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An Investigation of the Neutral Materials in the Benzene Extract of Aspenwood

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AN INVESTIGATION OF THE NEUTRAL MATERIALS IN THE BENZENE EXTRACT OF ASPENWOOD

A thesis submitted by

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INTRODUCTION

In recent years the dwindling supply of coniferous woods has forced many pulp manufacturers to turn to hardwoods for their supply of papermaking fibers. The abundance of aspen in the Lake States and the fact that it produces good quality fiber has led to its widespread use. One of the obstacles to its more widespread use is the fact that it contains large amounts of extractives, or resins, which are not easily removed during the cooking and bleaching operations. These extractives may lead to such problems as the accumulation of "pitch" at some stage in the papermaking operation or the development of self-sizing in papers intended for absorbent use.

Although the nature of softwood resins has been studied rather extensively in connection with the naval stores industry, little is known about the nature of hardwood resins. Almost nothing is known at the present time about the extractives of aspen. A knowledge of the chemistry of aspen extractives should lead to a better understanding of the problems encountered in the use of aspen as a pulpwood species.

The development of modern analytical techniques, especially chromatography, has made it possible to perform isolations and identifications that would have been impossible a few years ago. Of special interest to the worker in the field of natural products is the application of chromatographic methods to the separation of the highly complex mixtures so often encountered in plant chemistry. In addition, sensitive sprays often make it possible to detect the presence of minute amounts of a compound enabling us to check the progress of a separation or reaction each step of the way.

Although some recent work on the nature of the free fatty acids in Populus tremula has been reported (1), nothing is to be found concerning the nature of the neutral components of the extractives of aspen. Because of this, and because of the necessity of limiting the investigation to an area that can be covered practically, it was decided to investigate the neutral portion of the total extract.

It is hoped that the present investigation will give us at least a foothold on the chemistry of the aspen extractives.

HISTORICAL REVIEW

Although the resin content of a number of hardwood species has been reported in the literature, most of the data are scattered in isolated articles. A compilation of the work done on American pulpwoods in this field, prior to 1956, has been given by Isenberg, Buchanan, and Wise (2). It is significant that only five references were given for trembling aspen, and only one of these dealt with the isolation and identification of a pure compound. This was an article by Pearl and Beyer (3) on the isolation of p-hydroxybenzoic acid from Populus tremuloides. This compound was also isolated by Smith (4) from the European species, Populus tremula.

Preliminary work on the amounts of extractives in aspen was reported by Browning and Bublitz (5). They gave 3.8% for the total ether and alcohol-benzene extract of fresh aspenwood. Of the ether extract, 17.7% was unsaponifiable, 1.7% resin acid, and 72.4% fatty acid. Mutton (6), commenting on the resin acids reported by Browning and Bublitz, suggests that these are not acids of the abietic type. In a recent article, Buchanan, Sinnett, and Jappe (20) report the presence of typical resin acids in the benzene extract of Populus tremuloides and Betula papyrifera, the amounts contained in these species being very small, however.

In any case, the major portion of the hardwood resin consists of fatty acids, esters, and unsaponifiables.

The composition of the fatty acid fraction of the extract of Betula verrucosa is the subject of a paper by Kahila and Rinne (1) who found that the major acid isolated was linoleic acid. The saturated fatty acids

were also investigated and the following percentages given: myristic acid 1%, palmitic acid 37%, stearic acid 43%, arachidic acid 10%, and behenic acid 9% of the total saturated fatty acids. Although this seems to be the only quantitative work done on the fatty acids from common hardwoods, a number of papers have appeared recently describing the isolation of various fatty acids from hardwood extracts.

Hossfeld and Hunter (8) reported the isolation of lignoceric acid from the extract of trembling aspen bark, and Perilä and Toivonen (9) list the saturated fatty acids from Betula verrucosa. Buchanan, Sinnett, and Jappe (7) studied the fatty acids of Betula papyrifera and Populus tremuloides and found that the major acid was linoleic, whereas the major saturated acid was palmitic acid. Perilä (10) has examined the saturated fatty acids from Betula verrucosa and Populus tremula.

Although none of the foregoing authors mentioned the isolation of an odd-numbered fatty acid, Cooke and Hansen (11) recently reported the isolation of n-heptadecanoic acid from the tall oil of Pinus radiata.

A number of workers have reported the isolation of sterols from the unsaponifiable portion of hardwood extracts. Perilä (12) obtained a sterol from the unsaponifiables of the ether extract of Populus tremula which he called sitosterol. Kahila and Rinne (1) isolated a dextrarotatory sterol from the ether extract of Betula verrucosa, and Hossfeld and Hunter (8) reported the isolation of β-sitosterol and an unsaturated hydroxy sterol from trembling aspen bark. In addition, Kurth and Becker (13) have described the isolation of a phytosterol from the extract of Alnus rubra.

Another class of compounds recently reported in the extract of hardwoods is fatty alcohols. Hossfeld and Hunter (8) found ceryl alcohol in the extract of trembling aspen bark, and Pearl and co-workers (14) isolated ceryl alcohol and n-heptacosanoyl alcohol from aspen spent sulfite liquor.

Fatty alcohols have also been found in softwoods. Thus, Khäletskii and Solomonik (15) found fatty alcohols as well as sterols in the crude phytosterols from pine, and Sandqvist, Gorton, and Bengsston (16) found fatty alcohols in the extract of tall oil soap.

In addition to the fatty acids and fatty alcohols mentioned above, waxy hydrocarbons have been isolated from plants. Hellerqvist, Johnsson, and Bäcklund (17) reported finding such hydrocarbons in the crude phytosterols from sulfate soaps.

Buchanan, Sinnett, and Jappe (7) have reported the quantitative determination of glycerin from the saponification of the benzene extract of a number of woods including *Populus tremuloides* and *Betula papyrifera*. Their results indicate the presence of triglycerides as well as fatty acid esters of materials other than glycerin.

SUMMARY OF THE LITERATURE

Although the extractives of softwoods have been studied rather extensively and some work has been done on the extracts of certain hardwoods, little is known about the nature of aspen extractives.

EXPERIMENTAL PROGRAM

PREPARATION OF THE STARTING MATERIALS

PREPARATION OF THE WOOD

The wood used in this study, trembling aspen, was obtained in June from the Ripco Forest near Eagle River, Wisconsin. Several trees from different stands were felled and cut into four-foot bolts. The four-foot bolts were barked soon after cutting and stored in a warm room for rapid drying. This rapid drying is necessary to prevent the fungal attack to which aspen is especially prone.

The 30 pounds of wood used for each extraction were reduced to sawdust using a circular power saw and the sawdust, never prepared more than a day in advance of the extraction, stored in plastic bags as soon as cut. It was hoped that such treatment would preclude the possibility of any fungal attack on the freshly cut wood, and no such attack was noticed.

THE EXTRACTION PROCEDURE

Each 30-pound lot of sawdust was extracted in a 20-gallon, stainless steel extractor. The sawdust and 15-20 gallons of benzene were added to the extractor and allowed to stand for three days. At the end of three days, the benzene was drained off and fresh benzene added. The first and second batches of solvent, each having also been in contact with the wood three days, were concentrated under reduced pressure (8" Hg) and the recovered solvent re-used. The third batch of solvent was dilute and was not concentrated but was used as the

first-stage solvent for the next batch of sawdust. Each batch of sawdust was thus extracted successively with a dilute solution of extract and two fresh batches of benzene.

No attempt was made to keep the various batches separate as a representative sample was desired. The raw extract when dried was a dark amber wax with a characteristic woody odor. The yield of the raw extract was 1.27% based on the ovendry weight of the wood. Total weight of material obtained was 1258 g.

REMOVAL OF THE ACIDIC CONSTITUENTS

Inasmuch as severe emulsion troubles were encountered in trying to extract the benzene solution of the extract directly with 1% NaOH, some other procedure was necessary. A diagram of the apparatus used is given in Fig. 1. In this procedure 250 ml. of the benzene solution of the extract, containing approximately 20% solids, was placed in the lower separatory funnel and 1000 ml. of 1% NaOH slowly run into this from the upper separatory funnel being careful to allow no mixing of the two phases. At the end of 24 hours the lower phase was run out slowly and a fresh supply of 1% NaOH admitted. The lower phase was changed daily for a week at the end of which the benzene phase was sufficiently dilute in fatty acids to allow normal extraction.

After about one half of the total extract had been processed in this manner some more rapid method of removing the emulsion-causing fatty acids was sought. The method finally developed consisted of shaking the benzene solution of the raw extract with an excess of calcium hydroxide powder to which a small amount of water had been added

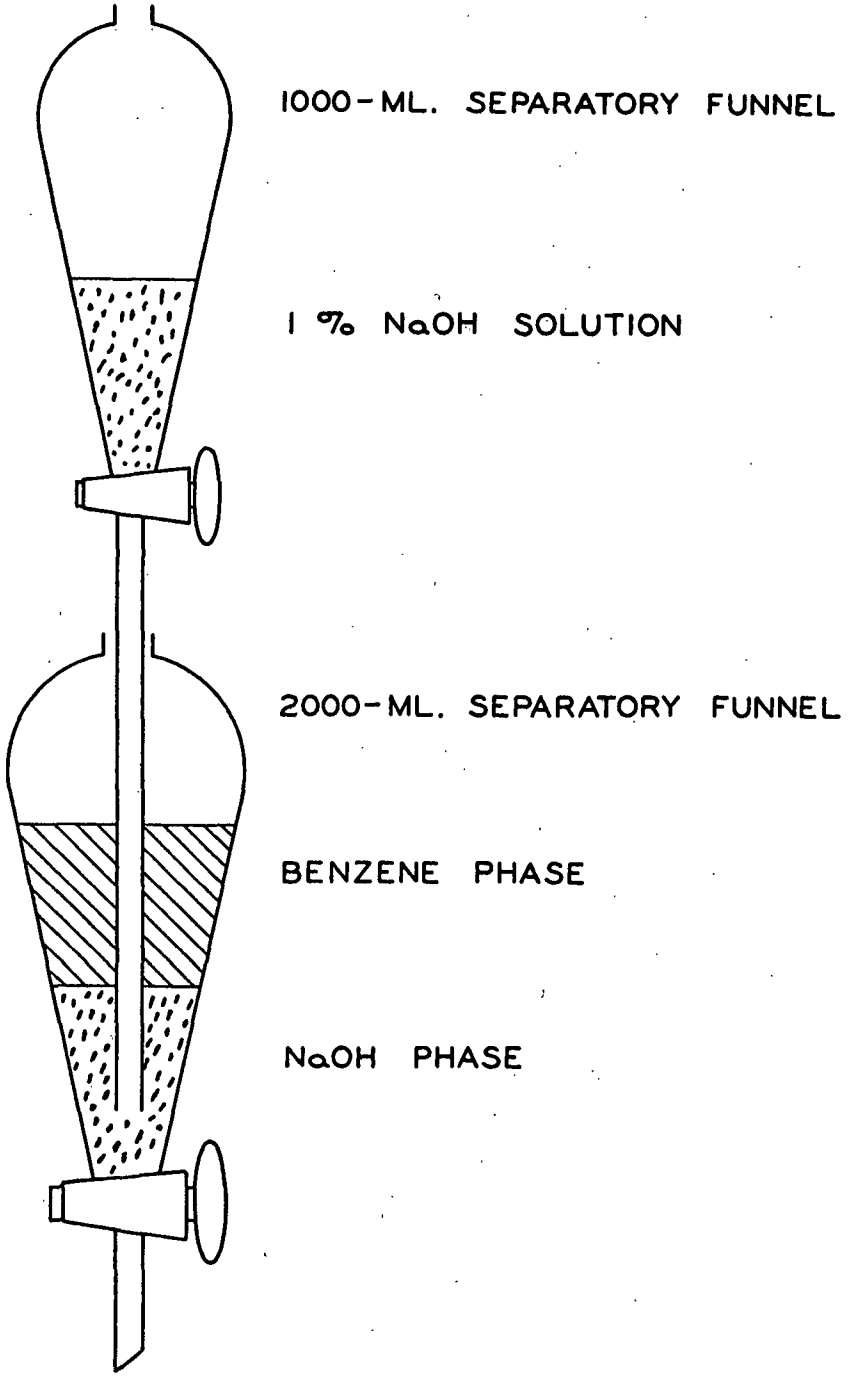

Figure 1. Extraction Apparatus

as a catalyst. The suspension was shaken for about one hour at the end of which it was centrifuged, the supernatant liquid poured off and the precipitate twice washed with fresh benzene. The resulting benzene solution could now be extracted directly with 1% sodium hydroxide without emulsion difficulties.

Following the extraction with 1% sodium hydroxide, the benzene solution was washed with dilute hydrochloric acid followed by distilled water until the wash water was neutral.

The remaining one half of the extract was processed by this latter method.

The materials remaining in the benzene solution following the extractions with sodium hydroxide and hydrochloric acid were the neutrals used for this study. The yield of neutrals was 0.624% based on the ovendry weight of the wood, or 616.4 g.

SEPARATION INTO GROUPS

A preliminary separation of the total neutrals on the basis of solubility was next carried out. The benzene solution was taken to dryness and extracted with boiling methanol until no further material was removed. The material soluble in methanol was called the methanol-soluble fraction, and the material insoluble in methanol was called the methanol-insoluble fraction. The yield of each of these fractions was methanol-insoluble 0.174% (172.5 g.) and methanol-soluble 0.450% (445.5 g.) based on the ovendry weight of the wood. These fractions were worked up separately inasmuch as their properties differ markedly.

A flow chart of the operations described thus far is given in Fig. 2.

THE METHANOL-INSOLUBLE FRACTION

INTRODUCTION AND PRELIMINARY SEPARATION

The methanol-insoluble fraction, a viscous, yellow oil, represents 28% of the total neutrals or 0.174% of the ovendry wood. The workup of this fraction is shown in Fig. 3.

This oil was chromatographed on alumina using benzene, chloroform, and alcohol containing 1% acetic acid, successively. The column was washed with four or five column volumes of each solvent and the solvents collected separately. The alumina used for all the chromatographic separations of this study was Fisher Adsorption Grade Alumina 80-200 mesh.

The material removed with benzene represented 67%, 115.6 g., of the methanol-insoluble fraction and was a clear, almost colorless, waxy solid melting at 60°C. to give a very viscous, tacky oil. The detailed investigation of this fraction will be described further on.

The material removed with chloroform represented 23% (39.7 g.) of the methanol-insoluble fraction. When this material was dissolved in methanol and the solution cooled, a precipitate appeared. Both the precipitate and the filtrate gave positive Liebermann-Burchard tests, suggesting the presence of steroidal materials. As only a small quantity of this material was available, no further work was done on it.

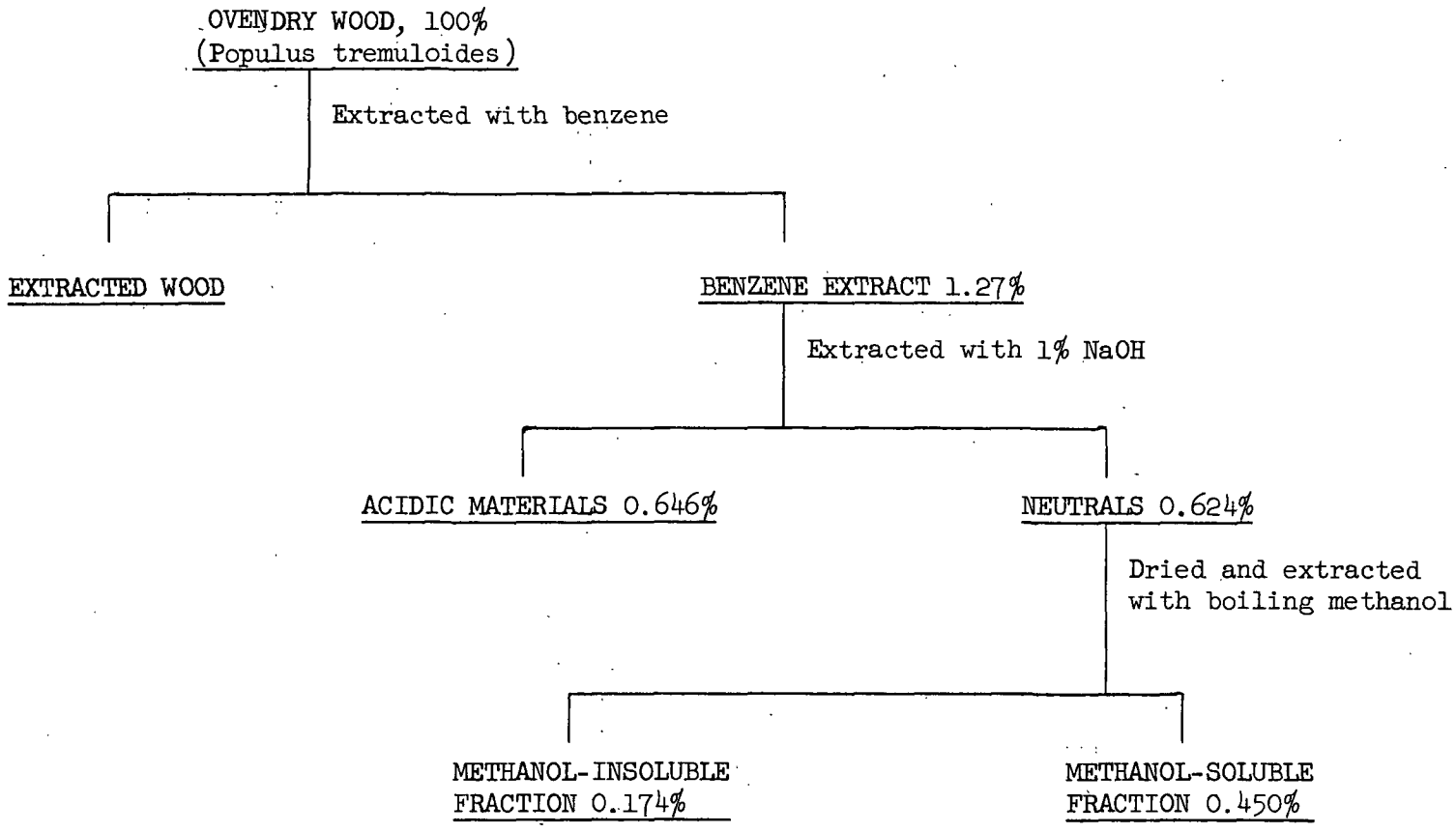

Figure 2. Flow Chart 1

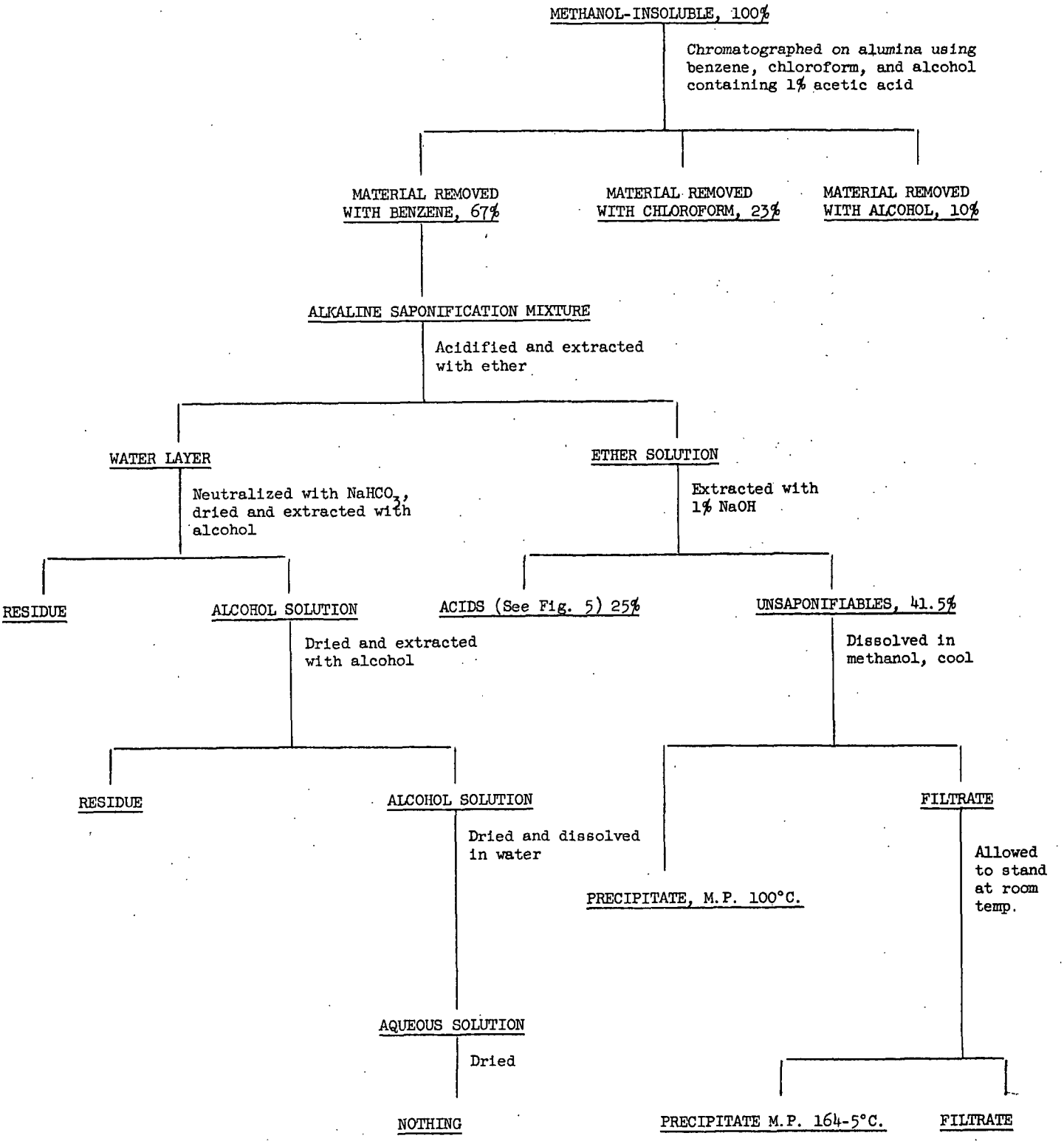

Figure 3. Flow Chart 2

The material removed with acidified alcohol represented about 10% (17.3 g.) of the methanol-insoluble fraction. Almost all the color was contained in this group, but the amount of this material was very small and no further work was carried out on it.

WORKUP OF THE MATERIAL REMOVED WITH BENZENE

Introduction

The material removed with benzene was saponified with 5% alcoholic potassium hydroxide under reflux for 24 hours. A small amount of benzene was added to the alcoholic potassium hydroxide to render the material soluble. At the end of 24 hours the alcohol was removed by boiling, water being added from time to time to maintain constant volume.

Because the presence of fatty acids again led to emulsion difficulties when trying to extract the alkaline saponification mixture directly with ether, the mixture was acidified with dilute sulfuric acid and then extracted with ether. The ether solution could now be extracted with 1% sodium hydroxide without undue difficulty.

The acids from this saponification represented 25%, 43 g., of the methanol-insoluble fraction and the unsaponifiables 41.5%, 71.6 g. These figures correspond to 37 and 62%, respectively, of the material removed with benzene.

The Water Layer

The water layer was neutralized with sodium bicarbonate and taken to dryness on the steam bath. The resulting solid was extracted with

95% ethanol, and this solution was in turn taken to dryness. The resulting residue was extracted with a small amount of 95% ethanol which was again taken to dryness. This residue was dissolved in water and run through a column containing Rohm and Haas MB-3, a mixed-bed resin containing both cationic and anionic exchange resins. When the resulting solution was evaporated, nothing remained.

Although it cannot be stated categorically that there are no glycerides in the methanol-insoluble fraction, the amount present, if any, must be small. Unfortunately, the lack of further amounts of methanol-insoluble material precluded the possibility of a chromatographic search of the water layer.

The Acid Fraction

Introduction

The 1% sodium hydroxide solution containing the acids from the saponification of the methanol-insoluble fraction was acidified with dilute sulfuric acid and extracted with ether. The resulting product, after removal of the ether, was a yellow oil solidifying slightly below room temperature.

Preliminary tests were made on this fraction using reverse-phase paper chromatography. For this procedure Whatman No. 1 paper was impregnated with mineral oil and developed with 85% acetic acid (18). Detection was accomplished using the mercuric salt technique described by Buchanan (19). The unsaturated acids were detected by exposing the paper to iodine vapors and then examining it under ultraviolet light (20). Under these conditions the unsaturated acids give dark spots.

The presence of both saturated and unsaturated fatty acids was indicated by the above-mentioned tests. Unfortunately, under the reverse-phase techniques described above a saturated fatty acid moves at the same rate as a monoenoic acid with two more carbon atoms. Palmitic and oleic acids are thus inseparable by this method. To avoid any subsequent confusion it was decided to try to separate the saturated acids from the unsaturated acids. Such a separation, it was felt, would simplify greatly the identification of the individual acids.

Separation of the Unsaturated and Saturated Acids

In the book, Fatty Acids, (21), a table is given listing the solubility ratios of palmitic and oleic acids in various solvents at various temperatures. From the table it may be seen that this ratio in ethyl ether at -40°C. is greater than 450:1. This very high ratio suggested a method for removing the unsaturated acids from the saturated acids.

The acids were dissolved in ether and placed in 250-ml. centrifuge bottles. The entire bottle was chilled to -70°C. in a dry ice, acetone bath and centrifuged at 2000 r.p.m. for one minute. At the end of this time the centrifuge was allowed to come to rest without braking. The supernatant liquid was carefully poured off without disturbing the precipitate and fresh ether added to the bottle. This process was repeated until no unsaturated acids were left in the precipitate.

The progress of the separation was checked by spotting a small amount of the precipitate each time on a small sheet of Whatman No. 1 paper, exposing it to iodine vapors and observing it under ultraviolet light (20). The presence of unsaturated acids gave a dark spot.

The procedure was checked using an equimolar mixture of palmitic acid and oleic acid, and in just two recrystallizations a palmitic acid was recovered having a melting point identical with the starting material and giving a negative test for unsaturated acids.

Two fractions were obtained by applying the separation described above to the total acids of this fraction, and these were worked up separately. The separations may be followed by referring to Fig. 4.

THE SATURATED FATTY ACIDS

Preliminary Work

The saturated fatty acid fraction was a white crystalline material melting at 51°C. When chromatographed using the reverse-phase paper chromatographic procedure described above, only one spot, corresponding to palmitic acid, was observed. Heavier loadings indicated the presence of several other higher molecular weight members of the fatty acid series. Some method for separating the various members of the series was sought.

Accordingly, the material was dissolved in ether and chilled to -40°C. at which time some material crystallized out. This was removed by centrifugation. The precipitate was labeled Fraction A. (See Fig. 4.) The supernatant liquid was reduced in volume and the solution again chilled to -40°C. This precipitate was labeled Fraction B. The procedure was repeated a third time and the precipitate from this step labeled Fraction C.

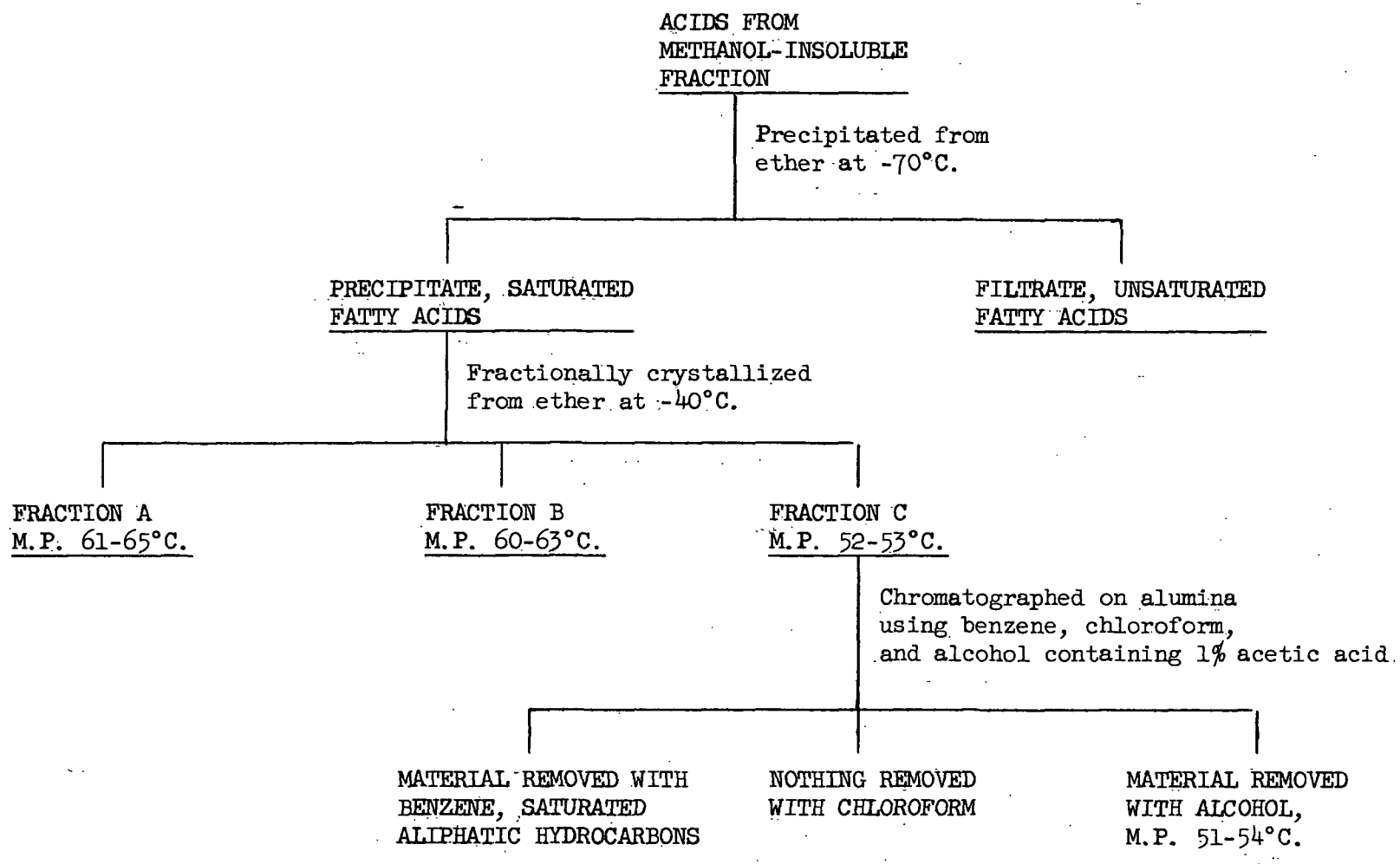

Figure 4. Flow Chart 3

Fraction A melted at 61-65°C. and, when chromatographed using reverse-phase paper chromatography, gave spots corresponding to arachidic, behenic, and lignoceric acids.

Fraction B melted at 60-63.5°C. and also gave spots corresponding to arachidic, behenic, and lignoceric acids.

Fraction C melted at 52.5°C. and, when chromatographed, showed only one spot corresponding to palmitic acid. The neutralization equivalent of this fraction, however, was 320 compared with the expected value of 256 for palmitic acid. This high value of neutralization equivalent suggested the presence of some nonacidic constituent not detected by the spray technique used on the chromatogram.

Because of this, Fraction C was dissolved in benzene and chromatographed on alumina. The column was washed with four or five column volumes each of benzene, followed by chloroform, and then by alcohol containing 1% acetic acid.

Evaporation of the benzene eluate yielded a semicrystalline solid melting at about room temperature. An infrared examination of this material revealed that it consisted of saturated aliphatic hydrocarbons. As only a small amount of this material was available, no further work was done on it.

The chloroform elution removed nothing.

The alcoholic eluate was evaporated and extracted with low-boiling petroleum ether.[a] This procedure is necessary, as some inorganic

[a] Throughout this thesis low-boiling petroleum ether refers to the 30-60°C. boiling range product.

material is removed from the alumina by the acidic alcohol. When the petroleum ether solution was dried in a rotary evaporator, the resulting product was a crystalline material melting at 51-54°C. and having a neutralization equivalent of 259. The infrared curve of this material was identical to that of an authentic sample of palmitic acid, but the low melting point was difficult to explain. The enigma was finally solved by the use of the gas chromatograph.

Gas Chromatography

Introduction

Gas chromatography has proved useful for the separation of complex mixtures of fatty acids and for the tentative identification of their individual components. In this apparatus the acids are separated as their methyl esters rather than as the free acids, and only very small amounts are required. Accordingly, small samples of Fractions A, B, and C were converted to their methyl esters and analyzed.

Preparation of the Methyl Esters

Only very small amounts of the acid were required for conversion to the methyl ester, one milligram being quite sufficient. Approximately one to five milligrams of the acid were dissolved in 1.5 ml. of methanol to which was added, then, four drops of a solution, freshly prepared, containing four grams of methanol to one gram of sulfuric acid. The resulting solution was boiled under reflux for approximately 45 minutes at the end of which the solution was diluted with water to 15 ml. and extracted with low-boiling petroleum ether. The petroleum ether solution

was next extracted with a 1% sodium hydroxide solution followed by distilled water until the wash water was neutral. The petroleum ether solution was then evaporated to the desired concentration.

Conditions Used with the Instrument

The conditions used with this Barber-Coleman instrument are as follows:

1.	Column length	6 ft.
2.	Column diameter	1/4 in.
3.	Solid phase	Johns Manville Chromsorb W
4.	Liquid phase	Cambridge Industries LAC 2R 446
5.	Temperature	213°C.
6.	Carrier gas	Argon
7.	Carrier gas flow rate	78 ml./min.
8.	Packing percentage	9% liquid phase based on total packing weight

Because only very small amounts of the methyl esters were used in this instrument, it was impossible to measure the amount added directly. For this reason the samples were dissolved in low-boiling petroleum ether and the solution introduced from graduated microsyringes. Retention times were measured from the air peak caused by the introduction of the sample to the peak of the curve.

Analysis of the Gas Chromatograms

In any given homologous series the retention time for each member of the series should be a logarithmic function of the molecular weight (22, 23). In the case of the methyl esters of the fatty acids this relationship holds true. Thus, if the logarithm of the retention time is plotted against the number of carbon atoms in the fatty acid, a straight line results. This relationship was checked using myristic,

n-pentadecanoic, palmitic, and stearic acids. The results are shown in Fig. 5, and it is apparent that the relationship is valid under the conditions employed.

In addition to the qualitative data which may be obtained as shown above, quantitative data are also obtainable with this apparatus. Whether quantitative data may be obtained, however, depends upon the constants employed with the instrument. Under certain conditions the areas under the curves traced by the detection apparatus are proportional to the amount of material introduced. Unfortunately, this work was carried out under conditions which do not give strictly quantitative data. Some idea of the order of magnitude of the various constituents in this mixture, however, may still be obtained by an examination of the relative areas under the curves.

Results and Discussion

A small amount of Fraction C was converted to its methyl ester and chromatographed in the instrument. This fraction, which had given only one spot corresponding to palmitic acid when examined by reverse-phase paper chromatography, gave three spots corresponding to palmitic, stearic, and arachidic acids. A plot of these results is given in Fig. 6.

A plot of the areas contained under each curve gave the following percentages of the total area: palmitic acid 77%, stearic acid 10%, and arachidic acid 13%. Accordingly, a mixture was prepared containing the relative percentages of the three components listed above. The melting point of this mixture was 55°C. compared with 53°C. found for Fraction C. A mixed melting point of equal amounts of Fraction C ...

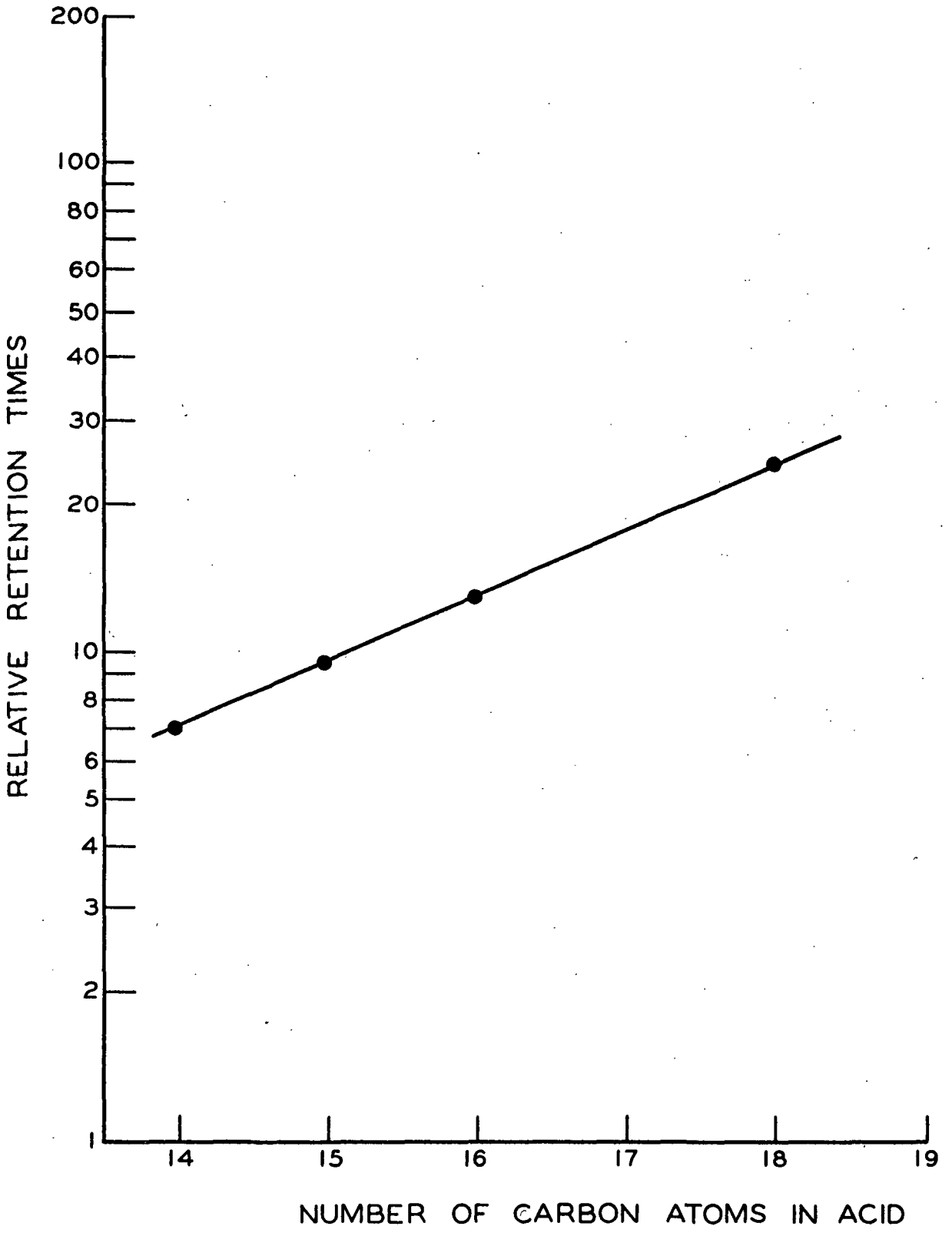

Figure 5. Retention Times of Known Acids

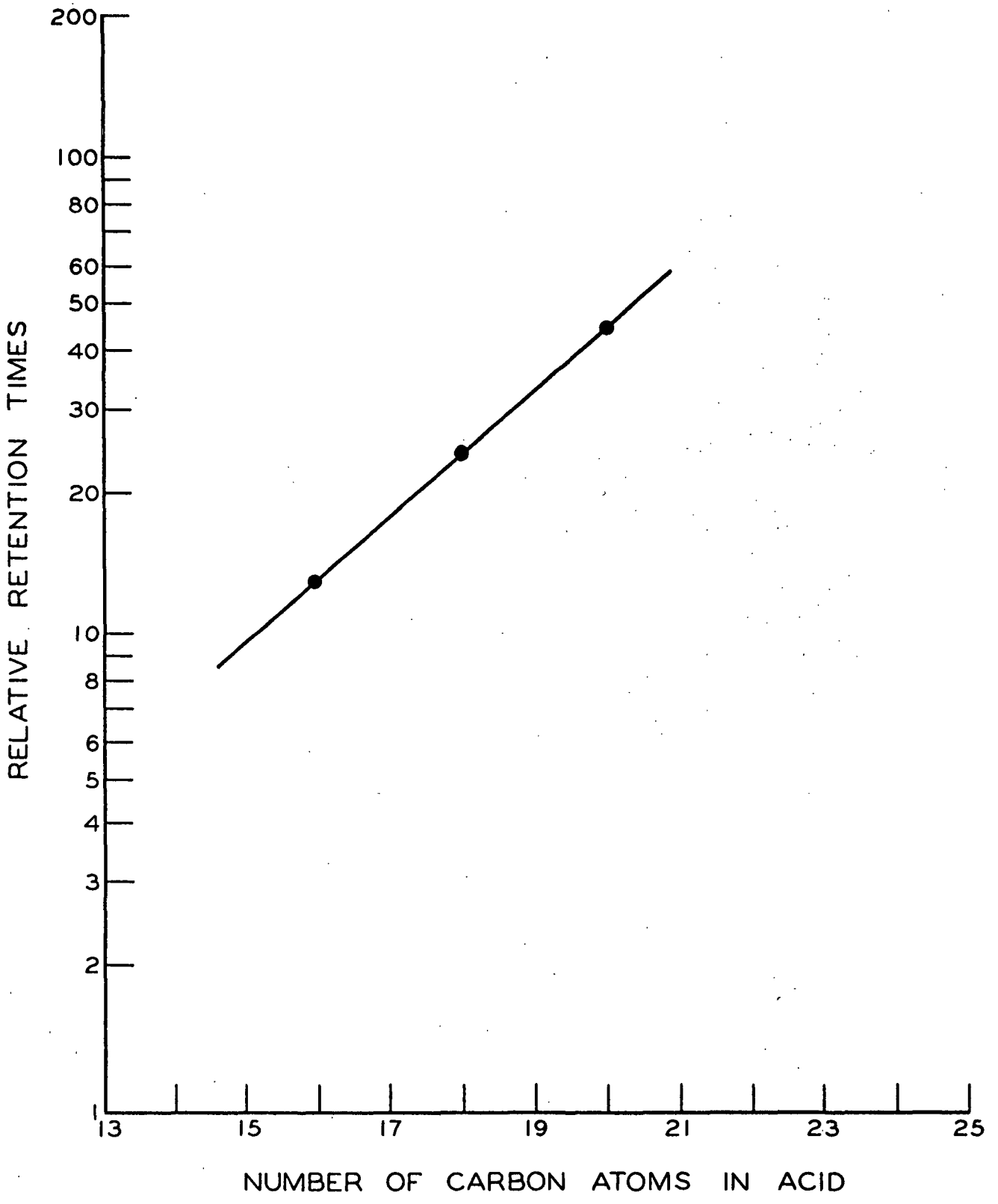

Figure 6. Retention Times for Fraction C

and the prepared mixture melted at 54°C. This agreement is rather good considering that the percentages indicated by an examination of the areas are only approximate.

The neutralization equivalent of Fraction C was 259 compared with the value of 266 to be expected from a mixture containing the relative amounts indicated above. Again, considering the fact that the percentages indicated by an examination of the areas are only approximate, this shows good agreement.

From the foregoing evidence, it seems certain that the major saturated fatty acid in the methanol-insoluble fraction is palmitic acid. It is interesting in this connection to note that Buchanan, Sinnett, and Jappe (7) found that the major saturated fatty acid in Populus tremuloides and Betula papyrifera was palmitic acid whereas Kahila and Rinne (1), studying the saturated fatty acids in the ether extract of Betula verrucosa, found approximately equal quantities of palmitic and stearic acids.

Fractions A and B were so similar that they may be considered together. When these fractions were chromatographed, the presence of all the saturated fatty acids from C_{12} to C_{24} was indicated. A plot of the results is given in Fig. 7.

The relative areas for each component are given for two runs in Table I. No values are given for C_{12}, C_{13}, and C_{14}, as these peaks, coming close to the beginning, are masked by the solvent peak.

The rather poor agreement shown for the two runs is not surprising if one remembers that the area measurements in this case give only

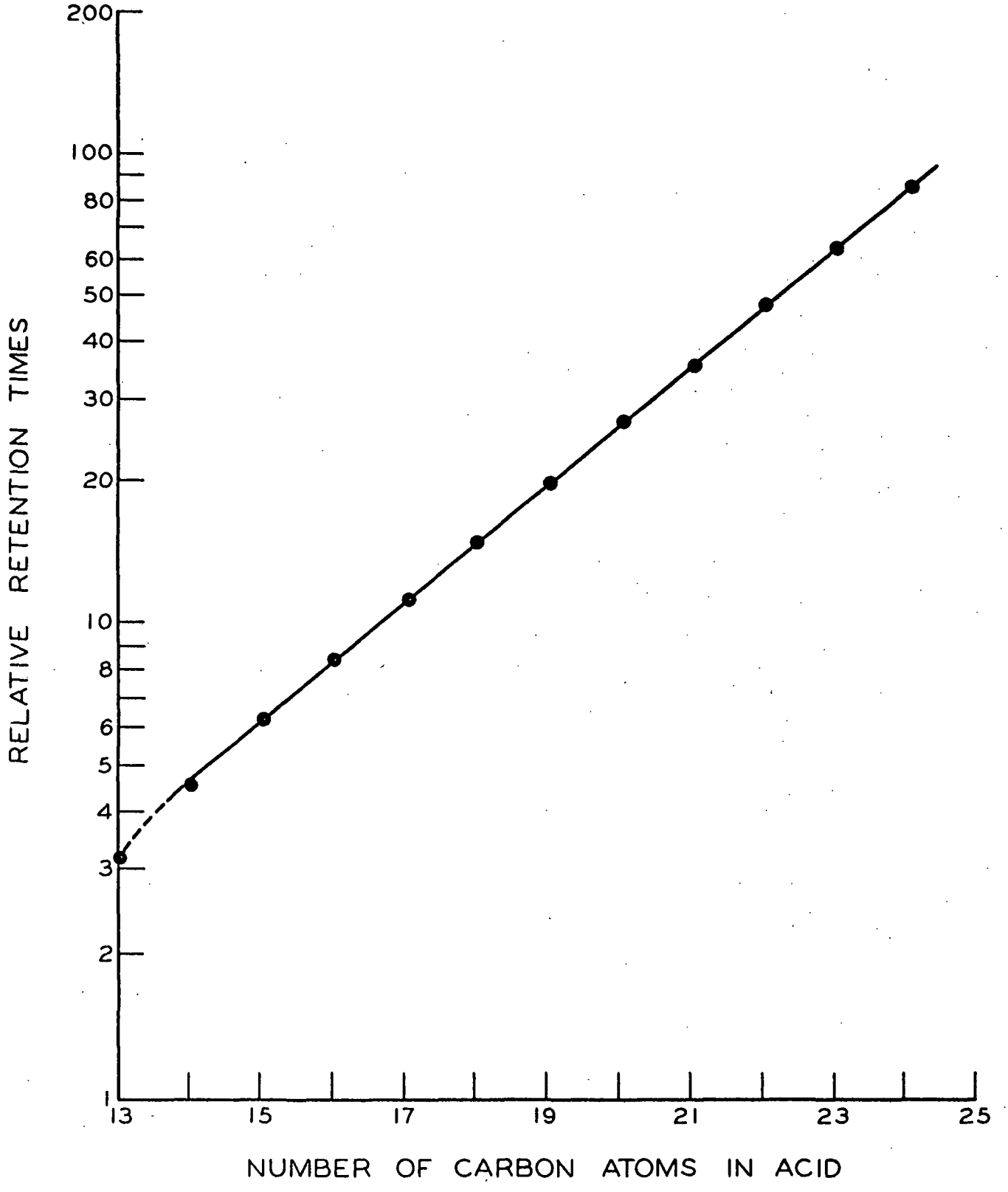

Figure 7. Retention Times for Fraction B

TABLE I

CHROMATOGRAPHIC DATA FOR
TWO ANALYSES OF FRACTION B

Number of Carbon Atoms	Total Area for Run 1, %	Total Area for Run 2, %
12	--	--
13	--	--
14	--	--
15	1.8	0.2
16	6.3	6.2
17	1.6	0.4
18	13.7	15.0
19	1.0	1.9
20	30.9	33.0
21	4.2	7.2
22	21.6	20.7
23	9.1	7.0
24	9.7	8.5
25	Too small to measure accurately	
26	Too small to measure accurately	

orders of magnitude. Although the actual percentages differ somewhat in the two runs, the orders of magnitude are about the same. The three major components of this fraction are thus stearic, arachidic, and behenic acids.

The presence of all the odd-numbered members of the fatty acid series is most interesting. Such complete series have been reported in human fat (24) and in butterfat (25), but as far as is known they have not been reported in plant extracts. Recently, Cooke and Hansen (11) isolated n-heptadecanoic acid from tall oil produced from *Pinus radiata*, but no other odd-numbered acids were indicated. It seems likely that, with the more widespread use of the gas chromatograph, other odd-numbered acids will be reported.

If a large-scale gas chromatograph were available, it should be possible to collect samples of each of these acids for melting point and carbon-hydrogen analyses. The amounts used with this instrument, a few micrograms, were too small to use for further characterization.

THE UNSATURATED FATTY ACIDS

The methyl esters of the unsaturated fatty acids were prepared using the method already described and then were chromatographed. The resulting chromatogram was very complex, the mixture apparently containing, in addition to some saturated fatty acids, both monoenoic and dienoic acids. An attempted graphic analysis of the peaks showed considerable scatter with no clear indication of an homologous series. No further work was done on this fraction.

THE UNSAPONIFIABLES

The ether solution of the unsaponifiables was taken to dryness, dissolved in methanol and chilled to 0°C. The white precipitate was removed by filtration. This material was a crystalline solid melting at 100°C. and giving a positive Liebermann-Burchard test indicating steroidal material. The amount precipitated represented only a small fraction of the unsaponifiables, and no further work was done on it.

When the filtrate was allowed to stand for several days at room temperature, however, a crystalline material slowly separated out. This material was recrystallized from methanol to constant melting point to yield a crystalline solid melting at 164-165°C. In contrast to the material described above which melted at 100°C., this material gave a negative Liebermann-Burchard test as well as a negative digitonin test. These two negative results indicate that the material is not a sterol. This material is optically active with a rotation of $[\alpha]_D^{25} = +16.7°$ ($\underline{c} = .02$) in chloroform. The infrared spectrum of this material, shown in Fig. 8, indicated the absence of carbonyl, conjugated unsaturation, and aromatic nuclei. There was some indication of cyclohexyl or cyclopropyl groups, but these bands are weak and difficult to assign with certainty. The one reactive group apparent in the infrared curve was hydroxyl. A Rast molecular weight was run and the indicated molecular weight was 372.

Further evidence of the absence of any conjugated unsaturation was provided by an examination of the ultraviolet spectrum of the material. The material was, in fact, virtually transparent in the ultraviolet region with no peaks evident.

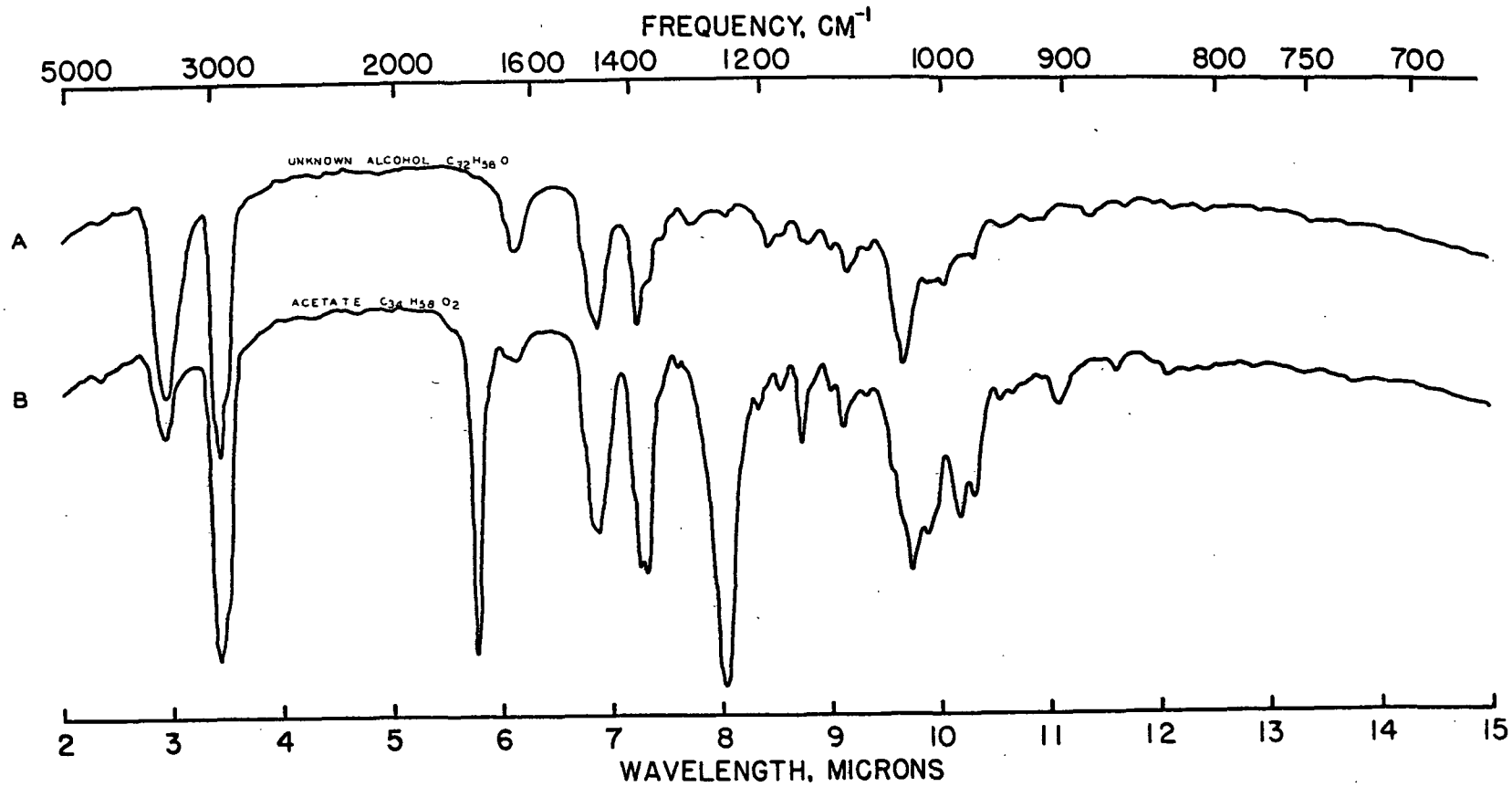

Figure 8. A. Spectrum of Unknown Alcohol. B. Spectrum of Acetate of Unknown Alcohol

That some unsaturation was present seemed likely from the subsequently determined formula, $C_{32}H_{54}O$. Such a formula demands that the total number of rings plus double bonds equal six. A test was, therefore, made for unsaturation using bromine, and the material was found to decolorize a carbon tetrachloride solution containing bromine. Unfortunately, insufficient material was available for a quantitative determination of the amount of bromine absorbed, but the presence of some unsaturation in the molecule is established.

An acetate of this alcohol was prepared using acetic anhydride in pyridine at room temperature as the acetylating agent. The resulting product was a crystalline solid melting at 164-165°C. At first it was thought that the material had not reacted, but a mixed melting point with the starting material was depressed 20 degrees. No rotation was run on this material, as the amount available was very small. The infrared spectrum of this material is also given in Fig. 8.

Carbon-hydrogen values were obtained on the acetate as well as on the starting material.[a] The values obtained on the parent compound were carbon 82.66 and 82.85, hydrogen 11.83 and 11.78, and those of the acetate were carbon 81.95 and 82.24, hydrogen 11.16 and 11.34. The analytical data suggest that the parent compound is a hemi-hydrate with the formula $C_{32}H_{54}O \cdot 1/2H_2O$. A summary of the data is given in Table II.

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[a] All carbon-hydrogen and acetyl values reported in this study were determined by Huffman Microanalytical Laboratories, P.O. Box 125, Wheatridge, Colorado.

TABLE II

CARBON-HYDROGEN RESULTS OF
THE UNKNOWN ALCOHOL AND ITS ACETATE DERIVATIVE

Compound	Calculated	Experimental	
(Alcohol) $C_{32}H_{54}O \cdot 1/2H_2O$	C, 82.94	82.66	82.85
	H, 11.87	11.83	11.78
(Acetate) $C_{34}H_{56}O_2$	C, 82.25	81.95	82.24
	H, 11.29	11.16	11.34

These values are in good agreement, and the assumption of the hemi-hydrate seems a logical one, inasmuch as methanol was used for all the recrystallizations with no attempt being made to exclude moisture from the system.

When an attempt was made to determine acetyl on the acetate by direct saponification followed by distillation of the volatile acids, three moles of acid were liberated instead of the expected one mole. The low oxygen content of the acetate, 7%, indicated by the carbon-hydrogen analysis precludes the possibility that the additional two moles of acid produced pre-existed as such in the acetate. That they were produced by oxidation during the alkaline saponification seems certain, but their place of origin is difficult to imagine, inasmuch as the infrared spectrum of the acetate did not reveal any easily oxidizable group.

THE METHANOL-SOLUBLE FRACTION

INTRODUCTION

The methanol-soluble fraction represents 72% of the total neutrals (445.5 g.) and is a viscous, light yellow oil with a characteristic woody odor. This material was saponified with 5% alcoholic potassium hydroxide under reflux for 16 hours at the end of which the alcohol was removed by boiling, water being added from time to time to maintain constant volume. As in the case of the methanol-insoluble fraction, trouble was encountered in trying to extract the alkaline saponification mixture directly with ether. Accordingly, the saponification mixture was first acidified with dilute sulfuric acid and then extracted with ether.

The subsequent workup of this fraction may be followed by referring to Fig. 9.

THE WATER LAYER

The acidic water layer was neutralized with sodium bicarbonate and taken to dryness on the steam bath. The resulting solid was extracted with 95% ethanol, and this solution was again taken to dryness. This solid was next extracted with a small quantity of 95% ethanol and again taken to dryness. The resulting material was dissolved in water and run through a column of Rohm and Haas MB-3, a mixed-bed resin containing both anionic and cationic-exchange resins. Evaporation of the resulting deionized solution yielded a thick sirup. The yield of this sirup was 1.54% based on the total methanol-soluble fraction or 6.86 g.

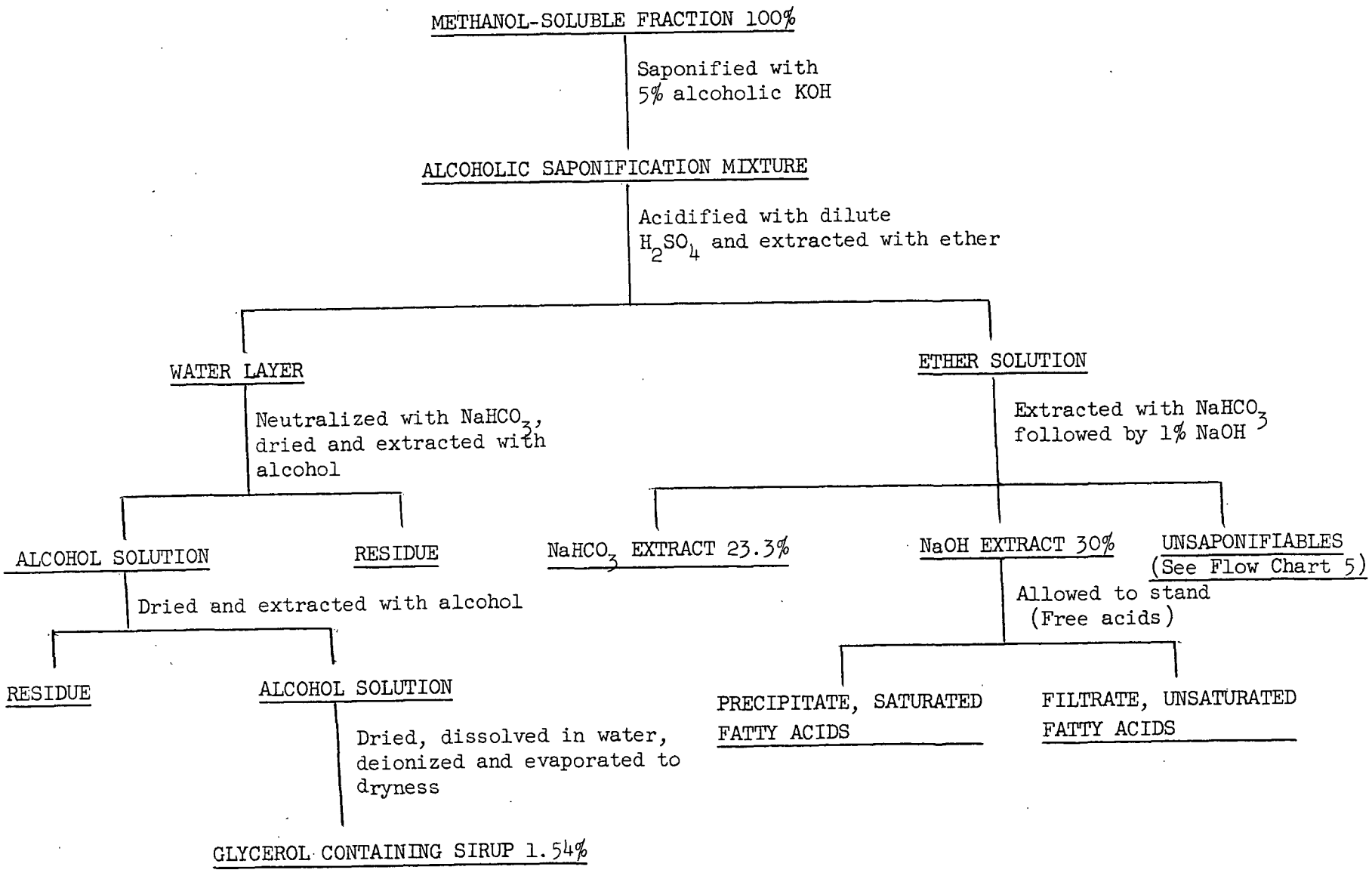

Figure 9. Flow Chart 4

This sirup was chromatographed using Whatman No. 1 paper with an 8:2:1 ethyl acetate:pyridine:water developer (26). An authentic sample of glycerol was spotted on the same sheet for comparison. After drying, the sheet was sprayed with the permanganate-periodate spray (27). The R_f values of both the unknown and the authentic glycerol spots were 0.415. The presence of glycerol was clearly indicated.

The tri-p-nitrobenzoate was prepared using p-nitrobenzoyl chloride in pyridine as the acylating agent. The melting point of the derivative was 185-188°C. (lit. 188°C.), and a mixed melting point with an authentic sample of glyceryl tri-p-nitrobenzoate was not depressed.

The yield of crude glycerol sirup was 1.54% compared to the yield of acids of 53%. It is possible that some glycerol was lost during the isolation procedure, and it is also possible that materials other than glycerol were contained in the crude sirup, although there were no reducing sugars; nevertheless, a comparison of the two yields is of some interest. The ratio of glycerol to acid in this case is 0.03 compared to the expected ratio in the case of triglycerides of approximately 0.10. This low ratio suggests the presence of the triglycerides as well as other fatty acid esters in the methanol-soluble fraction.

THE UNSAPONIFIABLES

The ether solution containing both the acids and the unsaponifiables from the saponification of the methanol-soluble fraction was extracted with 1% sodium hydroxide to remove the acids and then with distilled water until the wash water was neutral. The yield of unsaponifiables

based on the methanol-soluble group is 22.2% (98.9 g.) corresponding to 0.1% based on the ovendry wood. The workup of the unsaponifiables may be followed by referring to Fig. 10.

The ether solution was taken to dryness and the residue dissolved in low-boiling petroleum ether. This solution was chromatographed on alumina using petroleum ether, benzene, chloroform, and alcohol containing 1% acetic acid, successively.

The Fraction Removed With Petroleum Ether

The fraction removed with petroleum ether was found to contain esters that had escaped the saponification as well as hydrocarbons. The yield of this fraction was 0.43% (1.91 g.) based on the methanol-soluble group. The material was saponified with 10% alcoholic potassium hydroxide under reflux for four hours at the end of which the alcohol was removed by boiling again, and adding water from time to time to maintain constant volume. The mixture was acidified with dilute sulfuric acid and extracted with ether.

The ether solution was next extracted with 1% sodium hydroxide to remove the acids and washed with distilled water until the wash water was neutral. The ether solution of the unsaponifiables was next taken to dryness and the residue dissolved in low-boiling petroleum ether. When this solution was chromatographed on alumina, the portion removed with petroleum ether was found to contain a quantity of hydrocarbons. This material, a semi-solid similar to petroleum jelly, was examined by infrared absorption and found to contain only saturated aliphatic hydrocarbons.

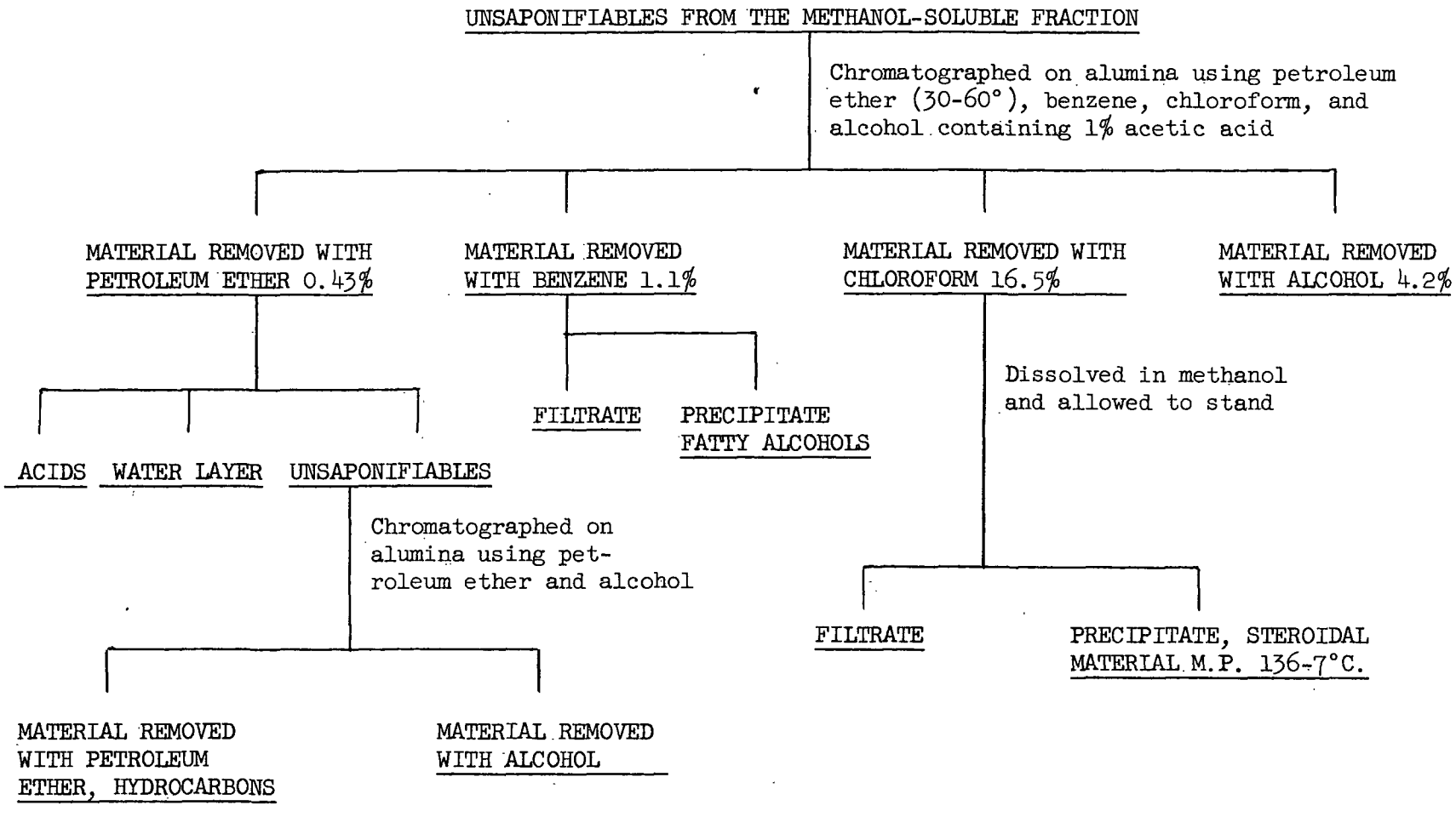

Figure 10. Flow Chart 5

No further work was done on the other fractions obtained on saponification of the fraction removed with petroleum ether.

The Fraction Removed With Benzene

The fraction removed with benzene represented 1.1% (4.9 g.) of the methanol-soluble fraction. When this fraction was dissolved in methanol and set aside, crystals appeared. Preliminary tests indicated that these materials might be fatty alcohols. An attempt then was made to separate and identify these materials by converting them to their acetates and chromatographing these in the gas chromatograph. The results of this attempt are unfortunately inconclusive, but the three major components indicated are the C_{24}, C_{26}, and C_{27} straight-chain fatty alcohols. These results must be considered tentative at the present time, as the peaks did not follow exactly the semilogarithmic relationship described earlier. Recently, however, Pearl and co-workers found a C_{26} and C_{27} fatty alcohol in aspen spent sulfite liquor (14), and Hossfeld and Hunter (8) reported ceryl alcohol (C_{26}) in the extract of trembling aspen bark.

The Fraction Removed With Chloroform

When the fraction removed with chloroform was dried, dissolved in methanol and allowed to stand, crystals separated out. This crystalline material was purified by recrystallization from methanol to constant melting point. The resulting material melted at 136-137°C., had a rotation in chloroform of $[\alpha]_D^{25} = -31$, $c = 0.02$, and gave positive Liebermann-Burchard and digitonin tests. The latter two tests suggest that the material is steroidal.

An infrared spectrum of this material shown in Fig. 11 revealed that although it contained no carbonyl, no conjugated unsaturation, and no benzene nuclei, it did contain hydroxyl. This infrared spectrum was found to be almost identical to that of β-sitosterol, the spectrum of which is included in Fig. 11 for comparison.

The acetate of this alcohol was prepared using acetic anhydride in pyridine at room temperature. The resulting product was purified by recrystallization from methanol to yield a crystalline solid melting at 117.5-118°C. The acetate was also optically active with a rotation of $[\alpha]_D^{25} = -28.4 \pm 0.8°$, $\underline{c} = 0.02$, in chloroform.

The benzoate was prepared using benzoyl chloride in pyridine at room temperature. The resulting product was purified by recrystallization from a methanol-benzene mixture, as this material is insoluble in methanol itself. The purified product was a crystalline solid melting at 147-148°C. and having a rotation of $[\alpha]_D^{25} = -14.3 \pm 0.5$, $\underline{c} = 0.02$, in chloroform.

Carbon-hydrogen analyses were performed on the two derivatives as well as on the parent compound, and the data suggest a C_{32} monohydroxy compound with 56-58 hydrogens. Although the carbon-hydrogen data summarized in Table III do not permit a clear choice of the number of hydrogens in the molecule, there are other factors which should be taken into consideration.

It has already been mentioned that the infrared spectrum of this alcohol is almost identical with that of β-sitosterol, a fact which may be ascertained by an examination of the two spectra given in Fig. 1.

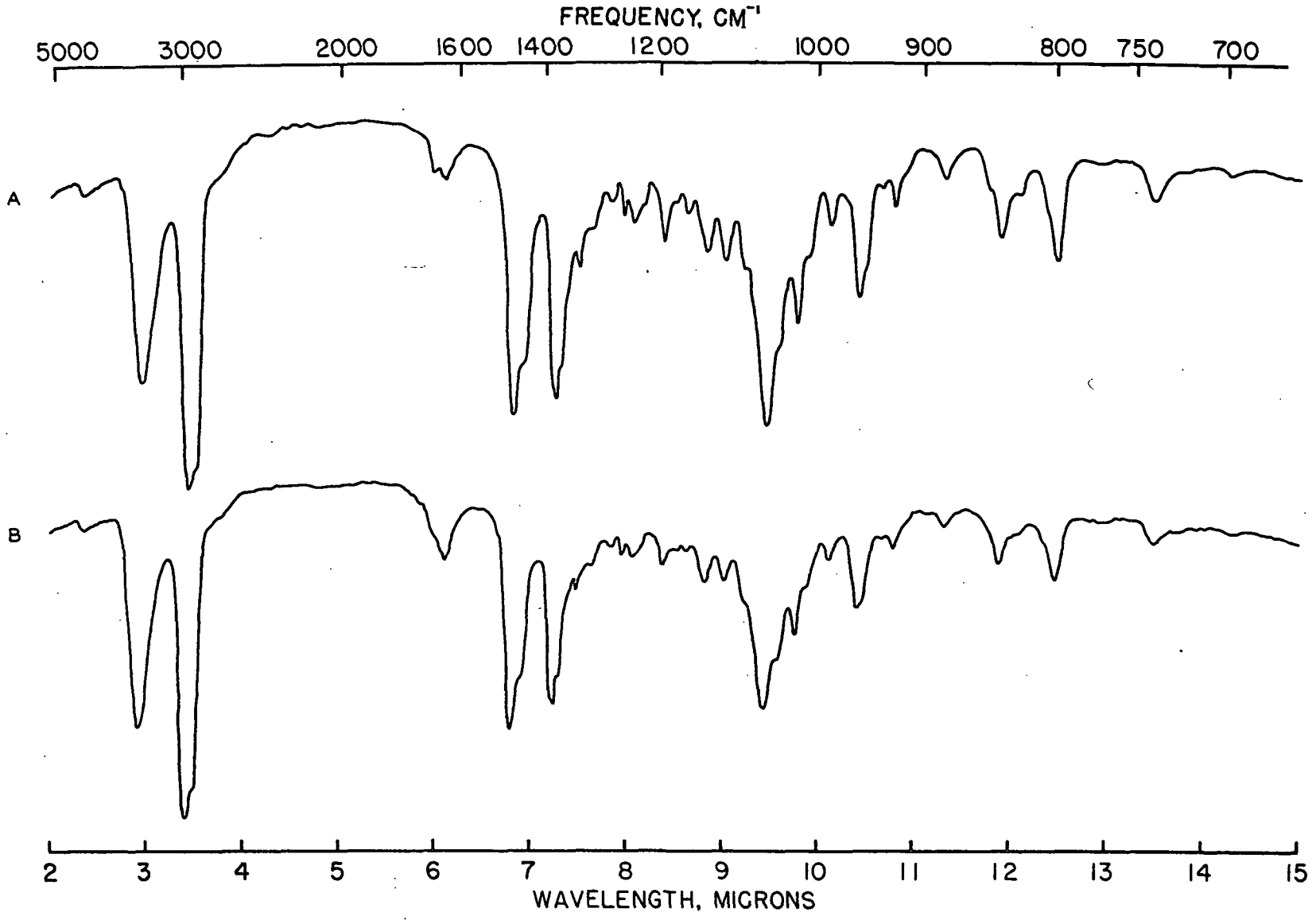

Figure 11. A. Spectrum of β-Sitosterol. B. Spectrum of Unknown Steroidal Material

TABLE III

CARBON-HYDROGEN RESULTS FOR THE
STEROIDAL MATERIAL AND ITS DERIVATIVES

Compound	Calculated for: R = 56,	R = 58	Experimental	
(Alcohol) $C_{32}H_{R}O \cdot 1/2H_2O$	82.58	82.22	82.00,	82.19
	12.26	12.63	12.35,	12.52
(Acetate) $C_{32}H_{R+2}O_2$	81.93	81.60	81.67,	82.02
	11.65	12.00	11.99,	12.02
(Benzoate) $C_{39}H_{R+4}O_2$	83.57	83.27	83.32,	83.15
	10.71	11.03	10.98,	10.98

The spectrum of the benzoate of this alcohol is also very similar to that of β-sitosterol benzoate, but although many similarities may be seen in the case of the acetates, sufficient differences may be noted to label this as a different compound. The curves for the acetates and benzoates are given in Figs. 12 and 13.

The correspondence of these infrared curves is not surprising in view of the fact that many sterols give similar infrared spectra. Beher, Parsons, and Baker (28) have pointed out that different sterols may give identical spectra and that the infrared spectrum alone is of little value in identifying a particular sterol. They show that β-sitosterol and cholesterol, for example, which differ in the side chain, give identical spectra.

The similarity of the infrared spectra to those of β-sitosterol and its derivatives and the positive diagnostic tests such as the Liebermann-

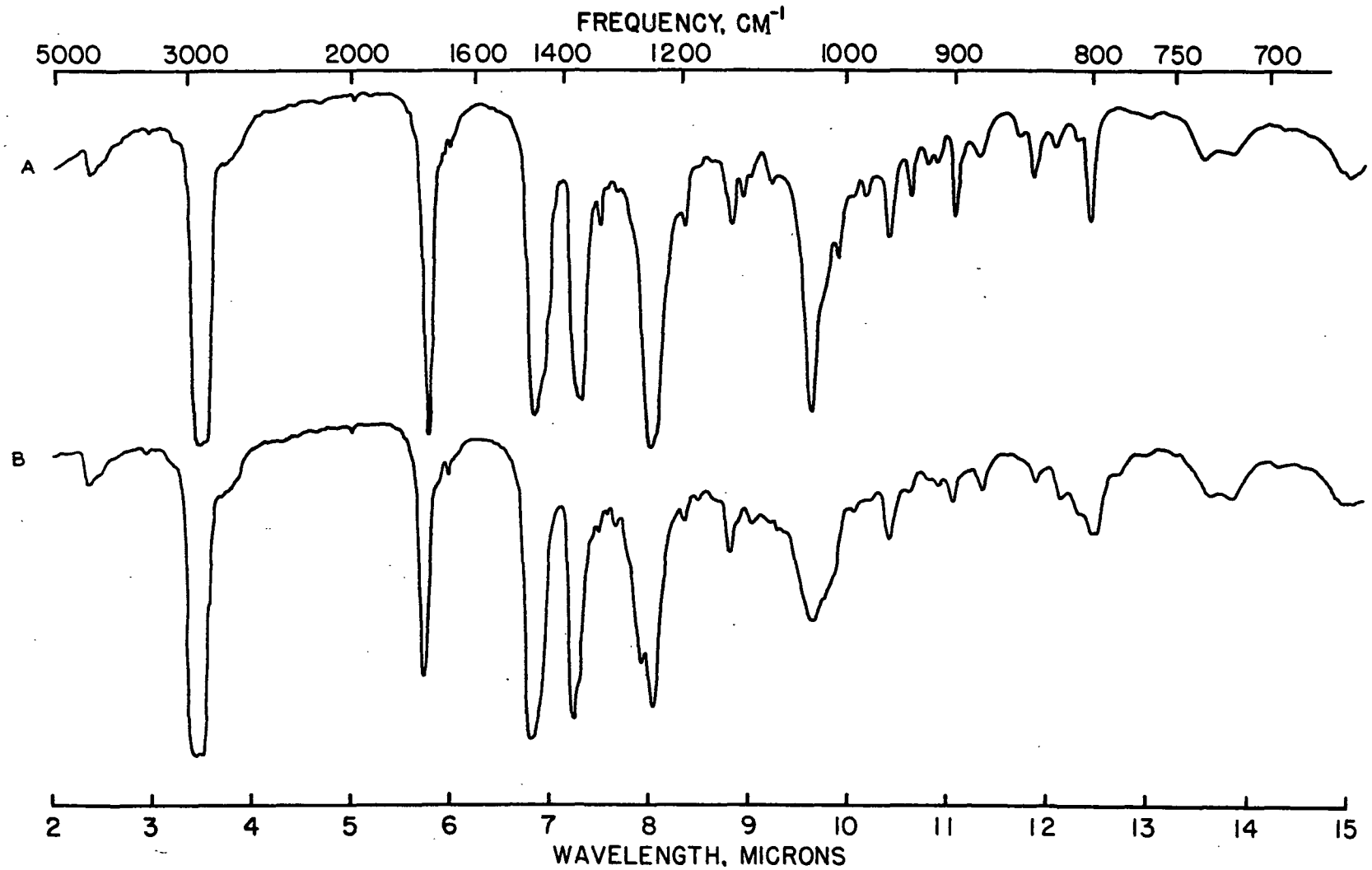

Figure 12. A. Spectrum of β-Sitosterol Acetate. B. Spectrum of Unknown Acetate

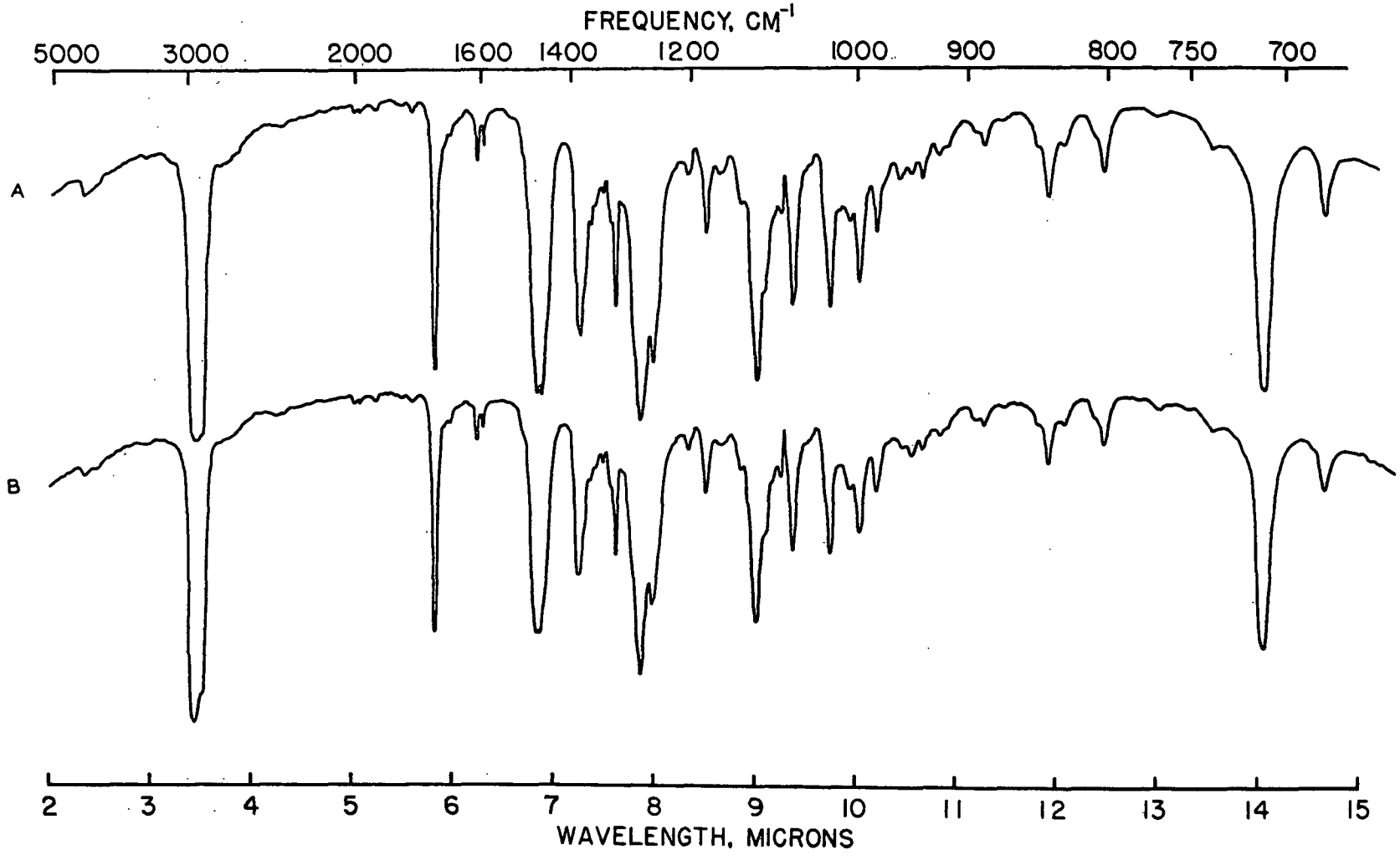

Figure 13. A. Spectrum of β-Sitosterol Benzoate. B. Spectrum of Unknown Benzoate

Burchard and the digitonin tests suggest that this material is a sterol. The presence of the sterol nucleus and concurrent unsaturation, indicated by the positive Liebermann-Burchard test, suggest a formula of $C_{32}H_{56}O$ for a monohydroxy, 32-carbon sterol. The two additional hydrogens suggested by the formula, $C_{32}H_{58}O$, would demand either a saturated molecule or the opening of one of the rings of the sterol nucleus. The former possibility seems ruled out by the positive Liebermann-Burchard test and the latter by the similarity of the infrared spectra to those of β-sitosterol and its derivatives.

The lack of any additional reactive unsaturation in this molecule was indicated by its failure to decolorize a carbon tetrachloride solution of bromine. If it is assumed that this molecule contains four rings, this latter bit of information places, in effect, a lower limit of 56 on the number of hydrogens in the molecule.

Some support for this formula, $C_{32}H_{56}O$, may be found by a look at the work of Perilä (12) who isolated a sterol glucoside from the European aspen, Populus tremula and suggested that it was a sitosterol glucoside with the formula $C_{35}H_{60}O_6$. This would give the sterol portion of the glucoside the formula $C_{29}H_{50}O$, the same as β-sitosterol. A carbon-hydrogen analysis of the glucoside, however, gave carbon 71.5% and hydrogen 10.3% compared with the calculated values of carbon 72.9% and hydrogen 10.4%. The difference in carbon content, 1.4%, is rather large.

If Perilä's data are reconsidered assuming a $C_{38}H_{60}O_6$ compound, one having a sterol portion with the formula $C_{32}H_{56}O$, the expected carbon-

hydrogen values are carbon 71.7% and hydrogen 10.7% compared with Perilä's experimentally determined values of carbon 71.5% and hydrogen 10.3%. These values are in better agreement than the values given by Perilä for the glucoside of a $C_{29}H_{50}O$ sterol.

Interestingly enough, the melting point of the parent sterol isolated by Perilä on acid hydrolysis of the glucoside and that of its acetate coincide with those found in this study for the unknown alcohol, $C_{32}H_{56}O$, and its acetate. In addition, Perilä found no unsaturation beyond the one double bond in the sterol nucleus, and this too coincides with the findings for this unknown alcohol.

The Fraction Removed With Alcohol

The fraction removed with alcohol represents 4.2% of the methanol-soluble fraction. This material was set aside and no further work done on it.

THE ACIDS

The 1% sodium hydroxide solution containing the acids from the saponification of the methanol-soluble fraction was acidified with dilute sulfuric acid and extracted with ether. The total yield of acidic material was 53.3% (237.5 g.) based on the methanol-soluble fraction. The acids were separated into two groups by extracting the ether solution with saturated sodium bicarbonate followed by 1% sodium hydroxide. The yield of bicarbonate-extractable acids was 23.3% (103.8 g.) based on the methanol-soluble fraction or 43.7% based on the total

acids. The yield of the sodium hydroxide-extractable acids was 30% (133.7 g.) based on the methanol-soluble fraction or 56.3% based on the total acids.

The Bicarbonate-Extractable Fraction

The sodium bicarbonate solution was acidified with dilute sulfuric acid and extracted with ether. A small amount of this ether solution was spotted on Whatman No. 1 paper, developed with a butanol:pyridine: water, 10:3:3 mixture (29) and sprayed with diazotized p-nitroaniline (33). There was no indication of any p-hydroxybenzoic acid.

The 1% Sodium Hydroxide Fraction

The 1% sodium hydroxide solution was acidified with dilute sulfuric acid and extracted with ether. When the ether was evaporated and the material allowed to stand, crystals appeared. These were washed with alcohol and recrystallized from an alcohol-water mixture. The melting point of the recrystallized material was 74°C. Preliminary tests showed this material to be a mixture of saturated fatty acids, so a quantity of this material was converted to its methyl ester by the procedures already described and analyzed by the gas chromatograph.

The results of the gas chromatograms indicated the presence of all the saturated fatty acids from C_{15} to C_{28} with the single exception of C_{27}, but the members below C_{20} were present in relatively small amounts and were obscured by the solvent peak under the conditions employed. A plot of the exit times is given in Fig. 14. Excellent agreement with the expected logarithmic relationship may be seen for the acids above C_{20}.

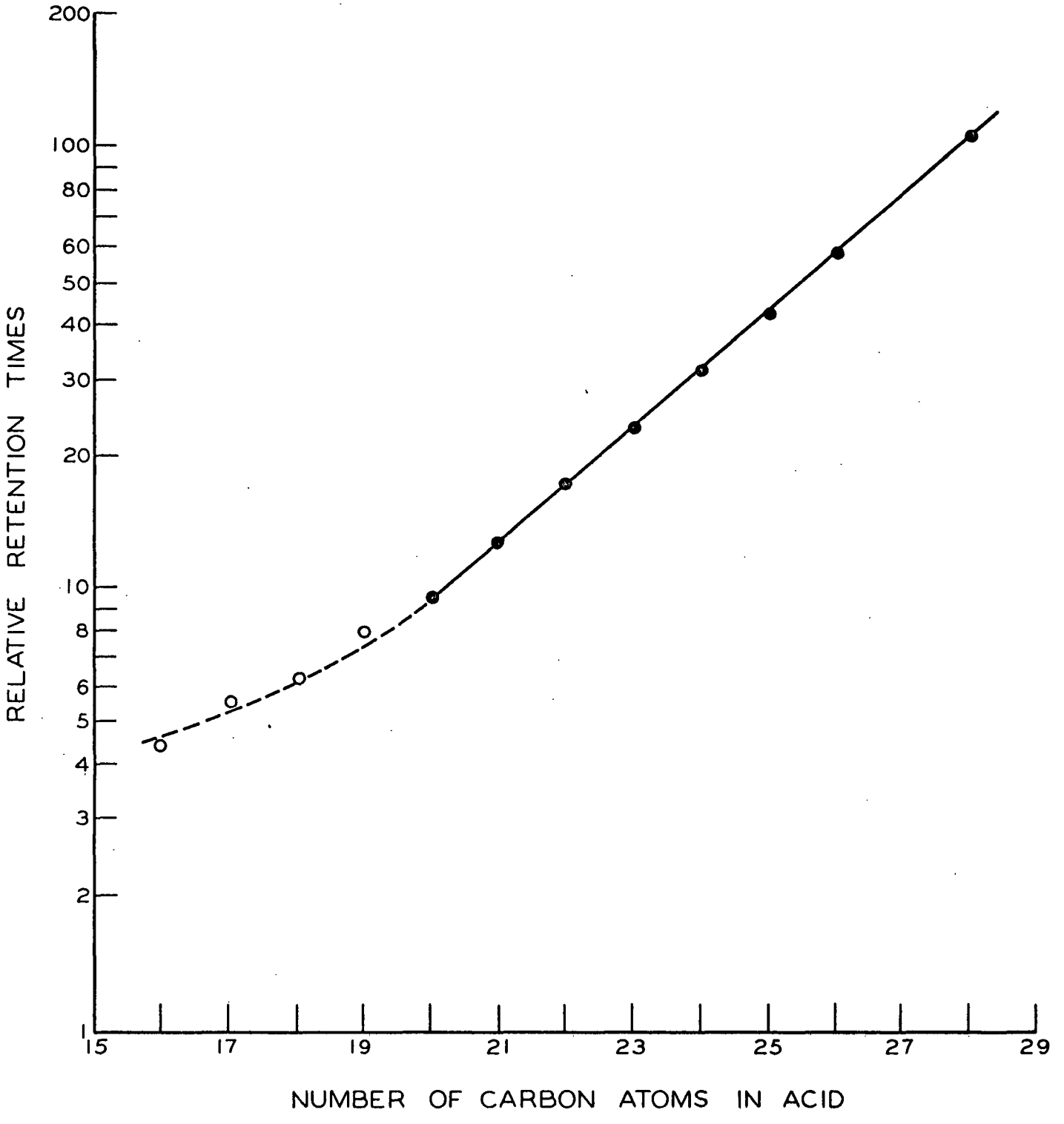

Figure 14. Retention Times For the Acids From Methanol-Soluble Fraction

The scatter shown for the members below this comes at least partly from their confusion with the solvent peak and from the fact that their presence in such small amounts made determination of the peak difficult.

It was previously stated that the conditions employed with this instrument did not permit the accumulation of strictly quantitative data, but some idea of the relative orders of magnitude may still be obtained by an examination of the relative areas under the curves. The relative areas for the members above C_{20} are given in Table IV.

TABLE IV

GAS CHROMATOGRAPHY DATA FOR THE SATURATED FATTY ACIDS FROM THE METHANOL-SOLUBLE FRACTION

Acid	Total Area, %
C_{20}	2.0
C_{21}	1.4
C_{22}	15.1
C_{23}	9.0
C_{24}	34.0
C_{25}	4.8
C_{26}	30.0
C_{27}	--
C_{28}	3.0

A small quantity of the mother liquor from which the saturated fatty acids crystallized was esterified and separated on the gas chromatograph. The results indicate that the major component of this fraction is linoleic acid, the doubly unsaturated C_{18} acid. Also

indicated are smaller amounts of oleic acid, the monounsaturated C_{18} acid and stearic acid, the saturated C_{18} acid.

The finding that the major acid from this fraction is linoleic acid is in accord with the conclusions of Buchanan, Sinnett, and Jappe (20) who found that the major acid in the extract of Populus tremuloides and Betula papyrifera was linoleic acid. This is also supported by the results of Kahila and Rinne (1) who found the major unsaturated acid of Betula verrucosa to be linoleic acid.

SUMMARY AND CONCLUSIONS

The neutral fraction of the benzene extract of aspenwood has been studied and several compounds isolated and identified. A preliminary separation was made on the basis of solubility in methanol and the two fractions, the methanol-soluble and the methanol-insoluble, studied separately.

The methanol-insoluble fraction was found to contain esters of fatty acids and an unknown alcohol, $C_{32}H_{54}O$. No glycerin was found on saponification of this fraction, but the method used to test for its presence may have been insufficiently sensitive.

The materials indicated by a vapor-phase chromatographic study of the acids from the saponification of this fraction included all the members of the saturated fatty acid series from C_{12} to C_{26} including the odd-numbered members. The unsaturated fatty acids from this fraction were found to be a complex mixture with no one predominant acid.

The unknown alcohol, assigned the formula $C_{32}H_{54}O$, gave negative results with the Liebermann-Burchard and digitonin tests. It gave a positive test, however, for unsaturation by the addition of bromine, but the infrared and ultraviolet spectra demonstrate that conjugated unsaturation is absent. An alkaline saponification performed on the acetate of this alcohol resulted in the liberation of two additional moles of volatile acids beyond the one mole expected from the monoacetate. No answer has been found for this behavior.

The methanol-soluble fraction was found to contain fatty acids, fatty alcohols, saturated aliphatic hydrocarbons, glycerol, and an unknown steroidal material, $C_{32}H_{56}O$.

Indications were again given by a gas chromatographic study for the presence of all the saturated fatty acids from C_{15} to C_{28} with the single exception of C_{27}. The unsaturated fatty acids were found to contain primarily linoleic acid with some oleic acid present.

Tentative chromatographic investigation of the fatty alcohols suggested the presence of C_{24}, C_{26}, and C_{27} alcohols. Although these results must be considered preliminary, they are supported by the findings of Pearl and co-workers (14) who isolated a C_{26} and C_{27} fatty alcohol from aspen spent sulfite liquor as well as by the findings of Hossfeld and Hunter (8) who isolated ceryl alcohol (C_{26}) from trembling aspen bark.

The saturated aliphatic hydrocarbons which were isolated from this fraction occurred as a complex mixture with the consistency of petroleum jelly.

The presence of glycerol was demonstrated chromatographically as well as by the preparation of its tri-*p*-nitrobenzoate.

Of interest in the present investigation is the discovery of several odd-numbered members of the fatty acid series. Although odd-numbered acids have been isolated from animal fats (24, 25, 31, 32) and *n*-heptadecanoic (margaric) acid has been reported in the tall oil from *Pinus radiata* (27), it is believed that this is the first time

that data indicating the presence of several members of the series in a plant extract have been reported. The increased sensitivity now possible with the use of the gas chromatograph may result in a more general finding of the odd-numbered members of the series.

The indicated presence of linoleic acid as the major constituent of the methanol-soluble fraction confirms the findings of Buchanan, Sinnett, and Jappe (7) who reported this acid as the major acid found on the saponification of the total extract of aspenwood. Kahila and Rinne (1) also reported finding linoleic acid as the major constituent of the European birch, Betula verrucosa. The presence of relatively large amounts of such unsaturated acids could lead to problems under the conditions used in papermaking.

The ratio of glycerol to total acids in the methanol-soluble fraction was 0.03 compared with the ratio in the case of the triglycerides of approximately 0.10. This low ratio suggests the presence of fatty acid esters other than glyceryl esters in addition to the expected triglycerides. The latter might be expected to survive the acid sulfite process and to be carried over into the pulp. Mutton (6) has shown that the amount of free fatty acids increases on aging, whereas the amount of combined fatty acid decreases. This finding suggests an hydrolysis of the naturally occurring fatty acid esters, and if this hydrolysis were to continue in the pulp it would supply a ready source of free fatty acids. This idea is supported by the work of Kahila and Rinne (1) who found a much higher proportion of free acids in the unbleached birch sulfite pulp resin than was found in the wood. The

suggestion has been made that the vapor-phase transfer of free fatty acids may be one cause of the development of self sizing in papers intended for absorbent use (33).

The similarity of the formulas given for the unknown alcohol isolated from the methanol-insoluble fraction and the steroidal material isolated from the methanol-soluble fraction suggest a possible relationship between the two, but without some clear idea of their respective structures nothing definite can be said.

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