-3-*O*-β-D-glucopyranoside	1.56	0.19
34	Quercetin-glycoside	0.65	+
35	Kaempferol-3-*O*-α-L-rhamnopyranoside	1.18	+
36	Quercetin-3-*O*-α-L-(4″-*O*-acetyl)-rhamnopyranoside	0.82	+
37	Kaempferol-glycoside	0.17	+
38	Kaempferol-glycoside	0.30	0
39	Kaempferol-glycoside	0.45	0
40	Naringenin	0.07	0
41	Flavanone	0.07	+
	Total content	44.70	27.05

[a] Refers to peak number in Fig. 1.
[b] + = traces of compounds (less 0.05 mg g^{-1}).
Values are the mean of three replicates.

The natural cinnamic acids apparently attain the *trans*-configuration but in UV light and during sample preparation *trans–cis* isomerization can occur. It has been postulated that the presence of *cis*-forms in plant cells is artifactual [20,21,27]. However, the study of *cis*-monolignol formation in the bark tissue of *Fagus grandifolia* has shown that it can be an enzyme-mediated process, because transformation of the *trans*-form to the *cis*-form occurred under conditions where photochemical isomerism was not observed [29]. Moreover, *cis*-coniferyl alcohol was the more preferred substrate for the glycosylation reaction than its *trans*-analog [30]. Thus, analysis of these data leads to the conclusion that *cis*-forms of *p*-coumaroylquinic acid are formed in the birch leaves *in vivo*, but the biochemical function of this process is not clear yet.

Compound 32

Compound 32 was isolated from *B. pendula* leaves. UV, MS and acid hydrolysis data showed that it is quercetin-rhamnoside. Earlier, quercetin-3-*O*-α-L-(4″-O-acetyl)-rhamnopyranoside was identified in the leaves of *B. pubescens* [11]. The distinctive peculiarity of the ^{1}H and ^{13}C NMR spectra of compound 32 was the lack of signals characteristic for the acetyl group (data not shown). Thus compound 32 was identified as quercetin-3-*O*-α-L-rhamnopyranoside.

3.4. *Quantitation and comparative analysis of the phenolics composition in the two birch species*

The total content of low-molecular-mass phenolics (as a sum of individual compounds) in *B. pubescens* leaves was 1.6 times higher than that of *B. pendula* (Table 2). The amounts of non-flavonoid compounds in white and silver birch leaves were 51 and 44%, respectively, of the total. There was no difference in the composition of non-flavonoids in the leaves of the birch species but concentrations of some individual compounds were different (Table 2).

The major phenolic compound in white birch foliage is chlorogenic acid. Its concentration reached 20 mg g^{-1} dry mass, or 46% of the total content of low-molecular-mass phenolics. However, in leaves of silver birch, the concentration of chlorogenic acid was less than 10% of that in white birch, and the major compound was 1-(4″-hydroxyphenyl)-3′-oxopropyl-β-D-glucopyranose (8.5 mg g^{-1} dry mass, or 31% of the total). The proportion of other non-flavonoids in the leaves of silver birch made up 12.2% of the total, which was twice the value in white birch (ca. 6%) (Table 2). The 3- and 5-*p*-coumaroylquinic acids were identified in both species. The pooled concentration of these esters in white birch leaves was two-fold that in the silver birch which, however, was found to contain only trace amounts of 1-*O*-galloyl-β-D-(2-*O*-acetyl)-glucopyranose (Table 2).

The composition of flavonoids in leaves of the two birch species does not differ much (Fig. 1, Table 2). One new flavonoid, quercetin-3-*O*-α-rhamnopyranoside, was found in leaves of the silver birch in addition to flavonoids which have been identified from white birch earlier [11]. Interestingly, the acetylated form of this compound is present in the leaves of white birch in a reasonable amount, but it was not detected in the leaves of silver birch at all. Two kaempferol-glycosides (38, 39) and naringenin were also absent and some flavonoids characteristic for white birch (kaempferol-3-*O*-β-glucopyranoside, kaempferol-glycoside (37) and flavanone) were present in leaves of silver birch in small or trace amounts (Table 2). Only one flavonoid, myricetin-3-*O*-α-L-(acetyl)-rhamnopyranoside, occurred in high concentrations in silver birch leaves (8.66 mg g^{-1} dry mass, or 32% of total).

Accordingly, the foliages of the two birch species have characteristic compositions of flavonoids and non-flavonoid phenolics. Due to the species-specific variation in phenols, widely used measures of total phenolics, pooling all the compounds, in botanical and ecological work may be misleading.

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