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Simultaneous Determination of Milk Fat (Butyric Acid) and Total Fat by 1-min Transesterification Directly in the Food

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Introduction

Milk fat contents in fatty foods and mixtures with other fats are commonly determined through butyric acid. *Molkentin* and *Precht* (1) recently reviewed methods for its analysis and found surprisingly large differences in the results (mean values between 3.06 and 3.71 g/kg for the same samples). Transesterification with sodium methylate turned out clearly superior to that with trimethyl sulphonium hydroxide or the analysis of free butyric acid. *Ulberth* (2) suggested to perform the analysis of methyl butyrate (E4) by headspace analysis with the advantage of shortening the analysis time.

This paper suggests to perform the analysis by a variation of the fast transesterification directly in the food previously described for the determination of the general fatty acid composition as well as of the fat content in terms of the sum of all the fatty acids (3, 4). This method has the following advantages:

- Extraction of the fat is circumvented.
- Transesterification takes 1 min at ambient temperature.
- «Fat» is clearly defined as the sum of all fatty acids.

The approach takes advantage of the fact that transesterification is faster than saponification and that fatty acid methyl esters (FAMEs) can be formed directly in the homogenized food sample, even if the latter mainly consists of water, such ice cream.

Below modifications are described adapting the method to include the determination of methyl butyrate (E4). Now it enables the simultaneous determination of the milk fat and total fat in a food: without previous isolation of the fat, samples like ice cream, infant formulas, sauces or chocolate are transesterified; E4 and the sum of all fatty acids are quantitated through internal standards.

The modified version could completely replace the previous method. However, the use of the more volatile Methyl tert. Butyl Ether (MTBE) requires that on-column injection is performed at an oven temperature not exceeding about 55 °C, which slows the analysis if there are no short chain fatty acids to be analyzed.

Transesterification in the food

The first paper (3) described the conditions suitable for transesterification at ambient temperature in 1 min. Dioxane is added to the homogenized sample as a mediator solvent to unify fat, methanol and possibly water into one phase. Then the transesterification is started by addition of methoxide in methanol. The speed of the transesterification was adjusted through the concentration of the methoxide. The ratio of the maximum amount of water in the sample (500 µl) and methanol added was chosen such that saponification remained negligible for several minutes. 60–90 s after adding the methoxide solution, aqueous hydrogencitrate is admixed to reduce the pH to 7–8 and stop the reaction. The FAMEs are extracted and analyzed by GC-FID.

The mediator solvent contains three or four internal standards. One is used for the calculation of the fat content, while two others enable to check transesterification, i.e. completeness of the reaction, and absence of relevant saponification. The last standard, a hydrocarbon eluted after the FAMEs, may be used to monitor discrimination during GC analysis, e.g. as a result of using split injection.

The second paper (4) described the application of the method to the determination of fat in a broad range of foods. Particular attention was paid to pre-treatments required for the solubilization of the fat in the homogenized food. Since transesterification occurs in a short time, the fat must be readily available to the reagents. Some foods (e.g. powdered milk) require formation of a slurry in water. For others, such as cheese, meat, and cereals, short refluxing in dimethylformamide (DMF) is effective. Fat contents thus obtained were compared with those from the standard methods of gravimetric fat determinations after acidic hydrolysis.

Butyric acid in milk fat

A problem inherent to all determinations through a marker compound, such as of milk fat through butyric acid, concerns the variability of the marker concentration in the material to be quantitated. According to IUPAC (5), a mean concentration of 3.2 g butyric acid is found in 100 g milk fat, corresponding to 3.72 g/100 g calculated as methyl ester. The Swiss Food Manual (6) gives a mean value of 3.74 with a range from 3.46 to 4.13 g/100 g (3.5 to 4.0 g/100 g in 95 % of all samples). For

butyric acid, *Souci-Fachmann-Kraut* (7) also lists 3.2 g/100 g milk fat. While there is good agreement among this data, there are also deviating results. In 1990, *Precht* (8) determined a mean concentration of 4.07 g/100 g in 83 samples of German milk fats, ranging from 3.54 to 4.50 g/100 g (methyl ester of butyric acid related to fat content). Recently, *Molkentin* and *Precht* (9) found in 136 samples of milk fats from all over Europe a mean concentration of 3.96 g/100 g, with results ranging between 3.56 and 4.35 g/100 g. An overview of related data, showing even broader deviations, was recently published by *Lipp* and *Anklam* (10). They also pointed out natural differences resulting from feeding, seasonal variations and the lactation period.

The methyl butyrate concentrations to be adopted for calculating milk fat depends on the aim of the analysis. For checking a minimum content of milk fat, the minimum E4 concentration may be most appropriate, i.e. a value of around 3.5 g/100 g. For the maximum content, an assumption of about 4.1 g/100 g might be more suitable.

Experimental

Materials

Gaschromatograph Mod. 8000 with on-column injector and autosampler AS800 equipped with a 5 µl syringe, FID, integration system ChromCard, all from CE Instruments (Milan, Italy); Blender (Büchi B-400); pipette for accurately dispensing 200 µl (e.g. Eppendorf); magnetic stirrer/heating plate; 50 ml Erlenmeyer flasks with glass stoppers and 10 ml screw cap flasks.

Standards

Triundecanin (tri-11), 1-tetradecene (C14:1), methyl nonanoate (E9), methyl butyrate (E4), tributyrin (tri-4), methyl valerate (E5), dimethylformamide (DMF) puriss, pentane, heptane purum and disodium hydrogen citrate purum, all from Fluka (Buchs, Switzerland); 30 g/100 ml methoxide in methanol, methanol p.A. and methyl-tert. butyl ether (MTBE) p.A. from Merck (Darmstadt, Germany).

Solutions

- *Sodium methoxide in methanol,* 5 g/100 ml. Should be at ambient temperature when applied, because the reaction is optimized to occur at about 25 °C.
- *Hydrogencitrate solution:* 100 g disodium hydrogen citrate and 150 g sodium chloride in 1 l of water
- *Extraction solvent,* 10 % (v/v) pentane in MTBE
- *Internal standard solution 1 (ISS1):* tri-11, E9, C14:1, and E5 in heptane, 0.50 g/100 ml. Discard when only 20 % filled (rapid reconcentration).
- *Internal standard solution 2 (ISS2):* tri-11, E9, C14:1, and E5 in MTBE, 50 mg/250 ml or ISS1 diluted 1 in 25. Discard when only 20 % filled.

- *Calibration rfE4/rfC18:* determination of the response factor of E4 (rfE4) and of the global response factor for C16/C18 fats and oils (rfC18):
  prepare a mixture of 5 g tri-4 in 100 ml of a vegetable oil; stir during 1 h at about 60 °C; can be stored in the dark over several years;
  analyze as a sample, i.e. accurately weigh in 50 mg of this mixture into a 50 ml Erlenmeyer flask, add 200 µl of ISS1 and transesterify as described below (add 5 ml of MTBE...). Solution freshly prepared for every series of analyses.
- *Calibration rfM and rfC:* global response factors for milk and cocos fat: analyze as a sample (accurately weigh in about 50 mg ...). Newly prepared for each series of samples.
- *Column test solution:* add 200 µl of ISS1 in a 50 ml Erlenmeyer flask and transesterify as described below (add 5 ml of MTBE...). Solution can be stored for years.
- *Blank test solution:* 2.5 ml of DMF in a 50 ml Erlenmeyer flask, add 200 µl of ISS1 and transesterify as described below (add 5 ml of MTBE...). If no DMF pretreatments are performed, leave DMF away. To be newly prepared whenever a solvent or reagent is changed.

Sample preparation

Sample size

Samples must be well homogenized since only 50–300 mg of material are analyzed. Two parallel analyses were performed for each sample. Amounts of sample weighed in were optimized to result in a suitable peak size for E4 without severe overloading of the column in the region of the C18-esters. Table 1 lists important types of foods as well as the suggested sample sizes such that they contain some 5–20 mg of milk fat and about 10 ng of E4 were analyzed by GC-FID. If merely 10 % of the fat consist of milk fat, the 10 ng of E4 go along with some 2.5 µg of other FAMEs, which is near the upper capacity limit of the column.

Table 1
Approximate fat and milk fat contents and suggested amounts of sample to weigh in

Product	*Fat (g/100 g)*	*Milk fat/fat (g/100 g)*	*Sample weighed in*
Butter	83	100	30 mg
Infant formula	25	80	50 mg
Margarine	80	10	100 mg
Milk chocolate	30–40	10–20	200 mg
Baker's ware	5–15	25–100	200 mg
Cream sauce, ice cream	3–15	25–100	300 mg

1.0 mg of the internal standards were added (200 µl of ISS1), corresponding to about 10 % of the milk fat content. Butter was the exception: for a sample size of 30 mg, 3 mg of standards were added (600 µl of ISS1).

Sample pretreatment

Table 2 shows the pre-treatments applied to the various types of foodstuffs (4). Slurries with water were prepared for some powders: 500 µl of water was added under vigorous stirring and the slurry allowed to stand for at least 5 min. Refluxing with 2.5 ml of dimethyl formamide (DMF) for 15 min and stirring was applied for samples with poorly accessible fat. Formation of lumps must be avoided, for which it is important to heat immediately after DMF has been added.

Table 2
Pre-treatments of food samples before transesterification

None	*Slurry with water*	*Refluxing with DMF*
milk, curd, yogurt	milk powder	cheese
unsweetened evaporated milk	infant formula	chocolate with nuts
ice cream	strongly sweetened milk products	cereals
chocolate without nuts	(e.g. sweet evaporated milk)	cocoa and malt drinks
sauces		

Transesterification

To the possibly pretreated sample, 200 µl of ISS1 and 5 ml of MTBE were added. After thorough mixing, dispersing a fine solid and possibly dissolving parts of the product (e.g. chocolate), 5 ml of 5 g/100 ml methoxide/methanol were added (vortex, 3 s). 60–90 s later, 20 ml of extraction solvent were admixed and the reaction stopped by addition of 20 ml hydrogencitrate solution. Since the liquids remained in one phase up to the addition of the aqueous solution, extraction was complete with little shaking. The supernatant was analyzed by on-column injection and GC-FID.

GC-analysis

The 25 m x 0.25 mm i.d. column, coated with an immobilized Carbowax of 0.25 µm film thickness, was designed for this analysis (BGB-Analytik, Anwil, Switzerland). It was equipped with a 50 cm x 0.53 mm i.d. deactivated precolumn (autosampler on-column injection). Inlet pressure, 80 kPa (hydrogen); injection at 40 °C (1.5 min), then 25 °/min to 180 °C and 15 °/min to 220 °C (3 min); injection volume, 0.6 µl. Injection 10 s after introduction of the syringe needle and the needle withdrawal 10 s after depression of the plunger. Figure 1 shows a chromatogram obtained from a milk chocolate containing about 4 % milk fat.

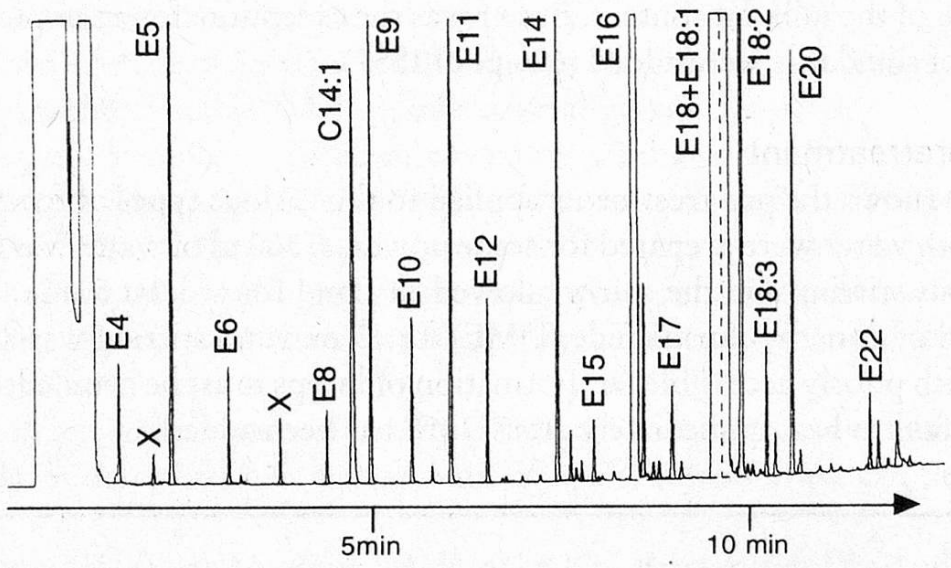

Figure 1 **Transesterified milk chocolate (200 mg) with 1 mg of the internal standards E5, C14:1, E9 and E11. x, impurities of MTBE**

Calculation of results

Results were calculated in an Excel table. This also facilitated the calculation of the ratios of the internal standards for the control of transesterification and GC analysis (see below).

E4 was calculated through E5. The response factor rfE4 was determined by transesterification of a mixture of tri-4 and vegetable oil in the same way as the samples. Milk fat contents were calculated on the basis of assumed concentrations of E4 or butyric acid in milk fat (see introduction).

Total fat contents were determined as described in (3), i.e. using E11 as internal standard and a global response factor for the type of fat present, distinguishing between C16/C18 fats/oils (rfC18), water-free milk fat (rfM) and cocos fat (rfC). rfC18 was obtained from the determination of rfE4 (only 95 % of the material weighed in). For mixtures of milk fat and other fats, mixed response factors were calculated. Since differences are small, a 10 % error in the mixing ratio results in less than 1 % difference.

Summarized procedure/small volume version

Table 3 summarizes the above procedure. For homogeneous samples, a smaller amount can be analyzed, saving solvent and enabling to work with 10 ml screw cap vials. The procedure for molten chocolate or margarine with an admixture of milk fat is summarized in table 4. It involves the more diluted solution of internal standards ISS2 in MTBE (caution: MTBE tends to reconcentrate rather rapidly). Weighing of the sample must occur at a precision of ± 0.1 mg or better, since 0.1 mg

Table 3
Summary of the procedure

1	Accurate weighing of about 30–500 mg of sample (table 1)
2	If necessary, pretreatment according to table 2
3	Addition of 200 µl of ISS1 (1 mg) and 5 ml of MTBE
4	Addition of 5 ml methoxide/methanol
5	Transesterification during 60–90 s
6	Addition of 20 ml extraction solvent, quick shaking
7	Addition of 20 ml hydrogencitrate solution
8	On-column injection of 0.6 µl and GC-FID analysis

corresponds to 0.5–0.7 % of the weight of the sample. Calibration of the response factors is also performed by the small scale procedure.

Optimization of accuracy

The accuracy achieved largely depends on the quality of the work performed by the analyst as well as his knowledge about the techniques involved. From estimated errors and observed standard deviations (see below) it is concluded that E4 can routinely be determined with an accuracy of 4 %, the total fat within 3 %. This presupposes, however, that no step of the procedure produces an error exceeding 1 % and most deviations remain below 0.5 %. GC analysis with a relative standard deviation of 1 % is possible, but requires careful control.

The analysis is based on two determinations requiring accuracy, i.e. the response factor for E4 and the global response factor for the total fat content. These solutions must be accurate (pure tri-4, no reconcentration during storage etc.). All other steps merely require reproducibility, since they are part of the comparison between standard solutions and the sample, and both analyses are performed by exactly the same procedure. However, since two analyses are compared, deviations add up.

Here the various steps are discussed regarding modifications compared to the procedure described previously and possible sources of error.

Sample preparation

Weighing of sample

If the sample is weighed in at an accuracy of ± 0.2 mg, the maximum error for 50 mg of sample is 0.4 %, which is the maximum tolerable. If the method is scaled down for homogeneous samples and only 15–20 mg are weighed in (table 4), accuracy must reach ± 0.1 mg.

Accuracy of the standard solutions

Frequent opening of the flask causes evaporation of solvent and a slow reconcentration of the standard solutions. In addition, flasks are usually not abso-

Table 4
Procedure recommended for homogeneous samples like molten chocolate

1	Accurate weighing of some 15–20 mg of sample or 1 droplet
2	Addition of 500 µl of ISS2 (0.10 mg)
3	Addition of 0.5 ml methoxide/methanol
4	Transesterification during 60–90 s
5	Addition of 2 ml extraction solvent, quick shaking
6	Addition of 2 ml hydrogencitrate solution
7	On-column injection of 0.6 µl and GC-FID analysis

lutely tight. However, slowly increasing concentrations in the ISS do not cause an error as long as the calibration of the response factors is performed with the same solution as the analysis of the sample; the mere increase of the response factors over some weeks has no negative effect. For this reason, but also because of drifting GC performance, response factors must be determined for every series of analyses using freshly prepared solutions.

Addition of ISS

For the addition of ISS, there is no need for accuracy – neither the solution must be accurate, nor the volume added; no high purity of the standard compounds is required. The addition must, however, be well reproducible. Pipetting devices should be tested on reproducibility, weighing 200 µl portions of the ISS solvent. Deviations should remain below 0.5 %.

Internal standards

The system of internal standards was based on that described previously (3), conceived to monitor as many critical steps as possible for every analysis. Tri-11 (E11) was the internal standard for the determination of the total fat content. C14:1 and E9 served to control transesterification (E11/C14:1) and saponification (E9/C14:1).

Commonly E5 is the internal standard for the quantitation of E4, following the rule that best results are obtained with an internal standard of a volatility similar to that of the component of interest. This is not necessarily important when using on-column injection. For a number of experiments, E4 concentrations were calculated by both E11 and E5. Relative standard deviations for sets of 5 injections of the same transesterified chocolate, analyzed at different days, are given in table 5. There is no significant difference whether calculating through E5 or E11. The data also shows that GC analysis caused standard deviations up to 1.0 %, partly because at that time a 10 µl syringe was used for the injection of 0.6 µl.

In another experiment, a transesterified test sample of 10 g/100 g milk fat in sunflower oil was injected ten times. Area ratios were determined in order to locate the major sources of deviations. As shown in table 6, area ratios E4/E11 and E4/E5 had

Table 5
Comparison of the relative standard deviations (%) obtained for the determination of E4 through E5 or E11 as internal standard

Experiment	*E4 calculated by*	
Nr.	*E5*	*E11*
1	0.65	0.55
2	0.43	0.41
3	0.99	1.02

Table 6
Relative standard deviations (%) for ratios of peak areas determined by 10 repeated injections of the same solution

	E4/E11	*E4/E5*	*E5/E11*	*E11/C14:1*	*E5/C14:1*
rel. st. dev (%)	1.01	0.96	0.35	0.13	0.36

relative standard deviations of around 1 %. Hence there was no difference whether E4 was quantitated by E5 or E11. The ratios of E5/E11 and E5/C14:1 had relative standard deviations of 0.35 %, indicating that the peak area of E4 was the main source of deviations.

It was concluded that under the conditions used, E11 was equally suited for determining E4 as E5. This may, however, be different when split injection is used. Furthermore, robust methods should be designed for non-optimal situations, such as adsorptive precolumns. In these situations, E5 may be preferable.

If E5 is used for quantitating E4, it should be added as trivalerin (tri-5). Firstly, tri-5 undergoes the same transesterification, i.e. compensates for some potential deviations, and, secondly, it does not evaporate from the standard solution. Presently, however, tri-5 is not available in adequate amounts.

The data again shows relative standard deviations reaching 1 %, confirming that GC is the most important source of error when the other steps are well under control.

In transesterified milk fat, E9 and E11 are present at concentrations of about 0.02–0.04 %. E9 and Tri-11 must, therefore, be added in amounts rendering this natural presence negligible. If less than 0.5 % error is accepted, the addition must result in a concentration related to the milk fat exceeding 8 %.

Transesterification

Homogeneous reaction phase

Fast transesterification presupposes that the fat of the sample and the reagent are brought together into a homogeneous phase. The mediator solvent previously used,

dioxane, is eluted from Carbowax columns after E4. Since this may be inconvenient, it was replaced by MTBE, as already used for transesterification involved in sterol analysis by on-line LC-GC (11). The lower boiling point of MTBE compels us to perform on-column injection at a lower oven temperature (maximum of around 55 °C), which is, however, anyway required for the analysis of E4.

Kinetics of transesterification

Reaction conditions must be such that transesterification reaches completion before relevant saponification occurs. For the fatty acids beyond E10, the suitable time frame ranged from 45 s to 3 min, independent of the amount of water present (maximum, 500 µl). Transesterification of tri-11 was slightly more rapid than that of the C18-esters (3), suggesting that it could be still faster for esters of butyric acid and that relevant saponification might occur before the higher fatty acids are fully transesterified.

Tests on saponification were performed adding 100 µl of E4, 0.5 mg/ml in MTBE, and 100 µl ISS1 to 5 ml MTBE. This mixture as well as analogous solutions with 100 or 500 µl of water were reacted with methoxide during 30, 60, 90, or 120 s. Table 7 shows ratios of peak areas, each representing means of two experiments (except of that without water with $n = 4$). After 30 s reaction time, transesterification of tri-11 was complete as concluded from the area ratio of E11/C14:1 reaching the maximum. After 90 s, the E11/C14:1 and E4/C14:1 ratios of the mixture containing 500 µl of water were slightly lower, probably as a result of some saponification. However, the E4/E11 ratio was not affected, indicating that the rates of saponification of E4 and E11 are not significantly different.

Analogous experiments in duplicates were performed with 50 mg of milk fat, i.e. on butyric acid being part of glycerides. After 90 and 120 s, the area ratio E11/C14:1 was slightly reduced (table 8), but the ratio of E4/E11 again remained constant.

Table 7
Ratios of peak areas obtained for an experiment designed to check the kinetics of saponification of E4

Test sample	*Duration*	*E11/C14:1*	*E9/C14:1*	*E4/C14:1*	*E4/E11*
E4 + 0 µl H_2O	60 s	0.70	0.70	0.48	0.68
E4 + 100 µl H_2O	30 s	0.71	0.70	0.48	0.67
E4 + 500 µl H_2O		0.70	0.69	0.47	0.67
E4 + 100 µl H_2O	60 s	0.71	0.70	0.48	0.68
E4 + 500 µl H_2O		0.70	0.69	0.47	0.67
E4 + 100 µl H_2O	90 s	0.71	0.70	0.48	0.68
E4 + 500 µl H_2O		0.68	0.67	0.46	0.67
E4 + 100 µl H_2O	120 s	0.71	0.69	0.48	0.68
E4 + 500 µl H_2O		0.68	0.66	0.46	0.68

Table 8

Ratios of peak areas obtained for an experiment on the reaction kinetics of butyric esters in milk fat. Ratios with E4 are corrected by the amount (mg) of milk fat weighed in

Test sample	*Duration*	*E11/C14:1*	*E9/C14:1*	*E4/C14:1*	*E4/E11*
+ 100 µl H_2O	30 s	0.76	0.71	0.39	0.514
+ 500 µl H_2O		0.74	0.70	0.38	0.515
+ 100 µl H_2O	60 s	0.76	0.71	0.39	0.515
+ 500 µl H_2O		0.74	0.70	0.38	0.516
+ 100 µl H_2O	90 s	0.75	0.71	0.39	0.517
+ 500 µl H_2O		0.73	0.69	0.38	0.516
+ 100 µl H_2O	120 s	0.75	0.71	0.39	0.517
+ 500 µl H_2O		0.73	0.69	0.38	0.516

It is concluded that the time window between 30 and 120 s provides correct results and that the window previously recommended (60–90 s) is also appropriate for the determination of E4.

Extraction from the transesterification medium

Extraction efficiencies of E4 from the transesterification medium were determined by second extractions with MTBE. Using pentane and the conditions used previously (3), yields of the first extracts for chocolate merely reached 92 % (table 9) and were poorly reproducible. Since those of E11 and the esters of larger molecular weight was better than 99 %, this resulted in a corresponding discrimination of E4. Generally E5 were extracted about 3 times more efficiently than E4, i.e. did not fully compensate for losses of E4. Addition of 20 g/100 ml sodium chloride to the citrate solution did not solve the problem.

With MTBE instead of pentane, yields were better. Substantial amounts of methanol were co-extracted, however, broadening the solvent peak and on certain

Table 9

Extraction efficiencies of E4 from the transesterification medium (means of 3–5 experiments)

	Extraction efficiencies of E4 (%)			
Sample	*Pentane*	*Pentane + NaCl*	*MTBE*	*MTBE + NaCl*
Standards	97.5	95		
Margarine			98.2	99.3
Milk fat		94.5		
Chocolate	91.9	96.2	97.7	98.8
Sauce powder			97.8	99
Sauce			97.1	98.1

columns distorting the E4 peak by partial solvent trapping (see below). This was improved by adding a doubled volume of hydrogencitrate solution and admixing 10 % (v/v) of pentane to the MTBE; extraction efficiencies remained unaffected. Since results shown under «MTBE» were still not considered satisfactory, 15 g/100 ml sodium chloride was added to the hydrogencitrate solution. This increased yields above 98 % even for a sauce containing a large amount of emulgators. Such extraction may still contribute a deviation of up to 1 %, but this was not considered sufficient reason to introduce a second extraction into the procedure.

Partial solvent trapping of E4

On-column injection frequently causes the type of peak distortion for E4 shown in figure 2 (strongly expanded first part of a chromatogram). The deformation results from partial (or incomplete) solvent trapping (12) occurring during solvent evaporation in the sample-coated column inlet. In the carrier gas stream, the solvent evaporates from the rear to the front of the sample layer. All but the most volatile solutes remain retained in the liquid until the solvent is fully evaporated and start chromatography as a sharp band (full solvent trapping). E4 is one of those volatile compounds which partly co-evaporate with the solvent. The co-evaporating material starts chromatography during a period of time corresponding to the duration of solvent evaporation and forms an initial band of a width which is proportional to the sample volume injected. The other (larger) part of E4 is fully trapped and released at the end of solvent evaporation, resulting in the sharp signal.

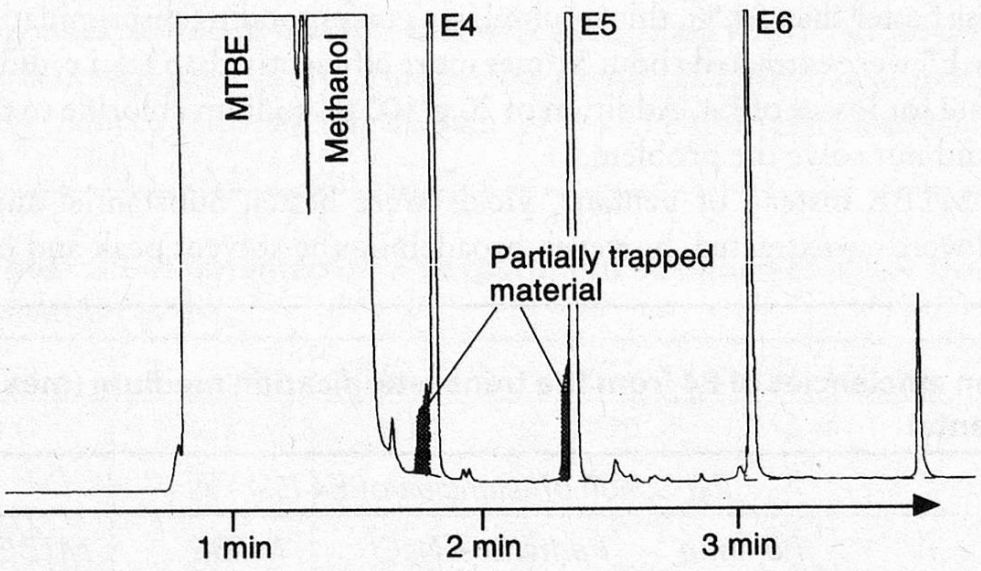

Figure 2 **The first part of a chromatogram expanded to show peak deformation by partial solvent trapping. Result obtained before optimisation of the extraction step**

Owing to its lower volatility, E5 is more strongly solvent-trapped than E4. The peak is, furthermore, sharpened by the temperature programming (cold trapping effect). The E6 peak is of perfect shape.

If the whole peak is integrated, quantitative determinations are not affected. In the rather extreme and unfortunate instance shown in figure 1, however, there was a small component on the shoulder that caused the integrator to split the E4 peak and cut off the incompletely trapped E4 material. Hence peak deformation can be tolerated as long as integration is not excessively disturbed. Correct positioning of the baseline is crucial for obtaining accurate results. Manual re-adjustment shows how rapidly peak areas change by 1 %.

Partial solvent trapping depends on the retention power of the solvent for the solute. Retention by hydrocarbons is weaker than that by MTBE. However, the MTBE extracts some methanol (the second part of the solvent peak), which again forms a weak solvent trap. This is why the extraction was adjusted (see above). Once the type of solvent is given, injection of a smaller sample volume is the only measure to improve peak shapes.

Injection into GC

On-column injection is the most accurate and reliable technique for sample introduction. It needs, nevertheless, some optimization. There is an interest in injecting the smallest possible volume of sample, firstly to minimize distortion of the E4 peak and, secondly, to reduce the dilution required, i.e. the amount of solvent for extraction or a separate dilution step. Too small a sample volume affects, however, the precision of the results: the mechanical transfer of the sample liquid from the needle tip to the capillary wall is no longer complete (13, 14). The sample remaining on the needle tip is partly evaporated in the stream of carrier gas, causing more of the volatile material to be analyzed than of the high boilers.

The minimum injection volume yielding precise data depends on the speed of the plunger during injection (insofar also on whether injection is performed manually or via autosampler), the size of the syringe (5 or 10 µl) and the shape of the needle tip. For the system used, it was determined through the standard deviations calculated for the area ratio of E4/E11 (table 10). Using a 10 µl syringe, about 0.4 µl more had to be injected to reach a similar reproducibility, which resulted in strong deformation of the E4 peak.

An injection volume of 0.6 µl was chosen. However, since injection performance may be different under other conditions, continuous monitoring by calculation of the E5/E11 ratio in the results obtained for each sample is important. The value obtained is less important than its stability: variations must remain below 1 %.

Using the on-column injector with the septum and the rotating switching valve (CE Instruments), the analysis of E4 may sensitively react to an untight septum. A strong leak upon opening the rotating valve causes a pressure drop and may result in carrier gas flowing backwards out of the column. Injection at that moment easily

Table 10
Relative standard deviations (RSD) for autosampler on-column injections with a 5 µl syringe; *n* = 10

Sample volume	*RSD*
0.3 µl	1.75 %
0.5 µl	0.54 %
0.7 µl	0.29 %

causes loss of sample material, primarily of volatiles. Even in presence of a substantial leak, loss of E4 is avoided by delaying the injection 10 s after introduction of the needle. In this way, the carrier gas flow into the column is re-established at a possibly lower inlet pressure before the injection occurs. The same was applied for the withdrawal of the syringe: in order to avoid an adverse effect by a pressure drop, the needle was left in the inserted position for 10 s after the injection.

Adsorption in the column

Adsorptivity in the GC column easily causes deviations in the order of a few percent and must be carefully kept under control. Even the rule that the first analysis of a series is frequently «wrong» belongs to this.

After some 50–100 analyses, the inlet of the precolumn usually became adsorptive, causing unequal losses throughout the chromatogram. At this stage, there were no obvious signs of adsorption (tailing peaks) in the chromatograms of samples. More sensitive testing was achieved by the transesterified ISS solution: adsorptivity caused tailing of the E11 peak and a reduced area compared to C14:1 or E5. In the chromatograms of the samples, no such tailing was observed, apparently because only the last peak of a mixture was affected, i.e. the E16 and E18 components temporarily deactivated the column (a phenomenon known from general column testing with methyl esters). Avoidance of systematic deviations through such adsorption phenomena was one of the reasons to perform calibration of rfE4 with a mixture containing vegetable oil. At a more advanced stage of column adsorptivity, the E18 peaks showed tailing with a shape as if peaks had feet. Finally there may be peak splitting, primarily on C14:1.

Adsorptivity is usually caused by a contamination of the column inlet and can be eliminated by removal of the first 10 cm of the precolumn or eventually by its replacement. More seldom the inlet of the coated column is the cause (remove 30 cm) or the outlet in the (over?)heated detector block. The separation columns usually lasted for a few thousand analyses (1–1.5 years).

Interfering peaks

To recognize «ghost peaks» or «memory effects», blanks should be tested by injection of a transesterified ISS solution with all the solvents and components in

use, possibly including DMF for sample pretreatment. DMF produces a large peak eluted before E8. Ghost peaks can be accepted provided they are disregarded when summing up areas for calculating the fat content.

On-column injection may cause memory effects by a contamination of the injector with components of intermediate volatility, particularly the C16 and C18 esters. If they reach about 0.5 % of the size of the peaks in the sample, the injector must be backflushed by carrier gas, introducing a small leak at its top and heating the oven to the maximum tolerated by the column in order to warm the injector.

Although seldom observed, there is a possibility that a sample produces peaks other than FAMEs, which requires that every chromatogram is examined for the peaks included when summing up the areas.

Validation of the method

Working range

The lower detection limit for milk fat in a mixture of fats and oils is determined by the amount of sample material that can be injected without causing unacceptable overloading effects, first affecting the E16/E18-region, later most of the peaks. For the column used, this capacity limit was reached with 3–5 µg of transesterified fat or oil. A 0.1 % concentration of milk fat results in an E4 peak of about 0.2 ng, which is well detected, but integration is less accurate (primarily depending on the adjustment of the baseline).

The upper limit of the method for the determination of milk fat in other fats results from the assumptions on the butyric acid concentration in the milk fat if the latter is not available for comparison. With more than about 50 % of an unknown milk fat in a mixture, the determination of the other fat through triglyceride analysis (15, 16) usually provides more accurate results.

Table 11
Analyses of test samples consisting of sunflower oil with a known milk fat being added at various levels

	Milk fat in oil (g/100 g)		*Rel. st. dev. (%)*	*Number of determinations*
	added	*determined*		
1	2	2.04	2.8	5
2	10	10.3	0.77	5
3	10	10.1	1.0	10
4	10	10.0	1.9	10
5	25	25.5	1.2	5
6	100	200.2	1.5	5
7	100	100.0	2.5	5

Linearity, accuracy

Test samples were prepared with 2, 10 and 25 g/100 g milk fat added to sunflower oil. The E4 content of the milk fat was determined and used for the calculations. The results shown in table 11 were obtained over several weeks, often with calibrations performed days before. The maximum deviation of the mean concentrations from the true result was 2.8 % (2 g/100 g milk fat added). The maximum relative standard deviation was 2.5 %.

Estimated accuracy

A statistical determination of accuracy and precision of a chromatographic method is of doubtful usefulness because the results depend too much on the daily performance of the system and the skill of the analyst. It is more useful to review the possible sources of deviations and to estimate their contributions.

- Weighing of a sample in the range of 50–300 mg occurs with an accuracy better than 0.5 %.
- For the addition of the internal standard (200 µl), precision rather than accuracy is required. Using a 100–1000 µl Eppendorf pipette, in two experiments relative standard deviations of 0.37 and 0.45 % (n = 10) were achieved after some optimization. Addition by a microliter syringe was less precise.
- Variations resulting from the transesterification process are difficult to estimate because they could only be determined by complete analyses. However, the fact that the standard deviations were similar for repeated total analyses as for repeated injections of the same solution suggested that transesterification made no relevant contribution to the error.
- GC analysis requires most attention. Using on-column injection, it can be optimized to result in a relative standard deviation of less than 1 %, but the uncertainty in real analysis is rather in the order of 2 %. Accurate integration (positioning of the baseline) and good column performance are most critical.

It is concluded that E4 determinations within 4 % from the true value are achievable, but also that values of ± 3 % are the best to hope for. Determinations of total fat contents are slightly more accurate.

Summary

The method for determining fat contents and fatty acid compositions through fast transesterification directly in the raw, homogenized food is extended to include the measurement of butyric acid. This enables the determination of contents of milk fat and total fat from the same analysis. The method needs some adjustments, primarily concerning the solvents. Possible sources of error are analyzed. GC analysis is the main source of uncertainly. Results with a reliable accuracy of ± 3–4 % can be achieved.

Zusammenfassung

Die Methode zur Bestimmung von Fettgehalt und Fettsäurezusammensetzung über eine schnelle Umesterung direkt im rohen, homogenisierten Lebensmittel wurde auf die Messung von Buttersäure ausgeweitet. Dies erlaubt, aus der gleichen Analyse sowohl den Milchfett- als auch den Gesamtfettgehalt zu berechnen. Dazu sind Anpassungen vor allem der Lösungsmittel nötig. Mögliche Probleme werden diskutiert. Die GC-Analyse ist die wichtigste Ursache von Unsicherheit. Die Resultate können innerhalb 3–4 % zuverlässig richtig sein.

Résumé

Le dosage de la teneur en matière grasse et de la composition en acides gras par une transestérification rapide et directe des aliments crus homogénéisés est la méthode qui a servi à la détermination de l'acide butyrique. Les teneurs en graisse de lait et graisse totale peuvent être calculées au moyen de la même analyse. On doit cependant introduire quelques modifications, surtout en ce qui concerne les solvants. Les causes potentielles d'erreurs font l'objet d'une discussion. L'analyse par chromatographie gazeuse est la principale source d'incertitudes. Des résultats corrects peuvent être obtenus avec une fiabilité de l'ordre de ± 3–4 %.

Key words

Fatty acid methyl esters, Milk fat in fat mixtures, Fat content in foods, Butyric acid, Transesterification of fats

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