A Study of Physical and Chemical Properties of the Fats from the Fat Lobes of the Moccasin (Agkistrodon Piscivorus) (Lacepede)

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TABLE OF CONTENTS

	Page
PREFACE	
INTRODUCTION AND REVIEW OF LITERATURE.....	1
Statement of Research Problem	1
Preparation of Moccasin Snake Oil	1
Methods of Separation of the Fatty Acids	2
Qualitative Investigation of Component Fatty Acids	4
Preparation of Mixed Fatty Acids from a Fat	5
Removal of Unsaponifiable Matter from the Mixed Fatty Acids	6
Removal of the Low Molecular Weight Fatty Acids	6
Chromatograph Absorption of Mixed Fatty Acids	7
Non-Solvent Crystallization	7
Solvent Crystallization	7
Separation of Polyethenoid Acids by Lithium Salts	8
Separation of Mixed Fatty Acids by Crystallization from Solvents at Low Temperatures	8
Distillation	8
Fractional Distillation of Higher Fatty Acid Esters at Low Pressures	14
Some Features of the Calculation of the Composition of Individual Ester Fractions	17
Details of the Method of Calculation Involved	18
Ester-Fractions of the More Saturated or "Solid" Acids	20

Page

Ester-Fractions of the More Unsaturated or "Liquid" Acids 20

Absorption Spectra 24

The Identification of the Fatty Acids 29

EXPERIMENTAL DATA AND DISCUSSION..... 31

Table I 31

The Significance of Table I 32

Ether-Insoluble Polybromides from Moccasin Fat 33

Distillation of Methyl Ester Fractions from Moccasin Fat 34

Table II 36

Table III 37

Table IV 38

Table V 39

Table VI 40

Table VII 41

Table VIII 42

Spectrophotometric Data and its Evaluation 44

Preparation of the Alkaline Glycol Solution 45

Table IX 46

Table X 47

Figure 1 50

Discussion of Figure 1 51

Determination of Linoleic, Linolenic, and Arachidonic Acids in Moccasin Snake Oil by Spectrophotometric Analysis 52

Page

Table XI 52

Figure 2 60

SUMMARY..... 62

Table XII 62

Table XIII 63

Table XIV 64

CONCLUSIONS..... 65

BIBLIOGRAPHY..... 66

ACKNOWLEDGMENT..... 72

BIOGRAPHICAL ITEMS..... 73

COMMITTEE REPORT..... 74

PREFACE

The material contained in this dissertation is presented in a format similar to that used for the articles in Chemical Reviews. This was found to be the most satisfactory for proper interrelation of the several parts of the investigation. The manner of listing references is the customary one for technical works in organic chemistry of comparable length and scope. Journal abbreviations are the official ones of Chemical Abstracts.

In conformity with present usage in research publications, all temperatures are on the Centigrade Scale.

INTRODUCTION

AND

REVIEW OF THE LITERATURE

STATEMENT OF RESEARCH PROBLEM

Snake oil, as used in this dissertation, refers to the glycerides obtained from the fat lobes of the moccasin. This research project involves a study of the chemical and physical properties of snake oil produced by cold-pressing the fat lobes from moccasin (Agkistrodon piscivorus)(Lacepede). The chief objective is a qualitative and quantitative analysis of the fatty acids present in moccasin snake oil.

For a summary of the work done on snake oils up to this time, see data by McLaughlin and Pollard[1] and that of Young and Pollard[2].

PREPARATION OF MOCCASIN SNAKE OIL

The original investigations in this laboratory, which were performed upon pooled samples of heat-rendered oils, indicate that heat-rendering produces some changes in the fatty acids originally present. Cold-pressing of the fat lobes of an individual species gives an excellent material for investigation. On the basis of the previous research by McLaughlin, Young, and Pollard, cold-pressed moccasin snake oil was determined to be more desirable for use in this investigation of fatty acids.

The fat lobes of the species Agkistrodon piscivorus (Lacepede), commonly known as the Cottonmouth Moccasin, were supplied by Mr. Ross Allen of the Ross Allen Reptile Institute of Silver Springs, Florida.

Upon removal of the lobes from the snakes, the blood was drained off, and the fat lobes were dried on filter paper. The lobes (in 25-gram quantities) were wrapped with extreme care in filter cloth so that the pressures used would not force large amounts of solids into the oil. The liquid pressed out contained snake oil, hemolyzed blood, blood cells, water, and some unbroken fat cells.

A centrifuge equipped with two 250 c.c. centrifuge tubes was operated at 1400 r.p.m. for two hours. Each run produced about 350 grams of purified snake oil.

METHODS OF SEPARATION OF THE FATTY ACIDS

Present-day knowledge of the fatty acids is the culmination of many years of study and research. No single factor has contributed more toward this knowledge than the many investigations which have been made upon the separation of the fatty acids. It is safe to conclude that future advancement in this field will be intimately associated with the development of more effective methods for the separation of these acids.

The many processes which have been investigated for fatty acid separation, such as crystallization of the acids or their derivatives, distillation of the acids or their esters, and other methods can be roughly divided into two groups. The first of these includes those processes which are useful for analytical or identification purposes only and which, because they are time-consuming or costly, have very

little or no commercial application. The second group consists of those processes which are adaptable to the large-scale preparation of the acids.

A rigid classification of the methods for fatty acid separation is not possible, since many of the commercial processes are also excellent laboratory procedures. Future research may convert some of the now strictly experimental methods into commercially feasible processes.

Among the procedures which have been suggested for the separation of the fatty acids are distilling, crystallizing, and centrifuging, together with combinations of these methods. No single procedure is entirely satisfactory for obtaining pure fatty acids. A combination of two or more procedures gives better results. This is particularly true in the laboratory-scale separation of fatty acid mixtures, since a number of crystallizations are often combined with several fractional distillations. Generally the methyl esters of the acids are employed in the distillation procedures. The choice of the method of separation depends upon the starting material available and upon the objective to be attained. In the following discussion it appears logical to consider some of the various unit processes separately. It should be realized, however, that they can frequently be advantageously combined and that the various methods differ greatly with regard to their practical importance.

Since this research is based upon analytical examinations of a natural fat, it seems desirable to include a discussion of the experimental methods which have so far found acceptance in the quantitative or semi-quantitative study of the natural fats. A fairly full account

of some of the experimental technique is included, although in other instances the treatment in this dissertation is more general. In particular, the more or less standard methods of determination of characteristics such as iodine and saponification values are not here given in detail. Publications of Lewkowitsch[3], Grun[4], Bolton[5], Kissling[6], Dunn[7] and Hilditch[8] give detailed discussions of various determinations.

Determinations of saponification equivalents and iodine numbers are made in practically all fat analyses. Saponification equivalents must be as accurate as possible, since subsequent calculations are based on them. Bond unsaturation is usually evaluated by iodine values, determined preferably by the Hanus method. Some cases may require special treatment. Mixtures of oleic, linoleic, and linolenic may require alkali isomerization followed by spectrographic analysis or determination of the Thiocyanogen Value.

QUANTITATIVE INVESTIGATION OF COMPONENT FATTY ACIDS

A mixture of higher fatty acids such as is usually present in natural fats is ultimately resolved by fractional distillation in a vacuum of the corresponding methyl ester into a series of saturated fractions which contain not more than two unsaturated and two homologous groups of unsaturated esters. It is rarely advisable, however, to convert the entire mixture of fatty acids into methyl esters and then attempt to separate the latter by fractionation. It is best to separate the mixture by use of the lead-salt-ether method and then esterify and distil.

Differences in the solubilities of the lead salts of saturated and unsaturated fatty acids have been utilized for separation. This separation is simplified if acids of low molecular weight are absent. Fractional crystallization from appropriate solvents at low temperatures may also be employed for separating mixed acids.

When fats contain appreciable amounts of acids of lower molecular weight than octanoic (caprylic), it is necessary to remove the more volatile acids of low molecular weight before proceeding to separate the higher saturated and unsaturated acids. This only applies to special types of fats such as milk fats and porpoise oils.

PREPARATION OF THE MIXED FATTY ACIDS FROM A FAT

The quantity of fatty acids required for an accurate analysis depends upon the complexity of the mixture of component acids. The amount runs from 20 grams for a simple type of fat to 500 grams for a complex type of fat such as fish oils.

Complete hydrolysis of the original fat is essential. To saponify 100 parts by weight of fat, add 30 parts by weight of potassium hydroxide in about 500 parts of alcohol (95-100 per cent), reflux for three hours, and then remove most of the alcohol by distillation. The soaps are dissolved in water, and unsaponifiable matter is removed at this stage, if necessary. The free fatty acids are formed by adding warm, dilute hydrochloric acid. If unsaturated acids are present in quantity, care must be taken to prevent oxidation. When the acids have been liberated

they are extracted with ether and are dried under vacuum at 100°C.

When highly unsaturated acids are present in quantity, it is not desirable to use too much alkali or to prolong the saponification. The highly unsaturated fatty acids easily undergo isomerization in the presence of excess alkali and lengthy heating. Therefore, with such acids it is desirable to use very little alkali in excess of the amount theoretically required and to heat under reflux only as long as is required.

REMOVAL OF UNSAPONIFIABLE MATTER FROM THE MIXED FATTY ACIDS

Most, if not all, of the unsaponifiable matter present in a fat tends to pass with the ether-soluble lead salts into the unsaturated or "liquid" fraction. If the amount of unsaponifiable matter in the fat is less than 0.7 per cent, removal is not necessary. In the moccasin snake oil, the unsaponifiable residue was not removed since the amount was only 0.46 per cent.

REMOVAL OF THE LOW MOLECULAR WEIGHT FATTY ACIDS

If appreciable quantities of lower molecular weight fatty acids are present, they should be removed before the higher fatty acids are separated. One method of removing the lower molecular weight fatty acids is by distillation in a current of steam.

The amount of low molecular weight fatty acids in moccasin snake oil is found to be very slight (0.13 per cent); therefore, it is not necessary to remove the low molecular weight fatty acids.

CHROMATOGRAPHIC ADSORPTION OF MIXED FATTY ACIDS

In this problem the use of chromatography appeared to offer no advantages over certain other procedures.

NON-SOLVENT CRYSTALLIZATION

The non-solvent separation of the fatty acids depends upon the fact that they are completely miscible in the liquid state. After the solutions are cooled, the higher melting acids crystallize, and they can be separated from the liquid acids by suitable mechanical means, such as pressing.

No complete investigation of non-solvent crystallization is given here since sufficient snake oil and equipment for low-temperature work were not available. It is probable that non-solvent crystallization would produce a good separation between the monoethenoid and polyethenoid acids present in snake oil.

SOLVENT CRYSTALLIZATION

One of the best preliminary methods of separating the various acids is by the use of the differing solubilities of the lead salts of the

fatty acids in ether. For a complete description of the technique used in the lead salt-ether method see the procedure given by the A.O.A.C.[9] and the work of McLaughlin and Pollard[1].

SEPARATION OF POLYETHENOIC ACIDS BY LITHIUM SALTS

Considerable work has been reported in the literature concerning the separation of polyethenoid acids by the use of lithium salts. The main solvent in use is the acetone-water solution. For a detailed summary of this procedure see Hilditch[10], Ralston[11], and Markley[12].

SEPARATION OF THE MIXED FATTY ACIDS BY CRYSTALLIZATION FROM SOLVENTS AT LOW TEMPERATURES

For a discussion of the advantages and disadvantages of this method see Hilditch[10]. Limitations of material and equipment precluded use of the method in this study. Separation of fatty acids by low-temperature, with or without solvents, is quite satisfactory when large amounts of fat are available. Its use when the quantity of fat is quite small is not advantageous.

DISTILLATION

The fractional distillation of fatty acid esters has been extensively employed as a means of separation of natural fats for analysis. The fractionation of the methyl esters is frequently used for the laboratory preparation of the individual acids. The most important earlier studies

concerned with the fractional distillation of fatty acids and their esters are those reported by Krafft and co-workers[13], who investigated the boiling points of the acids and their esters under high vacuum.

Caldwell and Hurtley[14] report the boiling points of lauric, myristic, palmitic, stearic, and oleic acids in a cathode vacuum, using a Gaede pump with a Crookes tube between the receiver and pump, and obtained values materially lower than those previously reported. These authors state that at a very low pressure a liquid has no boiling point; it sublimes or evaporates, just as water does in air, at a rate and temperature dependent upon the nature of the substance. Caldwell and Hurtley also describe a method for fractionation of the fatty acids of butter and coconut oil.

Brown[15] describes an apparatus for the distillation of the fatty acids at low pressures. The separation of palmitic and stearic acids by vacuum distillation was reported simultaneously by Krafft[16] and by Kreis and Hafner[17].

In 1906 the separation of the fatty acids of coconut oil[18] and of cod liver oil[19] by use of their methyl esters was first reported. The fatty acids of butter fat were separated by the fractional distillation of their methyl esters, and Elsdon[20, 21, 22, 23, 24, 25, 26] reports the separation of the fatty acids of both coconut and palm kernel oil by a similar method. The fractional distillation of methyl esters was also reported for the separation of fatty acids of cottonseed oil[27], hydrogenated herring oil[28], human milk[29], chaulmoogra oil[30],

and peanut oil[31]. Stokoe[32] described the fractional distillation of the methyl esters of cocomut oil fatty acids, and Armstrong, Allan, and Moore[33] reported the separation of the fatty acids of cocomut oil and palm kernel oil by the fractional distillation of their methyl esters.

In 1923, Brown and Beal[34] announced the separation of the methyl esters of the fatty acids of menhaden oil by vacuum distillation. They concluded that it is possible to make a rough separation of the acids according to their molecular weights by this method. Later Brown[35] stated that the separation of the methyl esters of the fatty acids of brain lipoids may be made by fractional distillation at pressures varying from 4 to 7 mm. The separation of the highly unsaturated fatty acids of beef brains by fractional distillation of their methyl esters did not succeed. Brown and Ault[36] stated that it is possible to distil fractionally the methyl esters of the debrominated acids of cattle, hog, and sheep brains.

The laboratory separation of fatty acids (as acids or as their esters) by the use of fractional distillation is considered to give incomplete separations. Channon, Drummond, and Golding[24] claim that the method is of little quantitative value. Since 1930 investigation of the fractional distillation of fatty acid mixtures by use of methyl esters has been intensified.

Equilibrium between the vapor and liquid phase is not obtained in the unpacked columns. Also, the reflux ratio and the rate of distilla-

tion are not under control. Separations performed in such equipment are consequently highly unsatisfactory. Improvements in laboratory distillation apparatus are described by a number of investigators[37,38,39,40,41]. In 1930 Jantzen and Tiedcke[42] developed an electrically heated, packed column which was enclosed in an evacuated jacket to protect it against temperature fluctuation. This apparatus was used in the separation of the methyl esters of palmitic and stearic acids, and the device was also employed by these authors for the fractionation of the high-melting acids of peanut oil. The apparatus was subsequently modified by later investigators[43, 44], and the improved version was used by Lepkovsky, Peskov, and Evans[45] for the seperation of caprylic, capric, lauric, myristic, palmitic, and erucic acids. Indications of association are observed in the separation of the higher acids, but they could be completely separated as their methyl esters.

Modern laboratory distillation apparatus employs an electrically heated, packed column which permits a high reflux ratio and an adjustable distillate collection. The column is packed in a well insulated outer shell to minimize thermal fluctuation.

Attached to the column are a distilling flask, receiver, and condenser. Standard taper, ground-glass joints are usually employed. The temperature at the top of the column is measured by a thermometer, and the pressure is indicated by a manometer. The distilling flask and the column are electrically heated, and a constant temperature is maintained by rheostats. The side arm is electrically heated in

order to prevent the distillate from solidifying. Low pressure is maintained by a standard vacuum pump attached to a large, evacuated steel drum since rapid pressure changes during the distillation are undesirable. The laboratory columns are usually three to four feet in length and are permanently mounted. Their efficiency depends largely upon the type of packing material employed, reflux ratio, etc.

Industrial columns are usually of the bubble-cap type. The various plates exert a scrubbing action and thus give the effect of multiple distillations. Hold-up in the apparatus is too great for laboratory distillations. It is adaptable only to large-scale, continuous work. Packed columns usually give materially less hold-up and are quite satisfactory in distilling small amounts of material. Numerous investigations of many different types of packing material have been reported. In 1932 Whitmore and Lux[46] described a column packed with Lessing rings (5 x 5 mm.) made of 50-mesh copper screen; and the next year Wilson, Parker, and Laughlin[47] described a column packed with a glass modification of the single- and double-turn wire helices which had been used earlier. Weston[48] reported a fractionating column which was a modification of that described by Cooper and Fasce[41].

The extensive investigations of Podbielenial[49] contributed much to the development of efficient, small-scale distillation equipment; and many of his results were summarized in his excellent article published in 1933. In this article he described his column and gave his recommendations for fractional distillation.

Klem[50] reported the separation of the methyl esters of palmitic, stearic, oleic, and elaidic acids by the use of the Podbielniak apparatus. Comparative studies of diversified packing material have been made by Fenske, Tongberg, and Quiggle[51]. These authors state that wire or glass helices, carding teeth, and jack chains were found to be the best packings.

The shape and distribution of the packing material are important determinative factors in the efficiency of fractionating columns. However, the importance of such factors as the contact surface and the proper drain-back cannot be over-emphasized. Tongberg, Quiggle and Fenske[52] describe an efficient fractionating column packed with one-turn glass helices. Packing material composed of wire helices is claimed to be extremely efficient[53, 54, 55]. A column packing of close-fitting wire helices was used by Schoenheimer and Rittenberg[56] for the fractionation of methyl palmitate from methyl stearate.

The use of fatty acid ester distillation methods of fat analysis was reviewed by Longenecker[57] in 1940.

The conical type of packing was described by Stedman[58, 59]. A detailed description of this type of packing and results of numbers of efficiency tests were given by Bragg[60].

In recent years the use of the molecular still has been of great importance in fractionating very high-boiling substances. Molecular distillation of the highly unsaturated acids of fish oils was reported by Farmer and Van den Heuvel[61].

FRACTIONAL DISTILLATION OF HIGHER FATTY ACID ESTERS AT LOW PRESSURES

As already stated, those portions of the mixed fatty acids which are to be further resolved by ester-fractionation are converted into methyl esters by boiling with about four times their weight of methyl alcohol in the presence of approximately one per cent by weight of concentrated sulfuric acid. The unesterified acid is removed by washing the ether solution of the esters with dilute potassium carbonate solution. The conversion into methyl esters is usually 92 - 98 per cent effective. If production is below this figure, the unesterified acids should be recovered and re-esterified.

The methyl esters are used primarily because of their slightly lower boiling points as compared with those of ethyl esters. With the operating pressures ranging from 0.1 to 0.2 mm. now readily obtainable with the ordinary rotary oil pumps, this factor is less significant than formerly. However, it is convenient to continue using the methyl esters since so much data already have been recorded for methyl esters.

The recorded boiling points at the head of the fractionating column are not of scientific significance in this type of distillation. However, the column head temperature should be systematically recorded; since, in conjunction with those of the heating bath, they afford a reliable indication of the smoothness of operation, and therefore of the efficiency of the fractionation.

At 0.2 mm. pressure, the column head temperatures for methyl laurate, methyl palmitate, and methyl oleate (or stearate) are approximately 75-80°, 110-15°, and 130-135°, respectively.

Unless a large fraction of known range is being collected for refractionation, the weight of any one ester-fraction should not greatly exceed about ten grams and may be as little as two or three grams. Even when it is reasonably certain that a mixture of constant composition is distilling in large quantity, it is better not to exceed the amount stated. Errors in the analytical determination of saponification equivalents or Iodine Values are minimized if carried out on several small fractions rather than on one very large cut. The minimum weight of a fraction is determined simply by the amount required for accurate determination of its analytical characteristics.

The unsaponifiable or non-fatty matter generally remains in the residual, undistilled ester fraction. The amount of unsaponifiable residue may be determined by removing it from the alkaline solution obtained after determination of the apparent equivalent of the residual ester. This is followed by recovery of the fatty acids and redetermination of their equivalents. The amount of esters in R grams of a residual ester is then:

$$R \times \frac{(\text{equivalent of recovered of fatty acid} + 14)}{\text{apparent equivalent of residual esters}}$$

The effect of heat upon unsaturated fatty esters during the fractionation is reported by Norris et al.[62] They report that esters of acids

with three or fewer unconjugated double bonds are substantially unaffected. It is their opinion that more unsaturated polyethenoids (e.g. fish oil) acids are also little altered, although slight rearrangement to conjugated isomerides, followed by some dimerization, may take place to a very small degree. Any material so altered appears in the residual, undistilled esters. These authors concluded that the changes induced in the course of a fairly prolonged distillation were insignificant. They state that the action of reagents (alkali) and solvents during hydrolysis and treatment of the fats prior to fractionation is a much more important potential source of structural alteration than any condition encountered during fractional distillation at low pressures. It should be pointed out that esters of conjugated higher fatty acids undergo polymerization and/or cyclization at the temperature at which they distill in heated fractionation columns of the described type and that such esters (e.g. methyl elaeostearates) cannot be distilled in a column at too high a temperature.

Norris and Terry[63] gave the precise boiling point data for the following esters at one mm. pressure: methyl myristate, 114°; methyl palmitate, 136°; methyl stearate, 155.5°; methyl oleate, 152.5°; and methyl linoleate, 149.5°. Althouse and Triebold[64] also reported some data on methyl oleate, methyl linoleate, and methyl linolenate. They claim that the boiling point of the three C_{18} acid methyl esters are in reverse order from that given by Norris and Terry. All agree that the boiling points of all three of the C_{18} acid methyl esters are extremely close.

SOME FEATURES OF THE CALCULATION OF THE COMPOSITION OF INDIVIDUAL ESTER-FRACTIONS

The purpose of the ester-fractionation procedure is the production of a series of ester-fractions for use in the analysis of a mixture of fatty acids. Each of these ester-fractions should contain not more than two unsaturated esters. The composition of such fractions can be directly calculated from their saponification equivalents and Iodine Values. This is true of most fats in which the only unsaturated components belong to the C_{18} series of acids (oleic, linoleic, and linolenic). When, as is usually the case, both oleic and linoleic acids are present, the mean Iodine Value of the C_{18} unsaturated esters can be determined. Except for a negligible error, it can be assumed that the distillation of these esters is proportionally constant throughout the operation.

The boiling point of methyl linoleate is very close to that of methyl oleate. If linolenic acid is the only poly-ethenoid acid present, a complete determination of such ester-fractions may be made. The determination may be made by use of Iodine Value and saponification equivalent in conjunction with the spectrophotometric determination of the linoleic acid.

In some fats, especially those from aquatic sources, the mixture of unsaturated acids, particularly in the C_{20} and C_{22} series, is very complex. The constitution of many of the poly-ethenoid acids is still uncertain, and it is necessary to adopt a different method of evaluating the ester-fractions.

DETAILS OF THE METHOD OF CALCULATION INVOLVED

As already stated, the composition of an ester-fraction which contains esters of only two saturated acids with the unsaturated acids all of the same carbon content can be deduced from its equivalent and Iodine Value by comparatively simple calculations as follows:

If a fraction of weight w, equivalent E_w, and Iodine Value I_w contains a weight u of unsaturated esters (of the same carbon content, e.g. C_{18}) with Iodine Value I_u and equivalent E_u,

$$u = w \left(\frac{I_w}{I_u} \right)$$

and the mean equivalent, E_s, of the saturated esters (w — u) present follows from the equation:

$$E_s = \frac{w - u}{\frac{w}{E_w} - \frac{u}{E_u}}$$

From E_s the weights of saturated esters are calculated as a binary mixture of the homologues (e.g. C_{16} and C_{18}) between the equivalents of which E_s lies.

Again, ester-fractions which include only unsaturated derivatives of acids of two groups in the homologous series can be evaluated directly from their saponification equivalent. In certain cases the computations become somewhat more complicated, since the weights of more than three independent components are involved.

If x, y, and z are the respective weights of the one saturated and the two unsaturated esters in a fraction of weight w, and E_x, E_y, E_z, E_w are the corresponding equivalents and I_y, I_z, I_w are the corresponding Iodine Values, then

$$(1)\quad x + y + z = w$$

$$(2)\quad x/E_x + y/E_y + z/E_z = w/E_w$$

$$(3)\quad y.I_y + z.I_z = w.I_w$$

(In equation (2) the saponification values V_x, V_y, V_z, V_w can be used if desired, the equation becoming:

$$x.V_x - z.V_x - y.V_z = w.V_w \quad)$$

The values of y and z, the unsaturated components, are thus determined while, from that of x, the binary mixture of saturated esters is evaluated directly from its equivalent (E_x).

It may be pointed out that, in use, the equations (1), (2), and (3) can be conveniently simplified by employing in equation (2) the reciprocal of the equivalents times 10^6.

The proper method of evaluation of the ester-fraction data in any given case depends upon the particular circumstances. In the majority of analyses, if the more efficient type of fractionating column has been used, it is not necessary to isolate and determine the equivalent of the saturated components of an ester-fraction.

ESTER-FRACTIONS OF THE MORE SATURATED OR "SOLID" ACIDS

The lead salts of tetra- and hexa-decenoic acids are freely soluble in ether. Since the small Iodine Values in fractions of equivalent below 296 may be attributed solely to methyl oleate, the calculation is straight-forward. This method has been used when "solid" esters have been distilled with a simple distillation apparatus.

When the electrically heated column is employed, it is evident that traces of these acids are detectable in the C_{14} and C_{16} ester-fractions. In such cases it may be assumed that the saturated and unsaturated portions of the fraction have the same equivalent.

In instances in which the saturated esters of a fraction have been isolated and their equivalent determined, the general equations given in the preceding paragraphs may be used with x as the weight of saturated esters of determined equivalents, E_x. The determined equivalent E_x may then be used to calculate x as a binary mixture of saturated esters.

ESTER-FRACTIONS OF THE MORE UNSATURATED OR "LIQUID" ACIDS

In fractions with equivalents below those of unsaturated C_{18} esters there are some palmitates and much larger amounts of myristates.

The saturated esters present in these fractions may be isolated and the components calculated by the general equations given. If an efficient fractionating column was used, calculations of the ester-fractions of equivalent below 270 on the assumption that their

saturated and unsaturated parts have the same equivalents is sufficient.

Ester-fractions with equivalents between 270 and 290 are calculated from the general equations. They are calculated as mixtures of palmitic, hexadecenoic, and unsaturated C_{18} esters. When lead salt-ether separation has been employed, the presence of stearic acid in the "liquid" acids may be considered to be of little importance.

Fractions consisting wholly of unsaturated C_{18} esters should be evaluated with the following facts in mind:

So long as the unsaturated C_{18} ester-fraction consists mainly of oleate, with not more than 10 to 20 per cent of linoleate, the determined equivalents correspond closely with those deduced from oleate and linoleate Iodine Values.

When the ester-fractions contain large proportions of linoleate, the determined equivalents of unsaturated C_{18} ester-fractions tend to be appreciably lower than those observed by the iodine values. The cause has not been proven, but it may be due to the presence of traces of autoöxidized esters which have undergone scission during refluxing with alcoholic potassium hydroxide. It should be borne in mind that the determined equivalents of the more unsaturated C_{18} ester fractions are usually slightly lower than the true values.

In ester-fractions immediately preceding those which consist wholly of unsaturated C_{18} esters, there are very small amounts of saturated esters. Actual calculations of the mean equivalents of these ester-fractions prove difficult, and results are not too reliable. It has been found that it is better to credit them as palmitate than to use their deduced equivalents.

When only linolenate and oleate are present, the proportion of each follows directly from the iodine values.

Fractions with equivalents above those of unsaturated C_{18} must be calculated to binary mixtures of C_{18} and C_{20}, or C_{20} and C_{22}, according to their observed equivalents.

In spite of the numerous variants and assumptions suggested in the preceding paragraphs, it has been found that reliable analyses may be obtained through calculation of ester-fractions. Each case must be considered upon its merits, and the calculations should take into account the methyl esters present in the mixture. The analyses of the more unsaturated fractions should be completed quickly, and care should be taken to avoid oxidation. With proper precautions, it is possible to obtain component acid data of the order of accuracy indicated below.

Having determined the approximate components of each fraction, it is a comparatively simple matter to obtain the composition of the "solid" and "liquid" esters. From this it is possible to calculate the amount of each fatty acid present in the original sample. In the case of

complex fats the operations are extremely lengthy and tedious, and a final accuracy of not more than ± 2 units may be reached in this calculation.

Even with the somewhat greater inaccuracy of the figures for some of the more highly unsaturated components of fish and allied fats, it has been claimed that the accuracy is comparable with that for many other natural products of a comparable complexity.

The "liquid" fatty acids obtained in a component acid analysis should be converted into methyl esters, and their fractional distillation carried through without delay.

Unless due precautions are observed, acids of the more unsaturated types are liable to undergo polymeric or other changes during the esterification. This has been shown to be especially likely to occur during concentration of the mineral acid by removal of the methyl alcohol. In such instances it is necessary to (a) employ the minimum concentration of sulfuric acid to secure nearly complete esterification (probably as little as 0.5 per cent), and (b) remove no unchanged methyl alcohol by distillation, but to pour the reaction mixture into cold water and proceed at once to extract the esters from the dilute alcohol with ether. The latter precaution is very important.

The "solid" acids obtained in separations carried out as above contain the whole of any stearic or higher saturated acids present, nearly all the palmitic acid, a considerable proportion of any myristic acid present, and smaller proportions of any lower saturated

acids. They will also include, of unsaturated acids, minor percentages of oleic acid. Mono-ethenoid acids of the C_{16} or lower series, and poly-ethenoid acids of any carbon content will, on the other hand, pass almost entirely into the "liquid" acid group.

In addition to nearly all the oleic acid present, the "liquid" acids may include most of the more unsaturated acids of the C_{18} or higher series; the mono-ethenoid acids of the C_{16} or lower series; much of the saturated C_8, C_{10}, C_{12}, minor amounts of myristic; and traces of the palmitic.

ABSORPTION SPECTRA

Owing to the presence of the double bond in the carbonyl group, the saturated fatty acids show a typical absorption spectrum. The ethylenic acids not only exhibit an absorption due to the carbonyl group but also show characteristic absorption values dependent upon the number and the relative positions of the double bonds.

Since the ultraviolet absorption properties of the saturated fatty acids are dependent upon the presence of the chromophoric carbonyl group, it is evident that the absorption characteristics of the saturated acids should be qualitatively similar. Ramart-Lucas and associates[65] investigated the ultra-violet absorption properties of formic, acetic, butyric, caproic, caprylic, lauric, myristic, palmitic, and stearic acids between the wave lengths of 2000 and 2555 Angstroms. They stated that the absorption coefficients of

the higher members are essentially similar. These authors concluded that since the length of the chain does not materially affect the absorption coefficient, methyl and methylene groups cannot be considered as chromophores in this region of the spectrum. Bielecki and Henri[66] had studied a number of saturated acids in the range 2144 to 2600 Angstroms earlier and had contended that an increase in the length of the hydrocarbon chain was attended by a shift in the absorption band toward the red and also by an increase in the magnitude of total absorption. However, Ley and Arends[67] stated that this generalization does not apply to the higher saturated acids. Burr and Miller[68] compared the values of λ for acetic and palmitic acids for various values of the molecular extinction coefficient ϵ.

Studies of the absorption coefficients of the saturated acids have led to the postulate that in the liquid state the acids exist in a dissociated and in a non-dissociated form[69, 70]. Ester formation does not materially affect the character of the absorption curve. It has been shown that the values of the various esters differ only slightly from those of the parent acids[66], but absorption is radically changed by salt formation[67].

Unsaturation in the hydrocarbon chain produces a change in both the intensity and the position of the absorption bands, the absorption being due not only to the carbonyl group but also to the ethylenic bands. Conjugation is attended by a marked shift in the absorption band toward the red. This phenomenon recently has been the subject of

extensive study and has been proposed for use in distinguishing conjugated from unconjugated acids or esters[71, 72, 73]. After a study of the absorption spectra of unsaturated acids Van der Hulst[74] reported characteristic maxima which depended upon the number and relative positions of the ethylenic bonds. Dingwell and Thompson[75] reported the presence of a maxima at 2700 Angstroms for alpha-eleostearic acid. An isolated double bond between carbon atoms produces an absorption maxima at 1800 Angstroms. Mitchell and Kraybill[72] stated that from theoretical considerations, absorption bands in the spectral region of wave lengths longer than 2200 Angstroms are not characteristic of non-conjugated linoleic or linolenic acid. Burr and Miller[68] recently extended the absorption studies of the unsaturated acids to 2100 Angstroms and noted that the unsaturated acids gave an absorption curve that is a combination of the saturated acid absorption curve plus an absorption curve due to the ethylenic group. The new band for oleic acid occurs at a longer wave length than that observed for 3-heptene, and a comparison of the absorption spectra of oleic, linoleic, and linolenic acids shows that the addition of ethylenic bonds produces a further shift of this characteristic band toward the longer wave lengths.

Determination of the infrared spectra are frequently useful in establishing the structure of isomeric unsaturated compounds[76, 77, 78]. Cis acids show a strong infrared absorption, but this absorption is absent in the trans form.

Studies of the ultraviolet absorption spectra of the saturated fatty acids and of oleic, linoleic, linolenic, and arachidonic acids have been extended to 2100 Angstroms by Burr and associates[79]. Since none of the unsaturated acids reaches a peak at 2100 Angstroms, these studies were later extended to the extreme ultraviolet; and many interesting observations were made[80]. A comparison of the absorption spectra of myristic acid dissolved in ethanol, iso-octane, and heptane shows that the polar solvent, ethanol, shifts the absorption maximum to a lower wave length and gives lower absolute values for extinction coefficients. All the saturated acids have a broad band with the maximum at about 2050 Angstroms and an intense absorption between 1850 and 1730 Angstroms. Unsaturation in the fatty acids produces absorption similar to that of the unsaturated hydrocarbons. Elaidinization of oleic acid shifts the absorption curve toward the visible end of the spectrum. The available spectrophotometric data on the fats, fatty acids, and their esters have recently been compiled[81].

Brice[82] continued his research on existing spectrophotometric methods, modified for application primarily to the detection and estimation of low proportions of conjugated and non-conjugated components in fats, oils, and soaps. The method is also applicable to fatty materials having high proportions of those components.

Modifications include corrections for absorption by interfering substances, use of alkaline glycol as an isomerization medium in the analytical procedure, and correction of absorption data on the

isomerized product. The absorption data correction is needed to compensate for the absorption by conjugated components which were in the material before the isomerization.

Results on some new spectrophotometric analyses of fatty acids were reported by O'Connor[83, 84].

In 1946 Lundberg[85] stated that there were various spectrophotometric changes in fats during rancidification.

Further work has been done on the spectrophotometric studies of the oxidation of fats through the oxygen absorption and chromophore production in fatty esters. Holmen[85, 86] investigated the relationship of oxygen absorption to absorption band development in the esters of the common unsaturated fatty acids. The data reported must be evaluated carefully before any conclusions are reached.

Beadle[87] recently published a method for the use of ultraviolet spectrophotometry in the study of changes in the double-bond system of fatty acids. His procedure is claimed to be of special help in cases where both conjugated and non-conjugated components occur.

Hilditch and Shrivastava[88] reported that the determination of linolenic acid by the spectrophotometric method gave a higher value than the amount of linolenic acid present should give. No explanation was offered for this excess over the true values.

O'Connor[89] obtained results in the determination of the alpha and beta isomers of eleostearic acid in tung oil by the use of a

spectrophotometer. The results obtained from spectrophotometric and chemical analyses were not in complete agreement.

In 1948 Hendrickson et al.[90] discussed the application of ultraviolet spectrophotometry in drying oil research. In their article they traced the development of tests for the determination of fatty acids by spectrophotometric methods.

THE IDENTIFICATION OF THE FATTY ACIDS

The methods used for the identification of pure organic compounds and for ascertaining the components of mixtures are generally familiar to all chemists. Physical constants such as the melting points, refractive indices, and specific gravities are universally recognized as contributing to the identification of organic compounds. The preparation of a characterizing derivative frequently offers further proof of the identity and purity of the compound in question.

Unfortunately, very few acceptable derivatives of the unsaturated fatty acids have been described. A number of investigators have developed derivatives of the saturated acids which have proved to be very useful in the identification of these compounds. The close structural similarity of the various series of fatty acids has made possible the use of a number of analytical procedures which have proved to be of great value.

Such constants as the neutralization equivalents, the Iodine Number, the acetyl value, and the more recent thiocyanate number are

well-known terms which have been applied to the fats and fatty acids for many years. Without attempting to detract from the usefulness of such analytical constants, it should be pointed out that they are not, per se, proof of the identity or purity of an individual acid. For instance, an equimolecular mixture of palmitic and stearic acids will possess the neutralization equivalent of margaric acid, and a mixture of unsaturated acids may possess the theoretical iodine number of oleic acid.

EXPERIMENTAL DATA AND DISCUSSION

EXPERIMENTAL DATA AND DISCUSSION

TABLE I

Data on Oil from Cold-Pressed Fat Lobes of Moccasin (Agkistrodon Piscivorus)(Lacepede)

Moisture and Volatile Matter	Less than 0.1 %
Specific Gravity (25°/4°)	0.9268
Index of Refraction (25°)	1.4690
Saponification Number (Koettstorfer Number)	192.6
Iodine Number (Hanus)	104.4-109.1
Soluble Acids	0.13 %
Insoluble Acids	94.85 %
Reichert-Meissl Value (Soluble Volatile Acids)	0.07 %
Polenske Value (Insoluble Volatile Acids)	0.04 %
Saturated Acids	22.7 %
Unsaturated Acids	72.7 %
Free Fatty Acids	0.31 %
Acetyl Value	4.1
Unsaponifiable Residue	0.46 %
Nitrogen	0.00 %
Phosphorus	0.00 %
Specific Rotation	-0.12°
Thiocyanogen Value	77.2

THE SIGNIFICANCE OF TABLE I

From the data in Table I, it is seen that the amount of soluble acids is 0.13 per cent, the soluble volatile acids are 0.07 per cent, and the insoluble volatile acids are 0.04 per cent. This information shows that the amount of low molecular weight fatty acids in moccasin snake oil is very low. Low molecular weight fatty acids means those acids of lower molecular weight than lauric acid.

The unsaponifiable residue is important since the amount of higher molecular weight alcohols present is shown by the quantity of the residue. Data indicate that the amount of higher molecular weight alcohols (of the cholesterol type) present in moccasin snake oil is 0.46 per cent. Several color tests run on the unsaponifiable matter show that cholesterol-type alcohols are present.

An acetyl value of 4.1 was found. This value is actually the "apparent acetyl value" rather than the true acetyl value[3]. After subtracting the acetylation due to the free fatty acid, the acetylation due to the free hydroxy groups of the glycerol (produced when the free fatty acids were formed), and taking into account the experimental error in the determination of acetyl values, it was found that the "true acetyl value" is very small. It is evident that the amount of hydroxy acids present in moccasin snake oil is very slight. The acetyl value is used for the purpose of determining the amount of hydroxy acids present in moccasin snake oil.

It should be pointed out here that the difference between the Iodine Value and the thiocyanogen value of an unsaturated acid mixture is a measure of the poly-unsaturated acids present[91]. Since the thiocyanogen value of the moccasin snake oil is 77.2 and the Iodine Value of the moccasin snake oil is 104.4, it is apparent that the amount of poly-unsaturated acids present should be about 26.8 per cent. The analysis of the fatty acids present in moccasin snake oil substantiates this finding.

It must be pointed out that the iodine number of a compound is not always a true measure of its unsaturation. An examination of the theory behind the use of iodine numbers in determining the unsaturation of different compounds showed several important factors. Reports have stated that conjugated double bonds fail to give the theoretical iodine number and that the distance of the double bond from the carboxyl and the methyl group in any acid affects the iodine addition.

ETHER-INSOLUBLE POLYBROMIDES FROM MOCCASIN FAT

The percentage of ether-insoluble polybromides, determined as described by Lewkowitsch[3], gives a preliminary estimate of the amount of unsaturated C_{20} and C_{22} fatty acids. From 63 grams of "liquid" acids (obtained from the lead salt-ether method of separation) there were produced a total of 15 grams of ether-insoluble polybromides. From this mixture of polybromides was obtained a fraction which began to blacken at 200°C., and on further heating the fraction decomposed

on further heating. According to Lewkowitsch, this is typical of arachidonic acid. When Lewkowitsch made this statement it was thought that the empirical formula of clupanodonic acid was $C_{18}H_{28}O_2$ and that of arachidonic acid was $C_{20}H_{32}O_2$. At the present time the empirical formula of clupanodonic acid appears to be $C_{22}H_{34}O_2$, and that of arachidonic acid is still considered to be $C_{20}H_{32}O_2$.

On the basis of the amount of ether-insoluble polybromides obtained, it was calculated that the original snake oil contains at least 7 per cent of the highly unsaturated C_{20} and C_{22} fatty acids.

It should be pointed out that the ether-insoluble polybromides do not always give the actual quantity of the highly unsaturated C_{20} and C_{22} fatty acids present[92].

DISTILLATION OF METHYL ESTER FRACTIONS FROM MOCCASIN FAT

Fifty grams of "liquid" fatty acids, obtained by the use of the lead-salt-ether method, were added to 200 grams of methanol and 3 grams of concentrated sulfuric acid. The mixture was boiled for about fifteen minutes, and then the excess methanol distilled off until about 80 per cent of the methanol was removed. The mixture was then poured into 1 liter of distilled water, and 200 grams of ether were added. The ether layer was then washed with a dilute solution of potassium carbonate. After the unreacted fatty acids had been removed from the esters, by washing repeatedly with potassium carbonate solution,

and the ether was distilled off, leaving the methyl esters. A 92.4 per cent conversion into the methyl esters resulted.

The data in Table II were obtained when 45.3 grams of methyl esters were distilled at about 1 mm. pressure in a Lecky and Ewell[93] type column. The methyl esters were obtained from the "liquid" acids separated by use of the lead-salt-ether procedure. The distillation fractions were examined for refractive indices, saponification equivalents, and Iodine Values. With this information it was possible to use the Hilditch method of calculation for the composition of the individual ester-fractions[8] according to the procedure as discussed in the review of the literature.

TABLE II

Data Obtained from Distillation at 1 mm. of 45.3 Grams of Methyl Esters of "Liquid" Acids.

"Liquid" Acids Produced by Lead-Salt-Ether Method

Fraction	Temperature (°C.)	Weight of Fraction (in g.)	n_D^{25}	Saponification Equivalent	Iodine Value (Hanus)
1	107-117	0.2	1.4452	-	56.3
2	117-128	0.7	1.4445	230.0	50.8
3	128-132	1.0	1.4449	250.0	63.6
4	133-138	1.7	1.4460	263.5	70.3
5	138-148	3.7	1.4483	280.0	80.1
6	149-153	13.2	1.4520	298.2	102.9
7	153-157	8.1	1.4532	302.1	104.9
8	158-162	4.2	1.4542	293.5	104.9
9	162-165.5	1.2	1.4598	296.8	87.4
10	165.5-168	0.9	1.4679	290.0	120.3
11	168-169	1.9	1.4679	276.0	109.1
12	170-200	4.3	1.4805	313.3	212.0
13 (Residue)	-	4.2	-	-	-

From the saponification equivalents, refractive indices and Iodine Numbers, the break-down of the various fractions are given in Table III

TABLE III

Ester Composition of Fractions Obtained from Distillation at 1 mm. of 45.3 grams of Methyl Esters of "Liquid" Acids. "Liquid" Acids Produced by Lead-Salt-Ether Method

Sample	Weight of Fraction (in g.)	Amount of Esters (in g.)					
		Methyl myristate	Methyl palmitate	Methyl hexadecenoate	Methyl oleate	Methyl linolate	Methyl arachidonate & Methyl clupanodonate
1	0.2	0.08		0.12			
2	0.7	0.32		0.38			
3	1.0	0.22	0.11	0.67			
4	1.7	0.11	0.33	1.26			
5	3.7		0.44	1.67	1.59		
6	13.2				10.56	2.64	
7	8.1				6.48	1.62	
8	4.2				3.36	0.84	
9	1.2					1.2	
10	0.9					0.9	
11	1.9					1.9	
12	4.3					0.86	3.44
13	4.2[a]						3.9
TOTAL	45.3	0.73	0.88	4.10	21.99	10.26	7.34

[a] The 4.2 g. present is 0.3 g. of unsaponifiable residue and 3.9 g. of of residue composed of highly unsaturated acids

The "liquid" fatty acid fraction obtained by the lead salt-ether method is 71.0 per cent of the total snake oil. On converting the values for the various methyl esters, as shown in Table III, the following percentages of acids are obtained:

TABLE IV

Percentage Composition of "Liquid" Fatty Acid Fraction

(Calculated from Data in Tables II and III)

Name of Acid	Percentage
Myristic	1.15
Palmitic	1.39
Hexadecenoic	6.43
Oleic	34.48
Linoleic	16.00
Arachidonic and Clupanodonic	10.98
Unsaponifiable Residue	0.57
TOTAL	71.00

The data in Table V was obtained by fractionating the methyl esters from the "solid" acids.

TABLE V

Data Obtained from Distillation at 1 mm. of 20.0 Grams of Methyl Esters of "Solid" Acids.

"Solid" Acids Produced by Lead-Salt-Ether Method

Fraction	Temperature (°C.)	Weight of Fraction (in g.)	n_D^{25}	Saponification Equivalent	Iodine Value (Hanus)
1	121-124	0.2	1.4358	-	3.2
2	125-128	0.3	1.4365	-	0.7
3	129-139	4.1	Solid	270.9	1.7
4	140-143	4.5	Solid	275.3	0.3
5	144	3.3	Solid	280.2	0.0
6	145-150	1.2	1.4398	277.0	6.5
7	150-154	1.0	Solid	281.9	11.7
8	154-164	3.9	Solid	299.5	8.4
9	Residue	0.7	Solid	292.5	9.3

From the saponification equivalents, refractive indices, and Iodine Values shown in Table V the break-down of the various fractions given in table VI has been calculated.

TABLE VI

Ester Composition of Fractions Obtained from Distillation at 1 mm. of 20.3 grams of Methyl Esters of "Solid" Acids. "Solid" Acids Produced by Lead-Salt-Ether Method

Sample	Weight of Fraction (in g.)	Methyl myristate	Methyl hexadecenoate	Methyl palmitate	Methyl oleate	Methyl stearate
1	0.2	0.194	0.006			
2	0.3	0.298	0.002			
3	4.1		0.073	4.027		
4	4.5			3.645		0.855
5	3.3			2.079		1.221
6	1.2			0.972	0.091	0.137
7	1.0			0.575	0.063	0.367
8	3.9				0.390	3.510
9	0.7			0.140	0.061	0.499
TOTALS	19.2	0.492	0.081	11.438	0.605	6.589

The "solid" fatty acids fraction obtained by the lead-salt-ether method is 24.4 per cent of the total snake oil. On converting the values for the various methyl esters, as shown in Table VI, into the percentages of the original snake oil the following results are obtained:

TABLE VII

Percentages of Fatty Acids in the "Solid" Fraction

(Calculated from Table VI)

Name of Acid	Percentage
Myristic	0.62
Hexadecenoic	0.10
Palmitic	14.54
Oleic	0.77
Stearic	8.37
TOTAL	24.40

When the acid percentages from the "liquid" and "solid" fatty acids fractions were added the data given on the following page in Table VIII were obtained.

TABLE VIII

Percentage of Fatty Acids in Combined "Liquid" and "Solid" Fractions of Moccasin Snake Oil.

Fractions Obtained by the Lead-Salt-Ether Method

Name of Acid	Percentage
Myristic	1.77
Palmitic	15.93
Stearic	8.37
Hexadecenoic	6.53
Oleic	35.25
Linoleic	16.00
Arachidonic and Clupanodonic	10.97
TOTAL	94.82

Table III is based upon the distillation data shown in Tables II to VII, inclusive.

EXPERIMENTAL DATA — THE SATURATED FATTY ACIDS

The data obtained from the fractional distillation of the "solid" fraction of the moccasin snake oil is given in the section on distillation.

After separation of the "solid" fatty acids from the "liquid" fatty acids by use of the lead-salt-ether procedure, it was found that the Average Molecular Weight of the "solid" fatty acid fraction is 264.1, while that of the "liquid" fatty acid fraction is 290.

Before examining the data obtained from the lead-salt-ether separation, the sample was tested for possible oxidation of the unsaturated acids. The following data were obtained:

Iodine Number (Hanus), Original Moccasin Snake Oil...107.1

Iodine Number (Hanus), "Solid" Fraction..... 9.7

Iodine Number (Hanus), "Liquid" Fraction.....145.6

The combined Iodine Values of the "liquid" and "solid" fatty acid fractions account for 99.2 per cent of the unsaturation of the original snake oil.

Work with the "solid" acid fraction showed that it contained a high percentage of palmitic and stearic acids. Therefore, it was desirable to have a fraction which contained only palmitic and stearic acids. This purpose was achieved by use of a solution of methanol and water of a specific gravity of 0.911 at 0°C. The solution was saturated with palmitic and stearic acid, and to this saturated solution was added 0.9132 g. of the "solid" acids from moccasin snake oil. After the mixture had remained overnight in a Frigidaire, the precipitate was weighed; and a total of 0.8234 g. of fatty acids was obtained. Under the conditions of the experiment, only a mixture of palmitic and stearic acids should have precipitated.

The freezing point of the precipitate was determined to be 54°C. According to Ralston[96], the freezing point of a binary mixture of palmitic and stearic acids in which the mole fraction of palmitic is 0.7 and the mole fraction of stearic is 0.3, is 54°C. The average

molecular weight of Ralston's mixture was 265.6, while the actual average molecular weight of the precipitate was 265.2.

SPECTROPHOTOMETRIC DATA AND ITS EVALUATION

The spectrophotometer used was a Beckman Model DU with a No. 2501 Ultraviolet Accessory Set attached. The ultraviolet accessory set provided the necessary equipment for making measurements in the wave length region of 350 to 220 millimicrons.

The above apparatus was tested and runs were made to standardize and check it for efficiency of operation. For the standard methods of operation the reader is referred to the various Beckman Bulletins.

The procedure used in this phase of the research is one which was devised by Mitchell, Kraybill, and Zscheile[94] and was later elaborated upon by Beadle and Kraybill[95]. They report that it is possible to determine the percentage of linoleic, linolenic, and arachidonic acids by isomerizing the double bonds into conjugated positions in an alkaline solution of ethylene glycol.

The method used was as follows:

Between 0.1 and 0.2 g. of moccasin snake oil was accurately weighed and placed in the type of small vial used for Iodine Number determination. In a 15 x 2.5 cm. (6 x 1 in.) test tube were placed 10 ml. of alkaline glycol reagent. The test tube was covered loosely with a glass cap and was kept immersed at a constant depth in an oil bath at 180°C. When the temperature of the reagent in the test tube

had reached 180°C. the vial containing the moccasin snake oil was dropped into the tube. The test tube was swirled three times at one-minute intervals to mix the fat with the glycol solution.

After 25 minutes the tube was removed from the bath and was cooled rapidly under the tap. The contents of the tube were transferred quantitatively to a 250-ml. volumetric flask. Ethanol was used to wash out the tube, and the volume was diluted with 99 per cent ethanol.

The samples stood in a refrigerator overnight. The materials removed from the tube in the hot alkali solution had precipitated. The solution in the volumetric flask was brought to room temperature, and a portion of it was filtered. Proper dilution for absorption measurements were made with 99 per cent ethanol.

It was necessary to carry a blank solution of alkaline glycol throughout the whole procedure, including dilution, for use in the solvent cell.

PREPARATION OF THE ALKALINE GLYCOL SOLUTION

Potassium hydroxide (7.5 g. assaying 85 per cent) was added to 100 ml. of ethylene glycol, resulting in a 1.3 M potassium hydroxide solution. While preparing the reagent, the glycol solution was boiled in an Erlenmeyer flask until the temperature reached 190°C. Most of the water was removed by boiling, so that it was possible to maintain a temperature of 180°C. while the sample was being heated.

The formula used to determine specific alpha is:

$$\text{Specific alpha} = \frac{\log_{10} I_o/I}{c\,l}$$

Alpha = absorption coefficient

I_o = intensity of radiation transmitted by the solvent

I = intensity of radiation transmitted by the solution

c = concentration of solute in grams per 1000 ml.

l = length in centimeters of solution through which the radiation passes.

Table IX gives the standard values reported by Beadle and Kraybill[95] for the determination of fatty acid mixtures containing linoleic, linolenic, and arachidonic acids:

TABLE IX

Reference Values for Use in Spectrophotometric Analysis

Isomerized fatty acid soap	Specific Absorption Coefficient			
	2340 Å	2680 Å	3010 Å	3160 Å
Arachidonic	59.3	53.4	25.8	22.6
Linolenic	60.9	53.2		
Linoleic	86.0			

In order to evaluate the spectrophotometric data from the isomerized moccasin snake oil it was necessary to run a sample of the original oil. After investigation it was found that diethyl ether is a good solvent. In spectrophotometric work it is necessary to have a solvent which does not absorb ultraviolet light so that all absorption shown will be that of the compound to be examined. The original concentration was 10 grams of moccasin snake oil to which was added enough diethyl ether to make a total volume of 100 ml. This gives a 10 per cent solution of moccasin snake oil.

TABLE X

Spectrophotometric Data on Pure, Cold-pressed Moccasin Snake Oil

Wave Length in Angstroms	Alpha	Log $\frac{I_o}{I}$	Slit Width	Concentration	Log Specific Extinction coefficient
3500	0.00167	0.167	0.8 mm.	C[a]	- 2.7773
3400	0.00227	0.227	0.8 mm.	C	- 2.6440
3300	0.00326	0.326	0.8 mm.	C	- 2.4864
3200	0.00534	0.534	0.8 mm.	C	- 2.2733
3180	0.00586	0.586	0.8 mm.	C	- 2.2321
3150	0.00614	0.614	0.8 mm.	C	- 2.2118
3130	0.00610	0.610	0.8 mm.	C	- 2.2147
3120	0.00605	0.605	0.8 mm.	C	- 2.2147

[a] In the concentration column, C indicates 10 per cent moccasin snake oil-diethyl ether solution.

TABLE X (Continued)

Spectrophotometric Data on Pure, Cold-pressed Moccasin Snake Oil

Wave Length in Angstroms	Alpha	Log $\frac{I_o}{I}$	Slit Width	Concentration	Log Specific Extinction coefficient
3100	0.00617	0.617	0.8 mm.	C	- 2.2097
3050	0.00782	0.208	0.8 mm.	C/4	- 2.1040
3000	0.01050	0.262	0.8 mm.	C/4	- 1.9798
2950	0.01328	0.332	0.8 mm.	C/4	- 1.9721
2900	0.02005	0.501	0.8 mm.	C/4	- 1.6990
2850	0.03668	0.917	0.8 mm.	C/4	- 1.4318
2800	0.0512	0.321	0.8 mm.	C/16	- 1.2907
2750	0.0547	0.342	0.8 mm.	C/16	- 1.2620
2700	0.0685	0.427	0.8 mm.	C/16	- 1.1643
2680	0.0774	0.121	0.8 mm.	C/16	- 1.1113
2650	0.0665	0.104	0.8 mm.	C/64	- 1.1772
2600	0.0742	0.117	0.8 mm.	C/64	- 1.13
2550	0.1414	0.221	0.8 mm.	C/64	- 0.85
2500	0.4147	0.167	0.8 mm.	C/64	- 0.40
2450	0.888	0.347	0.8 mm.	C/256	- 0.05
2400	1.270	0.494	0.8 mm.	C/256	+ 0.10
2350	1.515	0.592	0.8 mm.	C/256	+ 0.18
2300	1.567	0.612	1.0 mm.	C/256	+ 0.20
2250	1.472	0.575	1.5 mm.	C/256	+ 0.17
2200	1.510	0.590	2.0 mm.	C/256	+ 0.179

In order to graph the data given in Table X it is necessary to use the Log Specific Extinction Coefficient. This makes it possible to place all of the graph on one curve. The Log Specific Extinction Coefficient is obtained by taking the Log to base 10 of the Alpha value in Table X. For this graph see Figure 1.

FIGURE 1. ULTRA-VIOLET ABSORPTION SPECTRA OF MOCCASIN SNAKE OIL IN DIETHYL ETHER.

DISCUSSION OF FIGURE 1

The absorption at 2340 Angstroms is caused by the diene conjugation. The alpha value is 1.525. If 100 per cent conjugated linoleic acid gives an alpha of 86.0, if 100 per cent conjugated linolenic acid gives an alpha of 60.9, and if 100 per cent conjugated arachidonic acid gives an alpha of 59.3, then it is possible to calculate the amount of conjugation present from the results found. If all the absorption at 2340 Angstroms is assumed to be linoleic, then there is present in the original moccasin snake oil 1.77 per cent of a conjugated linoleic acid. By the same procedure it was found that the percentage of conjugated trienes in the original moccasin snake oil was 0.15, and the percentage of conjugated tetraene present in the original moccasin snake oil was 0.04. The triene absorption shows at the 2680 Angstroms wave length. The tetraene absorption shows at the 3010 Angstroms and the 3160 Angstroms wave length.

DETERMINATION OF LINOLEIC, LINOLENIC, AND ARACHIDONIC ACIDS IN MOCCASIN SNAKE OIL BY SPECTROPHOTOMETRIC ANALYSIS

The snake oil used in this determination was isomerized in an alkaline glycol solution in accordance with the method of Mitchell, Kraybill, and Zscheile[94], which recommends a sample of from 0.1 to 0.2 gram. A sample of moccasin snake oil, 0.1355 gram, was made up to a volume of 100 ml. with 99 per cent ethanol, according to the procedure which was described in detail in the preceding section. This solution of 0.1355 gram of snake oil per 100 ml. is represented in the table by C in the concentration column.

A Beckman Spectrophotometer was used, and readings were recorded at intervals of 10 Angstroms throughout the range from 3500 to 2170 Angstroms. The results are reported in Table XI. Since the log alpha is the same as the log Specific Extinction Coefficient, the log alpha for each wave length was not tabulated.

TABLE XI

Determination of Linoleic, Linolenic, and Arachidonic Acids in Moccasin Snake Oil by Spectrophotometric Analysis

Wave Length in Angstroms	Alpha	Log $\frac{I_0}{I}$	Slit Width	Concentration	Log Specific Extinction Coefficient
3500	0.80	0.108	0.8 mm.	C/10	- 0.0969
3490	0.84	0.114	0.8 mm.	C/10	- 0.0757
3480	0.86	0.117	0.8 mm.	C/10	- 0.0655

TABLE XI (Continued)

Wave Length in Angstroms	Alpha	Log $\frac{I_o}{I}$	Slit Width	Concentration	Log Specific Extinction Coefficient
3470	0.86	0.117	0.8 mm.	C/10	- 0.0655
3460	0.84	0.114	0.8 mm.	C/10	- 0.0755
3450	0.80	0.108	0.8 mm.	C/10	- 0.0969
3440	0.75	0.102	0.8 mm.	C/10	- 0.1249
3430	0.70	0.094	0.8 mm.	C/10	- 0.1549
3420	0.64	0.087	0.8 mm.	C/10	- 0.1938
3410	0.61	0.083	0.8 mm.	C/10	- 0.2147
3400	0.61	0.082	0.8 mm.	C/10	- 0.2147
3390	0.61	0.083	0.8 mm.	C/10	- 0.2147
3380	0.64	0.087	0.8 mm.	C/10	- 0.1938
3370	0.68	0.092	0.8 mm.	C/10	- 0.1675
3360	0.73	0.099	0.8 mm.	C/10	- 0.1367
3350	0.79	0.107	0.8 mm.	C/10	- 0.1024
3340	0.86	0.116	0.8 mm.	C/10	- 0.0655
3330	0.93	0.126	0.8 mm.	C/10	- 0.0315
3320	1.02	0.138	0.8 mm.	C/10	- 0.0086
3310	1.04	0.141	0.8 mm.	C/10	- 0.0170
3300	1.06	0.144	0.8 mm.	C/10	+ 0.0253
3290	1.08	0.147	0.8 mm.	C/10	+ 0.0334
3280	1.10	0.149	0.8 mm.	C/10	+ 0.0414
3270	1.11	0.150	0.8 mm.	C/10	+ 0.0453

TABLE XI (Continued)

Wave Length in Angstroms	Alpha	Log $\frac{I_0}{I}$	Slit Width	Concentration	Log Specific Extinction Coefficient
3260	1.12	0.152	018 mm.	C/10	+ 0.0492
3250	1.16	0.157	0.8 mm.	C/10	+ 0.0645
3240	1.24	0.168	0.8 mm.	C/10	+ 0.0934
3230	1.34	0.182	0.8 mm.	C/10	+ 0.1271
3220	1.50	0.203	0.8 mm.	C/10	+ 0.1761
3210	1.70	0.232	0.8 mm.	C/10	+ 0.2304
3200	1.92	0.260	0.8 mm.	C/10	+ 0.2833
3190	2.15	0.292	0.8 mm.	C/10	+ 0.3324
3180	2.38	0.322	0.8 mm.	C/10	+ 0.3766
3170	2.53	0.343	0.8 mm.	C/10	+ 0.4031
3160	2.61	0.354	0.8 mm.	C/10	+ 0.4166
3150	2.60	0.353	0.8 mm.	C/10	+ 0.4150
3140	2.52	0.342	0.8 mm.	C/10	+ 0.4014
3130	2.39	0.323	0.8 mm.	C/10	+ 0.3784
3120	2.23	0.302	0.8 mm.	C/10	+ 0.3483
3110	2.08	0.282	0.8 mm.	C/10	+ 0.3181
3100	1.99	0.269	0.8 mm.	C/10	+ 0.2989
3090	1.94	0.263	0.8 mm.	C/10	+ 0.2878
3080	1.99	0.268	0.8 mm.	C/10	+ 0.2989
3070	2.08	0.282	0.8 mm.	C/10	+ 0.3181
3060	2.23	0.302	0.8 mm.	C/10	+ 0.3483

TABLE XI (Continued)

Wave Length in Angstroms	Alpha	Log $\frac{I_o}{I}$	Slit Width	Concentration	Log Specific Extinction Coefficient
3050	2.41	0.326	0.8 mm.	C/10	+ 0.3820
3040	2.59	0.352	0.8 mm.	C/10	+ 0.4133
3030	2.74	0.372	0.8 mm.	C/10	+ 0.4378
3020	2.86	0.388	0.8 mm.	C/10	+ 0.4564
3010	2.90	0.393	0.8 mm.	C/10	+ 0.4624
3000	2.85	0.386	0.8 mm.	C/10	+ 0.4548
2990	2.73	0.370	0.8 mm.	C/10	+ 0.4362
2980	2.60	0.353	0.8 mm.	C/10	+ 0.4150
2970	2.47	0.334	0.8 mm.	C/10	+ 0.3927
2960	2.38	0.322	0.8 mm.	C/10	+ 0.3766
2950	2.35	0.318	0.8 mm.	C/10	+ 0.3711
2940	2.42	0.328	0.8 mm.	C/10	+ 0.3838
2930	2.56	0.347	0.8 mm.	C/10	+ 0.4082
2920	2.77	0.376	0.8 mm.	C/10	+ 0.4425
2910	3.06	0.415	0.8 mm.	C/10	+ 0.4857
2900	3.36	0.455	0.8 mm.	C/10	+ 0.5263
2890	3.74	0.507	0.8 mm.	C/10	+ 0.5729
2880	4.15	0.563	0.8 mm.	C/10	+ 0.6180
2870	4.59	0.622	0.8 mm.	C/10	+ 0.6618
2860	4.00	0.677	0.8 mm.	C/10	+ 0.6990
2850	5.44	0.737	0.8 mm.	C/10	+ 0.7356

TABLE XI (Continued)

Wave Length in Angstroms	Alpha	Log $\frac{I_o}{I}$	Slit Width	Concentration	Log Specific Extinction Coefficient
2840	5.90	0.800	0.8 mm.	C/10	+0.7709
2830	6.32	0.856	0.8 mm.	C/10	+0.8007
2820	6.61	0.896	0.8 mm.	C/10	+0.8206
2810	6.94	0.940	0.8 mm.	C/10	+0.8414
2800	7.03	0.952	0.8 mm.	C/10	+0.8470
2790	7.03	0.952	0.8 mm.	C/10	+0.8470
2780	7.00	0.948	0.8 mm.	C/10	+0.8451
2770	6.96	0.943	0.8 mm.	C/10	+0.8426
2760	6.89	0.933	0.8 mm.	C/10	+0.8382
2750	6.96	0.943	0.8 mm.	C/10	+0.8426
2740	7.08	0.960	0.8 mm.	C/10	+0.8500
2730	7.36	0.997	0.8 mm.	C/10	+0.8669
2720	7.65	1.037	0.8 mm.	C/10	+0.8837
2710	7.88	1.068	0.8mm.	C/10	+0.8965
2700	8.34	1.130	0.8 mm.	C/10	+0.9212
2690	8.31	1.126	0.8 mm.	C/10	+0.9196
2680	8.18	1.108	0.8 mm.	C/10	+0.9128
2670	8.17	1.107	0.8 mm.	C/10	+0.9122
2660	7.55	1.023	0.8 mm.	C/10	+0.8779
2650	7.28	0.986	0.8 mm.	C/10	+0.8621
2640	6.92	0.938	0.8 mm.	C/10	+0.8401
2630	6.73	0.912	0.8 mm.	C/10	+0.8280

TABLE XI (Continued)

Wave Length in Angstroms	Alpha	Log $\frac{I_o}{I}$	Slit Width	Concentration	Log Specific Extinction Coefficient
2620	6.60	0.895	0.8 mm.	C/10	+ 0.8195
2610	6.61	0.896	0.8 mm.	C/10	+ 0.8202
2600	6.64	0.900	0.8 mm.	C/10	+ 0.8222
2590	6.60	0.895	0.8 mm.	C/10	+ 0.8195
2580	6.57	0.890	0.8 mm.	C/10	+ 0.8176
2570	6.46	0.875	0.8 mm.	C/10	+ 0.8102
2560	6.37	0.863	0.8 mm.	C/10	+ 0.8041
2550	6.31	0.855	0.8 mm.	C/10	+ 0.8000
2540	6.35	0.861	0.8 mm.	C/10	+ 0.8028
2530	6.57	0.892	0.8 mm.	C/10	+ 0.8176
2520	7.02	0.950	0.8 mm.	C/10	+ 0.8463
2510	7.64	1.030	0.8 mm.	C/10	+ 0.8831
2500	8.18	1.116	0.8 mm.	C/10	+ 0.9128
2490	9.74	0.132	0.8 mm.	C/100	+ 0.9886
2480	10.63	0.144	0.8 mm.	C/100	+ 1.0265
2470	11.66	0.158	0.8 mm.	C/100	+ 1.0668
2460	12.62	0.171	0.8 mm.	C/100	+ 1.1011
2450	13.43	0.182	0.8 mm.	C/100	+ 1.1281
2440	14.26	0.193	0.8 mm.	C/100	+ 1.1535
2430	14.91	0.202	0.8 mm.	C/100	+ 1.1735

TABLE XI (Continued)

Wave Length in Angstroms	Alpha	Log $\frac{I_o}{I}$	Slit Width	Concentration	Log Specific Extinction Coefficient
2420	15.64	0.212	0.8 mm.	C/100	+ 1.1942
2410	16.38	0.222	0.8 mm.	C/100	+ 1.2143
2400	17.12	0.232	0.8 mm.	C/100	+ 1.2335
2390	17.86	0.242	0.8 mm.	C/100	+ 1.2519
2380	18.45	0.250	0.8 mm.	C/100	+ 1.2660
2370	19.04	0.258	0.8 mm.	C/100	+ 1.2797
2360	19.50	0.264	0.8 mm.	C/100	+ 1.2900
2350	19.78	0.268	0.8 mm.	C/100	+ 1.2963
2340	20.00	0.271	0.8 mm.	C/100	+ 1.3010
2330	20.15	0.273	0.8 mm.	C/100	+ 1.3043
2320	20.22	0.274	0.8 mm.	C/100	+ 1.3058
2310	20.15	0.273	1.0 mm.	C/100	+ 1.3043
2300	19.78	0.268	1.0 mm.	C/100	+ 1.2963
2290	19.71	0.267	1.0 mm.	C/100	+ 1.2947
2280	19.04	0.258	1.0 mm.	C/100	+ 1.2797
2270	18.60	0.252	1.0 mm.	C/100	+ 1.2695
2260	18.30	0.248	1.5 mm.	C/100	+ 1.2625
2250	17.48	0.237	1.5 mm.	C/100	+ 1.2425
2240	16.83	0.228	1.5 mm.	C/100	+ 1.2261
2230	16.02	0.217	1.5 mm.	C/100	+ 1.2046

TABLE XI (Continued)

Wave Length in Angstroms	Alpha	Log $\frac{I_o}{I}$	Slit Width	Concentration	Log Specific Extinction Coefficient
2220	15.50	0.210	1.5 mm.	C/100	+1.1903
2210	14.98	0.203	1.5 mm.	C/100	+1.1756
2200	14.61	0.198	2.0 mm.	C/100	+1.1647
2190	13.95	0.189	2.0 mm.	C/100	+1.1446
2180	13.35	0.181	2.0 mm.	C/100	+1.1255
2170	12.77	0.173	2.0 mm.	C/100	+1.1062

Some of the data from Table XI is presented graphically in Figure 2.

The Specific Absorption Coefficients found from Table XI were as follows:

At 2340 Angstroms, the value obtained was 20.00,

At 2680 Angstroms, the value obtained was 8.18,

At 3010 Angstroms, the value obtained was 2.90,

At 3160 Angstroms, the value obtained was 2.61.

From Table IX it was found that the above values were equivalent to the following percentages of acids:

At 2340 Angstroms, 15.4 per cent of linoleic acid,

At 2680 Angstroms, 3.9 per cent of linolenic acid,

At 3010 Angstroms, 11.24 per cent of arachidonic acid,

At 3160 Angstroms, 11.57 per cent of arachidonic acid.

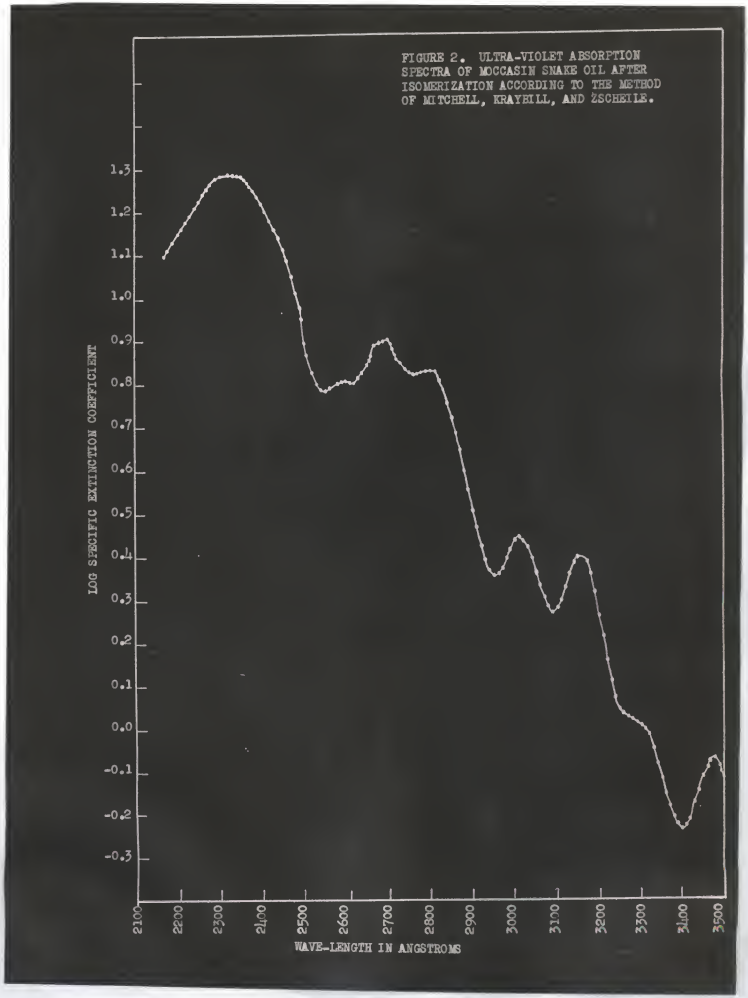
FIGURE 2. ULTRA-VIOLET ABSORPTION SPECTRA OF MOCCASIN SNAKE OIL AFTER ISOMERIZATION ACCORDING TO THE METHOD OF MITCHELL, KRAYHILL, AND ZSCHEILE.
LOG SPECIFIC EXTINCTION COEFFICIENT
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
2100
2200
2300
2400
2500
2600
2700
2800
2900
3000
3100
3200
3300
3400
3500
WAVE-LENGTH IN ANGSTROMS

An examination of the data indicates that the following values are more probably correct:

Linoleic acid..... 15.4 per cent

Arachidonic and clupanodonic acids.. 11.4 per cent

From spectrophotometric data for the range from 2400 to 2800 Angstroms it appeared that the absorption may have been caused by a vitamin or by vitamin-like substances. It is probable that this is the reason that Hilditch and Shrivastava[88] found too high a value for linolenic acid by the spectrophotometric method. Vitamin D, which contains three double bonds in conjugated position, would be expected to show up in this range on the spectrophotometer[97].

SUMMARY

The following qualitative and quantitative analyses of moccasin snake oil are based on research reported in this dissertation:

TABLE XII

Percentage of Fatty Acids in Moccasin Snake Oil

Name of Acid	Percentage
Myristic	1.77
Palmitic	15.93
Stearic	8.37
Hexadecenoic	6.53
Oleic	35.25
Linoleic	15.70
Arachidonic and Clupanodonic	11.18
TOTAL	94.73

Two partial tests of the reliability of this analysis are:

(1) The Iodine Value of the sum of fatty acids found as compared to the original moccasin snake oil, and

(2) The saponification equivalent of the sum of the fatty acids found as compared to the original moccasin snake oil.

Tables XIII and XIV give the results obtained.

TABLE XIII

Percentage Composition and Iodine Values of Fatty Acids of Moccasin Snake Oil

Name of Acid	Percentage of Total	Theoretical Iodine Value
Myristic	1.77	0.000
Palmitic	15.93	0.000
Stearic	8.37	0.000
Hexadecenoic	6.53	0.0653 gram
Oleic	35.25	0.317 gram
Linoleic	15.7	0.285 gram
Arachidonic and Clupanodonic	11.4	0.381 gram
TOTAL		1.048 grams

The total Iodine Value of the original moccasin snake oil was 1.071 grams, which is 0.023 gram greater than the total for the acids. These determinations were based upon a one-gram sample of the original moccasin snake oil.

TABLE XIV

Percentage Composition and Saponification Equivalents of the Fatty Acids of Moccasin Snake Oil

Name of Acid	Percentage of Total	Theoretical Saponification Equivalent
Myristic	1.77	4.4
Palmitic	15.93	34.9
Stearic	8.37	16.5
Hexadecenoic	6.53	14.4
Oleic	35.25	70.1
Linoleic	15.7	31.4
Arachidonic and Clupanodonic	11.4	21.0
TOTAL		192.7

The total saponification equivalent of the original moccasin snake oil was 192.6, which is 0.1 gram less than that of the acids. These calculations were based on a one-gram sample of the original moccasin snake oil.

CONCLUSIONS

Based upon the data shown in this dissertation, the following conclusions are indicated:

(1) Highly unsaturated fatty acids are not easily distilled, even at pressures as low as 0.1 mm. to 0.2 mm.

(2) In spectrophotometric determination of fatty acids extreme care must be taken to evaluate the data obtained.

(3) In any determination of fatty acids in moccasin snake oil, no single method is absolutely reliable.

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This dissertation was prepared under the direction of the Chairman of the candidate's Supervisory Committee and has been approved by all members of the Committee. It was submitted to the Graduate Council and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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