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Journal of Chromatography A, 864 (1999) 283–291

JOURNAL OF CHROMATOGRAPHY A

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Characterisation of hydrolysable tannins from leaves of *Betula pubescens* by high-performance liquid chromatography–mass spectrometry

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Received 20 May 1999; received in revised form 22 September 1999; accepted 27 September 1999

Abstract

A high-performance liquid chromatography–electrospray ionisation mass spectrometry (HPLC–ESI-MS) method, assisted by diode array detection, for the characterisation of individual hydrolysable tannins in birch leaves was developed. With the method, it was found that birch (*Betula pubescens*) leaves contained an exceptionally complex mixture of hydrolysable tannins; 14 gallotannins and 20 ellagitannins were identified. The developed HPLC–ESI-MS method allows the qualitative and quantitative determination of individual gallotannins and ellagitannins directly from crude birch leaf extract. This is important in studying ecological functions of these phenolic compounds, especially their role in the resistance of birch leaves against insects.

Keywords: *Betula pubescens*; Hydrolysable tannins; Gallotannins; Ellagitannins

1. Introduction

Hydrolysable tannins (HTs) represent a large group of polyphenolic compounds that are widely distributed in the plant kingdom. They are esters of a polyol (most often β-D-glucose) with either gallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins). These ester forms vary from simple compounds such as β-D-glucogallin to compounds with M_r values in excess of 2500 [1]. When the glucose core is esterified with five or fewer galloyl groups, the resulting compounds are defined as gallotannin precursors. The first true gallotannin is hexagalloylglucopyranose that is formed by attachment of additional galloyl unit to pentagalloylglucopyranose (Fig. 1A) core via so-called *meta*-depside bond. Ellagitannins, in turn, are supposed to result from oxidative C–C coupling between two spatially adjacent galloyl groups of pentagalloylglucopyranose to form hexahydroxydiphenoyl (HHDP) units as in trigalloyl-HHDP-glucopyranose (Fig. 1B) [2]. In this paper the terms gallotannins and ellagitannins are used in a liberal sense, although some of them indeed are precursors of these classes of tannins.

Earlier, 11 phenolic compounds of birch leaves were preliminarily identified as gallotannins [3]. It was shown that these phenolics decline the suitability of birch leaves for larvae of the geometrid moth

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)01036-5

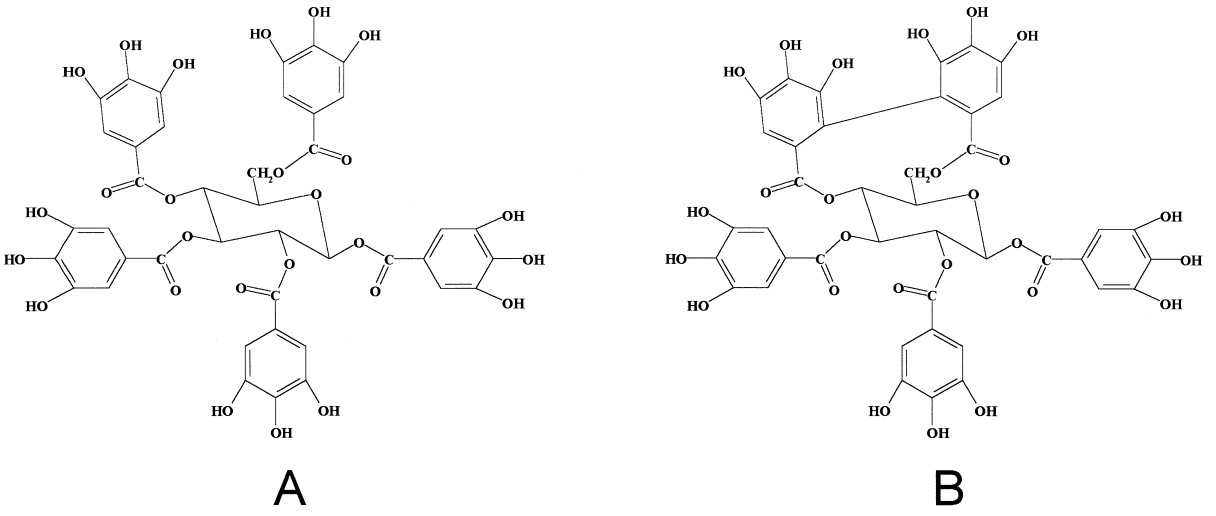

Fig. 1. Structures of pentagalloylglucopyranose (A, gallotannin) and trigalloyl-HHDP-glucopyranose (B, ellagitannin).

Epirrita autumnata [4,5]. They are supposed to decrease the nutritive value of young birch leaves and to contribute to leaf toughness by transformation into insoluble cell wall-bound forms during leaf maturation [3].

In order to clarify the true role of HTs in regulation of plant–herbivore relationships, it is necessary to have a reliable method for quantitation of both individual gallo- and ellagitannins. Until recent years, most of the methods for determination of HTs have included the measurement of the total contents of gallo- or ellagitannins only. These methods have been based either on the analyses of gallic and ellagic acids in hydrolysates of plant extracts [6,7] or on the protein precipitation capacity of extracts [8,9]. However, some chromatographic methods have been developed also for the analysis of individual gallotannins, mainly by reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection [3,10]. RP-HPLC has been claimed to allow even the separation of galloylglucopyranose isomers with the same degree of galloylation [10].

Electrospray ionisation (ESI) is a gentle ionisation method in mass spectrometry. It generates mainly deprotonated molecules $[M-H]^-$ of the compounds analysed in the negative ion mode of ESI-MS [11]. When an ESI interface is connected to a HPLC system, these deprotonated molecules allow a rapid determination of the molecular mass of a compound directly after its elution from the HPLC column [12]. The use of ion trace analysis in HPLC–ESI-MS makes it possible to detect and quantify compounds having different mass values, even if they were co-eluting in HPLC.

In this paper, we report a fast and efficient method for the qualitative and quantitative analysis of hydrolysable tannins, including all individual isomers, from crude birch leaf extracts using HPLC–ESI-MS. Also a simple method for preliminary identification of HTs using HPLC with diode array detection (DAD) is presented.

2. Experimental

2.1. Sample preparation

Leaves of white birch (*Betula pubescens*) were sampled in Turku, South West Finland, for analysis at the end of May. Leaves at this time are young and have been shown to have maximum concentration of gallotannins [3]. The collected plant material was vacuum dried and homogenised into fine powder.

Fine powder of birch leaves (100 g dry mass) was suspended in 700 ml of 70% acetone (containing 0.1% ascorbic acid to prevent oxidation) and shaken for 3 h. After centrifuging at 4000 *g* for 10 min, the insoluble residue was re-extracted four times with

the same solvent. The combined extract was evaporated to dryness under reduced pressure at 35°C. The residue was dissolved in 50 ml water and water-insoluble compounds (chlorophyll, lipids, resins) were separated by centrifuging.

The resulting extract was applied into Sephadex LH-20 column (40×3.8 cm I.D., Pharmacia, Umeå, Sweden) and fractionated by consecutive elution with water, 30% and 50% MeOH, and 10%, 30%, 50% and 70% Me_2CO. The acetone fractions containing hydrolysable tannins were purified further by preparative HPLC.

2.2. Standards and solvents

Gallic acid was purchased from Sigma (St. Louis, MO, USA). Acetone, acetonitrile and methanol were from Lab-Scan (Dublin, Ireland); formic and orthophosphoric acids were from FF Chemicals (Yli-Ii, Finland). Pure water was delivered by a Milli-Q Water purification system.

2.3. Equipment

2.3.1. HPLC–DAD analysis

Analytical chromatographic analysis was performed with an HPLC system consisting of a Merck–Hitachi L-7100 HPLC pump (Hitachi, Tokyo, Japan) and a Perkin-Elmer LC-235 diode array detector (Perkin-Elmer, Norwalk, CT, USA) connected to a Graphic Printer GP-100 (Perkin-Elmer, Beaconsfield, UK). Injections were made via a Rheodyne rotary switching valve (Cotati, CA, USA) with a 20-μl loop. The column was Superspher 100 RP-18 (75×4 mm I.D., 4 μm, Merck, Germany). Preparative HPLC was performed using the Diasorb-130-C16T column (250×15 mm I.D., 6 μm, BioChimMac, Russia–Austria–Germany). The sample was applied to the column through a 500-μl loop valve.

2.3.2. HPLC–ESI-MS analysis

HPLC–ESI-MS analysis was performed using a Perkin-Elmer Sciex API 365 triple quadrupole mass spectrometer (Sciex, Toronto, Canada). This instrument is equipped with an ionspray (pneumatically-assisted electrospray) interface and a Macintosh data system. The HPLC system consists of two Perkin-Elmer Series 200 micro pumps connected to a Series 200 autosampler (Perkin-Elmer). The eluate passes through a Superspher 100 RP-18 column (75×4 mm I.D., 4 μm, Merck) and the UV trace is recorded with a 785A UV–Vis detector (Perkin-Elmer). After UV detection, part of the eluate was split off and introduced into the ESI-MS system. The mass spectrometer was operated both in the negative mode and in the positive mode. Mass spectra were obtained by acquiring data between 100 and 1100 atomic mass units.

2.4. Chromatographic conditions

2.4.1. Analytical HPLC–DAD analysis

Two solvents were used: (A) 0.1 *M* H_3PO_4; (B) acetonitrile. The elution profile was: 0–3 min, 98% A (isocratic); 3–22 min, 2–20% B in A (linear gradient); 22–30 min, 20–30% B in A (linear gradient). Flow-rate, 1 ml min^{-1}; detection at 280 nm. The acquisition of UV spectra (200–360 nm) was done automatically at the apex of each peak.

2.4.2. Preparative HPLC analysis

Two solvents were used for the first step: (A) 0.1 *M* H_3PO_4; (B) acetonitrile. The elution profile was: 0–30 min, 100% A (isocratic); 30–160 min, 0–15% B in A (linear gradient); 160–180 min, 15% B in A (isocratic). Flow-rate, 5 ml min^{-1}; detection at 280 nm. The second step was also performed using two solvents: (A) 2.5% formic acid; (B) acetonitrile. The elution profile was: 0–10 min, 100% A (isocratic); 10–80 min, 0–30% B in A (linear gradient). Flow-rate, 4 ml min^{-1}; detection at 280 nm.

2.4.3. HPLC–ESI-MS analysis

Two solvents were used: (A) 0.4% formic acid; (B) acetonitrile. The elution profile was: 0–3 min, 100% A (isocratic); 3–30 min, 0–30% B in A (linear gradient); 30–35 min, 30–40% B in A (linear gradient). Flow-rate, 1 ml min^{-1}; detection at 280 nm.

The following conditions were used in ESI-MS. Negative ion experiments: needle voltage, −4000 V; heated nitrogen gas temperature, 300°C; orifice plate voltage, −35 V; ring voltage, −220 V; nebulizer gas at position 8; curtain gas at position 10. Positive ion experiments: needle voltage, 5200 V; heated nitrogen

gas temperature, 300°C; orifice plate voltage, 45 V; ring voltage, 220 V; nebulizer gas at position 8; curtain gas at position 10.

3. Results

3.1. Fractionation and purification of extract

Plant extracts are traditionally fractionated by column chromatography with Sephadex LH-20. We performed the fractionation by consecutive elution with water (fraction 1); 30% and 50% MeOH (fractions 2–3); and 10%, 30%, 50% and 70% Me_2CO (fractions 4–7). Caffeoylquinic acids, coumaroylquinic acids and flavonol glycosides were the main compounds in fractions 1–4. HTs eluted mainly in fractions 5–7 in the order of degree of galloylation of the glucose core. Fractions 5–7 were then further purified using preparative HPLC.

Mono- and digalloyl derivatives that eluted in fractions 1–4 are shown in Fig. 2A (crude birch leaf extract) and the main hydrolysable tannins in Fig. 2B (combined HT fractions 5–7). Some HTs in Fig. 2B are included also in Fig. 2A where they are, however, mainly overlapped by numerous other phenolics that were earlier identified in birch leaves [13–15].

3.2. HPLC–DAD

The UV spectrum of HTs is important when determining the aglycone nature of the molecule. In gallotannins the aglycone is gallic acid and it was mainly responsible for the shape of the UV spectra of gallotannins as can be seen in Fig. 3 (e.g., pentagalloylglucopyranose, peak 19). The differences in UV spectra among individual ellagitannins were caused by different numbers of C–C linkages between galloyl moieties in the tannin. This observation led us to the conclusion that the intensity of absorbance maximum in the region from 275 to 285 nm was dependent on the number of galloyl groups in relation to HHDP groups present in the molecule (see Fig. 3, peaks 6, 17, 10 and 18 having zero, one, two and three galloyl groups, respectively). The values of absorbance maxima were always greater for gallotannins in comparison to ellagitannins.

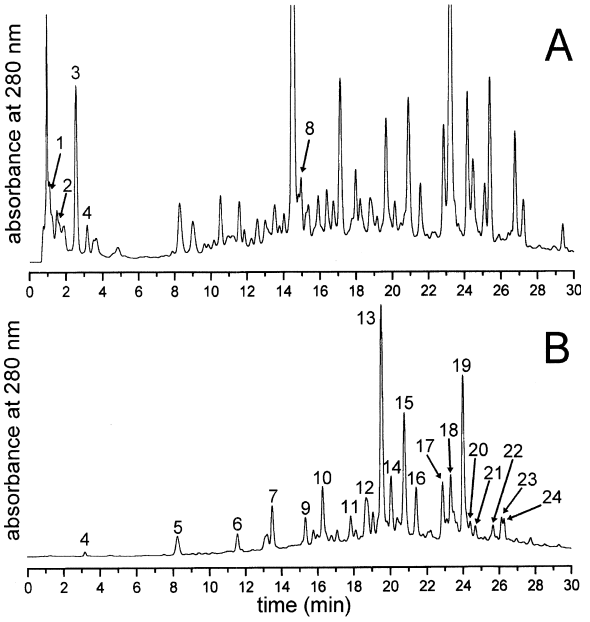

Fig. 2. HPLC traces of crude birch leaf extract (A) and purified fraction of hydrolysable tannins from birch leaves (B). Superspher 100 RP-18 column (75×4 mm I.D., 4 μm, Merck, Germany) at flow-rate of 1 ml min^{-1} with gradient of acetonitrile in 0.1 *M* H_3PO_4. Peaks: 1 and 2, HHDP-glucopyranose isomers; 3, galloylglucopyranose; 4, gallic acid; 5 and 6, bis-HHDP-glucopyranose isomers; 7 and 10, digalloyl-HHDP-glucopyranose isomers; 8, digalloylglucopyranose; 9, galloyl-HHDP-glucopyranose; 11, trigalloylglucopyranose; 12, 15 and 16, tetragalloylglucopyranose isomers; 13, 14 and 17, galloyl-bis-HHDP-glucopyranose isomers; 18, trigalloyl-HHDP-glucopyranose; 19 and 21, pentagalloylglucopyranose isomers; 20 and 22, tetragalloyl-HHDP-glucopyranose isomers; 23 and 24, hexagalloylglucopyranose isomers.

3.3. HPLC–ESI-MS analysis

In order to get information about the molecular masses of HTs preliminarily detected by HPLC–DAD, HPLC–ESI-MS analysis of the fractionated and partially purified extract was carried out. However, it was not possible to use the same chromatographic conditions as in HPLC–DAD because H_3PO_4 is not a preferred solvent in HPLC–ESI-MS. For this reason 0.1 *M* H_3PO_4 was replaced with 0.4% HCOOH.

The main peak in the mass spectra of an individual HT in the negative ion ESI-MS was the deprotonated molecule $[M-H]^-$. Almost all the HTs also gave information about $[M-2H]^{2-}$ or $[2M-H]^-$ ions

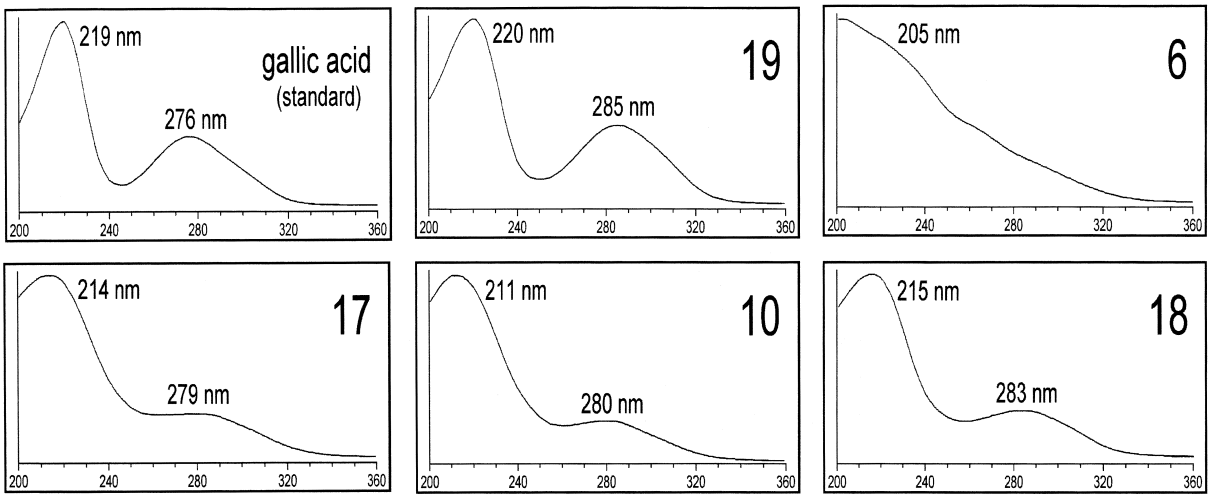

Fig. 3. Examples of characteristic UV spectra of hydrolysable tannins in birch (*Betula pubescens*) leaves. Numbers in spectra are related to the peaks in Fig. 2.

depending on the mass of the compound. On the basis of HPLC–ESI-MS analysis the total number of different HTs in the young birch leaves was found to be over 30, tetra- and pentagalloyl derivatives being the most numerous ones. The main m/z values and the number of isomers of HTs obtained from negative ion HPLC–ESI-MS are shown in Table 1.

Although electrospray is a very gentle ionisation method [11], it was possible to find some fragmentation patterns for HTs even in simple MS mode. For gallotannins the fragmentation involved losses of one or more galloyl groups (152 mass units) and the loss of 170 mass units corresponding to gallic acid. The fragmentation of pentagalloylglucopyranose (5GG) and hexagalloylglucopyranose (6GG) is shown in Fig. 4 and the corresponding mass peaks are shown in Fig. 5. The fragmentation pattern shown in Fig. 4 is postulative since we were not able to find any differences in the cleavability of different galloyl groups, e.g., in 5GG. However, in the case of 6GG we did not observe a loss of 170 mass units in addition to the loss of 152 mass units. This indicates

Table 1
The main m/z values and the number of isomers observed from negative ion experiments with HPLC–ESI-MS for hydrolysable tannins in birch leaves

Compound	m/z values	Number of isomers
Galloylglucopyranose	331, 663	2
HHDP-glucopyranose	481, 963	3
Digalloylglucopyranose	483, 967	2
Galloyl-HHDP-glucopyranose	633	2
Trigalloylglucopyranose	635, 465	2
bis-HHDP-glucopyranose	783, 391	2
Digalloyl-HHDP-glucopyranose	785, 392	2
Tetragalloylglucopyranose	787, 393, 617	4
Galloyl-bis-HHDP-glucopyranose	935, 467, 391	5
Trigalloyl-HHDP-glucopyranose	937, 468, 392	2
Pentagalloylglucopyranose	939, 469, 769, 393	2
Digalloyl-bis-HHDP-glucopyranose	1087, 543	1
Tetragalloyl-HHDP-glucopyranose	1089, 544	3
Hexagalloylglucopyranose	1091, 469, 545, 939, 393, 769	2

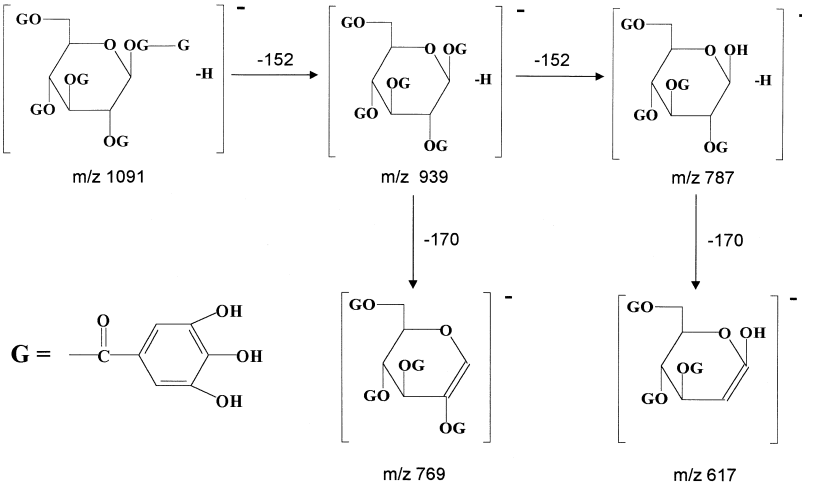

Fig. 4. Tentative fragmentation of hexa- (m/z 1091) and pentagalloylglucopyranose (m/z 939) in negative ion HPLC–ESI-MS.

that when a gallotannin has galloyl unit(s) attached to a 5GG core via a *meta*-depside bond these galloyl units are more cleavable in negative ion ESI-MS than galloyl units attached directly to the glucose core. Fig. 5B demonstrates further that in case of fragmentation the intensity of $[M-152-2H]^{2-}$ ion (m/z 469) is much greater than the intensity of $[M-152-H]^{-}$ ion (m/z 939) whereas in case of $[M-2H]^{2-}$ (m/z 545) and $[M-H]^{-}$ (m/z 1091) ions the ratio of the intensities is the opposite.

The fragmentation pattern of ellagitannins was less clear. In mass spectra of compounds 13, 14 and 18 (all pentagalloyl derivatives) it was possible to obtain m/z values 391, 391 and 392 respectively. These values corresponded to the loss of one galloyl group and were presented by $[M-2H]^{2-}$ ion. Similarly, in the case of compounds 5 and 6, m/z value 481 indicated loss of a hexahydroxydiphenoyl group.

Experiments in the positive ion mode of ESI-MS did not give either $[M+H]^{+}$, $[M+2H]^{2+}$ or $[2M+H]^{+}$ ion values for HTs. Instead, for gallotannins it gave m/z values corresponding to the loss of 170 mass units. For ellagitannins, that had at least one galloyl group attached to glucose, loss of 152 and in some cases also 170 mass units were obtained. These results suggest that positive ion ESI-MS can be a helpful addition to the negative ion experiments, although the information obtained is not so easily interpreted. This is mainly because of the lack of m/z values corresponding to the protonated molecule $[M+H]^{+}$.

Ion trace analysis is a useful tool for determining the concentrations of individual HTs even from crude birch leaf extracts. The measurement of peak areas related to ionisation intensities of deprotonated molecules of each individual HT is a suitable method for this purpose unless there are compounds present in the crude extract that have the same mass values as HTs. Fig. 6 shows the ion trace chromatograms of tetra-, penta- and hexagalloyl derivatives obtained from a combined HT fraction. So far we were not able to measure the concentrations of individual compounds because standards were not commercially available.

4. Discussion

On the basis of HPLC–ESI-MS analysis the abundance of individual HTs in birch leaves was confirmed. If individual HTs have specific effects like in birch–herbivore interactions, the above observation emphasises the importance of analysing at least the relative concentrations of all these individual compounds. This is especially important because the relative concentrations of gallo- and

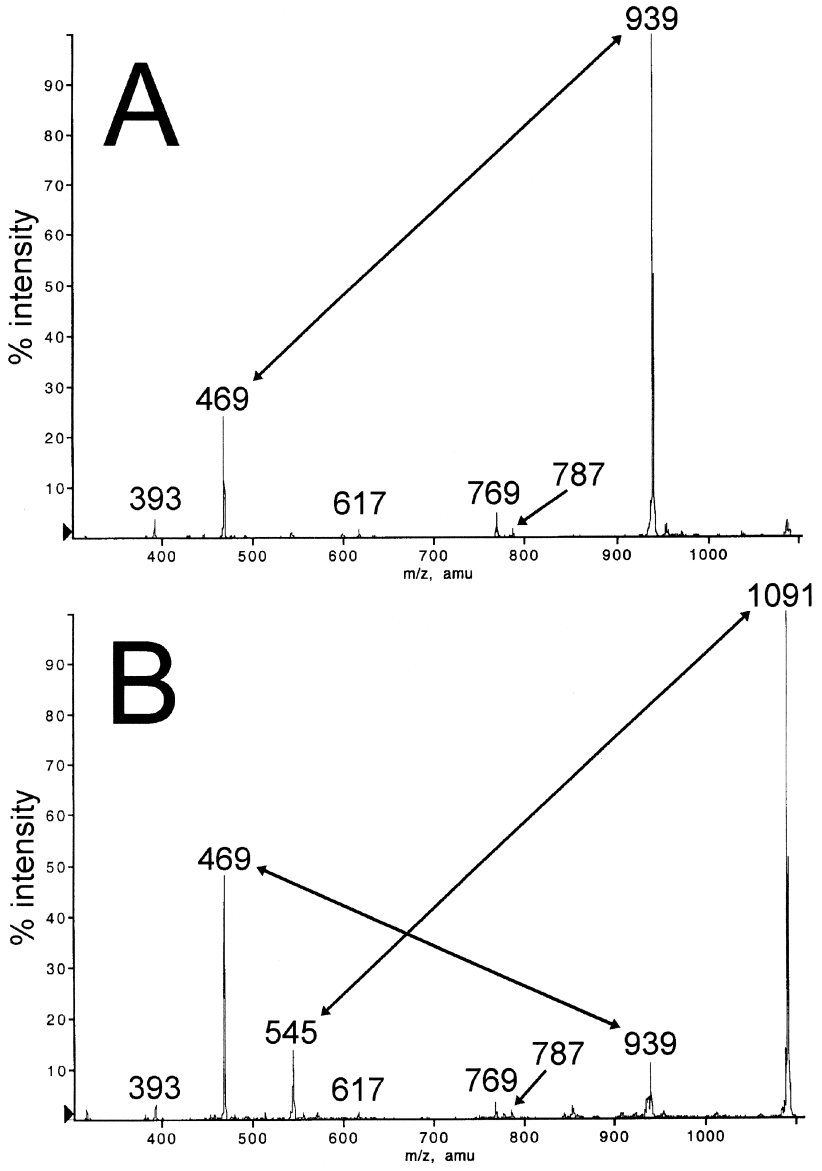

Fig. 5. The main m/z values for penta- (A) and hexagalloylglucopyranose (B) from negative ion HPLC–ESI-MS. (A) 939=$[M-H]^-$, 469=$[M-2H]^{2-}$, 787=$[M-152-H]^-$, 393=$[M-152-2H]^{2-}$, 769=$[M-170-H]^-$, 617=$[M-152-170-H]^-$; (B) 1091=$[M-H]^-$, 545=$[M-2H]^{2-}$, 939=$[M-152-H]^-$, 469=$[M-152-2H]^{2-}$, 787=$[M-304-H]^-$, 393=$[M-304-2H]^{2-}$, 769=$[M-152-170-H]^-$, 617=$[M-304-170-H]^-$.

ellagitannins may change during leaf growth and development as has been observed in *Liquidambar formosana* [16].

The isolation and purification of HTs from crude birch leaf extracts is necessary to achieve standards for the measurement of absolute concentrations. At the same time the determination of structural differences between isomers with ^{1}H nuclear magnetic

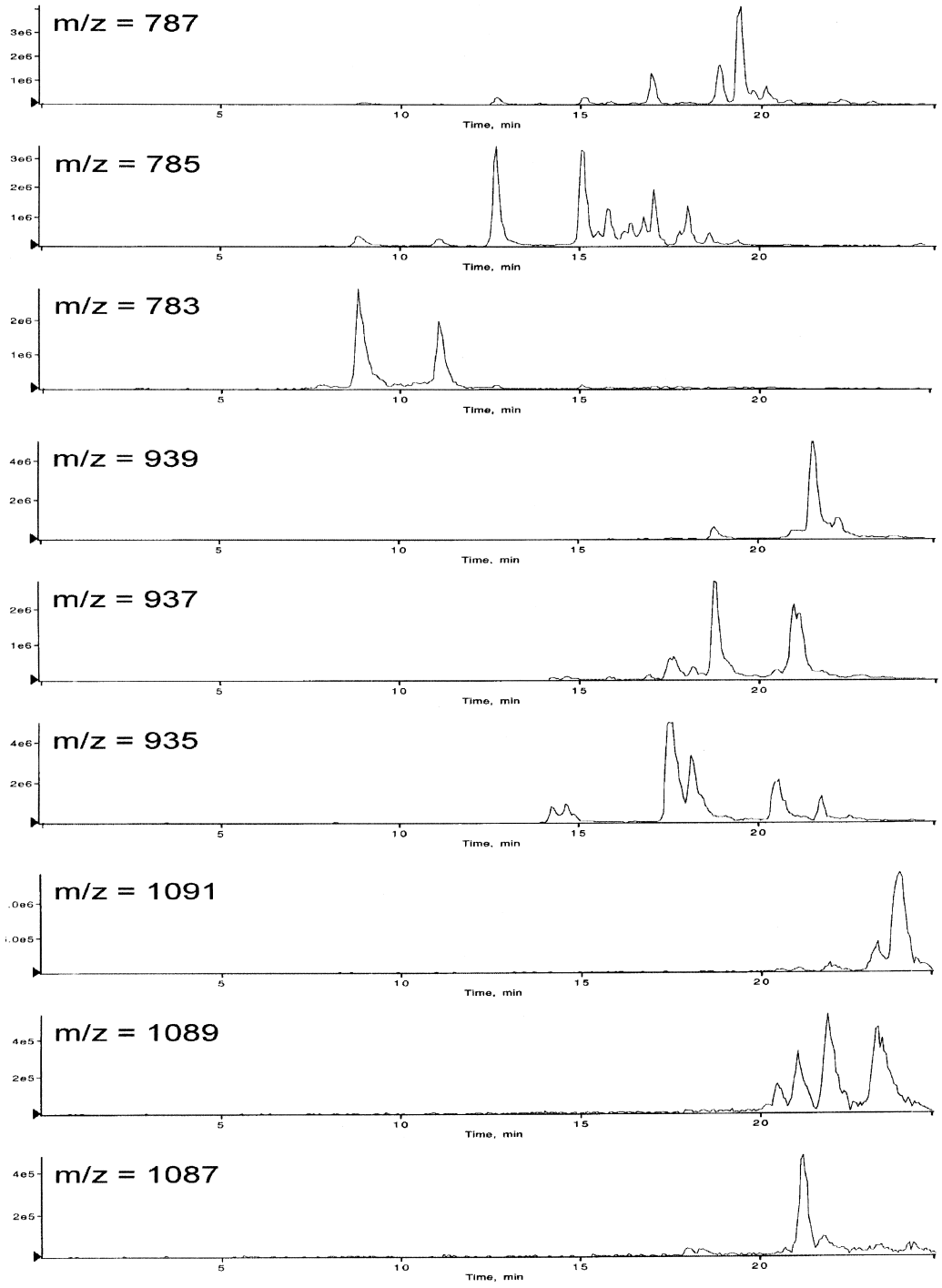

Fig. 6. Ion trace chromatograms of tetra- (m/z 783–787), penta- (m/z 935–939) and hexagalloyl (m/z 1087–1091) derivatives. The corresponding compounds and the number of isomers are shown in Table 1.

resonance (NMR) analysis will be crucial if e.g., the biosynthetic pathways of gallotannins and especially ellagitannins in birch (*Betula pubescens*) leaves are to be clarified.

Acknowledgements

This study was funded by a grant from the Academy of Finland to E.H.

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