Carter proposed the name "phytoglycolipides" for this class of sphingolipides.

The determination of ethanolamine and serine in hydrolysates usually has been done by assay of ammonia, which is liberated upon oxidation by periodate (25, 26). The assumption however that periodate liberates ammonia only from compounds having the amino group and a hydroxyl group on adjacent carbon atoms (*e.g.*, ethanolamine and serine) does not hold. Choline also develops ammonia on oxidation with periodate. Therefore choline has to be removed from the hydrolysate prior to de-amination with periodate (21c).

The isolation of individual, intact phospholipides is a prerequisite for the elucidation of chemical structures and for work in intermediary metabolism that involves tracer techniques. Combinations of countercurrent distribution and chromatography undoubtedly will lead to a more comprehensive knowledge of the structure and more accurate analysis of this interesting and important class of lipides.

Summary

A survey is given of the micro determination of three major lipide classes: cholesterol, triglycerides, and phospholipides. The merits of a popular direct method for serum cholesterol and a system for rapid chromatographic separation of esterified cholesterol from other lipides have been discussed.

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Analysis of Plant Waxes by Means of Chromatography and X-Ray Diffraction

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COLUMN CHROMATOGRAPHY of a number of plant waxes by means of Al_2O_3 led to the isolation of fractions containing paraffins, esters, alcohols, or acids, as main constituents. In the fractions these substances could be identified by means of x-ray analysis. The waxes that were studied were derived from Candelilla, carnauba palm, sugar cane, and kapok.

Introduction

Cole (6) applied chromatography on columns of Al_2O_3 Woelm, anionotropic pH 4[1] to various plant waxes and succeeded in separating them into functionally homogeneous groups. However the activity of the alumina is not described entirely in the customary way (4), and the percentage of water which it contained is not mentioned. Preliminary experiments with a mixture of known composition were carried out on Al_2O_3 (Fisher).

Schuette and Baldinus (15) have analyzed the paraffins occurring in Candelilla wax, qualitatively as well as quantitatively, by chromatography on Al_2O_3 grade F_{20} (Al Ore Company). They too failed to record the activity of the Al_2O_3 in the customary way.

[1] Supplied by M. Woelm, Eschwege.

It seemed desirable to continue Cole's attempts to separate the plant waxes in functionally homogeneous groups and to use the same separation, *viz,* Al_2O_3 Woelm, anionotropic pH 4[1] but to standardize it according to Brockmann and Schodder (4).

For identifying the main components in the waxes themselves, as well as in the fractions that had been obtained chromatographically, x-ray analysis seemed to be the most suitable method.

In crystallized normal long-chain aliphatic products the chain molecules are arranged into piles of uni-molecular layers of parallel chains in which the chain direction is perpendicular or at an oblique angle to the basal planes of the layers. Consequently the thickness of the layers depends on the chain length of the compound in question and on the angle of the chains with the basal plane. This thickness can be measured by x-ray diffraction as the so-called long spacing. Therefore, provided a reference standard is availalbe, the x-ray long spacing affords a means of determining the chain length of an unknown member of a homologous series of n-aliphatic long-chain compounds.

It is known that in this way the normal aliphatic wax components may be identified, not only after isolation (5, 12, 13, 14, 17) but occasionally in the

native product, *viz.*, if they are present in the latter as the main component (9, 10). However, if the composition of the waxes happens to be very heterogeneous, data from the x-ray diagram is not very conclusive. We have selected several such waxes with a heterogeneous composition in order to find out if it is possible to arrive at conclusive x-ray data after a separation has been made by means of the chromatographic method. Also waxes with a less heterogeneous composition have been studied by combining the two methods since this might yield new data in addition to those afforded by x-ray diffraction alone.

Methods

Chromatography. Columns with a diameter of 2–4 cm. and a length of about ten times this size were used. These columns were filled with a slurry consisting of heptane and Al_2O_3. As it appeared impossible to avoid breaks in the adsorption column if the experiments were carried out at 50°C., most separations were made at room temperature. Our standard preparation always was Al_2O_3 Woelm, anionotropic pH 4, grade I,[1] which contained no water. By adding 3, 6, 10, and 15% water, as recommended by the manufacturer, less strongly polar adsorbents were obtained. The activities according to the standard of Brockmann and Schodder (4) thus became grades I, II, III, IV, and V, respectively.

By applying solvents with an increasingly polar character more strongly adsorbed (more strongly polar) wax components could be eluted from the various columns. Following Cole (6), we used heptane, heptane with 1–5% ether, heptane with 1–5% propanol, and heptane with 1–5% glacial acetic acid as solvents. All these fluids were used in a water-free condition. As a rule, Al_2O_3 grade II was used in combination with the following series of solvents: heptane, heptane with 4% ether, heptane with 4% propanol, and heptane with 4% glacial acetic acid.

The paraffin-fraction was usually chromatographed a second time on Al_2O_3 grade I, with heptane as the solvent.

Special care was required to deposit the dissolved wax on the column. About 0.5–1 g. of wax was dissolved in 20 ml. of hot heptane and poured on a column irradiated at the top by an infrared lamp. As soon as the solution has disappeared entirely into the column, the lamp is removed, and elution with cold solvent is started.

As a control, a mixture consisting of paraffins (mp. 55–60°C.), cetylstearate, octadecanol, and stearic acid was separated on the column. In Table I the exact composition of the mixture is given with the amounts recovered; the latter amounts, on the average, to about 90%. Identification was made by means of saponification number, acid number, etc., according to the D.G.F.—Einheitsmethoden (3).

X-Ray Analysis. The x-ray diagrams were obtained by means of Cu–Kα radiation (30–35 kV/300–350 mA) from powdered specimens with a thickness of about 0.5 mm. on flat films. The distance to the preparation was either 40 or 80 mm. The collimator consisted either of a capillary of lead glass, 50 mm. in length and ¼ or ½ mm. in diameter, or of a wedge collimator for small-angle photographs according to the principle of Lely and Van Rijssel (11), constructed as described by Kreger and Schammhart (10). In order to ensure micro-cristallinity of the powdered specimens, the wax was melted on a thin brass plate and frozen quickly by putting the plate on a block of ice.

TABLE I

Separation on Al_2O_3 of a Synthetic Mixture of Wax Components

Solvent	ml.	Yield
		grams
Heptane	450	0.43 paraffin
Heptane + 4% ether	500	0.46 cetylstearate
Heptane + 4% propanol	600	0.53 octadecanol
Heptane + 4% glacial acetic acid	650	0.46 stearic acid

Column 35 × 2.5 cm., containing 75 g. of Al_2O_3, anionotropic pH 4, grade II,[1] mixture deposit on the column consisting of 0.50 g. of paraffin, 0.50 g. of cetylstearate, 0.60 g. of octadecanol and 0.50 g. of stearic acid.

Results

a) *Candelilla Wax.* This wax was chosen because its composition is already fairly well-known (1, 5, 15) so that it could serve as a test of our methods.

The long-chain reflections shown by the raw commercial product agree with those of a normal C_{33} paraffin. Elution from a column of Al_2O_3 Woelm, anionotropic pH 4, grade III,[1] with heptane yielded a paraffin whereas with heptane + 2.5% glacial acetic acid a fraction consisting of wax acids was obtained. In both fractions the components were easily identifiable by means of the x-ray diagram. The first-named fraction showed a long-spacing of 41.7Å (± 0.2), which corresponds with the spacing to be expected for a mixture of C_{31} and C_{33} paraffins in the proportion 90:10 (13). The acid fraction showed a long spacing of 81.0 Å (± 0.2), corresponding with that of an equimolecular mixture of C_{30}, C_{32}, and C_{34} acids (14). The presence of free alcohols could not be demonstrated.

Two experiments, the one made with 663 and the other with 952 mg. of wax, yielded 35% paraffin upon elution with heptane from a column of Al_2O_3 Woelm, anionotropic pH 4, grade I.[1] A control with pure paraffin eluted in the same manner resulted in a 99% yield. These results are in good agreement with the data obtained by Chibnall *et al.* (5), Schuette and Baldinus (15), and Alcocer and Sanders (1).

b) *Carnauba Wax.* According to Warth (18), this wax contains 84–85% alkyl esters and 0.5–3% paraffins, mainly with 27 C atoms. The x-ray diagram of the raw commercial product does not show distinct long-spacing reflections and does not permit any definite conclusion.

By chromatography on Al_2O_3 Woelm, anionotropic pH 4, grade I,[1] and elution with heptane a very small fraction with a long-spacing of 39.9 Å (± 0.2) was separated indicating the presence of C_{29} and C_{31} paraffins in the proportion 75:25 (13). In the fraction obtained by elution with heptane plus 5% ether, the main component appeared to be an ester with a long spacing of 77.1 Å (± 0.2), which according to the formula given by Kreger and Schamhart (10) indicates a chain consisting of 58 C atoms. The main fractions could not be identified by means of the x-ray diagrams.

A sample of so-called white carnauba wax appeared to consist of 75% paraffins.

c) *Sugar-Cane Wax.* A considerable amount of data is available on the chemistry of refined sugar-cane wax (2), but with regard to the composition of the cuticular wax of this plant little is known. Kreger (9) and Wijnberg (19) reported that the latter con-

TABLE II

X-Ray Long-Spacings of Waxes Obtained from the Cuticle of Different Clones of *Saccharum officinarum* and from that of *S. spontaneum*

	Name of clone	Origin	Long spacing	Suggested alcohols C_{24} C_{26} C_{28} C_{30}
Saccharum officinarum	?	cult. Delft 1947	75.2 Å ± 0.3 (9)	25:75
Saccharum officinarum	?	cult. Delft 1957	79.2 Å ± 0.4	40:60
alcohol fraction of the same; separation on Al_2O_3			79.7 Å ± 0.4	30:70
Saccharum officinarum	2426 P.O.J.	cult. Java 1913	76.0 Å ± 0.4+	90:10
Saccharum officinarum	Fidji (red)	cult. Java 1913	76.2 Å ± 0.7+	90:10
Saccharum spontaneum	.....	coll Java 1913	70.9 Å ± 0.3+	95: 5
Saccharum spontaneum	.....	cult. Delft 1954	70.9 Å ± 0.3	95: 5
Saccharum spontaneum	.....	cult. Delft 1947	70.1 Å ± 0.4 (9)	35:65

sists mainly of long-chain primary alcohols. With regard to the exact length of the chain there is however as yet no unity of opinion.

Wijnberg (19) carried out a chemical analysis of the wax that was scraped from the stems of cultivated sugar cane *(Saccharum officinarum)* and found C_{30} primary alcohol as its main component. Kreger (9) as well as Horn and Matic (8) studied this wax by means of x-ray analysis and arrived at the conclusion that C_{28} alcohol is its main component. Findley, quoted by Spickett (16), finds for the alcohol fraction a chain length of 29 to 30 C-atoms; as the wax alcohols generally contain an even number of these atoms (5), this probably means that it is a mixture of C_{28} and C_{30} with the latter in excess. Apart from the alcohols the last-named investigator finds 2.3% of paraffin and 28% of ketone; the latter is supposed to have no less than 61 C atoms.

With wax scraped from *Saccharum spontaneum* (wild sugar cane) Kreger (9) observes x-ray reflections which indicate a mixture of C_{24} and C_{26} alcohol with C_{26} as the main component.

A wax sample scraped from sugar cane *(S. officinarum)*, grown in the hothouse belonging to the Delft laboratory, was chromatographically analyzed in the manner described above. From a column of Al_2O_3 Woelm, anionotropic pH 4, grade II,[1] elution with heptane yielded an unknown, according to the x-ray diagram presumably nonaliphatic compound. Elution with heptane plus 4% ether yielded a considerable fraction which contained a substance, according to the x-ray diagram, presumably an ester, with a long spacing of 77.0 Å (± 0.3), indicating a chain consisting of 58 C atoms. The fraction obtained by elution with heptane plus propanol consisted mainly of alcohols. It comprised 63% of the starting material. The long spacing was 79.7 Å (± 0.4), indicating a mixture of alcohols with 30 and 28 C atoms in the proportion 60:40 (14). Prior to the chromatographic separation this wax showed a long spacing of 79.2 Å (± 0.4). An x-ray diagram made of a sample that had been studied by Kreger (9) in 1947 and that had been obtained also from sugar cane grown in the hothouse at Delft, revealed a long spacing of 75.2 Å, in agreement with the earlier measurement.

In Table II these data are given in combination with similar ones obtained from some other kinds of sugar cane. Those that are marked with a + sign were obtained from wax that had been scraped from stems collected in 1913 in Java and preserved in formalin. The estimations were carried out on x-ray diagrams made available by D. R. Kreger.

With regard to the wax derived from the cuticle of various clones of *S. officinarum* it can be concluded that the primary alcohol which constitutes its main component may have 28 as well as 30 C atoms and that this apparently depends on the nature of the clone and perhaps also on the environment.

The data pertaining to the wax of *S. spontaneum* indicate so far a less variable composition of the alcohol fraction, with C_{26} alcohol as the main constituent and, in addition, with C_{24} or a slight amount of C_{28} alcohol.

In considering the ratios between the components as they are recorded in Table II, it should be borne in mind that the mixture might not be a binary but a ternary one and that an alcohol with the next larger or smaller even number of C atoms may have been present in addition. Such a mixture too would cause a single series of long spacings (14). Under these circumstances the alcohol indicated as the main component of the binary mixture would constitute a considerable proportion of the ternary mixture too.

The U.V. absorption-spectrum of the wax of *S. officinarum* (hothouse, Delft, 1957), recorded by means of a Beckman spectrophotometer, type DU G 4700 (solvent iso-octane), showed peaks at 232, 260, 270, 281, 303, and 318 mμ. The peaks at 270 and 281 mμ indicate perhaps the presence of a conjugated triene, and those at 318 and 232 μ an 1-2-unsaturated ketone (7).

The U.V. spectrum of the wax of *S. spontaneum* showed no peaks.

d) *Wax of Java Kapok (Ceiba pentandra).* Comparatively little is known of the wax of Java kapok. According to Warth (18), it would contain myricyl alcohol and a phytosterol. In view of the meagerness of these data a study of this wax by the methods described above was undertaken.

By extracting 300 g. of kapok in a Soxhlet's apparatus by means of benzene-alcohol 2:1, 4 g. of wax were obtained. Prior to the chromatography it did not show distinct long-spacing reflections.

When subjected to chromatography in the manner described above, the heptane fraction proved to contain 8.8% of the original amount; the x-ray analysis of this fraction revealed a sharp paraffin diagram. A long-spacing of 37.8 Å was found, which corresponds with a mixture of C_{27} and C_{29} paraffin in a proportion of about 70 to 30. The fraction obtained with heptane plus propanol revealed the presence of a compound which, judging from the x-ray diagram, was not aliphatic and which was not further identified but may have been a phytosterol. It therefore appears that paraffins are present in the wax of Java kapok also.

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Gas-Liquid Chromatography of Fatty Derivatives. II. Analysis of Fatty Alcohol Mixtures by Gas-Liquid Chromatography[1]

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IN A RECENT REPORT from this laboratory (3) gas-liquid chromatography was applied to the analysis of saturated fatty alcohols. The separations were made by using either silicone grease or Carbowax 4000 monostearate as the partitioning agent and C-22 firebrick as the inert support. The analysis of the mixed saturated and unsaturated components in alcohols derived from soybean and linseed oil was only partially successful in that the separation of the unsaturated alcohols was incomplete.

The analysis of mixtures of saturated and unsaturated fatty acids in the form of methyl esters has recently been made practical by Lipsky (4) and by Orr and Callen (6) through the use of certain polyesters as partitioning liquids. Preliminary work showed that these polyesters appeared to be singularly selective toward unsaturated components in mixtures with saturates. It was logical therefore to expect that they would perform similarly toward the unsaturated alcohols if they, in turn, were converted to esters.

In this study it was demonstrated that these polyesters are effective in the separation of both saturated and unsaturated fatty alcohols and that vapor-phase chromatography can be the basis for an analysis which would otherwise be impractical, if not impossible.

Experimental

Preparation of Alcohol Standards. In order to prove the feasibility of separating quantitatively mixtures of unsaturated alcohol acetates on polyester columns, it was necessary to prepare the individual alcohols in as nearly pure a state as possible. Reduction of the corresponding methyl esters by lithium aluminum hydride was chosen as the approach for two reasons: a) the methyl esters are more easily purified than the alcohols; and b) the reduction is essentially quantitative. The methyl esters were supplied by the Hormel Institute. Methyl linoleate and linolenate as supplied were made by debromination of tetrabromostearic and hexabromostearic acids, respectively.

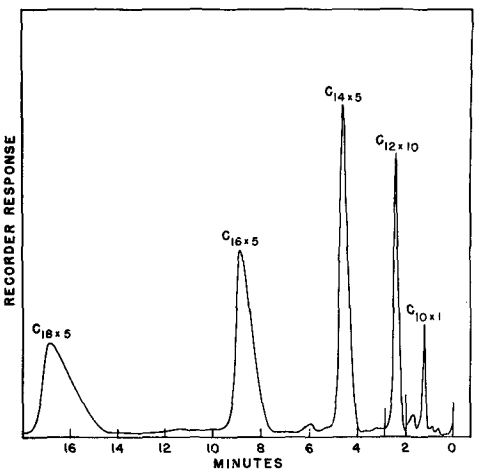

FIG. 1. Chromatogram of a mixture of normal C_{10} through C_{18} alcohol acetates, attenuation as noted.

In order to check the purity of the methyl esters they were chromatographed according to the method described by Orr and Callen (6). The methyl stearate, methyl oleate, and methyl linolenate showed only one component by gas chromatography; the methyl linoleate contained 1.5% of methyl oleate. A correction was applied for this oleate contamination in subsequent studies of blends.

The procedure used for the lithium aluminum hydride reduction was essentially that of Nystrom and Brown (5). Ethyl ether was refluxed over lithium aluminum hydride and distilled into a second flask containing twice the theoretical amount of lithium aluminum hydride required for the reduction. After solution was effected, the methyl ester was added

[1] ADM Technical Talk No. 166.