



# DETECTION AND QUANTIFICATION OF COCOA BUTTER EQUIVALENTS IN MILK CHOCOLATE

## VALIDATED METHOD

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European Commission  
Directorate-General Joint Research Centre  
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## 1 Scope

This 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 down to a level of 0.5 g CBE/100 g milk chocolate and quantified well around levels of 5 % CBE addition to milk chocolate with a prediction error of 0.7 g CBE/100 g milk chocolate.

**NOTE: In the European Union chocolate and cocoa based products are currently governed by Directive 2000/36/EC [1], which authorizes the replacement of cocoa butter (CB) by vegetable fats other than CB up to 5 % of the total weight, provided that their labelling is supplemented by the statement "Contains vegetable fats in addition to cocoa butter". Six vegetable fats (so called cocoa butter equivalents, CBEs), clearly specified in Annex II of the Directive, can be used singly or in blends, i.e., Illipé (*Shorea spp.*); palm oil (*Elaeis guineensis*, *Elaeis olifera*); sal (*Shorea robusta*); shea (*Butyrospermum parkii*); kokum gurgi (*Garcinia indica*); and mango kernel (*Mangifera indica*). The intended application of the standard is to assess correct labelling of milk chocolate according to Directive 2000/36/EC [1]. To assess correct labelling of dark chocolate the use of the following standards is recommended [2-5].**

## 2 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 molecular weight 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), 1,2-dioleoyl-3-palmitoyl-glycerol (POO), 1,3-distearoyl-2-oleoyl-glycerol (SOS) and 1,2-dioleoyl-3-stearoyl-glycerol (SOO) are used

- (i) to calculate the MF content in the chocolate fat (g MF/100 g chocolate fat),
- (ii) 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 in case the detection approach indicates that the sample is not pure CB,
- (iii) to quantify the amount of the CBE admixture in chocolate fat (g CBE/100 g chocolate fat) using a partial least squares (PLS) regression model using six input variables, i.e., the five TAGs POP, POS, POO, SOS, SOO normalized to 100 % and the determined MF content of the chocolate fat.

To control correct labelling of milk chocolate, the obtained results related to chocolate fat are converted into g MF/100 g chocolate and g CBE/100 g chocolate, requiring the accurate determination of the total fat content of the chocolate using a Soxhlet extraction procedure [6]. In case the detection approach proves the absence of CBEs in chocolate fat, the quantification of CBEs and the determination of the total fat content of chocolate are obsolete.

### 3 Reagents, solutions and standards

Use only reagents of recognised analytical grade, unless otherwise stated.

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

**3.1 Cocoa butter Certified Reference Material (IRMM-801)** [7], for calibration purposes and system suitability tests.

**3.2 Pure milk fat**, for system suitability tests.

**3.3 1-Palmitoyl-2-stearoyl-3-butyroyl-glycerol (PSB)**, for calibration purposes.

Dissolve ca. 40 mg of PSB in a 50 mL volumetric flask with *iso*-octane resulting in a stock solution of ca.  $c = 0.8$  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 IRMM-801 (3.1) into 25 mL volumetric flasks and adding the respective volumes of the PSB stock solution as given in Table 1. Fill up to the mark with *iso*-octane.

**Table 1. Masses of IRMM (3.1) and volumes of PSB stock solution for preparation of series of PSB calibration solutions in matrix**

Calibration solution	IRMM-801 (3.1) weighed into 25 mL volumetric flask [mg]	Volume taken from PSB stock solution [mL] and added to 25 mL volumetric flask	$c_{\text{PSB}}$ in calibration solution $i$ [mg/mL]	Final $c_{\text{IRMM-PSB}}$ solution [mg/mL]
1	ca. 250	4	0.128	ca. 10
2	ca. 250	3	0.096	ca. 10
3	ca. 250	2	0.064	ca. 10
4	ca. 250	1	0.032	ca. 10
5	ca. 250	0.5	0.016	ca. 10

**For cold on-column (OCI) injection:** Dilute each calibration solution 1:5 with *iso*-octane to obtain a final IRMM-PSB concentration ( $c_{\text{IRMM-PSB}}$ ) of ca. 2 mg/mL in each solution and PSB concentrations ( $c_{\text{PSB}}$ ) ranging from 0.0256 (calibration solution 1) to 0.0032 mg/mL (calibration solution 5).

**For split injection (e.g. split ratio of 1:10):** Dilute each calibration solution 1:2 with *iso*-octane to obtain a final IRMM-PSB concentration ( $c_{\text{IRMM-PSB}}$ ) of ca. 5 mg/mL in each solution and PSB concentrations ( $c_{\text{PSB}}$ ) ranging from 0.064 (calibration solution 1) to 0.008 mg/mL (calibration solution 5).

**Note:** The final PSB concentrations have to be calculated using the actual weighed mass in the stock standard solution.

**3.4 alpha-Cholestane,** used as internal standard.

Dissolve ca. 50 mg alpha-cholestane in 50 mL of *iso*-octane.

**For cold on-column injection:** Dilute 1:250 ( $c = 0.004$  mg/mL).

**For split injection (e.g. split ratio of 1:10):** Dilute 1:100 ( $c = 0.01$  mg/mL).

**3.5 Fat solvent,** non-chlorinated solvents (e.g. petroleum ether, *n*-hexane, *n*-heptane, *iso*-octane).

**3.6 Hydrochloric acid,**  $c = 4$  mol/L.

## 4 Apparatus and equipment

**4.1 Analytical balance,** with a readability of 0.1 mg.

**4.2 Drying oven.**

A dry heater block may be used.

**4.3 Filter paper,** 15 cm.

(e.g. S&S 589<sup>1</sup> black ribbon paper is an example of a suitable product commercially available)

**4.4 Food grater,** a kitchen blender with a design featuring the motor above the receiving container to avoid melting the samples (e.g. Philips HR2833).

#### 4.5 Rotary evaporator.

Alternative evaporation procedures may be used.

#### 4.6 Evaporation block, with nitrogen supply.

#### 4.7 Desiccator.

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

#### 4.9 Volumetric flasks, of capacity 10, 25, 50 and 100 mL (or other capacities if needed).

#### 4.10 Pipettes, of capacities ranging from 1 to 10 mL (or other capacities if needed).

#### 4.11 Microsyringe, with maximum volume 10 µL, graduated to 0.1 µL, or automatic sample injector.

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

**NOTE: 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 9.2.**

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

GLC column:	CB-TAP 25 m x 0.25 mm i.d., fused silica coated with a medium polar thermo stable phenylmethylpolysiloxane stationary phase with a film thickness of 0.10 µm.
Oven programme for OCI:	100°C held for min 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 min 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 <sub>2</sub> (purity ≥ 99,999 %) with a constant flow rate of 3.5 mL/min (Another suitable carrier gas is helium)
Carrier gas for split:	H <sub>2</sub> (purity ≥ 99,999 %) with a constant flow rate of 2.5 mL/min (Another suitable carrier gas is helium)

**NOTE: Suitable columns and alternative experimental conditions, used in an international collaborative study [8], are listed in Annex A, Table A 1. Operating conditions may be changed to obtain optimum separation.**

#### 4.13 Chromatographic data system.

## 5 Sampling

Sampling is not part of this method.

## 6 Sample preparation

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

Before opening and using the IRMM-801 (3.1), the ampoule shall be warmed in an oven 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.

### 6.2 Preparation of pure milk fat for system suitability tests

If no pure milk fat is available it can be obtained from a butter sample by melting and passing the fat layer through a folded filter (4.3) at 50 °C in an oven.

### 6.3 Preparation of chocolate sample

Chill approx. 200 g of chocolate until hard, and grate to fine granular condition using a mechanical device (4.4). Mix thoroughly and preserve in a tightly stoppered bottle in a cool place.

#### 6.3.1 Rapid fat extraction

The chocolate fat is separated from 5 g grated chocolate (6.3) by extracting with two to three 10 mL portions of a suitable fat solvent (3.5). Centrifuge and decant. Combine the extracts and evaporate most of the fat solvent (4.5) and finally dry it under a stream of nitrogen (4.6).

**NOTE: 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. In case, no CBEs are detected the second part of the standard, i.e., quantification of CBEs around the statutory limit of 5 %, is not necessary. In case, CBEs are detected, the quantification part has to be performed using the same TAG profile as used for the CBE detection. However, in this case the accurate amount of total fat in chocolate has to be determined using the following procedure (6.3.2). Alternative extraction procedures may be used provided that the same results are obtained.**

#### 6.3.2 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 6.3) by Soxhlet extraction [6], as follows. Weigh 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 (3.6) and a few defatted boiling chips, or other antibumping agents, and stir. Cover with a watch glass, bring the solution slowly to boil, and boil gently for 15 min. Rinse the watch glass with 100 mL of water. Filter the solution

through a medium fluted filter paper (4.3), 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 (4.7) then weigh it. Place the thimble containing the dried sample in the Soxhlet apparatus (4.8), 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 >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 (4.7) to room temperature then weigh. 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 in percent of total fat in the chocolate,  $M_{\text{fat; choc}}$ , is calculated as follows:

**Equation 1:** 
$$M_{\text{fat; choc}} = \frac{w_{\text{fat}} \times 100}{w}$$

where

- $w$  is the mass of chocolate taken, in grams
- $w_{\text{fat}}$  is the mass of the total fat obtained from the chocolate by Soxhlet extraction [6], in grams
- $M_{\text{fat; choc}}$  is the mass fraction in percent of total fat in chocolate

**NOTE** 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 shall 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.

## 7 Procedure

### 7.1 Construction of calibration curve for determination of PSB content

Five calibration solutions containing different concentrations of PSB (3.3) but always the same concentration of alpha-cholestane (3.4) have to be prepared as follows:

#### For cold on-column (OCI) injection:

- **Calibration solution 1 (Final  $c_{\text{PSB } 1} = 0.0128 \text{ mg/mL}$ ;  $c_{\text{alpha-cholestane } 1} = 0.002 \text{ mg/mL}$ ):**  
Transfer 1 mL of calibration solution 1 ( $c_{\text{PSB } 1} = 0.0256 \text{ mg/mL}$ ; 3.3) in a test tube and add 1 mL of alpha-cholestane solution ( $c = 0.004 \text{ mg/mL}$ ; 3.4).
- **Calibration solution 2 (Final  $c_{\text{PSB } 2} = 0.0096 \text{ mg/mL}$ ;  $c_{\text{alpha-cholestane } 2} = 0.002 \text{ mg/mL}$ ):**  
Transfer 1 mL of calibration solution 2 ( $c_{\text{PSB } 2} = 0.0192 \text{ mg/mL}$ ; 3.3) in a test tube and add 1 mL of alpha-cholestane solution ( $c = 0.004 \text{ mg/mL}$ ; 3.4).
- **Calibration solution 3 (Final  $c_{\text{PSB } 3} = 0.0064 \text{ mg/mL}$ ;  $c_{\text{alpha-cholestane } 3} = 0.002 \text{ mg/mL}$ ):**  
Transfer 1 mL of calibration solution 3 ( $c_{\text{PSB } 3} = 0.0128 \text{ mg/mL}$ ; 3.3) in a test tube and add 1 mL of alpha-cholestane solution ( $c = 0.004 \text{ mg/mL}$ ; 3.4).
- **Calibration solution 4 (Final  $c_{\text{PSB } 4} = 0.0032 \text{ mg/mL}$ ;  $c_{\text{alpha-cholestane } 4} = 0.002 \text{ mg/mL}$ ):**  
Transfer 1 mL of calibration solution 4 ( $c_{\text{PSB } 4} = 0.0064 \text{ mg/mL}$ ; 3.3) in a test tube and add 1 mL of alpha-cholestane solution ( $c = 0.004 \text{ mg/mL}$ ; 3.4).
- **Calibration solution 5 (Final  $c_{\text{PSB } 5} = 0.0016 \text{ mg/mL}$ ;  $c_{\text{alpha-cholestane } 5} = 0.002 \text{ mg/mL}$ ):**  
Transfer 1 mL of calibration solution 5 ( $c_{\text{PSB } 5} = 0.0032 \text{ mg/mL}$ ; 3.3) in a test tube and add 1 mL of alpha-cholestane solution ( $c = 0.004 \text{ mg/mL}$ ; 3.4).

Inject 0.5  $\mu\text{L}$  of each calibration solution into the HR-GLC system using the cold on-column injection system.

#### For split injection:

- **Calibration solution 1 (Final  $c_{\text{PSB } 1} = 0.032 \text{ mg/mL}$ ;  $c_{\text{alpha-cholestane } 1} = 0.005 \text{ mg/mL}$ ):**  
Transfer 1 mL of calibration solution 1 ( $c_{\text{PSB } 1} = 0.064 \text{ mg/mL}$ ; 3.3) in a test tube and add 1 mL of alpha-cholestane solution ( $c = 0.01 \text{ mg/mL}$ ; 3.4).
- **Calibration solution 2 (Final  $c_{\text{PSB } 2} = 0.024 \text{ mg/mL}$ ;  $c_{\text{alpha-cholestane } 2} = 0.005 \text{ mg/mL}$ ):**  
Transfer 1 mL of calibration solution 2 ( $c_{\text{PSB } 2} = 0.048 \text{ mg/mL}$ ; 3.3) in a test tube and add 1 mL of alpha-cholestane solution ( $c = 0.01 \text{ mg/mL}$ ; 3.4).
- **Calibration solution 3 (Final  $c_{\text{PSB } 3} = 0.016 \text{ mg/mL}$ ;  $c_{\text{alpha-cholestane } 3} = 0.005 \text{ mg/mL}$ ):**  
Transfer 1 mL of calibration solution 3 ( $c_{\text{PSB } 3} = 0.032 \text{ mg/mL}$ ; 3.3) in a test tube and add 1 mL of alpha-cholestane solution ( $c = 0.01 \text{ mg/mL}$ ; 3.4).
- **Calibration solution 4 (Final  $c_{\text{PSB } 4} = 0.008 \text{ mg/mL}$ ;  $c_{\text{alpha-cholestane } 4} = 0.005 \text{ mg/mL}$ ):**  
Transfer 1 mL of calibration solution 4 ( $c_{\text{PSB } 4} = 0.016 \text{ mg/mL}$ ; 3.3) in a test tube and add 1 mL of alpha-cholestane solution ( $c = 0.01 \text{ mg/mL}$ ; 3.4).

- **Calibration solution 5 (Final  $c_{\text{PSB } 5} = 0.004 \text{ mg/mL}$ ;  $c_{\text{alpha-cholestane } 5} = 0.005 \text{ mg/mL}$ ):**  
Transfer 1 mL of calibration solution 5 ( $c_{\text{PSB } 5} = 0.008 \text{ mg/mL}$ ; 3.3) in a test tube and add 1 mL of alpha-cholestane solution ( $c = 0.01 \text{ mg/mL}$ ; 3.4).

Inject 1  $\mu\text{L}$  of the final test solution into the HR-GLC system using the cold on-column injection system.

**NOTE: Alternative sample amounts and injectors may be used provided that the detection system employed gives a linear response and the system suitability criteria (9.2) are met.**

### 7.2 Separation of individual TAGs of IRMM-801 by HR-GLC

The IRMM-801 (3.1) shall be warmed in a drying oven until completely melted. Pipettes (or similar equipment) used for transferring the sample during weighing operations should be brought to a temperature of ca. 55 °C in a drying oven in order to avoid partial fat fractionation during handling of samples.

**For cold on-column (OCI) injection:** Weigh ca. 0.1 g of IRMM-801 (3.1) in a 10 mL volumetric flask and dilute to the mark with *iso*-octane (3.5). Pipette 1 mL of the resulting solution in another 50 mL volumetric flask and dilute to the mark with the same solvent ( $c = 0.2 \text{ mg/mL}$ ). Inject 0.5  $\mu\text{L}$  of the final test solution into the HR-GLC system using the cold on-column injection system.

**For split injection:** Weigh ca. 0.1 g of IRMM-801 (3.1) in a 10 mL volumetric flask and dilute to the mark with *iso*-octane (3.5). Pipette 1 mL of the resulting solution in another 10 mL volumetric flask and dilute to the mark with the same solvent ( $c = 1 \text{ mg/mL}$ ). Inject 1  $\mu\text{L}$  of the final test solution into the HR-GLC system using the cold on-column injection system.

**NOTE: Alternative fat solvents, sample amounts and injectors may be used provided that the detection system employed gives a linear response and the system suitability criteria (9.2) are met.**

### 7.3 Separation of individual TAGs of pure MF by HR-GLC

**For cold on-column (OCI) injection:** Weigh ca. 0.05 g of pure milk fat (3.2) in a 50 mL volumetric flask and dilute to the mark with *iso*-octane (3.5) ( $c = 1 \text{ mg/mL}$ ). Transfer 1 mL of this solution in a test tube and add 1 mL of alpha-cholestane solution (3.4) (resulting test sample solution  $c = 0.5 \text{ mg/mL}$ ). Inject 0.5  $\mu\text{L}$  of the final test solution into the HR-GLC system using the cold on-column injection system.

**For split injection:** Weigh ca. 0.25 g of pure milk fat (3.2) in a 50 mL volumetric flask and dilute to the mark with *iso*-octane (3.5) ( $c = 5 \text{ mg/mL}$ ). Transfer 1 mL of this solution in a test tube and

add 1 mL of alpha-cholestane solution (3.4) (resulting test sample solution  $c = 2.5$  mg/mL). Inject 1  $\mu$ L of the final test solution into the HR-GLC system using the cold on-column injection system.

**NOTE: Alternative fat solvents, sample amounts and injectors may be used provided that the detection system employed gives a linear response and the system suitability criteria (9.2) are met.**

#### 7.4 Separation of individual TAGs of chocolate fat by HR-GLC

The test sample (chocolate fat extracted from milk chocolate by rapid fat extraction (6.3.1)) shall be warmed in a drying oven 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 ca. 55 °C in a drying oven in order to avoid partial fat fractionation during handling of samples.

**For cold on-column (OCI) injection:** Weigh ca. 0.1 g of chocolate fat (as obtained in 6.3.1) on an analytical balance (4.1) in a 100 mL volumetric flask and dilute to the mark with *iso*-octane (3.5) ( $c = 1$  mg/mL). Transfer 1 mL of this solution in a test tube and add 1 mL of alpha-cholestane solution (3.4) (resulting test sample solution  $c = 0.5$  mg/mL). Inject 0.5  $\mu$ L of the final test solution into the HR-GLC system using the cold on-column injection system.

**For split injection:** Weigh ca. 0.5 g of chocolate fat (as obtained in 6.3.1) on an analytical balance (4.1) in a 100 mL volumetric flask and dilute to the mark with *iso*-octane (3.5) ( $c = 5$  mg/mL). Transfer 1 mL of this solution in a test tube and add 1 mL of alpha-cholestane solution (3.4) (resulting test sample solution  $c = 2.5$  mg/mL). Inject 1  $\mu$ L of the final test solution into the HR-GLC system using the cold on-column injection system.

**NOTE: Alternative fat solvents, sample amounts and injectors may be used provided that the detection system employed gives a linear response and the system suitability criteria (9.2) are met.**

#### 7.5 Identification

Identification of 1-palmitoyl-2-stearoyl-3-butyroyl-glycerol (PSB) and alpha-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-oleoylglycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoylglycerol (POS), 1-palmitoyl-2,3-dioleoyl-glycerol (POO), 1,3-distearoyl-2-oleoylglycerol (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 (7.2).

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 (7.2) is given in Figure B 1. The elution order of the TAGs of an average pure MF (7.3) is given in Figure B 2.

## 8 Calculation

### 8.1 PSB and MF quantification in chocolate fat and chocolate

#### 8.1.1 Determination of PSB response factor

Determine the response factor of PSB by injection of the five calibration solutions (7.1) using experimental conditions identical to those used for the test sample. For each calibration solution a response factor for PSB,  $F_{PSB}$ , has to be calculated by

$$\text{Equation 2: } F_{PSB;i} = \frac{C_{PSB;i} \times A_{Cholestane;i}}{C_{Cholestane;i} \times A_{PSB;i}}$$

where

$A_{PSB;i}$	is the peak area of PSB in calibration solution i (7.1)
$A_{Cholestane;i}$	is the peak area of the internal standard alpha-cholestane in calibration solution i (7.1)
$C_{PSB;i}$	is the concentration [mg/mL] of PSB used in calibration solution i (7.1)
$C_{Cholestane;i}$	is the concentration [mg/mL] of the internal standard alpha-cholestane used in calibration solution i (7.1)
$F_{PSB;i}$	is the detector response factor of PSB in the calibration solution i

An average response factor for PSB,  $F_{PSB; mean}$ , obtained from the five calibration solutions has to be calculated and used for further calculations.

#### 8.1.2 PSB quantification in chocolate fat

The mass fraction in percent of PSB in the test sample (chocolate fat),  $M_{PSB; choc fat}$ , is calculated as follows:

$$\text{Equation 3: } M_{PSB; choc fat} = \frac{A_{PSB} \times C_{Cholestane} \times F_{PSB; mean} \times 100}{A_{Cholestane} \times C_{Sample}}$$

where

$A_{PSB}$	is the peak area of PSB in the test sample (7.4)
$A_{Cholestane}$	is the peak area of the internal standard alpha-cholestane in the test sample (7.4)

$F_{\text{PSB; mean}}$	is the average response factor for PSB (see 8.1.1)
$C_{\text{Cholestane}}$	is the concentration [mg/mL] of the internal standard alpha-cholestane in the test sample (7.4)
$C_{\text{Sample}}$	is the concentration [mg/mL] of the test sample (7.4)
$M_{\text{PSB; choc fat}}$	is the mass fraction in percent of PSB in the test sample

Report the results to two decimal places.

### 8.1.3 MF quantification in chocolate fat

The mass fraction in percent of milk fat in the chocolate fat,  $M_{\text{MF; choc fat}}$ , is calculated as follows:

**Equation 4:**  $M_{\text{MF; choc fat}} = 0.19 + (44.04 \times M_{\text{PSB; choc fat}})$

where

$M_{\text{PSB; choc fat}}$	is the mass fraction in percent of PSB in chocolate fat (see Equation 3)
$M_{\text{MF; choc fat}}$	is the mass fraction in percent of MF in chocolate fat

**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 [9].

Report the results to two decimal places.

### 8.1.4 MF quantification in chocolate

The mass fraction in percent of MF in chocolate,  $M_{\text{MF; choc}}$ , is calculated as follows:

**Equation 5:**  $M_{\text{MF; choc}} = \frac{M_{\text{fat; choc}} \times M_{\text{MF; choc fat}}}{100}$

where

$M_{\text{fat; choc}}$	is the mass fraction in percent of total fat in chocolate (see Equation 1)
$M_{\text{MF; choc fat}}$	is the mass fraction in percent of MF in chocolate fat (see Equation 4)
$M_{\text{MF; choc}}$	is the mass fraction in percent of MF in chocolate

## 8.2 CBE detection in chocolate fat

### 8.2.1 Determination of response factors for POP, POS and SOS

Determine the response factors of the TAGs i, i.e., POP, POS, and SOS, by injection of the IRMM-801 (7.2) solution using experimental conditions identical to those used for the test sample. Calculate the percentage of each of the three TAGs with respect to all TAGs present in IRMM-801 by the following equations:

**Equation 6:** 
$$P_{i;ref} = \frac{A_{i;ref}}{\sum A_{all\ TAGs;ref}} \times 100$$

**Equation 7:** 
$$F_i = \frac{M_{i;ref}}{P_{i;ref}}$$

where

$A_{i;ref}$	is the peak area of TAG i, i.e., POP, POS and SOS, in IRMM-801 (7.2)
$\sum A_{all\ TAGs;ref}$	is the sum of the peak areas attributed to all TAGs in IRMM-801 (7.2)
$P_{i;ref}$	is the percentage of TAG i, i.e., POP, POS and SOS, in IRMM-801 (see Equation 6)
$M_{i;ref}$	is the mass fraction in percent of TAG i in IRMM-801 as given in the certificate (POP=16.00 %, POS=39.40 %, SOS=27.90 %) [7]
$F_i$	is the detector response factor of TAG i, i.e., POP, POS and SOS, in IRMM-801

Report the results to two decimal places.

### 8.2.2 Calculation of mass percentages of POP, POS and SOS in chocolate fat

Calculate the mass percentage of the TAGs i, i.e., POP, POS and SOS, in the test sample with respect to all TAGs present in the test sample by

**Equation 8:** 
$$M_{i;total} = \frac{F_i \times A_i}{\sum A_{all\ TAGs}} \times 100$$

where

$A_i$	is the peak area corresponding to the TAG i, i.e., POP, POS and SOS, in the test sample (7.4)
$\sum A_{all\ TAGs}$	is the sum of the peak areas attributed to all TAGs in the test sample (7.4)
$F_i$	is the response factor for the TAG i, i.e., POP, POS and SOS (see Equation 7)
$M_{i;total}$	is the mass fraction in percent of TAG i, i.e., POP, POS and SOS, in the test sample

Report the results to two decimal places.

### 8.2.3 Correction for MF contribution

Calculate the contribution of the mass percentages of the TAGs POP, POS and SOS originating from MF by

**Equation 9:** 
$$M_{i;mf} = \frac{M_{MF;choc\ fat} \times M_{i;ref\ MF}}{100}$$

where

$M_{i, \text{ref MF}}$  is the average mass fraction in percent of TAG i in a MF, i.e., POP=3.99 %, POS=2.19 %, SOS=0.45 % (values obtained from database, [10])  
 $M_{\text{MF; choc}}$  is the mass fraction in percent of MF in the test sample (see Equation 4)  
 $M_{i, \text{mf}}$  is the mass fraction in percent of TAG i, i.e., POP, POS and SOS, originating from MF in the test sample

Subtract the obtained mass percentages of the three TAGs originating from MF (Equation 9) from the mass percentages of the three TAGs obtained for the test sample (Equation 8).

**Equation 10:**  $M_{i, \text{corr.}} = M_{i, \text{total}} - M_{i, \text{mf}}$

Normalize the obtained mass percentages of the three TAGs (Equation 10) to 100 % (Equation 12):

**Equation 11:**  $\text{POP}_{\text{corr.}} + \text{POS}_{\text{corr.}} + \text{SOS}_{\text{corr.}} = 100 \%$

Report the results to two decimal places.

#### **8.2.4 Decision whether chocolate fat is pure cocoa butter**

The variability of the TAG composition of CB is expressed by Equation 12 [11, 12].

**Equation 12:**  $\text{POP} - \% = 43.73 - 0.73 \times \text{SOS} - \%$  (residual SD = 0.125)

The principle of the method is that for pure CB samples POS is practically constant for wide variations of POP and SOS, resulting in a linear relationship (so-called “CB-line”, Equation 12) between POP and SOS. CBE and other fat admixtures will cause the TAG analysis to deviate from the “CB-line” to the extent that their POS value differs from the POS value of cocoa butter. For 99% of all analyses, pure cocoa butter complies with:

**Equation 13:**  $\text{POP}_{\text{corr.}} < 44.03 - 0.73 \times \text{SOS}_{\text{corr.}}$

A greater value of  $\text{POP}_{\text{corr.}}$ , as given by Equation 13, means that the sample is not pure CB.

**NOTE:** The advantage of the elaborated approach is that by using IRMM-801 (3.1) for calibration purpose, the mathematical expression can be used by individual testing laboratories for verifying the purity of CB, without tackling the problem of establishing a

“CB-line” as a prerequisite. Calibration by IRMM-801 (3.1) automatically links the results obtained in a laboratory to the cocoa butter TAG database and the elaborated decision rule (Equation 13).

### 8.3 CBE quantification in chocolate fat and chocolate

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

Determine the response factors of the TAGs j, i.e., POP, POS, POO, SOS and SOO, by injection of IRMM-801 solution (7.2) using experimental conditions identical to those used for the samples. Calculate the percentage of each of the five TAG fractions by the following equations:

$$\text{Equation 14: } P_{j, \text{ref}} = \frac{A_{j, \text{ref}}}{\sum A_{j, \text{ref}}} \times 100$$

$$\text{Equation 15: } F_j = \frac{M_{j, \text{ref}}}{P_{j, \text{ref}}}$$

where

$A_{j, \text{ref}}$	is the peak area of the TAG j, i.e., POP, POS, POO, SOS and SOO, in IRMM-801 (7.2)
$\sum A_{j, \text{ref}}$	is the sum of the peak areas attributed to POP, POS, POO, SOS, SOO, in IRMM-801 (7.2)
$P_{j, \text{ref}}$	is the percentage of TAG j, i.e., POP, POS, POO, SOS and SOO, in IRMM-801
$M_{j, \text{ref}}$	is the mass fraction in percent of TAG j, i.e., POP, POS, POO, SOS and SOO, in IRMM-801 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 % [7]
$F_j$	is the detector response factor of TAG j, i.e., POP, POS, POO, SOS and SOO, in IRMM-801

Report the results to two decimal places.

#### 8.3.2 Calculation of mass percentages of POP, POS, POO, SOS and SOO in chocolate fat

Calculate the mass percentages of the TAGs j, i.e., POP, POS, POO, SOS and SOO, in the test sample by

$$\text{Equation 16: } M_{j, \text{choc fat}} = \frac{F_j \times A_j}{\sum (F_j \times A_j)} \times 100$$

where

- $F_j$  is the response factor of the TAG  $j$ , i.e., POP, POS, POO, SOS and SOO, (see Equation 15)
- $A_j$  is the peak area corresponding to the TAG  $j$ , i.e., POP, POS, POO, SOS and SOO, in the test sample (7.4)
- $M_{j; \text{choc fat}}$  is the mass fraction in percent of TAG  $j$ , i.e., POP, POS, POO, SOS and SOO, in the test sample

Report the results to two decimal places.

### 8.3.3 CBE quantification in chocolate fat

The mass fraction in percent of CBE in the chocolate fat,  $M_{\text{CBE}; \text{choc fat}}$ , is calculated by using an expression (Equation 17), which was derived by PLS regression analysis of the relative proportions of the five main TAGs; i.e.,  $\text{POP}_{\text{choc fat}}$ ,  $\text{POS}_{\text{choc fat}}$ ,  $\text{POO}_{\text{choc fat}}$ ,  $\text{SOS}_{\text{choc fat}}$  and  $\text{SOO}_{\text{choc fat}}$  as determined in Equation 16 and the MF content of the chocolate fat, i.e.  $M_{\text{MF}; \text{choc fat}}$  as determined in Equation 4.

$$\text{Equation 17: } M_{\text{CBE}; \text{choc fat}} = -4.24 - (0.23 \times M_{\text{MF}; \text{choc fat}}) + (1.52 \times \text{POP}_{\text{choc fat}}) - (1.47 \times \text{POS}_{\text{choc fat}}) + (1.09 \times \text{POO}_{\text{choc fat}}) + (1.29 \times \text{SOS}_{\text{choc fat}}) + (0.26 \times \text{SOO}_{\text{choc fat}})$$

Report the result to one decimal place.

### 8.3.4 CBE quantification in chocolate

The mass fraction in percent of CBE in chocolate,  $M_{\text{CBE}; \text{choc}}$ , is calculated by applying Equation 18:

$$\text{Equation 18: } M_{\text{CBE}; \text{choc}} = \frac{M_{\text{fat}; \text{choc}} \times M_{\text{CBE}; \text{choc fat}}}{100}$$

where

- $M_{\text{fat}; \text{choc}}$  is the mass fraction in percent of total fat in chocolate (see Equation 1)
- $M_{\text{CBE}; \text{choc fat}}$  is the mass fraction in percent of CBE in chocolate fat (see Equation 17)
- $M_{\text{CBE}; \text{choc}}$  is the mass fraction in percent of CBE in chocolate

Report the result to one decimal place.

## 9 Procedural requirements

### 9.1 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 requirements of the system suitability tests (9.2) are met.

## 9.2 System suitability

### 9.2.1 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 (7.2) 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 (7.3) as shown in Figures B 2 to B 3.
- The HR-GLC separation system shall be capable of showing no co-elution for the internal standard alpha-cholestane. This requirement can be proven by using a MF fat sample (7.3) using alpha-cholestane as internal standard as shown in Figure B 4.

**NOTE: In the case of failure, the chromatographic conditions (e.g. sample size, column temperature, carrier gas flow, etc.) must be optimized.**

### 9.2.2 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 (7.2) 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. The stability of the system has to be verified by repeating analysis (at least triplicates). The obtained relative standard deviations of the determined detector response factors shall be less than 5 %.
- To check the stability of the separation system a calibration curve for PSB with alpha-cholestane as internal standard shall be established (at least duplicate injection of each calibration solution). The average detector response factor for PSB shall be calculated. RF values obtained on individual calibration solutions shall not deviate by more than 5 % from the average value.

**NOTE: In the case of failure, the chromatographic conditions (e.g. sample size, column temperature, carrier gas flow, etc.) must be optimized.**

## 10 Precision

### 10.1 Interlaboratory test

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

### 9.2 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, will in not more than 5 % of cases be greater than 0.3 g/100 g milk chocolate.

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

### 9.3 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, will 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 in Annex A.

## 11 Test report

The test report shall specify:

- all information necessary for the complete identification of the sample;
- the sampling method used, if known;
- the test method used, with reference to this standard
- all operating details not specified in this standard, or regarded as optional, together with details of any incidents which may have influenced the test results(s);
- 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 [8]. 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.

**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
<b>Carrier gas</b>												
- type	He	He	He	H <sub>2</sub>	H <sub>2</sub>	H <sub>2</sub>	H <sub>2</sub>	He	H <sub>2</sub>	H <sub>2</sub>	H <sub>2</sub>	H <sub>2</sub>
- if constant pressure (kPa)	100	180	-	-	-	-	150	135	130	140	-	-
- if constant flow (mL/min)	-	-	2.2	2	2	1.5	-	-	-	-	3.5	2
<b>Column characteristics</b>												
- stationary phase	Ultimeta I	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
- i.d. [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
<b>Temperature mode</b>												
- 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	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, hold time	360 / 10	340 / 30	340 / 17	340 / 10	340 / 15	340 / 13	340 / 10	340 / 30	370	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]	65-370	360	oven track	365	370	350		340	380	140-340	oven track	360
- Detector temperature [°C]	370	360	360	365	360	370	350	360	380	350	360	360
<b>Injection mode</b>												
- 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

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

<b>Sample</b>	<b>Chocolate samples <sup>(1)</sup></b>	<b>CBE type</b>	<b>CB [%]</b>	<b>CBE [%]</b>	<b>MF [%]</b>
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
	<b>Chocolate fat solutions</b>	<b>CBE type</b>	<b>CB [%]</b>	<b>CBE [%]</b>	<b>MF [%]</b>
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

<sup>(1)</sup> 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

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

<b>Sample</b>	<b>5</b>	<b>6</b>	<b>10</b>	<b>11</b>	<b>12</b>	<b>13</b>
<b>Number of laboratories</b>	12	12	12	12	12	12
<b>Number of outliers</b>	0	1	0	0	0	0
<b>Number of accepted laboratories</b>	12	11	12	12	12	12
<b>Mean value, g CBE/100 g chocolate</b>	5.20	4.08	4.62	5.81	5.35	8.23
<b>True value, g CBE/100 g chocolate</b>	5.11	3.95	4.81	5.99	6.02	8.41
<b>Bias, g CBE/100 g chocolate</b>	-0.09	-0.13	0.19	0.18	0.67	0.18
<b>Repeatability limit <math>r</math> [<math>r=2.8 \times s_r</math>], g/100 g</b>	0.28	0.15	0.26	0.19	0.19	0.23
<b>Repeatability standard deviation <math>s_r</math>, g/100 g</b>	0.10	0.05	0.09	0.07	0.07	0.08
<b>Repeatability relative standard deviation <math>RSD_r</math>, %</b>	1.9	1.3	2.0	1.2	1.2	1.0
<b>Reproducibility limit <math>R</math> [<math>r=2.8 \times s_R</math>], g/100 g</b>	0.59	0.53	0.50	0.46	0.53	0.52
<b>Reproducibility standard deviation <math>s_R</math>, g/100 g</b>	0.21	0.19	0.18	0.17	0.19	0.19
<b>Reproducibility relative standard deviation <math>RSD_R</math>, %</b>	4.1	4.7	3.8	2.8	3.5	2.2
<b>HorRAT value = <math>RSD_R</math>/predicted <math>RSD_R</math> <sup>(1)</sup></b>	1.30	1.45	1.21	0.93	1.13	0.77

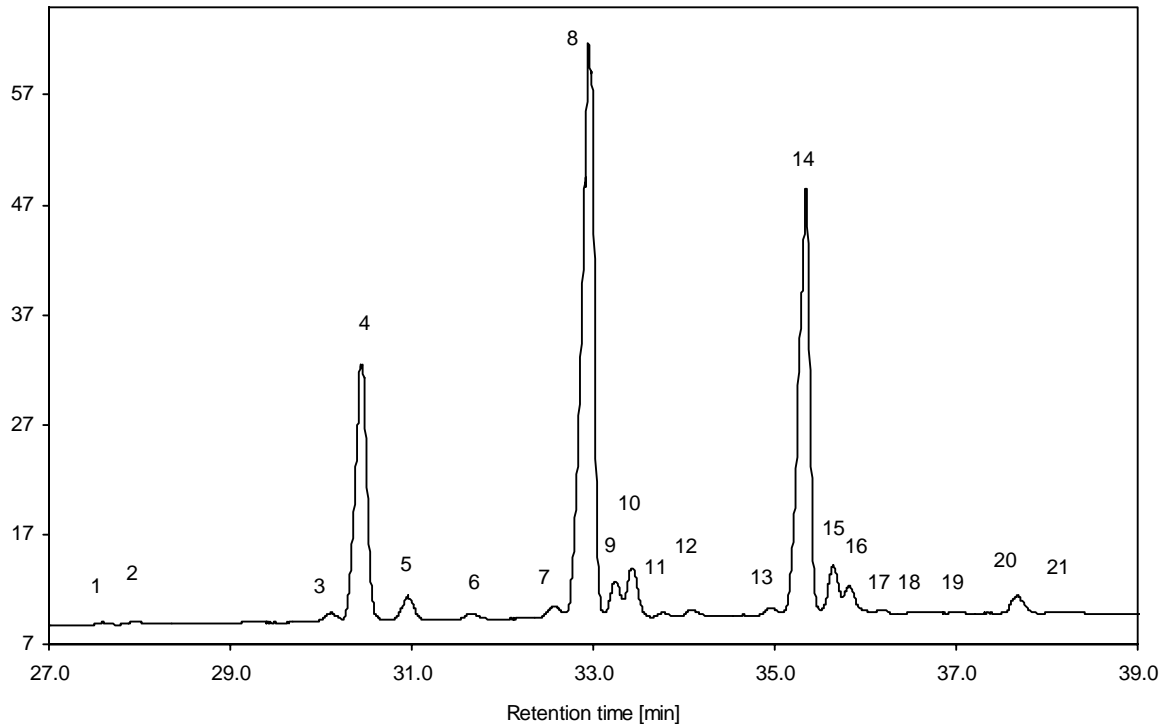
<sup>(1)</sup> predicted  $RSD_R = 2C^{-0.15}$ ; C = estimated mean concentration

**NOTE: Chocolate samples 1-4 and chocolate fat samples 7-9 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 %.**

# ANNEX B

(informative)

## Example chromatograms



**Peak identification:** 1, PPP; 2, MOP; 3, PPS; 4, POP; 5, PLP; 6, unidentified; 7, PSS; 8, POS; 9, POO; 10, PLS; 11, PLO; 12, unidentified; 13, SSS; 14, SOS; 15, SOO; 16, SLS + OOO; 17, SLO; 18, unidentified; 19, unidentified; 20, SOA; 21, AOO

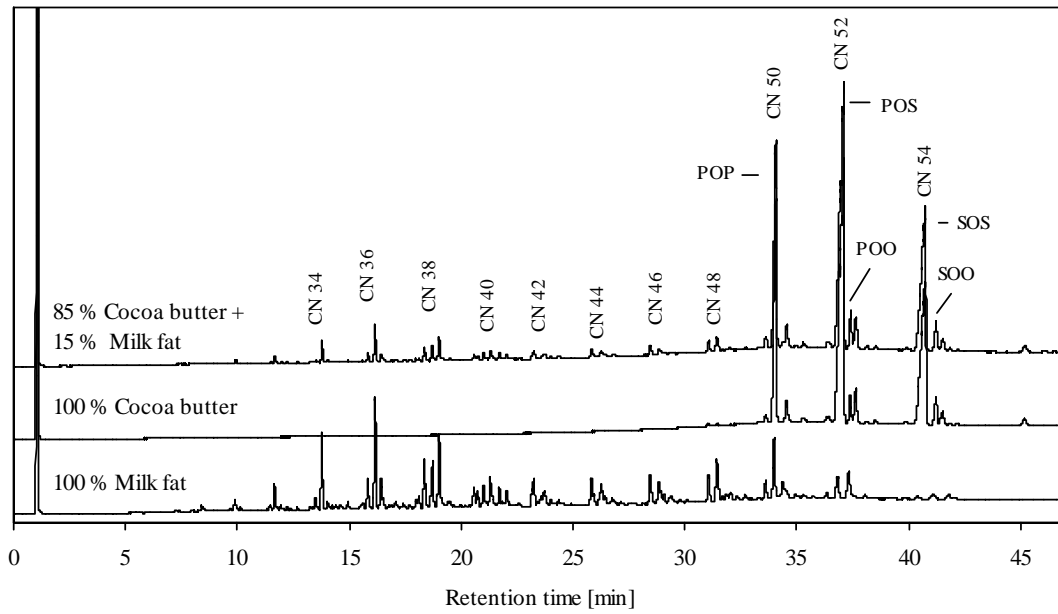
### Experimental conditions

GLC column: 25 m × 0.25 mm fused silica capillary column coated with 0.1 µm Chrompack TAP  
Temperature programme: 100 °C held for min 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<sub>2</sub> with a constant flow rate of 3.5 mL/min

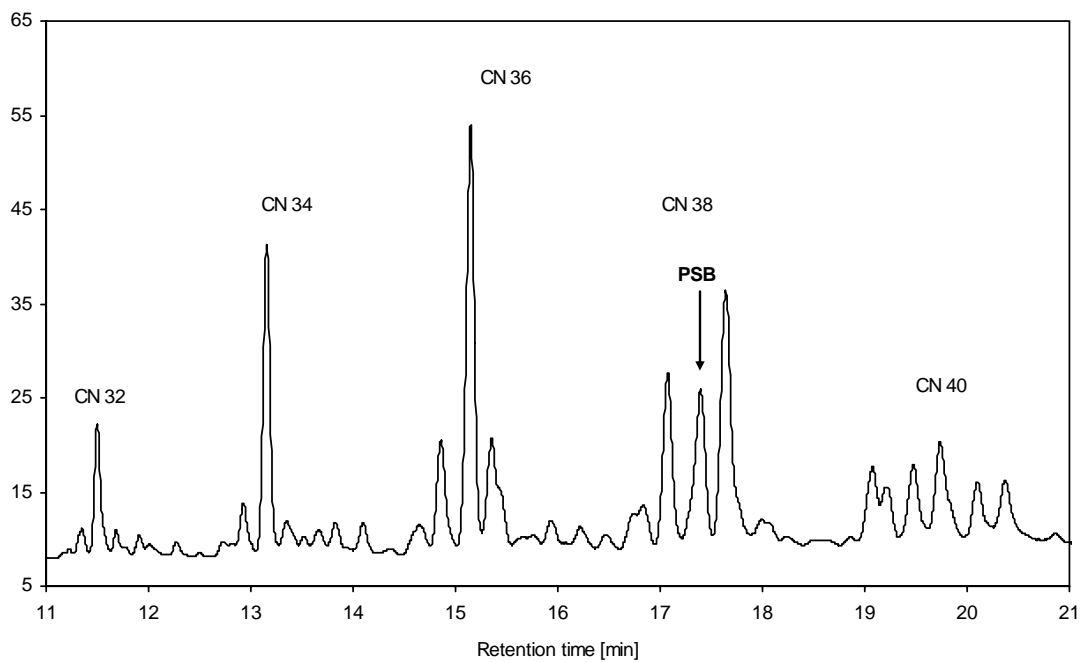
### Abbreviations:

PPP	Tripalmitin	SSS	Tristearin
MOP	1-Margaroyl-2-oleoyl-3-palmitoylglycerol	SOS	1,3-Distearoyl-2-oleoylglycerol
PPS	1,2-Dipalmitoyl-3-stearoylglycerol	SOO	1-Stearoyl-2,3-dioleoylglycerol
POP	1,3-Dipalmitoyl-2-oleoylglycerol	SLS	1,3-Distearoyl-2-linoleoyl glycerol
PLP	1,3-Dipalmitoyl-2-linoleoylglycerol	OOO	Triolein
PSS	1-Palmitoyl-2,3-distearoylglycerol	SLO	1-Stearoyl-2-linoleoyl-3-oleoylglycerol
POS	1-Palmitoyl-2-oleoyl-3-stearoylglycerol	SOA	1-Stearoyl-2-oleoyl-arachidoylglycerol
POO	1-Palmitoyl-2,3-dioleoylglycerol	AOO	1-Arachidoyl-2,3-dioleoylglycerol
PLS	1-Palmitoyl-2-linoleoyl-3-stearoylglycerol		

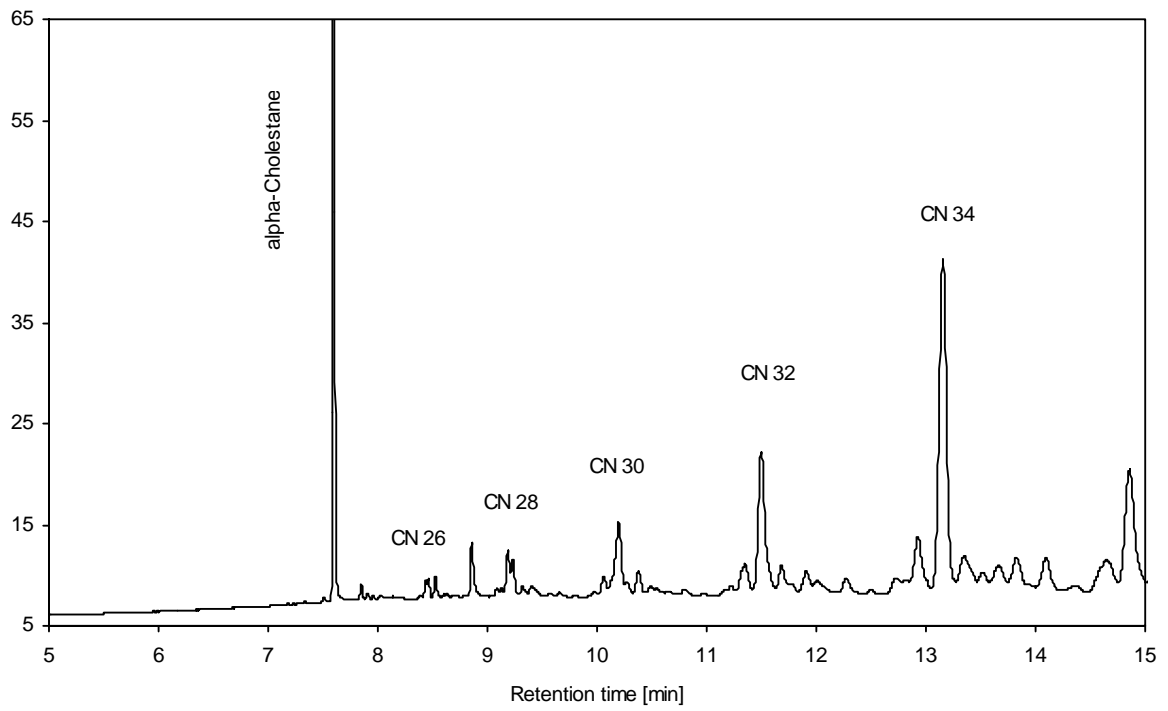
**Figure B 1: Triacylglycerol profile of IRMM-801**



**Figure B 2: Triacylglycerol profile of pure MF, pure CB and a mixture of CB with MF**



**Figure B 3: Triacylglycerol profile of pure milk fat: Chromatogram window for the part were PSB elutes**



**Figure B 4: Triacylglycerol profile of pure milk fat with the addition of alpha-cholestane: Chromatogram window for the part were alpha-cholestane elutes**

European Commission

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- VALIDATED METHOD

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