Application of Gas Liquid Chromatography–Mass Spectrometry to Analysis of Natural Products

Waxes from *Mangifera indica* and *Sesbania grandiflora*

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A homologous series of hydrocarbons (pentacosane to hexacontane), as well as octa- and nonacosanol, were identified as components of *Mangifera indica* L. and *Sesbania grandiflora* L. plant material extracts. In addition, evidence for the presence of waxes composed of esters formed between alcohols of C_{18}–C_{29} chain length to two major component acids, hexacosanoic and tetracosanoic acid, is presented.

The ethanolic extract of *Mangifera indica* L. fruit as well as a gall which occurs on the tree has been shown to exhibit antifungal and other medicinal properties. The ethanolic extract of the leaves and bark of *Sesbania grandiflora* plant (Papillionaccae subgroup of the Leguimunoceca family) also exhibits such properties. The fatty acid composition of *Mangifera indica* fruit kernel fat and Sesbania leaves has been studied (Pathak and Gunde, 1946), although no reports on the chemical composition of galls or of Sesbania bark extract have appeared. Although an unrelated species, evidence has been obtained that both plants contained similar compounds which exhibited medicinal activity (Yamamoto and Osima, 1932). Since the chemical nature of the pharmacologically active materials present in such extracts has not previously been investigated, the present study has determined the composition of the nonsaponifiable portion and waxes of *S. grandiflora* bark and *M. indica* galls as a portion of a program to characterize more completely the constituents of such plant extracts.

EXPERIMENTAL

Both the plants *Mangifera indica* L. and *Sesbania grandiflora* L. hereafter are designated as *A* and *B*, respectively.

Galls of *A* and bark *B* were collected in the vicinity of the tarai region of Kumaon, India, dried at room temperature, and ground to a fine powder. The powders of *A* and *B* were heated with 95% ethanol for 8 hours on a steam bath and filtered while hot. The filtrates were kept overnight and again filtered. Precipitates from both *A* and from *B* were separated, washed with 95% ethanol, and recrystallized with chloroform. The material *A* had a melting point of 64° C. and *B* of 74° C. and corresponded to an extracted yield of 0.20 and 0.07%, respectively.

Thin-layer chromatography of both of the compounds was carried out according to the method described by Stahl (1958), using glass plates covered with silica gel G.F. The solvent system which was employed for both mixtures was 65:10:20:5 Skelly F, ether, chloroform, and methanol, respectively.

Infrared spectra were determined in KBr pellets (1 mg. of sample per 200 mg. of KBr) using a Beckman Model IR-7 infrared spectrophotometer. Gas liquid chromatography of the extracts was carried out using a stainless steel 2-foot × 1/8-inch column packed with 5% SE-30–Chromasorb W 60- to 80-mesh with both an argon ionization and a flame detector system. Temperature programming was employed using a range of 150° to 350° C. at a program rate of 8° per minute.

The mixtures *A* and *B* were saponified by refluxing 10 to 50 mg. of sample in 25 ml. of 4*N* ethanolic potassium hydroxide solution for four hours. The saponifiable fractions of both *A* and *B* were separately treated with 4*N* hydrochloric acid to liberate the free acid. The acids so liberated, after extraction and drying, were then converted into their methyl esters using diazomethane.

Unsaponified materials were extracted from the alkaline hydrolysis mixture using diethyl ether. The alcohols so extracted (if any) were converted to their trimethyl silyl ether (TMS) derivatives using bistrimethyl silyl acetamide (BSA) (2 ml. BSA:10 mg. sample) and refluxing the reaction mixture for two hours at 165° C.

Mass spectra of the materials *A* and *B* were determined on a Hitachi–Perkin-Elmer RMU-6E Mass Spectrometer at 20 and 70 electron volts using a direct inlet and a gas chromatographic inlet (Khanna and Perkins, 1969).

RESULTS AND DISCUSSION

Preliminary results obtained using thin-layer chromatography indicated that extracts *A* and *B* were a mixture of at least four compounds each.

Examination of the mixture of compounds *A* and *B* in the infrared indicated that they both were esters with absorption at 1735 $cm.^{-1}$ The infrared spectrum of *B* indicated the presence of a free hydroxyl group at 3450 $cm.^{-1}$

Attempts to separate further the compounds contained in *A* and *B* proved fruitless using thin-layer and column chromatography on silica gel and silicic acid. A series of ill-defined spots appeared which, upon further chromatography, separated into even more components, indicating that the compounds present were probably a series of closely related species.

Gas liquid chromatography of the materials *A* and *B*, using a short nonpolar column (18-inch and 24-inch × 1/8-inch packed with 5% SE-30 on Chromasorb W), indicated the presence of a series of compounds which were eluted between 200° and 350° C. and corresponded to approximately 10% of the sample injected. The remainder of the material could not be eluted from the column. Both extracts were subjected to combined gas liquid chromatography-mass spectrometry (GLC-MS) using the column described

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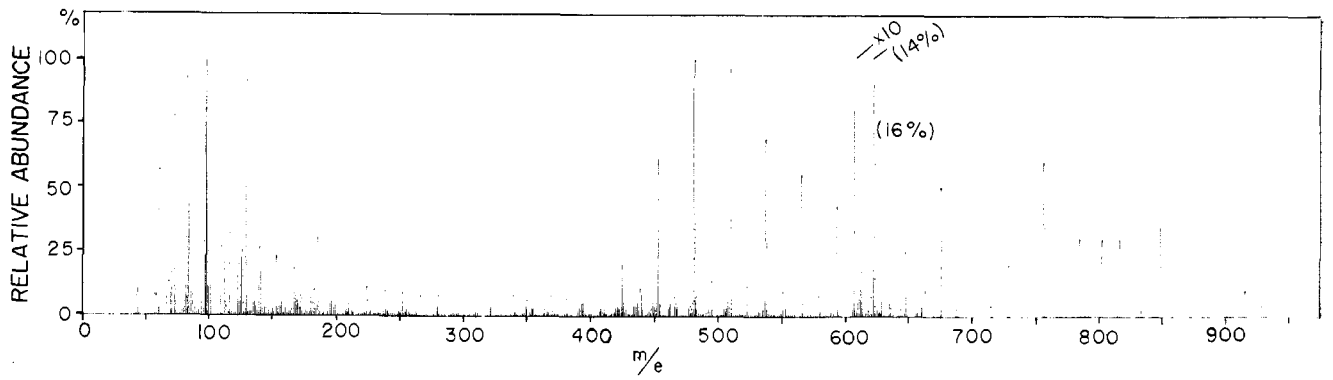

Figure 1. Mass spectrum of Wax A from *Mangifera indica* L.

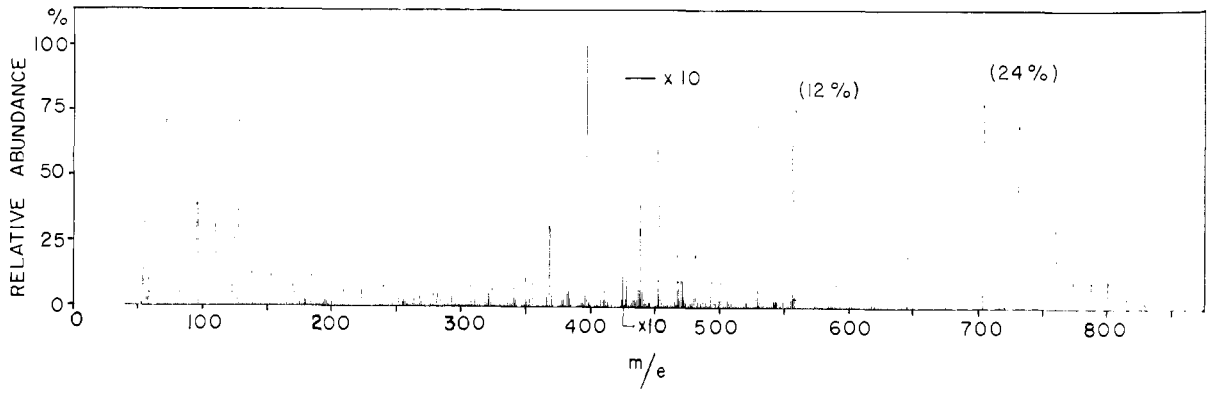

Figure 2. Mass spectrum of Wax B from *Sesbania grandiflora* L.

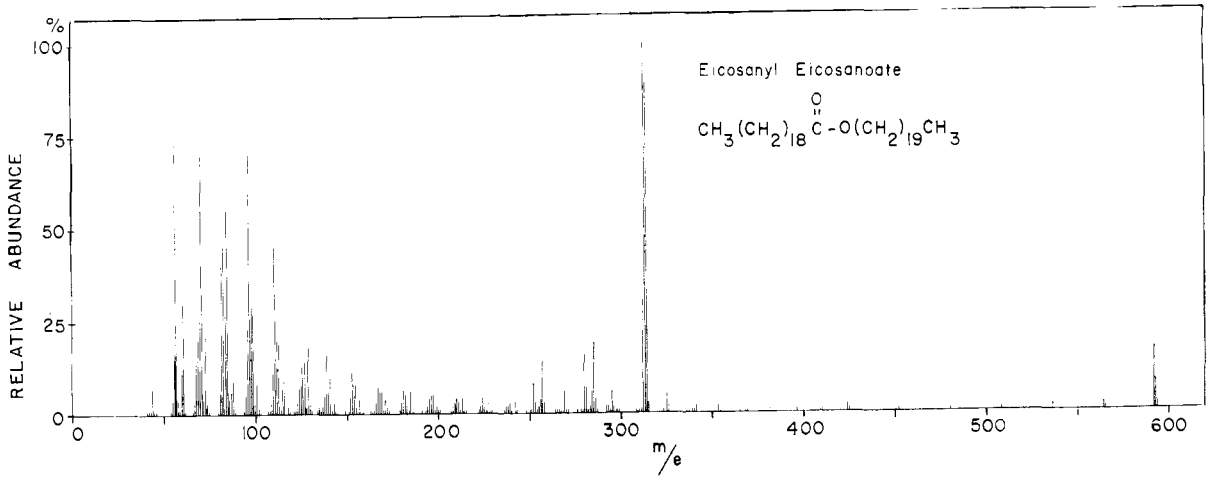

Figure 3. Mass spectrum of eicosanyl eicosanoate

above. A homologous series of long chain normal hydrocarbons of the structure C_nH_{2n+2} containing from 25 to 32 carbon atoms were identified from both extracts *A* and *B* (Table I). In all cases identifications were made by comparing the mass spectra with known hydrocarbons. When the unsaponifiable material from *A* and *B* were subjected to GLC-MS, the same series of peaks appeared with the addition of another major component (Table I) in each sample as well as many minor component peaks which appeared to be long chain alcohols. The unsaponifiable material was treated with bis-trimethyl silyl acetamide in order to convert any free hydroxyl groups to the corresponding trimethyl silyl ether (TMS) derivative. When this material was subjected to combined GLC-MS, the same series of peaks was found as previously observed for the nonsaponifiable material. All of the peaks except one major peak in *A* (45.2%) and a major component in *B* (43.4%) were again identified as hydrocarbons. The two anomalous peaks were identified as primary alcohols with the structure of $C_{28}H_{57}OH$ and $C_{29}H_{59}OH$ from *A* and *B*, respectively, with the aid of the TMS derivatives and of the mass spectra of the original materials. In addition several minor components were identified as alcohols with chain lengths from C_{18} to C_{30}. The fragmentation patterns observed for the TMS derivatives were similar to that reported by Sharkey *et al.* (1957).

The free carboxylic acid portions of *A* and *B* were esterified with diazomethane and subjected to GLC-MS. Two major peaks were eluted from the ester of *A*, their mass spectra had base peaks at m/e = 452 and 494; peaks representing *M*-29 and *M*-31, at m/e = 423 and 421, and 465, and 463, respec-

Table I. Composition of Volatile Material from *Mangifera indica* L. (*A*) and *Sesbania grandiflora* L. (*B*) extracts

Peak	Percentage	Molecular formula
	Sample *A*	
1	3.2	$C_{25}H_{52}$
2	8.5	$C_{27}H_{56}$
3	27.9	$C_{29}H_{60}$
4	2.2	$C_{31}H_{64}$
5	45.2	$C_{28}H_{57}OH$
6	12.4	$C_{36}H_{74}$
	Sample *B*	
1	9.0	$C_{25}H_{52}$
2	7.8	$C_{27}H_{56}$
3	10.0	$C_{29}H_{60}$
4	8.5	$C_{31}H_{64}$
5	43.4	$C_{29}H_{59}OH$
6	12.5	$C_{33}H_{68}$
7	8.6	$C_{35}H_{72}$

tively, were present. A series of fragments representing loss of alkyl ions $[M\text{-}(CH_2)_nCH_3]^+$ and the usual series of alkyl ion fragments of the structure $[CH_3(CH_2)_n]^+$ were also present. An intense peak at m/e = 74 representing the rearrangement ion due to cleavage alpha to a carbonyl group of methyl ester indicating the absence of alpha substitution was also present in both spectra (Ryhage and Stenhagen, 1960), as was the ion series $[(CH_2)_nCO_2CH_3]^+$. The spectra were consistent with those expected from methyl nonacosanoate and dotriacontanoate, and were verified by comparison with the spectra of the known saturated methyl esters.

Other peaks which were of lower concentration were identified as a homologous series of methyl esters ranging from methyl heptacosanoate through dotetracontanoate.

Two major peaks were present in the gas chromatogram of the methyl esters prepared from extract *B*. In both spectra, base peaks for the molecular ion were obtained at m/e = 410 and m/e = 382. Fragment ions were also present at *M*-29 and *M*-31, and at the values predicted for normal saturated methyl esters. The compounds were identified as methyl hexa- and tetracosanoate, respectively, and were verified by comparison with known standards.

The results thus far have indicated that the compounds isolated were mixtures of hydrocarbons and alcohols with the primary components occurring in the form of long chain esters—*i.e.*, waxes. From consideration of the data thus far obtained it appears that the structures of *A* and *B* are those of waxes containing a series of acids and alcohols interesterified among each other. This was further verified by high temperature mass spectrometry. Temperature programming of the sample in the mass spectrometer made it possible to fractionally remove the contaminating alcohols and hydrocarbon components and thus obtain the mass spectrum of the wax mixture. The mass spectrum of wax ester *A* and *B* is shown in Figures 1 and 2, respectively. Esters of long chain acids with long chain alcohols have characteristic mass spectra in which the base peak represents cleavage of the ester moiety and is the ester fragment (Ryhage and Stenhagen, 1959). This was verified by a comparison of the mass spectrum of eicosanyl eicosanoate ($C_{40}H_{80}O_2$) (Figure 3). The base peak in this spectrum is located at m/e = 313 corresponding to $[C_{20}H_{41}O_2]^+$, the acyl moiety of the ester. A substantial molecular ion at m/e = 592 (16.5%) was also present. In wax *A* (Figure 1), the base peak was found at m/e = 481, corresponding to an acyl moiety of 32 carbons. A fairly intense distribution of fragments from m/e = 411 to 607 at intervals of two methylene groups indicated the presence of fatty acids of chain length ranging from $CH_3(CH_2)_{25}CO_2H$ to $CH_3(CH_2)_{46}CO_2H$. At higher mass units m/e = 756 to 920 and at less intervals another distribution of fragment ions was found which indicated that the alcohol moieties present were homologous of greater and lesser chain lengths than $CH_3(CH_2)_{27}OH$, and was suggested also by the results obtained on the unsaponifiable material and trimethylsilyl derivative.

The mass spectrum of wax *B* (Figure 2) exhibited a base peak at m/e = 397 indicating the presence of an acid of the structure $CH_3(CH_2)_{24}CO_2H$ in the ester. Fragment ions were also found at m/e = 369 and higher mass values indicating the presence again of a series of acids differing by methylene groups. The presence of the C_{24} and C_{26} acid in the wax was verified by isolation and comparison with the spectra of the known acids outlined previously in this discussion. Other minor component acids composed the series from C_{22} to C_{31} in chain length. The identification of the alcohol moiety was not so clear. Small ions were present at values corresponding to increases in molecular size to m/e = 862. The major peak in the series was found at m/e 704. If it is assumed that this is derived from the major component ester fragment (C_{26}) then the predominant alcohol present is eicosanol. Additional evidence obtained using trimethyl silyl ether derivative formation indicated the presence of an alcohol of 29 carbons in length as well as others of higher molecular weight. Again the mass spectral pattern indicated the presence of a homologous series of molecular ions resulting with esterification of alcohols of C_{18}–C_{28} chain length with the two acid portions (C_{26} and C_{24}) which were found in major concentrations.

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