Analysis of Western Redcedar (*Thuja plicata* Donn) Heartwood Components by HPLC as a Possible Screening Tool for Trees with Enhanced Natural Durability

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Abstract

A method is described for the quantitative analysis of seven known compounds, specifically plicatic acid, thujaplicatin methyl ether, β-thujaplicin, γ-thujaplicin, β-thujaplicinol, thujic acid, and methyl thujate, in the ethanol extract of second growth western redcedar heartwood (*Thuja plicata* Donn) by high-performance liquid chromatography using diode array detection. The para bromo phenacyl ester of crotonic acid is synthesized for use as the internal standard for the method. Separation of compounds covering a wide range of polarities is achieved using an Inertsil ODS 3 3-µ column. Twenty seven second growth trees ranging in age from 40 to 125 years, originating from the coastal and interior regions of British Columbia, are selected for analysis and profiled using the described method. Samples consisting of five growth rings each are analyzed from the heartwood-sapwood boundary to the pith for each tree. Substantial variation in most heartwood compounds are detected within and between trees within a region. Significant variation in β-thujaplicin, the ratio between γ- and β-thujaplicin, and methyl thujate is detected between coastal and interior populations.

Introduction

Western redcedar (*Thuja plicata* Donn) is an important component of Pacific Northwest forests, both ecologically and economically. Its natural distribution includes both coastal and interior populations (1). Harvesting pressures have resulted in an extensive regeneration program, including the establishment of seed orchards for coastal British Columbia. Up to 10 million seedlings are planted annually in British Columbia, with over 80% of the seed coming from managed orchards.

Western redcedar is prized for its natural durability and is used in many exterior applications, such as fencing, roofing, and siding. The heartwood contains β-thujaplicin, γ-thujaplicin, and β-thujaplicinol, which are all excellent natural fungicides and are believed to be responsible for much of the wood's natural resistance to fungal attack (2–4).

Plicatic acid, though much less toxic to fungi, is approximately 10 times as abundant as the thujaplicins, giving it the potential to play an important role in protecting the tree from decay. The toxicity of β-thujaplicin and γ-thujaplicin have been compared with that of pentachlorophenol, and plicatic acid has been compared with zinc chloride, which is only one tenth as effective as pentachlorophenol (2). The role of thujaplicatin methyl ether, thujic acid, and methyl thujate in decay resistance is not known; however, they were included in the analytical data for future reference. Other, as yet unidentified compounds have also been detected, but are not reported here. The goal of this research was to provide a method to detect trees with elevated levels of heartwood compounds suspected to be responsible for natural durability. These selected trees will be incorporated into managed seed orchards and the resultant progeny used in the reforestation program. Future research will be conducted using nondestructive increment core sampling methods and focus on the bioactivity of as yet uncharacterized compounds as well as studies on the heritability of bioactive compound production.

Much of the original research on western redcedar extractives was done before the advent of modern analytical methods, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) (5). GC has been used to investigate the distribution of thujaplicins in old-growth and second-growth western redcedar (6,8–11). GC, while providing high resolution, cannot be used for the analysis of many of the lignans found in western redcedar without derivatization (7). In response to inquiries from the Forintek membership on the composition of Western redcedar extractives, an HPLC method was developed in 1998, subsequently modified and applied to the research reported here. The ethanol extract of western redcedar contained a wide range of compounds from the very polar and non-volatile

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plicatic acid to the nonpolar and volatile methyl thujate. HPLC with gradient elution was the method of choice for the direct analysis of complex extracts containing compounds with a wide range of polarity and volatility.

Experimental

Equipment

Analyses were performed on a Thermo HPLC consisting of a P4000 pump, AS3500 autosampler, and a UV6000LP photodiode array detector (Thermo Scientific, Palo Alto, CA). Data was captured and analyzed with ChromQuest V4.1 data system software (Thermo Scientific). The separation was performed on an Inertsil 3µ ODS3 150 × 4.6-mm column (GL Sciences, Torrence, CA) at 50°C. Extraction of samples was accomplished using a Branson B-52 ultrasonic bath (WA Brown Industrial Sales Inc., Orange, VA) in 20-mL pyrex vials (Wheaton # 986586). A Dispensette bottletop dispenser (BrandTech Scientific Inc., Essex, CT) was used to dispense extraction solvent.

Chemicals

Acetonitrile (HPLC grade, Anachemia Science, Richmond, B.C.), 18 MΩ water produced by a Millipore RO system with a 5 bowl polisher (Millipore, Billerica, MA), and 85% phosphoric acid (Mallinckrodt, AR) were used for all chromatographic analyses. Absolute ethanol (Commercial Alcohol, Langley, B.C.) was distilled in glass prior to use for extraction of the wood samples. Ethyl acetate (Accusolv) was purchased from Anachemia Science (Richmond, B.C.). β-Thujaplicin was purchased from TCI America (Portland, OR). Previously isolated and characterized samples of γ-thujaplicin, β-thujaplicinol, methyl thujate, and thujaplicatin methyl ether were available from research conducted at this laboratory (12–16). Plicatic acid was isolated by liquid–liquid extraction of the hot water extract of redcedar heartwood with ethyl acetate. The water was evaporated to dryness yielding chromatographically pure plicatic acid (15). The para bromophenacyl ester of crotonic acid was used as an internal standard for the chromatographic method (17).

Sample collection and preparation

A 15-cm disk was taken from breast height of 13 second growth trees from two coastal populations on southern Vancouver Island, and from two interior populations in the North Okanagan, British Columbia, varying in age from 40 to 125 years old. A radial section, 1-cm thick from pith to bark, was cut from the disk. Two 1 cm × 1 cm × tree radius samples were cut from the center section of the disks radial section. Each of the two samples was divided into identical five-ring blocks from the heartwood / sapwood boundary to the pith. Blocks, 1 cm × 1 cm × 5 rings, were recorded as H1-5, H6-10, H11-15, etc. from the heartwood/sapwood boundary to the pith (Figure 1). The two sets of edge-matched blocks were used for chemical analysis, and moisture content determination. The volume of each sample was recorded so that analyses could be expressed as concentration–volume and related to future analyses using increment cores as a non-destructive sampling method. The blocks for chemical analysis were cut into slivers for extraction. Moisture content was calculated on the oven-dried (at 105°C) sample weight. One gram samples were extracted with 10 mL of ethanol containing 200 µg/mL of crotonic acid *p*-bromophenacyl ester as the internal standard in 20-mL pyrex vials. The extraction was carried out in an ultrasonic bath for 2 h, during which time the bath temperature rose from approximately 20°C to a final temperature of 40°C.

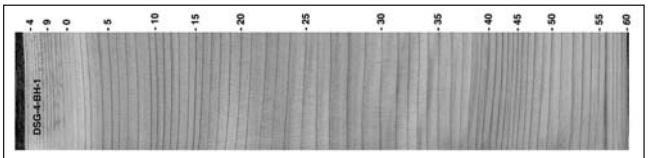

Figure 1. Radial section of Western redcedar tree used for analytical and moisture content samples. Zero is sapwood/heartwood boundary.

Calibration standards

Calibration curves were constructed for each component of interest to cover the expected concentration found in the heartwood of a typical tree (1, 2). Calibration standards were prepared in ethanol containing 200 µg/mL of internal standard (Table I). Multilevel calibration using the internal standard method was performed with ChromQuest software.

Chromatography

Chromatographic separation was achieved with an Inertsil

Table I. Calibration Standards µg/mL

	Level 1	Level 2	Level 3	Level 4
Plicatic acid	10.0	40	200	1000
Thujaplicatin methyl ether	5.0	20	100	500
γ-Thujaplicin	5.0	20	100	500
β-Thujaplicin	5.0	20	100	500
β-Thujaplicinol	5.0	20	100	500
Thujic acid	5.0	20	100	500
Methyl thujate	2.5	10	50	250

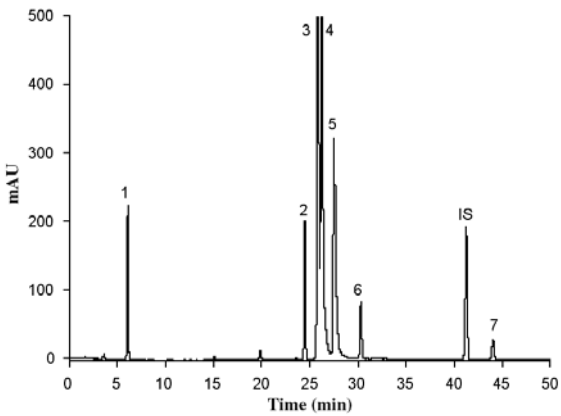

Figure 2. Calibration standard (level 3): plicatic acid, 1; thujaplicatin methyl ether, 2; γ-thujaplicin, 3; β-thujaplicin, 4; β-thujaplicinol, 5; thujic acid, 6; internal standard, IS; and methyl thujate, 7.

4.6 × 150 mm ODS3 3 µ column at 50°C. The mobile phase consisted of: mobile phase A, water–0.1% phosphoric acid (w/w, 85%) and mobile phase B, acetonitrile. Gradient elution from 10% B to 60% B in 50 min at a flow rate of 1.0 mL/min was used for all analyses. Injection volume was 10 µL. The injection volume was reduced to 5 µL where components exceeded the calibrated range. The limit of quantitation (LOQ) for the method was 200 µg/g of oven dry wood for each compound analyzed. The analytes were monitored at 230 nm, and spectra were collected from 200–360 nm (Figures 2 and 3). The spectral analysis component of ChromQuest was used to verify peak identity.

Statistical analysis

Volume-weighted averages for each five growth ring annulus were calculated, and these values were used to calculate the average level of each compound in the heartwood of the tree, assuming the tree was perfectly cylindrical. Values were expressed as µg/g of heartwood from the sapwood–heartwood boundary to the pith. Simple statistics on data from coastal and interior regions were calculated for each compound, and t-tests were performed for determining significance between means (18).

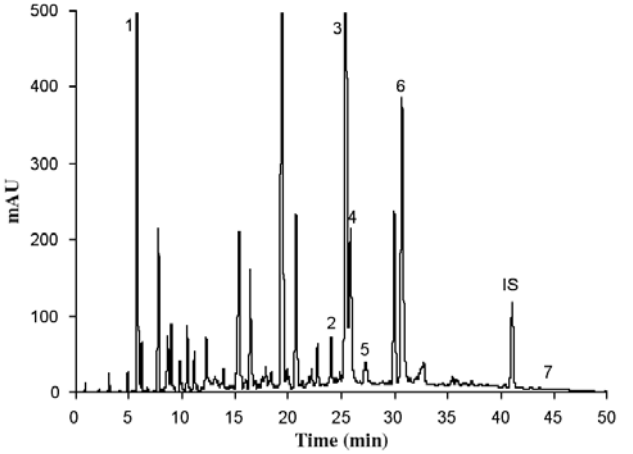

Figure 3. Typical ethanol extract of western redcedar heartwood: plicatic acid, 1; thujaplicatin methyl ether, 2; γ-thujaplicin, 3; β-thujaplicin, 4; β-thujaplicinol, 5; thujic acid, 6; internal standard, IS; and methyl thujate, 7.

Results and Discussion

There was considerable variation between 5-ring groups within a tree. Overall, the concentration of extractives was highest in the first third to half of the heartwood, gradually decreasing toward the pith of the tree (Figure 4). For three coastal trees exhibiting visible white rot decay in the inner heartwood, the level of methyl thujate was observed to increase from outer heartwood to inner heartwood, while thujic acid decreased, suggesting the methylation of thujic acid to form methyl thujate either by an organism or by the tree itself in response to the attack (Figure 5). Thus, although methyl thujate has not been reported as fungitoxic, it appears to be related to decay in some way. Methyl thujate has long been thought to be the main component of the odor in western redcedar and a substantial component of the heartwood; however, in many of the apparently healthy trees methyl thujate was not detected at all or occurred at very low levels. In addition, with trees exhibiting brown rot, the level of all extractives dropped to essentially zero within one or two growth rings of the decayed area, and the level dropped dramatically 10–30 growth rings from areas of visible white rot.

Variation in the level of all heartwood compounds between trees was high, with coefficients of variation (CV) ranging from 32% to 221% for coastal and 26% to 189% for interior (Table II). Gamma thujaplicin varied from 603 to 3659 µg/g and 526 to 2901 µg/g for coastal and interior trees, respectively, while β-thujaplicin varied from 361 to 4209 µg/g and 283 to 2024 µg/g. The ratio of γ-thujaplicin to β-thujaplicin ranged from 0.18 to 6.01 and from 0.09 to 3.81 for coastal and interior trees, respectively, (Table II). Substantial levels of phenotypic variation between western redcedar trees as shown here in conjunction

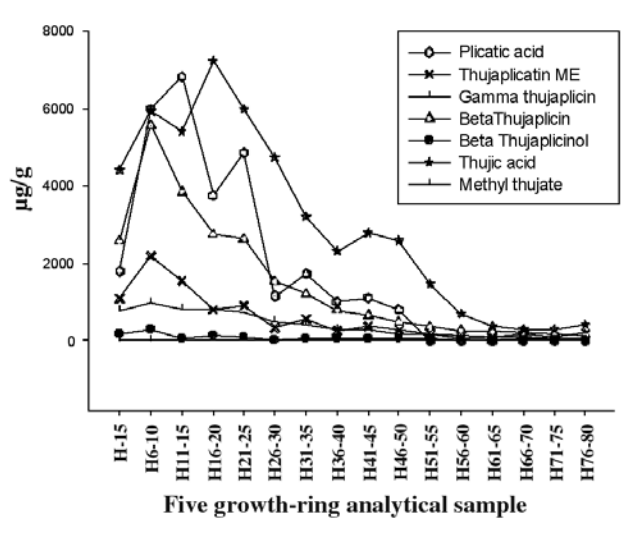

Figure 4. Radial distribution of compound in a typical tree.

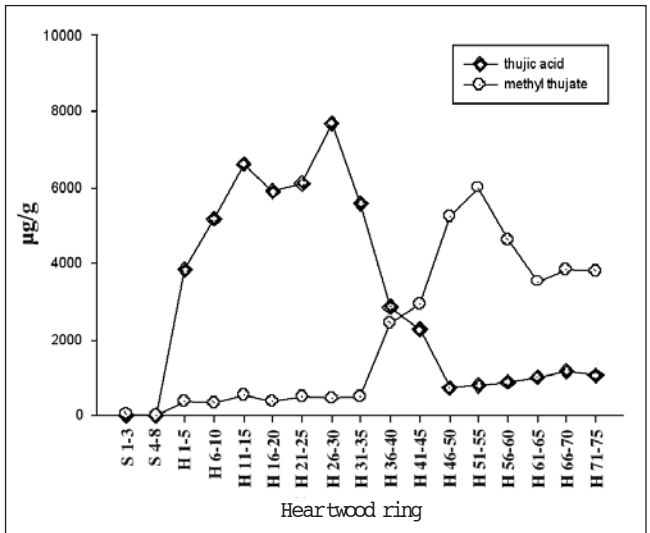

Figure 5. Thujic acid–methyl thujate relationship in tree with visible decay.

Table II. Volume Weighted Heartwood Averages µg/g

Compound (µg/g)	Coastal				Interior				t-Test
	Mean	Min	Max	CV (%)	Mean	Min	Max	CV (%)	
Plicatic acid	8532	836	21527	88	10050	1415	21934	75	ns*
Thujaplicatin methyl ether	1085	326	2957	60	1028	386	2020	50	ns
γ-Thujaplicin	1829	603	3659	49	1963	526	2901	36	ns
β-Thujaplicin	1595	361	4209	73	892	283	2024	64	†
β-Thujaplicinol	352	25	1027	87	406	86	920	57	ns
Thujic acid	5784	3189	8520	32	4583	2768	6437	26	ns
Methyl thujate	224	0	1803	221	5.14	0	32	189	‡
β–γ ratio	1.32	0.18	6.01	126	0.68	0.09	3.81	139	§

* ns = not significant.
† $p < 0.01$.
‡ $p < 0.001$.
§ $p < 0.05$.

Table III. Ratio of γ-Thujaplicin–β-Thujaplicin

Coastal		Interior	
Tree	Ratio	Tree	Ratio
DSG-1	3.37	HL-1	0.13
DSG-2	0.47	HL-2	0.61
DSG-3	0.69	HL-3	0.23
DSG-4	0.52	HL-4	1.17
DSG-5	0.44	HL-5	0.31
DSG-6	6.02	HL-6	0.19
YP-1	0.75	HL-7	0.09
YP-2	0.97	MC-1	0.29
YP-3	0.30	MC-2	0.53
YP-4	0.26	MC-3	0.90
YP-5	0.18	MC-4	3.81
YP-6	1.94	MC-5	0.52
YP-7	1.25	MC-6	0.39
	MC-7	MC-7	0.38

with high heritabilities reported for bioactive heartwood compounds with other conifer species (19), suggests that a genetic selection program for western redcedar durability is potentially feasible.

Mean differences between coastal and interior trees were significant for β-thujaplicin, methyl thujate, and the ratio of γ-thujaplicin to β-thujaplicin (Table III). The greater mean ratio of γ-thujaplicin to β-thujaplicin for coastal trees was a direct consequence of a higher mean concentration of β-thujaplicin. There was considerably more decay in the heartwood of interior trees (20), and this could be related to the lower levels of β-thujaplicin as compared to coastal trees.

Conclusion

The concentration of extractives was highest in the first third to half of the heartwood gradually decreasing toward the pith of the tree. Trees with visible signs of decay showed dramatically decreased extractive content both within and around the decayed regions. The relationship between susceptibility to fungal attack and extractive content is not clearly understood. Future research will need to be conducted to determine the threshold for individual compounds where no fungal growth occurs. Synergistic effects will also need to be investigated. The elevated levels of methyl thujate in trees exhibiting decay deserves further investigation.

Substantial tree-to-tree phenotypic variation is a good indication that a genetic selection program for producing western redcedar trees with improved durability is feasible. This seems particularly important in areas where decay is more prevalent and/or levels of fungi toxic compounds are historically low. Future research will be conducted on parent trees in the western redcedar tree improvement program using increment cores as a non-destructive sampling method and analyses based on concentration/volume of wood.

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