CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF UNSATURATED FATTY ACIDS

by

ALFRED THEODORE ERICSON

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INTRODUCTION

Rapid and complete resolution of fatty acid mixtures is an important problem that is yet unsolved. Many methods of chemical separation have been attempted but since the fatty acids differ very slightly in chemical properties from one another the separations based on chemical properties alone are neither rapid nor complete.

Recent experiments have indicated that separations based upon newly developed physical techniques should prove successful. The method of chromatography has been utilized in attempts to separate complex mixtures of saturated fatty acids. Oxidation and polymerization products have also been separated from fatty acids chromatographically. Even the purest unsaturated fatty acid samples that are now available and prepared by chemical methods, have been divided chromatographically into fractions having different iodine numbers. This would indicate that a chromatographic separation of unsaturated fatty acids should be possible.

Development of a rapid, quantitative separation of unsaturated fatty acids is highly desirable and as the method of chromatography seems to hold much promise, an investigation of the chromatographic separation of the unsaturated fatty acids was initiated.

HISTORY

Much research has been conducted in an attempt to find a rapid quantitative separation of fatty acids. The techniques utilized in these separations generally depend upon distillation, solubility, or adsorption of the fatty acids as such or after converting into esters, salts or halogenated derivatives. The present methods have been developed over a long period of time and although they are now more reliable than they were in the past they leave much to be desired in the way of simplicity and accuracy.

Chromatographic analysis has been applied to the separation and purification of various components of fats and oils. The method, in general, comprises solution of the material to be separated in a suitable solvent and passing the solution through a column of adsorbent. A difference in the degree with which different materials are adsorbed from the solvent by the column results in a separation of the materials.

A survey of the theory and application of ~~recent~~ adsorption procedures to problems in chromatography has been presented by Zeichmeister (20). In a ~~recent~~ article Williams (19) discusses the main advances in the application of chromatography to the analysis of fatty oils and emphasizes the principles which are involved. Deuel and Slater (8) in discussing the chemistry of lipids reviewed briefly the recent developments in fatty acid separations by chromatography, counter-

current distribution, and formation of urea addition complexes.

H. G. Cassidy (3) separated mixtures of higher saturated fatty acids chromatographically. In this separation carbon was used as the adsorbant and petroleum ether as the developer. It was found that a partial separation of lauric, stearic, and palmitic acids could be effected rapidly while a more complete quantitative analysis required a much longer period of time.

Attempts of chromatographic separation of fatty acids always meets with the problem that fatty acids are colorless. Graff and Skau (10) mixed phenol red with heavy magnesia before utilizing the magnesia as an adsorbant. When the fatty acid mixture came in contact with this adsorbant, yellow bands formed due to color changes of the phenol red. Graff and Skau were successful in separating an unsaturated fatty acid from a saturated one by this method. The separation was followed by the development of colored bands.

In an attempt to find an easy method of predicting if a particular adsorbant-solvent system could be utilized in the chromatographic separation of fatty acids, Cassidy (4) suggested the utilization of adsorption isotherms. This technique is based on the determination of the relative degree of adsorption of the pure fatty acids on the adsorbant. In a later work Cassidy (5) showed that the adsorption isotherms obtained for fatty acids from their pure solutions are of uncertain value as the fatty acids behave differently when mixed than in the pure state.

A method of separation of C_{10} to C_{19} saturated fatty acids by utilizing partition chromatography has been proposed by Ramsey and Patterson (16). The acids were separated on a column of silicic acid with methanol as the immobile solvent and 2,2,4 trimethyl pentane as the mobile solvent. Bromcresol green was used as an indicator. This method appears to be suitable for routine use in the study of the composition of certain fats.

R. T. Holman (11) used the method of displacement carrier chromatography to successfully separate mixtures of saturated fatty acids. Holman (12), in a preliminary note, reported the separation of unsaturated fatty acids by displacement carrier chromatography.

Reimenschneider et al,(17) has utilized chromatography for preparing samples of highly purified fatty acid from naturally occurring products. Spectroscopic investigation of these purified fatty acids indicates a higher purity than that of fatty acids purified by standard chemical methods.

Countercurrent distribution has been utilized by Craig (6) in a quantitative separation of complex mixtures of saturated fatty acids. This method involves distribution of the fatty acid sample between two immiscible liquids. As some of the fatty acids are dissolved more readily in one of the solvents than in the other, separations result when an upper phase containing the sample is passed countercurrent to the lower phase in the special apparatus utilized by Craig, et al, (7).

In work published after this investigation was initiated

Ahrens and Craig (1) succeeded in separating a complex mixture of oleic, linoleic, and linolenic acids by the method of countercurrent distribution. They used the method successfully to analyze a complex mixture of pig mesenteric fat. More recently Cannon, et al,(2) also successfully utilized the method of countercurrent distribution to separate a complex mixture of unsaturated fatty acids.

The results of the experiments utilizing adsorption techniques that have been referred to leads one to conclude that a separation of complex mixtures of fatty acids based upon adsorption methods should prove possible. Since Dutton (9) experienced considerable success in separating binary mixtures of methyl esters of fatty acids on an alumina column, it was proposed that chromatographic separation of unsaturated fatty acids on an alumina column be the aim of this investigation.

Several problems must be solved to achieve this goal. Preliminary experiments must be conducted: (a) to determine an adsorbant-solvent system that holds promise for such a separation, (b) to select a method of analysis for the presence of the different unsaturated fatty acids, and (c) to develop a method for the preparation of fatty acid samples for chromatographic analysis. The method which would be developed would then be utilized in attempts to separate natural and synthetic mixtures of fatty acids. The synthetic mixtures employed should prove valuable for determining the pathway of the resulting separations.

MATERIALS AND METHODS

The chromatographic column used in the separation of the unsaturated fatty acids was prepared by placing 30 ml. of purified acetone in a 50 ml. burette. The acetone was purified by distillation. When the more volatile two-thirds of the acetone was used, no interference with subsequent analysis resulted whereas, if the acetone was not purified in this manner, interference occurred. A glass cloth plug was placed in the bottom of the burette and tamped firmly into place with a glass rod. The purpose of the glass cloth was to prevent the fine particles of alumina from passing through the burette. Chromatographic alumina, Al-0109p, obtained from the Harshaw Chemical Company, was introduced into the burette through a funnel and then packed to give a uniform column by tapping the sides of the burette with a rubber mallet. It was found that 22 gms. of alumina were required for the preparation of a 10 inch column. This amount was used in all the experiments described. After the alumina was packed, the rate of flow of acetone through the column was adjusted to approximately 1 ml./minute. The column was then washed with 25 ml. of purified acetone before introducing the sample. When 2 ml. of acetone remained above the alumina column, the fatty acid sample dissolved in 5 ml. of purified acetone was introduced into the column.

In the chromatographic separation of the unsaturated fatty acids of corn oil the following method was utilized

in sample preparation. A 50 ml. sample of Mazola oil was saponified with alcoholic potassium hydroxide. The alcoholic potassium hydroxide solution was prepared by dissolving 75 gms. of reagent grade potassium hydroxide in 50 ml. of water followed by addition of 250 ml. of alcohol to the solution maintained at a temperature of 0° C by means of an ice bath. The mixture was placed under nitrogen and stored in the refrigerator over night at a temperature of 0° C. The saponification mixture was neutralized to litmus paper with sulfuric acid (1:4 by volume). This mixture was then diluted with warm water and the oily free fatty acid layer separated from the rest of the solution. This oil was separated from the rest of the solution by decantation, washed three times in a separatory funnel with warm water, and then stored at 0° C under nitrogen.

The corn oil fatty acids were separated from the pigments present in the Mazola oil by means of urea adducts. Ten ml. of the oily material from the saponification containing the fatty acids and the nonsaponifable substances was dissolved in 10 ml. of di-isopropyl ketone and stirred with 100 ml. of a urea solution for one half hour. This urea solution was a saturated solution prepared at 25° C and contained 10 gms. excess solid urea. The solid adduct which was formed was filtered from the solution and washed with three 10 ml. portions of di-isopropyl ketone. The solution was dried by spreading it out on filter paper. The adduct was decomposed by stirring with 50 ml. of hot water. The water

decomposed the urea adduct and the fatty acids were recovered in the upper layer. The fatty acids were washed with three 30 ml. portions of hot water and the remaining di-isopropyl ketone was removed under vacuum. The sample was prepared by dissolving .2 ml. of this corn oil fatty acid extract in 5 ml. of acetone.

Iodine numbers were determined by the following method. About .2 gm. of the fatty acid was accurately weighed and placed in a 500 ml. glass stoppered bottle. This sample was dissolved in 5 ml. of ethylene chloride, and 15 ml. of Hanus iodine solution was added. The mixture was stored in the dark for one half hour to complete the reaction. Ten ml. of 15% potassium iodide solution was added to the mixture followed by thorough shaking of the mixture. This solution was then diluted with 100 ml. of freshly boiled and cooled water and then titrated with .1 normal sodium thiosulfate. The grams of iodine adsorbed by 100 gms. of fat is the Hanus iodine number (15).

Highly purified fatty acids obtained from the Hormel foundation were used when artificial mixtures of unsaturated fatty acids were prepared. The Wijs iodine values of these fatty acids as determined by the Hormel foundation are listed in Table 1. The fatty acids were dissolved in 5 ml. of acetone to prepare the mixture for chromatographic analysis.

Table 1. Iodine numbers of fatty acids used in sample preparation

Acid	Iodine No.	Theoretical Iodine No.
Oleic acid	89.28	89.87
Linoleic acid	180.8	181.03
Linolenic acid	271.1	273.51

The sample was washed into the column with two 5 ml. potrions of acetone. The column was then washed with an eluting solution and the fatty acid was forced through the column. The eluting agent was placed in a separatory funnel reservoir above the column (Plate 1 and 2). A constant rate of liquid flow through the column was obtained by maintaining a constant liquid level above the column. The separatory funnel was connected to the burette with a short length of rubber tubing. The air pocket formed between the stem of the separatory funnel and the burette prevented reaction between the acetone-acetic acid mixture and the rubber tubing.

Samples were collected on a time basis. The rate of flow was adjusted, unless otherwise indicated, to give 10 ml. samples at 10 minute intervals. The first sample was collected when the eluting agent was added to the column.

In the first chromatographic experiments conducted, the samples were collected manually (Plate 1). This required the changing of sample bottles each 10 minutes. After collecting the samples, the sample bottles were filled with nitrogen and

Explanation of Plate I

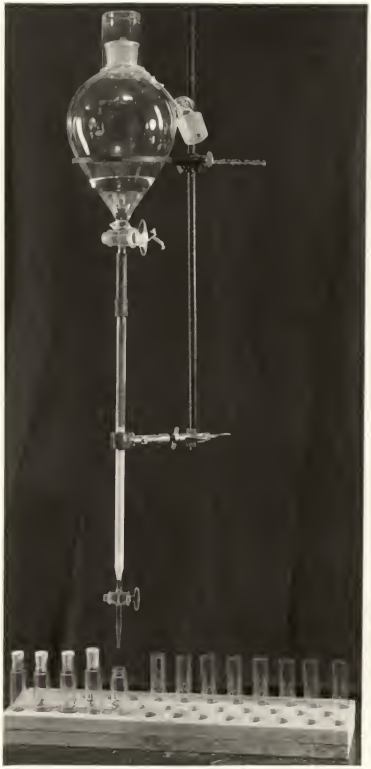
Diagram of apparatus utilized in the chromatographic investigations during which samples were collected manually.

A. Tube connecting reservoir and column.

B. Alumina column.

C. Glass cloth filter.

PLATE I



and stored. After all samples were collected the eluting agent was removed by heating the sample in warm water (60° C) under vacuum. After the evaporation had been completed it was possible to ascertain visually which sample bottles contained the fatty acid. In all cases no fatty acid was observed in samples number 1 through 15 thus eliminating need for further analysis of these samples.

In later experiments the method of sample collection was mechanized. The Technicon Time Flow Fraction Collector (Plate 2) was used, and sample tubes were mechanically changed at definite time intervals. The apparatus was attached to a vacuum line to keep air circulating through it constantly. The samples were stored under nitrogen after the run was completed. Samples were evaporated under vacuum in a dessicator heated with an infrared heat lamp.

Each sample was dissolved in 5 ml. of highly purified n-hexane preparatory to determination of optical densities at selected wavelengths. The n-hexane was obtained from Phillips Petroleum Company and purified as follows. One liter of 95 molecular per cent n-hexane was stirred with 300 ml. of concentrated sulfuric acid for 2 days. The n-hexane was separated from the sulfuric acid and washed with 500 ml. of water. The n-hexane was stirred with 500 ml. of a 20 per cent sodium hydroxide solution saturated with potassium permanganate for two days. The n-hexane was separated from the sodium hydroxide solution and washed with three 500 ml. portions of distilled water. The n-hexane was dried over anhydrous

Explanation of Plate 2

Picture of the Technicon Time Flow Fraction Collector.

PLATE II

sodium sulfate and distilled. This purified n-hexane proved a satisfactory solvent for the fatty acids in the spectral regions studied.

The ultraviolet spectra of the highly purified fatty acids and the corn oil fatty acid extract was carefully determined to find the wavelengths most suited for the spectral analysis of all samples collected in the chromatographic runs. A solution of .1 per cent fatty acid sample in highly purified n-hexane was prepared. The optical densities of the sample were determined between 300 and 222 millimicrons at increments of 2 millimicrons (Plate III). The corn oil fatty acid extract exhibited optical density peaks at wavelengths of 278, 268, and 258 millimicrons. Determination of optical densities of samples collected in chromatographic studies at these wavelengths should prove valuable as an indication of the amount of fatty acid present. The ratio of optical density at 228 to optical density at 258 are significantly different for the 3 different purified fatty acid samples and therefore, determination of optical densities at these two wavelengths should prove valuable as an indication of the particular fatty acid present in the samples investigated. From this experiment it was decided to analyze the fatty acid samples from the chromatographic runs at wavelengths of 228, 258, 268, and 278 millimicrons. If the optical density at any of these wavelengths was too high for an accurate determination the sample was further dilluted with highly purified n-hexane and the original optical density calculated.

Explanation of Plate III

Ultra violet spectra of fatty acid samples utilized in chromatographic investigations.

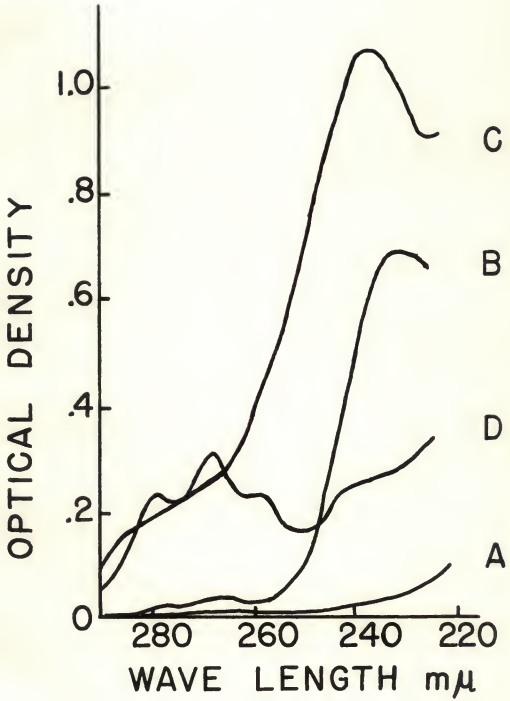
A. Oleic acid

B. Linoleic acid

C. Linolenic acid

D. Purified corn oil fatty acids

PLATE III



The method of analysis was illustrated with a chromatographic experiment using a sample of the corn oil fatty acid extract. When the optical density at constant wavelength was plotted against sample number, the resulting curve exhibited optical density peaks. (Plate IV). This indicated a separation. In some of the experiments conducted with artificial fatty acid mixtures the ratio of optical density at a wavelength of 228 millimicrons to the optical density at a wavelength of 268 millimicrons was plotted to illustrate graphically the separation. The ultraviolet spectra from 290 to 225 millimicrons of selected samples exhibiting optical density peaks or lying upon optical density plateaus were used to aid in determining the degree of separation. In several experiments the samples were evaporated in weighed sample bottles after spectral analysis and the weight of each sample was determined. This data was correlated with optical density data.

EXPERIMENTAL

Exploratory Experiments

A series of preliminary experiments were conducted to find an eluting agent which appeared suitable for a chromatographic separation of the unsaturated fatty acids.

The 25 cm. alumina chromatographic column was prepared as previously described. The column was washed with 25 ml. of Skellysolve B. A .5 ml. sample of corn oil fatty acids was

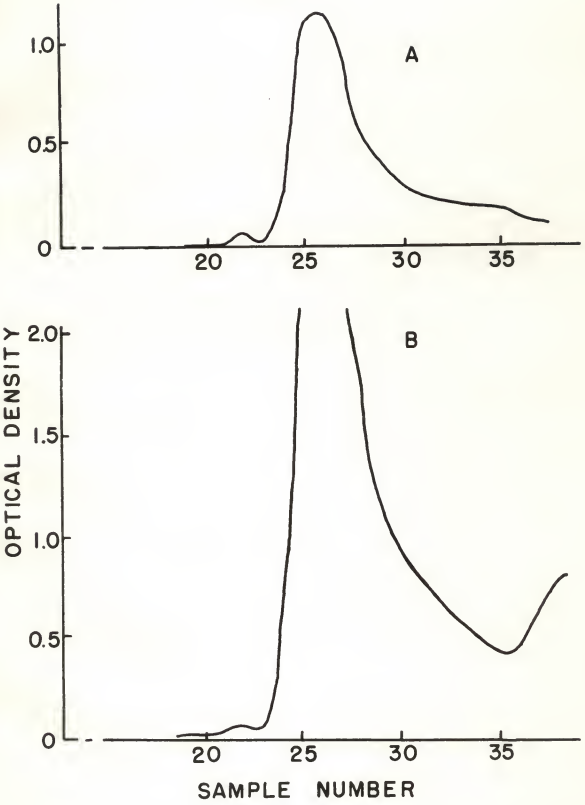
Explanation of Plate IV

Optical densities of samples collected in the chromatographic analysis of corn oil determined at wave lengths of:

A. 268 millimicrons

B. 228 millimicrons

PLATE IV



introduced into the column in Skellysolve B. The column was then eluted with 300 ml. of Skellysolve B. Upon determination of iodine numbers of samples collected it was found that no unsaturated fatty acids had been eluted.

In another experiment methyl alcohol was used in place of the Skellysolve B as the eluting agent. Iodine number determinations showed that the methyl alcohol would not elute the unsaturated fatty acids effectively.

The preceding experiment was repeated except a 15 per cent solution of ether in Skellysolve B was used in place of the Skellysolve B. Iodine number determinations showed no unsaturated fatty acid to have been eluted. The alumina column was carefully removed and divided into 7 equal portions. Iodine number determinations showed that the unsaturated fatty acids had moved down the column about 17 cm.

The chief problem in conducting the preceding experiments arose from the fact that the fatty acid sample developed no color as it passed through the column. By mixing the alumina with a small amount of phenolphthalien a red chromatographic column was prepared. This column was washed with 25 ml. of acetone. A .5 ml. sample of corn oil fatty acids was introduced into the column in 5 ml. of acetone. This fatty acid sample formed a white band at the top of the column. The column was eluted with 300 ml. of acetone. However, the fatty acids did not move down the column as indicated by the white band's stationary position.

The preceding experiment was repeated with the exception

that a small amount of water was added to the acetone. The white band indicated that this eluting agent moved the fatty acids down the column very little if at all.

It was decided that the addition of an organic acid, such as acetic, to a solvent such as acetone might be effective in eluting the fatty acids. It was reasoned that a stronger acid might force the weaker fatty acids off the column. A colored chromatographic column was prepared as previously described, and a .5 ml. sample of corn oil fatty acids was introduced into the column in 5 ml. of acetone. A one per cent solution of acetic acid in acetone was used as the eluting agent. The white band moved down the column. From iodine number determinations of samples collected it was found that unsaturated fatty acids were located at the front of the white band. Therefore, if the acetic acid-acetone mixture would not interfere with the analysis of samples, an acetic acid-acetone mixture should prove an effective eluting agent.

The preceeding experiment was repeated using in one case a .5 per cent solution of acetic acid in acetone and in the other case a .25 per cent solution of acetic acid in acetone as the eluting agent. The rate of movement of the white band of fatty acids down the column utilizing the .25 per cent solution of acetic acid appeared to be most desirable for the experiments to be conducted.

Since it was planned to utilize spectral analysis for determining the presence of unsaturated fatty acids in samples collected during chromatographic analysis, the following experiment was conducted to determine if the .25 per cent acetic

acid in acetone would interfere with spectral analysis. Ten ml. of a .25 per cent solution of acetic acid in acetone was evaporated under vacuum at 60° C in a 25 ml. sample bottle. Five ml. of highly purified n-hexane was then added to the tube and the optical densities at wavelengths of 228, 258, 268, and 278 was determined. It was found that the mixture of acetic acid in acetone would interfere with analysis (Table 2).

Table 2. Effect of purification of acetone upon optical densities.

A. Sample prepared from .25% acetic acid in acetone from stock solution.

B. Sample prepared from .25% acetic acid in acetone purified by passage through alumina.

C. Sample prepared from .25% acetic acid in acetone purified by distillation.

Wavelength millimicrons	Sample A	B	C
278	2.27	.427	.004
268	2.17	.412	.004
258	1.91	.392	.005
228	∞	1.10	.018

In an attempt to eliminate this interference the acetone was purified by the following methods: In the first method the acetone was percolated through a column of alumina. In the second method the acetone was distilled from a steam bath, the first two thirds of the acetone distilled being retained

for use as eluting agent. It was found that the acetone purified with alumina would interfere with spectral analysis while that purified by distillation would not interfere with spectral analysis (Table 2). Acetone used as eluting agent was therefore purified by distillation.

Separation of Corn Oil Fatty Acids from Pigments by Urea Adduct Formation

In investigating the chromatographic separation of the unsaturated fatty acids, the first problem was the separation of these acids from the impurities present in the fats and oils. These impurities would interfere with spectral analysis. Extracting the nonsaponifiable substances of the basic alcoholic solution resulting from the saponification of the oil (13) by means of ether is tedious and the separation is not considered satisfactory. Recently the method of separating aliphatic compounds by means of urea adduct formation has been used to partially separate the saturated and unsaturated fatty acids. (15) The separation is based upon the fact that linear, saturated compounds will form the adduct very readily, whereas unsaturated or branched chains will do so with difficulty or not at all. An investigation was initiated to determine if the unsaturated fatty acids could be satisfactorily removed from the colored impurities by urea adduct formation.

For this investigation corn oil was saponified with alcoholic potassium hydroxide and the fat-soluble material freed

from the resulting solution by dilution with water after neutralization in an ice bath with 20 per cent sulfuric acid. Corn oil was well suited to this investigation because the large amount of pigment present made the separation easy to follow. Ten milliliters of the fat-soluble material from the saponification were dissolved in 10 milliliters of di-isopropyl ketone and this solution stirred with 100 milliliters of a urea solution for one-half hour. The concentration of the urea solution employed in each case will be indicated subsequently. All urea solutions were prepared at 25 degrees C. The solid adduct was then filtered from the solution and washed with three 10 milliliter portions of di-isopropyl ketone. The solid was then spread on filter paper to dry.

The adduct was decomposed, preparatory to analysis, by stirring with 50 milliliters of hot water. The water decomposed the urea adduct and the fatty acids were recovered in the upper layer. The fatty acids were washed with three 30 milliliter portions of hot water and the remaining di-isopropyl ketone was removed under vacuum.

The ultraviolet spectra of the samples were determined by means of the Beckman, Model DU, Spectrophotometer. The results were reported on the basis of .1 per cent of fatty acid in highly purified n-hexane by volume. The Hanus iodine number was determined (15).

The following concentrations of urea were used in the first series. A saturated urea solution containing a large excess of solid urea, a saturated urea solution with no excess

solid urea, and a 90 per cent saturated urea solution. The iodine numbers of the fatty acids, after removal from the adduct, were determined in each case and the results are illustrated (Plate V). The iodine number of the fatty acids after separation from the adduct prepared from the 90 per cent of saturated urea solution was relatively low. This observation indicates that only the saturated fatty acids and part of the unsaturated fatty acids that are present in the corn oil fatty acid mixture were present in the adduct. With the saturated urea solution the iodine number of the fatty acids, after separation from the adduct, was increased slightly above that obtained with the 90 per cent urea solution. Therefore, more of the unsaturated fatty acids formed an adduct in the more concentrated urea solution. When the saturated solution contained a large excess of solid urea, the iodine number of the fatty acids, after removal from the adduct, was increased to that of the corn oil fatty acids prior to the adduct formation. From this it was concluded that all the corn oil unsaturated fatty acids had been removed in the adduct.

In all cases investigated it was found that the fatty acid extract was colorless. This showed that the pigments present in the naturally occuring corn oil did not form the adduct with urea.

The ultraviolet absorption spectra of the original corn oil, of the free fatty acids from the saponification of corn oil, and of the different extracts were determined. When at least ten grams of solid urea were present in the urea solution

Explanation of Plate V

Iodine Numbers of the fatty acids after removal from the urea adduct.

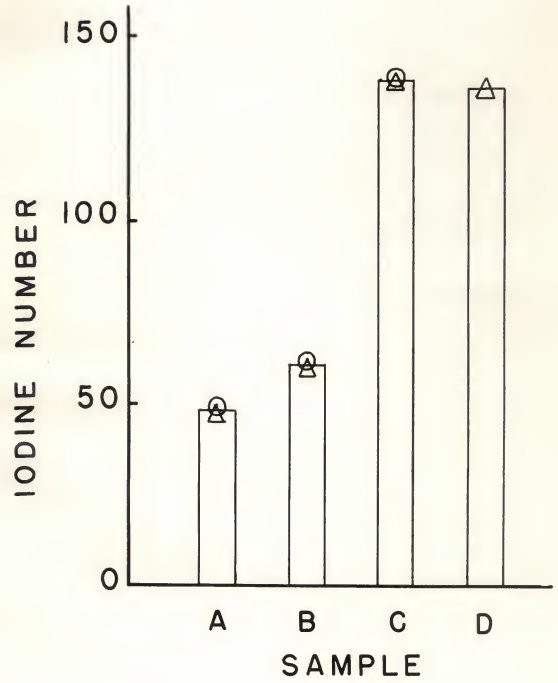
(A) 90 per cent urea solution.

(B) Saturated urea solution.

(C) Saturated urea solution plus large excess of solid urea.

(D) Free fatty acid mixture prior to urea treatment.

PLATE V



the spectra of the extract were the same as those of the original oil or the mixture of fatty acids prior to adduct formation (Plate VI). Therefore, all the unsaturated fatty acids of the corn oil were present in the adduct when large amounts of solid urea were present during adduct formation.

The iodine numbers determined in the first series indicated that a fractionation might be possible by varying the concentration of the urea solution. In a second series a more complete gradation of urea concentrations was used. Iodine numbers of the fatty acids after removal from the adduct are illustrated (Plate VII). It was found that when an 80 per cent saturated urea solution was used no adduct was formed either during the period of stirring the mixture or upon storing the mixture in the refrigerator overnight. When an 85 per cent saturated urea solution was used, no adduct formed during period of stirring the mixture, but the adduct did form when the mixture was stored in refrigerator overnight. The formation of adduct during storage in the refrigerator indicated that the reduction in temperature increased adduct formation, probably by increasing the degree of saturation of the urea solution. The iodine number of the fatty acid after it was removed from the adduct was determined and found to be relatively low. The value of the iodine numbers of the free fatty acid removed from the adduct showed increases similar to those of the first experiment. When the saturated urea solution contained ten or more grams excess of solid urea, the iodine number of the fatty acids, after separation from

Explanation of Plate VI

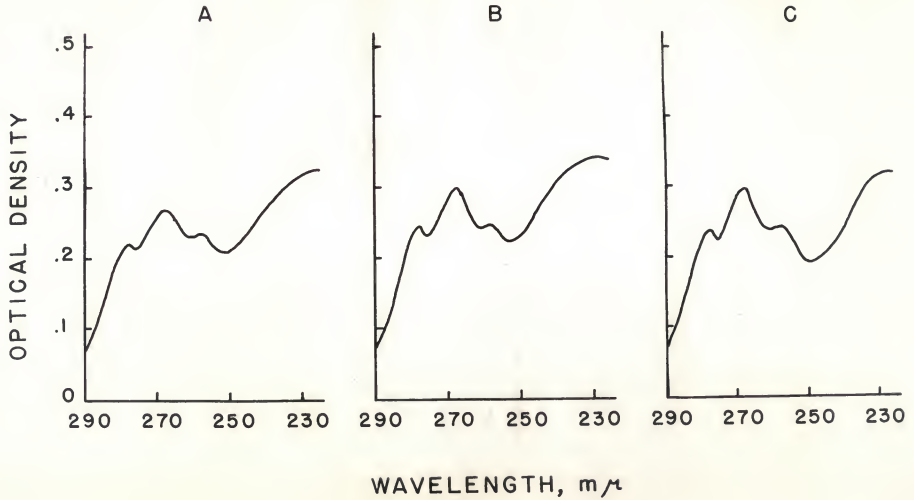
Ultra violet spectra of a 0.1 per cent solution in normal hexane.

(A) Corn oil.

(B) Free fatty acids from corn oil saponification.

(C) Free fatty acids after removal from adduct prepared using a saturated urea solution containing 10 grams excess solid urea.

PLATE VI


A
B
C
OPTICAL DENSITY
.5
.4
.3
.2
.1
0
290 270 250 230
290 270 250 230
290 270 250 230
WAVELENGTH, mμ

Explanation of Plate VII

Iodine numbers of the fatty acids after removal from adduct, zero values being used to indicate that no adduct formed.

(A) 80 per cent saturated urea solution stored in refrigerator over night.

(B) 85 per cent saturated urea solution.

(C) 85 per cent saturated urea solution stored in refrigerator over night.

(D) 90 per cent saturated urea solution.

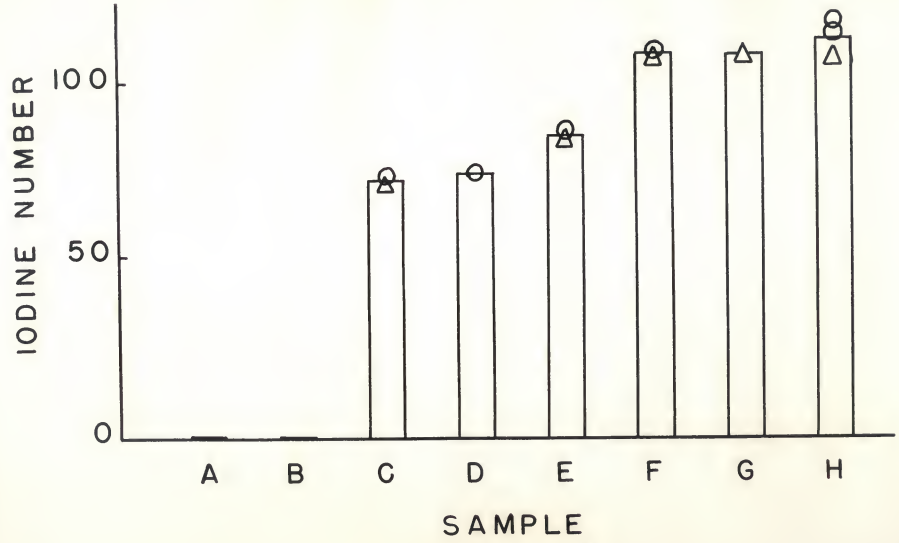
(E) Saturated urea solution.

(F) Saturated urea solution comtaining 10 grams excess of solid urea.

(G) Saturated urea solution containing large excess of solid urea.

(H) Free fatty acid mixture prior to urea treatment.

PLATE VII



the adduct, was found to be the same as that of the corn oil fatty acids before treatment with urea.

The ultraviolet spectra of the fatty acids, after separation from the adduct, was employed to determine if the adduct was subject to oxidation during storage. No significant change in spectra occurred during a six week period of storage. Therefore, the adduct can be stored for at least this period of time without oxidation of the fatty acids. Shlenk and Holman (18), upon the basis of peroxide values of stored adduct, reported similar results for a three week storage experiment.

Results in this investigation show that urea adduct formation is an effective means of separating fatty acids from the pigments present in corn oil. That fatty acids form the adduct more readily than these pigments can be explained on a basis of the urea forming adduct more readily with symmetrical molecules (21). Increasing the concentration of urea increases the probability that the unsaturated fatty acids will form the adduct, and a fractionation of unsaturated fatty acids by urea adduct formation might be possible by this method. When a saturated urea solution contains a large excess of solid urea, the adduct formed will contain the unsaturated fatty acids present in the original fatty acid sample, and a satisfactory separation from the pigments will be effected.

The optical densities of .05, .10, and .20 per cent solutions of the corn oil fatty acid extract in highly purified normal hexane was determined at wavelengths of 228, 258, 268

and 278 millimicrons (Table 3). It was found that the optical density varied directly with the concentration of the corn oil fatty acid extract in normal hexane, and therefore optical densities were utilized in the subsequent work as an analytical tool for determining the concentrations of fatty acid.

Table III. Optical densities of corn oil fatty acid extract with varying concentration.

Concentration	Wavelength Millimicrons			
	228	258	268	278
.05%	.157	.132	.158	.127
.10%	.301	.241	.301	.235
.20%	.575	.435	.492	.416

Chromatographic Separation of the Unsaturated Fatty Acids of Corn Oil

The separation of the corn oil fatty acids from the pigments present in the corn oil accomplished, the chromatographic separation of the corn oil fatty acid extract was now investigated.

Chromatographic experiments were conducted using .2 ml. of the corn oil fatty acid extract prepared by urea adduct formation dissolved in 5 ml. of acetone as the sample. A 25 cm. alumina chromatographic column was prepared by the method previously described. The column was washed with acetone preparatory to introducing the sample. A solution of

.25 per cent acetic acid in acetone was used to force the fatty acids through the column. The 10 ml. samples were collected manually in 25 ml. sample bottles one sample being collected each 10 minutes. The samples were prepared for spectral analysis as previously described.

Results of this experiment are graphically illustrated (Plate VIII). In the first run 32 ten ml. samples were collected. After evaporation of the acetone and acetic acid mixture from the sample bottles, it was found that samples 24 through 28 contained visible droplets of an oily liquid. Upon determination of optical densities it was found that these 4 samples exhibited an optical density peak at the wavelengths investigated. However, the optical density of sample number 32 was still relatively high indicating that more samples should have been collected. Upon examination of the ultraviolet spectra of selected samples from this experiment it was found that the spectra of the samples differed from the spectra of the origional corn oil fatty acid sample and that the spectra of the samples differed one from the other (Plate IX). This indicated that a separation was being effected.

Since analysis of the samples from the first run indicated that the collection of more samples would have been desirable, the experiment was repeated and 38 samples collected. Results of this run were in close agreement with those of the preceding one (Plate X). The droplets of oily liquid appeared in sample bottles 24 through 28. Spectral analysis showed the optical density to be very high for these 4 samples indicating a large amount of fatty acid present. The spectra of the

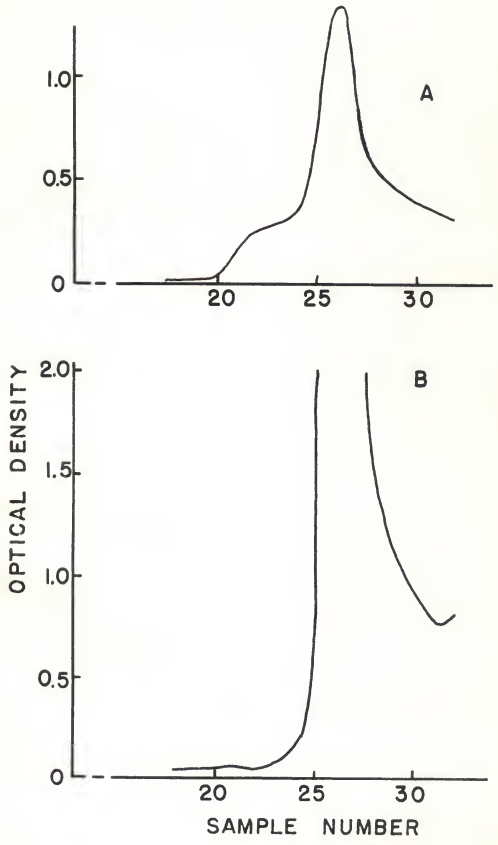
Explanation of Plate VIII

Optical densities of samples collected in the chromatographic analysis of corn oil determined at wave lengths of:

(A) 268 millimicrons

(B) 228 Millimicrons

PLATE VIII


1.0
0.5
0
A
20
25
30
OPTICAL DENSITY
2.0
1.5
1.0
0.5
0
B
20
25
30
SAMPLE NUMBER

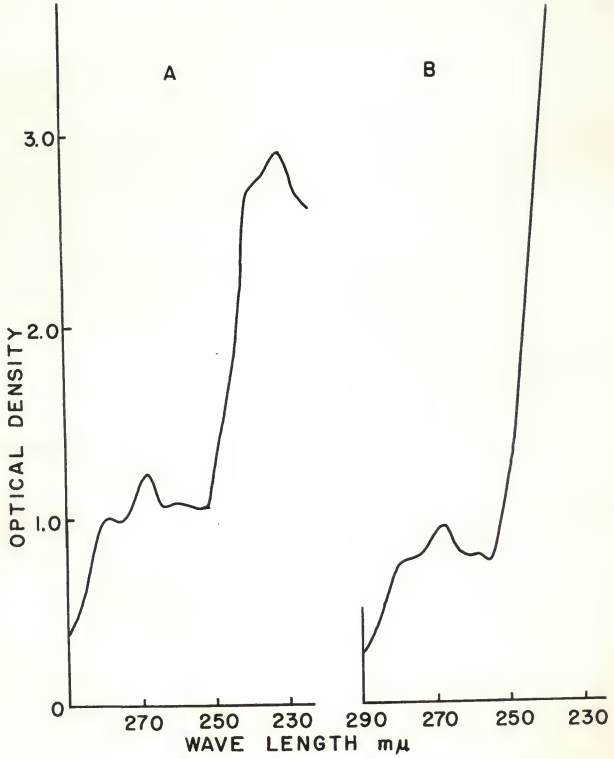
Explanation of Plate IX

Ultra violet spectra of two samples collected in the chromatographic investigation of the corn oil fatty acid extract.

(A) Sample 26

(B) Sample 27

PLATE IX


A
B
3.0
2.0
1.0
0
OPTICAL DENSITY
270
250
230
290
270
250
230
WAVE LENGTH mμ

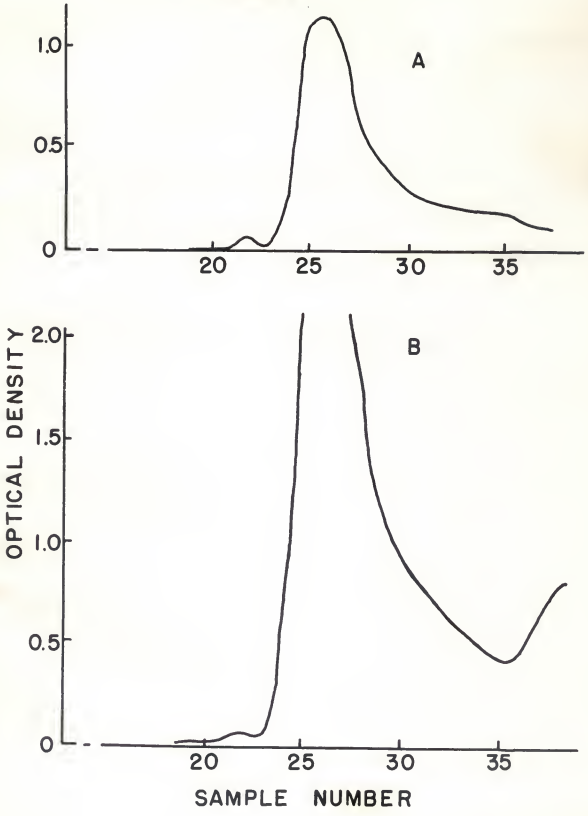
Explanation of Plate X

Optical densities of samples collected in the chromatographic analysis of corn oil determined at wavelengths of:

(A) 268 millimicrons

(B) 228 millimicrons

PLATE X


1.0
0.5
0
A
20
25
30
35
OPTICAL DENSITY
2.0
1.5
1.0
0.5
0
B
20
25
30
35
SAMPLE NUMBER

samples collected differed from that of the origional corn oil fatty acid and differed one from the other. This difference indicated that a separation was being effected. However, the complexity of the corn oil fatty acid mixture, which is composed of oleic, linoleic, palmitic, stearic, arachidic and lignoceric acids, made a quantitative treatment of the spectral data nearly impossible. It should, therefore, prove advantageous to investigate less complicated artificial mixtures of fatty acids to determine the degree of separations possible.

Chromatographic Separation of an Artificial Mixture of Oleic and Linolenic Acid

Since the corn oil fatty acid extract proved a too complicated mixture for accurate interpretation of spectral data after its chromatographic separation, less complex mixtures of highly purified unsaturated fatty acids were employed to determine the degree of separation possible.

The separation of highly purified oleic from linolenic acid was attempted in the first chromatographic experiment. These fatty acids were chosen for this experiment since they are well suited for determining the effect of degree of unsaturation upon the separation.

For the first chromatographic run conducted in this experiment the sample was prepared by dissolving .1 ml. of oleic acid in 5 ml. of acetone. A 25 cm. alumina chromatographic column was prepared as previously described and washed with acetone before introducing the sample. A solution of

.25% acetic acid in acetone was used to force the oleic acid through the column. The 10 ml. samples were collected mechanically with the Technicon Time Flow Sample Collector. One sample was collected at every 10 minute time interval. The samples were prepared for spectral analysis as previously described.

The purpose of this run was to determine the shape of the curves obtained when optical density is plotted against sample number at wave lengths of 228 and 258 millimicrons with oleic acid being the only acid present. The results of this run are graphically presented (Plate XI). From inspection of this graph it is apparent that the fatty acid sample was present mainly in samples 22 through 28. The curve at a wavelength of 228 is much more apparent than at a wavelength of 258 millimicrons as the oleic acid sample exhibits but very slight optical density at a wavelength of 258 millimicrons (Plate XI).

In the second chromatographic run the procedure was identical with that of the first. However the sample was prepared by dissolving .2 ml. of linolenic acid in 5 ml. of acetone.

This run was for the purpose of determining the shape of the curves obtained when optical density is plotted against sample number at wavelengths of 228 and 258 millimicrons linolenic acid being the only acid present. The results of this run are graphically presented (Plate XII). From an inspection of the graph it is apparent that samples 24 through 30 contained the largest amounts of linolenic acid and samples 31 through

Explanation of Plate XI

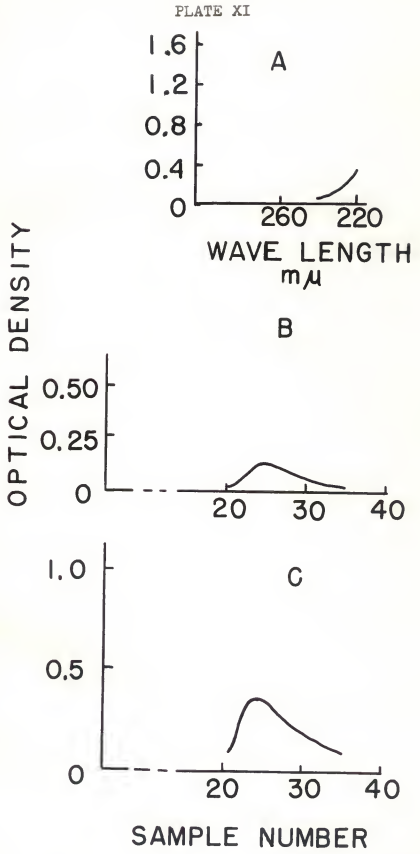
Optical density of samples collected in the chromatographic analysis of highly purified oleic acid.

A. Spectra of sample analyzed

B. Optical density at 258 millimicrons

C. Optical density at 228 millimicrons

PLATE XI

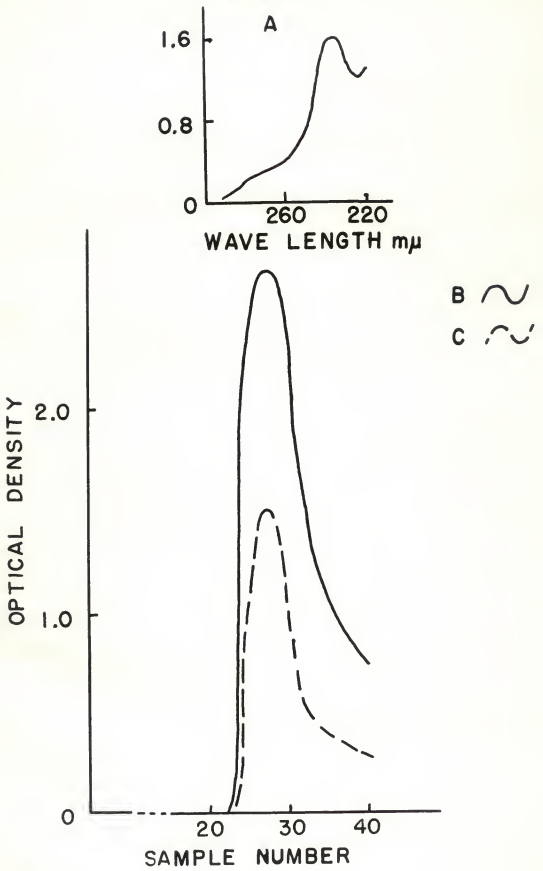

A
1.6
1.2
0.8
0.4
0
260
220
WAVE LENGTH
mμ
B
OPTICAL DENSITY
0.50
0.25
0
20
30
40
C
1.0
0.5
0
20
30
40
SAMPLE NUMBER

Explanation of Plate XII

Optical density of samples collected in the chromatographic analysis of highly purified linolenic acid.

A. Spectra of sample analyzed
B. Optical density at 258 millimicrons
C. Optical density at 228 millimicrons

PLATE XII


A
1.6
0.8
0
260
220
WAVE LENGTH mμ
B
C
OPTICAL DENSITY
2.0
1.0
0
20
30
40
SAMPLE NUMBER

50 also contained detectable amounts. The curve at a wavelength of 258 millimicrons is effectively the same as the curve at a wavelength of 228 millimicrons except its magnitude is only one half as great. This is in agreement with the ratio of optical density of linolenic acid at these two wavelengths (Plate XII).

Inspection of the results of the two preceding runs makes it apparent that difficulties will arise in the spectral analysis of the samples obtained from a mixture of oleic and linolenic acids. These difficulties will come from the fact that a very insignificant amount of linolenic acid will give a higher optical density at the wavelengths investigated than will a large amount of oleic acid. If the weight of fatty acid in each sample is determined, the comparison of the weight curve to the optical density curve will aid in showing the separation attained. The presence of oleic acid will effect the optical density curve slightly while its presence will show a marked effect on the weight curve.

In the final chromatographic run of this experiment the procedure was identical with that of the first 2 runs. However, the sample was prepared by dissolving .1 ml. of oleic acid and .1 ml. of linolenic acid in 5 ml. of acetone. After spectral analysis was completed the samples were transferred to weighed sample bottles, and the solvent n-hexane was evaporated under vacuum at about 60° C by the use of a vacuum dessicator and a heat lamp. The weight of fatty acid present in each

sample was then determined.

The optical densities at wavelengths of 228 and 258 millimicrons for the samples collected in this experiment are graphically compared (Plate XIII). The curve at a wavelength of 228 millimicrons exhibits one peak for sample number 25, another for sample number 30. The curve at a wavelength of 258 millimicrons exhibits a peak at sample number 26 and a plateau at sample number 30. This indicates the linolenic acid was separated from the oleic acid, sample number 26 being richest in linolenic acid and sample number 30 being richest in oleic acid. The ratio of the optical density at a wavelength of 228 millimicrons to the optical density at a wavelength of 258 millimicrons is plotted against sample number (Plate XIV). The value of this ratio for pure linolenic acid is 2.2 and the value for oleic acid is about 8. Inspection of this curve shows that sample number 26 is nearly pure linolenic acid and that sample number 30 is nearly pure oleic acid according to these optical density values. The curve obtained by weight analysis is also presented (Plate XIV). It appears upon inspection of this curve that the weight of the more strongly adsorbed acid per sample collected is not as great as the weight of the less strongly adsorbed acid per sample. This is in agreement with results presented by Dutton (9).

Separation of Linolenic from Linolenic Acid

In the preceding experiment the separation of a mixture

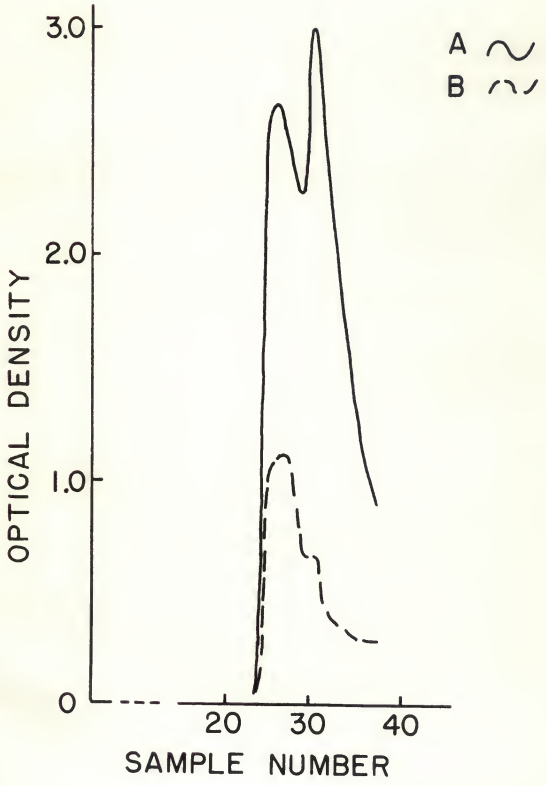
Explanation of Plate XIII

Optical densities of samples collected in the chromatographic analysis of a mixture of oleic and linolenic acids.

A. Optical density at 228 millimicrons

B. Optical density at 258 millimicrons

PLATE XIII



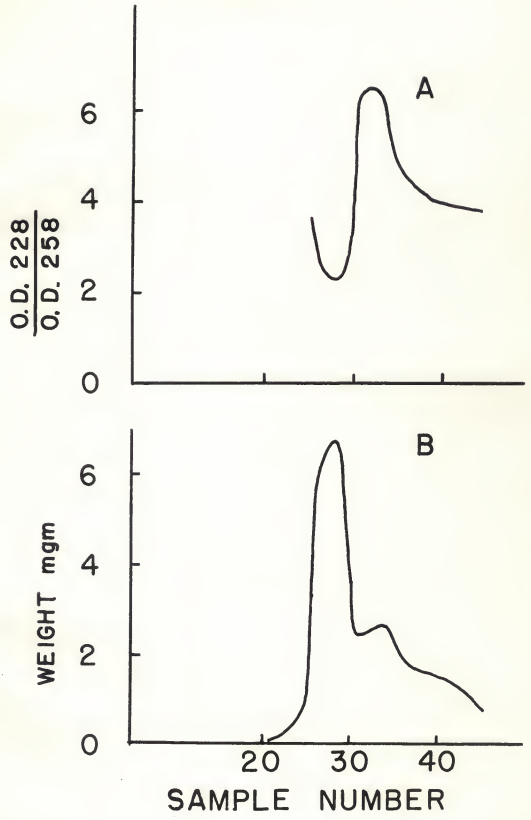
Explanation of Plate XIV

Chromatographic analysis of oleic and linolenic acids.

A. Optical density ratios

B. Weight analysis

PLATE XIV



of oleic and linolenic acids was investigated. It was found that although the acids were separated they were not separated as completely as desired. The reason for this seemed to be that the fatty acids were being forced from the column by the acetic acid-acetone mixture so rapidly that the system did not reach equilibrium at any one time. The separation of linolenic from linoleic acid was carried out using a reduced rate of flow of liquids through the column in an attempt to determine if the rate of flow in the preceding experiment was too rapid.

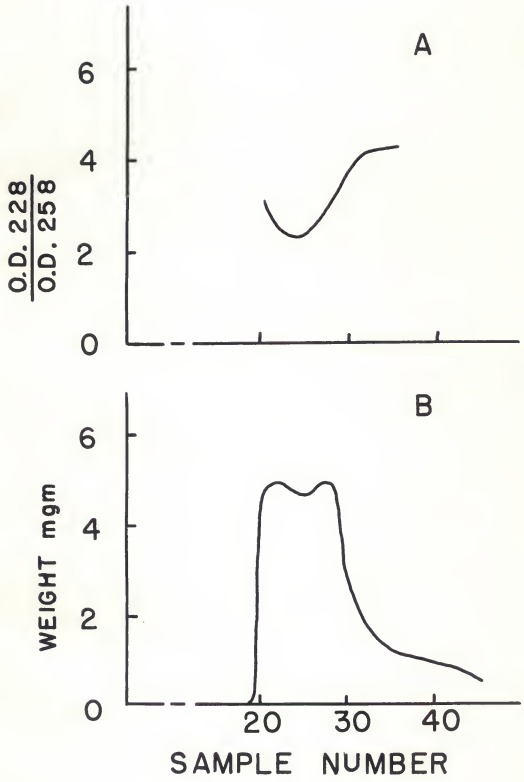
The fatty acid sample was prepared by dissolving .1 ml. of linoleic acid and .1 ml. of linolenic acid in 5 ml. of acetone. A 25 cm. alumina chromatographic column was prepared as in previous experiments. The column was washed with acetone preparatory to introducing the sample. A solution of .25 per cent acetic acid in acetone was used to force the fatty acid mixture through the column. The 10 ml. samples were collected mechanically with the Technicon Time Flow Sample Collector, one sample being collected each 15 minute time interval. The samples were transferred to weighed sample bottles and then evaporated under vacuum. The weight of each sample was then determined. The fatty acids remaining in each sample bottle was dissolved in 5 ml. of n-hexane. The optical density at wave lengths of 228 and 258 millimicrons was determined.

Results of this experiment are graphically represented (Plate XV). It is seen that even though the rate of flow was

Explanation of Plate XV

Optical density ratios and weight analysis for samples collected in the chromatographic analysis of a mixture of linoleic and linolenic acids.

PLATE XV



reduced significant amounts of fatty acids appear in samples 20 through 43. The shape of the curve obtained upon plotting weight of fatty acid sample against sample number shows that the two fatty acids were not as completely separated as desired during passage through the column. Results of optical density ratio calculations show that the first samples containing fatty acid were richer in linolenic and samples collected later were richer in linoleic acid. This agrees with the results of the preceding experiment.

Separation of Oleic, Linoleic, and Linolenic Acids

Since the separation of oleic from linolenic acid and linoleic from linolenic acid was partially effected, an attempt was made to separate a mixture of oleic, linoleic, and linolenic acids. These three unsaturated fatty acids occur as glycerides in fats and oils, and therefore an effective quantitative separation would prove highly desirable.

The fatty acid sample was prepared by dissolving .1 ml. of oleic acid, .1 ml. of linoleic acid, and .1 ml. of linolenic acid in 5 ml. of acetone. A 25 cm. alumina chromatographic column was prepared as previously described. The column was washed with acetone preparatory to introducing the sample. A solution of .25 per cent acetic acid in acetone was used to force the fatty acid mixture through the column. The 10 ml. samples were collected mechanically with the Technicon Time Flow Sample Collector, one sample being collected each 10 minute time interval. The samples were prepared for spectral analysis as previously described.

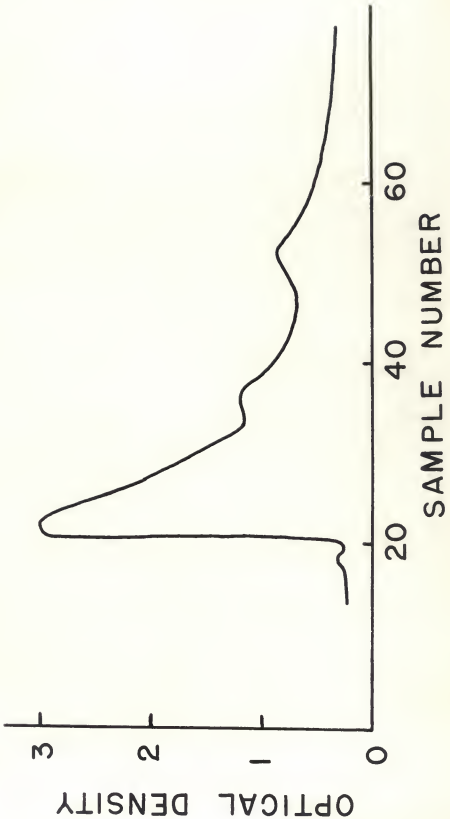
The purpose of this experiment was to determine if under the conditions of the experiment an effective separation was possible. Results of optical density determinations for samples collected in this experiment are illustrated (Plate XVI). The graph shows optical density at 228 millimicrons plotted against sample number. The first fatty acid sample appeared in sample bottle number 21 the unsaturated fatty acids appearing to be most concentrated in samples 21 through 32. The ratio of the optical density at 228 millimicrons to that at 258 millimicrons differs for these three acids having values of 2.2 for linolenic, 4 for slightly oxidized linoleic and about 8 for oleic acids. These values were utilized to show that a separation was effected. For samples 21 through 24 this ratio averaged 2.1, for samples 25 through 28 averaged 3.2, and for samples 29 through 32 averaged 3.8. This shows that the percentage concentration of linolenic acid is highest in the first samples, whereas the last samples contained a higher percentage of oleic acid. As the value of this ratio decreases on oxidation of the fatty acids, the values observed indicated some oxidation occured.

Even though 78 samples were collected traces of fatty acid were present in the last sample collected. This indicates the eluting agent did not force the more strongly adsorbed acids from the column in distinct bands. This condition might explain why the separation effected can not be complete since all fatty acids present exhibit a similar drifting trend. In this experiment it was shown that a chromatographic separation of the unsaturated fatty acids by the method developed was

Explanation of Plate XVI

Optical densities at a wave length of 228 millimicrons of samples collected in the chromatographic analysis of a mixture of oleic, linoleic, and linolenic acids.

PLATE XVI



possible.

The effect of varying the concentration of the different unsaturated fatty acids in the sample analyzed was also investigated. An attempt was made to determine the effect of quantitative variation of the concentration of fatty acids in the origional sample upon the separations obtained since in naturally occuring products the relative amounts of these acids varies.

In these experiments the alumina column was prepared and washed with acetone as previously described. The sample was prepared by weighing the unsaturated fatty acids into a weighed 10 ml. sample bottle. This sample was then dissolved in 5 ml. of acetone and transferred quantitatively to the column. The column was then eluted with a .25% solution of acetic acid in acetone. The rate of flow of eluting agent through the column was adjusted to .67 ml./ minute. The 5 ml. samples that were collected were evaporated, dissolved in normal hexane, and optical densities determined. The samples were then reevaporated in weighed containers, and weighed. The results are illustrated (Plate XVII). It was found that although optical densities are valuable in following the course of the separations, weight analysis adds much to the validity of the results since traces of impurities will have large effects upon the observed optical densities in the spectral region investigated.

It becomes apparent upon inspection of the data collected in this experiment that in both runs the first fractions which contained fatty acid were the heaviest (Plates XVII and XVIII).

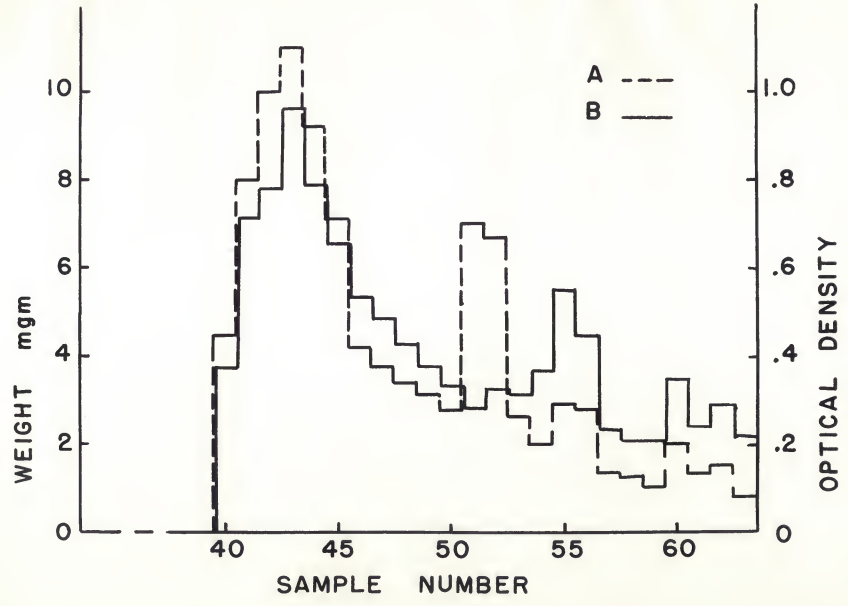
Explanation of Plate XVII

Weight and optical density curves for samples collected in the chromatographic analysis of a mixture of approximately 3 parts oleic acid, 2 parts linoleic acid, and 1 part linolenic acid.

A. Weight

B. Optical density

PLATE XVII


A
B
WEIGHT mgm
OPTICAL DENSITY
SAMPLE NUMBER
0
2
4
6
8
10
.2
.4
.6
.8
1.0
40
45
50
55
60

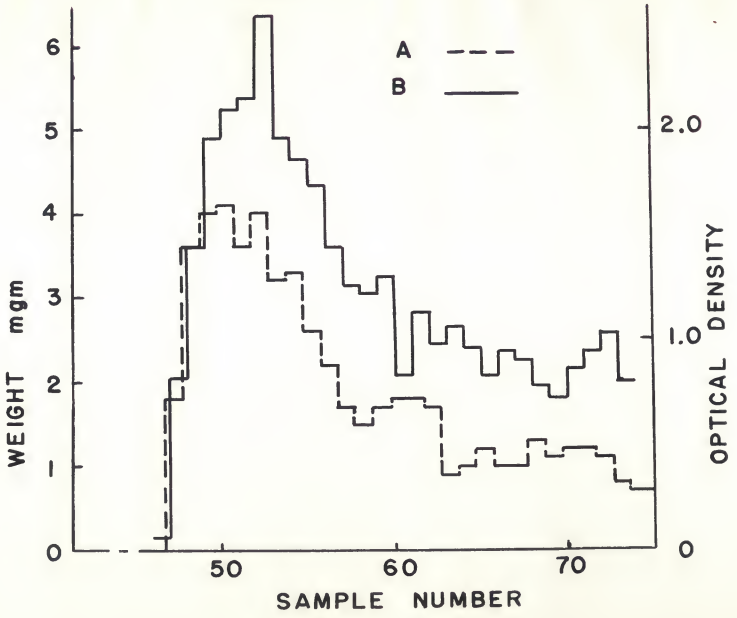
Explanation of Plate XVIII

Weight and optical density curves for samples collected in the chromatographic analysis of a mixture of approximately 1 part oleic acid, 2 parts linoleic acid, and 3 parts linolenic acid.

A. Weight

B. Optical density

PLATE XVIII


A
B
WEIGHT mgm
OPTICAL DENSITY
SAMPLE NUMBER
0
1
2
3
4
5
6
50
60
70
0
1.0
2.0

The concentration of oleic acid was highest in one run whereas the concentration of linolenic acid was highest in the second run.

The results of this experiment indicate that the concentration of the individual fatty acids exhibited a marked effect upon the shape of the curve. The less concentrated fatty acid were more strongly adsorbed on the alumina column, or the results indicate fractionation is very incomplete when the three unsaturated fatty acids are present in the original sample.

In either case these two separations appeared very incomplete with the added disadvantage that a considerable quantity of fatty acid was lost during passage through the column. At the time our investigation was being conducted a quantitative separation of the unsaturated fatty acids was effected by countercurrent distribution methods. Indeed distribution methods have proved highly satisfactory for the separation of complex mixtures of unsaturated fatty acids. On the other hand, the method investigated in this study apparently does not hold much promise for separating complex mixtures of unsaturated fatty acids and has been discontinued.

DISCUSSION

In this investigation a chromatographic method for the separation of unsaturated fatty acids has been investigated.

During preliminary experiments phenolphthalein colored alumina chromatograms were utilized to determine the solvents

that would be suitable as eluting agents for the separation of unsaturated fatty acid mixtures. A mixture of .25 per cent acetic acid in acetone was chosen as an eluting because it removed unsaturated fatty acids from the alumina column at a desirable rate.

As corn oil was a readily available source material for fatty acid sample, a method of purification of corn oil fatty acids based upon urea adduct formation was developed. Upon treatment of corn oil fatty acids with a saturated urea solution containing a large excess of solid urea the solid adduct formed had essentially the same fatty acid content as the original corn oil but colored pigments were removed. This purification eliminated colored material which interfered with subsequent analysis.

This purified corn oil fatty acid extract was investigated chromatographically utilizing an alumina column with .25 per cent acetic acid in acetone as the eluting agent. Optical density determinations conducted on the samples dissolved in n-hexane indicated that the corn oil fatty acid extract could be separated into fractions having different fatty acid content.

Artificial mixtures of highly purified unsaturated fatty acids were employed to gain a better knowledge of the pathway of the chromatographic separations obtained.

When highly purified oleic or highly purified linolenic acids were analyzed chromatographically only one nearly

symmetrical peak resulted upon plotting concentration of the sample against the sample number. When a mixture of one part of purified oleic acid with one part of purified linolenic acid was analyzed chromatographically two peaks resulted upon plotting concentration against the sample number, this showed that a separation was being effected. Further analysis showed the first peak to be composed mainly of linolenic acid, the second to be composed mainly of oleic. A mixture of one part of linoleic acid and one part of linolenic acid was similarly separated into two fractions the first being rich in linolenic acid, the second in linoleic.

Investigations showed that the chromatographic separations obtained with more complex ternary mixtures of unsaturated fatty acids of oleic, linoleic, and linolenic acids was not successful. Since binary mixtures of unsaturated fatty acids were successfully separated it is logical to conclude that a suitable variation of the method, a different adsorbant or another eluting agent would result in a final separation. Since the method does offer promise, it may be more extensively investigated at some future date. At the present time it has been discontinued.

During the time of these investigations, the immediate goal, the separation of complex mixtures of unsaturated fatty acids, was achieved in another way by other workers (1) (2) (12).

SUMMARY

I In preliminary work a mixture of .25 per cent acetic acid in acetone proved to be an effective eluting agent for fatty acids adsorbed from acetone upon an alumina column.

II Pigments present in a mixture of corn oil fatty acids were separated from the corn oil fatty acids by urea adduct formation, the corn oil fatty acid extract being more suitable for chromatographic investigation than the original material.

III The proposed chromatographic method was utilized in separating a corn oil fatty acid extract into samples having different fatty acid content.

IV Highly purified samples of oleic and of linolenic acids were analyzed by the chromatographic method under investigation and found to yield only one fraction of pure acid.

V A mixture of oleic and linolenic acids was separated chromatographically into two fractions, the first fraction being composed mainly of linolenic acid, the second of oleic acid.

VI A mixture of linoleic and linolenic acid was separated into one fraction rich in linolenic acid and a second fraction rich in linoleic acid.

VII Mixtures of more than two unsaturated fatty acids were not successfully separated by the chromatographic method under investigation.

ACKNOWLEDGEMENT

The author wishes to express his appreciation to Dr. Robert E. Clegg of the Department of Chemistry, Kansas State College, his major professor who through suggestions and guidance made this investigation possible. Thanks are also due Dr. R. E. Silker, Head, Department of Chemistry through whose efforts the necessary equipment for this investigation was made available.

LITERATURE CITED

(1) Ahrens, E. H. Jr. and Craig, L. C.
Separations of the higher fatty acids. The Journal of Biological Chemistry, March, 1952, 195:299

(2) Cannon, J. A., Zilch, K. T., and Dutton, H. J.
Countercurrent distributions of methyl esters of higher fat acids. Analytical Chemistry, October, 1952, 24:1530

(3) Cassidy, H. G.
Adsorption analysis IV separation of mixtures of higher saturated fatty acids. American Chemical Society Journal, October, 1941, 63:2735

(4) Cassidy, H. G.
Adsorption analysis II the adsorption of higher fatty acids. American Chemical Society Journal, November, 1940, 62:3037

(5) Cassidy, H. G.
Adsorption analysis III relation between adsorption isotherms and position on the adsorption column. American Chemical Society Journal, November, 1940, 62:3076

(6) Craig, L. C.
Partition chromatography and countercurrent distribution. Analytical Chemistry, November, 1950, 22:1346

(7) Craig, L. C., Hausmann, W., Ahrens, E. H. Jr., and Harfenist, E.
Automatic countercurrent distribution apparatus. Analytical Chemistry, September, 1951, 23:1236

(8) Deuel, H. J. Jr. and Slater, R. A.
The chemistry of lipids. Annual Review of Biochemistry, 1952, 21:109

(9) Dutton, H. J.
Adsorption analysis of lipids III synthetic mixtures of ethyl stearate, oleate, linoleate, and linolenate. Journal American Oil Chemistry Society, April, 1948, 25:120

(10) Graff, M. M. and Skau, E. L.
Colored chromatograms with higher fat acids. Industrial and Engineering Chemistry, Analytical Edition, May, 1943, 15:340

(11) Holman, R. T.
Displacement analysis of lipids IV carrier displacement separation of saturated fatty acids. Journal American Chemical Society, March, 1951, 73:1261

(12) Holman, R. T.
Fatty acid separation. Chemical and Engineering News, May 12, 1952, 30:1968

(13) Markley, Klare S.
Fatty acids, Interscience Publishers, Inc., New York, 1947, 579

(14) Newey, H. A., Shokal, E. C., Mueller, A. C., Bradley, T. F. and Fetterly, L. C.
Segragation of fatty acids and their derivatives by extractive crystilization with urea. Industrial and Engineering Chemistry, December, 1950, 42:2538

(15) Official Methods of Analysis of the Association of Official Agricultural Chemists, 1950, 7th Edition, 432

(16) Ramsey, L. L. and Patterson, W. I.
Separation and determination of the saturated straight chain fatty acids C_{11} to C_{19}. Journal Association of Official Agricultural Chemists, 1948, 31:441

(17) Reimenschneider, R. W., Herb, S. F., Nichols, P. L. Jr.
Isolation of pure natural linoleic and linolenic acids as their methyl esters by adsorption fractionation on silicic acid. Journal American Oil Chemists Society, July, 1949, 26:371

(18) Schlenk, H. and Holman, R. T.
The urea complexes of unsaturated fatty acids. Science, July 7, 1950, 112:19

(19) Williams, K. A.
Chromatographic analysis of fatty oils. Discussions of Faraday Society, 1949, 7:264

(20) Zeichmeister, L.
Progress in chromatography. New York: John Wiley and Sons Inc., 1950

(21) Zimmerschied, W. J., Dinerstein, R. A., Weitkamp, A. W., and Marschner, R. F.
Crystalline adducts of urea with linear aliphatic compounds. Industrial and Engineering Chemistry, July, 1950, 42:1300

APPENDIX

Table 4. Ultraviolet spectra of the highly purified fatty acid samples utilized in chromatographic studies.

Wavelength millimicrons	Fatty Acid- .1% solution in n-hexane			
	Oleic	Linoleic	Linolenic	Corn oil*
300	.0017	----	.033	.005
295	.0019	----	.043	.018
290	.0024	----	.085	.042
288	.0026	----	.113	.066
286	.0028	.002	.140	.103
284	.0029	.008	.160	.148
282	.0030	.014	.175	.200
280	.0034	.022	.190	.235
278	.0038	.023	.197	.237
276	.0039	.020	.205	.223
274	.0039	.022	.220	.224
272	.0041	.024	.231	.251
270	.0044	.028	.242	.290
268	.0045	.030	.255	.305
266	.0046	.036	.272	.283
264	.0047	.025	.300	.248
262	.0048	.024	.339	.229
260	.0050	.031	.395	.231
258	.0052	.039	.436	.230
256	.0053	.047	.485	.215
254	.0054	.073	.555	.178
252	.0058	.102	.614	.163
250	.0063	.152	.696	.156
248	.0075	.238	.790	.161
246	.0087	.314	.870	.166
244	.0102	.400	.945	.175
242	.0125	.463	1.00	.191
240	.0135	.537	1.05	.206
238	.0188	.594	1.06	.220
236	.0240	.648	1.07	.235
234	.0305	.685	1.07	.250
232	.0382	.695	1.04	.263
230	.0481	.690	1.00	.269
228	.0595	.682	.950	.270
226	.0725	.660	.905	.274

* Corn oil fatty acid purified by urea adduct method.

Table 5. Iodine Numbers of fatty acids after removal from the urea adduct.

Fatty acid sample	Average Iodine Numbers
Prepared using 90% urea solution	48.4
Prepared using a saturated urea solution	61.5
Prepared using a saturated urea solution containing a large excess of solid urea	135.5
Free fatty acid mixture prior to urea treatment.	121.6

Table 6.. Ultra violet spectra of (a) corn oil, (b) corn oil fatty acids and (c) purified corn oil fatty acids.

Wavelength millimicrons	Sample-.1% solution in n-hexane		
	a	b	c
290	.069	.082	.072
285	.117	.148	.142
280	.203	.224	.238
275	.205	.226	.241
270	.250	.274	.290
265	.230	.250	.260
260	.212	.234	.231
255	.196	.219	.190
250	.198	.236	.220
245	.229	.271	.226
240	.261	.308	.240
235	.288	.331	.262
230	.309	.348	.284
225	.312	.358	.325

Table 7. Iodine numbers of fatty acids after removal from the urea adduct.

Fatty acid sample	Average Iodine Numbers
Prepared using 80% saturated urea solution-mixture being stored at 3° C overnight.	*
Prepared using 85% saturated urea solution.	*
Prepared using 85% saturated urea solution-mixture being stored at 3° C overnight.	71.3
Prepared using 90% saturated urea solution.	77.2
Prepared using saturated urea solution.	84.0
Prepared using saturated urea solution containing 10 grams excess of solid urea.	111.4
Prepared using saturated urea solution containing large excess of solid urea.	110.3
Free fatty acid mixture prior to the urea treatment.	114.1

Table 8. Optical density of samples collected in the chromatographic investigation of the corn oil fatty acid extract.

Sample number	Wavelength millimicrons				
	228	246	258	268	278
1--15*					
16	.059	.031	.017	.013	.011
17	.071	.041	.027	.023	.019
18	.037	.020	.014	.010	.007
19	.065	.036	.020	.014	.012
20	.064	.035	.017	.012	.009
21	.071	.138	.253	.340	.374
22	.045	.056	.080	.100	.107
23	.095	.198	.349	.470	.520
24	.115	.087	.099	.108	.106
25	∞	1.20	.690	.765	.618
26	∞	1.62	1.13	1.35	1.18
27	1.43	2.01	.770	.819	.698
28	1.10	.658	.418	.481	.375
29	.921	.531	.335	.382	.299
30	.770	.503	.393	.472	.419
31	.820	.399	.249	.283	.221
32	.835	.439	.289	.316	.249

* As no material present in sample bottles after evaporation of the eluting agent, these samples were not analyzed.

Table 9. Ultra violet spectra of selected samples collected in the chromatographic investigation of corn oil fatty acids.

Wavelength millimicrons	Sample Number 21	26	27
300	.201	.203	.133
296	.224	.264	.180
292	.254	.333	.220
288	.281	.466	.316
284	.300	.721	.430
280	.314	.990	.656
276	.313	.980	.700
272	.304	1.06	.775
268	.284	1.23	.819
264	.264	1.06	.815
260	.238	1.07	.780
256	.206	1.06	.755
252	.167	1.05	.791
248	.139	1.40	1.50
244	.104	1.90	2.40
240	.095	2.70	2.92
236	.084	2.77	4.15
232	.077	2.90	∞
228	.070	2.70	∞
224	.066	2.60	∞

Table 10. Optical density of samples collected in the chromatographic investigation of the corn oil fatty acid extract.

Sample number	Wavelength millimicrons				
	228	246	258	268	278
1--15*					
16	.037	.030	.025	.021	.017
17	.065	.039	.027	.023	.020
18	.001	.007	.002	.000	.000
19	.034	.024	.016	.014	.011
20	.011	.014	.008	.007	.005
21	.016	.016	.006	.003	.001
22	.078	.063	.066	.067	.066
23	.028	.034	.034	.031	.042
24	.631	.359	.246	.280	.239
25	∞	1.41	.930	1.09	.873
26	∞	1.51	.965	1.13	.890
27	2.2	1.13	.755	.885	.720
28	1.7	.845	.445	.507	.405
29	1.11	.605	.343	.400	.316
30	.860	.458	.257	.290	.232
31	.795	.440	.245	.277	.176
32	.639	.357	.196	.221	.115
33	.447	.275	.195	.231	.162
34	.513	.298	.175	.196	.182
35	.393	.252	.182	.211	.105
36	.528	.274	.123	.130	.098
37	.561	.280	.117	.123	.086
38	.790	.384	.118	.106	.080

* As no material present in sample bottles upon evaporation of the eluting agent the samples were not analyzed.

Table 11. Optical density of samples collected in the chromatographic analysis of oleic acid.

Sample number	Wavelength millimicrons	
	228	258
1--17*		
18	.105	.015
19	.113	.015
20	.126	.017
21	.135	.025
22	.250	.081
23	.376	.126
24	.375	.121
25	.365	.131
26	.278	.099
27	.300	.110
28	.215	.060
29	.237	.064
30	.232	.063
31	.200	.060
32	.174	.049
33	.117	.032
34	.125	.042
35	.099	.029
36	.140	.047
37	.125	.035
38	.125	.035
39	.119	.034
40	.100	.028

* As no material present in sample bottles after evaporation of the eluting agent, these samples not analyzed.

Table 12. Optical density of samples collected in the chromatographic analysis of linolenic acid.

Sample number	Wavelength millimicrons			
	228	258	268	278
1--20*				
21	.013	.055	.068	.078
22	.007	.145	.190	.217
23	.124	.035	.031	.025
24	1.88	.571	.542	.423
25	2.35	1.18	1.06	.822
26	2.60	1.20	1.06	.800
27	2.80	1.52	1.40	1.09
28	2.60	1.65	1.48	1.16
29	2.70	1.25	1.18	.943
30	2.40	.960	.981	.832
31	1.30	.532	.570	.548
32	1.20	.400	.385	.341
33	1.50	.499	.470	.393
34	1.04	.334	.339	.304
35	1.29	.490	.509	.470
36	1.00	.488	.530	.509
37	.704	.245	.252	.233
38	.878	.485	.560	.555
39	.860	.348	.366	.345
40	.740	.331	.374	.365
41	1.24	.473	.490	.427
42	.580	.270	.305	.286
43	.735	.245	.238	.204
44	.640	.321	.356	.350
45	.582	.241	.264	.250
46	.683	.274	.274	.257
47	.762	.414	.464	.460
48	.560	.331	.396	.405
49	.545	.190	.115	.090
50	.630	.279	.297	.290

* As no material present in sample bottles after evaporation of the eluting agents the samples were not analyzed.

Table 13. Optical density and weight of samples collected in the chromatographic analysis of a mixture of oleic and linolenic acids.

Sample number	Wavelength millimicrons				Weight mgm.
	228	258	268	278	
1--20*					
21	----	----	----	----	---
22	----	----	.013	.015	.1
23	.047	.037	.050	.051	.3
24	.031	.050	.063	.066	.3
25	.665	.159	.145	.112	.6
26	2.60	.930	.820	.613	1.0
27	2.65	1.10	.960	.720	5.8
28	2.50	1.12	.980	.690	6.5
29	2.40	.895	.810	.596	6.8
30	2.25	.620	.560	.417	5.5
31	∞	.660	.671	.572	3.6
32	2.50	.429	.387	.290	2.5
33	1.80	.313	.271	.190	2.6
34	2.35	.385	.341	.261	2.6
35	1.61	.325	.270	.235	2.7
36	1.25	.340	.320	.195	1.7
37	1.05	.266	.260	.186	1.8
38	.892	.245	.224	.167	1.4
39	1.05	.241	.217	.160	1.6
40	.820	.229	.210	.165	1.7
41	.839	.200	.174	.131	1.3
42	.980	.240	.204	.154	1.3
43	.848	.205	.170	.128	1.7
44	.612	.178	.171	.129	.4
45	.887	.215	.190	.144	1.1
46	.785	.193	.170	.126	.8
47	.590	.147	.135	.105	
48	.610	.165	.142	.113	
49	.642	.186	.155	.113	
50	.510	.132	.123	.089	

* As no material present in sample bottles after evaporation of the eluting agent the samples were not analyzed.

Table 14. Optical densities and weights of samples collected in the chromatographic analysis of a mixture of linoleic and linolenic acids.

Sample number	Wavelength millimicrons		Weight
	228	258	mgm.
1--15*			
16	.044	.024	---
17	.195	.015	---
18	.042	.013	---
19	.056	.014	---
20	∞	.776	4.0
21	∞	1.15	5.0
22	∞	1.31	5.0
23	∞	1.46	4.0
24	∞	1.56	5.0
25	2.30	1.20	5.0
26	∞	1.07	4.0
27	2.30	.90	5.0
28	2.20	.98	5.0
29	2.20	.98	3.0
30	2.15	.81	3.0
31	1.60	.440	2.0
32	1.60	.363	2.0
33	1.50	.370	1.0
34	1.36	.370	1.0
35	.84	.195	1.0
36	1.56	.342	1.0
37	1.35	.279	1.0
38	1.07	.245	1.0
39	.700	.258	1.0
40	.510	.180	1.0
41	1.20	.120	1.0
42	1.22	.284	1.0
43	1.50	.283	.5
44	1.22	.340	.5
45	1.15	.286	.6

* Fatty acids not present therefore samples not analyzed.

Table 15. Optical densities of samples collected in the chromatographic analysis of oleic, linoleic and linolenic acid mixture.

Sample number	Wavelength millimicrons	
	228	258
1--16*		
17	.141	.021
18	.335	.078
19	.315	.086
20	.226	.073
21	3.10	1.14
22	3.20	1.35
23	3.40	1.91
24	2.80	1.35
25	2.50	.875
26	2.10	.642
27	2.10	.630
28	1.92	.532
29	1.86	.510
30	1.60	.399
31	1.54	.422
32	1.29	.370
33	1.145	.352
34	1.155	.313
35	1.180	.312
36	1.000	.285
37	1.530	.355
38	1.025	.292
39	.965	.242
40	.805	.206
41	.786	**
42	.845	
43	.715	
44	.740	
45	.740	
46	.670	
47	.630	
48	.688	
49	.617	
50	.710	
51	.780	
52	.905	
53	.680	
54	.540	
55	.453	
56	.890	
57	.523	

Table 15. (concl)

Sample number	Wavelength millimicrons	
	228	258
58	.457	
59	.477	
60	.540	
61	.425	
62	.455	
63	.432	
64	.385	
65	.430	
66	.390	
67	.583	
68	.342	
69	.493	
70	.352	
71	.430	
72	.320	
73	.352	
74	.320	
75	.282	
76	.328	
77	.292	

* Visual inspection of these samples after the eluting agent was evaporated indicated no fatty acids present.

** As visual inspection indicated no significant amount of material present after the eluting agent was evaporated samples were not analyzed at this wavelength.

Table 16. Optical densities and weights of samples collected in the chromatographic analysis of a mixture of .1028 gms. oleic acid, .0672 gms. linoleic acid, and .0311 gms. linolenic acid.

Sample number	Wavelength millimicrons		weight mgm**
	228	268	
1--36*			
37	.056	.032	.0
38	.052	.092	.0
39	.045	.039	.0
40	.382	.102	4.5
41	.725	.162	8.1
42	.790	.158	10.1
43	.975	.201	11.1
44	.812	.161	9.3
45	.663	.145	7.2
46	.538	.117	4.3
47	.500	.084	3.9
48	.435	.102	3.6
49	.390	.097	3.3
50	.340	.095	2.8
51	.295	.064	7.1
52	.342	.101	6.8
53	.323	.108	2.7
54	.363	.176	2.2
55	.565	.246	3.1
56	.447	.161	3.0
57	.230	.156	1.4
58	.211	.049	1.4
59	.215	.049	1.1
60	.365	.228	2.2
61	.238	.193	1.5
62	.298	.078	1.6
63	.213	.129	.9

* Visually no fatty acids present therefore only selected samples analyzed,

** Total weight .1032 gms.

Table 17. Optical densities and weights of samples collected in the chromatographic analysis of a mixture of .0227 gms. oleic acid, .0487 gms. linoleic acid, and .0666 gms. linolenic acid.

Sample number	Wavelength millimicrons		Weight mgm**
	228	268	
1--45*			
46	.061	.038	0.0
47	.815	.114	1.8
48	1.443	.196	3.6
49	1.960	.213	4.0
50	2.10	.235	4.1
51	2.15	.233	3.6
52	2.55	.264	4.0
53	1.96	.266	3.2
54	1.86	.216	3.3
55	1.73	.188	2.6
56	1.435	.153	2.2
57	1.250	.147	1.7
58	1.215	.153	1.5
59	1.297	.157	1.7
60	.840	.107	1.8
61	1.125	.137	1.8
62	.978	.113	1.7
63	1.060	.114	0.9
64	.960	.122	1.0
65	.825	.100	1.2
66	.940	.116	1.0
67	.900	.112	1.0
68	.785	.182	1.3
69	.722	.097	1.1
70	.865	.109	1.2
71	.927	.118	1.2
72	1.030	.125	1.1
73	.793	.113	0.8
74	1.040	.133	0.7
75	1.110	.146	0.7
76	.960	.122	0.6
77	1.098	.151	0.4
78	.975	.128	0.6
79	1.040	.153	0.6
80	1.060	.138	0.5
81	1.050	.148	1.2
82	.925	.130	0.8
83	.968	.147	0.9
84	.980	.150	0.8
85--100			8.6

* Visual inspection showed no fatty acids present in the sample bottles after eluting agent evaporated.

** Total weight .0710 gms.

CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF UNSATURATED FATTY ACIDS

by

ALFRED THEODORE ERICSON

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This investigation was initiated in an attempt to develop a satisfactory method for the separation of complex mixtures of unsaturated fatty acids. Results of previous work indicated that separations based upon chromatographic methods should prove possible; therefore, in this investigation the chromatographic separation of unsaturated fatty acids was investigated.

Preliminary experiments conducted utilizing phenolphthalein colored alumina chromatograms were conducted to determine what solvent would be a suitable eluting agent for unsaturated fatty acid mixtures. A mixture of .25 per cent acetic acid in acetone was chosen as the eluting agent for this investigation as it removed unsaturated fatty acids from the column at a desirable rate.

A method of purification of corn oil fatty acid based upon urea adduct formation was developed, corn oil being a readily available source material for fatty acid samples. Treatment of corn oil fatty acids with a saturated urea solution containing a large excess of solid urea caused the formation of a solid adduct which had essentially the same fatty acid content as the original corn oil but did not contain the colored pigments. This purification eliminated interference of colored material with subsequent analysis.

The purified corn oil fatty acid extract was chromatographically investigated utilizing an alumina column with .25 per cent acetic acid in acetone as the eluting agent.

The eluting agent was removed from the samples under vacuum the samples then being dissolved in n-hexane. Optical density values determined for these samples indicated that the corn oil fatty acid extract could be separated into fractions having different fatty acid content by this chromatographic method.

As interpretation of the data collected in the chromatographic investigation of corn oil proved nearly impossible due to the complexity of the corn oil fatty acid mixture, artificial mixtures of highly purified unsaturated fatty acids were employed in order to gain a better knowledge of the pathway of the chromatographic separations obtained.

When either highly purified oleic or highly purified linolenic acids were analyzed chromatographically only one nearly symmetrical peak resulted upon plotting concentration of the sample against the sample number. When a mixture of oleic acid and linolenic acid was analyzed chromatographically two peaks resulted upon plotting concentration against the sample number a separation being effected. Analysis showed the first peak to be composed mainly of linolenic acid; the second to be composed mainly of oleic. A mixture of one part of linoleic acid and one part of linolenic acid was similarly separated into two fractions, the first being rich in linolenic acid, the second in linoleic.

In investigating the chromatographic separations obtainable with more complex ternary mixtures of unsaturated fatty acids the separation of oleic, linoleic, and linolenic acids

was not successful utilizing the chromatographic method under investigation. As success was experienced in separations of binary mixtures of unsaturated fatty acids, it is logical to conclude that a suitable variation of the method, a different adsorbant or another eluting agent would result in a final separation.

In summary, the chromatographic method of separating unsaturated fatty acids under investigation proved suitable for separating binary mixtures prepared from highly purified oleic, linoleic, or linolenic acids, but was not effective in separating more complex ternary mixtures of these same acids.