A Rapid Low Temperature Method for Preparation of Methyl Esters of Fatty Acids[1,2]

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Abstract

A rapid method for preparation of methyl esters of fatty acids in lipids has been accomplished by forming the sulfuric acid complex of the lipid in ethyl ether at the temp of a dry ice-acetone bath. Decomposition of the complex with methanol results in direct formation of methyl esters of the fatty acids. A comparison was made of gas liquid chromatography (GLC) analysis of fatty acid composition of several fats using methyl esters prepared by this and by two other methods. Results of this comparison reveal that the method is not only rapid but provides complete reaction with no apparent changes in the fatty acids.

Introduction

THE PREPARATION of methyl esters of fatty acids for analysis by (GLC) has drawn the attention of many investigators. Evolution of methods processed from methods for esterification or interesterification by the more obvious means to more sophisticated means employing the shortest time with least modification or loss of product. Completeness of reaction, and sample size are also matters of great concern to investigators working with great numbers of samples from biological systems which can be sampled only in small volume or mass.

Two general approaches can be used. The most commonly used method involves liberation and isolation of fatty acids from lipids by saponification, acid hydrolysis, or enzymatic hydrolysis. The esters of the fatty acids may then be prepared by a variety of methods which require acid catalysis of the esterification reaction. The use of anhydrous HCl in methanol (16) boron trifluoride in anhydrous methanol (9) diazomethane (13) and sulfuric acid with methanol (14,2,11,12), have been used with varying success. The in-situ esterification, by methanolic HCl, of fatty acids held on a basic ion-exchange resin has also met with success (4). The possibility of conjugating the double bonds in polyunsaturated fatty acids during saponification has been suggested and a saponification method proposed (1) which negates this effect.

The second approach involves direct formation of esters by interesterification reactions brought about by basic catalysis (6,7,15).

A number of objections have been raised to the methods in most general use. These vary from the safety hazards involved in working with diazomethane to long reaction time, conjugation of double bonds, and incomplete reaction.

The technique and studies described here derive from an attempt to incorporate the advantages of time-saving, completeness of reaction, and minimum effect on components of a lipid system. They are based on the chemical behavior of esters in strong sulfuric acid.

Hantzsch (3) noted that most carboxylic acids behave as simple bases in strong sulfuric acid and are monoprotonated. Newman (10) postulated the formation of an acylium ion $RC{\equiv}O^+$ which occurs by a two-stage ionization when carboxylic acids are in the presence of strong sulfuric acids. He noted that treatment of acylium ion-sulfuric acid mixtures with alcohols lead to immediate formation of the ester in high yield. Kuhn and Corwin (5) found that esters of alochols with strongly electron attracting groups, e.g. β-Cl-ethylacetate and many esters of aromatic acids are readily hydrolyzed by dissolution in concentrated sulfuric acid and treatment with water or alcohols. Under these conditions acylium ions are converted to acids and esters, respectively. It appeared that if acylium ion-sulfuric acid complexes could be formed from the fatty acids in lipid moieties the formation of methyl esters would be possible. This was accomplished at low temp in solvent systems suitable for maintaining the lipid in solution at these temps.

Methods

Preparation of Methyl Esters

Base-Catalyzed Interesterification Method. The interesterification procedure described by Smith and Jack (15) was adapted for the preparation of methyl esters of some fats and oils. A 3.5 ml portion of neutral absolute methanol was added to 1 g of fat in 4 ml of pentane. To this mixture was added 0.2 ml 1.0 *N* KOH in absolute methanol. The mixture was gently swirled and allowed to stand in a dark cabinet at room temp (Ca. 22C) for 48 hr. The solution was then washed once with 20 ml of 0.06 *N* HCl and three times with 0.02 *N* HCl in a 50 ml separatory funnel. The samples were dried over sodium sulfate before they were concentrated at room temp, to a volume of 1 ml in a graduated 12 ml centrifuge tube.

Saponification and Esterification Method. A 200 mg sample of fat or oil was saponified with KOH as outlined by Ast (1). Methyl esters of the extracted free fatty acids were prepared by the method of Hornstein, et al. (4). Amberlite CG 400, 100-200 mesh was substituted for Amberlite IRA 400.

Low Temperature Sulfuric Acid Method. Sulfuric acid-methanol preparation of methyl esters. The amount of 200 mg of fat was dissolved in 20 ml of peroxide free diethyl ether in a 125 ml Erlenmeyer flask. The flask was placed in a dry ice-acetone bath and stirred by means of a magnetic stirrer. When the mixture had cooled to -60C, two ml of concentrated sulfuric acid were added from a 10 ml microburette at a rate of 1 ml/min. The bath temp was allowed to warm to -10C during a 10 min interval. The bath temp was again cooled to -60C; 15 ml

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TABLE I

Fatty Acid Composition of Butter Oil Determined by Base Catalyzed Interesterification, Saponification Followed by Esterification and by the Sulfuric Acid Method[a,b]

Fatty Acid[c]	Base-Catalyzed Interesterification	Saponification and Esterification	Sulfuric Acid-Methanol		
			Dry Ice Bath	Ice Bath	Room Temp
	%	%	%	%	%
6:0	2.5	1.2	1.4	1.9	2.4
8:0	1.7	1.4	1.4	1.5	1.7
10:0	3.7	3.4	3.4	3.5	3.3
11:0[d]	0.4	0.2	0.3	0.3	0.3
12:0	4.3	4.0	3.9	4.0	4.1
14:Br(?)[e]	trace[f]	trace	trace	trace	trace
14:0	12.6	13.1	12.4	12.3	12.2
14:1	1.6	1.8	1.7	1.6	1.7
15:0[d]	1.1	1.1	1.1	1.1	1.1
16:Br(?)[e]	trace	trace	trace	trace	trace
16:0	31.3	31.3	31.5	31.0	30.7
16:1	2.4	2.6	2.5	2.6	2.6
17:0	0.8	1.0	0.7	1.0	0.6
18:Br(?)[e]	trace	trace	trace	trace	trace
18:0	10.8	10.0	10.9	10.9	11.1
18:1	23.2	24.9	24.8	24.3	24.5
18:2	2.9	2.8	2.9	3.0	2.9
18:3	0.9	1.0	1.0	1.0	0.9

[a]Temp programmed 100-210C.
[b]Each column represents an average of two analyses.
[c]Number to left of colon indicates carbon number; number to the right indicates the number of double bonds.
[d]Identified by plotting retention time vs. carbon number.
[e]Br: Branched chain compound.
[f]Trace = les than 0.2%.

of absolute methanol followed by 13 ml of 35% methanolic-KOH were added. The mixture was removed from the dry ice bath and stirred by means of a magnetic stirrer until it reached room temp. Phenolpthalein was used to ascertain the presence of excess base. The mixture was quantitatively transferred to a 500 ml separatory funnel containing 150 ml of water. The methyl esters were extracted once with 30 ml of pentane and twice with 15 ml pentane. All samples were dried over anhydrous sodium sulfate before they were concentrated to a volume of 0.2 ml in graduated 12 ml centrifuge tubes.

Butter oil, coconut oil and soybean oil samples were esterified at room temp and in an ice bath by maintaining all of the above conditions with the exception of temp.

Thin-Layer Chromatography: Chromatographic

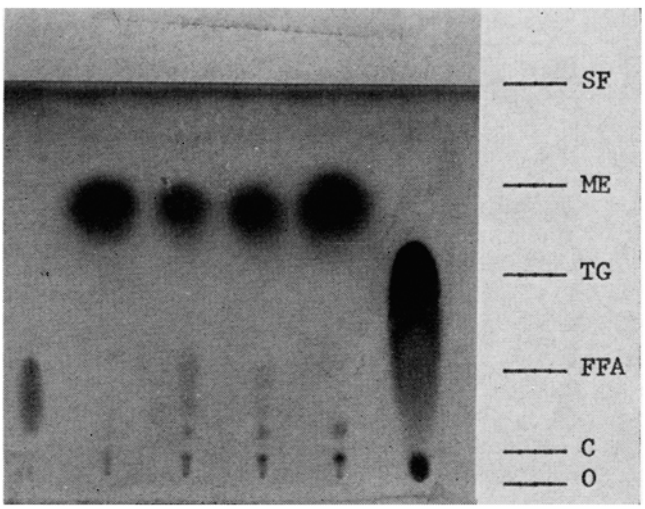

FIG. 1. TLC of butter oil methyl esters and nonesterified material. Solvent system: n-hexane·diethyl ether acetic acid 90:10:1 (v/v/v). Indicator: 50% sulfuric acid spray followed by charring. *A*: after Ast saponification; *B*: saponification + Hornstein esterification; *C*: H_2SO_4-methanol esterification at room temp; *D*: H_2SO_4-methanol esterification in an ice bath; *E*: H_2SO_4-methanol esterification in a dry ice-acetone bath; *F*: untreated butter oil + palmitic acid. Abbreviations: *SF* = solvent front; *ME* = methyl ester; *TG* = triglyceride; *FFA* = free fatty acid; *C* = cholesterol; *O* = origin.

TABLE II

Fatty Acid Composition of Coconut Oil Determined by Base Catalyzed Interesterification, Saponification Followed by Esterification, and by the Sulfuric Acid-Methanol Method[a]

Fatty Acid[b]	Base Catalyzed Interesterification[a]	Saponification and Esterification[c]	Sulfuric Acid-Methanol[d]		
			Dry Ice	Ice	Room Temp
	%	%	%	%	%
8:0	8.7	6.9	8.5	8.3	8.6
10:0	6.7	5.9	6.6	6.6	6.5
12:0	48.7	50.9	49.1	49.2	48.9
14:0	17.9	18.2	17.8	17.7	17.8
16:0	8.4	8.5	8.5	8.2	8.5
18:0	2.3	2.1	2.2	2.5	2.4
18:1	6.0	6.2	6.1	6.3	6.0
18:2	1.3	1.3	1.3	1.3	1.3

[a]Temp. programmed 150-210C.
[b]Number to left of colon = carbon number; number to right represents number of double bonds.
[c]Average two analyses.
[d]Average of three analyses.

plates were prepared with the Desaga applicator by making a slurry of 20 g Adsorbosil 1 with 35 ml of distilled water-acetone 2:1(v/v). Four plates were coated and activated at 110C for 35 min. The samples were spotted with a 10 ml Hamilton syringe and developed in an ascending manner in n-hexanediethyl ether-glacial acetic acid 90:10:1 (v/v/v). The plates were sprayed with 5% sulfuric acid followed by charring to visualize methyl ester spots and nonesterified material.

Gas Chromatography: An F and M Scientific Corp. Model 500 programmed temperature chromatographic unit with thermal detector was used with a 7 ft x 0.25 inch OD coiled column packed with 20% diethylene glycol succinate polyester and 1% phosphoric acid on 80/100 mesh acid-washed Chromosorb W. The helium flow rate was 75 ml/min. The detector temp and injection port temp were 250C. The column temp varied for the fat studied.

Results

In order to evaluate the effectiveness of the low temp sulfuric acid method for preparing methyl esters, a number of different fats were analyzed quantitatively by GLC for fatty acid composition. The composition of each fat was compared with the composition resulting from preparing methyl esters by one or two other methods. One method was that of Smith and Jack (15) which involves base-catalyzed interesterification. The other combined the rapid controlled saponification method of Ast for preparing fatty acids with the method using HCl-methanol on basic ion exchange resins developed by Hornstein et al. (4). This permitted comparison of some widely different methods commonly used in fatty acid analysis by GLC.

Butter oil was selected in order to test the efficiency on a fat having shorter chain fatty acids. The results are shown in Table 1. Generally the results from each method compared quite favorably. A comparison was also made of temp effect on prepar-

TABLE III

Fatty Acid Composition of Soybean Oil Determined by Base Catalyzed Interesterification, Saponification Followed by Esterification, and by the Sulfuric Acid-Methanol Method[a,b]

Fatty Acid[c]	Base Catalyzed Interesterification	Saponification and Esterification	Sulfuric Acid-Methanol	
			Dry Ice	Room Temp
	%	%	%	%
16	11.6	11.6	11.5	11.5
18	3.7	4.2	4.4	4.2
18:1	25.1	26.1	25.8	25.7
18:2	50.7	50.1	50.5	50.6
18:3	9.0	7.6	8.0	7.9

[a]Isothermal at 210C.
[b]Average of two analyses.
[c]Number to left of colon indicates carbon number; number to right indicates number of double bonds.

TABLE IV

Fatty Acid Composition of Lard, Tallow, and Corn Oil Determined by Saponification Followed by Esterification and by the Sulfuric Acid-Methanol Method

Fatty Acid[d]	Lard[a,c]		Tallow[b,c]		Corn oil[b,c]	
	Saponification and Esterification	Sulfuric Acid-Methanol	Saponification and Esterification	Sulfuric Acid-Methanol	Saponification and Esterification	Sulfuric Acid-Methanol
14:4	1.4[e]	1.5	3.4	3.5	t	t
14:1	—	—	2.9	3.0	—	—
15:0[e]	—	—	trace[g]	trace	—	—
16:0	25.0	24.6	25.4	26.2	13.1	13.6
16:1	2.9	3.0	11.1	10.8	—	—
17:0	—	—	trace	trace	—	—
18:Br(?)[f]	—	—	1.4	1.0	—	—
18:0	16.0	15.0	6.6	6.4	1.6	1.8
18:1	42.0	43.0	46.0	46.5	31.2	31.2
18:2	10.0	10.2	2.2	1.7	53.4	52.7
20	0.5	0.6	—	—	—	—
18:3	1.6	1.7	1.2	1.1	0.6	0.6
20:1[e]	0.4	0.5				
>20:1	0.4	0.6				

[a]Temp programmed 175-210C.
[b]Isothermal at 205C.
[c]Average of two analyses.
[d]Number to left indicates carbon number; number to right indicates number of double bonds.
[e]Identified by plotting retention time vs. carbon number.
[f]Br(?) = branched chain.
[g]Trace = less than 0.2%.

ing methyl esters by conducting the reactions at three temp—that of the dry ice-acetone bath, ice bath, and at ambient temp. While the results shown in Tables 1, 2 and 3 seemed to show little difference due to temp reaction, a monitor plate by thin layer chromatography (TLC), as shown in Figure 1, showed a more complete reaction at the lowest reaction temp. This was evidenced by complete absence of any spots on the plate in the free fatty acid or triglyceride region whereas these spots appeared in increasing intensity as the reaction temp was raised.

The fatty acid composition of coconut oil was determined after preparation of methyl esters by the three methods. Results are shown in Table II. This provided a study of a relatively saturated fat with a broad spectrum of fatty acids.

The effect of the method on a relatively highly unsaturated fat is shown by the results with soybean oil noted in Table III.

The data in Table IV show results of analysis of lard, tallow, and corn oil using the low-temp sulfuric acid method and the saponification-ion exchange resin method for preparation of methyl esters. Wide differences in composition due to method of methyl ester preparation were not observed with any of these fats.

Discussion

The low-temp sulfuric acid method for preparing methyl esters of fatty acids of lipids offers a number of advantages over other methods now commonly used. It permits direct conversion of the fatty acid components to methyl esters without need for saponification and isolation of the fatty acids. In this respect it resembles base-catalyzed interesterification. It offers another possible advantage here in that the acidic medium should not promote any conjugation of double bonds. That conjugation of double bonds occurs under conditions of base-catalyzed interesterification has not been established but conjugation during saponification has been discussed by Ast (1).

Sphingomyelins are notedly resistant to basic hydrolysis. Lipids having sphingomyelin components are less likely to have complete conversion of fatty acid components to methyl esters under conditions which involve saponification or which involve base-catalyzed interesterification. The time required for conversion is very short and permits preparation of a number of lipid samples for analysis within a relatively short time. The completeness of reaction is evidenced both by comparison of analyses of a number of fats using different methods for methyl ester preparation prior to GLC and by TLC.

Initial studies conducted with solutions of fat in pentane proved quite satisfactory for liquid fats (oils) such as safflower, corn, and soybean oil. However, incomplete reactions were commonly observed when lard, tallow, and butter oil were used. It was noted that these fats failed to stay in solution at the low temp utilized. Substitution of ethyl ether provided the solvent requirement to maintain solution and permit a complete reaction. This solvent was then adopted for all reactions and has proved to be satisfactory for all lipid systems studied to date.

It would appear that for many situations where absolute precision was not required a close analysis could be obtained by reacting at ice-bath temps or at ambient temps. It should be pointed out, however, that temp always rises when the sulfuric acid is added to the system and this may be a source of loss of short chain components when the temp is not sufficiently depressed. It is believed that the erratic results which were obtained for the butyric acid components of butter oil were occasioned by losses during the extraction and condensing steps. A real advantage for analysis of short chain components it to have methods of methyl ester preparation which eliminate these steps as proposed for the method using dimethoxy propane (8).

Occasionally, the GLC trace of esters, prepared by other methods from lipids which had been oxidized to varying degree, showed peaks which did not correspond with those for the known components of the lipid. It was observed that these extraneous peaks failed to appear when esters were prepared by the low-temp sulfuric acid methanolysis. The character of the extraneous peaks or the reason for their failure to be present when esters were prepared by this new method has not yet been investigated.

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