
**Animal and vegetable fats and oils —
Cocoa butter equivalents in cocoa butter
and plain chocolate —**

Part 2:
**Quantification of cocoa butter
equivalents**

*Corps gras d'origines animale et végétale — Équivalents au beurre
de cacao dans le beurre de cacao et dans le chocolat de ménage —*

Partie 2: Quantification des équivalents au beurre de cacao



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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 23275-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

ISO 23275 consists of the following parts, under the general title *Animal and vegetable fats and oils — Cocoa butter equivalents in cocoa butter and plain chocolate*:

- *Part 1: Determination of the presence of cocoa butter equivalents*
- *Part 2: Quantification of cocoa butter equivalents*

Introduction

“Cocoa butter equivalents” is the general term for fats used to replace cocoa butter in chocolate. They resemble the chemical composition and physical properties of cocoa butter very closely, making them therefore extremely difficult to quantify and even in some cases to detect. In principle, cocoa butter equivalents must by definition be fats low in lauric acid, rich in symmetrical mono-unsaturated triacylglycerols of the type 1,3-dipalmitoyl-2-oleoylglycerol, 1-palmitoyl-2-oleoyl-3-stearoylglycerol and 1,3-distearoyl-2-oleoylglycerol, miscible with cocoa butter, and obtained only by refining and fractionation.

Within the European Union, the following vegetable fats, obtained from the plants listed below, may be used singly or in blends, according to Directive 2000/36/EC [1]:

- illipé, Borneo tallow or tengkawang (*Shorea* spp.),
- palm oil (*Elaeis guineensis*, *Elaeis olifera*),
- sal (*Shorea robusta*),
- shea (*Butyrospermum parkii*),
- kokum gurgi (*Garcinia indica*), and
- mango kernel (*Mangifera indica*).

ISO 23275-1 specifies a procedure for the detection of these fats (restrictions are only made for pure illipé fat samples) in cocoa butter and plain chocolate. This part of ISO 23275 specifies a procedure allowing a reliable quantification of these fats at the level of 5 %, complying with the statutory limit laid down in Directive 2000/36/EC [1] of the European Parliament and the Council.

To facilitate the usage of both parts of ISO 23275, an analytical toolbox named “CoCal-1” has been established. “CoCal-1” contains the validated methods for detection (part 1) and quantification (part 2) of CBEs in plain chocolate, and also a certified cocoa butter reference material (IRMM-801) to calibrate the analyst’s instruments and an electronic evaluation sheet for Microsoft Excel® to calculate the final result. An analyst working on CBE detection and quantification has only to calibrate the gas chromatographic separation system using IRMM-801, separate the triglyceride fractions of the sample in question, and use the electronic evaluation sheet for subsequent data treatment to detect and quantify CBEs.

Animal and vegetable fats and oils — Cocoa butter equivalents in cocoa butter and plain chocolate —

Part 2: Quantification of cocoa butter equivalents

1 Scope

This part of ISO 23275 specifies a procedure for the quantification of cocoa butter equivalents (CBEs) in cocoa butter (CB) and plain chocolate by high-resolution capillary gas chromatography (HR-GC) of triacylglycerols, and subsequent data evaluation by partial least-squares regression analysis.

NOTE The presence of CBEs in CB and plain chocolate down to a level of 0,6 % (fat content of chocolate assumed to be 30 %) can be determined by using the procedure explained in ISO 23275-1. Differences in the procedure of the two methods exist in the number of individual triacylglycerols used for data treatment and in the mathematical evaluation principle of the data. The presence of CBEs is detected by linear regression analysis applied to the relative proportions of the three main triacylglycerol fractions of the fat analysed. The amount of the CBE admixture is estimated by partial least squares regression analysis applied to the relative proportions of the five main triacylglycerols.

2 Terms and definitions

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

2.1

cocoa butter equivalents

CBEs

fats detected in cocoa butter and plain chocolate

2.2

CBE content of cocoa butter

mass fraction of substances in cocoa butter determined by the procedure specified in this part of ISO 23275

NOTE It is expressed in grams per 100 g of cocoa butter.

2.2

CBE content of chocolate

mass fraction of substances in chocolate determined by the procedure specified in this part of ISO 23275

NOTE It is expressed in grams per 100 g of chocolate.

3 Principle

Cocoa butter, or the fat obtained from plain chocolate, is separated by HR-GC into triacylglycerol fractions according to their molecular mass and degree of unsaturation. The added amount of CBEs is estimated by partial least squares regression analysis applied to individual triacylglycerol fractions of the fat analysed.

4 Reagents and materials

Use only reagents of recognized 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.

- 4.1 **Cocoa butter Certified Reference Material (CRM)** IRMM-801 [2], for calibration purposes and system suitability check.
- 4.2 **Fat solvent**, non-chlorinated solvents (e.g. diethyl ether, *n*-heptane, iso-octane).
- 4.3 **Hydrochloric acid**, $c = 8 \text{ mol/l}$.
- 4.4 **Fluted filter paper**¹⁾, 15 cm.

5 Apparatus

- 5.1 **Analytical balance**, with a readability of 0,1 mg.
- 5.2 **Drying oven**, maintained at 55 °C.
A dry heater block may be used.
- 5.3 **Food grater**, a kitchen blender with a design featuring the motor above the receiving container to avoid melting the samples²⁾.
- 5.4 **Volumetric flasks**, of capacity 20 ml.
- 5.5 **Pipettes**, of capacity 1 ml.
- 5.6 **Microsyringe**, with maximum volume 10 µl, graduated to 0,1 µl, or **automatic sampler**.
- 5.7 **Gas chromatograph (GC)**, fitted with a cold on-column injection system and a flame ionization detector (FID).

Alternative injection systems [e.g. a split injector, 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:

- GC column: 25 m to 30 m length, with 0,25 mm i.d., fused silica coated with thermostable 50 % phenylmethylpolysiloxane to a film thickness of 0,1 µm to 0,15 µm.
- temperature programme: 100 °C (initial temperature), programme rate 30 °C/min to 340 °C (final temperature).
- carrier gas: helium or hydrogen (purity $\geq 99,999 \%$).

1) S&S 589 medium filter paper is an example of a suitable product available commercially.
2) Philips HR2833 is an example of suitable equipment commercially available.

This information is given for the convenience of the users of this part of ISO 23275 and does not constitute an endorsement by ISO of these products.

NOTE Suitable columns and alternative experimental conditions, used in an international collaborative study, are listed in Annex A. Operating conditions may be changed to obtain optimum separation of cocoa butter triacylglycerols.

5.8 Chromatographic data system.

5.9 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.

6 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this part of ISO 23275. A recommended sampling method is given in ISO 5555.

7 Preparation of test sample

7.1 Preparation of cocoa butter CRM for calibration purposes and system suitability check

Before opening and using the cocoa butter CRM (4.1), the ampoule shall be warmed in a drying 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 chocolate sample

Chill approx. 200 g of chocolate until hard, and grate to fine granular condition using a mechanical device (5.3). Mix thoroughly and preserve in tightly stoppered bottle in a cool place.

8 Procedure

8.1 Fat extraction

8.1.1 Separate the fat and determine the fat content in a sample of chocolate (prepared as described in 7.2) by Soxhlet extraction^[3], 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 (4.3) and a few defatted boiling chips, or other antibumping agent, and stir. Cover with a watch glass, bring the solution slowly to the boil, and boil gently for 15 min. Rinse the watch glass with 100 ml of water. Filter digest the solution through a medium fluted filter paper (4.4), or equivalent, rinsing the beaker three times with water. Continue washing until 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 then weigh it. Place the thimble containing the dried sample in the Soxhlet apparatus (5.9), supporting it with 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 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 total fat content, c_{fat} , of the chocolate, in grams per 100 g, is calculated as follows:

$$c_{\text{fat}} = \frac{m_{\text{fat}} \times 100}{m}$$

where

m_{fat} is the total fat obtained by extraction, in grams;

m is the mass of the test portion (chocolate), in grams.

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.

Report the fat content to two decimal places.

8.2 Separation of individual triacylglycerols by HR-GC

The test samples [cocoa butter, fat extracted from chocolate, and cocoa butter CRM (4.1)] 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 ca. 55 °C in a drying oven (5.2) in order to avoid partial fat fractionation during handling of samples.

Weigh ca. 0,2 g of test sample in a 20 ml volumetric flask (5.4) and dilute to the mark with a suitable fat solvent (4.2). Pipette (5.5) 1 ml of the resulting solution in another 20 ml volumetric flask and dilute to the mark with the same solvent.

Inject 0,5 µl to 1,0 µl of the final test solution ($\rho_{\text{fat}} = 0,5 \text{ mg/ml}$) into the HR-GC system using the cold on-column injection system.

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

8.3 Identification

Identification of the five major triacylglycerol fractions [1,3-dipalmitoyl-2-oleoylglycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoylglycerol (POS), 1-palmitoyl-2,3-dioleoylglycerol (POO), 1,3-distearoyl-2-oleoylglycerol (SOS) and 1-stearoyl-2,3-dioleoylglycerol (SOO)] is made by comparison of the retention times of the test samples with those of the cocoa butter CRM (4.1). In general, triacylglycerols 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 triacylglycerols of the cocoa butter CRM is given in the example chromatogram (Figure A.1).

9 Calculation

9.1 Determination of response factors

Determine the response factors of the triacylglycerols POP, POS, POO, SOS and SOO by injection of the cocoa butter CRM solution using experimental conditions identical to those used for the samples. Calculate the percentage of each of the five triacylglycerol fractions by the following equations:

$$P_{\text{ref},i} = \frac{A_{\text{ref},i}}{\sum A_{\text{ref},i}} \times 100 \% \quad (1)$$

$$F_i = \frac{w_{\text{ref},i}}{P_{\text{ref},i}} \quad (2)$$

where

$P_{\text{ref},i}$ is the percentage of triacylglycerol i in the cocoa butter CRM (from peak areas);

$A_{\text{ref},i}$ is the peak area of the triacylglycerol i in the cocoa butter CRM;

$\Sigma A_{\text{ref},i}$ is the sum of the peak areas attributed to POP, POS, POO, SOS, SOO in the cocoa butter CRM;

F_i is the detector response factor of triacylglycerol i in the cocoa butter CRM;

$w_{\text{ref},i}$ is the mass fraction, in percent, of triacylglycerol i in the cocoa butter CRM as given in the certificate [2].

Report the results to two decimal places.

9.2 Calculation of percentages of triacylglycerols

Calculate the percentages of the triacylglycerols POP, POS, POO, SOS and SOO in the test sample by

$$w_{\text{test},i} = \frac{F_i \times A_{\text{test},i}}{\sum (F_i \times A_{\text{test},i})} \times 100 \% \quad (3)$$

where

$w_{\text{test},i}$ is the mass fraction, in percent, of triacylglycerol i in the test samples;

$A_{\text{test},i}$ is the peak area corresponding to the triacylglycerol i in the test sample;

F_i is the response factor as determined in 9.1.

Report the results to two decimal places.

9.3 Calculation of CBE content in cocoa butter

The CBE content of the cocoa butter, $c_{\text{CBE,CB}}$, expressed in grams of CBE per 100 g of cocoa butter, is calculated by using a partial least squares regression analysis [Equation (4)] of the relative proportions of the five main triacylglycerols; i.e. %POP + %POS + %POO + %SOS + %SOO = 100 % as determined in Equation (3):

$$c_{\text{CBE,CB}} = 37,439 + 1,175 \times \text{POP} - 1,939 \times \text{POS} - 0,121 \times \text{POO} + 0,982 \times \text{SOS} - 0,097 \times \text{SOO} \quad (4)$$

Report the results to two decimal places in grams per 100 g.

Equation (4) was established by using a calibration set of 798 CB/CBE blends containing 10 %, 15 % and 20 % CBEs. The compulsory use of the cocoa butter CRM in this method for calibration purposes and system suitability check ensures a high comparability of the results between individual testing laboratories and commutability of the elaborated procedure [4].

In 99 % of cases where commercially available fats are used for blend formulation, the prediction error does not exceed $\pm 2,60$ % related to cocoa butter.

9.4 Calculation of CBE content in chocolate

The CBE content of the final product chocolate, $c_{\text{CBE,choc}}$, expressed in grams of CBE per 100 g of chocolate, is calculated by applying Equation (5):

$$c_{\text{CBE,choc}} = \frac{c_{\text{fat}} \cdot c_{\text{CBE,CB}}}{100} \quad (5)$$

where

c_{fat} is the total fat content of the chocolate, in grams per 100 g, as determined in 8.1;

$c_{\text{CBE,CB}}$ is the CBE content of the cocoa butter, in grams per 100 g, as determined in 9.3.

Report the results to two decimal places.

In 99 % of all cases where commercially available fats are used for blend formulation, the prediction error does not exceed $\pm 0,78$ % relative to chocolate (assumed fat content of chocolate 30 %).

10 Procedural requirements

10.1 General

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 (10.2) are met.

10.2 System suitability

The cocoa butter CRM (4.1) shall be used to check the suitability of the separation system.

a) Resolution

The HR-GC separation system shall be capable of separating the critical pairs POS/POO and SOS/SOO with a chromatographic resolution of at least 1,0. In the case of failure, the chromatographic conditions (e.g. sample size, column temperature, carrier gas flow) must be optimized.

b) Determination of detector response factors

To check that the assumption that flame-ionization detector response factors of triacylglycerols do not differ significantly from unity, the cocoa butter CRM shall be analysed applying standard GC conditions. Experience has shown that for a properly functioning chromatographic system the response factors for the five main triacylglycerols (POP, POS, POO, SOS, SOO) vary within a range of 0,80 to 1,20.

11 Precision

11.1 Interlaboratory test

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

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, will in not more than 5 % of cases be greater than 0,721 g/100 g relative to cocoa butter, and 0,216 g/100 g relative to the final product chocolate (with the assumption that the fat content of the chocolate is 30 %).

Values for the repeatability limit, r , as found in the validation study are summarized in Tables A.2 to A.4.

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, will in not more than 5 % of cases be greater than 1,719 g/100 g relative to cocoa butter, and 0,516 g/100 g relative to the final product chocolate (with the assumption that the fat content of the chocolate is 30 %).

Figures for the reproducibility limit, R , as found in the validation study are summarized in Tables A.2 to A.4.

12 Test report

The test report shall specify:

- 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 part of ISO 23275;
- d) all operating details not specified in this part of ISO 23275, 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.

Annex A (informative)

Results of interlaboratory test

The method was validated in 2002 in a European interlaboratory test with 13 participants [5] [6]. The method was validated for CBE levels approx. 5 % CBE in chocolate; i.e. admixture of 15 g, 25 g and 30 g CBE/100 g of cocoa butter corresponding to 4,5 g, 7,5 g and 9,0 g of CBE in chocolate (fat content of the chocolate assumed to be 30 %).

Table A.1 shows suitable GC conditions³⁾. An example of a triacylglycerol profile of the cocoa butter CRM is shown in Figure A.1. The precision results are given in Tables A.2 to A.4.

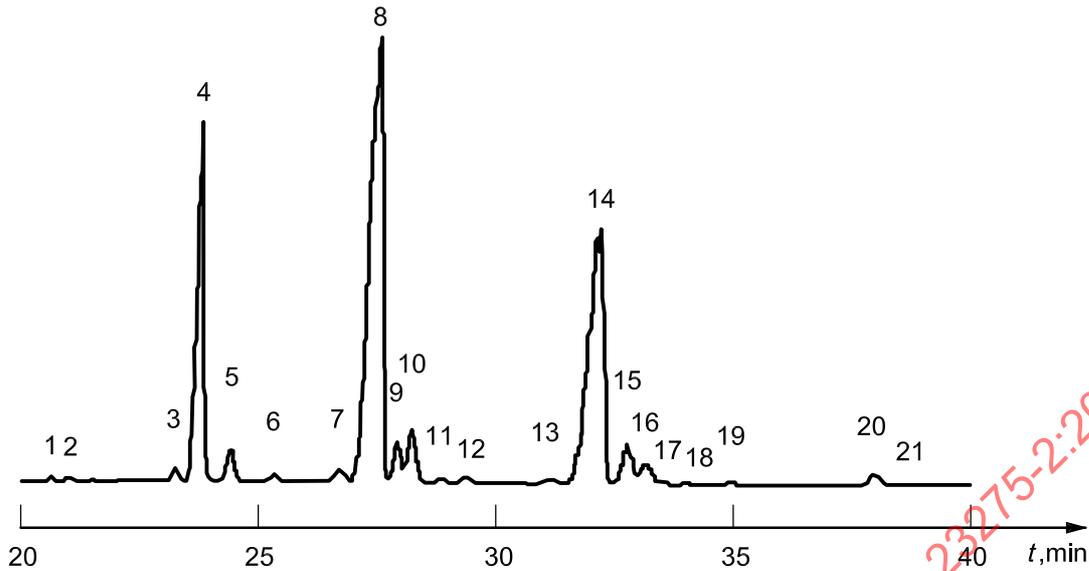
Table A.1 — Suitable GC conditions to be used for triacylglycerol analyses of cocoa butter, CBEs, CB/CBE blends and chocolate

Possible method	1	2	3	4	5
Column characteristics:					
— stationary phase	DB-17HT	RTx-65TG	CB-TAP	RTx-65TG	CB-TAP
— length (m)]	30	30	25	30	25
— i.d. (mm)	0,25	0,25	0,25	0,25	0,25
— film thickness (µm)	0,15	0,1	0,1	0,1	0,1
Temperature mode (oven):					
— injection temperature (°C)/hold time (min)	80/2	340/1	280/0	100/0,5	340/0
— programme rate 1 (°C/min)	50	1	10	50	1
— temperature 1 (°C)/hold time (min)	300/0	—	320/0	330/2	—
— programme rate 2 (°C/min)	30	—	2	1	—
— temperature 2 (°C)/hold time (min)	—	—	—	—	—
— programme rate 3 (°C/min)	—	—	—	—	—
— final temperature (°C)/hold time (min)	350/30	360/3	360/6	350/5	360/10
— injector temperature (°C)	oven track	390	370	oven track	360
— detector temperature (°C)	360	370	370	355	360
Injection mode	OCI	split	split	OCI	split
Carrier gas:					
— type	H ₂	H ₂	H ₂	He	He
— pressure (kPa)	—	120	100	—	150
— flow (ml/min)	0,8	—	—	0,8	—
Sample:					
— concentration (mg/ml)	0,3	50	12,5	0,3	
— volume injected (µl)	0,5	0,1	0,6	0,5	1

3) These types of columns are examples of suitable products available commercially. This information is given for the convenience of users of this part of ISO 23275 and does not constitute an endorsement by ISO of these products.

Table A.1 (continued)

Possible method	6	7	8	9	10	11
Column characteristics:						
— stationary phase	RTx-65TG	CB-TAP	DB-17HT	CB-TAP	CB-TAP	CB-TAP
— length (m)	30	25	30	25	25	25
— i.d. (mm)	0,25	0,25	0,25	0,25	0,25	0,25
— film thickness (µm)	0,1	0,1	0,15	0,1	0,1	0,1
Temperature mode (oven):						
— injection temperature (°C)/hold time (min)	200/0	100/0,1	50/2	200/2	100/1	200/2
— programme rate 1 (°C/min)	15	70	50	20	30	12
— temperature 1 (°C)/hold time (min)	360/0	—	300/1	320/0	300/2	—
— programme rate 2 (°C/min)	1	—	10	1	30	—
— temperature 2 (°C)/hold time (min)	—	—	340/2	—	—	—
— programme rate 3 (°C/min)	—	—	0,5	—	—	—
— final temperature (°C)/hold time (min)	370	350/21	345/26	360/10	340/35	350/10
— injector temperature (°C)	390	oven track	50	65-220-370	100	—
— detector temperature (°C)	390	360	360	370	360	360
Injection mode	split	OCI	OCI	OCI	OCI	hot OCI
Carrier gas:						
— type	H ₂	H ₂	H ₂	He	H ₂	H ₂
— pressure (kPa)	150	—	120	90	150	—
— flow (ml/min)	—	1	—	—	—	2,4
Sample:						
— concentration (mg/ml)	10	15	0,5	1 - 2	0,5	0,65
— volume injected (µl)	0,5	0,5	0,5	0,1	0,4	0,3



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

GC column: 25 m × 0,25 mm fused silica capillary column coated with 0,1 µm Chrompack TAP
 Oven temperature: 100 °C held for 1 min; 30 °C/min to 340 °C held for 35 min
 Injector: Cold on-column
 Detector (FID): 360 °C
 Carrier gas: H₂ at 1,6 bar head pressure
 Amount injected: 0,5 µl of a 0,5 mg/ml solution

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 A.1 — Triacylglycerol profile of the cocoa butter CRM