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Validated Method

Method Description for the Detection 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 detection 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 regression analysis. The method has been validated for the detection of 2% CBE admixture to cocoa butter corresponding to about 0.4% in chocolate (assumed fat content of chocolate 20%).

2   Principle

Cocoa butter, or the fat obtained by solvent extraction from plain chocolate, is separated by HR-GC into triglyceride fractions according to their molecular weight and degree of unsaturation. The presence of CBEs is detected by linear regression analysis applied to individual triglyceride fractions of the fat analysed.

3   Reagents and Materials

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

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

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

4   Apparatus

4.1 Balance, sensitivity ±1 mg
4.2 Volumetric flasks, of capacity 20 mL
4.3 Pipettes, of capacity 1 mL
4.4 Drying oven, maintained at 55 ºC (dry heater block may be used)
4.5 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 8.1*)

The separation and detection 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 to 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.
4.6 Chromatographic data system
4.7 Micro syringe: maximum volume 10 µl, graduated to 0.1 µl. (*Note: an automatic sampler may be used*)

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

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

Before opening and using the cocoa butter CRM (3.2), the ampoule has to be warmed in a drying oven (4.4) 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.

6 Preparation of the test sample

According to AOAC Official Method 970.20 [1] chill ca 200 g chocolate until hard, and grate to fine granular condition using a mechanical device (4.8). Mix thoroughly and preserve in tightly stoppered bottle in a cool place.

7 Procedure

7.1 Fat extraction

Obtain the fat from the chocolate prepared as described in (6) according to the AOAC Official Method 920.75 [2]. That is to say, fat is separated from 10 − 40 g grated chocolate by extracting with two or three 100 mL portions of ether. (*Note: alternative extraction procedures may be used, e.g. by Soxhlet, by supercritical carbon dioxide or by using microwaves, provided that the same results are obtained*).

7.2 Separation of individual triglycerides by HR-GC

The test samples (cocoa butter, fat extracted from chocolate, cocoa butter CRM (3.2)) have to be warmed in a drying oven (4.4) until completely melted. If the liquid sample contains 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.

Weigh ca 0.2 g test sample in a 20 mL volumetric flask (4.2) and bring to volume with a suitable fat solvent (3.1). 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 (8.1) are met*).

7.3 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 (3.2). The elution order of the triglycerides of the cocoa butter CRM is given in the example chromatogram (Annex A, Figure A.1).

7.4 Calculation

7.4.1 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:

$$AR_i \; [\%] = \frac{AR_i}{\sum AR_i} \times 100 \qquad \textbf{[Equation 1]}$$

$$RF_i = \frac{MR_i \, [\%]}{AR_i \, [\%]} \qquad \textbf{[Equation 2]}$$

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 [3]

AR$_i$ [%] ..... area-% of triglyceride i in the cocoa butter CRM

RF$_i$ detector response factor of triglyceride i in the cocoa butter CRM.

7.4.2 Calculation of weight percentages of triglycerides

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

$$MS_i \; [\%] = \frac{RF_i \times AS_i}{\sum (RF_i \times AS_i)} \times 100 \qquad \textbf{[Equation 3]}$$

AS$_i$............ area under the peak corresponding to the triglyceride i in the test sample

RF$_i$............ response factors as determined in 7.4.1

MS$_i$ [%] ..... mass-% of triglyceride i in the test samples.

7.4.3 Decision if sample is pure cocoa butter

The variability of the triglyceride composition is expressed by Equation 4 using the normalised triglycerides, i.e. %POP+%POS+%SOS=100% as determined in Equation 3:

POP = 43.734 – 0.733 x SOS (residual standard deviation = 0.125)   **[Equation 4]**

This equation was established by using a standardised data base of the triglyceride profile of 74 individual genuine cocoa butters evaluated and in-house validated by the authors. The cocoa butter CRM (3.2) was used to standardise the applied analytical methodology for the determination of the triglyceride profile of the cocoa butters.

For 99% of all analyses, pure cocoa butter complies with:

$$POP < 44.025 - 0.733 \times SOS \qquad \text{[Equation 5]}$$

A greater value of POP, as given by Equation 5, means that the sample is not pure cocoa butter.

8 Notes on Procedure

The details of the chromatographic procedure depend, among other factors, on the equipment, the type, age, and supplier of the column, the means of introduction of the test solution, the sample size, and the detector. Different column lengths and brands may be used, and injection volumes may be varied, if the requirements of the system suitability tests (8.1) are met.

8.1 System suitability

The cocoa butter CRM (3.2) has to be used to check the suitability of the separation system.

8.1.1 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.

8.1.2 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.

9 Validation data

The analytical approach was validated in a European collaborative study with 13 participants [4]. The results derived from this interlaboratory test demonstrate that the procedure performs well with a detection limit of at least 2% CBE admixture to CB corresponding to 0.4% CBE in chocolate (assumed fat content of chocolate 20%) without giving false-positive or false-negative results.

Note: As an aid to verify the proper functioning of the chromatographic system precision data for the three triglycerides POP, POS and SOS (normalised to 100%) are given below. Details of the collaborative study on the precision of the method are listed in Annex A, Tables A.2 to A.5.

9.1 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.

For POP values ranging from *18.99 to 25.37 g/100 g:*   *r = 0.514 g/100 g*

For POS values ranging from *43.76 to 47.73 g/100 g:* $r = 0.293 \, g/100 \, g$

For SOS values ranging from *30.87 to 33.80 g/100 g:* $r = 0.621 \, g/100 \, g$

9.2 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.

For POP values ranging from *18.99 to 25.37 g/100 g:* $r = 0.741 \, g/100 \, g$

For POS values ranging from *43.76 to 47.73 g/100 g:* $r = 0.588 \, g/100 \, g$

For SOS values ranging from *30.87 to 33.80 g/100 g:* $r = 0.782 \, g/100 \, g$

Note: Weight percentage data for the three major fractions POP, POS and SOS of the test sample have to be calculated and normalised to 100%.

Repeatability limits r and reproducibility limits R for individual triglycerides derived from this collaborative study may be applicable to mean values beyond the given range.

Reference:

[1] Official Methods of Analysis of AOAC International (1995). AOAC Official Method 970.20 - Cacao Products. Preparation of Sample.

[2] Official Methods of Analysis of AOAC International (1995). AOAC Official Method 920.75 - Separation of Fat in Cacao Products.

[3] R. Koeber, M. Buchgraber, F. Ulberth, R. Bacarolo, A. Bernreuther, H. Schimmel, E. Anklam, J. Pauwels (2003) The certification of the content of five triglycerides in cocoa butter (in press).

[4] M. Buchgraber, E. Anklam (2003). Validation of a method for the detection of cocoa butter equivalents in cocoa butter and plain chocolate. Report on the validation study. EUR 20685 EN, ISBN 92-894-5510-1.

Annex A

(informative)

Table A. 1: Suitable GC conditions to be used for triglyceride analyses of cocoa butter

Method possibility	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

| *Method possibility*	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

Retention time [min]
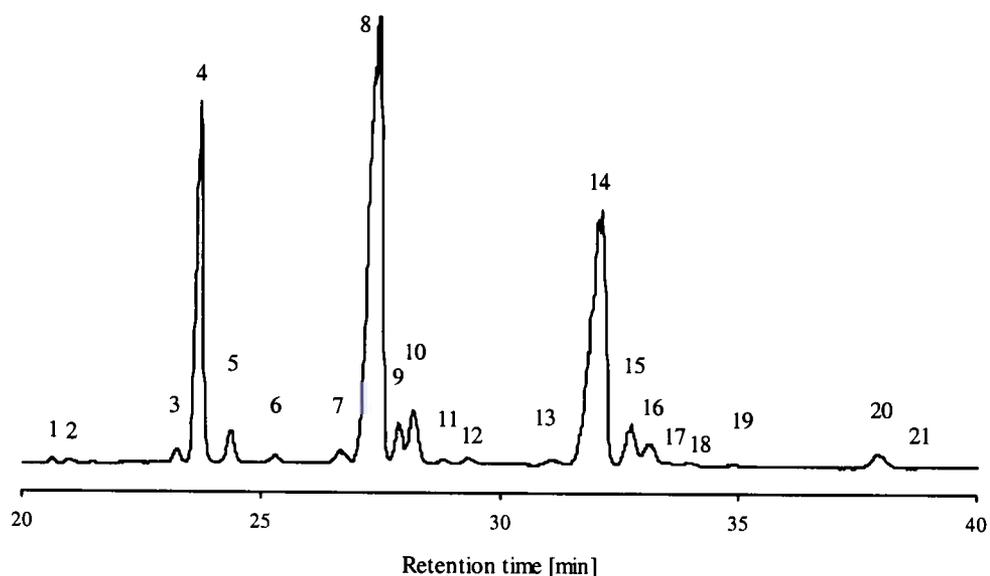
Figure A. 1: Triglyceride profile of the cocoa butter CRM

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

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Table A. 2: Precision data – pure cocoa butter samples

Year of collaborative study	pure CB [1]			pure CB [2]			pure CB [3]		
	2002								
	POP	POS	SOS	POP	POS	SOS	POP	POS	SOS
Number of laboratories	13	13	13	13	13	13	13	13	13
Number of outliers	1	1	1	1	1	1	1	1	1
Number of accepted results	12	12	12	12	12	12	12	12	12
Mean value (g/100 g)	20.40	47.73	31.87	19.49	47.42	33.09	18.99	47.21	33.80
Repeatability standard deviation s_r, g/100 g	0.06	0.06	0.09	0.07	0.09	0.10	0.09	0.08	0.15
Repeatability relative standard deviation RSD_r, %	0.3	0.1	0.3	0.4	0.2	0.3	0.5	0.2	0.5
Repeatability limit r [r = 2.8 x s_r], g/100 g	0.16	0.16	0.26	0.19	0.27	0.27	0.26	0.24	0.43
Reproducibility standard deviation s_R, g/100 g	0.14	0.11	0.17	0.08	0.15	0.17	0.12	0.09	0.17
Reproducibility relative standard deviation RSD_R, %	0.7	0.2	0.5	0.4	0.3	0.5	0.6	0.2	0.5
Reproducibility limit R [R = 2.8 x s_R], g/100 g	0.40	0.30	0.47	0.23	0.41	0.46	0.34	0.25	0.47

(1) Country origin: Grenada
(2) Country origin: Ghana
(3) Country origin: Ivory Coast

Table A. 3: Precision data – real chocolate samples

Year of collaborative study	chocolate [CBE added]			chocolate [no CBEs added]		
	2002					
	POP	POS	SOS	POP	POS	SOS
Number of laboratories	13	13	13	13	13	13
Number of outliers	2	2	2	1	1	1
Number of accepted results	11	11	11	12	12	12
Mean value (g/100 g)	25.37	43.76	30.87	19.74	47.40	32.86
Repeatability standard deviation s_r, g/100 g	0.13	0.10	0.18	0.09	0.07	0.07
Repeatability relative standard deviation RSD_r, %	0.5	0.2	0.6	0.5	0.1	0.2
Repeatability limit r [r = 2.8 x s_r], g/100 g	0.36	0.28	0.50	0.25	0.20	0.21
Reproducibility standard deviation s_R, g/100 g	0.26	0.21	0.20	0.12	0.11	0.11
Reproducibility relative standard deviation RSD_R, %	1.0	0.5	0.6	0.6	0.2	0.3
Reproducibility limit R [R = 2.8 x s_R], g/100 g	0.74	0.59	0.55	0.34	0.30	0.31

Table A. 4: Precision data – cocoa butter with ca 2% CBE admixture

Year of collaborative study	2.06% CBE/CB [1]			1.98% CBE/CB [2]			2.04% CBE/CB [3]			2.09% CBE/CB [4]		
	2002											
	POP	POS	SOS	POP	POS	SOS	POP	POS	SOS	POP	POS	SOS
Number of laboratories	13	13	13	13	13	13	13	13	13	13	13	13
Number of outliers	0	0	0	1	1	1	2	2	2	1	1	1
Number of accepted results	13	13	13	12	12	12	11	11	11	12	12	12
Mean value (g/100 g)	20.61	47.08	32.31	19.94	46.80	33.27	19.70	46.62	33.68	19.86	46.65	33.50
Repeatability standard deviation s_r, g/100 g	0.18	0.10	0.22	0.05	0.07	0.08	0.09	0.06	0.14	0.03	0.08	0.08
Repeatability relative standard deviation RSD_r, %	0.9	0.2	0.7	0.3	0.1	0.2	0.5	0.1	0.4	0.1	0.2	0.2
Repeatability limit r [r = 2.8 x s_r], g/100 g	0.51	0.27	0.62	0.14	0.20	0.23	0.26	0.18	0.38	0.08	0.23	0.22
Reproducibility standard deviation s_R, g/100 g	0.19	0.13	0.25	0.12	0.09	0.18	0.11	0.14	0.15	0.09	0.12	0.18
Reproducibility relative standard deviation RSD_R, %	0.9	0.3	0.8	0.6	0.2	0.5	0.6	0.3	0.4	0.5	0.3	0.6
Reproducibility limit R [R = 2.8 x s_R], g/100 g	0.54	0.37	0.70	0.33	0.25	0.51	0.32	0.40	0.42	0.26	0.33	0.52

(1) CB from Grenada mixed with CBE type PMF/(Shea + Illipé) [35/65]
(2) CB from Ghana mixed with CBE type PMF/Shea [50/50]
(3) CB from Ivory coast mixed with CBE type PMF/Sal/Mango [50/25/25]
(4) CB from Ivory coast mixed with CBE type PMF/Shea/Illipé [50/25/25]

Table A. 5: Precision data – cocoa butter with ca 4% CBE admixture

	4.05% CBE/CB [1]			3.96% CBE/CB [2]			4.04% CBE/CB [3]			4.00% CBE/CB [4]		
Year of collaborative study	2002											
	POP	POS	SOS	POP	POS	SOS	POP	POS	SOS	POP	POS	SOS
Number of laboratories	13	13	13	13	13	13	13	13	13	13	13	13
Number of outliers	0	0	0	1	1	1	1	1	1	1	1	1
Number of accepted results	13	13	13	12	12	12	12	12	12	12	12	12
Mean value (g/100 g)	20.75	46.34	32.91	20.35	46.18	33.48	20.42	46.08	33.50	20.76	46.10	33.14
Repeatability standard deviation s_r, g/100 g	0.14	0.08	0.17	0.07	0.05	0.07	0.12	0.10	0.13	0.11	0.08	0.13
Repeatability relative standard deviation RSD_r, %	0.7	0.2	0.5	0.4	0.1	0.2	0.6	0.2	0.4	0.5	0.2	0.4
Repeatability limit r [$r = 2.8 \times s_r$], g/100 g	0.39	0.22	0.48	0.21	0.15	0.21	0.32	0.29	0.38	0.31	0.22	0.35
Reproducibility standard deviation s_R, g/100 g	0.16	0.18	0.28	0.21	0.07	0.21	0.16	0.15	0.20	0.16	0.13	0.23
Reproducibility relative standard deviation RSD_R, %	0.8	0.4	0.8	1.1	0.1	0.6	0.8	0.3	0.6	0.8	0.3	0.7
Reproducibility limit R [$R = 2.8 \times s_R$], g/100 g	0.44	0.51	0.78	0.60	0.18	0.60	0.44	0.41	0.55	0.46	0.35	0.63

(1) CB from Grenada mixed with CBE type PMF/(Shea + Illipé) [35/65]
(2) CB from Ghana mixed with CBE type PMF/Shea [50/50]
(3) CB from Ivory Coast mixed with CBE type PMF/Sal/Mango [50/25/25]
(4) CB from Ivory Coast mixed with CBE type PMF/Shea/Illipé [50/25/25]

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