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Title THE SEPARATION AND PURIFICATION OF THE FATTY AND HYDROXY FATTY ACIDS OF WHITE FIR BARK WAX

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This investigation was undertaken to develop a procedure by which the fatty acids could be separated from the hydroxy fatty acids of bark waxes. The specific hexane-insoluble, benzene-soluble wax used in this research was obtained from the bark of white fir, Abies concolor (Lindl. and Gord.).

The acids were removed from the other components of the wax by a process of saponification, then acidification, followed by extraction with benzene, then hexane. These crystals were resaponified, extracted with hot benzene, and then acidified. This procedure resulted in two crude free acid fractions, I and II.

Then separately, fractions I and II were subjected to a purification procedure which was designed to yield pure acids. This procedure consisted first of forming the methyl esters of the crude free acid mixture and crystallizing them from hexane. These methyl ester fractions were then saponi-

fied and again crystallized from hexane. Some of the resulting acid fractions were then combined on the basis of simolar melting points. These fractions were then subjected to an acetylation procedure which would attach an acetate grouping to any hydroxyl groups which were present. In this way the hydroxy acids could be separated from the fatty acids by the relative solubility of the acetates and acids in hexane. In this solvent the acetates were the more coluble components. These acetate and acid fractions were then saponified using a quantitative procedure, so that the approximate molecular weight of the fraction could be obtained. At this point after recrystallization from hexane, the fraction was considered to be pure. However, a gas-liquid chromatography analysis showed that the samples were not pure but contained up to 30% of contaminating acids.

But despite this fact, the major acid was separated and had the following properties: melting point 87.5-89°C., methyl ester melting point 70-74°C., apparently no hydroxyl group present, and the approximate molecular weight of the methyl ester 345.3. This acid was obtained from the crude free acid fraction I. One other acid from this same fraction showed these definite properties: melting point 88.5-90°C., methyl ester melting point 71-72°C., acetate melting point 72-74°C., and approximate molecular weight of the acetate 400. There were other acids obtained from this Fraction I, but they were present in small quantities and were not completely characterized.

From the crude free acid mixture II a C_{16} dicarboxylic acid was isolated. Its properties were the following: melting point 124-125°C., dimethyl ester melting point 54-55°C., and no hydroxyl group present. No other acids were isolated from this fraction II.

This research has shown that the procedure which was developed for the purification of these acids was only partially successful. However, the major acids could be obtained relatively pure, but there were other smaller fractions which might contain mixtures of these major acids or different acids.

THE SEPARATION AND PURIFICATION OF THE FATTY AND HYDROXY FATTY ACIDS OF WHITE FIR BARK WAX

by

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I. INTRODUCTION

Statement of Problem

The subject of this research concerned the fatty and hydroxy fatty acids which were obtained from the hexane-insoluble, benzene-soluble fraction of the bark of white fir (*Abies concolor* (Lindl. and Gord.). The major emphasis was placed on the extraction of these acids from the other components that are present in the bark and the purification of the individual acids. Therefore a procedure needed to be developed by which these acids could be obtained in relatively pure form.

Reasons for Study

This study was undertaken for several reasons. An earlier research of the white fir bark disclosed that only two saturated acids were present, behenic and 13-hydroxy myristic (14); however, further work by E. F. Kurth indicated that the hezane-insoluble wax contained a complex mixture of fatty acids (27).

The utilization of the bark of sawlogs and peelers has long been a problem of sawmill and plywood plant operators. This problem may become more acute in the immediate future, since the State Sanitary Authority of the state of Oregon has proposed that no more conical burners be constructed and that the burning of waste in those that are now in use be carefully controlled. These proposed regulations were designed to control or prevent air pollution (37). Therefore it appears that more economically feasible uses need to be developed for the consumption of bark. At this time chemical utilization of bark includes such things as the Silvacon products of Weyerhaeuser Timber Company for the production of thermosetting compounds and cold-molded plastics (2). Other uses include products for oil-well drilling muds, for leather tanning, for water purification treatment, for the flotation process, for ceramic binders, and for phenolic resins (34,45). Another use of some importance is the combination of bark and wood which serves as a suitable raw material for hardboard. Approximately 20-45% of the weight of the hardboard can be bark (1). However, agricultural uses for mulches and soil-conditioners is probably the largest use (25).

In Table I are shown the percentages of fatty acids in waxes of certain species of trees and the percentages

of wax in the barks of these trees. From this data it can be seen that fatty and hydroxy fatty acids are actually available from the barks of these species. The feasibility of commercial utilization is again another question.

Uses of Fatty Acids

Since there are many uses for fatty acids and their derivatives, any additional source may be desirable. Fatty acids seem to be utilized mainly in the form of metal salts and other derivatives. For example, the heavy metal soaps find uses as coloring agents in varnishes and other coatings (43,p.896-898), mordants for dyes (43,p.896-898) agents for the treatment of leather and various fabrics (43,p.896-898) ingredients for cosmetics (16), and additives for lubricants (40). Copper and mercury salts find uses in fungicidal sprays (23), mercury soaps in salves and ointments (10), and zinc soaps in dusting and drying powders (8). Calcium soaps are incorporated in the waterproofing of paper (6), in waxes (9), in paints (4), and in cleansing compounds (39). The acid chloride derivatives of the fatty acids find uses as intermediates in the preparation of chemicals which possess wetting and detergent properties (43, p.812), and with naphthalene or petroleum oil fractions they can be used as addends to lubricating oils (33).

TABLE I. PERCENTAGES OF FATTY ACIDS IN WAX AND WAX IN BARK OF VARIOUS SPECIES

% fatty acids in wax	% wax in bark	Species	ref.
25 h.i.[a] 60 h.s.[b]	3.6-6.0 h.s.	Douglas fir	24,30
54.7 h.s. 61.5 h.i.	2.5 h.s. 1.0 h.i.	white fir	14
	5.0 h.s.	Ponderosa pine	28
57.8 b.s.[c]	1.52 h.i.	*Pinus densiflora*	11
	2.5 h.s.	red fir	3
	3.32 h.s.	Mountain hemlock	26
63 h.s.	3.8 h.s.	Aspen	15
20.7 h.s.	3.5 h.s.	*Euphorbia cerifera*	44

[a]hexane-insoluble wax

[b]hexane-soluble wax

[c]benzene-soluble wax

The sodium sulfates of the fatty acids find their largest use as wetting agents and detergents (5), in combination with higher alcohols or mineral oils they can be used as flotation agents (32), and they can also be used as insecticides as both the emulsifying and toxic agent (7).

Therefore from the standpoint of chemical utilization there is a demand for fatty acids and a demand for more utilization of barks.

Previous Work in This Area

Much of the work concerning the extractives of conifer barks has been done by Kurth and co-workers. Their work has included various species of firs and Douglas fir (24, 12,13,14,30,31,3), pines (28,29), cedar (46), and hemlock (26). Some other workers have also been interested in pine (11,42), oak (20,21,22), and birch (19,18). A short summary of some of the pertinent papers follow.

Kurth has reported the composition of the hexane-soluble wax fraction of Douglas-fir bark (24). His procedure included the extraction of the bark in a stainless steel extractor with hot hexane. The wax which remained after the evaporation of the solvent was then saponified with ethanolic potassium hydroxide. The saponification mixture was then extracted with hexane in a separatory funnel to remove unsaponifiable compounds. This unsaponi-

fiable fraction was evaporated to dryness and crystallized from acetone. The white crystalline solid was identified as lignoceryl alcohol. Then the filtrate from the lignoceryl alcohol crystallization, after evaporation of the acetone and recrystallization from dilute alcohol, was identified as phytosterol which is a mixture of α- and β-sitosterols. This lignoceryl alcohol and phytosterol are present to the extent of approximately 20% in the wax. The major portion of the wax was obtained in the alkaline solution from the separation of the unsaponifiable matter. This solution was acidified with sulfuric acid, extracted with hexane, and recrystallized from acetone and hexane. The white crystals which were obtained were identified as lignoceric acid. There was a small amount of yellow solid residue left in the filtrate from this crystallization which was characterized as oleic acid. The lignoceric acid and small amount of oleic acid composes approximately 60% of the wax. In the aqueous layer after the extraction of the lignoceric acid a brown resin remained suspended. This resin was extracted with diethyl ether and recrystallized from hot benzene and dilute ethanol which gave yellow prisms. These crystals were identified as ferulic acid (4-hydroxy-3-methoxy-cinnamic acid). This constituent accounted for approximately 20% of the wax. It was also mentioned that the benzene-soluble, hexane-insoluble wax appeared to be a more complex mixture.

In a paper by Hergert and Kurth the cork fraction from the Douglas-fir bark was studied (13). The extractives were separated according to their solubilities in hexane, benzene, diethyl ether, ethanol, and hot water. The hexane and benzene fractions were waxes. The hexane-soluble wax was analyzed and shown to contain lignoceric acid 49.3%, lignoceryl alcohol 27.5%, ferulic acid 9.8%, phytosterol 0.6%, hexane-insoluble, benzene-soluble acid 8.1%, and benzene-insoluble phenolic material 3.6%. The benzene-soluble wax had a more complicated composition. Again phytosterol 0.21%, lignoceryl alcohol 2.3%, and lignoceric acid 19.2% were obtained. Also a hydroxy palmitic acid 7.30%, and an unsaturated hydroxy acid 36.88% were isolated. Other unidentified products were an ether-soluble acid 6.35%, an ethyl acetate-insoluble material 4.57%, and an acetone-soluble phlobaphene 27.37%. Also present in the mixture was glycerol 5.92%.

Hergert and Kurth also examined the extractives of the total bark of white fir (14). Bark samples were taken from the top, middle, and bottom sections of the trees to determine the distribution of the extractives. Again the extractives were separated according to their solubilities in hexane, benzene, diethyl ether, ethanol, and hot water. The hexane-soluble wax was dissolved in hot acetone and allowed to cool, forming a white precipitate of uncombined alcohols. This precipitate was recrystallized from acetone

and characterized as lignoceryl alcohol. The filtrate from the above precipitation was dissolved in diethyl ether and extracted with 5% potassium carbonate to remove any free acids. These potassium salts were then acidified with hydrochloric acid, the solids collected on a filter and recrystallized from acetone. The white crystalline product was identified as behenic acid and its near homologs. The ether layer which remained after the extraction of the free acids was evaporated to dryness, then saponified with alcoholic potassium hydroxide. After saponification the alcohol was replaced with water, and this solution was extracted with diethyl ether to remove any unsaponifiable matter. This unsaponifiable fraction was a cream-colored wax which was again subjected to saponification, ether extraction, and recrystallization from acetone to yield a white crystalline substance identified as lignoceryl alcohol. Also in this fraction was found a small amount of phytosterol which was identical to that obtained from the Douglas-fir bark. The potassium salt mixture which remained after the extraction of the unsaponifiable matter was acidified and extracted with diethyl ether to remove the initial combined acids. The ether layer was evaporated and the residue was crystallized from hexane and acetone to give a white crystalline product which was identified as behenic acid. Therefore the total composition of this wax was as follows:

lignoceryl alcohol 34.78%, behenic acid 50.09%, phytosterol 1.26%, unsaturated alcohols 1.82%, and unsaturated acids 9.85%. The hexane-insoluble, benzene-soluble wax appeared to be more complex than the hexane-soluble wax. Its insolubility in cold sodium bicarbonate, sodium carbonate, or sodium hydroxide indicated the absence of free acids or acidic groupings. Therefore this wax was saponified in 40% alcoholic potassium hydroxide after which the alcohol was removed and the aqueous solution extracted with diethyl ether to remove the unsaponifiable matter. This fraction was crystallized from acetone and characterized as lignoceryl alcohol. The potassium salts were then acidified, the solids collected, and air-dried. This precipitate was extracted with hot hexane giving a soluble fraction and an insoluble fraction. The soluble portion was allowed to cool to 30°C., at which temperature a white crystalline hydroxy acid precipitated. This fraction was identified as 13-hydroxymyristic acid. The filtrate contained behenic acid. The hot hexane-insoluble fraction was extracted with benzene again giving soluble and insoluble portions. The soluble fraction was identified only as an unsaturated acid, whereas the insoluble fraction was extracted with diethyl ether, again yielding an insoluble residue which was characterized only as a phenolic acid. Therefore the total composition of this wax was as follows:

lignoceryl alcohol 5.35%, phytosterol 0.3%, 13-hydroxymyristic acid 48.8%, behenic acid 3.02%, a phenolic acid 32.52%, unsaturated acids 9.69%, and unsaturated alcohols 0.3%.

A study was made of the chemical composition of the cork fraction of white fir bark (12). The acids were obtained in much the same way as was previously described (14). The hydroxy fatty acid fraction was found to be a mixture rather than a single compound. The major acid was again reported as 13-hydroxymyristic. A hydroxy arachidic acid and a dihydroxy dicarboxylic acid were also present. It was mentioned that the separation was not complete and that there might possibly be other acids present.

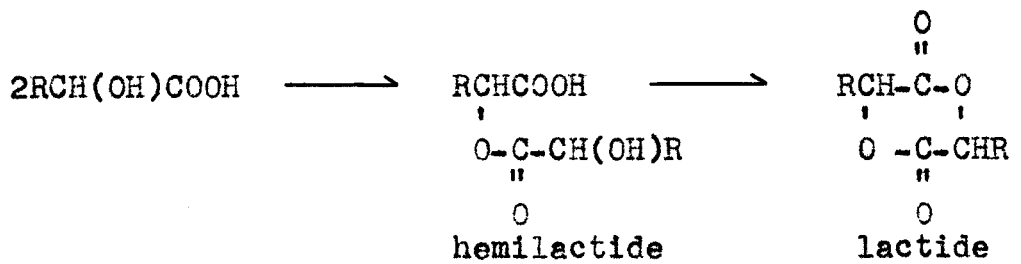
As can be seen from these summaries a somewhat successful method of separation of the fatty acids from other constituents has been described. A method for the purification of the fatty acids needs to be proposed, and the fatty acids from the white fir bark have not been completely isolated and characterized.

Problems of Hydroxy Fatty Acids

Since some hydroxy fatty acids are present in the bark wax, it might be of interest to review several difficulties which this second functional group introduces into the problem. Lactone formation can occur, the ease of which varies with the position of the hydroxyl group on the chain.

The γ- and δ-lactones are the most stable. Therefore, with the hydroxyl group attached to the 4- or 5-carbon atom, the lactones form easily; and the free acid is difficult to obtain.

If α-hydroxy acids are present, two such molecules can become linked to form a hemilactide or a lactide.



Most hydroxy acids will dehydrate on heating to form ethylenic acids.

Etholide formation can also occur; but, for the most part, these linkages should be broken by the saponification procedure.

$$HOCH_2 (CH_2)_x COOH + HOCH_2 (CH_2) x COOH \longrightarrow$$
$$HOCH_2 (CH_2)_x CH_2 COOCH_2 (CH_2)_x COOH + H_2O$$

As can be seen from these reactions, care must be taken when isolating unknown hydroxy fatty acids.

II. SEPARATION OF ACIDS FROM OTHER COMPONENTS OF THE BARK

White fir bark had been previously extracted with hexane and benzene, so that the actual starting material was the hexane-insoluble, benzene-soluble wax. Since it has been previously reported (14) that the white fir hexane-insoluble, benzene-soluble wax also contains lignoceryl alcohol, phytosterol, and unsaturated alcohols besides the fatty acids, these materials must first be removed as the initial step in the purification of the acids. Therefore to achieve this objective these operations were performed on the wax as shown in Figure 1.

In this procedure 200 grams of the hexane-insoluble, benzene-soluble wax were heated in a 10% sodium hydroxide solution. This mixture was boiled approximately four hours until excessive foaming occurred. The source of heat was removed, and the mixture was acidified with dilute hydrochloric acid, yielding a brown, curdy precipitate. After cooling, the precipitate was collected on a Büchner funnel. The filtrate, that is the water solution, contained the inorganic salt and some dark coloring matter. The precipitate was then boiled in benzene for several minutes. Most of the solids dissolved, however, approximately 25% of the materials was insoluble in the hot benzene. These were the dark-colored phenolics. The solvent was completely removed from the

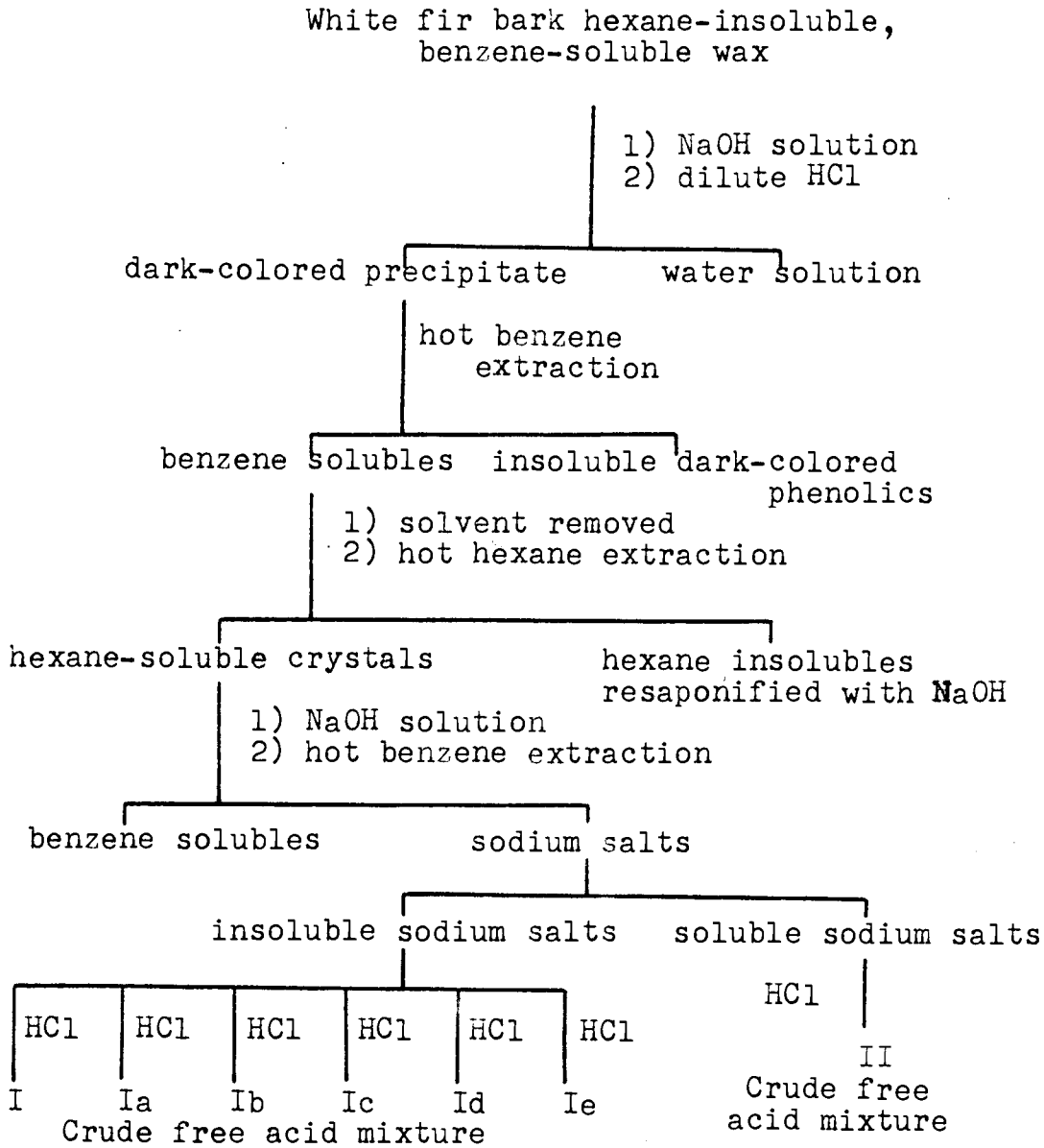


Figure 1. Flow diagram for the separation of acids from other materials in bark wax.

benzene soluble fraction leaving a cream-colored wax. This wax was then boiled in hexane for several minutes, again yielding a soluble fraction and an insoluble fraction. Both fractions were saponified in sodium hydroxide solution. The hexane-insoluble fraction was present as the minor constituent at this point. After saponification of the hexane-soluble crystals, these salts were extracted with hot benzene in a separatory funnel. The benzene-soluble fraction contained neutrals such as lignoceryl alcohol. The aqueous layer contained the sodium salts, some of which were soluble in the water solution and some of which were insoluble. The soluble-sodium-salts fraction was acidified with dilute hydrochloric acid to give a crude free acid mixture which was designated by the Roman numeral II. The insoluble-sodium-salts fraction was further separated depending on the relative insolubility of the sodium salts in the water solution. Then each fraction was acidified with dilute hydrochloric acid to give crude free acid mixtures designated by the following numerals and letters: I, Ia, Ib, Ic, Id, Ie. By this procedure the long chain ester linkages of the wax were broken to give the fatty acids, and the fatty acids were further separated from the impurities of the wax.

With this method of extracting the total fatty acids from the wax several difficulties were encountered which were not immediately apparent. The procedure appeared to

be rather simple and straightforward; however, separations at several points were not definite and complete. These stumbling blocks will be discussed in detail in Section IV of this paper.

III. PURIFICATION OF COMPLEX ACID MIXTURE

The crude free acid mixtures, I, Ia, Ib, Ic, Id, and Ie which were obtained from the previous procedure were subjected to a new type of procedure which would separate the individual fatty acids from each other and separate the fatty acids from the hydroxy fatty acids.

Each mixture I-Ie was separately extracted with hexane. The crude mixture I was entirely soluble in the solvent. The fractions were obtained from crystallization from the hexane. One fraction precipitated from the solvent at room temperature. The second fraction separated from the hexane at refrigerator temperature, and the last fraction remained in the filtrate and could only be obtained as a waxy solid after complete evaporation of the solvent. This was the pattern which was followed in all crystallizations of the acid and their derivative mixtures. Therefore in the following explanations R. T. will be used to signify those crystals which separate from the solvent at room temperature, I. B. to represent those crystals which separate from the solvent at refrigerator (ice box) temperature, and F. will indicate the crystals which remain in the filtrate.

The crude acid mixtures Ia, Ib, Ic, Id, and Ie gave two fractions when boiled with hexane: one which was soluble in solvent and one which was not. The insoluble ones

were then recrystallized from benzene. Figure 2 is a flow diagram showing the procedure used on the fraction Ia for separation of the acids. This same procedure was employed for the other crude acid mixtures also. The melting points of the different fractions which were obtained (shown in Figure 2) were also typical of the melting points of the fractions obtained from the other crude acid mixtures. Fraction Ie also followed this pattern; however, when the insolubles were recrystallized from benzene, a part of the sample did not dissolve. See Figure 3. As can be seen from Figures 2 and 3, the fractions after recrystallization from either hexane or benzene were then subjected to a methylating reagent which formed the methyl esters of the acids. The methyl esters were not formed from the filtrates, because they were, for the most part, yellowish-orange, sticky, low-melting substances which gave a positive test for unsaturation with potassium permanganate solution. Therefore, all these filtrates were combined and set aside and not further characterized. They formed only a small part of the total. Also methyl esters were not made from any fraction which was less than 0.3 grams. For example, the methyl ester of the I. B. fraction of the hexane soluble portion of Ie was not formed. See Figure 3.

The methylating agent used was a solution of hydrogen chloride gas introduced into anhydrous methanol. The hydrogen chloride gas was present to the extent of 4-5%. This

reagent was prepared by boiling a concentrated hydrochloric acid solution and allowing the hydrogen chloride gas to pass through a calcium chloride drying tube and bubbling into the anhydrous methanol. The gas was allowed to enter one liter of methanol for approximately forty-five minutes to one hour.

The actual methylation procedure included the addition of 50 milliliters of methanol-HCl reagent to each sample along with five to ten milliliters of benzene to insure the dissolution of the acid. This solution was then refluxed for two hours. The average weight of the samples was 2.34 grams; however, these sample weights were distributed in the range 0.34-6.01 grams. After the two-hour reflux period the solutions were allowed to cool, and then they were poured into cold distilled water to precipitate the methyl ester. These solids were then removed by a vacuum filtration on a Büchner funnel. After evaporation of the filtrate no methyl esters were found to remain in this solution. Therefore each precipitate was crystallized from hexane again giving three fractions: R. T. crystals, I. B. crystals, and a filtrate. After dissolving the solids in the hot hexane, a small, darkbrown, sticky residue usually remained in the beaker which indicated that the acids were purified by forming the methyl ester derivative. Usually the methyl esters were white crystals or had only a slightly tan discoloration whereas the crude acid mixture had a

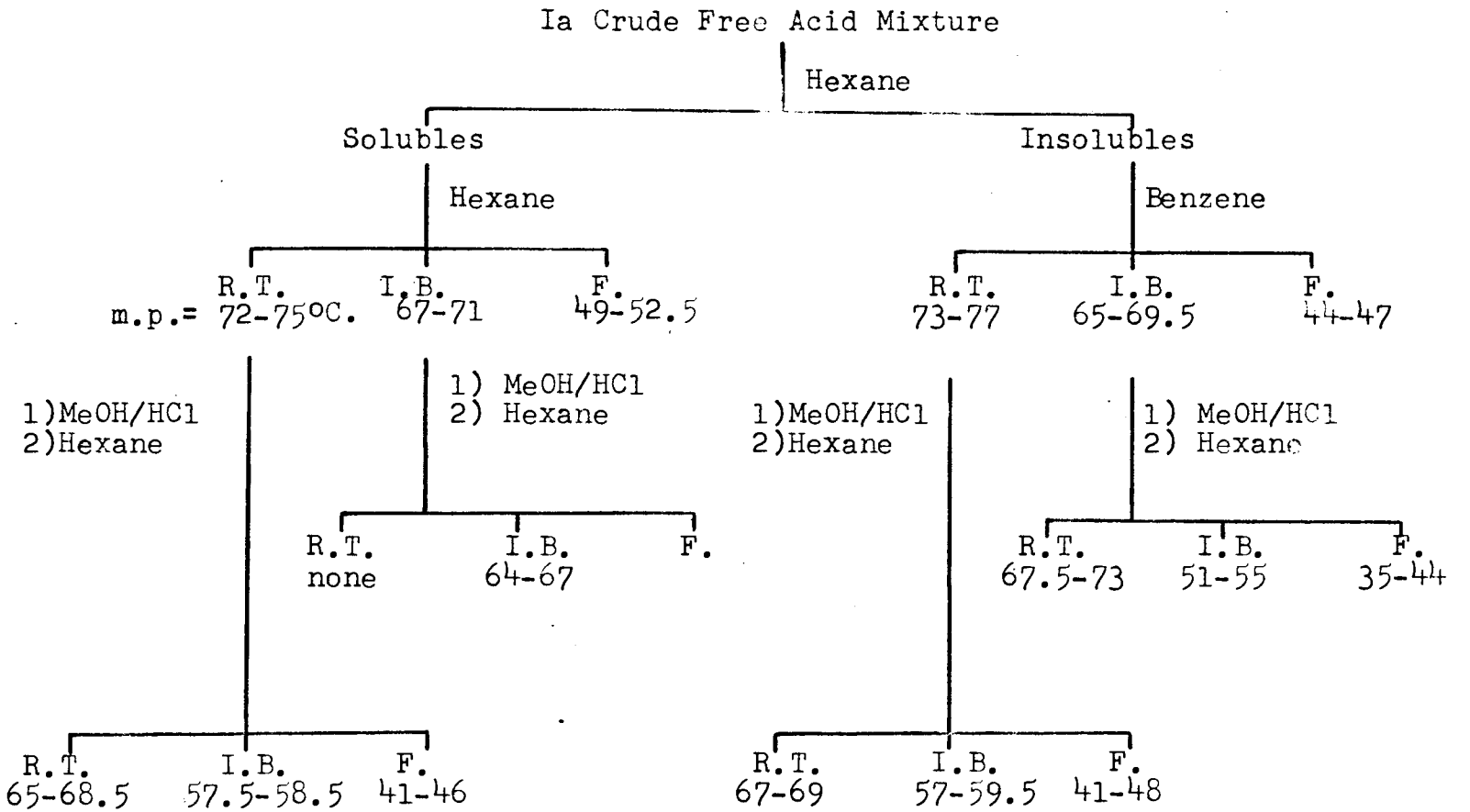


Figure 2. Flow Diagram of Methylation of fraction Ia.

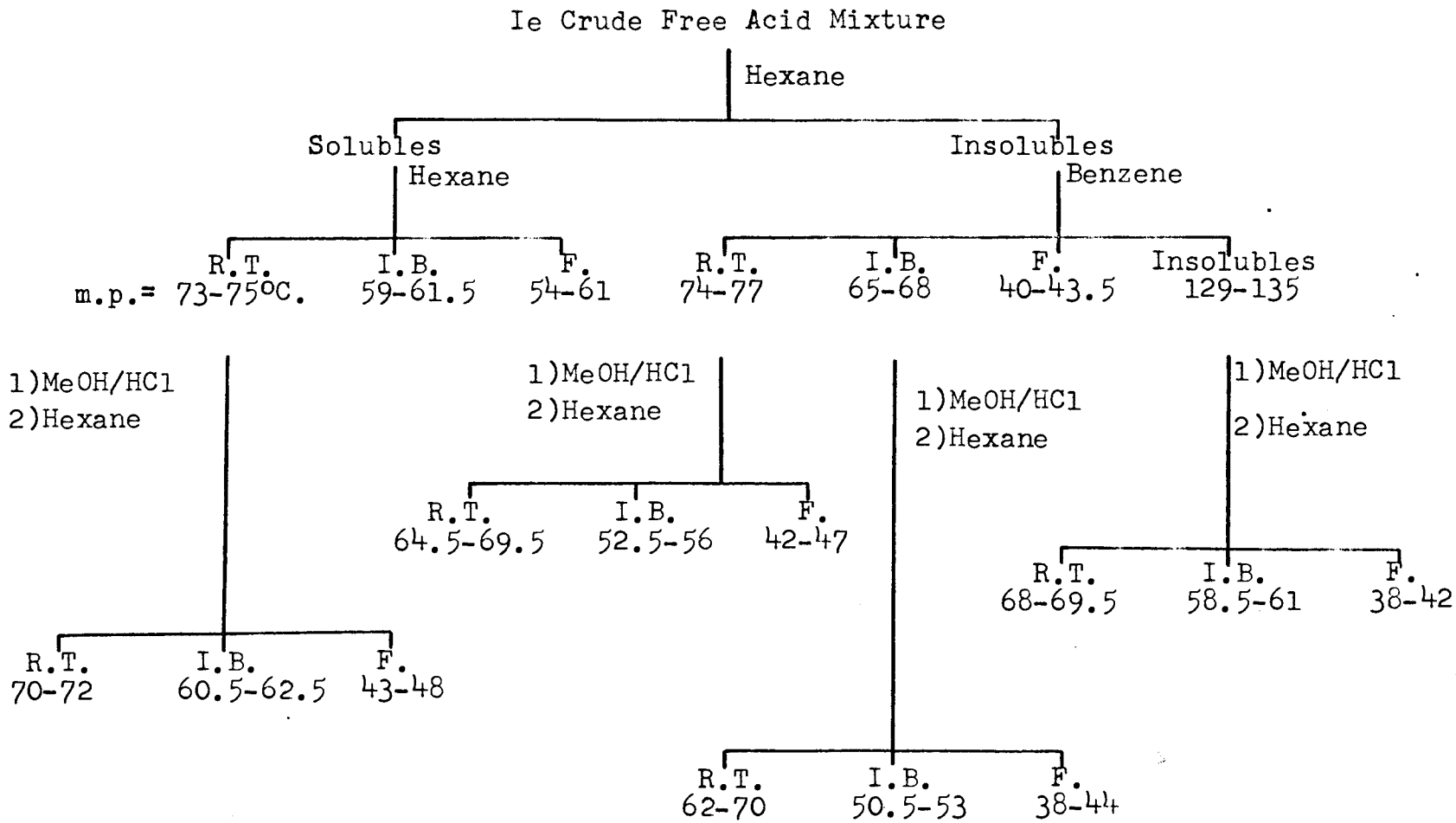


Figure 3. Flow Diagram of Methylation of fraction Ie.

definite light brown color.

The next operation which was performed was to combine all the methyl esters from the hexane-soluble portions with similar melting points and to combine all the methyl esters from the hexane-insoluble portions with similar melting points. The combination from all the crude acid mixtures I-Ie are shown in Figure 4. There were then sixteen samples as indicated by the numbers on the horizontal lines. The melting points of these samples varied from 31-74°C.

These sixteen samples were then subjected to a quantitative saponification procedure which served as an indication of their molecular weights. This procedure consisted of weighing out carefully 0.5-2.0 grams of the sample and refluxing it for one hour with an alcoholic potassium hydroxide solution. This caustic solution was prepared by dissolving reagent grade potassium hydroxide in 95% ethanol and allowing the insoluble carbonates to settle to the bottom of container overnight. The clear supernatant liquid was decanted into a two-liter bottle. The concentration of this solution was then found by titration with a previously standardized sulfuric acid solution. The actual concentrations of the potassium hydroxide solutions used in this work was 0.4041N and 0.4225N. Standard sulfuric acid solutions were 0.2059N and 0.1990N. After the one-hour reflux period the salt was back-titrated with standard sulfuric acid solution. The titration was accom-

plished while the solution was still hot, so that the salts were still dissolved in the reaction mixture. After the pH had been adjusted to the phenolphthalein endpoint by the sulfuric acid, two milliliters of the alcoholic potassium hydroxide solution were added, and the reaction mixture was placed on the hot plate for several minutes. A back-titration to the phenolphthalein endpoint with the sulfuric acid was again performed. Then another two milliliters of alcoholic potassium hydroxide solution was injected into the mixture and the process repeated for the last time. By performing the titrations in this manner, the experimenter could check to see whether the methyl ester had been completely converted to the salts. Also three values for the saponification equivalent were obtained which could be averaged for a more accurate determination.

The following expression was used for the calculation of the saponification equivalent:

$$\frac{\text{Wt. of methyl ester used}}{\text{meq. KOH which reacted with methyl ester}} \times 1000 = \text{S. E.}$$

In this instance the saponification equivalent was actually the molecular weight of the ester since the reaction which took place is represented by the equation:

$$R - \overset{O}{\overset{\|}{C}} - OCH_3 + KOH \longrightarrow R - \overset{O}{\overset{\|}{C}} - OK + CH_3OH$$

After the samples had been saponified and back-titrated, approximately 10-20 milliliters of dilute hydrochloric acid were added to completely convert the salts

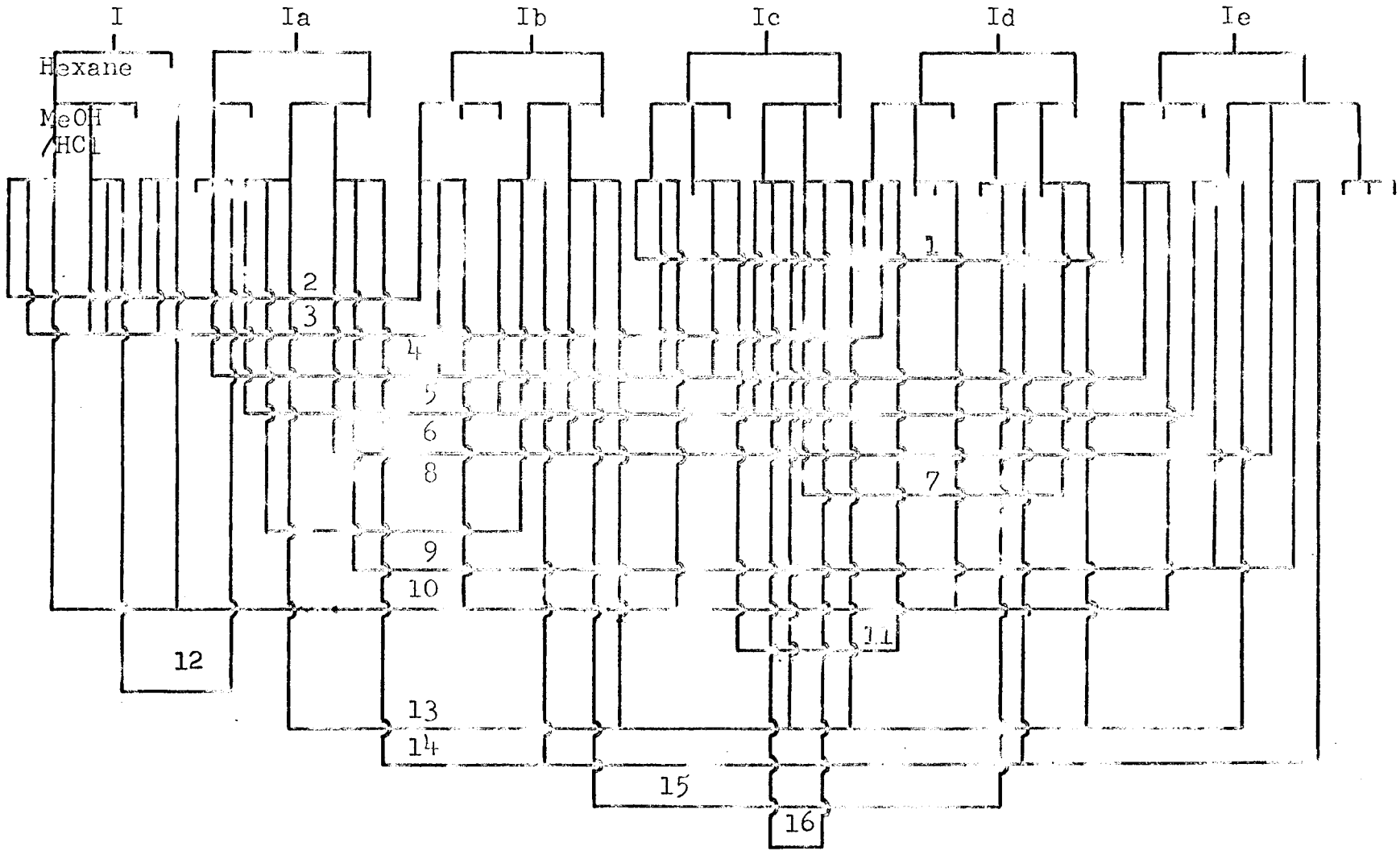


Figure 4. Flow diagram representing the combination of methyl esters

to the acids. Then approximately 25% of the solution was allowed to evaporate, after which the white precipitate was collected on a Büchner funnel. The crystals were air-dried and then recrystallized from hexane. Again from the majority of the samples three fractions were obtained: R. T. crystals, I. B. crystals, and a filtrate. With the appearance of these three fractions, the R. T. and I. B. fractions being about equal in amount, it would seem that the acids were not yet pure or perhaps some lactone, lactide, or etholide formation occurred with the hydroxy acids. Several of these fractions were combined on the basis of their approximately equal saponification equivalent and their almost identical melting points. The saponification equivalent of the methyl esters ranged from 199-409.7 which indicated that the chain lengths of these substances to be between 10 and 26 carbon atoms. The melting points of these recovered acids ranged from 38-86°C.

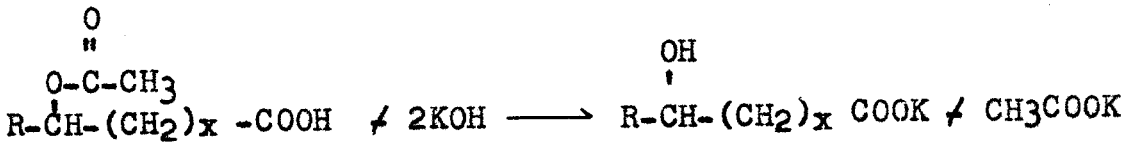
The next operation which was performed on these acids was to prepare the acetates of any hydroxy acids which were present. The acid samples which ranged from one to two grams were placed in test tubes, and approximately 20 milliliters of acetic anhydride-pyridine(4:1, v/v) were added. The test tubes were then placed in a hot water bath for thirty minutes. During this time the acids were completely dissolved in the acetic anhydride -pyridine reagent. After the thirty-minute reaction time the test tubes were emptied

into beakers which contained cold distilled water. A white precipitate was formed. After cooling this water solution, the solids were collected by a vacuum filtration on a Büchner funnel. After being air-dried, the white curds were crystallized from hexane. Again three fractions were obtained, depending on the relative solubility in the hexane. It should be noted here that if the original acid sample contained a hydroxyl group, the acetate which was formed was more soluble in the hexane than the original acid. Usually if an acetate were formed, the melting point of the crystals would be approximately 10-15 degrees lower than the melting point of the acid. If the acid did not contain a hydroxyl group, the melting point of the acid sometimes was raised four to five degrees. Therefore, the primary basis on which a certain sample was designated as having a hydroxyl group was the comparison of the melting point of the original acid and the melting point of the substance after treatment with acetic anyhdride-pyridine. However, solubilities were also considered, and a third method of determining the presence of the hydroxyl group was the saponification of the acetic anhydride-pyridine-treated crystals.

The acetic anhydride-pyridine-treated fractions were combined only according to their melting points. The saponification equivalents of the methyl esters were not taken into consideration; since three fractions were obtained

from the acid crystallization and three fractions were obtained from the "acetate" crystallization, the methyl esters could not have been pure, and the saponification equivalents only indicated the vicinity of the molecular weights. After this preliminary combination of the "acetates", these samples were then recrystallized from hexane. In some cases three fractions were obtained, but in others only one or two fractions were isolated. It was found that again some of these fractions could be combined on the basis of their melting points. At this point there were thirteen definite fractions with their melting points ranging from 32-79°C.

These thirteen samples, some of which were acetates and some of which were acids, were subjected to the saponification procedure which has been previously described. The saponification equivalents of the acetates could not be considered the molecular weight of the substance, because the reaction is represented by the following equation:



Therefore the saponification equivalent which was obtained must be multiplied by two to determine the molecular weight of the acetate. Forty-two was then subtracted from this value to obtain the molecular weight of the hydroxy acid. From this saponification data the chain length of the

acids present was indicated to be between 14 and 26 carbon atoms. The acids which were recovered from the saponification procedure were recrystallized from hexane, and the various fractions were combined according to their melting points and their saponfication equivalents. These combinations were again recrystallized from hexane and recombined to yield ten fractions, four of which contained workable amounts of sample, that is, one to four grams. The other six fractions were present in amounts less than one gram.

One of these four samples was again methylated with the methanol-HCl reagent and the product recrystallized from hexane. The melting point of the methyl ester crystals was 70-74°C. A quantitative saponification was then carried out on the methyl ester which gave a saponification equivalent of 345.3. The recovered and recrystallized acid melted at 87.5-89°C. This sample is mentioned specifically because it appears to be present to the extent of at least 50%. This sample which appeared to be pure is shown as Sample 8 in Table II. In recrystallizing the methyl ester of this sample, a second fraction appeared which was designated as Sample 7.

It should be mentioned at this point that throughout this entire procedure for the purification of the acids, the small amounts of sample which remained in the beakers after recrystallization were washed out with hexane and placed in a residue beaker to be purified by this procedure

TABLE II. PROPERTIES OF SAMPLES (FIRST TRIAL)

Sample	Acid m.p.[a]	"Acetate"[b] m.p.	M.E.[c] m.p.	S. E.[d]
1	70-74	none	62-64	228.3(NE)[e]
2	82.5-84.5	none		268.7(NE)
3	87-88	65-66.5	68-69	196.5(Ac)[f]
4	88.5-90	72-75	78-79	230.7(Ac)
5	85-86.5	none	77-78	214.7(NE)
6	68-70	57.5-59		196 (Ac)
7	84-85	75-77	71-74	325.5(ME)[g]
8	87.5-89	none	70-74	345.3(ME)
9	91.5-94.5	72-74	72-76.5	354.0(ME)
10	86-90	70-77	70-71	290 (ME)
11	84.5-87.5	69-73	68-69	187.5(Ac)
12	84.5-87.5	52-61.5	68-69	185 (Ac)
13	86-89	66-73	71-72	206 (Ac)
14	88.5-90	67-68.5	74-75	200.7(Ac)
15	63-68	52-55	45-49	170.2(Ac)
16	85-86.5	59.5-63	69-70	186.3(Ac)
17	86-87.5	64.5-66	68-69	190.1(Ac)

[a] melting points are given in degrees Centigrade

[b] "Acetate" indicates substance obtained after addition of acetic anhydride-pyridine reagent

[c] M.E. indicates methyl ester

[d] S.E. indicates saponification equivalent

[e] NE actually indicates a neutralization equivalent

[f] Ac indicates a S. E. taken on an "Acetate"

[g] ME indicates a S. E. taken on a methyl ester

later. Therefore samples numbered 13-17 were obtained from the contents of the residue beaker. Samples 9 and 10 were isolated from the remaining three samples which were previously mentioned. Samples 11 and 12 were obtained from the Ie hexane-insoluble, benzene-insoluble fraction by this same procedure which has been discussed. (Refer to Figure 3). Samples 1-6 were fractions which were present in amounts of less than one gram.

From Table II it can be seen that many of the samples have similar properties. Perhaps these samples could be combined. This possibility will be discussed in the section entitled Gas-Liquid Chromatography Analysis.

This same procedure with a few slight modifications was utilized to purify another sample of the crude free acids in order to compare the results. This time, however, only the hexane-insoluble, benzene-soluble fractions of Ia, Ib, Ic, and Id were used. The melting range of this group of acids was 73-79°C. Fifty-nine grams of this fraction was treated with the methanol-HCl reagent, refluxed for three hours, cooled; and the solids were collected on a Büchner funnel. The filtrate was evaporated to dryness, yielding a dark-colored, wax-like substance. Both fractions were then washed with water twice to remove any residual methanol-HCl reagent. These solids were then air-dried and crystallized from hexane, each yielding the

three fractions: R. T. crystals, I. B. crystals, and a filtrate. Each of these fractions was then recrystallized from methanol, again each giving three fractions. Some of the fractions were combined on the basis of their melting points, and the combined fractions were recrystallized from hexane. Then some of these fractions were combined according to their melting points. Now at this point there were ten distinct fractions with a melting point range of 36-69°C. The melting points of some of the fractions overlapped the melting points of the other fractions. The intermediate step of recrystallizing the methyl esters from the methanol also allowed for more separation of the samples. The ten fractions were then saponified, not by the quantitative procedure, but by a not-so-laborious method of addition of solid potassium hydroxide to approximately a 30% ethanol solution of the methyl ester. The saponification was allowed to proceed for one hour, after which dilute hydrochloric acid was added to convert the salts to acids. The white precipitate was then collected by a vacuum filtration, air-dried, and crystallized from hexane. The methyl esters were not subjected to the quantitative saponification procedure since the data obtained would only have been a rough estimation of the molecular weight of the esters, and at this point the esters were still very much a mixture. After crystallization of the acids and combination according to melting points, there were seven fractions. These seven

fractions were then subjected to the acetic anhydride-pyridine reagent for 30 minutes. The products of this treatment were then recrystallized from hexane twice and combined on the basis of their melting points and according to the fraction from which they originated. Now there were fourteen fractions which were then subjected to the quantitative saponification procedure for an indication of the molecular weights of the samples. After recrystallization of the acids, these fractions were again methylated with the methanol-HCl reagent to give a methyl ester melting point for these purified samples. Table III shows the properties of these fourteen samples. Sample A essentially has the same properties as Sample 8 in Table II. The only discrepancy is in the saponification equivalent of Sample A which may be explained by inadequate washing of the acetic anhydride-pyridine-treated acid. Samples B and C were combined after the last methyl ester formation and then saponified with potassium hydroxide to give an acid which melted at 84-85°C. This fraction is probably identical to Sample 7 (Table II) even though there is a slight discrepancy in melting points of the methyl esters and acetates. Samples F and L, and N even after the acetylation operation appeared to contain yellow impurities. With such a high melting range in comparison to the other acids which were isolated, these fractions may contain a dicarboxylic acid, which was isolated from the soluble-sodium-salts fraction. (Refer

TABLE III. PROPERTIES OF SAMPLES (SECOND TRIAL)

Sample	Acid m.p.[a]	"Acetate"[b] m.p.	M.E.[c] m.p.	S.E.[d]
A	84-86	80-83	70-72	237 (Ac)[e]
B	73-75	57-59	77-80	186.7(Ac)
C	82-84	62-63	77-79	190.3(Ac)
D	69-74	60-66	75-76	180 (Ac)
E	67-73	68-70	72-74	172 (Ac)
F	97-107	74-77	71-73	177 (Ac)
G	73-77	49-50	68-71	158.5(Ac)
H	77-78	76-79	68-69	200.8(Ac)
I	75-77	52-53	56-58	173.2(Ac)
J	78-84	72-76	67-70	191.8(Ac)
K	66-70	50-55	64-68	173.2(Ac)
L	98-102	79-83	52-53	161 (Ac)
M	68-70	51-58	65-68	158.2(Ac)
N	104-111	80-87		150.5(Ac)

[a]melting points are given in degrees Centigrade

[b]"Acetate" indicates substance obtained after addition of acetic anhydride-pyridine reagent

[c]M.E. indicates methyl ester

[d]S.E. indicates saponification equivalent

[e]Ac indicates a S. E. taken on an "Acetate"

to Figure 1.) The other samples, D. E, G, H, I, and J contained such small amounts of material that further purification by this method would not have been feasible. They were included in this Table to indicate the number of fractions obtained by this procedure and the small differences in their properties. Sample K appeared to be identical with Sample M except in the value of the saponification equivalent. However, 15 units difference in the saponification equivalent may not be critical at this point, since the inadequate washing of the acetate can greatly affect this data.

Just as this procedure was used to purify the crude acid mixture designated as I, it was also used to purify the crude acid mixture II which was obtained from the soluble-sodium-salts fraction. A single acid was isolated from this fraction which melted at 124-125°C. From neutralization equivalent data a dicarboxylic acid was indicated which contained 16 carbon atoms. The dimethyl ester melted at 54-55°C. and no acetate was formed after treatment with acetic anhydride-pyridine reagent. In his book Ralston reports these properties for thapsic acid, a straight chain C_{16} dicarboxylic acid: acid melting point 125-125.2°C. (43, p.245) and dimethyl ester melting point 51.6°C. (43,p.513).

Generally speaking, the procedure used for the purification of these acids can be represented by the flow diagram shown in Figure 5. The abbreviations used in this

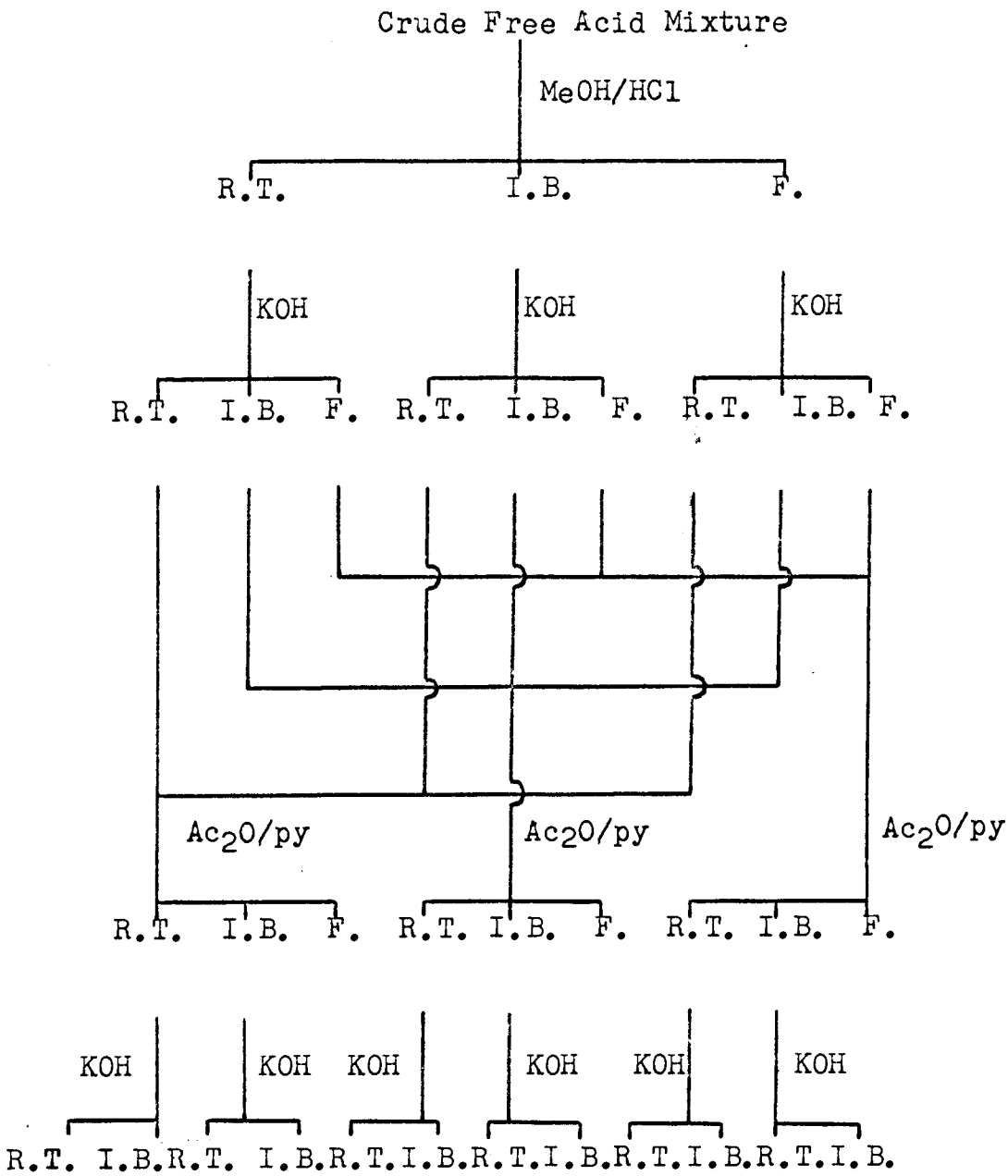


Figure 5. Generalized procedure for purification of fatty and hydroxy fatty acids.

diagram are the same as those which have been described earlier. But to summarize, R. T. represents the crystals which separate from the solvent at room temperature, I. B. are those crystals which separate from the solvent at refrigerator temperature, F. are the crystals which are left in the filtrate, KOH indicates a saponification procedure which is quantitative (although this procedure need not be quantitative when converting the methyl esters to the acids), Ac_2O/py indicates the acetylation procedure which attaches an acetate grouping to any hydroxyl groups which may be present, and MeOH/HCl indicates formation of the methyl esters. It should be noted that after each acid, methyl ester, or acetate has been treated with a reagent, it is crystallized from hexane. After the methylation step the fractions can be crystallized from methanol and then from hexane to allow for more separation.

After the first saponification procedure some of the acids may be combined as indicated by their melting points. Generally, all the R. T. crystals were combined, all the I. B. crystals were combined, and all the filtrates were combined. However, that was not always true. Sometimes combinations could not be made and sometimes R. T. crystals could be combined with I. B. crystals. Combinations were also made after the acetylation procedure and sometimes after the second saponification. The filtrates

obtained after the acetylation were usually so small that they were washed into a residue beaker to be characterized later. Therefore Figure 5 summarizes the procedure for the purification of fatty acids and hydroxy fatty acids of white fir bark wax as it was utilized in this research.

IV. PROBLEMS OF PURIFICATION

Separation of Neutrals

There were many difficulties encountered using this procedure which weren't immediately apparent. One of these problems was the incomplete separation of the neutrals from the acids. (Refer to Figure 1.) With the large amount of material (200 grams of wax) which was saponified the sodium salts, which are bulky, tend to occlude some of the long chain fatty alcohols. For example, Sample K (Table 3) after the final methyl ester formation was again saponified with the alcoholic potassium hydroxide solution and then back-titrated just to the phenolphtyalein endpoint with sulfuric acid. This solution was then evaporated to dryness and the white crystalline residue was extracted with hot hexane in a Soxhlet extractor. The potassium salts were not appreciably soluble in the hot hexane but the fatty alcohol was. After the extraction was completed and the alcohol crystallized, it was found that the previously-thought pure Sample K was actually about 25% fatty alcohol and 75% fatty acid. This long chain alcohol might possibly be lignoceryl alcohol, that is, a 24-carbon atom alcohol, since this substance was reported by Hergert & Kurth in their study of the extractives of white fir bark(14). However, this alcohol which was isolated may be impure since its melting pt. was 68-69°C. whereas lignoceryl alcohol melts

at 73-74°C. On the other hand, Sample B was also converted to the salt form and extracted with hexane, but no alcohol was recovered from the solvent. Therefore it seems that some acids have a greater tendency to occlude the alcohols than others, and caution must be exercised when considering whether a sample is strictly an acid.

Separation of Soluble and Insoluble Sodium Salts

Another difficulty occurred with the separation of the soluble and insoluble sodium salts. (Refer to Figure 1.) At first it appeared that this separation was sharp, that is, two fractions were obtained: the salts which formed a precipitate and those which remained in the water solution. However, as one continued through the procedure, invariably several samples appeared in the insoluble-sodium-salt fraction which had higher melting point ranges. These acids were impure since they appeared as a very pale yellow powder which indicated that some phenolic materials were still present. But their crystal structure, that is, a very fine powder was the same as the dicarboxylic acid which was obtained from the soluble-sodium-salt fraction. These impure, insoluble-sodium-salt fractions, after being converted to the acid, usually melted in the range 95-110°C. whereas the pure dicarboxylic acid melted at 124-125°. However, some of these impure fractions also showed indications of a hydroxyl group present. Therefore, these fractions may not be entirely the dicarboxylic

acid. But this impure acid fraction was definitely distinct from the other acids in the insoluble-sodium-salt fraction in both its melting characteristics and its crystal structure, no matter what the crystallizing conditions were. The other acids which were present in this fraction had definite melting points not above 95°C. and their crystals were more like short needles.

Melting Points

Another problem arose in the fact that the melting points of the fatty and hydroxy fatty acids occur within a considerably narrow range. The crude acids melted in three ranges: 72-77°C., 65-71°C., and 45-53°C. The methyl esters melted in these ranges: 67-73°C., 57-60°C., and 42-48°C. And the acetates melted at 50-55°C. and 44-47°C. Although the individual samples had definite, sharp melting points, when considered as a group they tend to fall within these ranges. If one acid sample melted at 66-68°C. and another melted at 70-71°C., were they two different acids or were they the same acid, or perhaps were they still both mixtures? Therefore, when acid fractions were combined, they were not done with complete certainty that the acid samples were the same. There was also this same element of doubt in the combination of the methyl esters and acetates.

Incomplete Saponification

Although the hexane-insoluble, benzene-soluble wax underwent saponification twice (See Figure 1.) and the acid mixture again was saponified twice (See Figure 5.) this was sometimes not enough. For example, on an isolated fraction of a crude acid which was later to be purified and designated as Sample 8, a neutralization equivalent was taken on the acid after the formation of the methyl ester, and this value was 384. However, after this the methyl ester was again formed, and a saponification equivalent was taken on this sample. The new value indicated the molecular weight of the acid to be 331, at which point this sample was designated as Sample 8. Therefore the samples needed to be resaponified until consistent neutralization and saponification data were obtained.

V. THIN-LAYER CHROMATOGRAPHY ANALYSIS

Thin-layer chromatography was used as a means of determining the purity of a sample. The number of spots observed after development would indicate the number of compounds present in a particular sample.

Since the fatty acids were white crystalline materials they would not be detectable on the white silica gel plate. Therefore, in the preparation of the plates a small amount of fluorescein was added to the silica gel. The plates were then a pale yellow color, and the acid spots could be detected under an ultraviolet light.

The samples were prepared by dissolving approximately three milligrams of sample in five millliliters of diethyl ether. Some samples needed to be heated before they would dissolve in the solvent. Approximately five to ten microliters of each sample was then spotted on the plates. The plates were then placed in a developing chamber for 20 minutes. The developing solution consisted of hexane, diethyl ether, and acetic acid in a ratio of 60:40:2, v/v. If a hexane:diethyl ether (9:1, v/v) solvent was used, the acids did not move from their original spot. Therefore, the eluting solution was made more polar by the addition of the extra diethyl ether and acetic acid.

Figure 6 is a diagram of a developed plate showing placement of the acid samples. The 1st row of spots which

appeared after developing seemed to indicate that the entire original spot moved about 1-1.5 centimeters. The appearance of these displaced spots were identical to the original ones, that is, the fluorescein had been removed from the center. Sample 10 was the only one that did not show this phenomenon. Aside from this fact only one other spot was shown for the samples except 2, 5, and 11 which showed two spots. This might tend to indicate that these samples were relatively pure. However, these spots appeared at the same distance from the original for all the samples which would indicate that all the samples were the same acid. But this did not seem logical, since the properties of each sample were different from any other. Therefore thin-layer chromatography was abandoned in favor of gas-liquid chromatography which could give a more complete picture of the compounds present.

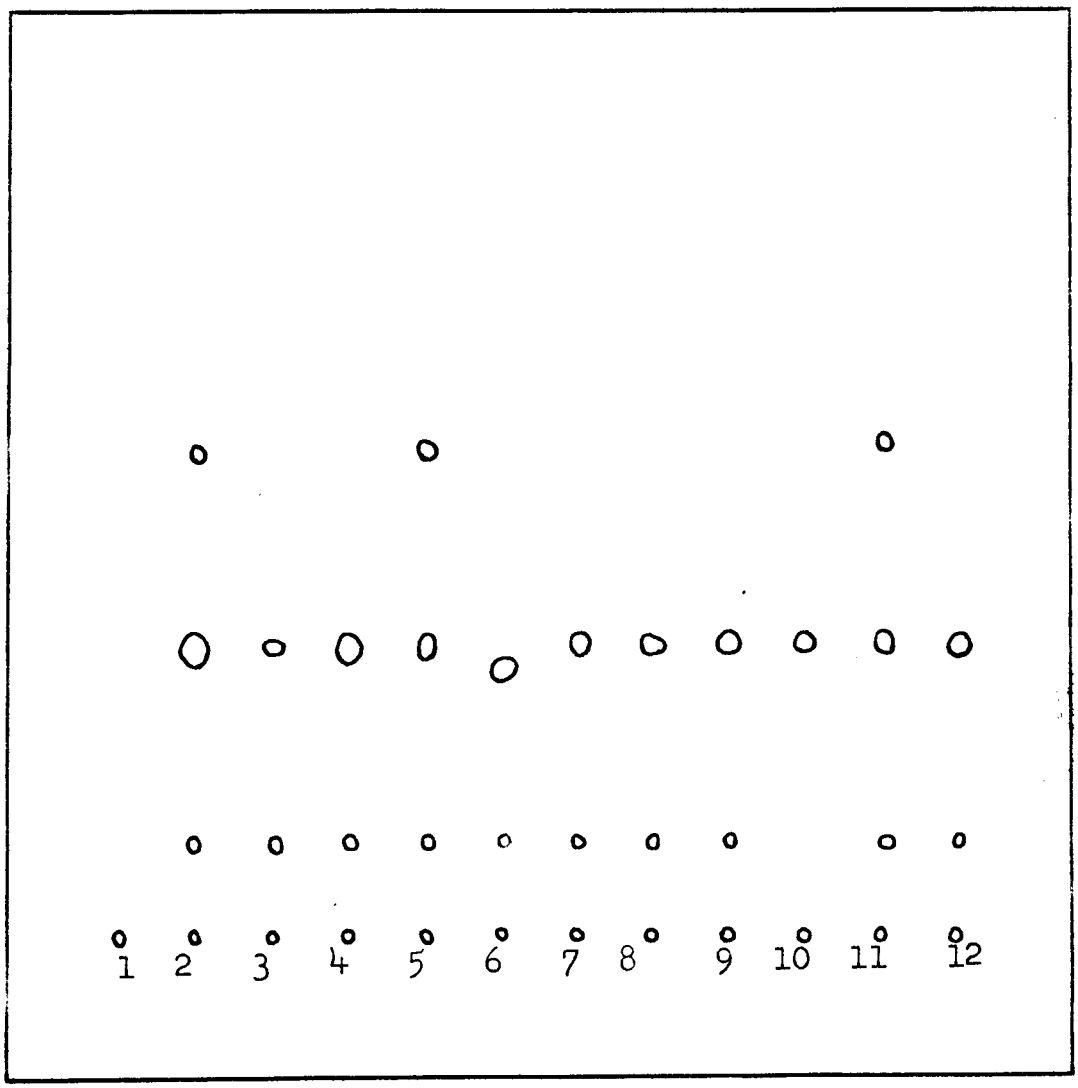


Figure 6. Diagram of developed thin-layer chromatography plate of fatty acids. Developing solvent was hexane diethylether:acetic acid, 60:40:2,v/v.

VI. GAS-LIQUID CHROMATOGRAPHY ANALYSIS

Six samples from Table II were analyzed by gas-liquid chromatography. The samples were 3, 4, 9, 13, 14, and 17. These samples were chosen because their properties were nearly identical. It was thought that perhaps they were all the same acid or the same mixture.

The acid samples were converted to the methyl esters by the procedure already described. These methyl esters were then injected one at a time into a 1/8" x 6' stainless steel column containing 15% ethylene glycol succinate on Chromosorb P. The column temperature was 190°C., and the injection temperature was 268°C. The flow rate of the helium was 35 ml/min. Detection was by a hydrogen flame ionization unit. Methyl palmitate and methyl stearate were used as standards.

Figure 7 shows the gas chromatogram for Sample 13. The other five chromatograms were comparable. Table IV shows the retention time of the four peaks relative to methyl palmitate and the percentage of each peak for each sample. The last column shows the average percentage of each peak if all the samples were considered as one. Figure 8 shows the plot of retention time vs the number of carbon atoms. By using the carbon number method of Woodford and van Gent (49) peak 1 has a carbon atom number of 18.9, peak 2 20.0, peak 3,21.2, and peak 4,22.4. Therefore

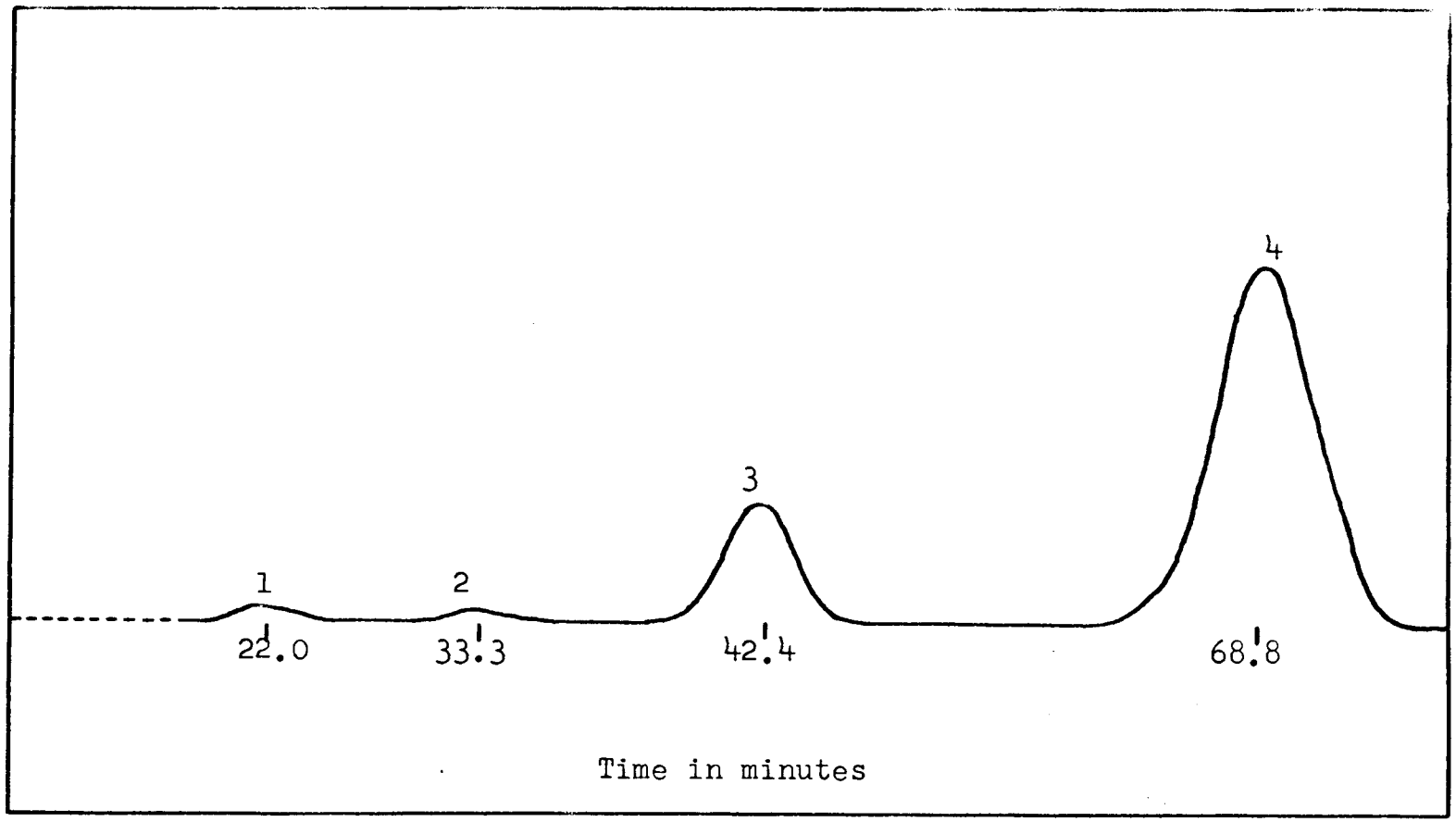


Figure 7. Gas chromatogram of methyl ester form of Sample 13 on 1/8" x 6' stainless steel column containing 15% ethylene glycol succinate on Chromosorb P. Column temperature 190°C., flow rate of the gas 35 ml/min, and detection by hydrogen flame ionization.

it would seem that peak 2 indicated the presence of a straight chain C_{20} acid, and peak 4 indicated not a straight chain C_{22} acid, but probably a hydroxy acid. Since peak 4 represented the major components, it is then consistent with other data obtained on this sample to consider this peak as indicative of a hydroxy acid. However, peaks 1 and 3 could indicate acids of 19 and 21 carbon atoms, but it is more likely that these peaks were due also to hydroxy acids since other data showed the presence of a hydroxyl group or groups.

It has been found that the position of the hydroxyl on a carbon chain affects the retention time of the substance (41,47). Data concerning the retention times and carbon numbers of methyl hydroxypalmitates and methyl hydroxystearates have been recorded in the literature (41,47). However, hydroxyl groups attached to other long chain fatty acids have not been thoroughly investigated. Therefore, at this time peaks 1 and 3 will remain unassigned.

TABLE IV. PERCENTAGES OF METHYL ESTERS IN SAMPLES 4, 13, 14, 17, 9, and 3.

peak no.	retention time relative to methyl palmitate	Sample 4	Sample 13	Sample 14	Sample 17	Sample 9	Sample 3	Ave.
4	7.9	72.1%	71.6%	78.5%	71.2%	79.6%	69.5%	73.8%
3	5.2	7.5%	21.4%	14.7%	23.6%	15.7%	23.8%	17.8%
2	3.6	20.3%	3.0%		5.3%	4.7%	6.8%	6.6%
1	2.5		4.0%	6.8%				1.8 %

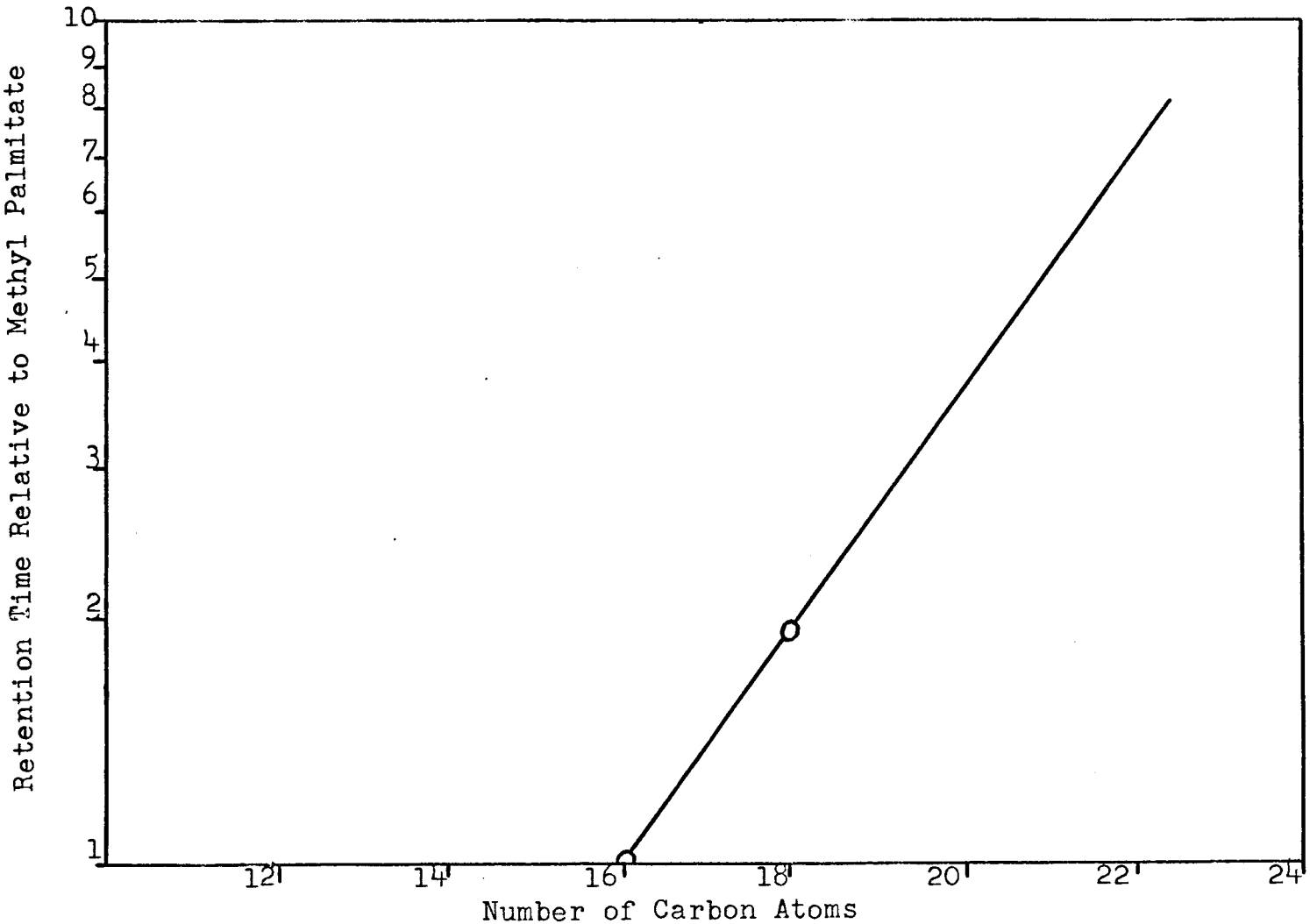


Figure 8. Semilogarithmic plot of retention time relative to methyl palmitate vs. number of carbon atoms

VII. RESULTS AND CONCLUSIONS

In concluding it might be well to consider the procedure for purifying the crude free acid mixture. The first step of this procedure was the formation of the methyl esters. At this point the slight yellow coloring matter was removed, and the largest fraction (approximately 60%) separated from the hexane at room temperature. Then the greatest portion of this fraction (approximately 80%) was purified and designated as Samples 8 and A. These samples displayed the properties of behenic acid, although the melting point of the acid was slightly higher (4-5 degrees) than that accepted for behenic acid (79.7°C.). The methyl ester showed a melting point 20 degrees higher than methyl behenate (53.3°C.), but the methyl ester may have been incompletely formed and acid may still have been present. However, even with these inconsistencies it would appear that this fraction was behenic acid as has been previously reported (14).

The gas-liquid chromatography analysis of the six samples showed that this purification procedure gives incomplete separation of the various components which are present in small amounts. However, most of the major acid was removed after the methyl ester formation by this procedure.

One of the hydroxy acids contained in this white fir bark wax might possibly be a hydroxybehenic acid. Samples 3, 4, 9, 13, 14, and 17, although still mixtures as shown by the gas-liquid chromatography analysis, seemed to display properties of a 22-hydroxybehenic acid which was isolated by Jensen (20). However, since these samples were mixtures, it cannot be stated definitely that 22-hydroxybehenic acid was present in the white fir bark until further work can be completed.

The one acid which seemed to be identified definitely was the C_{16} dicarboxylic acid obtained from the soluble sodium salt fraction.

As can be seen from the data and results, this procedure was not highly successful for the isolation of pure compounds. But the major acid could be removed after the first operation. The other fractions could be obtained somewhat pure, but there always seemed to be small amounts of other acids present in the "purified" sample. However, this procedure could be used successfully for rough separations of the various acids.

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APPENDIX

APPENDIX

Since the body of this paper had been written, further work on these acids has been completed.

The sample designated as 8 and A, which formed the major part of the hexane insoluble acid fraction, was subjected to a gas chromatography analysis. First, it was found that the methyl ester which melted 72-74°C. was actually incompletely methylated. Therefore it was remethylated, forming a methyl ester melting 67-68°C. This methyl ester was then injected into the ethylene glycol succinate gas chromatography column. The chromatogram showed three peaks. (Refer to Table V.) Of these three peaks only the 5.47 peak could have been methyl behenate and that constituted only five percent of the mixture. Therefore the presence of three compounds in this fraction explained the variation of its properties from those of methyl behenate.

Some fractions were subjected to the cycle of methylation, neutralization, acetylation, and neutralization twice and the resulting six fractions were analyzed by gas-liquid chromatography. Data concerning these fractions are shown in Table V. Even after this extensive treatment three compounds were found in some of the fractions.

The filtrates, designated MEF, from the methyl ester crystallization were combined and injected into the gas

chromatograph. Interestingly, the results indicated six components, two of which were the same as found in the higher melting fractions.

Again this data indicated that the purification procedure which was developed was inadequate for the preparation of single, pure fatty acids from the white fir bark wax.

TABLE V.

Sample		Acid Melting Point	Methyl Ester melting point	Saponification Equivalent of methyl ester	Retention time relative to methyl palmitate
A ≠ 8		87.5-88.5	67-68	348	4.67 5.47 7.52
B1		87.5-89	68-69	356	4.82 7.71 12.9
A15-A21		83-85	67-68	346.3	4.60 7.35 11.9
A4 ≠ A7	RT IB	67-68.5 45-48	48-50	277	12.4
A11 ≠ A8		89.90	63-65	301	4.80
A13		74-76	49-50	256	4.90 13.5
A22		79-80	58-59	298	4.87 7.75 13.3
MEF	RT IB	75-77 67-68	43-48	272.3	3.47 12.9 4.83 15.7 6.67 9.06