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Title: Chemical Characterization of Breakdown Products Involved in Detection of Ingipient Decay of Wood

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Incipient decay in Douglas-fir [Psuedotsuga menziesii (Mirb.) Franko] and Southern Yellow Pine (Pinus spp.) has been shown to be detected with infrared (IR) analysis. A characteristic absorption peak at 1720 cm^{-1} occurs in warm water extracts from decayed wood, but is absent in extracts from non-decayed wood. The objective of this study was to analyze the chemical source of the 1720 cm^{-1} peak.

Because the IR peak at 1720 cm^{-1} is characteristic of carbonyl groups, a technique was developed to isolate carbonyl compounds in incipiently decayed wood. Decayed wood was ground to 40 mesh and soxhlet extracted with 95 percent ethanol. After concentration on a rotary evaporator and drying, a portion of the extract was dissolved in ethanol and streaked on Whatman #1 or 3MM paper. The chromatograms were eluted upfield with 50 percent ethanol for nine hours. When dried, the chromatograms were viewed in a UV lightbox where their bands were marked with soft pencil.

Chromatograms of the extract from Douglas-fir wood incipiently decayed by brown and white rot fungi revealed changes in the wood's chemical structure when a comparison to the chromatograms resulting from the extract of end-matched non-decay wood was made. To isolate the bands containing carbonyl-bearing decay compounds, chromatograms of decayed wood extract were cut to separate the individual bands. The compounds were extracted with ethanol and dried for IR analysis.

Detection of carbonyl functional groups was made by the absorbance of IR light at 1720 cm^{-1}. IR analysis of the compounds extracted from the bands reveals that the carbonyl-bearing compounds have low motility under this solvent system. The IR spectra of these compounds exhibits both acid and aromatic character, indicating that the decay compounds probably arise from an enzymatic oxidative degradation of the lignin polymer.

Chemical Characterization of Breakdown Products Involved in Detection of Incipient Decay of Wood

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INTRODUCTION

There is no doubt that wood is a valuable natural resource. Wood is renewable; easy to gather and process; can be physically or chemically altered to yield thousands of different products; is fairly abundant; and is relatively durable. Unfortunately, as a biological material it is subject to natural decay processes that would ultimately return its components to the air and soil.

Decay organisms that attack wood include bacteria, fungi, and various species of the class Insecta. Of the three, decay fungi probably have the greatest impact on wood in service. Wood in contact with the soil or in any other environment where excess moisture is available, conditions which wood in service is often exposed to, creates a potential for the germination and spread of decay fungi.

Decay of wood in service by wood destroying fungi is a well known phenomenon that presents both economic and safety problems. Ideally, the fungal decay of wood should be prevented; either by treating the wood or maintaining its local environment at conditions that will not support the growth of fungi. When decay is detected, the wood should be reconditioned or replaced. However, the early or

incipient stages of decay are usually invisible to a simple visual inspection. Studies have shown that even though the decay is invisible, wood suffers a significant loss of strength.

There are two overriding definitions of incipient decay. In the laboratory, where the initial weight of small wood samples is usually known before degradation is initiated, incipient decay is usually defined as the stage of fungal decay in which five percent or less of the initial weight of the wood has been lost. In field samples, incipient decay is defined as the stage of decay in which decay fungi are present but their damage is not yet visible.

Many methods have been used to detect the presence of decay fungi in wood. Most detection methods require a great deal of skill, equipment, or time to obtain results. Incipient decay is an indicator of chemical changes in the wood. Gibson (1984) noted that infrared spectra of warm-water extracts from wood in the incipient stage of decay revealed an absorbance peak at 1720cm^{-1} that increased as decay (weight loss) progressed, but was never present in spectra of extracts from non-decayed, control samples.

My thesis is an examination of the chemical nature of the by-products of wood produced during fungal decay with the possibility that this information could lead to a method to detect incipient decay of wood that is easy to perform, inexpensive, and give relatively fast results.

OBJECTIVES

The objectives of this thesis are:

1. To isolate the compounds found in extracts of incipiently decayed wood from Douglas-fir [Psuedotsuga menziesii (Mirb.) Franko] and Southern Yellow Pine (Pinus spp.) that give rise to an absorbance peak of 1720 cm^{-1} in the infrared range.
2. To attempt to identify the above compounds.

LITERATURE REVIEW

Chemical Changes in Decaying Wood

The major result of fungal degradation of wood is a localized to extensive depolymerization of one or more of the three major wood components: cellulose, the hemicelluloses, and lignin. Reactions involved in wood degradation are usually accomplished through enzymatic pathways, although it will be discussed later that there is evidence that both brown and white rot fungi produce free radicals by which to initiate attack on crystalline cellulose. Enzyme catalyzed reactions for lignin are mostly oxidative, with initial hydrolysis to de-methylate the side chains. Polysaccharides are enzymatically hydrolyzed to shorten the chain length or to release monosaccharides, although some oxidation to glyconic acids occurs.

In a study by Philippou and Zavarin (1984), the effect of peroxide oxidation of wood and wood components was studied. The results (Figure 1) show that lignin, upon strong oxidation, (analogous to enzymatic oxidation), exhibits a marked increase in carbonyl content. Wood treated in a similar manner also yielded a higher carbonyl content, but cellulose exhibited very little change in its IR spectrum. The conclusion is that lignin, of the major wood components, displays marked changes due to oxidative degradation.

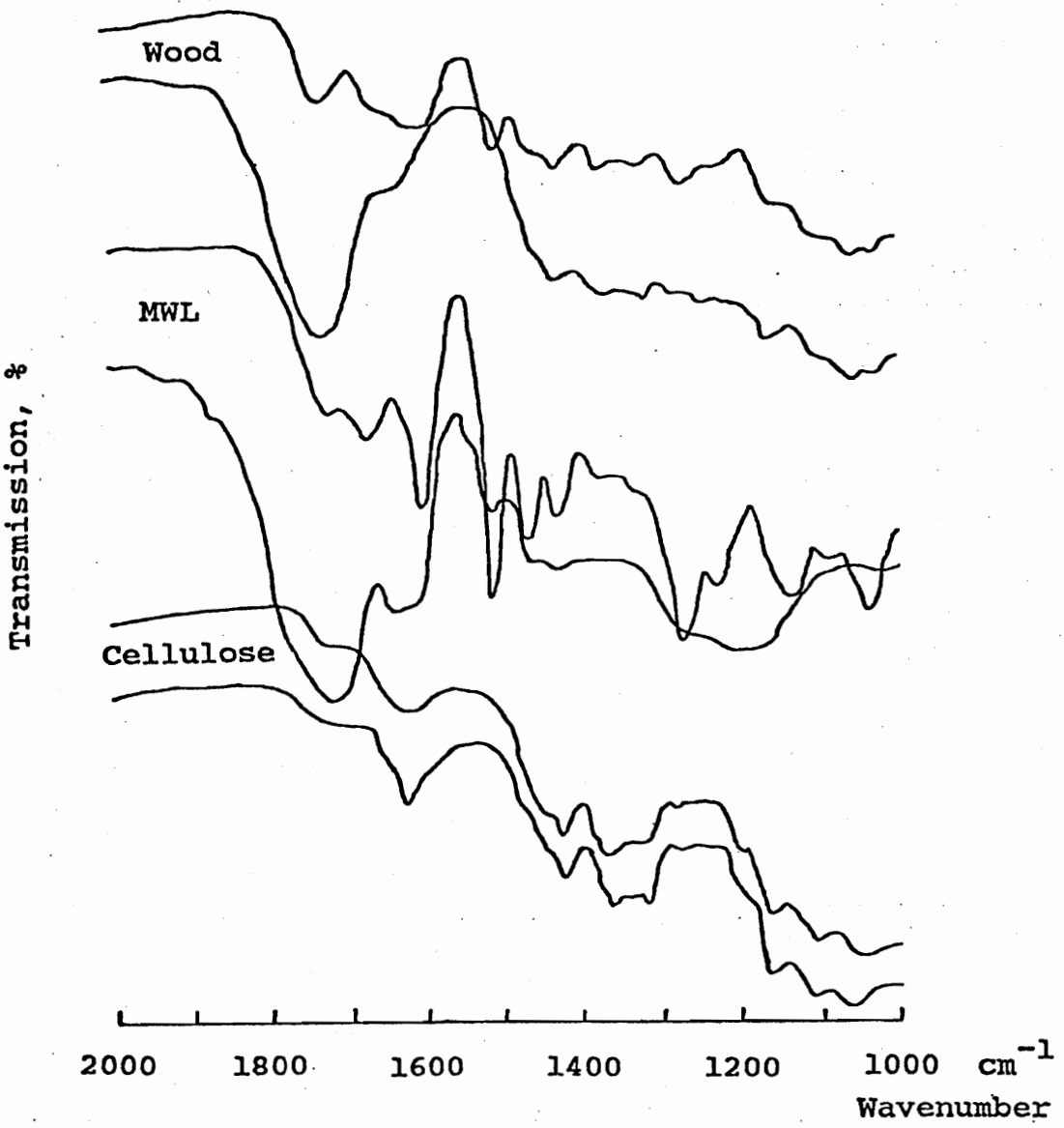

Figure 1. The effect of peroxide oxidation on wood, lignin, and cellulose. The lower trace of each pair is the spectrum of the sample after oxidation.

Decay by Brown Rot Fungi

Brown rot fungi are those whose overall effect on wood is the decomposition and removal of wood carbohydrates. In advanced decay, the residue is a dark-brown, friable mass that has a characteristic cubically broken, ember-like appearance that resembles burned wood. Decay can progress to a point where the weight of the residue is almost that of the lignin portion of the original undecayed wood (Crawford, p. 48).

Invasion of the wood cell by brown rot hyphae is almost entirely through the pit apertures during incipient decay (Wilcox, 1968). There is variation in the order of attack on the wall lamellae of softwoods, but it appears that the S2 layer is attacked first and then the S1 and S3 follow. The more highly lignified the cell wall, the more resistant the wall is to brown rot fungal attack.

Brown rot fungi cause a rapid and extensive depolymerization of cellulose (Kirk, 1975). Concurrently, significant changes are made to the lignin fraction. Although the weight of lignin remains about the same during degradation, many of the side chain carbons and methoxyl carbons are lost while an extensive amount of oxygen is added to the modified polymer; up to 35 percent more than in unmodified lignin (Crawford, p. 51). Kirk (1975) found that lignin isolated from Spruce wood decayed to about 70 percent weight loss was approximately one atom richer in

oxygen, and 35 percent deficient in methoxyl content per C_9 ("monomer") unit.

The oxygen added to the polymer appears as alpha carbonyl and carboxylic acid groups with some new phenolic hydroxyls. A loss of aromaticity was also noted.

In a study by Highley, et al (1983) cotton fibers decayed by brown rot fungi were observed by scanning electron microscope (SEM) and transmission electron microscope (TEM). Although random growth of the fungi was found over the fiber surface, no bore holes or erosion troughs were visible. However, degradation of the cellulose within the fiber was extensive. The hyphal sheath was found to often extend away from the hyphae and sometimes encircled the fiber. The sheath has been proposed to facilitate cellulolytic action (non-cellulase) by aiding transport of non-enzyme degrading agents to the substrate (Jutte and Sachs, 1976). Because the crystalline structure of cellulose precludes the intrusion of large molecule enzymes, a possible method of swelling the cellulose structure by enzymatically produced extracellular hydrogen peroxide appears responsible. Hydrogen peroxide in the presence of bi-valent metal ions, (Fe^{++}, Cu^{++}, Co^{++}, etc.), decomposes into hydroxyl free radicals that could easily penetrate the crystalline cellulose structure causing rapid, widespread depolymerization (Koenigs, 1974; Halliwell, 1978). Highley (1982) detected hydroxyl free radicals in brown rot fungi filtrates, but could not find

the parent peroxide.

Decay by White Rot Fungi

White rot fungi are those that are able to exclusively decompose all of the important structural components, including both cellulose and lignin. In advance decay, the residue is a spongy or stringy mass that is usually white or grayish white. In addition, white rot fungi are characteristically producers of extracellular phenol oxidases by which they can be distinguished from brown rot fungi (Crawford, P. 38). When cultured on malt agar, white rot fungi can be readily distinguished from brown rot fungi by their development of a blue color when treated with an alcoholic solution of gum guaiac (Boyce, 1961).

Invasion of the wood cell is done through both the pit apertures and bore holes during incipient decay (Wilcox, 1968). Decay progresses from the cell lumen towards the compound middle lemella, leading to a gradual thinning of the cell wall. In some instances, lignin is degraded first; in others the lignin and cellulose are degraded simultaneously (Panshin and deZeeuw, p. 344).

Different species of white rot fungi degrade the various components of wood at different rates, although all degrade lignin and cellulose. Crawford (p. 39) gives a compendium of many authors' results on the preference of removal of lignin and cellulose by different white rot

fungi. Some degrade either lignin or cellulose at a faster rate, while some degrade both at about the same proportional rate. Under the proper environmental conditions, white rot fungi completely degrade all structural components, with ultimate formation of carbon dioxide and water (Cowling, 1961).

As in the case with brown rot fungi, lignin degraded by white rot fungi looses many of its side chain carbons and methoxyl carbons, while an extensive amount of oxygen is added to the modified polymer. Kirk and Chang (1974) found that lignin isolated from Spruce wood decayed to about a 50 percent weight loss was approximately one atom richer in oxygen and 25 percent deficient in methoxyl content per C_9-unit. The oxygen added to the polymer as alpha carbonyl and carboxylic acid groups with no significant increase in phenolic hydroxyls. An extensive loss of aromaticity was noted without complete loss of the polymeric structure (Kirk and Chang, 1975). The evidence indicates that white rot fungi demethylate native lignin with rapid oxygenolytic cleavage of the polyhydroxylated aromatic rings to form aliphatic products; probably unsaturated carboxylic acids. Further reactions release these fragments from the lignin macromolecule. Because of the polarity of carbon--oxygen bonds, the lignin fragments produced by white rot degradation are more easily extracted with polar solvents such as dioxane and ethanol. This increased extractability of degraded lignin was noted by

Kirk and Chang (1974) and Hatta (1966).

In a study by Highley and Murmanis (1984) cotton fibers decayed by white rot fungi were observed by SEM and TEM. Of the five fungi tested, four were found to have penetrated the fibers, while Phanerochaete chrysosporium was limited strictly to surface degradation. Localized degradation within the cellulose was severe. The results demonstrate that a diffusible cellulose degrading system is responsible. This evidence supports Cowling (1961) who found cell wall thinning in cells without hyphae proceeding at about the same rate as cells with hyphae. In addition, Wilcox (1970) noted that white rot fungi produce cavity shaped fissures in the S2 layer of the secondary wall at some distance from the hyphae. A possible degradation method involves radicals generated by oxidative enzymes. The radicals would include superoxide anion ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), and hydroxyl radicals ($OH^{\cdot}$). Nakatsubo, et al (1981) report singlet oxygen formation by Ph. chrysosporium and provide evidence that it plays an integral role in lignin biodegredation. Eriksson (1981) found that superoxide anion is produced extracellularly by some wood destroying fungi.

The role of the sheath in cell wall degradation is unclear. Murmanis, et al (1984) declared that enzyme attack was initiated through the sheath because degradation of the cell wall under the sheath was evident without the presence of a hyphal strand. Highley and Murmanis (1984)

concluded that the sheath function was probably for attachment, nutrition, and protection since the sheath did not restrict the cellulytic system of white rot fungi. The sheath may also serve as a transport system for enzymes from the hyphal membrane. Lobarzewski (1984) reported that immobilized cell membrane fragments of Trametes versicolor were capable of an enzymatic release of simple phenolic compounds from Na-lignosulfonates; mainly members of the ferulic and vanillic acid families. His results agree with the compounds observed in extracts from wood decayed by white rot fungi (Kirk and Chang, 1975).

The requirement of a co-substrate for the degradation of lignin by white rot fungi is well evidenced. Kirk, et al (1977) found that white rot fungi would not grow on lignin alone. Ander and Eriksson (1977) point out that lignin degradation has always been shown to occur simultaneously with degradation of at least one of the wood polysaccharides. Finally, Ruel, et al (1984) found that Spruce wood impregnated with a two percent solution of glucose before decay by Sporotrichum pulverulentum had a markedly increased decay rate with an inhibition of cellulase and hemicellulase production. The result was extensive lignin degradation with little loss of cellulose. Most of the cellulose remained as large bundles or elementary fibrils as decay progressed.

Incipient Decay

The early stage of decay is called incipient decay. In this stage, decay fungi actively spread through the wood. Visible indication of incipient decay, if any, is a slight discoloration that is often mistaken for chemical stain (Panshin and DeZeeuw, p. 344). A microscopic examination of incipiently decayed wood reveals hyphae in the cell lumens and penetration of the cell walls by hyphae, predominantly through pits at weight losses below five percent and through bore holes at more advanced stages of decay.

Incipient decay indicates that wood is in an environment which supports decay, especially temperature and moisture content. Incipient decay is also harbinger to severe changes in the physical properties of the wood. Gibson (1984) reported that test beams decayed by brown and white rot fungi showing weight losses of 0.00 to 5.43 percent and 0.00 to 5.30 percent, respectively, yielded mean strength losses of 30.06% MOR, 7.67% MOE and 3.79% MOR, 5.05% MOE, respectively. Of the individual fungi tested, Poria placenta, a brown rot fungi, was found to have caused an average strength loss of 50.19% MOR and 14.04% MOE in small Douglas-fir heartwood beams with an average weight loss of 1.88 percent. Coriolus versicolor, a white rot fungus, caused an average weight loss of 4.20 percent and strength losses of 4.31% MOR and 7.31% MOE.

These differences can be explained by their mode of attack. Brown rot fungi, in incipient stages, decay or degrade wood saccharides very quickly. This loss, especially of cellulose, leads to a rapid loss of strength; most notably toughness. Also, some enzymes produced by brown rot fungi appear to act at a distance from the hyphae in order to swell cellulose and begin its depolymerization. White rot fungi are also found to act on cellulose at points that the hyphae are not in direct contact with, but attack is usually localized. White rot fungi metabolize cellulose and lignin simultaneously, and lignin is apparently metabolized in situ. Therefore, white rot fungi must actively degrade cell wall components in close proximity to the hyphal strand. The result is that brown rot fungi destroy the crystalline structure of cellulose rapidly, causing a rapid decrease in strength, while white rot fungal attack is more localized to the hyphae and proceeds at a slower rate.

It should be noted that once decay fungi germinate in wood, incipient decay has begun. To date, the only reliable methods of detecting the non-visible stages of decay are to microscopically examine the hypha growing in apparently sound wood or to culture increment cores or wood shavings and examine the hyphae that emerge for wood-decaying strains. In culture, wood destroying Basidiomycetes mycelia tend to appear silky in comparison to non-decay fungi mycelia of the same family. Under the

microscope, wood destroying fungi often exhibit "clamp connections" (Lloyd, 1972).

Many methods of microscopic examination for the detection of incipient decay have been evaluated. The degree of decay by brown rot fungi is particularly difficult to evaluate at weight losses below five percent because decay occurs in non-proximal areas to the hyphae (Wilcox, 1968). Staining of the wood and/or hyphal tissues has also been tested with varying degrees of success. Recently, acridine orange was used to evaluate early brown rot decay, (Krahmer, et al, 1982). Color change from green-yellow to orange-yellow was most obvious, through fluorescence microscopy, at a weight loss of about three percent, but was not considered practical below that level.

As stated earlier, Gibson (1984) noted that infrared spectra of warm-water extracts from wood in the incipient stage of decay revealed an absorbance peak at 1720 cm^{-1} that increased as decay (weight loss) progressed, but was never present in spectra of extracts from non-decayed, control samples. This peak is in the signature range of compounds containing carbonyl constituents.

IR spectrophotometry

The use of IR spectrophotometry may see increasing use as a valuable tool in the detection of incipient decay, especially for that caused by brown rot fungi. Gibson (1984) reports that for most of the fungi tested, the absorption peak at 1720 cm^{-1} from warm-water extracts of decay specimens was observed before weight loss was detectable.

IR spectrophotometry is very useful in organic chemistry. Within the IR range of 4000 to 600 cm^{-1}, a great deal of the chemical structure and functionality of an organic compound can be determined. Detection of compounds containing oxygen is particularly sensitive because oxygen is a strong electron withdrawer; producing strong bipolar bonds.

An IR spectrophotometer is basically a simple machine. It is composed of an IR source (a hot, glowing tungsten filament), a diffraction grating that separates the wavelengths of light passing through it, a slit that allows only a small portion of the IR wavelengths to pass it at any given time, and a photo-detection tube (PDT). A broad-spectrum beam of IR light from the source, (glower), passes through a sample to the diffraction grating. The diffraction grating, a form of prism, separates the individual wavelengths by bending light of different wavelengths different amounts, producing an IR "rainbow".

The slit is a very thin window that only allows a tiny portion of the "rainbow" to pass through it to a series of mirrors that eventually brings the light to a PDT. The PDT converts the light striking it into an electric current. Variation in the intensity of light absorbed by the PDT causes a variation in the current produced that can be recorded on a strip chart recorder. In a dual beam IR spectrophotometer like the Beckman IR-20A, a second beam of light from the source passes through a similar diffraction grating to the mirrors. In the mirror assembly is a chopper that passes alternating pulses of light from the sample and dual beam. In this case, the PDT measures the difference in intensities of light that have passed through the "control" and the sample. The difference between the two produced currents is what is recorded.

When IR light is passed through an organic sample, various wavelengths of the light are absorbed. Heat, in this case IR light, causes molecular motion. Most of the bonds in organic compounds absorb IR light of specific frequencies and convert that input of energy into stretching motion of the bonds or twisting motion of double bonds. In a dual beam IR spectrophotometer, the resulting loss of light intensity for a particular wavelength from the sample beam is found when the PDT compares it to the intensity from the "control" beam. The difference is charted as an absorbance peak by the recorder.

As stated, bonds containing oxygen are particularly sensitive to IR analysis. Their strength allows them to absorb a great deal of energy to convert to motion and most of the frequencies of IR light that they absorb do not coincide with those absorbed by other bonds. These frequencies are: 3600-3350 cm^{-1} for O--H bond in alcohols; 3500-2500 cm^{-1} broad peak for O--H of carboxylic acids; 1780-1690 cm^{-1} for C==O carbonyls which include aldehydes, ketones, carboxylic acids and their derivatives (esters and anhydrides); 1300-1080 cm^{-1} for C--O of alcohols, carboxylic acids, esters, and ethers; and a related peak of 2800-2700 cm^{-1} for C--H of aldehydes (hydrogen attached to a carbonyl). The functionality of oxygen contained in an organic compound is readily apparent by the combination of absorption peaks it causes in the IR spectrum.

MATERIALS AND METHODS

Isolation of Compounds

Extraction

Three matched samples of Douglas-fir wood meal, decayed by brown and white rot fungi, were extracted by the warm water method of Gibson (1984), Gibson's method modified to ethanol, and the Soxhlet method given below. Unless otherwise noted, 95 percent ethanol (ethanol) was used as a solvent. All ethanol used, whether new or recovered, was glass-distilled before use. This was the solvent of choice for four reasons: it has a lower boiling point than water which makes it easier to remove from the extract; it is commonly available to laboratories in large quantities at a fairly low price; it is mostly recoverable from the extracts by using a rotary evaporator; and it gives an extract that yields a stronger IR absorption peak at 1720 cm^{-1} than a warm water extract of a matched sample.

For the first test, Douglas-fir wood samples incipiently decayed by both brown and white rot fungi (Gibson, 1984) were split into match-stick sized pieces and ground to 40 mesh in a small Wiley mill. 25 g of the wood meal were put in a 43x123 mm cellulose thimble, covered with a piece of glass fiber filter paper, and extracted with 250 ml of ethanol in a Soxhlet apparatus. A quantitative extraction of the decay compounds was desired.

Therefore, the extraction process was run for a three hour minimum time. Beams decayed by brown and white rot fungi were not segregated because Gibson's experiments had shown that both classes of decay fungi caused the expression of an absorbance peak at 1720 cm^{-1} in the IR range, and a large quantity of extract containing the compounds that gave rise to this peak was desired.

The extract was concentrated on a rotary evaporator equipped with a safety trap and a 60-65^{o} water bath. The thick syrup was thinned with 5 ml of ethanol and transferred to a 35 ml glass scintillation vial with a Pasteur pipette. Three 5 ml aliquots of ethanol were used as washes to complete the quantitative transfer of the extract to the scintillation vial. After drying in a 70^{o} oven, the tar was solidified in a vacuum oven at 75^{o}. The resulting dry extract was tightly capped for future use.

For subsequent samples of southern yellow pine, the extraction procedure was identical. Before being ground, southern yellow pine wood samples were separated by specie of fungi by which they were decayed. The fungi tested were Gloeophyllum saepiaria, G. trabeum, Poria placenta, P. radiculosa, and Lentinus lepideus.

Chromatography

A small amount of the dried extract was dissolved in 10 ml of ethanol to be used for chromatography. The liquid was applied with a streaking pipette to half sheets of Whatman #1 paper cut to 28.5x48 cm. Best results were obtained when the streak ran from edge to edge in the machine direction due to the slow soluability of a line streaked four or five times. The chromatogram was developed with 50 percent ethanol eluting upfield for about nine hours. After air drying, the chromatogram was viewed in an ultraviolet lightbox for identifiable regions which were delineated with a soft pencil and numbered consecutively where one equals the origin and the last number equals the last band at the solvent front.

The bands were separated and cut into smaller pieces, and placed in separate 500 ml Erlenmeyer flasks. When the bands from five or six similar chromatograms had been collected, separated, and placed in their own individual flasks, they were extracted with ethanol. Warming the ethanol/paper mixture on a hotplate seemed to improve the extraction process. The strips were extracted four times with enough ethanol to cover them in their flasks. The solutions were filtered through Whatman #1 paper to remove any loose paper fibers and collected in a 100 ml round bottom flask. The procedure was repeated for each remaining collection of band pieces. When filtration was complete, the extract was concentrated on a rotary

evaporator in the same method used for the initial wood meal extract.

It was later found that Whatman 3MM paper, a thick version of the #1 paper, gave similar separation of the wood meal extract. Being thicker, it held two or three times as much material in a streak but developed at a slower rate.

Characterization of Compounds

Indicator Reagents

A number of common indicator reagents were tested for their sensitivity to compounds in the extracts. Most were used as sprays on the chromatograms.

Aniline hydrogen phthalate is an indicator for reducing sugars. To make the indicator, 1.0 g of freshly distilled aniline and 1.6 g of phthalic acid are dissolved in 100 ml of water-saturated 1-butanol.

Bis-diazotized benzidine is an indicator for phenolic hydroxyl groups, especially those of condensed tannins and other flavonoid compounds. The indicator, which must be made fresh before use, is made by thoroughly mixing three parts of benzidine reagent with two parts of ten percent (w/w) sodium nitrite. The benzidine reagent is made by stirring 5 g benzidine or 6 g benzidine hydrochloride with 14 ml concentrated HCl and then dissolving the suspension in 980 ml of water (Roux and Maihs, 1960).

Bromcresol green is a pH indicator that is yellow at pH 3.8 and blue-green at pH 5.4. When the spraying apparatus was cleaned, it was discovered that at pH 7.0, this indicator is bright blue. To make the indicator, 0.04 g of the dry powder is added to 100 ml ethanol. When used in this study, the solution was neutralized with a few drops of one percent sodium hydroxide until a deep green color was developed.

Alizarine red S is a pH indicator that is yellow at pH 3.7 and purple at pH 5.2. The working indicator is a one percent (w/w) aqueous solution of the dry powder.

2,4-dinitrophenylhydrazine is an indicator for carbonyl compounds. The reaction of this reagent with a carbonyl results in the formation of a C==N bond in a 2,4-dinitrophenylhydrazone; usually a bright yellow to orange precipitate. The indicator is made by placing 4 g of 2,4-dinitrophenylhydrazine in a 250 ml Erlenmeyer flask and adding 20 ml concentrated sulfuric acid. Water (30 ml) is added slowly while stirring until solution is complete. To the warm solution is added 100 ml of ethanol. If the powdered reagent used is the more common moist powder containing 20 percent water, the amount of powder used is 5 g. In use, a solution of 0.5 g carbonyl compound in 20 ml ethanol is mixed with 15 ml of the indicator reagent. Crystallization usually occurs in ten minutes, although standing overnight may be necessary.

Infrared Spectroscopic Analysis

To characterize the extracts of the chromatographic regions, (bands), a Beckman IR-20A double beam infrared spectrophotometer was used. This instrument scans the range of wavenumbers between 4000 and 250 cm^{-1}. The extracts were examined in solid phase using KBr as a pelletizing vehicle. Approximately 200 mg of KBr was initially mixed with 2-3 mg of dried band extract in an agate mortar, then powdered in a Wig'L'Bug vibrating ball mill. The powder was pressed in a 13 mm pelletizing die at 25,000 pounds per square inch (psi) for two minutes, then immediately raised to 40,000 psi for one minute. The resulting pellet was usually about 0.5 mm thick.

RESULTS AND DISCUSSION

Isolation of Compounds

Extraction

Three methods of extraction were evaluated for their ability to remove the compounds from wood that give rise to an absorbance peak at 1720 cm^{-1} in the IR range. The concentrated and dried extracts were analyzed with IR spectrophotometry. A comparison of the IR spectra shows several differences (Figure 2). All three showed a strong O--H bond absorbance of hydrogen-bonded alcohols and phenols. Dried warm water extract of decayed Douglas-fir shows a strong absorbance at 1050 cm^{-1} that is probably due to C--O bonding and a weak absorbance at 1730 cm^{-1} due to carbonyl groups (C=O). This extract showed very weak aromatic character; expected absorbance would be in the 870-675 cm^{-1} range and a stronger peak at 1600-1500 cm^{-1}. The extract from the warm ethanol method gave the strongest carbonyl absorbance and a strong C--O bond absorbance response in the range of 1300-1080 cm^{-1}. The aromatic character of this extract was stronger than that of the warm water extract, but not as strong as that of the extract obtained by the Soxhlet method. The Soxhlet extract also exhibited strong carbonyl character and C--O bond absorbance.

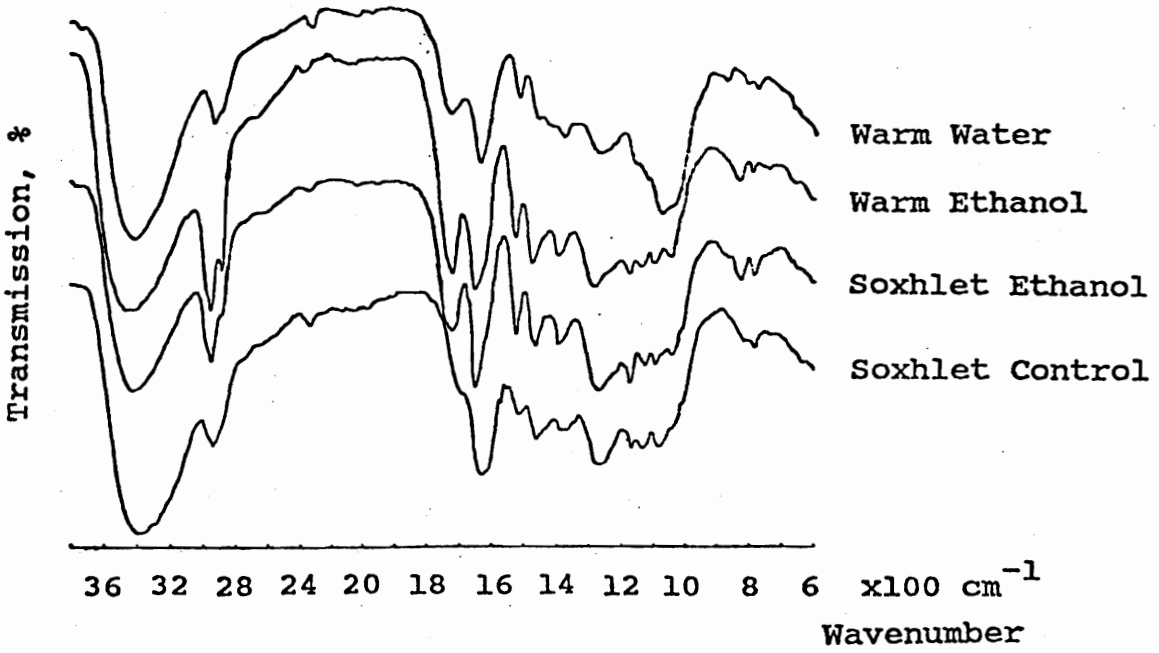

Figure 2. Infrared spectra of wood extracts prepared by different methods.

The ethanol Soxhlet method of wood meal extraction was chosen for this investigation for the following reasons:

1. Carbonyl-containing compounds were removed in high yield.
2. The sample is continuously flushed with fresh ethanol which should have fairly quantitatively removed the carbonyl-containing compounds (in comparison to the other two methods).
3. It is an easy procedure to perform.
4. Severe bumping was experienced with the warm ethanol method that resulted in wood meal deposited on the walls of the vessel of solvent. This would result in incomplete extraction of the wood meal.

Chromatography

In an attempt to find the best procedure for separating the decay extract compounds, a number of methods and solvent systems were tested. Eluting paper from the top with 1-butanol/acetic acid/water in a 4:1:5 v/v ratio produced a fast chromatogram but had little separation. All of the material was washed far downfield in a single band with an R_f of about 0.9. Eluting a similar paper with water, from the top, yielded good separation. Developing time was, however, very slow and a comparison of five runs showed problems with reproducibility. Running a paper chromatogram upfield in the manner of TLC plate improved reproducibility dramatically. Since the extraction

procedure was an ethanol modification of the warm water method of Gibson (1984), various aqueous concentrations of ethanol were tested as the solvent system. When side-by-side tests were run on identical chromatograms using 25, 50, 75, and 95 percent ethanol as the eluent, 50 percent ethanol was found to give good results fairly quickly and with good separation. By eluting for nine hours, two batches of chromatograms could be run in one day, the second being started in late evening to run overnight. On Whatman #1 paper, the solvent front would move about 20 cm in the nine hour running time. Similar results were obtained with Whatman 3MM paper, but running time increased one to two hours.

When the dried chromatograms of wood extracts heavily streaked on Whatman #1 or 3MM paper were examined in an ultraviolet (UV) lightbox, bands of colored fluorescence were detectable (Figure 3).

An extract of Douglas-fir control samples caused chromatograms with eleven separate bands of color. In contrast, an extract of combined Douglas-fir decay samples yielded a chromatogram having eight separate bands of color. The chemistry of the extractable portion of Douglas-fir wood appears to have changed greatly. In the control example, bands five through seven have combined or disappeared as has band eleven. Of the remaining bands, the colors of fluorescence have changed; most notably bands three, four, and nine.

Douglas-fir decay

Bands 1 2 3 4 5 6 7 8

Douglas-fir non-decay

Bands 1 2 3 4 5 6 7 8 9 10 11

Southern Yellow Pine non-decay

Bands 1 2 3 4 5 6 7 8

Southern Yellow Pine decayed by Gloeophyllum saepiaria

Bands 1 2 3 4 5 6 7

Southern Yellow Pine decayed by Gloeophyllum trabeum

Bands 1 2 3 4 5 6 7

Southern Yellow Pine decayed by Poria placenta

Bands 1 2 3 4 5 6 7

Southern Yellow Pine decayed by Poria radiculosa

Bands 1 2 3 4 5 6 7 8 9

Southern Yellow Pine decayed by Lentinus lepideus

Bands 1 2 3 4 5 6 7

Figure 3. Chromatograms viewed in an ultraviolet lightbox.

An extract of southern yellow pine control samples cause chromatograms having eight bands. In contrast, extracts of each of the five decay samples yielded chromatograms having seven bands of color with the exception of the chromatogram for the extract of pine wood decayed by Gloeophyllum saepiaria. This extract has an additional band of color within band five. The band may be present, but undetectable, in the other pine chromatograms because the G. saepiaria extract was streaked on at a much higher loading rate than the others.

From this analysis, it does not appear that the complexity of southern yellow pine extractable material is greatly altered by fungal decay. This does not mean that the compounds in the extract are unchanged; nor does it preclude the addition of compounds rendered soluble by fungal decay. This is evidenced by the change in fluorescence coloration of bands one, three, and four.

It should be noted that fluorescence coloration is due to both structure and chemical composition of a compound. Therefore, a band-color change observed in a UV lightbox is indicative of a change in a band's composition, structure, or constitution.

Characterization of Compounds

Indicator Reagents

A number of common indicator sprays were tested for their sensitivity to compounds in the extracts. A heavily streaked chromatogram developed with 50 percent ethanol was cut into inch wide strips, marked under UV light to reveal their zones, and sprayed with indicator. Those indicator solutions that gave results were aniline hydrogen phthalate, bis-diazotized benzidine, bromcresol green, and alizarine red S. The reaction pattern of the chromatograms to the sprays is given in Figure 4. In the figure, the bands are arranged in seven columns. The columns demonstrate the groupings of bands from each of the chromatograms that exhibit similar reactions to sprayed-on indicator reagents. The only exception to this column grouping was the reaction of Douglas-fir to aniline hydrogen phthalate. 2,4-dinitrophenylhydrazine could not be used as a spray reagent. Instead it was used as a reagent solution.

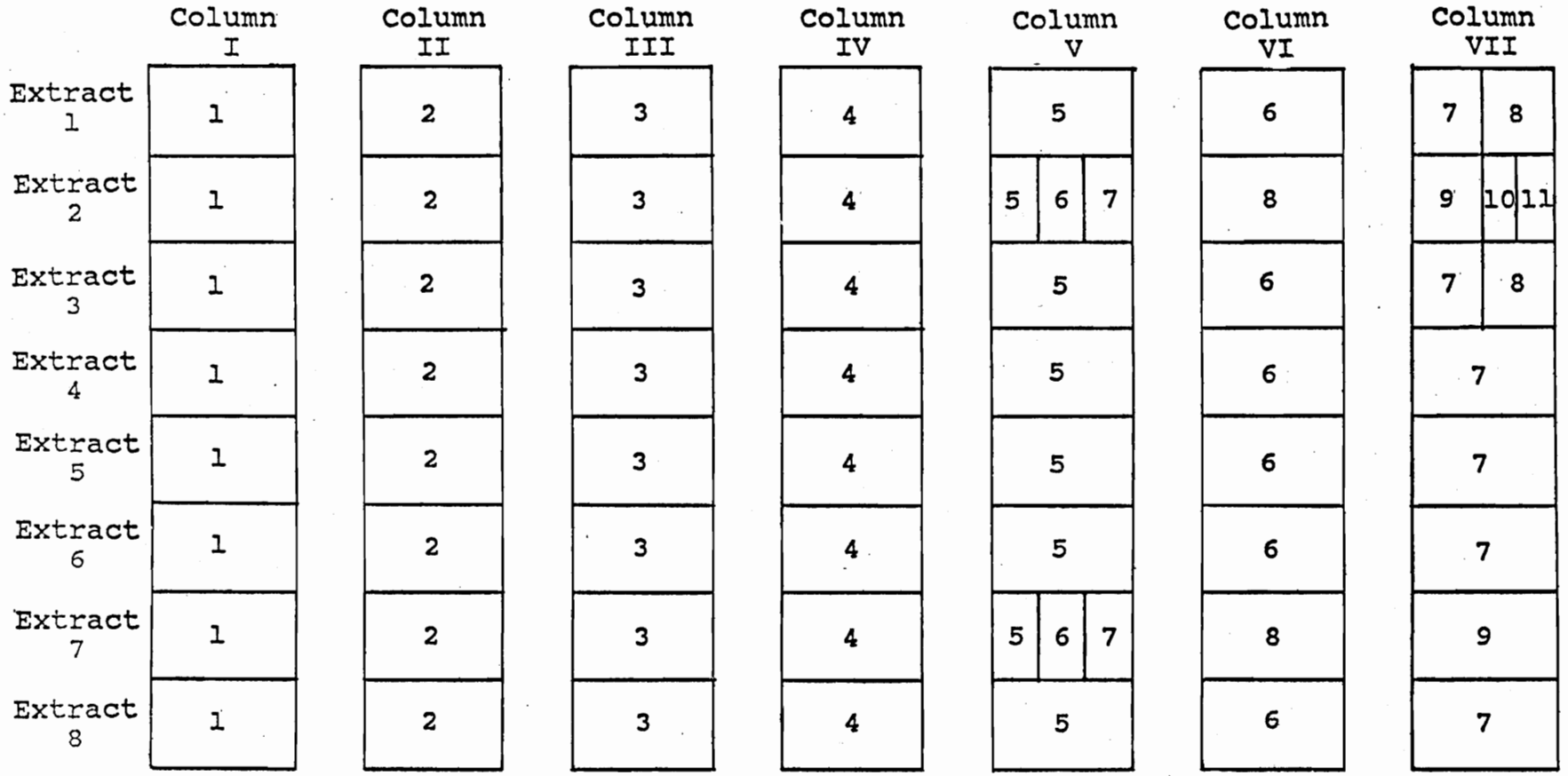

Figure 4. Reaction pattern of chromatograph bands to indicator reagent sprays. Extracts are labeled as follows: 1 = Douglas-fir decay, 2 = Douglas-fir non-decay, 3 = Southern Yellow Pine non-decay, and Southern Yellow Pine decayed by: 4 = Gloeophyllum saepiaria, 5 = Gloeophyllum trabeum, 6 = Poria placenta, 7 = Poria radiculosa, 8 = Lentinus lepideus.

Aniline hydrogen phthalate is an indicator for reducing sugars. Reducing polysaccharides, including cellulose, react with this indicator, but only weakly because of the low concentration of reducing ends when compared to the number of non-reducing monosaccharides in the polymer. Upon heating, reducing sugars are revealed as light brown areas on a light yellowish cream colored background in the portion of the chromatogram that was washed by the solvent. The unwashed portions of the paper develop a light buff color to this spray.

When this spray was used on a chromatogram of southern yellow pine extract, reduction occurred in band six and was visible as a light brown area. When the spray was used on the five chromatograms of decayed pine wood extract, each showed the same reaction. All reacting bands were found at about the same R_f value whether decayed or non-decayed. In contrast, a chromatogram of non-decayed Douglas-fir extract gave a very strong reaction at bands nine and ten. Unlike the pine samples, decayed Douglas-fir extract chromatograms did not react to this spray. It would appear from these results that there is a fundamental difference in the fungal action on these two wood species. Whereas the decay of southern yellow pine wood does not seem to greatly reduce the number of aldehyde groups, including free sugars, in the soluble portion of the wood, fungal action in Douglas-fir appears to destroy or alter most of these groups.

Bis-diazotized benzidine is an indicator for phenolic hydroxyls, especially those of condensed tannins and other flavonoid compounds. Immediately upon spraying, any areas that react must be marked because the spray reacts with paper as it dries to give a buff color, or a dark orange-brown background if the paper is heated to dry the spray. The color is strong enough to mask any weakly reactive areas that might appear when the chromatogram is first sprayed. Reactive areas are usually a red-brown color.

All of the eight extracts tested showed a slight reaction at band one and the two non-decayed extracts reacted at band three. All of the chromatograms showed a moderate reaction in the bands that align with bands seven and eight of the decayed Douglas-fir extract, and a variable reaction in the column of band six. In this column, the non-decayed Douglas-fir sample reacted in a very strong red-brown color while the similar band six of the decayed specimen reacted only moderately. The sample from non-decayed southern yellow pine showed a weak reaction in this band, but each of the decayed samples developed a pale purple color in this band. This spray readily demonstrates that the composition of the extractable portion of wood changes upon fungal degradation by the loss of band three from both non-decayed samples and the color changes that were found in the other reactive bands. The reaction at the higher band numbers is probably indicative of condensed tannins found in the extractable

portion of wood.

Bromcresol green is a pH indicator that is yellow at pH 3.8 and blue-green at pH 5.4. When the spraying apparatus was cleaned, it was discovered that at pH 7.0, this indicator is bright blue. When this is sprayed on chromatographic paper and allowed to dry, the paper develops a light yellow-green color. While still wet, the paper is the same color as the spray; yellow. The portion of the paper that was unwashed by the solvent develops a greenish blue color.

While being sprayed, all of the chromatograms developed a bright blue color in the column containing bands seven and eight of decayed Douglas-fir. All of the chromatograms showed acidic character in bands one through four, giving these bands a light greenish-yellow color. The exception to this was the non-decayed pine which showed very strong acid character in these bands. The use of this spray on chromatograms does not significantly appear to differentiate samples of decayed and non-decayed wood.

Alizarine red S is a pH indicator that is yellow at pH 3.7 and purple at pH 5.2. When this is sprayed on chromatographic paper, the washed portion is yellow-green and the unwashed portion is the same with included spots of weak purple.

In Douglas-fir samples, a strong purple color was developed in band eight of the decayed and bands ten and eleven of the non-decayed samples. In all of the pine

samples, a strong purple color developed in the bands of the final column. All of the decayed pine samples also showed weak purple spots in the column containing band six of the non-decayed sample. All other bands in all of the chromatograms were a yellow-green color. Although some differences were observed with this spray, its use as an indicator of decay is questionable. One point to note is, however, that pH of bands one through four appears to be within the range of 3.7-3.8.

2,4-dinitrophenylhydrazine is an indicator for carbonyl compounds. The reaction of this reagent with a carbonyl results in the formation of a C==N bond in a 2,4-dinitrophenylhydrazone; usually a bright yellow to orange precipitate. Because the reaction is acid catylized, the reagent cannot be used as a spray on paper chromatograms. Instead, the reagent is mixed with the ethanol extract to form hydrazones with any carbonyls present in solution. This reagent is unreactive to carboxylic acids.

When the extracts of Douglas-fir were tested with this reagent, no reaction was observed in either the decayed or the non-decayed extract. When the reagent was used with southern yellow pine decayed wood extracts, however, a dark precipitate was rapidly formed. The color was not in the expected range but the extract itself was highly colored. It is believed that the dark color of the formed hydrazone was due to the colored compounds present in the extracts. When the reagent was used on the non-decayed extract, no

reaction was observed. The results indicate that a rapid method of detecting incipient decay in souther yellow pine exists.

Infrared Spectroscopic Analysis

Infrared spectra of ethanol extracts from non-decayed and decayed Douglas-fir samples are shown in Figure 2. Gibson (1984) found that the absorption peak at 1720 cm^{-1} was present in spectra of extracts from decayed wood, but was absent in spectra from all non-decayed wood extracts. He notes that this peak was often found in the spectra of samples before a measurable weight loss occurred and became more prominent as weight loss increased. Kirk (1975), and Kirk and Chang (1974,1975) also reported a strong absorbance at 1720 cm^{-1} in their studies of fungally decayed lignin.

Infrared analysis of chromatographic bands was limited to those from decayed Douglas-fir extracts. In keeping with the objectives of this thesis, the bands were analyzed for compounds that absorbed in the carbonyl range. Before the analyses began, an extract was made of a whole chromatogram to confirm that the carbonyl-containing compounds were recoverable.

To narrow down the bands that would give carbonyl absorption peaks, the bands from a chromatogram were extracted in three separate groups. The first group contained bands one, two, and five. A dried ethanol

extract of this group gave a strong carbonyl absorbance, as did the extract of the second group. The second group contained bands three and four. The last group contained bands six, seven, and eight, and gave a very weak carbonyl absorbance peak.

The criteria for grouping the peaks was based on their fluorescence under ultraviolet light. The bands of the first group had a fluorescence that was not much different than the background fluorescence of the chromatographic paper. The remaining bands were grouped by their proximity to one another. The results of the re-extraction and IR analysis indicated that the decay compounds containing carbonyl functional groups had not moved very far from the chromatogram's origin when 50 percent ethanol was used as the eluent.

After enough chromatograms of the decayed wood extract were collected, an extract of each band was made. Figure 5 shows the spectra for the eight bands. All of the bands show the strong absorbances in the O--H region (3600-3350 cm^{-1}) of hydrogen-bonded alcohols and phenols and the C--H region (3100-2900 cm^{-1}) of alkanes.

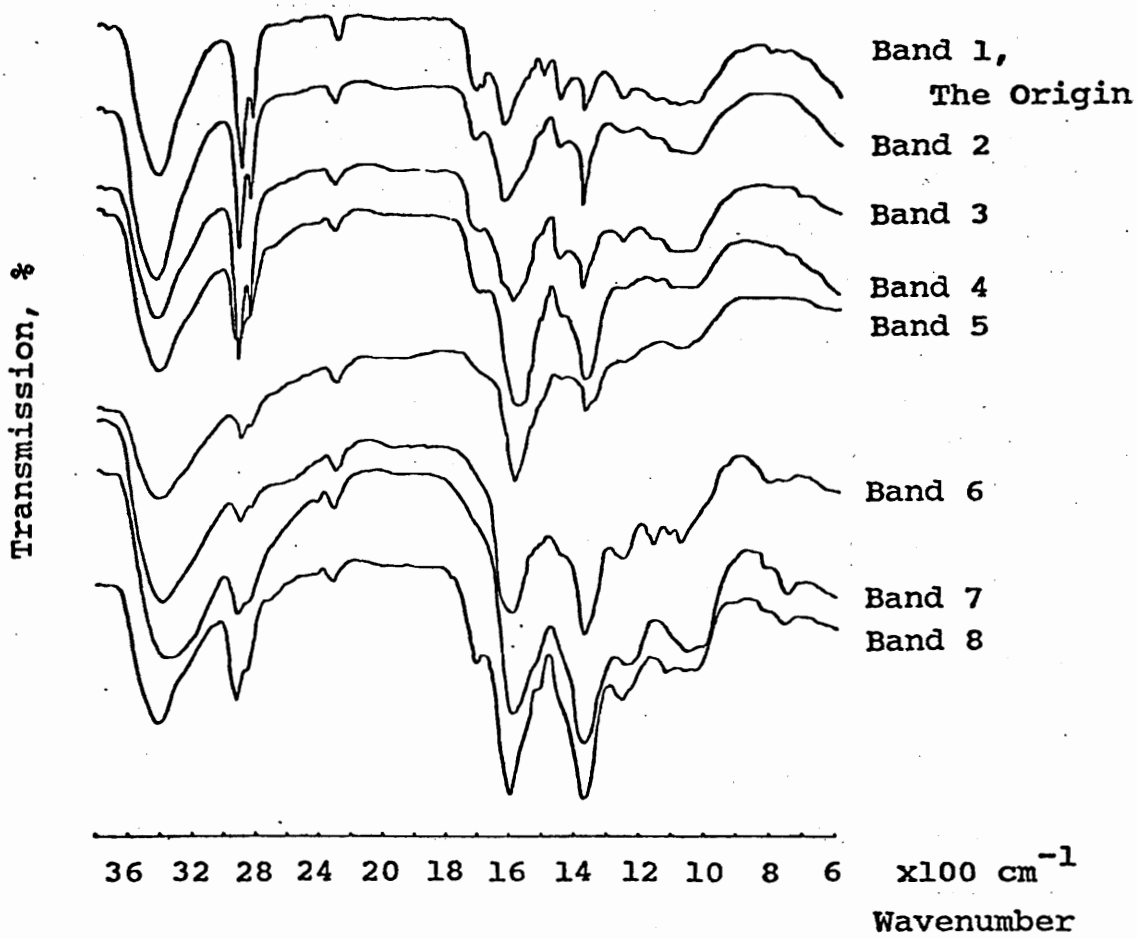

Figure 5. Infrared spectra of the eight chromatographic bands of decayed Douglas-fir wood extract.

Chromatograph band one is the only band that shows an even remotely strong carbonyl character (1720 cm^{-1}), although an earlier spectrum of band two run at three percent concentration in the pellet also showed a strong absorbance in this region. Bands three and eight show weak absorbance in the carbonyl region which might indicate a low concentration of this functional group in those bands. A lower concentration might be present in bands two and four, but no real beginnings of an absorbance peak are apparent. For the most part, carbonyl-containing compounds do not appear to be very mobile under the conditions of this chromatography system. The lack of mobility of these compounds is, however, advantageous. Although the band extracts were not weighed to measure their yields, it appeared that the volume of extracts from bands one through four comprised less than half of the total recoverable extract from all bands. Therefore, about half of the extract could be separated from the carbonyl-containing portions and discarded.

Enzymatic oxidase activity would be expected to produce carbonyls and carboxyls in the polysaccharide fraction of wood; especially in the initial stages when the polymer is being opened by oxidative radicals. However, these compounds should be extremely short-lived in an enzymatic environment. To cleave an aromatic ring, at least two phenolic hydroxyls are required; preferably vicinal. In a polysaccharide, virtually all of the carbons in the

skeleton are bound to oxygen. This makes polysaccharides and especially free monosaccharides extremely susceptible to decomposition; not oxidation to acids or carbonyls, but the complete oxidation to carbon dioxide and water.

The spectra obtained of the bands having carbonyl absorbance appear to be of lignin origin. The strength of the absorbance peaks and the wavenumbers they occur at is in good accordance with the IR spectra of lignin given in Figure 1. This should not be surprising because the high temperature peroxide degradation of lignin (Philippou and Zavarin, 1984) should closely resemble the enzymatic oxidative degradation of lignin. The spectra obtained from the carbonyl-containing bands also strongly resemble those obtained by Kirk and Chang (1974, 1975) in their work on lignin heavily decayed by white or brown rot fungi. Kirk and Chang attributed the carbonyl absorbance at 1720 cm^{-1} to to the formation of alpha-keto and carboxyl groups formed by the <u>in situ</u> enzymatic oxidation of the lignin polymer.

CONCLUSIONS

The following conclusions were drawn from this study:

Of the methods tested, an ethanol Soxhlet extraction was found to be the most practical method to remove soluble decay compounds from wood. This method also appears to yield a larger portion of the compounds that give rise to an absorbance peak of 1720 cm^{-1} in the infrared range (carbonyl containing compounds).

Paper chromatograms developed upfield with 50 percent ethanol displayed good separation of the compounds in the extracts that were streaked on them. About half of the extract could be separated as non-carbonyl bearing by this elution system.

Compounds containing carbonyls appear to have low mobility under the conditions of this system. Carbonyl absorbance in the infrared range was found in bands one through four of chromatograms streaked with decayed Douglas-fir extract.

The carbonyl-containing compounds that are detected probably derive from the enzymatic oxidative degradation of lignin. The spectra of these compounds, or the bands containing them, closely resemble the spectrum produced by milled wood lignin that has been oxidatively degraded by hydrogen peroxide at high temperatures and the spectrum produced by heavily degraded lignin extracted from wood with a dioxane and water mixture.

The fungal decay of Douglas-fir appears to destroy the soluble sugars originally present in the extractable portion of the wood. If any reducible compounds remain in the extractable portion, they are in a low enough concentration to not be detectable on a chromatogram sprayed with aniline hydrogen phthalate.

2,4-dinitrophenylhydrazine may be a valuable reagent for the detection of incipient decay in southern yellow pine wood extracts. The reagent was not indicative of decay when used with extracts of Douglas-fir.

RECOMMENDATIONS

The results and conclusions of this study indicate a need for more research in the following areas:

A better method of isolating the carbonyl-containing compounds in decayed wood extracts should be found. Column chromatography and HPLC are two methods that will probably give better separation and allow larger amounts of extract to be processed at one time.

An identification should be made of the chemical compounds containing carbonyls that are produced during the incipient decay of wood. The literature indicates that vanillic acid is one of the compounds produced during decay of wood. This should be confirmed.

The reaction of carbonyl compounds in decayed wood extracts with amines or azides may make the separation of these compounds easier. A suggestion is the use of 2,4-dinitrophenylhydrazine in an acidic medium which reacts with carbonyls to produce a bright orange to yellow precipitate. Initial tests with this reagent demonstrated its ability to detect decay in ethanol extracts of southern yellow pine wood. The detection limits should be established.

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