PARTITION CHROMATOGRAPHY OF FATTY ACIDS

by

KHUSHMANLAL PRANJIVANDAS PATEL

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

at the

UNIVERSITY OF WISCONSIN

1954

ACKNOWLEDGEMENT

The author wishes to express his sincere appreciation and thanks to Dr. T. Higuchi for suggesting this problem and guiding it to completion.

Appreciation is also expressed to the Wisconsin Alumni Research Foundation for grants which helped to make this investigation possible.

Approved by Takeru Higuchi

Assoc. Prof. of Pharmacy

Date May 28th 1954

UNIVERSITY OF WISCONSIN LIBRARY
MANUSCRIPT THESES

In presenting this thesis in partial fulfillment of the requirements for an advanced degree at the University of Wisconsin I agree that the Library shall make it freely available for inspection. I further agree that permission for extensive copying or publication of this thesis for scholarly purposes may be granted by my major professor, DR. TAKERU HIGUCHI, or, in his absence by the Dean of the Graduate School. It is understood that any copying or publication of this thesis for financial gain shall not be permitted without my written permission.

May 28, 1954
Date

Khushmanlal P. Patel
Author

FOR BORROWERS OF THIS THESIS

Unpublished theses deposited in the University of Wisconsin Library may be used only with due regard for the rights of the authors. Permission for the extensive copying or publication of this thesis in whole or in part must be obtained in advance in all cases from the major professor named above, or, in his absence, from the Dean of the Graduate School. Extensive copying or publication of this thesis for which there might be financial return to the borrower must have the written permission of the author.

This thesis has been used by the following persons, whose signatures attest their acceptance of the above restrictions.* A library which borrows this thesis for the use of its patrons is required to secure the signature of each user.

Name & Address of User	Date Used

TABLE OF CONTENTS

PAGE

I. INTRODUCTION..... 1

II. NEED FOR ANALYTICAL METHODS FOR FATTY ACID MIXTURES..... 3

III. REVIEW OF METHODS PROPOSED FOR ANALYSIS OF FATTY ACIDS... 9

A. METHODS FOR DETERMINATION OF PHYSICAL AND CHEMICAL PROPERTIES OF MIXTURES..... 9

B. ANALYTICAL METHODS FOR DETERMINATION OF CHEMICAL COMPOSITION.....10

1. Spectrophotometric Methods.....11

2. Solubility Methods.....11

3. Distillation Methods.....12

a. Steam Distillation.....12

b. Vacuum Distillation.....13

c. Molecular Distillation.....15

4. Chromatographic Methods.....16

a. Adsorption Chromatography.....16

(1) Elution Analysis.....16

(2) Frontal Analysis.....18

(3) Displacement Analysis.....19

(4) Carrier Displacement Analysis.....20

b. Partition Chromatography.....22

(1) Partition Chromatography of Silica Gel.....23

(2) Gas-liquid Partition Chromatography.....26

(3) Reversed Phase Partition Chromatography.....27

(4) Paper Chromatography.....28

TABLE OF CONTENTS

(Continued)

PAGE

c. Chromatography of the Sample.....84

(1) Apparatus.....84

(2) Reagents.....84

(3) Packing of the Columns.....85

(4) Sample Addition and Elution.85

(5) Analysis of Eluates.....86

(6) Calculations.....87

4. Results and Discussion.....88

VI. GENERAL CONCLUSION.....90

VII. SUMMARY.....91

BIBLIOGRAPHY.....93

APPENDIX..... i

TABLE OF CONTENTS

(Continued)

PAGE

(5) Comparison of Different Methods.....30

IV. THEORETICAL ASPECTS.....32

A. SIMPLIFIED THEORY OF PARTITION CHROMATOGRAPHY.....32

B. THEORETICAL LIMITATION OF PARTITION CHROMATOGRAPHIC METHODS PRESENTLY IN USE FOR HIGHER SATURATED FATTY ACIDS.....41

V. EXPERIMENTAL STUDIES.....49

A. ATTEMPTED MODIFICATION OF RAMSEY AND PATTERSON'S METHOD.....50

B. ATTEMPTED USE OF ION-EXCHANGE RESINS IN PARTITION CHROMATOGRAPHY.....54

1. Ion-exchange Partition Chromatography of Higher Fatty Acids.....54

a. Introduction.....54

b. Theory.....55

c. Results and Discussion.....56

2. Ion-exchange Partition Chromatography of Lower Dicarboxylic Acids.....61

C. SEPARATION METHODS BASED ON CONVERSION OF FATTY ACIDS TO AMINES.....67

1. Theoretical and Experimental Basis for the Method.....67

2. Development of Partition Chromatographic Methods for the Separation of Mixed Esters of C_6-C_8 and C_8-C_{11} Saturated Fatty Acids..75

3. Recommended Procedure.....82

a. Preparation of Reagents.....82

b. Procedure for Carrying out the Reaction.....83

I. INTRODUCTION

Despite the importance of saturated fatty acids and their derivatives to the food, drug and chemical industries, no satisfactory method of analysis for mixtures of these acids is presently available. This paper will attempt to show the theoretical limitations of the current methods, largely chromatographic, and to present the results of a search for a better method.

The higher fatty acids are usually separated by fractional distillation of their methyl or ethyl esters. Low temperature crystallization is also being used more frequently for fatty acid purification, as well as for separation. However, these techniques are not readily adapted for routine analytical procedures and especially for small amounts of materials.

Refinements in apparatus and improvements in the above procedure have somewhat increased the reliability of these methods. Nevertheless, they are either time consuming or require special apparatus. Furthermore, they do not produce sharp separations and are consequently not suitable for quantitative determinations.

In searching for more reliable and simpler procedures for smaller quantities, many workers have investigated chromatographic techniques. Fatty acids have been separated by these methods, but the results leave much to be desired. The classical chromatographic method is that of Ramsey and Patterson (1). Although it can be used for small quantities of higher saturated fatty acid homologues, it is time consuming and laborious. The recoveries range over 100 ± 10%. Only the separation of even-numbered carbon atom acids from each other or odd-numbered carbon atom acids from each other can be achieved fairly

completely. Obviously there is a need for a rapid routine method for analyzing mixtures of these fatty acids; especially when mixtures of odd- and even-numbered carbon atom fatty acids are involved.

These investigations were a part of a larger program devoted to exploration of methods and means of utilizing partition chromatographic methods for analysis of substances of pharmaceutical nature. Previous studies have shwon the applicability of this approach to the aspirin, phenacetin and caffeine combinations and to mixtures of -hydroxy-benzoate esters. The results of the latter studies are included in this report in forms of reprint.

II. NEED FOR ANALYTICAL METHODS FOR FATTY ACID MIXTURES

There are innumerable potential uses for a good method of fatty acid analysis. These include product control in the pharmaceutical and cosmetic industries; the determination of composition of flavors, both natural and synthetic; the application to studies of fermentation and food decomposition; and many others.

Despite the long history of fatty acid investigation, not many workers have been attracted to its analytical aspects. Because of the apparent affinity of the members of the fatty acid series for one another, their mixtures are difficult to separate without elaborate techniques. These techniques have included fractional distillation, selective crystallization, and chromatography.

Another obstacle has been the lack of pure materials. In the cosmetic industry, for example, it has been postulated that the gelling agent in most solid colognes is sodium stearate. Depending upon the quality of the stearic acid used, there have been varying amounts of gelling. Thus, Hystrene T-70 (Atlas Powder Company stearic acid) produces better gelling than triple-pressed stearic acid. Analysis of Hystrene T-70 showed it consisted of 1% or less of oleic acid and about 70% of stearic acid. Triple-pressed stearic acid, on the other hand, contained about 5% of oleic acid and about 45% of stearic acid. Hence the superior gelling with Hystrene T-70 over that with triple-pressed stearic acid corroborates the above hypothesis.

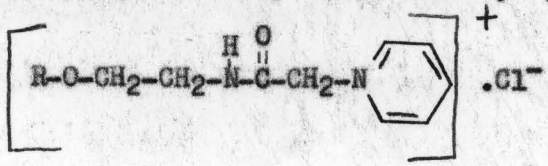
Commercial stearic acid is prepared in large quantities and is graded according to its melting point. Depending upon the type fat

used and the method of preparation, different grades contain more or less stearic acid together with palmitic acid and unsaturated acids. The different grades are designated as: "Single pressed (M.P. 125-126° C)," "Double pressed (M.P. 129-130° C)," and "Triple pressed (M.P. 130-131° C)."

Surface active agents prepared from fatty acids consume well over two billion pounds a year of fatty acids. Soap--the alkaline salt of fatty acids--accounts for the major portion of such products. The unique straight chain hydrocarbon radical with the terminally reactive group is ideal for achieving the maximum in desired surface active properties. The sodium salts of fatty acids ranging from C_{12} to C_{18} are excellent detergents, particularly when selected and blended for the specific purpose required, while those of shorter chain acids impart more rapid solubility and low temperature performance. Hence from the control viewpoint, a good method of fatty acid analysis would be of great practical value.

Surface active agents produced from fats and oils have been classified as cationic, anionic, and non-ionic agents.

The cationic agents are essentially substituted ammonias. Certain salts of primary amines made from fatty acids are excellent cationic surface active agents. Quarternary ammonium salts are likewise cationic and are used extensively as germicides and fungicides. Epstein, Harris and Katzman (2) varied the fatty acids from C_8 to C_{18} and determined the bactericidal values of the compounds produced. Their results showed that varying the fatty acid radical, R, in



where R = $CH_3-(CH_2)_n-\overset{O}{\overset{\|}{C}}-$, had a decided effect on the bactericidal potency and that the C_{14} homologue had the maximum bactericidal activity. The sequence of diminishing bactericidal power was as follows:

$$C_{14} > C_{12} > C_{16} > C_{18} > C_{10} > C_8.$$

Although cationic agents have received much wider acclaim, there are also numerous effective germicides among the anionic surface active agents. In this group of surface active agents, fatty alkyl sulfates have been most widely studied. The straight chain primary C_{12} to C_{18} compounds are effective against Gram-positive bacteria and much less effective against Gram-negative types.

Non-ionic agents have been used in pharmaceutical and cosmetic industries, primarily in emulsion formation. Thus, the glyceryl monostearate emulsifiers have been used to prepare emulsions of castor oil, mineral oil, etc., for internal medication.[7] Cod liver oil and vitamin oil emulsions have been prepared with Sorbitol oleate emulsifiers.

In all of these surface active agents, the purer the fatty acid used, the more specific is the property of the product. Hence for effective quality control in manufacturing to produce uniform, reproducible products from different cheap raw materials, accurate analytical procedures would be extremely valuable.

Besides the uses of surface active agents in pharmacy, their uses in laundries and as cleaning and sanitizing agents are too well known to require further comment other than to point out that such usage exceeds in volume any other use for surface active agents. They have

also been used in the textile industry for scouring wool, in kier-boiling of cotton, etc., as lubricants for wire drawing, as emulsifiers for waxes and polishes, and many others. The list of uses is inexhaustible, but this gives one some idea of the scope of diverse uses of different fatty acids which have resulted out of better methods of fractionation of fatty acid mixtures.

As newer methods of analysis became available, previously unreported components of fatty acids of some oils were discovered. Wikoff, Kaplan and Berman (3), in trying to investigate the cause of spoilage of peanut butter, reported the discovery of lauric and caprylic acids in peanut oil in 1944. Previously to this in the literature, no mention had been made about the presence of these acids in the analysis of peanut oil.

Thaunahauser, Setz and Bennetti (4) found on analysis that spleen contained palmitic, stearic and lignoceric Sphingomyelins in the ratio of 2:2:1. The analysis of Sphingomyelin from red blood cells showed that it was composed mainly of lignoceric Sphingomyelin. This fact had some physiological significance which they were not able to explain.

Bacteria are known to ferment glucose to 2,3-butanediol and as metabolic products, all form organic acids. These acids are likely to be acetic, n-butyric, formic, succinic and lactic acids. For analysis of these acids, a method by partition chromatography was developed.

In studying the volatile flavor and odor constituents of pineapple, small amounts of ester mixtures of fatty acids were obtained which were difficult to separate sharply. Subsequent hydrolysis gave a mixture of fatty acids which Kirchner et al (5) were not able to

separate and purify at that time. This gave them an impetus to develop a method of analysis for that particular purpose.

Fatty acids are basic building blocks of polymerizable protective coatings. Accordingly it is absolutely necessary to gain some insight, first of all, in the composition of fatty acid mixtures obtained by the hydrolysis of the oil; secondly, how these fatty acids undergo the process of polymerization. Finally, it is also of interest to inspect the protective coatings which are based on the chemistry of fatty acids and on their ability to polymerize. On these considerations, tung oil, castor oil, sardine oil, and other marine oils have been successfully used in the paint industry.

Practically all tradings in oils and oil bearing materials, as well as many products derived from them, e. g., fatty acids, are now conducted in an orderly manner, under the trading rules maintained by trade associations and governmental agencies. Many of the rules and the contracts for purchases specify the use of analytical methods for evaluation purposes which have been investigated and adopted by trade associations. The assurance with which these methods are used has been instrumental in establishing greater confidence between buyers and sellers, thus almost completely eliminating trade controversies. A further use of such analytical methods is in the field of research on oil bearing materials, fats and oils of the products derived from them. By providing standardized means of evaluation, these methods permit the properties of similar products to be measured and compared, process control in research and production to be followed, and fundamental research results of different workers to be correlated.

Practically any of the uses of fatty acids and derivatives

thereof, which have been so briefly described could be elaborated at length. Every use that has been mentioned is commercially significant. As the result of appearance of new fatty acid derivatives each year, we have an expanding field of uses developing. Without entering into a discussion of fatty acids technology, one may emphasize that whether in obtaining proper solubility and detergency properties for soaps, in achieving proper polymerization characteristics for drying oils or in attaining desirable plastic and organoleptic qualities for shortening, knowledge of fatty acid composition in the raw materials is of greatest value. In many instances, mere knowledge about fat will suffice, but very often knowledge of different fatty acids in respective fat is very important. As long as one fat is more available and another more suitable, there will be the desire for modification and substitution of one for the other. It is by the combination of improved methods of fatty acid modification in a fat with a greater knowledge of fat composition that good cause of flexibility in choice of fatty raw materials will be advanced.

III. REVIEW OF METHODS PROPOSED FOR ANALYSIS OF FATTY ACIDS

The available analytical techniques may be divided into two broad classifications: those designed for (1) determination of physical and chemical properties; (2) determination of chemical composition. These are reviewed in turn in the following sections.

A. Methods for Determination of Physical and Chemical Properties of Mixtures

These methods are employed usually for the purpose of characterizing mixed fatty acids and their derivatives as such. They include such classical methods of fat and oil analyses as iodine number, acid number, thiocyanogen number, saponification equivalent, neutral equivalent, etc. Physical properties such as melting point, boiling point, setting point, transition temperature, and refractive index are also used wherever possible. These methods are not used for the determination of molecular compositions of these mixtures. These methods can be used in certain isolated cases for determination of exact composition.

When the mixture is known to contain two saturated homologous acids and an unsaturated acid, e.g., oleic acid, then the amount of oleic acid is determined by means of iodine and thiocyanogen numbers and amount of saturated acids is determined by the observed neutralization equivalent or molecular weight which will lie in between the respective values for two homologues. In case of esters, the unsaturated component may be destroyed by permanganate oxidation

prior to determining the saponification value of the mixture.

If the unsaturated component is not oleic acid but one with higher unsaturation, then the calculation of the composition of the mixture becomes more involved. Hilditch (6) has shown various mathematical treatment for such calculations.

Longenecker (7,8) et al have pointed out the difficulties of determining the composition of mixed fatty acids by these methods. These authors have shown that in a mixture of methyl palmitate and methyl stearate, saponification equivalent must be determined accurately to $\pm$ 0.28 units in order to calculate the composition to $\pm$1.0% of the true value. The authors also have proposed a method of determining the composition of methyl esters based on refractive indices with a high degree of accuracy, provided there is available a refractometer of the requisite precision. They have determined the refractive indices of known binary mixtures of methyl esters of higher saturated fatty acids (C_{10}-C_{18}) as reference standards. On the basis of this work, it was found that the composition of a binary mixture could be determined to an accuracy of $\pm$ 0.5% with an Abbe refractometer.

B. Analytical Methods for Determination of Chemical Composition

These could be divided into four general classes, all involving separation, except the first:

1. Spectrophotometric methods
2. Solubility methods
3. Distillation methods
4. Chromatographic methods.

1. Spectrophotometric Methods. (9)

The measurement of absorption spectra for fatty acids in the far region of ultra-violet has resulted in the development of the methods for quantitative calculations of the fatty acids with two or more double bonds present in fatty acids. The analysis of unsaturated fatty acids is beyond the scope of this study and therefore the method has not been discussed in detail.

2. Solubility Methods. (10-16)

There are available various methods for the separation of fatty acid mixtures which depend on relative solubilities of individual acids, their salts, or bromo derivatives in different solvents or in the same solvent at different temperatures. These methods make it possible to separate a mixture of fatty acids into saturated and unsaturated fractions and to separate the unsaturated acids into subfractions of different degrees of unsaturation or into individual components. None of the methods is strictly quantitative owing to the mutual solubility effects exerted by one component of the mixture on the solubility of the other components or vice versa. However, they afford a means of resolving relatively complex mixtures into simpler ones which can in turn be separated further by other means.

These methods, however, cannot be used for the separation of the mixtures of saturated fatty acids, and therefore have not been discussed here. However, it will be worthwhile to mention the low temperature crystallization process. This solubility method is not universal in its application, but with mixed acids of many fats and

oils rather good separations of saturated and unsaturated acids can be obtained.

According to trade literature (17) Emery Industries, Inc. began investigations on the solvent fractionation of fatty acids in 1934 which culminated in the development of a pilot plant and subsequently a semi-commercial unit for effecting this operation. In 1942, the first of two commercial units for production of a variety of fatty acid fractions by low temperature fractionation was placed in operation. The process has been applied to the production of stearic and oleic acids from Twitchellized and distilled tallows at a rate of approximately two million pounds per month. It is claimed that the process has been applied to the separation of solid and liquid acids of linseed, soybean, palm, sardine and cottonseed oils.

3. Distillation Methods.

Distillation of mixed fatty acids and their derivatives may be carried out to effect one of the several different types of separation, namely, (a) to separate so-called volatile from non-volatile acids by means of steam (18,19,20); (b) to separate relatively non-volatile members of a homologous series by vacuum distillation; and (c) to separate a relatively non-volatile acid from a non-distillable component such as an oxidized or polymerized component by molecular distillation.

a. Steam distillation. Most monographs (18, 19) and methods of analysis (20) contain directions for conducting the separation of so-called volatile acids from non-volatile acids by steam distillation. This method is of some utility as in the case of milk fats,

nearly all of which contain short chain acids such as butyric, caproic, caprylic, etc. Cocoanut, palm kernel and related fats also contain small amounts of acids which are somewhat more volatile than the long chain acids with which they are associated.

Steam distillation may serve to effect a partial separation of the shorter chain from the longer chain acids. The volatilized acids may then be recovered from the condensed distillate by extraction with ethyl ether. After removal of the ether, the acids may be further separated by fractional distillation through an efficient distilling column.

b. Vacuum distillation. Vacuum distillation of fatty acids and ester mixtures constitutes one of the most important methods of separation of these products. Only in the past few years have these phases of fatty acid investigation received attention commensurate with their importance in the laboratory and industry.

A distillation unit (10) of whatever design must be of necessity a compromise between theory and practice, but it is possible to design and operate such equipment with a relatively high degree of efficiency. Such a unit consists of four essential parts, namely, a boiler and a source of heat, a column including the packing and insulation, a still head including a condenser, reflux controller and a fraction cutter, and a means of producing and controlling the vacuum.

Under ideal conditions of fractional distillation, it should be possible to resolve a mixture of components by completely removing successive molecular species at fixed temperatures and pressures. Also ideally, it might be assumed that one component of a given system

should distil at a fixed temperature and pressure until all of that component has been removed, following which the temperature of the condensate vapor should rise to that of the next higher boiling component and again remain constant until it has been removed. Such ideal conditions seldom prevail and in case of routine fatty acid or ester fractionation, they are quite frequently not even approximated. Distillation is more often than not carried out over a range of temperatures and pressures without any very exact knowledge of the manner in which these conditions are varying. The resulting fractions cannot therefore, be expected to be composed of a single component, or if the original mixture is very complex, even of a very small number of components. This is true in many cases, as is evident from the examination of published data on acid and ester fractionation.

Wyman and Barkenleus (21) found no very great difficulty in separating binary mixtures consisting of methyl esters of myristic, palmitic and stearic acids but with a three component system it was quite difficult to obtain complete separation. Each fraction, though predominant in one ester, was contaminated little with others. A second fractionation gave better separation.

Norris and Terry (22) have likewise discussed at length the requisite of good ester fractionation and illustrated with examples of the degree of separation which is obtainable with binary mixtures of methyl esters of oleic and stearic acids, oleic and linoleic acids and with U.S.P. stearic acids.

Since knowledge of composition of a mixture of fatty acids or esters is dependent to a large extent upon the known efficiency of

any given fractionation apparatus and technique employed, there is much need for additional systematic and painstaking investigation in the field. All of the elements, including relatively pure acids and esters, many types of distillation equipment and a fundamental knowledge of the theory of distillation are at hand for undertaking such investigations.

c. Molecular distillation. This differs from ordinary distillation by virtue of the fact that evaporation and condensation occur in a highly evacuated atmosphere (0.001 mm or less), between surfaces separated by a very short distance, usually of the order of 1-2 cm, and by the further fact that evaporation of the distilland occurs from the surface of thin films (0.01-0.02 mm). Under these conditions, separation of normally non-distillable, high molecular weight and thermally unstable substances is possible.

From time to time, reviews (10) covering this technique have appeared, and many types of distillation equipment have been described, together with their application to the separation of a variety of substances.

Although molecular distillation has been carried out in the separation of unsaturated fatty acids, no work has been reported in the literature about the separation of higher saturated fatty acids.

4. Chromatographic Methods.

The theory and practice of chromatography have been summarized in several major publications (23-26). Although chromatography is a routine method in separations and analysis in some fields, it has not generally reached that stage of development in its application to the analysis of mixtures of fatty acids.

From the scattered observations which are reproduced in the following discussions, it will be apparent, however, that chromatography is an effective tool when applied to some specific problems. If the tendency to systematize the chromatographic information about the analysis of mixtures of fatty acids is accelerated, this method might prove to be a very popular and powerful analytical method for the mixtures of fatty acids.

The chromatographic techniques thus far developed for use in fatty acid analysis fall into two general classes: a. adsorption chromatography; b. partition chromatography.

a. Adsorption chromatography. This general technique, which is fundamentally dependent on the differential adsorbability of the homologous acids, can be subdivided into several methods,which are taken up in the following sections. These methods generally suffer from a number of drawbacks. They are long involved, laborious, and tedious procedures and take as long as three weeks to complete the analysis of binary mixtures of fatty acids, such as mixtures of stearic and palmitic acids. Results leave much to be desired.

(1) Elution analysis.

In this method, the sample is applied to the top of an adsorption column in the form of a solution. When additional solvent is added,

the adsorbed solutes migrate slowly down the column, the rate of migration being dependent upon the relative ease with which a particular solute is desorbed by the solvent or "eluate." By the elution procedure, the adsorbed zones are separated one from the other and can be washed from the column, one at a time. The least adsorbed substance moves most rapidly and emerges first from the column. The most adsorbed substance is the last to emerge.

Graff and Skau (27) have separated partially oleic and stearic acids on magnesium oxide columns, colored by the addition of phenol red. The acids from visible zones which may be eluted by dissolving the magnesia in dilute hydrochloric acid. Cassidy (28) described the separation of lauric and stearic acids, and palmitic and stearic acids on a charcoal column using light petroleum as solvent. However, these experiments take many days or even weeks, and yields of pure acids leave much to be desired (ca 60%).

Stearic acid was separated from abietic acid by adsorption on charcoal and elution with a variety of solvents in an investigation by Papps and Othmer (29). 1-Nitropropane gave by far the best results.

Using magnesium sulfate as adsorbent and petroleum ether as solvent, Manunta (30) was able to segregate a model mixture of equal parts of palmitic, stearic and oleic acids. Oleic acid was least adsorbed and palmitic acid was most adsorbed in this system. Mazumdar and Goswami (31) found alumina and magnesia to be the best adsorbents for separating oleic and stearic acids, whereas Dutton (32) achieved good separation of these two acids using activated carbon.

Dutton and Reinbold (33) investigated the separation of ethyl stearate, oleate, linoleate, linolenate in all possible combinations of two. They used alumina as adsorbent and 1.75% ethyl ether in petroleum ether as solvent. They observed fractionation in all six systems, the purity of the first component varying from 68 to 83% and the second component varying in purity from 61% to 76%. In all cases the second component was more unsaturated ester (more strongly adsorbed).

This type of chromatography has also been used for fractionating methyl esters of fatty acids from beef adrenal lipids (34), in preparing highly purified methyl linoleate and methyl linolenate from methyl esters of linseed and perilla oil (35), in separating oxidation products of unsaturated fatty acids (36-38), and in scrubbing out free fatty acids from an oil (39).

Above are given but a few examples which will serve to illustrate the wide range of usefulness of elution chromatography in fatty acid analysis.

(2) Frontal analysis.

When a solution containing a mixture of solutes is passed continuously through a column of adsorbent, the solutes are adsorbed from the solution and are retarded to different degrees. The components appear in the effluent in order of their adsorbability. If successive small increments of the effluent are analyzed by physical or chemical means, a front will be observed for each component in the mixture. When the last front appears, the composition of the percolate is the same as that of solution pressed in. The only component which

is separated pure is that portion of the least adsorbed substance which appears ahead of the second front. This technique is obviously not intended as a preparative procedure. Its value lies chiefly in appraising a mixture for its complexity.

Claesson (40) has studied the separation of model mixtures of fatty acids, ethyl esters of fatty acids, dibasic acids on active carbon. With silica gel dried at 800° C as adsorbent, the unsaturated, saturated and branched chain acids could be separated. With charcoal as adsorbent, he has separated saturated acids, the adsorption increasing with the chain length.

(3) Displacement analyses.

If a sample to be analyzed is dissolved in a solvent and pressed into a chromatographic column, and a solution of a more strongly adsorbed substance is pressed in after it, the solutes arrange themselves in zones on the column in order of adsorbability. As the "displacer" substance advances down the column, the substances in the sample arrange themselves in zones ahead of it, each in turn displacing the next more weakly adsorbed substance. Each displaced substance emerges from the column in a characteristic concentration, and in the more general cases, if concentration of solute is plotted against the volume of effluent, a stepwise curve is obtained. The height of the step is characteristic of the substance in a given chromatographic system, and the area under the step is proportional to the quantity of the component.

Holman and Hagdahl (41) have carried out separations of higher fatty acids using this technique. Palmitic acid (0.5 per cent in

alcohol) serves as displacer for myristic and lauric acids, but picric acid is the most useful displacing agent found. The authors also showed that the displacement of linoleic acid is difficult, as the acid leaves the column only very slowly, mixed with the displacing agent. The recoveries of this acid were only 70%.

A study of the effect of unsaturation upon adsorbability of fatty acids on charcoal has been made by Holman and Williams (42). Saturated and unsaturated acids of the same chain length have been separated by displacement in the range of 4 through 18 carbon atoms. Butyric and crotonic acids were separated by displacement with caproic acid in water solution. Valeric, 2-pentenoic, and caproic acids were separated in aqueous solution. Isovaleric, α,β-unsaturated isovaleric and caproic acids were separated similarly. Caproic and sorbic acids were easily separable in 40% aqueous ethanol by displacement with caprylic acid. Undecenoic and undecanoic acids were separated in 50% aqueous ethanol solution by displacement with lauric acid. Palmitoleic and palmitic acids were separated in 95% ethanol solution by displacement with stearic acid.

In C_{18} series, oleic, petroselinic and linoleic acids have been separated from stearic acid by displacement. The separation of linoleic acid, its conjugated isomer and stearic acid was achieved by displacement with 0.9% arachedic acid in absolute ethanol. Impurities in fatty acids which could not be removed by repeated fractional distillation were detectable by displacement chromatography.

(4) Carrier displacement analysis.

This technique was introduced by Tiselius and Hagdahl (43). In this procedure, a proportionately large quantity of "carrier" sub-

stances is added to the limited amount of sample to be separated. When the sample plus carrier is displaced, the carriers and components of the sample arrange themselves in zones. The carriers form broad zones and the sample substances form narrow zones, between the carriers. Thus, the components of the sample are carried at the fronts of the carriers, and small quantities of substances, too small to be detected as individual zones, can be separated.

Hagdahl and Holman (44, 45) have described the separations of all the straight chain fatty acids from C_1-C_{20}, using a mixture of 1 part Darco G-60 and two parts Hyflo Super Cel. The best displacer for a group of acids is the next higher acid, and as solvent, mixtures of water and ethyl alcohol are used. Although under the correct conditions, each acid gives a definite step, detectable optically as well as by titration, Holman (46) in a later paper improves upon the separation and obtains quantitative recoveries by the use of carrier displacement. (Table 1).

Table 1
Recoveries of individual and total acids in carrier displacement separations. Solvent, 0.05% methyl stearate.

Acid		Experiment 1	Experiment 2
Lauric	Sample, mg.	4.75	3.85
	Recovery, %	100.0	93.5
Myristic	Sample, mg.	10.2	9.6
	Recovery, %	100.4	100.1
Palmitic	Sample, mg.	14.0	14.5
	Recovery, %	100.2	98.5
Stearic	Sample, mg.	14.4	14.8
	Recovery, %	98.0	100.3
Total	Recovery, %	100.1	100.1

If the small quantities of the acids are mixed with ten times the weight of their methyl esters in 95% ethanol, the acids emerge immediately in front of their methyl esters. Titration of the effluent shows recoveries of 93.5-100.3%. A displacer for methyl esters must also be employed, and Holman uses the next higher methyl ester. For example, in separation of myristic, palmitic and stearic acids, methyl laurate, myristate and palmitate are used as carriers and 1% methyl stearate as the displacer.

The separation of branched and unbranched saturated fatty acids as palmitic and isopalmitic acids has been achieved by carrier displacement. Using similar systems, the separability of four different branched isomers of stearic acid has been demonstrated (47).

Displacement chromatography, although a new technique, has proved to be very useful in many types of separations, in some cases quantitatively too. It has been applied to fatty acids, esters, triglycerides, and sterol separations (72).

b. Partition chromatography. This modification of chromatographic procedure utilizes the differences in partition coefficients of several solutes between two liquid phases. One liquid phase is held immobile by a solid carrier, the other is allowed to flow past the immobile phase. In practice, partition chromatography is very similar to elution chromatography. The basic difference is that in partition, chromatography, the immobile phase is liquid and the separations depend upon the partition coefficients of solutes in the two liquid phases. Several variations of this basic method have recently evolved.

(1) Partition chromatography or silica gel.

Smith (48) applied partition chromatography on silica gel to the separation of volatile fatty acids and obtained qualitative separations of formic, acetic, propionic, butyric and valeric acid. The technique has been further developed by Elsden (49). As formic acid is strongly adsorbed, it must first be eliminated. The other four acids are dissolved in chloroform containing 5% of butanol and passed through a column of silica gel, which acts as the support for the stationary phase of water, colored with bromocresol green. The different acids are retained in order of their partition coefficients: acetic acid at the top of the column and the other distributed down the column in order of increasing chain length, each acid forming a yellow zone. The determination of the R_f values allows the identification of the acids. On washing the column with chloroform-20% butanol, each zone passes out of the column in the filtrate. The zones are estimated quantitatively by titration of the eluates with an accuracy of 0.5-1.5%. Elsdon was not successful in eluting acetic acid out.

Several other workers have improved on the method of Elsdon. Neish (50) substituted benzene-butanol and Nijkamp (51) CCl_4-butanol for Elsdon's $CHCl_3$-butanol and obtained quantitative estimations of acetic acid.

Scarisbrick et al (52,53) have obtained quantitative separations ($100 \pm 4\%$) of the saturated volatile fatty acids (C_2 to C_8), using three or at the most four acids in one column. Columns were silica gel partition columns, having aqueous buffers as the stationary phase and chloroform-butanol mixture as the mobile phase.

No indicators were used on the column, but the column eluate was titrated directly with 0.005 N KOH. Straight and branched chain isomers, however, could not be separated.

Fairbairn and Harpur (54) used two columns of silica gel in series. The upper column retained C_2 and C_3 acids and the lower separated C_4-C_8 acids. By disconnecting the two columns after the C_4-C_8 acids have passed through the first, quantitative estimations were carried out with an accuracy of $100 \pm 2\%$.

By using first an unbuffered column and rechromatographing the higher acids on buffered columns, Gray et al (55) have achieved the separation of isomeric acids as well as obtaining quantitative results for all the acids present in rumen distillates. Ramsey (56) has also obtained a partial separation of n- and isobutyric acids on silica gel-methanol partition column, using isooctane as eluant. Isomeric C_5 acids were separated by Bueding and Yale (57) on Celite columns buffered at pH 6.5 with phosphate buffer and by using 5% to 20% butanol in chloroform mixtures as eluants for different acids.

Ramsey and Patterson (58) have used a column of silicic acid saturated with methanol (containing bromocresol green as indicator) and a mobile phase of isooctane to give the separations of the straight chain fatty acids from C_5 to C_{10} with an accuracy of $100 \pm 10\%$. In a later publication (1) the range was extended to the C_{11} to C_{19} acids (odd carbon number acid mixtures or even carbon number acid mixtures, but not all in a mixture) by using a silicic acid column with a mixture of furfuryl alcohol and 2-aminopyridine as the stationary phase, and n-hexane as the mobile phase. Recoveries here

were also 100 $\pm$ 10% (Tables 2 and 3). Quantitative separations of straight chain fatty acids from C_2 to C_{10} have been obtained by Peterson and Johnson (59) using a celite column with 27 to 35 N sulfuric acid as the stationary phase and benzene as the mobile phase.

Table 2 (1)

Recoveries of lauric, myristic, palmitic, stearic acids when present in admixture.

	Acid (Name)	Added (mg)	Found (mg)	Recovery (%)
Mixture 1	Stearic	10	10	100
			9.3	93
	Palmitic	10	9.7	97
			9.8	98
	Myristic	10	9.7	97
			9.7	97
	Lauric	10	9.4	94
			10.0	100
Mixture 2	Stearic	10	9.3	93
	Palmitic	20	18.8	94
	Myristic	5	5.5	110
	Lauric	3	3.1	103

Table 3 (1)

Recoveries of nonadecanoic, margaric, pentadecanoic, tridecanoic, hendecanoic acids when present in admixture.

	Acid (Name)	Added (mg)	Found (mg)	Recovery (%)
Mixture 1	Nonadecanoic	10	9.4	94
	Margaric	10	9.4	94
	Pentadecanoic	10	10.0	100
	Tridecanoic	10	9.5	95
	Hendecanoic	10	9.3	93
Mixture 2	Nonadecanoic	20	18.8	94
	Margaric	20	19.7	98
	Pentadecanoic	20	19.4	97
	Tridecanoic	20	19.1	95
	Hendecanoic	20	18.9	94

Ramsey and Patterson's (58) method was employed for the detection of n-pelargonic, n-caprylic and n-oenanthic acids in the degradation of a glycolipid by Jarvis and Johnson (60) and for oenanthic acid in the degradation of trans-11-octadecenoic acid by Bumpus et al (61). Peterson, Johnson, and Price (62) also used this method for analysis of fatty acids in cheese.

(2) Gas-liquid partition chromatography.

Partition chromatography on silica gel has been extended by James and Martin (63) to columns using a stationary liquid phase and a mobile gas phase (nitrogen). The columns consist of 4 ft.

or 11 ft. lengths of 4 mm internal diameter glass tube packed with kieselgühr (size graded celite 545) which acts as the support for the liquid phase, which for the separation of the volatile fatty acids, is a mixture of DC 550 silicone and stearic acid (10% w/w). The end of the column is drawn down to a capillary and by means of a thin rubber gasket fits into a bottom of a titration cell containing aqueous indicator (0.01% phenol red). The mixture of the acids is applied by means of a micropipette to a fibreglass plug at the far end of the column packing. A stream of nitrogen gas from a manostat is then passed down the column. The acids move down the column generally in the order of the molecular weight and are absorbed from nitrogen gas stream by the water in the titration cell. The acids are continuously titrated by means of an automatic recording burette controlled by a green sensitive photocell and an amplifier.

By the use of this technique, all the n- and iso- acids from formic to dodecanoic acid can be separated and roughly quantitatively estimated in micro amounts. All the isomers of valeric acid (trimethylacetic, isovaleric, methylethylacetic, and n-valeric acid) can also be separated.

K. El-Shazly (64) was successful in separating lower volatile fatty acids in rumen of the sheep, kept on a variety of diets by gas-liquid partition chromatogram of James and Martin (63).

(3) Reversed phase partition chromatography.

This technique differs from ordinary partition chromatography in that it uses a non-polar solvent as the immobile phase. Boldingh (65) showed the separation of the C_8-C_{18} acids, using a column of

vulcanised Hevea rubber as support for benzene. By decreasing the water content of aqueous methanol, acids of increasing chain length could be extracted from the column. However, the operation of the rubber columns is critical, for example, the temperature must be kept between 21-23° C.

A simpler reversed phase column was described by Howard and Martin (66) who made Hyflo supercel kieselguhr water-repellant by exposing it to vapors of dimethyl-dichloro-silane. The non-polar phase is readily supported by this material and the fractions of the eluant flowing out of the column were titrated and showed almost complete separations of lauric, myristic, palmitic and stearic acids.

(4) Paper chromatography.

Paper chromatography is in reality one dimensional or two dimensional partition chromatography on a paper support.

The fatty acids from C_1-C_9 were separated by various workers (67-71) using butyl alcohol containing ammonia. The detection of the spots was carried out by spraying the paper with an indicator of the right pH range to show up the pH difference between the neutral background and the spots of the ammonium salts of the fatty acids. Bromocresol green and bromothymol blue (40 mg in 100 ml H_2O) (68) as well as bromophenol blue (50 mg and 200 mg citric acid in 100 ml H_2O) (70) were recommended. R_f values are also given by authors.

Quantitative estimation by measurement of the spot areas was achieved by Reid and Lederer (71) after spraying the paper with alcoholic bromocresol purple and exposing for a short time to NH_3 fumes. Sharply defined yellow spots on a blue background appear, which can

be traced onto graph paper, giving an accuracy of 2 to 5% when three standards were run together with triplicate analyses on the same sheet. This method was found satisfactory for the analysis of fatty acids in the rumen of sheep after distillation, also for the detection of fatty acids in nematode parasites (71).

Fink and Fink (72) converted volatile and non-volatile fatty acids to the corresponding potassium hydroxamates and separated them on the paper and visualized by treatment with ferric chloride which develops purple spots on a yellow background. Thompson (73) developed this technique especially for fatty acids and by employing amyl alcohol-acetic acid-water mixture or benzene-formic acid-water mixture, achieved good separations of all acids from C_1 to C_9. The hydroxamic acids of saturated fatty acids from C_2 to C_{22} have R_f values such that they are separable in an aqueous butanol system, but the higher homologues were inseparable (74).

Owing to their high solubility in lipoid solvents, the acids from C_{10} upwards are not readily separated on paper partition chromatograms. Boldingh (75, 76) achieved good separations of ethyl esters on paper impregnated with rubber latex. On such rubber coated paper strips, separations are possible with methanol and methanol-acetone as solvents. R_f values are only approximate as they depend on the amount of rubber in the paper.

The behavior of high molecular weight fatty acids was studied by Kaufman and Budwig (77) who found filter paper a suitable medium for a radiometric determination of fatty acids by converting them to radioactive salts and carrying out a micro-determination of the iodine value. The authors were successful only in separating pairs

of acids, as oleic and butyric acids, oleic and linoleic acids, and oleic and stearic acids by using 20-30% methanol for separating a high molecular weight acid from a low one and 80-99% methanol for two high ones.

Paper chromatograms are relatively inaccurate when compared with column chromatograms. However, it is apparent that paper chromatograms are adaptable to microanalysis of fatty acids; it seems likely that within the next few years, it will be an area of intense research.

5. Comparison of Different Methods.

It is of somewhat interest to compare directly the relative merits and demerits of the several general methods considered above. No one technique can be said to be generally superior over the rest. Each method appears to have usually its particular area of utility. This is often the case when no single good method is available.

In the present instances we are not particularly concerned with methods based on solubility or low temperature crystallization, as these yield only semi-quantitative data and are not suited for analytical purposes. These methods, moreover, are designed for differentiation, in main, between saturated and unsaturated acids.

Distillation methods are presently probably more widely used in the fats and oils field than all others combined. They are relatively simple in theory and require only normal manipulative skill. To obtain reasonable analytical results, however, considerable investment in distilling equipment is necessary. Furthermore, relatively large samples of the order of a hundred grams are usually used. The results, moreover, are not too satisfactory from an analytical standpoint because of the overlap between components. The method

also requires an inordinate amount of time.

Wherever chromatographic methods have been developed, they are the methods of choice for the analysis. They require samples of the order of milligram quantities. Results obtained by these methods have been shown to be very reliable (Tables 1, 2, 3). One or more various types of chromatography have been used to effect separations of closely similar substances differing by one or more carbon atoms. Sharpness of separation of compounds having slight molecular differences exceeds that of most methods of separation. However, sometimes it takes 15 days to finish one analysis by chromatography. The present state of knowledge of chromatographic methods' application to the field of fatty acid analysis is a little confusing because few experiments could be compared. However, recently some systematized experiments have aided in organizing the information. If this tendency to systematize the chromatographic information is followed, the method will prove to be very powerful analytical method in the analysis of fatty acid mixtures.

IV. THEORETICAL ASPECTS

A. Simplified Theory of Partition Chromatography

A review of the theory of partition chromatography is presented below since it forms the basis for later discussions of various attempts to develop optimum conditions for the method as applied to the fatty acids. The discussion follows essentially that of Craig (79) and Martin and Synge (80).

The treatment of the associated systems* is original with this paper.

The purpose of partition chromatographic separation may be compared with that of continuous liquid-liquid extraction. For the chromatographic method, the laborious procedure of continuous liquid-liquid extraction has been, however, simplified, and improved by immobilizing one phase on a mechanical support, known to have none or weak adsorptive properties.

This analogy permits the calculation of concentrations of solute at any time and place in a chromatographic column. The chromatographic column, for this purpose, is regarded as being divided up into successive layers of such thickness that the solution issuing from each is in equilibrium with the mean concentration of the solute in the non-mobile phase throughout the layer. The thickness of such a layer is termed "H.E.T.P." (height equivalent to one theoretical plate). For the equations to be manageable, certain simplifying assumptions have been made. It is assumed that the diffusion of

*The author is indebted to Mr. Eino Nelson for the calculations done on the electronic computer machine.

the solute between two phases is independent of its concentration, that is, the partition isotherm is linear and is independent of pressure of other solutes.

On the basis of the above assumptions, the following equations were derived.

Let C_u = concentration of solute per unit volume, in the internal phase (non-mobile) in one theoretical plate

C_L = concentration of solute per unit volume in external phase (mobile) in one theoretical plate

V_u = volume of internal phase in one theoretical plate

V_L = volume of external phase in one theoretical plate

U = total amount in internal phase in one theoretical plate

L = total amount in external phase in one theoretical plate

k = partition coefficient

$$= \frac{\text{concentration of solute in internal phase}}{\text{concentration of solute in external phase}}$$

$\therefore$ Total amount in internal phase in one theoretical plate

$$C_u \times V_u = U$$

Similarly, total amount in external phase in one theoretical plate

$$C_L \times V_L = L$$

$\therefore$ Total amount U L unity, $\therefore$ U and L will be fractions

and by definition, $K = \frac{C_u}{C_L}$

Let $r = \frac{V_u}{V_L}$

Now $U + L = 1$ $\therefore$ $V_u C_u + V_L C_L = 1$ ($\therefore$ $U = V_u C_u$ and $L = V_L C_L$)

Substituting C_L by $\frac{C_u}{k}$ and V_L by $\frac{V_u}{r}$, we get

$$V_u C_u + \frac{V_u}{r} \cdot \frac{C_u}{k} = 1$$

$$\therefore V_u C_u \left(1 + \frac{1}{kr}\right) = 1 \qquad \therefore U \left(1 + \frac{1}{kr}\right) = 1$$

$$\therefore U = \frac{1}{1 + \frac{1}{kr}} = \frac{kr}{kr\ 1} = \text{Fraction of solute in internal phase}$$

$$\therefore L = 1 - \frac{kr}{kr+1} = \frac{1}{kr+1} = \text{Fraction of solute in external phase.}$$

Consider the case where unit mass of a single solute is put into the first plate and is then followed by pure solvent. We can draw up a table showing the quantity of solute in each plate.

No. of transfers, n	Serial no. of plate, t from top of the column				
	1	2	3	4	5
0	1	0	0	0	0
1	U	L			
2	U^2	2UL	L^2		
3	U^3	$3U^2L$	$3UL^2$	L^3	
4	U^4	$4U^3L$	$6U^2L^2$	$4UL^3$	L^4

Let $U = Y$ and $L = X$

We see that the quantity in each plate is a term of binomial expansion, $(y+x)^n$ so that when n successive transfers, i. e., when n

successive certain volumes of solvent have passed,

$$T_{t,n} = \frac{n!}{t!(n-t)!} \cdot y^{n-t} \cdot x^{t}$$

where $T_{t,n}$ = amount in plate t after n such successive volumes of solvent have passed, or after n transfers.

When n and t are very large, as is usual in a chromatographic column, it becomes cumbersome to work with the above equations. However, Stirling's approximations could be applied. Upon simplification,

$$n! \cong n^{n} \cdot e^{-n} \cdot \sqrt{2\pi n}$$

$$t! \cong t^{t} \cdot e^{-t} \cdot \sqrt{2\pi t}$$

and $$(n-t)! \cong (n-t)^{(n-t)} \cdot e^{-(n-t)} \cdot \sqrt{2\pi(n-t)}$$

$$\therefore T_{t,n} = \frac{n^{n} \cdot e^{-n} \sqrt{2\pi n}}{t^{t} \cdot e^{-t} \sqrt{2\pi t} \cdot (n-t)^{(n-t)} \cdot e^{-(n-t)} \cdot \sqrt{2\pi(n-t)}} \cdot y^{(n-t)} \cdot x^{t}$$

$$\cong \left(\frac{nx}{t}\right)^{t} \left(\frac{ny}{n-t}\right)^{n-t} \sqrt{\frac{n}{2\pi t\,(n-t)}}$$

Let $\delta = t - nx = t - n(1-y) = t - n + ny$

$$\therefore t = nx + \delta$$

$$\therefore \left(\frac{nx}{t}\right)^{t} = \left(\frac{nx}{nx+\delta}\right)^{nx+\delta} = \left(\frac{nx+\delta}{nx}\right)^{-(nx+\delta)} = \left(1 + \frac{\delta}{nx}\right)^{-(nx+\delta)}$$

simly $$\left(\frac{ny}{n-t}\right)^{n-t} = \left(\frac{ny}{ny-\delta}\right)^{ny-\delta} = \left(\frac{ny-\delta}{ny}\right)^{-(ny-\delta)} = \left(1 - \frac{\delta}{ny}\right)^{-(ny-\delta)}$$

Simly,

$$\sqrt{\frac{n}{2\pi(nx+\delta)(ny-\delta)}} = \sqrt{\frac{n}{2\pi nx\,ny\left(1+\frac{\delta}{nx}\right)\left(1-\frac{\delta}{ny}\right)}}$$

$$= \frac{1}{\sqrt{2\pi nxy\left(1+\frac{\delta}{nx}\right)\left(1-\frac{\delta}{ny}\right)}}$$

let $B = \sqrt{2\pi nxy\left(1+\frac{\delta}{nx}\right)\left(1-\frac{\delta}{ny}\right)}$

Then $T_t \cdot B = \left(1+\frac{\delta}{nx}\right)^{-(nx+\delta)} \cdot \left(1-\frac{\delta}{ny}\right)^{-(ny-\delta)}$

and $\log T_t \cdot B = -(nx+\delta)\log\left(1+\frac{\delta}{nx}\right) - (ny-\delta)\log\left(1-\frac{\delta}{ny}\right)$

Assuming, $|\delta| < nxy$, so that

$$\left|\frac{\delta}{nx}\right| < 1 \quad \text{and} \quad \left|\frac{\delta}{ny}\right| < 1$$

it will permit us to write the 2 convergent series,

$$\log\left(1+\frac{\delta}{nx}\right) = \frac{\delta}{nx} - \frac{\delta^2}{2n^2x^2} + \frac{\delta^3}{3n^3x^3} - \cdots\cdots$$

$$\& \log\left(1-\frac{\delta}{ny}\right) = -\frac{\delta}{ny} - \frac{\delta^2}{2n^2y^2} - \frac{\delta^3}{3n^3y^3} - \cdots\cdots$$

Then,

$$\log T_t \cdot B = -(nx+\delta)\left(\frac{\delta}{nx} - \frac{\delta^2}{2n^2x^2} + \frac{\delta^3}{3n^3x^3}\right) - (ny-\delta)\left(-\frac{\delta^2}{2n^2y^2} - \frac{\delta}{ny} - \frac{\delta^3}{3n^3y^3}\right)$$

Expanding we get,

$$\log T_t \cdot B = -\delta - \frac{\delta^2}{nx} + \frac{\delta^2}{2nx} + \frac{\delta^3}{2n^2x^2} - \frac{\delta^3}{3n^2x^2} - \frac{\delta^4}{3n^3x^3} + \delta - \frac{\delta^2}{ny} + \frac{\delta^2}{2ny} - \frac{\delta^3}{2n^2y^2} + \frac{\delta^3}{3n^2y^2} - \frac{\delta^4}{3n^3y^3}$$

Now $-\frac{\delta^2}{nx} + \frac{\delta^2}{2nx} = -\frac{\delta^2}{2nx}$; $-\frac{\delta^2}{ny} + \frac{\delta^2}{2ny} = -\frac{\delta^2}{2ny}$

and $\frac{\delta^3}{2n^2x^2} - \frac{\delta^3}{3n^2x^2} - \frac{\delta^3}{2n^2y^2} + \frac{\delta^3}{3n^2y^2} = \frac{3\delta^2y^2 - 2\delta^3y^2 - 3\delta^3x^2 + 2\delta^3x^2}{2.3\,n^2x^2y^2}$

$$= -\frac{\delta^3(x^2-y^2)}{2.3\,n^2x^2y^2}$$

$$\therefore \log T_t \cdot B \overset{n}{=} -\frac{\delta^2}{2nxy} - \frac{\delta^3(x^2-y^2 \text{------}}{2.3\,n^2x^2y^2}$$

as $|\delta| < nxy$, $\therefore \log T_t \cdot B \overset{n}{=} -\frac{\delta^2}{2nxy}$

$$\text{or } T_t = \frac{1}{\sqrt{2nxy\pi}} \cdot e^{-\frac{\delta^2}{2nxy}}$$

$$\therefore T_t = \frac{1}{\sqrt{2\pi nxy}} \cdot e^{-\frac{(t-nx)^2}{2nxy}}$$

This equation tells us the amount in plate t after n such successive volumes of solvents have passed.

A convenient graphical method for representing such a distribution results when the fraction or percentage present in a tube is plotted as ordinate against the serial number of the plate as abscissa. This gives a distribution, shown in Fig. 1, for a partition ratio of 1 and for theoretical plates. It will be noted that the curve is perfectly symmetrical. For higher number of theoretical plates and transfers, the curve becomes normal curve of error.

From the above equation, it is possible to find which theoretical plate will have the maximum concentration of the solute.

Near maximum concentration,

$$T_{n,t} \simeq \frac{n!}{t!(n-t)!} \cdot y^{n-t} \cdot x^{t} \simeq T_{n,(t+1)} \simeq \frac{n!}{(t\ 1)!(n-t-1)!} \cdot y^{n-t-1} \cdot x^{t+1}$$

Simplifying, we get

$$\frac{y}{x} = \frac{n-t}{t+1} \quad \therefore \quad \frac{y}{x} = \frac{kr/kr\ 1}{1/kr\ 1} = kr$$

$$\therefore \quad kr = \frac{n-t}{t+1}$$

$$\therefore \quad krt + kr = n-t$$

$$\therefore \quad t(kr+1) = n-kr$$

$$\therefore \quad t = \frac{n-kr}{(kr\ 1)} \quad \text{when n is very large,}$$

$$t = n.\frac{1}{kr\ 1}$$

$t \simeq n.x$ where x fraction which is in the mobile phase.

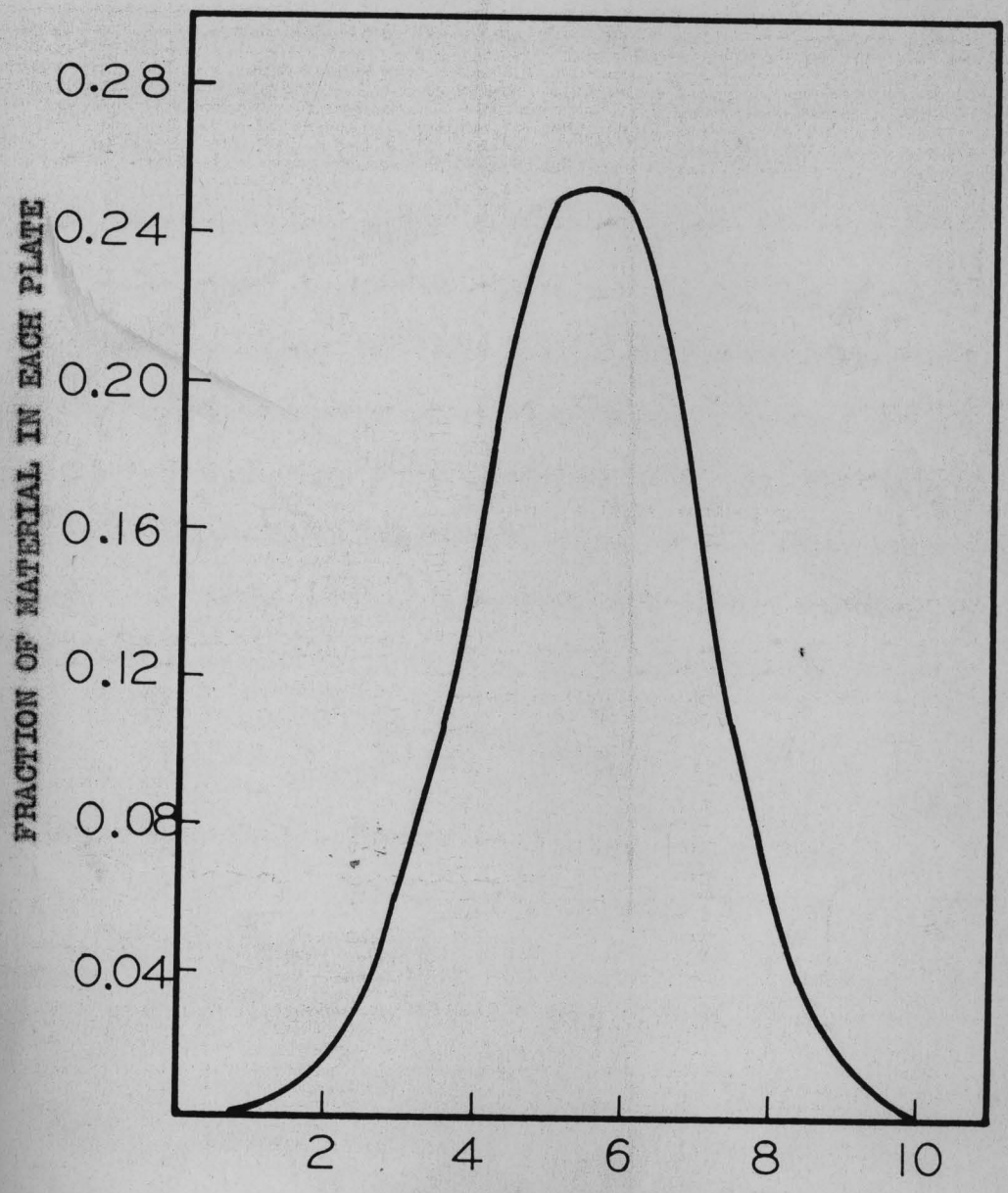

FRACTION OF MATERIAL IN EACH PLATE
0.28
0.24
0.20
0.16
0.12
0.08
0.04
2
4
6
8
10
No. of theoretical plate

Fig. 1.

Here n could be taken as rate of flow of the pure solvent. X is sometimes referred to as R_f value in the literature.

From this equation, we see that as n increases, i.e., as more and more solvent is poured through, the band of the solute in the column will move down at a certain rate. The rate of travel of the band t, is then given by rate of flow of the solvent times the fraction of the material in the external phase.

There are two disadvantages of increasing n:

(1) As more and more solvent is passed through the column, the solution becomes progressively more dilute because the solute becomes scattered in more and more tubes as distribution proceeds (Table 4). In Fig. 2, it is seen that for 24 transfers (Curve A), the maximum tube contains 16.4% of the original while in 200 transfers (Curve D), it contains only 5.6%. In such processes, progressive dilution cannot be prevented, since it is fundamentally a part of the process.

Table 4

No. of transfers	Curve spread	Total tubes occupied by band %
25	15	60
50	21	42
100	30	30
200	42	21
400	60	15
1000	95	9.5

DISTRIBUTION PATTERNS FOR
INCREASING NUMBER OF TRANSFERS

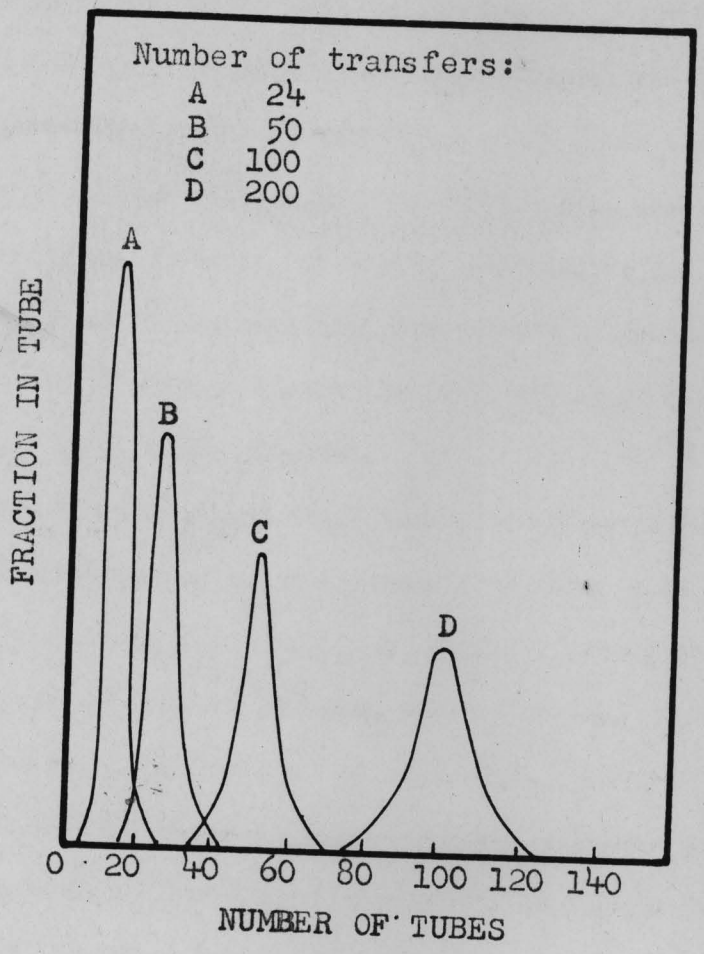


Fig. 2

(2) As a result of (1), broadening of the band occurs because the solute is scattered over a large number of tubes. But in practice, the polarity of the eluant is generally increased so that the substance elutes out over a small range of tubes. This is shown in Fig. 3.

In the resolution of two or more substances by chromatography, it may be considered that the separation depends upon the relative rate of migration of independently migrating band of one substance as compared to that of the other. As seen previously, the rates are governed by $t = Rx$. However, at times, progressive broadening of the band which is so pronounced when the partition isotherm is non-linear, operates in such a manner that the effect of different rates of migration is in part defeated.

All the above equations have been derived considering the liquid-liquid distribution of solute between 2 phases, throughout t no. of plates. In case of chromatography, besides having the distribution, fractions of aliquot portions are collected. This means after a solute passes the t^{th} plate, it comes out. This is analogous to the single withdrawal method in liquid-liquid extraction. If these withdrawn fractions are plotted against the amount in that respective fraction, the curve represents a Pascal distribution curve, as shown in Fig. 3. In case of liquid-liquid distribution, the distribution curve is symmetrical, but Pascal's distribution curve tails a little in the later part.

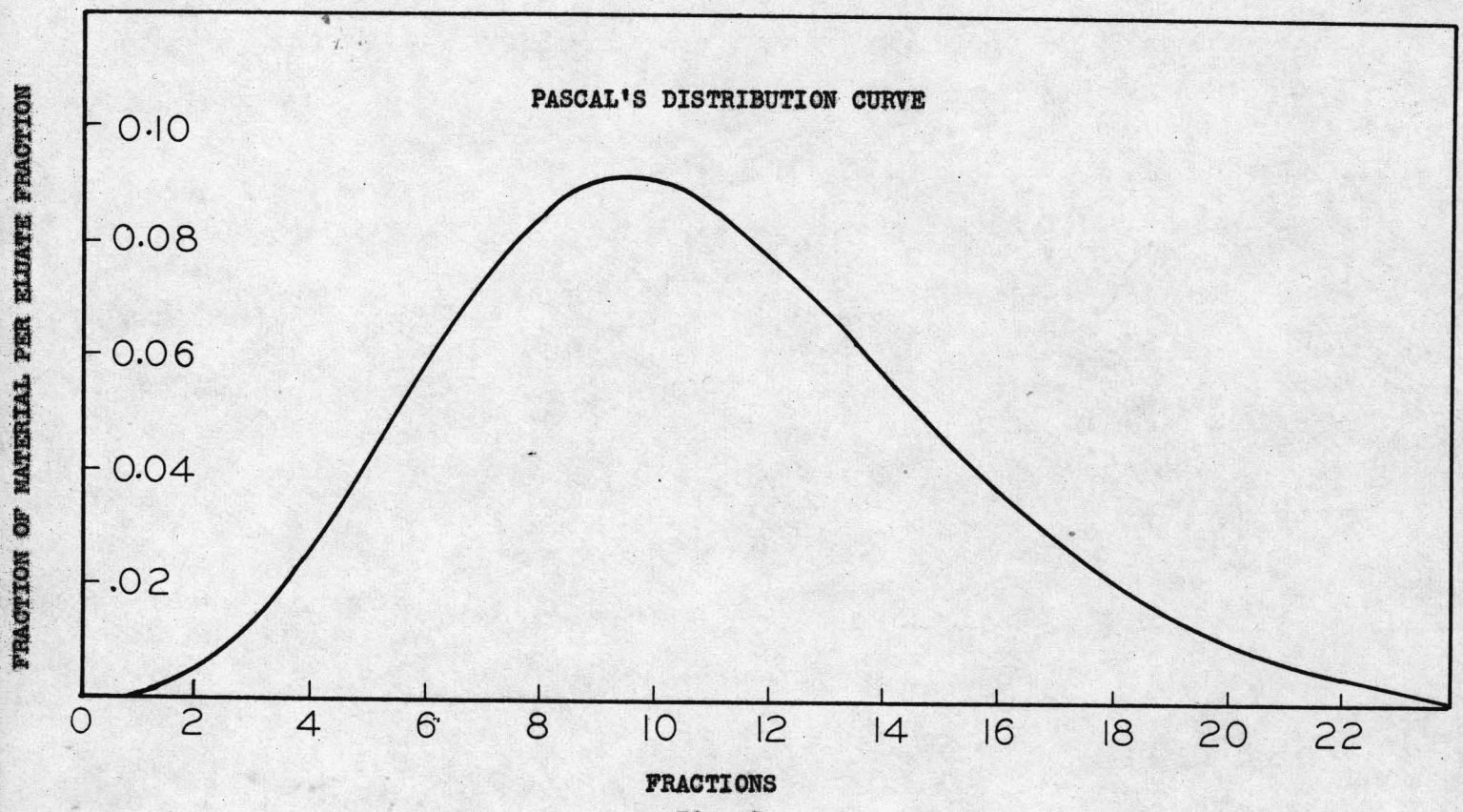

PASCAL'S DISTRIBUTION CURVE
FRACTION OF MATERIAL PER ELUATE FRACTION
0.10
0.08
0.06
0.04
.02
0
2
4
6
8
10
12
14
16
18
20
22
FRACTIONS

Fig. 3

B. Theoretical Limitations of Partition Chromatographic Methods Presently in Use for Higher Saturated Fatty Acids

In all cases of separation of higher fatty acid mixtures by partition chromatography, no method of separation or analysis of mixtures of higher fatty acids above C_9 differing by only one methylene group has been reported. One possible reason for the difficulty of such a separation may lie in the fact that fatty acids tend to dimerize in non-polar solvents like n-hexane (81, 82). The effect of this dimerization is reflected in the partition isotherm of the fatty acids in such a manner that it becomes parabolic. The deviation from linearity, it will be shown, results effectively in broadening of the elution peak in partition chromatography.

In previous theoretical discussions of the process of partition chromatography, it was assumed that the partition isotherm of the substance was linear. In the above case, however, as the partition isotherm is non-linear, the distribution of the solute which results from the liquid-liquid countercurrent distribution process like chromatography is not calculable by means of the expansion of $(y+x)^n$. The use of this expansion is limited to those cases only where x and y, the fractions of solute in external and internal phases in one theoretical plate, are expressible in terms of an unchanging partition coefficient and the volume ratio of the phases in contact.

As the dimerization occurs in one of the phases, then the fraction in either phase can only be expressed in terms of the partition coefficient, volume ratio of the phases in contact, and the concentration or amount in either phase. This follows from the expression for

equilibrium, for example, when dimerization occurs in the lower phase:

$$K_{p.c.} = \frac{C_u}{C_L(1-\alpha)}$$

where $K_{p.c.}$ = partition coefficient of the solute between external and internal phases

C_u = concentration of solute in internal phase per unit volume

C_L = concentration of solute in external phase per unit volume

α = degree of association

$$\therefore (K_{p.c.})^2 = \frac{(C_u)^2}{C_L(1-\alpha)}$$

$$\therefore (K_{p.c.})^2 \times (1-\alpha) = \frac{(C_u)^2}{C_L}$$

$$= \frac{U^2/(V_u)^2}{L/V_L}$$

$$= \frac{U^2}{L} \cdot \frac{V_L}{V_u \cdot V_u}$$

$$= U^2 \cdot \frac{1}{V_u} \cdot \frac{1}{r}$$

where V_u volume of internal phase

V_L volume of external phase

$r = \frac{V_u}{V_L}$

$$\therefore (K_{p.c.})^2(1-\alpha)(V_u)(r) = \frac{U^2}{L}$$

Putting $(K_{p.c.})^2(1-\alpha)(V_u)(r) = K$, we get $K = \frac{U^2}{L}$

where U and L are respectively the amounts in the internal and external

phases, and K is the product of true partition coefficient squared, volume ratio, difference between degree of association and unity, and the volume of internal phase.

Therefore the fraction of the material in the internal phase is

$$y = \frac{U}{U + L}$$

Substituting for L by $\frac{U^2}{K}$ $\left(\because K = \frac{U^2}{L}\right)$

we get, $y = \frac{U}{U + \frac{U^2}{K}}$

$$= \frac{K}{U + K}$$

Similarly it can be shown that the fraction of the material in the external phase will be,

$$x = 1 - \frac{K}{U + K}$$

$$= \frac{U}{U + K} .$$

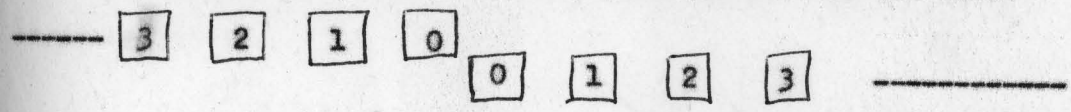
Since U is an unknown function of n and r, the expansion of $(y + x)^n$ will only hold for n equal to 0 or 1.

In view of the above consideration, it was decided to calculate the distribution, which would result in the case where partition isotherm is parabolic, by a numerical method. Since such a calculation for any system of an appreciable number of theoretical plates would be extremely tedious, it was decided to program the calculation for the electronic computer, available at this institution. (IBM CPC model 2).

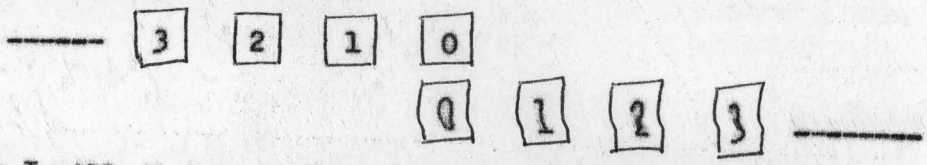
The programming for the computer calculations followed the usual way of showing the liquid-liquid countercurrent distribution scheme. For the ease of calculations and understanding, "theoretical plates"

are designated as "number of tubes" and so internal and external phases are divided into discrete portions, and r is taken as unity.

Beginning with the tubes in the position of zero transfer:



Let us put the discrete portions of internal phase into the upper row of tubes and discrete portions of external phase into the lower row of the tubes. When both the rows of the tubes are in the above position, upper 0 tube contains some initial amount, say I. Now when we bring the lower row of tubes in the following position, (this transfer is called zero transfer)



the amount I will divide itself into two, upper and lower 0 tubes according to partition coefficient value. Say amount in upper zero tube is U_o^o and amount in the lower tube is L_o^o after equilibrium has been reached.

$$\text{therefore } U_o^o + L_o^o = I$$

Substituting $\frac{U_o^{o2}}{K}$ for L_o^o in the above expression, we get

$$U_o^o + \frac{U_o^{o2}}{K} = I \qquad \left(\because K = \frac{U_o^{o2}}{L_o^o}\right)$$

$$\therefore U_o^o K + U_o^{o2} = KI$$

$$\therefore U_o^{o2} + U_o^o K - KI = 0$$

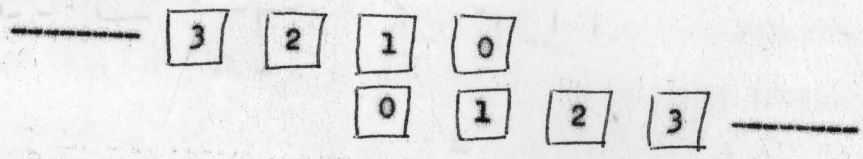
Solving this quadratic equation, we get

$$U_o^o = \frac{-K \pm \sqrt{K^2 + 4KI}}{2}$$

$$U_o^o = -\frac{K}{2} + \frac{1}{2}\sqrt{K^2 + 4KI}$$

and $L_o^o = I - U_o^o$.

After one transfer, the lower row of the tubes is shifted again to the left to bring it in the position shown below: (This is called first transfer).



The amount U_o^o in the upper 0 tube will divide itself between upper 0 tube and lower 1 tube.

$\therefore U_o^1 + L_1^1 = U_o^o$ where U_o^1 = amount in the upper 0 tube after first transfer

L_1^1 = amount in the lower 1 tube after first transfer

U_o^o = amount in the upper 0 tube after zero transfer.

$\therefore$ substituting $U_o^{1^2}$ for L_1^1, we get

$$U_o^1 + \frac{U_o^{1^2}}{K} = U_o^o$$

Solving the above quadratic equation, we get

$$U_o^1 \cdot K + U_o^{1^2} - U_o^o K = 0$$

$$\therefore U_o^{1^2} + U_o^1 \cdot K - U_o^o \cdot K = 0$$

$$\& \quad U_o^1 = \frac{-K \pm \sqrt{K^2 + 4KU_o^o}}{2}$$

$$\therefore U_o^1 = \frac{-K}{2} + \frac{1}{2}\sqrt{K^2 + 4KU_o^o}$$

The value for U_o^o is available from the calculation from zero transfer. In the computer programming, this value was stored in a memory register

for use in the next calculation. The value of $U_o^{1\,2}$ divided by K gave the value L_1^1, where L_1^1 amount in the lower 1 tube after first transfer.

The machine was programmed to repeat these calculations for upper 0 tube through n transfers. In order that all calculations could be performed in terms of U, the values of $\frac{U_o^n}{K}$ were machine punched into summary cards to be used in calculations of U_1^n. Likewise, values of any U_t^n were punched into summary cards. In the machine procedure used, all the values of U_o^n were calculated before proceeding with the calculations of values of U_t^n.

The calculations of the value of U_t^n after the first transfer proceeded as follows: for example, for U_1^2, for upper 1 tube after two transfers,

the material balance for the upper 1 tube and lower 1 tube which are in contact is:

$$U_1^2 + L_1^2 = U_1^1 + L_1^1$$

but $L_1^2 = \frac{(U_1^2)^2}{K}$ and $L_1^1 = \frac{(U_o^1)^2}{K}$

$$\therefore \quad U_1^2 + \frac{(U_1^2)^2}{K} = U_1^1 + \frac{(U_o^1)^2}{K}$$

$$\therefore \quad KU_1^2 + (U_1^2)^2 = KU_1^1 + (U_o^1)^2$$

$$\therefore \quad (U_1^2)^2 + KU_1^2 - \left[KU_1^1 + (U_o^1)^2\right] = 0$$

Solving the above quadratic equation, we get,

$$U_1^2 = \frac{-K \pm \sqrt{K^2 + 4\left[KU_1^1 + (U_o^1)^2\right]}}{2}$$

$$= \frac{-K}{2} + \frac{1}{2}\sqrt{K^2 + 4\left[KU_1^1 + (U_o^1)^2\right]}$$

In this expression, U_o^1 was available from information punched into summary cards, and U_1^1 was available from information obtained in the preceding calculation and stored in a memory register.

The relations just shown are sufficient for the calculation of the liquid-liquid countercurrent distribution for any number of theoretical plates. The type of distribution which results in the case of a parabolic partition isotherm under consideration, is shown in Fig. 4-10, for three different partition coefficients, 2/3, 1 and 6 2/3. Fig. 4-7 show the distribution of three substances with these three partition coefficient values after 12, 24, 48, and 96 transfers respectively. The development of distribution of substances having the above partition coefficient values in shown in Fig. 8-10.

It becomes immediately apparent from an examination of the curves in Fig. 4-10, that the deviation of the isotherm from linearity results in tailing of the band. Therefore, no small number of transfers will result in effecting a separation of materials whose partition isotherms are non-linear. Even though there is tenfold difference in partition coefficients of two substances (2/3 and 20/3 in Fig. 7), after 96 transfers, in which case best separation would be expected, about 15% of one material is admixed with the other. While in the case of higher homologues of fatty acids where the difference in the partition coefficients of two successive fatty acids is only about one

and one-half fold or less, it would be extremely difficult to separate them on chromatographic column. As the partition coefficients of two substances come closer and closer, separation of the mixture of the two becomes worse and worse.

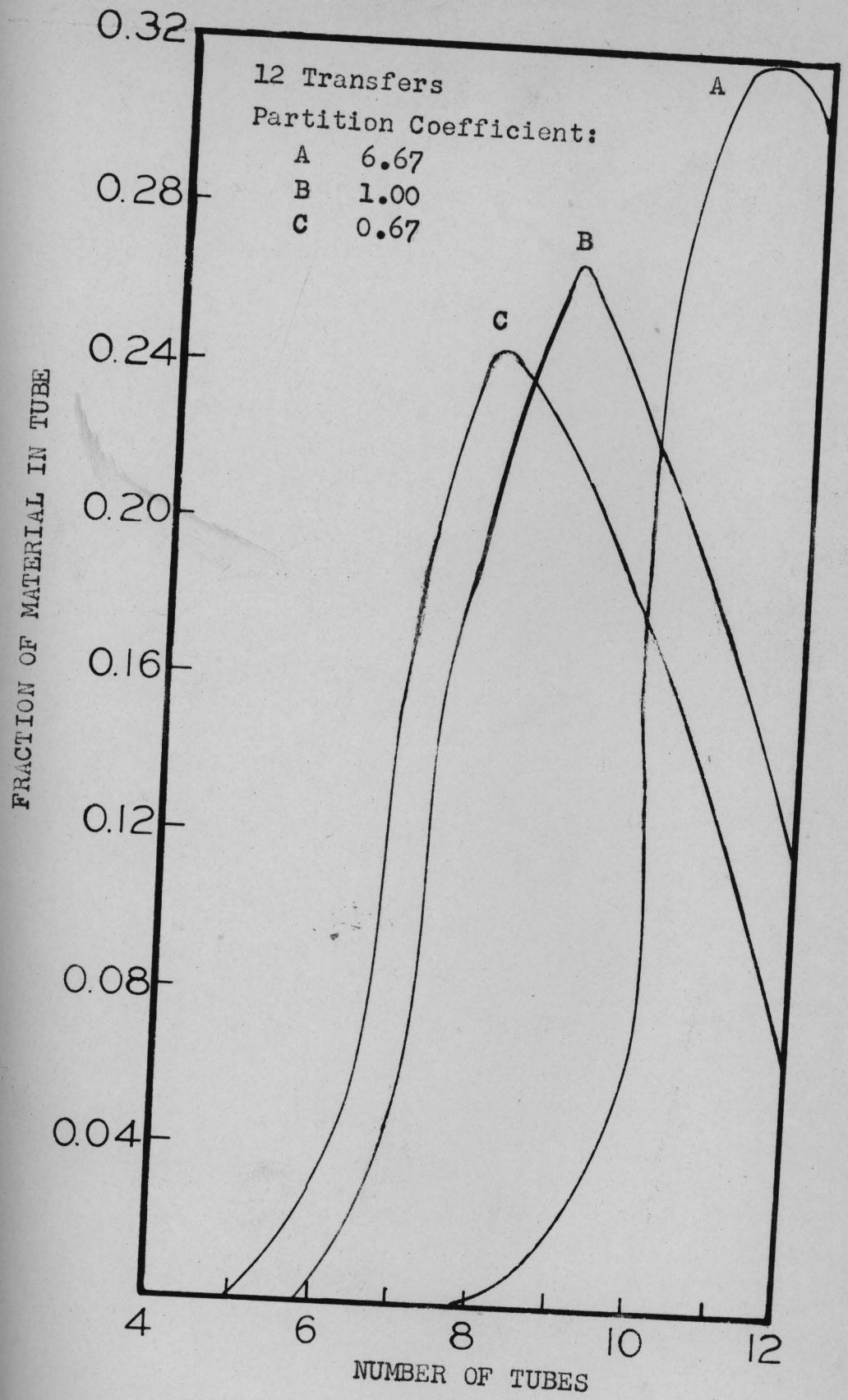

12 Transfers
Partition Coefficient:
A 6.67
B 1.00
C 0.67
A
B
C
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
FRACTION OF MATERIAL IN TUBE
4
6
8
10
12
NUMBER OF TUBES

Fig. 4

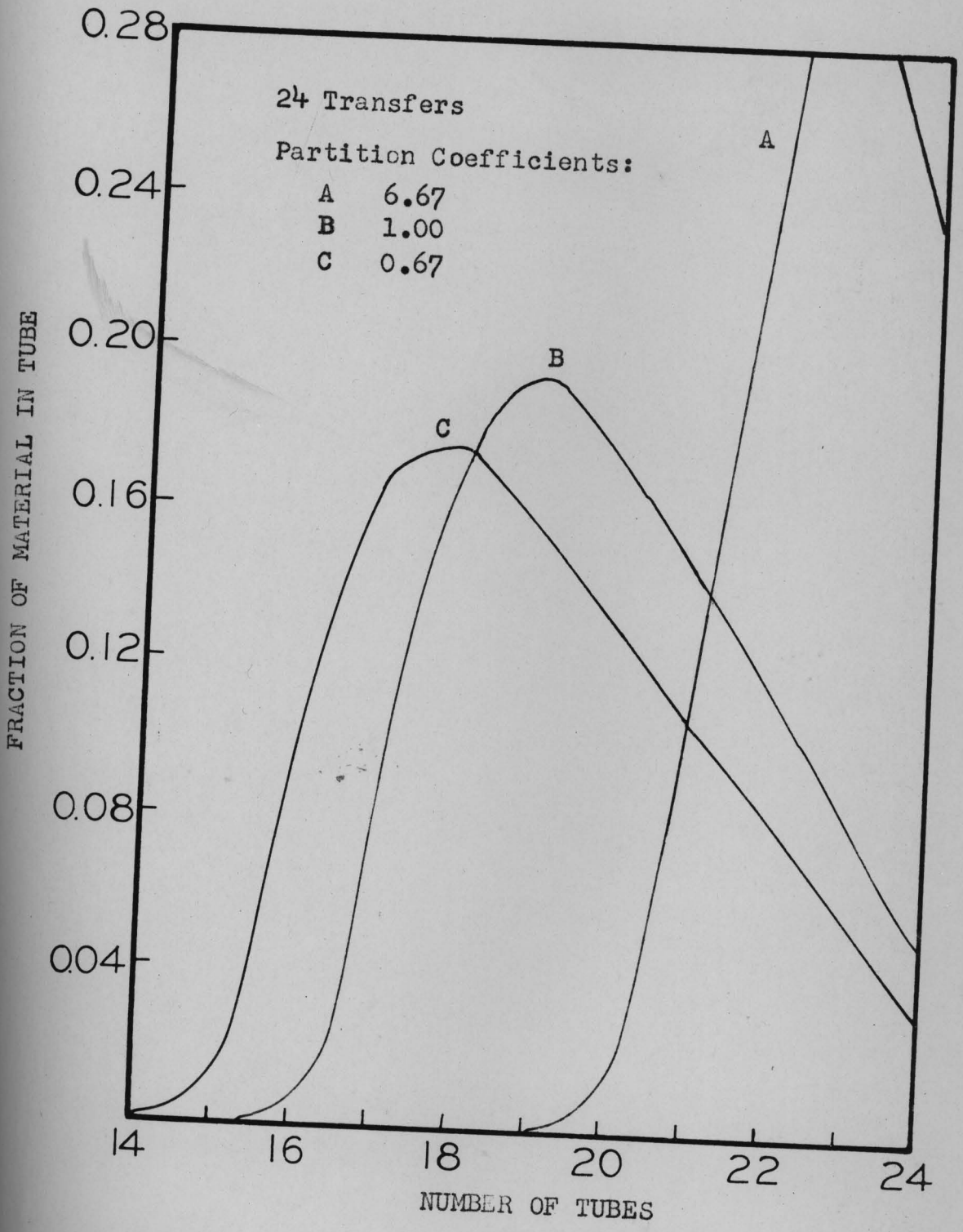

24 Transfers
Partition Coefficients:
A 6.67
B 1.00
C 0.67
A
B
C
0.28
0.24
0.20
0.16
0.12
0.08
0.04
FRACTION OF MATERIAL IN TUBE
14
16
18
20
22
24
NUMBER OF TUBES

Fig. 5

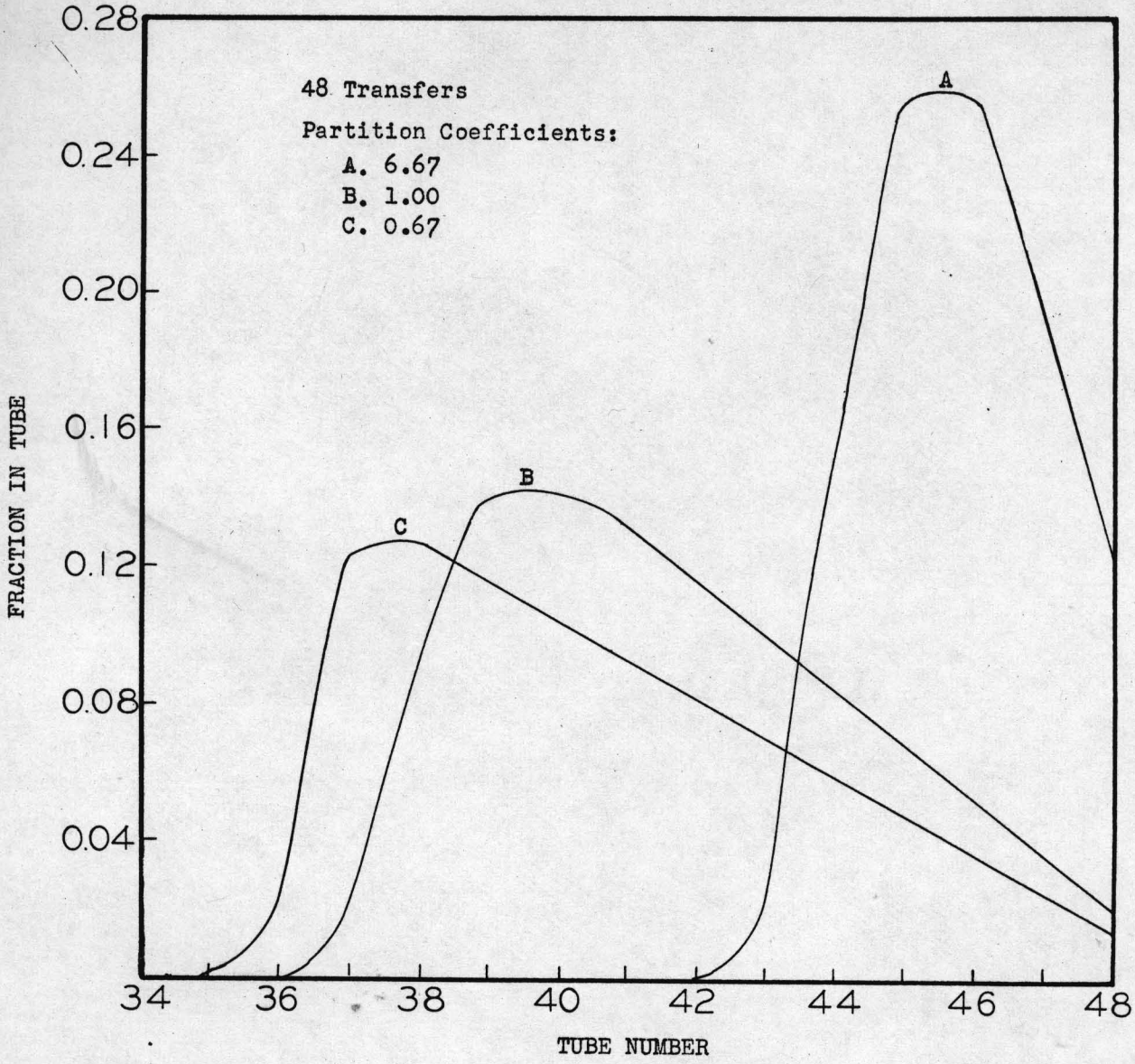

48 Transfers
Partition Coefficients:
A. 6.67
B. 1.00
C. 0.67
A
B
C
0.28
0.24
0.20
0.16
0.12
0.08
0.04
FRACTION IN TUBE
34
36
38
40
42
44
46
48
TUBE NUMBER

Fig. 6

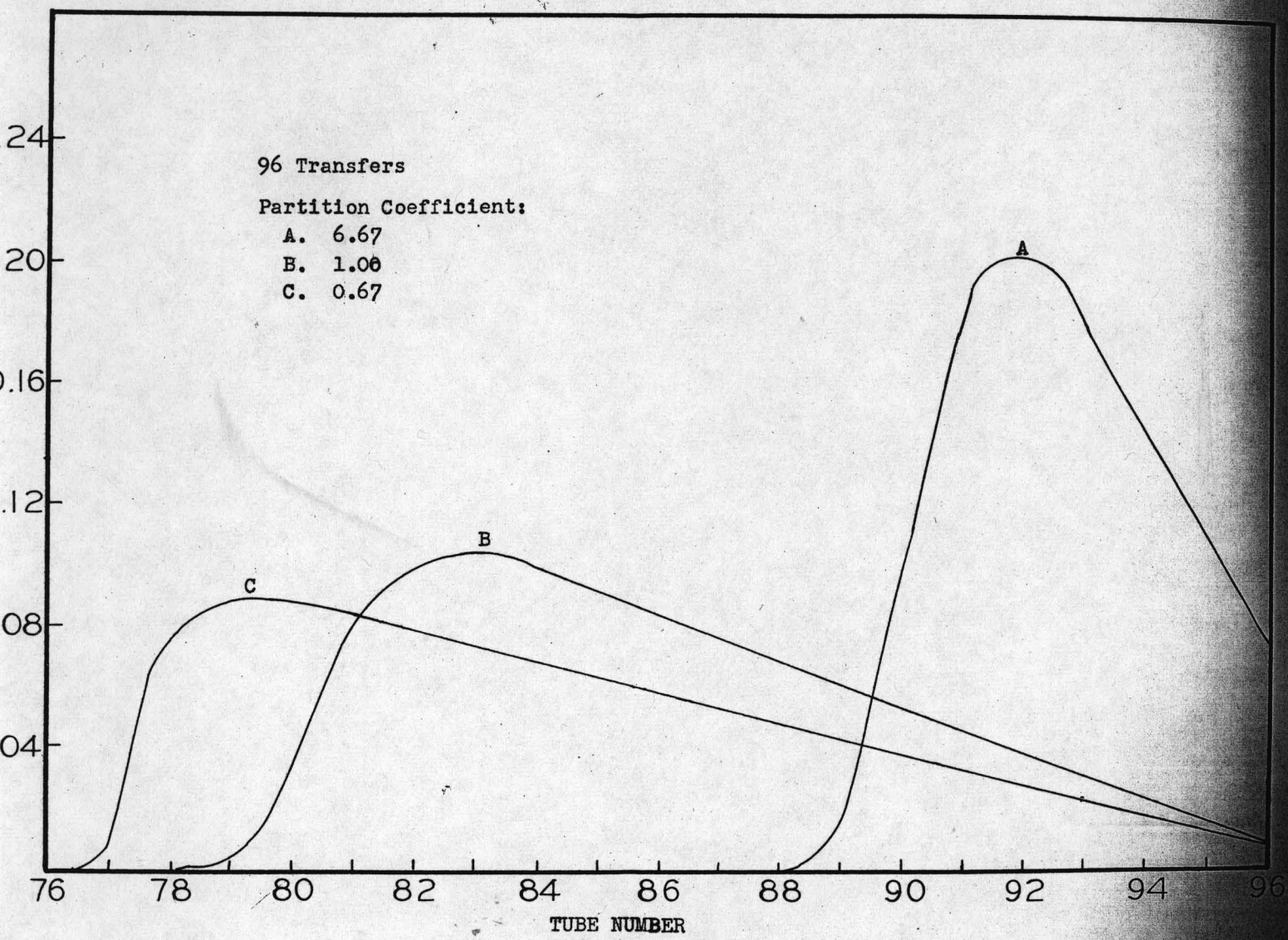

96 Transfers
Partition Coefficient:
A. 6.67
B. 1.00
C. 0.67
A
B
C
.24
20
.16
.12
08
04
76
78
80
82
84
86
88
90
92
94
96
TUBE NUMBER

Fig. 7

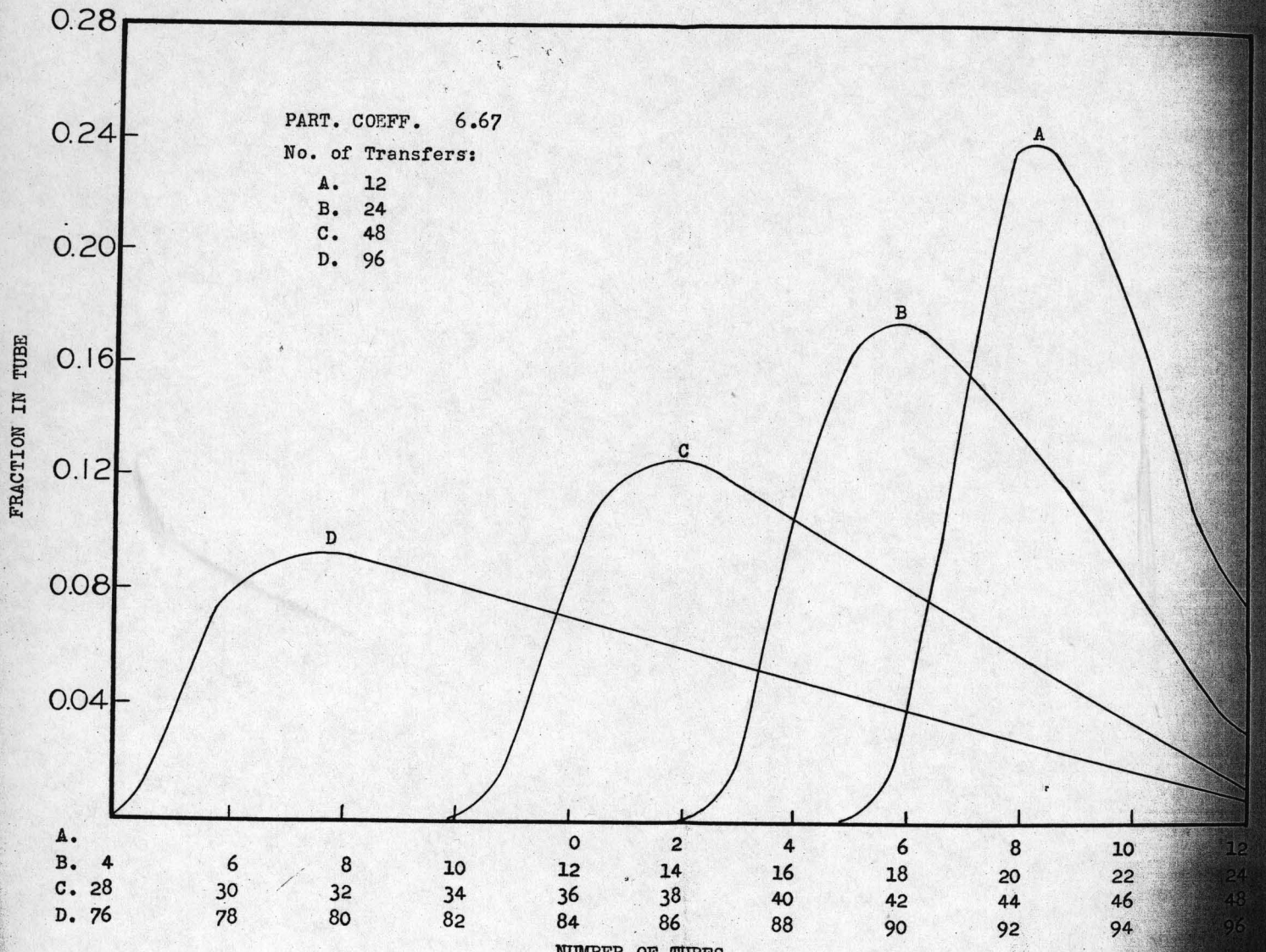

0.28
0.24
0.20
0.16
0.12
0.08
0.04
FRACTION IN TUBE
PART. COEFF. 6.67
No. of Transfers:
A. 12
B. 24
C. 48
D. 96
A
B
C
D
A.
B. 4 6 8 10 12 14 16 18 20 22 24
C. 28 30 32 34 36 38 40 42 44 46 48
D. 76 78 80 82 84 86 88 90 92 94 96
0 2 4 6 8 10 12
NUMBER OF TUBES

Fig. 8

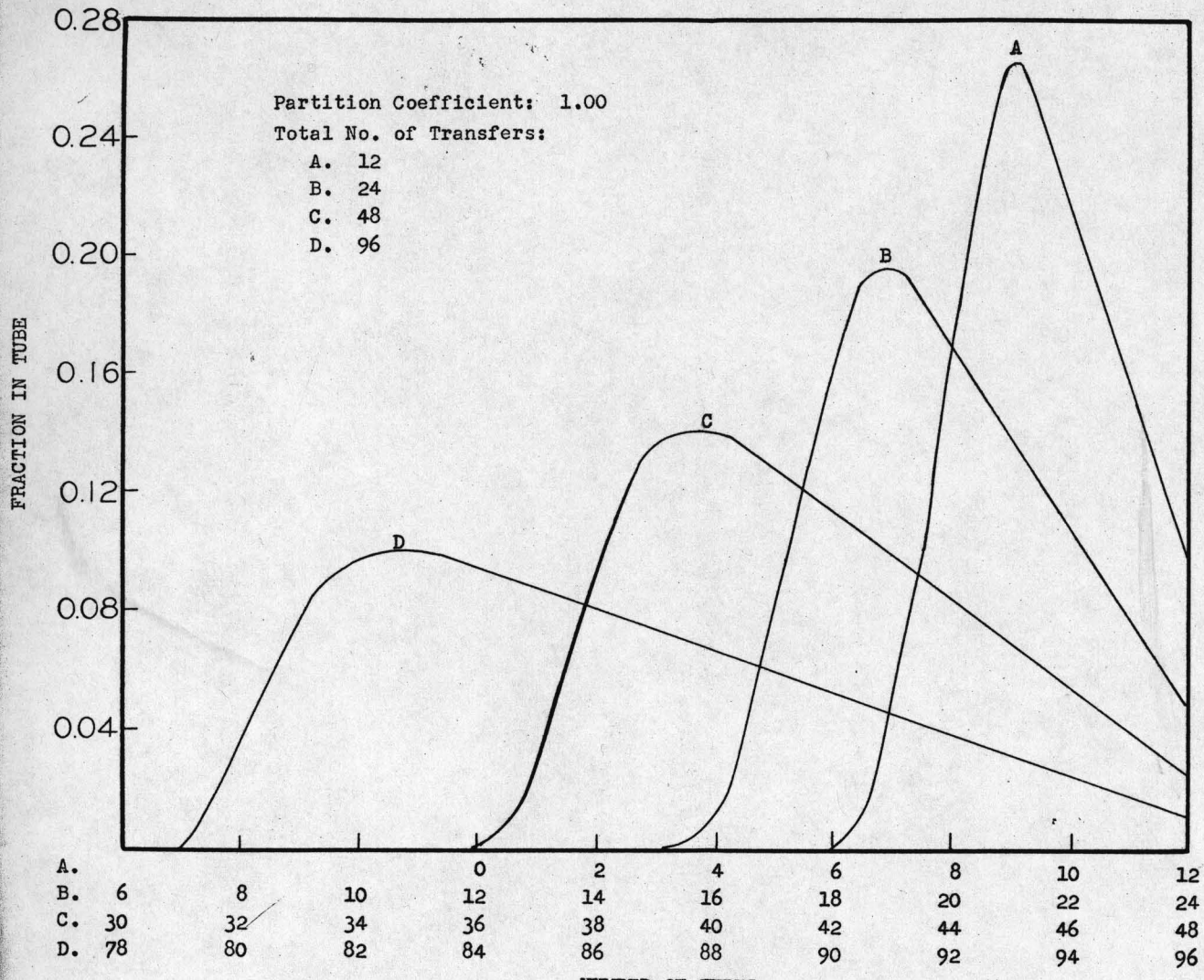

Partition Coefficient: 1.00
Total No. of Transfers:
A. 12
B. 24
C. 48
D. 96
FRACTION IN TUBE
0.28
0.24
0.20
0.16
0.12
0.08
0.04
A
B
C
D
A. 0 2 4 6 8 10 12
B. 6 8 10 12 14 16 18 20 22 24
C. 30 32 34 36 38 40 42 44 46 48
D. 78 80 82 84 86 88 90 92 94 96
NUMBER OF TUBES

Fig. 9

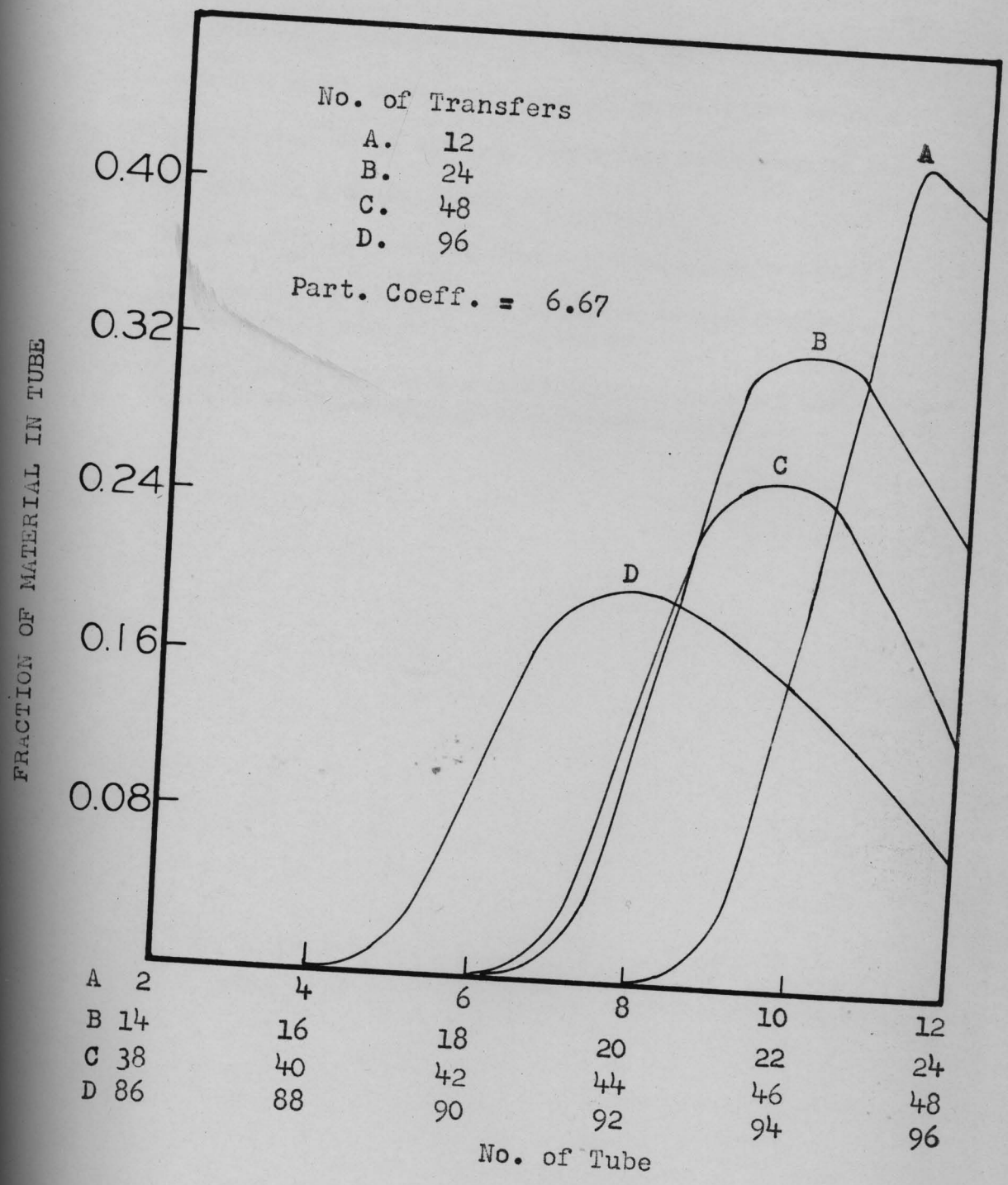

No. of Transfers
A. 12
B. 24
C. 48
D. 96
Part. Coeff. = 6.67
A
B
C
D
0.40
0.32
0.24
0.16
0.08
FRACTION OF MATERIAL IN TUBE
A 2 4 6 8 10 12
B 14 16 18 20 22 24
C 38 40 42 44 46 48
D 86 88 90 92 94 96
No. of Tube

Fig. 10

V. EXPERIMENTAL STUDIES

In the present investigation, the search for a suitable analytical procedure for mixed saturated higher fatty acids was directed along several lines. These studies, which were restricted to partition chromatographic methods, included:

A. Attempted improvement of Ramsey and Patterson's method

B. Utilization of ion-exchange resins as combined holder and buffer in partition chromatography

C. Investigation of a method based on conversion of the acids to more easily separated chemical form.

A. Attempted Modifications of Ramsey and Patterson's Method

Since from previous discussions (page 24), the method of Ramsey and Patterson (1) appeared most promising for analysis of mixtures of higher homologues of fatty acids, possible improvements on this method were sought, so that more quantitative results could be obtained. In Ramsey and Patterson's method, fatty acids are separated on a silicic acid column using 50:50 mixture of furfuryl alcohol and 2-aminopyridine as the internal phase and n-hexane as the external phase. The fractions, 2 ml each, are collected and titrated cumulatively against standard sodium ethylate, using phenolphthalein as indicator.

For comparison purposes later on, a run was made, based on Ramsey and Patterson's method. Each fraction of 10 ml was collected and titrated against sodium ethylate, using bromocresol purple as indicator and a stream of nitrogen as stirrer. Fig. 11 shows the results obtained. It becomes apparent that the separation of the mixture of acids is not complete and therefore quantitative results are difficult to calculate.

A possible theoretical drawback in the method of Ramsey and Patterson may lie in the fact that the internal phase, although alkaline, is not buffered. In a non-buffered system, fatty acids will ionize, and ionic dissociation in internal phase will deform the partition isotherm from linearity and will cause the zones to tail. This ionic effect may be controlled by using an unextractable buffer mixture because the pH will be constant in presence of a buffer,

since $\frac{[H^+][A^-]}{[HA]} = \text{constant}$

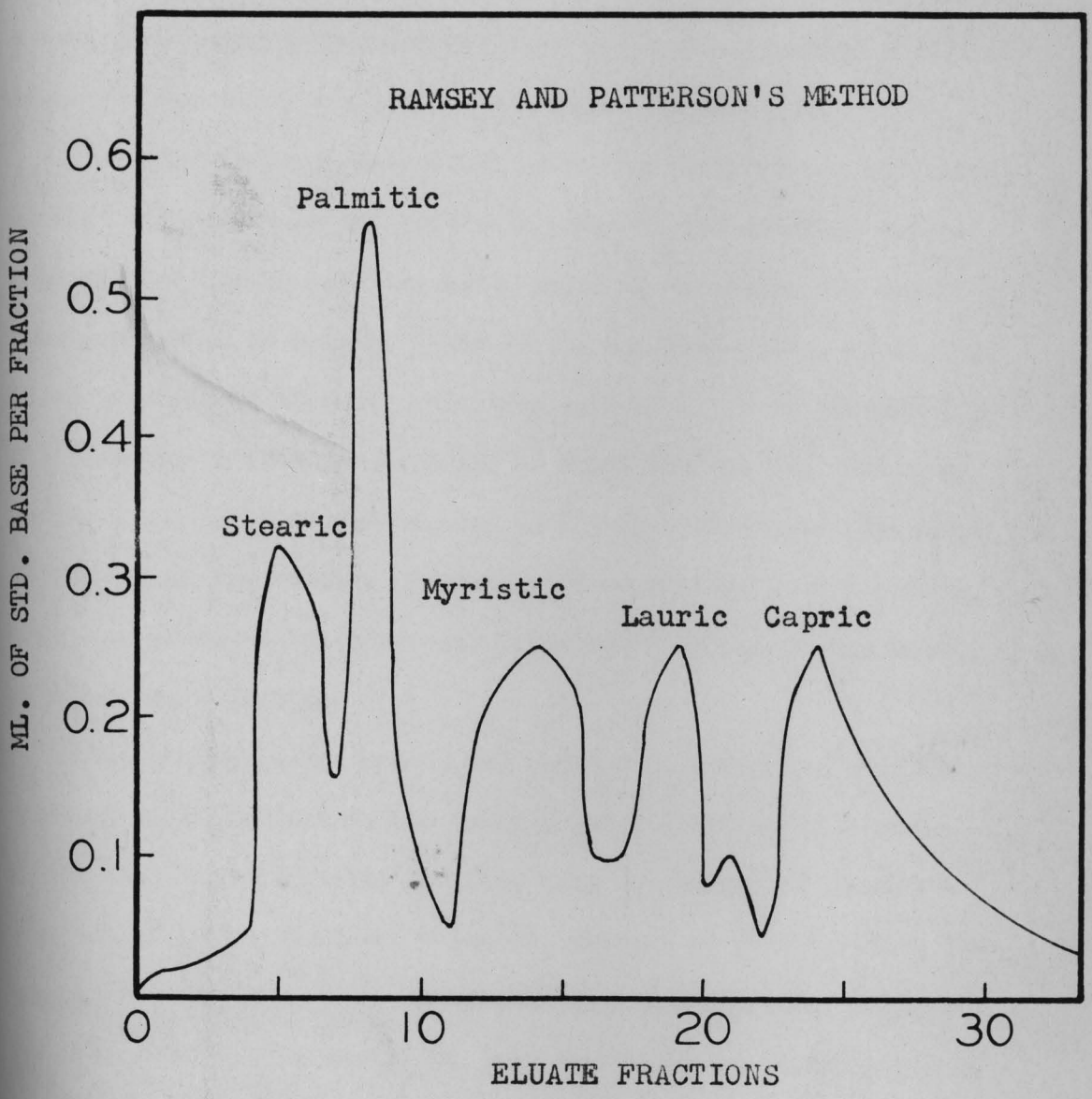

RAMSEY AND PATTERSON'S METHOD
ML. OF STD. BASE PER FRACTION
0.6
0.5
0.4
0.3
0.2
0.1
Palmitic
Stearic
Myristic
Lauric
Capric
0
10
20
30
ELUATE FRACTIONS

Fig. 11

therefore $\frac{\text{unionized acid}}{\text{ionized acid}} = \text{constant.}$

Thus by using buffered columns, the bands of ionizing substances may be rendered sharp by promoting linearity in the effective distribution isotherm and hence sharp peaks should be obtained.

In order to study the effect of buffer composition, different amounts of glacial acetic acid were added to the internal phase. When glacial acetic acid was added equal to one-tenth the amount of 2-aminopyridine on a molar basis in the internal phase, separation of the mixture of stearic, palmitic, myristic, lauric and capric acid was only partially obtained, as shown in Fig. 12. Besides, Skelly B was fast evaporating and as a result deposited some acid at the tip of the column. However, the separation looked better than that achieved by Ramsey and Patterson's method in the hands of the present worker.

Evaporation of the eluant was deduced by using Skelly C instead of Skelly B because the boiling point of Skelly C is much higher than that of Skelly B. This time the amount of glacial acetic acid in the internal phase was reduced to one-fifteenth the amount of 2-aminopyridine, calculated on a molar basis. Eluting the higher acids with Skelly C, good separation of the mixtures of stearic, palmitic, myristic and lauric acids was obtained (Fig. 13). This separation is best achieved, so far, using a proper buffer of furfuryl alcohol-2-aminopyridine and glacial acetic acid. However, no attempts were made to check the quantitative results.

In trying to find some other solvent combinations, unbuffered

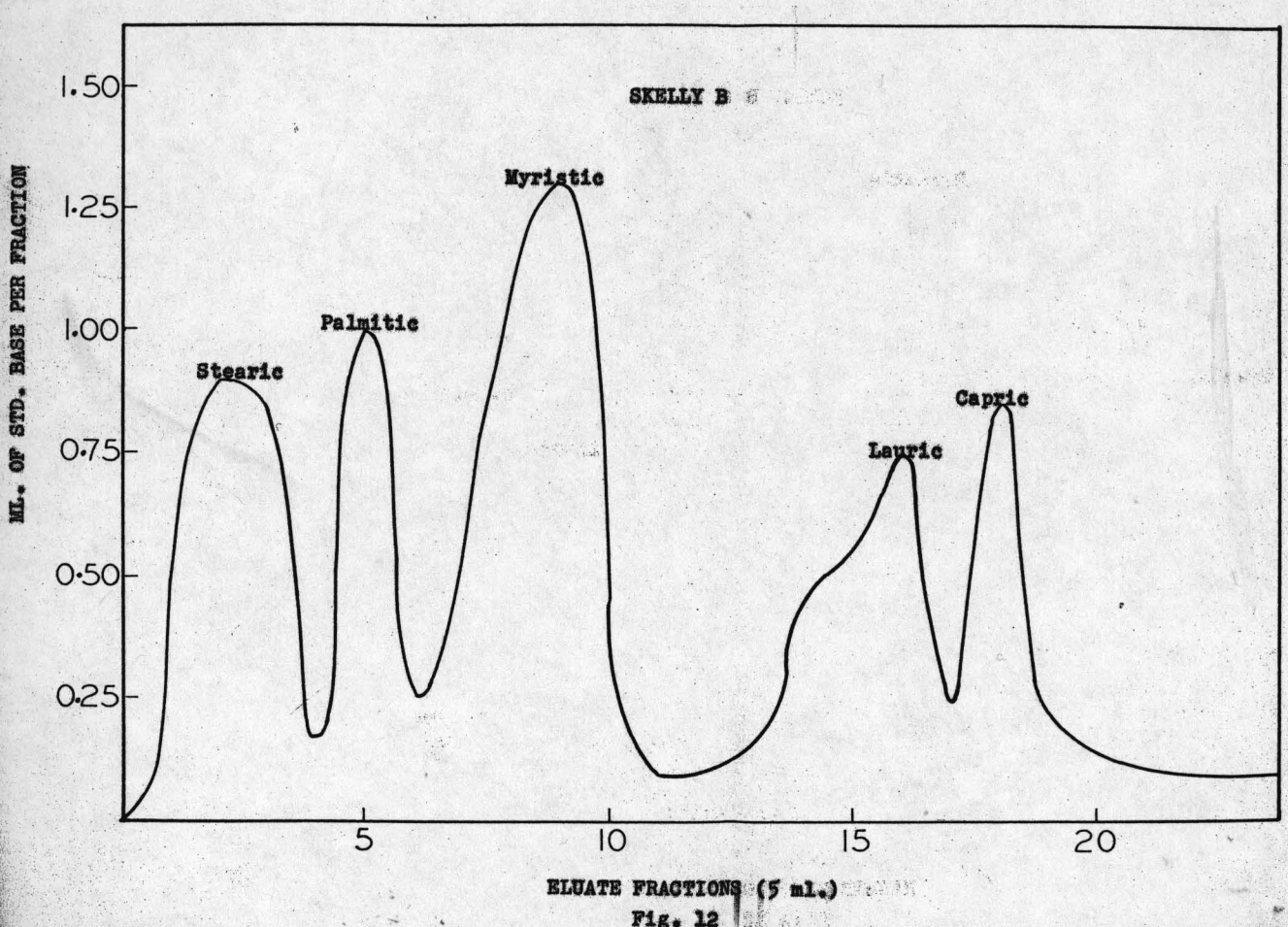

SKELLY B
ML. OF STD. BASE PER FRACTION
1.50
1.25
1.00
0.75
0.50
0.25
Stearic
Palmitic
Myristic
Lauric
Capric
5
10
15
20
ELUATE FRACTIONS (5 ml.)

Fig. 12

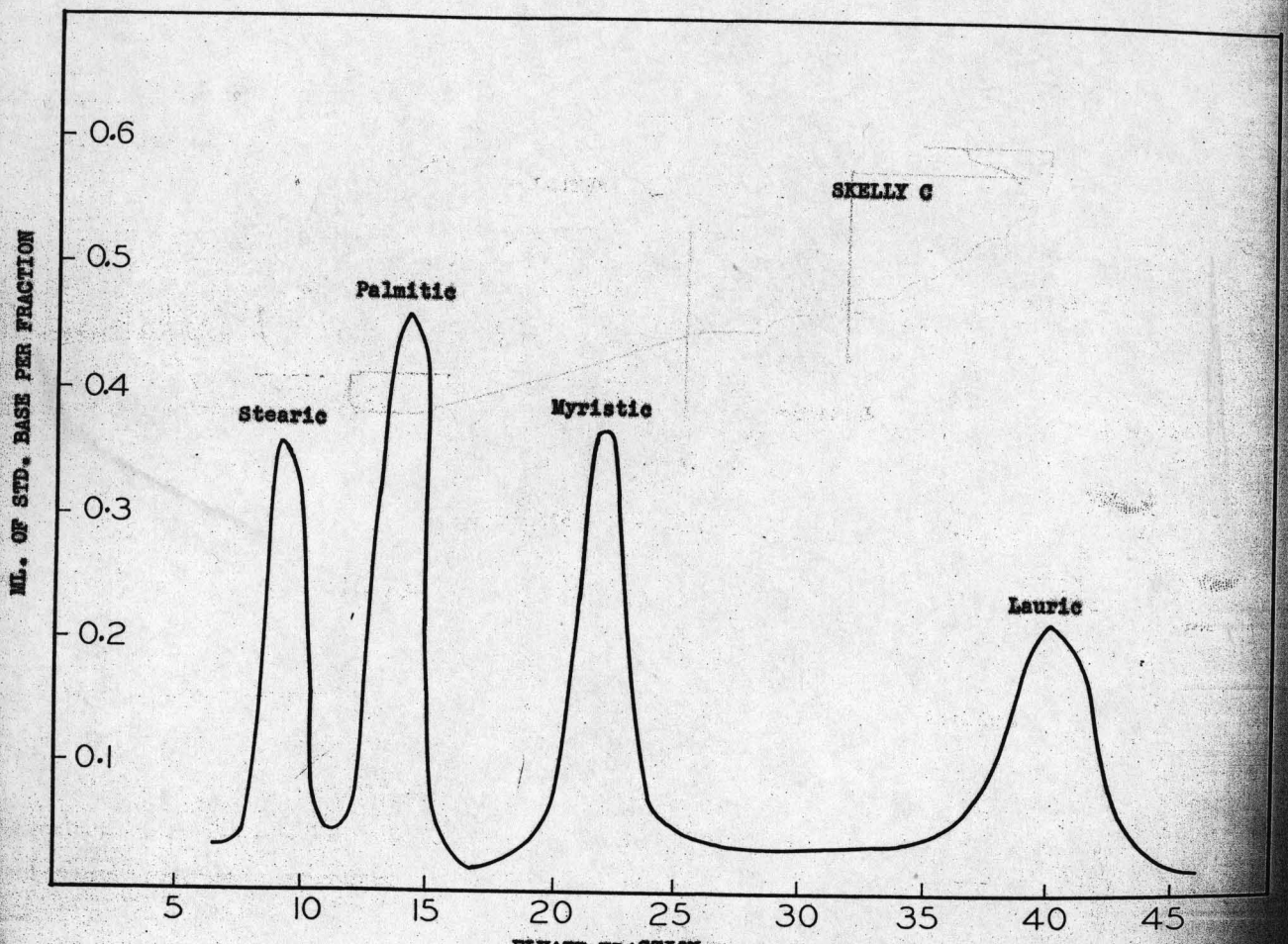

SKELLY C
Palmitic
Stearic
Myristic
Lauric
0.6
0.5
0.4
0.3
0.2
0.1
ML. OF STD. BASE PER FRACTION
5
10
15
20
25
30
35
40
45
ELUATE FRACTION

Fig. 13

triethylene tetramine was used as internal phase on silicic acid. When with this system, Skelly C was used as eluant, acids were found to distribute over a wide range of fractions, and separations did not take place. Failure of separation probably may lie in two reasons: (1) a buffer was not used; (2) external phase being Skelly C, fatty acids may tend to dimerize. Both promote non-linearity of the effective distribution isotherm of fatty acids, thus helping elution curves to tail.

Hence a buffer composed of

10 ml ethylene glycol
10 ml triethylene tetramine
different amounts of glacial acetic acid

was used as internal phase. Ethylene glycol was used to promote the solubility of fatty acids in the internal phase. Diisopropyl ether was used as external phase so that fatty acids may not dimerize.

It was found, however, that when the amount of glacial acetic acid was 3.9 ml, not a single acid was eluted out. As the amount of glacial acetic acid was slowly increased, fatty acids were found to distribute over a wide range of fractions, and no separation was achieved. When the amount of glacial acetic acid in the internal phase was 5.46 ml, all acids were eluted out in the first 100 ml of the eluant.

A number of other aqueous alkaline buffers (up to pH 9.0) were used as internal phases, but all the fatty acids were eluted out right away by diisopropyl ether, and even by Skelly C. However, it was certain that a highly alkaline buffer was necessary to hold the acids in the internal phase, if diisopropyl ether is used as external

phase to stop the dimerization effect of fatty acids. As it was not possible to use highly alkaline buffer with silicic acid because of mutual solubility, another support had to be found.

B. Attempted Use of Ion-exchange Resins in Partition Chromatography

1. Ion-exchange Partition Chromatography of Higher Fatty Acids.

a. Introduction. In the previous section, which deals with the modification of Ramsey and Patterson's method, it was shown that aqueous buffers up to pH 8 were not able to hold the acids in the column, and the acids were eluted out very quickly by diisopropyl ether. This indicates the necessity of high alkaline buffers as internal phase. With such highly alkaline buffers, however, silicic acid cannot be used as the mechanical support, because of the incompatibility of the two. A possible solution to the problem was the use of finely powdered ion-exchange resins as the mechanical support.

Chromatography on ion-exchange resins is of comparatively recent origin, partly because the commercial production of suitable resins has awaited definition of properties desirable in a resin which is to be used for chromatography. A range of resins is now available, strongly acidic, weakly acidic, strongly basic and weakly basic.

Ion-exchange chromatography, which has proved useful in other separation processes, has found little application in the separation or analysis of fatty acids. The common fatty acids have nearly the same ionization constants, and therefore it becomes somewhat difficult to separate them on ion-exchange resins as anions. However, there is a possibility of separating fatty acid mixtures on the basis of differential adsorption of fatty acids on ion-exchange

resins.

Ion-exchange resins, so far, have been used as ion-exchanging resins. There have been no reports made in the literature of the use of ion-exchange resins as mechanical support for the internal phase in the partition chromatography. Because ion-exchange resins are insoluble, high molecular weight polyelectrolytes with their acidic or basic groups free, capable of ionization, advantage could be taken of this ionizing property of the resins in that while being used as mechanical support for the internal phase, they themselves could be used as buffers by adding either the alkali or the acid according to desired pH buffer.

Thus having the possibility of buffering ion-exchange resins at high alkaline pH (11-12), it appeared possible to separate higher homologues of fatty acids. Unlike silicic acid, resins are stable at highly alkaline internal phase. Diisopropyl ether was used as external phase to avoid the dimerization of fatty acids.

b. Theory. As the ion-exchange resin particle is surrounded by aqueous phase which is buffered, and an organic phase is moving by, the situation has been created just like counter current liquid-liquid extraction. As from one side, elute is being taken out, it behaves like the single withdrawal method, and the mathematics is like the one discussed previously. The curve thus obtained will be similar to Pascal's distribution curve.

Whatever ion exchange resins were used, they were ground to 80-100 mesh powder. The result of grinding is obviously that the smaller the particle size, the greater the surface area. Generally,

40 gm of powder was well mixed with a sufficient amount of aqueous internal phase so that the powder just becomes wet and not slurry-like. To this wet mass, external phase is added, and slurry is made. This slurry is packed in the column, taking the usual precautions in packing of chromatographic columns.

Amberlite IRA-400, a strong base resin (Rohm and Haas); Amberlite XE-64, a weak carboxylic acid type (Rohm and Haas); Amberlite IR-120, a nuclear sulfonic (Rohm and Haas) were tried as supports as well as buffering agents. Stationary or internal phase was alkaline or acidic solution of different pH. Generally, diisopropyl ether was used as eluant. As the substances used were acidic, eluates were easily analyzed by titrating them with standard alcoholic base, using 0.1% metacresol purple solution as indicator and bubbling N_2 as stirrer.

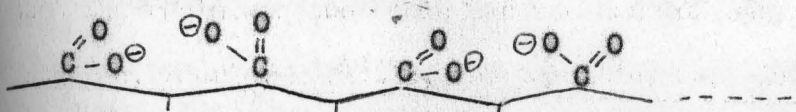
c. Results and discussion. Amberlite IRA-400 (in the free base form) was used with distilled water and 50% diethylenetetramine in water as internal phases. In both cases, it was found that the resin was too basic because none of the four acids (stearic, lauric, myristic, and palmitic) was eluted out, even with 20% chloroform in diisopropyl ether. A number of other aqueous solutions down to pH 7.91, all containing about 25% ethylene glycol, were tried as internal phases. Still the acids were not eluted out.

It seems that actually ion-exchange took place. Fatty acid anions replaced OH^- ions from resin, and therefore nothing was eluted out with 20% chloroform-diisopropyl ether. Probably more polar external phase would have eluted them out.

As amberlite IRA-400 was found very basic, amberlite XE-64, which is a weakly acidic resin, was used. Using different internal phases of different pH values up to 12.00 and eluting with diisopropyl ether, it was found that everything was eluted out right away. This happened because as soon as the internal phase was mixed with the resin, it being weakly acidic, the final pH of internal phase became acidic, around pH 5.00. As a result, fatty acids became overwhelmingly soluble in diisopropyl ether and were eluted out right away.

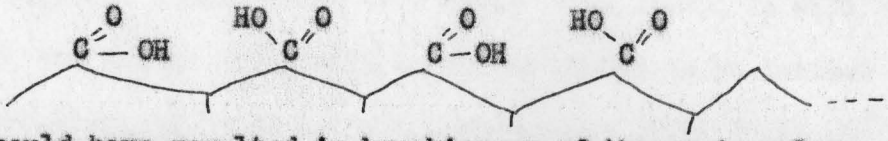
Considering the above results, it was decided to neutralize the acidic groups of resins by washing the resin in 10% aqueous NaOH. To the neutral resin, now internal phases with different pH values were tried.

Internal phase was made up of 80% ethylene glycol and 20% aqueous phase. When the pH of internal phase was from 7 to around 11.0, on addition of the sample of acids, it was found that the channels were formed in the body of the resin column. Probably, this could be explained as follows:



When the state of the resin was in alkaline condition, the carboxyl groups were ionized and the two negative charges on the carboxyl groups repelled each other, keeping the structure of the molecule extended, as shown above. But on addition of acidic samples, charges on the carboxyl groups were neutralized in some cases and in some cases were partly neutralized. Again the resin, being weakly acidic, cannot ionize. As a result, there was no force left between 2

carboxyl groups as a repellent, and the structure collapsed to its original shape.



This could have resulted in breaking up of the resin column.

When the pH of the internal phase was increased a little higher, between 11.5 to 12.00, it seemed as if it was transitional pH. Around pH 11.5, once, every acid was eluted out, and once nothing came out. Results were not consistent and reproducible. Channeling did not quite seem to take place at this pH. Around pH 12.00, after getting about 50 ml of eluate, the column stopped and no more eluate came through. The reason for this behavior became clear when the column was run at pH 12.55. At this pH, a cake-like material was formed on the top of the column when the sample was added. This blocked the passage of the eluant going through. Even under the pounds of pressure of nitrogen, not a single drop came through. This explains why the column stopped trickling at pH 12.00.

This cake formation may be due to the surface active property of the soaps of higher fatty acids. These soaps in high concentration form micelles or gel-like structures in aqueous phase. When the sample of higher fatty acids was added on the top of the column, due to the high alkaline pH of the internal phase, soaps were formed and these soaps were concentrated on the top; as a result, micelles might have formed.

As seen above, too high or low pH resulted in gel formation or channeling, respectively. Where neither occurred, there was also no separation of fatty acids.

Caking is due to the surface active property of the fatty acids, and therefore very hard to eliminate, but channeling could be eliminated if a stronger acidic resin were used. Even though it will be neutralized, the acidic group will be strong enough to be ionized and thus channeling could at least be prevented.

On this basis, amberlite IR-120, a nuclear sulfonic resin, was used. Here also, the internal phase was made up of 80% ethylene glycol plus 20% aqueous phase of different pH values.

As it was not desirable to use the acidic resin as such, it was decided to neutralize the resin by washing it with 10% NaOH solution and to use the sodium salt of the resin. When the neutral salt was buffered at different pH range, the same difficulties were encountered as in the case of neutral amberlite XE-64. At low pH, below 11.50, everything was eluted out.

The range between pH 11.5 and 11.6 was found critical. At 11.52, once the separation of the mixture of myristic and palmitic acids was achieved, but the results were not reproducible. At 11.57, every acid was eluted out, and there was no separation.

When the pH was higher than 11.60, all the acids were held in the column. External phase did come through. At pH 11.95, micelle formation probably occurred because the solvent flow was stopped.

Amberlite IRA-120 behaved just like amberlite XE-64, except at low pH, channeling did not take place, because it was a stronger acid. In the case of high pH, almost invariably, cake-like material formation occurred.

However, this does not exclude the possibilities of using ion-exchange resins in any other manner. If ion-exchange did take place

in case of stronger basic resins like amberlite IRA-400, then by eluting with aqueous solutions of different pH, it ought to be possible to elute different acids out of the column. Whether the separation of the mixtures of fatty acids could be effected or not, is hard to predict because the ionization constants of the higher fatty acids are very close, and therefore it becomes difficult to separate them as anions. However, there is a possibility of separation on the basis of differential adsorption.

In a single run with amberlite IR-120, separation of myristic and palmitic acids was effected at pH 11.52. If optimum conditions, such as pH, packing of the column, rate of flow of eluant, etc., are employed, separation of these acids may, therefore, be obtained. Since these conditions appear to be so critical, this approach is probably not too feasible.

As additional ion-exchange resins become available, the scope of partition chromatography on ion-exchange resins may be expected to broaden. Although attempts to analyze the mixtures of higher fatty acids on ion-exchange resins were not successful, the feasibility of such methods in the analytical field has been demonstrated by the separation of lower dicarboxylic acids, as discussed in the next section.

2. Ion-exchange Partition Chromatography of Lower Dicarboxylic Acids.

The feasibility of using powdered ion-exchange resins as the adsorbent for the internal phase in partition chromatography is demonstrated in this study. It must be kept in mind that this system is quite unlike the usual ion-exchange chromatography composed of one liquid phase, the external solvent and a solid phase, the resin. In the application of the resins to partition chromatography, one is dealing with a triphase system, composed of two liquids and a solid phase. Although extensive studies on simple ion-exchange chromatography have been made, no work has apparently been done with the partition system.

To illustrate the workings of the method, it has been applied to the separation of mixtures of lower dicarboxylic acids. Higuchi et al (83) have reported a method of separation of dicarboxylic acids (C_4 to C_{10}) by partition chromatography in 1952. They have used silicic acid for the support of the internal phase, which is 1 M citrate buffer, pH 5.20. By eluting with different eluants, they have separated benzoic, sebacic, azelaic, suberic and pimelic acids. Eluants used were increasing amounts of butanol in chloroform, different percentages for different acids.

It was decided to use amberlite XE-64, a weak carboxylic acid resin, powdered to around 100 mesh instead of silicic acid. To buffer the resin at pH 5.20, following procedure was adopted. Thirty grams of resin was suspended in distilled water and was titrated potentiometrically against 1 M NaOH. It was found that 13.5 milliequivalents of NaOH was required to raise the pH from 2.50 to 5.20.

This provided a means of obtaining any proportion of aqueous phase, as desired. Generally in these experiments, 30 grams of resin was mixed with 25 ml of internal phase, composed of 13.5 ml of 1 M NaOH and the remainder, distilled water.

The sample was prepared as in the earlier method (83) by dissolving sebacic, suberic and pimelic acids in t-amyl alcohol by heating and then making up to the volume with chloroform. This sample, when chromatogrammed on a column, prepared as previously described (refer to the last section), gave an elution chromatogram shown in Fig. 14. It is evident from the figure, that the mixture was completely resolved. Sebacic acid, however, seemed to have yielded two distinct peaks. A second run was made with very slow rate of elution. Sebacic acid again gave two peaks. It could be possible that 5% n-butanol in chloroform might have washed out some acidity from the resin, and it might have come out where sebacic acid was being eluted out.

Washing the ion-exchange resin with pure n-butanol four to five times should take acidity out, if any. When this butanol-washed resin was used, it was found that the sample of sebacic, suberic and pimelic acids still gave four peaks instead of three (Fig. 15).

There are a few possibilities of what the first two peaks could be. (a) The first peak could be either C_{11} dicarboxylic acid or sebacic acid, and the second peak could be either sebacic acid or azelaic acid; or (b) While trying to dissolve the acids in the t-amyl alcohol by heat, acids might be forming monoesters with t-amyl alcohol. Therefore, the first peak could be monoesters, and the second

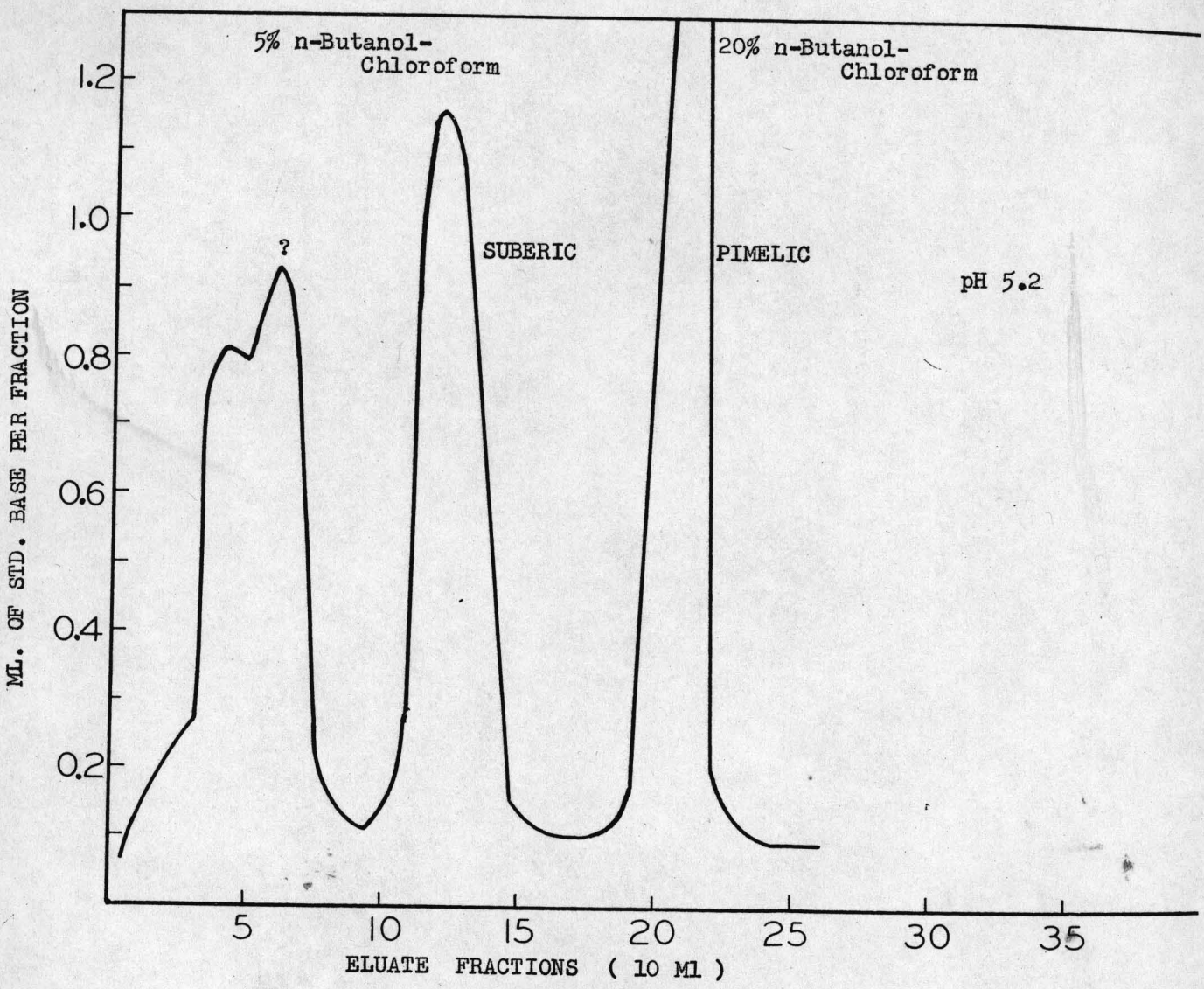

5% n-Butanol-
Chloroform
20% n-Butanol-
Chloroform
?
SUBERIC
PIMELIC
pH 5.2
ML. OF STD. BASE PER FRACTION
1.2
1.0
0.8
0.6
0.4
0.2
5
10
15
20
25
30
35
ELUATE FRACTIONS (10 Ml)

Fig. 14

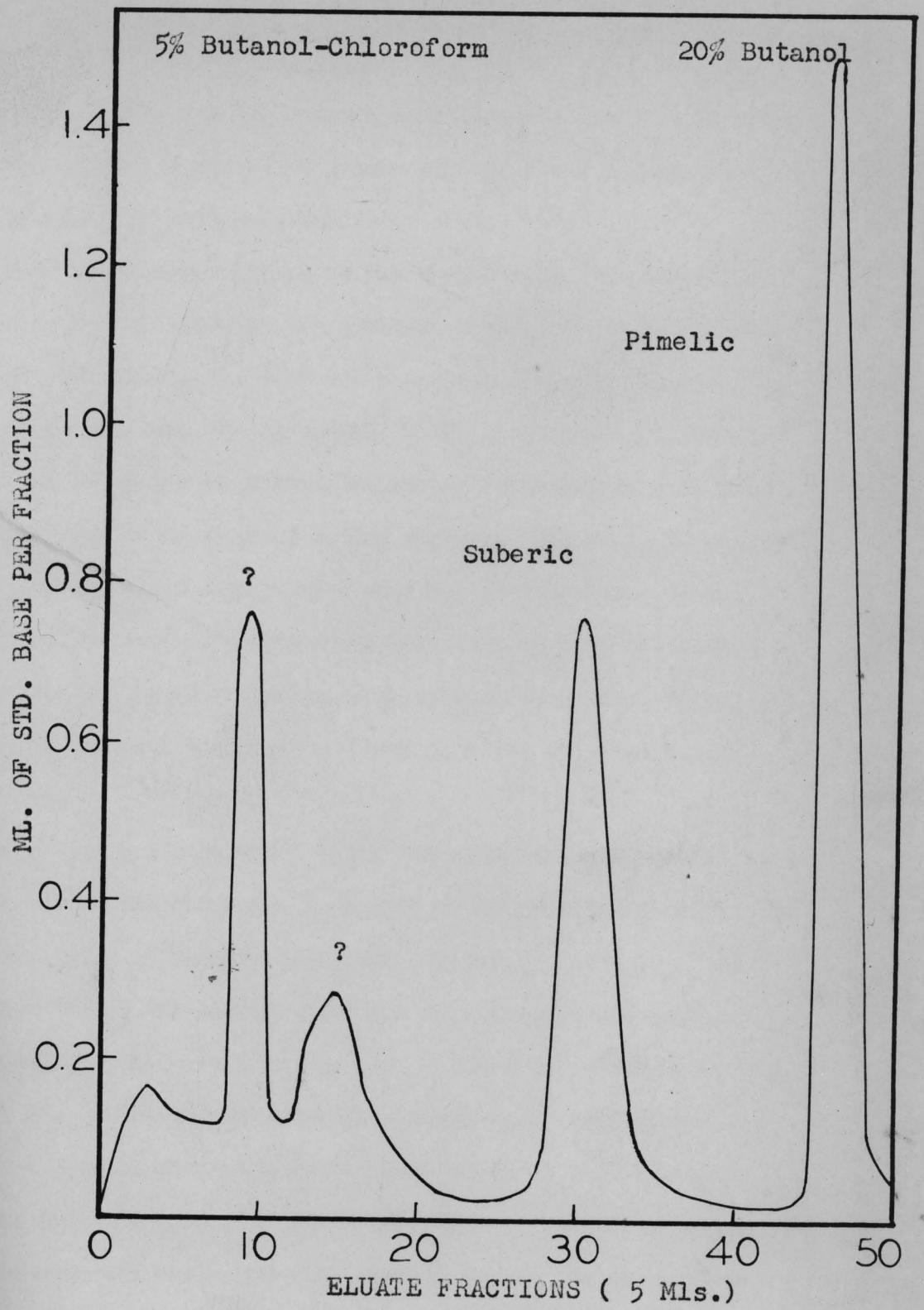

5% Butanol-Chloroform
20% Butanol
Pimelic
Suberic
?
?
ML. OF STD. BASE PER FRACTION
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
ELUATE FRACTIONS (5 Mls.)

Fig. 15

peak, sebacic acid. As the recoveries of suberic and pimelic acids are quantitative, it seems, only sebacic acid forms monoesters with t-amyl alcohol, if this possibility is true.

In the first possibility, as the azelaic acid was available, pure and recrystallized, it was decided to add some azelaic acid to the sample of sebacic, suberic and pimelic acids. If the impurity in the above chromatogram is azelaic acid, four peaks will be obtained, and if it is not azelaic acid, the chromatogram will show five peaks because of five molecular species. Figure 16 shows the chromatogram obtained with such a sample. It shows five peaks. This means that sebacic acid cannot have azelaic acid as impurity. As C_{11} dicarboxylic acid was not available at this time, it was not possible to find out whether the first peak was C_{11} dicarboxylic acid or not.

Out of these experiments, still two possibilities were left. Either C_{11} dicarboxylic acid is present or monoesters of acids, mainly monoester of sebacic acid, are present.

Since it was not possible to have C_{11} dicarboxylic acid, it was decided to prove the identity of the first two peaks by characterizing it. A few columns were run, using only sebacic acid as sample. A typical chromatogram has been shown in Fig. 17. The fractions under the two different peaks were collected and acidified. Liberated acid was extracted with diethyl ether. Acids were recrystallized from water. Melting point of sebacic acid reported in the literature is 133° C. The acid recovered from the first peak gave 130°-131° C as melting point, and the acid from the second

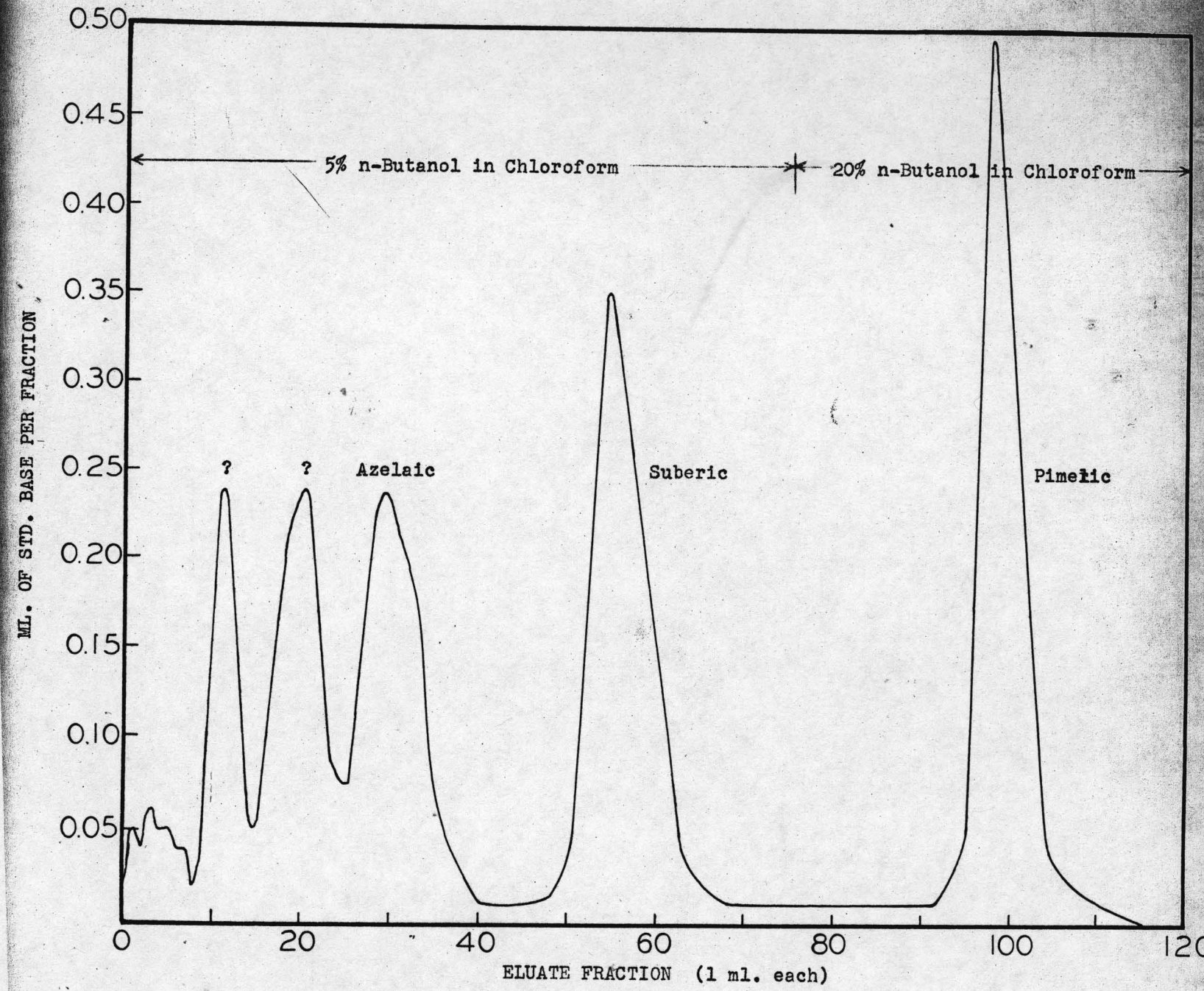

0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
ML. OF STD. BASE PER FRACTION
5% n-Butanol in Chloroform
20% n-Butanol in Chloroform
?
?
Azelaic
Suberic
Pimelic
0
20
40
60
80
100
120
ELUATE FRACTION (1 ml. each)

Fig. 16

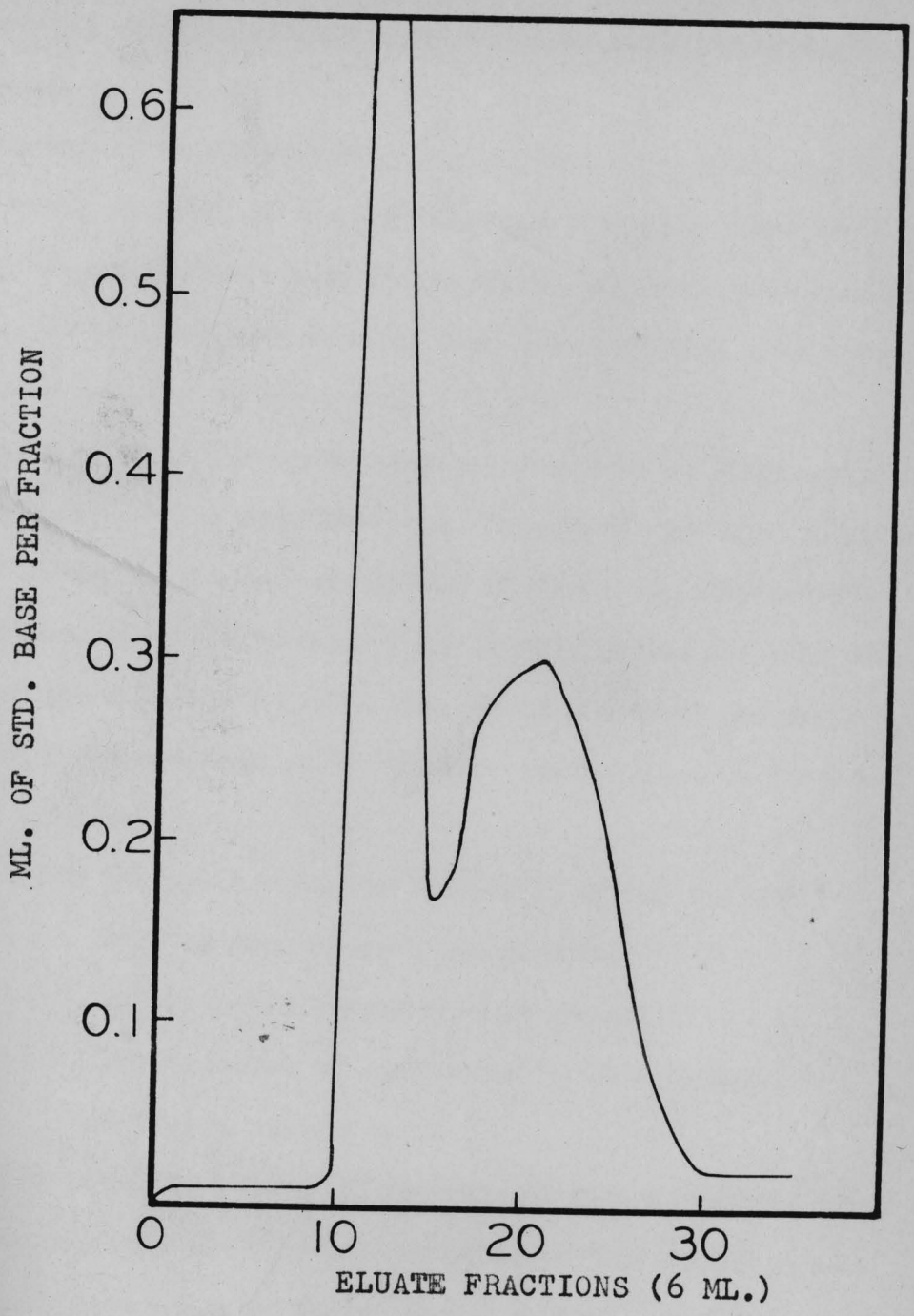

0.6
0.5
0.4
0.3
0.2
0.1
ML. OF STD. BASE PER FRACTION
0
10
20
30
ELUATE FRACTIONS (6 ML.)

Fig. 17

peak gave 129.5° to 130.5° C. This shows that probably both acids are the same, and this did not turn out to be a good characterizing method.

However, by chromatogramming the samples over again through the two different columns, if the two acids are different molecular species, they will come out as before at two different positions on elution. If both are sebacic acid, then they both will give peaks at the same position on the graph.

Figure 18 shows the curves obtained from the two different acids. Both gave peaks at the 23rd fraction. This proof that both acids are the same is definitely more positive. If at all C_{11} dicarboxylic acids or monoesters are present, their melting points would be different and on the column, they will come out at different positions too. Therefore, there are some other phenomena taking place in the ion-exchange columns.

The other phenomena which are showing up may be due to:

(a) rate of flow of the external phase

(b) the amount of internal phase present

(c) the formation of equilibrium between monomers and dimers of sebacic acid.

(a) To check the effects of the rate of flow on sebacic acid, several columns were run at different rates of flow of the eluant.

When fractions were collected at the rate of 5 ml in 15 minutes, as in the above cases, sebacic acid splits up in about half and half into two different peaks. When the column flow was extremely slow, about 1 ml in half an hour, the amount of acid in each fraction was so little that it became very difficult to distinguish between the

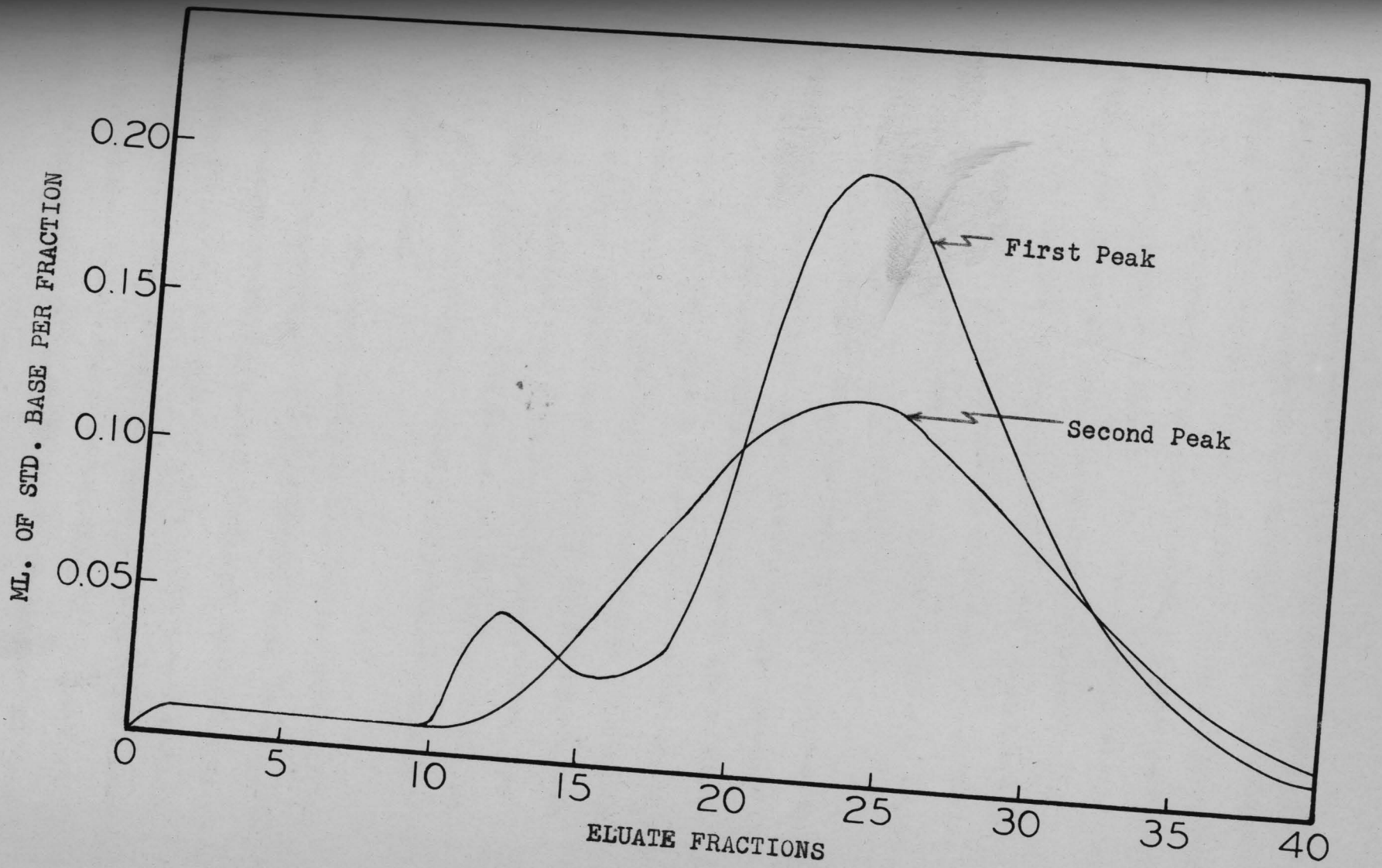

0.20
0.15
0.10
0.05
ML. OF STD. BASE PER FRACTION
First Peak
Second Peak
0
5
10
15
20
25
30
35
40
ELUATE FRACTIONS

Fig. 18

separations of sebacic, suberic and pimelic acids. When 4 ml were collected in half an hour, sebacic acid splits up again into 50:50 portions in two different peaks. The only difference observed was, when the rate of flow was fast, peaks were sharp; when the rate of flow was slow, peaks were broad. This shows that the rate of flow of the eluant is not the cause for the split of sebacic acid.

(b) As the rate of flow and the splitting of sebacic acid were not correlated, different amounts of internal phase with the same buffer capacity were used to investigate the cause of splitting of sebacic acid.

When 5 ml of internal phase was used, splitting of sebacic acid occurred, but the separation between sebacic and suberic acids did not take place. Increasing the amount of water in the internal phase, separation between suberic and sebacic became better and better, but sebacic acid was always split into 2 peaks, about half and half up to 25 ml of internal phase. When 30 ml of internal phase were used, sometimes one peak of sebacic acid was obtained, sometimes splitting occurred. This showed that the amount of the internal phase had some effect on sebacic acid which caused it to split into two peaks.

(c) There is one more possibility that the equilibrium is taking place between monomers and dimers of sebacic acid. Sebacic acid samples, prepared by dissolving the acid in t-amyl alcohol by heat, were analyzed immediately and after 24 hours of its preparation, 30 ml of internal phase were used all the time. If equilibrium between monomers and dimers reaches after a certain time, it will show in different chromatograms. It was found that in the samples analyzed

immediately, occasionally splitting of sebacic acid occurred, and occasionally it did not. The same results were found in the analysis of the samples after 24 hours.

The sample size used in the above case was from 5 to 15 mgm of sebacic acid. Splitting did occur, no matter what sample size was used.

It seems that the amount of internal phase is probably the only factor responsible for splitting sebacic acid. Higher amounts of aqueous phase could not be used because the mass became too wet, which eventually might clog up the column. However, no matter what the conditions were, when splitting occurred, only sebacic acid split up and not azelaic, suberic or pimelic acids. This was very noticeable.

From the separation of mixtures of sebacic, azelaic, suberic and pimelic acids on ion-exchange columns, it is apparent that the ion-exchange resins could be used successfully in partition chromatography for dual purposes:

(a) as a mechanical support

(b) as a buffering agent with the internal phase.

There are all kinds of resins available, and if attempts are made to systematize the chromatographic information thus obtained, this method of separation by partition chromatography using ion-exchange resins might prove to be very popular and powerful analytical method.

C. Separation Methods Based on Conversion of Fatty Acids to Amines

1. Theoretical and Experimental Basis for the Method.

In the several methods discussed in the preceding sections, chromatographic separations have been applied directly to the mixtures of free fatty acids. There are, however, a number of possible advantages which can be gained by resorting to chemical conversion prior to the separatory process. In the following, the results of an investigation of one promising procedure based on this approach are presented.

There are several criteria that a new chemical form of the sample must meet, if it is to permit superior chromatographic separation and analysis. These are:

1. It must be readily and quantitatively formed from the fatty acid sample.

2. It must be readily assayable after elution from a partition chromatographic column.

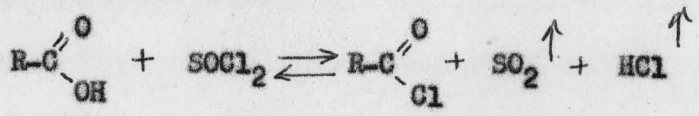
3. It must exhibit no great tendency to undergo association in the selected solvent pair.

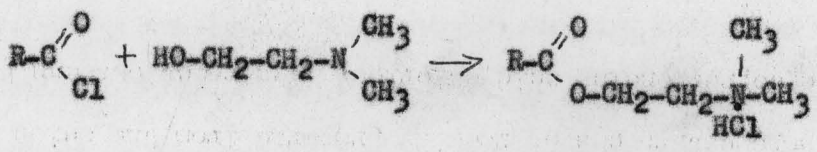
4. Its partition coefficient in a suitable partition system must show great sensitivity to chain length.

5. A suitable adsorbent for the internal phase must be available. The dimethylaminoethyl esters of the fatty acids appear to fulfill these requirements.

These esters can be readily prepared from the acids by the way of the corresponding acid chloride. The reactions can be represented

by the following equations:

(a) 

(b) 

The first reaction corresponding to the conversion of acid to acid chloride by use of thionyl chloride can be rendered essentially quantitative in the presence of excess reagent. Since two of the products are volatile and progressively removed from the reaction site, the reaction is driven to completion. The possible important sources of lowered yield in this step may lie in insufficiency of reaction time and in degradation of the acid chloride after its formation.

The second reaction takes place very rapidly and can reasonably be expected to be quantitative, especially in presence of a great excess of the amino alcohol and when carried out in anhydrous media. Since the amine is a tertiary amine, no amide formation can be expected. Moreover, the presence of the basic group would lead to Schotten-Baumann reaction conditions, further facilitating the esterification stop.

To determine the optimum conditions for the acid chloride formation, a series of experimental runs were made under the following conditions:

About 0.2 meq. of each fatty acid sample was reacted individually with 2 millimoles of thionyl chloride in 2 ml of Skelly C. After

letting the reaction proceed for the prescribed time and temperature, excess dimethylaminoethyl alcohol was added to the reaction mixture. Five ml of 1 M bicarbonate buffer, about pH 9, was added and esters were extracted by Skelly C. The extractants were collected in a 25 ml volumetric flask. Five ml of this solution was used as sample in the chromatographic column for the analysis of their ester content. Internal phase was 1 M citrate buffer at pH 4.50. Esters of different acids were eluted by different solvents as shown under "Recommended Procedure" (page 82).

Preliminary studies indicated that at high temperatures, irregular recoveries were obtained. At 60° C, recoveries of all the acids tried (Tables 6-9) were 97% or better within 4 hours, except those of nonanoic acid, which was later found to be impure (Table 12). Per cent recoveries reported in these tables are the ratio of the amount of the ester recovered after chromatographic procedure to the amount of the total acidity in the starting fatty acid samples. Lower recoveries may have been due to the impure starting material.

Table 6

Undecanoic Acid (at 60° C)

Run no.	No. of hrs. refluxed	Meq. acid reacted	Meq. ester recovered after chromatography	% Recovery
1	4	0.2088	0.2084	99.8
2	6	0.2088	0.2050	98.0
3	8	0.2088	0.1903	91.2
4	10	0.2088	0.1968	94.1
5	12	0.6420	0.5520	86.0

Table 7

Decanoic Acid (at 60° C)

Run no.	No. of hrs. refluxed	Meq. acid reacted	Meq. ester recovered	% Recovery
1	3	0.3056	0.3030	99.3
2	4	0.3056	0.2996	97.7
3	5	0.3056	0.3056	100.0
4	6	0.3056	0.3010	98.8

Table 8

Nonanoic Acid (60° C)

Run no.	No. of hrs. refluxed	Meq. acid reacted	Meq. ester recovered	% Recovery
1	3	0.2464	0.1623	65.8
2	3	0.2464	0.1620	65.6
3	4	0.2464	0.1629	66.0
4	4	0.2464	0.1650	67.0
5	4	0.2464	0.1525	62.0
6	5	0.2464	0.1657	67.2

Table 9

Octanoic Acid (60° C)

Run no.	No. of hrs. refluxed	Meq. acid reacted	Meq. ester recovered	% Recovery
1	3	0.2783	0.2692	96.7
2	4	0.2783	0.2680	96.3
3	5	0.2783	0.2716	97.5

The second criterion of the derivative form, i. e., relative ease of its analysis, has been already described in part in the preceding section. The esters are readily analysed in the solution in eluates by titrating with standard perchloric acid in glacial acetic acid. Of the several indicators tried, quinaldine red and benzeneazodiphenylamine were found more suitable than others. The color change of both the indicators was sharp in Skelly C as well as in chloroform, but whenever diisopropyl ether was present, only quinaldine red did not give a sharp color change. About 1 micro-equivalent of excess perchloric acid was sufficient for the sharp color change. Benzeneazodiphenylamine became the indicator of choice because of sharp color change in all different solvents used.

To determine whether there was any significant tendency for the ester molecules to undergo association, a study of partition coefficients of these amino esters was carried out as follows:

Suppose C_9 ester is being partitioned. 100 ml of C_9 ester solution in diisopropyl ether was shaken with 100 ml of aqueous buffer, pH 4.6. It was found that partition coefficient was 1.087, where

$$K = \frac{\text{concentration in aqueous phase.}}{\text{concentration in organic phase}}$$

When the same solution of diisopropyl ether was again shaken with fresh buffer, however, it was found that this time partition coefficient was 0.748. On successive fractionation, it was found that the value was stabilized at 0.6. This showed that the partition isotherm was linear in case of amino esters.

Table 10 and Fig. 19 show such constant partition coefficients

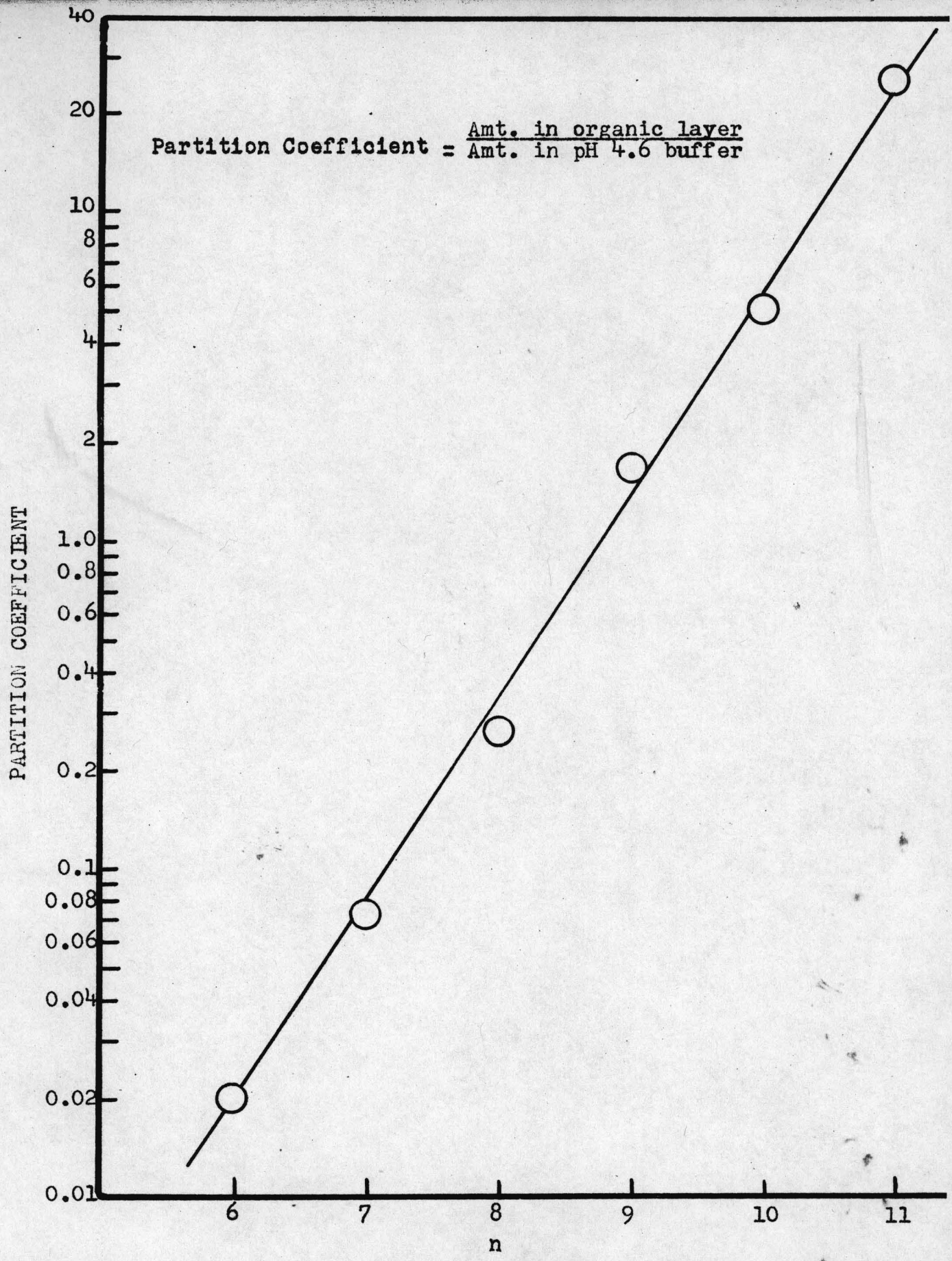


Fig. 19. Partition Coefficient of Dimethylaminoethyl ester of C_n ac

Table 10

Ester of C_n acid where C_n =	K*	K#
C_{11}	25	63
C_{10}	5	13
C_9	1.67	4.2
C_8	.27	.7
C_7	.07	.2
C_6	.02	.05

* K = $\frac{\text{concentration in diisopropyl ether}}{\text{concentration in aqueous pH 4.6 buffer}}$

\# K = $\frac{\text{concentration in diisopropyl ether}}{\text{concentration in aqueous pH 5.00 buffer}}$

(calculated from K values at pH 4.6)

of different esters. It is apparent from the slope obtained that the partition coefficients increased by a factor of four approximately for each carbon atom increase in the fatty acid chain.

In case of Ramsey and Patterson's (1) method, partition coefficients increased by a factor of approximately two for every two carbon atom increase in the chain length of the fatty acid. Thus the partitioning behavior of amino esters is considerably more sensitive to chain length than the corresponding fatty acids.

Because of the acidic nature of the aqueous solution used as the internal phase, it was possible to use silicic acid as adsorbent. It was later found, however, that the esters were significantly adsorbed on silicic acid, resulting in somewhat poorer separation in many cases. No special study, however, was made about adsorbents.

2. Development of the Partition Chromatographic Methods for the Separation of Mixed Esters of C_6-C_8 and C_8-C_{11} Fatty Acids.

On the basis of previous discussions, two methods for the separation of mixed fatty acid esters were developed.

First, the mixtures of esters of C_8, C_9 and C_{10} acids were used for separation. In the chromatographic column, the rate of movement of each ester is governed by $t = R_1 x$

where t = rate of movement of the ester

R_1 = rate of movement of the solvent

$$x = \frac{Kr}{Kr + 1}$$

K = partition coefficient = $\frac{\text{conc. in organic phase}}{\text{conc. in aqueous phase}}$

$r = 1$ (approximately)

When 30 grams of silicic acid are packed in the column, the height of the column was about 25 cm, and it held approximately 25 ml of external phase. Therefore, $R_1 = 1$ cm/ml.

$\therefore$ For C_8 ester, $x = \frac{0.675}{1.675} = 0.403$

and $t = 1 \times 0.403 = 0.403$ cm/ml of eluant

$\therefore$ to travel 25 cm in the column, $\frac{25}{0.403} = 62d$ ml of eluant.

So at around 62d ml of the eluant, we should find the maximum concentration of C_8 ester.

Similarly, for C_9 ester,

$t = R_1 x$ $\qquad x = \frac{4.17}{5.17} = 0.806$

$\therefore$ $t = 1 \times 0.806 = 0.806$ cm/ml of eluant

$\therefore \frac{25}{0.806}$ = 25th ml of the eluant.

The peak of C_{10} ester should be at 25th ml of the eluant.

From the above calculations, it is expected that separation might not take place between C_{10} and C_9 esters, because they both will be coming out together, while separation should take place between C_8 and C_9 esters.

Figure 20 shows the chromatogram obtained by running a silicic acid column (30 grams), using 1 M citrate buffer at pH 5.00 as internal phase. The external phase was diisopropyl ether. Each fraction contained about 5 ml of eluant.

When these data were compared with the theoretical calculations, it was found that C_{10} had a peak at 40th ml, while theoretically it should have a peak at about 25th ml of the eluant. Similarly, C_9 ester had a peak around 70th ml of the eluant while it should have a peak around 31st ml of the eluant, and C_8 ester had a peak around 160th ml instead of theoretical 62d ml of the eluant.

Theoretical calculations were strictly based on partition effect only, but from experimental results, it was found that the esters were held back more than expected by partition effect. It was evident, then, that some other phenomenon was playing a part in the separation of esters. As dimerization of esters in diisopropyl ether is not possible, adsorption of esters on silicic acid looked very probable because esters being amines could be adsorbed by silicic acid. Also the skewedness in the elution curves of C_9 and C_8 esters showed that besides partition effect, there was some other effect playing a part.

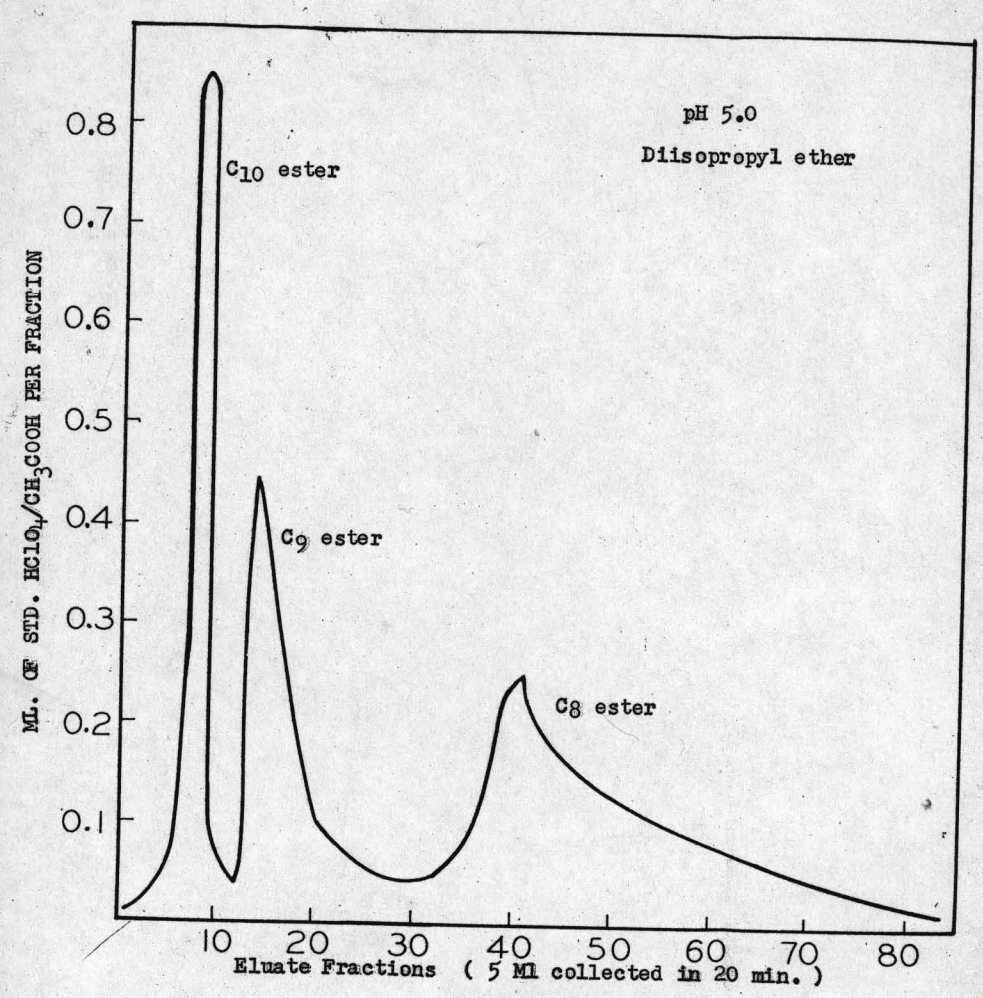

pH 5.0
Diisopropyl ether
C_{10} ester
C_9 ester
C_8 ester
ML. OF STD. $HClO_4/CH_3COOH$ PER FRACTION
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
80
Eluate Fractions (5 Ml collected in 20 min.)

Fig. 20

As the separation of C_8, C_9 and C_{10} esters was achieved by using 1 M citrate buffer, pH 5.00, on silicic acid, it was decided to add C_{11} ester to the sample and separate all 4 esters. As the separation of C_{11} ester from C_{10} ester was not possible at pH 5.00, pH of the internal phase was decreased to 4.5.

Figure 21 shows the elution curves of C_{11} and C_{10} esters. It was evident from the curve obtained that as the pH of the internal phase was decreased, the adsorption of esters on silicic acid became more significant. Hence the tailing effect was observed. When Skelly C was used as external phase, separation between C_{11} and C_{10} esters was effected (Fig. 22).

Figure 23 shows the elution curves of C_{10} and C_9 esters when the pH of the internal phase was 4.5 and external phase was diisopropyl ether. C_9 ester shows a strong adsorption effect and hence tailing. This adsorption effect was partly counteracted by using more polar solvent. Thus when 15% chloroform-diisopropyl ether was used as eluant, C_9 ester was eluted out sharply. (See Fig. 24).

Final procedure for the separation of C_8, C_9, C_{10} and C_{11} esters turned out as follows:

Using silicic acid as support for internal phase, pH 4.5 1 M citrate buffer sample in Skelly C is added at top. The chromatogram is developed with Skelly C until C_{11} ester is eluted out. After that, diisopropyl ether is added and C_{10} ester is eluted out. After that, 15% chloroform in diisopropyl ether is added to elute out C_9 ester and then 50:50::$CHCl_3$ in diisopropyl ether is added to elute out C_8 ester. A complete separation of 4 esters is shown in Fig. 24. These solvent combinations were obtained after trying out many

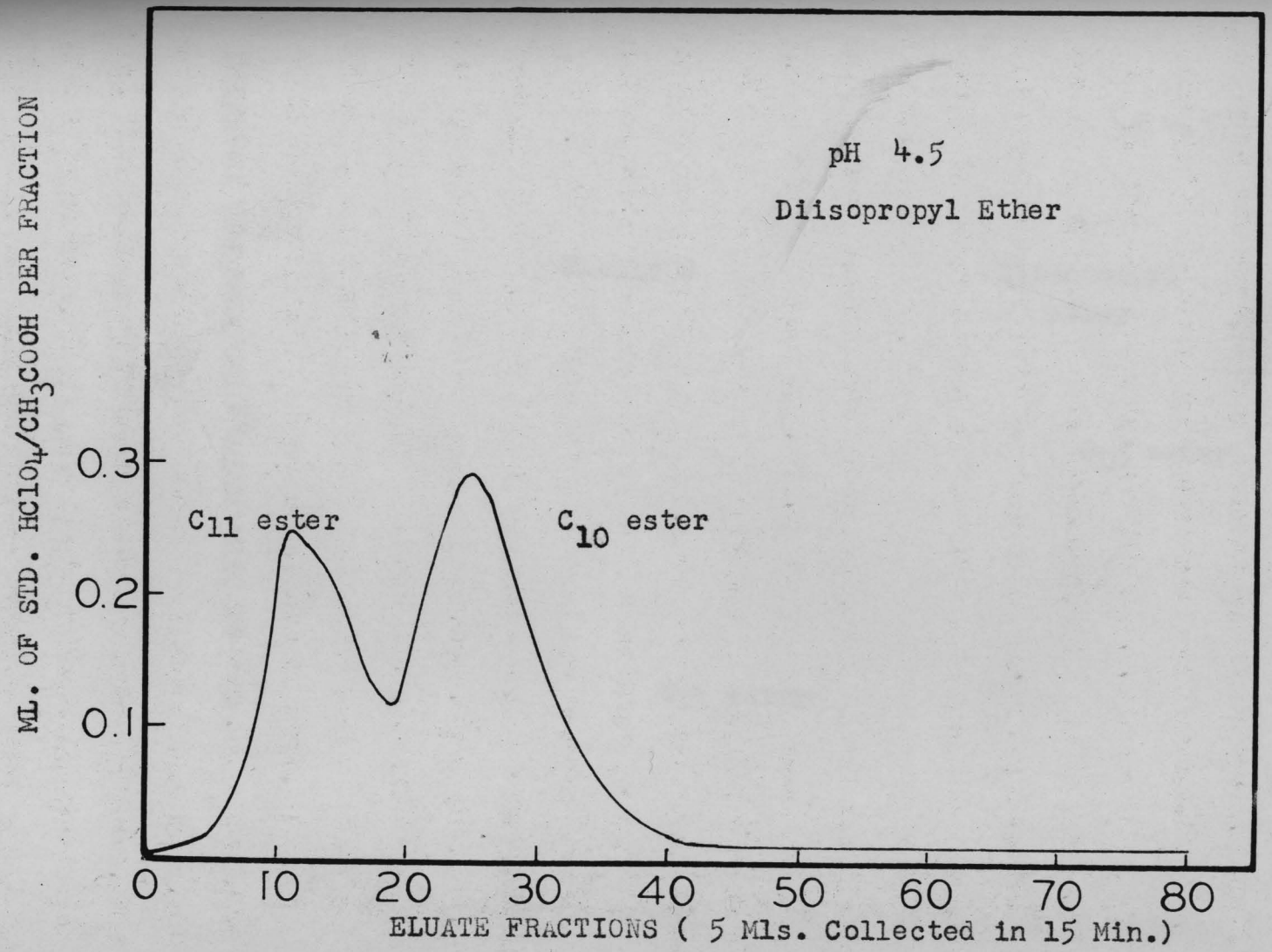

pH 4.5
Diisopropyl Ether
ML. OF STD. $HClO_4/CH_3COOH$ PER FRACTION
0.3
0.2
0.1
C_{11} ester
C_{10} ester
0
10
20
30
40
50
60
70
80
ELUATE FRACTIONS (5 Mls. Collected in 15 Min.)

Fig. 21

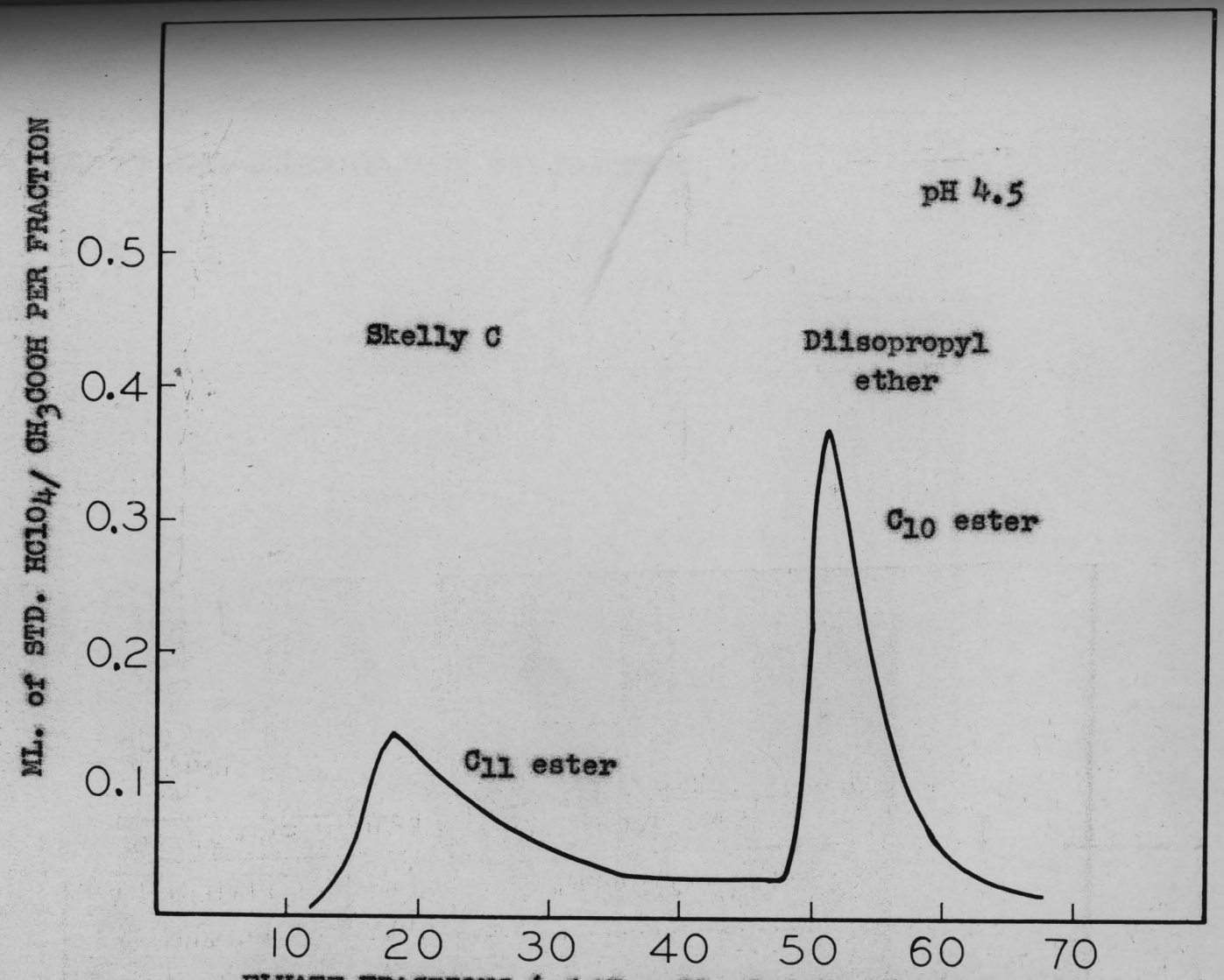

pH 4.5
Skelly C
Diisopropyl ether
C_{10} ester
C_{11} ester
ML. of STD. $HClO_4$/ CH_3COOH PER FRACTION
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
ELUATE FRACTIONS (5 Ml collected in 15 minutes)

Fig.22.

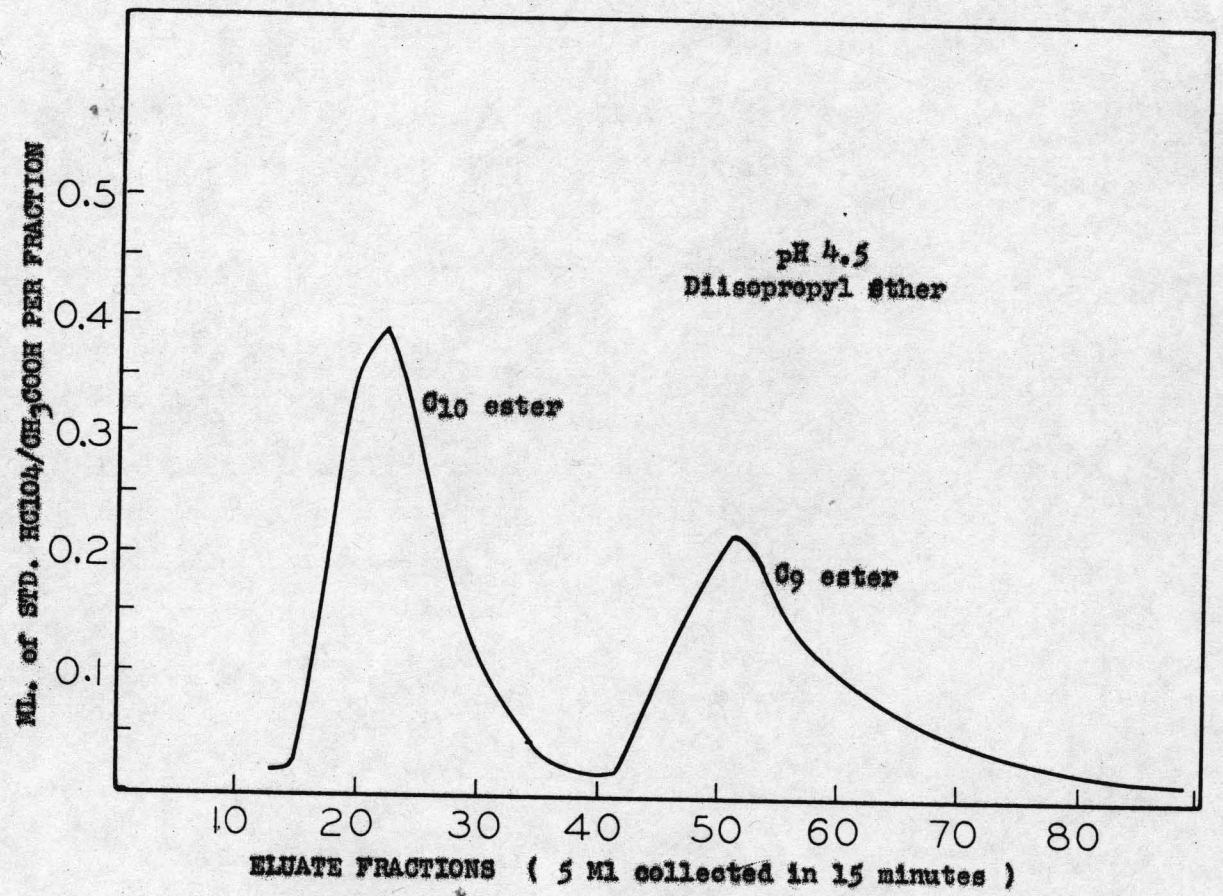

ML. of STD. HClO4/CH3COOH PER FRACTION
0.5
0.4
0.3
0.2
0.1
pH 4.5
Diisopropyl Ether
C10 ester
C9 ester
10
20
30
40
50
60
70
80
ELUATE FRACTIONS (5 Ml collected in 15 minutes)

Fig.23 .

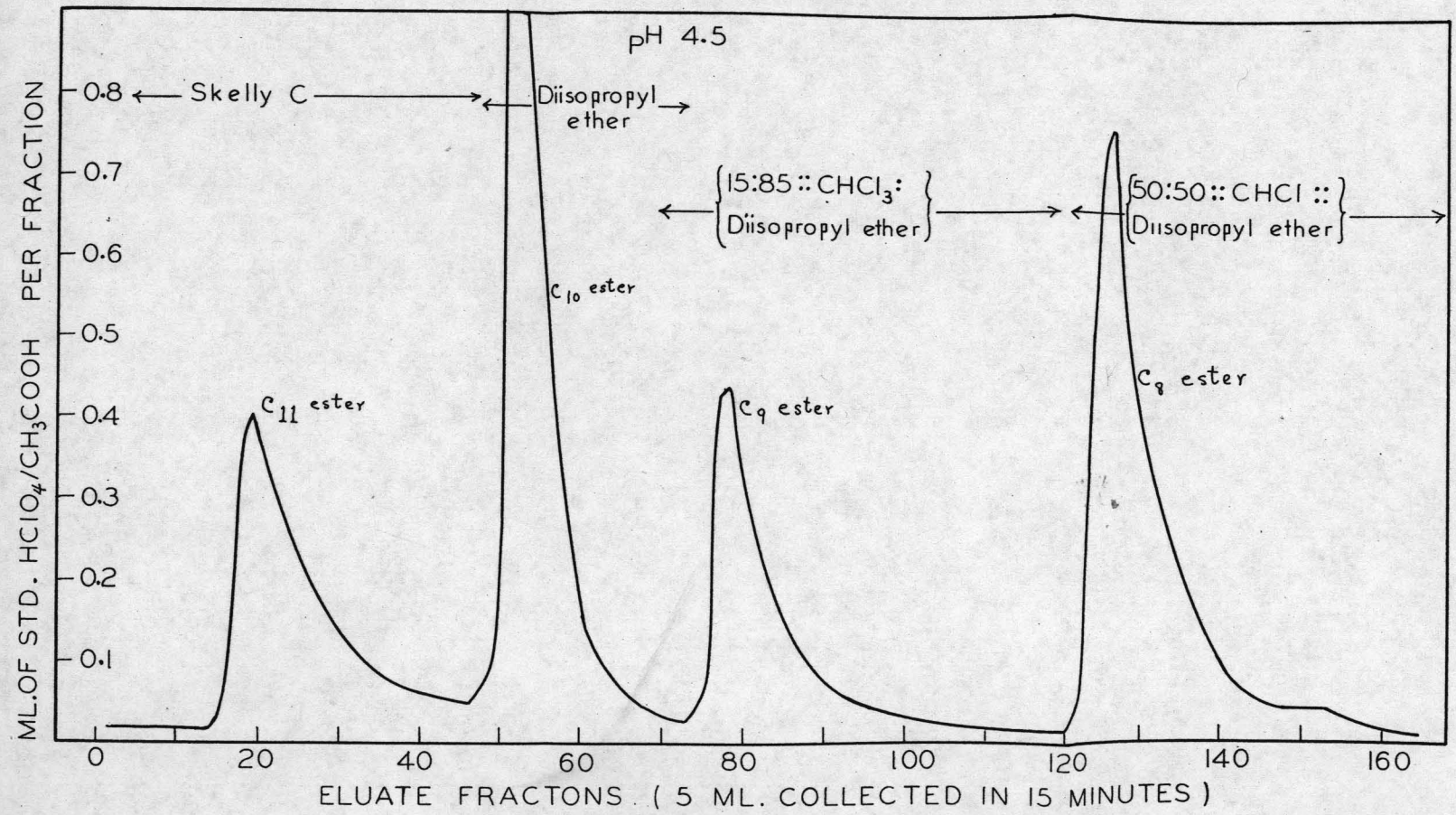

pH 4.5
Skelly C
Diisopropyl ether
15:85 :: CHCl3 : Diisopropyl ether
50:50 :: CHCl :: Diisopropyl ether
C11 ester
C10 ester
C9 ester
C8 ester
ML. OF STD. HClO4/CH3COOH PER FRACTION
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
20
40
60
80
100
120
140
160
ELUATE FRACTONS (5 ML. COLLECTED IN 15 MINUTES)

FIGURE 24

different combinations of solvents so that the sharpest peak for each could be obtained.

After achieving the separation of C_8, C_9, C_{10} and C_{11} esters, it was decided to separate C_{12} ester with above mixture.

From Fig. 24, it was evident that at pH 4.50, separation between C_{12} ester and C_{11} esters was not possible because of strong adsorption. A column was run, however, to see the results. Figure 25 shows the elution curves for C_{12} and C_{11} esters. Separation did not take place.

Figure 26 shows the elution curves of C_{12} and C_{11} esters when the pH of the internal phase was 4.0 and external phase was Skelly C. It was clear that as the pH was made more acidic, adsorption of esters became significant. If there had been no adsorption, it would have been possible to separate C_{12} and C_{11} esters at pH 4.0.

Different solvent combinations and different pH were used in an attempt to separate the C_{12} ester from C_{11} ester. Figures 27-29 show the elution curves of C_{12} and C_{11} esters. Separation did not take place. Wherever chloroform was used in the external phase, regardless of its concentration, always one peak was obtained.

Attempts were made to minimize the adsorption effect in the case where partial separation of C_{12} and C_{11} esters was obtained as in Fig. 26. Silicic acid had strong affinity for amino esters. If this affinity for esters could be preferentially satisfied by adding some other amine to the internal phase, probably the adsorption effect could be cut down. Figure 30 shows the partial separation obtained when 2% dimethylaminoethyl alcohol was added to the internal phase, pH 4.02. Separation was better but was not complete.

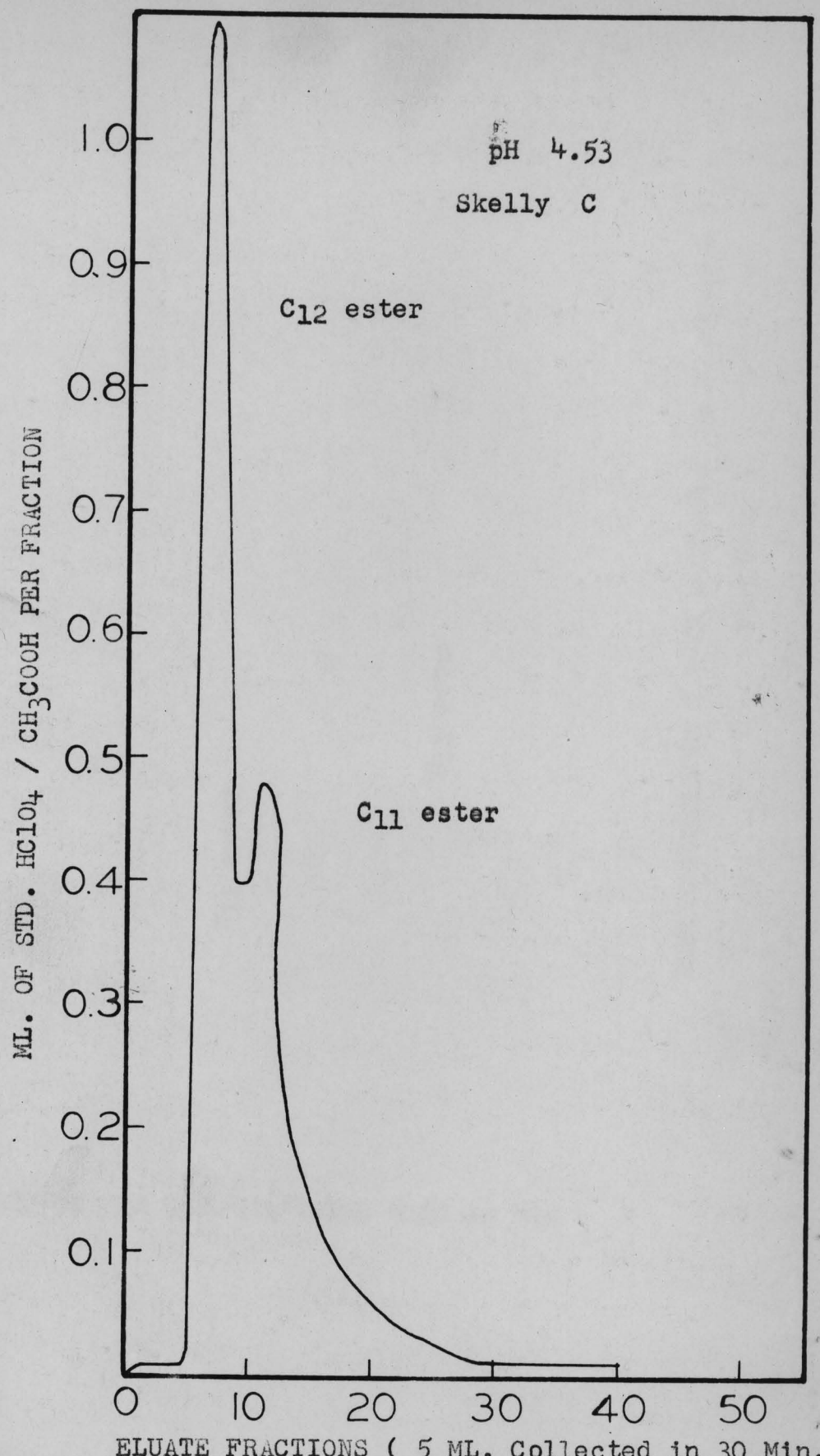

pH 4.53
Skelly C
C_{12} ester
C_{11} ester
ML. OF STD. $HClO_4$ / CH_3COOH PER FRACTION
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
ELUATE FRACTIONS (5 ML. Collected in 30 Min.)

Fig. 25

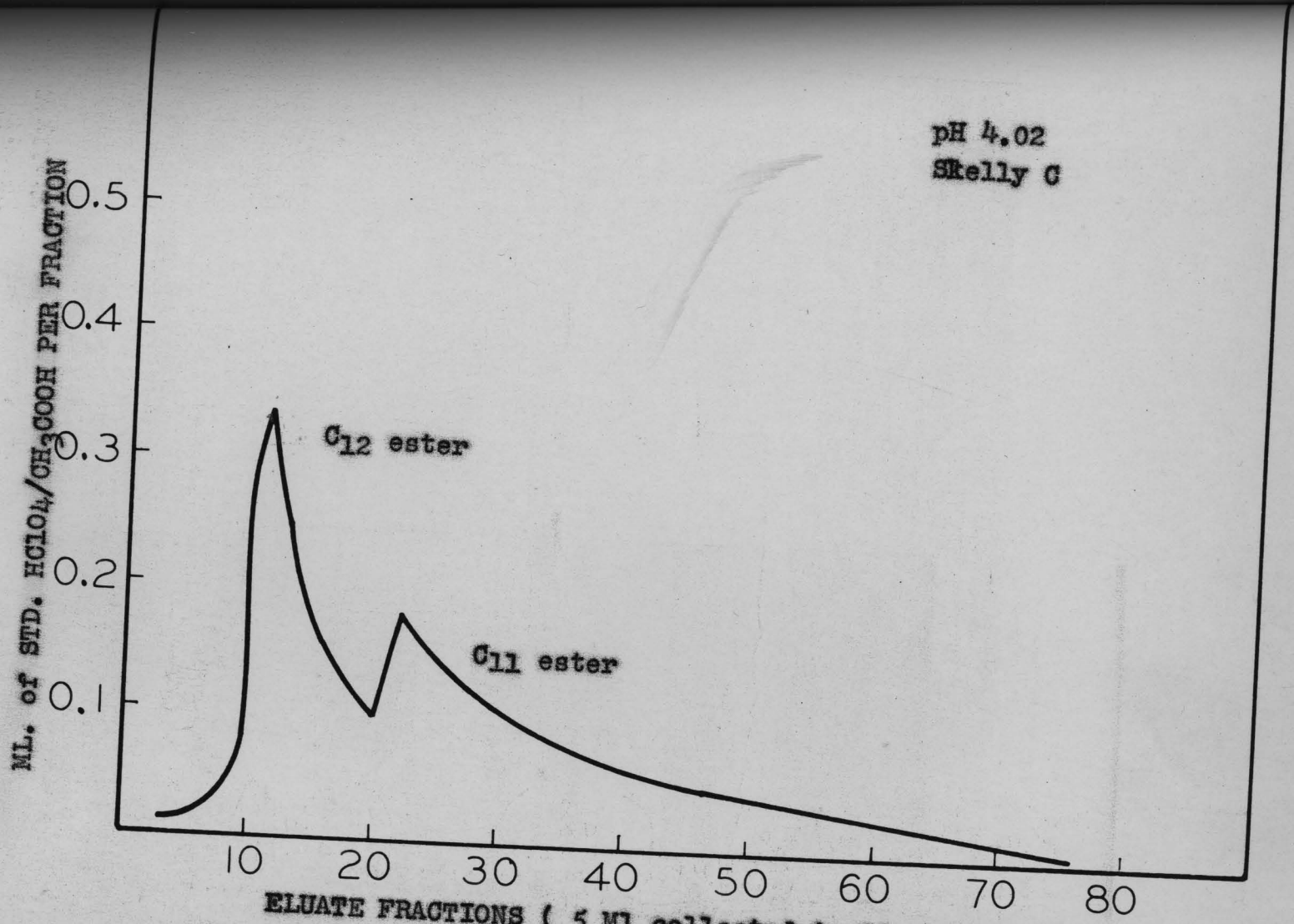

pH 4.02
Skelly C
ML. of STD. $HClO_4/CH_3COOH$ PER FRACTION
0.5
0.4
0.3
0.2
0.1
C_{12} ester
C_{11} ester
10
20
30
40
50
60
70
80
ELUATE FRACTIONS (5 Ml collected in 20 minutes)

Fig.26 .

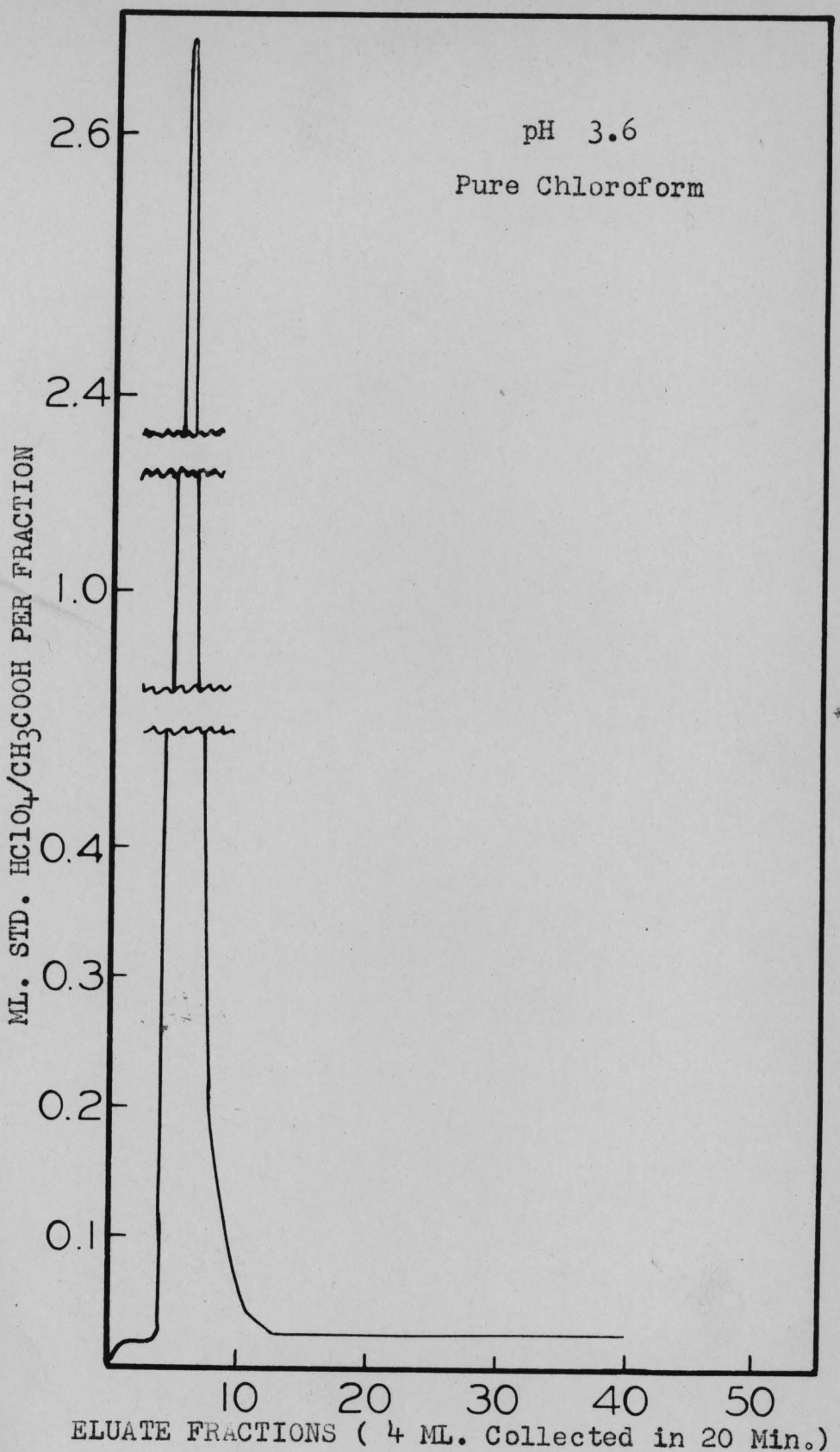

pH 3.6
Pure Chloroform
2.6
2.4
1.0
0.4
0.3
0.2
0.1
ML. STD. HClO4/CH3COOH PER FRACTION
10
20
30
40
50
ELUATE FRACTIONS (4 ML. Collected in 20 Min.)

Fig. 27

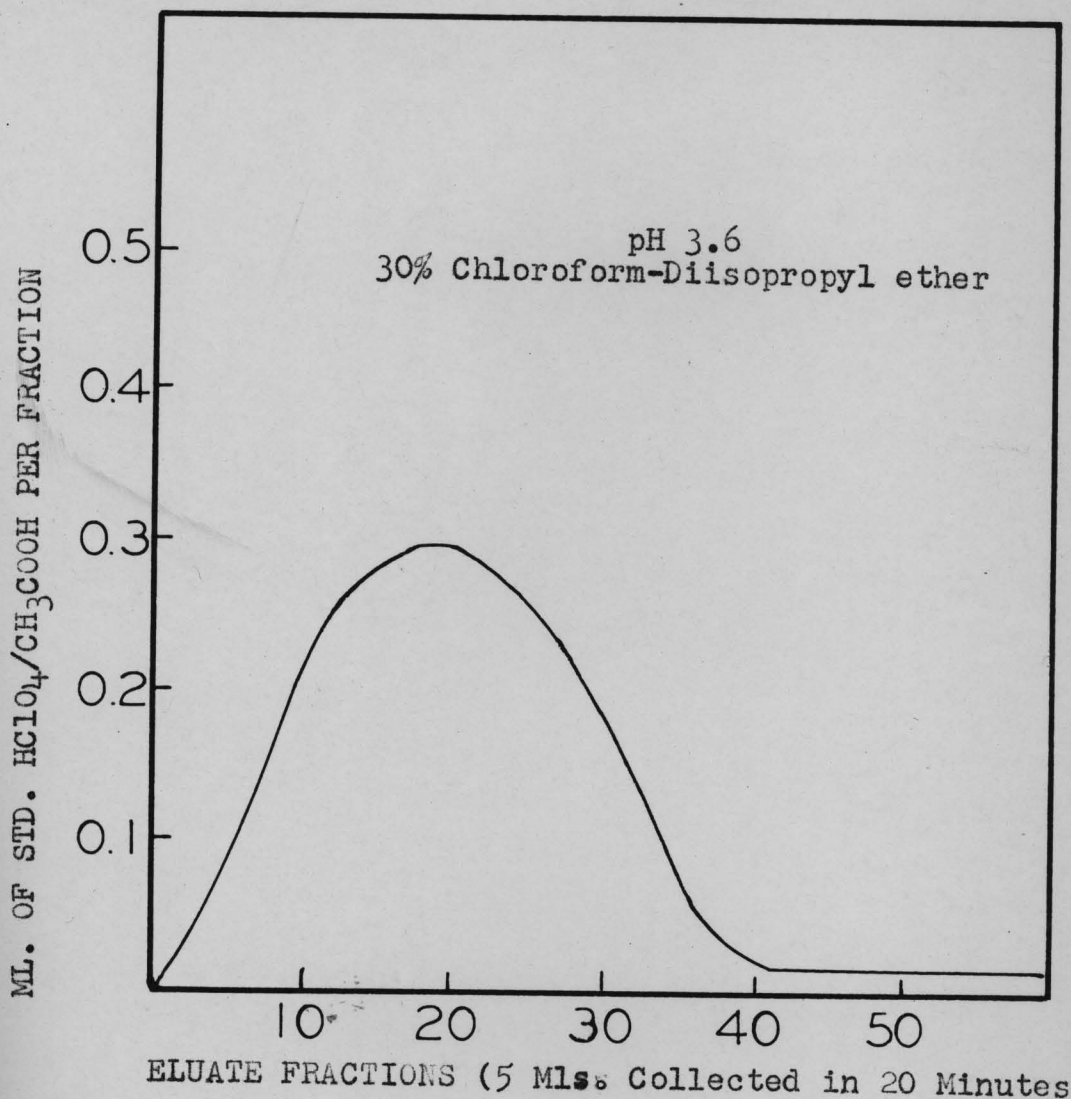

pH 3.6
30% Chloroform-Diisopropyl ether
ML. OF STD. $HClO_4/CH_3COOH$ PER FRACTION
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
ELUATE FRACTIONS (5 Mls. Collected in 20 Minutes)

Fig. 28

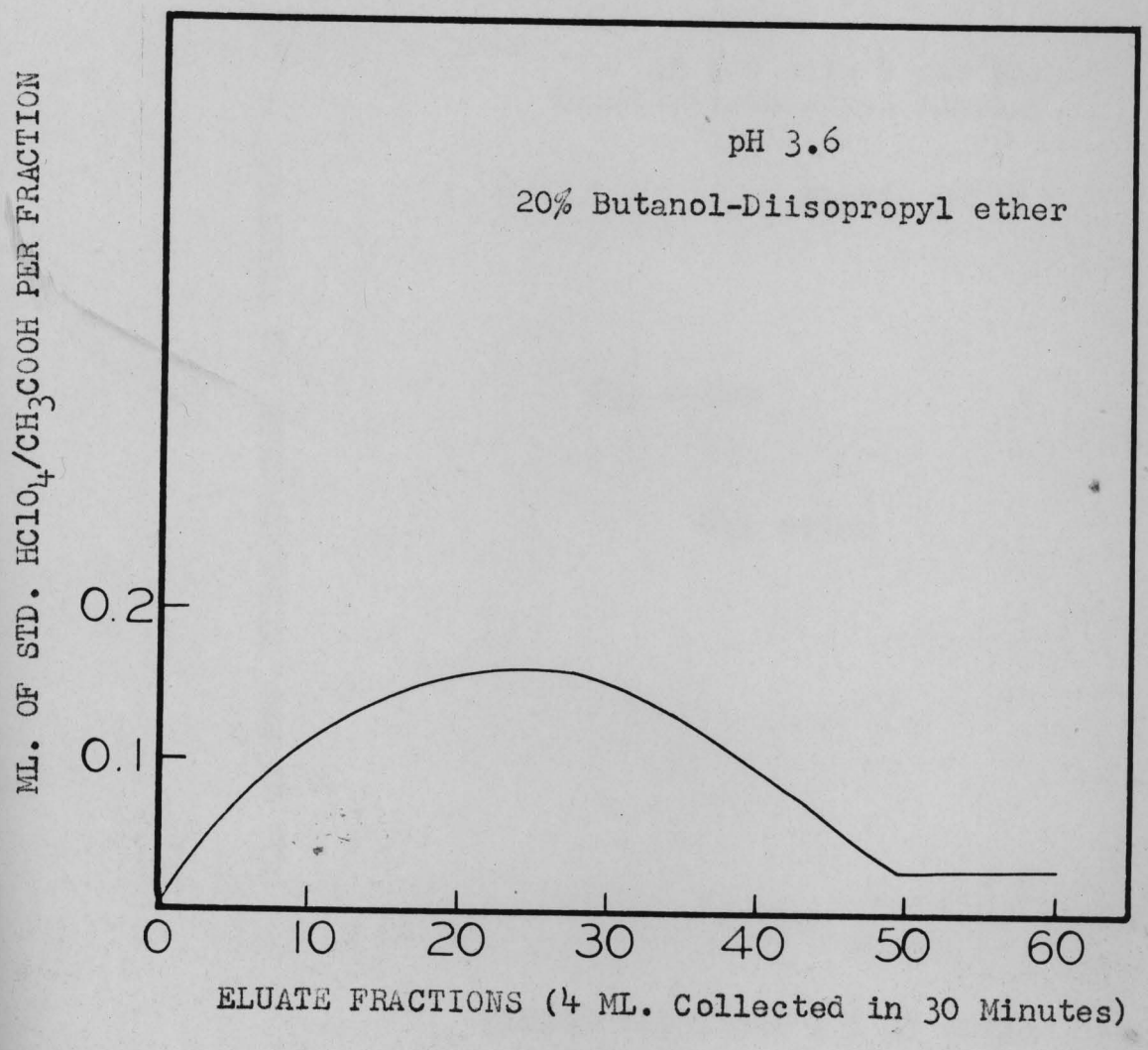

pH 3.6
20% Butanol-Diisopropyl ether
ML. OF STD. $HClO_4/CH_3COOH$ PER FRACTION
0.2
0.1
0
10
20
30
40
50
60
ELUATE FRACTIONS (4 ML. Collected in 30 Minutes)

Fig. 29

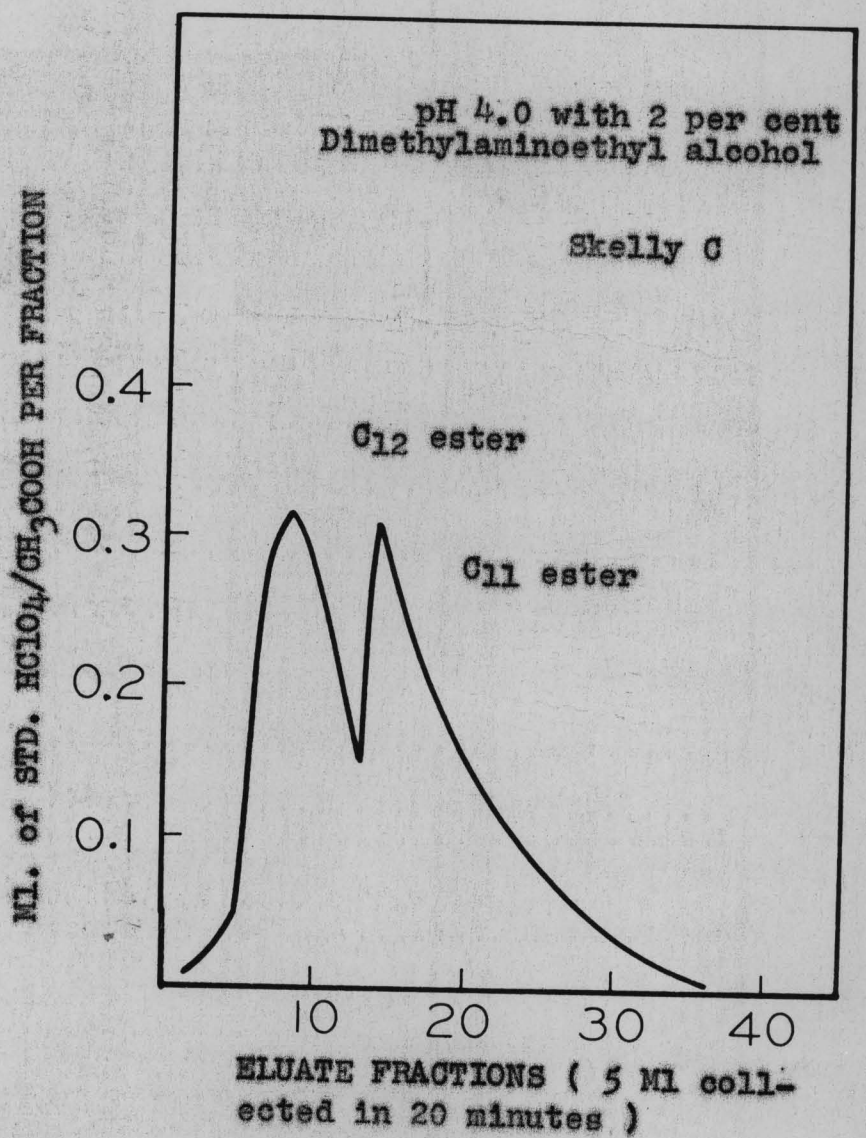

pH 4.0 with 2 per cent
Dimethylaminoethyl alcohol
Skelly C
C_{12} ester
C_{11} ester
Ml. of STD. $HClO_4/CH_3COOH$ PER FRACTION
0.4
0.3
0.2
0.1
10
20
30
40
ELUATE FRACTIONS (5 Ml collected in 20 minutes)

Fig.30 .

Another way to eliminate adsorption is to add methyl alcohol in the internal phase. Methyl alcohol solubilizes the amino esters, thus preventing them from being adsorbed by silicic acid. On this basis, a column was run with 10% methyl alcohol in the internal phase, pH 4.0. Skelly C was used as external phase. Figure 31 shows the elution curves obtained. Instead of expected separation, strong adsorption was noted.

To see what happens to partition coefficient values of C_{12} and C_{11} esters when methyl alcohol is present in the internal phase, determinations of the partition coefficient values were carried out between different pH and Skelly C. Aqueous phase was made up of 75% methyl alcohol plus 25% buffer. Figure 32 and Table 11 show the results obtained. It becomes evident that methyl alcohol reduces the difference between partition coefficient values of two successive members of esters used. Hence it should be avoided.

Another method to reduce adsorption is the use of some support which does not adsorb these esters. Solka-floc, a highly purified wood cellulose, was used as support. C_{11} and C_{10} esters were not separated by using 1 M citrate buffer, pH 4.5, and Skelly C (Fig. 33). These solvent combinations worked when silicic acid was used as support.

Because a method was developed on silicic acid for the separation of esters of C_8, C_9, C_{10} and C_{11} acids, it was decided to develop a method using Solka-floc for the separation of the esters of lower acids. When the solvent combination, pH 4.50, and Skelly C were used, separation of C_8 and C_7 esters was effected. Figure 34 shows the elution curves of C_8 and C_7 esters. As the pH found in

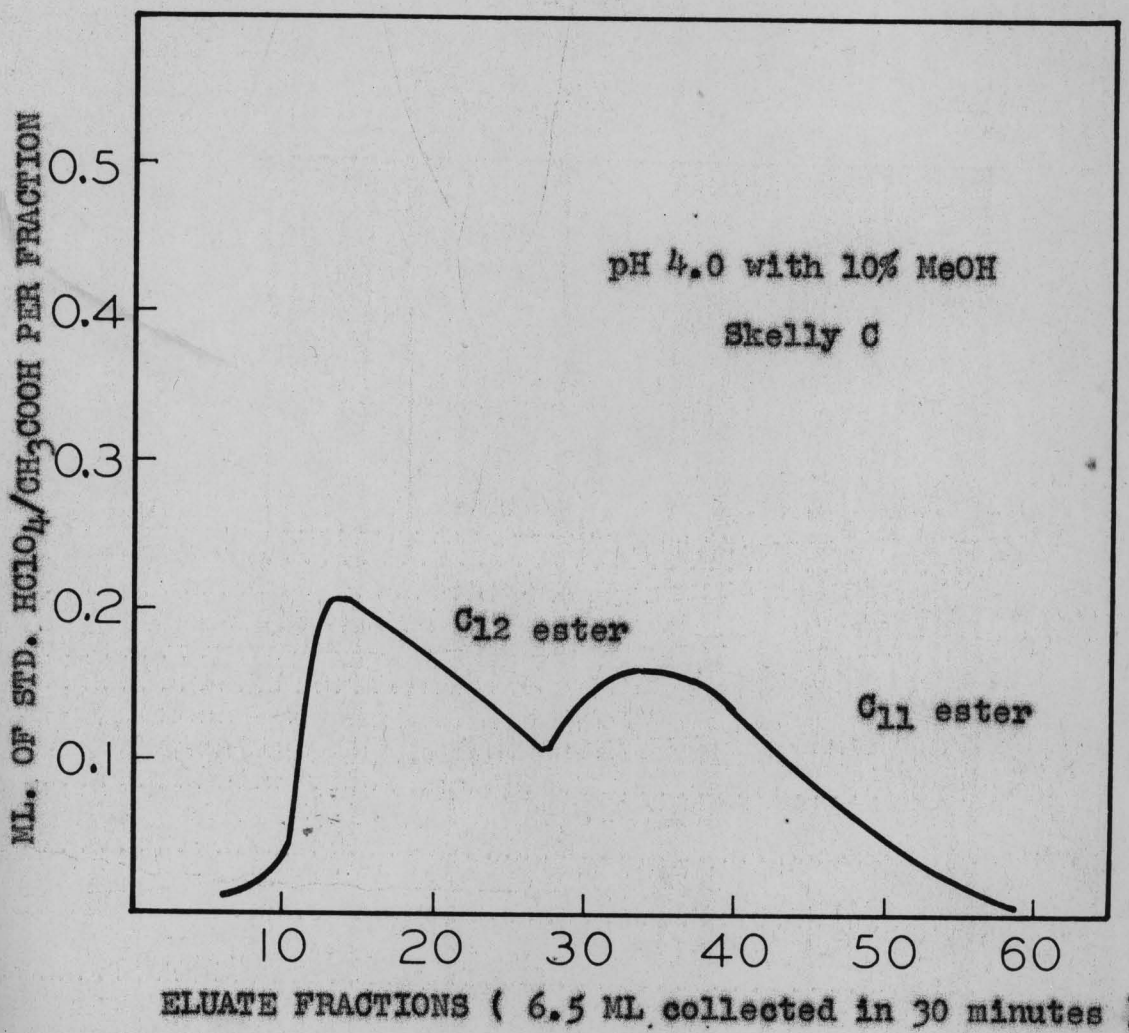

ML. OF STD. $HClO_4/CH_3COOH$ PER FRACTION
0.5
0.4
0.3
0.2
0.1
pH 4.0 with 10% MeOH
Skelly C
C_{12} ester
C_{11} ester
10
20
30
40
50
60
ELUATE FRACTIONS (6.5 ML collected in 30 minutes)

Fig .31.

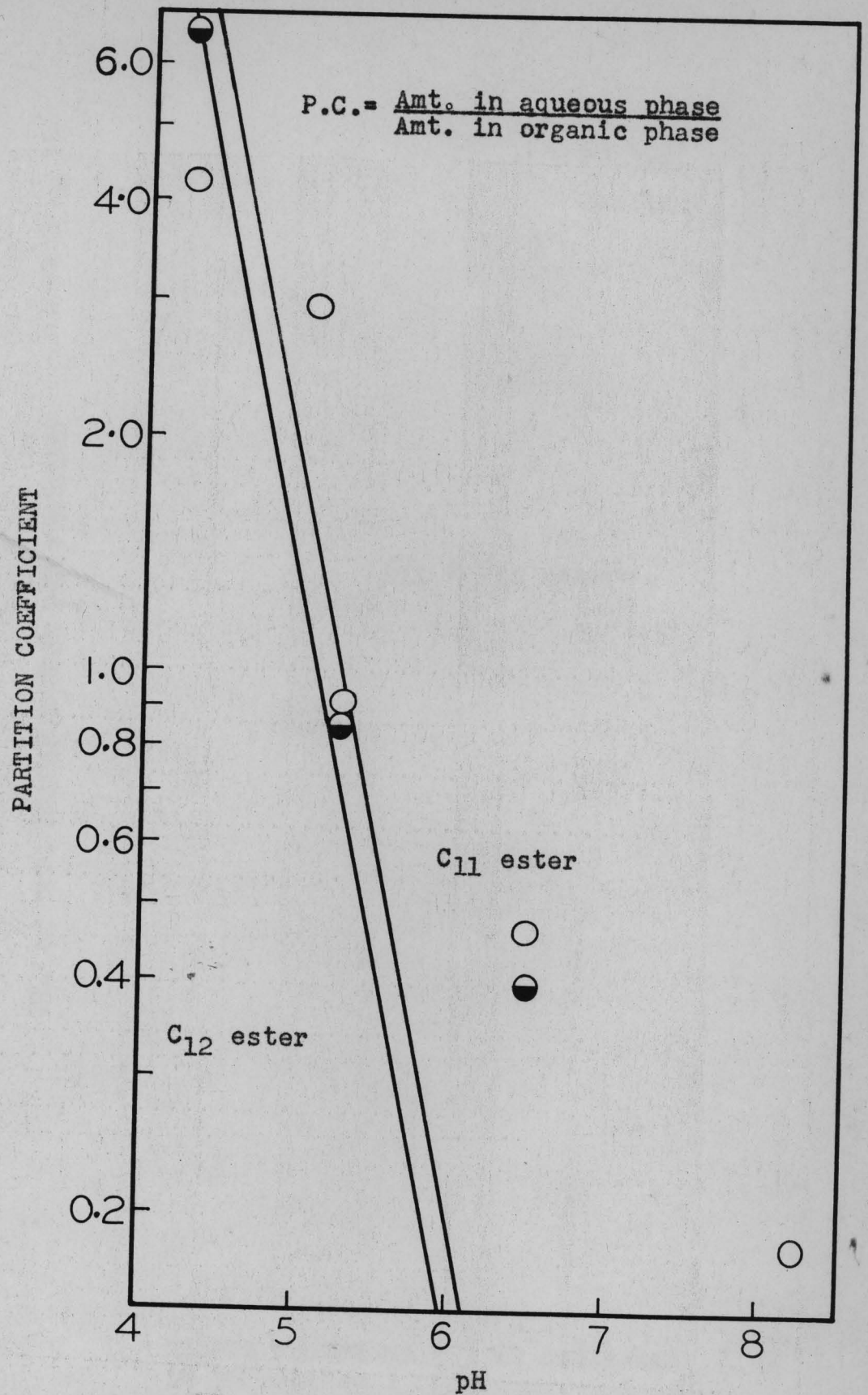


Fig.32 . Partition Coefficient vs pH.

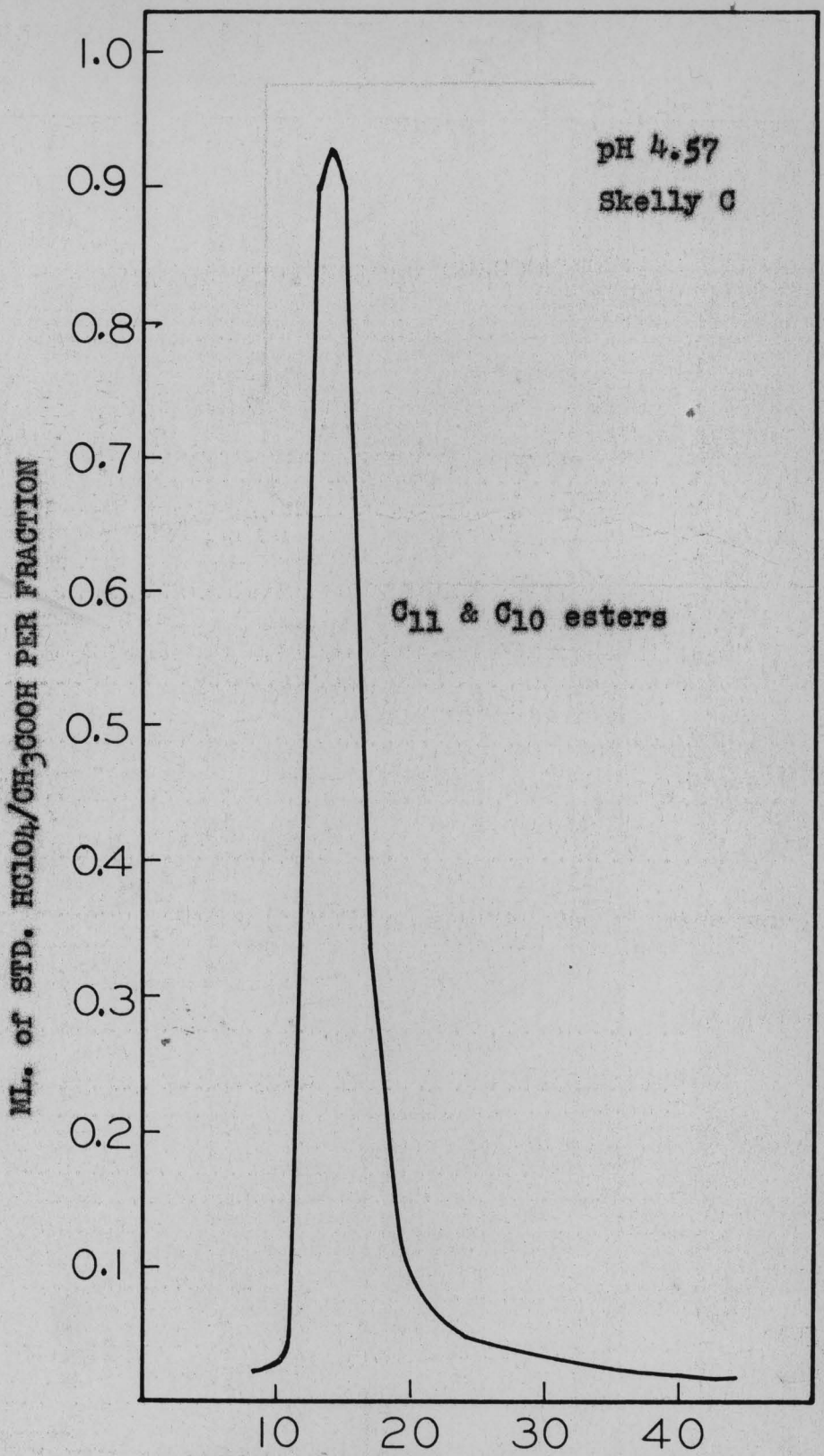

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ML. of STD. HClO4/CH3COOH PER FRACTION
pH 4.57
Skelly C
C11 & C10 esters
10
20
30
40
ELUATE FRACTIONS (3 Ml collected in 30 minutes)

Fig. 33.

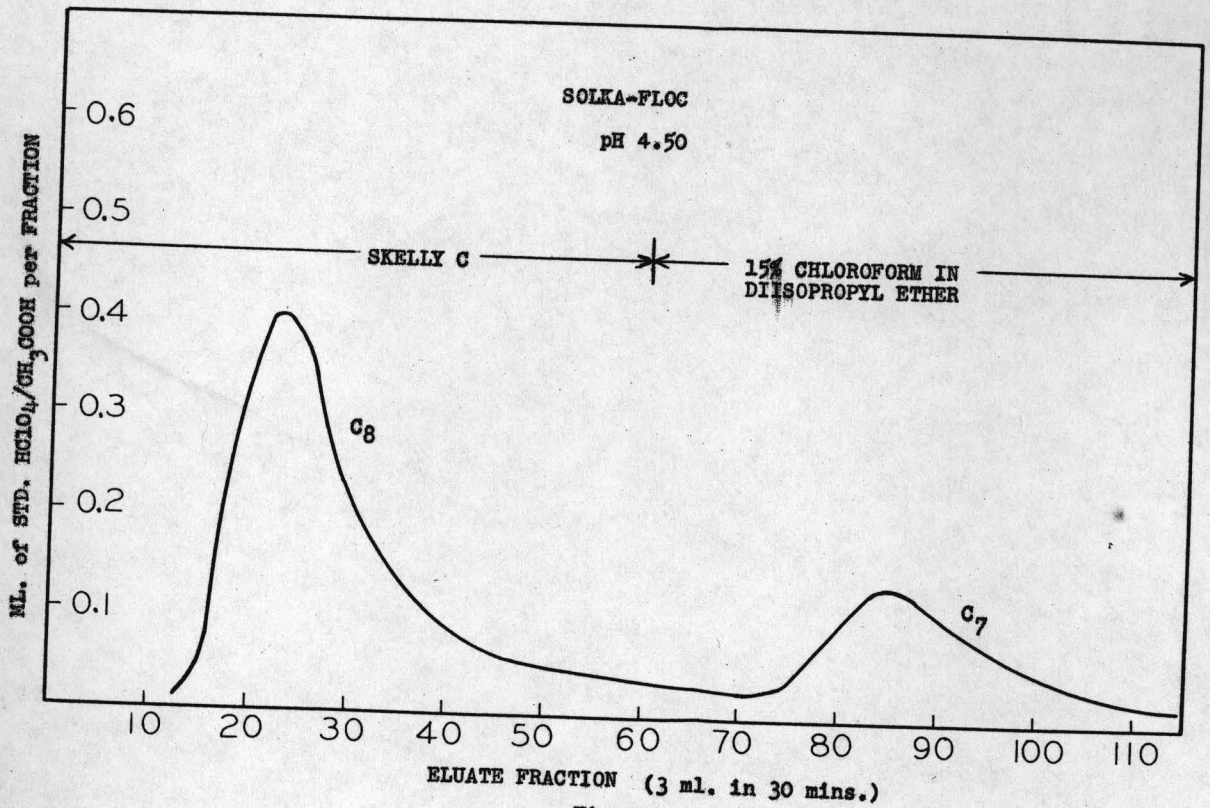

SOLKA-FLOC
pH 4.50
SKELLY C
15% CHLOROFORM IN DIISOPROPYL ETHER
ML. of STD. $HClO_4/CH_3COOH$ per FRACTION
0.6
0.5
0.4
0.3
0.2
0.1
C_8
C_7
10
20
30
40
50
60
70
80
90
100
110
ELUATE FRACTION (3 ml. in 30 mins.)

Fig. 34

Table 11

$$K = \frac{\text{aqueous}}{\text{organic}}$$

	pH	C_{12} ester	C_{11} ester
Citrate	4.28	4.24	6.58
	5.09	2.98	2.09
	5.28	0.92	0.85
	6.48	0.47	0.40
Phosphate	8.23	0.18	0.14
	8.99	0.16	0.14

this case was high because the esters were held in column strongly, it was decided to use pH 5.0. Sharp peaks were obtained with Skelly C (Fig. 35). Figure 36 shows the elution curves of C_7 and C_6 esters. As C_6 is strongly held in the column, also is more adsorbed. Therefore, 50:50 chloroform-diisopropyl ether was used to elute C_6 ester.

Figures 37 and 38 show two curves obtained when rates of movement of the solvent in both cases were different. Figure 37 shows the elution curves of C_6, C_7 and C_8 esters when 7 ml of eluant were collected in half an hour. Figure 38 shows when 5 ml of eluant were collected in half an hour. When the rate is fast, sharper peaks are obtained, less adsorption takes place, but there are possibilities that the separation might become worse. When the rate is slow, broader peaks are obtained, significant adsorption is observed, but separation is complete. So by trial and error, one has to find such a rate of flow of eluant that one gets sharpest peaks, yet separation is complete.

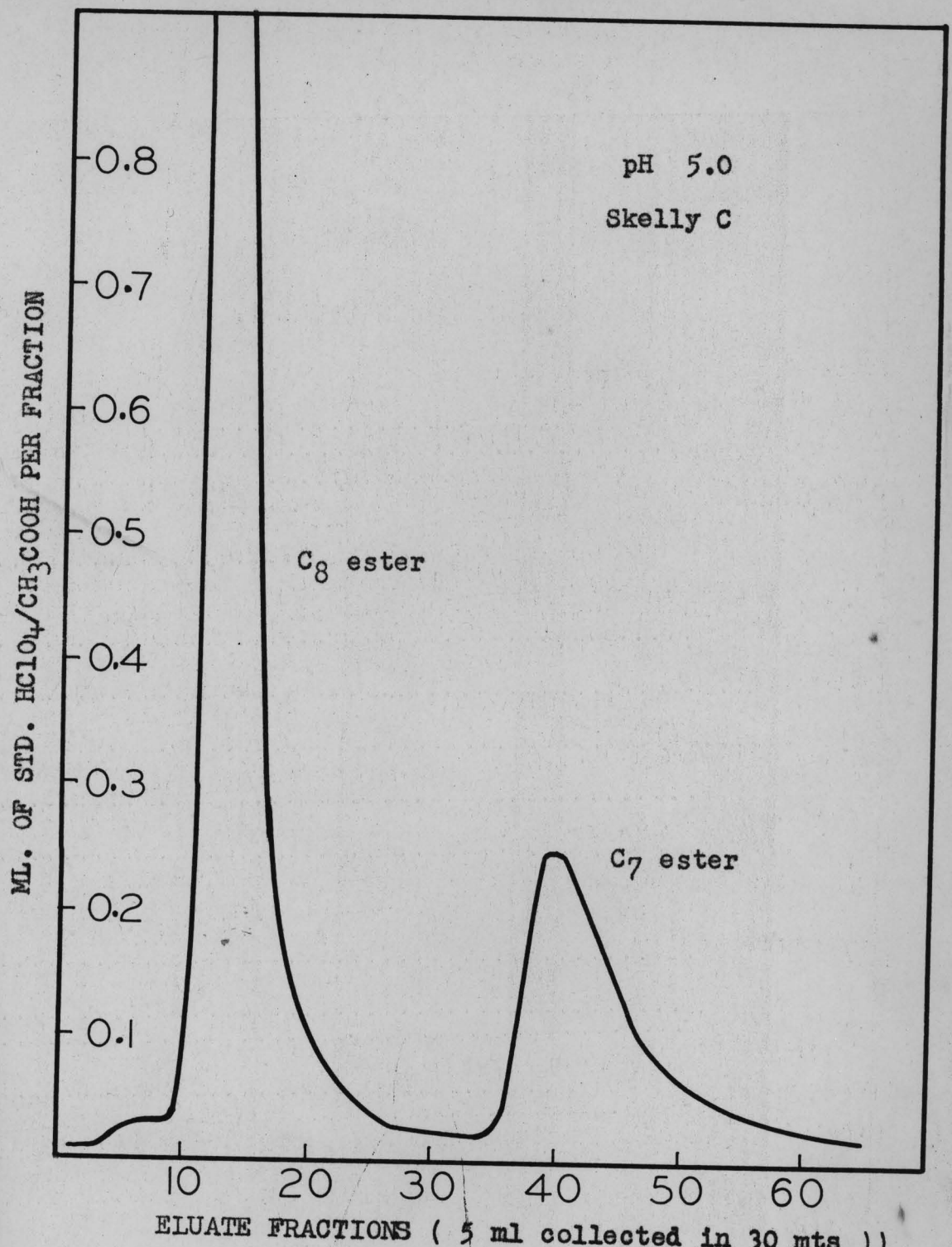

pH 5.0
Skelly C
C_8 ester
C_7 ester
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ML. OF STD. $HClO_4/CH_3COOH$ PER FRACTION
10
20
30
40
50
60
ELUATE FRACTIONS (5 ml collected in 30 mts))

FIG. 35

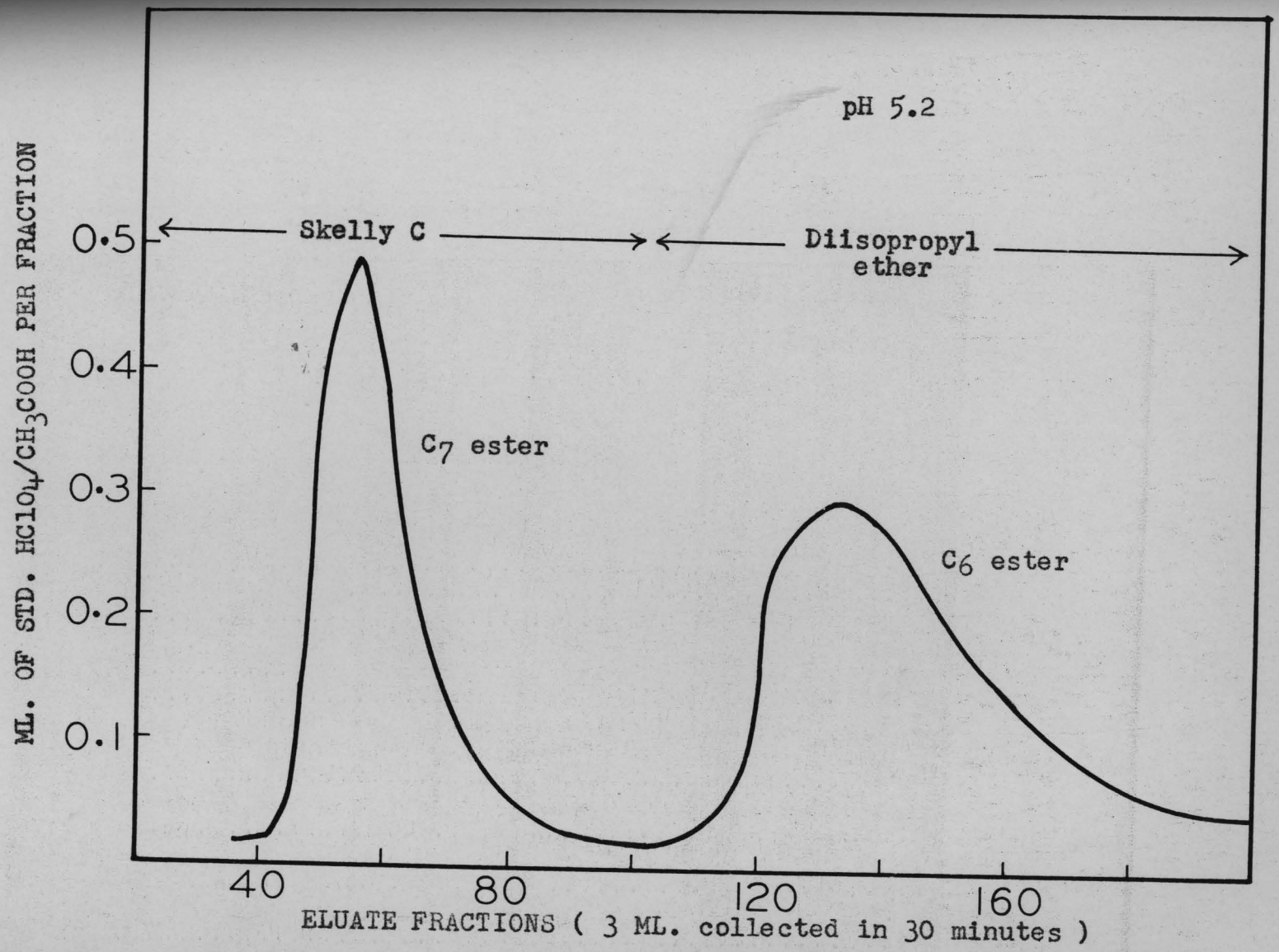

pH 5.2
Skelly C
Diisopropyl ether
C_7 ester
C_6 ester
ML. OF STD. $HClO_4/CH_3COOH$ PER FRACTION
0.5
0.4
0.3
0.2
0.1
40
80
120
160
ELUATE FRACTIONS (3 ML. collected in 30 minutes)

Fig.36 .

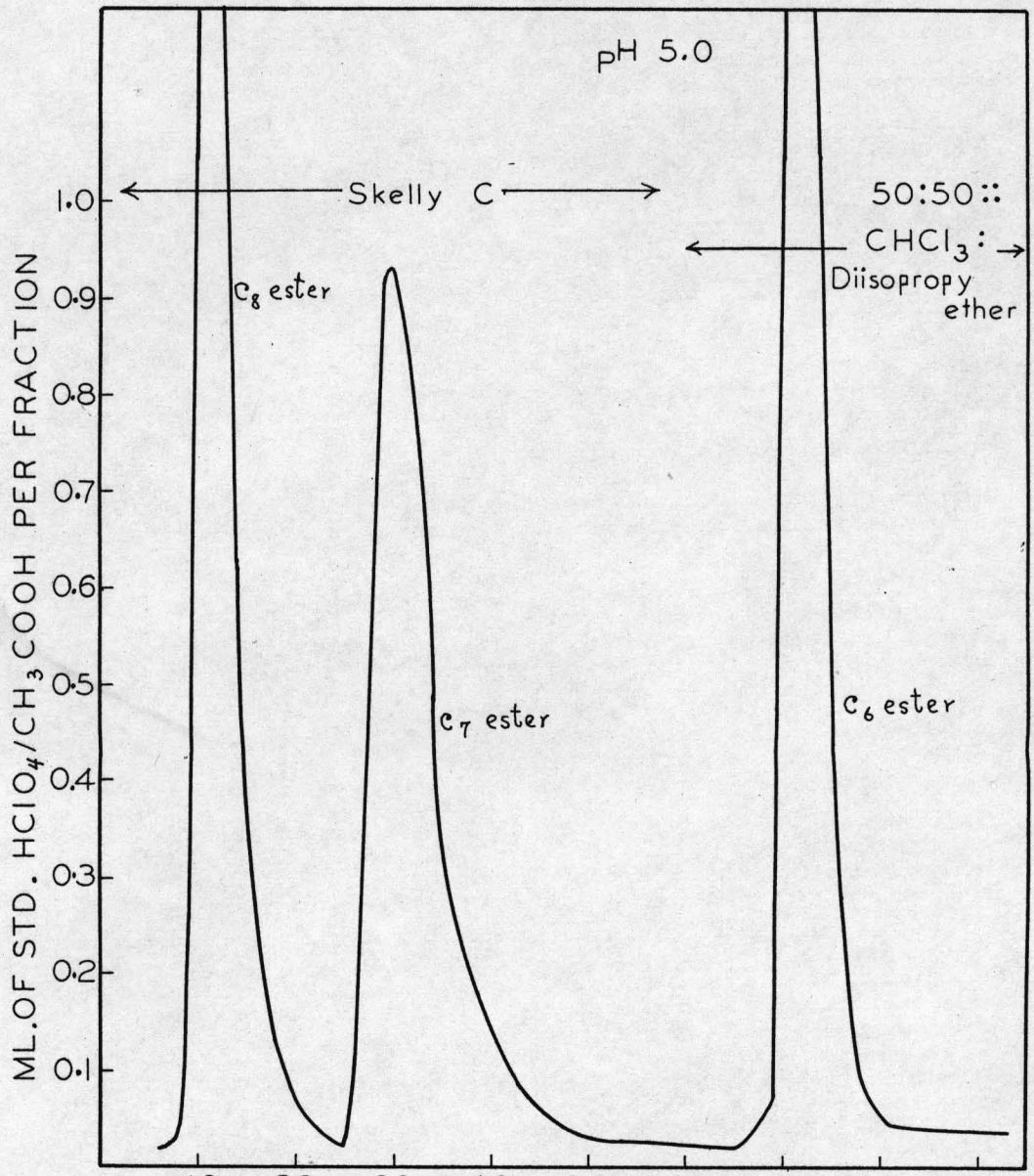

pH 5.0
Skelly C
50:50 ::
$CHCl_3$:
Diisopropy ether
C_8 ester
C_7 ester
C_6 ester
ML. OF STD. $HClO_4/CH_3COOH$ PER FRACTION
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
80
90
ELUATE FRACTIONS (7 ML. collected in 30 Minutes)

FIGURE 37

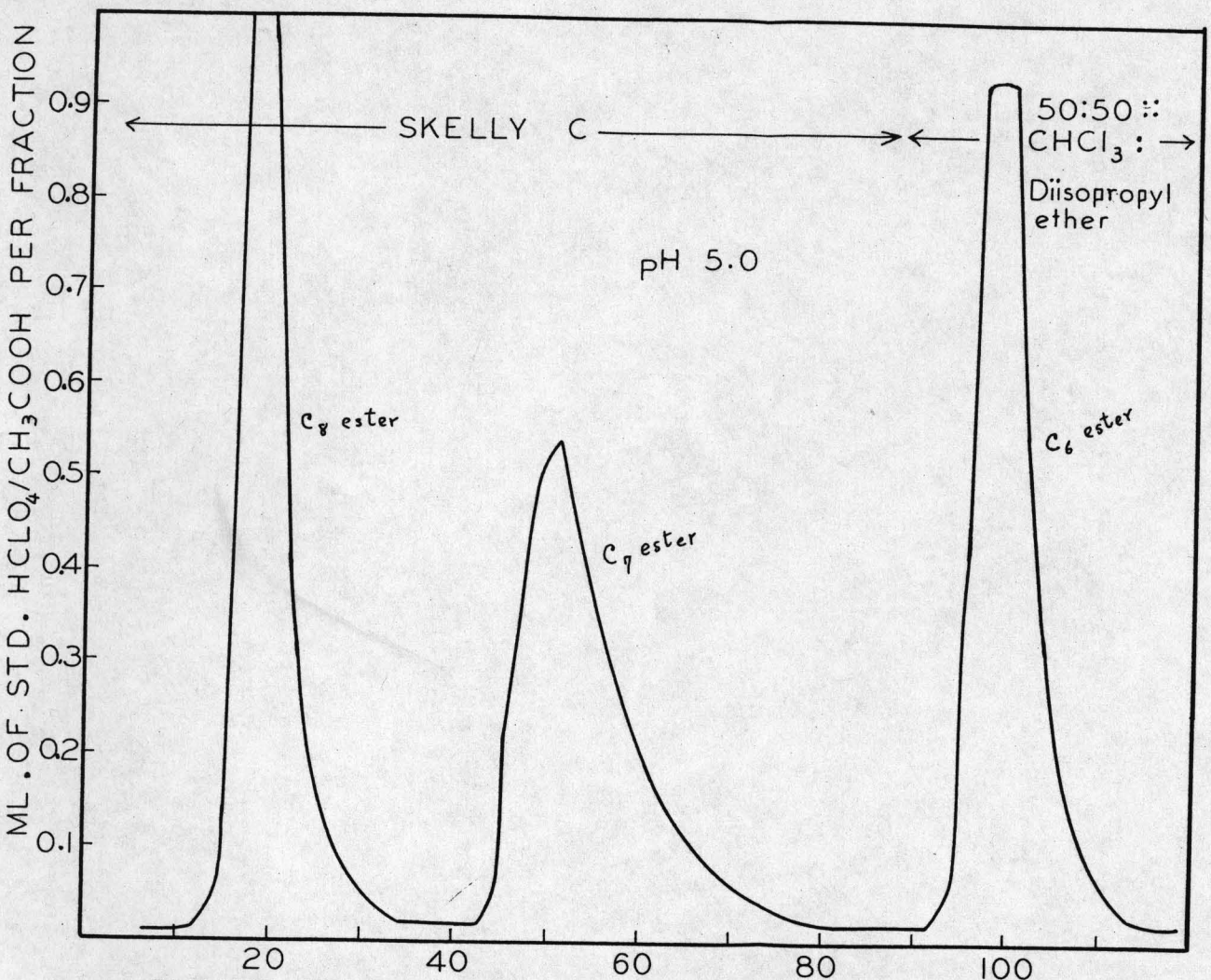

ML. OF STD. $HCLO_4/CH_3COOH$ PER FRACTION
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
SKELLY C
50:50 :: $CHCl_3$: →
Diisopropyl ether
pH 5.0
C_8 ester
C_7 ester
C_6 ester
20
40
60
80
100
ELUATE FRACTIONS (5 ML. COLLECTED IN 30 MINUTES)

FIGURE 38

3. Recommended Procedure for Analysis of Mixed Fatty Acids, C_6-C_8 or C_8-C_{11}.

The following method, on the basis of preceding discussion, is recommended for the analysis of mixed fatty acids, C_6-C_8 or C_8-C_{11}.

a. Preparation of reagents.

(1) Preparation of known solutions of fatty acids:

A separate solution of each fatty acid expected in the unknown is made up in Skelly C solvent, the solution to contain about 40 to 50 milligrams of the acid per milliliter. Standardization of each solution is carried out by titrating an aliquot portion of the fatty acid solution against standard alcoholic NaOH.

(2) Preparation of the unknown sample solution:

A solution in Skelly C of the unknown sample is made in such a manner that each milliliter contains about 0.2 to 0.25 milli-equivalents of the total acids.

(3) Preparation of thionyl chloride solution:

A solution of thionyl chloride is made in Skelly C, such that each milliliter of this solution contains 2 to 2.5 millimoles of thionyl chloride.

(4) Preparation of 1M bicarbonate buffer:

0.466 gram of Na_2CO_3 and 8.4 grams of $NaHCO_3$ is dissolved in 100 milliliters of distilled water.

(5) Preparation of 0.01 N perchloric acid in glacial acetic acid:

8.5 ml of 72% perchloric acid is mixed with 200 or 300 ml of glacial acetic acid and 20 ml of acetic anhydride. Dilute to one liter with glacial acetic acid and allow to stand overnight to per-

mit complete reaction of acetic anhydride with the water present. This solution of perchloric acid in glacial acetic acid is about 0.1 N. Ten milliliters of this solution is diluted to 100 milliliters with glacial acetic acid and standardized against potassium acid phthalate in glacial acetic acid as directed below.

About 0.05 gram of potassium acid phthalate is weighed accurately and added to 20 ml of glacial acetic acid. The mixture is refluxed gently a few minutes to effect solution. After cooling, methyl violet (0.2% in glacial acetic acid) is added and the solution is titrated with perchloric acid to the first disappearance of the violet tinge.

(6) Preparation of indicator:

Ten milligrams of benzenazodiphenylamine is dissolved in 100 ml of glacial acetic acid.

b. Procedure for carrying out the reaction.

Four milliliters of one known fatty acid solution or one milliliter of each solution of different fatty acids under investigation are pipetted directly into the cold-finger apparatus. Total acid concentration, therefore, will be 0.8 to 1.0 milliequivalent.

If the fatty acid solution is of unknown sample, four milliliters of unknown solution are pipetted into the cold-finger apparatus.

To the fatty acid solution in cold-finger apparatus, four milliliters of thionyl chloride solution is added. The apparatus is closed by means of the condenser and is dipped into an oil-bath, heated electrically, at 60° C. The reaction is allowed to proceed for approximately 3 to 4 hours. After this time, the cold-finger

COLD-FINGER APPARATUS

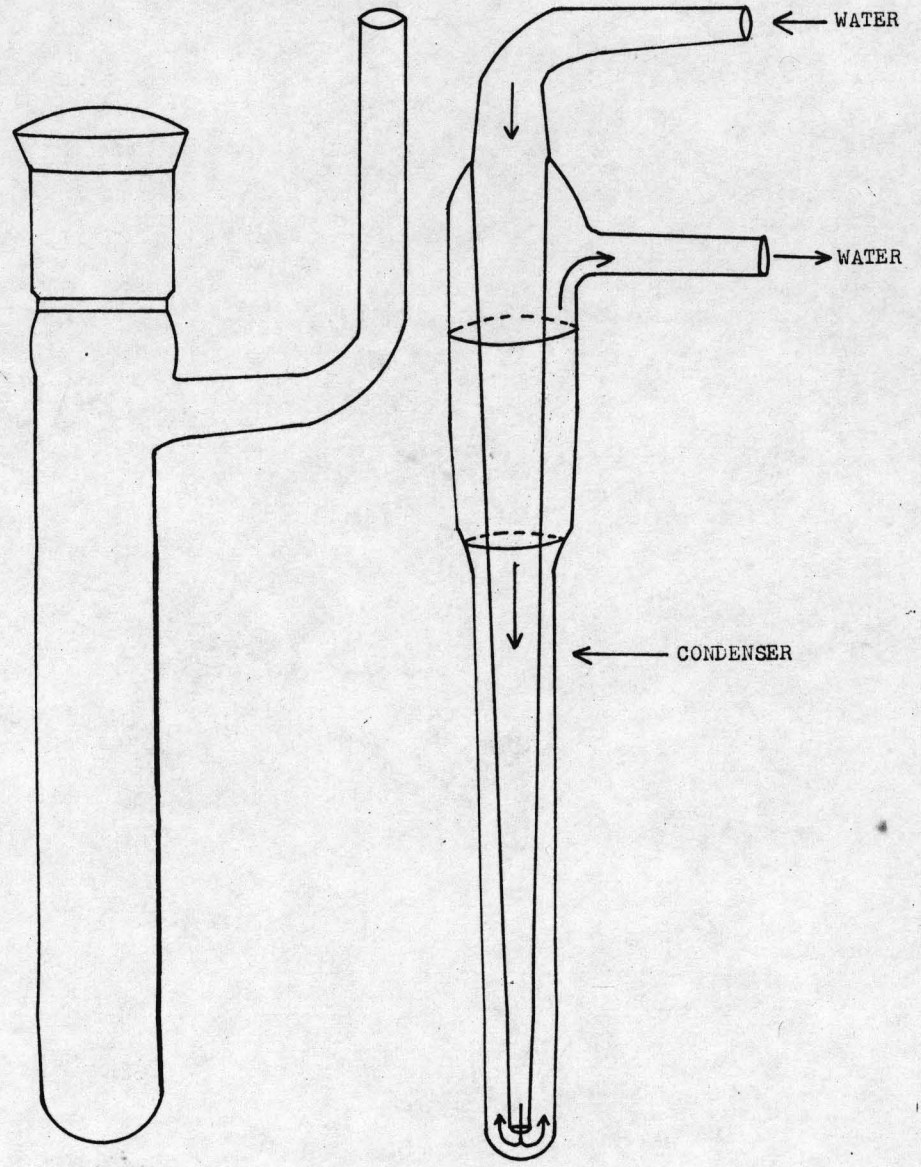


Fig. 39

apparatus is taken out of the oil-bath and is cooled by dipping it into the cold water. The condenser is slowly lifted up and simultaneously washed by about two milliliters of Skelly C. There is no danger of acid chlorides being volatalized because the boiling points of these compounds are generally around 200° C. 5.2 ml of dimethylaminoethyl alcohol is added slowly to the reaction mixture, with cooling and swirling to avoid any local overheating.

After about fifteen minutes, 5 ml of 1 M bicarbonate buffer is added and the whole reaction mixture is shaken. When the two layers separate, upper Skelly C layer which contains dimethylamino ethyl esters of the fatty acids, is pipetted out with a long medicinal dropper into a 25 ml volumetric flask which already contains 5 ml of distilled water. About two milliliters of pure Skelly C is added to the reaction mixture, the whole reaction mixture is shaken by swirling, and upper layer is again pipetted with the long medicinal dropper into the volumetric flask. Extractions are continued until 25 ml are made up. These 25 ml consist of 20 ml of Skelly C solution of esters of fatty acids and 5 ml of water. Five ml of Skelly C solution is used for the chromatographic purpose.

c. Chromatography of the sample.

(1) Apparatus:

20 mm pyrex chromatographic column, 45 cm long is used. A close-fitting glass plunger is employed in packing the columns.

(2) Reagents:

Silicic acid, chromatographic grade (Mallinckrodt Co.)

Solka-floc, BW 200 (Brown Co.)

1 M citrate buffers, pH 4.50; and pH 5.00

Freshly distilled diisopropyl ether

Chloroform (analytical reagent grade)

Skelly C

Glacial acetic acid.

(3) Packing of the columns:

(a) For higher fatty acid esters:

Thirty grams of silicic acid and 30 ml of 1 M citrate buffer, pH 4.50, are thoroughly worked together in a beaker with a test tube or a thick glass-rod. Roughly about 200 ml of Skelly C are added and a homogeneous slurry formed by vigorous stirring. This slurry is packed incrementwise into the column, care being taken to prevent formation of air pockets or other forms of heterogeneity. Each increment is packed down, uniformly quite firmly with the plunger.

(b) For lower fatty acid esters:

The procedure is the same as preceding except 30 grams of Solka-floc and 30 ml of 1 M citrate buffer, pH 5.00, are used instead of silicic acid and pH 4.50 buffer respectively. In packing each increment, care should be taken not to squeeze water out of Solka-floc, as it is easy to squeeze water out by applying high pressures in packing. Pressure is applied only up to the point where water is seen being squeezed out.

(4) Sample addition and elution:

In both the cases, 5 ml sample of the Skelly C solution of esters is pipetted into respective columns. Sample is allowed to run through completely and then 2 ml of pure Skelly C solvent is

pipetted in, washing the sides of the column down. After this portion is completely absorbed, column is set up on the fraction collector and different fractions are collected in test tubes.

In case of higher fatty acid esters, dimethylaminoethyl undecylate is eluted out with Skelly C, dimethylaminoethyl caprate is eluted out with freshly distilled diisopropyl ether, dimethylaminoethyl nonanoate is eluted out with 15% chloroform in diisopropyl ether, dimethylaminoethyl octanoate is eluted out with 50% chloroform in diisopropyl ether.

As a modification of above elution process, if heptanoic acid is present, then dimethylaminoethyl octanoate is eluted out with ½% phenol in benzene and dimethyl heptanoate is eluted out with 50% chloroform in diisopropyl ether.

In case of lower fatty acids, dimethylaminoethyl octanoate and heptylate both are eluted out with Skelly C and dimethylaminoethyl hexanoate is eluted out with 50% chloroform in diisopropyl ether.

As a modification of above procedure, if valeric acid is present, then dimethylaminoethyl hexanoate is eluted out with diisopropyl ether and dimethylaminoethyl valerate is eluted out with pure chloroform.

(5) Analysis of the eluates:

To each test tube in the eluate, equal amount of glacial acetic acid is added. This mixture is titrated with standard perchloric acid solution in glacial acetic acid, using 0.01% solution of benzeneazodiphenylamine as indicator. Nitrogen gas is bubbled through in each tube for stirring purposes only.

(6) Calculations:

Readings of all the fractions containing only one ester are added together. Blank for each is subtracted. Then, say X ml of 0.01 N $HClO_4$/HAc are required for one ester,

then, $\frac{X \times 4 \times 0.01 \times 100}{Y}$ = % recovery

where Y = millimoles of the acid used.

The above relationship is true because on mole of acid will give one mole of ester.

4. Results and Discussion.

Results obtained by analyzing three synthetic mixtures of C_6, C_7 and C_8 fatty acids by recommended procedure are shown in Table 12. Percentage yield reported here is the ratio of the ester recovered after chromatography to the acidity in the starting sample of fatty acid used. Reproducible results have been obtained.

Table 13 shows the composition of nonanoic sample used throughout the investigation of this report. Table 14 shows the result of one synthetic mixture of C_8-C_{11} fatty acids. Although the results are in harmony with the results obtained when each individual acid was esterified and chromatogrammed (Tables 6-9), further studies of this procedure are necessary.

In the separation of esters of mixed fatty acids, only partition effect is supposed to take place. It was found, however, that significant adsorption of esters on silicic acid also took place. Due to this adsorption effect, C_{12} ester was not separated from C_{11} ester. Attempts to minimize the adsorption effect were not successful. Even though Solka-floc was found less adsorptive than silicic acid, further studies about none or weakly adsorptive supports are highly desirable.

As the partition coefficient values of amino esters of fatty acids are found to differ by a factor of four to every one carbon atom increase in chain length, it would be possible to separate the amino esters by counter-current distribution. In counter-current distribution, there would be no adsorption effect encountered, as in chromatography.

Table 12

Analysis of Synthetic Mixtures of Lower Fatty Acids

Run no.	Components of mixture	Meq. acid added	Meq. ester recovered	% Recovery
1	*Octanoic acid	0.2783	0.2822	101.3
	*Heptanoic acid	0.3078	0.2866	93.2
	*Hexanoic acid	0.3582	0.3290	92.0
2	Octanoic acid	0.2783	0.2800	102.0
	Heptanoic acid	0.3078	0.2864	93.3
	Hexanoic acid	0.3582	0.3316	92.5
3	Octanoic acid	0.2783	0.2824	101.8
	Heptanoic acid	0.3078	0.2896	94.0
	Hexanoic acid	0.3582	0.3264	91.3

*Eastman Kodak, White Label Grade

Table 13

Nonanoic Acid
(Refluxed at 60°, 5 hours)

Run no.	Components of mixture	Meq. acid reacted	Meq. ester recovered	% Recovery
1	Undecanoic acid	0.2464	none	-
	Decanoic acid		none	-
	Nonanoic acid		0.1604	65.0
	Octanoic acid		0.0378	15.4

Table 14

Analysis of One Synthetic Mixture
(at 60° C, 5 hours)

Run no.	Components of mixture	Meq. acid reacted	Meq. ester found	% Recovery
1	Undecanoic acid	0.2054	0.2074	101.0
	Decanoic acid	0.3056	0.3100	101.0
	Nonanoic acid	0.2464	0.1680	68.1
	Octanoic acid	0.2783	0.3064	110.0

VI. GENERAL CONCLUSION

On the basis of the preceding discussions and experimental results, the following conclusions seem to be warranted.

1. The method of Ramsey and Patterson for analysis of mixed higher fatty acids is improved by the addition of a water-soluble organic acidic agent to the internal phase.

2. A new method of partition chromatography based on the use of ion-exchange resins both as mechanical support and as buffer has been developed.

3. It has been shown that dimethylaminoethyl esters of C_8, C_9, C_{10}, and C_{11} acids can be synthesized in quantitative yields by the method described above.

4. These esters can be separated completely on a silicic acid column using, as the internal phase, 1 M citrate buffer at pH 4.50. The column is eluted by a succession of organic solvents.

5. The esters of the lower fatty acids C_6, C_7, and C_8 have been separated on Solka-floc columns. The internal phase was 1 M citrate buffer at pH 5.00. The external phase was Skelly C and 50% chloroform-diisopropyl ether.

6. Adsorption of esters on silicic acid and Solka-floc has been minimized by the addition of dimethylaminoethyl alcohol to the internal phase.

VII. SUMMARY

Several different approaches were made in attempts to develop better methods of analyses of saturated higher fatty acids. Studies were made of separations based on (1) modifications of Ramsey and Patterson's method; (2) partition chromatography on ion-exchange resins; and (3) conversion of fatty acids to amines.

Somewhat better separations of mixtures of higher even numbered carbon atom fatty acids was achieved when furfuryl alcohol, 2-amino-pyridine, glacial acetic acid buffer was replaced in Ramsey and Patterson's method. Successive members of higher homologues of saturated fatty acids were not separated.

The essential feasibility of using ion-exchange resins for general partition chromatographic purposes has been demonstrated by complete separation of mixtures of lower dicarboxylic acids. The separation of higher saturated fatty acids on ion-exchange columns was, however, unsuccessful, apparently due to the surface active property of higher fatty acids.

Dimethylaminoethyl derivatives of fatty acids have been prepared. Preliminary studies showed the conversion of fatty acids to amino esters to be essentially quantitative. Partition coefficients of the esters were determined between aqueous buffer, pH 4.6, and diisopropyl ether, and from these values theoretical calculations for the elution of different esters were made. Although the theoretical calculations based on strictly partition process did not check well with the experimental results obtained on silicic acid columns (because of somewhat concurrent adsorption) they were

helpful in the development of proper solvent combinations.

Two methods of separation of fatty acid esters have been developed: (1) Esters of C_8-C_{11} acids were separated on silicic acid column. (2) Esters of C_6-C_8 acids were separated on Solka-floc columns. Significant adsorption of esters occurred on silicic acid and somewhat less on Solka-floc. Attempts to minimize this adsorption by adding dimethylaminoethyl alcohol and methyl alcohol in the internal phase were not fruitful. Although the separation achieved was complete and could be carried out with small amounts of fatty acids up to C_{11} acid, the tailing effect due to the adsorption process hindered the separation of acids of high molecular weight.

APPENDIX

STUDIES ON SEPARATION AND ANALYSIS OF HIGHER DICARBOXYLIC ACIDS BY PARTITION CHROMATOGRAPHY

An analytical procedure (1) for separation and determination of C_4-C_{10} dicarboxylic acids by partition chromatography has been reported in the literature. This method is being used for control purposes in industry. Attempts were made in our laboratories to extend this method to determination of higher straight-chained dicarboxylic (C_{10}-C_{14}) acids.

To test the feasibility of this approach, partition coefficients of C_{10}, C_{11}, C_{13}, and C_{14} dicarboxylic acids were determined between 5% n-butanol in chloroform and buffers of different pH. Results are shown in Table I and Figure 1. As expected, a slope of 2 was obtained. In case of C_{13} and C_{14} dicarboxylic acids, pH range was too low to give reliable data, since the determinations were carried out in the systems in which the acids were nearly all in the organic layer.

Figure 2 shows the elution curves of C_{10}, C_{11}, C_{13} and C_{14} dicarboxylic acids when internal phase was 1 M phosphate buffer, pH 6.4 on silicic acid and external phase was 5% n-butanol in chloroform. Instead of four different peaks, only three peaks have been obtained. This means two acids have come out together. From partition coefficients data, it was clear that separation between C_{13} and C_{14} dicarboxylic was not possible. Therefore, the first peak is composed of C_{13} and C_{14} dicarboxylic acids, the second peak is

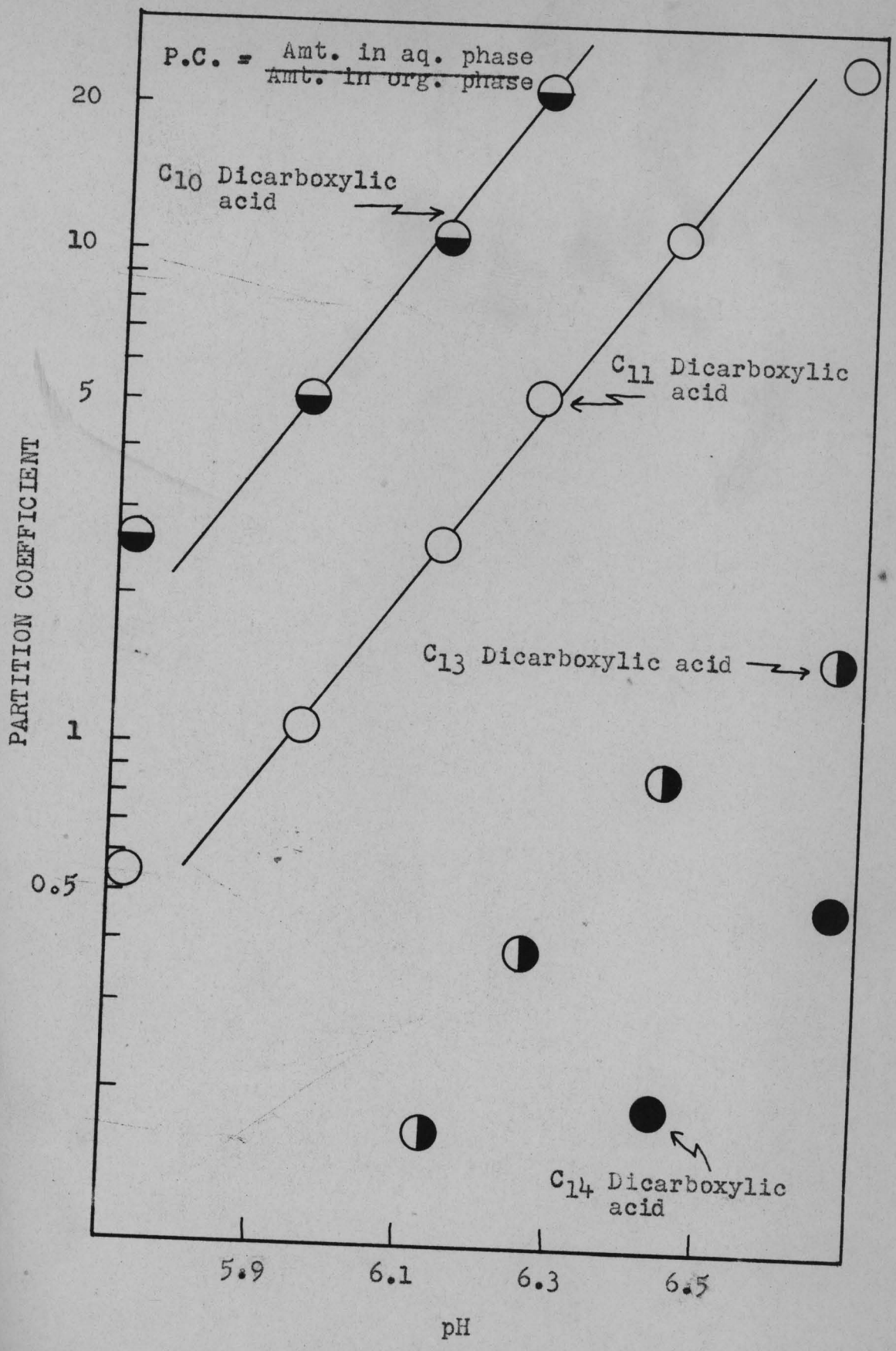

P.C. = Amt. in aq. phase / Amt. in org. phase
20
10
5
1
0.5
PARTITION COEFFICIENT
C10 Dicarboxylic acid
C11 Dicarboxylic acid
C13 Dicarboxylic acid
C14 Dicarboxylic acid
5.9
6.1
6.3
6.5
pH

Fig. 1

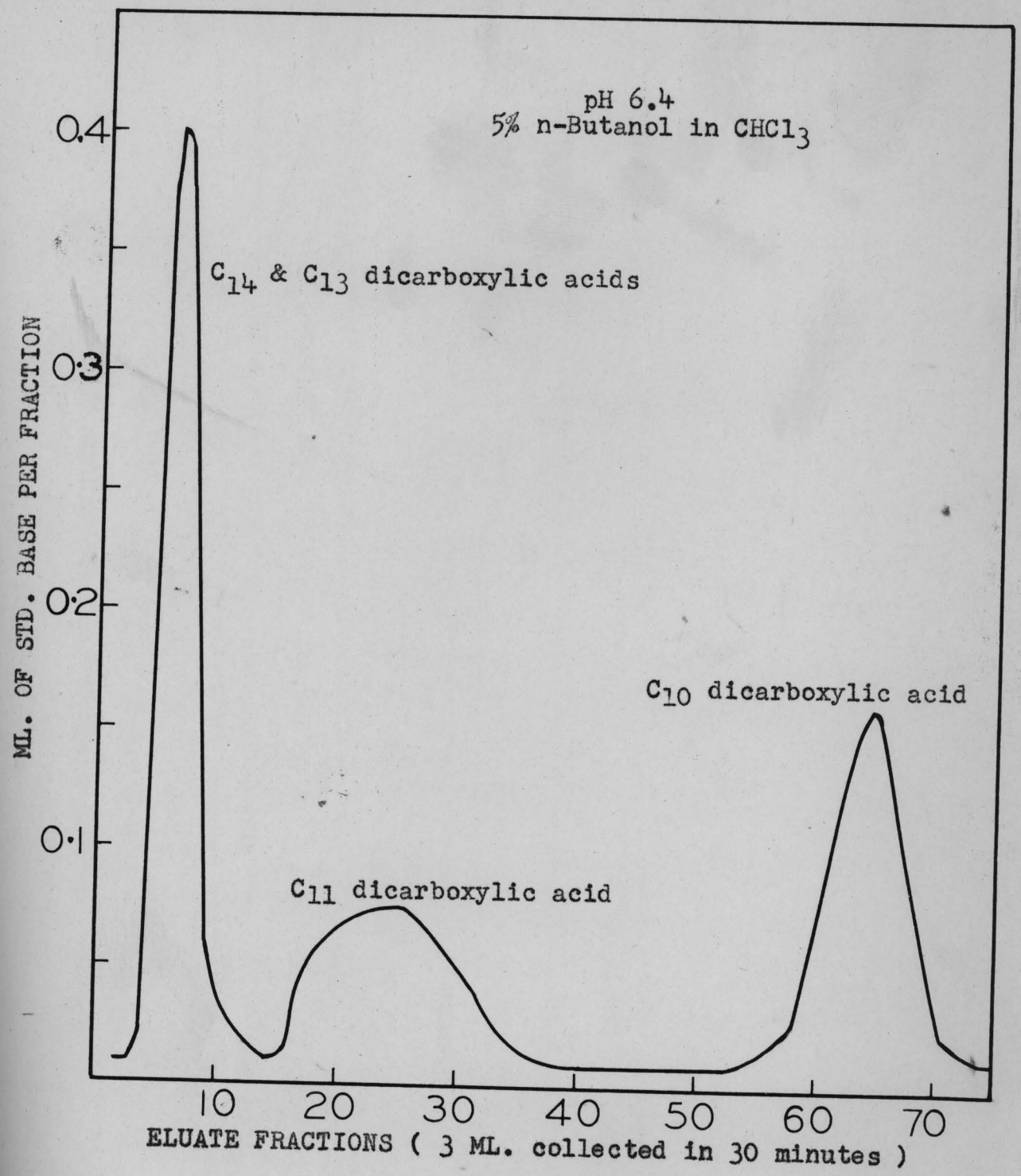

pH 6.4
5% n-Butanol in $CHCl_3$
C_{14} & C_{13} dicarboxylic acids
C_{11} dicarboxylic acid
C_{10} dicarboxylic acid
ML. OF STD. BASE PER FRACTION
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
ELUATE FRACTIONS (3 ML. collected in 30 minutes)

Fig. 2.

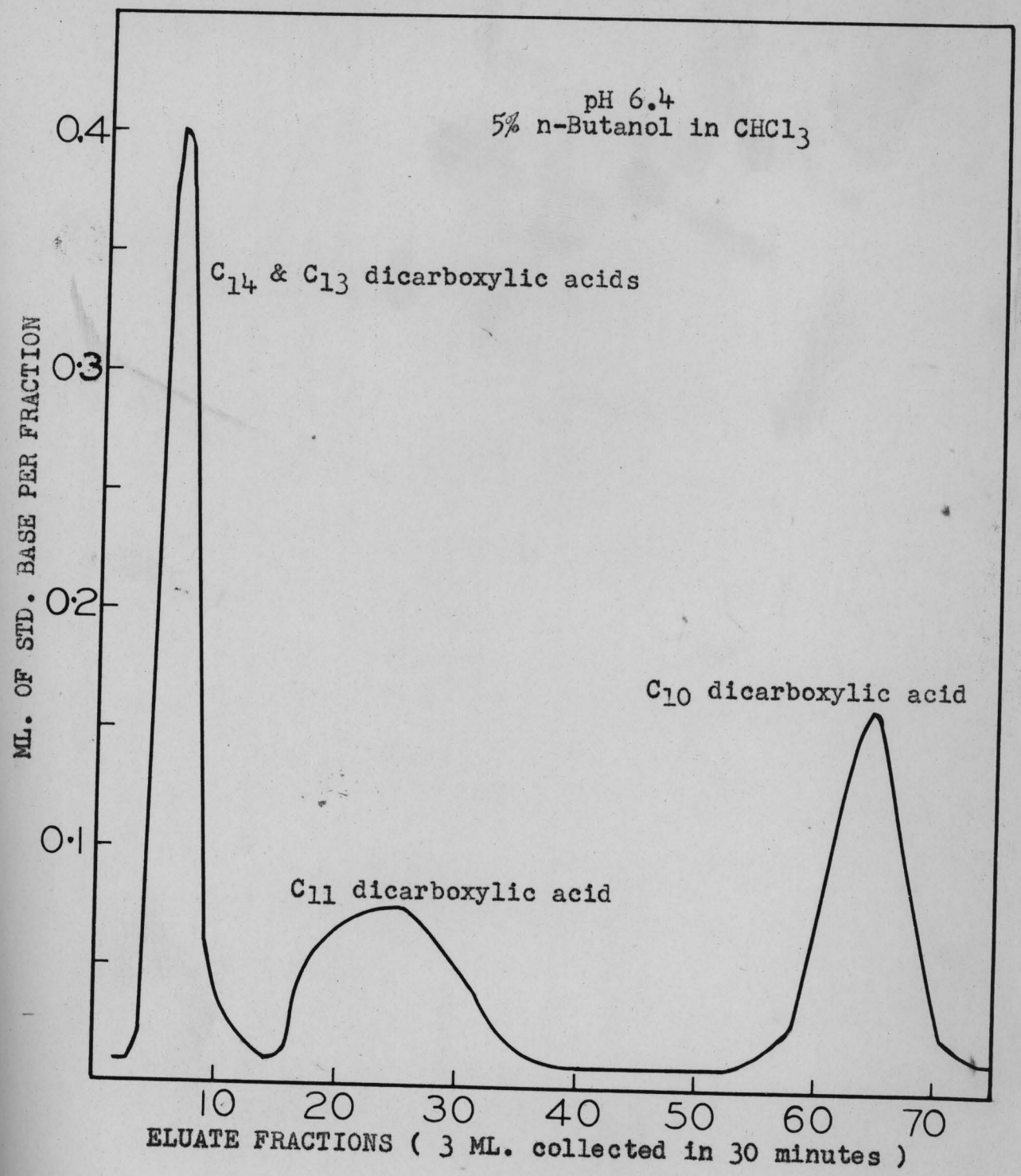

pH 6.4
5% n-Butanol in $CHCl_3$
C_{14} & C_{13} dicarboxylic acids
C_{11} dicarboxylic acid
C_{10} dicarboxylic acid
ML. OF STD. BASE PER FRACTION
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
ELUATE FRACTIONS (3 ML. collected in 30 minutes)

Fig. 2.

TABLE I

PARTITION COEFFICIENTS OF DICARBOXYLIC ACIDS

BETWEEN DIFFERENT pH AND 5% BUTANOL-$CHCl_3$

pH	Sebacic Acid			C_{11} dicarboxylic acid			C_{13} dicarboxylic acid			C_{14} dicarboxylic acid		
	low conc.	high conc.	Average K*	low conc.	high conc.	Average K*	low conc.	high conc.	Average K*	low conc.	high conc.	Average K*
5.72	2.8	2.4	2.6	0.53	0.56	0.55						
5.95	5.4	4.9	5.1	1.04	1.16	1.10						
6.13	11	11	11	2.5	2.7	2.6	0.17	0.17	0.17			
6.26	20	24	22	5.1	5.6	5.3	0.46	0.34	0.40			
6.44				12	11	11.5	0.97	0.81	0.89	0.20	0.19	0.19
6.67				26	23	24.5		1.60	1.60	0.50	0.50	0.50
7.01							2.38		2.38	1.24		1.24

* $K = \frac{\text{Conc. in aqueous phase}}{\text{Conc. in organic phase}}$

that of C_{11} dicarboxylic acid, and the third one is that of C_{10} dicarboxylic acid.

Separation between C_{14} and C_{13} could be achieved either by raising the pH of internal phase or by decreasing the polarity of the external phase. Determination of partition coefficient values of C_{14} and C_{13} dicarboxylic acids between 7.01 pH buffer and 5% n-butanol-$CHCl_3$ gave the values as 1.24 and 2.38, respectively (Table I). This means separation should take place at pH 7.00 by eluting with 5% n-butanol-chloroform.

Figure 3 shows the elution curves for C_{10}, C_{11}, C_{13} and C_{14} dicarboxylic acids when pH of internal phase was 7.01 and external phase was different percentages of n-butanol in chloroform for different acids. There was no separation between C_{14} and C_{13} dicarboxylic acids.

The difficulty was found to have resided in the addition of sample. Sample was usually prepared in 5% t-amyl alcohol-$CHCl_3$ solution. Usually 5 ml sample was used. Now when sample in 5% alcohol-chloroform solution was added on the top of the column, C_{14} and C_{13} being too soluble in 5% alcohol were eluted out right away, although 1%, 2%, or 3% butanol-chloroform solvents were used as eluants. That sample of 5 ml containing 5% t-amyl alcohol-$CHCl_3$ had enough polarity to elute out C_{14} and C_{13} acids together. This was found out when samples were prepared in pure $CHCl_3$, and only two milliliters of sample were used, so that a sharp band might form on the top of the column.

Figure 4 shows the chromatogram obtained when internal phase was pH 7.00, 1 M phosphate buffer, eluant was pure chloroform and

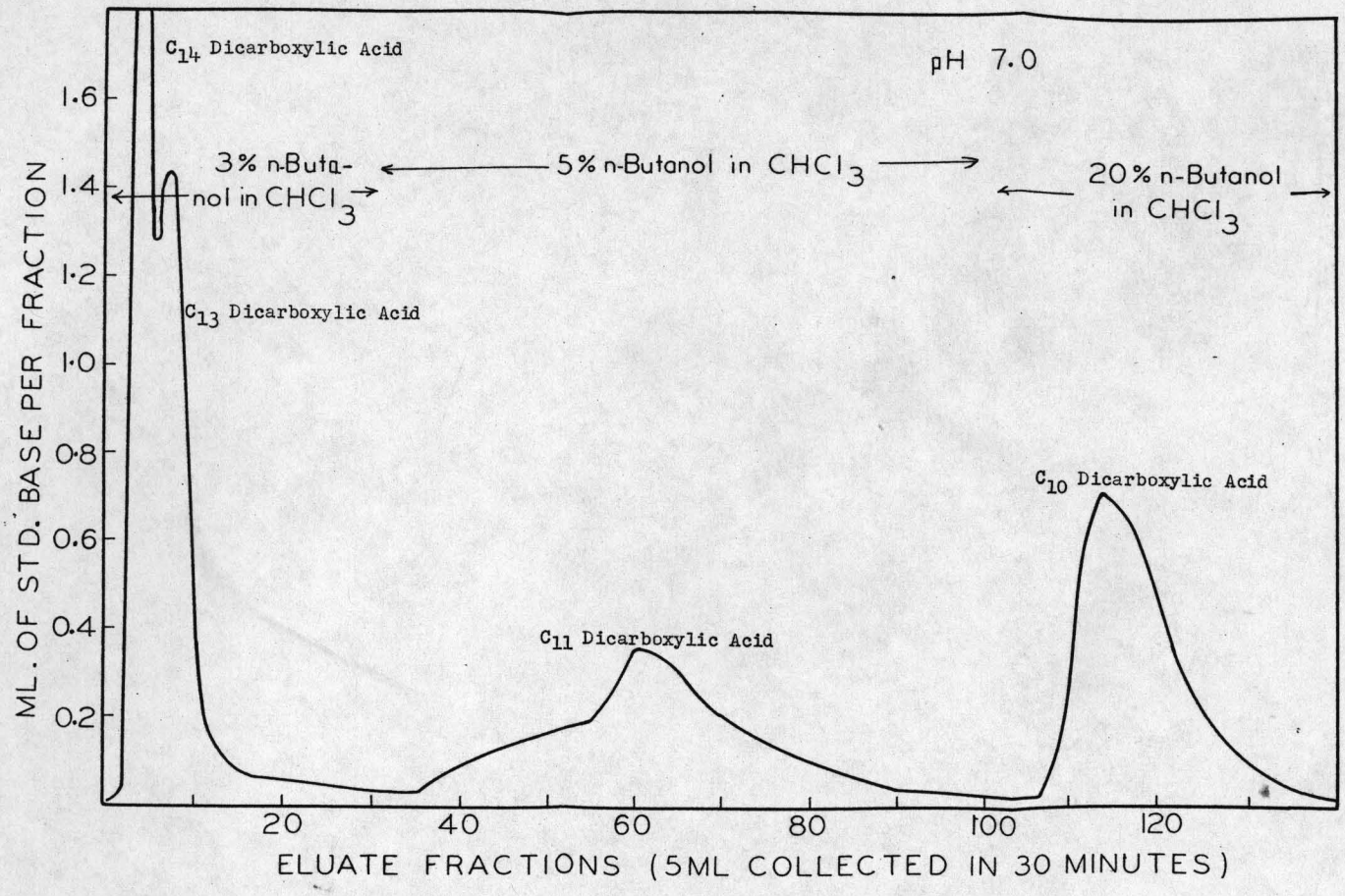

C_{14} Dicarboxylic Acid
pH 7.0
3% n-Butanol in $CHCl_3$
5% n-Butanol in $CHCl_3$
20% n-Butanol in $CHCl_3$
C_{13} Dicarboxylic Acid
C_{10} Dicarboxylic Acid
C_{11} Dicarboxylic Acid
ML. OF STD. BASE PER FRACTION
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
20
40
60
80
100
120
ELUATE FRACTIONS (5ML COLLECTED IN 30 MINUTES)

FIGURE 3

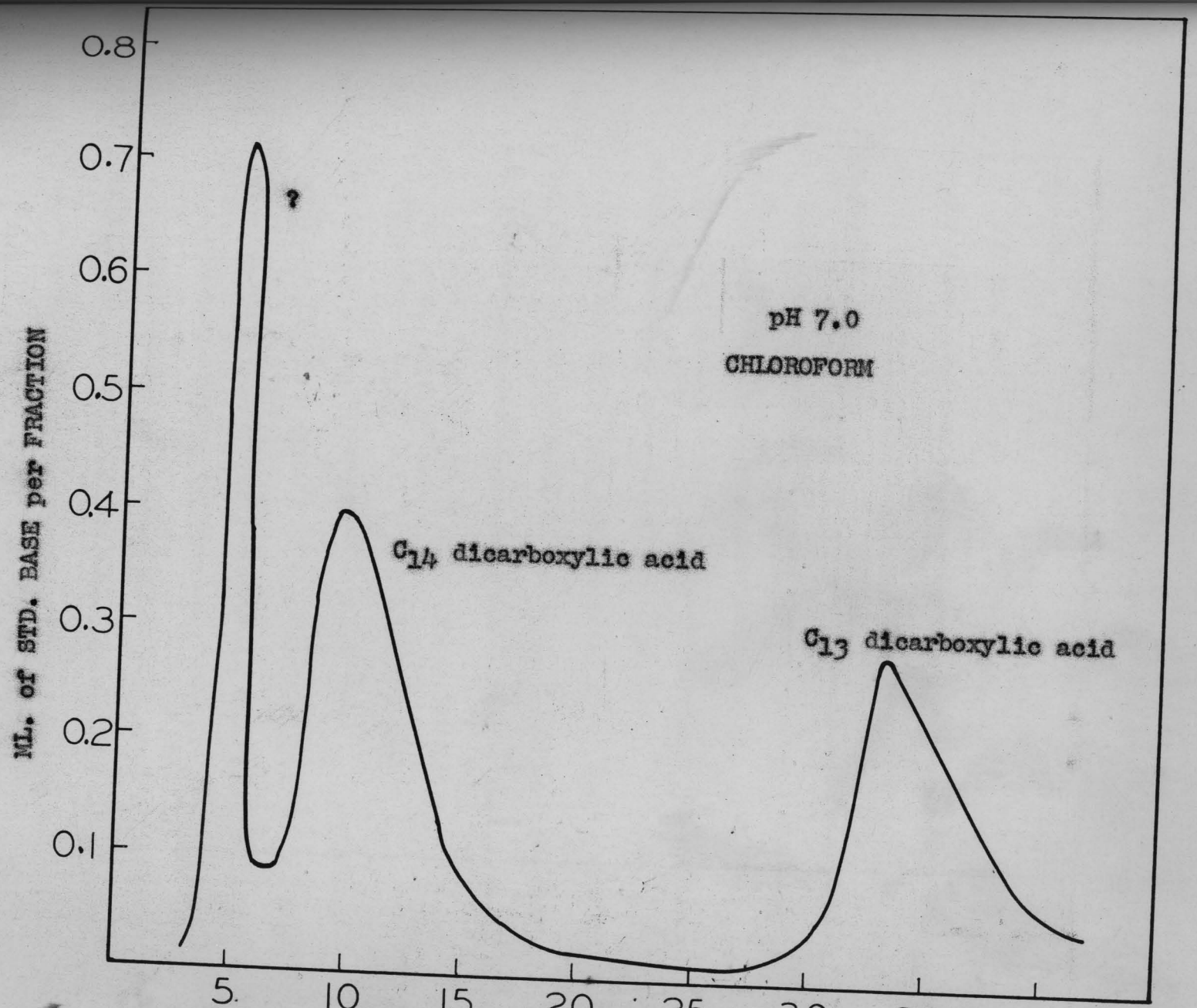


Fig.4. ELUATE FRACTIONS (5 Ml collected in 25 minutes)

sample was added in pure chloroform. It has certainly resolved the mixture of C_{14} and C_{13} acids, but a new peak appeared this time.

If this new peak would not have appeared, it would have been easier to separate C_{14}, C_{13}, C_{11} and C_{10} dicarboxylic acid using pH 7.00 buffer on silicic acid and adding sample in pure chloroform, but evidently this new peak should be investigated first.

This new peak in front of C_{14} dicarboxylic acid could be C_{15} dicarboxylic acid impurity or it might be a monoester of either C_{13} or C_{14} dicarboxylic acid with t-amyl alcohol. Characterization of all three peaks were carried out as follows:

Three acids from three peaks were extracted with diethyl ether after acidification of the fractions containing respective acids and their melting points were determined. Results are as follows:

1st peak: Not enough . ˙. could not be determined.
2nd peak: 115-119° C.
3rd Peak: 90-102° C.

Melting point of C_{14} dicarboxylic acid was found to be 116-121° C, therefore second peak was that of C_{14} dicarboxylic acid. Melting point of C_{13} dicarboxylic acid was found to be 70-100° C, therefore, the third peak was that of C_{13} dicarboxylic acid.

All three acids were rechromatogrammed on three different columns and Figure 5 shows the curves obtained. This proves that all the three acids are different molecular species. Second and third peaks are C_{14} and C_{13} dicarboxylic acids, respectively, but as the melting point of the first was not determined because of insufficient material, it could not be decided.

Considering the recoveries of C_{14} and C_{13} dicarboxylic acids, only 75% of C_{14} acid was recovered, while 96% of C_{13} dicarboxylic

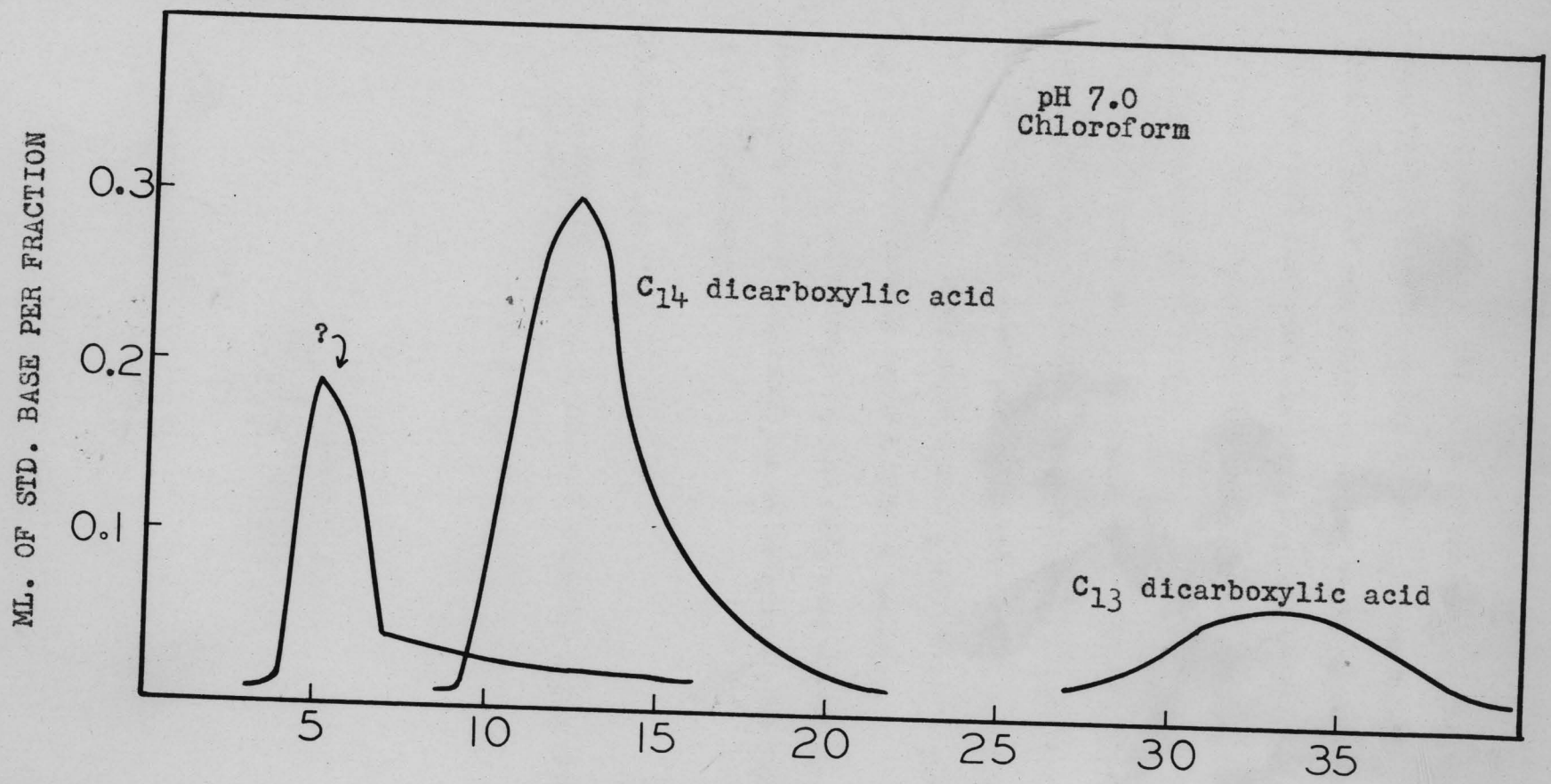

pH 7.0
Chloroform
0.3
0.2
0.1
?
C_{14} dicarboxylic acid
C_{13} dicarboxylic acid
5
10
15
20
25
30
35
ML. OF STD. BASE PER FRACTION
ELUATE FRACTIONS (5 ML.)

Fig. 5.

acid was recovered, therefore if the acid in the first peak is monoester at all, it should be of C_{14} dicarboxylic acid mainly. Calculation of recoveries on weight basis shows that the amount in the first peak plus the amount in the second peak exceeds 100% by 75%. Therefore, the first peak could not be monoester of C_{14} dicarboxylic acid.

This leaves only one alternative; that the first peak could be C_{15} dicarboxylic acid. Calculating as C_{15} dicarboxylic acid, the amount in the first peak plus the amount in the second peak is around 100%. This shows that C_{14} dicarboxylic sample used had C_{15} dicarboxylic acid (around 25%) as impurity.

However, no attempt has yet been made either to separate C_{15} dicarboxylic acid completely or to determine quantitative recoveries of all four acids.

References: —

(1) Higuchi, T., Hill, N. C., and Cororan, G. B., Anal. Chem., 24, 491 (1952).

BIBLIOGRAPHY

(1) Ramsey, L.L. and Patterson, W. I., J. Assoc. Official Agri. Chem., 31, 441 (1948).

(2) Epstein, A.K., Harris, B.R. and Katzman, M., Soc. Exptal. Biol. Med. 53, 238 (1943).

(3) Wikoff, H.L., Kaplan, J.M. and Berman, A.L., J. Biol. Chem. 153, 227 (1944).

(4) Thannahauser, S., Setz, P. and Benetti, J., J. Biol. Chem. 126, 785 (1938).

(5) Kirchner, J. G., Prata, A.N. and Haagen-Smit, A.J., Ind. Eng. Chem., Anal. Ed., 18, 31 (1946).

(6) Hilditch, T.P. "The Chemical Constitution of Natural Fats" Wiley.

(7) Longenecker, H.E. and Mattil, K.F., Oil and Soap 21, 16 (1944).

(8) Longenecker, H.E. and Baldwin, A.R., Oil and Soap 22, 151 (1945).

(9) Mitchell, J.H., Kraybill, H.R. and Zscheille, F.P., Ind. Eng. Chem. Anal. Ed. 15, 1 (1943).

(10) Markley, K.S. "Fatty Acids" p. 583.

(11) Gusserow, C.A., Arch. Pharm. 27, 153 (1828).

(12) Twitchell, E., J. Ind. Eng. Chem. 13, 806 (1921).

(13) Kerr, R.H., Ind. Engr. Chem. 8, 904 (1916).

(14) Thomas, A.W. and Yn, C.L., J. Amer. Chem. Soc. 45, 113 (1923).

(15) Voorhies, S.T. and Bauer, S.T., Oil and Soap 20, 175 (1943).

(16) Brown, J.B. and Stoner, G.C., J. Amer. Chem. Soc., 59, 3 (1937).

(17) Kistler, R.E., Muckerheide, V.J. and Meyers, L.D., Oil and Soap 23, 146 (1946).

(18) Jamieson, G.S., Vegetable Oils and Fats, p. 400.

(19) Hilditch, T.P., "The Chemical Constitution of Natural Fats" Wiley.

(20) "Official and Tentative Methods, Amer. Oil Chem. Soc. 1946.

(21) Wyman, F.W. and Barkenlens, C., Ind. Eng. Chem. Anal. Ed. 12, 658 (1940).

(22) Norris, F.A. and Terry, D.E., Oil and Soap 22, 41 (1945).

(23) Strain, H.H., "Chromatographic Adsorption Analysis, 1942.

(24) Zechmeister, L. and Cholnoky, L., "Principles and Practice of Chromatography", 1941.

(25) Zechmeister, L. and Cholnoky, L., "Progress in Chromatography", 1941.

(26) Cassidy, H.G., "Adsorption of Chromatography", 1951.

(27) Graff, M.M. and Skau, E.L. Ind. Eng. Chem. Anal. Ed. 15, 340 (1943).

(28) Cassidy, H.G., J. Amer. Chem. Soc. 63, 2735 (1941).

(29) Papp, G. and Othmer, D.F., Ind. Eng. Chem. Ind. Ed. 36, 430 (1944).

(30) Manunta, C., Helv. Chim. Acta 22, 1156 (1939).

(31) Mazumdee, B.K. and Goswami, M.N., Indian Soap J. 12, 227 (1947).

(32) Dutton, H.J., J. Phys. Chem. 48, 179 (1944).

(33) Dutton, H.J. and Reinhold, C.L., J. Amer. Oil Chem. Soc. 25, 120 (1948).

(34) White, M.F. and Brown, J.B., J. Amer. Chem. Soc. 70, 4269 (1948).

(35) Reimenschneider, R.W., Herb, S.F. and Nichols, P.F., J. Amer. Oil Chem. Soc. 26, 371 (1949).

(36) Farmer, E.H. and Sutton, D.A., J. Chem. Soc. 139 (1942).

(37) Hilditch, T.P. and Atherton, D., J. Chem. Soc. 105 (1944).

(38) Bergstrom, S., Nature 156 (717) (1945).

(39) Kaufman, H.P. and Schmidt, O., Fetti u Seifen 47 294 (1940).

(40) Claesson, S., Arkiv. Kemi, Mineral Geol. 15H, No. 9 (1941).

(41) Holman, R.T. and Hagdahl, L. Arch. Biochem. 17, 301 (1948).

(42) Holman, R.T. and Williams, W.T., J. Amer. Chem. Soc. 73, 5285 (1951).

(43) Tiselins, A. and Hagdahl, L., Acta Chim. Scand. 4, 394 (1950).

(44) Hagdahl, L. and Holman, R.T., J. Amer. Chem. Soc. 72, 701 (1950).

(45) Hagdahl, L. and Holman, R.T., J. Biol. Chem. 182, 421 (1950).

(46) Holman, R.T., J. Amer. Chem. Soc. 73, 1261 (1951).

(47) Holman, R. T., J. Amer. Chem. Soc. 73, 5289 (1951).

(48) Smith, E.L., Biochem J., 36, XXII (1942).

(49) Elsden, S.R., Biochem. J. 40, 252 (1946).

(50) Neish, A.L., Can. J. Research 27B, 6 (1949).

(51) Nijkamp, H.J., Chem. Weekblav 45, 480 (1949).

(52) Scarisbrie, R., Baldwin, E. and Moyle, V., Biochem. J. 43, 308 (1948).

(53) Scarisbrie, R., Baldwin, E. and Moyle, V., Biochem. J. 42, XIV (1948).

(54) Fairbairn, D. and Harpur, R.P., Nature 166, 789 (1950).

(55) Gray, F.R., Pilgrim, A.F., Rodda, H.J. and Weller, R.A., Nature 167, 954 (1951).

(56) Ramsey, L.L., J. Assoc. Official Agr. Chem. 31, 164 (1948).

(57) Bueding, E. and Yale, H.W., J. Biol. Chem., 193, 411 (1953).

(58) Ramsey, L.L. and Patterson, W.I., J. Assoc. Official Agr. Chem., 31, 139 (1948).

(59) Peterson, M.H. and Johnson, M.J., J. Biol. Chem. 174, 775 (1948).

(60) Jarvis, F.G. and Johnson, M.J., J. Amer. Chem. Soc. 71, 4124 (1949).

(61) Bumpus, F.M., Taylor, W.R. and Strong, F.M., J. Amer. Chem. Soc. 72, 2116 (1950).

(62) Peterson, M.H. Johnson, M.J. and Price, W.V., J. Dairy Science 32, 862 (1949).

(63) James, A.T. and Martin, A.J.P., Biochem. J. 50, 679, (1952).

(64) El-Shazly, K., Biochem. J., 51, 640 (1952).

(65) Boldingh, J., Rec. trav. Chim. 69, 247 (1950).

(66) Howard, G.A. and Martin, A.J.P., Biochem. J. 46, 532 (1950).

(67) Brown, F., Biochem. J. 47, 598 (1950).

(68) Brown, F. and Hall, L.P., Nature 166, 66 (1950).

(69) Hiscox, E.R. and Berridge, N.J. Nature 166, 522 (1950).

(70) Kennedy, E.P. and Barker, H.A., Anal. Chem. 23, 1033 (1951).

(71) Reid, R.L. and Lederer, M., Biochem. J. 50, 60 (1952).

(72) Fink, K. and Fink, R.M., Proc. Soc. Exp. Biol. Med. 70, 654 (1949).

(73) Thompson, A.R., Australian J. Sci. Res. 134, 180 (1951).

(74) Move Y and Noda, M., J. Agr. Chem. Soc. Japan 18, 294 (1950).

(75) Boldingh, J., Experimentia 4, 270 (1948).

(76) Boldingh, J., Rec. trav. Chim. 69, 247 (1950).

(77) Kaufmann, H.P. and Budwig, J., Fette u Seifen 53, 408 (1951).

(78). Claesson, S., Arkiv Kenn Min Geol. 23A No. 9.

(79) Craig, L.C., "Technique of Organic Chem." Vol. III, p.171.

(80) Martin, A.J.P. and Synge, R.L.M., Biochem. J. 35, 1358 (1941).

(81) Ward, A.F.H. and Tordai, L., Rec. trav. Chim. 71, 482 (1952).

(82) Toshi, S. and Minorn, O. Kagaku, 20, 187 (1950) thru C.A. 46, 3360 h.

(83) Higuchi, T. et al, Anal. Chem., 24, 491 (1952).