Sauro Bianchi*, Florian Zikeli, Vittorio Vinciguerra, Ivana Kroslakova, Ingo Mayer, Frédéric Pichelin and Giorgio Matteucci

Characterization of *Pinus nigra* var. *laricio* [Maire] bark extracts at the analytical and pilot scale

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Abstract: *Pinus nigra* var. *laricio* bark and its hot-water extracts (HWE) obtained at an analytical and pilot plant scale have been characterized in terms of phenolic extractives, condensed tannins (CTs), carbohydrates and inorganic compounds. Analytical extractions with aqueous acetone were also performed for comparison with HWE. The bark contains 35.5 g kg^{-1} CT, and two-thirds of it could be extracted. Analytical HWE at 75°C led to a total yield of 56.4 g kg^{-1}. The extracts are mainly composed of phenolic compounds (50.7%) and pectins (19.7%). CTs amount to 17.9% of the extracts and are procyanidins with a mean degree of polymerization (DP) of about 9. Non-tannin phenolic oligomers also occurred in the extracts, which could be identified by pyrolysis gas chromatography mass spectrometry (Py-GC/MS) as lignin fragments. Matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF MS) revealed that the CT is a flavanol derivative in methylated form. Further characterization and tailoring of the HWE properties is needed in the context of their specific application.

Keywords: acetone extractions, bark extractives, carbohydrates, condensed tannins, hot-water extractions, MALDI-TOF MS, *Pinus nigra* var. *laricio*, Py-GC/MS, softwood bark, thiolysis, pectins

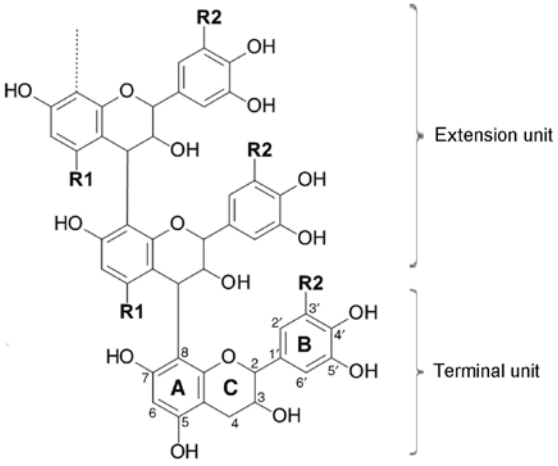
***Corresponding author: Sauro Bianchi,** Bern University of Applied Sciences, Institute for Materials and Wood Technology, Biel, Switzerland, e-mail: sauro.bianchi@bfh.ch.
https://orcid.org/0000-0002-6979-3622
Florian Zikeli and Vittorio Vinciguerra: Università degli Studi della Tuscia, Department for Innovation in Biological Systems, Food and Forestry, Viterbo, Italy
Ivana Kroslakova: Zurich University of Applied Sciences, Institute of Chemistry and Biotechnology, Wädenswil, Switzerland
Ingo Mayer and Frédéric Pichelin: Bern University of Applied Sciences, Institute for Materials and Wood Technology, Biel, Switzerland
Giorgio Matteucci: Consiglio Nazionale delle Ricerche, Istituto per i Sistemi Agricoli e Forestali del Mediterraneo (CNR-ISAFOM), Rende, Italy

Introduction

The Calabria region in South Italy has a forest coverage of about 613 000 ha, corresponding to 40% of the land surface, which is the highest coverage along the Italian Peninsula. Calabria is underdeveloped against other Italian and European regions and the local forest and wood industry suffers from technological, logistical and economic problems. The *Pinus nigra* var. *laricio* [Maire] (for brevity: *P. nigra*) is a widely exploited but scarcely investigated softwood (SW) species of the Calabrian forest. Its bark is a largely available by-product of the local wood industry, which is usually burned for energy production or is used as fertilizer in horticulture. The recovery of phenolic compounds and condensed tannins (CTs) from *P. nigra* barks through hot-water extractions (HWE) would be an opportunity for its valorization within the chain of forest biorefinery with a positive impact on the Calabrian industry.

CTs are polydisperse homo-oligomers made of flavanol units, which are almost ubiquitous in plant tissues (Porter 1989). Depending on the hydroxylation pattern of the flavanol units, they are classified as fisetinidins, robinetinidins, procyanidins and prodelphinidins, or based on the hydroxylation pattern of the phenolic A-ring, there are resorcinol or phloroglucinol type CTs (Figure 1).

CTs are suited as additives in the formulation of thermosetting wood adhesives for plywood, particleboards and fibreboards, as well as in cold setting resin systems for laminated timber (Porter and Hemingway 1989; Pizzi 2006). These CTs are mostly isolated via HWE from tropical or sub-tropical broad-leaved trees, such as quebracho (*Schinopsis lorentzii* [Engl.] and *balansae* [Engl.]) and black wattle (*Acacia mearnsii* [De Wild.]), but the bark of European SW species might also be a source of CTs for wood adhesive formulations (Weissman 1985; Dix and Marutzky 1987; Roffael et al. 2000). A problem is the higher viscosities and lower reaction yields toward formaldehyde than in the case of tropical CTs (Weissmann 1981; Roffael et al. 2000). This is due to both the molecular structure of SW bark CTs, which favors their rearrangement in less reactive macromolecules (Pizzi and Stephanou 1994a,b), and the presence of a considerable amount of non-tannin



R1	R2	Monomer unit	Oligomer	Class
H	H	fisetinidol	fisetinidin	resorcinol tannin
H	OH	robinetinidol	robinetinidin	
OH	H	(epi)catechin	procyanidin	phloroglucinol tannin
OH	OH	(epi)gallocatechin	prodelphinidin	

Figure 1: Schematic chemical structure of condensed tannins.

compounds, in particular carbohydrates, which can develop colloidal interactions with the CTs (Pizzi 1982; Weissman 1985; Garnier et al. 2001).

To evaluate the suitability of *P. nigra* bark as a source of adhesive additives, more knowledge is needed concerning the compositions of bark extractives and the molecular structure of the bark CTs. Extractions with different solvents should be performed and the efficiency of CT extraction estimated. HWE at both analytical and pilot plant scales should be analyzed. The characteristics of the *P. nigra* bark HWE should then be compared with other extracts with regard to their specific application.

Materials and methods

Material preparation: For the analytical and pilot scale extractions, 10 kg of *P. nigra* bark flakes were provided in February 2017 by the sawmill Fabiano Legnami S.r.l. (Chiaravalle Centrale, Italy) and dispatched to the Bern University of Applied Sciences (Biel, Switzerland). The bark flakes were oven-dried at 70°C for about 12 h until a moisture content (MC) of 3% was reached. Percentage data in the present work refer to the weight percent based on dry material, i.e. dry bark and dry extractives, respectively. The dried bark was then hammer-milled using a 2-mm sieve (Friedli THM-A, Burgdorf, Switzerland) and finally stored at room temperature (rT) protected from air and light.

Extraction: For analytical scale extractions, the hammer-milled bark was powdered to an average size of about 50 µm in a vibratory disk mill (Herzog HSM 100H, Osnabrück, Germany). Extractions were performed with petrol ether, aq. acetone (acetone:water 9:1, v/v) and hot water (HW) at various temperatures in an accelerated solvent extraction (ASE) system (ASE200 Dionex, Sunnyvale, USA) equipped with 22 ml cells, each of them loaded with 2 g of powdered bark. All extractions consisted of an initial cell heating, two extraction cycles of 10 min and final rinsing and purging. Petrol ether, acetone/water (9:1) and water extracts, respectively, were evaporated under hood at rT, dried under vacuum at 40°C and freeze-dried. The dry extracts were finally stored at rT protected from light and air.

For pilot scale extractions, a 15 l laboratory reactor (miniPilot Büchi AG, Uster, Switzerland) was filled with 7 l of water and heated to 75°C. After temperature stabilization, 1 kg of hammer-milled bark was soaked for 30 min in HW under moderate stirring. The extract was collected by filtration of the bark slurry through a sequence of metal filters (mesh sizes of 500, 200, 100, 32 µm). The retained bark was rinsed with 3 l of fresh water, squeezed with a masher and the recovered liquid filtered and combined with the extract. The solution was then concentrated at 40°C under vacuum to a volume of about 2 l, freeze-dried and stored at rT protected from air and light.

Analysis (see Figure 2): The concentration of the total phenolic compounds in the extracts was estimated by the Folin-Ciocalteu (FC) assay (Singelton et al. 1999) on 0.2 g l^{-1} aqueous solutions of the extracts. Calibration was performed with highly purified CT from quebracho heartwood (Fintan QP, Silvateam, Italy) as the reactivity of CT oligomers toward the FC assay is lower than the sum of the reactivities of their single units (Everette et al. 2010). The absorbance of a 1 g l^{-1} solution of (–)-epicatechin (HPLC grade, Sigma-Aldrich, Switzerland) equals that of a 1.39 g l^{-1} solution of the highly purified quebracho CT.

Solid phase extraction (SPE) was performed according to Sun et al. (1998) with slight modifications. An SPE cartridge (Agilent Bond-Elute C18 10 mg) was preconditioned with 15 ml of MeOH and 15 ml of distilled water, in sequence, and then loaded with 10 ml of a 1 g l^{-1} aqueous solution of bark extract. Elutions were successively performed with 15 ml of distilled water (F0), 20 ml of ethyl acetate (F1) and 20 ml of MeOH (F2). The collected F1 was vacuum-dried at 40°C, newly dissolved in 10 ml of water and loaded in a new and conditioned SPE cartridge, which was successively eluted with 20 ml of diethyl ether (F1A) and 20 ml of ethyl acetate (F1B). Fraction F0 was freeze-dried. Fraction F1A was evaporated under hood at rT. Fractions F1B and F2 were vacuum-dried at 40°C. The four fractions were roughly attributed to phenolic acids (F0), phenolic monomers (F1A), small phenolic oligomers, e.g. dimers, trimers (F1B) and large phenolic oligomers, e.g. CT (F2). The concentrations of phenolic compounds were determined by FC assay after their re-dissolution in 50 ml of distilled water.

The amount, monomeric composition and degree of polymerization (DP) of the CTs were determined by acid thiolysis according to Bianchi et al. (2016). In brief, for the determination of total CT in bark (CT_{total}), 200 mg of powdered bark was dispersed in 20 ml acidic MeOH in the presence of cysteamine HCl (50 mg) and kept at 65°C for 60 min. A 0.4 ml aliquot of the thiolysate was then recovered, quenched with 1.0 ml of distilled water and poured in air-tight vials for the high performance liquid chromatography-ultraviolet (HPLC-UV_{280nm}) analysis. For the determination of CT in HWE, 0.2 ml of an MeOH solution of the extracts (1 g l^{-1}) were mixed with 0.2 ml of acidic MeOH containing cysteamine HCl (5 g l^{-1}). The mixture was then processed in the same way as for the bark thiolysis. A total of 20 µl of the quenched thiolysate was injected into the HPLC

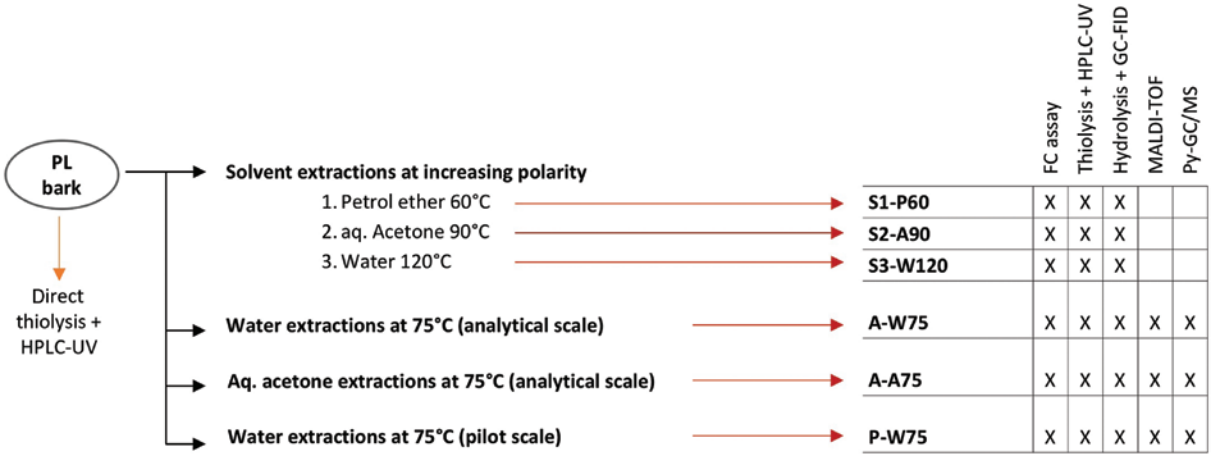


Figure 2: Diagram of analytical procedure applied for the analysis of *P. nigra* bark and bark extracts.

system (Agilent HP 1200, Santa Clara, CA, USA) equipped with a Cosmosil Protein-R Ø4.6×250 mm column. Elution was performed at 1 ml min^{-1} with a gradient from 100% of acidic water to 33.5% of acidic acetonitrile:water (4:1, v/v) (Bianchi et al. 2016). Identification and quantification of the cleaved units were performed by comparison with analytical standards (Sigma Aldrich, Switzerland). During thiolysis, CT terminal units (with hydrogen in position 4, Figure 1) are released in their native state, while all the others appear as thioether adducts. The DP was determined via HPLC:

$$DP = (\Sigma[T] + \Sigma[E]) / \Sigma[T], \quad (1)$$

where [T] and [E] are the molar concentrations of terminal and extension units, respectively.

Matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF MS) experiments were performed according to Bianchi et al. (2015). In brief, acetone solution of the extract was mixed with the matrix (2,5-dihydroxybenzoic acid) and spiked with KCl. Drops of the mixture were deposited on a steel plate and measurements were carried out in a linear positive mode on the instrument equipped with a 355 nm Nd:YAG solid state laser (Bruker Daltonik Autoflex speed, Bremen, Germany) in the 300–5000 m/z range.

Total carbohydrates (CH_{total}) in the extracts were determined by acid hydrolysis followed by silylation and gas chromatography-flame ionization detector (GC-FID) according to Willför et al. (2009). The dried extracts (30.0 mg) were diluted in 6 ml of 0.5 M · H_2SO_4 and autoclaved for 50 min at 120°C. After cooling, the hydrolysate was neutralized with pyridine, and the solution brought to 30 ml with MeOH:water 4:1, v/v. A 100 µl aliquot of the hydrolysate was then dried under vacuum at 40°C. Silylation was performed at 70°C for 1 h with 80 µl of pyridine, 240 µl of N,O-bis(trimethylsilyl)trifluoroacetamide and 10 µl of chlorotrimethylsilane. An amount of 150 µl of hexane was then added to the silylated sample and the hexane phase was recovered for analysis. For GC-FID analysis, 3 µl of the sample was injected at 260°C in split mode (1:20) into an Agilent HP-5 column (Santa Clara, CA, USA), 30 m×0.320 mm i.d. and coated with a 5-µm thick film of 5% phenyl-methyl polysiloxan. Hydrogen was the carrier gas (2 ml min^{-1}). Temperature program: 130°C (holding time 5 min)→180°C (3°C min^{-1}), holding time 3 min→240° (12°C min^{-1}), holding time 1 min→290°C (50°C min^{-1}), holding time 5 min. The FID was set at 300°C. The quantification of the CH residues was performed by comparison with standards of D-arabinose (Ara), L-rhamnose (Rha), D-(+)-xylose (Xyl), D-(+)-galactose (Gal), D-(+)-mannose (Man), D-(+)-glucose (Glc) and galacturonic acid (GalA) (Sigma-Aldrich, Switzerland).

The free monosaccharide fraction of the CH (CH_{mono}) was evaluated by analyzing a 1 g l^{-1} solution of the extracts in MeOH:water 4:1, v/v without hydrolysis. A 100 µl aliquot of the extract solution was dried under vacuum at 40°C and successively silylated and analyzed by GC-FID as described above for the hydrolysate. The bound CH fraction (CH_{bound}) was calculated as the difference between CH_{total} and CH_{mono}, which is related to both oligo/polysaccharides and glycosyl residues.

For pyrolysis gas chromatography mass spectrometry (Py-GC/MS), the pellets of the extracts (1.5–2.0 mg) were directly pyrolyzed at 450°C in a furnace chamber (SGE Pyrojector II, Ringwood, Australia). The GC instrument (Agilent HP 5890 Series II Plus, Santa Clara, CA, USA) was equipped with a Restek Rtx®-1701 column, 30 m×0.25 mm i.d. Helium was the carrier gas, with 100 kPa in the pyrolyzer and 70 kPa in the GC injector (280°C, 1:20 split ratio). The temperature gradient was as follows: 45°C 4 min holding time→240°C (4°C min^{-1})→280°C (39°C min^{-1}). The GC was coupled with a HP 5971A-MSD (Agilent Technologies, Santa Clara, CA, USA) working in the EI mode (70 eV). Scans from 35 to 500 m/z were run in 0.7 s cycles, and the mass spectra were compared with NIST and Wiley computer libraries and the reference literature (Faix et al. 1990a,b, 1991a,b).

Total inorganic compounds in the bark and extracts were measured using thermogravimetry (TG). About 10 mg sample was placed on the balance plate of a TG system Mettler-Toledo TC10A/TC15 (Columbus, OH, USA), heated at a rate of 20°C min^{-1} and held at 550°C for 10 min. The remaining ashes were designated as inorganic compounds.

Results and discussion

Bark characterization

The total amount of extractives in *P. nigra* bark was 134.1 g kg^{-1} (b.o. dry bark), (Table 1), which was determined

Table 1: Total bark extractives in *P. nigra* bark recovered through successive extractions with petrol ether (S1-P60), aq. acetone (S2-A90) and water (S3-W120).

	Total yield ($n=5$)	Phenolic comp.[a] ($n=3$)		CT ($n=1$)		Monosacch. ($n=1$)		Bound CH. ($n=1$)	
Extract	(g kg^{-1})	(g kg^{-1})	(%)	(g kg^{-1})	(%)	(g kg^{-1})	(%)	(g kg^{-1})	(%).
S1-P60	22.0 ± 0.4	–	–	–	–	–	–	–	–
S2-A90	63.8 ± 3.3	36.3 ± 2.0	59.6 ± 1.3	18.4	28.8	0.7	1.1	5.0	7.8
S3-W120	48.3 ± 1.6	17.6 ± 0.6	36.5 ± 0.5	4.8	9.9	0.7	1.5	13.8	28.6
SUM	134.1 ± 3.7	53.9 ± 2.1	40.2 ± 1.5	23.2	17.3	1.4	1.9	18.8	14.0

[a]Phenolic compounds expressed as g_{QE}; "–" not determined. Mean ± StD (StD, standard deviation) of *n* measurements.

by successive extractions on an ASE system with petrol ether at 60°C (S1-P60), aq. acetone at 90°C (S2-A90) and water at 120°C (S3-W120). The S2-A90 fraction was the largest one (63.8 g kg^{-1}), closely followed by the S3-W120 fraction (48.3 g kg^{-1}). S1-P60 containing mostly apolar extractives like fatty and resin acids was the minor fraction (22.0 g kg^{-1}). The S2-A90 fraction showed a total phenolic concentration of 56.9% (b.o. dry extracts), and ca. half of it were CTs (Table 1). In S3-W120, total phenolics and CT concentrations decreased to 36.5 and 9.9%, respectively (Table 1). Most of the phenolic extractives were thus recovered by aq. acetone.

From the sum of S2-A90 and S3-W120 yields, the total amount of phenolics was estimated to be 53.9 g kg^{-1} and the total extractable CT as 23.2 g kg^{-1} (Table 1). However, direct thiolysis on *P. nigra* bark indicates that the CT_{total} is 35.6 g kg^{-1} (Table 2). Accordingly, a large part of CT was not extractable, as already indicated by Matthews et al. (1997a) and Bianchi et al. (2016) for other SW species.

CT_{total} were procyanidins, i.e. oligomers made of epicatechin units and a high frequency of catechin in the terminal unit (Table 2). Similar structures were observed in most bark CTs in other SW species (Matthews et al. 1997a; Bianchi et al. 2015) with exception of CT from radiata pine (*Pinus radiata* [D.Don]) bark, which is mainly composed of catechin units regardless of the position along the polymer chain (Matthews et al. 1997a). The DP of *P. nigra* bark CT was around 14 (Table 2), which is a high value in view of the DPs observed in other SW species (3–8) (Matthews et al. 1997a; Bianchi et al. 2015).

The CT data from the aq. acetone and water extracts did not appreciably differ from those observed in CT_{total}, with exception of the slightly lower DP in the S2-A90 fraction (Table 2). Therefore, the molecular structure of CT does not appear to significantly influence their extractability. Nevertheless, physical entanglements between CT and other bark constituents might constrain the extraction (Stafford and Cheng 1980; Matthews et al. 1997a).

The contribution of CH_{mono} and CH_{bound} in S2-A90 was 8.9%, (1.1% + 7.8%, Table 1). Typical is the presence of bound glucose and rhamnose (Table 3), i.e. the occurrence of phenolic glucosides and rhamnosides seems to

Table 2: CT monomeric composition and mDP in *P. nigra* bark and various *P. nigra* bark extracts estimated by thiolysis followed by HPLC-UV.

		Cleaved flavanol					
Sample		CATt	ECATt	CATe	ECATe	Total	DP
Bark ($n=3$)	(g kg^{-1})	2.1 ± 0.6	0.4 ± 0.2	6.5 ± 1.0	26.5 ± 1.2	35.6 ± 1.7	13.8 ± 0.7
S2-A90 ($n=1$)	(%)	1.76	1.07	2.65	23.32	28.80	10.2
	(g kg^{-1})	1.12	0.68	1.69	14.88	18.37	
S3-W120 ($n=1$)	(%)	0.43	0.26	0.98	8.23	9.90	14.3
	(g kg^{-1})	0.21	0.13	0.47	3.98	4.78	
A-W75 ($n=1$)	(%)	1.28	0.63	1.46	14.41	17.87	9.3
	(g kg^{-1})	0.72	0.36	0.82	8.13	10.03	
A-A75 ($n=1$)	(%)	2.06	0.63	1.66	15.89	20.24	7.5
	(g kg^{-1})	1.74	0.53	1.41	13.46	17.14	
P-W75 ($n=1$)	(%)	1.14	0.49	1.30	12.00	14.93	9.2
	(g kg^{-1})	0.32	0.14	0.36	3.36	4.18	

Terminal units: CATt, catechin; ECATt, epicatechin. Extension units: CATe, catechin; ECATe, epicatechin. Mean ± StD (StD, standard deviation) of *n* measurements.

Table 3: Carbohydrate monomeric composition in various *P. nigra* bark extracts determined by hydrolysis followed by GC-FID.

	Monomeric residues (%)						
Extract	**Ara**	**Rha**	**Xyl**	**Gal**	**Glc**	**Man**	**GalA**
S2-A90							
CH_{mono}	0.40	<0.01	<0.01	<0.01	0.71	<0.01	<0.01
CH_{bound}	0.45	1.72	0.46	<0.01	5.16	<0.01	<0.01
S3-W120							
CH_{mono}	1.20	<0.01	<0.01	<0.01	0.32	<0.01	<0.01
CH_{bound}	8.51	1.18	0.96	3.78	6.83	4.05	3.24
A-W75							
CH_{mono}	0.53	<0.01	<0.01	<0.01	1.30	<0.01	<0.01
CH_{bound}	3.74	1.61	0.91	2.46	8.10	1.56	1.35
A-A75							
CH_{mono}	0.32	<0.01	<0.01	<0.01	0.49	<0.01	<0.01
CH_{bound}	0.19	1.39	0.27	<0.01	3.63	<0.01	<0.01
P-W75							
CH_{mono}	0.24	0.05	0.12	1.69	0.93	0.75	0.22
CH_{bound}	3.94	0.58	0.19	2.49	6.72	0.49	1.16

($n=1$). CH, Carbohydrates.

be probable. CH_{mono} and CH_{bound} in S3-W120 correspond to 1.5% and 28.6%, respectively (Table 1), with mainly bound arabinose > glucose > mannose > galactose > galacturonic acid (Table 3). The presence of water extractable pectins like arabinans, glucans, arabinogalactans and galactoglucomannans, typical for SW barks (Weissmann 1981, 1985; Le Normand et al. 2014; Kemppainen et al. 2014), was thus indicated also for *P. nigra* bark.

Analytical water extractions

HWEs were performed at 75°C as this temperature seems to be promising based on preliminary experiments (data not shown), providing a maximum on CT yields and a minimum of undesired by-products, such as pectins. This experience was also made with HWE of *Picea abies* [Karst] bark (Bianchi et al. 2016).

Analytical scale HWE (A-W75) achieved a total yield of 56.4 g kg^{-1}, with phenolic compounds representing almost half of the extractives (Table 4). According to the SPE analysis (Table 5), most of the phenolic molecules were oligomers (fraction F2). Thiolysis of this fraction indicated, however, that only half of these oligomers are CTs. Py-GC/MS of A-W75 (Table 6) showed the distinctive presence of catechol (peak 32, 13.0%) and 4-methyl-catechol (peak 34, 2.4%), which are pyrolysis products of procyanidin oligomers (Galletti et al. 1995; Diouf et al. 2013). However, the spectra also showed the occurrence of guaiacol (peak 16, 6.4%), 4-methyl-guaiacol (peak 22, 1.1%), 4-ethyl-guaiacol (peak 27, 1.7%), 4-vinyl-guaiacol (peak 29, 6.9%), *trans*-isoeugenol (peak 33, 0.7%), acetoguaiacone (peak 37, 1.6%) and dihydroconiferyl alcohol (peak 40, 10.9%), which are typical pyrolysis products from SW lignin (Obst 1983). Non-tannin phenolic oligomers might then be associated with lignin fragments, the detection of which in HWE of SW bark extracts was seldom reported in previous studies.

The concentration of CT in A-W75 was 17.9%, which corresponds to a CT yield of 10.0 g kg^{-1} (Table 2). This value is less than a third of the CT_{total} in *P. nigra* bark (Table 2) and about the half of the extractable fraction (Table 1).

Table 5: Phenolic compounds and CT in analytical *P. nigra* bark water extracts and their SPE fractions corresponding to phenolic acids (F0), phenolic monomers (F1A), small phenolic oligomers (F1B), large phenolic oligomers (F2).

	Total phenolic comp. ($n=3$)		CT ($n=1$)	
Extract	**(g kg^{-1})**	**(%)**	**(g kg^{-1})**	**(%)**
Crude A-W75	30.2 ± 0.3	50.7 ± 0.5	10.6	17.8
- F0	3.1 ± 0.1	5.1 ± 0.1	<0.1	<0.1
- F1A	1.7 ± 0.1	2.8 ± 0.1	0.1	0.2
- F1B	6.4 ± 0.1	10.7 ± 0.1	1.9	3.2
- F2	17.9 ± 0.2	29.9 ± 0.3	9.6	16.1

Mean ± StD (StD, standard deviation) of *n* measurements.

Table 4: Yields and composition of various *P. nigra* bark extracts.

	Total yield (n^a)	Phen. comp.[b] ($n=3$)		CH_{mono} ($n=1$)		CH_{bound} ($n=1$)		Inorg. comp. ($n=3$)		Non-polar comp. ($n=1$)	
Extract	**(g kg^{-1})**	**(g kg^{-1})**	**(%)**	**(g kg^{-1})**	**(%)**	**(g kg^{-1})**	**(%)**	**(g kg^{-1})**	**(%)**	**(g kg^{-1})**	**(%)**
A-W75	56.4 ± 4.2	28.6 ± 2.1	50.7 ± 0.5	1.0	1.8	11.1	19.7	1.9 ± 0.4	3.3 ± 0.7	<0.1	<0.1
A-A75	84.7 ± 4.2	38.8 ± 1.2	45.8 ± 0.3	0.7	0.8	4.6	5.5	<0.1	<0.1	22.0	25.9
P-W75	28.0	11.9 ± 0.2	42.6 ± 0.6	1.1	4.0	4.4	15.6	1.7 ± 0.1	6.2 ± 0.3	–	–

[a]A-W75: ($n=6$); A-A75: ($n=6$); P-W75: ($n=1$); [b]phenolic compounds expressed as g_{QE}; "–", not determined; CH, carbohydrates. Mean ± StD (Std, standard deviation) of *n* measurements.

Table 6: Pyrolysis products of various *P. nigra* bark extracts ($n = 1$).

	Pyrolysis product	RT (min)	A-W75 (%)	P-W75 (%)	A-A75 (%)
1	Furan	1.50	0.4	0.6	0.0
2	Acetone	1.63	1.6	1.5	10.7
3	2-Methylfuran	1.86	1.2	1.1	0.4
4	2,5-Dimethylfuran	3.19	0.3	0.3	0.1
5	Acetic acid	3.42	1.2	2.2	1.1
6	3-Butynoic acid	7.95	0.7	0.9	0.0
7	Furfural	9.13	1.1	0.7	0.0
8	Limonene	12.09	0.3	0.2	4.5
9	*p*-Cymene	12.86	0.6	0.5	0.7
10	1,2-Cyclopentanedione	13.55	0.4	0.4	0.0
11	5-Methyl-furfural	14.61	0.5	0.3	0.0
12	3-Methyl-2-cyclo-penten-1-one	15.10	0.5	0.8	0.0
13	*o*-Allyltoluene	16.11	0.5	0.3	0.4
14	Corylone	17.42	0.5	0.5	0.0
15	Phenol	19.10	3.9	4.9	2.1
16	Guaiacol	19.24	6.4	6.8	5.7
17	*o*-Cresol	20.86	0.6	0.3	0.2
18	endo-Borneol	21.17	0.8	0.0	0.4
19	Terpineol	21.56	0.6	0.0	0.3
20	Dihydrocarveol	22.05	0.5	0.6	0.2
21	*p*-Cresol	22.18	1.8	2.4	0.6
22	4-Methyl-guaiacol	23.04	1.1	1.1	3.6
23	2,4-Dimethyl-phenol	23.15	0.3	0.3	0.4
24	Myrtenol	24.90	0.5	0.0	0.0
25	4-Ethyl-phenol	25.27	0.5	0.7	0.4
26	Benzoic acid	25.56	0.4	0.0	0.0
27	4-Ethyl-guaiacol	26.00	1.7	1.6	2.7
28	Sugar, unknown	27.72	2.1	2.5	0.0
29	4-Vinyl-guaiacol	27.98	6.9	9.0	9.0
30	4-Vinyl-phenol	28.28	1.5	1.6	0.0
31	5-Hydroxymethyl-furfural	29.62	0.0	0.0	0.0
32	Catechol	29.92	13.0	8.6	24.0
33	Isoeugenol (trans)	32.26	0.7	1.1	1.3
34	4-Methylcatechol	32.39	2.4	2.0	3.4
35	Vanillin	32.95	0.4	0.7	1.2
36	4-Propylguaiacol	34.87	0.7	1.2	0.0
37	Acetoguiaiacone	35.47	1.6	1.4	1.0
38	3,4-Dimethoxy-phenol	35.60	3.2	4.1	1.4
39	Levoglucosan	40.05	10.8	10.7	7.9
40	Dihydroconiferylalcohol	40.69	10.9	13.4	8.6

A large fraction of CT remained, therefore, still available in the *P. nigra* bark after water extractions at 75°C, i.e. the HWE bark residues are still CT-rich substrates that could be extracted under harsher conditions and stronger solvents, or valorized in other applications that do not require the extraction of CT, e.g. heavy metal removal in polluted waters (Seki et al. 1997; Martin-Dupont et al. 2002; Liang et al. 2014).

Thiolysis of A-W75 confirmed that *P. nigra* CTs are procyanidins with a very high DP (≈9) (Table 2). The high DPs might be advantageous during curing but hamper the penetration into wood cell wall and the cross-linking reactions. Pre-polymers in synthetic phenolic adhesives have usually Mw between 500 and 1000 Da (Dargaville et al. 1997) corresponding to flavanol dimers and trimers.

The procyanidin chain character of the CT was further confirmed by MALDI-TOF MS. The mass spectrum of A-W75 (Figure 3) shows a series of intense mass peaks (905.9, 1194.2, 1482.2, 1770.8, 2059.0, 2347.3 m/z, etc.), i.e. with average mass differences of 288.3 m/z. This mass matches with the molecular mass of a catechin or epicatechin monomeric unit (290.3 – 2H = 288.3 Da) in CT. The peak series can be described:

$$[M + K^+] = x \cdot 288.3 + 39 + 2, \quad (2)$$

where x represents the number of (epi)catechin units in the oligomer, 39 is the mass of the cationization agent (K^+) and 2 is the mass of the two hydrogen atoms on the first and the last flavanol units. Accordingly, procyanidins with a degree of polymerization up to 17 units could be detected in the A-W75 mass spectrum ($17 \times 288.3 + 39 + 2 = 4942.1$ Da≈4936.6 m/z). Such large oligomers were never identified in MALDI-TOF mass spectra of other SW bark extracts. This indicates that the DP of *P. nigra* bark CT is higher than usual.

CH_{bound} represented 19.7% of A-W75 (Table 4) and comprised mostly glucose, arabinose and galactose residues (Table 3). The concurrent extraction of pectins, like glucans, arabinans and arabinogalactans was evidenced. Free CH_{mono} were a minor fraction of the extract (1.8%, Table 4) and were mainly constituted of glucose and arabinose (Table 3). The detection of Py-GC/MS peaks associated to furan, 2-methylfuran, 2,5-dimethylfuran, furfural, 1,2-cyclopentanedione, 5-methyl-furfural, 3-methyl-2-cyclopenten-1-one and levoglucosan were indicative of the presence of CHs in the extract (Table 6).

Inorganic compounds (ashes) amounts to 3.3% of the A-W75 extract (Table 4), corresponding to a yield of 1.9 g kg^{-1}, a value almost constant among the HWEs of SW barks (Bianchi et al. 2015).

Analytical aq. acetone extraction

Analytical extraction with aq. acetone at 75°C (A-A75) led to extractives with considerably different characteristics. Higher yields of total extractives, phenolics and CT were observed (84.7, 38.8 and 17.1 g kg^{-1}, respectively) alongside a strong decrement in yield and concentration of CH_{bound} (4.6 g kg^{-1} and 5.5%, respectively) (Tables 2 and 4). No residual ashes were detectable.

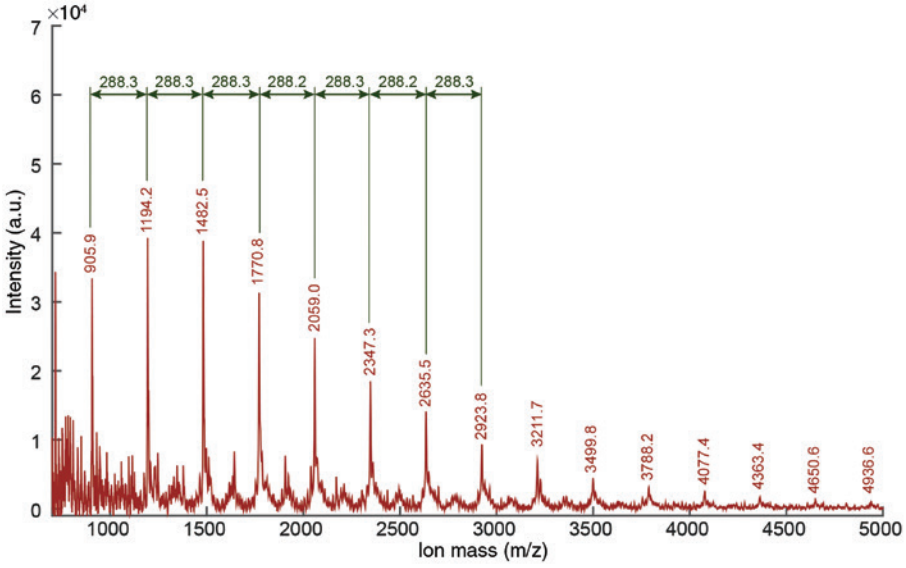


Figure 3: MALDI-TOF mass spectrum between 700 and 5000 m/z of 75°C water extracts at the analytical scale (A-W75) from *P. nigra* bark. The labelled peaks correspond to procyanidin oligomers.

However, the low yield of CHs did not elevate the concentration of phenolics (Table 4). A successive petrol ether extraction at 60°C of the bark residues (after aq. acetone extraction) showed that apolar compounds were no more detectable. It can be safely concluded that all apolar compounds (22.0 g kg^{-1}, Table 1) were extracted with aq. acetone. A-A75 was thus assumed to consist of 25.9% nonpolar extractives (Table 4). The presence of apolar compounds in A-A75 was not detectable with Py-GC/MS with the exception of the increment of limonene content (peak no. 8, 4.5% – Table 6).

Thiolysis indicated the presence of procyanidins in A-W75 with DPs of 7.5 (Table 2). The MALDI-TOF mass spectrum (Figure 4) shows an improved signal-to-noise ratio, probably due to lower CH_{bound} concentration. A main homogeneous procyanidin series (905.7, 1194.2, 1482.5, 1770.9, 2058.9 m/z, etc.) was detected. A second peak series (1040.8, 1329.8, 1618.4, 1905.9, 2195.0 m/z, etc.) with a splitting of about +136 m/z from the main series (Figure 4) suggests the presence of di-hydroxybenzoate ester groups (154.1 – 18 = 136.1 Da), based on the observation of Ucar et al. (2013), who extracted CT from Turkish pine (*Pinus brutia* [Ten.]) bark. Nevertheless, this interpretation needs to be confirmed.

Following each procyanidin peak, secondary peaks of lower intensity were observed with splits of +14, +27 and +39 m/z, which are indicative of methyl (+14 Da), dimethyl (+28 Da) and other derivatives of the flavanol units. Guaiacol and 4-methyl-guaiacol groups were observed via Py-GC/MS but could be as well belong to SW lignin fragments. Therefore, the interpretation of the methyl and dimethyl groups in the context of CT mass spectrum is not straightforward. The low intensity peak with –17 m/z in the context of procyanidin series (Figure 4) might be due to CT lacking of a hydroxyl group, e.g. containing an epiafzelechin monomeric unit. Phenol, *p*-cresol and 4-vinylphenol, seen in the pyrograms (Table 6), can be either lignin fragments or fragments of flavanol units with a phenol B-ring.

Only traces (0.8%) of free monosaccharides were detected in A-A75, which were due to glucose and arabinose (Table 3) like in the A-W75 fraction. Bound carbohydrates are composed of glucose and rhamnose residues (Table 3). The presence of arabinans and arabinogalactans, as seen in A-W75, can thus be excluded in A-A75.

Pilot scale extraction

Pilot scale HWE at 75°C (P-W75) gave rise to a total extraction yield of 28.0 g kg^{-1}, which is about the half of the yield obtained at the analytical scale (Table 4). The yields of phenolic compounds, CT and bound carbohydrates were about 2.5 times lower than that in the analytical extracts (Table 4). Their composition showed only subtle variations to that of A-W75 (Tables 2 and 3). Monosaccharides and inorganic compounds were detected in similar yields for analytical and pilot scale extraction (Table 4). Monosaccharides occur in decreasing order:

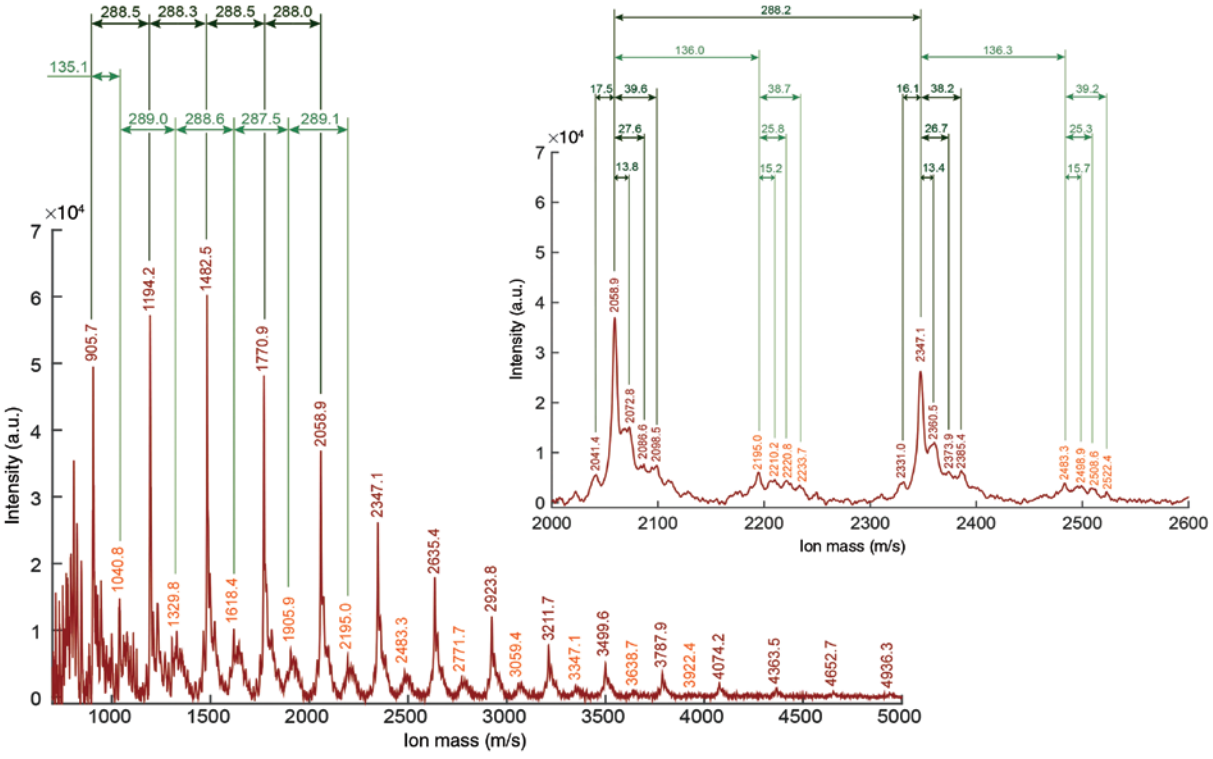


Figure 4: MALDI-TOF mass spectrum between 700 and 5000 m/z of analytical scale 75°C aq. acetone extracts (A-A75) from *P. nigra* bark. The dark red labelled peaks correspond to procyanidin oligomers. The orange labelled peaks correspond to procyanidins with a linked moiety of about 136 m/z. The MALDI-TOF mass spectrum between 2000 and 2600 m/z with the distances between main and secondary peaks is reported in particular.

galactose > glucose > mannose > arabinose > galacturonic acid (Table 3). Accordingly, the P-W75 and A-W75 are very similar, which is also demonstrated by Py-GC/MS (Table 6).

The yield difference might be partially related to particle size differences (approx. 50 µm for analytical extraction and about 2 mm for the pilot extraction). This assumption was supported by an analytical extraction with water at 75°C of the hammer-milled bark (like in P-W75), which has a total yield of 41.5 g kg^{-1}, a value between the yield data of A-W75 and P-W75.

Conclusions

HWE of *P. nigra* bark contains up to 50.7% of phenolic compounds. Almost one half of it is constituted of CTs, more precisely procyanidins with DPs of about 9. A part of the phenolic extractives are lignin-like. MALDI-TOF MS results could be partly interpreted as CTs containing methylated flavanols. The presence of pectins (e.g. arabinans, arabinogalactans, glucans) in concentrations between 15.6 and 19.7% was also observed. These characteristics of *P. nigra* bark HWE suggested a high viscosity and a partially constrained reactivity in adhesive formulations. Further research is necessary to optimize the extraction process and the extract properties and to suit them better for adhesive applications.

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