# SOME REACTION CONDITIONS FOR METHYLATION OF TRIGLYCERIDES AND PHOSPHOLIPIDS

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ABSTRACT

SOME REACTION CONDITIONS FOR METHYLATION OF TRIGLYCERIDES AND PHOSPHOLIPIDS

by Barbara Jeanne Zook

Determination of conditions for the most complete methylation of triglycerides and phospholipids by varying the proportions of potassium hydroxide and sulfuric acid and the reaction time in the low temperature-sulfuric acid method were the aims of this study. Neutral lipids methylated included corn, butter and coconut oils, lard and tallow. The phospholipids phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin as well as a mixture of phospholipids, all prepared from egg yolk, and soy phospholipid were also methylated. Portions of each lipid were methylated by the low temperature-sulfuric acid method under differing reaction conditions. Thin layer chromatography was used to judge the completeness of methylation. Gas liquid chromatography was used to ascertain whether differences in treatment resulted in differences in fatty acid composition.

The study showed 1) that when no sulfuric acid was used, methylation of both neutral and phospholipids was complete at all levels of potassium hydroxide. 2) Times of 3, 20

and 40 minutes between addition of methanol and addition of potassium hydroxide did not appear to produce different amounts of methyl esters in triglyceride samples (as judged by thin layer chromatography) while with the phospholipid samples a reaction period of 20 minutes resulted in more methyl esters than a 3 minute period (as judged by thin layer chromatography). 3) Differences in reaction conditions did not affect the apparent fatty acid composition of either triglycerides or phospholipids.

Evidence is given for the proposal that the mechanism is actually base-catalyzed interesterification.

# SOME REACTION CONDITIONS FOR METHYLATION OF TRIGLYCERIDES AND PHOSPHOLIPIDS

By

Barbara Jeanne Zook

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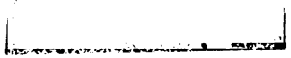

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# INTRODUCTION

Lipids are a heterogeneous group of organic compounds characterized by a variable solubility in organic solvents and insolubility, for the most part, in water. Bloor (1943) included two additional criteria: namely, lipids must be actual or potential esters of fatty acids, and they must be utilizable by living organisms.

Thus, lipids are important compounds biologically where they serve several functions. In the form of simple triglycerides they are a concentrated energy source which can be rapidly mobilized and degraded in response to the cells' demands for energy. They protect delicate body organs from mechanical shock and also help maintain body temperature through their insulating effect. The more complex phospholipids, on the other hand, are found as structural components of nervous tissue and sub-cellular particles and in egg yolk. They may also be involved in transport across cell membranes.

There is a wide distribution of lipids in natural foodstuffs. Animal products are well known for their fat content, but even most fruits and vegetables contain between 0.1 and 1% total lipid. In addition, fats are often used in preparation of foods, as flavoring agents, and are carriers for the fat soluble vitamins.

Since the characteristics of a given lipid are determined largely by the kind and proportion of fatty acids and other hydrolysis products, a means of quantitatively identifying the acids present is important in understanding variations in properties. Most of the currently used methods for characterization involve reaction of the lipid to form methyl esters of the fatty acids and subsequent identification of these with the aid of gas liquid chromatography (GLC).

The purpose of this research was to determine the conditions for the most complete methylation of triglycerides and phospholipids by varying the proportions of potassium hydroxide and sulfuric acid and the reaction times in the low temperature-sulfuric acid method of McGinnis and Dugan (1965). Thin layer chromatography (TLC) was used to judge completeness of reaction and GLC data from samples prepared under the varying conditions were compared.

# REVIEW OF LITERATURE

Although some workers (Dugan et al., 1966; Luddy et al., 1960) have methylated lipids containing biological materials without prior extraction, it is more common to first extract the lipids. The most widely used method for extraction of total lipids has been dexcribed by Folch et al. (1957). A tissue sample was homogenized and extracted with a mixture of chloroform-methanol (2:1, v/v) and then mixed thoroughly with 0.2 volume of water or a salt solution to make the final proportion of chloroform:methanol:water 8:4:3 by volume. The system separated into two phases with the lower phase containing the lipid extract.

The crude lipid extract can be fractionated in several ways. (1) Precipitation of the phospholipids from the lipid extract with acetone (Lea et al., 1955). (2) Column chromatography of the extract on silicic acid (Lea et al., 1955) or Florisil (Carroll, 1963) followed by elution with combinations of solvents to separate the various lipid classes. (3) Application of the crude lipid extract to a preparative thin layer chromatographic (TLC) plate (0.5-1.0 mm thick) and development in a suitable solvent system. The bands, visualized under ultraviolet (UV) light after spraying the plate with 2',7'-dichlorofluorescein, can be scraped from the plates and the components eluted with appropriate solvents.

Until recent years the isolation and identification of the component fatty acids from natural products was a difficult process, especially if the quantitative composition of the fatty acid mixture was desired. The available techniques, often used in combinations, involved separation by (1) distillation, (2) solubility, and (3) adsorption of fatty acids themselves or their esters, salts or halogenated derivatives. Once a fatty acid mixture was resolved into its component acids, it was characterized by (1) determination of one or more physical properties such as melting point, boiling point, refractive index, etc., (2) determination of such chemical properties as the absorption of iodine, and (3) preparation of a characteristic derivative, usually an ester, and determination of the properties of the derivative. Such procedures were time-consuming and tedious.

The separation of polyunsaturated fatty acids (PUFA) was especially difficult. Since the early methods of separating fatty acids were primarily dependent on chemical differences, and since the distinctions between PUFA were not expressed primarily by differences in reactivity, chemical methods were rather non-specific. The differences between the PUFA were expressed more subtly by small differences in physical properties. Analysis methods based on differences in vapor pressure, partition coefficients, absorbabilities, ionization fragmentation, electron resonance and in UV absorption contributed much to the realization of the abundance of fatty acids.

The last technique was particularly useful. Since the most common PUFA contained methylene-interrupted double bonds, they did not show absorption peaks in the usual UV range. Thus the acids were converted to conjugated isomers by heating with alkali (alkali isomerization) and the resulting selective absorption was used as a measure of concentration (Pitt and Morton, 1957).

A new era in lipid chemistry began when James and Martin (1952) described the gas chromatographic separation of fatty acids ranging from $C_1$ to $C_{14}$. Cropper and Heywood (1953) introduced an even more fruitful approach by converting the acids to methyl esters before chromatography. The esters were more volatile than the free acids and did not dimerize. Other workers have refined the technique so that, with the proper choice of operating conditions, the methyl esters of both saturated and unsaturated fatty acids can be analyzed quickly and accurately.

An essential prerequisite for the analysis of lipid mixtures of biological origin by gas liquid chromatography (GLC) was the quantitative formation and isolation of the methyl esters of the constituent fatty acids. Early methods for the preparation of methyl esters used large samples and harsh reaction conditions, but more recently small samples and short reaction times, resulting in little if any modification or loss of sample have been used.

Two general approaches to the formation of methyl esters are commonly used. The most commonly used methods involve

liberation and isolation of fatty acids from lipids by saponification or acid hydrolysis. The esters of the fatty acids may then be prepared by a variety of methods. Acidic reagents such as anhydrous hydrochloric acid in methanol, boron trifluoride in anhydrous methanol, sulfuric acid with methanol at various temperatures, and diazomethane have been used. The methylation of fatty acids on a basic ion-exchange resin with methanolic hydrochloric acid has also been used.

In the second approach the esters are formed directly by interesterification reactions brought about by basic catalysts such as potassium or sodium methoxide and potassium hydroxide.

Stoffel and co-workers (1959) developed a methylation procedure using hydrochloric acid in anhydrous methanol. A sample containing 1-10 mg of acids or esters was dissolved in a mixture of 5% hydrochloric acid in superdry methanol and dry benzene in a microsublimation tube. A condenser fitted with a calcium chloride drying tube was attached and the mixture refluxed at 80-100$^{o}$C for 2 hours. It was then cooled to room temperature and two volumes of water were added. The methyl esters were extracted with petroleum ether. The pooled extracts were dried over a sodium sulfate-sodium bicarbonate mixture for 1 hour, and then after the esters had been quantitatively transferred with petroleum ether to a second microsublimation tube, the solvent was evaporated to

dryness at reduced pressure. Finally the methyl esters were subjected to sublimation.

Metcalfe and Schmitz (1961) developed an esterification procedure using a boron trifluoride-methanol reagent. To prepare the reagent, 1 liter of reagent grade methanol in a 2 liter flask was weighed and cooled in an ice bath. Boron trifluoride was bubbled through a glass tube into the alcohol until 125 g were taken up. Three ml of this reagent were added to a test tube containing 100-200 mg of fatty acids and the solution was boiled on a steam bath for 2 minutes. The esters were extracted with petroleum ether. The total time of preparation was about ten minutes.

A more recent paper from Metcalfe's group (1966) described the extension of their method to the direct formation of methyl esters in triglycerides, phospholipids and other lipids. The rapid saponification procedure of Ast (1963) was used. Four ml of 0.5 N methanolic sodium hydroxide were added to ca. 150 mg of lipid in a 50 ml volumetric flask, and the mixture was heated on a steam bath until the fat globules dissolved. Then 5 ml of the $BF_3$-methanol reagent were added and the mixture boiled for 2 minutes. Sufficient saturated salt solution was added to float the methyl esters up into the narrow neck of the flask where they could be easily withdrawn. With the use of a saturated salt solution, the quantitative recovery of acids down to the $C_4$ range was effected.

Morrison and Smith (1964) found that different reaction times were required for different lipid materials. The method was essentially the same as that of Metcalfe but, in this case, $BF_3$-methanol reagent at a concentration of 140 g $BF_3$/liter methanol was added in the proportion of 1 ml reagent/4-16 mg lipid.

A method for the methylation, on a small scale, of fatty acids with diazomethane was reported by Schlenk and Gellerman (1960). The apparatus consisted of three test tubes with side arms connected in series. A stream of nitrogen, saturated with ether in the first tube, carried the diazomethane, generated in the second tube, into the third tube where the esterification took place. When a tinge of yellow appeared in the third tube, the reaction was discontinued and the slight excess of reagent was removed either in a stream of nitrogen or by adding a dilute solution of acetic acid in ether. Diazomethane is toxic and explosive and must be handled with care.

The procedure developed by Boyle and Ludwig (1962) utilized sulfuric acid. The fatty acids were esterified using a 1% solution of sulfuric acid in methanol and a 2 hour refluxing period. The methyl esters were then extracted from this solution with petroleum ether.

McGinnis and Dugan (1965) also used sulfuric acid for their methylation procedure, but used a low temperature. A 200 mg fat sample in a 125 ml Erlenmeyer flask was dissolved

in 25 ml of peroxide free diethyl ether. The solution was placed in a dry ice-acetone bath, was stirred by a magnetic stirrer, and after the mixture reached a temperature of -60 C, 2 ml of concentrated sulfuric acid were added at the rate of 1 ml per minute. The temperature of the bath was allowed to rise to -10 C during a 10 minute period, and then the bath was again cooled to -60 C. A 15 ml portion of absolute methanol followed by 13 ml of 35% methanolic potassium hydroxide were added. The flask was removed from the dry ice bath and the stirring was continued until the sample reached room temperature. Phenolphthalein was used to ascertain the presence of excess base. The mixture was then extracted with petroleum ether.

The method offered several advantages over other methods. First there was direct conversion of the glycerol esters to methyl esters without a step involving saponification and isolation of the fatty acids. In this respect, it resembled base-catalyzed interesterification. Secondly, the acid milieu and low temperature should prevent the isomerization of double bonds which had been reported to result from saponification. An important convenience factor was that the total preparation of sample took ca. 20 minutes. TLC analysis has shown the reaction to be complete, and comparisons of the fatty acid composition of several fats using this method and two others were favorable.

The methylation procedure developed by Hornstein and co-workers (1960) used a basic ion-exchange resin. The resin

(10 g of Amberlite IRA-400) was pretreated by stirring with 25 ml of 1 N sodium hydroxide for 5 minutes. After settling, the supernatant was discarded, and the resin was washed first with successive portions of distilled water to remove any free alkali, and then with three 25 ml portions of anhydrous ethanol to remove water. Finally it was washed with three 25 ml portions of petroleum ether to remove the alcohol. A sample containing 0.1-1 g of free fatty acids was dissolved in about 40 ml of petroleum ether. This solution was decanted onto 10 g of the pretreated resin in a 250 ml Erlenmeyer flask. Two 5 ml portions of petroleum ether were used to rinse the original flask. After stirring the solution and the resin with a magnetic stirrer for 5 minutes, the resin was allowed to settle and the supernatant was discarded. Three 25 ml portions of petroleum ether were used to wash the residue free of fat. The absorbed fatty acids were directly converted to the methyl esters on the resin, and after adding a 25 ml portion of anhydrous methanol-hydrochloric acid (5-10% acid), the mixture was stirred for 25 minutes. The methanol solution was then decanted through a rapid filter paper into a 250 ml separatory funnel. The resin was washed by stirring for 5 minutes with two successive 15 ml portions of anhydrous methanol-hydrochloric acid, again decanting each wash through the filter. Ten ml of distilled water were added to the combined methanol extracts and the solution was extracted with 50 ml of petroleum ether. The aqueous phase

was drained into a second separatory funnel and extracted twice with 20 ml portions of petroleum ether. Finally the petroleum ether extracts were combined, washed with water and dried over anhydrous sodium sulfate. This method is useful for the determination of free fatty acids in the presence of fat.

Vorbeck and co-workers (1961) published a paper in which they compared the results of three of the above methods: the microsublimation technique of Stoffel; a diazomethane method; and the ion-exchange method of Hornstein. It had been suggested that methylation with diazomethane gave poor quantitative results because of the possibility of the polyenoic acids forming addition products at the double bonds. Vorbeck's group found no proof for this theory. On the contrary, their results indicated that the diazomethane procedure was superior to the other two when there was present a high percentage of short chain fatty acids. The microsublimation technique in particular gave low yields of the shorter chain fatty acids. However, the three methods were comparable when the longer chain fatty acids were analyzed.

A second approach to the formation of methyl esters involves the direct formation of esters by transesterification in the presence of a basic catalyst. The method described by Smith and Jack (1954) was carried out with sodium hydroxide as the catalyst. A 200 g milk fat sample was dissolved in 800 ml of pentane. A 700 ml portion of neutralized absolute

methanol and 1 ml of 1 N potassium hydroxide were added to the mixture which was then gently swirled and allowed to stand at room temperature for 48 hours. Finally the solution was divided into two equal volumes, each of which was washed once with 250 ml of 0.06 N hydrochloric acid and three times with 0.02 N hydrochloric acid.

Luddy and co-workers (1960) used either potassium or sodium methoxide as the catalyst in their procedure. A 10-50 mg lipid sample dissolved in 5 ml of petroleum ether was placed in a small round-bottom flask equipped with a condenser. A known volume of a standard methoxide solution was added by pipette and the mixture was refluxed for various times, depending on the nature of the lipid material. While the reaction mixture was still warm, a 0.5 N methanolic solution of sulfuric acid was added through the condenser in an amount in slight excess over the amount calculated to neutralize the methoxide. The contents were cooled, transferred to a separatory funnel, and the methyl esters were extracted with petroleum ether.

# MATERIALS AND METHODS

## Materials

Corn oil, coconut oil, lard, beef tallow, butter oil, soy phospholipid (Centrolux P containing mainly phosphatidyl choline and phosphatidyl enthanolamine), egg yolk phospholipid and individual phospholipids separated from the egg yolk were used as samples.

## Methods

(1) Preparation of lipid from egg yolk.

The method was based on that of Lea *et al.* (1955). Twelve egg yolks were homogenized in chloroform:methanol (2:1, v/v) for 30 sec and the final volume of the solvent adjusted to 1400 ml. After shaking, the homogenate was filtered with mild suction through Whatman No. 1 filter paper. Two hundred fifty ml of aqueous 0.01 M calcium chloride were added to the filtrate to form a mixture which was shaken and then allowed to separate into two phases. The upper aqueous methanol layer was discarded while the lower chloroform layer was passed through a column of cellulose powder (packed as a slurry in chloroform) 3 cm in diameter and ca. 30 cm high to remove the non-lipid contaminants. The column was washed with an additional 200 ml of chloroform and the combined effluents, after being concentrated under nitrogen, were

poured with stirring into 500 ml of ice cold acetone. The supernatant was discarded and the residue was dissolved in 100 ml of diethyl ether. As before, the ether solution was poured into 500 ml of ice cold acetone and the precipitate was dissolved in chloroform for chromatography.

(2) Preparation of phospholipid from crude egg phospholipid.

An adaption of the Florisil method of Carroll (1963) was used. An aliquot of the lipid solution in chloroform was added to 5 g of acid-washed Florisil. After standing for several minutes, this Florisil-lipid mixture was transferred to a Büchner funnel equipped with a fritted glass disk (medium porosity) and was rinsed with a total of 100 ml of chloroform. The chloroform eluant (containing the neutral lipids) was discarded and the phospholipids were eluted with 100 ml of methanol. Finally the solvent was evaporated under nitrogen and the sample was redissolved in petroleum ether for TLC.

(3) Preparative thin layer chromatography.

A sample of the crude phospholipid extract was applied as a streak to plates coated with a layer of silica gel G 500 μ thick. The plates were developed in an equilibrated tank containing a solvent system of chloroform:methanol:water (65:25:4, v/v/v) until the solvent had almost reached the top of the plate. After the solvent had evaporated, the plates were sprayed with 2',7'-dichlorofluorescein and the

bands, visualized under a UV light, were scraped off. The individual phospholipid classes were methylated directly without elution from the silica gel G. The identity of the bands (phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelin) was checked by spraying a reference plate with ninhydrin and with the molybdate spray reported by Dittmer and Lester (1964) and by then comparing the $R_f$ values of the bands detected in this way with those seen under the UV light.

(4) Methylation of lipids.

The basic methylation procedure was that of McGinnis and Dugan (1965). The triglyceride samples (3 drops or ca. 20 mg) and mixed phospholipid samples (aliquots of a phospholipid solution) were methylated in diethyl ether solution. The individual phospholipid samples were scraped from the preparative TLC plates and methylated directly without elution from the silica gel G. In each case, the lipid, in a 125 ml Erlenmeyer flask, was dissolved in 25 ml of diethyl ether. The solution, stirred by a magnetic stirrer, was placed in a dry ice-acetone bath, and after a temperature of -60 C was reached, 2 ml of concentrated sulfuric acid were added at the rate of 1 ml per minute. The temperature was allowed to rise during a 10 minute period. A 15 ml portion of absolute methanol, followed after 3, 20 or 40 minutes by 15 ml of methanol containing various amounts of potassium hydroxide, was added. The potassium hydroxide used was at least 85%

pure and was expressed as grams of potassium hydroxide per 15 ml of methanol. The flask was removed from the dry ice bath and stirring was continued until the sample reached room temperature. The methyl esters were extracted once with 30 ml of petroleum ether and twice with 15 ml of petroleum ether. The samples were dried over anhydrous sodium sulfate and concentrated to ca. 1 ml.

(5) Analytical thin layer chromatography.

TLC was also used to check the samples for completeness of reaction. Samples were spotted on silica gel G plates 0.25 μ thick and developed in a neutral lipid solvent system containing petroleum ether:diethyl ether:acetic acid (90:10:1, v/v/v). In addition, phospholipids were developed in a polar system containing chloroform:methanol:water (65:25:4, v/v/v). Spots were visualized by spraying with 50% sulfuric acid followed by charring.

(6) Gas liquid chromatography.

GLC was carried out on a dual column Beckman GC-5 gas chromatograph equipped with a thermal conductivity detector. The 6 ft. x 1/8" (O.D.) stainless steel coiled columns were packed with 20% diethylene glycol succinate (DEGS) and 1% phosphoric acid on 80-100 mesh acid-washed Chromosorb W. The detector was maintained at 250 C while the column temperature was 184 C. The methyl ester peaks were identified by comparison with standards.

## RESULTS AND DISCUSSION

### Thin Layer Chromatographic Analysis

Corn oil was used for the study of the effect of quantity of potassium hydroxide on methylation of fatty acids in triglycerides. Table 1 shows the treatments used while Figure 1 shows the results of TLC analysis of sets 1 and 2. Distinct spots are indicated by solid lines and faint spots and streaking by broken lines. With sulfuric acid at a level of 2 ml and with increasing amounts of potassium hydroxide (set 1), the spots representing both triglycerides and the products such as mono- and diglycerides and free fatty acids that result from partial methylation appeared to become smaller while the spots corresponding to methyl esters increased in area. Since the original samples were of approximately the same size, and since the methylated samples were concentrated to equal volumes, an estimate of the degree of methylation could be obtained from the relative size of the spots. Reaction seemed most complete with potassium hydroxide at the level of 5.5 g. When no sulfuric acid was used (set 2), any amount of potassium hydroxide appeared to effect complete methyl ester formation.

As shown in Figure 2, when the amount of sulfuric acid was increased in the absence of potassium hydroxide (set 3),

Table 1. Reaction conditions used for methylation of corn oil.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Set 1 | Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| | ml $H_2SO_4$ | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| | g $KOH_a$ | 0 | 1 | 2 | 3 | 4 | 5 | 5.5 |
| Set 2 | Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| | ml $H_2SO_4$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | g $KOH_a$ | 0 | 1 | 2 | 3 | 4 | 5 | 5.5 |
| Set 3 | Treatment | 1 | 2 | 3 | 4 | 5 | 6 | |
| | ml $H_2SO_4$ | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | |
| | g $KOH_a$ | 0 | 0 | 0 | 0 | 0 | 0 | |
| Set 4 | Treatment | 1 | 2 | 3 | 4 | 5 | 6 | |
| | ml $H_2SO_4$ | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | |
| | g $KOH_a$ | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | |

a g KOH/15 ml methanol

Table 2. Reaction conditions used for methylation of butter oil, tallow, lard and coconut oil.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| ml $H_2SO_4$ | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| g $KOH_a$ | 0 | 1 | 2 | 3 | 4 | 5 | 5.5 |

a g KOH/15 ml methanol

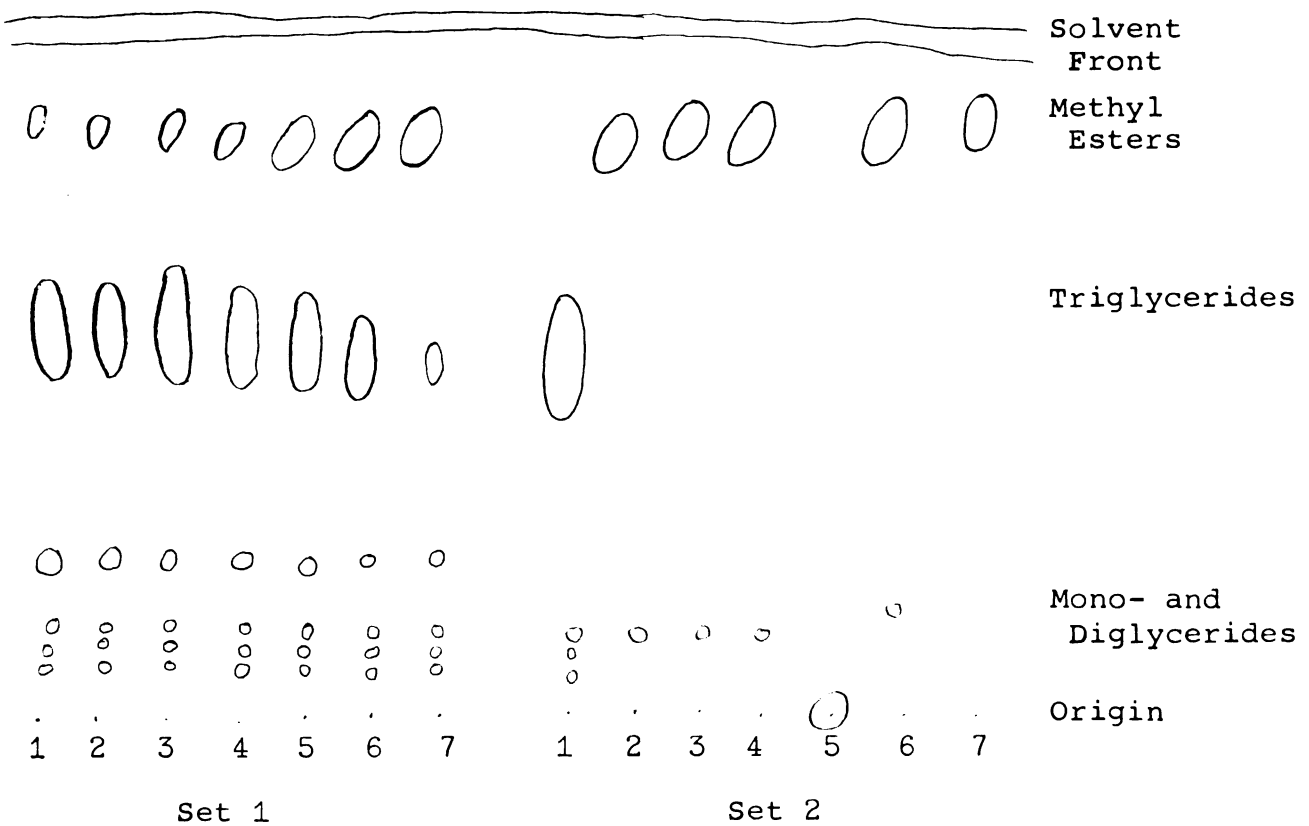



Figure 1. TLC of corn oil methylated according to the reaction conditions given in Table 1, Set 1 (acid and increasing base) and Set 2 (no acid and increasing base). Developing solvent: petroleum ether:diethyl ether:acetic acid 90:10:1 (v/v/v). Indicator: 50% sulfuric acid spray followed by charring.

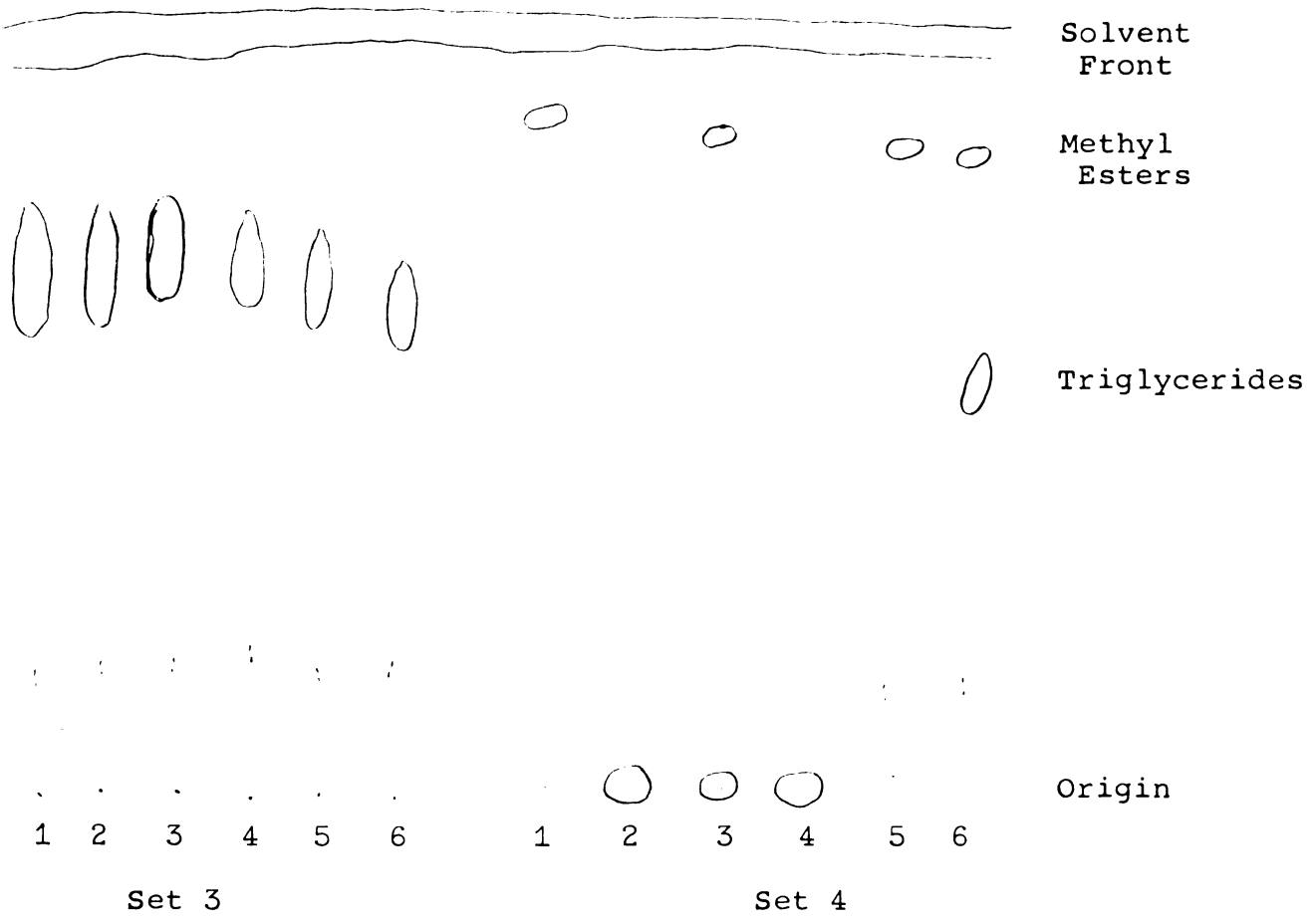



Figure 2. TLC of corn oil methylated according to the reaction conditions given in Table 1, Set 3 (no base and increasing acid) and Set 4 (base and increasing acid). Developing solvent: petroleum ether:diethyl ether:acetic acid 90:10:1 (v/v/v). Indicator: 50% sulfuric acid spray followed by charring.

only triglyceride spots were observed on the TLC plate. However, with potassium hydroxide at the level of 5.5 g (set 4), methylation appeared complete at all levels of sulfuric acid except at 2.5 ml where the triglyceride spot was still relatively small. Samples corresponding to 0.5, 1.0 and 1.5 ml of sulfuric acid gave only a large spot at the origin. It was speculated that this spot was due either to soaps or to potassium sulfate.

Butter oil, tallow, lard and coconut oil were given the treatments shown in Table 2, and the TLC results are shown in Figures 3 and 4. Again the trend of increasing amounts of methyl esters with increasing amounts of potassium hydroxide was seen. Potassium hydroxide at a level of 5.0 or 5.5 g seemed to provide satisfactory yields of methyl esters. An interesting variation from the usual pattern was the large methyl ester spot found in the butter oil and lard samples that were methylated without using any potassium hydroxide for neutralization. However, in another lard sample methyated under the same conditions (see Table 3, treatment 1), no methyl ester spots were obtained. Lard and coconut oil were also methylated according to the conditions indicated in Table 3 to test the effects of longer reaction times on completeness of methylation. Figures 5 and 6 show the TLC analysis. The treatments in set 1 appeared to leave much of the triglyceride unreacted while the other treatments seemed to give more complete reaction. There appeared to be

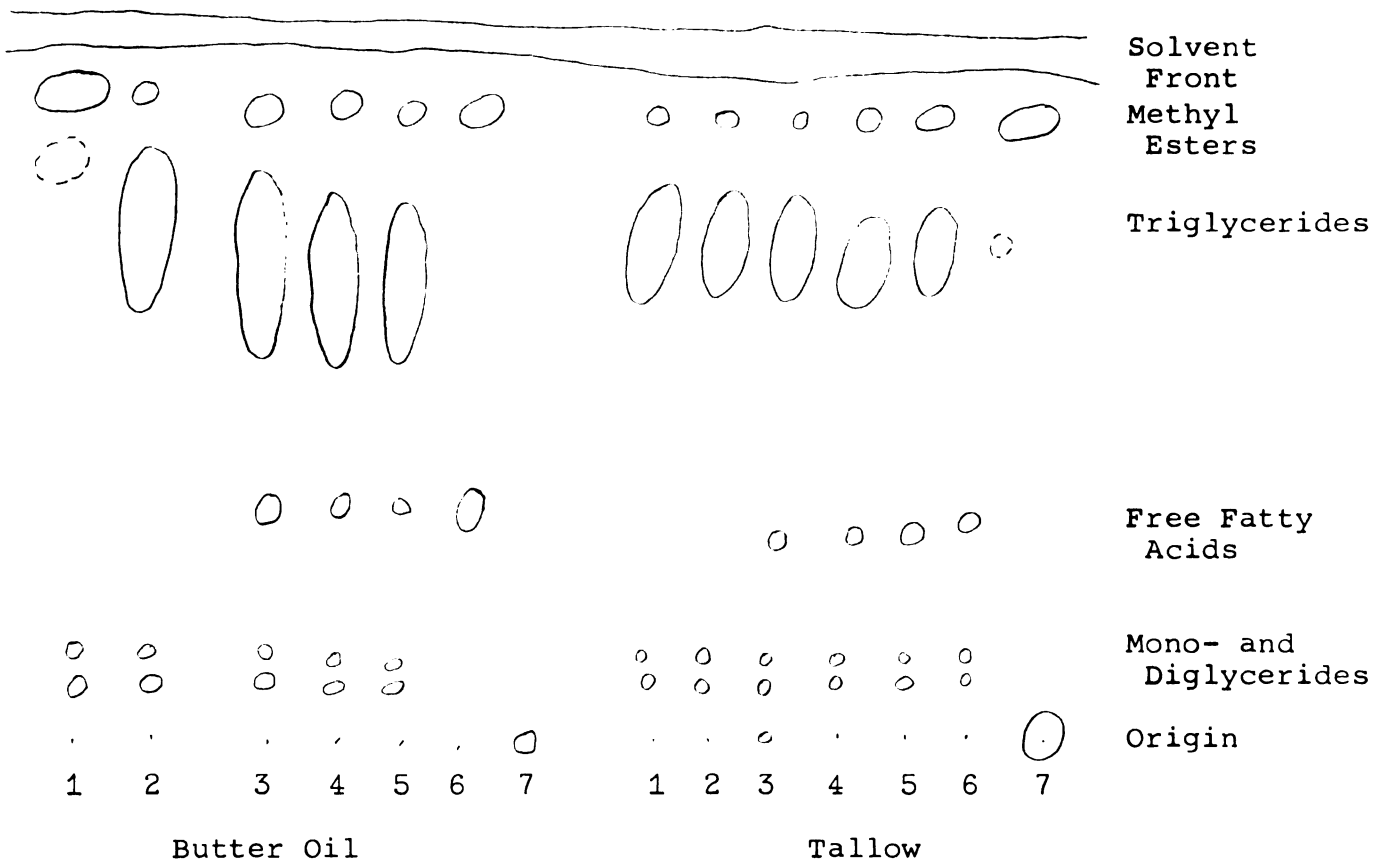



Figure 3. TLC of butter oil and tallow methylated according to the reaction conditions given in Table 2 (acid plus increasing amounts of base). Developing solvent: petroleum ether:diethyl ether:acetic acid 90:10:1 (v/v/v). Indicator: 50% $H_2SO_4$ spray followed by charring.

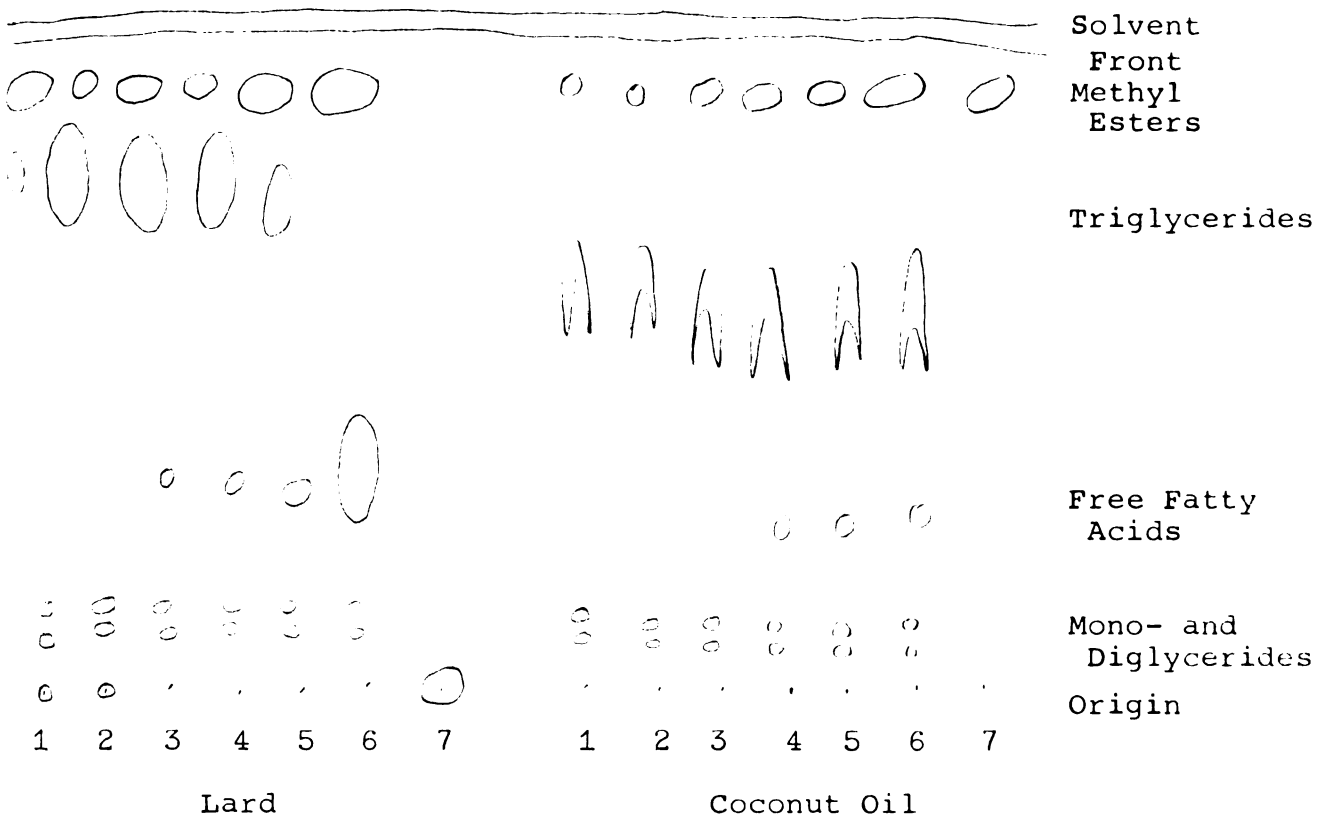



Figure 4. TLC of lard and coconut oil methylated according to the reaction conditions given in Table 2 (acid plus increasing amounts of base). Developing solvent: petroleum ether: diethyl ether:acetic acid 90:10:1 (v/v/v). Indicator: 50% $H_2SO_4$ spray followed by charring.

Table 3. Reaction conditions used for methylation of lard and coconut oil.

| | Set 1 | | | Set 2 | | | Set 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| ml $H_2SO_4$ | 2 | 2 | 2 | 0 | 0 | 0 | 2 | 2 | 2 |
| g KOH[a] | 0 | 0 | 0 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 |
| min.[b] | 3 | 20 | 40 | 3 | 20 | 40 | 3 | 20 | 40 |

a g KOH/15 ml methanol
b minutes between the addition of methanol and the addition of the methanolic KOH


Figure 5. TLC of lard methylated according to the reaction conditions given in Table 3. Developing solvent: petroleum ether:diethyl ether:acetic acid 90:10:1 (v/v/v). Indicator: 50% sulfuric acid spray followed by charring.

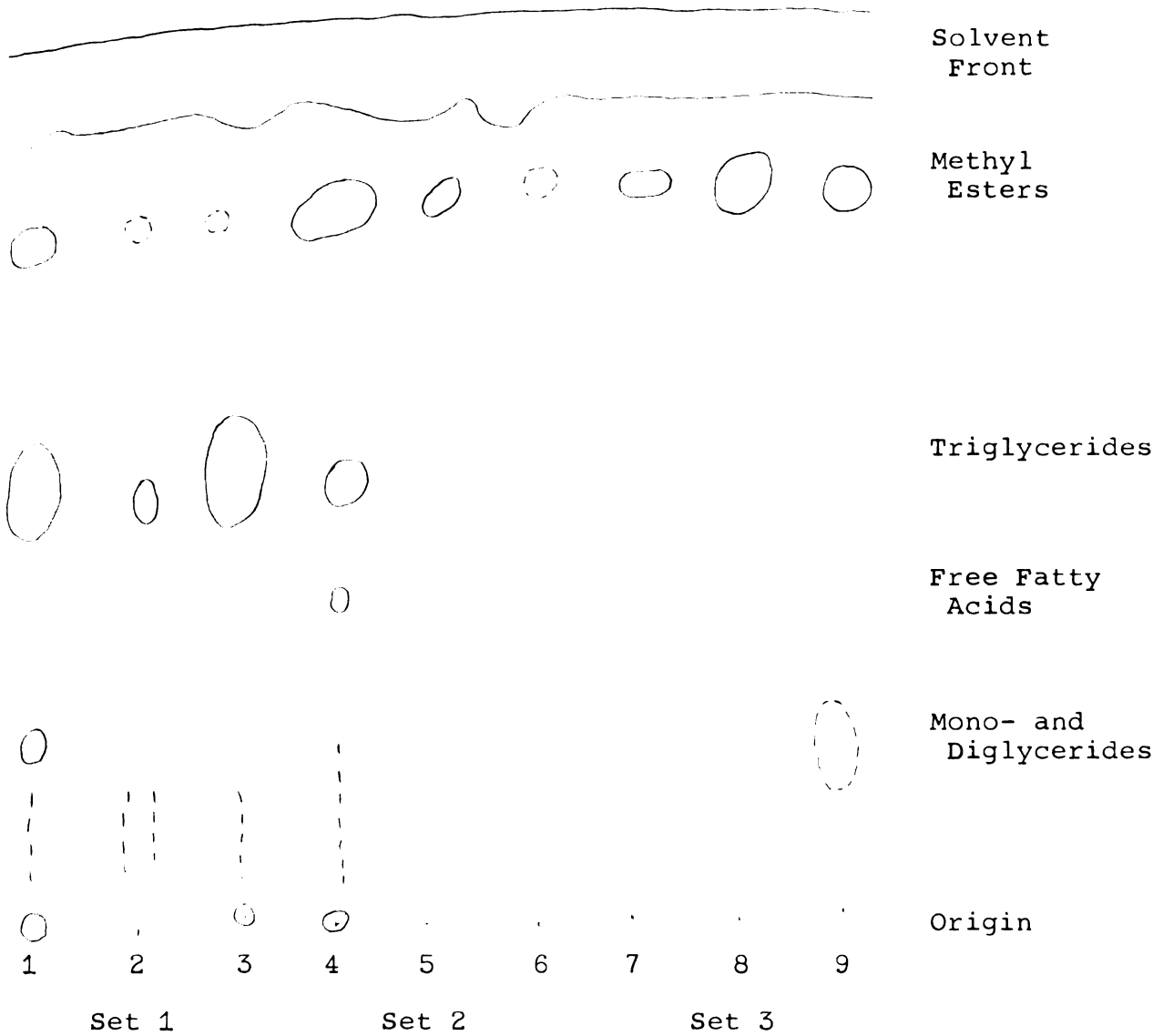



Figure 6. TLC of coconut oil methylated according to the reaction conditions given in Table 3 (Set 1: acid only, Set 2: base only, Set 3: acid and base). Developing solvent: petroleum ether:diethyl ether:acetic acid 90:10:1 (v/v/v). Indicator: 50% sulfuric acid spray followed by charring.

little difference within a given set due to differences in reaction time. It should be noted that in set 1 which corresponded to treatment 1 in Table 2, there was a mixture of methyl ester, triglyceride and mono- and diglyceride spots rather than the large methyl ester spot found in the previous treatment. The fact that all three samples in set 1 had similar patterns indicated that the methylation of the previous sample resulted in some kind of artifact.

Soy phospholipid (Centrolux P) which contained mainly phosphatidyl choline and phosphatidyl ethanolamine was used as a source for the study of the effects of potassium hydroxide on phospholipids. Table 4 shows the various treatments used, while Figures 7 and 8 show the results of the TLC analysis. These samples were developed in two solvent systems: the first (see Figure 7), the neutral lipid system mentioned above, was used to ascertain the completeness of methylation; the second one (see Figure 8), a combination of the polar solvents chloroform, methanol and water in the proportions 65:25:4 (v/v/v), was used to check for the presence of phospholipid spots. Treatments D, E, F, G, and L appeared to give complete methylation as judged by the absence of non-methyl ester spots in the neutral solvent and by the relative absence of spots in the polar solvent. There appeared to be little difference in degree of methylation among the rest of the treatments. The trend of increasing amounts of methyl esters with increasing amounts of potassium

Table 4. Reaction conditions used for methylation of soy phospholipids.

| Treatment | A | B | C | D | E | F | G | H | I | J | K | L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ml $H_2SO_4$ | 2 | 2 | 2 | 2 | 0 | 0 | 1 | 4 | 1 | 2 | 3 | 2 |
| g $KOH_a$ | 0 | 2 | 4 | 5.5 | 2 | 4 | 5.5 | 5.5 | 0 | 0 | 0 | 5.5 |
| $min._b$ | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 20 |

a g KOH/15 ml methanol
b minutes between the addition of methanol and the addition of the methanolic KOH

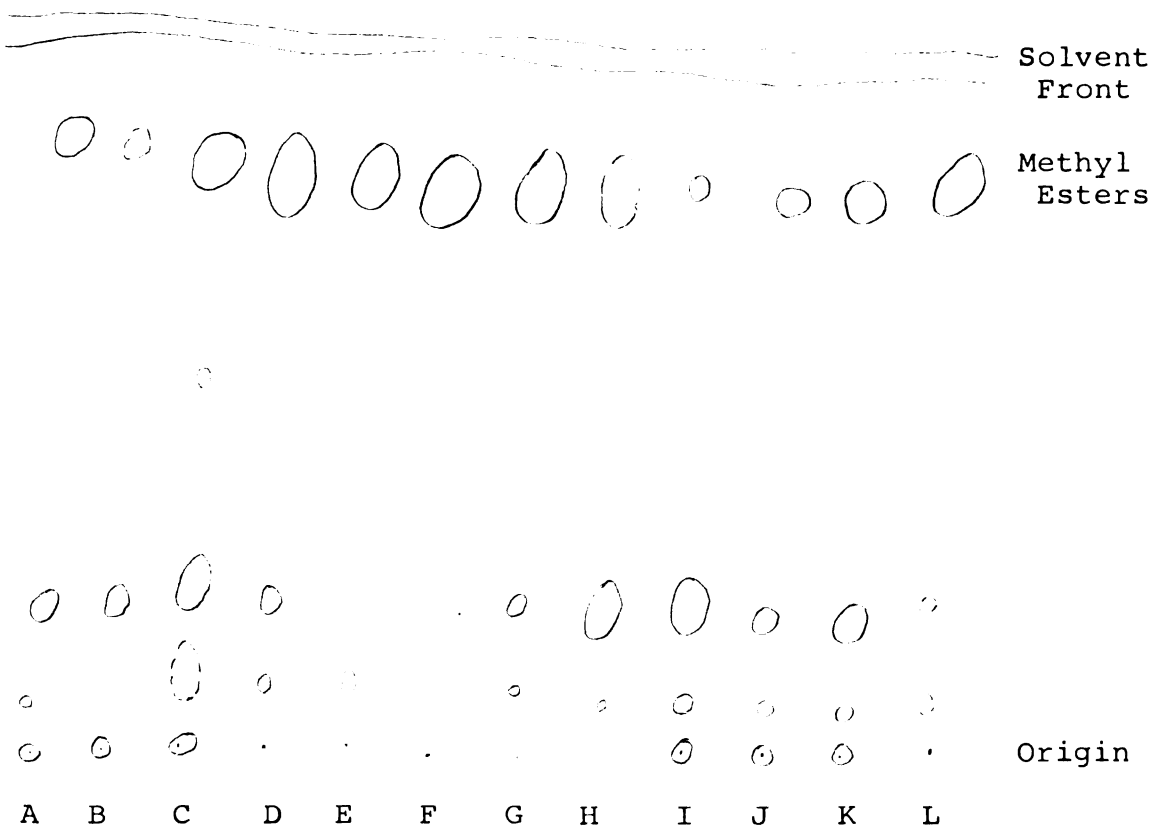



Figure 7. TLC of soy phospholipid methylated according to the reaction conditions given in Table 4. Developing solvent: petroleum ether:diethyl ether:acetic acid 90:10:1 (v/v/v). Detector: 50% sulfuric acid spray followed by charring.

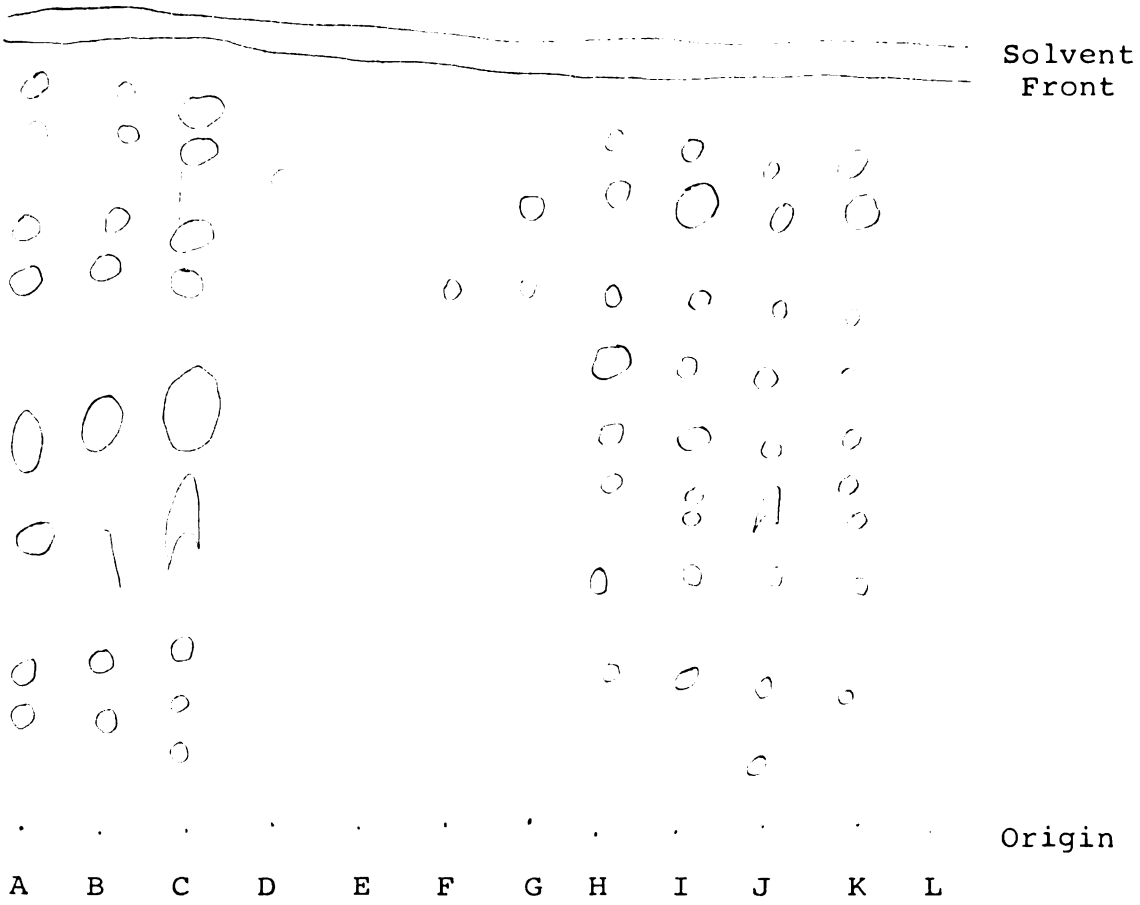



Figure 8. TLC of soy phospholipids methylated according to the reaction conditions given in Table 4. Developing solvent: chloroform:methanol:water 65:25:4 (v/v/v). Detector: 50% sulfuric acid spray followed by charring.

hydroxide was not observed with the soy phospholipid samples (treatments A, B, C and D).

Phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin as well as a mixture of phospholipids, all from egg yolk, were methylated using the treatments shown in Table 5. The TLC results shown in Figures 9, 10 and 11 were obtained with the neutral solvent system and showed that increasing potassium hydroxide concentration did not result in an increase in the yield of methyl esters. Some methyl esters were present in all samples, but no treatment resulted in complete methylation. These results agree with those obtained for the soy phospholipid samples and indicate that different conditions are required for phospholipids and triglycerides. Even the phospholipid samples in which there was a 20 minute period between the addition of methanol and the addition of potassium hydroxide did not result in a substantial increase in methyl esters.

Gas Liquid Chromatographic Analysis

GLC analysis of the triglyceride samples shown in Figures 12 and 13 gave some evidence of differences between treatments although there was no visible trend. An analysis of variance of the ratio of 18:2 to 18:0 in the corn oil samples showed no significant differences between treatments in spite of the large variations in completeness of reaction seen on TLC.

Table 5. Reaction conditions used for methylation of egg phospholipids.

| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| ml $H_2SO_4$ | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| g KOH[a] | 0 | 1 | 2 | 3 | 4 | 5 | 5.5 | 5.5 |
| min.[b] | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 20 |

a g KOH/15 ml methanol
b minutes between the addition of methanol and the addition of potassium hydroxide

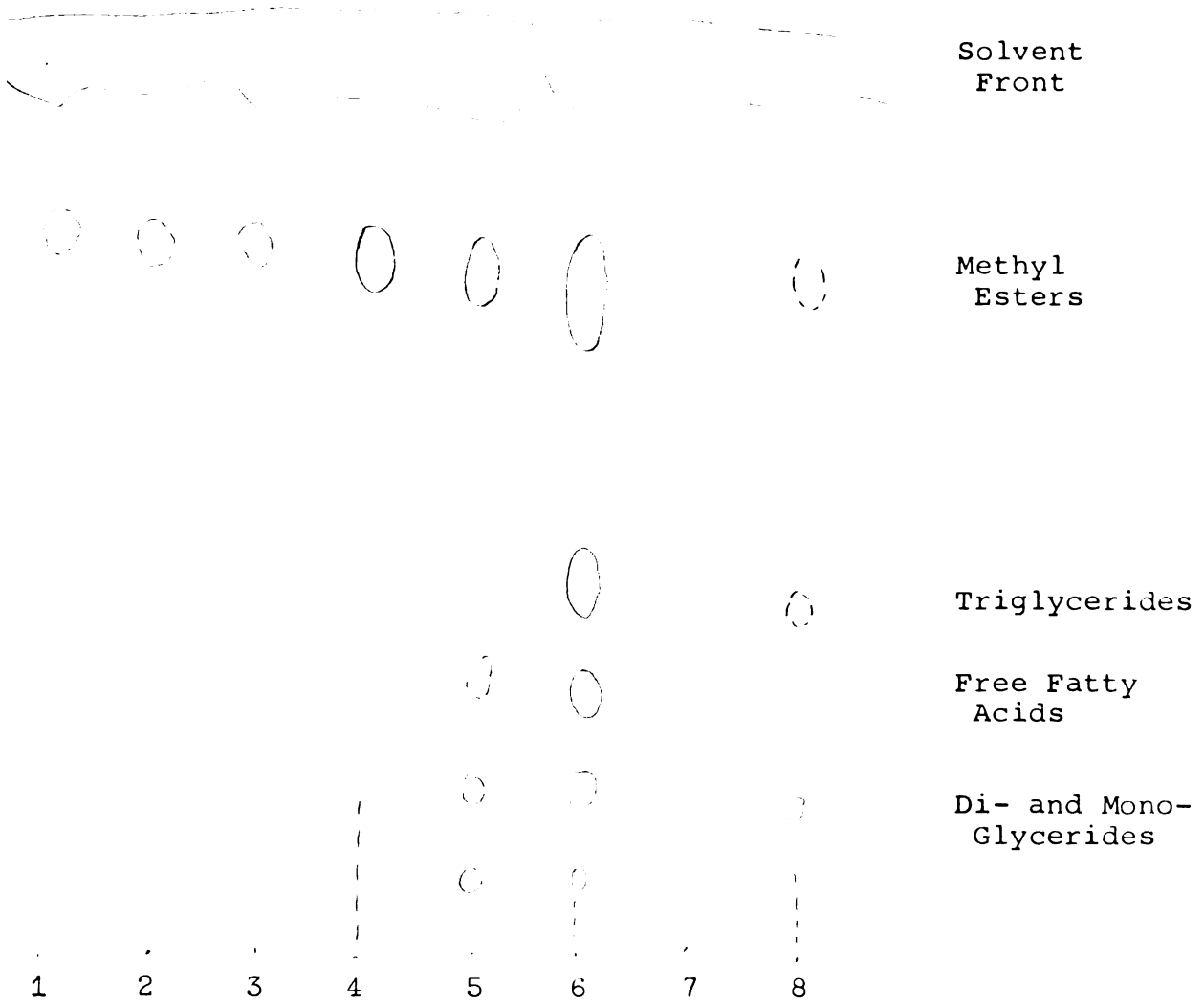



Figure 9. TLC of egg phospholipid methylated according to the reaction conditions given in Table 5 (acid plus increasing amounts of base). Developing solvent: petroleum ether:diethyl ether:acetic acid 90:10:1 (v/v/v). Detector: 50% sulfuric acid spray followed by charring.

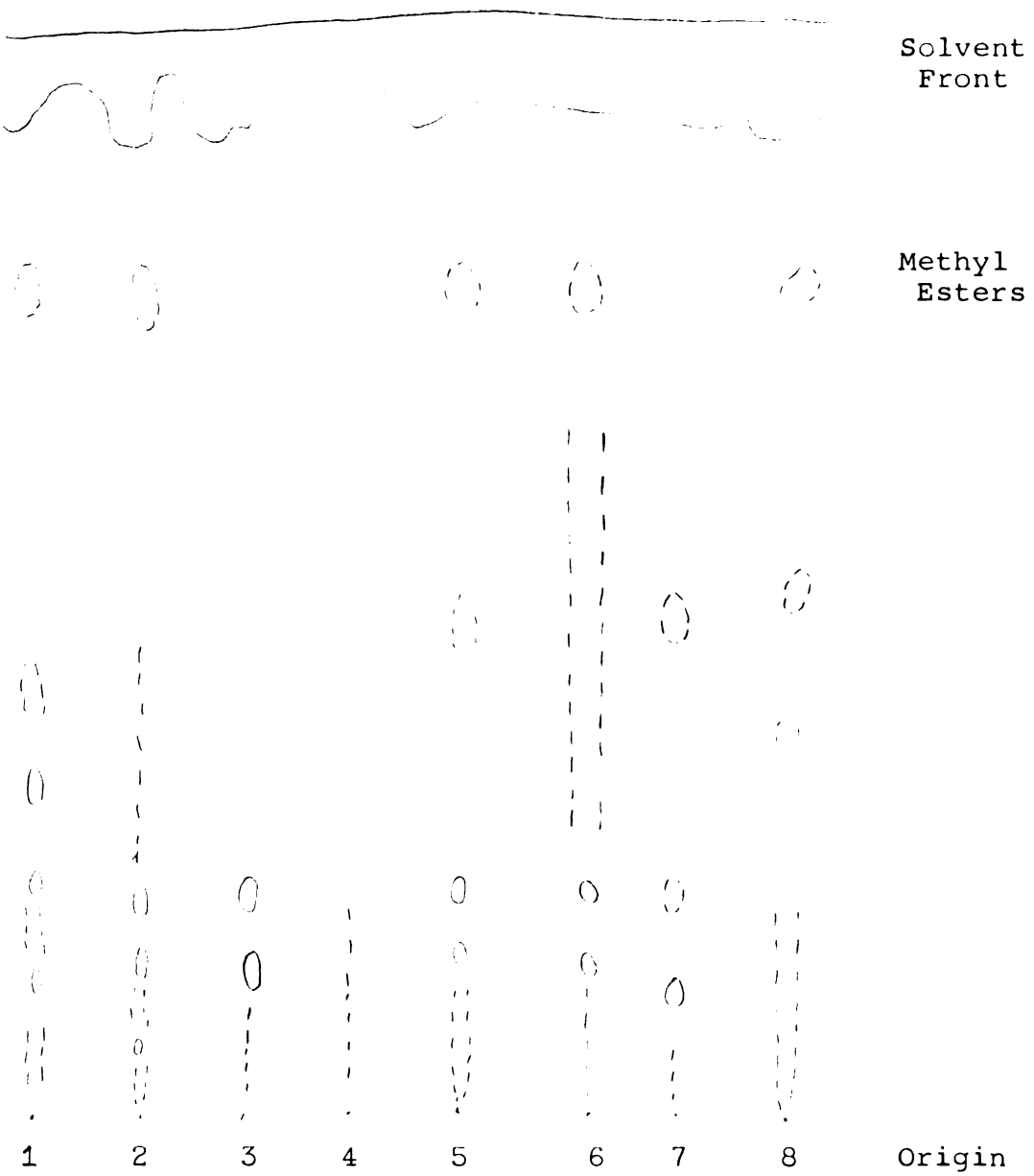



Figure 10. TLC of egg phosphatidyl choline methylated according to the reaction conditions given in Table 5 (acid and increasing amounts of base). Developing solvent: petroleum ether:diethyl ether:acetic acid 90:10:1 (v/v/v). Detector: 50% sulfuric acid spray followed by charring.

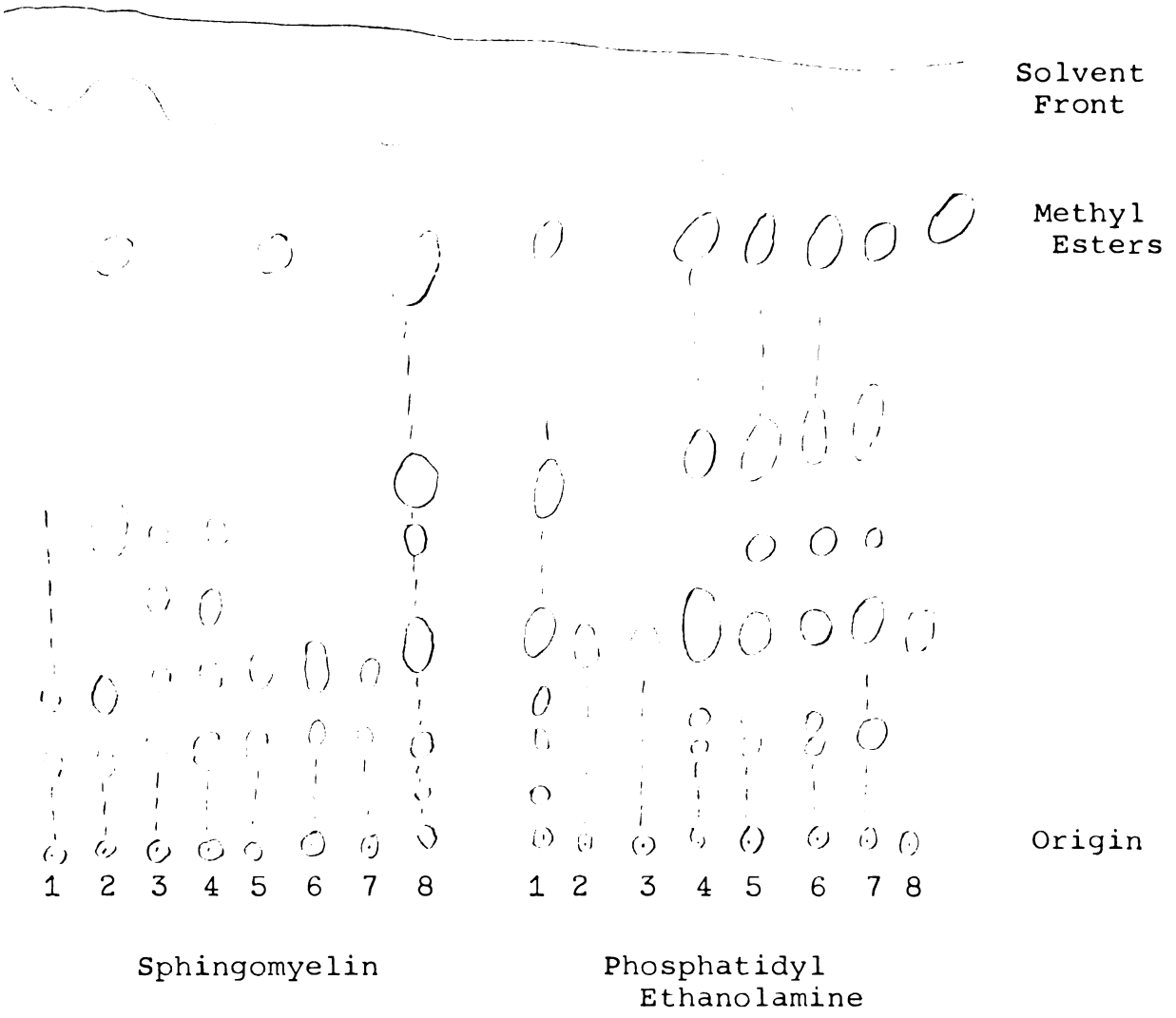



Figure 11. TLC of egg phosphatidyl ethanolamine and sphingomyelin methylated according to the reaction conditions given in Table 5 (acid and increasing amounts of base). Developing solvent: petroleum ether:diethyl ether:acetic acid 90:10:1 (v/v/v). Detector: 50% sulfuric acid spray followed by charring.

Figure 12. Fatty acid composition of corn oil (area %).

Set 1

| Fatty acid | g KOH (in addition to 2 ml $H_2SO_4$) 2.0 | 3.0 | 4.0 | 5.0 | 5.5 |
|---|---|---|---|---|---|
| 14:0[a] | --- | 2.03 | 1.12 | --- | --- |
| 16:0 | 12.28 | 12.38 | 12.05 | 13.16 | 20.27 |
| 18:0 | 2.24 | 1.69 | 1.73 | 1.74 | 14.45 |
| 18:1 | 27.92 | 26.62 | 27.28 | 27.42 | 15.27 |
| 18:2 | 56.84 | 57.28 | 57.83 | 57.68 | 50.01 |
| 18:3 | 0.26 | --- | --- | --- | --- |
| ? | 0.46 | --- | --- | --- | --- |

Set 2

| Fatty acid | g KOH (no sulfuric acid added) 1.0 | 2.0 | 3.0 | 5.5 |
|---|---|---|---|---|
| 16:0 | 12.96 | 11.73 | 12.18 | 11.72 |
| 18:0 | 2.06 | 1.98 | 1.83 | 1.74 |
| 18:1 | 28.23 | 26.68 | 26.48 | 26.28 |
| 18:2 | 56.18 | 58.77 | 57.90 | 59.45 |
| 18:3 | 0.20 | 0.29 | 0.57 | 0.27 |
| ? | 0.38 | 0.55 | 1.04 | 0.53 |

Set 4

| Fatty acid | ml $H_2SO_4$, 5.5 g KOH 0.0 | 2.0 | 2.5 |
|---|---|---|---|
| 16:0 | 12.98 | 12.28 | 13.72 |
| 18:0 | 2.05 | 2.24 | 2.46 |
| 18:1 | 25.52 | 27.92 | 30.45 |
| 18:2 | 58.10 | 56.84 | 52.71 |
| 18:3 | 0.56 | 0.26 | 0.17 |
| ? | --- | 0.46 | 0.49 |

a number to left of colon indicates carbon number; number to the right indicates the number of double bonds.

NOTE: Set 3 is not shown because none of the samples were methylated.

Figure 13. Fatty acid composition of butter oil, tallow, lard and coconut oil (area %).

| | g KOH/15 ml methanol | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Butter oil | | | | Tallow | Coconut Oil | | Lard | | |
| Fatty acid | 0.0 | 1.0 | 4.0 | 5.0 | 5.0 | 4.0 | 5.0 | 0.0 | 3.0 | 4.0 |
| 12:0[a] | 4.84 | 4.65 | 4.38 | 5.13 | --- | 42.15 | 44.40 | --- | --- | --- |
| 14:0 | 13.97 | 13.28 | 14.47 | 14.65 | 4.49 | 41.58 | 20.54 | 1.72 | 3.38 | 4.48 |
| 16:0 | 35.99 | 36.36 | 36.60 | 36.82 | 31.03 | 5.71 | 23.71 | 26.59 | 25.01 | 26.32 |
| 16:1 | 3.44 | 2.28 | 2.98 | 3.25 | 3.84 | --- | --- | 2.69 | 2.82 | 3.26 |
| 18:0 | 12.28 | 14.08 | 12.60 | 36.82 | 24.86 | 1.81 | 3.12 | 20.56 | 10.61 | 10.45 |
| 18:1 | 26.82 | 29.35 | 28.96 | 26.55 | 33.93 | 5.25 | 6.24 | 39.97 | 47.96 | 45.59 |
| 18:2 | 2.65 | --- | --- | 2.04 | 1.84 | 3.05 | 1.99 | 8.46 | 10.24 | 9.90 |

a number to left of colon indicates carbon number; number to right indicates the number of double bonds.

NOTE: Only samples showing large amounts of methyl esters on TLC were chromatographed.

It was thought that perhaps at low concentrations of potassium hydroxide the shorter chain and polyunsaturated acids would be preferentially methylated. The ratio of 18:2 (or another polyunsaturated acid) to 18:0 should be a valid measure to test this hypothesis. However, the GLC data did not confirm this idea. Although there were differences, they were not significant.

Butter oil, coconut oil, tallow and lard samples gave similar results. Only the samples which had relatively large amounts of methyl esters were chromatographed since the others gave only an extremely large peak initially with extensive tailing before returning to the base line. As with the corn oil samples, there were differences between treatments, but no trend that could be related to concentration of potassium hydroxide was apparent. An analysis of variance of the ratio of 18:2 to 18:0 showed that there were no significant differences between treatments for a given fat.

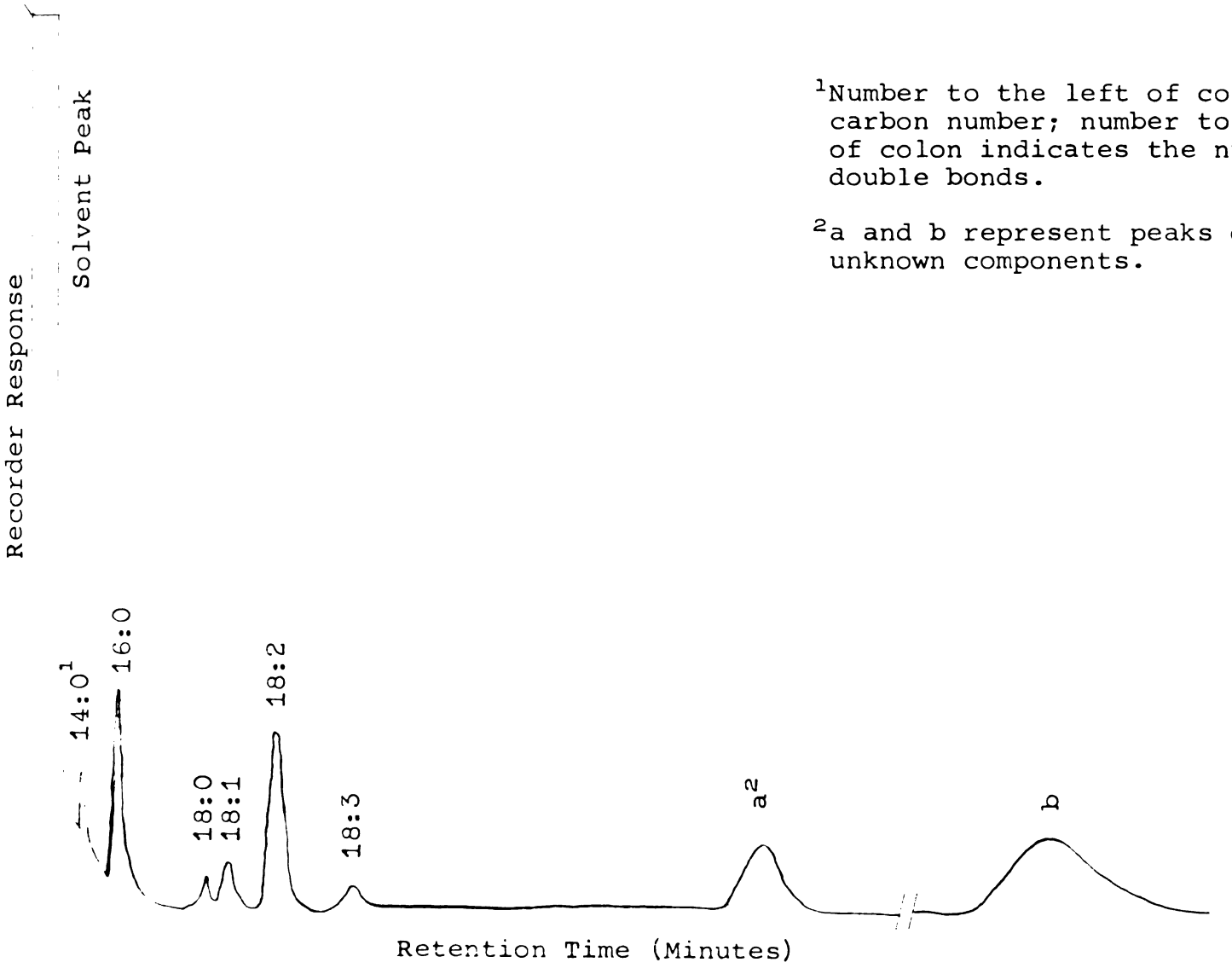
GLC analysis of the soy phospholipid samples gave the results shown in Figure 14. A representative chromatogram from one of these samples is given in Figure 15. An interesting point was the absence of unknown peaks a and b in samples D, E, F, G and L, the samples which, according to TLC analysis, were completely methylated. An analysis of variance of the ratio of 18:3 to 18:0 (Figure 16) yielded no significant differences (Figure 17). The analysis

Figure 14. Fatty acid composition of soy phospholipid (area %).

| Fatty acid | Treatment (see Table 4) B | C | D | E | F | G | H | J | K | L |
|---|---|---|---|---|---|---|---|---|---|---|
| 14:0 | --- | 1.21 | 0.66 | --- | 0.75 | 0.89 | 0.25 | 2.34 | --- | 0.98 |
| 16:0 | 3.22 | 19.19 | 21.89 | 20.15 | 17.24 | 19.86 | 20.04 | 10.06 | 10.91 | 19.33 |
| 18:0 | 2.23 | 4.06 | 4.34 | 4.48 | 4.87 | 4.57 | 4.44 | 2.14 | 2.04 | 4.75 |
| 18:1 | 18.76 | 8.25 | 10.10 | 10.14 | 10.62 | 10.06 | 9.49 | 4.15 | 4.26 | 10.71 |
| 18:2 | 5.79 | 50.20 | 55.13 | 57.23 | 59.35 | 56.47 | 49.77 | 20.21 | 22.39 | 56.40 |
| 18:3 | 4.22 | 3.64 | 7.88 | 8.00 | 7.54 | 8.15 | 6.87 | 2.49 | 3.34 | 7.82 |
| a | 65.79 | 3.64 | --- | --- | --- | --- | 0.30 | 18.13 | 16.63 | --- |
| b | --- | 6.74 | --- | --- | --- | --- | 8.85 | 40.49 | 40.42 | --- |

NOTE: Sample I gave no GLC response.

Figure 15. Gas liquid chromatogram of soy phospholipid methylated with 2 ml sulfuric acid and no potassium hydroxide (Treatment J).




[1]Number to the left of colon indicates carbon number; number to the right of colon indicates the number of double bonds.

[2]a and b represent peaks due to unknown components.

Figure 16. The ratio of 18:3 to 18:0 fatty acids in soy phospholipid samples methylated according to the reaction conditions in Table 4.

| 18:3/18:0 Ratio | Treatment (see Table 4) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | B | C | D | E | F | G | H | J | K | L |
| 18:3/18:0 Ratio | 1.90 | 1.66 | 1.82 | 1.79 | 1.55 | 1.78 | 1.57 | 1.17 | 1.63 | 1.65 |

Figure 17. Analysis of variance of the 18:3/18:0 ratio of the fatty acids of soy phospholipid samples methylated according to the reaction conditions given in Table 4.

| Source of variation | d.f. | S.S. | M.S. | F |
|---|---|---|---|---|
| Between treatments | 9 | 0.49 | 0.05419 | 2.572 |
| Within treatments | 11 | 0.23 | 0.02 | |
| Total | 20 | 0.72 | | |

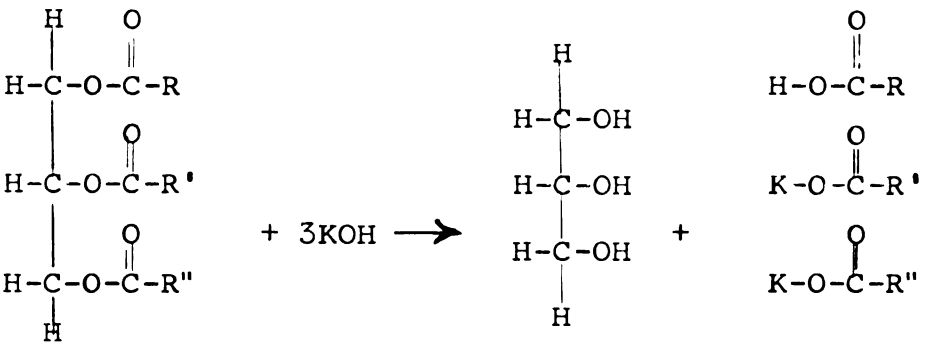
F(0.05, 9, 11) = 2.90
F(0.01, 9, 11) = 4.63

indicated, however, that although the treatment effect was not significant at the 5% level of probability, it was close to this level of significance. Analysis using Duncan's Multiple Range test indicated that treatment J resulted in a significantly lower ratio when compared with the other ($P < 0.05$) treatments, but no other pairs of treatments differed significantly.

Discussion

It appears that the quantity of potassium hydroxide used to neutralize the sulfuric acid in the low temperature-sulfuric acid method does not significantly affect the apparent fatty acid composition of either triglycerides or phospholipids as measured by GLC of the corresponding methyl esters in spite of differences in completeness of reaction.

However, two other factors must be considered. First, when excessive amounts of potassium hydroxide were used, there was a tendency for a foamy emulsion to form during the process of extracting the methyl esters. This foam was thought to be due to the formation of soaps and was indicated on TLC by large spots which remained at the origin. Soaps, defined chemically as metallic salts of long chain fatty acids, can be formed by the following reaction:



triglyceride glycerol soaps

This reaction is known as saponification. The potassium soaps which form are fairly soluble in water and, if present, are ordinarily separated from the methyl ester-ether layer during the extraction process. But soaps can also act as emulsifiers, and it appears to be in this capacity that they cause the foamy layer. When the ether layer including the foam was dried over anhydrous sodium sulfate in the usual manner, some water and perhaps even more soaps could be included in the final solution. Either one or a combination of both soaps and water could cause the large TLC spots at the origin. The samples that had only large spots at the origin on TLC were the same ones that produced the single large peak on GLC. Thus, it is preferable to use the lowest possible concentration of potassium hydroxide to keep the extraction process free of difficulty. Secondly, if the results of a lipid fatty acid analysis are to be absolutely quantitative rather than a measure of the relative amounts of the various fatty acids present, it is essential to have complete methylation. Therefore, at least with triglyceride

samples, reaction conditions must be chosen with the end results in mind. With phospholipid samples, conditions must be chosen to avoid the production of extraneous peaks which can radically change the apparent fatty acid composition.

In the original method as presented by McGinnis and Dugan (1965) the function of the potassium hydroxide was to neutralize the excess sulfuric acid in order to prevent the reversion of the fatty esters to the acid form. Table 6 shows the theoretical calculations for the neutralization of 2 ml of 98% sulfuric acid by potassium hydroxide (computed on the basis of 85% purity) and the difference between the number of milliequivalents of each at various levels of potassium hydroxide. According to these calculations, ca. 2.5 g potassium hydroxide should neutralize 2 ml of sulfuric acid. The fact that methylation did not appear to be complete until almost twice this amount had been added indicated that the reaction was probably base catalyzed. If the reaction is base catalyzed, it is unique in that other base catalyzed interesterification procedures usually have involved higher temperatures than used here (Luddy et al., 1960) or longer reaction times (Smith and Jack, 1954).

The theoretical basis of the method, as originally proposed, was the chemical behavior of esters in strong acids. The formation of an acylium ion-sulfuric acid complex which would readily react with methanol to form methyl esters was suggested. Since methylation of the corn oil samples when no

Table 6. Neutralization of 2 ml 98% sulfuric acid by potassium hydroxide (85% purity).

| g KOH | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 | 4.0 | 4.5 | 5.0 | 5.5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| meq KOH | 0.0 | 7.6 | 15.2 | 22.8 | 30.4 | 37.9 | 45.6 | 53.2 | 60.7 | 68.3 | 75.8 | 83.5 |
| meq $H_2SO_4$ | 37.3 | 37.3 | 37.3 | 37.3 | 37.3 | 37.3 | 37.3 | 37.3 | 37.3 | 37.3 | 37.3 | 37.3 |
| Excess acid | 37.3 | 29.7 | 21.2 | 14.5 | 6.9 | ---- | ---- | ---- | ---- | ---- | ---- | ---- |
| Excess base | ---- | ---- | ---- | ---- | ---- | 0.6 | 8.3 | 15.9 | 23.4 | 31.0 | 38.5 | 46.2 |

sulfuric acid and increasing amounts of potassium hydroxide were used (Table 1, set 2) was complete at all levels of alkali, it appeared that sulfuric acid was not acting as a catalyst. If it were acting as a catalyst, the treatments in Table 1, set 3 should result in the formation of methyl esters. If the lack of potassium hydroxide for neutralization of the excess sulfuric acid did cause some reversion, large amounts of free fatty acids would be expected rather than the unreacted triglycerides that were found. No free fatty acids were observed on TLC. Among the soy phospholipid samples that were completely methylated (treatments D, E, F, G and L, Table 4), treatments E and F included no sulfuric acid in the reaction while treatment G used only 1 ml. Thus, both the triglyceride and phospholipid samples tend to support the conclusion that the reaction is essentially a base-catalyzed interesterification reaction.

Kurz (1937) found that potassium hydroxide catalyzed alcoholysis consisted of two reactions, namely, saponification followed by re-esterification. When the reaction was conducted at 20 C, he found an increase in the velocity of the saponification reaction with an increase of water in the alcohol and with an increase in the alkali content of the reaction mixture. When methylated samples in this study were poured into water at the beginning of the extraction process, many of the samples reacted exothermally with the water. It is possible that the combination of excess alkali and water causes the

saponification and subsequent re-esterification with methanol that Kurz reported.

When operating conditions are adjusted satisfactorily and peak parameters are measured carefully, the accuracy of quantitative analysis by GLC should be within 1%. Absolute peak areas produced by different compounds do not change relative to one another when either flow rate or temperature are varied and no effect has been observed on relative areas. The internal normalization method can be used to compute the per cent of each component in the mixture without regard for the absolute amount of material present. The areas of all the peaks are summed and this value is divided into the area of the individual peaks. The amount of each component is then represented as area per cent. If peak areas are calculated directly from the chromatogram without correction for variations in detector response, satisfactory results are obtained only when detector response does not vary much from compound to compound. Thermal conductivity detectors are unsatisfactory in this respect although differences are smaller when helium or hydrogen rather than nitrogen is used as the carrier gas.

Too much reliance should not be placed on the results of the analysis of variance of the GLC data. To be most valid statistically, such an analysis should include data from many samples. In this research there were a limited number of samples which decreases the applicability of the statistical analysis.

In these studies TLC was used to judge completeness of reaction and GLC was used to see if any differences in composition could be attributed to treatment effects. The GLC data show differences, but a larger number of samples are needed to determine the statistical significance of the variations between samples. The present data indicate that the ratios of a polyunsaturated fatty acid to stearic acid were not significantly affected by differences in treatments. The agreement of the ratio of 18:3 to 18:0 among the soy phospholipid samples is especially good in view of the wide range of completeness of methylation.

## SUMMARY

1) The quantity of potassium hydroxide used in the low temperature-sulfuric acid methylation procedure of McGinnis and Dugan (1965) does not affect the apparent fatty acid composition of either triglycerides or phospholipids as measured by GLC of the resulting methyl esters.

2) As judged by TLC, increasing amounts of potassium hydroxide resulted in increased amounts of methyl esters and more complete methylation of triglyceride samples.

3) Sulfuric acid in the absence of potassium hydroxide resulted in no methyl ester formation in the phospholipid samples.

4) Sulfuric acid in the absence of potassium hydroxide resulted in no methyl ester formation in triglyceride samples except in one lard and one butter oil sample where the anomaly is thought to be an artifact since it was not found in a duplicate lard sample.

5) When no sulfuric acid was used, methylation of both phospholipid and triglyceride samples was complete at all levels of potassium hydroxide.

6) Evidence is given for the proposal that the mechanism is actually a base-catalyzed interesterification.

7) No significant differences in the ratio of 18:2 to 18:0 (corn oil samples) or 18:3 to 18:0 (soy phospholipid samples) were observed. This indicates that the polyunsaturated fatty acids are not preferentially methylated.

8) While the time between addition of the methanol and the addition of potassium hydroxide did not appear to change the amount of methyl esters produced from triglyceride samples, a reaction period of 20 minutes resulted in complete methylation of a soy phospholipid sample.

9) Virtually complete methylation of triglyceride samples can be effected at a level of 5.0 g KOH/15 ml methanol and sulfuric acid at a level of 2 ml or less or by using from 1-5.5 g KOH/15 ml methanol and no sulfuric acid.

10) Complete methylation of phospholipids can be effected by using no sulfuric acid and 2-4 g KOH/15 ml methanol, by using 1-2 ml sulfuric acid in conjunction with 5.5 g KOH/15 ml methanol or by using 2 ml sulfuric acid, 5.5 g KOH/15 ml methanol and a 20 minute period between the addition of methanol and potassium hydroxide.

11) The conditions chosen for a triglyceride sample depend on the kind of analysis desired.

12) The conditions given above for the phospholipid samples (see 10) are recommended in order to avoid the appearance of the unknown peaks a and b which radically alter the apparent fatty acid composition.

## LITERATURE CITED


Ast, H. J. 1963. Inadvertent isomerization of polyunsaturated acids during ester preparation. Anal. Chem. 35:1539.

Bloor, W. R. 1943. Biochemistry of the fatty acids. Reinhold Publishing Corp., New York. Pp. 1-2.

Boyle, J. J. and E. H. Ludwig. 1962. Analysis of fatty acids of continuously cultured mammalian cells by gas-liquid chromatography. Nature 196:893.

Cropper, F. R. and A. Heywood. 1953. Analytical separation of the methyl esters of the $C_{12}$-$C_{22}$ fatty acids by vaporphase chromatography. Nature 172:1101-1102.

Dittmer, J. and R. L. Lester. 1964. A simple specific spray for determination of phospholipid on thin-layer chromatography. J. Lipid Res. 5:126-127.

Dugan, L. R., Jr., G. W. McGinnis and D. V. Vadehra. 1966. Low temperature direct methylation of lipids in biological materials. Lipids 1:305-308.

Folch, J., M. Lees and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-509.

Hornstein, I., J. A. Alford, L. E. Elliot and P. F. Crowe. 1960. Determination of free fatty acids in fats. Anal. Chem. 32:540-542.

James, A. T. and A. J. P. Martin. 1952. Gas-liquid chromatography: the separation and microestimation of volatile fatty acids from formic acid to dodecanoic acid. Biochem. J. 50:679-690.

Lea, C. H., and D. N. Rhodes and R. D. Stoll. 1955. Phospholipids. 3. On the chromatographic separation of glycerophospholipids. Biochem. J. 60:353-363.

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

Kurz, H. 1937. In Fatty Acids. K. S. Markley. Interscience Publishers, Inc., New York. Pp. 299-300.

McGinnis, G. W. and L. R. Dugan, Jr. 1965. A rapid low temperature method for preparation of methyl esters of fatty acids. J. Am. Oil Chemists' Soc. 42:305-307.

Metcalfe, L. D. and A. A. Schmitz. 1961. The rapid preparation of fatty acid esters for gas chromatographic analysis. Anal. Chem. 33:363.

Metcalfe, L. D., A. A. Schmitz and J. R. Pelka. 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem. 38:514.

Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethyl acetals from lipids with boron fluoride-methanol. J. Lipid Res. 5:600.

Pitt, G. A. J. and R. A. Morton. 1957. Progress in the chemistry of fats and other lipids. Vol. 4. Ultraviolet spectrophotometry of fatty acids. Pergamon Press, New York. Pp. 227.

Schlenk, H. and J. L. Gellerman. 1960. Esterification of fatty acids with diazomethane on a small scale. Anal. Chem. 32:1412.

Smith, L. M. and E. L. Jack. 1954. The saturated fatty acids of milk fat. I. Methyl ester fractionation and isolation of monoethanoid constituents. J. Dairy Sci. 37:380.

Stoffel, W., F. Chu and E. H. Ahrens, Jr. 1959. Analysis of long-chain fatty acids by gas-liquid chromatography. Micromethod for preparation of methyl esters. Anal. Chem. 31:307.

Vorbeck, M. L., L. R. Mattick, F. A. Lee and C. S. Pederson. 1961. Preparation of methyl esters of fatty acids for gas-liquid chromatography. Quantitative comparison of methylation technique. Anal. Chem. 33:1512.