

HPLC-MS/MS INVESTIGATION AND EXTRACTION OPTIMIZATION OF BEECH (*FAGUS SYLVATICA* L.) BARK POLYPHENOLS

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INTRODUCTION

Beech is a widely used material in wood industry with several application fields [1]. During the processing of beech logs, considerable amount of bark waste is produced which is difficult to utilize. However, the bark tissues of trees can be a rich source of extractives, mainly of polyphenols. Using appropriate clean-up methods these compounds could be extracted and utilized for industrial applications (e.g. food antioxidants, wood preservatives, nutrition supplements, etc.). The aim of the present research was the HPLC-MS/MS assessment of beech bark polyphenols, as high-performance separation and multistage MS characterization of these compounds has not been carried out yet. Using -ESI ionization with a triple quadrupole/linear ion trap hybrid MS detector (+)-catechin, (-)-epicatechin, quercetin-O-hexoside, taxifolin-O-hexosides (3), taxifolin-O-pentosides (4), B-type (6) and C-type (6) procyanidins, as well as other compounds with defined [M-H]⁻ m/z values and MS/MS spectra, yet up-to-now unsolved structures have been identified. The comparison of different extraction methods (stirring, sonication, microwave assisted extraction) using different solvents (water, 4:1 methanol:water, 4:1 ethanol:water) and time/temperature schedules was also carried out in order to investigate optimum extraction efficiency. Methods were compared basing on total phenol contents (Folin-Ciocalteu) and MRM peak areas of the identified compounds. It has been established that respecting extraction efficiency pure water can be as good as mixtures containing organic solvents under certain circumstances, which also supports the concept of green extraction methods. Too long extraction times and high temperatures can result in the decrease of the concentrations. Further research is needed to determine antioxidant capacity of the extracts and to assess utilization potentialities using biological tests (wood rotting fungi, bacterial species).

SAMPLING SITE, TIME

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SAMPLE PREPARATION

Debarking of the stems. Microwave pretreatment of the bark (enzyme inactivation) for 2 min. with 750 W microwave energy. Rasing of the inner bark tissues.

EXTRACTS PREPARATION

Extraction of 0.15 g bark with 15 ml solvent. Extraction solvents: (4:1) Methanol: Water, 4:1 Ethanol: Water and Water)

EXTRACTION METHODS:

Stirring: 2 h, 5 h, 24 h at room temperature.

Microwave assisted extraction:

10 and 20 min at 60°C, 80°C, 100°C, 120 °C.

Sonication: 10 min, 20 min, 30 min at room temperature.

DETERMINATION OF TOTAL PHENOLICS

Total phenol content was measured using the Folin-Ciocalteu assay [2] applying quercetin as standard. Absorbance was measured at 760 nm.

RESULTS

The total phenol content of the extracts:

Total phenolics (mg g ⁻¹ dried bark) In the case of STIRRING			
t (min)	T (°C)	4:1 Methanol:Water	4:1 Ethanol:Water
2 h		35.84 ± 2.00 ^A	36.43 ± 4.29 ^A
5 h		36.41 ± 3.70 ^A	48.30 ± 2.12 ^D
24 h		42.97 ± 3.44 ^E	46.39 ± 4.94 ^D
Total phenolics (mg g ⁻¹ dried bark) In the case of SONICATION			
t (min)	T (°C)	4:1 Methanol:Water	4:1 Ethanol:Water
10 min		39.93 ± 7.88 ^{AB}	49.90 ± 2.77 ^E
20 min		42.66 ± 7.45 ^{DE}	48.94 ± 4.23 ^{DE}
30 min		30.88 ± 2.56 ^C	44.49 ± 5.71 ^{BD}
Total phenolics (mg g ⁻¹ dried bark) In the case of MICROWAVE ASSISTED EXTRACTION			
t (min)	T (°C)	4:1 Methanol:Water	4:1 Ethanol:Water
10	60	40.49 ± 1.87 ^{BCD}	43.03 ± 2.43 ^{BCDE}
10	80	42.45 ± 6.70 ^{BCDE}	45.44 ± 4.60 ^{CDEF}
10	100	45.98 ± 2.46 ^{DEF}	46.42 ± 2.29 ^{DEF}
10	120	47.55 ± 4.00 ^{EF}	53.55 ± 7.25 ^{GH}
20	60	43.94 ± 2.31 ^{BCDE}	47.34 ± 4.24 ^{EF}
20	80	44.63 ± 1.66 ^{BCDE}	45.60 ± 3.84 ^{CDEF}
20	100	55.08 ± 5.15 ^{HI}	57.28 ± 10.17 ^J
20	120	58.08 ± 0.92 ^J	65.22 ± 13.84 ^J

Table 1. Total phenol content of beech bark measured with different extraction methods. Different letters in one table indicate significant difference at p < 0.05 level.

CONCLUSIONS

- 4:1 ethanol:water and water proved to be the best solvents for the extraction. The efficiency of the solvents depends on the extraction time and temperature significantly.
- Due to the excessive mechanical impacts and durations, the total phenol content of the aqueous extracts was observed to decrease with stirring (5h → 24h). This can be explained by a possible decomposition of polyphenols.
- With higher temperatures the total phenol content increases in MW extraction which proved to be the most efficient method.
- Pure water at high temperatures can be as effective as the mixtures containing organic solvents. This can support the implementation of green extraction technologies for beech bark in future.

HPLC-MS/MS MEASUREMENTS

HPLC measurements were carried out on a Shimadzu LC-20 equipment coupled with a DAD detector and an AB Sciex 3200 QTRAP[®] triple quadrupole/linear ion trap LC/MS/MS detector.

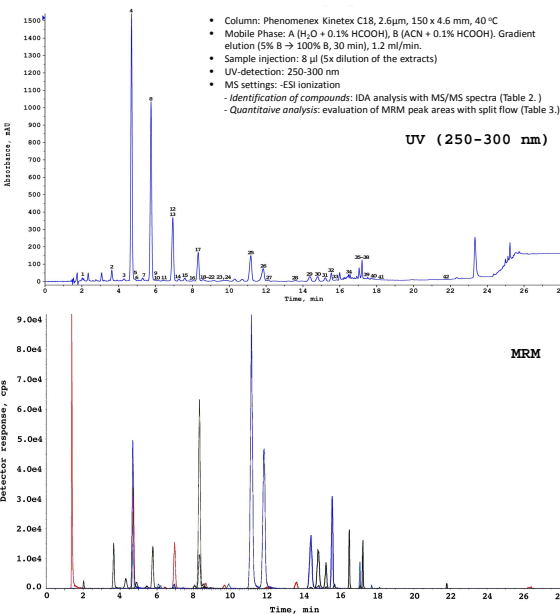
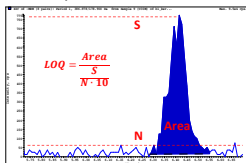


Figure 1. Typical UV and MRM chromatograms obtained during the chromatographic separation process

RELATIVE QUANTITATIVE EVALUATION OF THE IDENTIFIED COMPONENTS IN SELECTED EXTRACTION METHODS

Amounts of individual compounds were compared by their respective MRM peak areas (Table 3).

Limits of quantitation (LOQ) were evaluated by the rule S/N>10.



CONCLUSIONS

- Ethanol solutions and short extraction times favour the extraction of (+)-catechin and (-)-epicatechin possibly because of the oxidation of these compounds in aqueous media.
- Procyanidins are more favourably extracted at ambient conditions in ethanolic solutions.
- Higher temperatures favour the extraction of the taxifolin glycosides.
- For other compounds microwave assisted extraction proved to be the most efficient in general.
- Pure water as an extraction solvent is best at 120 °C for 10 min with microwave extraction, extracting a broad range of phenolic compounds efficiently, except of procyanidins.
- For the optimum extraction of beech bark polyphenols microwave assisted extraction (or the use of respective pressurized hot liquids) is proposed using water with 0-10% ethanol content for 10 minutes. For ambient condition extractions the same solvent with the use of short extraction times (sonication: 10 min, stirring: 5 hours) are the best.
- Future investigations will focus on the assessment of antioxidant capacity (DPPH, ABTS, FRAP) of the extracts and finding connections between AO values and the relative concentrations of individual compounds.

IDENTIFICATION OF COMPOUNDS VIA MS/MS SPECTRA

Peak	t _r (min)	Compound name	[M-H] ⁻	MS/MS	MRM (Q1/Q3)
1	2.03	procyanidin C trimer 1	865	865, 739, 695, 577, 407, 125	865.1/125.1
2	3.66	procyanidin B dimer 2	577	577, 289, 407, 425, 451, 125	577.1/125.1
3	4.34	procyanidin B dimer 2	577	577, 289, 407, 425, 451, 125	577.1/125.1
4	4.7	(+)-catechin	289	289, 245, 203, 109, 221	288.9/108.9
5	4.72	confenifer isomer 1*	387	387, 341, 207, 179, 164	386.9/178.9
6	4.92	procyanidin C trimer 2	865	865, 739, 695, 577, 407, 125	865.1/125.1
7	5.47	procyanidin C trimer 3	865	865, 739, 695, 577, 407, 125	865.1/125.1
8	5.81	syringin*	417	417, 371, 209, 179, 161	416.9/209
9	6.14	procyanidin B dimer 3	577	577, 289, 407, 425, 451, 125	577.1/125.1
10	6.14	procyanidin C trimer 4	865	865, 739, 695, 577, 407, 125	865.1/125.1
11	6.46	ui. caffeic acid-hexoside*	387	387, 341, 207, 179, 164	386.9/178.9
12	6.95	confenifer isomer 2*	387	387, 341, 207, 179, 164	386.9/178.9
13	6.96	(-)-epicatechin	289	289, 245, 203, 109, 221	288.8/108.9
14	7.31	procyanidin B dimer 4	577	577, 289, 407, 425, 451, 125	577.1/125.1
15	7.46	procyanidin B dimer 5	577	577, 289, 407, 425, 451, 125	577.1/125.1
16	8.09	ui. catechin derivative 1	631	631, 289, 327, 245, 203	631.1/289.0
17	8.28	ui. catechin derivative 2	551	551, 419, 373, 359, 401	551.1/289.0
18	8.28	coumaric acid-O-dihexoside	487	487, 325, 235, 163, 143	487.1/58.9
19	8.64	procyanidin C trimer 5	865	865, 739, 695, 577, 407, 125	865.1/125.1
20	8.68	taxifolin-O-hexoside 1	465	465, 285, 303, 339, 257, 151	465.0/151.0
21	8.81	ui. catechin derivative 3	631	631, 289, 327, 245, 203	631.1/289.0
22	8.96	procyanidin C trimer 6	865	865, 739, 695, 577, 407, 125	865.1/125.1
23	9.71	taxifolin-O-hexoside 2	465	465, 285, 303, 339, 257, 151	465.0/151.0
24	9.93	procyanidin B dimer 6	577	577, 289, 407, 425, 451, 125	577.1/125.1
25	11.16	taxifolin-O-pentoside 1	435	435, 285, 303, 179, 151	434.9/151.0
26	11.86	taxifolin-O-pentoside 2	435	435, 285, 303, 179, 151	434.9/151.0
27	12.14	taxifolin-O-hexoside 3	465	465, 285, 303, 339, 257, 151	465.0/151.0
28	13.62	unidentified	441	441, 330, 205, 133, 997	440.9/59.1
29	14.38	taxifolin-O-pentoside 3	435	435, 285, 303, 179, 151	434.9/151.0
30	14.83	ui.(-)-epicatechin-(O-hexoside) 1	551	551, 419, 373, 359, 401	551.1/289.0
31	15.22	ui.(-)-epicatechin-(O-hexoside) 2	551	551, 419, 373, 359, 401	551.1/289.0
32	15.54	taxifolin-O-pentoside 4	435	435, 285, 303, 179, 151	434.9/151.0
33	15.67	quercetin-O-hexoside	463	463, 300, 301, 271, 255	462.9/299.8
34	16.49	ui.(-)-epicatechin-(O-hexoside) a	553	553, 421, 406, 373, 391	553.1/59.0
35	17.06	syringic acid-O-dihexoside 1	521	521, 359, 341, 299, 239	521.0/340.9
36	17.07	ui.(-)-epicatechin-(O-hexoside) 3	551	551, 419, 373, 359, 401	551.1/289.0
37	17.17	ui.(-)-epicatechin-(O-hexoside) b	553	553, 421, 406, 373, 391	553.1/59.0
38	17.22	caffeic acid-O-pentose-O-hexoside 1	491	491, 311, 296, 179, 108	491.0/107.9
39	17.68	syringic acid-O-dihexoside 2	521	521, 359, 341, 299, 239	521.0/340.9
40	17.85	caffeic acid-O-pentose-O-hexoside 2	491	491, 311, 296, 179, 108	491.0/107.9
41	18.13	syringic acid-O-dihexoside 3	521	521, 359, 341, 299, 239	521.0/340.9
42	21.77	ui.(-)-epicatechin-(O-hexoside) 4	551	551, 419, 373, 359, 401	551.1/289.0

Table 2. Identification of compounds with IDA analysis by recording MS/MS spectra. MRM: Q1/Q3 transitions used for quantitative analysis. *detected as [M - H + HCOOH] adduct; ui. unidentified

Peak	Compound name	LOQ (area) %/N ²	U10M	U10E	U20M	U20E	U30E	S2E	S5E	S5W	S24W	M120 W10	M120 E10	M120 E20	M120 E30	M120 E10
4	(+)-catechin	500	220 000	234 000	208 333	239 667	215 000	214 000	308 667	89 133	72 300	225 667	206 667	255 333	260 333	239 333
13	(-)-epicatechin	250	68 967	78 300	56 000	62 000	74 300	142 700	179 300	37 471	104 000	96 867	92 000	92 333	92 333	92 333
2	procyanidin B dimer 1	2500	79 400	94 767	71 567	88 033	70 900	63 500	85 800	7842	7113	27 133	12 033	30 767	25 533	27 400
3	procyanidin B dimer 2	2500	18 067	18 333	16 813	21 733	14 987	23 233	35 433	<LOQ	<LOQ	18 697	20 333	34 100	28 333	32 433
9	procyanidin B dimer 3	2500	35 067	40 433	25 700	26 833	24 834	27 077	5880	<LOQ	<LOQ	<LOQ	<LOQ	2723	<LOQ	<LOQ
14	procyanidin B dimer 4	2500	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
15	procyanidin B dimer 5	2500	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
21	procyanidin C trimer 4	2500	10 687	12 987	8910	14 165	8647	11 333	15 200	<LOQ	<LOQ	<LOQ	<LOQ	10 147	8270	7160
1	procyanidin C trimer 1	2500	7597	5827	7007	6263	4423	5290	7370	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6	procyanidin C trimer 2	2500	19 833	23 533	16 787	21 700	16 703	13 667	22 100	<LOQ	<LOQ	<LOQ	<LOQ	3917	2607	3880
7	procyanidin C trimer 3	2500	6203	8397	5133	7657	4910	6333	9560	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
10	procyanidin C trimer 4	2500	26 337	4280	<LOQ	2930	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
19	procyanidin C trimer 5	2500	5607	6523	4209	7267	4757	6107	8760	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
22	procyanidin C trimer 6	2500	16 937	20 467	11 206	14 893	14 783	1503	4760	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Σ flavanols			492 091	548 919	434 568	515 000	457 142	359 253	522 857	104 030	85 506	389 261	341 120	601 946	594 022	573 359
25	taxifolin-O-pentoside 1	2000	420 333	358 000	432 000	516 333	434 667	706 667	576 667	269 333	336 333	836 333	731 333	696 667	745 333	698 000
26	taxifolin-O-pentoside 2	2000	222 333	201 333	218 667	274 000	239 667	371 333	382 667	284 333	329 333	816 333	700 333	573 333	636 333	591 333
29	taxifolin-O-pentoside 3	2000	94 167	77 700	99 467	117 867	98 567	166 000	202 000	123 000	123 000	224 000	187 333	224 000	207 000	194 000
32	taxifolin-O-pentoside 4	2000	96 633	84 100	9											