

AN ABSTRACT OF THE THESIS OF

HENRY HAI-LOONG FANG for the MASTER OF SCIENCE  
(Name of student) (Degree)

Wood Science  
in (Wood Chemistry) presented on April 20, 1971  
(Major) (Date)

Title: DOUGLAS-FIR BARK; n-HEXANE-SOLUBLE FRACTION

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

Douglas-fir bark was extracted with n-hexane yielding a light colored, "wax-like" solid as the n-hexane-soluble fraction. The chemical constituents of this n-hexane-soluble fraction were partially resolved by column chromatography using Silica Gel G as stationary phase and chloroform-n-hexane (3:1 v/v) as developing solvent. Several bands were observed under ultraviolet light. Two narrow bands which were close to each other, but well separated from the other bands, were collected and designated as the "yellow band."

The chemical constituents of the "yellow-band" were partially separated by thin-layer chromatography using 14 different solvent systems. Of these, chloroform-carbon tetrachloride (6:1 v/v) and diethyl ether-n-hexane (1:4 v/v) were the best. Fourteen compounds were detected. Two of the compounds were identified as  $\beta$ -sitosterol and campesterol.

The "yellow band" fraction was well resolved by gas-liquid chromatography using four different liquid phases and a variety of temperatures. The liquid phases SE-52, OV-17, and UC-W98 provided good resolution, but the phase Hi-EFF 8BP showed poor separation. Gas-liquid chromatography showed the presence of at least 20 compounds, two of which were identified as  $\beta$ -sitosterol and campesterol.

Positive identification of the presence of  $\beta$ -sitosterol and campesterol in the "yellow band" fraction was accomplished by gas-liquid chromatography combined with rapid scan mass spectrometry. The data from the mass spectra also indicated the presence of other sterols as well as several terpenes. The presence of terpenes in the n-hexane soluble fraction of Douglas-fir bark has not been previously reported.

DOUGLAS-FIR BARK; n-HEXANE-  
SOLUBLE FRACTION

by

Henry Hai-Loong Fang

A THESIS

submitted to

Oregon State University

in partial fulfillment of  
the requirements for the  
degree of

Master of Science

June 1971

APPROVED:

Signature redacted for privacy.

---

Associate Professor Forest Products Chemistry  
in charge of major

Signature redacted for privacy.

---

Head of Department of Forest Products

Signature redacted for privacy. /

---

Dean of Graduate School

Date thesis is presented April 20, 1971.

Typed by Opal Grossnicklaus for Henry Hai-Loong Fang

COMMITTEE MEMBERS

Ray F W  
Signature \_\_\_\_\_  
El Will  
Signature \_\_\_\_\_  
Signature redacted for privacy.  
Signature redacted for privacy.

Date: April 20, 1971

## ACKNOWLEDGEMENTS

I express my most sincere thanks to my major professor, Dr. Murray L. Laver, for his ever present interest and guidance in the research program and for his many constructive criticisms in preparing the thesis manuscript.

Appreciation is extended to Dr. Harvey Aft for his early guidance in the research work, and to Dr. Robert L. Krahmer and Dr. Leonard M. Libby for their invaluable assistance and counsel.

Special thanks are extended to Dr. and Mrs. K. C. Lu for their constant inspiration and encouragement. From the time I arrived in this country they have taken me as one of their family, giving me the warmest feeling I have ever had.

To my fiancée, Miss Ta-Yun Wu, for her understanding and help, thanks are extended.

Special gratitude is expressed to my dearest mother, Chi-Ping Fang, for her never-ending encouragement, patience, and sacrifice. Her love has given me tremendous strength. This thesis is affectionately dedicated to her.

## TABLE OF CONTENTS

I.	INTRODUCTION	1
II.	HISTORICAL REVIEW	3
III.	EXPERIMENTAL	23
	Collection of Bark Samples	23
	Sample Preparation and <u>n</u> -Hexane Extraction	23
	Separation of the <u>n</u> -Hexane-Soluble Components	25
	Separation by Solvent Extraction	25
	Separation by Column Chromatography	25
	Separation by Thin-Layer Chromatography	29
	Separation by Paper Chromatography	32
	Separation by Gas-Liquid Chromatography	33
	Identification by Gas-Liquid Chromatography	
	Combined With Rapid-Scan Mass Spectrometry	37
IV.	RESULTS AND DISCUSSION	40
	Collection of Bark Samples	40
	Sample Preparation and <u>n</u> -Hexane Extraction	40
	Separation of the <u>n</u> -Hexane-Soluble Components	41
	Separation by Solvent Extraction	41
	Separation by Column Chromatography	42
	Separation by Thin-Layer Chromatography	43
	Separation by Paper Chromatography	53
	Separation by Gas-Liquid Chromatography	53
	Identification by Gas-Liquid Chromatography	
	Combined With Rapid-Scan Mass Spectrometry	69
	Combined Gas-Liquid Chromatography and	
	Mass Spectrometry of Unidentified Compounds	74
V.	SUMMARY AND CONCLUSIONS	80
	BIBLIOGRAPHY	82

## LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. Anatomy of Douglas-fir bark.	4
2. Ultraviolet spectrum of the <u>n</u> -hexane-soluble fraction of Douglas-fir bark.	24
3. Column chromatogram of the <u>n</u> -hexane-soluble fraction from Douglas-fir bark.	27
4. Two-dimensional thin-layer chromatography of the "yellow-band."	54
5. Gas-liquid chromatographic separation on SE-52 of the silylated mixture of the "yellow-band."	58
6. Gas-liquid chromatographic separation on SE-52 of the nonsilylated mixture of the "yellow-band."	59
7. Gas-liquid chromatographic separation on UC W-98 of the nonsilylated mixture of the "yellow-band."	60
8. Gas-liquid chromatographic separation on UC W-98 (temperature programmed) of the nonsilylated mixture of the "yellow-band."	61
9. Gas-liquid chromatographic separation on OV-17 of the nonsilylated mixture of the "yellow-band."	62
10. Gas-liquid chromatographic separation on OV-17 (temperature programmed) of the nonsilylated mixture of the "yellow-band."	63
11. Gas-liquid chromatographic separation on Hi-EFF 8BP of the nonsilylated mixture of the "yellow-band."	64
12. Gas-liquid chromatographic separation on UC-W-98 of the silylated mixture of the "yellow-band." Identification by rapid scan mass spectrometry.	65



## LIST OF CHARTS

<u>Chart</u>		<u>Page</u>
1	Separation of the components of benzene-soluble "wax" from Douglas-fir cork.	13
2	Extraction methods to separate the <u>n</u> -hexane-soluble fraction of Douglas-fir bark.	26

# LIST OF TABLES

<u>Table</u>		<u>Page</u>
1.	EXTRACTIVES IN BARK FROM 80 to 95-YEAR OLD TREES	6
2.	EXTRACTIVES CONTENT OF THE BAST FIBERS OF DOUGLAS-FIR BARK	14
3.	FRACTIONATION OF <u>n</u> -HEXANE-SOLUBLES FROM NEWLY FORMED INNER BARK OF DOUGLAS-FIR	15
4.	COLUMNS USED IN GAS-LIQUID CHROMATOGRAPHY	34
5.	CONDITIONS FOR SEPARATION OF THE "YELLOW BAND" BY GAS-LIQUID CHROMATOGRAPHY	36
6.	THIN-LAYER CHROMATOGRAPHY OF SAMPLES A AND B FROM SOLVENT EXTRACTION	41
7.	THIN-LAYER CHROMATOGRAPHY OF THE "YELLOW BAND" FROM THE <u>n</u> -HEXANE-SOLUBLE FRACTION OF DOUGLAS-FIR BARK	44
8.	THIN-LAYER CHROMATOGRAPHY RESULTS OF PLATES IMPREGNATED WITH SILVER NITRATE	51
9.	R <sub>f</sub> VALUES OF $\beta$ -SITOSTEROL AND CORRESPONDING SPOT FROM THE "YELLOW BAND"	67
10.	GLC COMPARISON OF THE "YELLOW BAND" WITH KNOWN CAMPESTEROL AND $\beta$ -SITOSTEROL	68

## DOUGLAS-FIR BARK; n-HEXANE-SOLUBLE FRACTION

### I. INTRODUCTION

The chemical components which comprise the bark of Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] are not well understood. Two to three million tons of the bark are generated each year from forest commerce, but a relatively small portion of this natural raw material finds commercial outlets. Most of the bark is wasted. It is usually disposed of by burning which contributes to a general air pollution problem. Before new and more valuable uses of bark can be introduced, a better understanding of its chemical constituents and physical properties are needed.

Douglas-fir bark is complex, and detailed information about its chemical constituents has proven difficult to obtain. However, the constituents can be partially resolved by extraction with common organic solvents such as n-hexane, benzene, petroleum ether, methyl ethyl ketone and others. The n-hexane-soluble fraction appears to be the best separated. It is a light colored, "wax-like" material that has been the subject of interest as a potential "vegetable wax" for use in carbon papers, hot melt adhesives, shoe polishes, water-proofing agents and similar products. However, there has been little effort to understand the material and to associate its properties with its chemical constitution. The work herein reported

is a detailed chemical investigation of some components in the n-hexane-soluble fraction of Douglas-fir bark. The study involves the use of modern techniques of separation, purification and characterization of chemical compounds. The advent of these modern methods allows a more comprehensive investigation than was previously possible.

## II. HISTORICAL REVIEW

In some of the early literature, authors refer to Douglas-fir as Pseudotsuga taxifolia (Poir.) Britt. and other names. However, the presently preferred botanical name is Pseudotsuga menziesii (Mirb.) Franco. The names all refer to the same genus and species. Mention of this is made to avoid confusion about the exact species investigated.

Several authors also report results on specific anatomical parts of Douglas-fir bark, and so a brief description of bark anatomy is herein included for purposes of definition. For a detailed anatomical description of Douglas-fir bark, reference is made to Grillos (21), Grillos and Smith (22), and Chang (14). Briefly, however, bark can be considered to consist of inner bark and outer bark (Figure 1). The inner bark (phloem cells) is the portion from the vascular cambium to the cork cambium of the innermost cork layer. The outer bark (rhytidome) is everything to the outside of the innermost cork cambium (Figure 1).

The inner bark comes from the vascular cambium, that layer of living cells between the wood and bark which divide to form wood to the inside and bark to the outside. The inner bark is composed mainly of sieve cells, axial and ray parenchyma, and sclereids. Much of the inner bark is living in the living tree because many

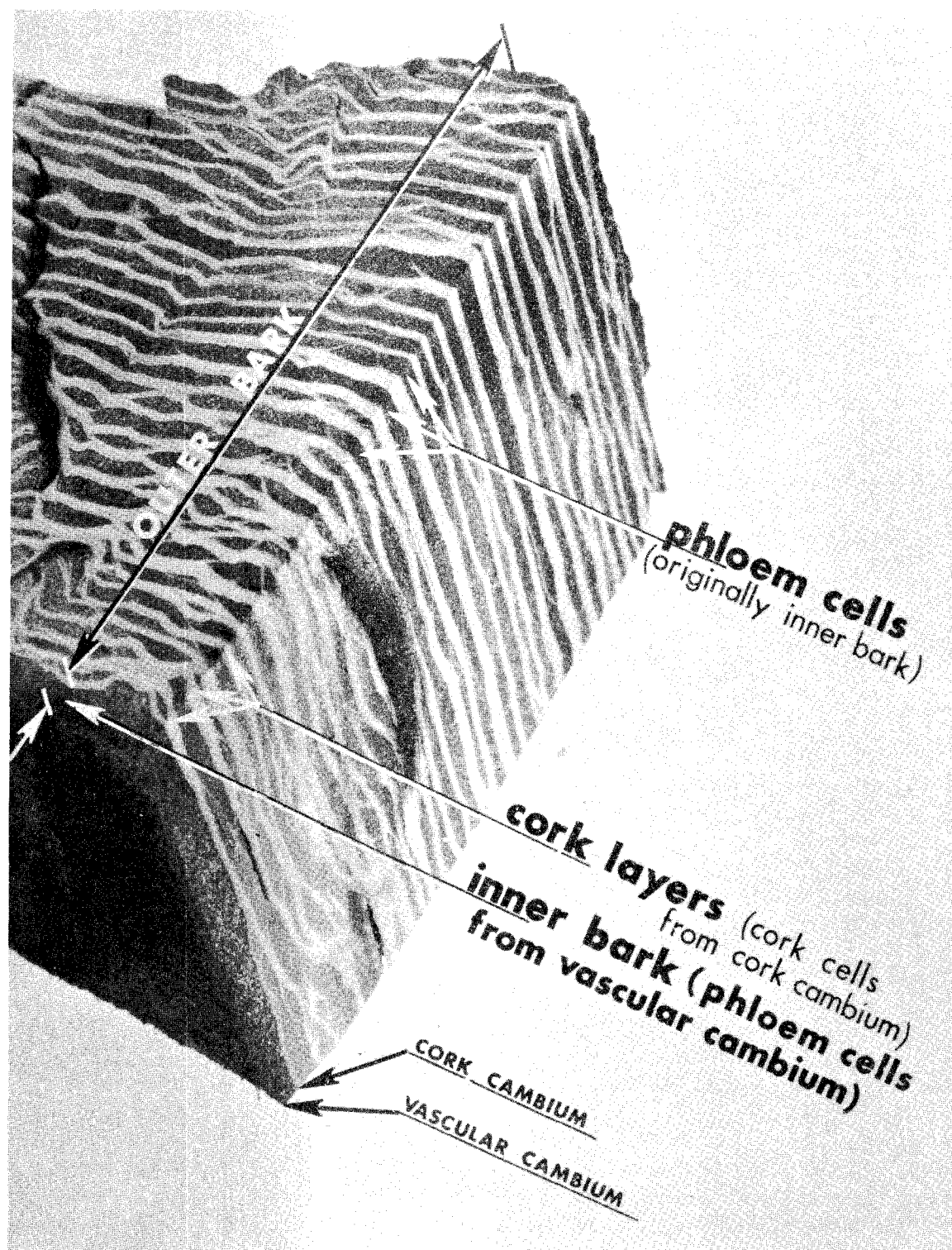


Figure 1. Anatomy of Douglas-fir bark.

of the parenchyma and sieve cells remain alive as long as they are components of the inner bark.

Douglas-fir sclereids are short, sharply pointed, spindle-shaped fibers of a red brown color. They are often referred to as bast fibers. They are lignified cells and develop from axial parenchyma cells some distance from the vascular cambium. In becoming sclereids, axial parenchyma cells approximately 0.1 to 0.5 mm in length elongate to 1 to 2 mm by apical intrusive growth, and form thick walls. The sclereids are commonly straight and somewhat cigar-shaped. Kiefer and Kurth (32) and Ross and Krahmer (51) describe and illustrate the general appearance and position of the sclereids in Douglas-fir bark.

The outer bark of Douglas-fir consists of layers of cork in which growth layers are usually visible [Ross and Krahmer (51)]. Interspersed among the corky layers are areas of phloem tissue that contain the sclereids and other cell types found in the inner bark (Figure 1). The cork layers form from the cork cambium (Figure 1) which are cells that were once living parenchyma cells of the inner bark. New cork cambia form in the inner bark and cut away part of the inner bark, which now becomes part of the outer bark. The cork cambium produces cork cells to the outside and a few storage cells to the inside. Cork cells have thin cellulose walls which are coated with suberin. Suberin is believed to consist largely of esters of

hydroxy fatty acids (31, p. 633). The cork layers may also contain tannin and starch. All cells outside the innermost cork cells are dead because no food supply can pass through this layer of cork cells. This then results in an outer bark composed of cork cells and dead phloem cells, which were once inner bark.

Kurth and Kiefer (39) found that the chemical components of the whole bark could be partially separated by extraction with various solvents. Table 1 indicates the nature and yields of the Douglas-fir extractives which they removed by successive extraction with the solvents shown.

TABLE 1. EXTRACTIVES IN BARK FROM 80 TO 95-YEAR OLD TREES.

Extractive	Yield % <sup>a</sup>	Solvent
Light-colored "wax"	5.47	Hexane
Light-brown "wax"	2.52	Benzene
Dihydroquercetin	5.95	Ethyl ether
Tannin and phlobaphene	7.70	Acetone
Tannin and carbohydrates	6.68	Hot water
Sum of five extractives	28.32	

<sup>a</sup>Percentage based on oven-dry weight of bark.

Although Kurth and co-workers use the term "wax" throughout their publications, there is some conflict as to a precise definition



of wax. Noller (44, p. 205) reports that

. . . a practical definition of a wax might be that it is anything with a waxy feel and a melting point above body temperature and below the boiling point of water. Thus the term paraffin wax is used for a mixture of solid hydrocarbons, beeswax for a mixture of esters, and carbowax for a synthetic polyether. Chemically, however, waxes have been defined as esters of long-chain ( $C_{16}$  and above) monohydric (one hydroxyl group) alcohols with long-chain ( $C_{16}$  and above) fatty acids. Hence they have the general formula of a simple ester,  $RCOOR$ . Actually the natural waxes are mixtures of esters and frequently contain hydrocarbons as well.

Other authors similarly define waxes. Mutton (43, p. 348)

writes that

. . . fatty acids are long, straight chain aliphatic monocarboxylic acids which usually occur in esterified form. Esters of fatty acids and glycerol are called fats or oils, whereas esters of fatty acids and high molecular weight monohydric alcohols are known as "waxes".

Hart and Schuetz (24, p. 221) contend that "waxes cannot be transformed into water-soluble products when boiled with alkali. They are saponified but, unlike glycerol from fats and oils, the long-chain alcohols are insoluble in water." They also write that "together with the esters, waxes frequently contain small quantities of saturated hydrocarbons, free fatty acids, alcohols and sterols."

It is evident that the use of the term "wax" in the early literature on the hexane solubles and benzene solubles of Douglas-fir bark encompassed the broadest, practical meaning, and was not reserved to a precise chemical definition.

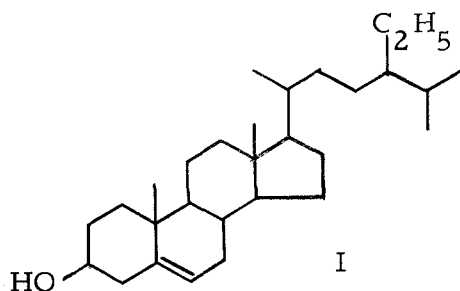
The light-colored "wax" referred to by Kurth and Kiefer (39) in Table 1 was extracted with hot n-hexane in a stainless steel extractor. The hot solvent was pumped upward through the bark and into an evaporator where the solvent was removed from the extracted wax by steam distillation.

A moisture content range from 1.7% to 44.9% had little effect on the efficiency of extraction. However, bark which had a moisture content above 40% was found to require a slightly higher solvent-to-bark ratio to give comparable "wax" extraction yields. It appeared that a flow rate in the range of 5 to 7 linear feet per hour and a solvent-to-bark ratio of 3 to 3.5 gallons per pound of dry bark was satisfactory. These ratios were also found to be the most satisfactory in the extraction of "wax" from Douglas-fir lignin residues (39).

The light-brown "wax" referred to by Kurth and Kiefer (39) in Table 1 was obtained by benzene extraction of the bark residue remaining from the n-hexane extraction. In a nonpressure extraction vessel, the optimum conditions for extraction with benzene appeared to be as follows:

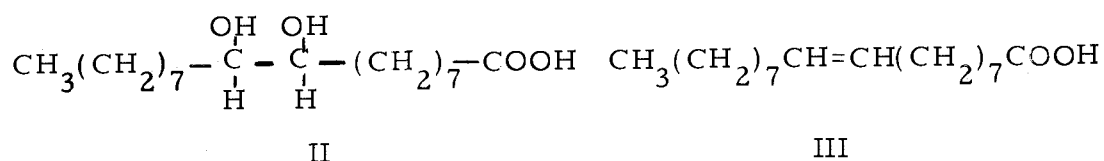
Moisture content of bark	10% to 40%
Extraction time	3 hours
Flow rate of solvent	35 gallons per hour
Temperature	Above 76.7° (170° F)
Solvent-to-bark ratio	3.1 gallons per pound of dry bark

Kurth, in a separate publication (35), reported on the chemical composition of the "waxes" in Douglas-fir bark. The n-hexane-soluble wax was saponified by refluxing a mixture of 20 g of the wax, 20 g of potassium hydroxide and 300 ml of 70% ethanol for three hours. Following this, 100 ml of water was added, the alcohol was removed by evaporation, and the residue extracted with n-hexane in a separatory funnel to remove unsaponifiable compounds. The unsaponifiable matter was dried and recrystallized from acetone. A white crystalline solid was obtained which was identified as lignoceryl alcohol, also named tetracosanol-1, a straight-chained, saturated alcohol,  $C_{24}H_{50}OH$ . The yield was 19.5% of the "wax." The filtrate from the lignoceryl alcohol crystallization gave a positive Liebermann-Burchard test for sterols (17, p. 80; 18, p. 100) and a precipitate with digitonin. After removal of the acetone, the residue was crystallized as white needles from dilute alcohol. The material was identified as "phytosterols." The yield was 0.3% of the "wax." "Phytosterols" are now known to be mixtures of closely related sterols of which the most common one in bark is  $\beta$ -sitosterol (I).



$\beta$ -Sitosterol

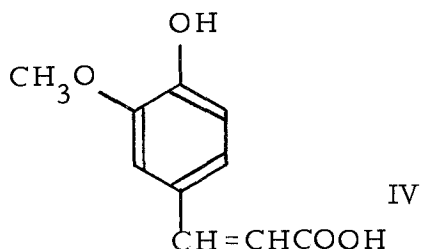
The alkaline solution from the separation of the unsaponifiable matter was acidified with sulfuric acid and extracted with n-hexane in a separatory funnel. The n-hexane solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was recrystallized from acetone and then from n-hexane. The white crystals were identified as lignoceric acid, also named tetracosanoic acid, a straight-chained, saturated acid,  $C_{24}H_{48}O_2$ . The yield was 60.5% of the "wax." The small amount of yellow solid residue left in the filtrate from this crystallization was oxidized with cold potassium permanganate to dihydroxystearic acid; m.p.  $129^\circ - 130^\circ$  (II). This indicated the presence of oleic acid (III) in the original wax.



Dihydroxystearic acid

Oleic acid

A brown resin remained suspended in the aqueous liquor after the n-hexane extraction of the lignoceric acid. The resin was extracted with diethyl ether. Evaporation of the ether left a brown residue which crystallized from benzene as fine crystals. Recrystallization from dilute ethanol gave yellow prisms. These crystals were identified as ferulic acid (4-hydroxy-3-methoxy cinnamic acid), (IV). The yield was 22% of the original wax.



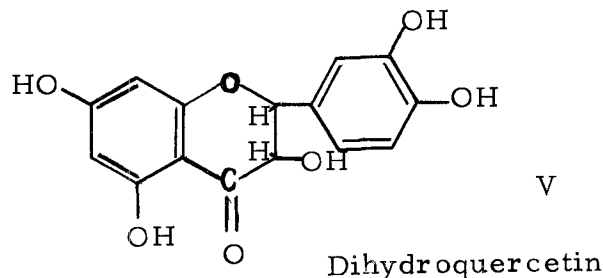
4-Hydroxy-3-methoxy cinnamic acid

That fraction of the bark which was n-hexane insoluble but benzene soluble (benzene-soluble "wax") was reported by Kurth and co-workers (28, 36) to possess a more complicated composition than the n-hexane-soluble "wax." It had a melting point of 60° to 63°, and Kurth tentatively isolated, after saponification, a fatty acid mixture in about 25% yield, a dark-colored phlobaphene in 24% yield, a dark colored diethyl ether-soluble acid fraction in 26% yield, unsaponifiable matter in 5% yield and glycerol.

The cork fraction of Douglas-fir bark was studied by Hergert and Kurth (28). The extractives were separated according to their solubilities in n-hexane, benzene, diethyl ether, ethanol and hot water. The n-hexane and benzene fractions were "waxes." The yield of n-hexane-soluble "wax" was 5.78% and of benzene-soluble "wax" was 1.75% based on an average of nine trees sampled. The n-hexane-soluble "wax" was found to contain lignoceric acid, 49.3%; lignoceryl alcohol, 27.5%; ferulic acid, 9.8%; "phytosterol," 0.6%; and n-hexane-insoluble, benzene-soluble acidic material, 8.1%; a benzene-insoluble phenolic material, 3.6%. The n-hexane-insoluble

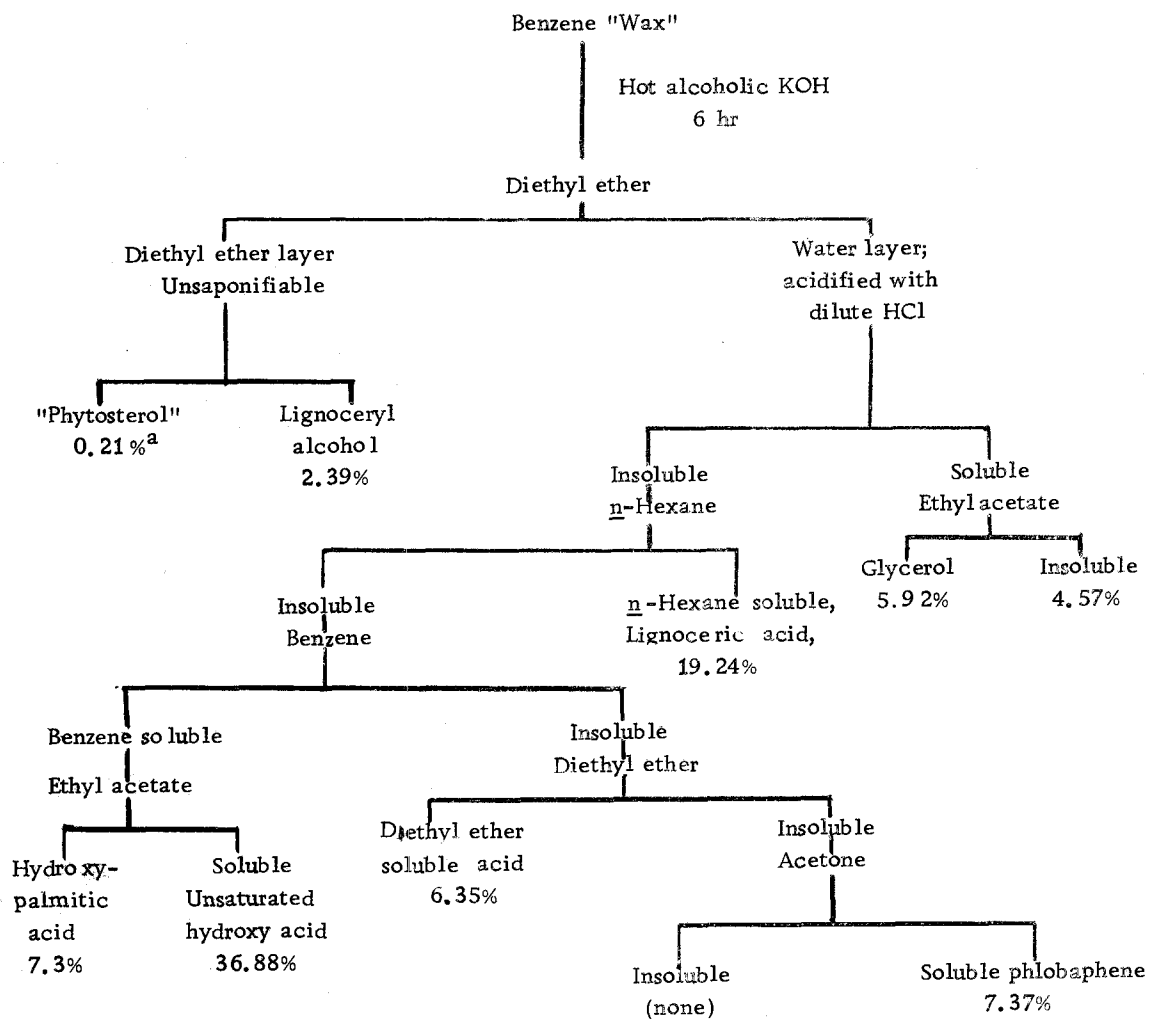
but benzene-soluble "wax" proved to have a more complicated composition and was studied as shown in Chart 1, p. 13. The authors did not locate the position of the hydroxyl group in the hydroxypalmitic acid,  $C_{16}H_{32}O_3$ , but reported that it was not terminal.

It is interesting to note that dihydroquercetin (V) was the extractive present in greatest amount from the cork. Although not extracted with n-hexane or benzene, and not considered a part of the "wax-portion," Douglas-fir bark represents a major source of this compound. It is easily obtained from the "wax"-free (benzene extracted) bark or cork by extraction with diethyl ether. Upon evaporation of the diethyl ether it can be recrystallized from hot water as long white



needles, m.p. 241-248 (39). The quantity of dihydroquercetin in cork varies from 5% in samples of cork from second-growth trees to 23% in cork from mature trees (27). This indicates the desirability of separating the cork from the other bark components when the extraction of dihydroquercetin is intended.

In 1967 Kurth reported more recent work on the n-hexane-insoluble, benzene-soluble "waxes" of Douglas-fir bark and cork (36). In addition to the compounds previously mentioned (Chart 1, p. 13) he



<sup>a</sup> Percentages based on oven-dry weight of wax.

Chart 1. Separation of the components of benzene-soluble "wax" from Douglas-fir cork.

found an hydroxybehenic acid,  $C_{22}H_{44}O_3$ , and an hydroxyarachidic acid (hydroxyeicosanoic acid),  $C_{20}H_{40}O_3$ .

The hydroxybehenic acid was crystalline but gave a range of melting points depending upon how it was recrystallized. Kurth comments that this is characteristic of those hydroxy-fatty acids that form lactones and lactides. He did not report the position of the hydroxyl group. Likewise he did not report the exact position of the hydroxyl group in the hydroxyarachidic acid. He also mentions the presence of a dicarboxylic acid of tentative formula  $C_{20}H_{38}O_4$  but did not report further characterization.

The chemical composition of the bast fibers of Douglas-fir bark were also studied by Kieffer and Kurth (32). Table 2 shows the yields of extractives resulting from successive extractions with the solvents shown.

TABLE 2. EXTRACTIVES CONTENT OF THE BAST FIBERS OF DOUGLAS-FIR BARK.

Solvent	Yield % <sup>a</sup>	Nature
<u>n</u> -Hexane	1.59	Light yellow "wax"
Benzene	0.95	Brown "wax"
Diethyl ether	0.38	Dihydroquercetin
Hot water	9.61	Tannin, Carbohydrate, etc.
Ethanol	0.96	Phlobaphine

<sup>a</sup> Percentages based on oven-dry weight of bast fibers.



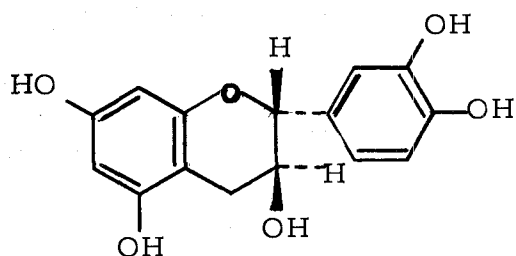
They indicated that the bast fibers did not contain as much extractive material, particularly dihydroquercetin and "wax," as did the cork fraction of Douglas-fir bark. Further separation of the extractives into their individual components was not reported.

Holmes and Kurth (29, 30) investigated the chemical composition of the newly formed inner bark of Douglas-fir. The n-hexane-soluble extractives are listed in Table 3.

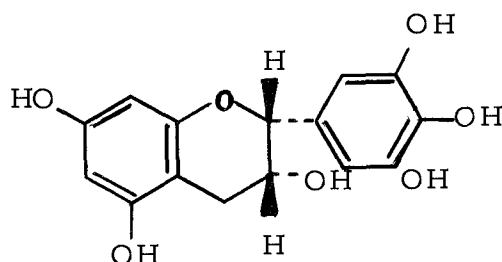
TABLE 3. FRACTIONATION OF n-HEXANE-SOLUBLES FROM NEWLY FORMED INNER BARK OF DOUGLAS-FIR.

Fractionation	Yield %	Description
<u>n</u> -Hexane-solubles	100	Tan-colored, sticky "wax"
Free acids	3	Dark-brown "wax"
Unsaponifiables	5	Light-brown "wax"
Saponifiables		
Diethyl ether solubles	66	Dark-brown sirup
Acetone solubles	27	Dark-brown amorphous powder

It is interesting to note that the authors report that the diethyl ether solubles of the outer bark contained dihydroquercetin (V, p. 12) while the inner bark contained d-catechin (VI) and l-epicatechin (VII) (mature inner bark contained a trace of d-dihydroquercetin) [present terminology is (2R, 3S)-(+)-catechin and (2R, 3R)-(-)-epicatechin (12)].



VI  
(+)-Catechin

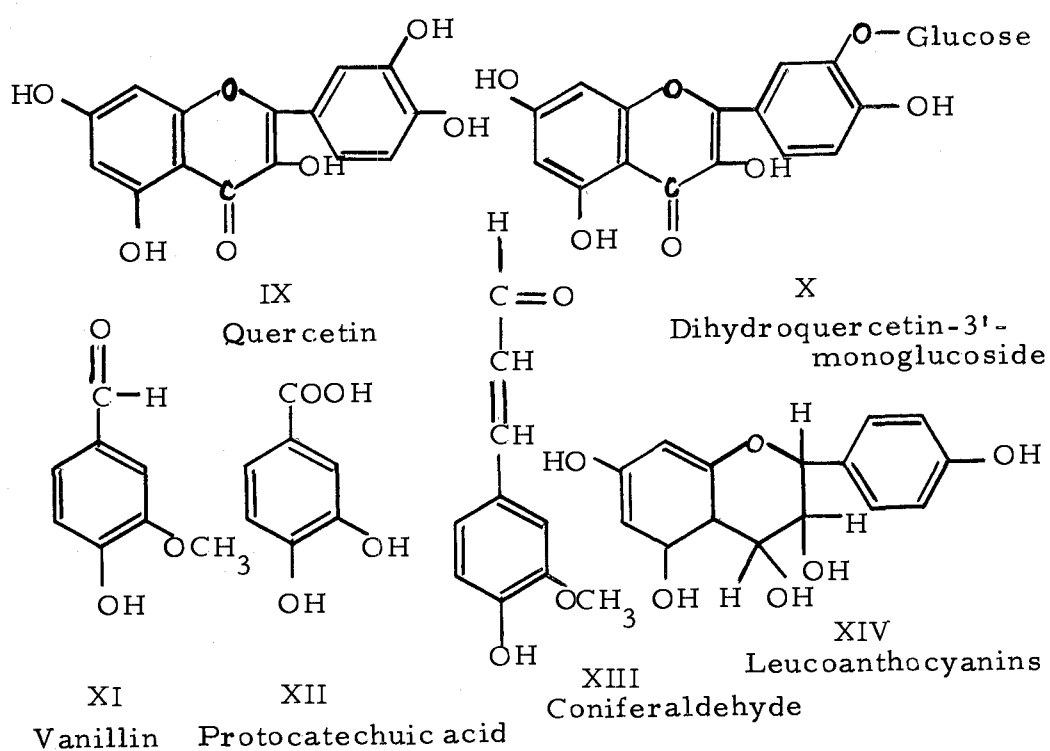


VII  
(-)-Epicatechin

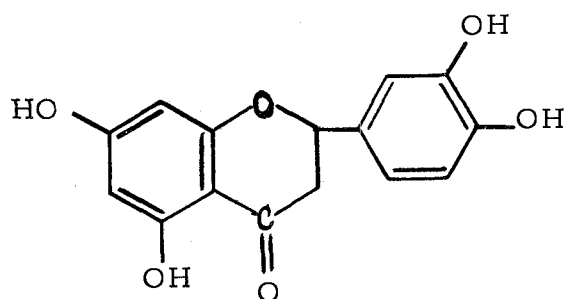
The authors state that the presence of the catechins in the living inner bark, and their absence in the dead outer bark, implies that they are the precursors of the polyphenol components of either bark or wood. However, the biosynthetic pathway of polyphenols in plants still is not understood.

Manners (41) reports a detailed chromatographic examination of Douglas-fir bark as part of a comparative study of other barks of the genus Pseudotsuga. He successively extracted whole bark with n-hexane, benzene, diethyl ether, ethanol and water. Although there was some separation made with these solvents, the overlap of compounds into different solvents was considerable and most of the materials identified were present in the n-hexane-soluble fraction to a more or less degree. The compounds were identified by chromatographic techniques and ultraviolet spectral studies.

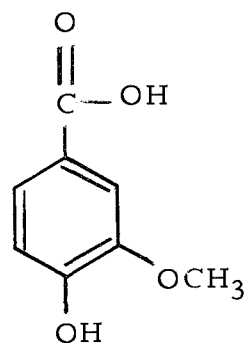
Compounds which were identified but had previously been reported were: dihydroquercetin (V, p. 12), quercetin (IX), dihydroquercetin-3'-monoglucoside (X), d-catechin (VI, p. 16), 1-epicatechin (VII, p. 16), vanillin (XI), protocatechuic acid (XII), coniferaldehyde (XIII) and some leucoanthocyanins (XIV) (flavan-3,4-diols).



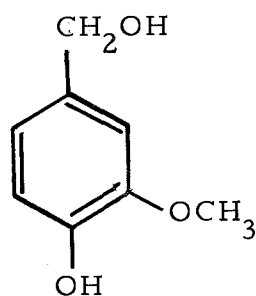
Compounds which were found and had not been previously reported were: eriodictzol (XV), vanillic acid (XVI), vanillyl alcohol (XVII), acetovanillone (XVIII), and two unidentified esters of ferulic acid (IV, p. 11).



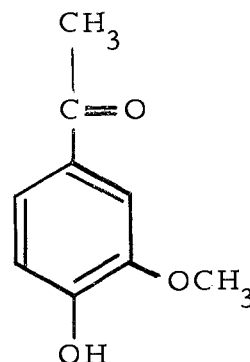
XV  
Eriodictzol



XVI  
Vanillic acid



XVII  
Vanillyl alcohol



XVIII  
Acetovanillone

The chemical composition of several barks other than Douglas-fir bark have been studied (6, 13, 27, 31, 37, 38, 39). The results are of interest here because compounds reported in other barks are quite likely to be found in Douglas-fir bark. Knowledge of these compounds is helpful in a systematic approach to a thorough understanding of bark components.

The n-hexane-soluble "wax" from the bark of white fir [Abies concolor (Gord. and Glend.) Lindl.] was shown by Hergert (26) to

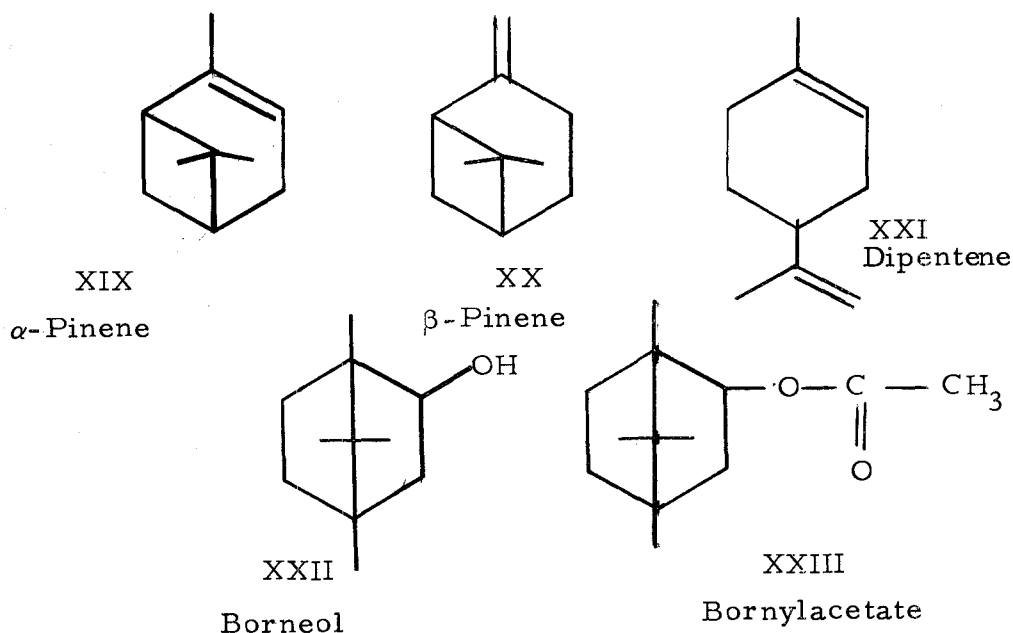
contain lignoceryl alcohol, 34.78%, behenic acid, 30.09%, "phyto-sterol," 1.26%, unsaturated alcohols, 1.82%, and unsaturated acids, 9.85%. The n-hexane-insoluble, benzene-soluble "wax" appeared to be more complex than the n-hexane-soluble "wax." Its insolubility in cold sodium bicarbonate, sodium carbonate, or sodium hydroxide indicated the absence of free acids or acidic groups. Lignoceryl alcohol, 5.35%, 13-hydroxymyristic acid,  $\text{CH}_3\text{CHOH}(\text{CH}_2)_{11}\text{COOH}$ , 48.8%, behenic acid, 3.02%, "phytosterol," 0.3%, a phenolic acid, 32.52%, unsaturated acids, 9.69% and unsaturated alcohols, 0.3% were found in the benzene-soluble "wax." The composition of the cork fraction of white fir bark was shown (27) to contain 13-hydroxymyristic acid, an hydroxyarachidic acid  $\text{C}_{20}\text{H}_{40}\text{O}_3$  and a dihydroxydicarboxylic acid.

The results showed that the "wax" of white fir was somewhat different from the "wax" of Douglas-fir. The aliphatic acid obtained in largest yield from the Douglas-fir n-hexane-insoluble, benzene-soluble "wax" was lignoceric acid, while the aliphatic acid obtained in largest yield from white fir "wax" was an hydroxypalmitic acid,  $\text{C}_{16}\text{H}_{32}\text{O}_3$ . The latter "wax" also contained a large amount of a  $\text{C}_{16}$  dicarboxylic acid; mp  $124^\circ - 125^\circ$ . The white fir "wax" did not appear to contain lignoceric acid but did contain some behenic acid.

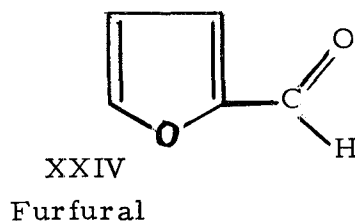
The "wax" of the bark of mountain hemlock [Tsuga mertensiana (Bond.) Carr.] was entirely soluble in n-hexane. The chief

components of this "wax" were lignoceric acid and lignoceryl alcohol. It did not appear to contain any of the hydroxy-fatty acids or the dicarboxylic acids found in the n-hexane-insoluble "waxes" from Douglas-fir or white fir.

The volatile materials in bark have not been as thoroughly studied as the non-volatiles. However, Kurth and Hubbard (38) showed that the n-hexane extract of ponderosa pine [Pinus ponderosa (Laws.)] contained 0.2% of a volatile oil recovered by steam distillation. The authors did not attempt to separate the oil into its components but did report that it had similar properties to the volatile oil from ponderosa pine wood which was found by Adams (1) to consist of  $\alpha$  - and  $\beta$ -pinene (XIX and XX), dipentene (limonene, XXI), borneol (XXII), and bornyl acetate (XXIII).



The n-hexane extract of the bark of incense cedar [Libocedrus decurrens (Torr.)] was shown by Smith and Kurth (57) to contain 0.95% of a volatile material. The major components of the oil had been characterized many years before by Schorger (55) as furfural (XXIV),  $\alpha$ -pinene (XIX, p. 20), dipentene (XXI, p. 20), bornyl acetate (XXIII, p. 20), and free borneol (XXII, p. 20). Carlberg and Kurth (13) also make reference to early work on the bark of the "true firs" which are said to contain mixtures of various terpenes and terpene alcohols.



A review article by Jensen, Fremer, Sierila, and Wartiovaara (31) outlines some of the reported work on the terpenes in bark. The hydrocarbon solvents extract small amounts of volatile oil from bark which appear to consist principally of monoterpenes. However, the literature contains evidence that the unextracted terpenes of the bark are mainly of the triterpene type.

From the above evidence it is considered likely that Douglas-fir bark contains terpenes, and that they would be extracted into the n-hexane fraction. However, Kurth and co-workers (28, 35, 36, 39) did not report any. This could be because the amounts are too small to be detected by their saponification, and extraction techniques.

"Phytosterols" have also been found in bark.  $\beta$ -Sitosterol (I, p. 9) is common, but the reported  $\alpha$ -sitosterol (28, 35, 36) was in reality a wrong name (4). The limited methods of separation and purification available at the time resulted in these workers investigating mixtures rather than pure compounds.

The recent advances in chromatographic separations coupled with the physical techniques now available to characterize materials, allows for a thorough study of bark components. It is possible to determine if terpenes exist in Douglas-fir bark, and what kinds of sterols besides  $\beta$ -sitosterol are present. The experimental work reported in this thesis represents an effort to clarify many of these questions.



### III. EXPERIMENTAL

#### Collection of Bark Samples

Bark samples used in this study were collected from indigenous sources of Douglas-fir. They consisted of bark from trees of various ages and diameters. The specimens were stripped from the trees, sealed in polyethylene bags, and brought to the Forest Research Laboratory, Oregon State University.

#### Sample Preparation and n-Hexane Extraction

Bark chips were ground to pass a screen of ten meshes to the inch (The W. S. Tyler Company, Cleveland, Ohio). Since it was not necessary to dry the bark before extraction (39), the ground bark was packed into a Soxhlet thimble, placed in a Soxhlet extractor and extracted for 48 hours with n-hexane. The solvent was evaporated under aspirator vacuum on a rotary evaporator (Büchi, Rotavapor, Switzerland). The residual "wax-like" solid was transferred to a sample jar with a minimum of n-hexane solvent. The excess solvent was evaporated by passage of a stream of nitrogen. The ultraviolet absorption spectrum of the extracted solids was measured in a chloroform-n-hexane (3:1 v/v) solution (Figure 2), (Beckman DB spectrophotometer).

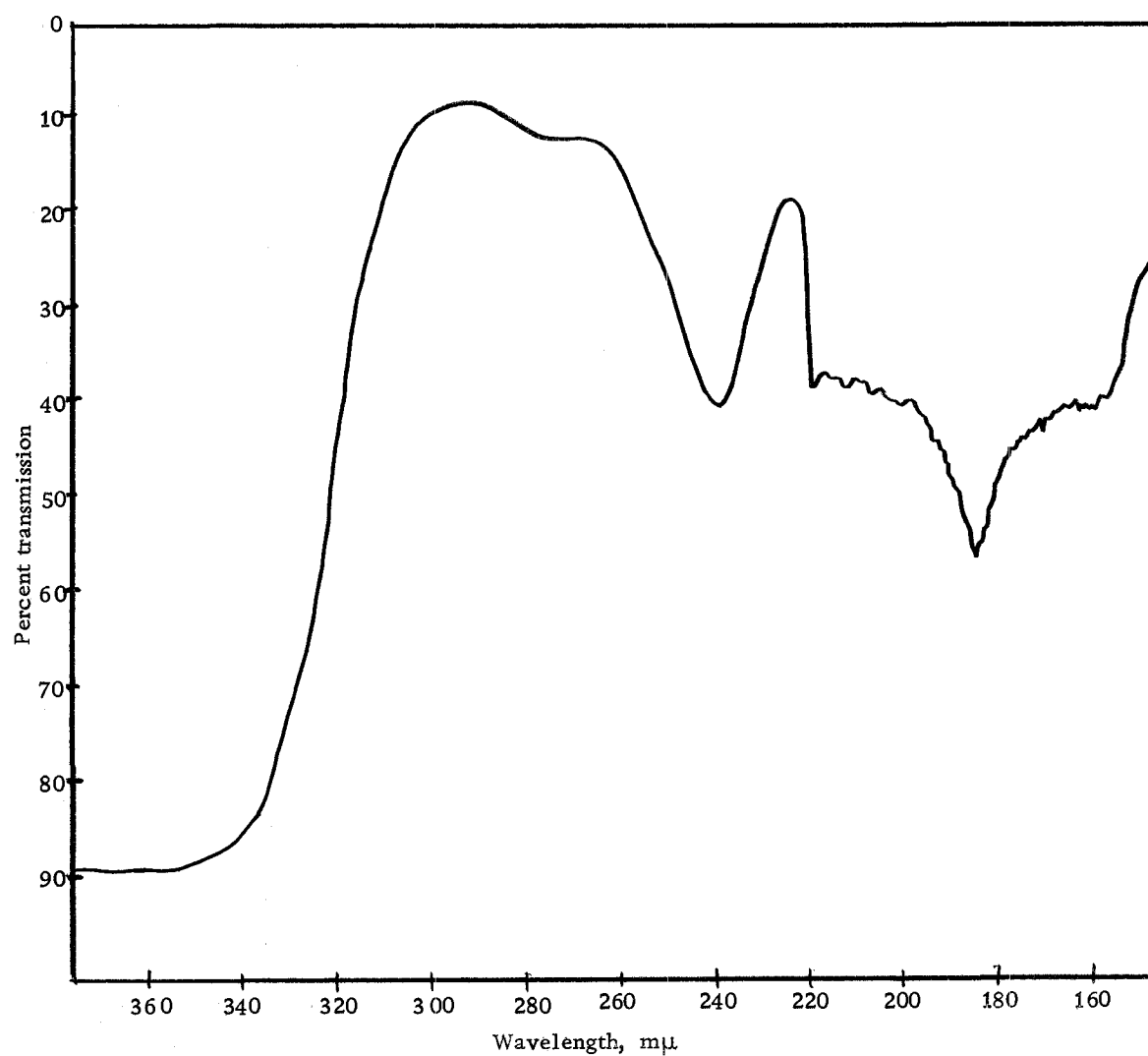


Figure 2. Ultraviolet spectrum of the n-hexane-soluble fraction of Douglas-fir bark.  
Solvent; chloroform-n-hexane (3:1 v/v).

## Separation of the *n*-Hexane-Soluble Components

### Separation by Solvent Extraction

To determine if the components were neutral or acidic, extraction methods were used as illustrated in Chart 2.

Samples A and B were subjected to thin-layer chromatography to determine the number of compounds present. The thin-layer chromatograms consisted of eight-inch square glass plates which supported a layer of Silica Gel G (E. Merck Ag, Darmstadt, Germany). The layer was applied as an aqueous mixture (15 g Silica Gel G per 35 ml of water) by spraying through an ordinary chromatography sprayer. The plates were dried and activated for one hour at 105°.

The developing solvent used for samples A and B was chloroform-carbon tetrachloride (6:1 v/v). Detection of the spots was accomplished by ultraviolet light and by exposure to iodine vapor.

The thin-layer chromatograms of bark samples A and B showed a complex number of spots. Therefore samples A and B were not further investigated.

### Separation by Column Chromatography

Column chromatography was used as a first step in separating the *n*-hexane-soluble materials. A typical separation is described

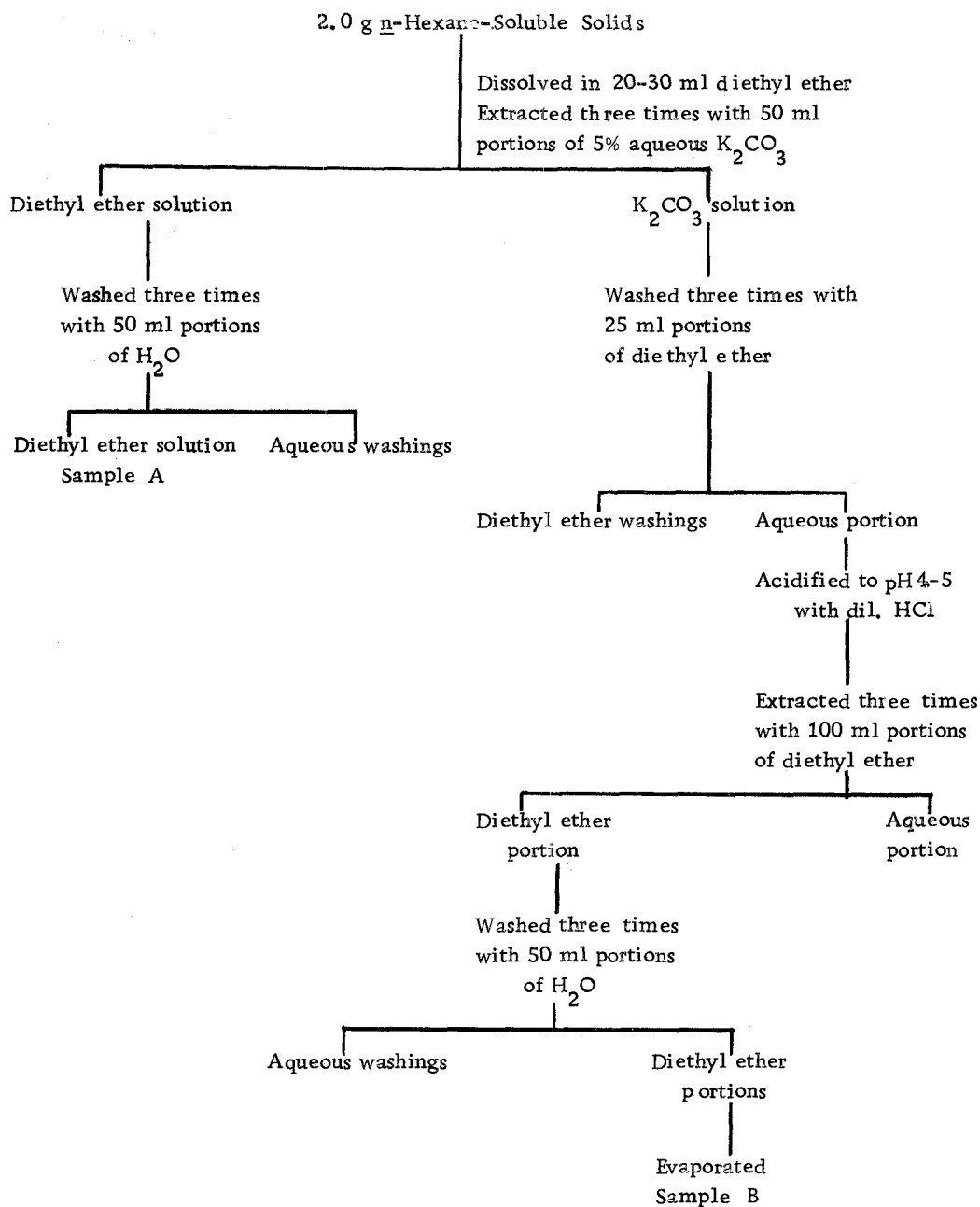


Chart 2. Extraction methods to separate the n-hexane-soluble fraction of Douglas-fir bark.

below. Silica Gel G was packed uniformly into a glass column two feet in length and one inch in diameter. A portion (2.00 g) of the n-hexane-soluble fraction was dissolved in chloroform (15.0 ml) and added to the top of the Silica Gel G column. Chloroform-n-hexane (3:1 v/v) was used as the developing solvent. A series of bands were located by use of ultraviolet light (Figure 3).

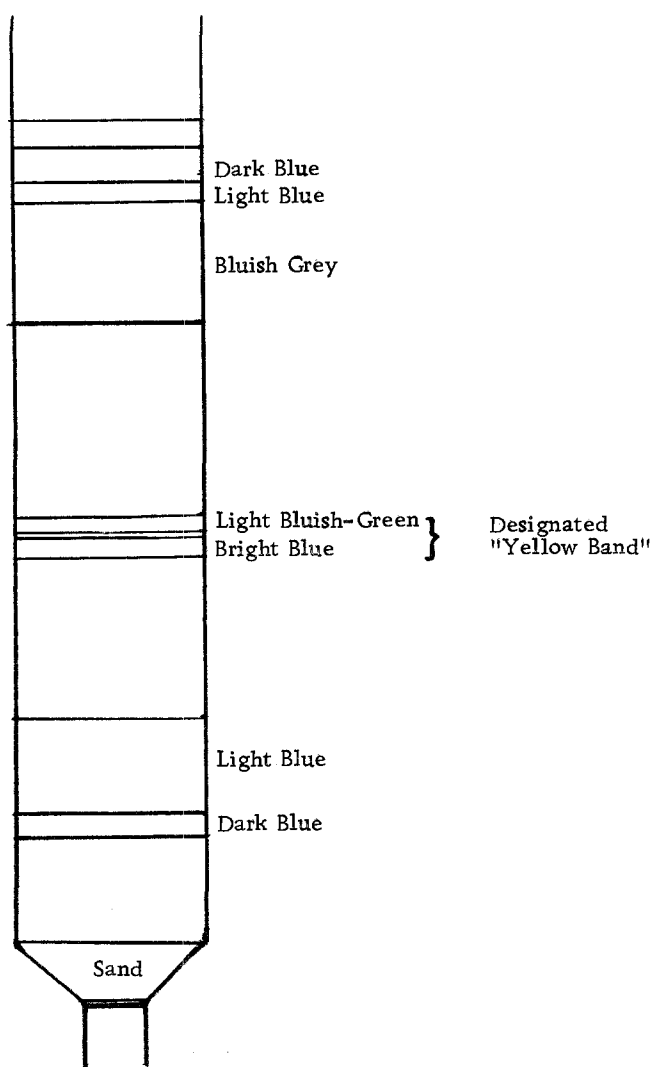


Figure 3. Column chromatogram of the n-hexane-soluble fraction from Douglas-fir bark.

The fastest moving two bands (blue and light blue bands) have been investigated by Aft, H.(2). The present investigation is concerned with the next two fastest moving bands, the light bluish-green band and the bright-blue band (designated in combination as the "yellow band"). To collect these bands the fastest moving zones were first washed from the column and stored. Because of the difficulty of separating the next two bands, they were washed from the column and collected as a mixture. The mixture was added to the top of a second Silica Gel G column and developed with the same solvent system. When the bright-blue band (fastest moving) approached the sand in the bottom of the column, fractions of effluent (5.0 ml) were collected. These fractions were tested by thin-layer chromatography. The thin-layer plates were prepared from Silica Gel G as previously described. The fractions were tested in two different solvent systems: diethyl ether-n-hexane (1:4 v/v) and chloroform-carbon tetrachloride (6:1 v/v). The chromatograms showed several spots for each fraction, indicating that the column chromatographic separation was incomplete.

However, as a source of starting material for further separations, the "yellow band" (Figure 3, p. 27) was collected from several Silica Gel G columns.

The Liebermann-Burchard test (17, p. 70; 18, p. 100) was employed to determine if the material contained unsaturated sterols.

A sample of the "yellow band" from the column chromatographic separation (Figure 3, p. 27) was dissolved in chloroform. An aliquot (1.0 ml) of the solution was added to a test tube and dried. Acetic anhydride (2.0 ml) was added followed by the dropwise addition of concentrated sulfuric acid. A dark green color change indicated a positive test.

#### Separation by Thin-Layer Chromatography

The "yellow band" from the column chromatographic separation (Figure 3, p. 27) was subjected to thin-layer chromatography in several solvent systems in an effort to more completely resolve the mixture. The thin-layer plates were prepared with Silica Gel G as previously described.

Silver nitrate was used with Silica Gel in some thin-layer separations. The silver nitrate (1.58 g) was dissolved in water (4.5 g) and absolute methanol (40.5 ml) was added. "Eastman Chromatogram Sheets" (6061 Silica Gel, Eastman Kodak Company, Rochester, N. Y.), which were already layered with Silica Gel, were wetted in the solution by dipping for several seconds. The sheets were air-dried in the dark, activated by placing in an oven at 105° for ten minutes, and used within one hour.

Solutions of the samples to be chromatographed were applied to the thin-layer plates with capillary tubes in such a way that the

surfaces of the plates were disturbed as little as possible. The chromatography tanks used for irrigating the thin-layer plates were saturated with vapors from the developing solvent at least two hours before the plates were placed in the tanks.

Aliquots of the "yellow band" (Figure 3, p. 27) in chloroform-n-hexane (3:1 v/v) solution were added to the thin-layer plates and developed with the following solvent systems (all hexane solvents were n-hexane):

1. Chloroform-carbon tetrachloride (6:1 v/v)
2. Chloroform-carbon tetrachloride (1:1 v/v)
3. Chloroform-carbon tetrachloride (1:4 v/v)
4. Chloroform-hexane (4:1 v/v)
5. Benzene-hexane (2:1 v/v)
6. Diethyl ether-hexane (3:1 v/v)
7. Diethyl ether-hexane (1:4 v/v)
8. Chloroform-benzene (6:1 v/v)
9. Chloroform-benzene (1:1 v/v)
10. Chloroform-benzene (1:4 v/v)
11. Benzene-methanol-acetic acid (45:8:4 v/v/v) (41, p. 24)
12. Hexane-diethyl ether-acetic acid (70:30:1 v/v/v) (41, p. 24)
13. Hexane-diethyl ether-acetic acid (85:15:1 v/v/v) (53, p. 1282)
14. Chloroform-ethyl acetate-formic acid (5:4:1 v/v/v) (3, p. 22)

Some thin-layer plates were developed two dimensionally in the



following solvent systems:

n-hexane-diethyl ether-acetic acid (70:30:1 v/v/v) in one direction followed by chloroform in the second direction; diethyl ether-n-hexane (1:4 v/v) in one direction followed by chloroform-carbon tetrachloride (6:1 v/v) in the second direction.

After development, the thin-layer plates were dried at room temperature. Those plates which were impregnated with silver nitrate were dried in the dark. The following methods were used to examine the developed plates:

1. Ultraviolet light
2. Ultraviolet light with the plate in ammonia vapors
3. Sprayed with 30% aqueous sulfuric acid, heated for 5 to 10 minutes at 105° in an oven and finally viewed under ultraviolet light
4. Exposure to iodine vapors (8, p. 85)
5. Sprayed with bromothymol blue solution (50 mg bromothymol blue, 1.25 g boric acid, 8 ml 1 N sodium hydroxide and 112 ml water, or 40 mg bromothymol blue and 100 ml 0.01 N sodium hydroxide) (47, p. 128)
6. Sprayed with p-nitroaniline solution ((a) p-nitroaniline, 0.3% in 8% (w/v) HCl (25 ml.) and NaNO<sub>2</sub> (5% w/v) (1.5 ml.) mixed immediately before spraying. (b) Na<sub>2</sub> CO<sub>3</sub> (20% w/v)). The two solutions were used successively (10).

Authentic  $\beta$ -sitosterol (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was chromatographed by one-dimensional thin-layer chromatography simultaneously with the "yellow band" (Figure 3, p. 27) in each of the 14 solvent systems listed above. The spots were located by exposure to iodine vapor. The separation by every solvent system showed a compound in the "yellow band" (Figure 3, p. 27), which migrated the same distance as the authentic  $\beta$ -sitosterol.

#### Separation by Paper Chromatography

The "yellow band" from the column chromatographic separation (Figure 3, p. 27) of the n-hexane-soluble fraction was also subjected to paper chromatography in an effort to improve the resolution. Whatman No. 1 filter paper was employed in conjunction with the following solvent systems:

1. 1-Butanol-acetic acid-water (4:1:5 v/v/v)
2. Glacial acetic acid (25)
3. 1-Butanol-acetic acid-water (6:1:2 v/v/v) (25)
4. Benzene-acetic acid-water (125:20:3 v/v/v)

Two-dimensional paper chromatography was also used. The solvent systems were:

1. 1-Butanol-acetic acid-water (4:1:5 v/v/v) (upper layer) in one direction followed by 2.0% aqueous acetic acid in the second

direction.

2. Benzene-acetic acid-water (125:20:3 v/v/v) in the one direction followed by 2.0% aqueous acetic acid in the second direction.

The papers were equilibrated for six to eight hours in an atmosphere of the developing solvent before irrigation. An exception was the glacial acetic acid which tended to degrade the paper.

After development, the papers were removed from the developing tank and air-dried. The following methods were used to examine the chromatograms:

1. Ultraviolet light
2. Ultraviolet light with the paper in ammonia vapors
3. p-Nitroaniline spray (10)

#### Separation by Gas-Liquid Chromatography

Gas-liquid chromatography was used to test the purity of the samples separated by column chromatography and thin-layer chromatography and to collect pure samples. A Hewlett-Packard 5750 Research Chromatograph (Hewlett-Packard Company, Palo Alto, Calif.) and an Aerograph 200 (Varian Aerograph, Walnut Creek, Calif.) were used. Both instruments were equipped with flame ionization detectors and used helium as the carrier gas. Stainless steel columns (1/8 in. O.D.) were filled with the packing material of choice. A glass wool plug was inserted in the end of the column

and the material was packed to a uniform tightness by vibrating the column with a small vibrating tool. Four different column packings were used in an attempt to find a column packing which would give good resolution. Details of the four columns are listed in Table 4.

TABLE 4. COLUMNS USED IN GAS-LIQUID CHROMATOGRAPHY.

Col. no.	Length ft.	O.D. <sup>a</sup> in.	Liquid coating	Concentration	Solid support	Mesh.	Instrument
1	12	1/8	SE-52	10 wt. %	Gas Chrom. W.	60/80	Aerograph 200
2	6	1/8	OV-17	3 wt. %	Gas Chrom. Q.	100/120	Hewlett-Packard
3	6	1/8	UC W-98	10 wt. %	Silicone Gum	80/100	Hewlett-Packard
4	6	1/8	Hi-EFF 8 BP	3 wt. %	Gas Chrom Q.	100/120	Hewlett-Packard

<sup>a</sup> Outside diameter.

The columns were put on the instrument for conditioning for 12 to 18 hours at a temperature of 10° above the maximum temperature used for resolution. Various temperatures, flow rates, sensitivities and chart speeds were tested. Both temperature programming and isothermal methods were used.

Most of the samples were directly injected into the gas chromatograph in n-hexane solution. However, because some of the compounds were thought to contain hydroxyl groups the trimethylsilyl ethers of several of the fractions were prepared in an attempt to improve resolution. These trimethylsilyl ethers were made by reacting dry samples for 5 to 10 minutes at room temperature with hexamethyldisilazane and trimethylchlorosilane in pyridine (2:1:10 v/v/v) (33). The pyridine solution was injected directly into

the gas chromatograph.

The "yellow band" from column chromatography (Figure 3, p. 27) was investigated. Four different columns were used (Table 4, p. 34). The experimental conditions are shown in Table 5 (p. 36) and the chromatograms are shown later in Figures 4 through 10, (pp. 58-65).

Two bands from the thin-layer chromatographic separation [developer, diethyl ether-n-hexane (1:4 v/v)] of the "yellow band" (Figure 3, p. 27) were tested for purity by gas-liquid chromatography. The two bands were readily observed on the thin-layer plates by ultraviolet light. The faster moving band showed a yellow-blue color and the slower moving band showed a bright-blue color. These two bands were separately scraped from the thin-layer plates, and the organic compounds extracted from the solid Silica Gel G with n-hexane. The compounds were separated by gas-liquid chromatography on the following column packings: SE-32; UC W-98; OV-17 (Table 4, p. 34). Both silylated and non-silylated samples were injected under the general conditions outlined in Table 5 (p. 36).

An authentic sample of  $\beta$ -sitosterol was subjected to gas-liquid chromatography using the columns and the conditions shown in Table 5, (p. 36). The retention times were compared with those for the peaks in Figures 4 through 10 (pp. 58-65).

A "peak enhancement" study was conducted by adding a small

TABLE 5. CONDITIONS FOR SEPARATION OF THE "YELLOW BAND" BY GAS-LIQUID CHROMATOGRAPHY

Sample	Figure no.	Detector temp. °C	Injection port temp. °C	Column	Column temp. °C	Flow rate ml/min	Range setting	Attenuation setting	Instrument
Silylated Mixture	5	265	277	SE-52	Isothermal 255	60	1	4	Aerograph
Mixture	6	265	277	SE-52	Isothermal 255	60	1	4	Aerograph
Mixture	7	270	275	UC W-98	Isothermal 275	28	10 <sup>2</sup>	16	Hewlett-Packard
Mixture	8	255	235	UC W-98	Program 148 ~300 2°/min	28	10 <sup>2</sup>	16	Hewlett-Packard
Mixture	9	278	272	OV-17	Isothermal 255	30	10 <sup>2</sup>	16	Hewlett-Packard
Mixture	10	260	245	OV-17	Program 170 ~300 2°/min	30	10 <sup>2</sup>	16	Hewlett-Packard
Mixture	11	275	270	Hi-EFF 8 BP	Program 140 ~300 30°/min	30	10 <sup>2</sup>	16	Hewlett-Packard

quantity of authentic  $\beta$ -sitosterol to the n-hexane solution of the "yellow band" and injecting the solution into the gas chromatograph under the conditions outlined in Table 5 (p. 36).

A known sample of stigmasterol (Aldrich Chemical Co., Milwaukee, Wis.) was chromatographed on SE-52 and OV-17 columns under the conditions outlined in Table 5 (p. 36).

Identification by Gas-Liquid Chromatography Combined  
With Rapid-Scan Mass Spectrometry

Gas-liquid chromatography in combination with mass spectrometry was used to separate and identify a range of compounds present in the n-hexane-soluble fraction of Douglas-fir bark. The following operating conditions were used:

Gas-Liquid Chromatography

Instrument	F & M 810 (Hewlett-Packard)
Detector	Hydrogen flame ionization
Detector temperature	248 °
Injection sample	4 $\mu$ l of the silylated mixture
Injection port temperature	270 °
Column	UC W-98
Column temperature	Isothermal at 265 °
Carrier gas flow rate	30 ml/min of helium

### Mass Spectrometry

Filament current	70 eV source, 40 $\mu$ A
Electron voltage	20 eV and 70 eV
Analyzer pressure	$1 \times 10^{-6}$ mm Hg
Multiplier voltage	3.00 KV
Scanning speed	6.5 seconds from <u>m/e</u> 24 to 500

The mass spectrometer used was an Atlas CH-4, Nier-type (nine inch, 60 degree sector) single-focusing instrument. The trimethylsilyl ethers of the fractions of interest were separated by gas-liquid chromatography and a portion of the effluent was passed directly into the dual ion source of the mass spectrometer. The gas-liquid chromatograph was fitted with a 5:1 (EC-1 valve:flame) splitter. The mass spectrometer was equipped with an EC-1 throttle valve which was adjusted to permit approximately 10% of the remaining column effluent to enter the ionization chamber, the remaining effluent being vented into the air through a heated tube. The effluent was further split in the ion source with 50% going to the 70 eV source. The 20 eV source operated at less than the ionization potential of the carrier gas (helium), but above that of organic compounds, and was therefore used as a continuous total ionization readout without any contribution from ionized helium. The 70 eV source provided the



ionization used to obtain the mass spectra. The spectra were recorded on a Honeywell 1508 oscillograph.

#### IV. RESULTS AND DISCUSSION

##### Collection of Bark Samples

Since a reasonably representative sample of Douglas-fir bark was desired, small amounts were taken from a number of trees in the area of Corvallis, Oregon. Because of the number of trees sampled and because these samples were thoroughly mixed into a larger collection, no effort was made to ascertain the diameter or age of the trees.

##### Sample Preparation and n-Hexane Extraction

The n-hexane extraction closely followed the method of Kurth and Kieffer (39). These authors reported the n-hexane-soluble material to be a light colored "wax," whereas other solvents such as benzene extracted dark-colored materials. The light color indicated a less complex fraction which was more desirable for an initial study.

The ultraviolet spectrum (Figure 2, p. 24) was identical to that obtained by Manners (41) indicating that the n-hexane extraction was reproducible.

## Separation of the n-Hexane-Soluble Components

### Separation by Solvent Extraction

The n-hexane-soluble fraction was separated into a neutral portion (soluble in diethyl ether, sample A) and an acidic portion (soluble in potassium carbonate, sample B) (Chart 2, p. 26). Both samples A and B were tested by thin-layer chromatography. The results are shown in Table 6.

TABLE 6. THIN-LAYER CHROMATOGRAPHY OF SAMPLES A AND B FROM SOLVENT EXTRACTION.

	Sample A				Sample B		
Spot No.	1	2	3	4	1	2	3
R <sub>f</sub> value	0.02	0.20	0.23	0.48	0.02	0.03	0.09
Color under UV <sup>a</sup>	Blue	Blue	Blue	Blue	Blue	Blue	Blue

<sup>a</sup>Ultraviolet light.

The results showed that the n-hexane-soluble fraction contained both acidic and neutral compounds. It was also evident that both sample A and sample B were mixtures which still required further separation by chromatographic techniques. Thus it was considered that the solvent extraction offered little improvement as a method

of separation and that it was best to go directly to chromatographic methods. Therefore, samples A and B were not further investigated.

### Separation by Column Chromatography

Column chromatography on Silica Gel G was used to provide a coarse separation of the n-hexane-soluble compounds. A number of distinct bands were evident as the column developed (Figure 3, p. 27). The fastest moving bands labeled "dark blue" and "light blue" have been investigated by Aft, H. (2).

The following bands labeled "light bluish green" and "bright blue" (designated in combination as the "yellow band") are the subject of this investigation. This "yellow band" was eluted from the column and tested for purity by thin-layer chromatography. The better resolving power of the thin-layer technique showed that the "yellow band" was a mixture of several chemical compounds. Some thin-layer plates showed as many as 11 distinct spots plus a sizable spot which remained at the origin. The spot at the origin contained an undetermined number of compounds.

A solution of the "yellow band" showed a positive Lieberman-Burchard test (17, p. 70; 18, p. 100) indicative of the presence of sterols.

A quantity of the "yellow band" was accumulated from several columns and the mixture was further separated by thin-layer, paper,

and gas-liquid chromatography.

### Separation by Thin-Layer Chromatography

The results of one-dimensional thin-layer chromatography of the "yellow band" from column chromatography (Figure 3, p. 27) are shown in Table 7 (pp. 44-50).

The results of two-dimensional thin-layer chromatography are illustrated in Figure 4 (p. 57).

The "yellow band" was also separated on plates which were impregnated with silver nitrate. The results are shown in Table 8 (p. 51).

The three plates were examined in an iodine chamber. However, no spots were observed, not even the one detected by the other indicators.

The separation on thin-layer plates impregnated with silver nitrate showed no significant improvement over the separation on plates of Silica Gel G only.

The extensive thin-layer chromatographic study was conducted to find out how many compounds were in the "yellow band" (Figure 3, page 27) and to find a solvent system which gave good separation which could be used as a method to collect pure compounds.

The 14 solvent systems employed are widely used for the separation of lipids (3, 28, 41, 53) as well as for sterols and terpenes. The solvent systems composed of chloroform-carbon tetrachloride

TABLE 7. THIN-LAYER CHROMATOGRAPHY OF THE "YELLOW BAND" FROM THE n-HEXANE- SOLUBLE FRACTION OF DOUGLAS-FIR BARK.

Solvent	Method of Detection	$R_f$ and Color <sup>g</sup> of Spots									
1 CHCl <sub>3</sub> -CCl <sub>4</sub> (6:1 v/v)	1 <sup>a</sup>	.00	.06	.45	.67	.72	.76	.80			
		Y	PY	B	BB	YB	DB	B			
	2 <sup>b</sup>	.00	.06	.34	.45	.55	.67	.72	.76	.80	
		Y	YG	YG	B	LB	YG	B	DB	B	
	3 <sup>c</sup>	.50	.61	.73	.80						
		Pr	Pr	Bn	Bn						
2 CHCl <sub>3</sub> -CCl <sub>4</sub> (1:1 v/v)	4 <sup>d</sup>	.00	.06	.34	.41	.45	.49	.51	.61	.67	.72
											.76
											.80
	5 <sup>e</sup>	.41	.50	.70							
		Y	Y	Y							
	6 <sup>f</sup>	.46	.75								
		DPr	DPr								
	1	.00	.13	.22	.46	.55	.62	.68	.71	.76	
		PY	DB	DB	BB	LB	YB	B	DB	LB	
	2	.00	.13	.22	.46	.58	.70				
		Y	DB	DB	YGr	LB	B				
	3	.26	.41	.51	.67	.74					
		Pr	Pr	Pr	Bn	Bn					
	4	.00	.13	.22	.39	.46	.55	.62	.68	.71	.76
	5	.00	.11	.26	.40	.52	.69				
		Y	Y	Y	Y	Y	Y				
	6	.21	.29	.41	.63						
		DBn	Bn	DPr	DPr						

TABLE 7. Continued.

Solvent	Method of Detection	R <sub>f</sub> and Color of Spots									
$\text{CHCl}_3$ - $\text{CCl}_4$ $\text{CHCl}_3$ - $\text{CCl}_4$ (1:4 v/v)	1	.0	.12	.17	.23	.28	.35	.44			
		Y	B	BB	G	YB	B	B			
	2	.0	.03	.06	.17	.23	.28	.35	.44		
		Y	Y	B	YG	G	YB	B	B		
	3	.08	.18	.29	.37						
		Pr	Pr	Bn	Bn						
	4	.0	.03	.06	.12	.19	.23	.28	.35	.44	
	5	.0	.08	.18	.27		.37	.45			
		Y	Y	Y	Y		Y	Y			
	6	.09	.22	.37	.52						
		DBn	DPr	DPr	Bn						
$\text{CHCl}_3$ - $n\text{-C}_6\text{H}_{14}$ $\text{CHCl}_3$ - $n\text{-C}_6\text{H}_{14}$ (4:1 v/v)	1	.0	.06	.76	.81	.84	.86	.89	.91		
		Y	DB	BB	PB	B	DB	B	PB		
	2	.0	.76	.81	.84	.90					
		Y	YG	B	DB	B					
	3	.49	.64	.74	.84	.90					
		LBn	Pr	LBn	Bn	Bn					
	4	.0	.07	.49	.58	.65	.72	.76	.81	.84	.86
	5	.56	.66	.73	.84						
		Y	Y	Y	Y						
	6	.45	.55	.63	.72	.77	.84	.89			
		DBn	Bn	DPr	Bn	Bn	DPr	DBn			

TABLE 7. Continued.

Solvent	Method of Detection		R <sub>f</sub> and Color of Spots				
5							
C <sub>6</sub> H <sub>6</sub> -n-C <sub>6</sub> H <sub>14</sub> (2:1 v/v)	1	.0	.07	.17	.22	.45	
		Y	BB	YB	B	DB	
	2	.0	.07	.17	.22	.45	
		Y	YG	LB	B	B	
	3	.0	.06	.16	.17	.21	.46
		Y	Bn	Bn	Pr	Pr	LG
	4	.0	.07	.17	.18	.22	.45
	5	.07	.17	.18	.46		
		Y	Y	Y	Y		
	6	.07	.17	.19	.45		
		Bn	DPr	Bn	DBn		
6							
Et <sub>2</sub> O-n-C <sub>6</sub> H <sub>14</sub> (3:1 v/v)	1	.73	.78	.81	.82	.84	
		BB	YB	B	DB	LB	
	2	.73	.78	.81	.82	.85	
		YG	B	B	DB	B	
	3	.73	.76	.82			
		Bn	Pr	DBn			
	4	.73	.76	.82			
	5	.72	.75	.81			
		Y	Y	Y			
	6	.73	.75	.82	.84		
		Bn	DPr	DPr	DBn		



TABLE 7. Continued.

Solvent	Method of Detection		$R_f$ and Color of Spots						
<b>7</b>									
$\text{Et}_2\text{O}-\text{n-C}_6\text{H}_{14}$ (1:4 v/v)	1	.0	.06	.39	.49	.58			
		Y	BB	B	YB	DB			
	2	.0	.06	.39	.49	.58			
		Y	BB	B	YB	DB			
	3	.06	.23	.30	.39	.49	.59		
		Bn	Pr	Pr	Bn	Bn	Bn		
	4	.0	.06	.14	.23	.30	.39	.49	.59
	5	.06	.24	.40	.49	.59			
		Y	Y	Y	Y	Y			
	6	.06	.23	.39	.48	.58			
		DPr	Pr	DPr	Bn	DBn			
<b>8</b>									
$\text{CHCl}_3-\text{C}_6\text{H}_6$ (6:1 v/v)	1	.0	.06	.23	.62	.68	.72	.77	.84
		Y	PY	DB	BB	YB	B	DB	LB
	2	.0	.06	.23	.62	.68	.72	.77	.84
		Y	PY	DB	BB	LB	B	DB	LB
	3	.48	.56	.62	.69	.72	.84		
		Pr	Bn	Bn	Bn	Bn	DBn		
	4	.42	.48	.56	.62	.68	.72	.77	.84
	5	.48	.56	.63	.69	.79			
		Y	Y	BB	Y	Pr			
	6	.42	.49	.56	.63	.72	.85		
		Bn	DPr	Bn	Bn	DPr	DBn		

TABLE 7. Continued.

Solvent	Method of Detection		R <sub>f</sub> and Color of Spots						
9									
CHCl <sub>3</sub> -C <sub>6</sub> H <sub>6</sub> (1:1 v/v)	1	.0	.04	.1	.36	.44	.52	.68	.72
		Y	YG	DB	DB	BB	YB	B	LB
	2	.0	.04	.1	.36	.44	.52	.68	.72
		Y	YG	DB	DB	YG	LB	B	LB
	3	.37	.45	.52	.68	.72			
		Pr	Bn	Bn	Bn	DBn			
4	.0	.04	.1	.36	.44	.52	.68	.72	
5	.36	.44	.52	.67	.72				
	Y	Y	Y	Y	Y				
6	.37	.44	.50	.52	.67	.72			
	DPr	Bn	Bn	Bn	DPr	DBn			
10									
CHCl <sub>3</sub> -C <sub>6</sub> H <sub>6</sub> (1:4 v/v)	1	.0	.18	.25	.26	.33	.42	.51	
		Y	DB	BB	YB	DB	B	DB	
	2	.0	.18	.25	.26	.33	.42	.51	
		Y	DB	YG	LB	DB	B	DB	
	3	.26	.27	.40	.48	.51			
		Pr	Bn	Bn	Bn	DBn			
4	.0	.18	.25	.26	.33	.42	.51		
5	.25	.26	.40	.50					
	Y	YB	Y	Y					
6	.25	.33	.48	.51					
	DPr	Bn	DPr	DBn					

TABLE 7. Continued.

Solvent	Method of Detection	R <sub>f</sub> and Color of Spots								
11 $\text{C}_6\text{H}_6$ -MeOH- HOAc (45:8:4 v/v/v)	1	.80	.84	.89						
		BB	DB	LB						
	2	.80	.84	.89						
		BB	DB	B						
	3	.81	.84	.90						
		Bn	Br	Bn						
12 $\text{n-C}_6\text{H}_{14}$ -Et <sub>2</sub> O- HOAc (70:30:1 v/v/v)	4	.80	.84	.90						
	5	.81	.84	.88						
		Y	Y	Y						
	6	.80	.84	.88						
		DPr	Pr	DPr						
	1	.0	.26	.57	.62	.69				
12 $\text{n-C}_6\text{H}_{14}$ -Et <sub>2</sub> O- HOAc (70:30:1 v/v/v)		Y	BB	B	YB	DB				
	2	.0	.26	.57	.62	.69				
		Y	BB	B	LB	DB				
	3	.51	.57	.62	.69					
		Pr	Bn	Bn	Bn					
	4	.0	.13	.25	.39	.48	.51	.57	.62	.70
12 $\text{n-C}_6\text{H}_{14}$ -Et <sub>2</sub> O- HOAc (70:30:1 v/v/v)	5	.26	.51	.58	.62	.69				
		Y	Y	Y	Y	Y				
	6	.19	.27	.39	.51	.58	.64	.70		
		Bn	DPr	Bn	DPr	DPr	Bn	DBn		

TABLE 7. Continued.

Solvent	Method of Detection	R <sub>f</sub> and Color of Spots						
13 $\underline{n}\text{-C}_6\text{H}_{14}\text{-Et}_2\text{O-}$ HOAc (85:15:1 v/v/v)	1	.0	.12	.20	.42	.47	.60	
		Y	BB	B	B	YB	DB	
	2	.0	.12	.20	.42	.47	.60	
		Y	BB	B	B	YB	DB	
	3	.12	.20	.37	.42	.48	.61	
		Bn	Bn	Pr	Bn	Bn	Bn	
	4	.0	.12	.20	.37	.42	.47	.60
	5	.12	.20	.37	.42	.47	.61	
		Y	Y	Y	Y	Y	Y	
	6	.13	.22	.40	.45	.49	.63	
		Bn	Bn	Pr	DPr	Bn	DBn	
14 $\text{CHCl}_3\text{-Et}_2\text{O-}$ HCOOH (5:4:1 v/v/v)	1	.83	.87	.90				
		YB	B	LB				
	2	.83	.87	.90				
		YB	B	B				
	3	.83	.87	.91				
		Bn	Bn	Bn				
	4	.83	.84	.87	.90			
	5	.85	.89					
		Y	Y					
	6	.83	.84	.89				
		DPr	Pr	DBn				

<sup>a</sup>Ultraviolet light. <sup>b</sup>Ultraviolet light with the plates in ammonia vapors. <sup>c</sup>Plates sprayed with 30% aqueous sulfuric acid, heated for 5 to 10 minutes at 105° in an oven and viewed under ultraviolet light. <sup>d</sup>Plates exposed to iodine vapors. <sup>e</sup>Plates sprayed with bromothymol blue solution. <sup>f</sup>Plates sprayed with *p*-nitroaniline solution. <sup>g</sup>The spots detected are color coded as follows: DB, dark blue; YB, yellow-blue; YG, yellow-green; Y, yellow; Bn, brown; DBn, dark brown; Pr, purple; DPr, dark purple; LB, light blue; PY, pale yellow; BB, bright blue; LBn, light brown; LG, light green.

TABLE 8. THIN-LAYER CHROMATOGRAPHY RESULTS OF PLATES IMPREGNATED WITH SILVER NITRATE,

Solvent	Methods of Detection	$R_f$ and Color <sup>e</sup>						
$\underline{n}\text{-C}_6\text{H}_{14}\text{-Et}_2\text{O-}$ HOAc (70:3:1 v/v/v)	1 <sup>a</sup>	.13	.22	.41	.49	.55	.59	.83
		DB	BB	B	DB	LB	B	DB
	2 <sup>b</sup>	.13	.22	.41	.50	.56	.59	.83
		DB	BB	B	DB	B	B	DB
	3 <sup>c</sup>	.41	.59	.84				
		Pr	Bn	DBn				
	5 <sup>d</sup>	.13	.22	.43	.59	.64	.84	
		Bn	Bn	Y	DBn	Y	DBn	
$\text{CHCl}_3\text{-CCl}_4$ (6:1 v/v)	1	.53	.71	.79				
		DB	YB	DB				
	2	.53	.71	.79				
		DB	YB	DB				
	3	.52	.71	.80				
		Pr	DPr	DBn				
	5	.53	.71	.80				
		Y	DBn	DBn				
$\text{Et}_2\text{O-}\underline{n}\text{-C}_6\text{H}_{14}$ (1:4 v/v)	1	.17	.21	.36				
		B	B	LB				
	2	.17	.21	.36				
		B	B	B				
	3	.22	.36	.49				
		Pr	Bn	DBn				
	5	.23	.36	.49				
		Y	Bn	Y				

<sup>a</sup>Ultraviolet light. <sup>b</sup>Ultraviolet light with the plates in ammonia vapors. <sup>c</sup>Plates sprayed with 30% aqueous sulfuric acid, heated for 5 to 10 minutes at 105° in an oven and viewed under ultraviolet light. <sup>d</sup>Plates sprayed with bromothymol blue solution. <sup>e</sup>The spots detected are color coded as follows: DB, dark blue; YB, yellow-blue; YG, yellow-green; Y, yellow; Bn, brown; DBn, dark brown; Pr, purple; DPr, dark purple; LB, light blue; PY, pale yellow; BB, bright blue; LBn, light brown; LG, light green.

(6:1 v/v), diethyl ether-n-hexane (1:4 v/v), n-hexane-diethyl ether-acetic acid (70:30:1 v/v/v) were the best. Of these three, chloroform-carbon tetrachloride (6:1 v/v) produced more spots than the other two, but each spot had considerable tailing. The diethyl ether-n-hexane (1:4 v/v) system gave very good separation and although the chromatogram showed fewer spots than the solvent system of chloroform-carbon tetrachloride (6:1 v/v) the amount of tailing was reduced. The addition of acetic acid to the ether and n-hexane solvent systems [n-hexane-diethyl ether-acetic acid (70:30:1 v/v/v), and n-hexane-diethyl ether-acetic acid (85:15:1 v/v/v)] did not show improvement. Solvent systems with higher polarity such as benzene-methanol-acetic acid (45:8:4 v/v/v) and chloroform-diethyl ether-formic acid (5:4:1 v/v/v) showed poor separation and fewer spots could be observed than with the less polar solvent systems.

Exposure of the thin-layer plates to iodine vapor proved to be the best method of detecting the spots. It was the easiest to use and showed more spots than the other methods tried. Simple exposure to ultraviolet light was the second best method of spot detection. The combination of ammonia vapors and ultraviolet light did not show any improvement over the use of ultraviolet light alone. The other methods, 30% aqueous sulfuric acid, bromothymol blue spray, and p-nitroaniline spray showed no improvement over the iodine vapor method and were more difficult to use.

Chromatogram sheets impregnated with silver nitrate are known to slow down the migration of compounds with double bonds (4). The  $\pi$  electrons are held by the  $\text{Ag}^+$  ions and so movement is slower. Thus, the more double bonds that a compound has, the lower the  $R_f$  will be. However, in the present investigation the silver nitrate plates did not show any improvement in resolution over Silica Gel G alone. This may indicate a lack of double-bond character in the compounds.

#### Separation by Paper Chromatography

Separation of the "yellow-band" (Figure 3, p. 27) by paper chromatography showed incomplete resolution. None of the four solvent systems completely separated the "yellow-band." The reason seems to be that the highly polar solvent systems did not separate the non-polar n-hexane-soluble fractions.

#### Separation by Gas-Liquid Chromatography

Four columns (Tables 4 and 5, pp. 34 and 36) were used in an attempt to completely resolve the compounds in the "yellow-band" from the column chromatograph (Figure 3, p. 27).

The Hi-EFF 8 BP column did not give good resolution. Even with temperature programming, it showed only five peaks (Figure 11, p. 64). The other three columns showed no great difference in

resolving power although a comparison of Figures 6, 7, and 9 (pp. 59, 60, 62) shows that the SE-52 column did give more peaks than the others. This might be due to greater resolving power of the liquid phase or due to the fact that the column was two times longer than the other three and hence had more theoretical plates (45, p. 60).

Silylation of the mixture before injection into the gas chromatograph seemed not to be necessary. In fact, the silylated mixture gave fewer peaks than the fractions without silylation. A comparison of Figures 5 and 6 ( pp. 58, 59) shows that the silylated mixture in Figure 5 yielded seven peaks, from 5-a to 5-g (the rest of the peaks were due to the silylating reagents), while Figure 6 shows 16 peaks marked from 6-a to 6-p. The last two peaks (peak 6-o and 6-p) in Figure 6 never showed under the conditions used for Figure 5, even after running for seven hours.

Silylation usually enhances the separation of compounds that contain hydroxyl groups. The compounds become more volatile and easier to elute off the column. The reason that fewer peaks showed for the silylated mixture than for the non-silylated mixture is not clear at present. It is possible that silylation made the compounds more similar in boiling points and in polarities, so that the column did not separate them. Although silylation gave fewer peaks, it did provide better resolution between peaks 5-f and 5-g (Figure 5, p. 58) than between peaks 6-m and 6-n (Figure 6, p. 59). This



better resolution proved to be very helpful for later mass spectrometry work.

Temperature programming methods were used because of the wide boiling-point ranges of the compounds and because there were so many compounds. Figures 8, 10 and 11 (pp. 61, 63, and 64) show these chromatograms. The results in Figure 8 indicate that the mixture may have consisted of three groups of compounds. The first group (peaks 8-a to 8-d) had elution temperatures below 148°. The second group contained four compounds (peaks 8-e to 8-h) which had elution temperatures between 210° and 248°. The third group contained high boiling compounds with elution temperatures over 260°. These three figures showed a rise in the base line as the temperature increased. This is characteristic of temperature-programmed gas-liquid chromatography (16, p. 466). A rate of temperature increase of 2°/min. proved to give better resolution than more rapid increases in temperature.

Separation of the "yellow band" from the column chromatograph (Figure 3, p. 27) by thin-layer chromatography prior to injection into the gas chromatograph yielded improved separation. The "yellow-blue band" (high  $R_f$ ) from the thin-layer plates was shown to contain two compounds by gas-liquid chromatography. Their retention times were identical with peaks 8-h and 8-i (Figure 8, p. 61) under the same gas-liquid chromatographic conditions. It is quite possible

that these peaks represent the compounds in the n-hexane solubles which give the "yellow-blue band" on thin-layer chromatography.

However, there were no peaks by gas-liquid chromatography which could be attributed to the "bright-blue band" (low  $R_f$ ) from the thin-layer plates. It may be that the "bright-blue band" contained high-boiling compounds which did not elute under the conditions used.

The results of gas-liquid chromatography showed that at least 20 compounds were jammed into the "yellow band" from column chromatography (Figure 3, p. 27). Nineteen of these are shown in Figure 8, (p. 61) and there is a minimum of one in the "bright-blue band" from the thin-layer plates which did not show in Figure 8, making a minimum of 20 compounds. These materials must be similar in chemical nature and structure. This further increases the difficulty of separation and purification. The identification of every compound shown by gas-liquid chromatography is often impossible, mainly because of the time and difficulties involved and the minute amounts of material which can be detected (9, p. 130).

However, the combination of thin-layer chromatography and gas-liquid chromatography produced evidence for the identity of some of the compounds.

Previously published papers (35, 36) mentioned that phytosterol was present in the n-hexane-soluble fraction of Douglas-fir bark. The phytosterol was said to be a mixture of  $\alpha$  - and  $\beta$ -sitosterol.

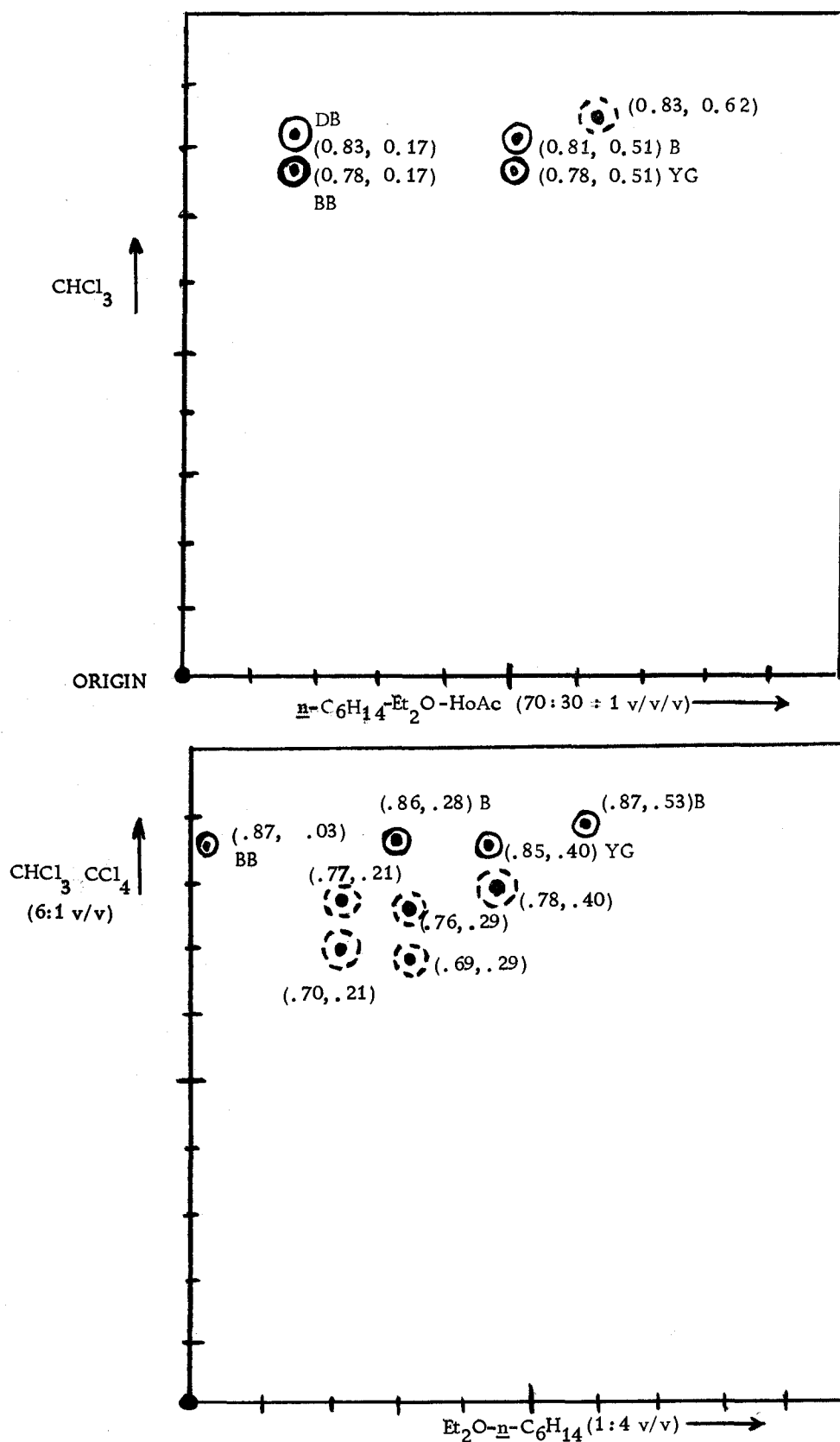


Figure 4. Two-dimensional thin-layer chromatography of the "yellow-band."  $\odot$  = observed under ultraviolet light;  $\odot$  = observed on exposure to iodine vapors.

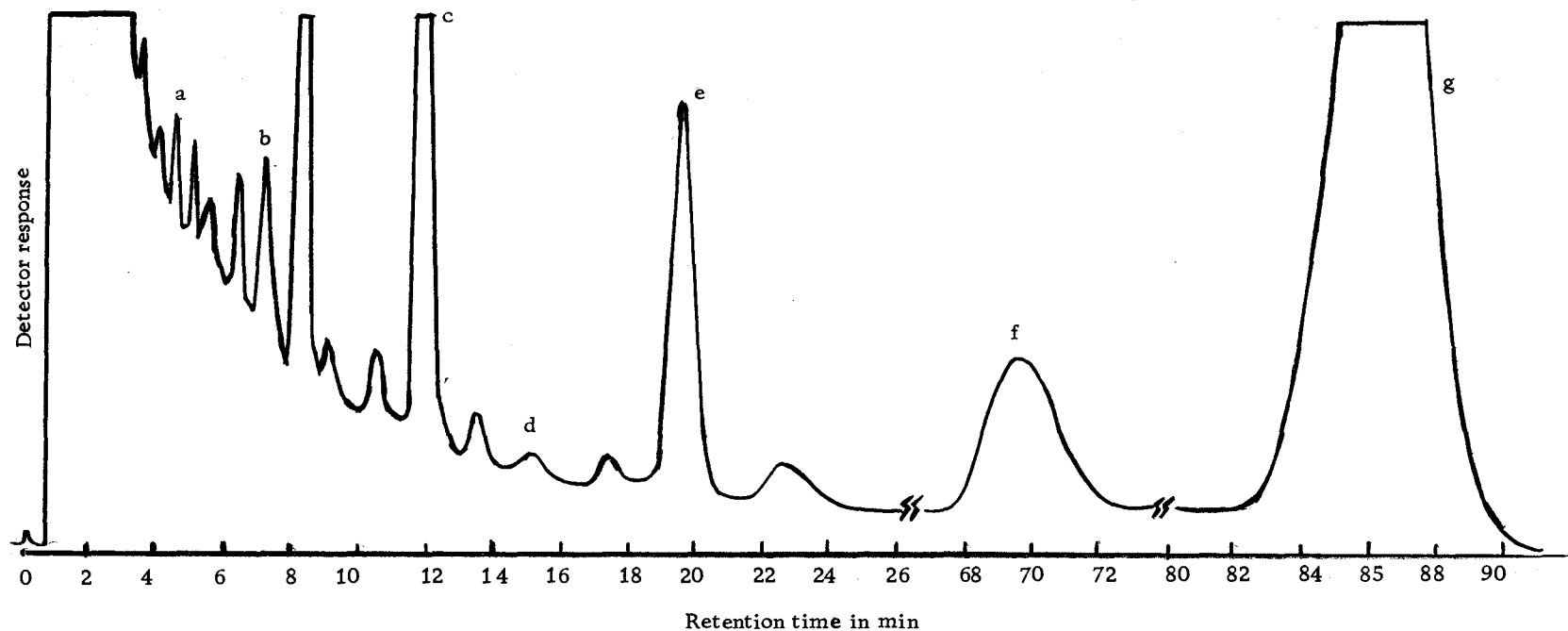


Figure 5. Gas-liquid chromatographic separation on SE-52 of the silylated mixture of the "yellow-band." Peaks "a, b, c, d, and e" are unidentified; "f" is campesterol, "g" is  $\beta$ -sitosterol. Unlettered peaks are solvent. Conditions: Column, 10% SE-52 on 60/80 mesh Gas Chrom. W, 12 ft x 1/8 in O.D., stainless steel; injection port 277°, detector 265°, column temp. 255° isothermal; helium flow rate 60 ml/min; range setting 1, attenuation setting 4; instrument, Aerograph.

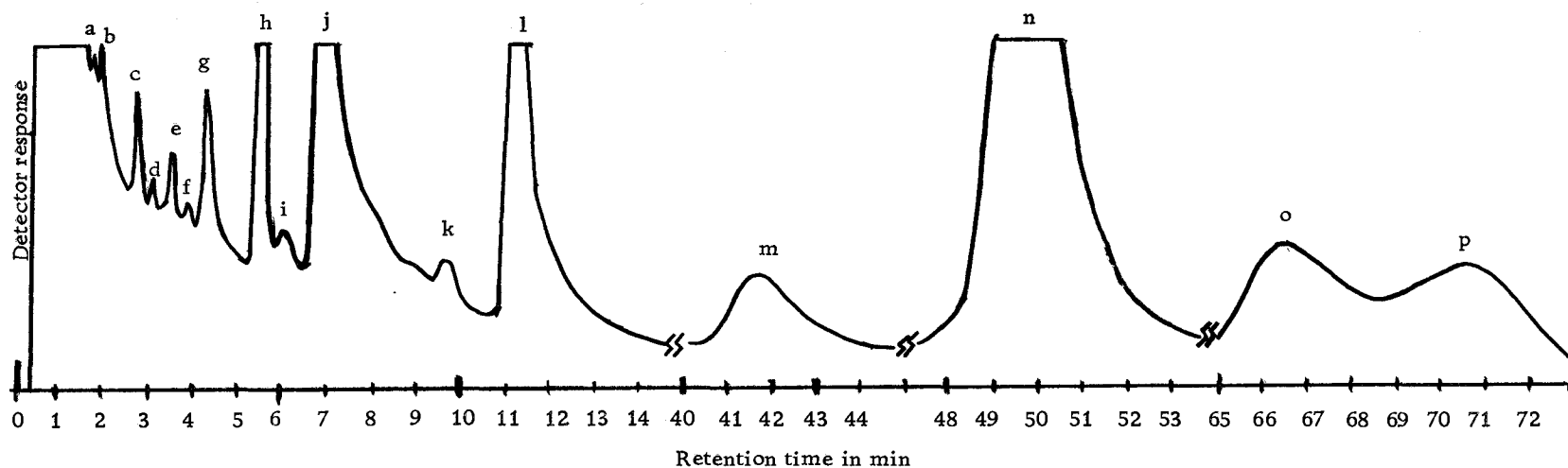


Figure 6. Gas liquid chromatographic separation on SE-52 of the nonsilylated mixture of the "yellow-band." Peaks "a, b, c, d, e, f, g, h, i, j, k, l, o, and p" are unidentified, "m" is campesterol, "n" is  $\beta$ -sitosterol. Unlettered peaks are from solvent. Conditions: Column, 10% SE-52 on 60/80 mesh Gas Chrom. W, 12 ft x 1/8 in O.D., stainless steel; injection port 277°, detector 265°, column temp. 255° isothermal; helium flow rate 60 ml/min; range setting 1, attenuation setting 4; instrument, Aerograph.

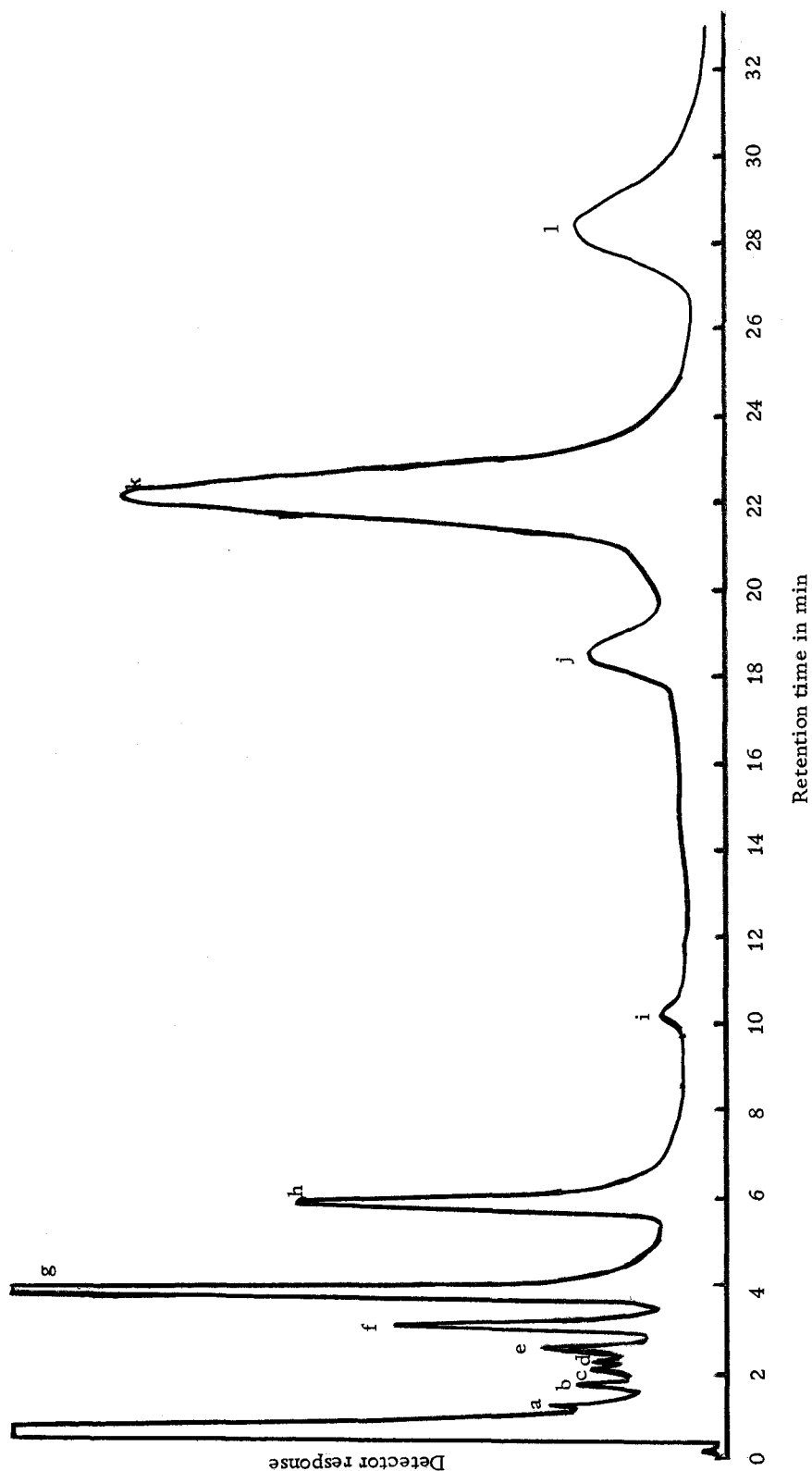


Figure 7. Gas-liquid chromatographic separation on UC W-98 of the nonsilylated mixture of the "yellow band." Peaks "a, b, c, d, e, f, g, h, i, and l" are unidentified; "j" is campesterol; "h" is  $\beta$ -sitosterol. Conditions: Column, 10% UC W-98 on 80/100 mesh Silicone Gum, 6 ft x 1/8 in O.D., stainless steel; injection port 275°, detector 270°, column temp. 275° isothermal; helium flow rate 28 ml/min; range setting 10<sup>2</sup>, attenuation setting, 16; instrument, Hewlett-Packard.

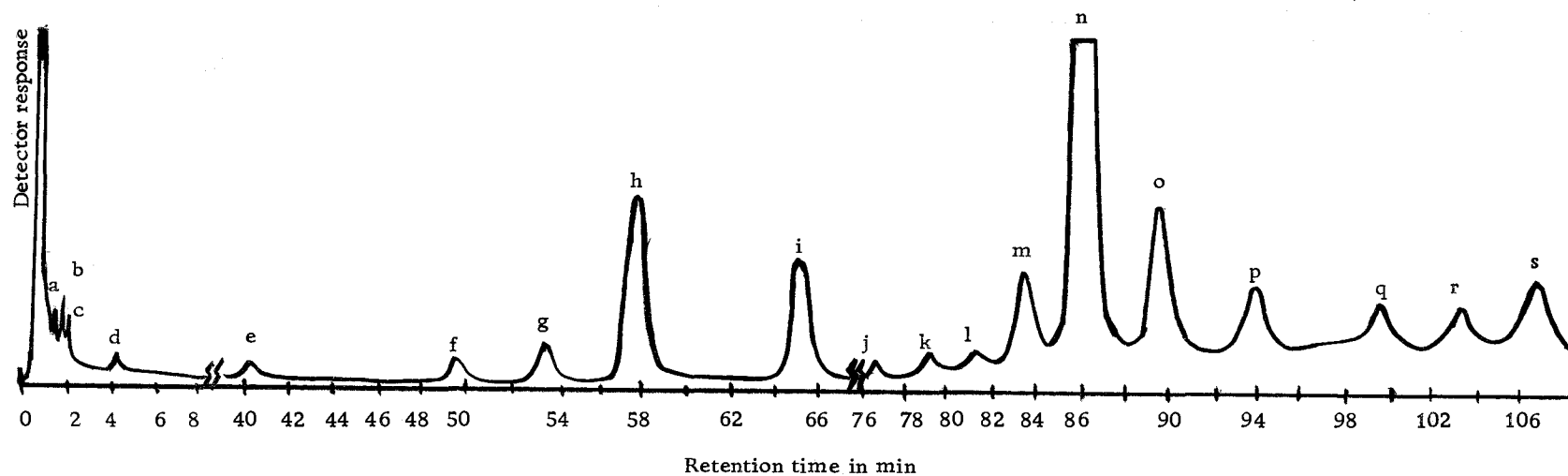


Figure 8. Gas-liquid chromatographic separation on UC W-98 (temperature programmed) of the nonsilylated mixture of the "yellow-band." Peaks "a, b, c, d, e, f, g, h, i, j, k, l, o, p, q, r, and s" are unidentified, "m" is campesterol, "n" is  $\beta$ -sitosterol. Unlettered peaks are from solvent. Conditions: Column, 10% UC W-98 on 80/100 mesh Silicone Gum, 6 ft x 1/8 in O. D., stainless steel; injection port 235°, detector 255°, column temp. held at 148° for 10 min, then programmed at 2°/min to 300°; Helium flow rate 28 ml/min; range setting  $10^2$ , attenuation setting 16; instrument, Hewlett-Packard.

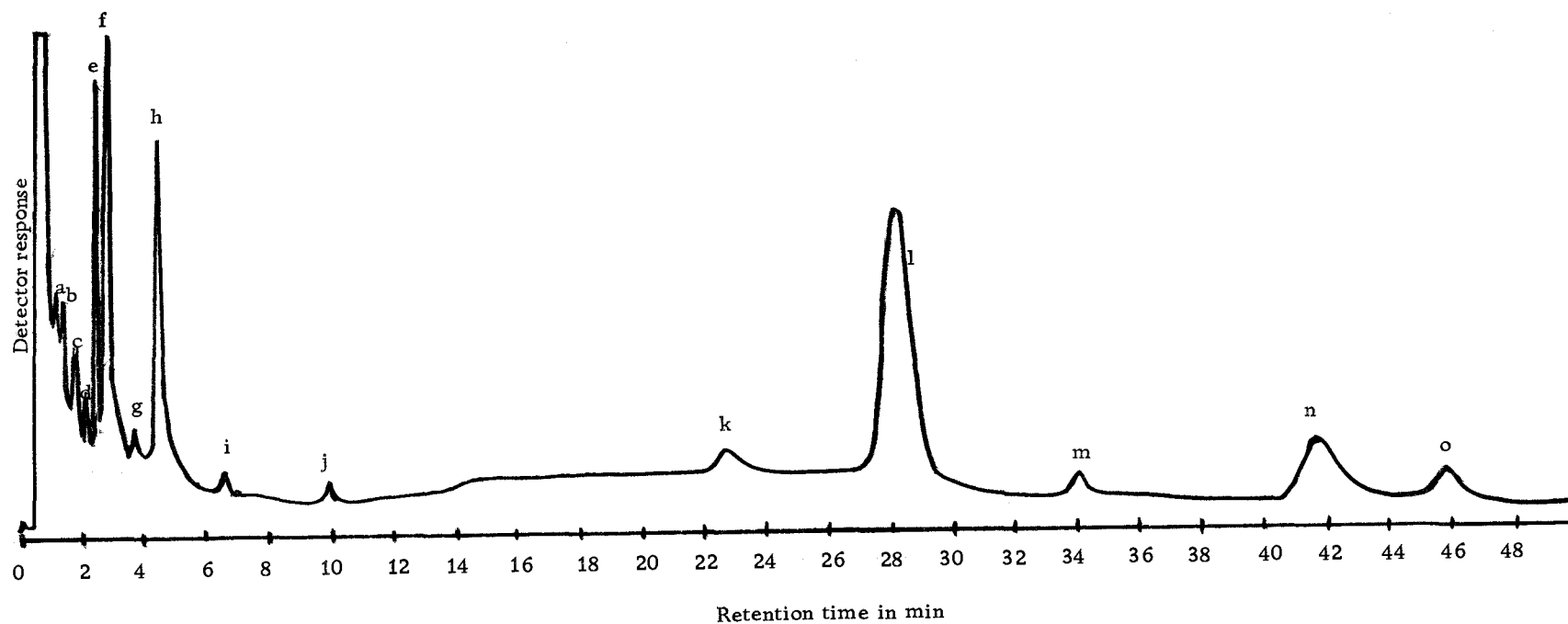


Figure 9. Gas-liquid chromatographic separation on OV-17 of the nonsilylated mixture of the "yellow-band." Peaks "a, b, c, d, e, f, g, h, i, j, m, n, and o" are unidentified, "k" is campesterol, "l" is  $\beta$ -sitosterol. Unlettered peaks are from solvent. Conditions: Column, 3% OV-17 on 100/120 mesh Gas chrom Q 6 ft x 1/8 in O.D., stainless steel; injection port 272°, detector 278°, column temp. 255° isothermal; helium flow rate 30 ml/min; range setting 10<sup>2</sup>, attenuation setting 16; instrument, Hewlett-Packard.



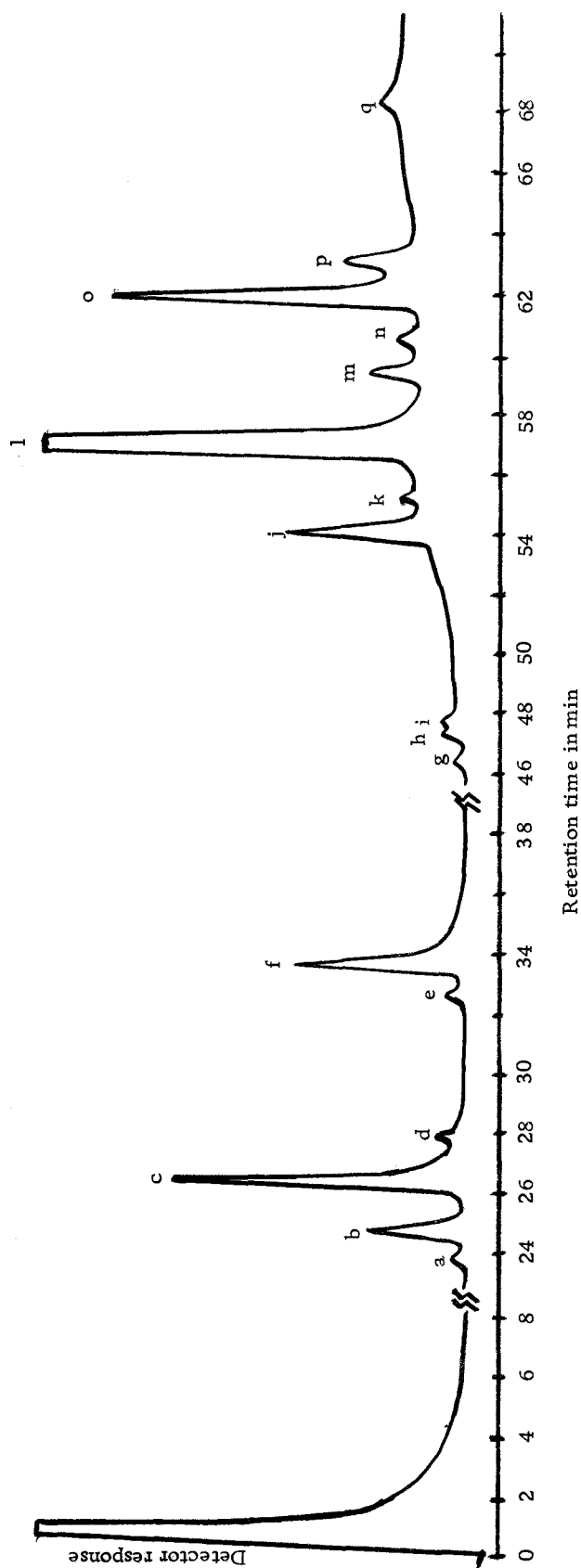


Figure 10. Gas-liquid chromatographic separation on OV-17 (temperature programmed) of the nonsilylated mixture of the "yellow-band." Peaks "a, b, c, d, e, f, g, h, i, k, m, n, o, p, and q" are unidentified, "j" is campesterol, "l" is  $\beta$ -sitosterol. Unlettered peaks are from solvent. Conditions: Column, 3% OV-17 on 100/120 mesh GasChrom Q, 6 ft x 1/8 in O.D. stainless steel; injection port 245°, detector 260°, column temp. programmed from 170° at 2°/min to 300°, helium flow 30 ml/min; range setting 10<sup>2</sup>, attenuation setting 16; instrument, Hewlett-Packard.

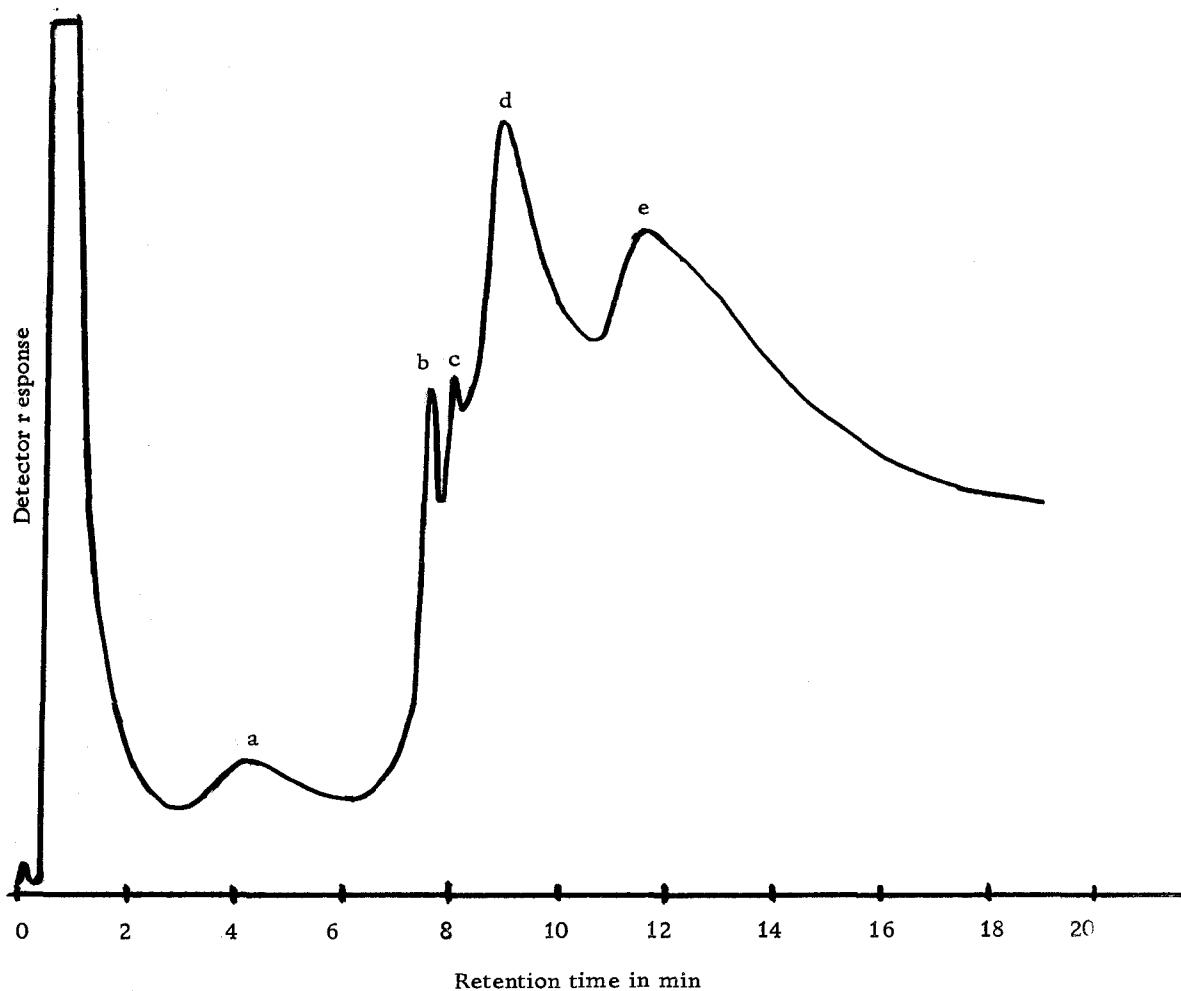


Figure 11. Gas-liquid chromatographic separation on Hi-EFF 8BP of the nonsilylated mixture of the "yellow-band." Peak "a" is campesterol, peak "d" is  $\beta$ -sitosterol, peaks "b, c, and e" are unidentified. Unlettered peaks are from solvent. Conditions: Column, 3% Hi-EFF 8BP on 100/120 mesh Gas Chrom Q, 6 ft x 1/8 in O.D., stainless steel; injection port 270°, detector 275°, column temp. programmed from 140° at 30°/min to 300°; helium flow 30 ml/min; range setting 10<sup>2</sup>, attenuation setting 16; instrument, Hewlett-Packard.

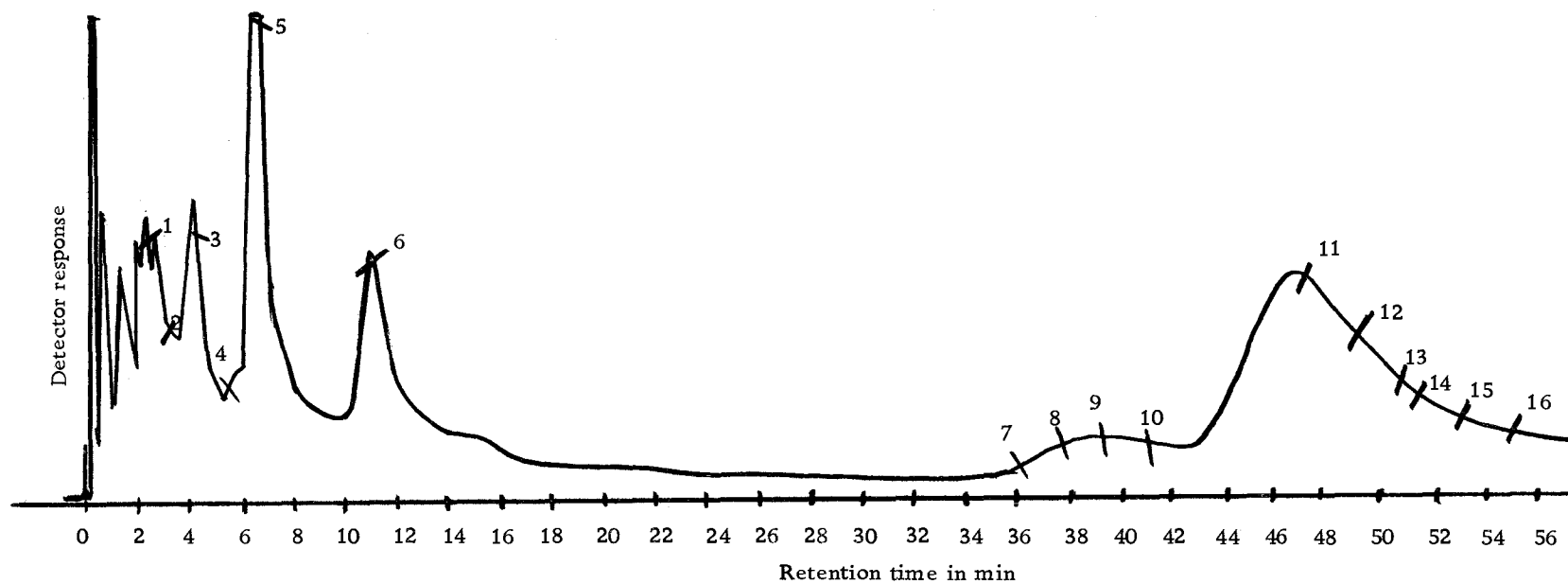


Figure 12. Gas-liquid chromatographic separation on UC W-98 of the silylated mixture of the "yellow-band." Identification by rapid scan mass spectrometry. Mass spectrum scans were taken where the numbers indicate. Conditions: Column, 10% UC W-98 on 80/100 mesh Silicone Gum, 6 ft x 1/8 in O.D., stainless steel; injection port 270°, detector 248°, column temp. 265° isothermal; helium flow rate 30 ml/min; instrument F&M 810 (Hewlett-Packard).

However, it has later been found that  $\alpha$ -sitosterol is not an entity in itself but is a mixture of several sterols (4).

The positive result of the Liebermann-Burchard test in the present work indicated that the "yellow-band" isolated by column chromatography (Figure 3, p. 27) contained unsaturated sterols. This "yellow band" was shown by thin-layer chromatography to contain a compound which migrated the same distance as authentic  $\beta$ -sitosterol. The comparison was made in 14 different solvent systems. The  $R_f$  values of authentic  $\beta$ -sitosterol and the corresponding spot in the "yellow band" are shown in Table 9.

The sample of  $\beta$ -sitosterol from the Aldrich Chemical Co. was also subjected to gas-liquid chromatography using the columns and conditions outlined in Table 5 (p. 36). The sample was shown to be not completely pure  $\beta$ -sitosterol but contained a small amount of a material with the same retention time as campesterol (20). A comparison of the retention time of campesterol and  $\beta$ -sitosterol with those compounds in the "yellow-band" showed that with each column separation there was a peak in the "yellow-band" with the same retention time as the knowns. The results are given in Table 10.

A "peak enhancement" study, performed by adding an aliquot of known campesterol and  $\beta$ -sitosterol to the solution of the "yellow-band," showed no new peaks in the gas-liquid chromatographic

TABLE 9.  $R_f$  VALUES OF  $\beta$ -SITOSTEROL AND CORRESPONDING SPOT FROM THE "YELLOW BAND"

Solvent system	$\beta$ -Sitosterol "Yellow Band" <sup>h</sup>	
$\text{CHCl}_3^a$ - $\text{CCl}_4^b$ (6:1 v/v)	0.50	0.51
$\text{CHCl}_3$ - $\text{CCl}_4$ (1:1 v/v)	0.41	0.46
$\text{CHCl}_3$ - $\text{CCl}_4$ (1:4 v/v)	0.19	0.19
$\text{CHCl}_3$ - $\underline{n}\text{-C}_6\text{H}_{14}^e$ (4:1 v/v)	0.65	0.65
$\text{C}_6\text{H}_6^d$ - $\underline{n}\text{-C}_6\text{H}_{14}$ (2:1 v/v)	0.18	0.18
$\text{Et}_2\text{O}^e$ - $\underline{n}\text{-C}_6\text{H}_{14}$ (3:1 v/v)	0.76	0.76
$\text{Et}_2\text{O}$ - $\underline{n}\text{-C}_6\text{H}_{14}$ (1:4 v/v)	0.23	0.23
$\text{CHCl}_3$ - $\text{C}_6\text{H}_6$ (6:1 v/v)	0.48	0.48
$\text{CHCl}_3$ - $\text{C}_6\text{H}_6$ (1:1 v/v)	0.37	0.36
$\text{CHCl}_3$ - $\text{C}_6\text{H}_6$ (1:4 v/v)	0.25	0.25
$\text{C}_6\text{H}_6$ - $\text{MeOH}^f$ - $\text{HOAc}^g$ (45:8:4 v/v/v)	0.79	0.79
$\underline{n}\text{-C}_6\text{H}_{14}$ - $\text{Et}_2\text{O}$ - $\text{HOAc}$ (70:30:1 v/v/v)	0.51	0.51
$\underline{n}\text{-C}_6\text{H}_{14}$ - $\text{Et}_2\text{O}$ - $\text{HOAc}$ (85:15:1 v/v/v)	0.37	0.37
$\text{CHCl}_3$ - $\text{Et}_2\text{O}$ -Formic acid (5:4:1 v/v/v)	0.84	0.84

<sup>a</sup> $\text{CHCl}_3$ -chloroform<sup>b</sup> $\text{CCl}_4$ -carbon tetrachloride<sup>c</sup> $\underline{n}\text{-C}_6\text{H}_{14}$ - $\underline{n}$ -hexane<sup>d</sup> $\text{C}_6\text{H}_6$ -benzene<sup>e</sup> $\text{Et}_2\text{O}$ -diethyl ether<sup>f</sup> $\text{MeOH}$ -methanol<sup>g</sup> $\text{HOAc}$ -acetic acid<sup>h</sup>These  $R_f$  values are also shown in the respective solvent systems in Table 7 (p. 44).

spectra. The peaks thought to be campesterol and  $\beta$ -sitosterol (Table 10) were considerably larger than when no known compounds were added indicating that the retention times were identical.

TABLE 10. GLC<sup>a</sup> COMPARISON OF THE "YELLOW BAND" WITH KNOWN CAMPESTEROL AND  $\beta$ -SITOSTEROL.

Figure for GLC separation of "yellow band"	Peak with same retention time as campesterol	Peak with same retention time as $\beta$ -sitosterol
Figure 5, p. 58	5-f	5-g
Figure 6, p. 59	6-m	6-n
Figure 7, p. 60	7-j	7-k
Figure 8, p. 61	8-m	8-n
Figure 9, p. 62	9-k	9-l
Figure 10, p. 63	10-j	10-l
Figure 11, p. 64	11-a	11-d

<sup>a</sup>Gas-liquid chromatography.

It is concluded from the thin-layer and gas-liquid chromatographic evidence that the n-hexane-soluble fraction from Douglas-fir bark contains campesterol and  $\beta$ -sitosterol.

It is now known that often the fraction which in the earlier literature was called  $\alpha$ -sitosterol was in reality a mixture of campesterol and stigmasterol (4). Since campesterol was found in the "yellow-band" from column chromatography (Figure 3, p. 27), it was considered possible that stigmasterol was also present. However, the retention time for known stigmasterol by gas-liquid chromatography did not match the retention times for any of the

compounds separated from the "yellow-band" (Figures 5 through 11, pp. 58-64). Therefore, it was thought unlikely that stigmasterol was present in the "yellow-band" fraction.

Since iso-fucoesterol and fucoesterol are considered to be biosynthetic precursors of  $\beta$ -sitosterol (20), it was thought that these compounds might be present with  $\beta$ -sitosterol in the "yellow-band" fraction (Figure 3, p. 27). The Hi-EFF 8 BP column has been reported (20) to separate fucoesterol from  $\beta$ -sitosterol. The chromatogram from this column (Figure 11, p. 64) did show a peak (peak 11-e) which had a close relative retention time (11.37 min) to that published for fucoesterol (11.33 min) (20). Therefore, it is possible that fucoesterol is present in the n-hexane-soluble fraction but more definitive evidence is required. A sample of pure fucoesterol could not be obtained for this work.

#### Identification by Gas-Liquid Chromatography Combined With Rapid Scan Mass Spectrometry

A portion of the gas effluent from the gas-liquid chromatographic column was passed directly into the ion-source of the mass spectrometer. The remaining portion of the gas effluent from the column was passed through a flame ionization detector which produced an electrical signal which was recorded on an X-Y strip recorder. In this way when a peak appeared on the recorder, a portion

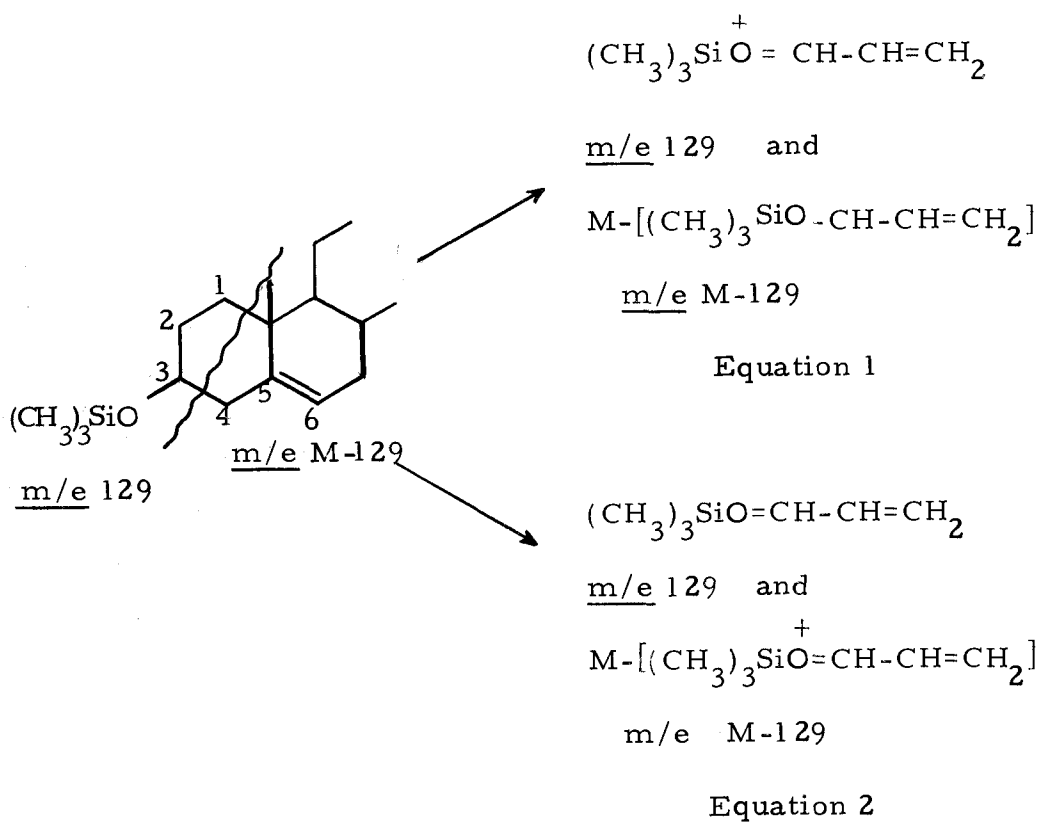
of the compounds causing the peak was also in the mass spectrometer and a rapid scan by the mass spectrometer produced a fragmentation spectra for that compound. Thus the gas-liquid chromatograph separated the mixture into pure compounds and the mass spectrometer yielded information as to the identity of each pure compound.

An aliquot of the trimethylsilyl ethers of the known campesterol and  $\beta$ -sitosterol mixture was injected into the gas chromatograph. As the peaks appeared on the recorder, a scan was made by the mass spectrometer. In this way standard mass spectra were obtained for known  $\beta$ -sitosterol and campesterol for reference purposes.

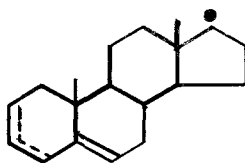
In the same way, the trimethylsilyl ethers of the "yellow-band" (Figure 3, p. 27) were injected into the gas chromatograph. The gas-liquid chromatographic spectrum was scanned by mass spectrometry in the positions shown in Figure 12, (p. 65). In the mass spectrum of the trimethylsilyl ether of  $\beta$ -sitosterol (from Figure 12, scan 15), characteristic peaks were found at m/e 486, 396, 381, 357, 255 and 129. This cracking pattern was found to be in good agreement with that published by Eneroth, Hellstrom and Ryhage (15) although the relative intensities of the peaks were slightly different. This was attributed to the fact that the experimental conditions were not identical. The base peak at m/e 129, which is a characteristic fragment for a 5-en-3-ol steroid trimethylsilyl ether (46, p. 38) was very obvious. In the fragmentation reaction the trimethylsilyl



ether derivative is split into two parts, one part which is charged and the other which is neutral. The charge can reside on the m/e 129 fragment (Equation 1) or the m/e M-129 fragment (M is the molecular ion mass) (Equation 2). Therefore, both fragments are recorded, but usually the intensities are different. This fragmentation is demonstrated below.



A peak at m/e 255 was also intense and was considered to represent the nuclear fragment 1



Fragment 1

The mass spectra for scans 7, 8, 9 and 10 (Figure 12, p. 65) were not as conclusive for campesterol as might be expected from the retention times. However, peaks at  $m/e$  129 and 255; which are characteristic for sterols, were observed in spectra numbers 8, 9 and 10. These spectra indicated mixtures of terpenes and sterols. Spectrum number 8 showed considerable evidence for terpenes (p. 79) while spectra numbers 9 and 10 showed less evidence for terpenes and more for sterols.

Peaks in the high mass range ( $m/e > 300$ ) were too weak to be seen. Thus, no molecular ion peaks for  $\beta$ -sitosterol or campesterol were evident. There are two reasons for the weak spectrum in this mass range. First, both campesterol and  $\beta$ -sitosterol are high molecular weight, high boiling compounds, and it proved to be very difficult to get all of the sample into the ionization chamber. A portion of the sample probably condensed in the interface between the gas chromatograph and the mass spectrometer, so that only a portion of the sample slowly and gradually passed into the ionization chamber. Therefore, although the gas-liquid chromatographic resolution was good, the total ionization record from the mass spectrometer was somewhat smeared and the spectra were weak. This is why the best spectrum for  $\beta$ -sitosterol was obtained at scan 15 of Figure 12 rather than at scan 11 or 12. Secondly, campesterol is present in much smaller amounts in the mixture injected than is  $\beta$ -sitosterol. Therefore, the sample injected into the gas-liquid chromatograph contained little campesterol and thus very little material was available for the

mass spectrometer even though the gas-liquid chromatographic detection was adequate. Add to this the condensation problem discussed above, and the result was that very, very little campesterol entered the ionization chamber.

A 2-1/2 times increase in the amount of the mixture injected into the gas-liquid chromatograph did not solve the problem, since the amount of  $\beta$ -sitosterol then became so large that it overlapped the campesterol peak on gas-liquid chromatography. With the larger sample gas-liquid chromatographic resolution was poorer, and the mass spectra showed gross contamination of the components with each other. Difficulty in obtaining a good spectrum for campesterol was also experienced by Eneroth, Hellstrom and Ryhage (15). They published a spectrum from an incompletely resolved sample and, therefore, their spectrum also represents a mixture.

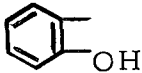


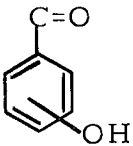
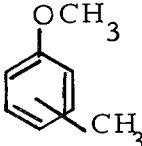
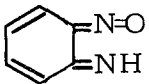
The mass spectral data support the gas-liquid chromatographic retention data as to the presence of campesterol. The ratio of campesterol to  $\beta$ -sitosterol is about 1 to 8 as estimated by areas under the gas-liquid chromatographic curves.

The identification of campesterol and  $\beta$ -sitosterol in the n-hexane-soluble fraction of Douglas-fir bark has proven very interesting because it leads to a search for other sterols and the part which they might play in bark. The physiological role of these materials is largely unknown, although Rowe (52) has suggested that  $\beta$ -sitosterol may have a function in cell-wall permeability.

Combined Gas-Liquid Chromatography Mass Spectrometry of Unidentified Compounds

Several mass spectra were obtained to help elucidate the structures of the unidentified peaks of Figure 12, (p. 65); positions where spectra were obtained are indicated as shown in Figure 12. All mass spectra originally showed peaks at  $m/e = 18, 28, 32, 40$  and  $44$ , originating, respectively, from  $H_2O$ ,  $N_2$ ,  $O_2$ , Ar and  $CO_2$ .

Spectrum #1. In this spectrum, intense peaks were noted at  $m/e$  93 and 121 and, in fact,  $m/e$  93 was the base peak. Silverstein and Bassler (56, p. 62) suggest the following structures for  $m/e$  93 and 121:

$m/e$	Ions
93	$CH_2Br$ ,  $C_7H_9$ ,  $C=O$
	 $C_7H_9$ (terpenes)
121	   $C_9H_{13}$ (terpenes)

Considering the source of the sample, and other fragment ions of the spectra, a terpene structure seems more probable than any of the other structures listed above.

Ryhage and von Sydon (54) obtained the mass spectra of 17 monoterpene hydrocarbons and both  $m/e$  93 and 121 were found in

every case. With a few exceptions, many monoterpene hydrocarbons had m/e 93 as the base peak. Ryhage and von Sydow described it as a characteristic peak of terpenes. McLafferty indicated (42, p. 40) that m/e 93.0704 can arise from monoterpenes ( $C_{10}H_{16}$ ), sesquiterpenes ( $C_{15}H_{26}$ ), or cyclohexenyl- $Y_2$ - $Y_3$  (where  $Y_n$  is one or more functional groups). The probability of monoterpenes was given as 0.66 while the relative probability of sesquiterpenes was 0.06. Therefore, the total probability of m/e 93 in spectrum 1 being a terpene is 0.72. McLafferty also indicated that m/e 121 can be a fragment of monoterpenes (42, p. 51). A. F. Thomas and B. Wilhalm also found m/e 93 and 121 to be characteristic ions for terpenes (58, p. 475). The present study found an ion at m/e 136, which is also indicative of terpenes (58, p. 475).

Gas-liquid chromatography of several representatives of the terpene classes indicated that the monoterpenes eluted first, the sesquiterpenes next, then the diterpenes, and lastly the triterpenes (4). Compound No. 1 (Figure 12, p. 65) showed similar retention times by gas-liquid chromatography to the data accumulated for the known monoterpenes. Therefore, it is thought that the peak of m/e 136 is likely to be the molecular ion peak of a monoterpene hydrocarbon.

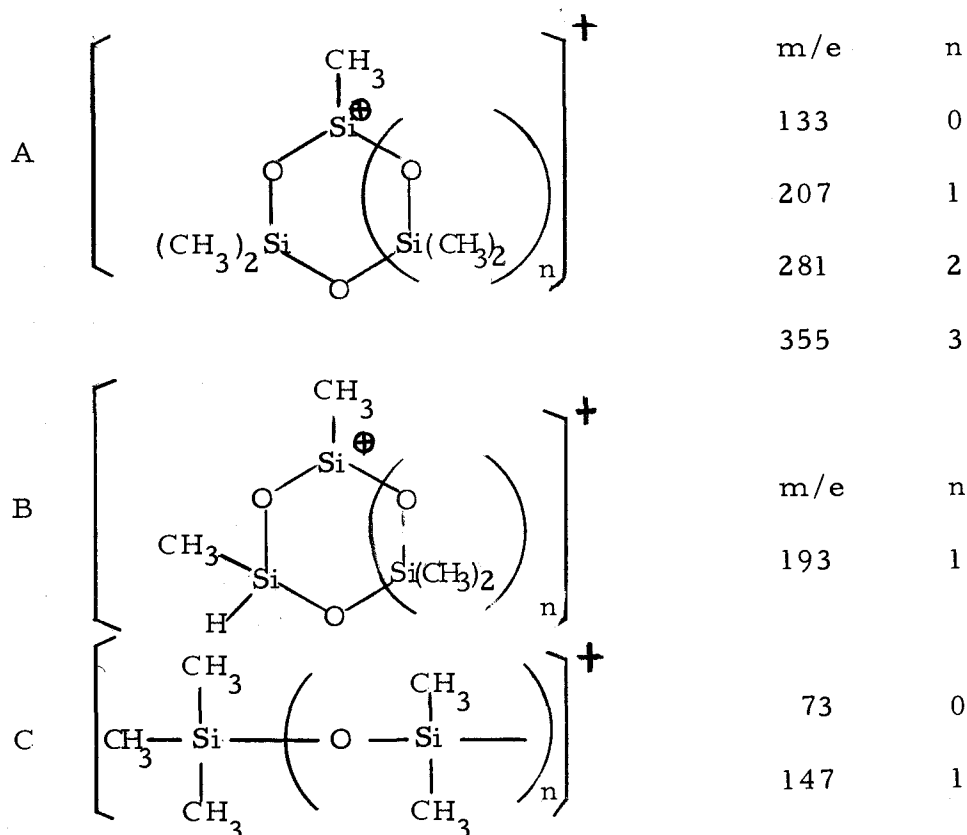
Conjugated double bonds can make a compound more stable under electron bombardment than the same compound without

conjugation (48, p. 99). Therefore, a conjugated compound is likely to have a larger parent peak. The small parent peak of compound No. 1 (Figure 12, p. 65) suggests that this compound does not have conjugated double bonds (48, p. 99).

If m/e 136 is due to the parent ion, then the peak at m/e 93 is due to M-43, from the loss of an isopropyl group or indirectly from the M-15 moiety. With a parent ion at m/e 136, the ion at m/e 121 is due to M-15. A peak at m/e 91 was also observed for compound No. 1 (Figure 12, p. 65). This ion is also common for many monoterpene hydrocarbons (54). This ion at m/e 91 corresponds to the stable tropylium ion, arising from m/e 93 through the loss of two hydrogen atoms or a hydrogen molecule (11, p. 141). An ion at m/e 80 was also observed. This ion can arise from monoterpene hydrocarbons although it is usually of low intensity. Since pyridine was used as the solvent for the silylation reaction, m/e 80 can also arise from the  $N^{15}$  in the M + 1 parent peak of pyridine. From the above data, it is quite possible that compound No. 1 is a monoterpene hydrocarbon. Peaks at m/e 41, 55, and 105 were also observed. These peaks are common in monoterpene hydrocarbon mass spectra (54).

Because the sample was silylated and because silicone compounds were used as the stationary phase in the gas-liquid chromatographic column, a number of peaks corresponding to silicon

compounds were observed. These triplet ions were noted at m/e 73-75, 133-135, 193-195, 207-209, and 281-283 (7, p. 171). These ions are due to the species represented as A, B and C below.



An intense ion at m/e 327 was also thought due to a fragment that contains silicone, but it was not in the above series (40).

Since the gas-liquid chromatographic column temperature was over 240°, monoterpenes might be expected to be decomposed. Therefore, this spectrum is likely to contain peaks of thermally decomposed monoterpene hydrocarbons. This was demonstrated by the peaks at m/e 119 and 134, probably arising from a metal catalyzed thermal dehydrogenation. The former was due to (M-2)-15

and the latter was due to M-2. The 119 fragment was much larger than m/e 136, but the m/e 134 ion had the same intensity as the 136 ion. The relative intensities of the fragments originating from the thermally undecomposed molecules are unchanged, which makes an identification quite feasible even if some thermal decomposition has occurred (54).

Spectrum #2. Spectrum #2 was scanned at the valley between two peaks. Very little information can be obtained from the data.

Spectrum #3. This spectrum showed prominent ions at m/e 41, 43, 55, 69, 70, 80, 91, 112, 121 and 136, and the ion at m/e 41 was the base peak. The fragment ion at m/e 41 very likely corresponds to  $C_3H_5^+$ , but its origin may involve hydrogen rearrangement if the parent compound is a terpene (11, p. 142). If the ion of very low intensity at m/e 136 is the molecular ion, the weak intensity likely indicates that the double bonds in this compound are not in conjugation. This spectrum was very similar to the published spectrum of myrcene (54). However, when authentic myrcene was compared with the sample by gas-liquid chromatography at 140° column temperature, it showed a different retention time.

Spectrum #3 also contained large triplet peaks at m/e 355-357 which were very likely due to a silicone compound. The possible structure is shown on page 77, where n is equal to 3.



Spectra #4, 5 and 7. Little information can be obtained from these spectra and the results will not be discussed here.

Spectra #6 and 8. In spectrum #6 prominent ions were found at m/e 41, 55, 69, 91, 93 and 121. In spectrum #8 intense ions were found at m/e 41, 55, 57, 93, 121 and 135. These two spectra probably contain terpenes, but there is insufficient evidence to say that they are monoterpenes. Both spectra contained a prominent ion at m/e 383. This is almost certainly due to a silicone compound and has the structure shown on page 77, where n is equal to 4.

## V. SUMMARY AND CONCLUSIONS

1. The n-hexane solvent extracted a light-colored, "wax-like" fraction from Douglas-fir bark.

2. The n-hexane-soluble fraction was not well resolved by partition between diethyl ether solvent and a 5% aqueous potassium carbonate solution.

3. The n-hexane-soluble fraction was partially resolved by column chromatography using Silica Gel G as stationary phase and chloroform-n-hexane (3:1 v/v) as developing solvent. Several bands were observed on the column with ultraviolet light as detecting agent. Two narrow bands which were close to each other but well separated from the remaining bands were collected as a fraction. This fraction was designated as the "yellow band."

4. The "yellow band" fraction was further separated by thin-layer chromatography on Silica Gel G using 14 different solvent systems. Of these, chloroform-carbon tetrachloride (6:1 v/v) and diethyl ether-n-hexane (1:4 v/v) were the best. Exposure of the plates to iodine vapors proved to be the best method for detecting the spots. Fourteen different compounds were detected. Two of the compounds were identified as  $\beta$ -sitosterol and campesterol.

Neither two-dimensional chromatography nor chromatography on plates impregnated with silver nitrate showed an improvement

over the solvent systems indicated above.

5. Resolution of the "yellow band" was attempted by paper chromatography using four different solvent systems. However, separation was incomplete and considerable overlapping of compounds was observed.

6. The "yellow band" fraction was resolved by gas-liquid chromatography using four different liquid phases and a variety of temperatures, including temperature programming. The liquid phases SE-52, OV-17, and UC-W-98 provided good resolution, but the phase Hi-EFF 8BP showed poor separation. Gas-liquid chromatography showed the presence of at least 20 compounds, two of which were identified as  $\beta$ -sitosterol and campesterol.

7. Positive identification of the presence of  $\beta$ -sitosterol and campesterol in the "yellow band" fraction was accomplished by gas-liquid chromatography combined with rapid scan mass spectrometry. The data from the mass spectra also indicated the presence of several terpenes, two of which are monoterpenes.

## BIBLIOGRAPHY

1. Adams, M. Composition of wood turpentine. *Journal of Industrial and Engineering Chemistry*. 7:957-960. 1915.
2. Aft, H. Associate Professor, Department of Chemistry, University of Maine at Farmington, Farmington, Maine. Personal Communication. 1969.
3. Aida, K. Ethanolysis products from bark flavonoids and polymeric phenolics. Master's thesis. Corvallis, Oregon State University, 1968. 79 numb. leaves.
4. Baisted, D. J. Associate Professor, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon. Personal Communication. 1970.
5. Banks, R. C. Isolation of certain toxic compounds of kraft mill waste and attempts to determine their structure. Doctoral thesis. Corvallis, Oregon State University. 1968. 109 numb. leaves.
6. Becker, Edward S. and E. F. Kurth. The chemical nature of the extractives from the bark of red fir. *Tappi* 41:380-384. 1958.
7. Biemann, K. Mass spectrometry: Organic chemical applications. New York. McGraw-Hill Book Company, 1962. 370 p.
8. Bobbitt, J. M. Thin layer chromatography. New York, N. Y. Reinhold Publishing Co. 1963. 208 p.
9. Bobbitt, J. M.; A. E. Schwarting and R. J. Gritter. Introduction to chromatography. New York, N. Y. Reinhold Publishing Co. 1968. 160 p.
10. Bray, H. G., W. V. Thorpe and K. White. The fate of certain organic acids and amides in the rabbit. 10. The application of paper chromatography to metabolic studies of hydroxybenzoic acids and amides. *Biochemical Journal* 46:271-275. 1950.

11. Budzikiewicz, H., C. Djerassi and D. H. Williams. Structure elucidation of natural products by mass spectrometry. Vol. II: Steroids, terpenoids, sugars, and miscellaneous classes. San Francisco. Holden-Day, Inc. 1964. 690 p.
12. Cahn, R. S. An introduction to the sequence rule. Journal of Chemical Education 41:116-125. 1964.
13. Carlberg, G. L. and E. F. Kurth. Extractives from the western true firs. Tappi 43:982-988. 1960.
14. Chang, Y. Bark structure of North American conifers. (U. S. Dept. of Agriculture, Technology Bulletin 1095). 1954. 86 p.
15. Eneroth, P., K. Hellstrom and R. Ryhage. Identification and quantification of neutral fecal steroids by gas-liquid chromatography and mass spectrometry: studies of human excretion during two dietary regimens. Journal of Lipid Research 5:245-257. 1964.
16. Ewing, G. W. Instrumental methods of chemical analysis. New York, N.Y. McGraw-Hill, Inc. 1969. 627 p.
17. Fieser, L. F. Experiments in organic chemistry. 3rd ed. Boston, D. C. Heath and Company. 1957. 353 p.
18. Fieser, L. F. and Fieser, Mary. Natural products related to phenanthrene. 3rd ed. New York, N.Y. Reinhold Publishing Co. 1949. 704 p.
19. Gage, T. B., C. D. Douglass and S. H. Wender. Identification of flavonoid compounds by filter paper chromatography. Analytical Chemistry 23:1582-1585. 1951.
20. Goad, L. J. and T. W. Goodwin. The biosynthesis of sterols in higher plants. Biochemical Journal 99: 735-746. 1966.
21. Grillos, S. T. Structure and development of the bark of Douglas-fir, Pseudotsuga menziesii (Mirb.) Franco. Doctoral thesis. Corvallis, Oregon State University, 1956. 67 numb. leaves.
22. Grillos, S. T. and F. H. Smith. The secondary phloem of Douglas-fir. Forestry Science. 5:377-388. 1959.

23. Harborne, J. B. The chromatography of flavonoid pigments. *Journal of Chromatography* 2:581-604. 1959.
24. Hart, H. and R. D. Schuetz. Organic chemistry. 3rd. ed. Boston, Houghton Mifflin Company. 1966. 353 p.
25. Hathway, D. E. and J. W. T. Seakins. Hydroxystilbenes of Eucalyptus wandoo. *Biochemical Journal* 8:335-339. 1958.
26. Hergert, H. L. The chemical nature of the extractives from White Fir bark. *Tappi* 36:137-144. 1953.
27. Hergert, H. L. Chemical composition of cork from White Fir bark. *Forest Products Journal* 8:335-339. 1958.
28. Hergert, H. L. and E. F. Kurth. The chemical nature of the cork from Douglas-fir bark. *Tappi* 35:59-66. 1952.
29. Holmes, G. W. The chemical composition of newly formed inner bark of Douglas-fir Pseudotsuga menziesii (Mirb) Franco. Doctoral thesis. Corvallis, Oregon State University, 1961. 70 numb. leaves.
30. Holmes, G. W. and E. F. Kurth. The chemical composition of the newly formed inner bark of Douglas-fir. *Tappi* 44:893-898, 1961.
31. Jensen, W., K. E. Fremer, P. Sierilä and V. Wartiovaara. The chemistry of bark. In: *The chemistry of wood*, ed. by B. L. Browning, New York, N. Y. Interscience Publishers, 1963. p. 587-666.
32. Kieffer, H. J. and E. F. Kurth. The chemical composition of the bast fibers of Douglas-fir bark. *Tappi* 36:14-19. 1953.
33. Krahmer, R. L., R. W. Hemingway and W. E. Hillis. The cellular distribution of lignans in Tsuga heterophylla wood. *Wood Science and Technology* 4:122-139. 1970.
34. Kurth, E. F. Wax from lignin residue in ethanol production. Paper read before the meeting of the northwest section of the Forest Products Research Society, Portland, Oregon, May 19, 1948.

35. Kurth, E. F. The composition of the wax in Douglas-fir bark. *Journal of the American Chemical Society* 72:1685-1686. 1950.
36. Kurth, E. F. The chemical composition of conifer bark waxes and corks. *Tappi* 50:253-257. 1967.
37. Kurth, E. F. and J. K. Hubbard. Extractives from Ponderosa Pine bark. *Industrial and Engineering Chemistry* 43:896-900. 1951.
38. Kurth, E. F., J. K. Hubbard and J. D. Humphrey. Chemical composition of Ponderosa and Sugar Pine barks. *Paper Trade Journal* 130:37-42. 1960.
39. Kurth, E. F. and H. J. Kieffer. Wax from Douglas-fir bark. *Tappi* 33:183-186. 1950.
40. Libbey, L. M. Associate Professor, Department of Food Science and Technology, Oregon State University, Corvallis, Oregon. Personal Communication. 1970.
41. Manners, G. M. The chemical composition of the bark extractives of four species of the genus *Pseudotsuga*. Master's thesis. Corvallis, Oregon State University. 1965. 73 numb. leaves.
42. McLafferty, F. W. Mass spectral correlations. Washington, American Chemical Society. 1963. 117 p.
43. Mutton, D. B. Wood Resins. In: *Wood extractives and their significance to the pulp and paper industry*, ed. by W. E. Hillis, New York, N. Y. Academic Press Inc., 1962. p. 331-363.
44. Noller, C. R. *Chemistry of organic compounds*. 3rd ed. Philadelphia, W. B. Saunders Company, 1965. 1114 p.
45. Pecsok, R. L. and L. D. Shields. *Modern methods of chemical analysis*. New York, N. Y. John Wiley and Sons, Inc., 1968. 480 p.
46. Pierce, A. E. *Silylation of organic compounds*. Rockford, Illinois. Pierce Chemical Co., 1968. 487 p.
47. Randerath, K. *Thin layer chromatography*. New York, N. Y. Academic Press, Inc. 1963. 250 p.

48. Reed, R. F. Application of mass spectrometry to organic chemistry. New York, N. Y. Academic Press, Inc. 1966. 256 p.
49. Reio, L. A method for the paper-chromatographic separation and identification of phenol derivatives, mould metabolites and related compounds of biochemical interest, using a "reference system." *Journal of Chromatography* 1:338-373. 1958.
50. Roberts, E. A. H. and D. J. Wood. Separation of tea polyphenolics on paper chromatograms. *Biochemical Journal* 53: 332-336. 1953.
51. Ross, W. D. and R. L. Krahmer. Some sources of variation in the structure of Douglas-fir bark. Accepted for publication in *Wood and Fiber*. Vol. 3. 1971.
52. Rowe, J. W. The sterols of pine bark. *Phytochemistry* 4: 1-10. 1965.
53. Ruggieri, S. Separation of methyl esters of fatty acids by thin layer chromatography. *Nature* 193:1282-1283. 1962.
54. Ryhage, R. and E. von Sydön. Mass spectrometry of terpenes I. Monoterpene hydrocarbons. *Acta Chemica Scandinavica* 17: 2025-2035. 1963.
55. Schorger, A. W. Oils of the coniferae: V-The leaf and twig, and bark oil of incense cedar. *Industrial and Engineering Chemistry* 8:22-24. 1916.
56. Silverstein, R. M. and G. C. Bassler. Spectrometric identification of organic compounds. 2nd ed. New York, John Wiley and Sons, Inc., 1967. 256 p.
57. Smith, J. E. and E. F. Kurth. The chemical nature of cedar barks. *Tappi* 36:71-78. 1953.
58. Thomas, A. F. and E. Willhalm. Les spectres de masse des hydrocarbures monosterpéniques. *Helvetica chimica acta* 47:465-487. 1964.