



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements
Food Safety and Quality Unit
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Validated Method

Method Description for the Quantification of Cocoa Butter Equivalents in Cocoa Butter and Plain Chocolate

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1 Scope and Field of Application

This Standard specifies a validated procedure for the quantification of cocoa butter equivalents (CBE) in cocoa butter (CB) and plain chocolate by high-resolution capillary gas chromatography (HR-GC) of triglycerides and subsequent data evaluation by Partial Least Squares regression analysis. The intended application of the method is the assessment of compliance with the statutory limit of 5% CBE addition to as laid down in the European Chocolate Directive 2000/36/EC [1]. The compulsory use of the cocoa butter Certified Reference Material (IRMM 801) in this Standard for calibration purpose and system suitability check ensures high comparability of the results between individual testing laboratories and ensures the commutability of the elaborated procedure.

Note: The presence of CBEs in CB and plain chocolate down to a level of 0.4% (fat content of chocolate assumed to be 20%) can be determined by a procedure elaborated and validated by the Joint Research Centre [2].

2 Principle

Cocoa butter, or the fat obtained from plain chocolate according to the Soxhlet principle [3], is separated by HR-GC into triglyceride fractions according to their molecular weight and degree of unsaturation. The added amount of CBEs is estimated by Partial Least Squares regression analysis applied to individual triglyceride fractions of the fat analysed.

3 Definition

For the purpose of this procedure, the following definitions apply:

CBE content of cocoa butter: The mass fraction of substances determined by the procedure specified in this method description. It is expressed in gram per 100 grams cocoa butter.

CBE content of chocolate: The mass fraction of substances determined by the procedure specified in this method description. It is expressed in gram per 100 grams of chocolate.

4 Reagents and Materials

All reagents shall be of recognised analytical grade, unless otherwise stated.

4.1 Fat solvent (non-chlorinated solvents e.g. *n*-heptane, *iso*-octane)

4.2 Cocoa butter Certified Reference Material (IRMM-801), for calibration purposes and system suitability check

5 Apparatus

5.1 Balance, sensitivity ± 1 mg

5.2 Volumetric flasks, of capacity 20 mL

5.3 Pipettes, of capacity 1 mL

5.4 Drying oven, maintained at 55 °C (dry heater block may be used)

5.5 Gas chromatograph (GC): a chromatograph fitted with a cold on-column injection system and a flame ionisation detector (FID). (*Note: alternative injection system, 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 9.1*)

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

- GC column: 25 - 30 m x 0.25 i.d. fused silica coated with thermo stable 50 % phenylmethylpolysiloxane with a film thickness of 0.1 – 0.15 μm (*Note: Suitable columns are listed in Annex A, Table A 1*)
- temperature programme: 100 °C (initial temperature), programme rate 30 °C/min to 340 °C (final temperature). (*Note: Operating conditions may be changed to obtain optimum separation of cocoa butter triglycerides*)
- carrier gas: helium or hydrogen (purity ≥ 99.999 %)

Alternative experimental conditions, used in an international collaborative study, are listed in Annex A, Table A 1.

5.6 Chromatographic data system

5.7 Micro syringe: maximum volume 10 μl , graduated to 0.1 μl (*Note: an automatic sampler may be used*)

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

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

Before opening and using the cocoa butter CRM (4.2), the ampoule has to be warmed in a drying oven (5.4) until the contents have melted. When a clear solution is obtained, mix the contents by repeated inversion for not less than 20 sec., open and transfer the contents to a clean vial, which can be tightly sealed and preserved in a cool place for future usage.

7 Preparation of chocolate samples prior to extraction

According to AOAC Official Method 970.20 [4] chill ca 200 g chocolate until hard, and grate to fine granular condition using a mechanical device (5.8). Mix thoroughly and preserve in tightly stoppered bottle in a cool place.

8 Procedure

8.1 Fat extraction

Obtain the fat and determine the fat content from chocolate prepared as described in (7) by Soxhlet extraction according to the AOAC Official Method 963.15 [3]. (*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*).

8.2 Separation of individual triglycerides by HR-GC

The test samples (cocoa butter, fat extracted from chocolate, cocoa butter CRM (4.2)) have to be warmed in a drying oven (5.4) 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.

Weigh ca 0.2 g test sample in a 20 mL volumetric flask (5.2) and bring to volume with a suitable fat solvent (4.1). Pipette 1 mL of the resulting solution in another 20 mL volumetric flask and bring to volume with the same solvent.

Inject 0.5-1.0 µL of the final test solution (0.5 mg fat/mL) into the HR-GC 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 (9.1) are met)

8.3 Identification

Identification of the five major triglyceride 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 samples with those of the cocoa butter CRM (4.2). The elution order of the triglycerides of the cocoa butter CRM is given in the example chromatogram (Annex A, Figure A 1).

8.4 Calculation

8.4.1 Determination of response factors

Determine the response factors of the triglycerides 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 area percentage for each of the five triglyceride fractions by:

$$AR_i [\%] = \frac{AR_i}{\sum AR_i} \times 100 \quad \text{[Equation 1]}$$

$$RF_i = \frac{MR_i [\%]}{AR_i [\%]} \quad \text{[Equation 2]}$$

AR_i area under the peak corresponding to triglyceride i in the cocoa butter CRM

$\sum AR_i$ sum of the areas under the peaks attributed to POP, POS, POO, SOS, SOO in the cocoa butter CRM

MR_i [%] mass% of triglyceride i in the cocoa butter CRM as given in the Certificate [5]

AR_i [%] area% of triglyceride i in the cocoa butter CRM

RF_i detector response factor of triglyceride i in the cocoa butter CRM.

8.4.2 Calculation of weight percentages of triglycerides

Calculate the weight percentage of the triglycerides POP, POS, POO, SOS and SOO in the test sample by

$$MS_i [\%] = \frac{RF_i \times AS_i}{\sum (RF_i \times AS_i)} \times 100 \quad \text{[Equation 3]}$$

AS_i area under the peak corresponding to the triglyceride i in the test sample

RF_i response factors as determined in 8.4.1

MS_i [%]..... mass% of triglyceride i in the test samples.

8.4.3 Calculation of %CBE in cocoa butter

The CBE content of the cocoa butter, expressed in grams CBE per 100 grams of cocoa butter, is calculated by using a Partial Least Squares regression analysis (Equation 4) of the relative proportions of the five main triglycerides, i.e. POP+POS+POO+SOS+SOO=100% as determined in Equation 3.

$$\%CBE \text{ in cocoa butter} = 37.439 + 1.175 \times POP - 1.939 \times POS - 0.121 \times POO + 0.982 \times SOS - 0.097 \times SOO \quad \text{[Equation 4]}$$

This equation was established by using arithmetically simulated CB/CBE blends calculated on the basis of a standardised database of the triglyceride profile of a broad range of cocoa butter and CBE samples [6]. The cocoa butter CRM (4.2) was used to standardise the applied analytical methodology for the determination of the triglyceride profiles.

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

8.4.3 Calculation of %CBE in chocolate

The CBE content of the final product chocolate, expressed in grams CBE per 100 grams of chocolate, is calculated by applying Equation 5.

$$\%CBE \text{ in chocolate} = \%fat \times \%CBE \text{ in cocoa butter}/100 \quad \text{[Equation 5]}$$

%fat g fat/100 g chocolate as determined in 8.1

%CBE in cocoa butter as determined in 8.4.3

In 99% of cases where commercially available fats are used for blend formulation the prediction error does not exceed $\pm 0.4\%$ related to chocolate.

9 Notes on Procedure

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 (9.1) are met.

9.1 System suitability

The cocoa butter CRM (4.2) has to be used to check the suitability of the separation system.

9.1.1 Resolution

The HR-GC separation system must be capable of separating the critical pairs POS/POO and SOS/SOO with a chromatographic resolution of at least 1.0. In case of failure, the chromatographic conditions (e.g. sample size, column temperature, carrier gas flow, etc) have to be optimised.

9.1.2 Determination of detector response factors

Experience has shown that for a properly functioning chromatographic system the response factors for the five main triglycerides (POP, POS, POO, SOS, SOO) vary within a range of 0.8 – 1.2.

10 Validation data

The analytical approach was validated in a European collaborative study with 13 participants. The method was validated for CBE levels around the statutory addition limit of 5% CBE in chocolate, i.e. admixture of 15, 25 and 30 g CBE/100 g cocoa butter corresponding to 3, 5 and 6 g of CBE in chocolate (fat content of the chocolate assumed to be 20%). Details of the collaborative study on the precision of the method are listed in Annex A, Tables A 2 to A 4.

10.1 Repeatability

The absolute difference between two single test results found on an identical test material by one operator using the same apparatus within the shortest feasible interval will exceed the repeatability limits r for individual triglycerides in not more than 5% of the cases. Figures for r as found in the validation study are summarised in Table A 2 to A 4. Based upon these results the repeatability limit is 0.8 g/100 g related to cocoa butter and 0.1 g/100 g related to the final product chocolate with the assumption that the fat content of the chocolate is 20%.

10.2 Reproducibility

The absolute difference between two single test results on an identical test material reported by two laboratories will exceed the reproducibility limits R for individual triglycerides in not more than 5% of the cases. Figures for R as found in the validation study are summarised in Table A 2 to A 4. Based upon these results the reproducibility limit is 1.5 g/100 g related to cocoa butter and 0.3 g/100 g related to the final product chocolate with the assumption that the fat content of the chocolate is 20%.

Reference:

- [1] Directive 2000/36/EC of the European Parliament and the Council of 23 June 2000 relating to cocoa and chocolate products intended for human consumption. OJ L197, 19-25.
- [2] M. Buchgraber, E. Anklam (2003). Validation of a method for the detection of cocoa butter equivalents in cocoa butter and plain chocolate. EUR 20685 EN.
- [3] Official Methods of Analysis of AOAC International (1995). AOAC Official Method 963.15 - Fat in Cacao Products.
- [4] Official Methods of Analysis of AOAC International (1995). AOAC Official Method 970.20 - Cacao Products. Preparation of Sample.
- [5] R. Koeber et al. (2003). The certification of the content of five triglycerides in cocoa butter. EUR 20781 EN.
- [6] M. Buchgraber et al.. Publication in preparation.

Annex A

(informative)

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

<i>Method possibility</i>	1	2	3	4	5
<u>Column characteristics</u>					
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
<u>Temperature mode</u>					
- 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
<u>Injection mode</u>					
	OCI	split	split	OCI	split
<u>Carrier gas</u>					
type	H ₂	H ₂	H ₂	He	He
pressure [kPa]	-	120	100	-	150
flow [mL/min]	0.8	-	-	0.8	-
<u>Sample</u>					
concentration [mg/mL]	0.3	50	12.5	0.3	
volume injected [µl]	0.5	0.1	0.6	0.5	1

<i>Method possibility</i>	6	7	8	9	10	11
<u>Column characteristics</u>						
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
<u>Temperature mode</u>						
- 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
<u>Injection mode</u>						
	split	OCI	OCI	OCI	OCI	hot OCI
<u>Carrier gas</u>						
type	H ₂	H ₂	H ₂	He	H ₂	H ₂
pressure [kPa]	150	-	120	90	150	-
flow [mL/min]	-	1	-	-	-	2.4
<u>Sample</u>						
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

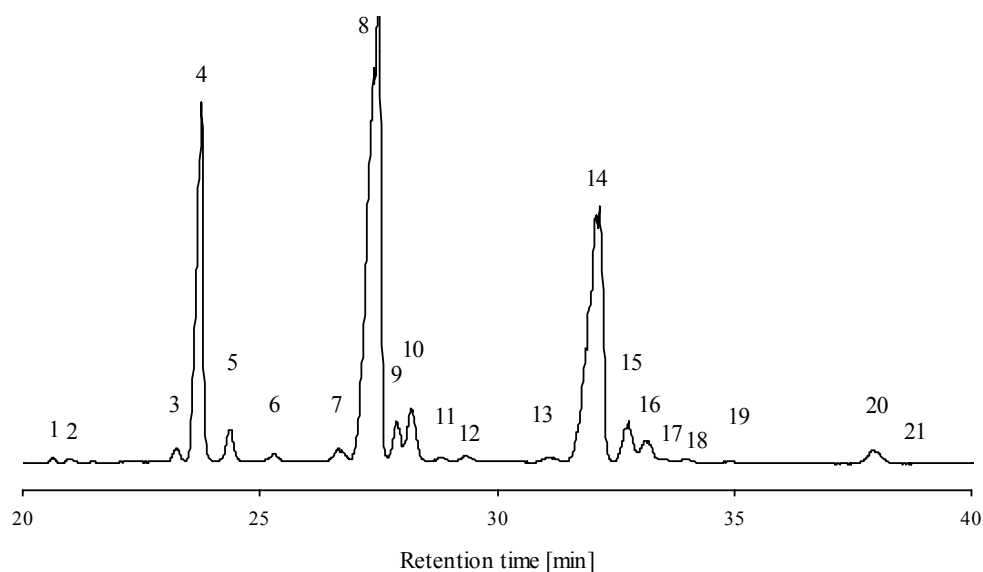


Figure A. 1: Triglyceride profile of the cocoa butter CRM

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 x 0.25 mm fused silica capillary column coated with 0.1 μm Chrompack TAP
Oven temperature	100 °C held for min, 30 °C/min to 340 ° held for 35 min
Injector	Cold on-column
Detector (FID)	360 °C
Carrier gas	H ₂ at 1.6 bar head pressure
Amount injected	0.5 μl of a 0.4 mg/mL solution

Abbreviations:

PPP: tripalmitin
MOP: 1-margaroyl-2-oleoyl-3-palmitoylglycerol
PPS: 1,2-dipalmitoyl-3-stearoylglycerol
POP: 1,3-dipalmitoyl-2-oleoylglycerol
PLP: 1,3-dipalmitoyl-2-linoleoylglycerol
PSS: 1-palmitoyl-2,3-distearoylglycerol

POS: 1-palmitoyl-2-oleoyl-3-stearoylglycerol
POO: 1-palmitoyl-2,3-dioleoylglycerol
PLS: 1-palmitoyl-2-linoleoyl-3-stearoylglycerol
PLO: 1-palmitoyl-2-linoleoyl-3-oleoylglycerol
SSS: tristearin
SOS: 1,3-distearoyl-2-oleoylglycerol
SOO: 1-stearoyl-2,3-dioleoylglycerol
SLS: 1,3-distearoyl-2- linoleoyl glycerol
OOO: triolein
SLO: 1- stearoyl -2-linoleoyl-3-oleoylglycerol
SOA: 1-stearoyl-2-oleoyl-arachidoylglycerol
AOO: 1-arachidoyl-2,3- dioleoylglycerol

Table A 2: Precision data – Chocolate sample and CB/CBE blends prepared with a CB from Grenada and a CBE of the type PMF/(Shea + Illipé) [35/65]

<i>Sample</i>	<i>Chocolate sample</i>	<i>Low level</i>	<i>Medium level</i>	<i>High level</i>
Year of collaborative study	2002			
Number of laboratories	13	13	13	13
Number of outliers	1	1	0	0
Number of accepted results	12	12	13	13
Mean value, g CBE/100 g CB	13.99	15.14	24.80	29.56
True value, g CBE/100 g CB	-	14.79	24.92	30.01
Bias, g CBE/100 g CB	-	-0.35	0.12	0.45
Repeatability standard deviation s_r , g/100 g	0.26	0.17	0.18	0.22
Repeatability relative standard deviation RSD_r , %	1.8	1.1	0.7	0.8
Repeatability limit r [$r = 2.8 \times s_r$], g/100 g	0.72	0.47	0.51	0.62
Reproducibility standard deviation s_R , g/100 g	0.61	0.36	0.41	0.54
Reproducibility relative standard deviation RSD_R , %	4.4	2.4	1.7	1.8
Reproducibility limit R [$R = 2.8 \times s_R$], g/100 g	1.72	1.00	1.15	1.51
HORRAT value	1.6	0.9	0.7	0.8

Table A 3: Precision data – CB/CBE blends prepared with a CB from Ghana and a CBE of the type PMF/Shea [50/50]

<i>Sample</i>	<i>Low level</i>	<i>Medium level</i>	<i>High level</i>
Year of collaborative study	2002		
Number of laboratories	13	13	13
Number of outliers	2	1	0
Number of accepted results	11	12	13
Mean value, g CBE/100 g CB	16.63	25.87	30.69
True value, g CBE/100 g CB	15.07	24.90	30.01
Bias, g CBE/100 g CB	-1.56	-0.97	-0.68
Repeatability standard deviation s_r , g/100 g	0.21	0.26	0.16
Repeatability relative standard deviation RSD_r , %	1.3	1.0	0.5
Repeatability limit r [$r = 2.8 \times s_r$], g/100 g	0.60	0.72	0.44
Reproducibility standard deviation s_R , g/100 g	0.23	0.43	0.45
Reproducibility relative standard deviation RSD_R , %	1.4	1.7	1.5
Reproducibility limit R [$R = 2.8 \times s_R$], g/100 g	0.64	1.20	1.26
HORRAT value	0.5	0.7	0.6

Table A 4: Precision data – CBE/CB blends prepared with a CB from Ivory Coast and a CBE of the type PMF/Sal/Mango [50/25/25]

<i>Sample</i>	<i>Low level</i>	<i>Medium level</i>	<i>High level</i>
Year of collaborative study	2002		
Number of laboratories	13	13	13
Number of outliers	0	1	0
Number of accepted results	13	12	13
Mean value, g CBE/100 g CB	15.65	24.17	28.53
True value, g CBE/100 g CB	14.97	24.97	29.96
Bias, g CBE/100 g CB	-0.68	0.8	1.43
Repeatability standard deviation s_r , g/100 g	0.22	0.19	0.22
Repeatability relative standard deviation RSD_r , %	1.4	0.8	0.8
Repeatability limit r [$r = 2.8 \times s_r$], g/100 g	0.63	0.53	0.62
Reproducibility standard deviation s_R , g/100 g	0.31	0.36	0.41
Reproducibility relative standard deviation RSD_R , %	2.0	1.5	1.4
Reproducibility limit R [$R = 2.8 \times s_R$], g/100 g	0.87	1.00	1.14
HORRAT value	0.7	0.6	0.6