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# Antioxidant properties and detailed polyphenol profiling of European hornbeam (*Carpinus betulus* L.) leaves by multiple antioxidant capacity assays and high-performance liquid chromatography/multistage electrospray mass spectrometry

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ABSTRACT

According to recent studies, the antioxidant and anticancer related properties of European hornbeam (*Carpinus betulus* L.) leaf extracts are remarkable, making it a possible raw material for medicine, requiring the identification of major active compounds and the assessment of the antioxidant properties of the extracts. In the present work the high-performance liquid chromatographic/multistage mass spectrometric characterization of the antioxidant polyphenolic compounds as well as the evaluation of the seasonal changes in the major antioxidant capacity and related parameters (DPPH, ABTS, FRAP, total phenol content, total flavonoid content and total flavan-3-ol content) have been carried out for the leaves of European hornbeam for the first time. The extracts exhibited the highest antioxidant capacity in August, basing on the FRAP (106.24 ± 3.10 mg AAE/g dw.) and DPPH ($IC_{50}$: 4.63 ± 0.88 μg/ml) assays and in May, based on the ABTS (329.78 ± 23.89 mg TE/g dw.) method. From the August extract a total of 171 compounds, including phenolic acids, flavonoid glycosides, tannins, catechins and procyanidins have been characterized and identified. According to the HPLC-PDA peak areas, the most abundant polyphenolic compounds in the August extracts were chlorogenic acid, ellagic acid, ellagitannins, myricetin-, luteolin-, quercetin- and apigenin glycosides. Further research is needed for the elucidation of the compounds which are primarily responsible for the antioxidant properties.

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## 1. Introduction

The leaf extracts of various tree species (Frédérich et al., 2009; Katsube et al., 2009; Germanò et al., 2012; Reinoso et al., 2012; Santos et al., 2013; Liu et al., 2015; Pereira et al., 2015; Talhaoui et al., 2015) have been known to exhibit several benefical effects (antioxidant, anticancer, anti-inflammatory, antidiabetic, etc.) on human health on consumption. This makes the investigation, identification and quantitative determination of the molecular constitutents with powerful antioxidant-and related properties highly important in these extracts. The group of plant extractives, primarily responsible for these health effects are the polyphenols (Watson, 2014). Plant polyphenols have not only a salutary effect on human health but also play a major role in the defense reactions in the tissues of their origin, against biotic and abiotic stress (e.g. infestation, climatic adaptation, etc.), which is another reason for the importance of their research as metabolomic indicators and biomarkers (Cadahía et al., 2015; Griesser et al., 2015).

Hornbeams are typical tree species of the Northern Hemisphere. Altogether 30–40 species are known and European hornbeam (*Carpinus betulus* L.) and to a lesser extent Oriental hornbeam (*Carpinus orientalis* L.) are the most common and widespread across much of Europe (Molnár, 2004). According to recent findings, the antioxidant capacity values of European hornbeam leaves are remarkably high compared to data of the leaves of other Hungarian forest tree species (Nebehaj et al., 2015). Previous research of Kuiters and Sarink (1986) revealed that the extracts of the October leaf litter of European hornbeam trees contain very high amounts of total phenols and phenolic acids compared to other common coniferous and deciduous trees. Moreover, the anticancer properties of European hornbeam leaf extracts have been also reported to be exceptional (Cieckiewicz et al., 2012). All these results make European hornbeam leaf a promising renewable biomass resource for the biorefinery utilization of its antioxidant extractives in the

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future for healthcare and medical products. This requires, however that the antioxidant properties of the leaves and the compounds responsible for the beneficial health effects be thoroughly investigated and identified.

The aim of the present research was the investigation of the antioxidant capacity as well as of the total amounts of polyphenols, flavonoids and flavan-3-ols in European hornbeam leaf extracts throughout the growing season (May–September) to find the time of the year when antioxidant properties are the best and to track which group of phenolics are the most abundant in the extracts. Antioxidant capacities were assayed with the DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (ferric reducing ability of plasma) and ABTS (2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid)) methods. From the extract showing the best antioxidant parameters, the detailed high-performance liquid chromatographic separation and multistage mass spectrometric (HPLC–MS/MS) characterization of individual compounds has been carried out for the first time. Compounds were characterized and identified by their mass spectra and most characteristic fragments, providing a database for the future investigation of the role of individual compounds in determining antioxidant and related properties in European hornbeam leaves.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Water for the extraction and HPLC analyses was produced with double distillation using conventional distillation equipment. Acetonitrile (LCMS grade) and methanol (HPLC grade) were obtained from VWR-International (Budapest, Hungary). (+)-Catechin, quercetin, rutin, ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulfate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-*S*-triazine (TPTZ), iron(III)-chloride, *p*-dimethylaminocinnamaldehyde (DMACA), acetic acid, sodium acetate, potassium acetate, hydrochloric acid, sulfuric acid, sodium carbonate, potassium hydrogen phosphate and potassium dihydrogen phosphate were obtained from Sigma-Aldrich (Budapest, Hungary). Folin-Ciocâlteu reagent was purchased from Merck (Darmstadt, Germany).

### 2.2. Sample collection and processing

Leaf samples were collected in the Botanical Garden of the University of West-Hungary, Sopron (Hungary), between May and September 2014, on 5 occasions (between the 15th and 22nd of each month). One healthy tree was sampled by taking 15 shade- and 15 sun leaves from different parts of the tree. Leaves were combined, treated with microwave irradiation for 1 min at 700 W in a household microwave oven to ensure the inactivation of polyphenol-oxidizing enzymes (Wang et al., 2008; Makk et al., 2013; Nebehaj et al., 2013) and ground. No further drying of the samples was carried out. Extraction was done immediately after the processing. Samples were extracted as follows: 0.2 g leaf powder with 20 mL methanol:water 80:20 v/v solution, for 20 min in an ultrasonic bath (Elma Transsonic T570 ultrasonic bath, Elma Schmidbauer GmbH, Singen, Germany). Extracts were filtered using 0.45 μm cellulose-acetate syringe filters prior to analyses.

### 2.3. Antioxidant capacity and total contents of phenols

Assays were run with a Hitachi U-1500 type spectrophotometer (Hitachi Ltd., Tokyo, Japan) at the respective wavelengths. All measurements were run in triplicate. The respective antioxidant capacity parameters were also determined for the reference compounds trolox, (+)-catechin and rutin.

#### 2.3.1. FRAP assay

The FRAP antioxidant capacity assay was run as described by Benzie and Strain (1996) using ascorbic acid as a standard; absorbance was measured at 593 nm. Results were determined in mg equivalents of ascorbic acid/g dry leaf units (mg AAE/g dw.).

#### 2.3.2. ABTS assay

The assay was run as described by Stratil et al. (2007) using the $ABTS^{+}$ radical ion and trolox standard for 10 min of reaction time; absorbance was measured at 734 nm. ABTS antioxidant power was given in mg equivalents of trolox/g dry leaf units (mg TE/g dw.).

#### 2.3.3. DPPH assay

The DPPH assay was carried out using the slightly modified method of Sharma and Bhat (2009) by mixing 2090 μL unbuffered methanol, 900 μL $2 \times 10^{-4}$ M methanolic DPPH solution and 10 μL extract. After incubation for 30 min at room temperature in the dark, the decrease in absorbance was measured at 515 nm. Results were calculated as $IC_{50}$ (50% inhibition concentration) values in μg extractives/ml assay (μg/ml) units, representing the amount of extractives which will react with 50% of the added DPPH radicals in the total volume of the assay (3 ml) under these conditions.

#### 2.3.4. Total phenol content

Total phenol content was measured using the Folin-Ciocâlteu assay (Singleton and Rossi, 1965) applying quercetin as the standard at 760 nm. Total phenol content was expressed in mg equivalents of quercetin/g dry leaf units (mg QE/g dw.).

#### 2.3.5. Total flavonoid content

The total flavonoid assay was run as descibed by Kalita et al. (2013) using quercetin as the standard at 415 nm. The total flavonoid content was expressed in mg equivalents of quercetin/g dry leaf units (mg QE/g dw.).

#### 2.3.6. Total flavan-3-ol content

The total flavan-3-ol content was determined using the *p*-dimethylaminocinnamaldehyde (DMACA) assay which gives a highly specific reaction with catechin-type compounds and with procyanidins (Treutter, 1989). The assay was run as follows: 2350 μL of methanol was mixed with 100 μL DMACA-reagent (2.0 m/v% DMACA dissolved in methanol: 6 N $H_2SO_4$ 50:50 v/v solution) and with 20 μL sample. After the reaction at room temperature for 20 min under minimal light conditions, the reaction mixture was subjected to spectrophotometric analysis at 640 nm. (+)-Catechin was used as the standard. Total flavan-3-ol content evaluated in mg equivalents of (+)-catechin/g dry leaf units (mg CE/g dw.).

### 2.4. Determination of total extractive content

5 ml of the extracts were evaporated to dryness at 80 °C in a drying chamber and the remaining solids were weighed. The total extractive content was calculated in mg extractives/ml extract units. The results were used for the calculation of the DPPH $IC_{50}$ values.

### 2.5. HPLC–MS/MS identification

HPLC measurements were carried out using a Shimadzu LC-20 type liquid chromatograph coupled with a Shimadzu SPD-M20A type diode array detector (PDA) (Shimadzu Corporation, Kyoto, Japan) and an AB Sciex 3200 QTrap triple quadrupole/linear ion trap

LC/MS/MS detector (AB Sciex, Framingham, USA). A Phenomenex Luna C18, 250 mm × 4.6 mm, 5 μm column was used for the separation with a Phenomenex SecurityGuard ULTRA LC type guard column (Phenomenex Inc., Torrance, USA) at 40 °C. The injection volume was 8 μL. The mobile phase consisted of A ($H_2O$ + 0.1% HCOOH) and B ($CH_3CN$ + 0.1% HCOOH). A gradient elution was run with 1.2 mL/min flow-rate using the following time gradient: 3% B (0–4 min), 5% B (22 min), 15% B (45 min), 57% B (83 min), 100% B (90–98 min), 3% B (99–106 min). In order to monitor basic separation quality of peaks, PDA detection was carried out in a wavelength range giving the highest absorbance (250–380 nm). In all of the measurements, negative electrospray ionization mode was used for the MS detector. Because of the relatively high flow rate of the mobile phase, flow-splitting was applied using a split valve, which allowed 0.6 mL/min flow to enter the MS ion source. Chromatographic data were acquired and evaluated using the Analyst 1.6.1 software. Polyphenols were identified with the Information Dependent Analysis (IDA) scanning function of the mass spectrometer which utilizes time programing and the linear ion trap function of the MS detector to perform automatic on-line MS/MS experiments during the chromatographic separation: survey (Q1) scans were performed between 150 and 1300 *m*/*z*. After selection of a particular *m*/*z* ion and Q2 fragmentation, the dependent (Q3) product ion scans were performed between 80 and 1300 *m*/*z*. In the ion source ion spray voltage was set at −4500 V, the curtain gas ($N_2$) pressure was set at 40 psi, spray gas ($N_2$) pressure at 30 psi, drying gas ($N_2$) pressure at 30 psi, and ion source temperature at 500 °C. The recorded MS/MS spectra were evaluated using the RIKEN tandem mass spectral database (Sawada et al., 2012), MassBank database (Horai et al., 2010), by scientific data found in the literature and by the use of fragmentation rules (McLafferty and Tureček, 1993).

### 2.6. Statistical evaluation of measured data

For the comparison of respective chemical parameters throughout the vegetation period, ANOVA analysis was run using Statistica 11 (StatSoft Inc., Tulsa, USA) software applying the Tukey HSD calculation method for the post-hoc test. In order to fulfill the requirements of the ANOVA analysis, values of the measurements were first checked for normal ditribution, then the variables were checked for the homogeneity of variances using Bartlett's Chi-square test.

## 3. Results and discussion

### 3.1. Seasonal changes in major chemical parameters

Table 1 summarizes the results of seasonal changes of the total phenol-, total flavonoid- and total flavan-3-ol contents, as well as the values of the DPPH, FRAP and ABTS antioxidant capacities. The total phenol content increased from May until July and decreased afterwards. The total amounts of flavan-3-ols and flavonoids reached maximum values in July and August respectively. With temperate-zone tree species, similiar seasonal tendecies were found for the leaves of European beech (*Fagus sylvatica* L.) showing increasing total phenol levels from early spring to late summer with maximum values in September (Pirvu et al., 2013). According to the literature, the tendecy of the seasonal changes in the total phenol content of plant leaves can also depend on species, site conditions (altitude, solar irradiation), the position of the leaves and variety (Zhang et al., 2010; Vagiri et al., 2015). Increasing total phenol concentrations were accompanied by stronger antioxidant capacities. Overall the best FRAP and DPPH levels were measured in August. It must be noted, however, that in the DPPH assay the alterations during the vegetation period were found not to be significant at the applied significance level. During the August measurements, the amount of total flavonoids reached maximum values too, indicating the determinant role of these compounds in the DPPH and FRAP antioxidant power of the extracts. The ABTS antioxidant capacity reached its highest value in May. Considering reference compounds, it was concluded that European hornbeam leaf extracts have medium to high antioxidant power compared to respective DPPH $IC_{50}$ (trolox: 4.29 μg/ml, (+)-catechin: 7.40 μg/ml, rutin: 13.94 μg/ml) FRAP (trolox: 550 mg AAE/g, (+)-catechin: 524 mg AAE/g, rutin: 295 mg AAE/g) and ABTS ((+)-catechin: 3514 mg TE/g, rutin: 1760 mg TE/g) values. Different antioxidant capacity assays can be specific or rather unspecific for certain types and classes of compounds (Frankel and Meyer, 2000); thus non-phenolic substances can influence measured values and the results will not necessarily correlate with total phenol levels. Compared with the total phenol concentration in the same month, the high ABTS antioxidant capacity of May leaf samples can not be explained by the high concentration of phenolic antioxidants solely, but presumably by the presence of antioxidants with different chemical structures, too.

Comparing total flavonoid and flavan-3-ol concentrations with the total phenol content of a given sample, we concluded that the flavan-3-ols are present only in minor concentrations and besides the flavonoids other classes of polyphenols (e.g. phenolic acids, tannins, stilbenes, etc.) must also be present in high amounts in the leaves of European hornbeam trees.

Several different types of compounds can account for the antioxidant properties of plant tissues, which requires the separation, characterization and identification of these molecules. In the present work this was done using high performance liquid chromatography coupled with multistage mass spectrometry, which resulted a database of retention times and MS/MS fragmentation data, suitable for the future investigation of the role of individual compounds in determining antioxidant and related properties of European hornbeam leaf extracts.

### 3.2. Identification of C. Betulus *L. leaf polyphenols by HPLC–MS/MS*

HPLC–MS/MS analysis was carried out for the detailed structural identification of major polyphenols and extractives in European hornbeam leaves for the first time. Analysis was done from the August extracts. Using the triple quadrupole/linear ion trap function of the mass spectrometer, on-line automated MS/MS experiments were run during the separation which enabled the recording of $MS^n$ spectra and the later structural elucidation of the molecules. The basic separation quality and retention behaviour of peaks was monitored using UV detection between 250 and 380 nm (Fig. 1.).

The detected and identified compounds are listed in Table 2. together with the corresponding retention and MS/MS fragmentation data.

Previous investigations, carried out on European hornbeam leaf litter extracts revealed the presence of phenolic acids (gallic acid, gentisic acid, vanillic acid, protocatechuic acid, *p*-coumaric acid, *o*-coumaric acid) and aldehydes (*p*-hydroxybenzaldehyde, syringaldehyde) by the use of silylation derivatization and gas chromatographic separation/flame ionization detection (Kuiters and Sarink, 1986). As partially degraded leaves (leaf litter) were used for the investigations, these compounds can be both native extractives and degradation products.

Based on the present HPLC separation and the evaluation of the MS/MS spectra, leaves of European hornbeam were found to contain a great variety of polyphenolic compounds including phenolic acids, ellagitannins, gallotannins, flavonoids, catechins, procyanidins as well as other unidentified compounds with defined $[M-H]^-$

**Table 1**
The total phenol content, total flavonoid content, total flavan-3-ol content and antioxidant capacity (DPPH, FRAP, ABTS) of European hornbeam leaf extracts (mean ± standard deviation). Different combinations of capitals indicate significant differences at $p < 0.05$ level. For the results of the DPPH assay significance level is $p < 0.02$.

| | May | June | July | August | September |
|---|---|---|---|---|---|
| Total phenol (mg QE/g dw.) | $78.81 \pm 0.59^{A}$ | $93.08 \pm 3.29^{B}$ | $105.93 \pm 5.57^{C}$ | $94.27 \pm 5.38^{B}$ | $80.81 \pm 3.05^{A}$ |
| Total flavan-3-ols (mg CE/g dw.) | $0.47 \pm 0.02^{A}$ | $0.73 \pm 0.01^{B}$ | $1.82 \pm 0.02^{E}$ | $1.66 \pm 0.01^{D}$ | $1.10 \pm 0.05^{C}$ |
| Total flavonoids (mg QE/g dw.) | $11.05 \pm 0.52^{B}$ | $11.03 \pm 0.75^{B}$ | $10.03 \pm 0.68^{B}$ | $12.84 \pm 0.28^{C}$ | $8.42 \pm 0.26^{A}$ |
| DPPH $IC_{50}$ (μg/ml) | $6.87 \pm 0.39^{A}$ | $6.37 \pm 1.90^{A}$ | $5.51 \pm 0.85^{A}$ | $4.63 \pm 0.88^{A}$ | $4.69 \pm 0.28^{A}$ |
| FRAP (mg AAE/g dw.) | $78.79 \pm 3.05^{AB}$ | $77.89 \pm 1.21^{A}$ | $84.04 \pm 2.67^{B}$ | $106.24 \pm 3.10^{D}$ | $92.08 \pm 1.21^{C}$ |
| ABTS (mg TE/g dw.) | $329.78 \pm 23.89^{C}$ | $315.18 \pm 3.21^{BC}$ | $280.83 \pm 4.57^{B}$ | $293.92 \pm 14.53^{B}$ | $236.20 \pm 5.68^{A}$ |

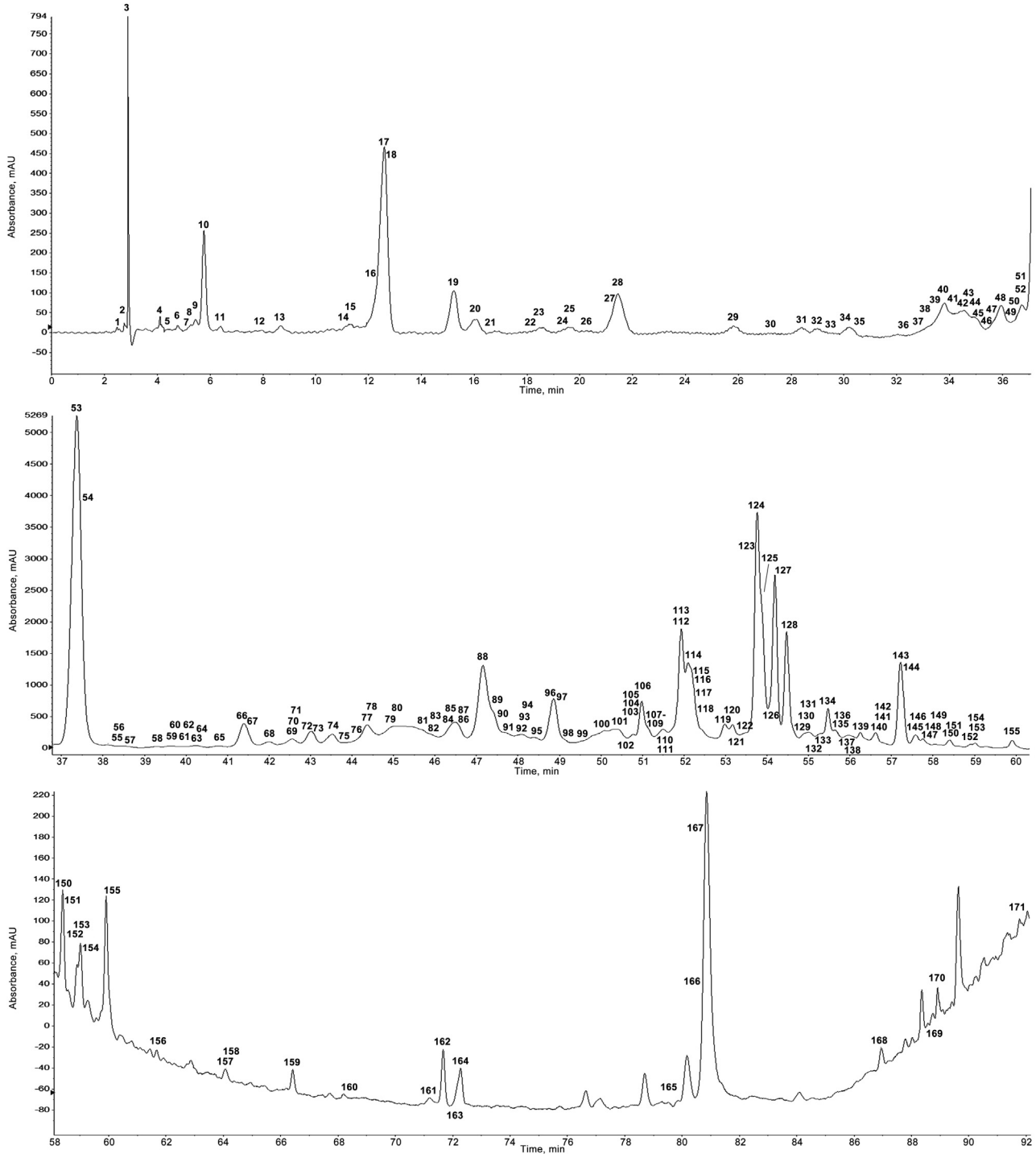


**Fig. 1.** The UV (250–380 nm) chromatogram of the *Carpinus betulus* leaf extract.

**Table 2**
Identified polyphenols from European hornbeam leaf extracts – chromatographic and mass spectrometric data.

| Peak | $t_r$ (min) | Compound name | $[M-H]^-$ $m/z$ | MS/MS $m/z$ |
|---|---|---|---|---|
| 1 | 2.63 | Unidentified-*O*-hexoside | 355 | 235, 193, 179, 161, 143, 125, 113, 101 |
| 2 | 2.76 | Unidentified | 405 | 387, 191 |
| 3 | 2.90 | Unidentified | 209 | 191, 173, 159, 141, 129, 111 |
| 4 | 4.10 | Unidentified | 337 | 277, 174, 127 |
| 5 | 4.32 | Monogalloyl glucose | 331 | 271, 211, 169, 151, 125 |
| 6 | 4.80 | Unidentified | 225 | 179, 115 |
| 7 | 5.27 | Unidentified | 517 | 499, 454, 411, 395, 355, 249, 223, 205, 190, 179 |
| 8 | 5.31 | Monogalloyl glucose | 331 | 271, 211, 169, 151, 125 |
| 9 | 5.45 | Galloylquinic acid isomer | 343 | 191, 169, 125, 111, 107 |
| 10 | 5.79 | Unidentified | 355 | 337, 311, 293, 274, 249, 237, 205, 193, 187, 179, 161, 159, 133, 121, 105 |
| 11 | 6.41 | Monogalloyl glucose | 331 | 271, 211, 169, 151, 125 |
| 12 | 7.9 | Monogalloyl glucose | 331 | 271, 211, 169, 151, 125 |
| 13 | 8.68 | Gallic acid | 169 | 125 |
| 14 | 11.20 | Galloylquinic acid isomer | 343 | 211, 191, 173, 169, 137, 125, 93 |
| 15 | 11.30 | Hydroxydihydrocaffeoylquinic acid | 371 | 353, 341, 191, 179, 173, 161, 135, 111 |
| 16 | 12.20 | Hydroxydihydrocaffeoylquinic acid | 371 | 353, 341, 191, 179, 173, 161, 135, 111 |
| 17 | 12.60 | Galloylquinic acid isomer | 343 | 211, 191, 173, 169, 137, 125, 93 |
| 18 | 12.68 | Hydroxydihydrocaffeoylquinic acid | 371 | 353, 341, 191, 179, 173, 161, 135, 111 |
| 19 | 15.27 | Unidentified gallic acid derivative | 345 | 183, 169, 125, 113 |
| 20 | 16.13 | Unidentified-*O*-hexoside | 345 | 331, 285, 225, 197, 183, 139, 107 |
| 21 | 16.69 | Unidentified caffeoylquinic acid derivative | 371 | 197, 191, 179, 173, 135, 111, 93 |
| 22 | 18.30 | Gallocatechin | 305 | 261, 219, 179, 167, 137, 125 |
| 23 | 18.60 | (iso)Vanillic acid | 167 | 152, 123, 108 |
| 24 | 19.35 | Unidentified caffeoylquinic acid derivative | 371 | 197, 191, 179, 173, 135, 111, 93 |
| 25 | 19.70 | Galloylshikimic acid isomer | 325 | 281, 169, 155, 137, 125, 111 |
| 26 | 20.25 | Methoxy-benzoic acid-*O*-hexoside derivative | 345 | 183, 168, 139, 124 |
| 27 | 21.40 | Methoxy-benzoic acid-*O*-hexoside derivative | 345 | 183, 168, 139, 124 |
| 28 | 21.60 | Galloylshikimic acid isomer | 325 | 169, 155, 125, 111, 107, 93 |
| 29 | 25.82 | Syringic acid-*O*-hexoside | 359 | 197, 182, 167, 153, 138, 123, 95 |
| 30 | 27.25 | Protocatechuic acid-*O*-pentoside | 285 | 153, 152, 135, 109, 108 |
| 31 | 28.41 | Monogalloyl glucose | 331 | 271, 211, 169, 151, 125 |
| 32 | 28.98 | Syringic acid-*O*-hexoside | 359 | 239, 203, 197, 182, 167, 153, 138 |
| 33 | 29.66 | Galloylshikimic acid isomer | 325 | 169, 137, 125, 111, 107 |
| 34 | 30.16 | Methyl gallate | 183 | 168, 139, 124 |
| 35 | 30.38 | Syringic acid-*O*-hexoside | 359 | 269, 239, 197, 182, 167, 153, 138 |
| 36 | 32.25 | Unidentified-*O*-hexoside | 389 | 343, 227, 212, 209 |
| 37 | 32.82 | Unidentified-*O*-hexoside | 389 | 343, 227, 212, 209, 179, 152, 137 |
| 38 | 33.08 | Epigallocatechin | 305 | 261, 219, 179, 167, 165, 137, 125 |
| 39 | 33.62 | Procyanidin B dimer | 577 | 533, 425, 407, 289, 245, 125 |
| 40 | 33.76 | Unidentified | 337 | 277, 249, 237, 219, 205, 193, 177, 161, 149, 133 |
| 41 | 33.80 | Chlorogenic acid isomer | 353 | 191, 179, 135 |
| 42 | 34.60 | Caffeic acid-*O*-hexoside | 341 | 179, 135 |
| 43 | 34.78 | Procyanidin B dimer | 577 | 533, 425, 407, 289, 245, 125 |
| 44 | 34.85 | Unidentified | 401 | 239, 221, 203, 177, 161, 149, 144, 121 |
| 45 | 35.00 | (+)-Catechin | 289 | 245, 221, 203, 125, 109 |
| 46 | 35.35 | Unidentified | 509 | 465, 273, 191 |
| 47 | 35.45 | Unidentified | 417 | 285, 241, 152, 108 |
| 48 | 35.92 | Digalloyl-HHDP glucose | 785 | 633, 615, 483, 419, 313, 301, 275, 249, 205, 169 |
| 49 | 36.17 | Unidentified | 417 | 285, 241, 152, 108 |
| 50 | 36.59 | Unidentified | 307 | 263, 219, 203, 174, 157 |
| 51 | 36.72 | Unidentified | 423 | 337, 293, 249, 205 |
| 52 | 36.8 | Digalloylquinic acid | 495 | 343, 337, 325, 191, 169, 125, 107 |
| 53 | 37.39 | Chlorogenic acid isomer | 353 | 191, 179, 135 |
| 54 | 37.53 | Unidentified mw: 1084 | 1083 | $[M-2H]^{2-}$: 541 |
| 55 | 38.37 | Unidentified | 401 | 293, 269, 161, 101 |
| 56 | 38.40 | Digalloyl glucose | 483 | 439, 331, 313, 287, 271, 169, 125 |
| 57 | 38.59 | Unidentified | 527 | 509, 479, 313, 169, 125 |
| 58 | 39.30 | Sinapaldehyde | 207 | 192, 177, 163 |
| 59 | 39.63 | Trigalloyl glucose | 635 | 483, 465, 313, 271, 211, 169, 125 |
| 60 | 39.74 | Galloylquercetin | 453 | 327, 313, 301, 300, 297, 285, 169, 125 |
| 61 | 39.93 | Unidentified | 291 | 247, 219, 191 |
| 62 | 40.06 | Monogalloyl glucose-quinic acid ester | 505 | 331, 313, 245, 177, 173, 169, 135, 125, 111 |
| 63 | 40.25 | Unidentified | 1127 | |
| 64 | 40.3 | Galloylquercetin | 453 | 327, 313, 301, 300, 297, 285, 169, 125 |
| 65 | 40.81 | Unidentified gallic acid derivative, mw: 652 | 651 | 481, 453, 437, 409, 381, 355, 275, 231, 169, 125 |
| 66 | 41.4 | Chlorogenic acid isomer | 353 | 191, 179, 135 |
| 67 | 41.42 | Unidentified-*O*-hexoside | 437 | 317, 275, 274, 257, 218, 190 |
| 68 | 42.04 | Unidentified-*O*-hexoside | 431 | 385, 265, 223, 205, 161, 153, 138, 113, 101 |
| 69 | 42.56 | Unidentified, mw: 1084 | 1083 | $[M-2H]^{2-}$: 541 |
| 70 | 42.57 | Unidentified-*C*-hexoside | 361 | 343, 271, 241, 223, 199 |
| 71 | 42.60 | Unidentified-*O*-hexoside | 437 | 317, 275, 274, 257, 218, 190 |
| 72 | 42.93 | (−)-Epicatechin | 289 | 245, 221, 203, 125, 109 |
| 73 | 43.03 | Galloyl-HHDP-glucose | 633 | 463, 418, 331, 301, 275, 273, 241 |
| 74 | 43.53 | Digalloyl-HHDP glucose | 785 | 633, 615, 483, 419, 313, 301, 275, 249, 205, 169 |
| 75 | 43.8 | Unidentified | 431 | 385, 205, 179, 153 |
| 76 | 44.12 | Unidentified gallic acid derivative | 477 | 325, 201, 169, 125 |

Table 2 (*Continued*)

| Peak | $t_r$ (min) | Compound name | $[M-H]^-$ $m/z$ | MS/MS $m/z$ |
|---|---|---|---|---|
| 77 | 44.3 | Trigalloyl glucose | 635 | 483, 465, 313, 271, 211, 169, 125 |
| 78 | 44.37 | Unidentified gallic acid derivative, mw: 652 | 651 | 481, 453, 437, 419, 331, 313, 275, 169, 125 |
| 79 | 44.93 | p-Coumaroylquinic acid isomer | 337 | 191, 173, 163, 119 |
| 80 | 45.04 | Unidentified | 345 | 271, 241, 212 |
| 81 | 45.7 | Apigenin-di-*C*-hexoside | 593 | 557, 503, 473, 413, 383, 353, 325, 269 |
| 82 | 45.94 | Unidentified | 431 | 223, 205, 187, 179, 135 |
| 83 | 45.95 | Trigalloyl glucose | 635 | 483, 465, 313, 271, 211, 169, 125 |
| 84 | 46.38 | Unidentified ellagitannin, mw: 1110 | 1109 | 1049, 973, 935, 765, 633, 615, 501, 463, 301, 275, 249, 205 |
| 85 | 46.39 | Unidentified | 337 | 249, 205, 190, 177, 163, 161, 133 |
| 86 | 46.60 | Caffeoylshikimic acid isomer | 335 | 179, 161, 155, 135, 111, 93 |
| 87 | 46.60 | Unidentified | 1127 | |
| 88 | 47.18 | Unidentified ellagitannin, mw: 952 | 951 | 933, 915, 765, 615, 463, 343, 301, 273, 245 |
| 89 | 47.44 | Quercetin-*O*-hexoside | 463 | 343, 301, 300, 272, 257, 215, 191 |
| 90 | 47.6 | Unidentified ellagitannin, mw: 936 | 935 | $[M-2H]^{2-}$: 467; 765, 301, 275, 247, 169 |
| 91 | 47.71 | Trigalloyl glucose | 635 | 483, 465, 313, 271, 211, 169, 125 |
| 92 | 48.1 | Digalloyl-HHDP glucose | 785 | 633, 615, 483, 419, 313, 301, 275, 249, 205, 169 |
| 93 | 48.2 | Quercetin-*O*-hexoside | 463 | 343, 301, 300, 272, 257, 215, 191 |
| 94 | 48.27 | Unidentified | 511 | 327, 183, 168, 124 |
| 95 | 48.40 | Unidentified | 275 | 257, 229, 219, 203, 201, 191 |
| 96 | 48.8 | Unidentified-*O*-hexoside | 437 | 275, 274, 257, 229, 218, 203, 190 |
| 97 | 48.88 | Unidentified ellagitannin, mw: 1110 | 1109 | 1049, 973, 935, 765, 633, 615, 501, 463, 301, 275, 249, 205 |
| 98 | 49.1 | Myricetin-*O*-rhamnoside derivative | 643 | 489, 463, 343, 317, 316, 287, 271, 179, 135 |
| 99 | 49.55 | Caffeoylshikimic acid isomer | 335 | 179, 161, 155, 135 |
| 100 | 50.06 | Unidentified ellagitannin, mw: 1110 | 1109 | 1049, 973, 935, 765, 633, 615, 501, 463, 301, 275, 249, 205 |
| 101 | 50.34 | Unidentified ellagitannin, mw: 954 | 953 | 935, 765, 615, 463, 419, 301, 275, 247 |
| 102 | 50.52 | Unidentified-*O*-hexoside | 579 | 459, 417, 181, 166 |
| 103 | 50.78 | Myricetin-*O*-hexoside | 479 | 317, 316, 287, 271, 179, 151 |
| 104 | 50.8 | Coumaroyl-*O*-galloyl-glucose | 477 | 433, 331, 313, 295, 211, 169, 163, 125 |
| 105 | 50.86 | Unidentified | 343 | 284, 269, 239, 211 |
| 106 | 51.01 | Quercetin-*O*-hexoside | 463 | 343, 301, 300, 272, 257, 215, 191 |
| 107 | 51.14 | Coumaroyl-*O*-galloyl-glucose | 477 | 433, 313, 241, 211, 169, 163, 125, 119 |
| 108 | 51.16 | Myricetin-*O*-hexoside | 479 | 317, 316, 287, 271, 179, 151 |
| 109 | 51.30 | Unidentified-*C*-hexoside | 359 | 341, 329, 323, 299, 269, 257, 239, 211, 197, 113 |
| 110 | 51.5 | Myricetin-*O*-rhamnoside derivative | 643 | 495, 489, 463, 452, 343, 317, 316, 287, 271, 135 |
| 111 | 51.50 | Unidentified | 1127 | |
| 112 | 51.96 | Unidentified ellagitannin, mw: 954 | 953 | $[M-2H]^{2-}$: 476; 935, 765, 633, 615, 463, 419, 301, 275, 247 |
| 113 | 51.96 | Unidentified ellagitannin, mw: 1106 | 1105 | $[M-CO_2-2H]^{2-}$: 530; 891, 767, 425, 392, 313, 301, 273, 169, 125 |
| 114 | 52.11 | Trigalloyl-HHDP-glucose | 937 | $[M-2H]^{2-}$: 468; 785, 767, 635, 465, 313, 301, 275, 169 |
| 115 | 52.21 | Quercetin-*O*-pentoside | 433 | 301, 300, 272, 257, 216 |
| 116 | 52.22 | Unidentified ellagitannin, mw: 1106 | 1105 | $[M-CO_2-2H]^{2-}$: 530; 891, 767, 425, 392, 313, 301, 273, 169, 125 |
| 117 | 52.28 | Unidentified | 985 | |
| 118 | 52.35 | Tetragalloyl glucose | 787 | 635, 617, 465, 447, 313, 215, 169 |
| 119 | 53.00 | Tetragalloyl glucose | 787 | 635, 617, 465, 447, 313, 215, 169 |
| 120 | 53.07 | Isorhamnetin-*O*-hexoside | 477 | 315, 314, 300, 299, 271 |
| 121 | 53.13 | Unidentified | 413 | 369, 351, 311, 269, 161, 125, 99 |
| 122 | 53.5 | Myricetin-*O*-pentoside | 449 | 317, 316, 287, 271, 269, 259, 243, 209, 179, 151, 137 |
| 123 | 53.63 | Tetragalloyl glucose | 787 | 635, 617, 465, 447, 313, 215, 169 |
| 124 | 53.78 | Myricetin-*O*-rhamnoside | 463 | 359, 317, 316, 287, 271, 259, 242, 191, 179 |
| 125 | 53.90 | Ellagic acid | 301 | 284, 257, 229, 185 |
| 126 | 54.10 | Quercetin-*O*-hexoside | 463 | 343, 301, 300, 272, 257, 215, 191 |
| 127 | 54.22 | Trigalloyl-HHDP-glucose | 937 | $[M-2H]^{2-}$: 468; 785, 767, 635, 465, 313, 301, 275, 169 |
| 128 | 54.50 | Luteolin-*O*-hexoside | 447 | 327, 285, 284, 217, 199, 175, 151, 133 |
| 129 | 54.76 | Unidentified | 509 | 473, 461, 367, 327, 313, 179, 163, 149, 119, 107 |
| 130 | 54.89 | Unidentified | 415 | 369, 179 |
| 131 | 55.00 | Myricetin-*O*-hexoside-derivative | 657 | 547, 515, 479, 401, 369, 317, 316, 271, 220, 179, 151 |
| 132 | 55.04 | Isorhamnetin-*O*-hexoside | 477 | 315, 314, 300, 299, 271 |
| 133 | 55.38 | Unidentified | 331 | 287, 243, 177, 165, 133, 121, 109 |
| 134 | 55.49 | Pentagalloyl glucose | 939 | $[M-2H]^{2-}$: 469; 769, 629, 617, 465, 313, 169, 125 |
| 135 | 55.64 | Quercetin-*O*-hexoside | 463 | 343, 301, 300, 272, 257, 215, 191 |
| 136 | 55.67 | Myricetin-*O*-hexoside-derivative | 657 | 547, 515, 479, 401, 369, 317, 316, 271, 179, 151 |
| 137 | 55.81 | Digalloyl glucose | 483 | 439, 331, 313, 287, 271, 169, 125 |
| 138 | 56.04 | Unidentified | 579 | 459, 417, 402, 387, 181, 166 |
| 139 | 56.28 | Digalloyl-bis-HHDP-glucose | 1087 | $[M-2H]^{2-}$: 543; 451, 425, 399, 313, 301, 169, 125 |
| 140 | 56.49 | Pentagalloyl glucose | 939 | $[M-2H]^{2-}$: 469; 769, 629, 617, 465, 313, 169, 125 |
| 141 | 56.62 | Quercetin-*O*-pentoside | 433 | 301, 300, 272, 257, 243, 229, 179, 151 |
| 142 | 56.65 | Isorhamnetin-*O*-pentoside | 447 | 315, 314, 300, 299, 285, 271, 243 |
| 143 | 57.22 | Quercetin-*O*-rhamnoside | 447 | 343, 301, 300, 272, 255, 243, 179, 151 |
| 144 | 57.30 | Apigenin-*O*-hexoside | 431 | 431, 341, 311, 269, 268, 225, 201, 151, 149 |
| 145 | 57.5 | Unidentified | 477 | 315, 314, 297, 271 |
| 146 | 57.60 | Unidentified | 429 | 249, 205, 187, 161 |
| 147 | 57.8 | Pentagalloyl glucose | 939 | $[M-2H]^{2-}$: 469; 769, 629, 617, 465, 313, 169, 125 |
| 148 | 57.88 | Unidentified | 1173 | |
| 149 | 58.06 | Unidentified gallic acid derivative | 461 | 446, 313, 298, 283, 269, 255, 169, 125 |
| 150 | 58.43 | Unidentified gallic acid derivative | 461 | 446, 328, 313, 298, 285, 270, 255, 169, 125 |
| 151 | 58.45 | Unidentified | 539 | 503, 397, 343, 328, 179, 164, 146, 134 |
| 152 | 58.9 | Unidentified | 463 | 301, 245 |
| 153 | 59.03 | Unidentified gallic acid derivative, mw: 970 | 969 | $[M-2H]^{2-}$: 484; 799, 645, 408, 325, 169, 125 |

Table 2 (*Continued*)

| Peak | $t_r$ (min) | Compound name | $[M-H]^-$ *m/z* | MS/MS *m/z* |
|---|---|---|---|---|
| 154 | 59.07 | Eucaglobulin | 497 | 482, 411, 327, 285, 183, 169, 125 |
| 155 | 59.94 | Kaempferol-*O*-rhamnoside | 431 | 327, 285, 284, 255, 229, 227, 211, 187 |
| 156 | 61.57 | Unidentified | 389 | 251, 227, 207, 183, 165 |
| 157 | 63.99 | Unidentified | 327 | 283, 254, 211, 196 |
| 158 | 64.07 | Unidentified | 585 | 509, 371, 359, 195, 165 |
| 159 | 66.42 | Unidentified | 853 | 785, 723, 365, 343, 217, 197 |
| 160 | 68.21 | Unidentified | 617 | 197, 182, 151, 137, 125 |
| 161 | 71.3 | Unidentified | 221 | 177, 149, 147, 135, 121 |
| 162 | 71.7 | Kaempferol-di-*O*-rhamnoside | 577 | 431, 413, 285, 284, 255, 227, 145, 119 |
| 163 | 72.06 | Unidentified | 369 | 277, 241, 211 |
| 164 | 72.32 | Kaempferol-di-*O*-rhamnoside | 577 | 431, 413, 285, 284, 255, 227, 145, 119 |
| 165 | 79.72 | Unidentified | 287 | 269, 241, 223, 155, 115 |
| 166 | 80.67 | Unidentified | 287 | 269, 241, 223, 155, 115 |
| 167 | 80.70 | Unidentified | 243 | 243, 175 |
| 168 | 86.90 | Unidentified | 345 | 277, 197, 134, 127 |
| 169 | 88.70 | Unidentified | 539 | 471 |
| 170 | 88.93 | Unidentified | 243 | 197 |
| 171 | 91.98 | Unidentified | 455 | 409, 391 |

*m/z* values and MS/MS spectra. Altogether 171 compounds have been characterized and identified.

Respecting phenolic acids and flavonoids it was found that the majority of these compounds are present in form of glycosides. The most frequent sugar moieties conjugated to phenolic substances were pentoses, hexoses and rhamnose. The identity of the sugar part was verified by fragmentation data. Neutral losses of $[M-H-120]^-$ and $[M-H-162]^-$ (loss of hexosyl unit) and $[M-H-180]^-$ (loss of hexose sugar) indicated hexose sugars, while $[M-H-132]^-$ and $[M-H-150]^-$ the presence of a pentose moiety. The presence of rhamnose as a sugar unit was proven by the neutral loss of $[M-H-146]^-$. Considering the linkage of the sugar unit, glycosides can be either *C*-glycosides or *O*-glycosides which can be differentiated by the fragmentation characteristics of the sugar unit (Cuyckens and Claeys, 2004; Vukics and Guttman, 2010).

### 3.2.1. Phenolic acids and derivatives

One of the most abundant groups of phenolics found in the leaf extracts, were phenolic acids and their derivatives. Most of the phenolic acids (caffeic acid, methoxybenzoic acid, syringic acid, protocatechuic acid, gallic acid, ellagic acid) can be found in glycosylated form or conjugated to either shikimic acid or quinic acid. Gallic acid (**13**), ellagic acid (**125**), (iso)vanillic acid (**23**) were also found in underivatized form.

According to Fig. 1 the chlorogenic acid (caffoylquinic acid) isomer **53** can be found in high concentration in the extracts, while isomers **41** and **66** have lower concentrations. The $[M-H]^-$ ion at *m/z* 353 and fragments at *m/z* 191 [quinic acid−H]$^-$, 179 [caffeic acid−H]$^-$, and 135 [caffeic acid−H−$CO_2$]$^-$are characteristic of caffeoylquinic acid isomers. The high concentrations of ellagic acid (**125**) could be also verified from the chromatogram via peak heights.

Caffeoylshikimic acids (**86**, **99**) were identified by caffeoyl residues (*m/z* 179, 161, 135) and by the neutral loss of the shikimate moiety $[M-H-156]^-$ = *m/z* 179 as well as by the fragments of shikimic acid at *m/z* 155 [shikimic acid−H−$H_2O$]$^-$ and *m/z* 111 [shikimic acid−H−$H_2O$−$CO_2$]$^-$.

Peak **42** was identified as caffeic acid-*O*-hexoside, by caffeic acid fragments and the neutral loss of $[M-H-162]^-$ = *m/z* 179 [caffeic acid−H]$^-$.

Peaks (**15**, **16**, **18**) were tentatively identified as hydroxydihidrocaffeolyquinic acid isomers, as they show peaks similar to caffeolyquinic acids (*m/z* 353, 191, 179, 135), yet have an $[M-H]^-$ ion at *m/z* 371. The mass difference between 371 and 353 = 18 *m/z* was explained by the additional presence of two *H* atoms and one *O* atom as an addition to chlorogenic acid structure.

Peaks **21** and **24** were also identified as caffeoylquinic acid derivaties because of caffeic acid fragments (*m/z* 179, 135) and quinic acid fragments *m/z* 191 and *m/z* 173 [quinic acid−$H_2O$−H]$^-$.

Two methoxybenzoic acid-*O*-hexoside derivatives were found (**26**, **27**), which were identified by the neutral loss of $[M-H-162]^-$ = *m/z* 183 proving the *O*-hexoside moiety and the aglycone (*m/z* 183), as well as by the *m/z* 168 $[M-H-162-CH_3]^-$, and *m/z* 162 $[M-H-162-CO_2]^-$ ions.

Syringic acid was found in its *O*-hexoside conjugates (**29**, **32**, **35**). The aglycone as well as the presence of the *O*-hexoside moiety was proven by the $[M-H-162]^-$ = *m/z* 197 ion and by the *m/z* 182 [syringic acid−H−$CH_3$]$^-$, *m/z* 167 [syringic acid−H−2$CH_3$]$^-$ and *m/z* 153 [syringic acid−H−$CO_2$]$^-$ ions.

One protocatechuic acid-*O*-pentoside was identified (**30**) by the ions *m/z* 153 $[M-H-132]^-$ = [protocatechuic acid−H]$^-$, *m/z* 135 [protocatechuic acid−H−$H_2O$]$^-$ and *m/z* 109 [protocatechuic acid−H−$CO_2$]$^-$.

As opposed to earlier results (Kuiters and Sarink, 1986), no syringaldehyde was found in the extracts, instead sinapaldehyde (**58**) was shown.

### 3.2.2. Tannins

European hornbeam leaves contain a wide variety of hydrolysable tannins, gallotannins and ellagitannins. Gallotannins were characterized by their typical *m/z* 169 and 125 MS/MS fragments which correspond to [gallic acid−H]$^-$ and [gallic acid−H−$CO_2$]$^-$ fragment ions respectively and by fragments of glucose linked to different numbers of galloyl units. The loss of galloyl moieties was tracked by the mass differences of *m/z* 152 and the neutral loss of gallic acid was indicated by differences of *m/z* 170 in the MS/MS spectra. Ellagitannins were characterized by the presence of the *m/z* 301 [ellagic acid−H]$^-$ fragment ion as well as by the typical loss of one or more HHDP (hexahydroxydiphenoyl) units, producing $[M-H-302]^-$ fragments in the mass spectrum (Sanz et al., 2010). Large-sized ellagitannins often produced doubly charged molecule ions ($[M-2H]^{2-}$) which was proven by the mass difference of the isotopic peaks of the molecule ion (0.5 *m/z* instead of 1 *m/z*). The typical residue of the cleavage of both gallotannins and ellagitannins were the *m/z* 313 ion corresponding to [galloyl glucose−$H_2O$]$^-$ ion and the *m/z* 331 ion [galloyl glucose−H]$^-$.

Monogalloyl glucoses (**5**, **8**, **11**, **12**, **31**) were identified by the *m/z* 331 $[M-H]^-$ ion and the presence of fragments *m/z* 169, 125 (gallic acid) as well as by the neutral loss of 120 (*m/z* 271) and 162 (*m/z* 169) units which correspond to the presence of a hexose moiety.

Galloyl-HHDP-glucose at *m/z* 633 (**73**) was identified by *m/z* 463 [M−H−gallic acid]$^-$ and $[M-H-HHDP]^-$ = *m/z* 331 fragments.

Digalloyl glucose isomers (**56**, **137**) at *m/z* 483 were characterized by the fragment at *m/z* 313 [M−H−gallic acid]$^-$ ion as well as with *m/z* 169 and 125 ions (Wang et al., 2011).

The digalloyl-HHDP-glucose isomers (**48**, **74**, **92**) at *m/z* 785 were identified by the presence of *m/z* 615 [M−H−gallic acid]$^-$, *m/z* 313 [M−H−gallic acid−HHDP]$^-$ and *m/z* 301 ions.

One digalloyl-*bis*-HHDP-glucose compound was found (**139**) with *m/z* 1087 [M−H]$^-$ and *m/z* 543 [M−2H]$^{2-}$ and was verified by *m/z* 313 [M−H−HHDP−HHDP−gallic acid]$^-$ and by *m/z* 301 [ellagic acid−H]$^-$ ions. This compound was also confirmed in the leaves of *Betula pubescens*, which is a closely related species to European hornbeam (Salminen et al., 1999).

Four isomers of trigalloyl glucose at *m/z* 635 were identified (**59**, **77**, **83**, **91**) by fragment ions *m/z* 483 [M−H−galloyl]$^-$ and *m/z* 313 [M−H−galloyl−gallic acid]$^-$. Two trigalloyl-HHDP-glucose isomers (**114**, **127**) were identified by the *m/z* 785 [M−2H]$^{2-}$ doubly charged molecule ion and by the fragments *m/z* 767 [M−H−gallic acid]$^-$, *m/z* 465 [M−H−gallic acid−HHDP]$^-$ fragments and by the *m/z* 313 [M−H−gallic acid−HHDP−galloyl]$^-$ ions.

Tetragalloyl glucose isomers at *m/z* 787 were found at peak positions **118**, **119** and **123**. Characteristic fragmentation of these compounds involved *m/z* 635 [M−H−galloyl]$^-$, *m/z* 617 [M−H−gallic acid]$^-$, *m/z* 465 [M−H−gallic acid−galloyl]$^-$, *m/z* 447 [M−H−gallic acid−gallic acid]$^-$, and *m/z* 313 [M−H−gallic acid−galloyl−galloyl]$^-$ ions.

Pentagalloyl glucose compounds (**134**, **140**, **147**) with *m/z* 939 [M−H]$^-$ and *m/z* 469 [M−2H]$^{2-}$ showed typical fragments at *m/z* 769 [M−H−gallic acid]$^-$, *m/z* 617 [M−H−gallic acid−galloyl]$^-$, *m/z* 465 [M−H−gallic acid−galloyl−galloyl]$^-$ and *m/z* 313 [M−H−gallic acid−galloyl−galloyl−galloyl]$^-$.

Unidentified gallic acid derivatives were found at [M−H]$^-$ *m/z* 345 (**19**), *m/z* 461 (**149**, **150**), *m/z* 477 (**76**), *m/z* 651 (**65**, **78**) and *m/z* 969 (**153**) showing the typical fragments of gallic acid in their mass spectra.

Unidentified ellagitannins were indicated with [M−H]$^-$ *m/z* 935 (**90**), *m/z* 951 (**88**), *m/z* 953 (**101**, **112**), *m/z* 1105 (**113**, **116**) and *m/z* 1109 (**84**, **97**, **100**) showing the typical fragments of both gallic acid and ellagic acid in their mass spectra.

No clear fragmentation was recorded for compounds at *m/z* 985 (**117**), *m/z* 1083 (**54**, **69**), *m/z* 1127 (**63**, **87**, **111**, **148**) because of too weak signal. These compounds need to be investigated in the future.

Several low molecular weight gallic acid conjugates were identified. Monogalloyl glucose-quinic acid ester (**62**), galloylquinic acid isomers (**9**, **14**, **17**), digalloylquinic acid (**52**) showed characteristic fragments of gallic acid (*m/z* 169, 125) and quinic acid (*m/z* 191, 173). Galloylshikimic acid isomers (**25**, **28**, **33**) were identified by galloyl residues (*m/z* 169, 125) as well as by the neutral loss [M−H−156]$^-$ = *m/z* 169 of the shikimate moiety and by the fragments of shikimic acid (*m/z* 155, 111).

Methylgallate was identified by *m/z* 168 [M−H−$CH_3$]$^-$ and by *m/z* 139 [M−H−$CO_2$]$^-$ ions. Two galloylquercetin isomers were found (**60**, **64**) showing characteristic fragments of gallic acid (*m/z* 169, 125) and quercetin (*m/z* 301, 300). The compounds **104** and **107** were identified as coumaroyl-*O*-galloyl-glucose isomers (Wang et al., 2011). Eucaglobulin (**154**), a special monoterpene glycoside conjugate of gallic acid was also evidenced in European hornbeam leaf. This compound has been already identified in the leaves of *Eucalyptus globulus* and characterized by MS/MS fragmentation (Boulekbache-Makhlouf et al., 2013).

According to Fig. 1 and peak heights the most abundant tannin compounds were unidentified ellagitannins (**88**, **97**, **112**, **113**), trigalloyl-HHDP-glucose (**114**, **127**) and tetragalloyl glucose (**123**).

*3.2.3. Flavonoid glycosides*

A wide variety of flavonoid glycosides and derivatives of these compounds were identified in the leaf extract of European hornbeam. All of the flavonoids were either *C*- or *O*-glycosylated with sugar units, free aglycones were not indicated.

Altogether eight quercetin-*O*-glycosides were identified, out of which five were quercetin-*O*-hexosides at *m/z* 463 (**89**, **93**, **106**, **126**, **135**); two were identified as quercetin-*O*-pentoside isomers at *m/z* 433 (**115**, **141**) and one was quercetin-*O*-rhamnoside at *m/z* 447 (**143**). The gallic acid conjugates of quercetin (galloylquercetin: **60**, **64**) were already mentioned earlier. The presence of quercetin as an aglycone was verified by the strong *m/z* 301 and *m/z* 300 ions, as well as by the MS/MS fragments at *m/z* 272, 257, and 255. A wide variety of quercetin glycosides were also found in the leaves of *Betula pendula*, which belongs to the same family as hornbeams (Germanò et al., 2012).

Respecting myricetin compounds altogether four glycosides were found. The presence of myricetin as the aglycone was confirmed by the characteristic *m/z* 316 and *m/z* 317 fragment ions as well as the *m/z* 287, 271 and 179 ions. Altogether two myricetin-*O*-hexosides (**103**, **108**), one myricetin-*O*-pentoside (**122**) and one myricetin-*O*-rhamnoside (**124**) were identified. In addition two derivatives of myricetin-*O*-hexoside at *m/z* 657 (**131**, **136**) and two derivatives of myricetin-*O*-rhamnoside at *m/z* 643 (**98**, **110**) were tentatively identified with hitherto unknown structures. The mass spectra of these compounds contain all *m/z* fragments of the aglycone myricetin, as well as the [M−H]$^-$ ion of the respective glycoside, yet they also contain “additional” fragments at higher *m/z* values, suggesting that these compounds are derivatives of these gylcosides.

Kaempferol glycosides were detected as kaempferol-*O*-rhamnoside (**155**) and kaempferol-di-*O*-rhamnoside (**162**, **164**). Aglycone was verified by strong *m/z* 285, 284 as well as by *m/z* 255, 227 fragment ions. The di-*O*-rhamnoside moieties were verified by the consecutive neutral losses of 146 amu units (*m/z* 577 → 431 → 285) proving that two different *O* atoms of the aglycone are glycosilated (Cuyckens and Claeys, 2004; Vukics and Guttman, 2010).

One luteolin glycoside, luteolin-*O*-hexoside was detected (**128**) according to the *m/z* 447 [M−H]$^-$ ion and fragmenation data. Although kaempferol and luteolin have identical molecular masses and very similar MS/MS spectra, and kamepferol glycosides were identified from the sample, peak **128** was tentatively identified as a hexoside conjugate of luteolin. Mass spectrum of kaempferol contains intense *m/z* 285 and *m/z* 284 peaks and the characteristic *m/z* 255 and *m/z* 227 fragments, while the fragmentation of luteolin yields intense *m/z* 285 and *m/z* 284 ions, but a lot of small intensity fragments too (*m/z* 227, 199, 175, 151, 133) (Sánchez-Rabaneda et al., 2003).

Two apigenin-glycosides were detected. One of them was an apigenin-di-*C*-hexoside (**81**) at *m/z* 593 [M−H]$^-$. The *C* linkage of the sugar units was evidenced by the fact that in the MS/MS spectrum between [M−H]$^-$ and *m/z* 431 [M−H−162]$^-$ several sugar fragments (*m/z* 503 [M−H−90]$^-$, *m/z* 473 [M−H−120]$^-$) were detected with high intensities, which is characteristic of *C* hexosides (Cuyckens and Claeys, 2004; Vukics and Guttman, 2010). The loss of another *C*-linked hexose moiety was evidenced by *m/z* 383 and *m/z* 353 ions ([M−H−120−−90]$^-$and [M−H−120−120]$^-$respectively. Their structure was also confirmed by literature data (Benayad et al., 2014).

The other apigenin derivative was tentatively identified as apigenin-*O*-hexoside (**144**), showing characteristic fragmentation of *O*-hexosides and of the aglycone apigenin (*m/z* 269, 225, 201, 151, 149).

Altogether three isorhamnetin-*O*-glycosides, including two isorhamnetin-*O*-hexosides (**120**, **132**) and one isorhamnetin-*O*-pentoside (**142**) were identified. Characteristic peaks of isorhamentin aglycone are *m*/*z* 315 and 314, as well as *m*/*z* 300 and 299 due to the loss of a $CH_3$ group from the molecule ion.

According to the peak heights in Fig. 1 the most abundant flavonoid compounds in the August extracts were quercetin-*O*-hexoside (**89**, **106**), quercetin-*O*-pentoside (**115**), myricetin-*O*-rhamnoside (**124**), luteolin-*O*-hexoside (**128**), quercetin-*O*-rhamnoside (**143**) and apigenin-*O*-hexoside (**144**).

### 3.2.4. Catechins and procyanidins

Although the presence of catechins and polymeric procyanidins, including (+)-catechin (**45**), (−)-epicatechin (**72**), gallocatechin (**22**), epigallocatechin (**38**) and procyanidin B dimers (**39**, **43**) were evidenced in the extract, their concentrations were not high according to the chromatogram, compared to phenolic acid and flavonoid compounds. These results are in accordance with the data in Section 3.1, indicating that the majority of the polyphenolic compounds in the August extracts of European hornbeam leaves are not of the flavan-3-ol structure (catechins, procyanidins).

### 3.2.5. Unknown compounds

The structure of the compounds marked as unidentified (**2**, **3**, **4**, **6**, **7**, **10**, **40**, **44**, **46**, **47**, **49**, **50**, **51**, **55**, **57**, **61**, **63**, **75**, **80**, **82**, **85**, **87**, **94**, **95**, **105**, **111**, **117**, **121**, **129**, **130**, **133**, **138**, **145**, **146**, **148**, **151**, **152**, **156**, **157**, **158**, **159**, **160**, **161**, **163**, **165**, **166**, **167**, **168**, **169**, **170**, **171**) have not yet been revealed. Among the unidentified-*O*-hexosides (**1**, **20**, **36**, **37**, **67**, **68**, **71**, **96**, **102**) and unidentified-*C*-hexosides (**70**, **109**) only the presence, type and the linkage of the sugar moiety could be evidenced.

Further research is needed to elucidate the structures of the compounds marked as unidentified, basing on the MS/MS data in Table 2. Although 82 out of the 171 compounds have hitherto unknown or partially identified structures, their structural elucidation is essential for future research, as they may contain such non-polyphenolic type, or low-concentration compounds too, which could have significant bioactive effects. The data in Table 2. represents a database which provides basis for the future quantitative determination, extraction optimization, and the structural analysis of special target compounds, responsible for the antioxidant, anticancer and various other potentially beneficial health effects of European hornbeam leaf extracts.

## 4. Conclusion

In this work the seasonal changes of the antioxidant capacity and related parameters of European hornbeam leaves as well as the high performance liquid chromatographic separation/multistage mass spectrometric characterization and identification of the leaf polyphenols have been carried out. Altogether 171 compounds, including gallotannins, ellagitannins, flavonoid glycosides, phenolic acids and derivatives, flavan-3-ols as well as other unknown compounds with defined $[M-H]^-$ *m*/*z* values and MS/MS spectra have been identified and characterized. The most abundant compounds in the best DPPH and FRAP antioxidant capacity August extracts were chlorogenic acid, ellagic acid, ellagitannins, myricetin-, luteolin-, quercetin- and apigenin glycosides, which are supposed to be mostly responsible for the excellent antioxidant properties. Further research is needed for the elucidation of the compounds which are responsible for the remarkably high ABTS antioxidant capacity of the May extracts.

The DPPH, FRAP and ABTS methods were suitable for tracking the complex and comprehensive seasonal alterations of the antioxidant capacity of European hornbeam leaves, while total phenol, flavonoid-, and flavan-3-ol assays enabled the monitoring of the seasonal changes of different types of phenolic compounds.

The extensive database, set up by the detailed mass spectrometric profiling of the leaf extractives can serve as a base for determining seasonal changes of the concentrations of individual compounds, for tracking their role in antioxidant, anticancer and other health effects and for extraction optimization.

The wide variety of polyphenolic compounds, the high antioxidant capacity and the recent results on the anticancer potential could make the leaves of European hornbeam a promising renewable biomass resource of antioxidant extractives in the future for the production of healthcare and medical products.

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