DOUGLAS-FIR BARK. III. STEROL AND WAX ESTERS OF THE *n*-HEXANE WAX[1,2]

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ABSTRACT

Sterol and wax esters were isolated as chemically intact compounds from the *n*-hexane-soluble fraction of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] bark in a continuing effort to elucidate the properties of this wax. The sterol esters were composed of sitosterol and campesterol esterified to any or all of the fatty acids, *n*-tridecanoic acid, *n*-hexadecanoic acid, *n*-heptadecanoic acid, *cis*-9-octadecenoic acid, *n*-nonadecanoic acid, *n*-eicosanoic acid, *n*-docosanoic acid, and *n*-tetracosanoic acid. The wax esters were composed of 1-docosanol and 1-tetracosanol, also esterified to the above-named acids. The fact that any or all of the fatty acids can be esterified to each of the sterols and alcohols, producing a great mixture of different sterol and wax esters, may partly account for the softness and low melting point of Douglas-fir *n*-hexane wax.

Keywords: Sterol esters, wax esters, sitosterol, campesterol, Douglas-fir bark, *Pseudotsuga menziesii*, *n*-hexane wax.

INTRODUCTION

The wax from the bark of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco], a high quality vegetable wax that can be used in formulating polishing waxes and manufacturing carbon paper (Good and Trocino 1974; Trocino 1975), has been of commercial interest for some 35 years (Hall 1971). The presence of wax in Douglas-fir bark was recognized as early as 1923 (Howard 1923) but attracted little attention until the late 1940s when Clark et al. (1947) extracted the bark with benzene and reported "wax-like brown and black substances." Kurth (1950a) and Kurth and Kiefer (1950) did pioneering studies on Douglas-fir bark wax; extracting bark from 80- to 95-year-old trees with *n*-hexane gave a light-colored "hexane wax" (5.47% yield), and extracting the residue with benzene gave a light-brown "benzene wax" (additional 2.52% yield). Patents were granted in the early 1950s for extraction and refining of the wax (Kurth 1950b, 1953, 1954) and, more

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[1] First in series: Laver, M. L., C.-H. Chen, J. V. Zerrudo, and Y.-C. Lai. 1974. Carbohydrates of the inner bark of *Pseudotsuga menziesii*. Phytochemistry 13:1891–1896. Second in series: Fernandez, E. C., and M. L. Laver. 1985. Douglas-fir bark. II. Isolation and characterization of a glucomannan. Submitted to Wood and Fiber Science.

[2] Taken in part from the Ph.D. thesis of Henry H.-L. Fang, Oregon State University, Corvallis, OR, 1974. FRL 1671, Forest Research Laboratory, Oregon State University. The mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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recently, for extraction and utilization with new solvent systems and processes (Trocino 1971, 1973). A commercial plant (Bohemia Lumber Company, Incorporated, Eugene, OR) for extracting Douglas-fir bark wax was started in 1975 and operated for 6–7 years.

At this laboratory, Laver et al. (1971) reported the presence of uncombined sitosterol and campesterol in the *n*-hexane-soluble fraction (*n*-hexane wax) as well as some unidentified terpenes and "steroid-like" compounds. Loveland and Laver (1972a, b) showed that upon chemical fragmentation by saponification, the *n*-hexane-insoluble but benzene-soluble materials (benzene wax) of Douglas-fir bark yielded *n*-fatty acids, $\alpha\,\omega$-dicarboxylic acids, ω-hydroxy fatty acids, and some fatty alcohols. However, these previous studies involved identification of components that existed in the free state in the original wax and of components that resulted from degradation when the wax was saponified. No effort was made to determine the functional groups or linkages uniting these substances in the original wax. The work reported here emphasizes the isolation of major components—sterol and wax esters—as they exist in their combined forms in the *n*-hexane wax in a continuing effort to elucidate the properties of Douglas-fir bark wax and to aid in its possible modifications for commercial use.

ANALYTICAL TECHNIQUES

Thin-layer chromatography (TLC) used Silica Gel G (type 60) (E M Laboratories Inc., Elmsford, NY) as the solid phase (0.25 mm thick); two solvent systems, (A) diethyl ether-*n*-hexane (1:4 v/v) and (B) chloroform-carbon tetrachloride (6:1 v/v); and two indicator methods, (1) ultraviolet (UV) light and (2) iodine vapors.

Gas-liquid chromatography (GC) was performed on a Hewlett-Packard 5750B gas chromatograph equipped with flame ionization detectors. Three column systems were tested: (1) stainless steel column 1.829 m long × 2.159 mm inside diameter packed with 10% UC W-98 on Silicone Gum 80/100 mesh; (2) stainless steel column (same dimensions as column system 1) packed with 3% OV-17 on Gas Chrom Q 100/120 mesh; and (3) stainless steel column 3.658 m long × 2.159 mm inside diameter packed with 10% SE-52 on Chromosorb G (high performance) 80/100 mesh. Preparative GC used a stainless steel column 1.829 m long × 5.334 mm inside diameter packed with 3% OV-17 on Gas Chrom Q 100/120 mesh. For preparative work, the gas chromatograph was fitted with an effluent splitter, which divided the effluent stream after elution from the column such that $\frac{1}{6}$ went to the detector and the other $\frac{5}{6}$ went to the outlet and was collected, usually in a liquid nitrogen trap.

Liebermann-Burchard tests for sterols were performed by placing a few milligrams of dry sample into a test tube and adding a few milliliters of acetic anhydride. Concentrated sulfuric acid was added dropwise and the color observed. The test is positive if the colorless solution first turns purple, then blue, then dark green (Fieser 1968).

For saponification, the material of interest was dissolved in 95% ethanol containing 10% potassium hydroxide and refluxed gently for 3.75 h. The reaction products were extracted three times in a separatory funnel with 10-ml portions of *n*-hexane. The *n*-hexane layer contained the unsaponifiables (alcohols), the ethanol layer the saponifiables (salts of carboxylic acids). The ethanol layer was acidified to pH 3–4 with concentrated sulfuric acid and extracted three times in

a separatory funnel with 10-ml portions of diethyl ether, resulting in diethyl ether solubles (carboxylic acids) and ethanol-water solubles (inorganic salts).

Purified samples were subjected to mass spectrometry (MS). Mass spectrometry spectra were obtained on a Finnigan 1015C mass spectrometer by the direct ion source method (filament current 400 A, electron voltage 70 eV, analyzer pressure 10^{-7} atm). Because this was a low-resolution instrument, the masses of the ions were determined only to the closest whole-mass unit. However, this proved sufficient for unambiguously identifying the compounds when the Finnigan mass spectrometer just described was attached in tandem to a Varian Aerograph 1400 gas chromatograph (GC/MS), and the MS and chromatographic data were both used.

Melting points were obtained by the hot stage method on a Fischer-Johns melting point apparatus and are uncorrected.

ISOLATING THE *n*-HEXANE WAX

The bark sample[3] was collected from a dominant 58-year-old Douglas-fir tree (diameter at breast height 37.6 cm, overall height 31.1 m, bark thickness 3.8 cm) in the George T. Gerlinger Experimental Forest near Falls City, OR, which is operated by the College of Forestry, Oregon State University, in cooperation with the State Forestry Department of Oregon. The bark was ground in a disk grinder until small enough to pass through a screen with openings 12.7 mm square. A part (1974.76 g; moisture content 11.2%, oven-dry method) was extracted for 36 h with *n*-hexane in a Soxhlet apparatus. The solvent was evaporated under aspirator vacuum on a rotary evaporator. Final drying was by passage of a stream of dry nitrogen; yield was 100.91 g (5.11%) of a light yellow, waxlike solid. This yield is similar to the 5.47% reported by Kurth and Kiefer (1950) and the 4.75% (old-growth Douglas-fir bark) and 4.80% (second-growth Douglas-fir bark) reported by Manners (1965). Thus, a value near 5% seems consistent.

ISOLATING AND IDENTIFYING THE STEROL WAX ESTERS

A part (17.0 g) of the wax was chromatographed on a column prepared by slurrying 700.0 g of Silica Gel G with 2,000.0 ml of chloroform-*n*-hexane (3:1 v/v) solvent and 72.0 ml of water and pouring the slurry into a glass column (75.0 mm inside diameter × 1.0 m long). The column was irrigated with chloroform-*n*-hexane (3:1 v/v) and yielded a series of bands that fluoresced under UV light (Fig. 1). This paper deals only with the fastest moving fluorescent band. Investigations of other bands will be described in subsequent publications.

The fastest moving band was collected in 12 fractions (425 ml, 352 ml, 9 × 100 ml, 50 ml) analyzed by TLC (solvent systems A and B; indicator method 1 followed by 2). Fractions 1–6 showed two concentrated colored areas although each area showed tailing. The later fractions to elute demonstrated more colored areas, indicating an increasingly complex mixture. None of the fractions had spots that migrated the same distance as authentic sitosterol or campesterol on cochromatography. Liebermann-Burchard tests were conducted on fractions 1, 2, 4, 6, 9, and 11. Fractions 1–9 were positive; the most prominent color was from

[3] A voucher specimen (no. 140826; Douglas-fir bark) from this geographical region is deposited at the Herbarium of the Department of Botany, Oregon State University, Corvallis, OR.

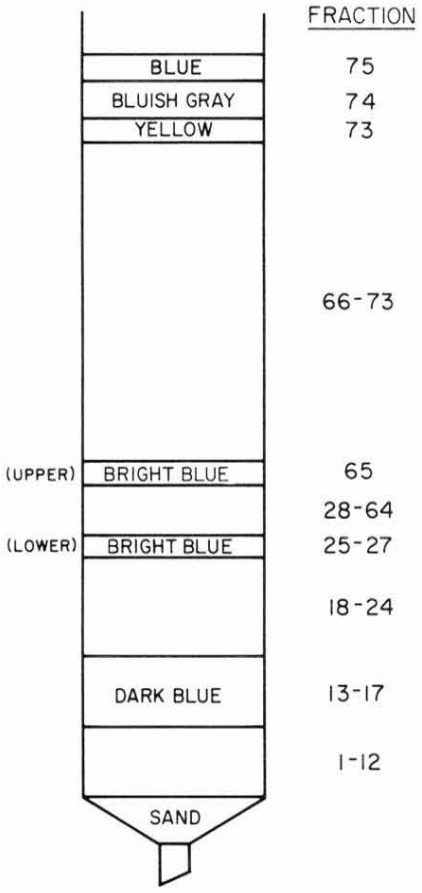


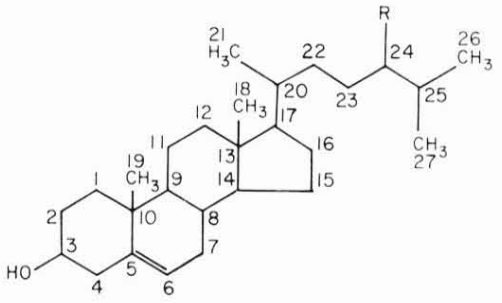
FIG. 1. Column chromatogram of the *n*-hexane-soluble fraction from Douglas-fir bark. Solvent system: chloroform-*n*-hexane (3:1 v/v); detection UV light.

fraction 2, the least prominent from fraction 9 (estimated visually). Gas chromatography of the fractions revealed no peaks at all, even though column systems (1, 2, and 3) and conditions (column temperature 240 C, detector temperature 270 C, injection port temperature 260 C) known to separate free sterols were used.

Fraction 2 from the fastest moving fluorescent band was saponified. Thin-layer chromatography (solvent system A) of the unsaponifiables showed a spot (R_f = 0.15) that migrated the same distance as authentic sitosterol on co-chromatography. Gas chromatography (column system 1: 240 C isothermal, detector 270 C, injection port 255 C, helium carrier gas 35 ml/min; column system 2: 240 C isothermal, detector 265 C, injection port 260 C, helium carrier gas 35 ml/min) of the unsaponifiables showed peaks with the same retention time as authentic 1-docosanol (6.0 min on column system 1, 4.0 min on column system 2), 1-tetracosanol (10.0 min on column system 1, 6.7 min on column system 2), campesterol (35.0 min on column system 1, 35.2 min on column system 2), and sitosterol (43.0 min on column system 1, 43.5 min on column system 2). Some minor peaks also were evident but were not identified. Peak enhancement tech-

niques showed no additional peaks, and the peaks corresponding to campesterol and sitosterol became larger when these authentic compounds were added. The areas under the GC peaks showed the ratio of the alcohols to be: 1-docosanol 4.0, 1-tetracosanol 4.6, campesterol 1.0, and sitosterol 4.6. Thus, fatty alcohols were present in somewhat greater total quantity than sterols.

Solids of the fractions whose peaks had the same retention times as those of campesterol and sitosterol were collected by preparative GC, and microgram amounts of each solid were individually analyzed by MS. The MS spectrum of the material of the preparative GC peak with the retention time of campesterol (Fig. 2A) showed mass-to-charge ratio (*m/z*) peaks at 400 (molecular ion peak, or $M^{+\cdot}$, of I, $C_{28}H_{48}O$, requires 400), 385, 382, 367, 315, 289, 274, 273, 261, 255, 246, 231, 229, and 213. Although numerous peaks were below *m/z* 200, the characteristic ones were at 145, 119, 107, 95, and 81 (base peak). The MS spectrum of the material of the preparative GC peak with the retention time of sitosterol (Fig. 2B) showed *m/z* peaks at 414 ($M^{+\cdot}$ of II, $C_{29}H_{50}O$, requires 414), 399, 396, 381, 329, 303, 288, 275, 273, 255, 246, 231, 229, and 213. Again, numerous peaks were below *m/z* 200, but the characteristic ones, including the base peak, were the same as for I. Peaks representing *m/z*s of $[M - CH_3]^+$, $[M - H_2O]^+$, and $[M - CH_3 - H_2O]^+$ also were evident in each spectrum (Fig. 2A, B). These are general phenomena for sterols such as I (campesterol, when $R = CH_3$) and II (sitosterol, when $R = C_2H_5$):



I. R = CH_3 = CAMPESTEROL
II. R = C_2H_5 = SITOSTEROL

The MS spectrum in Fig. 2A indicated that the compound was a Δ^5-monounsaturated sterol. The peaks at *m/z* $[M - 85]^+$ and $[M - 111]^+$ are known to occur mainly in Δ^5-monounsaturated sterols and serve to distinguish this class from the corresponding Δ^7-monounsaturated sterols. In a Δ^5-monounsaturated sterol such as I, the *m/z* $[M - 85]^+$ peak would arise from loss of a fragment or fragments containing C_2 to C_6 or C_3 to C_7 plus two hydrogen atoms, the *m/z* $[M - 111]^+$ peak from loss of a fragment or fragments containing C_1 to C_7. The high intensity of these two peaks indicated that the sterol was in the free form (Knights 1967). The *m/z* positions of the mass spectrum (Fig. 2A) were identical to those published for campesterol (Stenhagen et al. 1974). On the basis of GC and MS data, the compound was identified as I.

The MS spectrum in Fig. 2B indicated that this compound also was a Δ^5-monounsaturated sterol. Just as for I, the *m/z* peak at $[M - 85]^+$ would arise from a sterol such as II from loss of a fragment or fragments containing C_2 to C_6

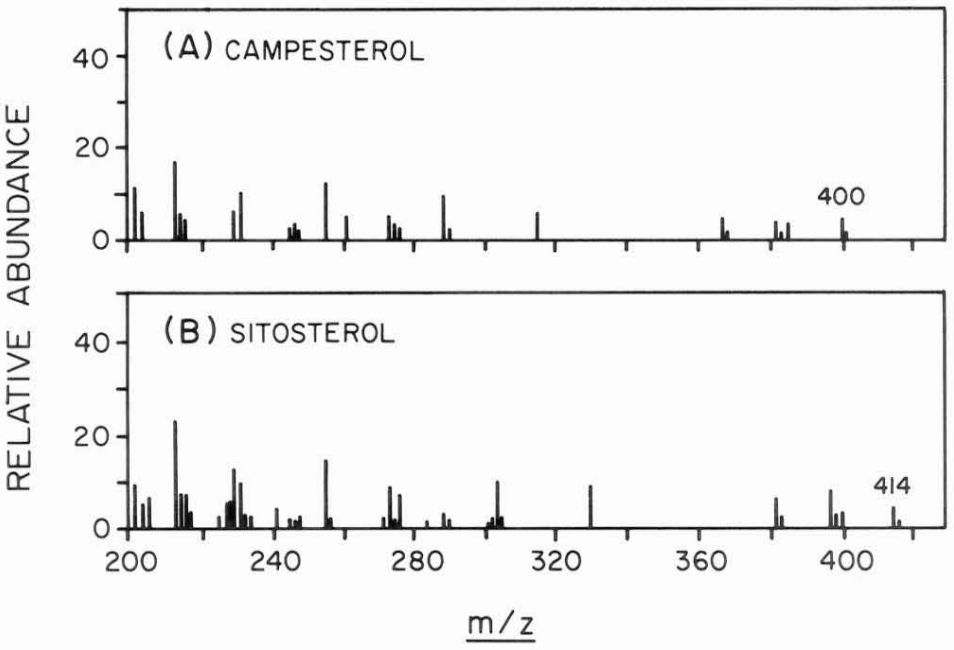


FIG. 2. Mass spectrum of the material of the preparative GC with the retention time of (A) campesterol and (B) sitosterol. The material in (A) was identified by GC and MS as campesterol, that in (B) as sitosterol.

or C_3 to C_7 plus two hydrogen atoms, the m/z $[M - 111]^+$ peak from loss of a fragment or fragments containing C_1 to C_7. These peaks, like those of I (Fig. 2A), also had high intensities, indicating a free sterol. The m/z peaks 329, 303, 288, and 285 were 14 mass units higher than the corresponding peaks in the spectrum of I (Fig. 2A), suggesting that the molecule contained one more methylene group than I, which is consistent with formulae I and II. The m/z positions of the mass spectrum (Fig. 2B) were identical to those published for sitosterol (Stenhagen et al. 1974), although the heights of some peaks differed because of different analytical conditions, primarily in the MS voltages (Pecsok and Shields 1968). On the basis of TLC, GC, and MS data, the compound was identified as II.

The saponifiable diethyl ether-soluble materials from saponified fraction 2, which were considered fatty acids, were methylated with diazomethane (Vogel 1956; Fieser 1957). The methylated materials were chromatographed by GC on column system 1 (temperature programmed from 150 to 240 C at 4 C/min, detector 270 C, injection port 255 C, helium carrier gas 35 ml/min) (Fig. 3A) and column system 2 (temperature programmed from 140 to 240 C at 2 C/min, detector 265 C, injection port 255 C, helium carrier gas 35 ml/min) (Fig. 3B). The spectra (Fig. 3A, B) showed major peaks whose retention times were the same as those of the authentic compounds listed in the figure captions.

Authentic methyl esters of saturated fatty acids of chain lengths C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, and C_{24} were analyzed by GC on column system 2 (190 C isothermal, detector 220 C, injection port 220 C, helium carrier gas 35 ml/min) and their retention times determined. Plotting $\log_{10}$retention time vs. carbon chain length

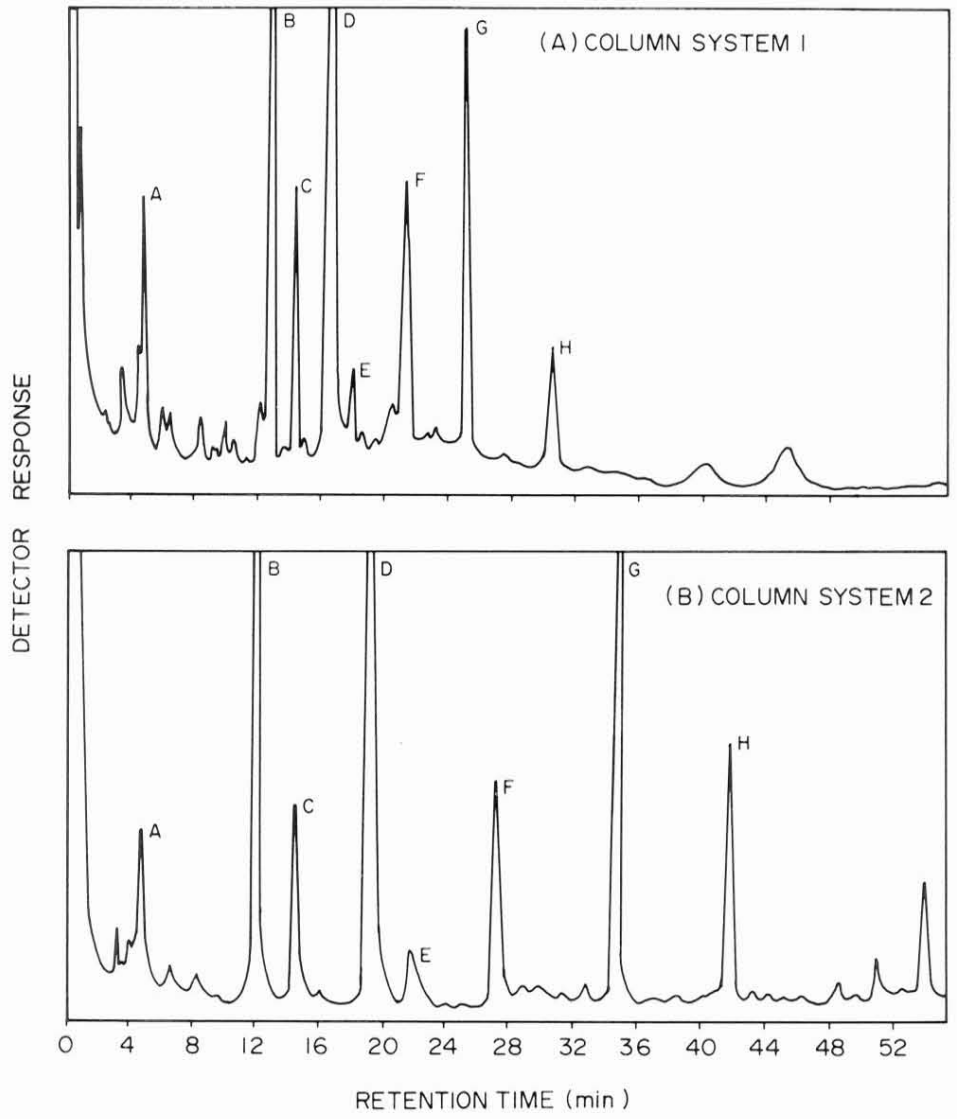


FIG. 3. GC separation of the methylated saponifiable and diethyl ether-soluble materials from fraction 2 for (A) column system 1 and (B) column system 2. Peaks A–H have retention times identical to those of authentic compounds as follows: peak A methyl *n*-tridecanoate; B methyl *n*-hexadecanoate; C methyl *n*-heptadecanoate; D methyl *cis*-9-octadecenoate; E methyl *n*-nonadecanoate; F methyl *n*-eicosanoate; G methyl *n*-docosanoate; H methyl *n*-tetracosanoate.

yielded a straight line. Under identical conditions, GC analysis of the methylated saponifiable and diethyl ether-soluble materials from fraction 2 showed peaks whose retention times, when applied to the above-derived straight line, indicated methyl esters of saturated fatty acids with carbon chain lengths of C_{13}, C_{16}, C_{17}, C_{19}, C_{20}, C_{22}, and C_{24}. These data reinforced the identifications of the compounds authenticated in Fig. 3A and B with the exception of methyl *cis*-9-octadecenoate, which is unsaturated and whose retention time therefore did not fit the straight

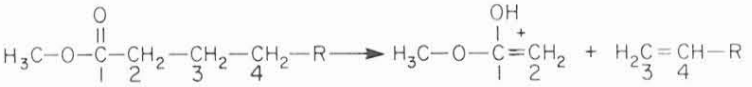
TABLE 1. *GC/MS data of the methylated saponifiable and diethyl ether-soluble materials from fraction 2.*

Compound (formula)	Peak[a]	Retention time (min)[b]	Molecular ion peak	Observed peaks
				(*m/z*)
Methyl *n*-tridecanoate ($C_{12}H_{25}COOCH_3$)	A	4.50	228	228, 199, 185, 171, 157, 143, 129, 115, 101, 87, 74 (base peak)
Methyl *n*-hexadecanoate ($C_{15}H_{31}COOCH_3$)	B	11.75	270	270, 241, 239, 227, 213, 199, 185, 171, 157, 143, 129, 115, 101, 87, 74 (base peak)
Methyl *n*-heptadecanoate ($C_{16}H_{33}COOCH_3$)	C	14.25	284	284, 255, 241, 213, 199, 185, 143, 129, 115, 101, 87, 74 (base peak)
Methyl *cis*-9-octadecenoate ($C_{17}H_{33}COOCH_3$)	D	19.00	296	296, 264, 222, 180, 115, 101, 91, 87, 74, 69, 55 (base peak)
—	E[c]	21.63	—	—
Methyl *n*-eicosanoate ($C_{19}H_{39}COOCH_3$)	F	26.88	326	326, 297, 295, 281, 241, 213, 199, 185, 143, 129, 115, 101, 87, 74 (base peak)
Methyl *n*-docosanoate ($C_{21}H_{43}COOCH_3$)	G	34.50	354	354, 325, 323, 311, 297, 295, 281, 241, 213, 199, 185, 143, 129, 115, 101, 87, 74 (base peak)
Methyl *n*-tetracosanoate ($C_{23}H_{47}COOCH_3$)	H	41.75	382	382, 339, 325, 311, 297, 295, 281, 241, 213, 199, 185, 143, 129, 115, 101, 87, 74 (base peak)

[a] Peaks are those in Fig. 3B.
[b] Retention times are those of the peaks in Fig. 3B.
[c] Too small for MS detection.

line. The methylated saponifiable and diethyl ether-soluble materials from fraction 2 were analyzed by GC/MS with column system 2 and the GC conditions described for Fig. 3B. The GC spectrum was identical to that of Fig. 3B; MS results of the compounds causing the peaks in Fig. 3B are given in Table 1.

MS spectra of the GC peaks A, B, C, F, G, and H (Fig. 3B) all showed similar *m/z* fragments in the low mass end (Table 1). All of the spectra had a base peak at *m/z* 74; the peak arises from ions formed by a C_2–C_3 cleavage with respect to the carbonyl group with simultaneous migration of one hydrogen atom from the fragments that were lost. The reaction thought to occur is as shown, where III goes to IV and V (Chapman 1965):



III IV V

The hydrogen atom was taken from C_4 and migrated to the oxygen atom (Sharkey et al. 1959). IV has been shown to have the hydroxyl rather than an alternative keto form. The alkyl fragment formed a stable 1-olefin, V. Peaks at *m/z* 87, 101, 115, 129, and 143, due to the ion $[CH_3OOC(CH_2)_n]^+$ where n = 2, 3, 4, 5, and 6, respectively (Dinh-Nguyên et al. 1960), also were evident.

The spectra showed some common characteristics in the high mass end as well. All had *m/z* values representing $[M - CH_2CH_3]^+$, $[M - CH_2CH_2CH_3]^+$, and $[M - CH_2CH_2CH_2CH_3]^+$ from the hydrocarbon end of the saturated chain. The spectrum of each GC peak had a molecular ion peak that exactly matched the

molecular weight of the suspected methyl ester (Table 1). Although the molecular formulae could not be ascertained with high precision because the mass spectrometer was a low-resolution instrument, the whole-number molecular weights matched. Mass spectrometry spectra for the compounds thought to be methyl *n*-tridecanoate, methyl *n*-hexadecanoate, methyl *n*-heptadecanoate, methyl *n*-eicosanoate, methyl *n*-docosanoate, and methyl *n*-tetracosanoate were identical in the position of their *m/z* fragment peaks to those published for the authentic compounds (Stenhagen et al. 1974); thus, the GC and GC/MS results unambiguously identified those six compounds. Therefore, the saponifiable diethyl ether solubles from fraction 2 of the *n*-hexane wax contained *n*-tridecanoic, *n*-hexadecanoic, *n*-heptadecanoic, *n*-eicosanoic, *n*-docosanoic, and *n*-tetracosanoic acids.

The GC retention time of peak E in both Fig. 3A and B was the same in each column system as that of authentic methyl *n*-nonadecanoate, though the amount was too small for obtaining a good GC/MS spectrum. However, on the basis of GC evidence, we believe that the saponifiable diethyl ether solubles from fraction 2 of the *n*-hexane wax contained *n*-nonadecanoic acid.

The GC peak D (Fig. 3B), with a retention time identical to that of authentic methyl *cis*-9-octadecenoate, showed an MS spectrum with a base peak at *m/z* 55. Peaks at *m/z* 74, 87, 101, and 115 were due to the same fragments as described earlier for the methyl esters of the saturated fatty acids. The peak at *m/z* 91 is typical for unsaturated fatty acid esters (Ryhage and Stenhagen 1959). Hallgren et al. (1959) suggested that the fragment results from tropylium ions formed through extensive rearrangement and cyclization. The molecular ion peak of *m/z* 296 matched the molecular weight of a methyl octadecenoate. On the basis of the MS data, the compound was considered to be the methyl ester of a fatty acid containing 18 carbon atoms, with one double bond in the chain; its MS spectrum was identical in *m/z* fragment peaks to that published for authentic methyl *cis*-9-octadecenoate (Stenhagen et al. 1974). However, the position of the double bond and whether the hydrogens are *cis* or *trans* cannot always be clearly ascertained from GC and MS data. Therefore, a sample of peak D (Fig. 3B) was collected by GC and mixed with authentic methyl *cis*-9-octadecenoate for a mixed melting point test; there was no depression of the melting point, indicating the compounds were identical. On the basis of mixed melting point, GC, and GC/MS data, the compound of peak D (Fig. 3B) was identified as methyl *cis*-9-octadecenoate. Therefore, the saponifiable diethyl ether solubles from fraction 2 of the *n*-hexane wax contained *cis*-9-octadecenoic acid.

The peaks of Fig. 3B with retention times of 6.6 and 8.3 min were essentially traces. Detecting the peaks above the baseline was difficult, and good MS spectra of these peaks could not be obtained, probably because the small amounts of vapors did not pass the interface from gas chromatograph to mass spectrometer. The peaks did not show in the 190 C isothermal GC spectrum, probably because the higher initial temperature did not allow resolution from the larger methyl *n*-tridecanoate peak; therefore, the materials could not be identified. However, because they eluted between the methyl *n*-tridecanoate and methyl *n*-hexadecanoate peaks (peaks A and B, Fig. 3B), they were thought to be methyl *n*-tetradecanoate and methyl *n*-pentadecanoate.

Two peaks (retention times 40.5 and 45.5 min) in Fig. 3A and three peaks (retention times 48.8, 51.0, and 54.0 min) in Fig. 3B eluted late. Although MS

spectra could not be obtained, these peaks were undoubtedly due to the fatty acid methyl esters with carbon chain lengths longer than the C_{24} of peak H.

The ratios of the peak sizes from GC were: methyl *n*-nonadecanoate 1.0 (the smallest, and the basis for the rest); methyl *n*-tridecanoate 1.2; methyl *n*-heptadecanoate 2.3; methyl *n*-eicosanoate 2.8; methyl *n*-tetracosanoate 3.0; methyl *n*-hexadecanoate 4.7; methyl *n*-docosanoate 6.0; methyl *cis*-9-octadecenoate 12.1. Therefore, the *n*-hexane wax contained components that, as usual, were composed predominantly of fatty acids of even-numbered carbon chain lengths, although those of odd-numbered lengths certainly were present.

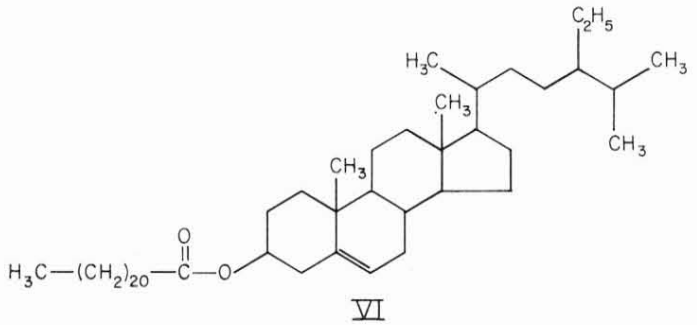
DISCUSSION

Column chromatography was the method of choice to resolve the wax components in quantities great enough for structural studies. In previous studies (Laver et al. 1971), obtaining definitive resolution of the various column chromatographic bands had proven difficult. However, adding water to the slurry of Silica Gel before packing it into the column improved band separation: 10 clearly resolved bands were evident under UV light (Fig. 1).

Fractions 1–9 from the fastest moving band (Fig. 1) had positive Liebermann-Burchard tests. Because both free sterols and sterol esters can show positive Liebermann-Burchard tests, one or both types of compounds could have been present in these fractions. However, TLC showed no spots migrating the same distance as authentic free sterols and GC showed no peaks for free sterols, although they had been reported in other chromatographic fractions of *n*-hexane wax (Laver et al. 1971). Therefore, we conclude that the *n*-hexane wax contained sterol esters.

Fraction 2 of the fastest moving band (Fig. 1) was investigated because it showed the strongest Liebermann-Burchard test and therefore was concluded to be richest in sterol esters. Saponification of the materials therein resulted in the release of the sterols campesterol and sitosterol and the alcohols 1-docosanol and 1-tetracosanol. Also released were several fatty acids; those positively identified were *n*-tridecanoic, *n*-hexadecanoic, *n*-heptadecanoic, *cis*-9-octadecenoic, *n*-nonadecanoic, *n*-eicosanoic, *n*-docosanoic, and *n*-tetracosanoic acid. The presence of others was demonstrated.

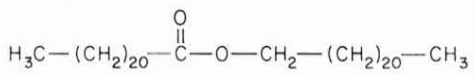
We conclude that the sterols were chemically bonded to the fatty acids by ester linkages. A typical sterol ester is shown as VI,



VI

which upon saponification followed by acidification would yield sitosterol and *n*-docosanoic acid. Because two sterols and eight fatty acids were identified, the original *n*-hexane wax apparently contained a complex mixture of sterol esters.

The alkyl alcohols 1-docosanol and 1-tetracosanol, identified in the unsaponifiables from fraction 2 along with campesterol and sitosterol, undoubtedly were also chemically bonded to the fatty acids by ester linkages. These ester compounds would be waxes. A typical wax molecule, an ester of a long-chain fatty acid and a long-chain fatty alcohol, is shown as VII,



VII

which upon saponification followed by acidification would yield *n*-docosanoic acid and *n*-docosanol. Because two fatty alcohols and eight fatty acids were identified, the original *n*-hexane wax also apparently contained a complex mixture of wax compounds.

Saturated fatty acids such as *n*-octadecanoic acid (setting point 69.3 C, melting point 69.9 C) (Francis et al. 1937) are solids at room temperature, whereas unsaturated fatty acids of the same carbon chain length such as *cis*-9-octadecenoic acid (setting point 13 C, melting point 16 C) (Robinson and Robinson 1925) are liquid. The fact that an unsaturated fatty acid (*cis*-9-octadecenoic acid) predominated in the saponifiables from fraction 2 and thus was part of a large portion of the sterol and wax esters might explain why *n*-hexane wax from Douglas-fir bark is soft and of low melting point (60–63 C) (Kurth 1950a). The fatty acid portions of the sterol and wax esters are, for the most part, also relatively short in carbon chain length, with C_{24} the longest carbon chain of any consequence. Short-chain fatty acids tend to be of lower melting point than those of longer chain length—which may also contribute to the softness and the low melting point of the *n*-hexane wax from Douglas-fir bark.

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