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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

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

Partie 1: Détermination de la présence d'équivalents au beurre de cacao

Reference number
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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 23275-1 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*).

This part of ISO 23275 specifies a procedure for the detection of these fats (restrictions are only made for pure illipé fat samples) in cocoa butter and plain chocolate. ISO 23275-2 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.

Information on "CoCal-1" is available on the website of the Institute for Reference Materials and Measurements: http://www.irmm.jrc.be.

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

1 Scope

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

The method is applicable for the detection of 2 % CBE admixture to cocoa butter, corresponding to about 0,6 % CBE in chocolate (i.e. the assumed fat content of chocolate is 30 %).

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 according the procedure specified in this part of ISO 23275

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

3 Principle

Cocoa butter, or the fat obtained by solvent extraction from plain chocolate, is separated by HR-GC into triacylglycerol fractions according to their molecular mass and degree of unsaturation. The presence of CBEs is detected by linear 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).

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, i.e. a kitchen blender with a design featuring the motor above the receiving container to avoid melting the samples[1)].

5.4 Rotary evaporator.

Alternative evaporation procedures may be used.

5.5 Pipettes, of capacity 1 ml.

5.6 Volumetric flasks, of capacity 20 ml.

5.7 Microsyringe, with maximum volume 10 µl, graduated to 0,1 µl, or **automatic sampler**.

5.8 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 detection have been found 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 $\geqslant$ 99,999 %).

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.

5.9 Chromatographic data system.

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.

1) Philips HR2833 is an example of suitable equipment available commercially.

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 this product.

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

Separate the fat from 10 g to 40 g of grated chocolate (as described in 7.2) by extracting with two or three 100 ml portions of a fat solvent (4.2). Centrifuge and decant. Combine the extracts and evaporate most of the fat solvent (5.4) then finally dry it under a stream of nitrogen.

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.

8.2 Separation of individual triacylglycerols by HR-GC

The test samples [cocoa butter, fat extracted from chocolate, cocoa butter CRM (4.1)] shall be warmed in a drying oven (5.2) 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 (5.2) in order to avoid partial fat fractionation.

Weigh ca. 0,2 g of test sample in a 20 ml volumetric flask (5.6) and dilute to the mark with a suitable fat solvent (4.2). Pipette (5.5) 1 ml of the resulting solution into 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 (ρ_{fat} = 0,5 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:

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

$$F_i = \frac{w_{\text{ref},i}}{P_{\text{ref},i}} \tag{2}$$

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 and SOS in the test sample by

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

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 Decision if sample is pure cocoa butter

The variability of the triacylglycerol composition of cocoa butter is expressed by Equation (4) using the normalized triacylglycerols, i.e. %POP + %POS + %SOS = 100 % as determined in Equation (3):

$$\text{POP} = 43{,}734 - 0{,}733 \times \text{SOS} \tag{4}$$

(residual standard deviation = 0,125)

The principle of the method is that for cocoa butter samples POS is practically constant for wide variations of POP and SOS, resulting in a linear relationship [so-called “CB-line”, Equation (4)] between POP and SOS. All CB/CBE mixtures will cause the triacylglycerol analysis to deviate from the “CB-line” to the extent that their POS value differs from the POS value of cocoa butter. Equation (4) was established by using a standardized database of the triacylglycerol profile of 74 individual genuine cocoa butters evaluated and in-house validated [3]. The cocoa butter CRM (4.1) was used to standardize the applied analytical methodology for the determination of the triacylglycerol profile of the cocoa butters.

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

$$\mathrm{POP} < 44{,}025 - 0{,}733 \times \mathrm{SOS} \qquad (5)$$

A higher value for POP, as given by Equation (5), means that the sample is not pure cocoa butter. The advantage of the approach elaborated is that by using the cocoa butter CRM for calibration purposes, the mathematical expression can be used by individual testing laboratories for verifying the purity of cocoa butter, without tackling the problem of establishing a “CB-line” as a prerequisite. Calibration by the cocoa butter CRM automatically links the results obtained in a laboratory to the cocoa butter triacylglycerol database and the elaborated decision rule [Equation (5)].

10 Procedural requirements

10.1 General considerations

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

NOTE As an aid to verify the proper functioning of the chromatographic system, precision data for the three triacylglycerols POP, POS and SOS (normalized to 100 %) are given in 11.2 and 11.3.

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

r = 0,514 g/100 g	for POP values ranging from 18,99 g/100 g to 25,37 g/100 g
r = 0,293 g/100 g	for POS values ranging from 43,76 g/100 g to 47,73 g/100 g
r = 0,621 g/100 g	for SOS values ranging from 30,87 g/100 g to 33,80 g/100 g

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

R = 0,741 g/100 g	for POP values ranging from 18,99 g/100 g to 25,37 g/100 g
R = 0,588 g/100 g	for POS values ranging from 43,76 g/100 g to 47,73 g/100 g
R = 0,782 g/100 g	for SOS values ranging from 30,87 g/100 g to 33,80 g/100 g

NOTE Repeatability limits and reproducibility limits derived from this interlaboratory test are indicative values and may be applicable to triacylglycerols values beyond the given range (data from extended study [3]).

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 [4] [5]. 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 0,6 % CBE in chocolate (assumed fat content of chocolate 30 %) without giving false-positive or false-negative results.

Table A.1 shows suitable GC conditions[2]. 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.5.

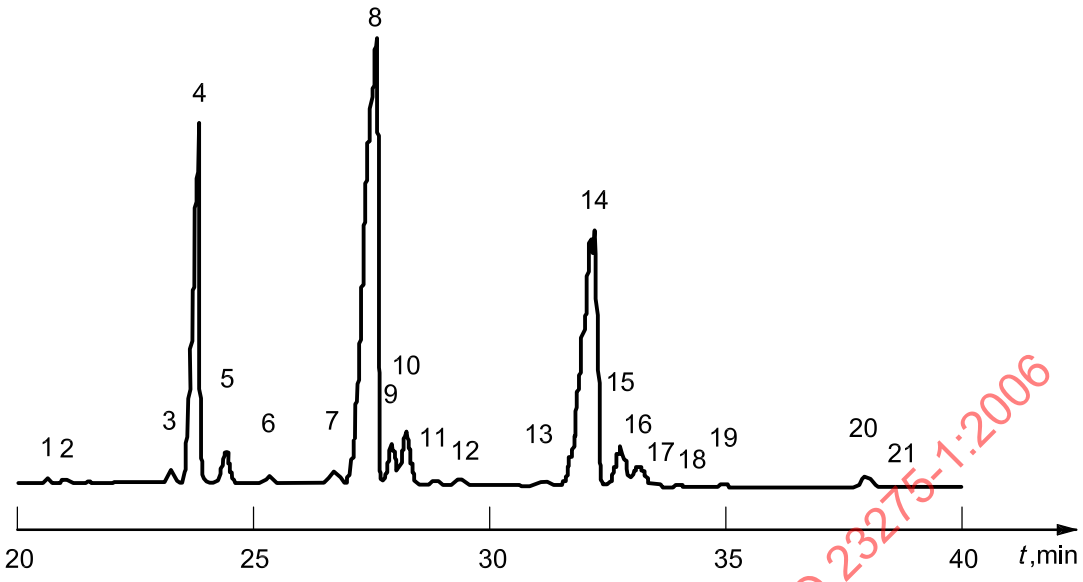
Table A.1 — Suitable GC conditions to be used for triacylglycerol analyses of cocoa butter, CBEs, CB/CBE blends and chocolate

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

2) 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:	H_2 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

Table A.2 — Precision data for pure cocoa butter samples

	Pure CB [a]			Pure CB [b]			Pure CB [c]		
	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)	20,396	47,731	31,873	19,491	47,421	33,089	18,991	47,210	33,799
Repeatability standard deviation, s_r, g/100 g	0,057	0,056	0,093	0,069	0,095	0,097	0,092	0,084	0,154
Repeatability relative standard deviation, %	0,28	0,12	0,29	0,36	0,20	0,29	0,49	0,18	0,45
Repeatability limit, r [= 2,83 $\times s_r$], g/100 g	0,160	0,157	0,261	0,194	0,265	0,271	0,258	0,236	0,430
Reproducibility standard deviation, s_R, g/100 g	0,142	0,108	0,169	0,081	0,148	0,166	0,120	0,090	0,168
Reproducibility relative standard deviation, %	0,70	0,23	0,53	0,42	0,31	0,50	0,63	0,19	0,50
Reproducibility limit, R [= 2,83 $\times s_R$], g/100 g	0,397	0,302	0,473	0,227	0,413	0,464	0,337	0,253	0,471

[a] Country origin: Grenada
[b] Country origin: Ghana
[c] Country origin: Ivory Coast

Table A.3 — Precision data for real chocolate samples

	Chocolate [CBE added]			Chocolate [no CBEs added]		
	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)	25,375	43,757	30,868	19,740	47,401	32,859
Repeatability standard deviation, s_r, g/100 g	0,128	0,101	0,180	0,089	0,070	0,074
Repeatability relative standard deviation, %	0,50	0,23	0,58	0,45	0,15	0,23
Repeatability limit, r [= 2,83 $\times s_r$], g/100 g	0,358	0,282	0,503	0,250	0,195	0,208
Reproducibility standard deviation, s_R, g/100 g	0,265	0,210	0,198	0,120	0,106	0,111
Reproducibility relative standard deviation, %	1,04	0,48	0,64	0,61	0,22	0,34
Reproducibility limit, R [= 2,83 $\times s_R$], g/100 g	0,741	0,588	0,553	0,335	0,298	0,310