Analysis of Polymeric Extractives of Western Juniper (Juniperus occidentalis) Heartwood

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ABSTRACT

Extractives of Western Juniper (*Juniperus occidentalis*) have been isolated and separated by chromatographic means. Since this species is durable, bioactivity of several fractions has been determined. The bioactivity has been determined by the brine shrimp lethal bioassay. Less polar compounds with a low molecular weight average possess a high toxicity therefore contribute to the overall durability of this species. More polar fractions with higher molecular weight averages are non-toxic. The polymeric material with M_w = 13,550 g/mole, termed phlobaphene, has been purified. The analyses of western juniper phlobaphene by Fourier Transformed Infrared spectroscopy and 2D HMBC NMR (two dimensional Heteronuclear Multiple Bond Correlation) show thatat this is a heterogeneous polymeric material which reveals three long-range correlations. A long range labelled carbonyl correlation at around δ = 6.00 ppm corresponding to α-proton of β-*O*-4 type ether structures which is comparable to similar correlations found in lignins.

The two other long-range correlations at around $\delta = 5.00$ ppm and $\delta = 5.30$ ppm are not assignable to any lignin-like structure. The 2D HMBC experiment can be used as an improved quanlitative NMR technique for the assessment of labelled lignins and phlobaphenes. The ^{13}C NMR has a potential applicability as an improved technique for quantitative analyses of labelled lignins.

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INTRODUCTION

Western juniper (*Juniperus occidentalis*) occupies the dry forest sites of the Pacific Northwest, where stands of juniper are mixed with ponderosa pine (*Pinus ponderosa*). The species has recently spread to moister sites and border plains of sage brush (*Artemisia tridentata*), particularly in central Oregon and Washington (Gholz, 1980). Furthermore, western juniper has taken over more than 400,000 acres in Idaho, in sites that had previously been occupied by stands of sage brush and grass. In the past, the spread of juniper into the adjacent zones has been controlled with the practice of suppressive fires, but this practice has only allowed juniper to invade traditionally nonjuniper communities (Burkhart and Tisdale 1969, 1976).

Juniper is well adapted to the harsh environment of the high desert region of the western United States. The development of a stubby growth form and taper makes it difficult for commercial exploitation by the forest products industry and undesirable for range managers because it competes successfully with grasses (Britton and Sneva, 1981). Therefore, the rapidly increasing area of invasion has caused a great deal of concern, especially for its effects on livestock forage supplies.

Several studies were carried out to gain an understanding of the ecology and management of juniper rangelands in this region of the country. Areas of growth were determined, a policy of eradication was established, and recommendations were applied for a more beneficial control of juniper on invaded sites (Burkhart and Tisdale, 1969). Since this report, many control techniques have been applied to junipers with different degrees of success. The use of various herbicides commonly used for the control of undesirable species have been evaluated, these treatments did not effectively control western juniper and caused damage to the neighboring vegetation (Britton and Sneva, 1979). Due to the existing areas occupied by western juniper, some alternatives for the improvement and utilization of western juniper by ranchers were achieved as a ranch manager option in the early 80's. Evaluating the cost/effect of the techniques applied led to the conclusions that mechanical control should be used for big trees, and herbicide applications could be used for smaller ones. Unfortunately, in wood harvesting for these woodlands, only 42% of the entire woody biomass was marketed (Young *et al.*, 1982).

Eradication policies, in general, have not been successful for reducing the expansion of the range of western juniper. It is well adapted to survive in the high desert regions of the Western United States. Its resistance to control implies that this species possesses some biological or chemical advantage. One of the most interesting aspects of the chemical nature of western juniper is its natural durability. Heartwood extracts have been found to have anti-insecticidal and antimicrobial activity (Carter, 1974; Oda *et al.*, 1977; Scheffer, 1965; McChesney and Adams, 1985; and Adams *et al.*, 1988). An alternative approach to the eradication of the species may be to capitalize on the plethora of natural products produced by this species as a source of biocides, medicinals, or other specialty chemicals. One area of particular interest would be the use of chemicals such as those found in western juniper as an alternative wood preservation method.

The chemicals utilized in wood preservation are mostly organic or petroleum based materials with potentially harmful consequences on the environment and public health. Moreover, the use of inorganic salts such as Chromated Copper Arsenate (CCA) and Chromated Copper Borate (CCB) as water-borne preservatives is been banned in several countries of Europe. There is thought that it might occur in USA as well. Logical approaches to the replacement of these chemicals with less injurious compounds have been attempted by isolating wood extractives from durable woods and impregnating them into less durable woods (Carter, 1982).

The extraction of bioactive compounds from western juniper and their introduction into non-durable woods is probably not an economically feasible alternative to current preservation practices. However, the isolation and characterization of the extractives present in the heartwood of western juniper can provide an insight into the mechanism by which this species attains its high level of durability. In characterizing these materials, one may also discover extracts which could eventually find their way into the market place as medicines or biocides. This will have the net result of increasing the economic value of this species, thereby providing an alternative for simple eradication.

One of the more interesting aspects of heartwood formation in trees is the production of high molecular weight materials. The role of these polymeric compounds in heartwood is not known. These materials are typically not investigated although their concentrations are significant (Hergert, 1989). This present work deals with the polymeric heartwood extractives of western juniper. Fractions have been isolated and submitted to various chromatographic and spectroscopic studies. These efforts have provided the first chemical evaluation of these materials in a Juniper species.

PREVIOUS WORK AND PRESENT OUTLOOK

Wood can be described on the molecular level as a biopolymer composite. The structural strength required for upright growth is brought about by the covalent and/or non-covalent associations of cellulose, hemicellulose and lignin (Fengel and Wegener, 1989). Extractives such as resin acids, fats, terpenes, lignans, phenolic compounds, etc. are present in relatively low concentrations and serve as food reserves or protectants from pathogen attack (Sjostrom, 1993).

The xylem of a young living tree is mainly made up of sapwood, which accomplishes three vital functions: first, transport water, salts and gases from the roots to all parts of the tree; secondly, provide a strong structural support for the tree; and thirdly, serve as a reservoir for water, energy, minerals and solutes. As the tree ages, physiological changes take place which ultimately result in cell death. The initiation of this aging process is governed by an intricate balance between physical and biochemical conditions and varies widely between species. It is believed to be genetically controlled, (Duchense *et al.*, 1992) and can be influenced by the rate of the growth as well as environmental conditions. Cell death implies that there will be no biological processes available to the tree if pathogen invasion occurs. Biochemical processes have therefore evolved to prevent degradation of the non-living portions of the tree. This process is termed heartwood formation. In some species, insect attack, pruning, and cambial damage can induce heartwood formation as well (Hillis, 1987).

During heartwood formation, extractives are biosynthesized in bordered pits and parenchyma cells, predominantly at the heartwood/sapwood interface. This process is considered to be control by enzymatic and/or non-specific oxidative transformation. The polyphenols are initially produced within a parenchyma cell compartment (vacuole or amyloplast) where controlled biosynthetic transformations convert sugars to polyphenols. Upon death of the cell, compartmentalization disintegrates and mixing of the polyphenols and the cytoplasmic constituents occurs. Some of the enzymes present originally in the cytoplasm can then react with the polyphenols affording non-specific transformations. Once free of the boundaries of the cell, the polyphenols can move by diffusion processes and further react non-specifically with other extractives as well as cell wall components. The products of the non-specific reactions are often termed phlobaphenes.

A large proportion of the extractives may be removed from wood by means of polar and non-polar solvents. Their composition and concentration varies upon the site, species, part of the tree and age of the wood (Fengel and Wegener, 1987). Commonly, one fraction is named "resin" which refers to a mixture of different compounds that mutually inhibit crystallization (Sandermann, 1960). These compounds include the terpenes, lignans, stilbenes, flavonoids and other aromatics. Other organic compounds that are present in the extractives encompass fats, waxes, fatty acids and alcohols, steroids and higher hydrocarbons.

One of the most interesting classes of extractives are the so-called polyphenols. These compounds all originate from the shikimic acid pathway (Figure 1), with subsequent transformations affording hydrolyzable (e.g. gallotannins, ellagitannins) and condensed tannins (e.g. dihydroquercetin, epicatechin, etc.), lignans (e.g. pinoresinol, neolignans and norlignans).

It has been proposed that the purpose of polyphenol production is to assist trees in passive resistance to pathogen attack (Appel and Schultz, 1992; Scalbert, 1992). Trees have two mechanisms of protection from pathogen attack. **PASSIVE** defenses, employed in the heartwood and the bark of several species, act as a toxic barrier to the attack of natural wood enemies. Since the bulk of a tree is composed of non-living tissue, this passive mechanism protects wood where biological responses are not possible. This mechanism relies on the formation of extractives in the heartwood (Laks, 1991), with the decay resistance due principally to the biocidal activity of these extractives toward fungi as well as other pathogens such as termites. **ACTIVE** responses occur in the living portions of the tree and are a response to a mechanical wound or to the action of an invading organism.

Natural products of forest plants such as those present in bark and heartwood have found a widespread use as perfumes, spices, medicines and drugs for centuries. One of the earliest uses of tannins for instance, was for production of leathers from animal hides. Natural products chemists worldwide have been searching for new and improved economically important compounds from the plants of the world. In relation

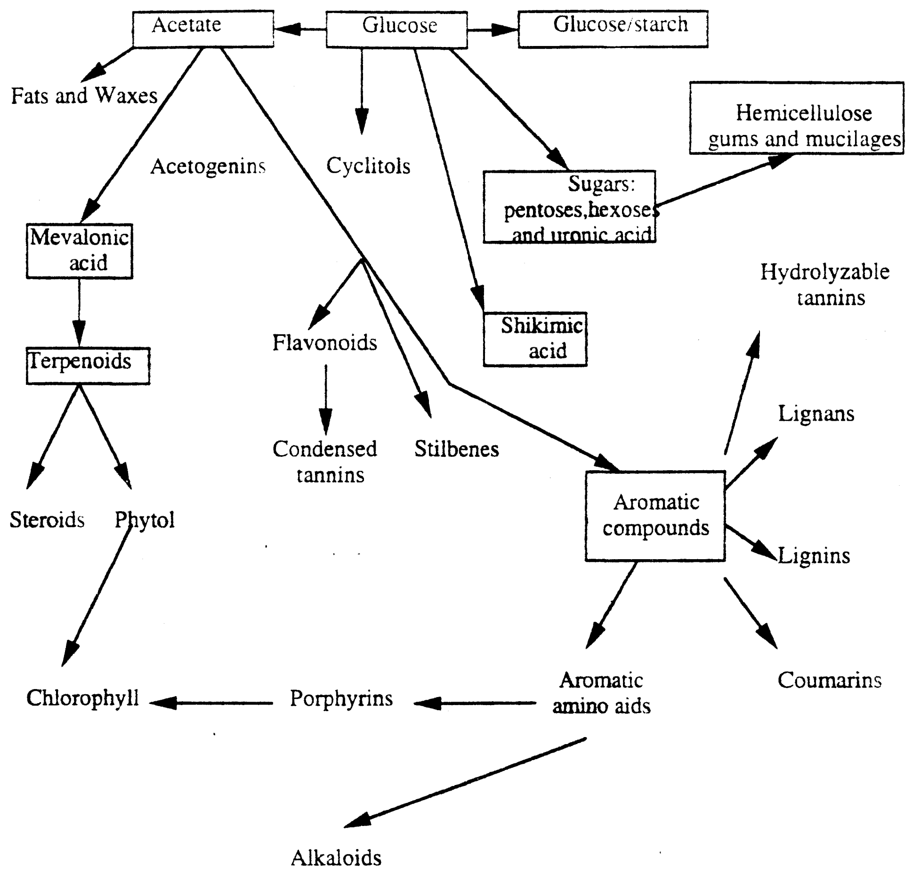


Figure 1. General Biosynthetic Pathway for Extractive Production in Plants

to juniper, the most notable attribute is its high resistance to decay (Scheffer and Cowling, 1965). Understanding its mechanism of decay resistance may lead to new environmentally-benign treatment practices for wood preservation. In this context, the identification of the major constituents of juniper heartwood is of vital importance.

Regarding western juniper, several chemical analyses have been performed to determine its chemical composition and to extract the volatile components from its leaves and wood (Kurth and Ross, 1948; Fahey and Kurth, 1955; Vasek and Scora, 1967; Tatro *et al.*, 1973; von Rudloff *et al.*, 1980). It was found that the yield of volatile oils from the sapwood and heartwood was 0.9-1.25% (based on the weight of dry wood), with the main constituents being cedrol and cedrene (Kurth and Lackey, 1948). Other extractives found in western juniper wood were fatty acids, phytosterol, and catechol phlobaphenes (Kurth and Ross, 1954). A later analysis of volatile oils of foliage and branches obtained by steam distillation established that the main compounds were borneol, present in the free form and as the acetate (22-25%), cadinene 20-25%, and other volatile oils such as: α–pinene (**1**, p. 24), β–phellandrene, sabinene, terpinolene, α-terpinene (**2**, p. 24), borneol (**3**, p. 24), etc. (Fahey and Kurth, 1955). Its heartwood extracts have been studied mainly as a possible cedarwood oil source (Adams, 1987). The analysis of volatile oils western North American junipers by gas-liquid chromatography has also been performed as a means of taxonomic differentiation among the members of the Cuppressaceae family (Vasek and Scora, 1967; Adams *et al.*1983, MacKenzie and Holme 1984).

The development of chromatographic methods and IR spectroscopy contributed substantially to the taxonomic differentiation among six species of junipers. Results of a study reported 37 detected components and the identification of 9 chromatographic patterns. Although, major compounds where not unambiguously identified, some were tentatively characterized as anethole, terpineol, neryl formate, estragole, cadinene, sabinol, bornyl acetate, furfural and linaool (Vasek and Scora, 1967). Chemosystematic studies of western North American junipers validated the chemical composition of western juniper volatile leaf oils as being mainly comprised of sabinene, myrcene, 3-carene, limonene, α–terpinene, terpinolene, borneol, bornyl acetate, thymol, δ–cadinene, calamanene, ε–cadinene; and cadinol isomers (Adams *et al.*, 1983), as described in Table I.

In the same way, volatile heartwood oils of 11 taxa of *Juniperus* were chromatographed to determine the yield and the major components of commercial value: α-cedrene, β-cedrene, thujopsen, cuparene, cedrol and widdrol. Cedrol was found to be the main compound accounting for the 38.9 % of a total yield of 1.68% (Adams, 1987).

Seasonal variations of oil composition were similarly investigated. A study of leaf oils of three juniper species (*occidentalis, osteosperma*, and *californica*) determined that the yield of the monoterpene volatile fraction is a short-term temperature phenomenon (diurnal fluctuation) rather than by a long term seasonal effect (Tatro *et al.*, 1973). The variation of oil terpene composition was found to be dependent on the geographical population of the species as well (von Rudloff *et al.*, 1980). Yields of hexane and methanol soluble material from sequential 6-h Soxhlet extractions (hexane followed by methanol) of heartwood and bark/sapwood of western juniper were reported on an oven dry weight basis as being 1.89% hexane extract and 2.76% methanol extract; and, 0.46% hexane extract and 3.25% methanol extract; respectively (Adams, 1987).

A new trend in the investigations of wood extractives involves the use of assays for the determination of biocidal activities against insects and other pathogens. Temperate and tropical wood species such as *Juniperus virginiana* and others were analyzed to gain a better understanding of the relationship between subterranean termites and chemical constituents of wood extractives (Carter, 1974). The active insecticidal principles in the heartwood of *Juniper recurva* Buch. were found to be thujopsene and 8-cedren-13-ol and some sesquiterpenes (Oda *et al.*, 1977).

McChesney and Adams (1985) developed a procedure for evaluating the antibacterial, antifungal and toxic activities of 80 plant species from the southern United States and southern Great Plains. From the Cupressaceae family only *Juniperus monosperma* was studied. Polar, intermediate and non-polar extracts were screened against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Mycobacteium smegmatis*.

Table I. Chemical Composition of Western Juniper Extracts Adapted from different sources (Fahey and Kurth, 1955; Vasek and Scora, 1967; Tatro *et al.*, 1973; von Rudloff *et al.* , 1980; Adams, 1987)

COMPOUND	TOTAL OIL (%)	COMPOUND	TOTAL OIL (%)
Tricycline	traces - 2.3	Linalool	traces - 0.5
α–Thujene	0.8 - 1.2	Pinene hydrate	traces
α-Pinene	2.5 - 7.85	cis-Sabinene hydrate	0.6
Camphene	0.2 - 1.0	trans-Sabinene hydrate	0.9
Sabinene	8.4 - 37.85	Borneol	1.3 - 3.6
Myrcene	1.1 - 2.12	4-Terpineol	0.4 - 6.6
α–Phellandrene	0.5 - 1.1	Isothimol	0.9
β-Phellandrene	1.0 - 2.3	Thymol	0.5
3-Carene	0.3 - 7.8	Eugenol acetate	0.9
α-Terpinene	traces - 3.14	α–Murolene	0.9
p-Cymene	0.6 - 12	α–Cardinene	2.7
Limonene	1.2 - 2.2	Calamanene	0.6
γ–Terpinene	1.8 - 4.4	β-Cadinene	2.6 - 5.3
p-Menth-1(7),3-diene	0.7	Cadinol isomer	2.6 - 5.3
Terpinolene	0.7 - 1.1	4 Terpinenyl Acetate	0.7
4-Terpinenyl Acetate	0.7		

The reported results indicate that the non-polar and intermediate extracts of this species possessed antibacterial activity and antifungal activities against all the microorganisms screened. In the case of *Juniperus occidentalis*, it was reported that heartwood, bark/sapwood and leaf extracts of this species all showed termicidal activity. It was noticed that organic solvents such as hexane and methanol did not remove all of this activity (Adams *et al.*, 1988). It was also shown that hexane heartwood, bark/sapwood and leaf extracts from *Juniperus occidentalis* possess a greater antibacterial activity than the corresponding methanol extracts. In relation to antifungal activity, only the hexane and methanol heartwood extracts exhibited activity (Clark and McChesney, 1990).

Lately, several studies have been performed in other species of the *Cupressaceae* family, specifically *Juniperus thurifera* and *Juniperus chinesis*. Chemical analysis of the crude extracts of the first species have resulted in the isolation and identification of several pimarane acids (**4**, **5**, p. 24) and labdane acids (**6**, **7**, p. 24) also called diterpenes. The structural elucidation was determined from spectral data of their corresponding methyl esters (San Feliciano *et al.*, 1993 **a**). Likewise, a method for the structural elucidation of these compounds was developed based on one-bond and long-range heteronuclear 2D NMR correlations obtained for methyl abietate (San Feliciano *et al.*, 1993 **b**).

Moreover, hexane extracts of *Juniperus thurifera* leaves have yielded several new diterpenoids of the labdane, pimarane and abietane skeletons as well as several sesquiterpenoids. Their characterization was performed mainly by ^{1}H and ^{13}C NMR techniques as well as chemical transformations. The new assignments were: 8-hydroxy-labda-13(16),14-dien-19-yl *O*-acetyl-*Z*-coumarate (**8**, p. 25) and *O*-acetyl-E-coumarate (**9**, p. 25) (San Feliciano *et al.*, 1988). The isolation and characterization of a number of lignans of this hexane extract were also described (San Feliciano *et al.*, 1989).

Fifteen lignans (three new compounds) were obtained from polar extracts, by Soxhlet extraction with chloroform after depletion with hexane. Again, assignment of the ^{13}C spectrum of β–methyl peltatin was performed through one-bond and long-range heteronuclear 2D NMR experiments (San Feliciano *et al.*, 1992). These hexane and chloroform extracts have both shown significant cytotoxic and cytostatic effects (San Feliciano *et al.* unpublished data).

Additionally, acetone extracts of the bark from *Juniperus chinesis* have been analyzed, and several diterpenes including semperviranes, abietanes, totaranes and a new chinane-type compound having isopropyl groups at C-11, C-12, C-13, and C-14 positions were isolated and purified by silica gel chromatography. The identification was accomplished by means of mass spectroscopy, UV, IR and ^{1}H NMR techniques (Jim-Min *et al.*, 1993). It was also mentioned that the leaf extracts of this species were subjected to similar methods and eleven additional abietanes were found. Some of the assignments for the different compounds were determined with ^{13}C NMR and Nuclear Overhauser Effect experiments (Ching-Kuo *et al.*, 1993)

Recently, the leaves of 30 Japanese conifers have been tested to obtain bioactive compounds by a paper disk method. The system consists of a platinum loop which holds the fungi *Ceratocystis picea, Tricophyton rubrum*, and *Epidermophyton floccosum*. These fungi are inoculated on a petri dish which contains a medium of commercial potato dextran agar and Sabouraud's agar. Ohashi *et al.* (1994) have characterized these antifungal components as being sesquiterpenes. After screening, *Juniperus chinesis* var. *pyramidalis* was selected for further investigation. The three antifugal sesquiterpenes were obtained from the hexane soluble part of the methanol extract. Their structures were assessed to be 8-acetoxyelemol (**10**, p. 25), 8-hydroxyelemol (**11**, p. 25) and hinokiic acid by instrumental analysis.

In contrast to the previous mentioned studies, investigations concerning the polar polymeric extractives of wood are relatively rare. These materials are termed phlobaphenes, a loose categorization to describe the polymeric, water-insoluble, methanol extractable materials present in many species. Although, efforts have been made to procure these materials (Swan, 1963; Zavarin, 1965), the identification of their structures and the interaction with the co-occurring extractives has yet to be accomplished. This information would lead to a better understanding of the role phlobaphenes play in natural wood durability, and also improve our understanding of heartwood formation.

Natural phlobaphenes are found in the bark and wood of most woody plants (Laks and Hemingway, 1987). These polyphenolic materials are present in the wax-free alcohol extracts from wood and bark, often accompanying tannins (Herbert and Kurth, 1952). The term "natural phlobaphenes" is applied to the water insoluble polymeric phenolics obtained from plants by extraction with neutral organic solvents (Zavarin and Snajerk, 1963). They are also referred to as reddish purple pigments and are considered to be a result of the condensation of tannins through enzymatic oxidation or dehydration (Buchanan *et al.* 1944). In contrast, "synthetic" phlobaphenes called 'tanner's reds" are formed by precipitation during treatment of extracts with mineral acid in the tanning industry (Haslam, 1966 and Ishak, 1972). Recently, it has been reported that the phlobaphene formation also occurs during sulphite-water extraction of tannins from pine bark (Sealy-Fisher and Pizzi, 1992).

There is speculation as to the differences between the "natural" phlobaphenes which may include extraneous matter such as lignans, and those formed by acid treatment (Sealy-Fisher and Pizzi, 1992). The study of polymeric materials such as phlobaphenes is quite challenging. Their complete purification is laborious to accomplish as the material remains bound to chromatographic supports. The random non-enzymatic oxidation reactions which lead to their formation affords a polymer which makes chemical elucidation difficult.

Early studies have reported the presence of phlobaphenes only as a means to balance the yields of other extractives for both softwoods and hardwoods. The chemical nature of the cork from Douglas-fir bark has been described as being composed of lignoceric acid, lignoceryl alcohol, phytosterol, glycerol and phlobaphenes among other materials (Hergert and Kurth, 1952). They pointed out that these compounds are similar to those which cement the cork cells together (in the same way as lignin forms the middle lamella in wood) and appears to be the precursor of these substances. Likewise, Kurth and Becker (1953) reported the presence of a catechol nucleus as well as the presence of a benzene nucleus and an absorption peak of 1700 cm^{-1} characteristic for carbonyl group and an intense OH band in the bark and heartwood extractives of Red Alder (*Alnus rubra*).

Chemical analysis of the bark of Red Fir (*Abies magnifica* A. Murr.) stated that phlobaphene present in the ether and alcohol extract was dewaxed, washed with warm water and saturated with chloride to precipitate the tannins, which were extracted with benzene to remove waxy materials, recrystallized and chromatographed, resulting in almost pure catechin (Becker *et al.*, 1958). It was also found that phlobaphenes absorb UV radiation at a wave band similar to that of catechol tannins indicating that these compounds are structurally related (Graham and Kurth, 1959).

Additionally, in western true firs (*Abies amabilis* Loud, *Abies procera* Rehd; *Abies concolor* Lindl. and Gord; and *Abies grandis* Lindl.) it was concluded that flavonoids, phlobatannins and phlobaphenes are related in structure, considering that phlobatannins are the condensation products of catechin-like products and phlobaphenes are further condensation products of phlobatannins (Carlberg and Kurth, 1960). It has also been suggested that natural phlobaphenes from heartwood of Redwood (*Sequoia sempervirens* Endl.) resulted from tannins through oxidation or dehydrogenation (Zavarin and Snajberk, 1963). Later, it was pointed out that these polymers contained guaiacol and veratrol units, which seemed to be responsible for the high methoxyl values and polarity of some of the fractions (Zavarin *et al.*, 1965). Subsequently, the term phlobaphene was applied to any colored material from bark, suggesting that the coloration is due to the presence of a cation in the molecule which presented similar IR and UV characteristics to cyanidins. In any event, phlobaphene appeared to be a heterogeneous phenolic polymer containing both an organic cation and an organic anion (Swan, 1963).

The need to apply modern methods that facilitate the study of these materials led to the use of conductometric titration to determine the phenolic groups in polymeric phenolic compounds (Baeza *et al.*,1985). As a result, relative molecular masses of 6500 and 2300; and, 53 and 23 total phenolic groups per mole for phlobaphene and tannin molecules respectively, were determined. The monomeric unit has a relative molecular mass of 320 corresponding to a molecular formula of $C_{16}H_{16}O_7$. The value for OH group per monomeric unit is 2.61 and 2.83 for phlobaphenes and tannins respectively (Baeza *et al.*, 1986).

The most recent data published for molecular weight distribution of polymeric polyphenols from mature heartwood accounts for conifer condensed tannins of the heartwood and outer bark of several species (Hergert, 1992). Hergert found out that western red cedar heartwood extracts possessed a M_n = 1,575, M_w = 4,525, M_z = 2.87; tannins of mature California Redwood heartwood (*Sequoia sempervirens*) derived from *p*-hydroxyphenol and presented a M_n = 1,105, M_w = 1,476, and M_z = 1,911, polydispersity = 1.336 (strong linearity); western hemlock heartwood showed a lignin-like material which had incorporated lignans with a M_n= 2,219, M_w = 4,223, and M_z = 7,634, and polydispersity = 1.9; and finally, "tannins" (proanthocyanidins) of red spruce outer bark had a M_n = 1,300, M_w = 3,554, M_z = 8,259,and polydispersity index = 2.73.

Since phlobaphenes have been defined as lignin-like materials, a molecular weight comparison between these polymeric materials would be appropriate. Molecular weight distribution for several industrial lignins has been performed. The results showed a range of M_w = 3,000 - 20,000 g/mole; a M_n from 1,200 to 20,000 g/mole and polydispersity indexes from 2 to 12 (Glasser *et al.*, 1993), somewhat higher than that observed for phlobaphenes.

A study of the polymeric extractives of Douglas-fir has recently been performed on Sephadex LH-20 in methanol (Foo and Karchesy, 1989). Although six fractions were obtained, a substantial portion of phlobaphenes was retained on the column. After IR and ^{13}C NMR, it was revealed that the complex mixture contained condensed tannins, carbohydrates, methoxyl groups, and dihydroquercetin. The degree to which these compounds were covalently linked was not determined . It appeared that 50% of the polyphenols in the outer bark were polyflavonoids of the procyanidin type, whereas the rest seemed to be oligomers and polymers formed by oxidative coupling.

Low molecular weight polyphenols are thought to be precursors to phlobaphene formation (Zavarin *et al.*, 1965; Kurth *et al.*, 1968; Ishak, 1974; Malan *et al.*, 1992). Chemical shifts characteristic of isolated flavanoids, flavonoid glycosides, procyanidins and lignans in ^{13}C NMR have been observed in ^{13}C NMR of phlobaphene polymers from Douglas-fir outer bark (Malan *et al.*, 1992). Efforts have also been made to elucidate the chemical structure of natural "phlobaphene" condensed tannins by Botha *et al.*, 1981; van der Westhuizen *et al.*, 1981; Hundt and Roux, 1981; McGraw and Hemingway, 1982;

Steenkamp *et al.*, 1985; Young *et al.*,1985; Steynberg *et al.*, 1986; Laks and Hemingway, 1987. Foo and Karchesy (1989) concluded that "phlobaphenes are a crude extract preparation that is complex in structure, heterogenous in composition, and variable from source to source". No characterization of the phlobaphenes of *Juniperus* species has yet been performed. The advent of improved chromatographic methods combined with modern spectroscopic techniques can provide new insights into these materials leading to a improved understanding of their structure and biogenesis.

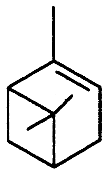
Adsorption chromatography on silica gel and Sephadex LH-20 has been extensively used for separation of crude extracts of woody plants. These packings afford a strong adsorbitivity of compounds such as tannins, which makes quantitative recovery difficult (Okuda *et al.*, 1989), and also reduce the lifetime of the solid support. These disadvantages can be overcome by use of a vinyl polymer gel such as Toyopearl HW-40.

Toyopearl chromatography is unquestionably one of the major methods for separation of complex organic mixtures in natural products chemistry. In addition to the ability of these resins to separate mixtures according to their molecular weight (gel permeation chromatography), it works also as an adsorption chromatography media. The sample moves through the adsorbent bed and it is induced to move through the bed by means of pressured flow or capillary action. As the sample moves through the adsorbent bed, its various components will be held (adsorbed) at the adsorbent surface to a greater or lesser extent, depending upon the chemical nature of the component. Toyopearl resins, which are hydrophilic microporous packings, are generally used for bioprocessing chromatography, their characteristics are bed stability, and high overall sample capacity (Tosohass, 1989). Tightly adsorbed materials can be eluted by treatment with aqueous base, which also cleans the packing material. Thus the ability to use a wide variety of solvents and conditions (acid, base) makes the support extremely versatile. Separation of low molecular weight compounds from the high molecular materials in wood extractives depends upon the degree of adsorption versus the effects of size exclusion (Okuda *et al.*, 1989). Toyopearl chromatography is extensively applied in protein purification (Kato *et al.*, 1981; Yoshitomo *et al.*, 1982; Shigeta *et al.*, 1982; Ihara *et al.*, 982; and, Aoyama *et al.*, 1982; Ozawa *et al.*, 1982), and it has also been applied in the analysis of proanthocyanidins and ellagitannins (Okuda *et al.*, 1989; Zucker, 1983; Haslam 1979).

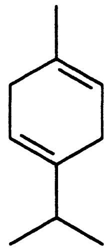
Early work on separation of the phlobaphene fraction of juniper was accomplished mainly by extraction of the dark-colored residues from the volatile oils with solvent-based strategies (Mulick, 1969) followed by chemical modification such as methylation with dimethyl sulphate in alkaline solution and/or oxidation with potassium permanganate (Zavarin *et al.*, 1955; Ishak, 1974). These techniques are time consuming and often troublesome. Extraction with organic solvents in Soxhlet extractors using hot water have the disadvantage that high temperature could directly affect phlobaphene structure. Paper chromatography can be helpful in detecting the presence of certain compounds, but quantitation and identification are difficult. When chromatographic separations were attempted, a portion of the material remained bound on the solid support, it was necessary the use very polar solvents such as acetic acid, which can cause degradation. Acetylationdeacetylation cycles were also considered as means of purification, resulting in low recoveries and loss of part of the product on the chromatographic column.

Another problem that was encountered during the chemical treatments was that the material sometimes polymerized during the purification. Destructive techniques, such as pyrolysis have also been employed, and chromatography and spectroscopy (IR and UV) have allowed the detection and identification of compounds such as carvacrol, derivatives of pyrogallol and catechin among other groups (Kurth and Lackey, 1948; Hergert and Kurth, 1953; Swan, 1963; Ishak, 1974).

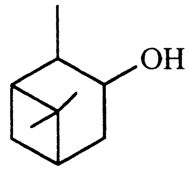
Attempts to improve phlobaphene analyses techniques continued with the practice of ethanolysis and methanolysis (Zavarin and Snajberk, 1963; Kurth *et al.*, 1968) in combination with analytical methods such as thin layer chromatography (one-dimensional and two-dimensional). In the pyrolysis of the phlobaphenes from redwood, it was found that these materials represent a mixture of soluble lignin and condesation products of tannin, composed of guaiacol-type units (Zavarin and Snajberk, 1965). Kurth *et al.* (1968) stated that phlobaphenes of conifer barks are polymers of catechins and related flavonoids. The molecular weight distribution of pine extracts has been determined by vapor pressure osmometry in methanol at 45°C and gel permeation chromatography, reporting relative molecular masses of 6,500 for phlobaphenes (Baeza *et al.*, 1986). Evidences of biphenyl linkages in phlobaphenes have been reported recently, accounting for a phenolic oxidative coupling in phlobaphene formation (Malan *et al.*, 1992). These previous investigations have shown that gymnosperm phlobaphenes are highly variable materials which depend upon the source and whose structure is related to the co-occurrent compounds such as catechol and benzene nuclei (heartwood extractives of Red Alder - Kurth and Becker, 1953); tannins and catechol tannins (bark of Red Fir - Becker *et al.*, 1958; Redwood - Zavarin and Snajberk, 1963, Western Red Cedar - Hergert, 1992; California Redwood heartwood - Hergert, 1992); catechin-like structure (western True Firs - Carlberg and Kurth, 1960); heterogeneous polyphenolic polyesters (western Red Cedar bark - Swan, 1963); guaiacol and veratrol units (Redwood heartwood Zavarin *et al.*, 1965); cyanidin units (Mountain Hemlock - Kurth *et al.*, 1968); lignin-like units which incorporated lignans (Western Hemlock heartwood - Hergert, 1992); proanthocyanidins units (Red Spruce outer bark - Hergert, 1992); and, complex mixtures of condensed tannins, carbohydrates (glucosides), flavonoid methyl ethers and/or lignans, and dihydroquercetin (Foo and Karchesy, 1989) . Additional information is needed to more fully understand the role of phlobaphenes in woody cell wall maturation. Efforts of this thesis center on the phlobaphene formation of *Juniperus occidentalis*, a highly durable species of the Great Basin region of the U. S.



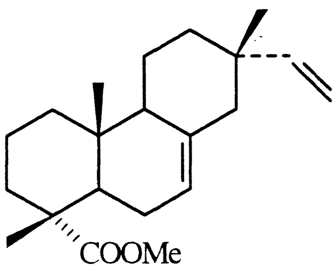
(**1**)
α-pinene



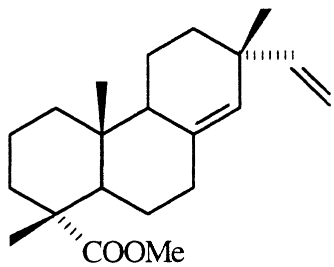
(**2**)
terpinene



(**3**)
borneol

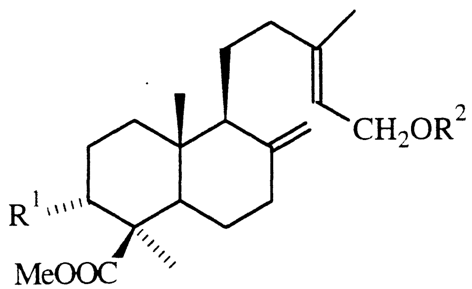


(**4**)



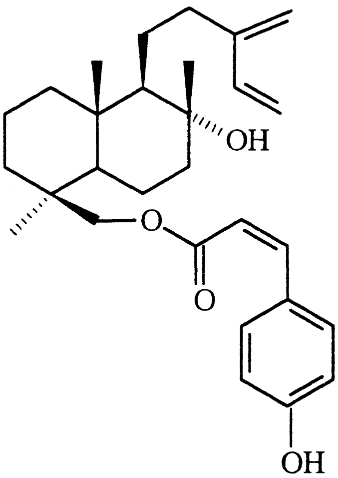
(**5**)

pimarane acids



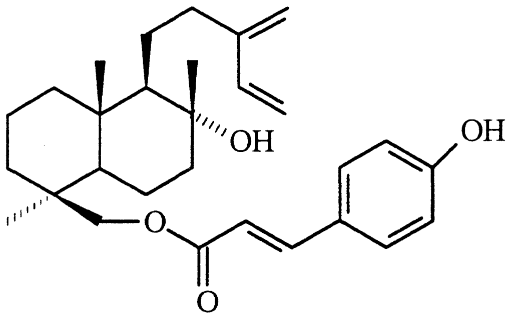
(**6**) $R^1 = H$, $R^2 = Ac$
(**7**) $R^1 = H$, $R^2 = H$

labdane acids



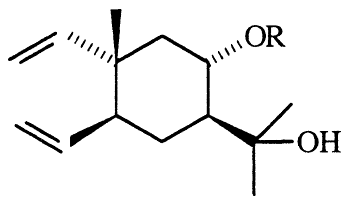
(**8**)

8-hydroxy-labda-13(16),14-dien-
19-11 *O*-acetyl-*Z*-coumarate



(**9**)

8-hydroxy-labda-13(16),14-dien-
19-11 *O*-acetyl-*E*-coumarate



(**10**) R = Ac
(**11**) R = H

(**10**) 8-acetoxyelemol
(**11**) 8-hydroxyelemol

OBJECTIVES

Wood extractives research has typically been focused on the structural elucidation of trace components, while the study of the composition of major constituents such as the phenolic polymers in heartwood has been paid little attention. These polar fractions are called Brauns' native lignin, secondary lignin, polylignan, or lignin-like materials or phlobaphenes, and there is disagreement as to whether these constituents are secondary metabolites or degradation products of lignin resulting from acidic compounds present in heartwood (Hergert, 1989).

Polar solvent extraction of sapwood, heartwood or rhytidome bark yields a colored solid which is insoluble in water. Therefore, its biosynthesis should be in the same way as other heartwood extractives, since solubility characteristics would prevent translocation from the sapwood or cambium. These phenolic polymers have structural characteristics which are species dependent, and are theorized as forming polylignans. Heartwood lignans are optically active lignin dimers which have been β–β–coupled (Ayres and Loike, 1990) suggesting that they are biosynthesized by a different mechanism than lignin, involving stereospecific enzymatic controlled reactions. Neolignans differ from lignans in that any type of stereospecific coupling other than β–β is present. While dimers are the predominant species found in woody plants, oligomeric materials continue to be discovered (Whiting, 1990). On the other hand, polyphenolic polymers based on flavan-3,4 diol or gallic acid derivatives co-occur with heartwood solvent-soluble lignin-like polymers (Stafford, 1989). Substantial quantities of all three types of polymers (polylignans, lignans and tannins) are deposited in heartwood and bark in a form that depicts them insoluble in neutral solvents. By definition, they are secondary metabolites but in analytical procedures they act as cell-wall constituents. At the moment there are no suitable chromatographic methods for isolation of these cell-bound polymers without structural alteration or degradation. This area of wood extractives chemistry for both angiosperms and gymnosperms needs clarification.

These observations combined with the fact that *Juniperus occidentalis* is a very durable species, have led to the development of a study for juniper extractives in an attempt to:

- Develop a chromatographic method to isolate crude heartwood extracts (polar and non polar fractions) by using Toyopearl resins.
- Purify low molecular weight fractions by a combination of Toyopearl, flash, and preparative thin layer chromatography (TLC).
- Isolate and determine the molecular weight of some fractions by size exclusion chromatography.
- Determine the bioactivity of several fractions by the brine shrimp bioassay (McLaughlin,

1991), in order to identify the active fractions and whether the polymeric materials contribute to western juniper durability.

- Identify functional groups in some of the polymeric fractions by spectroscopic methods, such as FT-IR.

- Characterize high molecular fractions with the aid of nuclear magnetic resonance (NMR).

MATERIALS AND METHODS

Isolation of the crude extracts

Heartwood juniper chips were obtained from the Caroll Ranch, 6 miles west of Mitchell, Oregon. Bark, wood and needle samples have been deposited in the Oregon State University herbarium. Juniper heartwood chips (1 Kg) were subjected to 3 successive 48 to 72 hr. room temperature extractions with methanol. This extraction was performed in triplicate. The extracts were obtained by filtering and evaporating under reduced pressure at 40 °C. The methanol solubles were separated into polar and non-polar fractions by liquid-liquid extraction with hexane. Both extracts, methanol-solubles and hexane solubles were dried by evaporation under methanol reduced pressure at 40 °C and freeze-dried.

Extractive-free biomass analysis

The extracted wood chips were wiley-milled to pass a 1 mm screen and subjected to a Soxhlet extraction with methanol 48 hrs. The carbohydrate, Klason-lignin and acid-soluble lignin contents were determined by standard procedures (Kaar *et. al.*, 1991). The moisture content of the extractive free chips was determined by oven drying at 105 °C.

Fractionation of the crude extract

Toyopearl Chromatography

Preparative Toyopearl chromatography was performed with the methanol-soluble, hexane-insoluble fraction on a glass column (70 x 7.5 cm) packed under pressure (10 p.s.i. N_2) with Toyopearl resin TSK-GEL HW-40F (2.31 L) in filtered and degased 95% ethanol, prepared as recommended by manufacturers (TosoHass, 1989). The column was connected to a pump (Waters model) which was maintained at 2.5 mL/min flow rate. A UV monitor (LDC III) was connected to the outlet of the column and operated at 254 nm and 2.048 AUFS. Methanol soluble extract (15 g) was added to the column and the aliquots of 20 mL were collected. The fractionation was monitored by thin layer chromatography (TLC) using Al Sil G/UV Plates (UV_{254}) developed in chloroform:methanol:acetic acid:water $CHCl_3$:MeOH:AcOH:H_2O (85:15:10:2) (McGraw and Hemingway, 1982). Visualization was either by UV light or staining with phosphomolibdic acid in ethanol.

Small scale Toyopearl chromatography was performed in two small columns (40 x 4 and 30 x 2 cm), packed in the same way as the preparative column. Elution was performed with 90% ethanol. Aliquots of 8 mL were collected using the same procedures described.

Flash Chromatography (Silica gel Chromatography)

The flash chromatography was set up following the standard procedure (Still *et al.*, 1978; Majors and Enzweiler, 1980), and elution was accomplished with mixtures of $CHCl_3$:Isopropanol or $CHCl_3$:MeOH .

Thin-Layer Chromatography

Attempts to purify some fractions were carried out by preparative thin layer chromatography in plates of silica gel 60 (20x20 cm) developed in a chamber with $CHCl_3$:Isopropanol in different mixtures of increasing polarity (100:2, 50:2).

Molecular Weight Distribution

Acetylation

Acetylation of the fractions was accomplished by standard means with acetic anhydride and pyridine. The sample was dissolved in pyridine and acetic anhydride was added. The mixture was stirred for 12 hours, the reaction was quenched with absolute ethanol. The mixture was then diluted with ethyl acetate and washed with aqueous NH_4Cl (twice). The sample was dried with $MgSO_4$, filtered and solvent eliminated by evaporation under reduced pressure. The remaining pyridine was removed by repeated additions and evaporations of toluene (co-distillation). The samples were then dried under high vacuum or freeze-dried.

Liquid Size Exclusion Chromatography (Gel Permeation Chromatography)

The molecular weight distribution was determined following the standard procedure described in D 3593 - 80 (ASTM). This test method allows the determination of molecular weights for macromolecules which are soluble in tetrahydrofuran by liquid size exclusion chromatography. A universal calibration plot was performed using monodisperse polystyrene standards (molecular weights: 162; 1,700; 5,000; 9,000; 12,000; 22,000) and synthetic compounds previously prepared (R. F. Helm) L162; L9K; L1700; LRH5M/ 4-acetoxyacetovanillone (**12**), M_w = 208 g/mole; LRH7M/ 4-benzyloxyacetovanillone (**15**), M_w = 256 g/mole, LRH85N/ 1-(4-benzyloxy-3-methoxyphenyl)-2-(2-Methoxyphenoxy)ethanone (**16**), M_w = 378 g/mole; LRH28A1D/ isotaxiresinol penta-acetate (**17**), M_w = 556 g/mole; LRH139N/ 2-(4-benzyloxy-3-methoxybenzoy)-2-(2-methoxyphenoxy)ethyl2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranoside (**18**), M_w = 987 g/mole (Helm. et al., manuscript in preparation); L22K, L7K, LSK, and L12K. Chemical structures for these compounds are depicted in pages 35 and 36

The standard used for the plotting of the molecular weight distribution of juniper fractions was 1-(4-benzyloxy-3-methoxypheny)-2-(2-methoxyphenoxy)ethanone with a M_w = 378 g/mole.

Bioactivity Assays

The biological activity of the fractions was accomplished following the brine shrimp lethal bioassay method used for higher plant screening (McLaughlin, 1991).

Juniper crude fractions were tested at concentrations of 10, 100, and 1000 ppm (μg/L) in vials containing 10 brine shrimp in each replicate. Survivors were counted after 24 h. Analyses were performed in Oregon State University - Department of Forest Products.

Spectroscopy

The IR spectra of crude and acetylated western juniper fractions were recorded on a Nicolet 5 SXC FT-IR spectrometer, as KBr pellets. ^{1}H and ^{13}C NMR of the acetylated fractions were run on a Varian 400 using the default pulse programs. The solvent was *d*-acetone which also served as internal reference central solvent peak (^{1}H, 2.04 ppm; ^{13}C, 29.8 ppm).

^{13}C-Labelled Phlobaphene Acetates

Acetylation

The acetylation of the high molecular weight phlobaphene fraction, was performed as follows. The sample (150 mg) was dissolved in tetrahydrofuran under a flow of nitrogen, and 4-dimethylaminopyridine and acetyl-1-^{13}C chloride were added. After one hour, pyridine (6 mL) was added to improve the solubility of the starting material. Four hours later, the reaction was quenched with absolute ethanol. The mixture was extracted with ethyl acetate versus. 3% HCl.; and the organic layer was then washed with aqueous NH_4Cl (twice). The ethyl acetate layer was then dried with $MgSO_4$, and evaporated under reduced pressure at 40 °C. Traces of pyridine were co-distilled with repeated additions and evaporations of toluene (four times). The sample was then dried under high vacuum. The process had an overall yield of 82%.

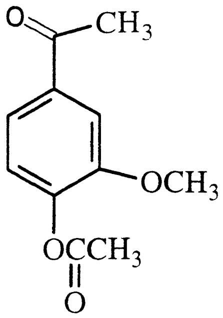
Lignin Acetylation with Acetyl-1-^{13}C Chloride

Both lignin alkali and lignin organosolv (0.05 g) obtained from the Biobased Material Research Center were dissolved in pyridine (3 mL), under continuous stirring. Acetyl-1-^{13}C chloride (0.35 mL) and dimethyl amino pyridine (0.025 g) were added. The reaction was stirred for 48 hours, at which time methanol (150 μL) was added, the mixture reacted for 12 hours. The solution was added drop wise to a flask which contained 0.1 N HCl (20 mL). The precipitate was filtered in a membrane filter, washed with 0.1 N HCl followed by distilled water. The peracetate was then quantitatively transferred to a vial and dried under high vacuum for approximately 12 hours. The yields for the acetylations of lignin alkali and lignin organosolv are 132% and 87%, respectively.

Long-range C-H Correlation

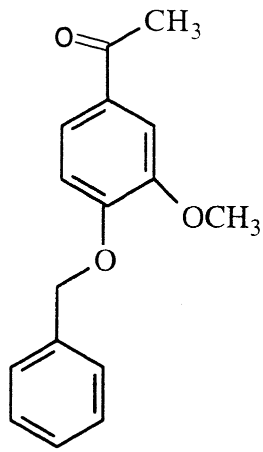
(Heteronuclear Multiple Bond Correlation HMBC)

NMR experiments were performed with a Varian 400-MHz instrument fitted with a 5-mm probe (normal geometry). The standard conditions used for all analyses were 24-80 mg of material in 500 μL of d_6-acetone, with a central solvent peak used as internal reference (δ_H=2.04 ppm and δ_C=29.8 ppm). Long-range correlations were obtained with 8-16 scans/ 200 increments at a temperature of 27°C, and correlation delays of 0.055 and 0.100 sec.



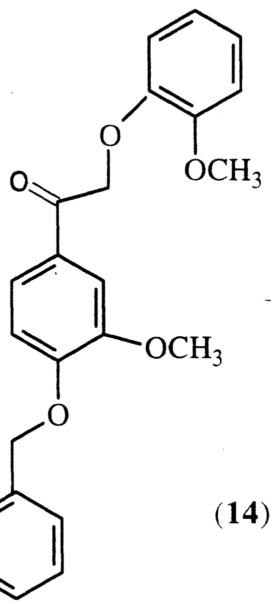
(**12**)

4-acetoxyacetovanillone



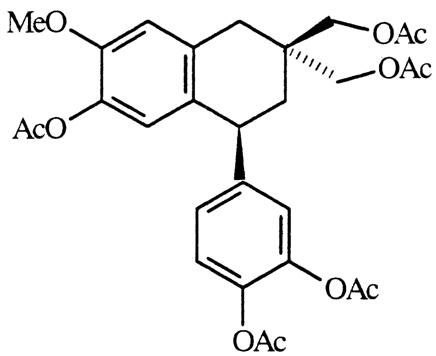
(**13**)

4-benzyloxyacetovanillone

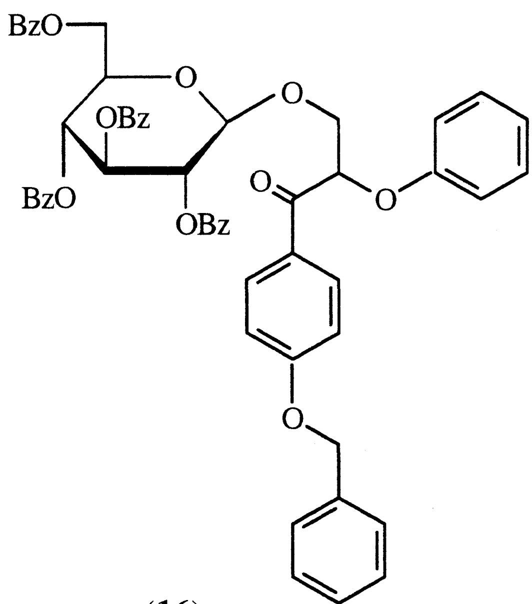


(**14**)

1-(4-benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)ethanone)



(**15**) Isotaxiresinol penta-acetate



(**16**)

2-(4-benxyloxy-3-methoxy benzoyl phenoxy) ethyl
2, 3, 4, 6 -tetra-*O*-benzoyl-β-**D**-glucopyranoside

RESULTS AND DISCUSSION

Isolation of the crude extracts

The general protocol used for isolation and purification is described in Figure 2. The first step in the study was to extract the heartwood with an appropriate solvent. The testing of various solvents indicated that the highest recoveries were obtained with methanol (Table II). On these small scale trials, three successive extractions with methanol afforded a 5.93% yield of solubles. Large scale extractions with methanol (1 kg wood) were performed in triplicate affording a MeOH crude extract yield of 5.3, 5.87, and 5.97%, respectively.

The general extraction of methanol and hexane solubles of western juniper is depicted in Figure 3. The methanol soluble fraction was extracted with hexane obtaining final yields of 3.5%, 3.52% and 2.47% for hexane extractables; 1.8%, 2.35% and 3.5% for methanol solubles. The first sample resulted in a lower amount of methanol extract because the extraction with hexane was performed several times until no more material was present in the hexane layer. The increasing percentage in the results is due to the period of extraction. The longer the period of extraction time the better the extraction yield.

Table No. II. Yields of the isolation of Western Juniper Extractives with Different Solvents

	YIELD (%)		
Extraction	Methanol	Hexane	Ethyl-Ether
First	4.77	0.75	1.76
Second	0.97	0.14	0.54
Third	0.19	0.05	0.02
Total	5.93	0.94	2.22

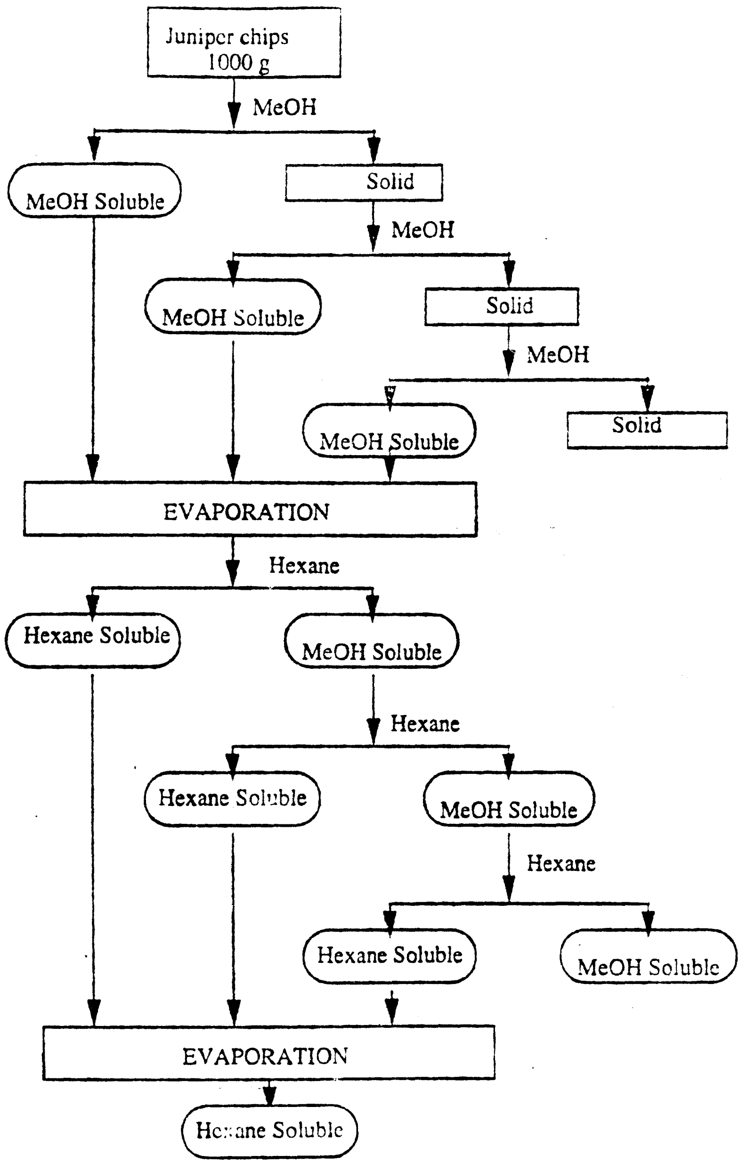


Figure 2. Isolation of MeOH and Hexane Soluble Fractions from Juniper (Triplicate)

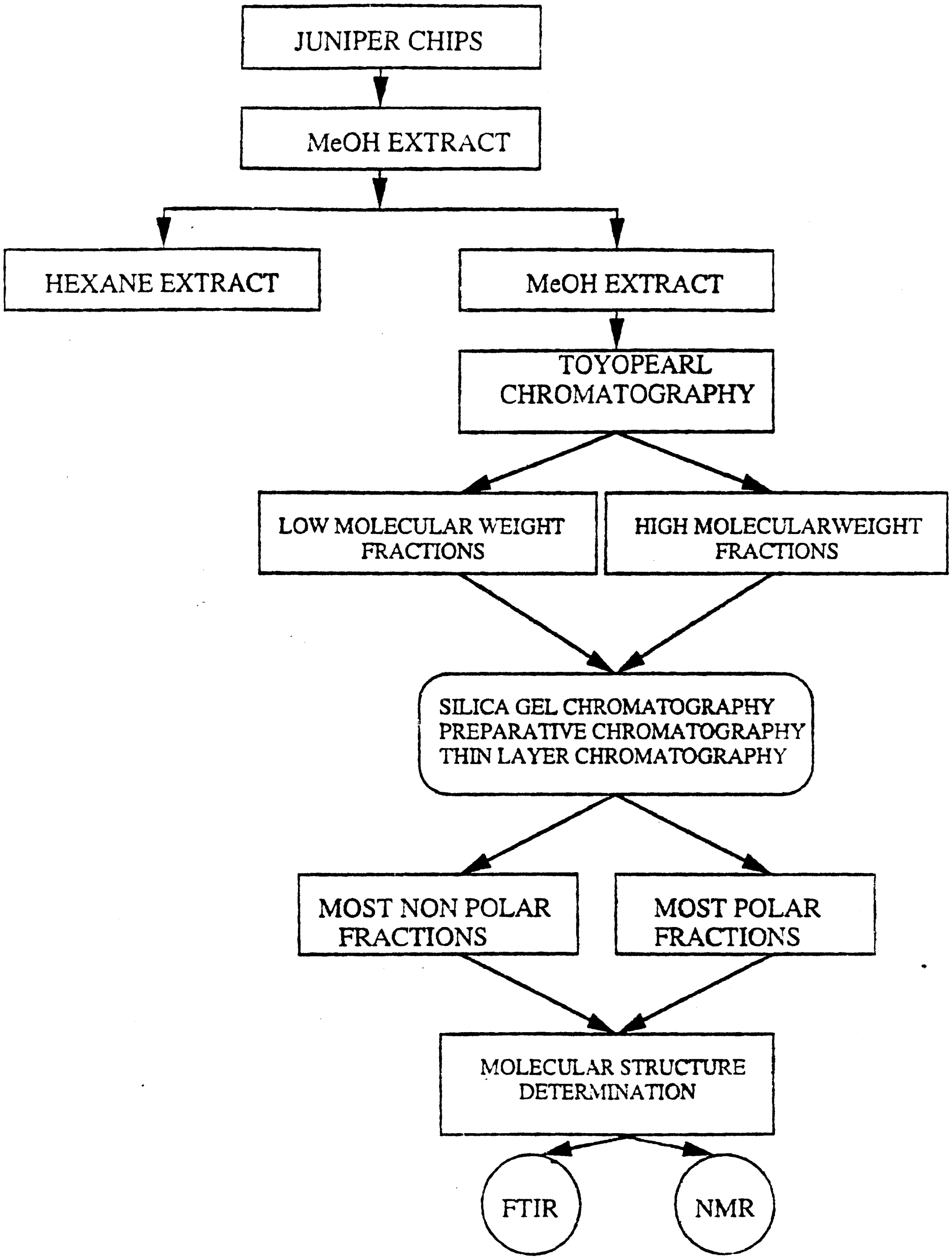


Figure 3. Isolation and Purification of Heartwood Extractives from Western Juniper

Extractive-free biomass analysis

The moisture content of the extractive-free biomass was 6.5%. A Soxhlet extraction was performed on this material to see how much extractive material remained, and to eliminate as much of it as possible prior to chemical analysis. The yields of the Soxhlet extraction were 1.28%, 1.32%, and 1.25%, giving a total extractive content of 6.58%, 7.19%, and 7.22%, respectively. Therefore 19.45%, 18.35%, and 17.07% of the extractables were not removed by room temperature extraction. Although, it is apparent that the Soxhlet extraction would have provided for a more efficient removal of the extractives, the effect of heat on extractive structure is not known. Therefore the decision was made to avoid the use of heat. The extractive free wood was subsequently submitted to biomass analysis and found to contain 56.6% carbohydrate and 34.8% lignin as shown in Table III. Chemical analysis data published in the literature for western juniper show 5.23%, 64.74%, and 35.18% for total extractives, hollocellulose and lignin, respectively, for air-dried samples under 10% moisture content (Kurth, 1948). The reason for the significant differences in carbohydrate contents is not known.

Fractionation of the crude extract

Toyopearl Chromatography

The experiment is summarized in Figure 4, the overall yield for this fractionation was 96.24% which indicates that little or almost no material was bound to the chromatographic resin. This represents a significant improvement in separation and purification of these types of extractives. Eleven fractions were collected as is described in Table IV, based upon the thin layer chromatograms on silica gel plates developed in $CHCl_3$:MeOH:AcOH:H_2O (85:10:15:2). Although, fraction c and d eluted with 95% ethanol (less polar fractions) representing 46.76% of the total extractives, fractions eluted with acetone:water (70:30) also are significant, representing 22.77%.

The UV chromatogram of the separation is shown in Figure 5, and is a typical chromatogram for the heartwood extracts. The column was eluted with ethanol 95% until the eluent showed relatively little absorbance in the UV. In order to remove the more polar fractions, the solvent was changed to acetone:water (70:30).

Similarly, the elution was performed until no more material eluted from the column. The chromatogram presents a irregular region between fractions i and j, probably due to the presence of some air bubbles trapped in the bed of the column or in the solvent. The dashed line represents the normal trend of the curve (based upon several other separation performed in the laboratory).

Table III. Duplicate Chemical Analysis of Extractive-free Western Juniper Heartwood (oven dried basis)

Compound	%	%
Glucose	29	33.19
Xylose	9.1	9.37
Galactose	6.24	4.44
Arabinose	traces	traces
Mannose	12.25	9.92
Total Sugar Content	56.59	56.91
Klason Lignin	34.46	34.46
Acid Sol. lignin	0.39	0.39
Total Lignin	34.85	34.85
5-(Hydroxymethyl)-2-furfuraldehyde (HMF)	0.2	0.22
2-Furfural	0.32	0.32

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Table IV. Toyopearl Chromatography - Fractionation of MeOH extract of Heartwood Juniper

FRACTIONS	TUBES	RECOVERY (%)	FORM
a	1 - 10	0.01	Solid
b	11 - 30	1.20	Solid
c	31 - 47	9.51	Syrup
d	48 - 74	37.25	Syrup
e	75 - 90	7.40	Oil
f	91 - 131	7.94	Syrup
g	132 - 190	7.16	Syrup
h	191 - 231	0.66	Solid
i	232 - 251	4.20	Solid
j	252 - 270	15.69	Solid
k	271 - 332	5.38	Solid
	TOTAL	96.30	

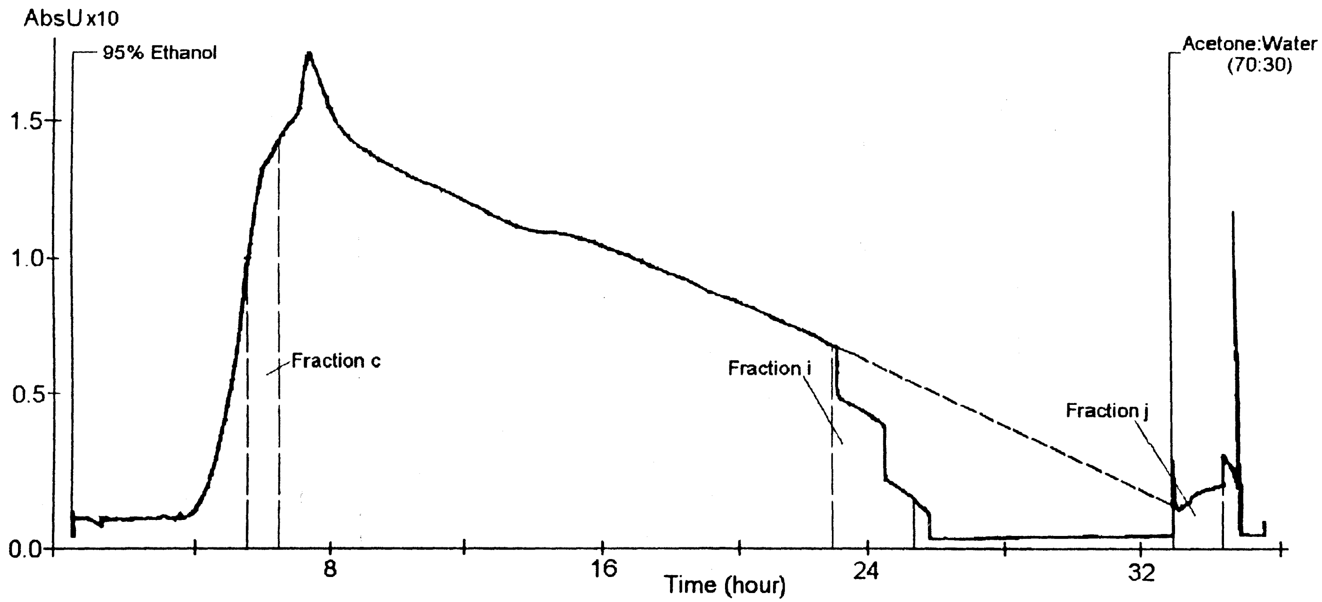


Figure 5. UV Chromatogram of the Toyopearl Chromatography of Western Juniper Extracts

Flash Chromatography (Silica gel) and Thin Layer Chromatography of the Less Polar Fractions

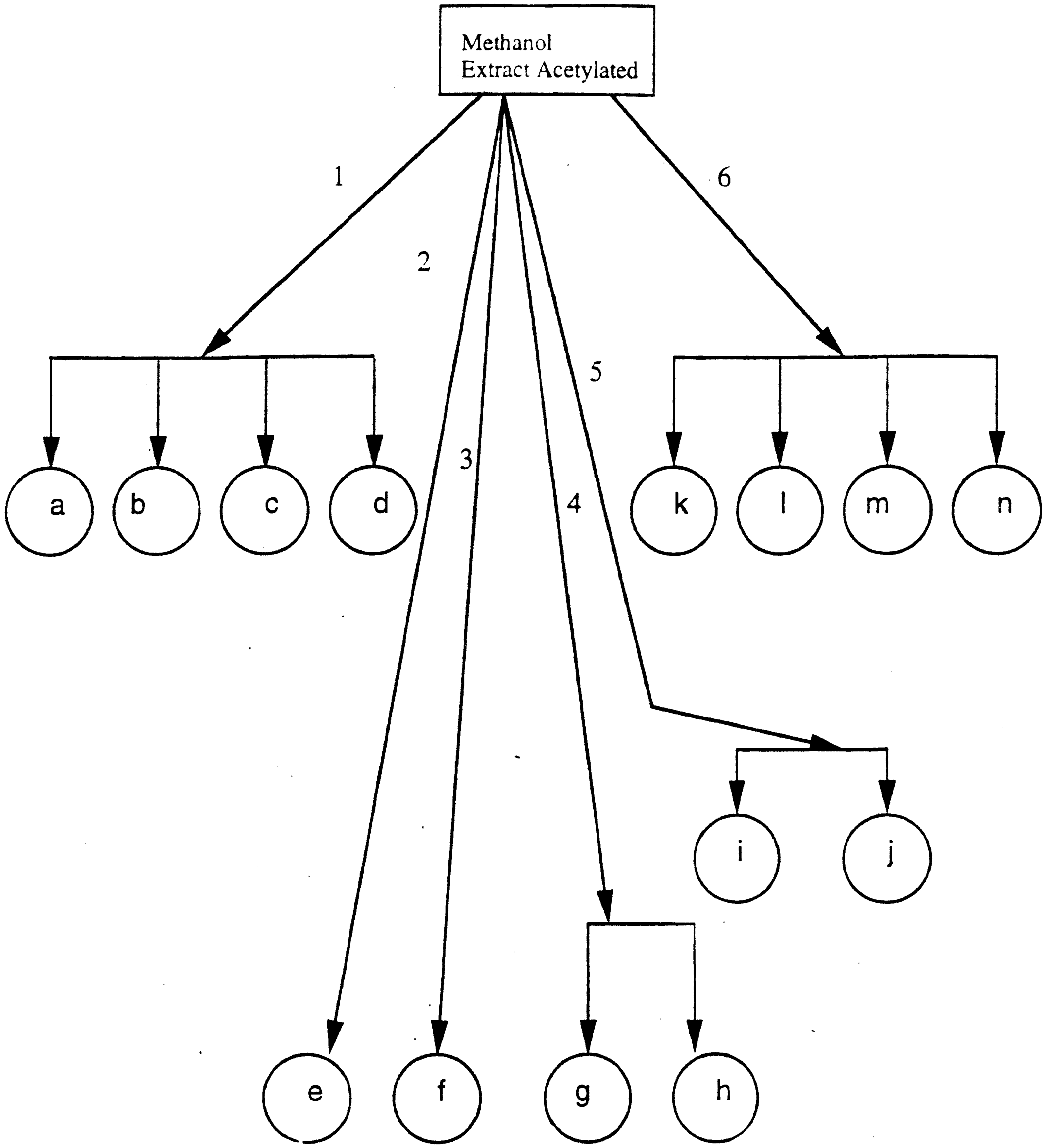
Less polar fractions eluted from the Toyopearl column were acetylated and attempts to purify them were performed following the procedure depicted in Figure 6. The chromatography of the fractions on silica gel column had a yield between 96-98% of recovery. Fractions were pooled according to their TLCs, in all cases the fractions eluted from the column were complex mixtures.

Based upon the TLC, attempts to a further purification of some fractions were performed. For instance, crude fraction d was rechromatographed in a silica gel column eluted with mixtures of $CHCl_3$:Isopropanol (ascending polarity) and $CHCl_3$:MeOH to afford five fractions. The TLC exhibited by fraction d revealed a single spot at $R_f = 0.4$; however, a ^{1}H NMR spectrum of this fraction revealed a polymeric material. The overall yield of this chromatographic separation had a recovery of 96.30 %.

Fractionation of the peracetate methanol extract on silica gel chromatography eluted with less polar solvents such as Hexane:EtoAc (19:1) resulted in 4 oily colorless fractions. When the solvent was changed to $CHCl_3$:Isopropanol (in ascending polarity), nine additional fractions were obtained: four waxy and five solid fractions. Since fraction d contained crystals, a recrystallization in absolute ethanol yielded long transparent crystals which appreared to be a terpene according to the ^{1}H NMR spectrum of this compound. No effiorts for further identification were made.

While the analysis of non-polar fractions by TLC gave a rapid assessment of complexity, the analysis of the polymeric fractions by this technique was not a useful screening method. These materials remained at the origin of plate due to their strong binding to the silica gel media. The acetylation of these materials decreased their polarity which allowed purification either by preparative TLC or Toyopearl chromatography. The yield for preparative chromatography was 47.42%. A rechromatography in Toyopearl columns (small scale) of these fractions eluted with the same solvent also yielded a low recovery (47.8%).

Purification of the phlobaphenes was performed in a small scale Toyopearl chromatography eluted with filtered and degassed ethanol 90%, acetone:water (70:30), and pure acetone. Three fractions were collected according to the UV chromatogram (Figure 7). This chromatogram clearly shows a first eluted purer polymeric fraction (a); secondly, a mixture of polymeric fractions and low molecular weight compounds (fraction b); and finally, another polymeric fraction (c).



1 = CHI_3 : Isopropanol (50:1)
2 = $CHCl_3$: Isopropanol (19:1)
3 = $CHCl_3$: Isopropanol (9:1)
4 = $CHCl_3$: MeOH (6:1)
5 = CHC_{l3} : MeOH (4:1)
6 = $CHCl_3$: MeOH (1:1)

Figure 6. Flash Chromatography of acetylated methanol extract on silica gel. Elution with solvent systems 1, 2, 3, 4, 5, and 6.

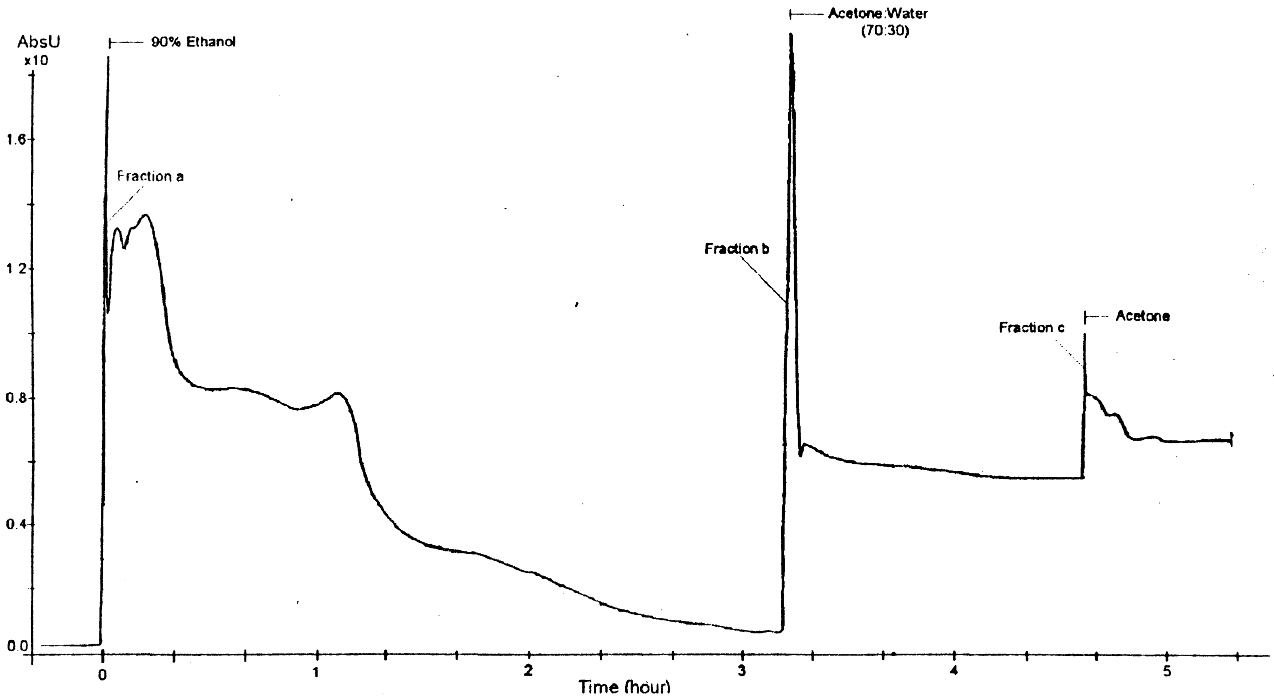


Figure 7. UV Chromatogram of Polymeric Phlobaphene Purification on Toyopearl Chromatography

Molecular Weight Distribution of Heartwood Extractives

Average molecular weight were determined on several fractions as the peracetates derivatives. The crude fractions were acetylated following the standard procedure for wood products. The fractions were subjected to the "standard test method for molecular weight averages and molecular weight distribution of certain polymers by Liquid Size Exclusion Chromatography (Gel Permeation Chromatography GPC) using universal calibration standard (D 3593 - 80 Reapproved 1986). The results for three representative fractions are summarized in Table V. Values for M_w fall between the range of 341.3 and 22,210 g/mole. A typical low molecular weight fraction can be represented by fraction c (M_w = 426.5 g/mole); an intermediate molecular weight fraction 3 by fraction i (M_w = 1165 g/mole); and a high molecular weight fraction by fraction j (M_w = 13,550 g/mole). The polydispersities range between 1 and 9.

Figure 8 shows the Molecular Weight Distribution for these three fractions compared to the acetylated MeOH extract acetylated. The low molecular weight fraction appears to correspond to the approximate molecular weights of lignans (M_w = 399 - 444) or resin acids (M_w = 412) (Kuo and Yu, 1993, San Feliciano *et al.*, 1988, 1990). The intermediate molecular weight fraction possesses a M_w 3-4 times that of the low molecular weight fraction.

Table V. Mn, Mw, Polydispersity and a of Some Fractions of Heartwood Western Juniper.

	M_n (g/mole)	M_w (g/mole)	Polydispersity	α
MeOH Extract peracetate	497	1,340	2.695	0.269
Low Molecular Weight fraction	350	426	1.216	0.326
Intermediate Molecular Weight fraction	1,018	1,665	1.635	0.176
High Molecular Weight fraction	1,400	13,550	9.679	0.357

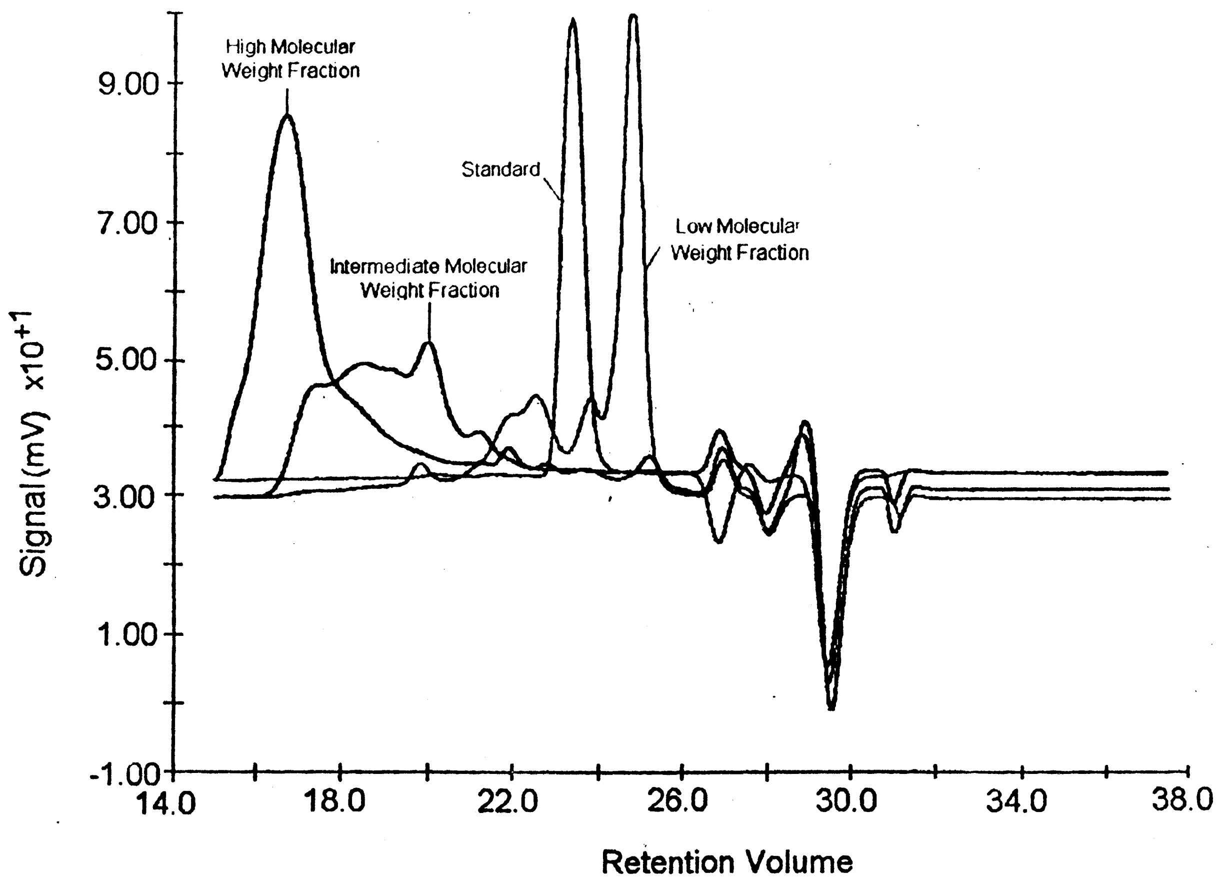


Figure 8. Molecular weight Distribution of heartwood juniper fractions

The high molecular weight fraction corresponds to a phlobaphene of relatively high molecular weight, and in fact the molecular weight approaches that of isolated lignins which range from 3,000 to 20,000 g/mole (Glasser *et al.*, 1993). This fraction represents one of the highest molecular weight extracts yet to be analyzed in a woody species.

Bioactivity Assays

It is important to assess which fractions are the most toxic. The simplest method of doing this is the brine shrimp method of McLaughlin (1991). Methanol crude extract, hexane crude extract, methanol extract peracetate; low, intermediate, and high molecular weight crude fractions were submitted to this test. Results are reported as the lethal concentration (concentration at which half of the tested polpulation dies). The general relationship between toxicity and LC_{50} is that a compound or extract is considered toxic if the $LC_{50} < 100$, and non-toxic if $LC_{50} > 1000$ (Table VI). The toxicity results obtained are shown in Table VII. Hexane extract presented a $LC_{50} = 46$ comparable with the peracetate methanol extract which showed a $LC_{50} = 34$, demonstrating a high toxicity. Figure 9 exhibits the relationship between the molecular weight of the fractions and the LC_{50}, and Figure 10 displays the dose-response relationship. The low molecular weight fraction of western juniper had presented a LC_{50} smaller than of the crude hexane extract, which suggests that the most toxic components are present in this fraction. The intermediate molecular weight fraction (LC_{50} = 1,165) and the high molecular weight fractions (LC_{50} = 9,679) are relatively non-toxic. Therefore it appears that these materials are not present in heartwood to afford resistance to biological decay. They are either by-products of wood ageing or serve some other unknown role.

Table VI. Toxicity and LC_{50} relationship

LC_{50}	< 100	< 300	< 500	> 1000
Toxicity	High	Moderate	Low	Non Toxic

Table VII. Toxicity of some of the fractions of Western juniper extractives

			MAXIMUM RESPONSE (%)			
	M_w (g/mole)	LC_{50} (μg/L)	0 (μg/L)	10 (μg/L)	100 (μg/L)	1000 (μg/L)
MeOH		-	0	10	80	100
Hexane		43	0	20	90	100
Low Molecular. Weight Fraction	426.5	16	0	25	70	100
Intermediate Molecular Weight Fraction	1165	1356	0	35	50	80
High Molecular Weight Fraction	13550	1623	0	10	60	70

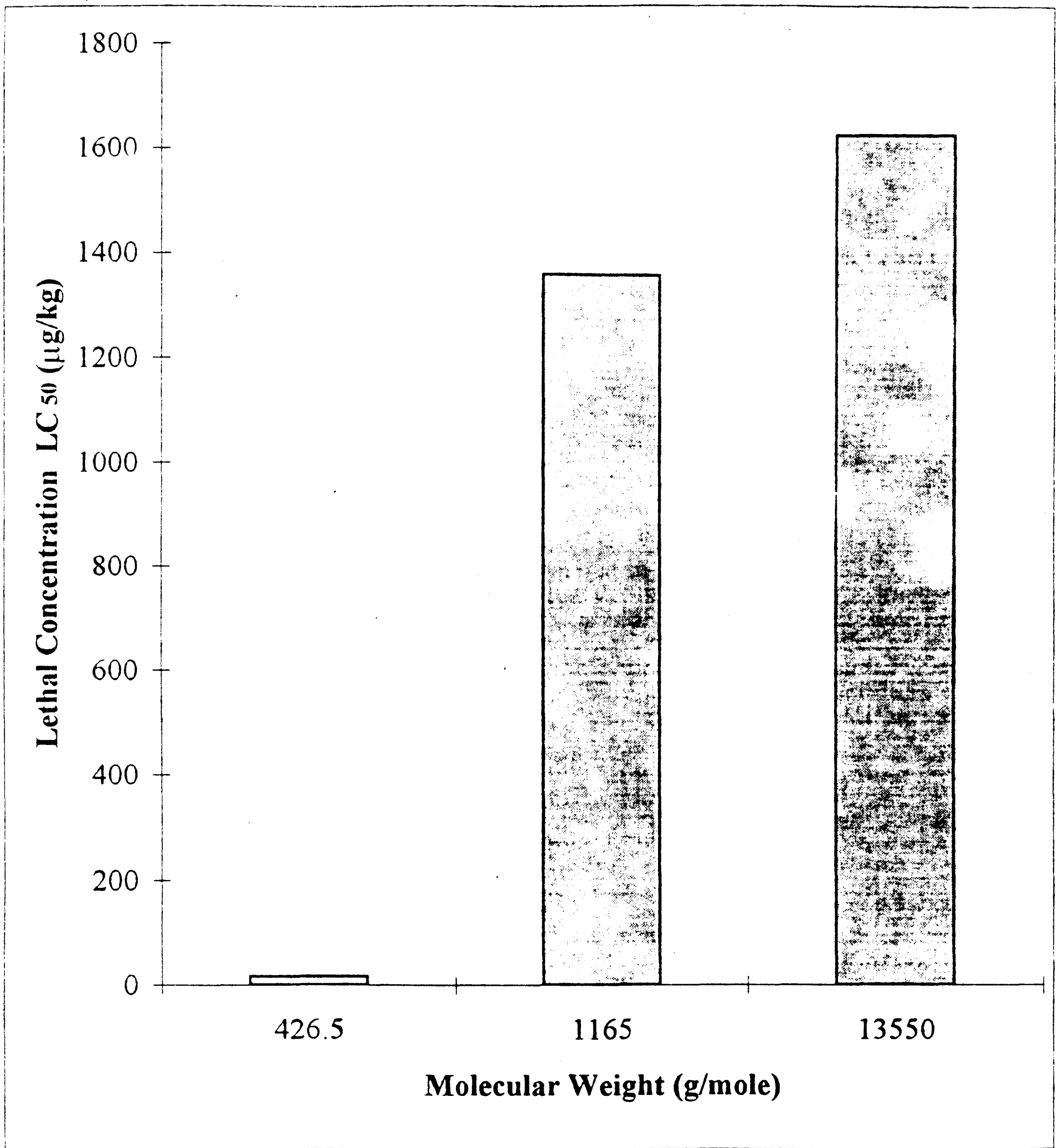


Figure 9. The Relationship between Molecular Weight distribution and Toxicity for Several Heartwood Fractions

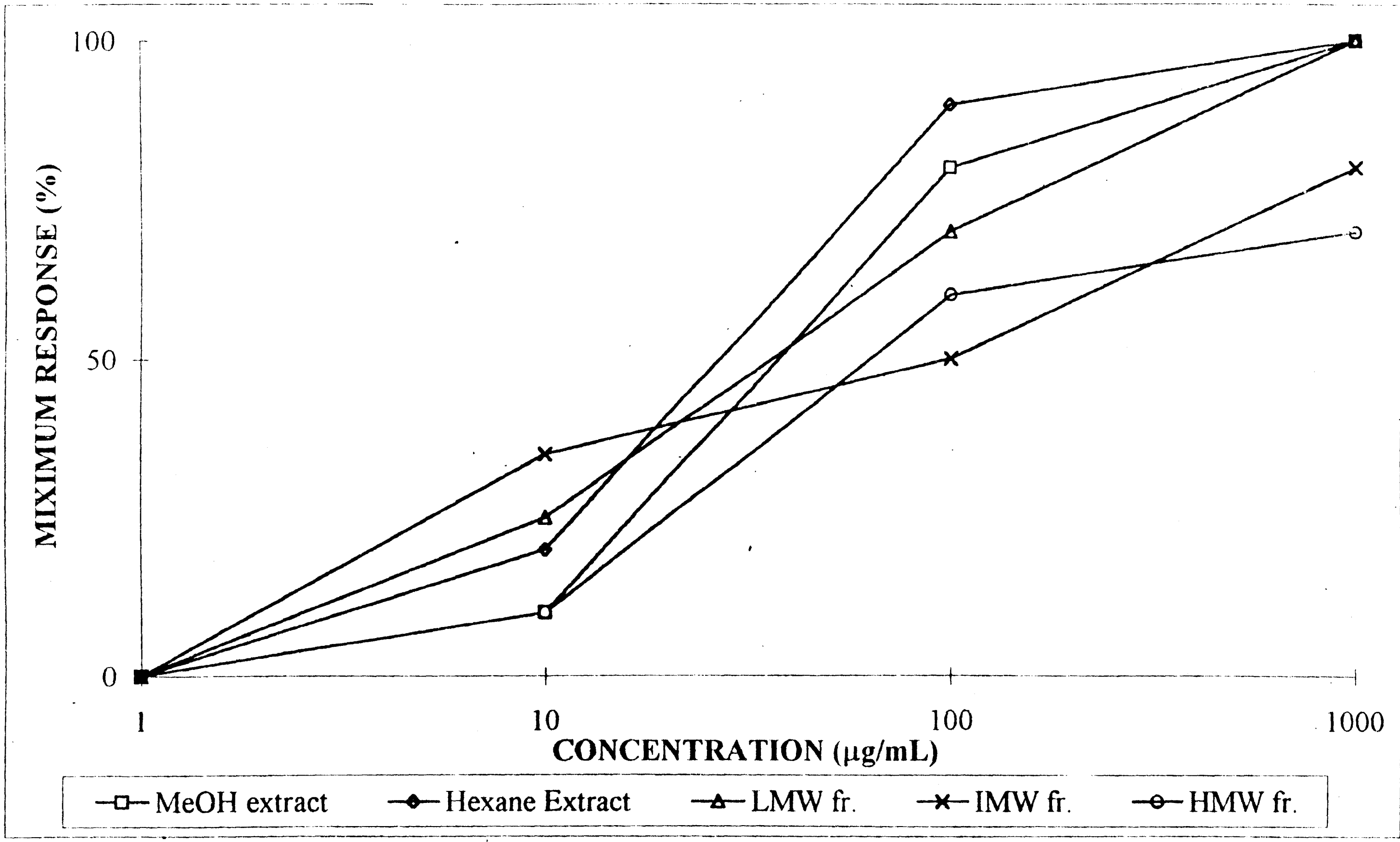


Figure 10. Dose-response Relationship of Juniper Extracts

Characterization of chemical structure of some fractions

Low Molecular Weight Fractions

FT-IR Spectroscopy, ^{1}H and ^{13}C NMR

Figure 11 displays the FT-IR spectra of the methanol crude extract, the low molecular weight, intermediate molecular weight and high molecular weight fractions. These spectra show a band at 3,300-3,400 cm^{-1} which corresponds to hydroxyl groups. The low molecular weight fraction spectrum reveals resolved peaks at 2,800-2,900 cm^{-1} which suggest the presence of CH_3 and/or CH_2, and C-H stretch.

According to the IR spectra (Figure 12 and Table VIII) of the peracetate derivatives, the main difference between these spectra is the expected decrease in the OH band upon acetylation. The strong bands at 2,830-2,810 cm^{-1} and 2,740-2,720 cm^{-1} suggest the presence of a carboxylic group and/or aliphatics (C-H stretch). The band at 1,740-1,738 cm^{-1} corresponds to aliphatic acids and their esters. The bands at 1,603 and 1,465 cm^{-1} suggest an aromatic guaiacyl-type functional group confirmed by the band at 1,267 cm^{-1}, typical for guaiacyl units.

In relation to the juniper phlobaphene, the FT-IR spectra of the crude polymeric material are compared to the FT-IR spectra of alkali and organosolv lignin (Figure 13). Table IX describes the characteristic bands for these spectra and also for their peracetates (Figure 14), reflecting the following: Acetylation of the phlobaphenes and lignins is revealed by the decline of the hydroxyl band at approximately 3,400 cm^{-1} and the appearance of resolved ester bands at around 1,765-1,740 cm^{-1}, corresponding to the aromatic and aliphatic alkanoxy groups, respectively (Glasser and Jain, 1993). The main difference between lignin and phlobaphenes is the band around 1,200-1,400 cm^{-1} which suggests a C-O-C antisymmetric stretch in ester groups.

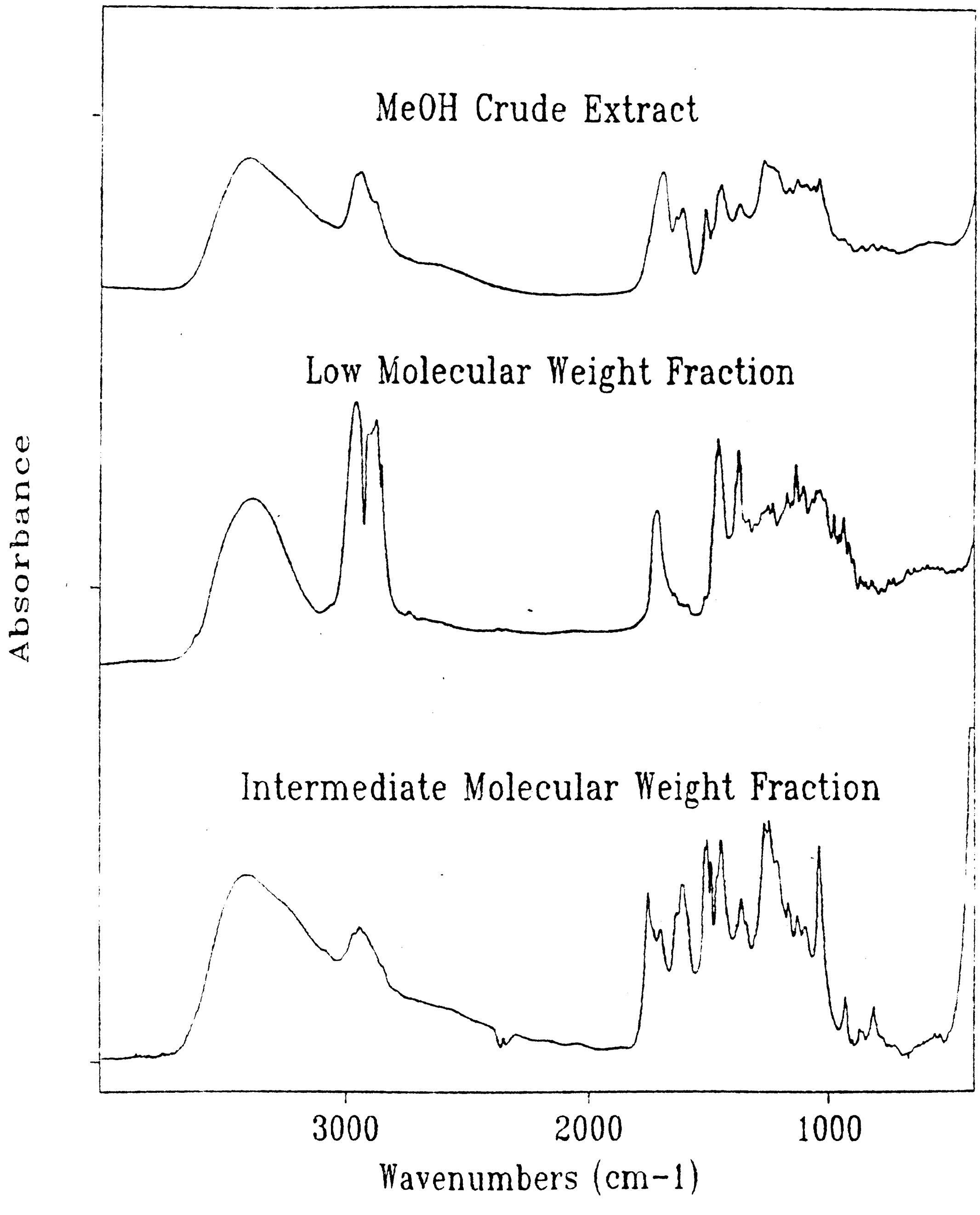


Figure 11. **FTIR Spectra of Crude Fractions of Heartwood Juniper Extracts**

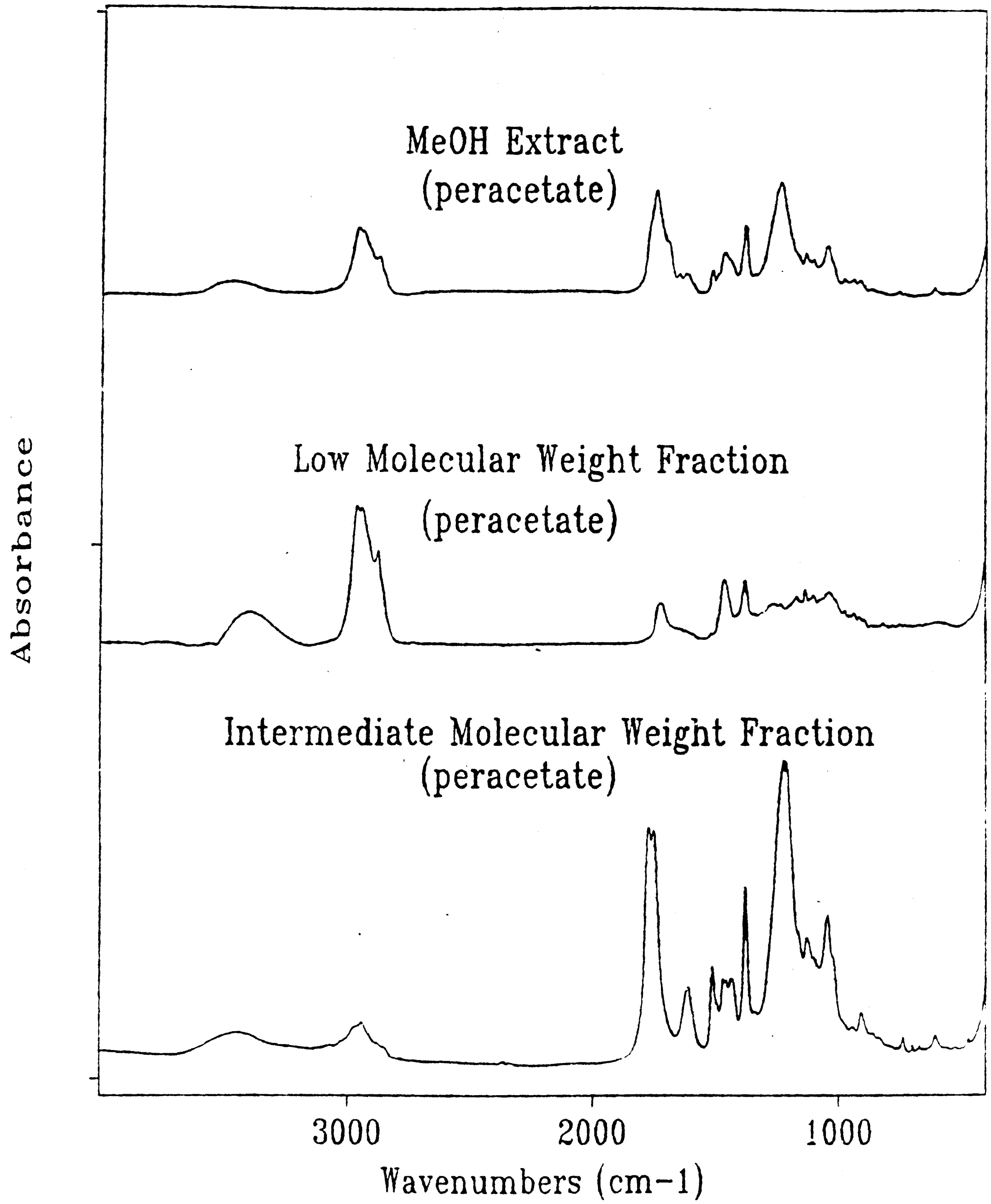


Figure 12. FTIR Spectra of Heartwood Juniper Fraction (peracetates)

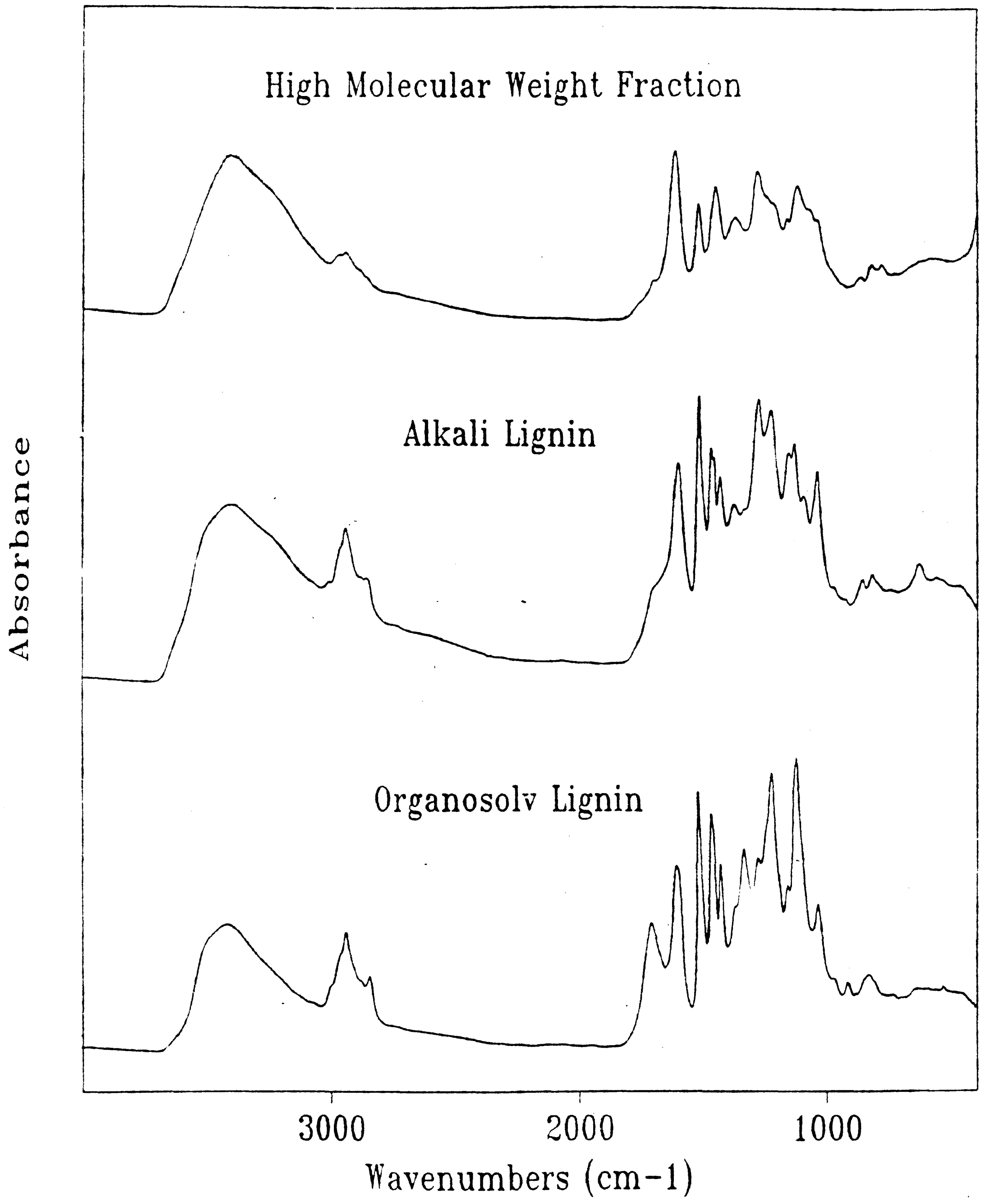


Figure 13. FTIR Spectra of Crude Lignins and Juniper Phlobaphene

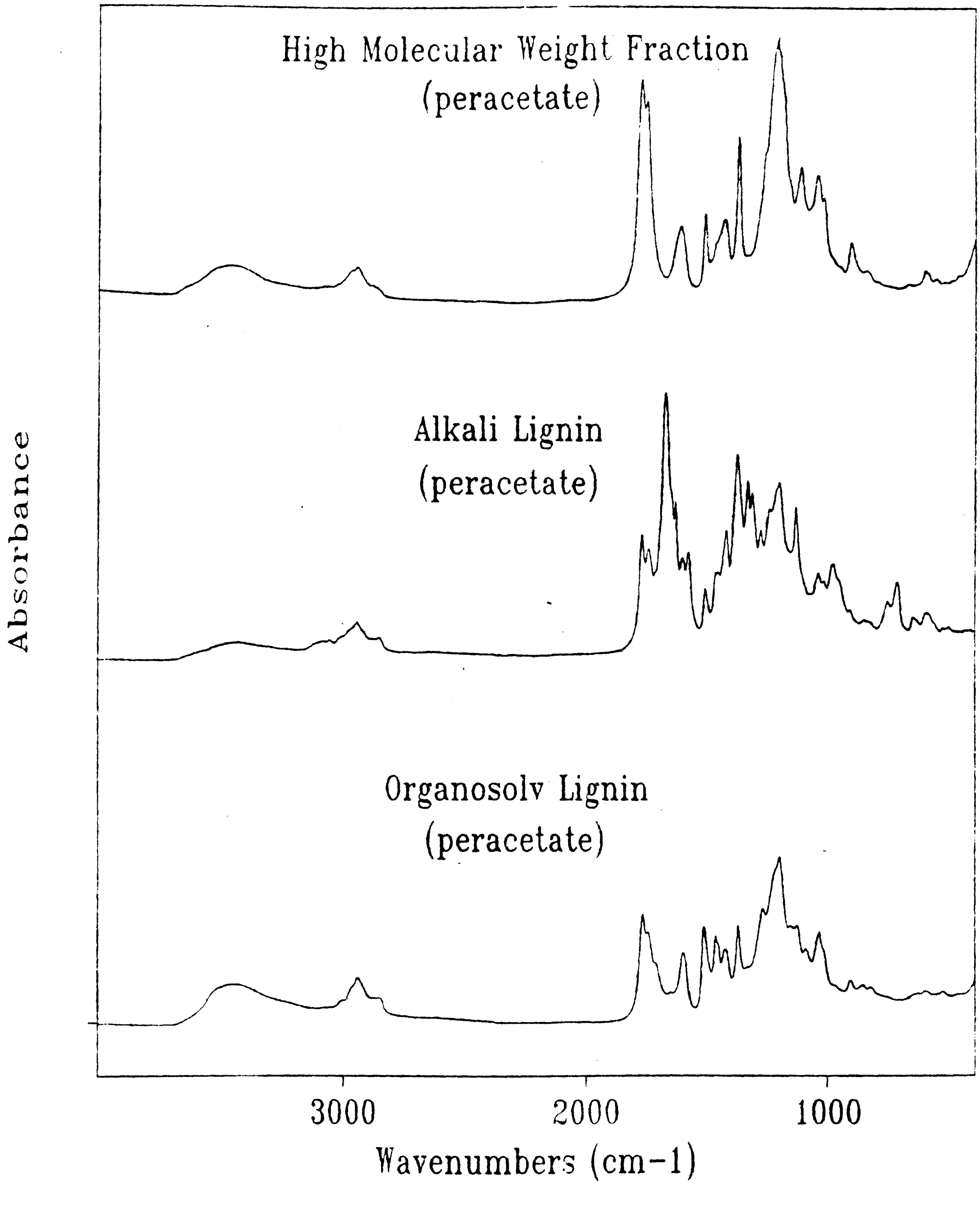


Figure 14. FTIR Spectra of Lignin and Juniper Phlobaphene (peracetates)

Table VIII. FT-IR Spectra of Extractives from Western Juniper Heartwood

WAVE-NUMBER cm^{-1}	BAND	a	b	c	d	e	f
3394	O-H stretch	Strong	-	Strong	Weak	Weak	Weak
2957	CH_3, CH_2	Strong	Strong	Strong	Strong	Weak	Weak
2929	C-H stretch	Strong	Strong	Strong	Strong	Weak	Weak
1710-1738	C=O stretch aldehyde & carboxylic acid	Very Strong	Very Strong	Strong	Strong	Weak	Weak
1600-1690	Aromatic skeletal vibrations plus C=O stretch	Very Strong	Strong	Strong	Strong	Very Strong	Strong
1520-1506	Aromatic skeletal vibration	-	-	Weak	Strong	Weak	Weak
1450-1490	C-H deformation asymmetry in CH_3 & CH_2	Weak	Strong	Strong	Strong	Weak	Weak
1365	Aliphatic C-H stretch in CH_2	Strong	Strong	Strong	Strong	Weak	Weak
1266-1252	Guaiacyl rings plus C=O stretch	Shoulder	Resolved	Weak	Weak	Strong	Weak
1220-1230	C-C plus C-O plus C=O stretch	not resolved	Strong	Weak	Weak	-	-
966-990	C-H out-of-plane deformation	Weak	Weak	Weak	Weak	Very Strong	Very Strong
915-925	C-H out-of-plane aromatic	Weak	Weak	Weak	Weak	Strong	Strong

[a] Crude methanol extract

[b] Acetylated methanol extract

[c] Low molecular weight fraction

[d] Acetylated low molecular weight fraction

[e] Intermediate molecular weight fraction

[f] Acetylated intermediate molecular weight fraction

Table IX. FT-IR Spectra of Phlobaphenes and Alkali and Organosolv Lignin (crude and acetylated)

WAVE-NUMBER cm^{-1}	BAND	a	b	c	d	e	f
3394	O-H stretch	Strong	Weak	Strong	Weak	Weak	Weak
2957	CH_3 and CH_2	Weak	Weak	Weak	Weak	Weak	Weak
2929	C-H stretch	Strong	Strong	Strong	Strong	Strong	Weak
1710-1738	C=O stretch aldehyde & carboxylic acid	-	Strong	-	-	-	-
1600-1690	Aromatic skeletal vibration plus C=O stretch	Strong	Strong	Shoulder	Resolved	-	-
1520-1506	Aromatic skeletal vibration	Strong	Weak	Strong	Weak	Strong	Weak
1450-1490	C-H deformation asymmetry in CH_3 & CH_2	Strong	Weak	Strong	Shoulder	Weak	Weak
1365	Aliphatic C-H stretch in CH_2	Weak	Weak	Weak	Shoulder	Weak	Weak
1266-1252	Guaiacyl rings plus C=O stretch	Shoulder	Shoulder	Strong	Strong	Shoulder	Strong
1220-1230	C-C plus C-O plus C=O stretch	Strong	Strong	Strong	Not Resolved	Strong	Strong
966-990	C-H out-of-plane deformation (trans)	Weak	Weak	Weak	Weak	Weak	Weak
915-925	C-H out-of-plane aromatic	Weak	Weak	Weak	Weak	Weak	Weak

[a] Crude Phlobaphene [c] Alkali Lignin [e] Organosolv Lignin

[b] Acetylated Phlobaphene [d] Acetylated Alkali Lignin [f] Acetylated Organosolv Lignin

NMR Analyses of Phlobaphenes and Lignins

The one dimensional ^{1}H and ^{13}C NMR experiments of phlobaphene peracetate did not provide enough information for the analysis of this polymeric material (Figure 15, 16). In addition, the ^{13}C-NMR experiment was accomplished in 12 hours, due to the low sensitivity. For this reason, the decision to perform two dimensional experiments with enriched samples of phlobaphene was made. From the results, it was observed that juniper phlobaphene spectra have characteristics comparable to acetylated lignin. Thus an analysis of labelled lignin was thought to be appropriate.

The ^{1}H and ^{13}C NMR spectra of unlabelled and labelled phlobaphene peracetate (Figure 17, 18) showed that the peaks around δ = 3.6-4 ppm and δ = 167-172 ppm, respectively, correspond to the aromatic acetate (carbonyl region), defining the region of interest.

Acetylation of Phlobaphenes, Lignin Alkali and Lignin Organosolv with Acetyl-1-^{13}C Chloride

The acetylation of phlobaphenes is easily carried out in the conditions already described yielding a brown solid which is easy to handle. The yield of the acetylation of phlobaphenes (82%) suggests losses due to the extraction. Losses on the acetylation of organosolv lignin may be due to the solubilization (87% recovery). Alkali Lignin showed a higher yield (132%).

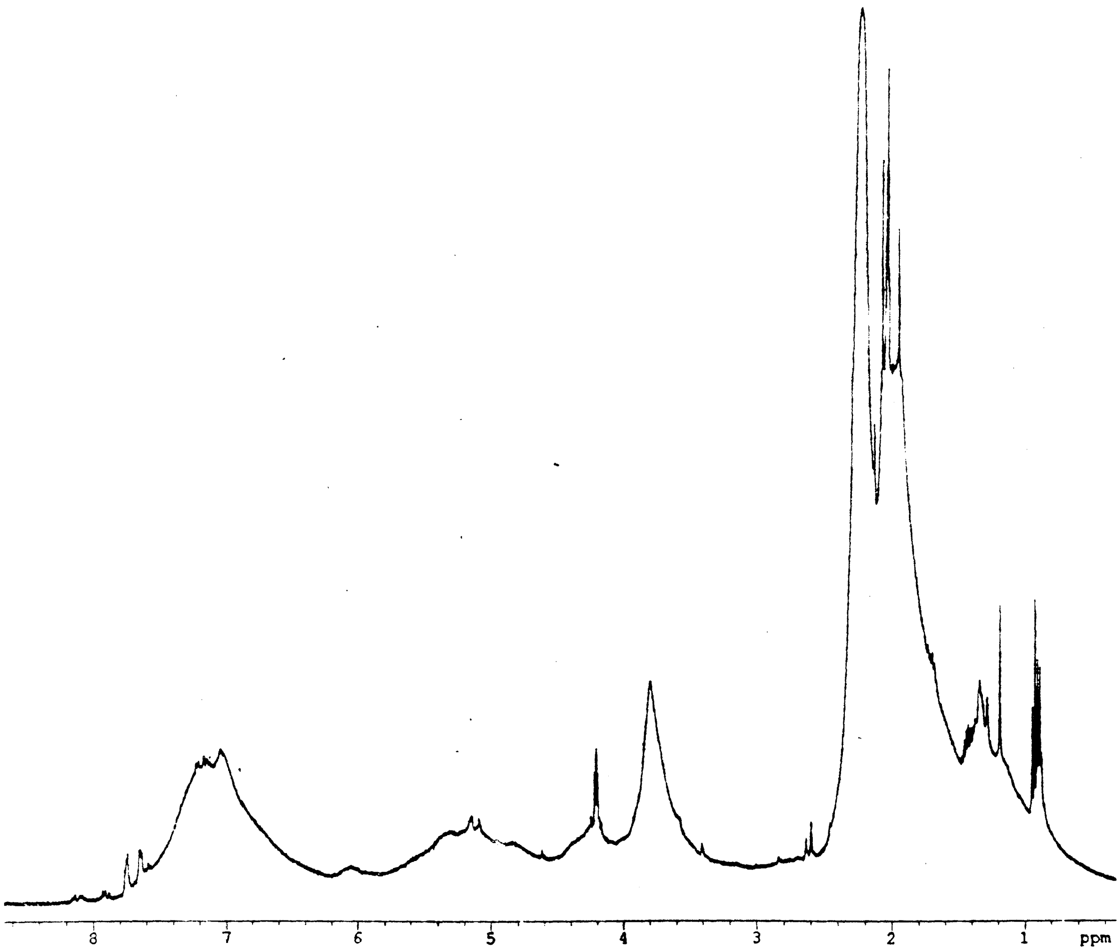


Figure 15. ^{1}H NMR Spectrum of Acetylated Phlobaphene

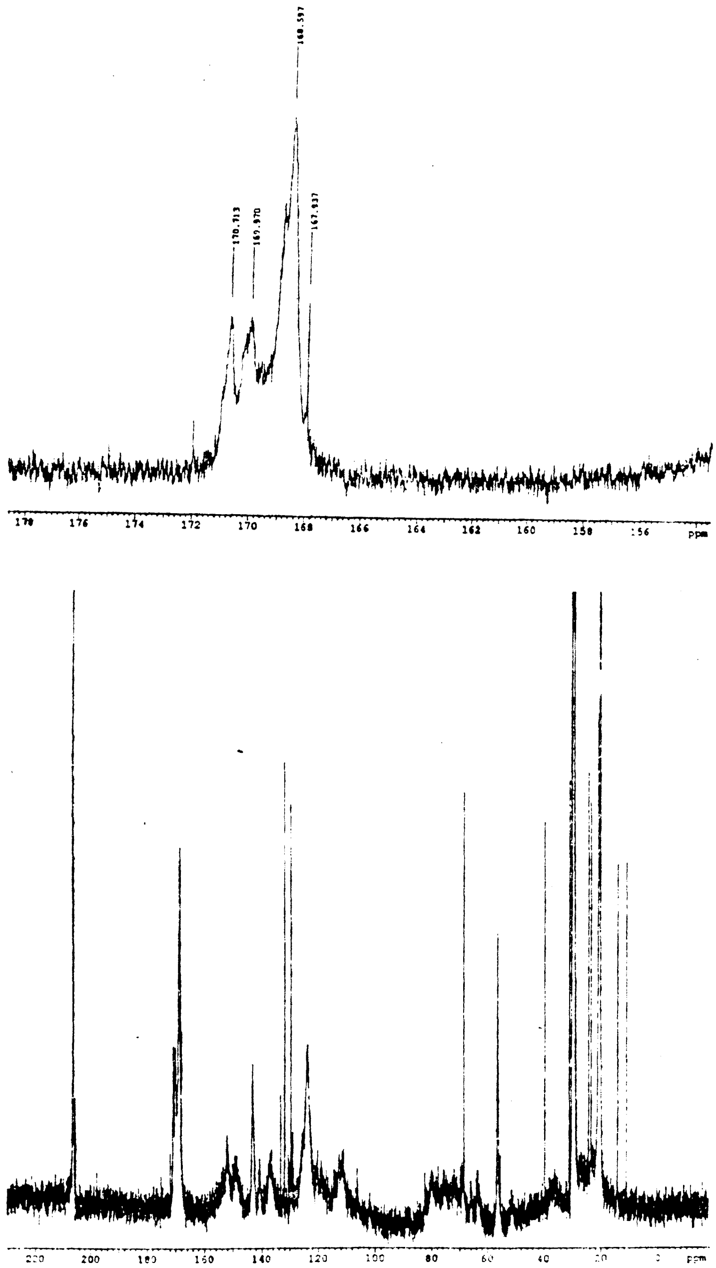


Figure 16. ^{13}C NMR Spectrum of Acetylated Phlobaphene

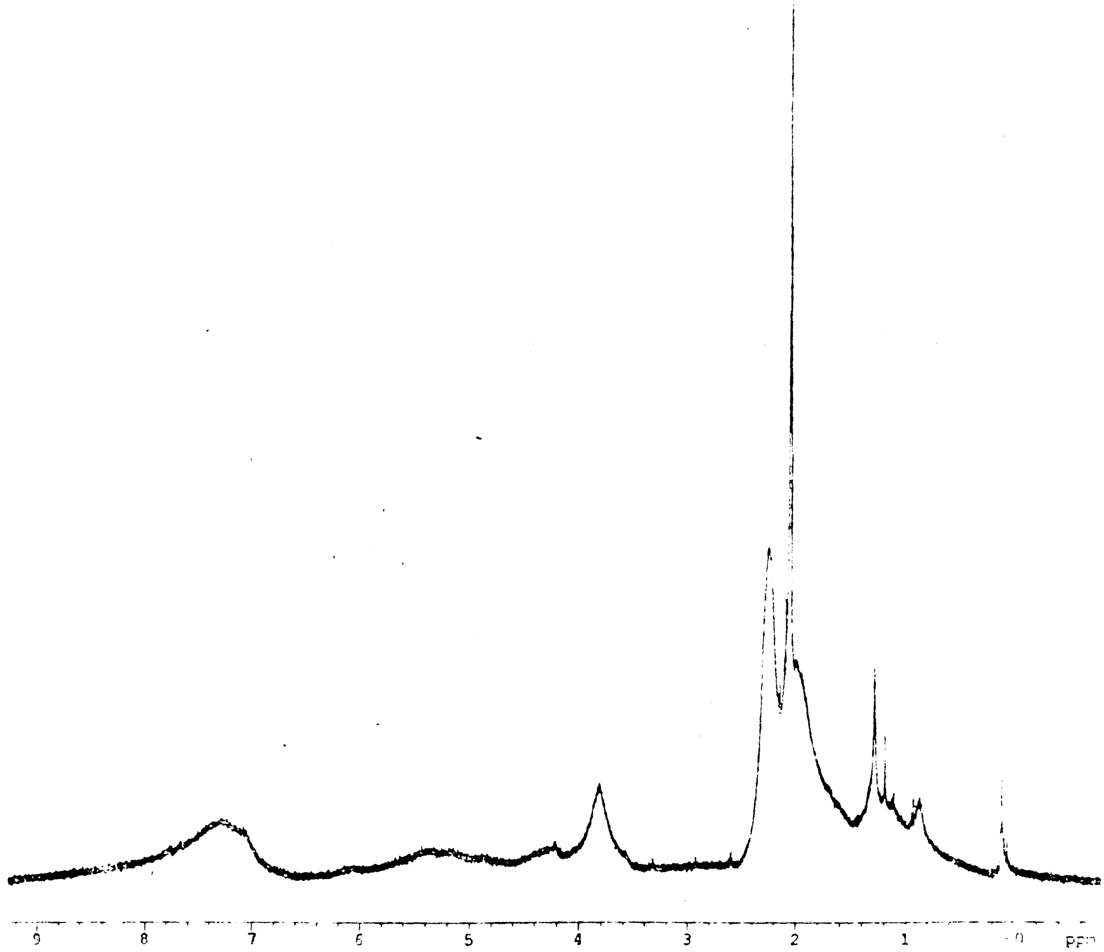


Figure 17. ^{1}H NMR Spectrum of Labelled Phlobaphene

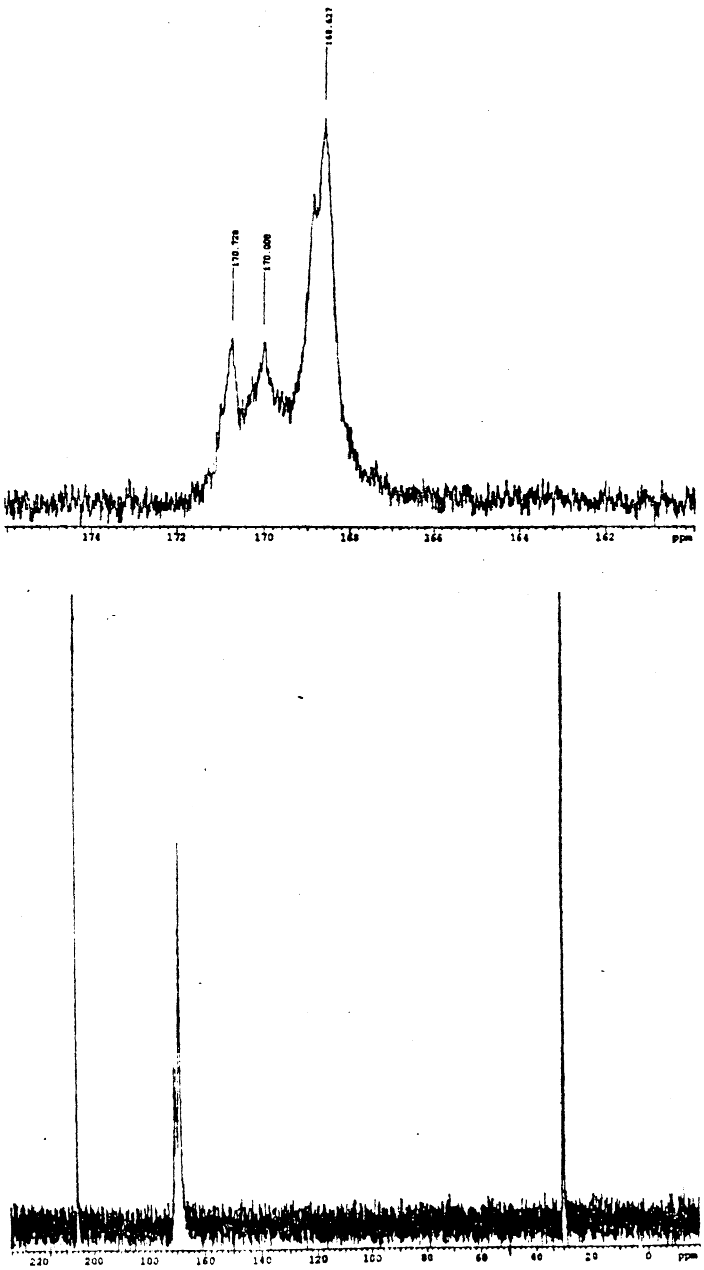


Figure 18. ^{13}C NMR Spectrum of Labelled Phlobaphene

Heteronuclear Multiple Bond Correlation Experiment

Figures 19, 20, 21 display the long-range multiple bond correlation (HMBC) spectra of labelled phlobaphene, lignin alkali and lignin organosolv peracetates. The main difference among these three NMR spectra is the absence of the correlation at around $\delta = 3.5\text{-}4$ ppm for the labelled phlobaphene peracetate, accounting for the lack of methoxyl (OCH_3) groups. This suggests different chemical environments for phlobaphenes and lignins. Table X describes the C-H correlation in the region of interest. Only the correlation at around $\delta = 6.01 - 6.06$ ppm and $\delta = 169.34 - 168.96$ ppm, appears in the spectra of the three polymeric materials, and corresponds to the long range correlation between the labelled carbonyl and the α-proton on β-*O*-4 type esther structures (Ralph *et al.*, 1994) suggesting a similar situation for juniper phlobaphene. In contrast, labelled juniper phlobaphene peracetate presents two other C-H correlations in the same region, one at $\delta = 5.00 - 5.01$ ppm and $\delta = 169.00 - 169.68$ ppm; and the other at $\delta = 5.02 - 5.03$ ppm and $\delta = 169.92 - 169.18$ ppm. These correlations are not assignable to any lignin-like structure and are novel to the juniper phlobaphene. ^{13}C-NMR lignin spectra show other correlations at 168.961, 169.977, and 168.74 ppm which correspond to primary, secondary and aromatic phenolic groups, respectively (Faix et al., 1994).

New Lignin Analysis Method - Quantitative NMR

This analytical method turns out to be useful for quantitative ^{13}C-NMR experiments for polymeric lignins, such as OMe values and total OH (phenolic and aliphatic) determination. The experiment can be accomplished in 10 minutes in the described conditions versus the 30-33 hours of the currently in use method (Figure 22). This technique can be very helpful for lignin chemists.

Table X. Long-Range Correlations of Phlobaphenes, Lignin Alkali, and Lignin Organosolv Peracetates (2D HMBC) in the region 160-175 ppm

C-H Correlations	Phlobaphenes Peracetate δ (ppm)	Lignin Alkali Peracetate δ (ppm)	Lignin Organosolv Peracetate δ (ppm)
^{1}H	5.00 - 5.05	-	-
^{13}C	169.85-169.01	-	-
^{1}H	5.30 - 5.35	-	-
^{13}C	169.90-169.26	-	-
^{1}H	6.01 - 6.12	6.00 - 6.10	6.00 - 6.10
^{13}C	169.35-168.84	169.20-168.60	169.20-168.60
^{1}H	-	6.66 - 6.78	-
^{13}C	-	169.10-168.40	-
^{1}H	-	4.28 - 4.46	4.28 - 4.46
^{13}C	-	170.10-169.40	170.10-169.40

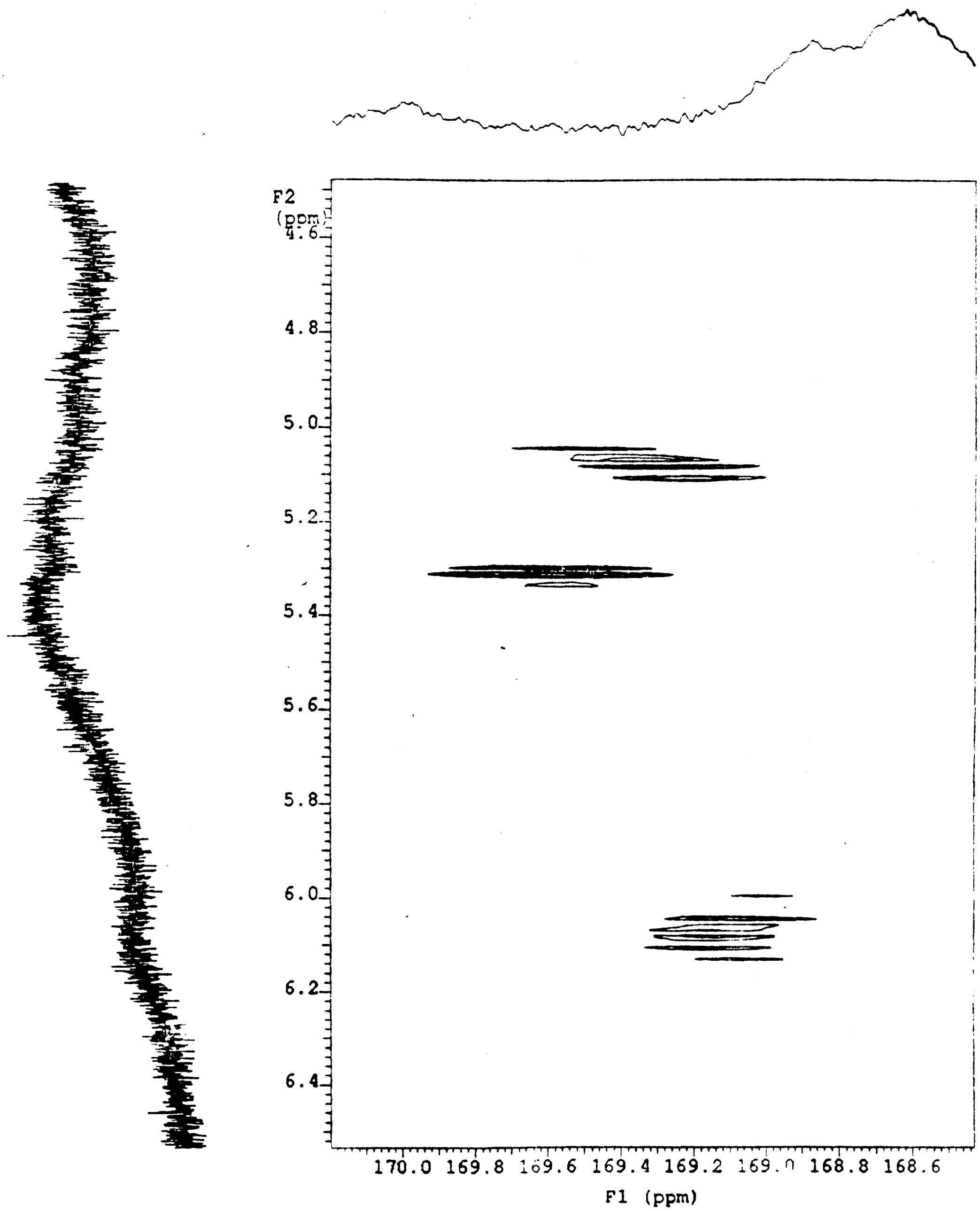


Figure 19. HMBC Spectrum of Labelled Phlobaphene

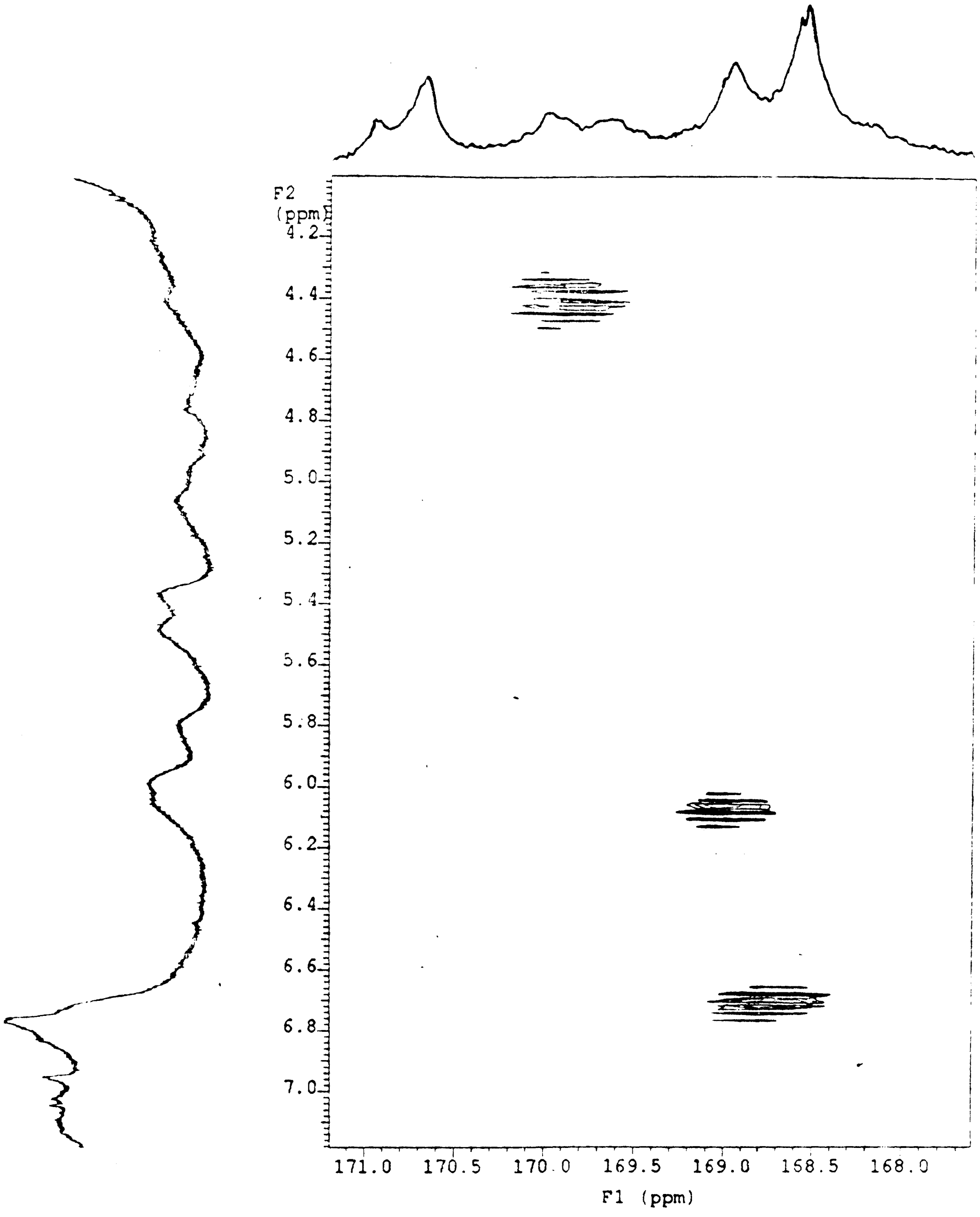


Figure 20. HMBC Spectrum of Labelled Lignin Alkali

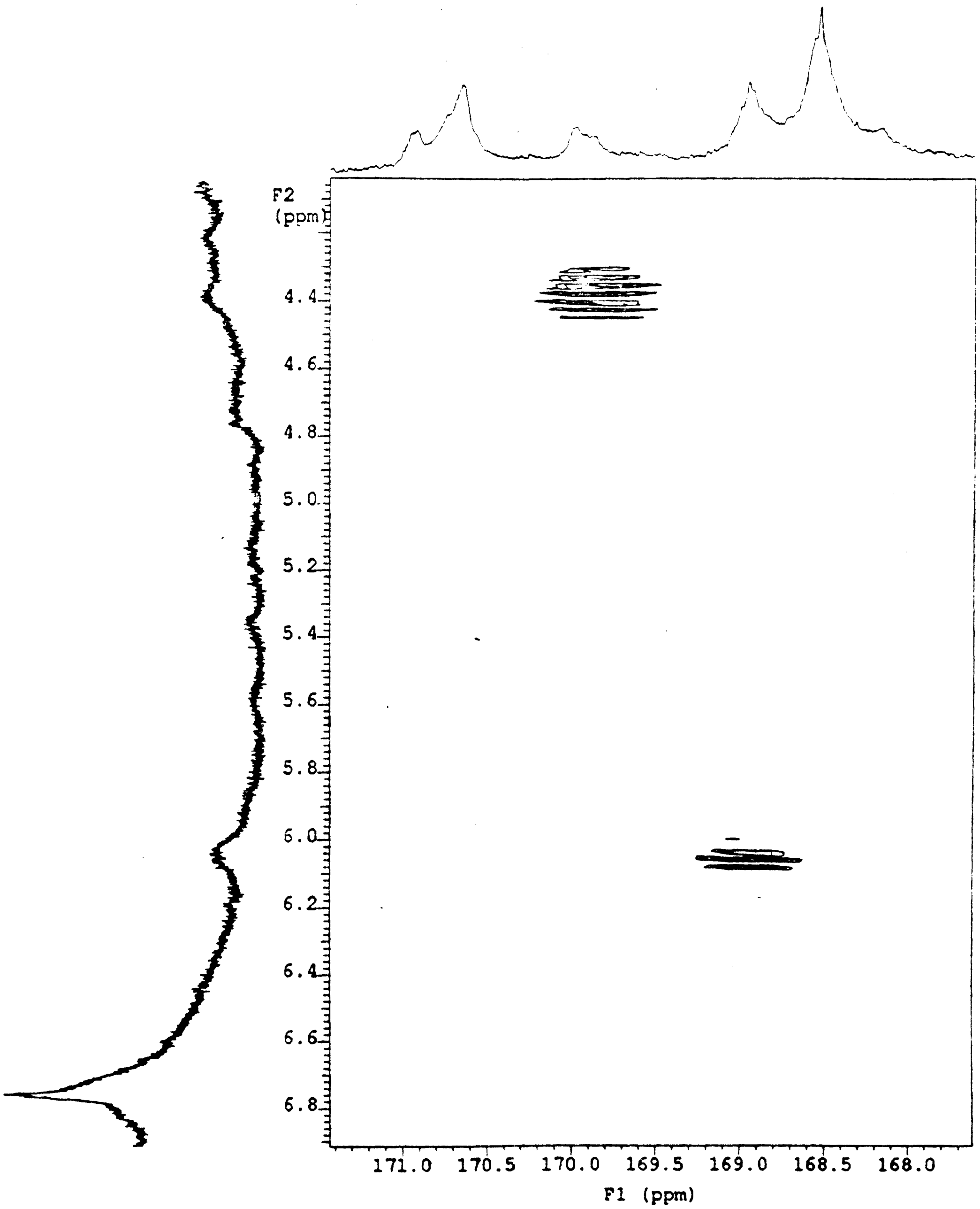


Figure 21. HMBC Spectrum of Labelled Lignin Organosolv

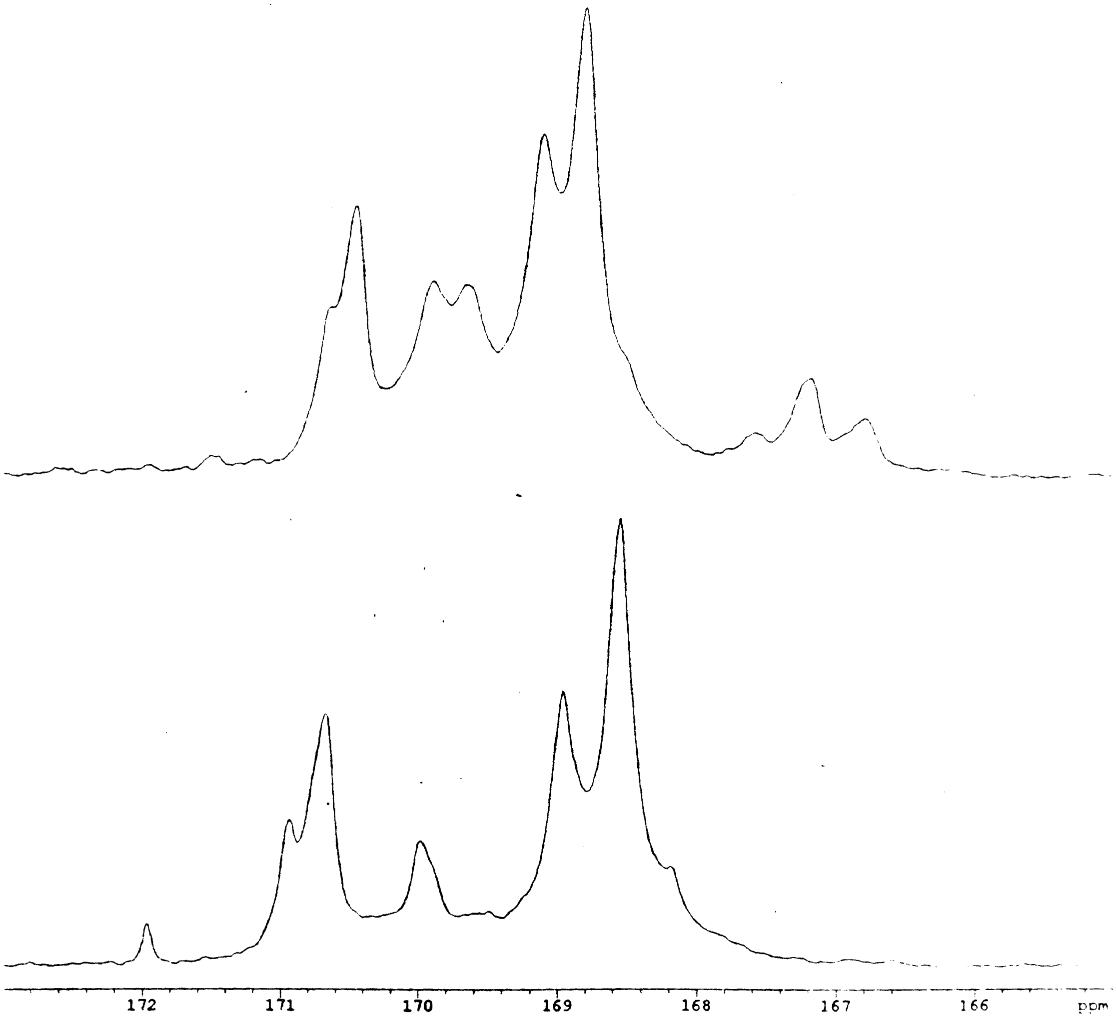


Figure 22. Quantitative ^{13}C NMR Spectra of Alkali (top) and Organosolv (bottom) Lignins .

CONCLUDING REMARKS

Methanol crude extract was isolated from western juniper chips. A liquid-liquid extraction was then performed with hexane. The methanol-layer was subsequently submitted to a preparative Toyopearl chromatography. Several of the resulting fractions were subjected to a brine shrimp bioassay. The less polar fractions are relatively toxic, whereas more polar fractions are non-toxic. Thus it appears that the later do not contribute to the durability of this species, being a biosynthetic by-product or serving an unknown role. The average molecular weight of several fractions was also determined by size exclusion chromatography, resulting in ranges of M_w=340-22,000 g/mole. The most toxic fraction has an average molecular weight of M_w= 450 g/mole. This is comparable with some lignans and resin acids previously determined for other juniper species. The more polar fractions, termed phlobaphenes, were also the higher molecular weight fractions. Their purification was accomplished by means of Toyopearl and silica gel chromatography. Western juniper phlobaphenes can be described as heterogenous polymeric material with molecular weights ranging between M_w=13,350-22,000 g/mole, which possess α-protons of the β–O-4 type ether structures similar to lignins, but also have unknown correlations not attributable to a lignin-like structure.

Fourier Transformed Infrared and two dimensional NMR spectroscopy (Heteronuclear Multiple Bond Correlation experiments) showed to be helpful in the identification of functional groups and C-H correlations to define the chemical environment for both phlobaphenes and lignins. The 2D HMBC experiment can be used as a fast and novel technique for quanlitative assessment of lignins, while the quantitaive ^{13}C method affords a major improvement over currently employed techniques.

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