⁂Analysis of Fatty Acid Methyl Esters with High Accuracy and Reliability. IV. Fats with Fatty Acids Containing Four or More Carbon Atoms

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ABSTRACT

Those aspects of the quantitative methanolysis of fats with fatty acids containing four or more carbon atoms and the gas liquid chromatographic analysis of the methyl esters which have not been adequately dealt with to date were investigated. Accurate analysis of the esters requires the optimization of the total chromatographic system using a carefully prepared methyl ester primary standard and applying, as the only correction factors, the theoretical flame ionization detector response factors of Ackman and Sipos. These factors now have also been shown to be highly accurate for short chain saturated fatty acid methyl esters, in particular methyl butyrate and methyl caproate. Under the conditions specified by the international methods for the methylation (methanolic KOH) of fats with fatty acids containing four or more carbon atoms, saponification of the esters, once formed, proceeds much more rapidly for short chain than for long chain esters. This problem is easily overcome by neutralizing the catalyst, which then leads to very stable solutions and contributes to column life. This step should be mandatory in the international procedures. Tristearin methylated more slowly than other triacylglycerols under the standard conditions, but there was no difficulty in the case of unsaturated fatty acids of this chain length. Satisfactory results could not be obtained when sodium methoxide was the catalyst. The optimum procedure rendered unnecessary the use of methyl pentanoate as an internal standard for the accurate quantitation of methyl butyrate.

INTRODUCTION

The quantitative analysis of the fatty acid composition of fats with fatty acids containing four or more carbon atoms, which we refer to herein as short chain fats (SCF), by gas liquid chromatography (GLC) of the fatty acid methyl esters (FAME), has long been known to present special difficulties compared with the analysis of other fats and oils. These difficulties arise from the presence in SCF of significant amounts of short chain fatty acids, in particular butyric and caproic acids, and special precautions must be taken to ensure that errors are not introduced into the analysis as a consequence of this. Such errors may result from:

(a) Failure to methylate quantitatively;

(b) Subsequent saponification of the esters when alkaline methylation media are used;

(c) Failure to transfer the short chain esters quantitatively from the aqueous into the organic layer when aqueous extraction procedures are used;

(d) Preferential evaporative losses of the short chain esters during work up or storage;

(e) Discrimination in GLC analysis;

(f) Loss of accuracy due to inadequate resolution of the short chain esters during GLC;

(g) Failure to apply accurate response factors to allow for the differing responses of the various esters in the flame ionization detector (FID), for which the short chain esters require the largest corrections;

(h) Inaccurate integration of peak areas.

Attempts to deal with the above problems have been widely reported in the literature. That the methodology for the analysis of SCF is not yet adequate, however, is supported by the results of an Association of Official Analytical Chemists (AOAC) collaborative study (1) which included the analysis of butterfat and triacylglycerol primary standard mixtures simulating butterfat using the methodology (2,3) of the International Union of Pure and Applied Chemists (IUPAC). These studies indicated that methyl butyrate in the above samples was determined with much poorer precision than were long chain esters of similar concentration in other oils, and that serious loss of methyl butyrate occurred in some cases. The authors recommended that the IUPAC methodology for milk fats not be adopted as official first action in the Official Methods of Analysis and that separate methodology should be developed.

Badings and De Jong (4), in recognizing the widespread occurrence of inconsistent and erroneous results, recently carried out the only study to date with all of the above considerations in mind of the methodology of Christopherson and Glass (5) and the subsequent GLC analysis; this methodology constitutes the basis of the present International Standards (2,3,6,7). They reported improvements to the methodology which gave improved accuracy and precision, but consideration of the findings of our work, now reported, indicates that they did not resolve the problems completely.

The aim of our present investigations has been to resolve those aspects of the above considerations, (a)-(h), which have not been dealt with adequately to date.

(a) and (b). With regard to quantitative methylation, it is well established that base-catalyzed methanolysis proceeds rapidly at room temperature, and it is apparent that the typical reaction times of about 1 hr, especially with high temperature, used by the earlier workers were excessive. Reaction times of 1-5 min have been shown by Christopherson and Glass (5), Shehata et al. (8) and Glass (9) to give high yields of methyl esters from milkfat or other oils on the basis of thin layer chromatographic evidence and by Bannon et al. (10) on the basis of GLC evidence. Little is known, however, about the rates of methanolysis as a function of chain length. A closely related question concerns the rate of saponification of the methyl esters, once formed, as a function of chain length. Glass (9) demonstrated that saponification inevitably occurs in the presence of base, and Christie (11) showed that saponification was very rapid with polar lipids. Badings and De Jong (4) recommended against the storage of reaction mixtures for long periods because of possible saponification, but did not study the problem extensively.

In the present work, we have carried out studies into aspects of both the methanolysis and saponification as a function of chain length of a primary standard mixture containing saturated triacylglycerols ranging in chain length from C4 to C18 inclusive. The role of unsaturation in the methanolysis reaction was also studied using a mixed primary/secondary triacylglycerol standard.

(c) and (d). Losses of short chain esters during extraction from aqueous media and/or evaporative losses during handling have long been known to present difficulties in the preparation of milk fat methyl esters (12). In an attempt to avoid both sources of loss, DeMan (13) described, in 1964, the first of the techniques in which methanolysis was carried out in a sealed tube, and no aqueous extraction was used. Data later presented by DeMan (14) demonstrated

that aqueous extraction procedures were very unsatisfactory and that the sealed tube technique gave much higher yields of the short chain esters. A much faster version of a sealed tube methanolysis was described, in 1969, by Christopherson and Glass (5), and this method (methanolic KOH) constituted the basis for the international procedures (2,6). As it is clear that sealed tube procedures are vastly superior to those using aqueous work up, this aspect of the methodology required no further investigation.

(e) and (f). With regard to the chromatographic aspects of the analysis, discrimination of the sample components during introduction onto the column always has the potential to introduce difficulties in the case of mixtures containing components of widely differing volatilities. No studies have determined if discrimination can be avoided in the case of butterfat methyl esters. To this end we have carried out studies using primary standard mixtures of saturated FAME ranging in chain length from C4 to C18 inclusive. The second aspect of the chromatography, resolution of the short chain esters, is readily achieved by temperature programming and needed no further attention.

(g) and (h). The question of FID response factors, in particular for the short chain esters, is one which has not been adequately dealt with. In 1964, Ackman and Sipos (15) proposed that the response of saturated FAME was proportional to the weight percent carbon content of the ester, excluding the carbonyl carbon atom. Few studies have been directed toward this problem as the usual practice is to apply individual correction factors to each ester which take into account not only the FID response, but all other systematic errors incurred in preparation of the esters, chromatography and peak measurement. Shehata et al. (8) reported good agreement between the observed and calculated theoretical response factors for a number of esters, including methyl butyrate and methyl caproate. This agreement, however, could not be considered sufficiently good for high accuracy work. Badings and De Jong (4) rightly drew attention to the problem of response factors and determined overall correction factors for the various FAME which gave improved accuracy and precision. However, we believe the determination of arbitrary factors to be improper. Thus, in previous studies (16), we confirmed that the theoretical factors were highly accurate for all saturated FAME down to and including methyl caprylate and also have recently demonstrated this for olefinic, unsaturated FAME (17). It was concluded that these factors should be used at all times and that other system parameters should be adjusted accordingly such that accurate results are then obtained for an appropriate primary standard FAME mixture. We argue that the need to use factors other than the theoretical indicates that systematic errors exist in the methodology, and that such errors must exist, therefore, in the Badings and De Jong (4) methodology. In the present study, primary standard methyl ester mixtures were used to extend our previous studies by investigating the responses for methyl butyrate and methyl caproate.

The final consideration above, accurate integration of peak areas, is readily achieved with modern computing integrators, and this aspect of the methodology needed no further consideration.

As it is impossible to investigate the methylation procedure without establishing the accuracy of the GLC analysis, the chromatographic aspects were investigated first. It was then possible to carry out the investigations into the methylation procedure.

EXPERIMENTAL PROCEDURES

Materials

Isooctane (2,2,4-trimethylpentane), anhydrous methanol, potassium hydroxide and hydrochloric acid were all Pronalys analytical reagent grade (May and Baker, West Footscray, Victoria, Australia). A purity check of the isooctane by GLC under conditions similar to those used throughout the experiments showed that there were no peaks on the solvent tail that coincided with any short chain methyl esters. Sodium was analytical reagent (BDH, Poole, England). Sodium sulphate was Univar reagent grade (Ajax, Sydney, Australia). Anhydrous pyridine was laboratory reagent (May and Baker, Dagenham, England). Hexamethyldisilazane and trimethylchlorosilane were both specially purified grade (Pierce, Rockford, Illinois, U.S.A.). 2 M sodium methoxide was prepared by dissolving sodium (4.6 g) in anhydrous methanol (100 ml). The trimethylsilylating reagent was a mixture of hexamethyldisilazane (10 ml) and trimethylchlorosilane (5 ml) in anhydrous pyridine (50 ml).

Reference esters were methyl butyrate, methyl caproate, methyl caprylate, methyl caprate, methyl laurate, methyl myristate (2 samples), methyl stearate (all puriss grade, Fluka, Buchs, Switzerland) and methyl palmitate (Sigma, St. Louis, Missouri, U.S.A.). The purity of each ester was checked for the presence of other esters and free fatty acids. To check for other esters, samples were analyzed under the same GLC conditions used throughout the experiments as described below. To check for free fatty acids, the ester (10-15 mg) was treated with the trimethylsilylating reagent (0.5 ml) for 5 min in an oven at 100 C and the sample analyzed on a Hewlett-Packard model 5880A gas chromatograph fitted with a 12 m × 0.20 mm I.D. fused silica capillary column coated with SE-30. Two μl of sample was injected using a split ratio of ca. 100:1 and a temperature program appropriate for the particular ester.

Reference triacylglycerols were tributyrin, tricaproin, tricaprylin, tricaprin, trilaurin, trimyristin, tripalmitin and tristearin and were all puriss grade (Fluka, Buchs, Switzerland). Each was checked for purity as described previously (18). Crude sunflower oil was obtained locally (Meggitt, Sydney, Australia). Butter was purchased locally and the fat isolated for analysis by melting and filtration.

Methods

GLC. GLC was carried out on a Varian Model 2700 chromatograph fitted with an FID to which two minor modifications had been made as previously described (16). The glass column (2 m × 4 mm I.D.) was packed with 10% DEGS-PS on 80-100 mesh Supelcoport (Supelco, Bellefonte, Pennsylvania, U.S.A.). The carrier gas was high purity nitrogen at a flow rate of 30 ml/min, which was the van Deemter optimum for the column. High purity hydrogen was supplied at a flow rate of 45 ml/min to the FID from a Mark V Elhygen hydrogen generator (Milton Roy, Riviera Beach, Florida, U.S.A.) and compressed, oil-free air was supplied at a flow rate of 500 ml/min. The injector and detector temperatures were 175 C and 200 C, respectively, and the column oven temperature was programmed from 50 C to 165 C at 8 C/min. Samples were injected with a 5 μl plunger-in-barrel syringe fitted with a 9 cm needle (S.G.E., Melbourne, Australia) using a 2.5 μl injection volume and a "cold needle" injection technique, by which is meant that the plunger was depressed as soon as the needle had been inserted completely into the injection port without allowing time for the needle to heat up. Variations on the sample introduction technique are outlined later. The output signal from the detector was amplified at an electrometer sensitivity of 10^{-9} A/mV, and the electrometer signal was attenuated by a 1:10 voltage divider before A/D conversion. Peak areas were measured using a Hewlett-Packard Model 3354 Laboratory Automation System operating in a tangent skim mode for the short chain fatty ester peaks on the solvent tail. The

apparent weight-% composition of the methyl ester solutions was determined by the same automation system after applying the following theoretical relative response factors calculated using the method of Ackman and Sipos (15): methyl butyrate 1.5396, methyl caproate 1.3084, methyl caprylate 1.1927, methyl caprate 1.1233, methyl laurate 1.0771, methyl myristate 1.0440, methyl palmitate 1.0193 and methyl stearate 1.0000. Analyses were evaluated by determining the grade of analysis which is defined as follows:

$$\text{Grade} = 100 - \Sigma \mid c_i - c_i' \mid$$

where c_i = % content of ester determined
c_i' = % content of ester known

A grade of at least 99% was required before an analysis was considered to be satisfactory.

Standard Mixtures

Primary standard mixtures of saturated FAME were prepared from the reference esters. The first and second mixtures had chain length distributions simulating that of butter fatty acids, while the third was similar, but excluded methyl butyrate. The compositions were corrected according to the purity checks by the procedure described previously (16). The mixtures were analyzed as 10% solutions in isooctane.

The primary standard mixture of saturated triacylglycerols was prepared analogously to the FAME standards. The mixed primary/secondary standard was prepared by weighing the reference tributyrin and trimyristin into crude sunflower oil such that the concentrations of butyric and myristic acids were similar to those in butter fatty acids. The actual fatty acid composition of the long chain fatty acids in this mixture, including methyl myristate, was determined by the technique previously described (10).

Investigation of GLC Analysis

Optimization of the GLC component of the total analysis was carried out using a FAME standard (Standard No. 1). Variations in the sample introduction technique comprised the use of a "slow" injection and of "sample prime" and "solvent prime" loading of the sample in the syringe. By "slow" injection is meant that the syringe plunger was depressed during a period of approximately 1 sec instead of instantaneously. By sample prime is meant that the syringe needle was filled with sample before taking up the nominal injection volume of 2.5 µl. By solvent prime technique is meant that the syringe needle plus 0.5 µl was filled with solvent before taking up the nominal injection volume of 2.5 µl of sample.

Determination of Low Concentrations of Methyl Butyrate

To investigate the determination of methyl butyrate at lower concentrations, mixtures were made up of two primary FAME standards which were of similar composition, except that one contained methyl butyrate (Standard No. 2) and one did not (Standard No. 3). Solutions of 0, 10, 20, 50 and 100% of Standard No. 2 in Standard No. 3 were analyzed.

Investigation of the Methanolysis

By "quantitative methylation" is meant that a solution of the esters was obtained with a fatty acid composition representative of that of the original sample.

Studies into the rates of methanolysis of the two triacylglycerol standard mixtures and of the subsequent saponification of the methyl esters were carried out initially using the conditions specified in the international procedures, but reacting for various periods of time. Ambient temperature was approximately 20 C. Modifications to the technique included the use of sodium methoxide as catalyst and neutralization of the catalyst after 6 min by the addition of 2 N hydrochloric acid.

The repeatability of the total optimized procedure for the methanolysis of SCF and GLC of the methyl esters was determined by carrying out 10 analyses and calculating the coefficient of variation (CV) for the estimates of the major esters.

Optimized Methylation Procedure

SCF (ca. 200 mg) is dissolved in isooctane (ca. 2 ml) in a glass-stoppered test tube of approximately 10 ml capacity. 2N methanolic potassium hydroxide (ca. 100 µl) is added, the mixture shaken vigorously for 30 sec and allowed to react for a total of 6 min at room temperature (ca. 20 C). The catalyst is neutralized immediately by adding 2N hydrochloric acid with shaking, to the methyl orange end-point, and the small lower layer is allowed to separate (ca. 10 sec). The upper layer is injected into the chromatograph. (N.B. The upper layer will remain unchanged in composition for more than 24 hr while kept stoppered in the test tube, or it may be kept indefinitely sealed in glass ampoules stored at -20 C).

RESULTS AND DISCUSSION

Purity Checks on Esters

The results of purity checks on the reference esters are given in Table I.

TABLE I

Purity Checks on Reference Esters

	Composition by GLC analysis (%)									
	Fatty acid methyl ester									
Reference ester	4:0	6:0	8:0	10:0	12:0	14:0	16:0	18:0	Fatty acid[a]	Irrelevant peaks
Methyl butyrate	99.92									0.08
Methyl caproate		99.74	0.02						0.24	
Methyl caprylate			99.94	0.06						
Methyl caprate				99.71	0.13					0.16
Methyl laurate				0.04	99.60	0.36				
Methyl myristate (A)				0.10	0.94	98.74	0.22			
Methyl myristate (B)				0.07	0.64	99.03	0.18	0.03		0.05
Methyl palmitate						0.03	99.87	0.03		0.07
Methyl stearate								98.54	0.97	0.49

[a]Same chain length as principal ester.

These results are included to illustrate the need for stringent purity checks of the reference esters if highly accurate results are to be achieved, in particular the need to check for free fatty acid, which was detected in two instances.

Sample Introduction Technique for GLC

The results of GLC analyses of FAME Standard No. 1 using several variations of the sample introduction technique are given in Table II.

Under the initial set of conditions, it was not possible to obtain satisfactory results for the standard mixture. Loss of methyl butyrate was particularly severe, underlining one of the difficulties which commonly will be encountered in analyzing SCF methyl esters. This loss might have been attributed to:

(i) Discrimination in sample introduction;

(ii) Loss on the column;

(iii) Detector response other than that predicted by the theoretical response factors which were used to calculate the above results, or

(iv) Inaccurate integration of the peak area.

The assumption was made initially that the loss of butyrate was due to discrimination in sample introduction, in particular to either or both of two well-known causes of discrimination, viz. back diffusion after the sample has vaporized in the injection zone and/or discrimination in the syringe needle. The results in Table II illustrate the effect of techniques designed to overcome these problems. To attempt to reduce back diffusion, two measures were adopted. First, a "slow" injection technique, in which the syringe plunger was depressed during a period of approximately 1 sec, was used in order to limit the rate of vaporization of the sample and thus allow the carrier gas more time to sweep the sample onto the column. Second, the injection zone of the column was filled with column packing rather than glass wool in order to inhibit by chromatographic means any back diffusion of methyl butyrate. The effect of the progressive implementation of these measures is seen in Table II. It was apparent that the most important factor in obtaining higher accuracy than that obtained under the initial conditions was the use of the slow injection technique. A further improvement resulted from packing the injection zone with column packing, but there was no apparent benefit from the use of the solvent prime injection technique. Even better results were obtained on a second GLC column which showed improved chromatographic resolution compared with the first column. These results are shown in Table III, which deals with the analysis of low concentrations of methyl butyrate. Because of the high accuracy which was achieved using only the theoretical

TABLE II

Analysis of the First Primary Standard Methyl Ester Mixture with Variations of GLC Sample Introduction Technique

GLC technique[a]		Composition (%) FAME 4:0	6:0	8:0	10:0	12:0	14:0	16:0	18:0	Grade of analysis (%)
1. Initial	Known	5.86	2.84	1.94	2.99	5.11	11.43	31.81	38.01	
	Found	4.89	2.53	1.88	2.88	5.22	11.47	32.31	38.83	97.08
2. A		5.39	2.73	1.96	2.92	5.21	11.37	31.96	38.47	98.56
3. B		4.99	2.58	1.91	2.92	5.28	11.45	32.12	38.75	97.58
4. A+B		5.57	2.82	2.00	2.97	5.26	11.31	31.81	38.24	99.11
5. B+C		5.25	2.69	1.93	2.89	5.19	11.30	31.94	38.80	98.00
6. A+B+C		5.54	2.86	2.03	3.01	5.31	11.37	31.72	38.17	99.04

[a]Initial: "Fast" injection; glass wool packing in injection zone; sample prime.
A, "Slow" injection in place of "Fast" injection.
B, Column packing in place of glass wool packing.
C, Solvent prime in place of sample prime.

TABLE III

Analysis of Mixtures of Primary Methyl Ester Standards Containing Variable Amounts of Methyl Butyrate

Concentration of second primary standard in third (%)		Composition (%) FAME 4:0	6:0	8:0	10:0	12:0	14:0	16:0	18:0	Grade of analysis (%)
0 (Standard No. 3)	Known		4.79	3.10	3.62	7.20	14.25	27.98	39.07	
	Found (1)		4.76	3.10	3.61	7.29	14.15	27.99	39.10	99.73
	Found (2)		4.83	3.14	3.64	7.32	14.24	27.88	38.95	99.55
10	Known	0.64	4.62	2.99	3.65	7.00	14.15	28.15	38.81	
	Found (1)	0.61	4.60	3.00	3.66	7.08	14.11	28.17	38.76	99.74
	Found (2)	0.60	4.61	3.01	3.66	7.08	14.09	28.16	38.77	99.73
20	Known	1.29	4.45	2.87	3.69	6.79	14.04	28.32	38.56	
	Found (1)	1.22	4.43	2.90	3.69	6.92	14.01	28.32	38.51	99.67
	Found (2)	1.20	4.40	2.88	3.68	6.93	14.03	28.35	38.52	99.62
50	Known	3.22	3.94	2.54	3.79	6.18	13.73	28.82	37.79	
	Found (1)	3.10	3.95	2.56	3.78	6.27	13.67	28.84	37.83	99.63
	Found (2)	3.09	3.99	2.57	3.81	6.31	13.73	28.77	37.74	99.54
100 (Standard No. 2)	Known	6.43	3.10	1.97	3.96	5.16	13.21	29.66	36.51	
	Found (1)	6.25	3.08	1.97	3.92	5.23	13.20	29.80	36.56	99.49
	Found (2)	6.32	3.09	2.00	3.99	5.31	13.23	29.58	36.48	99.54

TABLE IV

Methanolysis of the Triacylglycerol Primary Standard Mixture Using Potassium Hydroxide as the Catalyst and Subsequent Saponification of the Methyl Esters

Time (hr)		Composition (%) FAME 4:0	6:0	8:0	10:0	12:0	14:0	16:0	18:0	Grade of analysis (%)
	Known	5.57	3.08	2.38	3.13	4.73	11.26	33.95	35.90	
0.1	Found	6.06	3.23	2.55	3.37	5.02	11.60	34.71	33.37	94.93
2		5.43	3.21	2.51	3.38	5.04	11.83	35.31	33.30	94.51
3		5.34	3.14	2.48	3.34	5.00	11.81	35.47	33.42	94.58
5		4.54	2.85	2.33	3.19	4.88	11.52	34.91	35.78	97.15
6		4.57	2.86	2.34	3.23	4.86	11.50	35.02	35.61	96.91
24		2.72	2.24	2.09	3.05	4.84	11.69	35.68	37.69	91.90

response factors of Ackman and Sipos to correct peak areas, it was concluded that the results indicated that these factors were highly accurate for all saturated straight-chain FAME down to and including methyl butyrate.

The overall conclusion from the foregoing was that, in the GLC analysis of SCF methyl esters, several chromatographic parameters, including the quality of the column itself, can affect the accuracy of the analysis. It is likely that the significant parameters will vary considerably from one instrument to the next. Whereas the standard procedures require that the analysis be standardized with a FAME mixture of accurately known composition, we maintain that these standards are generally improperly used. Thus, the usual practice is to determine "correction factors" which compensate for the total of all systematic errors. In contrast, we believe that such standards should properly be used to adjust chromatographic parameters such that accurate results are obtained using only the theoretical FID response factors of Ackman and Sipos to correct peak areas.

Determination of Low Concentrations of Methyl Butyrate

The determination of low concentrations of methyl butyrate is of interest for two reasons. First, it is required to evaluate accurately parameters which may give rise to losses of methyl butyrate in SCF methyl esters during preparation, handling and storage of the esters. Second, it is required for the accurate analysis of SCF when present in blends with other oils. The problem was investigated using blends of two primary FAME standards of similar composition, except that one contained methyl butyrate (Standard No. 2) and one did not (Standard No. 3). Results of analyses carried out under the optimum GLC conditions determined above are given in Table III.

The results in all cases were excellent, indicating that methyl butyrate could be determined accurately over the range of concentrations of interest.

Investigation of Methylation Technique

The quantitative aspects of the methanolysis reaction and the subsequent saponification of the methyl esters were investigated simultaneously. In the initial series of experiments, the primary standard mixture of saturated triacylglycerols was methylated under the conditions set down in the international standard methods (2,6), which required the use of KOH as the catalyst. Samples were analyzed as a function of time using the optimized GLC conditions determined above, and the results are given in Table IV.

The results indicated, first, that saponification of the short chain esters, especially methyl butyrate, proceeded much more rapidly than for the long chain esters and, second, that tristearin methylated at a significantly slower rate than the other triacylglycerols. With regard to the saponification problem, it was apparent that reaction times exceeding about 15 min at room temperatures were not acceptable, and attempts were made to overcome the difficulty (a) by using sodium methoxide as the catalyst, which would be expected to reduce greatly the rate of saponification, and (b) by neutralizing the reaction mixture after a reaction period of 6 min, this time representing an optimum between a short reaction time in order to minimize saponification and a long reaction time in order to promote methanolysis of the long chain triacylglycerols. With regard to the slow rate of methanolysis of tristearin, it was necessary to determine the influence of unsaturation for triacylglycerols of the same chain length, as this would be of critical significance for typical SCF and other fats and oils. It was again possible to investigate the two above problems simultaneously by using the mixed primary/secondary standard in which tributyrin and trimyristin were added to sunflower oil. The design of this mixture rested on the use of trimyristin as an internal standard to determine the rate of saponification of tributyrin on the one hand and the rate of methanolysis of the mainly unsaturated sunflower triacylglycerols on the other. The validity of using this mixture followed from the results in Table IV which indicated that the rate of saponification of trimyristin was negligible compared with that of tributyrin and that the methanolysis of trimyristin could be expected to be rapid and quantitative under the reaction conditions. The results of the studies concerning the saponification of methyl butyrate are given in Table V.

TABLE V

Saponification of Methyl Butyrate in the Mixed Primary/Secondary Standard

Catalyst	Time (hr)	Methyl butyrate (%)	
Potassium hydroxide		Known	4.82
	0.1	Found	5.05
	1.67		4.43
	5		3.62
	60		1.41
Sodium methoxide	0.1	Found	4.98
	1.67		4.65
	5		4.38
	24		4.34
Potassium hydroxide for 0.1 hr, then hydrochloric acid	0.1	Found	4.90
	1.67		4.95
	5		4.88
	24		4.91

The progressive saponification of methyl butyrate under the conditions specified in the international standard methods was again seen in these results. The use of sodium methoxide as the catalyst instead of potassium hydroxide, which was an alternative originally proposed by Christopherson and Glass (5), but not adopted in the international procedures, greatly reduced the rate of saponification of methyl butyrate. The use of this catalyst, however, was unacceptable because of the results given in Table VI concerning the methanolysis of the sunflower oil. Neutralizing the catalyst with a small amount of hydrochloric acid after 6 min was completely effective in stopping the saponification of methyl butyrate and gave a reaction mixture whose composition did not change during storage for 24 hr. The introduction of a small amount of water into the reaction mixture as a result of neutralizing with aqueous hydrochloric acid had no measurable effect on the concentration of methyl butyrate. Neutralization conveyed an additional benefit. In previous studies (10), we demonstrated that the repeated injection of reaction mixtures containing strong base leads to a rapid loss in column performance and the appearance of artifacts in the chromatograms. An easily recognized artifact eluted on DEGS-PS immediately after methyl caprate. This artifact was observed in the chromatograms of reaction mixtures using potassium hydroxide, and was larger again when sodium methoxide was used. It was absent, however, when the reaction catalyst was neutralized.

The results of the methanolysis studies for the longer chain triacylglycerols (sunflower oil) are given in Table VI. This table includes data from Table IV for the triacylglycerol primary standard recalculated excluding all fatty acids of chain length less than C14. In this respect, it was apparent that saponification of the short chain esters disproportionated the results for the long chain esters.

Further to the conclusions already drawn, the results for the primary standard indicated that quantitative methylation of tristearin was obtained only after reaction periods of the order of 24 hr under the conditions set down in the international standard methods. The results for the mixed primary/secondary standard indicated that this problem did not exist in the case of unsaturated fatty acids. Unexpectedly, sodium methoxide gave generally unsatisfactory results. Its use, therefore, appeared to be unacceptable on these grounds. Neutralization of the reaction mixture after 6 min led to excellent results and stable reaction mixtures, and it was concluded that this technique represented by far the best procedure for the methanolysis of SCF. Notwithstanding that unacceptable results were obtained for tristearin, the methylation of long chain saturated fatty acids appeared to be satisfactory in the presence of unsaturated fatty acids. This suggested that the problem may be specific for tristearin and that the method may thus not be acceptable for very hard fats, but this would have to be determined in individual cases.

Repeatability of Analysis of Typical Short Chain Fat

The optimum technique determined above for methanolysis and GLC analysis was applied to a butterfat sample in order to determine repeatability, and results for the principal fatty acids are given in Table VII.

CONCLUSIONS

The accurate quantitative analysis of the fatty acid composition of SCF requires initially that the chromatographic system be optimized and its quantitative performance verified using a carefully prepared primary standard mixture of saturated FAME with chain length ranging from C4 to C18 inclusive.

Adjustments to the system to optimize performance should be made such that accurate results are obtained for the primary standard using only the theoretical FID response factors of Ackman and Sipos to correct peak areas. These factors are highly accurate for all saturated FAME down to and including methyl butyrate and should be applied at all times.

The usual practice of applying "correction factors," which endeavor to compensate for the sum total of all errors due to deficiencies in the methylation technique, sys-

TABLE VI

Methanolysis of the Primary and the Mixed Primary/Secondary Triacylglycerol Standards

Catalyst	Time (hr)		Composition (%) FAME 14:0	16:0	18:0	18:1	18:2	20:0	18:3	22:0	Grade of analysis (%)
Primary standard		Known	13.88	41.86	44.26						
potassium	0.1	Found	14.56	43.56	41.88						95.24
hydroxide[a]	2		14.71	43.90	41.40						94.27
	3		14.63	43.95	41.41						94.31
	5		14.01	42.46	43.52						98.53
	6		14.00	42.64	43.36						98.20
	24		13.74	41.95	44.31						99.72
Mixed primary/		Known	15.01	5.51	4.77	16.17	56.92	0.28	0.36	0.62	
secondary standard,	0.1	Found	15.13	5.63	4.75	16.08	56.87	0.28	0.22	0.64	99.44
potassium hydroxide	1.67		15.05	5.54	4.79	16.13	56.88	0.32	0.38	0.63	99.76
	5		14.97	5.56	4.83	16.18	56.89	0.31	0.26	0.71	99.59
	60		14.71	5.58	4.95	16.44	56.76	0.32	0.27	0.70	98.81
Sodium methoxide	0.1	Found	16.11	6.14	4.74	16.00	55.28	0.29	0.25	0.62	96.31
	1.67		15.62	5.70	4.83	16.22	56.01	0.27	0.19	0.70	97.92
	5		15.37	5.63	4.86	16.28	56.29	0.30	0.25	0.72	98.46
	24		15.29	5.62	4.86	16.28	56.38	0.30	0.25	0.69	98.67
Potassium hydroxide	0.1	Found	15.05	5.51	4.78	16.18	57.03	0.31	0.28	0.60	99.70
for 0.1 hr, then	1.67		15.08	5.52	4.80	16.16	56.94	0.28	0.23	0.58	99.69
hydrochloric acid	5		15.01	5.52	4.78	16.15	56.89	0.32	0.30	0.66	99.79
	24		15.13	5.51	4.76	16.13	56.86	0.31	0.28	0.65	99.63

[a]Calculated from data in Table IV.

TABLE VII

Repeatability of Analysis of Typical Short Chain Fat Sample Using Optimized Procedure and Coefficients of Variation of Major Peaks

Fatty acid methyl ester	Concentration (%)	Coefficient of variation (%)
4:0	4.32	1.41
6:0	2.63	0.82
8:0	1.57	1.56
10:0	3.80	0.92
10:1	0.36	ND
12:0	3.79	1.11
12:1A	0.07	ND
12:1B	0.10	ND
13:0	0.20	ND
iso-14:0	0.13	ND
14:0	11.05	0.54
iso-15:0	0.30	ND
14:1 + anteiso-15:0	1.38	ND
15:0	1.16	ND
15:1	0.35	ND
16:0	25.17	0.44
16:1 + iso-17:0 + anteiso-17:0	2.49	ND
17:0	0.91	ND
17:1	0.36	ND
18:0	12.45	0.82
18:1	23.28	0.51
iso-18:1	0.59	ND
18:2	1.36	2.62
20:0	0.12	ND
18:3 + conjugated 18:2	2.04	0.90

ND = not determined.

tematic chromatographic errors, FID response and peak area measurement, is both unsound and unnecessary. All aspects of the analysis can and must be optimized so that the only correction factors which are required are the theoretical FID response factors.

Under the conditions specified for the international procedures for the methylation of fats and oils containing four or more carbon atoms, saponification of short chain esters, especially methyl butyrate, occurs much more rapidly than for long chain esters. The sample is no longer representative after as little as 15 min.

Saponification can be stopped by neutralizing the catalyst with hydrochloric acid when the methanolysis is complete (approx. 6 min). This recommended change to the international procedures is minor and retains the essential simplicity of the method, but conveys two important advantages. First, stable reaction mixtures and, in turn, highly accurate and reproducible quantitative results are obtained; the introduction of a small amount of aqueous phase results in no significant loss of methyl butyrate. Second, removal of the catalyst eliminates the inevitable progressive deterioration of the column caused by the injection of strong base.

The above procedures render unnecessary the use of methyl pentanoate as an internal standard for the accurate determination of methyl butyrate.

ACKNOWLEDGMENT

The Directors of Unilever PLC and of Unilever Australia Limited gave permission to publish this paper.

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[Received April 2, 1985]