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Determination of Total Fat, Milk Fat and Fatty Acid Composition Through 1-min Transesterification Directly in Foods: Collaborative Studies

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Introduction

Method I

In 1997, a method for the conversion of fatty acids of fat into their methyl esters was described that is performed directly in foods, even if the latter primarily consist of water (method I). It was conceived for determining the composition of the fatty acid methyl esters (FAME) as well as the fat content through the sum of all fatty acids. Conditions were adjusted such that up to 500 μ l of water could be tolerated and transesterification was complete in 1 min (1). A system of internal standards verifies the completeness of this conversion for each sample. The main advantages of the method are that no extraction of the fat is needed, transesterification is fast and that the fatty acid composition and the fat content are determined in the same analysis. The fast and mild reaction also protects the fatty acids from chemical modifications (such as oxidation of polyunsaturated fatty acids).

Fat contents were determined in a wide variety of foods and results compared to those obtained by the gravimetric methods most commonly used for the given type of food. No significant disagreement was found (2). For some products, a pretreatment is required for the solubilization of the fat.

Method II

Method I was adapted to include the measurement of butyric acid for the determination of milk fat in fats or foods also containing other fats (method II, [3]). The solvents dioxane and heptane were replaced by methyl-tert-butyl ether (MTBE). Further modifications concerned the reduction of the methanol content in the solution injected into the GC. Concentrations were adjusted such that methyl butyrate forms a sufficiently large peak. However, the steps of the procedure and the volumes of the media determining the kinetics of the transesterification were left unchanged.

Method II enables the direct determination of milk fat, total fat and fatty acid composition in products like ice creams, bakers' ware, cocoa drinks, margarines or chocolate. It could, in principle, replace method I, but practical advantages of method I recommend its further use for analyses not involving butyric acid.

- Extended life time for the uncoated precolumn because heptane is a weaker extraction solvent than MTBE (less co-extracted material).
- The higher boiling point of the solvent enables to use a higher oven temperature during on-column injection, which reduces the total analysis time by a factor of about two.
- The higher boiling solvents reduce the reconcentration of the standard solutions and the calibration mixture by solvent evaporation.

The two methods were validated through two collaborative studies. This paper summarizes the results and discusses the problems observed. It is intended to publish the final versions of the two methods in the Swiss Food Manual (4).

First ring test with method I

In 1997, a first study including 10 laboratories (industrial and government food control laboratories from Switzerland) was performed on the determination of the fat content as well as the fatty acid composition by method I. The following five samples were to be analyzed on fat content:

1. Coffee creamer with a declared content of milk fat of 15.0 g/100 g.
2. Plain chocolate free of milk fat, specially prepared by Halba AG, Wallisellen, Switzerland, with 30.5 g/100 g of cocoa fat (cocoa butter and fat in the cocoa mass).
3. Powder for cocoa drink ("Ovomaltine"), containing malt extract, fat-free milk powder, cocoa powder, eggs and some trace components, with a labelled fat content of 3.3 g/100 g.
4. Mayonnaise with a declared fat content of 82.7 g/100 g.
5. Ground meat ("meat cheese") with a labelled fat content of 28 g/100 g.

A sunflower oil and anhydrous milk fat were provided as reference fats for determining the global response factors. For the sunflower oil as well as samples two and four, also the fatty acid composition was asked.

The coffee creamer, the chocolate and the mayonnaise required no pretreatment, while the powder for the cocoa drink and the ground meat presupposed short

refluxing in dimethyl formamide (DMF). Every sample had to be worked up in duplicate and every transesterified sample analyzed twice, the mean of the latter two determinations being reported.

Results concerning fat content

Of 98 results received for the fat contents of the five samples, eight were considered outliers (Cochran and Dixon test). For all outliers it turned out that the procedure applied deviated from the method. Those results obtained by incorrect application of the method which did not result in outlying were left in the statistical evaluation.

As shown in table 1, most of the determined mean fat contents well agreed with the values expected from the specifications given above. The result for the creamer was 2 % higher than declared on the label (15.3 instead of 15.0 g/100 g), but this difference is not statistically significant. The result for the chocolate was 1.5 % above the recipe of the production. The fat content in the powder of the malt drink, however, was substantially below the declaration, but agreed with the result previously obtained for this product using the same method (2.8 g/100 g) or acid hydrolysis/gravimetry (2.7 g/100 g, see table 6, line 6 in ref. [2]). The result for the mayonnaise was 0.7 % above the declaration. Although statistically not significant, this confirms the tendency observed during routine analysis of dozens of other mayonnaises that fat contents determined by the FAME method are slightly higher (5).

The mean relative standard deviation (RSD) of the results obtained within each laboratory (intra-laboratory RSD or relative repeatability standard deviation) was highest for the malt powder and the meat product, which is probably due to insufficient homogeneity of the samples.

Table 1
Results of ring test 1 on fat contents by method I

	<i>Creamer</i>	<i>Chocolate</i>	<i>Drink</i>	<i>Mayonnaise</i>	<i>Meat</i>
Number of results	18	20	20	20	20
Number of outliers	—	—	4	2	2
Mean fat content (g fat/100 g)	15.3	30.5	2.72	83.4	26.8
Expected value (g fat/100 g)	15	30.0	3.3	82.7	28
<i>Repeatability</i>					
Standard deviation S_r	0.05	0.30	0.07	1.00	0.59
Repeatability ($r = 2.83 \times S_r$)	0.15	0.84	0.19	2.81	1.66
Coeff. of Variation (CV_r , %)	0.4	1.0	2.5	1.2	2.2
<i>Reproducibility</i>					
Standard deviation S_R	0.5	0.6	0.18	2.0	1.5
Repeatability ($R = 2.83 \times S_R$)	1.5	1.7	0.50	5.8	4.2
Coeff. of Variation (CV_R , %)	3.4	2.0	6.3	2.4	5.6

The RSD of the averaged results from the different laboratories (inter-laboratory RSD or relative reproducibility standard deviation) ranged between 2.0 and 3.4 % for three samples and reached 5.6 and 6.3 % for the two products, the homogeneity of which has probably been insufficient.

Problems encountered

On a meeting of the participants of the ring test organized after the evaluation of the results, the following sources of errors became apparent.

1. Some products need thorough homogenization, particularly since the amount of sample analyzed is small. This presupposes an efficient blender.
2. The amount of sample weighed in is limited to 500 mg when water is an important component. Otherwise the amount can be doubled at least, dispersion being the next limiting factor. For GC analysis, the extract must be diluted correspondingly. If homogeneity is still a problem, the whole procedure can be scaled up, multiplying the sample size and the volumes of reagents and extractants.
3. Pretreatment with DMF: heating should occur immediately after addition of DMF, because DMF tends to extract the water and produce hard lumps which are difficult to disperse.
4. Dioxane should be added to the DMF-treated sample before cooling because fat may precipitate and re-dissolve only slowly otherwise (particularly important for meat samples).
5. When adding up the FAMES, rather small peaks must be included in order to prevent errors from taking the minor FAMES into consideration to a variable extent. If components ≥ 0.2 % are quantitated, related problems become negligible.

Fatty acid composition

For three samples, the fatty acid composition had to be reported. Since the selection of the GC separation column was left to the participants, the whole range of polarities of the stationary phase was used. Results for four major fatty acids are summarized in table 2. Absolute and relative standard deviations (SD and RSD)

Table 2
Fatty acid compositions: mean concentrations (g/100 g), standard deviations (SD, g/100 g) and relative standard deviations (RSD, %)

Sample	Sunflower oil			Chocolate			Mayonnaise		
	Mean	SD	RSD	Mean	SD	RSD	Mean	SD	RSD
FAME 16:0	6.30	0.14	2.2	25.35	0.43	1.7	6.43	0.13	2.0
FAME 18:0	3.81	0.10	2.6	35.68	0.43	1.2	4.32	0.12	2.8
FAME 18:1	25.74	0.46	1.8	33.45	0.43	1.3	22.01	0.25	1.1
FAME 18:2	63.07	0.53	0.84	3.48	0.12	3.4	65.90	0.73	1.1

show that there was no difference whether an oil or a more complex food sample was analyzed. The major contribution to the standard deviation originated from a different treatment of minor components: for some results, the sum of the four fatty acids was 100 %, for others merely 97 %.

Second ring test with method II

In 1999, a second ring test was organised which concentrated on the determination of milk fat and total fat by method II. It involved eight Swiss laboratories, three of which had participated on the first study. However, only in one laboratory the same analyst had performed the work in the first study.

Four samples were sent out.

1. *Margarine containing milk fat*, specially prepared by Morgia AG, Lyss, delivered together with the dry milk fat used for this sample. For the determination of the milk fat content in the margarine, butyric acid had to be measured in the milk fat and in the margarine. Hence the final result based on two determinations. Transesterification was performed without pretreatment.
2. *Milk chocolate*, specially prepared by Halba AG, Wallisellen, as well as the milk powder used. Contents of milk fat and total fat in the chocolate were required. Butyric acid had to be measured in the fat of the milk powder, which presupposed the determination of the fat content. Then the contents of butyric acid and total fat were to be measured in the chocolate. Hence, the determination of milk fat required three analyses, that of the fat content a single one. Transesterification of the milk powder involved the formation of a slurry in water, while the chocolate could be worked up directly after dispersion in MTBE.
3. *Powder of a cream sauce* from the market, from which the contents of milk fat and total fat were asked. Both parameters required a single analysis, since an assumption of the butyric acid content in the milk fat was given. This sample required a DMF pretreatment.
4. *Biscuit*, to be analyzed for the same parameters and by the same approach as the cream sauce.

Samples had to be prepared in duplicate and every transesterified sample analyzed twice, the latter results being averaged.

Results

The results are shown in table 3. They were evaluated without eliminating outliers.

1. For the margarine, the mean result for milk fat (MF), 13.4 g/100 g, was close to the 13.5 g/100 g in the mixture. Five of the eight laboratories obtained a result within the range of ± 5 % of this concentration, but one mean value was too high by 18 %.
2. The total fat content determined in chocolate was 1.7 % lower than indicated by production. The largest deviation reported from the mean value was 1.8 %, the

Table 3

Contents of milk fat (MF) and total fat (TF) (g/100 g) determined in the second ring test. Evaluation of eight results each without eliminating outliers

Probe	Margarine	Chocolate	Cream sauce		Biscuit	
	MF	TF	MF	TF	MF	TF
Mean (g fat/100 g)	13.4	28.2	3.5	15.6	12.3	10.8
Median (g fat/100 g)	13.3	28.2	3.6	15.6	12.4	11.0
Values $< \pm 5$ %	5	8	6	8	8	7
Values $> \pm 10$ %	1	0	0	0	0	0
Composition (g/100 g)	13.5	28.7	3.5	unkn	unkn	11.5
RSD (%)	7.8	1.05	6.3	2.5	2.2	4.0

unkn = unknown

RSD 1.05 %. The measured milk fat content (involving three analyses) quite exactly corresponded to that of the production, but two results deviated by almost 10 %.

- For the cream sauce, all results were well within the 5 % limit of the mean value, with standard deviations of 2.2 and 2.5 %.
- The biscuit turned out to be the most difficult sample: RSDs for the total fat and the milk fat were 4.0 and 10.7 %, respectively. One of the results for the milk fat content was 24 % too low.

Problems

On a meeting of participants, the following main problems were recognized:

- The most important errors originated from GC analysis, as concluded from the deviations being similar for the two GC analyses of the same solution as for the two complete determinations.
- The largest deviations were encountered with small peaks of methyl butyrate. The absolute peak area of methyl butyrate in the transesterified samples must not be substantially smaller than in the calibration mixture.
- Often integration was not checked with sufficient care. A small change in the assumed baseline easily causes a deviation by several percents, particularly if peaks are small.
- Methyl butyrate and methyl valerate (the internal standard) are highly volatile and easily lost if transesterified samples are not handled and stored appropriately. Trivalerate, recently made commercially available by Fluka, should replace methyl valerate as internal standard added to the sample.
- Mixing of biscuits easily separates fat from the sample. It must be restricted to a minimum and warming of the sample avoided (mixing of cooled samples?).

Conclusions

Among the participants of the collaborative study, the methods found much positive echo. They massively reduce sample preparation work and provide reliable results for almost the whole range of foods. They are particularly advantageous when both the fat content and the fatty acid composition must be determined, such as for nutritional declaration or when milk fat and total fat are required (e.g. chocolate).

It was also mentioned, on the other hand, that the large increase in productivity is only achieved if a minimum number of samples is analyzed since capillary GC and the training of a specialist are prerequisites. This does not apply, however, to milk fat determinations through butyric acid since this requires GC analysis anyway.

Quantitative performance

The results of the two ring tests were taxed satisfactory. There is room for improvement since nearly all participants used the ring test for introducing the method into their laboratory and, hence, lacked prior experience.

Table 4 compares the results of the milk fat determinations in the ring test with the last two FAPAS performance tests (Food analysis performance assessment scheme, Central Science Laboratory, CSL, Sand Hutton, UK). The FAPAS test required the determination of butyric acid in pure milk fat (single analysis), whereas the ring test was on foods and required up to three analyses for the final result. Nevertheless, 66 % of the results from the ring test were less than 5 % off the mean value, compared to 40 and 51 % of the FAPAS test. Merely 7 % of the ring test results deviated by more than 10 %, while it was 33 and 24 % for the FAPAS test.

The largest error component in the determination of butyric acid is the GC analysis. Hence, the lower precision observed for the FAPAS test primarily shows poorer GC analysis or that the ring test participants were more careful about this aspect. This also suggests that sample preparation through transesterification directly in the food does not introduce a significant error.

Table 4

Comparison of the results on butyric acid and milk fat determinations of the ring test with the last two FAPAS performance tests. Results grouped as less than 5 % off the mean value ($< \pm 5$ %), 5 to 10 % off (± 5 –10 %) and more than 10 % off ($> \pm 10$ %)

	FAPAS 1411 11.1998	FAPAS 1413 7.1999	Ring test 1999
Sample	Butter fat	Butter fat	Foods
Results	55	51	32
$< \pm 5$ %	40 %	51 %	66 %
± 5 –10 %	27 %	25 %	28 %
$> \pm 10$ %	33 %	24 %	7 %

Definition of "fat"

Fat determination through the sum of the transesterified fatty acids not necessarily provides the same results as gravimetry, which yields a subject for extended discussions and speculations. By the gravimetric method, compounds other than acylglycerols and fatty acids esters are co-extracted. Direct transesterification provides a clearly defined result: the sum of transesterified fatty acids calculated as triacylglycerols.

The ring tests confirmed previous results (2) that for the broad range of foods tested, the differences between fat contents obtained by direct transesterification and gravimetry are too small to be significant. The largest differences are observed for foods containing little fat (the malt drink powder not belonging to this group because acid hydrolysis/gravimetry produced the same low result as the FAME method).

Recent work by *Boppart* (6) investigated the behavior of various components on whether and to what extent they were included into "fat" determined by transesterification or acid hydrolysis/gravimetry. Fatty acids from partial glycerides, glycolipids or phospholipids are included by both methods. With transesterification, the carbon atom of the glycerol the fatty acid is attached to is always included. With gravimetry, the glycerol is included for the triglycerides, while it is removed by acid hydrolysis of mono- or diglycerides as well as of glycolipids or phospholipids. Fatty acids bonded in sterol or wax esters are only partly determined by the FAME method because their transesterification is substantially slower. Gravimetry, on the other hand, fully includes them together with their alcohol moiety. However, all these differences are too small to be of practical significance.

As the FAME method involves alkaline transesterification, free fatty acids are not recorded. This may be a significant shortcoming for aged cheese (e.g. parmiggiano) or meat products (e.g. salami), maybe also for beef cubes. For these applications, an acidic (trans-)esterification with hydrochloric acid, as proposed by *Bohnert et al.* (7), might be preferable.

Fat determinations through transesterification directly in the food and summing the FAMEs resembles the method required by the US-FDA (8), but is far shorter. The US method requires the isolation of the fat by classical means, thus hydrolysis, and extraction. Then the fat is saponified and methylated before GC analysis to sum up the FAMEs. It includes the free fatty acids as well as the acids from waxes and sterol esters, but for the large majority of foods this does not justify the lengthy procedure.

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Summary

Results of two collaborative studies, which have been performed on the determination of total fat and milk fat as well as fatty acid composition by direct transesterification in the food, are summarized. Method I was designed for the determination of fat contents and fat compositions. Method II is a variation of method I directed to the measurement of methyl butyrate and milk fat. The results demonstrate that the shortened sample preparation (elimination of hydrolysis and isolation of the fat phase) does not influence negatively the quantitative results.

Zusammenfassung

Die Resultate zweier Ringversuche werden zusammengefasst, welche zur Bestimmung von Gesamtfett, Milchfett und Fettsäurezusammensetzung mittels direkter Umesterung im Lebensmittel durchgeführt wurden. Methode I wurde zur allgemeinen Bestimmung von Fettgehalt und Zusammensetzung entwickelt. Methode II ist eine Variante davon für die Messung des Gehaltes an Buttersäuremethylester und damit von Milchfett. Die Resultate weisen nach, dass sich die verkürzte Probenaufarbeitung (Eliminierung der Hydrolyse und Fettextraktion) nicht negativ auf die quantitativen Resultate auswirkt.

Résumé

Ce travail résume les résultats de deux études interlaboratoires portant sur la détermination de la teneur globale en graisse, de la graisse lactique et de la composition des acides gras par trans-estérification directe dans l'aliment. La méthode I permet la détermination de la teneur globale en graisse ainsi que la composition des acides gras. La méthode II est une adaptation de la méthode I pour déterminer le méthylate de l'acide butyrique et donc la graisse lactique. Les résultats montrent que le prétraitement réduit de l'échantillon (suppression de l'hydrolyse et de l'extraction de la graisse) n'a pas d'influence négative sur les résultats quantitatifs.

Key words

Fatty acid methyl esters, Fat content in foods, Fatty acid composition, Collaborative study

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