AN ABSTRACT OF THE THESIS OF

HONG-KEUN SONG (Name of student) for the degree of **DOCTOR OF PHILOSOPHY** (Degree)

in FOREST PRODUCTS (Major department) presented on December 16, 1987 (Date)

Title: **CHEMISTRY OF PHENOLIC STAINS IN DOUGLAS-FIR SAPWOOD.**

Abstract approved: Signature redacted for privacy.
Murray L. Laver

Dihydroquercetin [(2,3-trans)-3,3',4',5,7-pentahydroxy-flavanonol] was shown to be the precursor to the phenolic stains that often develop in Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] sapwood. Douglas-fir sapwood was extracted with methanol and the methanol solubles were fractionated by solvent partition, thin-layer chromatography, silica-gel column chromatography, and Sephadex gel-permeation chromatography. Tyrosinase, a commercially available catechol oxidase enzyme, was used to locate and trace the precursors to color formation in the solubilized materials. Careful control of the separations and careful testing of the fractions for color formation resulted in the identification and isolation of dihydroquercetin as the precursor of the stains.

It was established that neither oregonin, the color precursor of stains in red alder wood and bark, nor catechin, a flavonoid that has been associated with stains

in western hemlock wood, were involved in the staining of Douglas-fir sapwood.

The inhibitors, ethylenedinitrillotetraacetic acid disodium salt (EDTA), phenylthiourea, mercaptobenzothiazole, and Chloramine-T proved ineffective in preventing color formation when the methanol-extracted materials from Douglas-fir sapwood were treated with tyrosinase.

An improved method of isolating dihydroquercetin from Douglas-fir bark was developed. This pure crystalline dihydroquercetin was reacted with tyrosinase to simulate the reactions that might lead to color formation in Douglas-fir sapwood. The reaction mixture became brown in color and a dark brown polymeric precipitate formed. The brown polymer was recovered and was shown to have a number of characteristics similar to the stains in Douglas-fir sapwood by ultraviolet and infrared spectroscopy. The compounds 3,4-dihydroxybenzoic acid (protocatechuic acid), 3,4-dihydroxyphenylacetic acid, and quercetin were found in the reaction products of dihydroquercetin and tyrosinase. They were considered to be degradation products of dihydroquercetin and possible intermediates in polymeric stain formation. Thus, a relationship between the presence of dihydroquercetin, enzyme action, and the phenolic stains that often develop in Douglas-fir sapwood was established.

Chemistry of Phenolic Stains in Douglas-Fir Sapwood

by

Hong-Keun Song

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Date thesis is presented December 16, 1987

Typed by Hong-Keun Song for Hong-Keun Song

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INTRODUCTION.

Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] is the principal commercial softwood of the Pacific Northwest, accounting for nearly three quarters of the conifer inventory. At advanced ages, Douglas-fir trees may attain diameters of 1.8 meters and heights of 75 meters. These trees typically are grown in a rotation of 50-80 years, and the timber is used for a substantial part of the pulp and paper, lumber, plywood, furniture, and particleboard produced in the United States.

The stains which develop on commercial Douglas-fir sapwood are problems of great economic importance. Blue stain is caused by development of certain fungi in sapwood. However, some other stains, nonfungal and usually brown, may appear on exposed surfaces of green lumber as it dries. These stains may occur in commercially important western woods such as sugar pine, white pine, ponderosa pine, redwood, western hemlock, and red alder. Some studies have produced data to support the chemical or enzymatic stains in red alder, western hemlock and some other Japanese commercial woods. (Karchesy et al., 1974; Karchesy, 1975; Takahashi et al., 1983; Terazawa et al., 1984a, b; Hrutfiord

et al., 1985; Takahashi and Ogiyama, 1985a, b; Takahashi and Ogiyama, 1986.)

The staining of Douglas-fir that is due to phenolic compounds is confined to the sapwood and it develops mostly on the surfaces. The discoloration usually is some shade of brown, yellowish-brown, orange-brown, and, in rare cases, bright yellow or orange on end grain surfaces where staining tends to be most intense. Discoloration on ends that have been sprayed with red coating are sometimes blackish (Miller et al., 1983).

Once the stain has developed, the best way to remove it is by physical sanding or planing. This can be expensive and does not assure that the stain will not reform on the fresh surface. Therefore, prevention of the formation of the stain is the desired approach. A study of the chemistry of the stain is intended to show the precursors of the problem and what actually constitutes the stain.

The objectives of this work were to test, with tyrosinase enzyme, the phenolic compounds resulting from methanol extraction of Douglas-fir sapwood, to find the precursors that develop into the stains, and to determine the chemical mechanism of stain formation to find a method to prevent the formation of the stains on Douglas-fir sapwood.

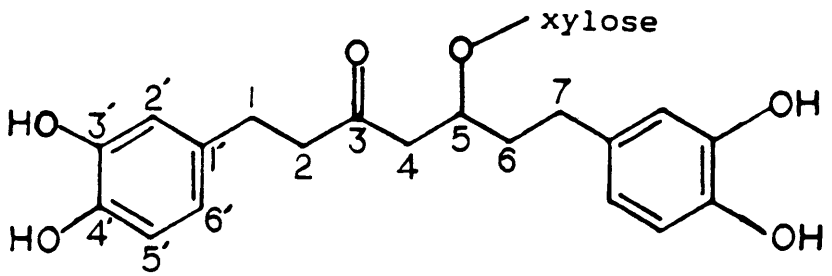
HISTORICAL REVIEW

The decorative value and color stability of wood surfaces are of economic importance, considering the increasing competition from alternative materials. Kurth and Becker (1953) initiated studies to determine the chemical nature of the coloring matters in red alder. They found the coloring matters to be in much higher concentration in the bark than in the wood and subsequently used bark as a source of their extractives. Although they did not identify any specific phenolic compounds, they did find that the major component of the ethyl acetate extract was a phenolic xyloside. Karchesy et al.(1974) and Karchesy (1975) more throughly investigated the phenolic compounds involved in the coloring of red alder. They found that oregonin, [1,7-bis-(3,4-dihydroxyphenyl)heptan-3-one-5-xylopyranoside] (I) represents a precursor of the reddish-brown stain. They also reported that condensed tannins did not contribute to the staining phenomenon of red alder. More recently Terazawa et al. (1984) demonstrated clearly that oregonin is the precursor for the reddish-brown discoloration in other species of alder in Japan.

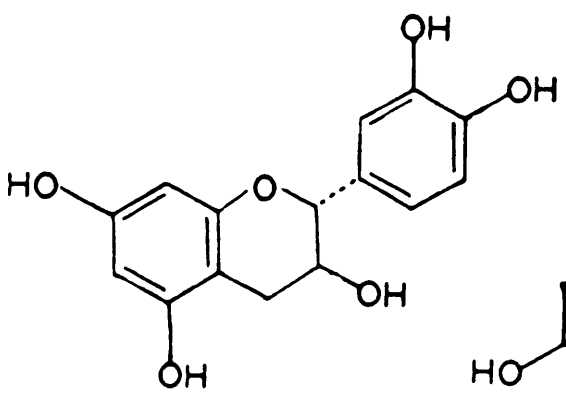
There are several additional investigations of phenolic compounds involved in the discoloration of wood. Sugar pine brown stain has been the subject of considerable research

and of a number of papers (Millet, 1952; Stutz, 1959). Evans and Halvorson (1962) initiated studies in the early nineteen sixties to determine the cause and control of brown stain in western hemlock. They found the coloring matters to be in the sapwood only, and to be most intense in lumber from the inner sapwood of the tree. Although they did not identify specific phenolic compounds, they did suggest that leucoanthocyanins become condensed in the presence of bacterial enzymes to form soluble polymers which then migrate to the surface of the wood during drying, and are oxidized to dark brown polymers when in contact with air. Recently Hrutfiord et al. (1985) studied the color formation in western hemlock chips used for mechanical pulps. They found that (+)-catechin (II) concentration declined during storage. The decline in (+)-catechin concentration was found to correlate closely to the initial loss of brightness of the mechanical pulps. They further ascertained that (+)-catechin in fresh chips became oxidized, giving brown colored polymers which in turn were retained in the wood fibers.

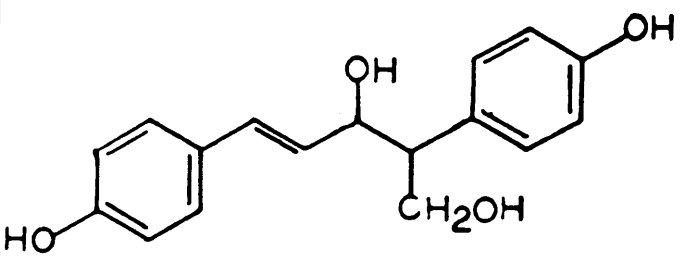
There are sequential studies of phenolic compounds in discolored sugi (Cryptomeria japonica D. Don) sapwood. Takahashi et. al. (1983) and Takahashi and Ogiyama (1985a, b, 1986) suggested that yateresinol (III), 1,4-bis-(p-hydroxyphenyl)butadiene (IV), sequirin-c (V) and agatharesinol (VI) may be responsible for the discoloration in several sugi sapwoods infected by different bacteria.



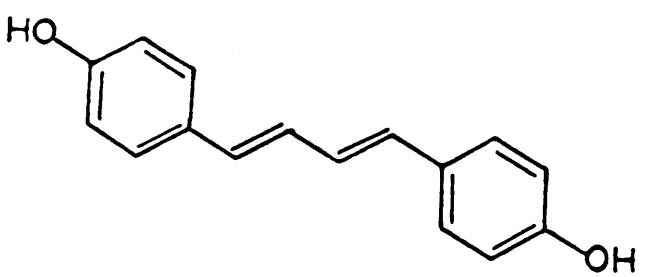
1,7-bis-(3,4-dihydroxyphenyl)heptan-3-one-5-xylopyranoside



II. (+)-catechin



III. yateresinol



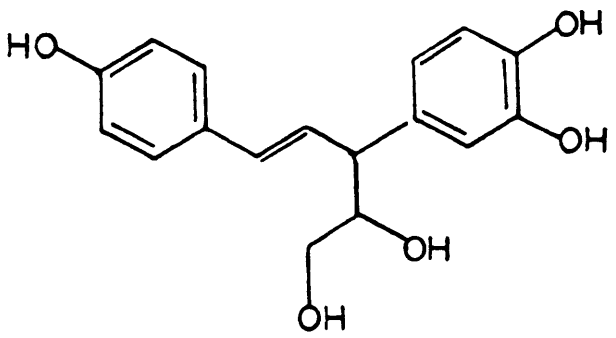
IV. 1,4-bis-(p-hydroxyphenyl)-butadiene

Yazaki et. al. (1985) reported that three different types of components could be involved in the discoloration of ilomba wood. These are (-)-epicatechin (VII), (+)-catechin (II), and the intensely red, water soluble component of the methanol extract of discolored ilomba wood (Pycnanthus angolensis Exell).

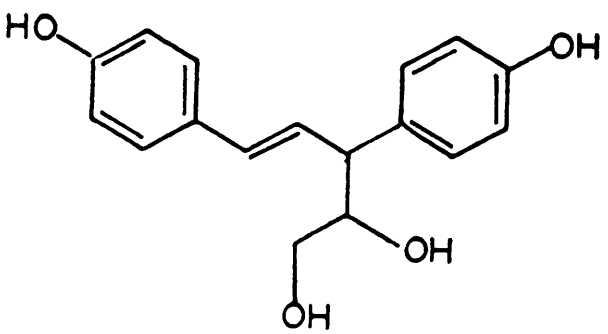
Miller et. al. (1983) studied the prevention of stain in Douglas-fir sapwood during drying by altering physical treatments. However, no one has systematically studied the chemical precursors to the stain in Douglas-fir sapwood, nor the stain itself, nor prevention of the stain by simple chemical treatments.

It is clear that any of the above phenolic compounds may be involved in the brown and reddish stain phenomena. The original phenolic compounds are colorless. The development of colors from colorless compounds requires chemical changes due to reactions by light, pH change, enzymatic oxidation, bacterial infection or combinations of these. For example, the color of anthocyanins is significantly influenced by the pH of the medium in which they are dissolved (Figure 1), (Iacobucci and Sweeny, 1983).

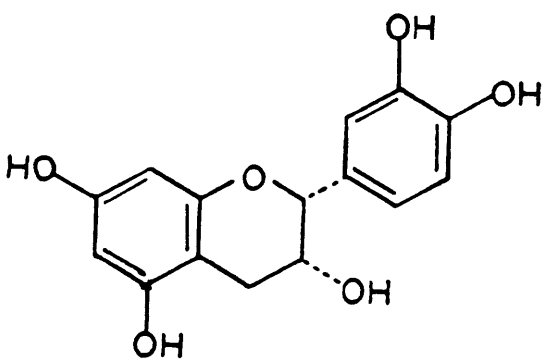
Mibayashi et. al. (1985) studied the discoloration of wood by acid. They isolated and identified the compounds which are responsible for the discoloration by acid. They found that pinosylvin (VIII) and its monomethyl ether (IX) were largely responsible for discoloration of Pinus densiflora heartwood. They also suggested that sugiresinol



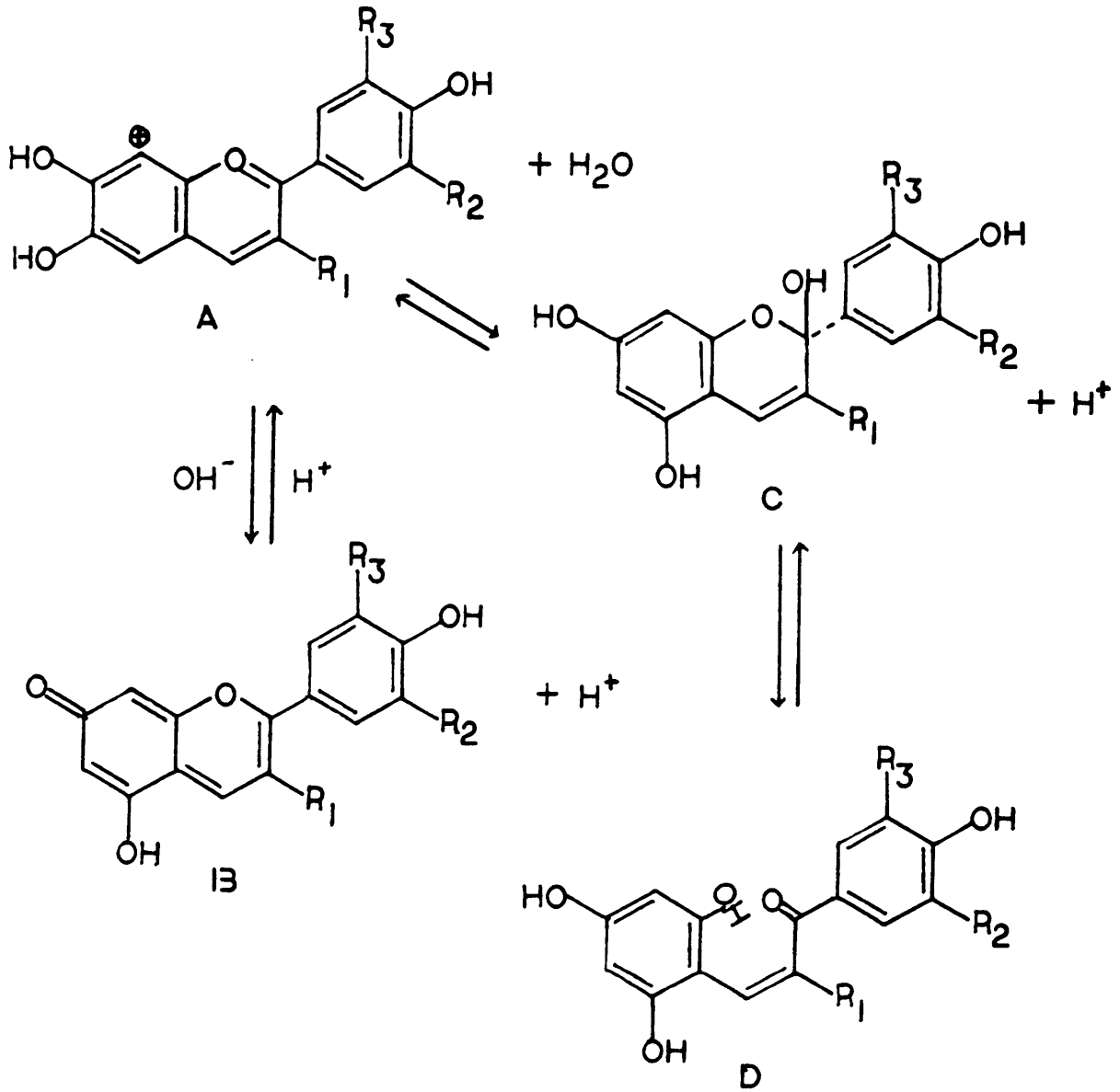
V. sequirin-c



VI. agatharesinol



VII. (-)-epicatechin



A: R_1=O-sugar, red; R_1=H, yellow at pH<2
B: red or blue
C and D: colorless

Figure 1. Structural transformation reactions of anthocyanidins.

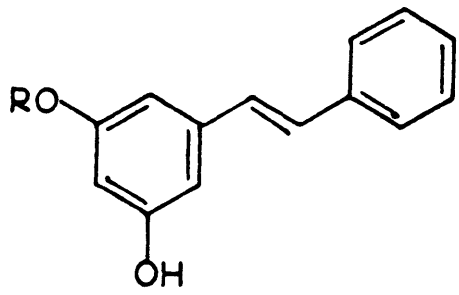
(X) and hydroxysugiresinol (XI) may be active in the discoloration.

Bauch et al. (1985) reported the influence of pH in the discoloration of ilomba wood. They found that the discoloration of ilomba wood was caused by chemical reactions of some compounds due to a pH change from acid to alkali by bacteria.

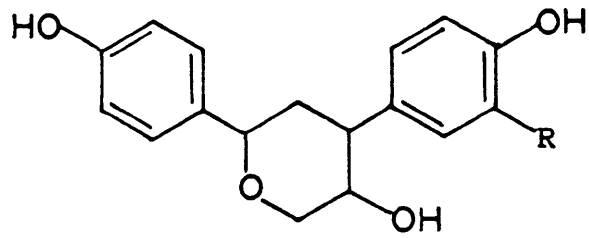
Takahashi et al. (1983) and Takahashi and Ogiyama (1985a, b, 1986) studied bacterial infected sugi wood and concluded that some phenolic compounds were chemically changed because of bacterial activities. Recently Morrell (1987) reported the discoloration of red alder due to the fungus Ceratocystis picea.

Because of the widespread occurrence of the catechol functional group, such as in catechin (II), dihydroquercetin (XII) and catechol (XIII), and because of the presence of catechol-oxidizing enzyme systems in nature, there has long been an investigative interest in the chemical reactions involved in the oxidative transformation of such compounds into dark insoluble pigments.

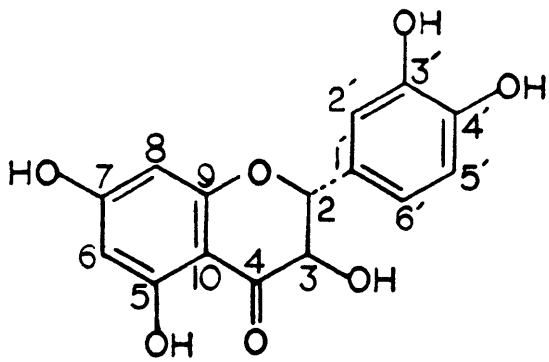
It has been known for many years that o-benzoquinone is one of the initial products of the oxidation of catechol-group compounds (Pugh and Raper, 1927; Wagreich and Nelson, 1939; Dawson and Tarpley, 1938; Mason, 1949). o-Benzoquinone is very unstable in aqueous systems at physiological pH (between pH 4 and pH 7) (Dawson and Nelson, 1938; Nelson and Dawson, 1944) and such quinone systems,



VIII. R=H, pinosylvin
IX. R=CH_3, pinosylvin monomethyl ether



X. R=H, sugireninol
XI. R=OH, hydroxysugiresinol



XII. (2,3-<u>trans</u>)-3,3′,4′,5,7-pentahydroxyflavanonol (dihydroquercetin)

prepared either enzymatically of by chemical oxidizing agents, deposit a dark insoluble precipitate on standing (Figure 2).

Forsyth and Quesnel (1957) found that under conditions of very low catechol concentration and optimum oxygen uptake only one intermediate, a purple-red pigment which was not structurally identified could be detected, using two dimensional paper chromatography to trap the intermediates in the enzymatic oxidation of catechol. However, at higher than 1 X 10^{-3}M catechol concentration, three isomeric tetrahydroxydiphenyls and a yellow quinone were trapped and structurally identified (Figure 3). The yellow quinone was identified as diphenylenedioxide-2,3-quinone and was shown to be derived from 2,3',4'-trihydroxydiphenyl ether by oxidation (Figure 4) (Forsyth et al., 1960).

An investigation completed by Dawson and Tarpley (1963) reported that the aerobic oxidation of catechol as catalyzed by the enzyme tyrosinase under physiological conditions (between pH 4 and pH 7) resulted in the formation of a dark, insoluble, humic-like or melanin-like pigment. They found that the stability of o-benzoquinone in aqueous systems is markedly affected by the pH, the catechol concentration, the buffer concentration, the buffer type and in general by the availability of nucleophiles in the system. Figure 5 shows their proposed mechanism of catechol oxidation.

In 1966 Waiss et al., investigated the oxidative condensation of catechol and resorcinols. They found that

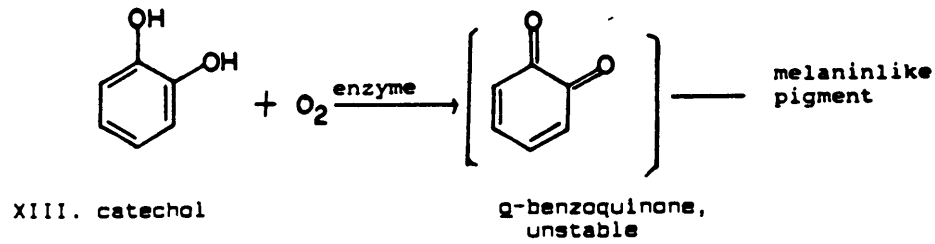


Figure 2. The enzymatic oxidation of catechol.

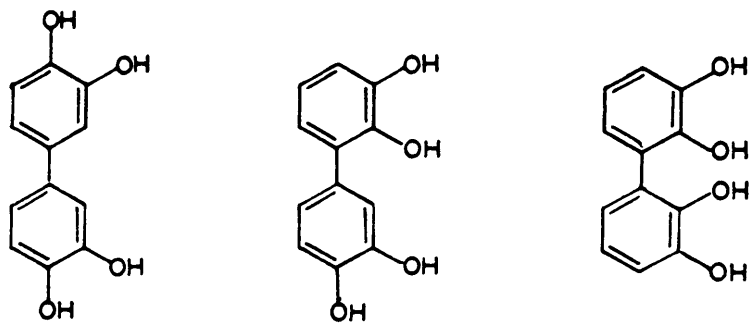


Figure 3. The isomeric tetrahydroxydiphenyls.

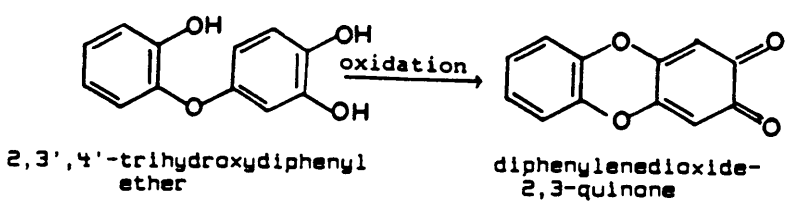


Figure 4. The yellow quinone.

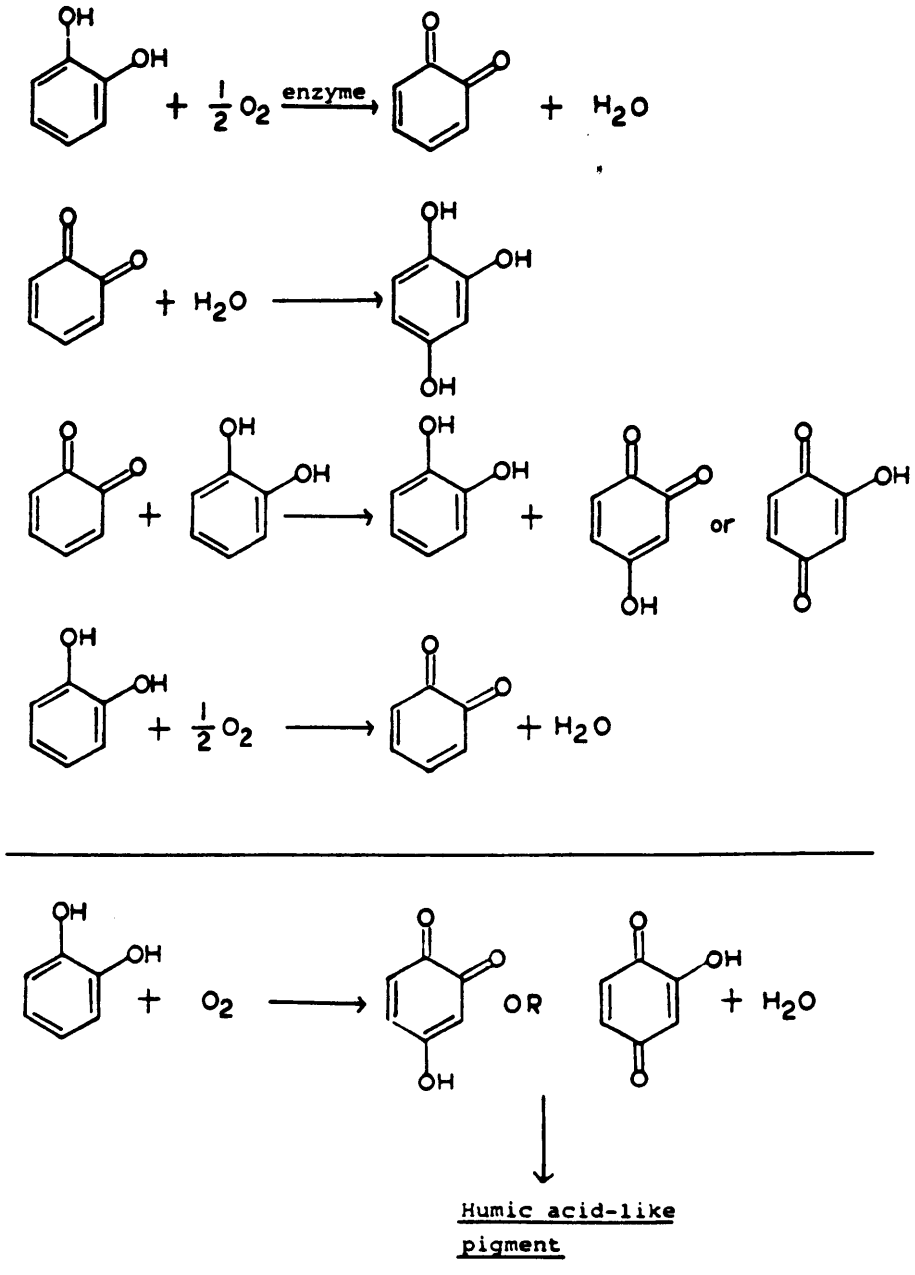


Figure 5. The water-reaction mechanism of catechol.

an equimolar solution of 3-isopropylcatechol and orcinol mixed with 1 N sodium hydroxide in the presence of air rapidly formed a dark green color and gave three different types of self-condensation products (Figure 6).

In addition to these investigations of catechol oxidation reactions, several investigators studied polyphenol oxidation reactions. Hathway and Seakins (1955, 1957a, b) studied sequentially the autoxidation of catechin in neutral aqueous solution, the enzymatic oxidation, and the chemical oxidation. They found that an o-quinone was an intermediate in the polymerization of catechin just as it was in catechol. They also suggested that the polymerization of catechin was due to the head-to-tail C-C linkage between C-6′ and C-8 (Figure 7).

Weinges and coworkers (1969, 1970, 1971) studied the mechanism of the enzymatic oxidation of catechin. They isolated dehydrodicatechin A which is structurally isomeric with the procyanidins of group A, as isolated from plants, from the reaction products of catechin with various phenol oxidases. In addition to these investigations, they reported four additional dehydrodicatechins B1-B4 and a dehydrotricatechin through spectroscopic studies. They suggested the mechanism of the enzymatic oxidation of catechin shown in Figure 8.

Work initiated at the Oregon Forest Products Laboratory in 1947 by Kurth and co-workers demonstrated that Douglas

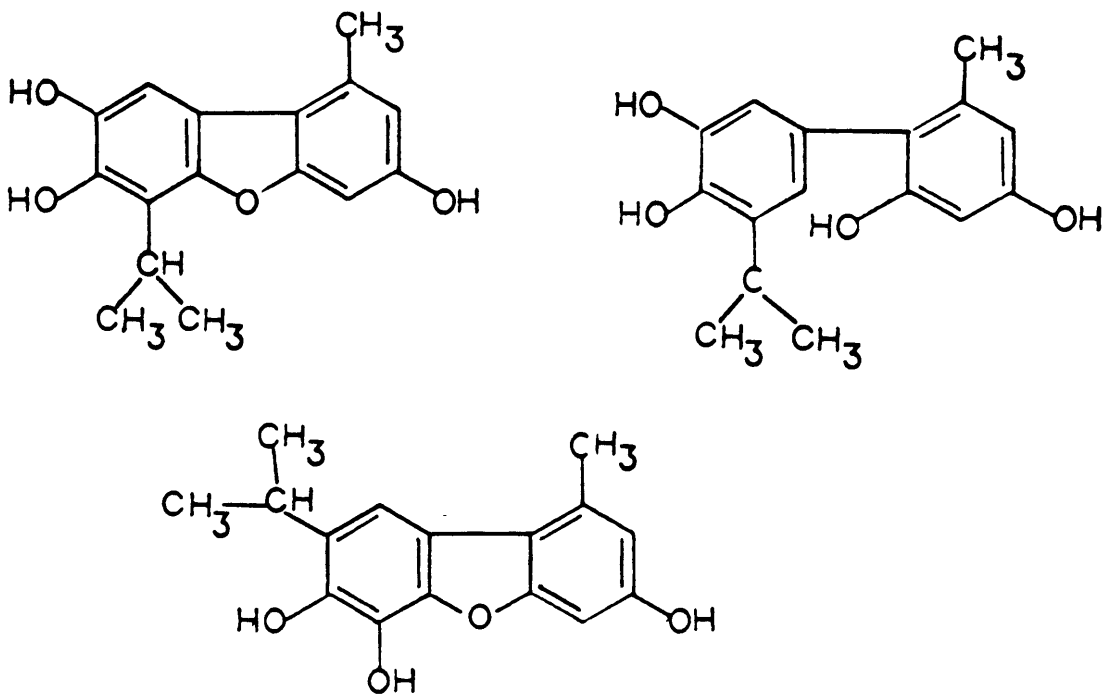


Figure 6. The structures of the 3-isopropylcatechol-orcinol condensation products.

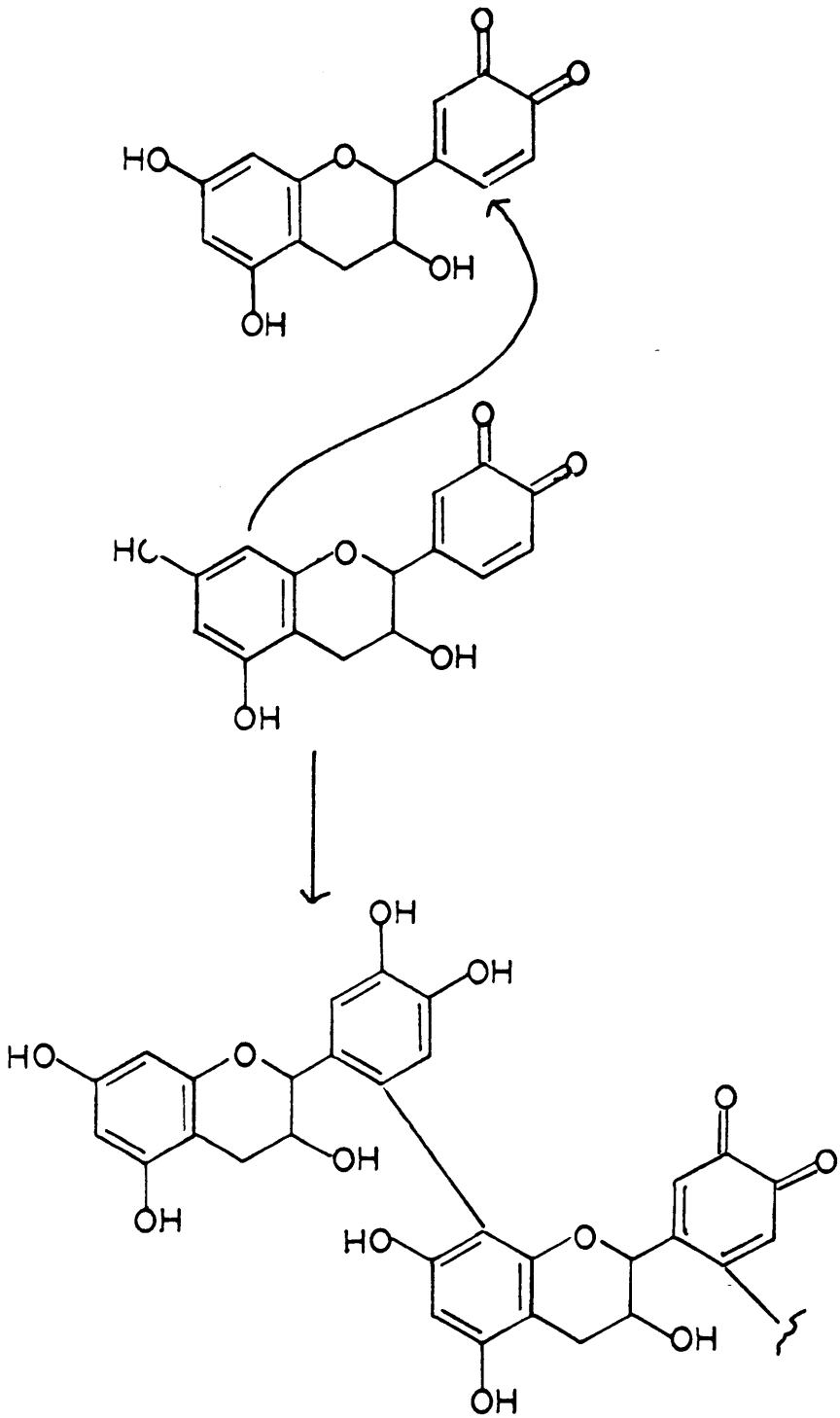


Figure 7. The polymerization of catechin autoxidation.

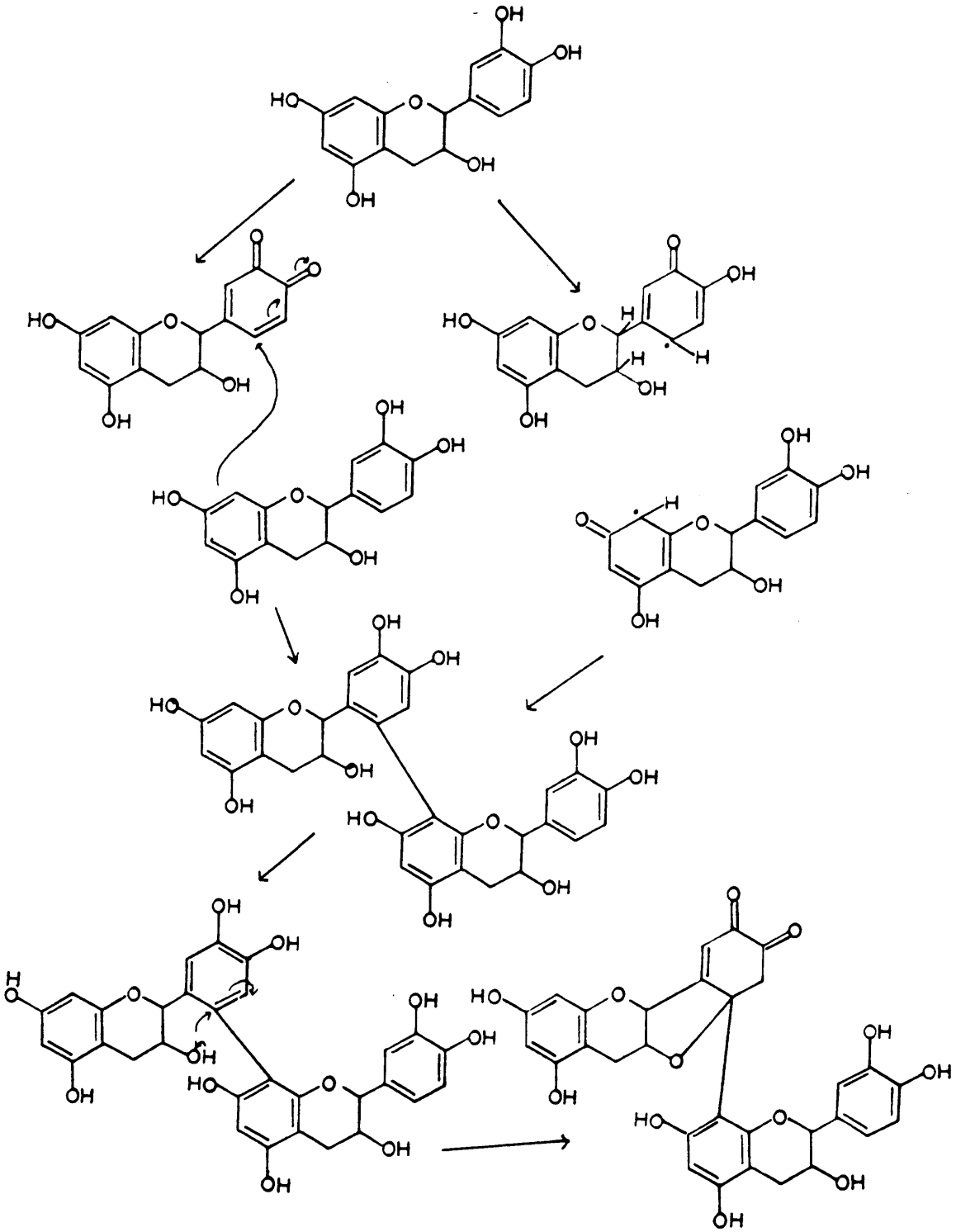
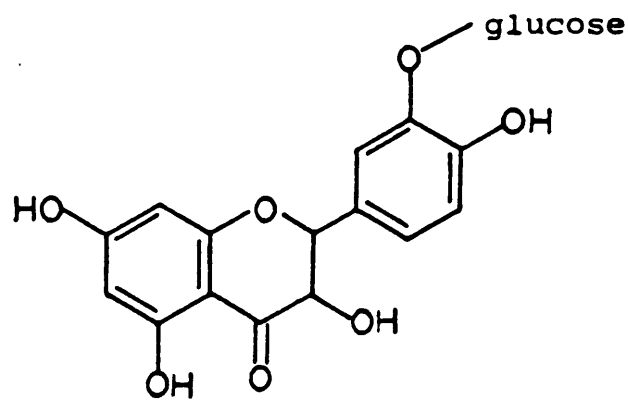


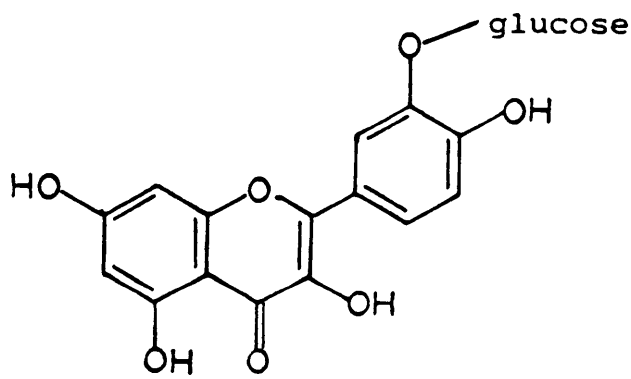
Figure 8. The mechanism of catechin oxidation.

fir bark contained a variety of useful chemicals, among which was dihydroquercetin [(2,3-trans)-3,3′,4′,5,7-pentahydroxyflavanonol]. Since its initial discovery in Douglas-fir heartwood (Pew, 1948; Graham and Kurth, 1949) and in the bark of Douglas-fir (Kurth et al., 1948; Hubbard and Kurth, 1949), dihydroquercetin has been reported to be present in the wood or bark of many other coniferous species, either as the aglycone (XII) or as the 3′-glucoside (XIV) or as the 3′-glucoside of quercetin (XV). (Hergert and Goldschmid, 1958). Several procedures for the isolation of dihydroquercetin from Douglas-fir bark have been developed (Hergert and Kurth, 1952; Kurth and Becker, 1953; Kurth and Chan, 1953; Kurth et al., 1955; Kurth, 1956; Roberts and Gregory, 1958).

Gardner and Barton (1960) studied the variation of dihydroquercetin between trees and within trees in Douglas-fir. They found that sapwood contained dihydroquercetin quantities that ranged from 0 to 0.56 precent. Several other investigators (Rhydholm, 1965; Squire et al., 1967; Hemingway and Hillis, 1970; Karchesy et al., 1976) have also studied Douglas-fir flavonoids.



XIV. 3'-O-glucoside of dihydroquercetin



XV. 3'-O-glucoside of quercetin

EXPERIMENTAL

1. Bark of Red Alder

1.1. Collection of Bark

Red alder (Alnus rubra Bong.) bark was collected from a freshly cut tree taken from McDonald Forest, Benton County, Oregon in May of 1985. The tree was approximately 11.2 cm diameter at breast height (DBH) and the tree was cut 30 cm high from the ground. The bark was stripped from the lower trunk. The bark was chopped prior to extraction.

1.2. Extraction

Chopped bark was extracted immediately with 100% ethanol to prevent stains from developing by enzymes. It was stored in a 5.0-liter flask for 3 weeks. The extracts were filtered through number 1 filter paper and evaporated under reduced pressure at 35-40^{o} C. A dark, paste like syrup was obtained.

1.3. Separation of Oregonin

1.3.1. Chromatography

1.3.1.1. Thin-Layer Chromatography

Extractives of red alder bark were chromatographed on silica-gel thin-layer chromatograms (TLC) (Merck, Silica-Gel 254F) with two different solvent systems: (1) acetone-ethyl acetate-water (10:10:1, v/v) (Terazawa et al., 1984a); (2) chloroform-methanol-acetic acid-water (85:15:10:4, v/v) (Karchesy, 1975).

Chromatographic detection methods used were: (A) 37% formaldehyde-sulfuric acid-water (11:5:5, v/v) (Sears and Casebier, 1968) spray followed by heating at 105^{o} C for a few minutes, (B) ultraviolet light (UV).

1.3.1.2. Enzymatic Detection of Color Precursors

Tyrosinase enzyme (Sigma Chemical Co.) was used to check the presence of color precursors. The enzyme tests were conducted in Sorensen's citrate II buffer solution prepared according to the Handbook of Biochemistry (J-234) as follows: 21.0 g of citric acid [1 H_2O] was dissolved in 200 ml of 1 N sodium hydroxide solution and diluted to 1000 ml in a volumetric flask. This provided a 0.1 M disodium

citrate solution (Solution A). An amount of 69.3 ml of solution A was added to 31.7 ml of a 0.1 N sodium hydroxide solution (Solution B) to give Sorensen's citrate II buffer solution with a pH of 5.6 as measured by a pH meter.

1.3.1.3. Column Chromatography

An aliquot of the syrup (section 1.2) was mixed with chloroform and filtered. The residue was dissolved in ethyl acetate and tested by TLC (solvent 1). The ethyl acetate solution was concentrated on a rotary evaporator and injected onto a Silica-Gel G-7 (J.T. Baker Chemical Co.)(<40 micrometer) column. It was chromatographed with a water-saturated ethyl acetate solution. Eluents were collected by a Gilson FC-100 automatic fraction collector in 5-ml aliquots. Every third aliquot was tested by silica-gel TLC (solvent 1) and where the results were identical the aliquots were combined. Two hundred individual aliquots were collected and 12 combined fractions were obtained. A small part (2.0 ml) of each of the 12 combined fractions was added to 5.0 ml of Sorensen's citrate II buffer solution in a 20.0 ml test tube and color-tested by adding one or two drops of tyrosinase enzyme solution which had been prepared by dissolving 4.0 mg of tyrosinase powder in 10.0 ml of Sorensen's citrate II buffer solution. Color changes were observed visually. A small part (2.0 ml) of each of the 12

combined fractions was set aside as a control sample. The results are shown in Table I.

The treated solutions changed color immediately, but the controls changed only after a lengthy exposure to light and air for about two weeks. Combined aliquots 80-94 and 95-156 (Table I) whose test samples became bright golden yellow immediately after treatment with tyrosinase were further analyzed by high performance liquid chromatography (HPLC).

1.3.2. Analyses of Oregonin by HPLC

1.3.2.1 Column and Equipment

Two chromatographic pumps (Gilson, Model 302) were used to pump the eluent solvents which were water and methanol. The detection system was a Gilson Model 111B ultraviolet absorption monitor. A linear recorder was used to give a recording spectrum of the absorptions. A prepacked column, reverse-phase C-18 (Waters), was used. The flow rates were automatically controlled by an Apple II microcomputer. Preparative HPLC of oregonin also used the same system except the column was a preparative, reverse phase C-18.

Table I. Color Test of Red Alder Extract.

Aliquot Numbers	Control[a]	Combined Aliquot Color[b]
1-14	no	no
15-22	no	no
23-36	no	no
37-47	no	no
48-61	no	trace yellow
62-67	pale yellow	pale yellow
68-76	pale yellow	pale yellow
77-79	yellow	yellow
80-94	bright golden yellow	bright golden yellow
95-156	bright golden yellow	bright golden yellow
157-180	pale red	pale red
181-200	pale yellow	pale yellow

a: No treatment with tyrosinase. The color of the control samples was observed after two weeks from sample preparation.

b: Combined aliquot color was observed immediately after the addition of tyrosinase.

1.3.2.2. Optimum Operating Conditions

The flow rate of solvents for qualitative analyses was 1 ml/min of 40:60 (v/v) methanol-water. A wavelength of 280 nm was used to detect phenolic compounds. The sensitivity of the detector was set at 0.2 AUFS and the chart speed was 2 mm/min.

The flow rates of solvents for preparative HPLC was 8 ml/min of 40:60 (v/v) methanol-water. A wavelength of 280 nm was used to detect oregonin. The sensitivity of the detector was set at 0.5 AUFS and the chart speed was 4 cm/hr. Combined aliquots 80-94 and 95-156 (Table I) were analyzed by HPLC. Two major peaks were collected and labeled Fraction I and Fraction II. These fractions were freeze-dried. The overall schematic diagram is shown in Figure 9.

1.4. Identification of Oregonin

Fraction I and Fraction II were tested by TLC with solvent systems (1) and (2) described in section 1.3.1.1. Fraction I and Fraction II showed a single spot on TLC. The R_f values were R_f (2) = 0.35 (system 2) and R_f (1) = 0.61 (system 1) for a single spot. The freeze-dried samples were dissolved in acetone-D_6 for proton and carbon-13 NMR spectroscopy. A Bruker FT 400 MHz NMR spectrometer was used. Found: proton NMR (acetone-D_6); 6.71-6.69 ppm (4H,

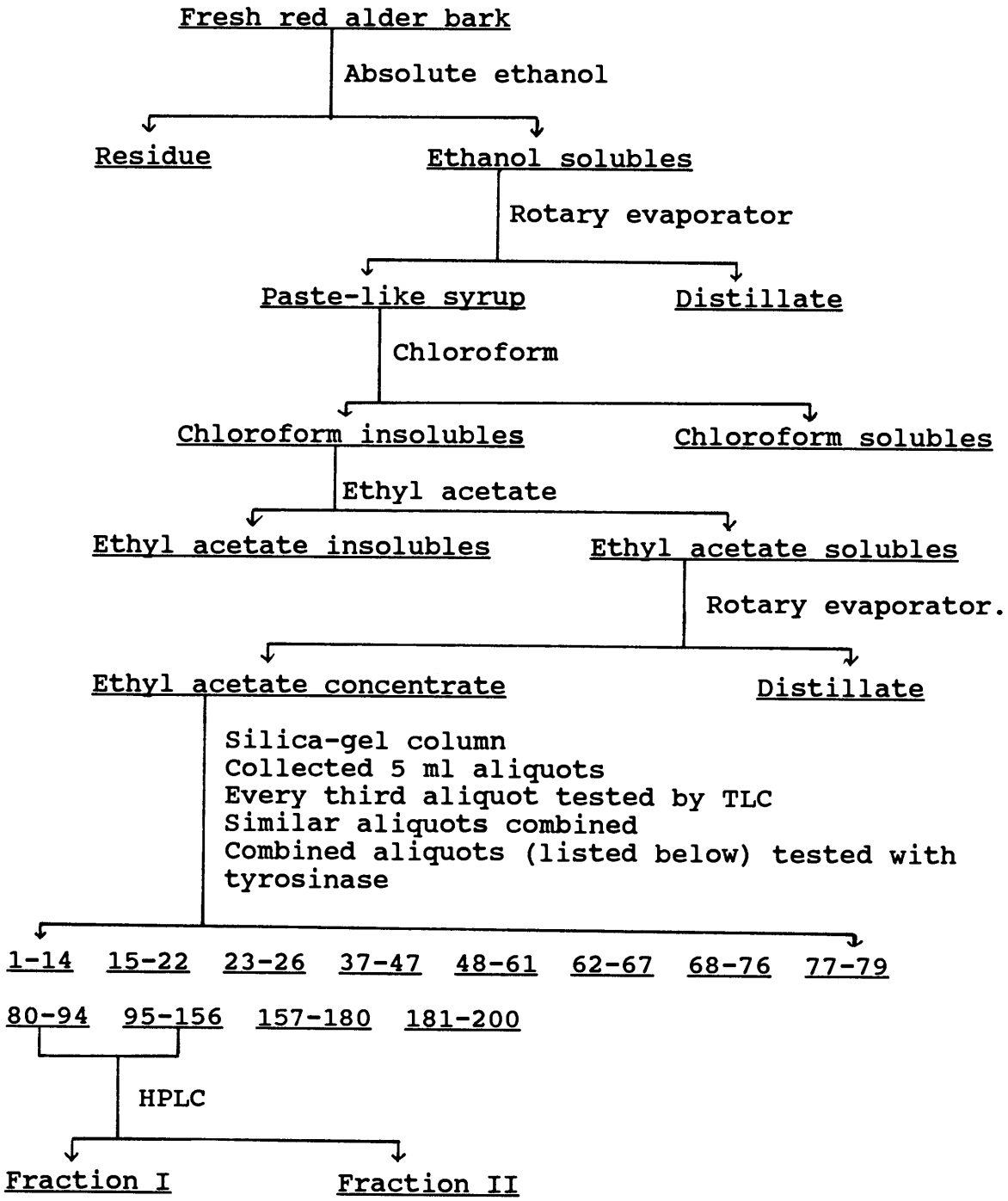


Figure 9. Isolation of oregonin.

dd, J = 2, 8 Hz, for C-2'and 5'), 6.50-6.52 ppm (2H, d, J = 2, 8 Hz, for C-6'), 4.07-4.14 ppm (1H, q, J = 7.2 Hz, for C-5), 2.50-2.83 ppm (8H, m, for C-1,2,4,and 7), 1.74-1.78 ppm (2H, dd, J = 3, 6 Hz, for C-6), 4.11-4.13 ppm (1H, ms, equatorial H for C-5"), 3.40-3.53 ppm (1H, ms, axial H for C-5"), 3.14-3.23 ppm (3H, m, for C-2", 3", and 4"), 4.31-4.33 ppm (1H, d, J = 8 Hz, for C-1"). carbon 13 NMR (acetone D_6); 145.63 and 145.66 ppm (2 C-4'), 143.74 and 143.94 ppm (2 C-5'), 133.84 and 133.85 ppm (2 C-1'), 115.92-120.38 ppm (6 C-2', 3' and 4'), 209.55 ppm (C-3), 77.57 ppm (C-5), 48.04 ppm (C-1), 46.00 ppm (C-7), 38.24 pp(C-4), 31.27 ppm (C-2), 29.25 ppm (C-6), 103.93 ppm (C-1"), 66.44 ppm (C-5"), 70.83 ppm (C-3"), 74.66 ppm (C-4"), 75.91 ppm (C-2").

2. Sapwood of Douglas-fir

2.1. Collection

A Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] tree was cut from McDonald Forest, Benton County, Oregon in January of 1985. The tree was approximately 23 cm DBH and the sapwood was cut from the upper trunk. The sapwood was freshly sawed without heartwood. The sapwood was passed through a Wiley mill to shred it prior to extraction.

2.2. Extraction

The shredded sapwood was added immediately to distilled methanol to prevent enzyme action. The extraction mixture was stored in a 6-liter flask for 3 weeks. The extracts were filtered through number 1 filter paper and evaporated on a rotary evaporator at 35-40^{o} C. A dark, red-colored paste-like material (EX-1) was obtained. The red colored material was washed into a separatory funnel with water and extracted with chloroform (3 times). The chloroform layer was separated (EX-1-A).

The residue was extracted with ethyl acetate. The ethyl acetate layer was reduced in volume under reduced pressure at 35-40^{o} C (EX-1-B).

The residue was dried on a rotary evaporator at 35-40^{o} C (EX-1-C). The schematic diagram of the extraction procedure is shown in Figure 10.

2.3. Color Test of Solubles with Tyrosinase

Aliquots (2.0 ml) of the methanol solubles (EX-1), the chloroform solubles (EX-1-A), the ethyl acetate solubles (EX-1-B), and the water solubles (EX-1-C) (Figure 10) were tested with tyrosinase enzyme solution and color changes were observed visually. Additional aliquots (2.0 ml) of each of these solutions were set aside as controls without tyrosinase addition. The results are given in Table II.

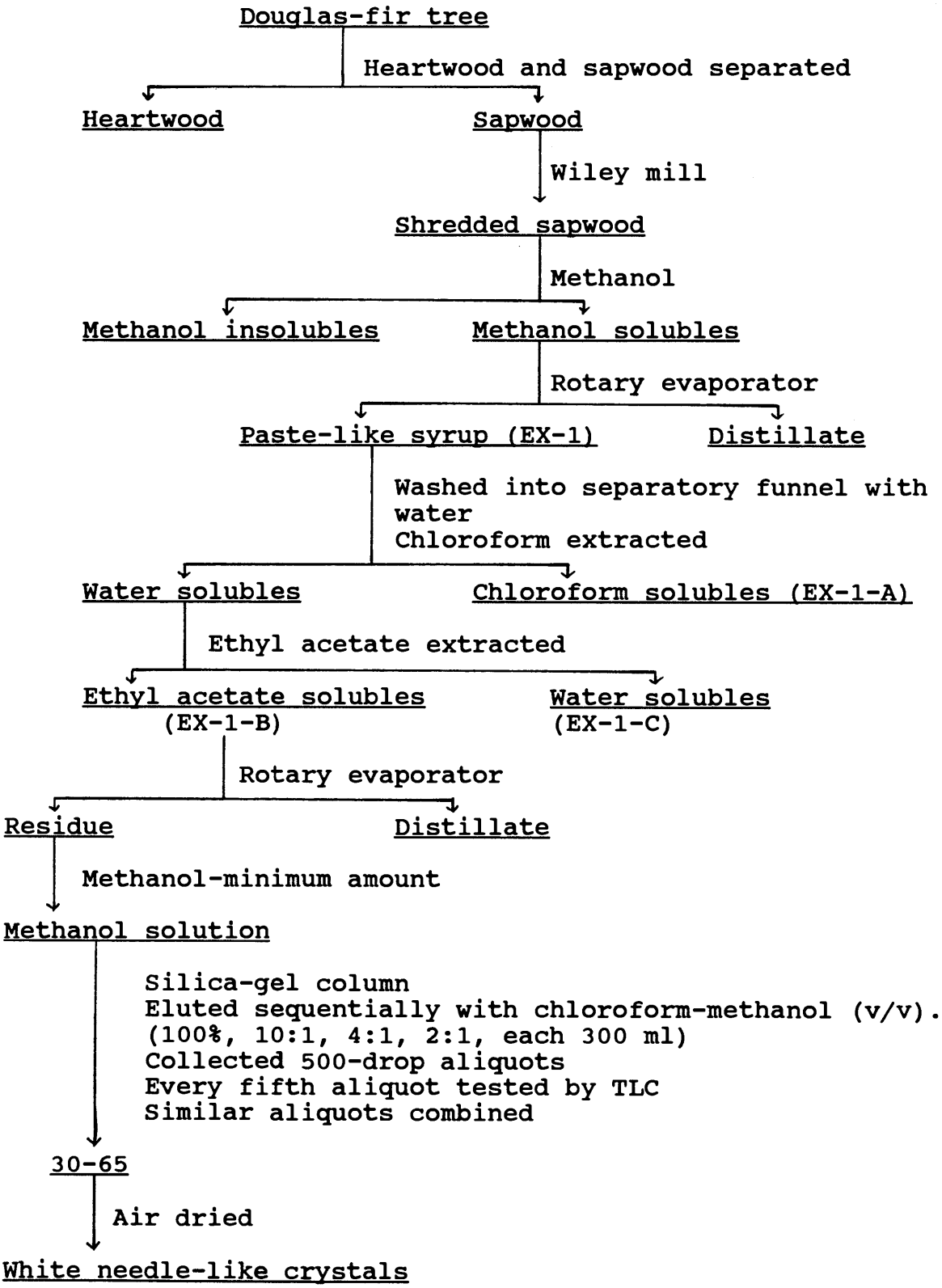


Figure 10. Isolation of dihydroquercetin.

Table II. Color Test of Douglas-fir Extract.

Fraction	Control#	Fraction Color*
methanol extract (EX-1)	orange	intensified orange color
chloroform extract (EX-1-A)	no color	no color
ethyl acetate extract (EX-1-B)	yellow	bright golden yellow
water solubles (EX-1-C)	orange	intensified orange color

#: No treatment with tyrosinase. The color of the control samples was observed after two weeks from sample preparation.

*: Tyrosinase treated samples developed color immediately.

2.3.1. Test of Inhibitors to Color Formation

Solutions of oregonin and Douglas-fir sapwood extract (EX-1, EX-1-B and EX-1-C, Figure 10) in Sorensen's citrate II buffer solution were examined for color inhibition by the addition of known inhibitors. The inhibitors ethylenedinitrillotetraacetic acid disodium salt (EDTA), phenylthiourea, mercaptobenzothiazole, and Chloramine-T were added as a few crystals of solid material. The procedure closely followed that of Terazawa et al. (1984b).

2.4. Separation of the Stain Precursors

2.4.1. Thin-Layer Chromatography

Silica-gel TLC plates (20X20 cm) (Merck) were precoated with silica-gel 60 F-254. Cellulose TLC plates (20X20 cm) were precoated with F 1440/LS 254 (Schleicher & Schuell Co.). Short ultraviolet wavelength was used for detection.

Solvent systems for silica-gel TLC were as follows;

(i). Ethyl acetate-chloroform-88% formic acid (6:3:1,v/v)

(ii). Benzene-methanol-acetic acid (45:8:4,v/v)

(iii). Benzene-ethyl acetate-88% formic acid (40:10:5,v/v)

Solvent systems for cellulose TLC were as follows;

(iv). t-Butanol-acetic acid-water (3:1:1,v/v)

(v) . 6% Acetic acid

2.4.2. Silica-Gel Column Chromatography

2.4.2.1. Packing Materials, Columns and Separations

Silica-gel 7G, (J.T.Baker Chemical Co.), was slurried with ethyl acetate. The slurried silica-gel was packed into a glass column (3X25 cm) by gravity. Each separation column was prepared with a new silica-gel slurry.

The methanol solubles (EX-1, Figure 10) were directly applied to the top of a silica-gel column after they were dissolved into a minimum amount of methanol. The column was developed with water-saturated ethyl acetate solution under gravity. Aliquots of 25 ml each were collected by an automatic fraction collector. A total of 20 aliquots was collected and every third aliquot was tested by TLC (system 1) and tyrosinase. A yellow-like color developed in aliquots 4-6 and 7 upon the addition of tyrosinase. Aliquots 4-6 and 7 showed different spots on TLC (system 1).

A second aliquot of EX-1 (Figure 10) was applied to the top of a silica-gel column after dissolution in a minimum amount of methanol. The acetone-ethyl acetate-water (10:10:1, v/v) solvent was eluted by gravity and the eluent was collected in aliquots of 300 drops (ca 2.5 ml) in tubes by an automatic fraction collector. A total of 275 aliquots were collected. Every fifth aliquot was tested by TLC

(system 1) and combined into fractions according to the separation patterns. The results are shown in Table III.

Fraction 2 containing aliquots 11-27 collected from the column (Table III) was extracted with diethyl ether. The diethyl ether soluble portion was rechromatographed on the same type of column. The solvent was sequentially eluted with chloroform, chloroform-methanol (4:1, v/v), and methanol. The chloroform eluent was tested by tyrosinase to check for a change of color. No color developed.

The chloroform-methanol (4:1, v/v) eluent was collected in samples of 500 drops and tested by TLC (system i) and tyrosinase. Samples numbered 10-35 reacted with tyrosinase immediately and strongly to give a brown-red color.

The methanol eluent was collected and tested with tyrosinase.

2.5. Isolation of Dihydroquercetin (DHQ)

2.5.1. Isolation

In the preliminary testing described above, the methanol solubles (EX-1, Figure 10) from Douglas-fir sapwood were dissolved in three different organic solvents, methanol, chloroform, and ethyl acetate (Figure 10). The chloroform solubles (Ex-I-A) were not investigated because

Table III. Color Test of Fraction EX-1 (Figure 10) (Dark Red Colored)[a]

Fraction Number	Aliquots Contained	Control[b]	Fraction Color[c]
1	1-10	no	no
2	11-27	no	bright golden yellow
3	28-51	no	no
4	52-63	no	no
5	64-80	no	no
6	81-275	no	no

a: Separated on a silica-gel column with acetone-ethyl acetate- water (10:10:1, v/v).

b: No treatment with tyrosinase.

c: Tyrosinase treated samples developed color immediately.

they showed no color formation with tyrosinase enzyme (Table II). The ethyl acetate solubles (Ex-I-B, Figure 10) were chromatographed on a silica-gel column by sequential elution with variable mixtures of chloroform-methanol (100%, 10:1, 4:1, 2:1, and 100 %, v/v, each 300 ml.)

EX-I-B (Figure 10) was dissolved in a minimum amount of methanol and placed on the top of a silica-gel column. The solvents were eluted by gravity and collected in aliquots of 500 drops. Two hundred such aliquots were collected. Every fifth aliquot was tested by TLC (system i) and combined according to the resulting pattern of spots. Aliquots 1-29 were not further investigated because they were chloroform soluble and thus the residues of EX-1-A (Figure 10). White crystals formed in the fraction composed of the original aliquots 30-65 on evaporation in air. The schematic diagram is shown in Figure 10.

2.5.2. Identification of Dihydroquercetin

The needle-like crystals (section 2.5.1, Figure 10) were examined by melting point, UV spectra, proton, and carbon-13 nuclear magnetic resonance (NMR) spectra according to methods published by Markham (1983). The melting point was 238-240^{o} C and was identical to that of authentic dihydroquercetin supplied by ICN. K&K Laboratories, Inc.. The literature value for the melting point of dihydro-quecetin is 240-242^{o} C (Kurth, 1956). There was no

reduction of melting point on admixture with authentic dihydroquercetin. The UV spectrometer used was a UV 265 FW (Shimadzu Co., Kyoto, Japan). A Bruker FT 400 MHz NMR spectrometer was used. Found: UV spectra; λmax (MeOH) 324.0 and 289.0 nm; (MeOH + NaOH) 326.0, 244.4 (sh) nm; (MeOH + $AlCl_3$) 381.4, 313.6 nm; (MeOH + $AlCl_3$ + HCl) 378.4, 310.6 nm; (MeOH + NaOAc) 326.6, 291.4 (sh) nm; (MeOH + NaOAc + H_3BO_3) 326.6 (sh), 291.4 nm. Proton NMR; 5.93 ppm (1H, d, J = 2 Hz, for 6-H), 5.97 ppm (1H, d, J = 2 Hz, for 8-H), 7.05 ppm (1H, d, J = 2 Hz, for 5'-H), 6.88 and 6.90 ppm (1H, dd, J = 2, 8 Hz, for 6-H'), 6.83 and 6.85 ppm (1H, d, J = 8 Hz, for 2'-H), 4.99 and 5.02 ppm (1H, d, J = 11.3 Hz, for 2-H), 4.58 and 4.61 ppm (1H, d, J = 11.3 Hz, for 3-H). Carbon 13 NMR; 198.11 ppm (C-4), 167.85 ppm (C-7), 165.0 ppm (C-5), 164.06 ppm (C-7), 146.51 ppm (C-4'), 145.69 ppm (C-3'), 129.67 ppm (C-1'), 120.75 ppm (C-6'), 115.77 and 115.66 ppm (C-2', 5'), 102.0 ppm (C-10), 96.98 ppm (C-6), 95.96 ppm (C-8), 84.42 ppm (C-2), 73.06 ppm (C-3).

2.6. Isolation of a Glycoside of Dihydroquercetin

2.6.1. Liquid Chromatography on Silica-Gel Columns

The water-solubles from the methanol extract of Douglas-fir sapwood (Fraction EX-I-C, Figure 10) were dissolved in a small amount of water. The sticky solution was stored in a separatory funnel for one day. The

precipitate that formed was removed by filtration and discarded. The filtrate was chromatograhed on silica-gel TLC (system i). The TLC chromatogram showed two major spots which were dihydroquercetin and an unknown.

The filtrate was evaporated on a rotary evaporator under reduced pressure. The sticky syrup that remained was dissolved in a minimum amount of methanol and added to the top of a silica-gel column. The column was sequentially eluted with solutions of chloroform-methanol (v/v) of 4:1 and 2:1, each 400 ml respectively (Figure 11).

The eluent was collected in 900 drop aliquots with an automatic fraction collector. Every fifth aliquot was tested by TLC (system i) and combined according to similar spot patterns. Aliquots 21-27 (Figure 11)contained a small amount of quercetin and a major amount of dihydroquercetin.

Aliquots 28-32 (Figure 11) showed an unknown spot on TLC. The aliquots were combined and chromatographed on preparative silica-gel TLC with chloroform-ethyl acetate-88% formic acid (6:3:1 v/v) by multiple development (two times). There were two strips collected by extraction with methanol. This method was not suitable for their separation and was not further investigated.

2.6.2. Liquid Chromatography on Sephadex LH-20 Columns

A glass column (2.6X60 cm), manufactured by Kontes Glass Co., was slurry-packed with Sephadex LH-20 (Sigma Co.) after the packing material had soaked in 95% ethanol for 24 hr. The 95% ethanol eluent was pumped at a rate of 2.8 ml/min with an FMI Lab pump (Model RP-SY) manufactured by Fluid Metering Inc.. The eluent was monitored by a UV detector (Gilson Co., Model 111B) at 280 nm wavelength. The recorder was manufactured by Linear Co.

The combined aliquots 28-32 (Figure 11) from the silica-gel column were chromatographed on the Sephadex LH-20 column (Figure 11). The eluent was collected according to the peaks indicated by the UV monitor. Six peaks were collected. Each peak was tested by TLC (system i). Peak IV (Figure 11) contained the major component. It was rechromatographed on the same column to eliminate residual impurities. After the eluent was collected the sample was tested by TLC (system i). The TLC chromatogram showed only one spot with an R_f=0.39. The sample was freeze-dried. The overall schematic diagram is shown Figure 11.

2.6.3. Identification

A part of the freeze-dried sample (section 2.6.2, Figure 11) (2.0 mg) was hydrolyzed by heating at 100^{o} C in 2

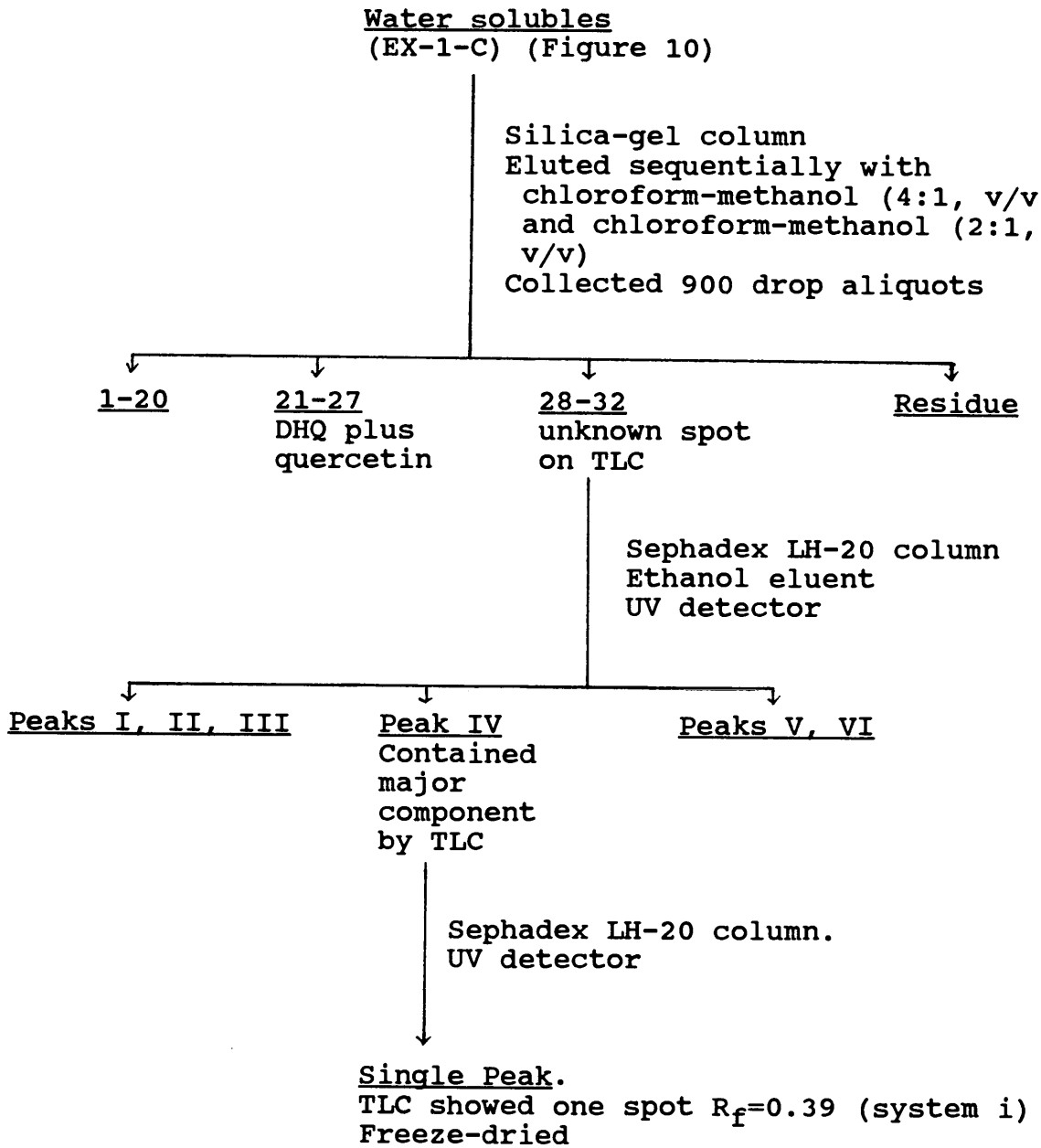


Figure 11. Isolation of dihydroquercetin glycoside.

N hydrochloric acid-ethanol (1:1, v/v, 10 ml) in a stoppered tube (Harborne, 1965). The hydrolyzate was extracted with ethyl acetate in a separatory funnel. The ethyl acetate layer was chromatographed on silica-gel TLC (system i) and cellulose TLC (systems iv and v). The TLC's showed a single spot which migrated the same distance as authentic dihydroquercetin. The water layer was neutralized with a small amount of ammonium hydroxide, then taken to dryness on a rotary evaporator. The dried water layer was dissolved in a minimum amount of methanol to eliminate ammonium chloride salts. The methanol solubles were chromatographed on paper and cellulose TLC (ethyl acetate-pyridine-water, 8:2:1, v/v) with authentic samples of D-glucose, D-mannose, D-galactose, D-xylose and L-arabinose. The chromatograms were sprayed with aniline hydrogen phthalate indicator (1.66 g of phthalic acid dissolved in 100 ml of water-saturated n-butanol containing 0.93 g of aniline) and showed the presence of glucose only.

A second portion of the freeze-dried sample (section 2.6.2, Figure 11) was dissolved in acetone D_6 for NMR. A UV spectrum was also obtained in methanol.

The UV spectra, proton and carbon 13 NMR spectra showed that the material was a glycoside of dihydroquercetin. Found: UV spectra, λmax, (MeOH) 324.6 (sh), 290 nm; (MeOH + NaOH) 325.0, 246.0 nm; (MeOH + $AlCl_3$) 378.6, 313.6, 280 (sh) nm; (MeOH + $AlCl_3$ + HCl) 378.0, 312.7, 282 (sh) nm; (MeOH + NaOAc) 325.2, 286.0, 250 (sh) nm; (MeOH + NaOAc + H_3BO_3)

325.0 (sh), 291.0 nm. Proton NMR: 7.36 ppm (1H, d, J = 2 Hz, for 5'-H), 7.09 and 7.07 ppm (1H, dd, J = 2, 8 Hz, for 6'-H), 6.90 ppm (1H, d, J = 2 Hz, for 2'-H), 5.93 ppm (1H, d, J 2 Hz, for 6-H), 5.97 ppm (1H, d, J = 2 Hz, for 8-H), 4.99 and 5.02 ppm (1H, d, J = 11.3 Hz, for 2-H), 4.85 ppm (1H, d, J=7.4 Hz, for 1"-H), 4.58 and 4.61 ppm (1H, d, J = 2 Hz, for 3-H), 3.89 ppm (1H, d, J=11 Hz, for 3"-H), 3.67 ppm (1H, dd, J=5,12 Hz, for 5"-H), 3.39-3.57 ppm (4H, m, for 2", 4", 6"-H). Carbon-13 NMR: 198.10 ppm (C-4), 168.47 ppm (C-7), 164.94 ppm (C-5), 164.13 ppm (C-9), 148.83 ppm (C-4'), 146.31 ppm (C-3'), 129.85 ppm (C-1'), 124.65 ppm (C-6'), 118.23 ppm (C-2'), 116.99 ppm (C-5'), 103.79 ppm (C-1"), 101.74 ppm (C-10), 97.44 ppm (C-6), 96.40 ppm (C-8), 84.61 ppm (C-2), 78.04 ppm (C-3"), 77.42 ppm (C-5"), 74.69 ppm (C-2"), 73.33 ppm (C-3), 71.30 ppm (C-4"), 62.40 ppm (C-6").

3. Preparation of Dihydroquercetin from Douglas-fir Bark

3.1. Bark Extraction

A dihydroquercetin rich sample of bark material, supplied by Weyerhaeuser Company, was slurried with chloroform and filtered. The residue was slurried with ethyl acetate and filtered. The ethyl acetate filtrate was dried in the hood. A dark brown-red paste-like material resulted (Figure 12).

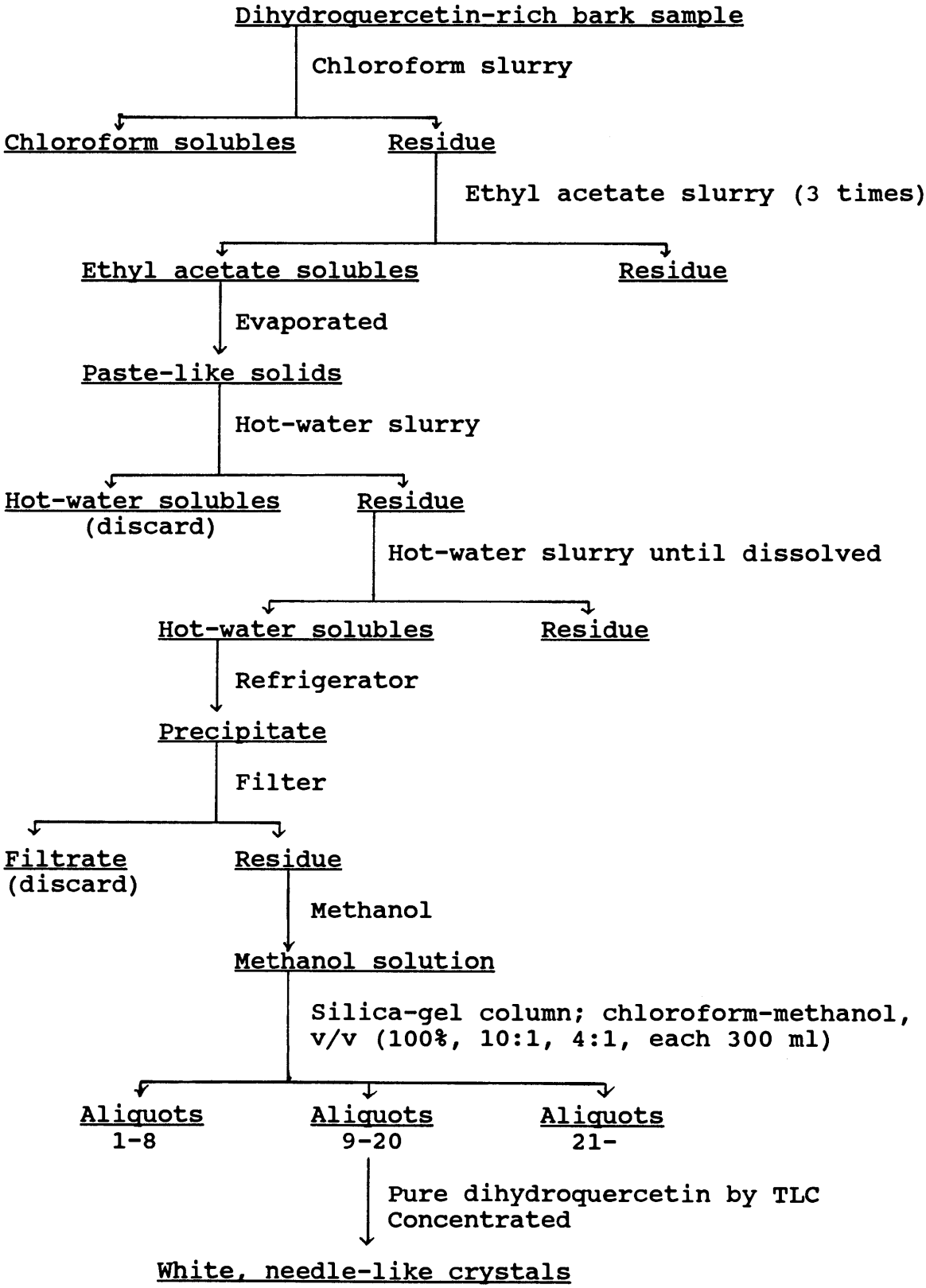


Figure 12. Crystallization of dihydroquercetin.

3.2. Purification of Dihydroquercetin

The dried ethyl acetate soluble material (Figure 12) was treated with hot water on a steam bath. A rich colored portion dissolved initially and a white colored solid remained on the bottom of the flask. The colored solubles were decanted and discarded. The remaining solids were treated with a fresh addition of hot water. The hot water solubles were recovered as a filtrate and additional hot water was added to the solids. This procedure was repeated until all of the solids had dissolved in hot water. The hot water solutions (with the exception of the initial highly colored solution) were combined and stored in the refrigerator. A yellow precipitate formed which was recovered by filtration. TLC (system i) showed this material to be rich in dihydroquercetin.

3.3. Crystallization of Dihydroquercetin

The dried precipitate described above (Figure 12) was dissolved in a minimum amount of methanol and loaded on a silica-gel column (4X30 cm) which was slurry-packed with Silica-Gel 7G (J.T. Backer Co.). The Silica-Gel 7G had been previously soaked in chloroform-methanol (10:1, v/v). The column was eluted sequentially with solutions of chloroform-methanol (v/v) (100%, 10:1, 4:1, each 300 ml) and the eluent was collected by an automatic fraction collector at the rate

of an aliquot every hour. Aliquots 9-20 (Figure 12) showed pure dihydroquercetin by TLC (system i). After aliquots 9-20 were reduced in volume in a dark hood, needle-like crystals appeared. The liquid above the needles was decanted and the crystals were collected. The white needle-like crystals had a mp of 238-240^{o} C. TLC (system i) of the crystals showed a single spot that moved the same distance as dihydroquercetin on cochromatography. A schematic diagram is shown in Figure 12.

4. Reaction of Dihydroquercetin with Tyrosinase

4.1..Reaction of Dihydroquercetin with Tyrosinase

An amount of 5.0 g of dihydroquercetin which had been isolated from Douglas-fir bark (Figure 12) was dissolved in 40 ml of acetone in a 500 ml flask. An amount of 320 ml of distilled deionized water was added. Tyrosinase (12 mg) was dissolved in 32.0 ml of Sorensen's citrate II buffer solution of pH 5.6 (Handbook of Biochemistry, J-234) and added to the dihydroquercetin solution. An amount of 4.0 ml of 3.0% hydrogenperoxide was added to the reaction flask. The same amount of tyrosinase (12.0 mg) and hydrogenperoxide solution (4.0 ml) were added every day and the reaction was stirred continuously. Because of the low solubility of

dihydroquercetin in water, 4.0 ml of acetone was added every second day.

The color of the reaction changed from colorless to brown with time and a brown precipitate formed. After 30 days the reaction was stopped because all of the dihydroquercetin had disappeared as analyzed by TLC (system i).

The Sorensen's citrate II buffer solution used above was prepared as follows: 21.0 g of citric acid [1 H_2O] was dissolved in 200 ml of 1 N sodium hydroxide solution and diluted to 1000 ml in a volumetric flask. This provided a 0.1 M disodium citrate solution (Solution A). An amount of 69.3 ml of solution A was added to 31.7 ml of a 0.1 N sodium hydroxide solution (Solution B) to give Sorensen's citrate II buffer solution with a pH of 5.6 as measured by a ph meter.

4.2. Separation of Reaction Products

The mixture resulting from the reaction of dihydroquercetin and tyrosinase was separated by filtration with Whatman Number 1 filter paper. The filtrate was removed for further investigation. An attempt was made to dissolve the residue by passing methanol through the residue on the filter paper. The methanol solution that passed through was a bright red color (F-1). The residue that

remained was methanol insoluble and was dark brown in color (R) (Figure 13).

The filtrate from the initial filtration was reduced in volume under reduced pressure at 35-40° C, then extracted with ethyl acetate (6 times with 200 ml) in a separatory funnel. The ethyl acetate layer was dried with sodium sulfate powder and concentrated to dryness on a rotary evaporator at 35-45° C. The dried material was redissolved in methanol (F-2) (Figure 13). The water layer was evaporated of residual ethyl acetate (F-3) (Figure 13).

4.2.1. Gel-Permeation Chromatography

Four different types of packing material were used to separate and to analyze the reaction mixture of dihydroquercetin and tyrosinase.

For analytical purpose a 100 angstrom micro-styragel prepacked column and a 500 angstrom phenogel prepacked column were connected in tandem with the micro-styragel column (Waters) in front followed by the phenogel column (Waters). The eluent solvent for these columns consisted of N,N-dimethylformamide (DMF) to which was added 3.0 ml of ammonium formate per 1.0 liter of DMF to accelerate the ionizing power. The solvent was stirred continuously overnight, filtered and degased by vacuum. The eluent was pumped through the columns by a Model 6000A pump (Waters) at a flow rate of 1.0 ml/min.

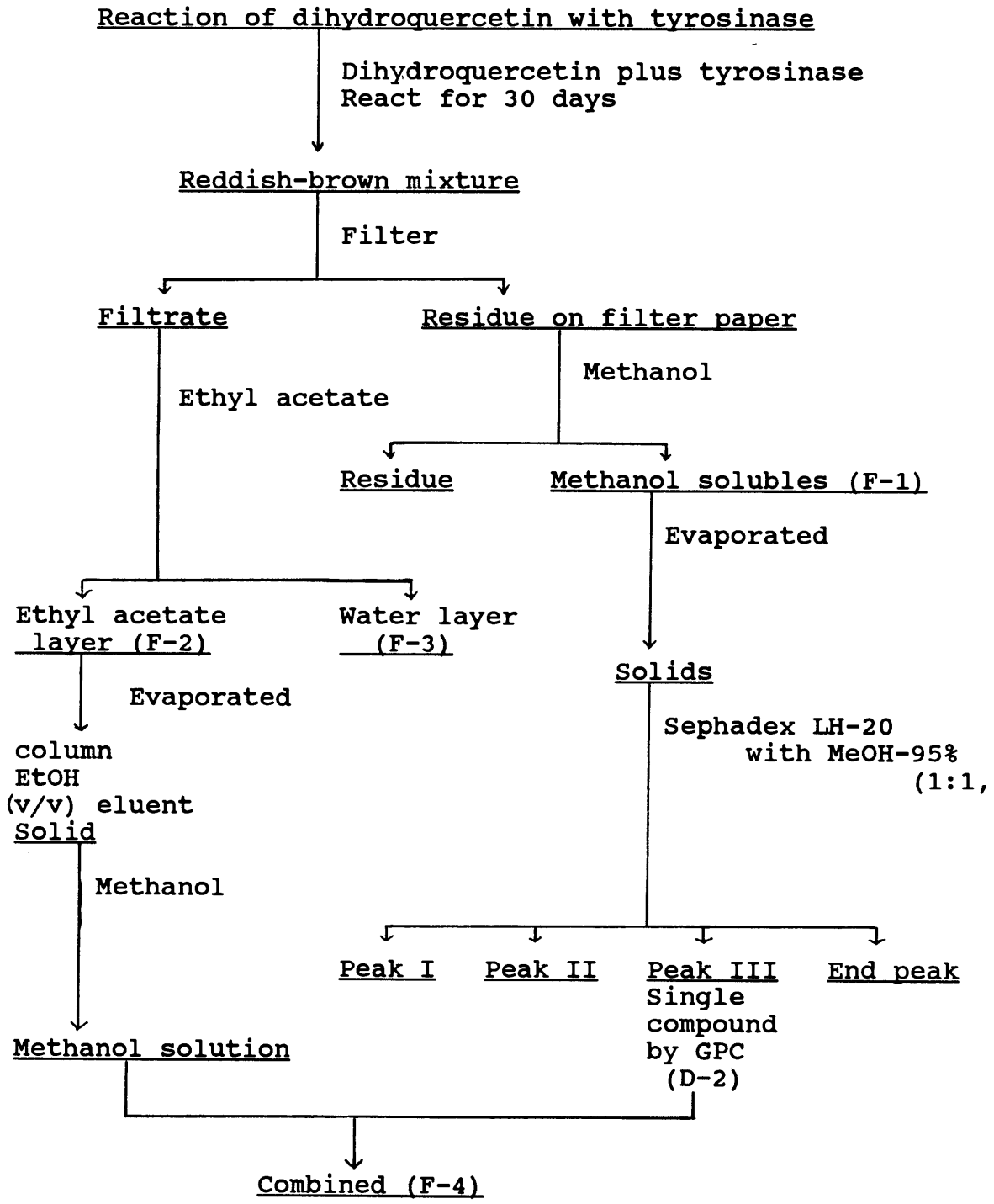


Figure 13. Reaction of dihydroquercetin with tyrosinase (continued on next page).

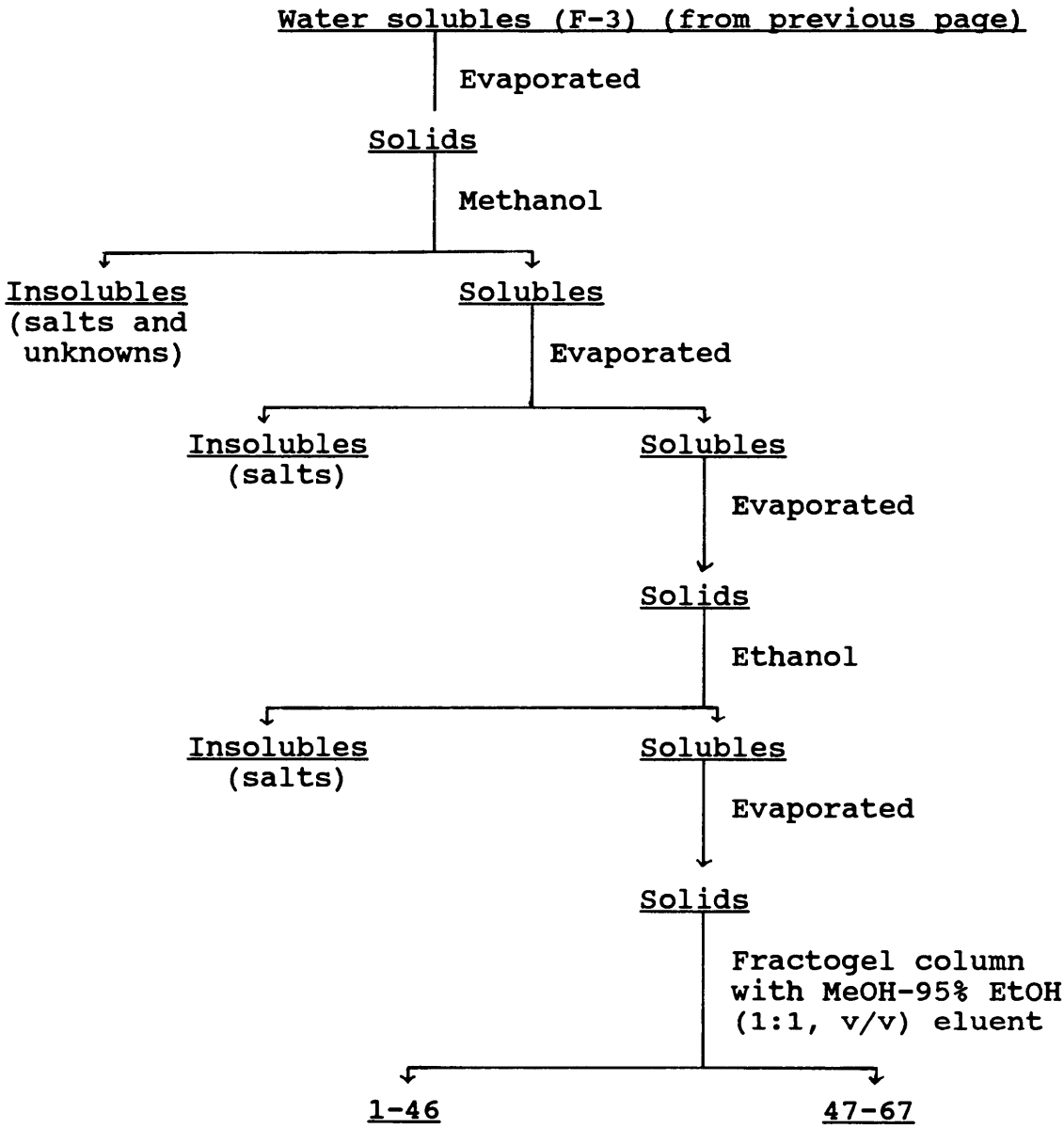


Figure 13 (continued). Reaction of dihydroquercetin with tyrosinase (continued on next page).

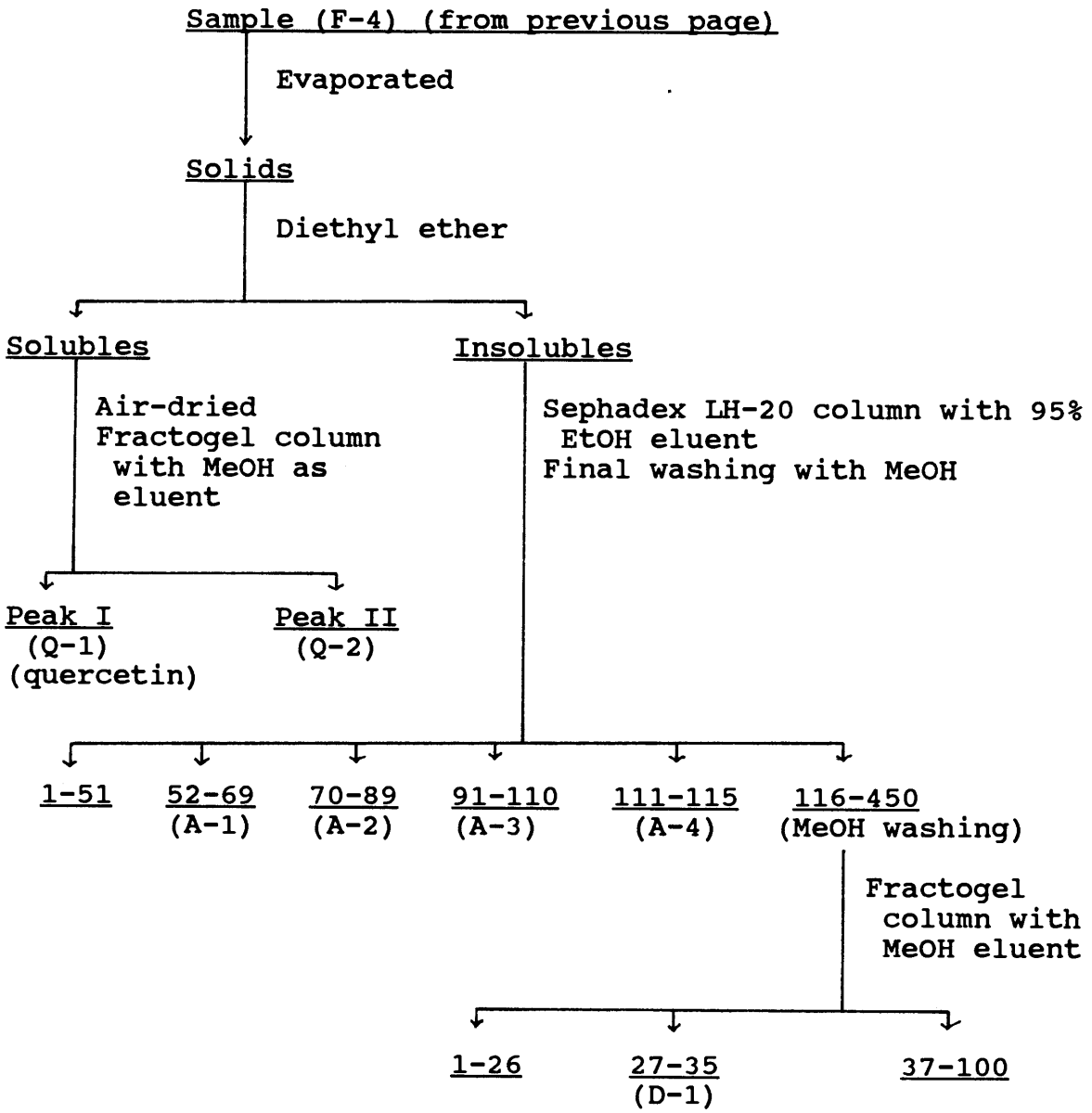


Figure 13 (continued). Reaction of dihydroquercetin with tyrosinase.

The detection system was a Gilson two wavelength ultraviolet absorption monitor (Model 111B). The 280 nm wavelength was used. The sensitivity of the detector was set at 0.5 AUFS. A Spectra-physics SP4200 integrator with the attenuation set at 8 was used to provide recorded spectra of the absorptions. This system was used to trace the reaction products of dihydroquercetin and tyrosinase and to analyze the eluent aliquots from the larger preparative columns. The system will be referred to as the GPC analytical system.

For preparative purposes, Sephadex LH-20 (Sigma Co.) and Fractogel TSK-HW-40F supplied by EM Science were used as packing materials. The Sephadex LH-20 column (8 X 60 cm) was eluted with 95 % ethanol with a 1.0 ml/min flow rate to separate low molecular weight compounds (150 MW). The eluent was collected with an automatic collector (Model FC-100), by Gilson Co.. The Fractogel TSK-HW-40F column (1.5 X 60 cm) was used to separated the medium range molecular weight compounds (MW 300-600) with methanol-95 % ethanol (1:1, v/v) and methanol as solvents. The flow rate was 0.5 ml/min. The eluent was collected by an automatic collector as before. The solvents were pumped by a Model M-45 Waters pump.

The samples, F-1, F-2, and F-3 (Figure 13) were initially analyzed on the GPC analytical system. The remainder of F-1 (Figure 13) was dried on a rotary evaporator and dissolved in 10 ml of methanol-95 % ethanol

(1:1, v/v). The prepared sample was chromatographed on a Sephadex LH-20 column with methanol-95% ethanol (1:1, v/v) as eluting solvent. The eluent was monitored by a Gilson 111B UV detector and collected according to the peaks which appeared. Each peak was analyzed by the GPC analytical system. Peak III (D-2, Figure 13) was shown to contain primarily one compound on analyses by the GPC analytical system. It had a 14.88 min retention time.The other peaks contained mixtures.

The other mixtures were concentrated and combined with F-2, the ethyl acetate soluble materials (Figure 13), because of the similarity of their GPC spectra. These combined samples were labeled F-4 (Figure 13). After evaporating the ethyl acetate, the combined, dried sample was extracted with 30 ml of diethyl ether. The insoluble portion was dissolved in 10 ml of 95 % ethanol, loaded on a Sephadex LH-20 column and eluted with 95% ethanol solvent. The eluent was collected with an automatic collector in 650 drop aliquots. Final washing of the column was with methanol (aliquots 116-450). Every fifth aliquot was analyzed by the GPC analytical system and combined according to the similarity of the results. The combined aliquots were aliquots 52-69 (A-1), aliquots 70-89 (A-2), aliquots 91-110 (A-3), aliquots 111-115 (A-4), and methanol washings (Figure 13). Fractions, A-1, A-2, A-3, and A-4, were freeze-dried and analyzed by UV, IR and NMR spectroscopy.

The methanol washings (Figure 13) were rechromatographed on a Fractogel column with methanol as the solvent. The eluent was collected with an automatic collector in aliquots of 350 drops. Aliquots 1-26 contained residual low molecular weight compounds. Aliquots 27-35 contained one compound (D-1) which had a 15.13 min retention time on the GPC analytical system. After aliquot 36, the aliquots showed several other compounds which were only in trace amounts. They were detected on the GPC analytical system only and amounted to less than 2.0 mg. The rest of the compounds were of higher molecular weight, mainly trimeric compounds as indicated by the GPC analytical system and were not separated.

The diethyl ether solubles (Figure 13) were chromatographed on a Fractogel column with methanol as an eluent. The eluent was collected according to the peaks detected by a UV detector at 254 nm wavelength. There were two peaks, Peak I and Peak II. These were analyzed by the GPC analytical system. After evaporation of the solvent, Peak I was dissolved in diethyl ether and dried in a vacuum desiccator (Q-1). After evaporation of the solvent, Peak II was dissolved in water and freeze-dried (Q-2) (Figure 13).

The water-soluble portion, F-3 (Figure 13), was dried on a rotary evaporator at 35-40^{o} C. The dried material, which has mostly salts and higher molecular weight compounds, was extracted with methanol. The methanol-soluble portion was dried, then extracted with absolute

ethanol to eliminate the salts. This was repeated three times. The ethanol-soluble material was chromatographed on a Fractogel column with methanol-95 % ethanol (1:1, v/v) as eluent. The eluent was collected by an automatic collector in aliquots of 400 drops. Aliquots 47-67 contained one chromatographically pure compound which had a 14.06 min retention time on the GPC analytical system (D-3). The overall schematic diagram is shown in Figure 13.

4.3. Identification of Reaction Products

Sample R, which remained on the filter paper in Figure 13, was tested in various solvents, including benzene, chloroform, ethyl acetate, acetone, methanol, and water, to test its solubility. The residue R did not dissolved in any of the solvents and the dried residue had a blackish color. The residue was examined by IR and reflectance UV spectroscopy after it was completely dried. The samples for UV and IR were prepared in potassium bromide pellets. The spectroscopic data were as follows;
Found: IR νmax (KBr); 3460, 3380, 2924, 2366, 1649, 1536, 1516, 1450, 1390, 1250, 1157, 699 cm^{-1}. reflectance UV λmax (KBr); general reflectance in visible region.

Stained Douglas-fir sapwood was also investigated by IR and reflectance UV after the stained surface was scraped with a razor blade. The samples for spectroscopic analyses were made with potassium bromide pellets. Found: IR νmax

(KBr); 3460, 3380, 2924, 2366, 1728, 1640, 1516, 1450, 1370, 1270, 1157, 1065, 699 cm^{-1}. reflectance UV λmax (KBr); general absorption in visible region.

The samples, A-1, A-2, A-3 and A-4 (Figure 13) were dissolved in acetone-D_6 for NMR spectroscopic analyses. A Bruker FT 400 MHz NMR spectrometer was used for analyses. The NMR spectra of the samples revealed that A-2 and A-3 were mixtures of A-1 and A-4. A-1 also contained a reasonable amount of A-4. The compound 3,4-dihydroxybenzoic acid was found to be in A-4 as analyzed by two dimensional cellulose TLC with authentic 3,4-dihydroxybenzoic acid (solvent iv and v). The retention time of 15.98 min was the same as authentic 3,4-dihydroxybenzoic acid by the GPC analytical system. The results of the analyses were as follows: Found: 3,4-dihydroxybenzoic acid, UV λmax (MeOH); 291.8 nm, 257.0 nm. IR νmax (KBr); 3423.4, 3254.7, 1693.7, 1693.7, 1609.4,1525.0, 1447.6, 1370.3, 1293.0, 1236.7, 1194.5, 1117.2, 1089.1, 941.41, 772.66, 625.0 cm^{-1}. Proton NMR; 7.51 ppm (1H, d, J=1.8 Hz for 2-H), 7.45-7.47 ppm (1H, d.d, J=2, 8 Hz for 6-H), 6.89 ppm (1H, d, J=8 Hz for 5-H). Carbon-13 NMR; 167.62 ppm (C-7), 150.73 ppm (C-4), 145.57 ppm (C-3), 123.57 ppm (C-1), 123.11 ppm (C-6), 117.47 ppm (C-2), 115.67 ppm (C-5).

3,4-Dihydroxyphenylacetic acid, UV λmax (MeOH); 288.6 nm, 259.2 nm. IR νmax (KBr); 3423.4, 3254.7, 1707.8, 1679.7, 1609.4, 1525.0, 1447.6, 1405.0, 1384.4, 1293,0, 1282.5, 1250.8, 1201.6, 1117.2, 764.6, 625.0 cm^{-1}. Proton

NMR; 6.81 ppm (1H, d, J=1.8 Hz for 5-H), 6.7 ppm (1H, d, J=8 for 2-H), 6.6 ppm (1H, d.d, J=1.7, 8 Hz for 6-H). Carbon-13 NMR; 173.7 ppm (C-8), 146.69 ppm (C-3), 144.77 ppm (C-4), 127.25 ppm (C-1), 121.49 ppm (C-1), 117.23 ppm (C-5), 115.89 ppm (C-2), 40.83 ppm (C-7).

The sample, Q-1 (Figure 13) was dissolved in methanol-D4 and analyzed on a Varian FT-80 MHz NMR spectrometer. The proton NMR spectrum of quercetin (Q-1) (Figure 13) which was the major compound of the reaction products was identical with Markham's result (1975) and gave the same spot with authentic quercetin by TLC (solvent i).

Quercetin. Proton NMR; 7.7 ppm (1H, d.d, J=2,8 Hz for 6'-h), 7.58 ppm (1H, d, J=2 Hz for 2'-H), 6.85 ppm (1H, d, J=12 for 5'H), 6.83 ppm (1H, d, J=2 Hz for 8-H), 6.2 ppm (1H, d, J=2 Hz for 6-H).

The unidentified quercetin-type dimeric compound, Q-2 (Figure 13), was analyzed on the Varian FT 80 MHz spectrometer. The sample of Q-2 changed in the NMR tube for some unknown reason during the week of waiting to run the 400 MHz spectra. After testing on the Bruker FT 400 MHz spectrometer, it was found that the compound which had a 14.34 min retention time on the GPC analytical system was changed. After analyses by NMR spectroscopy, the sample was again analyzed on the GPC analytical system. The chromatogram showed that four major compounds had resulted from the original compound Q-2 (Figure 13).

Found: Proton NMR; 7.9 ppm (1H, m for H-6′), 7.82 ppm (1H, m for H-2′), 6.65 ppm (1H, dd for H-5′), which are the B ring protons of the monomeric quercetin unit, 7.2 ppm (1H, m for H-5′), 7.1 ppm (1H, m for H-6′), 7.0 ppm (1H, m for H-2′), which are the B ring protons of a monomeric dihydroquercetin-type of unit, 6.35 ppm (1H, dd, for H-8), 6.15 ppm (1H, dd, for H-6), which are the A ring protons of a monomeric quercetin-type unit, 5.75 ppm (1H, s for either H-8 or H-6), which are A ring protons of a monomeric dihydroquercetin-type unit.

The other separated samples, D-1, D-2 and D-3 (Figure 13) were not investigated further.

4.4 The Reaction of Dihydroquercetin with Tyrosinase on Douglas-fir Sapwood.

Douglas-fir 2X4 inch lumber without stains on the surface was used as a testing material. Dihydroquercetin (1 mg) was dissolved in 10 ml of methanol. This was uniformly sprayed on the surface of the sapwood and dried at room temperature. Tyrosinase (0.4 mg) was dissolved in 10 ml of distilled, deionized water. After the dihydroquercetin solution had completely dried the tyrosinase solution was sprayed on top of it on the wood. The color of the wood surface became brown after 30 minutes. The wood surface was periodically moistened with a water spray, and the color intensified with time. In a parallel experiment tyrosinase

solution was sprayed on the surface of wood pieces which had not been previously treated with dihydroquercetin. The color of the wood surfaces did not change to any great extent, but did become slightly greenish-grey in color after one day. It was found that the color of this sample was somewhat intensified after three weeks. The pieces that had been treated with dihydroquercetin only were observed at the same time. There was no change in the surface color.

RESULTS AND DISCUSSION

1. Bark of Red Alder

The purpose of this work was to isolate and identify oregonin, the known precursor in the red alder staining phenomenon. The objective was to compare oregonin to the possible precursors of the Douglas-fir sapwood stain.

1.1. Collection of Bark

The bark samples were collected from a freshly fallen tree to reduce chances of contamination and decomposition from organisms and enzymes. The samples were collected in the spring of the year when it was easy to separate the bark from the wood.

1.2 Extraction

Generally, acetone, methanol or ethyl acetate extracts are prepared by cold solvent percolation of crushed plant material (Haslam, 1966). Karchesy (1975) had found that oregonin was extracted into acetone. However, it was difficult to collect the bark without change of the constituents because the bark easily turned dark red after exposure to air. In view of the known reactivity of some of

the polyphenol oxidases, more convenient extraction conditions were practiced. In the present work red alder bark which had been freshly stripped from the wood was extracted with absolute ethanol. Chloroform was used to remove less polar materials from the ethanol extract.

1.3 Separation of Oregonin

Karchesy (1975) isolated oregonin by partition between water and ethyl acetate followed by methylation. It was necessary in the present work to isolate pure oregonin without derivatization in order to test it with an enzyme for color reactions. There are two papers (Terazawa et al., 1984a; Hrutfiord and Luthi, 1982) which have reported methods that do not require derivatization. In the present work, the method used by Hrutfiord and Luthi (1982) was used.

1.3.1 Chromatography

1.3.1.1 TLC Chromatography

It had been established that oregonin was separated from other compounds by silica-gel TLC with acetone-ethyl acetate-water (10:10:1, v/v) (Terazawa et al., 1984a). Karchesy (1975) also reported that silica-gel TLC was good for separating oregonin from other compounds with

chloroform-methanol-acetic acid-water (85:15:10:4, v/v) as solvent.

Detection of phenolic compounds on TLC chromatograms is commonly achieved by various spray reagents or with ultraviolet light. Because oregonin contains two catechol groups, 37% formaldehyde-sulfuric acid-water (11:5:5, v/v), and ultraviolet light were used.

The major spot on TLC (Figure 14) was oregonin, exactly as reported by Karchesy (1975) and Terazawa et al. (1984a).

1.3.1.2. Enzymatic Detection of Color Precursors

Tyrosinase enzyme was selected to trace the presence of color precursors. The precursors to color formation were usually colorless and an indicator method was needed to tell if they might change to colored materials. Tyrosinase is a catechol oxidase enzyme known to oxidize catechol groups to ortho-quinones as shown in Figures 2, 4 and 5. Terazawa et al (1984a, b) used these enzyme systems very effectively to trace and identify the color precursors in the genus Alnus. If buffered solutions of compounds that contained catechol groups were treated with tyrosinase enzyme, a color, usually yellowish to red, developed immediately.

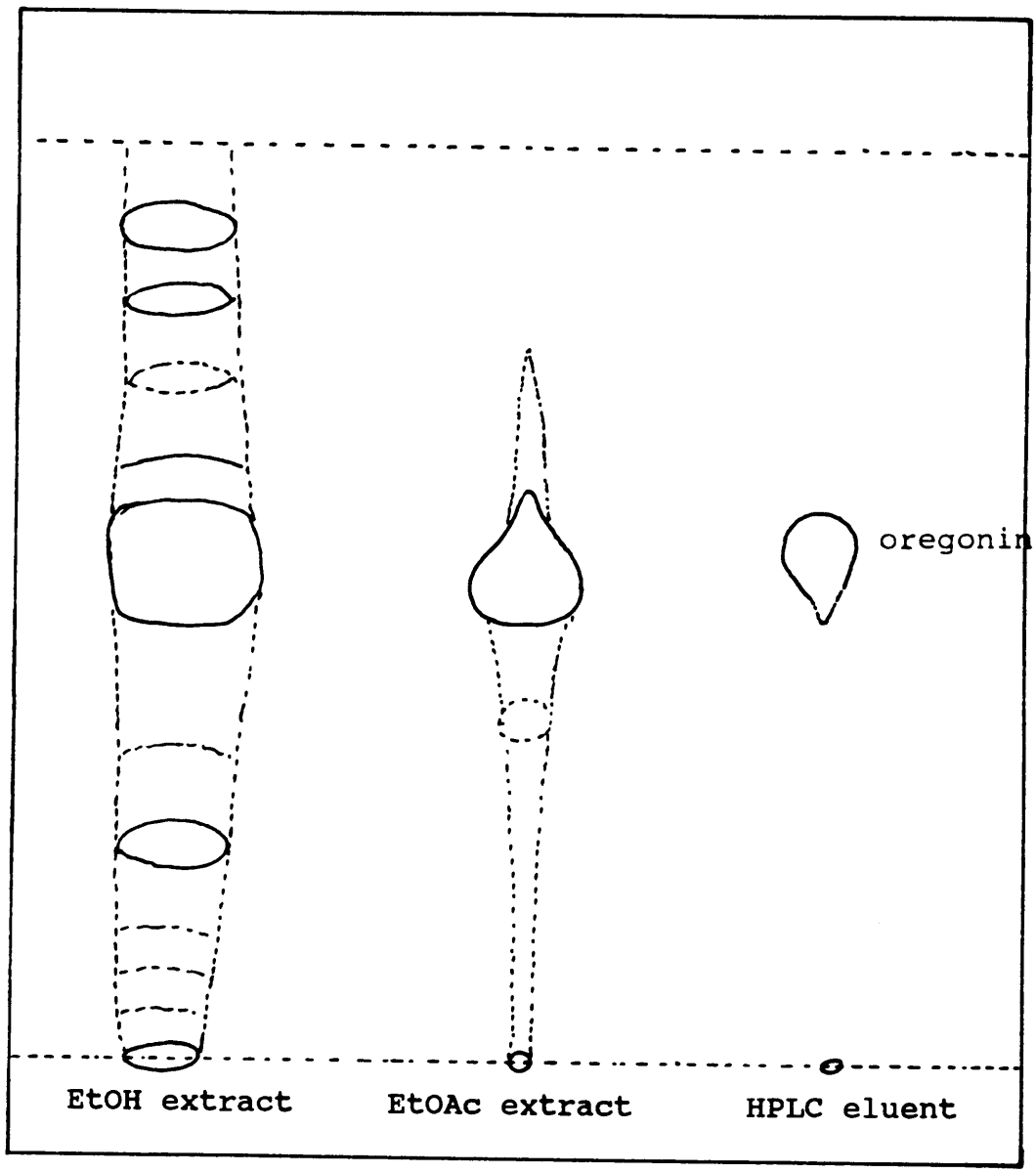


Figure 14. TLC chromatogram of oregonin from red alder bark.

1.3.1.3 Column Chromatography

There are numerous packing materials available to separate phenolic compounds. In the present work, silica-gel was chosen because of its success on TLC plates. The size of the particle was important to the isolation of pure oregonin. Several particle sizes were examined. Silica-Gel 7-G (40 micrometer) was chosen to separate oregonin because of the high resolution rather than a packing of larger size. The disadvantage of this type of packing material was that it required a long time for the elution of samples.

Twelve combined fractions were examined by tyrosinase in order to identify oregonin fractions after these fractions had been tested by TLC (Figure 9). After eluting 200 aliquots the more polar compounds still remained on the column. The results of the reaction with tyrosinase are shown in Table 1.

Fractions 9 and 10 were strongly colored and the TLC's of these were matched with the result of the TLC chromatogram on Figure 14. Treated solutions of fractions 9 and 10 immediately changed color from colorless to bright golden brown by tyrosinase, while the control was changed by exposure to light and air only after a long time.

1.3.2. Analyses of Oregonin by HPLC

1.3.2.1. Column and Equipment

Reverse-phase C-18 columns were used for both analytical and preparative HPLC of oregonin-rich solutions. Hrutfiord and Luthi (1982) had shown that oregonin could be separated and isolated by HPLC.

1.3.2.2. Optimum Operating Conditions

Methanol-water (40:60, v/v) proved to be the best solvent system for the separation and isolation of oregonin by HPLC. When these optimum conditions were used (Figure 9), two peaks related to oregonin were detected (Figure 15). Oregonin (I) possesses a chiral carbon at the point of the glycosidic attachment to the xylose portion, and the two peaks may have been due to the stereoisomers that could result from this chiral center.

1.4. Identification of Oregonin.

After the collection of Fractions I and II (Figure 9 and 15), they were chromatographed on silica-gel TLC. Each fraction gave an identical single spot. The R_f value of the single spot was identical with Karchesy's result (1975) and

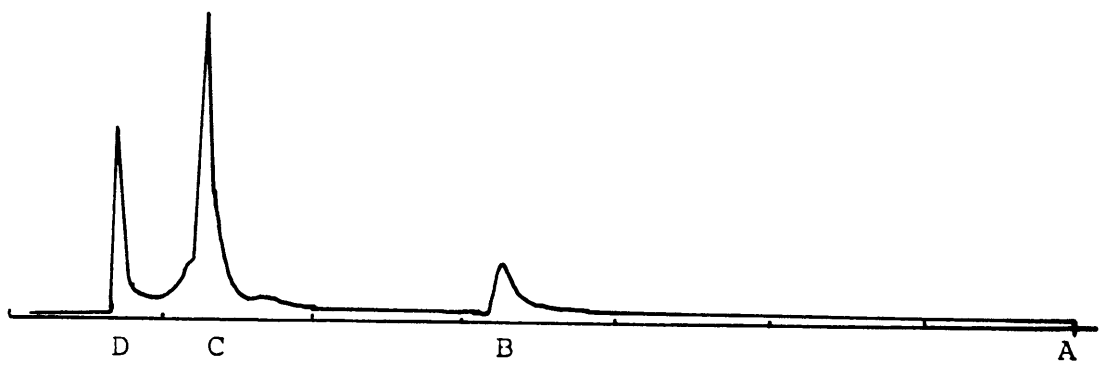


Figure 15. HPLC spectrum of oregonin on a C-18 reverse phase column. A, injection point; B, unknown; C and D, from oregonin

Terazawa's value (1984a) for oregonin. The Rf values were: R_f (system 1) = 0.61; R_f (system 2) =0.35.

The NMR spectra of natural oregonin without derivation have not been previously published. The proton NMR spectrum of oregonin (Figure 16) showed signals for aromatic protons equivalent to six at 6.50-6.72 ppm. A quintet (J=7 Hz) at 4.07-4.14 ppm corresponded to one proton on C-5. Multiplets from 2.50-2.83 ppm corresponded to protons on C-1,7 and C-2,4. There were 8 protons because of the similar environment which consisted of four deshielded methylene protons by phenolic rings and four by the ketone and the oxygen in the glycosidic bond on C-5. A double-doublet at 1.74-1.78 ppm (J=3.2 Hz and J=6.0 Hz) was one deshielded methylene group on C-6. Protons on C-2, C-3 and C-4 of the xylopyranose ring of oregonin resonated in multiplets (3.14-3.23 ppm) which were a typical xylose pattern. Two quartets, 3.40-3.53 ppm and 3.84-3.88 ppm, were due to the two C-5 protons of the xylopyranose, which are in the axial and equatorial positions, respectively. The anomeric xylosyl proton that showed at 4.31-4.33 ppm was deshielded more than the other xylopyranose protons by the two acetal oxygens. The above results are quite well matched with the reports of Karchesy et al. (1974), Karchesy (1975) and Terazawa et al. (1984a).

The carbon-13 NMR spectrum of oregonin (Figure 17) showed signals of aromatic carbons equivalent to twelve. Shifts of the aromatic carbon atom attached directly to the

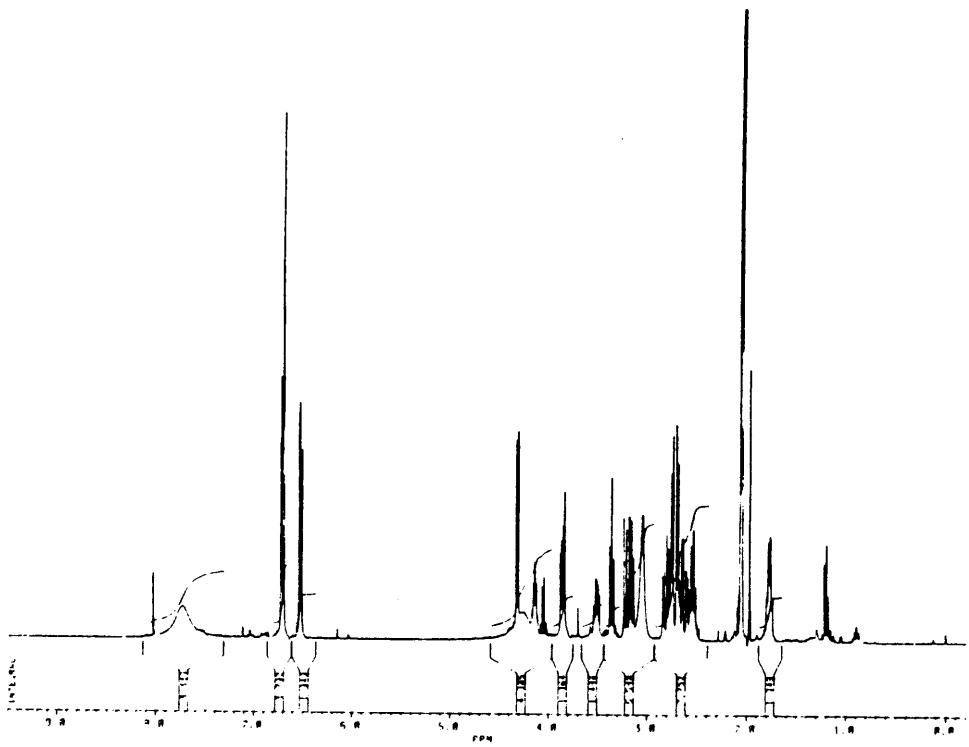


Figure 16. Proton NMR spectrum (acetone-D_6) of oregonin.

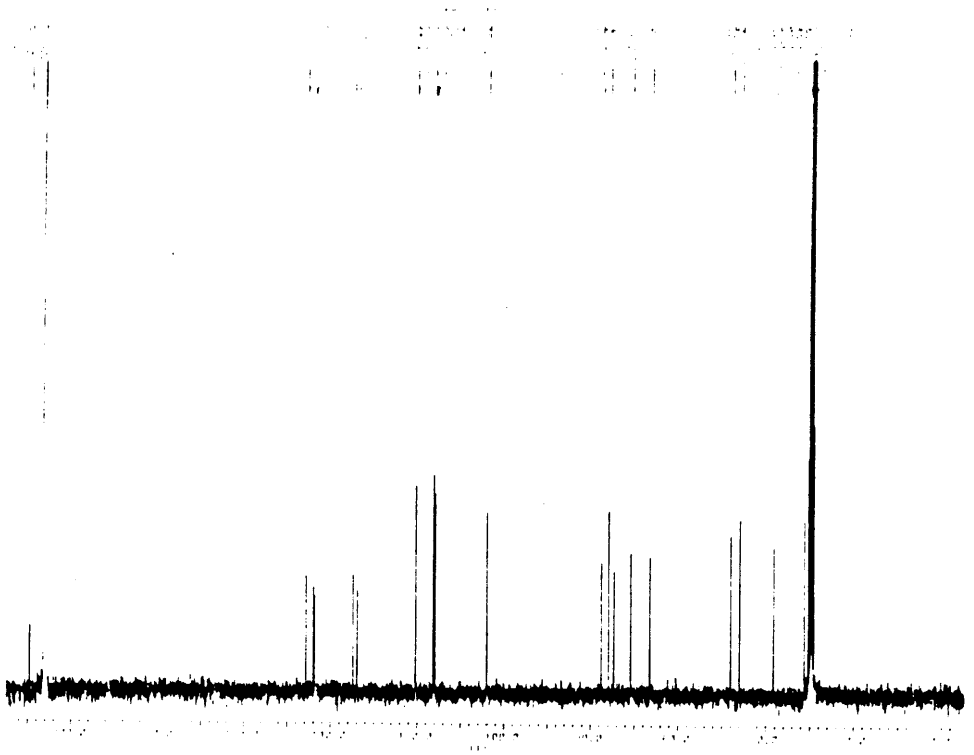


Figure 17. Carbon-13 NMR spectrum (acetone-D_6) of oregonin.

substituent have been correlated with substituent electronegativity after correcting for magnetic anisotropy effects; shifts at the para aromatic carbon have been correlated with the Hammett's constant. Meta shifts are generally small-up to several ppm for a single substituent. Ortho shifts are not readily predictable and range over -15 ppm (Silverstein et al, 1981). Therefore, two peaks at 145.63 and 145.66 ppm corresponded to two para-carbons (C-4) on the aromatic ring. Meta-carbon atoms C-5 on the aromatic ring were distinguished from para carbons at 143.74 and 143.94 ppm. Two C-1 atoms corresponded to 133.85 and 134.84 ppm. Others (C-2, 3, and 4) carbons on the aromatic rings were shown in the 115.92-120.38 ppm range. The peak at 209.55 ppm corresponded to C-3 of the ketone group. Only one methine carbon peak on the heptane skeleton of oregonin (Figure 18) was the C-5 carbon atom shown at 77.57. Two peaks at 48.04 and 46.00 ppm which corresponded on C-1 and C-7 were deshielded by phenyl groups and upshielded by the ketone and acetal oxygen linkage, respectively. C-4, C-2 and C-6, three methylene carbon peaks, were shown at 38.24, 31.27, and 29.25 ppm, respectively. A peak at 103.93 ppm corresponded to the C-1" xyloside carbon atom which was deshielded by the two acetal oxygens. Only one methylene carbon atom at 66.44 ppm was due to C-5" of xylopyranose ring which was shielded by the oxygen in the pyranose ring. The other three methine carbons at 70.83, 74.66, and 75.91 ppm corresponded on C-3", C-4", and C-2", respectively.

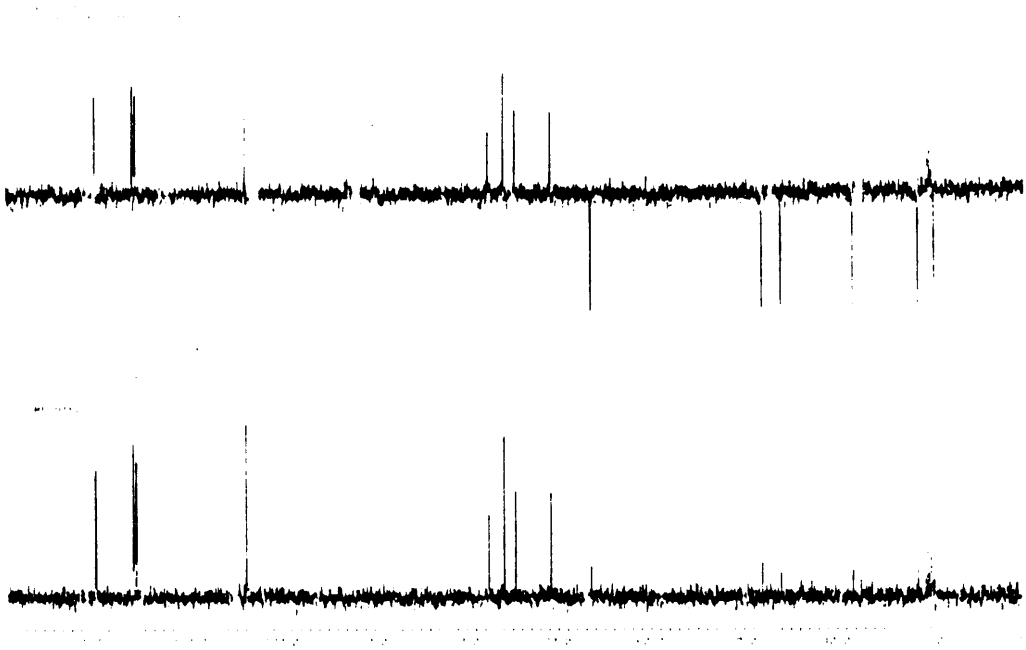


Figure 18. DEPT of oregonin.

There was no difference between Fractions I and II (Figure 9) by NMR spectroscopy. The presence of two peaks by HPLC analyses (Figure 15) was not resolved.

2. Sapwood of Douglas-fir

The purpose of this work was to isolate and identify the possible precursors to the Douglas-fir sapwood staining phenomenon.

Anthocyanin, anthocyanidin, flavonoids and oregonin were considered the likely precursors to the color pigment. Oregonin was a precursor of the stain of red alder (Karchesy, 1975) and to keyamahannoki (Alnus hirsuta) and hannoki (Alnus japonica, [Betulaceae] (Terazawa et al., 1984a). Fustin was a precursor to the discoloration of hazenoki (Rhus succedaned L.) (Kondo and Imamura, 1985). Yateresinol and 1,4-bis-(p-hydroxyphenyl)-butadiene also may be responsible for the discoloration to sugi (Cryptomeria japonica D. Don) (Takahashi et al., 1983).

Structural elucidation of the phenolic compounds, therefore, would indicate their possible involvement in the staining reaction and would provide a basis for subsequent work on elucidation of the exact mechanisms of the staining reaction.

2.1 Collection

Variations in extractives within the stem are principally associated with their presence in the heartwood. Extractives are usually much lower in the sapwood than in the heartwood. It has been generally known that Douglas-fir heartwood is more highly colored than the sapwood because of the amount of extractives.

Because of the above reasons, it was considered that the place from which the sample was taken was important. The sample was collected from the higher trunk after the tree was freshly cut. The higher trunk was stored in the cold and dark room until the sample was prepared.

2.2 Extraction

There are numerous solvent systems to extract phenolic compounds from natural plants. Generally, acetone, methanol or ethyl acetate extracts are prepared by cold solvent percolation of crushed plant material (Haslam 1966).

In the present work, it was performed with ethanol or methanol in order to prevent enzymatic reaction during the extractions.

Because the extracted solution contained many different compounds, it was preliminarily separated into three fractions by three different solvents, chloroform, ethyl

acetate and methanol according to the difference in their polarities.

Most of the non-polar compounds, waxes, terpenes, and fats were isolated with chloroform (Fraction EX-1-A, Figure 10). Even though some phenolic compounds which are methoxylated flavonoids also might be extracted in it, the test with tyrosinase and exposure to light revealed that there were no compounds which could be precursors of the stains.

Almost all of the monomeric flavanols were extracted with ethyl acetate (EX-1-B, Figure 10). When EX-1-B was tested by tyrosinase the color of the solution changed immediately from colorless to a strong bright golden yellow or brown (Table IV).

The water solubles (EX-1-C, Figure 10) contained mainly condensed tannins and flavanol glycosides which were not dissolved in the ethyl acetate. When EX-1-C (Figure 10) was reacted with tyrosinase it gave a strong color (Table IV).

Because tyrosinase was a catechol oxidase enzyme that reacted with catechol groups to give red colors, it was used as an indicator to detect possible precursors of stains. The enzymatic tests were conducted on three fractions and several known compounds and with some well-known inhibitors. The results are shown Table IV.

Three facts were shown from these results: 1), none of the possible precursors of the color in Douglas-fir sapwood were oregonin nor any type of cinnamic acid which possessed

Table IV. Color Test of Phenolic Compounds with and without Inhibitor.

Phenolic Compounds	A	B	C	D
oregonin	yellow	no color yellow*	yellow	yellow
methanol extract (EX-1, Figure 10)	yellow brown*	no color yellow*	no color yellow*	yellow
ethyl acetate extract (EX-1-B) (Figure 10)	brown	yellow	yellow	yellow
water solubles (EX-1-C, Figure 10)	tan color	tan color	tan color	tan color
3,4-dihydroxy cinnamic acid	tan color	no color dark brown*	yellow dark brown*	-
3-methoxy-4-hydroxy cinnamic acid	no color	no color yellow*	no color yellow*	-
para-hydroxy cinnamic acid	no color	no color light yellow	no color brown*	-

*: Observed color after two weeks.

A: Ethylenedinitrillotetraacetic acid disodium salt (EDTA).

B: Phenylthiourea.

C: Mercaptobenzothiazole.

D: Chloramine-T

Sample was treated with tyrosinase after inhibitor added.

a C_6-C_3 skeleton; 2), the methanol solubles (EX-1), the ethyl acetate solubles (EX-1-B) and the water solubles (EX-1-C) of Figure 10 potentially contained precursors of stains; 3), the inhibitors shown in Table IV were not suitable inhibitors for these compounds.

2.3. Test of Inhibitors to Color Formation

Preliminary tests were made of some well known inhibitors to the activity of phenolic oxidase enzymes such as tyrosinase. The inhibitors were selected from the work of Terazawa et al. (1984b) who investigated the inhibition to color formation in several Alnus species. The results shown in Table IV indicate that none of the inhibitors had a lasting effect on preventing the color formation of precursors extracted from Douglas-fir sapwood.

However, the patterns of color that developed from tyrosinase addition were different depending on the compounds and the inhibitors. Ethylenedinitrillotetraacetic acid disodium salts (EDTA) developed color immediately after the addition of tyrosinase in all solutions of compounds but the intensity of color was the lightest of all the solutions for a long period (one month). Phenylthiourea was the best inhibitor for short periods (1-2 days), but the intensity of color was the same as the treated samples after one week. Mercaptobenzothiazole containing solutions developed color immediately after adding the enzyme the same as the enzyme

treated solutions. Chloramine-T developed mild colors with time. The results of enzymatic tests showed that samples EX-1, EX-1-B, and EX-1-C (Figure 10) contained compounds that could potentially be precursors of stains.

2.4 Separation of the Stain Precursors

Paper chromatography is probably the most generally useful chromatographic technique available to flavonoid chemists. Preliminary analyses of a plant extract for the presence of flavonoids is conveniently accomplished by this method or the similar thin-layer chromatography.

Column chromatography for large-scale separations was investigated depending on the packing materials and several different solvent systems.

2.4.1. Thin-Layer Chromatography

It was established from preliminary analyses of the extraction of Douglas-fir sapwood that dihydroquercetin-type flavonoids were present. Chromatographic analyses of the methanol extract (EX-1), the chloroform solubles (EX-1-A) and the ethyl acetate solubles (EX-1-B) (Figure 10) of Douglas-fir sapwood were undertaken in order to locate color precursors and to devise methods for their isolation.

In general, two dimensional paper chromatography has been employed in the preliminary qualitative analyses of flavonoids and related phenolic compounds (Markham, 1983). Although paper chromatography of flavonoids is a good method with high resolution, it is very time consuming. In the present work, TLC was used instead of paper chromatography because of several advantages. TLC is particularly useful for investigating solvents for possible use in column chromatography, for analyses of fractions from column chromatography, for fast development and for easy detection with ultraviolet light.

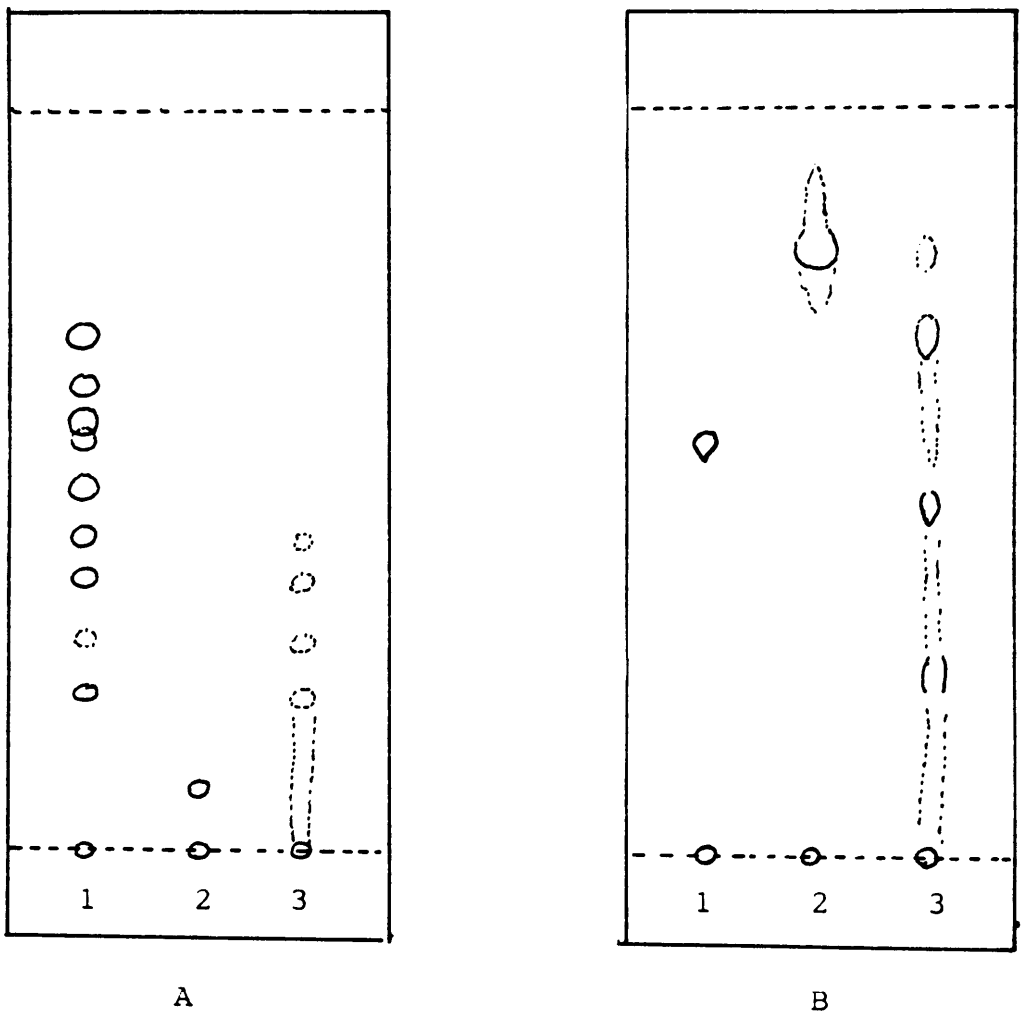
Two dimensional cellulose TLC has been employed in the preliminary qualitative analyses of flavonoids. (Markham, 1983) Numerous solvent systems have been employed, most of which are based on solvent pairs which effect separation in one direction by partitioning effects and in the other by adsorption effects. Tertiary-butanol-water-acetic acid (3:1:1, v/v, TBA) in the first direction followed by six to fifty percent acetic acid in the second direction is usually the most effective for polar compounds (Markham, 1983).

Silica-gel TLC was used to analyze for the comparatively less polar compounds in wood extractives and flavonoid glycosides. Several solvent systems were tested in the present work. Benzene-ethyl acetate-88% formic acid (45:10:5, v/v) and chloroform-ethyl acetate-88% formic acid (6:3:1, v/v) gave the best resolution for the less polar compounds which were the monomeric flavonoids.

Detection of phenolic compounds on chromatograms is commonly achieved by various spray reagents or with ultraviolet light.

EX-1-A and EX-1-B (Figure 10) were chromatographed under the same conditions with authentic oregonin by acetone-ethyl acetate-water (10:10:1, v/v) in order to be sure whether or not oregonin was present in the extracts of Douglas-fir sapwood. EX-1 and EX-1-B were also chromatographed with authentic catechin which was a precursor of the stain on hemlock (Hrutfiord et al., 1985) by benzene-ethyl acetate-88% formic acid (45:10:5, v/v). The results are shown in Figure 19.
After these primary tests, it was revealed that neither oregonin nor catechin were precursors of the stains in Douglas-fir sapwood.

The chloroform solubles (EX-1-A, Figure 10) were best resolved with benzene-ethyl acetate-88% formic acid (45:10:5, v/v). Since chloroform solubles (EX-1-A, Figure 10) gave no color reaction with tyrosinase or light, no further study was done. The ethyl acetate solubles (EX-1-B, Figure 10) and water solubles (EX-1-C, Figure 10) were best resolved with both silica-gel TLC and cellulose TLC. The solvent systems for the two types of TLC were: Ethyl acetate-chloroform-88% formic acid (6:3:1, v/v, ECF) for silica-gel TLC, and tertiary butanol-acetic acid-water (3:1:1, v/v, TBA) and 6% acetic acid for cellulose TLC. The ECF system was more suitable for ethyl acetate solubles



1. methanol extract EX-1 (Figure 10)
2. catechin
3. ethyl acetate solubles EX-1-B (Figure 10)

1. oregonin
2. chloroform solubles EX-1-A (Figure 10)
3. ethyl acetate solubles EX-1-B (Figure 10)

A: Benzene-ethyl acetate-88% formic acid (45:10:5, v/v)
B: Ethyl acetate-acetone-water (10:10:1, v/v)

Figure 19. TLC chromatograms of the extracts of Douglas -fir sapwood.

(Figure 20) and the TBA and acetic acid system was better for water solubles. The major spots on the silica-gel TLC (Figure 20) were dihydroquercetin (spot A) and dihydroquercetin glycoside (spot B) whose R_f values were 0.61 and 0.39, respectively. Freshly prepared extracts gave no sign of containing free quercetin on TLC.

2.4.2. Silica-Gel Column Chromatography

The technique basically involves application of a flavonoid mixture (in solution) to a column of powdered adsorbent (such as cellulose, silica or polyamide) and sequential elution of individual components with appropriate solvents. It is generally considered that separations are based on partitioning, i.e, mostly cellulose and silica chromatography (Markham, 1983).

The range of available column packings is vast. In the present work, five types of column packings including cellulose, silica, polyamide, Sephadex LH-20, and Fractogel were investigated.

2.4.2.1. Silica-Gel Column Chromatography

For medium polar flavonoids, silica packing was superior to cellulose and polyamide by preliminary analyses of the extracts of Douglas-fir sapwood. Column packings should be carefully chosen for column chromatography because

the particle size is important. If it is too small, the flow rate of the eluting solvent may be unacceptably slow, and if too large the chromatographic separation of components may be poor. Two sizes of packings were tested with three different solvent systems, which were water-saturated ethyl acetate, acetone-ethyl acetate-water (10:10:1, v/v) and chloroform-methanol mixtures. The packing materials were 70-230 mesh silica gel that is generally used for column chromatography and 40 micrometer silica-gel that is generally used for TLC. The 70-230 mesh silica-gel gave an unacceptably fast flow rate and poor resolution with the three solvent systems. The 40 micrometer silica-gel gave good separation of the components. The techniques of column packing and sample application followed those outlined by Markham (Markham, 1983).

In general, the solvent was determined experimentally and it was often simplest to explore possibilities by TLC prior to running a column. According to the TLC chromatography, water-saturated ethyl acetate, acetone-ethyl acetate-water (10:10:1, v/v) and chloroform-methanol, v/v (100 %, 10:1, 4:1, 2:1, 1:1, 100 % methanol) were chosen because of the polarity and the solubility of the samples.

The flow rate of the eluting solvent was fastest with the water-saturated ethyl acetate system. The resolution of the sample, however, was the poorest. This solvent system was not acceptable for this work. Acetone-ethyl acetate-

water (10:10:1, v/v), which had a medium range of eluting power, gave moderate resolution. It could separate materials into three different portions, nonpolar, medium polar, and highly polar compounds that did not elute with it. The best results were obtained with the chloroform-methanol systems because the less polar and the medium polar components were eluted first with the 100 % chloroform. The sequential change of mixing ratio from 10:1 (v/v) to 100 % methanol separated the components with reasonably purity. However, the compounds which were more polar than the dihydroquercetin glycosides did not have an acceptable range of separation.

2.5. Isolation of Dihydroquercetin

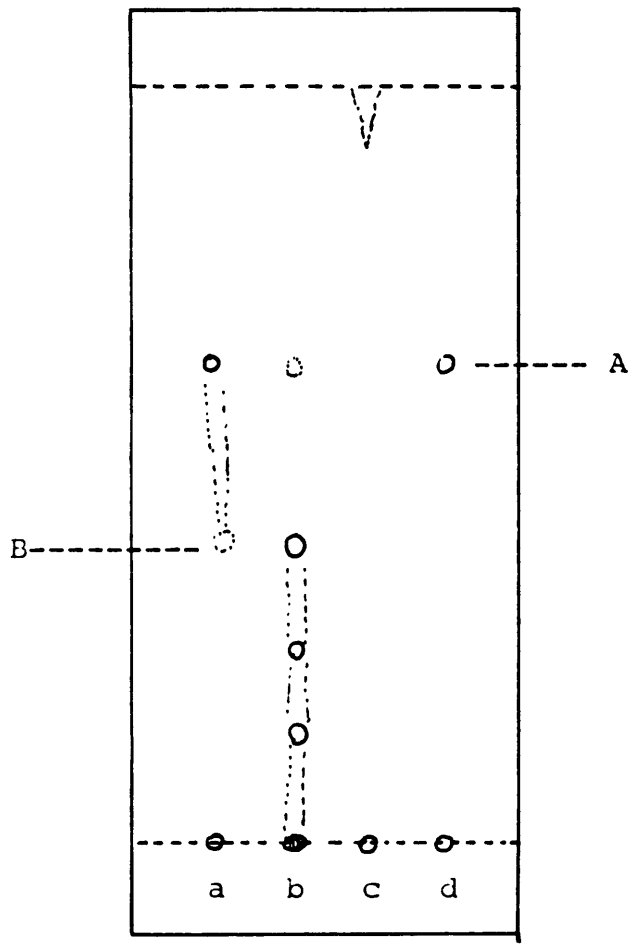
2.5.1. Isolation

Fractions EX-1-B and EX-1-C (Figure 10) were studied carefully with several types of chromatography. Fraction EX-1-B (Figure 10) was chromatographed on silica-gel column with two solvent systems, acetone-ethyl acetate-water (10:10:1, v/v) and sequential chloroform-methanol mixtures. After Fraction EX-1-B was chromatographed with the acetone-ethyl acetate-water (10:10:1, v/v) solvent system six portions were collected. The second portion (11-27 aliquots) strongly reacted with tyrosinase and changed color from colorless to brown. TLC of this second portion showed

several spots which indicated the presence of less polar and medium polar components. Additional purification was achieved by rechromatographing on a new silica-gel column with 300 ml of chloroform and 500 ml of a chloroform-methanol (4:1, v/v) mixture. The 10-35 aliquots were identical with the major spot shown in Figure 20 and changed color with tyrosinase. The other aliquots after aliquot 35 also reacted with tyrosinase and gave a color. Since they were not pure enough to identify, additional investigations were carried out to find better systems.

Freshly prepared Fraction EX-1-B (Figure 10) had no free quercetin in the aliquots collected from column chromatography. However, quercetin was detected by TLC in the aliquots from fraction EX-1-B (Figure 10) in aqueous methanol solutions after several days from the preparation of the sample. Dihydroquercetin readily converts to quercetin under various mild, oxidative processes (Hergert,1962). Kurth discovered (Kurth and Chan, 1953b: Kurth, 1956) that quercetin was formed upon heating an aqueous solution of dihydroquercetin and sodium bisulfite. According to the present work, however, dihydroquercetin easily yields quercetin without any treatment except that of light in polar solution, such as alcohol and water.

After several investigations, it was found that the following method was satisfactory for the present work. Figure 10 shows the analysis scheme outlined in the experimental section. Aliquots 6-29 contained quercetin as

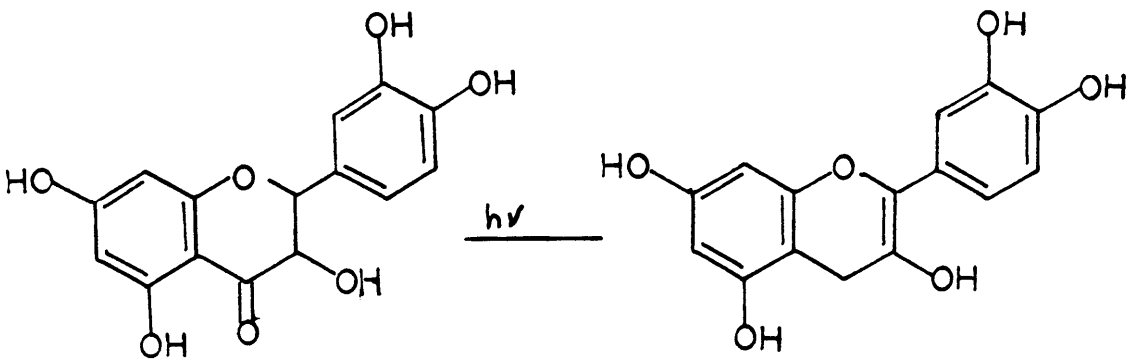


Ethyl acetate-chloroform-88% formic acid (6:3:1, v/v)

a: ethyl acetate solubles EX-1-B (Figure 10)
b: water solubles EX-1-C (Figure 10)
c: chloroform solubles EX-1-A (Figure 10)
d: authentic dihydroquercetin

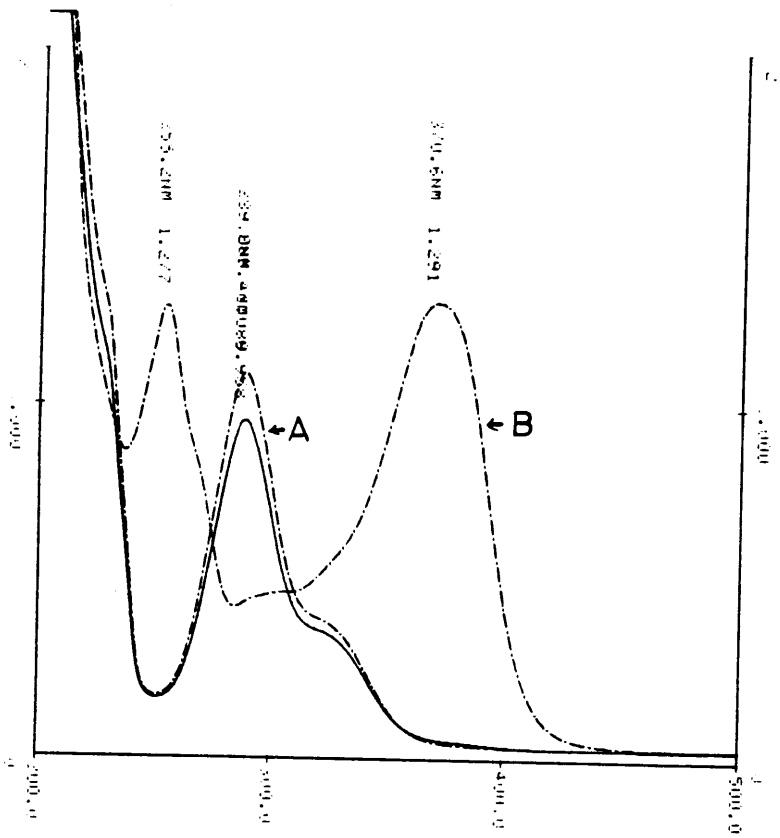
Figure 20. TLC chromatogram of the ethyl acetate extract of Douglas-fir sapwood.

analyzed by TLC. Aliquots 30-65 contained a pure compound which formed white, needle-like crystals. The crystals were very stable in air and light. The purity of the crystals was over 99% as checked by a GPC analytical system.



2.5.2. Identification of Dihydroquercetin

The white, needle-like crystals were examined by melting point, R_f values by TLC (system i), ultraviolet spectra, proton NMR and carbon-13 NMR to identify it. The melting point was 238-240° C, identical to authentic dihydroquercetin. Kurth (1956) reported a melting point of 240-242° C for dihydroquercetin. Neutral and alkaline ultraviolet curves were identical with those of authentic dihydroquercetin (Figure 21). Proton NMR of dihydroquercetin (Figure 22) showed a typical hydrogen bonding signal at 11.71 ppm between the 5-OH and 4-C=O. Because the C ring is a rigid system there is no free rotation of the C-4 ketone group. It caused strong hydrogen bonding between the C-5 hydroxyl and the C-4 ketone group. In general, the



A : Dihydroquercetin crystals in methanol
B : Authentic dihydroquercetin in methanol
B : Authentic quercetin in methanol

Figure 21. UV spectra of dihydroquercetin and quercetin in methanol.

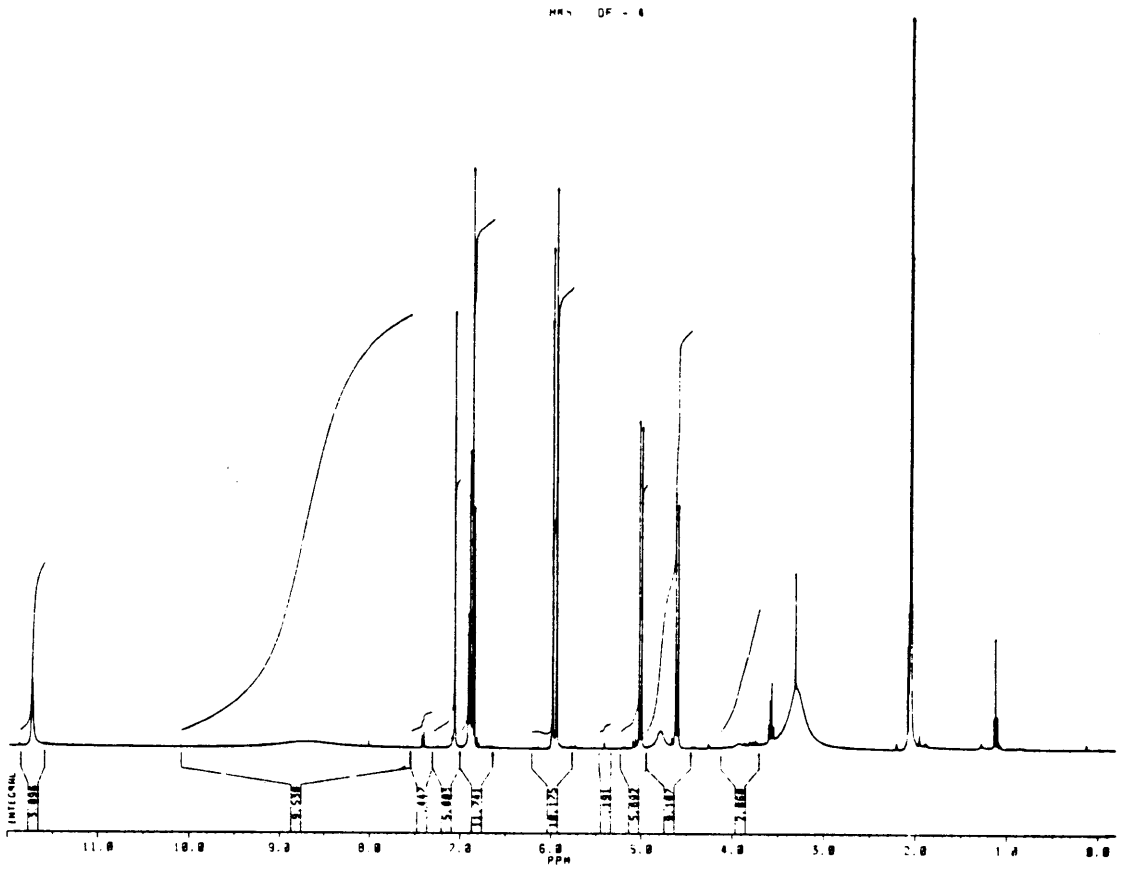


Figure 22. Proton NMR spectum (acetone-D_6) of dihydroquercetin.

ring protons of an aromatic system have resonance near 7.2 ppm; however, electron-withdrawing ring substituents will move the resonance of these protons downfield, and electron-donating ring substituents will move the resonance of these protons upfield (Pavia et al., 1979) Therefore, the 6-H and the 8-H on the A ring were moved upfield to 5.93 ppm, J=2 Hz, and to 5.97 ppm, J=2 Hz, respectively, by the ortho and para ether and hydroxyl groups. The spectrum showed signals due to the three aromatic protons of the B ring (7.05 ppm, doublet, J=2 Hz for 5'-H; 6.88 and 6.90 ppm, double-doublet, J=2, 8 Hz for 6'-H; 6.83 and 6.85 ppm, doublet, J=8 Hz for 2'-H). The stereochemistry of dihydroquercetin (C-2 and C-3, trans) was deduced from the proton NMR signals; a doublet at 4.99 and 5.02 ppm (J=11.54 Hz) was characteristic of the C-2 proton, and a doublet at 4.58 and 4.61 ppm (J=11.33 Hz) was due to the C-3 proton. Proton spin-spin-coupling constants (J=11.4 Hz) imply that the vicinal 2-H and 3-H are both in axial positions and that the 2-B ring and the 3-hydroxyl groups are in equatorial positions (Silverstein et al., 1981). Carbon-13 NMR (Figure 23) confirmed the carbon skeleton and also confirmed that no functional groups contained carbon atoms such as alkyl groups. The spectrum showed signals that were attributed to a carbonyl at C-4 (198.11 ppm), a methine carbon at C-3 (73.06 ppm) adjacent to the carbonyl and an aromatic ring with a 3',4'-dihydroxy substitution pattern. The 5-OH group deshielded the carbonyl resonances by 2.85 ppm due to chelation effects.

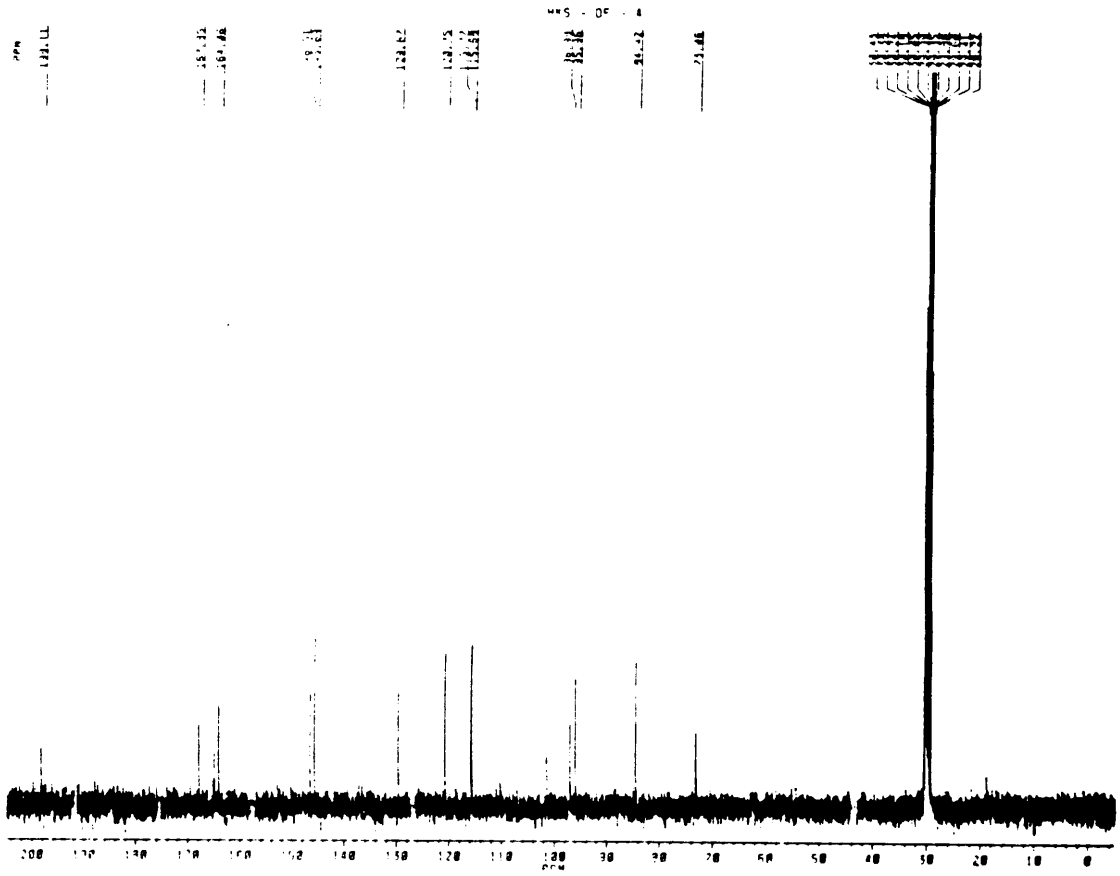


Figure 23. Carbon-13 NMR spectrum (acetone-D_6) of dihydroquercetin.

Among the C-5, C-7 and C-9 carbons which usually appear between 157-167 ppm, the C-7 signal always appears at the lowest field in 5,7-oxygenated flavonoids (Herz et al., 1980; Huckerby and Sunman, 1979; Iinuma et al., 1980a, b; Nomura and Fukai, 1979; Pelter and Ward, 1978). For a large number of cases, C-6 and C-8 resonances appear between 90-100 ppm and the C-6 signal is always at a downfield position with respect to C-8. The chemical shift difference is small (ca. 0.9 ppm) in flavanones and larger (ca. 4.5 ppm) in flavones and flavonols (Agrawal and Rastogi, 1981) All signals were well matched with Markham's report (Markham, 1983) and were the same as the authentic dihydroquercetin carbon-13 NMR spectrum.

2.6. Isolation of a Glycoside of Dihydroquercetin

2.6.1. Liquid Chromatography on Silica-Gel Columns

Investigations of the fractions EX-1-B and EX-1-C (Figure 10) of Douglas-fir sapwood revealed that glycosidic flavonoids were present. The method which was discussed in the experimental (section 2.6.1) was excellent to separate aglycones and glycosides. The preparative TLC described in the experimental section was not suitable because glycosides may be partially hydrolyzed and oxidized during development of the chromatograms.

2.6.2. Liquid Chromatography on Sephadex LH-20 Columns

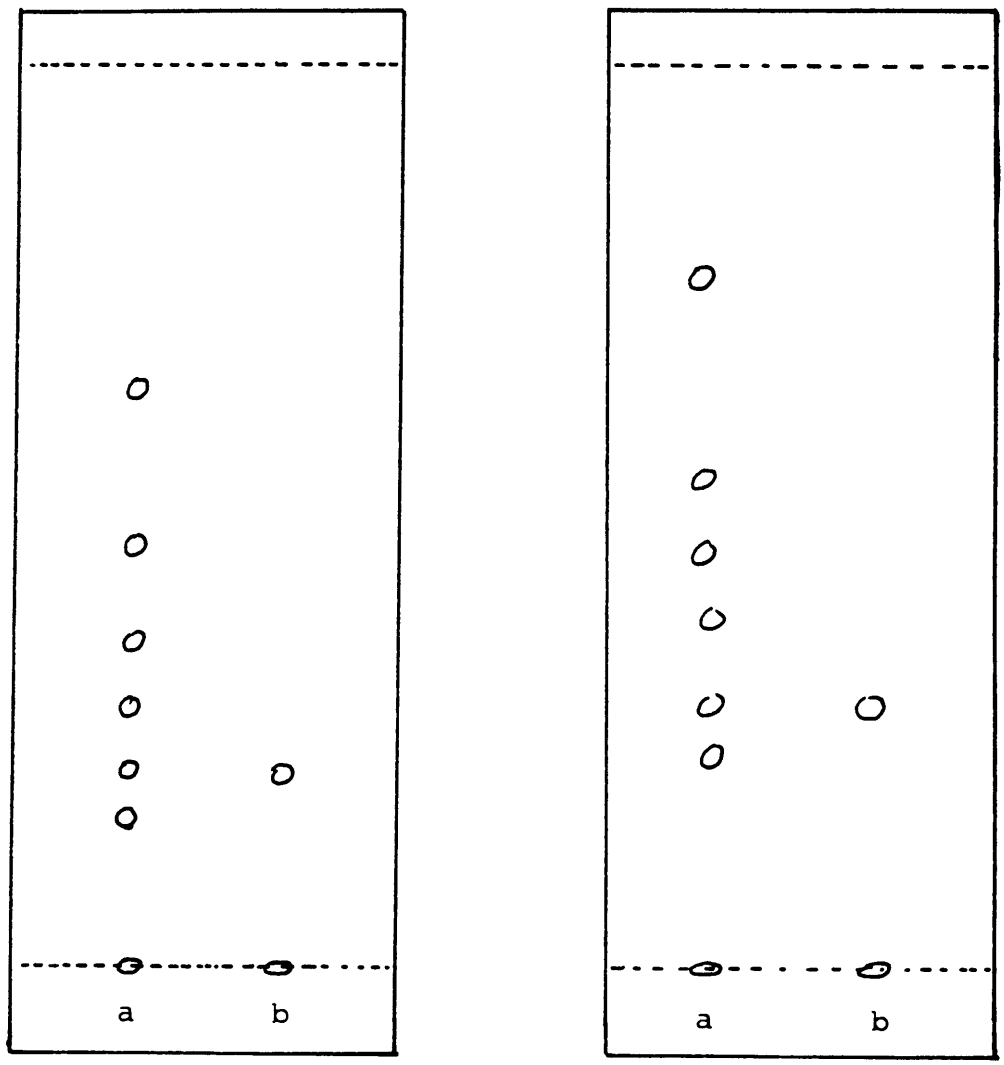
It was found that Sephadex LH-20 column chromatography was an excellent system for the isolation of glycosides. Ethanol (95%) was a better solvent than methanol or water for the isolation. Aliquots 28-32 from the silica-gel column (Figure 11) were rechromatographed on a Sephadex LH-20 column with 95% ethanol as eluent (Figure 11). Six peaks were collected. Peak 4 (Figure 11) contained the major components in aliquots 28-32 and strongly showed one spot which had an R_f=0.39 on silica-gel TLC (system i). Peak 4 (Figure 11) was rechromatographed on the same column under the same conditions. This gave one pure compound.

2.6.3. Identification

Harborne (1965) described the characterization of flavonoid glycosides by acidic hydrolysis. Before hydroysis of the sample, it was tested by paper chromatography and no free sugar or aglycone was present. After hydrolysis the solution was neutralized with ammonium hydroxide to prevent further hydrolysis and unexpected degradation of compounds during concentration. The hydroysis solution was extracted with ethyl acetate in a separatory funnel to extract the aglycone. The ethyl acetate layer was tested with silica-gel and cellulose TLC to identify the aglycone. The TLC

chromatogram of the aglycone showed the same R_f value as authentic dihydroquercetin . The water layer was reduced in volume and dissolved in a minimum amount of methanol because the salt of ammonium chloride interfered with sugar analyses by paper and cellulose TLC. Figure 24 shown the results of paper chromatography and cellulose TLC. The glycosidic unit of the glycoside of the flavonoid in Douglas-fir sapwood was glucose.

The freeze-dried sample (Figure 11) was tested with UV spectroscopy. Neutral and alkaline ultraviolet curves of the sample was identical with those of dihydroquercetin. Ultraviolet spectroscopic analyses are well-known methods for the identification of the hydroxylation pattern of flavonoids using shift reagents. Sodium acetate causes significant ionization of only the most acidic of the flavonoid hydroxyl groups. Thus it is used primarily to detect the presence of a free 7-hydroxyl group. Sodium acetate-boric acid bridges the two hydroxyls in an ortho-dihydroxyl group and it is used to detect their presence. By forming acid-stable complexes between hydroxyls and neighboring ketones, and acid-labile complexes with ortho-dihydroxyl groups, aluminium chloride and aluminium chloride-hydrochloric acid reagents can be used to detect both groups. The $AlCl_3$ spectrum thus represents the sum effects of all complexes on the spectrum, whilst the $AlCl_3$-HCl spectrum represents the effect only of the hydroxy-ketone complexes. The UV spectra of the dihydroquercetin



paper chromatogram TLC chromatogram

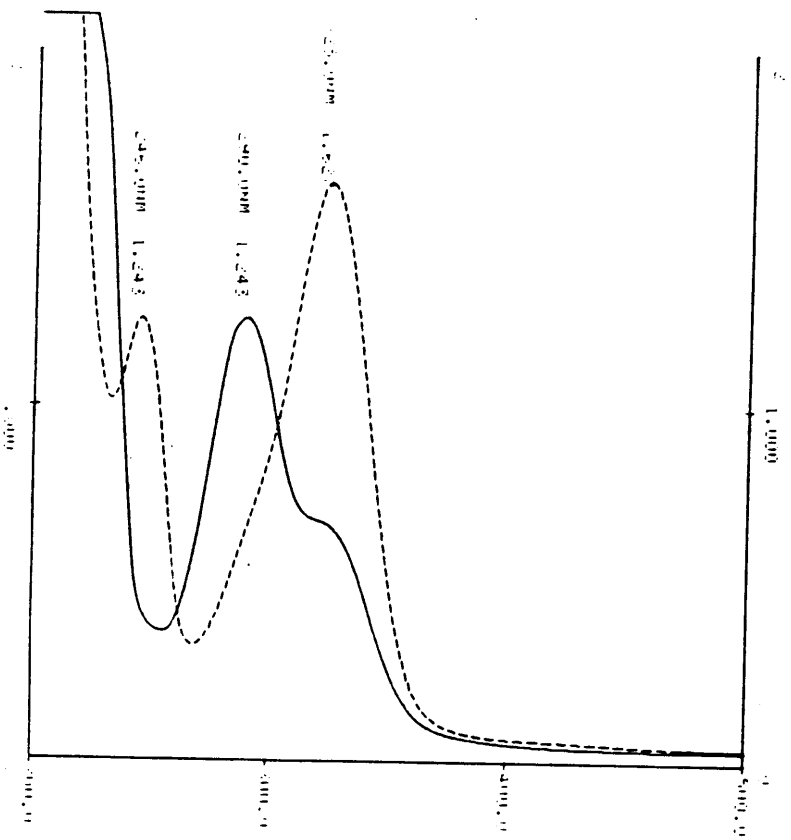
a: authentic carbohydrates
b: hydrolyzate of the DHQ glycoside

Figure 24. Paper and TLC chromatograms of the hydrolyzate of the dihydroquercetin glycoside.

glucoside (Figures 25, 26, and 27) demonstrated clearly the hydroxyl pattern of that compound. Figures 25 and 27 explained that the 7-hydroxyl group was not substituted because of a bathochromic shift in alkaline and sodium acetate reagents. Figure 26 showed that the 5-hydroxyl group was not substituted because there was no difference between the spectrum of $AlCl_3$ and the spectrum of $AlCl_3$-HCl. Figure 27 demonstrated that the ortho-dihydroxyl group was substituted by the glucose unit because the spectrum of NaOAc showed a bathochromic shift to 325 nm and the spectrum of NaOAc-H_3BO_3 did not change band II which was identical with the original spectrum.

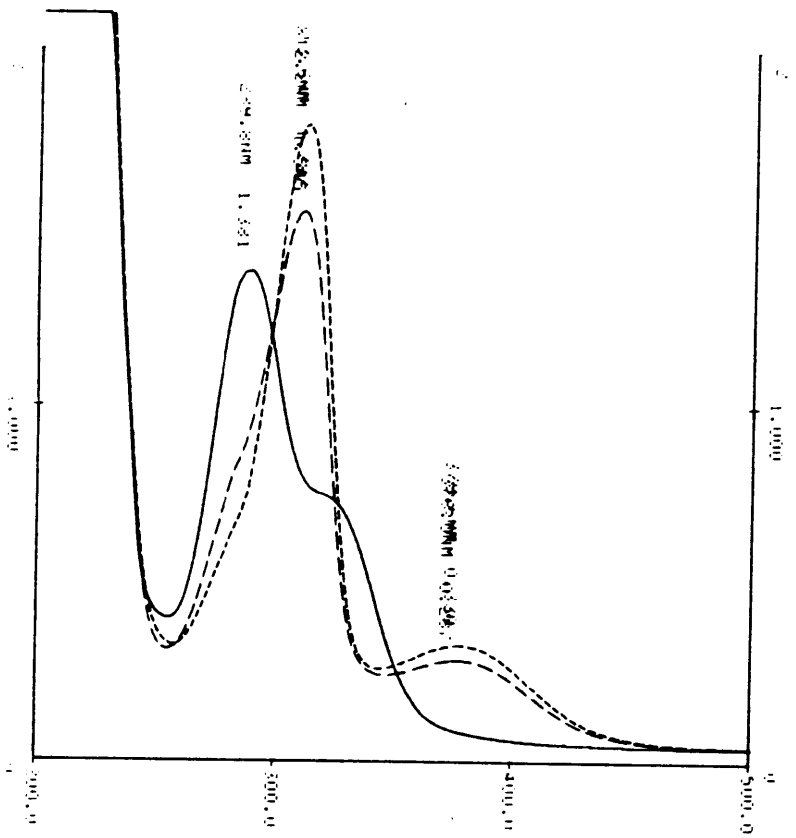
The proton NMR spectrum of the sample (Figure 28) supported the structure of the glycoside. Since the 6-H and 8-H on the A ring were identical with those of dihydroquercetin then the 5 and 7-hydroxyl groups were not substituted. The stereochemistry and the signals of dihydroquercetin 3′-glucoside (C-2 and C-3, trans) were also identical with those of dihydroquercetin. However, the signals due to the three aromatic protons on the B ring (7.36 ppm, doublet, J=2 Hz for C′-5; 7.09 and 7.07ppm, double-doublet, J=2, 8 Hz for C′-6; 6.90 ppm, doublet, J=2 Hz for C′-2) were changed because of substitution on either the 3′ or 4′-hydroxyl group by a glucosyl unit. The O-glucosyl group moved the resonance of the proton downfield.

Recently carbon-13 NMR has been used as a powerful tool to define the sugar moiety and its substituent position on



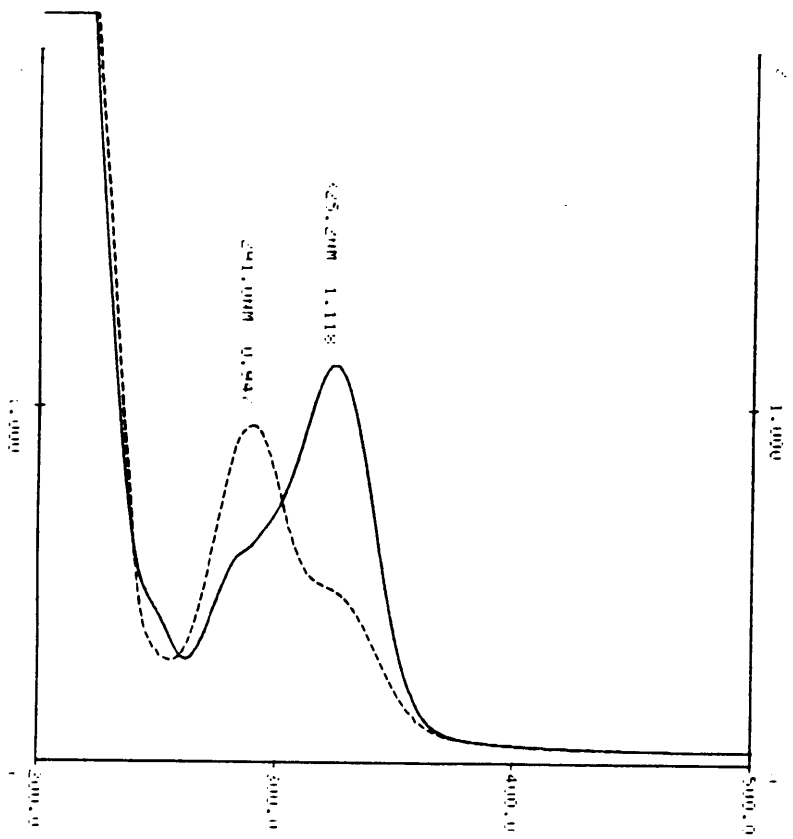
———: Dihydroquercetin glucoside in methanol
-·-·-·-: Dihydroquercetin glucoside in methanol with sodium hydroxide

Figure 25. UV spectra of the dihydroquercetin glucoside with an alkaline shift reagent.



——— : Dihydroquercetin glucoside in methanol
----- : Dihydroquercetin glucoside in methanol with aluminum chloride
— — — : Dihydroquercetin glucoside in methanol with aluminum chloride and hydrochloric acid

Figure 26. UV spectra of the dihydroquercetin glucoside with aluminium chloride with and without hydrochloric acid.



: Dihydroquercetin glucoside in methanol with sodium acetate
: Dihydroquercetin glucoside in methanol with sodium acetate and boric acid

Figure 27. UV spectra of the dihydroquercetin glucoside with sodium acetate with and without boric acid.

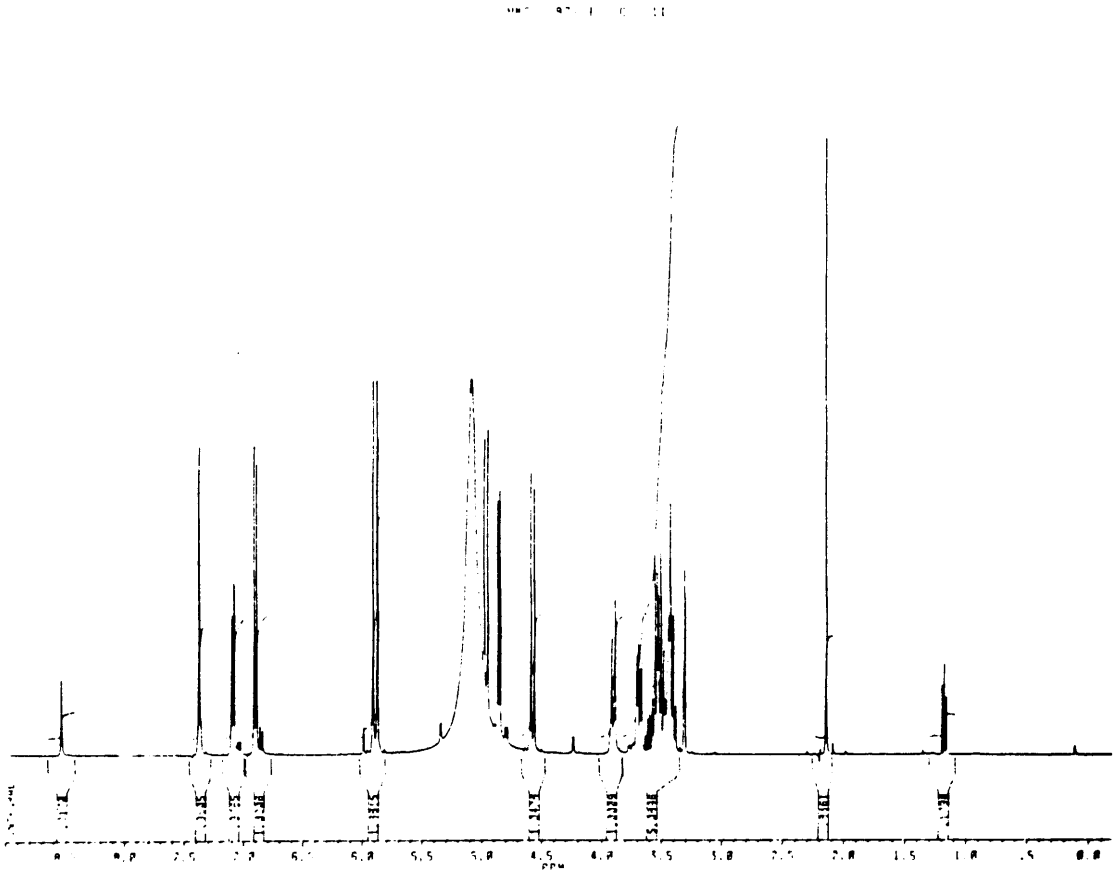


Figure 28. Proton NMR spectrum (acetone D_6) of dihydroquercetin-3'-O-glucopyranoside.

glycosides of flavonoids. Glycosylation of a phenolic hydroxyl induces an upfield shift in the resonance of the carbon directly involved in the derivatization. (Tori, et. al., 1977) O-Glucosylation of a flavonoid hydroxyl produces an upfield shift of 1.4-2.0 ppm in the signal of the adjacent flavonoid carbon, whereas the signal of ortho carbons are shifted downfield to a lesser extent (0.5-1.0 ppm) than the para signal which is shifted downfield (α-effect) by 1.5-1.8 ppm (Wenkert and Gottlieb, 1977). Glycosylation at either of the positions in 3′, 4′-dihydroxylated flavonoids produces upfield shifts of the respective glycosylated carbon analogous to those mentioned above. However, the α-effect on hydroxyl-bearing ortho carbons is more marked and significant than the other ortho unsubstituted carbons (Markham et al., 1978)

In the carbon-13 NMR spectrum of the sample (Figure 29), the signals of the B ring showed similar shifts to those mentioned above. The C-6′ signal which is on a para position in relation to the C-3′-O-glucose was shifted downfield by more than the 1.5 ppm of the ortho carbons C-2′ and C-4′. Table V shows the difference in the signals of the B ring of dihydroquercetin and those of dihydroquercetin-3′-O-glucopyranoside.

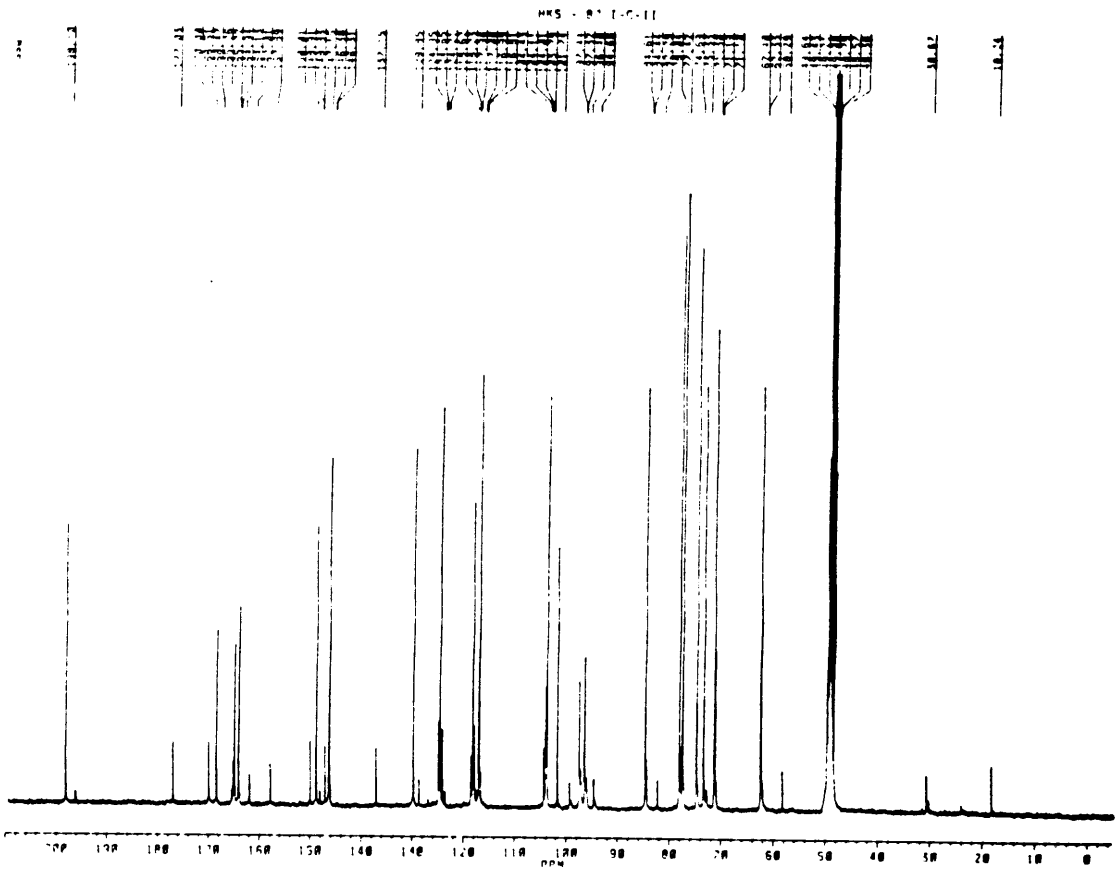


Figure 29. Carbon-13 NMR spectrum (acetone D_6) of dihydroquercetin-3'-O-glucopyranoside.

Table V. Comparison of the Chemical Shifts of the B Ring Carbons in Dihydroquercetin 3′-O-glucopyranoside

	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
DHQ	129.67	115.77	145.69	146.51	115.66	120.75
DHQ-G	129.85	118.23	146.31	148.83	116.99	124.65
-	-0.18	-2.46	-0.62	-1.72	-1.33	-3.90

DHQ; Dihydroquercetin.

DHQ G; Dihydroquercetin-3′-O-glucopyranoside.

-; Difference between DHQ and DHQ-G

The hydroxyl-bearing ortho carbon C-4′ had a more marked α-effect than the other ortho unsubstituted carbon, C-2′ as shown in Table V. The glycosylated carbon, C-3′, produced only a 0.62 ppm downfield shift which is less by 3.28 ppm than the C-2′, C-4′, C-5′ and C-6′ carbons.

Carbon-13 NMR spectra can be applied to determine the ring size of the sugar and its anomeric configuration (Bock and Pederson, 1974, 1975, Bock et al., 1973). Glycosylation of a sugar hydroxyl produces a sizable downfield shift in the resonance of this hydroxy-bearing carbon (Colson et al., 1974). Brewster et al. (1979) reported that the sugar C-1" signal of β-anomers appears at 100-102 ppm whereas the corresponding signal in α-anomers appears at ca. 95 ppm. In the carbon-13 NMR spectrum of the sample, the signal of the

C-1" appeared at 103.79 ppm. The result implied that the anomeric configuration of the glucose involved a β-linkage. The other carbon signals of the glucose unit were well matched with those reported by Cussans and Huckerby (1975).

3. Preparation of Dihydroquercetin from Douglas-fir Bark

3.1. Bark Extraction

Dihydroquercetin is a compound of central importance to the chemistry and biochemistry of procyanidins, the most widely distributed class of condensed-tannins. Recently (2R, 3R)-dihydroquercetin was a precursor in the synthesis of the (2R, 3S, 4R)-3,3',4,4',5,7-hexahydroxyflavan which is the intermediate compound in the polymerization of procyanidins (Porter and Foo, 1982).

The direct synthesis of well-characterized natural dimeric procyanidins from dihydroquercetin requires the isolation of considerable amounts of this chemical from natural sources. There is some information available on the isolation of dihydroquercetin (Hergert and Kurth, 1952; Kurth et al., 1948; Kurth and Becker, 1953; Kurth and Chan, 1953; Kurth et al., 1955; Kurth, 1956; Roberts and Gregory, 1958; Vercurysse et al., 1985). Dihydroquercetin is relatively difficult to purify by regular procedures because it is very unstable in most solvents. It is easily oxidized

and changed to quercetin in air or in solution by light. It is, therefore, very important to make pure crystals for easy storage and handling.

3.2. Purification of Dihydroquercetin

In the present work, the method describes the crystallization of dihydroquercetin from the bark of Douglas-fir by silica-gel column chromatography. It is generally accepted that silica-gel is a useful adsorbent for the separation of flavone aglycones with chloroform-methanol solvent mixtures (Karl et al., 1977; Higuchi and Donnelly, 1978; Tschesche et al., 1980) and it is shown here that it can be equally useful for the crystallization of pure dihydroquercetin.

In 1956, Kurth tried to purify dihydroquercetin for preparative purposes from the bark of Douglas-fir and Jeffery pine by a sulfite reaction. In this procedure the steps are complicated for a preparative method and dihydroquercetin is easily oxidized to quercetin in the aqueous solution during recrystallization. Quercetin, which is formed in aqueous solution, is very difficult to remove from crystalline dihydroquercetin by any method except the chromatographic method described in this work. Also, dihydroquercetin isolated from aqueous solution is very unstable to light and humidity. It was very important to find a method which can make crystalline dihydroquercetin.

The crystalline dihydroquercetin in this work is quite stable to light, humidity and air. The crystalline material was formed on elution from the column (Figure 12). Recrystallization failed with any other solvent system than that used in the column chromatography because of the instability of dihydroquercetin in solution. Dihydroquercetin is easily oxidized to quercetin and quercetin interferes with the crystallization.

The crystals were tested by melting point, TLC chromatography, UV, and NMR spectroscopy. The results are the same as those for dihydroquercetin described in the Results and Discussion, section 2.5.2.

4. Reaction of Dihydroquercetin with Tyrosinase

4.1. Reaction of Dihydroquercetin with Tyrosinase

The polymeric brown pigments produced by phenolase enzymes or other oxidative reactions of phenolic compounds in plant products have not been studied to a great extent. More attention has been paid to substrate and reaction conditions than to the relatively intractable brown product. As mentioned in the historical review, there are some investigations of substrates that produce discolorization of wood.

Compounds that contain catechol groups, such as catechin and dihydroquercetin, also act like free catechol with tyrosinase and these make brown pigments. It was necessary to establish an illustration of the reaction. In preliminary work, dihydroquercetin was reacted with tyrosinase and it changed color from colorless to a brownish color. In this section, therefore, the reaction of dihydroquercetin with tyrosinase was investigated in more detail.

Because dihydroquercetin, unlike catechin, was difficult to dissolve in water at room temperature (Hergert, 1962), the reaction conditions of Weinges et al. (1969) were modified by the addition of a small amount of acetone during the reaction. However, dihydroquercetin did not completely dissolve in the reaction mixture at first. After one day the color of the reaction mixture became yellow. A brown precipitate formed and so the reaction mixture contained two solids, the undissolved dihydroquercetin and the brown reaction product. The brown precipitate increased with time and the amount of solid dihydroquercetin decreased. Aliquots of the reaction solution were analyzed periodically by TLC (system i) for the presence of dihydroquercetin. When dihydroquercetin was no longer detected by TLC the reaction was stopped (30 days).

4.2. Separation of Reaction Products

The brown-colored precipitate was of interest because the stains on the surface of Douglas-fir sapwood could not be removed by ordinary extraction methods. The stains were very stable. The reacted solution was filtered to isolate the insoluble material, R (Figure 13).

After the separation of R, the solvent soluble portion was separated into three fractions by their differences in solubility in three different solvents which were ethyl acetate (F-2, Figure 13), methanol (F-1, Figure 13) and water (F-3, Figure 13). These three fractions were further investigated by various separation methods.

4.2.1. Gel-Permeation Chromatography

Analyses of each fraction were achieved by a GPC analytical system which consisted of a 100 angstrom micro-styragel column and a 500 angstrom phenogel column attached in tandem. Fraction F-1 (methanol soluble compounds, Figure 13) contained low molecular weight compounds (M.W. < 200) and oligomeric compounds. Fraction F-2 (ethyl acetate solubles, Figure 13) contained mainly low molecular weight compounds and a small amount of dimeric and trimeric compounds. Fraction-3 (water layer, Figure 13) contained mainly salts of the buffer solution, small amounts of

dimeric and trimeric compounds, and mostly oligomeric compounds.

Fraction F-1 (Figure 13) was investigated on Sephadex LH-20 columns with methanol-95% ethanol (1:1, v/v) as eluent. One compound which had a 14.88 min retention time on the GPC analytical system was separated (D-2, Figure 13). The other fractions were mixtures of compounds. The rest of the portion was extracted with ethyl acetate to extract low molecular weight compounds which had mainly 15.09-17.00 min retention times on the GPC analytical system.

The ethyl acetate soluble materials of F-1 (Figure 13) were combined with F-2 (Figure 13) and labeled Fraction F-4 (Figure 13). Because this had mainly quercetin-type compounds and the rest were unknown, a preliminary extraction was performed with diethyl ether to isolate quercetin (Figure 13). Most of the quercetin-type compounds were extracted into the diethyl ether. The diehyl ether soluble compounds had two distinct major peaks on the GPC analytical system. These had retention times of 15.13 and 14.14 min. Additional purification was achieved by chromatography on a Fractogel column with methanol. Samples, Q-1 and Q-2 (Figure 13), obtained in this way were freeze-dried to investigate their structures.

The diethyl ether insoluble compounds of F-4 (Figure 13) were chromatographed on a Sephadex LH-20 column with 95% ethanol. Most of the low molecular weight (150<M.W.<180) compounds were separated from the higher molecular weight

(M.W.>300) compounds. The eluent from the Sephadex LH-20 column was analyzed by the GPC analytical system and fractions were combined according to their retention times because TLC analyses was not satisfactory for these analyses. The fractionated samples, A-1, A-2, A-3 and A-4, (Figure 13) were collected for the purpose of investigation of structures. Samples obtained in this way were freeze-dried.

The higher molecular weight compounds remained on the column from the above chromatography and they were removed by eluting the column with methanol (Figure 13). The methanol washings were further separated on a Fractogel column with methanol as eluent. There were several different compounds in the eluent. The amount of samples, however, were less than 2 mg each except for two samples. The retention time of the samples were 13.87, 13.43, 14.14, 14.67, and 15.13 min. A residual trace amount of dihydroquercetin was also detected with a 15.02 min retention time. Since the samples always contained small amounts of impurities, there was no possible way to identify their structures. Samples which had retention times of 14.14 and 15.13 min were combined with the rest of the diethyl ether soluble materials. After the samples were dried, the retention times of the samples changed to 14.34 and 15.40 min. These are listed as Q-1 and Q-2 (Figure 13).

The water solubles (F-3, Figure 13) were extracted with methanol after evaporation of the water. The methanol-

soluble portion still contained large amounts of salts so it was extracted with absolute ethanol and filtered through a microfilter system (0.2 micrometer pore size). After most of the salts were eliminated in this way, the samples were chromatographed on a Fractogel column with methanol-95% ethanol (1:1, v/v) as eluent. The low-molecular weight compounds were eluted first, then one major compound which had a 14.06 min retention time was collected. The collected sample was freeze-dried for further investigation (D-3) (Figure 13).

4.3. Identification of Reaction Products

The air-dried residue R (Figure 13) had distinctive characteristics. It was blackish in color and had very low solubility in common solvents. Therefore, it was very hard to define the exact structure by normal methods. The only possible methods were UV and IR spectroscopic methods.

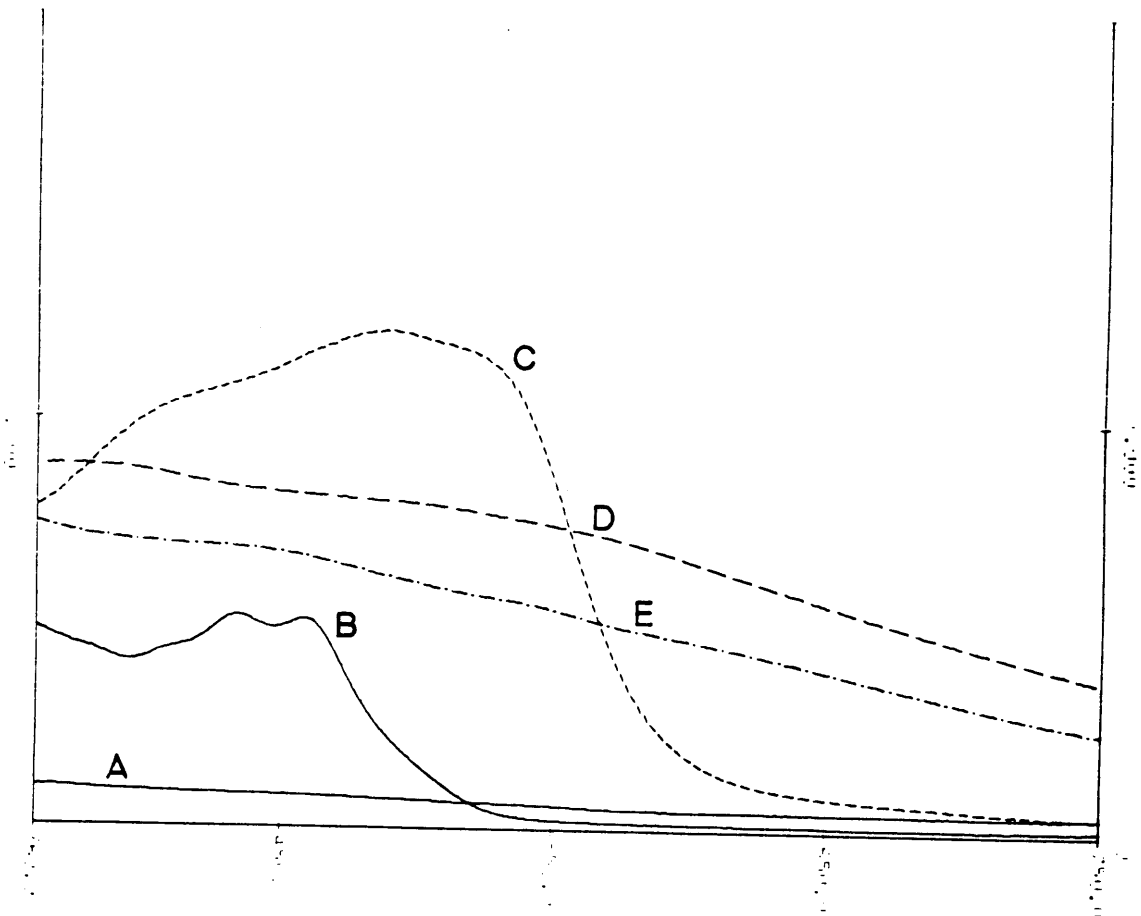
Stained sapwood of Douglas-fir was also tested with various solvents in an attempt to solubilize the color. There was no evidence of extraction of the colored material from the wood surface. To identify and compare the stained sapwood with R (Figure 13), it was necessary to make potassium bromide (KBr) pellets. The colored portion of the wood was scraped with a razor blade to obtain small particles of stained sapwood (SW). Blois (1969) and two others (Wilczok et al., 1984; Garcia-Borron et al., 1985)

used this method to investigate melanin by making KBr pellets.

R (Figure 13) and SW had similar characteristics when investigated by UV spectroscopy. Figure 30 shows a general absorption in the visible region for the KBr pellets of R (Figure 13) and SW. The IR spectra of R and SW (Figures 31, and 32) showed more clearly the relationship between them. Basically the difference is the amount of acetal and-or ketal groups in the 1064 cm^{-1} range due to celullose and other carbohydrates in the wood sample.

Figure 33 shows the difference between the spectrum of stained wood and the spectrum of regular wood. As shown in Figure 33 the strong C-0 stretching in the 1064 cm^{-1} region comes from normal wood pieces. The samples were collected from the same piece of wood that developed stain on the surface. The carboxylic pattern of SW (1650-1720 cm^{-1} region) might be a little different from that of R because R was prepared under better conditions of oxidation than the naturally developed stain.

The samples, A-1, A-2, A-3 and A-4 (Figure 13) were investigated structurally by NMR spectroscopy. It was found that A-4 was reasonably pure and that A-1 through A-3 were mixtures of A-4 and other compounds. The proton and carbon-13 NMR spectra of A-1 and A-4 are shown in Figures 34, 35, 36, and 37. A comparison of the spectra of A-1 and A-4 revealed that A-1 contained A-4 in the same amount as A-1. Figure 38 shows the correlation between proton bearing



A: KBr pellet
B: Yellow paper
C: Orange paper
D: Sample R
E: Sample SW

Figure 30. UV spectra of the polymer R resulting from the reaction of dihydroquercetin with tyrosinase, and the stained wood (SW).

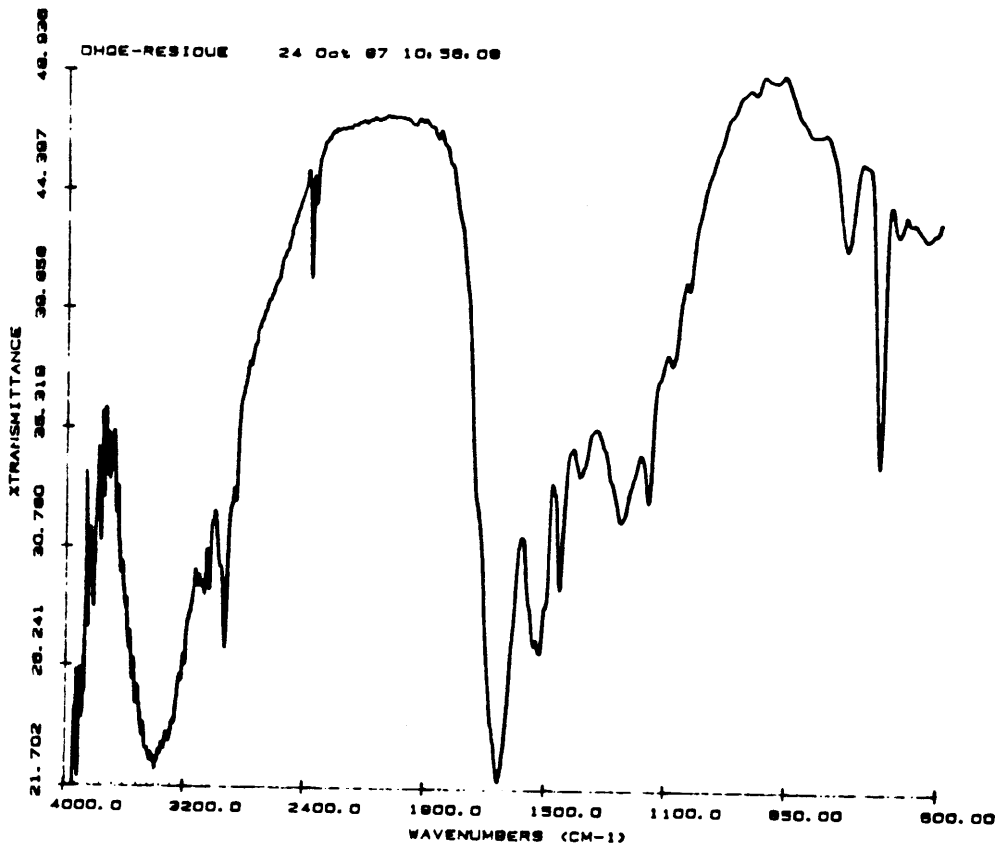


Figure 31. IR spectrum of the polymer R of dihydroquercetin reaction products with tyrosinase.

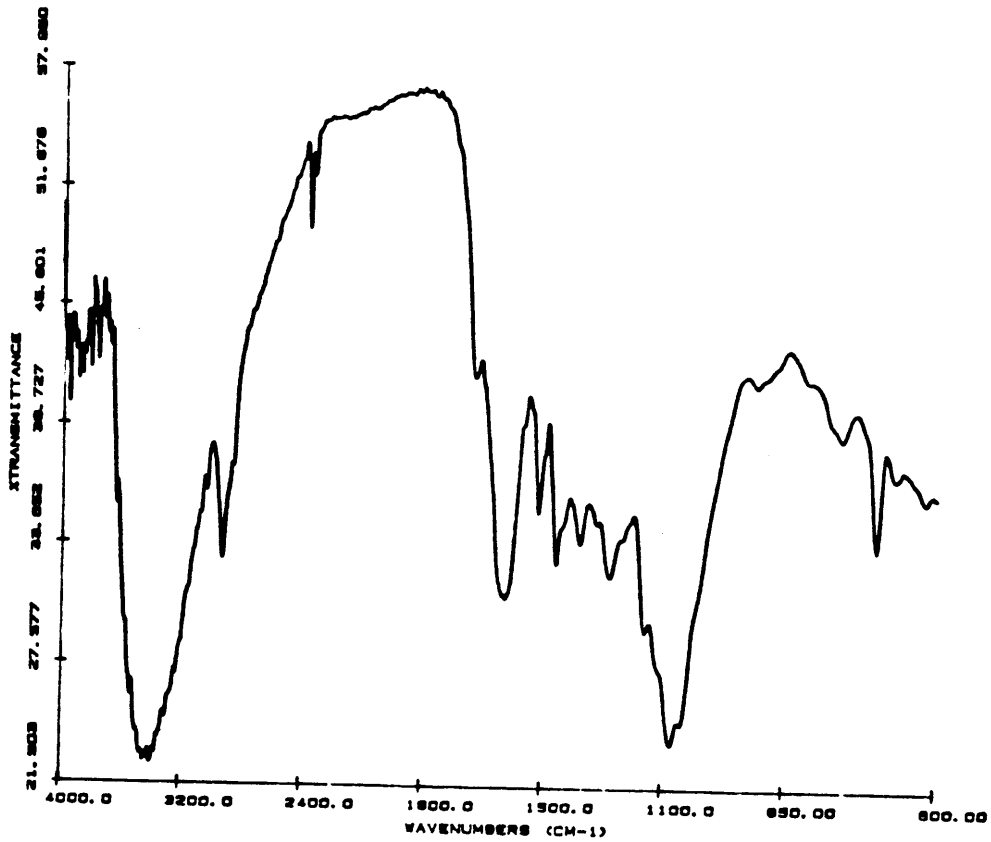
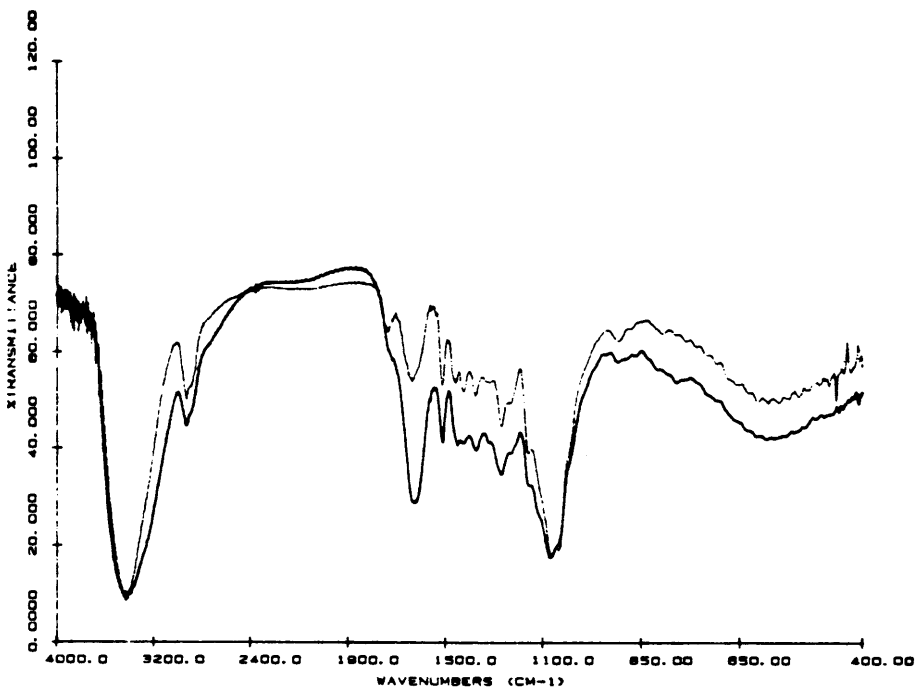


Figure 32. IR spectrum of stained wood.



A: Stained wood (SW)
B: Normal wood.

Figure 33. IR spectrum of stained wood and of unstained Douglas-fir sapwood.

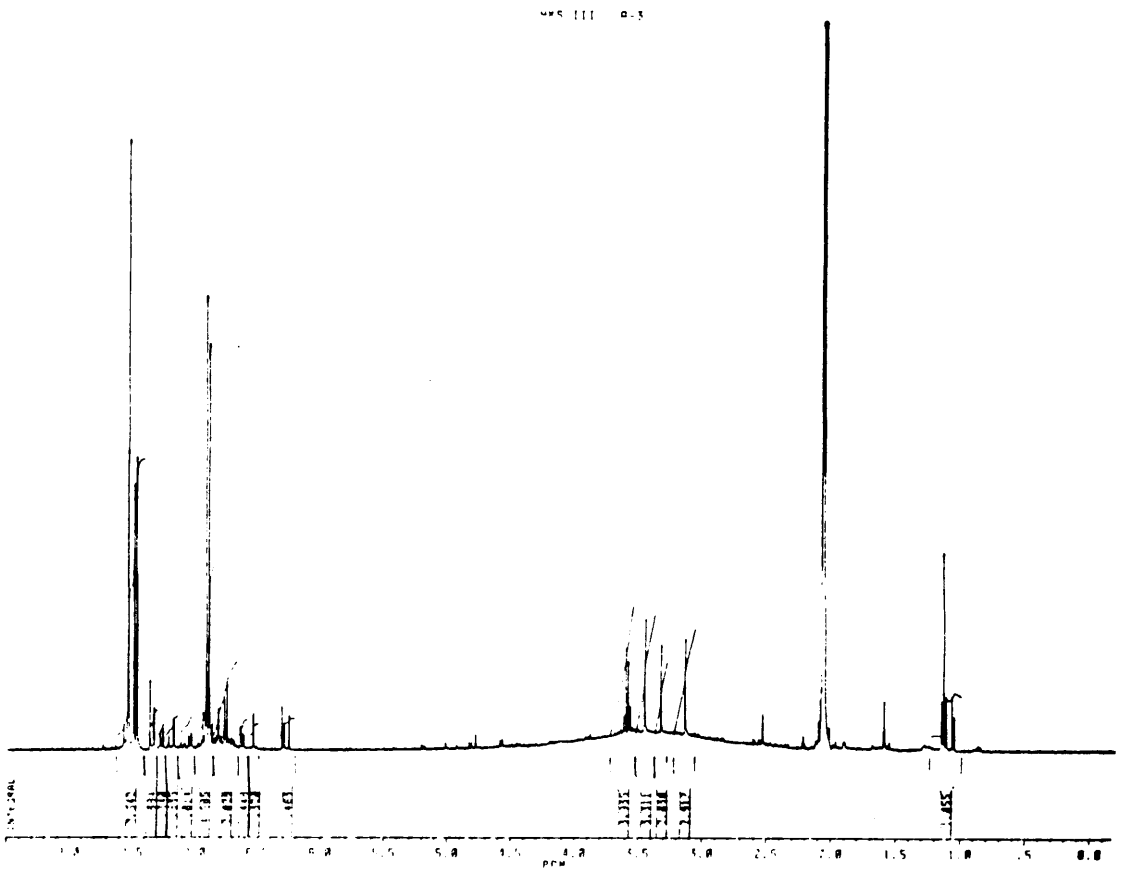


Figure 34. Proton NMR spectrum (acetone D_6) of sample A-4 (3,4-dihydroxybenzoic acid).

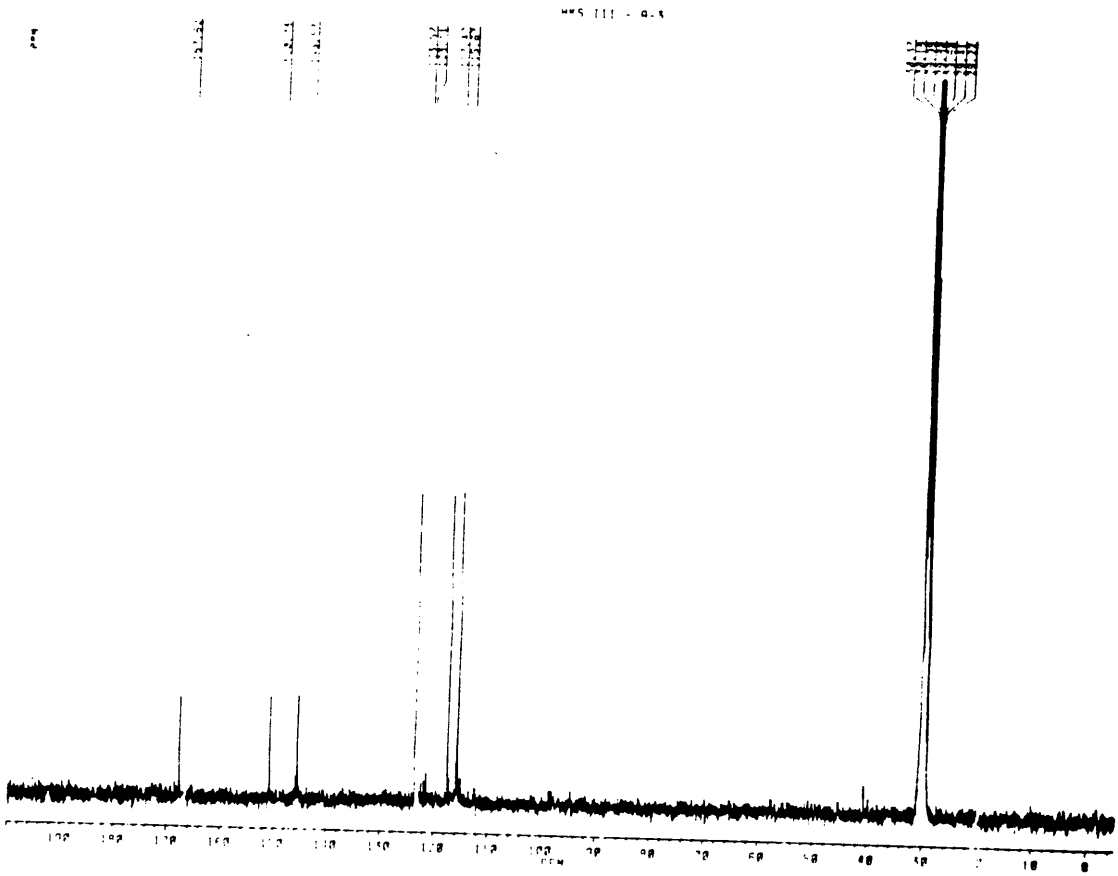


Figure 35. Carbon-13 NMR spectrum (acetone D_6) of sample A-4 (3,4-dihydroxybenzoic acid).

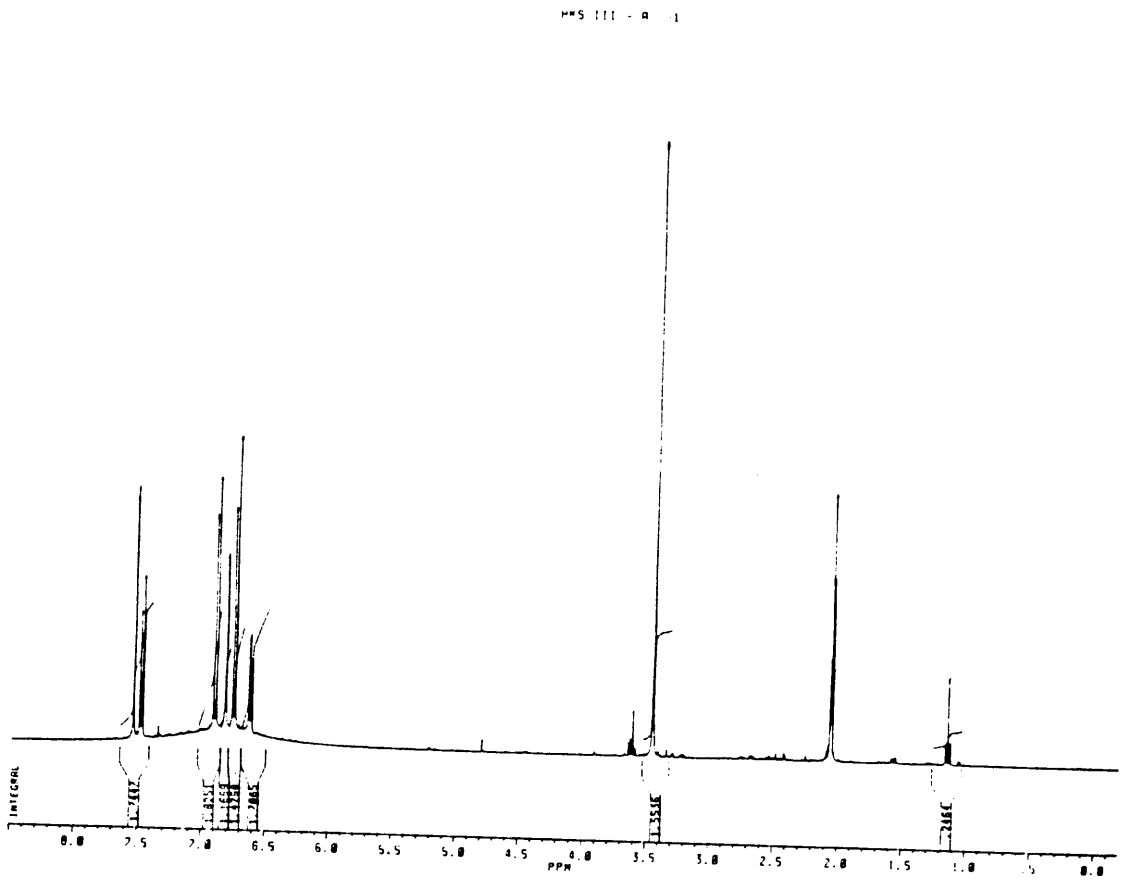


Figure 36. Proton NMR spectrum (acetone D_6) of sample A-1 (3,4-dihydroxyphenylacetic acid and 3,4-dihydroxybenzoic acid).

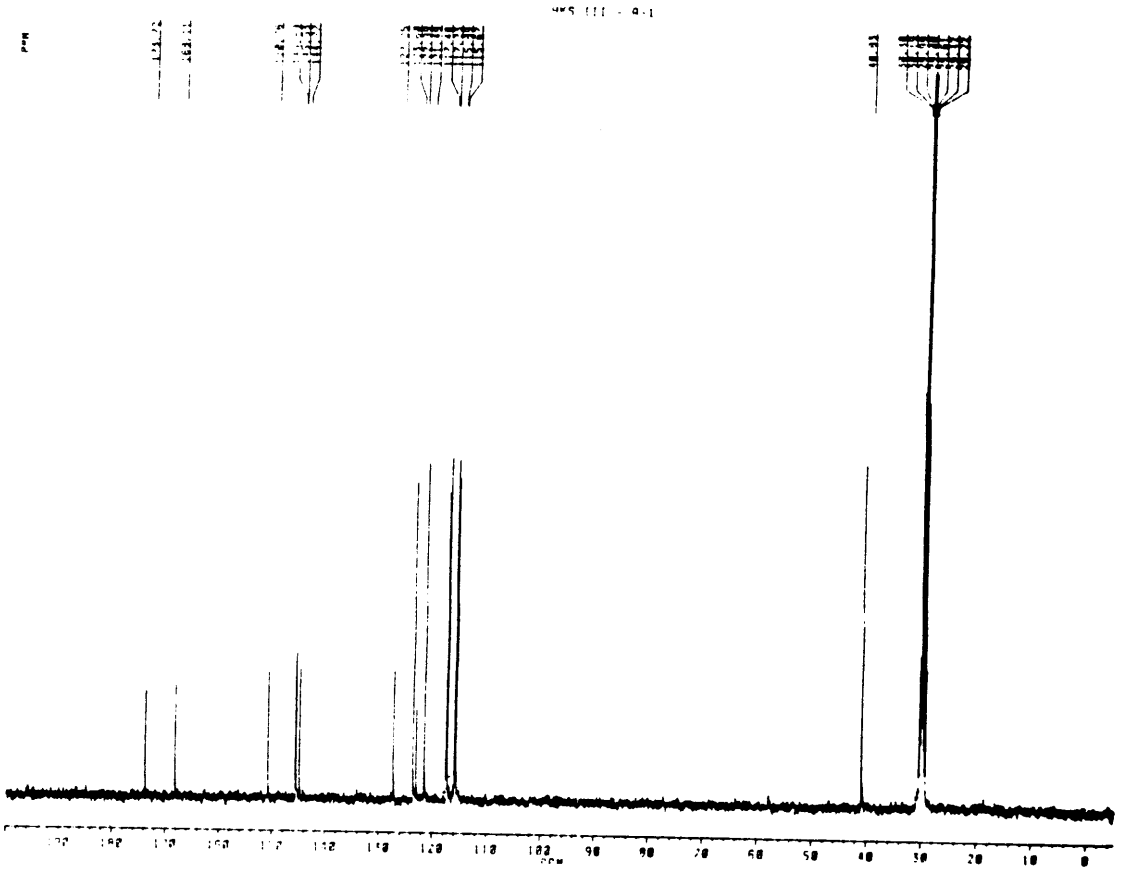


Figure 37. Carbon-13 NMR spectrum (acetone D_6) of sample A-1 (3,4-dihydroxyphenylacetic acid and 3,4-dihydroxybenzoic acid).

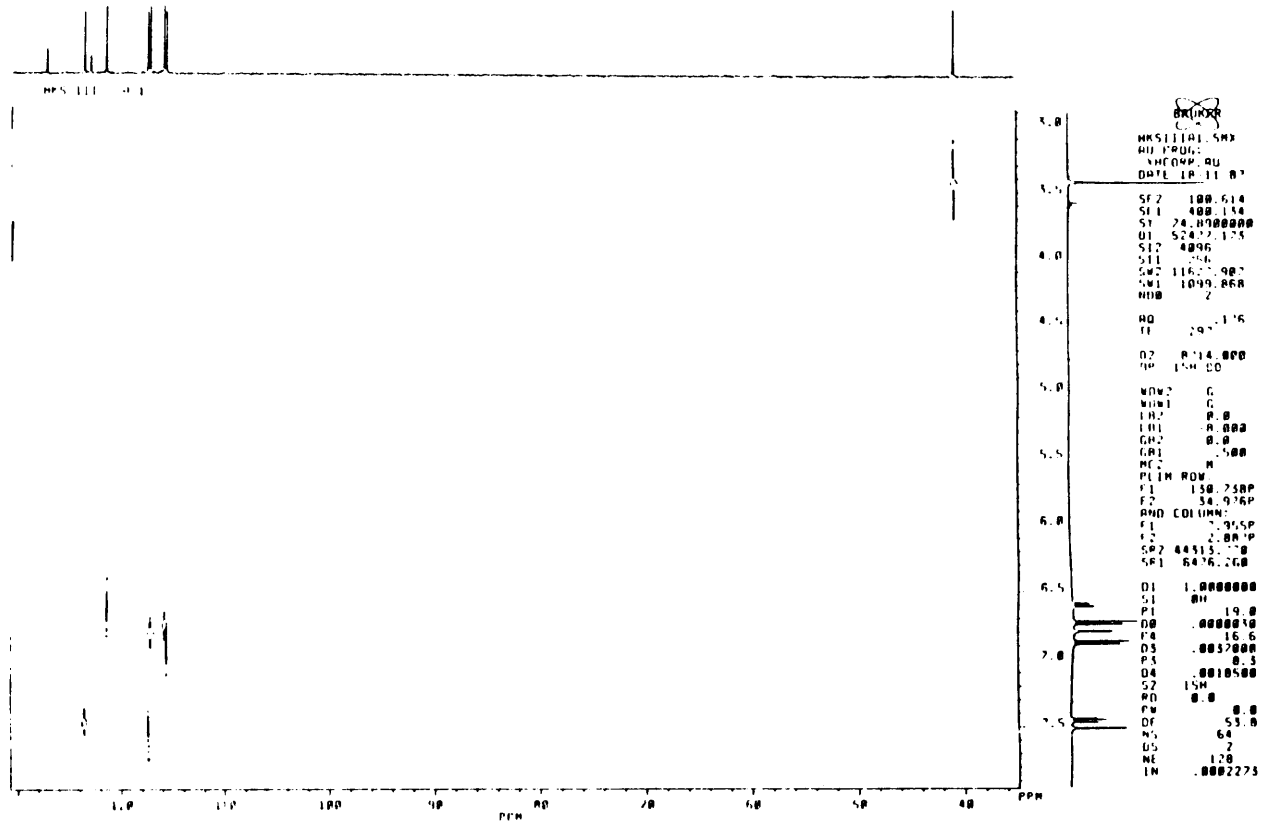


Figure 38. Heteronuclear correlated (HETCOR) spectrum of sample A-1 (3,4-dihydroxybenzoic acid and 3,4-dihydroxyphenylacetic acid).

carbons and protons. The three benzene ring carbons, 117.47, 123.11, and 115.67 ppm, which come from A-4 were correlated with three protons, 7.51, 7.46, and 6.89 ppm, respectively. The other three benzene ring carbons at 115.89, 121.49, and 117.23 ppm and the methylene carbon at 40.83 ppm were correlated with protons at 6.7, 6.6, and 6.81 ppm, respectively. Figure 39 shows the types of carbons and the carbon-13 NMR spectrum also shows the number of carbons. From this information it was concluded that compound A-4 had seven carbons and that three of them bore one proton each and that four tertiary carbons were present. The IR spectra of A-1 and A-4 (Figures 40, and 41) showed a carboxyl group at 3500-2500, 1707.8, 1405, 1293 and 1282 (doublet) cm^{-1}. One tertiary carbon was derived from the carboxylic carbon and the other three carbons were from the benzene ring. The UV spectra of A-1 and A-4 (Figures 42, and 43) showed that the compound had an ortho-dihydroxy group on the benzene ring because the treatment with NaOAc-H_3BO_3 caused a bathochromic shift of +14 (Markham, 1983). According to these results, the compound was a dihydroxybenzoic acid. The calculation of chemical shifts by empirical rules for NMR spectra give the last information needed to assign the position of attachment of the hydroxyl groups on the benzene ring. The structure of A-4 is 3,4-dihydroxybenzoic acid. Because of the similarity of the two compounds that was evident from the NMR, UV, and IR spectra (Figures 34, 35, 36, 37, 40, 41, 42, and 43) the structure

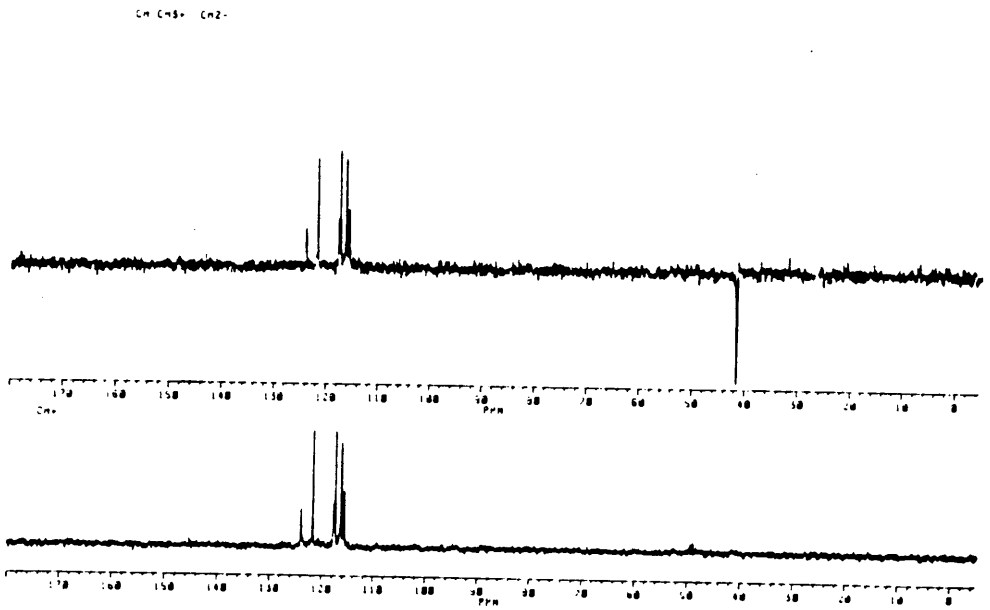


Figure 39. DEPT of sample A-1 (3,4-dihydroxybenzoic acid and 3,4-dihydroxyphenylacetic acid).

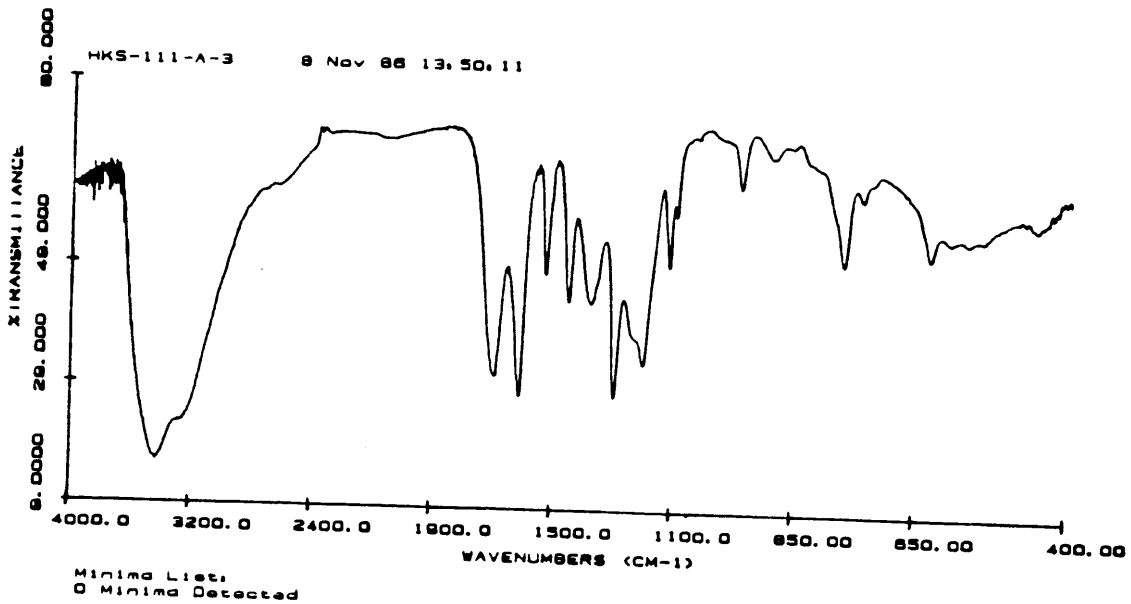


Figure 40. IR spectrum of sample A-4 (3,4-dihydroxybenzoic acid).

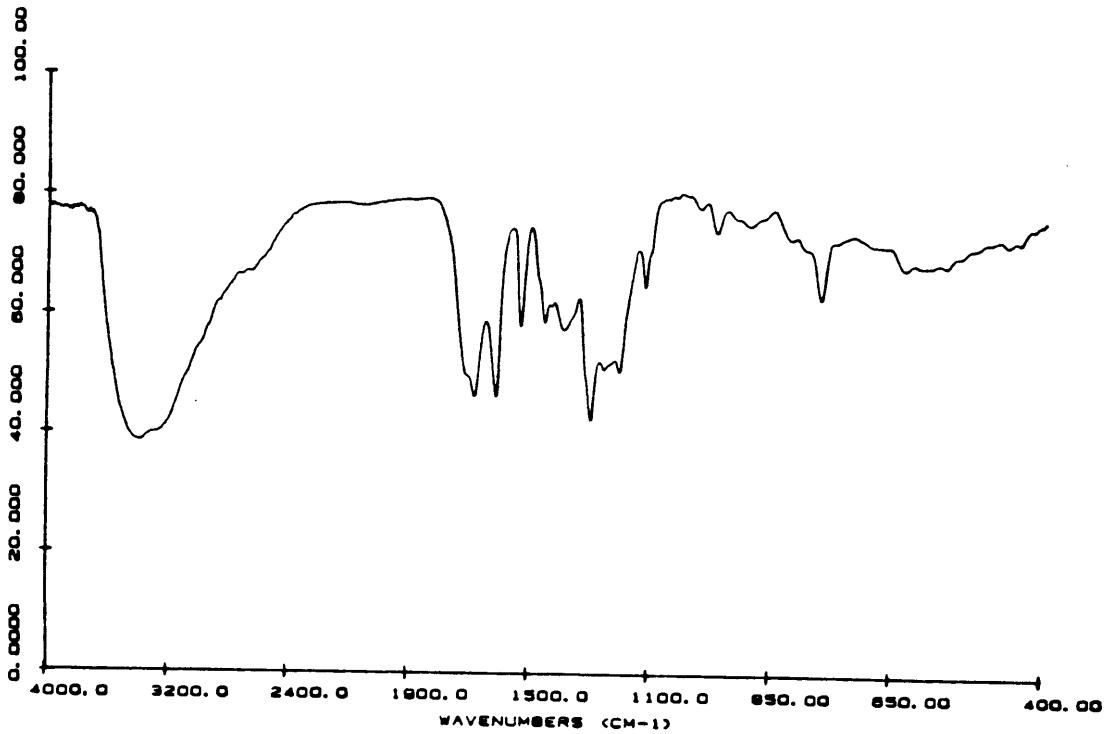
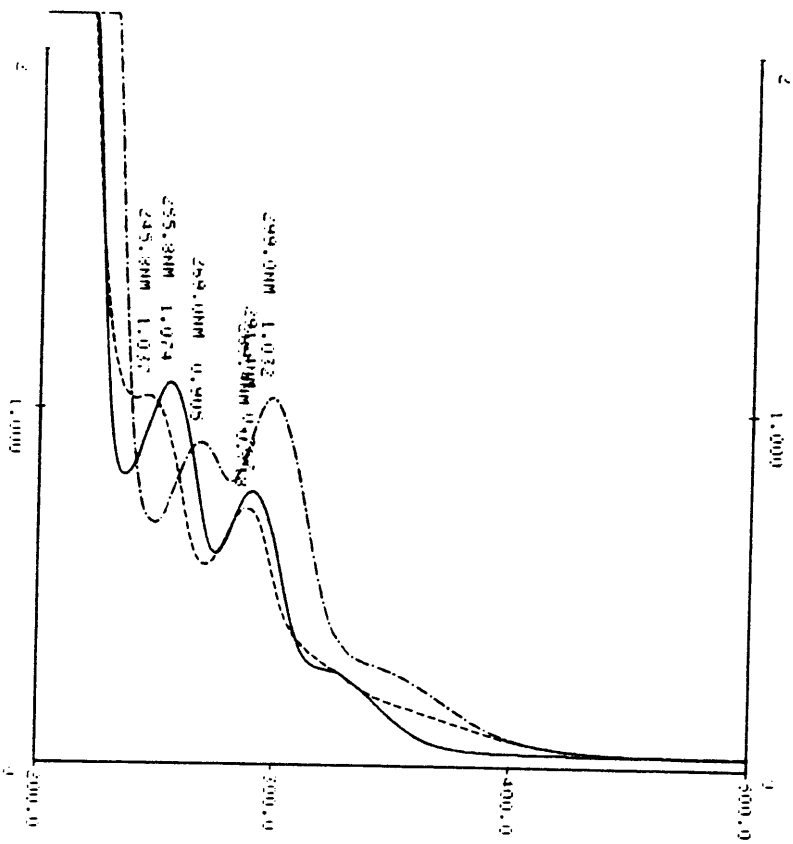
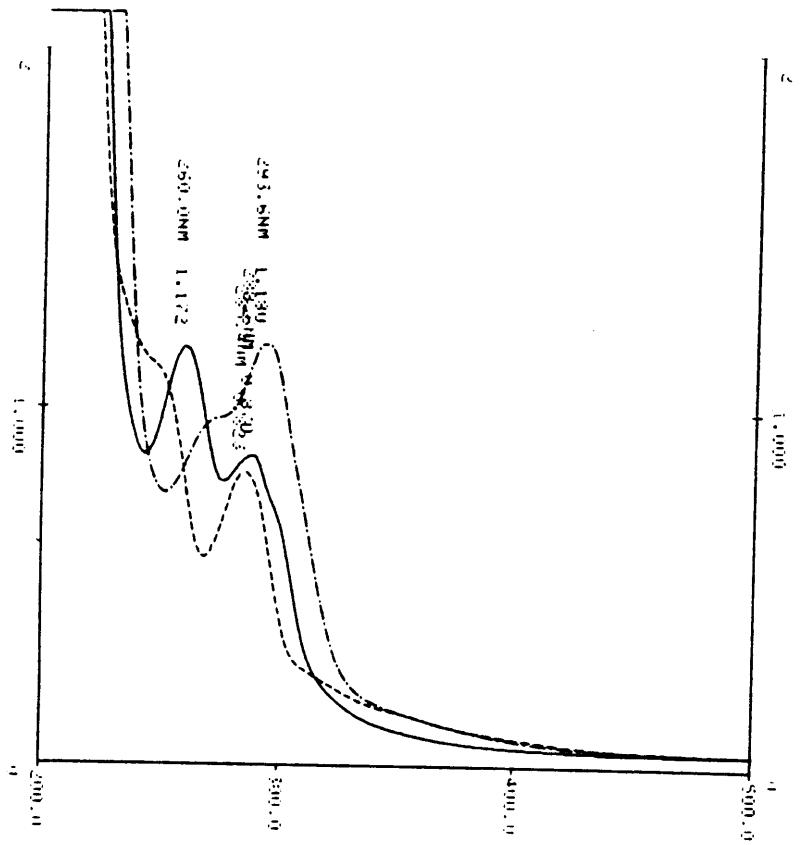


Figure 41. IR spectrum of sample A-1 (3,4-dihydroxyphenyl-acetic acid and 3,4-dihydroxybenzoic acid).



——— : 3,4-dihydroxybenzoic acid in methanol
----- : 3,4-dihydroxybenzoic acid in methanol with sodium acetate
-·-·- : 3,4-dihydroxybenzoic acid in methanol with sodium acetate and boric acid

Figure 42. UV spectra of sample A-1 (3,4-dihydroxybenzoic acid) with sodium acetate with and without boric acid.



——— : A-1 in methanol
----- : A-1 in methanol with sodium acetate
-·-·-· : A-1 in methanol with sodium acetate and boric acid

Figure 43. UV spectra of sample A-1 (3,4-dihydroxybenzoic acid and 3,4-dihydroxyphenylacetic acid) with and without boric acid.

of the second compound in A-1 was easily deduced from the NMR spectra by calculation of the chemical shifts. The structure of this second compound in A-1 was 3,4-dihydroxyphenylacetic acid. The structure of 3,4-dihydroxybenzoic acid was confirmed by TLC and GPC analyses of authentic 3,4-dihydroxybenzoic acid. The R_f values and retention times were the same for A-4 and authentic 3,4-dihydroxybenzoic acid. The proton NMR spectrum of A-4 was the same as for authentic 3,4-dihydroxybenzoic acid (The Aldrich library of NMR spectra, 1986).

According to these results, dihydroquercetin was degraded by tyrosinase during the reaction. The cleavage pattern of degradation is shown in Figure 44.

The diethyl ether soluble portion (Figure 13) showed two interesting phenomena. It contained mainly two compounds with retention times of 14.14 and 15.13 min by the GPC analytical system. After the compounds were dried in air, the retention times were changed to 14.34 and 15.40 min on the GPC analytical system. The diethyl ether soluble compounds were chromatographed on a Fractogel column with methanol (Figure 13). The two compounds were separated into peaks which were labeled Q-1 (retention time, 15.40 min by the GPC analytical system) and Q-2 (retention time, 14.34 min by the GPC analytical system).

A phenomenon of interest occurred during the NMR analyses of sample Q-2 (Figure 13). Figure 45 is the proton NMR spectrum of freshly dissolved Q-2 in methanol D_4. It

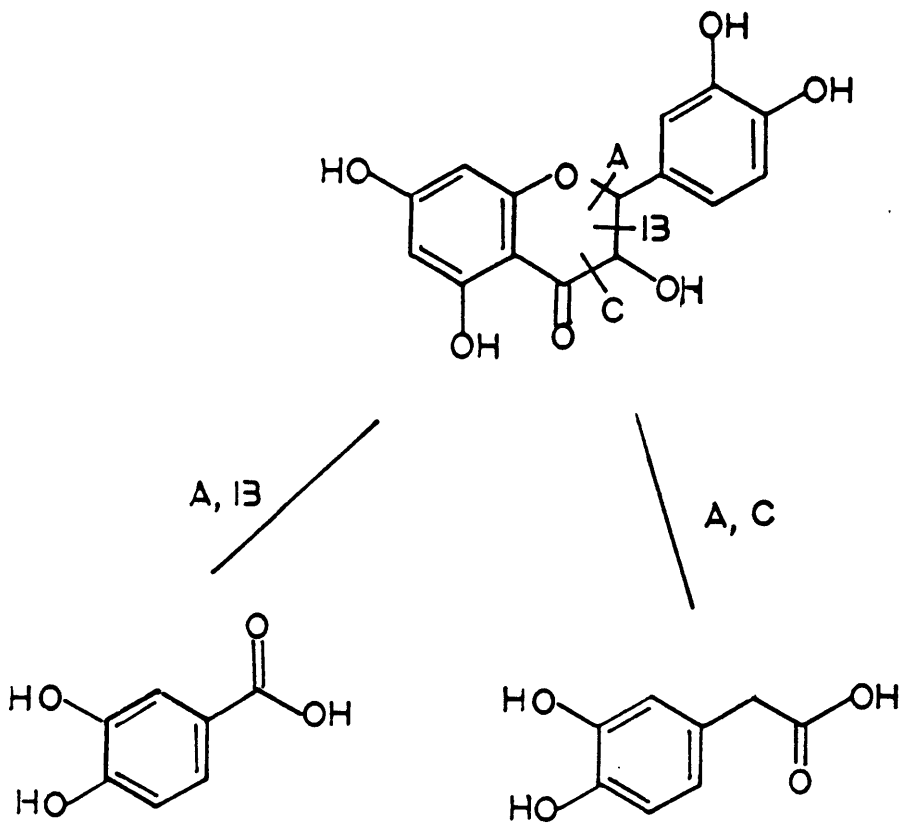


Figure 44. Degradation pattern of dihydroquercetin by tyrosinase.

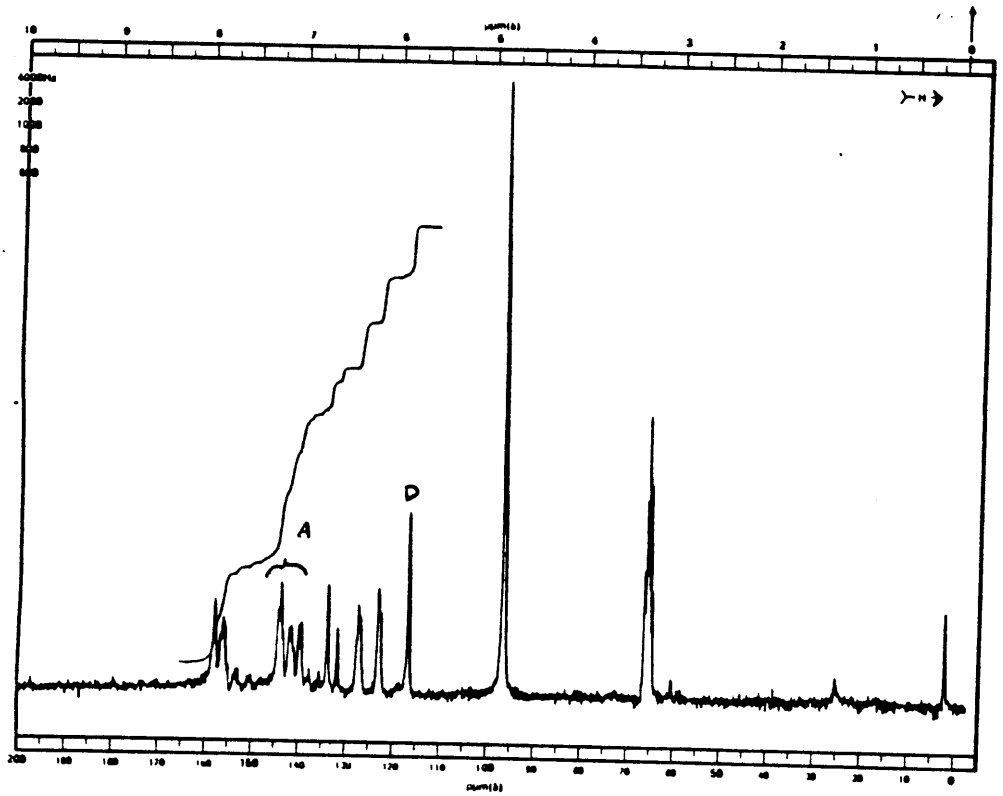


Figure 45. Proton NMR spectrum (methanol D_4) of Q-2 (Figure 13).

was necessary to wait for about one week to obtain a carbon-13 NMR spectrum of the same sample. At the end of the waiting period the sample, still in the NMR tube and still in methanol D_4, had changed color from yellow to brown and a precipitate had formed. The proton NMR was again obtained on the same sample in the same NMR tube (Figure 46). Clearly the original Q-2 material had undergone change. The changed material was poured from the NMR tube and investigated.

Figure 47 is the analytical GPC chromatogram of freshly dissolved Q-2 and shows one major peak at a retention time of 14.35 min. The retention time and the NMR spectra indicated that the original Q-2 material was a dimeric compound. The evidence for this was derived from the proton NMR spectra of Figures 45 and 48. In Figure 45, the typical quercetin chemical shifts were easily identified by comparison with the proton NMR spectrum of quercetin (Figure 48). The chemical shifts were slightly changed and H-6′ and H-2′ were slightly downfield and H-5′, H-8 and H-6 were slightly upfield. The coupling patterns of H-6′, H-2′, H-8 and H-6 were also changed to multiplets in Figure 45 as compared to Figure 48. The coupling for H-5′ was not changed. These alterations of the proton NMR shift positions reinforced the concept that the original Q-2 material was a dimer, but with considerable quercetin character so that one of the units of the dimer was most likely quercetin. The other unit of the dimer possessed an

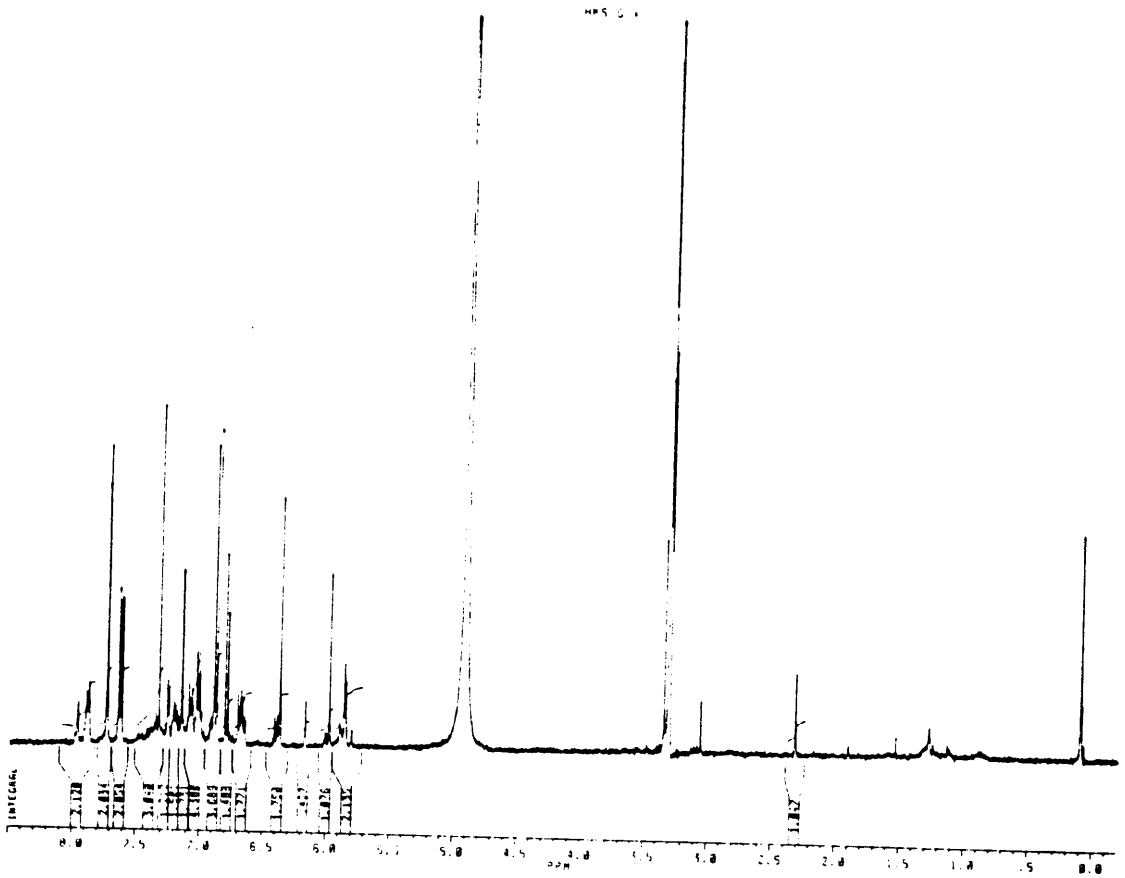


Figure 46. Proton NMR spectrum (methanol D_4) of Q-2 after standing for one week.

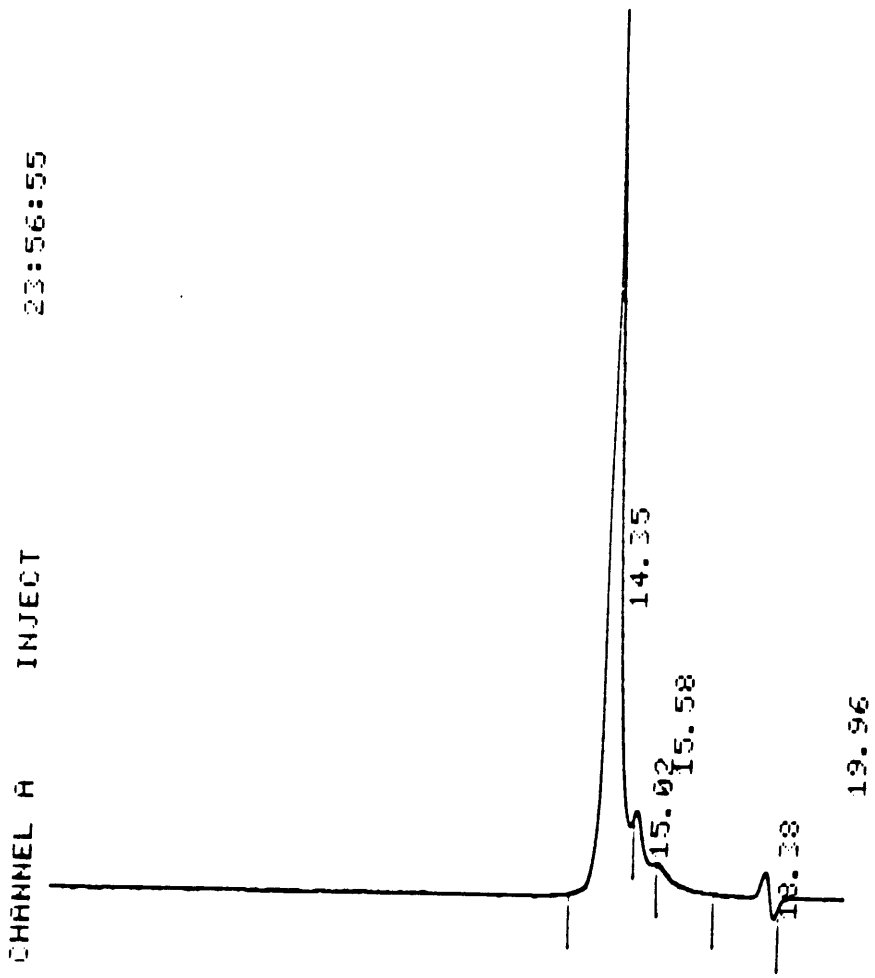


Figure 47. GPC spectrum of Q-2 (Figure 13).

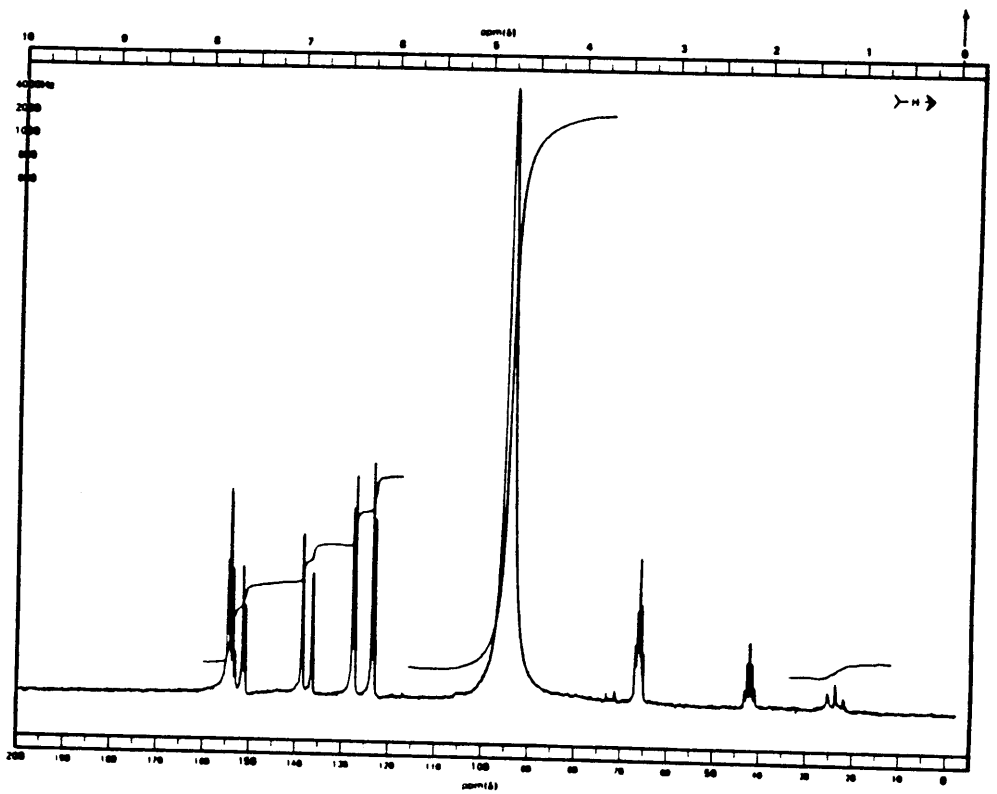


Figure 48. Proton NMR spectrum (methanol D_4) of sample Q-1 (quercetin).

open C-ring, possibly of the chalcone type derived from dihydroquercetin. The chalcone-type unit may be attached to quercetin through either its C-6 or C-8 carbon. This chalcone-type unit had dihydroquercetin characteristics except for the C ring. In Figure 45, the peaks designated as A were the chemical shifts of the B-ring pattern of dihydroquercetin except they were slightly downfield the same as for the quercetin unit. Peak D (Figure 45) was well matched with the calculated position of the A-ring proton of the phloroglucinol ring form according to empirical rules. Because the C-ring proton did not show in the correct position for dihydroquercetin it implied that the C ring of dihydroquercetin was changed.

Figure 49 is the GPC analytical spectrum of the material that had been in methanol D_4 for one week. The retention time of 15.42 min (Figure 49) was the same as that for authentic quercetin so the original Q-2 material had undergone reaction to produce quercetin plus other substances. The peak at 14.19 min retention time (Figure 49) was possibly due to the original Q-2 but in an ortho-quinone form because the retention time was the same as the retention time of Q-2 it was before dried. The peak at 14.96 min in Figure 49 may be due to a chalcone-type compound in which one of the units came from Q-2. The earliest peak to elute, 13.58 min retention time (Figure 49) is possibly a trimeric-type compound.

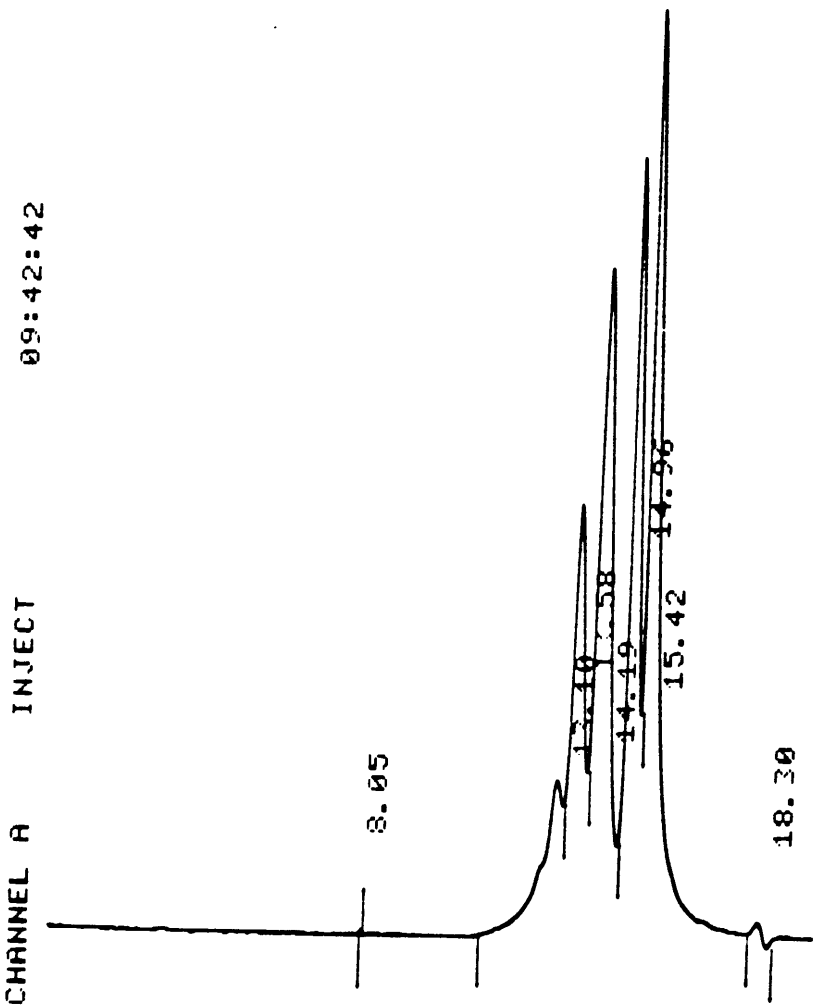


Figure 49. GPC spectrum of Q-2 after standing for one week.

Unfortunately sample Q-2 changed and polymerized on standing so that a meaningful carbon-13 NMR could not be obtained. Therefore, it was not possible to identify the linkage pattern between the quercetin and dihydroquercetin-type compounds. However, a possible scheme for the formation of these materials is shown in Figure 50.

4.4. Reaction of Dihydroquercetin with Tyrosinase on Douglas-fir Sapwood

When unstained Douglas-fir sapwood was first treated with dihydroquercetin and then tyrosinase a brown stain appeared on the wood surface within 30 minutes. Unstained sapwood was treated with tyrosinase only and no significant coloration developed. This indicated that dihydroquercetin is a likely precursor to the stains. Both dihydroquercetin and an enzyme appear necessary to bring about staining. Since the wood was open to the air and the room's humidity it is quite likely that dihydroquercetin, an enzyme, oxygen and moisture are all necessary for stains to develop.

4.5 Discussion

The IR spectra of the black polymers from animals, plants, and the autoxidation of catechol, hydroquinone, or dopa are all very similar (Andrews and Pridham, 1967; Blois, 1969; Wilczok et al., 1984; Garcia-Borron et al., 1985).

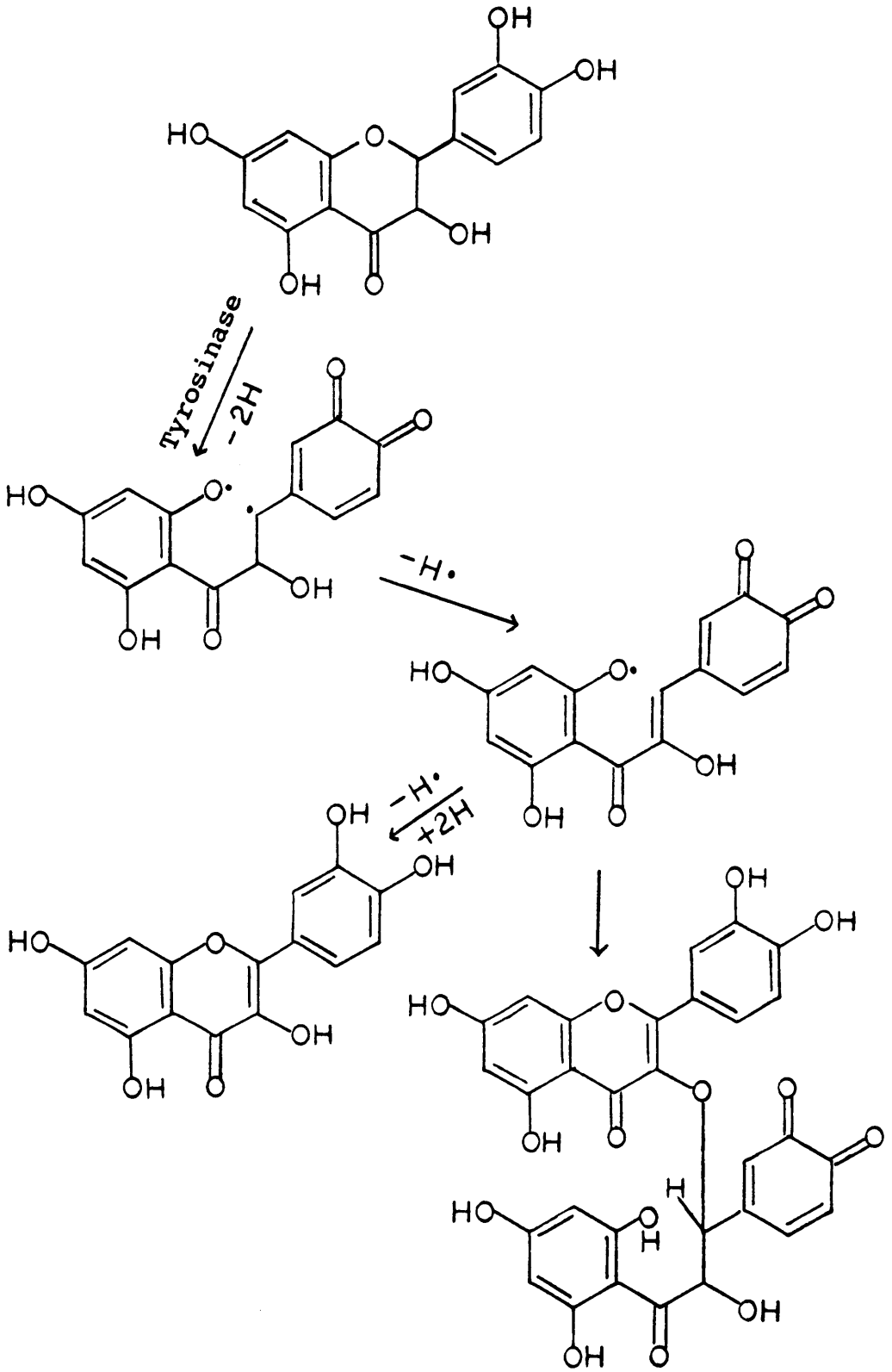


Figure 50. Scheme for the proposed products of the reaction of dihydroquercetin with tyrosinase.

Quinone bands, which are shown in the IR spectra (Figures 31 and 32) in the 1650-1600 cm^{-1} region, are present. Absorption bands attributable to carboxyl are present in the natural plant pigments and are weak but not absent with autoxidized catechol (Singleton, 1972). In the present work, a weak absorption band of carboxylic acid appeared as a shoulder peak in the 1700 cm^{-1} region in Figure 31.

Several studies have been carried out to investigate the structure of brown pigments in plant. Alkaline fusion of the natural fungal and higher plant black pigments has produced mostly 3,4-dihydroxybenzoic acid (protocatechuic acid), 2-hydroxybenzoic acid (salicylic acid) and catechol as the main products (Andrews and Pridham, 1967; Nicolaus, 1966; Nicolaus et al., 1964; Piattelli, et al., 1965). These are also the products of alkaline fusion of the pigment produced by alkaline autoxidation of catechol (Nicolaus, 1966). Salicylic acid is believed to result from fission of partial structures including ether linkages (Piattelli, et al., 1965).

In the present work, 3,4-dihydroxybenzoic acid and 3,4-dihydroxyphenylacetic acid were found as free compounds in the reaction products of dihydroquercetin and tyrosinase. There is no sign of phloroglucinol in the original reaction products by TLC analyses. The phloroglucinol unit was found in a dimeric compound (Q-2, Figure 13). Andrews and Pridham (1967), and others reported that phloroglucinol was not

found in the pigment of several plant species (Nicolaus, 1966; Nicolaus, et al., 1964).

5. SUMMARY AND CONCLUSIONS

1. Neither oregonin nor catechin were precursors to the phenolic stains in Douglas-fir sapwood.
2. Douglas-fir sapwood was extracted with methanol and the methanol solubles were fractionated by solvent partition, TLC, silica-gel column chromatography, and Sephadex LH-20 column chromatography.
3. Tyrosinase, a commercially available catechol oxidase enzyme, was used to locate and trace the precursors to color formation in the methanol-soluble materials of Douglas-fir sapwood.
4. The well known inhibitors, ethylenedinitrillotetraacetic acid disodium salt (EDTA), phenylthiourea, mercaptobenzothiazole, and Chloramine-T proved ineffective in preventing color formation when the methanol soluble materials from Douglas-fir sapwood were treated with tyrosinase.
5. Dihydroquercetin was shown to be a precursor to the stain in Douglas-fir sapwood.
6. A dihydroquercetin glycoside identified as the previously known dihydroquercetin-3'-O-glucopyranoside was also found in the methanol-solubles of Douglas-fir sapwood.
7. An improved method of isolating dihydroquercetin from Douglas-fir bark was developed. The procedure used silica-gel column chromatography and sequential elution

with chloroform-methanol (v/v) solvents (100%, 10:1, 4:1, each 300 ml). Evaporation of the solvent yielded crystals of pure dihydroquercetin.

8. Pure dihydroquercetin was reacted with tyrosinase in a citrate buffer at pH 5.6. The dihydroquercetin slowly disappeared and a dark brown polymeric precipitate formed.

9. The brown polymer was recovered and was shown to have a number of characteristics similar to the stains in Douglas-fir sapwood by UV and IR spectroscopy.

10. The known compounds, 3,4-dihydroxybenzoic acid (protocatechuic acid), and 3,4-dihydroxyphenylacetic acid, were found in the reaction products of dihydroquercetin and tyrosinase. They were considered to be degradation products of dihydroquercetin and possible intermediates in polymeric stain formation.

11. Several of the soluble products of the reaction between dihydroquercetin and tyrosinase had short lifetimes and underwent additional reactions in water and methanol. They were considered to be intermediates in polymeric stain formation and to be dimers, trimers and other oligomers of dihydroquercetin and dihydroquercetin derivatives.

12. Unstained Douglas-fir sapwood was sprayed with dihydroquercetin solution and then treated with tyrosinase. Stains developed within 30 minutes, thus

lending support to the concept that dihydroquercetin reacts with enzymes in the sapwood to produce stains.

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