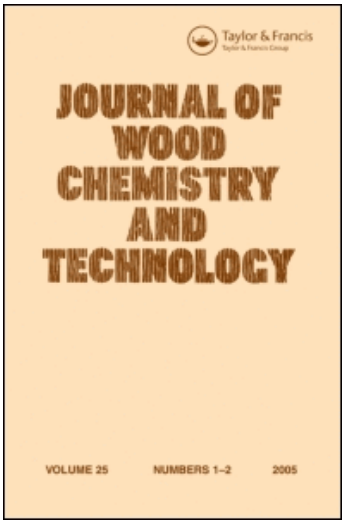

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Aspen Knots, a Rich Source of Flavonoids

Suvi P. Pietarinen[a]; Stefan M. Willför[a]; Frank A. Vikström[a]; Bjarne R. Holmbom[a]
[a] Process Chemistry Centre, Laboratory of Wood and Paper Chemistry, bo Akademi University, Turku/bo, Finland

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Aspen Knots, a Rich Source of Flavonoids

Suvi P. Pietarinen, Stefan M. Willför, Frank A. Vikström, and Bjarne R. Holmbom

Process Chemistry Centre, Laboratory of Wood and Paper Chemistry, Åbo Akademi University, Turku/Åbo, Finland

Abstract: Hydrophilic extractives in heartwood, sapwood, and knots of three aspen species were analyzed by gas chromatography, gas chromatography-mass spectrometry, and high-performance size-exclusion chromatography. The amounts of flavonoids were considerably higher in the knots relative to the stem wood. Flavonoids identified were dihydrokaempferol, naringenin, kaempferol, catechin, and taxifolin. In addition, glycosides of dihydrokaempferol, naringenin, and kaempferol were identified by mass spectrometry and shown to be glucosides by enzymatic hydrolysis by β-D-glucosidase. Dihydrokaempferol and its glucoside dominated in all knot samples although there were variations in the amounts and composition of the extractives. The total amounts of flavonoids varied between 11 and 43 mg/g in *Populus tremula*, 12 and 62 mg/g in *Populus tremuloides* and 47 and 82 mg/g in *Populus grandidentata*. The aspen knots were found to be a rich source of bioactive flavonoids.

Keywords: Aspen, *Populus grandidentata*, *Populus tremuloides*, *Populus tremula*, sapwood, heartwood, knots, hydrophilic extractives, flavonoids

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Address correspondence to Suvi P. Pietarinen, Process Chemistry Centre, Laboratory of Wood and Paper Chemistry, Åbo Akademi University, Porthansgatan 3, FIN-20500, Turku/Åbo, Finland. E-mail: suvi.pietarinen@abo.fi

INTRODUCTION

A knot is the part of a branch that is encased in the tree stem. The knots of several softwood species (*Picea abies*, *Pinus sylvestris*, and several *Abies spp*.) contain exceptionally large amounts of bioactive polyphenols, especially lignans.[1–4] The amount of phenolic extractives has been found to be 2–10 times larger in knots of *Salix caprea* than in its stemwood.[5]

Aspen wood is an excellent raw material for paper pulp. The lipophilic constituents of aspen, however, can cause deposit problems in papermaking, and pulping, and have therefore been thoroughly studied.[6–8] Flavonoids in pollen and buds have been analyzed as taxonomic markers in the genus *Salicaea*.[9] In the stemwood of European aspen, *Populus tremula*, the total amount of hydrophilic ethanolic extractives has been reported to be 1.7–3.0% of dry weight.[10] Low molecular weight aryl extractives, such as benzyl alcohol, *p*-ethyl phenol, phenol, β-phenylethanol, and sinapyl alcohol, have been found in wood of trembling aspen, *P. tremuloides*.[11] *p*-Hydroxybenzoic acid has been identified in the wood of bigtooth aspen, *P. grandidentata*[12] and flavonoids naringenin, kaempferol, dihydrokaempferol, and methyl ethers of kaempferol and naringenin were identified in the wood of *P. tremuloides* by Fernandez et al.[13] Naringenin has been found in sulphite pulping liquors from *P. tremuloides*.[14]

We recently studied the lipophilic extractives in the stem wood and knots of *P. tremuloides* and *P. grandidentata*.[8] The objective of this study was to determine the amount and composition of phenolic substances in knots and stemwood of *P. tremula*, *P. tremuloides*, and *P. grandidentata*. Our particular interest was to assess whether the knot material could also be a source of valuable bioactive compounds.

MATERIAL AND METHODS

Wood Material

Two mature *Populus tremula*, *P. tremuloides*, and *P. grandidentata* trees were felled and samples of stemwood and knots were sawn out, frozen, and shipped frozen to our laboratory. The *P. tremula* trees were felled in August 2000 in Houtskär in southwestern Finland, and the *P. tremuloides* and *P. grandidentata* trees in August 2002 in Cape Breton, Nova Scotia, Canada. Samples of heartwood and sapwood, as well as living and dead knots, were taken from each tree (Table 1). Living knots had a live branch attached, whereas dead knots had either a dead branch attached or the branch had fallen off. *Populus tremula* trees did not have a sharp boundary between sapwood and heartwood; thus, the six innermost annual rings were analyzed as heartwood. The two other tree species had distinctive heartwood areas. Heartwood samples were further distinguished visually by light microscopy using differences in vessel cell structures. Heartwood samples of

Table 1. Attributes of *Populus tremula, P. tremuloides*, and *P. grandidentata* samples

	P. tremula		*P. tremuloides*		*P. grandidentata*	
	Tree 1	Tree 2	Tree 1	Tree 2	Tree 1	Tree 2
Number of annual rings	30	31	71	58	32	37
Number of heartwood annual rings	19	12	33	24	22	26
Length of the tree/m	13	17	21	20	9.4	14.1
Stemwood disc height/m	1.5	4.5	1.5	2.3	1.5	2.1
Diameter of the stemwood disc/cm	19	17	29	43	18	29
Living knot height/m	5.0	6.0	11	11	6.4	6.1
Dead knot height/m	3.0	4.0	4.6	4.8	2.6	3.0

P. tremuloides contained discolored, decayed wood that was analyzed separately. The decay was probably caused by false tinder fungus (*Phellinus tremulae* [Bond]) or poplar peniophora (*Peniophora polygonia* [Pers.:Fr.]). These decays occur in about 5–13% of this species in western Canada.[15] The sapwood sample of one *P. grandidentata* tree contained a red stain that was also analyzed separately.

Preparation of the Wood Extracts

Wood samples were splintered, freeze-dried, and ground in an analytical laboratory mill (Pulverisette 19, Fritsch). A second freeze-drying step after grinding ensured almost complete removal of volatile compounds. Sequential extraction was carried out in an ASE apparatus (Accelerated Solvent Extractor, Dionex Corp.) according to Willför et al.[1] Lipophilic extractives were first extracted with *n*-hexane (solvent temperature 90°C, pressure 13.8 MPa, two 5 min static cycles) and then hydrophilic extractives were extracted with acetone:water (95:5, v:v) (solvent temperature 100°C, pressure 13.8 MPa, two 5 min static cycles).

Analysis by Gas Chromatography (GC), GC-Mass Spectrometry (GC-MS), and High-Performance Size-Exclusion Chromatography (HPSEC)

After evaporation of the extract solutions and silylation, the extractives were analyzed by GC (Perkin Elmer AutoSystem XL instrument) on a 25 m × 0.20 mm i.d. column coated with cross-linked methyl polysiloxane (HP-1) with a film thickness of 0.11 μm. The column oven parameters were: 120°C, 6°C/min −300°C (10 min); carrier gas H_2 (20 mL/min); split injector (1:20) 260°C; FID detector 300°C; injection volume 1 μL.[16] The

extractives were silylated by addition of 80 μL bis-(trimethylsilyl)-trifluoroacetamide, 40 μL trimethylchlorosilane, and 20 μL pyridine. The reaction was completed by keeping the test tubes in an oven at 70°C for 45 min. Heneicosanoic acid and betulinol were used as internal standards and no FID correction factors were used. The method is not perfect as a quantitative analysis of sugars, but the sugar amounts in different samples can be compared with each other. The limit of quantitation was approx. 0.01 mg/g, but compounds of smaller amounts could be detected. These are reported as trace amounts. All results were calculated on a freeze-dried wood basis.

Flavonoid glycosides were analyzed on a short GC 6 m × 0.53 mm i.d., 0.15 μm HP-1 column using cholesteryl heptadecanoate as an internal standard. No FID correction factors were used. The gas chromatograph was a Varian 3400 instrument and the column oven parameters were: 100°C (1.5 min), 12°C/min −340°C (5 min); carrier gas H_2 (20 mL/min); SPI (Septum-equipped Programmable Injector) 80°C (0.5 min) −200°C/min −340°C (18 min); FID detector 340°C; injection volume 0.4 μL. Identification of individual components was performed by GC-MS analysis of the silylated components with an HP 6890-5973 GC-quadrupole-MSD instrument. Both a similar 25 m HP-1 GC column as above and a 15 m × 0.25 mm i.d., 0.1 μm MXT-65TG column were used, which allowed elution of the silylated flavonoid glycosides.

Flavonoid glycosides were quantified after enzymatic hydrolysis using β-glucosidase (β-glucosidase from almonds, BioChemica). Extracts were evaporated, solubilised in water, and β-glucosidase was added. Samples were kept at 35°C for 24 h. After the hydrolysis was completed samples were freeze-dried, silylated, and analyzed with GC as explained earlier.

Identification of the aglyconic flavonoids was based on silylated reference compounds, using both their mass spectra and retention times.

The molar-mass distribution of the extractives was determined by high-performance size-exclusion chromatography (HPSEC) using a Spark Holland Marathon-XT Auto Sampler, Pharmacia LKB – HPLC Pump 2248, 30 cm × 7.8 mm i.d., 5 μm; TSK G5000, TSK G3000, and TSK G1500 HHR columns with a guard column (4 cm × 6 mm i.d.) and a Pharmacia LKB 2142 differential refractometric detector. The solvent (tetrahydrofuran, THF) flow rate was 1 mL/min and the concentration of each sample was adjusted to a concentration of 1 mg/mL of flavonoids. The injection volume was 100 μL. This method has been used successfully for samples containing polyphenols, such as oligolignans[1] and bi- and triflavonoids;[17] however, the solubility of large polymers, such as tannins, in THF can be low.

RESULTS

The amounts of hydrophilic extractives were larger in the knots than in the stemwood samples (Table 2). However, there were considerable differences

Table 2. Gravimetric amounts (mg/g freezedried wood) of hydrophilic extractives in *P. tremula, P. tremuloides*, and *P. grandidentata*

	Sapwood		Heartwood		Blackwood		Living knot		Dead knot		Red part
	Tree 1	Tree 2	Tree 1	Tree 2	Tree 1	Tree 2	Tree 1	Tree 2	Tree 1	Tree 2	Tree 2
Populus tremula	14	16	18	19	—	—	45	100	86	85	—
Populus tremuloides	11	15	11	9.3	7.9	14	65	63	82	161	—
Populus grandidentata	9.1	6.7	39	20	—	—	150	52	77	85	6.9

between the amounts in knots within species. The variation was smaller in stemwood samples. Heartwood and sapwood samples of *P. tremula* and *P. tremuloides* contained similar amounts of extractives, while the extractives were slightly greater in *P. tremula* than in *P. tremuloides*. In *P. tremuloides*; decayed wood contained similar amounts of extractives as healthy stemwood samples. In contrast to *P. tremula* and *P. tremuloides*, the heartwood of *P. grandidentata* contained larger amounts of hydrophilic extractives than the sapwood.

GC-MS analysis of the extracts indicated that, in addition to simple acids, sugars, sugar alcohols, and flavonoids, extracts contained flavonoid glycosides. Hence, the extracts were hydrolyzed enzymatically with β-glucosidase. After this treatment, glycosides were not observed (Figure 1).

The predominant compound in knot extracts of *P. tremula* was the flavanone dihydrokaempferol (Table 3). Amounts of dihydrokaempferol were 50–3000 times larger in knots than in stemwood. Dihydrokaempferol amounts, however, did not increase with enzymatic hydrolysis. This indicates that glucosidic dihydrokaempferol was not present. Another flavanone, naringenin, was the second most abundant compound, and in one sapwood sample it was dominant. Naringenin amounts increased slightly with enzymatic hydrolysis of living knot samples. Overall, dead knots contained larger amounts of dihydrokaempferol and naringenin than living knots.

Kaempferol and taxifolin were also identified in the samples. Although the amounts of these two flavonoids were small compared to dihydrokaempferol and naringenin, accumulation in knots could be observed and during hydrolysis the aglyconic taxifolin was liberated and the amounts increased considerably. The aglyconic flavonoids that were identified in this study are shown in Figure 2.

Dihydrokaempferol was dominant among the aglyconic flavonoids in *P. tremuloides*, as it was in *P. tremula* samples (compare Tables 3 and 4). Amounts of dihydrokaempferol were 50–900 times larger in the knots than in the stemwood. The glucosidic dihydrokaempferol was enzymatically hydrolyzed and amounts increased in all samples except in the dead knot of tree 1. Naringenin, kaempferol, and taxifolin were also identified in the samples (Table 4) and taxifolin was liberated by hydrolysis of the stemwood samples. Amounts of flavonoids in sapwood and in heartwood were the same in both trees. One of the dead knots only contained about one-third of the aglyconic flavonoids than the other. In addition, the amounts in this knot did not increase with enzymatic hydrolysis, whereas in the other dead knot the flavonoid amounts were roughly doubled. The composition and amounts of flavonoids in decayed wood was similar to that in healthy stemwood.

Living knots of *P. grandidentata* contained large amounts of glucosides. The enzymatic hydrolysis increased the flavonoid amounts from 30 mg/g to 80 mg/g (Table 5). Dihydrokaempferol dominated as it

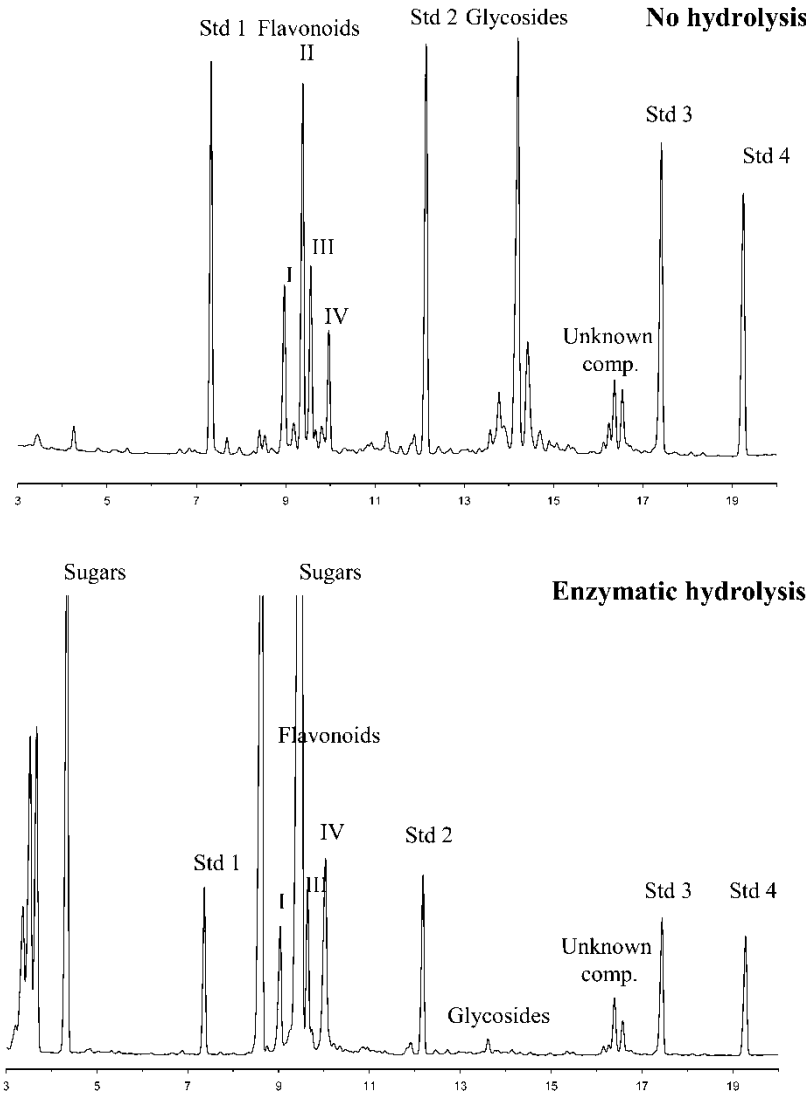

Figure 1. GC short column chromatograms before and after enzymatic hydrolysis of extract of *P. grandidentata* living knot of tree 1. Flavonoids identified in the samples are: I naringenin, II dihydrokaempferol, III taxifolin, and IV kaempferol. Internal standards were: Std 1. heneicosanoic acid, Std 2. betulinol, Std 3. cholesteryl heptadecanoate, and Std 4. 1,3-dipalmitoyl-2-oleyl glycerol.

did in the two other species, but the amounts of naringenin, taxifolin, and catechin were larger than in the other species. Heartwood contained remarkably large amounts of flavonoids before and after hydrolysis compared to sapwood, although the amounts in the heartwood were smaller than in the

Table 3. Amounts of hydrophilic extractives (mg/g freezedried wood) in *Populus tremula* determined by GC

	Sapwood				Heartwood				Living knot				Dead knot			
	Tree 1		Tree 2		Tree 1		Tree 2		Tree 1		Tree 2		Tree 1		Tree 2	
Simple acids																
Benzoic acid	+[a]		+		+		+		0.03		0.01		0.02		0.04	
3-hydroxybenzoic acid	0.01		+		0.01		0.01		0.02		0.01		0.07		0.05	
4-hydroxybenzoic acid	0.05		0.05		0.03		0.02		0.65		0.27		0.53		0.82	
Σ simple acids	0.07		0.07		0.08		0.06		0.73		0.31		0.69		1.0	
Σ sugars and sugar alcohols	1.4		1.8		0.37		0.19		4.3		2.5		4.2		4.1	
Flavonoid aglycones	Hydrolysis		Hydrolysis		Hydrolysis		Hydrolysis		Hydrolysis		Hydrolysis		Hydrolysis		Hydrolysis	
Naringenin	0.21	0.21	0.41	0.41	0.03	0.03	0.07	0.07	0.11	0.33	0.13	0.23	6.0	6.0	4.0	4.7
Dihydrokaempferol	−	0.01	0.01	0.04	−	0.06	0.21	0.34	8.4	8.4	7.9	7.9	31	31	26	26
Kaempferol	+	0.03	+	0.04	+	0.03	+	0.02	0.24	0.33	0.18	0.23	0.58	0.58	0.42	1.42
Taxifolin	0.01	0.36	0.01	0.36	+	0.29	+	0.25	0.29	2.4	0.24	2.2	0.77	5.18	0.33	2.4
Σ flavonoid aglycones	0.22	0.61	0.43	0.85	0.03	0.41	0.28	0.68	9.0	11	8.5	11	38	43	31	34

[a]Trace amounts.
[b]Enzymatic hydrolysis.

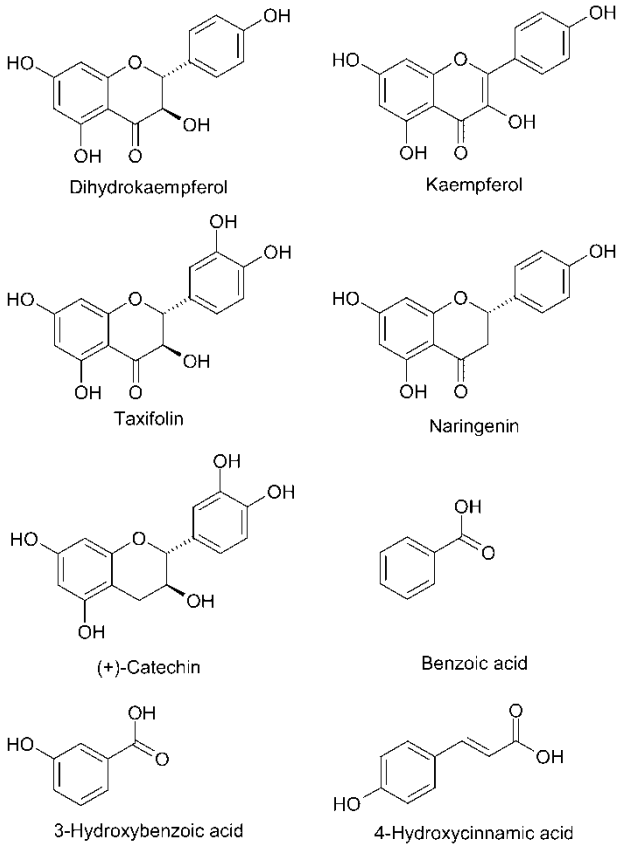

Figure 2. Structures of some extractives identified in knots of *P. tremula*, *P. tremuloides*, and *P. grandidentata.*

knots (Table 5). This difference in concentration between heartwood and sapwood was not observed in the other two species. Living and dead knots contained similar amounts of free flavonoids, but the amounts in dead knots were smaller after hydrolysis. Red-stained sapwood contained smaller amounts of hydrophilic extractives than the normal sapwood whereas the glucoside amounts were similar.

The amounts of simple acids were larger in knots of *P. tremula* and *P. tremuloides* than in stemwood, whereas the amounts in knots of *P. grandidentata* were similar to the stemwood (Tables 3, 4, and 5). Benzoic acid, 3-hydroxybenzoic acid, and 4-hydroxycinnamic acid were identified in *P. tremula* extracts. *P. tremuloides* and *P. grandidentata* contained malic acid, 4-hydroxybenzoic acid, and 4-hydroxycinnamic acid.

Sugars and sugar alcohols accumulated in the knots of *P. tremula* and *P. tremuloides*, whereas sapwood of *P. grandidentata* contained similar

Table 4. Hydrophilic extractives (mg/g freezedried wood) in *Populus tremuloides* determined by GC

	Sapwood				Heartwood				Blackwood				Living knot				Dead knot			
	Tree 1		Tree 2		Tree 1		Tree 2		Tree 1		Tree 2		Tree 1		Tree 2		Tree 1		Tree 2	
Simple acids																				
Malic acid	+[a]		+		+		+		+		+		0.01		0.01		0.01		0.01	
4-hydroxybenzoic acid	0.02		0.03		0.07		0.04		0.05		0.07		0.01		0.01		0.04		0.05	
4-hydroxybenzoic acid	0.01		0.01		+		0.01		0.01		0.01		0.18		0.14		0.68		2.3	
Σ simple acids	0.03		0.04		0.08		0.05		0.07		0.08		0.20		0.16		0.72		2.4	
Σ sugars and sugar alcohols	0.38		0.50		0.18		0.12		0.27		0.16		1.9		1.1		2.4		3.5	
Flavonoid aglycones			Hydrolysis		Hydrolysis[b]		Hydrolysis		Hydrolysis[b]		Hydrolysis		Hydrolysis[b]		Hydrolysis		Hydrolysis[b]		Hydrolysis	
Naringenin	0.06	0.00	0.03	0.05	0.02	0.33	0.04	0.29	0.04	0.29	0.04	0.13	5.2	8.7	5.2	5.2	4.1	4.1	11	11
Dihydrokaempferol	0.00	0.11	0.03	0.10	0.02	0.23	0.02	2.1	0.05	0.30	0.13	1.2	7.7	9.1	6.1	18	6.8	6.8	18	34
Kaempferol	0.02	0.05	0.01	0.05	0.01	0.02	0.01	0.05	0.02	0.02	0.02	0.18	0.78	0.78	0.64	0.64	1.0	1.0	1.7	15
Taxifolin	0.01	0.35	+	0.25	+	0.01	+	0.27	+	0.15	+	0.21	0.45	0.54	0.33	3.6	0.31	0.31	1.1	2.6
Σ flavonoid aglycones	0.09	0.51	0.07	0.45	0.05	0.59	0.07	2.7	0.11	0.51	0.28	1.7	14	19	12	27	12	12	32	62

[a]Trace amounts.
[b]Enzymatic hydrolysis.

Table 5. Hydrophilic extractives (mg/g freezedried wood) in *Populus grandidentata* determined by GC

	Sapwood				Heartwood				Living knot				Dead knot				Red stain	
	Tree 1		Tree 2		Tree 1		Tree 2		Tree 1		Tree 2		Tree 1		Tree 2		Tree 2	
Simple acids																		
Malic acid	0.01		+[a]		0.01		0.01		+		0.01		0.01		0.01		+	
4-hydroxybenzoic acid	0.11		0.09		0.10		0.15		0.03		0.07		0.05		0.04		0.03	
4-hydroxybenzoic acid	0.01		0.01		0.01		0.02		0.06		0.07		0.01		0.13		0.03	
Σ simple acid	0.13		0.11		0.12		0.18		0.09		0.15		0.07		0.17		0.07	
Σ sugars and sugar alcohols	3.5		2.0		1.3		1.0		2.1		2.1		2.9		3.4		0.88	
Flavonoid aglycones			Hydrolysis		Hydrolysis[b]		Hydrolysis		Hydrolysis[b]		Hydrolysis		Hydrolysis[b]		Hydrolysis		Hydrolysis[b]	
Naringenin	0.03	0.03	0.03	0.03	2.6	2.6	1.5	2.2	5.4	6.9	6.9	12	6.4	9.0	8.1	8.1	+	0.03
Dihydrokaempferol	0.10	0.11	0.08	0.23	6.7	6.7	3.4	3.4	13	42	13	50	13	40	12	27	+	0.20
Kaempferol	0.05	0.05	0.02	0.07	0.05	0.08	0.02	0.09	0.03	0.38	0.04	0.62	0.03	0.20	0.01	0.40	0.01	0.13
Taxifolln	0.01	0.17	0.01	0.37	0.51	0.51	0.24	0.89	3.6	17	2.8	8.5	2.0	6.0	2.1	4.7	+	0.41
Catechin	+	0.03	+	0.06	2.2	2.2	+	0.80	7.8	16	8.7	8.7	6.5	6.5	7.2	7.2	+	0.07
Σ flavonoid aglycones	0.19	0.39	0.14	0.76	12	12	5.2	7.4	30	82	31	80	28	62	30	47	0.01	0.84

[a]Trace amounts.
[b]Enzymatic hydrolysis.

amounts of sugars and sugar alcohols as the knots (Tables 3, 4, and 5). Heartwood of all species contained less sugar than sapwood.

No oligomeric or polymeric material was observed by HPSEC analysis in any of the extracts. If tannin would have been present in the samples, the mono- and dimeric units, as gallic acid and ellagic acid or biflavonoids would have been observed in GC analysis.

DISCUSSION

The flavonoid concentrations in the knots of the aspen species were considerably higher than in the stemwood. This is similar to what has been reported for knots of *Salix caprea*[5] and several softwoods, where the accumulating compounds are mostly lignans. These compounds probably function as a defense against invading micro-organisms and fungi. An open wound is formed when a branch is broken, which then can be colonized by micro-organisms and fungi. Hardwood knots are particularly sensitive to infections due to a gelatinous layer in the tension wood that can easily be degraded by brown rot fungi.[18] Phenolic extractives can be formed as a response to external stress, which is certainly the situation at the base of the branch.[19,20]

Malterud et al.[21] studied the flavonoids present in *Salix caprea* and found that of six identified flavonoids (dihydrokaempferol, naringenin, aromadendrin, taxifolin, prunin, and (+)-catechin) naringenin was most effective against both micro-organisms and fungi. Naringenin, dihydrokaempferol, and taxifolin have previously been identified in black galls occurring on *P. tremuloides* stems.[22] Naringenin, naringenin chalcone, catechin, and pinocembrin chalcone have been found in bud exudates of *P. tremula* and *P. tremuloides*.[9]

There were no considerable differences between living and dead knots, except for the *P. tremula* trees that had smaller amounts of flavonoids in living knots than in dead knots.

The amount of flavonoids in heartwood samples differed markedly from that in sapwood of *P. grandidentata*, but not in the other species. Decayed wood of *P. tremuloides* contained amounts of flavonoids similar to healthy parts of the stemwood, but a larger proportion of these were in the aglyconic form. The degree of glycosylation affects the antioxidative properties of flavonoids. For example, the antioxidative activity of glycosides of taxifolin and myricetin is lower than the corresponding aglycones.[23]

The amounts of flavonoids in knots are so large that isolation of these compounds is feasible by extraction and chromatographic purification. The isolated flavonoids can be utilized for example as technical antioxidants. Although these knots can be considered harmful in the paper industry,[8] they could provide a rich source of bioactive chemicals.

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