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An analysis of the oil of *echinocystis fabacea* seeds

Francis Warren Sayre
University of the Pacific

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AN ANALYSIS OF THE OIL
OF
ECHINO CYSTIS FABACEA SEEDS

A Thesis
Presented to
the Faculty of the Department of Chemistry
College of the Pacific

In Partial Fulfillment
of the Requirements for the Degree
Master of Arts

By
Francis Warren Sayre
June 1951

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AN ANALYSIS OF THE OIL
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FORWARD

Echinocystis fabacea, commonly called "Man Root" because of the peculiar shape of the root, not unlike that of a man, finds abundant occurrence in the central valleys of California. This plant, a member of the wild cucumber family, is of particular interest because it is an oilseed plant. The variety *agrestis* is especially common around Stockton. Other varieties are found throughout the Sacramento and San Joaquin Valleys and in the Coast Range Mountains.

Oils have played an important part throughout history and are continuing to do so in our present economy. An investigation of the oils of *Echinocystis fabacea* seeds proved to be of considerable interest.

An extensive search of the literature revealed that only a slight amount of work has been done on these oils. M. B. Daughters made a very brief study of them in 1918.

This paper will discuss several of the analytical methods employed in the analysis of fats and oils and

evaluate them in terms of their applicability to the oils of wild cucumber seeds.

USAGE OF FATS AND OILS

Man has been familiar with the fats and oils since prehistoric time. Long before he understood the nature of them he recognized differences in their properties and behavior under different environmental conditions. When and how man first became familiar with fats and oils and their manifold uses is lost in antiquity, but it is known that they were widely used among primitive peoples as medicinals, in cosmetics, in religious ceremonies, as illuminants, fuels, lubricants, and for many other purposes.

Klemgard (1937) mentions the fact that the Egyptians used olive oil as a lubricant for moving large stones, statues, and building materials, and that axle greases consisting of a fat and lime, together with other materials, were used in lubricating Egyptian chariots as early as 1400 B. C. Sarton's Introduction to the History of Science (1927) contains many references to the use of fats and oils in art, technology, and medicine from the beginning of the Greek and Hebrew knowledge (ninth and eighth centuries, B. C.) to the middle ages. Candles made from beeswax and tallow were used by the Romans.

Waxes, which are fatty acid esters of the higher alcohols, were used by early shipbuilders as protective coatings. They have also been found in early Egyptian mummy cases and wall paintings. Natural pigments were used in

combinations with the waxes in many of the early art forms. Tempra, one of these early art forms, employed an emulsion of wax or oil, water, pigments, and some emulsifying agents such as vegetable gum or egg yolk.

The paint industry had its beginning in early Egyptian history when varnishes were first employed on mummy cases. Many of these varnishes are insoluble and their method of preparation is still unknown. Others are known to have been made from nut oils. Transparent varnish recipes dating from the eighth and ninth centuries B.C. are known to contain linseed oil in combination with natural resins. Thus one of our most important modern industries had its beginning with the oils and waxes several thousand years ago.

The common expression "pour oil on troubled waters" had its origin with the Greek sailors who used oil for subduing waves during a storm.

From the accrued evidence we can see that the peoples of all early civilization were acquainted with the fats and oils and used them for a great number of different purposes. It is of interest to note that today the primary applications of these substances is much the same as those which prevailed in antiquity. In addition to the very old uses we have added new ones. Through the methods of modern technology, fats are now being used in the production of explosives, synthetic resins, fibers and rubbers, flootation

agents for separation of mineral material from ores, de-emulsifiers in the petroleum industry, insecticides, weed killers, and many other products and processes. With each year industry is finding new and diverse uses for the fats and oils. The increased use of such substances has sprung primarily from the increased knowledge of the nature and structure of these and related materials.

The discovery of glycerol in 1779 when Scheele heated olive oil with litharge was the beginning of modern fat chemistry. The next major step in the understanding of fats and oils was the discovery, by Chevreul (1813-1823) that fats were actually the fatty acid esters of glycerol, and that soaps were the metallic salts of these fatty acids. Prior to this time soaps had been used for thousands of years both as cleansing agents and as medicinals, but the chemical nature of these substances was entirely unknown. After these two important steps in the evolution of fat chemistry, comparatively little was done in the field up until the beginning of the present century, even though the nineteenth century has been classified as the golden age of organic chemistry.

The reason for the lag in fat chemistry is understandable. Man had known about the fats and used them since the days of antiquity. A great many industries were built around them on a purely empirical basis. There were no troublesome

excesses of fats so that they did not accumulate, hence there was no special need for the study of them as was the case with the coal tar industries. They presented no serious disposal problems, nor were they a cheap source of raw material.

From an academic standpoint the fats were not attractive substances to deal with because of the difficulty of separating the individual fatty acids and the difficulty of preparing crystalline derivatives once they were separated. They were considered to be merely simple esters of glycerol, or at best mixtures of triglycerides, and were thought of as possessing very few definite physical or chemical properties by which they could be classified. They were incapable of separation into definite chemical entities by any of the regular methods such as crystallization or distillation. At this period the petroleum industry was still in its infancy and very little was known about the chemistry of high molecular weight polymers. The development of the petroleum industry was accompanied by the development of numerous new products from the hydrocarbons and also from simple organic molecules. This perhaps was the greatest single factor that caused the organic chemist to turn to the natural fats and their derived fatty acids for the development of similar products. Whether this was the reason or not is difficult to say, but during the twenties

a new emphasis began to be placed on lipid chemistry. Since that time research along these lines has been increasing steadily. A number of scientific journals have appeared dealing solely with this branch of chemistry. With the increased research and interest in this area it was soon realized that the chemistry of the fats and fatty acids was not as simple as had been previously thought. It became necessary to revise a number of old ideas concerning the nature and reactivity of the substances in this field.

OCCURRENCE OF THE FATS AND OILS

The fats and oils are found almost universally distributed in plant and animal life. In the animal body fats are stored in the fatty tissues which, except for the water present, are almost pure fat. Since fats give about two and one-fourth times as much energy as either proteins or carbohydrates, they are the most economical way, as far as weight goes, of storing energy. They permit the body to maintain a considerable amount of reserve energy with a minimum of weight. Fats serve also as heat insulators in the animals since they are poor conductors of heat. Biosynthesis of fats from carbohydrates during the ripening of the seeds and the reverse process during germination have been demonstrated in plants (Du Sablon, Ivanov, and others). This would indicate that the function of the fats and oils in

plants is one of energy storage very similar to that in animals. The term fats is generally applied to substances which are solid at 20°C., and oils to those which are liquid at the same temperature.

The term "fats" is usually used to signify triglycerides of fatty acids, however in the plant kingdom another very important group of closely related compounds, the waxes, is found. Waxes differ from the fats in that they are fatty acid esters of the higher alcohols. They serve important protective functions to the plants. The familiar carnuba wax comes from the leaves of the carnuba palm in Brazil and is used extensively in waxes and polishes. Many fruits such as apples and citrus fruits have wax coatings that protect them from drying out and from organisms causing rot. Wax coatings protect many plants against disease producing fungi and bacteria.

SIGNIFICANCE OF FATS AND OILS

Oils and the products of oils play an extremely important role in our national economy. The known fats and oils of economic and industrial importance probably do not exceed a few hundred, though many more have been examined more or less superficially and have so far been of academic interest only. Due to the widespread distribution of the fats and oils, they offer an almost inexhaustible supply of

long chain aliphatic compounds comparable only to petroleum. Petroleum has had the advantage over the natural fats up to the present time due to its relative cheapness, but once petroleum is withdrawn from the earth, it is non-replaceable. The natural fats have the advantage that there is a continuous perennial supply of them and that they are more reactive chemically. As the supply of petroleum shrinks, the price can be expected to rise considerably. Such a turn of events will undoubtedly enable the natural fats and oils to compete successfully with petroleum products. They have proved superior in many respects for lubricating oils. Castor oil and sperm oil were used almost exclusively for the breaking in of airplane motors during the second world war.

We were by no means the only nation to recognize the value of the natural lipids during wartime. In French West Africa all Moroccan subjects were required to cultivate ten percent of their land in oilseeds such as linseed, sunflower, castor bean, colza, sesame, or peanut, provided that they farmed more than five hectares, about 12.3 acres (Foreign Commerce Weekly, September 5, 1943). Long before the war actually started, Germany had realized the need for fats and oils and had reopened the Antarctic whaling fleet after a lapse of seventy years (Foreign Commerce Weekly, May 29, 1943). Whale oil may be stored safely longer than most fats

and oils. The German oil supply was considerably bolstered with the seizure of the rich oilseed producing area of the Ukraine, the whale and fish oil in Norway, and the large stocks of vegetable oil in the crushing mills of the Scandinavian and low countries. Many other nations were known to be growing oilseed crops for the oil which could be extracted or expressed from them.

Our country turned to the natural fats and oils for a great many different uses during the period that it was impossible to get enough of these materials from existing sources. Sperm oil was widely used in production as a lubricant for breaking in motors, making machine tools, rifling guns, tanning leathers, and as a finishing agent in textile manufacture. Castor oil was dehydrated to replace the unavailable tung oil from China and perilla oil from Manchuria, both of which were used extensively in the paint and finishing industries. With the supply of these materials cut off, it became imperative that new materials be developed to replace them. At that time dehydrated castor oil was found to be the best substitute for tung oil.

The figures for the production of soy bean oil during this period are indicative of the increased reliance upon the natural plant products for oil in the United States. Production of soy beans increased from less than five million bushels in 1925 to almost two hundred million bushels in 1943,

and during the same period the production of soybean oil increased from about two and one half million pounds to around a billion and a quarter pounds (Markey, 1947). Corresponding increases in the production of various other seed-oils were shown.

The tremendous demand for glycerine forced us to turn to the fats and oils as a source of this critical material. Existing production was entirely inadequate to meet the increasing demands for glycerine in the production of munitions. The problem became so serious that all waste fats, including kitchen fats, were collected in order that the glycerine could be recovered from them.

In view of the demonstrated ability of man to expand the production of the oilbearing crops in many parts of the world to supply almost any conceivable demand, and of the dwindling supply of irreplaceable petroleum raw materials, it seems natural that it will be but a matter of time until industry will look toward the perennial oil crops as a source of long chain carbon compounds, both saturated and unsaturated. These compounds may be broken down by pyrolysis or other processes to give shorter chain compounds, or they may be polymerized and condensed to give longer ones. The diversity and number of products that can be built up from these sources appears to rival or surpass anything that the petroleum industry has ever done. The potentialities are limited only by the lack of research in this field.

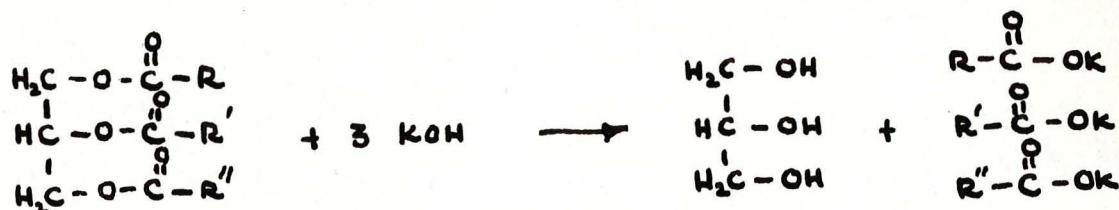
CHEMICAL NATURE OF FATS AND OILS

Fats and oils, like other esters, can be readily hydrolyzed, usually by aqueous alkali, in which case the products are glycerol and the alkali salts of the fatty acids which are known as soaps. The free fatty acids may be liberated by dilute mineral acid and subsequently extracted with ether and further purified by fractional crystallization, esterification and distillation, or by carrier displacement separation (Holman, 1951). The glycerol remains in the aqueous layer. This process of hydrolysis by alkali is known as saponification.

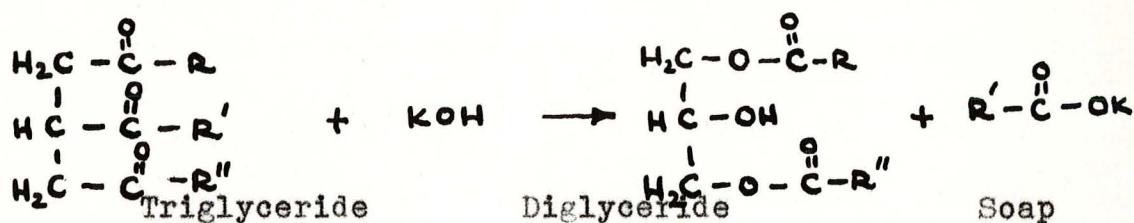
Saponification is a very old process and was employed by the ancients in the production of detergents as has been mentioned previously. There is evidence to indicate that soaps were used by the Romans. Pliny has described their production by the Gauls who heated goat tallow with wood ashes, the action being the hydrolysis of the fats by potassium carbonate from the wood ash. Soap boiling has been a common practice in Germany since the ninth century. Even though the process of soap production was widely utilized, it was not until Scheele's discovery of glycerol that the nature of the process involved was at all understood. Chevreul's work explained that the soaps were actually the metallic salts of the fatty acids. The work of these two men laid the foundation for later investigation to be carried on.

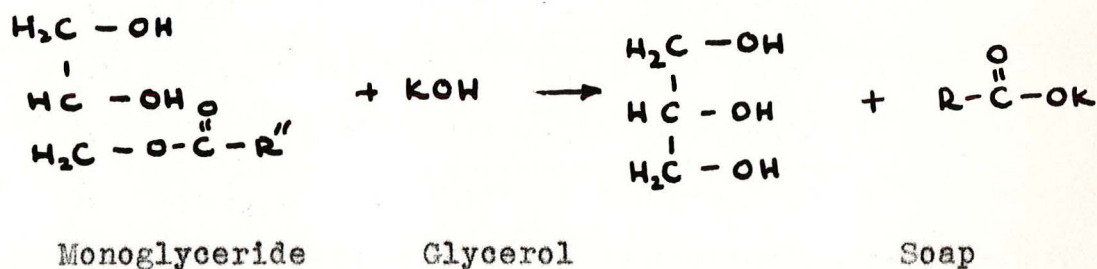
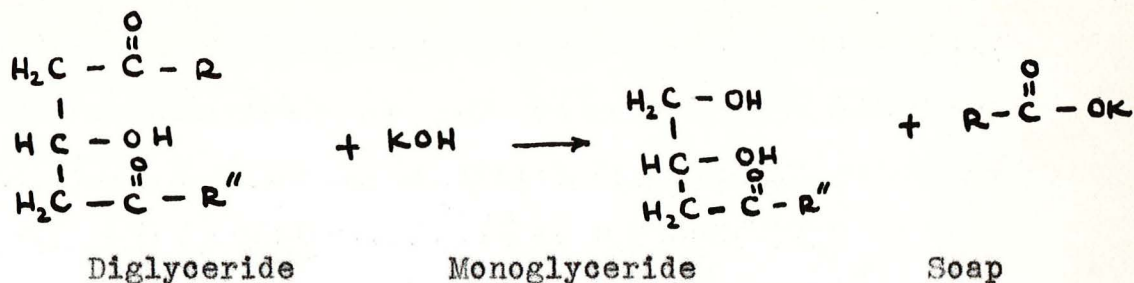
The hydrolysis of fats has been affected in several ways, but basically they can all be broken down into four general procedures, acid hydrolysis, basic hydrolysis, direct hydrolysis by live steam, and enzymatic hydrolysis. Fundamentally these reactions do not vary as the actual hydrolyzing agent is probably water in all cases.

The actual mechanism involved in hydrolysis of fats has been the subject of much debate. There are two possible methods by which this can be accomplished. The triglyceride could be broken all at once or it could be broken in a step-wise manner with the intermediate formation of a diglyceride and a mono-glyceride, prior to the formation of glycerol and the soap. The two mechanisms can be represented in the following manner.



Simultaneous removal of fatty acids





There appears to be little doubt at the present time that the stepwise procedure is the one followed. The work of Geitel (1897), Lewkowitsch (1898), Balbiano (1902), and Kremann (1906) all tend to support the stepwise theory. Studies by Meyer (1911) further substantiate the stepwise theory. It is probable that the reactions follow similar paths, irrespective of whether they are conducted in the presence of excess acids or bases, or catalytic amounts of these substances. Despite considerable investigation, the exact mechanism of saponification and other hydrolytic actions of the glycerides still remains unknown. The results of Rowe (1933) upon saponification in the presence of varying amounts of alkali indicate that the alkali plays a

catalytic role in the hydrolysis of the ester. In this case the basic reaction would be between the fat and water. When the reaction is carried out in an alcoholic caustic solution, the reaction proceeds much more rapidly than it does in aqueous medium, probably owing to the increased solubility of the fat in alcohol. Fundamentally the solvent should not alter the reaction, however it has been shown by Kurz (1937) that when the reaction is carried out in alcohol the rate is greatly increased if there is water present. This observation was verified in our own research.

Upon liberation of the free fatty acids, a mixture of all those contained in the original glyceride is obtained. The problem of separating these is one of considerable magnitude and complexity.

PREPARATION OF THE OIL

The oil was prepared by solvent extraction of the seeds of *Echinocystis fabacea*, a perennial from a large fusiform root usually located ten and a half to twelve inches below the surface of the ground. The roots vary in size from about the size of a small carrot when young, to very large as they approach maturity. One root dug by Dr. Stocking of the Botany Department, College of the Pacific, near the College weighed 149 pounds. A considerably smaller one dug along the Calaveras River, near the College, weighed fifteen

and one-fourth pounds. The plant has small white flowers with yellow centers which precede the fruit, a small spiny basket about two inches in diameter at maturity, and containing usually four seeds. Other varieties of *Echinocystis*, or *Marah* as it is being called in the more recent literature, have more seeds in the fruit (Jepson). As the seeds are very rich in oils, rodents feed on them. In order to collect seeds for investigation it is necessary to bag them or select an area where the rodents are controlled.

Extraction of the ground seeds was carried out in a Soxhlet extractor by the use of a solvent. Alcohol was found to give a fair extraction, however ether was found to be a much more satisfactory solvent. Prior to extraction the whole seeds were ground through a sixty mesh screen in an Arthur Thomas laboratory mill. The ground seed was placed in the extraction thimble and extraction was allowed to continue for at least eight hours of reflux time. This was found to be the optimum time for complete extraction under the conditions employed.

The ether extract was vacuum filtered through a Whatman number five filter paper in a Buchner funnel to remove any loose material that happened to get into the solvent during extraction. It was found that this step simplified the subsequent purification considerably. The ether solution was refiltered, this time through a piece of absorbent

cotton in a small funnel to remove the water present. The method proved very effective. The solution was then transferred to a distilling flask and the ether distilled. The last traces of ether were driven off by heating on a water bath after transfer to a 250 ml. beaker. The above procedure gave a fairly pure product. Individual yields varied from twenty-six per cent to thirty-two per cent with an average value of about thirty per cent. This is considerably higher than the yields from several oilseed plants that are grown commercially for their oils (Bulletin, Agricultural Extension Service, University of California).

ANALYSIS OF THE OIL

The highly complex nature of the naturally occurring fat mixtures makes necessary a number of physical and chemical tests in order to gain approximate ideas about their composition. Physical constants that are of value in ascertaining the composition of fats and oils are the melting point, boiling point, refractive index, absorption spectra, specific gravity, and viscosity. Though it is often impossible to get one or two of these, the ones that can be determined give valuable indications as to the nature of the individual components of the mixture. Chemical tests of value include saponification equivalent, iodine number, acid number, and other tests that have been applied to the

fats for years. While many of these values have little quantitative significance, they are indicative of the characteristics of the mixture. Many of these tests are also applicable to the separated fractions.

Physical examination of the oil yielded the following results: It had a reddish brown color with a green bloom, characteristic of many oils. It possessed a distinctive, rather pleasant odor and taste. Physical measurements gave the following results:

$$n_{20^{\circ}\text{C}} = 1.4695$$

$$d_{20^{\circ}} = 0.9074 \text{ (Westphal balance)}$$

There was no optical rotation.

Boiling point - unobtainable due to decomposition.

It was readily shown that the oil was a triglyceride by heating a small amount of it with potassium bisulfate. Upon fusion with this chemical, glycerol esters are broken down to give acrolein, a substance with a pungent disagreeable odor that is unmistakable.

The acrolein formed in this reaction is the result of dehydration of some of the glycerol released by the high temperature hydrolysis of fats. Glycerol is readily extracted from the saponification mixtures. When the free fatty acids are extracted with ether, the glycerol remains in the aqueous layer because of its highly polar nature. If the water is removed by distillation glycerine remains. This was a very important method of preparing technical glycerine during the last world war.

The saponification equivalent provides a measure of the relative size of the molecule. Echinocystis oil was found to have a saponification equivalent of 278 by the diethylene glycol method (Chriner, Fuson). Work of Hedemann and Lucas showed that saponification of most esters proceeds almost instantaneously and difficult ones in three minutes in alkaline diethylene glycol (Markley, 1949, p. 573). Most of the literature values report this determination in terms of saponification number which is the number of milligrams of potassium hydroxide that are required to completely saponify one gram of the oil. The saponification number was calculated from these data to be 202. This value compares favorably with the value reported by M. R. Daughters in 1918. Daughter's work was done on Echinocystis oregana, another variety of the same plant, which he found to have a saponification number of 193.

The saponification equivalent enables calculation of the average molecular weight of the individual fatty acids comprising the mixture in the oil. Since the oil is a triglyceride the molecular weight will be three times the equivalent weight, or 834. The average molecular weight of the individual fatty acids was determined to be approximately 263 in the following manner:

M. Weight Oil Molecule	=	834	
M. Wt (Glycerol)	=	<u>92.09</u>	
		742	= Wt. fatty acids -1 OH group each
		<u>51</u>	= 3 x 17 (for 3 OH groups)
		793	
		<u>-3</u>	= 3 x 1 for each H of glycerol removed during esterification
		790	= Sum of Molecular Weights of the fatty acids

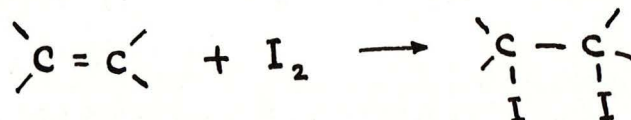
$$790 \times \frac{1}{3} = 263 \text{ (approx) = Average Molecular Weight of fatty acids}$$

The iodine number provides a measure of the unsaturated acid content of the material. The iodine number is the number of grams of iodine that will combine with one hundred grams of fat. Hanus iodine solution was used in the determination of the iodine number. The method employs iodine monobromide in glacial acetic acid at room temperature for thirty minutes. After the time has elapsed, potassium iodide is added to remove the bromine from solution as bromine replaces iodine from its salts. The free iodine is then titrated with standard sodium thiosulfate solution,

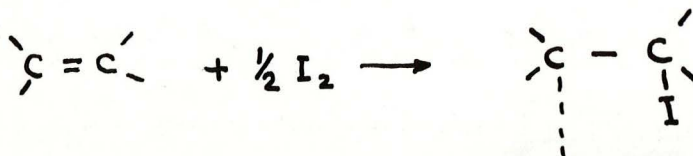
using starch as an indicator. The amount of halogen added in the original solution and the amount remaining in the final solution being known, the amount absorbed by the weighed sample of oil may be calculated. Both iodine and bromine may be absorbed by the oil, however the results are expressed as the number of grams of iodine absorbed by one hundred grams of oil. The oil was found to have an iodine number of 108. This value is a little lower than that reported by M. E. Daughters (116), however it should be noted that neither the variety nor the climatic conditions were the same in the two researches. It is well known that iodine values of oils derived from the same plant species vary widely with climatic conditions. A striking example of this is evidenced in the following comparison; a sample of linseed oil grown in the cold climate of Switzerland had an iodine value of 190, whereas the same stock grown in a Berlin greenhouse at a temperature of 25-30°C. showed an iodine value of 93 (Fieser & Fieser, 1951). Other examples confirming the climatic effect upon degree of unsaturation can be readily cited.

Further consideration of the iodine number and the molecular weight indicate that the molecule will add an average of seven equivalents of iodine per mole of oil. This does not necessarily mean that each mole of glyceride will add seven equivalents, but it is an average value in-

dicating that one mole probably adds six equivalents and another eight, so that the overall average is seven. This appears reasonable as the structure of the double bond is such that it should add an even number of halogens per mole.



Addition of 2 halogens



Addition of a single halogen

This is an extremely unlikely structure

It is safe to assume that an even number of halogens add to each unsaturation.

A smear of the oil was made on a microscope slide to determine whether or not the oil was a drying oil. The formation of a film would be noted if it were. After five days no film was formed, hence it was concluded that the oil was not a drying oil. The iodine number indicated that there was a considerable amount of unsaturation though not enough to make it a drying oil. Drying oils are generally of a conjugated nature and have considerably higher iodine numbers.

The acid number tells the amount of free acid found in an oil and is determined by titrating a weighed sample of

it in a solvent against 0.1 N potassium hydroxide in neutral alcohol. Results are expressed as the number of milligrams of potassium hydroxide required to neutralize the free fatty acids in one gram of fat. A high acid number would be expected in a rancid fat due to decomposition. The acid number of Echinocystis oil was found to be less than two milligrams of potassium hydroxide. This is a fairly low value and indicates that there is not much free fatty acid present. Acid numbers range from 0.25 for beef tallow to 59.8 per cent for wool fat (Anderson, 1947).

The next step after the analysis of the oil in its natural state is to break it down chemically and study its component parts. Several qualitative tests were run in order that an idea might be gained of the types of fatty acids that could be present. The oil was tested for hydroxyl groups by adding a ferric chloride solution to an ether solution of oil. The presence of hydroxy acids would cause the solution to turn a deep brown. A negative test indicated the absence of hydroxy groups. A confirmatory test using ferric ammonium nitrate, which should give a dark color in the presence of hydroxyl groups also showed negative results. This rules out the presence of ricinoleic acid or any other hydroxy acid. This also helps to explain why there is no optical rotation. Should hydroxy acids be present, they can be quantitatively estimated by determining their acetyl

number. In determining this value the fat or oil is first treated with acetic anhydride, which reacts with each hydroxyl group, introducing an acetyl group. A weighed sample of the acetylated fat is then saponified with alcoholic potassium hydroxide and the fatty acids are liberated from their soaps by adding mineral acid equivalent to the amount of potassium hydroxide used in saponification. Acetic acid is a soluble fatty acid and can be separated from the rest by filtration and determined by titration with standard alkali. In this determination a blank must be run on the unacetylated fat since most fats contain some soluble fatty acids other than acetic. The acetyl number is defined as the number of milligrams of potassium hydroxide required to neutralize the acetic acid obtained by saponifying one gram of acetylated fat. Most fats and oils have a low acetyl number, averaging between three and fifteen. Castor oil which is high in ricinoleic acid, has a value of about 150 (Anderson, 1947).

Isolation and identification of the component fatty acids comprising a natural fat or wax is generally a difficult process, particularly if it is to be done quantitatively. Fats and oils are of all degrees of complexity and may be composed of fatty acids of widely differing properties, e.g. they must comprise readily volatile and highly nonvolatile acids, or completely saturated and highly unsaturated acids.

On the other hand they may comprise a homologous series and differ only slightly from member to member in chemical and physical properties, thus affording no ready means of sharp separation or of preparing characteristic derivatives unequivocally differentiating them from the neighboring members of the series.

Due to the complexity of the mixture of the fatty acids occurring in the natural fats and oils, no single method is capable of effecting a separation, but a method or combination of methods must be adapted to the purpose for which separation is being made. Often it is impossible to effect complete separation and identification. The methods available are the culmination of a long period of time and at the present are more reliable than at any previous time, however they still leave much to be desired in the way of simplicity and accuracy.

The available techniques may be divided into three general types, distillation, solubility separation, and adsorption of fatty acids, *per se*, or of their methyl or ethyl esters. Recently a great deal of work has been done on carrier displacement separation of the fatty acids (Holman, R. T. 1951).

.....The most satisfactory system of carriers has been found to be the methyl esters of the fatty acids themselves. With a dilute solution of the displacer substance as solvent for all operations, it has been found that recoveries

of 5-15 milligram quantities of lauric, myristic, palmitic, and stearic acids approach 100 per cent. This type of chromatographic separation thus offers the possibility of a clear cut separation and determination of small quantities of these acids, and it seems likely that carrier displacement can be applied to other substances.

A number of methods are available for characterization of a particular fatty acid after its separation from the mixture of acids. These methods are: 1. determination of the physical properties such as boiling point, refractive index, density, specific gravity, optical rotation, etc.; 2. determination of chemical properties; 3. preparation of a characteristic derivative and determination of its properties.

A satisfactory derivative should be a well defined, easily prepared crystalline solid. In addition its melting point should differ sufficiently from closely allied compounds that it can be used as a basis for distinguishing between them and its mixed melting point should be decidedly different than the pure derivative. The problem of finding satisfactory derivatives for the lower members of the saturated series has not been particularly difficult, but that of finding satisfactory derivatives for the higher ones has proved exceedingly difficult. Many of them are so closely related that, in spite of the fact that a number of derivatives have been proposed, few, if any, have proved satisfactory. The same problem has been encountered in the pre-

paration of derivatives of the higher unsaturated fatty acids and attempts to find characterizing derivatives have proved even more futile. Amides, anilides, p-toluidides, o-toluidides, o-bromo toluidides, p-bromoanilides, 2,4,6, tribromoanilides, and several others have been proposed, however the same difficulties have been found in all of them (Ralston, 1947). Firstly they do not differ by systematic increments in melting points as a homologous series is ascended and the melting points are entirely unpredictable on a structural basis in most of them. Secondly many of the series have the same melting point as that of the compound above or below it, thus affording no means of identification. Many other derivatives have been tried with varying degrees of success, however none have been found that are entirely satisfactory. One of the best is the 4,4'-diaminodiphenylmethane derivative which was proposed in 1939 by Ralston and McCorkle. The diamides are prepared by heating one part of 4,4'-diaminodiphenylmethane with slightly more than two parts of the acids. Although the lower acids require refluxing, a few minutes heating is sufficient to prepare the diamides of the higher acids. The products are crystallized from a mixture of benzene and 1-butanol. The diamides are easily prepared and purified and have proved to be reasonably satisfactory derivatives, according to the authors.

Saponification is a valuable tool in the analysis of fats as it gives the alkali salts of the free fatty acids and glycerol. It is then possible to prepare the free fatty acid mixture simply by acidification of the saponification mixture and extraction of the fatty acids with ether. In the laboratory saponification was effected by using an isopropanol solution of potassium hydroxide and a few milliliters of water. Water hastens the saponification process if it is present in small amounts (Markley, 1947). The mixture was allowed to stand overnight after refluxing for three hours.

The unsaponifiable matter is the part of a fat or oil which is insoluble or incapable of forming a soluble soap with alkalies. Pure edible fats and oils usually contain only a very small amount, one to two per cent, of unsaponified matter. If hot water is added after the fat has been saponified, the unsaponifiable matter appears as oily drops or as a milkiness. It can then be extracted with petroleum ether. Echinocystis oil was found to have almost no unsaponifiable matter. This is an important component as it has been shown that certain fat soluble vitamins and sterols are important constituents of the unsaponifiable matter.

The free fatty acids were precipitated from the hydrolysis mixture by acidification with dilute sulfuric acid. They behaved very much like butter upon separation. The fatty

acids were repeatedly washed with distilled water until all traces of acidity were removed from the washings, and were then air dried.

An attempt was made to separate the mixture of free fatty acids by fractional crystallization at low temperatures. The mixture of fatty acids was dissolved in acetone warmed to a temperature of 25-30°C. As the solution cooled to room temperature the first fraction separated out and was filtered off. The filtrate was further cooled in an acetone and solid carbon dioxide bath (temperature about -78°C.) and at about -39°C. a second portion was removed. A third fraction separated out at -60°C. Filtrations were made through a cold filter constructed by placing a small funnel within a larger funnel, the small one being held in place by a cork which also served to retain the cold medium around the inner funnel. The method proved very successful for cold filtration. In this manner a crude separation of the fatty acids was effected. An attempt was made to determine the fatty acids present in each of the fractions separated by fractional crystallization without further purification.

The first fraction (removed from acetone at room temperature) was found to have an equivalent weight of 313 when titrated with 0.1 N potassium hydroxide in alcohol using ether as a solvent for the fatty acid and titrating to a phenolphthalein endpoint. Since it was a solid at room

temperature it was assumed to be a saturated fatty acid. Arachidic acid ($C_{19}H_{39}COOH$) has a molecular weight of 312.52, a value very close to that found here. It is quite probable that arachidic acid is present as it is found in many seed oils, though usually in small quantities. Arachidic acid is found also in peanut oil (3.3%), the amount varying slightly with the climatic conditions under which the peanuts are grown.

The second fraction, separated at $-59^{\circ}C.$, when titrated in the same manner gave an equivalent weight of 291. It was suspected that this acid was steric acid, the molecular weight of which is 284.5. One explanation of the high value could be that the stearic acid contained some arachidic acid as the two are quite closely related structurally, and hence difficult to separate. This would lead to an apparent increase in the equivalent weight of the stearic acid.

The third fraction separated at a temperature below $-60^{\circ}C.$ was found to have an equivalent weight of 280 and an iodine number of 173. This is probably linoleic acid which has a molecular weight of 280 and a theoretical iodine number of 181.3.

An attempt was made to separate the solid from the unsaturated fatty acids by the lead salt differential solubility method. The theory behind this separation is that the lead salts of the saturated fatty acids are insoluble.

in ether while those of the unsaturated acids are soluble. (Lewkowitsch, 1922, page 557). Results of the lead salt separation showed 9.5 per cent saturated fatty acids and 73.8 per cent unsaturated acids. These values were based on the original amount of oil taken before saponification, hence are somewhat lower than would have been obtained if the mixed fatty acids had been used as a starting point. The latter case would have yielded 11.5 per cent saturated and 88.5 per cent unsaturated acids. This result may be slightly low for the saturated acids and high for the unsaturated ones as the method is not quantitative due to the fact that the lead salts of some of the saturated fatty acids are slightly soluble in ether. These values must then be accepted as indications rather than as absolute. The results do indicate that there is a considerably higher content of unsaturated fatty acids than of saturated present in the oil.

The above analyses indicate that there is arachidic acid (312.52), stearic acid (284.5), and linoleic acid (280.4) present. These are all considerably higher than the average molecular weight of the fatty acids as determined by the saponification equivalent. This means that there must be a considerable portion of lower fatty acids which have not as yet been accounted for to balance the high molecular weight fatty acids believed to be present.

Another method of determining the fatty acids present

in a mixture is the preparation of the methyl or ethyl esters of the individual acids. This may be done through the acid chloride obtained from the free acid by treatment with phosphorus trichloride, or by direct ester interchange using a catalyst. The latter method was used.

The ethyl esters of the fatty acids were prepared by ester interchange between the oil and an excess of ethanol, concentrated sulfuric acid being used as a catalyst. The mixture was refluxed for approximately five hours at which time the mixture had become homogenous. The excess ethanol was removed by distillation and the ester extracted from the concentrated mixture with petroleum ether. The ether extract was washed, first with water, then with dilute sodium carbonate to neutralize the acid, and finally with water to remove any remaining salts. The ether extract was filtered through absorbent cotton to remove any moisture remaining. The petroleum ether was removed by distillation leaving the ester. Final traces of ether were removed by heating on a water bath. The product obtained was a light yellow, pleasant smelling liquid having a refractive index of 1.4550 at 20°C. Due to small yields, no boiling point data were obtained.

An attempt to prepare the methyl esters by direct ester interchange using sulfuric acid as a catalyst proved unsuccessful, however, the methyl esters were obtained by using an excess of methanol and about 0.2 gram of potassium hydroxide. The esters were extracted with ether, neutralized,

washed, and finally obtained in a fairly pure form after distillation and final removal of the ether on a water bath. The mixture of esters was found to have a refractive index of 1.3562 at 20°C. Vacuum distillation of the methyl esters gave the following four fractions:

1. 6 ml. at 182°C at 8 mm. Hg pressure
2. 1 ml. at 196°C at 8 mm. Hg pressure
3. 1 ml. at 200°C at 8 mm. Hg pressure
4. About 0.5 ml. at 220°C at 8 mm. Hg pressure

The first fraction is probably methyl palmitate which has a boiling point of 180°C at 10 mm. Hg. This acid is undoubtedly present as it is widely distributed in plant oils and is one of the lower weight fatty acids.

Methyl linoleate has been found to have a boiling point of 198°C at 10 mm. Hg, so the second fraction is probably that ester. This tends to confirm previous work which suggests linoleic acid as one of the fractions separated by fractional crystallization in the cold. The two pieces of evidence are almost conclusive that linoleic acid is one of the fatty acids present in the original ester. Linoleic acid is known to occur rather widely in vegetable seed oils, hence it is entirely within reason that it should be found here.

The third fraction appears to be methyl oleate which is reported in the literature to have a boiling point of 201°C at 10 mm. Hg. This fraction was not found in the

fractional crystallization, however that does not mean that oleic acid is absent, due to the crude nature of the separation and the difficulty of separating closely related unsaturated fatty acids. The fourth fraction is unaccounted for.

It has been suggested that absorption spectra are of value in determining the structure of fats and oils so a group of absorption spectra were prepared to see if there might be any indication of the composition of echinocystis oil from them. Absorption spectra were run on echinocystis oil, oleic acid, peanut oil, and linseed oil, using the Beckman Spectrophotometer, Model B. Echinocystis oil and linseed oil were found to give absorption bands at 670 μ and at first this was believed to be significant. Markley, however, reports that the fatty acids show no absorption in the visible region, but that carotene has a characteristic absorption band at 450 μ and chlorophyll has one at 660 μ . It is probable that chlorophyll is responsible for this band rather than any similarity in the structure of the two oils. Echinocystis oil, peanut oil, and oleic acid all show absorption bands at 925 μ and since peanut oil is known to contain about 60 per cent oleic acid, there is a good possibility that this may mean that echinocystis oil does also. This would tend to confirm the data from the ester fractionation. Aside from this, little information can be drawn from the

absorption spectra. The absorption spectra of the four oils are plotted in Figure 1.

Per Cent Transmittance

TRANSMITTANCE
VS
WAVELENGTH μ

LEGEND:

- ENCHINO CYSTIS FABACEA
- OLEIC ACID
- PEANUT OIL
- LINSEED OIL

300

850

900

950

1000



SUMMARY OF FINDINGS

Physical properties of oil

$n_{20^{\circ}\text{C}}$ = 1.4695

$d_{20^{\circ}\text{C}}$ = 0.9074 (Westphal balance)

Color = reddish brown with green bloom

No optical rotation

Boiling point - unobtainable due to decomposition

Chemical properties of oil

Acid value = 2 mgm KOH

Saponification equivalent = 278

Saponification number = 202

Iodine number = 108

Analytical findings

The oil is believed to contain:

Arachidic acid

Stearic acid

Linoleic acid

Oleic acid

Palmitic acid

Other low molecular weight acids, undetermined

Esters prepared:

Ethyl esters of mixture, $n_{20^{\circ}\text{C}}$ = 1.4550

Methyl esters $n_{20^{\circ}\text{C}}$ = 1.3562

Methyl linoleate B.P. 198°C , 8 mm. Hg

Methyl palmitate 182°C , 8 mm. Hg

Methyl oleate 200°C , 8 mm. Hg

DISCUSSION OF FINDINGS

The methods used and the results obtained in this laboratory gave a fairly good indication of the nature of the oil extracted from *Echinocystis fabacea* seeds, as far as they went, however it was impossible to carry them to completion owing to time, equipment, and chemical availability limitations. There are however a number of conclusions which can be drawn from the work that has been done.

1. Qualitative analysis shows that the oil probably contains linoleic, oleic, palmitic, stearic, arachidic, and some lower molecular weight fatty acids esterified with glycerol.

2. The oil is a semi drying oil, but will not form a film even upon prolonged exposure to the atmosphere.

3. The oil can be extracted from the seeds of *Echinocystis fabacea* and probably other *Echinocystis* varieties with a good yield. Since the plant grows readily it is possible that it might become economically important should the need arise to find new sources of oils.

4. It is quite possible that the oil from *Echinocystis* seeds could be used as an edible oil as it possesses a unique and interesting flavor and has been demonstrated to have no deleterious effects upon animals.

SUMMARY

A good yield of oil has been extracted from the seeds of *Echinocystis fabacea* (Average = 30 per cent), which exhibits the following properties:

Acid value	= 2 mgm. KOH
$n_{20^{\circ}\text{C}}$	= 1.4695
$d_{20^{\circ}\text{C}}$	= 0.9074
Saponification equivalent	= 278
Saponification number	= 302
Iodine number	= 108

Qualitative analysis of the oil revealed that it contained glycerol esters of linoleic, oleic, palmitic, stearic, arichidic, and some lower molecular weight fatty acids.

No attempt was made to analyze the fatty acids quantitatively.

The oil is non toxic and has a rather unique and interesting flavor.

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