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CHROMATOGRAPHIC INVESTIGATION OF THE TRACE LIPIDS IN HONEY

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I hereby recommend that this dissertation prepared under my direction by Milton R. Smith entitled Chromatographic Investigation of the Trace Lipids in Honey be accepted as fulfilling the dissertation requirement of the degree of DOCTOR OF PHILOSOPHY

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SIGNED: Milton R. Smith

To

Sue and Dirk

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ABSTRACT

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Honeys from seven different floral sources were assayed for antibacterial activity. The antibacterial factor was present in all honeys tested, dialyzable, retained by a Seitz filter, more effective against _M_. _flavus_ and _S_. _lutea_ than _B_. _cereus_ and _B_. _subtilus_, not duplicated by high carbohydrate concentration or acidity, not extractable with Skellysolve B or diethyl ether, and not distillable from cotton honey. During investigation of the antibacterial factor in honey a lipid material was isolated. Since little or no information on the nature of the lipids in honey appears in the literature, a study of the composition of the cotton honey lipid extract was conducted.

Qualitative tests on the crude lipid extract of cotton honey revealed the presence of glycerides, sterols and possible phospholipids. Palmitic and oleic acid in relative concentrations of 27 and 60% respectively, along with small amounts of lauric, myristoleic, stearic and linoleic were identified in the crude extract by gas-liquid and paper chromatography. Twelve compounds, ten unsaturated, one acidic, and three reactive to antimony trichloride were separated by thin-layer chromatography. The lipids in the particulate matter of cotton honey were similar to those of the crude lipid extract as indicated by thin-layer chromatography.

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INTRODUCTION

Research on the composition of honey is quite extensive as evidenced by the reviews of White (44) and Pryce-Jones (35). These workers report the identification of a considerable number of sugars, vitamins, minerals, free acids and proteins in honey. However, no study has been made of the lipid components of honey. With the advent of new methods for lipid analysis, such as vapor-phase and thin-layer chromatography, which allow separation of minute amounts of lipids, a study of the trace lipid components of honey was made possible.

Various workers have reported the presence of free short chain acids in honey. In 1908 Farnsteiner (15) showed that formic acid was present. Later Heiduschka (18) reported the presence of the volatile acids butyric, valeric, caproic, capric and the non-volatile acids lactic, malic, tartaric, oxalic and succinic. Nelson and Nottern in 1931 (30) isolated citric, malic and succinic acid and identified them by crystallography and melting points of derivatives. Using paper chromatography to identify the acids in honey Vavruch (42) reported the presence of malic, tartaric, and citric acid in all samples and traces of succinic and lactic in some. Stinson _et al_. (38) made an extensive study of the acids in clover honey, isolated by ion-exchange adsorption, and separated by silicic acid partition chromatography and ion-exchange chromatography ten acids. These acids were identified by paper chromatography and infrared spectra of the sodium salts and other derivatives. Acids definitely identified were butyric, acetic, formic, lactic, succinic, pyroglutamic, malic, citric and gluconic. Oxalic acid was tentatively identified. These workers proposed that gluconic acid was the principal acid in honey. Dörrscheidt and Friedrich (14) separated the odorous substances of several honeys by gas-liquid chromatography. With carbowax as the stationary phase 31 components were separated. Among these methyl formate, methyl acetate, methyl propionate,methyl butyrate, methyl valerate and methyl caproate were identified by retention times.

All previously mentioned workers examined the free or volatile acid content of honey. However none studied the release of acids after saponification, except Heiduschka and Kaufmann (19) who reported in 1911 that more volatile acids and more formic acid were obtained in every case by steam distillation after saponification. However Heiduschka did not study the release of higher molecular weight fatty acids.

Dold *et al*. (13) in 1937 was the first to report the presence of a thermolabile, dialyzable, and photolabile antibacterial substance in honey, "inhibine." Prica (34) confirmed these findings and reported that neither the acid nor sugar content of honey was responsible for the observed inhibition. Plachy (33) also confirmed the work of Dold and indicated that formic and malic acid were not responsible for the antibacterial activity of honey. In addition Dold *et al*. (13), Plachy (33), and Stomfay-Stitz (39) reported that the antibacterial factor of honey was retained by a Seitz or asbestos filter.

Since the first report by Dold many workers have studied "inhibine" in honey although no information on its actual nature has been published until the recent report by Schuler and Vogel (36). They reported that the antibacterial factor in honey was an ethereal oil obtained by distillation or by extraction of honey with diethyl ether. The oil contained unsaturated compounds as indicated by decolorization of iodine-potassium iodide. In some cases a positive terpene reaction was obtained. On the other hand White *et al*. (45) reported that "inhibine" is hydrogen peroxide formed by action of the enzyme glucose oxidase on glucose according to the equation:

$$\text{glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{glucoholactone} + H_2O_2$$

These authors state that accumulation of hydrogen peroxide in diluted honey accounts for all of the reported properties of "inhibine."

Possibly the antibacterial property of honey is due, in part, to the presence of pollen. Chauvin _et_ _al_. (7, 8, 9) report that alcohol, diethyl ether or boiling water extracts of several pollens show antibiotic activity against some but not all bacteria tested. Further evidence for an antibacterial substance in pollen was presented by Michalski (28). This worker was able to extract and chromatograph on paper a growth inhibiting and stimilating factor from _Corylus_ _avellana_ pollen.

Pollen may be the source of trace lipids in honey since a variety of compounds have been isolated and identified from ether and alcohol extracts. Studying corn pollen Anderson (1) isolated noncosane, phytosterol palmitate and a saturated C_{30} alcohol. Kiesel and Rubin (22) found heptocosane in sugar beet pollen in a yield of 2% of the dry pollen. In the non-saponifiable fraction of hazel pollen Sosa and Sosa-Bourdouil (37) found tricosane, one alcohol $C_{16}H_{32}O$ and two unsaturated sterols. Also palmitic acid, one C_{12} acid and one branched chain C_{23} acid were isolated. Using mass spectrometry Nilsson _et_ _al_. (31) were able to identify 1-octacosanol, 1-hexacosanol, 1-tetracosanol in _Pinus_ _montana_; pentacosane and heptacosane from *Zea mays*; and heptacosane, nonacosane and traces of tricosane and pentacosane from *Alnus glutinosa*.

Recently Barbier *et al*. (3, 4) have identified 24-methylenecholesterol in the pollen of various plants. According to the authors this is the first incidence of this sterol in the plant kingdom.

Several higher fatty acids have been identified recently in pollen. Ching and Ching (10) isolated and analyzed by gas-liquid chromatography the pollen of some coniferous species. The major components in *Pseudotsuga* were oleic, palmitic and linoleic acids, whereas in *pinus* they were linolenic, oleic, palmitic and stearic. The methyl esters of the fatty acids of corn pollen were prepared and fractionated by Barr *et al*. (5). These workers identified palmitic, stearic, oleic, linoleic and linolenic acid by the melting points of the p-bromophenacyl esters of the saturated acids and of the hydroxy and bromine addition compounds of the unsaturated acids.

The investigations reported above indicate that little is known of the trace lipids in honey. The purpose of this investigation was to establish definitely that fatty acids are present in cotton honey, determine which ones are present, test the antibacterial properties of the lipid material, and separate and characterize some of the other components in this lipid.

METHODS

I. Studies on the antibacterial factor in honey

A. Pad-plate antibiotic method

To test for the presence of the antibacterial property of honey in various solutions the pad-plate method of Grady and Williams (17) with modifications was used. In this method 1/4 inch paper disks were dipped in the test solution and placed on agar plates previously seeded with *Sarcina lutea*, *Micrococcus flavus*, *Bacillus cereus* or *Bacillus subtilus*. Penassay seed agar[1] was used as the assay medium and to maintain stock cultures. The broth medium consisted of 3% BBL Trypticase Soy Broth. Optimum growth was obtained at 27°C for *S. lutea* and *M. Flavus*, and 37°C for *B. cereus* and *B. subtilus*.

B. Contribution of colloidal material to inhibiting effect of honey

To determine the inhibiting effect contributed by the colloidal material of honey, different honeys were treated as follows: cotton honey was diluted to 40% with water and centifuged at 17,000 rpm for 30 min.; 40% cotton

[1]Difco Laboratories, Detroit, Michigan.

honey was filtered through a Seitz filter, and undiluted Sweet Clover honey was filtered through a sintered glass filter. Also 25 ml of 100% cotton honey was dialyzed in cellulose membrane against an equal volume of water. Each of the resulting solutions was compared with a similar dilution of untreated honey by the pad-plate method.

C. Determination of the relative buffer capacity of honey

It was of interest to determine the relative buffer capacity of honey, since a solution strongly buffered to an acid pH above the optimum pH of the organism would inhibit its growth. The capacity of the buffer system in honey was determined in 8 ml of a 1:1 dilution of honey, buffered control sugar solution and a non-buffered control sugar solution by titration with 0.04 N NaOH. The results were expressed as the ml of 0.04 N NaOH required to bring the solution to pH7, divided by the total change in pH. The buffered control sugar solution, pH 3.6 was made up from McIlvaine's standard buffer solutions (21) with citric acid and disodium phosphate. This in turn was used to make a sugar solution, equivalent to honey, containing 32% glucose, 39% fructose, 8% maltose, 2% raffinose, 2% sucrose, and 17% water as 3.6 pH buffer. When distilled water replaced the buffer in this solution, it was referred to as the control sugar solution.

D. Contribution of pH to the antibacterial effect of honey

The contribution of pH to the antibacterial effect of a given honey was determined by comparison of growth inhibition caused by the control sugar at pH 6.3 and 4.2, buffered control sugar at pH 7.0 and 3.6 and honey at pH 4.1 (pH of honey in natural state) and pH 7.3. The pad-plate method was used in this test.

E. Distillation of the antibacterial ethereal oil from honey

Schuler and Vogel (36) reported distillation of an ethereal oil from honey which inhibited the growth of *Escherichia coli*. Based on this work attempts were made to distill the antibacterial factor from cotton honey. About 300 ml of cotton honey was placed in a round bottom flask, and distilled under a vacuum of about 16 mm. of Hg. Two fractions were collected, one at 43° and another over the range of $50-60^{\circ}C$. Ice water was circulated through the condenser in this experiment. In a second distillation two dry ice traps were used to collect the oil and three fractions were collected. These distilled at 25°, $50-60^{\circ}$ and $60-95^{\circ}C$ at approximately 16 mm. of Hg. Each of these fractions was tested for antibacterial activity by the pad-plate method.

F. Chromatography of the antibacterial factor in honey

Paper chromatography in combination with the pad-plate method has been used for the separation and detection of antibiotics such as penicillins and streptomycins (6). Studies were carried out to determine whether the antibacterial factor in honey could be separated by the same method. Cotton honey was diluted to 50% with water and spotted on Whatman No. 1 paper. These papers were developed for 16 hours in a 4:1:5 butanol-acetic acid-water solvent system, air-dried, placed on an agar plate seeded with S. lutea and stored in the refrigerator for 30 min. The paper chromatograms were then removed from the agar and the plates were incubated at 30°C for about 15 hours. They were then examined for zones of inhibition, which would correspond to the position of the inhibiting fractions on the chromatogram. To determine the position of the sugars on the paper a control strip, previously inoculated with 50% honey, was cut off prior to placing the rest of the chromatogram on the agar, and sprayed with aniline hydrogen phthalate reagent (32). Also multiple development and excessive developing times, up to 48 hours, were used to separate the sugars and antibacterial factor.

G. Test for antibacterial factor in lipid extract of cotton honey

Cotton honey (500 ml) was diluted to 50% and extracted three times with diethyl ether. The diethyl ether extract was evaporated to about 100 ml and tested by means of the pad-plate method for the antibacterial factor.

II. Studies on the lipids in cotton honey

A. Extraction procedure

Cotton honey was diluted to 50% (w/w) with distilled water, filtered through cheese cloth and extracted three times with re-distilled Skellysolve B (b.p. 60-70°C) and diethyl ether. Emulsions were broken with absolute ethanol. The extract was washed at least three times with distilled water to remove any non-lipid material, dried over sodium sulfate over night, and concentrated by rotary evacuation at 30-40°C. The residue was weighed, taken up in dry Skellysolve B and stored in the deep freeze until needed. This fraction is referred to as the crude extract.

The Molisch test (43) was performed on all crude extracts to determine whether carbohydrate still remained in the sample. In this test thymol was used in place of naphthol. Normal washing of the extract was found to remove all the carbohydrate.

In addition the infrared absorption spectrum was determined on the crude extract and other subsequent fractions. For analysis a 2% solution of the sample dissolved in spectral quality chloroform was placed in a sodium chloride cell and the spectrum recorded on a Perkin-Elmer Infracord (Model 137).

B. Qualitative methods

A spot test for glycerol or glycerides (16) made on the crude extract was positive. In this test acrolein, formed by heating the sample with potassium bisulfate, was detected by a blue color that turns violet red with alkali when treated with an aqueous solution of sodium nitroprusside containing a little piperidine.

Also a spot test for phosphorous (16) on the crude extract was positive. This test was performed by ignition with lime to form the tertiary calcium phosphate which is converted to ammonium phosphomolybdate by the action of a nitric acid solution of ammonium molybdate and is identified by the very sensitive, benzidine reaction.

The Liebermann-Burchard Test for sterols performed on the crude extract was positive. A chloroform solution of the sample when treated with acetic anhydride and sulfuric acid gives a blue-green color if sterols are present.

The ninhydrin test for protein or alpha amino acids was negative. This test was performed by adding 0.5 ml of 0.1% aqueous solution of triketohydrindene hydrate to the test solution and heating. If the solution turned blue upon cooling, the test was positive.

C. Quantitative methods

The crude extract was analyzed for the amount of non-esterified fatty acids (12) by titration with NaOH; and the uEq/L of acid was determined by comparison with a curve based on the titration of palmitic acid.

The hydroxamate reaction (2) was used to determine the ester groups in the crude extract, in which carboxylic acid esters were reacted with hydroxylamine in alkaline solution to form the hydroxamic acids. Subsequent reaction of the hydroxamic acids with ferric chloride produced a red to violet color, which was proportional to the amount of ester present.

D. Gas-liquid chromatography

To prepare a sample for analysis on gas-liquid chromatography the crude extract was subjected to transesterification (23) to convert sterol esters, phospholipides and triglycerides to their respective methyl esters. In this reaction the sample was refluxed in excess methanol and approximately 0.4 N. sodium methoxide to convert rapidly to the methyl esters. This product was further treated by elution from a column of silicic acid-filter aid (80:20) to obtain methyl esters free of unsaponifiables or other impurities. The "purified" methyl esters were then subjected to analysis on an Aerography model A 90 C gas chromatograph. The columns used were 20% diethyleneglycol succinate (D E G S) on acid-washed fire brick (60/80 mesh). The length and diameter of the columns varied from 5 to 10 feet and 1/4 to 1/8 inch respectively, however the calculation of the equivalent chain length (E.C.L.) cancels out any variations caused by changes in length, diameter and pressure (29).

To determine the relative concentration of the purified methyl esters we used a Research Specialties Co. model 604 gas chromatograph with an ionizing detector and a 10% D E G S column on chromosorb w, acid-washed (60/80 mesh).

E. Paper chromatography

Since it was necessary to obtain the free fatty acid for paper chromatography, the crude extract was saponified with 0.5 N alcoholic potassium hydroxide by refluxing 2 hours and standing over night in the dark (20). Diethyl ether was added in an amount equal to the total volume and then distilled water until two layers formed. The diethyl ether layer was decanted in a separatory funnel and extracted three times with distilled water to remove any fatty acid salts. These washings were combined with the original aqueous layer, acidified with hydrochloric acid, and the free fatty acids extracted three times with Skellysolve B. This fraction was then washed once with 1% sodium carbonate, three times with distilled water and then dried over sodium sulfate over night in the refrigerator. Before chromatographing, the sample was concentrated on a rotary evaporator at 35-40°C.

For chromatography of the fatty acids Whatman No. 1 paper was impregnated with 5% silicone (Dow Corning 555) in diethyl ether (26). After application of the unknown sample and appropriate standards to a single strip of paper, the chromatogram was developed for 15 to 18 hours in 85% acetic acid in distilled water. The paper was then air-dried, and washed three times for three minutes in distilled water. Finally the paper was soaked 5 minutes in 1% lead acetate and again three times in distilled water. The chromatogram was again air-dried and exposed to hydrogen sulfide gas which indicated the presence of fatty acids by brown spots on a white background. To detect unsaturated fatty acids the chromatogram was washed in distilled water, dried and exposed to iodine vapors. Under these conditions unsaturated fatty acids produced yellow spots on a white background.

F. Thin-layer chromatography

1. General procedure

The lipid extracted from various samples were separated and studied by means of thin-layer chromatography. Glass plates (20 x 20 cm) were prepared with thin layers of Silica Gel G according to Mangold (25). A slurry of Silica Gel G and distilled water (1:2 v/v) was mixed, poured into the applicator[1] and applied to the glass plates. The chromatoplates were air-dried for 10-20 min. and then activated by heating in an oven to 110° - 120°C for 1-2 hours. After cooling the plates were kept in a storage cabinet. Samples of 100-500 γ were applied to the plates with a 10 λ micropipette as 1% solutions in Skellysolve B. The solvent system most generally used was Skellysolve B-diethyl ether-acetic acid, 90/10/1, v/v/v. To separate the slow-moving compounds Skellysolve B-diethyl ether, 3:7, v/v was used (11). In all cases the developing jar was lined with filter paper to maintain uniform

[1]Research Specialties Co., Richmond, Calif.

saturation of solvent vapor in the jar. Prior to chromatography all samples were dissolved in Skellysolve B and stored in the refrigerator.

2. Spray reagents

Various spray reagents were used on the chromatoplates to locate compounds and determine reactive groups (25). To locate compounds aqueous sulfuric acid, 1/1, v/v or concentrated sulfuric acid saturated with potassium dichromate were used. Then the plates were heated for 5-10 min. at 100°C to char any organic compounds. Exposure of the chromatoplate to iodine vapors revealed any unsaturated compounds by forming yellow spots on a white background. Bromcresol green (0.1%) in n-butanol indicated the presence of acidic spots, when the chromatoplate was free of background acid. 2', 7'-dichlorofluorescein, 0.2% in methanol spray reagent revealed under ultra violet light, all non-polar lipids, saturated and unsaturated. A saturated solution of antimony trichloride in chloroform revealed terpenes, aliphatic lipids and steroids (41).

3. Preparation of samples

A 20g. sample of cotton honey was diluted to 50% and centrifuged for 30 min. at 17,000 RPM. The supernatant was poured off and 1ml. of distilled water plus

1ml. of Skellysolve B-diethyl ether, 1:1 v/v was added to the residue and mixed. Any emulsion formed was broken with absolute ethanol and the upper organic layer was then drawn off with a pipette. After three extractions the extracts were combined and concentrated by evaporation. This extract was applied directly to the thin-layer chromatoplate.

To extract the lipid from cotton pollen a weighed pollen sample was allowed to stand over night in Skellysolve B-diethyl ether, 1:1, v/v. The solvent was filtered off and the pollen extracted two more times for about 5 min. The solvent was removed in a rotary evaporator, the yield determined, and enough Skellysolve B added to make a 1% solution. This solution is referred to as the pollen lipid extract.

The lipids of cotton pollen were saponified with 0.5 N alcoholic potassium hydroxide by refluxing 2 hours and standing over night in the dark. The solution was acidified and extracted with diethyl ether to obtain both saponifiable and unsaponifiable material. The diethyl ether solution was dried over sodium sulfate, concentrated and made up to 1% with Skellysolve B. This solution is referred to as the saponified pollen lipids.

For thin-layer chromatography the crude extract of cotton honey was made up in a 1% solution of Skellysolve B and stored in the refrigerator.

A lipid extract of the particulate matter of cotton honey was obtained by the following method: cotton honey was diluted to 60%, w/w, filtered through cheese cloth and filtered through a Seitz filter under vacuum. After about 700 ml of honey solution had passed through the filter the particulate matter on the filter was washed three times with distilled water and then three times with Skellysolve B and diethyl ether to extract the lipid. The ether solution thus obtained was taken to dryness in a rotary evaporator to determine the yield and made up to 1% in petroleum ether for chromatography. This solution is referred to as the filter extract.

4. Elution of unknown compounds

Compounds eluted from thin-layer chromato-plates were located after development by spraying part of the plate with 50% sulfuric acid or a saturated solution of potassium dicromate in concentrated sulfuric and heating at 100°C for 5 min. Then the areas which contained the compound to be eluted were stripped off and eluted with diethyl ether. After elution the unknown compounds were subjected to the following analysis: determination of infrared spectra, Liebermann-Burchard Test for sterols, spot test for phosphorous (16), and spot test for esters of carboxylic acids (16).

G. Staining of pollen in honey

Diluted samples of honey were checked for pollen content by staining with aqueous fuchsin according to the following method (46): a drop of diluted honey was placed on a clean microscope slide and mixed with a drop of hot melted glycerin jelly containing aqueous fuchsin. Before the drop on the slide had time to jel it was covered with a cover slip and observed under 10X magnification. Pollen grains present in the honey stained purple.

RESULTS AND DISCUSSION

The inhibiting effect of the various honeys as determined by the pad-plate procedure is shown in Table 1. All honeys at the 100% level inhibited growth of _M. flavus_ and _S. lutea_, and sweet clover or cotton honey showed inhibition down to 10% concentration. The control sugar solution showed very slight inhibition of all four bacteria. It is obvious from these results that _M. flavus_ and _S. lutea_ are more sensitive to the antibacterial activity of honey than _B. cereus_ and _B. subtilus_. If the antibacterial factor of honey is hydrogen peroxide, as according to White (45), this difference in sensitivity is difficult to explain, since hydrogen peroxide is a a non-specific germicide and would inhibit the growth of bacteria to the same degree. Possibly this discrepancy could be explained by the spore-forming bacteria, such as _B. cereus_ and _B. subtilus_, being more resistant to hydrogen peroxide.

The results of the study on the contribution of colloidal material to the inhibiting effect of honey are shown in Table 2. When cotton honey, was diluted to 40% with water and centrifuged to remove the particulate matter

Table 1. Inhibiting effect of honey

Honey, %	*B. cereus*					*B. subtilus*					*M. flavus*					*S. lutea*				
	100	40	20	10	1	100	40	20	10	1	100	40	20	10	1	100	40	20	10	1
Sweet Clover, Alfalfa (Budge)	S	-	-	-	-	S	-	-	-	-	+	+	+	+	-	+	+	+	S	-
Tulip	+	+	-	-	-	+	-	-	-	-	+	+	+	-	-	+	+	-	-	-
Xtra white orange (citrus)	+	-	-	-	-	S	-	-	-	-	+	+	-	-	-	+	+	-	-	-
Tupelo	+	-	-	-	-	S	-	-	-	-	+	+	+	-	-	+	-	-	-	-
Buckwheat	+	S	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-
Sweet Clover, Alfalfa (Powers)	+	S	-	-	-	-	-	-	-	-	+	+	S	-	-	+	+	+	+	-
Fall Flower	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	+	-	-	-
Cotton	+	-	-	-	-	+	-	-	-	-	+	+	+	S	-	+	+	+	+	-

Note: + definite inhibition
- no inhibition
S slight inhibition

Table 2. Comparative antibacterial effects of untreated honey and clarified or dialyzed honey

	Diameter of Inhibiting Ring				
	Replicates				Average
	mm.	mm.	mm.	mm.	mm.
Cotton honey, 40%	9.1	8.8	8.9	8.6	8.8
Cotton honey, centrifuged, 40%	8.4	8.1	8.0	8.1	8.2
Cotton honey, 40%	8.6	8.8	8.9		8.8
Cotton honey, filtered[1], 40%	0	0	0		0[3]
Sweet Clover honey, 100%	11.6	16.0	18.1		15.2
Sweet Clover honey, filtered[2], 100%	10.6	12.6	13.2		12.1
Cotton honey, dialyzed	8.8	8.1	8.1		8.3
Dialysate of Cotton honey	8.0	7.6	7.9		7.8
Cotton honey, 100%	10.9	16.1	14.0		13.7

[1]Seitz filter

[2]Sintered glass funnel, medium porosity

[3]Some slight inhibition

the inhibition of S. lutea was unchanged. However, filtering a 40% solution of cotton honey through a Seitz filter reduced the inhibition almost to zero. Also, the inhibition of S. lutea by sweet clover honey was lowered by filtering through a sintered glass filter. On the other hand, the inhibiting factor could be dialyzed through a cellulose membrane. These results confirm those of Dold *et al.* (13), Prica (34), Plachy (33), and Stomfay-Stitz and Kominos (39).

The capacity of the buffer system was determined as previously described. The results were as follows:

	pH	capacity ml./pH unit
control sugar	4.5	0.10
buffered control sugar	3.6	1.57
cotton honey	3.8	1.27
sweet clover honey	3.9	.47

The buffer capacitites of the two honeys tested showed considerable differences. However, the buffer capacity of the honey was less in both cases than the buffered control sugar.

The effect of pH on bacterial inhibition was studied with S. lutea as the test organism for the pad-plate test. Buffered and non-buffered control sugar solution were compared to sweet clover honey as shown in Table 3. The only control sugar solution which caused inhibition was that buffered to pH 3.6, indicating the relation of acidity and inhibition. The 100% sweet clover honey had almost the same inhibiting power as the control sugar buffered to pH 3.6, but the buffered control had a much stronger buffering system than sweet clover honey. Also, the 80% sweet clover honey at pH 7.3 had almost the same inhibiting effect as it did at pH 4.1, indicating that the acidity of honey was not responsible for the inhibition of bacterial growth. This confirms the results of Dold *et al.* (13), Prica (34) and Plachy (33), who found that to produce inhibition more acid had to be present in artificial honey than was found in natural honey.

The results of the attempt to distill the antibacterial factor from cotton honey were in general negative. The fraction collected in the first distillation at 43°C showed very slight inhibition. However, none of the other fractors showed any antibacterial activity, but the honey remaining in reaction flasks was still active in each case. It would appear that the antibacterial factor in cotton honey is not as volatile as the factor reported by Schuler and Vogel (36).

Chromatography of the antibacterial factor of cotton honey on paper and location by the pad-plate method did not

Table 3. Effect of pH on bacterial inhibition

	pH	Diameter of Inhibition Rings			
		Replicates			Average
		cm.	cm.	cm.	cm.
Control sugar	6.3	0	0	0	0
Control sugar	4.2	0	0	0	0
Buffered control sugar	7.0	0	0	0	0
Buffered control sugar	3.6	1.56	1.45	1.68	1.56
Sweet Clover, 80%	7.3	1.02	1.29	1.01	1.11
Sweet Clover, 80%	4.1	1.01	0.98	0.89	0.96
Sweet Clover, 100%	4.7	1.42	1.32	1.48	1.41

yield very consistent results. One paper chromatogram developed 15 hours in butanol-acetic acid-water showed inhibition of S. lutea at an R_f of 0.25 and another at 0.14 (.10-.18) and 0.14 (.09-.20). Spraying with aniline hydrogen phthalate revealed high concentrations of sugars within these areas of inhibition. Multiple development and excessive developing times separated the sugars on one chromatogram and produced areas of inhibition at 16.0 to 22 cm, and 31.6-40.3 cm, from the origin. The area of inhibition closest to the origin (16.0 to 22 cm) did not contain any sugars, indicating that the antibacterial factor could be separated from the sugars of honey. In general, the areas of inhibition were quite diffuse and difficult to see. However, if the antibacterial factor is hydrogen peroxide, as previously mentioned, no migration would be expected on paper chromatography.

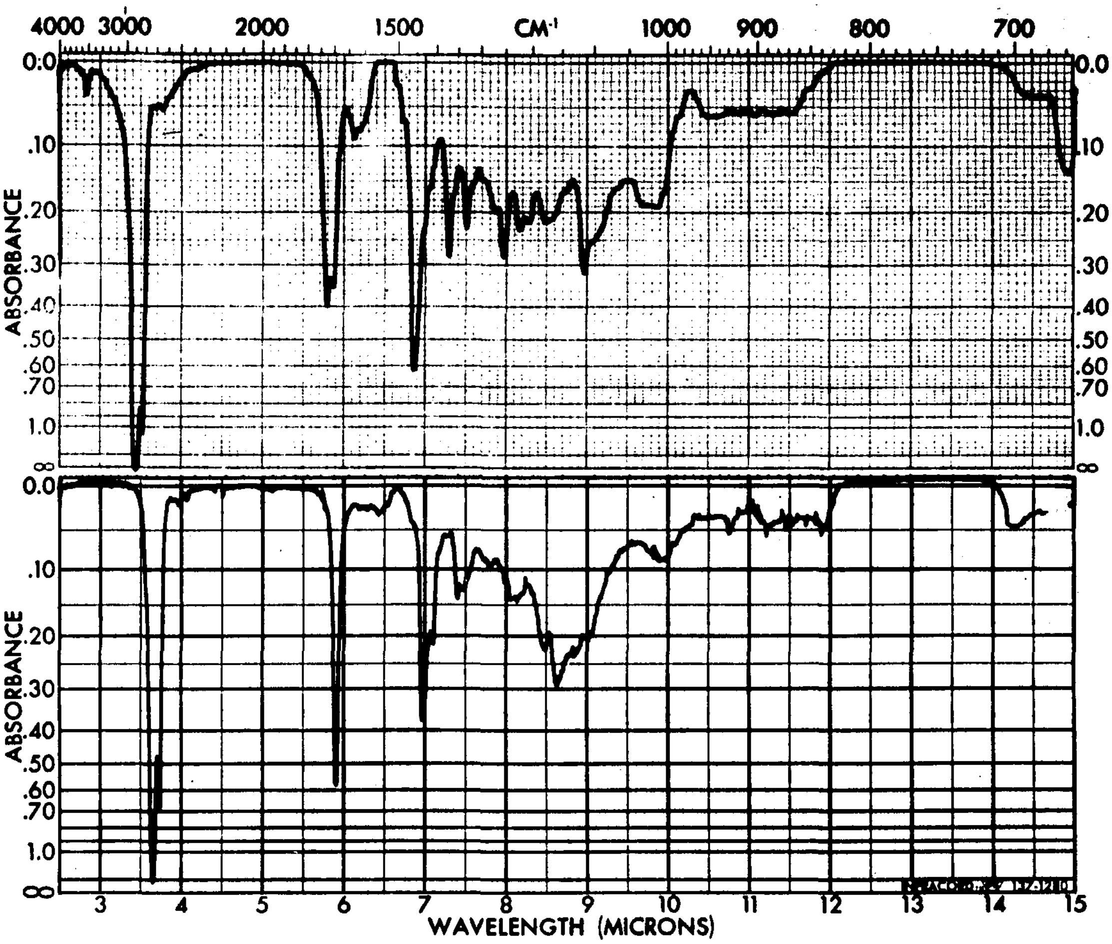
Assay of the diethyl ether extract of cotton honey by the pad-plate method for the antibacterial factor was negative. The antibacterial factor is therefore not soluble in diethyl ether. It was of interest, however, to investigate this lipid extract since no reports appear in the literature as to its composition.

Extraction of 1-2 kg. of cotton honey with Skellysolve B and diethyl ether produced a yield of 0.015%

of a yellow, lipid residue. This residue, referred to as the crude extract, was used in the spot tests and analyzed for fatty acids.

The spot tests for glycerides, phosphorus and sterols on the crude extract of cotton honey were all positive. However, the blank of the phosphorus spot test also gave a slightly positive test. The ninhydrin reaction for protein or amino acids and the Molisch test for carbohydrate were negative. The tests indicate the presence of triglyceride, sterols and possibly phospholipids. In addition the quantitative method for non-esterified fatty acid based on a palmitic acid standard curve showed an average of 20.7 mEq/L of acid. Hydrolysis of the sample may have caused these results to be high. The results of the hydroxamate test for ester groups showed 1.0 μEq/mg. of ester.

The infrared absorption spectrum of the crude extract as shown in Figure 1, showed sharp absorption bands at 3.5, 5.9 and 6.9 microns which are typical of alkane and ester groups (27). On the other hand, the bands indicating carboxyl groups are absent. The presence of a sharp band at 2.8μ indicated a free OH group, however this band disappeared after transesterification and elution on silicic acid. The purified methyl esters, eluted from silicic acid, gave the characteristic infrared spectrum of



top: crude extract

bottom: purified methyl esters

Figure 1. Infrared spectra of cotton honey extracts

fatty acids. The slight shifting of some bands was caused by mechanical error.

Table 4 shows the results of gas-liquid chromatography of the purified methyl esters from honey. By comparison with known methyl esters methyl laurate, myristoleate, palmitate, stearate, oleate and linoleate are definitely shown to be present. In the purified methyl esters the E.C.L. of 8.6 may be the unsaturated analogue of carprylic acid, octenoic acid. Methyl myristate and palmitoleate may be present, but not enough observations were made to be certain. The purified methyl esters may contain either methyl arachidate (saturated C-20) or linolenate, since the E.C.L.'s are similar it is not possible to determine whether one or both of the methyl esters are present. One E.C.L., 17.2, was omitted from Table 4 because it does not correlate with any of the standard methyl esters. This methyl ester was observed only once using the Aerograph gas chromatograph, however the ionizing detector gas chromatograph showed a very small amount of a similar fatty acid at an E.C.L. of 17.4. A typical gas chromatogram of the honey methyl esters is shown in Figure 2.

The percentage concentrations of the purified methyl esters were determined by means of a gas chromatograph with an ionizing detector and an integrater. By multiplying the

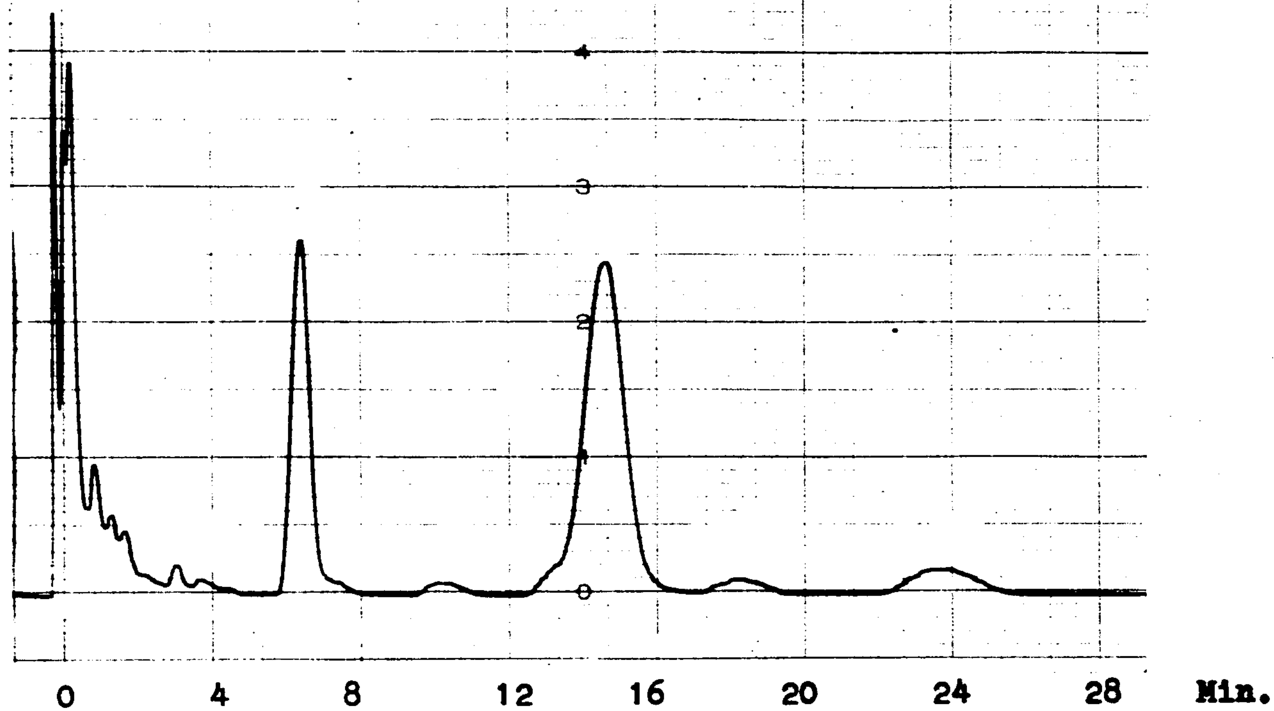
Table 4. Gas chromatography of fatty acids isolated from cotton honey

	Equivalent Chain Length			
	Methyl Ester Standards		Honey Methyl Esters	
Caprylate	8.1 ± .30[a]	(3)[b]	8.6[c] ± .21[a]	(6)[b]
Caprate	10.0 ± .10	(3)		
Laurate	11.9 ± .10	(4)	11.5 ± .45	(5)
Myristate	14.0 ± .09	(7)	13.7	(1)
Myristoleate	14.7 ± .07	(7)	14.8 ± .10	(2)
Palmitate	16.0 ± .08	(7)	16.0 ± .20	(7)
Palmitoleate	16.6 ± .11	(7)	16.7	(1)
Stearate	18.0 ± .11	(7)	18.0 ± .25	(4)
Oleate	18.6 ± .11	(7)	18.6 ± .25	(7)
Linoleate	19.2 ± .15	(7)	19.1 ± .18	(5)
Arachidate	20.0 ± .15	(5)	20.0 ± .29	(5)
Linolenate	20.0 ± .17	(3)		
Vaccinate	18.3	(1)		
Behenate	21.9	(1)		

[a]Standard deviation.

[b]Number of observations

[c]Significantly different from the corresponding methyl ester at the 5% level.



Research Specialties Co. model 604 gas chromatograph

Column temperature: 170°C, Pressure: 3.7 lb.

Figure 2. Chromatogram of purified methyl esters from cotton honey.

value of the integrater times the molecular weight of each methylester a factor is obtained which is directly proportional to the relative percentage composition. The results of these calculations are shown in Table 5. The purified methyl ester fraction contains approximately 60% methyl oleate and 27% methyl palmitate.

After saponification the fatty acids of the crude extract were subjected to reverse phase paper chromatography. The fatty acids of cotton honey have the same R_fs as that of palmitic and oleic acid as seen in Table 6. Even the colors of the spots on the paper correspond, palmitic acid being slightly darker than oleic acid. Use of iodine vapors confirms the presence of oleic acid at R_f .37. Palmitic and oleic acid cannot be separated by this method, however Table 7 shows that the crude extract of cotton honey contains both palmitic and oleic acid as evidenced by comparison of R_f values and treatment with various indicators. The fatty acid indicated by iodine vapors with an R_f of .55 may be linoleic acid. Increased concentration did not yield additional fatty acids.

The diluted cotton honey after extraction with Skellysolve B and diethyl ether was made alkaline, refluxed for 2 hours, cooled and extracted again with Skellysolve B and diethyl ether. This was done to determine if all the

Table 5. Relative concentration of honey fatty acids determined by intergrater technique with gas-liquid chromatography

Methyl Ester Standards		Honey Methyl Esters		
	E.C.L.	Retention time, min.	E.C.L.	Relative % concentration
Methyl myristate	14.0	3.3	13.9	1.0
		4.0	14.5	1.1
Palmitate	16.0	6.7	16.0	26.7
Palmitoleate	16.5	7.7	16.5	1.0
		10.5	17.4	
		13.3	18.1	1.7
Oleate	18.5	14.9	18.5	60.3
Linoleate	19.2	18.5	19.2	1.8
Arachidate	20.0	24.0	20.0	6.5

Table 6. Paper chromatography of honey fatty acids

Sample	R_f Indicator: H_2S
Honey fatty acid	.44 (.39-.49)
Honey fatty acid	.40 (.35-.45)
Stearic acid	.29 (.26-.32)
Palmitic acid	.43 (.41-.46)
Oleic acid	.40 (.38-.42)

Table 7. Paper chromatography of honey fatty acids using two indicators

Sample	R_f
	Indicator: H_2S[a]
Honey fatty acids	.37 (.32-.42)
Honey fatty acids	.37 (.31-.44)
Palmitic and oleic acid	.40 (.36-.44)
	Indicator: I_2[a]
Honey fatty acids	.37 (.43-.39) and
	.55 (.52-.58)
Palmitic and oleic	.37 (.33-.42)

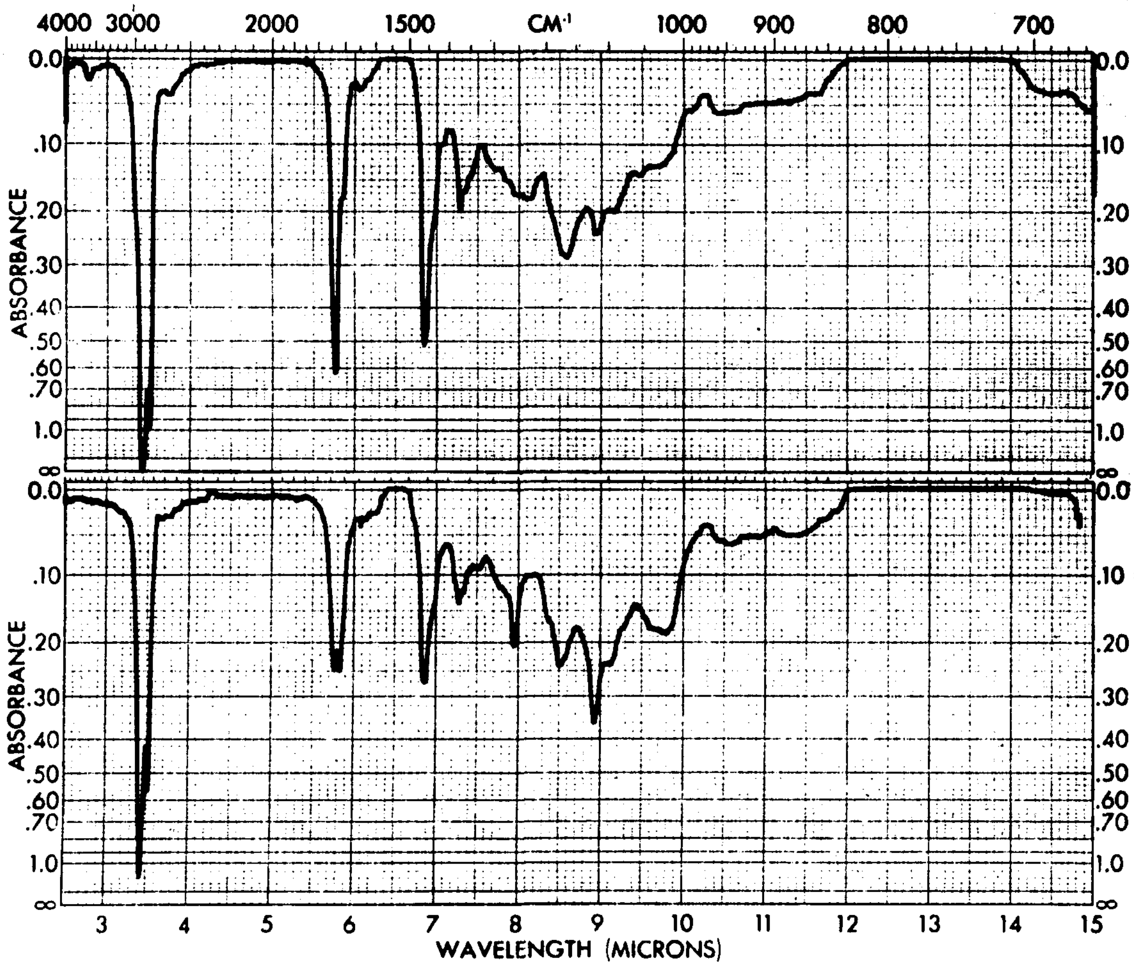
[a]Chromatogram cut in half for treatment with two indicators.

lipid was extracted initially. However, the extract contained decomposed carbohydrate which could not be removed by successive washings. Therefore, since these analysis are primarily qualitative rather than quantitative, the alkaline extract was not investigated further.

Thin-layer chromatography of the cotton honey lipid extract, obtained by centrifugation, revealed one spot at R_f .36. This spot appeared in the same position as a spot found in the crude extract and in high concentration in the filter extract. Further investigation of this extract was discontinued because of insufficient material.

The filter extract was obtained from cotton honey in a yield of approximately 20 mg.%. The infrared spectrum of this extract as shown in Figure 3, displayed bands indicating the presence of alkane and ester groups. Figure 4 shows a chromatoplate of crude extract and filter extract after spraying with 50%. sulfuric acid. Distinct similarities can be seen between the two extracts although concentrations of the separated compounds vary.

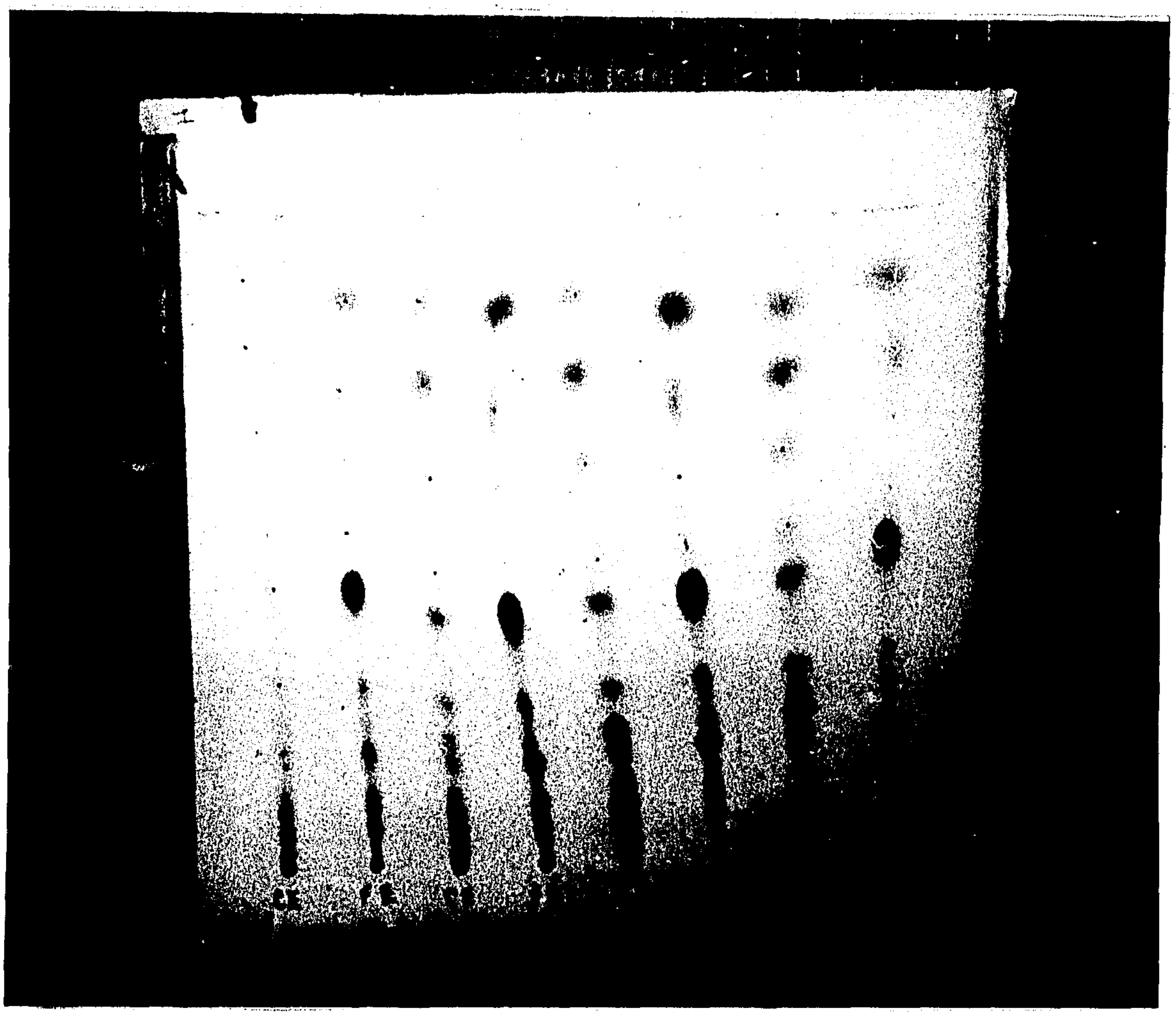
Table 8 shows the average R_f values of the crude extract and filter extract determined on thin-layer chromatography. According to Malins and Manglold (24), although the pattern of separation remains the same, the R_f values increase with increasing concentration, therefore



top: filter extract

bottom: eluent I

Figure 3. Infrared spectra of cotton honey extracts.



100 γ per spot

Developing Solvent: 90:10:1
Skellysolve B: diethyl ether: acetic acid

Figure 4. Chromatoplate of crude extract and filter extract.

Table 8. Thin-layer chromatography of cotton honey extracts. Comparison of average R_f values and reactions to various reagents.

R_f Values	
Crude Extract	**Filter Extract**
.02 ±.00[a](17),[b] I,[c] F[d]	
.06 ±.01 (16), I, F	.06 ±.01[a](18),[b] F[d] .07 ±.00 (2)
.09 ±.01 (17), A,[e] F	.10 ±.01 (18), F
.12 ±.01 (17)	
.19 ±.03 (12), I, F	.19 ±.04 (9), I,[c] F
.22 ±.03 (12), I, F	.24 ±.07 (10), I, F
.28 ±.03 (15), I, F	.28 ±.03 (16), I, F
.42 ±.04 (17), I, F	.44 ±.03 (17), I, F
.49 ±.04 (13), I, F	.53 ±.05 (5)
.62 ±.04 (17), I, F	.61 ±.03 (13), F
.74 ±.04 (17), I, F	.71 ±.04 (17), I, F
.86 ±.04 (17), I, F	.85 ±.03 (17), I, F

[a]Standard deviation.

[b]Number of observations.

[c]Positive reaction with iodine vapor.

[d]Showed fluorescence under ultra-violet light after spraying with 2', 7' -dichlorofluorescein.

[e]Positive reaction with bromcresol green spray.

each sample was applied to the chromatoplate as 10 λ of a 1% solution or 100 γ . The similarities between the crude extract and the filter extract are again demonstrated by this table. Ten spots in the filter extract correspond to spots in the crude extract. Two spots in the crude extract at R_f .02 and .12 do not appear in the filter extract, while only one spot at R_f .07 in the filter extract failed to appear in the crude extract. The compound in highest concentration in the crude extract appears at R_f .44. Similarity in the two extracts was observed also in the reactions to the various spray reagents. Most of the spots fluoresced under ultra-violet light after spraying with 2', 7' -dichlorofluorescein indicating non-polar lipids, saturated and unsaturated. Also most of the spots reacted with iodine vapors indicating unsaturation. One spot at R_f .09 in the crude extract reacted to the bromocresol spray indicating the presence of a carboxyl group.

The lipid extracted from cotton pollen displayed two spots in high concentration at R_f .61 and .96 as shown in Table 9. Higher concentrations of this lipid produced a streak on the chromatoplate hindering the determination of other R_f values. The infrared absorption spectrum of this extract showed bands indicating the presence of alkane and ester groups.

Table 9. Comparison of cotton honey and cotton pollen lipid extracts. Average R_f values and reaction to various reagents on thin-layer chromatography.

Crude Extract	Saponified Pollen lipids	Pollen lipid Extract
.02 ±.00[a](10),[b]I,[c]F[d]		
.05 ±.00 (10), I, F	.05 ±.01[a](11),[b]I,[c]F[d]	
.08 ±.01 (10), F, A[e]	.08 ±.01 (10), F	
.11 ±.01 (7), F	.11 ±.00 (3)	.11 ±.00(1),F[d] .15 ±.00(2),I,F
.18 ±.04 (6), I, F	.19 ±.03 (7), I, F, A[e]	.19 ±.01(3),I,F
.21 ±.06 (4), I, F	.23 ±.03 (6), I, F	
.28 ±.02 (5), I, F	.28 ±.01 (2)	
.42 ±.04 (10), I, F		
.50 ±.02 (2), F		
.61 ±.02 (10), I, F		.61 ±.01 (6),I,F
.74 ±.05 (10), I, F		
.86 ±.04 (10), I, F	.86 ±.03 (10) F	
	.98 ±.00 (8), I, F	.96 ±.01(6), I,F

[a]Standard deviation.

[b]Number of observations.

[c]Reacted with iodine vapor.

[d]Fluoresced under ultra-violet light after spraying with 2', 7' -dichlorofluorescein.

[e]Reacted with bromcresol green spray.

The saponified pollen lipids, which contained both saponified and unsaponified material, showed five more spots on the chromatoplate than the pollen lipid extract, five of these were produced by the saponification. The spot at R_f .19 contains a carboxyl group and is probably a fatty acid. Two spots, which appeared in the pollen lipid extract, did not appear in the saponified pollen extract, indicating that these compounds were destroyed during saponification. Three of the eight compounds in the saponified pollen lipids coincide with and gave reactions similar to compounds in the crude extract of honey, indicating that the source of these lipids may be cotton pollen.

Thin-layer chromatography of the crude extract, upon exposure to antimony trichloride, revealed three spots at R_f 0.5, 0.7 and .36, colored pink, pink and brown respectively. Table 10 shows the R_f values and color reactions of several pure compounds. None of the pure compounds tested coincided with those in the crude extract when compared on the basis of R_f, color complex with antimony trichloride and rate of color formation. Cholesterol at R_f .06 formed an orange spot immediately upon exposure to antimony trichloride, where as the reactive compounds in the crude extract appeared very slowly. The reactive compound at R_f .05 in the crude extract was quite

Table 10. Average R_f values and color reactions of pure compounds run by thin-layer chromatography

Pure Compounds	Average R_f	Color complex with $SbCl_3$
Cholesteryl acetate	.49	purple
Cholesterol	.06	orange
Methyl oleate	.57	
Methyl palmitate	.52	
Palmitic Acid	(.19-.34)[a]	
B Carotene	.85	blue
Vitamin A acetate	.35	dark blue

[a]Streak instead of spot.

weak and faded in a short time. The spot at R_f .36 appeared to be the same as the one reported at R_f .28 in Table 8. Since higher concentration of crude extract were used when spraying with antimony trichloride the R_f would be slightly higher. To elute from the chromatoplate the compound which formed a stable, pink complex with antimony trichloride the solvent system was changed to 3:7 Skellysolve B-diethyl ether. Using this system the reactive compound, referred to as eluent I, moved to an R_f of about .60. The other eluted compounds were separated using the solvent system 90:10:1, Skellysolve B-ether-diethyl ether-acetic acid.

Table 11 shows the results of tests on five eluted compounds from the crude extract. None of these was a sterol since each gave a negative Liebermann-Burchard Test. All the eluents showed infrared absorption spectra indicating alkane and small amounts of carbonyl groups, although eluent II gave a negative test for ester groups, This compound was yellow in the untreated state and brown in the presence of antimony trichloride. Eluent I had the sweet smell similar to natural honey and may be the compound reported by Schuler and Vogel (14) which appeared in their ethereal oil and gave a positive terpene reaction with antimony trichloride. The infrared spectrum of eluent I is shown in Figure 3. The spot tests for phosphorus were not very reliable since the blank also gave

Table 11. Results of tests on the eluted compounds from the crude extract

Eluent	Esters of Carboxylic Acids	Liebermann-Burchard Test	R_f	Reaction to $SbCl_3$	Test for Phosphorus
I	+	-	.07	pink	-
II	-	-	.28	brown	+
III	+	-	.61	none	-
IV	+	-	.74	none	-
V	+	-	.86	none	-

a slight positive test.

The most probable source of lipid in cotton honey is the pollen. However, it must not be in the granular form since Todd and Varsell (40) report no pollen grains in 32 samples of cotton nectar; and also no cotton pollen grains were found in the cotton honey used in the above experiments. In addition the results of the thin-layer chromatography of the filter extract indicate that the particulate matter contains much of the lipid material. Other sources which must be considered are the nectar, the bee and beeswax; although oleic acid which was found in high concentration in the purified methyl ester is not generally found in beeswax.

SUMMARY AND CONCLUSIONS

1. The antibacterial factor was present in all honeys tested.

2. The antibacterial factor in honey was more effective against _M_. _flavus_ and _S_. _lutea_ than against _B_. _cereus_ and _B_. _subtilus_.

3. The antibacterial factor was dialyzable and was retained on a Seitz filter.

4. Neither the acidity nor the high carbohydrate concentration in honey were responsible for the antibacterial effect of honey.

5. The antibacterial factor was not separated from cotton honey by extraction with diethyl ether or vacuum distillation.

6. The crude lipid extract of cotton honey gave positive spot tests for glycerides, sterols and phosphorus. Quantitative methods showed 20.7 mEq/L. of free acid and 1.0 uEq/mg. of ester groups. The infrared absorption spectrum showed the typical bands of alkane and ester groups.

7. Gas-liquid and paper chromatography revealed palmitic and oleic acid in relative concentrations of 27 and 60% respectively, along with small amounts of lauric,

myristoleic, stearic, and linoleic acids.

8. Thin-layer chromatography of the crude extract revealed twelve lipids: ten unsaturated, one acidic, and three reactive to antimony trichloride.

9. With thin-layer chromatography similarities were noted between the lipids of the crude extract and the particulate matter (filter extract) of cotton honey, indicating pollen as the probable source of the trace lipids.

10. Five compounds in the crude extract were isolated by elution from thin-layer chromatograms. Four contained ester groups and two formed a color complex with antimony trichloride.

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