REVIEW

Preparation of Fatty Acid Methyl Esters for Gas-Chromatographic Analysis of Lipids in Biological Materials

Ke-Shun Liu*
Soyfood Laboratory, Jacob Hartz Seed Co., Inc., Stuttgart, Arkansas 72160

Theoretically, preparation of fatty acid methyl esters (FAMEs) deals with reversible chemical reactions in a complex system. Methodologically, there are numerous ways, generally characterized by the type of catalysts used and steps involved. Although there are more than a half dozen common catalysts, the majority fall into either acidic (HCl, H_2SO_4 and BF_3) or alkaline types ($NaOCH_3$, KOH and NaOH), with each having its own catalytic capability and application limitations. In terms of steps, many conventional methods, including those officially recognized, consist of drying, digestion, extraction, purification, alkaline hydrolysis, transmethylation/methylation and post-reaction work-up. Although these methods are capable of providing reliable estimates if some precautions are taken, they are cumbersome, time-consuming and cost-inefficient. A new approach has been to transmethylate lipids *in situ*. Due to its simplicity, high sensitivity, comparable reliability and capability to determine total fatty acids, the method of direct transmethylation is finding a unique place in lipid determination. Regardless of which method is used, quantitative methylation requires chemists to take precautions at every step involved, particularly during FAME formation and subsequent recovery steps. Evidently, there is an urgent need for more systematic studies, guided by the chemical principle of reactions involved and physicochemical properties of regents and end products, into factors affecting these steps. Hopefully, this will lead to an improved method, which measures lipid composition in biological materials not only with high accuracy but also with high efficiency and minimum costs.

KEY WORDS: Derivatization, fatty acid methyl esters, gas chromatography, lipid analysis.

The use of gas chromatography (GC) to characterize fatty acid profiles of lipids in biological materials (including human food) has been routine in laboratories of various scientific institutions and industrial organizations. A necessary procedure associated with this analysis is lipid derivatization. This process changes the volatility of lipid components, and improves peak shape and thus provides better separation. Although there are many derivatization procedures described in the literature, the majority involve conversion of fatty acid components into corresponding esters, usually methyl esters (1–3). Even with this conversion, the method has varied considerably in terms of steps involved, solvents and reagents used, conditions applied and size of samples tested. This variation in methodology may be necessary for various types of biological samples and laboratory conditions, but it could lead to confusion as well as difficulty in comparing data from different studies.

The common procedures for preparing fatty acid methyl esters (FAMEs), including those officially recognized (4–9), have traditionally consisted of extraction of lipids out of biological materials and transesterification of extracted lipids. Many of these methods are capable of producing reliable data if some special precautions are taken (2). However, due to involvement of multiple steps to complete each procedure, the conventional procedure is complex, inefficient and, in some cases, impractical. To overcome these problems, a growing number of investigators have described an alternative procedure that combines extraction and transmethylation into one step (10–24). Although the emergence of this so-called *in situ* or direct transmethylation method adds more diversity to the methods of FAME preparation, the attempt has met with varying degrees of success.

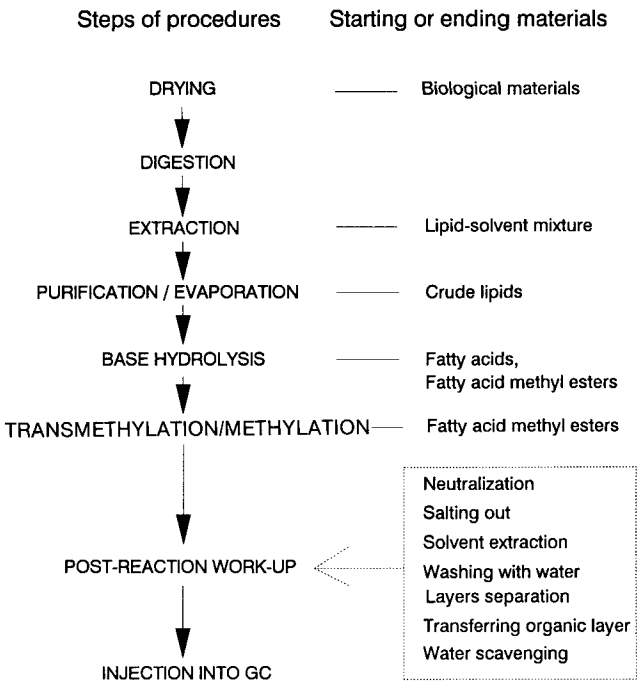
Over the years, there have been many review articles dealing with FAME preparation (3,25–31). However, the majority covered this subject under a general topic of lipid derivatization. Even on FAME preparation, all the articles, except for a recent one by Christie (31), dealt exclusively with the conventional method. In terms of research on this subject, emphasis has been given on achieving high accuracy and reliability in recent years (29,32–35). However, with increased evidence of the relationship between diet and heart disease and with recent enactment of the Nutritional Labeling and Education Act (NLEA) by the U.S. Congress, there is an urgent need for a method that measures lipids and their composition in biological materials not only with accuracy and reliability but also with convenience, cost efficiency and environmental soundness. In line with these facts, the present review on FAME preparation attempts to cover both conventional and direct transmethylation methods with respect to principles of reactions, critical parameters and limitations of their application. It also attempts to provide some useful information or stimulate focused research for laboratory chemists on application and development of a lipid derivatization method. Because space does not permit sufficient details to be given here for many procedures under discussion, readers are encouraged to refer to the original articles.

CONVENTIONAL METHOD

As outlined in Scheme 1, the common procedure for preparation of FAMEs out of biological samples traditionally consists of many steps. These may include drying, digestion, solvent extraction, purification/evaporation, alkaline hydrolysis, transmethylation/methylation and post-reaction work-up, depending on the type of samples to be analyzed and the particular method to be used.

Lipids extraction. Lipid extraction can be carried out with or without prior acid digestion, but samples must be thoroughly dried to facilitate solvent penetration. The purpose of digestion is to free "bound lipid" from the lipid-containing material. A digestion step is normally performed by heating samples with a high concentration of an acid solution in the presence of methanol (5,36). Lipids are then extracted with diethyl ether under refluxing (5). In addition to the ether, many other organic solvents or mixtures of them have been used, including chloroform,

*Address correspondence at Jacob Hartz Seed Co., Inc., 901 North Park Ave., Stuttgart, AR 72160.



SCHEME 1

dichloromethane, hexane, toluene, benzene and methanol (30,36–39). When choosing a solvent for a particular application, one needs to consider the potential hazard and costs of the solvent in addition to its effectiveness of extraction. Furthermore, an antioxidant is commonly added to the solvent to prevent lipid peroxidation, unless the sample contains sufficient natural antioxidants.

After extraction, the solvent and other volatile substances in the extract mixture are evaporated by mild heating under a stream of nitrogen gas. Prior to this step, solvent washing to remove nonlipid impurities may also be performed. The remaining part is referred to as crude lipids. At this stage, fractionation into polar and nonpolar lipids and separation of lipids into different classes may also be performed. If concerns are only with relative composition of fatty acids, no vigorous extraction is necessary. In some cases, the solvent–lipid solution is used directly for transmethylation/methylation without a parification/evaporation step (21,38,40).

TRANSMETHYLATION/METHYLATION

Formation of FAMEs is normally accomplished in the presence of a catalyst mixed with or dissolved in methanol. A heat treatment (commonly by a metal heating block) may be applied, depending on which catalyst is used and how long the reaction is allowed to proceed. There are more than a half-dozen catalysts. The majority can be characterized as either acidic (HCl, H_2SO_4 and BF_3) or alkaline ($NaOCH_3$, KOH and NaOH).

Terminology and principle. Lipids are mainly a mixture of esters, and preparation of FAMEs actually involves converting one ester to another. The reaction is referred to as "transesterification" in general and "transmethylation" in particular. Because the reaction involves cleavage of an ester by an alcohol, transesterification is also referred to as "alcoholysis" and transmethylation as "methanolysis." When FAMEs are formed from interactions between fatty acids and methanol, the reaction is termed as "methylation" ("esterification"). Therefore, strictly speaking, methylation and transmethylation (or esterification and transesterification) refer to different reactions, although in the literature these terms are used interchangeably.

According to the principle of organic chemistry, both transesterification and esterification are reversible reactions. However, transesterification can be catalyzed by either an acid or a base:

$$\text{R'-CO-OR''} + \text{R-OH} \underset{}{\overset{\text{H}^+ \text{ or } {}^-\text{OCH}_3}{\rightleftharpoons}} \text{R'-CO-OR} + \text{R''-OH} \quad [1]$$

whereas esterification cannot be catalyzed by a base but can be by an acid:

$$\text{R'-CO-OH} + \text{R-OH} \overset{\text{H}^+}{\rightleftharpoons} \text{R-CO-OR} + \text{HOH} \quad [2]$$

Under certain conditions, these reactions reach an equilibrium. To shift the equilibrium to the right, it is necessary to use a large excess of the alcohol whose esters we wish to make, or else remove one of the products from the reaction. The second approach is better when feasible because this way the reaction can be driven to near-completion.

The presence of water in the reaction system interferes with both transesterification and esterification. This is because esters, once formed, can undergo hydrolysis, which is the reverse reaction of esterification. However, unlike esterification, hydrolysis can occur in the presence of either acid or alkali. For acid hydrolysis, the reaction is reversible as shown in Reaction 2, while for alkaline hydrolysis:

$$\text{R'-CO-OR''} + \text{HOH} \overset{\text{OH-}}{\rightarrow} \text{R'-CO-O}^- + \text{H-OR''} \quad [3]$$

The reaction is essentially irreversible because the resonance-stabilized carboxylate anion shows little tendency to react with alcohol. Instead, it reacts readily with Na^+ or K^+ present in the reaction mixture to form a stable salt known as soap:

$$\text{R'-CO-O}^- + \text{Na}^+ \rightarrow \text{R'CO-O}^-\text{Na}^+ \quad [4]$$

Therefore, the alkaline hydrolysis of esters is also referred to as saponification.

Acid-catalyzed transmethylation/methylation. Acidic catalysts not only transesterify triglycerides and other complex lipids but also esterify free fatty acids in the presence of methanol. Three commonly used acid reagents are HCl, H_2SO_4 and BF_3, all in methanol. Heating is required to speed up the reactions. The temperature may range from 60–90°C, and duration may last from a few minutes to several hours. For this group of reagents, care should be taken to avoid concentrations higher than commonly recommended. Otherwise, undesirable side reactions could occur. One obvious cause of these side reactions is the loss of unsaturated esters (41).

Transesterification of fatty acids with anhydrous HCl/MeOH for gas–liquid chromatography was introduced about 35 years ago (42). It is one of the milder

reagents and has been claimed to be the best general-purpose esterifying agent (31). The reagent can be prepared either by bubbling anhydrous hydrogen chloride gas into methanol or by adding liquid acetyl chloride slowly to methanol (43). The concentration is normally 5%. Limited stability of the reagent was reported by Kishimoto and Radin (44), who found that half the titratable acid was lost at room temperature in six weeks. Alternatively, aqueous HCl in methanol (36% HCl solution/MeOH = 4:1, vol/vol) has also been used (45).

An alternative acid catalyst is sulfuric acid in methanol. The reagent can be readily prepared by adding concentrated sulfuric acid directly to methanol. The reported concentration ranges from 1–2%, but in our laboratory, this concentration range was found to cause a higher estimate of linolenic acid when a lipid sample was heated in an open tube at 90°C (Liu, K.-S., unpublished data). Under such a condition, a 0.5% concentration gave better results. Although the reagent has been cited for decomposing polyunsaturated fatty acids under certain conditions and requiring extensive work-up (26), these problems also occur with other acidic reagents (41). In fact, because of its easy preparation and its similarity to HCl/MeOH, H_2SO_4/MeOH was officially recognized by the Association of Official Analytical Chemists in 1965 (4).

Boron fluoride alcoholate is a strong Lewis acid. Its use for esterifying carboxylic acids in an analytical procedure with Karl Fischer regent was described as early as 1940 (46), but its use for preparing FAMEs from free fatty acids was not reported until 1961 (47). Later, Morrison and Smith (41) conducted an excellent study on transmethylation of lipids by BF_3-methanol reagent and found that its effect on neutral lipids could be greatly enhanced by the presence of an organic solvent such as benzene. Because BF_3/MeOH has a higher esterifying than transesterifying capability, Metcalfe *et al.* (48) described a method in which alkaline hydrolysis was combined with BF_3-catalyzed esterification. As a result, quantitative formation of FAMEs requires only 10 min. Since then, the procedure has quickly gained popularity. One strong indication is its adoption by the American Oil Chemists' Society (AOCS) in 1969 (6) to replace the H_2SO_4-methanol procedure. In fact, BF_3/MeOH is now the most commonly used catalyst for FAME preparation (2). The concentration usually ranges from 6 to 14%, reaction temperatures from 80 to 100°C, and time from 2 to 60 min.

The popularity of BF_3/MeOH does not necessarily mean that the reagent is the best. This is because BF_3 is toxic and expensive. It also has a limited shelf life. Furthermore, there have been reports that indicate the appearance of artifacts associated with the use of this reagent (49–51). Because it is prepared by bubbling BF_3 gas into cooled methanol, a good fume hood and special care are required to avoid white fumes emerging from the flask.

Alkali-catalyzed transmethylation. Compared with acid catalysts, alkaline catalysts transesterify neutral lipids in anhydrous methanol medium at a much faster speed. However, they are unable to esterify free fatty acids. Furthermore, the reaction requires more rigid anhydrous conditions because the presence of water leads to irreversible hydrolysis of lipids (see Reactions 3 and 4). A comprehensive review on this group of catalysts can be found in the literature (29).

Among the alkaline reagents, sodium methoxide in anhydrous methanol is the most popular one. Others include potassium hydroxide and sodium hydroxide in methanol. Potassium methoxide can also be used, but there is danger associated with handling the metallic potassium. With these reagents, many rapid procedures (29,52–54), including the alternative procedure of AOCS Ce 2-66 (7), have been reported, with alkali concentrations ranging from 0.2 to 3.3 N, reaction temperatures from ambient to refluxing, and reaction time from a few seconds to an hour.

Two great shortcomings associated with alkaline catalysts are their inability to esterify free fatty acids and their requirement of a more rigid anhydrous condition. Therefore, the method can be applied only to purified fats and oils with an acid value <2. Although this limitation does not prevent the method from being widely used in fats and oils industries after free fatty acids and water are removed from crude lipids during the refining process, the restrictions should be taken into consideration seriously when other lipid samples are to be tested. Otherwise, significant errors could result.

Sodium methoxide is prepared by dissolving sodium metal in dry methanol as shown in the following reaction:

$$2\ CH_3OH + 2\ Na \rightarrow 2\ Na^+OCH_3^- + H_2 \qquad [5]$$

When potassium or sodium hydroxide is used as the alkaline catalyst, a small amount of water is produced as a result of the following reaction:

$$Na^+OH^- + H\text{-}OCH_3 \rightleftharpoons Na^+OCH_3^- + HOH \qquad [6]$$

The $Na^+OCH_3^-$ formed catalyzes transesterification according to Reaction 1. However, the presence of OH^- in the medium also leads irreversibly to saponification as shown in Reactions 3 and 4.

Because formation of a soap could promote emulsification and delay the separation of the organic layer during extraction of esters, Bannon *et al.* (29) speculated that it would be desirable to use methoxide rather than hydroxide as the catalyst. They later (35) recommended the use of sodium methoxide under a refluxing temperature instead of hydroxide/ambient temperature when samples to be analyzed contain high levels of long-chain fatty acids.

Alkaline hydrolysis before methylation. It is not absolutely necessary to hydrolyze lipids to free fatty acids before methylation because most lipids can be transmethylated directly. However, many classical methods, particularly those using BF_3 as methylation reagent, still employ alkaline hydrolysis. This is because the alkali-catalyzed reaction can lead to near-completion of lipid hydrolysis, and free fatty acids released can then be esterified with an acid reagent at a faster rate. As a result, the whole operation time is shortened (48). Because the alkaline reagent is also capable of catalyzing transmethylation, it should be emphasized that during the alkaline hydrolysis step, some lipids are also transmethylated.

Alkaline hydrolysis is commonly carried out by refluxing extracted lipids with 0.5 N NaOH/MeOH solution for a few minutes (5). Other reagents, such as KOH/MeOH and tetramethyl ammonium hydroxide, work as well (45). Regarding the effects of time and temperature on saponification, Jham *et al.* (45) reported that no significant differences were noted in the relative percentage composi-

tion of the fatty acids, but the highest response was obtained by 5 min at a refluxing temperature. Nevertheless, excessive alkaline concentrations and high temperatures during saponification are not recommended because they may cause formation of conjugated double bonds (27).

Diazomethane. Diazomethane is another methylation reagent. It is generally prepared in ethereal solution by the action of alkali on a nitrosamide (e.g., *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) in the presence of an alcohol (55). Compared with acid catalysts, diazomethane in ether esterifies fatty acid at a much faster rate, although it has no ability to catalyze transesterification:

$$\text{R-CO-OH} + \text{CH}_2\text{-N}_2 \rightarrow \text{R-CO-OCH}_3 + \text{N}_2 \qquad [7]$$

Because of this feature, it has successfully been used in conjuction with alkaline reagents to compensate for the shortcoming of the latter (54). When diazomethane is used for preparation of FAMEs out of lipids, an alkaline hydrolysis step must precede (18,55). Because the reagent is highly toxic and potentially explosive, care must be taken during its preparation, and strong light and apparatus with ground glass joints must be avoided. In addition, the reagent has a short shelf life. In most cases, it needs to be freshly prepared. All these shortcomings prevent diazomethane from being used as commonly as other catalysts.

A general comparison of the seven commonly used transmethylation/methylation catalysts discussed above is summarized in Table 1 in terms of their catalytic capacity, common concentrations, reaction conditions and other features.

Lipid type and presence of a solvent. There are various types of lipids from biological materials, including triglycerides, phospholipids, sterols and sphingolipids. Except for sphingolipids, which contain N-acyl bonds, most others contain O-acyl bonds. Because almost all catalysts are carried in methanol as a transmethylation/methylation reagent, the rate of the reactions depends on not only the type of catalyst and reaction conditions but also on the solubility of a particular lipid in the methanol medium. For polar lipids, such as free fatty acids and phospholipids, which are well dissolved in methanol, the transmethylation/methylation reaction can proceed rapidly. However, for nonpolar lipids such as triglycerides, because of their poor solubility in methanol, the reaction will not proceed at a reasonable rate. This problem is effectively solved by adding another solvent into the reaction system, which helps dissolve lipids. In addition, in theory, this solvent should withdraw some esters formed in the reaction mixture and, therefore, help the reversible reactions (Reactions 1 and 2) shift toward ester formation. For the N-acyl type of lipids, a high concentration, vigorous refluxing conditions and a long reaction time are also required (16,26,41).

Under given transesterification/esterification conditions, the effects of solvent type and its ratio to methanol have been studied (17,41,56). Among solvents used during FAME formation are benzene, toluene, diethyl ether, ethyl chloride, dichloromethane, tetrahydrofuran, hexane, heptane and chloroform. In general, the type of lipids determines the solvent to be added to the reaction mixture, while the effectiveness of a solvent depends on its ability to solubilize lipids and its ability to mix with methanol. In addition, the tendency to form artifacts and potential risks of explosion and poison should also be considered. There are methods in which the same solvent is used for facilitating FAME formation and for extracting the FAMEs. Addition of extracting solvent is then omitted during post-reaction work-up step.

Qualitative methylation. Characterization of lipids includes qualitative and quantitative analyses. By definition, "qualitative analysis" refers to the measurement of the relative percentage of fatty acids in samples whereas "quantitative analysis" measures the amount of individual fatty acid per unit of a given lipid-containing sample. Bannon *et al.* (33) defined "quantitative methylation" as "a solution of the esters obtained with a fatty acid composition representative of that of the original sample." In this context, even for qualitative analysis, quantitative methylation should be ensured.

To obtain quantitative methylation, many methods described in the literature have emphasized the completion of transmethylation/methylation. This approach is undoubtedly useful for both quantitative and qualitative analyses. However, because Reactions 1 and 2 are reversible, theoretically they do not proceed to absolute completion, even in the presence of a large amount of methanol. Instead, they come to an apparent halt or an equilibrium at some point between 0 and 100% comple-

TABLE 1

A General Comparison Among Some Commonly Used Transesterifying/Esterifying Reagents

Features	Acid in MeOH			Alkali in MeOH			In ether
	HCl	H_2SO_4	BF_3	$NaOCH_3$	KOH	NaOH	CH_2N_2
Common concentration	5%	1–2%	6–14%	0.2–2 N			
Common reaction temperature	60°C–refluxing			ambient–refluxing			ambient
Common reaction time	30 min–2 h		2–10 min	a few seconds—1 h			a few minutes
Esterifying power	medium	medium	high	no	no	no	high
Transesterifying power	low	low	low	high	high	high	no
Form of starting material	gas/liquid	liquid	gas	metal	pallet	pallet	gas
Ease of preparation	no	yes	no	no	yes	yes	no
Water introduced during preparation	no	yes	no	no	yes	yes	no
Potential hazard associated with preparation	yes	no	yes	yes	no	no	yes
Saponification after reaction	no	no	no	yes	yes	yes	no
Sensitive to water interference	low	low	low	high	high	high	low

tion. When equilibrium is reached under given conditions, the net velocity of the reaction is zero because the absolute velocity in the forward direction exactly equals the absolute velocity in the reverse direction. At this point, the concentrations of the reactants and products remain constant with time.

The position of the equilibrium is generally described by an equilibrium constant, K_{eq}, which is a ratio of the rate constant of the forward reaction and that of the reverse reaction. K_{eq} is affected by reaction temperature and concentrations of reactants and products, but not by a catalyst. A catalyst only shortens the time required for a reaction to reach an equilibrium. This is because the catalyst speeds up not only the forward reaction but also the reverse reaction. In the real reaction system, more complexity is obvious due to the presence of the reaction solvent and other substances. In addition, when an alkaline catalyst is used, the situation becomes even more complex because there is an irreversible saponification involved. This subject will be discussed further under a separate section.

Post-reaction work-up. Preparation of FAMEs is finalized by some work-up procedures immediately after transmethylation/methylation. The objectives of this step are threefold. First, to transfer FAMEs formed in the reaction mixture quantitatively into an organic solvent; second, to purify the FAME-solvent solution so that injection of this solution produces few artifacts as well as little damage to column performance; and third, to prevent any undesirable side reaction that may continue to occur after the main reaction. Although there is great variation among methods, the work-up procedures may include neutralizing the reaction mixture, extracting FAMEs with an organic solvent, salting out with a salt solution, washing with water, separating layers of solvents by centrifugation or long standing, and drying the organic mixture with sodium sulfate. Solvent evaporation may also become necessary if one needs to increase FAME concentration or recover FAMEs for quantitative analysis.

According to Bannon *et al.* (33), there are four possible errors associated with FAME preparation. These are (i) failure to methylate quantitatively, (ii) failure to transfer esters quantitatively into an organic layer, (iii) evaporative losses of esters during work-up or storage and (iv) saponification of the esters after methylation when an alkaline reagent is used. Among these four errors, three are closely related to post-reaction work-up. Therefore, the post-reaction work-up is a crucial step in obtaining an accurate and reliable analysis, even though it may be time-consuming, tedious, and, in some cases, unnecessary. Unfortunately, many generally recognized procedures tend to emphasize small details in describing the work-up step. Some even regard the work-up as unnecessary and attempt to by-pass it. One major reason might be that the importance of this step is not well recognized. Another reason is that, in some situations, there are conflicts among minimizing work-up, ensuring quantitative FAME recovery and reducing damage to the GC column. For example, extensive work-up may reduce column damage and maximize transfer, but it may also lead to high evaporative and oxidative losses of esters and long operation time.

For quantitative transfer of FAMEs into an organic solvent, small precautions can actually make a big difference in some cases. For example, Bannon *et al.* (32) found that, for the AOCS method with BF_3 as a catalyst, extraction of FAMEs under tepid conditions (30–40°C) plus shaking of more than 15 s at the work-up step reduced a significant loss of low-molecular weight FAMEs. The extracting solvent should also play a key role here, even though it has been given little emphasis in the literature. The ideal solvent should provide similar solubility for all FAMEs formed in the reaction mixture, so that FAMEs extracted represent the real composition in the mixture. It should also have low solubility in methanol so that there is a clear separation between the two layers. To reduce the tendency of saponification after alkali-catalyzed reaction, neutralizing the reaction mixture, or washing repeatedly with water has been practiced. Further minimization of possible alkaline hydrolysis can be achieved by avoiding long-time storage of reaction mixtures (5). Another way to avoid irreversible alkaline hydrolysis is to use an acid catalyst. And finally, to mimimize the presence of any artifacts, one needs to have pure solvents and a clear separation of the organic layer from the methanol layer just before transfer. All these beneficial steps can also reduce damage to column life.

Lipids containing short-chain fatty acids. Lipids containing short-chain fatty acids, such as milk fat, can be transesterified by either acid or alkaline catalysts. However, quantitative transfer of esters from the reaction medium into an organic solvent is difficult unless special precautions are taken. This is because esters of short-chain fatty acids are both highly volatile and readily soluble in water. High volatility requires low reaction temperature, short reaction time and a minimal work-up step, whereas high polarity prevents any aqueous washing.

A method described by Christopherson and Glass (52) involving alkaline transmethylation under ambient temperature is well known for best meeting these criteria. In addition, the procedure uses stoichiometric amounts of the alcoholic base to avoid a large methanol peak, whose appearance makes estimation of methyl butyrate difficult. However, because the procedure uses an alkaline reagent, and irreversible saponification is thus inevitable, its reliability has been questioned by Bannon *et al.* (33). They found that the composition of the analytical sample by this procedure changed significantly in as little as 15 min, and they attributed this finding to the fact the shorter-chainlength FAMEs saponify faster than do those of longer chainlength. To overcome this problem, the authors suggested neutralizing the alkaline catalyst after 6 min reaction with 2.0 N HCl solution. This duration represents an optimum between a short reaction time to minimize saponification and a long reaction time to promote methanolysis of the long-chain triglycerides. Because of its convenience and simplicity, the method of Christopherson and Glass (52) has been used for preparation of other types of lipids as well. In fact, it is the basis for the alternative procedure of AOCS Ce 2-66 (7).

DIRECT TRANSMETHYLATION

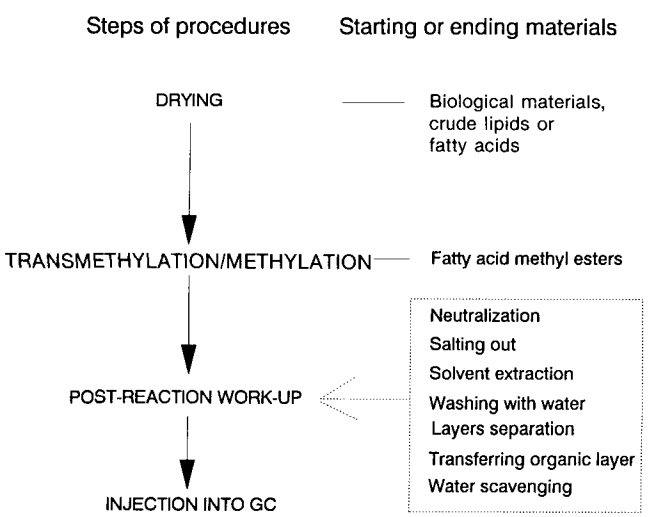
The method involving extraction and derivatization of lipids into FAMEs, discussed above, has represented a classical solution for many GC analyses of lipids in biological samples. Yet, this multistep methodology is often time-consuming and cumbersome. Generally, it requires large volumes of reagents and large sample sizes. In addition,

the multistep procedures may lead to introduction of contaminants and losses of esters. Therefore, it is impractical in those laboratories where the number of tests is relatively large and availability of samples is rather limited.

In an effort to overcome the limitations of the conventional method, many investigators have developed new procedures in which lipids are transmethylated *in situ* (11–24,57–59). As a result, the outlined procedure in Scheme 1 becomes simplified into the one shown in Scheme 2. Because of its simplicity, high sensitivity, high efficiency and relative reliability, there is a growing number of laboratories, including our laboratory, that use this *in situ* transmethylation procedure for preparation of fatty acid methyl, although no official recognition has been given so far.

Variation in terminology. Methods that involve simultaneous extraction and transmethylation of lipids in biological materials have been referred to in various terms, such as "direct transmethylation," "one-step extraction/methylation," "one-step extraction and esterification," "combined tissue digestion and esterification," "direct extraction derivatization," "direct methylation" and "*in situ* methylation." However, strictly speaking, some of these terms are incorrect, particularly those containing the word "methylation" or "esterification." The reason is that the major reaction that occurs in the system is transesterification rather than esterification. In addition, the term "direct esterification" or "direct methylation" has a connotation that refers (in organic chemistry) to conversion of a carboxylic acid to an ester without first converting it to an acid chloride.

Development history. As early as 1963, using BF_3-methanol, Abel *et al.* (10) succeeded in transmethylating lipids in bacteria *in situ* without prior extraction. The effectiveness of this method was later confirmed in both bacteria (57) and plant tissues (12). In 1966, Dugan *et al.* (11) directly transmethylated lipids in animal tissues by H_2SO_4/MeOH in diethyl ether at low temperature. With the same reagent, *in situ* transesterification of lipids also succeeded in cereal grains (13), soybean seeds (21) and rice bran (60), although the purpose of the last application was to facilitate removal of free fatty acids from the rice bran rather than lipid analysis.



SCHEME 2

In 1976, Outen *et al.* (15) described a method which directly transmethylated long-chain fatty acids in feeds, digesta and feces. The method involved mixing dried test materials, benzene and 5% methanolic HCl in screw-capped bottles, heating at 70°C for 2 h, and extracting formed FAMEs out of the mixture. Sukhija and Palmquist (20) adopted the method with modifications limited to post-reaction work-up. The method was further modified by Ulberth and Henninger (24) for determining the fatty acid composition of processed foods, following a suggestion by Sukhija and Palmquist (20) that toluene is preferred over benzene. The validity of this method was also confirmed by others (22). About the same time as Outen *et al.* (15), another one-step procedure for transmethylation of lipids in mammalian tissues was reported (14,16). The method involves the direct reaction of anhydrous HCl-methanol with lipids in approximately 10 mg of tissue or 0.1 mL of serum after removal of water from samples by reacting with 2,2′-dimethoxypropane. Acetone and methanol, produced during the reaction, as well as the excess dimethoxypropane, are evaporated by lyophilization prior to transesterification to eliminate the formation of artifacts from the solvents. In a later report, BF_3-MeOH was used as the reagent to directly transmethylate the dimethoxypropane-treated and lyophilized plasma samples (23).

Lepage and Roy (17) described an *in situ* method for characterization of triglycerides in mammalian tissues. Although their method advocates the same reagent as the one of Outen *et al.* (15), it differed from the latter in that acetyl chloride is added to the mixture of benzene–methanol instead of methanol alone. The reaction conditions were also changed from 2 h at 70°C to 1 h at 100°C. This method was later modified by the same authors for *in situ* transesterification of all classes of lipids (58) and for plasma nonesterified fatty acids (59). Browse *et al.* (18) described a method in which digestion and FAME formation from fresh leaf tissue were combined, also with methanolic HCl. No solvent is present during the reaction, but FAME recovery is carried out by a combination of hexane and water. They reported that the direct procedure led to reduced recovery of FAMEs, but the fatty acid composition obtained was comparable to that of conventional methods.

For transmethylation of lipids in rapeseeds, Hougen and Bodo (38) found that the methanolic sodium methoxide could be added to the sample either at the end or at the beginning of the solvent extraction without significantly affecting the final results. This might be the first report of an alkaline reagent for *in situ* transmethylation. Later, Long *et al.* (19) reported a procedure with methanolic KOH for directly transmethylating lipids in corn and soybean meals.

Because of the diverse methods reported in the literature and because many reporters used or modified a procedure for FAME preparation without giving a reference, it is difficult to trace the first report on direct transmethylation. It is also difficult to find out the original procedure on which a modification is based. Regardless of these difficulties, and regardless of variations in procedures described, the direct transmethylation generally involves adding an organic solvent and a methanolic-catalyst reagent to a small amount of sample, preferably dried, in a test

tube and heating the mixture for a certain time period (10 min to several hours), depending on heating temperature (65–100°C) and lipid composition. For quantitative analysis, an internal standard is commonly added. After transmethylation, the reaction mixture is then subjected to a work-up similar to that discussed for conventional methods.

Alkaline vs. acidic catalysts. Among the direct transmethylation methods reported, only a few used alkali catalysts (19,38) while the majority used acid catalysts. The theory behind this trend is that, unlike acid reagents, alkaline reagents not only have no capacity to methylate free fatty acids but also promote irreversible hydrolysis (saponification) of esters after their formation. As a result, the reaction system requires rigid anhydrous conditions. Consequently, in spite of the fact that the use of alkaline reagents for refined lipids (with acid value <2) has the advantage of being rapid, their use in biological materials for direct transmethylation is not recommended. Evidence supporting this recommendation includes (i) the yield of FAMEs from fresh leaf tissues, transmethylated directly by sodium methoxide (0.5M), is less than 10% of that by methanolic HCl (18); and (ii) *in situ* transmethylation of lipids in corn and soybean meals by methanolic KOH is hindered by the presence of more than 8% moisture in the samples)19).

Studies into interferences. A biological material is a complex mixture of chemical substances. Thus, there are concerns that *in situ* transmethylation of lipids may encounter many interferences or side reactions. Even with acidic reagents, possible water interference has been a concern for many method developers. For examples, several investigators (14,16) treated their lipid-containing samples with a water scavenger, 2,2′-dimethoxypropane before adding extraction/methylation reagents. Browse *et al.* (18) included 2,2′-dimethoxypropane into their catalytic reagent. Outen *et al.* (15) suggested that samples must be dried over P_2O_5 for about 12 h before testing. However, these practices have been cited as unnecessary, based on results of the following studies: (i) Lepage and Roy (58) reported that recoveries (>95%) of standards were unaffected by the presence of 5% water added; (ii) Sukhija and Palmquist (20) found that either freeze-dried or oven-dried (55°C) samples gave reproducible results because methanol in their reaction system could adequately absorb up to 100 mg of water (20% of their sample weight); and (iii) Ulberth and Henninger (24) added water directly to known amounts of a standard, which was a soya–maize–oil blend and found that the transesterification reaction was not hindered if the added water was below 100 mg. They also tested oven-dried liver sausage samples and found that up to 28.3 mg water added (corresponding to a moisture content of 40.7%) did not interfere with the formation of methyl esters from acyl glycerols. Nevertheless, it is the author's opinion that test samples should be as dry as possible regardless of which method (conventional or direct transmethylation) is applied.

Besides possible water interference, there are also concerns of interference by other substances present in the testing sample, such as proteins and carbohydrates. Fortunately, these substances appear to exert little effect on quantitative transmethylation. This conclusion is based on the fact that, compared with the conventional method, the majority of direct transmethylation methods described above have produced similar estimates of relative fatty acid composition. Some are even claimed to be more accurate (58). The theory behind this fact is rather difficult to explain. It is assumed that for reversible reactions like transmethylation of complex lipids an interference may lead to shift of the equilibrium for the formation of a given FAME, but at the same time this interference exerts a similar effect to the formation of all other FAMEs. As a result, the relative composition of the FAMEs is little affected.

Capability to determine total fatty acids. The direct transmethylation method requires a small sample size (as little as milligrams) and fewer reagents for lipid determination. At the same time, it allows chemists to have high throughput with a reasonable accuracy. The cost reduction is thus obvious. Furthermore, when an internal standard is used, the method has the capability to determine both an individual acid and total fatty acids in a given sample simultaneously. As several researchers have pointed out (19–21,24), this capability of direct transmethylation turns out to be one of its great advantages. Here is the reason. The lipid content is normally determined by solvent extraction. This represents the content of crude lipid, which is methodologically defined as the proportion of a given sample that is soluble in an organic solvent or mixture. This proportion is in fact a mixture of components that includes triglycerides, phospholipids, fatty acids, sterols, waxes and pigments. Because the fatty acid constituents are nutritionally important, the analysis of lipid content by solvent extraction has been criticized for its failure to accurately estimate nutritional values in biological materials. Therefore, a more appropriate method is one that estimates the content of total fatty acids.

Use of an internal standard. For some transmethylation/methylation reactions, particularly those catalyzed by acid reagents, it may take many hours to reach near completion. By using an internal standard, it is believed that quantitative analysis can be assured without requiring the reaction to go to completion (59) or worrying about losses of esters when more vigorous transmethylation conditions are applied (18). Here, an assumption is that the relative concentration of FAMEs formed at a given time after the reaction is initiated represents the true composition of fatty acids in the system. However, it should be emphasized that, for a given reaction system, this assumption may be true only after the system reaches equilibrium.

Many internal standards have been used in conjunction with the direct transmethylation method to quantitate lipid components, including tridecanoic acid (C18:3) (58), pentadecanoic acid (C15:0) (59), heptadecanoic acid (C17:0) (15,21) and nonadecanoic acid (C18:0) (20,24). Heptadecanoic acid methyl ester has also been used when estimation of FAME yield is an objective (18,21). When choosing an internal standard for a particular application, one needs to obtain a balance between the following: (i) it represents major fatty acids of interest in the lipids in terms of molecular size and structure, and (ii) its chromatographic peak is not in the region where a number of peaks are in close proximity. In addition, the internal standard selected should be absent from the biological samples to be tested. When an alkaline catalyst is used, a free fatty acid cannot be used as an internal standard. A triglyceride can substitute for it (19). Regardless of which internal

standard is to be used, it should be added at the beginning of the reaction. By doing so, it is taken through all the steps of the procedure. Consequently, the need to account for completion of methylation and the dilution of the GC injection volume is obviated.

FUTURE RESEARCH NEEDS

At present, analysis of fatty acid composition of lipids in biological materials is commonly carried out by GC after converting lipid components into corresponding methyl esters. There are many methods described in the literature to accomplish this conversion. Many of them are modifications of previously developed methods to meet an individual investigator's needs. Despite their great variations, these methods can generally be classified by the type of catalytic reagents used and steps of procedures involved. In terms of reagent type, there are more than a half dozen catalysts that are commonly used. The majority fall into either an acidic type (HCl, H_2SO and BF_3) or an alkaline type (sodium methoxide, sodium/potassium hydroxide), with each having its own catalytic capability and application limitations. Many common methods traditionally consist of multiple steps, including drying, digestion, extraction, purification, base hydrolysis, transmethylation/methylation and post-reaction work-up. A new approach for FAME preparation has been to transmethylate lipids *in situ*. By doing so, all steps (except for drying and post-reaction work-up) in the conventional method are combined into one. The new method requires less sample, less reagents and less work, but at the same time it has comparative reliability and capacity to determine total fatty acids in a given amount of sample. The improvement of analytical efficiency is obvious.

Regardless of how well a GC can perform and regardless of which method is used, quantitative methylation is critical for both qualitative and quantitative analyses of fatty acids in lipid-containing materials. In theory, preparation of FAMEs out of lipids deals with reversible chemical reactions in a complex system. Thus, factors affecting quantitative methylation include the type of lipids, the type of reagents, concentration of reagents, presence of an organic solvent, reaction temperature, reaction time and substances present in lipid-containing samples (such as water). In addition, at the post-reaction work-up step, quantitative transfer of FAMEs into an organic solvent without evaporative losses and side effects has proved to be a difficult task. Other concerns are irreversible saponification of esters when alkaline catalyst is used, and possible damage to the column by substances in the final FAME solution. In practice, FAME preparation requires chemists to take precautions at every step involved, particularly during FAME formation and subsequent recovery steps. Although collaborative or comparative studies are useful to find out which method is better than the other and how reliable a particular method is, additional studies into factors affecting each step of FAME preparation are urgently needed. Rather than by some empirical data, these studies should be guided by chemical principles of transmethylation and methylation reactions and by physicochemical properties of reagents and end products.

It is hoped that this review will not only provide some useful information to laboratory chemists on selecting a method for a particular application but also stimulate some worthwhile research into FAME preparation. Consequently, it may lead to an improved method that measures lipids in biological materials not only with high accuracy but also with high efficiency and minimum costs.

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