Chromatographic separation of the high molecular weight straight chain fatty acids
by Thomas E Shellenberger

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree pf Master of Science in Chemistry
Montana State University

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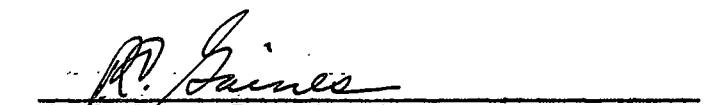

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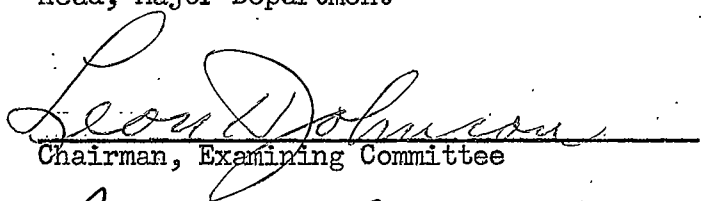

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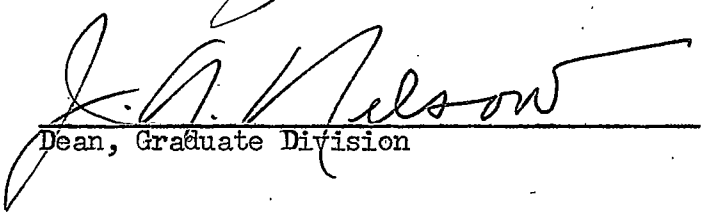

Dean, Graduate Division

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ABSTRACT

An attempt has been made to find a chromatographic system which will effectively separate high molecular weight straight chain fatty acids. Charcoal, alumina, magnesium oxide, urea, nylon, paper, and silicone coated paper were tested as column materials. The columns were developed using various alcohols in combination with acetone, chloroform and saturated hydrocarbons. Analysis of the effluent was accomplished by titration and all results were expressed graphically to observe elution peaks of the fatty acids. No effective separation was found with these systems.

INTRODUCTION

One of the many problems in biochemistry is the quantitative isolation of the high molecular weight fatty acids present in natural waxes and fats. The acids present in any material may vary from readily volatile to nonvolatile acids and from completely saturated to highly unsaturated acids. In most cases, the acids present are members of a homologous series whose chemical and physical properties vary only slightly from one another. This makes sharp separations of the acids or their derivatives difficult.

The techniques usually employed for the separation and isolation of fatty acids are divided into three general classes: (1) distillation, (2) solubility, and (3) chromatography (20). This work is concerned only with chromatography.

HISTORICAL REVIEW

The use of chromatography as an analytical tool has progressed far from the first paper absorption procedure originated by Schöbein or the columnar adsorption method perfected by Tswett (33). Since then, chromatography has had wide application in the field of inorganic chemistry and has been successfully applied to separations of amino acids, carbohydrates, and lipids. The application of chromatography to the separation of fatty acids, on the other hand, has just begun to be investigated to any extent.

Much of the work in identification of fatty acids by paper chromatography has been done by H. P. Kaufmann and J. Budwig (13, 14, 15, 16) although other investigators (3, 11, 12, 18, 29) used paper and paper-

partition chromatography to great advantage. The work of Kaufmann deals mainly with the identification of different fatty acids, C_3 - C_{18}, or their metal soaps by colored spot tests. Inouye and Noda (11, 12) have applied paper chromatography to saturated C_4 - C_{22} and unsaturated fatty acids C_{12} - C_{22} by developing a descending chromatogram with a methanol: petroleum hydrocarbon bp 140-70 mixture, as the solvent system. Long, Quayle, and Stedman (18), Reid and Lederer (29) and Duncan and Porteous (3) have used alcohol-ammonium hydroxide mixtures to obtain partition separations of the lower fatty acids, C_2 - C_7, on paper.

Column chromatography has been investigated much more, possibly because it can be made to give an easy quantitative as well as a qualitative measure. R. T. Holman, in conjunction with J. G. Hamilton and W. T. Williams (4, 6, 7, 8, 9) have used columns of 1 part Darco G-60 with 2 parts Hyflo Supercel to effect separations of C_{12} - C_{18} saturated fatty acids. He and his associates used modified Tiselius-Claesson interferometric absorption analysis apparatus to detect differences in carrier displacement separations of fatty acids.

Much work has been done to separate the fatty acids by partition chromatography on columns of silicic acid and silica gels. Muzumdar and Boswami (22) used a silica gel column in their separation of stearic and oleic acids. They also used alumina, magnesium oxide and activated carbon for column materials. Ramsey and Patterson, in two of their publications (27, 28) reported separations of all fatty acids from C_5 - C_{19} on columns of silicic acids using methanol and 2,2,4,-trimethyl pentane as solvent

for C_5 - C_{10} and a mixture of furfuryl alcohol and 2 aminopyridine with hexane for C_{11} - C_{19}. Nijkamp (23, 24, 25) has also worked out a separation of the fatty acids from C_4 - C_{20} on silica gel columns using isooctane saturated with an excess of 95% methanol as the solvent. A reversed-phase partition method advanced by Howard and Martin (10) was reported successful using kiesselguhr treated with dichlorodimethylsilane vapor to render it "unwettable ". They found the most suitable solvent systems for acids from lauric to stearic were aqueous methyl alcohol-octane or aqueous acetone-medicinal paraffin. This same reversed phase system was employed by Silk and Hahn (32) to separate mixtures of C_{16} - C_{24} fatty acids. Harris and Wick (5) have presented a procedure designed to give a silica gel suitable for chromatography.

Alumina (Al_2O_3) has been used extensively in columns for the separation of the fatty acids. Asahara and associates (1) used petroleum ether system to obtain separations while Ruiz and Munoz (30) found that the saturated acids accumulate in the upper parts of these columns. The former also used magnesium oxide and calcium oxide mixed with ethanol as adsorbents for column chromatography. Acetone was employed by DiModica and Rossi (2) to elute the unsaturated acids linolenic, linoleic, and oleic from a cold alumina column.

A micro method has been worked out by Mai (19) to separate the fatty acids from C_8 - C_{18} by partition chromatography on nylon thread. The solvent he employed was a 1:1:1 mixture of ethanol:water:acetone.

Separations of steroids by Kritchevsky and Tiselius (17) was ac-

complished by reversed phase partition chromatography on silicone treated paper. A two phase solvent system resulted from his solvent system of ethanol, water, and chloroform. The chromatograms were developed with the polar phase after saturating the paper with the non-polar solvent. This publication, although not dealing directly with fatty acids, was reviewed for possible application to fatty acid separation.

EXPERIMENTAL TECHNIQUE AND PROCEDURE

The chromatographic techniques employed in this investigation were (1) direct adsorption of acid to column material, (2) partition separation between a polar and non-polar solvent system, where the polar solvent is the immobile phase, and (3) reversed phase partition chromatography where the non-polar solvent is the immobile phase. Regardless of the technique used, the column materials were packed in glass chromatographic tubes with sintered glass plates at the bottom and the acids eluted from the column using proper solvents or solvent systems. By employing an automatic fraction cutter, fractions of nearly equal volumes of effluent were collected and analyzed for the fatty acid content of each. The acids were identified by an increase in titer when the amount of acid present in each fraction was graphed versus the total volume of effluent. In this manner, several column materials and solvent systems were tested in an attempt to find a combination that would give an effective separation of fatty acids from myristic to arachidic.

The method of packing the columns varied with the kind of material used, the size of particles in the material, the solvent or solvents em-

ployed, the diameter of tube, and the height to which the tube was packed. In general, it was found that a thick slurry of the column material and solvent system should be suspended in the chromatographic tube to obtain best results. This slurry can be packed to give a homogeneous column by tamping or by using 5-10 lb. pressure from a pressure tank. It was also found that the longer columns did not require as tight packing as the shorter columns. The small diameter columns also required less packing. The amount of packing required for different column materials varied considerably. When using nylon for the column material, a much tighter packing was necessary to give a reasonable flow rate than with paper and its modifications. The solvents used with the paper columns had an effect on flow rate but no prediction as to which system would require a loose or tight pack could be made. Column packing is a developed art and reasonably reproducible columns can only be achieved after acquiring some experience in packing methods.

The flow rate of any column type was dependent upon (1) the height of the column, (2) the tightness of pack, (3) the material in the column, (4) the solvent system employed, and (5) the height of the solvent level above the top of the column. The first four criteria were decided when the columns were packed but the latter was varied after development of the column had commenced. For best results, a flow rate of .5 to 3 cc./15 min. should be maintained by raising or lowering the solvent reservoir. (In some cases, a reduced head of pressure was necessary to give a reasonable flow; that is, top of the solvent level was below the top of the column.)

The fraction cutter was made in this laboratory and consisted of a rotating turntable timed to move a glass receiving tube under the chromatographic column every 15 minutes. The turntable has an 80 tube capacity.

The effluent was analyzed for fatty acids by titration with .01N potassium hydroxide. This was used since the potassium salt of a fatty acid is slightly more soluble than the sodium salt in an alcohol solution. All titrations were carried out in an alcohol media and titrated to a phenolphthalein end point. The graphs were made by plotting volume potassium hydroxide on the vertical axis against total volume of effluent on the horizontal axis. The position of the acid as it elutes from a particular column system is characteristic for each acid. The term "elution position" is used to indicate the volume of effluent that has emerged from the column prior to elution of the fatty acid under examination.

The fatty acids were applied to the columns in solutions containing 10 mg. of the fatty acid in one of the constituents of the solvent system under investigation.

RESULTS

The first column material utilized was 1 part Darco G-60 mixed with 3 parts Hyflo supercel. The solvent system employed was ethanol and ethanol:water mixtures. Stearic acid, when developed with ethanol on charcoal, eluted from the column at 12 cc., just behind the solvent front. Single acids were used in cursory examinations of column materials. This was done so as to observe the placement of individual acid peaks. When the

solvent system was changed to 80% ethanol, stearic acid was not eluted from the column. A mixture of methanol, isopropanol, and water was next tried on a charcoal column and stearic acid again had not eluted from the column at 150 cc. At this point the flow rate dropped and the column began to dry and pull away from the side of the chromatographic tube. No success was apparent so the use of this material was abandoned.

Aluminum oxide (Al_2O_3) was tried next for possible separation properties. Stearic acid again was developed with 95% ethanol in a cursory examination. A sharp increase in titer was noted at a point corresponding to about 700 cc. of effluent. Normal propanol and butanol were employed as solvents in an attempt to bring this peak closer to solvent front but no improvement was noted. Stearic acid had not eluted from the column at 900 cc. using butanol as solvent. In these trials, when using butanol and propanol, the solvent was evaporated and ethanol added before attempting the titration. Because of the large volume of effluent required, it was felt that columns using alumina would be difficult to duplicate; therefore, alumina was rejected as a column material.

Next attempts at acid separations were made on nylon columns. The columns were packed using ground Zytel nylon resin provided by DuPont. The nylon was kept cold with dry ice during grinding to minimize shredding due to heat produced during the grinding process. The nylon was ground until it would pass a 1/2 mm. screen. This was still too coarse and resulted in a high flow rate. The solvents used were combinations of ethanol, acetone, and water. The column diameter in all trials was 1.1 cm.

and the column lengths were always approximately 9-10 inches (23-25.5 cm.). Table I shows the relative positions of the fatty acids with different solvent combinations. Trials I-J and K-L are shown as complete plots on figures 1 and 2.

TABLE I

EFFECT OF SOLVENT COMPOSITION ON ELUTION OF FATTY ACIDS

TRIAL	ETHANOL:ACETONE:WATER RATIO	ACID	ELUTION POSITION
A	1:1:1	C_{18}	11.5
B	1:1:1	C_{18}	12.0
C	0:1:1	C_{18}	No elution
D	1:1:0	C_{18}	Front
E	1:0:1	C_{18}	No elution
F	4.5:1:4.5	C_{18}	No elution
G	2:1:2	C_{18}	No elution
H	1:1:1	C_{20}	Front
I	3:2:3	C_{16}	15.5
J	3:2:3	C_{16}	12.5
K	5:4:5	C_{14}	12.0
L	5:4:5	C_{18}	12.0

In all trials on nylon columns, the higher homologs come out of the column before the next lower acid indicating a reversed phase chromatographic system in operation. The nylon columns showed some tendency to affect separations of fatty acids. All attempts to move the acid fronts back further from the solvent front did not succeed so this material was rejected as a column material.

The next trials were made on columns packed with Whatman Cellulose Powder Standard Grade #21480 Cellulose Powder for Chromatography. This was an attempt to adapt the paper separation method of Inouye and Noda

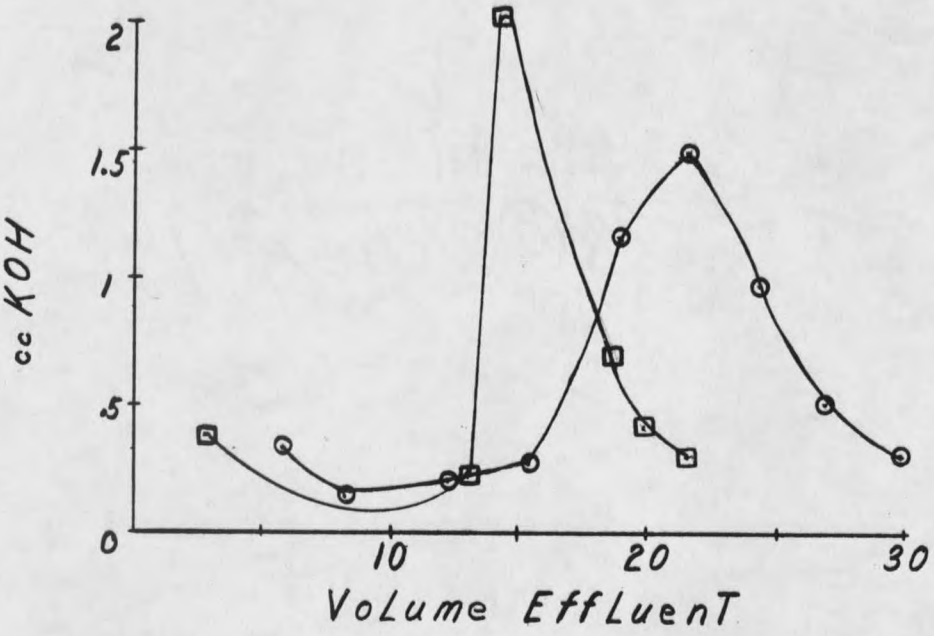

Figure 1. Nylon column. Solvent: ethanol:water:acetone in ratio of 3:3:2. ⊙- Myristic acid ⊡- Palmitic acid

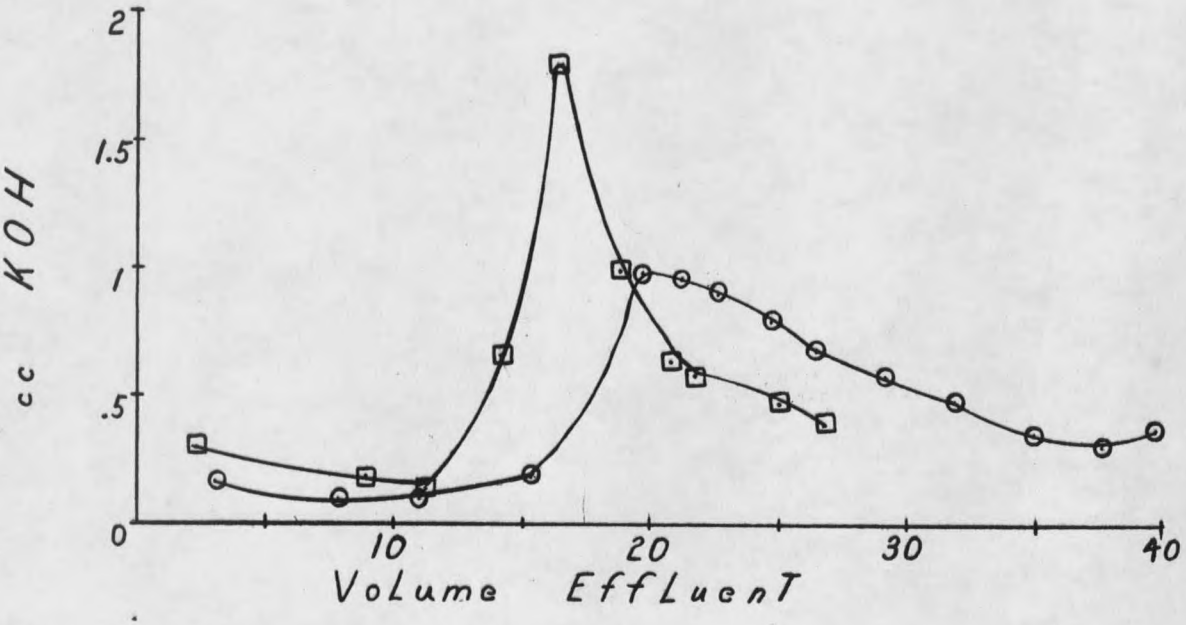

Figure 2. Nylon column. Solvent: ethanol:water:acetone in ratio of 5:5:4. ⊙- Myristic acid. ⊡- Palmitic acid

(11, 12) to column procedures. Figure 3 shows the results when stearic, arachidic, and a mixture of stearic and arachidic acids are developed on a paper column using a 1:1 mixture of methanol:Skellysolve B. The column was 1.1 cm. wide and was 14.5 inches long. Stearic acid shows an elution position of 18 cc. and arachidic has an elution position of 20 cc. The acids showed a slightly different elution position which also can be seen by the sudden increase in the titrations curve for the mixture. It was hoped that a longer column would resolve the acids better. With this in mind, a column .9 cm. wide and 35 1/2 inches long was set up and the acids were developed with the same solvent system. The results are shown in figure 4. As indicated on the graph, the elution position of both acids was the same.

As a result of the data illustrated in figures 3 and 4, the ratio of methanol to Skellysolve was changed in further trials. Figure 5 shows the results obtained with a solvent composition of 3 parts methanol to 1 part Skellysolve. The acids again showed a definite tendency to overlap and the solvent combination was changed to 4:1 methanol:Skellysolve. The results obtained with this combination showed overlapping of the acid peaks with their position directly behind the solvent front. The peaks obtained with the 3:1 combination were set back from the front about 10 cc.

A solvent combination of 3 parts methanol to 1 part heptane was tried on a paper column. Palmitic and stearic acids were used in this development. Both acids peaked very well but eluted from the column right behind the solvent front.

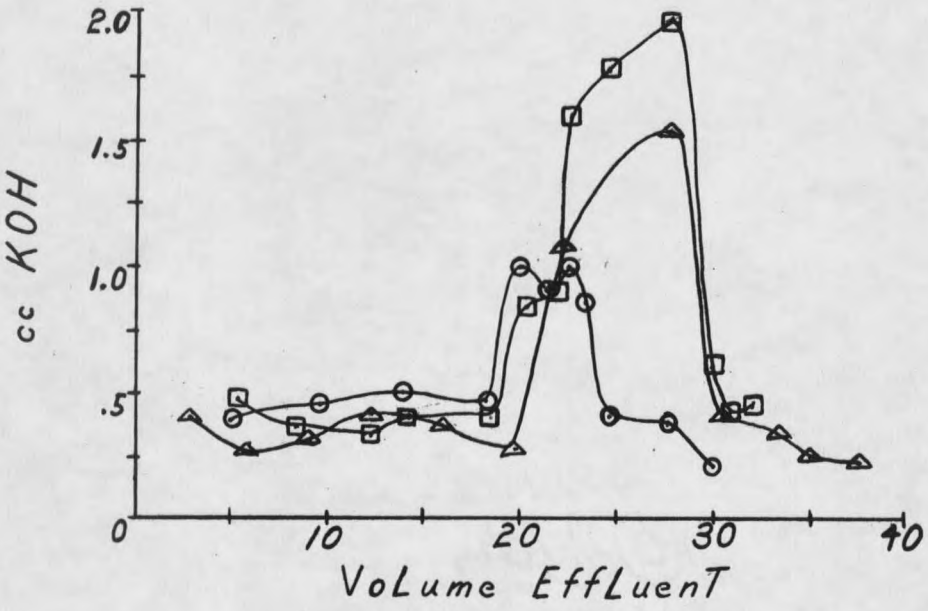

Figure 3. Paper column. Solvent: methanol:Skellysolve B in ratio of 1:1 ⊙- Stearic acid. △- Arachidic acid. ⊡- Mixture of stearic and arachidic acid.

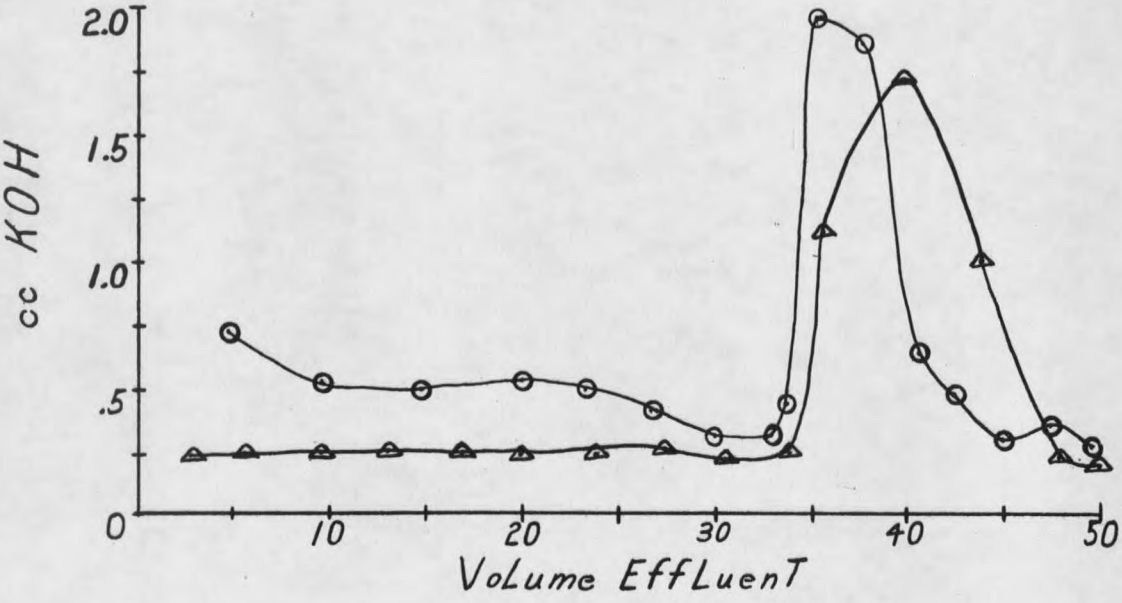

Figure 4. Paper column. Solvent: methanol:Skellysolve B in ratio of 1:1. ⊙- Stearic acid. △- Arachidic acid.

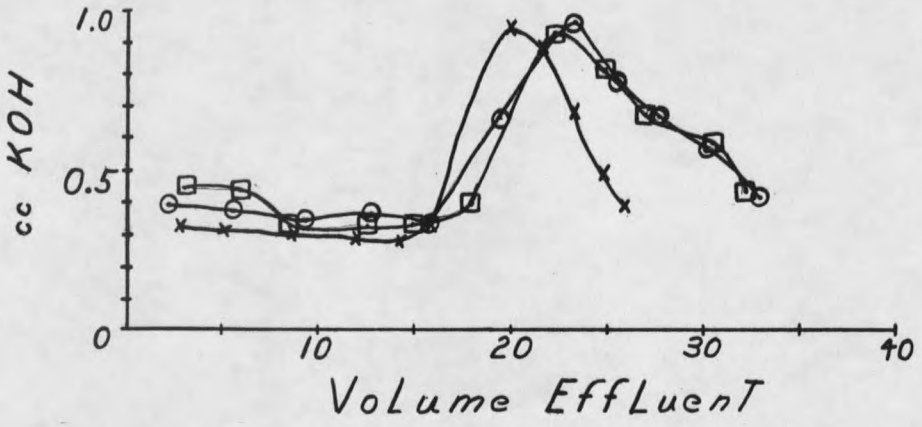

Figure 5. Paper column. Solvent: methanol:Skellysolve B in ratio of 3:1. x - Palmitic acid. ⊙ - Stearic acid. ⊡ - Arachidic acid.

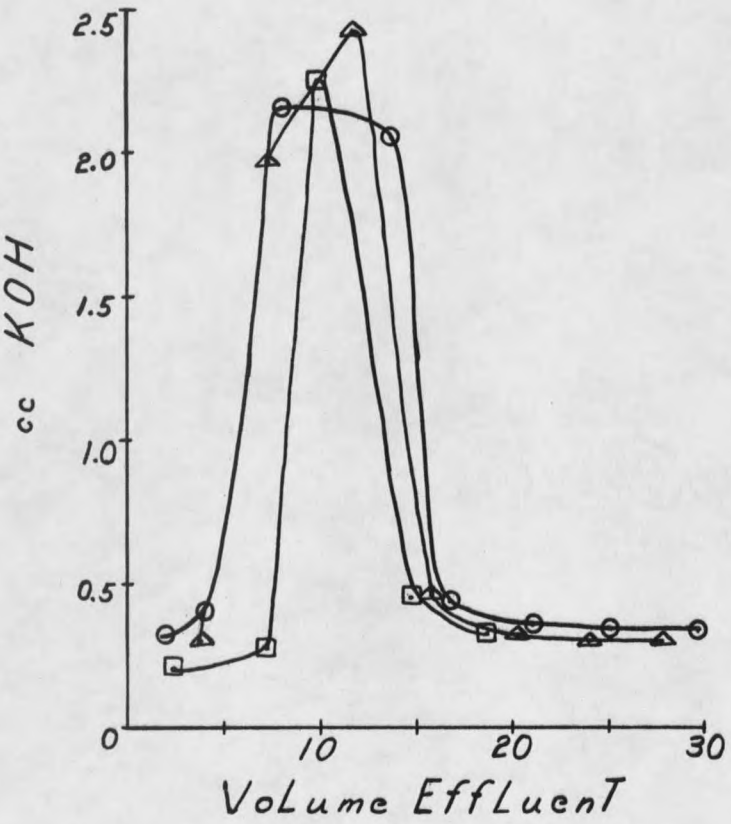

Figure 6. Paper column. Solvent: n-propanol:Skellysolve B in ratio of 3:1. ⊙- Palmitic acid. ▲- Stearic acid. ⊡ - Arachidic acid.

Different alcohols in combination with Skellysolve B were tried next on paper packed columns. N-propyl, allyl, and benzyl alcohols in a ratio of 3:1 with Skellysolve B were used to try to move the acids back from the solvent front. The curve obtained with n-propyl:Skellysolve B as a solvent is shown in figure 6. Palmitic, stearic, and arachidic acids were not moved back from the solvent front and did not show any tendency to separate. The same types of curves were obtained with allyl and benzyl alcohols. The results indicated on paper columns were not promising so the material was rejected as unlikely to affect fatty acid separations.

A 1:1 mixture by weight of magnesium oxide and Hyflo supercel was utilized next for column material using a solvent combination of Skellysolve B and acetone. This procedure is used by this laboratory in the routine analysis of plant material for carotene and it was hoped that fatty acids could also be separated by the system. Several trials were made using a mixture of acids. Elution peaks were noted in one trial but these results were not duplicated in repeat columns. This seems to indicate that any results obtainable on a magnesium oxide-Hyflo supercel column would be unpredictable and make this type of column very difficult to use.

In 1940, Bengen discovered the phenomena of urea complex formation with normal aliphatic compounds. Schlenk and Holman (31) have utilized this for a method of separating and stabilizing fatty acids. An attempt was made to adapt this method to column chromatography. Ethanol and isopropanol were used as solvents after saturation with urea. The columns

were packed using a slurry of urea in the alcohols. The acids came out with the solvent front but it was noted that only a small proportion of the applied acids emerged with the effluent solvent. It was further noted that as the length of the carbon chain of the acid increased the amount of acid eluted from the column diminished. This effect can be seen in figure 7.

The titer required for equivalent amounts of stearic acid was less than that for palmitic acid and the titer required for palmitic acid was less than that needed for myristic acid.

Attempts to adapt the method of Kritchevsky and Tiselius (17) for steroid separations to fatty acid separations gave the best indication of a system suitable for this type of chromatography.

Dow Corning Antifoam A was used as the silicone source. The paper was coated by suspending the antifoam in diethyl ether and mixing in sufficient quantities of the paper. The ether was then evaporated to leave the paper evenly coated with the silicone. The solvent system originally employed was ethanol:chloroform:water in the ratio of 10:10:6. This formed a two phase system. The coated paper was packed in the chromatographic columns using the lower phase. After addition of the acids by an alcohol solution, the columns were developed using the upper phase. It was found that a new column had to be prepared for each new run since the chloroform tended to remove the silicone from the paper. The first column was short, 7 1/2 inches long, and the mixture of acids showed a tendency to separate as shown in figure 8. This result indicated the need for a