1962

Glyceride study of lard before and after molecular rearrangement

Avery Dean Milloy
Iowa State University

GLYCERIDE STUDY OF LARD BEFORE AND AFTER MOLECULAR REARRANGEMENT

by

Avery Dean Milloy

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Chemical Engineering

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1962

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

INTRODUCTION

Lard enjoyed the position of being the premium shortening until vegetable shortenings slowly started taking over in the 1920's and 1930's. Vegetable shortening manufacturers found that hydrogenated vegetable oils were superior to lard for certain culinary uses, especially in the baking of cakes. These facts were made known to the homemaker and commercial bakers in such a convincing manner that hydrogenated vegetable oil shortenings soon cut deeply into the sales of lard as a shortening. Vegetable oil shortenings soon surpassed lard in sales and have continued to do so until the present time.

This competition in the shortening field caused considerable research to be conducted to determine why lard should be inferior to hydrogenated vegetable oil shortenings and what could be done to improve lard for culinary purposes.

Hawley and Holman (29) give the following four shortcomings of natural lard as compared to good hydrogenated vegetable shortenings:

1. a poor oxidative stability, creating off-flavor problems in storage and in high temperatures uses such as frying and pastry;
2. a grainy, translucent appearance and texture that is unattractive compared to the smooth, white appearance of vegetable shortenings and causes performance

difficulties in some baking uses;

3. a poor plastic range, that is, it usually is too soft at warm temperatures and too hard at cold temperatures; and
4. non-uniformity in consistency and flavor, which can vary with the season, area, and rendering method.

Oxidation stability was improved through the use of antioxidants and by partial hydrogenation. Random interesterification of lard can cure the graininess problem. The poor plastic range can be improved by the addition of 9-12% completely hydrogenated fat (usually cottonseed oil) to the randomized fat or better yet according to Hawley and Holman (29), by directed interesterification in which the amount of trisaturated glycerides is increased and the amount of disaturated monounsaturated glycerides decreased.

Rearranged lard is formed (4) by heating lard at a temperature of anywhere just above its melting point to about 250°C in the presence of a catalyst. There are many possible catalysts of which the alkali metal derivatives are the most effective. A commonly used catalyst in the laboratory is sodium methoxide. If the temperature is high enough to insure maintaining a single phase reaction mixture, the rearrangement is random. That is, the fatty acid radicals move from site to site in the mixture in a completely random manner until the catalyst is killed by addition of a weak acid solution or

water depending upon the catalyst used. Therefore a random fatty acid distribution on all the possible sites is obtained. But if the temperature is allowed to decrease below the melting point of the trisaturated glycerides, the trisaturated glycerides are removed from the reaction phase by crystallization and therefore the reaction is directed toward the removal of the saturated acids from the reaction phase. This removal of the trisaturated glycerides from the reaction phase causes the relative amounts of GS_2U, disaturated-monounsaturated; GSU_2, monosaturated-diunsaturated; and GU_3, triunsaturated glycerides to change and the final glyceride structure of the fat is different for the directed rearrangement than for the random rearrangement. Hawley and Holman (29) point out that the plastic range of lard is greatly affected by the amount of disaturated glycerides remaining in lard as they give little or no heat resistance to the shortening because their melting point is approximately that of room temperature.

By directed rearrangement of lard, a product is obtained which compares well with hydrogenated vegetable shortenings in all respects. One of the major problems of lard producers today is to convince the housewife and the bakers that the modified lard is as good as the vegetable shortenings. As early as in 1953, modified lard was being put on the market to compete with hydrogenated vegetable shortenings as is pointed out by Slater (87) in that a plant for producing a

randomly rearranged lard is described as being in operation by Armour and Company. In 1957, Placek and Holman (71) reported that their directly interesterified lard, Fluffo, was second in sales only to their own all vegetable shortening, Crisco, and also describe an all purpose shortening being sold under the tradename of Pertex and another product for baking cakes which contains monoglycerides and goes under the tradename of Selex. Both of the latter products are directly interesterified lard products produced by Procter and Gamble.

In the course of developing modified lard, one of the most important questions was what makes the rearranged lard better than natural lard. One way to answer this question might lie in obtaining complete chemical composition of the lard and the glyceride composition before and after rearrangement. The difficulty here, of course, lies in that there are not adequate analytical methods today to obtain completely such data. The chemical composition (glycerol and the various fatty acid radicals) can be determined by methods already developed and in common usage, but the glyceride composition determination before and after molecular rearrangement is an entirely different matter. The methods of glyceride analysis are very limited and at best, only a rough estimation can be made as to the types of glycerides present.

The purpose of this study was to use the best methods available today to determine the glyceride structure of lard before and after molecular rearrangement.

LITERATURE REVIEW

A natural fat may be defined as a mixture of mixed triglycerides. The problem here is therefore the analysis of the triglyceride mixtures before and after rearrangement. There has been a considerable amount of work on glyceride analysis but these studies have included the mono-, di-, and triglycerides. The main concern here is with only the triglycerides.

Hilditch (38) gives a general review of the development of the methods of glyceride studies from which some of the main developments described by him are presented here.

Early Developments

It was observed by Chevreul in 1823 that natural fats were glycerol esters of fatty acids and it was assumed that the glycerides were simple triglycerides. In 1897, Heise, and in a few years later, Kilmont, Bomer and others started studies which showed that natural fats were not simple triglycerides and that simple triglycerides were the exception rather than the rule.

The first reports of attempts to separate and isolate glycerides by low temperature crystallization appeared in the early 1900's. The work often consisted of elaborate crystallization procedures which in the case of Bomer actually involved many hundreds of crystallizations. This work resulted

In the isolation of the major triglyceride constituents from fats made up of a limited number of fatty acids. It is interesting to note that Bomer and his collaborators recorded the isolation of two palmitostearins from lard in 1913 by these methods and in 1923, Amberger and Wieshahn obtained similar results for lard and in addition reported 2% oleodistearin and 11% oleopalmitostearin.

Between 1920 and 1930, fractional crystallization techniques were employed after either hydrogenating or brominating the unsaturated portion of the fat. The results were qualitative in nature and did little more than to help confirm the generalization that natural fats are mixtures of mixed triglycerides and that simple triglycerides are very seldom found naturally.

From about 1927 on, the major contributions in the field of the glyceride study of fats came out of the laboratory of Hilditch. Hilditch summarizes this work by listing the methods of investigation developed at Liverpool as having evolved as follows:

I. Determination of the fully saturated glycerides present in natural fats.

II. Determination of the triunsaturated C_{18} glycerides (oleic, linoleic, etc.) in fats:

(a) by estimation of the tristearin content of the completely hydrogenated fat;

(b) by study of the glyceride composition of the fat after hydrogenation to varying extents.

III. (a) More detailed examination of the component glycerides in solid seed or animal fats by separating the latter into fractions varying in solubility in acetone, each fraction being examined separately for its fatty acid composition and, if desired, its fully saturated glyceride content, content of triunsaturated C_{18} glycerides, etc.

(b) Similar examination of liquid vegetable or animal fats by systematic crystallization from acetone or ether at suitable temperatures down to -70°C, each fraction being examined separately for its fatty acid composition.

Chemical Oxidation

A method for the determination of the fully saturated glycerides in a fat was worked out by Hilditch and Lea (35) in which a fat is oxidized in an acetone solution by the addition of powdered potassium permanganate until all the unsaturated carbon to carbon double bonds have been severed. The saturated fatty acid radicals are unaffected by this treatment and therefore the trisaturated glycerides are unaffected by the oxidation. It was found that the trisaturated glycerides could be separated from the azelaoglycerides by washing

with alkali if proper precautions were taken.

This chemical oxidation method was modified somewhat by Kartha (45) who added acetic acid to the reaction mixture along with the potassium permanganate to maintain a slightly acidic solution during the oxidation so that the potassium hydroxide formed during the reaction was neutralized and therefore not free to cause hydrolysis of the azelaoglycerides as Kartha claimed was taking place in the Hilditch and Lea procedure. Kartha also worked out a method of separation of the oxidation products by taking advantage of the difference of the solubility of their magnesium salts. In this method, the trisaturated glyceride content must be determined by an independent method, usually by crystallization, and the results of the method are an estimation of each of the four major types of glycerides present in the fat; GS_3, GS_2U, GSU_2, and GU_3 where the G, S, and U represent the glycerol radical, saturated fatty acid radical, and unsaturated fatty acid radical respectively.

Progressive Hydrogenation

Of the studies in which a series of progressively hydrogenated fats was prepared and studied to determine the component glycerides of the fat, the study which is of interest here is that of Hilditch and Stainsby (37). Here the fat used was the inner back fat of a sow (component acids:

myristic 2.8, palmitic 27.3, stearic 14.4, oleic 40.9, linoleic 13.5, unsaturated C_{20-22} 1.1 mol percent). The original inner back fat contained 5.6 mol percent of fully saturated components (component acids: plamitic 54.0, stearic 46.0 mol percent). Crystallization of the fully saturated glycerides produced in the hydrogenated fats gave 2-palmitodistearin (m.p. 67.0-67.5°C), in amounts corresponding to 80 percent or more of the total palmitodistearin present in these fully saturated portions of the fat. That the original pig fat therefore contained 2-palmitoglycerides was confirmed later by Meara (59).

Low Temperature Crystallization

When solid carbon dioxide became available for common usage in the laboratory, low temperature fractional crystallization techniques were developed to a much higher degree than the isolation techniques used by the early workers. Again Hilditch and his collaborators were the leaders in the field. In general, the methods worked out were the fractionation of a fat into several fractions starting out as low as -70°C in some cases and obtaining fractions at successively higher temperatures by crystallization of the lower melting glycerides in either acetone or ether solutions. Each of the fractions obtained was further investigated for its fatty acid composition. By assuming that each fraction contained no more than two of the four major glyceride types, the overall

glyceride composition in terms of GS_3, GS_2U, GSU_2, and GU_3 was determined. In cases in which the number of component fatty acids in the fat was relatively few, the individual component glycerides of a fat were stated with some degree of precision. But in the more common situation in which the number of fatty acids was relatively greater, the results were less definite. Hilditch and Pedelty (36) investigated pig perinephric and back fat and obtained the results given in Table 1.

Table 1. Fatty acid composition and major glyceride type composition of pig depot fat by Hilditch and Pedelty (37)

	Pig	
	Perinephric	Back
Saturated fatty acids (mol %)		
Lauric	0.5	---
Myristic	1.3	1.3
Palmitic	31.1	29.0
Stearic	17.6	13.8
Total	50.5	44.1
Unsaturated fatty acids (mol %)		
Oleic	40.6	43.9
Linoleic	5.3	7.2
Total	49.5	55.9
Glycerides		
GS_3	9	5
GS_2U	46	36
GSU_2	40	52
GU_3	5	7

American workers in the field followed the same general procedure of fractional crystallization worked out by Hilditch and his collaborators. Of these, several investigations have involved lard. In 1946, Riemenschneider et al. (79) obtained seven fractions of lard and gave the fatty acid and glyceride compositions for the lard as shown in Table 2.

Table 2. Fatty acid and major glyceride type compositions of lard by Riemenschneider et al. (79)

	Wt % found	Mol % found	Mol % random (Calc'd)
Fatty acid composition			
Linoleic	12.75		
Linolenic	0.82		
Arachidonic	0.42		
Oleic	49.6		
Saturated	36.1		
Glycerides			
GS_3	1.9	1.9	5.2
GS_2U	25.7	25.9	26.3
GSU_2	54.4	54.6	44.0
GU_3	18.0	17.7	24.5

In 1953, Quimby et al. (75) carried out extensive crystallizations of lard, beef tallow, and mutton tallow. Fractions which were representative of each of the four major glyceride types present were obtained. Studies of these fractions were

made which involved obtaining the melting point, X-ray diffraction pattern, and cooling curve of completely hydrogenated fractions before and after random rearrangement. The results of this study confirmed the findings of Hilditch in that it was concluded that lard contains 2-palmitoglycerides and very little if any 1-palmitoglycerides.

Low Temperature Crystallization and Chemical Oxidation Compared

In 1954, Luddy *et al.* (51) determined the glyceride composition of four fats by both the oxidation and crystallization methods. Lard was one of the four fats used in the work. The results of this work are given below in Table 3.

Table 3. Oxidation and fractional crystallization methods of determining the four glyceride types of lard compared, Luddy *et al.* (51)

	Found		Calculated[a]	
	Oxid.	Cryst.	Restricted random	Random
Glycerides				
GS_3	2.8	2.8	2.8	5.8
GS_2U	24.6	27.4	32.4	27.7
GSU_2	58.9	54.8	43.1	43.6
GU_3	13.7	15.0	21.7	22.9
Sm[b]	38.8	39.4	38.8	38.8

[a]Calculated from oxidation experimental data.

[b]Sm = mol % of saturated acids (as glycerides).

The results of Table 3 were obtained using a fractional crystallization procedure somewhat different than that in the work of 1946 but essentially the same type of fractions were obtained and the same assumption that only two of the four types of glycerides were present in any one of the fractions was used. Only five fractions are obtained in the fractional crystallization procedure used in this work.

This work showed that the oxidation and crystallization methods gave essentially the same results when applied to lard, chicken fat, and cottonseed oil but that substantially different results were obtained for palm oil by these two methods.

Microscopy

Microscopy has been employed to study lard before and after rearrangement by Hoerr and Waugh (39) and by Herb et al. (31). It is useful in studying the type of crystals formed under different conditions of crystallization and also to study the incorporation of air into cake batters when different types of shortenings are used.

Partial Enzymatic Hydrolysis

In recent years there has been a considerable amount of work conducted in the study of natural fats by carrying out a partial hydrolysis of the fats in the presence of an enzyme

and analyzing the hydrolysis products. In 1948, Desnuelle et al. (24) studied the enzymatic hydrolysis of triolein and peanut oils in which they concluded that at a pH of 7.0 in the absence of calcium salts, the hydrolysis products consisted of mainly triglycerides and diglycerides but only small amounts of monoglycerides. At a pH of 8.0 the results were similar but a more complete hydrolysis of triglycerides took place. At a pH of 8.0 in the presence of calcium salts the triglycerides and diglycerides were more completely hydrolyzed and monoglycerides accumulated in the hydrolysis products. In 1950, Desnuelle et al. (22) showed that as the quantity of calcium salts are increased in the enzymatic hydrolysis reaction mixture, the hydrolysis gradually passed from the type "generator of diglycerides" to the type "generator of monoglycerides." They also demonstrated that calcium ions are much more effective than calcium oleate. In 1951, Desnuelle et al. (23) described conditions which lead to hydrolysis of about one-third of the triglycerides molecules and which resulted in formation of considerable quantities of glycerol. The outstanding feature of these conditions was the low fat concentration in the over-all hydrolysis reaction emulsion.

After it had been learned that lipolyses carried out under different conditions in the presence of pancreatic lipase could be made to yield different amounts of hydrolysis products it became necessary to develop better methods of analysis of

the products of hydrolysis. Mattson et al. (56) demonstrated the determination of 1-monoglycerides by the use of the periodic acid oxidation method of Pohle and Mehlenbacher (72) and the determination of the total monoglycerides by first converting 2-monoglycerides to 1-monoglycerides and the 1-monoglycerides again determined as before. The 2-monoglycerides were obtained by the difference of the 2 results. The 2-monoglycerides were converted to 1-monoglycerides by use of 55% perchloric acid. The results of their work showed that almost all of the monoglycerides formed were of the 2-monoglycerides configuration. They also suggested that the diglycerides were probably of the 1,2-configuration.

Desnuelle and Constantin (21) presented microanalysis techniques to determine the amounts of liberated fatty acids, monoglycerides, diglycerides, triglycerides and glycerol in the enzymatic lipolysis products. This work also showed that in vitro and intraluminar lipolysis possess the same characteristics.

In 1952, Borgstrom (9) described the separation of free fatty acids and glycerides from cholesterol esters by use of a silicic acid column. Separation of the fatty acids from the glycerides was accomplished using anion exchange resin to adsorb the fatty acids as first described by Cason et al. (15). Borgstrom (11) also carried out in vivo and in vitro studies

of enzymatic hydrolysis and among other results he found that in both cases there was a re-synthesis of glyceride ester-bonds by the use of C_{14} labeled free palmitic acids added to the glyceride samples. Borgstrom (10), published methods for the separation of tri-, di-, 1-mono-, and 2-monoglycerides. The tri-, di-, and monoglycerides were separated on a silicic acid chromatography column. Borgstrom found that 2-monoglycerides are partially isomerized to 1-monoglycerides in passing through the column. He presented a method by which the monoglycerides can be separated from the tri-, and diglycerides by partition chromatography. The di- and triglycerides can then be separated on a silicic acid column. The monoglyceride fraction can be further treated by periodic acid oxidation of the 1-monoglyceride and separation of the oxidation product and 2-monoglycerides on a silicic acid column. As in previous work the free fatty acids are adsorbed on anion exchange resin columns. Passage of synthetic 2-monopalmitin through Amberlite IRA-400 anion exchange resin did not cause any detectable isomerization to 1-monoglycerides. The partition column separation used the solvent system of heptane-80% aqueous ethanol developed by Zilch and Dutton (100) and used for Craig separations by Mattson et al. (56).

In 1953 and 1954 Borgstrom (7, 12, 13, 8) published work on more detailed studies of the mechanism and condition for enzymatic hydrolysis. Borgstrom (13) found that about 20%

of the monoglycerides formed during enzymatic lipolysis are of the 1-monoglyceride configuration. He also showed that the diglycerides formed are of the 1,2-diglyceride type. The 1,2-diglycerides were distinguished from the 1,3-diglycerides by chromic acid oxidation. By use of labeled free fatty acids added to glycerides fed to rats (8) it was found that there was a real synthesis of glyceride-ester bonds and not only trans-esterification. In a study of the effect of bile acids on the hydrolysis and the effect of the pH of the reaction mixture (7), it was found that a plot of percent hydrolysis vs. pH curves for different concentrations of tauro-cholic acid, a maximum at pH = 7.2 with 0.5% tauro-cholic acid, a maximum at pH = 6.7 with 0.1 tauro-cholic acid, and a maximum at about pH = 6 for 0.2% tauro-cholic acid. With increasing concentration of tauro-cholic acid, a second optimum also appeared at a pH of about 9. This second pH optimum starts to show up at a tauro-cholic acid concentration of 0.1%. In a more detailed study (12) it was found that there is an exchange between the free fatty acids and the fatty acids in the 1- and 3-position of the glycerides but no exchange was detected for the 2-position fatty acids. There was also evidence that part of the exchange between the free fatty acids and the glycerides was due to re-synthesis of glyceride-ester bonds. But no re-synthesis appeared to take place with glycerol and the free fatty acids, therefore indicating that the overall hy-

drolysis of glycerides proceeds toward completion *in vitro*. This study also confirmed that the hydrolysis proceeds from 1,2-diglycerides to both 1- and 2-monoglycerides with the 2-monoglycerides prevailing. The pH vs. activity curves for hydrolysis and re-synthesis of glyceride ester bonds in the absence of bile acids are parallel and have maximums at a pH of about 8. The effect of calcium ions was to accelerate the rate of hydrolysis and the increased rate of hydrolysis due to the calcium ions was parallel to a decreased rate of re-synthesis of glyceride-ester bonds. This work also indicated that different fatty acids are built into glycerides during hydrolysis at different rates.

Savary and Desnuelle (83) describe their method of hydrolysis product analysis by free fatty acid adsorption on Amberlite IRA-400 and separation of mono-, di-, and triglycerides on an inverse phase partition column using silica kieselguhr as solid support, cychohexane as the stationary phase and ethanol-water as eluant. The separations were satisfactory except when the glycerides contained short-chain fatty acids (C_{12}) and the more usual long chain fatty acids (C_{16} and C_{18}). They point out though that alcohol causes isomerization of some 2-monoglycerides to 1-monoglycerides during passage through the column but that the overall monoglyceride content remains unchanged. Isomerization can be avoided by replacing alcohol with acetone.

Savary and Desnuelle (85) studied the hydrolysis of synthetic triglycerides by the action of pancreatic lipase and some of their results are presented in Table 4.

Table 4. Pancreatic lipase hydrolysis products of synthetic mixed triglycerides, Savary and Desnuelle (85)

Glyceride employed	Duration of lipolysis	Composition of products obtained (moles/100 moles theoretically liberated)				Unsaturation of fractions (Chains oleic for 100 chains)			
		acids	MG	DG	TG	Acids	MG	DG	TG
POO	2 hr. 45 min.	29	19	45	35	58	80	71	65
POP	2 hr. 45 min.	38	30	40	25	5	87	48	32
OPP	2 hr. 45 min.	28	21	36	41	51	4	20	34

These results confirm that the hydrolysis hydrolyzes the 1- and 3-position fatty acids in preference to the 2-position fatty acids. Their work also showed that 1-monoglycerides arise from the action of the lipase and that isomerization of 2-monoglycerides to 1-monoglycerides during hydrolysis was of secondary importance. Details of results not reported in the paper indicated the 1-oleic acid was detached by the lipase a little faster than the 1-palmitic acid but that this "specificity of chain" was less clear than the specificity of position.

In 1955, Mattson and Beck (54) carried out detailed studies of digestion *in vitro* of triglycerides by pancreatic lipase. The conclusions of this study were that the hydroly-

sis progresses more rapidly in an electrolyte concentration of 1-2 molar, bile salts at low concentration promote hydrolysis but are inhibitory at higher levels, and that an increase in the amount of enzyme used increases the amount of digestion products but that the effect was not linear. Hydrolysis of 2-oleodipalmitin resulted in a diglyceride of oleopalmitin and the monoglyceride was monoolein. From the data presented for the 2-oleodipalmitin hydrolysis it was calculated that 9.0 mol percent of the fatty acids freed were oleic and that 88.8 mol percent of the monoglycerides formed were monoolein. By the varying of the length of time of hydrolysis they found that appreciable quantities of 2-monoglycerides are present for times of less than 60 minutes but that at 60 minutes only the equilibrium mixture of 1- and 2-monoglycerides was present. They concluded that 1-monoglycerides are a result of isomerization of 2-monoglycerides.

By 1956, enough was understood about partial hydrolysis of triglycerides in the presence of pancreatic lipase to use the method for the study of the structure of natural triglyceride mixtures. In 1956, Mattson and Beck (55) studied the specificity of pancreatic lipase for the primary hydroxyl groups of glycerides. Their study included hydrolysis of 2-oleodipalmitin, 2-oleodistearin, 2-palmitodiolein, and 1-oleodipalmitin. Study of these materials confirmed previous

work that the hydrolysis proceeded in steps from triglyceride to 1,2-diglyceride to 2-monoglyceride and that this course was followed for 16 and 18 carbon saturated fatty acids and 18 carbon unsaturated acids regardless of their location in the glycerides. To further show that the type of fatty acid was not important as compared to its location in the molecule, natural lard and randomly rearranged lard were hydrolyzed and the results compared. The uniform content of fatty acids in each of the fractions for the randomized lard showed that the enzyme presents no specificity for any one of the acids. Data for the lard samples are presented in Table 5.

Table 5. Fatty acid composition of enzymatic hydrolysis products of lard and randomly rearranged lard by Mattson and Beck (55)

Hydrolysis products	Iodine value	Percent composition				
		Saturated	Oleic	Linoleic	Linolenic	Arachidonic
Crude lard						
Unhydrolyzed	71.0	36.0	50.3	12.7	0.5	0.4
Free fatty acids	87.5	21.2	59.8	17.1	0.6	0.3
Monoglycerides	32.6	71.1	22.5	6.3	0.3	0.0
Randomly rearranged lard						
Unhydrolyzed	70.3	36.7	49.9	12.5	0.6	0.4
Free fatty acids	69.6	38.6	46.6	14.0	0.7	0.2
Monoglycerides	68.5	39.0	47.3	12.9	0.6	0.2

The data of Table 5 show that crude lard has a saturated acid content in the 2-position of more than twice that of unsaturated acids in this position. As the palmitic acid content of lard (about 25%) is usually about twice that of stearic

acid (stearic acid about 12%) and usually the only other saturated acid of greater than trace amounts is myristic acid (2-4%), this work definitely confirms the work of Hilditch and Stainsby (37), Meara (59), and Quimby *et al.* (75), in which it was found that the glycerides of lard were of the 2-palmito type rather than 1-palmitoglycerides.

Savary and Desnuelle (82) also carried out specificity studies during the enzymatic hydrolysis of triglycerides in 1956. Three pure glycerides, 2-oleoyl dipalmitin, 2-palmito-oleopalmitin, and 2-oleooleopalmitin, and several mixtures of glycerides with random distribution of the chains were studied. Analysis of the hydrolysis products confirmed that the 1- and 3-position fatty acids are hydrolyzed much more rapidly than the 2-position fatty acids but that the 2-position fatty acids are not completely inert in that 10-30% of the monoglycerides are 1-monoglycerides. They state that it is more probable that the occurrence of the 1-monoglycerides is due to direct action of the enzyme system at the 2-position than due to an isomerization of 1,2-diglycerides and 2-monoglycerides. Mattson and Beck (54) attributed 1-monoglyceride formation to mainly isomerization. Savary and Desnuelle also concluded that the degree of unsaturation of the fatty acids (0-2 double bonds) and their length (C_{18} to C_{12}) did not have any appreciable influence on the rate of hydrolysis. The specificity is due much more to position than nature of the chain.

In 1957, Savary et al. (84) published the results of the study of the structure of fats employing pancreatic lipase. They found in all of the animal depot-fats and vegetable fats of seeds and fruits that the distribution of fatty acids in the outer and inner positions of the glycerides were not random. More specifically, in all the cases studied with the exception of pig fat, it was found that there was a great excess of unsaturated acids in the 2-position and that the predominant isomer for the disaturated-monounsaturated glycerides was of the symmetrical one and that for diunsaturated-monosaturated glycerides, the asymmetrical isomer predominated. For pig depot-fat regardless of where it occurred in the body or the state of fattening of the animal, the situation was exactly reversed. Palmitic and stearic acids were found to occupy the same positions except in pig fat in which palmitic occurred in the 2-position to a much higher degree than stearic. The distribution of oleic acid and linoleic acid in pig fat occupied the same positions.

Mattson and Lutton (57) studied the distribution of fatty acids in the glycerides of 12 vegetable fats and 8 animal fats of which lard was one. The study was made using enzyme hydrolysis of the fat and an analysis was made of the fatty acids freed and the monoglycerides formed. The data for pig fat is essentially the same as that obtained by Mattson and Beck (55). They also concluded that natural occurring tri-

glycerides have a high degree of specificity in their fatty acid distribution and that natural fats whether vegetable or animal do not have random distributions. The vegetable fat saturated acids occurred predominantly in the 1- and 3-positions but a similar generalization could not be made for animal fats. Lard was found to be an exception to all the other fats studied in that it was the only one in which the saturated fatty acids were found predominantly in the 2-position. Lard was also an exception to the strong tendency of linoleic acid being found in the 2-position of animal fats.

Desnuelle and Savary (25) wrote a review of their work on the glyceride structure of some natural fatty substances in 1959. They review the development of work done in the study of the structure of fats and outline the methods of analysis employed by themselves in these studies. In their discussion they point out that none of the natural fats they studied and that were studied by Mattson and Lutton (57) conformed to a random distribution. The specificity of position appeared to be due to whether the fatty acid was saturated or unsaturated. In vegetable fats the saturated acids were always located preferentially on the external positions with cocoa butter being an extreme in that only unsaturated acids are found in the 2-position. With animal fats the principle is less well followed but in general the unsaturation in the external positions is greater than in the internal position. Pork was

found to be an exception; the saturated acids were found to be preferentially on the internal positions. They also found that in some cases the length of the saturated chains and the degree of unsaturation corresponded to a given position. Examples of this were the relatively greater proportion of palmitic acid in the internal position than stearic acid in pork and the greater abundance of linoleic acid in the internal position than oleic acid in rat, dog, human, and horse glycerides. Linoleic and oleic acids appeared to occupy the same positions in pork as was previously reported by Mattson and Lutton (57).

Savary and Desnuelle (81) made a study of cocoa butter and the depot fats of mutton and pork by separating the 2-monoglyceride fraction after enzymatic hydrolysis. This was accomplished by periodic acid treatment of all the hydrolysis products followed by column separation. The monoglyceride fraction consists of 2-monoglycerides only as the 1-monoglycerides are destroyed by the periodic acid. As reported in previous work by them, cocoa butter was found to have 100% unsaturated acids in the 2-position. Mutton was found to have 80-90% unsaturated acids in the 2-position whereas in pork 80-90% of the 2-position fatty acids are saturated. Of these saturated acids in the 2-position of pork, palmitic acid was found more frequently than stearic acid.

The work of Reiser and Reddy (78) was very similar to that which was done at the same time by Mattson and Lutton (57). It was somewhat more detailed in that the depot fat was partially separated by fractional crystallization techniques. The fat was obtained from an animal which had been fed for three months on a diet containing only 1% fat and 0.2% dienoic acid. The results of their study were that oleic acid was found in the 1-position of each glyceride molecule and the residual oleic acid was found in the 3-position. Linoleic acid was found in the 2-position of the resultant dioleins and that saturated acids complete the structure.

Method of Calculating the Glyceride Structure from Minimum Data

Vander Wal (91) has presented a method of calculating the distribution of the saturated and unsaturated acyl groups in fats from a minimum of data. He summarizes the method as follows: "Evidence has been presented that the proportions of the glyceride types and isomeric forms in some fats in which C_{16}-C_{18} chains are greatly in excess can be determined by calculations based on a) the percentage of saturated acyl groups in the whole fat, b) the percentage of saturated groups in the 2-monoglycerides which can be derived from the fats by hydrolysis, c) the assumption that all the saturated and unsaturated acyl groups (S and U) present in each of the three positions in the molecules are dispersed therein at

random, and d) the assumption that the proportions of S and U in the 1-position are identical with those in the 3-positions. The method may apply to all predominately C_{16}-C_{18} fats and to other fats as well." At the time he wrote this paper, there was no experimental data for comparison of his calculated values for the isomeric glyceride types. Since, Youngs (98) has presented experimental data for comparison. These results are given in Table 6.

Table 6. Comparison of calculated and experimental values for the six glyceride types present in lard, Youngs (98) and Vander Wal (91)

	SSS	SSU	SUS	SUU	USU	UUU
Youngs experimental	8	29	0	15	36	12
Vander Wal Dist.	6	29	2	12	36	15

Combined Chemical Oxidation and Enzyme Hydrolysis Study

Youngs (98) presented a method for determination of the structure of fats by a combination of several techniques used to study fats. He first quantitatively oxidized the unsaturated acids and separated the oxidation products on a liquid-liquid partition column into two fractions. Enzymatic hydrolysis of each fraction, the oxidized sample, and the original sample was carried out followed by analysis of the fatty acids freed during the hydrolysis. Fatty acid analyses

of each fraction, the oxidized sample, and the original sample and the free fatty acid analyses of the fatty acids freed during hydrolysis furnished enough data to calculate the amounts of each of the six types of triglycerides present in the original fat. These six glycerides types were SSS, SSU, SUS, SUU, USU, UUU where S corresponds to a saturated fatty acid and U corresponds to an unsaturated fatty acid. Of all the glyceride structure studies so far mentioned, this was the first work that employed gas-liquid partition chromatography for the fatty acid analyses.

Column Chromatography Studies

Separations of mono-, di-, and triglycerides by column chromatography have already been mentioned in the literature on enzymatic hydrolysis. These were methods presented by Borgstrom (10) who used a silicic acid column and Savary and Desnuelle (89) who used an inverse phase partition column using a solid support of silica kieselguhr and a stationary phase of cyclohexane. More recently, Quinlin and Weiser (76) and Ravin et al. (77) separated mono-, di-, and triglycerides on a silica gel column. Different solvent systems were used but both gave good separations.

Separation of dicarboxylic acids and silicic acid columns have been described. Higuchi et al. (34) demonstrated the separation of the dicarboxylic acids C_4 to C_{10} in 1952

and Corcoran (17) extended the separations from C_{11} through C_{16} as well as separating the straight chain saturated monocarboxylic acids C_1 through C_{10}. In 1960, Smith (88) described techniques for silicic acid column separation of C_4 through C_{12} straight-chain dicarboxylic acids which were believed to be considerably easier to use.

Savary and Desnuelle (83) described a method of saturated and unsaturated fatty acid separations by use of inverse phase partition chromatography and permanganate hydroxylation of the unsaturated acids. Of particular interest in this paper were the details given on the chromatographic techniques. These details may be of interest to anyone unfamiliar with the apparatus required, methods of filling the column, charging the column, elution of the sample, fraction collection, and analysis of the fractions.

In autoxidation studies on fatty acids and their methyl esters, Frankel *et al.* (26) describe a liquid partition chromatographic method to isolate and determine hydroperoxides, unoxidized fatty acids or methyl esters, and secondary and polymeric decomposition products.

In 1960, Wren (97) published a review on the chromatography of lipids on silicic acid. In this 22 page review a table listing the order of elution of lipids was given and is shown on the following page.

Table 7. Order of elution of lipids from silicic acid, Wren (97)

Hydrocarbons
Esters other than steryl esters and diglycerides
Steryl esters
Fatty aldehydes (?)
Triglycerides
Long chain alcohols
Fatty acids
Quinones
Sterols
Diglycerides
Monoglycerides
Glycolipids
Lipoamino-acids
Bile acids
Glycerophosphatidic acids
Inositol-containing lipids
Phosphatidylethanolamines
Lysophosphatidylethanolamines
Lecithins
Sphingomyelins
Lysolecithins
Ethers
Miscellaneous lipids

It is also pointed out in the review that the terms silica, silica gel, and silicic acid are used synonymously in the literature and are all powdered solids of silicon dioxide with varying amounts of water.

Thin Layer Chromatography

The most recent development in the use of silica gel or silicic acid has been in its application in very thin layers on strips of plate glass. The lipid to be fractionated is

then spotted on one end of the plate glass and the spotted end of the plate immersed in a suitable solvent and developed in an ascending manner. Mangold and Malins (53) demonstrated the separation of fats, oils, and waxes into their constituent lipid classes. Malins and Mangold (52) further separated classes of lipids obtained from thin layer chromatography into their constituents by use of siliconized silicic acid plates, gas-liquid chromatography, and paper chromatography. Privett et al. (74) separated mono-, di-, and triglycerides by thin layer chromatography and molecular distillation and obtained good agreement with known compositions. Densitometric analysis of the plates after the separations have been made was described. Privettand Blank (73) reported a method for the analysis of component mono-, di-, and triglycerides by the use of thin layer chromatography. Samples were first reacted with ozone which attacked the carbon-carbon double bonds and catalytic hydrogen reduction of these ozonides to aldehyde groups. Separation of the resultant glyceryl residues was performed. Procedures for the analysis of four monoglyceride types, six of the seven possible diglyceride types and four of the six possible triglyceride types were demonstrated. Results obtained for a sample of lard are given in Table 8. Wollish et al. (96) describe several pieces of improved equipment in detail and review the current literature and applications in different fields.

Table 8. Analysis of the triglyceride types of lard, Privett and Blank (73)

Triglyceride Type	Found Percent	Literature values (25)	(17)
GS_3	6.7	2.4	2.8
GS_2U	34.6	28.0	27.4
GSU_2	42.7	40.1	54.8
GU_3	16.0	29.5	15.0

Fatty Acid Analysis

In carrying out glyceride structure studies, fatty acid analyses are quite often desired. In the Hilditch laboratory, methods for fractional distillation of methyl esters of the fatty acids were developed. With the development of the ultraviolet spectrophotometric method of determining the polyunsaturated acids, a powerful new analytical tool was available to the fat chemist. The ultraviolet method was rapidly standardized and it has become one of the official methods of the American Oil Chemists' Society (2). Bergstrom and Borgstrom (6) in reviewing recent developments in methods present sections on isolation of fatty acids and separation of fatty acids. These methods are mainly of the liquid-liquid extraction and column chromatography types. The separations in general are difficult and very often not complete. For example, stearic and oleic acid are very difficult to separate

or if the separations can be easily made on the basis of different degrees of unsaturation, then difficulty is often encountered with overlap between a shorter chain fatty acid of lower degree unsaturation and a longer chain fatty acid of higher degree unsaturation. In general these types of separations require several hours.

The latest development in fatty acid analysis has been in the field of gas-liquid partition chromatography. The first work with fatty acids was by James and Martin (44) in 1952 when they separated the volatile fatty acid esters from formic to dodecanoic acids. In 1956, James and Martin (43) presented data for formic to octadecanoic acids. In 1956, Cropper and Heywood (20) presented data for separation of methyl esters of the C_{12}-C_{22} fatty acids. In 1958, several papers on fatty acid analysis appeared. Orr and Callen (69) gave the preliminary results obtained in the screening of partition agents for the fatty acid methyl esters and named Reoplex 400, a polyoxyalkalene adipate, as the best found to date. Craig and Murty (19) describe preliminary work with polyesters as partition liquids and Stoffel et al. (89) describe the satisfactory separation of highly unsaturated fatty acid methyl esters of C_{16}-C_{22} without decomposition or reaction of these fatty acid methyl esters. In 1959, Orr and Callen (68) presented detailed results of their screening of many partition liquids and reported that Reoplex 400 was the

best partition liquid of all those tested. Lipsky and Landowne (46) also presented their findings in screening of various polyesters as partition liquids for fatty acid analysis and describe a polyester called Lac-4-R777, a succinate polyester of diethylene glycol, which gave results comparable to those of Reoplex 400.

In 1959, Lipsky et al. (47) gave more results on the study of different stationary liquids for separation of the long chain fatty acids commonly found in nature. They found that alkyd resins prepared from shorter dibasic acids than sebacic and azelaic acids gave better separation of oleic and stearic acid as well as being more rapid. They found that diethylene glycol succinate polyester was one of the best liquid substrates for excellent resolution and comparatively short retention times for chain lengths to C_{26}. They also reported that columns which yielded the highest number of theoretical plates were not necessarily the most efficient ones for separating two closely related compounds.

Craig and Murty (18) also made a study in 1959 of the quantitative fatty acid analysis of vegetable oils by gas-liquid chromatography. They found that the thermal stability of the polyester polymers could be improved by using 1,4-butanediol or ethylene glycol instead of diethylene glycol without loss of resolution. Iodine values calculated from gas-liquid chromatography analyses were in good agreement with

measured iodine values and indicated that gas-liquid partition chromatography was accurate to within one unit percentage.

The use of gas-liquid chromatography has grown into a field involving a variety of types of detectors used. In the field of lipids, Lipsky et al. (48) demonstrated the use of an ionization detection system and the use of capillary columns with which very difficult separations such as the separation of cis-trans isomers a C_{18} fatty acid with one double bond (methyl elaidate and methyl oleate) were separated.

Herb et al. (33) showed that gas-liquid chromatography results obtained using thermal conductivity detectors compared well with known percentage model mixtures and with analysis by spectrophotometric methods for fatty acid methyl esters of fats and oils. They also were unsuccessful in trying to determine the very small amounts of arachidonic and pentaenoic acids in lard by gas-liquid chromatography. Herb et al. (32) presented more information about the number of fatty acids found in lard triglycerides when a detailed study of the trace quantities was made. Quantitative evidence was obtained for over 20 esters ranging in chain length from 10 to 22 carbon atoms. Included were the esters of the following: saturated acids (10, 12, 14, 15, 16, 17, 18, 19, and 20 carbon-atoms), monounsaturated acids (14, 16, 18, and 20 carbon-atoms), polyunsaturated acids (18:2, 20:2 (?), 18:3, 20:4, 20:5, 22:5).

Peaks for several additional trace components were also observed.

Ackman et al. (1) reported that short chain dicarboxylic acid esters decompose during separation on polyester gas chromatography media whereas with silicone grease as the partition medium, satisfactory results were obtained. The decomposition occurred for methyl esters of oxalic and malonic acids; longer chain dicarboxylic acids were not decomposed. This becomes especially important in structure studies of polyethylenic fatty acids when oxidation products are analyzed to determine the position of double bonds.

The direct analysis of fatty acids has been reported by Metcalfe (61). The equipment required is the same as for methyl ester analyses with the exception of the column, which is treated with phosphoric acid to prevent tailing. If this method catches on, it would save much time which is normally required for preparing the methyl esters of the fatty acids. This paper is an extension of his original announcement (62).

After chromatographs are obtained, the calculation of the sample composition must be made. The normal procedure is to find the area under each peak and set area percent equal to weight percent of the peak component. Craig and Murty (18) and Herb et al. (33) found that area percent calculations gave good results on known samples. Areas can be measured by use

of a planimeter or by drawing tangents through the points of inflection of the peaks and using the base times height of the triangle formed for calculation of the areas.

The work of Horrocks et al. (41) shows that peak area is not a simple function of the concentration in mol percent or weight percent. The percentage compositions calculated from uncorrected areas was high for low molecular weight esters and low for high molecular weight esters. They calculated relative response values for each fatty acid from experimental data as compared to palmitate to be used for correcting the measured areas of a chromatogram. The magnitude of these corrections can be seen from the data presented in the table 9.

Table 9. Effect of relative response on the composition of known methyl ester mixtures determined by gas-liquid chromatography, Horrocks et al. (41)

Ester	Known	Found[a]	Percent error	Corrected[b]	Percent error
Butyrate	4.6	5.9	28.3	4.7	2.2
Octanoate	9.9	11.5	16.2	10.0	1.0
Laurate	10.5	11.3	7.6	10.5	0
Palmitate	25.2	24.7	2.0	24.7	2.0
Arachidate	49.8	46.6	6.4	50.0	0.4
Decanoate	11.0	12.2	10.9	10.6	3.6
Myristate	22.8	24.6	7.9	23.0	0.9
Stearate	20.5	20.7	1.0	20.7	1.0
Behenate	45.7	42.5	7.0	45.7	0

[a]Calculated from uncorrected areas.

[b]Corrected for relative response.

In studies involving the oxidation of double bonds in fatty acids, the resulting oxidation products contain dicarboxylic acids of varying chain lengths depending upon the position of the double bonds. Nowakowska et al. (64) reported the preliminary study of the oxidation products of soybean oil fatty acids, oleic acid, linoleic acid, and linolenic acid by use of a gas-liquid partition column of Dow Corning silicon, high-vacuum grease on Celite 545. Several unexpected peaks were obtained. Further work with oxidation products by Frankel et al. (27) identified an unknown gas chromatographic peak which appeared near palmitate when Craig polyester succinate was used as the stationary liquid phase. This peak appeared in autoxidized samples of soybean oil methyl esters and was identified as methyl azelaaldehydate. This component is formed when the oxidation of the double bond in the 9,10-position of a fatty acid produces an aldehyde instead of a fatty acid group.

Glyceride Separation by Gas-Liquid Chromatography

Gas-liquid partition chromatography has been very successful in making fatty acid analyses a routine procedure but has had only limited success in analyzing higher forms of lipids. There has been some work done with mono-, di-, and triglycerides but much more needs to be done before glyceride analyses can be made by gas-liquid partition chromatography.

McInnes et al. (58) described the application of gas-liquid partition chromatography to the quantitative estimation of monoglycerides. The monoglycerides were first converted to allyl esters by reacting with mesyl chloride in the presence of pyridine and the resulting dimesyl derivatives treated with sodium iodide in acetone. When 1- and 2-monoglycerides were determined, 2 samples were run; the first for total monoglycerides and the second for just 2-monoglycerides by first destroying 1-monoglycerides with periodic acid. The 1-monoglycerides were obtained by difference.

In 1959, Huebner (42) reported a preliminary study on mono- and diglycerides by gas liquid partition chromatography. The mono- and diglycerides could not be eluted as such but acetylation with acetyl chloride assured their complete elution from the column.

Fryer et al. (28) reported for the first time quantitative separation of the triglycerides tributyrin through tristearin. They also presented "fingerprints" of several edible oils. They also state that "The main problem in obtaining acceptable curves of the higher homologs is in vaporizing the oil without thermal decomposition. For triglycerides up to trimyristin this was obtained by increasing the injection block temperature. However the higher homologs do not respond as well, and increasing flow rate must be combined

with the higher temperatures to obtain the desired result even though these changes have not given good curves for molecular weights above 800."

Table 10. Quantitative interpretation of gas-liquid partition chromatography elution, tributyrin through trimyristin, Pryer et al. (28)

Triglyceride	Standard mixture		Curve I	Curve II
	Mol %	Weight %	Area %	Area %
Butyric	14.9	8.11	8.15	12.2
Hexanoic	10.3	7.18	11.2	11.8
Octanoic	8.54	7.25	14.2	10.0
Decanoic	17.9	17.93	21.2	23.4
Lauric	22.3	25.71	29.7	26.0
Myristic	25.9	33.82	15.3	16.4

The data presented in Table 10 show that the method is not developed well enough to be very useful for quantitative analysis of even a simple mixture of trisaturated glycerides. Much work needs to be done in this area to include the unsaturated fatty acid and mixed glycerides.

A review (3) of gas-liquid partition chromatography preliminary studies on triglycerides was published in 1961. Of particular interest is a chromatogram of lard triglycerides using temperature programing furnished by Huebner and is from results which were presented at the American Oil Chemists' Society Short Course in Rochester. A 30 inch column, $\frac{1}{4}$ inch O.D., packed with 3% SE-30 silicone oil on 100/120 mesh Gas-

Chrom P was used with a helium flow rate of 145 ml/min and a flash heater temperature of 370°C. The components were trapped as they emerged from the chromatograph and were established to be triglycerides. The total time of analysis was 12 minutes and the temperature range of 260 to approximately 390°C was used. Four peaks were obtained and the total number of carbons in the triglyceride of each peak reported. These results are presented below and were taken from the chromatograph presented.

Table 11. Triglyceride elution of lard with temperature programed gas-liquid partition chromatography (3)

Column Temp., °C.	Size of peak[a]	No. of carbons
353	3-4%	48
359	10-15%	50
365	60-80%	52
369	10-15%	54

[a]These percents were merely estimated to give an indication of the relative areas of the peaks. The percents were not published in the article cited.

Other chromatograms presented are of butter, trisaturated glyceride mixture, palm kernel oil, triolein, and trilinolein.

As more work is carried out with gas-liquid partition chromatography, the studies become more detailed and cover a much narrower area of study. Several examples of this type

of work follow. The identification of fatty acids by use of identification constants for mono- and dicarboxylic acids was described by Miwa et al. (63). Orr (70) considers recorder-integrator errors in gas chromatography area measurements. Bens (5) studied the absorption characteristics of C-22 firebrick, glass spheres, and Tide when used as chromatographic supports.

Fatty Acid Methyl Ester Preparation

In making fatty acid methyl ester analyses, one of the major concerns is the preparation of the methyl esters. Several papers have been published describing different methods of preparation and comparison of results obtained using different methods.

Stoffel et al. (90) describe a method in which the esters or acids to be methylated are treated with 5% hydrochloric acid (dry) in superdry methanol. The esters are extracted with ether, dried and neutralized over a mixture of sodium sulfate and sodium bicarbonate and the methyl esters finally recovered by microsublimation.

Hornstein (40) presented the method of adsorbing free fatty acid on anion exchange resin, washing the resin free of any other fatty material and finally direct conversion of the adsorbed fatty acids on the resin to methyl esters by the use of anhydrous methanol-hydrochloric acid.

Metcalfe and Schmidtz (60) prepared methyl esters by using boron trifluoride-methanol which converted fatty acids to methyl esters in about 2 minutes. Comparison to preparation by use of anhydrous methanol-hydrochloric acid and by diazomethane showed good agreement of the results. This method has an advantage over the use of diazomethane in that the boron trifluoride-methanol solution can be stored whereas diazomethane must be freshly prepared just before it is used.

Schlenk and Gellerman (86) describe the preparation and use of etheral diazomethane for preparation of methyl esters for microanalysis. They found that the reaction was slow in etheral diazomethane but proceeds very rapidly in the presence of 10% methanol. They also reported there was no reaction of the double bonds in linoleic, linolenic, and arachidonic acids when methylated by their method.

Luddy et al. (50) described substantially complete esterification of fatty acids in cholesteryl esters, phospholipides, and glycerides by methanolysis with a large excess sodium or potassium methoxide in absolute methanol. Glycerides required only 5 minutes refluxing time. Quantitative conversion of uncombined fatty acids was accomplished by direct esterification with absolute methanol containing 4% hydrochloric acid or sulfuric acid and by methylation with diazomethane.

Vorbeck et al. (95) compared four methods of preparation

of methyl esters. The methods compared were methyl esters prepared by 1) diazomethane, 2) methanol-hydrochloric acid with sublimation, 3) methanol-hydrochloric acid on ion exchange resin, and 4) methanol-boron trifluoride. The methods were comparable for higher molecular weight fatty acids mixtures but diazomethane appeared to give the best results when lower weight fatty acids were present.

EXPERIMENTAL STUDIES AND RESULTS

Introduction

In general, the details of the experimental work are given as it was conducted in connection with the major phases of the research. The major phases of the research were considered to be 1) Kartha's chemical oxidation method to determine the four major glycerides types, 2) low temperature fractional crystallization to determine the four major glyceride types, and 3) Youngs' method of glyceride structure study utilizing chemical oxidation and enzymatic hydrolysis. The low temperature crystallization study is further broken down into a number of phases based upon the method used or studied for analyzing the lard fractions.

Details of the construction and operation of equipment are given in appendices. Some procedures, such as methyl ester preparation, common to several phases of the work are also given in an appendix when the procedure itself was not a subject of study.

The lard before and lard after samples used in this work were obtained from the Rath Packing Company of Waterloo, Iowa. Some of the lard before rearrangement sample was used to prepare the rearranged lard sample. In this work, the terms "lard before", "lard after", "lard B", and "lard A" are used to refer

to the lard samples before and after rearrangement. The letters "B" and "A" are also used to designate whether fractions were obtained from the lard B or lard A samples.

The lard was obtained from the fat of hogs by digestion in water by bubbling 40-60 psig steam through a fat-water mixture. The water was removed by centrifuging. The lard before sample was obtained at this point of the processing. The lard after sample was prepared by first slightly hydrogenating the fat, drying the fat under vacuum to less than 0.005% water, adding 0.02% by weight sodium methoxide (methyl alcohol solution), and allowing the reaction to take place for 15-20 minutes. The reaction mixture started at about 100°F and was allowed to cool during the reaction. At the end of the reaction, the catalyst was destroyed by adding water.

The Kartha oxidation was carried out on different samples than the fractional crystallizations No. 1 and 2. Fractional crystallizations No. 3, 4, and 5 were carried out on a third set of lard B and lard A samples.

Kartha Acetic Acid-Acetone Permanganate Oxidation

When this glyceride study of lard was started, the use of gas-liquid chromatography and lipase hydrolysis methods were in the development stage. Kartha's oxidation and low temperature crystallization were the best methods of determin-

ing the structure of a fat. The details of Kartha's procedure and methods of calculation are given in detail by Kartha (45).

The procedure involved cleavage of the carbon-carbon double bonds in the fat sample, separation of magnesium salts of the oxidation products into a precipitate and a filtrate fractions, and separation of the saturated acids in each fraction by magnesium salt precipitation. The weight, iodine value, and saponification value of the saturated acid fractions were determined. From this data and an independent determination of the trisaturated glycerides in the fat, the amounts of GS_3, GS_2U, GSU_2, and GU_3 in the fat sample were calculated. The trisaturated glycerides were determined by crystallization from acetone as described by Luddy and Riemenschneider (49).

The oxidation and separation of saturated acids from the two fractions was successful but poor saponification and iodine values were obtained. The results did not check published results of Luddy et al. (51). Not enough sample remained for more iodine and saponification value determinations. As the work was for familiarization with techniques used in this field and since the results obtained would have little value other than to confirm other workers results, more oxidations were not conducted. The considerable amount of time required would have been great compared to the value of good results

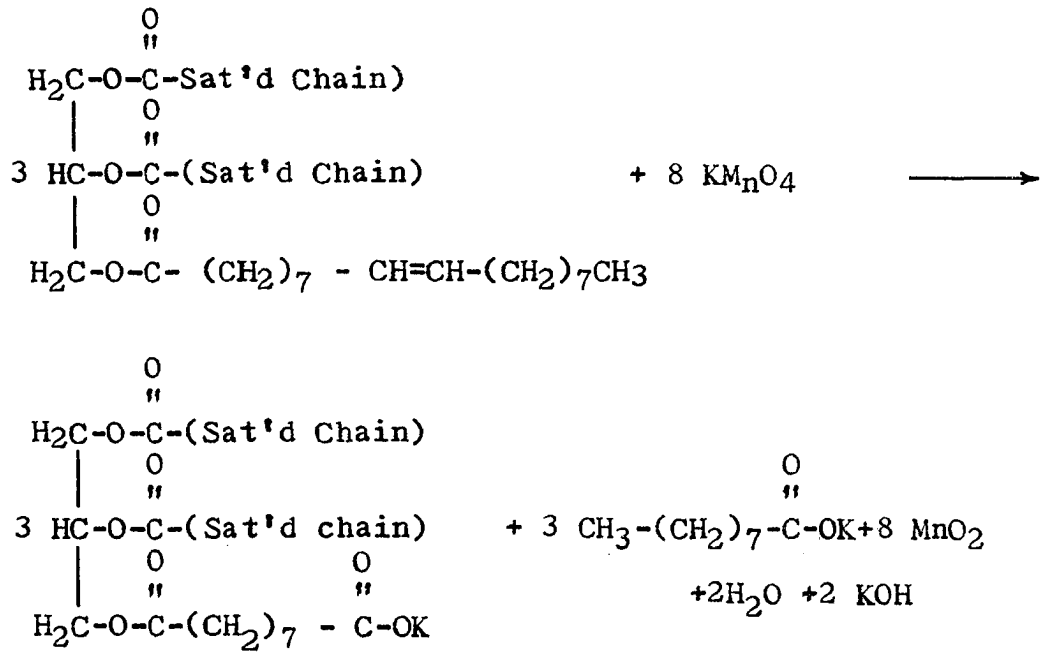
obtained.

A number of preliminary oxidations were carried out before the procedure was carried through to the end results. Some observations were made which are not given in the published procedure but could be helpful in carrying out potassium permanganate oxidations by this procedure.

The oxidation is carried out by refluxing an acetone solution of the fat and periodic additions of acetic acid and potassium permanganate. The oxidation is considered complete when the addition of one gram of permanganate maintains a colored solution for 45 minutes. A bulk grade acetone of unknown purity sold at Iowa State University Chemical Stores was used. When one gram of permanganate could not be made to maintain a colored solution for 45 minutes, the acetone was suspected of having permanganate consuming impurities or the acetone itself was actually being attacked. Performance of analytical reagent grade acetone compared to the bulk grade acetone showed no detectable difference. Test samples of acetic acid, acetic acid-acetone, and acetone were refluxed with small quantities of permanganate. These qualitative tests indicated that there was very little reaction with glacial acetic acid alone, but acetone alone did consume permanganate and a mixture of glacial acetic acid in acetone reacted more rapidly than either of the solvents alone. In trying to maintain a colored solution for 45 minutes, it was

found that as much as over 100 grams of permanganate had to be added for a 5 gram sample of lard while about 60 grams of permanganate were actually sufficient.

The time which a colored solution was maintained upon addition of one gram of permanganate after a given amount of total permanganate had been added varied with different runs on the same sample and with the rate of reflux. A better criterion for insuring complete oxidation appears to be the total amount of permanganate added. The reaction of permanganate can be represented by the following equation.



The theoretical amount of permanganate based on an approximate iodine value of the fat could be calculated and an excess then used.

After oxidation, the manganese dioxide precipitate causes a black, viscous, heterogenous solution to form. The acetone

was removed under vacuum and a suspension of the residue in water was treated with sodium bisulfite and sulfuric acid to dissolve the manganese dioxide. It was found that to completely dissolve the black precipitate required 2-3 hours with heating. There is a point at which the suspension of manganese dioxide breaks up and settles out and a clear light yellow fatty layer forms on top of the solution. There does not appear to be any need of going beyond this point in dissolving the manganese dioxide. The fatty material may be extracted with ether. Carrying the dissolution of manganese to completion results in the precipitation of large amounts of sodium sulfate which is just as troublesome in the extraction of the fatty material with ether as is the undissolved manganese dioxide.

Low Temperature Fractional Crystallization of Lard

Introduction

The details of the low temperature crystallization cabinet as it was constructed and modified for different crystallizations and its operation are given in an appendix. The crystallization scheme used was that presented by Luddy *et al.* (51) which results in a total of five fractions.

The low temperature cabinet was constructed to maintain a constant sample chamber temperature by turning on and off a blower fan which forced air through a basket of crushed Dry

Ice and circulated the air between the sample chamber and the Dry Ice chamber.

Several crystallizations were carried out to obtain essentially the same fractions but the fractions were analyzed by different methods. Details of the fractionation scheme and the procedure are given in an appendix.

The large amounts of dried acetone required for solvent were obtained by refluxing bulk grade acetone over calcium chloride to remove water followed by distillation of the acetone from the calcium chloride, Details of the apparatus and procedure are given in an appendix.

Fractional crystallization No. 1. Analysis by iodine and saponification values

Six 50-gram samples, three lard samples before and three lard samples after rearrangement, were run simultaneously. Iodine and saponification values were run for each sample but calculations for the four major glycerides types did not agree with other published data for lard. The iodine and saponification values were not reliable. Table 12 is given for the purpose of demonstrating how the iodine and saponification values varied when more than one analysis was made for a given sample.

Table 12. Iodine and saponification values of lard fractions

Sample no.		Before			After		
		1	2	3	4	5	6
P - 3	S.V.	195.9 197.6	195.3 195.3	188.0 189.9 219.0	167.6 170.0 186.8	178.1 177.9	179.2 177.1
	I.V.	27.4 28.3	27.3 26.2	29.2 25.7 25.2	15.0 12.9 15.3	14.6 14.9	15.9 13.9 12.6
P - 6	S.V.	199.8 203.2	192.8 194.3	192.2 194.5	192.4 194.6	198.7 201.1	197.2 197.0
	I.V.	34.3 31.9 34.3	42.3 44.4	40.7 40.4	42.5 37.8 33.1	44.7 33.4	36.1 35.2
F - 6	S.V.		171.1 157.3		194.4 192.5	191.3 192.4	184.3 194.7
	I.V.	55.1 55.7	57.4 55.9	57.9 57.1	53.1 28.2 52.8	36.6 51.4 50.8	52.3 50.9
F - 5	S.V.	197.3 209.3	199.4 201.4	174.7 197.3	201.1 202.0	178.7 173.7	191.5 198.6
	I.V.	67.2 71.5 63.0 66.0	72.1 73.3	70.6 71.3	68.7 68.1	72.2 62.7 67.5	65.6 66.3
F - 4	S.V.	230.1 233.9	221.7 221.3	222.8 236.5	206.5 207.7	207.1 199.5	209.1 240.2
	I.V.	78.9 65.1 78.9	96.3 86.1 73.1 72.4	73.1 78.8 66.1 65.2	80.7 76.7 68.9 74.3 62.7	65.9 66.3	63.4 71.1

Fractional crystallization No. 2. Analysis by iodine and saponification values and poly-unsaturated acids by ultraviolet spectrophotometry

Experience from work with the chemical oxidation and the first crystallization indicated that triplicate analyses were not necessary because the methods being used do not give

precise results and also because of the added work involved in trying to run 6 samples simultaneously instead of just 2 samples.

The low temperature cabinet was modified to accommodate just 2 samples. Analysis of the fractions obtained by iodine and saponification values were again poor and the results compared poorly with published data.

An attempt to use ultraviolet spectrophotometry for poly-unsaturated acid analysis also resulted in poor results. One of the major problems involved in the use of this method is that an experienced operator is needed in order to obtain reproducible results. In this method, oleic acid is obtained by difference between the amount of unsaturation found by the iodine value and the amount of unsaturation found by spectrophotometry for the poly-unsaturated acids. Saturated acids are then found by difference between the sample weight and the weight of unsaturated acids determined from the iodine value and spectrophotometry.

Analysis of the progress being made on this research problem again indicated that the work and time involved in working out the techniques involved to obtain good results were not justified by the value of the results obtained. Therefore further work using iodine values and ultraviolet spectrophotometry were discontinued. (It should be pointed

out that the ultraviolet spectrophotometer being used was not located in the laboratory in which the research was being conducted which presented some problems in carrying out the analyses.)

In the analysis for unsaturation by iodine values and by spectrophotometry it was learned that sample storage prior to analysis was important and that the size of the samples being stored is also an important factor. Samples of lard stored in large quantities (one pound or more) under refrigeration at 35-40°F did not undergo appreciable oxidation but small samples such as 0.15 gram samples for iodine values and 80 milligram spectrophotometry samples showed large changes when left at room temperature and open to the atmosphere for several days. The importance of sample size very probably lies in the relative amount of sample as compared to exposed surface area. A lard sample for iodine value (iodine value 61.2) left out on the laboratory bench for 5 days gave an iodine value of 56.6. One of the major reasons for the poor ultraviolet spectrophotometric results was thought to be because the 80 milligram samples were stored in a desicator at room temperature for several days. It should be noted that the two pound lard samples stored under refrigeration for over one year smelled rancid but their overall fatty acid compositions had not changed appreciably.

Fractional crystallization No. 3. Fraction analyses by gas-liquid chromatography and pancreatic lipase hydrolysis

By the time this work was started, papers had started to appear on the use of enzyme hydrolysis as a method of study of fat glyceride structure and papers on successful quantitative separation by gas-liquid chromatography of the common fatty acids found in fats had been published. Therefore it was decided to incorporate these methods into the study of lard.

Two lard samples, before and after rearrangement, were fractionally crystallized. Fresh lard samples were obtained from the Rath Packing Company of Waterloo, Iowa for this work.

Work was started on constructing a gas chromatograph suitable for fatty acid methyl ester analysis. The first column oven and sample injection systems which were constructed did not work too well. Details of the changes made or where better separations were required are given in an appendix.

Methyl esters were prepared by refluxing samples with large excesses of approximately 5% anhydrous hydrochloric acid-methanol solution. The methyl esters were recovered by first evaporating most of the excess methanol, adding water and extracting four times with ethyl ether. The ether solutions were combined, washed with distilled water until neutral to litmus paper, the ether solution dried over granular sodium

sulfate, and the dried ether solution transferred to a round bottom flask and evaporated. The methyl ester samples were then transferred to screw top glass vials and stored under refrigeration until run on a gas chromatograph.

This method of methyl ester preparation is suitable for glycerides as well as fatty acids. Care must be taken to maintain anhydrous conditions for methyl ester preparation of glycerides by this method (see appendix on methyl ester preparation). The hydrochloric acid-methanol solution was prepared as described by Vogel (92) and is presented in detail in an appendix.

The hydrolyses carried out here were of 30 minutes duration following the procedure of Mattson and Beck (54) except that no bile salts were used. Analysis of the hydrolysis products was made by use of Amberlite IRA-400 to adsorb the fatty acids freed during hydrolysis followed by use of a 4 x 4 completion of squares liquid-liquid extraction method as was used by Mattson and Beck (54). More details of the hydrolysis procedure are given in the section on Fractional crystallization No. 5.

Separation of free fatty acids from the lipase hydrolysis products using Amberlite IRA-400 anion exchange resin In all the references made to the use of anion exchange resin for adsorption of fatty acids, reference is made to Cason et

al. (15) and very little detail about the techniques involved in its use are described. Borgstrom (9) and Savary and Desnuelle (80) give some information.

Cason et al. (15) gave the following information. "The wet ether solution was passed through a column of 225 grams (wet weight) of Amberlite IRA-400 which had previously been washed with alkali and then washed with water until the eluate was neutral. The eluate was passed through the column a second time, then the column was washed with two 100 ml portions of ether, the second portion leaving only 0.2 grams of residue on evaporation. Removal of ether from the total eluate left a residue of 21.1 grams of amide,..... Elution of the ion exchange column with a mixture of ether, methanol, and aqueous hydrochloric acid yielded 15.3 grams of mixed acid....."

Borgstrom (9) reported more details: "The ion exchanger IRA-400 was slurried into chromatogram tubes with a diameter of 20 mm. Usually a column of 100 mm height was used. The capacity of such a column was about 1 gram of oleic acid. The preparation of the exchanger was made according to the preparation of Cason, Sumrell, and Mitchell. Wet ether was used as solvent for the lipid mixture to be separated. The fatty acids were eluated with a mixture of 80 parts ethanol and 20 parts concentrated hydrochloric acid."

The article by Savary and Desnuelle (80) had not been translated at the time this portion of the research was carried out but was translated at a later date while making a more detailed search of the literature on enzymatic hydrolysis techniques. The portions pertaining to the ion exchange resin are given here for comparison.

"Ten grams (resin saturated with water) of Amberlite IRA-400 are put in suspension in 1 N sodium hydroxide and the suspension is filtered into a column (12 mm ID) through a small plug of glass wool. The resin is washed with water until neutral, then it is washed 3 times with 40 cc of 95% ethanol and 3 times with 30 cc of ethyl ether free of peroxides. After the washing, the formation of air bubbles is prevented by chasing the bubbles by gently agitating a fine glass rod. A sample of the mixture studied (containing a maximum of 300 mg of fatty acids) is dissolved in 25 cc of ethyl ether and the solution is poured into the column whose effluent is regulated by a stopcock at an average of 1 drop per second. Three washes with 15 cc of ethyl ether suffices to carry down quantitatively the glycerides. The acids, which are then eluted (in a partially esterified state) by washing the column with a mixture of 95% ethanol (50 vol), 10 N hydrochloric acid (10 vol), and ethyl ether (75 vol)."

After running some test columns of equal weights of oleic acid and lard, the following procedure was thought to be nec-

essary to obtain complete separation of the fatty acid and glycerides and then to recover all of the fatty acids from the resin.

1. Dissolve the hydrolysis products (from 2 gram lard sample) in 50 ml of ethyl ether.
2. Pass the solution through an Amberlite IRA-400 column packed to a depth of 120 mm (24 mm ID). A glass stopcock in a rubber stopper supporting a glass wool plug was used to regulate the flow.
3. Pass the solution through the column a second time.
4. Pass 75 ml of ethyl ether through the column to wash the glycerides through the column.
5. Combine the ether solutions, dry over granular sodium sulfate, evaporate the ether and set aside the residue for further analysis.
6. Elute the column with three 50 ml portions of 80% ethanol--20% concentrated hydrochloric acid.
7. Dilute the alcohol solution with 150 ml of distilled water.
8. Extract the free fatty acids 3 or 4 times with a total of 175-225 ml of ethyl ether. Wash the ether extracts with distilled water until neutral to litmus paper, dry over granular sodium sulfate, evaporate the ether and weigh the residue.
9. Wash the column with 100 ml of 15% sodium hydroxide.

Wash the column with distilled water until the effluent water is neutral to litmus paper.

The weights of the fatty acids recovered are given in the table below. A test sample was run with the hydrolyzed lard and lard fractions as a check.

Table 13. Free fatty acids recovered from hydrolysis products using Amberlite IRA-400 columns[a]

Sample	Wt. of hyd. sample	Wt. of free fatty acids recovered	Wt. F.F.A. x 100 / orig. sample wt.
Trial hyd No. 1 Lard B	2.003	0.806	40.3
Trial hyd No. 2 Lard A	2.001	0.526	26.4
Lard B	2.004	0.640	36.9
Lard A	2.005	0.547	27.3
P-6 B	1.504	0.259	17.2
P-6 A	2.005	0.299	14.9
F-6 B	2.002	0.1895	9.5
F-6 A	2.002	0.381	19.0
F-5 B	2.000	0.159	7.97
F-5 A	2.000	0.212	10.6
F-4 B	1.506	0.149	9.9
F-4 A	2.009	0.377	18.8
Test sample: Lard oleic 2.007 2.015	Not hydrolyzed	Lard 1.954 Oleic 1.437	Lard 94.5 Oleic 71.4

[a]P-3 fractions were not hydrolyzed because their melting points were above the temperature at which the hydrolyses were conducted (40°C).

The trial No. 1 lard B and lard B samples were run on the same column; the trial No. 2 lard A and Lard A samples were run on another column. The remainder of the samples were run on separate (freshly prepared resin) columns. The results show very clearly that when approximately the same degree of hydrolysis was obtained in each sample the fatty acid recovery was very poor. In other work with these columns it has been found that the glycerides wash through the column satisfactorily but the fatty acids are not completely adsorbed and/or are not completely recovered. The recovery of the fatty acids from the ethanol-hydrochloric acid solution by ether extraction may not be 100% complete but would not account for the large loss of fatty acids found in the data.

The question now arises as to whether the fatty acids which were recovered were representative of the fatty acids freed during hydrolysis. A second question is whether all of the free fatty acids were removed from the glycerides.

The second question was answered by weighing the glycerides product of F-6 B which passed through the column, dissolving it in ether and stirring for 5 minutes with 15 grams of Amberlite IRA-400 resin. The solution was filtered and resin washed with ether, the ether dried over granular sodium sulfate, the ether evaporated and the residue weighed. This residue was treated by the same procedure a second time. The

results of these tests are given in Table 14.

Table 14. Free fatty acid adsorption on Amberlite IRA-400 for the F-6 B sample

	Weight in grams
Weight of material passing through Amberlite IRA-400 column	1.8234
Weight of glyceride products after first treatment with 15 grams of Amberlite IRA-400	1.6653
First loss in weight	0.1581
Weight of glyceride products after second treatment with 15 grams of Amberlite IRA-400	1.6060
Second loss of weight	0.059
Total loss in weight	0.2171
Weight of fatty acids recovered using the Amberlite IRA-400 column	0.1895
Total weight of free fatty acids adsorbed and recovered from the Amberlite IRA-400	0.4066

To check the question as to whether partial recovery of the free fatty acids yields a representative sample of the fatty acids freed during hydrolysis, methyl esters were prepared of the 3 fatty acid fractions recovered from the F-6 B hydrolysis products and a gas-liquid chromatogram obtained. The apparatus being used at this time was a Podbielniak Chromacon gas chromatograph located in the Dairy Industry Department in a laboratory of Dr. E. G. Hammond. The column being used, Craig polyester (butanediol succinate) on acid

washed Chromosorb, had previously given satisfactory resolution of palmitate-palmitoleate and of stearate-oleate. When these samples were run, good resolution was not obtained and the results for the amounts of material of different chain length but not for difference in degree of unsaturation are given in Table 15.

Table 15. Fatty acid methyl ester composition of free fatty acid fractions recovered from the hydrolysis products of sample F-6 B

	Free fatty acid fraction composition, weight percent		Calculated composition of combined fractions, weight percent	Composition of F-6 B crystallization fraction, weight percent
	Column	1st 15 gram resin treatment	2nd 15 gram resin treatment		
C_{14}	4.4	2.2	2.7	3.3	2.5
C_{16}	17.4	38.6	42.6	29.2	32.2
C_{18}	78.1	59.3	54.6	67.5	65.3

The original F-6 B sample analysis and the fatty acids freed during hydrolysis should be expected to differ more because of the expected difference in the 1- and 3-position fatty acid composition as compared to the 2-position fatty acid composition. But the large difference in the free fatty acid fractions definitely shows that incomplete recovery of the free fatty acids will not result in a representative sample of the free fatty acids.

From the data obtained for the study of the resin up to this point, it could not be determined for certain whether the resin is selective for fatty acids of varying chain lengths and degree of unsaturation or whether the composition of the small quantities of fatty acids was being altered because of losses of some of the acids during the ether extraction of the fatty acids from the ethanol-hydrochloric acid solution. The resin selectivity was thought to probably be the cause but the extraction procedure could not be ruled out on the basis of any of the data. Therefore a series of samples of the same composition were treated with different amounts of Amberlite IRA-400 resin by stirring the sample and resin in a solution of ether (15 minutes), filtering through a fluted filter paper, washing the resin with two 50 ml portions of ether, drying the ether solutions over granular sodium sulfate, and weighing the material which was not adsorbed on the column. The fatty acids were recovered from the resin by treating it with three 50 ml portions of 80% ethanol-20% concentrated hydrochloric acid. Because of the limited solubility of the fatty acids in ethanol-hydrochloric acid, ethyl ether was used in all cases for the final wash of the resin and in some of the latter samples, the ether was added to the ethanol-hydrochloric acid wash solutions before treating the resin. Methyl esters of the recovered fatty acids were formed by refluxing with 4% anhydrous hydrochloric acid-methanol solution

and the methyl esters recovered by extraction. The results of these tests are given in Table 16.

A check was made after these fatty acids had been recovered to determine qualitatively the solubility of fatty acids in 80% ethanol-20% concentrated hydrochloric acid. A sample of 1.003 grams of lard B fatty acids could not be dissolved in 190 ml of the ethanol-hydrochloric acid solution at room temperature with stirring. Addition of just a few ml of ethyl ether did not dissolve all of the fatty acids but 40 ml of ether added to the solution did dissolve all of the fatty acids. Therefore it was apparent that ether was necessary for complete recovery of the fatty acids from the resin. Even though the acids are desorbed from the resin, they may not be washed from the resin because of their limited solubilities.

The samples to be treated with the resin consisted of one gram of fatty acids of lard B and one gram of mixed glycerides of lard A.

The lard fatty acid sample was prepared by saponifying 20-25 grams of lard B with 0.5 N alcoholic potassium hydroxide and recovery of the fatty acids by acidifying, extracting with ethyl ether, drying over sodium sulfate and evaporating the ethyl ether.

The mixed glycerides of lard were prepared by refluxing 21 grams of lard A with 7 grams of glycerol in the presence of a spatula tip of sodium methoxide in 200 ml of dioxan-1,4 for 5 hours. Acetic acid was added to destroy the catalyst, the dioxane evaporated, the product diluted with 4-5 times its volume of water, extracted with 100 ml, 50 ml, and 50 ml portions of ethyl ether. The ether extracts were washed with sodium bicarbonate 2 times, then twice with water, the ether solution dried over granular sodium sulfate, and the ether evaporated. A silica gel column separation of this product 3 months later showed that the weight percent of each of the glycerides present was: 3.5% monoglycerides, 19.4% diglycerides, and 77.1% triglycerides.

Table 16. Amberlite IRA-400 capacity and selectivity test

Sample	Grams resin	Fatty acid		Wt % F.A. rec'd	Lard glyc added	F.A.+lard glyc		Overall % rec'd
		Added	Recov.			Added	Rec'd	
A	1	1.011	0.097	9.6	0.999	2.010	1.984	98.7
B	5	1.002	0.381	38.0	1.013	2.015	1.975	98.0
C	10	1.006	0.691	68.7	1.014	2.020	1.946	96.3
D	15	1.007	0.960	95.4	1.009	2.016	1.919	95.2
E	30	1.004	0.940	93.6	1.002	2.006	1.872	93.3
F	30	1.004	0.987	98.3	1.097	2.101	1.928	91.7

Samples E and F of Table 16 were run to give some idea of the reproducibility of the results.

The fatty acid methyl ester analysis using gas-liquid chromatography on a butanediol succinate column, 20% liquid substrate on acid washed 60/80 mesh Chromosorb. The column dimensions were 8 ft x $\frac{1}{4}$ in OD copper tubing.

Table 17. Resin selectivity test; methyl ester composition of lard fatty acids recovered from Amberlite IRA-400; Ethanol-hydrochloric acid recovery

Sample	F.A. mix	Mixed glyc	A	B	C	D	E	F	Average E and F
Myr.	1.4	1.7	3.6	2.8	2.1	1.5	1.6	1.5	1.6
Pal.	27.6	26.8	40.9	36.3	31.0	26.9	26.4	26.7	26.6
P.O.	2.9	3.5	3.2	3.4	3.4	3.2	3.6	2.9	3.3
Stear.	11.4	12.2	11.4	9.0	10.5	11.2	11.2	11.8	11.5
Oleic	45.7	46.7	34.4	39.3	43.2	46.6	47.4	46.6	47.0
Linol.	11.1	9.0	6.5	9.2	9.8	10.6	9.8	10.5	10.2
Gms of resin			1	5	10	15	30	30	30

Several months later, another series of samples were run to test the resin selectivity. In this test more attention was paid to controlling the volume of the solutions more closely to insure similar conditions for each sample. The procedure followed was that described by Hornstein et al. (40) in which the fatty acids are converted to methyl ester on the resin by methanol-hydrochloric acid treatment after adsorption of the fatty acids. Details of this procedure are given in

the section on Fractional crystallization No. 4. The results of this test are given in Table 18.

Table 18. Resin selectivity test; methyl ester composition of fatty acids recovered from Amberlite IRA-400; Methyl esters prepared on the resin

Sample	A	B	C	D	E	F
Myr.	4.0	3.1	1.7	1.5	1.2	1.4
Pal.	34.2	32.1	26.8	26.0	26.2	23.8
P.O.	4.7	4.0	3.1	2.8	2.4	3.1
Stear.	8.0	9.0	12.4	12.8	12.2	11.8
Oleic	37.8	40.7	45.3	46.4	47.7	45.3
Linol.	11.3	11.1	10.8	10.5	10.2	10.1
Gms of resin	1	5	10	15	30	0

The results in Tables 17 and 18 are combined into Figure 1 to show more clearly the trends in the compositions and the comparison of the results of the two tests.

The results of these 2 tests indicate that resin is selective: a) it is more selective for short chain fatty acids than longer chain fatty acids and b) it is more selective for unsaturated acids than for saturated acids of the same chain length.

The data also indicate the amount of resin required to adsorb one gram of fatty acids under the conditions of these tests is between 10-15 grams. The resin capacity lies between 0.067 to 0.100 grams of fatty acids (C_{14}-C_{18}) per gram of resin. Based on a molecular weight of 280, the capacity be-

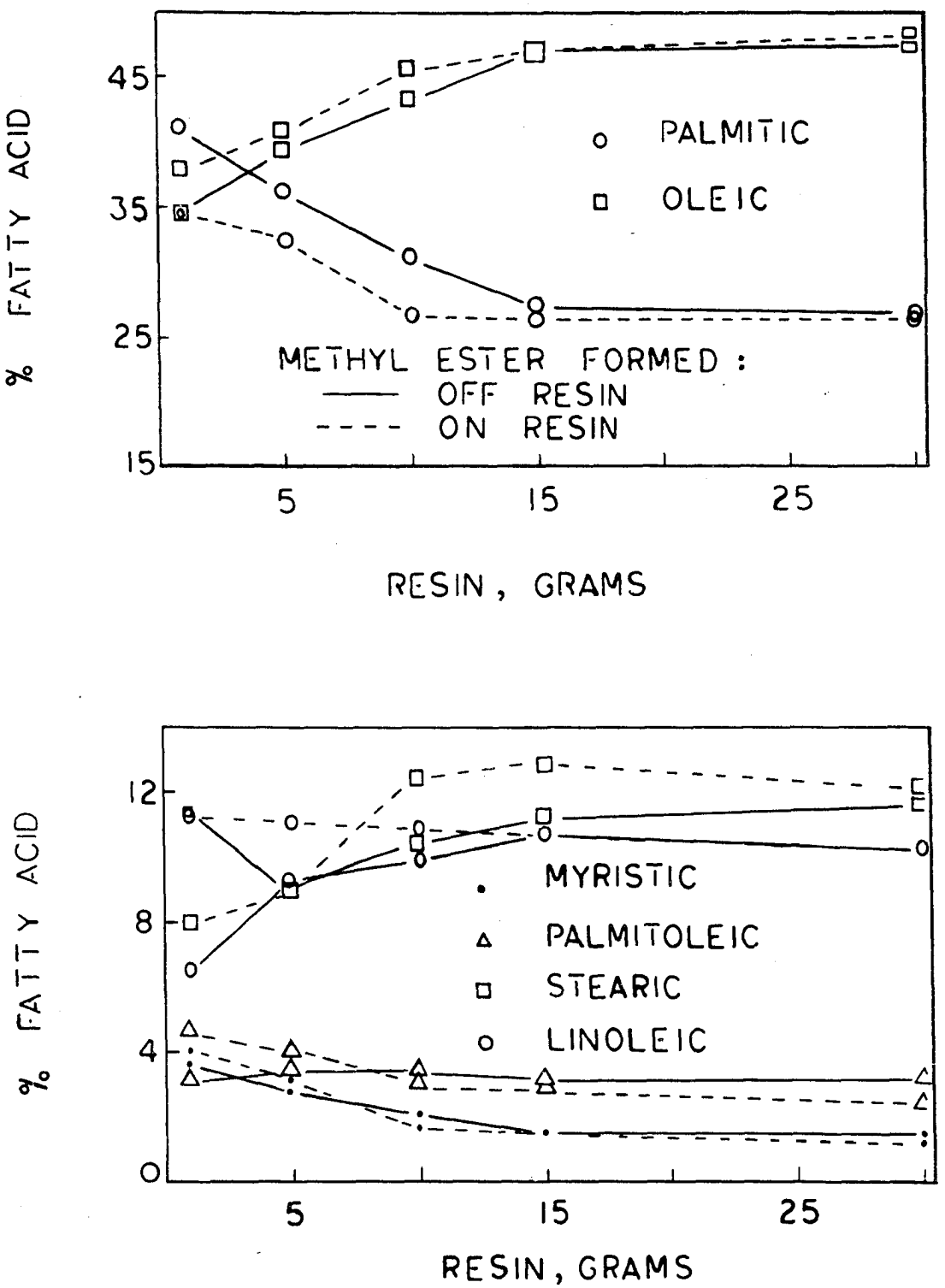


FIG. 1. RESIN SELECTIVITY

comes 0.24 to 0.36 milliequivalents per gram of resin. The reagent bottle gives the total exchange capacity as 3.3 milliequivalents per gram based on dry resin. The weight in the milliequivalents is based on a wet resin. Assuming the water content of the resin was 50% the capacity for fatty acids would still be only 0.48 to 0.72 milliequivalents of C_{18} fatty acid per gram of resin. This is only 1/7 to 1/5 of its total rated capacity.

After having made the above tests and having obtained such poor adsorption and recovery of fatty acids from the hydrolysis products by the use of resin columns, it was concluded that theoretically the methods being used were adequate but in practice another factor predominates all others. This factor is the packing of the column and its condition during adsorption and subsequent fatty acid removal.

Amberlite IRA-400 (-OH form) was slurried into the columns using ethyl ether. Bubbles were removed either by use of a brass wire used to stir the resin in the column, or by repeatedly inverting the column to first wash all the resin into the top part of the column and then reversing the inversion process to work the resin back into the lower part of the column. In this process of inverting the column repeatedly, entrapped air was allowed to escape from the resin. After these columns were allowed to stand for a few minutes, bubbles

would usually start forming throughout the column. These bubbles were probably caused by the formation of ethyl ether vapors. Although it was never tried with the resin columns, a cooling water jacket might slow down or prevent this bubble formation. Similar difficulty with silica gel columns was eliminated by the use of a water jacket on the column. But the cooling might not be as effective with the resin because it was observed that the resin columns proved to be difficult while silica gel columns at the same temperature (and using ethyl ether for elution) behaved very well. Some resin columns formed bubbles much worse than others. The reason for this was never determined. As a result of the bubble formation, poor contact between the fatty acid-glyceride ether solution and the resin was obtained. This resulted in some of the fatty acids passing through the column without being adsorbed and requiring that the sample solution be passed through the column more than once. The number of passes required depended upon how bad the bubble formation was.

None of the three references (9, 15, 80) describing the use of the resin columns make any reference to difficulties as encountered here. Whether they had any difficulties with bubbles or not, therefore, is not known.

Prior to the publication of Hornstein's method (40) of using this resin to recover fatty acids from fats, some addi-

tional investigations were carried out to develop a suitable method of recovering free fatty acids from the hydrolisis products based on a single batch contact of the free fatty acid-glyceride solution with the resin.

The resin selectivity tests showed that 15 grams of resin could be expected to adsorb approximately 1 gram of free fatty acids in the presence of an equal amount of mixed glycerides. The glycerides could be quantitatively recovered by washing the resin with portions of ethyl ether.

The recovery of the adsorbed fatty acids was made by the use of an ether-acetic acid solution. The fatty acids are much more soluble in this solution than in the ethanol-hydrochloric acid solution and has the added advantage of being able to evaporate both the ether and the acetic acid leaving a residue of just the fatty acids. With the ethanol-hydrochloric acid solution, the fatty acids were recovered by extracting the fatty acids with ethyl ether. One disadvantage of the ether-acetic acid solution as compared to the ethanol-hydrochloric acid solution is that the acetic acid is weak acid and requires the use of either more contacts or a higher acetic acid concentration to recover all the fatty acids from the resin.

Tests were conducted using a commercial grade oleic acid. One gram samples of oleic acid were dissolved in 50 ml of wet

ethyl ether and the oleic acid adsorbed by stirring the solution with 15 grams of Amberlite IRA-400 (-OH form) for 10 minutes. The ether was decanted from the resin and the resin was washed with 25 ml of ether. The ether was evaporated and the weight of the residue determined. The oleic acid was recovered from the resin by treating it with 50 ml portions of ethyl ether-acetic acid solution. The resin and each ether-acetic acid solution were stirred for 10 minutes and then decanted from the resin. The ether-acetic acid solutions were evaporated and the weight of the recovered oleic acid was determined. A summary of some of the tests conducted are summarized in the following table.

Table 19. Recovery of oleic acid from Amberlite IRA-400 using ethyl ether-acetic acid solution

Sample	A	B	C	D	E
Wt of oleic acid added	0.970	1.010	1.032	1.010	1.001
Wt of resin	15	15	15	15	15
Wt of mat'l not exch'd	0.000	0.001	0.001	0.000	0.001
No. of ether-HAc treatments	1	2	2	2	2
Strength (vol:vol) ether: HAc	2:1	2:1	1:1	1:1	1.5:1
Wt of oleic acid recovered	0.730	0.863	0.958	0.938	0.878

The results of Table 19 show that the use of 1:1, ethyl ether:acetic acid (volume basis) was suitable for the recovery of up to one gram of a commercial grade oleic acid sample adsorbed on 15 grams of Amberlite IRA-400 by 10 minute contacts with stirring of the resin with two 50 ml portions of the solution.

Separation of monoglycerides from di- and triglycerides using 4 x 4 completion of squares liquid-liquid extraction

After the hydrolysis products of the crystallization fractions and the lards had been passed through Amberlite IRA-400 (-OH form) columns, the fatty acids which remained in the hydrolysis products were adsorbed on 15 grams of resin. The material which was not adsorbed was recovered. No attempt was made to recover the fatty acids from the resin.

The monoglycerides in the remaining hydrolysis products were recovered by liquid-liquid extraction as shown schematically in Figure 2 by using four 250 ml separatory funnels. The solvents were prepared by shaking 80% aqueous ethanol with Skellysolve F. The layers were separated and these solutions were used as solvents and are referred to as the alcohol and the Skellysolve F solvents even though each solution was saturated with the other. The glycerides from which the free fatty acids had been removed, were dissolved and transferred to the first funnel with enough Skellysolve F to obtain a

solution containing 2-2.5% glycerides. For 1.6 grams of hydrolysis products and a solution containing approximately 2.5% glycerides, 1.6 x 40 = 64 ml of Skellysolve F were required. The 64 ml of Skellysolve F permitted using sample sizes ranging from 1.6 to 1.3 grams. This corresponds to 20 to 35% hydrolysis on a weight basis. The same volume of Skellysolve F was then put in each of the other three funnels. An equal volume of alcohol was added to the first funnel, shaken, allowed to settle, and the alcohol layer run into the second funnel. This alcohol layer was transferred successively through the four funnels. The alcohol solution obtained from the fourth funnel was called extract 1. A second volume of alcohol solution was similarly passed through the four funnels. This final alcohol solution was called extract 2. To finish the 4 x 4 completion of squares would require obtaining two more such alcohol extracts but as Mattson and Beck (54) describe in the procedures being used here, only two extracts were collected and analyzed. The combined alcohol extracts were evaporated, a few ml of water added, and the monoglycerides extracted with ethyl ether, the ether solution dried over granular sodium sulfate, and the ether evaporated. When emulsions formed, they were broken by adding a gram or so of sodium sulfate, shaking, and allowing the layers to separate. Methyl esters of the fatty acids in the monoglycerides were prepared by refluxing the samples with excess anhydrous

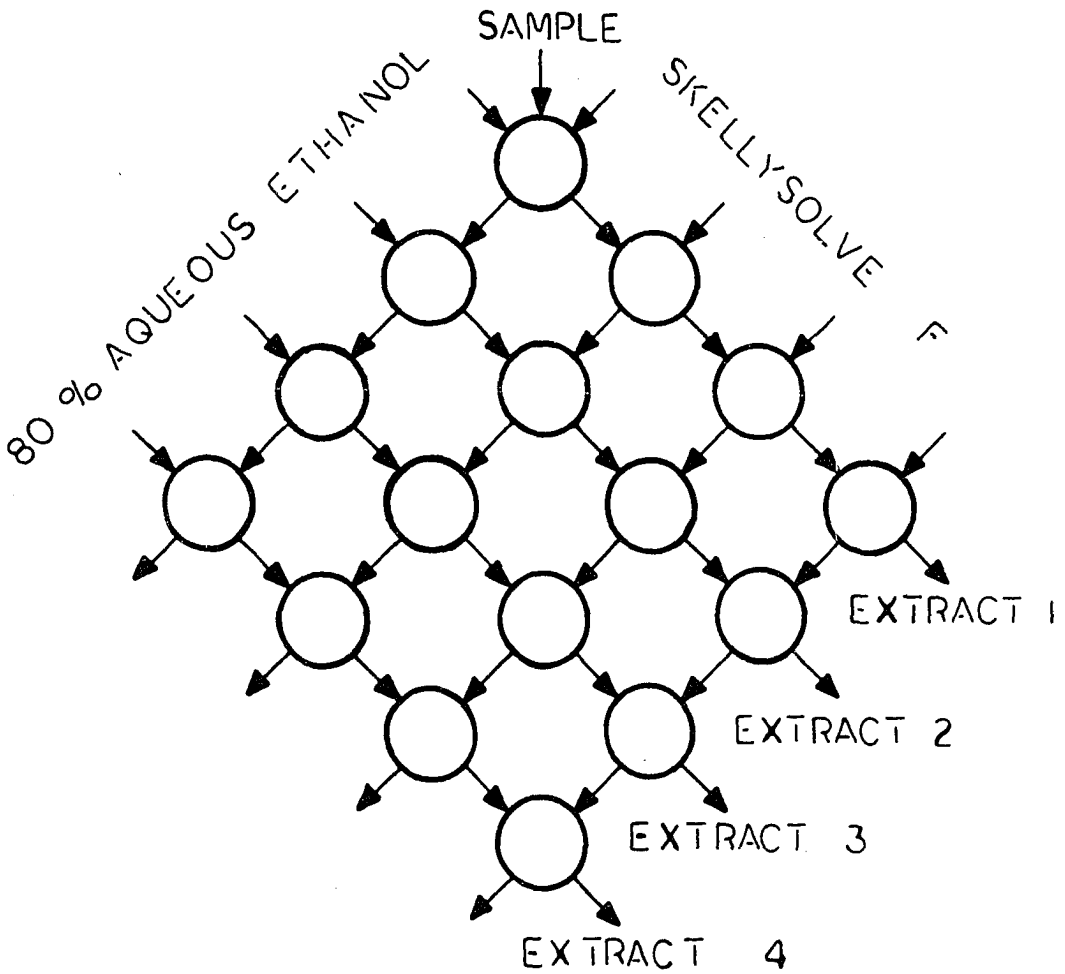


FIG. 2. LIQUID-LIQUID EXTRACTION OF MONOGLYCERIDES

methanol-hydrochloric acid solution and recovering the methyl esters by extraction with ether.

It was desired to get some idea of how complete the extraction of the monoglycerides was using this method of extraction. The hydrolysis products of the F-6 B and F-6 A crystallization fractions were extracted with two more 64 ml portions of ethanol solution to complete the extraction scheme for 4 x 4 completion of squares. The data obtained for these two samples are given in Table 20.

Table 20. The 4 x 4 completion of squares liquid-liquid extraction of monoglycerides from the hydrolysis products of F-6 B and F-6 A

Extract	1	2	3	4	Total
Wt of monoglycerides F-6 B	0.0436	0.0335	0.0240	0.0391	0.1402
Wt of monoglycerides F-6 A	0.1205	0.0771	0.0565	0.0438	0.2979
Residual glycerides F-6 B	------	-------	------	------	1.4814
Residual glycerides F-6 A	------	-------	------	------	1.0670

A study of the data in Table 20 show that considerable quantities of material were obtained in extracts 3 and 4. No tests were conducted to determine whether this material was monoglycerides or not. The final weights of the F-6 B and F-6 A hydrolysis products after the Amberlite IRA-400 treatments were 1.6060 and 1.3517 grams respectively. The combined residue and monoglyceride weights from Table 19 for these same samples were 1.6216 and 1.3649 respectively which correspond to increases in the weights of the samples of 9.72% and 9.76%. The most probable sources of these increases were stopcock grease from the separatory funnels and water left in the samples after evaporation of the solvent. These data indicate that considerable amounts of the monoglycerides were not recovered in the first two extracts. But very little if any separation of the component monoglycerides would be expected to take place in the four stages used in the extraction. As a quantitative recovery of the monoglycerides was not necessary, a representative sample of the monoglycerides is all that was necessary.

The F-6 B extracts were discarded without a fatty acid analysis having been made. The combined four extracts of F-6 A were analyzed for their component fatty acids.

Three samples, F-6 B, F-6 A, and lard B were hydrolyzed as before except that the time of hydrolysis was 15 minutes

instead of 30 minutes. The free fatty acids were removed from the hydrolysis products by batch contact with Amberlite IRA-400. The fatty acids were not recovered from the resin. The monoglycerides were separated from the hydrolysis products by the liquid-liquid extraction method just described.

Methyl ester of the monoglyceride fatty acids were prepared by refluxing with anhydrous methanol-hydrochloric acid and recovering the methyl esters by extraction. The fatty acid methyl esters were analyzed by gas-liquid partition chromatography on an ethylene glycol succinate column.

Results The results of the fractional crystallization and methyl ester fatty acid analyses of the lards, crystallization fractions, the fatty acids freed during hydrolysis, and the monoglycerides formed during hydrolysis are given in Tables 21 through 26.

Table 22 demonstrates that the fatty acid methyl ester analyses were not very accurate. The major reasons for the inaccuracy was due to poor baseline control and the imperfect separation of palmitate-palmitoleate and stearate-oleate peaks. The baseline usually changed during the time these peaks were being formed on the chromatograms of the chromatograph which was constructed. This caused errors in the area calculations because of the uncertainty of where the baseline should have been drawn for portions of the chromatogram in which peaks

were being formed and the trace did not return to the baseline between peaks. Several samples were run on a Podbielniak Chromacon gas chromatograph in the Dairy Industry Department. Triplicate analysis of lard before gave the average values listed in Table 22. These values were used for both lard before and after in calculations for Tables 21, 23, and 25. These values were considered to be more nearly correct than the individual analyses on the chromatograph which had been constructed. Actually the lard before and after have slightly different analyses because of the oxidation of some linoleic and oleic acids during the rearrangement processing.

In Tables 26 and 27, the fatty acid components designated by a question mark (?) corresponded to peaks resulting from the oxidation of the unsaturated acids in these samples. From work carried out with butanediol succinate columns a year later, it appears that the two peaks preceeding myristate correspond to succinate and pimelate. The first peak following myristate may have been due to a semialdehyde of suberate and the next three peaks suberate, semialdehyde of azelate, and azelate. These samples were run on an 8 ft x $\frac{1}{4}$ in OD copper tubing column. The butanediol succinate was supported on 48/65 mesh acid washed Chromosorb. Oxidation of the samples took place at room temperature while the samples of fatty acids adsorbed on Amberlite IRA-400 and the remaining hydrolysis

product residues were left sitting in beakers on the laboratory bench for several days.

Table 21. Fatty acid methyl ester compositions of lards and lard crystallization fractions

	Lard[a]		P-3		P-6		F-6		F-5		F-4	
	B	A	B	A	B	A	B	A	B	A	B	A
						Weight percent						
Myr.	1.7	Trace	1.8	1.8	1.6	2.5	1.9	2.4	1.7	2.3	1.7	
Pal.	26.8	39.6	55.6	45.8	48.7	29.8	24.4	10.7	15.4	10.9	10.0	
P.O.	2.9	--	--	--	--	2.4	3.5	7.4	5.7	11.3	6.6	
Stear.	12.4	39.6	33.2	25.0	22.5	7.0	10.3	3.7	6.0	2.8	1.3	
Oleic	46.2	20.9	9.3	27.5	27.2	53.9	52.8	63.6	57.9	50.5	61.9	
Linol.	10.0	--	--	--	--	4.4	7.1	12.4	13.3	22.1	18.5	
						Mol percent						
Myr.	2.0	Trace	2.1	2.1	1.9	3.0	2.3	3.0	2.1	2.8	2.0	
Pal.	28.5	42.0	58.0	48.1	51.0	31.6	26.0	11.4	16.5	11.7	10.8	
P.O.	3.1	--	--	--	--	2.6	3.7	8.0	6.2	12.1	7.2	
Stear.	11.9	37.9	31.2	23.6	21.2	6.6	9.9	3.6	5.8	2.7	1.2	
Oleic	44.6	20.2	8.8	26.2	25.9	51.9	51.1	62.0	56.5	49.1	60.6	
Linol.	9.8	--	--	--	--	4.2	7.0	12.1	13.1	21.6	18.3	

[a]See Table 22 and text for more details on the lard fatty acid compositions.

Table 22. Fatty acid methyl ester compositions of lard before and after molecular rearrangement

	Constructed chromatograph			Podbielniak chromacon
	Lard before	Lard after	Av. of lard B and A	Av. 3 chromatographs Lard B
Myr.	1.52	1.85	1.68	1.68
Pal.	26.58	26.80	26.69	26.79
P.O.	3.52	3.15	3.33	2.88
Stear.	10.33	11.28	10.80	12.43
Oleic	46.80	47.55	47.18	46.17
Linol.	11.40	9.36	10.38	10.02

Table 23. Mol percentage of saturated and unsaturated fatty acids in lard and each of the crystallization fractions

	Sample	Mol % S	Mol % U
Before			
	Lard	42.49	57.47
	P-3	79.90	20.16
	P-6	73.77	26.22
	F-6	41.19	58.73
	F-5	17.94	82.13
	F-4	17.23	82.77
After			
	Lard	42.49	57.47
	P-3	91.25	8.80
	P-6	74.08	25.86
	F-6	38.24	61.77
	F-5	24.36	75.69
	F-4	14.05	86.02

Table 24. Glyceride composition of lard before and after molecular rearrangement from fractional crystallization data

Fraction	Wt of fraction gms	Mol F.A. / 100 gm F.A.	Total wt % F.A. in glyc.	Mols of glyc.	Mol % of glyc. GS_3	GS_2U	GSU_2	GU_3	Mols of glyc. GS_3x 100	GS_2Ux 100	GSU_2x 100	GU_3x 100
Before												
P-3	9.59	0.3670	95.62	0.01122	39.5	60.6			0.444	0.680		
P-6	2.40	0.3709	94.70	0.00281	21.3	78.7			0.060	0.221		
F-6	26.84	0.3677	95.60	0.03144		23.7	76.3			0.746	2.400	
F-5	7.83	0.3630	95.64	0.00906			53.8	46.2			0.488	0.419
F-4	2.51	0.3645	95.67	0.00292			51.7	48.3			0.151	0.141
Totals												
	49.17			0.05745					0.504	1.647	3.039	0.560
							Mol %		8.75	28.65	52.85	9.75
After												
P-3	3.32	0.3742	94.57	0.00392	73.6	26.4			0.288	0.103		
P-6	8.35	0.3724	95.56	0.00990	22.4	77.6			0.222	0.769		
F-6	22.42	0.3655	96.89	0.02642		14.7	85.3			0.388	2.255	
F-5	9.11	0.3631	96.91	0.01059			73.1	26.9			0.774	0.285
F-4	6.01	0.3616	95.45	0.00690			42.1	57.9			0.291	0.399
Totals												
	49.21			0.05773					0.510	1.260	3.320	0.684
							Mol %		8.84	21.82	57.50	11.83

Table 25. Fatty acid balance for lard and the fractional crystallization fractions before and after molecular rearrangement

Sample	Wt in gms	Weight of fatty acids in grams Myr.	Pal.	P.O.	Stear.	Oleic	Linol	Total wt of F.A.
Before								
Lard	50.02	0.80	12.79	1.38	5.96	22.05	4.79	47.77
P-3	9.59	Trace	3.62	--	3.62	1.92	--	9.16
P-6	2.40	0.04	1.04	--	0.57	0.63	--	2.28
F-6	26.84	0.64	7.65	0.63	1.80	13.82	1.12	25.66
F-5	7.83	0.18	0.80	0.55	0.27	4.75	0.92	7.47
F-4	2.51	0.06	0.26	0.27	0.07	1.21	0.53	2.40
Totals	49.17	0.92	13.37	1.45	6.33	22.33	2.57	46.97[a]
After								
Lard	50.01	0.80	12.79	1.38	5.96	22.05	4.79	47.77
P-3	3.32	0.06	1.73	--	1.05	0.29	--	3.13
P-6	8.35	0.13	3.89	--	1.79	2.18	--	7.99
F-6	22.42	0.42	5.23	0.74	2.38	11.43	1.53	21.73
F-5	9.11	0.15	1.34	0.49	0.52	5.13	1.19	8.82
F-4	6.01	0.10	0.57	0.38	0.07	3.56	1.06	5.74
Totals	49.21	0.86	12.76	1.61	5.81	22.59	3.78	47.51[a]

[a]The weight percent fatty acids in lard before based on the weight of the glycerides was found to be 95.49%. This percentage times the total weight of lard fractions should agree with total weight of fatty acids in the fractions (last column in the tables). 49.17 x 0.9549 = 46.95;
49.21 x 0.9549 = 46.99
The 46.99 value does not agree very well with the 47.41 value obtained as a total in the "after" table. This is probably due to the use of the same lard fatty acid compositions for both the before and after samples. See Table 22.

Table 26. Composition of fatty acids liberated by enzymatic hydrolysis (mainly fatty acids from the 1- and 3- positions); analyses in wt percent fatty acids[a]

Fatty acid	Lard			P-6		F-6		F-5		F-4	
	B	B	A	B	A	B	A	B	A	B	A
Myr.	1.3	0.6	2.4	1.0	1.7	4.4	2.4	1.4	2.4	1.2	2.1
?	0.9	-	-	-	-	-	-	-	-	-	-
Pal.	6.0	7.4	19.4	15.9	23.8	14.8	24.0	14.7	18.8	19.1	10.3
P.O.	5.4	3.0	4.0	3.6	2.1	2.7	4.6	2.5	4.9	2.8	6.2
?	0.6	-	-	-	-	-	-	-	-	-	-
Stear.	8.5	14.1	10.1	15.5	8.7	13.6	10.5	16.8	10.8	15.1	9.8
Oleic	67.1	59.2	54.5	59.0	46.7	59.2	51.6	55.0	54.9	51.2	49.0
Linol	10.1	15.7	9.8	5.0	7.0	5.4	7.1	9.6	8.4	10.6	22.4

[a]The second lard before sample was obtained from hydrolysis products obtained from a 15 minute reaction time. All other samples were reacted for 30 minutes.

Table 27. Composition of fatty acids in monoglycerides obtained from enzymatic hydrolysis products; analyses in wt percent fatty acids

15 min:		2				6		8				
30 min:	1		3	4	5		7		9	10	11	12
	Lard			P-6		F-6			P-5		F-4	
Sample	B	B	A	B	A	B	A	A	B	A	B	A
?	-	-	-	-	-	-	-	-	-	1.5	-	1.4
?	-	-	-	-	-	-	Trace	-	1.7	0.9	-	Trace
Myr.	3.7	4.5	3.1	3.6	1.9	4.2	3.0	2.4	5.9	3.0	6.9	3.3
?	-	-	4.6	0.9	1.3	-	3.1	-	5.5	5.8	5.8	7.9
?	-	-	2.0	-	-	-	1.6	-	4.9	4.1	6.8	2.8
?	-	-	7.3	2.0	2.3	1.2	4.5	-	7.0	7.7	7.8	12.6
?	-	-	5.6	-	-	-	3.6	-	15.7	11.1	29.8	14.9
Pal.	83.9	67.2	44.5	78.6	62.1	72.8	41.1	36.6	23.7	25.2	17.0	17.6
P.O.	1.7	5.5	-	-	-	3.3	3.8	2.8	7.0	5.7	5.6	8.8
Stear.	3.5	2.2	12.7	9.8	26.3	6.5	14.5	12.2	4.9	6.0	-	-
Oleic	7.2	16.4	20.4	5.0	6.2	12.0	24.8	41.7	23.6	29.1	20.4	31.0
Linol.	-	4.3	-	-	-	-	-	4.2	-	-	-	-

Fractional crystallization No. 4. Fraction analyses by gas-liquid chromatography and pancreatic lipase hydrolysis

The purpose of this fractional crystallization and analyses was to obtain the same data as in fractional crystallization No. 3 and by using the same methods but to avoid oxidation of the unsaturated acids.

One major change in the procedures followed involved the recovery and methylation of the fatty acids freed during hydrolysis. The Amberlite IRA-400 columns were not very successful and the batch method followed by recovery of the acids from the resin by the use of a solution containing an organic solvent and an acid required considerable more time and chances for errors than the method used. The method used was that presented by Horstein et al. (40) in which the fatty acids are adsorbed on the resin and then converted to their methyl esters by treating the fatty acids while they are still on the resin with methanol-hydrochloric acid solution. Details of this procedure are given in an appendix.

The hydrolyses were again carried out according to Mattson and Beck (54) using a hydrolysis time of 15 minutes but again without bile salts. Two samples were worked on simultaneously and it was found that they could be hydrolyzed, the free fatty acids recovered, the monoglycerides extracted and their fatty acid methyl esters prepared in the course of about 10 hours.

This included transfer of the methyl esters to small vials for storage under refrigeration until chromatograms were prepared. This procedure was followed to keep oxidation of the samples to a minimum. The results for fractional crystallization No. 4 are given in Tables 29 through 37.

Table 36 is a summary of the fatty acid methyl ester analyses of the fatty acids freed during hydrolysis, the crystallization fractions (triglycerides) and the monoglycerides formed during the hydrolyses. The values are listed to make it easy to see whether the analyses followed the inverse lever rule; the triglyceride analyses should lie numerically between the free fatty acid and monoglyceride values. Actually the triglyceride value should be twice as far from the monoglyceride value as from the free fatty acid value. The free fatty acids represent the overall composition of 2 of the 3 possible triglyceride sites whereas the monoglycerides represent only 1 of the 3 possible positions. Therefore when mol percents are considered, the 2:1 relationship should be expected to hold. A glance at Table 36 shows that there is no need to see whether the 2:1 relationship is obeyed but whether the triglyceride composition falls between that of the free fatty acids and the monoglycerides. Not counting the diacid values and the P-3 column for which hydrolyses were not carried out, there are a possible 60 sets of values which may be compared and 32 of these do not conform to the correct relative order let alone

to the 2:1 relationship.

Oxidation was not the cause of this discrepancy in the data. Even though the hydrolysis is not 100% specific for the 1-, and 3-position fatty acids but probably in the range of 90% specific, the correct relative order of the values would be obeyed.

A study of the possible cause of these results involves 1) adsorption of the free fatty acids by the resin, 2) complete recovery of the fatty acids from the resin, 3) complete esterification of the recovered free fatty acids, 4) obtaining a representative sample of the monoglycerides, 5) complete methylation of the monoglyceride fatty acids, and 6) the proper hydrolysis techniques.

In carrying out fractional crystallization No. 5, each of the 6 points listed were considered and methods used to try to avoid errors from these sources.

In order to make the methyl ester analyses for this crystallization, a considerable amount of time was spent on improving the gas chromatograph and on separating the oxidation products from the monocarboxylic acids of the samples and identifying these oxidation peaks. The equipment changes are described in an appendix. Some of the work with the chromatograph is described below.

The gas chromatograph oven and sample injection system consisted of an insulated metal box attached to one end of the thermal conductivity cell for the column oven and a second smaller box attached to the column oven for the sample injection tube and heater. This system did not give satisfactory chromatograms so another oven and sample injection system were constructed. The oven consisted of an insulated cylindrical cover and a round base to house the column. The sample injection system was constructed such that its temperature changes would not affect the column temperature as was the case with the previous oven and sample injection system. Vapor lines were wrapped with resistance heating wire to prevent condensation of the methyl esters in these lines.

With this improved gas chromatograph, chromatograms were made using columns of 20% ethylene glycol succinate on acid washed 60/80 mesh Chromosorb. Preliminary analyses were made on a 6.5 ft x 3/16 in OD and 5 ft x 1/4 in OD copper tubing columns. Thermal conductivity cell bridge currents of 185-200 milliamperes were used and column temperatures of 210^{o}C. A Sargent SR recording potentiometer was used to make the chromatograms using a range plug of 5.0 millivolts. The carrier gas used was helium and the flowrates used were 70 ml per minute for the 6.5 ft x 3/16 in column and 65 ml per minute for the 5 ft x 1/4 in column. The separations obtained from the

6.5 ft x 3/16 in column did not appear to be any better than on the 5 ft x 1/4 in column even when the temperature and flowrate were varied. The main reason for this was probably because the 5.0 millivolt range plug was being used in the recorder and in order to obtain suitable peaks on the chromatograms, the sample sizes required were overloading the 3/16 in diameter column.

Figure 3 shows the effect of sample size on the separation of stearate-oleate. A sample of lard B was hydrolyzed by the method of Mattson and Beck (54) for 15 minutes but with no bile salts. The fatty acids freed during hydrolysis were recovered by use of Amberlite IRA-400 and methylating them on the resin. The resulting fatty acid methyl esters were analyzed. The chromatograph column temperature varied during the time required to run these two chromatograms but the change was only from about 215 to 218°C which accounts for the difference in the retention times for the two chromatograms. The conditions for the two chromatograms are listed in Table 28.

Table 28. Conditions for the two chromatograms of Figure 3

Chromatogram trace	Dashed	Solid
Recorder range plug, millivolts	5.0	2.5
Column temperature, °C	215-216	218
Thermal conductivity bridge current, milliamperes	200	200
Helium flowrate, ml per minute	80	80
Total area under peaks, square cm	68	56

As the recorder response for a 2.5 millivolt range is twice that for the 5.0 millivolt range, the areas put on the same basis of the 2.5 millivolt range are 136 square cm and 56 square cm which means that the dashed trace corresponds to a sample 2.4 times larger than the solid trace. A 10 microliter syringe was used for sample injection and the sample size was in the 1 microliter range. But the needle held about 1 microliter of sample when the plunger tip was at the zero of the syringe scale. When this needle full of sample was pushed into the sample injection septum as far as possible, about 0.8 microliter of sample was vaporized. But the amount of sample vaporized depended upon the length of time the needle was left in the injection system and with the amount of volatile solvent in the sample. It was difficult to reproduce sample sizes and to measure sample sizes less than 1 microliter up to about 2 microliters. Therefore the total peak area of each chromatogram is the best indication of the sample size used.

The column used for the analyses in Figure 3 was a 10 ft x 3/16 in butanediol succinate plus 1.5 ft x 3/16 in Dow Corning silicone grease column. This column was developed as described in the section on Fractional crystallization No. 5. Results of this later work are given here to help explain why a 6.5 ft column did not appear to give better separations than a 5 ft column.

Fig. 3. Effect of sample size on separation of methyl stearate and methyl oleate

Fig. 4. Mono- and dicarboxylic acid methyl ester chromatograms

Fig. 5. Chromatogram of crystallization fraction F-4 B

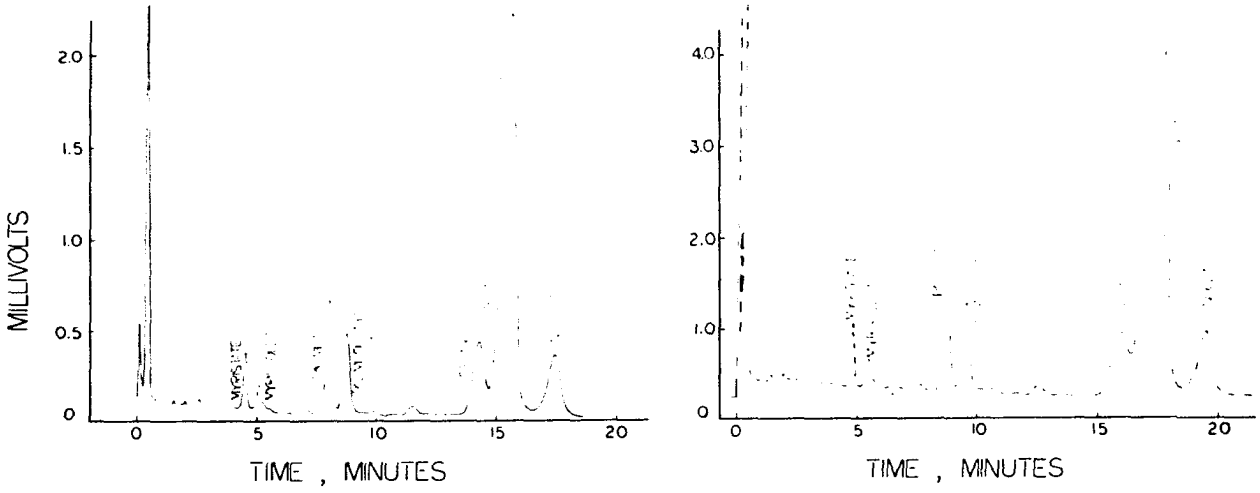


FIG . 3

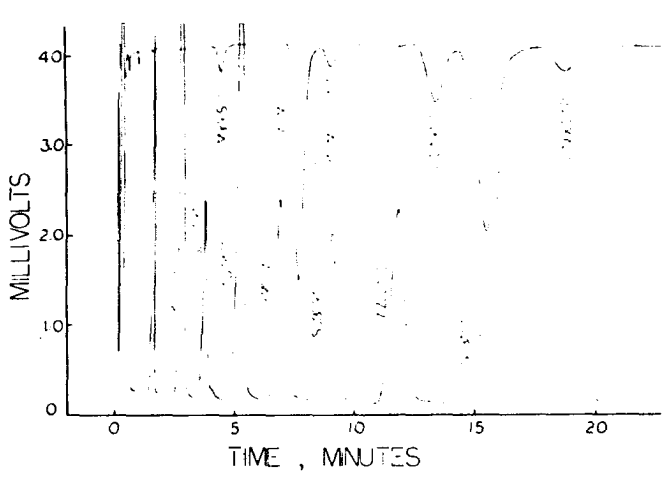


FIG . 4

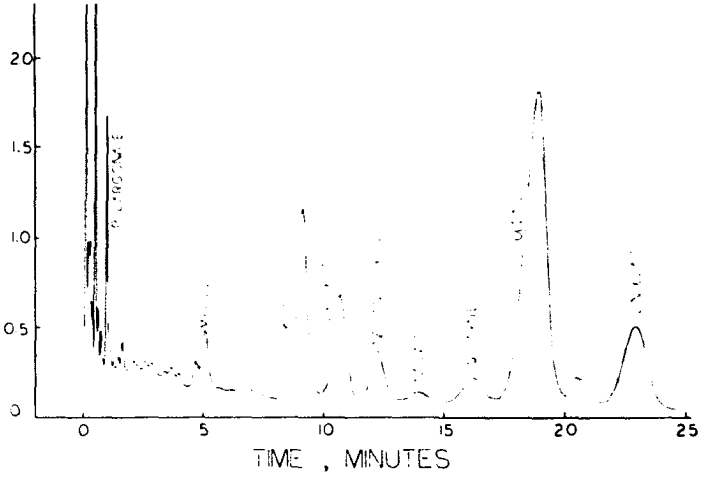


FIG . 5

The set of chromatograms used in the calculations were obtained using the 5 ft x 1/4 in OD copper tubing column repacked with new ethylene glycol succinate packing material. A temperature of approximately 210°C and a helium flowrate of about 60 ml per minute were used. The time of emergence of linoleate from the column was between 21 and 24 minutes. Figure 4 shows the relative positions of the mono- and dicarboxylic acids separated on a similar column several months after the samples of this crystallization were run. The chromatograms were obtained by running the two samples separately but recorded on the same sheet as shown. The chromatograph conditions were identical for both samples. A column temperature of 216°C, a helium flowrate of 67 ml per minute, a bridge current of 200 milliamperes, and a recorder range plug of 5.0 millivolts were used. Under these conditions, palmitoleate and suberate (DiC_8) overlap, oleate and sebacate (DiC_{10}) overlap, and linoleate and DiC_{11} would probably be only partially separated. Not shown on these chromatograms is an azelaaldehydate peak which would appear between suberate and azelate and would not overlap with any of the peaks shown.

Figure 5 is a chromatograph of the F-4 B crystallization fraction and shows the degradation of the sample due to oxidation by the presence of the diacid methyl ester peaks and the azelaaldehydate peak.

One of the objectives of this study of lard was to determine whether the fatty acid distribution in the 1- and 3-position is random or not. Consider the 6 possible types of glycerides in lard based upon saturated and unsaturated fatty acids. These are:

SSS	(GS_3)
SUS	(GS_2U)
USS	(GS_2U)
USU	(GSU_2)
SUU	(GSU_2)
UUU	(GU_3)

In the calculations for the 4 major types of glycerides in lard, (GS_3, GS_2U, GSU_2 and GU_3), it was assumed that there were no more than 2 of the major glyceride types in a given crystallization fraction. Based on this, there are three logical combinations to be expected from the crystallization separation which contain 2 major glyceride types. These are:

SSS	SUS	USU
SUS	USS	SUU
USS	USU	UUU
	SUU	

Suppose that the overall composition of the lard is 60 mol percent unsaturated and 40 mol percent saturated acids. Also suppose that of the 40% S, that 5% is myristic, 25% is palmitic, and 10% is stearic. If the lard were completely randomized, the composition found for the saturated acids in any one of the positions marked S would be 12.5% myristic, 62.5% palmitic, and 25% stearic. If all the acids in the

1-position of the SSS, SUS, and USS fraction were analyzed, the composition would depend upon the relative amounts of the three glyceride types because one of the glycerides contributes unsaturated acids. But the relative amounts of the saturated acids would remain the same. The relative amounts would be 5 : 25 : 10 = 1 : 5: 2 for myristic : palmitic : stearic. The same would be true for an analysis of the fatty acids in a given position for any of the fractions.

Now assume that the lard fatty acids are not completely randomized but that the composition of the fatty acids in the 1- and 3-positions is different than in the 2-position. Assume though that the distribution in the 1- and 3-positions is random and the distribution in the 2-position is random. The fatty acids for all 1- and 3-positions designated by a S should have the same composition. But for the fatty acids in the 1- and 3-positions of a fraction, the composition would vary with the relative amounts of each glyceride type present. But as in the case of the completely randomized lard, the relative amounts of the saturated acids would remain constant for all of the fractions. The same would be true of the 2-position fatty acids for each fraction.

These ratios of myristic : palmitic : stearic could be calculated for each of the lards and their fractions for both the 1- and 3-position and the 2-position fatty acids, but the

myristic peaks are quite small in many cases to be measured accurately and the stearic compositions may be in error in many cases because of the overlapping of stearate-oleate making accurate area measurement impossible.

The palmitic peak measurements were not hindered by either of the above difficulties. Therefore it would be better to find a method of checking the distribution of palmitic on a basis other than the myristic and stearic compositions alone. This can be accomplished by comparing the ratio of palmitic to stearic of the before fraction to the same ratio of the after fraction. As the after sample has been randomized, its ratio of palmitic to stearic should remain constant for all fractions. Errors in the stearic measurement could cause it to vary though. But the same type of variation should be present in the before ratio and therefore comparison of the before and after ratios should tend to cancel the errors in measuring the stearic peak if the measurements were made in a consistent manner. The errors in measuring the myristic peak would be expected to be more random in nature and not as likely to cancel.

Table 37 is a summary of the calculations of this type of ratio. It is clearly seen from the table that the ratios do not remain constant for all fractions which would indicate that the distribution of the saturated fatty acids in the 1-

and 3-positions and in the 2-position of lard B are not random. But as was shown by the figures in Table 36, the data for the free fatty acids and/or the monoglycerides is not reliable because the inverse lever rule is not obeyed. From the variations observed in Table 36 it is not surprising that the distribution ratios are not constant for all the fractions.

Table 29. Weights of crystallization fractions and the weights adjusted for losses during fractionation[a]

Sample	Wt of fraction recovered	Adjusted wt of fraction	Wt of fraction recovered	Adjusted wt of fraction
Lard	50.00	--	50.00	--
P-3	8.12	8.33	3.15	3.19
P-6	5.34	5.48	8.24	8.36
F-6	23.84	24.45	15.12	15.34
F-5	8.69	8.91	14.20	14.40
F-4	2.76	2.83	8.59	8.71
Total	48.75	50.00	49.30	50.00
Loss gms	1.25	--	0.70	--
% loss	2.5	--	1.40	--

[a]Weight of the original lard sample lost was divided between the fractions directly proportional to their weights.

Table 30. Mol percentage of saturated and unsaturated fatty acids in lards and crystallization fractions

	Sample	Mol % S	Mol % U
Before			
	Lard	40.90	59.10
	P-3	72.07	27.93
	P-6	65.84	34.16
	F-6	35.88	64.12
	F-5	15.89	84.11
	F-4	18.82	81.18

Table 30. (Continued)

Sample	Mol % S	Mol % U
After		
Lard	42.54	57.47
P-3	89.43	10.58
P-6	65.63	34.37
F-6	45.99	54.01
F-5	27.71	72.29
F-4	15.97	84.03

Fractional crystallization No. 5. Analyses by gas-liquid chromatography and pancreatic lipase hydrolysis

Introduction Before carrying out this crystallization several studies were made in an attempt to obtain results which would obey the inverse lever rule and give reliable data for determining the distribution of the fatty acids in the different types of glycerides. Six items were listed under Fractional crystallization No. 4 as being possible areas in which errors might be made. The 6 items involved are: 1) adsorption of free fatty acids by resin, 2) complete recovery of fatty acids from the resin, 3) complete methylation of the recovered free fatty acids, 4) obtaining a representative sample of the monoglycerides, 5) complete methylation of the monoglyceride fatty acids and 6) proper enzymatic hydrolysis techniques.

Table 31. Fatty acid methyl ester composition of lard crystallization fractions

	Lard		P-3		P-6		F-6		F-5		F-4	
	B	A	B	A	B	A	B	A	B	A	B	A
Weight percent												
Myr.	1.7	1.7	0.7	2.5	2.0	1.4	1.2	1.8	2.4	1.6	2.7	1.7
Pal.	26.2	26.8	34.6	53.1	39.6	39.8	26.9	29.7	10.3	17.9	12.8	11.5
P.O.	3.3	3.1	0.9	-	1.4	1.8	2.7	2.7	6.5	3.8	9.4	5.8
Stear.	11.5	12.5	36.1	33.4	23.2	23.4	6.1	13.0	2.1	6.9	2.4	1.9
Oleic	46.7	47.0	22.8	11.2	28.2	29.0	54.7	46.2	57.1	60.3	49.1	61.8
Linol	10.6	8.9	5.0	-	4.7	3.9	8.5	6.6	20.7	9.5	19.3	14.3
Semi-DiC_9	-	-	-	-	0.9	0.6	-	-	-	-	3.5	2.0
DiC_9	-	-	-	-	-	-	-	-	0.9	-	0.9	1.1
Mol percent												
Myr.	2.0	2.0	0.8	2.8	2.4	1.6	1.5	2.1	2.9	1.9	3.2	2.0
Pal.	27.0	28.5	36.7	55.2	41.4	41.7	28.6	31.4	10.9	19.2	13.4	12.2
P.O.	3.5	3.3	0.9	-	1.51	1.9	2.9	2.9	7.0	4.1	9.9	6.2
Stear.	11.1	12.0	34.6	31.4	22.0	22.3	5.8	12.5	2.0	6.7	2.3	1.8
Oleic	45.3	45.5	22.1	10.6	27.0	27.7	53.0	44.7	55.6	58.9	46.8	59.7
Linol.	10.4	8.7	4.9	-	4.5	3.8	8.3	6.4	20.3	9.4	18.5	13.9
Semi-DiC_9	-	-	-	-	1.2	0.9	-	-	-	-	4.9	2.9
DiC_9	-	-	-	-	-	-	-	-	1.2	-	1.2	1.4

Table 32. Glyceride composition of lard before and after molecular rearrangement

Fraction	Wt of fraction gms	Mol F.A. / 100 gm F.A.	Total wt % F.A. in glyc.	Mols of glyc.	Mol % of glyc. GS_3	GS_2U	GSU_2	GU_3	Mols of glyc. GS_3x 100	GS_2Ux 100	GSU_2x 100	GU_3x 100
Before												
P-3	8.33	0.3487	95.77	0.00927	16.21	83.79			0.1503	0.7769		
P-6	5.48	0.3532	95.73	0.00618		97.52	2.48			0.6023	0.0153	
P-6	24.45	0.3480	95.78	0.02717		7.64	92.36			0.2075	2.5091	
P-5	8.91	0.3463	95.80	0.00985			47.67	52.33			0.4697	0.5156
P-4	2.83	0.3540	95.78	0.00320			56.46	43.54			0.1806	0.1392
Totals	50.00			0.05567					0.1503	1.5867	3.1747	0.6548
							Mol %		2.7	28.5	57.0	11.8
After												
P-3	3.19	0.3556	95.68	0.00362	68.26	31.74			0.2470	0.1148		
P-6	8.36	0.3525	95.73	0.00940		96.89	3.11			0.9111	0.0292	
P-6	15.34	0.3491	95.75	0.01709		37.97	62.03			0.6490	1.0602	
P-5	14.40	0.3457	95.80	0.01589			83.13	16.87			1.3213	0.2681
P-4	8.71	0.3492	95.81	0.00971			47.91	52.09			0.4654	0.5060
Totals	50.00			0.05572					0.2470	1.6749	2.8761	0.7741
							Mol %		4.4	30.1	51.6	13.9

Table 33. Fatty acid balance of lard crystallization fractions[a]

	Wt in gms	Wt of fatty acids in grams						Total wt of all fatty acids
		Myr.	Pal.	P.O.	Stear.	Oleic	Linol.	
Before								
Lard	50.00	0.815	12.535	1.560	5.520	22.375	5.080	47.885
P-3	8.33	0.052	2.757	0.069	2.876	1.822	0.402	7.987
P-6	5.48	0.106	2.067	0.075	1.214	1.475	0.310	5.247
F-6	24.45	0.291	6.301	0.628	1.416	12.804	1.978	23.418
F-5	8.91	0.208	0.872	0.555	0.179	4.856	1.866	8.536
F-4	2.83	0.073	0.341	0.249	0.063	1.305	0.680	2.711
	50.00	0.730	12.338	1.576	5.748	22.262	5.236	47.890
After								
Lard	50.00	0.825	12.855	1.470	5.970	22.510	4.260	47.890
P-3	3.19	0.075	1.619	--	1.018	0.340	--	3.052
P-6	8.36	0.111	3.174	0.145	1.869	2.313	0.390	8.002
F-6	15.34	0.261	4.360	0.402	1.905	6.793	0.968	14.689
F-5	14.40	0.213	2.470	0.521	0.955	8.322	1.315	13.796
F-4	8.71	0.137	0.950	0.478	0.154	5.087	1.539	8.345
	50.00	0.797	12.573	1.546	5.901	22.855	4.212	47.884

[a]The weight of the oxidation products was converted to an equivalent weight of linoleic acid in these calculations.

Table 34. Composition of 1- and 3-position fatty acids in lards and crystallization fractions

	Lard		P-6		F-6		F-5		F-4	
	B	A	B	A	B	A	B	A	B	A
Weight percent										
Myr.	0.7	1.9	0.6	1.7	0.9	2.0	1.0	1.7	0.6	1.7
Pal.	6.9	27.3	15.1	40.1	11.2	28.4	11.9	25.9	8.8	14.1
P.O.	1.9	2.8	1.8	1.6	1.9	3.1	2.6	3.3	4.5	5.8
Semi-DiC_9	-	-	0.5	-	-	-	-	-	0.6	0.9
DiC_9	-	0.8	-	-	-	-	-	1.4	2.2	1.7
Stear.	15.7	16.6	22.9	26.8	13.6	15.2	13.7	19.3	9.2	5.3
Oleic	61.4	42.8	48.6	26.9	64.8	45.6	57.1	41.8	44.8	55.9
Linol.	13.5	7.8	10.5	2.9	7.6	5.8	13.7	6.5	29.3	14.7
Mol percent										
Myr.	0.8	2.3	0.7	2.0	1.1	2.4	1.2	2.0	0.8	2.1
Pal.	7.5	28.9	16.2	42.2	12.1	30.1	12.8	27.4	9.4	14.9
P.O.	2.0	3.0	1.9	1.7	2.1	3.3	2.9	3.5	4.8	6.2
Semi-DiC_9	-	-	0.8	-	-	-	-	-	0.9	1.2
DiC_9	-	1.0	-	-	-	-	-	1.9	3.0	2.3
Stear.	15.4	15.9	22.3	25.6	13.3	14.6	13.4	18.5	8.9	5.1
Oleic	60.8	41.3	47.7	25.8	63.9	44.1	56.2	40.3	43.6	54.0
Linol.	13.5	7.6	10.4	2.8	7.5	5.6	13.5	6.3	28.7	14.3

Table 35. Composition of 2-position fatty acids in lards and lard fractions

	Lard		P-6		F-6		F-5		F-4	
	B	A	B	A	B	A	B	A	B	A
Weight percent										
Myr.	4.7	2.4	5.5	2.6	4.9	2.9	5.8	2.5	4.5	2.2
DiC_7	-	-	-	-	-	-	-	-	0.8	0.5
Pal.	66.1	28.4	85.9	45.6	61.3	37.9	34.3	31.6	29.0	17.5
P.O.	5.4	4.1	-	2.8	4.3	3.0	9.1	3.6	10.4	5.4
Semi-DiC_9	-	1.9	-	1.5	-	1.0	2.3	3.4	2.3	2.9
DiC_9	-	0.5	-	-	-	-	0.9	0.7	1.4	0.8
Stear.	2.7	9.9	4.8	21.7	5.1	13.5	4.6	14.8	3.3	3.4
Oleic	16.0	44.2	3.8	23.8	21.0	37.7	35.8	38.7	30.3	55.1
Linol.	5.1	8.7	-	2.1	3.4	4.3	7.3	4.9	16.8	12.3
DiC_{12}[a]	-	-	-	-	-	-	-	-	1.9	-
Mol percent										
Myr.	5.3	2.8	6.2	3.0	5.6	3.4	6.6	2.9	5.1	2.5
DiC_7	-	-	-	-	-	-	-	-	1.2	0.7
Pal.	67.1	29.7	86.0	47.2	62.5	40.1	35.1	32.7	29.5	18.3
P.O.	5.6	4.3	-	2.9	4.4	3.2	9.4	3.7	10.7	5.7
Semi-DiC_9	-	2.6	-	2.1	-	1.4	3.2	4.8	3.1	4.1
DiC_9	-	0.7	-	-	-	-	1.2	0.9	1.8	1.1
Stear.	2.5	9.4	4.4	20.3	4.7	12.9	4.3	13.9	3.0	3.2
Oleic	14.8	42.2	3.4	22.5	19.6	34.9	33.4	36.5	28.1	52.6
Linol.	4.7	8.4	-	2.0	3.2	4.2	6.8	4.6	15.7	11.8
DiC_{12}[a]	-	-	-	-	-	-	-	-	2.0	-

[a]This peak had a retention time corresponding to DiC_{12} on a plot of logarithm of retention time ratio (unknown peak/palmitate) vs number of carbons for the diacid series. This was not sufficient evidence to prove this peak was DiC_{12} but was listed in this manner for identification.

Table 36. Summary of fatty acid compositions

	Lard		P-3		P-6		F-6		F-5		F-4	
	B	A	B	A	B	A	B	A	B	A	B	A
F.F.A.	0.8	2.3	--	--	0.7	2.0	1.1	2.4	1.2	2.0	0.8	2.1
Triglyc. Myr.	2.0	2.0[a]	0.8	2.8	2.4	1.6[a]	1.5	2.1[a]	2.9	1.9[a]	3.2	2.0[a]
2-mg	5.3	2.8	--	--	6.2	3.0	5.6	3.4	6.6	2.9	5.1	2.5
F.F.A.	7.5	28.9	--	--	16.2	42.2	12.1	30.1	12.8	27.4	9.4	14.9
Triglyc. Pal.	27.8	28.5[a]	36.7	55.2	41.4	41.7[a]	28.6	31.4	10.9[a]	19.2	13.4	12.2[a]
2-mg	67.1	29.7	--	--	86.0	47.2	62.5	40.1	35.1	32.7	29.5	18.3
F.F.A.	2.0	3.0	--	--	1.9	1.7	2.1	3.3	2.9	3.5	4.8	6.2
Triglyc. P.O.	3.5	3.3	0.9	--	1.5	1.9	2.9	2.9[a]	7.0	4.1[a]	9.9	6.2[a]
2-mg	3.6	4.3	--	--	--	2.9	4.4	3.2	9.4	3.7	10.7	5.7
F.F.A.	15.4	15.9	--	--	22.3	25.6	13.3	14.6	13.4	18.5	8.9	5.1
Triglyc. Stear.	11.1	12.0	34.6	31.4	22.0[a]	22.3	5.8	12.5[a]	2.0[a]	6.7[a]	2.3[a]	1.8[a]
2-mg	2.5	9.4	--	--	4.4	20.3	4.7	12.9	4.3	13.9	3.0	3.2
F.F.A.	60.8	41.3	--	--	47.7	25.8	63.9	44.1	56.2	40.3	43.6	54.0
Triglyc. Oleic	45.3	45.5[a]	22.1	10.6	27.0	27.7[a]	53.0	44.7[a]	55.6[a]	58.9[a]	46.8[a]	59.7[a]
2-mg	14.8	42.2	--	--	3.4	22.5	19.6	34.9	33.4	36.5	28.1	52.6
F.F.A.	13.5	7.6	--	--	10.4	2.8	7.5	5.6	13.5	6.3	28.7	14.3
Triglyc. Linol.	10.4	8.7[a]	4.9	--	4.5	3.8[a]	8.3[a]	6.4[a]	20.3[a]	9.4[a]	18.5	13.9
2-mg	4.7	8.4	--	--	--	2.0	3.2	4.2	6.8	4.6	15.7	11.8
F.F.A. Di-	--	1.0	--	--	0.8	--	--	--	--	1.9	3.0	2.3
Triglyc. acids	--	--[a]	--	--	1.2[a]	0.9	--	--[a]	1.2	--[a]	6.1	4.3
2-mg	--	3.3	--	--	--	2.1	--	1.4	4.4	5.7	8.1	5.9

[a]The triglyceride fatty acid composition does not lie between the free fatty acid (1- and 3-position) and the monoglyceride (2-position) fatty acid compositions.

Table 37. Saturated fatty acid distribution in lard crystallization fractions

	Lard		P-6		F-6		F-5		F-4	
	Position 2	Position 1 and 3	Position 2	Position 1 and 3	Position 2	Position 1 and 3	Position 2	Position 1 and 3	Position 2	Position 1 and 3
$\frac{Myr_B}{Pal_B}$ $\frac{Myr_A}{Pal_A}$	0.84	1.34	1.13	0.91	1.06	1.14	2.12	1.28	1.26	0.60
$\frac{Pal_B}{Stear_B}$ $\frac{Pal_A}{Stear_A}$	8.48	0.27	8.37	0.44	4.28	0.94	3.47	0.65	1.72	0.36
$\frac{Stear_B}{Myr_B}$ $\frac{Stear_A}{Myr_A}$	0.140	2.79	0.105	2.49	0.221	1.99	0.136	1.21	0.459	4.57

Items 1) and 2) were studied under Fractional crystallization No. 3. Items 3) and 5) both concern methylation and can be studied together. Items 4) and 6) are treated separately and in considerable detail.

Some qualitative information was also obtained about the oxidation of glyceride samples and fatty acid methyl esters during storage under refrigeration.

Again, improvements on the gas chromatograph were made and a search was made for a column which would give better separation of the monocarboxylic acid methyl esters and the methyl esters of the oxidation products. Although it was not absolutely necessary to obtain complete separation of all

the monocarboxylic acids and the oxidation product methyl esters for studying the lards, crystallization fractions and the 1- and 3-position and 2-position fatty acid methyl esters, it was anticipated that such a gas-liquid chromatography column would be needed for the next phase of the study in which complete oxidation of the unsaturated portions of the fat were to be studied.

In studying the distribution of the fatty acids in the different positions of the glycerides, it was assumed that the lard after sample was randomized. Even if the rearrangement as conducted by the Rath Packing Company were not completely random but was a directed rearrangement, the calculations would still be valid. As trisaturated glycerides were crystallized from the reaction mixture the proportion of saturated and unsaturated acids remaining in the liquid portion of the reaction mixture would change but the relative amounts of the saturated acids remaining in the liquid portion of the reaction mixture would remain constant because the interesterification process proceeds in a random manner. A similar statement would be true for the relative amount of unsaturated acids in the liquid phase.

In order to determine whether the lard after sample was completely randomized or directly rearranged it was compared to a sample of the lard before which was randomized such that

a second phase was not formed during interesterification. This randomized lard sample was carried through the same procedures as were the lard before and after samples. The randomized sample is referred to as "lard random" and "lard R". The letter "R" is used to designate fractions from this randomized lard.

Methylation of free fatty acids and glyceride fatty acids A check was made on whether or not the fractional crystallization No. 4 samples had oxidized during storage for approximately 9 months in 125 ml Erlenmeyer flasks under refrigeration. Seven to eight drops of F-4 A glycerides were refluxed with 5 ml of 0.5% anhydrous hydrochloric acid-methanol solution for 15 minutes, the alcohol solution evaporated under a water aspirator vacuum and the methyl esters transferred to a $\frac{1}{2}$ dram vial. A sample of F-5 A glycerides was treated in a similar manner. Table 38 includes the results for these two samples. The results compared to the original compositions of these two fractions indicated that there were now more oxidation products in the F-4 A sample but not in the F-5 A sample. A closer study of the results indicates that the F-5 A sample had considerably higher saturated acid compositions than the original analysis showed. The F-4 A sample compositions agreed fairly well except for the 18-carbon components. Here there are two effects involved. First is the expected decrease in the unsaturated acid compo-

sitions because of oxidation in which the linoleic acid composition would be expected to decrease by a greater amount than the oleic acid composition. Second is the effect of the baseline changing during the time a trace above the baseline is being made and the resultant error caused by trying to estimate where the baseline should be drawn. The second effect appears to have been predominate in the case of the F-4 A samples.

More F-5 A sample was available for study than the F-4 A sample and was used in continuing the study. Four samples were prepared by varying the amount and type of catalyst used for the methylation and by using different methods of recovering the methyl esters. The time of reflux for these four samples was 30 minutes.

In preparing these methyl esters, it was observed that the triglycerides were not completely soluble in the hydrochloric acid-methanol solution. As the time of reflux was increased from 15 to 30 minutes, the composition of oleic and linoleic acids increased in the methyl ester analysis. This indicated that under these conditions methylation of the saturated acids took place more readily than for the unsaturated acids. It was also observed that it took more methyl ester sample of column 3 (Table 38) than for the sample of column 4 to obtain the same total peak area on the chromato-

grams. This indicated that there had been incomplete methylation of the sample which did not vaporize and pass through the chromatograph column.

Luddy et al. (50) gave data which show that methanolysis of glycerides is complete in 5 minutes using a 0.2 N methanol solution of potassium methoxide but for the use of hydrochloric acid-methanol, the minimum time used in their study was 30 minutes for which complete methylation was accomplished.

The conclusions of these tests on the F-4 A and F-5 A samples were:

1. The glyceride fraction F-5 A had undergone some oxidation during storage as the linoleic acid composition dropped from 9.5 to 6.8%.

2. The methyl esters used for the original analysis of the F-5 A fraction were oxidized to about the same degree, as the same methyl ester sample run at a later date gave a linoleic acid composition of 6.6% as compared to 9.5% when originally run.

3. The amount of oxidation of the linoleic acid chains in the F-5 A triglyceride fraction was approximately 2.7% of the total fatty acids in the fraction and 28.4% of the linoleic acid originally present. It should be noted that the first methyl ester sample prepared (original methyl esters) were not chromatographed until about 4 months after they were

Table 38. Oxidation during storage and methylation tests[a]

	Composition of F-4 A triglycerides		Composition of F-5 A triglycerides						
			1	2	After storage of triglyc.				
	After storage	Orig. composition	Orig. methyl ester anal.	After storage of orig. methyl esters	3	4	5	6	7
Myr.	1.7	1.7	1.6	1.7	1.9	1.7	1.7	1.6	1.6
Pal.	11.8	11.5	17.9	18.9	24.6	19.2	18.7	19.9	19.1
P.O.	5.0	5.8	3.8	3.9	3.6	3.8	3.8	3.6	3.8
Semi-DiC_9	0.3	2.0	-	0.5	0.3	0.9	0.3	0.3	0.4
DiC_9	0.7	1.1	-	0.3	0.2	0.5	0.5	0.5	0.4
Stear.	1.6	1.9	6.9	6.7	12.3	7.6	7.3	8.5	7.1
Oleic	66.1	61.8	60.3	61.5	52.4	59.9	61.1	58.6	60.9
Linol.	12.9	14.3	9.5	6.6	4.7	6.5	6.7	6.9	6.8
No. chromatog.	1	3	3	2	2	3	2	3	2
Catalyst	0.5% HCl	About 5% HCl	About 5% HCl	About 5% HCl	0.5% HCl	2.4% HCl	2.4% HCl	0.5% HCl	$NaOCH_3$
Reflux, min.	15	over 60	over 60	over 60	15	30	30	30	30
Recovery[b]	Evap.	Extr.	Extr.	Extr.	Evap.	Extr.	Evap.	Evap.	Extr.

[a]Area percents.

[b]Evap. means methyl esters were recovered by evaporating the hydrochloric acid-methanol solution under vacuum. Extr. means the methyl esters were recovered by extracting from the reflux solution by use of ethyl ether.

prepared. The time of storage of the methyl esters after the first chromatogram was prepared until the second chromatogram was prepared was about 3 months.

4. In forming the methyl esters from the glycerides, at least 30 minutes reflux time should be used when anhydrous hydrochloric acid is used as the catalyst.

5. A hydrochloric acid concentration of 2.4% is preferable to 0.5%.

6. The methods of recovering the fatty acid methyl esters did not affect the compositions obtained; evaporation of the alcohol and extraction of the methyl esters gave the same compositions.

7. Incomplete methylation of the glycerides gave a different methyl ester composition than when the methylation was complete. Incomplete methylation gave high compositions for the unsaturated acids. No conclusions were drawn as to the effect of chain length of the fatty acids.

In preparing chromatograms of the methyl esters of the fatty acids freed during hydrolysis for fractional crystallization No. 4, it was observed that considerably more sample was required to obtain the desired peak areas for measurement. The methyl esters of the free fatty acids formed from F-6 A were particularly difficult in that this sample was always accompanied by bad baseline drift and an unsteady signal to the recorder.

The remaining methyl esters (one or two drops) of the free fatty acids from the F-6 A hydrolysis were refluxed for 1 hour with 10 ml of 2.4% anhydrous hydrochloric acid-methanol solution followed by evaporating the alcohol under vacuum. A peak now appeared before myristate and comparison to a known diacid methyl ester sample indicated that this new peak was dimethyl succinate (DiC_6). The results of four chromatograms of this sample compared to the original sample composition are given in Table 39.

Again, the data of Table 39 indicate that incomplete esterification gave high compositions for the saturated fatty acids and a low value for linoleic acid but very little difference for oleic. The differences between the compositions before and after re-esterification are quite small compared to the differences obtained for glycerides in Table 38.

Table 39. Re-esterification of the fatty acid freed during hydrolysis of F-6 A crystallization fraction

	Area percent					Original methyl esters
Chromatogram	1	2	3	4	Average	Av. of 3
DiC_6	1.78	1.63	1.60	1.63	1.7	-
Myr.	1.71	1.71	1.67	1.89	1.7	2.0
Pal.	27.67	27.19	27.54	27.16	27.3	28.4
P.O.	2.96	2.51	2.63	2.60	2.7	3.1
Semi-DiC_9	0.55	trace	0.41	0.25	0.4[a]	trace
DiC_9	0.36	trace	0.26	0.28	0.4[a]	trace
Stear.	13.54	13.91	13.79	**14.40**	13.9	15.2
Oleic	44.78	45.92	46.04	**45.38**	45.4	45.6
Linol.	6.64	7.13	6.04	**6.41**	6.6	5.8

[a]Calculated by putting 0.55 and 0.36 in the semi-DiC_9 and DiC_9 spaces for chromatogram 2 and normalizing the values in column 2 before calculating the average.

When Amberlite IRA-400 is used to recover free fatty acids from an ether solution of the free fatty acids and mixed glycerides, it becomes necessary to insure that all of the fatty acids are recovered. If not, a representative sample is not obtained as the resin selectivity tests showed. Therefore a series of samples were run to determine the best procedure to be followed when using Amberlite IRA-400 to recover the free fatty acids and when the methyl esters of the adsorbed fatty acids were formed by adding hydrochloric acid-methanol solution to the resin.

In the method used by Hornstein et al. (40), it was reported that 60-95% absolute recoveries of free fatty acids in fat were obtained. As their procedure was for the quantitative determination of free fatty acids in a fat sample, it was necessary for them to use an internal standard. A weighed amount of a fatty acid not present in the sample was added to the sample and the calculations then based upon the fat sample weight, the weight of free fatty acid added, and the composition of the fatty acids, including the internal standard, recovered from the fat.

As a quantitative determination of the free fatty acids in the hydrolysis products was not necessary, it was desired only to insure that a representative sample of the free fatty acids was obtained. Therefore an internal standard was not used.

The general procedure followed for all the samples in this series of tests was to use about 1 gram of fatty acids dissolved in 50 ml of ethyl ether, 15 grams of Amberlite IRA-400 (-OH form) added, stirred 5 minutes, the ether decanted from the resin, and the resin washed twice with 20 ml portions of ether. Methylation and recovery of the fatty acids adsorbed on the resin was accomplished by stirring 25 ml of hydrochloric acid-methanol solution and the resin for 25 minutes. The hydrochloric acid-methanol solution was decanted from the resin and the resin washed 2 times with either 15 ml portions of the hydrochloric acid-methanol solution and/or with 20 ml portions of ether. The combined hydrochloric acid-methanol solution and the washes were then treated to recover the methyl esters by evaporating the ether and methanol, adding 50 ml of ether to the residue, and washing the ether solution with water. The ether solution was then dried over anhydrous sodium sulfate and the methyl esters transferred to $\frac{1}{2}$ dram vials. Table 40 lists the procedure conditions and the chromatographic results of these tests.

Sample 1 was the preparation of the methyl esters of the lard fatty acid sample used in these tests to use for comparison of the methyl ester compositions obtained for the test samples.

For samples 2 and 3, the amount of methyl esters recovered indicated that neither of these samples had been completely

recovered. The amount of fatty acids which were not adsorbed was not determined but the amount of methyl esters transferred to the ½ dram vials was considerably less than the original sample. Chromatograms of all three samples were run on the same sheet of paper using the same sample size and the same chromatograph conditions. Sample 2 had about the same total area as sample 1 but sample 2 had a taller palmitate peak which the resin tests showed to be true when only part of a fatty acid sample was adsorbed. Sample 3 gave about 3/4 the area of samples 1 and 2 which indicated that the 1% hydrochloric acid-methanol solution had not been adequate in forming the methyl esters. Chromatogram peak areas were not calculated. The conclusions for samples 2 and 3 were that the resin had not adsorbed all the fatty acids and that the 1% hydrochloric acid-methanol solution did not completely methylate the adsorbed fatty acids.

Difficulty with the resin capacity dropping below its expected capacity of about 1 gram of fatty acids for 15 grams of resin had been observed in other work with the resin. The cause of this was never determined even though tests were conducted using different strengths of sodium hydroxide to convert the chloride form to the hydroxide from as well as different organic solvents for removing some of the water from the resin. It was found that satisfactory results could be obtained by preparing the resin just before it was needed but

if stored for more than several hours, the resin could not be used with confidence. In the remainder of these tests, the resin was prepared as it was used.

Sample 4 was run to determine whether resin from the batch used for samples 2 and 3 could be converted to the hydroxide form again and recover its lost capacity. The resin was treated by adding 50 ml of 5% aqueous sodium hydroxide to 15 grams of the resin and occasionally stirred during a 10 minute period. The resin was washed with distilled water until the wash water was neutral to litmus paper, washed 3 times with acetone and finally washed 2 times with ether. This resin wet with ether, was used without further drying. Sixty drops of oleic acid were then treated by the same procedure as were samples 2 and 3 using 9.6% hydrochloric acid-methanol solution. The ether decanted from the resin after adsorbing the fatty acids and the ether washes were combined and evaporated. There was only about one drop of fatty acids of the original 60 drop sample which had not been adsorbed. A chromatogram of the recovered methyl esters indicated that the sample had been completely methylated based on the area obtained from the injection sample size used. Peak areas were not calculated. The conclusion was that the resin could be regenerated to its original capacity and that 9.6% hydrochloric acid-methanol gave good results.

Sample 5 was run using resin prepared as described above and by using the conditions listed in Table 40. The 3% hydrochloric acid-methanol solution did not give complete methylation. A chromatograph indicated that the sample consisted of 1/5 or less methyl esters. The methyl ester sample was then treated with 5.0% hydrochloric acid-methanol to complete the methylation. The results of a chromatogram of the re-esterified sample are shown in Table 40 under sample 6.

Sample 7 was run using 5% hydrochloric acid-methanol which gave satisfactory results. Sample 8 demonstrated the use of 1-25 ml portion of 5% hydrochloric acid-methanol and 10 minutes contact time with the resin. The point being checked here was that the methylation actually continues during the evaporation of the ether and methanol and that the contact time with the resin needed to be only long enough for the resin color to change from dark yellow-brown to light yellow. There must be enough hydrochloric acid remaining in the solution to catalyze the methylation reaction after the resin has been completely converted to the chloride form. Ether was used to insure all the desorbed fatty acids were recovered from the resin. The results of the chromatogram of sample 8 indicated that the myristate, palmitate and palmitoleate values were slightly high. To check whether this was due to incomplete methylation, the methyl esters of sample 8 were again methylated and the results of this chromatogram are shown as sample 9.

The results of sample 8 and 9 are in good agreement indicating that the slightly high values were due to selectivity of the resin.

Sample 10 was run under the identical conditions of sample 8 except that 7.6% hydrochloric acid-methanol solution was used and the hydrochloric acid-methanol-ether solution was dried over anhydrous granulated sodium sulfate before evaporating the ether and alcohol. The methyl ester residues were not washed with water either before or after the evaporation. This test indicated that it was not necessary to wash the methyl esters after evaporating the ether and alcohol.

The conclusions of the study were:

1. The resin used to adsorb the fatty acids should always be checked to insure it has sufficient capacity to pick up all the fatty acids in the sample.

2. If all the fatty acids are not adsorbed from the solution by the resin, those adsorbed will not be a representative sample of the fatty acids. The shorter chain fatty acid compositions will be high.

3. A representative methyl ester sample can be obtained by the use of 5% or stronger hydrochloric acid-methanol solution. One 10 minute contact of the adsorbed fatty acids-resin with 25 ml of the 5% solution followed by 2 ether washes and evaporation of the solvents gives a satisfactory methyl ester

Table 40. Study of free fatty acid recovery on Amberlite IRA-400 and methylation on the resin

Sample no.	Procedure conditions 1	2	3	4	5	6	7	8	9	10
Fatty acid sample	Lard F.A.	Lard F.A.	Lard F.A.	Oleic acid	Lard F.A.	M.E. 5	Lard F.A.	Lard F.A.	M.E. 8	M.E. F.A.
Drops of sample not adsorbed	--	Not all adsorb.	Not all adsorb.	About 1	About 1-2	-	About 2	About 3	-	About 3
Drops of samples used	--	About 60	About 60	60	60	-	60	60	-	60
Conc. of HCl-MeOH	7.6%	9.6%	1.0%	9.6%	3.0%	5.0%	5.0%	5.0%	7.6%	7.6%
Portions of HCl-MeOH used/min stirred	--	1-25 ml 25 min 2-15 ml 5 min	1-25 ml 25 min 2-15 ml 5 min	1-25 ml 25 min 2-15 ml 5 min	1-25 ml 25 min	--	1-25 ml 25 min 2-15 ml 1 min	1-25 ml 10 min	--	1-25 ml 10 min
Ether washes	--	No	No	No	2-20 ml	--	1-15 ml	2-20 ml	--	2-20 ml
Water washes	yes	yes	yes	yes	yes	yes	yes	yes	yes	no
Methylation complete	yes	yes	no	yes	no	yes	yes	?	yes	yes

Table 40. (Continued)

Sample no.	Procedure conditions 1	2	3	4	5	6	7	8	9	10
	Chromatograph area percent									
DiC_4	0.2					0.3	trace	trace	0.4	0.3
Myr.	1.6					1.6	1.6	1.9	1.6	1.6
Pal.	27.5					26.9	27.6	28.3	28.3	28.8
P.O.	3.1					2.9	3.0	3.5	3.3	3.4
Semi-DiC_9	0.7					0.4	0.8	0.7	0.7	0.9
DiC_9	0.3					0.2	0.2	0.4	0.3	0.3
Stear.	12.8					12.6	12.8	12.4	13.3	12.5
Oleic	47.4					48.9	47.7	46.6	46.6	46.2
Linol.	6.5					6.3	6.3	6.2	5.5	6.0

sample. Washing with water to remove hydrochloric acid is not necessary for preparing gas-liquid chromatograph samples. Drying of the ether-methanol-fatty acid methyl ester solution over sodium sulfate may be used to remove water from the sample before evaporation.

The use of Amberlite IRA-400 for recovery of free fatty acids from a fat sample or from enzymatic hydrolysis products is probably the best method available to do the job when the actual separation is necessary. If the exact amount of the free fatty acids is not necessary, but the composition of the free fatty acids is desired, then another method is available which avoids the difficulties encountered with the resin but adds the hazards of a poisonous gas.

The method referred to is the use of diazomethane to form methyl esters of free fatty acids in the presence of mono-, di-, and triglycerides. Details of the preparation of ethereal diazomethane are given in an appendix describing methyl ester preparation.

This method of determining the free fatty acid composition involves treating a small sample of fat containing free fatty acids with an excess of freshly prepared ethereal solution of diazomethane. The ether and excess diazomethane are then evaporated leaving behind a residue consisting of the fatty

acid methyl esters and unreacted fat. A sample of this residue is injected into a chromatograph to analyze the methyl esters present. The mono-, di-, and triglycerides do not vaporize and remain in the first few inches of the column. The chromatograph sample size depends upon the amount of methyl esters in the sample. By comparing the total peak area of a pure methyl ester sample and the sample of fat and methyl esters, a rough approximation of the amount of fatty acids in the fat sample can be made. When the amount of methyl esters in the sample is low, the sample size injected into the chromatograph is several microliters. When several such large amounts of fatty material containing only a small amount of methyl esters have been injected into a column, the difficult separations become noticeably poorer and the column separation power can only be partially recovered by bleeding the column for several hours. By bleeding is meant to run helium gas through the column at its normal or slightly above operating temperature to elute material which is passing very slowly through the column and reducing its separation efficiency.

An estimation of the amount of free fatty acids in the fat may be made by this method. Comparison of the total peak areas obtained for identical sample sizes of the unknown sample and of a pure methyl ester sample gives an estimate of the amount of methyl esters in the unknown sample.

Monoglyceride recovery In recovery of the monoglycerides from the enzymatic hydrolysis products by liquid-liquid extractions, no tests were conducted to determine whether or not diglycerides were also extracted. The monoglycerides obtained by this method are probably representative of the monoglycerides in the sample regardless of whether or not all of them are recovered because of the difficulty of separating 1-mono- and 2-monoglycerides and of separating monoglycerides differing only in the length of chain or degree of unsaturation in the fatty acid chain.

Because of the uncertainty above, it was thought to be necessary either to study the monoglyceride extraction in more detail or to use a method for which the degree of separation was known. Later work anticipated was to involve the use of silica gel chromatographic columns. Therefore further study of the extraction method was not made but the use of silica gel columns was studied.

Quinlin and Weiser (76) and Ravin et al. (77) gave details of methods of separating mono-, di-, and triglycerides using silica gel columns but used different solvent. The method of Quinlin and Weiser (76) used a benzene-ethyl ether solvent system whereas Ravin et al. (77) used isopropyl ether-ethyl ether-isooctane-ethanol. Familiarization columns using the method of Quinlin and Weiser (76) were run. It was then de-

cided that it would be better to try to develop a solvent system in which benzene was eliminated because of its toxic effects. In analyzing a large number of samples, sufficient hood space was not available to handle both the columns and evaporation of the fractions collected which required running the columns on the laboratory bench open to the atmosphere unless hoods were constructed.

A solvent system of Skellysolve B and ethyl ether was developed to separate the mono-, di-, and triglycerides. The results of what appeared to be a satisfactory separation are shown in the data of Table 41 and Figure 6. The three major peaks, 2, 3, and 5, were expected to be mono-, di-, and triglyceride in that order, but the smaller peaks obtained required more study. Other chromatograms indicated that peak 2 could be further separated into two components. This rechromatographed peak is also shown on Figure 6. The problem now was to identify the component in each of these peaks. In looking for a method of analysis it was found that the sample size required for hydroxyl analyses by the method of Pohle and Mehlenbacher (72) and of Ogg et al. (67) was larger than the total weight of the smaller peaks. The use of infrared spectrophotometry was then considered. In a review of the use of infrared spectrophotometry in the fat and oil field, O'Connor (65) mentioned the work of O'Connor et al. (66) with mono-, di-, and triglycerides which gave qualitative results with the

possibility of being able to give quantitative results. Infrared spectra of the seven peaks were obtained through the Iowa State University Chemistry Department. These spectra are shown in Figures 7 and 8 for 6 of the total 7 peaks obtained in Figure 6. The portions of O'Connor et al. (66) used to interpret these spectra are quoted below.

> The 3.0 micron O-H stretching vibration. Two bands with maxima at about 2.7 and 2.8 microns are found in this region (Table III). The combined intensities of these bands are considerably greater in the spectra of the monoglycerides than in those of the diglycerides and they are completely absent in the spectra of triglycerides. The 2.7 micron band arises from a stretching vibration of a free O-H group. According to.... the 2.8 micron band most probably arises from an O-H stretching vibration of the single bridged dimer. The stretching vibration of more highly hydrogen bonded O-H is masked by the strong C-H stretching vibration at 3.3 microns.
>
> Observations of the infrared spectra of a triglyceride at these wave-lengths provide a ready check as to the presence of any mono- or diglyceride impurity. For example,....
>
> It would not be possible, from measurements in the 3 micron region, to determine whether or not the impurity in a triglyceride was a mono- or diglyceride. Nor could the presence of monoglyceride in diglyceride, or conversely the presence of diglyceride in a monoglyceride be detected. Fortunately, as will be shown, it does seem possible to make these distinctions in the region of C-O stretching."
>
> The 9.0 micron C-O stretching vibrations. All long-chain glycerides exhibit bands at 8.9 and 9.1 microns, as shown in Table IV. The weaker band at 8.9 microns is not observed in the spectra of the mixed short- and long-chain glycerides (TAble IV).
>
> Bands about the region 8.9 to 9.1 microns have been assigned to the C-O stretching vibration of the C-O-C ether

> group and probably arise in these compounds from stretching vibrations in the C-O-C portion of the COOR ester group. This group would give rise to two C-O stretching vibrations, $R-C^{1}-O_{2}-C$ (with C^{1} = O), (1) differing in frequency from (2) by reason of the unsaturated nature of the carbon atom.

It has been

> demonstrated that substitution on the carbon atom, alpha to the hydroxylated carbon, will produce a considerable shift of band maxima to lower frequencies and cause considerable overlapping of bands characteristic of the C-O stretching of the C-O-H group in the spectra of glycerides at longer wavelength. The bands are observed at 9.5 and 9.6 microns in the spectra of all glycerides which contain the C-O-H group.
>
> The band with maximum at 9.6 microns is observed only in the spectra of diglycerides (with the single exception of dipalmitolactin, the spectra of which resembles that of a diglyceride). It would appear logical therefore to assign this band to the C-O stretching of an alpha substituted secondary alcohol, ...The band arising from C-O stretching of alpha substituted primary alcohols (at 9.6 microns in the spectra of straight-chain unsubstituted compounds) would be shifted above the 9 micron region considered here. It is significant perhaps to observe that the spectra of all monoglycerides which contain a primary alcohol exhibit bands with maxima between 10.1 and 10.2 microns, not observed in the spectra of di- and triglycerides. In the spectra of the monoglycerides the shift in frequency of the C-O stretching band of the secondary alcohol might be expected to be somewhat different from that exhibited by the diglycerides. In the spectra of all monoglycerides a band is exhibited with maxima at 9.5 microns, and an inflection is noted at 9.4 microns. The band at 9.5 microns could be correlated with a C-O stretching of an alpha substituted secondary alcohol in a monoglyceride as distinguished from the similar stretching in a diglyceride at 9.6 microns.
>
> Whether these suggested correlations are entirely correct, a significant fact from the standpoint of use of the spectra for analytical purposes evolves. Mono-

glycerides can be detected by observation of a band with maximum at 9.5 microns and determined by consideration of the intensity of this band. Diglycerides can be detected and determined by use of the 9.6 micron band. Triglycerides can then be obtained by difference. Absence of any bands between 9.1 and 10.0 microns can be construed as evidence for a pure triglyceride.

The 10.0 micron C-H bending vibration about the trans C=C group. An absorption band with maximum at 10.3 microns, which has been shown to arise from C-H bending about a trans C=C (reference) has been used to determine quantitatively trans-isomers in monounsaturated fatty acids, esters, and glycerides.

Information obtained from two other sources are also applicable to the study of the glycerides. Chapman (16) studied the polymorphism of glycerides by infrared and noted that the H bonding to the OH groups was stronger in 1-monoglycerides as compared to 2-monoglycerides. The spectra of 1- and 2-monoglycerides of different polymorphic forms showed differences in the 3500-3650 cm^{-1} range. The chief difference between 2-monostearin, 2-monolaurin, 2-monopalmitin, and monolaurin was the number of bands in the 1350-1184 cm^{-1} region. Helme and Molines (30) mentioned absorption at the following wavelengths as corresponding to the group given with it. These were 1) presence of 0 compounds at 2.85 microns, 2) olefin bonds at 6.1 microns, 3) nonconjugated trans-isomers at 10.35 microns, and 4) cis-isomer at 10.95 microns.

A study of the spectra of peak 1 and 2 of Figure 6 showed that they were very similar. Neither had absorption bands in the 2.7-2.8 micron region which indicated that there were no

mono- or diglycerides present. The absorption maxima from 6.8 to 9.5 microns were very nearly the same except that for peak 1, the absorption was a little greater in general and the maximum at 9.0 microns was relatively a little less (for peak 1) than in peak 2. Comparison of spectra for pure tristearin, tripalmitin, and triolein absorption spectra given in O'Connor et al. (66) showed that the trisaturated glycerides had greater absorption maxima than triolein at 9.0-9.1 microns. If there were a separation of the triglycerides on the basis of unsaturation of the fatty acid chains present, then peak 1 should have been expected to have the greater maximum at 9.0 microns but the opposite was actually true. (Unsaturated material is more strongly adsorbed by silica gel than saturated material.) Therefore more must have been involved in the separation into peaks 1 and 2 than just the degree of unsaturation in the triglycerides. Neither peak 1 or 2 showed absorption at 10.3 microns. The infrared spectra for peak 1 was not shown because it was so nearly the same as peak 2.

The spectra for peak 2a was very nearly the same as for peak 2. The major differences occurred at 6.25, 9.0-9.2, and 10.3 microns. For peak 2a the maximum at 5.77 microns overlapped with the maximum at 6.25 which did not occur for the spectra of peaks 1 and 2. O'Connor et al. (66) listed absorption at 5.71-5.77 microns as being due to C=O stretching (COOR). This is a strong indication that unsaturated chains

which had been oxidized to give carboxylic acid groups had been separated into this peak and the additional absorption due to the C=O caused overlapping with the next maximum. The absorption maximum at 10.3 microns for peak 2a might have been an indication of trans-isomer of the C=C group but as will be seen when the other spectra are studied, the absorption maximum at 10.3 microns and the overlap of the C=O absorption maximum with the maximum at 6.25 microns always occurred together; neither were ever present alone. If the overlapping was actually being caused by the formation of more C=O groups, then possibly the maximum at 10.3 microns was being caused by something other than trans-isomers such as intermediate oxidation productions which had not undergone cleavage or the -COOH group formed upon cleavage.

The spectra of peaks 3 and 4 were very similar. Both had small maxima at 2.8 microns indicating the presence of mono- or diglycerides. Both had very small absorption maxima at about 9.5 and possibly at 9.65-9.7 microns but were not very clearly defined. Peak 4 had an absorption maximum at 10.3 microns with the accompanying overlap of the maxima at 5.77 and 6.25 microns. It would be very difficult to determine whether peaks 3 and 4 were mono- or diglycerides from the spectra alone. But because they are considerably different from the next peak spectra and because elution of known samples by other workers from silica gel columns have shown the order

of elution to be tri-, di-, and monoglyceride, it was concluded that peaks 3 and 4 were diglycerides.

Peak 5 and 6 also gave similar spectra. Both had pronounced maxima at 9.5-9.6 microns but that for peak 5 was 3 or 4 times larger. Peak 6 showed a maximum at 10.3 microns with the accompanying overlap between the 5.77 and 6.25 microns absorption maxima. Peak 5 had a much larger maxima at 2.8 microns than peaks 3 and 4 indicating that it was monoglyceride rather than diglyceride. The maximum at 2.8 microns for peak 6 was greater than for peaks 3 and 4 but less than for peak 5. Peak 5 also had a maximum at 10.1 microns indicating the presence of primary hydroxyl groups in monoglycerides whereas peak 6 did not exhibit absorption at 10.1 microns.

The conclusion of the study of the peaks obtained in the chromatogram of Figure 6 were that peaks 1, 2, and 2a consisted of triglycerides; peaks 3 and 4 consisted of diglycerides; and peak 5 consisted of monoglycerides. Peaks 2a, 4, and 6 probably consisted of tri-, di-, and monoglycerides respectively in which some of the unsaturated fatty acid chains had undergone oxidation. Peak 6 may have consisted of both mono- and diglycerides which had undergone oxidation; the diglycerides having been oxidized to a further extent than the monoglycerides. Probably little or no oxidized triglycerides were present in peak 6. Later work showed that glycerol would also appear in peak 6 if present.

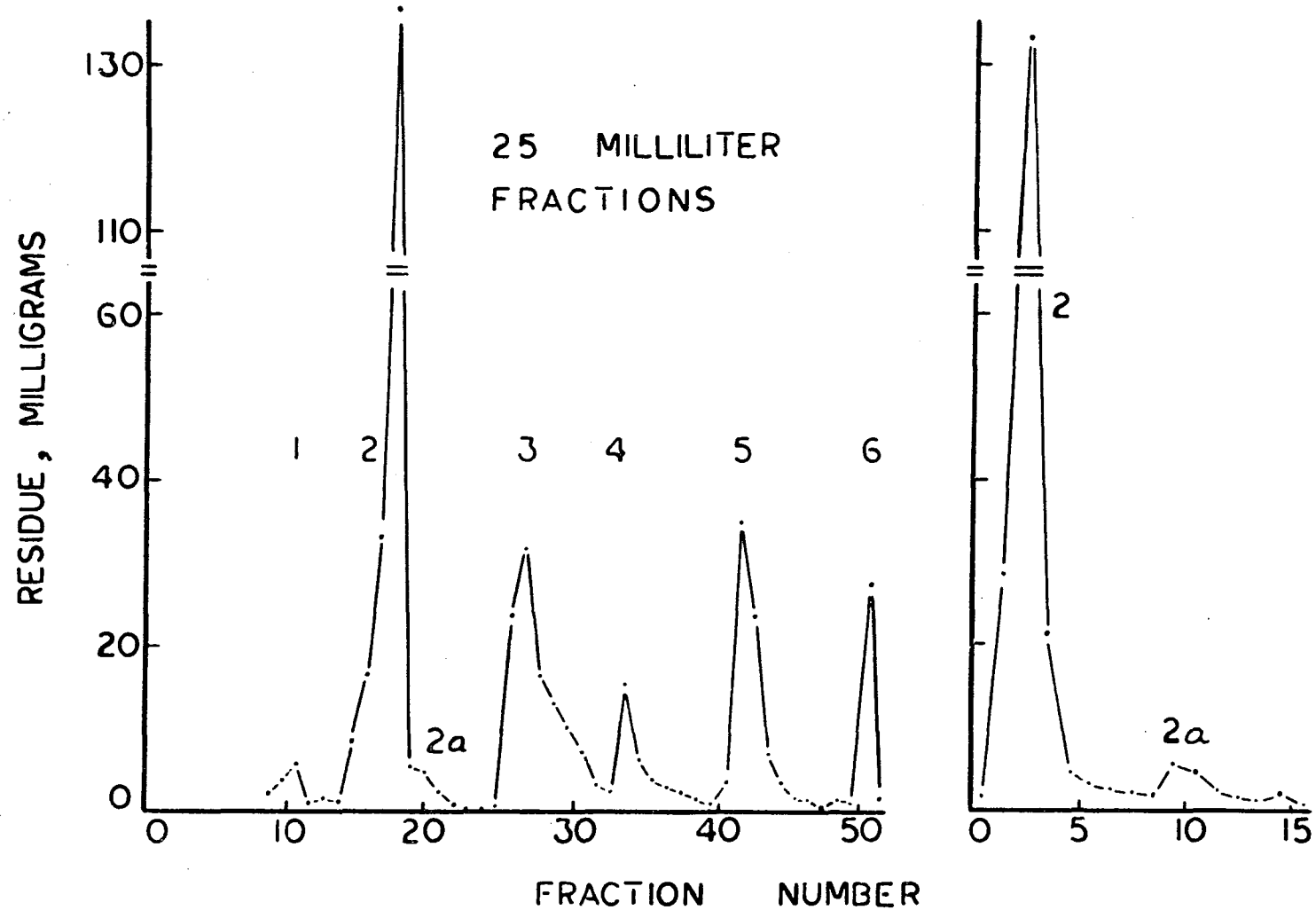


FIG. 6. SEPARATION OF LARD MIXED GLYCERIDES

Table 41. Separation of lard mixed glycerides on silica gel column[a]

Fraction	Eluting solvent	Flow rate ml/min	Residue, mg	Peak assign.
1	1.25%	1.7	-1.5	
2	ether-	1.7	-0.8	
3	Sk.B.	0.9	-0.9	
4		1.6	-0.9	None
5		1.6	-0.9	
6		1.7	-1.0	
7		1.4	-1.0	
8		1.3	-1.3	
9	7.5%	1.7	2.0	
10	ether-	1.7	4.2	
11	Sk.B.	1.7	10.4	1
12		1.7	0.9	
13		1.7	1.3	
14		1.6	1.0	
15		1.2	8.5	
16		1.7	16.3	
17	15%	1.8	33.2	
18	ether-	-	136.1	2
19	Sk.B.	1.8	5.2	+
20		1.7	4.6	2a
21		1.8	2.1	
22		1.7	0.5	
23		1.3	0.1	
24		1.1	0.1	
25	25%	1.8	0.3	
26	ether-	1.8	23.3	
27	Sk.B.	1.8	31.4	
28		1.7	16.0	
29		1.8	13.0	3
30		1.7	9.7	
31		1.3	6.9	
32		1.1	3.1	
33	50%	1.8	1.7	
34	ether-	1.8	15.3	
35	Sk.B.	1.9	5.9	
36		1.8	3.8	4
37		2.0	3.0	
38		1.9	1.7	
39		1.4	1.0	
40		1.2	0.6	

[a]Original lard mixed glyc. sample 497.5 mg; frac. vol, 25 ml.

Table 41. (Continued)

Fraction	Eluting solvent	Flow rate ml/min	Residue, mg	Peak assign.
41		1.9	3.9	
42	100%	2.4	34.9	
43	ether	2.1	23.7	5
44		2.4	6.4	
45		2.5	3.9	
46	100%	2.4	1.8	
47	ether	2.4	1.6	
48	———	1.7	0.0	5
49	100%	-	1.0	
50	MeOH	1.3	0.5	———
51		1.1	27.0	
52		-	1.5	6
53		-	0.45	
54		-	0.45	
		Re-chromatograph of peak 2-2a		
1	75%	2.5	1.1	
2	ether-	2.3	28.1	
3	Sk.B.	2.3	132.9	
4		2.2	20.9	
5		2.2	4.5	2
6		2.3	3.1	
7		2.3	2.5	
8	———	1.8	2.0	
9	15%	2.5	1.7	———
10	ether-	2.4	5.2	
11	Sk.B.	2.4	4.5	
12		2.2	2.2	
13		2.2	1.3	2a
14		2.5	1.0	
15		2.1	1.6	
16		-	0.4	

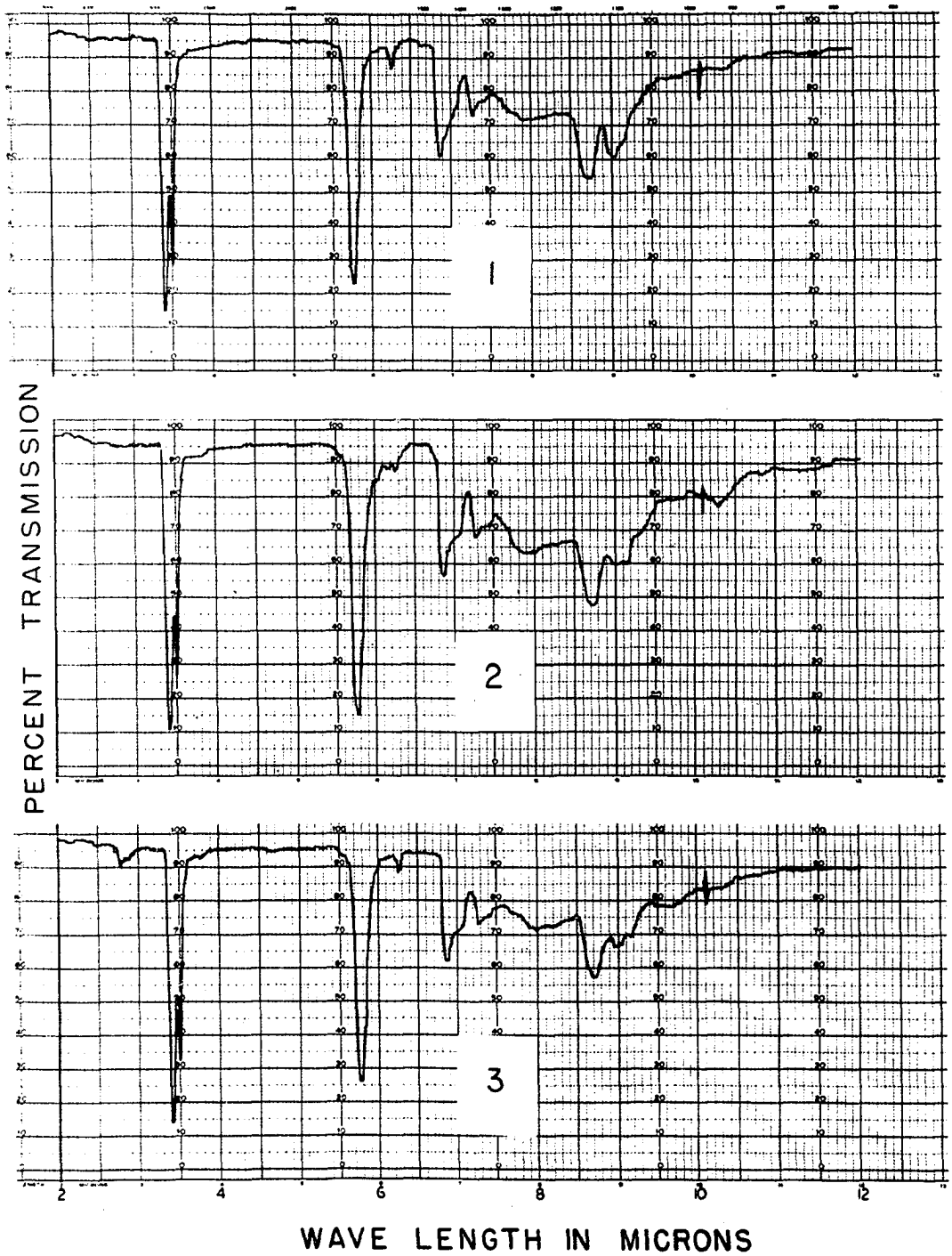


FIG. 7. INFRARED SPECTRA OF MIXED GLYCERIDE FRACTIONS

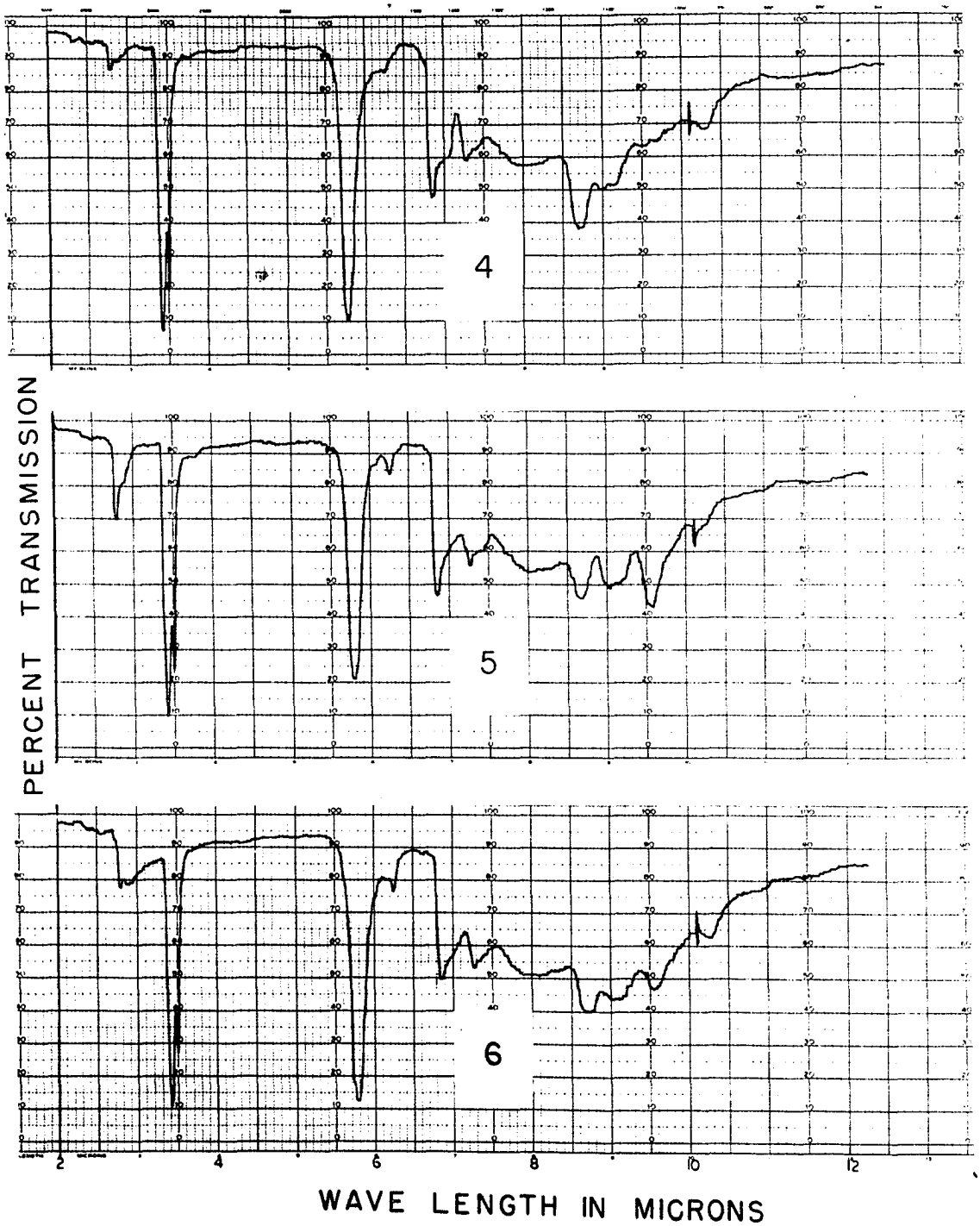


FIG. 8. INFRARED SPECTRA OF MIXED GLYCERIDE FRACTIONS

The mixed glyceride sample used for the silica gel column separations was prepared as described below. Twenty-one grams of lard A and 7.0 grams of glycerol were placed in a 50 ml round bottom flask with 42 milligrams of sodium methoxide and heated in a wax bath at 150-180°C for 3 hours. The mixture of lard and glycerol was agitated with a stream of nitrogen which also served to protect the lard from further oxidation. The first vapors from the lard consisted mainly of water but later were cloudy and had a rancid smell. The sodium methoxide was added after the water had been evaporated from the sample. After heating, the sample was allowed to cool under the nitrogen stream and then cooled by water to room temperature. Acetic acid, 4.5 ml of a 1% aqueous solution of the acid, was added to the sample to react with the catalyst. Ethyl ether was used to dissolve the fatty material and to transfer it to a 250 ml separatory funnel. The ether and aqueous acetic acid were shaken well and allowed to separate. There had been a glycerol layer under the fatty material layer after the glycerolysis reaction and most of the glycerol went into this aqueous acetic acid layer. The ether-fatty layer was washed several (3-4) times with water, the ether layer dried over sodium sulfate, and the ether evaporated. Emulsions formed during the washes were broken by the use of methanol, sodium sulfate granules, and by heating. The resulting triglyceride, diglyceride, and monoglyceride sample was stored in a rubber stoppered 125 ml Erlenmeyer flask under refrigeration. The

sample was reddish brown in color; the color developed during the 3 hour heating period at near 180°C.

The column used to carry out the separation of the mono-, di-, and triglycerides consisted of a 16 mm ID glass tube approximately 60 cm long. The silica gel was supported on a glass wool plug $\frac{1}{2}$ to 1 in thick. The flow through the column was regulated when needed by use of a glass stopcock held in place in the bottom of the column by a rubber stopper.

Davison Silica Gel, grade 922 (which passes through a 200 mesh screen) was screened to obtain the 200-250 mesh size particles. Thirty grams of the silica gel and 1.5 ml of water were stirred thoroughly in a 150 ml beaker with a glass stirring rod. Stirring was continued for a minute or two after all lumps had been completely broken into the 200-250 mesh size particles. The silica gel was suspended in Skellysolve B and the slurry poured into the column through a small funnel. The beaker and funnel were washed with Skellysolve B to recover all of the silica gel. The stopcock was then opened and the silica gel allowed to settle as the Skellysolve B was drained from the column. In earlier work with these columns, the column was tapped lightly to help pack the silica gel but it was found that the silica gel could become too tightly packed causing excessively slow flowrates. In later columns the column was not tapped at all. No difference was observed in the

separations obtained because of the difference in degree of packing. Care was exercised to not allow the liquid level to drop below the top of the silica gel bed. The weighed sample was then dissolved in the solution to be used first in eluting the column and poured into the column, using as little solvent as necessary. A 25 ml graduated cylinder was placed under the stopcock to collect the first fraction. The sample solvent was then allowed to drain into the packing, the walls of the top of the column washed down with small portions of solvent and after all of the sample had entered the silica gel, the top of the column was filled to the desired height to obtain the desired flowrate. Flowrates of 1.5-2.5 ml per minute were maintained. As 25 ml fractions were collected, 25 ml of solvent were added to the top of the column. The fractions were poured into weighed 50 ml beakers, the solvent evaporated and the residue weight obtained.

The evaporations were made on a steam plate in a hood. When the solvent was completely evaporated, the beakers were allowed to cool to room temperature and to equilibrate with the moisture in the air. The same method of drying the cleaned beakers was followed to insure against large differences in weight of the beakers because of the difference in their temperature and water vapor adsorbed on the surface of the glass. Beakers washed in soap and water, dried at 110^{o}C and

cooled as described above consistently weighed within about 0.5 milligram during day after day use. When washed and weighed twice on the same day the beaker weight normally checked within 0.1-0.2 milligram.

The silica gel was discarded after using once. Some checks were made to determine whether or not it could be used over again but the silica gel activity changed slightly and peaks came out slightly faster. Also samples with strongly adsorbed components are liable to accumulate in the silica gel.

The column run to obtain Figure 6 had first been eluted with 1.25% ether in Skellysolve B rather than the planned 2.5% ether solution. Another column was run and the 2.5% ether-Skellysolve B solution used and the results obtained are shown in Table 42 and Figure 9 which show clearly that peak 2-2a of Figure 6 did consist of separable materials without the need to rechromatograph the peak. Comparison of the weights of the combined peak fractions for the two column separations of the same sample are given in Table 43 and show good agreement considering that the errors of over 100 weighings are involved in the calculations for each sample chromatographed.

Methyl esters of each of the first 6 peaks of Figure 9 were prepared and chromatograms of the methyl esters prepared.

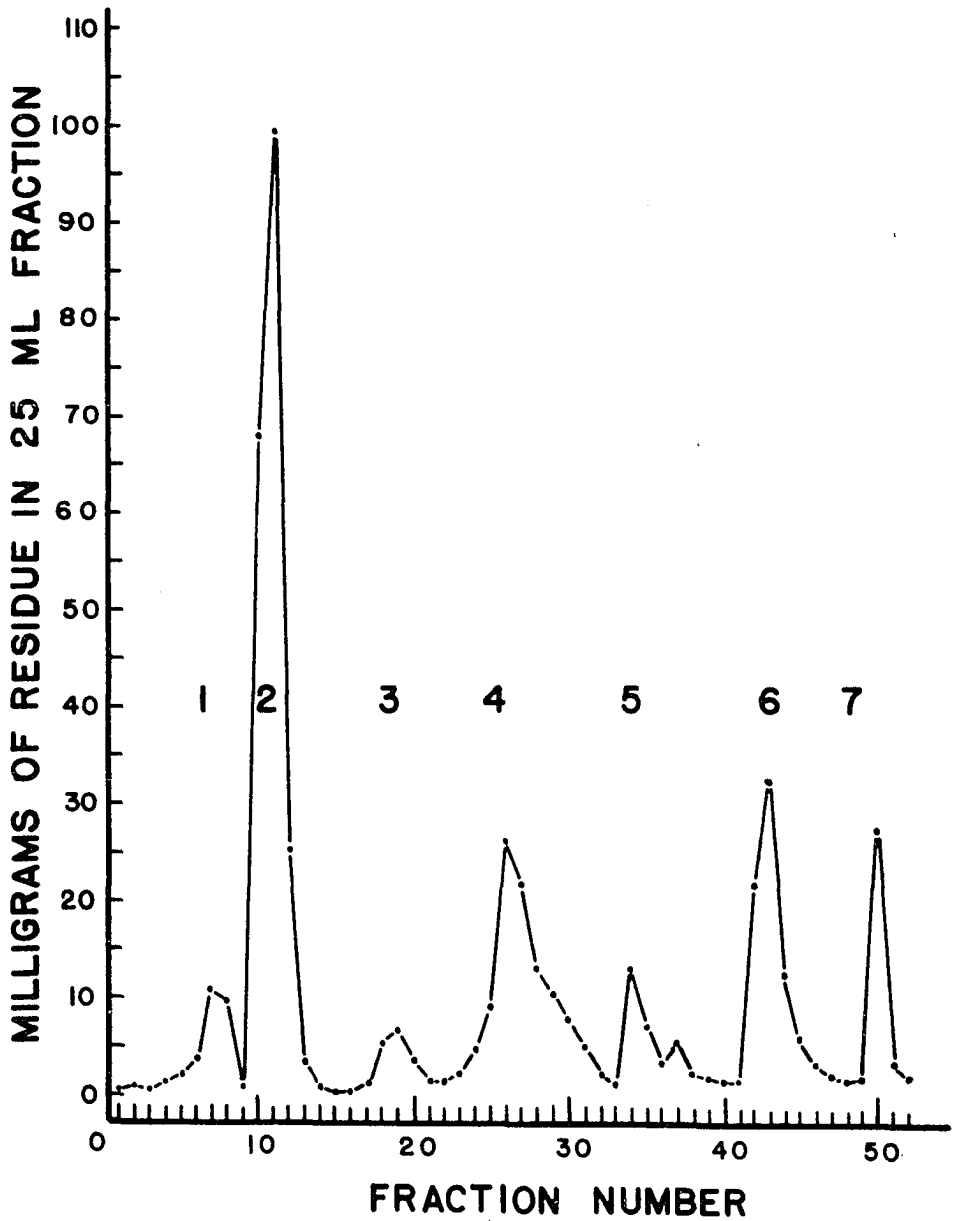


FIG. 9. SEPARATION OF LARD MIXED GLYCERIDES

Table 42. Separation of lard mixed glycerides on silica gel[a]

Fraction	Eluting solvent	Flowrate ml/min	Residue, mg	Peak assign.
1	2.5%	2.1	0.2	
2	ether-	2.1	0.8	
3	Sk.B.	2.1	0.3	
4		2.2	1.2	1
5		1.8	1.9	
6		1.9	4.2	
7		2.0	10.6	
8		1.6	9.7	
9		2.0	0.6	
10	7.5%	2.0	68.4	
11	ether-	2.1	99.5	
12	Sk.B.	2.2	25.2	
13		1.9	4.1	2
14		2.1	0.6	
15		2.0	0.2	
16		1.7	0.2	
17		2.1	1.0	
18		2.2	5.2	
19	15%	2.1	6.5	3
20	ether-	2.2	3.7	
21	Sk.B.	2.2	1.3	
22		2.2	1.3	
23		2.0	2.2	
24		1.5	4.7	
25		2.5	9.0	
26	25%	2.4	25.9	
27	ether-	1.9	21.9	
28	Sk.B.	2.1	13.2	4
29		2.0	10.5	
30		2.0	7.8	
31		1.9	5.0	
32		1.5	2.1	
33		1.9	1.0	
34	50%	2.3	13.4	
35	ether-	2.1	7.2	
36	Sk.B.	2.0	3.8	
37		2.3	5.4	5
38		2.2	2.0	
39		2.3	1.8	
40		1.7	1.2	

[a]500.0 mg sample of lard mixed glycerides; fraction volume, 25 ml.

Table 42. (Continued)

Fraction	Eluting solvent	Flowrate ml/min	Residue, mg	Peak assign.
41		2.1	1.2	———
42	100%	2.2	21.8	
43	ether	2.4	32.5	
44		2.4	12.6	6
45		2.6	5.7	
46		2.7	3.4	
47		1.2	2.0	
48	———	1.9	1.6	
49		1.4	1.7	———
50	100%	1.3	27.6	
51	MeOH	-	3.0	7
52		-	1.5	

The gas-liquid chromatogram results are given in Table 44. These results indicate that each peak had very nearly the same fatty acid composition. Notice that peak 1 did analyze a little higher in oleic acid than did peak 2 which was indicated by the infrared spectra study. Peak 7 which was eluted from the column using methanol was not analyzed at this time but gas-liquid chromatograms of the similar peaks were prepared for each of 3 such peaks obtained from later columns. Acetone was used in place of methanol for the elution of this peak. The gas-liquid chromatogram areas were not calculated but indicated that peak 7 of the silica gel column consisted mainly of pelargonic acid (C_9), and some glycerides containing oxidation products. No determination for glycerol was made.

From this data obtained for the lard mixed glycerides fractionations, the eluting solvent compositions were then selected to obtain the separation shown in Figure 10.

Table 43. Comparison of silica gel column chromatograms for lard mixed glycerides fractionation[a]

Chromatogram peak, Fig. 9		Fig. 6 wt %	Fig. 9 wt %	Fig. 11
0		-	-	1.2
1		4.2		6.8
2	(2+2a)	44.0	39.7	42.4
3			3.5	13.4
4		22.3	20.9	21.2
5		6.7	7.2	8.7
6		16.5	16.3	4.9
7		6.3	6.4	1.4

[a]Calculations based on individual residue weighings; combined peak fractions were not weighed. Calculations do not account for material which was not eluted from the column (more strongly adsorbed than MeOH or acetone).

Table 44. Fatty acid methyl ester composition of lard mixed glyceride fractions of Figure 6

	Silica gel column peak					
	1	2	2a	3	4	5
Myr.	0.6	1.7	1.4	1.6	1.9	1.4
Semi-DiC_9	-	-	-	-	1.0	-
DiC_9	-	-	-	-	0.7	-
Pal.	23.5	31.3	29.4	28.4	27.1	30.7
P.O.	2.2	3.1	3.1	3.1	3.4	2.8
Stear.	18.1	15.3	15.7	16.2	13.4	15.5
Oleic	52.5	43.0	46.7	46.1	44.1	45.6
Linol.	3.2	5.6	3.7	4.7	2.5	4.0
?	-	-	-	-	2.8	-
?	-	-	-	-	3.2	-
No. of chromatog.	2	2	2	1	2	2

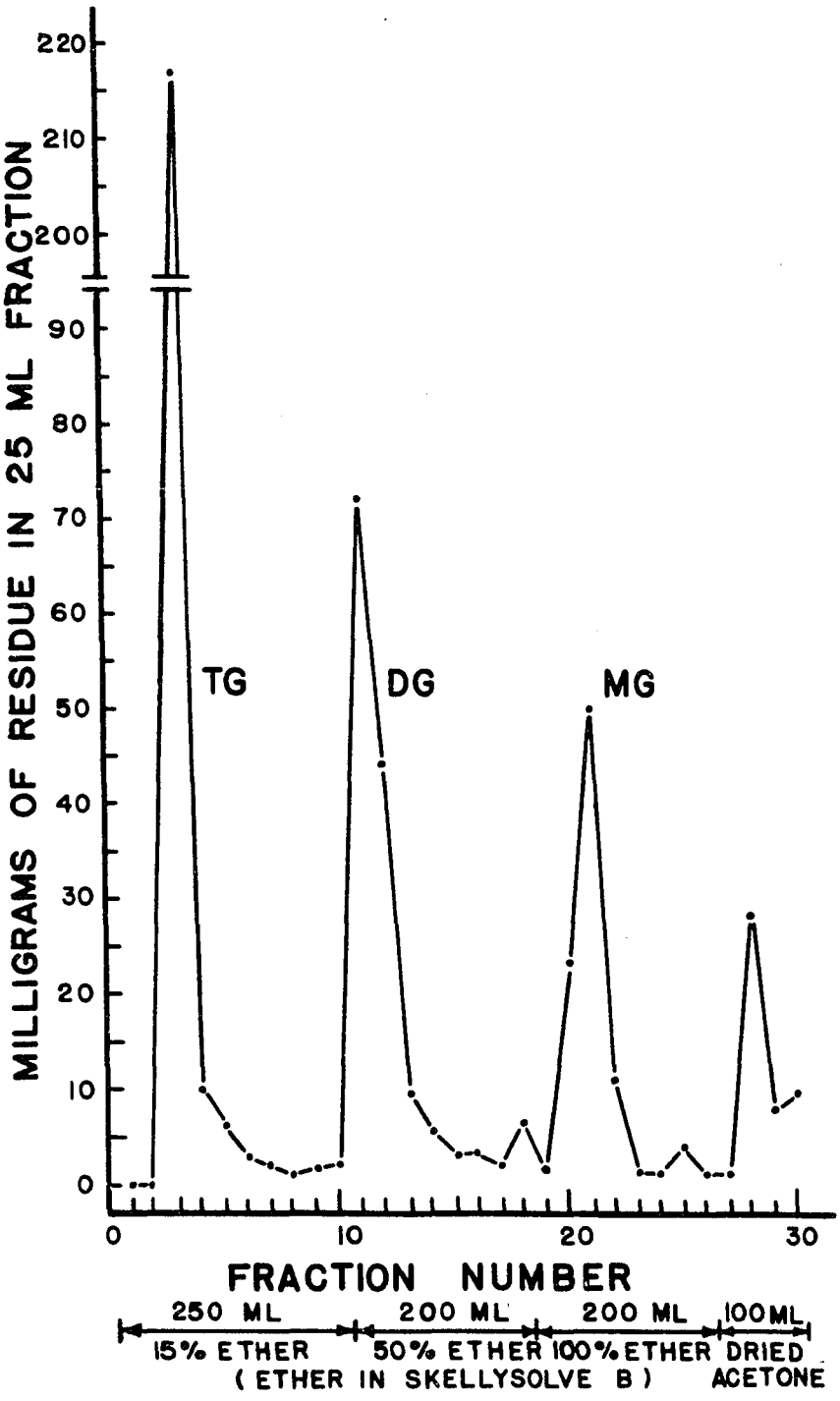


FIG. 10. SEPARATION OF LARD MIXED GLYCERIDES

In analyzing the hydrolysis products, it is desired to obtain the compositions of the 1- and 3-position fatty acids from an analysis of the fatty acids freed during the hydrolysis. The composition of the 2-position fatty acids may be obtained by an analysis of the monoglyceride fatty acids. But the hydrolysis is not 100% specific for the 1- and 3-position fatty acids and as a result, some 1-monoglycerides may be formed. If these 1-monoglycerides were separated from the 2-monoglycerides and only the 2-monoglycerides analyzed, the analysis should give better data for the 2-position fatty acid composition. Another factor involved is that the 2-monoglycerides are less stable than 1-monoglycerides. Savary and Desnuelle (81) pointed out that 2-monoglycerides isomerize easily when in contact with solvents containing alcohol. They also used the method of treating enzymatic hydrolysis products with periodic acid to destroy 1-monoglycerides and separation of the 2-monoglycerides from the mixture by use of a partition column. Their method does not separate the diglycerides, triglycerides, and oxidation products of the 1-monoglycerides. Use of their partition column required equipment to control the temperature of the column. Borgstrom (10) described the use of a silicic acid column for separation of mono-, di-, and triglycerides but it was found that 2-monoglycerides isomerized during passage through such a column. Where isomerization was not desired, a partition column separation of the monoglycerides from the di- and tri-

glyceride fraction was described.

From the separations obtained by silica gel columns in separating the lard mixed glycerides it was thought that separation of the tri-, di-, and 2-monoglycerides as well as the glycolic aldehyde formed from the 1-monoglycerides could be attained on one column. As the 1-monoglycerides are quantitatively destroyed before passage through the column, the only monoglycerides remaining in the hydrolysis products would be those of the 2-isomer. If isomerization occurred during passage through the column, it would not affect the results as both 1- and 2-monoglycerides appear in the same peak. Analysis of the fatty acids in the monoglyceride peak would be an analysis of the original 2-monoglyceride fatty acids.

This separation was found to be possible and is shown in Figure 11. The weight percentage of each peak is given in Table 43 for comparison to the same weight percents obtained for the peaks shown in Figure 9, a chromatogram of the same sample before the periodic acid oxidation. Data for Figure 11 are given in Table 45.

The separation of peaks 3 and 4 was not good in Figure 11 but the sample size used for that column was 945.8 milligrams. By decreasing the sample size to about 500 milligrams, much better separation is obtained. If the separation was still less than desired, more 15% ether-Skellysolve B could be used and

possibly use of a slightly lower ether content would aid in obtaining better separation. As the separation of the glycolic aldehyde was not necessary in this work, better separations were not studied but the data are presented here to show where the glycolic aldehyde emerges in relation to the monoglyceride peak 6. The data of Table 43 clearly indicate that much of the monoglyceride peak 6 of the sample before oxidation appears as peak 3 of the oxidized sample.

It should be noted here that the silica gel column work discussed so far as well as some preliminary work with these columns for separation of periodate-permanganate oxidized lard has been carried out prior to knowledge of the closely related work of Privett and Blank (73). Their work involved the use of thin layer chromatography and oxidation of the unsaturated fatty acid chains to yield aldehydes rather than the carboxylic acid groups obtained by the periodate-permanganate oxidation. They showed data for separation of 2-monoglyceride and the periodic acid oxidized 1-monoglyceride which confirmed the emergence of the glycolic aldehydes before monoglycerides, but they did not show the relation of these peaks to diglyceride emergence. This paper proved to be helpful in proceeding with the periodate-permanganate oxidized lard fractionation on silica gel columns.

It was found from experience with these columns that the results were reproducible in that the peaks emerged in the very

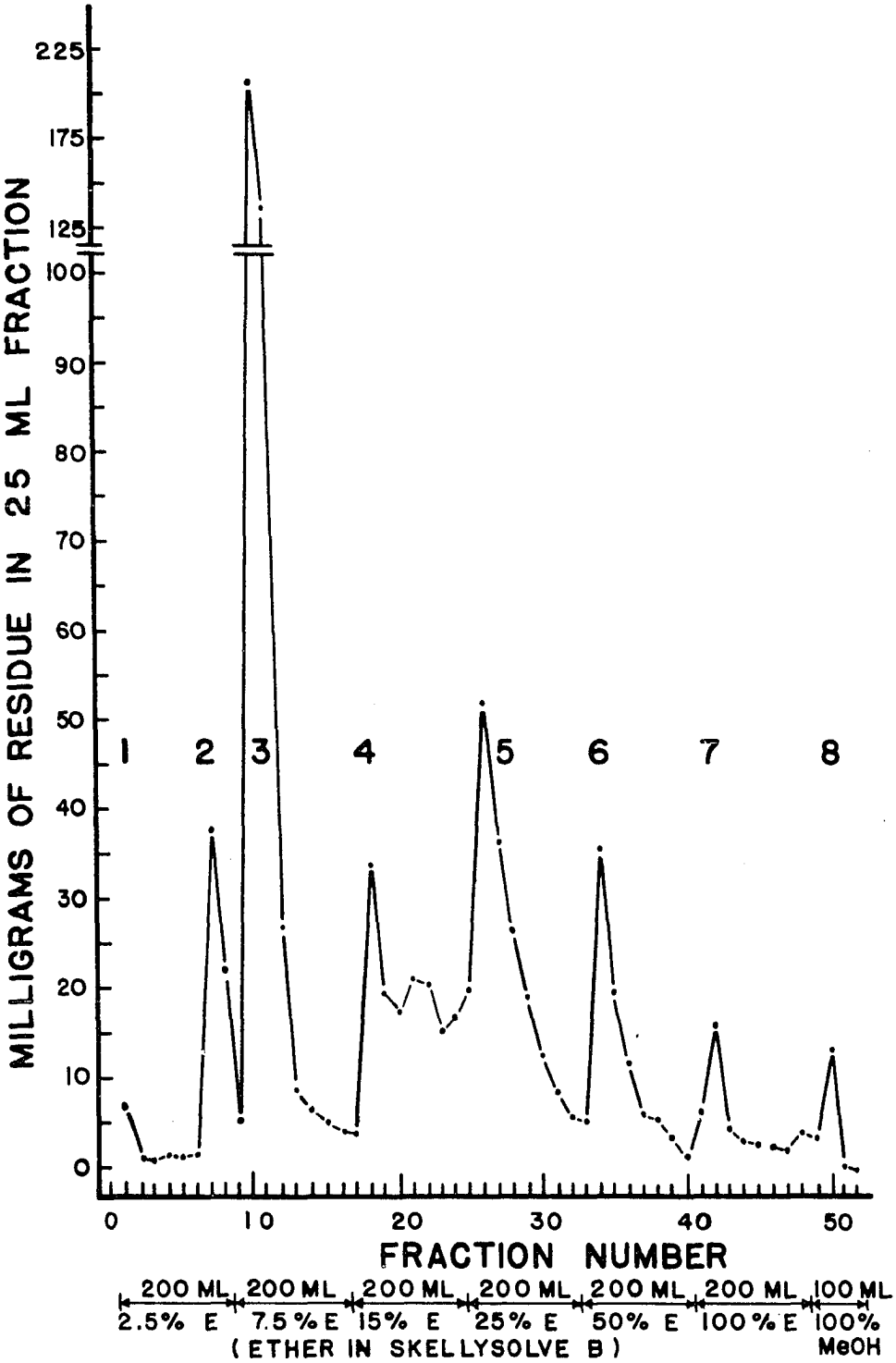


FIG. 11. SEPARATION OF PERIODIC ACID OXIDIZED LARD MIXED GLYCERIDES

Table 45. Separation of periodic acid oxidized lard mixed glycerides (Figure 11)

Fraction	Eluting solvent	Flowrate, ml./min.	Residue, mg.	Peak assign.
1		2.0	6.6	
2		1.9	0.9	
3	2.5%	1.8	0.6	
4	Ether-	1.9	1.0	
5	Sk.B.	2.0	0.9	
6		1.4	1.0	
7		2.4	37.5	
8		1.4	22.1	1
9		1.9	5.1	
10	7.5%	1.8	212.1	
11	Ether-	1.8	136.8	
12	Sk.B.	2.0	26.9	
13		2.0	8.7	2
14		1.9	6.5	
15		1.7	5.1	
16		1.5	4.4	
17		1.8	4.2	
18	15%	2.0	34.0	
19	Ether-	1.8	19.4	
20	Sk.B.	1.9	17.6	3
21		1.9	21.4	
22		1.9	20.6	
23		1.7	15.4	
24		1.4	16.7	
25		1.8	19.7	
26	25%	1.9	52.1	
27	Ether-	1.9	36.4	
28	Sk.B.	1.9	26.5	4
29		2.0	19.0	
30		1.7	12.5	
31		2.0	8.4	
32		1.6	6.0	
33		2.0	5.3	
34	50%	2.1	35.7	
35	Ether-	1.9	19.5	
36	Sk.B.	1.9	11.8	
37		1.9	6.0	5
38		1.8	5.4	
39		1.9	3.8	
40		1.7	1.3	

Table 45. (Continued)

Fraction	Eluting solvent	Flowrate, ml./min.	Residue, mg.	Peak assign.
41		1.8	6.4	
42	100%	2.5	16.1	
43	Ether	2.1	4.7	6
44		1.9	3.4	
45		2.0	3.1	
46		2.1	2.6	
47		2.0	2.2	
48		1.8	4.3	
49		2.1	3.9	
50	100%	1.3	13.6	
51	MeOH	1.4	0.1	7
52		-	-0.5	

same fractions when columns were run using the same techniques. Therefore, when a large number of samples need to be run, several columns can be run at one time by one operator. There is no need to collect 25 ml fractions but just one large fraction corresponding to the peak being eluted as shown by a chromatogram such as Figure 10. When only the monoglyceride peak is desired, then the initial eluting solution may be made strong enough to elute the diglycerides as well as the triglycerides and glycolic aldehydes, if present, into one fraction and then the monoglycerides in a second fraction. Figure 12 shows that this method is valid. Here, 250 ml of 50% ether-Skellysolve B were used to elute the tri- and diglycerides of the lard mixed glycerides sample into one fraction and then 25 ml fractions taken for the rest of the fractions to demonstrate that if the next eight fractions had been collected together, the pure mono-

glyceride peak would have been obtained. The acetone fractions collected were one of the three samples checked to determine the fatty acid composition of the peak following the monoglyceride peak as mentioned earlier.

One difficulty encountered with the use of the silica gel columns occurred whenever the temperature in the laboratory was about 85°F or above. When ethyl ether was being used in the solutions to elute the columns at this temperature, ether vapors formed in the column and appeared as white specks and/or streaks in the silica gel packing. They did not seem to affect the separations obtained unless the vapor formation was so bad that it actually caused shifting of the bed resulting in visible cracks in the bed. Later, in work on the separation of the periodate-permanganate oxidized lard, a water jacket was used on the column and the temperature of the water controlled by passing tap water through a coil (1/4 in copper tubing) submerged in a temperature controlled water bath.

The periodic acid oxidation of the 1-monoglycerides was carried out as described by Savary and Desnuelle (81) which is an application of the method as presented by Pohle and Mehlenbacher (72). The procedure is given below.

The hydrolysis products of 1.2 grams of fat were extracted, washed, and the solvent evaporated under vacuum. The residue

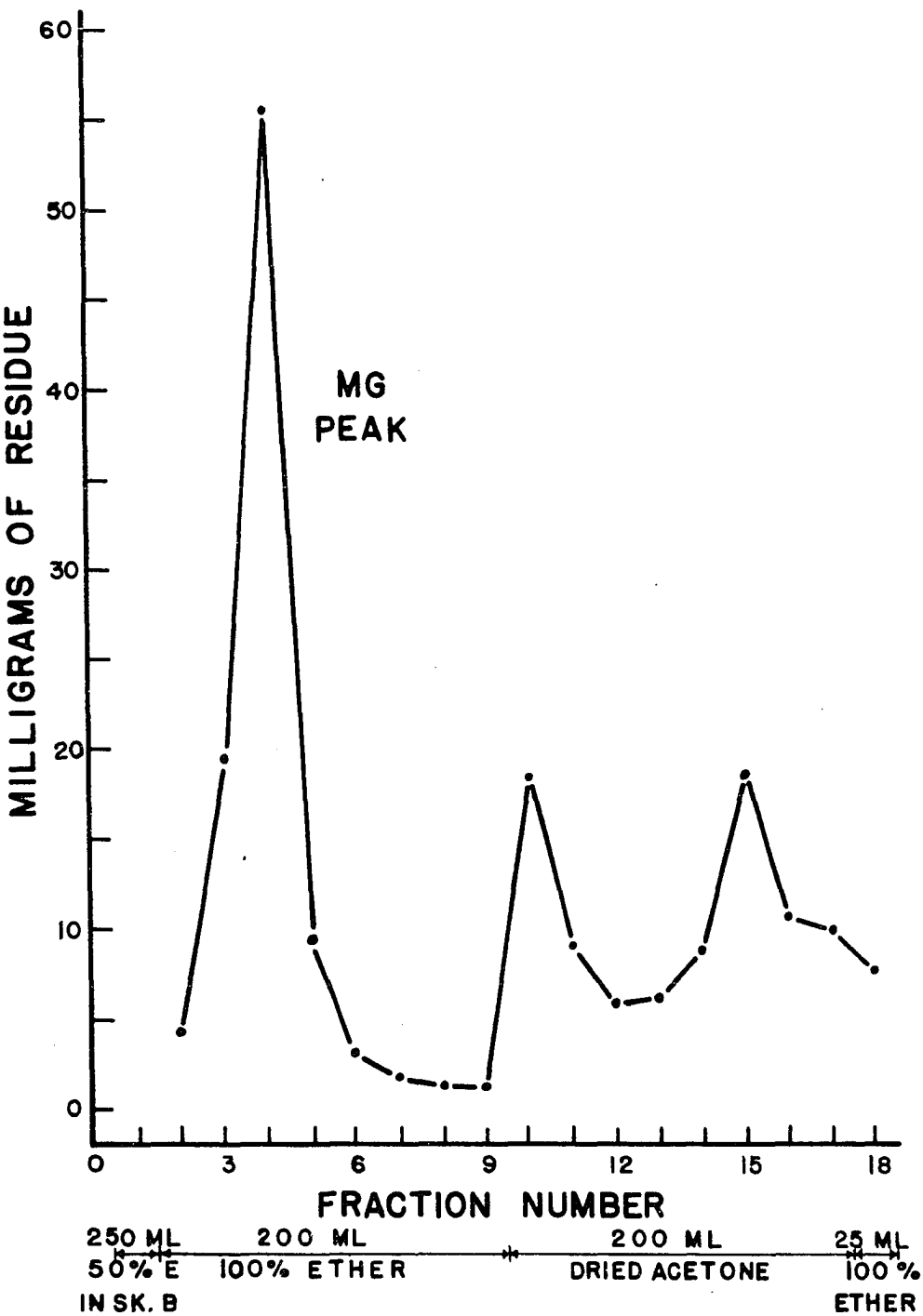


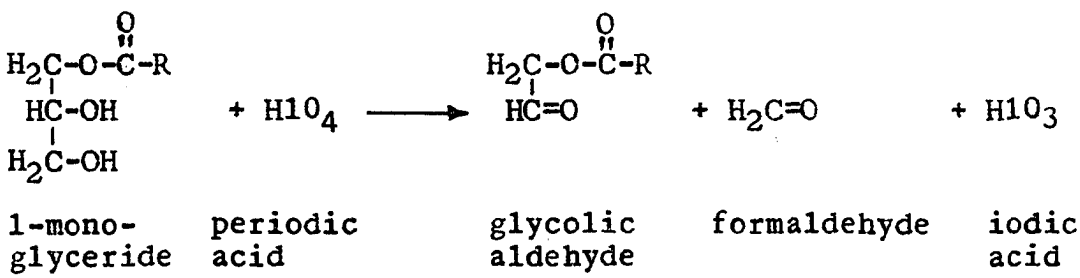
FIG. 12. COLLECTION OF THE MONOGLYCERIDE PEAK FRACTIONS

was taken up in 10-20 ml of 3:2 by volume acetic acid:chloroform, 1.5 times the necessary 0.2 N periodic acid was added and the mixture was allowed to react at room temperature for 30 minutes. The excess periodate was destroyed with 15% potassium iodide, 50 ml of water added, and the iodine formed was destroyed with sodium thiosulfate, a slight excess being used. All the operations were conducted at room temperature to prevent iodine from reacting with any unsaturated material present. The resulting solution was poured into a separatory funnel and the container was rinsed with three 5 ml portions of chloroform. The chloroform layer was separated from the aqueous phase and the aqueous phase extracted two more times with 5 ml portions of chloroform. The chloroform extracts were washed with an equal volume of water, the chloroform evaporated at 25°C under vacuum until there were only 3-4 ml of residue remaining. This residue was dissolved in 30 ml of ethyl ether and passed through an Amberlite IRA-400 column containing sufficient resin to adsorb all of the free fatty acids as well as the acetic acid remaining in the residue. The fatty material which passed through the column was recovered and separated on a chromatographic column to obtain the monoglyceride fraction.

The 0.2 N periodic acid solution was prepared by dissolving 0.7 grams of H_5IO_6 in 10 ml of water and 90 ml of glacial acetic acid. The thiosulfate solution used was 0.25 N and was prepared by dissolving 62.05 grams of $Na_2S_2O_3 \cdot 5H_2O$ in one liter of water.

In order to carry out the above procedure, it is necessary to determine the amount of periodic acid, potassium iodide, and thiosulfate solutions required for the sample. When running a sample, the expected amount of monoglyceride to be oxidized must be estimated and the required amount of periodic acid solution calculated. Based on the molecular weight of monoolein, 0.1373 ml of 0.2 N H_5IO_6 is required per milligram of 1-monoolein to be oxidized. The other two solutions may be very roughly estimated by the use of 0.238 ml of 15% KI per ml of 0.2 N H_5IO_6 used and 1.2 ml of 0.25 N $Na_2S_2O_3$ per ml of 0.2 N H_5IO_6 used.

The equation representing the oxidation of the 1-monoglyceride to glycolic aldehyde is shown below.



Enzymatic hydrolysis procedure The enzymatic hydrolysis procedures followed by several workers (11, 24, 54, 55, 82, 98) are included in an appendix. The hydrolyses conducted on the samples in Fractional crystallizations No. 3 and No. 4 followed the procedure of Mattson and Beck (54, 55).

The hydrolysis studies on fractionated natural fats and

oils gave very little data for the fatty acid compositions of the hydrolysis product components. When fatty acid composition data was given it was usually from a determination of the polyunsaturated acids by ultraviolet spectrophotometry and did not give the component saturated acid compositions.

It was desired to obtain more information about the fatty acid composition of the hydrolysis products obtained under different conditions. Samples of lard B were hydrolyzed by the method of Mattson and Beck (55) and by a somewhat modified method of Youngs (98).

Youngs (98) used a homogenizer but apparently did not control the temperature so samples were probably hydrolyzed at near room temperature. He also used a 30 minute hydrolysis as compared to 15 minutes in the Mattson and Beck procedure. As a homogenizer was not available for this work, the samples were run at 40°C and were stirred by use of an electric motor and glass stirring rod with an L-shaped bend at its end. Also, the amount of reaction mixture was made to correspond to approximately the same total volume of about 16.9 ml of the Mattson and Beck procedure. So in Youngs method, 18 ml of phosphate buffer, 120 milligrams of lard B and 48 milligrams of steapsin were used. Bile salts were not used in either method.

One change in the Mattson and Beck procedure was that the steapsin was added to the reaction mixture in its dry form

rather than suspended in 2.3 ml of water. The water was included in the reaction mixture.

A pH meter was used to follow the pH of the solution in both methods. The pH of the Mattson and Beck sample changed very rapidly at first and more slowly near the end of 15 minutes. The pH of the Youngs sample did not vary from 8.0. The samples were run in 50 ml beakers which permitted the use of 5 in pH meter electrodes.

The free fatty acids were recovered from the hydrolysis products of the 2 gram lard B sample run by the Mattson and Beck method by using 20 grams of Amberlite IRA-400 (-OH form). The adsorbed acids were recovered by treating the fatty acids-resin with 25-30 ml of 9.6% hydrochloric acid-methanol solution and washing the resin with ethyl ether. Part of the ether-methanol solution was then slowly evaporated to complete the methylation and remove some of the alcohol. The methyl esters were recovered by extracting with ether.

The free fatty acid recovery for the 120 milligram lard B sample was the same except that only 5 grams of resin were used and 7.6% hydrochloric acid-methanol solution were used.

The above two samples were run on one day and the same procedures carried out on two more samples the next day to check the reproducibility of the results. The results of these an-

alyses are shown in Table 46 which show that the results of the same sample run on different days checked very well but the results by the two methods differ considerably. The Youngs samples had more degradation products present than the Mattson and Beck samples. The Youngs samples contained more myristic, about the same palmitic, and less stearic acids than the Mattson and Beck samples. This indicated a difference due to chain length in the saturated acids. When the unsaturated acids are considered, the same trend is noted; the Youngs samples contained four times as much myristoleic, over twice as much palmitoleic, just a little more oleic, and less linoleic acids than the Mattson and Beck samples. The conclusion is that in Youngs method, more short chain fatty acids (both saturated and unsaturated) were hydrolyzed than in the Mattson and Beck sample. The unsaturated acids were more easily hydrolyzed than saturated acids of the same chain length in the Youngs samples than in the Mattson and Beck samples. No mention of this difference in composition of the free fatty acids obtained under different hydrolysis conditions had been made in the literature.

The problem then existed as to whether an extensive study of the enzymatic hydrolysis method should be made or to find a way of standardizing the conditions such that reproducible results could be obtained. Even though the results of a given sample might not be of much value in themselves, they could be used to compare with samples run under the same conditions. Be-

fore more hydrolyses were run, an analysis was made of the possible reasons for obtaining these differing results.

Table 46. Comparison of hydrolysis methods

Method:	Area percent Mattson and Beck		Youngs		Average	
Sample	1	3	2	4	M and B	Youngs
?	0.3	0.2	0.6	0.6	0.3	0.6
Myr.	1.3	0.9	2.7	2.9	1.1	2.8
M.O.	0.5	0.3	1.5	1.6	0.4	1.6
Pal.	6.8	7.3	6.9	6.8	7.0	6.8
P.O.	3.6	3.3	8.2	8.1	3.5	8.2
?	-	-	0.9	0.5	-	0.7
?	-	-	1.2	1.1	-	1.1
Stear.	14.7	15.1	6.9	6.8	14.9	6.9
Oleic	61.5	61.3	63.8	64.4	61.4	64.1
Linol.	11.4	11.7	7.2	7.4	11.5	7.3

Two factors which could account for different results for different hydrolysis conditions were that the enzyme was specific for fatty acids differing in type as well as position and that the specificity of the enzyme for the 1- and 3-positions was much less than 100%. Data are presented by Mattson and Beck (54, 55) which indicated that neither of these two factors are true for their hydrolysis conditions. They showed that the hydrolysis of 1-oleodipalmitin (OPP) was not more specific for either palmitic or oleic acids in the 1- and 3-positions. In the hydrolysis of 2-oleodipalmitin (POP) the iodine value of the free fatty acids indicated that there was 9 mol percent oleic acid which originated from the 2-position. Further, they showed

that the hydrolysis products of randomized lard had very nearly the same compositions; the monoglyceride fatty acids and the free fatty acids had the same compositions. They reported 21.2% saturated, 59.8% oleic, 17.1% linoleic, 0.6% linolenic and 0.3% arachidonic acids in the free fatty acids of crude lard. The corresponding values from Table 46 are 23.0% saturated, 61.4% oleic and 11.5% linoleic acids which is a good check for two entirely different lard samples.

Assuming that the Mattson and Beck sample free fatty acid compositions were correct, the reason for Youngs values being different has not been explained. The same results would be expected even though the same degree of hydrolysis had not been obtained in the samples run by the two different methods, unless one of the samples had been hydrolyzed beyond 67%. Hydrolysis beyond 67% would take place with the 2-position chains since all of the 1- and 3-position fatty acids would have been hydrolyzed. The data of Mattson and Beck (54) indicated that 68 mol percent of the original triglycerides had been hydrolyzed by the end of 15 minutes. If the Youngs sample had hydrolyzed to a greater degree, then the 2-position chains would have been hydrolyzed. The Youngs free fatty acid palmitic acid content should then have been expected to be greater than for the Mattson and Beck sample. Table 46 shows that the two palmitic compositions were very nearly the same. This indicates that there was a specificity on the basis of chain length and degree

of unsaturation of the 1- and 3-position fatty acids. The reason for this specificity can not be explained by the discussion above.

The modified Youngs method gave results for which there was no published data available to support or explain the results. Therefore the method of Mattson and Beck appeared to be the more reliable method to follow if a choice between the two was to be made. The choice was not this simple though because of the large difference in the amount of sample required by the two methods. The Mattson and Beck method required 2 grams whereas the modified Youngs method used only 0.12 gram. This became important in this work because some of the crystallization fractions were expected to be only 1.5-2.0 grams. It was therefore desirable to use a method which did not require such a large hydrolysis sample. One other disadvantage of the Mattson and Beck procedure was the use of electrodes to follow the pH throughout the hydrolysis. Because of the high concentration of the fat in the Mattson and Beck samples, fat coated the electrodes and often coated them so badly that pH measurements were not possible without cleaning the tip of the calomel electrode.

A series of hydrolyses were conducted under the conditions shown in Table 47. The procedures followed were the same as used for the first samples run to compare the methods of hy-

drolysis except for the changes appearing in Table 47. In general only one variable was changed in each run so that it could be compared to some other run to determine the effect of changing the variable.

Comparison of the results obtained for the Mattson and Beck samples to the Youngs run 2,4 indicated that by decreasing the amount of fat and steapsin (run 7) gave results comparable to those obtained by Youngs method. Decrease of just the amount of fat (run 5) or the fat and time (run 6) did not give the same results as the Youngs run 2,4. This indicated that the ratio of steapsin to fat is important and that the effect of the calcium chloride of the Mattson and Beck procedure did not give greatly different results than in the Youngs method (runs 7 and 2,4). These results also indicated that in the presence of calcium chloride and a high steapsin to fat ratio (run 5), more short chain and saturated fatty acids were obtained than in the Youngs run 2,4. This indicated that at low fat concentrations and conditions which increase the rate of hydrolysis, high steapsin to fat ratio and the presence of calcium chloride, the hydrolysis gave free fatty acids which had approached the overall lard composition (see Table 47). This indicated that the degree of hydrolysis was probably beyond 67%.

In comparison of the effects caused by variations in Youngs method, run 8 indicated that the use of bile salts increased the amount of all the saturated acids as compared to decreases

for the unsaturated acids except linoleic acid. The changes were not very great, on the order of 10%. Run 9 with bile salts, room temperature and 34 minutes hydrolysis time gave results very nearly the same as run 8.

Runs 10, 11, and 14 were identical except for temperatures of 40, 50, and 60°C. All three runs were for five minutes. At 50°C the short chain (C_{14} and C_{16}), both saturated and unsaturated, increased slightly as compared to the 40°C run and all three of the C_{18} fatty acids showed decreases. At 60°C greater increases in the C_{14} and C_{16} saturated and unsaturated acids were obtained but only the oleic acid content decreased (from 64.6 to 54.3%) whereas stearic and linoleic increased slightly. Comparison of the effect of increased temperature to the effect of the increased amount of steapsin in run 13 showed a major difference. Increased temperature increased the amounts of shorter chain fatty acids of both saturated and unsaturated chains whereas the increased amount of steapsin increased the amounts of all the saturated acids and these increases were greater for the long chain saturated acids. The increased amount of steapsin caused a decrease in the amounts of all the unsaturated acids; increased temperature had caused a decrease for all C_{18} acids at 50°C and for just oleic at 60°C. The effect of increased steapsin therefore appeared to be selective on the basis of chain length as well as on the basis of being saturated or unsaturated acids. The effect of temperature was

selective on the basis of chain length only.

Run 12 showed the effect of increasing the buffer solution concentration from 0.5 to 1.0 M. The changes observed were quite small but the trend was the same as for increased temperature; increase for all the C_{14} and C_{16} chains and decrease for all the C_{18} chains.

This study of the data of Table 47 indicated that a detailed examination of all the variables affecting the free fatty acid composition of the hydrolysis products was beyond the scope of this problem. It appeared at this point that the next best step to take was to run all samples under identical conditions and obtain results which were only relative rather than true compositions of the 1- and 3-position fatty acids.

As it was desired to use small fat samples and eliminate the need of using a pH meter for each run, Youngs method as it was first modified was considered the best method to follow. A series of samples were run in which the only variable was the time of hydrolysis. The purpose was to determine more accurately the effect of time and also to investigate the possibility of finding an area in which changes in time did not affect the free fatty acid compositions appreciably. The results of this series of samples are given in the first portion of Table 48. These data indicated that runs 10 and 2,4 did not fit in very well with the runs 15, 16, 17, 18, and 19 which were run together but at a different time than either run 10 or runs 2 and

4. When the compositions were studied leaving out runs 2,4 and 10, the changes with time were not very great. The myristoleic, and palmitoleic decreased with increase in time. Oleic remained constant and linoleic increased by a factor of almost two in 60 minutes. Myristic appeared to decrease slightly, palmitic remained nearly constant, and stearic stayed constant except for an increase of about 1/5 in going from 30 to 60 minutes.

The effect of time therefore did not seem to be very great for the saturated acids but did affect the unsaturated acid compositions appreciably. What appeared to be just as significant was that the two analyses carried out previously to the last five runs gave results which did not fit in with the other five results. One possible reason for the poor agreement was that the resin was not performing as expected. Poor resin performance could have been due to poor conversion to the -OH form and variations in the amount of ether used to dissolve the hydrolysis products for contact with the resin. As an equilibrium is established between the fatty acids on the resin and in the solution, changes in concentration could cause changes in the relative amounts of the fatty acids adsorbed.

The effects of the resin were then eliminated by using diazomethane to convert the free fatty acids to methyl esters without first separating them from the other hydrolysis products. The procedure followed was the same as in the modified Youngs method except that the volumes and weights of the reac-

tion mixture were decreased by a factor of six which made them the same as in the procedure given by Youngs. A temperature of 40°C and variable times were used. Experience showed that 7 drops of lard B weighed very close to 0.12 gram or 1 drop contained about 17 milligrams. Youngs method requires only that 10-20 milligrams be used. One drop lard B samples were used for the hydrolyses except the 15 minute sample for which a 7 drop sample was used with the corresponding increase in the other weights and volumes of materials used. The samples were run in 20 mm ID test tubes which had been cut off to a length of approximately 6.5-7.0 cm. Both the 1 drop and the 7 drop sample were run in these test tubes. A pH meter was not needed as the 60 minute sample of the previous series of samples showed that the pH did not change during the one hour hydrolysis. The results of this series of samples are given in the second portion of Table 48 and a graph of the fatty acid compositions vs time of hydrolysis is shown in Figure 13.

Figure 13 shows very definite trends in the fatty acid compositions as the hydrolysis time was increased. The unsaturated acid compositions each increased with time and the saturated acid contents decreased. The trend for myristic acid is not clear because of analytical errors in measuring this very small myristate peak of the gas chromatograms. A ten microliter syringe was used for sample injection into the gas chromatograph

and as has been previously explained, the exact amount of sample injected was not known. Therefore accurate data for the relative total peak areas obtained for the samples is not available. The amount of hydrolysis did not appear to have been as great as 67% for even the two hour sample.

The most apparent differences between these two series of hydrolyses for different lengths of time was the size of the samples and the method of obtaining the free fatty acid methyl esters. As will be discussed later, the difference in sample size was by far the more important factor.

An attempt to overcome the difficulties encountered with the modified Youngs method was made by trying to compromise between the conditions of the Mattson and Beck procedure and the modified Youngs procedure.

A summary of conditions used in different hydrolysis procedures is given in tabular form at the end of the appendix giving these procedures. From a study of these figures and consideration of the needs of this glyceride structure study the "proposed" hydrolysis conditions were listed. The sample size could be limited to 0.5 grams by using 5 ml of buffer solution. It was desired to use conditions more nearly the same as Mattson and Beck than Youngs. The use of the ammonium chloride-ammonium hydroxide buffer permitted the use of calcium chloride and the decreased fat concentration was made to help eliminate coating

of the pH meter electrodes with fat. No bile salts were used as they did not appear to have much effect on the hydrolysis. A series of samples were run using this compromise method and lard B samples sizes of 0.5 and 2.0 grams. As the pH meter electrodes could not be accommodated by the short test tubes used to run the 0.5 gram samples (1, 3, 5, 10, and 15 minute samples), the pH was allowed to decrease during the first 15 minutes. For the longer time samples (30, 60, 90, 120, and 300 minute samples), 2.0 grams of lard B were used and were run in 50 ml beakers which allowed the use of the pH meter electrodes. It was found that the pH dropped to about 7.0 during the first 15 minutes of hydrolysis. Therefore the pH was adjusted to 8.0 whenever it had dropped to 7.0 for the remainder of the samples. The free fatty acids were recovered by passing the hydrolysis products through 10 mm columns containing 10 grams of Amberlite IRA-400. A test column was run to check each batch of resin prepared to insure it had been properly activated and would pick up 500 milligrams of oleic acid. Twenty drops of the hydrolysis products of the 30-300 minute samples were passed through the resin columns. The hydrolysis products passing through the resin columns were then treated with periodic acid to destroy the 1-monoglycerides and the 2-monoglycerides were separated on silica gel columns. The results of this series of samples are given in Table 49.

There were variations in the compositions when compared with successive hydrolysis times but on the whole the compositions were fairly constant with increasing time. The erratic tendency of the data was not entirely due to the method used to adjust the pH. The first five samples were not adjusted. A closer study of the data also showed that both the free fatty acids and the 2-monoglycerides showed the same trends. For example, the stearic acid compositions for the five minute sample was greater than either the three or ten minute samples for both the free fatty acids and the 2-monoglycerides. This erratic tendency was due to the hydrolysis rather than the analytical techniques used for studying the hydrolysis products.

More hydrolyses were conducted in which erratic results were obtained. From the data presented and the erratic results obtained on lards and crystallization fractions, it was concluded that the one most important variation which would help account for the widely varying results involved the formation of the fat-aqueous solution emulsion. Looking back over some of the effects observed for variations of one variable at a time and keeping in mind that the better the emulsion the better the results, the following observations were made. Some of the variables which were studied might be expected to affect the ease of forming an emulsion. By increasing the temperature, fat near its melting point would become less viscous and would

be easier to break into smaller particles. The use of an emulsifying agent, bile salts, might help in forming the emulsion or stabilize the emulsion after it had been formed. Increasing the amount of electrolyte in the aqueous solution might help in forming better emulsions. Increasing the degree of agitation would be expected to be the most important variable of any mentioned so far. By varying the size and shape of the hydrolysis container, large variations in the mixing or shearing action of the stirrer could result. This appeared to be the case when the small volume solutions were run in test tubes and stirred with a L-shaped stirring rod. The velocity of the stirrer tip was decreased due to the shorter bottom leg used because of the decreased diameter of the container. So for a small stirrer, less finely divided emulsion was formed. Based upon the above, another series of samples were hydrolyzed with the only variable being time. The modified Youngs method was used. Bile salts were used as prescribed by Youngs and his proportions scaled up by a factor of six (conditions of a 120 milligram fat sample). The samples were run in 50 ml beakers and stirred with the largest L-shaped stirrer which could be used in the beakers without scraping the beaker wall. The glass rod diameter used was 3 mm. The samples were heated and stirred with the buffer-bile salt solution to form an emulsion before the dry steapsin was added. The stirrer was turned by a variable speed motor rated at a maximum speed of 833 rpm. The speed

used for stirring was the maximum and was decreased only when excessive foam was formed. Free fatty acid methyl esters were prepared using diazomethane. The results of this series of samples are shown in the third section of Table 48. There are slight variations in the compositions but in general the compositions did not vary with the time of hydrolysis. The first section of data in Table 48 was run under the same conditions except for the bile salts and the attention to the rate of stirring, but considerably different results were obtained for all of the components except palmitic and oleic acids.

Table 50 is a summary of the results obtained by the different methods of hydrolysis studied. All of the samples were for 15 minute hydrolyses except in column 5 in which the ten minute hydrolysis results are given. Column 3 is the 15 minute data for the same series of samples as column 5 but was not as representative of the series of samples as was the data of column 5. The data obtained by the Mattson and Beck procedure checks well with only the data of column 6. The data of column 6 were obtained using the modified Youngs hydrolysis with bile salts, 0.12 grams of fat, a 50 ml beaker for the hydrolysis container and rapid stirring. As the data of column 1 checked published data quite well, it was used as the basis of the comparisons and was considered to be correct. Therefore it was concluded that in order to obtain free fatty acids representative of the 1- and 3-positions, the Mattson and Beck procedure

Table 47. Enzymatic hydrolysis tests

	Hydrolysis conditions												
Method:	Mattson and Beck				Youngs								
Run:	1+3	5	6	7	2+4	8	9	10	11	12	13	14	
Lard B, gm.	2	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	
Steapsin, mg.	156	156	156	48	48	48	48	48	48	48	156	48	
Bile salts, mg.	0	0	0	0	0	30	30	0	0	0	0	0	
Temp., °C	40	40	40	40	40	40	rm.	40	50	40	40	60	
Time, min.	15	15	15	15	15	15	34	5	5	5	5	5	
Buffer conc. M.	1.0	1.0	1.0	1.0	0.5	0.5	0.5	0.5	0.5	1.0	0.5	0.5	
	Gas chromatogram area %												Lard B[a]
?	0.3	N.C.[b]	N.C.	--	0.6	N.C.	N.C.	N.C.	1.0	0.9	0.5	1.3	-
Myr.	1.1	1.8	2.5	2.3	2.8	3.1	2.8	2.8	3.3	3.4	3.0	3.7	1.5
M.O.	0.4	0.8	1.4	1.1	1.6	1.5	1.5	1.7	1.9	2.0	1.4	2.2	-
Pal.	7.0	12.4	7.0	7.9	6.8	7.7	7.6	7.2	7.5	7.4	12.3	10.1	26.1
P.O.	3.5	5.1	6.5	6.3	8.2	7.6	7.5	8.6	9.2	8.7	7.4	10.8	2.2
?	-	-	-	--	0.7	-	-	-	-	-	-	-	-
?	-	0.6	1.0	0.9	1.1	1.1	1.1	1.1	1.4	1.3	1.0	1.5	-
Stear.	14.9	14.1	8.5	9.8	6.9	8.0	7.8	6.6	5.8	5.9	13.6	7.5	12.3
Oleic.	61.4	56.3	63.0	61.9	64.1	62.1	62.6	64.6	63.7	63.7	54.3	55.4	46.9
Linol.	11.5	8.1	9.2	8.4	7.3	8.2	7.8	6.5	5.7	6.0	5.6	6.6	11.0
Arach.[c]	N.C.	0.8	1.0	1.5	N.C.	0.8	1.2	0.9	0.6	0.9	0.9	1.0	-

[a]Overall lard B composition given for comparison to other results.

[b]N.C. for not calculated.

[c]Arachidonic acid, C_{20} with 4 double bonds.

Table 48. Enzymatic hydrolysis time tests, modified Youngs procedure

	Free fatty acids--Amberlite IRA-400[a]							
Run:	15	16	10	17	2 and 4	18	19	1 and 3[b]
Time, min.:	1	3	5	10	15	30	60	15
	Gas chromatogram area %							
?	1.1	2.1	N.C.[c]	1.3	0.6	2.0	1.5	0.3
Myr	3.1	2.6	2.8	3.0	2.8	2.3	2.6	1.1
M.O.	1.8	1.3	1.7	1.5	1.6	0.9	0.9	0.4
Pol.	10.5	10.7	7.2	9.1	6.8	9.5	10.7	7.0
P.O.	8.7	8.1	8.6	7.4	8.2	6.6	5.5	3.5
?	-	-	-	-	0.7	-	-	-
?	1.4	1.3	1.1	1.1	1.1	1.1	0.7	-
Stear.	9.9	9.9	6.6	9.6	6.9	10.2	12.4	14.9
Oleic	57.6	56.8	64.6	58.7	64.1	59.1	56.2	61.4
Linol.	5.0	5.2	6.5	7.3	7.3	7.5	9.5	11.5
Arach.[d]	0.9	2.2	0.9	1.1	N.C.	0.8	0.6	N.C.
	Free fatty acids--diazomethane[e]							
Time, min.:	1	3	5	10	15	30	60	120
	Gas chromatogram area %							
Myr.	0.6	0.7	0.7	0.7	0.7	0.6	0.5	0.8
Pal.	29.5	24.4	20.2	16.4	14.0	10.8	9.3	12.0
P.O.	-	1.2	1.3	1.6	1.7	2.0	4.9	2.1
Stear.	39.9	35.6	30.5	27.0	23.7	21.5	18.4	18.2
Oleic	27.0	32.4	39.5	44.8	49.0	52.6	53.4	54.4
Linol.	3.0	5.8	7.9	8.9	10.2	11.6	12.4	11.8
Arach.	-	-	-	0.7	0.6	1.1	1.0	0.7

[a]Modified Youngs method, 120 milligram lard B sample, no bile salts.

[b]Mattson and Beck method.

[c]N.C., not calculated.

[d]Arach., arachidonic acid; C_{20}, 4 double bonds.

[e]Modified Youngs method, 10-20 milligram lard B sample, no bile salts.

Table 48. (Continued)

	Free fatty acids--diazomethane[f]									
Time, min.:	5	10	15	20	25	30	60	90	120	300
	Gas chromatograph area %									
?	0.5	0.4	-	Trace	0.4	0.6	-	-	0.2	-
Myr.	0.6	0.7	0.5	0.5	0.5	0.3	0.4	0.3	0.6	0.6
Pal.	10.4	8.5	8.4	8.7	8.7	8.5	9.3	9.7	8.4	9.2
P.O.	2.3	2.4	1.7	2.0	2.2	1.6	1.7	2.3	1.4	2.8
Stear.	16.0	14.1	14.5	14.1	16.3	14.1	14.5	14.7	11.6	14.1
Oleic	58.7	62.3	63.2	63.6	63.8	61.5	62.7	60.1	67.9	60.5
Linol.	11.6	11.8	11.7	11.2	8.1	13.4	11.3	13.0	10.0	12.9

[f]Modified Youngs method, 120 milligram lard B samples, 30 milligrams of bile salts, maximum stirring rate.

Table 49. Enzymatic hydrolysis time test, compromise of Mattson and Beck and of Youngs procedures

Time:	1	3	5	10	15	30	60	90	120	300
Peak:	Chromatogram area percent									
					Free fatty acids					
?	4.1	--	--	--	--	--	--	--	--	--
?	13.2	1.0	0.4	--	0.4	--	--	--	--	--
?	6.8	1.0	0.5	--	0.3	--	--	--	--	--
?	2.7	1.0	0.4	--	0.3	--	--	--	--	--
Myr.	--	--	--	--	--	--	Trace	Trace	Trace	0.4
Semi-DiC9	--	--	--	--	0.6	0.7	--	--	--	--
Pal.	14.2	6.4	7.3	5.6	5.4	5.2	8.5	9.4	11.0	8.4
P.O.	--	1.6	1.9	1.4	1.3	1.1	1.8	1.8	1.9	2.5
DiC10	10.1	--	--	--	--	--	--	--	--	--
DiC11	12.0	--	--	--	--	--	--	--	--	--
Stear.	16.6	23.1	20.8	22.5	21.7	24.6	21.3	20.4	18.8	9.7
Oleic	12.2	60.4	59.5	62.8	62.7	62.3	59.9	59.6	58.6	66.7
Linol.	7.2	5.6	8.1	7.7	7.2	6.1	8.5	8.8	9.8	12.3
					2-monoglycerides					
Myr.	4.0	2.6	2.7	3.1	2.8	4.4	3.2	4.3	3.8	3.3
Semi-DiC9	--	1.4	0.8	1.2	1.1	--	--	--	--	--
DiC9	--	0.7	0.7	0.7	--	--	--	--	--	--
Pal.	33.8	50.1	61.0	54.1	58.3	69.0	70.5	76.4	71.2	68.6
P.O.	8.4	2.9	2.8	4.0	3.9	4.5	3.4	2.9	5.1	4.4
?	--	--	0.8	--	1.2	--	--	--	--	--
DiC11	6.3	--	--	--	--	--	--	--	--	--
Stear.	15.0	11.6	9.8	9.1	8.4	3.5	3.1	3.8	3.7	4.2
Oleic	25.8	27.0	19.4	23.9	21.5	15.3	16.8	11.0	13.7	15.9
Linol.	6.7	3.8	2.1	3.9	2.9	3.3	3.0	1.7	2.5	3.5

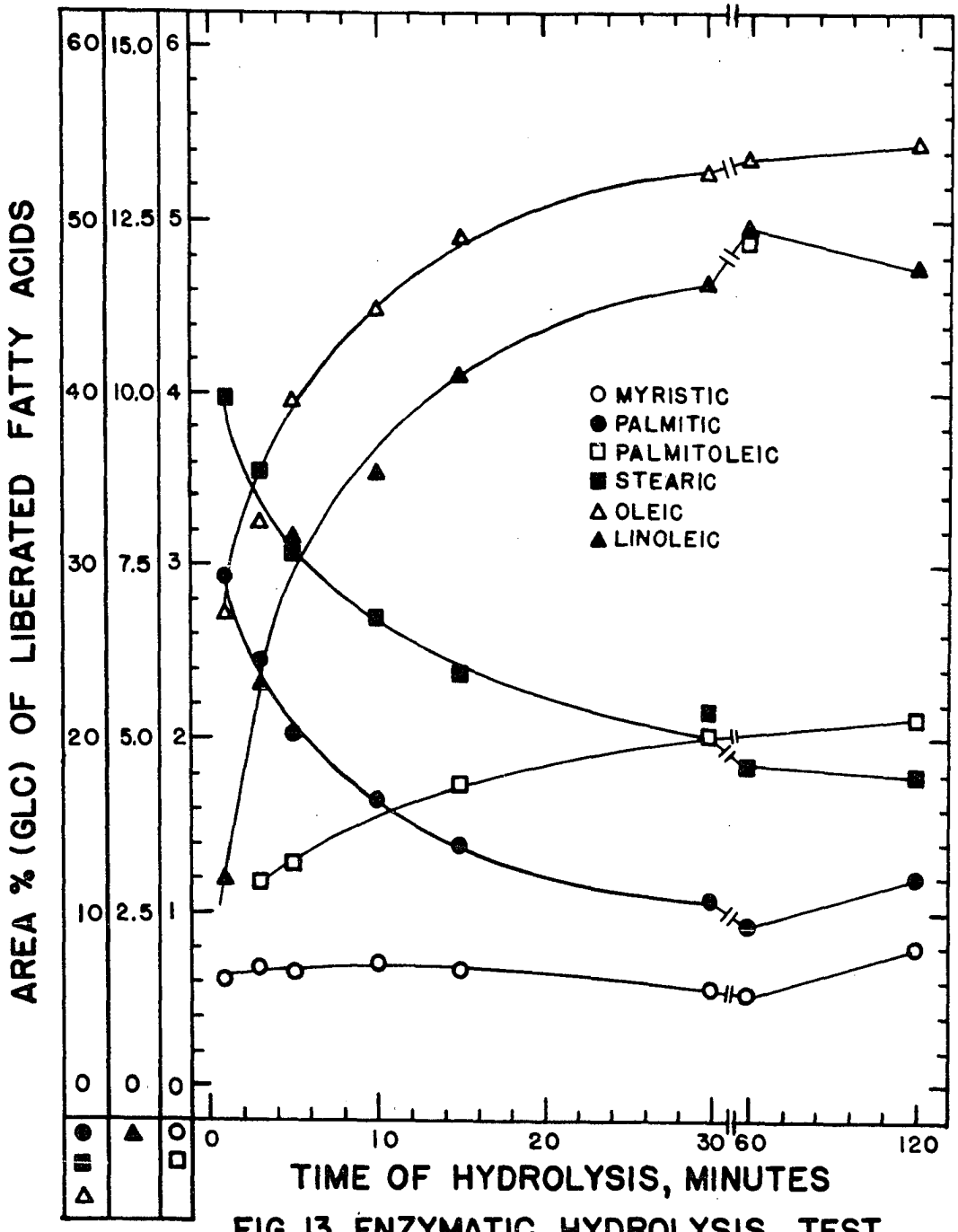


FIG. 13. ENZYMATIC HYDROLYSIS TEST, DIAZOMETHANE METHYL ESTERS

Table 50. Enzymatic hydrolysis tests, summary

Column:	1	2	3	4	5	6
Run:	1,3[a]	--	2,4[a]	17	--	--
Method	M and B	Comp.[b]	Mod.Ygs.		Modified Ygs.[c]	
Time	15	15	15	15	10	15
Sample size, gm.	2.0	0.5	0.12	0.12	0.010-0.020	0.12
Container	50 ml bkr.	Test tube	50 ml bkr.	50 ml bkr.	Test tube	50 ml bkr.
F.F.A.	Resin	Resin	Resin	Resin	CH_2N_2	CH_2N_2
			Chromatogram area percent			
Myr.	1.1	--	2.8	3.0	0.7	0.5
M.O.	0.4	--	1.6	1.5	--	--
Pal.	7.0	5.4	6.8	9.1	14.0	8.4
P.O.	3.5	1.3	8.2	7.4	1.7	1.7
Stear.	14.9	21.7	6.9	9.6	23.7	14.5
Oleic	61.4	62.7	64.1	58.7	49.0	63.2
Linol.	11.5	7.2	7.3	7.3	10.2	11.7
Other	0.3	1.6	2.4	3.5	0.6	--

[a]From Table 47.

[b]From Table 48.

[c]From Table 49.

or the modified Youngs procedure as conducted for the data of column 6 were the only suitable methods of those represented in Table 50.

Gas chromatography The gas chromatograph constructed and used in the previous fractional crystallizations gave excellent separation of the monocarboxylic acids found in lard when ethylene glycol succinate (EGS) substrate was used for the columns. But when the unsaturated monocarboxylic acids were oxidized, complete separation of all of the peaks could not be achieved by use of the EGS columns. Youngs (98)

obtained good separation of palmitate and azelate (DiC_9) using 15% butanediol succinate (BDS) on C-22 firebrick, but it was found here that columns of 15% BDS on Chromosorb did not give separation of the palmitate and azelate. After more study, it was concluded that the reason for the difference in the separations was not because of the equipment being used but because of the difference in the two inert supports used. A column of 15% BDS on C-22 firebrick was prepared later and the separations obtained by Youngs (98) could not be achieved. A column of 5% BDS on aqua regia treated C-22 firebrick gave good separation of the monocarboxylic acids and the dicarboxylic acid methyl esters but the retention times were somewhat longer than for the C-22 firebrick and Chromosorb columns because of the large pressure drop across the column due to the smaller mesh size of the solid support required to obtain good separations. BDS on Chromosorb W gave no separation of the palmitate-azelate pair and in general gave poorer separation of the C_{18} group. The column which gave the best all around performance was a column consisting of 10 ft x 3/16 in OD copper tubing with 15% BDS on 60/80 mesh Chromosorb plus 1.5 ft x 3/16 in OD copper tubing with 15% Dow Corning hi-vacuum silicone grease (DCSG) on 35/48 mesh aqua regia treated C-22 firebrick. The chromatograms of Figure 3 were obtained using such a column. The major disadvantage of this column was that the 1.5 ft section

of DCSG had the tendency to reverse the order of emergence of the saturated and unsaturated acids of a given chain length and therefore gave poorer separation of the stearate-oleate pair than BDS alone. But the DCSG was very effective in causing all of the dicarboxylic acids to emerge from the column in a shorter time than when the BDS column was used alone. By using the correct length of DCSG column, the desired separation of the myristate-pimelate(DiC_7) and palmitate-azelate (DiC_9) pairs could be obtained whereas with the BDS column alone, these separations were poor.

In the course of obtaining better separation of the monocarboxylic and dicarboxylic acid mixtures, several improvements were made in the gas chromatograph equipment. Details of these changes are given in the appendix. The improvements included the installation of a blower in the column oven to circulate the air and the use of a voltage regulator to help regulate the current supplied to the column oven heater. Baseline stability was improved by these changes but instability was not completely eliminated. The control of current to the oven heater could not overcome baseline instability due to changing ambient conditions. Rather than invest in equipment for a temperature control for the gas chromatograph, arrangements were made to use an F and M Model 500 gas chromatograph in the Chemical Engineering Department.

The F and M Model 500 gas chromatograph used Swagelok

fittings whereas the constructed gas chromatograph employed brass compression fittings using a single compression ring. These two fittings are not interchangeable. Therefore a new column consisting of 10 ft of BDS and 1.5 ft of DCSG was prepared. Quite different results were obtained using this new column. Comparable results were obtained by cutting off portions of the DCSG section until only 4 in of it remained. Similar columns prepared later also showed that the length of the DCSG section varied. This indicated that the same degree of packing was not obtained for the different BDS sections which resulted in different separations and therefore different lengths of DCSG were required.

One difficulty was encountered in the use of diazomethane for preparation of free fatty acid methyl esters for gas chromatograph samples. When the amount of esters in the samples was very low, larger injection samples were required. When the sample size required was over about one microliter, continued use of the same column gave progressively poorer separations. When the amount of esters was in the range of 40% of the sample, this difficulty was not noticeable. Some of the resolving efficiency could be recovered by several hours of bleeding the column at or slightly above the normal operating temperature but the columns did not return to their original efficiencies. The chromatograms obtained for the free fatty acid methyl esters in the hydrolysis (bile salts

and rapid stirring) products of the lards and the crystallizations fractions were not as good as desired. Figure 14 was typical of the separations obtained. The column used for Figure 14 was a 10 ft x 3/16 in OD copper tubing with 5% BDS on 80/100 mesh aqua regia treated C-22 firebrick. The conditions used were 220°C, helium flowrate of 54 ml per minute, and a bridge current of 200 milliamperes. The sample was run on the F and M Model 500 gas chromatograph and the sample size used was 0.5 microliters as measured by the use of a 1.0 microliter syringe.

The analyses of the lards and crystallization fractions were made on a 5 ft x 1/4 in OD EGS column and gave excellent separations as shown in Figure 4.

The data presented later for the fatty acid analyses of the 2-monoglycerides were obtained from a column consisting of 10 ft x 3/16 in OD copper tubing of 15% BDS on Chromosorb plus 4 in x 3/16 in OD copper tubing of 15% DCSG on 35/48 mesh aqua regia treated C-22 firebrick. Figure 15 shows the trace obtained for the 2-monoglyceride fatty acids methyl esters of F-4 R. The column temperature was 220°C, the helium flowrate 65 ml per minute, the bridge current 200 milliamperes, the sample size 0.6 microliters. The samples were run on the F and M Model 500 gas chromatograph.

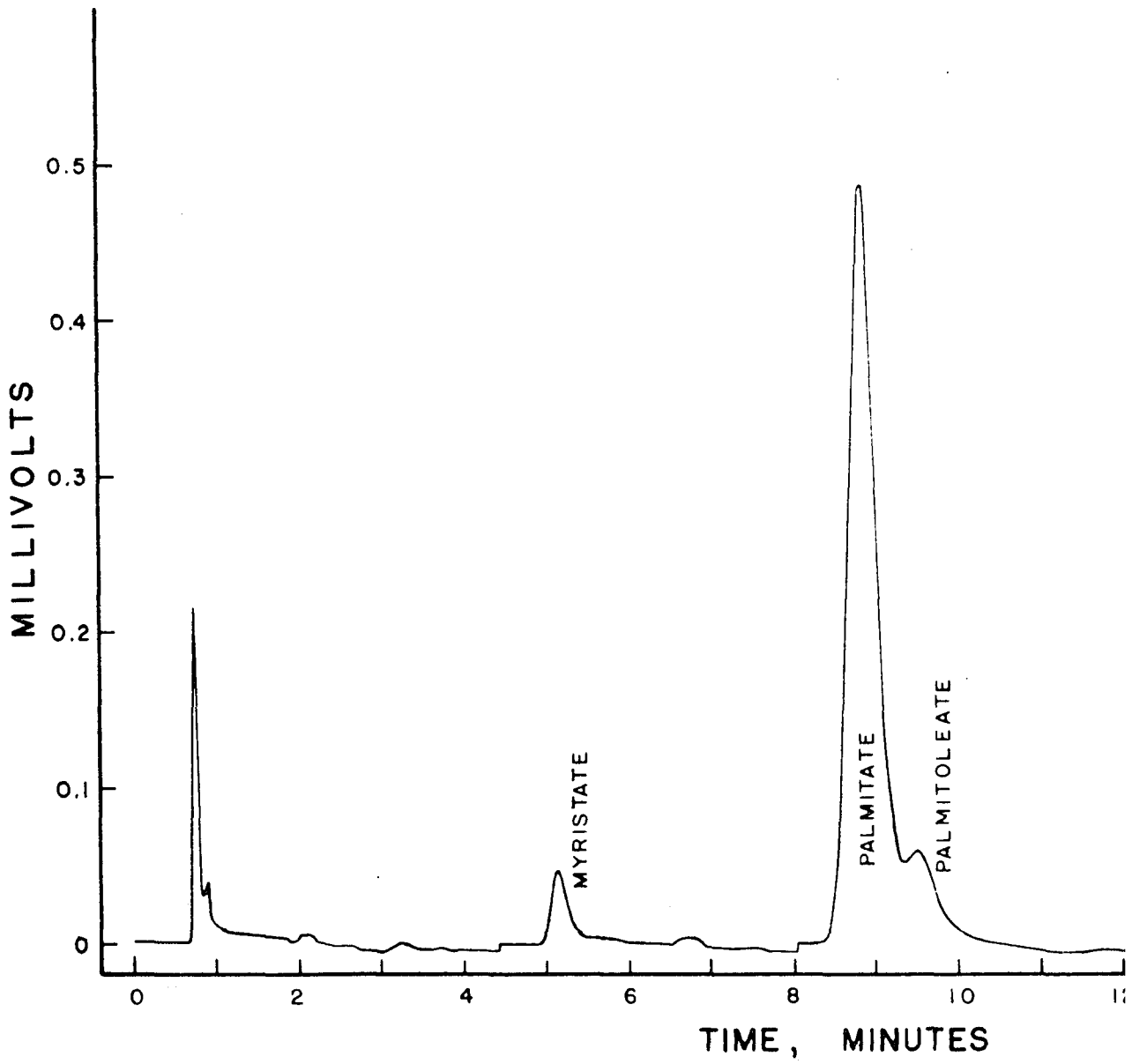
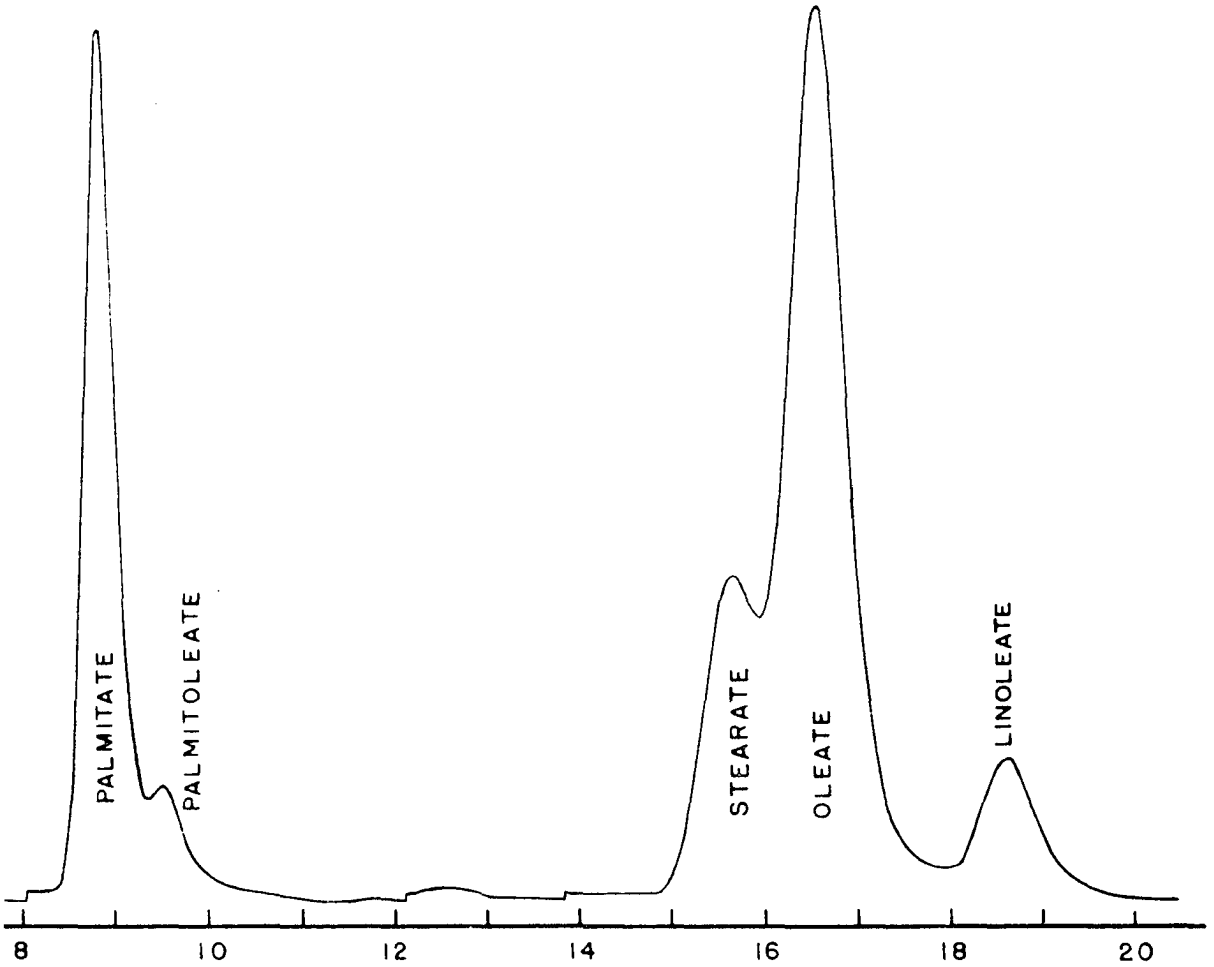


FIG. 14. FREE FATTY ACID METHYL ESTER ANALYSIS OF TI



ER ANALYSIS OF THE HYDROLYSIS PRODUCTS OF F-5 A

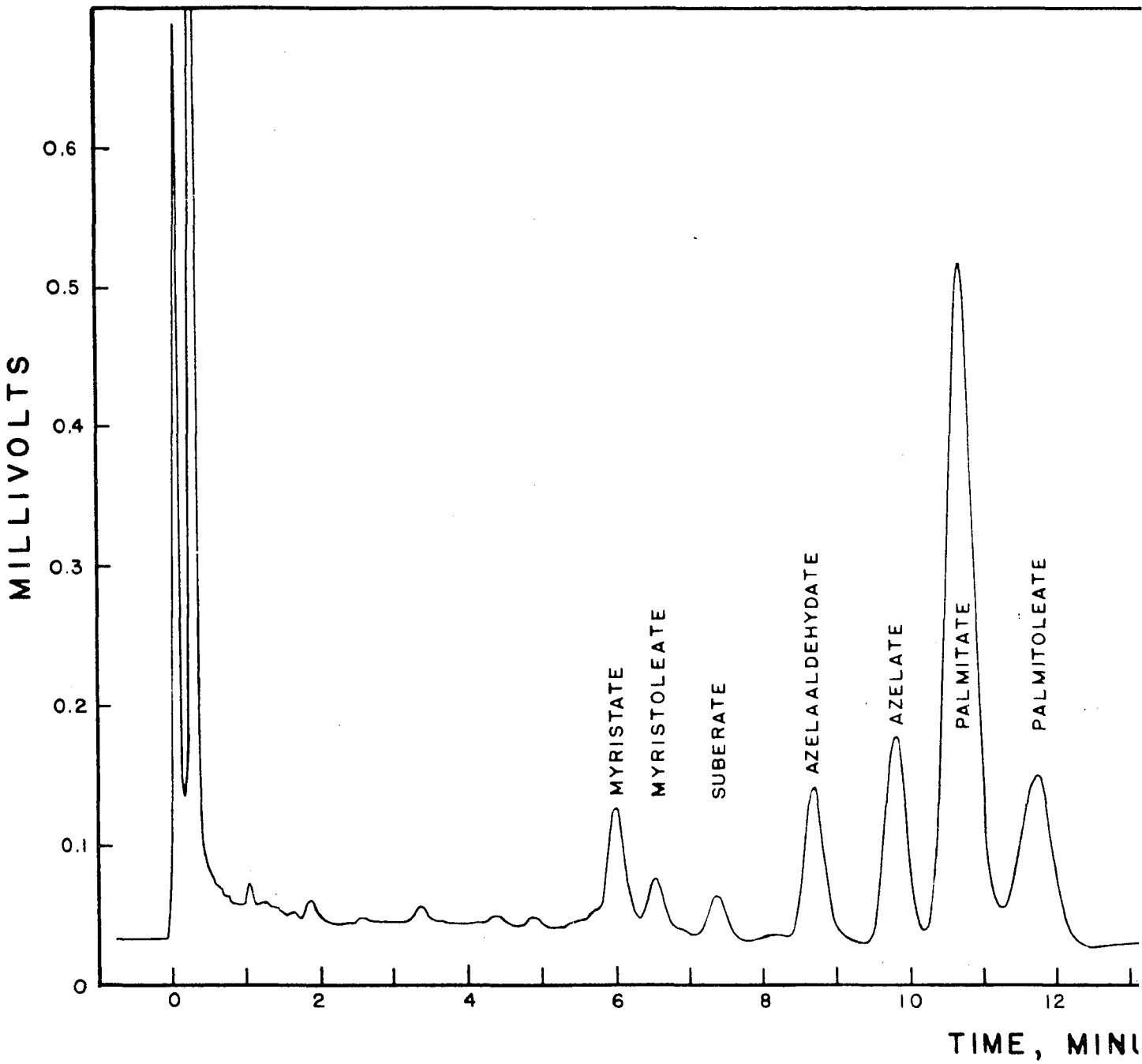
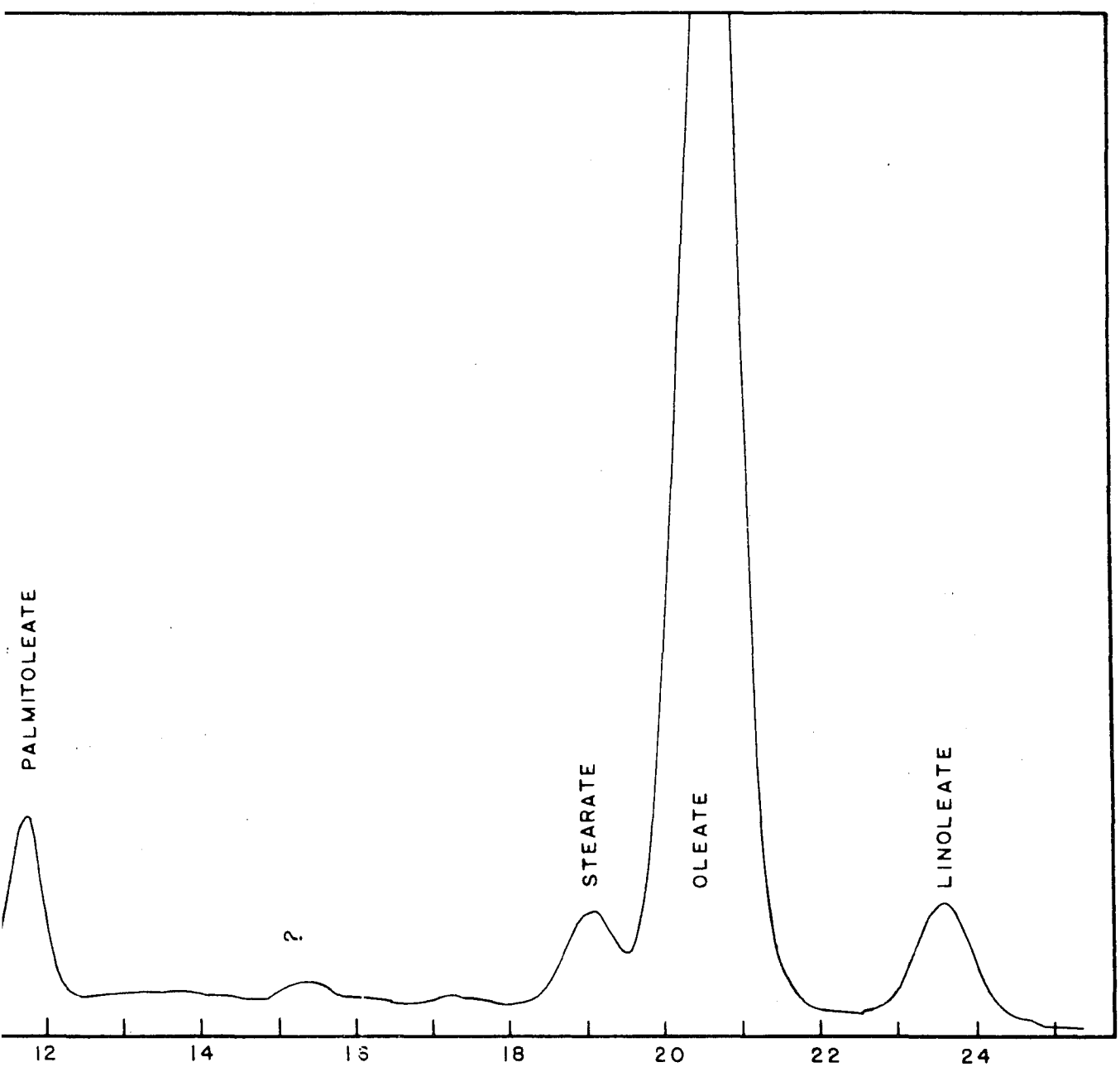


FIG. 15. 2-MONOGLYCERIDE FATTY A



E, MINUTES

ATTY ACID METHYL ESTERS OF F-4 R

Fractional crystallization, hydrolyses, and analyses

The fractional crystallization procedure was carried out simultaneously on three 25 gram samples; lard B, lard A, and lard R. The low temperature cabinet was modified slightly to accommodate the three samples instead of just two. Previous crystallizations had used flasks with outlets in the bottom and the filtrations were slow and difficult. In this crystallization, the samples were crystallized in beakers supported on a false bottom made of hardware cloth. The filtration flasks and funnels were kept in the Dry Ice chamber until needed for the filtration. They were then placed in the sample chamber one at a time and the clear acetone solution poured through the funnel before the precipitate was poured into the funnel. The filter cakes were pressed and washed as in previous crystallizations. An attempt to use filter sticks for the first filtration was not any better than using the flasks with outlets in the bottom. The filter sticks were only a few cm in diameter. They would have to be as large as the funnels required to do the same job if they were to be successful.

The randomized lard sample was prepared from 36.2 grams of lard A by first heating on a wax bath 30-45 minutes at 120-150^{o}C while bubbling nitrogen through the lard to remove water present in the lard. Sodium methoxide, 72 milligrams

(0.2% by weight of the lard), was then added to the lard and the heating continued for two hours at 115°C while bubbling nitrogen through the sample. The catalyst was destroyed by adding acetic acid. The sample was cooled, dissolved in ethyl ether and transferred to a 250 ml separatory funnel. The first water washing resulted in the separation of an ether and a water layer but the water layer was very milky. This milky water layer was separated and further treated. It was found to be alkaline or near neutral as blue litmus did not turn red. Dilute hydrochloric acid was added to acidify the water and the water layer was extracted twice with ether. The water layer became clear during the first extraction. These ether extracts were added to the lard sample in the separatory funnel and washed three more times with water. Emulsions formed which did not persist for more than a few minutes. The ether solution was then dried over anhydrous granular sodium sulfate and the ether evaporated. The last traces of ether were removed with the aid of a water aspirator vacuum. The sample was then stored under refrigeration in a 125 ml Erlenmeyer flask stoppered with a rubber stopper after flushing the flask with nitrogen.

Methyl esters of the fatty acids in each of the lards and the fractions were prepared using anhydrous 5% hydrochloric acid-methanol and the methyl esters were recovered by adding

water and extracting with ethyl ether. The methyl esters were transferred to 1/2 dram screw top vials and stored under refrigeration.

During the study of the hydrolysis procedures, each lard and its fractions were hydrolyzed under the compromise conditions. These samples were run in 10 ml beakers using 0.5 gram samples and the pH maintained at 8.0 throughout the 15 minute hydrolysis time. One change made here was the use of 2.0 M buffer solution instead of 1.0 M ammonium chloride-ammonium hydroxide buffer as was used before in this compromise method and as is used in the Mattson and Beck method. The pH was measured using a Beckman Model G (2-1/2 in electrodes) pH meter. As this pH meter uses a completely enclosed metal chamber to protect the electrode leads from stray currents and magnetic fields caused by relay coils, motor windings, etc., it was necessary to put a shield on the calomel electrode lead and ground this shield. The instrument case was also grounded. The pH meter operated very well when grounded in this manner even with a water bath motor only inches from the electrodes in the sample being maintained in the bath at 40°C. The shielding for the electrode lead was made from the flexible shielding removed from a piece of three strand shielded cable. Care must be taken to insure that the shield does not come in contact with the bare lead wire where

the pin is attached to the lead wire or that it does not touch the jack in the pH meter.

These conditions were used at that time to promote the formation of monoglycerides (see the hydrolysis procedures summary in the appendix on hydrolysis). The 1-monoglycerides in the hydrolysis products were destroyed by periodic acid oxidation and the 2-monoglycerides recovered on silica gel columns as previously described. It was thought that even though the free fatty acids liberated by the enzyme hydrolysis might not be representative of the 1- and 3-positions, that the 2-monoglycerides would be representative of the 2-position fatty acids. A study of the results showed that the inverse lever rule was not obeyed in many instances. The triglyceride (lards and crystallization fractions) fatty acid analyses and the free fatty acid analyses of the hydrolysis products were probably more reliable than these 2-monoglyceride fatty acid analyses. As was mentioned before, difficulty was encountered with ether vapors forming in the silica gel columns and when ten columns were run simultaneously, the flowrates may have been higher than used in the test columns in developing the method. Either of these two factors could have caused the triglycerides, fatty acids, glycolic aldehydes, and diglycerides peak to overlap the monoglyceride peak.

The lards and crystallization fractions were finally

hydrolyzed using the modified Youngs method described in the hydrolysis time tests. The conditions used were 0.12 gram sample size, 18 ml of buffer solution, a 50 ml beaker container, bile salts, and a maximum stirring rate to insure the formation of a finely divided, stable emulsion. The free fatty acids were converted to methyl esters using diazomethane.

The weights of the crystallization fractions obtained are given in Table 51 and a summary of the saturated and unsaturated fatty acid contents of the lards and their fractions is given in Table 52. The lards and their crystallization fraction fatty acid compositions are given in Table 53. The glyceride compositions obtained from the methyl ester compositions and weights of the fractions are given in Table 54. Table 55 shows the material balance obtained from the fraction weights and their fatty acid methyl ester compositions as compared to the lard compositions. Tables 56 and 57 give the methyl ester compositions of the hydrolysis product analyses of the liberated fatty acids and of the 2-monoglycerides. The free fatty acids and the 2-monoglycerides were obtained from different hydrolyses of the same samples. Table 58 is a summary of the compositions of the lards, crystallization fractions, the free fatty acids and the 2-monoglycerides arranged such that a check of the inverse lever rule can be easily obtained.

Table 51. Crystallization fraction weights[a]

	Before		After		Random	
	Frac. weight	Adj.frac. weight	Frac. weight	Adj.frac. weight	Frac. weight	Adj. frac. weight
P-3	4.219	4.223	1.675	1.681	1.558	1.567
P-6	2.460	2.462	5.534[b]	5.889	5.051	5.081
F-6	10.791	10.801	7.026	7.050	6.388	6.426
F-5	5.003	5.008	5.738	5.758	5.823	5.857
F-4	2.504	2.506	4.607	4.623	6.033	6.069
Total	24.977	25.000	24.580	25.001	24.853	25.000

[a]The loss in weight of the lard samples was proportioned between the fractions directly proportional to the fraction weights.

[b]Part of the P-6 A fraction bumped out of its container while solvent was being evaporated. The P-6 A fraction weight was estimated by subtracting the total fraction weights of lard A from the average total weight of the lard B and lard R fractions and adding the difference to the weight of the P-6 A remaining. The remaining weight of P-6 A was 5.534 grams and the difference added was 0.335 gram.

Table 52. Saturated and unsaturated acids in lards and lard fractions

	Mol % saturated			Mol % unsaturated		
	B	A	R	B	A	R
Lard	41.38	43.38	45.74	58.62	56.62	54.26
P-3	73.44	90.51	91.32	26.56	9.48	8.68
P-6	66.56	63.25	65.24	33.43	36.75	34.76
F-6	38.15	43.44	48.14	61.86	56.56	51.87
F-5	20.68	24.75	35.05	79.31	75.25	64.95
F-4	17.67	15.14	16.31	82.32	84.87	83.68

Table 53. Fatty acid methyl ester compositions of the lards and lard crystallization fractions

	Lard			P-3			P-6		
	B	A	R	B	A	R	B	A	R
				Weight %					
Myr.	1.50	1.45	1.73	0.59	1.93	2.02	1.70	1.58	1.46
Pal.	26.12	27.29	28.98	35.16	52.85	55.18	38.91	39.66	40.54
P.O.	2.21	2.61	3.28	0.71	Trace	0.23	0.72	1.18	1.74
Stear.	12.26	13.16	13.54	36.90	35.26	33.68	24.71	20.64	21.98
Oleic	46.92	46.86	46.87	22.91	9.42	8.89	29.04	32.28	31.01
Linol.	10.99	8.64	5.62	3.74	0.55	Trace	4.94	4.67	3.29
	100.00	100.01	100.02	100.01	100.01	100.00	100.02	100.01	100.02

	P-6			P-5			P-4		
	B	A	R	B	A	R	B	A	R
	1.71	1.67	1.66	1.76	1.57	1.63	2.05	1.60	1.60
	28.31	27.51	30.33	14.89	16.04	23.66	12.70	10.61	11.85
	1.97	2.24	2.78	4.96	3.69	4.31	8.82	5.63	7.00
	6.31	12.72	14.67	2.72	5.85	8.28	1.72	1.88	1.76
	55.93	49.65	45.72	59.15	63.13	54.85	51.35	62.77	68.42
	5.78	6.22	4.86	16.53	9.73	7.29	23.37	17.53	9.39
	100.01	100.01	100.02	100.01	100.01	100.02	100.01	100.02	100.02

	Lard mol %			P-3 mol %			P-6 mol %		
	B	A	R	B	A	R	B	A	R
Myr.	1.78	1.72	2.03	0.69	2.25	2.33	1.99	1.85	1.71
Pal.	27.78	28.99	30.71	37.29	55.01	57.30	40.99	41.74	42.61
P.O.	2.36	2.79	3.49	0.75	Trace	0.25	0.77	1.25	1.85
Stear.	11.82	12.67	13.00	35.46	33.25	31.69	23.58	19.66	20.92
Oleic	45.53	45.42	45.29	22.17	8.95	8.43	27.88	30.98	29.73
Linol.	10.73	8.42	5.47	3.64	0.53	Trace	4.78	4.52	3.18
	100.00	100.01	99.99	100.00	99.99	100.00	99.99	100.00	100.00

Table 53. (Continued)

	Lard mol %			P-3 mol %			P-6 mol %		
	B	A	R	B	A	R	B	A	R
Mol % total satd.	41.38	43.38	45.74	73.44	90.51	91.32	66.56	63.25	65.24

	F-6 mol %			F-5 mol %			F-4 mol %		
	B	A	R	B	A	R	B	A	R
Myr.	2.04	1.98	1.95	2.11	1.88	1.93	2.45	1.92	1.91
Pal.	30.05	29.22	32.13	15.94	17.19	25.16	13.55	11.39	12.69
P.O.	2.10	2.38	2.98	5.35	3.97	4.63	9.49	6.10	7.56
Stear.	6.06	12.24	14.06	2.63	5.68	7.96	1.67	1.83	1.71
Oleic	54.13	48.12	44.16	57.73	61.71	53.19	49.94	61.49	66.88
Linol.	5.63	6.06	4.73	16.23	9.57	7.13	22.89	17.28	9.24
	100.01	100.00	100.01	99.99	100.00	100.00	99.99	100.01	99.99
Mol % total satd.	38.15	43.44	48.14	20.68	24.75	35.05	17.67	15.14	16.31

Table 54. Glyceride composition of the lards

Fraction	Wt. of frac. gms	MCL F.A. / 100 gm F.A.	Total wt % F.A. in glyc.	Mols of glyc.	Mol % of glyc. GS_3	GS_2U	GSU_2	GU_3	Mols of glyc. $GS_3 \times 100$	$GS_2U \times 100$	$GSU_2 \times 100$	$GU_3 \times 100$
					Before							
P-3	4.22	0.3486	95.76	0.004696	[a]20.32	79.68			0.0954	0.3742		
P-6	2.46	0.3511	95.73	0.002756		99.71	0.29			0.2748	0.0008	
F-6	10.80	0.3484	95.76	0.012011		14.45	85.55			0.1736	1.0275	
F-5	5.01	0.3456	95.79	0.005529			62.05	37.95			0.3431	0.2098
F-4	2.51	0.3468	95.77	0.002779			53.01	46.99			0.1473	0.1306
	25.00			0.027771					0.0954	0.8226	1.5187	0.3404
									3.44%	29.62%	54.69%	12.26%
					After							
P-3	1.68	0.3552	95.68	0.001903	71.56	28.44			0.1362	0.0541		
P-6	5.89	0.3515	95.72	0.006606		89.75	10.25			0.5929	0.0677	
F-6	7.05	0.3481	95.75	0.007833		30.32	69.68			0.2375	0.5458	
F-5	5.76	0.3450	95.80	0.006346			74.25	25.75			0.4712	0.1634
F-4	4.62	0.3443	95.79	0.005079			45.42	54.58			0.2307	0.2772
	25.00			0.027767					0.1362	0.8845	1.3154	0.4406
									4.91	31.85	47.37	15.87
					Random							
P-3	1.57	0.3560	95.68	0.001783	73.96	26.04			0.1319	0.0464		
P-6	5.08	0.3518	95.71	0.005702		95.72	4.28			0.5458	0.0244	
F-6	6.43	0.3492	95.74	0.007166		44.42	55.58			0.3183	0.3983	
F-5	5.86	0.3478	95.76	0.006506		5.15	94.85			0.0335	0.6171	
F-4	6.07	0.3451	95.79	0.006689			48.93	51.07			0.3273	0.3416
	25.01			0.027846					0.1319	0.9440	1.3671	0.3416
									4.74	33.90	49.10	12.27

[a] Mols of glycerides $= \left(\frac{\text{wt of gly frac}}{1}\right)\left(\frac{\text{mols F.A.}}{100 \text{ gms F.A.}}\right)\left(\frac{\text{Total wt \% F.A. in gly}}{100}\right)\left(\frac{\text{mols of gly}}{\text{mol of F.A.}}\right) = (4.22)\left(\frac{0.3486}{100}\right)\left(\frac{95.76}{100}\right)\left(\frac{1}{3}\right) = 0.004696.$

Table 55. Fatty acid balance of lard crystallization fractions

Before	Wt, gms	Wt of fatty acids in gms						Total wt fatty acids
		Myr.	Pal.	P.O.	Stear.	Oleic	Linol.	
Lard	25.00	0.360	6.255	0.530	2.935	11.235	2.633	23.948
P-3	4.22	0.024	1.421	0.029	1.491	0.926	0.151	4.042
P-6	2.46	0.040	0.916	0.017	0.582	0.684	0.116	2.355
F-6	10.80	0.177	2.928	0.204	0.652	5.784	0.597	10.342
F-5	5.01	0.085	0.714	0.238	0.131	2.839	0.793	4.800
F-4	2.51	0.049	0.305	0.212	0.041	1.234	0.562	2.403
	25.00	0.375	6.284	0.700	2.897	11.467	2.219	23.942
								23.942
After								
Lard	25.00	0.348	6.533	0.625	3.150	11.218	2.068	23.942
P-3	1.68	0.031	0.850	Trace	0.567	0.151	0.009	1.608
P-6	5.89	0.089	2.236	0.067	1.164	1.820	0.263	5.639
F-6	7.05	0.113	1.857	0.151	0.859	3.352	0.420	6.752
F-5	5.76	0.086	0.885	0.204	0.323	3.484	0.537	5.519
F-4	4.62	0.071	0.469	0.249	0.083	2.778	0.776	4.426
	25.00	0.390	6.297	0.671	2.996	11.585	2.005	23.944
								23.944
Random								
Lard	25.00	0.415	6.938	0.785	3.240	11.218	1.345	23.941
P-3	1.57	0.030	0.829	0.003	0.506	0.134	Trace	1.502
P-6	5.08	0.071	1.971	0.085	1.069	1.508	0.160	4.864
F-6	6.43	0.102	1.867	0.171	0.903	2.814	0.299	6.156
F-5	5.86	0.091	1.328	0.242	0.465	3.078	0.409	5.613
F-4	6.07	0.093	0.689	0.407	0.103	3.978	0.546	5.816
	25.01	0.387	6.684	0.908	3.046	11.513	1.414	23.951
								23.952

Table 56. Fatty acid compositions of free fatty acids in hydrolysis products of lards and lard fractions

	Lard			P-3			P-6		
	B	A	R	B	A	R	B	A	R
				Weight %					
Myr.	0.5	1.5	1.7	0.3	2.4	2.0	0.3	1.6	1.1
Pal.	8.4	26.9	26.2	11.0	47.5	47.6	15.7	36.5	39.0
P.O.	1.7	2.6	3.0	1.0	0.9	1.6	1.1	1.6	2.6
Stear.	14.5	13.0	14.7	43.9	27.7	28.6	29.1	19.5	19.6
Oleic	63.2	48.9	50.2	37.3	19.2	18.7	45.7	35.4	33.5
Linol.	11.7	6.9	4.1	6.6	2.5	1.4	8.2	5.5	4.2
Total	100.0	99.8	99.9	100.1	100.2	99.9	100.1	100.1	100.0
				Mol %					
Myr.	0.6	1.8	2.0	0.4	2.8	2.1	0.4	1.9	1.3
Pal.	9.1	28.6	27.9	11.9	49.5	43.6	16.9	38.5	41.0
P.O.	1.8	2.8	3.2	1.1	1.0	1.5	1.2	1.7	2.8
Stear.	14.3	12.6	14.2	43.2	26.1	36.0	28.4	18.6	18.7
Oleic	62.5	47.5	48.7	36.9	18.3	15.6	45.0	34.0	32.2
Linol.	11.6	6.7	4.0	6.6	2.4	1.2	8.1	5.3	4.1
Total	99.9	100.0	100.0	100.1	100.1	100.0	100.0	100.0	100.1
Mol % total satd.	24.0	43.0	44.1	55.5	78.4	81.7	45.7	59.0	61.0

Table 56. (Continued)

	F-6			F-5			F-4		
	B	A	R	B	A	R	B	A	R
				Weight %					
Myr.	0.8	1.9	1.5	0.7	1.4	1.0	0.3	1.2	1.3
Pal.	11.6	31.3	31.0	11.5	24.6	27.7	8.4	14.4	12.5
P.O.	0.6	2.7	2.1	2.8	3.2	3.0	3.5	5.3	4.3
Stear.	12.1	17.3	20.6	7.4	14.1	12.4	5.8	2.9	4.1
Oleic	68.7	41.8	40.2	60.8	49.3	50.0	61.1	62.4	68.2
Linol.	6.2	4.9	4.6	16.7	7.4	5.9	20.9	13.8	9.5
Total	100.0	99.9	100.0	99.9	100.0	100.0	100.0	100.0	99.9
				Mol %					
Myr.	1.0	2.2	1.8	0.8	1.7	1.2	0.4	1.4	1.6
Pal.	12.6	33.1	32.9	12.4	26.2	29.4	9.1	15.4	13.4
P.O.	0.6	2.9	2.2	3.0	3.4	3.2	3.8	5.7	4.7
Stear.	11.9	16.6	19.8	7.2	13.6	11.9	5.7	2.8	4.0
Oleic	67.8	40.4	38.9	59.9	47.9	48.5	60.3	61.0	66.9
Linol.	6.2	4.8	4.5	16.6	7.2	5.8	20.8	13.6	9.4
Total	100.1	100.0	100.1	99.9	100.0	100.0	100.1	99.9	100.0
Mol % total satd.	25.5	51.9	54.5	20.4	41.5	42.5	15.2	19.6	19.0

Table 57. Fatty acid methyl ester compositions of the 2-monoglycerides of the hydrolysis products (mol %)

	Lard			P-3			P-6		
	B	A	R	B	A	R	B	A	R
Myr.	3.99	2.03	2.01	2.52	3.48	3.28	4.86	2.13	2.36
M.O.	-	-	-	-	-	-	-	-	-
DiC_8	-	-	-	-	-	-	0.19	-	-
Semiaz	2.94	0.71	1.90	-	2.81	-	0.24	-	0.73
DiC_9	-	0.46	1.93	3.65	0.83	0.62	-	-	-
Pal.	48.66	30.10	32.11	54.48	42.79	50.14	82.23	43.70	47.62
P.O.	2.24	3.52	2.52	1.25	1.64	1.52	0.57	1.93	1.60
DiC_{10}	0.58	-	-	-	-	0.84	0.68	-	0.68
Semi (DiC_{11})	0.78	-	-	0.78	1.95	-	-	-	-
DiC_{11}				1.00					
Stear.	8.06	12.24	12.52	24.83	23.58	27.67	6.46	17.58	21.53
Oleic	28.63	43.65	40.07	10.63	20.96	14.90	4.40	32.08	23.95
Linol	4.13	5.29	2.69	0.86	1.97	1.04	0.38	2.58	1.52
Linolen.	100.01	-	1.90	100.00	-	-	100.01	-	99.99
Arach.		2.00	2.35		-	-		-	
		100.00	100.00		100.01	100.01		100.00	
		F-6			F-5			F-4	
Myr.	5.21	2.65	2.66	5.01	2.26	2.26	4.20	2.08	2.50
M.O.	-	-	-	-	-	-	0.93	0.63	1.00
DiC_8	0.27	-	-	-	-	-	-	0.46	0.81
Semiaz	0.25	0.71	1.22	0.97	1.07	1.23	7.30	3.33	4.12
DiC_9	-	-	0.28	-	-	0.80	1.37	2.22	5.57
Semi (DiC_{11})	0.19	-	0.11	0.97	-	0.60	0.33	-	0.42
Pal.	73.79	32.20	38.73	38.75	12.75	20.80	27.04	13.51	17.59
P.O.	2.43	2.48	2.06	10.25	4.11	4.32	8.70	6.23	5.07
DiC_{10}	0.52	-	0.20	0.31	-	-	-	-	-
DiC_{11}									
Stear.	3.30	11.84	13.29	1.81	2.87	6.15	3.05	2.47	3.40
Oleic	12.49	47.03	39.33	35.52	66.71	58.07	37.55	53.92	54.83
Linol	1.55	3.08	2.12	6.41	6.69	5.78	9.53	8.56	4.70
Linolen.	100.00	-	100.00	100.00	1.39	100.01	100.00	2.70	100.01
Arach.		-			2.14			3.90	
		99.99			99.99			100.01	

Table 58. Summary of fatty acid compositions, mol percent

		Lard			P-3			P-6		
		B	A	R	B	A	R	B	A	R
Myr.	FFA	0.6	1.8	2.0	0.4	2.8	2.1	0.4	1.9	1.3
	TG	1.8	1.7	2.0	0.7	2.3	2.3	2.0	1.9	1.7
	2-MG	4.0	2.0	2.0	2.5	3.5	3.3	4.9	2.1	2.4
Pal.	FFA	9.1	28.6	27.9	11.9	49.5	43.6	16.9	38.5	41.0
	TG	27.8	29.0	30.7	37.3	55.0	57.3	41.0	41.7	42.6
	2-MG	48.7	30.1	32.1	54.5	42.8	50.1	82.2	43.7	47.6
P.O.	FFA	1.8	2.8	3.2	1.1	1.0	1.5	1.2	1.7	2.8
	TG	2.4	2.8	3.5	0.8	Trace	0.3	0.8	1.3	1.9
	2-MG	2.2	3.5	2.5	1.3	1.6	1.5	0.6	1.9	1.6
Stear.	FFA	14.3	12.6	14.2	43.2	26.1	36.0	28.4	18.6	18.7
	TG	11.8	12.7	13.0	35.5	33.3	31.7	23.6	19.7	20.9
	2-MG	8.1	12.2	12.5	24.8	23.6	27.7	6.5	17.6	21.5
Oleic.	FFA	62.5	47.5	48.7	36.9	18.3	15.6	45.0	34.0	32.2
	TG	45.5	45.4	45.3	22.2	9.0	8.4	27.9	31.0	29.7
	2-MG	28.6	43.7	40.1	10.6	21.0	14.9	4.4	32.1	24.0
Linol.	FFA	11.6	6.7	4.0	6.6	2.4	1.2	8.1	5.3	4.1
	TG	10.7	8.4	5.5	3.6	0.5	Trace	4.8	4.5	3.2
	2-MG	4.1	5.3	2.7	0.9	2.0	1.0	0.4	2.6	1.5
Diacids and others	FFA	-	-	-	-	-	-	-	-	-
	TG	-	-	-	-	-	-	-	-	-
	2-MG	4.3	3.2	8.1	5.4	5.6	1.5	1.1	0	1.4

Table 58. (Continued)

		F-6			F-5			F-4		
		B	A	R	B	A	R	B	A	R
Myr.	FFA	1.0	2.2	1.8	0.8	1.7	1.2	0.4	1.4	1.6
	TG	2.0	2.0	2.0	2.1	1.9	1.9	2.5	1.9	1.9
	2-MG	5.2	2.7	2.7	5.0	2.3	2.3	4.2	2.1	2.5
Pal.	FFA	12.6	33.1	32.9	12.4	26.2	29.4	9.1	15.4	13.4
	TG	30.1	29.2	32.1	15.9	17.2	252.	13.6	11.4	12.7
	2-MG	73.8	32.2	38.7	38.8	12.8	20.8	27.0	13.5	17.6
P.O.	FFA	0.6	2.7	2.2	3.0	3.4	3.2	3.8	5.7	4.7
	TG	2.1	2.4	3.0	5.4	4.0	4.6	9.5	6.1	7.6
	2-MG	2.4	2.5	2.1	10.3	4.1	4.3	8.7	6.2	5.1
Stear.	FFA	11.9	16.6	19.8	7.2	13.6	11.9	5.7	2.8	4.0
	TG	6.1	12.2	14.1	2.6	5.7	8.0	1.7	1.8	1.7
	2-MG	3.3	11.8	13.3	1.8	2.9	6.2	3.1	2.5	3.4
Oleic.	FFA	67.8	40.4	38.9	59.9	47.9	48.5	60.3	61.0	66.9
	TG	54.1	48.1	44.2	57.7	61.7	53.2	49.9	61.5	66.9
	2-MG	12.5	47.0	39.3	35.5	66.7	58.1	37.6	53.9	54.8
Linol.	FFA	6.2	4.8	4.5	16.6	7.2	5.8	20.8	13.6	9.4
	TG	5.6	6.1	4.7	16.2	9.6	7.1	22.9	17.3	9.2
	2-MG	1.6	3.1	2.1	6.4	6.7	5.8	9.5	8.6	4.7
Diacids and others	FFA	-	-	-	-	-	-	-	-	-
	TG	-	-	-	-	-	-	-	-	-
	2-MG	1.2	0.7	1.8	2.3	4.6	2.6	9.9	13.2	11.9

The inverse lever rule was not obeyed in many instances but as has been pointed out, the 2-monoglyceride fatty acid compositions are probably the least reliable data. A study of the 2-monoglycerides was desired so as to have a better check on the data by making material balances such as the 2:1 ratio of the mol percentage difference between the 2-monoglyceride and the triglyceride fatty acids and the difference between the free fatty acid and triglyceride fatty acid compositions. But the 2-monoglyceride compositions are not necessary to make the study. If the 2-monoglyceride compositions are desired they can be calculated from the triglyceride and free fatty acid compositions.

Ratios of the mol percentages of two fatty acids in each of the lards, lard fractions, free fatty acids, and the 2-monoglycerides were calculated. The ratios used here were myristic:palmitic, stearic:palmitic, palmitoleic:oleic, and linoleic:oleic. The purpose of the calculations was to determine whether or not the fatty acid distribution in the three different position groups (1-, 2-, and 3-positions; 1- and 3-positions; and 2-positions) was random for any of the three types of samples (before, after, and random). If the distribution for the acids was random, then the ratios would have been constants for the lard and each of its fractions. None of the three samples gave constant ratios for

each fraction. The before samples showed much greater differences in the ratios than the after and random samples. This indicated that none of the three lard samples was completely random as a whole or even in the 1- and 3-positions or in the 2-positions. The before samples were further removed from random distribution than were the after and random samples. These calculations have not been included here but the similar information is presented in a different manner. These ratios can be calculated from the data in Table 58 if desired.

Youngs and Sallans (99) presented a method of correlating fractionation data to provide information on the glyceride structure of fats and in particular showed the distribution of the unsaturated fatty acids among the GS_2U, GSU_2, and GU_3 glyceride types. They made a graph of the percentage of the major component fatty acid found in each fraction versus the iodine value of the fraction. This curve was compared to the straight line formed by plotting the calculated data for GS_3, GS_2U, GSU_2, and GU_3 obtained gy assuming the unsaturated acids were distributed at random in all of the glycerides. If the curve of the experimental results coincided with the calculated curve, then the distribution of the unsaturated acid plotted as the ordinate was distributed at random in the fat. They presented data and graphs for a completely random synthetic fat prepared from a mixture

of stearic, oleic, and linoleic acids; for sunflower oil; for cottonseed oil, and for beef tallow. All of these graphs gave experimental curves which coincided with the calculated curve based on random distribution of the unsaturated acids. This did not of course rule out the possibility of different distributions in the 1- and 3-positions and in the 2-position but did show that the overall distribution in the triglycerides was random for the unsaturated fatty acids. The data used for the beef tallow were obtained from the paper of Riemenschneider et al. (79). This same paper also gave data for the fractionation of lard but Youngs and Sallans (99) did not include the lard data in their study. These data have been presented here in Table 59 and a graph such as they suggested was prepared and is shown in Figure 16. The experimental and calculated curves clearly show that oleic acid did not correspond to a random distribution. Also included in Figure 16 are the same type of graphs for the lard B studied here. The before sample agrees very well with the sample of lard studied by Riemenschneider et al. (79).

Consider a sample in which the major component fatty acid is oleic acid and that it is distributed at random but that linoleic acid is not. The calculated iodine values for GS_3, GS_2U, GSU_2, and GU_3 assume a random distribution of both. Therefore a graph of oleic acid (the major component) composition versus iodine value would produce curves which coincide

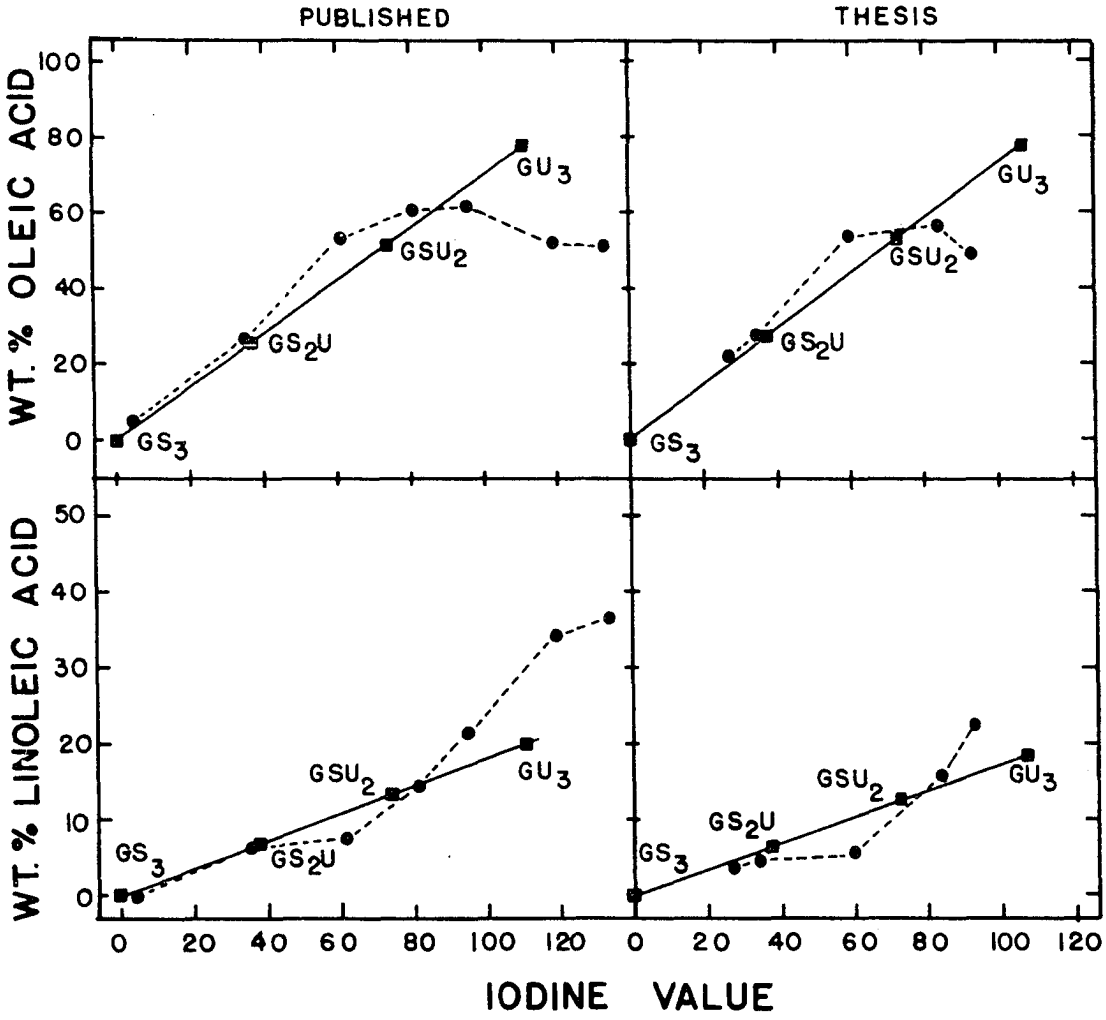


FIG. 16. FATTY ACID DISTRIBUTION IN LARD

Table 59. Data for graphs in Figure 16

Fraction	Wt. % oleic	Wt. % linol.	Iodine value
	Riemenschneider et al. (79) Experimental		
P-2	4.8	0.19	4.45
P-4	27.0	6.38	35.6
P-6	53.7	7.70	61.3
P-8	60.8	14.56	81.1
P-10	61.9	21.34	95.6
P-12	52.1	34.20	119.7
F	50.8	36.51	134.1

Calculated

	GS_3	GS_2U	GSU_2	GU_3
Wt. % oleic	0	25.9	51.8	77.7
Wt. % linol.	0	6.7	13.3	20.0
Iodine value	0	37.0	74.0	111.0

Thesis data Experimental

Fraction	Wt. % oleic	Wt. % linol.	Iodine value[a]
P-3	21.9	3.6	26.8
P-6	27.8	4.7	34.2
F-6	53.6	5.5	60.0
F-5	56.7	15.8	84.0
F-4	49.2	22.4	92.9

Calculated

	GS_3	GS_2U	GSU_2	GU_3
Wt. % oleic	0	27.0	53.0	78.0
Wt. % linol.	0	6.3	12.4	18.3
Iodine value	0	37.5	72.5	106.8

[a]Calculated from gas chromatogram peak areas.

and the conclusion at first might be that the unsaturated acids were distributed at random. But if a plot of the percentage linoleic acid composition versus iodine value were prepared, the experimental curve and the calculated curve would not coincide because the actual linoleic acid distribution was not random but the iodine value calculations for the calculated curve assumed that it was. The point being made here is that conclusions can be drawn only for whatever is plotted on the ordinate. Generalizations about the saturated or unsaturated acids can be made only if curves of each component are studied.

The choice of the abscissa for these graphs depends upon the type of fatty acid analyses made for the samples. If iodine values were obtained, then this choice is convenient. If a spectrophotometric determination of the polyunsaturated acids were made, then iodine values would have been necessary to obtain the amount of oleic acid. But if the analyses were made using a chromatographic column separation or by gas chromatography, then iodine values probably were not obtained and the choice of iodine value for the abscissa would be inconvenient. The iodine values may be calculated from the individual fatty acid compositions but this is really a step backwards as the iodine value does not give as much information as the individual composition itself. The total mol

percent of the unsaturated acids would serve the same purpose for these graphs. The iodine values would give an indication of whether the majority of the unsaturated acids were mono-, di-, tri-, etc. unsaturated fatty acids but this is not of any particular value in making these graphs or in their interpretation. Therefore the choice of the abscissa would be more logically some variable other than the iodine value which would have to be calculated. The choice could actually be any one of the individual fatty acids or any convenient group of acids. If an individual acid is chosen though, then both the ordinate and the abscissa would involve calculations from experimental data to obtain the calculated curve. The choice of either the total mol percentage saturated or unsaturated acids would eliminate the use of any experimental data for calculating the abscissa values of the calculated curve. This latter method was used to correlate the fractionation and hydrolysis data of this study.

Suppose that instead of plotting mol percentage of an individual fatty acid versus the total mol percentage saturated acids that the ordinate was chosen to be the ratio of the mol percentages of two fatty acids. Deviations of the experimental data from the calculated curve based upon random distribution of these two acids would be more difficult to interpret than a similar graph of just one of the individual

acids used as the ordinate. Therefore ordinates of the mol percentage of an individual acid and an abscissa of the total mol percentage saturated fatty acids was used to correlate the data.

An extension of the above method was made to include graphs based on just 1- and 3-position fatty acids. There are six types of glycerides possible when only the saturated and unsaturated acids are considered. These types are SSS, SUS, SSU, SUU, USU, and UUU. When only the 1- and 3-positions are considered, these become S-S, S-S, S-U, S-U, U-U, and U-U which reduce to only the three types S-S, S-U, and U-U. The data obtained for the free fatty acids of the hydrolysis products of the lards and lard fractions were used to prepare graphs of the mol percentage of an individual acid in the 1- and 3-positions versus the total mol percentage saturated acids in the 1- and 3-positions. The calculated curve has only three points representing the three possible combinations of saturated and unsaturated acids in the 1- and 3-positions. The calculated curve is based upon random distribution of the acid plotted on the ordinate and deviation of the experimental data from the calculated curve represents the amount of deviation from random distribution of this component in the 1- and 3-positions.

Graphs were prepared to show the distribution of each

of the six fatty acids of lard in each of the three lard samples and for both the lard and lard fraction triglycerides and the free fatty acids obtained from the hydrolysis of the lards and lard fractions. These graphs are shown in Figures 17-22. The data for the experimental curves were obtained from Tables 53 and 56. Data for the calculated distribution curves are given in Table 60.

Study of these figures showed that the distribution of the fatty acids in the 1- and 3-positions was very nearly the same as in the 1-, 2-, and 3-positions. Even though the ratios of saturated to unsaturated acids in the 1- and 3-positions and in the 2-positions are quite different for the lard B sample, these curves show that the distribution of the individual fatty acids in each of the two groups of positions was nearly the same.

The lard A and lard R samples appeared to be nearly identical. By studying the curves for palmitic and oleic acids (the two major component fatty acids) it was indicated that possibly the after sample was not quite completely randomized whereas the random sample was. Deviations due to experimental error may account for most of the differences observed but in particular, the triglyceride after curve for oleic acid showed that the oleic acid content in the first fraction on the left was low just as in the triglyceride be-

fore sample whereas the triglyceride random point was just barely below the random distribution curve. The free fatty acid after and random curves also showed this same tendency but was less pronounced. This indicated that the 1- and 3-position fatty acids were more easily randomized than the 2-position fatty acids.

None of the six fatty acids showed distribution curves which were random for the before samples. It is interesting to note though that all of the fatty acids in one of the two groups, saturated and unsaturated acids, did not deviate from a random distribution in the same manner. The saturated fatty acids, myristic and palmitic, showed similar trends and stearic showed the opposite trend. The amount of myristic and palmitic acid was high for the unsaturated fractions and low for the saturated fractions. The amount of stearic acid was low for the unsaturated fractions and high for the saturated fractions. These differences tended to balance each other such that it would not be expected to find the amount of any one of the six glyceride types to be higher or lower than what would be predicted from random distribution of saturated and unsaturated acids in the 1- and 3-positions and the 2-position for all six of the glyceride types. This was one of the assumptions made by Vander Wal (91) in his method of calculating the amounts of each of the six glyceride

types from just the mol percentage saturated acids in a fat and the mol percentage saturated acids in the 2-positions of the fat. This assumption does not exclude the possibility of the individual fatty acids having different distributions as has been found above for lard B. If all of the saturated acids showed the same deviation from random distribution, then the relative amounts of the six glyceride types would be different than if the deviations tended to balance each other. This can easily be seen by considering the glyceride types SSS, SSU, and SUS. Suppose that all of the saturated acids have higher than random amounts in these glycerides. Then there would be fewer saturated fatty acids remaining to form the SUU and USU glycerides than would be true if all of the fatty acid distributions were random.

A similar study of the unsaturated fatty acid distributions showed that the amounts of palmitoleic and linoleic acids were higher than the random distribution for the unsaturated fractions and oleic acid was low. Palmitoleic and linoleic acids showed the larger deviations from random than the major component fatty acid, oleic acid. Again the difference in the distributions tended to balance each other such that the unsaturated acids were distributed at random between all six of the glyceride types.

One other observation made was that the saturated acids

showed the largest deviations from random in the more saturated fractions and the unsaturated acids showed the largest deviations from random in the unsaturated fractions. Linoleic acid would be expected to decrease the melting point of a triglyceride more than oleic acid. Linoleic acid was found in less than random amounts in the saturated fractions. Similarly stearic acid was in lower than random amounts in the unsaturated fractions. The combined effects of these two deviations would be to give a fat with a higher melting point range than would be true in a fat which had random distributions.

Reiser and Reddy (78) found that linoleic acid in the triunsaturated fractions of the fat of a pig reared on a fat free diet was found mainly in the 2-position. The unsaturated fractions in this study contained nearly the same proportion of linoleic acid in the 1- and 3-positions and in the 2-positions but more linoleic acid in the 1- and 3-positions than in the 2-positions of the saturated portions (see Table 58 for these compositions).

The figures in Table 58 for the lard B sample also showed that myristic, palmitoleic, and palmitic acids were found more predominately in the 2-positions than in the 1- and 3-positions. Stearic, oleic, and linoleic acids were found to be more predominate in the 1- and 3-positions than

in the 2-positions. This indicated that chain length was more important in determining the position of a fatty acid than was the factor of whether the fatty acid was saturated or unsaturated. Published results for lard very often indicate that it is the saturated acids that are predominate in the 2-positions. This is true but might be misleading in that it is the short chain fatty acids which are found predominately in the 2-position and in lard the major C_{14} and C_{16} component is palmitic acid. Stearic acid makes up 11.8 mol percent of the total fatty acids in lard whereas palmitic accounts for 27.8 percent.

Table 61 gives the glyceride compositions found for the lards of fractional crystallizations No. 4 and No. 5. The data obtained by Riemenschneider *et al.* (79) are also given for comparison. The percentages of each glyceride type which would result if the saturated and unsaturated acids of each lard were completely randomized on all the available glyceride sites are also shown. The results of the two fractional crystallizations of lard B show good agreement; the lard A results are not in quite as good agreement in that the two GSU_2 compositions differ by 4.2% in a total of about 50%. The compositions for the lard A and lard R sample should be expected to agree more closely with the calculated random compositions than they did. The fatty acid compositions of the lards and lard fractions, the hydrolysis product free fatty

acid compositions, and the fatty acid distribution curves indicated that the lard A was very nearly randomized and that the lard R sample was completely randomized. The data of Table 61 indicate that either these samples were not randomized or that the fractional crystallization results for the GS_3 and GU_3 compositions were low. It is thought that the fractional crystallization method gave low results but no separations of a synthetic mixture were made by fractional crystallization to substantiate this.

If the assumption that the crystallization fractions contained no more than two major glyceride types was correct, then data for the fatty acid distribution in the SSU, SUS, SUU, and USU glyceride types could be obtained from the crystallization and hydrolysis data presented. The glycerides expected to be found in the three logically possible fractions would be:

SSS	SSU	USU
SSU	SUS	SUU
SUS	USU	UUU
	SUU	

The free fatty acids found in the hydrolysis products of the first fraction would contain unsaturated acids from only the SSU glycerides. The overall fatty acid composition of this fraction would include unsaturated acids from only the SUS fraction. Therefore from the total weight of the fraction and the fatty acid compositions, the amounts of each of the three

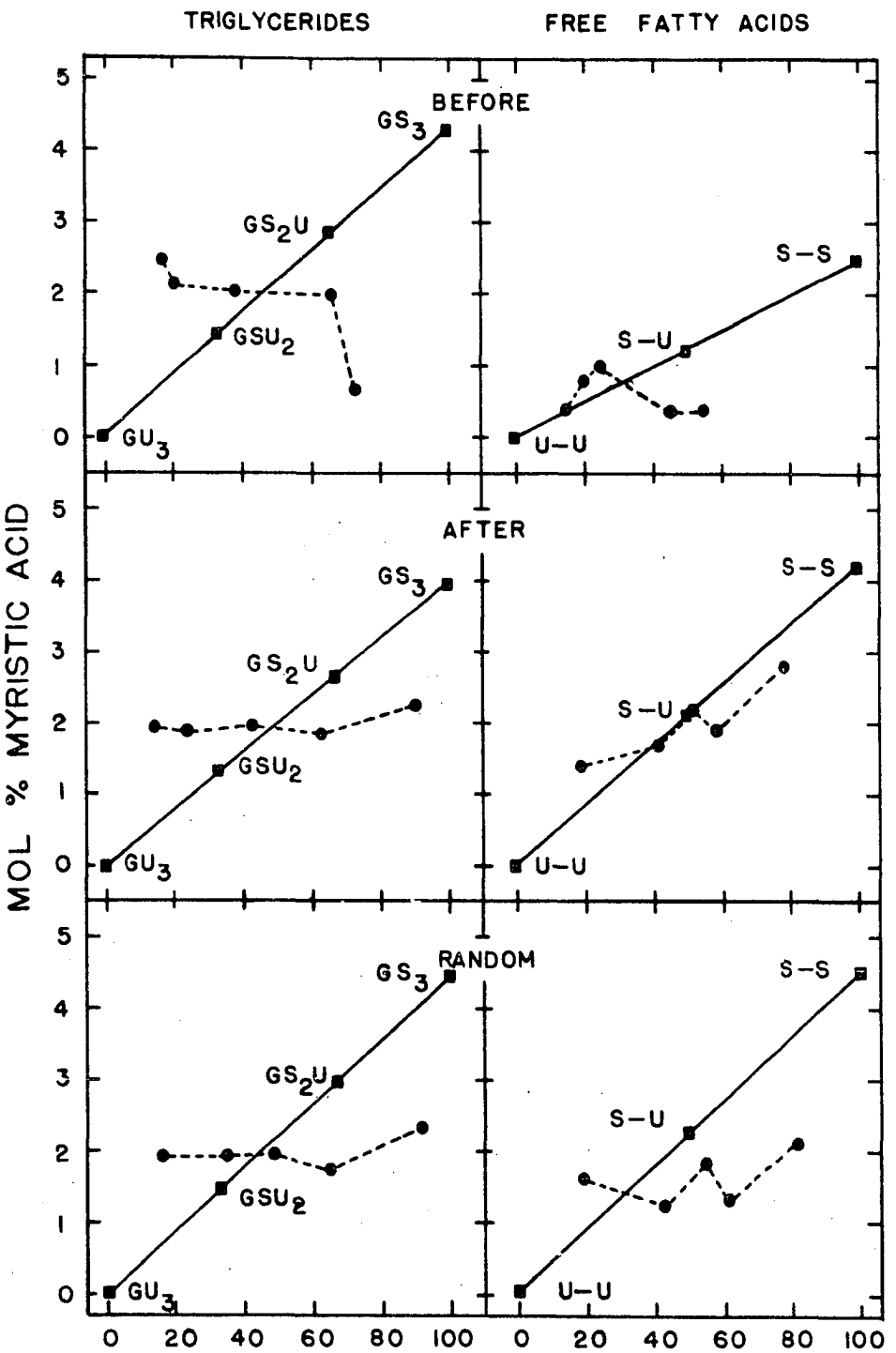


FIG. 17. MYRISTIC ACID DISTRIBUTION

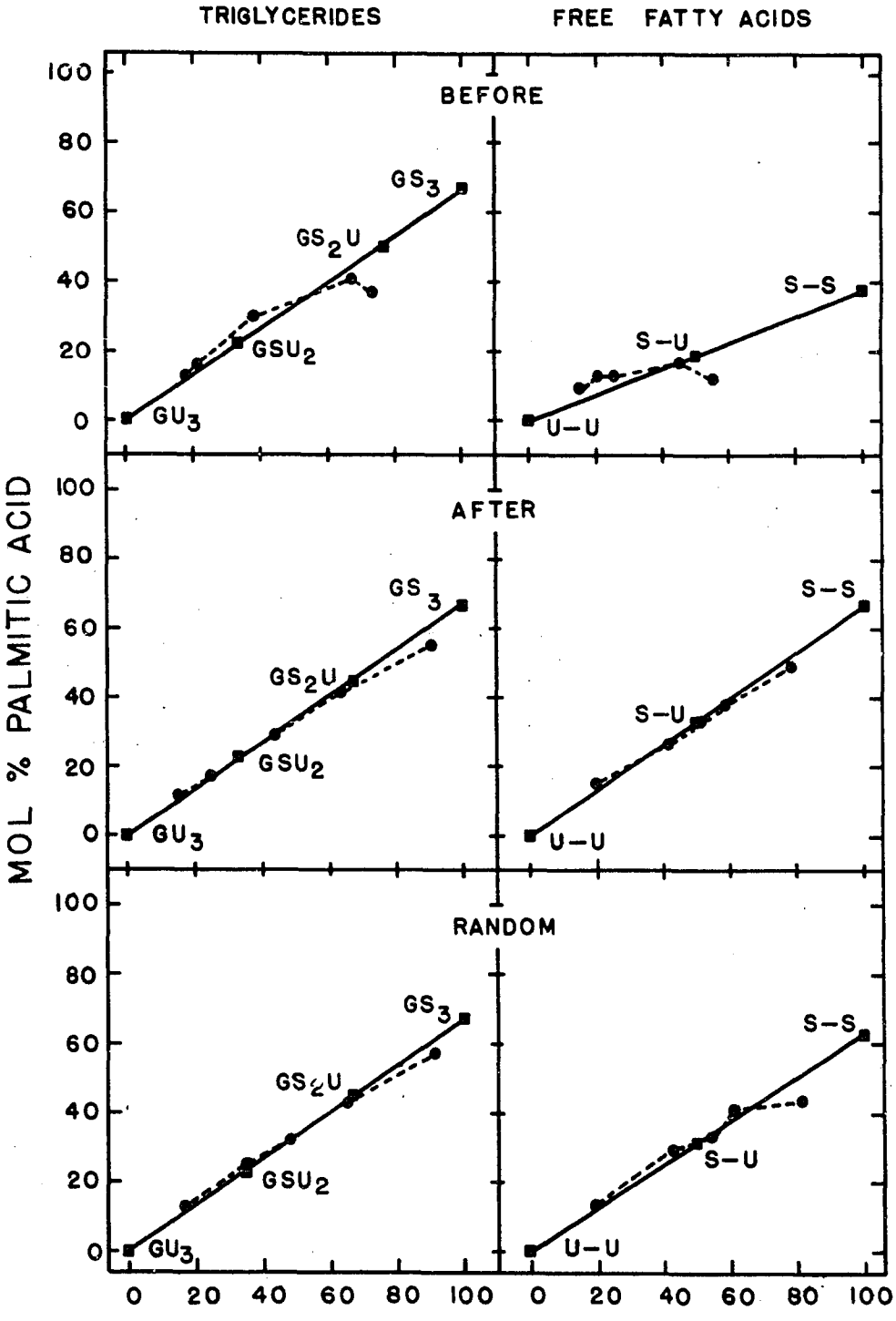


FIG. 18. PALMITIC ACID DISTRIBUTION

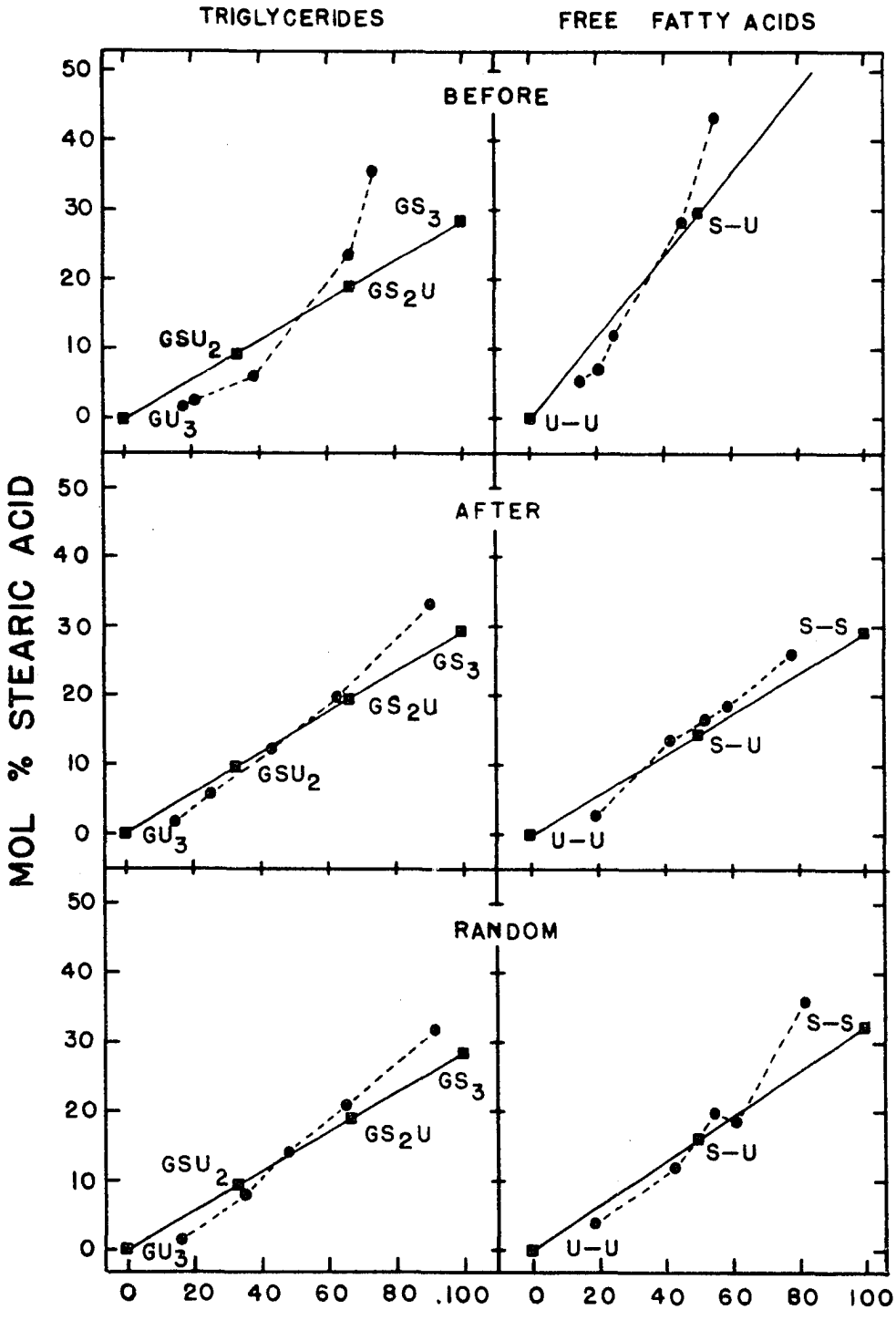


FIG. 19. STEARIC ACID DISTRIBUTION

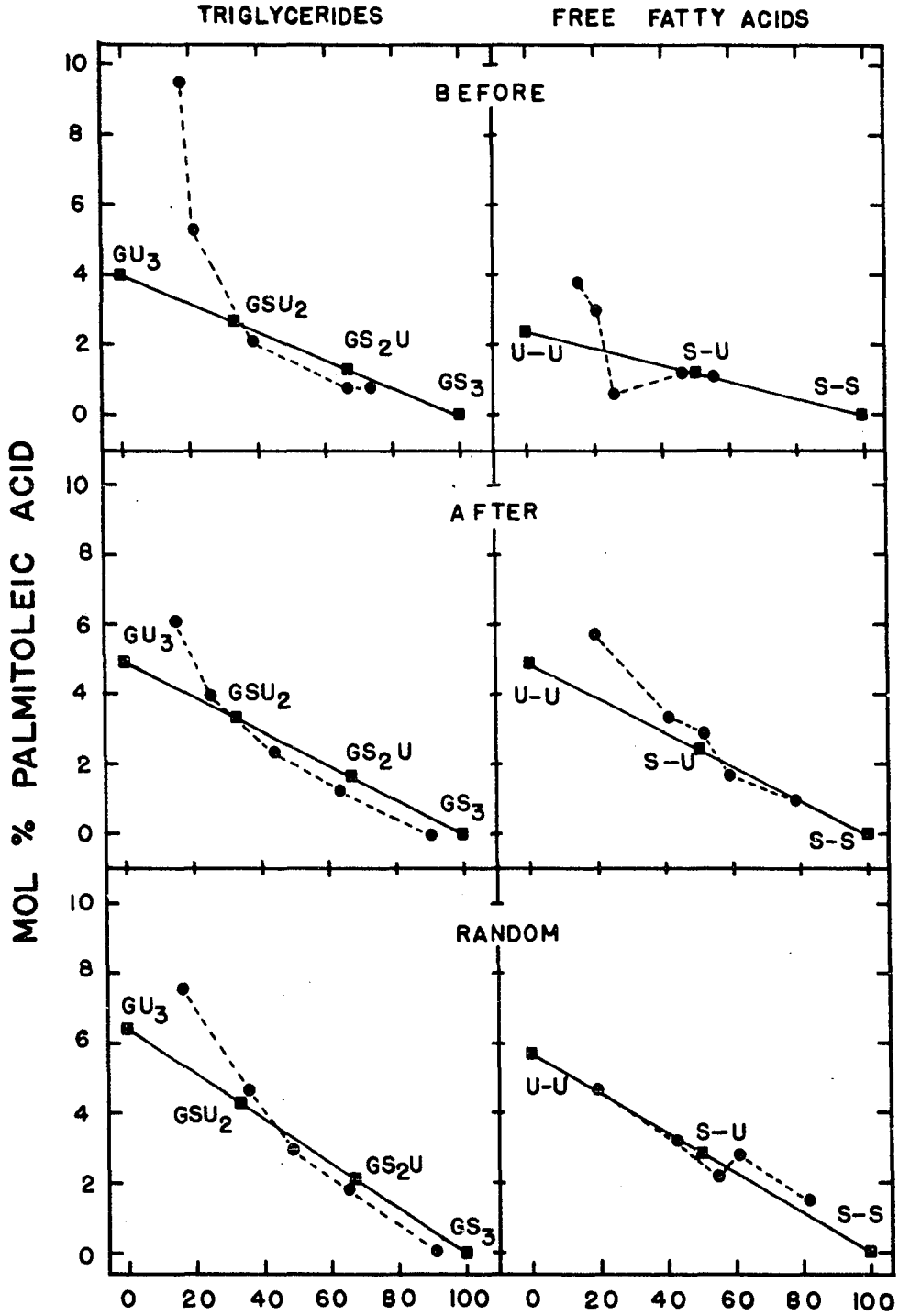


FIG. 20. PALMITOLEIC ACID DISTRIBUTION

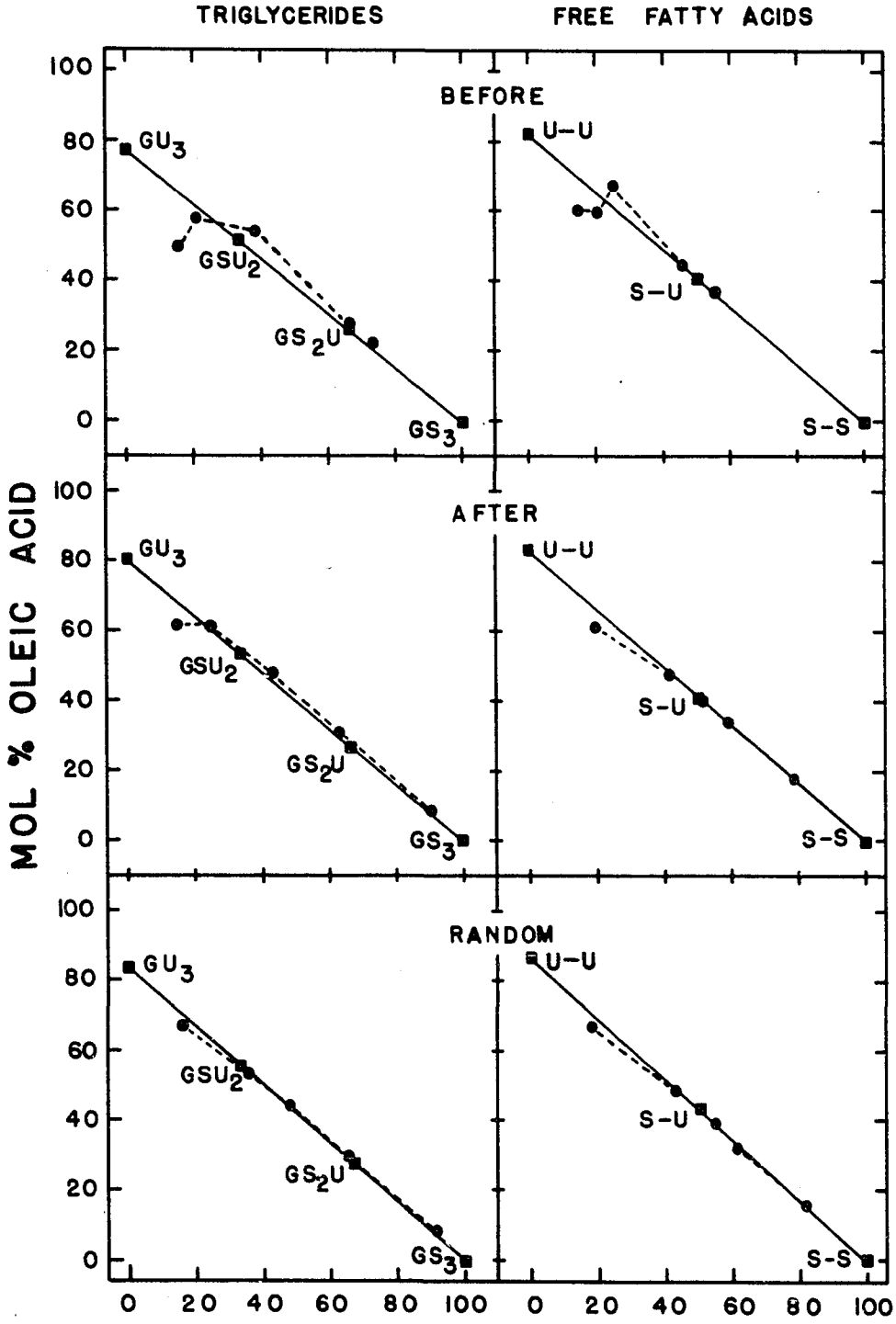


FIG.21. OLEIC ACID DISTRIBUTION

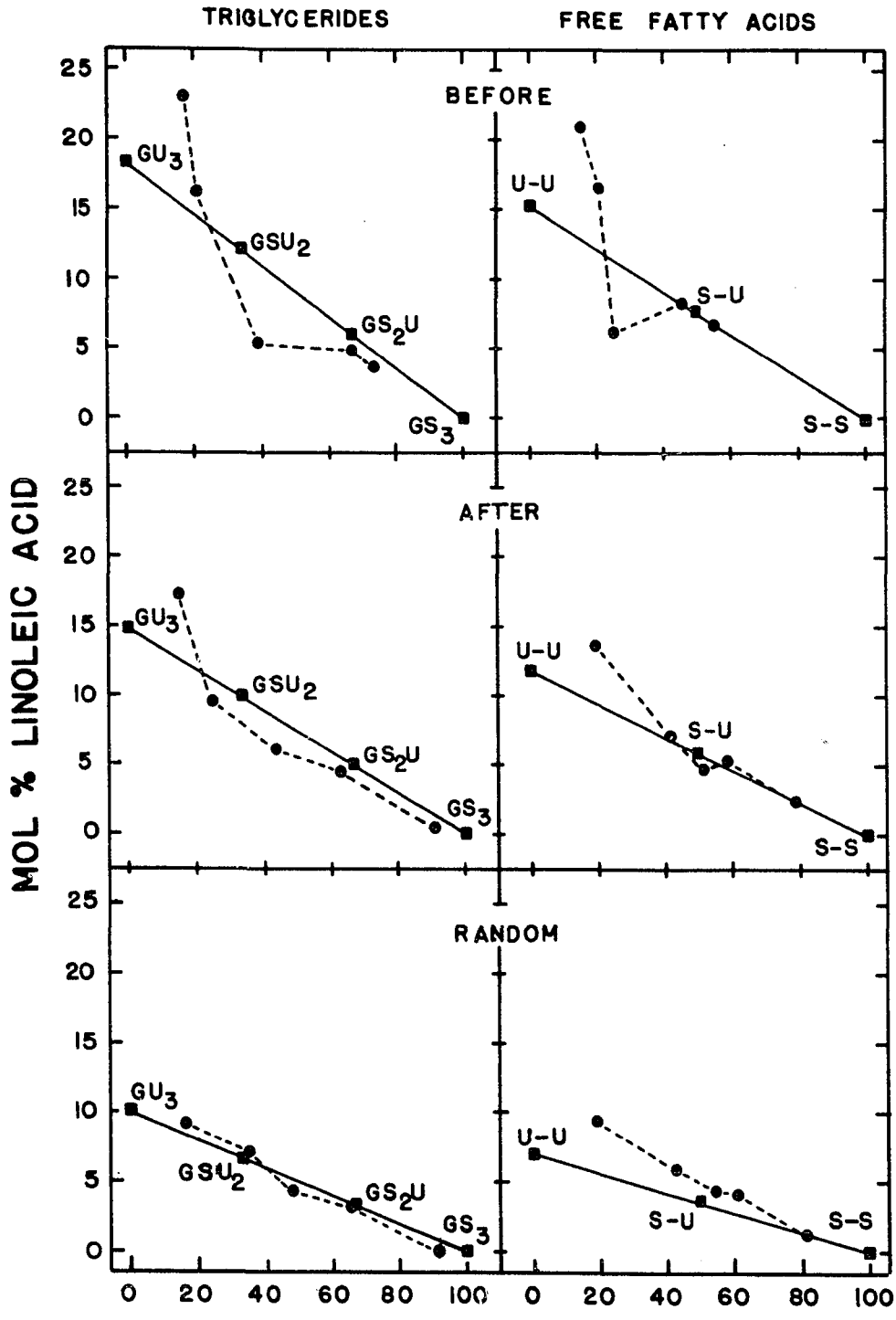


FIG. 22. LINOLEIC ACID DISTRIBUTION

Table 60. Data for calculated distribution curves of Figures 17-22 (all values for mol percentages)

	Lard			F.F.A. of lard		
	B	A	R	B	A	R
Myr.	1.78	1.72	2.03	0.6	1.8	2.0
Pal.	27.78	28.99	30.71	9.1	28.6	27.9
Stear.	11.82	12.67	13.00	14.3	12.6	14.2
P.O.	2.36	2.79	3.49	1.8	2.8	3.2
Oleic	45.53	45.42	45.29	62.5	47.5	48.7
Linol.	10.73	8.42	5.47	11.6	6.7	4.0
% total satd.	41.38	43.38	45.74	24.0	43.0	44.1
% total unsatd.	58.62	56.63	54.25	75.9	57.0	55.9
Myr.	4.30	3.96	4.44	2.5	4.2	4.5
Pal.	67.13	66.83	67.14	37.9	66.5	63.3
Stear.	28.56	29.21	28.42	59.6	29.3	32.2
P.O.	4.03	4.93	6.43	2.4	4.9	5.7
Oleic	77.67	80.20	83.48	82.3	83.3	87.1
Linol.	18.30	14.87	10.08	15.3	11.8	7.2

	Triglycerides				F.F.A.		
	GS_3	GS_2U	GSU_2	GU_3	S-S	S-U	U-U
Before							
Myr.	4.30	2.87	1.43	0	2.5	1.25	0
Pal.	67.13	44.75	22.38	0	37.9	18.95	0
Stear.	28.56	19.04	9.52	0	59.6	29.8	0
P.O.	0	1.34	2.69	4.03	0	1.2	2.4
Oleic	0	25.89	51.78	77.67	0	41.15	82.3
Linol.	0	6.10	12.20	18.30	0	7.65	15.3
After							
Myr.	3.96	2.64	1.32	0	4.2	2.1	0
Pal.	66.83	44.55	22.28	0	66.5	33.25	0
Stear.	29.21	19.47	9.74	0	29.3	14.65	0
P.O.	0	1.64	3.29	4.93	0	2.45	4.9
Oleic	0	26.73	53.47	80.20	0	41.65	83.3
Linol.	0	4.96	9.91	14.87	0	5.9	11.8
Random							
Myr.	4.44	2.96	1.48	0	4.5	2.25	0
Pal.	67.14	44.76	22.38	0	63.3	31.65	0
Stear.	28.42	18.95	9.47	0	32.2	16.1	0
P.O.	0	2.14	4.29	6.43	0	2.85	5.7
Oleic	0	27.83	55.65	83.48	0	43.55	87.1
Linol.	0	3.36	6.72	10.08	0	3.6	7.2
% total satd.	100.0	66.7	33.3	0	100.0	50.0	0

Table 61. Comparison of lard glyceride compositions

	Mol % satd. acids	Mol % unsatd. acids	Mol percent			
			GS_3	GS_2U	GSU_2	GU_3
Fractional crystallization No. 4						
Lard B, found	40.9	59.1	2.7	28.5	57.0	11.8
Calc. random	-	-	6.8	29.7	42.9	20.6
Lard A, found	42.5	57.5	4.4	30.1	51.6	13.9
Calc. random	-	-	7.7	31.1	42.2	19.0
Fractional crystallization No. 5						
Lard B, found	41.4	58.6	3.4	29.6	54.7	12.3
Calc. random	-	-	7.1	30.1	42.6	20.1
Lard A, found	43.4	56.6	4.9	31.8	47.4	15.9
Calc. random	-	-	8.2	32.0	41.7	18.1
Lard R, found	45.7	54.3	4.7	33.9	49.1	12.3
Calc. random	-	-	9.5	34.0	40.4	16.0
Riemenschneider *et al.* (79)						
Lard, found	37.4	62.6	1.9	25.9	54.6	17.6
Calc. random	-	-	5.2	26.3	44.0	24.5

glyceride types present could be calculated and the actual distribution of the unsaturated acids would be known. The same type of calculations could be made for the third type of fraction obtained but this time the actual distribution of the saturated acids in the USU and SUU glycerides would be obtained. The distribution curves showed that the fatty acid distributions for the triglycerides and the free fatty acids were very nearly the same. Therefore there was little point

in carrying out these calculations as the assumption may not be entirely correct.

Trisaturated glyceride determination

Luddy and Riemenschneider (49) presented a method for the determination of trisaturated glycerides in lard, hydrogenated lard, and tallow by crystallization from acetone. They used 25-50 grams of sample dissolved in dried acetone in the ratio of 40:1, ml of solvent:grams of fat. This solution was held at 15±0.3°C for 30 hours without stirring. The precipitate was filtered, pressed, washed and pressed again to remove the filtrate. The precipitate was then dissolved in dried acetone using a ratio of 60:1, ml of solvent:grams of fat. This solution was held at 15±0.3°C for 24 hours, filtered, pressed, washed, pressed again, and the remaining solvent removed under vacuum with heating on a water or steam bath.

They also gave several sets of crystallization conditions used which gave nearly quantitative precipitation of GS_3. The above procedure requires 2-3 days time; another set of conditions required only about one day. These conditions were to use 35 ml of dried acetone per gram of fat, hold at 10°C for two hours while stirring, filter, press, wash, and press the precipitate. The precipitate was then dissolved in dried

acetone using 200 ml of solvent per gram of precipitate. This solution was held at 10°C for two hours with stirring, filtered, pressed, washed and finally pressed as dry as possible. The remaining solvent was removed under vacuum with heating.

Samples were run for the before and after lard samples using both methods and are referred to as the short and long procedures below. The GS_3 precipitates were analyzed by forming methyl esters of the fatty acids and obtaining a gas chromatogram of these methyl esters. In calculating the amount of GS_3 in the precipitate, it was assumed that for every mol of unsaturated fatty acid there was a mol of monounsaturated disaturated triglyceride in the precipitate. The results of these determinations are given in Table 62 with the GS_3 determination from the complete lard fractional crystallizations No. 4 and No. 5.

Table 62. Lard trisaturated glyceride determination

	Mol percentage GS_3 in lard		
	B	A	R
Short procedure	2.0	3.6	--
Long procedure	2.1	3.2	--
Fractional crystn. No. 4	2.7	4.4	--
Fractional crystn. No. 5	3.4	4.9	4.7
Av. independent GS_3 determn.	2.1	3.4	--
Av. fractional crystn. determn.	3.1	4.7	4.7

The long and short procedures gave nearly the same results and the two fractional crystallizations gave nearly the same results, but the independent GS_3 determinations did not agree with the GS_3 determinations from the fractional crystallizations. The independent GS_3 determinations were 68% and 72% of the results from the fractional crystallizations for the before and after samples respectively.

The conclusions of these tests were that there was little advantage of using 54 hours crystallization time for the GS_3 determination as compared to just four hours crystallization time for the same determination. The independent GS_3 determination and the GS_3 determination obtained from fractionation of the lard samples did not agree. The independent determinations were 30% lower than the fractionation results. From these data alone, there was no way of knowing which results are more nearly correct.

One indication that these GS_3 determinations were low can be seen by the data given in Table 61. The calculated percentage of GS_3 for randomized samples were 40-50% higher than what was obtained from the fractional crystallization data. Two possibilities are that the lard random used was not completely randomized or that the fractional crystallization method gave low results. Probably the actual situation is between these two possibilities. The methyl ester compo-

sitions of the crystallization fractions indicated that the lard random sample was very nearly completely randomized when the fatty acid distribution curves were studied. It is therefore thought that the fractional crystallization gave low results and had more affect on the low results than did incomplete randomization of the lard sample.

Combined Oxidation-Hydrolysis Study

The fractions obtained from the fractional crystallization of lard were mixtures of two or more glyceride types. It was not possible to determine even the six glyceride types based on the saturated and unsaturated acids. Calculations were made for just the four major types of glycerides, GS_3, GS_2U, GSU_2, and GU_3.

Youngs (98) has described a method of determining the six glyceride types, SSS, SSU, SUS, SUU, USU, and UUU. The method involved oxidation of the fat to destroy all the carbon-carbon double bonds and separation of the oxidized fat into two fractions consisting of $GS_3 + GS_2A$ and $GSA_2 + GA_3$ where A represents the azelaic acid radical remaining after oxidation. Methyl ester analyses of the original fat, the oxidized fat and the two fractions were made. Samples of the original fat, oxidized fat, and the two fractions were hydrolyzed using pancreatic lipase and the free fatty acids converted to the methyl esters using diazomethane and analyzed by gas chromatography.

These eight analyses provided sufficient data to calculate the fraction of each of the six glyceride types present in the fat.

The above work suggested a method of studying the distribution of the individual saturated acids in lard. Only general information would be obtained for the unsaturated acids because they are destroyed during the oxidation and it is not possible to determine from which unsaturated acid the oxidation products originated. Youngs (98) used a partition column of 90% ethanol-10% water on silicic acid to separate the oxidized fat into two fractions. He presented a figure for the separation of oxidized cocoa butter into four fractions using countercurrent distribution between an alcohol phase and a Skellysolve B phase. He simplified the method by using a partition column and obtained only two fractions using the same solvent system in the column.

After having worked with silica gel columns on the separation of mono-, di-, and triglycerides it appeared that the oxidized fats could probably be separated into six fractions corresponding to the six glyceride types. The oxidized fat was found to be much more difficult to separate than the mixed glycerides but by using the solvent system of ethyl ether in Skellysolve B and acetic acid in ethyl ether the separations shown in Figures 23-26 were obtained. Tables 63-66 give the

details of the eluting solvents, flowrates, and the fraction weights obtained. Table 67 give the fractions combined to give the numbered peaks of the figures and the weights and weight percentages of each peak. Table 68 is a comparison of the weight percentage of each peak for the four figures. Tables 69-71 give the methyl ester compositions of the fatty acids found in each of the peaks for three of the figures. Table 72 gives the compositions of the lards before and after oxidation.

The columns used were 16 mm ID glass tubes about 60 cm long and were fitted with a water jacket. Tap water was passed through a copper coil in a temperature controlled water bath to maintain the temperature of the water at 21-24°C. Three and one-half ml of water were stirred very thoroughly with 35 grams of Davison Grade 923 (100/200 mesh) silica gel until no lumps remained. The silica gel was then slurried into the columns with Skellysolve B and allowed to settle of its own accord. The Skellysolve B was run from the column until it was just above the silica gel level. The first 25 ml of eluting solution was then used to transfer as much of the oxidized lard into the column as could be dissolved in the 25 ml. Each successive 25 ml of eluting solution was added to the sample container to dissolve more of the sample and poured into the column. As the amount of ether in the eluting

solution was increased more sample kept dissolving but the process was very slow. Therefore when 1 ml or so of ether was being used in the 25 ml of eluting solution, the ether was first put in the sample beaker to dissolve as much sample as possible and then the 24 ml or so of Skellysolve B was added to the sample beaker and this poured into the column. The material which was so difficult to dissolve was the components of very high azelaic acid content. As these components were held up in the very first cm or two of the silica gel until near the end of the eluting process, it was not necessary that they be put on the column with the first eluting solvent fraction. Twenty-five ml fractions were collected from the bottom of the column, the solution put in weighed 50 ml beakers, the solvent evaporated on a steam plate in a hood, cooled and weighed again to obtain the residue weights. The silica gel was supported on a glass wool plug and flow from the column controlled by the use of a stopcock and by the height of the solvent head on the column. The sample size for this column should be about 500 milligrams. Larger samples give overlapping peaks and with smaller samples the weighing errors and silica gel in the residue become proportionally larger.

The fat was oxidized under the conditions given by Youngs (98), who had modified the oxidation method worked out by von Rudloff (94). The oxidation was carried out at room

temperature and took several hours as von Rudloff presented the method. Youngs used the same conditions except that he refluxed the oxidation solution and obtained complete oxidation in one hour. The oxidized lard samples were prepared by weighing 1.0 gram of lard in a small beaker and using 100 ml of tertiary-butyl alcohol to transfer the lard to a one liter round bottom flask with a ground glass fitting. Sodium periodate, 5.484 grams, and 103.3 milligrams of potassium permanganate were dissolved in 268 ml of water and added to the lard solution in the one liter flask. Then 332 milligrams of potassium carbonate and 300 ml more of tertiary-butyl alcohol were added and the solution refluxed for one hour on a water bath (b.p. t-BuOH, 82.9°C). After the solution was cooled, sodium meta-bisulfite was added with stirring until the originally deep purple solution went through several color changes and finally became milky green. Ten percent sulfuric acid was added with stirring until the milky light green solution became clear light green. The fatty material was then extracted four times with ethyl ether. The ether-butanol extracts were combined and evaporated on a water bath with the aid of a water aspirator. The residue was dissolved in ether, washed with water until neutral to litmus, the ether solution dried over sodium sulfate and the ether evaporated.

Whenever a portion of the oxidized fat was needed, the sample was dissolved in ether and an aliquot of the solution

taken. If the sample was just melted and a few drops taken, a representative sample was not always obtained. The azelaic acid content of the sample taken was found to be low when the sample was not first dissolved in ether.

In carrying out the oxidation, the solution must remain deep purple in color. If not, the oxidation will not be complete. More periodate-permanganate must be added if the deep purple color disappears.

Sodium periodate was not available at the Iowa State University Chemical Stores but potassium periodate was. The sodium periodate is completely dissolved when used in the procedure above, but an equivalent molar amount of potassium periodate was only partially dissolved. It was found, however, that by swirling the container during the start of the refluxing until enough of the potassium periodate had reacted that all of the potassium periodate went into solution. Methyl ester analyses of oxidized lard at the end of one and two hours gave identical results and showed that the oxidation was complete in one hour (no oleic acid peak was obtained in the chromatogram).

The oxidized lard samples were separated on the silica gel columns in the same form as obtained from the oxidation procedure. But in order to hydrolyze the oxidized lard or its fractions, Youngs (98) showed that it was necessary to

methylate the acid groups formed during the oxidation by the use of diazomethane. If the oxidized samples were not methylated, it was found that the monocarboxylic acid chains were more easily removed than the azelaic acids.

Methyl esters of the fatty acids of the peaks of the chromatograms were prepared using anhydrous 5% hydrochloric acid-methanol. After refluxing 30-60 minutes, the alcohol was evaporated with the aid of a water aspirator vacuum. A total of about 15 ml of ethyl ether was used to wash down the distilling neck and walls of the 50 ml round bottom flask, sodium sulfate added and allowed to stand a few minutes. The ether was decanted and the sodium sulfate washed 3-4 times with more ether. The ether solutions and washes were combined and evaporated. The methyl ester residue was transferred to 1/2 dram screw top vials.

Enzymatic hydrolyses were carried out on the peaks obtained from the silica gel columns shown in Figures 23 and 24. The size of the samples was limited in many cases because both an overall composition methyl ester sample and a hydrolysis sample were required. The hydrolysis method of Youngs (98) was modified in that 40°C was used instead of room temperature and the samples were stirred at 833 rpm with a glass stirring rod in place of the homogenizer. The samples were run in the short test tubes, 20 mm ID x 6.5-7.0 cm.

Sample sizes ranged from 2.9 to 29.5 milligrams. Steapsin was weighed out for each sample and an amount equal to 40% of the weight of the fat was used. The time of hydrolysis was 15 minutes in each case. Samples which had high melting points were first heated in a water bath to melt the fatty material, the buffer and bile salts added and the mixture stirred vigorously to form an emulsion and then set in the 40°C bath to allow the sample and buffer temperature to become 40°C. The weighed steapsin was then added to the emulsion.

The hydrolysis products were extracted with ethyl ether, washed with water and the ether evaporated. The free fatty acid methyl esters were prepared using diazomethane. The results of the gas chromatograms indicated that the hydrolyses had proceeded much as the test samples of the second section of Table 48. The results for stearic acid appeared to be much too high and the palmitic acid compositions a little too high. A lard B sample hydrolyzed under the same conditions also gave similar high results for the stearic and palmitic acids. Therefore the results of the hydrolyses are not included.

The silica gel column for the oxidized lard R sample (Figure 26) was one of the first of the more successful attempts to obtain fractionation of the oxidized lards. The first five peaks were not completely separated. By making

the increase of ether in each of the successive 25 ml fractions of eluting solution in smaller increments, better separations were obtained. This figure also shows that the peaks tail badly (peak 5). The effect of tailing is shown by the data of Figure 24 for the fractionation of oxidized lard A. Peaks 8 and 9 are not completely separated here. But in Figure 25 these two peaks were separated much better. Data in Table 68 shows that the total weight of the two peaks (including the material between peaks) was 51.7 and 50.2 weight percent of the total samples but that the individual peaks were 33.3, 7.2 (between), and 11.2 weight percent for one and 27.5 and 22.7 weight percent for the other. Where the peak separation was very poor, the second peak weight percent was much too large because of the overlapping of the tail of the preceeding peak. No methyl ester analyses were made for the peaks of Figure 25 so the composition of the material between the peaks was not known.

Table 68 was made in an attempt to compare the peaks of the different silica gel column chromatographs. The comparison was difficult to make because different eluting solutions were used for each column. The results for the two oxidized lard A columns give some indication of the reproducibility of the results. In order to make an accurate comparison of the samples, columns would have to be run for each sample

using the same conditions and comparison made of the residues of the same fractions.

The oxidized lard B sample gave peaks which when analyzed, indicated that there was some oleic acid present in the sample. The oxidized lard B sample consisted of the combined residues remaining from two separate oxidations of lard B. One of these fractions had been checked for unoxidized material by gas chromatography and none was found. This fraction consisted of about 73% of the final sample. The other lard B sample was obtained by the use of potassium periodate and there was no indication of incomplete oxidation but a methyl ester analysis was not made of this fraction. It could have been incompletely oxidized.

In eluting the last two large peaks from the silica gel columns, it was necessary to use increasing amounts of acetic acid in ethyl ether. When these fractions containing acetic acid were evaporated on the steam plate, the evaporations took increasingly longer times. When pure glacial acetic acid was used, the 25 ml fractions required 15-20 hours to completely evaporate the acetic acid. The residues became tacky and very difficult to redissolve. The original sample was not completely soluble in Skellysolve B but dissolved easily in ethyl ether. The residues from the column after evaporation of the acetic acid could not be dissolved in ether.

They were more soluble in methyl aclohol and/or acetone but not completely. The methyl ester compositions of the last peak obtained were particularly low for azelaic acid which would be expected to be the major constituent if the last peak corresponded to GA_3. This indicated that the azelaic acid (and other diacids) had undergone reaction and was no longer methylated when treated with hydrochloric acid-methanol solution. A possible improvement in the evaporation of the acetic acid would be the use of a rotary vacuum film evaporator if available. This very probably would not prevent all of the diacids from reacting but might help considerably to reduce the amount which did react.

After this work had been started, the work of Privett and Blank (73) was studied. They used ozonolysis of the double bonds followed by reduction of the ozonides to aldehyde groups by selective hydrogenation. The separation of these aldehydes appeared to be much easier than the separation of the oxidation products containing carboxylic acid groups. Therefore even though thin layer chromatography was not used as in the work of Privett and Blank (73), the column separations could possibly be made without having to use acetic acid and better methyl ester compositions obtained. This method of oxidation was considered for this work but equipment for producing ozone and for carrying out the hydrogenations was

not available. There are several published methods for producing ozone. Two of these appeared to be of possible interest if equipment was to be built for producing ozone. In a paper by von Lindlar (93), the details of an ozonizer which can be built of glass was described. The major item for this apparatus appeared to be the equipment required to maintain the center electrode at 22,000 volts. A second paper, Bovee and Robinson (14), described the use of a small ultraviolet lamp, GE OZ4S11, to produce ozone.

The use of column chromatography has the advantage of being able to fractionate larger samples than thin layer chromatography but has the disadvantage of requiring much more time and considerable extra work in weighing and evaporating the fractions. The fractionation in Figure 23 required 22 hours eluting time plus several hours more time preparing for the run and finishing the weighings, combining samples, etc. This represents an extreme case but shows that the time invdved is in hours or almost days. The separation of the oxidation products containing the aldehyde groups and using thin layer chromatography required times measured in minutes as carried out by Privett and Blank (73). The disadvantage of thin layer chromatography as compared to column chromatography is that the sample size used in thin layer chromatography is on the order of 50 micrograms whereas about 500 milligrams were separated on the columns. This becomes important when it is

desired to hydrolyze each of the peaks (or spots) obtained as well as obtain the overall fatty acid methyl ester compositions.

In measuring the gas chromatogram peak areas for test samples containing both mono- and dicarboxylic acids, Youngs (98) found that the peak areas of the monocarboxylic acids (both saturated and unsaturated) corresponded to the weight of the components but that the diacids formed by the oxidation had peak areas consistently lower than for the monocarboxylic acids. Multiplication by a factor of 1.10 gave good results for the test sample mixtures over a wide range of compositions.

Table 72 gives the composition of the oxidized lards and gives the total saturated acid mol percentages and the total diacid mol percentages with and without the use of the 1.10 factor. The mol percentage of saturated and unsaturated acids for the original lard samples are also given for comparison. The total mol percentage of diacids obtained should agree with the total mol percentage of unsaturated acids in the original sample. The use of the 1.10 factor gave diacid compositions which were about half way between the diacid compositions calculated from the area percentages and the mol percentage of unsaturated acids in the original lards. This indicated that a factor of about 1.2 would be required to give diacid compositions corresponding to the unsaturated acid com-

position of the original lards. It was from calculations such as these that it was observed that the azelaic acid composition varied for analyses of the same sample indicating that the sampling technique was important. The samples of Table 72 were obtained by dissolving the oxidized lard in ether and taking an aliquot. The diacid contents still appeared to be too low. Starting with complicated mixtures such as these and trying to work out a multiplication factor for all of the diacids is subject to considerable error if more information about the response of the detector and recorder for each of the components is not known. Some additional work with the relative response of the mono- and diacids was conducted. The details of the work are given in the appendix on gas chromatography.

The Literature Review cited several examples of the separation of triglycerides using gas chromatography. If a mixture such as trimyristin, tripalmitin, and tristearin can be successfully separated by gas chromatography, then it does not seem unreasonable to expect that the oxidation products of lard could be separated in this manner. Rather than use the oxidized lard sample with the carboxylic acid groups at the points of cleavage, it would be better to use methylated samples or possibly completely hydrogenated oxidized lard samples. This appears to be a very good area for a problem using gas chromatography in the field of glyceride structure determination of fats and oils.

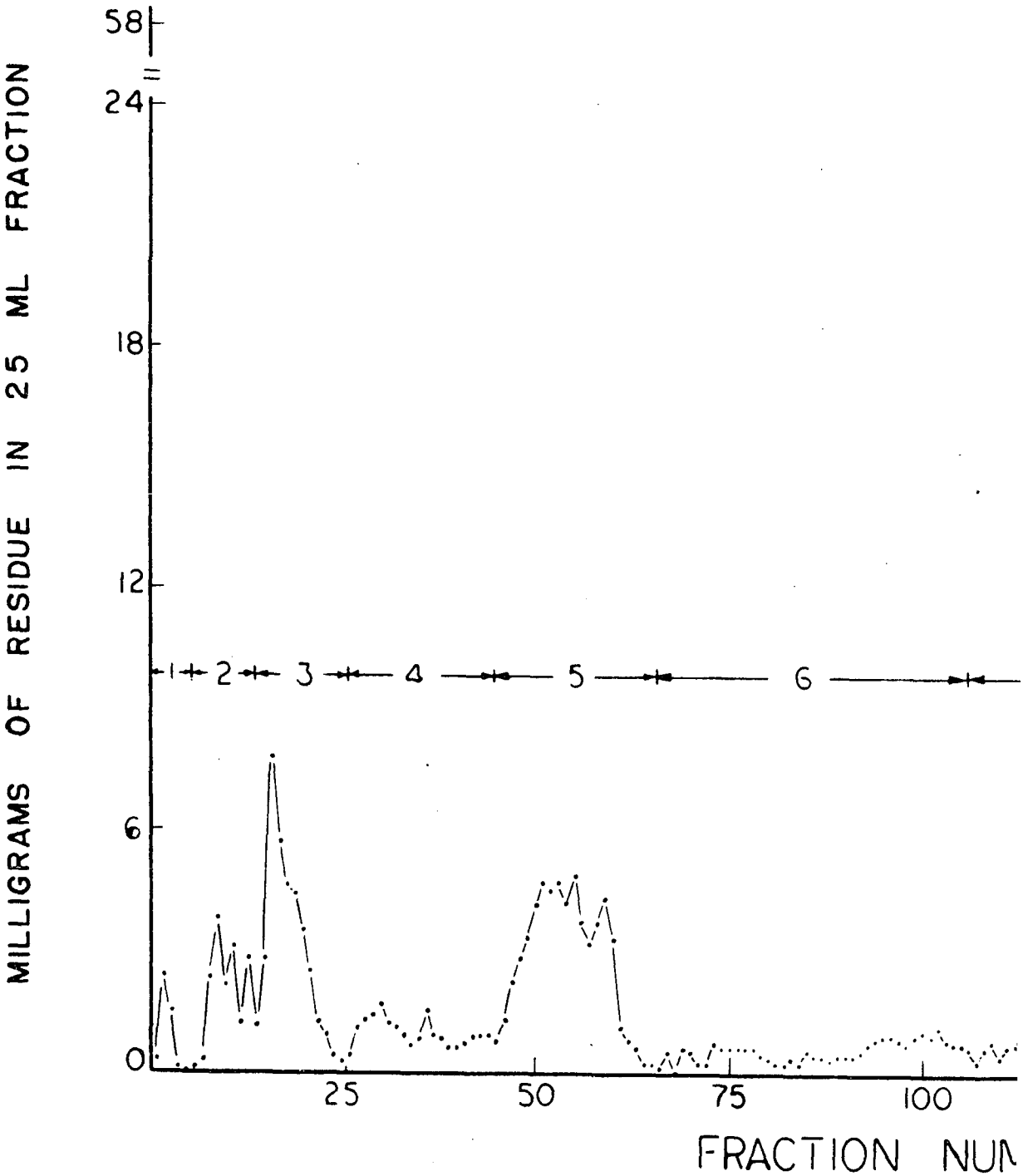
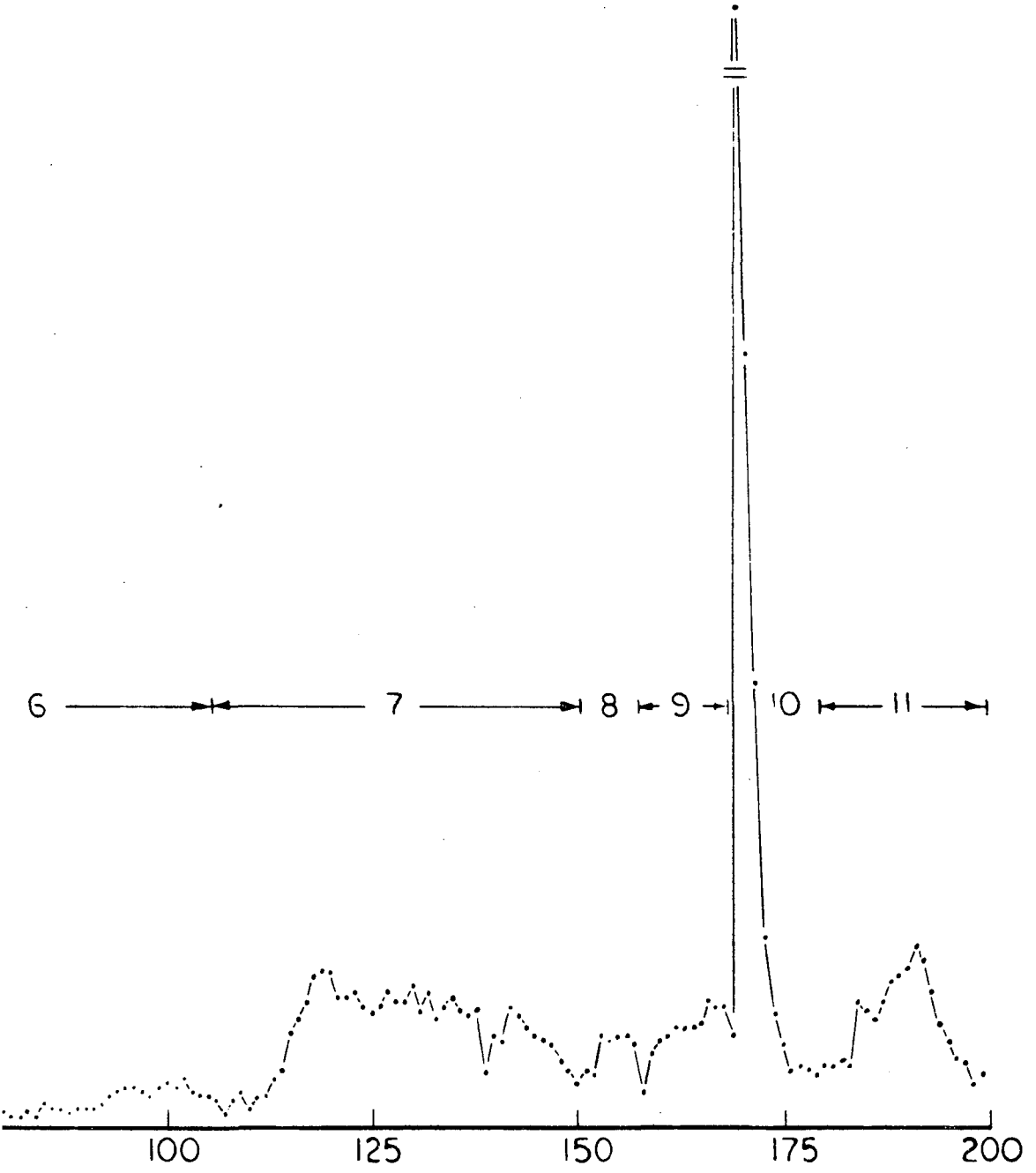


FIG. 23. FRACTIONATION



ACTION NUMBER

NATION OF OXIDIZED LARD B

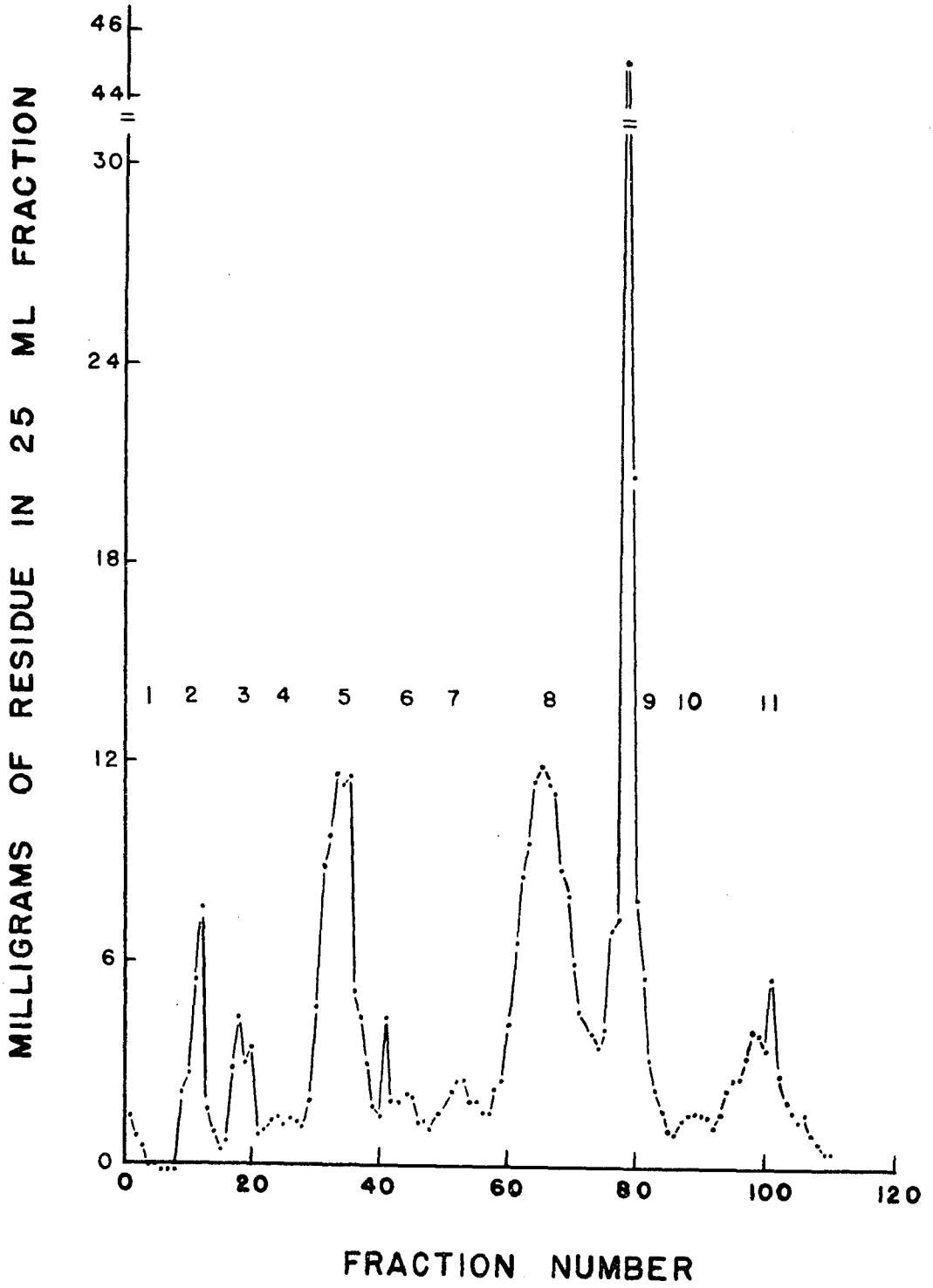


FIG. 24. FRACTIONATION OF OXIDIZED LARD A

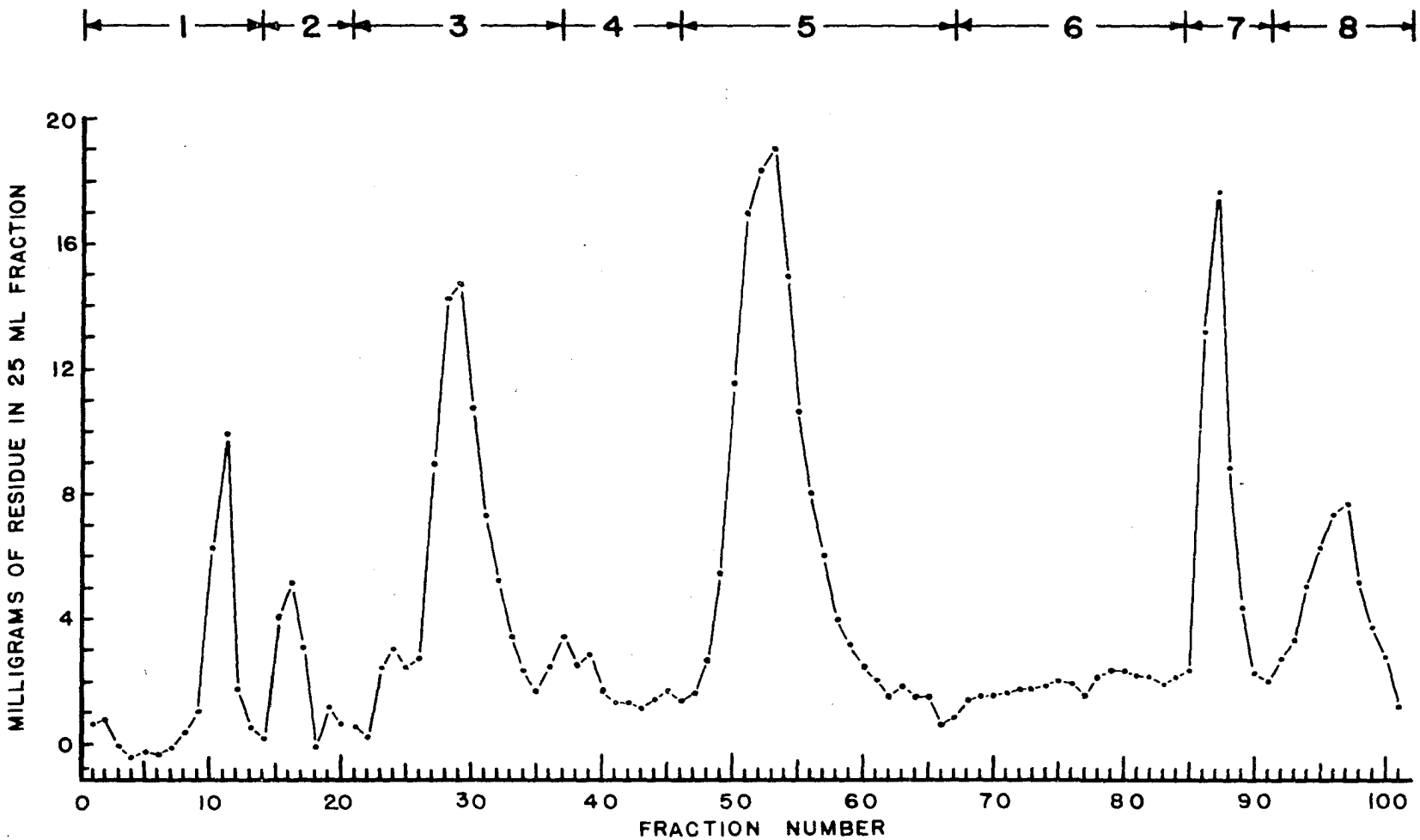


FIG. 25. FRACTIONATION OF OXIDIZED LARD A

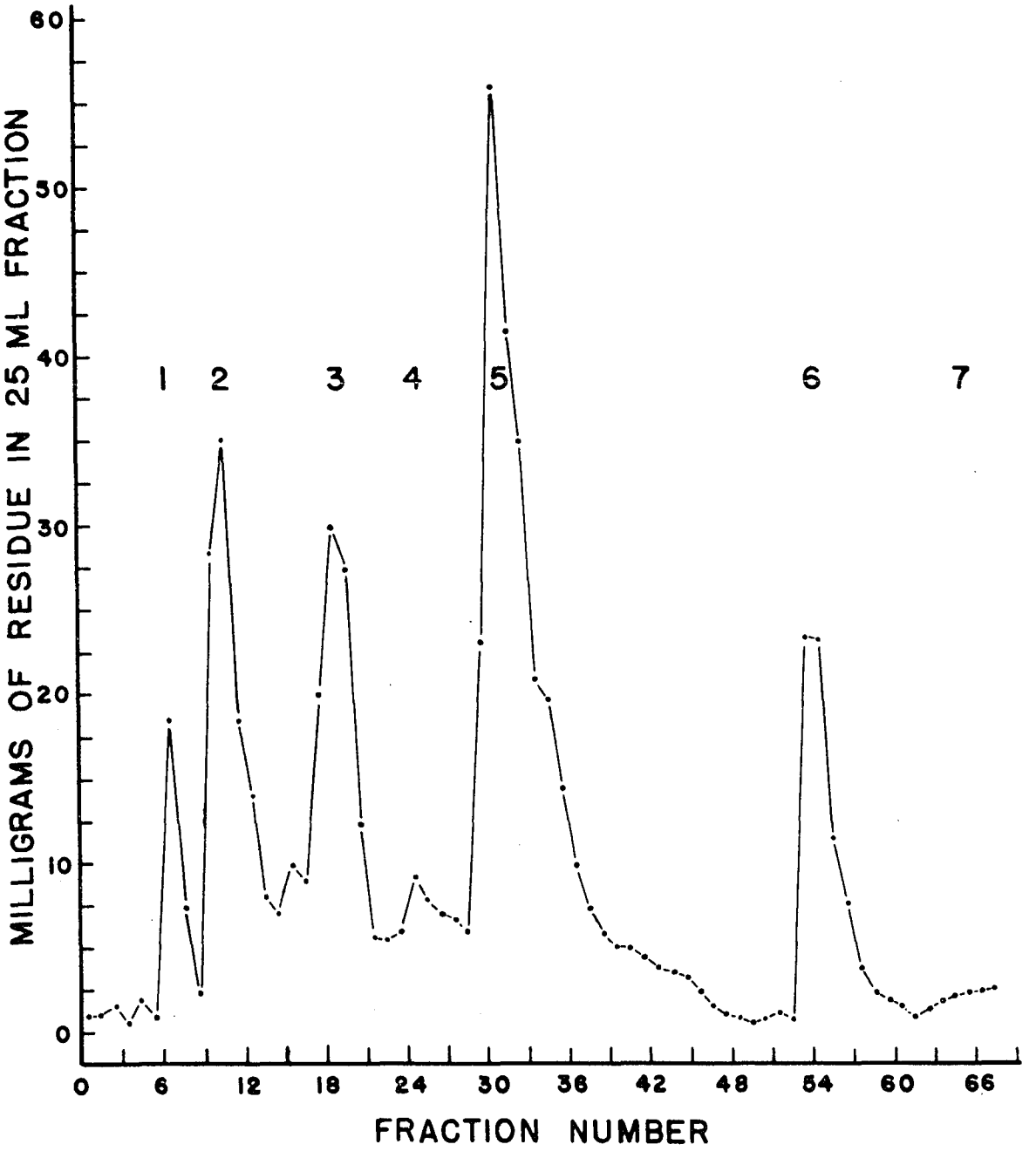


FIG. 26. FRACTIONATION OF OXIDIZED LARD R

Table 63. Fractionation data for Figure 23, oxidized lard B

Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg	Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg
1	0	5.0	0.3	46	20	4.3	1.3
2	1	4.3	2.4	47	20	4.2	2.2
3	2	5.3	1.5	48	20	4.0	2.8
4	3	5.6	0.1	49	21	3.9	3.3
5	4	5.2	0.0	50	21	3.9	4.1
6	5	5.2	0.1	51	21	3.8	4.7
7	6	3.6	0.3	52	21	3.8	4.5
8	7	3.8	2.3	53	22	3.6	4.7
9	8	3.4	3.8	54	22	4.0	4.2
10	9	3.7	2.1	55	22	3.5	4.9
11	10	4.0	3.1	56	22	3.8	3.7
12	11	3.9	1.2	57	23	4.2	3.2
13	12	4.0	2.8	58	23	4.0	3.7
14	12	3.1	1.1	59	23	4.2	4.3
15	12	3.3	2.8	60	23	3.9	4.3
16	12	3.0	7.8	61	24	3.9	1.1
17	13	3.5	5.7	62	24	4.0	0.8
18	13	3.7	4.6	63	24	3.7	0.6
19	13	2.7	4.4	64	24	3.5	0.2
20	13	2.6	3.5	65	25	3.4	0.2
21	14	3.7	2.5	66	25	4.5	0.1
22	14	3.9	1.2	67	25	4.4	0.5
23	14	4.0	0.9	68	25	4.4	0.0
24	14	3.8	0.4	69	26	3.8	0.6
25	15	4.4	0.3	70	26	3.9	0.5
26	15	4.5	0.4	71	26	4.0	0.2
27	15	4.5	1.1	72	26	4.0	0.2
28	15	3.7	1.3	73	27	3.6	0.7
29	16	4.0	1.4	74	27	4.3	0.6
30	16	4.4	1.7	75	27	5.0	0.6
31	16	4.9	1.2	76	27	4.0	0.6
32	16	3.7	1.1	77	28	4.3	0.6
33	17	4.3	0.9	78	28	4.4	0.6
34	17	4.5	0.7	79	28	4.4	0.4
35	17	4.2	0.8	80	28	3.7	0.3
36	17	3.9	1.5	81	29	4.4	0.2
37	18	3.9	0.9	82	29	4.4	0.2
38	18	4.0	0.8	83	29	4.1	0.3
39	18	4.0	0.6	84	29	4.1	0.2
40	18	4.4	0.6	85	30	4.1	0.5
41	19	4.0	0.7	86	30	4.2	0.4
42	19	4.3	0.9	87	30	4.6	0.4
43	19	4.4	0.9	88	30	3.8	0.3
44	19	3.9	0.9	89	31	4.2	0.4
45	20	4.0	0.8				

Table 63. (Continued)

Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg	Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg
90	31	4.2	0.4	135		3.9	3.0
91	31	4.3	0.4	136		4.5	2.7
92	31	4.0	0.5	137	43	4.2	2.6
93	32	4.3	0.7	138		4.2	2.7
94	32	4.2	0.8	139		4.2	1.3
95	32	4.2	0.9	140		3.8	2.1
96	32	4.2	0.9	141	44	3.8	2.0
97	33	4.4	0.8	142		4.4	2.8
98	33	4.0	0.7	143		4.7	2.6
99	33	4.3	0.9	144		4.2	2.3
100	33	4.2	1.0	145	45	4.2	2.1
101	34	4.2	0.9	146		4.1	2.0
102		4.2	1.1	147		4.3	1.9
103		4.2	0.8	148		4.2	1.5
104		4.2	0.7	149	46	3.2	1.3
105	35	4.1	0.7	150	47	3.9	1.0
106		4.2	0.6	151	48	3.6	1.3
107		3.7	0.3	152	49	3.7	1.2
108		4.0	0.6	153	50	4.2	2.1
109	36	3.9	0.8	154	51	3.6	2.0
110		4.3	0.4	155	52	4.0	2.1
111		4.3	0.7	156	53	3.8	2.1
112		4.4	0.7	157	54	4.1	1.9
113	37	4.0	1.1	158	55	4.0	0.8
114		4.0	1.3	159	60	3.9	1.7
115		4.0	2.2	160	65	4.0	2.0
116		3.9	2.5	161	70	4.3	2.1
117	38	3.8	2.9	162	75	4.2	2.3
118		4.5	3.5	163	80	4.2	2.3
119		4.2	3.6	164	85	4.3	2.3
120		4.1	3.6	165	90	4.5	2.4
121	39	3.3	3.0	166	95	3.7	2.9
122		4.2	3.0	167	100	4.6	2.8
123		4.2	3.1		% HA_C in ether		
124		3.9	2.8	168	2.5	4.2	2.8
125	40	4.2	2.7	169	5.0	4.6	2.1
126		4.2	2.8	170	7.5	4.5	58.4
127		4.0	3.1	171	10.0	4.1	18.1
128		4.5	2.9	172	12.5	3.9	10.4
129	41	4.2	2.9	173	15.0	3.7	4.4
130		4.5	3.3	174	17.5	3.6	2.6
131		3.9	2.7	175	20.0	3.3	1.9
142		3.9	3.1	176	22.5	3.7	1.3
133	42	4.1	2.5	177	25.0	3.7	1.4
134		3.9	2.8	178	27.5	3.4	1.3

Table 63. (Continued)

Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg	Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg
179	30.0	3.9	1.2	189	80	2.3	3.5
180	32.5	3.9	1.4	190	90	2.1	3.7
181	35	4.2	1.4	191	100	2.2	4.2
182	40	4.0	1.5	192	100	2.2	3.9
183	45	3.9	1.4	193	100	2.2	3.1
184	50	3.4	2.9	194	100	2.3	2.4
185	55	2.9	2.7	195	100	2.3	2.0
186	60	2.8	2.5	196	100	1.9	1.6
187	65	2.5	2.9	197	100	1.9	1.5
188	70	2.4	3.4	198	100	1.9	1.0
				199	100	1.9	1.2

Table 64. Fractionation data for Figure 24, oxidized lard A

Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg	Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg
1	0	5.8	1.5	24	19	5.3	1.5
2	1	5.0	0.9	25	19.5	5.1	1.3
3	2	5.0	0.6	26	20	5.2	1.5
4	3	5.7	0.0	27	20.5	5.2	1.4
5	4	5.8	0.0	28	21	5.1	1.2
6	5	5.4	-0.1	29	21.5	4.8	2.0
7	6	5.8	-0.1	30	22	5.1	4.8
8	7	5.4	-0.1	31	22.5	5.0	9.1
9	8	5.8	2.2	32	23	5.1	10.0
10	9	5.6	2.8	33	23.5	5.0	11.9
11	10	4.9	5.6	34	24	5.0	11.5
12	11	5.1	7.8	35	25	4.8	11.8
13	12	4.6	1.7	36	26	5.0	5.3
14	13	4.9	1.0	37	27	5.0	4.5
15	14	4.7	0.5	38	28	4.6	3.1
16	15	5.0	0.8	39	29	4.8	1.9
17	15.5	5.1	3.0	40	30	4.7	1.6
18	16	5.0	4.5	41	31	5.2	4.5
19	16.5	5.2	3.1	42	32	4.6	2.0
20	17	4.9	3.6	43	33	4.5	2.0
21	17.5	5.1	1.0	44	33.5	4.8	2.2
22	18	5.2	1.1	45	34	5.0	2.1
23	18.5	5.3	1.4	46	34.5	5.0	1.4

Table 64. (Continued)

Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg	Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg
47	35	5.0	1.4	87	30.0	4.0	1.5
48	36	4.9	1.2	88	32.5	5.1	1.7
49	38	5.0	1.7	89	35.0	4.2	1.7
50	40	5.1	1.9	90	40	4.1	1.7
51	41	5.1	2.3	91	45	4.0	1.6
52	42	4.9	2.6	92	50	3.7	1.3
53	43	4.9	2.4	93	55	3.8	1.7
54	44	4.6	2.0	94	60	3.5	2.4
55	45	4.8	2.0	95	65	3.2	2.7
56	45.5	4.4	1.7	96	70	2.9	2.8
57	46	4.6	1.7	97	80	2.7	3.4
58	46.5	4.8	2.2	98	90	2.4	4.2
59	47	4.7	2.7	99	100	2.1	4.1
60	48	4.7	4.3	100	100	2.1	3.6
61	50	5.0	6.8	101	100	2.3	5.7
62	52	4.9	8.8	102	100	2.3	2.9
63	54	5.1	9.8	103	100	2.2	2.1
64	56	5.0	11.7	104	100	2.1	1.8
65	58	5.0	12.1	105	100	2.1	1.5
66	60	5.1	11.6	106	100	2.2	1.7
67	62	5.2	11.3	107	100	2.2	1.1
68	64	4.8	9.0	108	100	2.1	0.9
69	66	4.8	8.2	109	100	2.0	0.6
70	68	4.9	8.2	110	100	2.1	0.6
71	70	4.8	4.8				
72	75	5.0	4.3				
73	80	5.2	4.1				
74	90	5.2	3.7				
75	100	5.4	4.2				
	% HA_C in ether						
76	2.5	5.2	7.2				
77	5.0	5.3	7.5				
78	7.5	4.7	45.4				
79	10.0	4.6	20.9				
80	12.5	4.5	8.1				
81	15.0	4.6	5.8				
82	17.5	4.3	3.3				
83	20.0	4.5	2.4				
84	22.5	4.2	1.8				
85	25.0	4.0	1.2				
86	27.5	4.0	1.1				

Table 65. Fractionation data for Figure 25, oxidized lard A

Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg	Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg
1	0	2.1	0.6	44	43	2.7	1.4
2	1	2.3	0.7	45	44	2.7	1.7
3	2	3.1	-0.1	46	45	1.6	1.4
4	3	2.9	-0.5	47	46	2.5	1.6
5	4	2.5	-0.3	48	47	3.3	2.6
6	5	2.5	-0.4	49	48	-	5.4
7	6	2.4	-0.2	50	49	-	11.5
8	7	2.3	0.3	51	50	-	16.9
9	8	2.1	1.0	52	51	2.7	18.3
10	9	2.3	6.2	53	52	2.5	19.0
11	10	3.6	9.9	54	53	2.6	14.9
12	11	2.6	1.7	55	54	2.7	10.6
13	12	2.4	0.5	56	55	2.7	8.0
14	13	2.5	0.1	57	56	2.4	6.0
15	14	2.2	4.0	58	57	2.4	3.9
16	15	2.4	5.1	59	58	2.3	3.1
17	16	2.2	3.0	60	59	2.6	2.4
18	17	2.2	-0.1	61	60	2.8	2.0
19	18	2.1	1.1	62	61	2.1	1.5
20	19	3.1	0.6	63	62	2.2	1.8
21	20	2.7	0.5	64	63	2.7	1.5
22	21	2.5	0.2	65	64	2.7	1.5
23	22	2.5	2.4	66	65	2.8	0.6
24	23	2.5	3.0	67	66	2.8	0.8
25	24	2.5	2.4	68	67	2.8	1.4
26	25	2.5	2.7	69	68	2.8	1.5
27	26	1.5	8.9	70	69	2.5	1.5
28	27	1.5	14.2	71	70	2.4	1.6
29	28	1.7	14.7	72	71	2.3	1.7
30	29	2.0	10.7	73	72	2.3	1.7
31	30	2.1	7.3	74	73	2.1	1.8
32	31	2.3	5.2	75	74	2.2	2.0
33	32	2.3	3.4	76	75	2.3	1.9
34	33	1.9	2.3	77	76	2.2	1.5
35	34	2.5	1.7	78	77	2.3	2.1
36	35	2.3	2.4	79	78	2.9	2.3
37	36	2.1	3.4	80	80	2.6	2.3
38	37	2.1	2.5	81	85	2.4	2.2
39	38	1.8	2.8	82	90	2.4	2.1
40	39	3.1	1.7	83	95	2.3	1.9
41	40	2.6	1.3	84	100	2.4	2.1
42	41	2.5	1.3		% HAc in ether		
43	42	2.6	1.1	85	5	2.4	2.3

Table 65. (Continued)

Fraction	% ether in Sk. B.	Flow-rate ml/min	Residue, mg	Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg
% HAc in ether (continued)							
86	10	2.3	13.1	94	80	1.6	5.0
87	15	2.3	17.6	95	100	1.9	6.2
88	20	2.3	8.8	96	100	1.6	7.3
89	25	2.5	4.3	97	100	2.2	7.6
90	30	2.3	2.2	98	100	2.2	5.1
91	40	2.2	2.0	99	100	2.2	3.7
92	50	1.9	2.7	100	100	-	2.8
93	60	1.8	3.3	101	100	-	1.2

Table 66. Fractionation data for Figure 26, oxidized lard R

Fraction	% ether in Sk. B.	Flow-rate ml/min	Residue, mg	Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg
1	0	2.3	0.9	26	50	2.3	7.7
2	2	2.6	0.9	27	52	2.3	7.1
3	4	2.0	1.5	28	54	1.9	6.7
4	6	1.9	0.3	29	56	2.4	6.0
5	8	2.0	1.9	30	58	2.4	23.1
6	10	2.9	0.5	31	60	2.3	56.2
7	12	2.7	18.5	32	62	-	41.4
8	14	3.0	7.3	33	64	2.3	35.2
9	16	1.7	2.0	34	66	2.3	21.1
10	18	2.0	28.0	35	68	2.3	19.8
11	20	1.9	35.3	36	70	2.3	14.6
12	22	2.5	18.1	37	72	2.3	9.8
13	24	1.9	14.2	38	74	2.3	7.5
14	26	2.5	8.0	39	76	3.3	6.0
15	28	2.3	7.0	40	78	2.9	5.2
16	30	2.3	9.6	41	80	2.8	5.1
17	32	2.2	8.6	42	82	1.9	4.6
18	34	1.9	20.0	43	84	2.9	3.9
19	36	2.1	29.8	44	86	2.2	3.1
20	38	3.0	27.2	45	88	2.2	2.9
21	40	2.3	12.3	46	90	2.1	2.5
22	42	2.3	6.0	47	92	1.7	2.0
23	44	2.3	5.8	48	94	2.6	1.5
24	46	2.3	6.1	49	96	2.2	1.4
25	48	2.3	8.9	50	98	2.1	1.0

Table 66. (Continued)

Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg	Fraction	% ether in Sk. B.	Flow-rate, ml/min	Residue, mg
51	100	2.0	1.1	61	30	1.7	1.7
	% HAc in ether			62	33	2.6	1.0
52	3	1.9	1.4	63	36	2.2	1.4
53	6	1.9	0.9	64	39	1.8	1.9
54	9	2.2	23.5	65	42	2.7	2.0
55	12	2.8	23.1	66	45	2.1	2.1
56	15	2.0	11.5	67	48	3.3	2.2
57	18	2.1	5.2	68	--	--	2.7
58	21	2.2	3.6				
59	24	2.2	2.5				
60	27	1.9	2.0				

Table 67. Data for peaks of the fractionated oxidized lards

Oxidized lard B, Figure 23				Oxidized lard A, Figure 24			
Fractions	Peak	Wt,mg	Wt %	Fractions	Peak	Wt,mg	Wt %
1-5	1	4.4	1.0	1-7	1	3.8	0.9
6-13	2	15.6	3.5	8-15	2	23.7	5.4
14-25	3	35.2	8.0	16-21	3	14.0	3.2
26-44	4	18.4	4.2	22-27	4	11.6	2.6
45-65	5	58.6	13.3	28-41	5	86.8	19.6
66-106	6	22.2	5.0	42-47	6	11.1	2.5
107-150	7	98.8	22.4	48-56	7	17.1	3.9
151-157	8	12.7	2.9	57-74	8	121.6	27.5
158-168	9	24.4	5.5	75-85	9	100.3	22.7
169-179	10	103.1	23.4	86-91	10	6.3	1.4
180-199	11	48.2	10.9	92-110	11	46.4	10.5
Total	-	441.6	100.1	Total	-	442.7	100.2
Oxidized lard A, Figure 25				Oxidized lard R, Figure 26			
1-12	1	28.1	6.7	1-9	1	33.8	4.4
13-20	2	20.7	4.9	10-15	2	110.6	14.5
21-36	3	88.3	21.0	16-23	3	119.3	15.6
37-45	4	18.4	4.4	24-29	4	42.5	5.6
46-66	5	140.2	33.3	30-53	5	271.3	35.6
67-84	6	30.1	7.2	54-62	6	74.1	9.7
85-90	7	47.1	11.2	63-69	7	12.3	1.6
91-101	8	48.0	11.4	---	Loss	99.2	13.0
Total	-	420.9	100.1	Total	-	763.1	100.0

Table 68. Comparison of the peaks of the oxidized lards

Figure:	23		24		25		26	
Oxid. lard:	B		A		A		R	
	Peak	Wt %	Peak	Wt %	Peak	Wt %	Peak	Wt %
	1	1.0	1	0.9				
	2	3.5	2	5.4	1	6.7	1	4.4
	3	8.0	3	3.2	2	4.9	2	14.5
	4	4.2	4	2.6				
	5	13.3	5	19.6	3	21.0	3	15.6
	6	5.0	6	2.5			4	5.6
			7	3.9	4	4.4		
	7	22.4	8	27.5	5	33.3	5	35.6
					6	7.2		
	8	2.9						
	9	5.5						
	10	23.4	9	22.7	7	11.2	6	9.2
			10	1.4				
	11	10.9	11	10.5	8	11.4	7	1.6
							loss	13.0
Original, mg	468.7		404.7		406.4		763.1	
Recovered, mg	441.6		446.4		420.9		663.9	

Table 69. Oxidized lard B, peak fatty acid compositions (Figure 23)

Silica gel col. peak:	1	2	3	4	5	6	7
Glc. Peak				Mol percent[a]			
DiC_7	-	T[b]	T	-	-	-	T
Myr.	-	3.1	2.6	1.1	1.6	1.2	1.8
DiC_8	-	T	T	-	-	3.3	0.9
Semi-DiC_9	-	-	T	-	-	-	T
DiC_9	-	-	4.0	2.9	34.7	48.2	40.3
Pal.	-	50.8	51.6	32.0	30.0	19.5	38.2
DiC_{10}	-	-	-	-	-	-	1.8
P.O.	-	-	-	3.5	1.4	2.5	-
Semi-DiC_{11}	-	-	0.6	-	-	-	T
DiC_{11}	-	-	T	-	T	2.4	6.1
Stear.	-	46.1	41.1	18.6	19.9	9.3	10.8
Oleic	-	-	-	39.6	12.5	13.6	T
Linol.	-	-	-	2.3	-	-	-

[a] Chromatogram area percent assumed equal to area percent and converted to mol percent.

[b] T = trace.

Table 69. (Continued)

Silica gel col. peak: Glc. Peak	1	2	3	4	5	6	7
			Mol percent[a]				
Mol % satd.	-	100.0	95.4	51.7	30.0	30.0	50.9
Mol % unsatd.	-	0	4.6	48.3	48.6	70.0	49.1

	8	9	10	11
DiC_7	T	T	T	-
Myr.	2.1	2.7	1.4	1.9
DiC_8	1.2	1.6	2.9	1.6
Semi-DiC_9	T	T	T	-
DiC_9	48.7	47.5	62.7	39.1
Pal.	39.7	40.7	20.8	30.1
DiC_{10}	1.9	T	1.3	-
P.O.	-	-	-	4.5
Semi-DiC_{11}	-	T	T	-
DiC_{11}	0.4	1.2	5.0	1.6
Stear.	5.7	6.0	4.9	10.2
Oleic	0.2	0.4	0.9	11.0
Linol.	-	-	-	-
Mol % satd.	47.5	49.4	27.1	42.2
Mol % unsatd.	52.5	50.6	72.9	57.8

Table 70. Oxidized lard A, peak fatty acid compositions (Figure 24)

Silica gel col. peak: Peak	1	2	3	4	5	6
			Mol percent			
DiC_7	-	T	T	T	T	T
Myr.	-	3.1	3.2	3.4	3.2	2.4
DiC_8	-	T	T	T	0.9	0.9
Semi-DiC_9	-	T	T	T	T	T
DiC_9	-	-	3.6	1.2	23.0	32.4
Palmitic	-	62.7	62.4	63.2	48.7	38.9
DiC_{10}	-	-	-	T	T	3.5
P.O.	-	-	-	-	-	-
DiC_{10}+P.O.	-	-	-	-	-	-
Semi-DiC_{11}	-	0.6	T	0.9	0.4	T

Table 70. (Continued)

Silica gel col. peak: Peak	1	2	3	4	5	6
			Mol percent			
DiC_{11}	-	-	T	-	2.0	2.1
Stear.	-	33.6	30.9	31.3	21.8	18.7
Oleic	-	-	-	-	-	0.9
Linol.	-	-	-	-	-	-
Mol % satd.	-	99.4	96.4	97.9	73.7	60.1
Mol % unsatd.	-	0.6	3.6	2.1	26.3	39.9

	7	8	9	10	11
DiC_7	T	T	0.5	T	0.8
Myr.	1.7	1.6	0.7	T	0.8
DiC_8	1.2	1.2	2.0	1.2	2.3
Semi-DiC_9	-	T	-	0.7	0.3
DiC_9	37.2	52.9	75.6	51.2	46.0
Pal.	34.9	26.6	9.5	16.4	19.4
DiC_{10}	-	1.3	0.8	-	-
P.O.	-	-	-	-	-
DiC_{10}+P.O.	2.3	-	-	1.9	2.3
Semi-DiC_{11}	-	-	-	T	1.2
DiC_{11}	3.1	4.6	7.1	7.4	4.3
Stear.	18.0	11.8	3.5	12.7	13.7
Oleic	1.6	-	0.4	8.6	9.0
Linol.	-	-	-	-	-
Mol % satd.	54.6	40.0	13.5	29.1	66.1
Mol % unsatd.	45.4	60.0	86.5	70.9	33.9

Table 71. Oxidized lard R, peak fatty acid compositions (Figure 26)

Silica gel col. peak: Glc. peak	1	2	3	4	5	6	7	2
				Mol percent				
Pelargonic	-	a	-	-	-	-	-	38.1
Myristic	3.4	4.1	3.5	2.4	1.5	-	-	2.5
DiC_8	-	-	-	1.8	1.7	9.6	3.5	-
DiC_9	-	-	19.6	29.4	55.3	71.4	12.0	-

[a]Pelargonic acid left out of these calculations. See last column for calculations with pelargonic acid included.

Table 71. (Continued)

Silica gel col. peak: Glc. peak	1	2	3	4	5	6	7	2
				Mol percent				
Palmitic	63.4	65.0	53.1	39.7	24.5	7.7	30.4	40.3
P.O.	-	-	-	-	-	-	3.1	-
DiC_{11}	-	-	-	4.3	5.7	5.9	1.6	-
Stearic	33.2	30.9	23.9	19.8	11.2	3.6	22.7	19.1
Oleic	-	-	-	2.7	-	2.0	25.1	-
Linol.	-	-	-	-	-	-	1.6	-

Table 72. Oxidized lard compositions and the saturated and unsaturated acids of the original lards

Lard	Oxidized lards: Before	Oxidized lards: After	Random
	Chromatogram area percent[a]		
Pelargonic(Cg)	5.9	2.2	2.5
	Mol percent[b]		
Myr.	2.0	2.8	2.1
DiC_8	1.9	1.9	2.0
DiC_9	45.1	43.3	41.0
Pal.	32.4	32.7	33.8
DiC_{10}	0.4	0.4	0.4
Semi-DiC_{11}	0.4	0.4	0.4
DiC_{11}	4.1	3.9	4.0
Stear.	13.9	14.8	16.3
Total satd.	48.2	50.2	52.2
Total diacid	51.8	49.8	47.8
	Diacids multiplied by 1.10		
Mol % satd.	45.9	47.8	49.8
Mol % diacid	54.1	52.2	50.2
	Original lards		
Mol % satd.	41.4	43.4	45.7
Mol % unsatd.	58.6	56.6	54.3

[a]Pelargonic acid is one of major short chain fatty acids cleaned from glyceride molecule during the oxidation. Some of the acid was not stripped from sample after oxidation. It was not included in calculations for glyceride residue composition.

[b]Chromatogram area percents were assumed to be equal to wt % and the mol % calculated from the wt %.

SUMMARY

The purpose of this research was to study the glyceride structure of lard before and after molecular rearrangement. The rearranged lard was prepared using sodium methylate. The methods used were fractionation of the lards by crystallization and the lard samples and crystallization fractions were further studied by employing enzymatic hydrolysis with steapsin. The fractional crystallizations were conducted in a low temperature cabinet cooled by circulating air through crushed Dry Ice. Details of the construction of the apparatus and the temperature control are described in an appendix. Fatty acid analyses were made by converting the acids to methyl esters and the methyl ester compositions determined by gas chromatography.

Methyl esters of the fatty acids of glycerides and of pure free fatty acids were prepared by the use of hydrochloric acid-methanol solution. Methylation of free fatty acids in a mixture of glycerides was conducted using ethereal solutions of diazomethane.

The hydrolysis products were analyzed by treating one sample with diazomethane and obtaining the free fatty acid compositions by gas chromatography. The free fatty acids in the hydrolysis products originated from the 1- and 3-positions of the original triglycerides. A second sample of the hy-

drolysis products was treated with periodic acid to destroy the 1-monoglycerides. The 2-monoglycerides were separated from the remainder of the hydrolysis products by use of silica gel column chromatography using ether-Skellysolve B for elution. The 2-monoglyceride fractions were analyzed for their fatty acid contents by gas chromatography of the fatty acid methyl esters. The 2-monoglyceride fatty acid composition data was not considered to be reliable because of ethyl ether vapor formation in the silica gel columns. Only the methyl ester compositions for the lard and lard fraction triglycerides and the free fatty acid methyl ester compositions of the hydrolysis products were studied in detail.

A study of different methods of hydrolysis using different conditions of hydrolysis was made. The conclusion was that all of the methods employed were satisfactory but the formation of the fat-buffer solution-steapsin emulsion was the determining factor in whether or not reliable results were obtained. If the emulsion was not finely divided and stable, the hydrolysis showed a specificity for the different types of fatty acid chains present in the triglyceride sample. The apparatus used to produce the emulsion therefore was more important than the hydrolysis conditions used.

The use of Amberlite IRA-400 anion exchange resin was studied for recovering free fatty acids from mixtures contain-

ing free fatty acids, mono-, di-, and triglycerides. The use of columns of resin were in general very troublesome and did not completely remove all of the fatty acids from the mixtures. The formation of ethyl ether vapor bubbles in the resin was the cause of the poor recoveries. One contact batch treatment with resin of a mixture of free fatty acids and glycerides in an ethereal solution was found to give better results. If enough resin was not used to recover all of the fatty acids, the resin was more selective for short chain fatty acids than long chains and more selective for unsaturated than saturated acids of the same chain length and the fatty acids recovered by the resin were not representative of the fatty acids originally present in the mixture. It was found that the most convenient method of recovering adsorbed fatty acids from the resin was to treat the resin-fatty acids with hydrochloric acid-methanol of 5% or higher acid concentration and to wash the resin with ethyl ether to insure all of the fatty acids and/or methyl esters were dissolved. The ether-hydrochloric acid-methanol solutions were then heated to complete the methylation and to evaporate the solvent. Traces of solvent and hydrochloric acid were removed under vacuum. The residue of methyl esters could then be injected directly into a gas chromatograph. If traces of water were present it was removed by adding anhydrous sodium sulfate to an ethereal solution of the methyl esters. The use of the resin was finally eliminated

by using diazomethane to obtain the free fatty acid compositions. The free fatty acids in the sample used to recover the 2-monoglycerides were eluted with the tri- and diglycerides and the glycolic aldehydes.

The analyses of the lard and lard fractions and of the free fatty acids formed during enzymatic hydrolyses gave data which were the basis of the following conclusions. The short chain fatty acids (both saturated and unsaturated) were found to be more predominate in the 2-position than in the 1- and 3-positions of the lard before rearrangement. Palmitic acid was by far the major short chain fatty acid (C_{14} and C_{16} chains). All of the C_{18} fatty acids of lard were found to be more predominate in the 1- and 3-positions of the lard before rearrangement. This included the saturated C_{18} acid, stearic acid, which was 12.3 mol percent of the total fatty acids. None of the individual fatty acids corresponded to a random distribution in the 1-, 2-, 3-positions or in just the 1- and 3-positions of lard fractions for the sample before rearrangement. The distributions for the individual fatty acids found in the 1-, 2-, and 3-positions and in the 1- and 3-positions showed similar deviations from random distribution for the same fractions.

The samples after rearrangement corresponded very closely to a random distribution of the individual fatty acids in the

lard fractions. The rearranged sample treated with sodium methylate for just 15-20 minutes indicated that the randomization may not have been quite complete. The 15-20 minutes rearranged sample after heating for 2 hours more with sodium methylate appeared to be completely randomized when the individual fatty acid distribution curves were studied. The fractional crystallization data gave lower trisaturated and triunsaturated glyceride contents for the rearranged samples than completely randomized samples would have but the differences were probably due to the assumptions made in making the glyceride composition calculations.

The distribution of the saturated acids among each of the six glyceride types, SSS, SSU, SUS, SUU, USU, and UUU appeared to be random even though none of the individual saturated acids showed a random distribution in these glycerides. This was possible because the deviations from random distribution for the individual saturated acids were not all higher or all lower than the calculated random distribution. The deviations of the individual fatty acids tended to cancel and the result was that the saturated fatty acid distribution tended to be random among the crystallization fractions. The same was found to be true for the unsaturated acids.

The deviations of the individual saturated fatty acids from random were in general larger for the saturated frac-

tions. The deviations of the individual unsaturated acids were larger for the unsaturated fractions and very nearly random for the saturated fractions.

Trisaturated glyceride determinations conducted as described in the literature by fractional crystallization gave results about 30% lower than obtained from the lard fractionation data. The amount of trisaturated glycerides calculated for random distribution of all the fatty acids on all the glyceride sites indicated that the fractionation data were also lower than the true value.

An attempt was made to study the individual saturated fatty acid distributions in each of the six glyceride types by first completely oxidizing the carbon-carbon double bonds of the lard to form carboxylic acid groups and fractionation of the oxidation products on silica gel columns. The fractionation of the oxidation products appeared to give separation into peaks corresponding to the six glyceride types but the methyl ester analyses did not indicate that pure fractions had been obtained except for the trisaturated glycerides. The diacid contents were probably unreliable because heating of the fractions during evaporation of solvent, particularly when acetic acid was present, caused the diacids to react. Methylation of the residues, after evaporation, using hydrochloric acid-methanol did not form methyl esters of all of the diacids originally present in the fraction before passage through the

silica gel column. Attempts to hydrolyze 3-30 milligram samples were also unsuccessful because the emulsions formed were not adequate. Recommendations for the continuation of this work are the formation of aldehyde groups instead of carboxylic acid groups in the oxidation step. These products should be more easily eluted and separated on silica gel columns and avoids the need to use acetic acid to elute the more polar components from the column. Adequate equipment to prepare emulsions of samples of approximately 3 ml in size would be necessary for carrying out successful hydrolyses.

The gas chromatograms obtained for most of the work were obtained from a gas chromatograph constructed in the laboratory. The final analyses used for the crystallization fractions were made on a F and M Model 500 gas chromatograph. Details of the various stages of construction of the gas chromatograph are described in an appendix. The best monocarboxylic acid separations were obtained using ethylene glycol succinate on acid washed Chromosorb. The separation of a mixture of mono- and dicarboxylic acids was accomplished by the use of 10 ft of 15% butanediol succinate on acid washed Chromosorb plus 4 to 18 in of 15% Dow Corning hi-vacuum silicone grease on aqua regia treated C-22 firebrick. The supports used for the liquid substrates were found to affect the separations obtained. The use of butanediol succinate on acid washed C-22

firebrick did not give satisfactory separations whereas aqua regia treated C-22 firebrick gave good separations. Butanediol succinate on Chromosorb W was found to be inadequate for separation of the C_{18} saturated and unsaturated series of fatty acid methyl esters and for the separation of pimelate-myristate and of azelate-palmitate.

LITERATURE CITED

1. Ackman, R. G., Bannerman, M. A., and Vandenheuvel, F. A. Decomposition of short-chain dicarboxylic acid esters during separation on polyester gas chromatography media. Anal. Chem. 32:1209. 1960.

2. A. O. C. S. Official Method Cd 7-58. Poly-unsaturated acids; ultraviolet spectrophotometric method. In American Oil Chemists' Society. Official and tentative methods of the American Oil Chemists' Society. 2nd ed. Chicago, Illinois, Author. Revision 1959.

3. Applied Science Laboratories, (State College, Pa.). Report on triglyceride analyses at Applied Science Labs brings reader comments. Gas-Chrom Newsletter 2, No. 4: 1. Sept. 1961.

4. Bailey, Alton E. Industrial oil and fat products. 2nd ed. New York, Interscience Publishers, Inc. 1951.

5. Bens, Everett M. Adsorption characteristics of some gas-liquid chromatographic supports. Anal. Chem. 33: 178-182. 1961.

6. Bergstrom, Sune and Borgstrom, Bengt. The intestinal absorption of fats. In Holman, Lundberg, and Malkin, eds. Progress in the chemistry of fats and other lipids. Vol. 3. pp. 351-394. New York, Pergamon Press. 1955.

7. Borgstrom, Bengt. Effect of tauro-cholic acid on the pH/activity curve of rat pancreatic lipase. Biochim. Biophys. Acta 13:149-150. 1954.

8. Borgstrom, Bengt. The formation of new glyceride-ester bonds during the digestion of glycerides in the lumen of the small intestine of the rat. Archives of Biochemistry and Biophysics 49:268-275.

9. Borgstrom, Bengt. Investigation on lipid separation methods; separation of cholesterol esters, glycerides, and free fatty acids. Acta Physiol. Scand. 25:111-119. 1952.

10. Borgstrom, Bengt. Investigation on lipid separation methods. III. Separation of tri-, di-, 1-mono-, and 2-monoglycerides. Acta Physiol. Scand. 25:231-239. 1953.

11. Borgstrom, Bengt. On the action of pancreatic lipase on triglycerides in vivo and vitro. Acta Physiol. Scand. 25:328-347. 1952.

12. Borgstrom, Bengt. On the mechanism of pancreatic lipolysis of glycerides. Biochim. Biophys. Acta 13:491-504. 1954.

13. Borgstrom, Bengt. On the mechanism of the hydrolysis of glycerides by pancreatic lipase. Acta Chem. Scand. 7: 557-558. 1953.

14. Bovee, H. H. and Robinson, Rex J. Sodium diphenylamine-sulfonate as an analytical reagent for ozone. Anal. Chem. 33:1115-1118. 1961.

15. Cason, James, Sumrell, Gene, and Mitchell, Robert S. Branched-chain fatty acids. XV. Syntheses of dimethyl-octadecanoic acids. J. Org. Chem. 15:850-859. 1950.

16. Chapman, I. D. Infrared spectra and the polymorphism of glycerides. J. Chem. Soc. (London) 1956:55-60. 1956.

17. Corcoran, Geraldine. Chromatographic separation and determination of straight-chain saturated monocarboxylic acids C_1 through C_{10} and dicarboxylic acids C_4 through C_{16}. Anal. Chem. 28:168-171. 1956.

18. Craig, B. M. and Murty, N. L. Quantitative fatty acid analysis of vegetable oils by gas-liquid chromatography. J. Am. Oil Chemists' Soc. 36:549-552. 1959.

19. Craig, B. M. and Murty, N. L. The separation of saturated and unsaturated fatty acid methyl esters by gas-liquid chromatography. Can. J. Chem. 36:1297-1301. 1958.

20. Cropper, R. R. and Heywood, A. The analysis of fatty acids and fatty alcohols by vapour phase chromatography. In Desty, D. H. Vapor phase chromatography. pp. 316-331. New York, Academic Press, Inc., Publishers. 1956.

21. Desnuelle, P. and Constantin, M. J. Formation de glycérides partiels pendant la lipolyse des triglycérides dans l'intestin. Biochim. Biophys. Acta 9:531-537. 1952.

22. Desnuelle, P., Naudet, M., and Constantin, M. J. Action des sels de calcium sur l'hydrolyse des triglycérides par la pancréatine et le suc pancréatique. Biochim. Biophys. Acta 5:561-568. 1950.

23. Desnuelle, P., Naudet, M., and Constantin, M. J. Sur un nouveau type de lipolyse *in vitro* une lipolyse "génératrice de glycérol". Biochim. Biophys. Acta 7: 251-256. 1951.

24. Desnuelle, P., Naudet, M., and Rouzier, J. Étude quantitative de la formation de glycérides partiels au cours de l'hydrolyse fermentaire des triglycérides. Biochim. Biophys. Acta 2:561-574. 1948.

25. Desnuelle, P. and Savary, P. Sur la structure glycéridique de quelques corps gras naturels. Fette, Seifen, Anstrichmittel 61:871-876. 1959.

26. Frankel, E. N., Evans, C. D., McConnell, D. G., and Jones, E. P. Analysis of lipids and oxidation products by partition chromatography; fatty acid hydroperoxides. J. Am. Oil Chemists' Soc. 38:134-137. 1961.

27. Frankel, E. N., Nowakowska, Janina, and Evans, C. D. Formation of methyl azelaaldehydate on autoxidation of lipids. J. Am. Oil Chemists' Soc. 38:161-162. 1961.

28. Fryer, F. H., Ormand, W. L., Crump, G. B. Triglyceride elution by gas chromatography. J. Am. Oil Chemists' Soc. 37:589-590. 1960.

29. Hawley, H. K. and Holman, G. W. Directed interesterification as a new processing tool for lard. J. Am. Oil Chemists' Soc. 33:29-35. 1956.

30. Helme, J. P. and Molines, J. Infrared spectra of linseed oil. Application to stand oils, glycerophihalate resin, and modified oils. Peintures, Pigments, Vernis. 33:524-543. 1957.

31. Herb, S. F., Audsley, M. C., and Riemenschneider, R. W. Some observations on the microscopy of lard and rearranged lard. J. Am. Oil Chemists' Soc. 33:189-193. 1956.

32. Herb, S. F., Magidman, Paul, Luddy, Francis E., and Riemenschneider, R. W. The fatty acid composition of lard including minor components. Paper presented at 52nd Annual Meeting of the American Oil Chemists' Society, St. Louis, Missouri, April 30-May 3, 1961. [Abstracted in printed program of the 52nd Annual Meeting] Chicago, Illinois, American Oil Chemists' Society. 1961.

33. Herb, S. F., Magidman, Paul, and Riemenschneider, R. W. Analysis of fats and oils by gas-liquid chromatography and ultraviolet spectrophotometry. J. Am. Oil Chemists' Soc. 37:127-129. 1960.

34. Higuchi, Takeru, Hill, Norman, and Corcoran, Geraldine. Chromatographic separation and determination of dicarboxylic acids C_4 to C_{10}. Anal. Chem. 24:491-493. 1952.

35. Hilditch, Thomas Percy and Lea, Colin Henry. Investigation of the constitution of glycerides in natural fats; a preliminary outline of two new methods. J. Chem. Soc. (London) 1927:3106-3117. 1927.

36. Hilditch, Thomas Percy and Pedelty, William Hopper. The body fats of the pig. V. Component glycerides of perinephric and outer back fats from the same animal. Biochemical Journal 34:971-979. 1940.

37. Hilditch, Thomas Percy and Stainsby, William John. The body fats of the pig. IV. Progressive hydrogenation as an aid in the study of glyceride structure. Biochemical Journal 29:90-99. 1935.

38. Hilditch, Thomas Percy. The chemical constitution of natural fats. 3rd ed. New York, John Wiley and Sons, Inc. 1956.

39. Hoerr, C. W. and Waugh, D. F. Some physical characteristics of rearranged lard. J. Am. Oil Chemists' Society. 32:37-41. 1955.

40. Hornstein, Irwin, Alford, John A., Elliott, L. E., and Crowe, P. E. Determination of free fatty acids in fat. Anal. Chem. 32:540-542. 1960.

41. Horrocks, Lloyd A., Cornwell, David G., and Brown, J. B. Quantitative gas-liquids chromatography of fatty acids methyl esters with the thermal conductivity detector. J. Lipid Research 2:92-94. 1961.

42. Huebner, V. R. Preliminary studies on the analysis of mono- and diglycerides by gas-liquid partition chromatography. J. Am. Oil Chemists' Soc. 36:262-263. 1959.

43. James, A. T. and Martin, A. J. P. Gas-liquid chromatography: the separation and identification of the methyl esters of saturated and unsaturated acids from formic acid to n-octadecanoic acid. Biochemical Journal 63:144-152. 1956.

44. James, A. T. and Martin, A. J. P. Gas-liquid chromatography; the separation and micro-estimation of volatile fatty acids from formic acid to dodecanoic acid. Biochemical Journal 50:679-690. 1952.

45. Kartha, A. R. S. The glyceride structure of natural fats. I. A technique for the quantitative determination of glyceride types in natural fats. (Edited and revised by R. J. Vander Wal). J. Am. Oil Chemists' Soc. 30:280-282. 1953.

46. Lipsky, S. R. and Landowne, R. A. Evaluation of a stationary phase for fatty acid analysis by gas-liquid chromatography. Annals of the New York Academy of Sciences 72:666-674. 1959.

47. Lipsky, S. R., Landowne, R. A., and Godet, M. R. The effects of varying the chemical composition of the stationary liquid on the resolution of the long chain saturated and unsaturated fatty acid esters by gas-liquid chromatography. Biochim. Biophys. Acta 31:336-347. 1959.

48. Lipsky, S. R., Landowne, R. A., and Lovelock, J. E. Separation of lipids by gas-liquid chromatography. Anal. Chem. 31:851-856. 1959.

49. Luddy, F. E. and Riemenschneider, R. W. Determination of trisaturated glycerides in lard, hydrogenated lard, and tallow. Oil and Soap 23:385-389. 1946.

50. Luddy, Francis E., Barford, R. A., and Riemenschneider, R. W. Direct conversion of lipid components to their fatty acid methyl esters. J. Am. Oil Chemists' Soc. 37:447-451. 1960.

51. Luddy, Francis E., Pertsch, George R., and Riemenschneider, Roy W. Glyceride composition of fats and oils determined by oxidation and crystallization methods. J. Am. Oil Chemists' Soc. 31:266-268. 1954.

52. Malins, Donald C. and Mangold, Helmut K. Analysis of complex lipid mixtures by thin layer chromatography and complementary methods. J. Am. Oil Chemists' Soc. 37: 576-578. 1960.

53. Mangold, Helmut K. and Malins, Donald C. Fractionation of fats, oils, and waxes on thin layers of silicic acid. J. Am. Oil Chemists' Soc. 37:383-385. 1960.

54. Mattson, F. H. and Beck, L. W. The digestion in vitro of triglycerides by pancreatic lipase. J. Biol. Chem. 214: 115-125. 1955.

55. Mattson, F. H. and Beck, L. W. The specificity of pancreatic lipase for the primary hydroxyl groups of glycerides. J. Biol. Chem. 219:735-740. 1956.

56. Mattson, F. H., Benedict, J. H., Martin, J. B., and Beck, L. W. Intermediates formed during the digestion of triglycerides. J. Nutrition 48:335-344. 1952.

57. Mattson, F. H. and Lutton, E. S. The specific distribution of fatty acids in the glycerides of animal and vegetable fats. J. Biol. Chem. 233:868-871. 1958.

58. Mc Innes, A. G., Tattrie, N. H., and Kates, M. Application of gas-liquid partition chromatography to the quantitative estimation of monoglycerides. J. Am. Oil Chemists' Soc. 37:7-11. 1960.

59. Meara, M. L. The configuration of naturally occurring mixed glycerides. Part II. The configuration of some monopalmitoglycerides from various sources. J. Chem. Soc. (London) 1945:23. 1945.

60. Metcalfe, L. D. and Schmidtz, A. A. The rapid preparation of fatty acid esters for gas chromatographic analysis. Anal. Chem. 33:363-364.

61. Metcalfe, Lincoln D. Direct analysis of fatty acids. Facts and Methods for Scientific Research 2, No. 1:1-3. New Castle, Delaware, F and M Scientific Corporation. Spring 1961.

62. Metcalfe, Lincoln D. Gas chromatography of unesterified fatty acids using polyester columns treated with phosphoric acid. Nature 188:142-143. 1960.

63. Miwa, Thomas K., Mikolajczak, Kenneth L., Earle, Fontaine R., and Wolff, Ivan A. Gas chromatographic characterization of fatty acids; identification constants for mono- and dicarboxylic methyl esters. Anal. Chem. 32:1739-1742. 1960.

64. Nowakowska, Janina, Melvin, E. H., and Wiebe, Richard. Separation of the oxidation products of fatty acids by means of gas-liquid partition chromatography. J. Am. Oil Chemists' Soc. 34:411-414. 1957.

65. O'Connor, Robert T. Infrared absorption spectra. J. Am. Oil Chemists' Soc. 32:624-633. 1955.

66. O'Connor, Robert T., DuPre, Elsie, F, and Feuge, R. O. The infrared spectra of mono-, di-, and triglycerides. J. Am. Oil Chemists' Soc. 32:88-93. 1955.

67. Ogg, C. L., Porter, W. L., and Willits, C. O. Determining the hydroxyl content of certain organic compounds. Ind. Eng. Chem., Anal. Ed. 17:394-397. 1945.

68. Orr, C. H. and Callen, J. E. Recent advances in the gas chromatographic separation of methyl esters of fatty acids. Annals of the New York Academy of Sciences 72: 649-665. 1959.

69. Orr, C. H. and Callen, J. E. Separation of polyunsaturated fatty acid methyl esters by gas chromatography. J. Am. Chem. Soc. 80:249. 1958.

70. Orr, Charles H. Recorder-integrator errors in gas chromatography area measurements. Anal. Chem. 33:158-159. 1961.

71. Placek, Chester and Holman, George W. Directed interesterification of lard. Ind. Eng. Chem. 49:162-169. 1957.

72. Pohle, W. D. and Mehlenbacher, V. C. A modification of the periodic acid method for the determination of monoglycerides and free glycerol in fats and oils. J. Am. Oil Chemists' Soc. 27:54-56. 1950.

73. Privett, O. S. and Blank, M. L. A new method for the analysis of component mono-, di-, and triglycerides. J. Lipid Research 2:37-44. 1961.

74. Privett, O. S., Blank, M. L., and Lundberg, W. O. Determination of mono-, di-, and triglycerides by molecular distillation and thin layer chromatography. J. Am. Oil Chemists' Soc. 38:312-316. 1961.

75. Quimby, O. T., Wille, R. L., and Lutton, E. S. On the glyceride composition of animal fats. J. Am. Oil Chemists' Soc. 30:186-190. 1953.

76. Quinlin, Patricia and Weiser, Jr., Herman J. Separation and determination of mono-, di-, and triglycerides in monoglyceride concentrates. J. Am. Oil Chemists' Soc. 35:325-327. 1958.

77. Ravin, Louis J., Meyer, Robert J., and Higuchi, Takeru. Chromatographic analysis of mixtures of mono-, di-, and tristearin containing mineral oil. J. Am. Oil Chemists' Soc. 34:261-263. 1957.

78. Reiser, Raymond and Reddy, H. G. Ramakrishna. The glyceride structure of swine depot fat. J. Am. Oil Chemists' Soc. 36:97-99. 1959.

79. Riemenschneider, R. W., Luddy, F. E., Swain, M. L., and Ault, W. C. Fractionation of lard and tallow by systematic crystallization. Oil and Soap 23:276-282. 1946.

80. Savary, P. and Desnuelle, P. Analyse chromatographique des mélanges contenant des acides gras, des monoglycérides, des diglycérides et des triglycérides. Bull. Soc. Chim. (France) 1954:936-940. 1954.

81. Savary, P. and Desnuelle, P. Étude des chaînes internes dans quelques triglycérides mixtes naturels. Biochim. Biophys. Acta 31:26-33. 1959.

82. Savary, P. and Desnuelle, P. Sur quelques éléments de spécificité pendant l'hydrolyse enzymatique des triglycérides. Biochim. Biophys. Acta 21:349-360. 1956.

83. Savary, P. and Desnuelle, P. Fractionnement quantitatif des acides gras saturés et insaturés par hydroxylation permanganique et chromatographie de partage en phases inversées. Bull. Soc. Chim. (France) 1953:939-945. 1953.

84. Savary, P., Flanzy, J., and Desnuelle, P. Emploi de la lipase pancréatique pour l'étude de la structure des corps gras naturels. Biochim. Biophys. Acta 24:414-423. 1957.

85. Savary, Pierre and Desnuelle, Pierre. Étude chromatographique de l'action de la lipase pancréatique sur des triglycérides mixtes. Comptes Rendus Hebdomadaires des Séances de L'Académie des Sciences 240:2571-2573. 1955.

86. Schlenk, Hermann and Gellerman, Joanne L. Esterification of fatty acids with diazomethane on a small scale. Anal. Chem. 32:1412-1414. 1960.

87. Slater, Lloyd E. Lard scores again. Food Eng. 25, No. 9:72-73, 100, 102-105. 1953.

88. Smith, Edgar G. Simplified chromatographic separation and analysis of C4 through C12 dibasic acids. Anal. Chem. 32:1301-1304. 1960.

89. Stoffel, W., Insull, Jr., W., and Ahrens, Jr., E. H. Gas-liquid chromatography of highly unsaturated fatty acid methyl esters. Proceedings of the Soc. for Experimental Biology and Medicine 99:238-241. 1958.

90. Stoffel, Wilhelm, Chu, Florence, and Ahrens, Jr., Edward H. Analysis of long-chain fatty acids by gas-liquid chromatography; micromethod for preparation of methyl esters. Anal. Chem. 31:307-308. 1959.

91. Vander Wal, R. J. Calculation of the distribution of the saturated and unsaturated acyl groups in fats, from pancreatic lipase hydrolysis data. J. Am. Oil Chemists' Soc. 37:18-20. 1960.

92. Vogel, Arthur I. Practical organic chemistry including qualitative organic analysis. 2nd ed. London, Longmans, Green and Co. 1951.

93. von H. Lindlar. Ein neuer Katalysator für selective Hydrierungen. Helvetica Chimica Acta 35:446-450. 1952.

94. von Rudloff, E. Periodate-permanganate oxidations. V. Oxidation of lipids in media containing organic solvents. Can. J. Chem. 34:1413-1418. 1956.

95. Vorbeck, Marie L., Mattick, Leonard R., Lee, Frank A., and Pedeson, Carl S. Preparation of methyl esters of fatty acids for gas-liquid chromatography; quantitative comparison of methylation techniques. Anal. Chem. 33: 1512-1514. 1961.

96. Wollish, E. G., Schmall, Morton; and Hawrylyshyn, Mary. Thin-layer chromatography; recent developments in equipment and applications. Anal. Chem. 33:1138-1142. 1961.

97. Wren, J. J. Review: Chromatography of lipids on silicic acid. J. Chromatography 4:173-195. 1960.

98. Youngs, C. G. Determination of the glyceride structure of fats. J. Am. Oil Chemists' Soc. 38:62-67. 1961.

99. Youngs, C. G. and Sallans, H. R. Fractionation and glyceride composition of fats. J. Am. Oil Chemists' Soc. 35:388-393. 1958.

100. Zilch, K. T. and Dutton, H. J. Analysis of fat acid oxidation products by countercurrent distribution methods. Anal. Chem. 23:775-778. 1951.

101. Zlatkis, Albert, Ling, Su-Yu, and Kaufman, H. R. Resolution of isomeric xylenes by gas-liquid chromatography. Anal. Chem. 31:945-947. 1959.

ACKNOWLEDGMENT

The author is grateful to Dr. L. K. Arnold for continued guidance, encouragement and help throughout the problem;

to the Chemical Engineering Department and the Iowa Engineering Experiment Station for financial support;

to the Rath Packing Company of Waterloo, Iowa and to the Procter and Gamble Company of Cincinnati, Ohio for research fellowships;

and to Mr. Paul Bond, Electrical Engineering graduate student, for advice on problems concerning electrical equipment.

APPENDIX A: LOW TEMPERATURE CRYSTALLIZATION EQUIPMENT AND PROCEDURE

The fractional crystallization procedure described by Luddy et al. (51) was followed. Figure 27 shows the steps and temperatures used in the fractionation scheme. Table 73 summarizes more clearly the nature of the glycerides in each of the fractions obtained. The P-3 fraction contains the glycerides with the lowest iodine value and fraction F-4 contains the glycerides with the highest iodine value. The P-6, F-6, and F-5 fractions each contain glycerides with progressively higher iodine values.

Table 73. Temperature range in which the glycerides of each fraction precipitate

Fraction	Contains glycerides which ppt.
P-3	above 15°C
P-6	between 15° to 0°C
F-6	between 0° to -25°C
F-5	between -25° to -45°C
F-4	below -45°C

The low temperature cabinet was constructed of 1/2 in plywood and 4 in corkboard insulation. The overall outside dimensions of the cabinet were 4 ft 9 in long, 2 ft 11 in high, and 2 ft 4 in wide. The cabinet was mounted on casters to facilitate moving it about in the laboratory.

The cabinet was divided into a sample section and a Dry

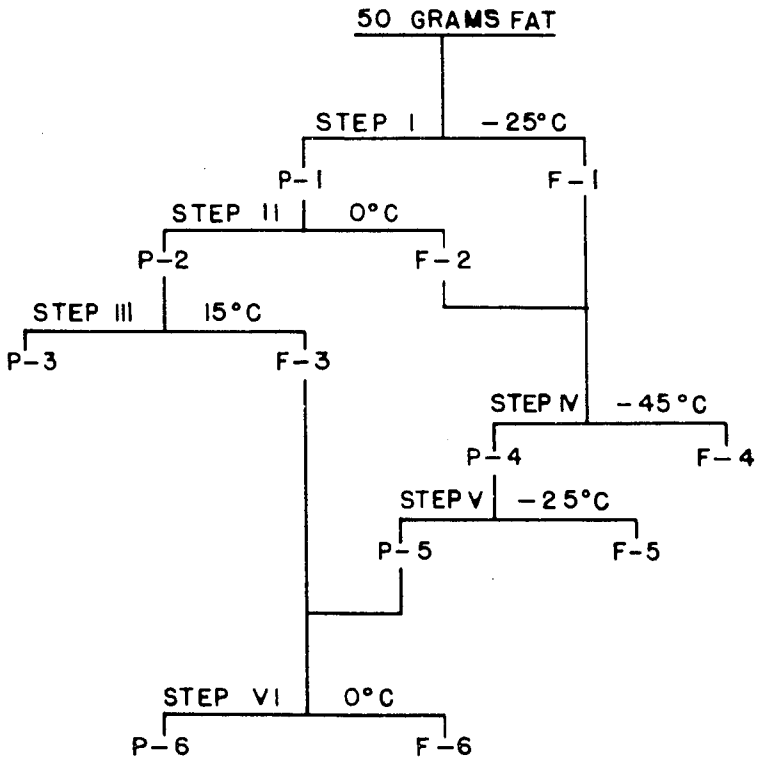


FIG. 27. STEPS IN THE FRACTIONAL CRYSTALLIZATION OF FATS FROM ACETONE. (30 ML/GM FOR EACH STEP).

Ice section by a partition wall. The Dry Ice side contained a wire basket for holding crushed Dry Ice and had an automobile heater fan and its housing mounted on the back wall of this section. The fan sucked air through a 4 in galvanized iron pipe which served as a duct from the sample section to the suction side of the fan. The fan forced this air to rise through the Dry Ice, causing the air to be cooled. This cooled air filled the Dry Ice section and passed into the sample chamber because of the small difference in pressure developed between the two sections by the Dry Ice fan.

The Dry Ice fan was turned by a V-belt which was driven by a 1/4 horse power electric motor mounted on the outside back of the cabinet. The power to this motor was on-off controlled by use of a bimetallic spiral thermoswitch connected in the electrical circuit shown in Figure 28. The series of relays was found to be necessary because of the low current capacity of the thermoswitch points which were rated at a maximum load of 1/8 watt. A mercury contact switch was used in the motor power supply line because the high inductive load of the motor caused other types of contact points to burn badly and eventually caused them to fuse together.

Because of vibration of the cabinet by motors mounted on the cabinet, the thermoswitch points chattered when they were very close to making or breaking contact. This caused the

electric motor to be turned on and off at a rate of many times per minute causing the motor to overheat and a thermoguard switch automatically broke the power line circuit of the motor. This difficulty was overcome by putting a time delay in the electrical system. This was accomplished through the use of a vacuum tube pentode with a grid bias consisting of a variable resistance and a capacitor. By varying the resistance the time of the delay could be varied from zero to over 1/2 minute. This resistance setting was made by trial and error such that the motor did not turn on and off rapidly enough to cause the thermoguard to break the power line circuit.

The pentode was driven by the diode in the 117L7/M7GT vacuum tube. The grid bias dc-source was originally supplied by the use of a transformer and a copper-copper oxide rectifier. But this was later changed to the use of a transformer and a transistor diode, GE 1N538. The change was made when the copper-copper oxide rectifier could not be made to rectify a dc supply sufficient to put a potential on the grid high enough to stop the flow of current through the pentode. The zero signal current flow through the pentode was large enough to hold the relay in the plate circuit closed and therefore the Dry Ice fan motor kept running when it should have been shut off.

The temperature of the samples were maintained by the

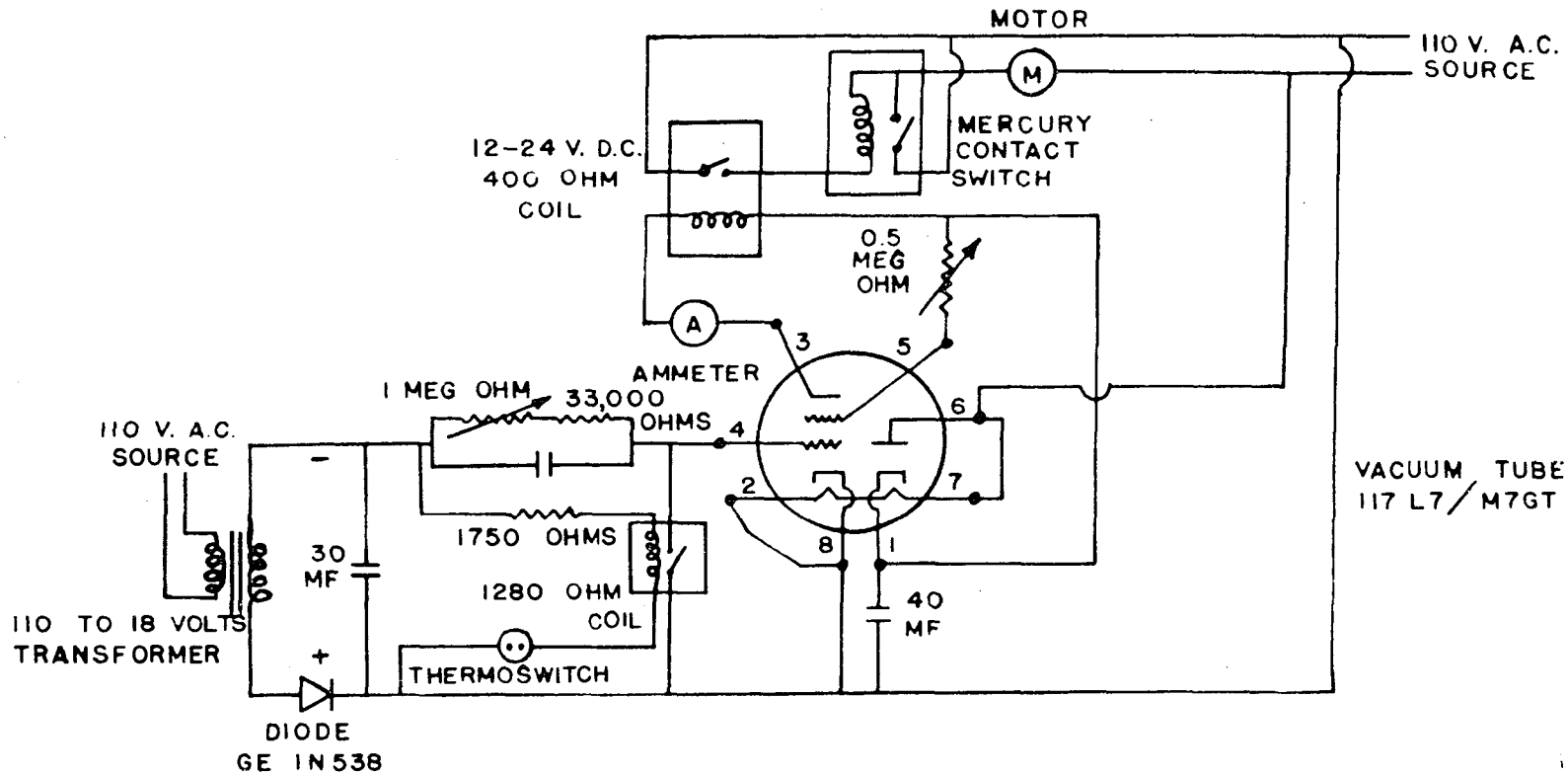


FIG. 28. SCHEMATIC WIRING DIAGRAM OF THE TEMPERATURE CONTROL WITH A TIME DELAY FOR THE LOW TEMPERATURE FRACTIONAL CRYSTALLIZATION CABINET

following series of steps. As the temperature in the sample chamber increased, the thermoswitch points were closed. This caused the relay coil in the same circuit as the thermoswitch to become energized and closed the contact points in the relay. These points were connected in a grid circuit and when closed, the grid potential became the same as the cathode potential. Current then passed through the tube and energized the relay coil in the plate circuit. The ammeter in this circuit was used to insure that the maximum tube plate current of 43 milliamperes was not exceeded. The plate current could be adjusted by varying the one-half million ohm variable resistance in the screen grid circuit. When the coil in the plate circuit relay was energized, the relay contact points in the Dry Ice fan motor circuit were closed and the Dry Ice fan was turned. Air sucked from the sample chamber passed up through Dry Ice in the wire basket and this cooled air was forced back into the sample chamber. When the sample chamber temperature decreased, the thermoswitch contact points opened. This caused the contact point of the relay in the thermoswitch circuit to open. The potential on the grid then changed from that of the cathode to a potential negative to that of the cathode by an amount equal to the output voltage of the transistor diode. This change in potential could be made to take place more or less rapidly by changing the variable resistance hooked in parallel with the 25 microfarad capaci-

tor. The time in seconds can be estimated from time in seconds equals resistance in ohms times the capacitance in farads. The 25 microfarad capacitor is charged when the relay contacts in the thermoswitch circuit are closed. When the relay contacts in the grid circuit open, the charged capacitor slowly discharges across the resistance in parallel with it. As the capacitor discharges, the potential on the grid slowly decreases to that of the diode output. The grid is hooked to the negative side of the diode and the cathode is hooked to the positive side. The grid is therefore negative with respect to the cathode and the current flow through the tube is cut to a very low value of only a few milliamperes. This small current is not enough to hold the relay in the plate circuit closed and the Dry Ice fan motor is shut off.

The sample section contained a fan which kept the air in this section well mixed. It was belt driven by a 1/4 horse power electric motor mounted on the outside back of the cabinet. The thermoswitch was located in this section near the opening in the partition through which air passed into the Dry Ice chamber to be cooled.

Round bottom flasks with outlets in the center of the bottom of the flasks were mounted in the cabinet using laboratory clamps. The outlets were fitted with short pieces of

rubber tubing and these were pinched shut by screw clamps. The screw clamps were brazed to metal mounting bars which were fastened to the cabinet walls. The screws were turned by means of number 9 wire handles passing out through the front wall of the cabinet.

The solutions in the flasks were stirred by means of stirrers mounted on a removable bar which was attached to the cabinet walls. The stirrer shafts and stirrer blades were made of stainless steel. The shafts passed through loose fitting porous metal bushings which were firmly mounted on the removable bar. The shafts were kept from sliding through the bushings by the pulleys mounted on the upper end of the shafts. The stirrers were turned by means of a belt made of 1/4 in round leather belting. The drive pulley on a 90 degree change of direction gear box was driven by means of a 1/4 horse power electric motor mounted on the outside back of the cabinet.

A Buchner funnel was mounted below each flask. The funnel stems were connected by means of rubber vacuum hoses and L-shaped adapters, which fit on the funnel stems, to copper tubes passing through the front wall of the cabinet. The external ends of the copper tubes were fitted with rubber vacuum hoses which were connected to filtration flasks. The filtration flasks were connected by means of rubber vacuum

hoses and a manifold to a single line leading to a water aspirator. Screw clamps were used to control the vacuum to each of the filtration flasks.

Iron-constantan thermocouples were used to measure the temperature of the solutions and the air at various points in the cabinet. The thermocouple wires were connected to a potentiometer through a multiple contact switch. The reference junction was placed in a thermosbottle filled with ice. To prevent rusting of the iron wire, the thermocouple was placed in a mercury well which was placed in the ice.

The entire top of the cabinet formed the lid for the cabinet and a seal between the lid and the box was made using strips of felt covered with polyethylene film and strips of rubber tubing placed on top of the felt. When the lid was latched down, the rubber tubing was crushed slightly and formed an air tight seal between the polyethylene cover and the underside of the lid.

The sample section was initially built to handle six samples. It was then modified to run only two samples by placing a partition in the sample chamber.

The partition between the sample chamber and the Dry Ice chamber had a 3-1/2 by 4 in hole to allow the cooled air in the Dry Ice chamber to pass into the sample chamber. When

the sample chamber was being maintained at near room temperature it was found that the circulation caused by the sample chamber fan was enough to keep the sample chamber at a temperature lower than desired without the Dry Ice fan ever running. This difficulty was overcome by threading the end of a 1/8 in welding rod. Nuts with threads of the same size were then brazed to a piece of galvanized iron sheeting cut just slightly larger than the hole in the partition. This plate and threaded rod were then mounted such that the rod extended through the front of the low temperature cabinet and when rotated, moved the plate across the hole in the partition. In this manner it was possible to vary the size of the opening without opening the low temperature cabinet.

The air temperature of the sample chamber cycled through several degrees of temperature when the low temperature cabinet was in operation. Thermocouples in the solutions indicated that the temperature of the two solutions in the small sample chamber could be maintained within approximately $\pm$ 0.3°C of the desired temperature. This temperature range varied with the temperature trying to be maintained. At -45°C, the variation from the desired temperature was more than the variation at near room temperature.

Samples were fractionated by dissolving the fat samples in dried acetone and diluting to the correct volume in ap-

propriate sized round bottom flasks. The flasks, Buchner funnels, stirrers, thermocouples, and acetone for washing the precipitates were all put in place in the cabinet. Dry Ice was crushed and placed in the wire basket. The lid was closed and the stirrers, circulating fan in the sample section, and the Dry Ice fan were turned on. The contact points on the bimetallic spiral thermoswitch were adjusted to maintain the proper temperature in the sample section. The temperatures of the solutions, measured by the use of thermocouples, were observed and used to determine the thermoswitch adjustments required.

After the samples were held at a constant temperature for the required length of time, the circulation fan in the sample section and the stirrers were turned off, the lid was opened, and the stirrers removed from the cabinet. The sample section was then covered with strips of plate glass and the Dry Ice section was covered with a large piece of pressed fiber board. This arrangement allowed the thermoswitch to maintain a constant temperature during the filtration step. The samples were filtered one at a time, the filter cakes pressed, and the cakes washed with 2-3 ml of precooled, dried acetone per gram of precipitate and the filter cakes were pressed again. The filter cakes were then transferred to flasks and the acetone removed by evaporation to determine the weight of the precipitates. Similarly, the filtrates

were evaporated to remove excess acetone in preparation for the next crystallization. Having once obtained the precipitate weights for each of the crystallization steps, use of these weights for another run on the same sample eliminated a considerable amount of evaporating and weighing of samples.

The longer the time allowed for the crystallization to take place, the better but some time limit had to be set. Approximate times used in this work were 15 hours for runs made at 0 and 15°C. and 8 hours for runs at -25 and -45°C. for preliminary fractionations. These times were picked to correspond favorably to the times used in other studies by other workers. In the case of lower temperatures the time was shorter because of the much higher Dry Ice consumption rates.

In Fractional crystallization No. 5 the low temperature cabinet was modified to accommodate three samples. This required reducing the lard samples from 50 to 25 grams in order to be able to put three samples in the small sample chamber previously used for running two samples. A third stirrer was added to the stirrer mount. A false bottom made of hardware cloth was constructed to support the sample solutions contained in beakers. The hardware cloth permitted the circulation fan below it to circulate the air in the sample chamber. The filtrations were conducted by placing a filtration

flask fitted with the appropriate sized Buchner funnel on the hardware cloth, decanting the clear solution into the funnel and finally the precipitate was poured into the funnel. The funnels and flasks were left in the Dry Ice chamber until needed for filtration.

The belt drives on the stirrer motor and the motor for the sample chamber circulation fan were also eliminated. They were replaced by direct couplings between the motor shafts and the fan and gear box shafts.

A total of 208 pounds of Dry Ice were required for fractional crystallization No. 5. Dry Ice was obtained from the Iowa State University Chemical Stores and stored in the empty sample chamber section until needed. Cooling of the sample chamber to -25°C for the first crystallization required considerably more Dry Ice than the -25°C crystallization step later in the procedure because the low temperature cabinet had to be cooled from room temperature. But once the cabinet was in operation and not allowed to come to room temperature, less Dry Ice was needed and the time required to cool to the crystallization temperature was shorter. Table 74 gives a summary of some of the fractional crystallization procedure used for the 50 and 25 gram lard samples.

Table 74. Fractional crystallization procedure information

Step	Fraction	Temp. of crystn., °C.	Time for cooling, hours	Time at crystn. temp., hours[a] Crystn. No. 4	Crystn. No. 5	Funnels for filtering[b] Lard B	Lard A
I	P-1	-25	$2\frac{1}{2}$	4	$4\frac{1}{4}$	No. 3	No. 3
II	P-2	0	1	12	$7\frac{1}{2}$	2A	2A
III	P-3	15	$\frac{1}{2}$	24	15	2A	1
IV	P-4	-45	2	8	8	3	3
V	P-5	-25	$1\frac{1}{2}$	9	8	3	3
VI	P-6	0	1	12	$17\frac{1}{2}$	1	2A

[a]In fractional crystallization No. 4, 50 gram lard samples were used. In fractional crystallization No. 5, 25 gram lard samples were used.

[b]The same size Buchner funnels were used in both the 50 gram and 25 gram lard sample fractionations. The same size funnels were used for the lard R sample as were used for the lard A sample.

APPENDIX B: DRY ACETONE

The acetone used as solvent in the fractional crystallizations was a commercial bulk grade acetone obtained by the Iowa State University Chemical Stores in 50 gallon drums. This acetone was dried over 8 mesh calcium chloride by refluxing 1600 ml of acetone with 50 grams of calcium chloride for 1-2 hours and distilling the acetone from the calcium chloride. Two-liter Erlenmeyer flasks were fitted with laboratory Liebig condensers and cork stoppers were used for making connections. A water bath was constructed which could handle six such flasks and condensers at one time. The bath was held off the floor by legs long enough to allow the reflux condensers to be removed from the rack above the water bath and used to condense the evaporated acetone and drain it into one gallon bottles sitting on the floor. The water bath was heated by passing 80-90 psig steam through a 1/4 in OD copper tube immersed in the water. The flow of steam was controlled by a solenoid operated valve and the solenoid was actuated by the opening and closing of a Fenwal thermoswitch connected in series with the solenoid and a 110 volt alternating current source. A second copper coil in the bath was used to rapidly cool the water bath in case refluxing became too vigorous by passing cooling water through the copper tubing. The dried acetone was stored in tightly capped one gallon bottles.

APPENDIX C: GAS CHROMATOGRAPHY

The major components of a gas chromatograph are the column and its oven, a detector to detect the separated sample components as they emerge from the column and an automatic recording device to produce a record of the variations in the signal produced in the detector. Also needed are a carrier gas supply for eluting the column and a device for introducing a vapor sample into the carrier gas just before the gas enters the column.

All of the gas chromatographs used in this work employed thermal conductivity cell detectors (TC cell). This type of detector requires a constant direct current source. The TC cell consists of a Wheatstone resistance bridge and is connected to the direct current source as shown in Figure 29. The TC cell used in the construction of the chromatograph was a Gow-Mac model TR-II-B, geometry 9193 thermal conductivity cell.

The power for the above TC cell was supplied by lead storage batteries. The resistances of the hot wire filaments, the bridge trimmer, and the current control required that a 12-volt source be used in order to obtain a bridge current of about 200 milliamperes. The batteries were charged after each few hours of use to maintain them at full charge to pre-

vent baseline drift due to slowly decreasing battery voltage. A six ampere-12 volt battery charger was used.

The current control and bridge trimmer for the TC cell were modified somewhat from what is shown in Figure 29 in the later stages of the work. The current control resistance consisted of a 30 ohm resistor and a 2 ohm resistor connected in series to allow easier adjustment of the TC cell current. The bridge trimmer (zero control) was left as a 2 ohm resistor, but a resistor was put in series with one of the lower resistance hot wire filaments to aid in balancing the bridge currents.

The carrier gas used was helium and the flowrate was controlled by a Matheson two stage regulator, model number 8. The flowrate was measured by means of a soap bubble flowmeter attached to the exit lines from the sample and reference gas lines of the TC cell.

In the early stages of the work with the gas chromatograph a Bristol model IPH-570 Dynamaster recording potentiometer was used. It had a minimum range of 5 millivolts for full scale deflection (0-5 millivolt range) to a maximum range of 50 millivolts for full scale deflection (0-50 millivolt range). It was desired to use a lower range than 0-5 millivolts for some of the methyl ester analyses. An unknown range of be-

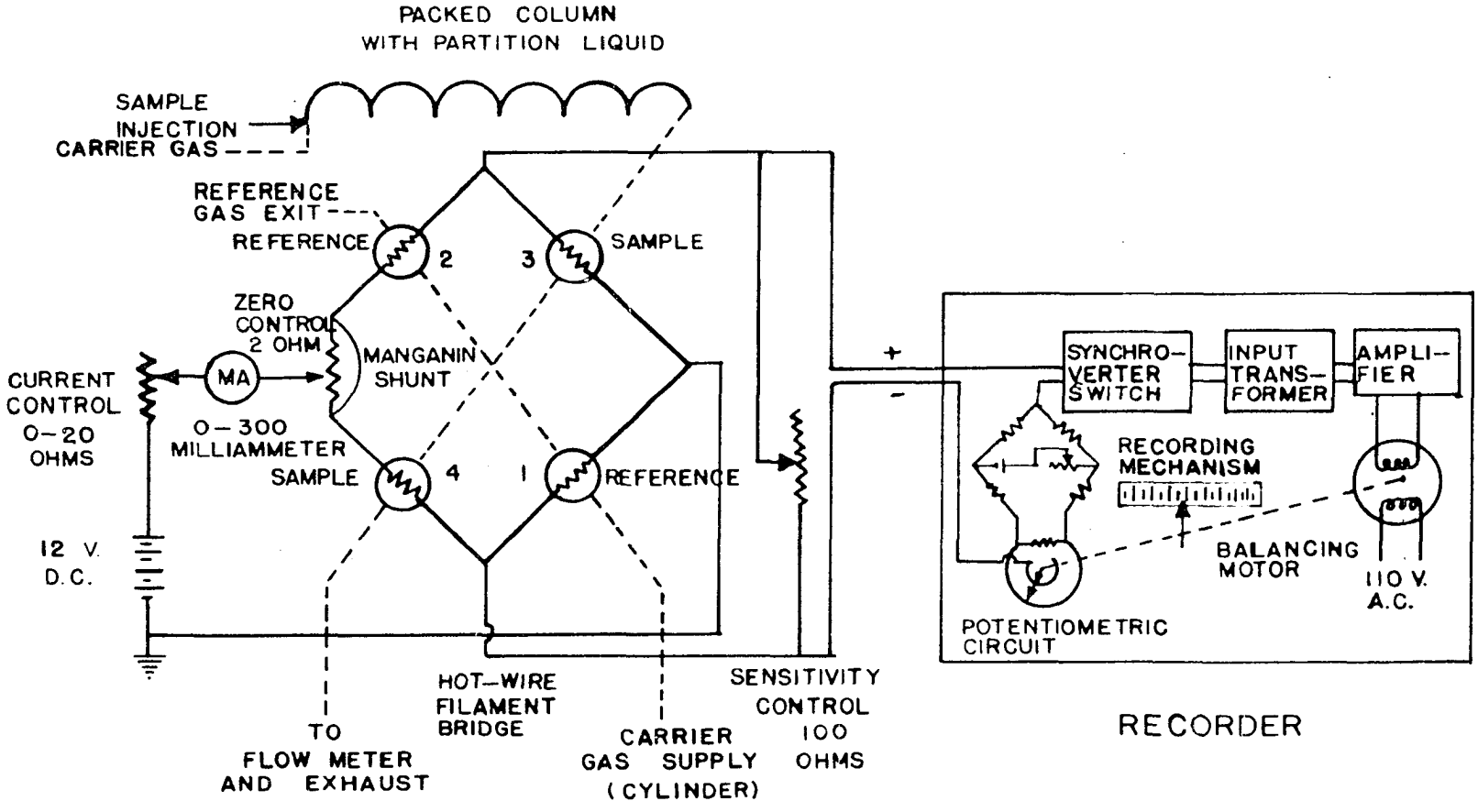


FIG. 29. SCHEMATIC OF GAS CHROMATOGRAPH OVERALL WIRING DIAGRAM AND FLOW OF CARRIER GAS

tween 0-1 and 0-3 millivolts was obtained by shorting out the resistance M in Figure 30. This recorder also had an automatic standardization mechanism which standardized the working cells against a standard cell every 15 minutes. The duration of the standardization was 20 seconds which often occurred when the trace was forming a peak. The automatic standardization was made inoperative by holding up a lever which rode on the edge of a rotating disc. Standardization took place when this lever dropped into a notch in the disc which caused the contact points of a relay to close.

For the methyl ester samples run for fractional crystallizations No. 4 and No. 5, a Sargent SR recording potentiometer was used. This instrument had range plugs of 1.0, 2.5, and 5.0 millivolts.

The column oven used for the crystallization No. 3 sample analyses consisted of a double walled metal box of 5 in x 5 in x 5 in inside dimensions and was attached to one end of the TC cell. The double walls were 1-1/2 in apart and were filled with glass wool insulation. The oven was heated by means of number 24 gage B and S, nichrome resistance wire wrapped on a 4-1/2 in length of iron pipe attached in a horizontal position on the inside wall opposite the TC cell. Two layers of glass cloth tape were used to insulate the heating wire from the iron pipe. Current to the oven was controlled

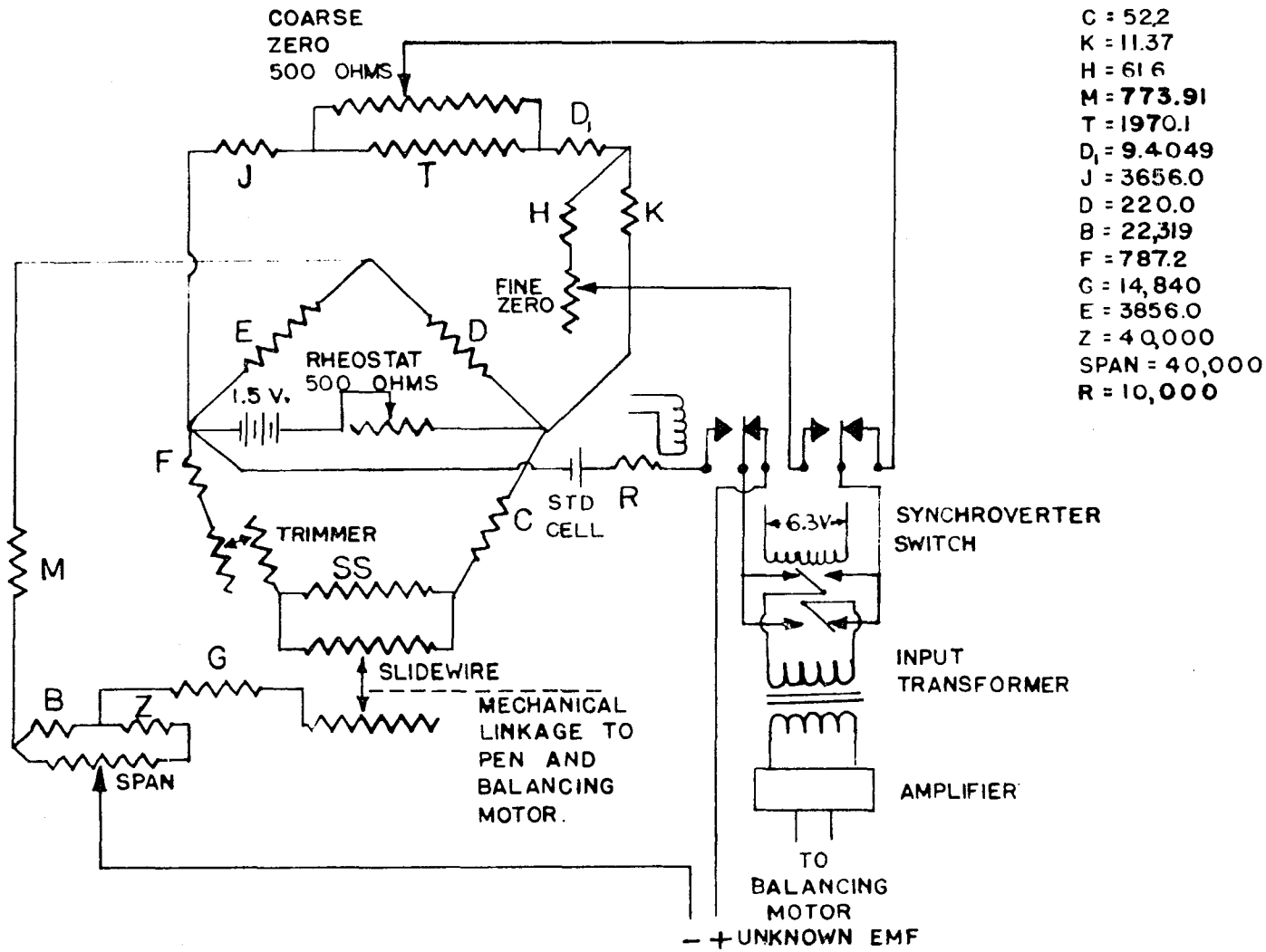
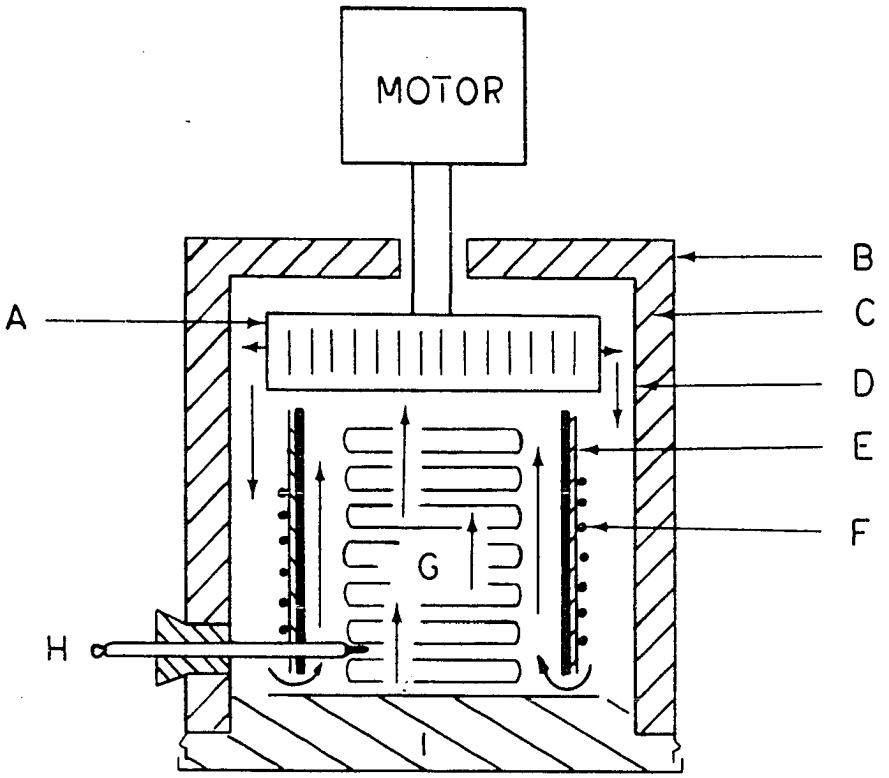


FIG. 30. POTENTIOMETRIC CIRCUIT OF THE BRISTOL MODEL 1PH—570 DYNAMASTER RECORDER

by use of a variable transformer. The temperature was measured by use of a thermometer. The oven had a removable top for access to the column. The coiled column was placed in the oven with the heater in the center of the coil.

A metal box 4 in x 4 in x 2-1/2 in was mounted on the outside wall of the oven opposite the TC cell and contained a short piece of pipe wrapped with glass cloth tape and resistance wire to form the sample injection tube heater. The injection tube extended through the heater pipe and the wall of the metal box. Glass wool insulation was packed around the heater. The end of the injection tube had one end of a threaded brass fitting brazed to the tubing. A small metal disc with a small hole in its center and a silicone rubber disc were held in place by a tubing brass fitting nut. Samples were injected into the gas stream by means of a syringe needle pushed through the silicone rubber disc. The injection tube temperature was measured by use of an iron-constantan thermocouple, a manual potentiometer, and a millivolt-temperature conversion table.

Results using this oven and injection system were not as good as published chromatograms using similar columns. Another column oven and injection system were constructed. These are represented in Figures 31 and 32. The dimensions of the column oven are not given but would accommodate columns of 1/4 in



A BLOWER

B OUTER WALL

C GLASS WOOL INSULATION

D INNER WALL

E HEATING WIRE SUPPORT

F INSULATED NICHROME RESISTANCE WIRE

G COLUMN

H THERMOMETER

I BASE

FIG. 31. CHROMATOGRAPH COLUMN OVEN

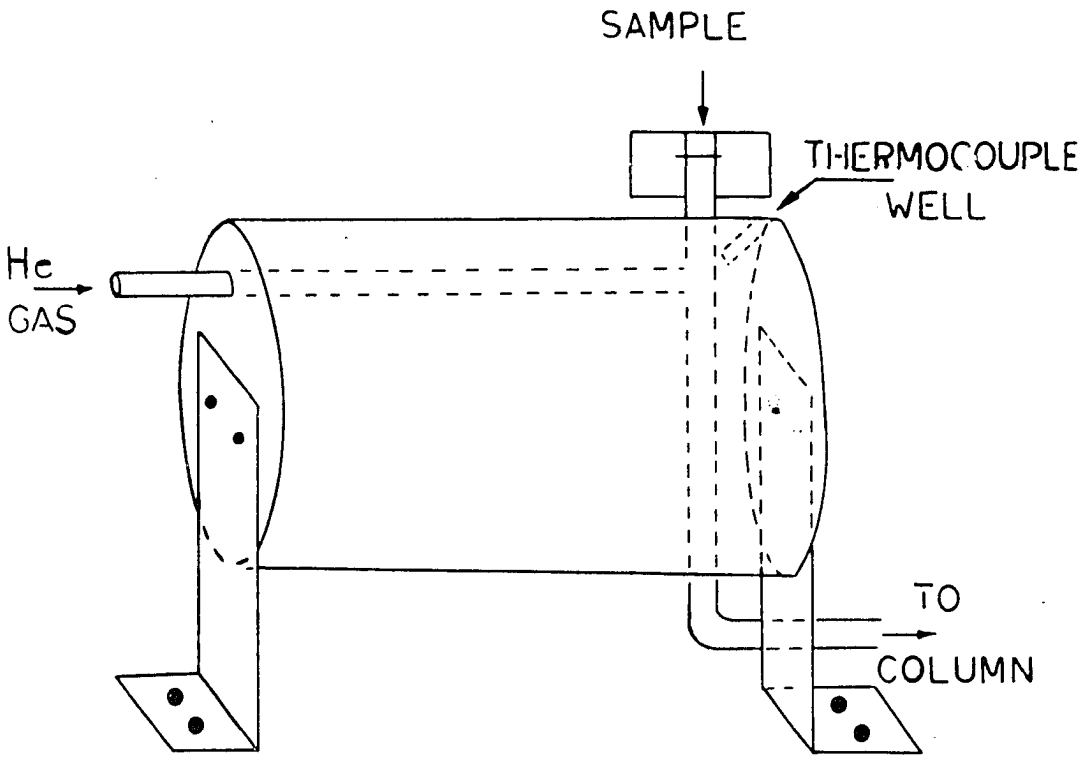


FIG. 32. SAMPLE INJECTION BLOCK

OD copper tubing which had been coiled on a 4 in OD mandrel. The column oven was first constructed with the resistance wire heater wrapped on pipe mounted in the center of the oven base and there was no blower in the oven. Later, better temperature control was desired and the oven was modified by the addition of the blower fan and the pipe heater was replaced by resistance wire wrapped around the 5 in diameter metal cylinder shown in Figure 31. Access to the column in the oven was possible by removal as a single unit the cylinders making up the oven walls and the motor mounted on top leaving only the base and column in place.

The injection block was made from a 3 in length of 2 in diameter solid brass. The gas passages were drilled as shown in Figure 32. The end of a threaded brass fitting silver soldered on top of the block and fitted with a metal disc with a hole in the center and a silicone rubber disc held in place by a brass fitting nut (for tubing fittings) permitted sample injection into the gas stream. A single turn of copper tubing was silver soldered to the brass nut and water was passed through the tubing to cool the silicone rubber disc. The brass block was electrically insulated by wrapping with two layers of glass cloth tape and then wrapped with nichrome resistance wire for heating the block. The block was mounted in a 5 in diameter x 4 in high cylindrical container and glass wool insulation was placed around the block.

A voltage regulator (Sorenson model 175, serial number 177, ac-voltage regulator, ± 0.2% rms regulation) was later used to help maintain a constant temperature by controlling the current input into the column oven heater. This worked quite well when there were no ambient air temperature and circulation changes.

The voltage to the column oven heater was controlled by the use of a variable transformer. The variable transformer was plugged into the voltage regulator.

The copper tubing lines between the sample injection block and the column, between the column and the TC cell, and the TC cell helium-sample gas exit line were insulated with two layers of glass cloth tape. These lines were then wrapped with nichrome resistance wire and this wire covered with about 1/2 in of glass wool insulation. The three line heaters were connected in series and their current controlled by the use of a variable transformer.

The current to the sample injection block heater was also controlled by a variable transformer. The transformer settings for the line heaters and the injection block were determined by using a thermocouple to measure temperatures at different settings.

Columns were prepared by packing copper tubing with

liquid substrates on inert supports. The packing material was poured into the tubing through a small funnel while vibrating the tubing with a laboratory vibrating tool or by holding the tubing against a short piece of flattened tubing turned rapidly by a laboratory stirrer motor. When the tubing was filled, it was tapped on the floor while being vibrated to aid in settling the packing until the desired degree of packing was obtained. The ends of the tubing were plugged with wads of glass wool to hold the packing in place.

Johns-Manville C-22 firebrick which had been crushed and screened to the desired size range was acid washed by treating with approximately 20% aqueous hydrochloric acid followed by washing with distilled water until the wash water was neutral to blue litmus paper. The firebrick was then dried by spreading into a thin layer and left overnight in an oven at 110°C. Aqua regia treated (ART) firebrick was suggested as a better support for some types of separation by Zlatkis et al. (101). It was prepared by covering firebrick with aqua regia (three parts hydrochloric acid-one part nitric acid) and allowed to react for one hour. Stirring during this time was necessary to allow the gas formed to escape from the slurry. The aqua regia was decanted from the packing, the packing washed several times with distilled water, and then washed with dilute sodium hydroxide solution until the wash solution was neutral to litmus paper. The firebrick was then washed several more

times with water, spread out in a thin layer and dried overnight in an oven at 110°C.

Dow Corning hi-vacuum silicone grease (DCSG) was prepared for use as a liquid substrate by suspending the grease in n-butyl acetate. The grease was coagulated by adding ethanol and the solution decanted from the grease. The grease was washed 4-5 times with ethanol to remove the butyl acetate. This grease was spread on ART C-22 firebrick by suspending a weighed amount of DCSG in toluene and then adding the desired weight of firebrick. The mixture was then stirred on a hot plate in a hood to remove the toluene.

Butanediol succinate (BDS or Craig polyester) was purchased from Wilkens Instrument and Research, Inc., P.O. Box 313, Walnut Creek, Calif. It was put on the supports by dissolving a weighed quantity in chloroform, adding solid support and the solvent evaporated while stirring until all of the solvent had been removed.

The methods available for obtaining the areas under the individual chromatogram peaks were by measurement with a planimeter or by drawing tangents through the points of inflection of the peak slopes and obtaining the product of the height and the width of the triangle formed by these two lines and the base line. Also checked was the product of the retention time times the height of the triangle constructed as

above. Percentages of each component were calculated from measurements by the three methods for four chromatograms of the same sample. The average of the result for the four chromatograms were found for each method and are listed in Table 75.

Table 75. Comparison of chromatograph peak measurement methods

Peak	Planimeter	Peak ht. x base width	Peak ht. x ret. time
Myristate	1.8	1.8	1.6
Palmitate	29.9	29.9	29.3
Palmitoleate	2.9	2.9	2.8
Stearate	13.0	13.3	13.6
Oleate	46.9	46.2	46.2
Linoleate	5.6	6.1	6.5

The three methods gave results which agreed very well. The planimeter method is by far the most tedious and therefore was not used. The method involving the retention time was also eliminated because it was more subject to errors such as slipping of the recorder paper or changes in temperature of the column during the running of a sample. The triangulation method was therefore used for measuring peak areas in this research. The F and M model 500 chromatograph used a disc integrator which should be expected to give the same results as the planimeter method although this comparison was not made.

On another series of eight samples, the area percentages

were measured by triangulation. The average component percentages were calculated and the individual determinations subtracted from the average value obtained. The deviations from the average value were then averaged irrespective of sign. The results of these measurements and calculations were myristate 1.75 ± 0.13, palmitate 26.40 ± 0.43, palmitoleate 3.05 ± 0.23, stearate 12.25 ± 0.53, oleate 46.85 ± 0.68, and linoleate 9.80 ± 0.35.

A sample containing 20% of each of five methyl esters shown in Table 76 was analyzed on a 5 ft x 1/4 in OD copper tubing column of 20% ethylene glycol succinate on 60/80 mesh acid washed Chromosorb. The sample was obtained from the Hormel Institute, Austin, Minnesota. The chromatograms showed only minute traces of oxidation product methyl esters present in the samples but this did not prove that the sample had not undergone oxidation as it was not methylated again before obtaining the chromatograms. The results of three chromatograms are given in Table 76 with the ratio of the known weight percentages to the chromatogram area percentages. These ratios were also calculated for data from the literature and are given for comparison. These figures showed that the results obtained on different chromatographs and columns show similar trends but do not give the exact same results. For precise work, then, calibration samples should be chromatographed.

Table 76. Relative mass response of monocarboxylic acids

	Chromatogram area percent				Known comp. wt. %	Known exper.	Ratio of known to exp. calc. from data in lit. Horrocks et al. (41)[a]	Craig+ Murty (19)	Herb et al. (33)
	1	2	3	Av.					
Pal.	22.6	22.6	22.5	22.6	20.0	0.885	0.885	0.882	0.855
Stear.	22.0	21.4	21.2	21.5	20.0	0.930	0.944	1.020	0.914
Oleic	22.3	22.2	22.6	22.4	20.0	0.893	1.004	0.955	0.979
Linol.	17.1	17.3	17.1	17.2	20.0	1.163	0.973	1.002	1.064
Lin	16.1	16.5	16.6	16.4	20.0	1.220	1.043	1.146	1.186

[a]The relative mass response values were divided into 100 and these numbers multiplied by 0.885 to put the ratios on the same basis as those calculated from the experimental data and known composition presented in the table.

Before this known composition sample had been obtained, comparison of the relative response of the mono- and dicarboxylic acids was made on a 5 ft x 1/4 in OD copper tubing column of 20% ethylene glycol succinate on 60/80 mesh acid washed Chromosorb. The monocarboxylic acids used were obtained by saponifying lard, recovering the fatty acids and methylating the recovered fatty acids. Samples of dicarboxylic acids of 95-98% purity were obtained and used to prepare a dicarboxylic acid methyl ester sample of unknown composition. A sample consisting of a mixture of the mono- and diacids was prepared by weighing portions of the above two mixtures. The monoacid-diacid mixture consisted of 38.1 milligrams of the monoacid sample and 44.6 milligrams of the diacid sample which corresponded to 46.07 weight percent

monoacids and 53.93 weight percent diacids. Chromatogram area percentages for these samples are given in Table 77.

Table 77. Change in response of mono- and diacids when mixed

	Chromatogram area %			Calcd. area % for mixture	Chrom. area % / Calcd. area %
	Mono-acid sample[a]	Diacid sample[b]	Mixture[c]		
DiC_2		5.17	2.33	2.38	0.979
DiC_4		18.56	9.84	8.54	1.152
DiC_5		6.31	3.55	2.91	1.220
Myr.	1.83		0.75	0.99	0.7576
DiC_6		20.48	12.43	9.44	1.317
DiC_7		10.94	6.57	5.04	1.304
Pal.	27.76		11.93	14.97	0.7969
P.O.	3.23				
DiC_8		12.13			
PO+DiC_8			8.70	7.33	1.187
DiC_9		17.68	10.89	8.15	1.336
Stear.	12.96		5.71	6.99	0.8169
Oleic	47.39				
DiC_{10}		7.62			
Oleic+DiC_{10}			24.19	29.07	0.8321
Linol.	6.83				
DiC_{11}[d]		1.12		4.20	
Linol+DiC_{11}			3.12	4.20	0.7429

[a]Average of 4 chromatograms.

[b]Average of 3 chromatograms.

[c]Average of 4 chromatograms.

[d]This component was an impurity in the DiC_{10} sample and had a retention time corresponding to that of DiC_{11}.

The expected area percentage of each peak of the mixture was calculated from the weight of the monoacid and of the diacid samples mixed and the chromatogram area percentages obtained for these two samples. The calculated area percentages

were compared with the actual areas measured for the mixture by calculating their ratio. The fact that the calculated and measured area percentages were different for each monoacid and for each diacid showed that the relative response of a component varied depending upon what other components were present in the sample. It is not known whether or not the relative response of a component would vary with its percentage in the mixture nor whether or not it would vary as the composition of some other component was varied. The ratio of the measured areas of the mixture to the calculated areas showed that the relative area of the monoacids was decreased and the areas of shorter chains were decreased more than the longer chains. The relative area of all the diacids except DiC_2 were increased and the increase was greater for the longer chains.

The results of the mono- and diacid sample mixture indicated that the areas obtained for the diacids were greater than the areas of the monoacids when compared on a weight basis. The paper of Youngs (98) indicated that the diacid peak areas had to be multiplied by a factor of 1.10 to obtain area percentages equal to the weight percentages of the components present in the sample. This is exactly the opposite of what the data of Table 77 indicated. This difference could be accounted for if the lard fatty acid methyl ester

sample contained a considerable amount of non-fatty acid material or if the fatty acids were not completely methylated.

Assume that the monoacid sample was not pure methyl esters. Then the ratio of the mixture chromatogram areas to the calculated areas would be expected to be less than 1.0 as was found to be true (range of 0.75 to 0.82). By multiplying the diacid ratios by the ratio for the nearest monoacid peak would give values for the diacid ratios based upon monoacid ratios of 1.0. That is:

$$1.2200 \times 0.7576 = 0.924 \ (DiC_5\text{-myristate})$$
$$1.3167 \times 0.7576 = 0.998 \ (DiC_6\text{-myristate})$$
$$1.3036 \times 0.7969 = 1.039 \ (DiC_7\text{-palmitate})$$
$$1.3362 \times 0.8169 = 1.092 \ (DiC_9\text{-stearate})$$

A graph of these four sets of values was prepared, adjusted ratio versus number of carbons in the diacid and values read for each of the diacids. These values were found to be:

DiC_5	0.924
DiC_6	0.998
DiC_7	1.039
DiC_8	1.067
DiC_9	1.092
DiC_{10}	1.112
DiC_{11}	1.127
DiC_{12}	1.135

These adjusted ratios indicate how much larger the areas obtained for the diacids were than obtained for an equal weight of a monoacid which appeared close to the diacid peak on the chromatogram.

Mixtures of a palmitic acid methyl ester sample and a

DiC_9 methyl ester sample were prepared. The palmitate sample contained impurities of laurate and myristate. The total area percentage of the monocarboxylic acids was measured and compared with the area percentage of the DiC_9 peak. These results are given in Table 78. These results again indicated that the area of the diacids was greater than for the monoacids on a weight basis. The data also indicated that the difference may change with composition. More work needs to be done to check these results. Pure methyl ester samples should be used. The purity of the samples used in this work was not known.

Table 78. Comparison of area percentages and known weight compositions

Sample:	1		2		3	
	Chrom. area %	Weighed	Chrom. area %	Weighed	Chrom. area %	Weighed
Monoacids	71.98	72.30	41.19	41.61	70.93	73.87
DiC_9	28.02	27.70	58.81	58.39	29.05	26.13
Number of chromatograms	3		3		1	
Correction factor-mult. x DiC_9 area	0.984		0.983		0.864	

Figure 33 is a graph of the logarithm of the retention time of the mono- and dicarboxylic acids versus the number of carbons in the chain. The saturated monocarboxylic acids lie on a straight line. Only two monounsaturated monocarboxylic

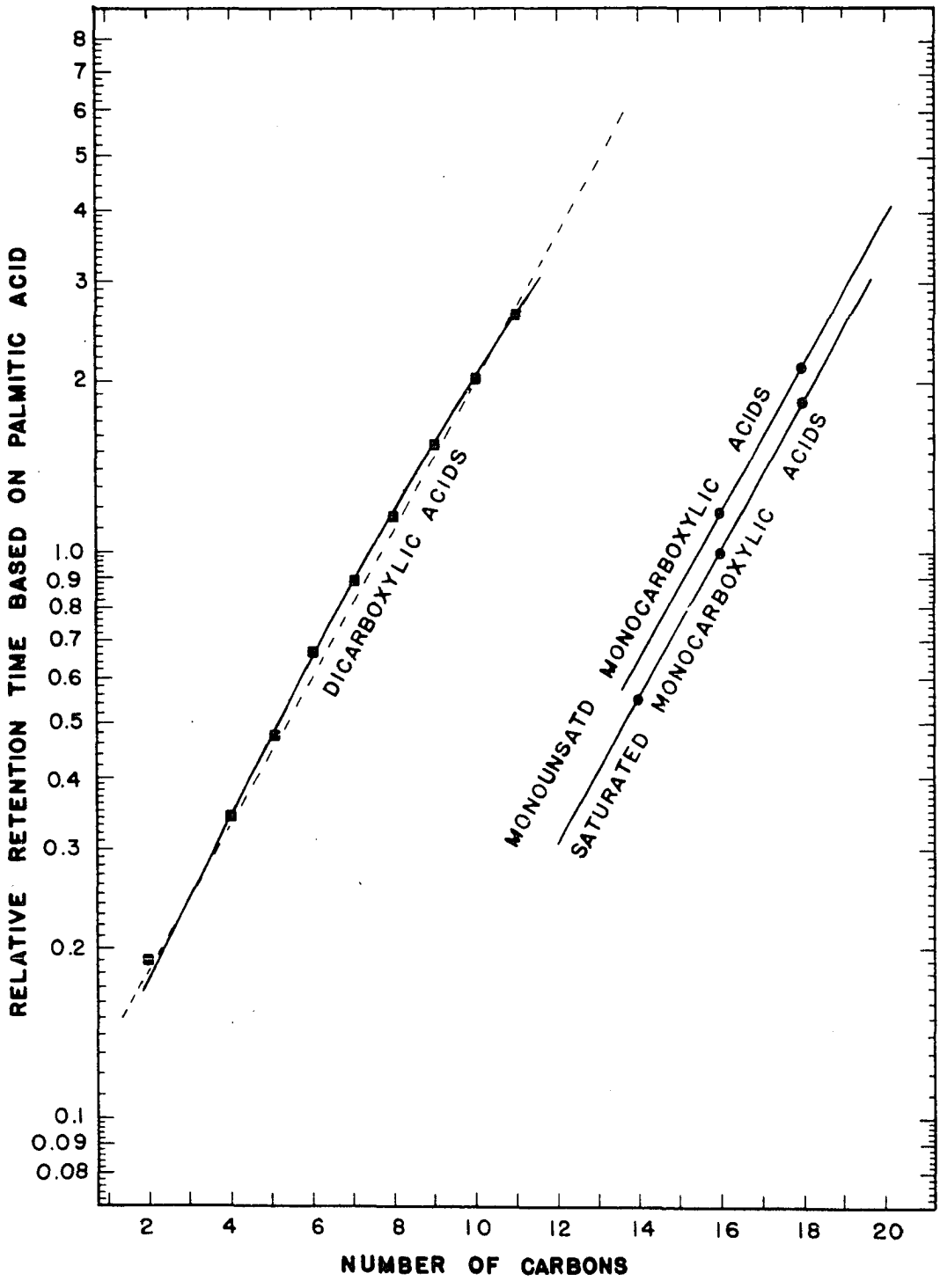


FIG. 33. RELATIVE RETENTION TIME VERSUS NUMBER OF CARBONS

acids are plotted but they lie on a line parallel to the saturated acids. The dicarboxylic acids lie on a curve above the monocarboxylic acid curves. The dotted line through DiC_9 is parallel to the saturated monocarboxylic acid line.

APPENDIX D: RESIN ACTIVATION

The resin used in this work was the anion exchange resin Amberlite IRA-400. Amberlite is a tradename of the Rohm and Haas Company, Philadelphia 5, Penn. This resin is a strongly basic, quanternary ammonium (polystyrene) type and was obtained in the chloride form. It was necessary to convert the resin to the hydroxide form before adsorption of fatty acids would take place. The chloride form of the resin was very stable even after several months storage in a tightly closed container but the hydroxide form prepared as described below was quite unstable. After standing in a closed container for several days, the hydroxide form of the resin smelled very strongly of ammonia. The fatty acid adsorption capacity of the hydroxide form decreased after standing just a few hours. The hydroxide form which had lost much of its capacity to adsorb fatty acids could be recovered by treating the resin in the same manner as in the conversion of the chloride form to the hydroxide form.

The chloride form of the resin was converted to the hydroxide form by treating the resin with an aqueous solution of sodium or potassium hydroxide. Solutions as strong as 15% by weight hydroxide were used but this strength hydroxide solution caused the resin to turn dark brown and the resin

appeared to break into finer particles more easily when used in columns or stirred in beakers with organic solvent solutions. It was found that treatment of the resin with 1 N sodium hydroxide (4%) with occasional stirring for 10 minutes gave satisfactory conversion without discoloration of the resin. The amount of hydroxide solution used was enough to give a liquid level one-half again as high as the resin in the container.

After the conversion to the hydroxide form had taken place, the hydroxide solution was decanted from the resin and the resin washed with distilled water until the wash water was neutral to red litmus paper. Water was partially removed from the resin by washing with an organic solvent which was miscible with water. Acetone or methyl alcohol were normally used. The amount of organic solvent used was enough to cover the resin in the container. The number of washes used depended upon how much water was desired to be removed from the resin. Two or three washes removed the free water in the resin. The acetone or alcohol were then washed from the resin by using the same solvent to be used to dissolve the fatty acids to be adsorbed on the resin. Ethyl ether was used in this work and was used to wash the resin three times. The resin was then spread on paper towels to allow the excess ether to evaporate. The resin was not recovered after it had been used once.

If a procedure for treating 10 grams of wet resin giving specific volumes of each solution is desired, see the details of the method followed by Hornstein *et al.* (40) given in Appendix F.

APPENDIX E: HYDROLYSIS PROCEDURES

The hydrolysis procedure followed by Mattson and Beck (54) was used in parts of the work in this thesis and is given as described by them. "The digestion conditions used in these studies were essentially those described by Desnuelle et al. (24), except that the buffer concentration was increased to 1.0 M. Unless otherwise indicated, the digestion mixture consisted of 13.6 ml of 1.0 M ammonium chloride-ammonium hydroxide buffer adjusted to pH 8.0, 0.3 ml of a 0.15 percent aqueous solution of bile salts (Difco), 1.0 ml of a 45 percent aqueous calcium chloride solution, 2.3 ml of an aqueous suspension of 156 mg of steapsin (Difico), and 2.0 gm of triglyceride. In all of the studies but one, 'winterized' cottonseed oil was the substrate used. During the digestion period (15 minutes) at 40 ± 0.5°C, the mixture was continuously agitated and a pH of 8.0 was maintained by the addition of 4 M ammonium hydroxide."

"At the end of the digestion period the pH was adjusted to 2 with N hydrochloric acid, 25 ml of ethyl alcohol were added, and the lipides were extracted with ethyl ether. The ethyl ether solution was washed with water and the lipides were treated in the same manner as in the in vivo studies."

In a later paper, Mattson and Beck (55) changed the conditions above slightly. "With two exceptions the digestion

conditions used in these studies were the same as those employed in the previous study (54). Since the earlier results had demonstrated that bile salts have little effect on the hydrolysis of triglycerides, bile salts were not added to the digest in the present studies. When 2-oleoyl distearin was used as the substrate, the temperature of the digest was increased to 45°C, since the melting point of this triglyceride is 43°C."

The description of the hydrolysis as given by Desnuelle et al. (24) is "1.2 grams of triolein are placed in a ground glass fitted tube with 6.5 ml of 0.1 M phosphate buffer, pH = 7.0, 7.8 mg of pancreatin (Byla) in suspension in 1.3 ml of water and 0.15 ml of a 10% solution of bile salts (Hoffman-la-Roche). The tube was supported in a 37°C bath and during the first hour was agitated frequently. After 18 hours contact, lipolysis was arrested by adding 1.3 ml of glacial acetic acid. The final pH of the hydrolysis mixture (before adding the acid to stop the hydrolysis) was 6.9. In cases in which calcium ions are used, the phosphate buffer can not be used and therefore a buffer of 0.1 M ammonium chloride-ammonium hydroxide at a pH = 8.0 was used and the pH maintained at 8.0 by the addition of 4 M ammonium hydroxide." Desnuelle et al. (22) correct an error in this procedure. The amount of pancreatin should be 78 milligrams, not 7.8 milligrams.

In a more recent paper Savary and Desnuelle (82) give the

following description of the hydrolysis. 'The glycerides POP, OPP, and POO have been treated by the enzymatic system of pancrease by 2 different methods. In the experiments 'without calcium' 1.2 grams of glyceride was hydrolyzed at 37°C in a small (ground glass) tube by pork pancreatin (78 mg in 1.3 ml of water) in the presence of 0.1 M phosphate buffer at pH = 7.0 (6.5 ml) and of bile salts of beef (0.15 ml of a 10% solution). In the experiments 'with calcium', the calcium ions (sensibly 1/2 atom per liberable chain) is applied by 1 ml of a solution of 24% calcium chloride, the phosphate is replaced by a buffer of 0.02 M ammonium chloride-ammonium hydroxide of pH = 8.0 and the pH is maintained constant by addition of 4 M ammonium hydroxide. After a vigorous agitation during the first minutes, the tube is agitated only occasionally during the rest of the test."

Borgstom (11) described the following hydrolysis method. "The pH of 80 ml of bile-pancreatic juice (from rats) was adjusted to pH = 6.3 by addition of 16 ml of 0.1 N hydrochloric acid. Six ml of this mixture were pipetted into Pyrex test tubes 16 x 150 mm and about 150 mg of the respective fat mixtures were added to eight tubes in each series. The tubes were immediately closed and placed in a water bath of 40°C equipped with arrangements for rotating the tubes with a speed of about 40 rpm. After 1/2, 1, 2, 3, 4, 6, 9, and 12 hours

one tube from each series was removed and analyzed for composition and isotope content as described above. The pH was measured in each tube after the end of the respective incubation times and was found to correspond well to the initial pH, the limits were 6.2-6.4."

The hydrolysis procedure followed by Youngs (98) is given below. "The proportions of the positional isomers in the fractions were determined by a specific hydrolysis of the 1- and 3-positions of the glycerides using pancreatic lipase. The lipase used was a commercial steapsin which had been hot extracted for six hours with Skellysolve F to remove residual fat. Ten to 20 mgs of fat, or of oxidized fat which had been methylated with diazomethane, were placed in the 'transition' flask of a VirTis 45 homogenizer, high speed, top entering blender manufactured by the VirTis Company, Gardiner, New York. Three ml of 0.5 M K_2HPO_4 buffered to pH = 8 with 0.5 M NaH_2PO_4 were added to the fat along with 5 mgs of bile salts. If necessary the flask was warmed until the fat sample was liquid and the homogenizer then run at high speed to give a stable emulsion. A weight of lipase equal to 40% of the weight of the fat was added and hydrolysis allowed to proceed for 30 minutes with the homogenizer running at low speed. At the end of the 30 minutes the reaction mixture was added to 3 ml of 1 N hydrochloric acid, the resulting solution saturated with sodium chloride and the total lipids recovered by 4 extractions

with ethyl ether. The ether was evaporated in a stream of air, the residue treated with an excess of diazomethane in ether and after again evaporating the ether and excess diazomethane a sample was injected directly into the gas chromatographic unit. Under the condition used only the hydrolysed fatty acids, converted to their methyl esters by the diazomethane, came through the column. The mono-, di-, and triglycerides in the sample remained on the column without any noticeable effect on the operation of the column even after a comparatively large number of samples had been run.

Comparison of peak areas for a given sample size of the hydrolyzed material with the areas for a similar sample size of pure fatty acids methyl esters showed that there was 30 to 40% hydrolysis under the conditions used."

Table 79 gives a summary of the hydrolysis conditions used by several different workers in their investigations. These conditions were studied to determine the "proposed conditions" which best suited the needs of this problem.

Table 79. Summary of hydrolysis conditions

	Glycerol generator	Mono-glyc. generator	Di-glyc. generator	Borg-strom	Mattson Beck	Youngs	Proposed condition
Reference:	(23)	(22)	(24)	(11)	(54)	(98)	--
Gms of fat	2.4	1.2	1.2	0.150	2.0	0.020	
Ml of soln.	125.8	7.95	7.95	6	17.2	3	
Buffer soln.molarity	0.02	0.10	0.10		1.0	0.5	2
Gms bile salts	1.8	0.015	0.015		0.00045	0.005	None
pH	8.0	8.0	7.0	6.3	8.0	8.0	8.0
Mg of pancreatin	360	78	78		156	8	
Temp. ^{o}C	40	37	37	40	40	Room temp.	40
$\frac{\text{Gm. steapsin}}{\text{Gm. fat}}$, %	15.0	6.5	6.5		7.8	40.0	10.0
$\frac{\text{Gm. fat}}{\text{Ml. aq. soln.}}$, %	1.91	15.1	15.1	2.5	16.3	0.667	10.0
$\frac{\text{Gm. steapsin}}{\text{Ml. aq. soln.}}$, %	0.286	0.982	0.982		0.907	0.266	1.0
$\frac{\text{Gm. bile salt}}{\text{Ml. aq. soln.}}$, %	1.43	0.189	0.262		0.262	0.167	None
$\frac{\text{Gm. } Ca^{++}}{\text{Ml. aq. soln.}}$, %	0.382	6.04	None	(8.5 mg %) 0.85	2.62	None	0.635

APPENDIX F: PREPARATION OF FATTY ACID METHYL ESTERS

The method used by Hornstein et al. (40) is quoted below.

"Pretreatment of Resin. Ten grams of Amberlite IRA-400 are stirred with 25 ml of 1 N sodium hydroxide for 5 minutes. The resin is allowed to settle and the supernatant liquid is discarded. The resin is successively washed with several portions of distilled water to remove free alkali, then with three 25 ml portions of anhydrous ethyl alcohol to remove water, and finally with three 25 ml portions of petroleum ether to displace the ethyl alcohol.

"Separation of Fatty Acids from Fat. If fat....For un-emulsified fat samples, 0.1 to 1.0 gram is dissolved directly in petroleum ether.

In either case 10 to 25 mg, accurately weighed, of n-heptadecanoic acid are added to the petroleum ether solution, which is decanted onto 10 grams of the pretreated resin in a 250 ml Erlenmeyer flask. Two 5 ml portions of petroleum ether are used to wash the residual solution into the flask. The solution, plus resin, is stirred (magnetic stirrer) for 5 minutes. The resin, plus adsorbed fatty acids, is allowed to settle and the supernatant liquid discarded. The residue is then washed free of fat by stirring with three successive 25 ml portions of petroleum ether and dsscarding the washings.

"Preparation of Methyl Esters. The adsorbed fatty acids are converted to the esters directly on the resin. A 25 ml portion of anhydrous methanol-hydrochloric acid is added to the resin and the mixture stirred for 25 minutes. The methanol solution is decanted through a rapid filter paper into a 250 ml separatory funnel. The resin is washed by stirring for 5 minutes with two successive 15 ml portions of anhydrous methanol-hydrochloric acid, again decanting each wash through the filter. Then 10 ml of distilled water are added to the combined methanol extracts and the solution is extracted with 50 ml portions of petroleum ether. The aqueous phase is drained into a second separatory funnel and extracted twice with 20 ml portions of petroleum ether. The combined petroleum ether extract is washed with 50 ml portions of water until free of acid, dried over anhydrous sodium sulfate, and then concentrated by a stream of dry nitrogen on a steam bath. The fatty acid esters are quantitatively transferred with petroleum ether to a 1 ml volumetric flask; an aliquot is then used for the gas chromatographic separations."

Throughout the procedure as given above, petroleum ether was replaced by ethyl ether. In drying the resin during pretreatment, methyl alcohol was used in place of ethyl alcohol and in recovery of the methyl esters from the methanol-hydrochloric acid solution, 100 ml of water were added instead

of just 10 ml and the extractions were made using ethyl ether. As quantitative determination of the fatty acids was not required here, the addition of a weighed quantity of an acid not present in the free fatty acids was not necessary.

Stoffel et al. (90) described the use of hydrochloric acid-methanol for preparation of fatty acid methyl esters of cholesterol esters, phosphatides, and glycerides. They stressed the need for the absence of water when the methylation takes place by interesterification as is the case with the three types of lipids mentioned. The desired reaction is

$$R_1COOR_2 + CH_3OH \xrightarrow{H^+} R_1COOCH_3 + R_2OH$$

but in the presence of water

$$R_1COOR_2 + H_2O \xrightarrow{H^+} R_1COOH + R_2OH$$

also takes place and gives low yields of methyl esters. Their micromethod is quoted below.

"Interesterification. The esters or acids to be methylated (1 to 10 mg) are dissolved in 4 ml of 5% hydrochloric acid in superdry methanol and 0.5 ml of dry benzene in a 15 ml microsublimation tube to which a condenser with a calcium chloride moisture trap is connected. The mixture is refluxed in a silicone bath at 80 to 100°C for 2 hours, with frequent shaking at the start to dissolve the lipide mixture. After cooling to room temperature, two volumes of water are added,

and the methyl esters are extracted three times with 3 ml of petroleum ether. The pooled extracts are simultaneously neutralized and dried over sodium sulfate-sodium bicarbonate mixture for 1 hour. The esters are then quantitatively transferred with petroleum ether to a second microsublimation tube and the solvent is evaporated to dryness at reduced pressure in a 40°C water bath.

"Microsublimation. After the microsublimation tube is fitted to the cold finger, a vacuum of 0.2 ± 0.15 mm of mercury is produced. The tube is then lowered into a silicone bath at 60 ± 2°C for 60 minutes. The assembly is disconnected after cooling, and the sublimed methyl esters are rinsed off with petroleum ether into a glass-stoppered tube. After evaporation of solvent, the preparation is now ready for application to the gas-liquid chromatography column."

The above method gives pure methyl ester samples but requires considerable time and equipment. Therefore methyl ester preparation of glycerides and of free fatty acids were prepared as described below.

Sample sizes of a few milligrams (20-100) and as large as 1 gram were refluxed with 15-20 ml of hydrochloric acid-methanol solution for 30 minutes in a 25 ml round bottom flask. A small boiling chip was used to prevent superheating of the

solutions. If the samples were not soluble in the methanol solution, a few ml of anhydrous ethyl ether were added to the sample. The reflux condenser was fitted with a calcium chloride drying tube. After refluxing, the alcohol (and ether if added) were partly evaporated and an equal or greater amount of water was added. The methyl esters were then extracted 3–4 times with ethyl ether, the ether extracts washed once or twice with water (wash water checked with blue litmus paper to insure hydrochloric acid was removed) and the ether solution dried over anhydrous granular sulfate. The ether was decanted from the sulfate and the sulfate rinsed three times with ether. The combined ether solutions were then evaporated and the methyl ester residues transferred to 1/2 dram screw top glass vials for storage until gas chromatograms were prepared.

It was found that this procedure could be shortened considerably by eliminating the extraction of the methyl esters with ether. After refluxing, the hydrochloric acid-methanol was evaporated using a vacuum to remove the last traces. Samples of the residue were injected directly into the gas chromatograph. Traces of hydrochloric acid, if present, did not interfere with the methyl ester analyses.

When it was desired to prepare methyl esters of free fatty acids in the presence of glycerides, ethereal diazomethane

was used. This method was also used to methylate the carboxylic acid groups of oxidized lard samples.

The diazomethane solution was prepared by the instructions furnished with diazald (N-methyl-N-nitroso-p-toluenesulfonamide) obtained from the Aldrich Chemical Company, 2369 North 29th Street, Milwaukee 10, Wisconsin.

"Preparation of ethereal-alcoholic solutions of diazomethane: Ethanol (95%, 25 ml) is added to a solution of potassium hydroxide (5 g) in water (8 ml) in a 100 ml distilling flask fitted with dropping-funnel and an efficient condenser set downward for distillation. The condenser is connected to two receiving flasks in series, the second of which contains 20-30 ml ether. The inlet tube of the second receiver dips below the surface of the ether, and both receivers are cooled to $0^{\circ}C$.

"The flask containing the alkali solution is heated in a water-bath to 65°, and a solution of 21.5 g (0.1 mole) of "Diazald" in about 130 ml of ether is added through the dropping funnel in about 25 minutes. The rate of distillation should about equal the rate of addition. When the dropping funnel is empty, another 20 ml of ether is added slowly and the distillation is continued until the distilling ether is colorless. The combined ethereal distillate contains about 3 g of diazomethane."

Milligram quantities of the material to be methylated were treated with enough of the ethereal diazomethane solution to have 300% excess diazomethane present at the start of the reaction. The methylation container was covered (not stoppered) with a piece of paper or a beaker to retard the escape of the diazomethane. A qualitative estimate of the amount of diazomethane in solution could be made by observing the yellow color of the solution. Freshly prepared ethereal diazomethane was straw yellow in color and became lighter yellow as the diazomethane reacted or escaped. A clear colorless ether solution remained when no diazomethane remained in the solution. Under the conditions used here, the solutions were used at room temperature and the yellow color persisted for longer than one hour. After one hour reaction time, the ether and diazomethane were evaporated leaving behind the methyl ester residue and any side reaction products which may have formed. Where desired, 10-15% methyl alcohol may be added to the reaction solution to insure complete reaction of the carboxylic acid groups in a shorter period of time as described by Schlenk and Gellerman (86).

APPENDIX G: PREPARATION OF ANHYDROUS HYDROCHLORIC ACID-METHANOL SOLUTION

The procedure used in this preparation was obtained from Vogel (92). The equipment consisted of common laboratory glassware, rubber stoppers, and Tygon tubing for making glass to glass tubing connections. Vogel presents a diagram of the apparatus if desired and the procedure is presented below.

One-hundred ml of concentrated hydrochloric acid were slowly run into 150 ml of concentrated sulfuric acid. The hydrochloric acid was placed in a small separatory funnel fitted with a 35-40 cm length of glass capillary tubing on the funnel stem. The sulfuric acid was placed in a second separatory funnel and the capillary tubing was extended through a rubber stopper to just a cm or two from the bottom of the sulfuric acid funnel. The hydrochloric acid vapors formed in the sulfuric acid funnel were conducted through a sulfuric acid trap to dry the gas, then through an empty safety trap and finally below the surface of anhydrous methyl alcohol in an Erlenmeyer flask cooled in an ice bath. The flow of hydrochloric acid through the capillary leg was started by raising the end of the capillary tubing out of the sulfuric acid until the hydrochloric acid had forced all of the air out of the capillary tubing. The capillary tubing was then put back in place. The flow of hydrochloric acid was controlled by use

of the stopcock in the hydrochloric acid funnel. Thirty-one to 33 grams of anhydrous hydrochloric acid were produced by this method. The sulfuric acid trap was fitted with a thistle tube extending just below the acid surface to prevent the build-up of pressure in the system. A precaution which is not mentioned by Vogel is that the capillary tube should extend to nearly the bottom of the sulfuric acid funnel to prevent the formation of two layers in this funnel. If two layers were suddenly mixed, a bad accident could result. The strength of the hydrochloric acid-methanol solution can be determined by titrating a sample with standard base or estimated from the increase in weight of the methyl alcohol.