RESOLUTION OF BEESWAX

A Thesis

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In Partial Fulfillment

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Master of Science

by

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To

my parents

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ABSTRACT

Beeswax has been subjected to thin-layer chromatography and found to contain hydrocarbons, esters, alcohols and acids. This was confirmed by chemical tests and infrared spectral data. The hydrocarbon fraction obtained pure by thin-layer chromatography was further analyzed by gas-liquid chromatography and found to contain C_{17} to C_{36} saturated paraffins. The esters similarly have been obtained pure by thin-layer chromatography and found to range from C_{32} to C_{52} determined by gas-liquid chromatography. The methyl esters of the fatty acids of beeswax determined by gas-liquid chromatography ranged from C_{14} to C_{28}.

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INTRODUCTION

The complete analysis of complex natural substances has been, and still is, of particular interest. Beeswax is a natural substance that workers have attempted to analyze by physical and chemical methods. The approach they have taken has been to identify particular components present in the wax (24, 64). Chibnall et al.(7) did an investigation of the alcohols, Damoy (13) of the acids, and Kebler (29) of the hydrocarbons present. Gascard (18) did extensive work on the melting points of several components of beeswax. Later investigators pointed out that the individual components referred to in the earlier literature are not single compounds, but almost invariably are mixtures of even-numbered honologues for the acids and alcohols and odd-numbered homologues for the hydrocarbons.

These complex mixtures seemed inseparable to these workers by the methods that were available to them. With the introduction of gas-liquid chromatography the separation became possible. So it was that in 1960 White et al. (66) reported the analysis of the hydrocarbons of beeswax, in which they showed the presence of the small amounts of hydrocarbons with even numbers of carbon atoms. Downing et al. (16) analyzed beeswax making extensive use of column chromatography and gas-liquid chromatography.

Another method of resolving mixtures is now widely

used, thin-layer chromatography. Its history goes as far back as 1938 when Izmailov and Shraiber (26) described the use of thin layers of adsorbent on glass plates for the separation of galenicals. Stahl made both equipment and chemicals commerically available through C. Desaga, Heidelberg, and following this, extensive and rapid development followed. At present an extensive literature is available, including several review articles (34, 41, 35) and books by Stahl (51), Bobbitt (2a), Marini-Bettolo (37), Randerath (45), and Truter (61). We have attempted to use this new method of separation for waxes, and primarily for beeswax.

A logical procedure for the systematic chromatographic analysis of beeswax should follow three steps: first, the complex mixture should be separated into classes of compounds having the same type and number of functional groups; second, there should be a subfractionation of each class of compounds into its individual homologues; last, the constituent parts making up the individual compounds should be determined, for example the acids and alcohols which make up the individual esters should be identified. We hoped to achieve this separation into classes of compounds by thin-layer chromatography. In this way wax constituents could be analyzed without change from the form in which they occurred naturally. Former techniques which involved preliminary saponification changed the constituents

chemically and left some doubt as to what they really were in the natural wax.

In our project we used three types of beeswax samples:

I. Beeswax, U.S.P. (White disc), Lot No. 722280, available from Fisher Laboratory Chemicals.

II. Wax Bees (yellow), Code 2408, available from Baker and Adamson Products, General Chemical Division, Allied Chemical Corporation, 40 Rector St., New York 6, N. Y.

III. Beeswax scales and combs from Italian strain, apis mellifera, available from Dr. Jay of the Entomology Department of the University of Manitoba.

These wax scales are the ones prepared by the bees along their lower abdomen, which drop off after they reach maturity. The clear scales were collected, with a pair of tweezers, from the bottom of the shipping cases after they arrived here. Similarly, the combs, that the bees prepare, were removed from those cases. Both of these samples were cleaned by washing them with water, followed by drying. They were then dissolved in chloroform and filtered through a glass wool filter. We were interested to find out whether some difference could be detected between the scale wax and the freshly prepared combs of the same bees. The bees are known to chew the wax scales to make them pliable and then form the comb.

LITERATURE REVIEW

An extensive analysis of beeswax was not done until the 1960's, when Downing et al.(16) published results of studies of wax produced by the Italian race Apis mellifica. Earlier workers identified only some of the components present in the wax. An extensive review of the earlier work is given by Warth (64) and his summary is given below.

Chemical Composition of Yellow Beeswax

Alcohol	60%
Acid	42.5%
Alkyl Esters of Fat and Wax Acids (combining wt. 700, m.p. 63.5°)	72%
myricyl palmitate	33%
lacceryl palmitate	9%
myricyl palmitoleate, m.p. 38°	12%
myricyl hydroxypalmitate	6%
myricyl cerotate	12%
Cholesteryl Esters of Fatty Acids	0.8%
cholesteryl palmitoleate, m.p. 40°	
Lactones	0.6%
-myristo-lactone, m.p. 41-42°	
Free Wax Acids	13-13.5%
(combining wt. 376.8, m.p. 77.5-79°)	
neocerotic acid, m.p. 77.8° (Gascard)	
cerotic acid (c), m.p. 82.5° (Brodie)	
montanic acid, m.p. 86.8° (Holde)	
melissic acid, m.p. 90° (Damoy)	
Hydrocarbons	12-12.5%
nonacosane, m.p. 63.5° (G)	
hentriacontane, m.p. 68.7° (G)	11%
Moisture	1-2%

Chibnall et al. (7) identified some alcohols (C_{24}-C_{36}), Kebler the percent hydrocarbons and Joyame and Hirai (59) identified 14-hydroxypalmitic acid in beeswax. Lately White et al. (66) determined the hydrocarbons of beeswax and found that the even-numbered hydrocarbons are definitely present, but in a very small concentration. The same was reported by Downing et al. (16) in 1961. The results of White and Downing are given on pages 98 and 99.

Downing et al. did a quantitative determination of the components of hydrolzed beeswax. They achieved a separation of the unsaponifiable fraction of beeswax into hydrocarbons, alcohols and "diols", and of the saponifiable fraction into acids and hydroxyacids. They reduced all alcohols and "diols" to the corresponding hydrocarbon mixture. Similarly the acids were reduced to the corresponding hydrocarbons. These prepared hydrocarbons were analyzed by gas-liquid chromatography. In their paper (16) they give the composition by weight of each homologue of the hydrocarbons, alcohols,"diols",acids and hydroxyacids.

PREPARATION OF THIN-LAYERS

The basic principle in the preparation of thin-layers consists of applying a thin coating of an adsorbent over a smooth flat surface, the surface being usually glass.

Glass plates

The glass plates used were obtained from a local hardware store. Variations of the surface of that glass did not exceed 0.001 in. Various sizes of the glass were cut, 5 x 20, 10 x 20, 15 x 20 and 20 x 20 cm. All edges of the plates were slightly beveled and smoothed. These glass plates were cleaned scrupulously, then placed for 24 hours in hot chromic-sulfuric acid followed by rinsing with distilled water and washing with alcohol. This procedure was repeated each time the plates were used.

Adsorbents

Silica Gel G - The Silica Gel G, "Merck" grade, made in Germany, was available from Kensington Scientific Corporation, 1717 Fifth Street, Berkeley, 10, California. The calcium sulfate (plaster of paris) content is about 13% and the average particle size 5 to 25 μ.

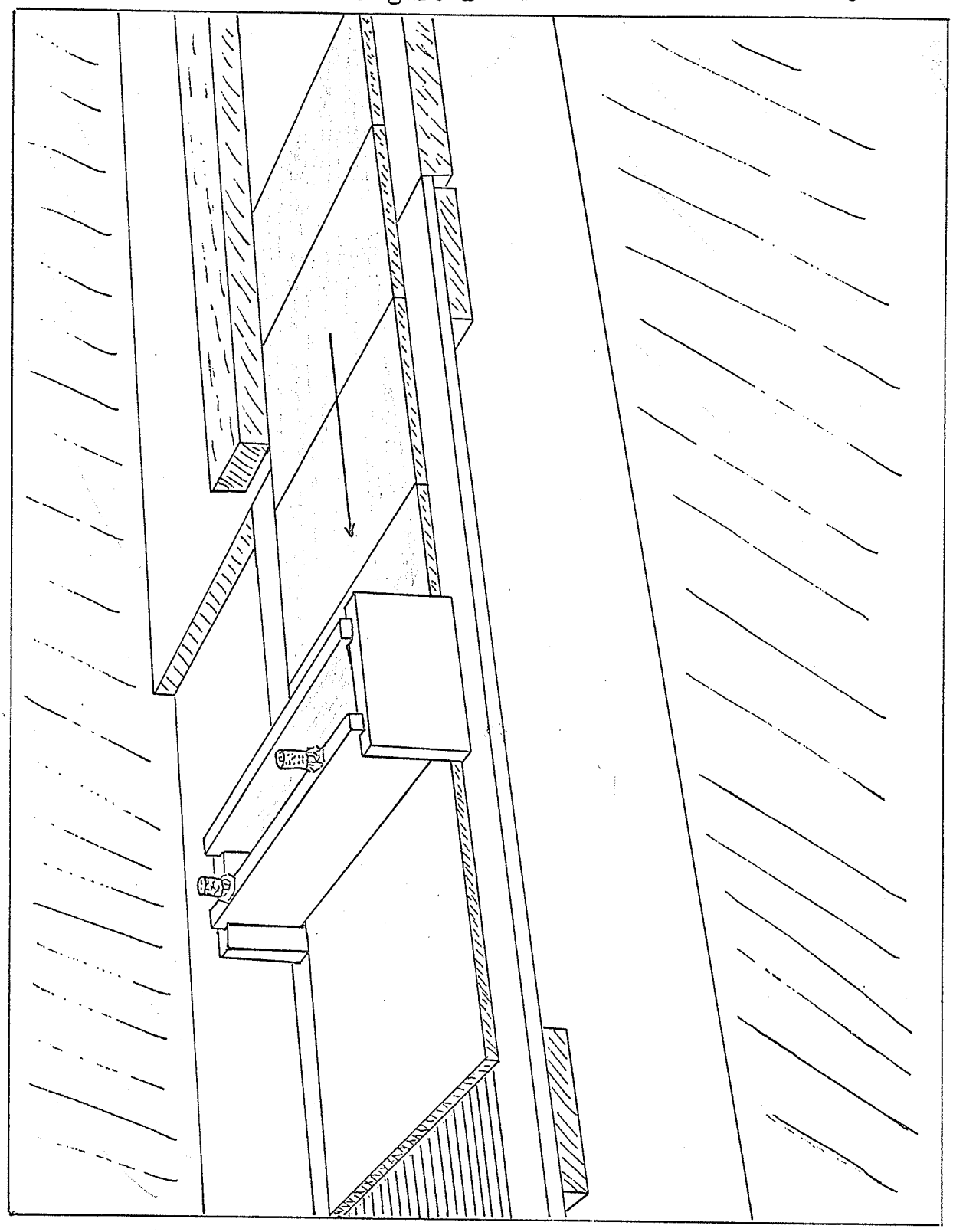
Aluminum Oxide G - The Aluminum Oxide G, manufactured by E. Merck, A.G. Darmstadt, Germany, was used. Its plaster of paris content and particle size is the same as that of Silica Gel G.

There are differences between the two adsorbents. Silica Gel G is essentially acid in character, whereas Aluminum Oxide G is essentially basic. Furthermore, Silica Gel G may function in both adsorption and partition chromatography, depending upon the solvent system used. Aluminum Oxide G on the other hand functions only in adsorption chromatography.

Porous Glass - Upon the suggestion of Dr. Gesser (20) investigations were made whether porous glass could be made applicable for thin-layer chromatography. The porous glass of mesh size 60 was available from Corning Glass Works, New York (Code 7930). It was ground to a mesh size 200 to 250 and mixed with plaster of paris of the same mesh size, in a ratio of 87 to 13.

Procedure using "Kensco" Equipment

The "Kensco" applicator available from Kensington Scientific Corporation, 1717 Fifth Street, Berkeley 10, California, was used for the preparation of consistent and uniform layers. This type of spreading device has a large base. There is a guide along one side and in the center is a stationary reservoir which has two movable sides that can slide up and down. One of these sides has two vertical holes through which adjustment screws are placed that control the thickness of the coating (Figure 1). A slurry of adsorbent and water is placed inside the reservoir and then the glass



KENSCO Applicator

plates are slowly passed beneath the reservoir. The width of the spreader is fixed, being 20 cm.

Thickness of the layer

One of the variables in thin-layer chromatography is the thickness of the coating applied. Experiments were made with several thicknesses ranging from 0.2 to 1 mm. The "thickness of the layer is unimportant with respect to the R_f value and the degree of separation" (2a). However, as the thickness of the coating is increased, cracks develop more easily on the surface of the adsorbent in the drying process and the surfaces are not smooth. It was Honegger (25) who showed that 5 mm was about the maximum thickness one could apply, whereas Stahl et al. (55) found that 0.15 mm was the minimum thickness to be used in order to obtain a reasonable separation. Most of our work was done with coatings 0.3 mm thick, because our work was largely qualitative. The only advantage of using a thicker adsorbent layer is in preparative thin-layer chromatography.

Preparation of Slurry

For adsorption thin-layer chromatography - A dry adsorbent such as Silica Gel G and Aluminum Oxide G is normally mixed with a liquid such as water, and then stirred vigorously to produce a uniform slurry. Just the right ratio has to be

used to obtain a viscosity suitable for spreading, neither too thin, which gives very thin films, nor too thick, which tends to form irregular patterns and lumpy spots. The recommended ratios (34) were found satisfactory, Silica Gel G/water 1/2 (w/v), and Aluminum Oxide G/water 1/2 (w/v).

For partition thin-layer chromatography - $AgNO_3$ impregnated plates. Silver nitrate impregnated plates were prepared in a similar manner as described above for Silica Gel G plates (1,39). An aqueous solution of silver nitrate of known strength was added to the adsorbent and made into a slurry and the latter applied to the plates with the applicator. The plates were prepared so that the percent silver nitrate by weight on the coating was either 11% or 20% which have been found to be satisfactory.

For partition thin-layer chromatography - Paraffin impregnated plates - It was found most convenient to dip previously prepared Silica Gel G plates cautiously into a 5% solution of paraffin oil in light boiling petroleum ether (B.P. 38 - 49°C). One end of the plate was placed first into a flat dish containing the paraffin oil, and then the whole plate was submerged. This gave a uniform coating as well as preventing the breaking up of the thin layer. The plates were removed from the bath and dried at room temperature.

Preparation of Porous Glass Slurry - The aforementioned

prepared mixture of porous glass and plaster of paris was mixed with distilled water in a ratio of 1/2, w/v. The slurry was applied in the same way onto the plates as was done with Silica Gel G.

Activation

The prepared plates were allowed to dry at room temperature for 2 to 3 hours, followed by activation at 120 to 140°C for 3 hours. These activated plates were stored in a desiccator until used.

Application of Sample

The choice of solvent to dissolve the beeswax - The sample to be applied onto the chromatogram should be dissolved in a suitable solvent to make up a 0.1 to 5% solution. This solvent should be low boiling, preferably between 50 and 100°C, to allow one to remove it quickly by blowing dry air over it; however, it should not evaporate too quickly. Furthermore, the solvent should be as non-polar as possible in order that the sample be concentrated at the center of the point of application and not at the periphery of the spot.

Solvents available to dissolve beeswax are chloroform, ether, and carbon tetrachloride (64). Beeswax is partially soluble in cold benzene or carbon disulfide, and sparingly soluble in cold alcohol. Since it is preferable to apply the

spots from a solution kept at room temperature, one is limited to chloroform, ether and carbon tetrachloride. Chloroform was preferred as a solvent for beeswax, because solutions of concentration greater than 5% could more easily be made than with the other solvents, and because it gave more uniformly distributed spots on the chromatogram than solutions in the other solvents.

Amount of sample applied - The amount of sample applied is based upon the purpose of the separation, whether it is for qualitative, quantitative or preparative chromatography. Furthermore, it depends upon the thickness of the coating. The thicker the coating, the more sample can be applied. For qualitative work any amount will do that gives a good resolution of the individual spots and shows up the faint spots distinctly. In the case of quantitative separations, a definite volume of a prepared solution is applied with a Hamilton syringe. For preparative chromatography as much as possible is applied which still yields a good separation.

Mechanism of application - The samples were applied at a definite distance, either 1.5 cm or 2 cm from the lower edge of the chromatoplate, by touching the tip of the Hamilton syringe to the adsorbent each time a drop was formed on the syringe. If several drops were applied to the same spot, the spot was

dried after each application by passing air over it. After the spots were applied, a line was drawn across the plate to mark off the height to which the developer was to migrate. For preparative and quantitative thin-layer chromatography individual spots spaced 9 mm apart were applied along the bottom of the chromatoplate. Better separations were obtained applying spots at a certain distance apart than when a continuous line of spots close together was placed along the bottom of the plate.

Ascending thin-layer chromatography

The prepared plates are placed in the chromatographic chamber, consisting of a jar in which a rack holding the plates is suspended. Developer is placed in the jar to a level which will reach halfway from the bottom of the plate to the spots. Along the inside wall of the developing chamber chromatographic paper is lined to insure saturation with solvent vapour. The plates are suspended for a few minutes inside the jar until the atmosphere within has reached saturation again. The rack is then lowered to the bottom by the protruding rods. The reason for allowing the chamber to reach saturation before the development process starts is to get a correct reading of the solvent front. In an unsaturated chamber, the solvent at the solvent front evaporates continuously, increasing the development time greatly. Such separations are equivalent to increasing the development distance, with several of the upper components crowded in the solvent front.

Reporting of R_f values

The R_f value is the ratio of the distance traversed by the substance to that traversed by the solvent, both of which are measured from the point of application. The R_f values obtained are related to the R_f values of test dyes under the same condition for sake of reproducibility. The test dye used was the dye system of Stahl (50) containing butter yellow, indophenol, and Sudan Red G. (Available from Desaga, G.m.b.H., Heidelberg, Germany. American agents, C. A. Brinkmann Co., Inc., Great Neck, Long Island, New York).

Methods of Detection

An extensive list of sprays that can be used in thin-layer chromatography is given by Waldi (63), Bobbitt (2b) and Randerath (45). Only a few reagent sprays proved useful for our work, such as iodine vapour, 2'7'-dichlorofluorescein, Rhodamine B, sulfuric acid and chromic-sulfuric acid for general detection of components, and silver nitrate-pyrogallol (63) specifically for acids. The method suggested by Ramsey and Patterson (44) for detecting acids with bromcresol green did not give a positive result of the acid fraction of beeswax, which was, however, observed with such a spray as silver nitrate-pyrogallol. We concluded that it was less sensitive and hence dropped it in favor of the silver nitrate-pyrogallol spray for detecting acids.

Eluting the spots

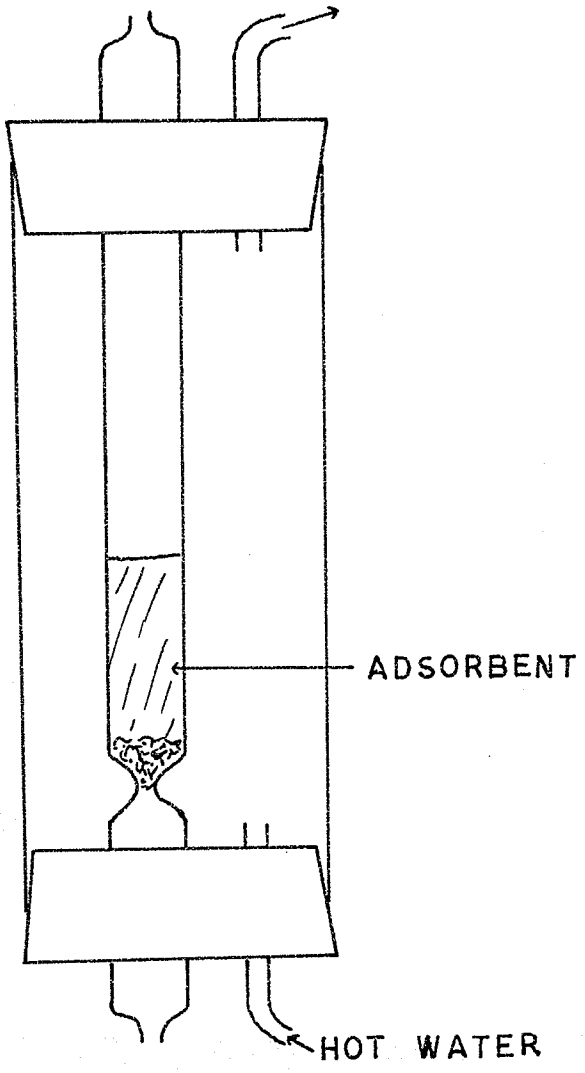
In preparatory and quantitative thin-layer chromatography one must remove the components from the adsorbent. This was

done by preparing a type of glass aspirator (68, 48). It consists simply of a glass tube drawn out at both ends with a restriction near one end of the tube, and glass wool placed at the restriction (see diagram below).

With this device one can collect the adsorbent quantitatively after it has been scratched loose with a microscope slide, by applying a vacuum on the right end. A non-destructive indicator, such as iodine vapour should be used to locate the position of the spots, which were then marked off. Often one developed spot was left on the plate, which was then sprayed with2'7'-dichloro-fluorescein followed by charring with chromic-sulfuric acid to confirm the first detection, i.e. the one with iodine for example.

To elute the components from the adsorbent in the aspirator, the glass aspirator was warmed by placing a warm water jacket about it (see diagram below). The solvent chloroform-acetone in a 50:50 ratio was passed through it slowly to dissolve the components out. The solvent chloroform-acetone was used because a chromatogram using this solution as developer moved all components to the top of the plate. Hence it was most useful in eluting all components quickly.

Two to 5 ml of the solvent were sufficient to elute the components from one spot.



QUALITATIVE ADSORPTION THIN-LAYER CHROMATOGRAPHY OF BEESWAX

The first step of the systematic chromatographic analysis of beeswax is the separation of the complex natural substance into classes of chemically similar compounds. Adsorption thin-layer chromatography can be used to effect such a separation. However, it is helpful to have an idea of the identity of the components present in the mixture. Former studies indicated that the following constituents are present in beeswax (16): hydrocarbons, esters, alcohols, "diols", acids, hydroxyacids, unsaturated acids, and 6% unidentified substance, probably propolis. The search for the right combination of adsorbent and solvent system for development is then greatly simplified.

The principle relating the three variables, adsorbent, solvent system and substance to be separated, is diagramatically represented in Fig. 2 (56).

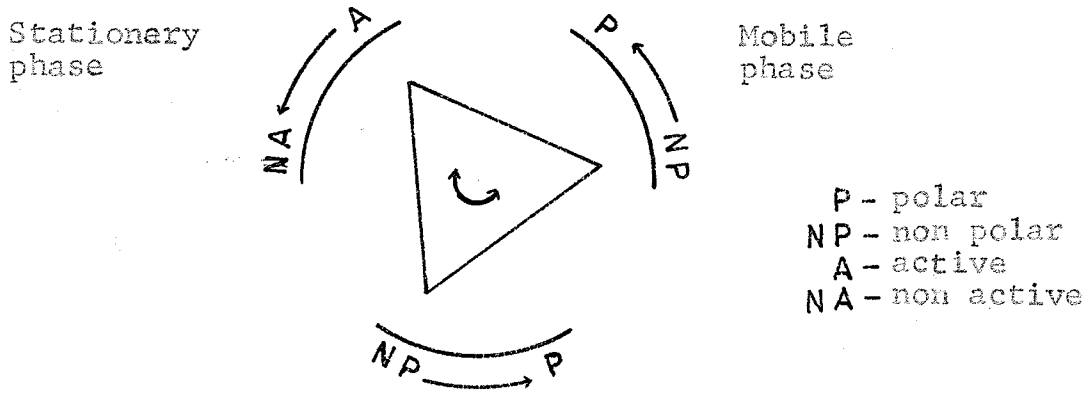


Fig. 2. Diagramatic representation of the three variables of thin-layer chromatography.

One can see from Fig. 2 that, as the polarity of the substance to be separated increases, the more polar the solvent must be, and the less active the stationary phase. In practice it is advisable to vary the mobile phase before changing the stationary phase.

Comparison of adsorbents

Experiments were conducted with the available adsorbents, Silica Gel G, Aluminum Oxide G and porous glass. Essentially, adsorption chromatography occurs on Aluminum Oxide G layer, hence it should separate the complex mixture into classes of compounds. Silica Gel G on the other hand gives either adsorption or partition chromatography, depending on the solvent system used.

For these adsorbents the water content is critical, because water blocks the active sites of the adsorbent and increases the degree of partition chromatography. For this reason some plates were activated while others were simply dried at room temperature. Plates dried at room temperature should give better separations of the more polar components in the mixture than of the less polar ones. This trend was observed; however, tailing increased correspondingly and, therefore, this technique was discontinued. Now since both activated Silica Gel G and Aluminum Oxide G plates were used, with not too polar solvents, primarily adsorption chromatography took place

on both types of plates resulting in almost identical separations. Silica Gel G plates were used more frequently.

Porous glass chromatoplates were used as well and their separations indicated that more partition than adsorption chromatography was taking place. Chromatograms of beeswax, bayberry wax and spermaceti using the same solvent system but different adsorbents showed that the waxes were separated into more spots on porous glass chromatoplates. See Fig. 3.

The porous glass adsorbent may be of considerable use in partition thin-layer chromatography, since the particle size, pore size (69) and active sites (65) can be controlled and varied. It has a great potential value for future investigations.

Comparison of developers

The Rf value of a component on a chromatogram may be increased by increasing the polarity of the solvent or developer. Several classical eluotropic series are known which can serve as guides in the choice of developer. Those by Trappe (60), Strain (57), Knight and Groennings (30), Neher and Arx (46), and from the Handbook of Physics and Chemistry (22), are only a few which may be applied to adsorption thin-layer chromatography. A good rule to observe in the choice of a solvent system is to keep it as simple as possible (34).

There are two generally accepted ways to do preliminary tests for which solvent will yield the desired separation, in this case the maximum number of distinct spots. By far, the

Figure 3. The development of three waxes, beeswax (a, d, g), bayberry wax (b, e, h) and spermaceti (c, f, i) is compared on three different thin-layer adsorbents, porous glass (a-c), Silica Gel G (d-f) and Aluminum Oxide G (g-i). The same eluent was used: Petroleum ether (b.p. 38° - 49° C)/ ether in the ratio 95/5 .

simplest test was initially reported by Izmailov and Shraiber (26), Crowe (10), and extended by Stahl (56). Several spots of the substance to be separated are dispersed on a thin-layer chromatogram, an inch or so apart. The solvent to be tested is dropped at the center of the spot until a fair size circle is formed. The periphery is marked and the solvent allowed to evaporate. The spots are made visible. That solvent, or solvent mixture, which gives the largest number of discrete concentric rings between the outer edge and the spot of application is the best developer. Although this method was tried repeatedly with beeswax, only blurs that proved inconclusive were obtained.

Due to the complexity of the beeswax, the second method was preferred. It consists simply of running several chromatograms in different solvents and thus by trial and error determining the most suitable solvent system. Narrow plates, 5 x 20 cm, were used for this purpose.

Results using different developers

Several chromatograms were made using carbon tetrachloride as the basic solvent. These are compared in Fig. 4. As the polarity of the developer was increased, the components moved up correspondingly. Therefore, optimum resolution should be possible when polar developers are used, which should, however, not be too polar, i.e.,above acetone in the eluotropic series. Since the solvent system carbon tetrachloride and benzene gave a fairly good separation, experiments were conducted to see what effect an increase in the benzene fraction had on the separation of beeswax. These results are shown in

Fig. 5.

According to Mangold (33), solvents containing petroleum ether, ether and acetic acid were successful in the separation of lipid mixtures. The reason for the addition of small amounts of acid and base is to facilitate the separation of acids and bases respectively and to reduce their "tailing." A comparison of the chromatograms in Fig. 6 will show the effect of the addition of acetic acid to the solvent system. When the proportions of petroleum ether/ether/acetic acid are changed, the Rf value of the components of beeswax may be increased as illustrated in Fig. 7. From Fig. 6 it may be concluded that the greater the acetic acid fraction in the developer the better is the resolution. However, from Fig. 7 the same is not true for increasing the ether fraction of the developer, but rather the resolution of beeswax on a thin-layer chromatogram did not improve at ether concentrations greater than 10%.

Increasing the development distance

Investigations were made as to the effect of an increase in the development distance. The results were encouraging when chromatograms were developed a distance of 15 cm instead of 10 cm. Many more spots appeared as seen in Fig. 8 and Fig. 9.

Increasing the thickness of the layer

As was mentioned in an earlier section, the separation is independent of the thickness. This was verified. Therefore, the only advantage of using thicker layers is in preparative thin-layer chromatography.

Reproducibility of adsorption thin-layer chromatography

Thin-layer chromatography has many advantages over paper chromatography such as faster development, higher sensitivity, noticeably sharper separations, and greater versatility in detection as far as sprays are concerned. However, it has one weakness in that it lacks reproducibility. Often identical plates which are chromatographed simultaneously with the same developer, give different Rf values. Although the Rf value may not be the same, it has been shown by several workers that the relative order of the spots do not change. The result of such thin-layer chromatographic separations of beeswax are shown in Fig. 10. Because of this fact, workers (15, 42, 50) in this field have suggested using universally available substances for comparison on chromatograms. The standards selected depend upon what solvent system and what adsorbent one uses. For the work on beeswax, the dye system of Stahl (50), namely butter yellow, indophenol, and Sudan Red G, was satisfactory. Fig. 11 gives a comparison of beeswax and this dye mixture on the chromatogram.

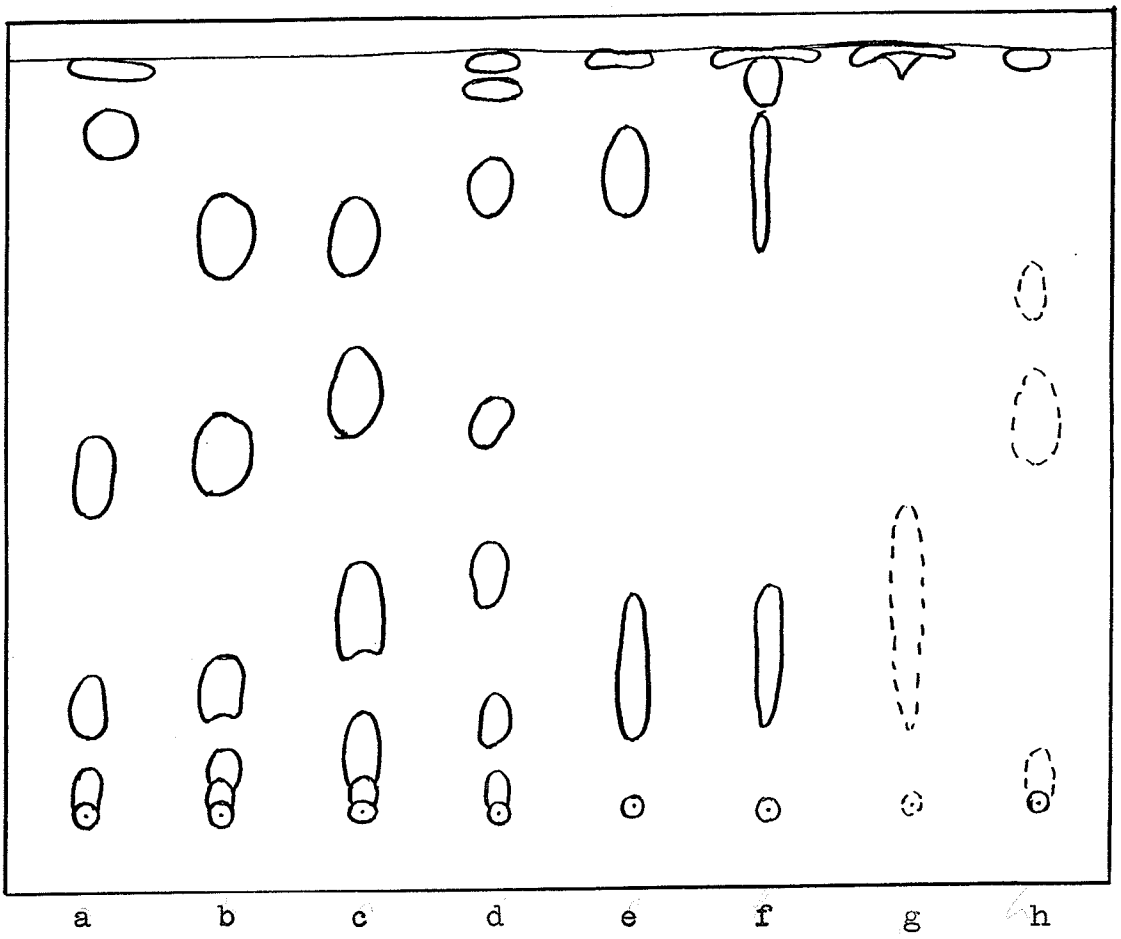


Figure 4 Chromatograms of beeswax on Silica Gel G plates using different solvent systems of which carbon tetrachloride is the basic solvent.

	Developer	Ratio	Time
-a-	carbon tetrachloride/ toluene	= 5/2	50 min.
-d-	carbon tetrachloride/dichloromethane	= 1/1	30 min.
-b-	carbon tetrachloride/benzene	= 5/2	31.7 min.
-c-	carbon tetrachloride/benzene/chloroform	= 5/2/1	26.7 min.
-e-	carbon tetrachloride/pyridine	= 20/1	70 min.
-f-	carbon tetrachloride/acetone	= 30/1	40 min.
-g-	carbon tetrachloride/acetone	= 15/1	40 min.
-h-	carbon tetrachloride/methylacetate	= 30/1	67 min.

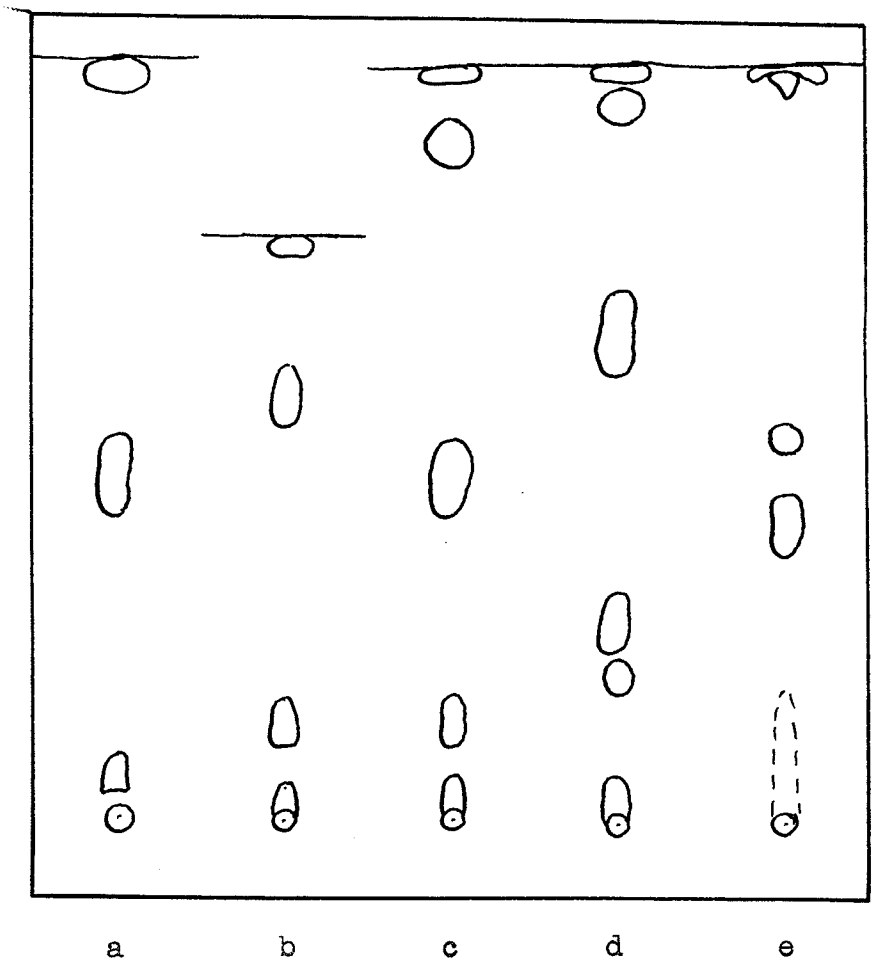


Figure 5 Chromatogram of beeswax on Silica Gel G plates using various ratios of carbon tetrachoride and benzene.

	Developer	Ratio	Time
-a-	carbon tetrachloride/benzene	= 14/1	40 min.
-b-	carbon tetrachloride/benzene	= 6/1	40 min.
-c-	carbon tetrachloride/benzene	= 5/2	45 min.
-d-	carbon tetrachloride/benzene	= 1/1	55 min.
-e-	carbon tetrachloride/benzene	= 1/7	60 min.

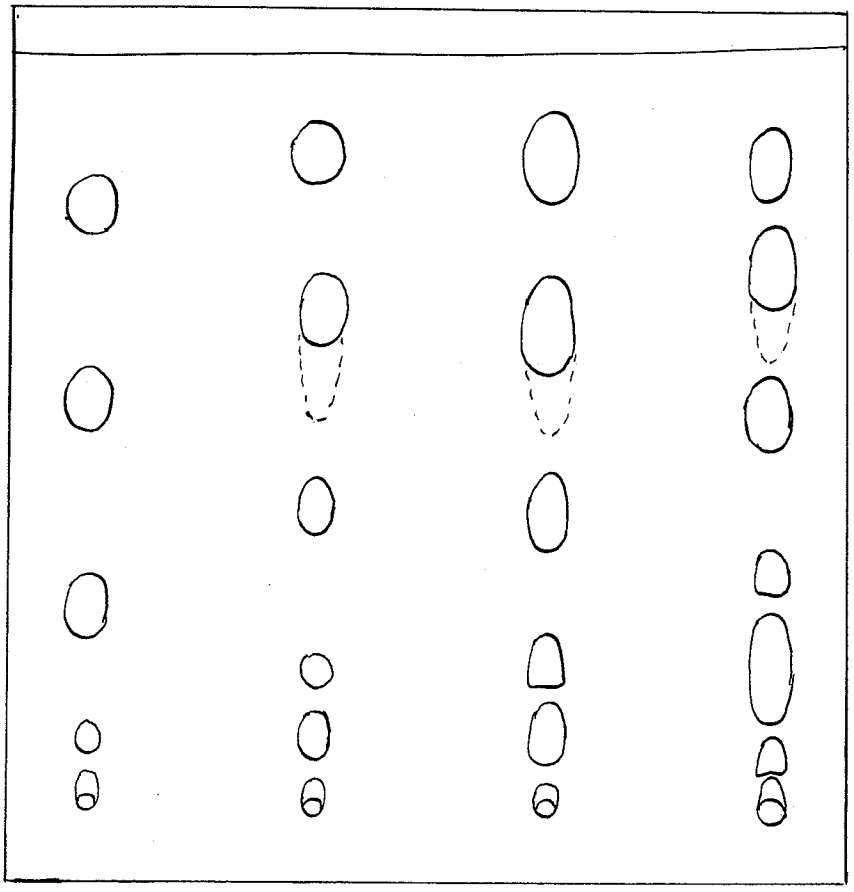
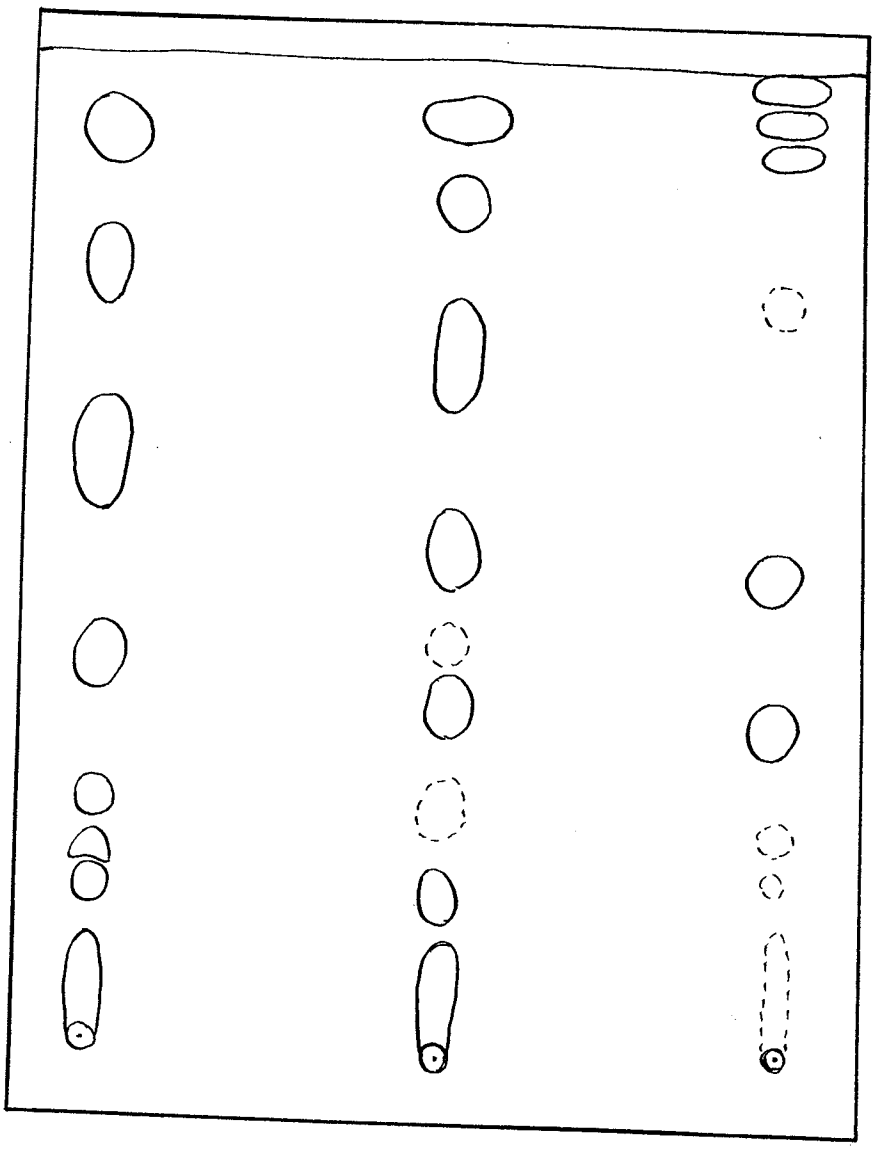


Figure 6 Chromatograms of beeswax on Silica Gel G plates in which increasing amounts of acetic acid (HAc) have been added to the developer petroleum ether/ether = 95/5 .

	Developer		Ratio	Time
-a-	petroleum ether/ether/	=	95/5	17 min.
-b-	petroleum ether/ether/HAc	=	95/5/½	15 min.
-c-	petroleum ether/ether/HAc	=	95/5/1	16.5 min.
-d-	petroleum ether/ether/HAc	=	95/5/1½	16.5 min.



a b c

Figure 7 Chromatograms of beeswax on Silica Gel G plates in which the ratio of petroleum ether/ether has been changed in the developer, petroleum ether/ether/acetic acid.

Developer:	Ratio:	Time:
-a- petroleum ether/ether/acetic acid	= 95/4/1	(41 min.)
-b- petroleum ether/ether/acetic acid	= 91/8/1	(35 min.)
-c- petroleum ether/ether/acetic acid	= 84/15/1	(50 min.)

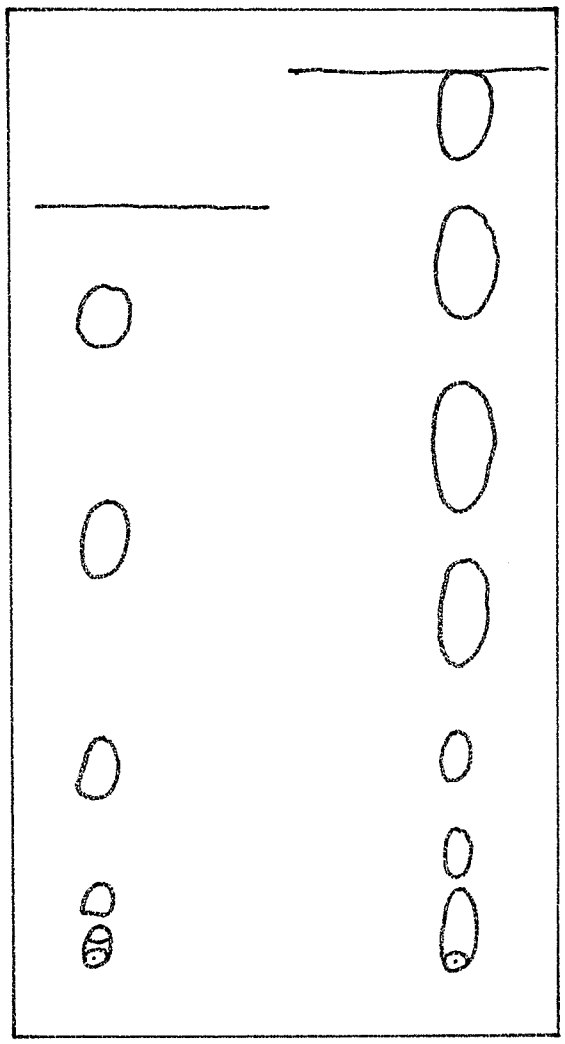


Figure 8 Chromatograms of beeswax on Silica Gel G plates using the developer petroleum ether/ ether. The development distance was 10 cm. in (a) and 12 cm in (b) . The corresponding development times were 21.6 min., and 30 min.

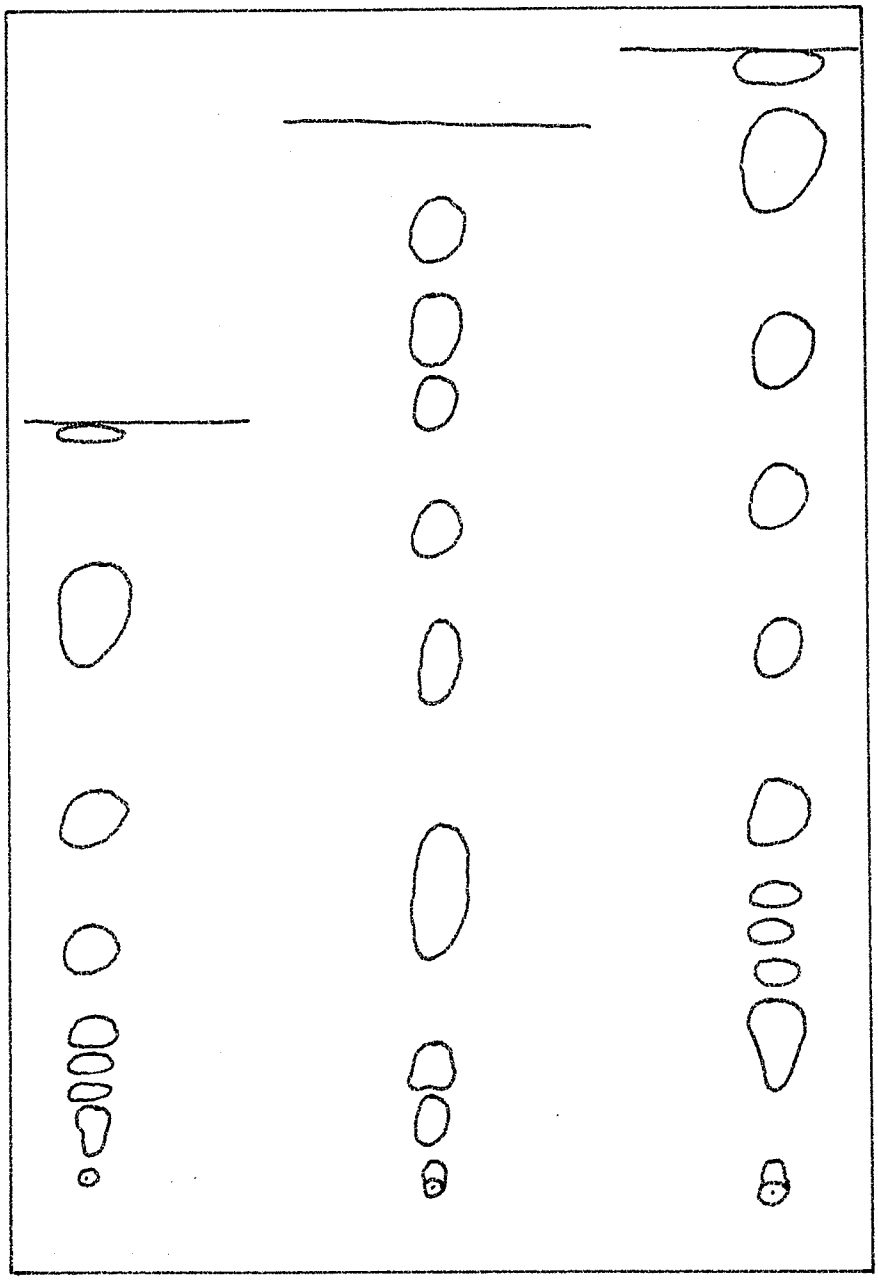


Figure 9 Chromatograms of beeswwax on Silica Gel G plates using the developer petroleum ether/ether/acetic acid = 95/5/1.

a- developed a distance of 10 cm taking 26 min.
b- developed a distance of 14 cm taking 32 min.
c- developed a distance of 15 cm taking 41 min.

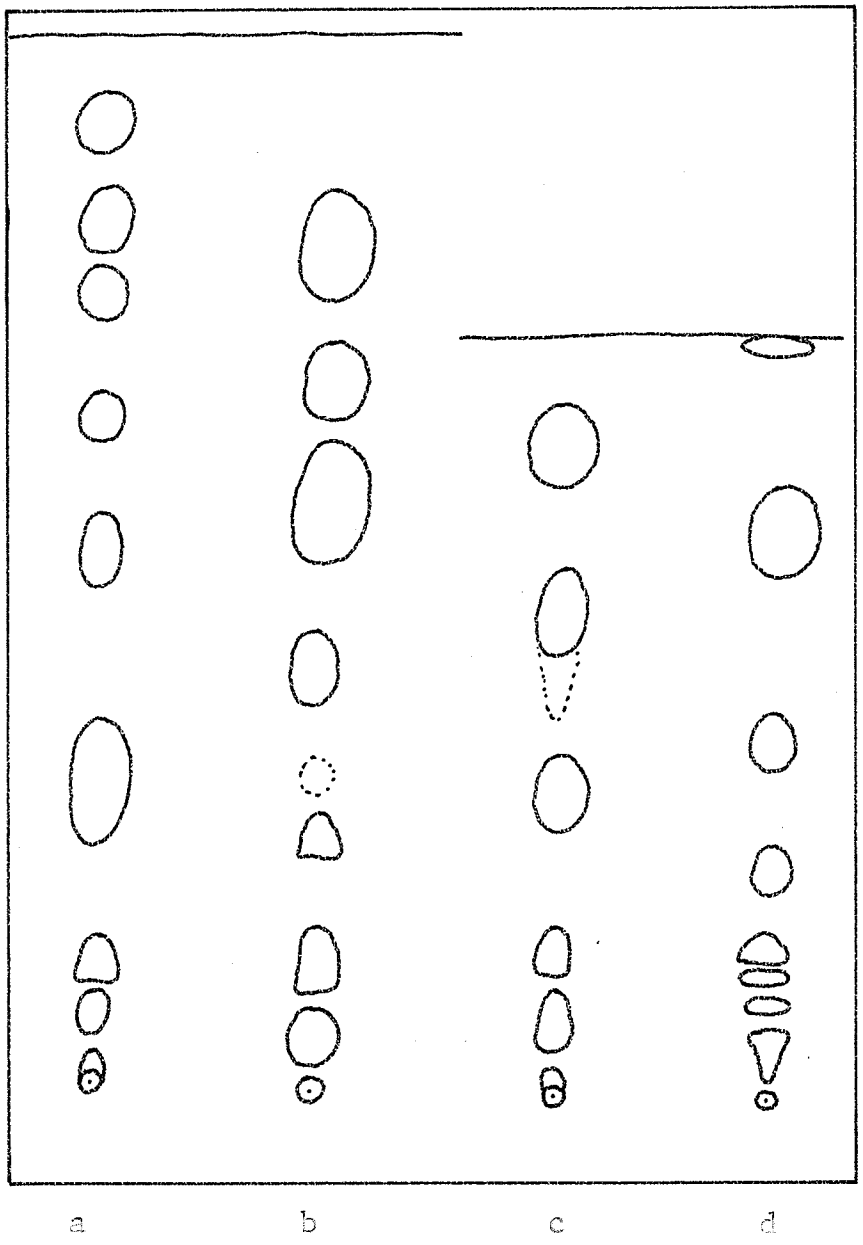


Figure 10 Four chromatograms of beeswax. Chromatogram a and b were chromatographed under identical conditions except their development times were different, 38 and 32 minutes respectively. Developer: petroleum ether/ether/acetic acid - 91/8/1. Development distance 14 cm.

Chromatogram c and d were chromatographed under identical conditions except their development times were different, 16.5 and 28 minutes respectively. Developer: petroleum ether/ether/acetic acid - 95/5/1. Development distance 10 cm.

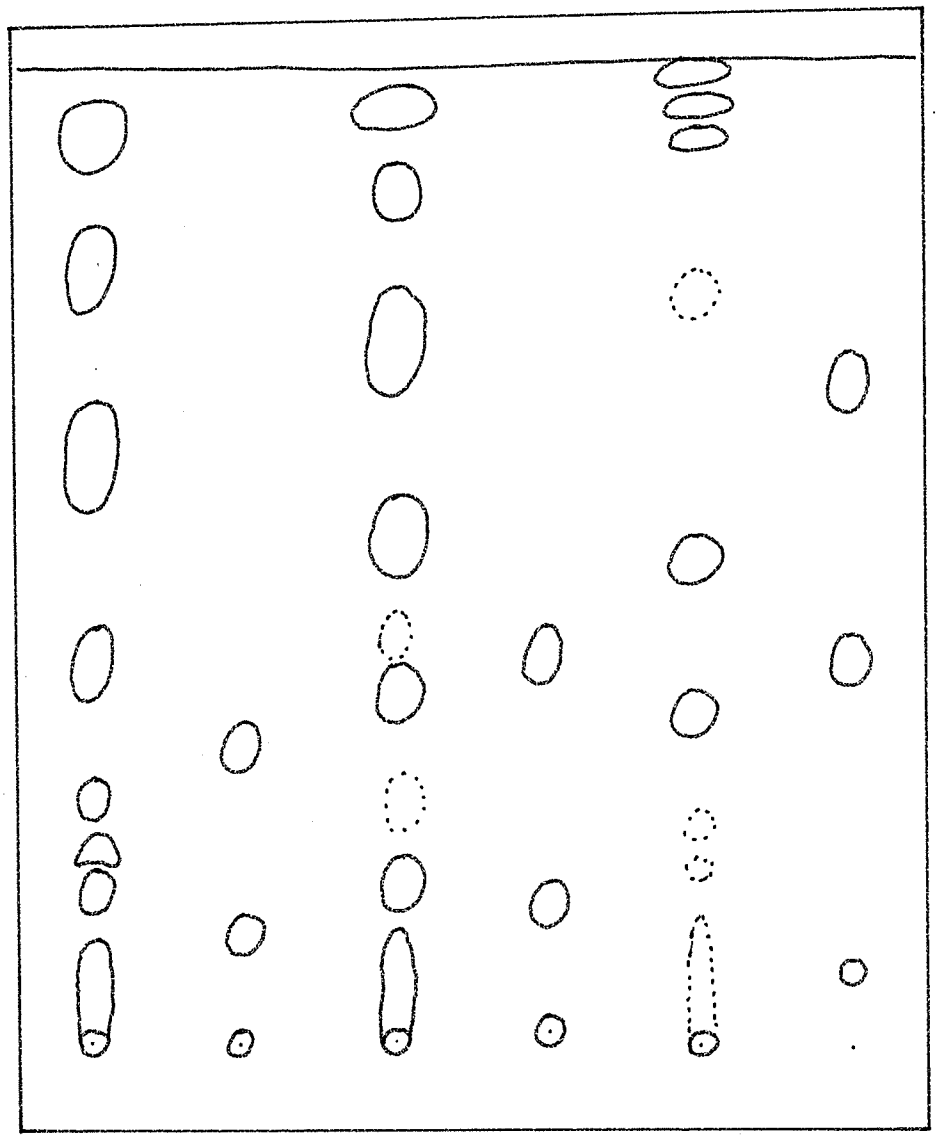


Figure 11 Chromatogram of beeswax (a,c,e) and the dye system of Stahl (Desago), (b,d,f) on Silica Gel G plates. The developers are petroleum ether/ether/ acetic acid = 95/4/1 for chromatograms a and b; petroleum ether/ether/acetic acid = 91/8/1 for chromatograms c and d; and petroleum ether/ether/ acetic acid = 84/15/1 for chromatograms e and f. The development distance for all separations was 13 cm. The development times are 41 min., 35 min., and 50 min. for chromatograms a and b, c, and d, and e and f respectively.

QUANTITATIVE ADSORPTION THIN-LAYER CHROMATOGRAPHY

Once the best solvent system and adsorbent were determined, the separation was made quantitative, by following the procedure outlined earlier (page 12). The results of two series of chromatograms will be given. In the first series, five activated Silica Gel G plates, 20 x 20 cm, were used on which a combined weight of 23 mg of beeswax (Fisher white beeswax) was chromatographed. The thickness of the applied coating was 0.3 mm. The plates were developed a distance of 13 cm with petroleum ether (B.P. 38 - 49°C), ether, acetic acid in the ratio 91:8:1. After the chromatograms were developed, the spots were visualized with iodine, the areas of the ten spots together with the point of application marked off, and the components eluted with a 50:50 mixture of chloroform and acetone. The solvent was evaporated at 65°C and the residue weighed.

A similar series with 10 plates was conducted on which a total of 37.9 mg of beeswax (Fisher white beeswax) was chromatographed. A typical separation with its corresponding Rf values is included in Table 1 which contains the experimental results of these two series.

The total substances eluted weighed consistently more than the sample applied. Extensive drying did not reduce the weight of the collected sample. Therefore, we eluted several 10 gm samples of Silica Gel G to find out how much impurities were dissolved from the plate coating with the mixture chloroform-acetone

(50 : 50). On the average, 4.2 mg of impurity was eluted from 10 gm of Silica Gel G. Honegger reported (24) that acetone eluted 10.5 mg of impurities from 50 gm of Silica Gel G, and chloroform 6.8 mg of impurities from the same amount of Silica Gel G. Table 1 includes calculations in which corrections are made for the impurities eluted. Recoveries of 97 and 99% of the beeswax were obtained in the two series.

TABLE 1

QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF BEESWAX

R_f	Typical separation		Series I Weights collected (mg)	Series I % by weight	Series II Weights collected (mg)	Series II % by weight	Average % by wt.
1.0							
0.92		1.	5.1	18.3	10.0	20.58	19.4
		2.	6.1	21.9	10.4	21.40	21.6
0.77		3.	3.8	13.6	3.9	8.02	10.8
		4.	1.2	4.3	3.0	6.17	5.2
		5.	0.4	1.4	2.0	4.12	2.8
0.57		6.	1.1	3.9	2.2	4.53	4.2
		7.	1.5	5.4	2.0	4.12	4.8
0.41		8.	1.8	6.4	2.2	4.53	5.5
0.28		9.	1.7	6.1	2.4	4.94	5.5
0.20		10.	3.6	12.9	4.5	9.26	11.1
0.13		11.	1.6	5.8	6.0	12.33	9.1
0.08			27.9		48.6		
0.03							
0.00							

	Series I	Series II
Total amount of material collected	27.9 mg	48.6 mg
Total amount of impurities collected	5.5 mg	11.1 mg
Total amount of sample collodted	22.4 mg	37.5 mg
Total amount of sample applied	23.0 mg	37.9 mg
Percentage recovery	97%	99%

PREPARATIVE CHROMATOGRAPHY

Since preparative adsorption thin-layer chromatography is time-consuming, preparative column chromatography of beeswax on activated alumina was attempted to obtain larger quantities of the desired fractions. The fractions obtained from column chromatography were subjected to thin-layer chromatography to check the effectiveness of the separation on the column.

Column chromatography

Material - Alumina - Adsorption Alumina for chromatographic use, 80 - 200 mesh, was available locally from the Fisher Scientific Co.

- Column - Pyrex glass column 1.3 cm i.d. x 30 cm with a 250 ml bulb on top, sintered glass disk and a ground glass stopcock, was used.

- Petroleum ether - Petroleum ether, Reagent, A.C.S. B.P. 38 - 49°C, Fisher Scientific Co.

Packing the column - A slurry of alumina and petroleum ether was made and added portionwise into the column. After each small addition of slurry, it was packed with a glass rod followed by packing with 5 lbs. air pressure, the bottom stopcock being open. The final column dimensions were 1.3 cm i.d. x 20 cm which contained 33 gm of alumina.

Chromatographing procedure and results - One gram of beeswax (Fisher's white beeswax, and beeswax scales, in separate experiments), dissolved in 30 ml of hot heptane was placed on the alumina column. The column required warming to pass the sample into the absorbent. Solvents of increasing polar character were then used to develop the beeswax. The procedure followed was similar to the one by Wiedenhof on candelilla wax (67). The solvent systems in the sequence used, and the volume taken are given below.

Solvent System	Volume
1) petroleum ether	500 ml
2) petroleum ether with 5% ether	400 ml
3) petroleum ether with 15% ether	400 ml
4) petroleum ether with 5% n-propanol	600 ml
5) petroleum ether with 5% acetic acid	200 ml
6) chloroform/acetic acid (50/50)	200 ml

Fractions of 100 ml each were collected and monitored on adsorption thin-layer chromatograms (see Fig. 12). Several of the fractions from column chromatography were further separated by preparative thin-layer chromatography.

Collecting 100 ml fractions was very much preferred to collecting the complete eluate from one particular solvent because better separations were effected. A thin-layer chromatographic separation of the fractions obtained verified the superiority of the separation with 100 ml fractions. Compare Fig. 13 with Fig. 12.

Previous workers used silica gel (11, 49) and alumina

columns (40) to separate the hydrocarbons from the saponified beeswax. It was Bruening (3) who suggested chromatographing beeswax and spermaceti directly without prior saponification on an alumina column to obtain the hydrocarbon fraction quantitatively. He used 400 ml of petroleum ether on a column similar to the one we used to obtain the hydrocarbons. This method was also adopted by White et al. (66). However, when their directions were followed, esters were obtained in the first 400 ml petroleum ether eluate collected as revealed by a thin-layer chromatogram (Fig. 13).

It has often been suggested (6) that fractions should be rechromatographed on a column to get a better separation. The results from rechromatographing the beeswax fractions on an alumina column showed little improvement. Therefore, to obtain a better separation thin-layer chromatography was employed, which proved more effective.

Figure 12 The various 100 ml fractions collected from preparative column chromatography were monitored on a thin-layer chromatogram. The developer was petroleum ether\ether\acetic acid = 95\5\1, the development time 22 minutes, and the development distance 10 cm.

Fractions 1 to 5 were eluted with petroleum ether (95\5)
Fractions 6 to 9 were eluted with petroleum ether\ether (85\15)
Fractions 10 to 13 were eluted with petroleum ether\ether\n-propanol (95\5)
Fractions 14 and 18 were eluted with petroleum ether\acetic acid (95\5)
Fractions 20 and 21 were eluted with chloroform\acetone (50\50)

Figure 12 THIN-LAYER CHROMATOGRAPHY OF THE TWENTY-ONE 100 ml FRACTIONS OF BEESWAX COLLECTED FROM AN ALUMINA COLUMN

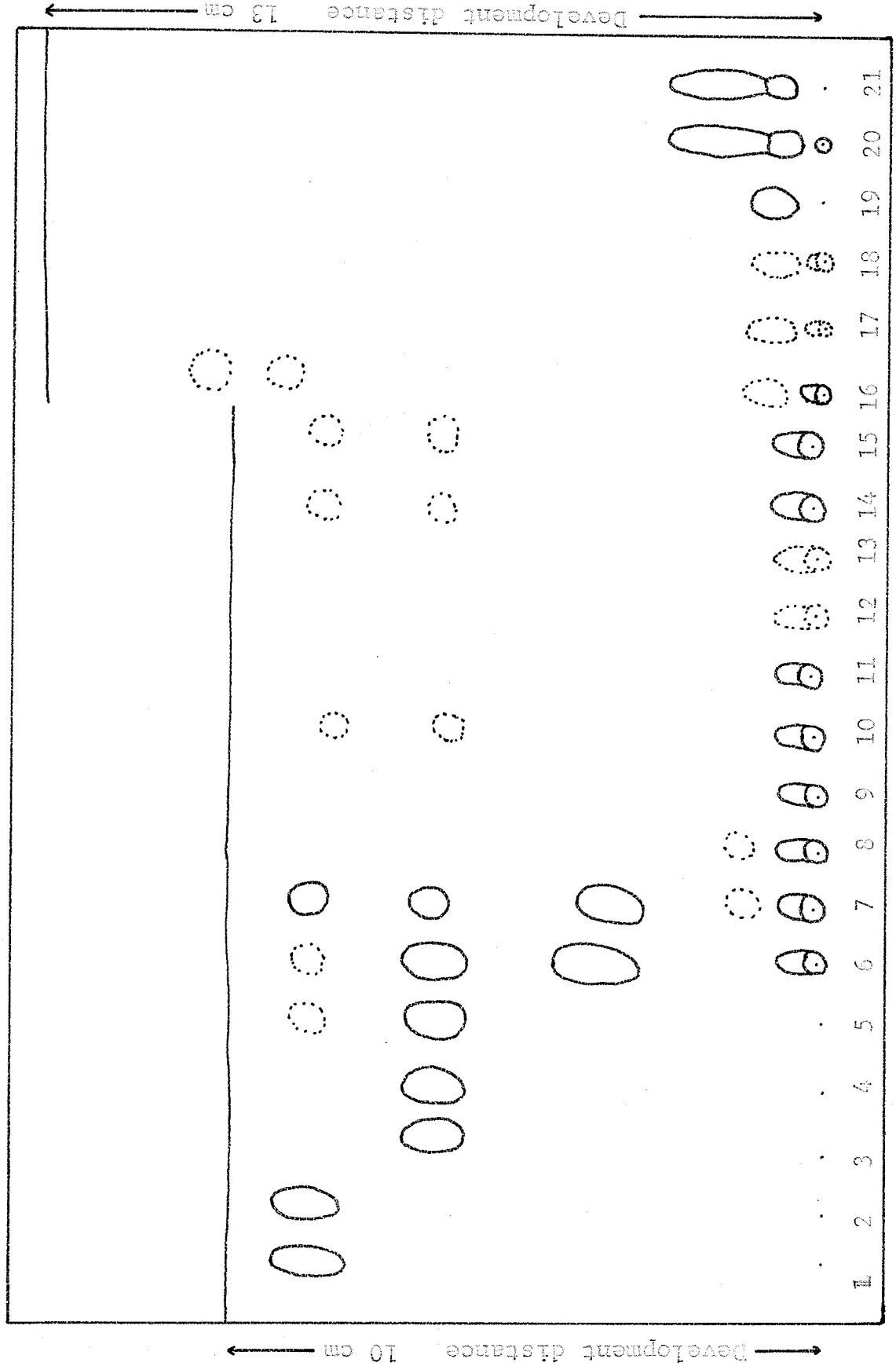
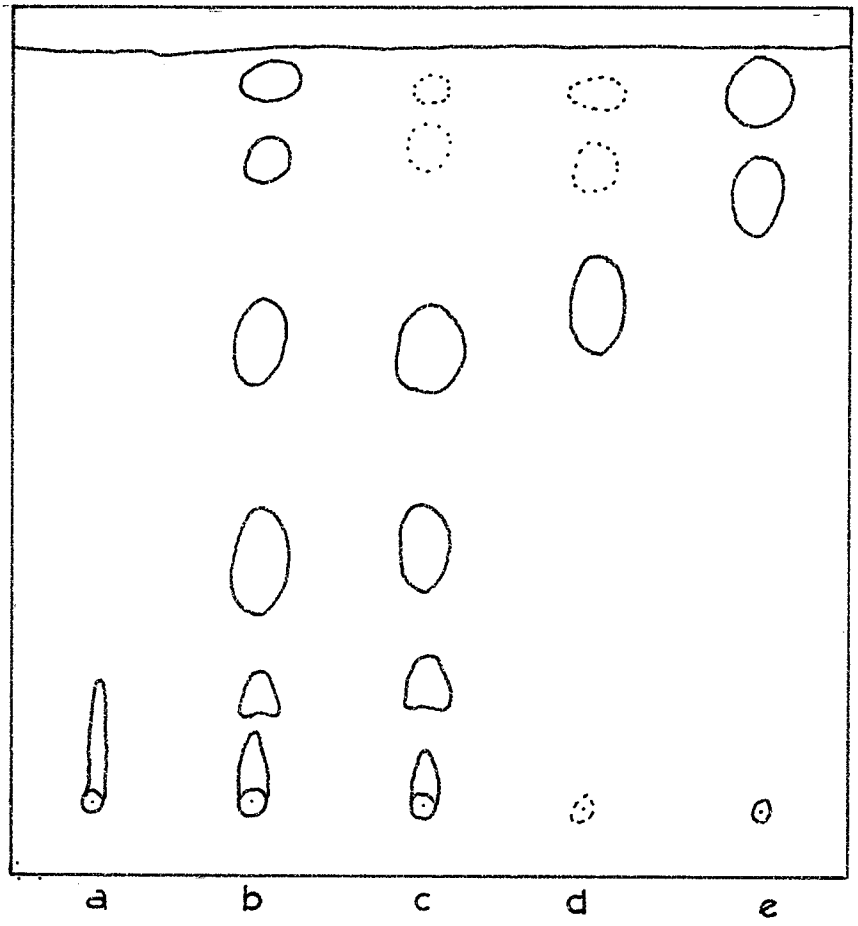


Figure 13

A THIN-LAYER CHROMATOGRAM OF THE FRACTIONS OF BEESWAX COLLECTED FROM COLUMN CHROMATOGRAPHY ON ALUMINA



The fractions collected from preparative column chromatography on Alumina were monitored on a thin-layer chromatogram. The developer was petroleum ether/ether/acetic acid = (95/5/1), the development distance 10 cm and the development time 25.5 minutes.

Chromatogram b: beeswax
e: eluate (400 ml) of petroleum ether
d: eluate (400 ml) of petroleum ether/ether (95/5)
c: eluate (400 ml) of petroleum ether/n-propanol (95/5)
a: eluate (400 ml) of petroleum ether/acetic acid (95/5)

IDENTIFICATION OF THE BEESWAX SPOTS SEPARATED BY ADSORPTION THIN-LAYER CHROMATOGRAPHY

A thorough study of natural substances, such as beeswax, should be centered around an identification of all components present as they occur in the natural state. Physical separations, which do not destroy the identity and character of the components present, are thus preferred. Adsorption thin-layer chromatography is one such physical separation technique. Beeswax was separated by this method into ten well defined spots, with something remaining at the point of application. Much time was spent in identifying the chemical character of the constituents of these spots. After this an attempt was made to identify the individual members of the classes of compounds separated by gas-liquid chromatography.

To determine the class of compound each spot belonged to, various methods were employed such as: conclusions drawn from the distance a component transversed on the plate, comparison with known compounds, chemical reactions with beeswax and the subsequent disappearance and appearance of spots, partition thin-layer chromatography, hydroxylamine hydrochloride reaction for esters, and infrared spectral data from each spot. These methods will be discussed in the order given, together with the respective conclusions reached.

Conclusions drawn from the R_f values of the various spots

Thin-layer adsorption chromatography of lipid classes

on Silica Gel G using the solvent mixture petroleum ether, ether and acetic acid in the ratio 90:10:1 (v/v/v) was done by Mangold (32, 34). From these chromatograms and others, one observes that strongly polar compounds such as acids and alcohols remain close to the starting line, whereas the least polar compounds such as the paraffins move close to the solvent front. The other classes of compounds are somewhere in between these extremes. They distribute themselves upward as follows: triglycerides, aldehydes, ketones, esters, unsaturated paraffins and saturated paraffins (47, 52).

Keeping this trend in mind, and knowing approximately what components to expect in beeswax, an examination of Fig.9c will tell us that possibly hydrocarbons, esters, hydroxy esters, acids, alcohols, hydroxyacids and "diols" are present, in the descending order given. Their approximate position is given in Fig. 14.

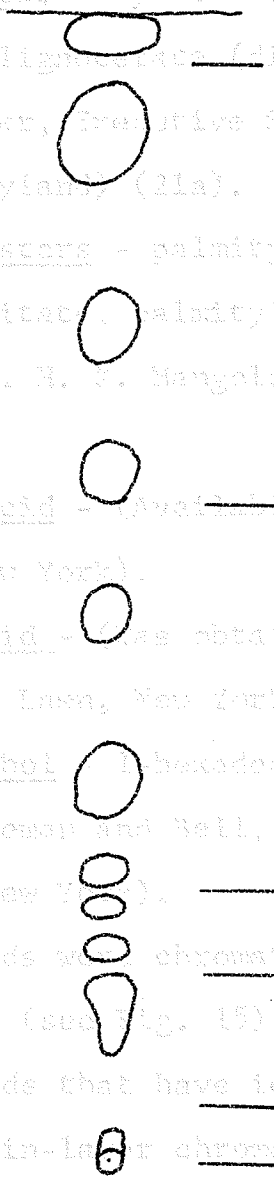
Comparison with known compounds

A variety of compounds was available for comparison:

1. Hydrocarbons - n-octadecane, n-nonadecane, n-eicosane, n-docosane, n-tetracosane, n-octacosane, n-dotriacontane, and n-hexatriacontane (available from Humphrey-Wilkinson Incorporated, North Haven, Conn.).

Figure 14

THIN-LAYER CHROMATOGRAM OF BEESWAX



In the figure the approximate positions of the classes of compounds separated under this chromatographic condition are given. The beeswax was developed on a Silica Gel G chromatogram with the eluate being petroleum ether/ether/acetic acid = 95/5/1. The development distance was 15 cm and it took 41 min.

2. Methyl esters - myristate, palmitate, stearate, arachidate, behenate and lignocerate (distributed by Metabolism Study Section, W. H. Goldwater, Executive Secretary, National Institutes of Health, Bethesda, Maryland) (21a).

3. Palmityl esters - palmityl laurate, palmityl myristate, palmityl palmitate, palmityl stearate. (These samples were donated by Dr. H. K. Mangold of The Hormel Institute, Austin, Minn.).

4. Palmitic acid - (Available from Eastman Kodak Company, Rochester, New York).

5. Stearic acid - (Was obtained from the Fisher Scientific Company, Fair Lawn, New York).

6. Cetyl alcohol - 1-hexadecanol, practical (Available from Matheson, Coleman and Bell, Norwood, Cincinnati, Ohio; East Rutherford, New York).

These compounds were chromatographed simultaneously with Fisher's white beeswax (see Fig. 15).

Compounds that have identical R_f values on the same adsorption thin-layer chromatogram very likely belong to the same class of compounds.

Chemical reactions, and the observed appearance and disappearance of spots

Saponification - Since esters are known to be present in beeswax, saponification of the wax and a subsequent comparison of this product of the saponified wax with the original beeswax

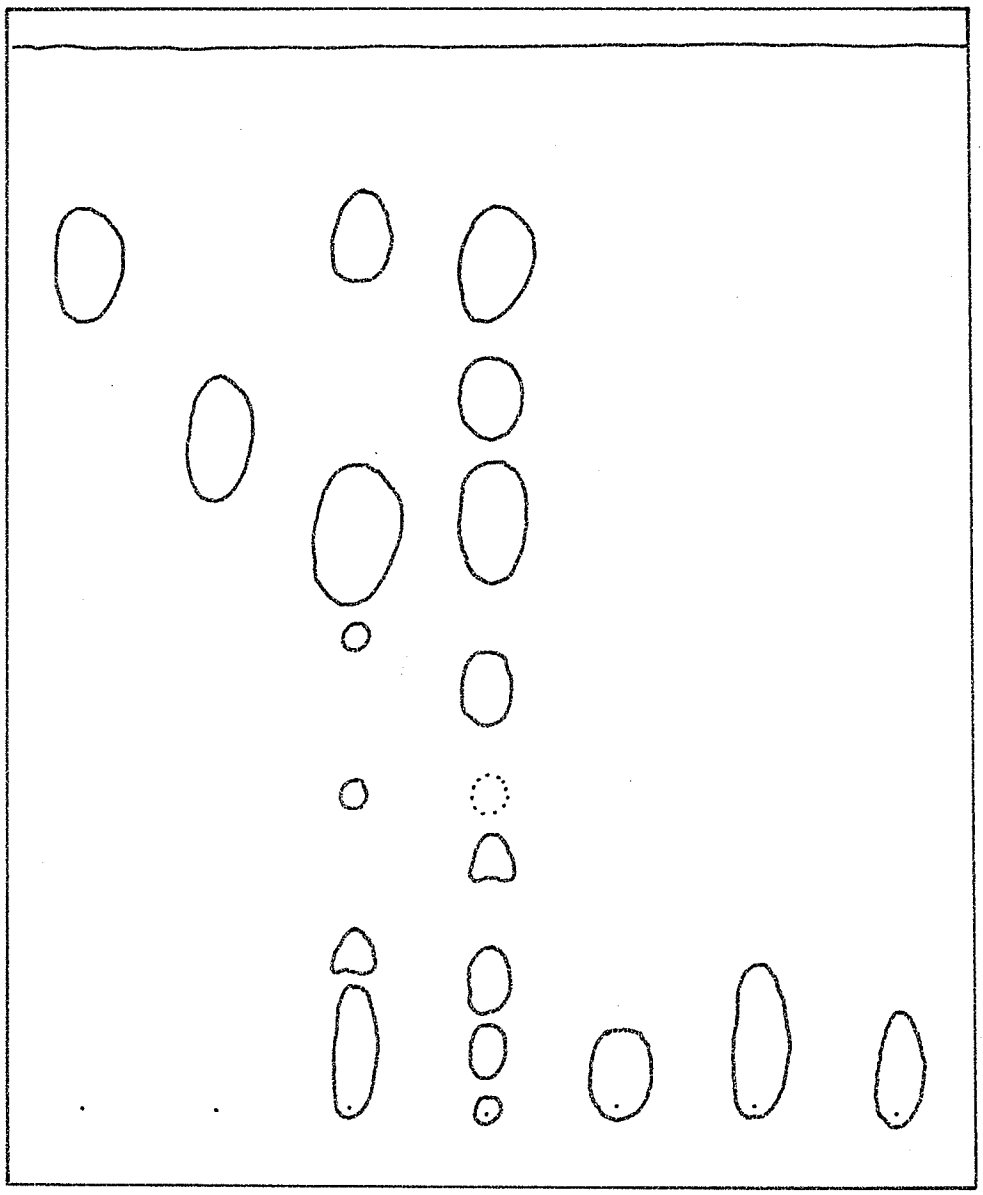


Figure 15 The spots are:

a) hydrocarbons (C_{18} - C_{36})
b) palmityl esters of stearic, palmitic, myristic and lauric acids.
c) methyl esters prepared from the fatty acids of beeswax
d) beeswax
e) cetyl alcohol
f) stearic acid
g) palmitic acid

Developer: Petroleum ether/ether/acetic acid= 91/8/1
Development time: 32 min.
Development distance: 14 cm.

sample on a thin-layer chromatogram should prove fruitful. Beeswax (Fisher's white beeswax) was saponified by the method described by Mangold (53). One gram of beeswax was placed in 15 gram of solution containing one part potassium hydroxide, one part distilled water and four parts methyl alcohol. This mixture was refluxed for 12 hours. The unsaponifiable material was then removed by diluting with water, and extracting with hot hexane (17). The hexane extracts were washed twice with 30% aqueous methyl alcohol, followed by evaporation to give a concentrated solution.

Methylation - To the saponifiable fraction a 10% aqueous calcium chloride solution was added (16), precipitating the calcium salts, followed by a filtering and drying of the salt. The methyl esters were formed directly from the calcium salt (17) by refluxing with benzene methanol 1:1 (200 ml) and sufficient sulfuric acid to liberate the acids and give a 1% sulfuric acid solution. This mixture was refluxed for 5 hours. After the period of refluxing, water was added and the benzene layer separated (top layer). Removal of the solvent gave the esters, which were then redissolved in chloroform.

The unsaponifiable fraction, the methyl esters prepared from beeswax, the palmityl esters and the methyl esters (standard) were all compared with the beeswax sample on two thin-layer chromatograms (Fig. 16). From the chromatograms it

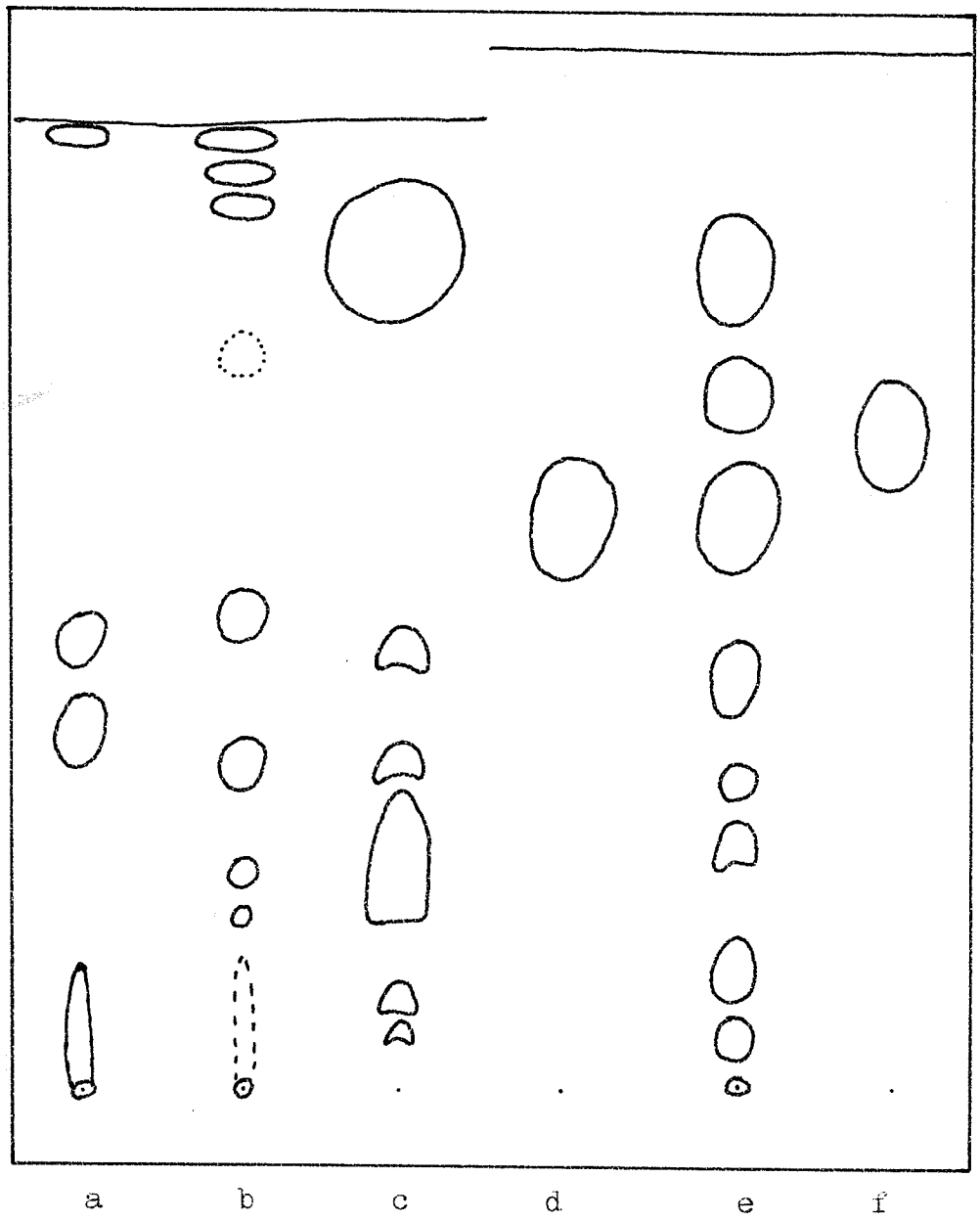


Figure 16 A thin-layer chromatographic separation of a) the unsaponifiable fraction of beeswax, b) beeswax and c) the prepared methyl esters. The developer was petroleum ether/ether/acetic acid = 84/15/1, the development time 50 min. and the development distance 13 cm.

On the other half of Fig. 16 is a separation of d) the methyl ester standard (C_{16} - C_{24} methyl esters), e) beeswax and f) the palmityl esters. The developer was petroleum ether/ether/acetic acid = 91/8/1, the development time 32 min. and the development distance 14 cm.

is seen that the methylation is not complete. Although the methyl esters are present to about 60% (determined by density comparison), the alcohol fractions are also present. The various spots shown have not all been identified. However, several spots disappeared in the unsaponifiable fraction, and these may have had ester properties.

Action of acetic anhydride with beeswax - To determine the free alcohol present in beeswax, we attempted to react beeswax with acetic anhydride in dry ether, refluxing the solution for 1 hour. Whether the reaction was quantitative or not was not known. However, from a thin-layer chromatogram that was made comparing the reacted beeswax with the original beeswax, one spot definitely disappeared. We were unable to tell whether the density of the ester spot increased or not (see Fig. 17).

Partition thin-layer chromatography

Silver-Nitrate impregnated plates - Silver nitrate impregnated plates have been successfully used to separate unsaturated from saturated lipids (62a, 62b). Unsaturated lipids form π-complexes with silver nitrate in the course of a chromatographic experiment and these complexes migrate more slowly than the saturated compounds of the same class. Since former workers have reported unsaturation to be present in beeswax, Marcusson and Böttger (36) in the hydrocarbons ($C_{30}H_{60}$) and Downing et al.

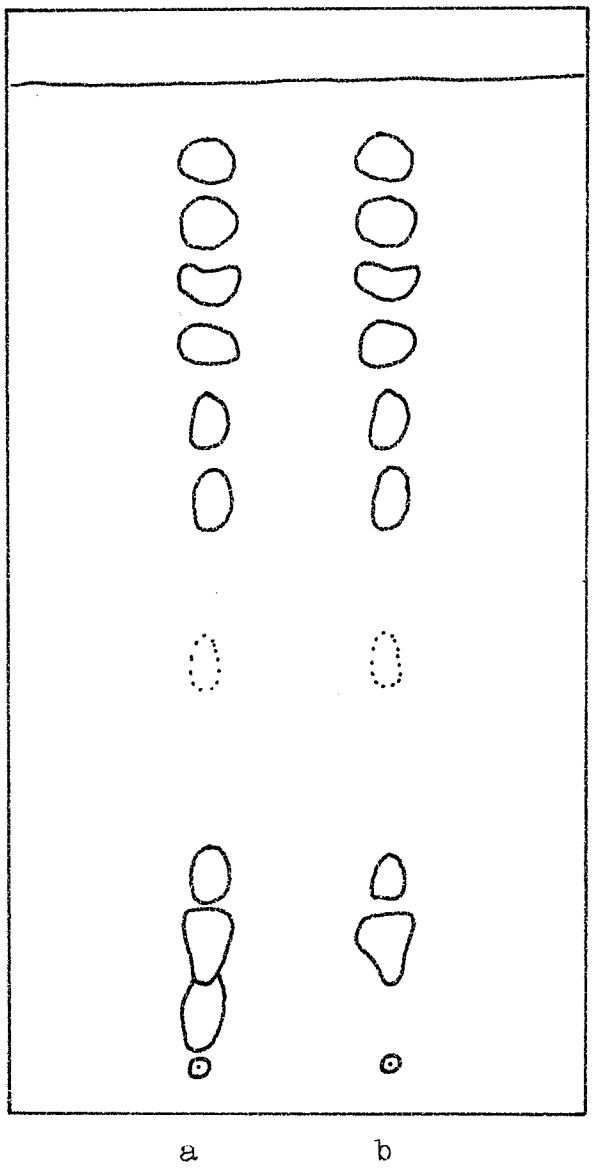


Figure 17 A chromatographic separation of beeswax (a) and acetylated beeswax (b) on a 20% silver nitrate impregnated Silica Gel G plate.
Developer: Petroleum ether/ether/acetic acid = 91/8/1
Developing time: 30 minutes
Developing distance: 13 cm

(16) in the acids (mono-unsaturated stearic acid:stearic acid in the ratio of 9:1), we thought that the appearance of more spots should give some indication of where the unsaturated components are located, i.e. in which class. Fig. 18 represents a chromatographic separation of beeswax on an 11% silver nitrate impregnated plate. Cetyl alcohol, the palmityl ester mixture, and a mixture of stearic and palmitic acid were chromatographed together with beeswax. This chromatogram resulted in a separation of stearic and palmitic acid. To test which spots corresponded to acids in beeswax, several chromatograms on 11 and 20% impregnated silver nitrate plates were made, and these were sprayed with a 0.0005 Molar pyrogallol solution in ethyl alcohol (54). See Fig. 19.

Paraffin impregnated plates - A separation of the acids has been achieved using paraffin impregnated plates (27). The acids are made visible by spraying the plates first with a silver nitrate solution followed by spraying with a pyrogallol solution. White spots on a dark background appear wherever acid spots are located. Figure 20 shows a partition thin-layer chromatogram of stearic acid, palmitic acid and the acid fraction from column chromatography purified by thin-layer chromatography. Partition thin-layer chromatography of the acids was achieved. However, it was not possible to identifiy the acids, since the separation looks more like tailing. The tailing arises because a homologous series of acids is present.

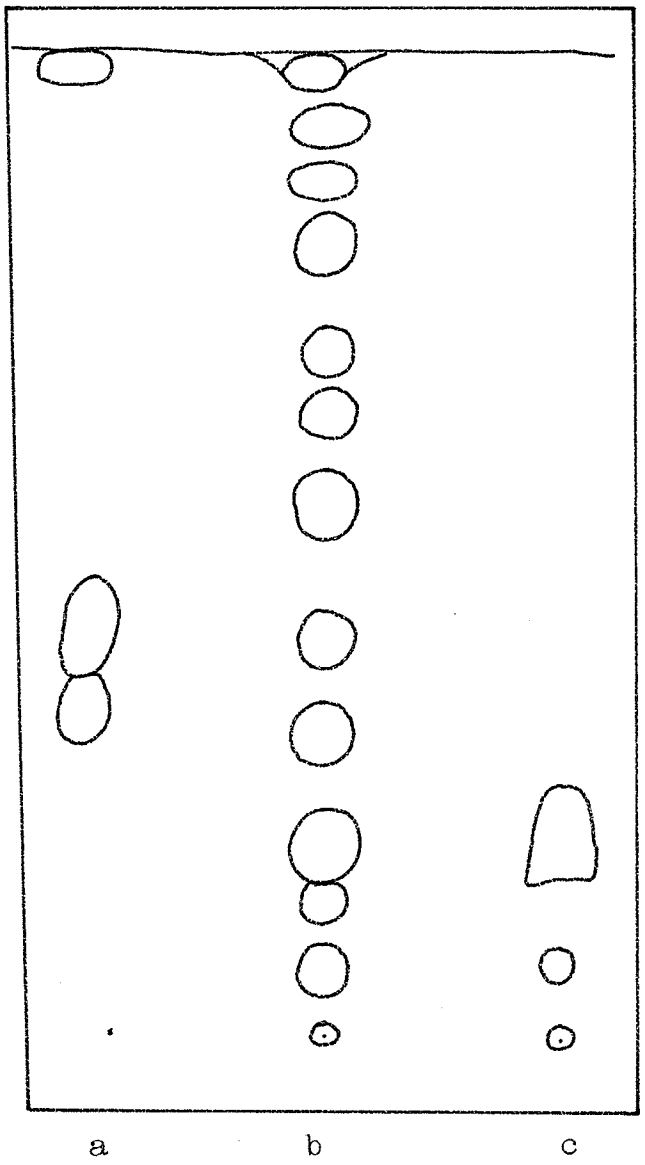


Figure 18 A chromatographic separation of a mixture of stearic acid, palmitic acid and the palmityl esters (a), of beeswax (b) and of cetyl alcohol (c) on an 11% silver nitrate impregnated Silica Gel G plate. The developer was petroleum ether/ether/acetic acid = 91/8/1. The development time was 49 minutes and the development distance 13 cm.

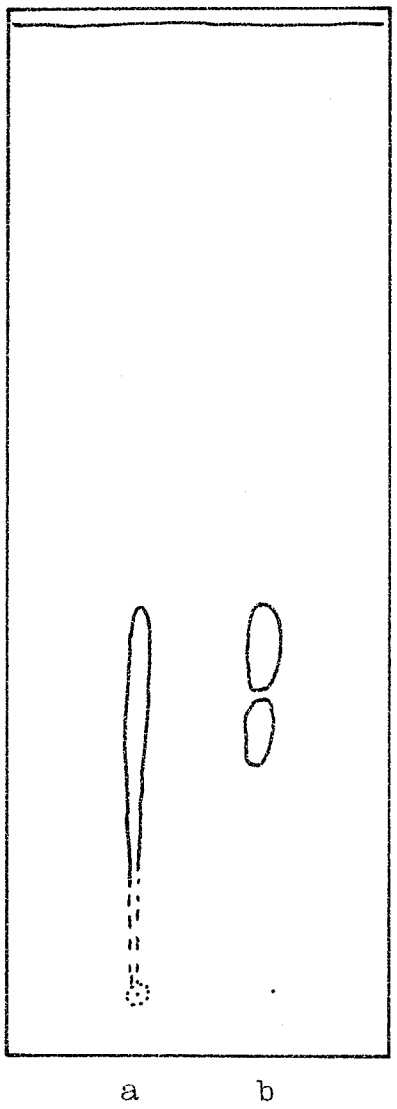


Figure 19 A chromatogram of a) beeswax and b) a mixture of stearic and palmitic acid on an 11% silver nitrate impregnated Silica Gel G plate.
To make the acids visible, the plate was sprayed with a pyrogallol solution.
Developer: Petroleum ether/ether/acetic acid = 91/8/1.
Development time: 37 minutes.

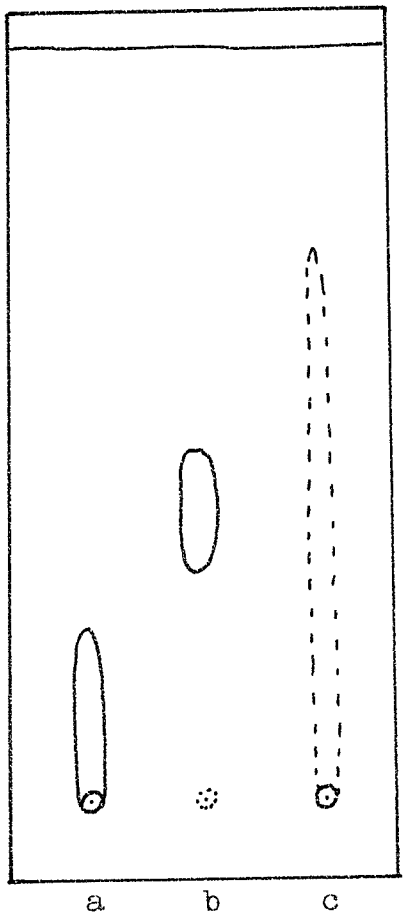


Figure 20 A partition thin-layer chromatogram of a) stearic acid, b) palmitic acid and c) the acid fraction from preparative column chromatography purified by thin-layer chromatography. The paraffin impregnated plate was developed with the eluate acetic acid/acetonitrile = 1/1 (v/v). The development distance was 10 cm and the development time took 66 minutes. The developed plates were sprayed with a silver nitrate solution followed by spraying with a pyrogallol solution to make the acids visible.

In all investigations with thin-layer chromatography one is very much at a loss if pure chemicals are not available for comparison. The method of comparison is by far the simplest and preferred method of identification. However, since such a comparison was not possible, a number of spots remained unidentified.

Estimation of esters by reaction with Hydroxylamine Hydrochloride

This method of analysis of fatty esters after they have been separated and purified by thin-layer chromatography has been employed by Vioque and Holman (61a). The procedure outlined by them was applied to the fractions obtained from preparative thin-layer chromatography of beeswax. See Table 1. The results of this determination of esters indicated that spots 2, 3, 4 and 5 were strongly absorbing whereas spots 8 and 9 absorbed weakly at 520 mμ in a Beckmann Spectrophotometer. Therefore, those spots where absorption occurred had ester properties. Spots 2, 3, 4 and 5 were concluded to be esters of long chain alcohols and long chain acids. Spots 8 and 9 could very likely be esters in which either the acid is a hydroxy acid or the alcohol a "diol", or both the acid and the alcohol contains a hydroxyl group.

Infrared Spectra - Infrared spectroscopic data are frequently used for identification of compounds. Since the infrared spectrum is characteristic of a compound, just as the melting point, boiling point, refractive index, X-ray powder photograph, etc., it may be used as a means of identifying the substance, as to its functional groups. The Infrared Spectrophotometer, Model 21, Perkin-Elmer Corporation, Norwalk, Connecticut, was used for this purpose.

The various thin-layer chromatographic fractions obtained from preparative thin-layer chromatography were dissolved in spectrograde carbon tetrachloride, Matheson, Coleman and Bell, Norwood, Cincinnati, Ohio, and the spectra were then obtained. This solvent was used because of solubility considerations and because it absorbs least in those ranges of the spectra under consideration.

The infrared spectrum of beeswax itself was very similar to the infrared spectrum of beeswax reported by Mainschein and Kenny (38). Figures 20a and 20b give the infrared spectra of all 10 spots (Table 1) separated by preparative thin-layer chromatography.

From the infrared data we concluded that the first spot, i.e. the spot with the largest R_f value, contained hydrobarbonx only. The second, third, fourth and fifth spots contained esters. Spot 6 contained compounds with strong acid and ester properties. Spots 8 and 9 contained acids, however, weak ester and alcohol properties were present in the spectra of both these spots. Spot 10 and the residue at the point of application, which is marked as the eleventh spot, contained compounds which had ester, acid and alcohol properties.

Conclusions Reached of the Chemical Nature of the Spots Beeswax was Resolved into by Preparative Thin-Layer Chromatography

The following will summarize the above findings of the spots into which beeswax was resolved by preparative thin-layer chromatography.

Spot 1 was confirmed to be hydrocarbons by the comparison with known compounds, the infrared spectrum and because this spot reappeared at the solvent front when the unsaponifiable fraction was chromatographed.

Spots 2 to 5 were shown to be esters by the hydroxylamine hydrochloride test, the infrared spectra, the disappearance of these spots after saponification, and partly by comparison with known compounds.

Spot 6 was shown to have compounds with strong ester and weak acid properties from the infrared spectrum. When the unsaponifiable fraction was chromatographed very little of the spot remained. The hydroxylamine hydrochloride test proved negative.

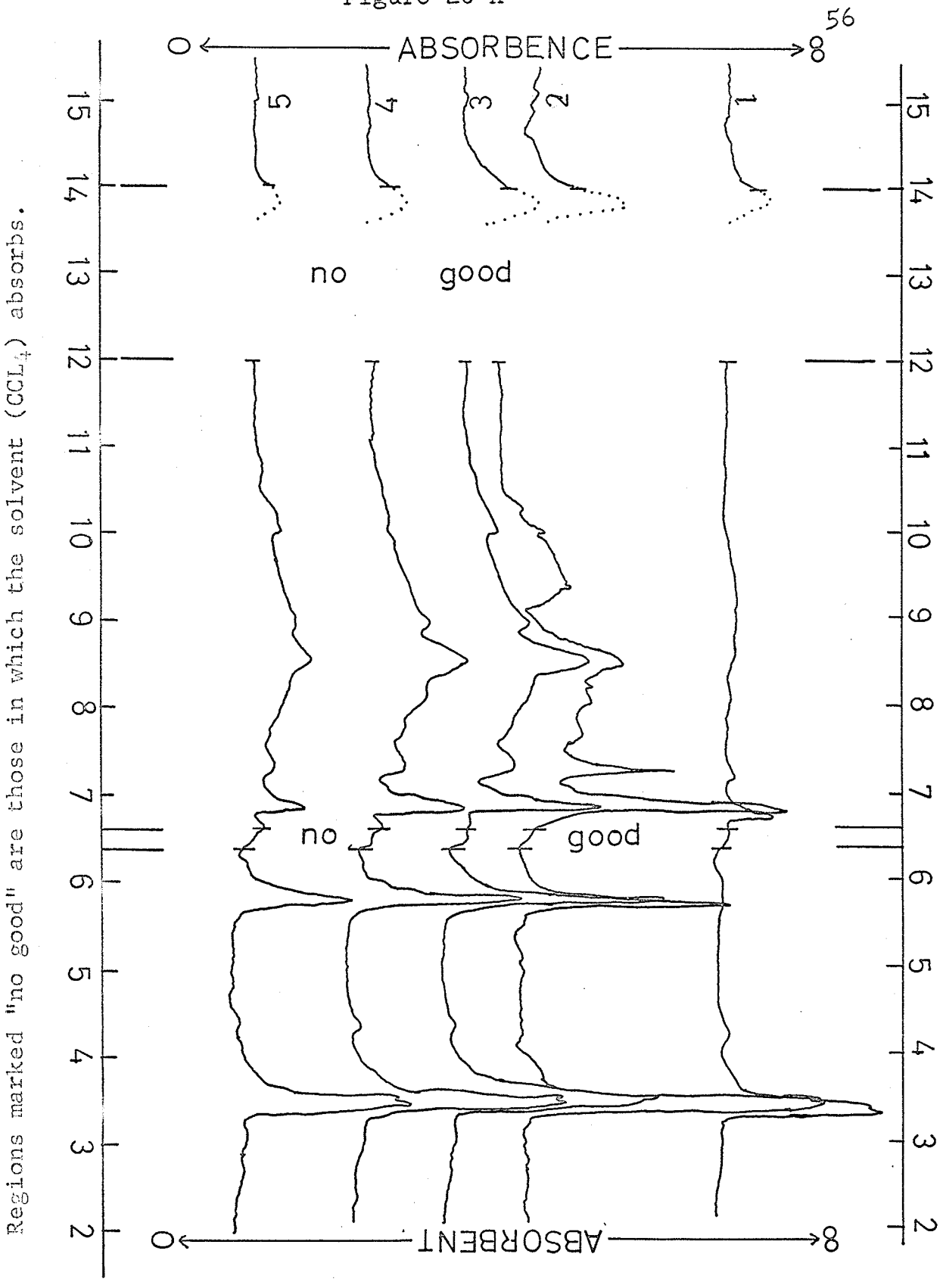
Spot 7 was shown to have strong acid and equally strong ester properties from the infrared spectrum. In the chromatographic separation of the unsaponifiable fraction, the spot partly disappeared. The hydroxylamine hydrochloride test was again negative.

Spots 8 and 9 showed weak ester and strong acid properties in their infrared spectra, and they gave a positive hydroxylamine hydrochloride test, indicating the presence of esters. Spot 9 had the same R_f value as stearic and palmitic acid. This spot was also present as a minor component in the unsaponifiable fraction of beeswax, indicating the presence of alcohols.

Spot 10 was shown to be alcohols from the acetic anhydride test. It contained acid, alcohol and ester properties as seen from the infrared spectrum. This spot had a comparable R_f value to cetyl alcohol, and in the unsaponifiable fraction it was present to a much larger concentration than in the original beeswax.

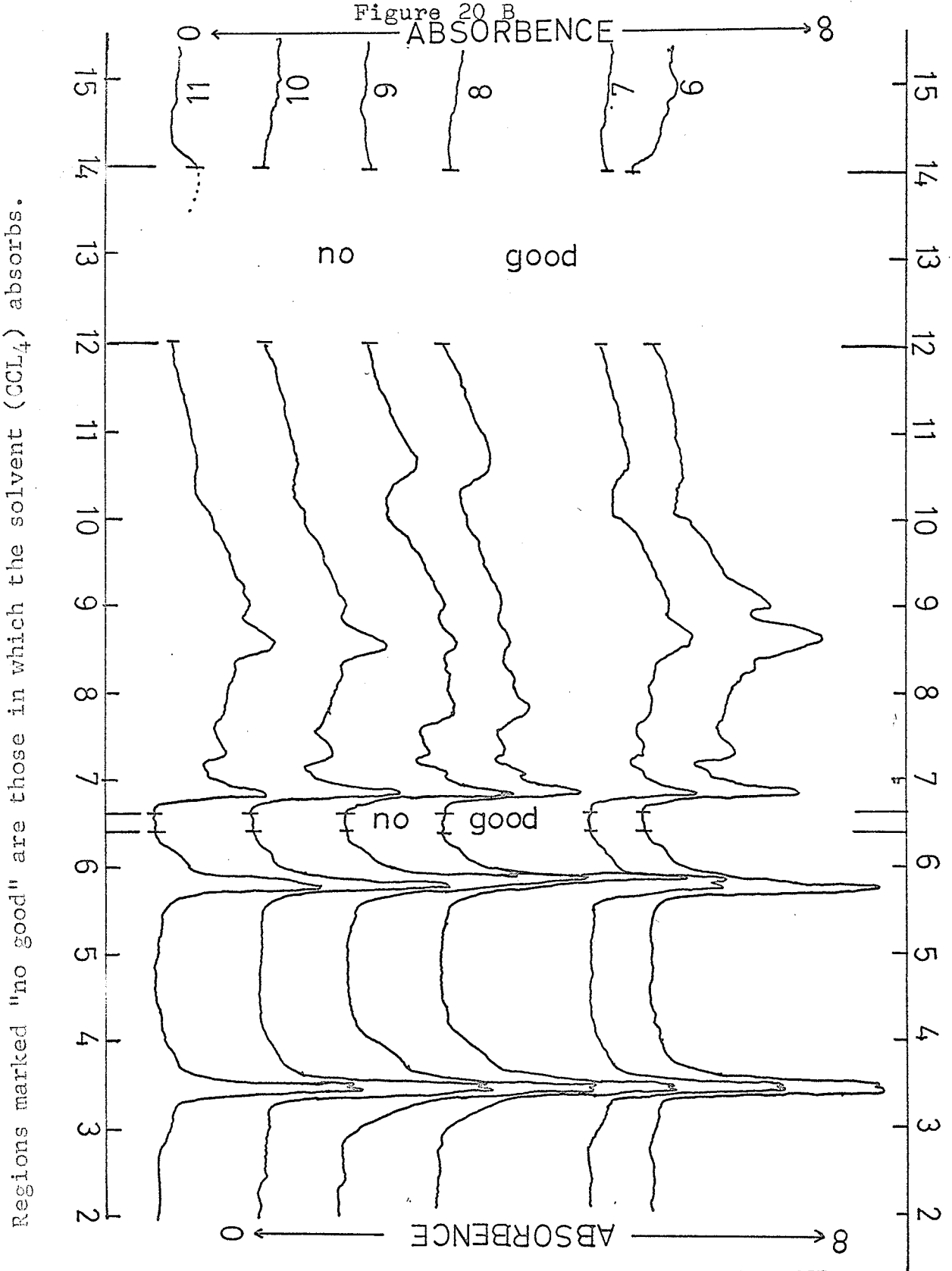
The residue at the point of application was shown to have alcohol, acid and ester properties as concluded from its infrared spectrum. The spot was also present in the chromatographic separation of the unsaponifiable beeswax fraction, hence confirmaing alcohol properties.

Figure 20 A



INFRARED SPECTRA OF LAYERS 1 TO 5 FROM A THIN-LAYER CHROMATOGRAM OF BEESWAX

Figure 20 B



Regions marked "no good" are those in which the solvent (CCL_4) absorbs.

INFRARED SPECTRA OF LAYERS 6 TO 11 FROM A THIN-LAYER CHROMATOGRAM OF BEESWAX

GAS-LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO THE ANALYSIS OF BEESWAX

Apparatus

The gas-liquid chromatograph used in our investigations was the Dynatronic Chrom Alyzer - 100, available from Dynatronic Instruments Corp., Electronics Division, Labline Inc., 3070 W. Grand Ave., Chicago, Ill. This instrument was equipped with a thermal conductivity detector, a power supply ("12 volts, D.C., solid state, 0.02% regulation, 400 milliamps."), a recorder ("Servo-type, null-balance potentiometric recorder for accurate, rectilinear, stripchart recording 1, 5, 10, 50 millivolts, 0.5, 1, 5, 10 volts full scale, Zener diode reference system, 2 chart speeds, 1 in. per minute and 16 in. per hour"), a flowmeter (twin rotameters - 6 in. scale - with needle valve calibration 0-100cc per minute) and a soap bubble type flowmeter (10 ml).

Carrier gas

Helium was used as carrier gas for all our work. The helium was available from the local Welders Supplies Ltd.

Support

Firebrick, non acid washed, 30/60 mesh, was obtained from Fisher Scientific Co., Ltd. This support was washed with concentrated hydrochloric acid, followed by several washings with distilled

water. Better results were obtained with acid washed firebrick. Furthermore, it was made sure that the smaller firebrick particles and the dust were removed by the washings. The support was dried at 110°C for several hours.

Stationary phase

Several stationary phases were used, including Apiezon L grease (Fisher Scientific Co., Ltd., Winnipeg), Silicone Stopcock Grease, (Dow Corning Corporation, Midland, Mich.), and Silicone Gum Rubber, SE-30 ("Methyl") (Wilkins Instrument and Research Inc., Box 313, Walnut Creek, California). The maximum temperature tolerated by these stationary phases is 300°C for Apiezon L grease, 350°C for Silicone grease, and 400°C for Silicone SE-30.

Silicone grease was purified as suggested by Cropper and Heywood (9). The grease was precipitated from ethyl acetate with ethyl alcohol, washed several times with ethyl alcohol, finally dried at 100°C and kept in a vacuum desiccator.

Preparation of the packing material

The stationary phase was dissolved in ether. The acid washed support was slowly added to the ether solution, while stirring constantly to obtain a uniform coating. The ether was evaporated by heating lightly as the stirring was continued, until no more ether appeared over the firebrick. Ether was completely

removed by heating the prepared packing material at 100°C for 1 to 2 hours.

Column

In all our work we used copper columns 3/16 in. o.d. and 1/8 in. i.d.. This copper refrigeration tubing (Noranda Copper and Brass, Ltd.) was available from a local firm.

Packing the column

The copper column to be packed was straightened, a glass wool plug placed in one end, and the prepared packing material was slowly added. The column was tapped continuously during the packing process. After the column was filled in this manner, it was placed overnight in a vibrator, made from a doorbell, to get a uniform packing, and to remove air pockets. A glass plug was then placed in the other end of the column and the latter was bent in a spiral to fit into the gas chromatograph.

Conditioning the column

All columns were conditioned by heating for an extended period of time at an elevated temperature, anywhere from 20 to 50°C higher than the temperature we expected to work at. Cason and Miller (9) emphasized the importance of proper conditioning of the column prior to use, in order that the volatile impurities be driven off. If this is not done, a signal corresponding to a continuous bleeding of the stationary phase will be noticed, resulting in a fluctuating base line.

Injection of Sample

All samples injected into the column were solutions in hexane, ether, chloroform or carbon tetrachloride. Hamilton syringes of 1, 10 or 50 microliters were used. The 10 microliter syringe with the 2 in. fixed needle was found to be most suitable for most of the work.

Reading of the retention times

The retention time is defined as the time for emergence of the peak maximum after injection of the sample. These were the values recorded for the various constituents from the gas chromatograph chart.

Quantitative Gas-Liquid Chromatography

In our work it was nearly impossible to prepare known concentrations of the unknowns collected from the thin-layer chromatograms by elution. Therefore, we measured the relative concentration by weight of each component by taking the ratios of the areas. This was justified since for a thermal conductivity cell, the areas under the curves are proportional to the weights, or in other words there is equal mass response (8). The areas under the curves of the chromatograph were measured with a compensating polar planimeter (Keuffel and Esser Co., Hoboken, N. J.).

Calculation of column efficiency

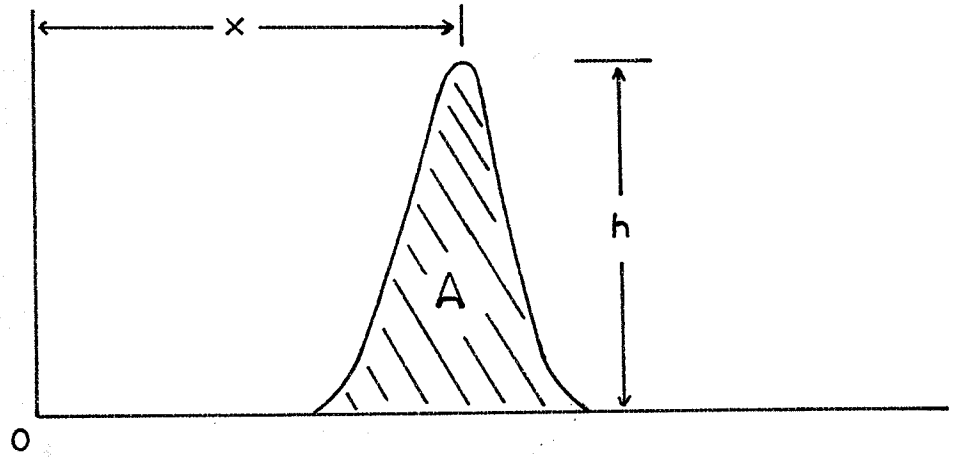
The "efficiency of gas chromatography columns is expressed most commonly as the number of theoretical plates N, which a column exhibits towards a specific solute under defined conditions of temperature, carrier flow rate, and sample size" (12). The average height equivalent to a theoretical plate, HETP, is then calculated knowing the column length (L).

$$H\ E\ T\ P = \frac{L}{N}$$

Where N is :

$$N = 2\pi\left[\frac{h\ (max)\ X}{A}\right]^2$$

In the diagram given below, the various parameters are represented graphically (12).



RESULTS WITH GAS-LIQUID CHROMATOGRAPHY

Resolution of the Hydrocarbon Homologues by Gas-Liquid Chromatography

High molecular weight hydrocarbons have been separated by gas-chromatography on packed columns as well as on coated capillary columns. Reference to such separations can be found in many of the recent books of gas chromatography such as those by Heftmann(23), Dal Nogare and Juvet (12), Burchfield and Storrs (4) and in the Gas Chromatography Abstracts (19). Particularly valuable were the conditions of separation of the hydrocarbons of beeswax reported by Downing et al. (16) and White et al. (66).

The hydrocarbon standards mentioned earlier were reported as 99% pure.

The hydrocarbons of beeswax were separated by thin-layer chromatography and these samples were analyzed by gas-liquid chromatography. In Table 2 the retention times of three different series of runs are reported with the weight percent of each component calculated from the areas under the curves. The weight percent values which are given are average values from a number of chromatograms.

Plots of the logarithm of the retention time vs. the carbon number is shown in Fig. 21 and Fig. 22. The former is a plot of the hydrocarbon standards, and the latter of the hydrocarbons of beeswax. From Fig. 22 we deduced that all hydrocarbons were saturated, since all the points fell on a straight line (14). A typical chromatograph of the beeswax hydrocarbons is given in Fig. 23.

Separation I : Chromatogram of the hydrocarbons of Fisher's white beeswax.
Purity : separated by thin-layer chromatography.
Column : 5 ft. copper column 1/8 in. i.d.
Stationary phase : 7% Apiezon L grease on acid washed firebrick, 30/60 mesh.
Inlet pressure : 18 psig.
Exit flow rate : 64.9 ml/min.
Column temperature : 306°C
Filament current : 250 milliamps
HETP : 3.24 x 10-3

Separation II : The column is the same as in Separation I except that :
Column temperature : 293°C
Inlet pressure : 25 psig.
Flow rate : 128 ml/min.
Filament current : 250 milliamps

Separation III: Chromatogram of the hydrocarbons of scale beeswax (Italian strain - apis mellifera).
Purity : separated by thin-layer chromatography.
Column : 4 ft. copper column 1/8 i.d.
Stationary phase : 5% Silicon SE-30 on acid washed firebrick, 30/60 mesh.
Inlet pressure : 20 psig.
Exit flow rate : 23.4 ml/min.
Column temperature : 299°C
Filament Current : 250 milliamps
HETP : 6.23 x 10-3

TABLE 2

HYDROCARBONS OF BEESWAX

n-paraffin	Separation I		Separation II		Separation III	
	t_R (min.)	% comp. by wt.	t_R (min.)	% comp. by wt.	t_R (min.)	% comp. by wt.
C_{17}	0.35	trace				
C_{18}	0.42	0.31	0.32	0.29		
C_{19}	0.58	0.58	0.48	0.74	0.59	0.15
C_{20}	0.73	0.34	0.60	0.50	0.69	0.05
C_{21}	0.94	1.03	0.81	1.30	0.81	0.70
C_{22}	1.20	0.69	1.07	0.79	0.99	0.35
C_{23}	1.58	4.36	1.41	4.73	1.18	4.18
C_{24}	2.05	1.31	1.83	1.47	1.46	1.04
C_{25}	2.66	10.62	2.44	11.71	1.81	10.80
C_{26}	3.45	2.21	3.17	2.12	2.24	2.09
C_{27}	4.50	29.89	4.24	32.43	2.82	27.53
C_{28}	5.75	1.81	5.48	2.72	3.46	2.44
C_{29}	7.47	16.50	7.28	16.16	4.36	16.38
C_{30}	9.60	1.90	9.49	1.59	5.42	2.44
C_{31}	12.40	15.93	12.50	14.41	6.84	15.70
C_{32}	16.00	1.79	16.30	1.46	8.45	1.74
C_{33}	19.40	8.86	21.05	7.25	10.20	7.32
C_{34}	26.80	1.64	28.80	0.85	13.20	1.04
C_{35}	35.00	trace	36.3	1.30	16.20	4.88
C_{36}					20.60	1.04

Figure 21 A PLOT OF THE LOG OF THE RETENTION TIME VS. THE CARBON NUMBER OF THE HYDROCARBON STANDARDS.

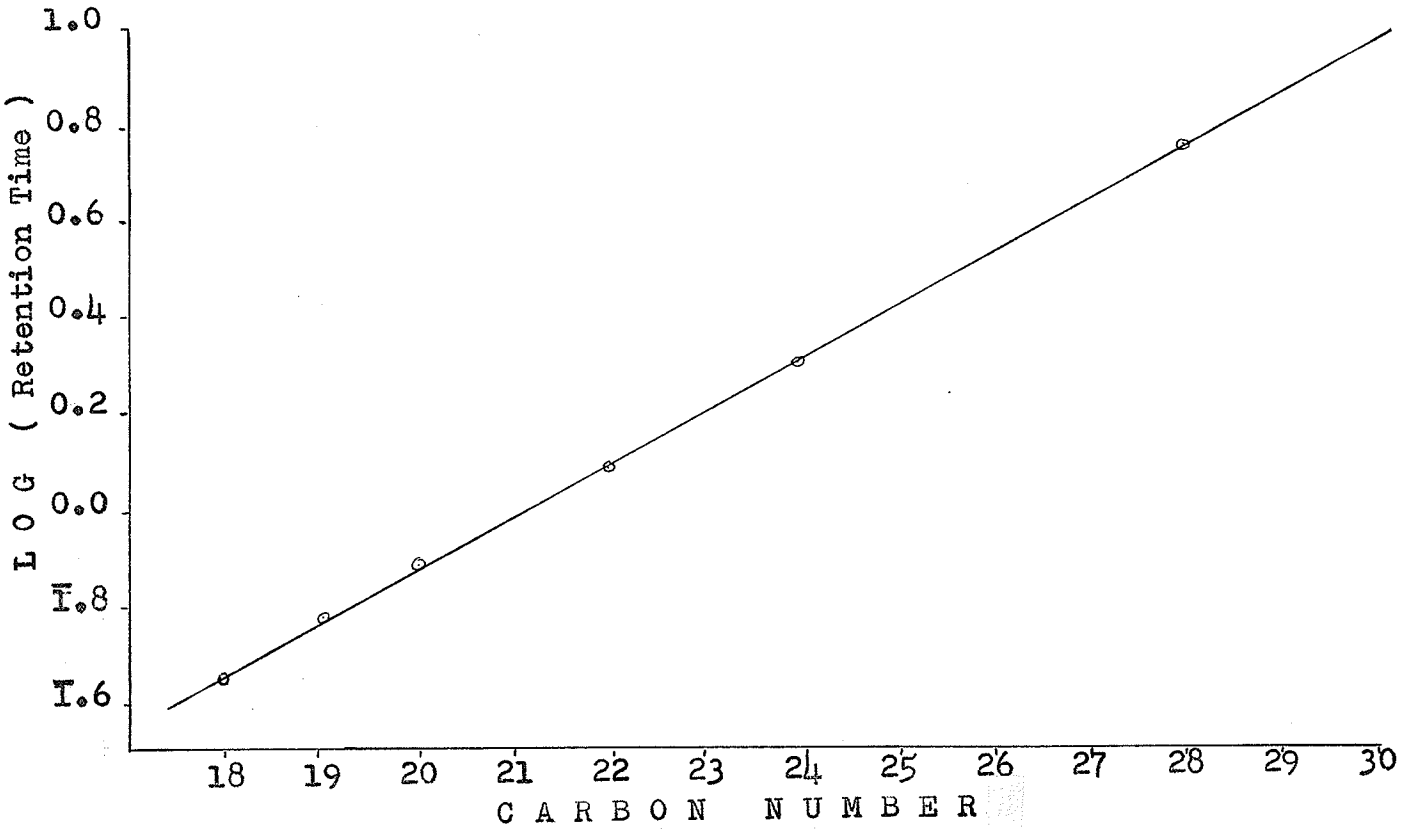
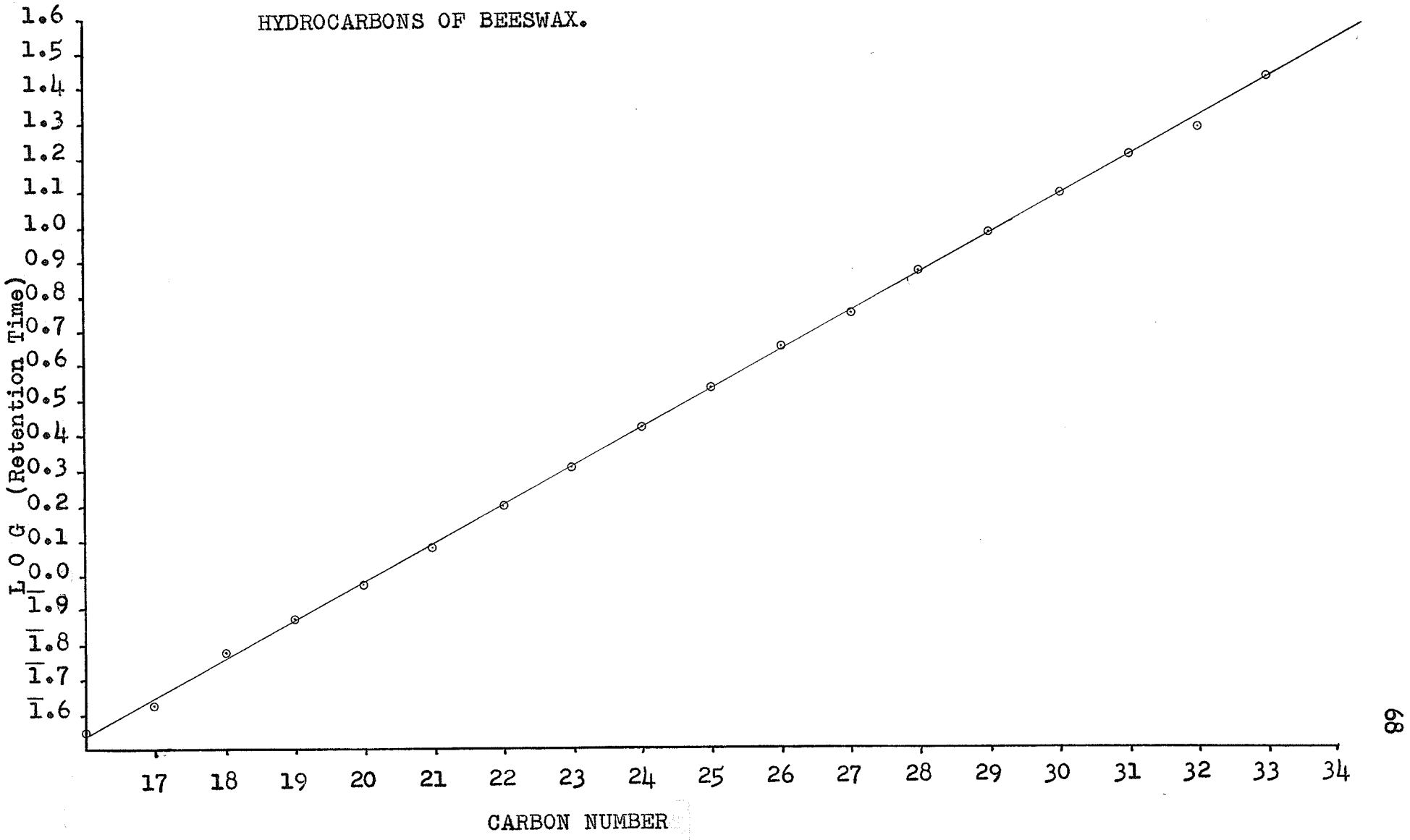


Figure 22 A PLOT OF THE LOG OF THE RETENTION TIME VS. THE CARBON NUMBER OF THE HYDROCARBONS OF BEESWAX.



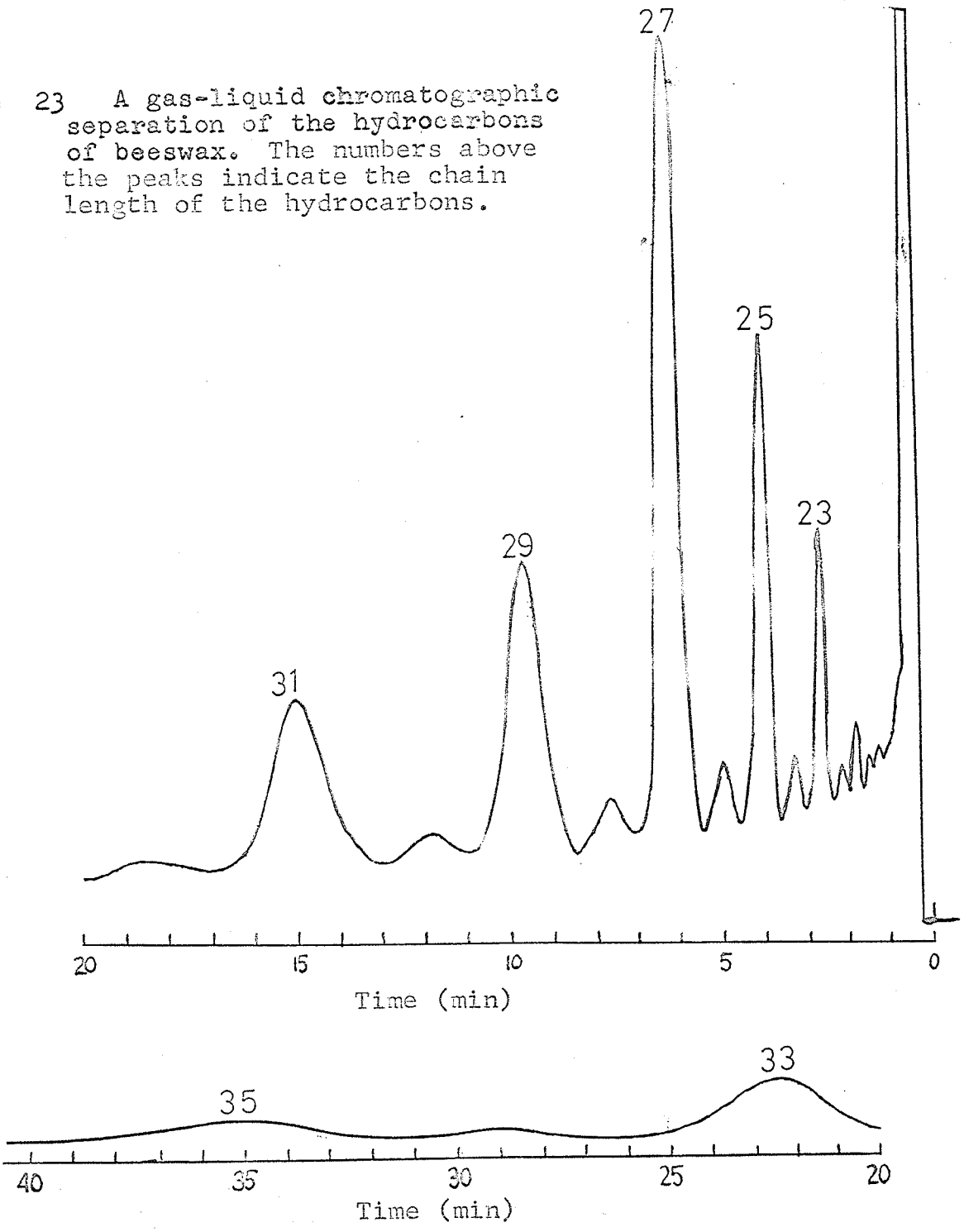


Figure 23 A gas-liquid chromatographic separation of the hydrocarbons of beeswax. The numbers above the peaks indicate the chain length of the hydrocarbons.

Resolution of Methyl Esters of Beeswax

The methyl esters prepared from the calcium salts of the fatty acids of beeswax (see page 46) were purified by thin-layer chromatography followed by elution with a solution of chloroform-acetone. These purified samples were injected into the gas-chromatograph.

The methyl ester standards used were obtained from Dr. Goldwater at The Heart Institute, Bethesda, Maryland. These included the methyl esters of myristic, palmitic, stearic, arachidic, behenic and lignoceric acids. Mixtures of these methyl esters are also available from The Heart Institute as reference standards in gas-liquid chromatography. Mixture F (21a), one of these mixtures, has the following composition by weight given in column A below. We have determined its percent composition by weight from several chromatograms (column B below). This was in good agreement with compositions reported by several other workers (21a).

	A % by wt.	B % by wt.
Methyl myristate	2.52	2.53
Methyl palmitate	4.18	6.06
Methyl stearate	7.31	7.07
Methyl arachidate	13.64	14.144
Methyl behenate	25.35	25.25
Methyl lignocerate	47.00	44.95
	100.00	100.00

In Table 3 the retention times and the percent composition by weight of the methyl esters of beeswax are

given. The retention times of the standards are given in parentheses. Fig. 24 gives a typical chromatogram of the methyl esters of beeswax. Again a plot of the log of the retention times vs. the carbon numbers of the methyl esters gives a straight line which indicates that the compounds are all methyl esters of the saturated fatty acid series (Fig. 24a).

Separation I : Chromatogram of the methyl esters of the fatty acids of beeswax.
Purity : separated by thin-layer chromatography
Column : 3 ft. copper column 1/8 cm. i.d.
Stationary phase : 5% Silicon SE-30 on acid washed firebrick, 30/60 mesh.
Inlet pressure : 10 psig.
Exit flow rate : 12.5 ml/min.
Column temperature : 275°C
Filament current : 250 milliamps
HETP : 4.23 x 10-2

Separation II : Similar to separation I, only :
Column : 4 ft. copper column.
Inlet pressure : 7 psig.
Exit flow rate : 12.4 ml/min.
Column temperature : 273.2°C
HETP : 1.72 x 10-2

TABLE 3

METHYL ESTERS OF THE FATTY ACIDS OF BEESWAX SEPARATED BY GAS-LIQUID CHROMATOGRAPHY

Methyl esters	Separation I			Separation II		
	t_R (min.)		% comp. by wt.	t_R (min.)		% comp. by wt.
C_{14}	0.64	(0.64)	0.28	1.37	(1.21)	0.77
C_{16}	0.89	(0.88)	40.99	1.74	(1.70)	41.62
C_{18}	1.29	(1.27)	51.03	2,64	(2.56)	52.58
C_{20}	1.94	(1.89)	3,67	4.02	(3.99)	2.78
C_{22}	3.03	(2.92)	1.16	6.50	(6.35)	1.14
C_{23}	3.60		trace			
C_{24}	4.34	(4.55)	1.27	10.30	(10.2)	1.08
C_{26}	7.45		0.99	17.10		1.28
C_{28}	12.03		0.61			

The numbers in brackets refer to the retention times of the methyl ester standards.

Figure 24. A gas-liquid chromatographic separation of the methyl esters of beeswax.

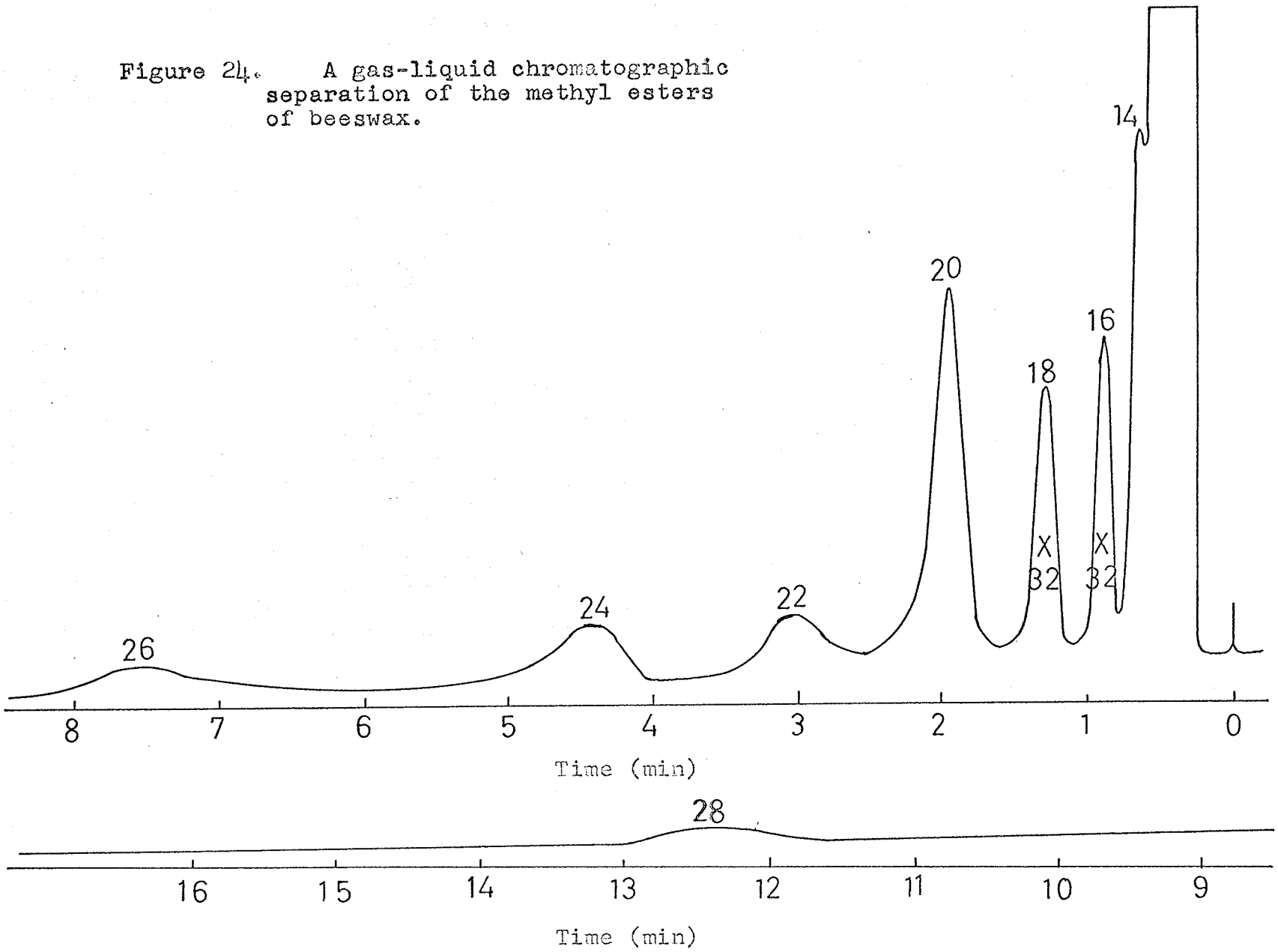
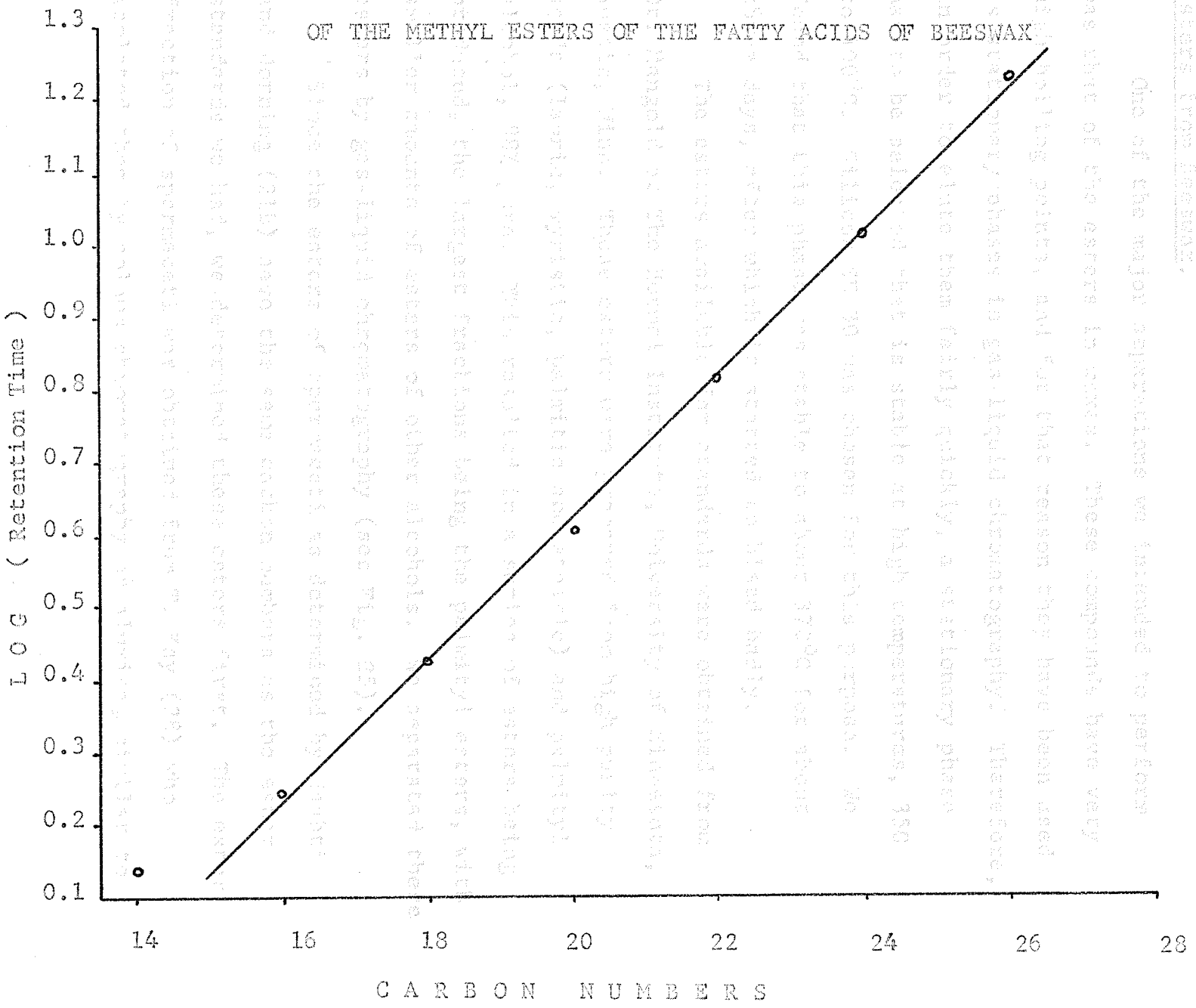


Figure 24a A PLOT OF THE LOG OF THE RETENTION TIME VS. THE CARBON NUMBER OF THE METHYL ESTERS OF THE FATTY ACIDS OF BEESWAX



Resolution of Palmityl Esters, Esters from Spermaceti and Esters from Beeswax.

One of the major separations we intended to perform was that of the esters in waxes. These compounds have very high boiling points, and for that reason they have been used as stationary phases in gas-liquid chromatography. Therefore, in order to elute them fairly quickly, a stationary phase has to be selected that is stable at high temperatures, 350 to 400°C. Silicon SE-30 was chosen for this purpose. We found that this phase was stable to about 370°C for about three days, after which it started to bleed badly.

The esters available for standards were obtained from Dr. Mangold at The Hormel Institute, University of Minnesota, Austin, Minn. These esters were prepared from high purity acids (lauric, myristic, palmitic and stearic) and palmityl alcohol, 98% pure. This resulted in a series of esters being produced, the largest fractions being the palmityl esters, with smaller amounts of esters of other alcohols. We separated these esters by gas-liquid chromatography (see Fig. 25).

Since the esters of spermaceti as determined by Haahti and Horning (21b) have the same carbon numbers as the ester standards we had, we determined these esters first. The ester fraction of spermaceti was obtained from T. Kay (28) who isolated them by column chromatography on alumina, similar to

our work on beeswax (page 35). The composition by carbon number of these esters is given in Table 4. In Fig. 26 a typical chromatogram of the esters of spermaceti is given, and in Fig. 27 a plot is shown of the logarithm of the retention time of these esters vs. their carbon number.

McCarthy and Kuksis (31) reported the separation of triglycerides up to 60 carbon atoms using a short column (18 in. x 1/8 in. in diameter) and a flame ionization detector. We were unable to achieve an effective separation of the esters using a ten and a sixteen inch column. However, we were able to obtain a separation with a 3 ft. column coated with 5% Silicon SE-30, at a temperature of 370°C. The column started to bleed excessively after the second day operating at these temperatures. The separation obtained from spots 2, 3, and 4 is shown in Figs. 28, 29 and 30. In Table 5 their retention times and percentage composition by weight are given. Figs. 31, 32 and 33 give plots of the retention times of beeswax esters vs. their carbon numbers.

From these separations it was further concluded that spots 2, 3 and 4 are esters. According to this separation partition chromatography of the esters was occurring on the thin-layer chromatograms using the developer petroleum ether, ether and acetic acid. A gas chromatographic separation of fraction 2

collected from an alumina column is shown in Fig. 34. The retention times and % compositions of these components are included in Table 5. This shows that the esters were partially separated by thin-layer chromatography according to chain length. Now since the esters were separated by partition chromatography, the shorter chain esters migrate further to the top on the chromatogram than the longer esters. It seemed surprising that we obtained such discrete ester spots on a thin-layer chromatogram, which proved to be only a partial separation of esters.

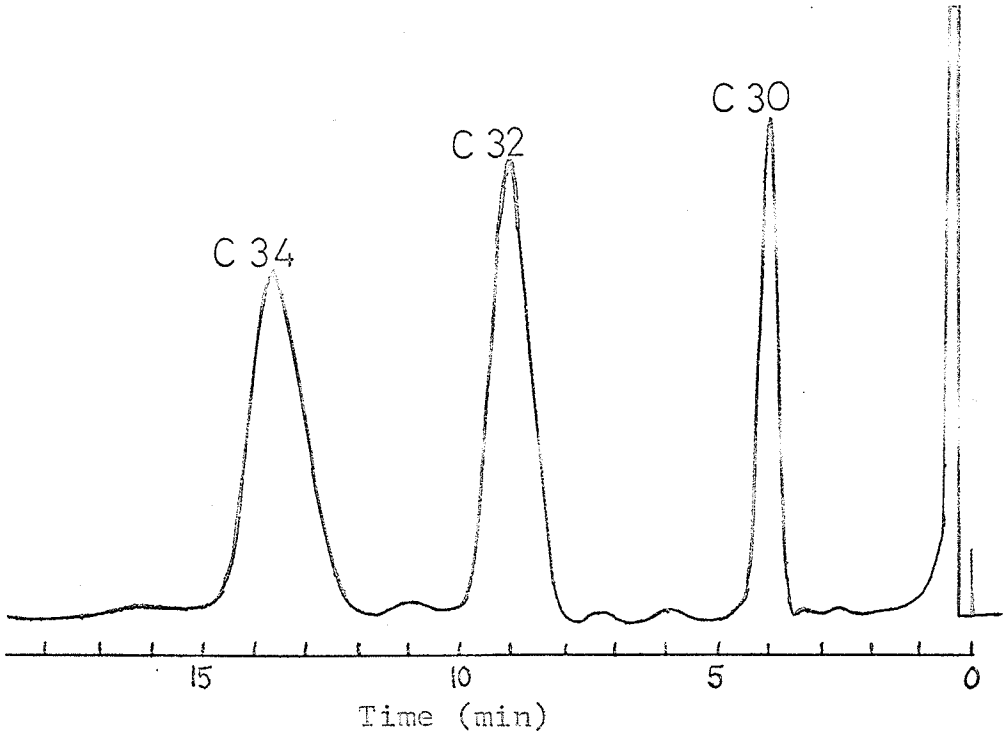


Figure 25 A gas-liquid chromatographic separation of a mixture of palmityl myristate, palmityl palmitate and palmityl stearate. These esters were 98 % pure.

Separation I : Chromatogram of the esters of spermaceti.
Purity : Column chromatography.
Column : 4 ft. copper column 1/8 in. i.d.
Stationary phase : 5% Silicon SE-30 on acid washed firebrick, 30/60 mesh.
Inlet pressure : 10 psig.
Exit flow rate : 16.3 ml/min.
Column temperature : 291.2°C
Filament current : 250 milliamps
HETP : 7.16 x 10-3

Separation II : Chromatogram of the esters of spermaceti.
The conditions were very similar to those above.
Column : 3 ft. copper column.
Stationary phase : same percentage and the same support.
Inlet pressure : 10 psig.
Exit flow rate : 22.4 ml/min.
Column temperature : 291°C
Filament current : 250 milliamps
HETP : 1.1 x 10-4

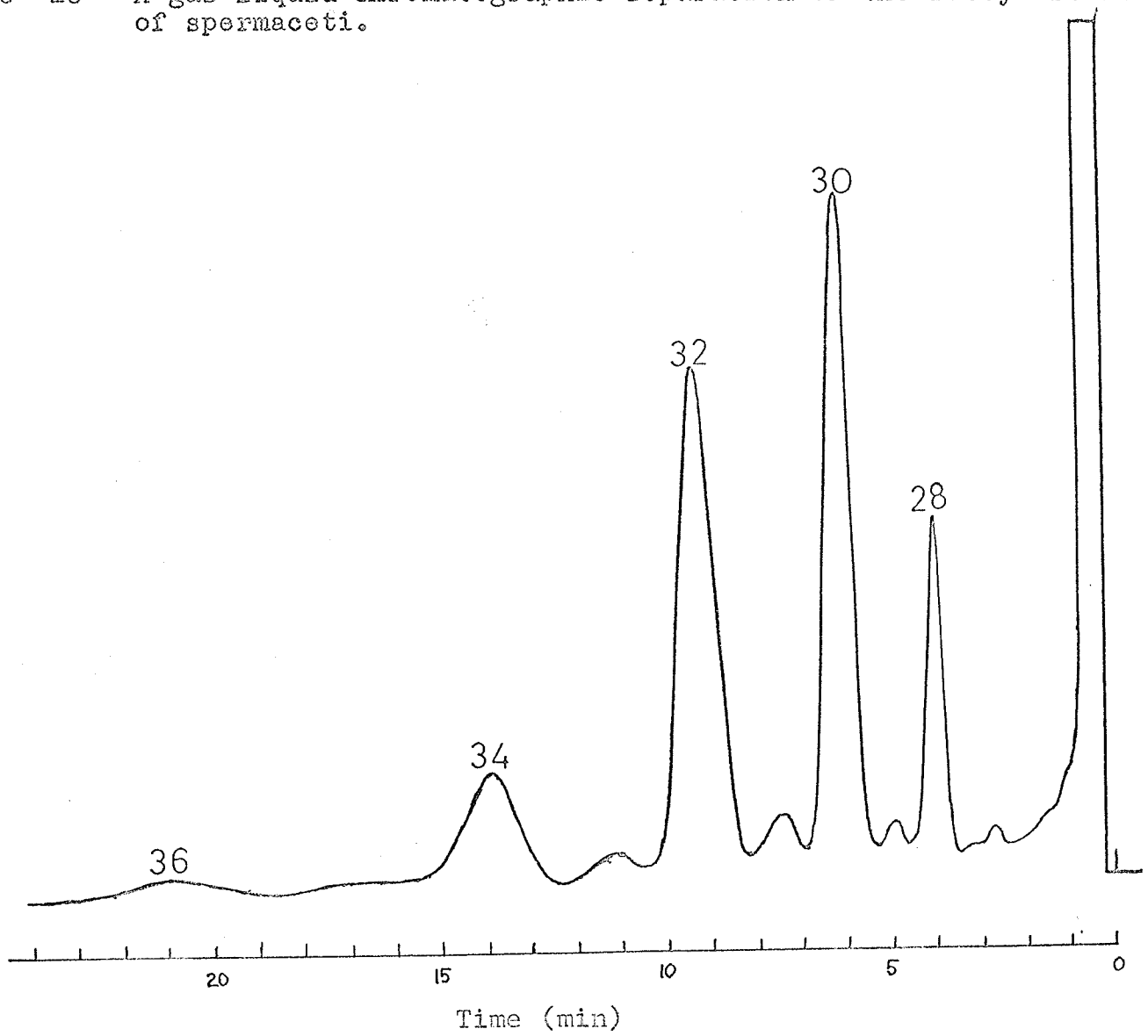
Separation III : Chromatogram of the esters of spermaceti.
Column, stationary phase, inlet pressure and detector current are the same as in separation II.
Exit flow rate : 12.5 ml/min.
Column temperature : 279°C
HETP : 8.45 x 10-2

TABLE 4

SEPARATION OF SPERMACETI ESTERS BY GAS-LIQUID CHROMATOGRAPHY

fatty esters	Separation I		Separation II		Separation III	
	t_R (min.)	% comp. by wt.	t_R (min.)	% comp. by wt.	t_R (min.)	% comp. by wt.
C_{23}					2.12	trace
C_{24}	1.09	trace	11.31	trace	2.88	trace
C_{25}	1.80	trace			3.82	trace
C_{26}	2.70	0.77	1.84	0.52	4.57	0.48
C_{27}	3.25	0.20	2.26	0.08	5.71	0.15
C_{28}	4.02(3.97)	11.51	2.88(2.83)	11.10	7.32	10.35
C_{29}	4.98	1.20	3.46	1.29	9.10	0.67
C_{30}	6.33(6.04)	29.61	4.49(4.33)	26.08	12.13	28.55
C_{31}	7.65	2.84	5.40	2.84	14.58	1.92
C_{32}	9.90(9.22)	38.57	6.93(6.60)	37.69	19.55	37.94
C_{33}	11.54	2.97	8.13	2.07	22.76	2.40
C_{34}	14.39(14.47)	10.75	10.28(10.04)	12.65	29.22	12.27
C_{35}	17.17	0.43	12.28	1.03	34.58	1.06
C_{36}	21.29	2.41	16.19	3.10	43.15	2.88
C_{37}	25.79	trace	22.71	trace	53.40	trace
C_{38}	31.18	trace	26.54	1.03		0.96
C_{39}			32.83	0.52		trace
C_{40}						0.27

Figure 26 A gas-liquid chromatographic separation of the fatty esters of spermaceti.



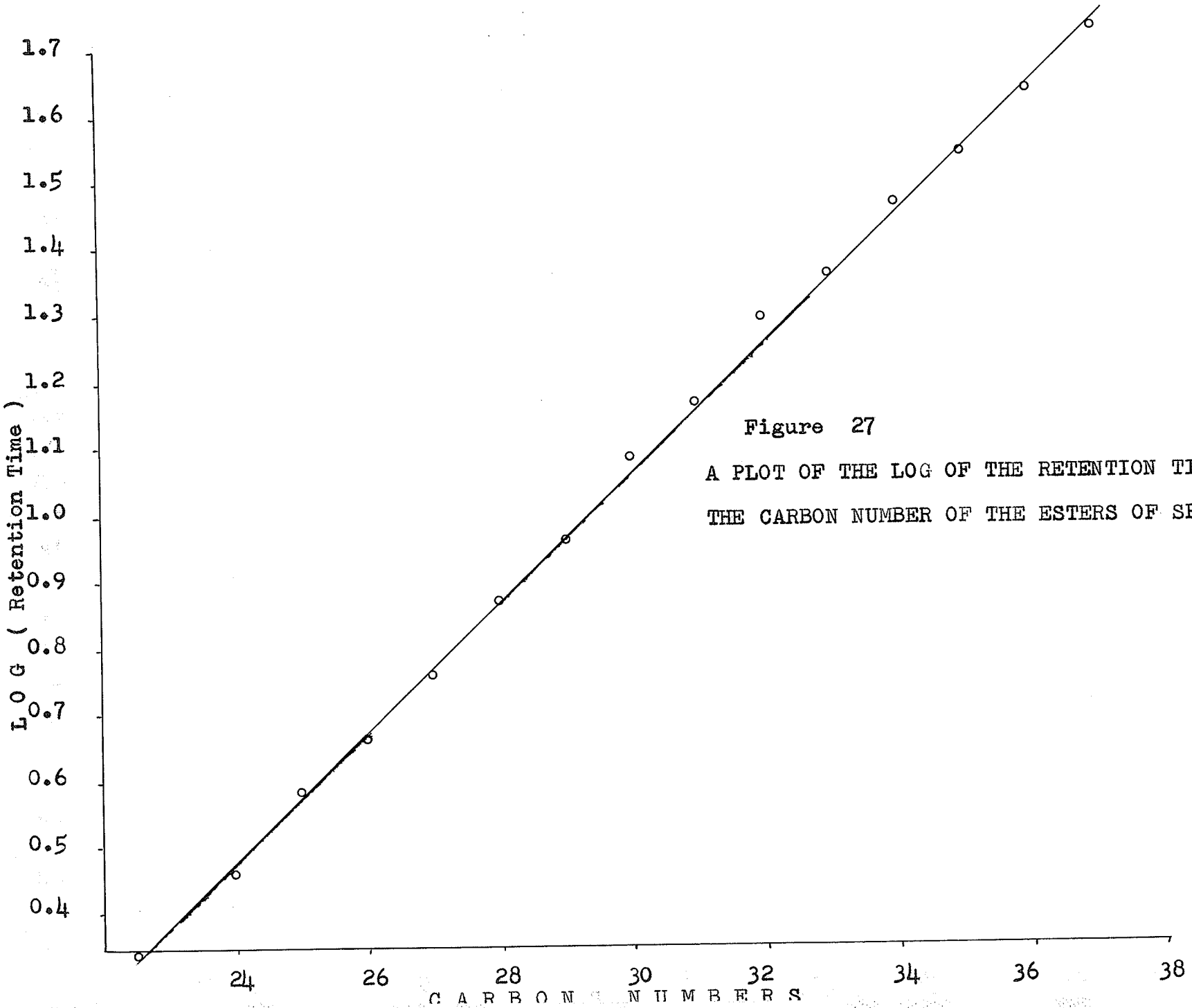
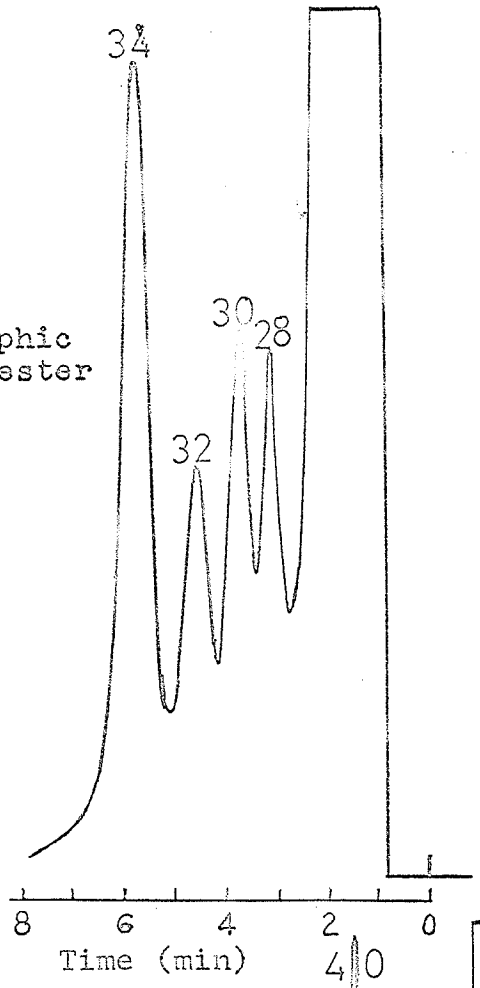


Figure 27

A PLOT OF THE LOG OF THE RETENTION TIME VS.

THE CARBON NUMBER OF THE ESTERS OF SPERMACETI.



A gas-liquid chromatographic separation of the palmityl ester standards.

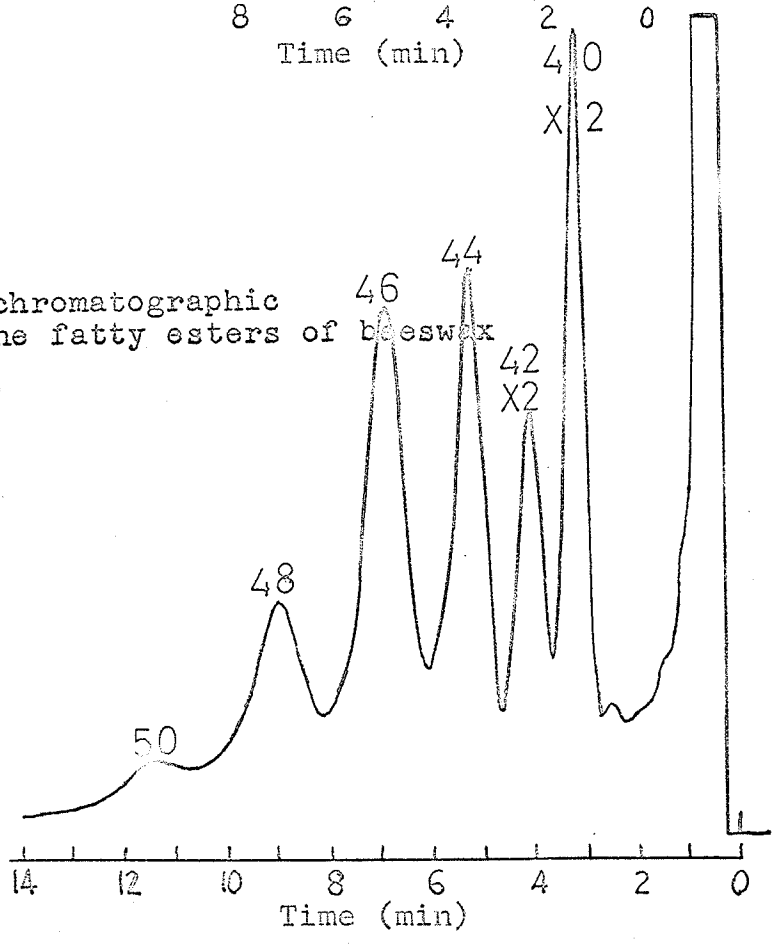


Figure 28

A gas-liquid chromatographic separation of the fatty esters of beeswax (Layer II.)

Figure 29 A gas-liquid chromatographic separation of the fatty esters of beeswax (layer III.)

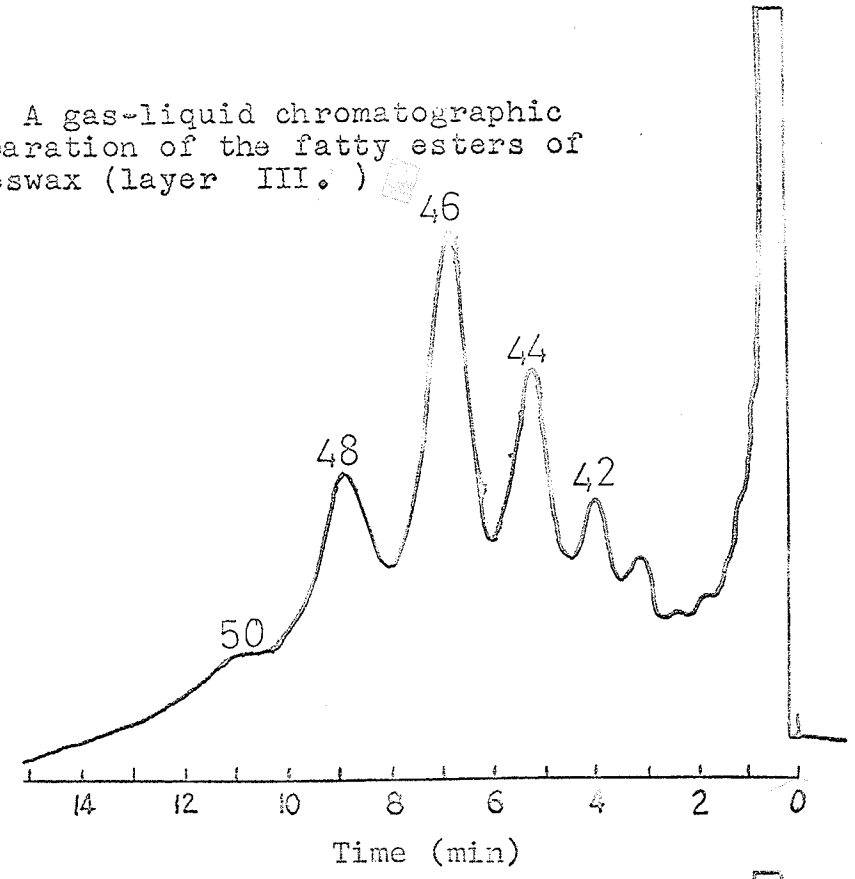
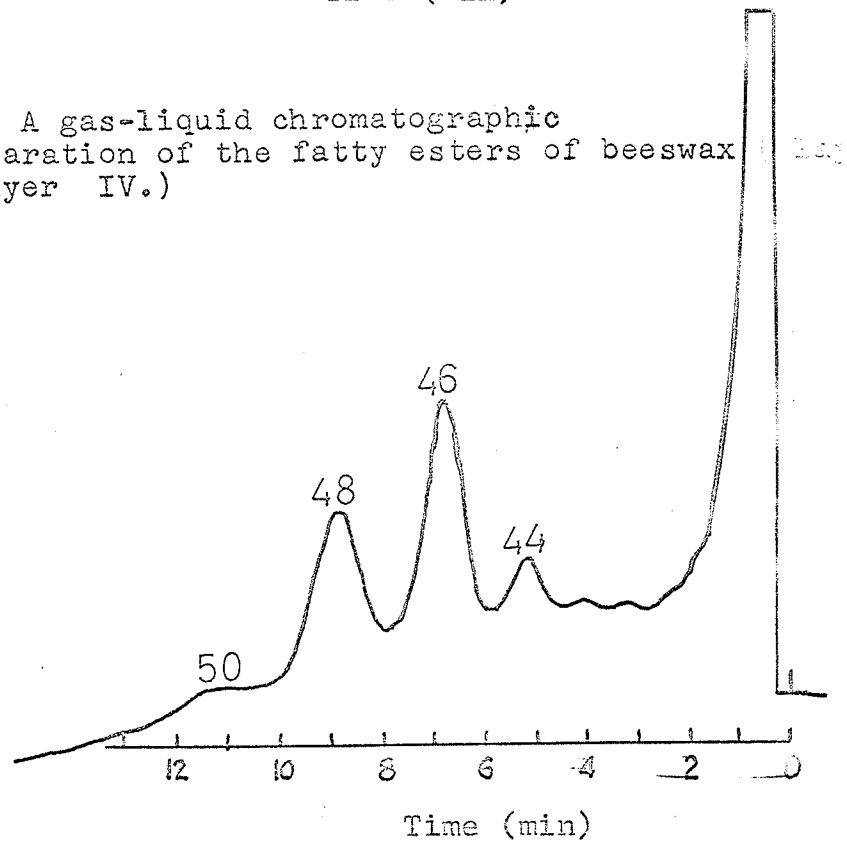


Figure 30 A gas-liquid chromatographic separation of the fatty esters of beeswax (layer IV.)



Separation I : Chromatograms of the second, third and fourth spot from a thin-layer chromatogram of beeswax and the second fraction from a separation of beeswax on alumina.
Column : 3 ft. column 1/8 in. i.d.
Stationary phase : 5% Silicon SE-30 on acid washed firebrick, 30/60 mesh.
Inlet pressure : 10 psig.
Exit flow rate : 10 ml/min.
Column temperature : 370°C
Filament current : 240 milliamps
HETP : 8.1 x 10-3

Separation II : The conditions are identical to those of Separation I except:
Exit flow rate : 25 ml/min.

Separation III : Chromatogram of the second layer from a thin-layer chromatogram of beeswax.
Column : 4 ft. copper column, 1/8 in. i.d.
Stationary phase : 5% Silicon SE-30 on acid washed firebrick, 30/60 mesh.
Inlet pressure : 10 psig.
Exit flow rate : 43.8 ml/min.
Column temperature : 320.5°C
Filament current : 250 milliamps
HETP : 1.32 x 10-1

Table 5

FATTY ESTERS OF BEESWAX SEPARATED BY GAS-LIQUID CHROMATOGRAPHY

Fatty Esters	Layer II						Layer III				Layer IV	
	Separation 1				Sep.2		Sep.3		Sep. 1		Sep. 2		Sep. 1	
	t_R		%		t_R	%	t_R	%	t_R	%	t_R	%	t_R	%
C_{28}		(0.83)												
C_{29}														
C_{30}		(1.00)							0.89	trace				
C_{32}	1.18	(1.20)	trace				1.23	trace	1.18	trace			1.23	trace
C_{34}	1.48	(1.48)	trace				1.84	trace	1.48	trace			1.48	trace
C_{36}	1.92		trace				2.66	trace	1.88	trace			1.80	trace
C_{38}	2.39		0.78				3.59	trace	2.36	trace			2.36	trace
C_{40}	3.22		32.55	1.54	19.59		5.17	22.3	3.10	3.01	1.58	2.70	3.10	2.33
C_{42}	3.97		21.18	1.99	23.71		7.38	16.8	4.00	9.04	2.07	7.21	4.00	4.66
C_{44}	5.13		15.69	2.64	20.62		10.49	12.1	5.17	22.89	2.69	18.92	5.11	15.12
C_{45}	5.89		trace						5.88	trace				
C_{46}	6.77		19.61	3.47	15.46		15.20	29.6	6.79	40.36	3.60	42.34	6.69	41.84
C_{47}	7.43		trace											
C_{48}	8.85		9.41	4.52	13.40		21.69	18.2	8.83	19.88	4.71	28.83	8.77	32.56
C_{50}	11.30		0.78	5.86	5.15				11.37	4.82			11.30	3.49
C_{52}				7.56	2.07				14.33	trace			15.63	trace

Table 5 cont.

Fatty Esters	Fraction Column			
	Separation 1		Separation 2	
	t_R	%	t_R	%
C_{28}				
C_{29}				
C_{30}				
C_{32}				
C_{34}				
C_{36}				
C_{38}	2.02	3.98		
C_{40}	3.22	17.05	1.48	20.33
C_{42}	4.14	9.66	1.91	11.29
C_{44}	5.36	10.23	2.54	12.43
C_{45}				
C_{46}	7.09	26.70	3.49	32.20
C_{47}				
C_{48}	9.29	26.70	4.55	21.47
C_{50}	11.85	5.68	5.69	2.28
C_{52}				

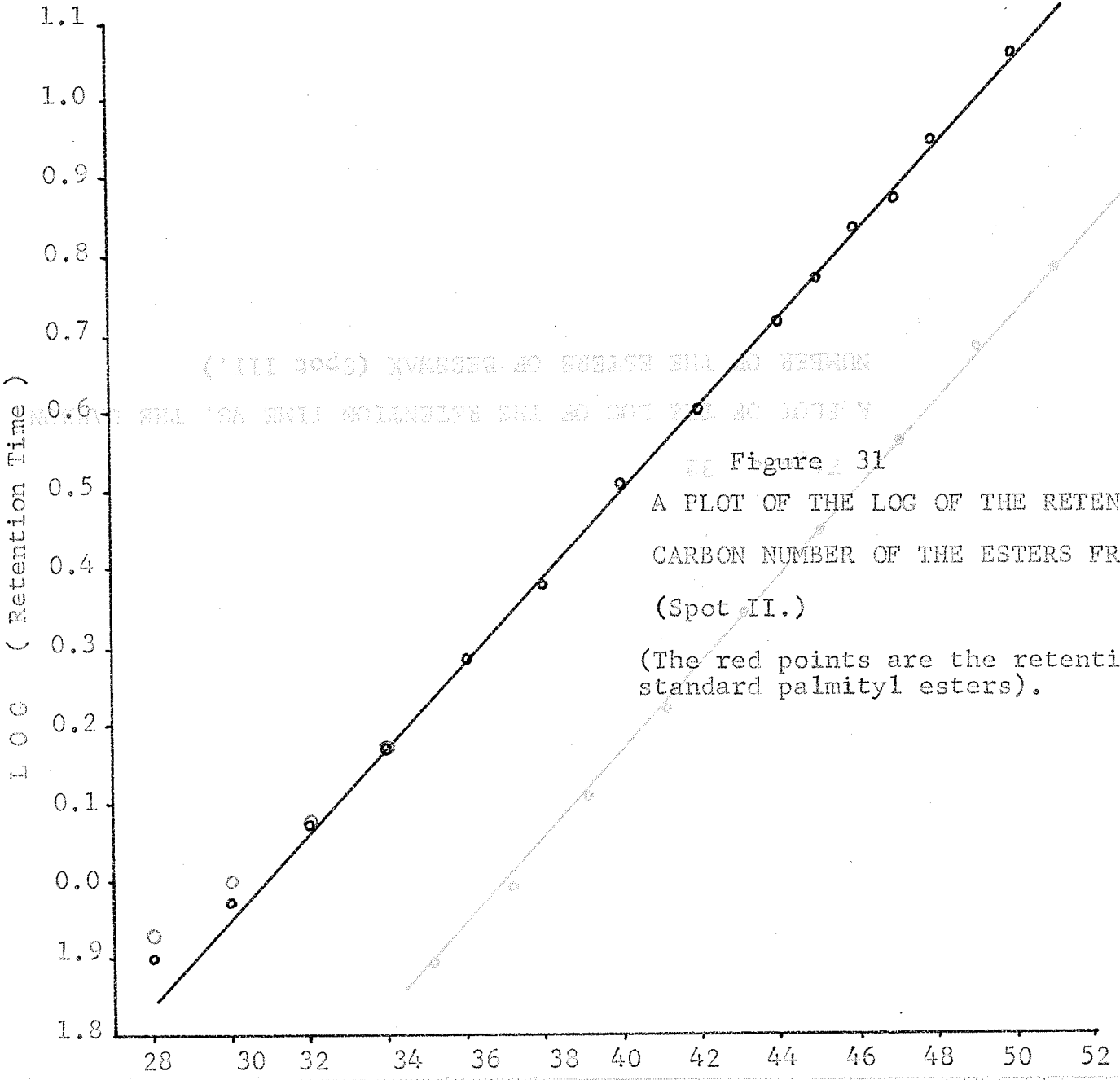


Figure 31

A PLOT OF THE LOG OF THE RETENTION TIME VS. THE CARBON NUMBER OF THE ESTERS FROM BEESWAX

(Spot II.)

(The red points are the retention times of the standard palmityl esters).

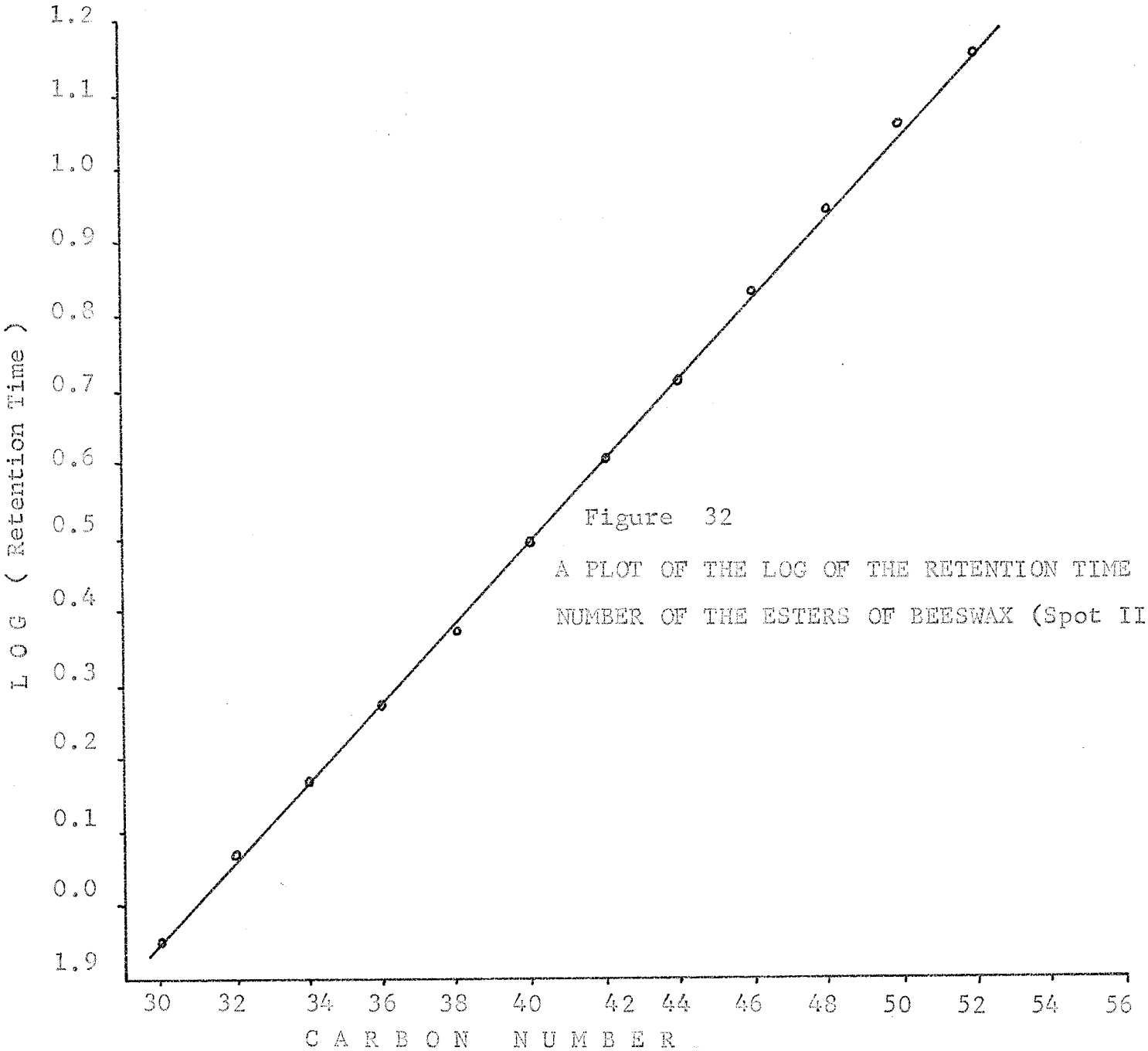


Figure 32

A PLOT OF THE LOG OF THE RETENTION TIME VS. THE CARBON NUMBER OF THE ESTERS OF BEESWAX (Spot III.)

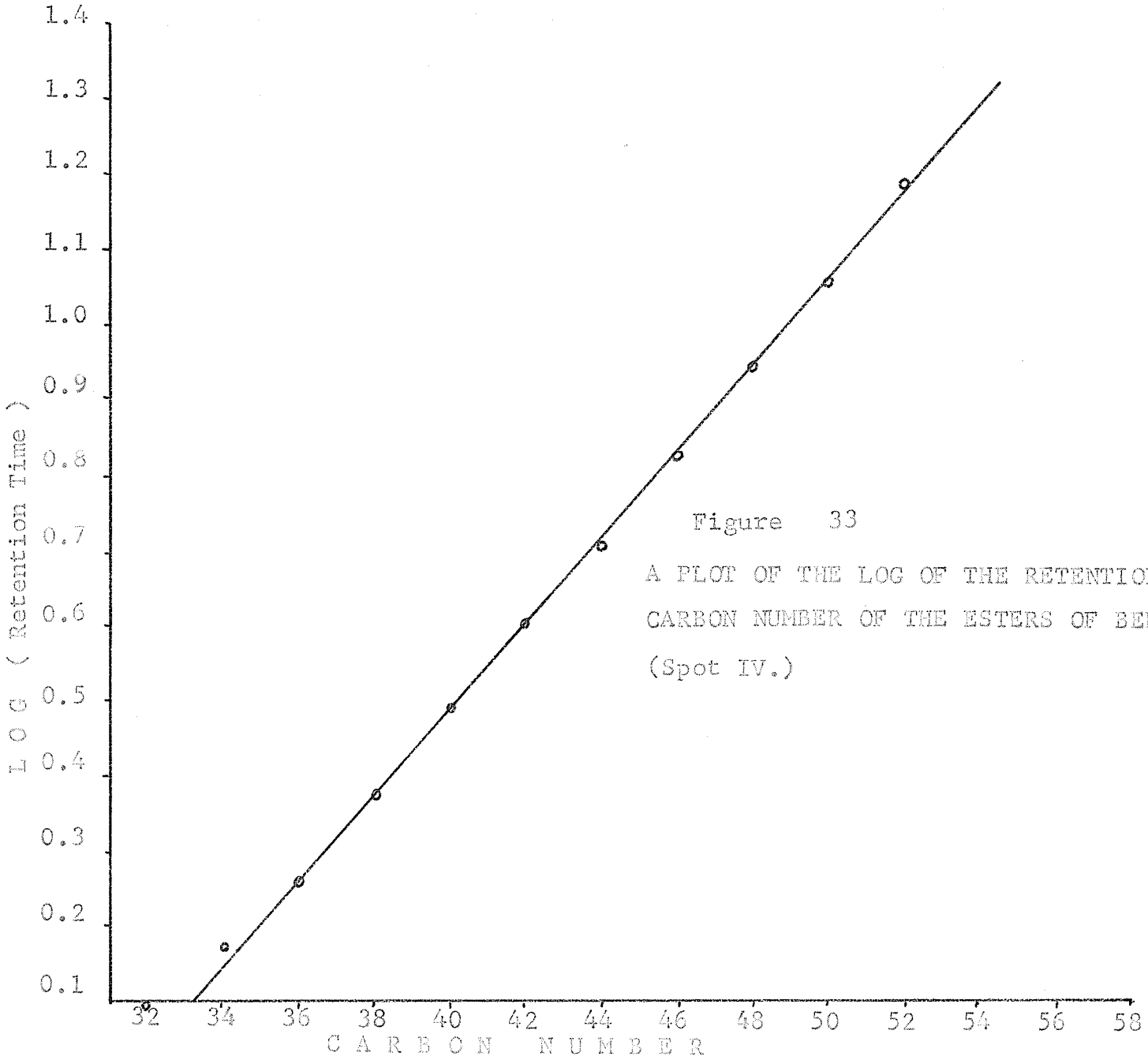
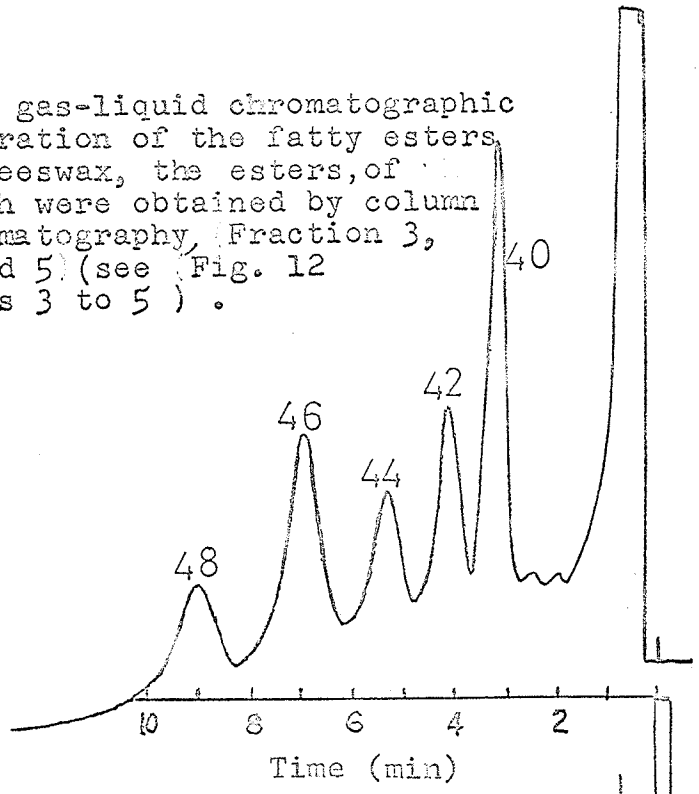


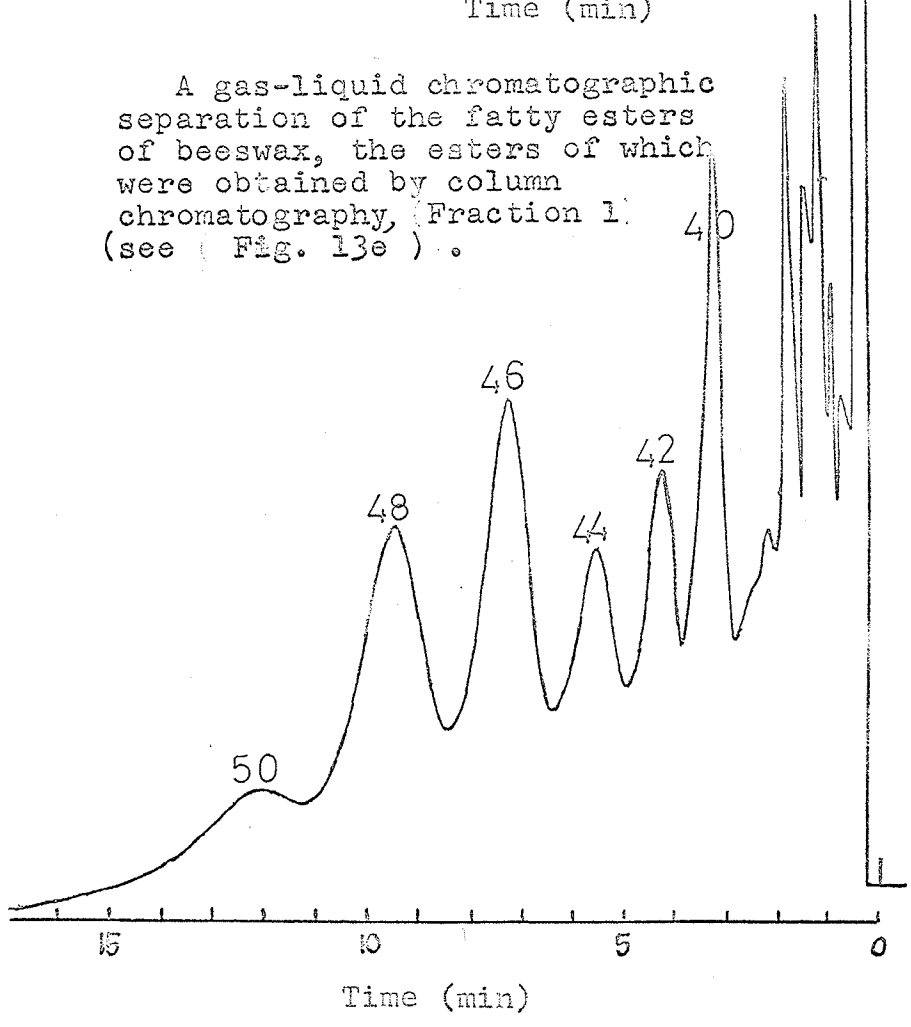
Figure 33

A PLOT OF THE LOG OF THE RETENTION TIME VS. THE CARBON NUMBER OF THE ESTERS OF BEESWAX (Spot IV.)

Figure 34 A gas-liquid chromatographic separation of the fatty esters of beeswax, the esters, of which were obtained by column chromatography, Fraction 3, 4 and 5 (see Fig. 12 spots 3 to 5).



A gas-liquid chromatographic separation of the fatty esters of beeswax, the esters of which were obtained by column chromatography, Fraction 1 (see Fig. 13e).



DISCUSSION

Extensive work on quantitative thin-layer chromatography of beeswax was done. This wax was separated into many spots, up to fourteen on a silver-nitrate impregnated plate (see Fig.18a). However, we tried to identify only the ten spots separated on a typical Silica Gel G chromatogram developed a distance of 13 cm with the developer, petroleum ether/ether/HAc = 95/5/1. The chemical nature of the ten spots into which beeswax was separated by thin-layer chromatography were according to our findings, starting from the top of the chromatogram and going down: I, hydrocarbons; II-V, esters; VI, esters with weak acid properties; VII, acids with equally strong ester properties; VIII, strong acids with weak ester properties; IX, acids with weak esters and alcohol properties and X, alcohols with weak ester and acid properties and the residue at the point of application contained alcohol acid and esters.

More work could be done trying to make the thin-layer chromatographic separations quantitative, followed by an extensive analysis of the spots by whatever methods are available.

The top spot of a thin-layer chromatogram of beeswax consisted of the hydrocarbons. The hydrocarbons analyzed by gas-liquid chromatography ranged from C_{17} to C_{36}. This is a further spread than was separated by either White *et al.* (66), C_{19} to C_{33}, or Downing *et al.* (32), C_{19} to C_{33}, although the same method of identification by gas-liquid chromatography was

used. However, we obtained the hydrocarbon fraction by a different method from that of the above mentioned workers. Whereas they separated the hydrocarbon fraction by column chromatography, we obtained this fraction by thin-layer chromatography.

The next spots were then analyzed by gas-liquid chromatography. Our results indicated that spots II, III and IV (see Table 1) were esters ranging from a totalcarbon number of 32 to 52. We made an interesting observation that, on the thin-layer chromatographs, partition thin-layer chromatography was occurring, with the shorter chain esters having a larger Rf value than the longer chain esters. However, when we compared the spots of the palmityl esters standards with the methyl ester standards, we found that the palmityl esters moved up further than the methyl esters on the chromatograms (Fig. 16). We were unable to explain this observation.

The methyl esters prepared from the fatty acids of beeswax were also determined by gas-liquid chromatography. The methyl esters were found to be ranging from C_{14} to C_{28}. This compared with C_{12} to C_{34} reported by Downing et al. (16). All methyl esters fractions prepared were checked as to their purity by thin-layer chromatography, and we found that the methylation techniques were not quantitative. We believe further work has to be done to see which methylation techniques will prove the most successful.

The results we obtained from the hydrocarbons, beeswax esters and methyl esters by gas-liquid chromatography are given in Table 6. In Tables 7 and 8 the values of White et al. (66) and Downing et al. (16) are given respectively for purposes of comparison.

This work on the analysis of waxes following the outline given in the introduction is of potential use in the complete identification of the complex wax mixtures. The fractions separated by thin-layer chromatography will have to be investigated further. Quantitative methylation techniques using minute quantities of material will have to be further investigated before each ester fraction can be saponified and the acids and alcohols determined quantitatively.

A problem we faced in the separation of the fatty esters by gas-liquid chromatography was that the thermal conductivity detector seemed not to be sensitive enough to detect components that remained in the column for more than 20 minutes, when the column was operating at high temperatures. One was unable to detect the peak of a component from the base line, even though sample sizes up to 50 microliters (50) were used, in which case the columns were definitely overloaded. There might be an advantage in using syringes which are designed to take solid samples. However, another way to eliminate this problem is to use a more sensitive detector, such as the flame ionization detector.

We had a gas chromatograph built, equipped with a flame ionization detector, by the technical staff from the Parker Laboratory, University of Manitoba. The design of the flame ionization detector is given in Fig. 35. We studied several designs, of which the one by Thompson (58) looked most promising. However, changes were made which we felt would improve the performance of the detector. The electrometer was also built by the technical staff, and it was a copy of the one by Thompson (58) with a $1 \times 10^{9}\,\Omega$, $2.3 \times 10^{9}\,\Omega$ and a $1 \times 10^{10}\,\Omega$ input resistance. A cross section of the gas chromatograph is given in Fig. 36.

Although the new gas-liquid chromatograph with the flame ionization detector is complete, we were unable to do any extensive work with it.

TABLE 6

SUMMARY OF THE HYDROCARBONS, METHYL ESTERS AND ESTERS OF BEESWAX DETERMINED BY GAS-LIQUID CHROMATOGRAPHY (% by wt.)

Carbon Number	Hydro-carbons	Methyl esters	Beeswax esters Spot II	Spot III	Spot IV
14		0.28			
15					
16		40.99			
17	trace				
18	0.31	51.03			
19	0.58				
20	0.34	3.67			
21	1.03				
22	0.69	1.16			
23	4.36	trace			
24	1.31	1.27			
25	10.62				
26	2.21	0.99			
27	29.89				
28	1.81	0.61			
29	16.50				
30	1.90			trace	
31	15.93				
32	1.79		trace	trace	trace
33	9.86				
34	1.64		trace	trace	trace
35	trace				
36			trace	trace	trace
37					
38			0.78	trace	trace
39					
40			32.55	3.01	2.33
41					
42			21.13	9.04	4.66
43					
44			15.69	22.89	15.12
45			trace	trace	
46			19.61	40.36	41.84
47			trace		
48			9.41	19.88	32.56
49					
50			0.78	4.84	3.49
51					
52				trace	trace

TABLE 7

THE HYDROCARBONS PRESENT IN BEESWAX AS DETERMINED BY WHITE *et al.* (66)

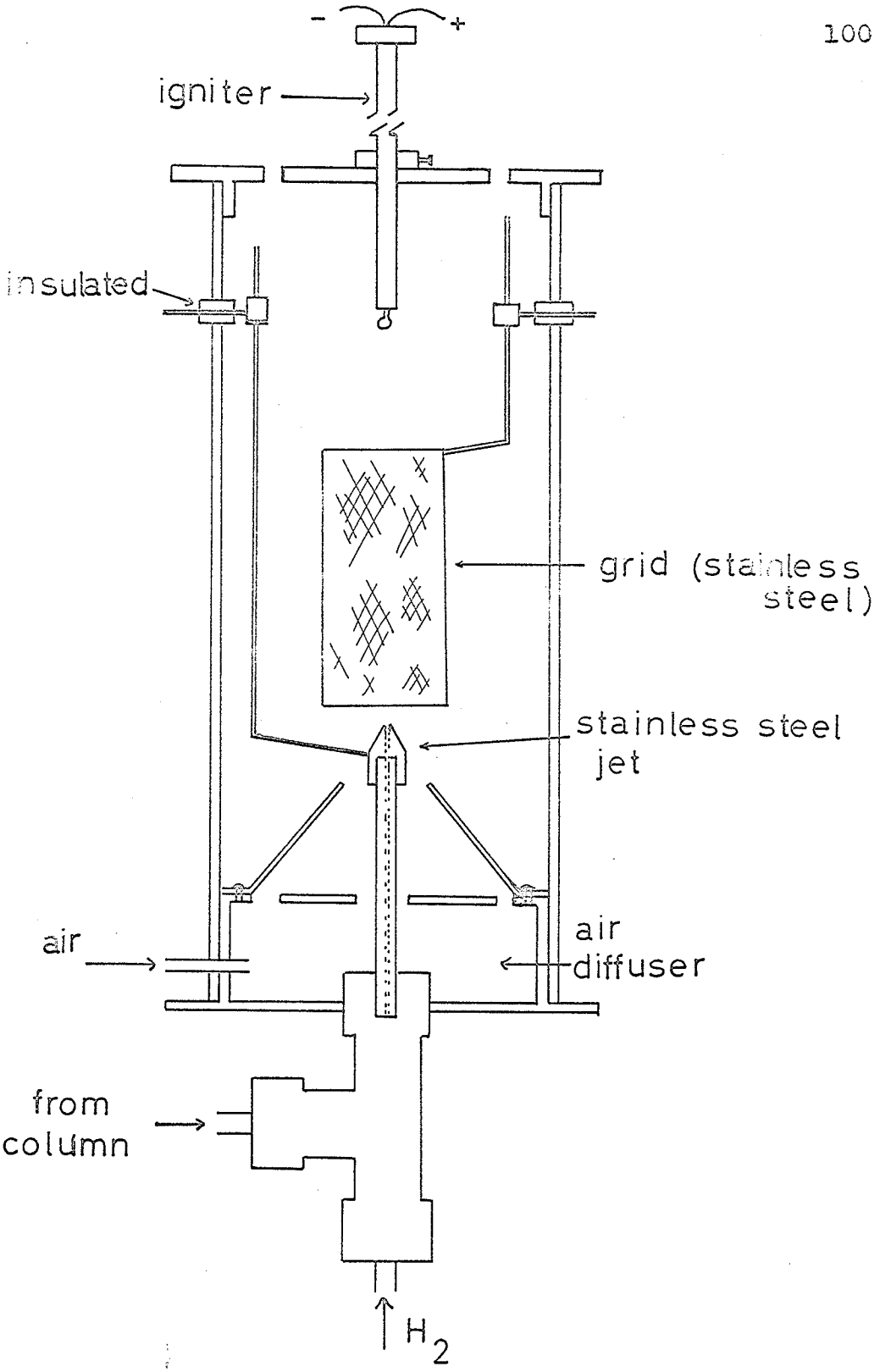
N-Hydrocarbons	Amount Present (%)
below 19	trace
19	0.26
20	0.01
21	0.63
22	0.29
23	3.79
24	0.29
25	11.6
26	1.21
27	40.7
28	1.42
29	25.2
30	trace
31	14.4
33	trace

Table 8

COMPONENTS PRESENT IN BEESWAX AS DETERMINED BY DOWNING *et al.* (16)

n-Paraffin Hydrocarbon Carbon No.	Hydrocarbons Wax A	Hydrocarbons Wax B	Monohydric Alcohols	"Diols"	Acids	Hydroxy-acids
12					0.3	0.4
14					0.8	1.5
16					50.5	58.5
17					0.3	0.4
18					8.5	9.8
19					-	trace
20	0.5	0.3		trace	0.9	4.1
21	0.8	0.8		-	-	-
22	0.3	0.2		trace	2.0	1.6
23	3.7	3.7		-	0.3	0.4
24	0.6	0.4	11.9	15.2	17.5	8.2
25	7.5	8.8	-	-	trace	0.5
26	1.2	1.0	10.1	19.6	4.9	3.7
27	26.8	30.1	trace	trace	trace	0.3
28	2.2	1.3	14.8	39.2	4.3	1.9
29	19.3	16.5	trace	2.6	trace	0.5
30	1.6	0.9	31.6	14.8	3.0	0.6
31	20.8	19.0	trace	2.2	trace	1.5
32	0.9	1.5	23.5	6.5	4.7	0.4
33	13.8	15.5	-		-	
34			5.4		2.0	
35			-		-	
36			2.7			

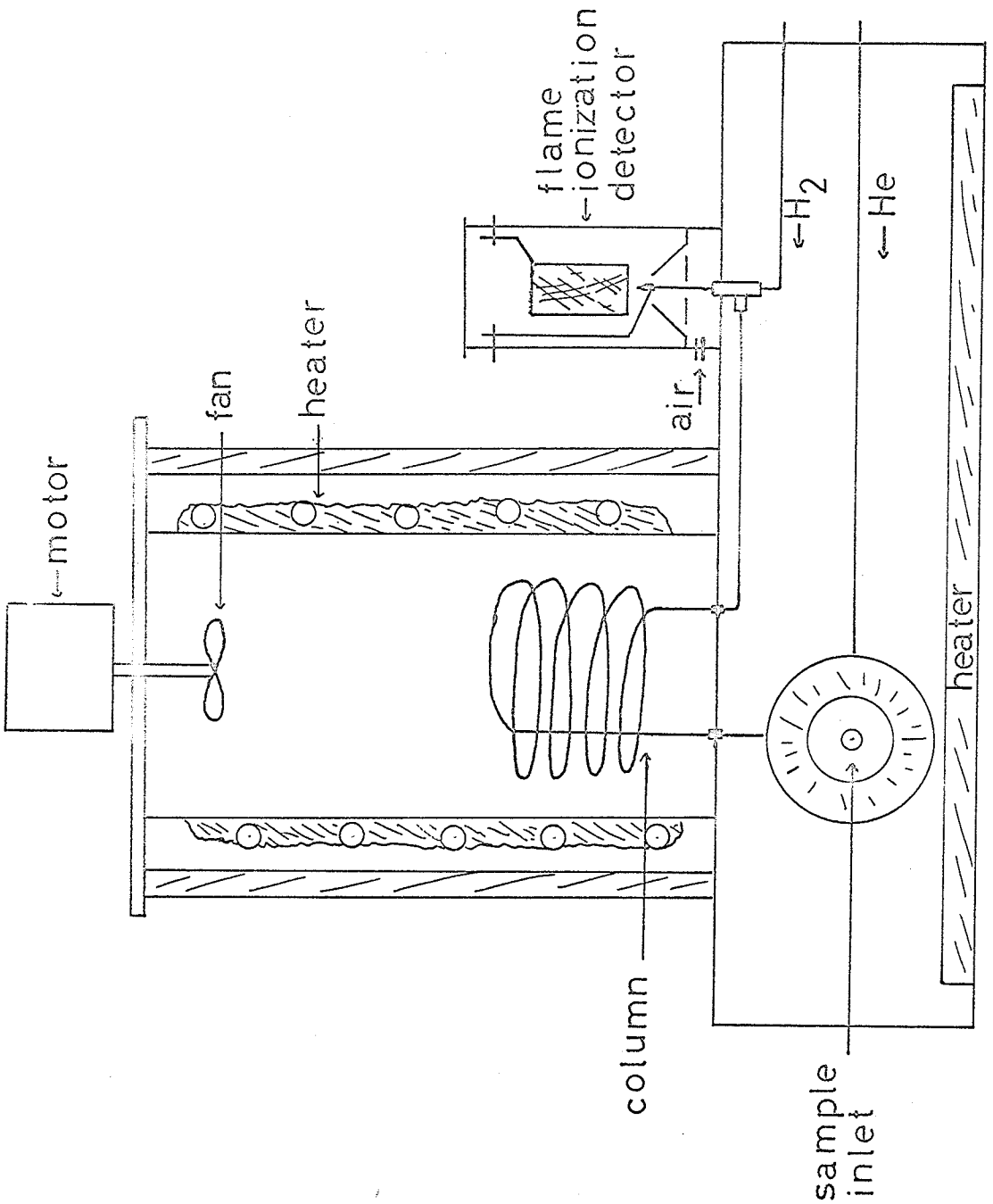
Figure 35



THE FLAME IONIZATION DETECTOR

Figure 36

GAS-LIQUID CHROMATOGRAPH WITH THE FLAME IONIZATION DETECTOR



CONCLUSION

Beeswax has been subjected to thin-layer chromatography and found to contain hydrocarbons, esters, alcohols and acids. This was confirmed by chemical tests and infrared spectral data. The hydrocarbon fraction was further analyzed by gas-liquid chromatography and found to contain C_{17} to C_{36} saturated paraffins. The ester fraction analyzed by gas-liquid chromatography gave esters ranging in carbon number from C_{32} to C_{52}. The methyl esters of the fatty acids of beeswax determined by gas-liquid chromatography ranged from C_{14} to C_{28}. No difference between the beeswax scales and combs of the apis mellifera was observed by thin-layer chromatography.

BIBLIOGRAPHY

1. Barrett, C. B., Dallas, M. S. J., and Padley, F. B., "The Separation of Glycerides by Thin-Layer Chromatography on Silica Impregnated with Silver Nitrate," Chem. and Ind. (London) 1962, 1050.

2. Bobbitt, J. M., Thin-Layer Chromatography, Reinhold Publishing Corporation, New York (1963).

3. Bruening, C. F., "Report on Cosmetic Creams, the Determination of Hydrocarbons in Cold Creams," A.O.A.C., 39, 391 (1956).

4. Burchfield, H. P., Storrs, E. E., Biochemical Applications of Gas Chromatography, Academic Press, New York and London (1962).

5. Cason, J., and Miller, W. J., "Versatility and Temperature Range of Silicone Grease as Partitioning Agent for Gas Chromatography", J. Org. Chem. 24, 1814 (1959).

6. Cassidy, H. G., Fundamentals of Chromatography, Technique of Organic Chemistry, Vol. 10, Interscience Publishers, Inc., New York (1957).

7. Chibnall, A. C., Piper, S. H., Pollard, A., Williams, E. F., and Sahai, P. N., "The Constitution of the Primary Alcohols, Fatty Acids and Paraffins present in Plant and Insect Waxes," Biochem. J., 28, 2189 (1934).

8. Condal-Bosch, L., XIV. "Some Problems of Quantitative Analysis in Gas Chromatography," J. of Chem. Ed. 41, A235 (1964).

9. Cropper, F. R., and Heywood, A., "Analytical Separation of the Methyl Esters of the C_{12} - C_{32} Fatty Acids by Vapour-Phase Chromatography," Nature 172, 1101 (1953).

10. Crowe, M. O'L., "Micromethod of Chromatographic Analysis," Anal. Chem., 13, 845, (1941).

11. Curylo, J., and Zalewski, W., Pszczel. Zes, Nauk, 1, 105 (1957), reported in Apicultural Absts. 37/60.

12. Dal Nogare, S., Juvet, R. S., Jr., Gas-Liquid Chromatography, Interscience Publishers, New York (1962).

13. Damoy, M. G., "Sur la Composition Chimique de la cire d'abeille," J. Pharm. Chim. 29, 148 (1924).

    "Sur la Composition Chimique de la cire d'abeille," J. Pharm. Chim. 29, 225 (1924).

14. Desty, D. H., and Whyman, B. H. F., The 21st Midyear Meeting of the Division of Refining, American Petroleum Institute, Montreal, May 14-17, 1956. "Application of Vapor Phase Chromatography to the Analysis of Liquid Petroleum Fractions," Anal. Chem. 28, 919 (1956).

15. Dhont, J. H., and de Rooy, C. de, "Chromatographic Behavior of 2,4,-dinitrophenylhydrazones on Chromatoplates," Analyst, 86, 74 (1961).

16. Downing, D. T., Kranz, Z. H., Lamberton, J. A., Murray, K. E., and Redcliffe, A. H., XVIII. "Beeswax: A Spectroscopic and Gas Chromatographic Examination," Aust. J. Chem., 14, 253 (1961).

17. Downing, D. T., Kranz, Z. H., and Murray, K. E., "XIV An Investigation of the Aliphatic Constituents of Hydrolysed Wool Wax by Gas Chromatography," Aust. J. Chem., 13, 80 (1960).

18. Cascard, A., and Damoy, G., "Chimie Organique - Sur les Acides de la cire d'abeilles," Comptes Rendus 177, 1222 (1923).

19. Gas Chromatography Abstracts, Ed. Knapman, C. E. H., Butterworths Scientific Publication, London.

20. Gesser, H. D., Associate Professor in Chemistry, University of Manitoba.

21a. Goldwater, W. H., Horning, E. C., Ahrens, E. H., Jr., Lipsky, S. R., Mattson, F. H., Mead, J. F., and Turner, D. A., "Quantitative Analysis of the Fatty Acids by Gas Chromatography," J. Lipid Research 5, 20 (1964).

21b. Haarth, E. O. A., and Horning, E. C., "The Separation of Human Skin Waxes by Gas Chromatography," Acta Chem. Scand., 15, 930 (1961).

22. Handbook of Physics and Chemistry, The Chemical Rubber Publishing Co., Cleveland, Ohio (1961).

23. Ed., Heftman, E., Chromatography, Reinhold Publishing Corporation, New York (1961).

24. Hilditch, T. P., The Chemical Constitution of Natural Fats, (Second edition), John Wiley and Sons Inc., 440 Fourth Avenue, New York (1949).

25. Honegger, C. G., "Präparative Dünnschichtchromatographie", Helv. Chim. Acta 45, 1409 (1962).

26. Izmailov, N. A., and Shraiber, M. S., Farmatsiya (Sofia), 3, 1 (1938); Chem. Abstr. 34, 855 (1940).

27. Kaufmann, H. P., and Ko, S. Y., "Thin-Layer Chromatography of Lipids. V. The Separation of Keto Acids, Hydroxy Acids, and Lactones." Fette, Seifen, Anstrichmittel 63, 823 (1961).

28. Kay, T., Graduate Student, University of Manitoba.

29. Kebler, L. F., "Notes on the Examination of Beeswax," Am. J. Pharm., 65, 585 (1893).

30. Knight, H. S., and Croennings, S., "Indicator Chromatographic Analysis of Organic Mixtures," Anal. Chem. 26, 1549 (1954).

31. Kuris, A., and McCarthy, M. J., "Gas-Liquid Chromatographic Fractionation of Natural Triglyceride Mixtures by Carbon Number," Can. J. of Biochem. and Physiol., 40, 679 (1962).

32. Malins, D. C., and Mangold, H. K., "Analysis of Complex Lipid Mixtures by Thin-Layer Chromatography and Complimentary Methods," J. Am. Oil Chemists' Soc. 37, 576 (1960).

33. Mangold, H. K., and Malins, D. C., "Fractionation of Fats, Oils, and Waxes on Thin Layers of Silicic Acid," J. Am. Oil Chemists' Soc. 37, 383 (1960).

34. Mangold, H. K., Lectures of the 1961 Short Course on Newer Lipid Analysis conducted by The American Oil Chemists' Society at the University of Rochester, Rochester, New York, July 24-26. Part III. "Thin-Layer Chromatography of Lipids." J. Am. Oil Chemists' Soc. 38, 708 (1961).

35. Mangold, H. K., "Thin-Layer Chromatography of Lipids," Lectures of the Twelfth Annual Short Course on Newer Lipid Analysis, American Oil Chemists' Society, Chicago 1, Ill., 1961 (extended), To be published in the J. Am. Oil Chemists' Soc.

36. Marcusson, J., and Bottger, "Die Zusammensetzung des Melens". Ber., 57B, 633 (1924).

37. Marini-Bettolo, G. B., Editor, Thin-Layer Chromatography, Elsevier Publishing Co., Amsterdam-London-New York-Princeton (1964).

38. Meinschein, W. G., and Kenny, C. S., "Analyses of a Chromatographic Fraction of Organic Extracts of Soils," Anal. Chem. 29, 1158 (1957).

39. Morris, L. J., "Fractionation of Cholesterol Esters by Thin-Layer Chromatography," J. Lipid Research 4,357 (1963).

40. Newburger, S. H., "The Analysis of Lanolin - Hydrocarbon Mixtures," A. O. A. C., 31, 670 (1948).

41. Peereboom, J. W. C., "Application of Thin-Layer Chromatography to the Analysis of Oils and Fats," Chem. Weekblatt 57, 625 (1961).

42. Peereboom, J. W. C., "The Analysis of Plasticizers by Micro-Adsorption Chromatography," J. Chromatography 4, 323 (1960).

43. The Structure of Glass, Translated from Russian by Uvarov, E. B., Proceedings of a Conference on the Structure of Glass, Leningrad, November 23-27, 1953. Academy of Sciences USSR Press, Moscow-Leningrad (1953). Consultants Bureau, Inc., New York (1958).

44. Kirchner, J. G., Miller, J. M., and Keller, G. J., "Separation and Identification of Some Terpenes by a New Chromatographic Technique," Anal. Chem. 23, 420 (1951).

45. Randerath, K., Thin-Layer Chromatography, translated by Libman, D. D., Verlag Chemie, GmbH., Weinheim, Bergstr., Academic Press, New York and London (1963).

46. Ibid., p 15.

47. Ibid., p 16.

48. Goldrick, B., and Hirsch, J., "A Technique for Quantitative Recovery of Lipids from Chromatograms," J. Lipid Research 4, 482 (1963).

49. Rosenberg, G. F. von, "Gemeinschaftsarbeiten der DGF, 34. Mitteilung. Neubearbeitung der Einheitlichen Untersuchungsmethoden für die Fette-und Wachsindustrie, XXIV: Analyse von Wachsen und Wachsprodukten IV". Fette, Seifen,Anstrichmittel 59, 28 (1957).

50. Stahl, E., "Dünnschicht-Chromatographie IV. Mitteilung: Einsatzschema, Randeffekt, 'Saure und Basische' Schichten, Stufentechnik" Archiv der Pharmazie 292, 11 (1959).

51. Stahl, E., Editor, "Dunnschichtchromatographie", Ein Laboratoriumshandbuch, Springer - Verlag, Berlin-Göttingen-Heidelberg (1962).

52. Ibid., p 139.

53. Ibid., p 150.

54. Ibid., p 514.

55. Stahl, E., Schröter, G., Kraft, G., and Renz, R., "Thin-Layer Chromatography," Pharmazie 11, 633 (1956).

56. Stahl, E., "Dunnschichtchromatographie," Phar. Rdschl, 2, 1 (1959).

57. Stain, H. H., Chromatographic Adsorption Analysis, Interscience Publishers, Inc., New York (1941).

58. Thompson, A. E., "A Flame Ionization Detector For Gas Chromatography," J. Chromatography 2, 148 (1959).

59. Toyama, Y., and Hirai, H., "Hydroxy Acids in Beeswax," J. Chem. Soc, Japan (Chem. Ind. Sect.), 54, 293 (1951).

60. Trappe, W., "Separation of Biological Fats from Natural Mixtures by means of Adsorption Columns," I. "The Eluotropic Series of Solvents." Biochem. Z. 305, 150 (1940).

61. Truter, E. V., Thin Film Chromatography, Cleaver-Hume, Press Ltd., London (1963).

62a. de Vries, B., "Quantitative Separations of Lipid Materials by Column Chromatography on Silica Impregnated with Silver Nitrate," Chem. and Ind., 1962, 1049.

62b. de Vries, B., "Quantitative Separations of Higher Fatty Acid Methyl Esters by Adsorption Chromatography on Silica Impregnated with Silver Nitrate," J. Am. Oil Chemists' Soc. 40, 184 (1963).

63. Waldi, D., Dünnschichtchromatographie, Editor, Stahl, E., Springer-Verlag, Berlin-Gottingen-Heidelberg (1962). pp. 497-515.

64. Warth, A. H., The Chemistry and Technology of Waxes, Reinhold Publishing Corporation, New York (1947).

65. Wartmann, H. J., "Organische Derivate des Silicagels," Doctor's Thesis, L. Speich, Reproduktionsanstalt, Brandschenkestr. 47/49, Zürich (1958).

66. White, J. W., Jr., Reader, M. K., Riethot, M. L, "Chromatographic Determination of Hyrdorocarbons in Beeswax," A. O. A. C., 43, 778 (1960).

67. Wiedenhof, N., "Analysis of Plant Waxes by Means of Chromatographic and X-Ray Diffraction," J. Am. Oil Chemists' Soc. 36, 297 (1959).

68. Beroza, M. and McGovern, F. P., "Transfer Tool for Thin-Layer Chromatography," Chem. Analyst, 52, 82 (1963).

69. Zhdanov, S. P., Kalmanovskii, V. I., Kiselev, A. V., Fiks, M. M., and Yashin, Ya. I., "Use of Porous Glasses as Adsorbents in Gas Chromatography," Russian Journal of Physical Chemistry 36, 595 (1962).