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Vegetable fats and oils — Determination of cocoa butter equivalents in milk chocolate

Corps gras d'origine végétale — Détermination des équivalents au beurre de cacao dans le chocolat au lait

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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.

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Vegetable fats and oils — Determination of cocoa butter equivalents in milk chocolate

1 Scope

This International 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 at a minimum level of 0,5 g CBE/100 g milk chocolate and quantified at a level of 5 % mass fraction CBE addition to milk chocolate with a predicted error of 0,7 g CBE/100 g milk chocolate.

2 Terms and definitions

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

2.1
milk fat content of milk chocolate
mass fraction of milk fat in milk chocolate determined by the procedure specified in this International Standard

NOTE The mass fraction is expressed in grams per 100 g of milk chocolate.

2.2
cocoa butter equivalents
non-cocoa vegetable oils and fats detected in milk chocolate in accordance with the procedure prescribed in this International Standard

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

2.3
cocoa butter equivalent content of milk chocolate
mass fraction of substances determined by the procedure specified in this International Standard

NOTE The mass fraction is expressed in grams per 100 g of milk chocolate.

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

a) to calculate the MF content in the chocolate fat (grams of MF per 100 g chocolate fat);

b) 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 if this procedure indicates that the sample is not pure cocoa butter (CB);

c) to quantify the amount of the CBE admixture in chocolate fat (grams of CBE per 100 g chocolate fat) using a partial least-squares (PLS) regression model with six input variables, i.e. the five TAGs, POP, POS, POO, SOS, and SOO, normalized to 100 % and the determined MF content of the chocolate fat.

To ensure the correct labelling of milk chocolate, the results obtained relating to chocolate fat are converted into grams of MF per 100 g chocolate and grams of CBE per 100 g chocolate, necessitating the accurate determination of the total fat content of the chocolate using a Soxhlet extraction procedure (based on AOAC Official Method 963.15[5]). When the detection procedure proves the absence of CBEs in the chocolate fat, the quantification and total fat content are not necessary.

4 Reagents, solutions and standards

NOTE Use only reagents of recognized analytical grade, unless otherwise specified.

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

4.1 Cocoa butter Certified Reference Material (IRMM-801)1) (see Reference [6]), for calibration purposes and system suitability tests.

4.2 Pure milk fat, for system suitability tests.

4.3 1-Palmitoyl-2-stearoyl-3-butyroyl-glycerol (PSB)2).

4.3.1 General

For calibration purposes, dissolve ~40 mg of PSB in a 50 ml volumetric flask (5.9) with isooctane resulting in a stock solution of ~ρ = 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 on an analytical balance (5.1) IRMM-801 (4.1) into 25 ml volumetric flasks (5.9) and adding the respective volumes of the PSB stock solution as given in Table 1. Make up to the mark with isooctane.

Table 1 — Masses of IRMM-801 and volumes of PSB stock solution for preparation of series of PSB calibration solutions in matrix

Calibration solution	IRMM-801 (4.1) weighed into 25 ml volumetric flask mg	Volume taken from PSB stock solution and added to 25 ml volumetric flask ml	Concentration of PSB in calibration solution $\rho_{\mathrm{PSB}i}$ mg/ml	Final IRMM-PSB concentration of solution $\rho_{\mathrm{IRMM\text{-}PSB}}$ mg/ml
1	~250	4	0,128	~10
2	~250	3	0,096	~10
3	~250	2	0,064	~10
4	~250	1	0,032	~10
5	~250	0,5	0,016	~10

1) Commercially available from the Institute for Reference Materials and Measurements (http://irmm.jrc.ec.europa.eu/), Belgium. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

2) Commercially available from Larodan (http://www.larodan.se/), Sweden. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

4.3.2 Cold on-column (OCI) injection

Dilute each calibration solution with isooctane, $\varphi = 1$ ml/5 ml, to obtain a final IRMM-PSB concentration ($\rho_{\text{IRMM-PSB}}$) of ~2 mg/ml in each solution and PSB concentrations (ρ_{PSB}) ranging from 0,025 6 mg/ml (calibration solution 1) to 0,003 2 mg/ml (calibration solution 5).

4.3.3 Split injection (e.g. split ratio of 1:10)

Dilute each calibration solution with isooctane, $\varphi = 1$ ml/2 ml, to obtain a final IRMM-PSB concentration ($\rho_{\text{IRMM-PSB}}$) of ~5 mg/ml in each solution and PSB concentrations (ρ_{PSB}) ranging from 0,064 mg/ml (calibration solution 1) to 0,008 mg/ml (calibration solution 5).

NOTE The final PSB concentrations shall be calculated using the actual mass in the stock standard solution.

4.4 α-Cholestane[3], $\rho = 100$ mg/100 ml, used as internal standard.

Dissolve ~50 mg α-cholestane in 50 ml of isooctane.

— For cold on-column injection: Dilute 1:250 ($\rho = 0{,}004$ mg/ml).

— For split injection (e.g. split ratio of 1:10): Dilute 1:100 ($\rho = 0{,}01$ mg/ml).

4.5 Fat solvent, non-chlorinated solvents (e.g. petroleum ether, *n*-hexane, *n*-heptane, isooctane).

4.6 Hydrochloric acid, $c(\text{HCl}) = 4$ mol/l.

5 Apparatus and equipment

5.1 Analytical balance, readable to the nearest 0,1 mg.

5.2 Drying oven. A dry heater block may be used.

5.3 Filter paper, diameter 15 cm [e.g. S&S 589/1[4]].

5.4 Food grater, a kitchen blender with a design featuring the motor above the mixing chamber to avoid melting the samples.

5.5 Rotary evaporator. Alternative evaporation procedures may be used.

5.6 Evaporation block, with nitrogen supply.

5.7 Desiccator, sealable enclosure containing desiccants used for preserving moisture-sensitive items.

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

5.9 Volumetric flasks, of capacity 10 ml, 25 ml, 50 ml and 100 ml (or other capacities if needed), ISO 1042[2] class A.

5.10 Pipettes, of capacities ranging from 1 ml to 10 ml (or other capacities if necessary), ISO 648[1] class A or ISO 8655-2[4].

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3) May be obtained from Sigma-Aldrich (http://www.sigmaaldrich.com/), Belgium.

4) S&S 589/1 black ribbon paper is an example of a suitable product commercially available. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

5.11 Microsyringe, with maximum volume 10 µl, graduated to 0,1 µl, or automatic sample injector.

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

NOTE 1 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 10.2.

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

GLC column:	CB-TAP 25 m × 0,25 mm i.d., fused silica coated with a medium polar thermostable phenylmethylpolysiloxane stationary phase with a film thickness of 0,10 µm
Oven programme for OCI:	100 °C held for at least 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 at least 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_2 (purity ⩾ 99,999 %) with a constant flow rate of 3,5 ml/min (another suitable carrier gas is helium)
Carrier gas for split:	H_2 (purity ⩾ 99,999 %) with a constant flow rate of 2,5 ml/min (another suitable carrier gas is helium)

NOTE 2 Columns and alternative experimental conditions, used in an international collaborative study (see Reference [7]), are listed in Table A 1. Operating conditions may be changed to obtain optimum separation.

5.13 Chromatographic data system.

6 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 5555[3]. A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

7 Sample preparation

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

Before opening and using the IRMM-801 (4.1), warm the ampoule in an 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 pure milk fat for system suitability tests

If no pure MF is available, it can be obtained from a butter sample by melting and passing the fat layer through a folded filter paper (5.3) at 50 °C in an oven (5.2).

7.3 Preparation of chocolate sample

7.3.1 General

Chill approximately 200 g of chocolate until hard, and grate to a fine granular condition using a food grater (5.4). Mix thoroughly and preserve in a tightly stoppered bottle in a cool place.

7.3.2 Rapid fat extraction

The chocolate fat is separated from 5 g grated chocolate (7.3.1) by extracting with two to three 10 ml portions of a suitable fat solvent (4.5). Centrifuge and decant. Combine the extracts and evaporate (5.5) most of the fat solvent and finally dry it under a stream of nitrogen (5.6).

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. When no CBEs are detected, the second part of the standard, i.e. quantification of CBEs around the statutory limit of 5 %, is not necessary. When CBEs are detected, the quantification part should be performed using the same TAG profile as used for the CBE detection. However, in this case, determine the accurate amount of total fat in chocolate using the procedure in 7.3.3. Alternative extraction procedures may be used provided that the same results are obtained.

7.3.3 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 7.3.2) by Soxhlet extraction (based on AOAC Official Method 963.15 [5]), as follows.

Weigh (5.1) 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 (4.6) and a few defatted boiling chips, or other antibumping agents, and stir. Cover with a watch glass, bring the solution slowly to the boil, and simmer for 15 min. Rinse the watch glass with 100 ml of water. Filter the solution through a medium fluted filter paper (5.3), 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 (5.7) then weigh (5.1) it. Place the thimble containing the dried sample in the Soxhlet apparatus (5.8), 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 more than 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 (5.7) to room temperature, then weigh (5.1). 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, expressed as a percentage, of total fat in the chocolate, $w_{\text{fat; choc}}$, is given by:

$$w_{\text{fat; choc}} = \frac{m_{\text{fat}} \times 100}{m} \tag{1}$$

where

m is the mass, in grams, of chocolate taken;

m_{fat} is the mass, in grams, of the total fat obtained from the chocolate by Soxhlet extraction (based on AOAC Official Method 963.15 [5]).

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 should 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.

8 Procedure

8.1 Construction of calibration curve for determination of PSB content

Five calibration solutions containing different concentrations of PSB (4.3) but always the same concentration of α-cholestane (4.4) are prepared as follows.

For cold on-column (OCI) injection:

— **Calibration solution 1** (Final $\rho_{PSB\ 1}$ = 0,012 8 mg/ml; $\rho_{\alpha\text{-cholestane}\ 1}$ = 0,002 mg/ml): Transfer 1 ml of calibration solution 1 ($\rho_{PSB\ 1}$ = 0,025 6 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,004 mg/ml; 4.4).

— **Calibration solution 2** (Final $\rho_{PSB\ 2}$ = 0,009 6 mg/ml; $\rho_{\alpha\text{-cholestane}\ 2}$ = 0,002 mg/ml): Transfer 1 ml of calibration solution 2 ($\rho_{PSB\ 2}$ = 0,019 2 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,004 mg/ml; 4.4).

— **Calibration solution 3** (Final $\rho_{PSB\ 3}$ = 0,006 4 mg/ml; $\rho_{\alpha\text{-cholestane}\ 3}$ = 0,002 mg/ml): Transfer 1 ml of calibration solution 3 ($\rho_{PSB\ 3}$ = 0,012 8 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,004 mg/ml; 4.4).

— **Calibration solution 4** (Final $\rho_{PSB\ 4}$ = 0,003 2 mg/ml; $\rho_{\alpha\text{-cholestane}\ 4}$ = 0,002 mg/ml): Transfer 1 ml of calibration solution 4 ($\rho_{PSB\ 4}$ = 0,006 4 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,004 mg/ml; 4.4).

— **Calibration solution 5** (Final $\rho_{PSB\ 5}$ = 0,001 6 mg/ml; $\rho_{\alpha\text{-cholestane}\ 5}$ = 0,002 mg/ml): Transfer 1 ml of calibration solution 5 ($\rho_{PSB\ 5}$ = 0,003 2 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,004 mg/ml; 4.4).

Inject 0,5 µl of each calibration solution into the HR-GLC system using the cold on-column injection system.

For split injection:

— **Calibration solution 1** (Final $\rho_{PSB\ 1}$ = 0,032 mg/ml; $\rho_{\alpha\text{-cholestane}\ 1}$ = 0,005 mg/ml): Transfer 1 ml of calibration solution 1 ($\rho_{PSB\ 1}$ = 0,064 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,01 mg/ml; 4.4).

— **Calibration solution 2** (Final $\rho_{PSB\ 2}$ = 0,024 mg/ml; $\rho_{\alpha\text{-cholestane}\ 2}$ = 0,005 mg/ml): Transfer 1 ml of calibration solution 2 ($\rho_{PSB\ 2}$ = 0,048 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,01 mg/ml; 4.4).

— **Calibration solution 3** (Final $\rho_{PSB\ 3}$ = 0,016 mg/ml; $\rho_{\alpha\text{-cholestane}\ 3}$ = 0,005 mg/ml): Transfer 1 ml of calibration solution 3 ($\rho_{PSB\ 3}$ = 0,032 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,01 mg/ml; 4.4).

— **Calibration solution 4** (Final $\rho_{PSB\ 4}$ = 0,008 mg/ml; $\rho_{\alpha\text{-cholestane}\ 4}$ = 0,005 mg/ml): Transfer 1 ml of calibration solution 4 ($\rho_{PSB\ 4}$ = 0,016 mg/ml; 4.3) in a test tube and add 1 ml of α-cholestane solution (ρ = 0,01 mg/ml; 4.4).

— **Calibration solution 5** (Final $\rho_{PSB\ 5}$ = 0,004 mg/ml; $\rho_{\alpha\text{-cholestane}\ 5}$ = 0,005 mg/ml): Transfer 1 ml of calibration solution 5 ($\rho_{PSB\ 5}$ = 0,008 mg/ml; 4.3) into a test tube and add 1 ml of α-cholestane solution (ρ = 0,01 mg/ml; 4.4).

Inject 1 µl of the final test solution into the HR-GLC system using the split injection system.

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

8.2 Separation of individual TAGs of IRMM-801 by HR-GLC

The IRMM-801 (4.1) shall be warmed in a drying oven (5.2) until completely melted. Pipettes (or similar equipment) used for transferring the sample during weighing operations should be brought to a temperature of ~55 °C in a drying oven to avoid partial fat fractionation during handling of samples.

For cold on-column (OCI) injection: Weigh (5.1) ~0,1 g of IRMM-801 (4.1) in a 10 ml volumetric flask (5.9) and dilute to the mark with isooctane (4.5). Pipette (5.10) 1 ml of the resulting solution into another 50 ml volumetric flask (5.9) and dilute to the mark with the same solvent (ρ = 0,2 mg/ml). Inject 0,5 µl of the final test solution into the HR-GLC system using the cold on-column injection system.

For split injection: Weigh (5.1) ~0,1 g of IRMM-801 (4.1) in a 10 ml volumetric flask (5.9) and dilute to the mark with isooctane (4.5). Pipette (5.10) 1 ml of the resulting solution into another 10 ml volumetric flask and dilute to the mark with the same solvent (ρ = 1 mg/ml). Inject 1 µl of the final test solution into the HR-GLC system using the split injection system.

Alternative fat solvents, sample amounts and injectors may be used provided that the detection system employed gives a linear response and conforms to the system suitability criteria (10.2).

8.3 Separation of individual TAGs of pure MF by HR-GLC

For cold on-column injection (OCI): Weigh (5.1) ~0,05 g of pure MF (4.2) in a 50 ml volumetric flask (5.9) and dilute to the mark with isooctane (4.5) (ρ = 1 mg/ml). Transfer 1 ml of this solution to a test tube and add 1 ml of α-cholestane solution (4.4) (resulting test solution ρ = 0,5 mg/ml). Inject 0,5 µl of the final test solution into the HR-GLC system using the cold on-column injection system.

For split injection: Weigh (5.1) ~0,25 g of pure MF (4.2) in a 50 ml volumetric flask (5.9) and dilute to the mark with isooctane (4.5) (ρ = 5 mg/ml). Transfer 1 ml of this solution to a test tube and add 1 ml of α-cholestane solution (4.4) (resulting test solution ρ = 2,5 mg/ml). Inject 1 µl of the final test solution into the HR-GLC system using the split injection system.

Alternative fat solvents, sample amounts and injectors may be used provided that the detection system employed gives a linear response and conforms to the system suitability criteria (10.2).

8.4 Separation of individual TAGs of chocolate fat by HR-GLC

The test sample [chocolate fat extracted from milk chocolate by rapid fat extraction (7.3.2)] 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 ~55 °C in a drying oven in order to avoid partial fat fractionation during handling of samples.

For cold on-column (OCI) injection: Weigh (5.1) ~0,1 g of chocolate fat (as obtained in 7.3.2) on an analytical balance (5.1) in a 100 ml volumetric flask (5.9) and dilute to the mark with isooctane (4.5) (ρ = 1 mg/ml). Transfer 1 ml of this solution to a test tube and add 1 ml of α-cholestane solution (4.4) (resulting test solution ρ = 0,5 mg/ml). Inject 0,5 µl of the final test solution into the HR-GLC system using the cold on-column injection system.

For split injection: Weigh (5.1) ~0,5 g of chocolate fat (as obtained in 7.3.2) on an analytical balance (5.1) in a 100 ml volumetric flask (5.9) and dilute to the mark with isooctane (4.5) (ρ = 5 mg/ml). Transfer 1 ml of this solution to a test tube and add 1 ml of α-cholestane solution (4.4) (resulting test solution ρ = 2,5 mg/ml). Inject 1 µl of the final test solution into the HR-GLC system using the split injection system.

Alternative fat solvents, sample amounts and injectors may be used provided that the detection system employed gives a linear response and conforms to the system suitability criteria (10.2)