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Helianthus Annuus - The Separation and Identification of a New Fatty Acid

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HELIANTHUS ANNUUS - THE SEPARATION AND IDENTIFICATION OF A NEW FATTY ACID

By

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INTRODUCTION

The term "fatty acids" was first applied principally to saturated, monobasic carboxylic acids and especially to long chain acids derived from animal and vegatable oils. As time passed and the knowledge of natural fats increased, polyunsaturated, hydroxy, keto and branched chain acids were also found as constituents of these fats and oils and these also came under the heading of fatty acids. Even acids containing alicyclic substituents were found.

More recently the term "fatty acid" has been applied to include saturated and unsaturated monobasic carboxylic acids and several other series of substituted acids having carbon skeletons similar to the normal saturated series.

Many different saturated and unsaturated fatty acids have been discovered as components of natural fats and oils, but this list is small compared to the list of those made in the laboratory by synthesis or from non-fat natural sources (waxes, fruit essences, fossiliferrous material).

Fats or oils are triglycerides or esters of fatty acids formed by the reaction between glycerol and three fatty acid molecules. A fat is considered to be a solid at room temperature while an oil is normally a liquid. The large number and complexity of the natural fats and oils is due to two things: the number, kind and arrangement of the individual fatty acids which are attached to the glycerol skeleton to form the specific glycerides, and the number and relative proportions of these

glycerides which are mixed or mutually dissolved to form the specific fat or oil.

The value of fats and oils has been known to man for a long time. At first, fats and oils were used for food. Later, as man recognized some of the physical properties, they were used as medicinals, in cosmetics, as illuminates and as lubricants. History records many references to the use of fats and oils from the 9th century B.C. through the Middle Ages.

The beginning of the 19th century saw many important discoveries concerning the chemical nature of fat. Chevrual established the glyceride nature of fats, prepared stearic, valeric, caproic and impure oleic acid. Toward the middle of the 19th century, Berthelot demonstrated the trihydric nature of glycerol. He synthesized mono-, di-, and triglycerides and suggested that natural fats consisted of heteroglycerides rather than mixtures of homoglycerides. From the middle of the 19th century until the 1920's, little progress was made in the field of fats and oils.

Starting in 1920, interest turned to the chemistry of fats and oils. New tools and techniques were introduced. These new procedures led to the discovery of new unknown substituted and isomeric acids which had been suspected but which could not be detected or identified with the previously available means. Biochemical and nutritional investigations multiplied and diversified. Shortly after 1920, it was discovered that fatty acids exhibited many reactions, (condensation, polymerization, isomerization, oxidation and reduction, addition and

substitution reactions) that were considered classical reations of organic chemistry.[10]

The production of fats and oils has grown to tremendous proportions. With the renewed interest in the chemistry of fats and oils came a great surge in the use of natural fats and oils. The estimated world production of fats and oils for the year 1959 was about 33.7 million short tons. The production of edible vegetable oils accounted for a little over one-third of the total fat and oil production. Sunflower oil accounted for about 1.6 million short tons. Only cottonseed, peanut and soybean oil were produced in greater quantities.[10] Sunflower oil, although relatively unknown as a food product in this country, has found great acceptance throughout Europe, especially in Russia.

HISTORICAL REVIEW

Sunflower oil has been investigated to some degree since it is used as a food throughout Europe. Linoleic and oleic form the two major component fatty acids. Together they account for about eighty-five per cent of the fatty acid content of the sunflower oil. Sunflower oil with a linoleic acid content up to seventy per cent is not unusual. The saturated acids account for twelve to fourteen per cent of the total acid content. About one-half of the saturated acids is palmitic acid, stearic acid forms one-quarter, and the remainder, smaller proportions of arachidic and behenic acids.[7]

In the last several years, the Department of Agriculture has been involved in a program to determine by chemical screening analysis, what amounts and general classes of fatty acids are contained in the seed oils of a large number and variety of uncultivated species. F. R. Earle and his co-workers[3] have reported in a series of papers the outcome of this screening analysis of the various seed oils. One of the tests included in this screening involved the hydrogen bromide uptake of the various oils. For convenience this hydrogen bromide uptake was calculated in terms of the percentage of oxirane oxygen as apparent epoxy oleic acid. The two samples of sunflower (Helianthus annuus) tested showed the presence of 2.9% and 3.1% epoxy oleic acid. The presence of this type of fatty acid in sunflower oil had not been previously reported in the literature.

Since the appearance of Earle's paper, C. R. Smith[17] and L. J. Morris[14] have demonstrated that the hydrogen bromide uptake is not specific for oxirane oxygen in epoxy fatty acids but also occurs in the presence of a cyclopropene ring or in the presence of a vinyl carbinol. Further, L. J. Morris[14] postulated the presence of a vicinally unsaturated hydroxy acid in several seed oils, one of which was sunflower oil (Helianthus annuus).

Therefore, with these facts in mind, the purpose of this investigation was to attempt to separate and identify the hydrogen bromide active unknown acid or acids present in the sunflower oil.

EXPERIMENTAL

Separation of the Unknown Fatty Acid

Preparation of the Sunflower Oil

The first step in the separation of the fatty acids of sunflower oil was the extraction of the oil from the seeds. Approximately five hundred grams of sunflower seeds were purchased locally. Forty gram samples of seed were ground at low speed for approximately one minute in a Waring Blender. The resulting seed meal was extracted with petroleum ether (b.p. 40-60°) for eight hours in a Soxhlet extraction apparatus. After the refluxing operation, the petroleum ether in the thimble chamber was washed into the receiving flask and the seed meal discarded. The petroleum ether was removed from the solution of the sunflower oil under vacuum at 40° by means of a Rinco rotating evaporator.

After four hours on the rotary evaporator, the flask containing the sunflower seed oil was removed and placed in a vacuum oven. The pressure in the oven was gradually reduced to between 0.2 - 0.5 mm. of Hg. without heating to prevent any violent eruptions of the oil. The oil remained under vacuum over night, approximately ten hours.

After removal from the vacuum oven, the oil was weighed, transferred to a storage flask, covered with nitrogen, and stored in a refrigerator at 5° until used in the next step.

Hydrogen Bromide Titration of the Sunflower Oil

Anhydrous hydrogen bromide was bubbled at a slow rate through glacial acetic acid until a normality of approximately 0.1N was attained. The hydrogen bromide was standardized by adding about 100 mg. of sodium carbonate, dried to constant weight, to 10 ml. glacial acetic acid and titrating to the blue-green end point of crystal violet indicator.

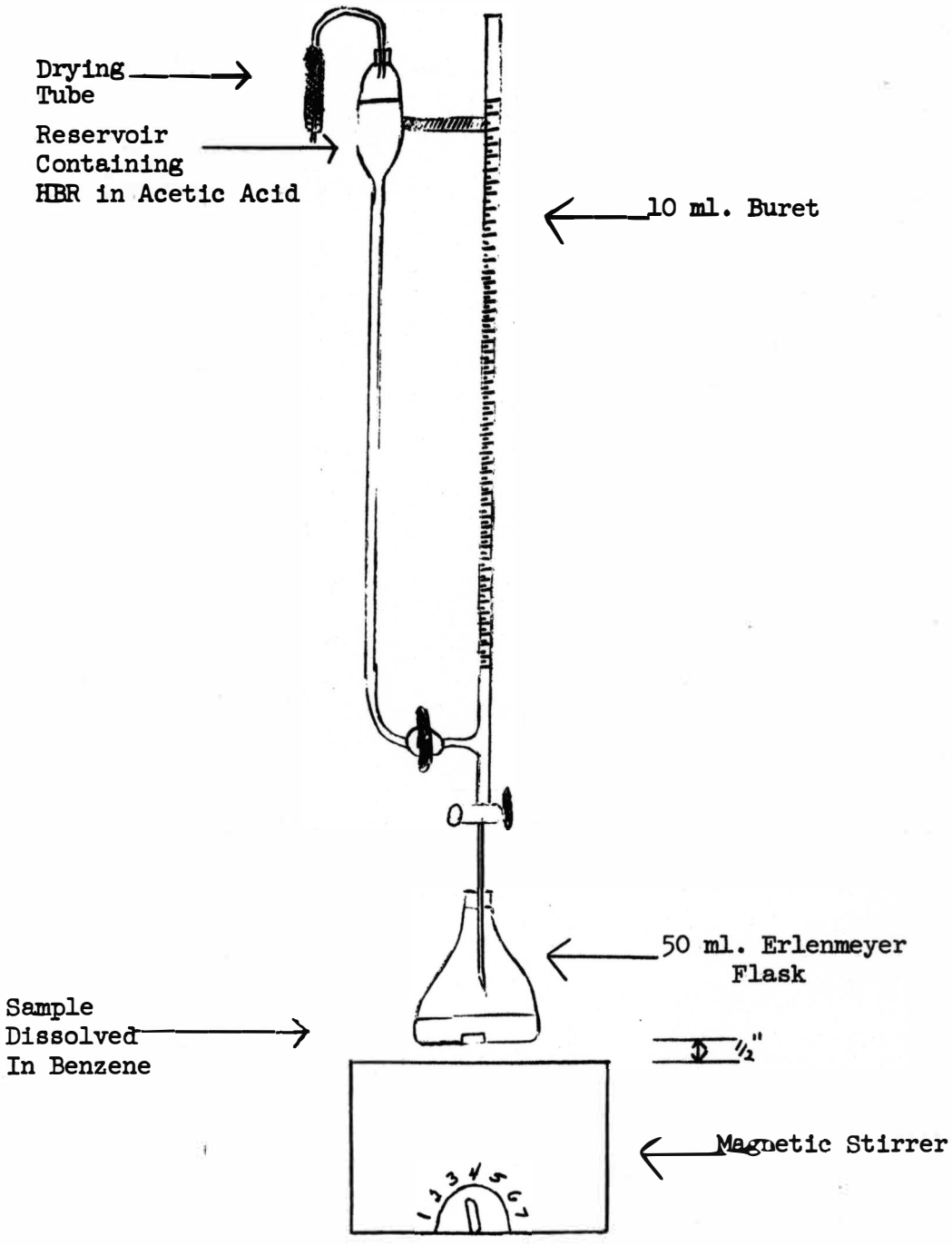
Three samples of oil of approximately 3 g. each were dissolved in benzene and titrated with the hydrogen bromide-acetic acid solution using the apparatus shown in Figure 1. In the first titration the end point continually faded after each addition of hydrogen bromide reagent and no stable end point could be obtained. However, it was noted during the titration that the solution temperature rose because of contact with the magnetic stirrer. When the warm solution was allowed to cool, the blue-green end point returned. In the remaining titrations the bottom of the flask containing the oils was held approximately one-half inch above the top of the magnetic stirrer, thus allowing circulation of air and the dissipation of the heat.

Preparation of Methyl Esters of the Sunflower Oil

The methyl esters of the sunflower oil were prepared by a process of interesterification. This method is similar to that used by C. R. Smith, Jr.,[18] for the preparation of the methyl ester of dimorphecolic acid. Alcoholysis of the oil was accomplished by

FIGURE 1

Durbetaki Titration Apparatus



refluxing on a steam bath 25 - 30 g. of oil in 200 ml. of 0.4% methanolic sodium methoxide for two hours. After the solution had cooled to room temperature it was made acid to litmus paper by the dropwise addition of glacial acetic acid. Four hundred ml. of water were added and the solution was extracted with 200 ml. of ethyl ether four times. The four ether extracts were collected and washed with 100 ml. portions of water until the rinse water no longer affected litmus paper. The ether solution was dried over approximately 50 g. of anhydrous sodium sulfate for a twenty-four hour period. The methyl esters were obtained by evaporation of the ethyl ether using a rotary evaporator under vacuum at a low temperature. The esters were placed in the vacuum oven and the pressure was reduced to 0.2 - 0.5 mm. of Hg. Finally the esters were weighed, transferred to a storage bottle, covered with nitrogen and stored in a refrigerator at 5° until further use.

Separation of Esters by Fractional Crystallization

The first attempt at separation of the esters was a short experiment in fractional crystallization: 16.7 g. of esters were dissolved in low boiling petroleum ether to make a 10% solution weight to volume. The ester solution was placed in a 250 ml. Erlenmeyer flask and suspended in a cooling bath composed of methanol and dry ice. This solution was maintained at -30° for thirty minutes. Since inspection showed no precipitation, the temperature of the cooling bath was lowered to -50° and maintained

at this temperature for two hours. At the end of the two hours, a precipitate had formed in the solution. This precipitate was removed without washing by filtering the solution under vacuum through a filter stick that had been previously cooled to -50°. The precipitate on the filter was allowed to come to room temperature and washed out of the filter with diethyl ether. The solution of the precipitate as well as the filtrate from the filtration were evaporated under vacuum to dryness using a rotary evaporator.

Column Chromatography of Methyl Esters

Separation of Esters on an Alumina Column

In the first attempt at separation of the methyl esters, elution chromatography from a column with alumina as the stationary phase was used. The column dimensions were 400 mm. x 25 mm. with 100 g. of alumina as the solid adsorbent. The sample was placed on the column and eluted with a hexane-ether mixture made increasingly more polar as time progressed by increasing the ratio of ether to hexane. The flow rate through the column was approximately 100 drops per minute and the effluent was collected in 10 ml. fractions.

For the first experiment, 3.0 g. of esters were placed on the column. Elution was started with 200 ml. of hexane followed by 100 ml. of each of mixtures of 1:9, 2:8, 3:7, 4:6 ether-hexane for a total elution of 600 ml. of solution. To insure complete removal of all material from the column, the column was finally washed with

100 ml. of ethyl ether collected in two 50 ml. portions. In order to determine the distribution of the esters, if any had occurred, the contents of the vials were evaporated and the residues weighed.

Since it was not feasible to use a rotary evaporator for each of the sixty separate fractions, the solvent was evaporated by use of nitrogen gas blowing on the solution in tared vials placed in a water bath maintained at a temperature of 40°. The temperature was kept as low as possible to prevent any degradation of the fatty acids. The final residue of solvent was removed by drying overnight in the vacuum oven at very low pressure (0.2 - 0.4 mm. Hg.). The vials were then weighed.

Location of the Unknown Fatty Acids

In order to determine the location of the unknown acid in the fractions collected, infrared spectra were determined for the samples 13, 29 and 55 as well as the two washings. All spectra were determined with a Perkin-Elmer Model 421 Infrared Spectrophotometer. Samples were spread in a thin film on NaCl crystal blocks, and scanned over a range of 2 - 15 μ.

Thin Layer Chromatography of the Fatty Acid Esters

Normal Silica Plates

The first thin layer chromatography system consisted of silica coated on glass plates. Silica gel G was used on all thin layer plates. Dry silica gel was mixed with water in a ratio of 1 to 2 and spread 0.5

mm. thick with a Desaga-Heidelberg spreader. Plate sizes were either 5 x 20 cm. or 20 x 20 cm. After being spread, the plates were dried at 100° for two hours, and stored in a closed case containing a desiccant. The solvent system consisted of ether-petroleum ether (20:80). Samples from the alumina column were dissolved in ethyl ether and 10 microliters were spotted on the plates. Developing time was approximately thirty minutes.

Two methods of developing the plates were tried. The first consisted of spraying the plates with 50% sulfuric acid and placing them in an oven at 100° for fifteen minutes. Heating charred all the organic material and anything organic appeared as a series of black spots. The second system consisted of placing the thin layer plates in an atmosphere saturated with I_2 vapors for thirty minutes.

Silver Nitrate Impregnated Silica Plates

Silver nitrate impregnated silica plates were prepared by mixing 30 g. of silica with 60 ml. of 12% w/v silver nitrate and spreading the plates with this suspension in the normal manner. These plates were dried for several hours at 105° and stored in an air-tight, dry, light-proof container. The first plates made were 0.25 mm. in thickness. Later the thickness of the silica layer was increased to 0.50 mm.

The developing solvents used in this system consisted of diethyl ether-hexane (10:90). Later the ratio of these solvents was changed

to (30:70). After the developing solvent had evaporated, the chromatograms were sprayed with a 0.1% aqueous-ethanol solution (1:1) of 2, 7--dichlorofluorescein. This spray rendered the spots visible under ultraviolet light at which time the position of any spots were marked by tracing the outline of the spot. A drawing was made of the chromatogram to be used as a record.

Further Chromatographic Runs Using the Alumina Column

In order to concentrate the unknown compounds, the alumina column was used for further rough separations. Samples of 10 - 15 g. were placed on the column. Since the unknown hydrogen bromide active compounds remained on the column until removed by pure ethyl ether, the column was eluted with the solutions previously mentioned, allowed to drain by gravity, and washed with 200 ml. of ethyl ether collecting this effluent. The ether solutions were evaporated in the usual manner and the residues stored under nitrogen in a refrigerator.

Silica Column Impregnated with Silver Nitrate

A silica column impregnated with silver nitrate was prepared. The adsorbent was prepared by mixing 100 g. of silicic acid with 200 ml. of 50% w/v aqueous solution of silver nitrate. The mixture was maintained, with frequent stirring, at 100° for 30 minutes. When cooled, it was filtered through a Buchner funnel and the silica-silver nitrate mixture dried for twenty-four hours at 110°.

A slurry was prepared by mixing 100 g. of silica adsorbent and 50 g. of Celite 535 with 200 ml. of petroleum ether. The slurry was heated for five minutes, cooled and poured into a column 28 mm. x 500 mm. The column had been previously covered with masking tape to protect the adsorbent from light. When the column was not in use, it was stored in a refrigerator at 5°.

A 1.5 g. sample of the methyl esters dissolved in hexane, was placed on the column. Elution was carried out by successive 30 ml. portions of various hexane-ether mixtures, starting with 5% ether solution and increasing the concentration of the ether by 5% every 30 ml. of eluting solvent. After ten 30 ml. fractions of solvent had been added, the eluting solvent was changed to 100% ethyl ether. A total of 300 ml. of ethyl ether was added to the column. Fractions were collected in 10 ml. samples at a rate of 0.5 ml. per minute for the first 300 ml. and 1.0 ml. per minute for the last 300 ml. The solvent was evaporated, the residue dried and weighed.

Another column was prepared as above but only 300 mg. of methyl esters were placed on the column. The concentration and volume of the eluting solvent were changed. Elution was started with 100 ml. of 30% ether-hexane followed by 100 ml. of 40%, 100 ml. of 50%, 100 ml. of 60% and finally 200 ml. of pure ethyl ether. The rate of collection was 1.0 ml. per minute throughout.

Liquid Partition Chromatographic Procedure

The final separation of the unknown fatty acids was achieved by using a liquid partition column. The column was prepared in the

following manner: a slurry of silicic acid and benzene was prepared by mixing 50 g. of silicic acid and 75 ml. of benzene in an Erlenmeyer flask. The stationary phase of 40 ml. of 20% methanol in benzene was added slowly to the flask while the flask was being shaken. This slurry was transferred to a column, 24 mm. x 400 mm., containing a fritted glass plate at the bottom and packed under air pressure until a constant height was attained. Approximately 50 - 75 ml. of mobile solvent, 2% methanol in benzene, was used to rinse the flask to get the remaining silicic acid into the column. A solvent head was maintained over the silicic acid.

A sample of the residue from the alumina column (varying between 300 - 700 mg.) dissolved in a small amount of mobile solvent, was placed on the column. As soon as the sample entered the column, the mobile solvent, 300 ml. of 2% methanol in benzene followed by 100 ml. of ethyl ether, was added and the system put under nitrogen pressure sufficient to maintain a rate of collection of approximately 3 ml. per minute. Samples were collected in 10 ml. fractions, the solvent evaporated, and the residues weighed.

Characterization of the Unknown HBr Active Fatty Acid

Physical Characteristics of the Unknown Compounds

The first step in the characterization of the unknowns consisted of determining their physical characteristics. The physical status of the compounds and the solubility properties were noted. The melting point of the solid sample was determined using a Thomas-Hoover capillary melting point apparatus. Elemental analysis for C, H, and O were run by the Physical and Analytical Section of The Upjohn Company. The molecular weight of compound A was determined using Mechrolab Vapor Pressure Osmometer Model 301.

Ultraviolet and Infrared Spectra of the Unknown Compounds

The ultraviolet spectra of the unknown compounds were obtained on a Cary, Model 15, Automatic Recording Spectrophotometer. Samples were dissolved in ethyl alcohol and read in the spectrophotometer from 400 to 220 mμ using 1 cm. cells.

The infrared spectra of the compounds were obtained with a Perkin-Elmer Infrared Spectrophotometer, Model 421, on a one percent solution in carbon tetrachloride in 1 mm. cells.

Gas Chromatographic Characterization of the Unknown Compounds

Two columns were prepared. The first column, 120 cm. in length, contained 15% Apiezon L on Celite (60-100 mesh). The polar column, 275 cm. in length, contained 20% LA C 446 on Celite (100-160 mesh). All chromatograms were run on a F. & M. Model 609, Gas Chromatograph.

A standard reference curve for each column was established by running chromatograms of a mixture of seven known methyl esters of fatty acids ranging from C_8 to C_{20} in carbon length. The log of the retention time was plotted versus the carbon length of the compound. The ECL values for the compounds were obtained by reading from the standard curve, obtained under the same operating conditions.

Nuclear Magnetic Resonance Analysis

The nuclear magnetic resonance (NMR) spectrum of compound A was obtained on a Varian A60 spectrophotometer. The solvent used was CD Cl_3 with tetramethyl silane used as an internal standard.

DISCUSSION AND RESULTS

Separation of the Unknown Fatty Acid

Preparation of the Sunflower Oil

The use of a vacuum oven to remove the last traces of petroleum ether from the sample was necessitated by the presence of petroleum ether in the sample, detected by its odor, even after four hours on the rotary evaporator. Because a temperature below 40° was maintained to prevent any structural changes in the unknowns, the reduced pressure used on the rotating evaporator was unable to remove all the solvent from the sample.

Since it was hoped that one to two grams of the unknown fatty acid could be separated from the other fatty acids of the sunflower seed oil, and since it accounted for approximately only 3% of the fatty acid content of the sunflower seed oil, a large amount of sunflower oil was accumulated. Since the sunflower oil comprised only between 25 - 50% by weight of the bulk of the sunflower seeds, twenty extractions were made. See Table 1. A total of 199.1 g. of sunflower oil was obtained with the average yield of 29.7% by weight. Oil from seeds purchased locally did not react with hydrogen bromide and was discarded. Oil from the U. S. Department of Agriculture seeds, which did react with hydrogen bromide, comprising a total of 138.6 g., was retained.

TABLE 1

Amount of Sunflower Oil Extracted From Seeds

Extraction No.	Lot No. of Seeds	Weight of Seeds Extracted -Grams	Weight of Oil Extracted -Grams	% Yield
1	Commercial seeds locally obtained	40.0	12.5	31.7
2	"	40.0	23.3	29.0
3	"	40.0		
4	"	40.0	24.7	30.1
5	"	40.0		
6	13,299	40.0	Oil lost -accident	
7	13,299	40.0		
8	13,299	40.0	13.3	33.3
9	13,299	40.0	12.9	32.3
10	13,296	40.0	36.4	30.3
11	13,296	40.0		
12	13,296	40.0		
13	13,302	40.0	28.1	23.4
14	13,302	40.0		
15	13,295	40.0		
16	13,295	40.0	28.1	23.4
17	13,290	40.0		
18	13,290	40.0		
19	13,290	40.0	19.8	24.8
20	13,290	40.0		
		Total	199.1	29.7% Average Yield
	(Weight of oils discarded #1-5)		-60.5	
	Total usable oil		138.6	

Discarded

Hydrogen Bromide Titration of the Sunflower Oil

From hydrogen bromide uptake, Earle[3] had reported that sunflower oil contained approximately 3% of a fatty acid calculated as a C_{18} epoxy acid. It seemed desirable in the beginning to check these figures using the sunflower seeds that were to be used in the work. For this reason, a Durbetaki[2] titration was performed on several samples of the sunflower oil.

Three samples of locally purchased oil were titrated and all these samples showed a negligible amount of hydrogen bromide uptake. Calculated as a C_{18} epoxy acid all samples showed less than 0.05% present.

Since Earle[3] had reported a content of approximately 3% it was necessary to obtain new samples of seeds and try again. C. R. Smith, Jr., obtained approximately 1000 g. of sunflower seeds from the Northern Regional Research Laboratory, Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

Oil from seeds obtained by C. R. Smith, Jr., showed the presence of 3.49% and 3.52% "epoxy acids", respectively, in two duplicate samples. These results compared favorably with the results obtained by the Northern Regional Laboratories.

No explanation of the absence of this hydrogen bromide uptake property in the locally purchased seeds can be given. Several things could influence the seeds however. The unknown acids are likely to be unstable in the presence of extremes of heat. The commercial seeds

may have been subjected to some process involving heat or cleaning that may have destroyed these unknown acids. Another possibility that must be considered is that these unknown acids are not found in all sunflower seeds produced throughout the world. Many natural seed oils have shown this property, that is, a variance of composition both in quality and quantity of fatty acids present in the seed oil.

Preparation of the Methyl Esters of the Sunflower Oil

The second step in the separation of the unknown fatty acids from the sunflower seed involves the cleavage of the oil molecule into its component fatty acids and glycerol. Since it is normally easier to separate the simple esters of fatty acids than the acids themselves, these acids were esterified first. This process can be accomplished in several ways. The first is to saponify the sunflower oil, extract the unsaponifiables from the soap solution and then liberate the fatty acids by adding a strong mineral acid. Methyl esters could then be prepared by esterification with diazomethane.

The second method involves the direct formation of the methyl esters from the glycerides by interesterification. This method was chosen because it was a less rigorous procedure and it combined both steps of the first procedure. Since these epoxy acids were uncharacterized and probably unstable in any extreme condition, the easier they could be handled, the better would be the method. The second method had one disadvantage in that it does not remove all of the unsaponifiables from the mixture of methyl esters.

The esters obtained are yellow liquids similar in appearance to the oil except the viscosity of the esters is somewhat lower than that of the sunflower oil. Altogether six interesterifications were performed. The results of these esterifications are shown in Table 2. A little over 118 g. of methyl esters were obtained.

Separation of Esters by Fractional Crystallization

A total of 1.5 g. of esters was recovered as the precipitate and 15.2 g. from the filtrate. In order to determine if any separation of the esters had occurred, the Durbetaki titration was performed on both samples and calculated as C_{18} epoxy acid. The titration indicated the presence of 3.74% unknown acid in the filtrate and 2.96% unknown acid in the precipitate. The original esters had an "unknown acid" content of 3.51% as determined by the Durbetaki titration. Some separation had occurred but it was not enough to continue further work on this method of separation. Better separation might have been accomplished if the solution had been cooled for a longer period of time. Other work in this type of separation showed that from six to forty-eight hours is necessary for equilibrium to be established between the precipitate and the remaining solution.

Column Chromatography of Methyl Esters

Separation of Esters on a Alumina Column

The results of chromatography on the alumina column are shown in Figure 2. The total weight of all the residues from the vials amounted

TABLE 2

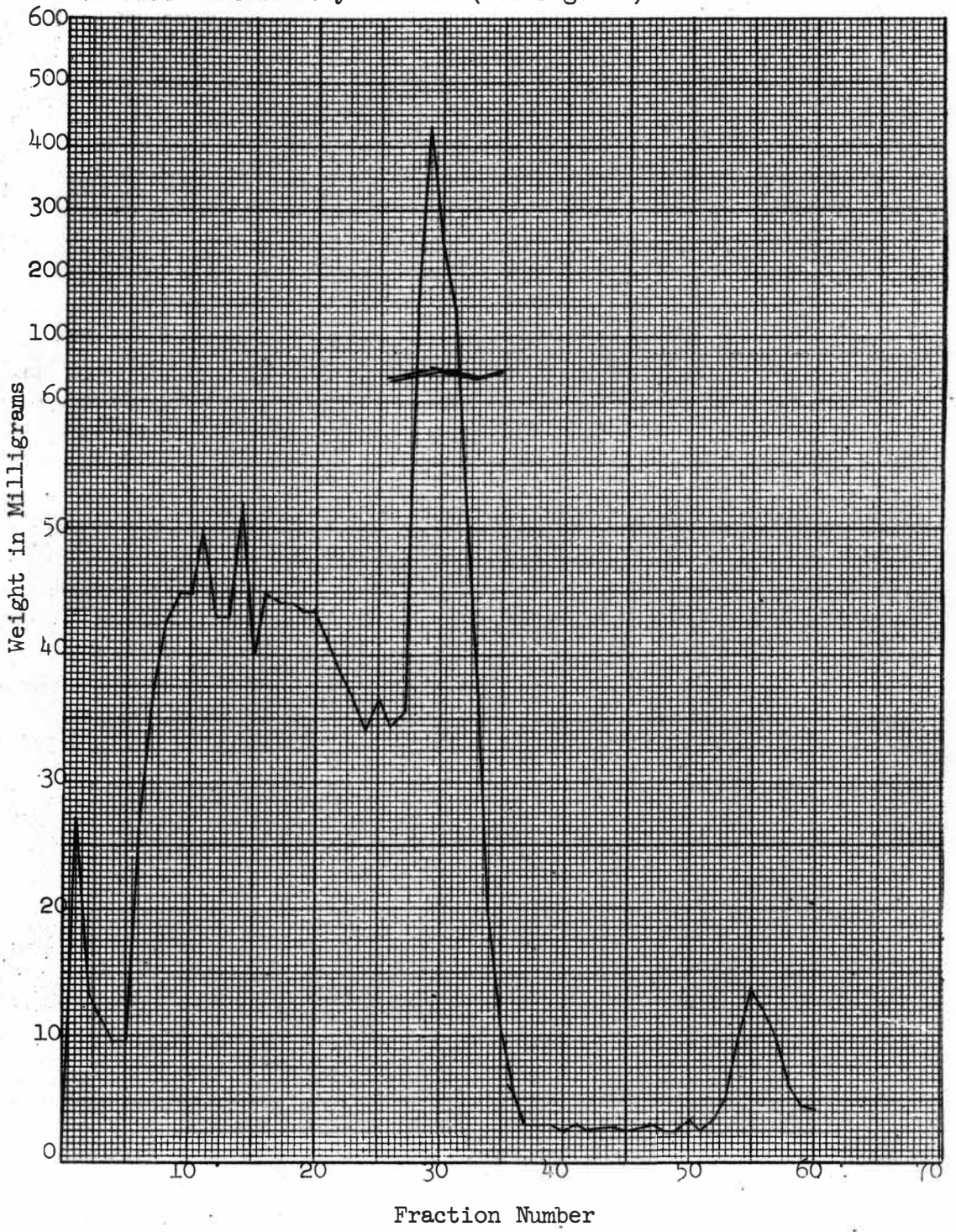
Alcoholysis of Sunflower Oil

Batch No.	Weight of Sunflower Oil -Grams	Weight of Methyl Esters -Grams
1	1.00	0.978
2	26.4	24.0
3	31.8	28.9
4	27.1	47.9
5	26.4	
6	18.7	16.8
	Total	118.58 g.

FIGURE 2

Fraction Weight Distribution Alumina Column

Solvent: Hexane-Ethyl Ether (See Page 11)



to 2.3 g. leaving an unaccounted for 700 mg. The two 50 ml. fractions of ether collected during the washing of the column were evaporated and they accounted for another 138 mg. of sample. Further washing with up to 300 ml. ethyl ether did not remove any more material from the column. This accounted for a total recovery of 2.45 g. of material from the original 3.0 g. placed on the columns.

Location of Unknown Fatty Acids

None of the original fractions showed any absorption in the infrared in the 2 - 3μ range, which would be indicative of a hydroxyl group in the structure. The residues washed off the column at the end of the chromatography run showed absorption in the hydroxyl range. This is not surprising that the unknown HBr active compound had been held up since an unsaturated fatty acid containing an hydroxyl group is more strongly polar than a normal unsaturated fatty acid.. The more polar solvent, pure ethyl ether, is able to elute the unknown acid off the column. Since the alumina column system gave an approximate sixteen-fold increase in the unknown fatty acid concentration by elimination of some of the components in the original mixture, it was considered worthy of more work with certain modifications. However, before this could be done, another method of detecting the presence of the unknown fatty acids had to be found since it became impractical to run infrared spectra on all the fractions collected.

Thin Layer Chromatography of the Fatty Acid Esters

Normal Silica Plates

The method which seemed the easiest as well as the fastest of keeping track of the unknown compounds was thin layer chromatography. While an individual sample might take two to three times longer to run than an infrared spectrum, thin layer chromatography offered the advantage of multiple separations at one time. Up to nine samples could be spotted on one large thin layer plate and be developed at the same time, thus giving considerable saving in time and energy.

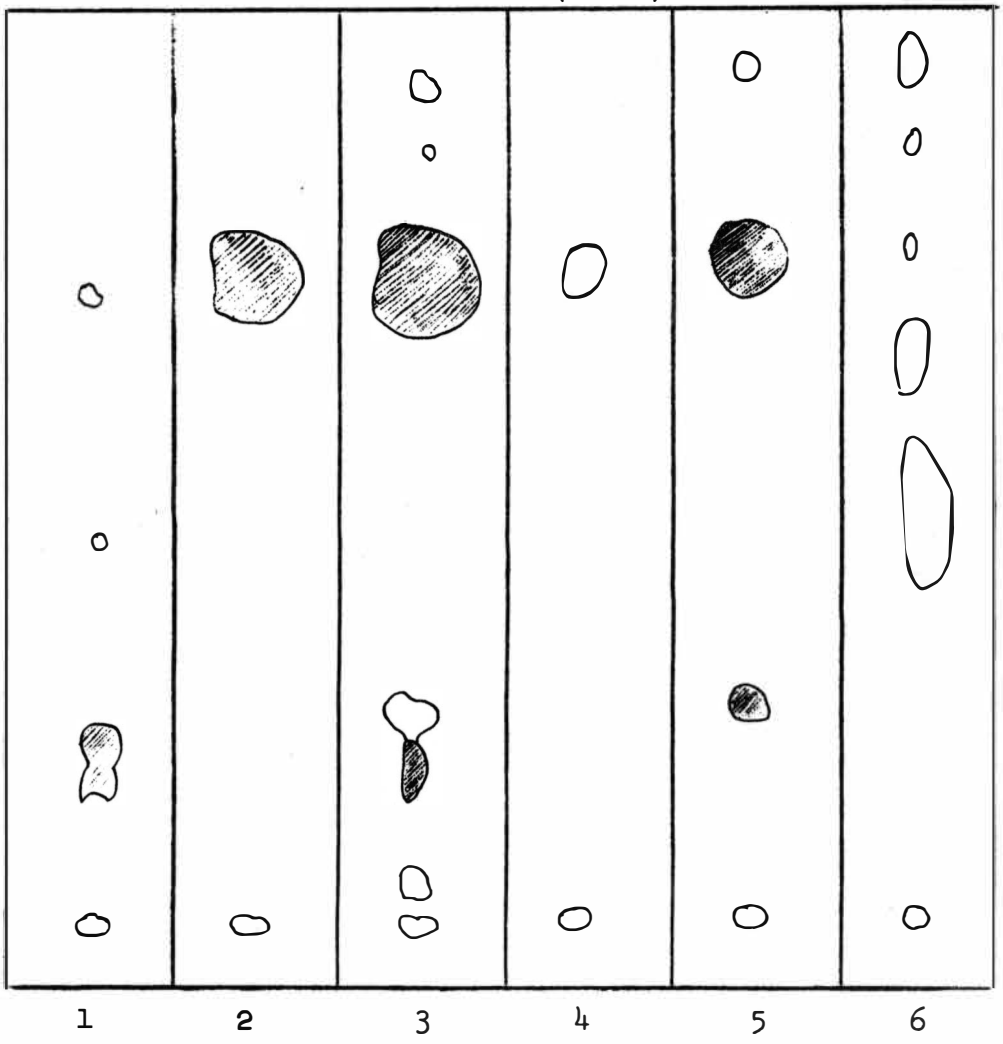
The first thin layer chromatography system was similar to that used by Morris[15] in his separation of mixed esters by thin layer chromatography.

Of the two methods of developing the plates tried, the first consisting of spraying the plates with 50% sulfuric acid, had a great disadvantage in that it was messy and difficult to handle. For this reason the second method, iodine absorption, was adopted. All unsaturated compounds strongly absorb I_2 vapors forming dark spots on the plate while saturated compounds absorb I_2 vapors to a much lesser extent, yet absorbing enough to make them visible. Since the spots on the plate, especially of the saturated compounds, will fade after exposure to normal atmosphere for several hours, the position of the spots must be marked in some manner or a drawing made of the respective plates. The advantage of ease in handling the I_2 vapor-developed plates outweighed this disadvantage. Figure 3 is a drawing of the results of the first silica plate. This plate represents fractions taken from the alumina column.

FIGURE 3

Thin Layer Chromatography Plate of Alumina Column Fractions

Solvent: Ether + Petroleum Ether (20:80)



1 Residue off column
2 Standard saturated methyl esters
3 Methyl esters of sunflower oil
4 Standard unsaturated methyl esters
5 Fraction #29
6 Fraction #55

In order to determine which fraction represented the hydrogen bromide active acid, the chromatographic pattern was compared with the infrared spectra obtained on the same samples. If the infrared spectra showed absorption in the hydroxyl range, the chromatogram of the same sample was studied. It was apparent that the fractions containing the unknown acids exhibited a characteristic pattern of movement. The spots representing hydrogen bromide active compound moved very little in this chromatographic system while the major components moved much faster (See #1. Figure 3). This chromatogram showed that the fraction containing hydrogen bromide active compounds still contained some impurities. Further, the spot representing the unknown indicates that the hydrogen bromide active compound may in fact be two or more compounds. It was necessary to obtain better differentiation of this unknown spot to determine if the unknown was a mixture of two or more components. The first thin layer system was sufficient for rough separation of the unknown but a better thin layer system was needed to characterize the pure compounds.

Silver Nitrate Impregnated Silica Plates

L. J. Morris[13] reported some successful separations of higher fatty acid isomers. He was able to separate by thin layer chromatography, using plates coated with silicic acid and impregnated with silver nitrate, the *cis* and *trans* isomers of mono-ethenoid fatty acids, and the vinylogues of non-oxygenated and of oxygenated fatty acids. Since his system was able to separate closely related compounds, it was probable that if the

spot shown to represent the unknown on the original thin layer plates actually represented two compounds, the silver nitrate impregnated silica plates would be able to resolve them.

The first plates made were only 0.25 mm. in thickness and the solvent front moved too quickly; consequently, there was little movement of anything away from the origin. On thickening the plates to 0.5 mm. good movement and separation was obtained.

The change to the silver nitrate impregnated plates also forced a change in the method of development of the plates. Iodine vapors could no longer be used on the silver nitrate impregnated plates because only negligible I_2 absorption took place. This necessitated the use of 2, 7---dichlorofluorescein, a common indicator used in the thin layer chromatography of fatty acids. After some experimentation the ratio of the ether-hexane solvent was changed from 10:90 to 30:70 because better separation occurred.

Figure 4 shows the results of one trial using the silver nitrate impregnated plates. It is evident that the former single spot has become two and the unknown compound is actually a mixture of at least two closely related compounds.

Further Chromatographic Runs Using the Alumina Column

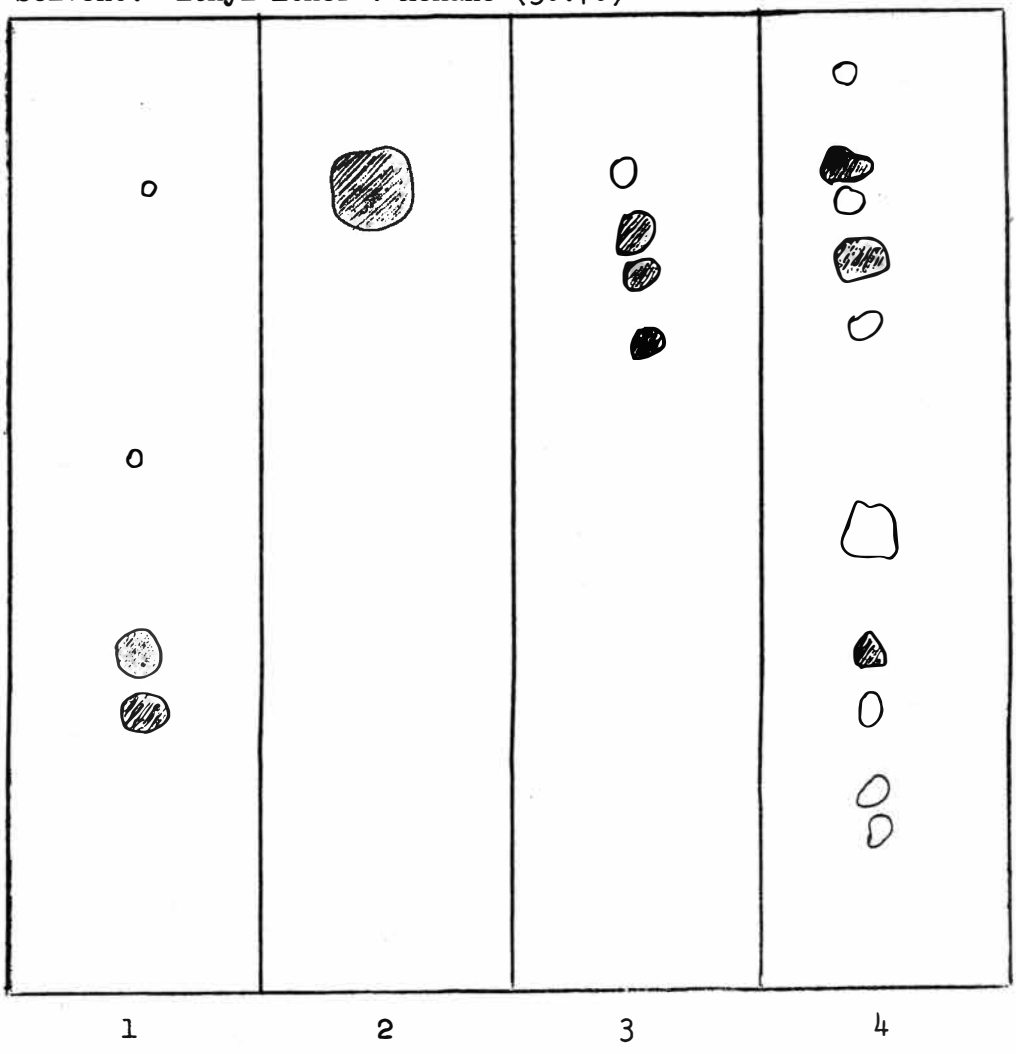
Since the original alumina column did give an approximate sixteen fold increase in the relative unknown constituent concentration, this method was sufficient, with some modifications, for a rough separation. Since the HBr active compounds represented only about 3% of the total fatty

FIGURE 4

Silver Nitrate Impregnated Silica

Thin Layer Chromatography Plate

Solvent: Ethyl Ether + Hexane (30:70)



1 Residue off Column
2 Standard Saturated Methyl Esters
3 Standard Unsaturated Methyl Esters
4 Methyl Esters of Sunflower Oil

acid content, it was desirable to remove as much and as many of the other fatty acids as easily as possible.

In order to make certain that none of the HBr active unknowns were lost, all solutions collected were spotted on thin layer plates before these solutions were discarded.

Table 3 shows the results of seven runs on the alumina column. A total of 98.5 g. of esters were passed through the column to yield a residue of about 4.6 g. This residue however, still contained impurities as well as a mixture of the two HBr active compounds.

Silica Column Impregnated with Silver Nitrate

Since the thin layer chromatography plates that had been coated with silica and impregnated with silver nitrate had been successful in separating the unknown compounds, it seemed reasonable to believe a silver nitrate impregnated silica column would do the same. DeVries[1] had done some similar work along this line obtaining quantitative separations of saturated cis and trans monoenoic and polyenoic methyl esters.

Figure 5 shows the result of the chromatographic run using the silver nitrate impregnated silica column. At first it appeared that separation had occurred. However, a thin layer chromatogram of the samples revealed that the unknown appeared to be equally distributed in all the fractions. Infrared spectra confirmed the presence of hydroxyl absorbing groups in all the fractions.

TABLE 3

Residues Collected From Alumina Column

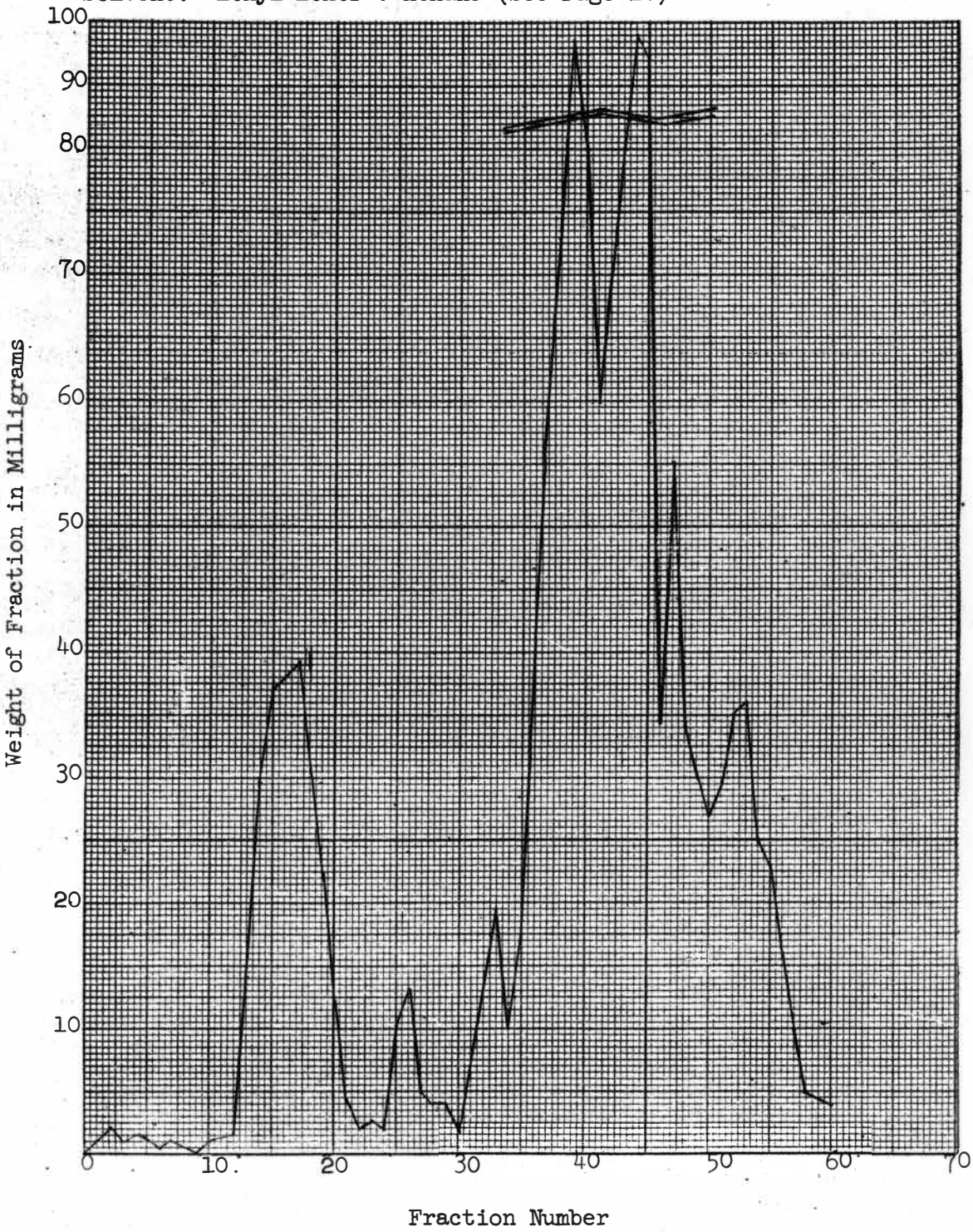
Run No.	Weight of Ester Placed on Column - Grams	Weight of Residue Obtained - Milligrams
1	10.47	488
2	10.75	480
3	15.07	793
4	16.43	819
5	13.09	534
6	14.80	649
7	17.86	849
Totals	98.47 g.	4,612 mg.

FIGURE 5

Fraction Weight Distribution

Silver Nitrate-Silica Column

Solvent: Ethyl Ether + Hexane (See Page 14)



The results of the second column were quite similar to the previous one. A plot of the weight distribution shows a separation, but thin layer plates show the separation is not of the right compounds. Apparently this case is one in which thin layer chromatography will work but similar adsorbents used in column chromatography will effect no separation.

Liquid Partition Chromatographic Procedure

E. N. Frankel[5] reported on the separation of hydroxy fatty acids using a liquid partition chromatographic procedure. Since the HBr active compounds were assumed to be hydroxy fatty acids, this method would seem applicable. Previous work by Frankel[4] had been the application of a liquid chromatography system to the determination of dimeric and polymeric products in fats and fatty acid hydroperoxides using 20% methanol as the starting phase and 2% methanol in benzene for the mobile solvent. In his new work he applied a similar system to the separation of some of the naturally occurring hydroxy fatty acids.

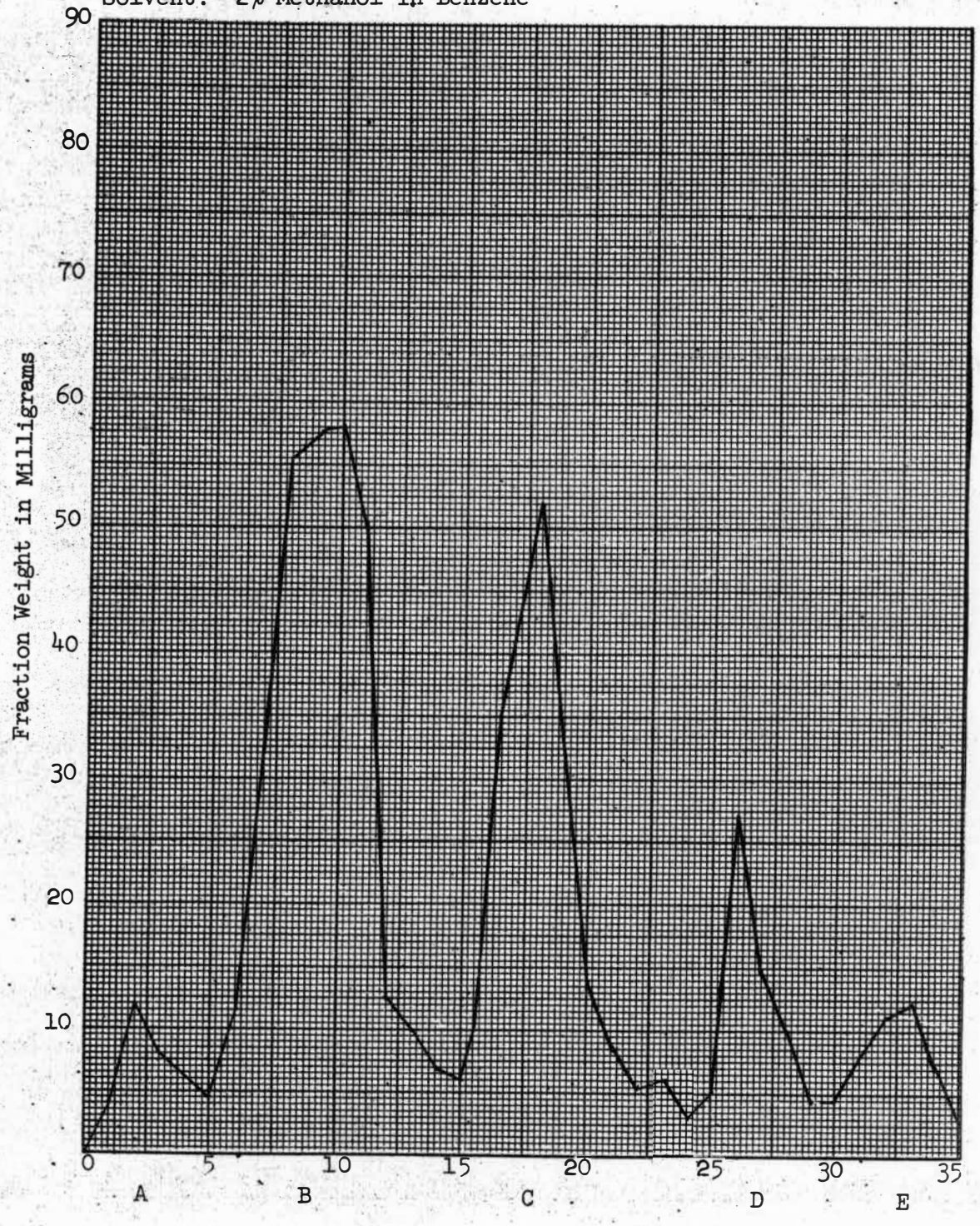
A typical plot of the weight of fractions versus the fraction number is shown by Figure 6. Several other components, other than the two HBr active unknowns have separated. Outright physical examination of the fractions make this obvious since the physical appearance of these fractions varied according to the place of elution from the column. Fractions A, D, and E were yellow liquids, similar in appearance to the sample esters. Fraction B was a yellow, waxy solid and fraction C was a white, waxy solid. Thin layer analysis of these fractions showed complete

FIGURE 6

Fraction Weight Distribution

Liquid Partition Column

Solvent: 2% Methanol in Benzene



separation of several components while other fractions remained as mixtures. Figure 7 is a drawing of a thin layer plate showing the separation achieved in the liquid partition column. One fraction, "B", consisted of a mixture of fractions A, C, and D. The peak corresponding to this fraction was probably due to overloading of the column. Subsequent re-chromatographing of this material on another column proved this theory correct, since the components now separated.

Although four relatively pure components were isolated using this liquid partition chromatographic system, infrared spectra showed absorption in the 2 - 3 μ range in only two of the components. One compound gave a spectrum showing strong hydroxy absorption while the other showed some absorption but a much weaker variety.

A total of eleven runs were made using the liquid partition column. Some of these runs included re-chromatograms of previously chromatographed material. If thin layer chromatography indicated a great proportion of the unknown HBr active compound still mixed with other components, that particular fraction was re-chromatographed.

Approximately 1.95 g. of "pure material" was separated, all of which showed some sort of hydroxy absorption by infrared spectroscopy.

Characterization of the Unknown Hydrogen Bromide Active Fatty Acid

Physical Characteristics of the Unknown Compounds

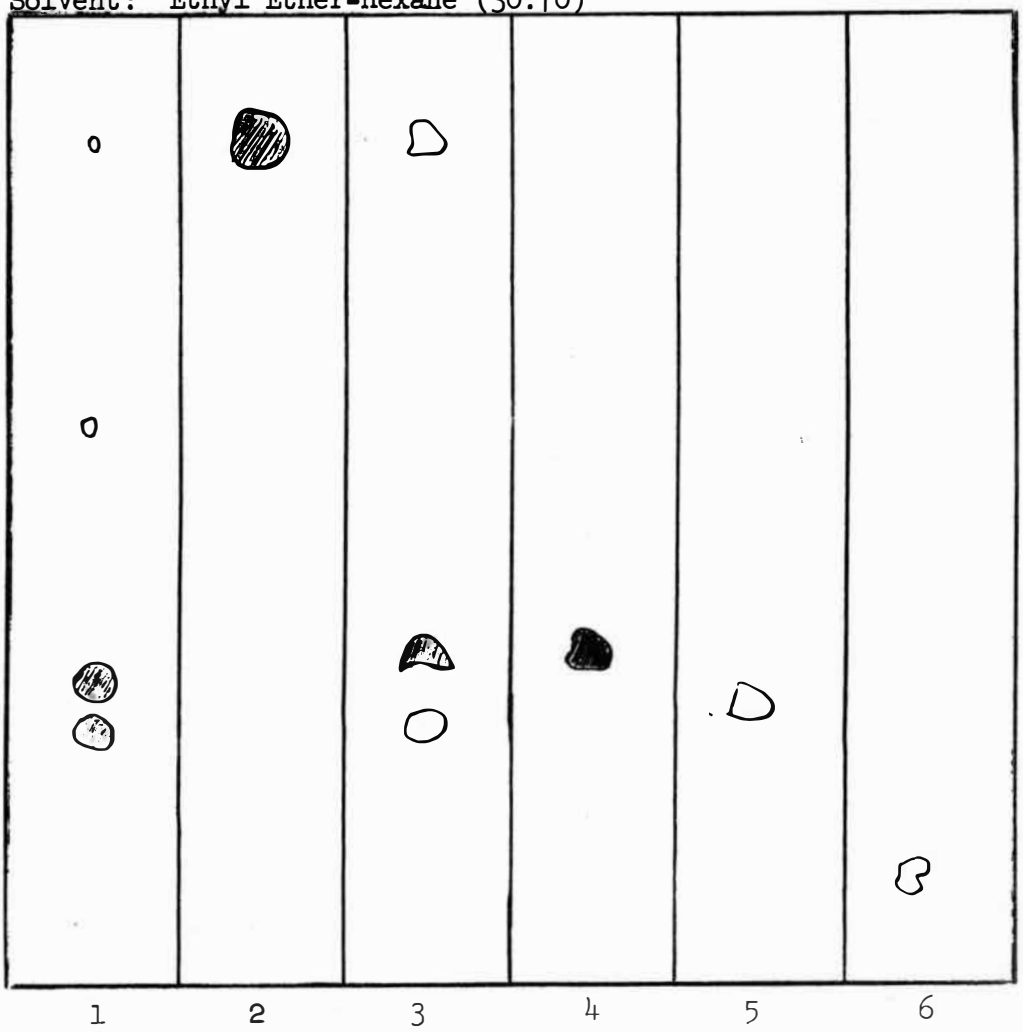
The 1.95 g. of material separated was composed of two components. Compound A accounted for about 900 mg. of the sample. This material

FIGURE 7

Thin Layer Chromatography Plate of Fractions

from Liquid-Partition Column

Solvent: Ethyl Ether-Hexane (30:70)



1 Reference Solution of Unknowns
2 Peak A
3 Peak B
4 Peak C
5 Peak D
6 Peak E

was a white, waxy solid which melted between 82.5° and 85.0°. This compound was soluble in all the common organic solvents. Elemental analysis of compound A showed C = 77.7%, H = 11.9%, O = 11.2%. An empirical formula of $C_{37}H_{67}O_4$ was calculated from this percentage composition. A molecular weight determination yielded a weight for compound A of 581. This compared favorably with the molecular weight of 575.9 calculated for $C_{37}H_{67}O_4$.

Compound B consisted of 1.05 g. of material and was a yellow, viscous liquid, similar in appearance to the unseparated methyl esters. Elemental analysis of compound B showed C = 68.7%, H = 10.1%, O = 22.9%

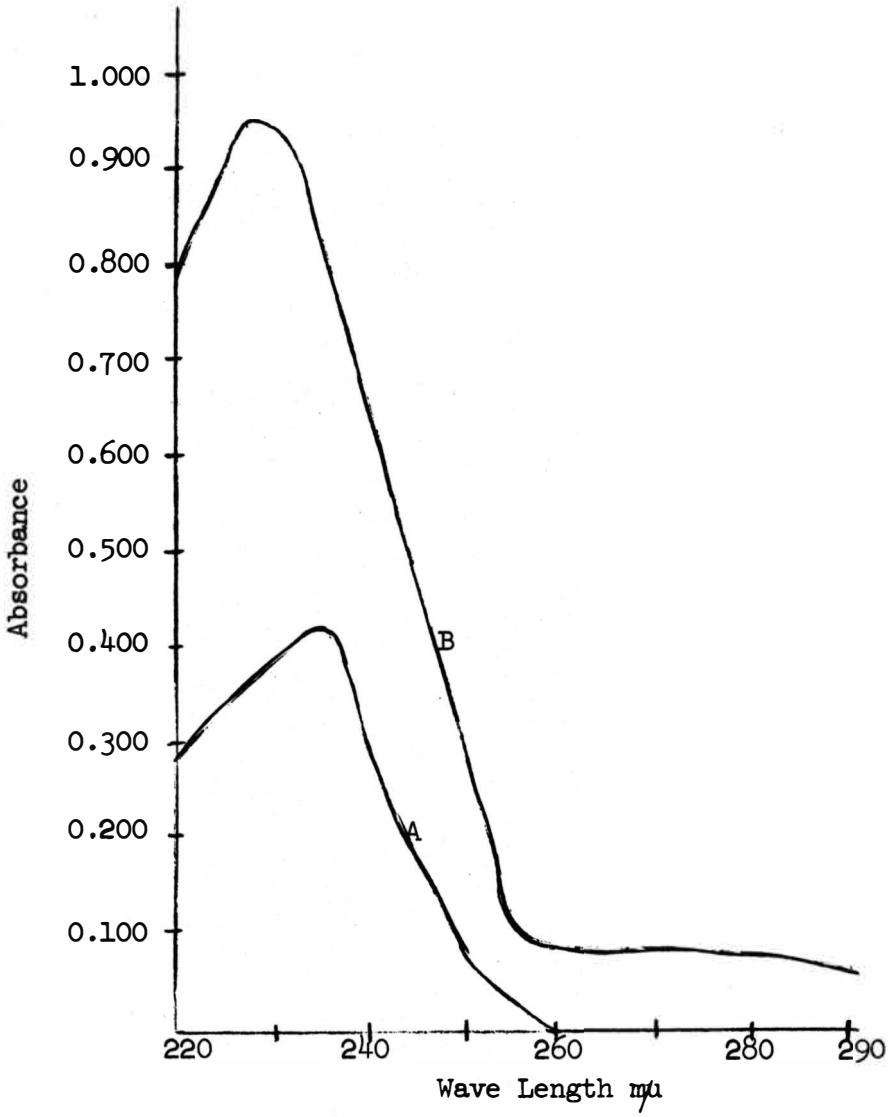
Ultraviolet and Infrared Spectra of the Unknown Compounds

The ultraviolet spectrum of sample A showed absorption at 234 mμ with an $E^{1\%}_{1\ cm.}$ of 412.0. Sample B showed absorption at 227 mμ with an $E^{1\%}_{1\ cm.}$ of 184.4. $E^{1\%}_{1\ cm.}$ is defined as the absorbance of a 1% solution in a 1 cm. light path. Diagrams of these curves are shown in Figure 8.

The presence of absorption around 230 mμ indicates the presence of a conjugated diene[8]. However, this is not a certainty since very small amounts of conjugated acids present as an impurity can give a large absorption in this region. Examples of this fact are the spectra of nominally pure linoleic and lineolenic acid which should be transparent above 220 mμ, yet often show absorption around 230 mμ. This absorption is due to the presence of small amounts of conjugated diene impurities in the linoleic and linolenic acid samples.

FIGURE 8

Ultraviolet Absorption Curves



Fraction	Concentration
A	1.0×10^{-3}%
B	5.0×10^{-3}%
Solvent:	Ethanol

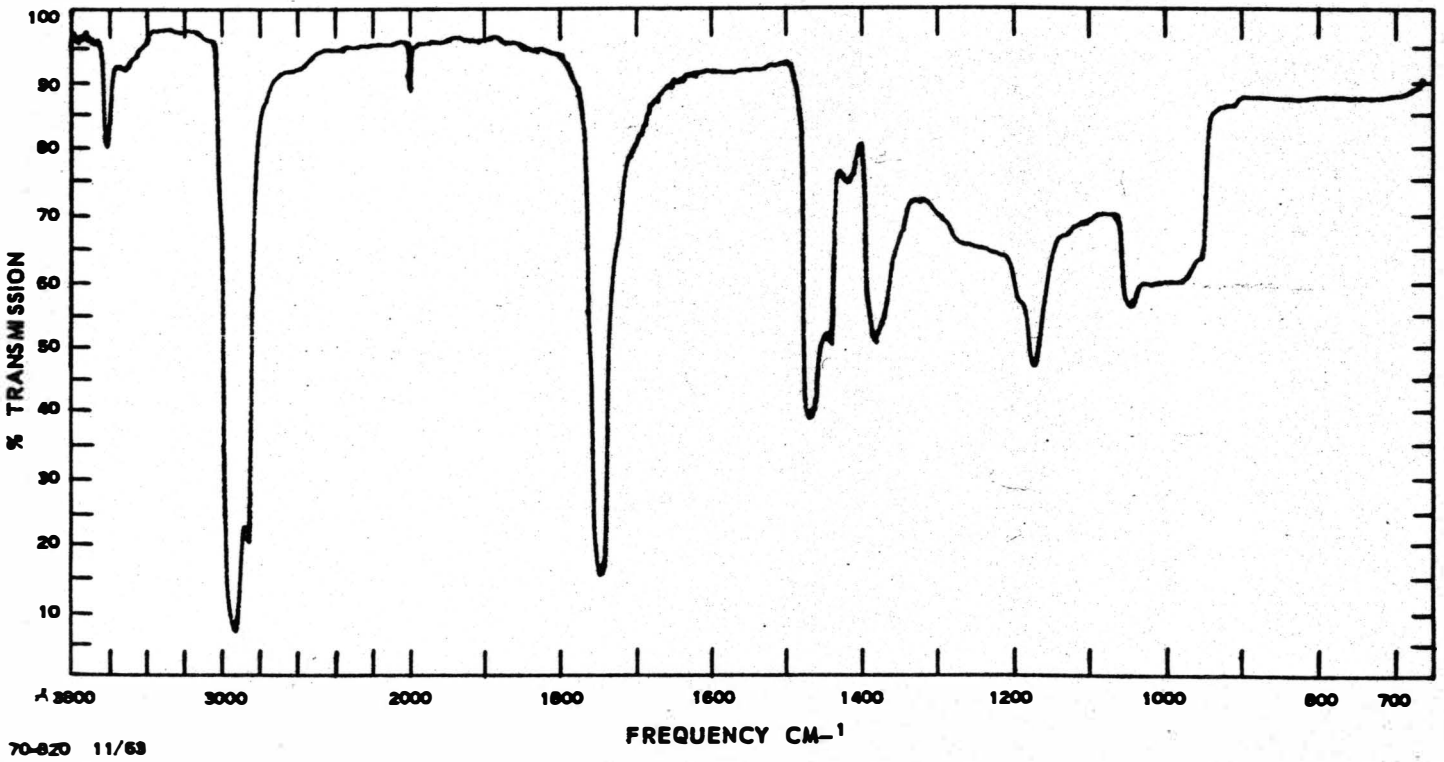
The infrared spectra obtained for compound A and compound B are shown in Figures 9 and 10 respectively. For comparison the spectrum of methyl ricinoleate (12-hydroxy-cis-9-octadecenoic acid) obtained under the same conditions is shown in Figure 11. Only a slight difference between this spectrum and the spectrum of compound A can be noted. A listing of some of the major and minor peaks and shoulders of absorption for compound A and compound B is given in Table 4 and Table 5 respectively.

Gas Chromatographic Characterization of the Unknown Compounds

Normally the identification of components on a gas chromatograph is established by the comparison of retention times or retention volumes with those of standards. In the screening of seed oils, it was necessary to obtain a value characteristic of each fatty acid which remained constant regardless of changes in experimental conditions. Thomas K. Miwa[12] reported on such a system. He characterized mono- and dicarboxylic methyl esters of fatty acids with a readily reproducible numerical constant. For a specific column packing and carrier gas these constants that he called "equivalent chain lengths" are independent of experimental conditions. A reference curve was established by plotting on a semi-log graph, the retention times (log scale) of two or more known normal, saturated monocarboxylic methyl esters against the number of carbons in the acid. A combination of two values obtained by use of a polar and non-polar packing was sufficient to characterize most of the fatty acids.

FIGURE 9

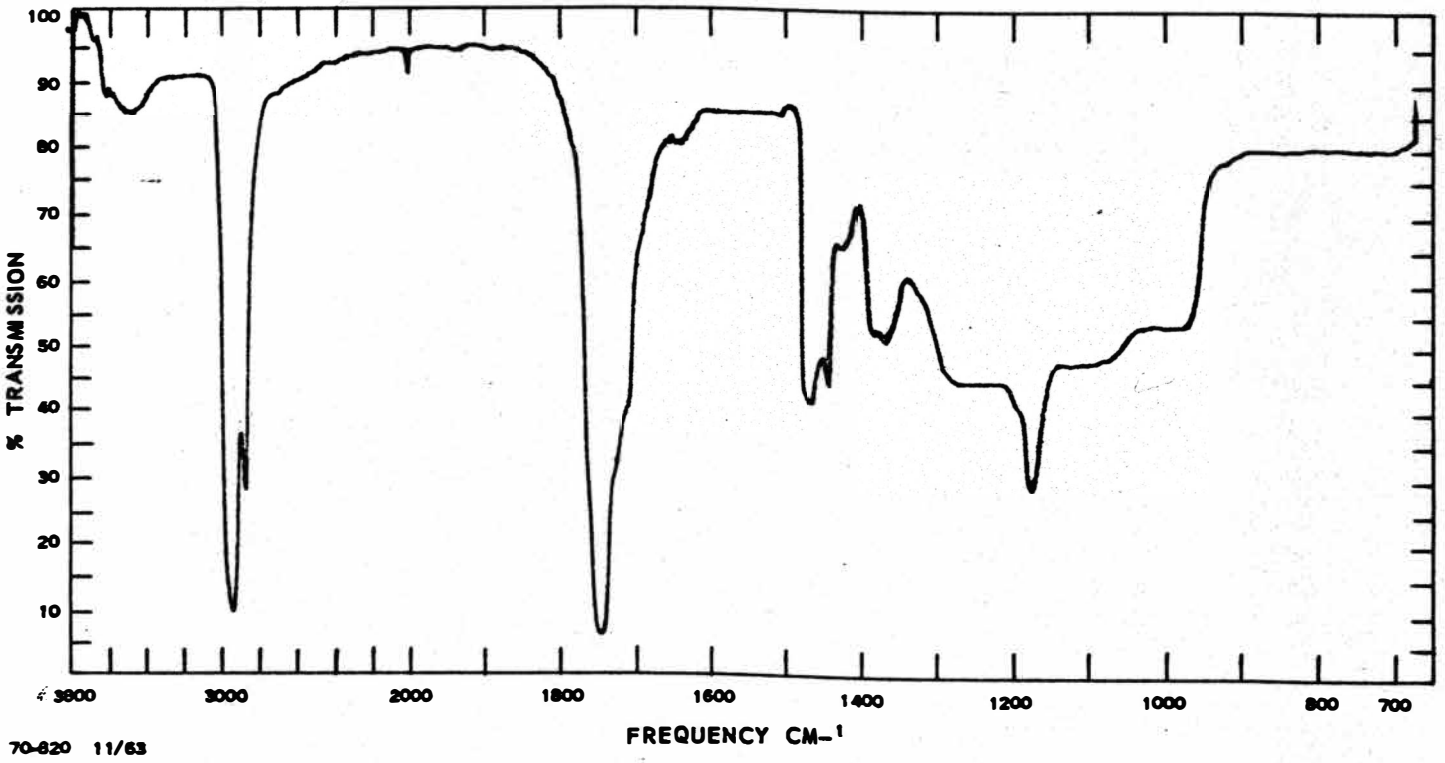
Infrared Absorption Spectrum for Compound A



Solvent: Carbon Tetrachloride

FIGURE 10

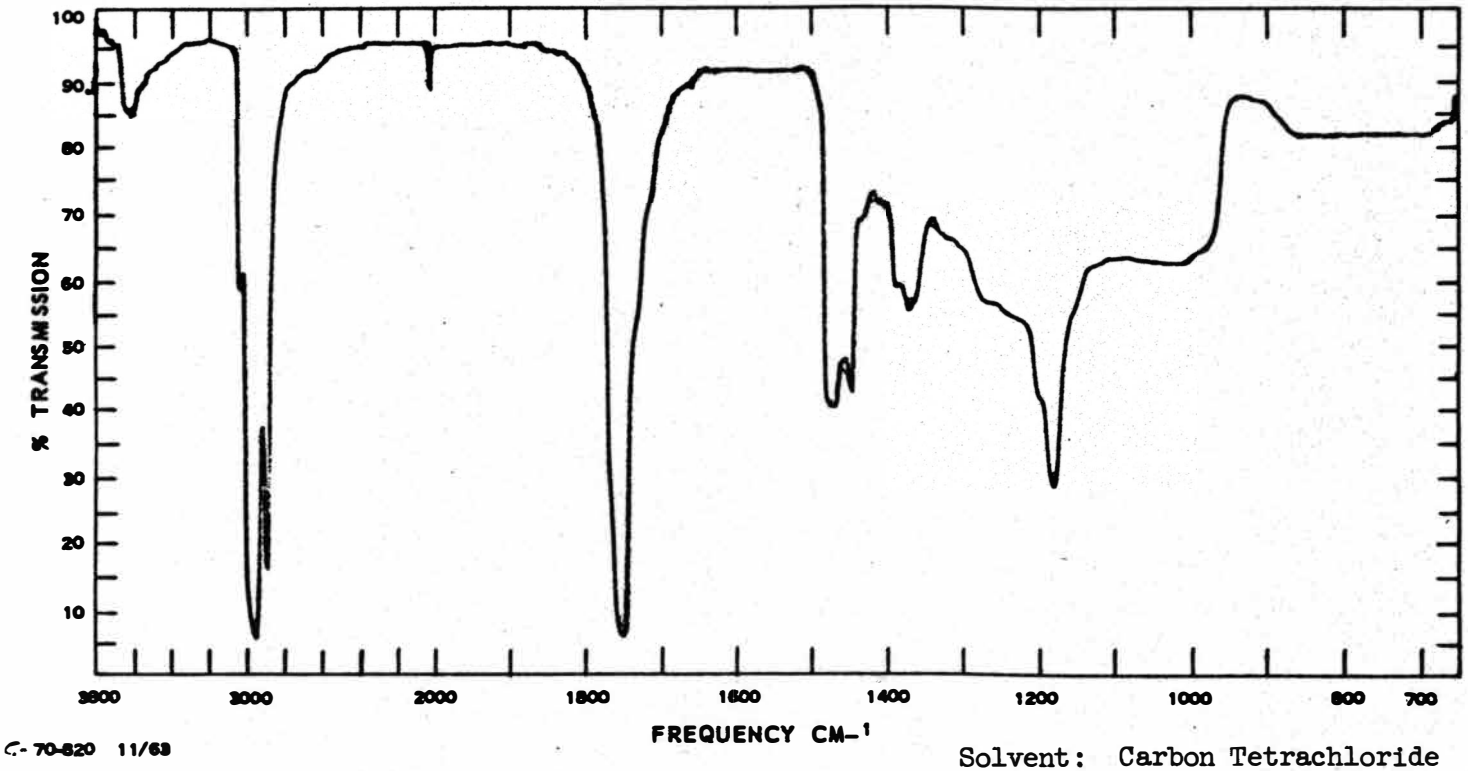
Infrared Absorption Spectrum for Compound B



Solvent: Carbon Tetrachloride

FIGURE 11

Infrared Absorption Spectrum for Methyl Ricinoleate



Solvent: Carbon Tetrachloride

TABLE 4

Major and Minor Peaks of Absorption

In The Infrared Spectrum of Compound A

Wave Length cm.$^{-1}$	Absorbance	Remarks	
3610	Strong	Free	Hydroxyl
2910	Strong	$-CH_2-$	Stretching
2840	Strong	$-CH_2-$	Stretching
1736	Strong	C=O-O	Ester Carbonyl Stretching
1460	Strong	$-CH_2-$	Bending
1441	Weak		
1431	Medium	$-CH_2-$	Bending
1411	Weak		
1375	Medium	$-C-CH_3$	Group
1363	Medium		
1190	Strong	C-O	Bonds stretching in methyl esters
1166	Strong		
1048	Strong		
1014	Weak		
979	Weak		
945	Medium		

TABLE 5

Major and Minor Peaks of Absorbance

In The Infrared Spectrum of Compound B

Wave Length cm.$^{-1}$	Absorbance	Remarks	
3530	Medium	OH	Bonded
2935	Strong	$-CH_2-$	Stretching
2860	Strong	$-CH_2-$	
1736	Strong	$-\overset{O}{\overset{\|}{C}}-O$	Ester Carbonyl Stretching
1635	Medium	C=C	
1460	Strong	$-CH_2-$	Bending
1453	Strong	$-CH_2-$	
1431	Strong	$-CH_2-$	Bending
1420	Medium		
1375	Strong	$-C-CH_3$	Group
1363	Strong		
1190	Strong	C-O	Bonds stretching
1166	Strong	C-O	in methyl ester
979	Weak		
945	Weak		

Compound A gave an ECL of 19.5 on the Apiezon L column and 23.0 on the LAC 446 column. Component B gave three peaks on each column. On the Apiezon L column these three peaks correspond to ECL's of 17.9, 19.5, and 21.8. On the polar LAC 446 column, the ECL values were 23.0, 26.3, and 27.3. This showed that component B was not pure as thought, but a mixture of compound A and two other materials. For this reason no further work was done on component B.

Nuclear Magnetic Resonance Analysis

Intrepretation of the nuclear magnetic resonance spectrum of Compound A showed several things.

1. The compound contained about seventy hydrogen atoms.
2. The presence of two, and possible three, methyl groups of the type CH_3-CH_2-alkyl.
3. A great deal of absorption due to different types of chains.
4. Two hydroxyl groups present (138 cps.).
5. A methyl ester linkage CH_3-O-$\overset{O}{\overset{\|}{C}}$- (220 cps.).
6. Two peaks at about 208 cps. and 249 cps. each thought to be due to a proton suggesting a structure such as -$\overset{H}{\underset{O}{C}}$- although other assignments are possible.
7. A complex region probably due to five vinyl protons.

SUMMARY AND CONCLUSION

The work in this paper describes the separation of a previously unreported fatty acid as a constituent of sunflower oil. Proceeding on the assumption that the hydrogen bromide uptake in sunflower oil was due to a hydroxy fatty acid as suggested by L. J. Morris[14] and C. R. Smith, Jr.,[19] rather than an epoxy fatty acid as normally would be indicated by this reaction, separation of the methyl esters was attempted.

By using an alumina column for a rough separation and a liquid partition column for the final purification, two compounds were obtained. The progress of separation of these components was followed by the use of thin layer chromatography and infrared spectral analysis.

In order to determine the structure of these compounds, the ultraviolet spectra of the two compounds were obtained. As noted, the ultraviolet spectra of both compounds indicated the presence of a conjugated diene moiety although this could not be definitely proved by ultraviolet absorption alone.

The infrared spectra of the two compounds showed that both compounds contained hydroxyl groups. Further analysis of the spectra showed that both of the compounds were esters, however, the spectra did not show any type of unsaturation in either compound. This fact did not rule out the presence of unsaturation, since in some molecules, e.g., methyl ricinoleate, unsaturation is not always seen in the infrared spectra. A comparison of the spectrum of compound A with the spectrum of methyl ricinoleate showed little difference.

Gas chromatographic analysis of the two compounds showed that compound B was in fact a mixture rather than a pure compound. The ECL data obtained on compound A did not correspond to any data of any known fatty acid as previously reported by Miwa.[12]

A high melting point indicated that the fatty acid was longer than the normal eighteen carbon fatty acids. Elemental analysis of compound A yielded results that indicated an empirical formula, $C_{37}H_{67}O_4$ which has a calculated molecular weight of 575.9. A molecular weight determination on compound A gave a value of 581 which agreed very closely with the theoretical result.

Nuclear magnetic resonance analysis added to the knowledge of compound A by indicating the presence of two hydroxy groups as well as confirming the presence of unsaturation in the compound. The NMR spectrum also indicated that approximately seventy hydrogen atoms were present in the compound. The possible existence of a vinyl carbinol structure was also indicated by the NMR spectrum as well as the possibility of branched chains.

In summary it can be stated that a methyl ester of a C_{36} dihydroxy unsaturated fatty acid has been isolated from sunflower oil. The determination of the exact position of the double bonds and the hydroxyl groups depends upon a more complete and complicated analysis of the compound.

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VITA

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The author is married and the father of three children.

APPROVAL OF EXAMINING COMMITTEE

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(Chairman)

Date _____