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Vegetable fats and oils — Determination of cocoa butter equivalents in milk chocolate

Corps gras d'origine végétale — Détermination des équivalents au beurre de cacao dans le chocolat au lait

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Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
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Foreword

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ISO 11053 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

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Vegetable fats and oils — Determination of cocoa butter equivalents in milk chocolate

1 Scope

This International Standard specifies a procedure for the detection and quantification of cocoa butter equivalents (CBEs) and milk fat (MF) in milk chocolate by triacylglycerol (TAG) profiling using high-resolution capillary gas-liquid chromatography (HR-GLC), and subsequent data evaluation by simple and partial least-squares regression analysis. CBE admixtures can be detected at a minimum level of 0,5 g CBE/100 g milk chocolate and quantified at a level of 5 % mass fraction CBE addition to milk chocolate with a predicted error of 0,7 g CBE/100 g milk chocolate.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
milk fat content of milk chocolate
mass fraction of milk fat in milk chocolate determined by the procedure specified in this International Standard

NOTE The mass fraction is expressed in grams per 100 g of milk chocolate.

2.2
cocoa butter equivalents
non-cocoa vegetable oils and fats detected in milk chocolate in accordance with the procedure prescribed in this International Standard

NOTE The result is expressed qualitatively, i.e. CBEs present/CBEs not present (YES/NO).

2.3
cocoa butter equivalent content of milk chocolate
mass fraction of substances determined by the procedure specified in this International Standard

NOTE The mass fraction is expressed in grams per 100 g of milk chocolate.

3 Principle

Test samples, i.e. chocolate fats obtained from milk chocolate using a rapid fat extraction procedure, are separated by HR-GLC into TAG fractions according to their relative molecular mass and degree of unsaturation. Individual TAG fractions, i.e. 1-palmitoyl-2-stearoyl-3-butyroyl-glycerol (PSB), 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (POS), 1-palmitoyl-2,3-dioleoyl-glycerol (POO), 1,3-distearoyl-2-oleoyl-glycerol (SOS), and 1-stearoyl-2,3-dioleoyl-glycerol (SOO) are used:

a) to calculate the MF content in the chocolate fat (grams of MF per 100 g chocolate fat);

b) to determine the presence/absence of CBEs in chocolate fat using a simple linear regression model based on the three TAGs, POP, POS, and SOS, corrected for the TAG contribution originating from MF, and if this procedure indicates that the sample is not pure cocoa butter (CB);

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c) to quantify the amount of the CBE admixture in chocolate fat (grams of CBE per 100 g chocolate fat) using a partial least-squares (PLS) regression model with six input variables, i.e. the five TAGs, POP, POS, POO, SOS, and SOO, normalized to 100 % and the determined MF content of the chocolate fat.

To ensure the correct labelling of milk chocolate, the results obtained relating to chocolate fat are converted into grams of MF per 100 g chocolate and grams of CBE per 100 g chocolate, necessitating the accurate determination of the total fat content of the chocolate using a Soxhlet extraction procedure (based on AOAC Official Method 963.15[5]). When the detection procedure proves the absence of CBEs in the chocolate fat, the quantification and total fat content are not necessary.

4 Reagents, solutions and standards

NOTE Use only reagents of recognized analytical grade, unless otherwise specified.

WARNING — Attention is drawn to the regulations which specify the handling of dangerous matter. Technical, organizational and personal safety measures should be followed.

4.1 Cocoa butter Certified Reference Material (IRMM-801)[1)] (see Reference [6]), for calibration purposes and system suitability tests.

4.2 Pure milk fat, for system suitability tests.

4.3 1-Palmitoyl-2-stearoyl-3-butyroyl-glycerol (PSB)[2)].

4.3.1 General

For calibration purposes, dissolve ~40 mg of PSB in a 50 ml volumetric flask (5.9) with isooctane resulting in a stock solution of $\sim\rho = 0{,}8$ mg/ml. Mix thoroughly until complete dissolution.

From this PSB stock solution prepare a series of five calibration solutions in matrix (IRMM-801) by weighing on an analytical balance (5.1) IRMM-801 (4.1) into 25 ml volumetric flasks (5.9) and adding the respective volumes of the PSB stock solution as given in Table 1. Make up to the mark with isooctane.

Table 1 — Masses of IRMM-801 and volumes of PSB stock solution for preparation of series of PSB calibration solutions in matrix

Calibration solution	IRMM-801 (4.1) weighed into 25 ml volumetric flask mg	Volume taken from PSB stock solution and added to 25 ml volumetric flask ml	Concentration of PSB in calibration solution $\rho_{PSB}i$ mg/ml	Final IRMM-PSB concentration of solution $\rho_{IRMM\text{-}PSB}$ mg/ml
1	~250	4	0,128	~10
2	~250	3	0,096	~10
3	~250	2	0,064	~10
4	~250	1	0,032	~10
5	~250	0,5	0,016	~10

1) Commercially available from the Institute for Reference Materials and Measurements (http://irmm.jrc.ec.europa.eu/), Belgium. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

2) Commercially available from Larodan (http://www.larodan.se/), Sweden. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

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4.3.2 Cold on-column (OCI) injection

Dilute each calibration solution with isooctane, $\varphi = 1$ ml/5 ml, to obtain a final IRMM-PSB concentration ($\rho_{IRMM\text{-}PSB}$) of ~2 mg/ml in each solution and PSB concentrations (ρ_{PSB}) ranging from 0,025 6 mg/ml (calibration solution 1) to 0,003 2 mg/ml (calibration solution 5).

4.3.3 Split injection (e.g. split ratio of 1:10)

Dilute each calibration solution with isooctane, $\varphi = 1$ ml/2 ml, to obtain a final IRMM-PSB concentration ($\rho_{IRMM\text{-}PSB}$) of ~5 mg/ml in each solution and PSB concentrations (ρ_{PSB}) ranging from 0,064 mg/ml (calibration solution 1) to 0,008 mg/ml (calibration solution 5).

NOTE The final PSB concentrations shall be calculated using the actual mass in the stock standard solution.

4.4 α-Cholestane[3], $\rho = 100$ mg/100 ml, used as internal standard.

Dissolve ~50 mg α-cholestane in 50 ml of isooctane.

- For cold on-column injection: Dilute 1:250 ($\rho = 0{,}004$ mg/ml).
- For split injection (e.g. split ratio of 1:10): Dilute 1:100 ($\rho = 0{,}01$ mg/ml).

4.5 Fat solvent, non-chlorinated solvents (e.g. petroleum ether, *n*-hexane, *n*-heptane, isooctane).

4.6 Hydrochloric acid, $c(HCl) = 4$ mol/l.

5 Apparatus and equipment

5.1 Analytical balance, readable to the nearest 0,1 mg.

5.2 Drying oven. A dry heater block may be used.

5.3 Filter paper, diameter 15 cm [e.g. S&S 589/1[4]].

5.4 Food grater, a kitchen blender with a design featuring the motor above the mixing chamber to avoid melting the samples.

5.5 Rotary evaporator. Alternative evaporation procedures may be used.

5.6 Evaporation block, with nitrogen supply.

5.7 Desiccator, sealable enclosure containing desiccants used for preserving moisture-sensitive items.

5.8 Soxhlet extractor, with standard taper joints, siphon capacity ~100 ml (33 mm × 88 mm extraction thimble), 250 ml Erlenmeyer flask, and regulated heating mantle (or equivalent).

5.9 Volumetric flasks, of capacity 10 ml, 25 ml, 50 ml and 100 ml (or other capacities if needed), ISO 1042[2] class A.

5.10 Pipettes, of capacities ranging from 1 ml to 10 ml (or other capacities if necessary), ISO 648[1] class A or ISO 8655-2[4].

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3) May be obtained from Sigma-Aldrich (http://www.sigmaaldrich.com/), Belgium.

4) S&S 589/1 black ribbon paper is an example of a suitable product commercially available. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

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5.11 Microsyringe, with maximum volume 10 µl, graduated to 0,1 µl, or automatic sample injector.

5.12 Gas chromatograph (GC), fitted with a cold on-column or a split injection system and a flame ionization detector (FID).

NOTE 1 Alternative injection systems [e.g. a programmed-temperature vaporizer (PTV) or a moving-needle injector] may be used provided the same results are obtained as indicated in 10.2.

The separation and quantification have proven to be satisfactory if the following experimental conditions are followed:

GLC column:	CB-TAP 25 m × 0,25 mm i.d., fused silica coated with a medium polar thermostable phenylmethylpolysiloxane stationary phase with a film thickness of 0,10 µm
Oven programme for OCI:	100 °C held for at least 2 min; 30 °C/min to 270 °C held for 1 min; 2,5 °C/min to 340 °C held for 7 min
Oven programme for split:	200 °C held for at least 1 min; 14 °C/min to 270 °C held for 1 min; 2,5 °C/min to 340 °C held for 10 min
Detector (FID):	360 °C
Carrier gas for OCI:	H_2 (purity ⩾ 99,999 %) with a constant flow rate of 3,5 ml/min (another suitable carrier gas is helium)
Carrier gas for split:	H_2 (purity ⩾ 99,999 %) with a constant flow rate of 2,5 ml/min (another suitable carrier gas is helium)

NOTE 2 Columns and alternative experimental conditions, used in an international collaborative study (see Reference [7]), are listed in Table A 1. Operating conditions may be changed to obtain optimum separation.

5.13 Chromatographic data system.

6 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 5555[3]. A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

7 Sample preparation

7.1 Preparation of IRMM-801 for calibration purposes and system suitability tests

Before opening and using the IRMM-801 (4.1), warm the ampoule in an oven (5.2) until the contents have melted. When a clear solution is obtained, mix the contents by repeated inversion for not less than 20 s. Then open and transfer the contents to a clean vial, which can be tightly sealed and preserved in a cool place for future usage.

7.2 Preparation of pure milk fat for system suitability tests

If no pure MF is available, it can be obtained from a butter sample by melting and passing the fat layer through a folded filter paper (5.3) at 50 °C in an oven (5.2).

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7.3 Preparation of chocolate sample

7.3.1 General

Chill approximately 200 g of chocolate until hard, and grate to a fine granular condition using a food grater (5.4). Mix thoroughly and preserve in a tightly stoppered bottle in a cool place.

7.3.2 Rapid fat extraction

The chocolate fat is separated from 5 g grated chocolate (7.3.1) by extracting with two to three 10 ml portions of a suitable fat solvent (4.5). Centrifuge and decant. Combine the extracts and evaporate (5.5) most of the fat solvent and finally dry it under a stream of nitrogen (5.6).

The chocolate fat obtained by rapid fat extraction is used for the final TAG analysis by HR-GLC. For the detection of CBEs in chocolate, the accurate amount of total fat in chocolate is not needed. When no CBEs are detected, the second part of the standard, i.e. quantification of CBEs around the statutory limit of 5 %, is not necessary. When CBEs are detected, the quantification part should be performed using the same TAG profile as used for the CBE detection. However, in this case, determine the accurate amount of total fat in chocolate using the procedure in 7.3.3. Alternative extraction procedures may be used provided that the same results are obtained.

7.3.3 Determination of total fat content

Separate the chocolate fat and determine the total fat content in a sample of milk chocolate (prepared as described in 7.3.2) by Soxhlet extraction (based on AOAC Official Method 963.15 [5]), as follows.

Weigh (5.1) 4 g to 5 g of chocolate into a 300 ml to 500 ml beaker. Add slowly, while stirring, 45 ml of boiling water to obtain a homogeneous suspension. Add 55 ml of HCl (4.6) and a few defatted boiling chips, or other antibumping agents, and stir. Cover with a watch glass, bring the solution slowly to the boil, and simmer for 15 min. Rinse the watch glass with 100 ml of water. Filter the solution through a medium fluted filter paper (5.3), or equivalent, rinsing the beaker three times with water. Continue washing until the last portion of filtrate is chlorine-free. Transfer the filter with the sample to a defatted extraction thimble and dry for 2 h in a small beaker at 100 °C. Place a glass wool plug over the filter paper. Add a few defatted antibumping chips to a 250 ml Erlenmeyer flask and dry for 1 h at 100 °C. Cool the flask to room temperature in a desiccator (5.7) then weigh (5.1) it. Place the thimble containing the dried sample in the Soxhlet apparatus (5.8), supporting it with a spiral or glass beads. Rinse the digestion beaker, drying beaker and watch glass with three 50 ml portions of petroleum ether, and add the washings to the thimble. Reflux the digested sample for 4 h, adjusting the heat so that the extractor siphons more than 30 times. Remove the flask and evaporate the solvent. Dry the flask at 102 °C to constant mass (1,5 h). Cool in the desiccator (5.7) to room temperature, then weigh (5.1). Constant mass is attained when successive 1 h drying periods show additional loss of $< 0,05$ % fat. Duplicate determinations should agree to within 0,1 % fat.

The mass fraction, expressed as a percentage, of total fat in the chocolate, $w_{\text{fat; choc}}$, is given by:

$$w_{\text{fat; choc}} = \frac{m_{\text{fat}} \times 100}{m} \quad (1)$$

where

m is the mass, in grams, of chocolate taken;

m_{fat} is the mass, in grams, of the total fat obtained from the chocolate by Soxhlet extraction (based on AOAC Official Method 963.15 [5]).

Alternative extraction procedures may be used (e.g. by accelerated solvent extraction, by supercritical carbon dioxide or by using microwaves) provided that the same results are obtained. The chocolate fat obtained by Soxhlet extraction should not be used for TAG analysis by HR-GLC since changes in the obtained TAG profile could be observed in some cases.

Report the result to two decimal places.

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8 Procedure

8.1 Construction of calibration curve for determination of PSB content

Five calibration solutions containing different concentrations of PSB (4.3) but always the same concentration of α-cholestane (4.4) are prepared as follows.

For cold on-column (OCI) injection:

— **Calibration solution 1** (Final $\rho_{PSB\ 1}$ = 0,012 8 mg/ml; $\rho_{\alpha\text{-cholestane}\ 1}$ = 0,002 mg/ml): Transfer 1 ml of calibration solution 1 ($\rho_{PSB\ 1}$ = 0,025 6 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,004 mg/ml; 4.4).

— **Calibration solution 2** (Final $\rho_{PSB\ 2}$ = 0,009 6 mg/ml; $\rho_{\alpha\text{-cholestane}\ 2}$ = 0,002 mg/ml): Transfer 1 ml of calibration solution 2 ($\rho_{PSB\ 2}$ = 0,019 2 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,004 mg/ml; 4.4).

— **Calibration solution 3** (Final $\rho_{PSB\ 3}$ = 0,006 4 mg/ml; $\rho_{\alpha\text{-cholestane}\ 3}$ = 0,002 mg/ml): Transfer 1 ml of calibration solution 3 ($\rho_{PSB\ 3}$ = 0,012 8 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,004 mg/ml; 4.4).

— **Calibration solution 4** (Final $\rho_{PSB\ 4}$ = 0,003 2 mg/ml; $\rho_{\alpha\text{-cholestane}\ 4}$ = 0,002 mg/ml): Transfer 1 ml of calibration solution 4 ($\rho_{PSB\ 4}$ = 0,006 4 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,004 mg/ml; 4.4).

— **Calibration solution 5** (Final $\rho_{PSB\ 5}$ = 0,001 6 mg/ml; $\rho_{\alpha\text{-cholestane}\ 5}$ = 0,002 mg/ml): Transfer 1 ml of calibration solution 5 ($\rho_{PSB\ 5}$ = 0,003 2 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,004 mg/ml; 4.4).

Inject 0,5 µl of each calibration solution into the HR-GLC system using the cold on-column injection system.

For split injection:

— **Calibration solution 1** (Final $\rho_{PSB\ 1}$ = 0,032 mg/ml; $\rho_{\alpha\text{-cholestane}\ 1}$ = 0,005 mg/ml): Transfer 1 ml of calibration solution 1 ($\rho_{PSB\ 1}$ = 0,064 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,01 mg/ml; 4.4).

— **Calibration solution 2** (Final $\rho_{PSB\ 2}$ = 0,024 mg/ml; $\rho_{\alpha\text{-cholestane}\ 2}$ = 0,005 mg/ml): Transfer 1 ml of calibration solution 2 ($\rho_{PSB\ 2}$ = 0,048 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,01 mg/ml; 4.4).

— **Calibration solution 3** (Final $\rho_{PSB\ 3}$ = 0,016 mg/ml; $\rho_{\alpha\text{-cholestane}\ 3}$ = 0,005 mg/ml): Transfer 1 ml of calibration solution 3 ($\rho_{PSB\ 3}$ = 0,032 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,01 mg/ml; 4.4).

— **Calibration solution 4** (Final $\rho_{PSB\ 4}$ = 0,008 mg/ml; $\rho_{\alpha\text{-cholestane}\ 4}$ = 0,005 mg/ml): Transfer 1 ml of calibration solution 4 ($\rho_{PSB\ 4}$ = 0,016 mg/ml; 4.3) in a test tube and add 1 ml of α-cholestane solution (ρ = 0,01 mg/ml; 4.4).

— **Calibration solution 5** (Final $\rho_{PSB\ 5}$ = 0,004 mg/ml; $\rho_{\alpha\text{-cholestane}\ 5}$ = 0,005 mg/ml): Transfer 1 ml of calibration solution 5 ($\rho_{PSB\ 5}$ = 0,008 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,01 mg/ml; 4.4).

Inject 1 µl of the final test solution into the HR-GLC system using the split injection system.

Alternative sample amounts and injectors may be used provided that the detection system employed gives a linear response and conforms to the system suitability criteria (10.2).

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8.2 Separation of individual TAGs of IRMM-801 by HR-GLC

The IRMM-801 (4.1) shall be warmed in a drying oven (5.2) until completely melted. Pipettes (or similar equipment) used for transferring the sample during weighing operations should be brought to a temperature of ~55 °C in a drying oven to avoid partial fat fractionation during handling of samples.

For cold on-column (OCI) injection: Weigh (5.1) ~0,1 g of IRMM-801 (4.1) in a 10 ml volumetric flask (5.9) and dilute to the mark with isooctane (4.5). Pipette (5.10) 1 ml of the resulting solution into another 50 ml volumetric flask (5.9) and dilute to the mark with the same solvent (ρ = 0,2 mg/ml). Inject 0,5 µl of the final test solution into the HR-GLC system using the cold on-column injection system.

For split injection: Weigh (5.1) ~0,1 g of IRMM-801 (4.1) in a 10 ml volumetric flask (5.9) and dilute to the mark with isooctane (4.5). Pipette (5.10) 1 ml of the resulting solution into another 10 ml volumetric flask and dilute to the mark with the same solvent (ρ = 1 mg/ml). Inject 1 µl of the final test solution into the HR-GLC system using the split injection system.

Alternative fat solvents, sample amounts and injectors may be used provided that the detection system employed gives a linear response and conforms to the system suitability criteria (10.2).

8.3 Separation of individual TAGs of pure MF by HR-GLC

For cold on-column injection (OCI): Weigh (5.1) ~0,05 g of pure MF (4.2) in a 50 ml volumetric flask (5.9) and dilute to the mark with isooctane (4.5) (ρ = 1 mg/ml). Transfer 1 ml of this solution to a test tube and add 1 ml of α-cholestane solution (4.4) (resulting test solution ρ = 0,5 mg/ml). Inject 0,5 µl of the final test solution into the HR-GLC system using the cold on-column injection system.

For split injection: Weigh (5.1) ~0,25 g of pure MF (4.2) in a 50 ml volumetric flask (5.9) and dilute to the mark with isooctane (4.5) (ρ = 5 mg/ml). Transfer 1 ml of this solution to a test tube and add 1 ml of α-cholestane solution (4.4) (resulting test solution ρ = 2,5 mg/ml). Inject 1 µl of the final test solution into the HR-GLC system using the split injection system.

Alternative fat solvents, sample amounts and injectors may be used provided that the detection system employed gives a linear response and conforms to the system suitability criteria (10.2).

8.4 Separation of individual TAGs of chocolate fat by HR-GLC

The test sample [chocolate fat extracted from milk chocolate by rapid fat extraction (7.3.2)] shall be warmed in a drying oven (5.2) until completely melted. If the liquid sample contains some sediment, filter the sample inside the oven to obtain a clear filtrate. Pipettes (or similar equipment) used for transferring the sample during weighing operations should be brought to a temperature of ~55 °C in a drying oven in order to avoid partial fat fractionation during handling of samples.

For cold on-column (OCI) injection: Weigh (5.1) ~0,1 g of chocolate fat (as obtained in 7.3.2) on an analytical balance (5.1) in a 100 ml volumetric flask (5.9) and dilute to the mark with isooctane (4.5) (ρ = 1 mg/ml). Transfer 1 ml of this solution to a test tube and add 1 ml of α-cholestane solution (4.4) (resulting test solution ρ = 0,5 mg/ml). Inject 0,5 µl of the final test solution into the HR-GLC system using the cold on-column injection system.

For split injection: Weigh (5.1) ~0,5 g of chocolate fat (as obtained in 7.3.2) on an analytical balance (5.1) in a 100 ml volumetric flask (5.9) and dilute to the mark with isooctane (4.5) (ρ = 5 mg/ml). Transfer 1 ml of this solution to a test tube and add 1 ml of α-cholestane solution (4.4) (resulting test solution ρ = 2,5 mg/ml). Inject 1 µl of the final test solution into the HR-GLC system using the split injection system.

Alternative fat solvents, sample amounts and injectors may be used provided that the detection system employed gives a linear response and conforms to the system suitability criteria (10.2)

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8.5 Identification

Identification of 1-palmitoyl-2-stearoyl-3-butyroyl-glycerol (PSB) and α-cholestane is made by comparison of the retention times of the test sample with those of the reference standards. Identification of the five major TAG fractions 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (POS), 1-palmitoyl-2,3-dioleoyl-glycerol (POO), 1,3-distearoyl-2-oleoyl-glycerol (SOS) and 1-stearoyl-2,3-dioleoyl-glycerol (SOO) is made by comparison of the retention times of the test sample with those of the IRMM-801 (8.2).

In general, TAGs appear in order of increasing number of carbon atoms and of increasing unsaturation for the same number of carbon atoms. The elution order of the TAGs of IRMM-801 (8.2) is given in Figure B.1. The elution order of the TAGs of an average pure MF (8.3) is given in Figure B.2.

9 Calculation

9.1 PSB and MF quantification in chocolate fat and chocolate

9.1.1 Determination of PSB response factor

Determine the response factor of PSB by injection of the five calibration solutions (8.1) using experimental conditions identical to those used for the test sample. For each calibration solution i, calculate a detector response factor for PSB, $F_{\mathrm{PSB};\,i}$, by:

$$F_{\mathrm{PSB};\,i} = \frac{\rho_{\mathrm{PSB};\,i}\ A_{\mathrm{cholestane};\,i}}{\rho_{\mathrm{cholestane};\,i}\ A_{\mathrm{PSB};\,i}} \qquad (2)$$

where

$A_{\mathrm{PSB};\,i}$ is the peak area of PSB in calibration solution i (8.1);

$A_{\mathrm{cholestane};\,i}$ is the peak area of the internal standard α-cholestane in calibration solution i (8.1);

$\rho_{\mathrm{PSB};\,i}$ is the concentration, in milligrams per millilitre, of PSB in calibration solution i (8.1);

$\rho_{\mathrm{cholestane};\,i}$ is the concentration, in milligrams per millilitre, of the internal standard α-cholestane in calibration solution i (8.1).

An average response factor for PSB, $\overline{F}_{\mathrm{PSB}}$, obtained from the five calibration solutions, shall be calculated and used for further calculations.

9.1.2 PSB quantification in chocolate fat

The mass fraction, expressed as a percentage, of PSB in the test sample (chocolate fat), $w_{\mathrm{PSB;\,choc\,fat}}$, is given by:

$$w_{\mathrm{PSB;\,choc\,fat}} = \frac{A_{\mathrm{PSB}}\ \rho_{\mathrm{cholestane}}\ \overline{F}_{\mathrm{PSB}} \times 100}{A_{\mathrm{cholestane}}\ \rho_{\mathrm{sample}}} \qquad (3)$$

where

A_{PSB} is the peak area of PSB in the test sample (8.4);

$A_{\mathrm{cholestane}}$ is the peak area of the internal standard α-cholestane in the test sample (8.4);

$\overline{F}_{\mathrm{PSB}}$ is the average response factor for PSB (9.1.1);

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$\rho_{\text{cholestane}}$ is the concentration, in milligrams per millilitre, of the internal standard α-cholestane in the test sample (8.4);

ρ_{sample} is the concentration, in milligrams per millilitre, of the test sample (8.4).

Report the results to two decimal places.

9.1.3 MF quantification in chocolate fat

The mass fraction of MF in the chocolate fat, $w_{\text{MF; choc fat}}$, expressed as a percentage, is given by:

$$w_{\text{MF; choc fat}} = 0{,}19 + 44{,}04\ w_{\text{PSB; choc fat}} \tag{4}$$

where $w_{\text{PSB; choc fat}}$ is the mass fraction of PSB in chocolate fat, expressed as a percentage [see Equation (3)].

NOTE The calibration function was established using data from an extensively tested database holding information on the TAG profile of more than 900 gravimetrically prepared CB-MF and CB-CBE-MF mixtures with known MF contents, simulating the composition of real chocolate fats (see Reference [8]).

Report the results to two decimal places.

9.1.4 MF quantification in chocolate

The mass fraction of MF in chocolate, $w_{\text{MF; choc}}$, expressed as a percentage, is given by:

$$w_{\text{MF; choc}} = \frac{w_{\text{fat; choc}}\ w_{\text{MF; choc fat}}}{100} \tag{5}$$

where

$w_{\text{fat; choc}}$ is the mass fraction of total fat in chocolate, expressed as a percentage [see Equation (1)];

$w_{\text{MF; choc fat}}$ is the mass fraction of MF in chocolate fat, expressed as a percentage [see Equation (4)].

9.2 CBE detection in chocolate fat

9.2.1 Determination of response factors for POP, POS, and SOS

Determine the detector response factor of each TAG i, i.e. POP, POS, and SOS, respectively, in IRMM-801, F_i, by injection of the IRMM-801 (8.2) solution using experimental conditions identical to those used for the test sample. Calculate F_i by:

$$P_{i;\text{ ref}} = \frac{A_{i;\text{ ref}}}{\Sigma A_{\text{all TAGs; ref}}} \times 100 \tag{6}$$

$$F_i = \frac{w_{i;\text{ ref}}}{P_{i;\text{ ref}}} \tag{7}$$

where

$A_{i;\text{ ref}}$ is the peak area of each TAG i, i.e. POP, POS, and SOS, respectively, in IRMM-801 (8.2);

$\Sigma A_{\text{all TAGs; ref}}$ is the sum of the peak areas attributed to all TAGs in IRMM-801 (8.2);

$P_{i;\text{ ref}}$ is the percentage of each TAG i, i.e. POP, POS, and SOS, respectively, in IRMM-801 (8.2) [see Equation (6)];

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$w_{i;\ \text{ref}}$ is the mass fraction, expressed as a percentage, of each TAG i, i.e. POP, POS, and SOS, respectively, in IRMM-801 (8.2) as given in the certificate (POP = 16,00 %, POS = 39,40 %, SOS = 27,90 %) (see Reference [6]).

Report the results to two decimal places.

9.2.2 Calculation of percentage mass fractions of POP, POS, and SOS in chocolate fat

Calculate the mass fraction, expressed as a percentage, of each TAG i, i.e. POP, POS, and SOS, respectively, $w_{i;\ \text{total}}$, in the test sample with respect to all TAGs present in the test sample by:

$$w_{i;\,\text{total}} = \frac{F_i\ A_i}{\Sigma A_{\text{all TAGs}}} \times 100 \tag{8}$$

where

A_i is the peak area corresponding to each TAG i, i.e. POP, POS, and SOS, respectively, in the test sample (8.4);

$\Sigma A_{\text{all TAGs}}$ is the sum of the peak areas attributed to all TAGs in the test sample (8.4);

F_i is the response factor for each TAG i, i.e. POP, POS, and SOS, respectively [see Equation (7)].

Report the results to two decimal places.

9.2.3 Correction for MF contribution

Calculate the contribution of the percentage mass fraction of each TAG i, i.e. POP, POS, and SOS, respectively, originating from MF, $w_{i;\ \text{MF}}$, by:

$$w_{i;\,\text{MF}} = \frac{w_{\text{MF; choc}}\ \overline{w}_{i;\,\text{ref MF}}}{100} \tag{9}$$

where

$\overline{w}_{i;\,\text{ref MF}}$ is the average percentage mass fraction of each TAG i in an MF, i.e. POP = 3,99 %, POS = 2,19 %, SOS = 0,45 % {values obtained from database (see Reference [9])};

$w_{\text{MF; choc}}$ is the percentage mass fraction of MF in the test sample [see Equation (4)].

Subtract the percentage mass fractions of the three TAGs originating from MF [Equation (9)] obtained from the percentage mass fractions of the three TAGs obtained for the test sample [Equation (8)].

$$w_{i;\,\text{corr}} = w_{i;\,\text{total}} - w_{i;\,\text{MF}} \tag{10}$$

Normalize the percentage mass fractions of the three TAGs [Equation (10)] obtained to 100 % [Equation (11)]:

$$w_{\text{POP; corr}} + w_{\text{POS; corr}} + w_{\text{SOS; corr}} = 100 \tag{11}$$

Report the results to two decimal places.

9.2.4 Decision whether chocolate fat is pure cocoa butter

In principle, the presence of CBEs in CB is detected by linear regression analysis applied to the relative percentage mass fractions of the three TAG components, i.e. POP, POS, and SOS. The variability of the TAG

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composition of CB is expressed by Equation (12) using the normalized TAG percentage mass fractions of POP, POS, and SOS, i.e. $w_{POP} + w_{POS} + w_{SOS} = 100$ (see References [10], [11]).

$$w_{POP} = 43{,}73 - 0{,}73 w_{SOS} \quad (s_{residual} = 0{,}125) \tag{12}$$

where $s_{residual}$ is the standard deviation of the residual.

The principle of the method is that, for pure CB samples, w_{POS} is practically constant for wide variations of w_{POP} and w_{SOS}, resulting in a linear relationship [the so-called "CB-line" represented by Equation (12)] between w_{POP} and w_{SOS}. CBE and other fat admixtures cause the TAG analysis to deviate from the "CB-line" to the extent that their w_{POS} value differs from the w_{POS} value of cocoa butter. Equation (12) was established by using a standardized database of the TAG profile of 74 individual genuine CBs evaluated (see Reference [10]). The IRMM-801 (4.1) was used to standardize the applied analytical methodology for the determination of the TAG profile of the CBs. For 99 % of all analyses, pure CB complies with:

$$w_{POP} < 44{,}03 - 0{,}73 w_{SOS} \tag{13}$$

A greater value of w_{POP}, as given by Equation (13), means that the sample is not pure CB. In the case of milk chocolate, use TAG percentage mass fractions corrected for the contribution of percentage mass fractions of the TAGs, POP, POS, and SOS, originating from MF [as determined in Equation (10)]. After normalizing the percentage mass fractions of the MF-corrected TAGs obtained to 100 % [Equation (11)], the same decision rules as for CB or dark chocolate [Equation (13)], to detect whether there are any CBEs present in the milk chocolate fat, can be applied.

$$w_{POP;\,corr} < 44{,}03 - 0{,}73 w_{SOS;\,corr} \tag{14}$$

A greater value of $w_{POP;\,corr}$ as given by Equation (14), means that the sample is not pure CB.

NOTE The advantage of the elaborated approach is that by using IRMM-801 (4.1) for calibration, the mathematical expression can be used by individual testing laboratories to verify the purity of CB, without tackling the problem of establishing a "CB-line" as a prerequisite. Calibration by IRMM-801 (4.1) automatically links the results obtained in a laboratory to the cocoa butter TAG database and the elaborated decision rule [Equation (13)].

9.3 CBE quantification in chocolate fat and chocolate

9.3.1 Determination of response factors for POP, POS, POO, SOS, and SOO

Determine the detector response factor of each TAG j, i.e. POP, POS, POO, SOS, and SOO, in IRMM-801, F_j, by injection of IRMM-801 solution (8.2) using experimental conditions identical to those used for the samples. Calculate F_j by:

$$P_{j;\,ref} = \frac{A_{j;\,ref}}{\Sigma A_{j;\,ref}} \times 100 \tag{15}$$

$$F_j = \frac{w_{j;\,ref}}{P_{j;\,ref}} \tag{16}$$

where

$A_{j;\,ref}$ is the peak area of each TAG j, i.e. POP, POS, POO, SOS, and SOO, respectively, in IRMM-801 (8.2);

$\Sigma A_{j;\,ref}$ is the sum of the peak areas attributed to all TAGs j, i.e. POP, POS, POO, SOS, and SOO, in IRMM-801 (8.2);

$P_{j;\,ref}$ is the percentage of each TAG j, i.e. POP, POS, POO, SOS, and SOO, in IRMM-801 (8.2);

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$w_{j;\,\mathrm{ref}}$ is the mass fraction, expressed as a percentage, of each TAG j, i.e. POP, POS, POO, SOS, and SOO, in IRMM-801 (8.2) as given in the certificate (POP = 18,14 %, POS = 44,68 %, POO = 2,26 %, SOS = 31,63 % and SOO = 3,29 %, i.e. normalized to 100 %) (see Reference [6]).

Report the results to two decimal places.

9.3.2 Calculation of percentage mass fractions of POP, POS, POO, SOS, and SOO in chocolate fat

Calculate the percentage mass fraction of each TAG j, i.e. POP, POS, POO, SOS, and SOO, respectively, in the test sample, $w_{j;\,\mathrm{choc\ fat}}$, by

$$w_{j;\,\mathrm{choc\ fat}} = \frac{F_j\ A_j}{\sum\left(F_j\ A_j\right)} \times 100 \qquad (17)$$

where

F_j is the response factor of each TAG j, i.e. POP, POS, POO, SOS, and SOO, respectively [see Equation (16)];

A_j is the peak area corresponding to each TAG j, i.e. POP, POS, POO, SOS, and SOO, respectively, in the test sample (8.4).

Report the results to two decimal places.

9.3.3 CBE quantification in chocolate fat

The mass fraction, expressed as a percentage, of CBE in the chocolate fat, $w_{\mathrm{CBE;choc\ fat}}$, is calculated using Equation (18), which was derived by PLS regression analysis of the relative proportions of the five main TAGs, i.e. $w_{\mathrm{POP;\,choc\ fat}}$, $w_{\mathrm{POS;\,choc\ fat}}$, $w_{\mathrm{POO;\,choc\ fat}}$, $w_{\mathrm{SOS;\,choc\ fat}}$ and $w_{\mathrm{SOO;\,choc\ fat}}$ as determined by Equation (17), and the MF content of the chocolate fat, i.e. $w_{\mathrm{MF;\,choc\ fat}}$ as determined by Equation (4).

$$w_{\mathrm{CBE;\,choc\ fat}} = -4{,}24 - 0{,}23\ w_{\mathrm{MF;\,choc\ fat}} + 1{,}52\ w_{\mathrm{POP;\,choc\ fat}} - 1{,}47\ w_{\mathrm{POS;\,choc\ fat}} + 1{,}09\ w_{\mathrm{POO;\,choc\ fat}} + 1{,}29\ w_{\mathrm{SOS;\,choc\ fat}} + 0{,}26\ w_{\mathrm{SOO;\,choc\ fat}} \qquad (18)$$

Report the result to one decimal place.

NOTE The quantification model was established using data from an extensively tested database holding information on the TAG profile of more than 700 gravimetrically prepared CB-CBE-MF blends varying in type and amount of CB, CBE and MF (see Reference [8]).

9.3.4 CBE quantification in chocolate

The mass fraction, expressed as a percentage, of CBE in chocolate, $w_{\mathrm{CBE;\,choc}}$, is calculated by Equation (19):

$$w_{\mathrm{CBE;\,choc}} = \frac{w_{\mathrm{fat;\,choc}}\ w_{\mathrm{CBE;\,choc\ fat}}}{100} \qquad (19)$$

where

$w_{\mathrm{fat;\,choc}}$ is the mass fraction, expressed as a percentage, of total fat in chocolate [see Equation (1)];

$w_{\mathrm{CBE;\,choc\ fat}}$ is the mass fraction, expressed as a percentage, of CBE in chocolate fat [see Equation (18)].

Report the result to one decimal place.

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10 Procedural requirements

10.1 General considerations

The details of the chromatographic procedure depend, among other factors, on equipment, type, age, and supplier of the column, means of injection of the test solution, sample size and detector. Different column lengths and brands may be used, and injection volumes may be varied, if the system conforms to the requirements of 10.2.

10.2 System suitability

10.2.1 Resolution

— The HR-GLC separation system shall be capable of separating the critical pairs POS/POO and SOS/SOO with a chromatographic resolution of at least 1,0. This requirement can be proven by using IRMM-801 (8.2) as shown in Figure B.1.

— The HR-GLC separation system shall be capable of separating PSB from neighbouring peaks within CN 38 group. This requirement can be proven by using a pure MF sample (8.3) as shown in Figures B.2 and B.3.

— The HR-GLC separation system shall be capable of showing no co-elution for the internal standard α–cholestane. This requirement can be proven by using a MF sample (8.3) using α-cholestane as internal standard as shown in Figure B.4.

In the case of failure, optimize the chromatographic conditions (e.g. sample size, column temperature, carrier gas flow, etc.) for greatest resolution.

10.2.2 Determination of detector response factors

— To check the assumption that flame-ionization detector response factors of TAGs do not differ by more than 20 % from unity, IRMM-801 (8.2) shall be analysed applying standard HR-GLC conditions. Experience has shown that for a properly functioning chromatographic system the response factors for the five TAGs (POP, POS, POO, SOS, SOO) vary within a range of 0,80 to 1,20. Verify the stability of the system by repeating the analysis (at least in triplicate). The coefficients of variation of the determined detector response factors obtained shall be less than 5 %.

— To check the stability of the separation system, a calibration curve for PSB with α-cholestane as internal standard shall be established (at least duplicate injections of each calibration solution). Calculate the average detector response factor for PSB. RF values obtained on individual calibration solutions shall not deviate by more than 5 % from the average value.

In the case of failure, optimize the chromatographic conditions (e.g. sample size, column temperature, and carrier gas flow).

11 Precision

11.1 Interlaboratory test

Details of the collaborative trial of the method are listed in Table A.1. The values derived from this interlaboratory study test may not be applicable to concentration ranges and matrices other than those specified.

11.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, shall in not more than 5 % of cases be greater than 0,3 g/100 g milk chocolate.

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Values for the repeatability limit, r, as found in the validation study are summarized in Table A.3.

11.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, shall in not more than 5 % of cases be greater than 0,6 g/100 g milk chocolate.

Values for the reproducibility limit, R, as found in the validation study are summarized in Table A.3.

12 Test report

The test report shall contain at least the following information:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this International Standard;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result(s) obtained or, if the repeatability has been checked, the final quoted result obtained.

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Annex A
(informative)

Results of interlaboratory test

The method was validated in 2006 in a European interlaboratory test with 12 participants (see Reference [7]). Method details as applied by the individual laboratories are given in Table A.1. Six tailor-made chocolate samples and seven chocolate fat samples varying in composition and levels of CBE were tested in the study (Table A.2). Precision data are summarized in Table A.3.

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Table A.1 — Suitable HR-GLC conditions to be used for TAG analysis of chocolate fats

Laboratory code	1	2	3	4	5	6	7	8	9	10	11	12
Carrier gas												
– type	He	He	He	H_2	H_2	H_2	H_2	He	H_2	H_2	H_2	H_2
– if constant pressure, kPa	100	180	—	—	—	—	150	135	130	140	—	—
– if constant flow, ml/min	—	—	2,2	2	2	1,5	—	—	—	—	3,5	2
Column characteristics												
– stationary phase	Ultimetal	CB-TAP	CB-TAP	CB-TAP	CB-TAP	CB-TAP	CB-TAP	CB-TAP	RTx-65TG	CB-TAP	CB-TAP	CB-TAP
– length, m	25	25	25	25	25	25	25	25	30	25	25	25
– internal diameter, mm	0,25	0,25	0,25	0,25	0,25	0,25	0,25	0,25	0,25	0,25	0,25	0,25
– film thickness, µm	0,05	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1
Temperature mode												
– Oven												
– injection temperature, °C – hold time, min	200 2	200 1	100 0,5	200 1	200 0	200 1	100 2	200 1	200 1	200 1	100 2	200 1
– programme rate 1, °C/min	20	14	40	14	20	24	30	14	15	30	30	14
– temperature, °C – hold time, min	320 0	270 0	280 1	270 0	270 0	270 0	270 1	270 0	360 0	270 0	270 1	270 0
– programme rate 2, °C/min	1	2,5	2,5	2,5	5	2,5	3	2	1	2,5	3,5	2,5
– temperature, °C – hold time, min	360 10	340 30	340 17	340 10	340 15	340 13	340 10	340 30	370 0	355 2	340 7	340 10
– programme rate 3, °C/min	—	10	—	—	25	—	—	—	—	—	—	—
– final temperature, °C – hold time, min	—	350 9	—	—	200	—	—	—	—	—	—	—
– Injector temperature, °C	65 to 370	360	oven track	365	370	350		340	380	140 to 340	oven track	360
– Detector temperature, °C	370	360	360	365	360	370	350	360	380	350	360	360
Injection mode												
– manual (M)/automatic (A)	M	A	—	A	A	A	M	A	A	A	A	A
– split/on-column/PTV	PTV	split	OCI	split	split	split	OCI	split	split	PTV	OCI	split
– if split [split ratio]	—	1:20	—	1:10	1:10	1:10	—	1:7	—	—	—	1:10

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Table A.2 — Composition of samples used in the interlaboratory study to test the method

Sample	Chocolate samples [a]	CBE type	CB %	CBE %	MF %
1	Milk chocolate, FCMP, no CBE	—	29,67	0,00	unknown
2	Milk chocolate, FCMP, CBE addition low level	50 % PMF + 50 % SOS rich fat	29,22	0,45	unknown
3	Milk chocolate, SKMP + MF, no CBE	—	25,70	0,00	unknown
4	Milk chocolate, SKMP + MF, CBE addition low level	50 % PMF + 50 % SOS rich fat	23,67	2,03	unknown
5	Milk chocolate, crumb + MF + FCMP + SKMP + WP, CBE addition at statutory level	50 % PMF + 50 % SOS rich fat	14,60	5,11	unknown
6	White chocolate, CBE addition at statutory level	50 % PMF + 50 % SOS rich fat	23,50	3,95	unknown
	Chocolate fat solutions	**CBE type**	**CB** %	**CBE** %	**MF** %
7	West African CB, no CBE	—	100,00	0,00	0,00
8	West African CB + mixture of 310 MF samples, no CBE	—	85,01	0,00	14,99
9	West African CB + mixture of 310 MF samples, CBE addition low level	70 % PMF + 30 % SOS rich fat	83,03	2,00	14,98
10	West African CB + mixture of 310 MF samples, CBE addition at statutory level	70 % PMF + 30 % SOS rich fat	68,95	16,03	15,02
11	West African CB + mixture of 310 MF samples, CBE addition at statutory level	70 % PMF + 30 % SOS rich fat	64,99	19,98	15,04
12	West African CB + mixture of 310 MF samples, CBE addition at statutory level	100 % soft PMF	64,94	20,08	14,99
13	West African CB + mixture of 310 MF samples, CBE addition at statutory level	70 % PMF + 30 % SOS rich fat	56,91	28,04	15,05

[a] Samples were made in test conches of 40 kg. FCMP = full cream milk powder; SKMP = skimmed milk powder; WP = whey powder; PMF = palm mid fraction.

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Table A.3 — Precision data for chocolate samples (samples 5 and 6) and for chocolate fat samples (samples 10 to 13)

Sample	5	6	10	11	12	13
Number of laboratories	12	12	12	12	12	12
Number of outliers	0	1	0	0	0	0
Number of accepted laboratories	12	11	12	12	12	12
Mean value, g CBE/100 g chocolate	5,20	4,08	4,62	5,81	5,35	8,23
True value, g CBE/100 g chocolate	5,11	3,95	4,81	5,99	6,02	8,41
Repeatability limit, r [$r = 2{,}8 \times s_r$], g/100 g	0,276	0,150	0,261	0,188	0,186	0,231
Repeatability standard deviation, s_r, g/100 g	0,098	0,054	0,093	0,067	0,067	0,082
Coefficient of variation of repeatability, CV(r), %	1,893	1,315	2,020	1,153	1,244	1,001
Reproducibility limit, R [$R = 2{,}8 \times s_R$], g/100 g	0,590	0,534	0,497	0,463	0,527	0,518
Reproducibility standard deviation, s_R, g/100 g	0,211	0,191	0,177	0,165	0,188	0,185
Coefficient of variation of reproducibility, CV(R), %	4,053	4,678	3,843	2,847	3,518	2,248

NOTE Chocolate samples 1 to 4 and chocolate fat samples 7 to 9 presented samples with low CBE additions to be used to test the detection approach. The efficiency of the detection approach (percentage of correctly classified samples) was 100 %.

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Annex B
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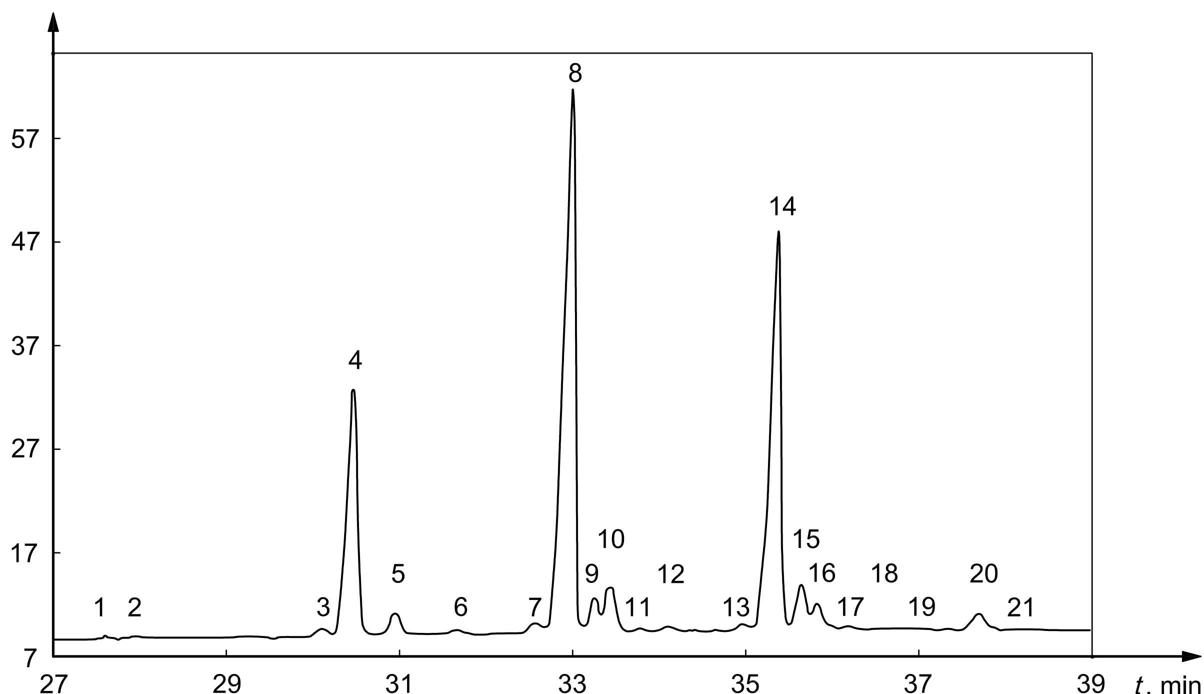
Example chromatograms



Key

t	retention time				
1	PPP	tripalmitin	12		unidentified
2	MOP	1-myristoyl-2-oleoyl-3-palmitoyl-glycerol	13	SSS	tristearin
3	PPS	1,2-dipalmitoyl-3-stearoyl-glycerol	14	SOS	1,3-distearoyl-2-oleoyl-glycerol
4	POP	1,3-dipalmitoyl-2-oleoyl-glycerol	15	SOO	1-stearoyl-2,3-dioleoyl-glycerol
5	PLP	1,3-dipalmitoyl-2-linoleoyl-glycerol	16	SLS	1,3-distearoyl-2-linoleoyl-glycerol
6		unidentified		OOO	triolein
7	PSS	1-palmitoyl-2,3-distearoyl-glycerol	17	SLO	1-stearoyl-2-linoleoyl-3-oleoyl-glycerol
8	POS	1-palmitoyl-2-oleoyl-3-stearoyl-glycerol	18		unidentified
9	POO	1-palmitoyl-2,3-dioleoyl-glycerol	19		unidentified
10	PLS	1-palmitoyl-2-linoleoyl-3-stearoyl-glycerol	20	SOA	1-stearoyl-2-oleoyl-arachidoyl-glycerol
11	PLO	1-palmitoyl-2-linoleoyl-3-oleoyl-glycerol	21	AOO	1-arachidoyl-2,3-dioleoyl-glycerol

Experimental conditions

GLC column:	25 m × 0,25 mm fused silica capillary column coated with 0,1 µm Chrompack TAP
Temperature programme:	100 °C held for at least 2 min; 30 °C/min to 270 °C held for 1 min; 2,5 °C/min to 340 °C held for 7 min
Injector:	Cold on-column
Detector (FID):	360 °C
Carrier gas:	H_2 with a constant flow rate of 3,5 ml/min

Figure B.1 — Triacylglycerol profile of IRMM-801

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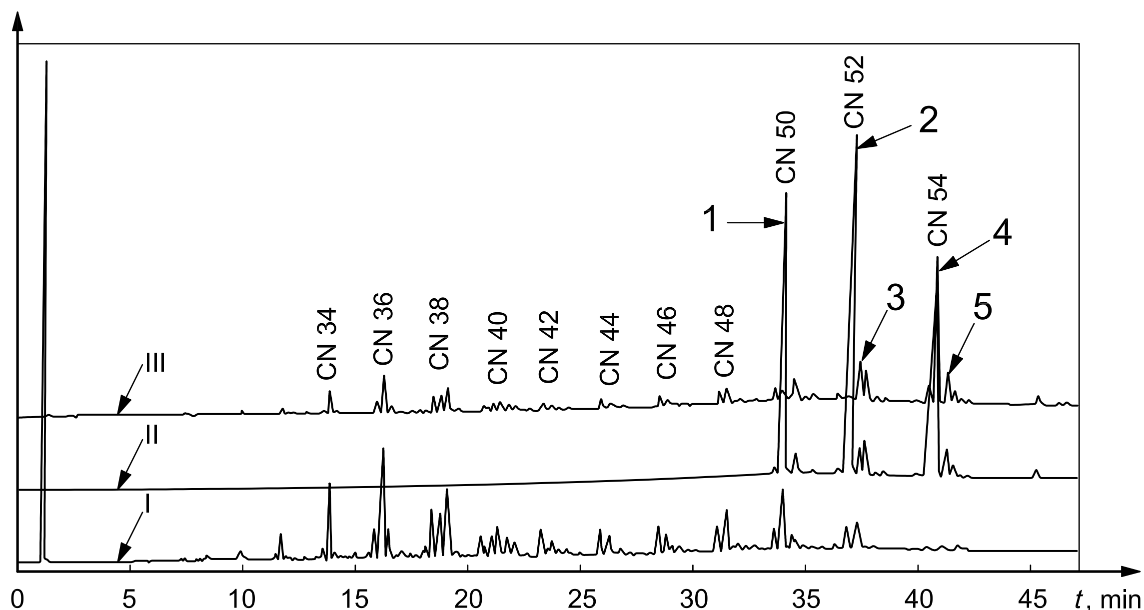

Key

t	retention time	
1	POP	1,3-dipalmitoyl-2-oleoyl-glycerol
2	POS	1-palmitoyl-2-oleoyl-3-stearoyl-glycerol
3	POO	1-palmitoyl-2,3-dioleoyl-glycerol
4	SOS	1,3-distearoyl-2-oleoyl-glycerol
5	SOO	1-stearoyl-2,3-dioleoyl-glycerol
I		milk fat 100 % mass fraction
II		cacao butter 100 % mass fraction
III		cacao butter 85 % mass fraction + milk fat 15 % mass fraction
CN 34 to CN 54		peak groups according to carbon number

Figure B.2 — Triacylglycerol profile of pure MF, pure CB and a mixture of CB with MF

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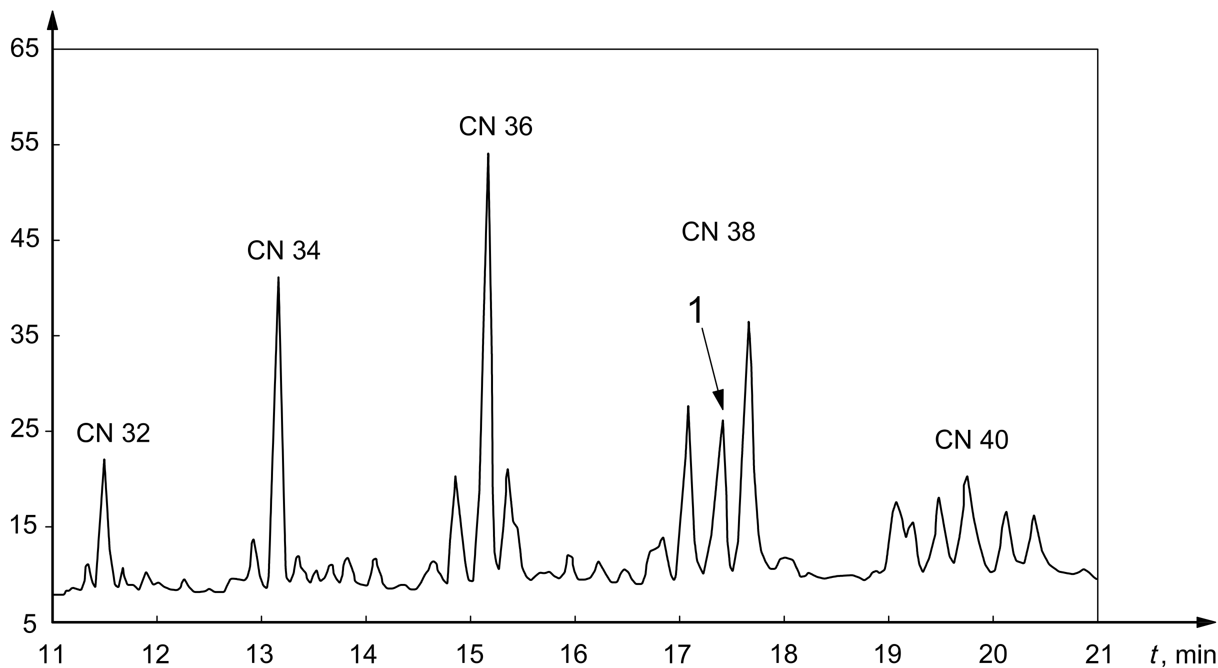


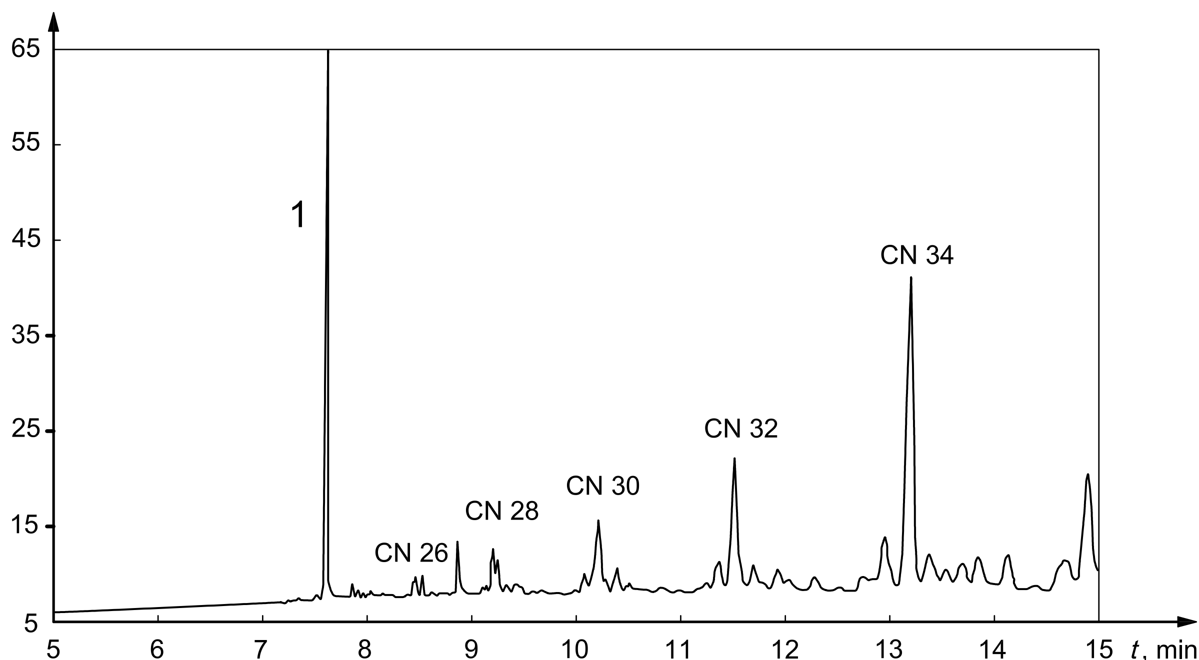
Key

t retention time

1 PSB 1-palmitoyl-2-stearoyl-3-butyroyl-glycerol

CN 32 to CN 40 peak groups according to carbon number

Figure B.3 — Triacylglycerol profile of pure MF: chromatogram window for the part where PSB elutes



Key

t retention time

1 α-cholestane

CN 26 to CN 34 peak groups according to carbon number

Figure B.4 — Triacylglycerol profile of pure MF with the addition of α-cholestane: chromatogram window for the part where α-cholestane elutes

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