Journal of Chromatography, 279 (1983) 493–506
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 201

GLASS CAPILLARY GAS CHROMATOGRAPHY OF FATTY ACID METHYL ESTERS. A STUDY OF CONDITIONS FOR THE QUANTITATIVE ANALYSIS OF SHORT- AND LONG-CHAIN FATTY ACIDS IN LIPIDS

H. T. BADINGS* and C. DE JONG
Netherlands Institute for Dairy Research (NIZO), Ede (The Netherlands)

SUMMARY

Methods of fatty acid analysis based on the capillary gas chromatography of fatty acid methyl esters (FAMEs) are now well established. However, several ring tests have shown that results obtained in various laboratories are unsatisfactory with regard to reproducibility, particularly in the analysis of lipids containing lower fatty acids (*e.g.*, milk fat). A rapid, reliable, and precise method for the quantitative analysis of FAMEs derived from lipids is described. Methods of preparing FAMEs sample introduction techniques, handling of glass capillary columns, and results of the quantitative analysis of fatty acids are presented.

INTRODUCTION

From the very beginning, gas chromatography (GC) has been an important aid in the analysis of fatty acids in fats and oils and other lipids[1]. Many papers have been published dealing with the GC analysis of fatty acids, particularly that of fatty acid methyl esters (FAMEs).

In order to improve the various methods further, two aspects have been given special attention: (a) the reliable conversion of free and bound fatty acids into FAMEs and (b) the accurate analysis of FAMEs by GC. Significant progress was made after the introduction of glass capillary gas chromatography on columns coated with suitable liquid phases having a high separating efficiency and selectivity. However, to ensure that fatty acid analyses are reliable, it is essential that none of the steps in the sequence of operations (from isolation and conversion into FAMEs to the final GC analysis) affect the qualitative and quantitative results. In particular, difficulties may be encountered in the analysis of lipids containing lower fatty acids (*e.g.*, milk fat) owing to the volatility of these fatty acids (and esters). Thus, special techniques are required for their conversion into FAMEs and for GC analysis.

This paper deals with the development of methods for the quantitative fatty acid analysis of lipids containing short- and long-chain fatty acids by means of glass capillary GC. In particular, the method of FAME preparation according to Christopherson and Glass[2] was re-evaluated. As this procedure lacks certain details, various modifications have been introduced in recent years, which have led to the misinterpretation of certain

0021-9673/83/$03.00

critical steps. As a result, inconsistent and erroneous results have been obtained that raised doubts as to the usefulness of the method.

We have re-examined the various steps in the method of Christopherson and Glass, optimized them, and have finally succeeded in achieving a detailed procedure that yields quantitative, precise and reproducible results. Also, conditions for accurate FAME analysis by glass capillary GC (injection techniques, types of column, etc.) are given.

EXPERIMENTAL

Reagents

Methanol containing $\leq 0.03\%$ of water, *n*-heptane $\geq 99.0\%$ and *n*-pentane $\geq 99.0\%$ were obtained from J. T. Baker.

2 M sodium methoxide in methanol. To a weighed amount of anhydrous methanol the appropriate amount of metallic sodium (cut into small pieces) is added slowly in order to limit temperature rise and hydrogen production during the reaction. The flask is provided with a drying tube to exclude water vapour.

Methanol–HCl (80:20, w/w). A weighed amount of anhydrous methanol is poured into a two-necked round-bottomed flask. One opening of the flask is used to insert a tube (reaching to the bottom of the flask) for the introduction of dry, gaseous hydrogen chloride from a cylinder. The other opening is provided with a drying tube. An empty wash bottle is connected between cylinder and flask as a safeguard in case the contents of the vessel are sucked back.

Conversion of lipid-bound fatty acids into FAMEs

A 100-mg amount of the anhydrous lipid sample is weighed into an 8-ml Sovirel culture tube with a screw-cap (PTFE liner) and 6 ml of heptane are added to dissolve the sample. Then 0.06 ml of 2 *M* sodium methoxide solution are added and the contents of the capped tube are stirred vigorously for 60 sec at room temperature with a Vortex mixer. The sediment of sodium glycerolate is separated by centrifugation at 1000 *g* for 3 min. A sample from the clear supernatant (usually 1 μl) is then taken for GC analysis of the FAMEs.

The following points should be given attention, in order to ensure correct performance of the procedure: (1) thorough shaking is essential to complete the reaction; (2) protect the reaction mixture against moisture from ambient air; (3) use pentane instead of heptane when analyses are carried out on narrow-bore capillary columns; (4) if the sample must be used for a longer period of time, the clear solution should be transferred from the tube (avoiding any contamination with sediment) to a small vial with a minimum of headspace.

Conversion of free fatty acids into FAMEs

A 100-mg amount of a sample of anhydrous free fatty acids (FFAs) (or the sodium salt) is weighed into an 8-ml Sovirel culture tube with a screw-cap (PTFE liner) and 6 ml of heptane are added. Then 1 ml of methanol–20% hydrochloric acid is added, the screw-cap is closed tightly and the Sovirel tube is mounted in a horizontal position in a shaking water-bath (temperature 85°C, shaking frequency 200/min).

After 15 min the tube is removed from the bath, cooled to 15°C and centrifuged

for 3 min at 1000 *g*. A sample is taken from the clear upper layer (usually 1 μl) for GC analysis of FAMEs.

For correct performance of the procedure, reference is made to points 1, 2 and 3 in the preceding section.

General procedure for the preparation of FAMEs from partly lipolysed lipid samples

Determination of the degree of lipolysis. Dissolve 100 mg of the lipid sample in 20 ml of ethanol–diethyl ether (1:1) and titrate with a 0.1 *M* methanolic sodium hydroxide solution, using thymol blue as indicator. Calculate mequiv. FFAs/100 mg of lipid = *f*.

Conversion of lipid-bound fatty acids into FAMEs. Weigh 100 mg of the anhydrous lipid sample into an 8-ml Sovirel culture tube and proceed as described in the first procedure, but add (*f* + 0.12) mequiv. sodium methoxide. If the composition of the lipid-bound fatty acids is to be determined, a sample can be taken from the clear supernatant for GC analysis.

Conversion of free and bound fatty acids into FAMEs. After the conversion step with sodium methoxide, open the Sovirel tube and add 1 ml of 20 % hydrochloric acid–methanol for esterification. Close the screw-cap tightly, shake the tube thoroughly with a Vortex mixer for 60 sec, and then mount it in a horizontal position in a shaking waterbath (temperature 85°C, shaking frequency 200/min).

After 15 min, remove the tube from the bath, cool it to 15°C, and subject its contents to centrifugation (3 min, 1000 *g*). Take a sample from the clear upper layer (usually 1 μl) for GC analysis of the FAMEs. For correct performance of the method, see points 1, 2 and 3 of the first procedure. The three procedures are summarized in Table I.

Glass capillary gas chromatography of FAMEs.

Analysis by making use of wide-bore capillary columns (WBCCs). A Varian

TABLE I

NIZO-PROCEDURE FOR ESTERIFICATION OF FATTY ACIDS TO METHYL ESTERS

Conversion of glyceride-bound fatty acids	*Conversion of free fatty acids*	*Combined procedure for conversion of glyceride-bound and free fatty acids*
100 mg fat	100 mg fat	Detn. of FFA value (*f* mequiv. 100 mg)
6 ml heptane	6 ml heptane	100 mg fat
0.06 ml 2 *M* $NaOCH_3$–CH_3OH	1 ml 20% HCl–CH_3OH	6 ml heptane
Shake 60 sec/20°C	Shake 15 min/85°C	(*f*+0.12) mequiv. 2 *M* $NaOCH_3$–CH_3OH
Centrifuge	Centrifuge (15°C)	Shake 60 sec/20°C
Inject ≈ 1 μl heptane phase for GC analysis	Inject ≈ 1 μl heptane phase for GC analysis	1 ml 20% HCl–CH_3OH
		Shake 15 min/85°C
		Centrifuge (15°C)
		Inject ≈ 1 μl heptane phase for GC analysis

Model 2740 gas chromatograph, equipped with a non-vaporizing on-column injector (own design)[3] for use with WBCCs and a flame-ionization detector, is used. The column is 40 m × 0.7 mm I.D. soda-glass, HCl-etched and coated with diethylene glycol succinate (DEGS) (film thickness, d_f = 1.2 μm). The carrier gas (helium) flow-rate is 7 ml/min. For the sample, draw 1.0 μl of the FAME solution, prepared as described above, into a 5-μl syringe (Hamilton, Type 85 SN, with a 70 mm/32 Ga needle, point style 3, ground-off square and bevelled). Inject at 30°C, raise the oven temperature to 180°C at a rate of 6°C/min and keep the column at this temperature for 18 min. The detector temperature is 220°C. Take care that the sample is injected correctly and handled adequately. Details of how to mount the injector in the oven and how to perform the procedure have been published earlier[3]. The original injector has been slightly modified, in order to allow coupling of the capillary column directly to the injector by means of ferrules. The adapted model is shown in Fig. 1. A small constriction in the vertical capillary of the injector, 1 cm below the tee, limits the extent to which the straightened capillary can be inserted into the aperture.

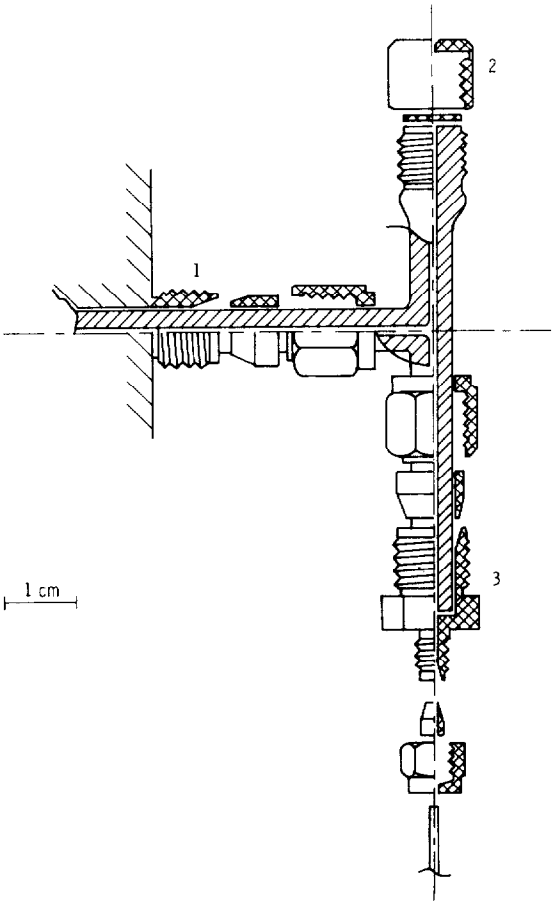


Fig. 1. Non-vaporizing on-column injector for wide-bore capillary columns. 1=Part of the tee inserted into the carrier gas inlet, from the inside of the column oven; 2=injector nut with septum; 3=mounting of straightened capillary.

Analysis by using narrow-bore capillary columns. A Carlo Erba Model 4160-01 gas chromatograph equipped with an on-column injector (automatic actuation) and a flame-ionization detector is used with a glass capillary column, 50 m × 0.32 mm I.D., coated with CP-Sil 88 (equivalent to Silar-10 or SP-2340; d_f = 0.2 μm) obtained from Chrompack (The Netherlands). The carrier gas (helium) flow-rate is 1.8 ml/min. The first coils of the capillary are washed with a solvent, in order to remove the stationary phase.

For the sample, 1.0 μl of the FAME solution is prepared as described above, but the dilution is 1:10. Pentane is used as solvent instead of heptane, in order to ensure a correct shape of the methyl butyrate peak*. A 10-μl syringe (Hamilton, Type 701 SN, with a needle of 75 mm length, 0.20 mm O.D. and point style 3) is used. Injection should take place at 30°C (with secondary air cooling for 7 sec) and the oven temperature is raised to 230°C at a rate of 5°C/min. The detector temperature is 250°C.

RESULTS AND DISCUSSION

Some considerations on the choice of methods of FAME preparation

Many methods for the esterification of fatty acids have been published. Most are based on diazomethanolysis[4], acid-catalysed methanolysis with hydrochloric acid–methanol[5], borontrifluoride–methanol[6] or sulphuric acid–methanol[7]. Also use is made of base-catalysed transesterification with sodium methoxide–methanol[2,8]. Other techniques involve saponification followed by either acid-catalysed esterification[9] or boron trifluoride–methanol esterification[10,11]. Recently, a method of base-catalysed transesterification with tetramethylammonium hydroxide has been published[12]. Reviews[13,14] have been published on methods for the preparation of fatty acid esters. Also, several studies have been published in which different methods are discussed and/or compared[11,15–19]. It must be concluded that a great many of the published methods have their disadvantages.

First, most of these methods are not applicable to the analysis of lipids which contain lower fatty acids, because low recoveries of these fatty acids and poor repeatability have been reported[11,15,16,18]. This holds true particularly for the procedures involving acid-catalysed methanolysis. Iverson and Shepard[17] have shown that the preparation of fatty acid *n*-butyl esters (FABEs) instead of FAMEs gives better results in the analysis of lower fatty acids. However, FABEs have a lower volatility, so that a higher temperature for GC and prolonged analysis times are needed. Other disadvantages have been pointed out by Timms[20].

Second, a number of methods for FAME preparation involve cumbersome procedures and/or the use of toxic reagents (diazomethane, boron trifluoride, etc.).

Some of the problems mentioned can be overcome by resorting to methods involving base-catalysed methanolysis. Lower fatty acids can also be analysed quantitatively by these methods, but FFAs are not converted. Also, the GC separation of the peaks for methyl butyrate and the methanol reagent (if present in larger amounts) may be difficult.

In order to cope with the last-mentioned problems, Christopherson and Glass[2] have developed a method of sodium methanolate-catalysed methanolysis in which a

* The use of very volatile solvents is not recommended in sampling techniques in general. To prevent overlap and peak distortion of the methyl butyrate peak, the use of pentane is a compromise.

small amount of methanol reactant is used. For the conversion of FFAs a second esterification step is carried out with methanol–hydrochloric acid.

The method published by Christopherson and Glass[2] lacks certain details. Therefore, a number of critical steps were re-examined in order to optimize them, and to formulate the present procedure which gives quantitative and precise results.

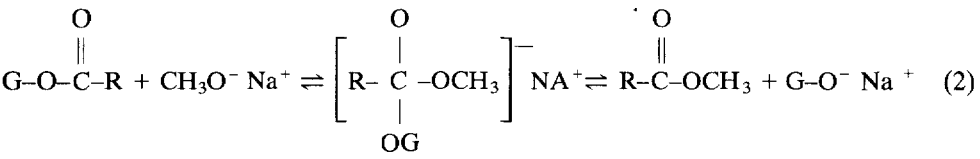
Optimization of the methanol–sodium methoxide esterification

The optimization of this procedure requires the following considerations.

(1) Glass[21] made an extensive study of alcoholysis and saponification reactions in methods for the preparation of FAMEs involving base-catalysed transesterification with sodium methoxide or hydroxide. In methanolic solutions of sodium hydroxide, methanolysis precedes the saponification process. This is due to the hydroxide–alkoxide equilibrium:

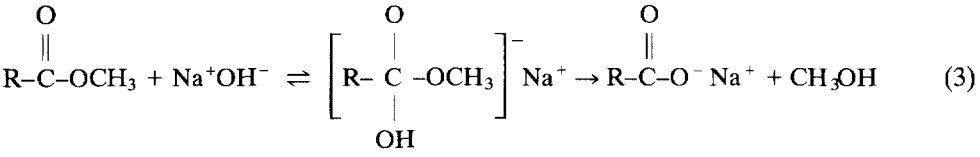
$$NaOH + CH_3OH \rightleftharpoons CH_3ONa + H_2O \quad (1)$$

which greatly favours methoxide formation, even in the presence of considerable amounts of water.



(2)

where G = glycerol/glyceride. However, if the solution is allowed to stand for some time, the esterification process is followed by a slower saponification reaction under the influence of hydroxide.



(3)

If a solution of sodium methoxide in anhydrous methanol is used, hydroxide formation and, hence, saponification will not take place. For this reason preference was given to the latter reagent in the method of esterification presented here. In accordance with the reaction mechanism outlined above, IR analysis showed that the sediment formed in the reaction is probably monosodium glycerolate.

(2) Although small amounts of water are apparently not deleterious, they may at length cause saponification. Fig. 2 shows the different reactions (methanolysis and saponification) that may occur during the base-catalysed conversion of glyceride-bound fatty acids into FAMEs.

A few studies have been made on the stability of the FAME reaction mixture. If the supernatant was left in contact with the sediment for 4 h, the FAME composition of the solution did not change. However, a long period of contact is not recommended. If the clear solution (separated from the sediment) was stored in a closed vial for several days, it was found to remain stable; the FAME composition of the solution did not change.

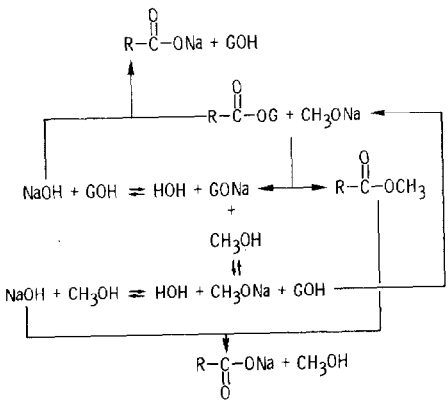


Fig. 2. Different pathways in the conversion of glyceride-bound fatty acids to FAMEs catalysed by sodium methanolate.

Although no extensive study of the stability of the reaction mixture after conversion with sodium hydroxide–methanol (possibly containing some water) was made, it was found that erroneous results could occur if the reaction mixture was stored.

(3) If the original Christopherson and Glass[2] procedure is used, the concentration of FAMEs formed is relatively high. It was found that the repeatibality in quantitative FAME analysis is low, when volumes < 1 μl are injected. The results appeared to be considerably better when only 100 mg of a lipid sample were dissolved in 6 ml of heptane and, after conversion into FAMEs, 1 μl of the solution was injected.

(4) Addition of 0.06 ml of 2 *M* sodium methoxide–methanol for esterification of 100 mg of milk fat in 6 ml of heptane yielded the best results. The reaction proceeded rapidly (completed in less than 60 sec) and the sediment could easily be separated from the supernatant. It should be noted that an amount of sodium methoxide has to be added which is approximately equimolar to the amount of glycerides, in order to complete alcoholysis. The procedure given in the experimental section is suitable for milk fat.

A larger volume of sodium methoxide of a lower concentration (*i.e.*, 0.12 ml, 1 *M*) results in a much longer reaction time and in the formation of a second layer of sediment and methanol. This layer may affect the FAME composition of the heptane phase.

(5) Vigorous shaking of the reaction mixture is essential to the complete conversion of both lower and higher fatty acids into FAMEs.

Esterification of free fatty acids

For the esterification of FFAs, which cannot be converted into FAMEs by base-catalysed methanolysis, Christopherson and Glass[2] and Glass[21] proposed a treatment of the lipid-containing heptane solution with 20% methanolic hydrochloric acid at room temperature for 1 h. However, in our hands, this method did not give satisfactory results; the degree of conversion was very low. A further study revealed that, in order to obtain good results, two factors are essential: (a) vigorous shaking of the heptane and 20% methanolic hydrochloric acid to increase the contact surface between the two phases (frequency 200/min) and (b) use of a higher temperature (85°C). The esterification of a known amount of FFA was studied at various reaction times. It was

found that 15 min were sufficient to obtain >99% conversion of FFAs into FAMEs. Even a period of 5 min gave satisfactory results, but as it is difficult to define the time required for the Sovirel tube containing the reaction mixture to warm up, a reaction time of 15 min was chosen.

Complete procedure for FAME preparation

If lipids containing both esterified and free fatty acids are to be analysed, their conversion into FAMEs must be carried out in two steps: (I) methanolysis with sodium methanolate (to convert the glyceride-bound fatty acids), followed by (II) methanol–hydrochloric acid esterification (to convert FFAs). As discussed before, the amount of sodium methanolate in the base-catalysed transesterification must be approximately equimolar to the amount of triglycerides in order to complete the reaction. In partly lipolysed lipids, an extra amount of sodium methanolate must be added to compensate for the neutralization of FFAs.

After the first step, the FFAs are present as sodium salts. For this reason, the contents of the reaction tube, including the sediment, must be used to perform the second step, the conversion of FFAs into FAMEs with methanol–hydrochloric acid.

The whole procedure was tested in a model experiment in which a mixture of glyceride-bound and free fatty acids of known composition was submitted to the two successive conversion steps. As can be seen from Table II, the results obtained come very close to the known composition of the sample.

In another experiment, the fatty acid composition of non-lipolysed milk fat was determined by step I and also, after complete saponification, by step II. The results of the two procedures were equal to within 0.1% difference for each fatty acid.

TABLE II

ANALYSIS OF A SAMPLE, B, CONTAINING GLYCERIDE-BOUND FATTY ACIDS (SAMPLE A) TO WHICH FFAs HAVE BEEN ADDED

Results obtained with step I should be equal to composition A, and results obtained with steps I and II should be equal to B. Results are expressed as mass-% fatty acids.

Fatty acid	*Known FA composition*		*Result of analyses of sample B after:*	
	A=milk fat	*B=A+ added FFAs*	*Conversion by steps I (CH_3OH–$NaOCH_3$)*	*Conversion by steps I and II (CH_3OH–$NaOCH_3$; CH_3OH–HCl)*
4:0	4.2	2.4	4.1	2.5
6:0	2.5	1.5	2.5	1.5
8:0	1.6	0.8	1.6	0.9
10:0	3.1	1.8	3.1	1.8
12:0	4.2	11.3	4.2	11.4
14:0	12.0	6.5	11.9	6.6
16:0	29.6	41.6	29.8	41.5
18:0	10.0	14.8	10.2	14.9
18:1	21.3	12.1	21.5	11.9
18:2	1.7	0.9	1.7	0.9
18:3	1.2	0.7	1.2	0.7
Others	8.6	5.6	8.2	5.4
Total	100.0	100.0	100.0	100.0

Finally, it was established that fatty acids, bound in phospholipids and cholesterol esters, can also be analysed by this procedure.

Glass capillary GC of FAMEs

Several papers[22–24] have shown that at present a high level of perfection has been reached in the GC resolution of very complex mixtures of FAMEs. All the same, the problems in the *quantitative* analysis of FAMEs are still underestimated. There are several factors that play a role (see also the next section). With regard to the quantitative GC analysis, it should be pointed out that unequal elution of sample components of different volatility from the syringe needle is a major cause of discrimination between sample components during injection of a sample in a heated (vaporizing) GC injector[25–27]. Serious errors in quantitative analysis are the results. We have experienced these phenomena in the analysis of FAMEs of milk fat (chain length between C_4 and C_{20}). The only solution to these problems is the use of a non-vaporizing on-column injector, as was explained by Grob and Grob[28]. For capillary columns of 0.3 mm I.D., such types of injector were designed by Schomburg *et al.*[29], Grob and Renhard[27,30] and Galli and Trestianu[31]. For wide-bore capillaries we have constructed a simple on-column injector (see Fig. 1 and ref. 3). This injector has been tested[3] and has been found to operate satisfactorily without sample discrimination. An example of the separation

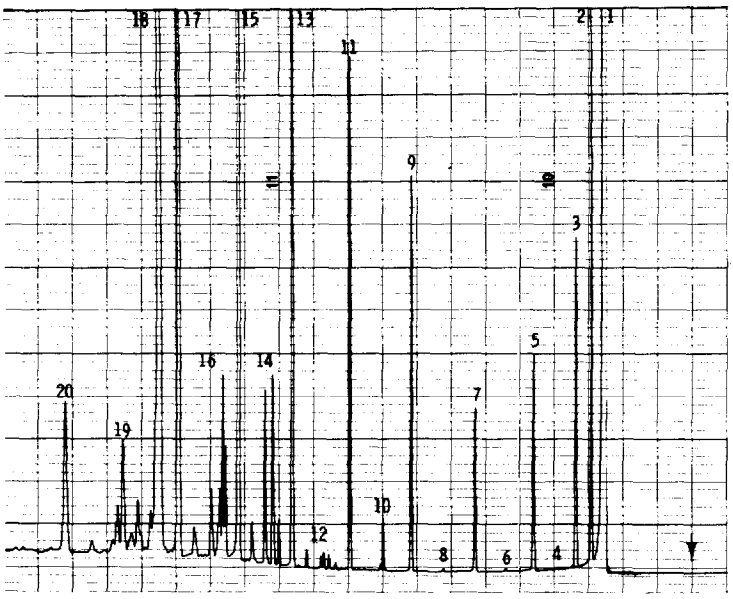


Fig. 3. Separation of fatty acid methyl esters from milk fat by capillary GC with the designed on-column injector for wide-bore capillary columns. For further experimental details see Experimental. Identity of peaks (determined by GC–MS analysis): 1=heptane; 2=methanol (solvent); 3=4:0; 4=5:0; 5=6:0; 6=7:0; 7=8:0; 8=9:0; 9=10:0; 10=10:1 followed by 11:0; 11=12:0; 12=peaks in succession: 13:br, 12:1, 13:0, un, 14:br, 13=14:0; 14=peaks in succesion: 15:br, 14:1, 15:0, 16:br + 15:1; 15=16:0; 16=peaks in succession: 16:1 + 17:br; 17:br, 17:0 + 16:1, 17:1 + 18:br; 17=18:0; 18=18:1 + 19:br; 19=peaks in succession: 19:0, un, 18:2, 20:0; 20=18:2 + 18:3. The first part of the code refers to the number of carbon atoms in the fatty acid chain. For the second part: 0=no double bonds in the chain; 1=one double bond in the chain, etc. br=branched-chain fatty acid; un=unknown fatty acid.

of FAMEs from milk fat is given in Fig. 3. Most of the peaks are separated satisfactorily and the solvent peaks do not interfere with the methyl butyrate peak.

It should be noted that it is essential to wash the first coils (1.2 m) of the capillary column with a solvent in order to remove the stationary phase because it was found that contact of the FAME reagent solution with the DEGS stationary phase leads to formation of artefacts. Peaks due to the presence of diethylene glycol, dimethyl succinate and other succinate esters could be demonstrated by GC–MS. One of these peaks coincides with the 16:0 FAME peak.

Similar problems were encountered by Timms[20], who used packed columns coated with cyanopropylsilicone phases. Timms also demonstrated that the artefact peaks observed by Iverson and Shepard[17] are due to the same phenomenon and are *not* caused by suspected artefacts of the alcoholysis procedure. Such artefacts were detected neither by Timms nor in this study.

Summary of factors affecting the accuracy and precision of FAME analysis by GC

To improve the results obtained with quantitative FAME analyses by GC in general, it is useful to present a summary of factors affecting the accuracy and precision of these types of analysis. This summary, given in Table III, is based on information from the literature and on results obtained in this work.

In many methods problems may arise from side-reactions and, in particular, from

TABLE III

FACTORS WHICH MAY AFFECT THE ACCURACY AND PRECISION OF QUANTITATIVE ANALYSIS OF FAMEs BY GAS CHROMATOGRAPHY

1. Conversion of fatty acids into FAMEs
1.1. Incomplete conversion
1.2. Side reactions (saponification, etc.)
1.3. Losses during preparation of FAMEs (evaporation, adsorption, extraction)

2. Sample introduction
2.1. Losses in the syringe
2.2. Sample discrimination during injection (selective evaporation)
2.3. Sample discrimination in splitters, other injector devices, etc.
2.4. Cold spots, back-diffusion, etc.
2.5. Pollution due to residues and decomposition products
2.6. Memory effects (caused by, among other things, 2.4 and 2.5)

3. Separation
3.1. (irreversible) adsorption; may cause losses, tailing, memory effects, etc.
3.2. Degradation or conversion (isomerization) of solutes
3.3. Influence of residence time in the column in combination with 3.1 and 3.2

4. Detector
4.1. Optimization, linearization of response, etc.
4.2. Pollution

5. Data acquisition
5.1. Interpretation of peak areas (baseline, drift, double peaks, etc.)
5.2. Further aspects of calculations
5.3. Response factors

losses of FAMEs during the conversion procedure, notably of lower fatty acids. These problems were discussed, and methods to overcome them were presented.

Sample introduction may also cause errors. Great care should be taken to prevent losses, pollution and memory effects. In particular, sample discrimination must be avoided. Apart from cold on-column injection, high precision and accuracy may be achieved with cold temperature-programmed splitless and split injection techniques, as discussed recently by Schomburg and co-workers[32,33]. In GC separation, errors may arise from losses of FAMEs during migration of compounds through the column. For this reason, it is important to use columns that are sufficiently inert and/or have been deactivated. Finally, attention should be paid to the risk of errors being introduced by certain methods of detection and data acquisition.

Determination of response factors

The accurate determination of response factors (RFs) for the different types of FAMEs is of primary importance in quantitative analysis. RFs are determined in the first place by differences in response between different FAME in flame-ionization detection. However, it should be noted that RFs are actually influenced by all factors mentioned in the preceding section. This means that RFs are instrument- and laboratory-dependent, even if the sample procedure and/or equipment is used. It is therefore important that each laboratory should determine its own RFs, taking into account that the methods used for RF determination and for actual FAME analysis should be identical.

For the present study, relative response factors (RRFs) for a number of FAMEs were determined by using triglyceride (TG) reference compounds which were subjected to the complete procedure. Before starting this work, the purity of the TG reference compounds and their exact composition had been determined by GC of the TGs and fatty acids, and by HPLC of the TGs. Reference TGs of minor fatty acids (branched-chain, unsaturated) were not usually available. The RFs of these fatty acids can be considered to be equal to those of the saturated fatty acids with the same carbon number.

Results are given in Table IV. RRFs for 14:0 and higher FAMEs come close to 1. This makes it clear that sample discrimination does not occur, and that the residence time of the FAMEs does not influence the RRFs. The latter observation indicates that the GC system is sufficiently inert so that compounds having a larger residence time are not lost to any great extent. If such a phenomenon should occur, the RRFs would steadily increase from 14:0 to 18:0 and higher FAMEs. Below 14:0, the RRF values increase with shorter chain length, as the relative amount of the FAME molecule that can be burnt in the flame-ionization detector is smaller for the lower fatty acids. It should be noted that RRFs are given with regard to 18:0 and are converted into values for mass-% fatty acid calculations.

Results of analyses of milk fat

The method presented here has now been used routinely for more than 3 years and hundreds of samples of milk fate have been analysed. The precision of the method was tested by analysing one sample of milk fat nineteen times. For each analysis the whole procedure, including the esterification step, was performed separately. The results are given in Table V.

TABLE IV

RELATIVE RESPONSE FACTORS OF THE FAMEs

Response factors are given in relation to 18:0, and are converted into mass-% fatty acid (means of 11 separate determinations).

Fatty acid	*RRF**	*S.D.***
4:0	1.43	0.032
6:0	1.18	0.024
8:0	1.09	0.017
10:0	1.05	0.013
12:0	1.03	0.008
14:0	1.02	0.010
16:0	1.01	0.017
18:0	1.00	—
18:1	1.00	0.019
18:2	1.00	0.003
18:3	1.00	0.003

* Relative response factors.
** Standard deviation of RFF.

The usefulness of the method was also tested by comparing the results of a ring test for one sample of milk fat, obtained in two separate laboratories. From Table VI it can be seen that the results were substantially improved after the present method had been introduced.

In conclusion, it should be mentioned that lipid reference materials of known fatty acid composition can be of great help in further improving the accuracy and pre-

TABLE V

ANALYSIS OF A SAMPLE OF MILK FAT

Average mass-% fatty acid (X), standard deviation (Sx) and number of independent determinations (n) used for the calculations are listed.

*Fatty acid**	*X*	*Sx*	*n*	*Fatty acid**	*X*	*Sx*	*n*
4:0	3.89	0.109	17	15:0	1.17	0.020	18
6:0	2.09	0.038	19	16:br	0.38	0.018	16
8:0	1.22	0.020	19	16:0	23.43	0.213	17
10:0	2.55	0.030	19	16:1	0.86	0.032	15
10:1	0.32	0.011	19	17:br	1.27	0.046	13
11:0	0.05	0.009	15	17:br	0.80	0.044	17
12:0	3.18	0.029	19	17:0+16:1	0.77	0.056	19
13:br	0.05	0.004	18	18:br+17:1	0.43	0.034	17
12:1	0.10	0.006	19	18:0	11.57	0.057	18
13:0	0.10	0.005	19	18:1	27.89	0.191	16
un	0.08	0.009	19	19:br	0.17	0.024	18
14:br	0.12	0.010	19	19:0	0.80	0.051	18
14:0	9.83	0.066	19	un	0.37	0.033	17
15:br	0.32	0.027	19	18:2	1.51	0.023	14
14:1	1.57	0.018	18	20:0	0.59	0.026	16
				18:3+18:2	2.52	0.058	19

* First part of code refers to the number of carbon atoms in the fatty acid chain. Second part: 0 = no double bonds in the chain; 1=one double bound in the chain; etc.; br=branched-chain fatty acid; un = unknown fatty acid.

TABLE VI

DETERMINATION OF THE FATTY ACID COMPOSITION OF A SAMPLE OF MILK FAT

Results of a ring test before and after introduction of the present method (note: the two samples are different).

Fatty acid	*Before introduction*		*After introduction*	
	Lab. A	*Lab. B*	*Lab. A*	*Lab. B*
4:0	2.6	3.3	3.7	3.9
6:0	1.6	2.0	2.1	2.1
8:0	1.0	1.2	1.2	1.2
10:0	2.2	2.6	2.6	2.6
12:0	3.0	3.4	3.2	3.2
14:0	8.4	9.6	9.9	9.8
16:0	25.9	26.7	23.6	23.4
18:0	12.0	11.1	11.4	11.6
18:1	29.2	26.6	27.2	27.3
Others	14.1	13.5	15.1	14.9
Total	100.0	100.0	100.0	100.0

cision of quantitative FA analyses by GC. Such reference materials are now in preparation by the EEC Bureau of References in Brussels and will become available in due course.

NOTE

After completion of this work, a paper by Bannon *et al.*[34] was published, dealing with the methoxide-catalysed methanolysis of fats and oils. Brief refluxing of the reaction mixture and removal of methoxide by aqueous extraction are recommended in this procedure. In our study the latter steps were not found necessary. They may also lead to losses of methyl butyrate.

REFERENCES

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 S. W. Christopherson and R. L. Glass, *J. Dairy Sci.*, 52 (1969) 1289.
3 H. T. Badings, C. de Jong and J. G. Wassink, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 644.
4 R. Roper and T. S. Ma, *Microchem. J.*, 1 (1957) 245.
5 W. Stoffel, F. Chu and E.H. Ahrens, Jr., *Anal. Chem.*, 31 (1959) 307.
6 W. R. Morrison and L. M. Smith, *J. Lipid Res.*, 5 (1964) 600.
7 *Official Methods of Analysis*, AOAC, Washington, DC, 10th ed., 1965, Sect. 266.052.
8 J. M. de Man, *J. Dairy Sci.*, 47 (1964) 546.
9 C. J. F. Böttcher, F. P. Woodford, E. Boelsma-van Haute and C. M. van Gent, *Rec. Trav. Chim. Pays-Bas*, 78 (1959) 794.
10 L. D. Metcalfe, A. A Schmitz and J. R. Pelka, *Anal. Chem.*, 38 (1966) 514.
11 D. van Wijngaarden, *Anal. Chem.*, 39 (1967) 848.
12 L. D. Metcalfe and C. N. Wang, *J. Chromatogr. Sci.*, 19 (1981) 530.
13 A. J. Shepard and J. L. Iverson, *J. Chromatogr. Sci.*, 13 (1975) 448.
14 R.G. Ackman, *Methods Enzymol.* 14 (1969) 329.
15 M. L. Vorbeck, L. R. Mattick, F. A. Lee and C. S. Pederson, *Anal. Chem.*, 33 (1961) 1512.
16 J. M. de Man, *Lab. Pract.*, 16 (1976) 150.

17 J. L. Iverson and A. J. Shepard, *J. Ass. Offic. Anal. Chem.*, 60 (1977) 284.
18 D. Firestone and W. Horowitz, *J. Ass. Offic. Anal. Chem.*, 62 (1979) 709.
19 H. Schuchman, *Kontakte (Merck)*, No. 2 (1975) 34.
20 R. E. Timms, Australian *J. Dairy Technol.*, 33 (1978) 4.
21 R. L. Glass, *Lipids*, 6 (1971) 919.
22 H. Jaeger, H. U. Klör and H. Ditschuneit, *J. Lipid. Res.*, 17 (1976) 185.
23 H. Heckers, F. W. Melcher and U. Schloeder, *J. Chromatogr.*, 136 (1977) 311.
24 H. T. Slover and E. Lanza, *J Amer. Oil. Chem. Soc.*, 56 (1979) 933.
25 K. Grob, Jr and H.P. Neukom, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 15.
26 K. Grob, Jr. and H. P. Neukom, *J. Chromatogr.*, 198 (1980) 64.
27 K. Grob, Jr. and S. Renhard, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 3 (1980) 627.
28 K. Grob and K. Grob, Jr., *J. Chromatogr.*, 151 (1978) 311.
29 G. Schomburg, R. Dielmann, H. Husmann and F. Weeke, *Chromatographia*, 10 (1977) 383.
30 K. Grob, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 1 (1978) 263.
31 M. Galli and S. Trestianu, *J. Chromatogr.*, 203 (1981) 193.
32 G. Schomburg, H. Husmann, H. Behlau and F. Schulz, *J. Chromatogr.*, 279 (1983) 251.
33 G. Schomburg, H. Husmann, F. Schulz, G. Teller and M. Bender, *J. Chromatogr.*, 279 (1983) 259.
34 C. D. Bannon, G. J. Breen, J. D. Craske, N. T. Hai, N. L. Harper and K. L. O'Rouke, *J. Chromatogr.*, 247 (1982) 71.