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Determination of fat content and fatty acid composition through 1-min transesterification in the food sample; principles

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Abstract Base-catalysed transesterification can occur in water-containing foods, such as ice-cream or milk, because it happens much faster than saponification. Under the conditions proposed, it takes 1 min at ambient temperature. The reaction must be stopped before relevant saponification occurs, reducing the pH. The method enables the determination of the fat content through the sum of all fatty acids as well as the fatty acid composition without previous extraction from the foodstuff. Some foods, such as meat or cheese, are heated for a short while in dimethylformamide (DMF) in order to solubilize the fat in the reaction medium. A system of four internal standards verifies the completeness of the transesterification for every analysis and checks for relevant saponification or discrimination by GC analysis.

Key words Transesterification in water-containing foods · Fat determination · Fatty acid composition · Verification through internal standards

Introduction

Fat analysis usually starts with the time-consuming hydrolysis of the foodstuff and extraction of the lipids (classically with a chloroform/methanol mixture). The fat content is determined gravimetrically, the fatty acid composition after formation of the methyl esters, commonly through drying of the extract and base-catalysed transmethylation or, more recently, using trimethylsulphonium hydroxide [1]. Since the gravimetric method is unspecific (all the components of low polarity extracted are quantitated as fat), more recent methods add GC analysis of the fatty acids, determining the fat content through the sum of all acids (e.g. [2]); however, this further lengthens the procedure.

As the determination of fat content, fat composition and fat quality are some of the most important methods in food analysis, various attempts have been made to shorten the analytical procedure, first of all to eliminate the time- and solvent-consuming extraction step. It was proposed to saponify the whole foodstuff and methylate the free acids with methanolic boron trifluoride [3, 4]–a procedure also called in situ transesterification [5]. Heating of fat-containing material in benzene with methanolic hydrochloric [6] or sulphuric [7] acid also enabled the determination of the composition of fatty acid methyl esters (FAME) without prior extraction, but no quantitation of the fat content. Recently, it was proposed that fat extraction could be avoided by lyophilizing the foodstuff (overnight) and performing base-catalysed transmethylation directly on this material [8, 9]. Furthermore, a method and instrumentation were introduced for saponification in the food sample and GC determination of the free acids thus generated (Büchi, Flawil, Switzerland, System B-815/820/821 consisting of an extraction unit and a dedicated gas chromatograph). It enables the determination of the fat content, but no detailed analysis of the fatty acid composition.

Base-catalysed transesterification

Usually methyl esters are prepared, because their analysis by GC is more accurate and separation more selective than that of the free fatty acids. During the last decade, base-catalysed transesterification has become the method most widely used. It turns all fatty acids into methyl esters, which are in the form of mono-, di-, and triglycerides, wax and sterol esters, or emulgators; however, it does not esterify free fatty acids. The background of transesterification has been discussed in several reviews [10–12].

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There are a large number of official methods for the transmethylation of fatty acids. Recommended temperatures range from ambient to reflux, the duration from minutes to several hours. Transesterification in 2 min at ambient temperature was described in 1969/70 [13, 14]. Nevertheless, the EU method [15] requires 2 h of reflux. These differences are partly the result of varying amounts of methoxide added, but probably also of a lack of optimization and the assumption that extra time would render the method more robust.

The methods for base-catalysed transesterification presently used are determined by the belief that the complete absence of water is a prerequisite. In fact, stoichiometrically some 6% of water in the sample is sufficient for complete saponification – butter and margarine contain almost three times as much. Thus, methods start with extraction of fat from water-containing foods and thorough drying. In this paper it will be shown that this is unnecessary: fat can be transesterified in foods even if they consist largely of water (such as milk or homogenized meat). This enables far more rapid determinations of fat contents and fatty acid compositions by transesterification directly in the foodstuffs.

Concept of the method

Base-catalysed transmethylation in water-containing media

As shown in Fig. 1, methoxide transesterifies triglycerides and other fatty acid esters into methyl esters. However, in the presence of water, methoxide also forms hydroxide, which may saponify the triglycerides or the newly generated methyl esters. While transesterification is a reversible reaction, saponification is irreversible and, hence, the end point of the reaction – if time is given to reach it.

More than 70 years ago, base-catalysed transesterification was shown to be some 1500 times faster than saponification [16–18]. In 1971, this was confirmed by Glass [19] and explained by the equilibrium between methoxide and hydroxide being on the side of the methoxide. It has also been shown that the rate of attack by methoxide is higher than that of hydroxide [20]. This enables the conversion of triglycerides and other esters of fatty acids into methyl esters even in presence of water, provided that the reaction is stopped before the methyl esters are saponified.

Elements of the new method

The method described below is characterized by the elements listed in Table 1. Conditions were optimized

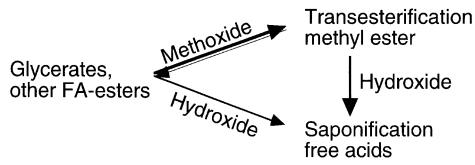

Fig. 1 Transesterification and saponification

Table 1 Key elements of direct transesterification

– Reaction in the homogenized food
– Fast transesterification: 1 min at ambient temperature
– Conditions providing robust optimum
– Stopping the reaction before relevant saponification occurs
– Internal standards verifying transesterification for each sample

to provide a fast reaction at ambient temperature. The goal was 1 min since this enables continuous work; usually four samples were worked up in parallel. Furthermore, conditions were selected to result in a sufficiently broad time window for the reaction so that good results are obtained even if the reaction time is not observed accurately and the temperature varies, as usually occurs if the laboratory is not temperature regulated. After complete transesterification, the mixture is acidified in order to stop saponification. Use of a triglyceride (triundecanin, tri-11) as an internal standard renders the method more robust: some deviation from a complete transesterification can be tolerated, because the internal standard is affected to a similar extent.

Mediator solvent

A mediator solvent is used to create a one-phase system of the fat with methanol and methoxide: solid fats must be dissolved in this solvent prior to transesterification. When a large proportion of the sample consists of water (e.g. milk, ice-cream), the mediator solvent also has to integrate the water into the one-phase system; triglycerides with long-chain saturated fatty acids in particular tend to precipitate and to react much slowlier then. Finally, it is advantageous if the mediator solvent still provides a one-phase system when the heptane or pentane is added for the extraction of the FAME, because this results in a very thorough extraction virtually without mixing when the subsequent addition of the aqueous solution splits the liquid into two phases.

Glass [19] proposed the use of benzene as a mediator solvent; other authors, dichloromethane, toluene, or tetrahydrofurane. These solvents fail, however, in presence of water. Dioxane was chosen, as it fulfils all the above requirements at modest concentrations, except the last point, when dimethylformamide (DMF) is used to solubilize enclosed fat. Dioxane has one flaw:

from polar GC columns it is eluted after methyl butyrate, i.e. it appears as a rather large peak (though well separated) if the milk fat content is determined through the concentration of methyl butyrate. If samples contain little water, such as chocolate, methyl-*tert* butyl ether (MTBE) may be used instead of dioxane.

Enclosed fat

Fat transesterification is a drawback if time is needed for solubilizing enclosed fat in the reagent mixture. In presence of water, the reaction time cannot be prolonged or temperature increased, i.e. solubilization must be achieved in a previous step. For certain solid samples, such as milk powder, preparation of a slurry with water before addition of dioxane and the reagent was successful. Other samples, such as cheese or meat, were refluxed in DMF for a few minutes. Owing to the high boiling point of DMF (150 °C), water evaporates inside the compartments and disrupts the structure enclosing the fat.

GC analysis

The separation of the fatty acids is performed according to the emphasis of the analysis. As our work was primarily aimed at the determination of the fat content, a short (3 m × 0.25 mm i.d.) apolar column was used. A more polar stationary phase and a somewhat longer column are needed for the separation of the saturates, monounsaturates and polyunsaturates, as required for the declaration of nutritional values. Finally, the analysis of minor components, such as the *trans* unsaturated acids, calls for a long, polar column.

Accurate determination of fat contents places a high level of demand on quantitative GC. Adsorptive effects in the column can be reduced by injection of amounts near to the capacity limit of the latter, which also facilitates accurate integration of the peak areas. Split injection may produce very reproducible results, as shown by Bannon et al. [21, 22]. However, on-column injection is more reliable since it avoids discrimination against higher-boiling-point FAMEs by selective elution from the needle as well as problems related to sample evaporation and splitting inside the injector [23].

On-column injection requires the column temperature during injection to be below the pressure-corrected boiling point of the sample [24]. Since small amounts of methanol and dioxane are co-extracted, forming an azeotropically boiling mixture, the boiling point of the extraction solvent is reduced. Usually heptane was used in order to enable injection at up to 90 °C (10 kPa inlet pressure). It is not suitable, however, if methyl butyrate is analysed with on-column injection: partial solvent trapping results in a peak with a chair-type deformation [25]. Since the analysis must start at a lower temperature, pentane is preferable: owing to faster solvent evaporation, partial solvent trapping no longer causes significant peak distortion. With columns of some 20 m in length, inlet pressures are sufficiently high to enable injection at 40–45 °C.

Verification of transesterification for each sample

Method validation is intended to ensure accuracy of analytical results through a method development that takes all possible variations of samples and conditions into consideration. The method described here adds an element to this, enabling transesterification and GC discrimination to be checked for each sample. It enables samples behaving abnormally or inappropriate practices to be recognized, i.e. ensures accuracy for each individual result.

Transesterification is checked by measuring the area ratios of three internal standards: methyl undecanoate (FAME-11), resulting from transesterification of tri-11; a FAME added to the sample (FAME-9); and a component not participating in the reactions (a hydrocarbon, HC). Incomplete transesterification yields an insufficient amount of FAME-11 compared to the HC. Too small a FAME-11 peak may, however, also be the result of saponification. Occurrence of the latter is recognized by a reduction of FAME-9, the methyl ester added as such. Hence reduced FAME-11 combined with complete FAME-9 indicates insufficient transesterification, while a reduction of both FAME-11 and FAME-9 shows advanced saponification. The HC was adjusted to the polarity of the stationary phase to be eluted between FAME-9 and FAME-11 and well separated from the FAMEs of the sample. Table 2 lists the hydrocarbons used during this work.

When classic or programmed temperature vaporizing (PTV) split injection is used, a fourth internal standard should be added, namely a HC eluted after most of the FAMEs, checking for discrimination mainly against the FAMEs-18. Since some discrimination may be unavoidable, it is essential to keep it reproducible: the area ratio of the two HC standards must be similar to that observed for calibration (see below).

Table 2 Hydrocarbons (HC) recommended as inert internal standards

Stationary phase	Hydrocarbon
100% methylsilicone (e.g. PS-255, DB-1, Rtx-1)	1-tetradecene (14 ene)
Carbowax 20'000	n-pentadecane
100% cyanopropyl polysiloxane (e.g. SP-2650, CP-88)	n-octadecane

Materials and methods

Materials

Gas chromatograph Mod. 8000 with on-column injector and FID, autosampler AS800, integration system ChromCard, all from CE Instruments (Milan, Italy), Blender (Büchi B-400), magnetic stirrer/heating plate, 50-ml Erlenmeyer flasks with glass stoppers, 20-ml screw cap flasks.

Triundecanin (tri-11), 1-tetradecene (C14:1), methyl nonanoate (FAME-9), heptane purum, DMF puriss, disodium hydrogen citrate purum, all from Fluka; 30% methoxide in methanol, methanol p.A., 1,4-dioxane p.A., all from Merck.

Solutions. Disodium hydrogen citrate in water, 15 g/100 ml; methoxide in methanol, 5 g/100 ml; internal standards (tri-11, FAME-9, C14:1) in dioxane, 100 mg/100 ml. The latter two solutions should be stored at ambient temperature, since the transesterification is thought to occur at this temperature.

Pretreatment

Non-homogeneous and solid samples are homogenized using a blender. The sample size is determined by its homogeneity and fat content (in the interests of obtaining fairly constant peak areas in GC). Of the less homogeneous samples, 50–500 mg (containing up to about 50 mg of fat) is accurately weighed into the 50-ml Erlenmeyer flask. For homogeneous samples, such as edible oils, some 15–50 mg is prepared in a 20-ml screw cap flask. For edible oils and molten fats, this means accurately weighing one drop from a Pasteur pipette.

The pretreatments recommended for various types of samples prior to transesterification are listed in Table 3. Samples with easily accessible fat (chocolate, milk, etc.) are directly transesterified. Milk powders and powders for infant foods are soaked with water: about 100 mg is stirred with 0.5 ml of water and allowed to stand for some 5 min. Other samples, such as cheese, meat, cereals, or nuts, are heated in DMF: 2.5 ml of DMF is added and the slurry refluxed, whilst being stirred, for 5–15 min. Before transesterification, samples are cooled to ambient temperature, e.g. in a water bath. This classification must be considered with some precaution. For instance, after a short period of heating in DMF, slightly higher fat contents were determined for certain ice-creams. Ground almonds or hazelnuts in chocolate contain fat which can only be reached through heat treatment with DMF. Dry meat or salami must be heated in DMF for as long as 45 min, presumably because the lack of water does not result in the opening up of the structure by evaporation.

Transesterification

To the larger sized samples, 5 ml of mediator solvent (usually dioxane) is added (Table 4), containing 5 mg of each of the internal standards tri-11, FAME-9, as well as one or two HCs adjusted to the GC stationary phase. After mixing and possibly dissolving any solid fat (e.g. chocolate), 5 ml of 5% methoxide/methanol is added (vortex, 3 s). Then, 60–90 s later, 25 ml of heptane is admixed. Immediately afterwards, the reaction (saponification) is stopped by addition of 10 ml of 15% disodium hydrogen citrate in water, reducing the pH to 7–8. Since samples without DMF remain in one phase with the extraction solvent until the addition of the aqueous solution, extraction is complete with little shaking. Samples containing DMF, however, form two phases when the extraction solvent is added, i.e. they must be thoroughly mixed on the vortex in order to extract the FAMEs. When the phases are separated, the supernatant is analysed by GC. Depending on the GC analysis of interest, as well as on whether split or on-column injection is applied, the sample needs further dilution. Using 0.5 µl on-column injection, 100 µl of FAME extract is pipetted into the autosampler vial and the vial filled up with about 1 ml of heptane.

Table 3 Pretreatment of foods for solubilization of the fat

None	Slurry with water	Refluxing with DMF
Milk	Milk powder	Meat/meat products
Yoghurt	Infant foods	Cheese
Curd		Soups/ready meals
Chocolate		Nuts
Ice-cream		Cocoa powder
		Cereals

Table 4 Transesterification for large sample sizes

1. If necessary: homogenize by blender
2. Accurately weight 50–500 mg sample into 50-ml flask; maximum 50 mg of fat
3. Pretreatment if necessary (Table 3): soak sample in 500 µl of water or reflux in 2.5 ml of DMF for 15 min and cool to ambient temperature
4. Add 5 ml of dioxane with internal standards (5 mg) and dissolve solid fat, e.g. of chocolate
5. Add 5 ml of methanol containing 5% methoxide, mix and allow to stand for 60–90 s
6. Add 25 ml of heptane, mix shortly, and add 10 ml of aqueous disodium hydrogen citrate (15%). Thoroughly mix if heptane did not form a one-phase system with the sample
7. GC analysis of the heptane solution: 0.5 µl on-column injection at 80–90 °C, possibly after dilution

Figure 2 shows a GC-FID chromatogram of butter fat obtained using a 3 m × 0.25 mm i.d. capillary column coated with an apolar stationary phase (PS-255) of 0.3 µm film thickness. 0.5 µl of the sample were injected by the on-column technique into a 20 cm × 0.53 mm i.d. silylated precolumn. The inlet pressure was 10 kPa (hydrogen). During injection, the oven temperature was 80 °C (1 min), then programmed at 20°/min to 220 °C. GC analysis took about 8 min.

For homogeneous samples, sample preparation can be scaled down by a factor of five. The most common analysis of this type concerns edible oils or molten fats, including samples such as plain chocolate. It is most convenient to weigh one droplet of oil, which amounts to around 15 mg (Table 5). If methyl butyrate is to be determined, FAMEs should be extracted with pentane instead of heptane and injection occurs at 40–45 °C.

Calibration

The FID responses of the various FAMEs differ somewhat from the response of the internal standard FAME-11 and correction factors may be needed. For the calculation of fat contents, global response factors are determined: the summed area of the FAMEs of the oil or fat is compared with that of the internal standard FAME-11. Since factors are not far from unity, it is sufficient to determine them for the most important types of fat, such as a vegetable oil, water-free milk fat, fish oil, and cocos fat. For mixtures of, for example, some 10% milk fat in cocoa butter (milk chocolate) or margarine, mixed correction factors are applied: if the factor for milk fat without FAME-4 and FAME-6 is 1.06 and that for vegetable oil 0.94, the correction factor will be 0.95.

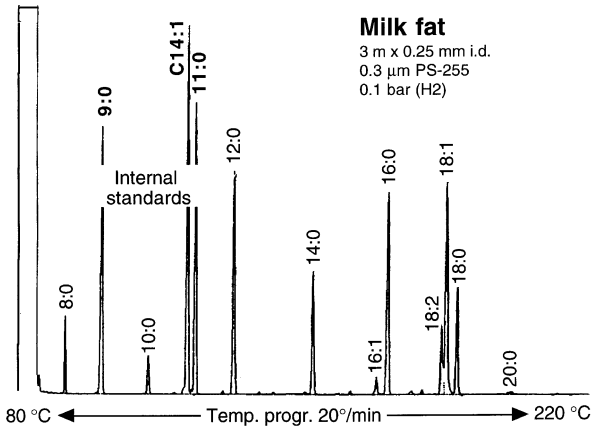

Fig. 2 GC-FID chromatogram of milk fat obtained from an apolar column of 3 m length. (*n*:*x* Fatty acid methyl ester with number of carbon atoms and double bonds in the acid). 9:0 and 11:0 are internal standards. C14:1, 1-tetradecene (inert internal standard)

Table 5 Transesterification for edible oils and fats, as well as homogeneous samples containing some 20% fat at least (e.g. chocolate)

1. If necessary: homogenize by blender
2. Accurately weigh 10–50 mg of sample into 20-ml screw cap vial; maximum 20 mg of fat. For edible oils and molten fats: one droplet from a Pasteur pipette
3. Add 1 ml of dioxane with internal standards (1.0 mg), mix and dissolve solid fats
4. Add 1 ml of methoxide solution and allow to stand for 60–90 s
5. Add 10 ml of heptane, mix shortly, add 2 ml of citrate solution, and mix thoroughly if heptane did not form a one-phase system
6. GC analysis of the heptane solution: 0.5 µl on-column injection at 80–90 °C

FAME-4 and FAME-6 of milk and cocos fat can either be analysed or compensated for by calibration. As the determination of FAME-4 greatly prolongs chromatography, the second option is usually preferable. Calibration and the analysis start at, for example, FAME-8; then, FAME-4 and FAME-6 are considered using a slightly larger correction factor. The two FAMEs not actually determined introduce an error limited to the variation of their small contribution to the total fat.

Area ratios for the internal standards indicating complete transesterification and no saponification were determined for a 1:1 mixture of FAME-9 and the HC, as well as for a 1:1 mixture of tri-11 and the HC after transesterification. FAME-9/HC was around 0.72; FAME-11/HC, 0.74. The purity of the standards is of limited importance since calibration finally relies on the oils and fats used for determining the response and correction factors. If split injection is applied and two internal standards are used for detecting discrimination, the area ratio of the hydrocarbons should be close to unity. A deviation from unity can be tolerated provided it is reproducible and calibration of the response factors occurred with the same discrimination.

Optimization of the method

Hydroxide or methoxide?

When transesterification is "overdone", saponification becomes a problem if water or hydroxide are present. For this reason, Bannon et al. [10] recommended the use of dry samples and methoxide as a catalyst rather than hydroxide. In the presence of water, hydroxide and methoxide are in an equilibrium, quite independent of whether either has been added. In fact, treatment of milk with sodium methoxide or potassium hydroxide resulted in identical rates of ester formation and saponification. On the other hand, for samples of water-free oil or fat, reaction with hydroxide resulted in the occurrence of noticeable saponification after 1 min. This is explained by a faster reaction if no water is present. Hence, methoxide was chosen as the catalyst so that the same reagent could be used for all types of samples.

The amount of methanol to be used

The amount of methanol added to the reaction mixture must result in a sufficiently large stoichiometric excess to keep the amount of monoglycerides in the interesterification equilibrium negligible. A total of 5 ml methanol for 50 mg of fat (proposed method) corresponds to an excess of roughly 1000, i.e. only about 0.1% of the fatty acids will be present as monoglycerides (for the internal standard as well as the fat of the sample). However, more importantly for water-containing samples is that an excess of methanol favours transesterification over saponification, i.e. this widens the window for acceptable reaction times.

Figure 3 shows transesterification and saponification rates observed for various volumes of methanol added to 500 mg of milk. The amount of methoxide was constant (125 mg, corresponding, for example, to 2.5% for 5 ml of methanol, i.e. only half of that which is suggested in the final method). The indicator for transesterification, the FAME-11/HC ratio, dropped rather rapidly with 1.25 ml of methanol, but remained near the maximum from 1 min to over 5 min when 5 ml was added. Hence, 5 ml of methanol is recommended in order to obtain a broad optimum for the duration of the reaction.

The area ratio for FAME-9/HC, informative about saponification, shows corresponding results: saponification is fast with 1.25 ml methanol, but slow with 5 ml. Since the amount of methoxide added was constant while the volume of the mixture varied from 6.75 to 10.5 ml, the reaction rates were somewhat higher for the low methanol volumes, thus accentuating the difference.

Concentration of methoxide

The concentration of methoxide is the most important factor influencing the reaction rate and was optimized to result in a 1 min transesterification time. The data shown in Fig. 4 were obtained with 100 mg sunflower

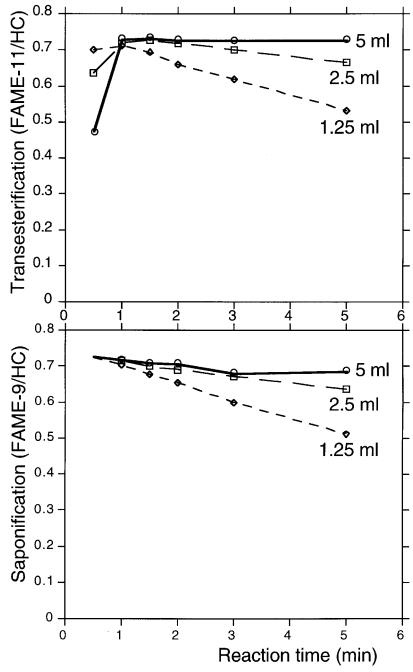

Fig. 3 Transesterification (area ratio of FAME-11/HC, *top*) and saponification (FAME-9/HC, *bottom*) in 500 µl of milk for varied volumes of methanol added

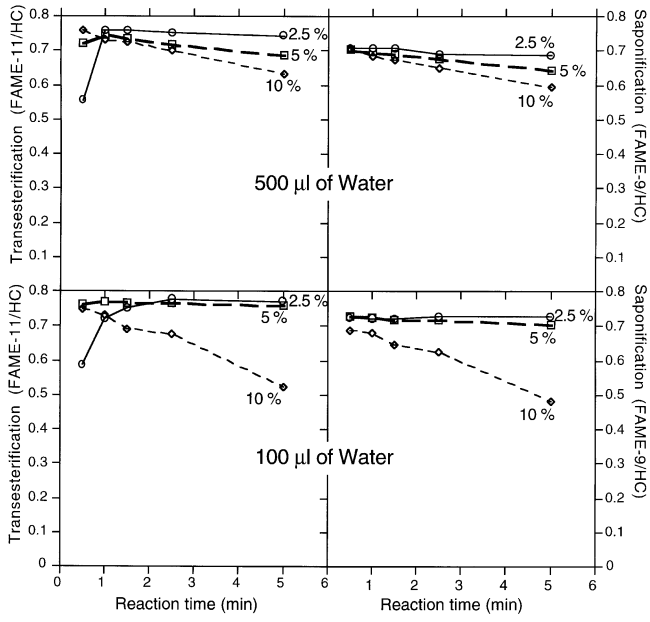

Fig. 4 Influence of the methoxide concentration in methanol (5 ml) on transesterification and saponification; 100 mg sunflower oil in 100 or 500 µl of water

oil, 5 ml methanol, and 500 µl (upper plots) or 100 µl (lower plots) water. The oil and the water were brought into the same phase by the dioxane.

With 500 µl water, the area ratio of FAME-11/HC shows transesterification with 2.5% methoxide to be

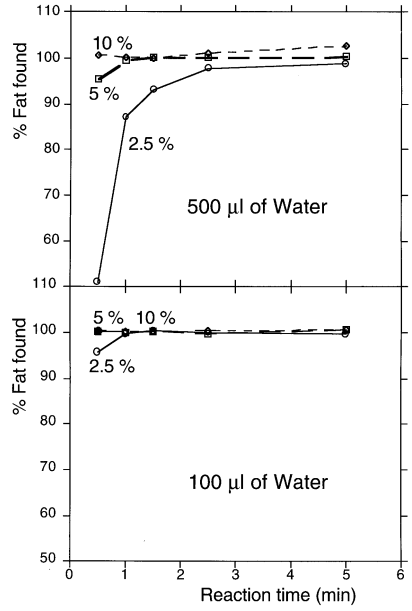

Fig. 5 Concentration of fat found in sunflower oil when varying the methoxide concentration and the reaction time; 100 and 500 µl of water added

only just about complete after 1 min. Since transesterification of long-chain fatty acids is slightly slower and the method should also be robust concerning temperature (i.e. transesterification be complete even if the temperature is below 20 °C), 5% methoxide was preferred. With this concentration, methylation was acceptable within a reaction time window of 30 s to more than 5 min. Higher concentrations resulted in faster saponification without significant advantages. As also shown by the area ratio FAME-9/HC, saponification with 10% methoxide reached some 15% after 5 min of reaction time.

Results with 100 µl water confirm that 5% methoxide suits the purpose. It is interesting to note that for 10% methoxide the rate of saponification is almost double that of the sample containing 500 µl water, whereas the reactions are clearly slower with 2.5% methoxide (see transesterification).

Figure 5 confirms the choices made for a simulated fat determination, i.e. the concentration of fat in sunflower oil (100%). With 500 µl water and 2.5% methoxide, only 97% fat was obtained after 90 s, owing to the somewhat slower reaction of the C18 triglycerides compared to tri-11. With 5% methoxide, the result was correct within a range of from 1 min to more than 5 min. After 5 min, 10% methoxide gave almost 103% fat, which reflects the slightly faster saponification of the FAME-11 compared to the FAME-18.

Extraction efficiency

After transesterification, the FAMEs are extracted into heptane or pentane. The yield was checked by

re-extraction with heptane for seven totally different types of samples. The second extract usually contained around 0.2–0.4% of the FAME of the first extract. Only for samples containing large amounts of emulsifiers, such as sauces, did the second extract contain up to 0.7% of the fat of the first extract. Since these losses similarly affect the internal standard, a single extraction was considered sufficient.

Results

Table 6 shows the reproducibility of transesterification and GC analysis for a sample (sunflower oil) causing no problems concerning solubilization of the fat into the reagent mixture. The relative standard deviation was below 0.5%. For ten samples of lard and butter fat, relative standard deviations were 0.9 and 1.2%, respectively. Since data obtained from 40 injections of the same transesterified sunflower oil had a relative standard deviation of 1.0%, GC was probably the most important source of the deviations. Injection, adsorption effects (as indicated by the variation of the response factors) and inaccurate integration contributed similarly and leave little room for the hope that precision could be substantially improved beyond a 1% relative standard deviation. However, at that level, inaccuracy of weighing the sample and variations owing to inhomogeneity of the sample also start to contribute.

Table 6 Reproducibility of results obtained by independent analyses

Sample	FAME-11 /HC	FAME-9 /HC	Fat %
1	0.76	0.71	100.08
2	0.76	0.71	99.48
3	0.76	0.71	100.9
4	0.76	0.71	99.7
5	0.76	0.71	100.07
6	0.76	0.71	100.44
7	0.76	0.71	100.61
8	0.76	0.71	99.99
9	0.76	0.71	99.76
Mean	0.76	0.71	100.11
R.S.D.			0.46%

Conclusion and outlook

Base-catalysed transesterification is possible in water-containing media, provided that the reaction is stopped before substantial saponification occurs. Conditions can be adjusted to result in a reaction taking 1 min at ambient temperature. Success can be verified by a set of internal standards for each sample. This enables fast determination of fat contents and fatty acid compositions through transesterification directly in the foodstuff, i.e. without hydrolysis and extraction of the lipids. The amounts of solvent involved are small and the stress on labile compounds, e.g. by acidic hydrolysis, is minimized.

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