Annals of Forest Science (2020) 77: 9
https://doi.org/10.1007/s13595-019-0916-x

RESEARCH PAPER

Quantitative and qualitative composition of bark polyphenols changes longitudinally with bark maturity in *Abies alba* Mill.

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Received: 13 August 2019 / Accepted: 23 December 2019 / Published online: 10 January 2020

Abstract

• ***Key message*** **Total bark extractive content increases at positions higher in the trunk of *Abies alba* Mill. trees. The greatest proportions of bark polyphenolic extractives are found in the lower section of the trunk, below the crown.**
• ***Context*** The bark of commercially grown softwood trees is a potentially valuable source of secondary metabolites including polyphenols such as tannins, used in the manufacture of adhesives and resins. There is little information about how the yield and composition of bark extracts vary longitudinally within trees and with respect to the presence or absence of branches.
• ***Aims*** We examined the variability of bark secondary metabolites in the softwood *Abies alba* both longitudinally within trees and among trees at specific sample heights. The aim was to determine whether specific bark fractions within this species contain more extractable secondary metabolites than others.
• ***Methods*** Eight trees of *A. alba* were harvested, and up to 13 discs were cut along the trunk from 30 cm above the ground to where the trunk was only 10 cm in diameter. Milled bark was extracted with water:ethanol (1:1) using an accelerated solvent extractor and the dry yield calculated. Extract composition was examined by liquid chromatography followed by mass spectrometry.
• ***Results*** Total extract yield increased from the base of the tree towards the top. The yield of the most abundant polyphenolic compounds decreased from the base of the tree towards the top, indicating the total extracts included compounds that were not detectable with the chromatographic method used.

Handling Editor: Leo Liu

Contribution of the co-authors The study was conceived of and designed by PG, FC and SD. Extract yields were measured by SC and MB. Mass spectrometry was done by CF and SD. NMR was done by SC and SD. MB did the data analysis and wrote the paper. PG and FC reviewed and commented on the paper. All authors have read and approved the final manuscript.

Data Deposition Information INCLUDES WEBLINK: https://urldefense.proofpoint.com/v2/url?u=http-3A__doi.org_10.7910_DVN_8C37DJ&d=DwIGaQ&c=vh6FgFnduejNhPPD0fl_yRaSfZy8CWbWnIf4XJhSqx8&r=c1RA_h1j7FQIIQH-dRZ3C0QCylXQl0RGQR7JUFl8yQQ&m=vVurUfxmz6YElpfji8HLuVbOnF7YB7oz8tjw_kKxAWQ&s=69XLdzLC6jcIi9itEkgrYtOW1bUKyYUWOEYQxZ5e1ls&e=

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• ***Conclusion*** Although extract yield is highest at the top of the tree, the composition of the extracts indicates that compounds with known marketable value, and present in detectable proportions, are found at the base of the tree.

Keywords Bark extractives · Catechin · LC-MS · Silver fir · Tannin · Within-tree variability

1 Introduction

Bark is the second most valuable product from forestry after wood, comprising 10 to 15% of stem volume. Bark is composed of all the tissues surrounding the vascular cambium and comprises the secondary phloem (inner bark) and the periderm (outer bark). The bulk of secondary metabolites in bark is formed in the polyphenolic parenchyma cells in the secondary phloem which is the layer directly surrounding the vascular cambium. A layer of these cells forms each season, and they remain viable even in the oldest (mature) bark layers (Krekling et al. 2000). As well as polyphenolic compounds, these cells also contain starch and lipids. Surrounding the secondary phloem is the periderm which includes the outermost layer covering the trunk. The greatest proportion of outer bark is found at the base of the trunk, which contains the bulk of mature bark, whereas the youngest (most recently formed) bark with the greatest proportion of inner bark is found at the top of the tree (Eberhardt 2013).

Valuable secondary metabolites have been found in bark extracts, with as many as 237 individual compounds identified (Jablonsky et al. 2017). Such compounds can be extracted using hot water or organic solvents and include tannins which form the major proportion of bark extractives, as well as lipophilic compounds and polysaccharides. Tannins are easily extracted with polar solvents such as water or water:ethanol mixtures and may constitute 15 to 30% of dry bark biomass in softwoods. Tannins are utilized in the manufacture of adhesives, resins and insulating foams (Feng et al. 2013; García et al. 2014; Lacoste et al. 2015). In Europe, tannins are typically sourced from exotic species such as the bark of black wattle (*Acacia mearnsii* De Wild.) or the heartwood of Quebracho (*Schinopsis lorentzii* Griseb.). Recently, bark of European softwoods including silver fir (*Abies alba* Mill.) has been shown to be potentially valuable source of tannins (Chupin et al. 2013; Bianchi et al. 2014; García et al. 2014; Kemppainen et al. 2014; Lacoste et al. 2015). In addition to the chemical manufacturing uses cited above, bark extracts including those from *A. alba* can be used as antioxidant nutraceuticals or find application in removing copper from polluted waters (Vasincu et al. 2013; Benković et al. 2014; Tofan et al. 2016). Factors preventing an efficient exploitation of tannins in European-grown softwood barks include a high demand for bark from other sectors such as the energy or horticultural sectors but also insufficient information about the availability, quality and biodiversity of tannins and other polyphenolics in these species.

Plant tannins are classified as either "hydrolysable" tannins, which are esters of gallic acid or ellagic acid, or "condensed" tannins which are also known as proanthocyanidins. Proanthocyanidin tannins are oligomers of flavan-3-ol units synthesized by the flavonoid pathway, and their biosynthesis can therefore be expected to be limited by the availability of photoassimilates (He et al. 2008) which may depend on canopy size, for example. The flavan-3-ols are first derivatized to epicatechin and catechin, then oxidized to quinones and polymerized (He et al. 2008; Vogt 2010). Prodelphinidin tannins are polymers of gallocatechin. It is unknown whether this polymerization is enzymatic or non-enzymatic; the degree of polymerization is typically between 2 and 17. The tannins of conifers have a lower degree of polymerization than angiosperms (Hernes and Hedges 2004). Condensed tannins have a high proportion of hydroxyl groups due to their phenolic constituents, which confer a high degree of reactivity towards compounds such as proteins, polysaccharides and aldehydes. Reactivity of the hydroxyl groups with formaldehyde makes condensed tannins particularly suitable for the efficient manufacture of adhesives, resins and foams (Pizzi and Stephanou 1994; Garnier et al. 2002). Identification of bark fractions containing high proportions of condensed tannins and tannins with high degrees of polymerization will facilitate the industrial valorization of bark co-products within the forest products supply chain.

There is some evidence that bark extractive yield, including polyphenols, depends on the height of the sample, tree age and tissue maturity (Stringer and Olson 1987; Vedernikov et al. 2011; Rizhikovs et al. 2015). If this variability actually occurs, it is important to quantify the availability and diversity of these compounds to determine which bark fractions within the tree are best suited for use in specific chemical industries. The supply chain has a hierarchical value structure, solid wood first, followed by industrial wood and finally wood for energy. The trunk segment (position within the tree), tree age and tree quality all influence the ultimate destination of the trunk segments. When considering softwood bark, young bark with a large proportion of secondary phloem can be found either on young trees and cut during thinning in young stands or on the upper logs recovered above the log dedicated to solid wood valorizations. They can be harvested before wood is processed in pulp and paper or panel plants. Conversely, old bark with a large proportion of outermost layers can be found at the base of logs from older trees dedicated to solid wood valorization. They can also be retrieved before wood processing in sawmills or in plants where slicing or rotary cuttings are operated.

Depending on the industrial process and the wood processed, different destinations of the bark co-products can be thus envisaged if the chemical compounds are different. If the composition does not vary in the different fractions of bark, a bulk collection of bark without any sorting is suitable.

Here, we tested the following hypotheses: (1) that there is little variation in the yield of bark extracts among *A. alba* trees from bark samples at the same positions within the stem, (2) that conversely, significant longitudinal (within-tree) variation exists in both the yield and composition of these bark extracts and (3) that different thinning management types could affect the yield and composition of bark extractives from these trees. To this end, we extracted bark samples and measured their yield as a proportion of dry bark mass from up to 13 heights from each of eight trees of *A. alba.* Water:ethanol (50:50, *v*/v) and successive extractions using pure organic solvents of decreasing polarity were used for extraction. The compositions of the water:ethanol extracts were examined using ultraviolet (UV) liquid chromatography followed by mass-spectroscopy (LC-MS) to identify and quantify which compounds were present in the extracts. UV-LC-MS was specifically chosen as tannins are UV-visible and are known to be particularly valuable components of softwood barks (Chupin et al. 2013; Bianchi et al. 2014; García et al. 2014; Kemppainen et al. 2014; Lacoste et al. 2015).

2 Material and methods

2.1 Tree growth and disc sampling

Trees of *A. alba* were grown in the Saint-Prix forest, a mixed stand with Norway spruce (*Picea abies* (L.) H.Karst.) and some broadleaves, at latitude north 46°59,550′, longitude east 4°3413′, elevation 750 m. Two silvicultural treatments were sampled: un-thinned and dynamic. Trees in the un-thinned stand had a mean radial growth of 2.5 mm per year between 1991 and 2011 and had a final stand density of 3233 stems ha^{-1} by the end of 2011. The dynamic treatment included two thinning events in 1991 and in 2009, and trees had a mean radial growth of 4.0 mm per year between 1991 and 2011. The un-thinned stand had a final stem density of 591 stems ha^{-1}.

From eight trees, up to 13 discs ~10 cm thick were harvested along the longitudinal axis, from the base (30 cm above ground) to the limit of wood suitable for industrial use as defined below. Three discs were harvested at 30, 80 and 130 cm above the ground for all trees, whereas the other samples were harvested according to chosen physiological and industry-relevant criteria. These were at the height where the diameter was 10 cm (the limit of wood used for industrial purposes such as panels, pulp and paper or energy purposes), midway between that height and 130 cm, at the height where the trunk diameter was 20 cm (the limit of wood used for construction), at the lowest green branch and at the base of the crown (i.e. the lowest whorl where ¾ of the branches are living). For these latter samples, discs were harvested both with branches and also at 10 cm above these positions (to give corresponding samples without branches). The heights of all samples taken for each tree, total tree height, age and management type are shown in Table 1.

2.2 Grinding, extraction and quantification

Bark from each disc was dissected into three aliquots, and each aliquot milled in a Fritsch pulverisette 9 mill (Fritsch, Idar-Oberstein, Germany) for 90 s at 1100 rpm. Milled bark was then dried at 103°C until constant weight (8 h). Triplicate samples of 2.00 g (accuracy ± 0.01 g) were prepared for subsequent extraction. The samples were extracted with water:ethanol (50:50, *v*/v) using a Thermo Scientific™ ASE™ 350 Accelerated Solvent Extractor. Each sample was transferred to a 34 ml stainless steel cylinder with 5 g of Fontainebleau sand over a cellulose filter paper (Thermo Scientific™ 056780). During extraction each cylinder was heated to 100°C and extracted three times at 1600 psi for 5 minutes each time, with a 60% fresh volume of water:ethanol (50:50, *v*/v) between each cycle. The extract was quantitatively transferred into a flask of known weight (± 0.0001 g) and the ethanol removed by rotary evaporation. Extracts were then freeze-dried (Martin Christ, Germany) and the flask weighed again. The yield was calculated as a dry weight percentage of the 2.00 g ground bark sample. A further sample of milled, dried bark (one 2.00 g sample) from each disc was also extracted successively with hexane (≥ 99.5%), followed by acetone (≥ 99.5%) and finally toluene:ethanol (50:50 *v*/v) (≥ 99.5%) in the ASE extractor three times each as above. The yield of each successive extraction was calculated as above.

2.3 LC-MS quantification and identification of compounds

The composition of polyphenolic compounds in the water:ethanol extracts was investigated using liquid chromatography followed by mass spectroscopy (LC-MS). To 10 mg of freeze-dried extract, 1 ml of water:ethanol (50:50, *v*/v) was added in a 1.5 ml clear glass autosampler vial (VWR, Radnor, PA, USA). Samples were solubilized in an ultrasonic bath (Decon FS100 Sonificator, Sussex, UK) for 2 min. Liquid chromatography was done with a Shimadzu LC-20A ultra-HPLC system (Shimadzu, Kyoto, Japan) equipped with an autosampler and interfaced with an SPD-20A photodiode-array (PDA) UV detector. Separation was done using a Luna C18 analytical column (i.d. 150 mm × 3 mm, Phenomenex, Torrance, CA, USA). The injection volume was 5 μl, the flow rate was 0.4 ml min^{-1}, and the gradient profile was the following: 0.0 to 10.0 min 5% (*v*/v) acetonitrile (≥ 99.9%, LC-MS

Table 1 Height (cm above the ground) of discs sampled within each tree and management type of the stand

Disc[a]	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	Tree 6	Tree 7	Tree 8
30 cm	30	30	30	30	30	30	30	30
80 cm	80	80	80	80	80	80	80	80
130 cm	130	130	130	130	130	130	130	130
MILAB	480		265		600	697	435	685
MILSB	490	330	277		605		445	
DEC20AB			465			1350	870	1318
DEC20SB	955		479			1365	885	1340
H1BVAB	1130	1136	950	947	720	495	515	703
H1BVSB	1140	1150	965	958	732	505	525	713
HBHAB	1365	1350	1095		780	795	765	890
HBHSB	1375	1361	1107		793	800	775	905
DEC10AB	1685	617	1509		1147	1745	1540	1720
DEC10SB	1705	629	1523		1154	1765	1525	1740
Total height	2240	1575	2050	1360	1699	2140	2010	2120
Management[b]	U	U	U	U	D	D	D	D
Age	50	44	51	57	48	43	43	44
DBH[c]	28.0	13.9	22.6	10.4	19.6	41.2	29.5	38.9

[a] 30 cm = 30 cm above ground; 80 cm = 80 cm above ground; 130 cm = 130 cm above ground; MILAB = mid-height between 130 and DEC10AB, with branches; MILSB = 10 cm above MILAB, without branches; DEC20AB = limit of wood for use in construction, with branches; DEC20SB = 10 cm above DEC20AB, without branches; H1BVAB = lowest green branch; H1BVSB = 10 cm above H1BVAB, without branches; HBHAB = base of the crown; HBHSB = 10 cm above HBHAB, without branches; DEC10AB = limit of wood for industrial use, with branches; DEC10SB = 10 cm above DEC10AB, without branches

[b] *U* un-thinned management; *D* dynamic management

[c] *DBH* Diameter at breast height (130 cm)

Chromasolv™) in water (LC-MS Chromasolv™), 10.0 to 18.0 min 30% acetonitrile, 18.0 to 19.0 min 80% acetonitrile and 19.0 to 20.0 min 5% acetonitrile containing 0.1% (v/v) formic acid. The UV-visible spectra were recorded between 190 and 800 nm. Mass spectrometry was done with a Shimadzu LC-MS 8030 triple quadrupole mass spectrometer. Positive and negative ion electrospray mass spectrometric analyses were carried out at a unit resolution between 100 and 2000 m/z at a scan speed of 15,000 U/s. The heatblock and desolvation line temperatures were 400°C and 250°C, respectively. Nitrogen was used as the drying (15 l min^{-1}) and nebulizing (3 l min^{-1}) gas. The ion spray voltage was ± 4500 V. Data were acquired and analysed with LabSolutions software (5.42SP4, Shimadzu). Identification was done by comparing peak retention times and UV-MS spectra to the literature and to standard solutions of (+)-catechin hydrate (98% HPLC, Sigma-Aldrich) and (−)-epicatechin (90%, Sigma Aldrich). Chromatogram peaks visible at 280 nm (phenolic absorption maximum) were numbered and defined according to their mass spectra. Each peak had characteristic ions or fragments which were used for identification, although the ions differed depending on adducts (sodium, glycosyl, etc.). Intensities of the ions varied among samples due to the presence of oligomeric catechins which caused an uneven baseline. Some peaks had more than one compound which co-eluted due to similar polarity, indicated by multiple ions characteristic of different compounds. Potential fragmentation induced by the ESI source has been excluded as the cause of the multiple ions. Numbered peaks, their retention times and characteristic ions in both positive and negative mode used for identification are given in Table 3. Oligomers (of three or more units) do not resolve well using this method, causing a rising baseline (Mueller-Harvey et al. 1987; Cadahia et al. 1997); oligomers were therefore quantified by integrating the total area under the baseline. The quantity (mg) of polyphenolic compounds represented by each peak was expressed in catechin equivalent weights and is therefore relative quantities rather than specific to each individual compound. All peak areas were within the linear range for quantification by this method. Catechin equivalents were calculated by dividing the mean peak areas of known amounts of the catechin and epicatechin standards above and dividing that value by the area of each peak in the chromatogram. This was then converted to a yield of mg per g of dry bark.

2.4 Nuclear magnetic resonance spectroscopy (^{13}C NMR)

As it became clear later in the study that the proportions of polyphenolic compounds did not necessarily represent the bulk of the extractives, nuclear magnetic resonance spectroscopy was used to analyse extracts for evidence of other types of compounds. Four water:ethanol extracts were chosen for structural analysis by NMR. These were from tree 2 and 6 (see Table 1), each at 130 cm high. Solid state cross-polarization/magic angle spinning (CP/MAS) ^{13}C NMR spectra were recorded directly on 250 mg of freeze-dried extracts packed in a 4 mm diameter ZrO_2 rotor using a Bruker MSL 300

spectrometer at 75.47 MHz. Acquisition time was 0.04 s, with number of transients 2048. All samples were run with a relaxation delay of 5 s, CP time of 1 ms and spectral width of 39,663 Hz. Spinning rate was 10 KHz. Interferograms were processed with TopSpin 3.6.2 using an apodization value of 21 Hz. Chemical shifts are parts per million (ppm).

2.5 Statistical analyses

Statistical analyses were done using R (R Development Core Team. 2008). All data are available online (Brennan et al. 2019). To investigate whether there were significant differences in yield among trees at each of the different disc types, an analysis of variance was used to test whether tree had a significant influence on yield. Where the effect of tree was significant, a post hoc Tukey's HSD test was used to determine which trees were significantly different from each other. To examine the relationship between yield and height across all trees, a linear model was built with the mean yield per disc as the response variable, with % total height or the second-degree polynomial of % total height and management type as predictor variables, with non-significant terms being iteratively dropped until the minimally adequate model was obtained. Inclusion of tree as a random effect was then tested in the model. To examine the compositional changes of the extracts, peak area was used to calculate yield (mg g^{-1} of bark), which was then used as the response variable. Linear models were built with the yield of each compound as the response variable with % total height, tree and presence or absence of branches as the predictor variables; the interaction with tree and height was also tested. Non-significant variables were dropped from the model until the minimally adequate model was obtained.

3 Results

3.1 Variation in yield of water:ethanol extracts

There were significant differences in yield among trees for discs at all heights sampled, except those at 80 cm above the ground, at mid-height without branches, or just above the crown base without branches Table 2. However, among the eight trees, often only one or two were different from each other.

Management type was not significant ($p < 0.9$) and was therefore not included in the final minimally adequate model, in which only the second-degree polynomial of % total height was a significant predictor variable ($p < 0.05$) as shown in Fig. 1. The points of samples with and without branches or samples grown under different management regimes are scattered throughout the plot indicating that there was no measurable effect of branch presence or management on extractive yield. When including the variable "tree" as a random effect, there was only minimal improvement in the model (AIC = 1295 without the random variable, AIC = 1290 with the random variable). Therefore although the form of relationship changed slightly among individual trees, overall the relationship of yield with height was reasonably consistent.

3.2 Successive extractions

Samples were successively extracted with solvents increasing in polarity. The yield from the first extraction with hexane had a significant linear increase with sample height, and the unthinned management programme significantly increased the mean yield by 1.7% over the dynamic management programme as shown in Fig. 2a. Neither sample height nor the management programme had a significant influence on the yield of the second extraction with acetone (Fig. 2b), the final extraction with toluene:ethanol (Fig. 2c) or all extract yields from the summed successive extractions (Fig. 2d).

3.3 Composition of extractives

A total of 12 peaks were quantified as indicated in the chromatogram shown in Fig. 3. The retention times and tentative peak assignments are shown in Table 3. Of the 12 peaks, almost all had a significant interaction between their yield and the second degree polynomial of % total height and tree, the polynomial relationship being more evident for some trees than others. The exceptions were a quercetin glycoside where the interaction was not significant but the variables were significant as main effects, isorhamnetin where only the tree effect was significant and peak 8 which had no significant variables influencing yield. Isorhamnetin glucoside and catechin additionally had the presence or absence of branches as a significant effect, where the presence of branches decreased yield for both peaks. The significant interaction between the second degree polynomial of % total height and tree meant that the form of the relationship with height changed among trees for peaks 1 (Fig. 4a), 2 (a dimer of gallocatechin), gallocatechin, gallocatechin gallate, epigallocatechin, peaks 7 (Fig. 4b) and 9 (a quercetin glycoside). The yield of a quercetin glycoside (peak 12) was significantly influenced by tree and % total height (Fig. 4c). There was also a significant interaction between % total height and tree on the yield of catechin oligomers (Fig. 4d), caused by trees 1 and 2 having only a slight increase in yield with tree height, compared to trees 5 and 8.

Not every tree had the same relationship between peak yield and sample height, which was also the case of total extract yield. However, the form of relationship between extract yield and height for each tree (Fig. 1) was not followed by individual peak yields and height (not shown). This discrepancy between increasing extract yield with increasing sample height, but decreasing polyphenolic content, indicates

Table 2 Differences[a] in water:ethanol extraction yield[b] among trees for each disc sampled

Disc[c]	Branch	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	Tree 6	Tree 7	Tree 8
30 cm	Without	17.38 ± 0.66 abc	20.90 ± 0.21 d	17.28 ± 0.42 abc	18.02 ± 0.68 bd	18.25 ± 1.82 cd	14.83 ± 0.58 a	15.13 ± 1.25 ab	17.88 ± 1.61 ad
80 cm	Without	18.30 ± 0.64	18.87 ± 1.03	18.08 ± 0.63	19.50 ± 1.50	18.70 ± 0.05	17.03 ± 1.84	17.28 ± 0.85	19.10 ± 1.05
130 cm	Without	18.27 ± 0.87 a	18.20 ± 0.91 a	19.78 ± 0.39 ab	20.45 ± 1.78 ab	16.45 ± 1.98 a	18.48 ± 1.79 a	20.17 ± 2.47 ab	23.83 ± 0.76 b
MILAB	With	22.25 ± 0.48 ac		21.38 ± 0.34 a		23.93 ± 0.64 bc	22.00 ± 0.61 ab	24.10 ± 1.19 c	27.20 ± 0.83 d
MILSB	Without	20.95 ± 3.67	18.77 ± 1.51	20.38 ± 0.11		22.43 ± 2.06		22.98 ± 2.37	
DEC20AB	With			23.83 ± 2.37 a			31.53 ± 1.38 b	23.75 ± 0.35 a	20.23 ± 1.00 a
DEC20SB	Without	17.60 ± 0.26 a		19.67 ± 0.29 ab			29.25 ± 1.06 d	20.90 ± 0.35 b	23.77 ± 1.45 c
H1BVAB	With	27.80 ± 0.60 a	21.78 ± 3.18 bc	21.98 ± 0.79 bc	25.42 ± 1.12ab	21.85 ± 0.79 bc	22.03 ± 1.69 bc	22.23 ± 1.63 bc	18.25 ± 1.74 c
H1BVSB	Without	22.58 ± 1.46 a	31.29 ± 2.67 b	20.37 ± 0.12 a	22.65 ± 1.15 a	21.00 ± 1.00 a	22.30 ± 2.61 a	20.25 ± 2.47 a	19.53 ± 0.91 a
HBHAB	With	26.00 ± 0.87 a	22.20 ± 1.98 ab	21.98 ± 0.38 bc		20.33 ± 1.88 bd	25.23 ± 0.25 ac	22.42 ± 0.38 ab	17.60 ± 2.16 d
HBHSB	Without	23.68 ± 0.71	29.38 ± 1.59	22.00 ± 2.44		23.07 ± 5.31	25.23 ± 0.87	26.98 ± 2.07	21.20 ± 1.34
DEC10AB	With	23.90 ± 1.10 ab	22.25 ± 0.90 bcd	25.55 ± 2.09 ac		23.93 ± 0.64 ab	20.12 ± 0.78 d	21.72 ± 1.14 bd	26.22 ± 1.75 a
DEC10SB	Without	26.93 ± 0.49 a	20.90 ± 0.84 b	12.08 ± 0.51 c		20.07 ± 1.72 b	20.67 ± 1.76 b	21.83 ± 0.67 b	28.38 ± 0.54 a

[a] Trees that do not share a letter are significantly different from one another as determined by Tukey's HSD test after an ANOVA showed that tree had a significant effect on yield for that disc. Samples without letters indicate ANOVA showed that tree had no effect on yield

[b] Yield is expressed as % by weight ± the standard deviation. $N = 3$

[c] 30 cm = 30 cm above ground; 80 cm = 80 cm above ground; 130 cm = 130 cm above ground; MILAB = mid-height of the tree, with branches; MILSB = 10 cm above MILAB, without branches; DEC20AB = limit of wood for construction use, with branches; DEC20SB = 10 cm above DEC20AB, without branches; H1BVAB = first green branch above the ground; H1BVSB = 10 cm above H1BVAB, without branches; HBHAB = base of the crown; HBHSB = 10 cm above HBHAB, without branches; DEC10AB = limit of wood for industrial use, with branches; DEC10SB = 10 cm above DEC10AB, without branches

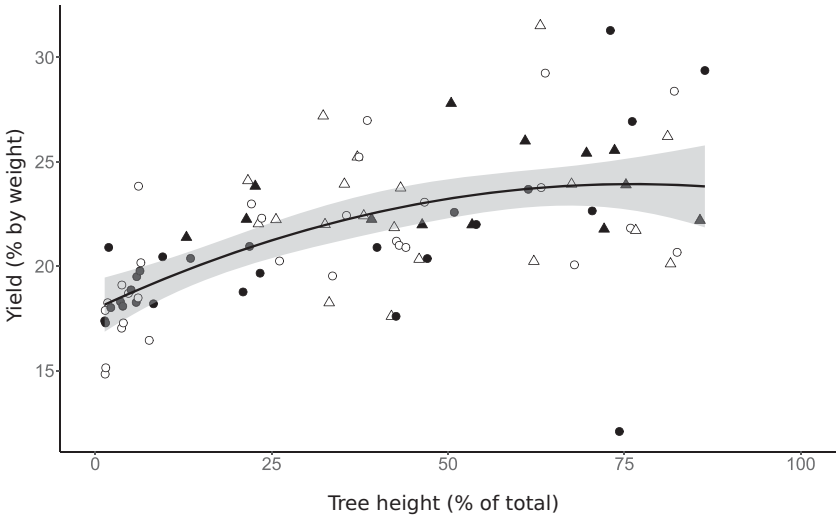

Fig. 1 Relationship between height of bark sampled and water:ethanol extractive yield across all eight trees. Filled circles = samples from the un-thinned stand without branches; open circles = dynamic management samples without branches; filled triangles = samples from the un-thinned stand with branches; open triangles = dynamic management samples with branches. The fitted line (yield = 17.951 + 0.157(% height) – 0.001(% height^2)) is shown in black, with the 95% confidence interval of the fitted line in grey shading

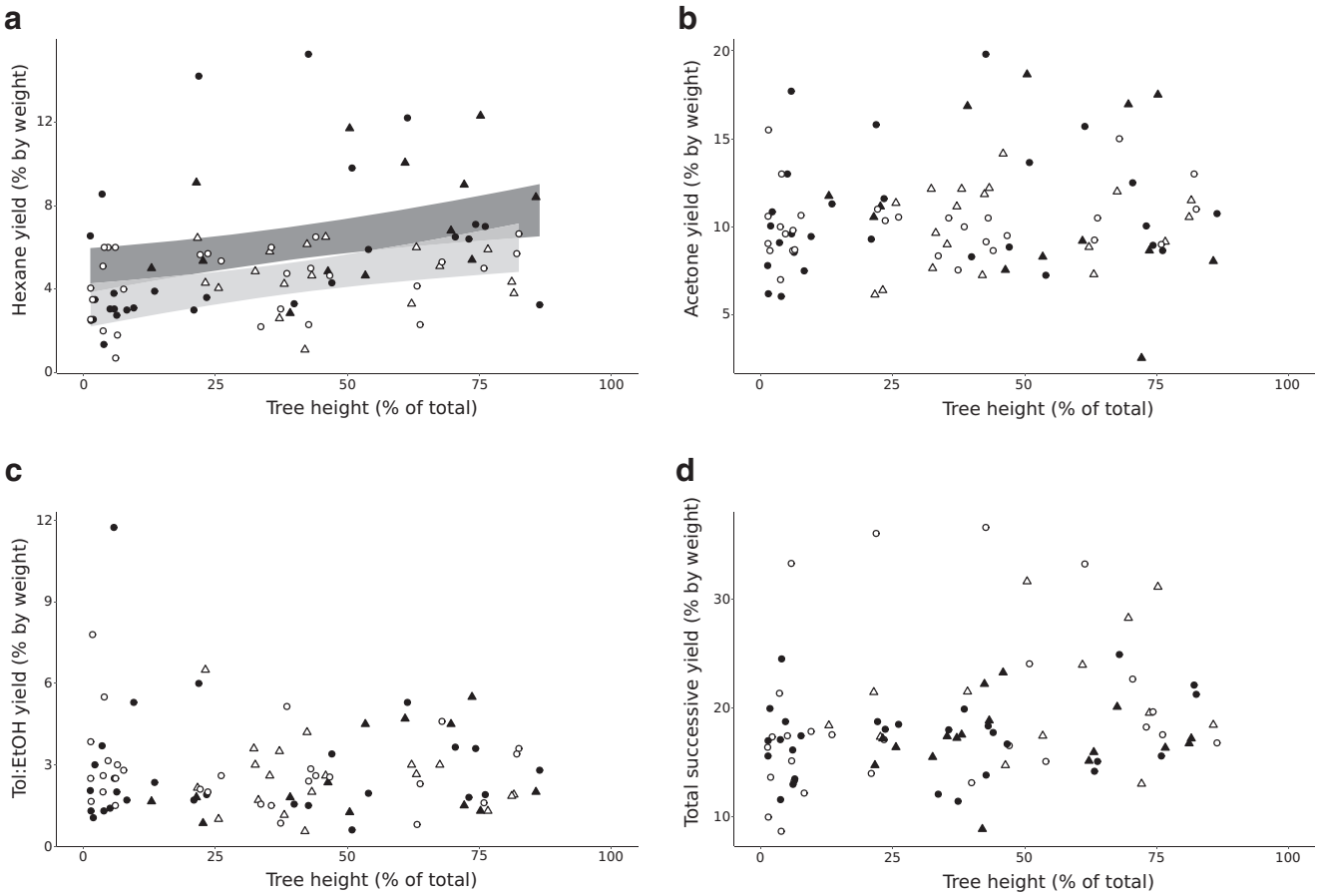

Fig. 2 Relationships between yield of extracts following successive extractions with solvents of increasing polarity. (a) There was a significant relationship between the yield of hexane extracts and sample height; the un-thinned management (yield = 3.20 + 0.03(% height), 95% confidence interval shown in dark grey) increased the mean yield by 1.7% over the dynamic management (yield = 4.86 + 0.03(% height), 95% confidence interval shown in light grey). (b) Acetone yield did not significantly increase with tree height. (c) Yield of toluene:ethanol extracts did not significantly increase with tree height. (d) The sum total of extracts from the successive extractions did not significantly increase with tree height. Filled circles = samples from the un-thinned stand without branches; open circles = dynamic management samples without branches; filled triangles = samples from the un-thinned stand with branches; open triangles = dynamic management samples with branches. The 95% confidence interval of the fitted model is shown in grey shading

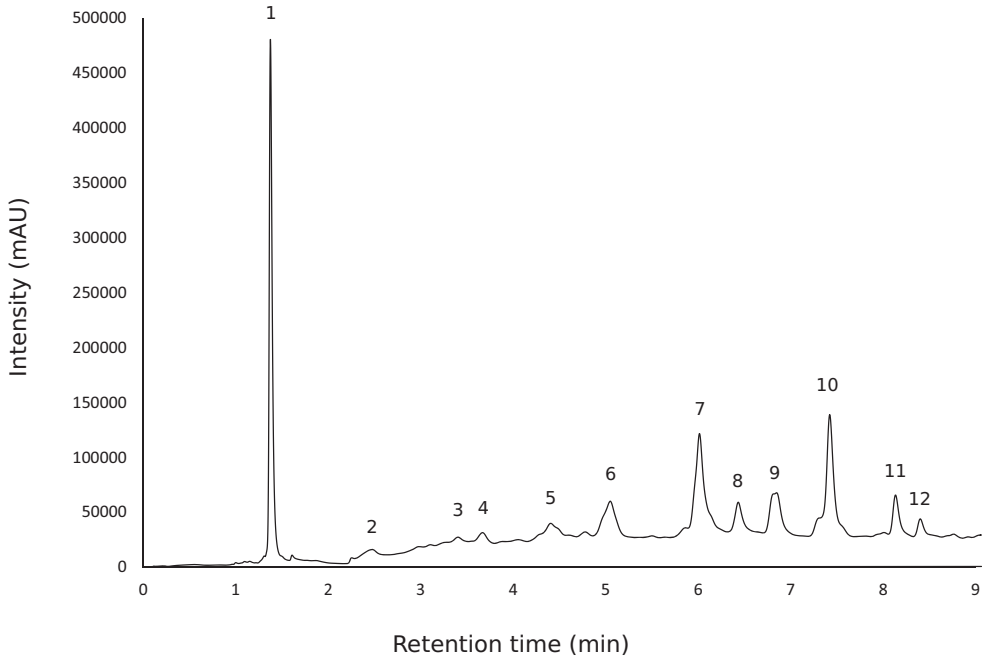

Fig. 3 Example UV-HP-LC chromatogram of tree 2, disc at 130 cm, indicating the relative retention positions of the 12 compounds investigated

that there must be compounds which are not UV-visible in the extracts. This was tested using ^{13}C NMR on samples at 130 cm from tree 2 and 6 (Fig. 5). Results indicated that there are significant peaks with shifts between 100 and 50 ppm, in particular at 75 ppm, which are indicative of the presence of carbohydrates (Inari et al. 2007). There are also signals at 155 ppm, 145 ppm and 130 ppm corresponding to the aromatic carbon linked to O, aromatic carbon linked to C and aromatic carbone linked to H present in the aromatic rings of polyphenolic compounds such as flavonoids and tannins (Inari et al. 2007). Signals between 50 and 10 ppm correspond to aliphatic carbons, which may be attributed in part to aliphatic carbons of heterocyclic flavonoid structures but also to suberin or its hydrolysis products (Gil et al. 1997). Therefore it may be that such compounds are contributing a greater proportion to extract yield at the top of the tree than at the base.

4 Discussion

Much of the scientific literature concerning bark extractives only concerns bark bulked from the segment of the trunk from the base of the tree to the base of the crown, as this is where the majority of valuable wood is present for the timber industry. By bulking, information about longitudinal variability is lost; however a previous study showed that in 10- to 12-year old trees of black locust (*Robinia pseudoacacia* L.), the hot water extractives of the stem (including wood and bark) increased significantly with % total tree height (Stringer and Olson 1987), although it was only the very top measure at 80% total tree height which differed significantly from the lower measurements. Our results are similar, as we have shown that in the bark of *A. alba*, the yield of water:ethanol extractives is typically highest in the region of the trunk from the base of the crown towards the top of the tree, rather than in the lower parts of the trunk. Previously, the total extraction yield of bark from the lowest 3 m of *A. alba* was reported to be 10.1% (Bianchi et al. 2014), which is lower than the 17 to 20% we found for samples below this height. Bianchi et al. (2014) noted that their yields were lower than for other softwoods and suggested this was caused by their low extraction temperature of 60°C.

Because the trunk base contains a greater proportion of mature outer bark, whereas wood towards the top has a greater proportion of younger inner bark, examining longitudinal variation in extractives allows us to determine indicators of bark maturity. The inner bark typically contains more extractives than the mature, outer bark (Eberhardt 2013). However, extractive yields and compositions change longitudinally even within these tissues. In silver birch (*Betula pendula* Roth) outer bark, the yields of ethanol-soluble extractives increased with height (10 to 90% total height), whereas the water-soluble extractives decreased with height (Vedernikov et al. 2011). In the inner bark, both the ethanol- and water-soluble extractive yields decreased with height; however the water soluble extractives were much higher in the inner bark than the outer bark (Vedernikov et al. 2011). This suggests a complex relationship between the synthesis, transport, storage and degradation of different extractable secondary metabolites between these tissues. Inner bark thickness remains relatively constant with tree height, but outer bark thickness is greatest at the tree base (Eberhardt 2013). Therefore differences in extractives between inner and outer bark tissues as a function

Table 3 Peak retention times (RT), characteristic ions and intensities used for peak identification[a] in positive and negative mode (±). Intensities are specific to an example spectrum of tree 2, at 130 cm height

Peak	Compound(s)[b]	RT range	RT mean		m/z (Intensity)
1		1.351–1.416	1.372	$[M+H]^+$	311.00 (100)*; 399.10 (79)**; 129.00 (34); 105.00 (17)**; 289.05 (17); 177.65 (10); 611.30 (9)*
				$[M-H]^-$	191.15 (100); 239.25 (41); 133.25 (33); 421.35 (25)*; 609.35 (6)*; 179.20 (5)
2	Gallocatechin (dimer)	2.063–3.015	2.480	$[M+H]^+$	311.00 (100); 105.20 (88)**; 102.50 (32)**; 122.90 (31); 339.10 (21); 195.00 (19); 114.70 (15); 611.30 (11)*; 219.10 (10); 289.10 (8)
				$[M-H]^-$	239.25 (100)**; 305.25 (90)*; 609.40 (31)*; 361.30 (28); 179.30 (21); 593.40 (19); 169.20 (19); 315.35 (17); 225.20 (13)
3	Gallocatechin[c]	3.091–3.917	3.280	$[M+H]^+$	214.15 (100)**; 307.10 (57)*; 234.60 (29); 595.30 (19)*; 217.15 (14); 215.25 (11)
				$[M-H]^-$	305.25 (100)*; 153.25 (22); 239.25 (12)**; 593.35 (10)*; 351.25 (5)
4	Gallocatechin gallate	3.127–4.155	3.526	$[M+H]^+$	105.15 (100)**; 102.35 (40)**; 122.80 (39); 595.30 (23)*; 208.05 (19); 611.25 (9)*
				$[M-H]^-$	593.40 (100)*; 153.25 (57); 609.40 (54)*; 239.25 (51)**; 594.40 (30); 321.25 (25); 211.25 (20); 179.15 (14); 305.35 (14); 331.25 (13); 377.00 (13); 427.35 (12)
5	Epigallocatechin[c]	4.082– 5.056	4.247	$[M+H]^+$	339.05 (100); 219.10 (95); 307.05 (94)*; 383.10 (63); 251.10 (60); 214.05 (36)**; 211.10 (35); 226.80 (26); 401.10 (24)
				$[M-H]^-$	305.20 (100)*; 361.30 (31); 113.35 (9)**; 351.45 (8); 327.30 (7); 593.35 (6); 175.05 (6)**; 239.35 (6)**; 611.25 (5)
6	(+)-Catechin	4.674–5.760	4.849	$[M+H]^+$	291.05 (100)*; 292.10 (53); 226.60 (37); 485.20 (34); 271.55 (18)
				$[M-H]^-$	289.25 (100)*; 290.30 (17); 335.25 (16); 507.40 (13)
7		5.138–6.830	5.781	$[M+H]^+$	531.30 (100)*; 317.10 (43)*; 499.25 (28)*; 532.25 (27); 528.40 (25); 369.15 (17); 299.15 (11)
				$[M-H]^-$	553.50 (100)*; 521.45 (57)*; 289.25 (38); 554.45 (30); 507.45 (20); 391.45 (18); 522.45 (16); 315.35 (14); 575.45 (11); 345.35 (9)
8		6.019–7.179	6.211	$[M+H]^+$	349.15 (100); 681.45 (67)*; 499.20 (59)*; 278.70 (39); 299.10 (31); 682.40 (26); 587.45 (21)*; 341.15 (17); 500.25 (14); 214.20 (14)**; 369.70 (13)
				$[M-H]^-$	475.40 (100)*; 521.40 (61)*; 657.50 (45)*; 311.30 (25)**; 476.40 (20); 658.55 (18); 522.40 (17); 329.30 (13); 589.50 (12)
9	Quercetin glycoside	6.431–7.558	6.625	$[M+H]^+$	549.30 (100)*; 303.60 (38); 546.40 (27); 283.10 (18); 551.25 (11); 611.30 (7); 163.10 (7)
				$[M-H]^-$	525.45 (100)*; 571.50 (29)*; 526.45 (25); 593.45 (19); 327.25 (9); 572.45 (9); 377.35 (6)
10	Isorhamnetin glucoside	6.985–8.071	7.190	$[M+H]^+$	563.35 (100)*; 515.25 (92)*; 307.15 (58); 512.35 (47); 317.10 (42)*;286.60 (38); 564.30 (27); 516.25 (23); 475.20 (22); 463.25 (20)
				$[M-H]^-$	537.45 (100)*; 491.40 (36)*; 538.45 (27); 315.35 (12); 585.50 (9); 492.40 (9); 559.50 (9); 197.25 (8); 539.50 (7); 509.40 (6)
11	Isorhamnetin	7.696–8.709	7.929	$[M+H]^+$	545.30 (100)*; 331.10 (56)*; 301.65 (50); 542.35 (41); 546.30 (29); 281.05 (19); 322.10 (16); 293.70 (15)
				$[M-H]^-$	567.50 (100)*; 568.55 (29); 569.50 (5)
12	Quercetin glycoside[d]	7.961–8.951	8.166	$[M+H]^+$	529.30 (100)*; 314.15 (50); 317.10 (49); 293.60 (30); 234.20 (27); 369.20 (22); 399.15 (12)
				$[M-H]^-$	551.45 (100)*; 505.45 (47); 315.30 (35); 552.45 (26); 316.90 (22); 175.50 (19)**; 345.25 (17); 113.30 (14)**; 506.40 (13)

[a] Ions listed are those used for peak identification but do not necessarily derive from the tentatively assigned compound, as detailed below. Ions without stars are likely to derive from water, formic acid, sodium or glycosidic compounds rather than the assigned compound

[b] Compounds listed have been tentatively identified based on ions characteristic of the associated peak

[c] It is not certain which peak is gallocatechin and which is epigallocatechin

[d] Quercetin-3-O-beta-glucopyranosyl-6′-acetate

* Ions which were characteristic of the tentatively assigned compound or compounds if more than one is present in each peak

** Ions which were used for peak identification but which were only potentially thought to derive from the tentatively assigned compound(s) due to intensity

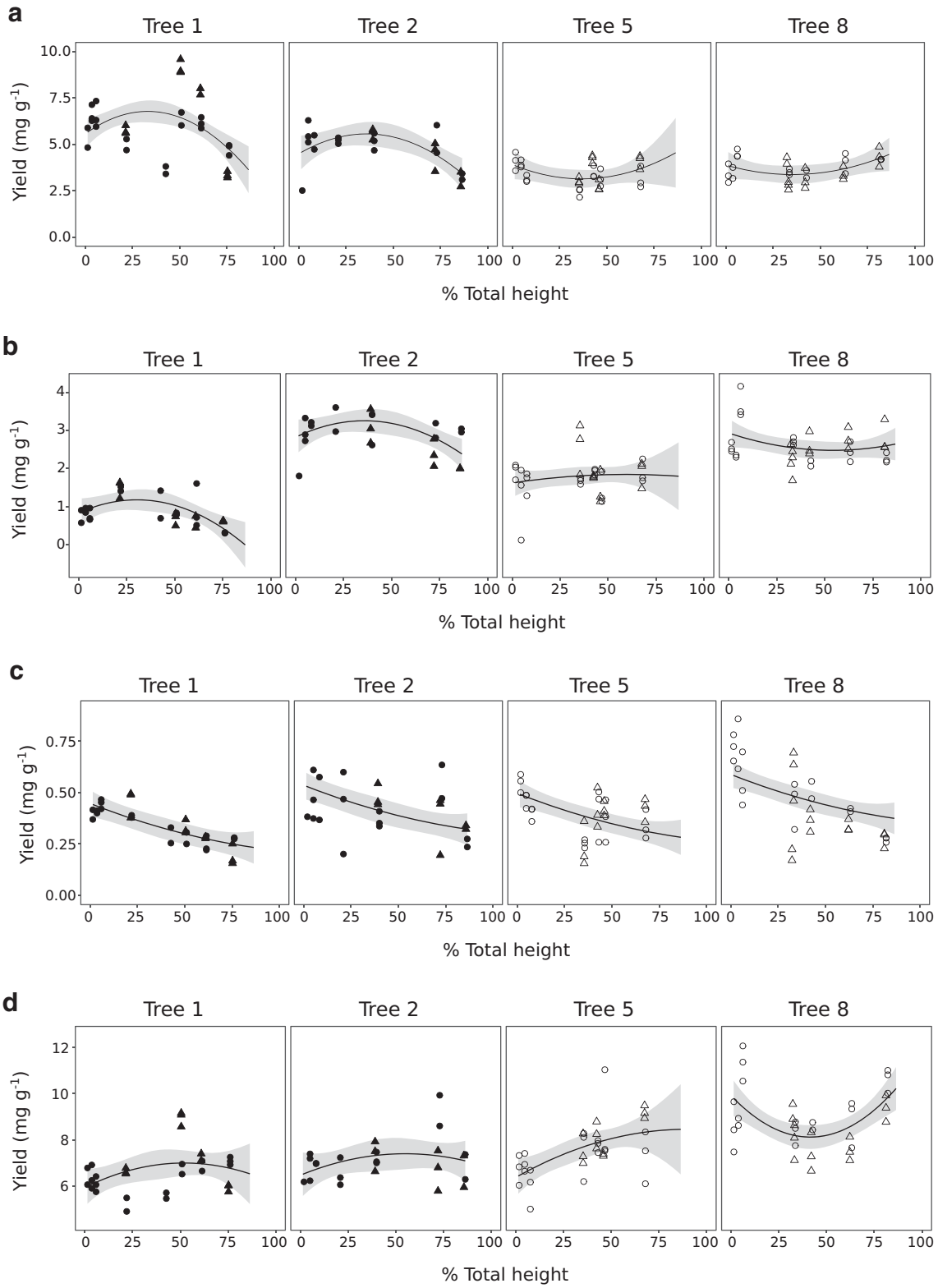

Fig. 4 Relationships between % total tree height and yields of selected peaks. Plots shown are the model results conditional on tree, which was a significant variable. (a) Peak 1, (b) peak 7, (c) peak 12 (quercetin glycoside), (d) oligomers of catechin. Trees 1 and 2 come from the un-thinned stand (filled shapes), and trees 5 and 8 come from the dynamically managed (open shapes). Samples without branches are denoted by circles, and samples with branches are denoted by triangles. The grey shading indicates a 95% confidence interval of the fitted values

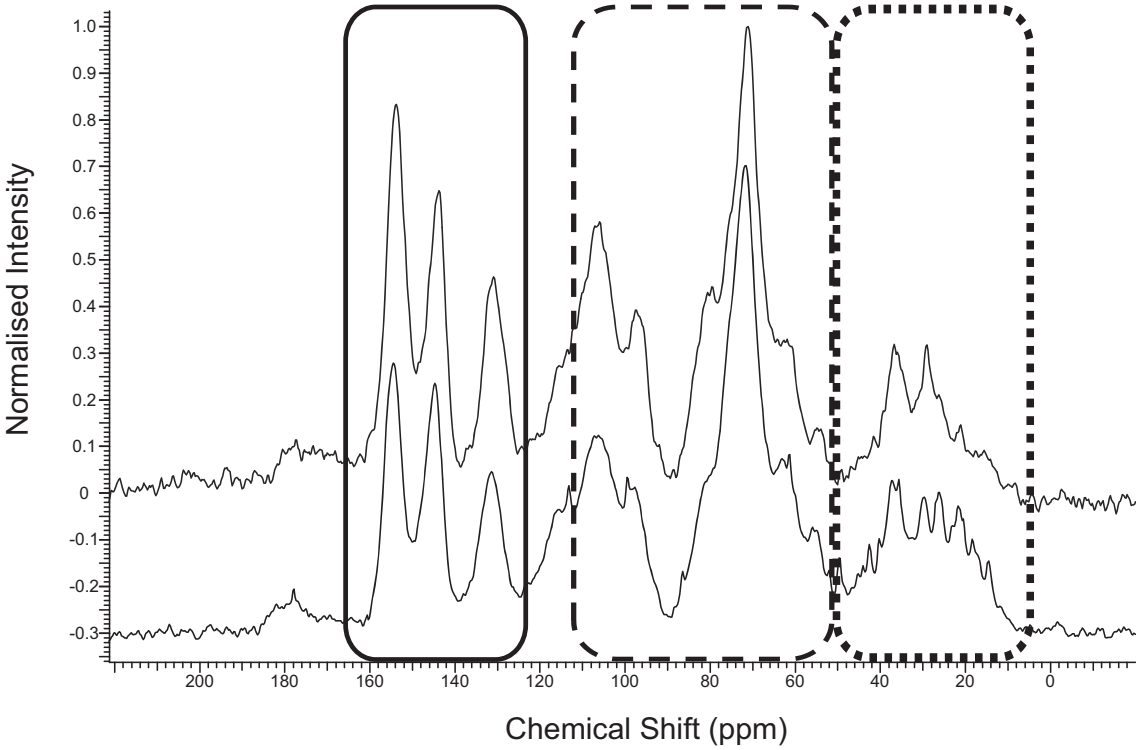

Fig. 5 For trees 2 and 6, CP/MAS ^{13}C NMR spectra were recorded for a sample at 130 cm height each. The top spectrum is tree 6, and the bottom spectrum is tree 2. The spectra indicate the presence of aromatic rings (165 to 125 ppm, solid line), carbohydrate moieties (110 to 50 ppm, dashed line) and aliphatic signals (50 to 10 ppm, dashed line)

of height indicates that yield depends on both tissue type and tissue maturity. Total extractive yield has been shown to decrease with age in downy birch (*Betula pubescens* Ehrh.) (Rizhikovs et al. 2015). In the present study, the observed differences among trees in the relationship between total extract yield and sample height are possibly due to a combination of differing proportions of inner and outer bark and tissue age, among the trees. In our study, the thinned trees had longer and larger crowns than in trees from the un-thinned stand. In leaves of quaking aspen (*Populus tremuloides* Michx.), partitioning of carbon to tannin synthesis is greater in source leaves than sink leaves (Kleiner et al. 1999), indicating tannin biosynthesis is directly affected by the availability of photoassimilates. That the effect of management type on water:ethanol extractive yield was not significant indicates synthesis of these secondary metabolites is unlikely to be limited by available photoassimilate in our study. Additionally, for the same disc types sampled, there were no differences in water:ethanol extractive yield among trees which were consistent with tree dimensions (i.e. diameter at breast height or total height). The results indicate that tree dimensions are likely to play a less significant role in the yield of hydrophilic extractives than bark tissue type or maturity.

We did find *n*-hexane extracts significantly increased with height, and the dynamic management scenario increased extract yields, indicating biosynthesis of bark lipophilic secondary metabolites is enhanced with increasing availability of photoassimilates. This small increase in yield may not be biologically significant. Our findings for the relationship between *n*-hexane extract yields and sample height contrast with the results of Krogell et al. (2012). They reported yields of 1.2% for the inner bark of *P. abies*, and 2.4% for the outer bark, which would give rise to the expectation of higher yields at the base of the tree than at the top. Yet in bark of poplar (*Populus* × euramericana "Guariento"), the yield of *n*-hexane extracts was also found to be higher (> 40%) at the top of the tree (diameter 11.2 cm) compared with the base of the tree 30 cm from the ground (> 30%) (diameter 25 cm). These yields were much higher than those observed in our study (~ 5%), likely typical of hardwood barks containing higher lipophilic extractives than softwood barks. The lipophilic extractives of *B. pendula* bark increased in the outer bark with tree height (16.3 to 27.5%) but decreased in the inner bark with tree height (3.2 to 2.5%) (Vedernikov et al. 2011). As the inner bark of *B. pendula* had much lower yields than the outer bark, the overall yield of lipophilic extractives would be expected to increase with height, similar to our observations for *n*-hexane extracts.

The polyphenolic parenchyma cells in secondary phloem are hypothesized to be the site where bark polyphenolics such

as tannins are synthesized (Franceschi et al. 1998). These cells remain viable even in extremely mature bark, and one layer of this cell type is produced every season (Krekling et al. 2000). Therefore the most mature bark should contain a greater number of these cells. Our LC-MS data indicated that although total extract yield is lowest in the base of the tree, the total proportion of the UV-visible phenolic compounds is often highest at the base of the tree and then decreases with tree height. Using ^{13}C NMR, we found that this discrepancy among the shape of yield relationships with height could be caused by compounds such as carbohydrates which are not visible in UV. Our method of LC-MS quantification was reliant on the use of catechin and epicatechin as standards to calculate catechin equivalents and may not be a fully accurate method of quantification for every compound. However, it was assumed that any imprecision would not change significantly with sample height so as to cause peak yield to appear constant, when it did in fact increase. More likely, the discrepancy is explained by the presence of compounds which are not visible in UV but which can be extracted from bark with water or ethanol. Such compounds include mono-, di- or oligosaccharides, stilbene glycosides and fatty acids. Except for a slight increase in yield of *n*-hexane extracts, we did not find increasing yields of lipophilic compounds with tree height, making fatty acids an unlikely cause of the observed increase in water:ethanol extract yields with height. Extracts from the bark of *A. alba* have previously been found to be composed of 29.1% carbohydrates, greater than the total of 27.9% polyphenolic compounds (Bianchi et al. 2015). The industrial bark of *P. abies* has been shown to contain 7.7 to 11.5% non-cellulosic glucose (Kemppainen et al. 2014), and the authors found they were unable to obtain pure extracts of only tannins or carbohydrates without also extracting impurities of the other compound type. Hot water extracts of *P. abies* inner bark also contain pectic polysaccharides, with the inner bark having a higher proportion of pectic polysaccharides than the outer bark (Krogell et al. 2012; Le Normand et al. 2012, 2014). It is therefore probable that in the present study, there were greater amounts of carbohydrates in extracts from the top of the tree which contain a greater proportion of inner bark, thereby increasing extract yield in a manner which does not contribute to the UV-visible compounds. The polyphenolic parenchyma cells of *P. abies* also contain starch granules (Krekling et al. 2000), although these were not found in extracts of *A. alba* (Bianchi et al. 2015). Carbohydrates can also be linked to UV-visible phenolic compounds and may contribute to the weight of extract yielded without the glycosyl residues contributing to the UV spectrum. Two flavonol glycosides were identified in our study, one of which, isorhamnetin glucoside, was present in higher proportions of catechin equivalents than the other peaks (except for polymeric polyphenols). Stilbene glycosides were not found in the bark of *A. alba* in the study of Bianchi et al. (2014), although they are common in species of spruce (*Picea* spp.) (Aritomi and Donnelly 1976; Krogell et al. 2012; Bianchi et al. 2014). The presence of lignans in *A. alba* bark extracts has previously been indicated (Benković et al. 2014; Bianchi et al. 2014), and although they were not identified in the present study, they may co-elute with other compounds in the unidentified peaks. The phloem parenchyma cells of *P. abies* (Li et al. 2012) and *Picea sitchensis* (Bong.) Carrière (Aritomi and Donnelly 1976) contain the stilbene glycoside astringent, which dimerizes in response to fungal attack.

5 Conclusion

Here we have clearly shown that there are few differences in extractive yield among trees of *A. alba* at specific heights. In contrast, there is significant longitudinal variation in extract yield and within trees of this species. Thinning management did not have any effect on the yield of water:ethanol extracts, although a small increase in yield was observed in hexane extracts in trees from the un-thinned stands. Although the yield of water:ethanol extracts of *A. alba* bark is typically higher towards the top of the crown, the yield of polyphenolic compounds tends to be higher towards the base of the stem. Therefore, more mature bark tends to have a greater proportion of polyphenolic compounds than younger bark, which may have a greater proportion of sugars. This study highlights the necessity of investigating the composition of bark extracts, not just yield but also in terms of chemical composition in order to most efficiently exploit the bark of commercially important species for the different chemical industries. For example, bark from the base of the stem is best suited to those industries that value polyphenolic compounds, such as those creating resins and adhesives.

Acknowledgements The authors warmly thank Antoine Billard, Rodolphe Bauer, Fleur Longuetaud and Frederic Mothe who managed the tree sampling and Adrien Contini, Antoine Sarrouille and Coline Lebreton for their technical support. Thanks also to ONF (the French National Forest Service) who provided the material from the Saint-Prix forest.

Funding The authors gratefully acknowledge the Lab of Excellence ARBRE for the post-doctoral fellowship granted to the first author. The UMR 1434 Silva and EA 4370 LERMAB are supported by a grant overseen by the French National Research Agency (ANR) as part of the "Investissements d'Avenir" programme (ANR-11-LABX-0002-01, Lab of Excellence ARBRE) in the frame of the project "BARK-TAN-BIO" included in the wider project "EXTRAFOREST" supported in addition by the French Ministry of Agriculture, the Lorraine-FEDER, ADEME and Grand Est Region.

Data availability The datasets generated and/or analysed during the current study are available in the Harvard Dataverse (Brennan et al. 2019) at https://doi.org/10.7910/DVN/8C37DJ

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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