Forintek Canada Corp.
Western Division
2665 East Mall
Vancouver, BC
V6T 1W5

Maximizing Natural Durability of Western Red-cedar: Beyond Thujaplicins

by

Jean Clark
Mycological Technologist
Durability and Protection

Bob Daniels
Wood Chemistry Analyst
Durability and Protection

Paul Morris
Group Leader
Durability and Protection

Prepared for

Recipient Agreement Number: R04-013
Research Program

Date: March 2004

C. R. Daniels
Acting Project Leader

Janet Ingram
Reviewed

Jean Cook
Department Manager

Summary

Western red-cedar (WRC, *Thuja plicata* Donn ex D. Don) wood was extracted sequentially with six solvents using two extraction methods. The extracts were prepared for subsequent bioassay and analysed by high performance liquid chromatography for known bioactive compound concentrations.

To focus identification of the extractives on those with bioactive properties, it was necessary to develop a micro-bioassay that would allow the biological activity of the unknown compounds present to be determined using minute quantities of each extracted constituent. The initial proposed technique utilised the loss of birefringence that occurs when decay fungi disrupt the crystalline cellulose structure as wood decays. Microtome sections of perishable sapwood were treated with microgram amounts of *T. plicata* heartwood compounds prior to exposure to decay fungi. The efficacy of the applied extract was then to be measured relative to the birefringence loss in untreated pine sapwood.

Validation of the technique required standardisation of a number of variables. Over 600 thin sections of ponderosa pine sapwood were cut and exposed to three different fungi, plus non-infected controls, under varying conditions of section thickness and orientation, media and growth conditions, viz, on grids or sterile microscope slides, with and without cover-slips, and with and without supplemental nitrogen, for six different incubation periods. Ultimately it was decided to test the extractives with two standard test brown rot fungi, *Coniophora puteana* and *Postia placenta*, using 25µ radial sections which were sterilised, dipped in Abrams's nutrient solution, and placed on a microscope slide prior to infection and incubation at 25 C, with a cover-slip placed over the inoculated section.

Analysis of the loss in birefringence was problematic and eventually abandoned due to time constraints. Towards the end of the project it was determined that the polarising filters in the microscope being used had been improperly manufactured. As a result, it was impossible to achieve 100% polarisation and we therefore could not examine loss in birefringence.

A commonly used antibiotic sensitivity test was modified to examine fungicidal efficacy of the extracts. Using 24-well tissue culture plates, four fungi were inoculated onto four media; one cellulose-based media was selected for use with *Perenniporia subacida* and *Cephaloascus albidus* isolated from a decaying second-growth WRC tree. Microlitre amounts of four known compounds dissolved in ethanol at three concentrations, plus the reference compound pentachlorophenol (PCP) and solvent controls, were pipetted onto paper disks and tested against the two fungi. Two of the extracts, ß-thujaplicin and thujic acid, plus PCP, were inhibitory to the fungi, verifying the methodology.

However, HPLC analysis of additional treated disks indicated that there was a substantial loss of chemical on the substrate over relatively short periods of time. This would indicate that the compounds were probably effective at lower concentrations than the targeted concentration. Volatilisation or decomposition when exposed to air and/or light was the likely cause of the observed mass loss of compounds. Recent tests have focused on the minimum time required for the ethanol solvent to evaporate when the extract is dispensed, so that disks may be rapidly moved onto the agar surface of the wells without any additional fungal toxicity from residual ethanol. A ventilation period of 30 minutes appears to be adequate for this. In addition, wood disks were found to be more effective than cellulose disks. In well tests, fungi grew well when wooden disks with ethanol were ventilated for 30 minutes. This test method will be used to bioassay isolated compounds.

Acknowledgements

The authors would like to acknowledge the funding provided by Forestry Innovation Investment in support of this research. Dr. Young Woon Lim of the Faculty of Forestry, University of British Columbia provided two of the fungal cultures used. The BC Ministry of Forests, and in particular Dr. John Russell, are thanked for providing funding for developmental stages of this research, test material, and encouragement throughout the process.

Table of Contents

List of Tables

List of Figures

1 Objectives

1. To develop a novel micro-bioassay to determine the wood preserving activities of all known and unknown (i.e., structurally uncharacterized) extractives in western red-cedar heartwood.
2. To determine the wood preserving activity of each distinct extractive present in western red-cedar heartwood.

2 Introduction

2.1 Rationale for the work

The total export value of western red-cedar (WRC, *Thuja plicata* Donn ex D. Don) products in British Columbia is $750 million, however, much of this value is predicated on a single attribute, its natural durability, which we do not fully understand. Second-growth WRC represents an increasing proportion of the annual allowable cut but its durability is under question. More than twelve million WRC seedlings are planted in British Columbia every year, but we do not know how resistant they will be to heart-rot and how durable the derived wood products will be. We do know that non-decay fungi invading standing trees can detoxify the known naturally-occurring protective chemicals, opening the way for heart-rot fungi to degrade the standing fibre. These non-decay fungi also create variability in the durability of the lumber product. We also know that second-growth WRC heartwood contains lower amounts of the known protective chemicals and in some cases higher amounts of as yet unknown extractives. For some second-growth trees there is a correlation between the amounts of known protective chemicals and durability, while for other trees the correlation breaks down, conceivably due to the presence of unknown bioactive compounds. The present lack of knowledge regarding the full spectrum of bioactive WRC heartwood constituents makes it difficult to rapidly screen propagation of planting stock for durability.

Presumably, one or more of the unknown compounds contribute substantially to the natural durability of WRC. It is also possible that these unknown compounds contribute to controlling the growth of fungi that are capable of detoxifying WRC heartwood constituents. Determining which of these unknowns are biologically active is the critical step towards understanding how natural durability works, and how to maintain and enhance it.

There has been a massive resurgence of interest in naturally durable wood species across the world over the last five years. Canada possesses the world's largest resource of naturally durable softwoods. For example, 82% of North America's saw-timber inventory of WRC grows in Canada. Research on bioactive WRC metabolites has been limited and fragmented over the past twenty years despite the fact that analytical instrumentation and chemical methods have advanced considerably.

Forintek was the driving force behind the founding of the Cedars Working Group (CWG) in June 2000. The group was formed to exchange information and foster synergistic research relationships focusing on

the properties and products of the North American cedar resource. The genesis of the CWG came from the recognition that major gaps exist in the understanding of cedar biochemistry, genetics and decay resistance. There are 16 interested clients from industry, universities, not-for-profit corporations/ associations and government research organisations as members of the CWG. The most recent meeting of this group was at Forintek on January 22, 2004. The minutes of this meeting are in Appendix F; a CD containing the presentations from that meeting is included with this report.

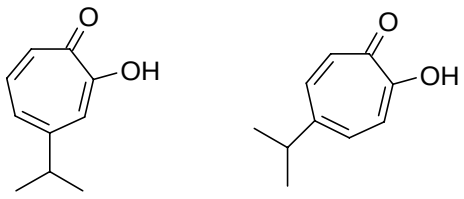
With the goal to identify prospective decay-resistance-plus trees for entry into a silviculture program, an existing collaborative project between Forintek and the British Columbia Ministry of Forests (BCMOF) is attempting to correlate the durability of *T. plicata* propagation stock and progeny with the chemical composition and content of the heartwood. The project is severely hampered, however, by a lack of chemical information on the entire range of potentially fungitoxic heartwood constituents. To address this problem, Forintek has developed HPLC methodology to quantitatively analyze small samples of *T. plicata* (eg, core borings) for α-, ß- and γ-thujaplicin, ß-dolabrin, nezukone, thujic acid, methyl thujate, plicatic acid, and thujaplicatin methyl ether, as well as ten structurally unidentified *T. plicata* metabolites.

Knowledge of which extractives have a role in durability will aid BCMOF in silvicultural selection of trees with high levels of these extractives. Using the methodology developed at Forintek, prospective plus trees can be analysed for extractive makeup prior to entry into the silvicultural program.

2.2 Background

2.2.1 Durability of Western Red-Cedar Heartwood

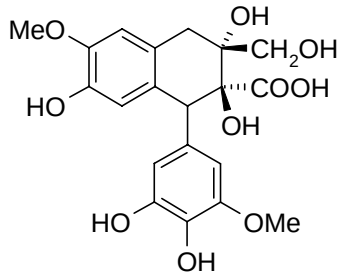
The decay resistance of WRC heartwood is commonly attributed to naturally-occuring fungitoxic compounds which are extractable from the fibrous wood matrix using organic solvents or hot water (Barton & Macdonald 1971). Accelerated decay experiments demonstrated that the pattern of heartwood decay resistance paralleled the variation in concentrations of natural fungicides determined analytically, particularly ß- and γ-thujaplicin (structures **1** and **2**, respectively), and led to the conclusion that the decay resistance of *T. plicata* is mainly due to the presence of these two tropolones (Rennerfelt 1948; Rudman 1962, 1963). This conclusion was supported by Jin *et al.'s* (1988a, 1988b) chemical and biological evidence demonstrating that fungi capable of metabolizing the thujaplicins compromise the decay resistance of *T. plicata* heartwood.



1 2

Earlier work by van der Kamp (1986), recent work by DeBell *et al.* (1997, 1999), as well as current work by Forintek, has brought into question the primary role attributed to ß- and γ-thujaplicin by Jin *et al.* (1988a), as well as others (Nault 1988), in providing decay resistance to WRC heartwood, both in living trees, as well as wood in-service. DeBell *et al.* (1997, 1999) demonstrated that while the presence of high levels (> 0.8% dry weight) of thujaplicins always correlate with fungal decay resistance, *T. plicata* heartwood containing low tropolone levels (< 0.25%) is also resistant to decay, albeit with highly variable results. Unpublished Forintek research has also shown that at a distance of 10 to 20 annual growth rings from visible stain or decay in some standing second-growth trees, the thujaplicin concentrations approach zero, while in other trees, the same compounds occur in significant amounts directly adjacent to the stained or decayed zone.[1] These results, when considered in combination with the presence of many unassigned peaks in HPLC chromatograms of *T. plicata* heartwood extracts, suggest that other (perhaps unidentified)[2] *T. plicata* metabolites also contribute to decay resistance.

Nearly fifty years ago Roff and Atkinson (1954) reported a water-soluble, non-volatile, polyphenolic *T. plicata* heartwood extract fraction – from which the thujaplicins had been carefully removed, and representing 4-5% of the oven dry heartwood weight – that possessed fungistatic properties.[3] While it is possible that the fungistatic activity of this fraction was due to plicatic acid (structure **3**) or one of the other lignans that have been subsequently characterized from *T. plicata* heartwood, the number of unknown peaks observed in HPLC chromatograms of heartwood extracts recently generated by Forintek suggest that at least ten novel (potentially biologically active) compounds remain to be characterized from WRC.[4]



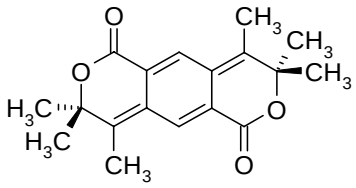
3

Isolation and identification of novel biologically active compounds from *T. plicata* heartwood, using bioassay-guided techniques coupled with modern isolation and molecular structure determination methods, represents the core of a proposed three-year project at Forintek.

[1] C. R. Daniels, Forintek Canada Corp., personal communication.
[2] Data recently generated by Forintek suggests that one unknown heartwood constituent may be a novel tropolone.
[3] Inhibited *Poria monticola* Murr. at a concentration of 2% in malt agar. Zinc chloride gave the same activity at 1% concentration.
[4] C. R. Daniels, Forintek Canada Corp., personal communication.

2.2.2 Microbial Colonization Sequence and Detoxification of *T. plicata* Metabolites

Information regarding the microbial deterioration of in-service *T. plicata* heartwood is generally lacking; only a few studies have been conducted on fungal species isolated from WRC utility poles (Scheffer *et al.,* 1984; Eslyn & Highley 1976, Morrell *et al.,* 2001), shingles and shakes (Smith 1970, Smith & Swan 1975, Setliff & Chung 1985). Additionally, in collaboration with the University of British Columbia, an intensive study on living, standing, trees was carried out by Forintek during the 1980's. In this study, Jin *et al.* (1988a) confirmed the hypothesis of MacLean and Gardner (1956a), wich had been expanded upon by van der Kamp (1975), that successive microbial colonization of standing *T. plicata* correlates with the metabolism/detoxification of *T. plicata* metabolites present in the heartwood. Jin *et al.* (1988a) demonstrated that colonization of living *T. plicata* involves a succession of microorganisms, beginning with a *Sporothrix* sp. capable of metabolizing ß- and γ- thujaplicin to thujin (structure **4**), followed by *Kirschsteiniella thujina* (Peck) Pomerleau & Etheridge, and finally by a *Phialophora* (DAOM 196486) species [initially identified as a *Cylindrocephalum* species by van der Kamp (1975)].[5]

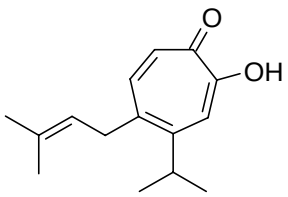


4

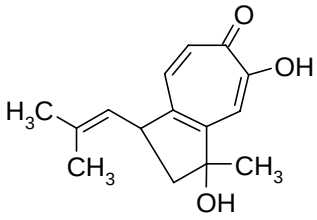
At present, it is not clear which microorganisms are able to grow on WRC wood in-service, or infect the standing trees. In order to design adequate measures to increase the durability of *T. plicata* products, it is imperative to generate comprehensive data on the microbial agents that cause failure.

It is anticipated that the chemical and microbiological methodology developed during this research on the chemical basis for WRC durability will be readily transferable to other durable cedar species, particularly eastern white-cedar (*Thuja occidentalis* L.) and yellow-cedar [*Chamaecyparis nootkatensis* (D. Don) Spach]. It has been found that α-, ß-, and γ-thujaplicin are present in eastern white-cedar heartwood (MacLean & Gardner 1956b, Behr 1976), and that yellow-cedar contains, in addition to chamic and chaminic acids, the tropolone derivatives nootkatin, and chanootin (structures **5** and **6**, respectively) (Barton 1976). Yellow-cedar is one of the most durable of the North American softwoods (Perry 1954, Barton 1976).

[5] It should be noted, in this context, that the fungal isolation conducted by Jin *et al.* (1988a) was carried out using a single 420 year-old WRC and did not address the initial mode of entry of the various fungi into the tree. Consequently, one may conclude that Jin *et al.*'s (1988a) results are limited and do not necessarily represent a general trend for decay of *T. plicata* heartwood by a succession of microorganisms.



5



6

2.3 Work Done in This Project

Research undertaken in the project reported here focuses on filling a key data gap in decision-making related to marketing of WRC wood products, selection of planting stock, and stand management. That is, identifying which of the unknown compounds detected in WRC heartwood are biologically active (capable of inhibiting the growth of wood-colonizing fungi) and thereby contribute to the natural durability of this important British Columbian forest resource.

Specifically, this research covers the preparation of extracts from previously characterized heartwood material; isolation and structure elucidation of the unknown bioactive compounds will be addressed in a future study. To evaluate bioactivity, a micro-bioassay for assessing naturally occurring chemicals responsible for the fungal decay resistance of WRC heartwood was developed and validated. The main requirement for the bioassay was that it be fast, at least semi-quantitative and require only a small amount of chemical. To verify the role of individual extractives, the very small quantities of extractives available were screened for fungicidal efficacy. This will assist in providing an answer to the key question of why the durability of WRC cannot be correlated with content of the known fungitoxic extractives.

The first objective of the project has essentially been achieved. There were major problems (detailed in 5.2.1) with the original proposed birefringence bioassay technique that necessitated abandoning it; thus a second method had to be developed. Delays were caused partly by equipment problems and a partly by a late start on the project. Verbal confirmation of funding was received on August 6, 2003 rather than April 1, 2003 as anticipated when the proposal was submitted. The second technique developed, a Multiwell agar test, can be utilised to screen both known compounds and solvent fractions containing mixtures of unidentified and uncharacterised compounds.

A number of unexpected results were obtained during development of the second bioassay method and resolving these issues took longer than anticipated. Most significant among them was the finding that mass loss of compounds occurred rapidly on a cellulose substrate when exposed to air and light. This could provide an answer for the many anomalous results reported in the literature.

Due to the additional effort required to achieve Objective 1, less progress was able to be made toward achieving Objective 2. Fractions were obtained using six solvents extracted by both Soxhlet and ultrasonic methods, and comparison of the extracts from the two methods was done. Fractions were prepared for bioassay but this could not be completed within the available time frame after the bioassay

method was developed. Additionally, larger quantities of the fractions have been prepared for the isolation of unknown compounds. This work was to have been directed by bioassay results but these were unavailable prior to the project funding termination on March 31, 2004.

3 Staff

Stephen Ayer	Organic Wood Chemist
Bob Daniels	Wood Chemistry Analyst, Interim Project Leader
Jean Clark	Mycological Technologist
Tony Vlachos	Durability and Protection Technologist
Paul Morris	Group Leader – Durability and Protection Group
Simmy Mukhija	Junior Mycological Technologist
David Liu	Visiting Scientist

4 Materials And Methods

4.1 Extractive Chemistry

4.1.1 Materials

All solvents were HPLC reagent grade with the exception of water and ethanol. Water was prepared using a Millipore RO system with a five-bowl MilliQ Plus system for final purification. Ethanol was purchased as absolute ethanol and was distilled in glass prior to use for extraction.

The old-growth and second-growth WRC wood used in this study was supplied by Dr. John Russell of the B.C. Ministry of Forests. Only heartwood was selected for extraction and the innermost 20-30 rings were discarded, as these rings contain much less extractive than the outer heartwood. The wood was ground in a Wiley mill to pass a forty-mesh screen.

The known compounds, plicatic acid, ß-thujaplicin, γ-thujaplicin, ß-thujaplicinol, thujic acid and methyl thujate, were selected for the development of the bioassay method. All were previously isolated from WRC heartwood at Forintek or its predecessor, the Western Forest Products Laboratory, with the exception of ß-thujaplicin which was purchased from TCI America. Pure thujic acid was prepared from its methyl ester by alkaline hydrolysis, and the free thujic acid was re-crystallized from hexane. Pentachlorophenol (PCP) was selected as a reference fungicide.

High performance liquid chromatography (HPLC) analyses were performed with a Thermo Finnigan HPLC equipped with a diode array detector (details of chromatographic method in press).

Extractions were accomplished with standard Soxhlet apparatus and an ultrasonic bath (Cole-Parmer).

Treated substrates were cellulose (Whatman #1 filter paper or Schleicher & Schuell ¼ inch antibiotic assay discs), Zitex (Teflon filter membrane), unknown hardwood (coffee stir stick), and spruce heartwood.

4.1.2 Methods

WRC wood meal, 330 grams in oven dry weight, was extracted sequentially with hexane, dichloromethane, ethyl acetate, ethanol, methanol and water using a 1L Soxhlet apparatus. A portion of WRC wood meal, 764 grams (oven dry weight), was also extracted in an ultrasonic bath at ambient temperature with the same solvent sequence. The two sets of extracts were compared for percent extractives and each of the twelve extracts analysed by HPLC to ensure that the extraction methods were approximately equivalent and that no artifacts (decomposition products) were formed. Significant differences between the two extraction methods would have indicated possible degradation of compounds. A portion of each extract was dissolved in ethanol to make a 10 mg/ml solution for subsequent bioassay.

Approximately 1.5 kg of old-growth wood and 1.5 kg of second-growth wood was extracted to provide the larger quantities of extract required for the isolation of unknown compounds and known compounds in short supply. The wood meal was extracted with hexane followed by dichloromethane in a 12L Soxhlet apparatus.

Initially five reference compounds, plicatic acid, ß-thujaplicin, thujic acid, methyl thujate, and PCP were selected for bioassay testing based on their suspected range of activity and availability. The five compounds were dissolved in ethanol to make the treating solutions for the bioassay disks. The compounds in ethanol solution were deposited on cellulose disks (S & S antibiotic assay discs) with an area of 0.3167 cm^2. Treatment levels are presented in 5.1, Table 4.

4.2 Bioassay

The original concept for the micro-bioassay for assessing naturally-occurring chemicals responsible for the fungal decay resistance of WRC heartwood was based on current understanding of wood-fibre decay by fungi, photomicroscopy, and by adaptation of computer-based imaging technology. The technique would allow the biological activity of the unknown compounds present in WRC to be determined, using minute quantities of each extracted constituent.

4.2.1 Birefringence Assay

Brown-rot decay is the most common and most destructive type of decay of wood in-service (Green III & Highley, 1997). When brown-rot fungi such as *C. puteana* decay wood, free radicals and extracellular cellulase are produced by the fungus to catalyze the conversion wood cellulose to glucose, with the net result being disruption of the crystalline cellulose matrix present in the S2 layer of the wood cell wall. This disruption of the crystalline cell wall material results in a loss of birefringence and the decayed cell wall, which, when viewed with a compound microscope and polarized light, will be darker than wood samples with non-disrupted cellulose (Anagnost, 1994, 1998; Wilcox, 1993a, 1993b). This loss of birefringence was to be utilized in the development of a rapid fungal wood-decay bioassay capable of evaluating milligram amounts of test sample.

4.2.1.1 Development and Validation of a Rapid Micro-bioassay

The proposed method for examining the fungicidal efficacy used microtome sections of perishable sapwood, microgram amounts of *T. plicata* heartwood extracts, chromatography fractions, and purified compounds applied to wood prior to exposure to decay fungi. The protective effect was then to be measured relative to the birefringence loss in untreated pine sapwood. Photomicroscope-based computer-aided image analysis was to be used to semi-quantify the amount of decay by measuring the percentage of the viewed area above and below a predetermined grey level.

An initial test using two brown rot fungi, *Postia placenta* (Fr.)Cke., Ftk 120F and *C. puteana* (Schum. ex Fr.) Karst., Ftk 9G, plus non-inoculated controls, was run to determine how well the fungi would grow on thin sections of untreated ponderosa pine (*Pinus ponderosa* Laws.) sapwood. Sterile plastic petri plates (Fisherbrand) containing 1.5% malt, 2% agar (Difco) were prepared, and thin sections of pine sapwood were placed between layers of Whatman #1 filter paper, moistened with distilled water and sterilised by autoclaving.

Two growth methods were examined. In the first, two sterile polypropylene grids, 6 x 6 x 0.22 cm (Cole Parmer), were placed on top of the agar and the plates centrally inoculated with one of the two fungi. Sterile pine thin sections were placed on the top grid, the plates sealed with Parafilm “M” laboratory film (American National Can) and incubated at 20 C.

The second growth method involved placing one sterile grid on the agar surface, then topping with a sterile microscope slide. Sterile pine thin sections were placed on the slide and topped with a sterile cover-slip positioned so one edge of the section was at the edge of the cover-slip. A very small piece of agar infected with one of the two test fungi was placed adjacent to the section edge. Plates were sealed with Parafilm “M” and incubated at 20 C.

After three weeks’ incubation, sections were removed, mounted on clean microscope slides with a drop of lactophenol cotton blue and a cover-slip, and observed with a compound microscope under brightfield and differential interference contrast illumination.

Having determined that it was possible to decay thin sections, a second test was initiated to optimise the test. A number of variables needed to be standardised as the micro-bioassay method was developed. The

main emphasis was on the growth method – whether to use sections on grids over infected malt agar or sections on sterile slides over water agar (2% agar, Difco); with or without cover-slips over sections on slides; whether there was a need for supplemental nitrogen (as a three second dip in Abrams nutrient solution - see Appendix A - followed by three seconds draining) or water on the section; and how long to incubate the test before removing sections for observation and analysis. A diagram detailing the combinations of variables examined is included in Appendix B. Ultimately three fungi were tested, *C. puteana*, *P. placenta* and *Perenniporia subacida* (Pk.)Donk.; the latter isolated from a decaying second-growth WRC tree. At this time we also purchased and accepted delivery on a research microscope with several illumination options including polarised light and a camera and image analysis system that was to be used to measure birefringence brightness.

4.2.2 Multiwell Test

Problems with the technique (covered under Results and Discussion 5.2.1) and time constraints forced temporary abandonment of the birefringence analysis technique. Because of the time remaining in the project and the need to generate efficacy data on identified extractives it was decided to modify an antibiotic sensitivity test wherein paper disks with known antibiotic loadings are exposed to bacteria and growth inhibition measured.

In the modified version the emphasis was placed on reducing the amount of extractive that would be required for a test. Using pre-sterilised 24-well polystyrene plates with a close fitting lid, (Multiwell, Becton-Dickson), 2-ml aliquots of media could be dispensed into the 3.6-ml wells. The test fungus was inoculated onto one side of the agar surface and a sterile paper disk with a measured amount of extractive placed on the opposite side. Initially, Whatman #1 filter paper disks (0.3318 cm^2) were used; later tests utilised Schleicher & Schuell ¼ inch antibiotic assay discs (0.3167 cm^2). Extractive concentrates were stored in the dark at –20 C and warmed to room temperature before use. Following a suitable incubation period, the amount of fungal growth across the agar and on the paper disk was rated relative to wells without disks, giving a measure of the inhibition attributable to the extractive. All testing was done in replicates of six except where noted.

4.2.2.1 Multiwell Test Development

In order to optimise the test method, a number of variables were examined, including growth media, test fungi, incubation time, and ability to determine a toxic threshold with known fungitoxic compounds.

In addition to the three fungi examined in the birefringence test, *C. puteana, P. placenta* and *P. subacida*, a fourth ascomycetous yeast, *Cephaloascus albidus* Kurtzman, also isolated from the decaying WRC tree, was evaluated. Identification of the two isolates from WRC had been confirmed by morphology and by large subunit DNA sequencing. The growth habit of these four cultures was evaluated on the four agar media being tested, as well as the time it took for the fungus to grow across the diameter of the well. The four media tested were Eggins and Pugh cellulose media without and with supplemental carbon (Appendix C), 1.5% malt agar (Appendix D) and Abrams nutrient media with trace metals (Appendix A).

A sterile non-treated paper disk (Whatman # 1 filter paper) was included as one of the variables. Also included in the testing were paper disks dipped in ethanol and vented for six days. Ethanol was the

solvent used to dilute the extractives and it was necessary to determine whether there was any residual toxicity in the disks from the solvent after venting.

Sterile paper disks (Whatman # 1 filter paper) charged with 1000 μg/cm^2 of ß-thujaplicin and vented for four to six days were also tested; it was anticipated that this level of the extract would prove toxic to the test fungi, thereby verifying the methodology. ß-thujaplicin was supplied at 5000 μg/ml in ethanol. Sterile disks were placed on the tip of a stainless steel pin while charging with chemical to avoid loss of chemical on a support structure such as a grid.

This test was done in replicates of three. Growth in the wells was rated at both 6 and 12 days following incubation at 25 C. A rating system was devised to describe the fungal growth.

4.2.2.2 Multiwell Method Confirmation

Using Eggins and Pugh media with supplemental carbon and *C. albidus* and *P. subacida* as the test organisms, a second test was done to examine whether it was possible to determine a toxic threshold for ß-thujaplicin and PCP, a known fungicide often included as a reference chemical in previous literature reports of WRC extract toxicity.

Both compounds were supplied at 5000 μg/ml in ethanol and tested at 100, 500 and 1000 μg/cm^2. Sterile disks (S & S antibiotic assay discs) were placed on the tip of a stainless steel pin while charging with chemical to avoid loss of chemical on a support structure such as a grid. Disks with chemical were vented for three days before placing in wells with fungi.

The wells had been inoculated with one of the fungi and incubated for two days prior to placing the treated disks on the agar surface. Well plates were then incubated at 25 C. Well plates infected with *P. subacida* were incubated for 11 days prior to assessing growth; those infected with *C. albidus* were incubated 27 days before rating.

4.2.2.3 Multiwell Analysis of Identified Extractives

The test followed the methodology outlined in 4.2.2.2 above. Methyl thujate and plicatic acid were tested at 1500, 2000 and 2500 μg/cm^2, using stock solutions supplied at 5000 μg/ml in ethanol. Thujic acid was also tested but because of the limited amount of extract available, it was only tested at 1183 and 1577 μg/cm^2, using a stock solution of 5000 μg/ml in ethanol. Well plates infected with *P. subacida* were incubated for 11 days prior to assessing growth; those infected with *C. albidus* were incubated 27 days before rating.

Anomalous analytical results detailed in Results and Discussion 5.1 caused reconsideration of the vent time between application of extractives to disks and planting disks in wells. Subsequent to the testing detailed in 5.1, 15 μl of ethanol was added to sterile 6 mm-diameter disks of spruce heartwood. Disks were vented for 30, 60 or 90 minutes prior to being placed in wells pre-infected with either *C. puteana* or *P. subacida*. A set of control wells was infected and left without disks. The top of the well plate was sealed with plastic kitchen film (Glad® Cling Wrap, The Chlorox Company of Canada Ltd.) and the lid replaced. Plates were then incubated at 25 C for 11 days prior to assessing growth.

5 Results and Discussion

5.1 Extractive Chemistry

As can be seen from the results in Tables 1 and 2, Soxhlet extraction is more thorough than ultrasonic extraction. The six Soxhlet and six ultrasonic extracts prepared for subsequent bioassay were analysed by HPLC (Appendix E, Figs. 1 – 12) and the results, expressed as a percentage of the extract, are shown in Table 3. The Soxhlet extraction method produced more suspected polymeric material with the most polar solvents, methanol and water, giving rise to a very broad peak from 15 – 40 min. as seen in Figs 10 and 12. Appendix E. The percentage of the known compounds in each extract is remarkably similar for both extraction methods with the exception of plicatic acid which is extracted less efficiently with ethanol and methanol but very efficiently with water using ultrasonic extraction.

Table 1: Extractive Yield by Solvent Using Ultrasonic Extraction

Extraction Solvent	Extractive (grams)	Extractive (%)
Hexane	1.3	0.2
Dichloromethane	13.7	1.8
Ethyl acetate	18.5	2.4
Ethanol	27.4	3.6
Methanol	32.3	4.2
Water	3.6	0.5
Total	96.8	12.7

Table 2: Extractive Yield by Solvent Using Soxhlet Extraction

Extraction Solvent	Extractive (grams)	Extractive (%)
Hexane	2.6	0.8
Dichloromethane	8.8	2.7
Ethyl acetate	15.6	4.7
Ethanol	14.7	4.5
Methanol	7.4	2.2
Water	6.2	1.9
Total	55.3	16.7

The hexane extract (10 g) from the second-growth WRC was subjected to liquid-liquid extraction with 2% sodium hydroxide. Pure (98%$^{+}$, 1.5 g) nezukone remained in the hexane layer while ß-thujaplicin, γ-thujaplicin, ß-thujaplicinol and thujic acid were extracted into the sodium hydroxide-water layer. Since substantial quantities of pure nezukone can now be isolated it will be included in future bioassays.

Table 3: Concentration of Known Compounds in Sequential Extracts Comparison Between Ultrasonic and Soxhlet Extraction

% of Extract	Hexane		DCM[1]		EtOAc[2]		Ethanol		Methanol		Water	
	US[3]	Sox[4]	US	Sox	US	Sox	US	Sox	US	Sox	US	Sox
Plicatic acid	0.00	0.00	0.00	0.00	22.28	22.15	22.28	41.75	34.93	48.47	59.56	15.14
γ-thujaplicin	0.30	0.74	0.48	0.42	0.00	0.00	0.00	0.12	0.09	0.05	0.00	0.05
ß-thujaplicin	11.48	16.55	15.41	13.50	2.31	2.57	2.31	0.07	0.51	0.05	0.04	0.00
ß-thujaplicinol	0.00	0.00	0.14	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00
Thujic acid	17.97	18.85	10.42	11.79	5.79	5.30	5.79	0.31	1.41	0.00	0.00	0.00
Methyl thujate	0.25	0.32	0.37	0.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Note:

1 Dichloromethane
2 Soxhlet extraction
3. ultrasonic extraction
4. ethyl acetate

A duplicate set of treated cellulose disks (S & S antibiotic assay discs), 0.3167 cm^2, had been prepared and left on a horizontal air bench for two months. It was decided to check the stability of the compounds on the unused cellulose disks. The disks were extracted in ethanol and analysed by HPLC, the results of which are presented below in Table 4.

Table 4: Recovery Levels From Cellulose Disks After 60 Days

	Solution Concentration (µg/ml)	Applied (µl)	Applied (µg)	Recovered (µg)	Recovery (%)
Pentachlorophenol	500	65	32.5	9.5	29.2
	500	65	32.5	10.7	32.9
	1000	65	65.0	28.6	44.0
	1000	65	65.0	23.8	36.6
	5000	65	325.0	238.0	73.2
	5000	65	325.0	242.0	74.5
ß-thujaplicin	500	65	32.5	3.4	10.5
	500	65	32.5	4.4	13.5
	5000	30	150.0	15.2	10.1
	5000	30	150.0	14.2	9.5
	5000	65	325.0	47.8	14.7
	5000	65	325.0	54.4	16.7
Methyl thujate	5000	95	475.0	13.4	2.8
	5000	95	475.0	13.8	2.9
	5000	125	625.0	25.0	4.0
	5000	125	625.0	15.0	2.4
	5000	160	800.0	29.8	3.7
	5000	160	800.0	36.6	4.6
Thujic acid	5000	75	375.0	229.0	61.1
	5000	75	375.0	209.0	55.7
	5000	100	500.0	248.0	49.6
	5000	100	500.0	203.0	40.6
	5000	100	500.0	176.0	35.2

	Solution Concentration (µg/ml)	Applied (µl)	Applied (µg)	Recovered (µg)	Recovery (%)
Plicatic acid	5000	95	475.0	112.0	23.6
	5000	95	475.0	142.0	29.9
	5000	125	625.0	214.0	34.2
	5000	125	625.0	206.0	33.0
	5000	160	800.0	220.0	27.5
	5000	160	800.0	260.0	32.5

These results indicate loss of compound by one or more of three mechanisms: 1) loss by evaporation (not an option for plicatic acid); 2) irreversible bonding to the cellulose surface, or; 3) decomposition/polymerization (probable in the case of plicatic acid).

To test if these results could be duplicated, a second set of treated disks was prepared using fresh solutions and two additional compounds. The treated disks were left on a horizontal air bench for 96 hours exposed to ambient light and temperature conditions after which they were analysed by HPLC. The results are presented below in Table 5.

Table 5: Recovery Levels From Cellulose Disks After Four Days

	Concentration (µg/ml)	Applied (µl)	Applied (µg)	Recovered (µg)	Recovery (%)
Pentachlorophenol	200	15.8	3.2	2.0	62.5
	1000	15.8	15.8	7.7	48.7
	1000	31.7	31.7	9.3	29.3
ß-thujaplicin	2000	15.8	31.7	16.5	52.1
	10000	15.8	158.4	59.4	37.5
	10000	31.7	316.7	121.3	38.3
γ-thujaplicin	2000	15.8	31.7	16.6	52.4
	10000	15.8	158.4	34.6	21.8
	10000	31.7	316.7	57.6	18.2
Methyl thujate	20000	39.6	791.8	36.1	4.6
	100000	15.8	1583.5	28.7	1.8
	100000	23.8	2375.3	50.1	2.1
Thujic acid	10000	31.7	316.7	64.2	20.3
	50000	12.7	633.4	251.4	39.7
	50000	19.0	950.1	563.0	59.3
ß-thujaplicinol	2000	15.8	31.7	6.5	20.5
	10000	15.8	158.4	33.9	21.4
	10000	31.7	316.7	61.4	19.4
	10000	63.3	633.4	66.8	10.5
Plicatic acid	50000	15.8	791.8	158.7	20.0
	50000	31.7	1583.5	494.6	31.2
	50000	47.5	2375.3	718.4	30.2

The results above verified that loss of compound was occurring. Teflon filter disks were then treated to eliminate the possibility of irreversible bonding to the substrate. The results of the treated Teflon disks are shown below in Table 6.

Table 6: Recovery Levels From Teflon Disks After One Day

	Concentration (µg/ml)	Applied (µl)	Applied (µg)	Recovered (µg)	Recovery (%)
Pentachlorophenol	1000	15.8	15.8	0.0	0.0
ß-thujaplicin	10000	15.8	158.4	1.4	0.9
γ-thujaplicin	10000	15.8	158.4	2.0	1.3
Methyl thujate	100000	15.8	1583.5	0.0	0.0
Thujic acid	50000	12.7	635.0	510.0	80.6
ß-thujaplicinol	10000	31.6	316.7	3.8	1.2
Plicatic acid	50000	31.7	1583.5	1163.0	73.4

The virtual total loss of compounds shown in this test indicates that, with the exception of plicatic acid, volatilisation is the main cause of poor recovery levels from cellulose and Teflon, since decomposition is unlikely to have occurred within one day. It is expected that plicatic acid will polymerize, affecting its recovery. Thujic acid is the least volatile of the other five compounds and this is reflected in its recovery.

In future testing, exposure to ambient conditions before plating on media for the bioassay will need to be kept to a minimum. However, the compounds used to treat the substrate are dissolved in ethanol. Based on the low recovery levels obtained from the studies outlined above, it is clear that enough time must be given between treatment and plating for the ethanol to evaporate so as not to affect fungal growth, but not so much time that compounds are lost through volatilization.

The application of treating solutions to the filter paper used for the substrate was tedious, as only small amounts of solution could be applied before drying. It was felt that the multiple application-drying procedure necessary to treat the substrate to the desired treatment level, coupled with the large surface area of the disk contributed to the volatilization of the compounds. The concentration of compounds in ethanol was therefore increased, allowing smaller volumes of treating solution to be applied to the substrate. Because of this it was decided to use wood as a substrate since 1 mm-thick wood would allow the application of much larger volumes of treating solutions before drying was necessary. Using wood is also much closer to providing "real life" conditions the fungi might encounter under natural conditions. A coffee stir stick (unknown hardwood) was cut into 1 x 5 x 5 mm squares. Two sets of the wood squares were treated at one treatment level with seven compounds. One set was air dried for 90 minutes, then sealed in vials, kept in the dark for 66 hours then extracted in an ultrasonic bath for 1 hour. The second set was left open to air in the dark for 66 hours then extracted with set 1 and analysed by HPLC. The results are presented below in Table 7.

Table 7: Recovery Levels From Unknown Hardwood Substrate

	Applied (µg)	Set 1		Set 2	
		µg Recovered	% Recovery	µg Recovered	% Recovery
Pentachlorophenol	16	16	100	14	88
ß-thujaplicin	160	122	76	69	43
γ-thujaplicin	160	143	89	119	74
Methyl thujate	1600	875	55	365	23
Thujic acid	650	614	94	590	91
ß-thujaplicinol	160	135	84	98	61
Plicatic acid	650	295	45	365	56

It is clear from the above results that evaporation plays a major role in the mass loss of the compounds left on the substrate after the evaporation of the ethanol used to dissolve the compounds. Since any residual ethanol would likely affect the growth of fungi, it was necessary to determine the shortest possible time to allow for the evaporation of ethanol from the substrate without preventing fungal growth. It was decided to use spruce heartwood as a substrate for all future method development. The spruce was cut into 1 mm-thick wafers from which 6 mm-diameter disks were punched. Four disks were treated with 15 µl of ethanol and weighed at time 0, time 20 min. and time 60 min. The results are shown below in Table 8.

Table 8: Ethanol Evaporation Rate From Spruce Disks

Disk	$T_{0\ min}$ (mg)	$T_{20\ min}$ (mg)	$T_{60\ min}$ (mg)
1	12.0	0.1	0.0
2	11.7	0.1	0.0
3	11.9	0.4	0.0
4	11.6	0.2	0.0

Since most of the ethanol had evaporated in the first 20 min., tests are currently underway to examine the minimum venting time necessary to allow evaporation of the solvent with minimum loss of extractive. Early indications are that venting for 30 minutes will remove all of the ethanol from the disks.

5.2 Bioassay

5.2.1 Birefringence

Based upon both availability and knowledge of its perishable nature, the decision was made to use sapwood of ponderosa pine for the thin sections. Extensive maintenance was required on the microtome slideway to ensure the sections were of uniform thickness and that the cutting motion was smooth,

without slicing artifacts that would interfere with viewing. In preliminary work, 15µ radial and cross sections were cut and used. The cross sections were deemed too fragile to withstand the handling needed in the method and radial sections were used for all subsequent tests. Sections 30µ and 40µ thick were also tried but these were deemed too thick for viewing under the compound microscope. After the initial set of tests, it was decided to use 25µ-thick sections as these were more robust but not too thick for observation.

Thin sections placed on grids on infected agar were heavily attacked by the decay fungi. However, the amount of aerial mycelium on the surface of the thin sections made it very difficult to view the wood beneath when placed on a slide, mounted in lactophenol, and observed with a compound microscope. Fungal hyphae were visible under the microscope on the sections placed on a slide on a grid over agar and infected at the edge of the thin section, but growth was not as vigourous as on the sections placed on the grids. Additionally, some of the sections appeared quite dry, which would prevent decay.

In a further test of the two methods, water agar replaced malt agar as the moisture reservoir under the slide-growth method as there was concern that the decay fungi or other contaminants could grow in the malt agar. Samples were removed for viewing every week for six weeks. The slide-growth method was preferred to the agar-grid method as viewing the wood under the microscope was much easier without the complication of aerial hyphae. All three fungi seemed to grow equally well, however, the slide-growth sections with a cover-slip appeared quite wet which may have retarded growth, while those without a cover-slip tended to be too dry. It may be necessary to briefly drain the dipped sections on a piece of sterile absorbent paper before adding a cover-slip and infecting.

Throughout this test, considerable problems were encountered with the new microscope and imaging system. It proved impossible to fully darken the viewing field under polarised light; this in turn meant that we could not measure birefringence. Because of these problems, it was not possible to accurately assess the decay in the various sections and we were unable to finalise on a growth method for infecting the sections. The limited time frame for the funding and the pressing need to develop an assay technique for use in measuring extractive efficacy required that the birefringence method be abandoned and the work shifted focus to developing the Multiwell test method. Near the end of the time frame for the project and after the well test work was underway, it was discovered that the polarisation filters had been misaligned before they were fused in place during manufacture of the microscope. The sections have all been retained in storage for possible future work.

5.2.2 Multiwell Test

5.2.2.1 Multiwell Test Development

Fungal growth on the four media was assessed by viewing under a low magnification on a dissecting microscope. Use of a microscope was necessary because the white or translucent mycelium of all the test cultures was very difficult to resolve visually on the white, cellulose-containing media without magnification. To aid in assessing the growth rate and vigour of the four isolates, a rating system (Table 9) was devised.

Table 9: Growth Rating System

0 = no growth on test media
1 = some growth on media, sparse, ≤ ½ distance to disk
2 = very good growth on media, close to disk
3 = growth on disk at the edge
4 = very good growth on disk

The growth results for the four fungi on the various media are presented in Table 10. *C. albidus* grew very slowly on all the media, but because of concerns that it could be involved in detoxification of extractives, it was decided to include it in future testing. Of the three decay fungi, the white rot *P. subacida* was the easiest to visualise and ultimately it was selected for use in further tests. The brown rot fungus *C. puteana* was selected as a backup, again due to ease of viewing. It has subsequently been decided to include both decay fungi in assessing extractive efficacy due to the desirability of having representatives of both rot types (and therefore different cellulose breakdown mechanisms) in the study.

Table 10: Average Fungal Growth in Multiwell Plates on Four Media With Three Treatments

Fungus	Treatment	Average Growth Rating on Media[1]			
		Eggins & Pugh Without Added Carbon	Eggins & Pugh With Added Carbon	1.5 % Malt	Abrams & Trace Metals
Cephaloascus albidus	Sterile Disk	2	1	2	1
	Sterile Ethanol-Dipped Disk	1.3	1	2	1
	Sterile Disk With 1000 µg/cm^2 ß-thujaplicin	1	1	1	1
Coniophora puteana	Sterile Disk	3	3	3	2
	Sterile Ethanol-Dipped Disk	3	2	3	3
	Sterile Disk With 1000 µg/cm^2 ß-thujaplicin	0	0	1	0
Perenniporia subacida	Sterile Disk	3	3.7	4	2.3
	Sterile Ethanol-Dipped Disk	2	2.7	4	3
	Sterile Disk With 1000 µg/cm^2 ß-thujaplicin	0	1	1	0
Postia placenta	Sterile Disk	2	1.7	4	1
	Sterile Ethanol-Dipped Disk	2.3	2	4	3.3
	Sterile Disk With 1000 µg/cm^2 ß-thujaplicin	0	0	0	0

[1] Average of three wells after 12 days' incubation.

In selecting a test media, it was decided that growth on the Abrams nutrient media was too sparse and it was removed from consideration. Conversely, the growth on the rich malt agar was too luxuriant and it was decided not to use it, as the fungi might grow well on the sugars in the media without being affected by the extractives under test. The growth on the Eggins and Pugh media with additional carbon in the form of yeast extract and asparagine gave slightly better aerial growth of the basidiomycetes than did the media without carbon, and was therefore easier to visualise. On that basis it was selected for use in further testing. It was also felt that the cellulose present in the Eggins and Pugh media would encourage

the production of cellulase enzymes in the test culture and this might more closely mimic what occurred in natural infection of trees.

Relative to the inhibition caused by ß-thujaplicin at 1000 µg/cm^2, the ethanol dip did not appear to greatly affect growth and it was decided that this was not likely to be a factor if extractive disks were well dried prior to placing them in the wells. The ß-thujaplicin completely stopped basidiomycete fungal growth, indicating that the method could be used to assess extractive efficacy. (*C.* albidus is not a decay fungus and was included in the test to assess its ability to grow in the presence of the test compounds.) It remained to be determined whether the method was sensitive enough to determine a toxic threshold for the compounds under test.

5.2.2.2 Multiwell Method Confirmation

The results of the test examining the toxic thresholds for ß-thujaplicin and PCP are presented in Table 11. It should be noted that, due to the two-day incubation of the well plates prior to adding the treated disks, the baseline growth rating for *C. albidus* was 0.2, and for *P. subacida* the baseline was 1.0. Both fungi showed a reduction in growth with increasing concentration of ß-thujaplicin, demonstrating that a toxic threshold could be obtained using the test method, but the lowest level of PCP tested was still inhibitory. Interestingly, previous reports in the literature (Rennerfelt, 1948; Rudman, 1963) rank the two chemicals as having very similar toxic thresholds.

Table 11: Average Fungal Growth in Multiwell Plates With Two Chemicals

Fungus	Treatment	Average Growth Rating[1]
Cephaloascus albidus	Sterile Disk Control	2.0
	Sterile Ethanol-Dipped Disk Control	2.0
	Sterile Disk With 100 µg/cm^2 ß-thujaplicin	1.8
	Sterile Disk With 500 µg/cm^2 ß-thujaplicin	0.2
	Sterile Disk With 1000 µg/cm^2 ß-thujaplicin	0.0
	Sterile Disk With 100 µg/cm^2 Pentachlorophenol	0.0
	Sterile Disk With 500 µg/cm^2 Pentachlorophenol	0.2
	Sterile Disk With 1000 µg/cm^2 Pentachlorophenol	0.2
Perenniporia subacida	Sterile Disk Control	4.0
	Sterile Ethanol-Dipped Disk Control	4.0
	Sterile Disk With 100 µg/cm^2 ß-thujaplicin	4.0
	Sterile Disk With 500 µg/cm^2 ß-thujaplicin	2.3
	Sterile Disk With 1000 µg/cm^2 ß-thujaplicin	1.0
	Sterile Disk With 100 µg/cm^2 Pentachlorophenol	1.0
	Sterile Disk With 500 µg/cm^2 Pentachlorophenol	1.0
	Sterile Disk With 1000 µg/cm^2 Pentachlorophenol	1.0

[1] Average of six wells after 11 days growth (*P. subacida*) or 27 days growth (*C. albidus*)

5.2.2.3 Multiwell Analysis of Identified Extractives

The results of the well test on four extractives and PCP at three concentrations is presented in Table 12 and Figures 1 and 2. At the concentrations tested, both ß-thujaplicin and thujic acid showed a concentration effect, with growth decreasing with increasing extractive concentration, as did PCP, the reference chemical. Neither methyl thujate nor plicatic acid inhibited growth at the concentrations tested. This was as expected, since literature reports indicate that methyl thujate and plicatic acid are not fungitoxic while the other two chemicals are. Again, PCP appeared more toxic than the ß-thujaplicin.

Table 12: Average Fungal Growth in Multiwell Plates With Four Extractives and Pentachlorophenol at Three Concentrations and Controls

Fungus	Treatment	Average Growth Rating[1]
Cephaloascus albidus	No Disk	1.3
	Sterile Disk	1.8
	Ethanol-Dipped Sterile Disk	2.0
	Sterile Disk With 100 µg/cm^2 Pentachlorophenol	0.0
	Sterile Disk With 500 µg/cm^2 Pentachlorophenol	0.2
	Sterile Disk With 1000 µg/cm^2 Pentachlorophenol	0.2
	Sterile Disk With 100 µg/cm^2 ß-thujaplicin	1.8
	Sterile Disk With 500 µg/cm^2 ß-thujaplicin	0.2
	Sterile Disk With 1000 µg/cm^2 ß-thujaplicin	0.0
	Sterile Disk With 1500 µg/cm^2 Methyl Thujate	2.0
	Sterile Disk With 2000 µg/cm^2 Methyl Thujate	2.0
	Sterile Disk With 2500 µg/cm^2 Methyl Thujate	2.0
	Sterile Disk With 1500 µg/cm^2 Plicatic Acid	1.8
	Sterile Disk With 2000 µg/cm^2 Plicatic Acid	1.3
	Sterile Disk With 2500 µg/cm^2 Plicatic Acid	1.7
	Sterile Disk With 1183 µg/cm^2 Thujic Acid	1.0
	Sterile Disk With 1577 µg/cm^2 Thujic Acid	0.9
Perenniporia subacida	No Disk	4.0
	Sterile Disk	4.0
	Ethanol-Dipped Disk	4.0
	Sterile Disk With 100 µg/cm^2 Pentachlorophenol	1.0
	Sterile Disk With 500 µg/cm^2 Pentachlorophenol	1.0
	Sterile Disk With 1000 µg/cm^2 Pentachlorophenol	1.0
	Sterile Disk With 100 µg/cm^2 ß-thujaplicin	4.0
	Sterile Disk With 500 µg/cm^2 ß-thujaplicin	2.3
	Sterile Disk With 1000 µg/cm^2 ß-thujaplicin	1.0
	Sterile Disk With 1500 µg/cm^2 Methyl Thujate	4.0
	Sterile Disk With 2000 µg/cm^2 Methyl Thujate	4.0
	Sterile Disk With 2500 µg/cm^2 Methyl Thujate	4.0
	Sterile Disk With 1500 µg/cm^2 Plicatic Acid	4.0
	Sterile Disk With 2000 µg/cm^2 Plicatic Acid	4.0
	Sterile Disk With 2500 µg/cm^2 Plicatic Acid	4.0
	Sterile Disk With 1183 µg/cm^2 Thujic Acid	2.3
	Sterile Disk With 1577 µg/cm^2 Thujic Acid	1.4

[1] Average of six wells after 11 days' growth (*P. subacida*) or 27 days' growth (*C. albidus*)

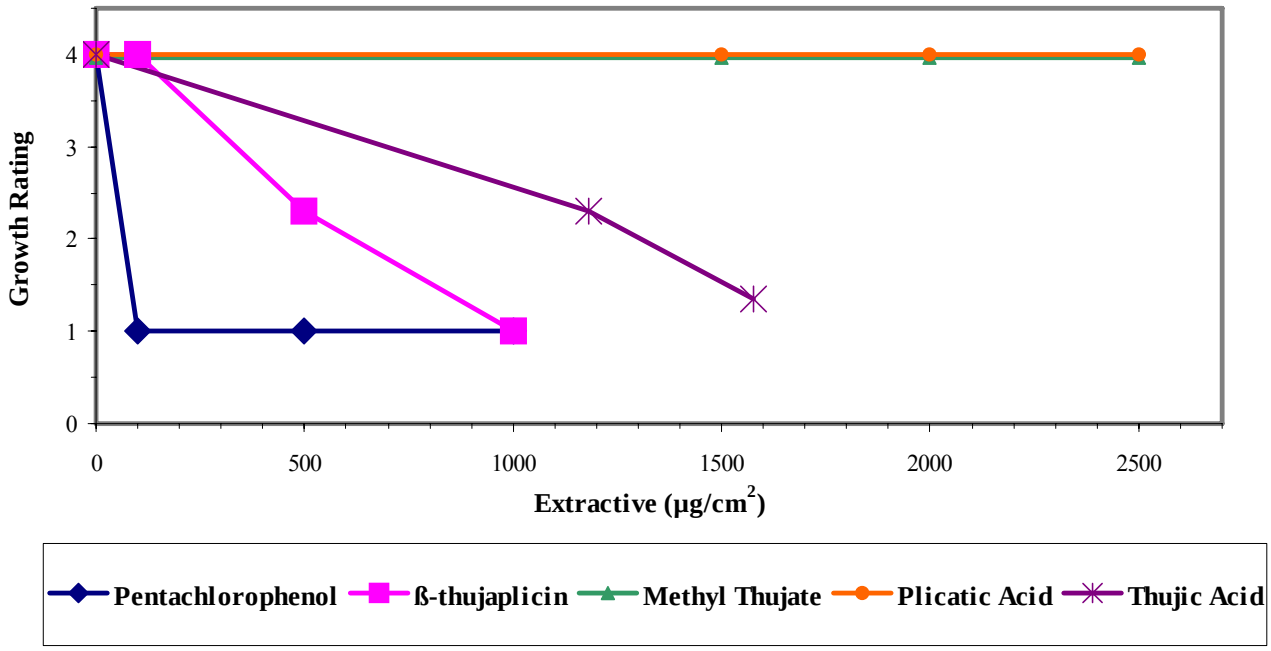


Figure 1: Average Growth Rates for P. Subacida Exposed to a Range of Concentrations of Western Red-cedar Extractives in a Multiwell Plate Test.

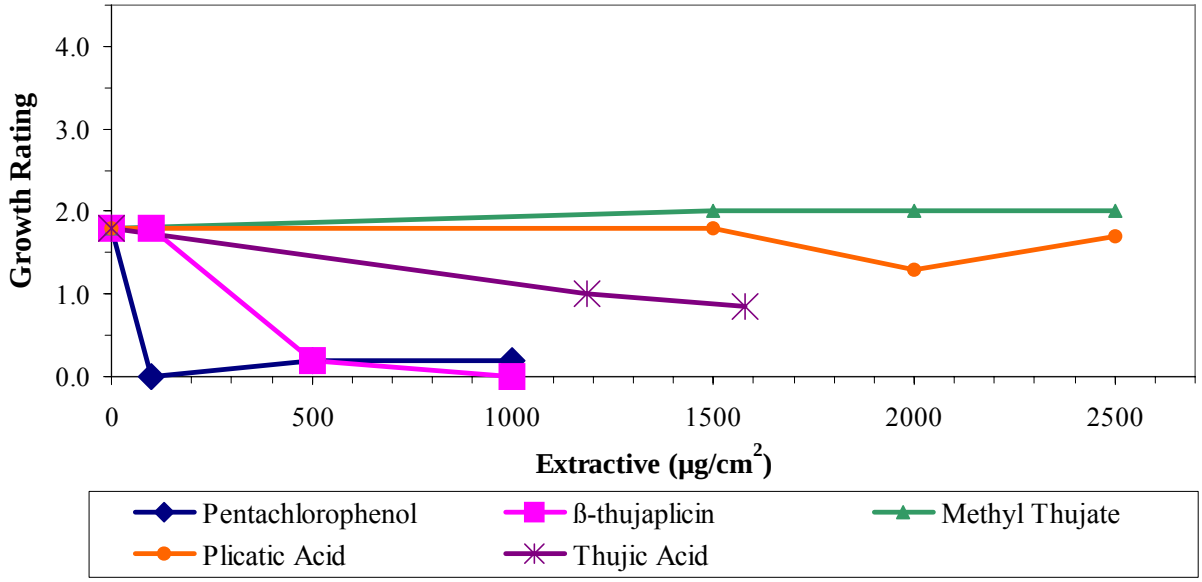


Figure 2: Average Growth Rates for C. Albidus Exposed to a Range of Concentrations of Western Red-cedar Extractives in a Multiwell Plate Test.

The growth ratings for the ethanol evaporation test are presented in Table 13. After 11 days' incubation, both fungi had grown across the agar and were growing on the spruce heartwood disks, irrespective of the ventilation time. The agar wells without a test disk were also overgrown at 11 days. Ventilation of ethanol-treated disks for 30 minutes was sufficient to remove any fungistatic effect of the ethanol.

Table 13: Average Fungal Growth in Multiwell Plates With Vented Ethanol-Treated Disks

Ventilation Time (min.)	*C. puteana* Growth Rating[1]	*P. subacida* Growth Rating[1]
No Disk	4.0	4.0
30	4.0	4.0
60	4.0	4.0
90	4.0	4.0

[1] Average of six wells after 11 days' growth.

The low recovery rates from the cellulose disks (Table 5) call into question the numbers reported in Table 12, although the conclusion that thujic acid and ß-thujaplicin are fungitoxic is still valid. It is likely that, due to the mass loss determined in 5.1, the extractives were tested at a lower concentration than reported. It may also help explain the variation in toxic threshold between PCP and ß-thujaplicin, and may call into question many of the reports in the literature of extractive toxic levels. We have been unable to locate reports in the literature of volatilisation or other loss of these extractives, nor have we found reports of testing protocols where these possibilities were taken into account.

6 Conclusions

- Soxhlet extraction of WRC wood meal was more thorough than ultrasonic extraction.
- WRC extractive solutions in ethanol placed onto cellulose disks showed rapid mass loss. This unexpected finding was a significant factor in method development.
- With the exception of plicatic acid, the observed mass loss of compounds on disks was due to evaporation.
- Loss of plicatic acid was likely due to polymerization and/or oxidation.
- The Multiwell test can be used to measure fungicidal efficacy of WRC extractives and to obtain toxic thresholds.
- ß-thujaplicin and thujic acid inhibit the growth of *P. subacida* and *C. albidus.*
- Extractive solutions in ethanol need to be vented for 25-30 minutes to remove ethanol; venting beyond this time will result in unacceptable levels of extractive loss.
- *C. puteana* (brown rot), *P. subacida* (white rot) and *C. albidus* (ascomycetous yeast with potential detoxifying ability) are suitable fungi for use in the Multiwell Test.
- Optimal incubation times in the Multiwell Test are approximately 10 days for decay fungi and 60 days for *C. albidus*.
- Wells should be inoculated two days prior to adding chemical.
- Eggins and Pugh cellulose media with supplemental carbon is an acceptable growth medium.
- Multiwell plates should be incubated at 25 °C.
- Equipment problems prevented full assessment of the birefringence assay.

7 Recommendations

Test disks with ethanol-based extractive solutions should be analysed to provide information on the evaporation rate of each compound. This information should be used to calibrate the bioassay and possibly develop a correction factor to normalise the amount applied, based on the expected loss due to evaporation. The effect on fungal growth of sealing the wells with plastic film to retard evaporation and cross-contamination by vapour in the Multiwell Test should be evaluated. Research should be done to determine whether the evaporation of volatile compounds is the same for pure compounds as it is for the compound in a whole extract.

Additional funding will be required to undertake the above work.

8 Literature Cited

Anagnost, S. E., J. J. Worrall and C. J. K. Wang. 1994. Diffuse cavity formation in soft rot of pine. Wood Sci. Technol. **28**(3):199-208.

Anagnost, S. E. 1998. Light microscope diagnosis of wood decay. IAWA Journal. **19**(2):141-167.

Barton, G. M. 1976. A review of yellow cedar (*Chamaecyparis nootkatensis* [D. Don] Spach) extractives and their importance to utilization. Wood Fiber Sci. **8**: 172.

Barton, G. M. and B. F. MacDonald. 1971. The chemistry and utilization of western red cedar. Canadian Forestry Service Pub. No. 1023.

Behr, E. A. 1976. Special physical and chemical properties of northern white cedar. *In* Proceedings of the National Northern White Cedar Conference, Cooperative Extension Service, Michigan State University, Department of Forestry.

DeBell, J. D., J. J. Morrell and B. L. Gartner. 1997. Tropolone content of increment cores as an indicator of decay resistance in western red-cedar. Wood and Fiber Sci. **29**: 364-369.

DeBell, J. D., J. J. Morrell and B. L. Gartner. 1999. Within-stem variation in tropolone content and decay resistance of second-growth western red-cedar. Forest Sci. **45**: 101-107.

Eslyn, W. E. and T. L. Highley. 1976. Decay resistance and susceptibility of sapwood of fifteen tree species. Phytopathology **66**: 1010-1017.

Green III, F. and T. L. Highley. 1996. Mechanism of brown-rot decay: paradigm or paradox. Internat. Biodeterioration and Biodegradation. **39**: 113-124.

Jin, L., B. J. van der Kamp, J. Wilson and E. P. Swan. 1988a. Biodegradation of thujaplicins in living western red cedar. Can. J. For. Res. **18**: 782-786.

Jin, L., J. W. Wilson, and E. P. Swan. 1988b. Thujin, a novel lactone isolated from the discoloured heartwood of Thuja plicata Donn. Can. J. Chem. **66**: 51-53.

MacLean, H. and J. A. F. Gardner. 1956a, Distribution of fungicidal extractives (thujaplicin and water soluble phenols) in western red cedar heartwood. For. Prod. J. 6: 510-516.

MacLean, H. and J. A. F. Gardner. 1956b. Analytical method for thujaplicins. Anal. Chem. **28**: 509.

Morrell, J. J., C. M. Freitag and C. Eddington. 2001. Effect of preservative treatment on survival of fungi in Western Red Cedar utility poles. For. Prod. J. **59**: 69-72.

Nault, J. 1988. Radial distribution of thujaplicins in old growth and second growth western red cedar (*Thuja plicata* Donn). Wood Sci. Technol. **22**: 73-80.

Perry, R. S. 1954. Yellow cedar. Its characteristics, properties, and uses. Bulletin No. 114. Forestry Branch of the Canadian Department of Northern Affairs and Natural Resources.

Rennerfelt, E. 1948. Investigations of thujaplicin, a fungicidal substance in the heartwood of *Thuja plicata* D. Don. Physiol. Plant. **1**: 245-254.

Roff, J. W. and P. M. Atkinson. 1954. Toxicity tests of a water-soluble phenolic fraction (thujaplicin-free) of western red cedar. Can. J. Bot. 32: 308-309.

Rudman, P. 1962. The causes of natural durability in timber. Part IX: The antifungal activity of heartwood extractives in a wood substrate. Holzforschung **16**: 74-77.

Rudman, P. 1963. The causes of natural durability in timber. Part XI: Some tests on the fungi toxicity of wood extractives and related compounds. Holzforschung **17**: 54-57.

Scheffer, T. C., B. S. Goodell and F. F. Lombard. 1984. Fungi and decay in western red-cedar utility poles. Wood and Fiber Sci **16**: 543-548.

Setliff, E. C. and W. C. Chung. 1985. Biological evaluation of western red cedar shakes after eleven years exposure. Unpublished report to the Canadian Forest Service. Forintek Canada Corp., Vancouver, BC. 6 pp.

Smith, R. S. 1970. Black stain in yellow cedar heartwood. Can. J. Bot. **48**: 1731-1739.

Smith, R. S. and D. W. Swan. 1975. Colonisation of western red cedar shingles and shakes by fungi. Mat. U Org. **3**: 253-262.

Van der Kamp, B. J. 1974. The distribution of microorganisms associated with decay of western red cedar. Can. J. For. Res. **5**: 61-67.

Van der Kamp, B. J. 1986. Effects of heartwood inhabiting fungi on thujaplicin content and decay resistance of western red-cedar (*Thuja plicata* Donn.). Wood and Fiber Sci. **18**: 421-427.

Wilcox, W. W. 1993a. Comparative morphology of early stages of brown-rot wood decay. IAWA Journal. **14**(2): 127-138.

Wilcox, W. W. 1993b. Comparison of scanning electron microscopy and light microscopy for the diagnosis of early stages of brown rot wood decay. IAWA Journal. **14**(3): 219-226.

Appendix A

Abrams Nutrient Solution

Chemical		Final Concentration
Ammonium Nitrate	NH_4NO_3	3.0 g
Potassium Dihydrogen Orthophosphate	KH_2PO_4	4.5 g
Magnesium Sulphate	$MgSO_4 \cdot 7H_2O$	2.0 g
Calcium Chloride	$CaCl_2$	0.1 g
Thiamine Hydrochloride		1 mg
Trace Metal Solution		1 ml
Distilled Water		1 litre

Autoclave and adjust to pH 5 using NaOH or HCl.
For agar media, add 2% Difco BactoAgar.

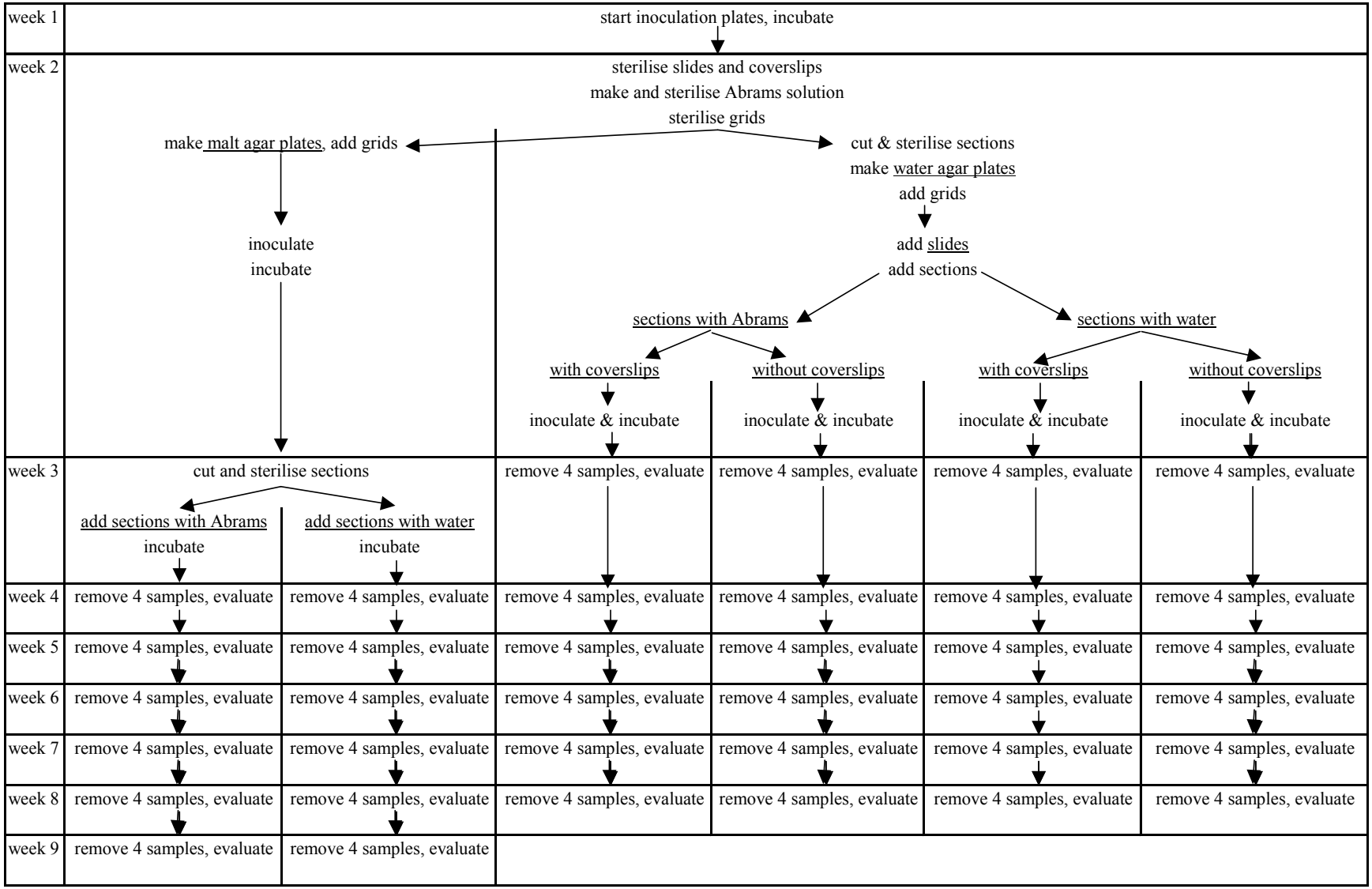
Trace Metal Solution

Boric Acid	H_3BO_3	0.6 mg
Ferric Chloride	$FeCl_3.6H_2O$	0.24 mg
Manganese Chloride	$MnCl_2.H_2O$	0.0361 mg
Ammonium Molybdate	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0184 mg
Zinc Sulphate	$ZnSO_4.7H_2O$	0.31 mg
Copper II Sulphate	$CuSO_4.5H_2O$	0.04 mg
Distilled Water		1 litre

Trace element solution from Dickenson, D.J. 1974. A new technique for screening fungicide for wood preservation. Int. Biodetn. Bull. **10**(2):49-50

Appendix B

Protocol for Testing Variables



Appendix C

Eggins and Pugh Cellulose Media With and Without Added Carbon

Chemical		Original Formulation With Added Carbon	Formulation as Modified by Bravery[1]
Ammonium sulphate	$(NH_4)_2SO_4$	0.5 g	0.543 g
Potassium dihydrogen orthophosphate	KH_2PO_4	1.0 g	1.0 g
Potassium chloride	KCl	0.5 g	0.5 g
(crystalline) Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	0.2 g	0.2 g
Calcium chloride	$CaCl_2$	0.1 g	0.1 g
L-asparagine		0.5 g	-
Difco yeast extract		0.5 g	-
Difco Agar		20 g	20 g
Distilled water		500 ml	500 ml
Thiamine hydrochloride		-	0.001 g

Mix the above in water, keep $MgSO_4 \cdot 7H_2O$ separate in a portion of the water.

Watman CF 11 cellulose powder	10 g	10 g
Distilled water	500 ml	500 ml

Soak the cellulose powder in the water with stirring 24 hours.

Combine two mixtures and autoclave Add sterile $MgSO_4 \cdot 7H_2O$ solution to mix after autoclaving and partially cooled. Mix well and dispense.

[1] Bravery's modification removes carbon sources other than cellulose.

Eggins, O.W. and Pugh, 1962. Isolation of cellulose decomposing fungi from the soil. Nature **193**:94.

Bravery, A.F. 1968. Microbiological breakdown of cellulose in the presence of alternative carbon sources. J. Science, Food and Agriculture. **19**:133-135.

Appendix D

Malt Agar - Forintek

Difco Malt Extract	15.0 g
Difco Bacto Agar	20.0 g
Distilled Water	1.0 l.

Autoclave, cool, mix well and dispense.

Appendix E
Extractive Chromatograms

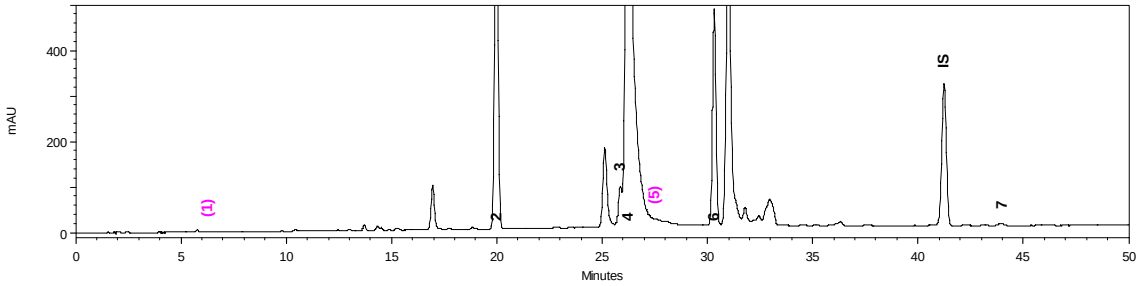


Fig. E-1. Hexane extract ultrasonic: 1- plicatic acid, 2- nezukone, 3- γ thujaplicin, 4- ß thujaplicin, 5- ß thujaplicinol, 6- thujic acid, 7- methyl thujate, IS- internal standard

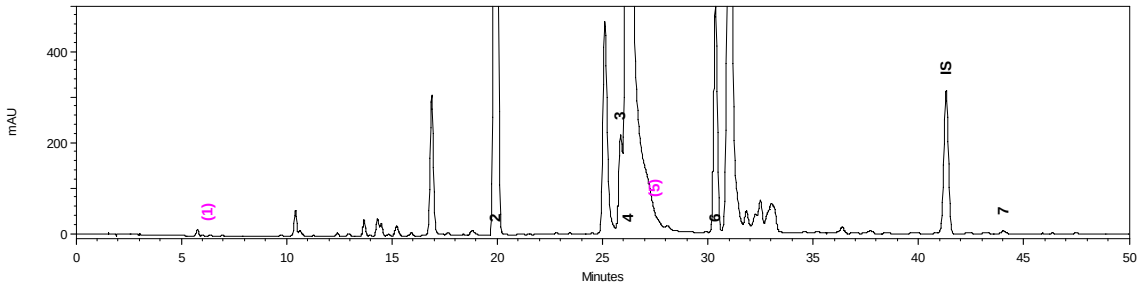


Fig. E-2. Hexane extract Soxhlet: 1- plicatic acid, 2- nezukone, 3- γ thujaplicin, 4- ß thujaplicin, 5- ß thujaplicinol, 6- thujic acid, 7- methyl thujate, IS- internal standard

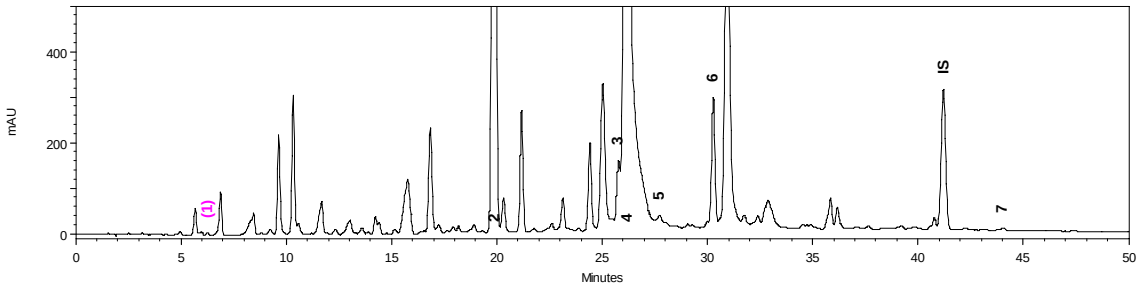


Fig. E-3. Dichloromethane extract ultrasonic: 1- plicatic acid, 2- nezukone, 3- γ thujaplicin, 4- ß thujaplicin, 5- ß thujaplicinol, 6- thujic acid, 7- methyl thujate, IS- internal standard

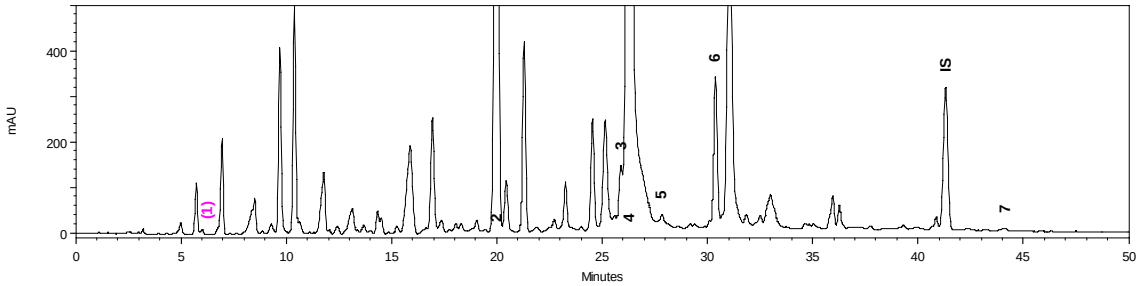


Fig. E-4. Dichloromethane extract: Soxhlet: 1- plicatic acid, 2- nezukone, 3- γ thujaplicin, 4- ß thujaplicin, 5- ß thujaplicinol, 6- thujic acid, 7- methyl thujate, IS- internal standard

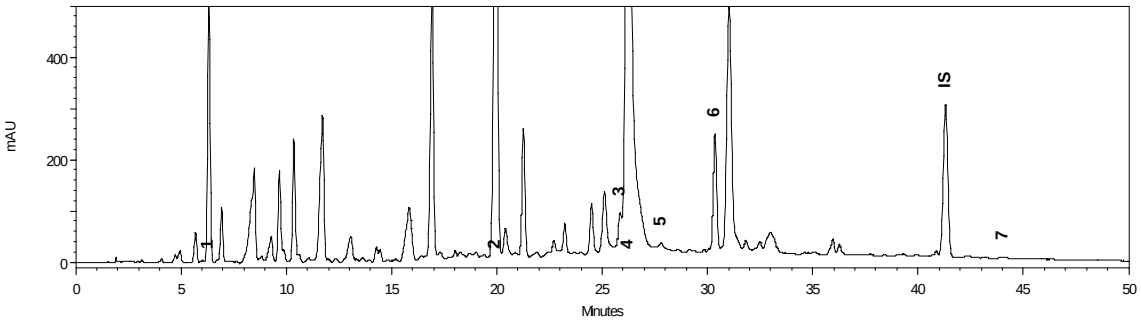


Fig.E-5. Ethyl acetate extract ultrasonic: 1- plicatic acid, 2- nezukone, 3- γ thujaplicin, 4- ß thujaplicin, 5- ß thujaplicinol, 6- thujic acid, 7- methyl thujate, IS- internal standard

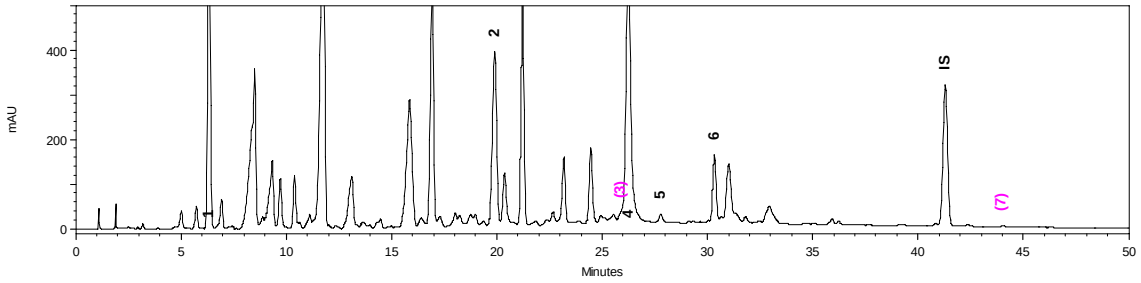


Fig. E-6. Ethyl acetate extract Soxhlet: 1- plicatic acid, 2- nezukone, 3- γ thujaplicin, 4- ß thujaplicin, 5- ß thujaplicinol, 6- thujic acid, 7- methyl thujate, IS- internal standard

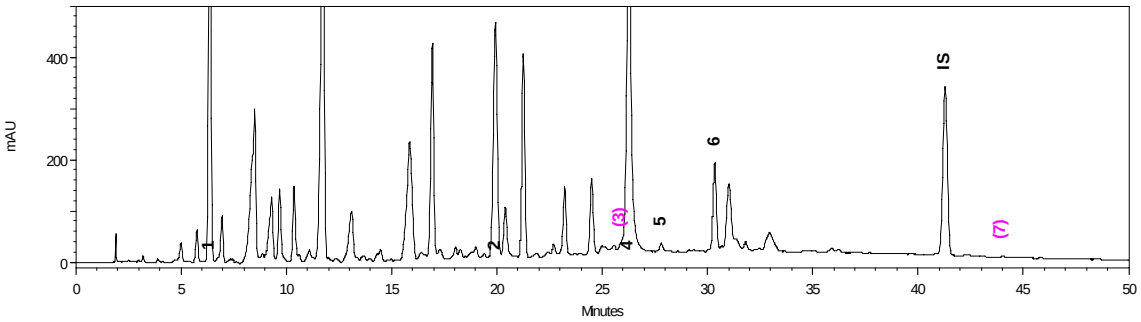


Fig. E-7. Ethanol extract ultrasonic: 1- plicatic acid, 2- nezukone, 3- γ thujaplicin, 4- ß thujaplicin, 5- ß thujaplicinol, 6- thujic acid, 7- methyl thujate, IS- internal standard

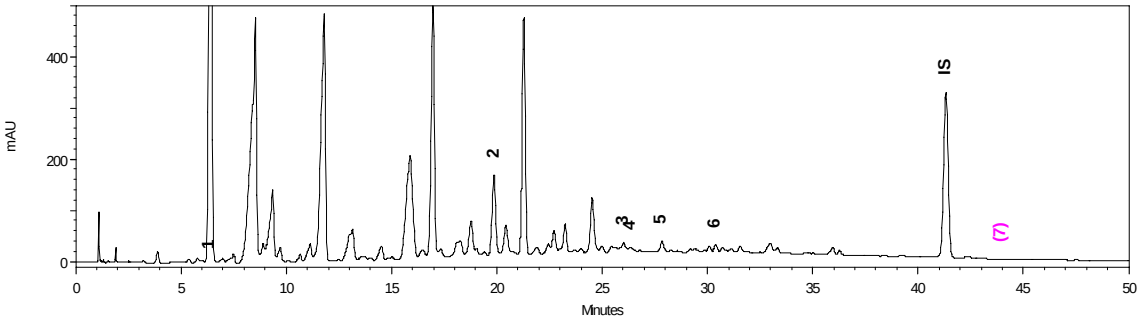


Fig. E-8. Ethanol extract Soxhlet: 1- plicatic acid, 2- nezukone, 3- γ thujaplicin, 4- ß thujaplicin,

5- ß thujaplicinol, 6- thujic acid, 7- methyl thujate, IS- internal standard

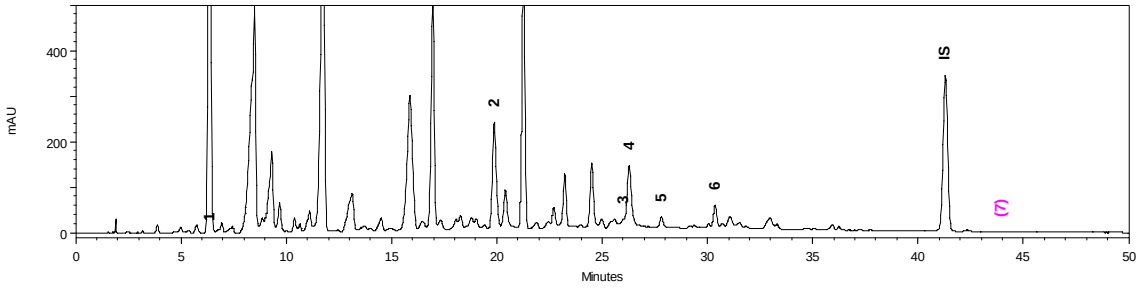


Fig. E-9. Methanol extract ultrasonic: 1- plicatic acid, 2- nezukone, 3- γ thujaplicin, 4- ß thujaplicin, 5- ß thujaplicinol, 6- thujic acid, 7- methyl thujate, IS- internal standard

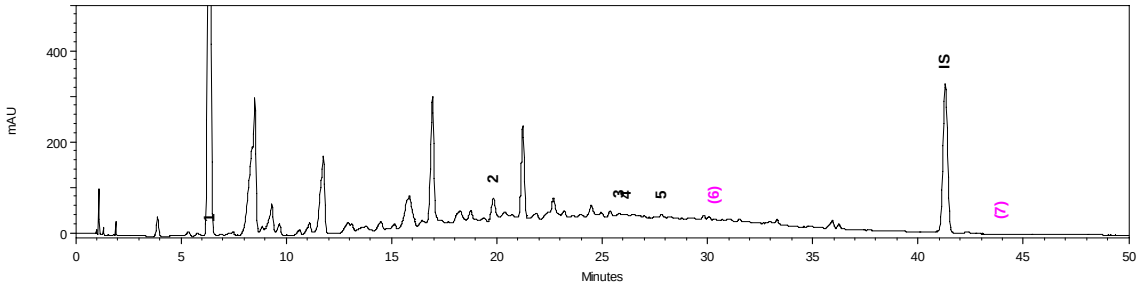


Fig. E-10. Methanol extract Soxhlet: 1- plicatic acid, 2- nezukone, 3- γ thujaplicin, 4- ß thujaplicin, 5- ß thujaplicinol, 6- thujic acid, 7- methyl thujate, IS- internal standard

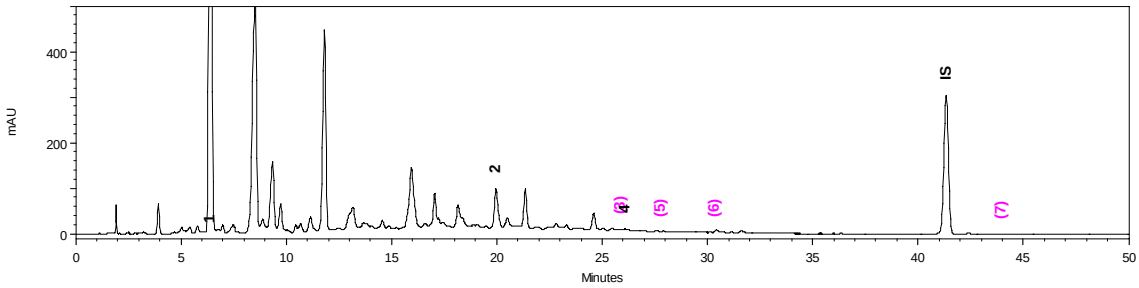


Fig. E-11. Water extract ultrasonic: 1- plicatic acid, 2- nezukone, 3- γ thujaplicin, 4- ß thujaplicin, 5- ß thujaplicinol, 6- thujic acid, 7- methyl thujate, IS- internal standard

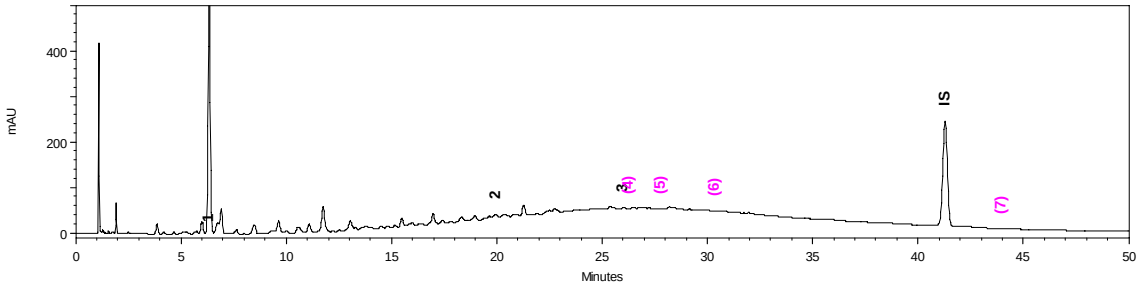


Fig. E-12. Water extract Soxhlet: 1- plicatic acid, 2- nezukone, 3- γ thujaplicin, 4- ß thujaplicin, 5- ß thujaplicinol, 6- thujic acid, 7- methyl thujate, IS- internal standard

Appendix F

Meeting Notes
Cedars Working Group

9.00 am – 4.00 pm January 22nd 2004
Boardroom
Forintek Canada Corp.
2665 East Mall, Vancouver, BC, V6T 1W5

Attendees

Dwayne Klassen	Louisiana Pacific
Aubrey Salon	Downie Timber
Benoit Schmitt	Maibec Industries
Charles Tardif	Maibec Industries
Inga Udluft	Chemco
David (Dai) Jones	Xylon Biotechnologies
Francois Saillant	Canadian Forest Service, Ottawa
Rona Sturrock	Canadian Forest Service, Victoria
John Russell	BC Ministry of Forests
Paul Morris	Forintek Canada Corp.
Bob Daniels	Forintek Canada Corp.
Jean Clark	Forintek Canada Corp.
Tony Byrne	Forintek Canada Corp.
Kevin Groves	Forintek Canada Corp.
Manon Gignac	Forintek Canada Corp.
Zhiming (David) Liu	Forintek Canada Corp.
Collette Breuil	University of British Columbia, Faculty of Forestry
Jae Jin Kim	University of British Columbia, Faculty of Forestry
Young Woon Lim	University of British Columbia, Faculty of Forestry
Russell Chedgy	University of British Columbia, Faculty of Forestry
Aron Ionut	UBC Research Forest
Adam Taylor	Oregon State University
Karen Bartlett	University of British Columbia, School of Occupational Health and Hygiene

Opening Remarks

Paul Morris added one item to the agenda in the section on current industry issues. Karen Bartlett on the issue of cedars and health.

Review of Six Directions for R&D

Paul Morris reviewed the 6 directions and asked are they still appropriate, are there other areas or activities to include? No other areas were suggested.

1. Growing Durability

Heritability of durability in Western red-cedar (see handout) – John Russell

Q. Effect of juvenile wood?
A. 20-30 years = juvenile
Q. Natural variation between trees – ecological variables?
A. yes – nutrition and water.
Dai Jones found stressed trees increase extractives – not as good for lumber but wanted for chemicals.
Q. Any co-operation with US?
A. yes, but they are more interested in Douglas-fir.
Q. Any characteristics profile with extractives?
A. Bob Daniels – no. Relationships among extractives don't stay the same (early days in data analysis). So far nothing is typical.

Fungal colonization in Western red-cedar trees (see handout) - Young Woon Lim

Q. Were the fungi growing inwards or outwards via the branch stub?
A. Probably outwards since the decay fungus attacks via roots.
Q. Relationship between presence of fungal DNA and amount of extractives?
A. Extractive analysis not done yet, potential for simple field test if absence of fungi correlates with high extractive content.

Maximizing natural durability of Western red-cedar – Extraction (see handout) – Bob Daniels

Q. Expand on differences from earlier work.
A. For example Nezukone. There is more present than originally thought. Some problems separating extractives. We also have several interesting peaks without identities.

Action item: Dai Jones may be able to provide some pure compounds as standards.

Maximizing natural durability of Western red-cedar – Bioassay (see handout) - Jean Clark

Dai Jones was interested in the data on Thujic Acid.

2. Enhancing Service Life

Laboratory testing of natural durability of Eastern white-cedar (see handout) – Manon Gignac

No questions

Field testing of Natural Durability –(handouts not reproducible) – Paul Morris

- 60 yr-old post test in Ontario. 18 yrs service for Eastern white-cedar compared to >45 years for treated pine.
- 30 yr-old shingle and shake test in BC. 20 yr service for untreated Western red-cedar, probably over 50 years when CCA treated.
- 20 yr-old decking test in BC. Some decay in Western red-cedar at 20 yrs.
- 12-yr-old decking test in BC. Some decay in Western red-cedar at 12 yrs.
- 6 yr-old above ground termite test of Western red-cedar in Ontario. Similar performance to CCA hem-fir.
- 3 yr-old post test of Western red-cedar old growth and 2nd growth with and without wrapping.
- 0.5 yr-old shingle test of western red-cedar with preservatives that could replace CCA in the long term.

Q. Is yellow-cedar more durable in sill plates than western red-cedar?
A. No difference in durability vs decay but yellow-cedar is more resistant to termites

Extractive depletion and fungal colonization- (see handouts) Young Woon Lim and Russ Chedgy

Bob Daniels mentioned water soluble and insoluble extractives were reversed.

Finishing properties of Western red-cedar (see handout) – Kevin Groves

Q. How to rate control?
A. Not all factors for finishes are applicable
Q. Amount applied?
A. As per manufacturers instructions – table is available.
Q. What was factory finish?
A. 2 coats of acrylic latex.
Q. Any effect of finish colour?
A. Tried to keep same colour – used white paint
Q. Effect of age of surface?
A. Apply ASAP after cutting – USFPL looked at surface age and found it was important. Now focusing on factory finishing – can do immediately after cutting – if the consumer finishes product, it may be too late – surface deactivation
Q. MC at time of coating? Dried to 10% in kiln
Q. Any extractive bleed problems?
A. Saw bleeding in first three months, gone at 6-month rating.
Aubrey Salon suggested should dry to service conditions at destination climate and applying coating at the ambient MC. It was noted that surface inactivation can occur during this time.

Effect of extractive content and finishes on dimensional stability – (no handout) Paul Morris

Study underway on light and dark cedar with and without coating measuring swelling under water. Dark cedar has fungi that reduce extractive content but may also change permeability. Work is being done using UBC equipment. Stephen Ayer the project leader is away on extended sick leave.

RC - Black fungal stain in wrc mainly *Aureobasidium pullulans*. Leaching and uv degradation.

3. Natural pesticides – (no presentations)

USFPL work by Stan Lebow is not continuing. Canfor also looked at this for sapstain, and a group in France is also working in the area. Concept was natural pesticide for wood. Dai Jones suggested extractives used as pesticides/preservatives are too expensive for industrial use Paul Morris mentioned they also need a PMRA label and this could prove difficult considering the number of unknowns. In Australia they are taking extractives from heartwood residues and putting them into sapwood of the same species in a closed loop process – may not require PMRA registration. Dai Jones mentioned the Haida are interested in using extractives for cultural and restorative uses. Joe Karchesi of Oregon State University is interested in the area.

4. Fine chemicals (no handout) – Dai Jones

Xylon – search coastal rain forest for natural theraputic compounds to commercialize. WRC - 12% of mass is extractive, can distinguish approx. 400 compounds as peaks but don't know what they all do. Use compounds occurring >1%, if <1% would need to synthesize. Greater than 1% are primarily tropolones (7-sided ring) - α-thujaplicin, β-thujaplicin, γ-thujaplicin. These are used in perfume. Lots of γ-thujaplicin available.

β-thujaplicin goes to market for traditional Japanese medicines, anti-inflamatory, anti-viral, anti-tumor drug. β-thujaplicin = $2000/kg, Unlike β-, γ-thujaplicin has no taste, colour and smell and price is higher.

Methyl thujate – fragrance derivatives
Thujic acid – insect repellent – amide
Nezukone – no known use
β-thujaplicinol – no known use
Plicatic acid – no known use – is a natural preservative for fats and oils

Q. β-thujaplicin – effect on skin?
Q. Permits for natural products vs synthetics? Same regulatory barriers for drugs – very high.

5. Reduced cost Pulping (no presentations**)**

Removing extractives prior to pulping would only be worthwhile if the size of the market increased dramatically and the fine chemicals industry expanded to match it. Lignol Innovations in the BC Research Building extract lignin from a variety of species and make alcohol from the residue using enzymes. The use of a catalyst to convert gasified wood to alcohol and water is another commercial alternative.

6. Colour Control/Stability (no presentations)

Forintek is working on natural finishes with a surface finish formulator and he had asked if there was any interest in water-based extractive blocking primers.

Downie Timber found oil-base is slow to dry. Moved to water-based. Wood must be dry.
Maibec uses water-base only – best for customer

Action item: Aubrey Salon to get info on water-base primers to Paul Morris.

Current Industry Issues

A. Proposed limits to second growth in production of Western red-cedar shakes and shingles

Relationship between growth rate and extractive content of Western red-cedar (see handout) Bob Daniels

No questions

Relationship between tree age and durability based on OSU data (see handout) Paul Morris

No questions

Definition/ identification of second growth (no handouts) _ John Russell

With regard to a scientific/academic definition, we cannot separate old growth from second growth in terms of ring width or extractive content. One possible definition of second growth is age is < 250 years but there are trees <250 yrs in old-growth forests.

If there is a need for a user/end-product definition, you could put limits on percent of sapwood or rings per inch. Some shingle mills consider fewer than 8 rings per inch to be second growth.

Q. Is there such a thing as an old growth tree? Isn't old growth about species diversity and a multi-layered forest canopy?
A. There seems to be a need for a user definition once the tree has been removed from the forest.

Heartwood formation and natural durability in Douglas-fir and Western red-cedar (see handout) Adam Taylor

Q. Were these stands from natural regeneration?
A. Yes.
Q. Can you correlate volume of sapwood and amount of extractives in the heartwood ring laid down in the same year?
A. Yes. Although cores were used, a calculation was done to convert to volume of annulus.

B. Consumer concern over health effects of naturally durable species (no handout) - Karen Bartlett

No reports of sensitization to whole wood products. WRC dust can give irreversible asthma, also contact dermatitis, irritation. Eastern white-cedar and redwood sawdust can also give respiratory allergy. Associated with fine dust. Scrubbing of cells is what releases agent (Plicatic acid is one agent). No carcinogenic outcome reported. Coarser dust is a lesser problem. Odour does not cause allergy or physiological reaction. May get pychosomatic effect. This is not the same as multiple chemical sensitivity. Caution in using cedar oils is recommended. A report has been drafted and will be available to members companies shortly.

Q. Does inhaled dust come back out of lungs?
A. Yes

Cedar Working Group web page (no handout) - Paul Morris

This is currently a page on the Forintek web site (www.forintek.ca) with a minimal amount of information on the group. Also see www.durable-wood.com Discussion on what people wanted on web. No suggestions received.

Wrap up (no handout) - Paul Morris

Paul Morris again reviewed the 6 research directions.
Are we covering high priority areas?
No additions suggested. Possibly remove reduced cost pulping and colour control/stability if no interest.
Are there others that should be brought into the group?
Possibly producers of fine chemicals from foliage oil should be added.
The next meeting is not scheduled. The CWG will meet when we have information to share.