Customs Laboratories
European Network

ILIADe 374: 2024 | CLEN method

Determination of milk fat content in fat extracted from food products

Version 13 February 2024

This table shows the most important changes that have been made compared with the latest former version	
Date of the latest former version: 14 May 2021	
Section	**Changes**
8. Precision	Update of precision data: repeatability and reproducibility

Determination of milk fat content in fat extracted from food products

1 Scope

The scope of this method is to determine the milk fat content of food products as outlined in Regulation (EC) No 900/2008. The milk fat content has to be known for correct tariff classification and for correct attribution of the Meursing code (Implementing Regulation (EU) No 514/2011).

This method is to be applied on the fat extracted from food samples but is not appropriate for samples containing a lot of free fatty acids. For the latter samples, it is better to use an acid catalysed transesterification which is not described here because food samples typically do not contain a significant amount of free fatty acids. Since valeric acid is used as internal standard, this method cannot be applied for products that already contain valeric acid in their fatty acid composition (e.g. fat obtained from goat milk).

2 Principle

The sample is the resultant fat fraction, obtained by the CLEN Method for Determination of Total Fat Content in Food Products (ILIADe 179) or by an alternative extraction technique (extraction in petroleum ether after acid hydrolysis).

In this method, the fat is dissolved in an appropriate organic solvent containing methyl valerate as internal standard. The fat is allowed to react in an alkaline methanol solution or sodium methylate to form FAMEs and glycerol (milk fat is transformed into methyl butyrate and glycerol). Methyl butyrate and methyl valerate (and potentially also other FAME's) are separated in a GC column and detected by a flame ionisation detector (FID). After the integration of the peak area of methyl butyrate and methyl valerate, the concentration of milk fat in the original sample can be calculated.

3 Reagents and Materials

3.1 Organic solvent to dissolve the fat fraction (e.g. hexane, heptane, tert-butyl methyl ether, iso-octane, …)

3.2 Methyl butyrate p.a. (for calibration)

3.3 Methyl valerate p.a. (as internal standard)

3.4 Methylation agent: Sodium methylate (0.5 to 5.4 M in methanol) or KOH (e.g. 2 M) in anhydrous methanol (transesterification), or equivalent.

3.5 Methanol (anhydrous)

3.6 Reagent to stop the transesterification reaction (e.g. $NaHSO_4.H_2O$ and/or Na_2SO_4). This reagent is essential when the GC analysis is not performed immediately.

4 Apparatus

4.1 Analytical balance with precision of 0.1 mg.

4.2 Vortex mixer

4.3 A gas chromatograph with split / split less injector, flame ionisation detector (FID)

4.4 GC capillary column: Gas chromatographic column (a polar capillary column, see examples in section 5.3)

4.5 Pipettes

4.6 Reaction tubes

4.7 GC vials

5 Procedure

5.1 Preparation of the internal standard solution and the calibration solution(s)

- Internal standard solution: Methyl valerate (internal standard) is diluted in the organic solvent to be used to dissolve the fat. A practical working concentration of methyl valerate is ca. 0.3 to 1.0 mg/mL.
- Calibration solution(s): Methyl butyrate is diluted in the internal standard solution. A practical working range of the concentrations of methyl butyrate is 0.1 – 1.5 mg/mL. Either a one point calibration or a multiple point calibration can be used. The calibration solution(s) also contain(s) the internal standard at the same concentration as used to dissolve the samples.

5.2 Preparation of fatty acid methyl esters

Because of the wide variety of methods in use, it is difficult to write down a standard procedure. First, the general handlings are written down, followed by three examples in more detail. Alternative (equivalent) procedures may also be possible.

In general: Weight on an analytical balance an appropriate amount of extracted fat (e.g. 50-200 mg) in a reaction tube. It might be helpful to warm the fat extract to 60°C to homogenize the sample. As the warmed fat has become liquid, a subsample can easily be weighed into a reaction tube using a pipette to transfer the subsample into the reaction tube.

In the following step, the fat is dissolved in the internal standard solution. Next, the methylation agent is added. The reaction tube is shaken vigorously on a vortex for 1 to 30 minutes and left to settle for about 10 minutes. Alternatively, the content of the reaction tube can be mixed by means of a laboratory shaker for up to 30 minutes. Subsequently the reaction is stopped by adding an appropriate reagent to stop the reaction (stopping the reaction is not always mandatory). The reaction tube is shaken vigorously for a minute (or by laboratory shaker for up to 30 minutes), followed by centrifugation (or allowed to settle until phases separate). The supernatant is pipetted into a GC vial.

Example 1: Weigh about 50 to 200 mg fat (depending on milk fat supposed concentration) in GC vials with cap. Add 200 µL of methyl valerate solution, 500 µL hexane and 500 µL methylation agent. Close carefully the vials and put them into an oven at 70 C for 7 minutes. Remove from the oven and vortex for 1 minute, place again in the oven for 7 minutes. Allow the sample to cool down to complete separation of the two phases. Take 200 µL from the upper phase, transfer it into a new GC vial and add 500 µL of hexane. Close, shake and inject in the GC.

Example 2: Weigh accurately about 100 mg extracted fat in a centrifugation tube. Dissolve in 5 mL of the methyl valerate (400 mg/L) solution. Add 250 µL KOH (2 M dissolved in methanol), followed by 2.4 mL hexane. Vortex for 1 minute. Add 500 mg $NaHSO_4$.H2O and vortex for 2 minutes until the supernatants becomes clear again. If not, repeat this step. Pipet the clear supernatants in a GC vial.

Example 3: Weight around 80 mg fat in an esterification tube. Add 4 mL methyl valerate (600 mg in 1 L hexane) and dissolve the fat. Add 200 µL KOH (2 M in methanol) and shake vigorously for 1 minute. Allow to rest for 15 to 30 minutes. Add around 100 mg $NaHSO_4$ and shake vigorously for 1 minute. Add around 100 mg Na_2SO_4 and shake vigorously for 1 minute. Centrifuge for 2 minutes at 2500 rpm. Transfer the supernatants in a GC vial.

Example 4: for edible fats and oils, refer to the procedures available in ISO 12966 part 2 method 5.4 or ISO 5509 & ISO 5508 (both expired).

5.3 GC analysis

The samples (calibration solution(s), control sample and samples) are measured using a GC-FID. Most GC software allow the input of weights and concentration (weight of extracted fat and concentration of methyl butyrate used for the calibration and methyl valerate used as internal standard).

Possible method conditions are shown in annex.

5.4 Quality control

The quality control can be performed by one (or a combination) of the following actions:

- Evaluation of measured peaks (symmetry, tailing, baseline separation of methyl butyrate and methyl valerate, …);
- Evaluation of blank sample (e.g. solvents used without C4, nor C5);
- Evaluation of the calibration;
- Evaluation of duplicate measurements;
- Measurement of a control sample: use of a CRM is advised to assess the quality of all the procedure.
- Baseline separation (RS value) of methyl butyrate and methyl valerate should be at least 1.0 and correlation coefficient of calibration curve should be 0.995.

6 Calculation

6.1 Calculation of concentration methyl butyrate

The corresponding peaks for methyl butyrate (C4-FAME) and methyl valerate (C5-FAME) are detected and their peak area is integrated. The peak area of methyl butyrate can be recalculated to a methyl butyrate concentration, using the methyl butyrate calibration line and using the internal standard.

The procedure below could be applied in order to calculate the result manually, if using a calibration curve:

- A calibration line is plotted with the area ratio (measured C4-FAME peak area / measured C5-FAME peak area) in function of the used quantity ratio (used C4-FAME concentration (mg/mL) / used C5-FAME concentration (mg/mL)). Calculate the slope of the linear regression;
- The area ratio of the sample is calculated (measured C4 peak area / measured C5 peak area);
- The quantity ratio of C4-FAME over C5-FAME in the sample is calculated using the calibration line;
- The mass fraction C4-FAME can be calculated by multiplying this quantity ratio by the quantity of C5-FAME in the amount of internal standard solution used for dissolving the sample, divided by the sample amount. The mass percentage (g methyl butyrate/100 g fat) is obtained by multiplying the mass fraction by 100%.

6.2 Conversion of methyl butyrate to milk fat

The milk fat content is calculated by multiplying the methyl butyrate content (g methyl butyrate / 100 g fat) by a factor and by the total fat content (expressed in %) and divided by 100.

The factor that has to be used, is 25 or 50 and depends on the composition of the sample. (cf. Implementing Regulation (EU) 2015/824 of 27 May 2015).

7 Expression of the results

The amount of milk fat is given with maximum 2 significant digits, with one decimal.

8 Precision

Nine CLEN inter laboratory tests were carried out between 2004 and 2023 including the determination of milk fat content in several matrices, with the participation of the EU Customs laboratories.
Based on the result of these inter laboratory tests, the following precision data are proposed as minimum requirement: every EU Customs Laboratory should be able to comply to these precision data.
The matrices “edible fats and oils” are not covered by the following precision data.

Repeatability (r) and reproducibility (R):

	Concentration % m/m	Repeatability (r) % m/m	Reproducibility (R) % m/m
BAME (C4-FAME)	0-1	0,1 absolute	0,3 absolute
BAME (C4-FAME)	1-5	10 relative	30 relative

	Concentration % m/m	Repeatability (r) % m/m	Reproducibility (R) % m/m
Milk Fat	0-15 15-40	10 relative 1.5 absolute	4 absolute 4 absolute

9 References:

9.1 European Commission Regulation (EC) No 900/2008 of 16 September 2008 (Laying down the methods of analysis and other technical provisions necessary for the application of the arrangements for imports of certain goods resulting from the processing of agricultural products)

9.2 European Commission Implementing Regulation (EU) 2015/824 of 27 May 2015 (amending regulation (EC) No 900/2008 laying down the methods of analysis and other technical provisions necessary for the application of the arrangements for imports of certain goods resulting from the processing of agricultural products

9.3 European Commission Implementing Regulation (EU) No 514/2011 of 25 May 2011 laying down the detailed rules for implementing the preferential trade arrangements applicable to certain foods resulting from the processing of agricultural products, as provided for in Article 7 (2) of Council Regulation (EC) No 1216/2009

Annex

(annex to section 5.3). Possible method conditions:

Column	GC-FID method
Permabond FFAP-DF-0.25, 25 m x 0.32 mm	carrier gas : He; temperature injector = 280°C; temperature detector = 250°C; gradient in column oven = 2 min at 50°C, till 80°C at 10°/min, till 220°C at 70°C/min, 220°C for 18.14 min; injection volume: 1µL with 3:1 split
Innowax 30 m x 0.25 mm, film thickness 0.25 µm	carrier gas: He; temperature injector = 240°C; temperature detector = 240°C; injection volume: 1µL with 50 split ratio
StabilWax capillary column, 30 m 0.25 mm ID, 0.25 µm DF	carrier gas: He; temperature injector = 220°C; temperature gradient in column oven: 4 min at 70°C, 15°C/min to 215°C, 20 minutes at 215°C; temperature detector = 230°C; injection volume: 1µL with 1:68.5 split ratio
StabilWax 30 m, 0.25 mm ID	Carrier gas: 95kPa He, temperature injector: 220°C, temperature detector: 230°C; gradient in column oven: 4 minutes at 70°C, from 70°C to 215°C by 15°C/min, 20 minutes at 215°C; injection volume: 1 µL with split 75mL/min (1:68.5)
Varian WCOT fused silica 25 m x 0.25 mm ID coating CP-wax 58 (FFAP) CB DF = 0.2, catalogue number: CP7717	carrier gas : He; temperature injector = 220°C; temperature detector = 240°C; gradient in column oven = 10 min at 40°C, 5 min from 40°C to 250°C and 2 min at 150°C; injection volume: 1µL with 1:50 split
Phenomenex Zebron ZB-waxplus, 30 m 0.32 mm ID, 0.25 µm film thickness	carrier gas : He; temperature injector = 300°C; temperature detector = 330°C; gradient in column oven = 7 min at 50°C, 50°C to 260°C at 45°C/min and 5 min at 260°C; injection volume: 1µL with split 80 mL/min
BPX70, 60 m 0.25 mm ID, 0.25 µm film thickness	Carrier gas : He constant flow rate 1mL/min; injector temperature 240°C; detector temperature 250°C ; column temperature 7min to 80°C, 20°C/min to 160°C,17 min to 160°C, 3°C/min to 200°C, 4min to 200°C
CP Sil 19 CB: 30 m length, internal diameter 0.25 mm; stationary phase film 0.25 µm	carrier gas : He; temperature injector = 180°C; temperature detector = 280°C; gradient in column oven = 15 min at 40°C, 50°C to 210°C at 20°C/min and 42 min at 210°C; injection volume: 1µL with split 1:20
SP 240, 60 m 0.32 mm ID, 0.20 µm film thickness	Carrier gas: He, injector temperature 230°C; detector temperature 230°C; gradient in column oven = 6min at 56^{o} C, step 5^{o} C / min to 200^{o} C and 15 min at 200^{o} C. Injection volume with split 1:10.
DB -23 – 30 m 0.25 mm ID 0.25 µm stationary phase	Carrier gas: He; injector temperature 250°C; detector temperature 280°C; gradient in column 40°C, step 8°C/min, to 120°C-step 12°C/min to 250°C; injection volume 1 µL, split 1:30