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THE LEAF POLYPHENOLS OF FOREST TREES: OPTIMIZATION OF THE EXTRACTION SOLVENT, SEASONAL CHANGES, AND STRUCTURAL ELUCIDATION BY HPLC-ESI-MS^n

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INTRODUCTION

Leaf extracts of some tree species can exhibit several beneficial effects (anti-oxidant, anti-inflammatory, anti-rheumatic, anti-cancerogenic) on human health. One of the most bioactive groups of extractives, responsible for these effects are the polyphenols. We have investigated the total phenol content of the leaves of some of the most important Hungarian tree species (*Fagus sylvatica*, *Carpinus (C.) betulus, Quercus (Q.) robur, Q. petraea, Q. cerris, Q. pubescens, Robinia pseudoacacia, Pinus (P.) sylvestris, P. nigra, Acer platanoides, Castanea sativa, Populus x euramericana*). **We have optimized extraction solvent composition** and have **determined seasonal variations** of the **total phenol contents**. The July and August leaves of ***Q. pubescens*** **and** ***C. betulus*** had the highest total phenol levels. Using **HPLC and MS/MS** measurements the **structural identification of leaf polyphenols** of these species have been carried out **for the first time**.

SAMPLING SITE, TIME

Botanic Garden of the University of West-Hungary, Sopron (H), between May and September 2014, on 5 occasions. One healthy tree from each species was sampled by taking 15 shade- and 15 sun leaves per occasion.

SAMPLE AND EXTRACTS PREPARATION

Leaf samples were treated with microwave irradiation for enzyme inactivation and ground to a fine powder. Extraction: 0.2 leaf powder + 20 ml 4:1 methanol:water/ 4:1 ethanol:water/ water, 20 min in ultrasonic bath.

DETERMINATION OF TOTAL PHENOLICS

Total phenol content was measured using the Folin-Ciocalteu assay [1] applying quercetin as standard. Absorbance was measured at 760 nm.

RESULTS

TOTAL PHENOLICS (mg*g^{-1} dried leaf) – *Extraction optimization*

SPECIES	4:1 METHANOL:WATER	4:1 ETHANOL:WATER	WATER
Fagus sylvatica	37.82 ± 1.01^{B}	**39.71 ± 1.33^{B}**	23.68 ± 1.09^{A}
Carpinus betulus	78.81 ± 0.59^{A}	76.84 ± 2.07^{A}	**86.75 ± 2.51^{B}**
Robinia pseudoacacia	**29.30 ± 0.99^{B}**	28.91 ± 0.12^{B}	23.55 ± 0.84^{A}
Acer platanoides	46.63 ± 2.80^{B}	**47.03 ± 1.28^{B}**	7.48 ± 0.00^{A}
Castanea sativa	**71.30 ± 3.53^{B}**	66.20 ± 1.33^{B}	38.67 ± 1.34^{A}
Populus x euramericana	29.03 ± 0.29^{B}	**31.22 ± 1.40^{B}**	25.55 ± 0.68^{A}
Quercus robur	**59.85 ± 2.27^{C}**	51.55 ± 2.16^{B}	37.14 ± 0.31^{A}
Quercus petraea	**56.47 ± 1.34^{C}**	47.62 ± 2.01^{B}	32.84 ± 0.72^{A}
Quercus pubescens	**37.52 ± 0.54^{B}**	29.33 ± 1.07^{A}	30.81 ± 0.81^{A}
Quercus cerris	**78.99 ± 3.35^{B}**	73.21 ± 5.50^{B}	51.68 ± 1.81^{A}
Pinus sylvestris	27.12 ± 0.78^{B}	**32.64 ± 0.63^{C}**	21.78 ± 0.24^{A}
Pinus nigra	**27.37 ± 0.97^{B}**	27.16 ± 0.91^{B}	22.38 ± 1.01^{A}

Table 1. Total phenol content of leaves measured with different extraction solvents. Different letters in one table indicate significant difference at p=0.05 level.

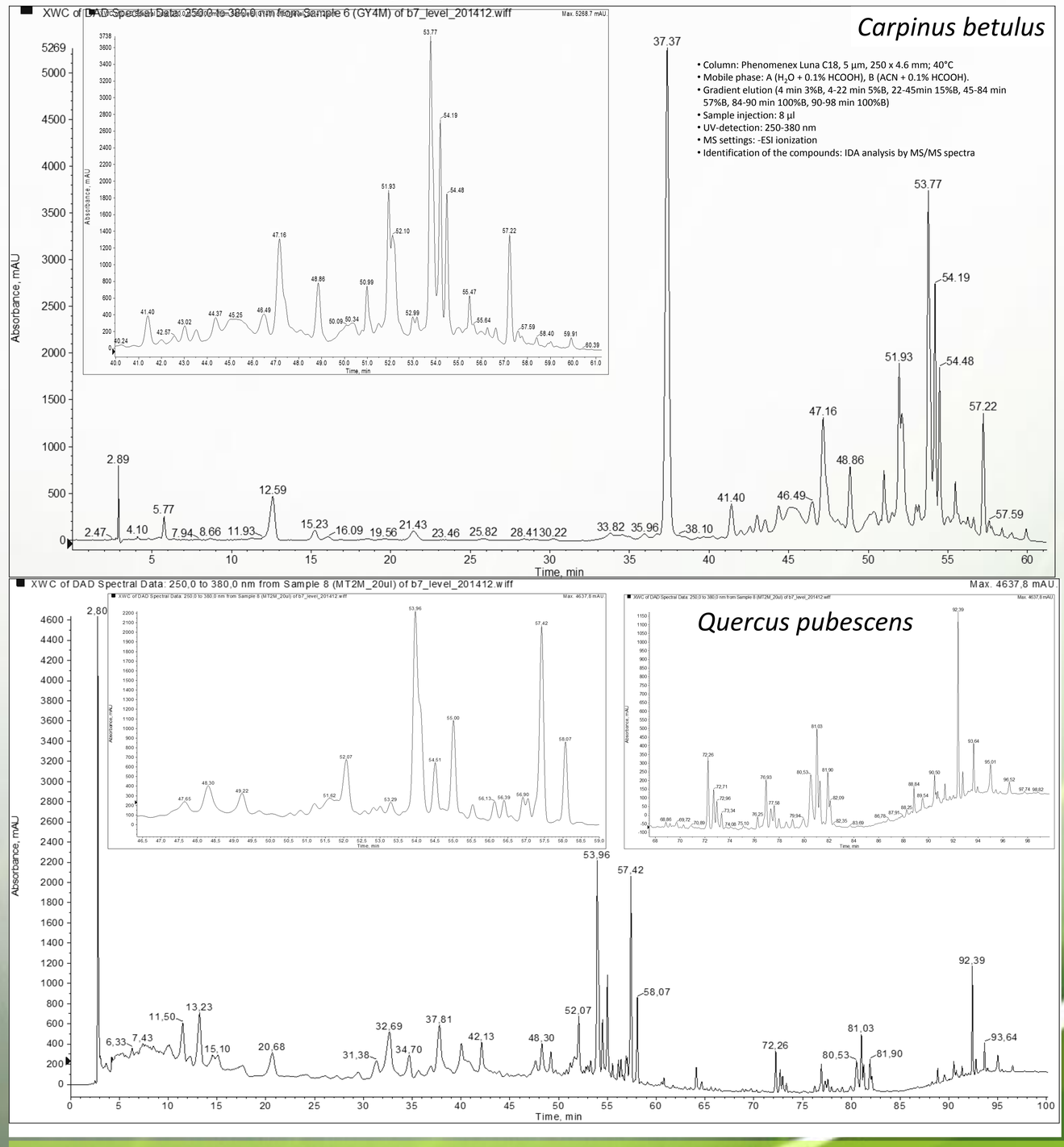
- In the case of most trees **methanol containing mixture** resulted the highest polyphenol yields, yet for *C. betulus* water worked best.
- The total phenol content **increased gradually from spring to autumn** in the leaves except of *Q. cerris*.
- Overall the highest values were measured in ***C. betulus*** **and** ***Q. pubescens*** followed by *Acer platanoides* and *Castanea sativa.*

TOTAL PHENOLICS (mg*g^{-1} dried leaf) – *Seasonal changes*

SPECIES	MAY	JUNE	JULY	AUGUST	SEPTEMBER
Fagus sylvatica	37.82 ± 1.01$^{A}_{B}$	56.83 ± 0.99$^{C}_{D}$	48.12 ± 1.28$^{B}_{BC}$	47.38 ± 3.14$^{B}_{B}$	57.71 ± 2.59$^{C}_{CB}$
Carpinus betulus	78.81 ± 0.59$^{A}_{D}$	**93.08 ± 3.29$^{B}_{H}$**	**105.93 ± 5.57$^{C}_{H}$**	**94.27 ± 5.38$^{B}_{G}$**	80.81 ± 3.05$^{A}_{E}$
Robinia pseudoacacia	29.30 ± 0.99$^{B}_{A}$	29.73 ± 0.73$^{B}_{A}$	43.25 ± 0.21$^{C}_{BA}$	19.89 ± 2.89$^{A}_{F}$	49.63 ± 1.84$^{D}_{B}$
Acer platanoides	46.63 ± 2.80$^{A}_{E}$	57.37 ± 1.98$^{B}_{D}$	80.21 ± 1.47$^{E}_{G}$	65.88 ± 2.83$^{C}_{DEF}$	73.73 ± 1.26$^{D}_{DE}$
Castanea sativa	71.30 ± 3.53$^{AB}_{F}$	78.52 ± 1.13$^{B}_{G}$	62.51 ± 1.59$^{A}_{DE}$	76.32 ± 5.25$^{B}_{F}$	75.49 ± 4.67$^{B}_{BD}$
Populus x euramericana	29.03 ± 0.29$^{A}_{A}$	53.63 ± 3.24$^{B}_{DC}$	73.73 ± 3.05$^{D}_{FG}$	61.96 ± 2.74$^{C}_{CDE}$	57.61 ± 0.58$^{BC}_{BC}$
Quercus robur	59.85 ± 2.27$^{B}_{C}$	58.46 ± 2.50$^{B}_{DE}$	48.32 ± 4.82$^{A}_{BC}$	49.36 ± 4.21$^{A}_{BC}$	72.21 ± 0.46$^{C}_{D}$
Quercus petraea	56.47 ± 1.34$^{A}_{C}$	51.23 ± 3.89$^{A}_{DC}$	59.15 ± 4.06$^{A}_{DE}$	53.70 ± 2.14$^{A}_{BCD}$	70.01 ± 2.42$^{B}_{D}$
Quercus pubescens	37.52 ± 0.54$^{A}_{B}$	71.68 ± 2.32$^{C}_{FG}$	63.80 ± 3.31$^{BC}_{E}$	**92.85 ± 3.67$^{D}_{G}$**	60.31 ± 5.73$^{B}_{C}$
Quercus cerris	78.99 ± 3.35$^{C}_{D}$	65.82 ± 1.59$^{AB}_{EF}$	65.89 ± 1.54$^{AB}_{EF}$	73.73 ± 5.81$^{BC}_{EF}$	59.69 ± 2.52$^{A}_{C}$
Pinus sylvestris	27.12 ± 0.78$^{A}_{A}$	42.56 ± 1.62$^{B}_{B}$	37.47 ± 3.95$^{B}_{A}$	24.63 ± 3.98$^{A}_{A}$	36.07 ± 1.74$^{B}_{A}$
Pinus nigra	27.37 ± 0.97$^{A}_{A}$	46.61 ± 4.23$^{B}_{BC}$	53.80 ± 1.56$^{B}_{CD}$	45.39 ± 5.51$^{B}_{B}$	35.78 ± 2.30$^{A}_{A}$

Table 2. Total phenol content of leaves in the case of different species. Different letters in one table (superscript: between species, subscript: between months)indicate significant difference at p=0.05 level.

From the extracts showing the highest total phenol levels (*C. betulus, Q. pubescens*) the HPLC-ESI-MS^n characterization of polyphenols has been carried out for the first time.



t_r (min)	COMPOUND NAME	[M-H]$^-$	MS/MS
5.8; 16.0	unknown-hexoside	355	337, 311, 161, 133, 121,
6.4; 7.9; 16.0; 28.4	monogalloyl glucose isomers	331	169, 125, 271, 331
8.7	gallic acid	169	125
11.3	chlorogenic acid derivative	371	353, 341, 191, 173, 161
18.3	gallocatechin	305	261, 219, 179, 167, 137
18.6	vanillic acid	167	167, 152, 123, 108
19.7; 21.6	galloyl-hexoside isomers	325	281, 169, 155, 137, 125
21.5; 30.2	methyl-gallate isomers	183	168, 139, 124
25.8	syringic acid	197	182, 167, 153, 138, 123
29.7	gallic acid derivative	325	169, 137, 125, 111, 107
30.4; 51.3	unknown-C-hexoside	359	341, 299, 239, 211, 197
33.1	epigallocatechin	305	261, 219, 179, 167, 137
33.1	chlorogenic acid isomer	353	191
33.7	dimeric procyanidin	577	533, 425, 407, 289, 245
33.8	chlorogenic acid derivative	706.6	533, 462.8, 353, 191, 179
34.6	dimeric procyanidin	577	533, 425, 289, 245, 124.9
35.1	(+)-catechin	289	245, 221, 203, 125, 109
35.8	chlorogenic acid derivative	706.6	706.6, 533, 462.8, 353, 191
36.8	gallic acid derivative	495	343, 337, 325, 191, 169, 125
35.9; 43.5	digalloyl-HHDP glucose isomers	785	633, 419, 301, 275, 169
37.2; 38.4;	digalloyl-glucose isomers	483	439, 331, 313, 301, 169
37.4	chlorogenic acid isomer	353	352.8, 191, 178.9, 135
37.8	chlorogenic acid derivative	707	533, 515, 353, 191, 179
39.7; 43.0	trigalloyl glucose isomers	635	483, 465, 271, 169, 125
42.9	(-)-epikatechin	289	245, 221, 203, 125, 109
43.1	unknown ellagic acid derivative	655	463, 331, 323, 301, 297

Table 3/A. The identified compounds in the case of *C. betulus*

t_r (min)	COMPOUND NAME	[M-H]$^-$	MS/MS
43.5	ellagic acid derivative	807	505, 445, 331, 301, 275
44.3; 46.0	trigalloyl glucose isomers	635	483, 465, 271, 169, 125
46.6; 47.7	trigalloyl glucose isomers	635	483, 465, 271, 169, 125
45.3; 46.4; 47.2	unknown galloyl glucose	953	909, 783, 615, 445, 301
46.4; 48.8; 50.1	unknown ellagic acid derivative	1108	1048, 765, 463, 301, 249
47.4; 54.1; 55.6	quercetin-O-hexoside	463	301, 300, 272, 215, 191
48.1	digalloyl-HHDP glucose	785	633, 615, 419, 301, 205
49.6	trigalloyl glucose isomer	635	483, 465, 271, 169, 125
50.1	gallic acid derivative	477	433, 313, 169, 125, 119
50.7	myricetin-O-hexoside	479	316, 271, 179
50.8	myricetin-O-galactoside	479	479, 316, 271, 287, 179
51.0	quercetin-O-hexoside	463	301, 300, 272, 257, 191
51.2	myricetin-O-galactoside	479	479, 316, 271, 287, 179
51.8	chlorogenic acid derivative	505	353, 191
52.1; 54.2	trigalloyl-HHDP glucose isomers	937	785, 465, 301, 275, 169
52.3	quercetin-O-pentoside	433	300, 301
52.4; 53.0; 53.6	tetragalloyl glucose isomers	787	635, 617, 465, 313, 169
53.5	myricetin-O-xyloside	449	316, 287, 271, 259, 243
53.8	myricitrin	463	359, 316, 287, 271, 259
53.9	ellagic acid	301	284, 257, 229, 185
54.5	luteolin-O-glucoside	447	447, 327, 285, 284, 256
56.6	quercetin-arabinoside/xyloside	433	301, 300, 255, 243, 179
57.2	quercetin-O-rhamnoside	447	301, 300, 272, 255, 243
57.3	apigenin-O-hexoside	431	431, 341, 311, 240, 225
58.9	ellagic acid hexoside	463	301, 245
59.9	kaempferol-3-rhamnoside	431	431, 285, 255, 227, 211

Table 3/B. The identified compounds in the case of *C. betulus*

t_r (min)	COMPOUND NAME	[M-H]$^-$	MS/MS
2.9; 3.0; 4.5	quinic acid isomers	191	173, 149, 127, 111, 93
6.4; 7.9; 16.0; 20.2	monogalloyl glucose isomers	331	169, 125, 271, 331
8.6	gallic acid	169	125
8.7; 11.5; 15.2	ellagtannin	1100	568, 493, 467, 301, 276
13.6; 21.1	ellagtannin, mw: 1866 Da	[M-2H]$^{2-}$: 932	914, 783, 631, 451, 301
18.1	gallocatechin	305	261, 219, 179, 167, 137
19.5; 21.6	galloyl-hexoside	325	281, 169, 155, 137, 125
23.9	gallocatechin-(4,8)-(epi)catechin	593	575, 425, 407, 289, 125
29.5; 30.4	methyl-gallate	183	168, 139, 124
31.9; 34.4	procyanidin B dimer	577	425, 407, 338, 289, 125
32.1	3-p-Coumaroylquinic acid	337	191, 173, 163, 155, 119
33.3	4-p-Coumaroylquinic acid	337	191, 173, 163, 155, 111
35.0	(+)-catechin	289	245, 221, 203, 125, 109
35.7; 43.5	digalloyl-HHDP glucose isomers	785	633, 419, 301, 275, 169
37.1; 38.1	digalloyl-glucose isomers	483	439, 331, 313, 301, 169
40.3	ellagtannin mw: 1116 Da	[M-2H]$^{2-}$: 557	932,5 465, 301, 181, 275
40.5; 42.2	ellagtannin, mw: 1866 Da	[M-2H]$^{2-}$: 932	914, 783, 631, 451, 301
42.9	(-)-epikatechin	289	245, 221, 203, 125, 109
45.9	trigalloyl glucose	635	483, 465, 313, 169, 125
47.4	quercetin-O-hexoside	463	301, 300, 243, 227
48.3; 49.4; 51.5	ellagtannin	1082	631, 586, 489, 301, 273
48.3	ellagtannin	499	499, 439, 341, 323, 301
48.9; 51.7; 52.5	Reginin A/Reginin D isomer mw: 1718 Da	[M-2H]$^{2-}$: 858	764, 613, 451, 781, 301, 275
49.4; 52.2	ellagtannin mw: 1106 Da	[M-2H]$^{2-}$: 552	631, 473, 467, 457, 451, 301,

Table 4/A. The identified compounds in the case of *Q. pubescens*

t_r (min)	COMPOUND NAME	[M-H]$^-$	MS/MS
50.7; 51.1	myricetin-O-hexoside	479	316, 287, 271, 242, 214
51.9	Quercetin-O-pentoside-O-hexoside	595	301, 300, 271, 255, 253
52.2	quercetin-O-pentoside	433	301, 300, 271, 255, 243
52.3; 53.0; 53.6; 54.0	tetragalloyl glucose isomers	787	635, 617, 465, 313, 169
52.7; 53.2	quercetin galloyl-hexoside	615	463, 301, 300, 255
52.9; 53.4; 54.7	galloyl-hexoside isomers	938.4	768.5, 616.5, 465, 429, 125
55.5; 55.8; 56.9	galloyl-hexoside isomers	938.4	768.5, 616.5, 465, 429, 125
53.7	myricitrin	463	317, 316, 288, 271, 259
53.9	ellagic acid	301	284, 257, 229, 185
54.0; 54.5	quercetin-O-hexoside	463	301, 300, 271, 255, 179
54.6; 54.8	kaempferol galloyl-hexoside	599	462, 447, 327, 313, 284
55.0	ellagitannin	499	343, 323, 301, 271, 215
55.5	trigalloyl-HHDP glucose	938	785, 615, 465, 419, 301
56.1; 56.8	kaempferol-O-hexoside	447	284, 285, 255, 227, 179
56.1	quercetin-O-pentoside	433	301, 300, 271, 255, 243
56.3	kaempferol galloyl-hexoside	599	462, 447, 327, 313, 284
57.0	isorhamnetin-O-rutinoside	623	315, 314, 299, 271, 255
57.2	quercetin-O-rhamnoside	447	301, 300, 271, 179, 151
57.3	isorhamnetin-O-hexoside_derivative	545	477, 357, 314, 257, 243
57.4	isorhamnetin-O-glucoside	477	357, 329, 285, 271,243
59.0	unknown-glycoside	447	314, 300, 285, 271, 243, 257
64.3; 64.6; 66.1	isorhamnetin-O-rutinoside	623	315, 314, 299, 271, 255
64.6; 65.1; 65.5; 66.5	kaempferol-O-hexoside-O-rhamnoside	593	447, 285, 284, 255, 227, 145, 119

Table 4/B. The identified compounds in the case of *Q. pubescens*

- *Carpinus betulus* contains a wide variety of polyphenols including **(+)-catechin, (-)-epicatechin, chlorogenic acid isomers, gallic acid, ellagic acid, ellagitannins and gallotannins, methyl-gallate, quercetin-, myricetin-, apigenin and kaempferol glycosides.**
- *Quercus pubescens* contains **gallic acid, ellagic acid, ellagitannins, and gallotannins, gallocatechin, (+)-catechin, (-)-epicatechin, dimeric procyanidins, caffeic acid derivatives, quercetin-, isorhamnetin-, kaempferol and myricetin-glycosides.**

[1] Singleton, J. A. Rossi. 1965. Colorimetry of total phenolics with phosphomolibdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture* 161: 144-158.

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