Journal of Chromatography, 247 (1982) 71–89
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,053

ANALYSIS OF FATTY ACID METHYL ESTERS WITH HIGH ACCURACY AND RELIABILITY

III. LITERATURE REVIEW OF AND INVESTIGATIONS INTO THE DEVELOPMENT OF RAPID PROCEDURES FOR THE METHOXIDE-CATALYSED METHANOLYSIS OF FATS AND OILS

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(Received May 26th, 1982)

SUMMARY

The literature leading to the development of rapid procedures for the preparation of fatty acid methyl esters for gas–liquid chromatography by methoxide-catalysed methanolysis of fats and oils is reviewed.

A rapid and reliable procedure utilizing the best features of the literature methods was developed and shown to give excellent quantitative results in the difficult case of a triacylglycerol primary standard mixture simulating coconut oil. Evidence is presented that brief refluxing of the reaction mixture is advantageous and that removal of methoxide by aqueous extraction at the completion of the reaction, as distinct from injecting the whole reaction mixture, is essential if good quantitative results are to be obtained. The method is faster and simpler than the existing international procedures, and the ester solution may be ready for injection into the chromatograph in about 2 min.

INTRODUCTION

The methylation of fats and oils for the purpose of determining the fatty acid composition by gas–liquid chromatography (GLC) is most commonly carried out by the boron trifluoride–methanol procedure as laid down in one of a number of essentially identical standards[1–4]. Although a rapid procedure for the methylation of free fatty acids using boron trifluoride–methanol alone was described in 1961[5], the first record of the technique for methylation of fats and oils was in 1966[6]. An indication of the rapid acceptance of the latter procedure was its approval as an American Oil Chemists' Society (AOCS) method in 1969[1]. In contrast, procedures utilizing base-catalysed methanolysis were carried out as early as 1956[7] for the purpose of GLC analysis, but gained official recognition only as recently as 1978[8] and 1979[9]. This

0021-9673/82/0000–0000/$02.75

situation appeared to us to be anomalous as there are now many examples in the literature describing the very rapid methylation of fats and oils using this technique which would appear to be especially advantageous to the fat and oil industry. It is recognized that base-catalysed methylation may be applied only to neutral fats and oils (acid value <2), but, for edible oil products, this may be considered an advantage, as it is the fatty acid composition of the acylglycerols that is of prime importance. Free fatty acids are removed from raw materials during refining, and the finished margarines and shortenings contain negligible amounts of these.

The slowness of official recognition of base-catalysed techniques has probably arisen out of the extraordinarily wide range of reaction conditions reported in the literature, which may well have caused confusion in the minds of many workers. Thus, solvents have varied in polarity from approximately 5% methanol in light petroleum to 100% methanol, base concentrations from 0.002 to 1 *N*, reaction temperatures from ambient to 90°C and reaction times from approximately 30 sec to 48 h.

It may appear even more anomalous that, despite this nebulous background, base-catalysed methanolysis is in fact widely used for the preparation of fatty acid methyl esters (FAME). To support this statement we quote data from the 1980–81 AOCS Smalley Gas Chromatography Check Program for Fatty Acid Analysis[10]. Of 472 samples for which information is available, 57% were methylated with boron trifluoride–methanol, 32% with sodium methoxide and 11% with other reagents. Thus, almost one third of the participants used base-catalysed methanolysis for the preparation of the esters. We take this to be an indication of the significant acceptance of the method, in one form or another, as an alternative to the boron trifluoride–methanol procedure.

The ultimate aim of this paper is to lead to improved standardized procedures and greater uniformity of technique amongst analysts working in the field. To this end we have endeavoured primarily to provide an overview of the experimental conditions which led finally to the development of very rapid methoxide-catalysed methylation techniques.

Following the review we evaluate a procedure which attempts to utilize the most desirable features of the many and varied literature methods and which we believe to be significantly more efficient than the current standard procedures[8,9] for the methylation of neutral fats. A shortcoming of all of the published methods is that their absolute accuracy is not demonstrated with triacylglycerol primary standard mixtures. We demonstrate that the developed procedure gives excellent quantitative results in the case of a triacylglycerol primary standard mixture simulating coconut oil, which we have previously shown[11] to be a particularly demanding test of the total procedure for the preparation and quantitation of the esters.

We also evaluate practices in the literature which receive little or no objective support. Thus, evidence is presented that brief refluxing of the reaction mixture is advantageous compared with carrying out the reaction at room temperature, and that the removal of the methoxide by aqueous extraction at the completion of the reaction, as distinct from injecting the whole reaction mixture, is essential if good quantitative results are to be obtained.

LITERATURE REVIEW

Nomenclature

Despite its widespread usage, no uniform definition of "transesterification" exists, the basic conflict being whether the term should be equated with ester interchange in the more general sense, or should mean only ester–ester interchange. Thus, three kinds of ester interchange are recognized in the general sense, these being ester–alcohol interchange or alcoholysis, ester–acid interchange or acidolysis and ester–ester interchange. Whereas Kirk-Othmer[12], Clark and Hawley[13], Christie[14], and many authors in the literature use it in this general sense, Bailey[15], Markley[16a] and Gunstone[17] equate it only with ester–ester interchange. We do not argue which definition or meaning is correct, but use the specific term "methanolysis" where a choice is possible.

Early developments

Glass[18] has rightly pointed out that a fundamental aspect, which continues to be sometimes misunderstood, of the behaviour of esters in solution in alcoholic base is that alcoholysis proceeds far more rapidly than saponification. The initial predominance of the alcoholysis reaction was recognized as long ago as 1898 by Henriques[19], who concluded that glycerides are first transformed by alcoholic potash into the ethyl esters which are then saponified. In 1920, Pardee and Reid[20] estimated from the data of Reid[21] and of Anderson and Pierce[22] that methyl benzoate was transformed into ethyl benzoate by methanolic base approximately 1500 times faster than it was saponified. While their investigations were concerned primarily with accounting for low saponification numbers which were often found for certain kinds of esters, this deduction underlined the high speed of the alcoholysis reaction relative to saponification. It is reasonable that similar behaviour could have been expected in the case of fats and oils under similar conditions, and subsequent studies clarified the practical considerations necessary to drive the alcoholysis towards completion for such materials with minimal saponification.

Toyama *et al.*[23] (1933) investigated the effect of various experimental conditions on the course of the ethanolysis and methanolysis of olive oil with alcoholic sodium hydroxide. They concluded that alcoholysis proceeded more rapidly with increasing amounts and concentrations of base and with increasing temperatures, and they also found that the reaction could readily be driven to completion by using the equivalent amount of sodium hydroxide. Soap formation was also promoted by the above factors, especially increasing temperature, and also by increasing water content, but was comparatively small. They also concluded that ethanolysis proceeded more rapidly than methanolysis.

Rowe[24] (1933) demonstrated that both the hydrolysis and alcoholysis of fats could be made to proceed with only catalytic amounts of base, as distinct from the relatively large amounts used by the Toyama group, and Kurz[25] (1937) complemented both the above studies by demonstrating that the splitting and, by implication, the methanolysis and ethanolysis of several oils could be driven virtually to completion using only catalytic amounts of alcoholic potash under suitable conditions. This finding was not the main objective of the work of Kurz, in which he unsuccessfully attempted to find ways of separating saturated from highly unsaturated fatty acids

and also monomeric from polymeric and condensed acids, but the experimental conditions were important as they were commonly used later as a means of preparing methyl esters for GLC purposes. Indeed, these results in some ways may have been counterproductive to the subsequent development of rapid transesterification methods as only long reaction periods of 24 h or more were generally associated with satisfactory, *i.e.*, near quantitative, yields. With hindsight, these long reaction periods may be seen to be associated with the generally low concentrations of base, the relatively low molar ratio of alcohol to substrate and the low reaction temperature of approximately 20°C used by Kurz, all of which were possibly selected with industrial practicalities in mind. A further significant conclusion reached by Kurz was that all conditions which decreased the saponification rate diminished the amount of potassium hydroxide necessary for complete transesterification.

Theory of the reaction

The above studies had thus elucidated the more important practical principles relating to the base-catalysed alcoholysis of fats and oils as early as 1937, but none of the above workers attempted to account for any of the observed behaviour. A comprehensive explanation, which was specifically framed in the context of the preparation of methyl esters for GLC, was not forthcoming until 1971, when Glass[18] assembled the relevant theoretical information which had accumulated in the intervening period. The essential features of the process are as follows.

First, the equilibrium shown in eqn. 1 lies well to the right, even in the presence of substantial amounts of water.

$$Na^+OH^- + ROH \rightleftharpoons Na^+OR^- + HOH \qquad (1)$$

Data to support this have been given by Caldin and Long[26,27]. Typically, in a solution made by dissolving sodium hydroxide in ethanol containing X % of water, the values of X and the percentages of the base present as OEt are, respectively, 0.2, 99; 0.5, 98; 1.0, 96; and 2.0, 94. This equilibrium alone largely accounts for the predominance of the alcoholysis reaction, especially in the case of methanolic potassium hydroxide in which the methanol is usually anhydrous.

Second nucleophilic attack by OR^- leads reversibly to alcoholysis as shown in the reaction

$$R'\text{–}CO\text{–}OR'' + Na^+OR^- \rightleftharpoons R'\text{–}\underset{\displaystyle OR}{\overset{\displaystyle O^-}{\overset{|}{\underset{|}{C}}}}\text{–}OR'' + Na^+ \rightleftharpoons R'\text{–}CO\text{–}OR + Na^+OR''^- \qquad (2)$$

On the other hand, attack by OH^- leads irreversibly to saponification as shown in the reaction

$$R'\text{–}CO\text{–}OR'' + Na^+OH^- \rightleftharpoons R'\text{–}\underset{\displaystyle OH}{\overset{\displaystyle O^-}{\overset{|}{\underset{|}{C}}}}\text{–}OR'' + Na^+ \rightarrow R'\text{–}CO\text{–}O^-Na^+ + R''OH \qquad (3)$$

The predominance of the alcoholysis reaction is further accounted for in the data of Bender and Glasson[28], who estimated that the relative rates of attack of OH^-, OMe^- and OEt^- on the ester, which is the rate-determining step in each case, are in the ratios 1:1.59:4.17, respectively, under ideal conditions.

Finally, the original base is regenerated from $Na^+OR''^-$ in eqn. 2 according to the reaction

$$Na^+OR''^- + ROH \rightleftharpoons R''OH + Na^+OR^- \quad (4)$$

The above theory accounts for the earlier findings and indicates that an efficient preparation of the esters should be achieved by displacing reaction 2 to the right and inhibiting reaction 3 for which the following steps may be taken:

(1) The concentration of OR^- should be maximized and the concentration of OH^- minimized. Even though the equilibrium in eqn. 1 is highly favourable, it would appear desirable to use methoxide rather than hydroxide as the base. Thus, the presence of even small amounts of soap could promote emulsification and delay the separation of the organic layer during extraction of the esters. Using methoxide, soap formation would be limited to that generated by free fatty acids in the substrate and to that resulting from the inevitable uptake of small amounts of moisture during the preparation and use of the methanolic reagent. It also follows that methoxide should be used at the highest concentration which does not lead to undesirable side reactions and at which it may be conveniently handled.

(2) The concentration of methanol should be as high as possible to displace reaction 4 to the right.

(3) The reaction should be carried out at the highest practical temperature which does not lead to undesirable side reactions.

Bearing in mind the above considerations, the various procedures described in the literature for the preparation of the methyl esters for GLC are now outlined with emphasis on the development of rapid methods. Most of the early workers were not concerned with optimizing the preparation, and the potential for rapid techniques was not effectively realized until 1968. It is possible that such developments were retarded by apparent misunderstandings of the earliest work. Markley[16b], for example, incorrectly states that "Kurz[25]... found in agreement with Rowe[24] and with Toyama *et al.*[23], that alcoholysis by potassium hydroxide consisted of two reactions, *viz.*, saponification followed by re-esterification". Glass[18] demonstrated the error in this statement, but appeared to be unaware that Markley had incorrectly reported the conclusions of the earlier workers. A similar misunderstanding appeared as recently as 1977[29], when it was stated, "When esters are prepared by transesterification techniques in which saponification and esterification are performed in the same vessel, the triglycerides must be quantitatively converted to salts which, in turn, must be quantitatively converted to esters". Thus, transesterification does not proceed by saponification at all.

Development of rapid procedures

The first preparation of the methyl esters by base-catalysed methanolysis of a fat or oil specifically for the purpose of analysis by GLC was carried out by James and Martin in 1956[7]. The esters of olive oil were prepared by the method of Kurz[25], but

the authors gave no experimental details. Kurz carried out this reaction in ethereal methanol with methanolic potassium hydroxide at room temperature such that the base concentration was approximately 0.006 *N* and the molar ratio of methanol to substrate was approximately 25 in the reaction mixture. The yield of liberated glycerol was 98.31% after 24 h, 98.78% after 48 h and 99.44% after 72 h. Assuming that James and Martin followed these experimental details, it is likely that they obtained an excellent yield of esters, but the preparation is marked by characteristics which were to appear commonly in later publications, *viz.*, a low concentration of base, a long reaction time, a low reaction temperature and the use of hydroxide rather than methoxide as the catalyst.

Craig and Murty[30] described the first use of methoxide for the preparation of esters in 1959. Six neutral vegetable oils were refluxed for 4 h in methanolic sodium methoxide at a substrate to catalyst ratio of 10. No comment was made on the yield of esters, which was probably good under the conditions used, but the authors did point out the speed and simplicity of the method.

In 1960 Luddy *et al.*[31], while investigating the transesterification of sterol esters, foreshadowed the development of rapid procedures for the transesterification of triglycerides when they concluded that this could be achieved by using many times the amount of sodium or potassium methoxide normally used for methanolysis. Thus a 98% yield of esters was achieved from sesame oil after refluxing for 5 min with a methanol to substrate ratio of about 180 and an amount of sodium methoxide around 1.5 times stoichiometric. This finding was essentially incidental to their work and appears to have escaped widespread observation as a number of subsequent papers described relatively inefficient methods[32–36].

Further to this earlier work, Luddy *et al.*[37] described the first rapid methylation procedure in 1968, detailing three versions of a sealed tube micromethod. For neutral glycerides 1–30 mg of substrate were heated at 65°C for a total of 2 min with a methanol to substrate ratio of at least 60 and, at the minimum, a stoichiometric amount of sodium methoxide. The esters were recovered by extraction with carbon disulphide and centrifugal separation, and a total preparation time of less than 6 min was claimed. The authors also stated that the transesterification reaction was complete in 30 sec under these conditions. This paper appeared to create a spate of interest in rapid methods and several other techniques soon appeared in the literature.

DeMan and colleagues developed a rapid version of a sealed tube method first devised fur butterfat by DeMan in 1964[38]. The original version used catalytic levels of sodium methoxide, methanol to substrate ratios of 20 and required heating for 1 h at 60°C. The latter method, described in 1970[39], used higher levels of sodium methoxide and methanol to substrate ratios and incorporated a mixed solvent of light petroleum and diethyl ether to enable the reaction to proceed homogeneously in 2 min at room temperature. Although described for butterfat, the method was stated to be suitable for other fats and oils, including hard fats. Reaction mixtures were injected directly into the chromatograph, and repeated injections were claimed not to affect the column adversely.

At about the same time, Glass and Christopherson published three versions of rapid transesterification procedures. The first appeared in 1969[40] and was a micromethod for the differential determination of esterified and free fatty acids in lipid mixtures. The lipid solution was treated with an excess of 1 *N* sodium methoxide over

that required to neutralize free fatty acids. After standing at room temperature 5 min, the free fatty acids could also be esterified by treatment with methanolic hydrochloric acid. Analysis of the neutralized and acidified materials gave the bound and total lipid fatty acid compositions, respectively. For neutral oils, only the first stage is required. The reaction mixtures were injected directly into the chromatograph, and it was maintained that the fatty acid soaps, when present, did not interfere with the chromatograph. The column could be purged by injection of methanolic hydrochloric acid at elevated oven temperatures.

The same authors[41] also described a room temperature method for the methanolysis of milk fat. Alcoholysis was shown by thin-layer chromatography to be complete after 2 min, and a total preparation time of less than 5 min was claimed. It was pointed out that direct injection of the reaction mixture was necessary to avoid loss of short-chain esters in an extraction step, and it was maintained that repeated injection did not adversely affect the column or detector. The "essentially non-alcoholic" solution aided resolution of methanol from the butyrate peak in the chromatogram. While the molar ratio of methanol to substrate of approximately 3 would appear to be less than adequate, an additional driving force for the reaction was the separation of glycerol from the reaction mixture due to its insolubility.

In 1971, Glass[18] discussed the theory of the transesterification reaction, the essential features of which have already been described. In this paper a further transesterification method for fats and oils was reported. The reaction was carried out at room temperature, with solubility of the glyceride enhanced by the inclusion of benzene in the solvent system. Triglycerides, phospholipids and wax esters were esterified in 1–2 min and sterol esters in 20 min at room temperatures. The reaction mixture was injected directly into the chromatograph with no reported damage to column or detector. The methanol to substrate ratio under these conditions lies between 600 and 60, in sharp contrast to the earlier methods. Base concentrations were 3–30 times stoichiometric, but lower levels were also found to be satisfactory for triglycerides and wax esters.

Hougen and Bodo[42] described both a "conventional methanolysis" and a "rapid methanolysis" for the methylation of rapeseed oil in 1973. The rapid method was notable for the use of a high concentration of base and the convenience of carrying out the preparation and extraction of the esters in the same reaction vessel.

Extraction procedures were also used by Zorawski[43] (1973) and Prevot *et al.*[44] (1975); the former used saturated sodium chloride to aid extraction of esters into isooctane while the latter first neutralized the reaction mixture with methanolic hydrochloric acid before addition of light petroleum and injection of the upper layer. Prevot stated that the total preparation time was very fast at 2.5 min.

Utrilla *et al.*[45] (1976) studied the formation of methyl esters from fats as a function of time using four catalysts, *viz.*, sulphuric acid, boron trifluoride, potassium methoxide and potassium hydroxide. Results were expressed as yields of esters formed relative to the maximum yield obtained in the reaction. The data obtained from the experiments with the two basic catalysts are pertinent to the review. For the methoxide reaction, which was based on the method of Luddy *et al.*[37], the relative abundance of esters dropped markedly from the 2 min to 30 min reaction period, although no explanation was given for this phenomenon. The potassium hydroxide method was based on a proposed UNE standard (Spain)[46] and on the method used

by Christopherson and Glass[41] for the rapid preparation of milk fat methyl esters. Utrilla *et al.*[45] found that the methylation of short-chain esters was almost instantaneous, whereas the long-chain esters required slightly longer. Soap formation was similarly faster for the short-chain esters.

The Christopherson and Glass procedure was also the basis for the method proposed by Bitner *et al.*[47]. The major improvement to the technique was to use a diethyl ether–methanol mixed solvent, eliminating hexane from the method in order to give a single phase and enhance the solubility of the oil.

The International Organization for Standardization (ISO)[8] and International Union of Pure and Applied Chemistry (IUPAC)[9] standard methylation procedures using base-catalysed methanolysis are identical and use potassium hydroxide rather than methoxide as the catalyst. There are three variants, which are given as "alternative methods not involving the use of boron trifluoride". The first variant is for neutral fats with an acid value less than 2 and uses a methanol to substrate ratio of about 90 and a base concentration of approximately 0.01 *N* in the reaction mixture. The second variant is for acid fats and oils, and the major difference is the use of more base (ca. 0.5 *N*) as much of the catalyst may be consumed by the free fatty acids. Both methods require reflux conditions and extraction of the esters formed into heptane. The third variant is for fats (such as butter fat) containing short-chain fatty acids, and is carried out at room temperature. The reaction mixture has a high level of heptane and low levels of methanol and potassium hydroxide catalyst. The driving force for the reaction is the separation of glycerol from the non-polar medium.

Discussion of literature review

The obvious feature of literature methods describing the methoxide-catalysed methanolysis of fats and oils is the extreme variability of the reaction conditions which have been used. Although many methods are obviously very inefficient, others appear to be highly efficient. The best of these are simpler and faster than the widely used boron trifluoride–methanol procedures[1–4] for the methylation of fats and oils, and should thus be more attractive for more widespread use in the fat and oil industry than presently may be the case. The currently described international standards for the methylation of neutral fats and oils by this method[8,9] appear to offer scope for improvement with regard to speed of reaction and simplicity of workup. In the following section we evaluate a procedure which attempts to overcome these shortcomings.

EXPERIMENTAL

Chemicals

All reagents were chemically pure. Isooctane (2,2,4-trimethylpentane) was of Baker Analyzed Reagent grade (J. T. Baker, Phillipsburg, NJ, U.S.A.). A purity check by GLC under conditions similar to those used throughout the experiments showed that there were no peaks on the solvent tail.

The primary standard mixture of triacylglycerols simulating coconut oil was the same as that previously described[11]. The various fats and oils were obtained from Edible Oil Industries Pty. Ltd. (Balmain, N.S.W., Australia).

Chromatography

GLC and processing of the data were carried out as described previously[48], except that samples were injected manually for the analysis of the primary standard and the soybean oil sample. For evaluation of the effect on the analysis and column of injecting the whole reaction mixture, samples were injected automatically. The detector was optimized for linearity as previously described[48].

Methylation procedure and related studies

Premolten fat or oil (14 drops or approximately 150 mg) was transferred to a dry, 50-ml volumetric flask fitted with a B14 ground-glass joint. The mixture was boiled under reflux for 30 sec with 5 ml of 0.25 *M* sodium methoxide in methanol–diethyl ether (1:1). The flask was removed from the heat source, 3 ml of isooctane and approximately 15 ml of saturated sodium chloride were added and the flask was stoppered and *shaken vigorously for 15 sec while tepid.* The liquid level was brought to the neck of the flask with more sodium chloride solution and the phases were allowed to separate. Approximately 2.5 μl of the upper layer were injected into the chromatograph.

The methylation procedure was evaluated by determining the means and standard deviations for each of the component esters of the triacylglycerol mixture and also by determining the grade of analysis[49] with respect to the known composition. The results were compared with those obtained using a modified boron trifluoride–methanol procedure[11] for both the primary standard and a soybean oil sample.

For the reaction rate studies, the methylation was carried out under the required conditions and the mixture worked up as above. The equivalent of about 50 mg of reaction product was treated with 1 ml of a solution of pyridine–hexamethyldisilazane–trimethylchlorosilane (10:2:1) and the total amounts of methyl esters, monoacylglycerols and diacylglycerols were determined simultaneously by GLC. Analysis was carried out on a Varian Model 1700 chromatograph fitted with a flame-ionization detector and a 2 m × 4 mm I.D. glass column packed with 3% OV-1 on 100–120-mesh Gas-Chrom Q, with nitrogen at 30 ml/min, hydrogen at 30 ml/min and air at 500 ml/min. The oven was temperature programmed from 100 to 300°C at 4°C/min, and peak areas were measured using a Hewlett-Packard Model 3354 Laboratory Automation System.

To determine if it was necessary to remove methoxide from the reaction mixture prior to GLC, the effects on analytical and chromatographic performance of repeated injections of methoxide-free and methoxide-containing solutions of coconut oil FAME on to a DEGS-PS column were determined. For this, a coconut oil sample was methylated, first by the procedure given above, in which methoxide is removed by aqueous extraction, and second by a procedure similar to that of Bitner *et al.*[47] in which no aqueous extraction was used. For the latter, five drops of coconut oil were shaken with 1.5 ml of 0.25 *M* sodium methoxide in methanol–diethyl ether (1:1) in a sealed vial and the mixture was allowed to stand at room temperature until required for analysis. Starting with the methoxide-free solution, 80 analyses of each were carried out. In addition, a methoxide-free solution of soybean oil FAME was analysed at the start of the experiment and then after every 20 injections of the coconut oil FAME solutions. Immediate and cumulative effects were considered. Immediate effects were assessed from the accuracy of the analytical results for the coconut oil

analyses. Cumulative effects were assessed from progressive changes in the accuracy of the analytical result for both the coconut oil and soybean oil FAME solutions and also from changes in chromatographic parameters for the soybean oil FAME chromatograms. The ability of the column to recover after repeated injections of the methoxide-containing solution was assessed by analysing the soybean oil FAME solution a further six times at the end of the experiment and examining the analytical and chromatographic performance. As the experiment required several days to complete, the soybean oil FAME solution was analyzed on a new DEGS-PS column after all other analyses had been completed to determine if the esters had remained unchanged throughout.

The chromatographic parameters for the soybean oil FAME chromatogram were determined using a Hewlett-Packard Model 3354 Laboratory Automation System. The parameters were effective theoretical plates for 18:0 and 18:3, separation factor for 18:0–18:1, resolution of 18:0–18:1 and tailing coefficient for 18:3.

The absolute accuracy of the above analyses for the coconut oil sample was supported by analysing methoxide-free and methoxide-containing solutions of the FAME of the triacylglycerol primary standard mixture. These solutions were also used to determine the effect on the analytical results of using phases other than DEGS-PS. The columns for these studies were 4.5 m × 4 mm I.D. glass packed with 10% Carbowax 20M on 100–120-mesh Gas-Chrom Q (220°C, isothermal) and 1.5 m × 4 mm I.D. glass packed with 15% Silar-10C on 230–270-mesh Gas-Chrom R (ULTRA-PAK, Applied Science Labs., State College, PA, U.S.A.) (180°C, isothermal).

RESULTS AND DISCUSSION

Selection of methanolysis reaction conditions

In selecting the particular reaction conditions and other details for the methylation procedure, the following considerations were taken into account. First, the method was designed primarily for use in the fat and oil industry where the available sample size is normally not limited, hence, the special adaptations of the various micromethods which appear in the literature were not considered. Thus, the proportions of analyte and reagents approximated those used in the standard procedures[8,9]. The method was similarly not meant for the analysis of butterfat, which requires special attention. Second, the improved solvent system of Bitner *et al.*[47], *viz.*, methanol–diethyl ether (1:1), was used to give a single phase and aid in dissolving the oil. Third, the reaction parameters were selected in order to displace the reaction towards completion as rapidly as possible in accordance with the principles proposed earlier in this paper in the theoretical discussion. Fourth, a vigorous extraction step was included to maximize the recovery of the short-chain FAME in accordance with our previous findings[11]. This aqueous extraction was also carried out to remove methoxide which we later show to be damaging to the analytical results if left in the reaction mixture.

Evaluation of methylation procedure for quantitative accuracy

The results of analysis of the triacylglycerol primary standard are given in Table I together with statistical data and the known composition of the standard.

TABLE I

ANALYSIS OF TRIACYLGLYCEROL MIXTURE AFTER METHOXIDE-CATALYSED METHANOLYSIS

Sample No.	*Composition by GLC (%)*							*Grade of analysis (%)*
	FAME							
	6:0	*8:0*	*10:0*	*12:0*	*14:0*	*16:0*	*18:0*	
1	0.68	8.25	6.32	47.62	19.14	8.34	9.64	99.19
2	0.80	8.32	6.36	47.61	19.07	8.26	9.58	99.50
3	0.77	8.44	6.42	47.63	19.00	8.23	9.50	99.19
4	0.79	8.32	6.37	47.65	19.03	8.26	9.58	99.46
5	0.74	8.35	6.37	47.75	19.03	8.24	9.53	99.41
6	0.74	8.31	6.35	47.51	19.15	8.30	9.65	99.20
7	0.76	8.25	6.33	47.57	19.13	8.32	9.64	99.26
8	0.82	8.29	6.33	47.79	18.89	8.26	9.63	99.35
Mean	0.76	8.32	6.36	47.64	19.06	8.28	9.60	99.32
S.D.	0.045	0.061	0.032	0.089	0.087	0.039	0.055	0.13
C.V. (%)	5.9	0.7	0.5	0.2	0.5	0.5	0.6	
Known %	0.93	8.31	6.20	47.72	19.08	8.18	9.58	
Δ*	−0.17	+0.01	+0.16	−0.08	−0.02	+0.10	+0.02	

* Difference between mean and known values.

The analytical results were in excellent agreement with the known composition and show excellent precision (repeatability). They were also marginally better than those recently reported for a modified boron trifluoride–methanol methylation procedure[11], because of slightly better recovery of methyl caproate, and were much better than those found for the AOCS and ISO boron trifluoride–methanol procedures.

The results of analyses for the soybean oil FAME are given in Table II together with the results after methylation with boron trifluoride–methanol.

The results were again all in excellent agreement, indicating that the strong base had no detectable effect on the polyunsaturated fatty acid components; the reaction products similarly showed identical absorptions in the UV region at 233 nm (conjugated dienes), or 262, 268 and 274 nm (conjugated trienes), when prepared using either boron trifluoride–methanol or methoxide.

Reaction rate studies

While it is generally recognized that methoxide-catalysed methanolysis of fats and oils is extremely rapid, even at room temperature, few studies have illustrated just how quickly the reaction may be carried out with assurance that it has gone to completion. Christopherson and Glass[41] demonstrated by thin-layer chromatography that conversion of milk fat into the methyl esters was complete in 2 min at room temperature. Glass[18] later investigated the saponification of corn oil with methanolic sodium hydroxide and similarly showed methanolysis to be complete in 3 min. Seemingly conflicting statements have, however, appeared in the literature, a serious instance being a report[50] that use of the IUPAC conditions for methanolysis[9] led after

TABLE II

DUPLICATE ANALYSES OF SOYBEAN OIL SAMPLE AFTER METHYLATION WITH BORON TRIFLUORIDE–METHANOL OR METHANOLIC METHOXIDE

Fatty acid methyl ester	*Composition by GLC (%)*			
	BF_3–methanol		*Methanolic methoxide*	
14:0	0.07	0.07	0.06	0.06
16:0	10.47	10.39	10.35	10.34
16:1	0.12	0.12	0.12	0.12
17:0	0.10	0.11	0.10	0.10
17:1	0.07	0.05	0.08	0.06
18:0	3.48	3.49	3.54	3.50
18:1	20.89	20.96	20.97	20.96
18:2	54.97	55.09	54.98	55.06
20:0	0.33	0.33	0.35	0.35
18:3	9.08	8.97	9.02	9.00
22:0	0.42	0.42	0.42	0.41

TABLE III

RATE OF METHYLATION OF FATS AND OILS

Fat, oil	*Temperature*	*Time (min)*	*Composition (%)*		
			FAME	*Monoacylglycerol*	*Diacylglycerol*
Soybean	Ambient	0.1	83.7	12.0	4.3
Soybean	Ambient	1	97.1	2.8	0.1
Soybean	Ambient	2	99.7	0.3	ND*
Soybean	Reflux**	0**	93.0	6.8	0.2
Soybean	Reflux	0.25	100.0	ND	ND
Soybean	Reflux	0.5	99.9	0.1	ND
Sunflower	Reflux	0.25	99.9	0.1	ND
Sunflower	Reflux	0.5	99.8	0.2	ND
Safflower	Reflux	0.25	99.5	0.5	ND
Safflower	Reflux	0.5	99.8	0.2	ND
Coconut	Reflux	0.5	100.0	ND	ND
Hydrogenated palm	Reflux	0.25	99.9	0.1	ND
Hydrogenated palm	Reflux	0.5	99.6	0.4	ND
Hydrogenated soybean/palm	Reflux	0.25	96.3	3.3	0.4
Hydrogenated soybean/palm	Reflux	0.5	99.7	0.3	ND
Tallow	Reflux	0.5	99.9	0.1	ND
Tallow stearin	Reflux	0.25	99.8	0.2	ND
Tallow stearin	Reflux	0.5	99.7	0.3	ND

* Not detected.

** Time required to reach reflux is approximately 0.25 min on a hot-plate and is not included in the reflux time.

15 min refluxing to incomplete methanolysis, with mono-, di- and triglycerides being detected in the reaction product.

Table III gives the results of a comparison of the rates of methanolysis of several fats and oils for various times and temperatures with other reaction conditions being as described in our procedure. The total concentrations of methyl esters, monoacylglycerols and diacylglycerols were determined simultaneously by normalization of the GLC results. The results do not take into account unreacted triacylglycerols, which were assumed to be negligible because of the very low levels of diacylglycerols, soaps, which Glass[18] has shown to be insignificant in the early stages of the reaction with neutral oils, and unsaponifiable matter.

The results confirm that methanolysis is essentially complete in 2 min at room temperature in the case of soybean oil, but also that reaction times less than this should not be used at this temperature. The results also indicate that, with refluxing, the reaction is complete after 15 sec with most fats and oils and that complete reaction is ensured in all cases after 30 sec. We have, therefore, included the use of refluxing in our own procedure in order to drive the reaction to completion in the shortest possible time. In addition, heating of the reaction mixture later ensures extraction of the FAME under tepid conditions which we believe promotes ease of quantitative extraction of the shorter chain FAME into the organic layer[11]. Using the procedure described, the FAME solution may be prepared ready for injection into the chromatograph in about 2 min.

Effects of injecting whole reaction mixtures

The literature methods for methoxide-catalysed methanolysis may be divided into two groups according to whether the reaction mixture is injected directly into the column, sometimes after neutralization or acidification with methanolic hydrochloric acid, or whether the mixture is diluted with water or brine and the esters recovered by extraction into a suitable solvent. The direct injection technique has advantages in time savings and in circumventing the possible loss of short chain FAME into the aqueous phase, but there is also the possibility of causing column and detector damage. There is disagreement in the literature in the latter respect. DeMan[38,51] found that repeated injection of a whole reaction mixture (not neutralized) had no harmful effect on the separating ability of a polyester (DEGS) column, provided his preferred transesterifying reagent (0.5 *N* methanolic sodium methoxide in light petroleum–diethyl ether) was used. However, he also reported that similar reagents containing benzene showed abnormal patterns for milk fat esters, especially when the sodium methoxide concentration was higher than 0.05 *N*, and serious losses of methyl oleate and other unsaturated fatty acids were also noticed in this instance. Christopherson and Glass[41] originally stated that repeated injection of a whole reaction mixture (whether acidified or not) did not adversely affect column (DEGS) or detector, but later indicated (for neutralized or acidified mixtures) that slow accumulation of material in the column gave rise to increasing bleed[40]. The column was restored by purging with injections of methanolic hydrochloric acid or by occasionally leaving at 220°C overnight at an increased carrier gas flow-rate. Glass[18], later again, indicated that no damage to column and detector resulted from direct injection of a (preferably neutralized) reaction mixture. In contrast, Iverson and Sheppard[29] found that injection of whole reaction mixtures onto a Silar 10C column resulted in

severe detector fouling, the generation of artifacts, unstable baselines, loss of resolution and a shortened column life.

Although the above workers had differing opinions about the effects of injecting whole reaction mixtures, none presented objective evidence to support their cases. In our studies, we were concerned with the effects of injecting reaction mixtures containing sodium methoxide, which is of particular interest for several reasons. First, methoxide might be expected to be particularly damaging to polyester phases by causing depolymerization. Second, it could catalyse transesterification between a polyester phase and the FAME. Third, it could lead to soap formation with the FAME in the hot injection zone. Finally, while some of the literature methods advise or specify that methoxide should be neutralized before the reaction mixture is injected, it is advantageous in time savings only if the whole reaction mixture is injected with no further treatment after completion of the methanolysis. Thus, the extraction procedure which we describe is rapid and may be carried out as simply as a neutralization step while having the advantage of removing inorganic reagents and the small amounts of soaps which are present.

To determine the effects of methoxide on the analytical and chromatographic performance of a DEGS-PS column, repeated injections were made of a solution of coconut oil FAME, first, when the reaction mixture was worked up by aqueous extraction and, second, when no such workup was carried out. The results of the evaluation procedure are given in Table IV for the analytical results on the coconut oil FAME, in Table V for the analytical results on the triacylglycerol primary standard, in Table VI for the analytical results on the soybean oil FAME and in Table VII for the chromatographic parameters for the soybean oil FAME chromatograms.

TABLE IV

ANALYSIS OF COCONUT OIL AFTER METHYLATION FOLLOWED BY AQUEOUS EXTRACTION OR NO EXTRACTION

Fatty acid methyl ester	*Composition by GLC (%)*										
	Injection No. (aqueous extraction)				*Injection No. (no extraction)*						
	1	*20*	*40*	*80*	*1*	*2*	*5*	*10*	*20*	*40*	*80*
6:0	0.63	0.62	0.65	0.64	0.36	0.31	ND*	0.31	0.34	ND	0.29
8:0	8.69	8.68	8.80	8.78	9.59	10.00	10.17	10.11	10.27	9.98	10.29
10:0	6.57	6.56	6.54	6.60	7.00	7.37	7.46	7.34	7.36	7.25	7.29
Unknown**	0.02	0.02	0.01	0.02	ND	0.20	0.26	0.32	0.57	0.51	0.67
12:0	46.92	47.00	46.66	46.83	49.78	50.81	50.87	50.19	50.04	49.95	49.95
14:0	17.54	17.56	17.71	17.62	17.41	17.10	16.91	16.91	16.43	16.82	16.42
16:0	8.74	8.71	8.77	8.70	7.53	6.90	6.77	6.84	6.38	6.92	6.64
18:0	2.63	2.63	2.64	2.62	1.86	1.63	1.55	1.55	1.37	1.58	1.46
18:1	6.40	6.44	6.47	6.43	5.04	4.40	4.09	4.05	4.01	4.23	4.04
18:2	1.64	1.64	1.64	1.63	1.32	1.12	1.07	1.07	1.14	1.11	1.07
Unknown***	0.07	0.07	0.06	0.06	0.11	0.17	0.86	1.30	2.08	1.65	1.88

* Not detected.

** Probably 11:0 for the aqueous extraction samples.

*** Probably 18:3 for the aqueous extraction samples.

TABLE V

ANALYSIS OF TRIACYLGLYCEROL PRIMARY STANDARD MIXTURE IN METHOXIDE-FREE (A) AND METHOXIDE-CONTAINING (B) SOLUTION ON DIFFERENT STATIONARY PHASES

Fatty acid methyl ester	*Composition by GLC (%)*				
	DEGS-PS (B)*	*Carbowax 20M*		*Silar 10C*	
		A	*B*	*A*	*B*
6:0	0.93	0.82	1.12	0.82	ND
8:0	9.77	8.35	9.73	8.31	9.45
10:0	7.19	6.34	7.16	6.34	7.07
12:0	52.14	47.58	50.26	47.65	52.20
14:0	18.10	19.23	18.77	19.25	19.02
16:0	6.15	8.19	6.48	8.23	6.62
18:0	5.16	9.43	6.11	9.39	5.56
Grade**	87.8	99.1	89.8	99.3	87.0

* Results for methoxide-free (A) solutions on DEGS-PS are those given in Table I.
** The known composition of the standard is given in Table I.

TABLE VI

RESULTS OF ANALYSES OF SOYBEAN METHYL ESTERS AFTER MULTIPLE INJECTIONS OF COCONUT OIL ESTER SOLUTIONS

Fatty acid methyl ester	*Composition by GLC (%)*										
	No. of prior injections of coconut esters									*Column recovery test*	*New column*
	0	*20*	*40*	*60*	*80*	*20*	*40*	*60*	*80*		
	Aqueous workup of reaction mixture					*No workup of reaction mixture*					
14:0	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.10
15:0	0.01	0.01	0.01	ND	0.01	ND	ND	0.02	0.01	0.01	0.01
16:0	11.13	11.12	11.12	11.12	11.12	11.28	11.30	11.27	11.25	11.26	11.09
16:1	0.14	0.13	0.13	0.13	0.13	ND	ND	ND	ND	ND	0.14
17:0	0.11	0.11	0.11	0.11	0.10	0.11	0.12	0.13	0.14	0.16	0.11
17:1	0.05	0.05	0.05	0.05	0.05	0.04	0.04	0.04	0.03	0.03	0.07
18:0	3.90	3.87	3.86	3.86	3.86	3.79	3.76	3.75	3.67	3.66	3.93
18:1	20.94	20.93	20.97	20.99	20.98	21.28	21.48	21.56	22.00	21.89	20.82
18:2	54.36	54.38	54.38	54.38	54.37	54.23	54.09	54.02	54.22	54.33	54.34
20:0	0.40	0.41	0.40	0.39	0.39	0.37	0.38	0.40	ND	ND	0.39
18:3	8.40	8.43	8.43	8.42	8.42	8.37	8.30	8.26	8.15	8.13	8.44
22:0	0.43	0.44	0.42	0.42	0.43	0.40	0.40	0.40	0.40	0.40	0.46
Grade	99.73	99.65	99.60	99.57	99.59	98.87	98.41	98.26	97.74	97.59	99.61

TABLE VII

EFFECT OF MULTIPLE INJECTIONS OF COCONUT OIL ESTER SOLUTION ON CHROMATOGRAPHIC PARAMETERS FOR SOYBEAN OIL METHYL ESTERS

Sample	*No. of prior coconut ester injections*	*Retention time of 18:1 (min)*	*Efficiency for 18:0 (effective plates)*	*Efficiency for 18:3 (effective plates)*	*Separation factor, 18:0 18:1*	*Resolution, 18:0-18:1*	*Tailing coefficient for 18:3*
Aqueous extracted coconut oil esters	0	15.2	1690	2720	1.18	1.56	1.02
	20	15.1	1550	2680	1.18	1.51	1.03
	40	15.2	1490	2610	1.18	1.47	1.04
	60	15.0	1520	2540	1.18	1.47	1.06
	80	15.1	1470	2620	1.18	1.46	1.05
Non-extracted coconut oil esters	20*	14.9	1500	2510	1.18	1.46	1.12
	40	15.1	1520	2560	1.18	1.47	1.10
	60	15.1	1620	2630	1.18	1.51	1.11
	80	15.2	1510	2530	1.18	1.47	1.15
Recovery test	–	15.1	1570	2540	1.18	1.47	1.18

* Zero sample identical with 80-injection results above.

Table V also includes results of analyses of the triacylglycerol primary standard on Carbowax 20M and Silar 10 C columns, which are discussed below.

The analytical results for the coconut oil FAME worked up by aqueous extraction indicate excellent analytical reproducibility even after 80 injections. As the results in Table I for the primary standard similarly worked up by aqueous extraction show excellent agreement with the known composition, it was concluded that these results for the coconut oil FAME were also highly accurate. In contrast, injection of the whole reaction mixture led to more variable and inaccurate results. That these results are inaccurate is confirmed by similar deviations in the results for the corresponding primary standard reaction mixture given in Table V.

Progressive deterioration of the system when the whole reaction mixture was injected was indicated by the generation of increasing amounts of the two unknowns indicated in Table IV (the first did not coincide with dimethyl succinate which has an equivalent chain length of about 12, but may have been monomethyl succinate). These compounds appeared in significant amounts from the second injection and soon increased to grossly unacceptable levels. If they were in fact stationary phase breakdown products, their formation may have occurred by way of methoxide-catalysed depolymerization of the DEGS-PS with the release with each fresh injection of methoxide of increasing amounts of both compounds as the overall extent of depolymerization proceeded.

Progressive deterioration of the system was further reflected in the decreasing accuracy of the results for the soybean oil FAME given in Table VI. Compared with the excellent reproducibility of the results following injection of the aqueous extracted coconut oil FAME, the latter group are more erratic and show rapid loss of grade to an unacceptable standard ($<99\%$). That the column had suffered permanent damage was indicated by the poor result for the recovery test sample, which was the last of the six consecutive soybean oil FAME samples injected at the end of the series. The validity of the data was confirmed by the excellent result obtained for the soybean oil FAME at the end of the series on a new column.

Surprisingly, only minimal evidence of column deterioration is seen in the chromatographic parameters given in Table VII, which remained almost constant except for a slight increase in the tailing coefficient of 18:3 for the second group. This possibly explains the literature reports that no loss of resolving power was apparent using direct injection techniques. Visual inspection of the chromatograms, however, showed that tailing of the major peak, 18:2, increased to the extent where 20:0 was not resolved, as indicated in Table IV, with consequent loss of accuracy of the results. From the above, it was concluded that an analysis may suffer quantitatively from the direct injection of the whole reaction mixtures while not necessarily showing obvious qualitative deterioration in the chromatograms.

The results of analyses of the whole reaction mixtures for coconut oil FAME in Table IV and for the primary standard in Table V are characterized by a relative increase in the short-chain esters and a corresponding decrease in long-chain esters. We have already indicated some possible ways in which methoxide could interfere when using a polyester stationary phase; however, the results in Table V further indicate that similar disproportionation occurs when other kinds of stationary phases are used. In order to account for this, it would appear that the principal disproportionating mechanism is soap formation between methoxide and the FAME in the hot

injection zone. Thus, transesterification of FAME with the stationary phase would not be expected with Silar 10C and should be negligible or greatly reduced with Carbowax 20M, and the observed disproportionation seen with DEGS-PS should not have occurred with these phases. Similarly, a mechanism involving isomerization of polyunsaturated fatty acids should have had no effect on the results for the primary standard which contains only saturated fatty acids. To account for the observed disproportionation, it is likely that soap formation occurs to a greater extent with long-chain esters because of their expected longer time of residence in or near the injection zone compared with the short-chain esters.

CONCLUSION

A procedure for the rapid methoxide-catalysed methanolysis of neutral fats and oils which endeavours to utilize favourable theoretical considerations together with the best features of the many literature methods and which is faster and simpler than the presently described international procedures was evaluated. Excellent quantitative results were obtained in the difficult case of a triacylglycerol primary standard mixture which simulated coconut oil, and results obtained for a soybean oil sample were identical with those obtained after methylation with boron-trifluoride–methanol. Evidence is presented that the reaction is complete after about 30 sec of refluxing under the conditions described. The ester solution is ready for injection into the chromatograph in about 2 min, and the method should be particularly suitable for routine use in the fat and oil industry.

The method includes an aqueous extraction step which we believe should necessarily be included in any procedure using methoxide. Thus, evidence if presented of the damaging effect of methoxide on quantitative results even though there may be little evidence of column damage indicated by chromatographic parameters.

ACKNOWLEDGEMENTS

This work was supported financially by the Australian Industrial Research and Development Incentives Board. Thanks are due to the Directors of Unilever for permission to publish this paper.

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