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Chemical characteristics of herbivore defenses in *Betula pendula* winter-dormant young stems

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ABSTRACT

Aqueous acetone-soluble phenolic glucosides, phenolic aglycones and triterpenoic components were extracted from winter-dormant *Betula pendula* twigs. The chemical composition was analyzed using gas chromatography for triterpenoids and most phenolics and high-performance liquid chromatography with ultraviolet–visible detection for phenolics, especially for platyfylloside. Gas chromatography–mass spectrometry and thermospray high-performance liquid chromatography–mass spectrometry were used for complete identification and structural elucidation. The analysis revealed five new components, salidroside, dehydrosalidroside, catechin pentoside, an isomer of catechin and deacetylpapyriferic acid, which have not previously been reported in the twigs of this species.

INTRODUCTION

In Finland, European white birch (*Betula pendula*, Betulaceae) is one economically promising deciduous tree species used in reforestation, and its demand in the plywood and pulp industry is increasing [1]. White birch is not preferred by forest owners, however, because they think it is very vulnerable to browsing mammals. Thus, to increase the distasteful or potentially toxic deterrent components in birch tissues and thereby their resistance to herbivore feeding (*e.g.* ref. 2), researchers and plant breeders have focused on the secondary phytochemicals in birch. More recently, studies on the Alaskan paper birch, *Betula resinifera*, have shown that a triterpene, papyriferic acid, is the main anti-feeding component in winter-dormant twigs [3]. On the other hand, a phenolic glucoside, platyfylloside, has been claimed to cause most of the low digestibility of *B. pendula* twigs in ruminants and to be responsible for sodium losses in hares and rabbits feeding on birch twigs [4].

In this paper we characterize phenolic and terpenoic components extracted

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from winter-dormant twigs of *B. pendula*. Our hare-feeding studies have indicated that several of these components may be negatively correlated with feeding by hares [5]. Betuloside, platyfylloside, (+)-catechin, papyriferic acid and deacetylpapyriferic acid have previously been identified by nuclear magnetic resonance (NMR) spectroscopy [4,6,7]; but there are no analytical data on papyriferic and deacetylpapyriferic acid identified by gas–liquid chromatography (GC) in any tree species. Moreover, the ultraviolet–visible (UV–VIS) spectral analysis of betuloside, platyfylloside and catechins are not found in previous publications. Throughout this study we have emphasized the use of gas chromatography–mass spectrometry (GC–MS) and thermospray high-performance liquid chromatography–mass spectrometry (HPLC–TSP-MS) systems for identification of compounds in birch. It should be noted that fragmentation routes presented are only tentative and based on the elemental principles of mass spectral fragmentations because it was not possible to perform metastable ion analyses. We describe the analysis of nine young stem components, of which four phenolic and one terpenoic phytochemical have not previously been reported in *B. pendula*.

EXPERIMENTAL

Materials

Stems from one-year-old seedlings of *B. pendula* were collected for phytochemical extractions. These seedlings were cultivated in a greenhouse on prefertilized commercial peat (VAPO) and allowed to remain in the unheated greenhouse until December in order to reach winter dormancy. The young stems were stored in sealed plastic bags at −20°C for a few months until extracted.

Equipment and chromatographic conditions

A Hewlett-Packard HPLC system (Hewlett-Packard, Avondale, PA, USA) was used which consisted of a quaternary solvent delivery system (HP 1050 Series) and an autosampler (HP 1050 Series). A photodiode array detector (HP 1040A Series) coupled with an HP data system–personal computer was used for recording chromatograms and UV–VIS spectra. The compounds were separated on a 60 mm × 4.6 mm I.D. column filled with HP Hypersil ODS II (3 μm) particles as the stationary phase. The samples were eluted (flow-rate 2 ml/min) using the gradient shown in Table I. To avoid the tailing of phenolic compounds, orthophosphoric acid was added to the solvent in order to adjust the pH to about 3. Before analysis, the samples were dissolved in water–methanol (1:1, v/v). The autoinjected volume was 10 μl. All runs were processed at the room temperature.

A capillary gas chromatograph (Hewlett-Packard Model 5890) equipped with a flame ionization detector and auto-injector (Model 7673A) was used. An OV-1 fused-silica capillary column (20 m × 0.32 mm I.D.) with a phase thickness of 0.25 μm was used throughout. The temperature programme was started at 210°C, and the temperature was raised to 318°C at a rate of 14°C/min. The detector and injector temperatures were 300 and 260°C, respectively. Helium was used as a carrier gas and the split-ratio was 1:15. The samples were derivatized with trimethylsilylimidazole in pyridine (Aldrich-Chemie, Steinheim, Germany). The sample vial was shaken vigorously for about 1 min and allowed to react for 10 h at 4°C. The autoinjected volume was 1 μl.

TABLE I

CONDITIONS USED IN HPLC GRADIENT ELUTION

Solvent A = aqueous 2% tetrahydrofuran + 0.25% orthophosphoric acid; solvent B = 100% methanol.

Time (min)	Solvent A (%)	Solvent B (%)
Initial	100	0
5	100	0
10	80	20
20	60	40
40	50	50
45	50	50
Rinsing	0	100
Equilibration	100	0

Sample preparation

Fresh-frozen stems (300–400 mg) were extracted in a clipping homogenisator three times for 5 min with 30 ml of 80% aqueous acetone (Merck, Darmstadt, Germany). The acetone was filtered, the residue washed with 20 ml of acetone and the whole extract evaporated using a vacuum evaporator. Aqueous acetone was shown to be the most effective and reproducible solvent for extracting both phenolic and terpenoic secondary components during the same extraction. Quantitatively, the solvent for slightly more complete extraction of terpenoic chemicals is 96% ethanol but it is a poor solvent for the phenolic components studied [8]. The sample was redissolved in methanol (Lab-Scan, Dublin, Ireland) and purified on Bond Elut C_{18} octadecyl (500 mg) solid-phase extraction columns. The eluent was used for HPLC, GC and GC–MS and HPLC–TSP–MS. All solvents used were HPLC grade.

Mass spectrometric measurements

GC–MS experiments were performed on a Jeol JMS D300 mass spectrometer coupled with a Carlo Erba Fractovap 4160 gas chromatograph and controlled by a Jeol JMA 2000H data system (Jeol, Tokyo, Japan). The column used was fused-silica capillary column with a chemically bonded SE-52 liquid phase (film thickness, 0.25 μm). Electron ionization spectra were obtained with an acceleration voltage of 3 kV, electron energy of 70 eV, ionization current of 300 μA and source temperature of 170°C. The following mass spectra of the trimethylsilyl (TMS) derivatives of the compounds identified were observed (m/z, relative intensity): salidroside, $M^{+\cdot}$ 660(0), 217(13), 206(9), 205(20), 204(100), 194(13), 193(67), 192(61), 147(9), 74(6), 73(53); dehydrosalidroside, $M^{+\cdot}$ 658(0), 361(11), 217(19), 206(9), 205(21), 204(100), 192(6), 191(36), 147(10), 129(14), 103(6), 73(40); betuloside, $M^{+\cdot}$ 688(0), 363(6), 362(65), 361(19), 221(15), 220(15), 206(10), 205(24), 204(100), 180(5), 179(34), 147(8), 103(8), 73(53); (+)-catechin $M^{+\cdot}$ 650(13), 383(10), 370(16), 369(34), 368(100), 357(5), 356 (12), 355(35), 297(7), 267(12), 179(8), 74(6), 73(72); pentose derivative of (+)-catechin, 577(6), 370(10), 369(20), 368(60), 355(6), 350(15), 349(33), 348(100), 283(10), 267(8), 259(11), 232(5), 218(6), 217(30), 179(5), 146(10), 129(10), 103(12), 75(6), 74(9), 73(89). Peaks with a relative intensity less than 5% are omitted. The 70-eV mass spectra of the TMS derivatives of papyriferic acid and deacetylpapyriferic acid are presented in Fig. 3 below.

The direct inlet probe at 200°C was used to introduce the isolated underivatized samples. The mass spectra obtained in this way were: papyriferic acid, $M^{+\cdot}$ 604(0), 442(8), 381(9), 191(9), 189(6), 144(10), 143(100), 135(7), 125(14), 109(6), 107(7), 95(7), 93(7), 85(14), 84(6), 81(8), 71(8), 69(7), 55(6), 44(17), 43(26); deacetylpapyfiferic acid, $M^{+\cdot}$ 562(0), 442(9), 381(8), 191(12), 189(8), 147(6), 144(10), 143(100), 135(10), 133(5), 125(17), 121(8), 119(6), 109(8), 108(5), 107(11), 105(6), 95(10), 93(10), 85(16), 84(6), 83(5), 81(11), 71(9), 69(10), 59(6), 55(8), 44(15), 43(31), 41(6).

LC–MS measurements were carried out using a VG thermospray–plasmaspray probe coupled to a VG Trio-2 quadrupole mass spectrometer. The measurements were run using the instrument in the thermospray ionization mode. The ion source temperature was 180°C, the vaporizer tip temperature 150°C and the repeller electrode potential 180 V. The HPLC system consisted of a Spectra-Physics Model SP8810 pump and Rheodyne 7125 injector with a 20-μl loop. The HPLC column was a HP Hypersil ODS (3 μm, 60 mm × 4.6 mm I.D.). The isocratic eluent system consisted of 0.1 *M* ammonium acetate–methanol (85:15, pH 7.2). The flow-rate was 1.0 ml/min.

RESULTS AND DISCUSSION

GC and GC–MS

The analysis of the aqueous acetone extract from winter-dormant *B. pendula* young stems by GC is shown in Fig. 1. The capillary column of fused silica coated with OV-1 used in this study gave satisfactory separation of TMS derivatives of phenolic aglycones, phenolic glucosides and triterpenoic compounds (Fig. 2). Simple soluble sugars, fructose (A), glucose (B, C), sucrose (D) and raffinose (E) (Fig. 1), could also be analyzed in the same run. All secondary components of birch stems, including salidroside, betuloside, (+)-catechin, catechin glycosides, dehydrosalidroside, papyriferic acid and deacetylpapyriferic acid, were eluted within 20 min. Only platyfylloside, found previously in *B. pendula* twigs, could not be analyzed by the GC

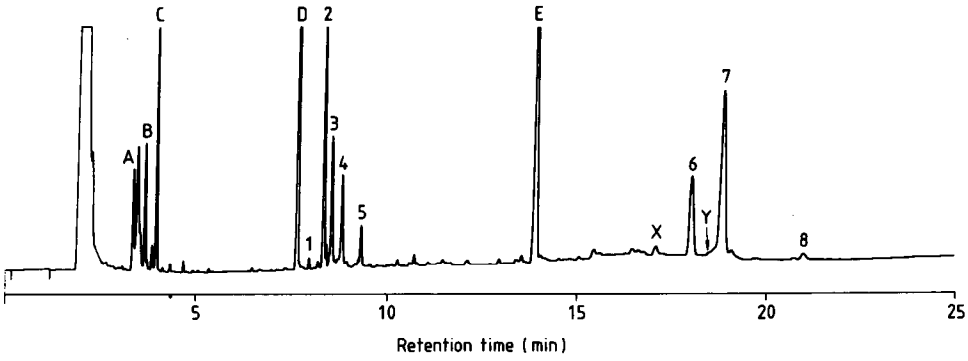
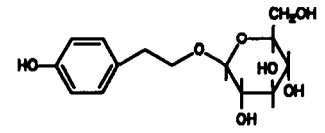
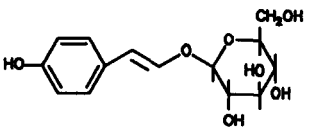


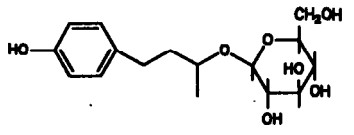
Fig. 1. GC trace of TMS derivatives of phenolic and terpenoic phytochemicals and sugars in the winter-dormant twigs of *B. pendula*. Peaks: 1 = salidroside; 2 = betuloside; 3 = (+)-catechin; 4 = isomer of (+)-catechin; 5 = dehydrosalidroside; 6 = catechin pentoside; 7 = papyriferic acid; 8 = deacetylpapyriferic acid; A = fructose; B and C = glucose; D = sucrose; E = raffinose; X and Y = unknown triterpenoic components.



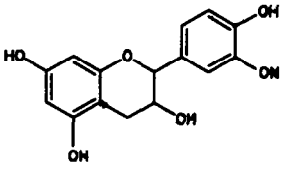
Salidroside



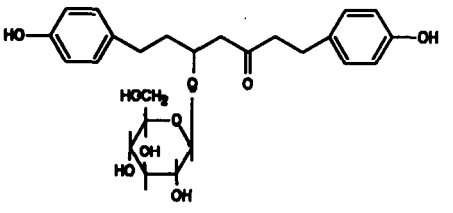
Dehydrosalidroside



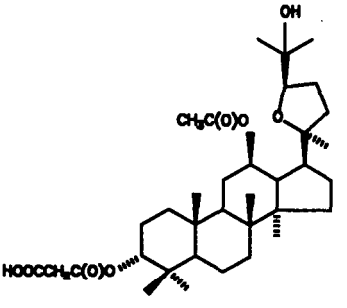
Betuloside



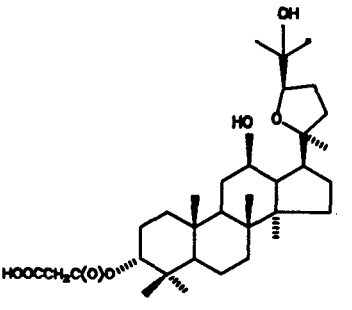
(+)-Catechin



Platyfylloside



Paryriferic acid



Deacetylpapyriferic acid

Fig. 2. Structures of the compounds identified in the aqueous acetone extracts of *B. pendula* twigs.

method. In addition to the identified components, the GC pattern also indicated the presence of minor unknown constituents.

The initial identification of the extracted components was based on their retention times. Identification was confirmed by GC–MS, which also allowed verification of some new components. Although the mass spectra did not always allow complete

assignment of structure, certain structural features were easy to identify. At least in cases where authentic reference compounds were available (sugars, Merck; salidroside; (+)-catechin, Sigma, St. Louis, MO, USA; papyriferic acid and deacetylpapyriferic acid) identification was simple and unambiguous.

Several of the most intense fragment ion peaks in the mass spectra of the phenolic glucosides, salidroside and betuloside (Fig. 1, components 1 and 2, respectively) were connected to the glucose part of the molecule and existed at *m/z* 451, 361, 217, 204 and 147. The intensity of these peaks varied considerably from compound to compound. The base peak, however, was always at *m/z* 204 representing the $[C_8H_{20}Si_2O_2]^+$ ion. Analogous fragmentations have previously been observed for platyphylloside [9]. Although no molecular ion peaks were present, it was possible to identify the aglycone moiety by means of the ions formed from the inductive cleavage of the acetal bond. The same bond was also cleaved with a simultaneous hydrogen transfer to the oxygen atom. These ions existed at mass numbers *m/z* 193 and 192 for salidroside and at *m/z* 221 and 220 for betuloside. In the case of betuloside, the length of the carbon chain allowed further elimination of propene from the *m/z* 193 ion, which led to formation of the *m/z* 179 ion. In the case of salidroside, the identification was verified by comparing its spectrum with that of the authentic compound.

The general appearance of the spectrum of component 5 (Fig. 1) resembled closely that of salidroside and betuloside. The spectrum was best rationalized as representing dehydrogenated salidroside, because the ion connected to the aglycone unit now existed at *m/z* 191 and the ion formed through a hydrogen atom rearrangement was totally absent. This is in agreement with the proposed structure since it is improbable that the necessary hydrogen would have been transferred from an ethylenic carbon.

Component 3 (Fig. 1) had a retention time and mass spectrum identical to that of authentic (+)-catechin. The molecular ion peak was clearly visible, accompanied with the $[M - 15]^+$ ion typical of TMS ethers. The main fragmentation was cleavage of the heterocyclic ring so that O–C-1 and C-2–C-3 bonds were broken, giving rise to the *m/z* 368 ion, which formed the base peak in the spectrum. Component 4 (Fig. 1) must be very similar to (+)-catechin. It is most probably an isomer of (+)-catechin because its spectrum was almost identical to that of (+)-catechin. It is noteworthy that this isomer was not (−)-epicatechin as the measurements with an authentic sample (Sigma) pointed out. In addition to the free catechin, a pentose derivative was also present (component 6, Fig. 1). Almost all the peaks present in the spectrum of the TMS derivative of (+)-catechin were also found in the spectrum of component 6. This can be rationalized as a formation of (+)-catechin tetra-TMS ether from its pentoside as a consequence of $OSi(CH_3)_3$ group migration from the pentose ring to the catechin moiety to replace the broken acetal bond. Related migrations have previously been observed for oligosaccharide TMS ethers [10]. Parallel to the fragmentations of phenolic glucosides (*cf.* salidroside and betuloside) the most abundant ions originated from the pentose moiety existing at *m/z* 349, 348 and 217.

As in Alaskan paper birch (*B. resinifera*) our samples also contained a triterpenoid papyriferic acid [6] (component 7, Fig. 1). The 70-eV mass spectrum of its TMS derivative (Fig. 3) resembled that of the underivatized acid in many respects and was identical to the spectrum of the compound isolated from Alaskan paper birch. With the underivatized compound, the tetrahydrofuran ring dominated the fragmentation

behavior, leading to formation of the *m/z* 143 ion as a consequence of α-cleavage reaction with respect to the ring oxygen atom. The related ion was also present in the spectrum of the TMS derivative at *m/z* 215. Silylation, however, altered the fragmentation behavior so that now the most important reaction was the α-cleavage reaction with respect to the tertiary alcohol group, which gave rise to formation of the ion at *m/z* 131. Other major fragment ions were practically the same for both the derivatized and underivatized compound. This is to be expected because they were formed by loss of the substituents in the ring system. Therefore the ions at *m/z* 442 and 381 can be rationalized as being formed through successive elimination of the malonyl and $C(OH)(CH_3)_2$ groups and the malonic acid, acetic acid and $C(OH)(CH_3)_2$ group, respectively.

Another triterpenoic compound with the longest retention time and hitherto unknown in *B. pendula* twigs was also found in GC analysis (component 8, Fig. 1). Its spectral similarity to papyriferic acid indicated that their structures are closely related. Based on comparison with an authentic sample, that component was identified as deacetylpapyriferic acid [7]. In the spectrum of the derivatized compound the only discernible difference from derivatized papyriferic acid (Fig. 3) was that the *m/z* 442 ion was now shifted to *m/z* 472. This is just the difference caused by substitution of the acetyl group in the molecule with the trimethylsilylated hydroxy group. The first suggestion was that this exchange took place during the derivatization process and that no deacetylpapyriferic acid was present in the original samples. This phenomenon was, however, never observed when authentic papyriferic acid was derivatized. So the compound detected must originate from the birch twigs. It is interesting to note that deacetylpapyriferic acid itself gave rise to a spectrum virtually identical to that of papyriferic acid. This was not expected because, analogous to the behavior described above, the *m/z* 442 ion now should exist at mass number *m/z* 400. The

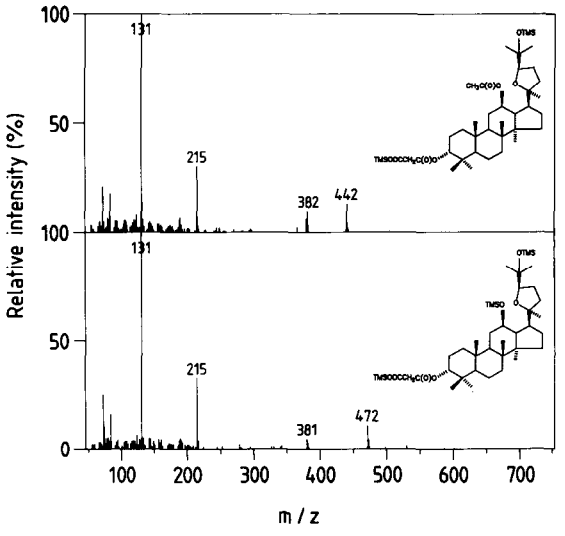


Fig. 3. Mass spectra (70 eV) of the TMS derivatives of papyriferic acid (top) and deacetylpapyriferic acid (bottom).

reason for this unexpected behavior is most probably explained by the effects of high temperature. At the elevated temperatures required to get the compound into the gas phase decarboxylation of the malonyl group took place, which led to the situation where there was an acetyl group at position 3 instead of the malonyl group. In this case the *m/z* 442 ion was formed by consecutive eliminations of hydroxy and $C(OH)(CH_3)_2$ groups. Of course, the same decarboxylation could also occur with papyriferic acid itself. Verification of this last assumption was, however, not possible. In both cases the relative intensity of the *m/z* 44 ion was decreased when the temperature of the direct inlet probe was programmed from 170 to 210°C, indicating that decarboxylation does take place.

In the GC–MS analysis two other unknown triterpenoic components were detected. The retention times of these were shorter than that of papyriferic acid, possibly indicating a shorter side-chain at position 3 (components X and Y, Fig. 1). The mass spectra of these components were identical to that of the TMS derivative of deacetyl papyriferic acid. As these components were not isolated, complete structural elucidation was not possible.

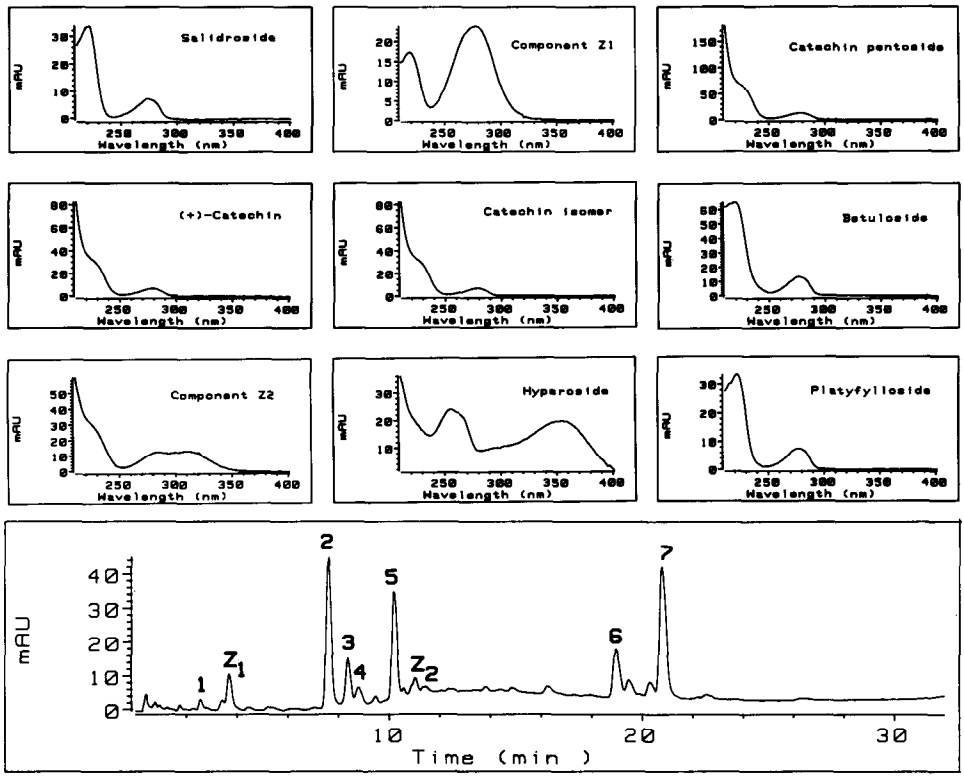


Fig. 4. HPLC trace and UV spectra of phenolic phytochemicals in the winter-dormant twigs of *B. pendula*. Peaks: 1 = salidroside; 2 = catechin pentoside; 3 = (+)-catechin; 4 = catechin isomer; 5 = betuloside; 6 = hyberoside; 7 = platyfylloside; Z_1 and Z_2 = unknowns.

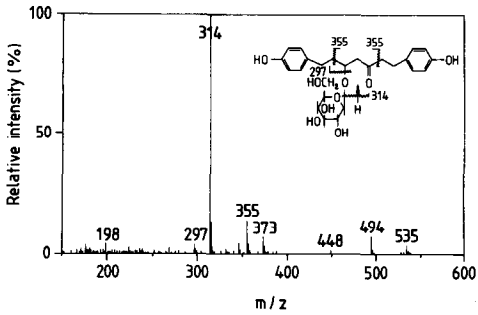


Fig. 5. Thermospray ionization mass spectrum of platyfylloside.

HPLC and HPLC–TSP-MS

An HPLC–UV–VIS profile and UV spectra of the components of the birch young stems extract are presented in Fig. 4. Most of the phenolic components in *B. pendula* winter-dormant stems found by GC are also easily detected and distinguished by their characteristic UV spectra. However, a more complete identification of the components in a chromatogram is possible by comparing their retention times and UV spectra with those of authentic and library components. Consequently, betuloside, platyfylloside, (+)-catechin and salidroside can easily be recognized as components of the extract (Fig. 4). The HPLC profile also indicated the presence of a few minor unknown components (Z_1, Z_2 and 6, Fig. 4), of which component 6 could be identified as the flavonoid glucoside hyperoside (Aldrich-Chemie). Based on retention time and spectral behavior, component Z_1 could be dehydrosalidroside.

LC–TSP-MS offers another possibility for the analysis of non-volatile and polar compounds directly without derivatization [11,12]. As platyfylloside was not detected by GC–MS this method was used to confirm its presence. The HPLC measurements, on the other hand, strongly indicated the presence of platyphylloside. The HPLC–TSP mass spectrum obtained (Fig. 5) shows an abundant ammonium adduct $[M + NH_4]^+$ ion peak at *m/z* 494. The ion at *m/z* 314 giving rise to the base peak and the *m/z* 297 ion were formed by cleavage of the glucosidic bonds. Corresponding fragmentations for a cyanogenic glucoside have been reported previously [13]. The ion at *m/z* 535 was presumably $[M + NH_4 + CH_3CN]^+$.

LC–TSP-MS was also used to verify the molecular weight of betuloside. In this case, almost the only adduct ions at *m/z* 346 and 387 were $[M + NH_4]^+$ and $[M + NH_4 + CH_3CN]^+$, respectively.

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