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Hot water extraction of Norway spruce (*Picea abies* [Karst.]) bark: analyses of the influence of bark aging and process parameters on the extract composition

DOI 10.1515/hf-2015-0160
Received July 15, 2015; accepted November 15, 2015; previously published online December 16, 2015

Abstract: The hot water (HW) extraction of Norway spruce (*Picea abies* [Karst.]) delivers condensed tannins and considerable amounts of other compounds. Yield and composition of the HW extracts were investigated as a function of natural weathering for up to 15 months. Total phenol monomers and oligomers were detected by Folin-Ciocaltau assay after fractionation by solid phase extraction (SPE). Procyanidins (PC) were determined by HPLC-UV after acid thiolysis and carbohydrates by HPLC combined with acid hydrolysis. Topochemistry of the bark before and after extraction was investigated by UV-microspectrometry (UMSP) and non-extractable PC analyzed by direct thiolysis on the bark. The influence of the parameters on the yield and composition of the extracts were evaluated, such as the extraction temperature, time and the addition of sodium sulfate and urea. Prolonged weathering resulted in a considerable decrease of the total extraction yield, partly because of leaching of phenolic monomers, mono- and oligosaccharides. The yield of phenolic oligomers also decreased at a moderate rate, while the yield of polysaccharides (pectins) was almost stable. Non-extractable and non-leachable compounds deposited in the cell lumens represent the majority of the phenolic extractives in spruce bark. Sequential extractions performed at increasing temperature proved to be a suitable method for the recovery of tannin-rich extracts.

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Keywords: bark aging, carbohydrates, condensed tannins, extraction parameters, face centered cubic statistical design, hot water extraction, non-extractable tannin, Norway spruce bark, response surface method, thiolysis, topochemistry, UMSP

Introduction

Condensed tannins are polyphenolic compounds abundant in the bark of several European softwoods (SWs) (Haslam 1989; Porter 1989; Matthews et al. 1997), which are composed of different procyanidin (PC) or prodelphinidin (PD) type building units (Figure 1). Bark tannins are well suitable for wood adhesive formulations (Yazaki and Collins 1994; Roffael et al. 2000; Bertaud et al. 2012). The annual volume of harvested SWs in Central and Northern Europe is about 25×10^7 m^3 (EUROSTAT 2015) and ca. 10% of this volume is bark, which is currently disposed or burned for energy recovery. In Germany, ca. 67% of forest is composed of spruce species and in northern-central Europe's forests the situation is similar (DESTATIS 2014). Thus Norway spruce (*Picea abies* [Karst.]) bark can be regarded as a largely available source of condensed tannins in Europe.

The main limitations in the exploitation of SW bark tannins are the low extraction yields and the high amounts of co-extracted non-tannin type compounds. In particular, the presence of carbohydrates is not desirable as they lower the concentration of reactive phenolics and increase the viscosity in tannin resin formulations (Weissman 1985; Garnier et al. 2001; Pizzi 2008). Stilbene glucosides and lignans were reported as typical phenolic extractives from spruce bark and root neck (Latva-Mäenpää et al. 2014). The purification of bark tannins from the co-extracted compounds furthers their exploitation field. Effective use of purified tannins and other phenolic bark extracts were identified in applications as radical scavengers (Makino et al. 2011), biological antioxidants (Telysheva et al. 2011), antifungal agents (Anttila et al. 2013),

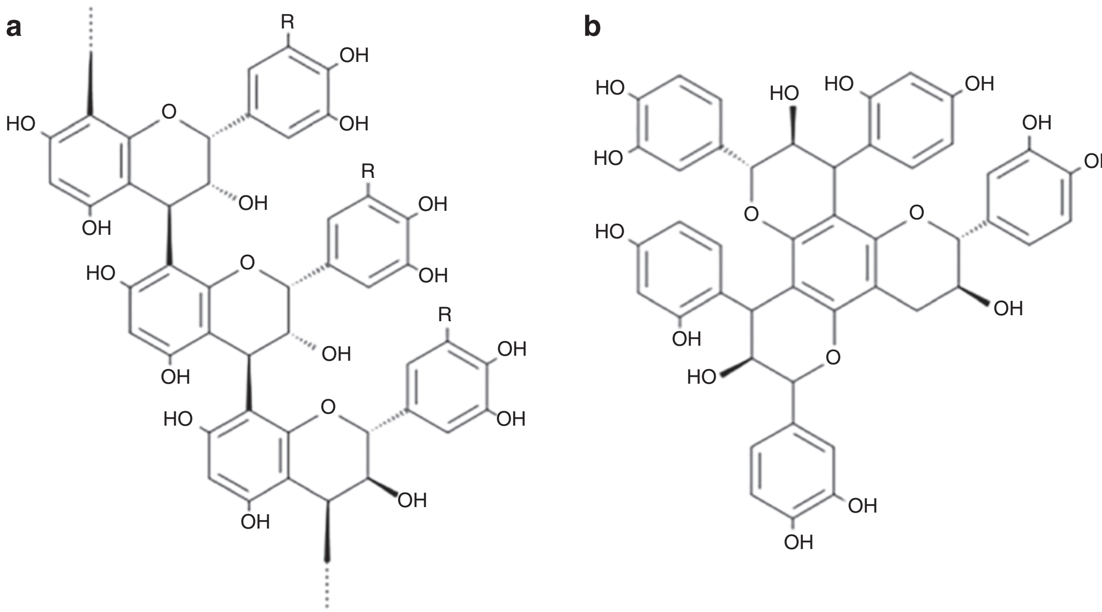

Figure 1: Examples of tannins with units of (a) procyanidins (a, with R=H) and prodelphinidins (a, with R=OH) and (b) phlobatannins (Steenkampf et al. 1985).

foamed resins (Lacoste et al. 2015) and heavy-metal chelation (Seki et al. 1997). Thermosetting and thermoplastic resins can be developed through hydroxypropylation of SW tannins (Garcia et al. 2014).

Isomerization and self-condensation of the tannins lead to the formation of phlobatannins with low solubility (Figure 1). In the worst case, phlobaphenes are formed, i.e. reddish-colored and non-soluble compounds with not yet clarified molecular structure, which may also occur naturally in bark. Phlobaphenes of Douglas fir bark (*Pseudotsuga menziesii* [Mirb.]) contain highly condensed polymers consisting of tannins, flavonoids, lignans, and carbohydrates (Foo and Karchesy 1989).

The extraction temperature influences essentially the tannin yield (Dix and Marutzky 1983; Jorge et al. 1999; Vázquez et al. 2001; Kemppainen et al. 2014). The addition of alkalis and sulfites to the hot water (HW), occasionally in combination with urea, also increases the extraction yield through inhibition of the phlobatannin rearrangements (Liiri et al. 1982; Dix and Marutzky 1983; Weissman 1983; Sealy-Fisher and Pizzi 1992). Huge variations in the extraction yields can be observed also within the same species. For example, the total yield of pure HW extractions from spruce bark ranged from 3.0 to 15.3 g kg^{1} dry bark (Weissman 1981; Bertaud et al. 2012; Kemppainen et al. 2014).

The age, storage conditions, harvesting time etc. of the bark influence heavily the yield and composition of the extracts. Matthews et al. (1997) highlighted the importance of age on the extractability of bark. Relevant differences in the extract yields from spruce bark collected at a sawmill and at a pulp mill were also observed, which are most likely related to different moisture conditions during log storage (Liiri et al. 1982). The stem heights are also influential in terms of polyphenols yields (König and Roffael 2002). The amount of co-extracted carbohydrates are influenced by the harvesting season (Weissman 1984).

In the present study, bark flakes should be extracted with HW, which were collected from Norway spruce log exposed to natural weathering. Topochemistry of the bark samples will be investigated by cellular UV microspectrometry (UMSP) before and after HW extraction. Extract composition should be characterized with regard to phenolic monomers, condensed tannins, mono-, oligo-, and polysaccharides. The influence of extraction temperature and time on the extract quality and on the ratio between tannins and carbohydrates will be in focus. The expectation is that the relationship between the extraction parameters and the tannin quality will be better understood and that an optimal set of extraction parameters will be found for tannin-rich extracts.

Materials and methods

Material collection: A Norway spruce log was felled end of October 2013 in a forest nearby Biel (Switzerland) at approximately 1000 m above mean sea level and exposed to natural weathering in a log yard. The tree was 120 years old, with a breast-high diameter (BHD)

of 50 cm and a total height of about 35 m. Bark flakes containing both inner and outer bark were collected periodically from 1 to 469 days after tree felling by handheld tools at 1 m and 10 m from the stump. Three other Norway spruce trees were also felled in March 2013 with BHD 40 and 60 cm in the same forest but at lower altitude (600 m). The logs were left on the ground close to the harvesting site and after 1–3 month barks were sampled. All the bark samples were deep-frozen and stored airtight on the day of collection.

Extraction: The bark flakes were milled to fine-powder in a swinging mill (Herzog HSM 100H, Osnabrück, Germany). The bark powder was then extracted with hot water (HW) by means of an accelerated solvent extraction device (Dionex ASE® 200, Sunnyvale, CA, USA) at specified temperatures, as reported previously by Bianchi et al. (2014). Unless reported otherwise, the extraction time was 10 min and the extraction temperature was 60°C. Some extractions were conducted with the addition of sulfites (Sodium sulfite, Sigma Aldrich, Switzerland) and urea (Sigma Aldrich, Switzerland) to HW as the extraction medium. The extract was finally freeze-dried and stored airtight in dark until analysis.

Analysis: The experimental design concerning the characterization of the spruce bark and the HW extracts is illustrated in Figure 2.

Scanning electron microscopy (SEM) samples were prepared from freeze-dried bark chips cutting the surface with a sliding microtome until cracks or damages were no longer visible. The samples were subsequently sputtered under vacuum with gold-palladium (10–15 nm in thickness) and observed by a table-top Hitachi TM3030 (Krefeld, Germany) SEM microscope (15 kV).

UV microspectometry (UMSP) was performed on samples prepared and analyzed as described by Koch and Kleist (2001). Briefly, freeze dried bark chips were dehydrated with acetone and subsequently impregnated with Spurr's epoxy resin. Semi-thin transverse sections (1 µm) were carefully cut with a calibrated ultra-microtome equipped with a diamond knife and transferred to quartz microscope slides. The thickness of the sample was regularly proofed by light reflection of the sections. UV absorbance scans at 280 nm on rectangular fields of the bark sections were recorded with a geometric resolution of 0.25 µm×0.25 µm by means of a Zeiss UMSP 80 instrument and APAMOS® (Carl Zeiss, Oberkochen, Germany) software. Three fields for each bark sample were analyzed. In addition, photometric

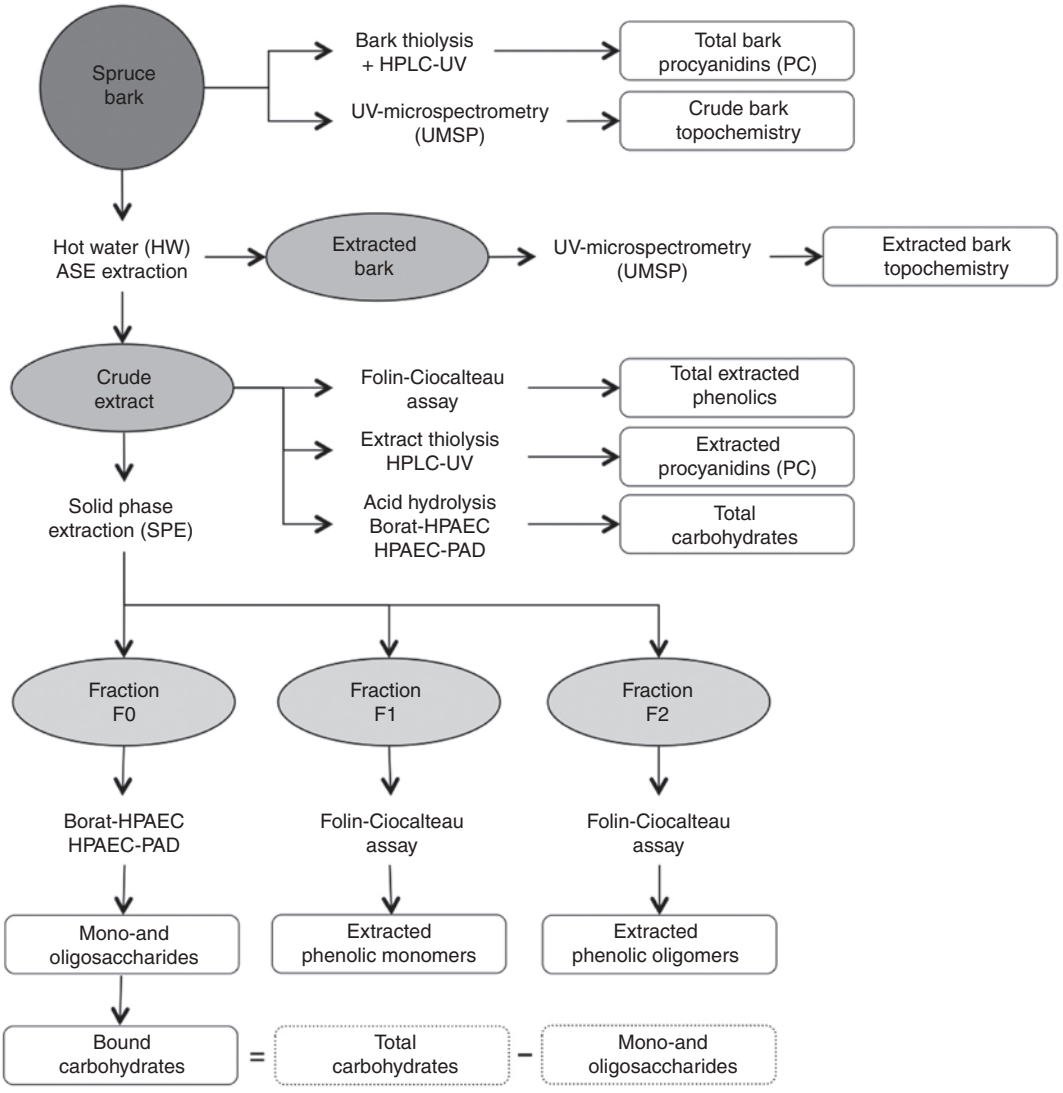

Figure 2: Flow diagram of the experimental design used in the characterization of spruce bark and extracts.

point measurements were performed on a spot size of 1 μm^2 between 240 and 500 nm (LAMWIN® software, Zeiss, Oberkochen, Germany). For comparative studies, at least 10 spectra were taken from each individual cell tissue or lumen deposit.

An aliquote of the crude freeze-dried extracts was split in three fractions (F0=carbohydrates and phenolic acids; F1=phenolic monomers; F2=phenolic oligomers) following the solid phase extraction (SPE) procedure proposed by Sun et al. (1998) with slight modification. Briefly, the SPE cartridges (Bond-Elute® 10 mg, Agilent, Santa Clara, CA, USA) were preconditioned in sequence with MeOH (Sigma Aldrich, Switzerland) and distilled water. Fifteen millilitre of an aqueous solution of the extract (1 g l^{-1}) was then loaded in the SPE cartridge. Sequential elution with 15 ml of distilled water, 20 ml of ethylacetate (Sigma Aldrich, Switzerland) and 20 ml of MeOH, was then performed in order to gain the fractions F0, F1, and F2, respectively. To the F0 fraction, 1 ml of sodium azide solution (60 mg/l, Sigma Aldrich, Switzerland) was added to stop any biological activity, and the volume was brought up to 30 ml. F1 and F2 fractions were vacuum dried at 40°C.

The total amount of phenolic compounds was determined by the Folin-Ciocalteu method (Singelton et al. 1999) on the whole extract (aqueous solutions 0.2 g l^{-1}) and on the fractions F1 and F2 (after dispersion of the vacuum dried samples in 20 ml of water). Three measurements were performed for each sample. Calibration was performed with (-)-epicatechin (Sigma Aldrich, Switzerland).

Tannin extraction yield was estimated by the methylcellulose precipitation method (Sarneckis et al. 2006). Briefly, 2.5 ml of aqueous extract (0.5 g l^{-1}) was mixed in sequence with 1.5 ml of 0.4% w/w aqueous methylcellulose (Sigma Aldrich, Switzerland) and 1.5 ml of saturated ammonium sulfate solution (Sigma Aldrich, Switzerland), and incubated for 30 min at room temperature. After centrifuging, the absorbance at 280 nm of the supernatant was measured and compared with a control solution. Calibration was performed with (-)-epicatechin.

The amount of PC and PD in the extracts, as well as their mean degree of polymerization (DP) was estimated by high performance liquid chromatography (HPLC) before and after acid thiolysis. The thiolysis was performed with cysteamine HCl (Sigma Aldrich, Switzerland) as described by Jerez et al. (2007), but the reaction time was 60 min. Reversed phase HPLC was done on an Agilent 1100 system (Santa Clara, CA, USA) with a Cosmosil Protein-R ø4.6×250 mm column (Nacalai Tesque, Kyoto, Japan) equipped with a UV diode array set at 280 nm. Elution (1 ml min^{-1}) was made with [A] 0.1% of trifluoracetic acid (TFA, Sigma Aldrich, Switzerland) inv water, and [B] 0.082% of TFA in acetonitrile:water (4:1 v/v), while the following gradient was applied: initial, 0% [B]; 0–5 min, from 0.0% to 7.5% [B]; 5–20 min, from 7.5% to 8.5% [B]; 20–30 min, from 8.5% to 13.5% [B]; 30–45 min, from 13.5% to 33.5% [B]. Identification and quantification of flavan-3-ols were performed by comparison with analytical standards (Sigma Aldrich, Switzerland). No standards were available for the flavan-3-ol thioethers, thus they were identified by MS with the same HPLC setting as described above, but on a Agilent 1290 Infinity HPLC system equipped to this purpose with a mass detector (Agilent 6130 quadrupole MS, Santa Clara, CA, USA). The quantification of the flavan-3-ol thioethers was performed assuming the same UV molar absorption factor of the corresponding flavan-3-ols. The occurrence of some known phenolic monomers, such as stilbene glucosides or flavonoids, were checked by analysis of the spectrum characteristics and the molecular masses detected by HPLC-MS in correspondence of some UV absorption peaks.

The total amount of PC and PD in bark, which includes both the extractable and the non-extractable molecules, was evaluated by direct thiolysis of the bark, as proposed by Matthews et al. (1997). The fine milled bark (50 mg) was dispersed in the thiolysis media (9.30 ml MeOH, 50 mg cysteamine HCl, 0.20 ml 37% HCl) and kept at 65°C for 60 min in an ultrasound bath. The extract solution was then filtered through a PTFE filter (0.37 µm). A 2.9 ml aliquot of it was thereafter recovered and diluted with water to 20 ml. The solution was then analyzed by HPLC-UV as specified for the extracts.

Total extracted carbohydrates were assessed after acid hydrolysis of the extracts. The freeze-dried extracts (50 mg) were suspended in 5 ml of water and then 0.9 ml of H_2SO_4 1 M was added. The samples were hydrolyzed in an autoclave at 120°C for 40 min. After cooling, the samples were brought to 50 ml with water and filtered on a no. 4 sintered glass crucible. The hydrolyzate monomeric carbohydrates glucose (Glc), xylose (Xyl), mannose (Man), galactose (Gal), arabinose (Ara) and rhamnose (Rha) were analyzed by Borat anion exchange chromatography (Borat-HPAEC) with post-derivatization and detection at 560 nm, as reported by Willför et al. (2009). Mannitol, 5-hydroxymethylfurfural (5-HMF), galacturonic acid (GalA) and glucuronic acid (GlcA) were detected by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), as described by Manns et al. (2014).

The free mono- and oligosaccharides (free carbohydrates) in the non-hydrolyzed water-eluted F0 fraction were quantified by the Borat-HPAEC method. Fructose (Fru), sucrose (Suc), stachyose (Sta) and raffinose (Raf) were also detected in this fraction by the HPAEC-PAD approach on a 4.0 mm Carbopac PA1 – 250 mm column (Thermo scientific, Germany). Isocratic elution (1.0 ml min^{-1} – 20 min) was performed with 75 mM NaOH. The compounds were detected by an ED50 electrochemical detector equipped with a disposable gold electrode and an Ag/AgCl reference electrode (Thermo scientific, Germany). The difference between total and free carbohydrates is termed "bound carbohydrates", which can be most likely assigned to polysaccharides and/or glycoside residuals.

Results and discussion

Topochemical analysis

Parenchyma cells, most of them considerably collapsed, represent the large majority of cells in inner and outer bark tissues (Figure 3 with SEM images). In the outer bark, several periderms bands are seen (Figure 3a, b). The innermost band corresponds to the newer periderms and it defines the boundary between inner and outer bark. The more external bands correspond to older periderm, as described in detail by Howard (1971). Each periderm band consists of four to five thick walled sclereids and, in the outer layer, of a few thin walled cork cells (phellem). Isolated sclereids were also observed among parenchyma cells, and in particular in the outer bark (Figure 3a, b). Solid deposits of extractives are present in the large majority of the lumens of sclereids (Figure 3d) and cork cells

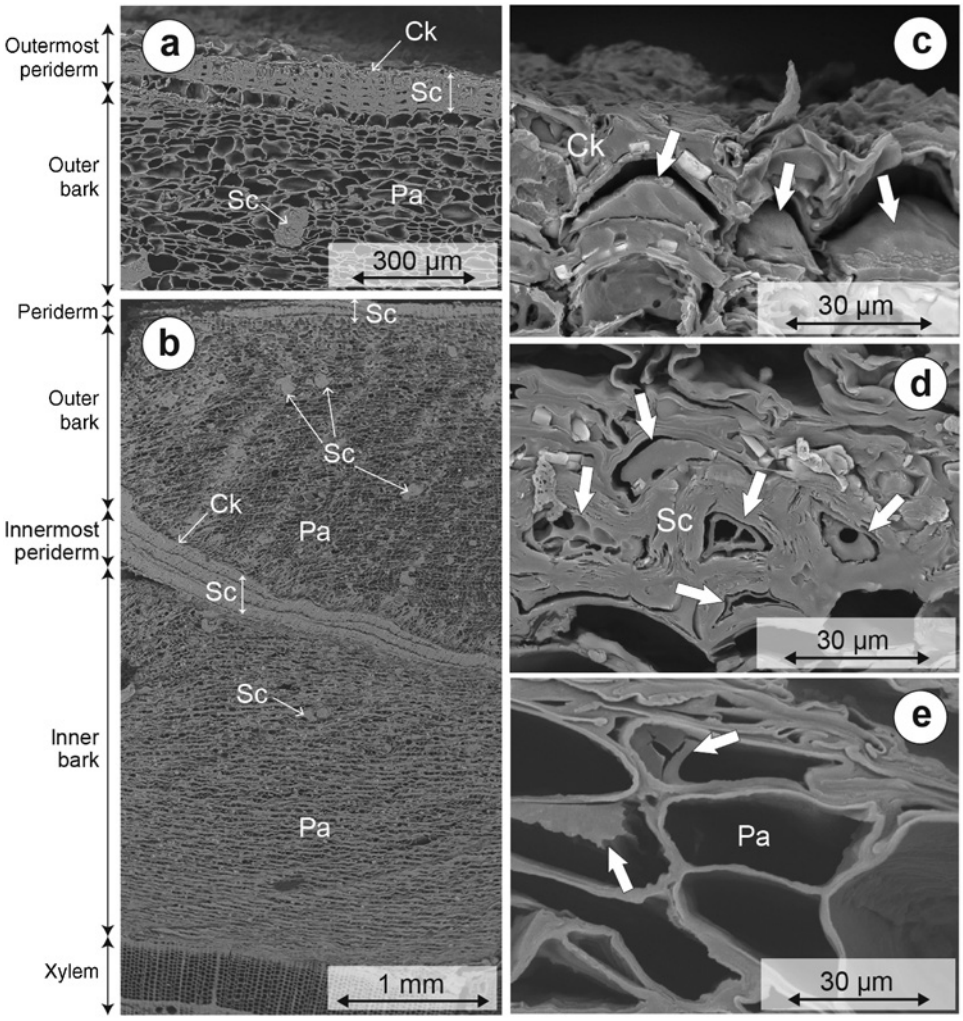

Figure 3: SEM pictures of Norway spruce bark (samples collected at 10 m in height 210 days after tree felling). Parenchyma cells (Pa), sclereids (Sc), cork cells (Ck), lumen deposits (thick white arrow). (a) Outermost periderm. (b) Inner and outer bark with two internal periderm bands. (c) Outermost cork cells with deposits in the lumen. (d) Particular of periderm sclereids with deposits in the lumen. (e) Outer bark parenchyma cells with deposits in the lumen.

(Figure 3c). They occur either as a thin layer attached to the lumen walls or as a bulky mass that fills the lumens almost completely. Deposits were also identified in the lumen of axial parenchyma cells (Figure 3e) but with a much lower frequency.

The distribution of the phenolic compounds was topochemically investigated by mapping at λ=280 nm (A_{280nm}) in selected samples (Figure 4). The cell walls (S2 layers) of the bark show distinct A_{280nm} between 0.32 and 0.78, which are considerably higher than the values reported by Koch and Kleist (2001) for spruce xylem cells (A_{280nm}=0.10 to 0.41). Point by point measurements of the S2 layer cell wall of sclereids and parenchyma cells (Figure 5) show UV absorbance spectra with a pronounced maximum at about 280 nm and a feeble local minimum close to 260 nm, typical of a guaiacyl lignin (Musha and Goring 1975). Accordingly, the S2 layers of bark cell tissues are highly lignified.

The point by point spectra of the cork cell S2 layers show a slight bathochromic shift of the maximum and a shoulder at ca. 330 nm (Figure 5). This spectral type is associated with the occurrence of conjugated double bonds or carboxylic groups (Goldschmid 1975). It can be suggested that condensed phenolic compounds, different from lignin, incrust the walls of spruce cork cells as described for beech bark (Prislan et al. 2012). The possibility that some monomers and smaller phenolic oligomers could penetrate the cell walls, especially in older and dead tissues, was discussed by Hillis (1985), though phenolic deposits are generally localized in the cell lumen of bark tissues.

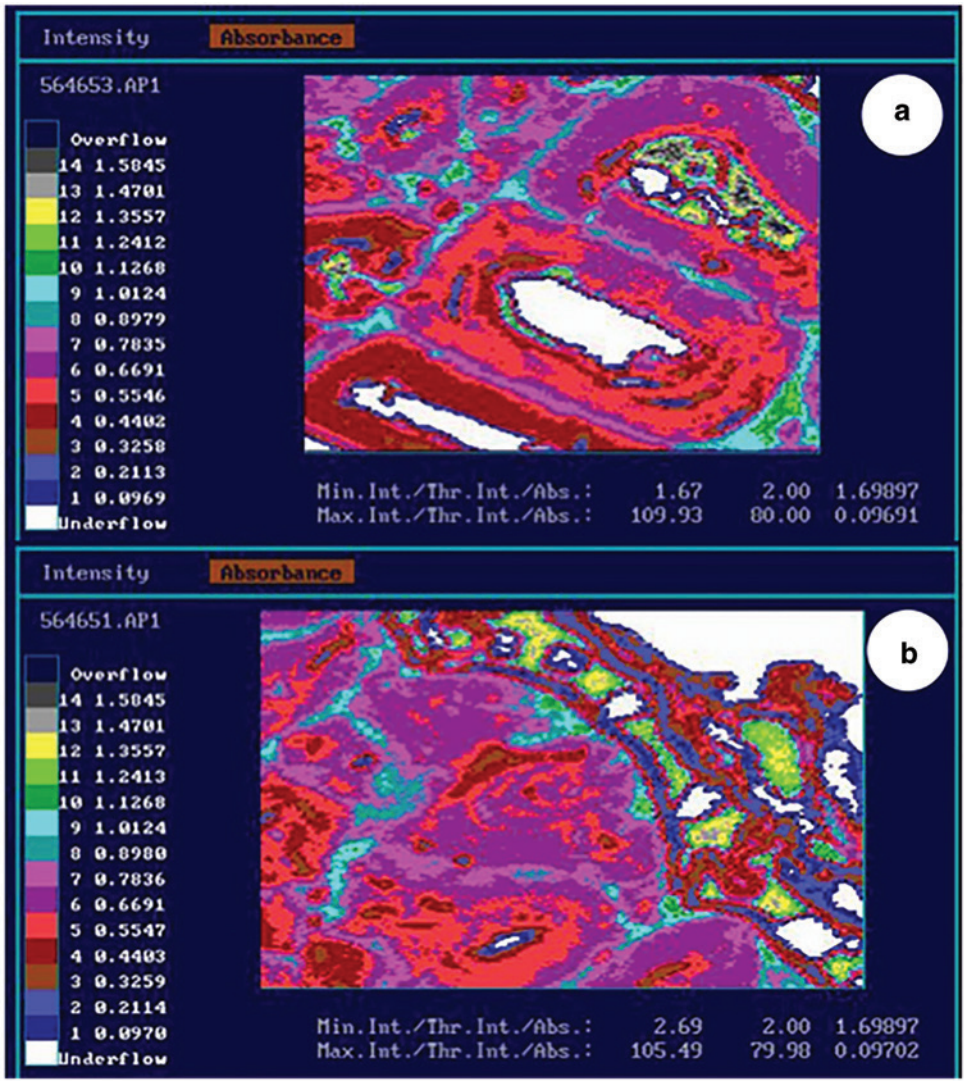

Figure 4: UMSP of Norway spruce bark. The color pixels represent different UV absorption values of the individual cell wall layers and extractives measured at 280 nm with a geometrical resolution of 0.25 μm×0.25 μm. (a) Highly lignified sclereids with phenolic deposits in the cell lumen. (b) Cork cells with distinct deposits of phenolic extractives.

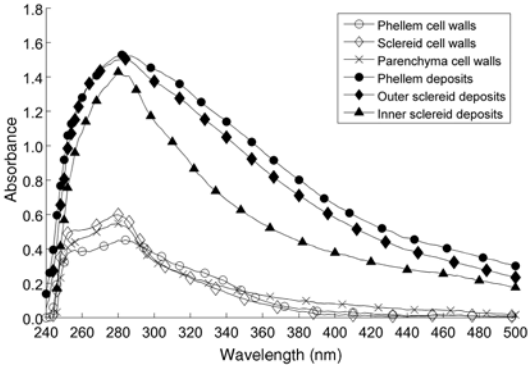

Figure 5: Representative UV absorbance spectra of cell wall (S2 layer) and phenolic deposits in Norway spruce bark (sample collected at 10 m in height 210 days after tree felling). Each line represents the mean of at least 10 spectra.

The deposits in the cell lumen are clearly silhouetted against the surrounding bark cell tissues with their distinctively higher absorbances (Figure 4). Their phenolic nature is evident. In the most internal sclereids, the spectra of the deposits (Figure 5) have a pronounced peak at 285 nm and a shoulder around 320 nm, which again is indicative to phenolic compounds with conjugated double bonds. Broad peaks are typical for the spectra of deposits in the outermost sclereids and cork cells, with conspicuous UV absorbances up to 400 nm (Figure 5). The interpretation is that the deposits are mostly condensed phenolics with conjugated double bonds, while the degree of condensation is increasing towards the most external periderm cell layers. These compounds could be associated to condensed tannins like PC and PD.

The presence of phlobaphene structures should, in fact, not be excluded.

Extract yield and composition along storage time

Spruce bark samples exposed to natural weathering were collected from 1 to 469 days after tree felling. The total extraction yield shows a noticeable decrease as storage time increased (Figure 6). The most relevant yield drop is detectable during the warm season between 100 and 265 days of weathering. The bark samples collected in 1 m height have lower yields that that in proximity to the first branches in 10 m height.

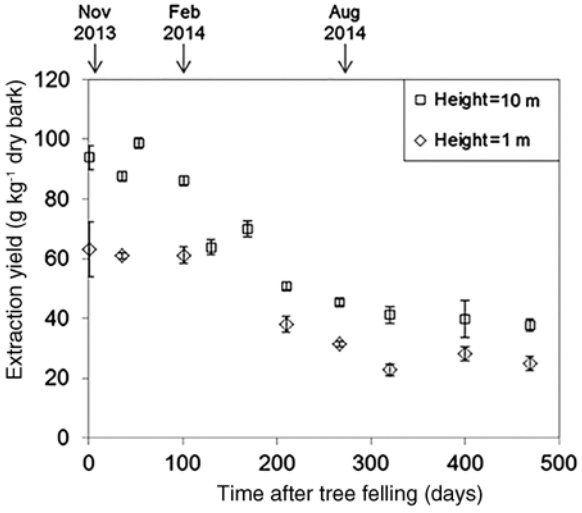

Figure 6: Variation of the total extraction yield from Norway spruce bark with extended storage time (after felling of the tree) at 1 m and 10 m heigths. Each point represents the average of n=5 extractions; bars indicate standard deviations.

The total extracted phenolic compounds, evaluated by the Folin-Ciocalteau assay, decreased with age in analogy to the total extraction yields (Table 1), i.e. the portion of total phenolics in the extracts is constant. The decrement of phenolics and condensed tannins as a function of natural weathering was already observed (Schofield et al. 1988; Kelsey and Harmon 1989), and this effect was mainly ascribed to their leaching. Matthews et al. (1997) and Maie et al. (2003) proposed a reduction of the phenolic yields through a progressive immobilization of the tannin molecules via condensation reactions.

Fresh bark samples contained an almost equal amount of phenolic monomers (F1 fraction) and phenolic oligomers (F2 fraction), while older samples contain mostly phenolic oligomers (Table 1). A faster depletion of the phenolic monomers than oligomers is obvious. In agreement with these results, HPLC-UV data of the extracts (data not reported) show before thiolysis a progressive peak intensity decrement with extended storage time associated with the decay of stilbene glucosides astringin and isorhapontin and the flavanonol taxifolin. In particular, the stilbene glucosides have a faster decay than taxifolin, which actually remained the only compound detectable by HPLC-UV at 469 days.

HPLC-UV after thiolysis on the collected samples revealed that condensed tannins are represented by PC, in agreement with previous works (Matthews et al. 1997; Bianchi et al. 2014). A considerable yield decrement of extracted PC with extended storage time (Table 1) is detectable, which occur more rapidly than the phenolic oligomers fraction (F2). A decrease of the DP of the extracted PC was also observed for the older samples

Table 1: Extracted total phenolics and extracted, total, and non-extractable procyanidins from Norway spruce bark samples after different storage days (d) at 1 and 10 m heights.

	Total phenolics extracted			Procyanidins				
				Extracted		Total		Residual
Parameters	Total gECE kg^{-1a}	Monom. (F1) gECE kg^{-1a}	Oligom. (F2) gECE kg^{-1a}	g kg^{-1a}	DP	g kg^{-1a}	DP	g kg^{-1a}
1 m								
1 day	22.1±1.1	10.3±0.6	11.3±0.5	8.6±0.9	6.3±0.9	22.8±1.2	7.2±1.1	14.2±1.5
210 days	10.1±0.4	3.3±0.3	6.2±0.3	2.8±0.3	4.7±0.7	17.5±0.9	7.7±1.2	14.6±0.9
469 days	6.5±0.3	1.8±0.2	4.0±0.1	1.3±0.3	4.8±0.7	14.4±0.7	7.7±1.2	13.0±0.8
10 m								
1 day	31.5±1.5	15.3±0.9	16.1±0.8	9.9±0.9	5.5±0.8	21.6±1.0	6.3±0.9	11.0±1.0
210 days	13.3±0.7	4.5±0.3	7.8±0.3	2.8±0.3	4.4±0.7	15.2±0.7	6.7±1.0	12.4±0.8
469 days	8.4±0.3	2.7±0.2	5.3±0.3	1.7±0.2	3.8±0.6	14.4±0.8	7.3±1.1	12.7±0.8

Extractions performed in water at 60°C. Mean±StD is presented. [a]Dry bark.

(Table 1). Clearly, phenolic oligomers extracted from spruce bark are not only composed of linear PC but also of thiolysis-resistant compounds, the preponderance of which is more pronounced at older samples. As suggested by Matthews et al. (1997), such compounds could be most likely associated to condensed tannins containing stilbene glucosides, but also lignans (Zhang and Gellerstedt 2008) or phlobatannins (Steenkampf et al. 1985) should be considered.

A sizable occurrence of mono and oligosaccharides (mainly glucose, fructose and sucrose) was observed only in the fresh (1 day) samples (Table 2). After 210 days, these compounds were detectable only in traces. This depletion is most likely related to their leaching, but biological degradation may have also contributed to this observation.

Bound carbohydrates also decreased with extended storage time, although less severely than mono- and oligosaccharides (Table 2). The main components of bound

Table 2: Composition of the carbohydrates in the bark extracts depending on the height and extraction time (at 60°C; mean and StD).

	Carbohydrates (g kg^{-1} dry bark)										
Parameters	**Glc**	**Gal**	**Fru**	**Xyl**	**Man**	**Ara**	**Rha**	**GalA**	**Suc**	**Raf**	**Sum**
1 m											
1 day											
Total	10.8±1.6	1.9±0.3	1.9±0.3	0.6±0.2	0.5±0.1	3.2±0.5	0.4±0.1	1.2±0.2	–	–	20.5±3.0
Free	1.2±0.5	0.1±0.1	1.7±0.6	<0.1	<0.1	0.1±0.1	<0.1	<0.1	2.1±0.4	0.1±0.1	5.4±1.3
Bound[a]	8.4±1.6	1.8±0.3	<0.1	0.6±0.2	0.5±0.1	3.1±0.5	0.4±0.1	1.2±0.2	–	–	16.1±3.2
1 m											
210 days											
Total	4.6±0.4	1.2±0.1	0.1±0.1	0.3±0.1	0.4±0.1	2.3±0.2	0.4±0.1	0.9±0.1	–	–	10.3±0.8
Free	0.5±0.1	<0.1	<0.1	<0.1	<0.1	0.1±0.1	<0.1	0.1±0.1	<0.1	<0.1	0.7±0.2
Bound[a]	4.1±0.4	1.2±0.1	<0.1	0.3±0.1	0.4±0.1	2.2±0.2	0.4±0.1	0.8±0.1	–	–	9.6±0.9
10 m											
1 day											
Total	17.0±0.8	2.8±0.2	2.1±0.2	0.7±0.2	0.7±0.2	4.9±0.3	0.7±0.2	1.8±0.2	–	–	30.8±1.5
Free	1.6±0.4	0.1±0.1	1.9±0.5	<0.1	<0.1	<0.1	<0.1	0.1±0.1	2.8±0.8	0.1±0.1	6.9±2.6
Bound[a]	13.8±0.9	2.7±±0.2	<0.1	0.7±0.2	0.7±0.2	4.8±0.3	0.7±0.2	1.7±0.2	–	–	25.1±2.8
10 m											
210 days											
Total	6.5±0.2	1.9±0.1	0.1±0.1	0.6±0.1	0.9±0.1	3.1±0.1	0.6±0.1	1.3±0.2	–	–	15.1±1.0
Free	0.5±0.1	<0.1	0.1±0.1	<0.1	<0.1	0.1±0.1	<0.1	<0.1	0.1±0.1	<0.1	0.7±0.2
Bound[a]	6.0±0.2	1.9±0.1	<0.1	0.6±0.1	0.9±0.1	3.1±0.1	0.6±0.1	1.3±0.2	–	–	14.4±1.0
30°C											
Total	3.8±0.3	1.4±0.1	0.5±0.1	0.4±0.1	0.3±0.1	1.6±0.2	0.3±0.1		–	–	8.3±0.7
Free	0.6±0.1	<0.1	0.6±0.1	<0.1	<0.1	0.2±0.1	<0.1		0.5±0.1	<0.1	2.6±0.2
Bound[a]	2.9±0.3	1.4±0.1	<0.1	0.4±0.1	0.3±0.1	1.4±0.1	0.3±0.1		–	–	6.7±0.6
45°C											
Total	0.9±0.2	0.4±0.1	<0.1	0.1±0.1	0.1±0.1	0.4±0.1	0.1±0.1		–	–	2.0±0.4
Free	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1		<0.1	<0.1	0.1±0.1
Bound[a]	0.8±0.2	0.4±0.1	<0.1	0.1±0.1	0.1±0.1	0.5±0.1	0.1±0.1		–	–	2.0±0.4
105°C											
Total	1.0±0.1	0.7±0.1	<0.1	0.2±0.1	0.3±0.1	1.4±0.2	0.1±0.1		–	–	3.7±0.5
Free	<0.1	<0.1	<0.1	<0.1	<0.1	0.2±0.1	<0.1		<0.1	<0.1	0.3±0.1
Bound[a]	1.0±0.1	0.7±0.1	<0.1	0.2±0.1	0.3±0.1	1.2±0.2	0.1±0.1		–	–	3.4±0.4
150°C											
Total	2.7±0.4	4.9±0.7	<0.1	2.6±0.3	2.1±0.3	9.4±1.2	0.7±0.1		–	–	22.2±2.9
Free	0.1±0.1	0.3±0.1	<0.1	0.1±0.1	<0.1	2.4±0.3	0.2±0.1		<0.1	<0.1	3.2±0.4
Bound[a]	2.5±0.3	4.6±0.6	<0.1	2.4±0.3	2.1±0.3	7.0±0.9	<0.1		–	–	19.1±2.5

The lower part of the table reports on data as a function of the extraction temperature. [a]Bound carbohydrates: difference between total and free carbohydrates. In the case of Glc, Gal, and Fru, the subtracted amount includes both the free monosaccharides and their monosaccharide units in the free oligosaccharides.

carbohydrate are after hydrolysis glucose, galactose, arabinose, and galacturonic acid. They could therefore be mostly associated to typical SW pectin-like polysaccharides like arabinans, arabinogalactans and glucans (Timell 1961; Kemppainen et al. 2014). Samples collected at 10 m height contain bound carbohydrates in considerably higher concentrations than samples collected at the 1 m height. The quicker drop in glucose yield compared to the other carbohydrate residuals is likely related to its association not only to polysaccharides but also to stilbene glucosides, which were rapidly decreased during aging.

Bark tissue variations along storage time and after extraction

The variation in the bark tissue during weathering was followed by UMSP and direct thiolysis. While the former focuses on the absorbance at 280 nm (associated to the total phenolics), the latter allows for estimating the total amount of PC as the sum of extractables and non-extractables (Matthews et al. 1997).

There are only small UV differences between 1 day old and 210 days old samples. A_{280nm} at 1 m height decreased from 0.65 to 0.61, while at 10 m the corresponding data varied from 0.61 to 0.56. Point spectra did not reveal either remarkable data variations with this regard (not shown). Direct thiolysis revealed more total PC with higher mDPs left in bark than those from extracted tannins (Table 1). The amounts of total PC decreased as function of storage time, which was also the case for extracted PC. The difference between total and extracted PC remained almost constant during aging (Table 1). At 469 days, the amount of total PC were nearly composed of non-extractable compounds, thus the weathering degrades or removes mainly the extractable materials. Benner et al. (1990) reported on similar results. The formation of additional secondary bonds between the molecules (condensation) as reported by Matthews et al. (1997) and Maie et al. (2003) seems of minor relevance. To collect more data with this regard, a bark sample collected after 1 day and at 10 m in height, was kept dry, at room temperature and protected from light for 15 months, and thereafter extracted. The extraction yield was to 85 g kg^{-1} dry bark and the total phenolic yield was 19.5 gECE kg^{-1}, i.e. these data are similar to those of fresh samples (Figure 6 and Table 1). Expectedly, without weathering and leaching there are no marked changes observable.

In this line are also the UMSP analyses of bark chips before and after extraction at 90°C, where no significant variations were detected. A point UV measurement evidenced just a minor decrease of the UV absorbance from unextracted samples (Figure 7). The cell closer to the extraction surface (cork cells and outermost sclereids) showed the most relevant changes. Shen et al. (1986) made similar observations for *Larix gmelinii* [Rupr.] bark and found that the extraction efficacy sharply declined

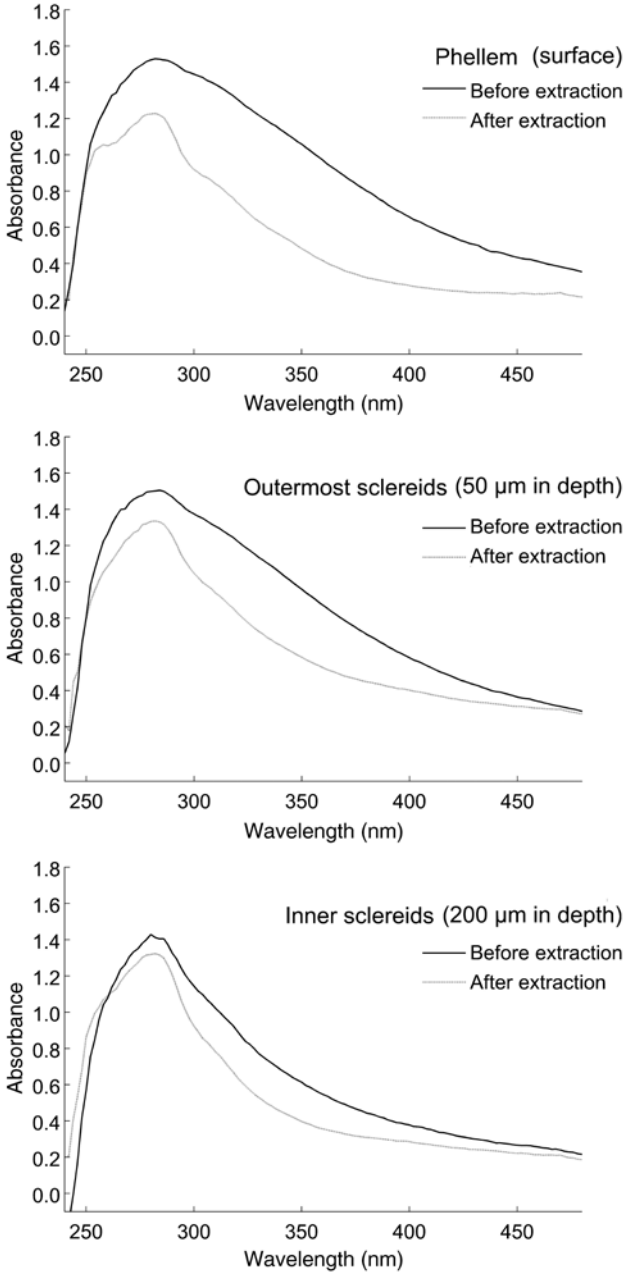

Figure 7: UV absorbance spectra of phenolic deposits before and after extraction in water at 90°C. Measurement performed in bark cells at different depths from the external surface. Each line represents the average absorbance of at least 10 individual point spectra.

moving back from the bark surface towards the inner cells.

Analysis of the extraction process parameters

The effects of extraction temperature (30–90°C), extraction time (2–60 min), addition of sulfites (0–5 g l^{-1}, as sulfite ions), and addition of urea (0–5 g l^{-1}) were studied by means of a face centered cubic statistical design fitted with a full quadratic model (Myers et al. 2009). A total of 24 extractions plus five repetitions of the design central point were performed. Precipitable tannins and total carbohydrate yields were chosen as responses. The choice of the precipitable tannin method was necessary because the Folin-Ciocalteau assay fails in presence of sulfites (Singelton et al. 1999).

Temperature, time and their interaction resulted in very significant factors ($P<0.001$) for both precipitable tannins and total carbohydrates. The addition of sulfites was only marginally significant in the interactions with temperature ($P=0.016$) and time ($P=0.019$). The benefits of sulfite were actually offset by the relevant increase of ashes in the extracts, as already pointed out by Jorge et al. (1999). Addition of urea did not show any significant effect, contrary to what has been reported for other SW barks (Sealy-Fisher and Pizzi 1992).

The fitting surfaces of precipitable tannins and total carbohydrates as a function of time and temperature are presented in Figure 8. As seen, tannins extraction is highly dependent on temperature, in agreement with the results of previous studies (Derkyi et al. 2011; Kemppainen et al. 2014). Total carbohydrates are already removed in a considerable amount at 30°C and their yields increased only slightly with temperature. The extraction temperature seems to be a suitable parameter for the separation of phenolics and carbohydrates in the bark extracts.

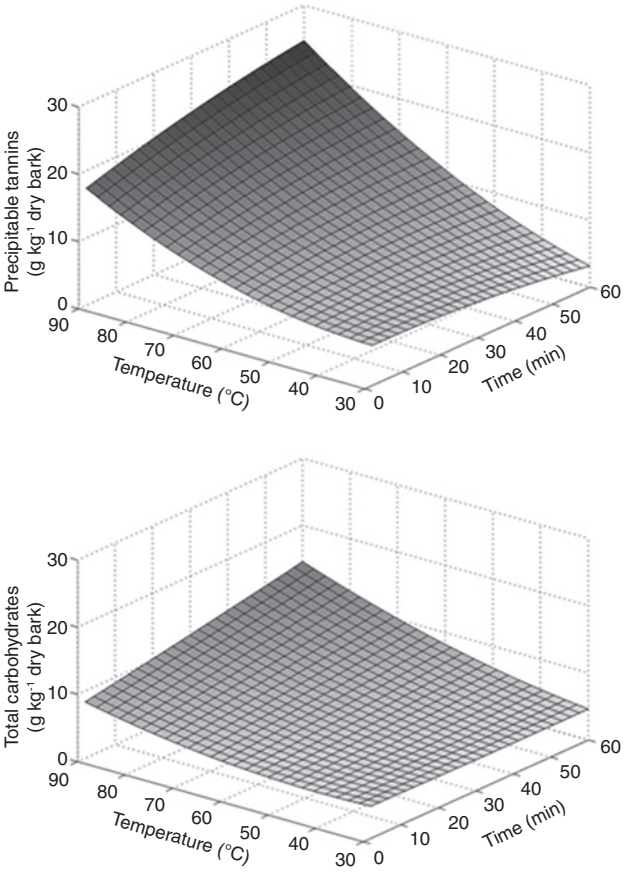

Figure 8: Yield fitting surfaces for precipitable tannins and total carbohydrates as a function of the extraction time and temperature (without addition of sulfites and urea).

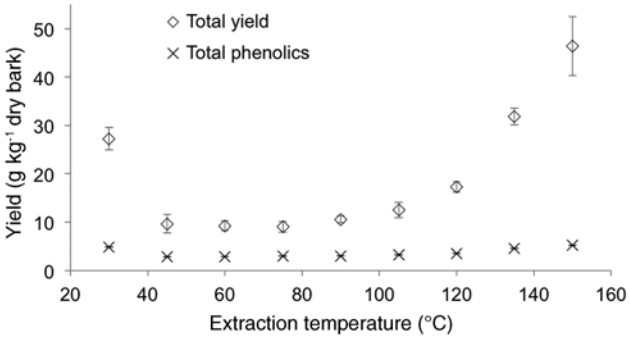

Figure 9: Total extraction and total phenolic yields in sequential extractions of Norway spruce bark at increasing temperatures. Phenolics are expressed as grams of (-)-epicatechin equivalents (gECE) after Folin-Ciocalteau assay.

Sequential extractions at increasing temperature

Norway spruce bark was successively extracted in steps of 15°C, from 30 to 150°C. The total yield at the first extraction step at 30°C was substantially higher than the following steps up to 105°C (Figure 9). Over 105°C, the total yield again remarkably increased. The amount of total phenolics as detected by Folin-Ciocalteau assay remained almost constant along the extraction steps (Figure 9). In the range between 30 and 105°C, the portion of phenolics in the extracts was more relevant, while out of these boundaries a more relevant extraction of carbohydrates occurred. The analysis of the hydrolyzed extracts (Table 2) shows that the total carbohydrate yield at 30 and 150°C is significantly higher than in the steps at 45 and 105°C.

The upsurge of carbohydrate yields over 100°C has been already reported by Kemppainen et al. (2014) and Makino et al. (2009).

The composition of the extracted carbohydrates in the course of the extraction steps (Table 2) shows that the free mono- and oligosaccharides (mostly glucose, fructose and sucrose) were almost completely extracted at 30°C. These components were detected only in traces in the following steps. The prevalence of glucose, galactose and arabinose monomeric residuals within the bound carbohydrates of all samples (Table 2) are indicative for the extraction of typical SW pectin-like polysaccharides like arabinans, arabinogalactans and glucans (Timell 1961) in all steps. At 150°C, a more conspicuous amount of xylose and mannose residuals can be interpreted as the additional extraction of typical SW bark hemicelluloses such as galactoglucomannans and arabinomethylglucuronoxylans (Timell 1961; Dietrich et al. 1978; Le Normand et al. 2012). At 150°C, the substantial amount of free arabinose is likely related to the degradation of some polysaccharides during extraction.

The phenolic fraction also varied in composition within the extraction steps. Stilbene glucosides and taxifolin were observed in HPLC-UV chromatograms of the extracts (not shown) from 30 to 75°C, with decreasing absorbance areas. The relatively higher concentration of bound glucose in the extracts at 30 and 45°C (Table 2) can be thus explained by the presence of stilbene glucosides. It is clear that phenolic monomers and mono- and oligosaccharides can be almost completely removed with cold or warm water, while in the following steps at higher temperature mostly tannin and polysaccharides (mostly pectins) are extracted. PCs were identified after thiolysis in all extraction steps. Their yield decreased almost constantly as a function of extraction temperature (Table 3), in discordance to what was observed for the total phenolics, i.e. in the extracts are more polymers not related to PC. A slight increase of the DP up to 75°C is visible in Table 3, indicating that higher temperatures are needed to extract PC with larger molecular mass. Over 105°C, the PC yield and their DP quickly dropped, while the amount of total phenolics considerably increased. At temperatures higher than 105°C, most of the phenolics in the extracts might be composed of oligomers not accessible to acid-thiolysis. These compounds are either native highly condensed polyphenolics or thermally modified condensed tannins as reported by Sealy-Fisher and Pizzi (1992).

Table 3: Total phenolics and procyanidins extracted from Norway spruce bark in sequential extraction at increasing temperatures.

		Procyanidins	
Temp.	Phen. total gECE kg^{-1a}	g kg^{-1a}	DP
30°C	4.9±0.3	1.6±0.2	5.7±0.9
45°C	2.8±0.2	1.0±0.1	6.3±0.9
75°C	3.0±0.3	0.8±0.1	7.2±1.1
105°C	3.2±0.2	0.8±0.1	6.7±0.9
150°C	5.2±0.3	0.3±0.1	2.6±.5

[a]Dry bark.

Conclusions

The composition of the HW extracts from Norway spruce bark varies considerably during prolonged bark exposure to natural weathering. These changes were mainly linked to the natural leaching of the most extractable compounds like mono- and oligosaccharides, phenolic monomers, and some condensed tannins. The physical and chemical characteristics of the extracts vary as well. A preliminary cold water extraction is able to remove the compounds more responsible for variations observed during bark aging. After HW treatments, the concentration of tannins in the extracts could be higher, but they occur always in combination with polysaccharides (mostly pectins). Extraction temperatures over 100°C are not recommended, due to the relevant amount of polysaccharides (from degradation of hemicelluloses) and highly condensed phenolic oligomers in the product. A shortcoming of the preliminary cold water extraction step is the loss of a considerable portion of tannins, which has to be carefully considered in the economic feasibility analysis of the process. Topochemical analysis of the bark tissues showed the considerable presence of non-extractable and non-leachable phenolic compounds, highlighting the potential of the extracted bark as a substrate rich in phenolic compounds, which are worth to be further valorized.

Acknowledgments: The authors would like to thank the Swiss National Research Program "Resource Wood" (NRP66) for their financial support; the Burgergemeinde Biel for providing the raw bark; Christina Hinterleitner from the Bern University of Applied Science for her great support in the development and implementation of the analytical work; Nicole Erasmy from the University of Hamburg, for her assistance in the carbohydrate analysis; Daniela Paul from Thünen Institute for her help in UMSP measurements.

References

Anttila, A.-K., Pirttilä, A.M., Häggman, H., Harju, A., Venäläinen, M., Haapala, A., Holmbom, B., Julkunen-Tiitto, R. (2013) Condensed conifers tannins as antifungal agents in liquid culture. Holzforschung 67:825–832.

Benner, H., Hatcher, P.G., Hedges, J.I. (1990) Early diagenesis of mangrove leaves in a tropical estuary. Bulk chemical characterization using solidstate ^{13}C NMR and elemental analysis. Geochim. Cosmochim. AC 54:2003–2013.

Bertaud, F., Tapin-Lingua, S., Pizzi, A., Navarrete, P., Petit-Conil, M. (2012) Development of green adhesives for fiberboard manufacturing using tannins and lignin from pulp mill residues. Cell. Chem. Techn. 46:449–455.

Bianchi, S., Gloss, A., Kroslokova, I., Mayer, I., Pichelin, F. (2014) Analysis of the structure of condensed tannins in water extracts from bark tissues of Norway spruce (*Picea abies* [Karst.]) and silver fir (*Abies alba* [Mill.]) using MALDI-TOF mass spectrometry. Ind. Crop Prod. 61:430–437.

Derkyi, N.S.A., Adu-Amankwa, B., Sekyere, D., Darkwa, N.A. (2011) Optimum acetone and ethanol extraction of polyphenols from *Pinus caribea* bark: maximizing tannin content using response surface methodology. Chem. Prod. Process Modelling 6(1), Article 9.

DESTATIS (2014) Land- und Forstwirtschaft, Fisherei. Forstwirtschaftliche Bodennutzung – Holzeinschlagsstatistik – Fachserie 3 Reihe 3.3.1. Statistisches Bundesamt, Wiesbaden, p. 8.

Dietrich, H.H., Garves, K., Behrensdorf, D., Sinner, M. (1978) Untersuchung über die Kohlenhydrate der Rinden einheimischer Holzarten. Holzforschung 32:60–67.

Dix, B., Marutzky, R. (1983) Untersuchungen zur Gewinnung von Polyphenolen aus Nadelholzrinden. Holz Roh Werkst. 41:45–50.

EUROSTAT. Agriculture Forestry and Fishery Statistics. 2014 Edition. European Union, 2015, pp. 141–165.

Foo, L.Y., Karchesy, J.J. (1989) Chemical nature of phlobaphenes. In: Chemistry and Significance of Condensed Tannins. Eds. Hemigway, R.W., Karchesy, J.J. Plenum Press, New York. pp. 109–118.

Garcia, D.E., Glasser, W.G., Pizzi, T.A., Osorio-Madrazo, A., Laborie, M.-P.G. (2014) Synthesis and physicochemical properties of hydroxypropryl tannins from maritime pine bark (*Pinus pinaster* Ait.). Holzforschung 68:411–418.

Garnier, S., Pizzi, A., Vorster, O.C., Halasz, L. (2001) Comparative rheological characteristics of industrial polyflavonoid tannin extracts. J. Appl. Polym. 81:1634–1642.

Goldschmid, O. (1975) Ultraviolet spectra. In: Lignins, Occurrence, Formation, Structure and Reactions. Eds. Sarkanen, K.V., Ludwig, C.H. Wiley Interscience, New York. pp. 241–266.

Haslam, E. (1989) Polyphenols – vegetable tannins. In: Plant Polyphenols: Vegetable Tannins Revisited. University Press, Cambridge, UK. pp. 1–13.

Hillis, W.E. (1985) Occurrence of extractives in wood tissues. In: Biosynthesis and Biodegradation of Wood Components. Ed. Higuchi, T. Academy Press, New York. pp. 209–288.

Howard, E.T. (1971) Bark structure of the southern pines. Wood Sci. 3:134–148.

Jerez, M., Selga, A., Sineiro, J., Torres, J.L., Núñez, M.J. (2007) A comparison between bark extracts from *Pinus pinaster* and *Pinus radiata*: antioxidant activity and procyanidin composition. Food Chem. 100:439–444.

Jorge, F.C., Brito, P., Pepino, L., Portugal, A., Gil, M.H., Irle, M.A., da Costa, R.P. (1999) Improved extraction of pine bark for wood adhesives. Proceedings of the 3rd European Panel Product Symposium, Llandudno, Wales. pp. 301–307.

Kelsey, R.G., Harmon, M.E. (1989) Distribution and variation of extractable total phenols and tannins in the logs of four conifers after 1 year on the ground. Can. J. Forest Res. 19:1030–1036.

Kemppainen, K., Siika-aho, M., Pattathil, S., Giovando, S., Kruus, K. (2014) Spruce bark as an industrial source of condensed tannins and non-cellulosic sugars. Ind. Crop Prod. 52:158–168.

Koch, G., Kleist, G. (2001) Application of Scanning UV microspectrometry to localise lignin and phenolic extractives in plant cell walls. Holzforschung 55:563–567.

König, B., Roffael, E. (2002) Zur reaktivität von Tanninen in Abhängigkeit vom Kambialalter. Holz Roh Werkst. 60:258.

Lacoste, C., Čop, M., Kempainnen, K., Giovando, S., Pizzi, A., Laboire, M.P., Sernek, M., Celzard, A. (2015) Biobased foams from condensed tannin extracts from Norway spruce (*Picea abies*) bark. Ind. Crop. Prod. 73:144–153.

Latva-Mäenpää, H., Laakso, T., Sarjala, T., Wähälä, K., Saranpää, P. (2014) Root neck of Norway spruce as source of bioactive lignans and stilbenes. Holzforschung 68:1–7.

Le Normand, M., Edlund, U., Holmbom, B., Ek, M. (2012) Hot-water extraction and characterization of spruce bark non-cellulosic polysaccharides. Nord. Pulp Paper Res. 27:18–23.

Liiri, O., Sairanen, H., Kilpeläinen, H., Kivistö, A. (1982) Bark extractives from spruce as constituents of plywood bonding agents. Holz Roh Werkst. 40:51–60.

Maie, N., Behrens, A., Knicker, H., Knaber-Kögel, I. (2003) Changes in the structure and protein binding ability of condensed tannins during decomposition of fresh needles and leaves. Soil Biol. Biochem. 35:577–589.

Makino, R., Ohara, S., Hashida, K. (2009) Efficient extraction of polyphenolics from the bark of tropical tree species. J. Trop. For. Sci. 21:45–49.

Makino, R., Ohara, S., Hashida, K. (2011) Radical scavenging characteristics of condensed tannins from bark of various tree species compared with quebracho wood tannin. Holzforschung 65:651–657.

Manns, D., Deutschle, A.L., Saake, B., Meyer, A.S. (2014) Methodology for quantitative determination of the carbohydrate composition of brown seaweeds (Laminariacee). RSC Advanced, 4:25736–25746.

Matthews, S.; Mila, I.; Scalbert, A.; Donnelly, D.M.X. (1997) Extractable and non-extractable proanthocyanidins in barks. Phytochemistry 45:405–410.

Musha, Y., Goring, D.A.I. (1975) Distribution of syringyl and guaiacyl moieties in hardwoods as indicated by ultraviolet microscopy. Wood Sci. Technol. 9:45–58.

Myers, R.H., Montgomery, D.C., Anderson-Cook, C.M. Response surface methodology: Process and Product Optimization Using Designed Experiments. 3rd Edition. John Wiley & Sons, Hoboken, NJ, 2009.

Pizzi, A. (2008) Tannins: major sources, properties and applications. In: Monomers, Polymers and Composites from Renewa-

ble Sources. Eds. Belgacem, M.N., Gandini, A. Elsevier, Oxford. pp. 179–199.

Porter, L.J. (1989) Condensed tannins. In: Natural Products of Woody Plants. Ed. Rowe, J.W. Springler-Verlag, Berlin, Germany. vol. 1. pp. 651–690.

Prislan, P., Koch, G., Schmitt, U., Gričar, J., Čufar, K. (2012) Cellular and topochemical characteristics of secondary changes in bark tissues of beech (*Fagus sylvatica*). Holzforschung 66:131–138.

Roffael, E., Dix, B., Okum, J. (2000) Use of spruce tannin as binder in particleboards and medium density fiberboards (MDF). Holz Roh Werkst. 58:301–305.

Sarneckis, C.J., Dambergs, R.G., Jones, P., Mercurio, M., Herderich, M.J., Smith, P.A. (2006) Quantification of condensed tannins by precipitation with methyl cellulose: development and validation of an optimised tool for grape and wine analysis. Aust. J. Grape Wine R. 12:39–49.

Schofield, J.A., Hagerman, A.E., Harold, A. (1988) Loss of tannins and other phenolics from willow leaf litter. J. Chem. Ecol. 24:1409–1421.

Sealy-Fisher, V.J., Pizzi, A. (1992) Increased pine tannins extraction and wood adhesives development by phlobaphenes minimization. Holz Roh Werkst. 50:212–220.

Seki, K., Saito, N., Aoyama, M. (1997) Removal of heavy metal ions from solutions by coniferous barks. Wood Sci. Technol. 31:441–447.

Shen, Z., Haslam, E., Falshaw, C.P., Begley, M.J. (1986) Procyanidins and polyphenols of Larix gmelini bark. Phytochemistry 25:2696–2635.

Singelton, V.L., Orthofer, R., Lamuela-Raventos, R.M. (1999) Analysis of total phenols and other oxid ation substrates and antioxidants by means of folin-ciocalteau reagent. Method Enzymol. 299:152–178.

Steenkampf, J.A., Steynberg, J.P., Brandt, E.V., Ferreira, D. Roux, D.G. (1985) Phlobatannins, a novel class of ring-isomerized condensed tannins. J. Chem. Soc., Chem. Comm. 1678.

Sun, B., Leandro, C., da Silva, J.M.R., Spranger, I. (1998) Separation of grape and wine proanthocyanidins according to their degree of polymerization. J. Agr. Food Chem. 46:1390–1396.

Telysheva, G., Dizhbite, T., Bikovens, O., Ponomarenko, J., Janceva, S., Krasilnikova, J. (2011) Structure and antioxidant activity of diarylheptanoids extracted from bark of grey alder (*Alnus incana*) and potential of biorefinery-based bark processing of European trees. Holzforschung 65:623–629.

Timell, T.E. (1961) Isolation of polysaccharides from the bark of gymnosperms. Sven. Papperstidn. 64:651–661.

Vázquez, G., González-Alvarez, J., Freire, S., López-Suevos, F., Antorrena, G. (2001) Characteristics of *Pinus pinaster* bark extracts obtained under various extraction conditions. Holz Roh Werkst. 59:451–456.

Weissman, G. (1981) Untersuchung der Rinderextrakte von *Picea abies* Karst. Holz Roh Werkst. 39:457–461.

Weissman, G. (1983) Studies on pine bark extracts. Int. J. Adhes. Adhes. 3:31–35.

Weissman, G. (1984) Zusammensetzung der wässrigen Rindenextrakte von *Picea abies* [Karst.] im Verlauf einer Vegetationperiode. Holzforschung 38:7–10.

Weissman, G. (1985) Untersuchung der Rinderextrakte von Lärchen. Holzforsch. Holzwert. 37:67–71.

Willför, S., Pranovich, A., Tamminen, T., Puls, J., Laine, C., Suurnäkki, A., Saake, B., Uotila, K., Simolin, H., Hemming, J., Holmbom, B. (2009) Carbohydrate analysis of plant materials with uronic acid-containing polysaccharides – A comparison between different hydrolysis and subsequent chromatographic analytical techniques. Ind. Crop Prod. 29:571–581.

Yazaki, Y., Collins, P.J. (1994) Wood adhesives based on tannin extracts from barks of some pine and spruce species. Holz Roh Werkst. 52:307–310.

Zhang, L., Gellerstedt, G. (2008) 2D heteronuclear (^{1}H-^{13}C) single quantum correlation (HSQC) NMR analysis of norway spruce bark components. In: Characterization of Lignocellulosic Materials. Ed. Hu, T.Q. Blackwell Publishing Ltd., Oxford, UK. pp. 3–12.