The free fatty acids and neutral lipids of the cuticular wax of the Mormon cricket, Anabrus simplex, Hald

by Joel Mackie Padmore

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Chemistry

Montana State University

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This thesis is dedicated to my wife, Carol, who has endured much and given her encouragement and moral support during the past four years.

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ABSTRACT

The composition of the free fatty acids and the neutral lipids of the Mormon cricket, Anabrus simplex, Hald., was studied by infrared spectroscopy and gas, column, and thin-layer chromatography. Free fatty acids were separated on the basis of unsaturation by column chromatography of their methyl esters on silver nitrate impregnated silicic acid. The presence of normal and branched chain saturated acids (C_8-C_{36}) and unsaturated acids (C_{14}-C_{24}) was demonstrated by gas chromatography on SE-30 silicone and EGS polyester columns. Structures of the major unsaturated acids, oleic, linoleic, and linolenic, were determined by infrared spectroscopy and reductive ozonolysis followed by gas chromatography. Neutral lipids were separated into chemical classes by column chromatography on various adsorbents. Wax esters, steryl esters, triglycerides, free sterols, diglycerides, monoglycerides, and traces of residual fatty acid were found. The fatty acid components were studied by transesterification followed by gas chromatography. The free sterols and non-saponifiables derived from the ester fractions were determined by gas chromatography on SE-30 and QF-1 silicone columns.

INTRODUCTION

The cuticular wax of insects has been found to be an important factor in the control of moisture balance in insects.[9,40] Abrasion of this layer frequently results in rapid dessication and death of the insect. Some insects, such as the cockroach, are restricted to moist habitats whereas other insects, such as the Mormon cricket, have the ability to survive in arid regions. A study of the nature of the cuticular lipids would lead to a greater understanding of one of the limiting factors in the geographical distribution of insects. Beament has postulated that retardation of water passage through the cuticle of the cockroach is due to the presence of a mono-layer of fatty alcohols at the lower interface of the cuticular wax.[7] In a recent study Gilby and Cox[18] showed that the lipids of the cockroach included fatty acids, aliphatic aldehydes, esters, and hydrocarbons, but no fatty alcohols; thereby partially refuting Beament's postulated mechanism of moisture control.

There have been few investigations into the chemical composition of cuticular waxes. Partial analyses have been carried out on cuticular waxes from ticks[17] and silkworm larvae.[2,32] Possibly the most complete study of an insect cuticular wax present in the literature is that of Gilby and Cox.[18] The nature of the hydrocarbons, aldehydes, and fatty acids were fairly completely elucidated. Little or no work was done to characterize the esters or to confirm the trace quantities of sterols reported.

The original investigation into the lipid composition of the cuticular wax of the Mormon cricket was initiated by J. H. Pepper and E. J. Hastings in the mid-1940's.[29] The object of the study was to learn more about the

control of water balance by studying the chemical composition of the insect cuticle. The Mormon cricket was selected because of its availability, large size, and economic importance in Montana. At that time insect waxes were generally believed to be relatively simple mixtures of long chain aliphatic alcohols, long chain aliphatic esters, and long chain aliphatic acids. The extracted cuticular wax was subjected to saponification, resulting in intractable emulsions. About 1957 the project was revived by Baker[5] who undertook a spectrophotometric and chromatographic study of the wax. This study showed the presence of esters, fatty acids, hydrocarbons, acidic resins, and possibly cholesterol. The acids and hydrocarbons were partially characterized, but no work was done on the esters and the presence of cholesterol was not confirmed.

The object of the present study has been to characterize as completely as possible the esters, free sterols, and free fatty acids. This has included the determination of double bond position and configuration of the major unsaturated acids and the identification and quantitative determination of the acids, fatty alcohols, and sterols of the various ester fractions. Methods were developed and/or modified to permit the analysis of the various fatty acids, sterols, and esters present.

EXPERIMENTAL PROCEDURE AND RESULTS

Isolation of Wax

Crickets used in this study were collected during the summers of 1961 and 1962 from various locations in southeastern Montana. The live crickets were quick frozen and stored in a freezer until excision of the cuticles. No selection was made as to sex but only adults past the fifth moult were used. The cuticles were excised under water and brushed lightly to remove adhering cellular material. The excised cuticles were dried and stored frozen until extracted.

Initial studies were performed on wax isolated by soxhlet extraction. The cuticles were held in a cup of chloroform-washed cheese cloth and extracted with chloroform for about twenty hours. The solvent was removed and the crude wax recovered.

Final studies were made on material extracted from 1962 cuticles at room temperature. Cuticles and chloroform were mixed in a large stainless steel blender and homogenized for five minutes The homogenate was allowed to stand for about ten minutes and was then filtered to remove the ground cuticles. The chloroform was reduced to about ten ml on a rotary evaporator under reduced pressure and then taken to dryness under a stream of nitrogen.

The wax isolated by both methods had similar characteristics. It was yellow-green in color, had a characteristic odor, and a softening range of 25-50 degrees. The crude wax obtained by either method was designated wax K.

Wax K was taken up in hexane and allowed to stand for about one hour. The hexane solution was filtered to remove the acidic resins and other

hexane insolubles. The filtered hexane solution was immediately passed through a Kies[22] countercurrent apparatus to remove the free fatty acids. The three stages contained 0.01N KOH, 0.005N KOH, and water respectively. Total time for an average Kies extraction was about 60 hours. Ethanol was added dropwise to the first and second stages as needed to reduce foaming.

The extracted hexane solution was taken to dryness and designated wax Kn. The infrared spectrum of Kn is shown in figure 1. The intensity of the bands at 720 and 730 cm^{-1}, the ratio of the carbon-hydrogen deformation bands at 1380 and 1470 cm^{-1}, and the intensity of the carbon-hydrogen stretching vibration near 2900 cm^{-1} indicate long hydrocarbon chains. The ester carbonyl at 1740 cm^{-1} is characteristic of that observed in triglycerides and steryl esters. The carbon-oxygen stretch near 1200 cm^{-1} is also similar in pattern to that observed for triglycerides. A small amount of fatty acid is probably still present as indicated by the shoulder at 1705 cm^{-1}, the characteristic carbonyl stretch of an aliphatic acid. Weak bands near 3300 cm^{-1} suggest the possibility of hydroxy compounds such as alcohols, sterols, monoglycerides, and diglycerides.

The combined aqueous solutions from the Kies apparatus were acidified to pH 3 with 1.0 N sulfuric acid and estracted three times with diethyl ether in 300, 300, and 200 ml portions. The combines ether extracts were washed with 25 ml of water and dried over anhydrous sodium sulfate. The ether was removed by evaporation on a rotary evaporator under reduced pressure to about 10 ml, then taken to dryness under a stream of dry nitrogen. The recovered acidic materials were designated was Ka. The infrared

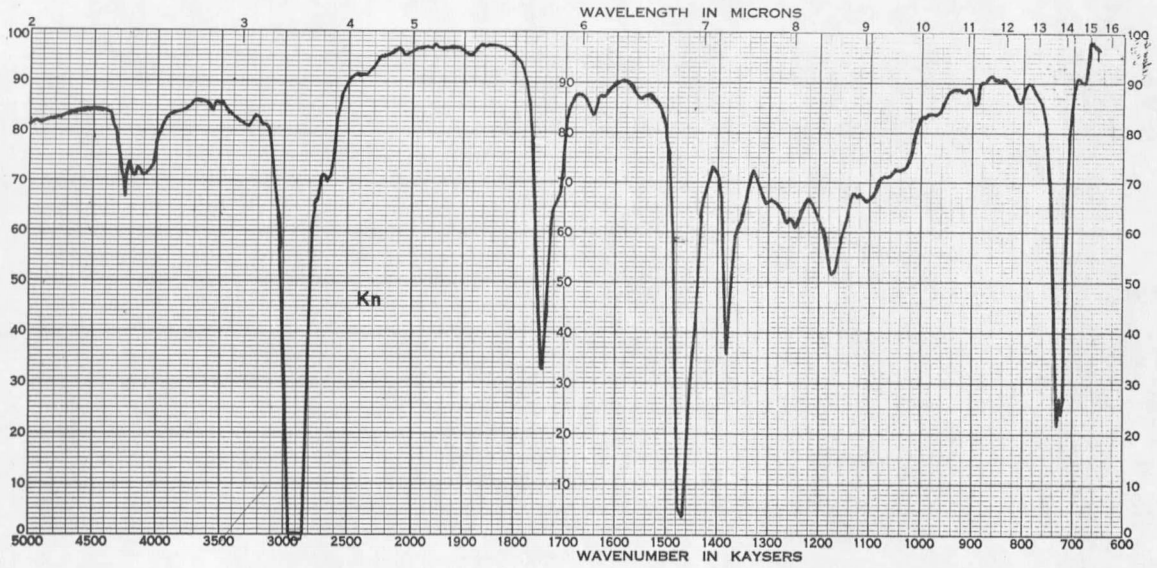

Figure 1. Infrared spectrum of Wax Kn (cap. film.)

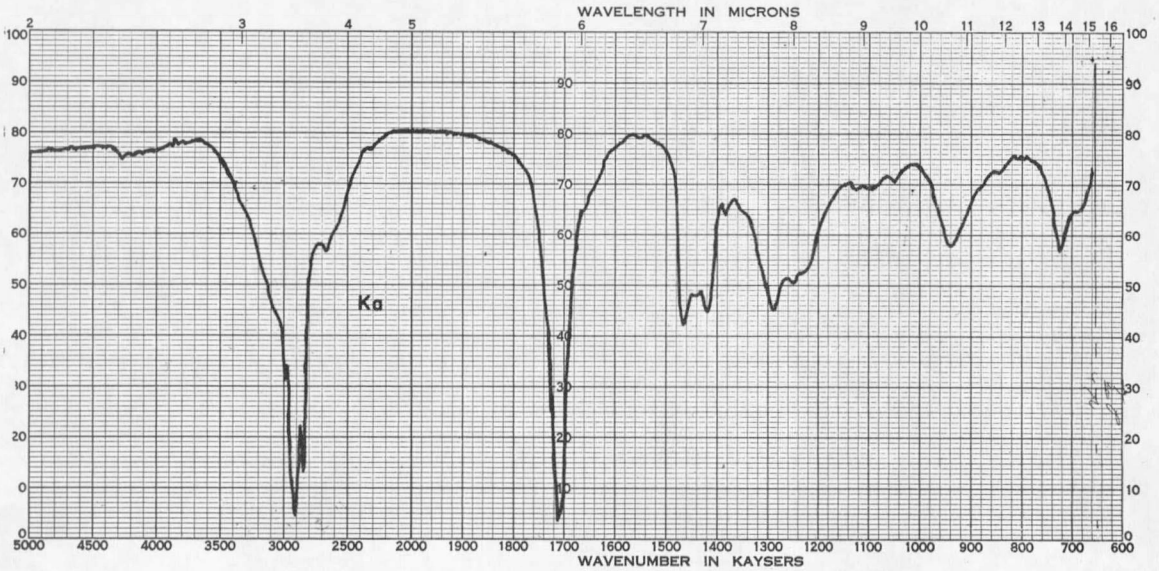

Figure 2. Infrared spectrum of Wax Ka (cap. film.)

spectrum, figure 2, is typical of spectra obtained from long chain fatty acids. The weak band just below 3000 cm^{-1} on the side of the carbon-hydrogen stretching vibration indicates the presence of unsaturation. Table I shows the relative amounts of hexane insolubles, neutrals, and acids obtained.

TABLE I

Composition of Wax K

Fraction	Weight Percent
hexane insolubles	1.3
Wax Ka	31.3
Wax Kn	67.4

Infrared Spectra

All infrared spectra were obtained on a Beckman IR-4 Spectrophotometer equipped with sodium chloride optics with presentation linear in wave-numbers. Spectra were taken as capillary films whenever possible. Spectra of solids were obtained in both carbon disulfide and carbon tetrachloride.

Column Chromatography

All chromatography columns, regardless of adsorbent used, were prepared in an identical manner. The weighed adsorbent was slurried with hexane and placed into a 1.2 cm column. The column was lightly tapped to insure uniform packing of the adsorbent; the adsorbent was covered with a filter paper

disk; and the prepared column washed thoroughly with hexane. The sample was introduced onto the column in a minimal amount of hexane and washed into the column with 1-2 m. of hexane. Care was taken at all times to insure that the column was covered with solvent.

In order to use a batch elution technique of column elution, each column was standardized for retention volumes of lipid classes. The lipid mixtures chosen for the standardization were selected to reflect as closely as possible the lipids to be encountered in the cuticular wax as determined by thin-layer chromatography. Sufficient columns were prepared for each separation to firmly establish retention volumes.

Thin-Layer Chromatography

Extensive use was made of thin-layer chromatography throughout the course of the investigation. Plates were prepared in a manner similar to that described by Mangold.[27] The adsorbent was slurried with water in a mortar for one minute, pouring the resultant slurry into a Desaga spreader, and rapidly spreading it over the plates. The plates were agitated for a few seconds while still wet to produce a more uniform layer. After air drying the plates were activated in an oven at 110°C for one hour. These activated plates were stored in a dessicator until used.

General lipid separations were performed on silica gel plates containing 0.04% Rhodamine 6 G indicator. The Rhodamine containing plates were prepared by adding the flourescent dye to the water used to prepare the slurry. The plates were developed with wicks in a 75/25/1 (v/v/v) hexane-diethyl ether-acetic acid solvent systerm. This solvent system gave a

separation in which steryl esters, triglycerides, sterols, diglycerides, and monoglycerides were identifiable when contained in the same mixture. Detection was made by viewing the developed plates under ultraviolet light both before and after exposure to iodine vapor followed by spraying with 50% sulfuric acid and charring in a drying oven. Detection levels for most lipids using this technique were around 0.1 microgram.[39]

Steryl esters and wax esters are not separable on silica gel by the usual solvents but may be separated without wicks using trichloroethylene as a solvent.[5] In this solvent system steryl esters migrate ahead of wax esters. It was discovered that a better separation could be affected by chromatographing steryl and wax esters on Anasil plates (Analabs, Hamden, Conn.) developed with wicks in a 90/10/1 (v/v/v) hexane-diethyl ether acetic acid solvent system. In this system wax esters migrate ahead of steryl esters. Detection was made by spraying the Anasil plates with dichlorofluorescein followed by iodine vapor and sulfuric acid as used with silica gel plates.

Mixtures of alcohols and sterols were separated by chromatography on silica gel plates developed without wicks in the solvent system 90/10 (v/v) benzene-ethyl acetate. Detection methods were similar to those previously described. The detection level of aliphatic alcohols was approximately 1 microgram and was in the neighborhood of 0.01 microgram for delta-5 sterols. During sulfuric acid char, sterols, and steryl esters develop an intense purple color. Straight chain alcohols develop a faint yellow color and lanosterol a reddish color.

Solvents

All solvents, whether technical or reagent grade, were found to contain several milligrams of impurities per liter[24] thus necessitating purification prior to use. Hexane (High Purity Normal Hexane, Phillips Petroleum Co.), reagent grade methanol, and reagent grade chloroform were found to be sufficiently purified by a simple distillation. Benzene was purified by stirring with concentrated sulfuric acid (5/1 v/v) for at least 8 hours prior to distillation. Diethyl ether was purified by distillation from freshly cut sodium metal. Purity of solvents was checked by concentrating 200 ml to less than 1 ml and chromatographing 50 microliters of the residue on silica gel thin-layer plates.

Gas Chromatography

Preliminary gas chromatograms were obtained using a Wilkens A90-C Aerograph equipped with a four filament thermal conductivity detector. Final gas chromatograms were obtained using an F&M Model 400 Biomedical Gas Chromatograph equipped with a flame ionization detector. Helium was used as a carrier gas at a standard flow of 65 ml per minute. Columns were constructed of 6 mm O.D pyrex glass tubing. Injection was made directly on column to circumvent the possible decomposition of sample in a hot metal injection block. Columns containing less than 3.0% liquid phase were packed on silanized 60/80 mesh Chromasorb W (John Mansville Inc.) and packed into silanized glass u-tubes. Hexamethyldisilazane was injected into the column prior to initial use in order to reduce sites which cause peak tailing and irreversible adsorption of sample. The 3.8% SE-30 column used in this study

was packed on 80/100 mesh Diatoport S (F&M Scientific Corp.). All other columns were packed on 60/80 mesh Gas Chrom P (Applied Science Labs.).

Preparation of Methyl Esters of Ka

In order to facilitate their handling in subsequent column and gas chromatograms the free fatty acids were converted to their methyl ester derivatives. The fatty acids were refluxed with boron trichloride-methanol or boron triflouride-methanol reagent (2 ml per 100 mg of acid) for five minutes. Identical results were achieved with either reagent. After reflux the reaction mixture was poured into 25 ml of water and extracted three times with 25 ml portions of hexane. The hexane extracts were combines, washed with 25 ml of water, then dried over anhydrous sodium sulfate. The hexane solution was evaporated to yield the fatty acid methyl esters, wax Kam.

The infrared spectrum of the methyl esters is shown in figure 3. Hydroxy methyl esters are indicated by the hydroxyl bands at 3350 and 3425 cm^{-1}. Unsaturation is indicated by the small band at 2980 cm^{-1}. Thin-layer chromatography on silica gel plates also indicated the presence of hydroxy methyl esters as well as more polar esters. The non-polar esters appeared to be unsaturated as indicated by the quantity of iodine vapor adsorbed on the above thin layer plate.[33]

Separation of Polar Methyl Esters

The methyl esters of the free fatty acids were chromatographed on a 20 g column of Florisil (Research Specialties, Richmond, Calif.) in order to separate the non-polar methyl esters from the polar methyl esters. The

non-polar methyl esters were eluted from the column with 100 ml of 10% diethyl ether in hexane. The hydroxy acid methyl esters and/or methyl esters of similar polarity were eluted from the column with 100 ml of 50% diethyl ether in hexane. The more polar methyl esters were eluted with 100 ml of 10% methanol in diethyl ether. A yellow pigment and other materials were not eluted from the column and not investigated. The three eluants from the florisil column were designated Kam-1, Kam-2, and Kam-3, respectively. The infrared spectra of the three methyl ester fractions is shown in figures 4 to 6. Table II shows the relative amounts of the individual methyl ester fraction derived from acids obtained from both 1961 and 1962 cuticles.

TABLE II

Composition of Methyl Esters, Kam

Year	% Kam-1	% Kam-2	% Kam-3	% not eluted
1961	83.9	3.9	0.6	11.7
1962	90.8	5.0	1.3	2.9

The fractions from the Florisil column were subjected to thin-layer chromatography on silica gel plates developed in a 75/25/1 (v/v/v) hexane diethyl ether-acetic acid solvent system. Fraction Kam-1 showed only a single spot characteristic of non-polar methyl esters such as methyl stearate or linolenate. Fraction Kam-2 showed a spot which migrated like methyl 12-hydroxystearate, plus other spots of a more polar nature. Kam-3

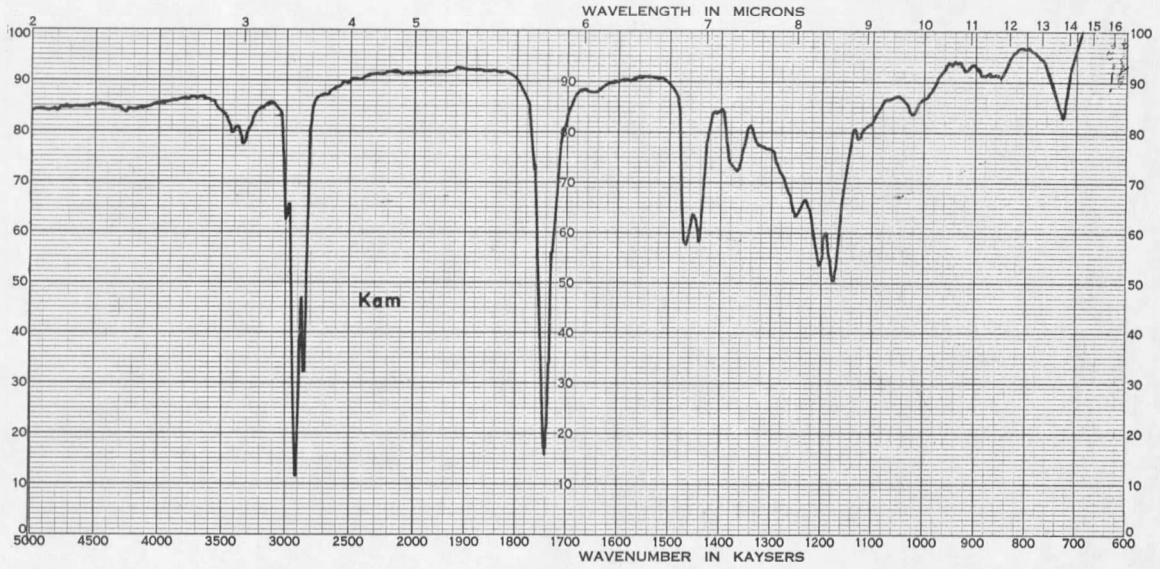

Figure 3. Infrared spectrum of Methyl esters Kam (cap. film)

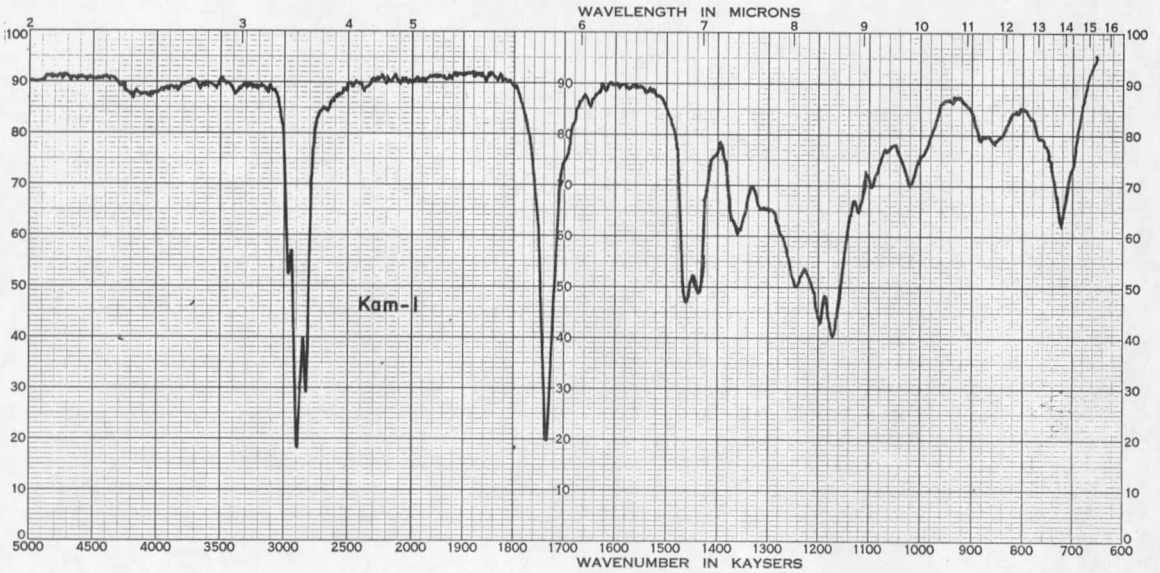

Figure 4. Infrared spectrum of Methyl esters Kam-1 (cap. film)

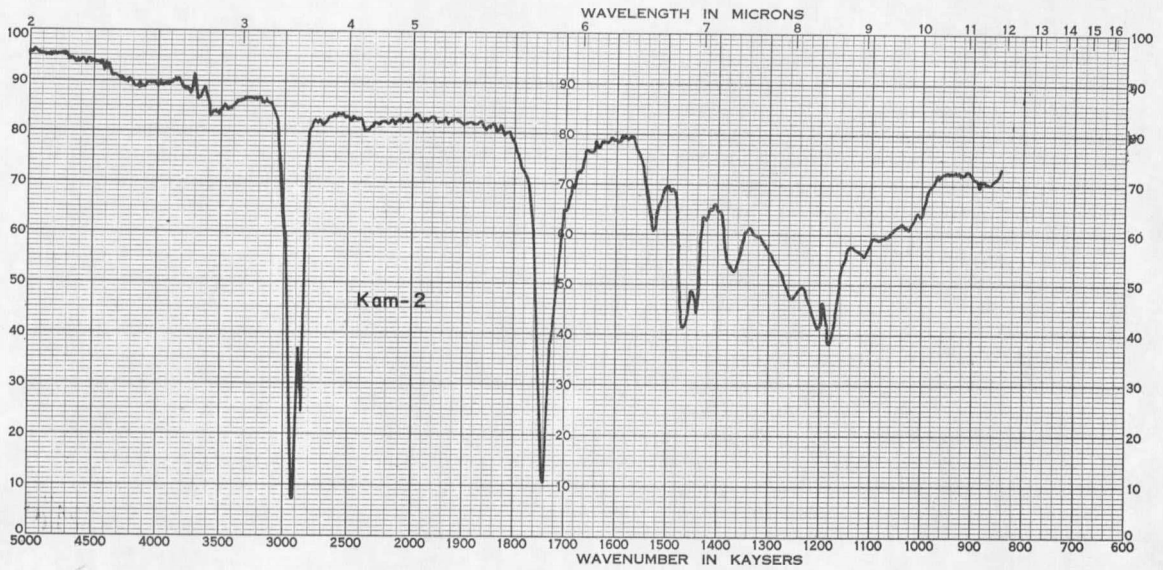

Figure 5. Infrared spectrum of methyl esters Kam-2 (CCl_4 sol.)

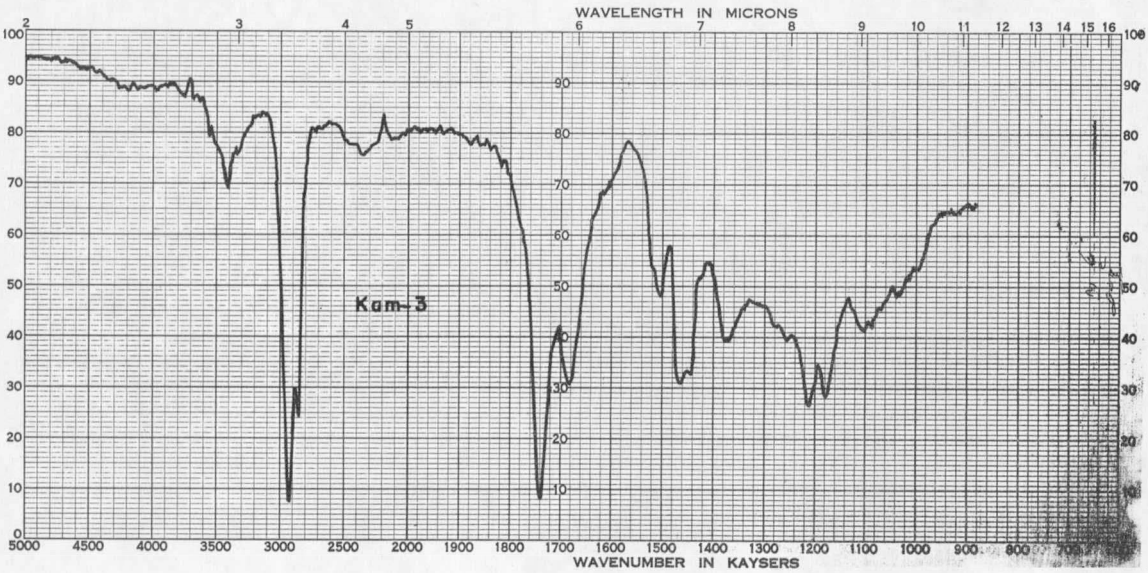

Figure 6. Infrared spectrum of methyl esters Kam-3 (CCl_4 sol.)

showed little material which migrated from the origin indicating materials of a high degree of polarity. Fraction Kam-1 was also chromatographed on silica gel plates containing 5% silver nitrate.[28] The plates were developed in a 50/50 (v/v) hexane-diethyl ether solvent system. The spots were detected by sulfuric acid char. Saturated, monoenoic, dienoic, and trienoic methyl esters were indicated.

Fractions Kam-2 and Kam-3 were analyzed by gas chromatography on a 4 foot 3.8% SE-30 silicone column at 195°C. The major peaks in the chromatogram of fraction Kam-2 appeared to be saturated and an unsaturated hydroxyoctadecanoate. Bromination of the sample followed by reinjection into the chromatograph brought about the disappearance of the peak thought to be the unsaturated hydroxy methyl ester. The assignments of chain length for the two methyl esters were based on the observation that the later major peak in the chromatogram had a retention time identical to that of methyl 12-hydroxy stearate. The peak immediately preceding it in the chromatogram, which disappears upon bromination, was also assigned the 18 carbon structure as unsaturated esters are eluted from SE-30 columns before their saturated analogs. No compounds were identified in fraction Kam-3. Representative chromatograms of these two fractions derived from 1961 acids are shown in figures 7 and 8. Corresponding fractions from 1962 acids exhibited minor quantitative differences.

Separation of Non-Polar Methyl Esters

Fraction Kam-1 was chromatographed on a 4 foot 15% ethylene glycol succinate polyester (EGS) column at 170°C. The chromatogram for the 1962

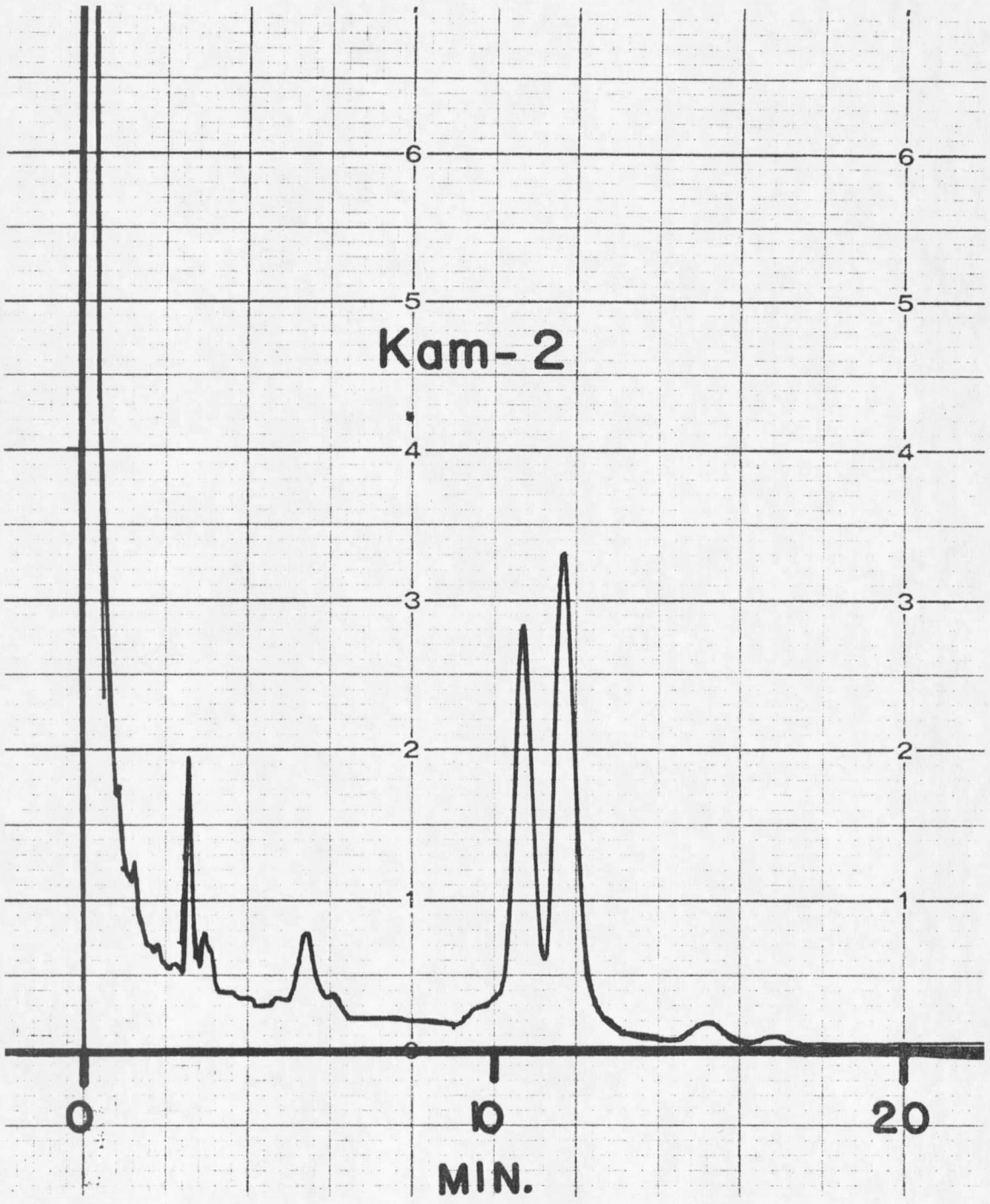

Figure 7. Gas chromatogram of Kam-2

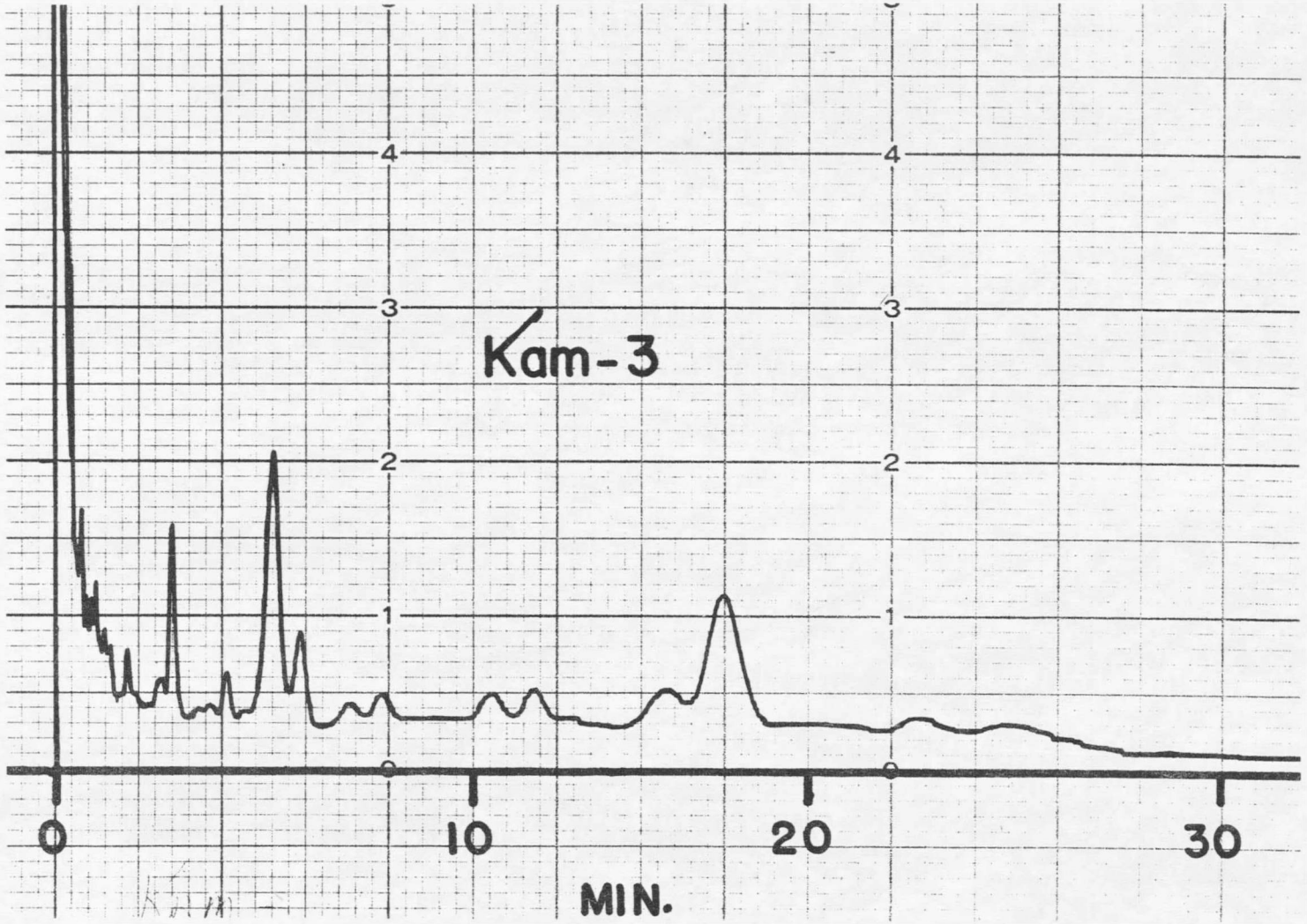

Figure 8. Gas chromatogram of Kam-3

methyl esters is shown in figure 9. Due to the complexity of the chromatogram obtained from the total non-polar ester fraction, it was decided to separate fraction Kam-1 into unsaturated and saturated methyl ester fractions. The methyl esters were accordingly chromatographed on a 25% silver nitrate-Adsorbosil column (Applied Science Labs, State College, Pa.).[13,14] Saturated methyl esters were eluted from the column with 40 ml of 18% benzene in hexane. Unsaturated methyl esters were isolated as a group by elution with 150 ml of 100% diethyl ether. The resultant fractions were designated Kam-1s and Kam-1u. The relative quantities of saturated and unsaturated fatty acids from 1961 and 1962 are shown in Table III. A flow diagram for the separation of methyl esters is shown in figure 10.

TABLE III

Composition of Fraction Kam-1

Year	% Kam-1s	% Kam-1u
1961	36.2	63.8
1962	24.0	76.0

Gas Chromatography of Saturated Methyl Esters, Kam-1

In order to determine the fatty acid methyl esters present in Kam-1s, they were chromatographed on EGS at 170°C. The resultant chromatograms indicated the presence of saturated esters from myristate to arichidate. No traces of unsaturated methyl esters could be detected by the disappearance or

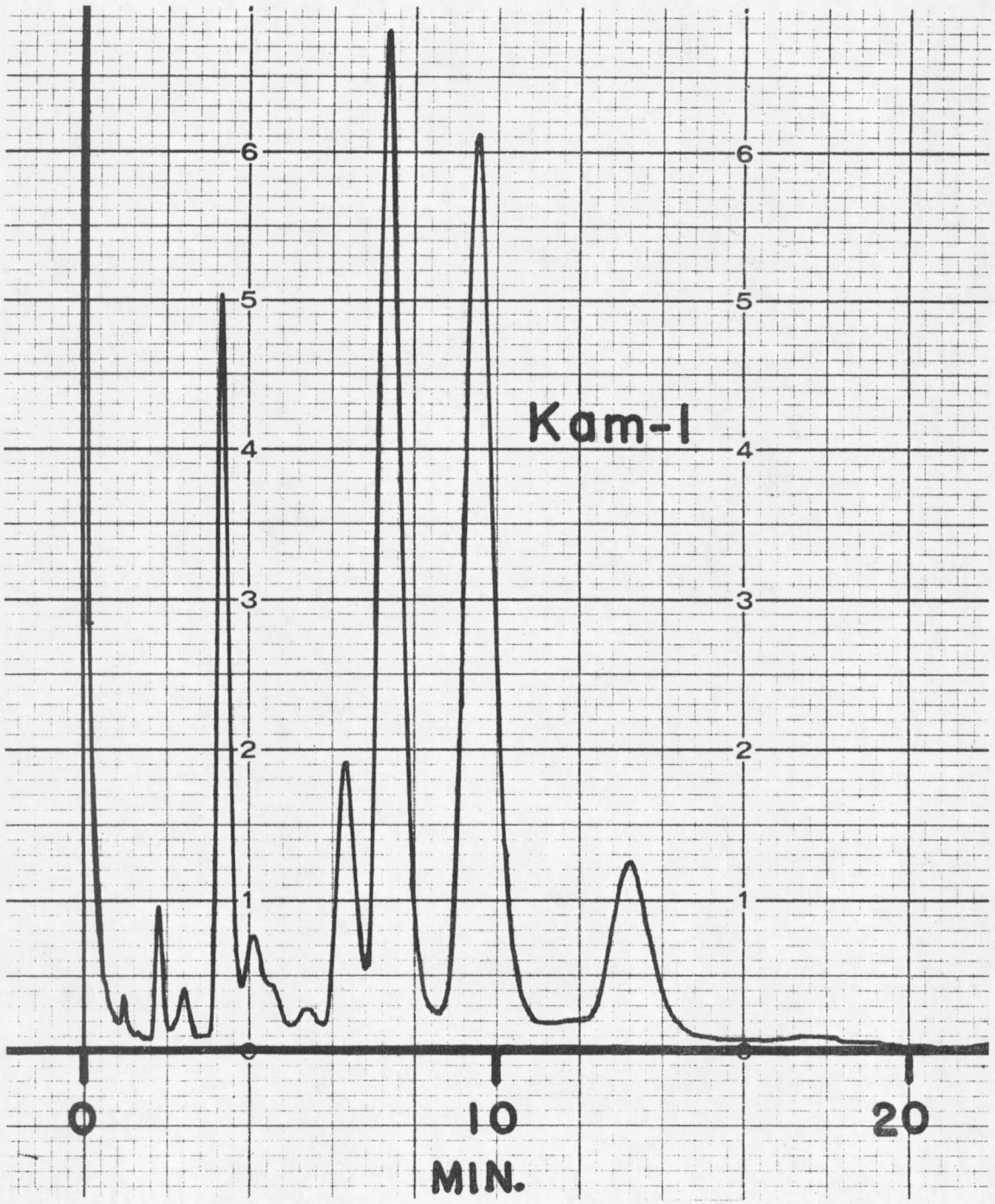

Figure 9. Gas chromatogram of Kam-1

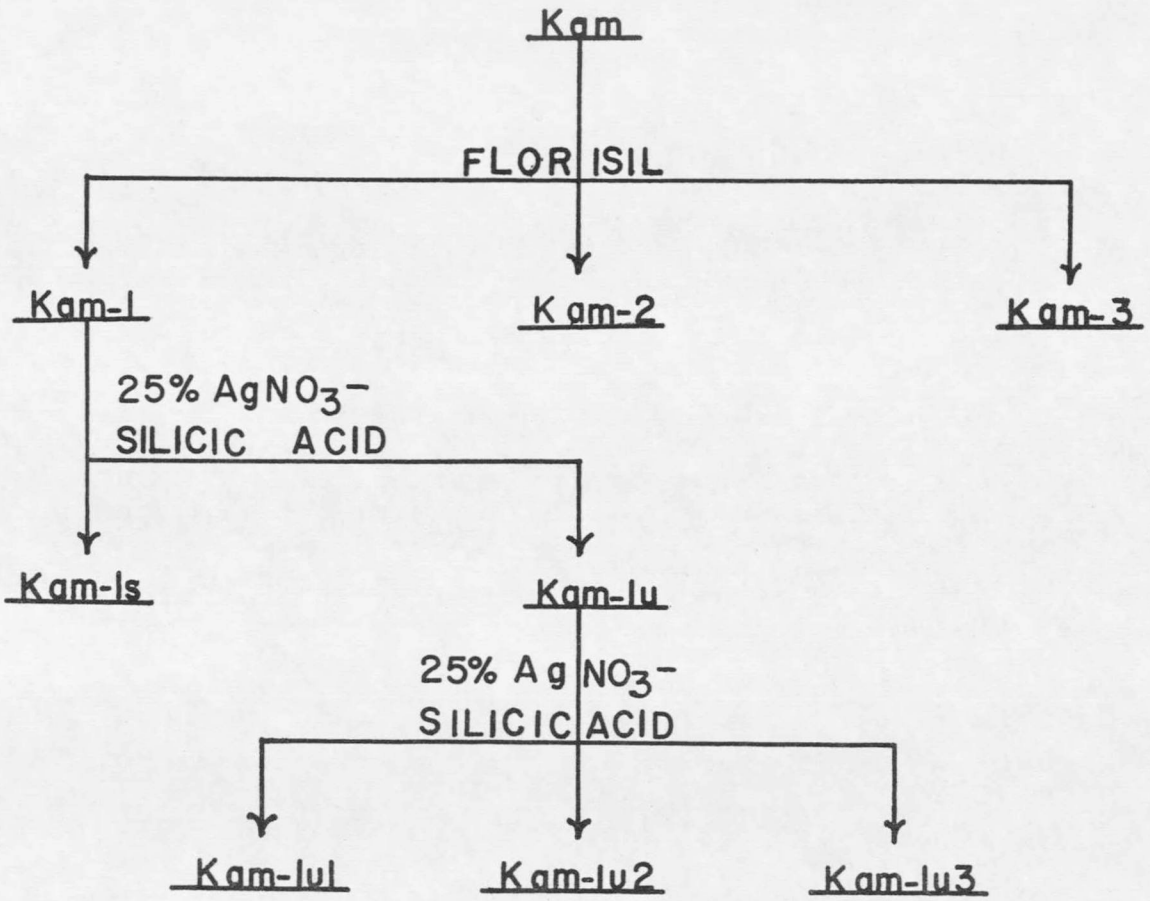

Figure 10. Chromatographic separations of free fatty acid methyl esters

shift of a peak when chromatographed subsequent to bromination of the sample. A representative chromatogram of 1962 methyl esters on EGS is shown in figure 11. Fraction Kam-1s was also chromatographed on SE-30. A programmed temperature gas chromatogram from 100 degrees to above 300 degrees shows the presence of saturated normal and branched chain methyl esters from laurate to tricontanate and above. A portion of the chromatogram from 1962 methyl esters is shown in figure 12. Isothermal chromatography on SE-30 at 80°C showed the presence of trace amounts of acids from C_8 to C_{11}. Programming on SE-30 from 200° indicated the presence of acid esters up to C_{36} and beyond.

Quantitative Analysis of the Saturated Methyl Esters

Fraction Kam-1s was quantitatively analyzed by a combination of gas chromatographic techniques. The normal acids from C_8 to C_{24} were analyzed by gas chromatography on EGS at 120, 170, and 195°C. Correlation was made between chromatograms by means of common peaks. Methyl laurate (C_{12}) was used to relate the chromatograms prepared at 120 and 170°C; methyl arachidate (C_{20}) was used to relate the chromatograms prepared at 170 to 195°C. Percentage composition was determined through the use of a Disk integrator attachment on a 1 mv Brown recorder. Chromatograms were prepared in triplicate and the values averaged. Data on branched chain methyl esters and normal esters of carbon numbers higher than C_{24} were obtained from the programmed temperature runs on SE-30. A portion of a typical chromatogram is shown in figure 11. Peak heights were taken to be proportional to the amount of component present in a sample as peak widths remained nearly constant

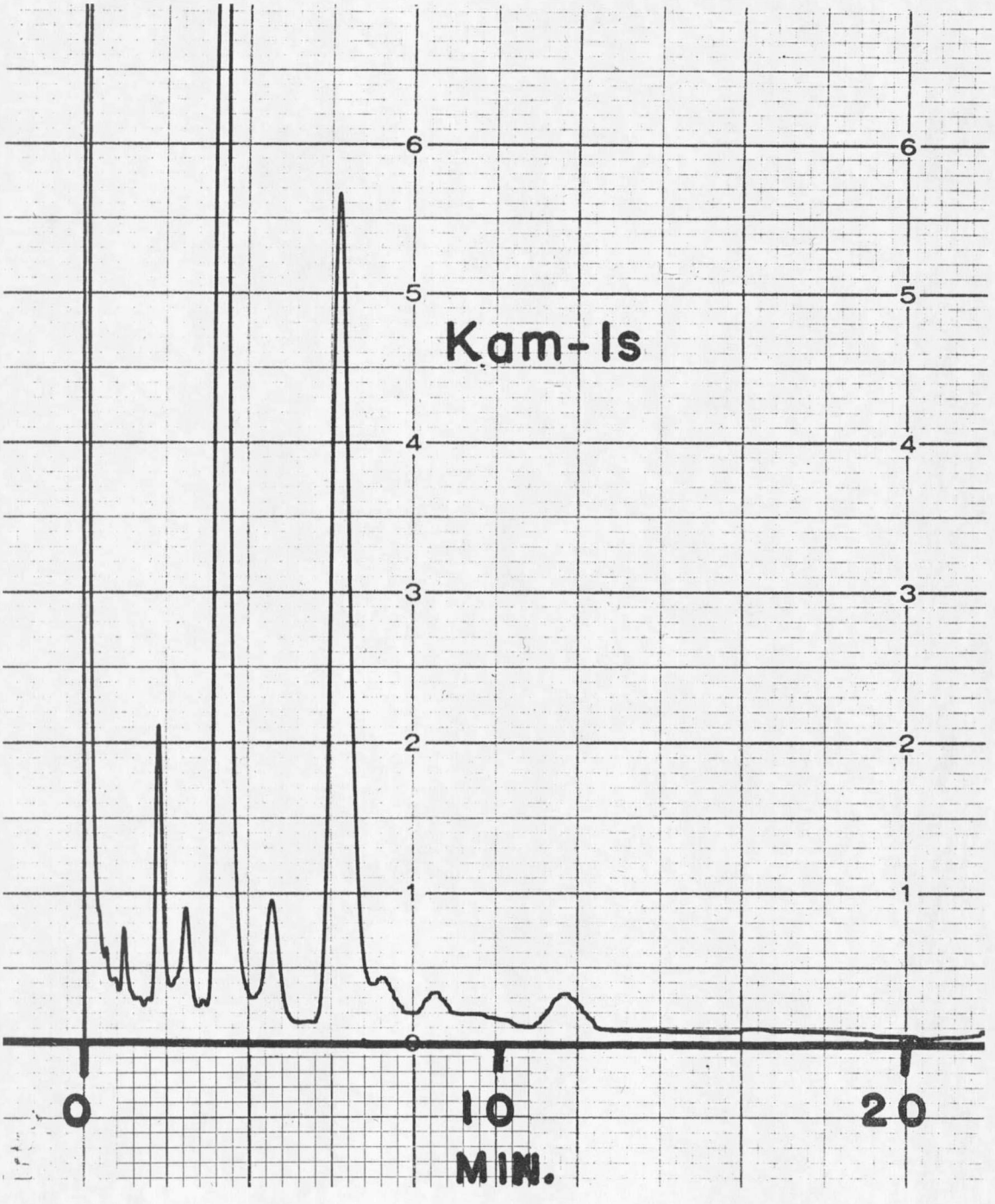

Figure 11. Isothermal gas chromatogram of Kam-ls

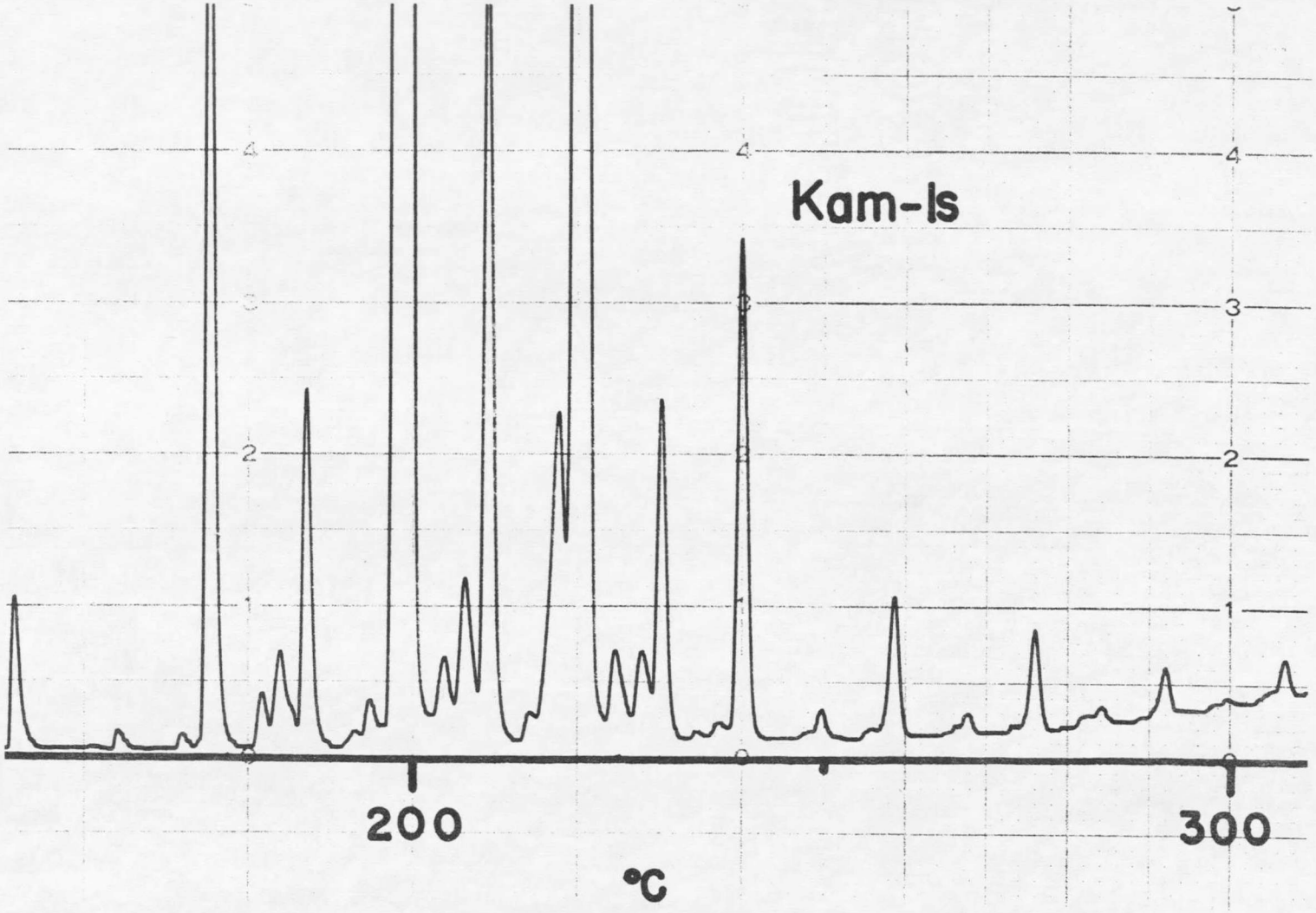

Figure 12. Programmed temperature gas chromatogram of Kam-ls

throughout the runs. The peaks of methyl myristate (C_{14}) and methyl arachidate (C_{20}) were used to correlate data from the programmed temperature chromatograms on SE-30 with the isothermal chromatograms on EGS. Normal short chain methyl esters from C_8 to C_{11} were present in trace quantities for both 1961 and 1962 acids. Corresponding branched chain methyl esters were not detected. Both normal and branched chain acids from C_{22} up to at least C_{36} were also present in trace quantities. The composition of the normal and branched chain saturated methyl esters from C_{12} up to C_{22} is shown in Table IV.

Gas Chromatography of Unsaturated Methyl Esters

To determine the unsaturated acids present methyl ester fractions Kam-lu from 1961 and 1962 were chromatographed on SE-30 at 195°C. Unsaturated acids from C_{12} to C_{24} were found. As SE-30 silicone does not resolve the various unsaturated acids of the same carbon number but containing different numbers of double bonds, fraction Kam-lu was chromatographed on EGS at both 170 and 195°C. A chromatogram of Kam-lu from 1962 acids is shown in figure 13. The mnemonic shorthand of Stoffel[35] and Farquhar[16] will be used to describe unsaturated fatty acid methyl esters. In this convention two numbers separated by a colon are used; the first number is the number of carbon atoms in the molecule and the second is the number of double bonds in the molecule. The major components as determined by peak sizes on SE-30 appeared to be C_{18} acids. The positions of the peaks on EGS indicated these methyl esters to be 18:1, 18:2, and 18:3. The retention times of the three methyl esters were identical to those obtained

TABLE IV

Composition of Saturated Methyl Esters, Kam-1

Carbon #	% 1961	% 1962
12	0.2	0.3
13br	T	T
13	T	T
14br	T	T
14	4.4	4.4
15br	0.1	0.2
15br	0.3	0.4
15	1.7	1.4
16br	0.1	0.1
16br	0.2	0.1
16	53.0	51.0
17br	0.5	0.4
17br	0.7	0.7
17	2.8	3.2
18br	0.5	0.1
18br	1.0	1.4
18	31.1	31.4
19br	0.3	0.2
19br	0.2	0.3
19	0.6	0.7
20br	T	T
20br	0.2	T
20	0.9	1.9
21br	T	T
21br	T	T
21	0.1	0.1
22br	T	T
22br	T	T
22	0.2	0.3

T denotes less than 0.1% of fraction
br denotes branched structures

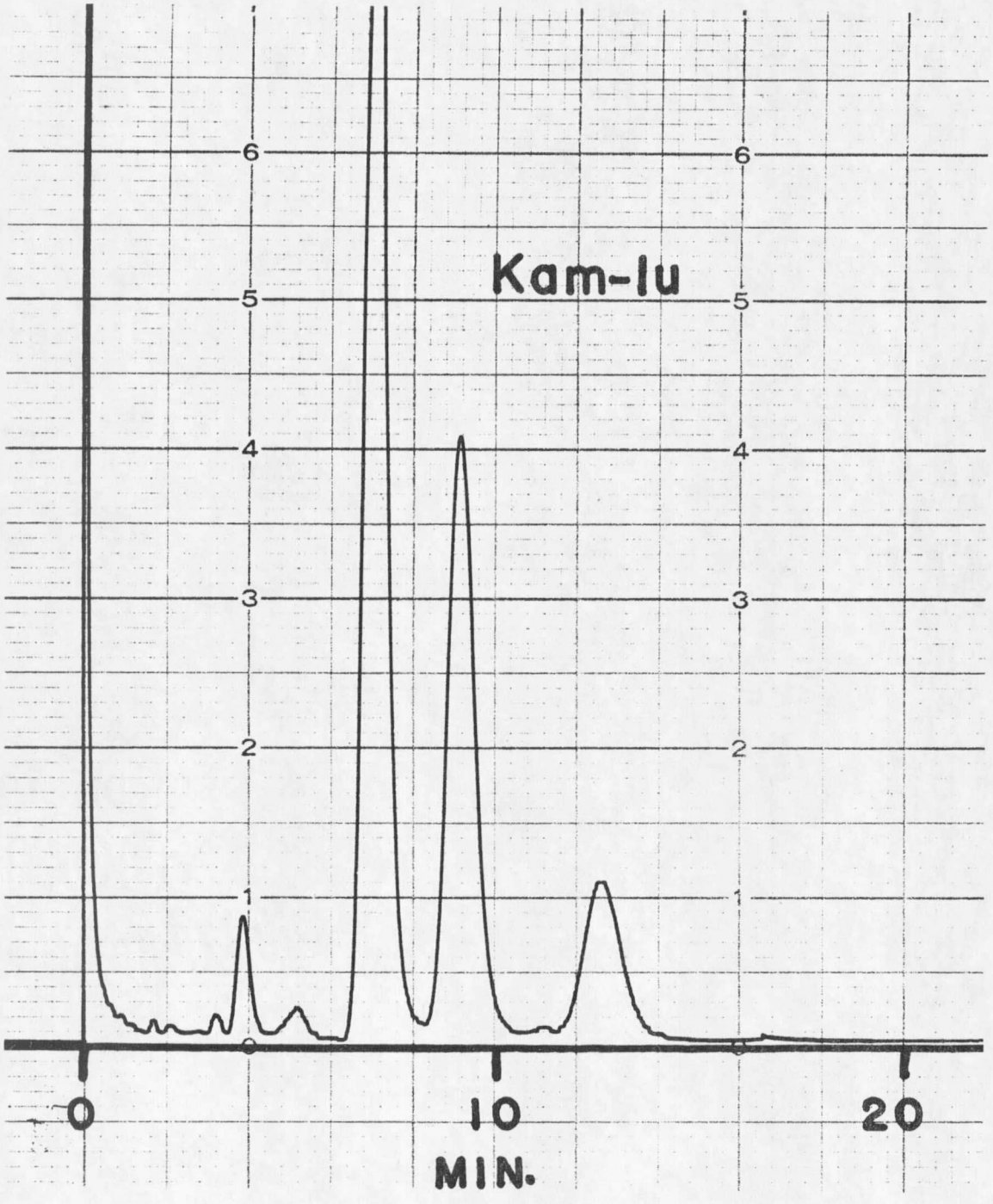

Figure 13. Gas chromatogram of Kam-lu

for oleate, linoleate and linolenate methyl esters.

Overlap of carbon numbers occur when chromatographing unsaturated acids on EGS, thus making peak assignments difficult. To make unequivocal assignments a portion of Kam-lu from 1961 was chromatographed on 25% silver nitrate-silicic acid column. Monoenoic acids were eluted with 100 ml of 3% diethyl ether in hexane, dienoic acids with 100 ml 12% diethyl ether in hexane, and trienoic acids with 200 ml 100% diethyl ether. The fractions so obtained designated Kam-lul, Kam-lu2, and Kam-lu3. Gas chromatography on the individual fractions on EGS showed that monoenoic esters present ranged from C_{14} to C_{24}, dienoic esters from C_{14} to C_{22}, and trienoic acids from C_{16} to C_{22}. The major methyl ester in each case was the C_{18} and accounted for at least 85% of the weight of the individual fractions. Gas chromatography of the unsaturated ester fractions indicated that the separations were about 95% complete.

Structure Determination of the Unsaturated Acids

Ozonolysis was used to determine the positions of the double bonds in the unsaturated acids. Ozonolysis has been extensively review as a technique for the determination of unsaturation in fatty acid methyl esters.[4] Both oxidative[1,12] and reductive [30,34] techniques have been used to cleave the ozonides formed from unsaturated fatty acid methyl esters. The reductive method afforded the best results when working with milligram quantities of unsaturated esters because the aldehydes and aldehyde esters produced may be directly analyzed by gas chromatography. In addition over-oxidation products are completely absent.[31,34]

The method used in this study was based on the method of Stein and Nicolaides.[34] The fatty acid methyl ester 1-2 mg, was dissolved in either 1 ml of dichloromethane at -70 or 1 ml of methyl caprate at -20°C. Ozone was bubbled into the solution through a capillary until the solution turned blue, indicating it was saturated with ozone. The excess ozone was removed by bubbling dry nitrogen through the reaction mixture for approximately 1 minute. A 2-3 molar excess of triphenyl phosphine was added directly to the reaction mixture to effect the reduction of the ozonides formed. After about 30 seconds the reaction mixture was removed from the cold bath, allowed to warm to room temperature, and a sample injected into the gas chromatograph.

The choice of solvent used was dictated by the nature of the fatty acid to be ozonized. Aldehyde products with a retention time shorter than that of caproaldehyde are buried in the solvent peak of dichloromethane and other volatile, ozone inert solvents. Thus polyunsaturated fatty acids often require ozonolysis in both solvents. The yield of aldehydes obtained by ozonolysis in methyl caprate is considerably lower than that obtained in dichloromethane due to the higher reaction temperatures and the decreased solubility of the triphenyl phosphine.

Each of the three unsaturated ester fractions gave the same aldehyde ester fraction upon ozonolysis in dichloromethane as determined by gas chromatography on EGS at 170°C. As this fragment was the same as that obtained from known samples of oleate, linoleate, and linolenate, each of the unknown C_{18} esters was assigned a 9,10 double bond. Thus the position of the double bond of the 18:1 methyl ester was the same as methyl oleate.

Chromatography of the reaction mixtures on EGS at 120°C showed the presence of pelargonaldehyde in the reaction mixture of Kam-lul, thus confirming the structure as a 9,10 double bond. No other short chain aldehyde was present in the mixture in detectable quantity. The only aliphatic aldehyde obtained from the reaction mixture of Kam-lu2 on EGS at 120°C was caproaldehyde, thus confirming its structure to be a 9, 12-octadienoic methyl ester. No short chain ester fragments were discernable for Kam-lu3 by chromatography on EGS. For this reason the trienoic acid fraction was also ozonized in methyl caprate and the reaction mixture analyzed by gas chromatography on a 20% XF-1150 silicone at 25°C. The only aldehyde found was propionaldehyde, thus confirming the structure as 9, 12, 15-octatrienoic methyl ester.

The infrared spectra of the three unsaturated methyl ester fractions correspond almost exactly to those obtained from methyl oleate, linoleate, and linolenate. The complete absence of a trans band at 960 cm^{-1} in the infrared spectra of the fractions Kam-lul and Kam-lu2 indicates that the double bonds must be in the cis configuration. A weak band near 960cm^{-1} in the spectrum of Kam-lu3 is not nearly as strong as that observed in the spectrum of methyl linolenate, so the majority of the double bonds in Kam-lu3 are probably in the cis configuration. Substantiating the cis configuration of the 18:1 is the occurence of only one spot corresponding to methyl oleate when the fraction is chromatographed on silica gel plates containing 5% silver nitrate. According to Morris[28] a mixture of oleate (cis configuration) and elaidate (trans configuration) would show two distinct spots. Thus the three major unsaturated acids are taken to be identical to oleic, linoleic,

and linolenic acids.

Quantitative Analysis of the Unsaturated Methyl Esters

Quantitative analysis of the methyl esters of the free unsaturated acids was performed on a 4 foot 15% EGS column at both 170 and 195°C. All chromatograms were run in triplicate and the values averaged. Correlation was made between the two sets of chromatograms by means of the common methyl linolenate peak. Table V shows the composition of the methyl esters derived from the unsaturated acids. Peaks not resolved have a combined percent listed. The major component in the unresolved peaks is underlined. In the case of the 18:1 and 18:3 peaks, the C_{18} acids make up more than 95% of the total peak as indicated by gas chromatograph of the monoenoic and trienoic methyl ester fractions.

TABLE V

Methyl Esters of the Free Unsaturated Acids, Kam-lu

	% Kam-lu 1961	% Kam-lu 1962
14:1	T	T
14:2	T	T
15:1	T	T
16:1	3.0	2.4
16:2	0.9	0.3
17:1	T	T
*18:1-16:3	54.3	39.9
18:2	29.1	44.8
*18:3-20:1	9.8	11.3
20:2	0.3	0.3
*22:1-20:3	1.0	0.7
22:2	1.3	T
*22:3-24:1	T	T

T denotes less than 0.1% of fraction
* not resolved under conditions used, major component underlined

Isolation of Neutral Lipids

Class separations of lipids are necessary to effectively study their composition by gas chromatography. A great deal of research has been devoted to the development of column chromatographic techniques for the separation of lipids. Most of these methods have been based on complete separations by one column.[8,10,20,21] Due to the complexity of the lipids obtained from the cuticular wax of the Mormon cricket, a more effective method has proved to be a combination of several columns in conjunction with preparative thin-layer chromatography.

The neutral lipids and hydrocarbons of wax Kn were chromatographed on a 4.5 g column of Unisil (Clarkson Chemical, Williamsport, Pa.), an activated silicic acid. Hydrocarbons were eluted from the column and accounted for 75% of wax Kn. The neutral lipids were eluted from the column in four fractions by the successive use of 18% benzene in hexane, 100 ml of 60% benzene in hexane, 75 ml of 100% benzene, and 100 ml 100% methanol.[8,21] The four fractions were designated Kno-1, Kno-2, Kno-3, and Kno-4, respectively. Thin-layer chromatography was used to analyze each of the fractions from the Unisil column. Kno-1 gave one spot corresponding to a mixture of wax and steryl esters plus a second spot which had migration characteristics similar to those of methyl or ethyl esters, or acetate esters of long chain alcohols. The infrared spectrum of Kno-1, figure 14, is similar to that obtained from known cholesteryl esters. The intense purple color developed by the spot on thin-layer after being sprayed with sulfuric acid and charred is indicative of delta-5 steryl esters. Kno-2 was found, by thin-layer chromatography, to be triglycerides. The infrared spectrum, figure 15, is similar to that of known triglyceride mixtures. Kno-3 was found to be composed of sterols and highly unsaturated triglycerides. Kno-4 appeared to be a mixture of monoglycerides, diglycerides, and free fatty acids. The infrared spectrum, figure 16, is similar to that of mono-olein.

Fraction Kno-1 was chromatographed on silica gel plates developed in trichloroethylene and on Anasil plates developed in 90/10/1 (v/v/v) hexane-diethyl ether-acetic acid in order to determine if wax esters were present. A spot was observed which migrated ahead of the steryl esters on the Anasil plates but behind the steryl esters on silica gel, thus indicating the

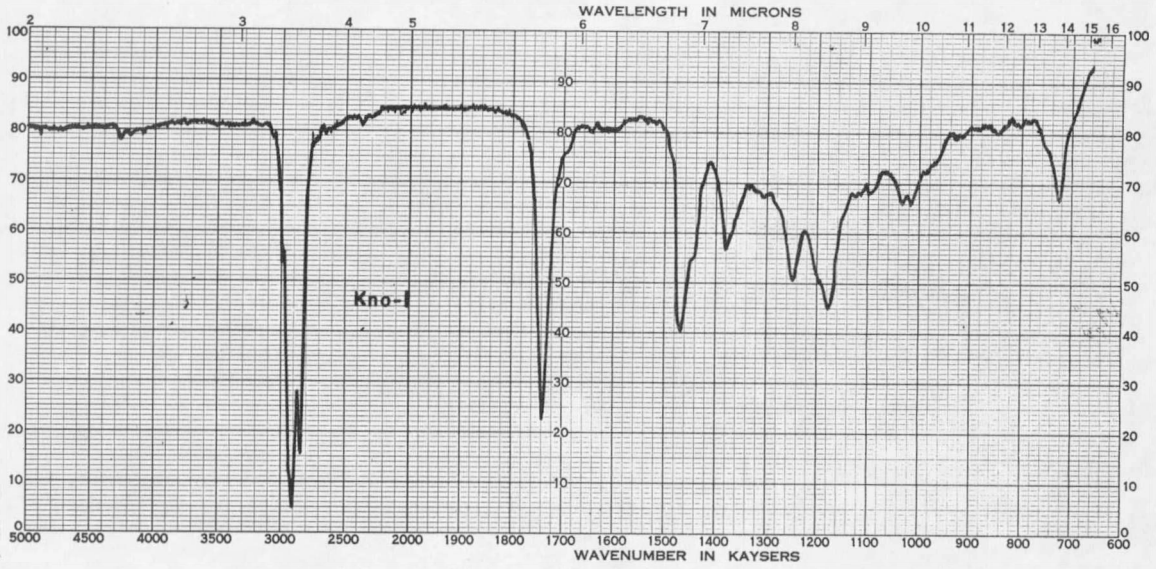

Figure 14. Infrared spectrum of Kno-1 (cap. film)

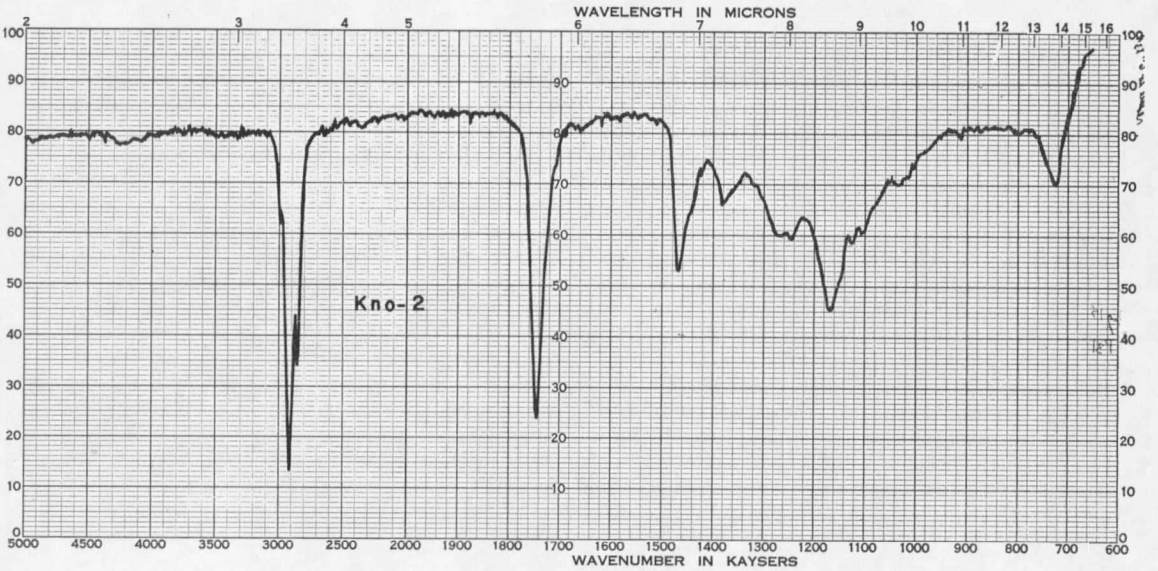

Figure 15. Infrared spectrum of Kno-2 (cap. film)

presence of wax esters.

Fraction Kno-1 was separated into two subfractions by means of preparative thin-layer chromatography on Adsorbisil -2 (no binder) plates. The Kno-1 was spotted in a continuous strip 2 cm above the bottom of the plate. The plate was developed in a 90/10/1 (v/v/v) hexane-diethyl ether-acetic acid solvent system until the solvent front had traveled 15 cm. The materials were located by chromatographing standards along the edges of the plate, spraying the standards with Rhodamine 6 G in ethanol, and viewing under ultraviolet light. The portions of the adsorbent containing the unknowns were scraped from the plate using the distances traveled by the standards as a guide. The adsorbent removed from the plate was slurried with diethyl ether and filtered through a fritted glass funnel of very fine porosity. The adsorbent was washed three times with 5 ml portions of diethyl ether. The solvent was taken to near dryness on a rotary evaporator, then taken to dryness under a stream of nitrogen. The wax and steryl ester fraction was designated Kno-1a and the second fraction, similar to acetates or methyl esters, was designated Kno-1b. No indication of silica gel was found in the isolated fractions. All materials in both fractions were soluble in hexane, leaving no undissolved residue. A blank plate run as a control failed to show the presence of either silica gel or extraneous lipids after elution.

The unsaturated triglycerides were removed from fraction Kno-3 by chromatography on an alumina column (Fischer, Fairlawn, N.J.). The triglycerides were eluted with 50 ml benzene and the sterols with 50 ml diethyl ether. The triglycerides recovered were added to fraction Kno-2.

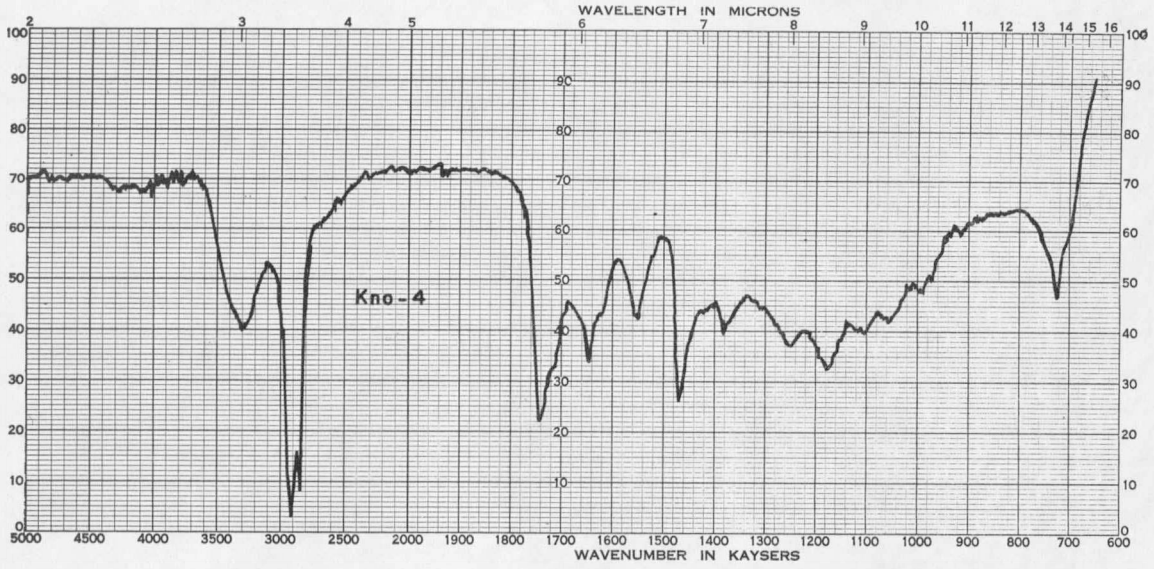

Figure 16. Infrared spectrum of Kno-4 (cap. film)

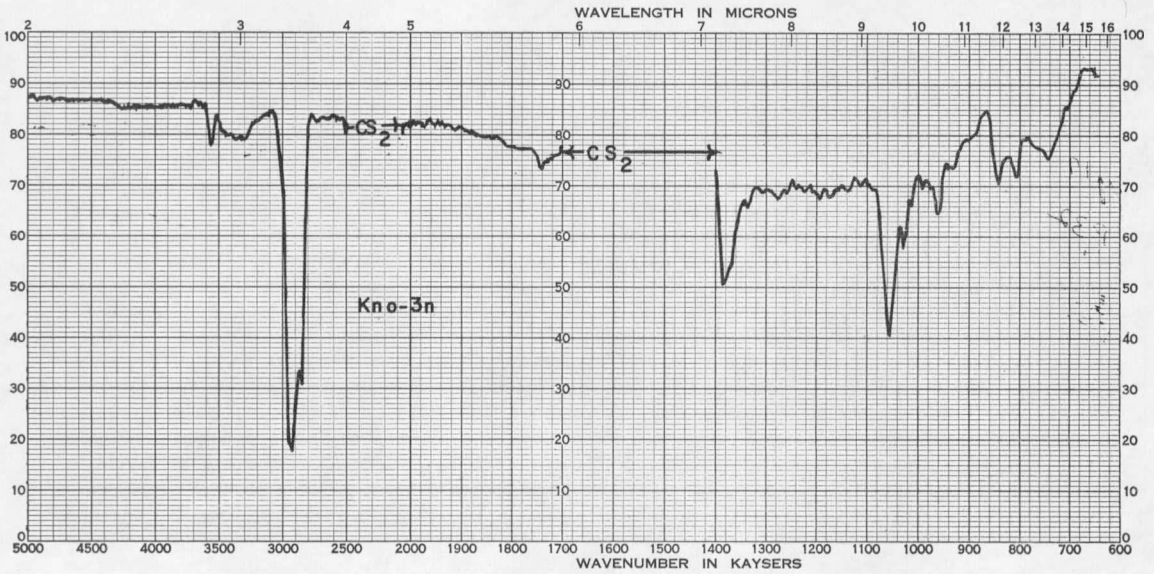

Figure 17. Infrared spectrum of Kno-3n (CS_2 sol.)

This fraction was then redesignated Kno-2m. The sterols contained very minor traces of diglyceride, indicating some hydrolysis of the triglycerides on the alumina column. The fatty acids released were not eluted from the column. The quantity of diglyceride present was just sufficient to give a faint spot when 75 micrograms of the sterol fraction was examined by thin-layer chromatography. The infrared spectrum of the sterols, Kno-3n, is shown in figure 17. The trace of diglyceride is indicated by the small ester carbonyl band near 1740 cm^{-1}. The remainder of the spectrum is identical to that obtained for cholesterol.

Fraction Kno-4 was chromatographed on a 5 g Florisil column to separate the monoglycerides, the diglycerides, and the fatty acids. Diglycerides were eluted with 100 ml 50% diethyl ether in hexane, monoglycerides by 100 ml 10% methanol in diethyl ether, and fatty acid by 50 ml 2% acetic acid in diethyl ether. The respective fractions were designated Kno-42, Kno-4b, and Kno-4c. The composition of Kno by lipid class is shown in Table VI. Figure 18 shows the separation scheme for the neutral lipids.

TABLE VI

Composition of Kno by Lipid Class

Fraction	Weight Percent
Kno-1a	22.6
Kno-1b	9.1
Kno-2m	36.5
Kno-3n	9.1
Kno-4a	4.9
Kno-4b	12.3
Kno-4c	5.5

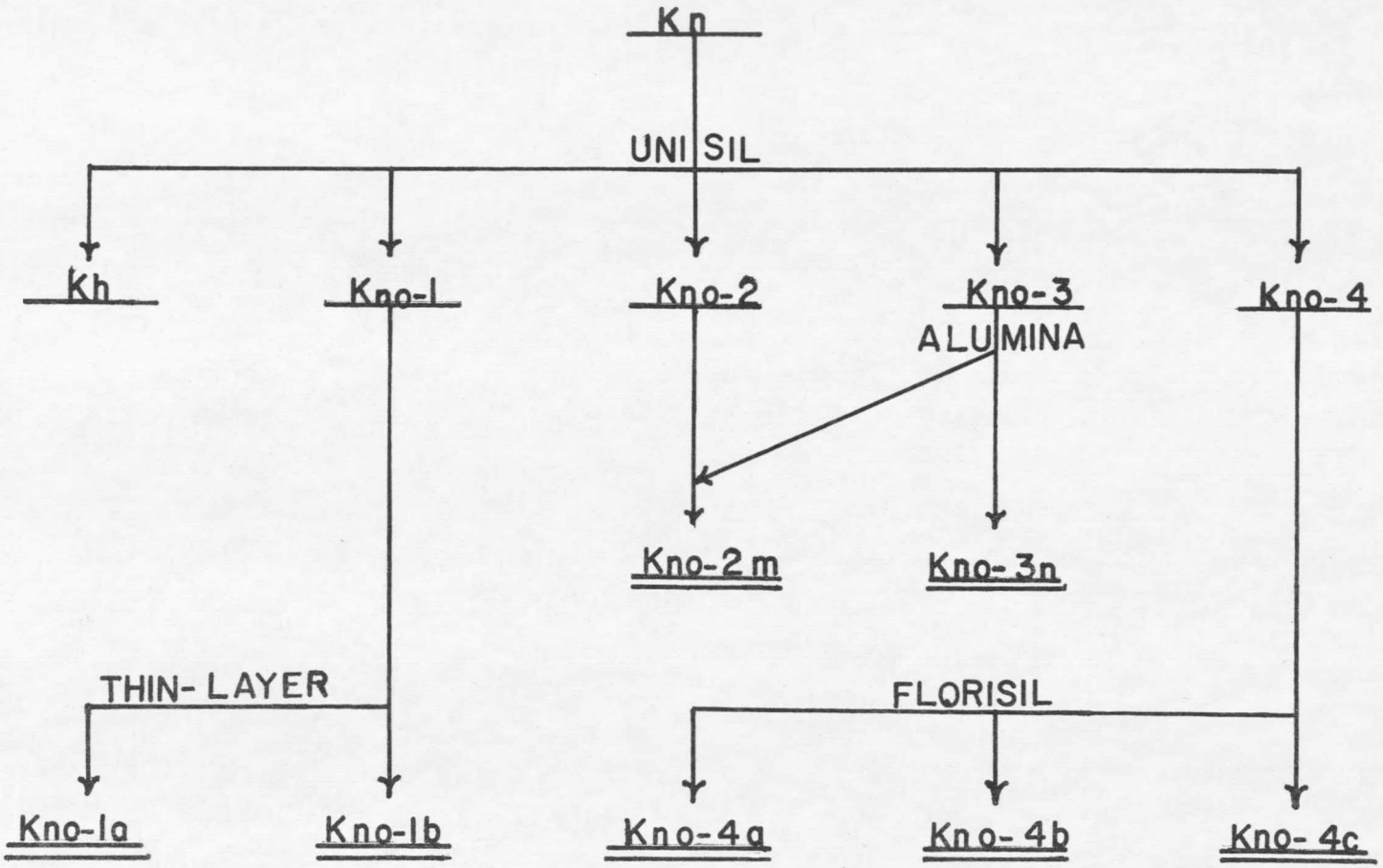

Figure 18. Chromatographic separations of the neutral lipids

Transesterification of Ester Fractions

The various ester fractions were transesterified by the method of Luddy et al.[26] by refluxing in sodium methoxide-methanol solutions. The sodium methoxide was freshly prepared immediately prior to use by dissolving sodium balls in redistilled methanol. Steryl and wax esters were transesterified with 10 ml of 0.4N solution for 1 hour. The other ester fractions were transesterified with 0.1N methoxide for fifteen minutes. The reaction mixtures were acidified with 0.5N sulfuric acid in methanol after reflux was completed. The acidified reaction mixtures were poured into 25 ml of water and extracted five times with 25, 25, 15, 15, and 15 ml portions of hexane. The hexane extracts were combined, washed with 10 ml of water, and dried over anhydrous sodium sulfate. The hexane was removed on a rotary evaporator to about 2 ml. Fractions Kno-2m, Kno-4a, Kno-4b were taken to dryness with a stream of nitrogen. The hexane solutions of Kno-1a and Kno-1b were chromatographed on Florisil to separate the non-saponifiables from the methyl esters. The methyl esters were eluted with 100 ml 15% diethyl ether in hexane and the non-saponifiables were removed with 100 ml 10% methanol in diethyl ether. The methyl esters so removed were designated Kno-1am and Kno-1bm; the nonsaponifiables were designated Kno-1an and Kno-1bn. Fraction Kno-4c was converted to its methyl esters with boron trichloride methanol reagent in a manner identical to that used for the free acids. Thin-layer chromatography was used to monitor each of the transesterification mixtures for completeness of reaction. All reactions went to apparent completion except for the wax and steryl esters. Small quantities of steryl ester could be detected in the reaction mixture after

one hour of reflux.

Quantitative Analysis of Methyl Esters of Neutral Lipids

The methyl esters from the transesterification were chromatographed on EGS at 170°C. Chromatograms obtained from the methyl esters are shown in figures 19 to 24. To simplify quantitative analysis the methyl esters were chromatographed on columns of 25% silver nitrate-Adsorbosil to separate the saturated acids from the unsaturated acids. The fatty acids were then gas chromatographed on EGS at both 170 and 195°C. Percentages were computed through the use of a Disk integrator. Correlation was made between chromatograms, prepared in triplicate, obtained at the two temperatures by the comparison of common peaks. Table VII shows the patterns of the saturated fatty acids of the neutral lipids. Table VIII shows the patterns of the unsaturated acids. Only crickets collected during the summer of 1962 were used for the study of the neutral lipids.

Analysis of Sterols and Alcohols

Free sterols and sterols from the steryl ester fractions were analyzed by gas chromatography on SE-30 and QF-1 silicone gas chromatography columns. Due to the peak overlap in the sterols, percentage composition was determined by triangulation of the peaks. Figures 25 and 26 show the chromatograms of the free and esterified sterols. The gas chromatograms were obtained on a 4 foot 3.8% SE-30 column at 250°C. The sterol fractions were also chromatographed on a 4 foot 0.75% QF-1 fluorinated silicone column at 202°C to substantiate the identification of the

TABLE VII

Saturated Fatty Acid Methyl Esters of the Neutral Lipids

Carbon No.	Kno-1a	Kno-1b	Kno-2m	Kno-4a	Kno-4b	Kno-4c
8 to 12	T	T	T	T	T	T
13	T	T	0.1	T	T	T
14	5.1	6.2	5.6	10.6	6.1	4.0
15	6.1	1.3	1.2	1.9	2.3	1.5
16	56.3	54.6	55.7	56.5	49.8	39.2
17	7.2	2.7	2.5	5.7	5.3	3.8
18	23.5	27.0	21.2	20.5	22.7	44.1
19	0.4	T	0.7	T	1.6	1.4
20	7.7	T	2.6	4.8	12.1	6.1

T denotes less than 0.1% of fraction

TABLE VIII

Unsaturated Fatty Acid Methyl Esters of the Neutral Lipids

	% composition					
Acid	Kno-1a	Kno-1b	Kno-2m	Kno-4a	Kno-4b	Kno-4c
14:1	0.7	T	T	T	T	T
14:2	0.4	T	T	T	T	T
15:1	0.3	T	T	T	T	T
16:1	7.1	1.8	2.5	4.3	4.9	2.4
16:2	1.0	0.4	0.3	T	T	T
17:1	T	T	T	T	T	2.6
*<u>18:1</u>-16:3	41.5	39.2	26.7	46.8	59.4	73.2
18:2	40.3	46.2	46.6	40.8	29.4	10.6
*<u>18:3</u>-20:1	7.6	2.1	11.6	6.4	5.6	3.3
20:2	0.2	0.2	0.2	1.6	T	5.1
*<u>22:1</u>-20:3	0.3	0.5	1.2	T	T	T
22:2	0.6	9.4	0.7	T	T	T
*<u>22:3</u>-24:1	T	T	T	T	T	T

T denotes less than 0.1% of fraction
* not resolvable under conditions used, major ester underlines

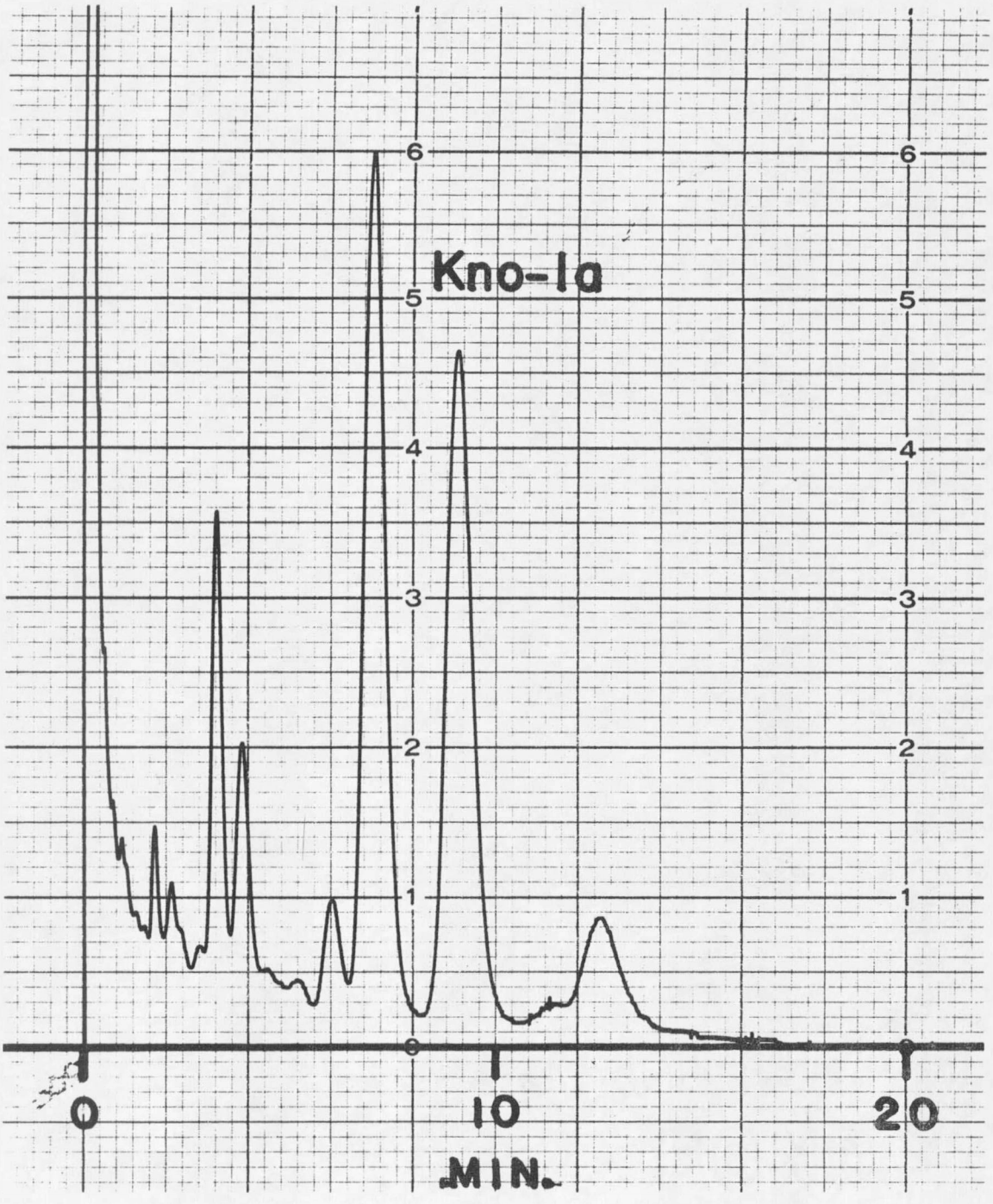

Figure 19. Gas chromatogram of the methyl esters of Kno-1a

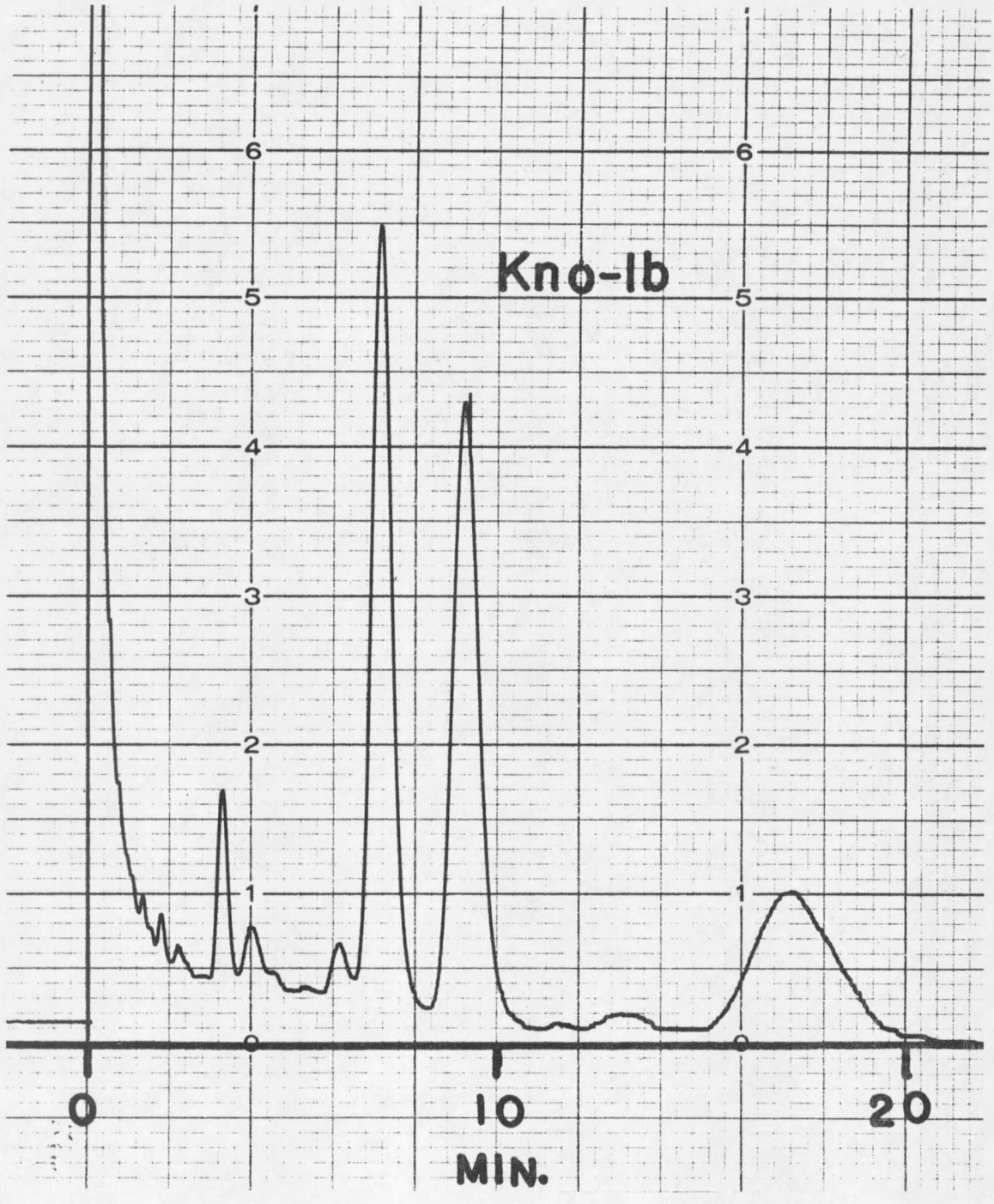

Figure 20. Gas chromatogram of the methyl esters of Kno-lb

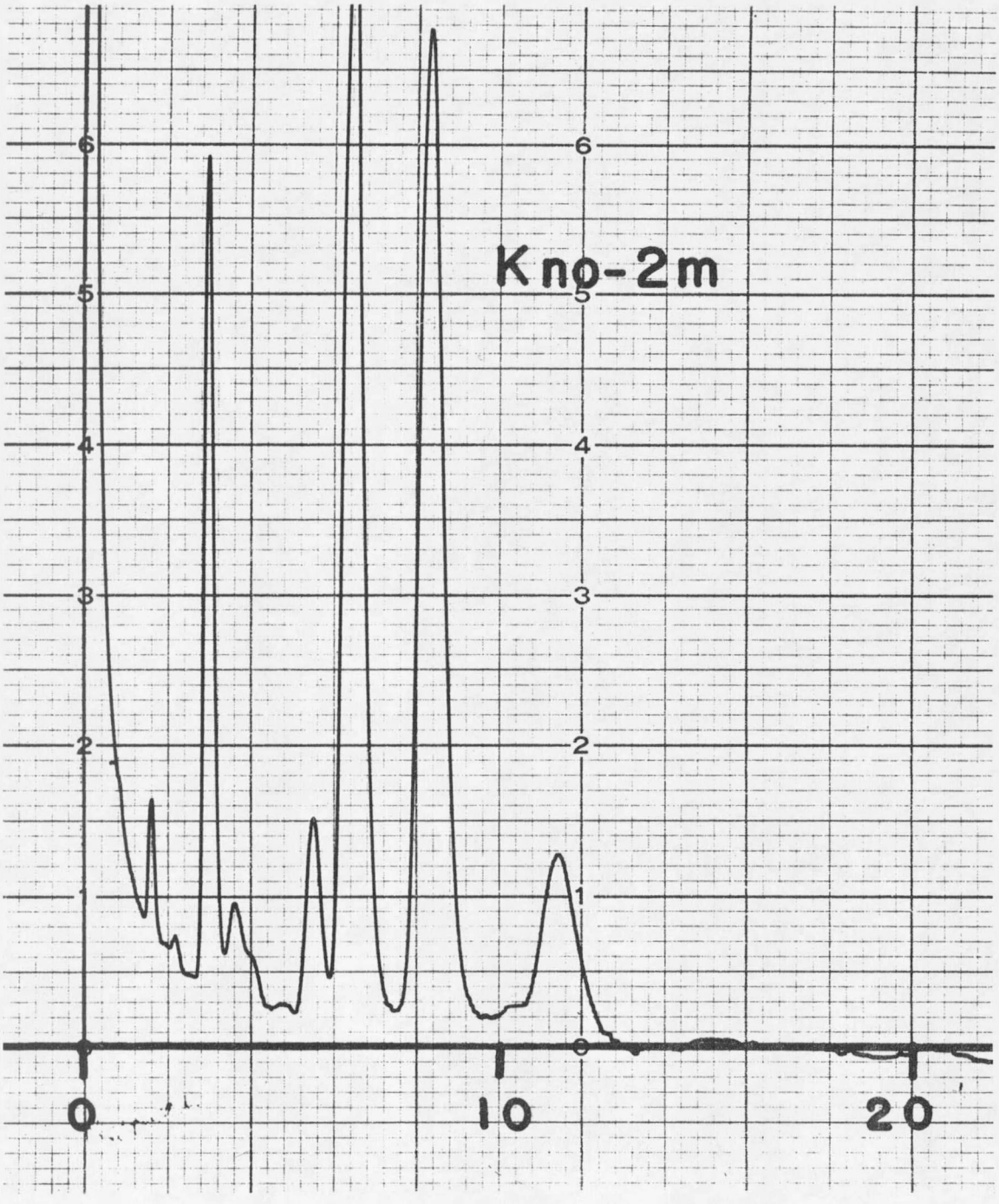

Figure 21. Gas chromatogram of the methyl esters of Kno-2m

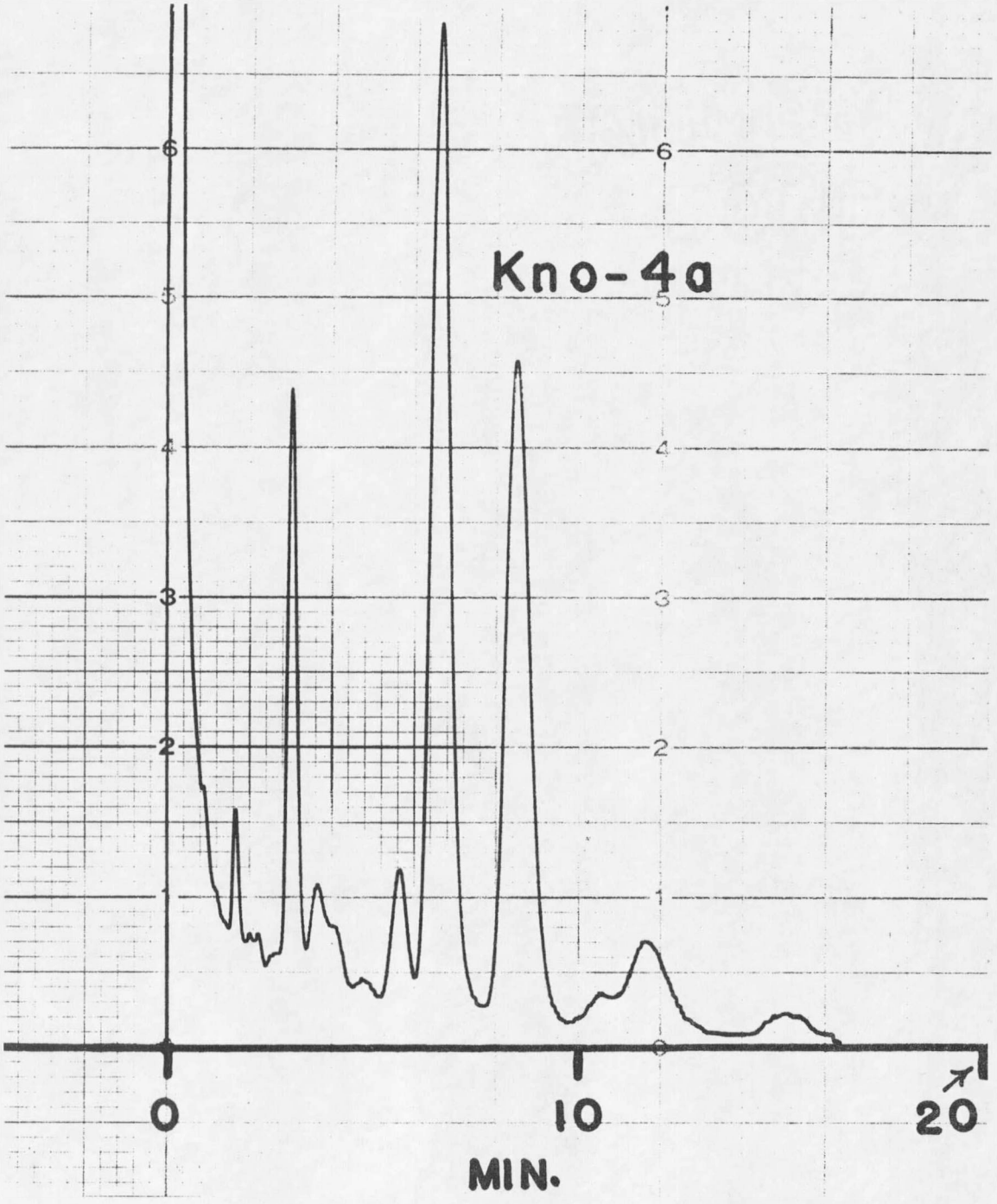

Figure 22. Gas chromatogram of the methyl esters of Kno-4a

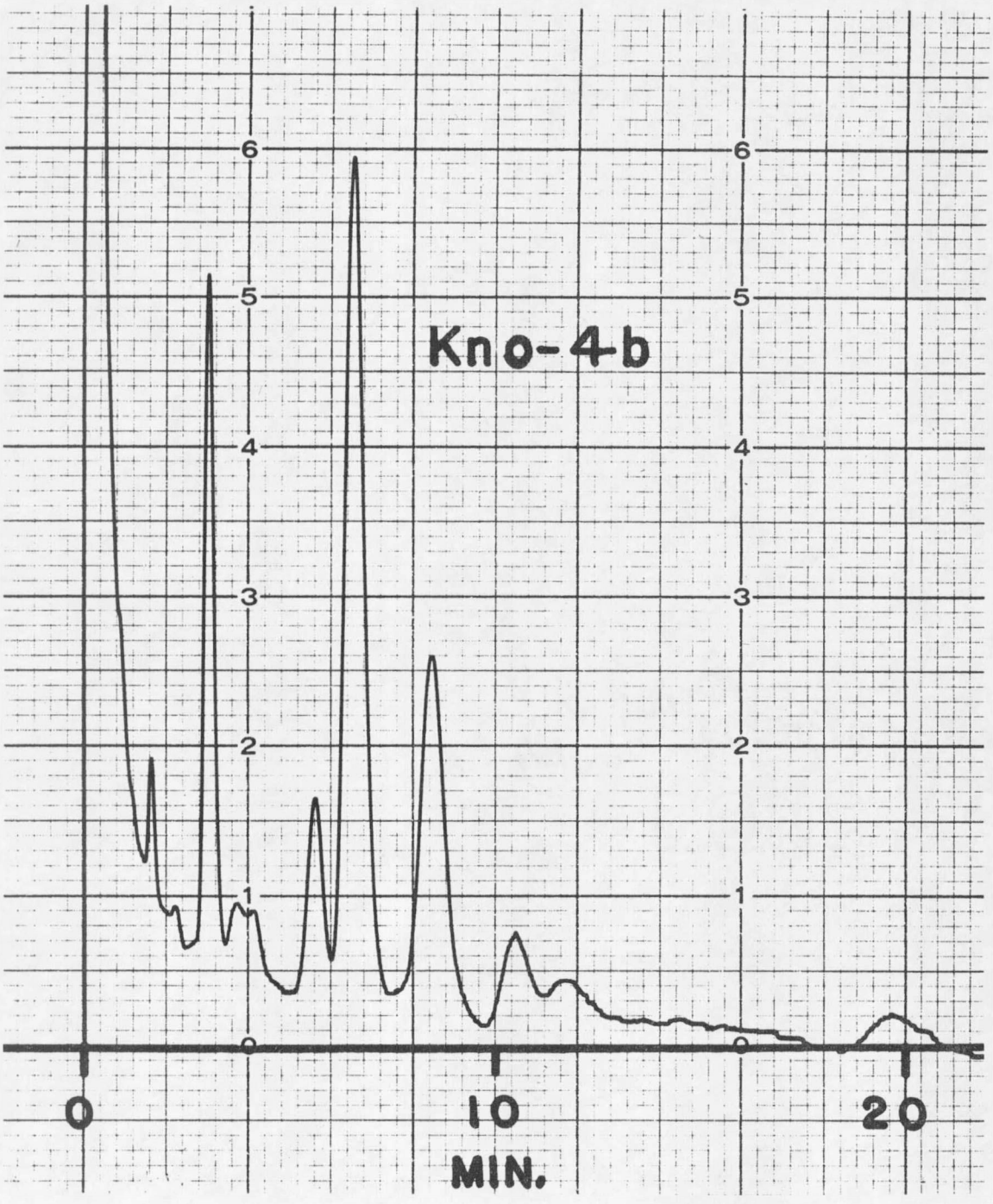

Figure 23. Gas chromatogram of the methyl esters of Kno-4b

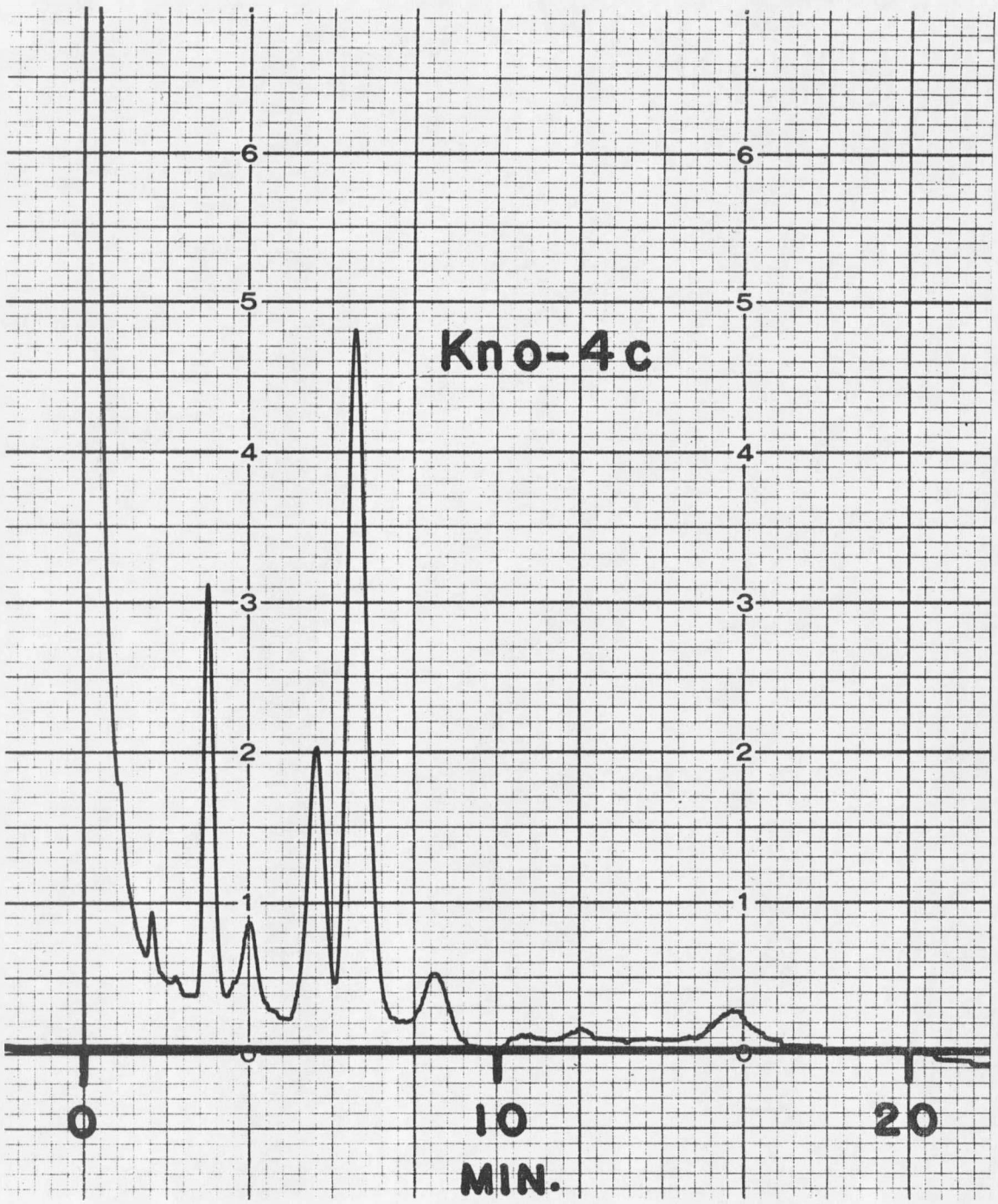

Figure 24. Gas chromatogram of the methyl esters of Kno-4c

sterols. Table IX shows the sterol composition of Kno-3n and Kno-1a. Unidentified structures are indicated by letter. Known samples of cholesterol, campesterol, and beta-sitosterol have retention times on both columns identical to those listed for the cuticular sterols which were identified.

TABLE IX

Sterol Composition of Fractions Kno-3n and Kno-1a

Sterol	% Kno-3n	% of sterols in Kno-1a	x Rt OF-1	y Rt SE-30
A	0.7	1.3	2.8	1.6
Cholesterol	92.2	86.2	3.1	1.8
B	0.9	2.7	3.6	2.0
Campesterol	2.5	3.5	4.1	2.3
β-sitosterol	3.7	5.5	5.0	2.9
C	T	0.8	6.0	3.2

T denotes less than 0.1% of fraction
x retention times relative to cholestane, t=3.2 minutes
y retention times relative to cholestane, t=6.2 minutes

The fatty alcohols of the non-saponifiables were chromatographed on SE-30 at 215°C for twenty minutes followed by temperature programming to 300°C. Figure 27 shows a portion of the gas chromatogram of the alcohols. Table X shows the composition of the fatty alcohols derived from the wax esters of Kno-1a. Unsaturation was determined by bromination followed by re-chromatography. Those peaks which disappeared or were shifted in position were taken to be unsaturated.

The non-saponifiables of Kno-1b were chromatographed on SE-30. The chromatogram is shown in figure 28. Cholesterol was present in the sample

as shown by the peak at 5.6 minutes, Rt=1.8. This assignment was confirmed by chromatography on QF-1. The peak at 9.1 minutes in figure 27, Rt=2.9, appeared to be either beta-sitosterol or C_{27} alcohol. Chromatography on QF-1, however, showed that this peak was neither beta-sitosterol or C_{27} alcohol as it had an Rt of 5.2. The peak at 9.8 minutes in figure 27 had the same retention time as sterol C in Table IX. Thin-layer chromatography on silica gel suggested that this fraction was mainly composed of materials similar to lanosterol. Gas chromatography on SE-30, however showed that none of the materials had the same retention time as lanosterol. None of the materials of this fraction, with the exception of cholesterol, were identified.

TABLE X

Fatty Alcohols of the Wax Esters

Carbon No.	% of Alcohols
16	T
17	T
18	1.6
19	T
20u	0.8
20	23.6
21	2.0
22u	13.6
22	17.4
23?	27.1
24	5.5
25	4.1
26	4.3

T denotes less than 0.1% of fraction
u denotes unsaturated structures
? indicates the peak does not fall on a log plot of either the saturated or unsaturated alcohols

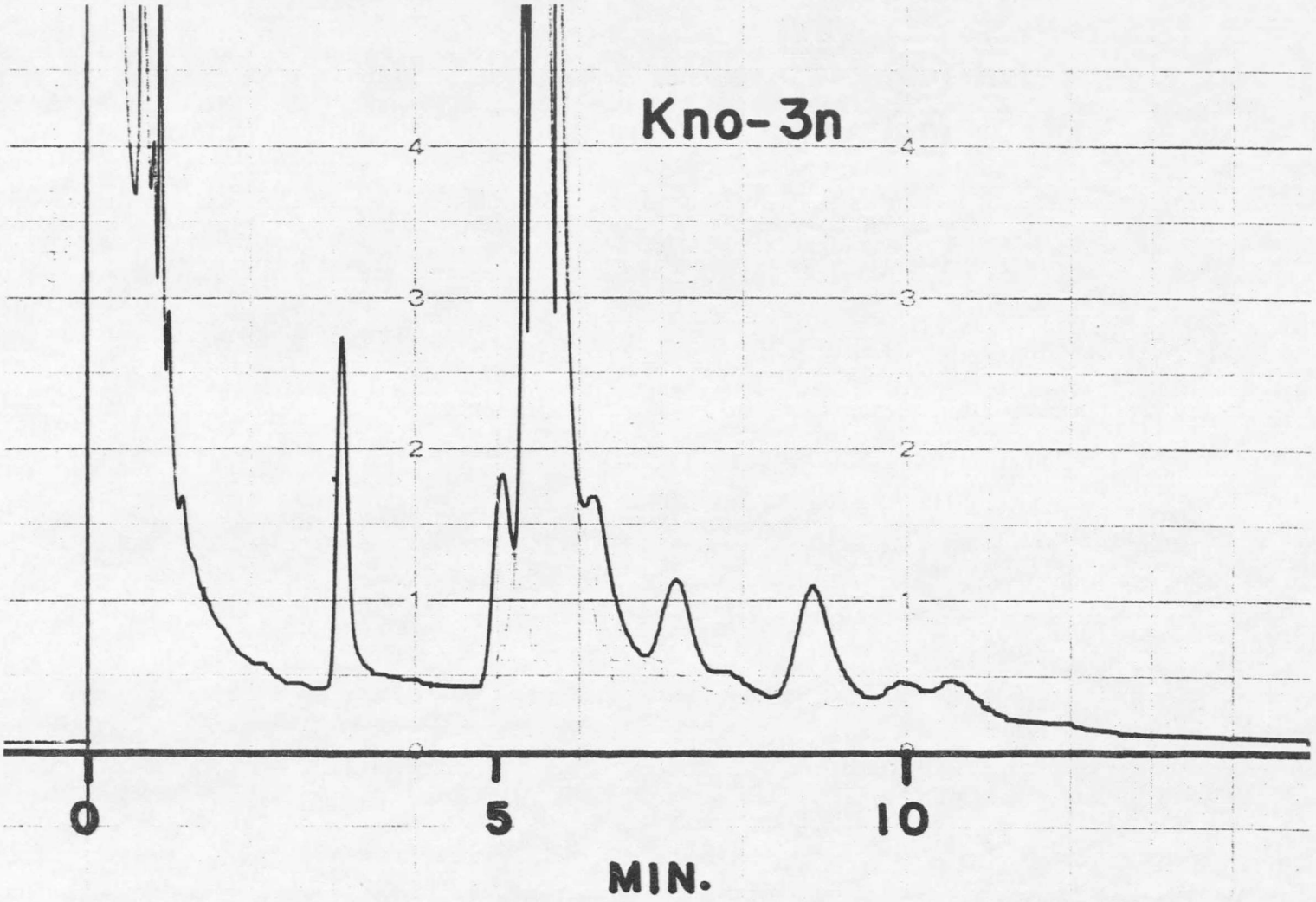

Figure 25. Gas chromatogram of the free sterols, Kno-3n

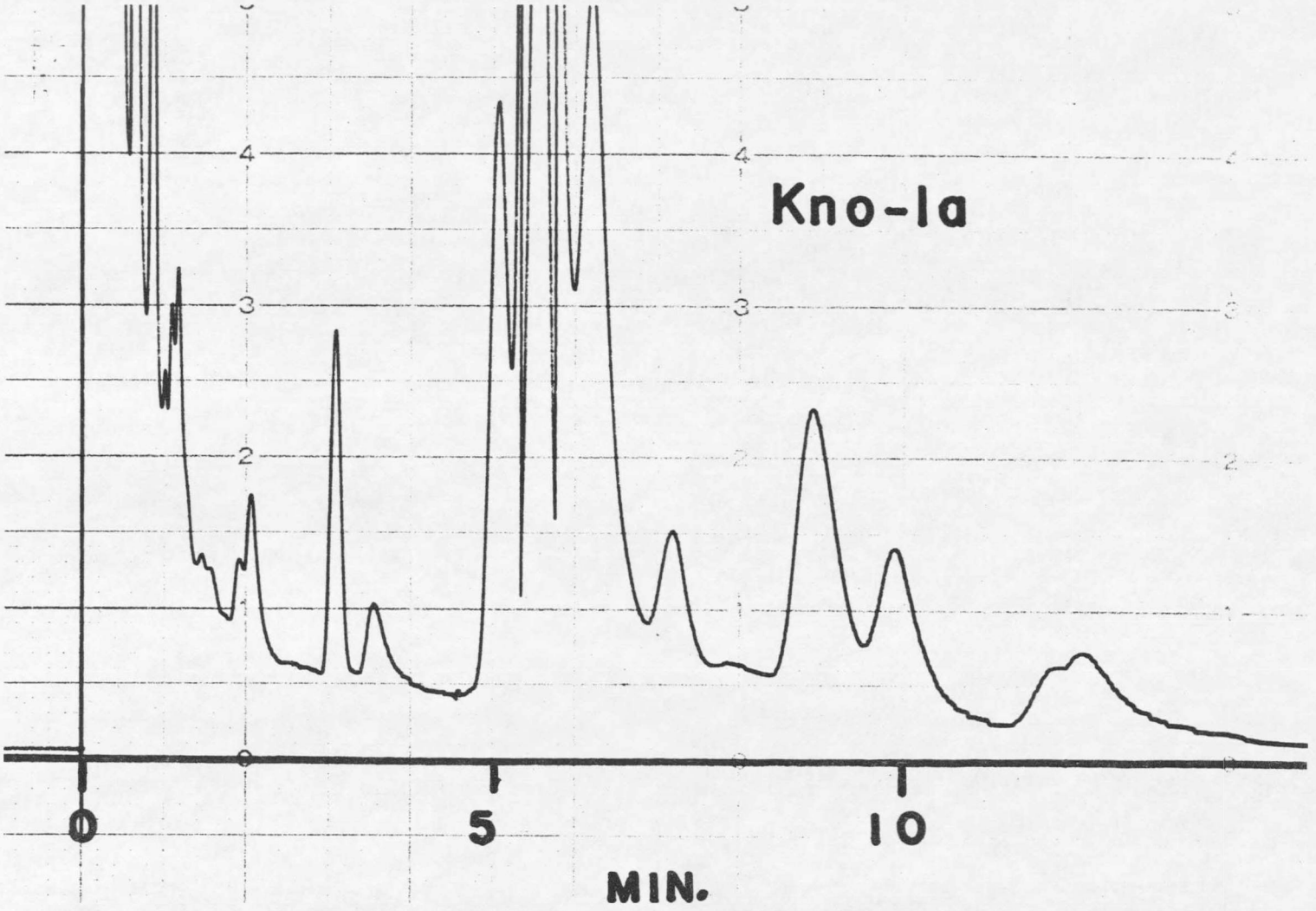

Figure 26. Gas chromatogram of the sterols of Kno-1a

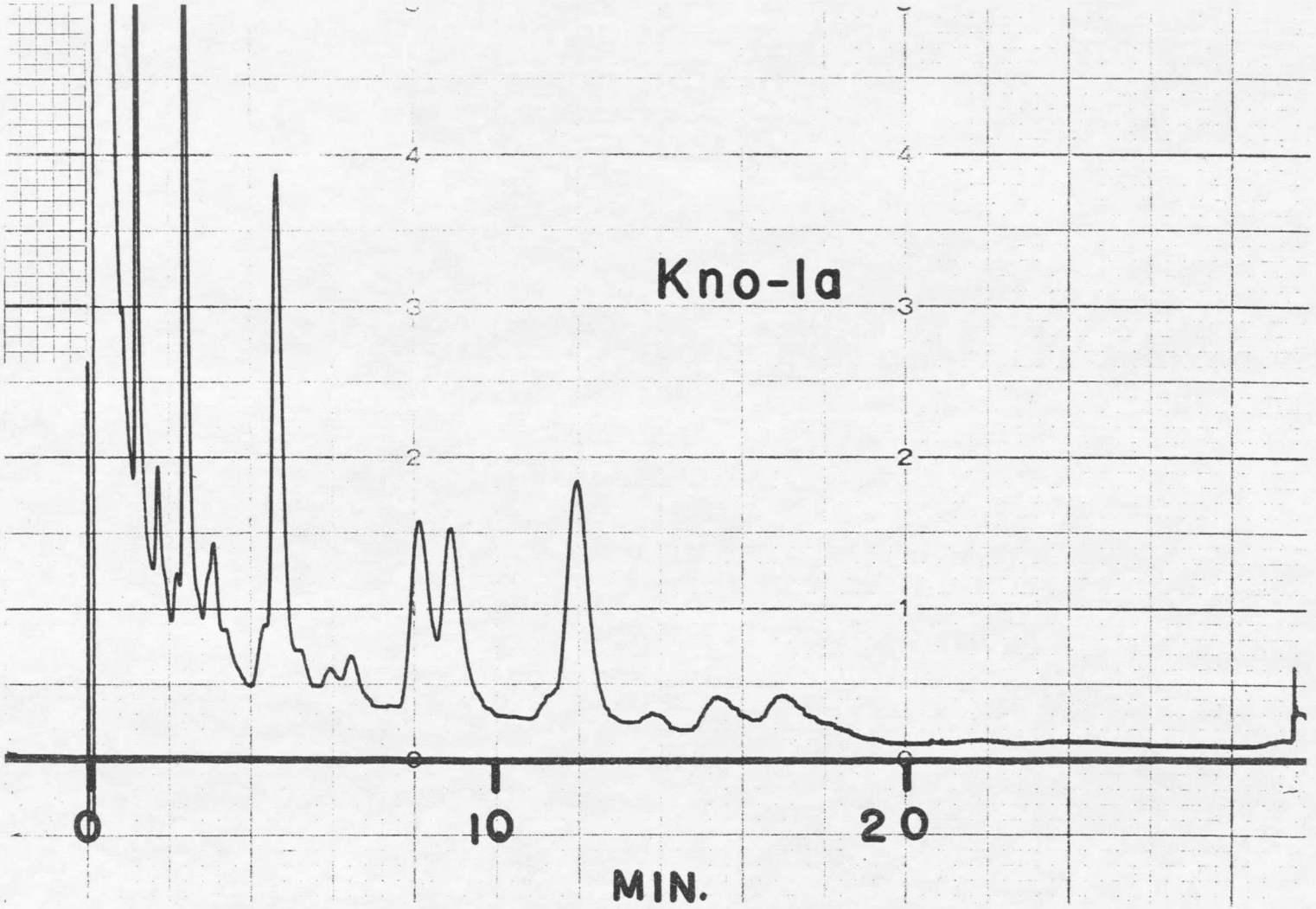

Figure 27. Gas chromatogram of the alcohols of Kno-la

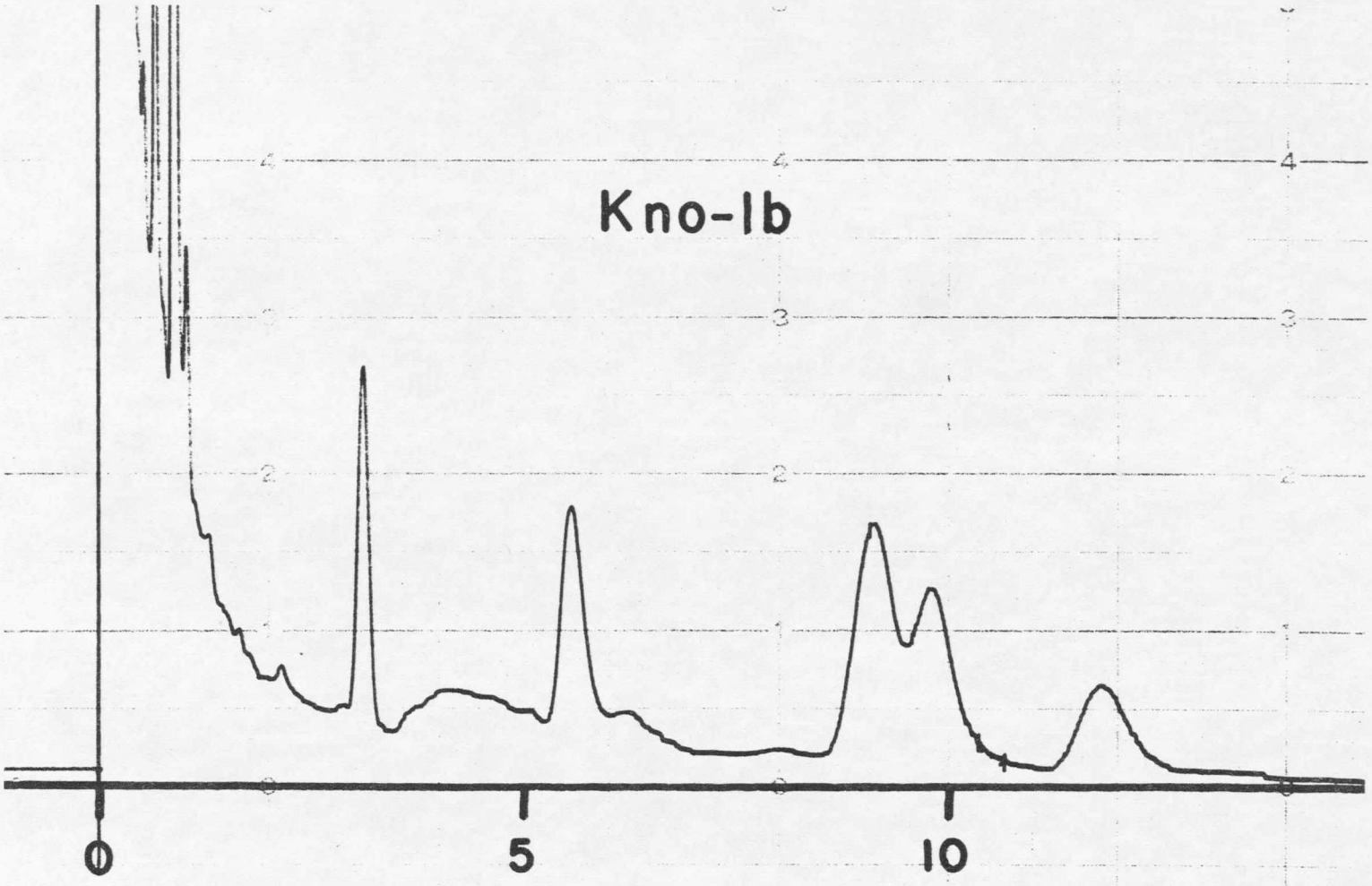

Figure 28. Gas chromatogram of the non-saponifiables of Kno-1b

DISCUSSION: LIMITATIONS OF RESULTS

Some of the results of this study were ambiguous and others were based on assumptions not indicated in the section on experimental procedure. In an attempt to indicate the nature of some of these assumptions and ambiguities the following discussion is included.

Percentage compositions for the various methyl ester fractions are based on the observation that the response of a flame detector is proportional to the number of carbon atoms in a molecule. The proportionality constant is the same for all members of a homologous series, thus the area percent computed from a recorder trace is directly convertible to weight percent. Ettre and Kabot[15] have shown that this same relationship holds for all types of non-polar methyl esters so that fatty acid methyl esters may be compared directly for weight percent from the detector response.

Tables IV and V list the relative percentages of the free saturated acids determined as methyl esters. Small differences may be noted between the saturated free fatty acids of 1961 and 1962. In almost all cases these deviations between the percentages computed for the two years fall within the standard deviations computed for the particular acid studied. In the case of the unsaturated acids the standard deviations do not account for the wide variance of percentage composition. These might represent differences in sample treatment. A second possibility is variation between the two years due to environmental selection. Observations on the big-headed grasshopper have shown differences in characteristics between isolated populations during the same year.[19] Svoboda[36] has found differences

in lipids between grasshopper eggs collected one mile apart on the same day. Thus the observed differences may be due to any one of several biological factors.

During the investigation into the nature of the free fatty acids it was noted that there were materials not eluted from the Florisil column used to separate the methyl esters into polar groups (Table II). The material not eluted could represent non-esterified fatty acids. This is unlikely as the infrared spectrum, figure 3, of the total methyl esters shows no evidence of an acid carbonyl at 1705 $cm.^{-1}$ In addition Vorbeck[38] has shown the methylation procedure used to be quantitative. A more likely possibility is that these materials are phosphatidic acids. Such materials would not be converted to methyl esters but would be retained on the column as magnesium salts. A third possibility is that these materials, as well as the polar methyl esters Kam-2 and Kam-3, represent oxidation products of the unsaturated acids. This is quite likely as the acids from 1961, which showed a high percentage of materials not eluted from Florisil, were collected from a number of Kies extractions and stored in water solution as sodium salts for several weeks. The fatty acids from 1962, which were converted to their methyl esters immediately upon isolation, showed a much lower percentage of materials not eluted.

In a recent study Ast[3] has found that methylene interrupted polyunsaturated fatty acids may be inadvertantly converted to the conjugated isomers during methyl ester preparation. No evidence for such isomerization was found in this study as conjugated structures would have given either longer aldehydes or aldehyde esters than the ones actually found.

No direct evidence was obtained for the assignments of branching to some of the saturated acids. These assignments were based on the generalization that branched chain structures are eluted before their straight chain counterparts during gas chromatography on SE-30 silicone. The gas chromatograms of the saturated acids show two series of branched chain compounds exist. These two series might represent iso and anteiso structures as Leibrand[23] showed the presence of structures of this type in the hydrocarbons of the Mormon cricket. It is also highly possible that the other highly branched acids exist but are masked by the peaks of the straight chain acids.[11] Conversion of the methyl esters to hydrocarbons followed by molecular sieve studies could be used to confirm the presence of branched chain acids. Mass spectral data on the branched chain methyl esters could be used to elucidate the branching if enough materials could be collected by preparative gas chromatography.

Wax and steryl esters could have been separated for independent study by preparative thin-layer chromatography on Anasil plates. This was not done in the present study due to the small amount of wax ester present, approximately 1/6 of the weight of fraction Kno-1a as estimated for thin-layer chromatography.

The sterols identified as cholesterol, campesterol, and beta-sitosterol could be other epimers. Variation in the configuration about one carbon atom would not be likely to change the relative retention times of the sterols. The sterol reported as campesterol in this study is usually reported as gamma-sitosterol. Thompson et al.[37], however, have recently shown that the supposed gamma-sitosterol to be campesterol. The campesterol

standard used in this study was the impurity in beta-sitosterol shown by Thompson to be campesterol.

SUMMARY

The cuticular lipids of the Mormon cricket were isolated by chloroform extraction followed by hexane precipitation. The crude lipids were separated into neutral lipids and fatty acids by a Kies countercurrent extraction.

The fatty acids were converted to their methyl esters and chromatographed on Florisil to effect a separation into polar groups. The non-polar methyl esters were further fractionated on silver nitrate-silicic acid columns into saturated methyl esters and unsaturated methyl esters. Quantitative analysis showed that the saturated acids ranged from C_8 to at least C_{36} and included both normal and branched chain structures. The major acids were found to be palmitic and stearic. The unsaturated acids were found to range from C_{12} to C_{24} with the major acids being identical with oleic, linoleic, and linolenic acids. The structures of the major unsaturated acids were determined by the use of reductive ozonolysis and infrared spectroscopy.

The neutral lipids were fractionated into chemical classes by a combination of column chromatographic methods. The neutral lipids included wax esters, steryl esters, triglycerides, free sterols, diglycerides, and monoglycerides. The free sterols were determined, and three of them were identified as being cholesterol, campesterol, and beta-sitosterol. The ester fractions were transesterified and the acids and non-saponifiables determined by gas chromatography. The methyl esters of the neutral lipids were found to be similar to those found in the free acids.

APPENDIX

TABLE XI

Retention Times*of the Major Acid Methyl Esters on EGS**

Figure No.	Methyl Ester 16:0	18:0	18:1	18:2	18:3
9	3.3	6.3	7.3	9.5	13.2
11	3.2	6.2	---	---	----
13	---	---	7.0	9.1	12.6
19	3.2	6.2	7.0	9.1	12.6
20	3.3	6.2	7.1	9.2	12.7
21	3.0	5.5	6.4	8.3	11.4
22	3.0	5.6	6.5	8.5	11.7
23	3.0	5.6	6.5	8.5	11.7
24	3.0	5.6	6.5	8.5	11.7

* Time in minutes from the solvent peak
** Column temperature--170°C

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