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Institute for Reference Materials and Measurements

Validation of an analytical approach to determine cocoa butter equivalents in milk chocolate

Report on the final collaborative trial

M. Buchgraber and S. Androni

European Commission
Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements, Geel, BE

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CONTENTS

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BO	butter oil
C	cochran
CB	cocoa butter
CBE	cocoa butter equivalent
CM	cocoa mass
CoCal	cocoa butter calculation toolbox
CR	crumb
DG	double grubbs
FCMP	full cream milk powder
FID	flame ionization detector
HR-GLC	high resolution gas liquid chromatography
IRMM	Institute for Reference Materials and Measurements
ISO	International Organization for Standardization
LEC	lecithin
MF	milk fat
MS	mean squares
OCI	cold on-column
PLS	partial least squares
PMF	palm mid fraction
POO	1,2-dioleoyl-3-palmitoyl-glycerol
POP	1,3-dipalmitoyl-2-oleoyl-glycerol
POS	1-palmitoyl-2-oleoyl-3-stearoyl-glycerol
PSB	1-palmitoyl-2-stearoyl-3-butyroyl-glycerol
PTV	programmed temperature vaporizer
r	repeatability
R	reproducibility
RE	relative error
RF	response factor
RSD_r	relative standard deviation of repeatability

RSD_R	relative standard deviation of reproducibility
SG	single grubbs
SKMP	skimmed milk powder
SOO	1,2-dioleoyl-3-stearoyl-glycerol
SOS	1,3-distearoyl-2-oleoyl-glycerol
s_r	repeatability standard deviation
s_R	reproducibility standard deviation
TAG	triacylglycerol
VAN	vanillin
WP	whey powder

1 INTRODUCTION

The European Parliament and Council adopted Directive 2000/36/EC [1], authorizing the replacement of cocoa butter (CB) by vegetable fats other than CB (so-called cocoa butter equivalents, CBEs), on 23 June 2000. The objective of the Directive was to simplify Community provisions concerning chocolate with a view to allowing the free movement of chocolate products within the Internal Market. Member States' laws, regulations and administrative provisions have to comply with the Directive since August 2003. A need has been recognized within official control laboratories for reliable analytical methods to prove label compliance to protect consumers from fraudulent malpractice. Due to the similar chemical composition and physical properties of CB and CBEs it is extremely difficult to quantify and in some cases even difficult to detect them.

The Institute for Reference Materials and Measurements (IRMM) of the European Commission's Directorate-General Joint Research Centre (EC-JRC) developed an integrated approach for determining CBEs in dark chocolate using triacylglycerol (TAG) profiling by high resolution gas liquid chromatography (HR-GLC) [2], which was subjected to validation by an international collaborative trial [3], allowing the implementation and enforcement of Directive 2000/36/EC [1] for dark chocolate. To facilitate the usage of the approach an analytical toolbox named "CoCal-1 (=cocoa butter calculation toolbox)" has been established by the IRMM, consisting of a validated method for detection of CBEs in dark chocolate [4], a validated method for quantification of CBEs in dark chocolate [5], both of them standardized by the International Organization for Standardization (ISO) [6-7], a certified cocoa butter reference material (IRMM-801) to calibrate the analyst's instruments [8], and an electronic evaluation sheet for Microsoft Excel® to calculate the final result [9].

So far, this standardized analytical approach established for dark chocolate was not applicable to milk chocolate since TAGs deriving from milk or milk fat

(MF) interfered with the detection and quantification of CBEs in chocolate. When milk chocolate is analysed it will be necessary to correct the observed TAG pattern for the presence of milk fat TAGs, requiring knowledge of the amount of MF present in the product. The problem of estimating the MF content in mixtures of fats or chocolates has already prompted a great deal of research [10-21]. Currently, the analysis of butyric acid in a mixed fat is a widely applied method [22-24] and has for instance already been applied to quantitate small amounts of MF in CB or chocolate fats [25-27]. However, with respect to the correct labelling of chocolate, this method can only provide an answer to one out of three questions, i.e., what is the content of MF in the chocolate fat. The method is not satisfactory for the other two questions, i.e., (i) is there any other fat in addition to CB present and if yes (ii) how much.

IRMM developed an improved analytical approach for the determination of MF in chocolate fats, which is based on a standardized database consisting of the TAG profile of genuine MF samples and mixtures thereof with chocolate fats. The TAG database, obtained by HR-GLC, was employed for the selection of a potential marker compound, i.e., 1-palmitoyl-2-stearoyl-3-butyroyl-glycerol (PSB), to be used to calculate the MF content in chocolate fats. PSB fulfilled the requirements (i) to be present in reasonable amounts allowing a reliable quantification of even low MF proportions in chocolate fats, (ii) to have an acceptable natural variability and (iii) to be present only in MF and no other fats. The advantage of the developed approach is that for further applications, i.e., determination of CBEs in chocolate fats, a single analysis is performed, whereas for the same purpose the butyric acid method requires two different analytical methods [28].

By using the obtained information from the MF quantification a modification of the existing approach for detection and quantification of CBEs in dark chocolate (CoCal-1) was developed for milk chocolate (CoCal-2) [29].

CoCal-2 is based on

(i) comprehensive databases covering the TAG composition of a wide range of authentic MF (n=310), CB (n=75) as well as CBE (n=74) samples and 947 gravimetrically prepared mixtures thereof,

(ii) the availability of a certified cocoa butter reference material (IRMM-801) for calibration,

(iii) an evaluation algorithm, which allows a reliable quantification of the MF content in chocolate fats using a simple linear regression model,

(iv) a subsequent correction of TAGs deriving from MF,

(v) mathematical expressions to detect the presence of CBEs in milk chocolate, and

(vi) a multivariate statistical formula to quantify the amount of CBEs in milk chocolate.

The elaborated approach has the advantage that by performing a single TAG analysis using HR-GLC several useful pieces of information can be determined, i.e.,

(i) the milk fat content in the sample,

(ii) the contribution of TAGs deriving from MF, and

(iii) the presence/absence of CBEs.

In case the detection approach indicates that the CB is not pure, a last question can be answered, i.e.,

(iv) how much CBE has been added,

allowing to control correct labelling of milk chocolate.

A substantial in-house testing of the approach formed the basis for the establishment of a draft method protocol (Annex A). On the basis of the in-house validated procedure full method validation by a collaborative trial was carried out. The results of the collaborative trial are presented in this report.

2 METHOD DESCRIPTION

Test samples, i.e., chocolate fats obtained from chocolate samples using a rapid fat extraction procedure, have to be separated by HR-GLC into TAG fractions according to their molecular weight and degree of unsaturation. In principle, the end user has only to determine seven peaks, i.e., alpha-cholestane (internal standard), PSB, 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (POS), 1,2-dioleoyl-3-palmitoyl-glycerol (POO), 1,3-distearoyl-2-oleoyl-glycerol (SOS) and 1,2-dioleoyl-3-stearoyl-glycerol (SOO). The obtained information is used

- to calculate the MF content in the chocolate fat via the determined PSB content (g MF/100 g chocolate fat) [28],
- 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 that are corrected for the TAG contribution originating from MF, and in case the detection approach indicates that the sample is not pure CB,
- to quantify the amount of the CBE admixture to chocolate fat (g CBE/100 g chocolate fat) using a partial least squares (PLS) regression model using six input variables, i.e., the five TAGs POP, POS, POO, SOS , SOO normalized to 100 % and the determined MF content of the chocolate fat [29].

Finally, to control correct labelling of milk chocolate, the obtained results related to chocolate fat have to be converted into g MF/100 g chocolate and g CBE/100 g chocolate, requiring the accurate determination of the total fat content of the chocolate using a Soxhlet extraction procedure [30]. In case the detection approach proves the absence of CBEs in chocolate fat, the quantification of CBEs and the determination of the total fat content of chocolate is not necessary. A detailed description of the whole approach and the performed in-house testing is given in [28-29].

3 PARTICIPANTS

3.1 Coordination of collaborative trial

European Commission, Directorate-General Joint Research Centre, Institute for Reference Materials and Measurements, Geel (BE)

3.2 Preparation of test samples

3.2.1 Chocolate samples

Barry Callebaut N.V., Lebbeke-Wieze (BE)

3.2.2 Chocolate fats in solution

European Commission, Directorate-General Joint Research Centre, Institute for Reference Materials and Measurements, Geel (BE)

3.3 Homogeneity testing of test samples

European Commission, Directorate-General Joint Research Centre, Institute for Reference Materials and Measurements, Geel (BE)

3.4 Distribution of test samples

European Commission, Directorate-General Joint Research Centre, Institute for Reference Materials and Measurements, Geel (BE)

3.5 Measurements

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Eurofins, Wiertz-Eggert-Jörissen GmbH, Hamburg (DE)

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Karlshamns Sweden AB, Karlshamn (SE)
Kraft Foods, Munich (DE)
Lebensmittelchemisches Institut des Bundesverbandes der Deutschen Süßwarenindustrie, Köln (DE)

3.6 Collation and statistical evaluation of results

European Commission, Directorate-General Joint Research Centre, Institute for Reference Materials and Measurements, Geel (BE)

4 TEST SAMPLES

The collaborative testing of a method of analysis requires considerable planning in terms of the design of the trial, the type of matrix or matrices to be analysed, the level of analytes of interest, and the numbers of samples that are to be included in the trial. Materials are required for which homogeneity of the analytes of interest during the period of the study have to be demonstrated.

On special request six milk chocolate samples (Tables 1-2) varying in composition and with known levels of CBEs were produced by Barry Callebaut N.V. (Lebbeke-Wieze, Belgium).

Furthermore, seven chocolate fat samples dissolved in *iso*-octane were prepared by the IRMM (Table 3). A representative MF sample for the preparation of the chocolate fat solutions was obtained by gravimetrically blending equal proportions of 310 individual MF samples collected in retail stores in 21 European countries over a period of 2001-2005 [28]. CB and part of the MF samples were provided by Kraft Foods (Väsby, Sweden) and the

CBE samples were obtained from Britannia Food Ingredients (Goole, United Kingdom).

Table 1. Composition of chocolate samples used for the study (prepared by Barry Callebaut N.V.)

Sa. No.	Chocolate type [1])	CBE type	Fat [%]	CB [%]	CBE [%]	MF [%] [2]
1	Milk chocolate, FCMP, no CBE	-	35.6	29.7	0.0	5.9
2	Milk chocolate, FCMP, CBE addition low level	50 % PMF + 50 % SOS rich fat	35.6	29.2	0.5	5.9
3	Milk chocolate, SKMP + MF, no CBE	-	32.0	25.7	0.0	6.3
4	Milk chocolate, SKMP + MF, CBE low level	50 % PMF + 50 % SOS rich fat	32.0	23.7	2.0	6.3
5	Milk chocolate, crumb + MF + FCMP + SKMP + WP, CBE addition at statutory level	50 % PMF + 50 % SOS rich fat	25.1	14.6	5.1	5.4
6	White chocolate, CBE addition at statutory level	50 % PMF + 50 % SOS rich fat	33.8	23.5	4.0	6.4

[1] Samples were made in test conches of 40 kg. FCMP = full cream milk powder; SKMP = skimmed milk powder; WP= whey powder; PMF = palm mid fraction

[2] MF contents are approximate values, i.e., estimates of the fat content of individual sample ingredients

Table 2. Detailed composition of chocolate samples used for the study

Sa. No.	Composition [%] [1]										
	Sugar	**CM**	**CB**	**CBE**	**BO**	**FCMP**	**SKMP**	**WP**	**CR**	**LEC**	**VAN**
1	42.6	11.1	23.6	0.0	0.0	22.0	0.0	0.0	0.0	0.8	0.0
2	42.6	11.1	23.1	0.5	0.0	22.0	0.0	0.0	0.0	0.8	0.0
3	46.5	8.6	21.0	0.0	6.1	0.0	17.4	0.0	0.0	0.5	0.0
4	46.5	8.6	19.0	2.0	6.1	0.0	17.4	0.0	0.0	0.5	0.0
5	29.2	8.0	9.0	5.1	2.4	0.0	11.2	5.1	29.2	0.6	0.0
6	48.2	0.0	23.5	4.0	0.0	23.7	0.0	0.0	0.0	0.6	0.1

[1] CM = cocoa mass; BO = butter oil; FCMP = full cream milk powder; SKMP = skimmed milk powder; WP= whey powder; CR = crumb; LEC = lecithin; VAN = vanillin

Table 3. Composition of chocolate fat solutions used for the study (prepared by IRMM)

Sa. No.	CB type	CBE type	MF type	CB [%]	CBE [%]	MF [%]
7	West African	-	-	100.00	0.00	0.00
8	West African	-	Mixture of 310 MF samples	85.01	0.00	14.99
9	West African	70 % PMF + 30 % SOS rich fat	Mixture of 310 MF samples	83.03	2.00	14.98
10	West African	70 % PMF + 30 % SOS rich fat	Mixture of 310 MF samples	68.95	16.03	15.02
11	West African	70 % PMF + 30 % SOS rich fat	Mixture of 310 MF samples	64.99	19.98	15.04
12	West African	100 % soft PMF	Mixture of 310 MF samples	64.94	20.08	14.99
13	West African	70 % PMF + 30 % SOS rich fat	Mixture of 310 MF samples	56.91	28.04	15.05

For the collaborative trial the participants received a shipment containing:

- six grated chocolate samples, from which the fat had to be extracted, and
- seven chocolate fat samples dissolved in 5 mL *iso*-octane.

Blind duplicates were provided for each sample (in total 26 test samples), which were coded by the coordinating laboratory.

Additionally,

- one ampoule of the cocoa butter certified reference material (IRMM-801),
- one ampoule of an average pure milk fat,
- one ampoule of PSB dissolved in 5 mL *iso*-octane,
- six ampoules of a mixture of CB with different levels of PSB dissolved in 5 mL *iso*-octane, and
- one ampoule of alpha-cholestane dissolved in 5 mL *iso*-octane

were provided for calibration purposes and system suitability checks.

4.1 Homogeneity study

Homogeneity of the chocolate samples (samples 1-6) was assessed by internationally agreed procedures [31]. From each chocolate sample 10

sample containers (units) were taken randomly from the sequence and the content of the container split into two equal parts (sub-samples). The fat from each sub-sample was extracted according to the AOAC Official Method 963.15 [30] and randomly subjected to TAG analysis by HR-GLC using a column from Varian-Chrompack (0.25 mm x 25 m, 0.1 µm CB-TAP, Varian, Inc., Middelburg, The Netherlands). Homogeneity of the chocolate samples was checked by determining the six major TAGs (PSB, POP, POS, POO, SOS and SOS) used for the determination of the milk fat content and the detection and quantification of CBEs in milk chocolate. The individual TAG results obtained for the duplicate set of values for each sample (replicate A and B) are given in Table B 1-6 (Annex B).

The within- and between-units standard deviations (SD) for the contents of PSP, POP, POS, POO, SOS and SOO were calculated by using one-way analysis of variance (ANOVA). The between-units standard deviation was used as an estimate of the inhomogeneity between-units and the within-units standard deviation as an estimate of the combined effects of the repeatability of the method and the possible within-unit inhomogeneity.

The ratios of the variances of the between- and within-unit series were compared by means of a Snedecor F-test to determine whether the between-unit variances differed significantly from the within-units variances. A sample batch is regarded as homogenous when the calculated F-value is smaller than the tabulated percentile point for the F-distribution (95 % confidence interval).

Table 4. Statistical results obtained by ANOVA for chocolate sample 1

Chocolate sample 1	**PSB [2]**	**%-POP [3]**	**%-POS [3]**	**%-POO [3]**	**%-SOS [3]**	**%-SOO [3]**
Mean	0.27	18.45	44.42	2.41	31.65	3.07
SD between-units	0.002	[1]	0.012	0.008	[1]	0.005
SD within-units	0.007	0.066	0.039	0.016	0.032	0.021
F	1.21	0.58	1.19	1.53	0.27	1.10
P-value	0.38	0.79	0.39	0.26	0.97	0.44
$F_{critical}$	3.02	3.02	3.02	3.02	3.02	3.02
$F<F_{critical}$	Yes	Yes	Yes	Yes	Yes	Yes

[1] Mean squares $(MS)_{between} < MS_{within}$; [2] g PSB/100 g chocolate fat; [3] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table 5. Statistical results obtained by ANOVA for chocolate sample 2

Chocolate sample 2	PSB [2]	%-POP [3]	%-POS [3]	%-POO [3]	%-SOS [3]	%-SOO [3]
Mean	0.29	18.69	44.00	2.55	31.57	3.18
SD between-units	0.004	[1]	0.008	[1]	0.018	[1]
SD within-units	0.005	0.033	0.016	0.032	0.058	0.026
F	2.35	0.97	1.49	0.95	1.19	0.46
P-value	0.10	0.51	0.27	0.52	0.39	0.87
$F_{critical}$	3.02	3.02	3.02	3.02	3.02	3.02
$F<F_{critical}$	Yes	Yes	Yes	Yes	Yes	Yes

[1] Mean squares $(MS)_{between} < MS_{within}$; [2] g PSB/100 g chocolate fat; [3] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table 6. Statistical results obtained by ANOVA for chocolate sample 3

Chocolate sample 3	PSB [2]	%-POP [3]	%-POS [3]	%-POO [3]	%-SOS [3]	%-SOO [3]
Mean	0.41	18.56	44.31	2.83	31.18	3.12
SD between-units	[1]	[1]	[1]	[1]	0.015	0.007
SD within-units	0.008	0.027	0.035	0.024	0.036	0.029
F	0.87	0.93	0.69	0.36	1.34	1.11
P-value	0.58	0.54	0.71	0.93	0.33	0.44
$F_{critical}$	3.02	3.02	3.02	3.02	3.02	3.02
$F<F_{critical}$	Yes	Yes	Yes	Yes	Yes	Yes

[1] Mean squares $(MS)_{between} < MS_{within}$; [2] g PSB/100 g chocolate fat; [3] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table 7. Statistical results obtained by ANOVA for chocolate sample 4

Chocolate sample 4	PSB [2]	%-POP [3]	%-POS [3]	%-POO [3]	%-SOS [3]	%-SOO [3]
Mean	0.39	20.04	41.70	3.29	31.44	3.53
SD between-units	0.003	0.005	0.012	0.028	0.013	[1]
SD within-units	0.006	0.022	0.027	0.045	0.016	0.024
F	1.51	1.12	1.40	1.81	2.30	0.68
P-value	0.26	0.43	0.30	0.18	0.11	0.71
$F_{critical}$	3.02	3.02	3.02	3.02	3.02	3.02
$F<F_{critical}$	Yes	Yes	Yes	Yes	Yes	Yes

[1] Mean squares $(MS)_{between} < MS_{within}$; [2] g PSB/100 g chocolate fat; [3] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table 8. Statistical results obtained by ANOVA for chocolate sample 5

Chocolate sample 5	PSB [2]	%-POP [3]	%-POS [3]	%-POO [3]	%-SOS [3]	%-SOO [3]
Mean	0.45	23.96	36.48	3.31	32.51	3.75
SD between-units	0.003	0.003	0.015	0.025	[1]	0.012
SD within-units	0.005	0.017	0.029	0.025	0.031	0.027
F	1.84	1.08	1.52	2.99	0.54	1.40
P-value	0.18	0.45	0.26	0.05	0.82	0.30
$F_{critical}$	3.02	3.02	3.02	3.02	3.02	3.02
$F<F_{critical}$	Yes	Yes	Yes	Yes	Yes	Yes

[1] Mean squares $(MS)_{between} < MS_{within}$; [2] g PSB/100 g chocolate fat; [3] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table 9. Statistical results obtained by ANOVA for chocolate sample 6

Chocolate sample 6	PSB [2]	%-POP [3]	%-POS [3]	%-POO [3]	%-SOS [3]	%-SOO [3]
Mean	0.30	21.55	40.06	2.78	32.28	3.33
SD between-units	0.004	0.009	0.007	[1]	0.004	0.014
SD within-units	0.005	0.015	0.018	0.026	0.033	0.025
F	2.26	1.68	1.33	0.95	1.03	1.61
P-value	0.11	0.21	0.33	0.53	0.48	0.24
$F_{critical}$	3.02	3.02	3.02	3.02	3.02	3.02
$F<F_{critical}$	Yes	Yes	Yes	Yes	Yes	Yes

[1] Mean squares $(MS)_{between} < MS_{within}$; [2] g PSB/100 g chocolate fat; [3] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

All tests (Tables 4-9) confirmed that the between-units inhomogeneity was insignificant (P>0.05). Therefore, the homogeneity of the chocolate samples was considered sufficient to be used as test materials for the validation study. The chocolate fat samples dissolved in *iso*-octane (samples 7-13) were considered to be homogeneous.

5 DESIGN OF THE COLLABORATIVE TRIAL

Fifteen laboratories from eight EU Member States with experience in TAG analysis were contacted to participate in the study. Of these, twelve laboratories submitted results.

For the collaborative trial the participants were provided with a method protocol (Annex A), collaborative study guidelines (Annex C) and the test samples (Tables 1, 3). The collaborators were requested to follow the method protocol exactly. However, the HR-GLC method gave some freedom to

choose procedural parameters (e.g. GC apparatus, column type, carrier gas, injection technique, etc.) within certain limits. Hence, in order to demonstrate that the HR-GLC methods applied were fit-for-purpose the participants had to meet predefined performance criteria (Table 10).

Table 10. Performance criteria for HR-GLC method

HR-GLC	Performance criteria	Tested with
Resolution	Separation of critical pairs POS/POO and SOS/SOO with a chromatographic resolution of at least 1.0.	IRMM-801
Resolution	Separation of PSB from neighbouring peaks within carbon number group 38.	Pure MF sample
Resolution	No co-elution of the internal standard alpha-cholestane with other TAGs	Pure MF sample + alpha-cholestane
Detector response factors (POP, POS, POO, SOS, SOO)	Flame-ionization detector response factors (RFs) of TAGs shall not differ significantly from unity. RSD of determined detector RFs shall be less than 5 %.	IRMM-801 (three replicates)
Detector response factors (PSB)	The relative error of the minimum obtained RF for PSB and the relative error of the maximum obtained RF for PSB shall be less than 5 % with respect to the average RF for PSB.	Six calibration solutions (duplicate injection) of a mixture of either PSB + alpha-cholestane for cold on column injection or CB + PSB + alpha-cholestane for split injection

In the case of failure, the chromatographic conditions of the applied HR-GLC system (e.g. sample size, column temperature programme, carrier gas flow, etc.) had to be optimized.

5.1 Methods used by individual laboratories

A brief outline of the HR-GLC methods used by the participants is given in Tables D 1-2 (Annex D).

All collaborators employed a flame ionization detector (FID) for detection purposes. All laboratories used narrow bore fused silica columns coated with medium-polarity stationary phases containing 50-65 % phenyl groups. The

columns used in the ring trial were either from Varian-Chrompack (0.25 mm x 25 m, 0.1 µm CB-TAP or 0.25 mm x 25 m, 0.05 µm Ultimetal) or from Restek (0.25 mm x 30 m, 0.1 µm Rtx-65TG). Different types of sample injection techniques, i.e., cold on-column (OCI) (3 labs), split (7 labs) and programmed temperature vaporizer (PTV) (2 labs) injection, were applied. Further controllable parameters, different in the individual methods, were the type of carrier gas, the carrier-gas flow rate and/or the inlet pressure and the temperature programming.

5.2 Analysis of test samples

The seven chocolate fat samples (provided as blind duplicates) dissolved in *iso*-octane had to be analysed once (in total 14 analysis). From each grated chocolate sample (provided as blind duplicates) the fat had to be obtained once by rapid fat extraction (12 extractions) and once by Soxhlet fat extraction (12 extractions). The obtained chocolate fats had to be dissolved in a proper solvent and each fat solution analysed once (in total 24 analyses). The samples had to be analysed by HR-GLC in random order.

A calibration curve and an average response factor for PSB using alpha-cholestane as internal standard had to be determined as described in the method protocol (Annex A). This had to be done before analyzing the first sample, after the 19th analysis and after the last test sample (in total three calibration curves). Laboratories employing a split injection technique had to use a mixture of CB and PSB dissolved in *iso*-octane in order to obtain stable RF values, whereas for cold on-column injection techniques PSB dissolved in *iso*-octane was sufficient.

Response factors for the five TAGs (POP, POO, POS, SOS, SOO) had to be determined before analyzing the first sample, after the 19th analysis and after the last test sample by using IRMM-801 (in total three determinations).

A flow-scheme detailing the handling of the samples is given in the collaborative study guidelines (Annex C).

5.3 Reporting of results

The results were reported by using an electronic reporting sheet (MS Excel® format) which was provided by the coordinator. The following information had to be filled into the evaluation sheet by the participants:

- Chromatographic conditions (column type, instrument, injection technique, etc.)
- Outcome of the system suitability check (system suitability resolution; system suitability IRMM-801; system suitability PSB)
- Data for the PSB calibration curves (calibration curve 1-3, and average RF 1 (mean RF obtained from calibration curve 1 and 2); average RF 2 (mean RF obtained from calibration curve 2 and 3))
- Obtained data for the test samples as follows (Analyses 1-19; Analyses 20-38):
    - Sample code as given on the sample label.
    - Final sample concentration (mg/mL) of the test samples and alpha-cholestane concentration (mg/mL).
    - Obtained total fat content of chocolate samples determined by Soxhlet extraction.
    - Average response factor determined for PSB and the obtained intercept and slope for the calibration curve.
    - Raw area counts of alpha-cholestane, PSB, POP, POS, POO, SOS, SOO.
    - Raw area sum of all TAGs.

6 RESULTS OF COLLABORATIVE TRIAL

The test samples, i.e., chocolate fats extracted from chocolate samples (samples 1-6) or chocolate fat solutions (samples 7-13), were separated by HR-GLC into TAG fractions according to their molecular weight and degree of unsaturation. The obtained TAG profiles were used

- to determine the PSB content in the chocolate fat (g PSB/100 g chocolate fat),
- to calculate the MF content in the chocolate fat via the determined PSB content (g MF/100 g chocolate fat),
- to determine the three TAGs POP, POS and SOS corrected for the contribution of the same TAGs originating from milk fat, to be used
- to determine the presence/absence of CBEs in chocolate fat (qualitative decision if sample is pure CB or not: yes/no)

In case the detection approach indicated that the sample is not pure CB, the TAG profiles were used

- to determine the five TAGs POP, POS, POO, SOS and SOO normalized to 100 %, and
- to quantify the amount of the CBE admixture to chocolate fat (g CBE/100 g chocolate fat).

However, to check label compliance of chocolate products, the results have to be expressed in g MF/100 g chocolate and g CBE/100 g chocolate. To this end, it was necessary to determine the accurate amount of the total fat content of chocolate.

Moreover, to see if the applied extraction procedures, i.e., rapid fat extraction or Soxhlet extraction, have an influence on the obtained TAG profile, the fats obtained by both methods were analysed by HR-GLC and subjected to statistical analysis.

6.1 Technical evaluation of submitted results

The results of the individual laboratories were examined along with the submitted raw data, chromatograms and the results of the system suitability check. All laboratories were able to demonstrate a correctly functioning chromatographic system by fulfilling the required performance criteria (Table 10).

Table 11. System suitability for the determination of an average RF for PSB

	Mean RF_{PSB}	SD RF_{PSB}	RSD RF_{PSB}	Minimum RF_{PSB}	Maximum RF_{PSB}	Minimum RE [(1)]	Maximum RE [(1)]
Lab 1	1.47	0.03	2.32	1.43	1.51	2.71	-2.66
Lab 2	2.37	0.07	2.90	2.29	2.47	3.33	-4.06
Lab 3	1.25	0.04	3.39	1.19	1.31	4.72	-4.59
Lab 4	1.28	0.01	1.13	1.26	1.30	1.62	-1.48
Lab 5	1.27	0.01	0.91	1.26	1.30	1.05	-1.63
Lab 6	1.50	0.02	1.26	1.47	1.52	1.89	-1.63
Lab 7	1.42	0.02	1.16	1.39	1.43	1.89	-1.12
Lab 8	1.52	0.02	1.21	1.49	1.54	2.08	-1.24
Lab 9	1.25	0.02	1.62	1.22	1.28	2.43	-2.11
Lab 10	1.36	0.03	2.09	1.33	1.41	2.33	-3.55
Lab 11	1.27	0.02	1.75	1.25	1.31	1.88	-2.86
Lab 12	1.32	0.02	1.26	1.30	1.35	1.50	-1.65

[(1)] RE = relative error

Table 12: System suitability for the determination of RF values for the five TAGs POP, POS, POO, SOS and SOO

RF	POP	POS	POO	SOS	SOO	RF	POP	POS	POO	SOS	SOO
	Lab 1						**Lab 7**				
Mean	0.8	1.0	1.0	1.2	1.3	**Mean**	1.0	1.0	0.9	1.0	0.9
RSD	1.58	0.46	0.97	0.57	0.39	**RSD**	1.01	0.18	2.81	0.05	1.53
	Lab 2						**Lab 8**				
Mean	0.8	1.0	1.0	1.2	1.5	**Mean**	0.8	1.0	1.0	1.2	1.3
RSD	0.86	0.32	1.54	0.11	1.68	**RSD**	0.22	0.13	1.70	0.12	0.47
	Lab 3						**Lab 9**				
Mean	0.9	1.0	1.0	1.1	1.2	**Mean**	0.9	1.0	1.2	1.1	1.2
RSD	0.32	0.17	1.20	0.18	0.63	**RSD**	0.45	0.41	0.13	0.29	0.85
	Lab 4						**Lab 10**				
Mean	0.9	1.0	1.0	1.1	1.1	**Mean**	0.9	1.0	0.8	1.1	1.0
RSD	0.39	0.09	0.51	0.10	2.90	**RSD**	0.46	0.11	0.44	0.46	0.14
	Lab 5						**Lab 11**				
Mean	0.9	1.0	1.0	1.1	1.1	**Mean**	1.0	1.0	1.0	1.0	1.0
RSD	0.21	0.10	0.46	0.15	0.41	**RSD**	0.23	0.01	1.55	0.15	1.37
	Lab 6						**Lab 12**				
Mean	0.8	1.0	0.8	1.3	1.1	**Mean**	0.9	1.0	0.9	1.1	1.1
RSD	0.41	0.02	0.37	0.35	0.97	**RSD**	0.30	0.18	1.85	0.08	0.98

For most of the laboratories the resulting average RF for PSB was close to the theoretical RF of PSB (1.27). Only laboratory 2 obtained a higher average RF for PSB (2.37). However, since the laboratory could demonstrate a good repeatability of the chromatographic analyses, i.e., a minimum and maximum relative error between ± 5 %, the data were accepted (Table 11). The same holds true for the determination of RF values for the five main TAGs. Some of the laboratories determined RF values for the five TAGs outside the suggested limit of 0.8 to 1.2, but all of them were able to determine them with appropriate precision (repeatability RSD < 2.9 %) (Table 12). Therefore, based on the technical evaluation of the submitted results all data sets from the 12 laboratories were accepted for the validation study.

6.2 Statistical evaluation of submitted results

The individual results as submitted by the participants and accepted on a technical basis are listed in Tables E 1-38 (Annex E). For each sample the PSB amount in the sample fat and the five major TAGs POP, POS, POO, SOS and SOO normalized to 100 % are given. Furthermore, the qualitative decision if the sample fat is pure CB or not, the MF and CBE amounts related to the chocolate fat and the final product chocolate, and the total fat content of the chocolate samples are shown. For all chocolate samples (samples 1-6) the results are given once by analyzing the fat obtained from rapid fat extraction (Tables E 1-12) and once by analyzing the fat obtained by Soxhlet extraction (Tables E 13-24). The resulting data for the chocolate fat samples (samples 7-13) are listed in Tables E 25-38.

The data sets accepted on technical grounds were subjected to statistical tests by procedures described in the internationally agreed *Protocol for the Design, Conduct and Interpretation of Method Performance Studies* [32].

In Tables F 1-8 (Annex F) all data accepted for technical reasons were included in the computation of precision figures, while Tables 13-18 contain the results of the statistical evaluation performed after removal of outliers detected by using the

- Cochran (C) test to identify outlying variances, and
- Single Grubbs (SG) and double Grubbs (DG) tests to detect outlying data set averages.

Graphs of the plotted laboratory means and the corresponding laboratory ranges of the main results, i.e., PSB content of chocolate fats (Figures G 1-12), MF content of chocolate fats (Figures G 13-24), CBE content of chocolate fats (Figures G 25-33), total fat content of chocolates (Figures G 34-39), MF content of chocolates (Figures G 40-51) and the CBE content of chocolates (Figures G 52-60) are given in Annex G. Additionally, the graphs are highlighting the data sets from individual laboratories that have been rejected for statistical reasons.

6.3 Final results

6.3.1 PSB content in chocolate fat

The comparison of results obtained for the PSB content in chocolate fat by analysing once the fat from the rapid fat extraction and once from the Soxhlet extraction shows that the comparability of PSB data obtained in different laboratories is significantly better by analysing the fat from the rapid fat extraction procedure. The relative standard deviation for reproducibility (RSD_R) without removing any outliers was < 7.3 % for all six chocolate samples (Table F 1, Annex F). Moreover, the results were in the same range as the results obtained for the pure chocolate fat solutions. By analyzing the fat obtained from Soxhlet extraction, the RSD_R was for all chocolate samples > 10.6 % (Table F 2, Annex F). Hence, to calculate the final precision figures for the PSB content in chocolate fat, the results from the rapid fat extraction method were used (Table 13).

Removal of outliers did not change the final picture. The RSD_R ranged from 2 to 4.6 %. The calculated HorRAT values, which can be used as a performance parameter indicating the acceptability of the precision of a method, ranged from 0.43 to 1.60, demonstrating an acceptable performance of the method.

Table 13. Statistical evaluation of PSB amounts in chocolate fat accepted on technical and statistical grounds (samples 1-6; fat for GLC analysis obtained by rapid fat extraction)

Sample number	**1**	**2**	**3**	**4**	**5**	**6**
Year of collaborative trial	2006					
Number of laboratories	12	12	12	12	12	12
Number of outliers	0	0	3	0	0	0
Identity of outlying laboratories			6, 1, 10			
Reason for removal			C, DG			
Number of accepted laboratories	12	12	9	12	12	12
Mean value, g PSB/100 g chocolate	0.26	0.29	0.44	0.43	0.49	0.33
Repeatability limit r [r=2.8 x s_r], g/100 g	0.04	0.03	0.02	0.03	0.03	0.01
Repeatability standard deviation s_r, g/100 g	0.01	0.01	0.01	0.01	0.01	0.01
Repeatability relative standard deviation RSD_r, %	5.7	3.1	1.5	2.6	2.3	1.6
Reproducibility limit R [r=2.8 x s_R], g/100 g	0.05	0.04	0.02	0.05	0.05	0.06
Reproducibility standard deviation s_R, g/100 g	0.02	0.01	0.01	0.02	0.02	0.02
Reproducibility relative standard deviation RSD_R, %	7.3	4.8	2.0	4.1	4.0	6.1
HorRAT value = RSD_R/predicted RSD_R (1)	1.49	0.99	0.43	0.89	0.90	1.29
Sample number	**8**	**9**	**10**	**11**	**12**	**13**
Year of collaborative trial	2006					
Number of laboratories	12	12	12	12	12	12
Number of outliers	0	1	0	0	0	0
Identity of outlying laboratories		7				
Reason for removal		C				
Number of accepted laboratories	12	11	12	12	12	12
Mean value, g PSB/100 g chocolate	0.35	0.36	0.35	0.35	0.35	0.35
Repeatability limit r [r=2.8 x s_r], g/100 g	0.03	0.01	0.03	0.03	0.03	0.03
Repeatability standard deviation s_r, g/100 g	0.01	0.00	0.01	0.01	0.01	0.01
Repeatability relative standard deviation RSD_r, %	2.6	1.2	2.7	3.5	2.8	3.3
Reproducibility limit R [r=2.8 x s_R], g/100 g	0.06	0.07	0.07	0.07	0.07	0.07
Reproducibility standard deviation s_R, g/100 g	0.02	0.03	0.03	0.03	0.02	0.03
Reproducibility relative standard deviation RSD_R, %	6.4	7.3	7.6	7.5	6.8	7.2
HorRAT value = RSD_R/predicted RSD_R (1)	1.36	1.57	1.62	1.60	1.44	1.53

(1) predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

6.3.2 MF content in chocolate fat

The MF content in chocolate fat was determined via the experimentally determined PSB content using a simple linear regression model developed and in-house validated by the IRMM (Method protocol - Annex A).

In Annex F, Table F 4 all data accepted on technical grounds were included in the computation of precision figures, while Table 14 contains the results of the statistical evaluation performed after removal of the detected outliers.

The resulting precision figures (RSD_r and RSD_R) were more or less the same as obtained for the PSB content (Table 13), due to the fact that the MF content is determined via the PSB amount. Only the HorRAT values (0.77-2.88) slightly increased since the resulting MF content in chocolate fat is much higher than the PSB content in chocolate fat, having an impact on the computed HorRAT value.

The obtained overall mean MF values for the chocolate fat solutions (samples 8-13) were in close agreement with the true MF values. The relative prediction errors were well within the expected range of +/- 10 % (-3.1 to -6.7 %). In case of chocolate samples 1, 2 and 6 the agreement between the predicted MF values and the given MF values was poor. However, this could be due to the fact that the known MF values are only approximate values, calculated from ingredient composition data. On the other hand, chocolate samples 3-5 showed excellent relative prediction errors between -1 to 2 %.

Table 14. Statistical evaluation of determined MF amounts in chocolate fat accepted on technical and statistical grounds (samples 1-6; chocolate fat for GLC analysis obtained by rapid fat extraction)

Sample number	1	2	3	4	5	6
Year of collaborative trial	2006					
Number of laboratories	12	12	12	12	12	12
Number of outliers	0	0	3	0	0	0
Identity of outlying laboratories			6, 1, 10			
Reason for removal			C, DG			
Number of accepted laboratories	12	12	9	12	12	12
Mean value, g MF/100 g chocolate	11.59	13.14	19.73	19.19	21.72	14.64
Approximate value, g MF/100 g chocolate [(2)]	16.56	16.56	19.56	19.56	21.51	18.81
Bias, g MF/100 g chocolate	4.97	3.43	-0.17	0.37	-0.22	4.17
Repeatability limit r [r=2.8 x s_r], g/100 g	1.81	1.13	0.84	1.38	1.37	0.64
Repeatability standard deviation s_r, g/100 g	0.65	0.40	0.30	0.49	0.49	0.23
Repeatability relative standard deviation RSD_r, %	5.6	3.1	1.5	2.6	2.3	1.6
Reproducibility limit R [r=2.8 x s_R], g/100 g	2.44	1.83	1.10	2.20	2.41	2.54
Reproducibility standard deviation s_R, g/100 g	0.87	0.65	0.39	0.79	0.86	0.91
Reproducibility relative standard deviation RSD_R, %	7.5	5.0	2.0	4.1	4.0	6.2
HorRAT value = RSD_R/predicted RSD_R [(1)]	2.71	1.83	0.78	1.60	1.57	2.32
Sample number	8	9	10	11	12	13
Year of collaborative trial	2006					
Number of laboratories	12	12	12	12	12	12
Number of outliers	0	1	0	0	0	0
Identity of outlying laboratories		7				
Reason for removal		C				
Number of accepted laboratories	12	11	12	12	12	12
Mean value, g MF/100 g chocolate	15.72	15.99	15.66	15.69	15.46	15.53
True value, g MF/100 g chocolate	14.99	14.98	15.02	15.04	14.99	15.05
Bias, g MF/100 g chocolate	-0.73	-1.01	-0.64	-0.65	-0.47	-0.48
Repeatability limit r [r=2.8 x s_r], g/100 g	1.11	0.54	1.12	1.49	1.15	1.35
Repeatability standard deviation s_r, g/100 g	0.40	0.19	0.40	0.53	0.41	0.48
Repeatability relative standard deviation RSD_r, %	2.5	1.2	2.6	3.4	2.7	3.1
Reproducibility limit R [r=2.8 x s_R], g/100 g	2.77	3.16	3.28	3.22	2.87	3.08
Reproducibility standard deviation s_R, g/100 g	0.99	1.13	1.17	1.15	1.02	1.10
Reproducibility relative standard deviation RSD_R, %	6.3	7.0	7.5	7.3	6.6	7.1
HorRAT value = RSD_R/predicted RSD_R [(1)]	2.38	2.67	2.83	2.77	2.50	2.68

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

[(2)] MF contents are approximate values; estimate of the fat content of individual sample ingredients

6.3.3 Detection of CBEs in chocolate fat

The outcome of the study was summarised as the number of "correct", "false positive" and "false negative" results. The efficiency of the detection approach (percentage of correctly classified samples) was 100 %. Pure CB (sample 7), CB-MF (sample 8), CB-CBE-MF (samples 9-13) blends as well as all milk chocolate samples (samples 1-6) were classified correctly. This suggests a detection limit of 1.3 % CBE in chocolate fat, resulting in 0.4 % CBE in milk chocolate assuming a fat content of 30 % of chocolate.

6.3.4 CBE content in chocolate fat

The next step was to determine for samples, for which CBEs were detected, the amount of it present in the samples. The five TAGs POP, POS, POO, SOS and SOO (normalized to 100 %) and the determined MF amount of the sample were subjected to a PLS model to calculate the final amount of CBE present in chocolate fat (Method protocol, Annex A). The TAG analysis for the chocolate samples was once performed using the obtained chocolate fat by rapid fat extraction and once by Soxhlet fat extraction.

The outcome was the same as described before for the analysis of PSB. The RSD_R for the quantification of CBEs in chocolate fats was significantly lower by using the TAG profile from the rapid extraction (Table F 4, Annex F) than from the Soxhlet extraction (Table F 5, Annex F). Therefore, the final precision figures for the quantification of CBEs in chocolate fat were calculated by using the TAG results obtained from the rapid fat extraction (Table 15). The outcome of the comparison of the two extraction methods by which the chocolate fat for HR-GLC analysis can be obtained, stresses the fact that the chocolate fat for HR-GLC analysis has to be obtained by rapid fat extraction (see method protocol, Annex). The Soxhlet procedure is only used for the accurate determination of the total fat content of the chocolate.

Table 15. Statistical evaluation of determined CBE amounts in chocolate fat accepted on technical and statistical grounds (samples 2-6; chocolate fat for GLC analysis obtained by rapid fat extraction)

Sample number	**2**	**4**	**5**	**6**	
Year of collaborative trial	2006				
Number of laboratories	12	12	12	12	
Number of outliers	0	1	1	0	
Identity of outlying laboratories		2	1		
Reason for removal		C	C		
Number of accepted laboratories	12	11	11	12	
Mean value, g CBE/100 g chocolate	1.63	5.87	20.55	12.07	
True value, g CBE/100 g chocolate	1.27	6.35	20.35	11.68	
Bias, g CBE/100 g chocolate	-0.36	0.48	-0.20	-0.38	
Repeatability limit r [r=2.8 x s_r], g/100 g	0.56	0.40	0.56	0.36	
Repeatability standard deviation s_r, g/100 g	0.20	0.14	0.20	0.13	
Repeatability relative standard deviation RSD_r, %	12.2	2.4	1.0	1.1	
Reproducibility limit R [r=2.8 x s_R], g/100 g	1.45	1.63	1.71	1.31	
Reproducibility standard deviation s_R, g/100 g	0.52	0.58	0.61	0.47	
Reproducibility relative standard deviation RSD_R, %	31.7	9.9	3.0	3.9	
HorRAT value = RSD_R/predicted RSD_R (1)	8.54	3.23	1.17	1.42	
Sample number	**9**	**10**	**11**	**12**	**13**
Year of collaborative trial	2006				
Number of laboratories	12	12	12	12	12
Number of outliers	1	0	0	1	0
Identity of outlying laboratories	7			6	
Reason for removal	C			C	
Number of accepted laboratories	11	12	12	11	12
Mean value, g CBE/100 g chocolate	1.60	15.39	19.38	17.78	27.44
True value, g CBE/100 g chocolate	2.00	16.03	19.98	20.08	28.04
Bias, g CBE/100 g chocolate	0.40	0.64	0.60	2.30	0.60
Repeatability limit r [r=2.8 x s_r], g/100 g	0.47	0.87	0.63	0.65	0.77
Repeatability standard deviation s_r, g/100 g	0.17	0.31	0.22	0.23	0.27
Repeatability relative standard deviation RSD_r, %	10.4	2.0	1.2	1.3	1.0
Reproducibility limit R [r=2.8 x s_R], g/100 g	1.27	1.66	1.54	1.78	1.73
Reproducibility standard deviation s_R, g/100 g	0.46	0.59	0.55	0.64	0.62
Reproducibility relative standard deviation RSD_R, %	28.5	3.8	2.8	3.6	2.2
HorRAT value = RSD_R/predicted RSD_R (1)	7.64	1.45	1.11	1.38	0.93

(1) predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

For samples 2, 4 and 9, in which the CBE addition was very low, i.e., less than 7 % (based on chocolate fat), the RSD_R was > 10 %. However, for samples, were the CBE addition was > 10 %, the RSD_R was in all cases less than 4 %. This is due to the fact that the established PLS model to calculate the final CBE addition was fitted to CBE amounts around the statutory level of

5 % of the final chocolate product. This would translate to a CBE addition of 15 % in chocolate fat, assuming a fat content of chocolate of 30 %.

The obtained overall mean values were in close agreement with the true values. With the exception of sample 12, the differences between the predicted values and the true values for all samples were between -0.38 and 0.6 %. Assuming a fat content of chocolate of 30 % this translates to ± 0.2 %. Sample 12, containing a soft palm mid fraction as CBE, shows a higher bias, i.e., 2.3 % (translating to 0.7 %, assuming a fat content of chocolate of 30 %).

6.3.5 Total fat content of chocolate samples

So far, all the results were expressed on the basis of chocolate fat. In the end, the results have to be expressed in g MF/100 g chocolate and g CBE/100 g chocolate, to check label compliance of chocolate products. To this end, it was necessary to determine the accurate amount of chocolate fat present in the chocolate samples. Even though sample 1 and 3 did not contain CBEs, the participants were asked to determine the total fat content of all chocolate samples (samples 1-6).

Procedures are in place for the extraction of total fat from chocolate. The most widely accepted method uses an acid digestion step to release bound lipids, followed by extraction with petroleum ether using a Soxhlet apparatus. The solvent is evaporated from the extract and the residue dried and weighed. The recommended procedure in the method protocol for the determination of the accurate amount of chocolate fat in chocolate was AOAC Official Method 936.15 [30]. However, alternative extraction procedures were allowed to use (e.g. by accelerated solvent extraction, by supercritical carbon dioxide or by using microwaves) provided that the same results were obtained.

In Table F 6 (Annex F) all data accepted on technical grounds were included in the computation of precision figures, while Table 16 contains the results of the statistical evaluation performed after removal of detected outliers. By

removing statistical outliers the RSD_R was reduced from 2.2 % to 1.2 %. The obtained HorRAT values of < 0.5 indicate that the laboratories have excellent experience with the applied methods. Moreover, the agreement between the determined mean value and the true value was very close.

Table 16. Statistical evaluation of determined total fat contents of chocolate samples accepted on technical and statistical grounds (total fat content determined by Soxhlet extraction)

Sample number	1	2	3	4	5	6
Year of collaborative trial	2006					
Number of laboratories	12	12	12	12	12	12
Number of outliers	3	1	0	0	2	1
Identity of outlying laboratories	3, 2, 4	3			3, 6	3
Reason for removal	C, DG	C			C, C	C
Number of accepted laboratories	9	11	12	12	10	11
Mean value, g total fat/100 g chocolate	34.71	35.63	31.93	32.11	25.28	33.86
True value, g total fat/100 g chocolate	35.56	35.56	31.95	31.95	25.11	33.81
Bias, g total fat/100 g chocolate	0.85	-0.07	0.02	-0.16	-0.17	-0.05
Repeatability limit r [$r=2.8 \times s_r$], g/100 g	0.31	0.19	0.71	0.34	0.33	0.46
Repeatability standard deviation s_r, g/100 g	0.11	0.07	0.26	0.12	0.12	0.17
Repeatability relative standard deviation RSD_r, %	0.3	0.2	0.8	0.4	0.5	0.5
Reproducibility limit R [$r=2.8 \times s_R$], g/100 g	0.43	0.71	0.82	0.70	0.72	1.11
Reproducibility standard deviation s_R, g/100 g	0.15	0.25	0.29	0.25	0.26	0.40
Reproducibility relative standard deviation RSD_R, %	0.4	0.7	0.9	0.8	1.0	1.2
HorRAT value = RSD_R/predicted RSD_R (1)	0.19	0.31	0.38	0.33	0.41	0.50

(1) predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

6.3.6 MF content in chocolate

By using the determined total fat contents of the chocolate samples (1-6) and an average assumed total fat content for the chocolate fat solutions (samples 8-13) of 30 %, the obtained results for the MF content based on chocolate fat (g MF/100 g chocolate fat) were converted to g MF/100 g chocolate.

The RSD_R for the chocolate samples (samples 1-6) showed a spread ranging from 2.1 to 7.1 %, whereas the RSD_R for the chocolate fat solutions (samples 8-13) ranged from 6.3 to 7.5 % (Table 17), demonstrating that the whole approach, which is based solely on chocolate fat blends (CB-CBE-MF mixtures), is applicable to real chocolate samples. Moreover, the results are suggesting that the additional analytical steps which have to be applied in

case of real chocolate samples, i.e., (i) the extraction of the chocolate fat from the chocolate samples by rapid fat extraction to be used for the TAG analysis and (ii) the determination of the total fat content of chocolate samples by Soxhlet extraction, do not degrade the final outcome.

Table 17. Statistical evaluation of determined MF amounts in chocolate accepted on technical and statistical grounds (samples 2-6, chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet extraction; samples 9-13, assumed fat content of chocolate = 30 %)

Sample number	**1**	**2**	**3**	**4**	**5**	**6**
Year of collaborative trial	2006					
Number of laboratories	12	12	12	12	12	12
Number of outliers	0	1	2	2	0	0
Identity of outlying laboratories		3	1, 10	1, 10		
Reason for removal		C	DG	DG		
Number of accepted laboratories	12	11	10	10	12	12
Mean value, g MF/100 g chocolate	4.08	4.69	6.31	6.17	5.50	4.95
Approximate value, g MF/100 g chocolate	5.89	5.89	6.25	6.25	5.40	6.36
Bias, g fat/100 g chocolate	1.81	1.20	-0.06	0.08	-0.10	1.41
Repeatability limit r [r=2.8 x s_r], g/100 g	0.45	0.30	0.28	0.52	0.33	0.38
Repeatability standard deviation s_r, g/100 g	0.16	0.11	0.10	0.19	0.12	0.14
Repeatability relative standard deviation RSD_r, %	3.9	2.3	1.6	3.0	2.1	2.8
Reproducibility limit R [r=2.8 x s_R], g/100 g	0.60	0.67	0.38	0.52	0.60	0.98
Reproducibility standard deviation s_R, g/100 g	0.22	0.24	0.13	0.19	0.22	0.35
Reproducibility relative standard deviation RSD_R, %	5.3	5.1	2.1	3.0	3.9	7.1
HorRAT value = RSD_R/predicted RSD_R (1)	1.63	1.61	0.70	1.00	1.27	2.26
Sample number	**8**	**9**	**10**	**11**	**12**	**13**
Year of collaborative trial	2006					
Number of laboratories	12	12	12	12	12	12
Number of outliers	0	1	0	0	0	0
Identity of outlying laboratories		7				
Reason for removal		C				
Number of accepted laboratories	12	11	12	12	12	12
Mean value, g MF/100 g chocolate	4.72	4.80	4.70	4.71	4.63	4.66
True value, g MF/100 g chocolate	4.50	4.49	4.51	4.51	4.50	4.52
Bias, g fat/100 g chocolate	-0.22	-0.31	-0.19	-0.20	-0.13	-0.14
Repeatability limit r [r=2.8 x s_r], g/100 g	0.33	0.16	0.34	0.45	0.28	0.41
Repeatability standard deviation s_r, g/100 g	0.12	0.06	0.12	0.16	0.10	0.14
Repeatability relative standard deviation RSD_r, %	2.5	1.2	2.6	3.4	2.2	3.1
Reproducibility limit R [r=2.8 x s_R], g/100 g	0.83	0.95	0.98	0.97	0.87	0.92
Reproducibility standard deviation s_R, g/100 g	0.30	0.34	0.35	0.35	0.31	0.33
Reproducibility relative standard deviation RSD_R, %	6.3	7.0	7.5	7.3	6.7	7.1
HorRAT value = RSD_R/predicted RSD_R (1)	1.99	2.23	2.36	2.31	2.12	2.23

(1) predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

6.3.7 CBE content in chocolate

By using the determined total fat contents of the chocolate samples (1-6) and an average assumed total fat content for the chocolate fat solutions (samples 8-13) of 30 %, the obtained results for the CBE content based on chocolate fat (g CBE/100 g chocolate fat) were converted to g CBE/100 g chocolate.

Table 18. Statistical evaluation of determined CBE amounts in chocolate accepted on technical and statistical grounds (samples 2-6, chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet extraction; samples 9-13, assumed fat content of chocolate = 30 %)

Sample number	**2**	**4**	**5**	**6**	
Year of collaborative trial	2006				
Number of laboratories	12	12	12	12	
Number of outliers	0	1	0	1	
Identity of outlying laboratories		2		3	
Reason for removal		C		C	
Number of accepted laboratories	12	11	12	11	
Mean value, g CBE/100 g chocolate	0.58	1.88	5.20	4.08	
True value, g CBE/100 g chocolate	0.45	2.03	5.11	3.95	
Bias, g fat/100 g chocolate	-0.13	0.15	-0.09	-0.13	
Repeatability limit r [r=2.8 x s_r], g/100 g	0.19	0.12	0.28	0.15	
Repeatability standard deviation s_r, g/100 g	0.07	0.04	0.10	0.05	
Repeatability relative standard deviation RSD_r, %	11.6	2.2	1.9	1.3	
Reproducibility limit R [r=2.8 x s_R], g/100 g	0.52	0.54	0.59	0.53	
Reproducibility standard deviation s_R, g/100 g	0.19	0.19	0.21	0.19	
Reproducibility relative standard deviation RSD_R, %	31.8	10.1	4.1	4.7	
HorRAT value = RSD_R/predicted RSD_R (1)	7.34	2.79	1.30	1.45	
Sample number	**9**	**10**	**11**	**12**	**13**
Year of collaborative trial	2006				
Number of laboratories	12	12	12	12	12
Number of outliers	1	0	0	0	0
Identity of outlying laboratories	6				
Reason for removal	C				
Number of accepted laboratories	11	12	12	12	12
Mean value, g CBE/100 g chocolate	0.48	4.62	5.81	5.35	8.23
True value, g CBE/100 g chocolate	0.60	4.81	5.99	6.02	8.41
Bias, g fat/100 g chocolate	0.12	0.19	0.18	0.67	0.18
Repeatability limit r [r=2.8 x s_r], g/100 g	0.14	0.26	0.19	0.19	0.23
Repeatability standard deviation s_r, g/100 g	0.05	0.09	0.07	0.07	0.08
Repeatability relative standard deviation RSD_r, %	10.4	2.0	1.2	1.2	1.0
Reproducibility limit R [r=2.8 x s_R], g/100 g	0.38	0.50	0.46	0.53	0.52
Reproducibility standard deviation s_R, g/100 g	0.14	0.18	0.17	0.19	0.19
Reproducibility relative standard deviation RSD_R, %	28.5	3.8	2.8	3.5	2.2
HorRAT value = RSD_R/predicted RSD_R (1)	6.37	1.21	0.93	1.13	0.77

(1) predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

The same observations were made as mentioned before for the determination of the MF content. The RSD_R for quantification of CBEs around the statutory limit of 5 % did not show any difference for real chocolate samples (samples 5-6) and for chocolate fat solutions (samples 10-13). The HorRAT values ranged from 0.77 to 1.45, suggesting excellent performance of the method (Table 18). The results of samples 2, 4 and 9 are just given as an example to show that the RSD_R in case of very low CBE additions, i.e., less than 2 %, increased dramatically. As already explained before, this is due to the fact that the established quantification approach is fitted to CBE additions around the statutory level of 5 %.

7 CONCLUSIONS

Validated analytical methods are those that have been subjected to collaborative trial assessment and for which performance characteristics such as bias, repeatability (r) and reproducibility (R) have been determined.

The obtained overall mean MF values for the chocolate fat solutions were in close agreement with the true MF values and the RSD_R was in the worst case 7.5 %. CBE admixtures were detected down to a level of 0.5 g CBE/100 g chocolate without false-positive or false-negative results. By using the newly developed quantification model based on PLS regression analysis the predicted CBE amounts were in close agreement with the true values. The applied model performed well at the statutory limit of 5 % CBE addition to chocolate with a prediction error of 0.7 %. High comparability of data between individual laboratories was demonstrated, resulting in excellent precision data. The RSD_R (< 5 %) for quantification of CBEs did not show any difference for real chocolate samples and for chocolate fat solutions, demonstrating that the whole approach, which is based solely on chocolate fat blends (CB-CBE-MF mixtures), is applicable to real chocolate samples. The HorRAT values were ranging from 0.77 to 1.45, suggesting a good performance of the whole approach.

The compulsory use of IRMM-801 for calibration purpose and system suitability check ensures high comparability of the results between individual testing laboratories. Moreover, commutability of the elaborated approach, which is based on reliable databases created under strict quality control schemes, reflecting as good as possible the natural variability of CBs, MFs and CBEs, is guaranteed.

The objective of the performed collaborative trial, i.e., to demonstrate that the defined method protocol produces acceptably accurate, repeatable and reproducible results even though applied by individual laboratories, was accomplished. The IRMM is currently transforming the method protocol (Annex A) into an ISO format and will submit it to ISO for adoption.

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ANNEX A – METHOD PROTOCOL

VALIDATION OF AN ANALYTICAL METHOD FOR THE DETECTION AND QUANTIFICATION OF COCOA BUTTER EQUIVALENTS IN MILK CHOCOLATE

Draft of an analytical method for the detection and quantification of cocoa butter equivalents in milk chocolate in a suitable format for intercomparison purposes.

1 Scope

This draft standard specifies a procedure for the detection and quantification of cocoa butter equivalents (CBEs) in cocoa butter-milk fat (CB-MF) mixtures and milk chocolate by triacylglycerol (TG) profiling using high-resolution capillary gas chromatography (HR-GC), and subsequent data evaluation by simple and multiple linear regression analysis.

2 Principle

The sample, i.e. a CB-MF mixture, a CB-CBE-MF mixture, or the fat obtained from milk chocolate is separated by HR-GC into triacylglycerol fractions according to their molecular weight and degree of unsaturation. The obtained triacylglycerol profile is used

- to determine the milk fat content
- to calculate the contribution of some triacylglycerols deriving from MF
- to determine the presence/absence of CBEs and if present
- to quantify the amount of the CBE admixture in the test sample.

The presence of CBEs is detected by simple linear regression analysis applied to the relative proportions of the three triacylglycerol fractions, i.e. POP, POS, SOS, which are corrected for the triacylglycerol amounts derived from MF. The amount of the CBE admixture is estimated by multiple linear regression analysis using the following variables: the relative proportions of the five triacylglycerols, i.e. POP, POS, POO, SOS, SOO and the milk fat content of the test sample.

Abbreviations:
POP 1,3-Dipalmitoyl-2-oleoylglycerol
POS 1-Palmitoyl-2-oleoyl-3-stearoylglycerol
POO 1-Palmitoyl-2,3-dioleoylglycerol
SOS 1,3-Distearoyl-2-oleoylglycerol
SOO 1-Stearoyl-2,3-dioleoylglycerol

3 Reagents and materials

Use only reagents of recognised 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.

3.1 **Cocoa butter Certified Reference Material (CRM) IRMM-801** [1], for calibration purposes and system suitability check.

3.2 **Pure milk fat,** for system suitability check.

3.3 **1-Palmitin-2-stearin-3-butyrin (PSB)**, for calibration purposes (6 different solutions).
Dissolve 50 mg in 50 ml iso-octane and dilute 1 ml of this solution to 100 ml resulting in a stock solution of c = 0.01 mg/ml. From this stock solution dilute 1.0, 2.0, 4.0, 6.0 and 8.0 ml to 10 ml. The solutions contain respectively 0.008, 0.006, 0.004, 0.002 and 0.001 mg PSB/ml iso-octane.

3.4 **alpha-Cholestane**, used as internal standard (c = 0.004 mg/ml).
Dissolve 40 mg alpha-cholestane in 100 ml of iso-octane and dilute 1 ml of this solution to 100 ml.

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

3.6 **Hydrochloric acid**, c = 8 mol/l.

4 Apparatus

4.1 **Analytical balance**, with a readability of 0.1 mg.

4.2 **Drying oven.**
A dry heater block may be used.

4.3 **Filter paper**, 15 cm.
(e.g. S&S 589[1] black ribbon paper is an example of a suitable product commercially available)

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

4.5 **Rotary evaporator.**
Alternative evaporation procedures may be used.

4.6 **Evaporation block**, with nitrogen supply.

4.7 **Desiccator**.

4.8 **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.

4.9 **Volumetric flasks**, of capacity 10, 50 and 100 mL (or other capacities if needed).

4.10 **Pipettes**, of capacities ranging from 1 to 10 mL (or other capacities if needed).

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

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

NOTE: 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 8.2.

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

GC column:	CP-TAP 25 m x 0.25 mm i.d., fused silica coated with a medium polar thermo stable phenylmethylpolysiloxane stationary phase with a film thickness of 0.10 µm. (Other suitable columns are e.g. Rtx 30 m x 0.25 mm x 0.10 µm or DB17-HT 30 m x 0.25 mm x 0.15 µm)
Temperature programme:	100°C held for 2 min; 30°C/min to 270°C held for 1 min; 2.5°C/min to 340°C held for 7 min
Injector:	Cold on-column
Detector (FID):	360°C
Carrier gas:	H_2 (purity $\geq$ 99,999 %) with a constant flow rate of 3.5 ml/min (Another suitable carrier gas is helium)

NOTE: Operating conditions may be changed to obtain optimum separation conditions.

4.13 **Chromatographic data system**.

5 Preparation of samples

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

Before opening and using the cocoa butter CRM (3.1), the ampoule shall be warmed in an oven 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.

5.2 Preparation of pure milk fat for system suitability check

If no pure milk fat is available it can be obtained from a butter sample by melting and passing the fat layer through a folded filter (4.1) at 50 ˚C in an oven.

5.3 Preparation of chocolate sample

Chill approx. 200 g of chocolate until hard, and grate to fine granular condition using a mechanical device (4.4). Mix thoroughly and preserve in tightly stoppered bottle in a cool place.

5.3.1 Rapid fat extraction

The fat is separated from 5 g grated chocolate (5.3) by extracting with two or three 10 mL portions of a suitable fat solvent (3.5). Centrifuge and decant. Combine the extracts and evaporate most of the fat solvent (4.5) and finally dry it under a stream of nitrogen (4.6).

NOTE: Alternative extraction procedures may be used provided that the same results are obtained. The rapid fat extraction is used for the detection of CBEs in chocolate (the accurate amount of fat in chocolate is not needed). In case, no CBEs are detected the second part of the standard, i.e. quantification of CBEs around the statutory limit of 5 %, has not to be carried out anymore. In case, CBEs are detected, the quantification part has to be performed and in this case the accurate amount of fat in chocolate has to be determined using the following procedure (5.3.2).

5.3.2 Fat extraction - Soxhlet

Separate the fat and determine the fat content in a sample of milk chocolate (prepared as described in 5.3) by Soxhlet extraction [2], 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 (3.6) 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 (4.1), 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 (4.7) then weigh it. Place the thimble containing the dried sample in the Soxhlet

apparatus (4.8), 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 (4.7) 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 mass fraction in percent of fat in the chocolate, $M_{fat;\,choc}$, is calculated as follows:

Equation 1: $$M_{fat;\,choc} = \frac{w_{fat} \times 100\,\%}{w}$$

where

w	is the mass of the test sample (chocolate) taken, in grams
w_{fat}	is the mass of the total fat obtained from the test sample by extraction, in grams
$M_{fat;\,choc}$	is the mass fraction in percent of fat in chocolate

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.

Report the result to two decimal places.

6 Procedure

6.1 Construction of calibration curve for determination of PSB content

Six calibration solutions containing different concentrations of PSB (3.3) but always the same concentration of alpha-cholestane (3.4) have to be prepared as follows:

- **Calibration solution 1 ($c_{PSB\ 1}$ = 0.005 mg/ml; $c_{alpha\text{-}cholestane\ 1}$ = 0.002 mg/ml):**
  Transfer 1 ml of the PSB solution (c = 0.01 mg/ml; 3.3) in a test tube and add 1 ml of alpha-cholestane solution (c = 0.004 mg/ml; 3.4).
- **Calibration solution 2 ($c_{PSB\ 2}$ = 0.004 mg/ml; $c_{alpha\text{-}cholestane\ 2}$ = 0.002 mg/ml):**
  Transfer 1 ml of the PSB solution (c = 0.008 mg/ml; 3.3) in a test tube and add 1 ml of alpha-cholestane solution (c = 0.004 mg/ml; 3.4).
- **Calibration solution 3 ($c_{PSB\ 3}$ = 0.003 mg/ml; $c_{alpha\text{-}cholestane\ 3}$ = 0.002 mg/ml):**
  Transfer 1 ml of the PSB solution (c = 0.006 mg/ml; 3.3) in a test tube and add 1 ml of alpha-cholestane solution (c = 0.004 mg/ml; 3.4).
- **Calibration solution 4 ($c_{PSB\ 4}$ = 0.002 mg/ml; $c_{alpha\text{-}cholestane\ 4}$ = 0.002 mg/ml):**

Transfer 1 ml of the PSB solution (c = 0.004 mg/ml; 3.3) in a test tube and add 1 ml of alpha-cholestane solution (c = 0.004 mg/ml; 3.4).

- **Calibration solution 5 ($c_{PSB\ 5}$ = 0.001 mg/ml; $c_{alpha\text{-}cholestane\ 5}$ = 0.002 mg/ml):**
  Transfer 1 ml of the PSB solution (c = 0.002 mg/ml; 3.3) in a test tube and add 1 ml of alpha-cholestane solution (c = 0.004 mg/ml; 3.4).
- **Calibration solution 6 ($c_{PSB\ 6}$ = 0.0005 mg/ml; $c_{alpha\text{-}cholestane\ 6}$ = 0.002 mg/ml):**
  Transfer 1 ml of the PSB solution (c = 0.001 mg/ml; 3.3) in a test tube and add 1 ml of alpha-cholestane solution (c = 0.004 mg/ml; 3.4).

Inject 0.5 µl of each calibration solution 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 (8.2) are met.

6.2 Separation of individual triacylglycerols of cocoa butter CRM by HR-GC

The cocoa butter CRM (3.1) shall be warmed in a drying oven until completely melted. 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. 0.1 g of test sample in a 10 ml volumetric flask and dilute to the mark with iso-octane (3.5). Pipette 1 ml of the resulting solution in another 50 ml volumetric flask and dilute to the mark with the same solvent (c = 0.2 mg/mL).

Inject 0.5 µl of the final test solution into the HR-GC system using the cold on-column injection system.

NOTE: Alternative fat solvents, sample amounts and injectors may be used provided that the detection system employed gives a linear response and the system suitability criteria (8.2) are met.

6.3 Separation of individual triacylglycerols of pure milk fat by HR-GC

Weigh ca. 0.1 g of pure milk fat (3.2) in a 10 ml volumetric flask and dilute to the mark with iso-octane (3.5). Pipette 1 ml of the resulting solution in another 10 ml volumetric flask and dilute to the mark with the same solvent (c = 1 mg/ml). Transfer 1 ml of this solution in a test tube and add 1 ml of alpha-cholestane solution (3.4) (resulting test sample solution c = 0.5 mg/ml).

Inject 0.5 µl of the final test solution into the HR-GC system using the cold on-column injection system.

NOTE: Alternative fat solvents, sample amounts and injectors may be used provided that the detection system employed gives a linear response and the system suitability criteria (8.2) are met.

6.4 Separation of individual triacylglycerols of test sample by HR-GC

The test sample (fat extracted from milk chocolate) shall be warmed in a drying oven 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. 0.1 g of test sample in a 10 ml volumetric flask and dilute to the mark with iso-octane (3.5). Pipette 1 ml of the resulting solution in another 10 ml volumetric flask and dilute to the mark with the same solvent (c = 1 mg/ml). Transfer 1 ml of this solution in a test tube and add 1 ml of alpha-cholestane solution (3.4) (resulting test sample solution c = 0.5 mg/ml).

Inject 0.5 µl of the final test solution into the HR-GC system using the cold on-column injection system.

NOTE: Alternative fat solvents, sample amounts and injectors may be used provided that the detection system employed gives a linear response and the system suitability criteria (8.2) are met.

6.5 Identification

Identification of 1-Palmitin-2-stearin-3-butyrin (PSB) and alpha-cholestane is made by comparison of the retention times of the test sample with those of the reference standards. Identification of the five major triacylglycerol 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 sample with those of the cocoa butter CRM (3.1).

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 Figure A 1. The elution order of the triacylglycerols of an average pure milk fat is given in Figure A 2.

7 Calculation

7.1 Determination of milk fat content in test sample

7.1.1 Determination of PSB response factor

Determine the response factor of the triacylglycerol PSB by injection of the six calibration solutions (6.1) using experimental conditions identical to those used for the test sample. For each calibration solution a response factor for PSB, F_{PSB}, has to be calculated by

Equation 2: $$F_{PSB;i} = \frac{C_{PSB;i} \times A_{Cholestane;i}}{C_{Cholestane;i} \times A_{PSB;i}}$$

where

$A_{PSB;\,i}$	is the peak area of the triacylglycerol PSB in the calibration solution i
$A_{Cholestane;\,i}$	is the peak area of the internal standard alpha-cholestane in the calibration solution i
$C_{PSB;\,i}$	is the concentration [mg/mL] of the triacylglycerol PSB used in the calibration solution i
$C_{Cholestane;\,i}$	is the concentration [mg/mL] of the internal standard alpha-cholestane used in the calibration solution i
$F_{PSB;\,i}$	is the detector response factor of PSB in the calibration solution i

An average response factor for PSB, $F_{PSB;\,mean}$, obtained from the six calibration solutions has to be calculated (Equation 3) and used for further calculations.

Equation 3: $$F_{PSB;mean} = \frac{F_{PSB;1} + F_{PSB;2} + F_{PSB;3} + F_{PSB;4} + F_{PSB;5} + F_{PSB;6}}{6}$$

7.1.2 Determination of PSB content in test sample

The mass fraction in percent of PSB in the test sample, $M_{PSB;\,choc}$, is calculated as follows:

Equation 4: $$M_{PSB;choc} = \frac{A_{PSB} \times C_{Cholestane} \times F_{PSB;mean} \times 100\,\%}{A_{Cholestane} \times C_{Sample}}$$

where

A_{PSB}	is the peak area of PSB in the test sample
$A_{Cholestane}$	is the peak area of the internal standard alpha-cholestane in the test sample
$F_{PSB;\,mean}$	is the average response factor for PSB (as determined in Equation 3)
$C_{Cholestane}$	is the concentration [mg/mL] of the internal standard alpha-cholestane in the test sample
C_{Sample}	is the concentration [mg/mL] of the test sample
$M_{PSB;\,choc}$	is the mass fraction in percent of PSB in the test sample

Report the results to two decimal places.

7.1.3 Determination of the milk fat content in test sample

The mass fraction in percent of milk fat in the test sample, $M_{MF;\,choc}$, is calculated as follows:

Equation 5: $M_{MF;choc} = 0.190 + (44.036 \times M_{PSB;choc})$

where

$M_{PSB;\,choc}$	is the mass fraction in percent of PSB in the test sample (as determined in Equation 4)
$M_{MF;\,choc}$	is the mass fraction in percent of milk fat in the test sample

Report the results to two decimal places.

7.2 Detection of CBEs in the test sample

7.2.1 Determination of response factors for three main triacylglycerols

Determine the response factors of the triacylglycerols POP, POS, and SOS by injection of the cocoa butter CRM solution using experimental conditions identical to those used for the test sample. Calculate the percentage of each of the three triacylglycerols with respect to all triacylglycerols present in the cocoa butter CRM by the following equations:

Equation 6: $P_{i;ref} = \frac{A_{i;ref}}{\sum A_{all\ TGs;ref}} \times 100\,\%$

Equation 7: $F_i = \frac{M_{i;ref}}{P_{i;ref}}$

where

$A_{i;\,ref}$	is the peak area of the triacylglycerol i in the cocoa butter CRM
$\Sigma A_{all\ TGs;\,ref}$	is the sum of the peak areas attributed to all triacylglycerols in the cocoa butter CRM
$P_{i;\,ref}$	is the percentage of triacylglycerol i in the cocoa butter CRM (from peak areas)
$M_{i;\,ref}$	is the mass fraction in percent of triacylglycerol i in the cocoa butter CRM as given in the certificate [1], i.e. POP=16.00 %, POS=39.40 %, SOS=27.90 %
F_i	is the detector response factor of triacylglycerol i in the cocoa butter CRM

Report the results to two decimal places.

7.2.2 Calculation of mass percentages of three main triacylglycerols

Calculate the mass percentage of the triacylglycerols POP, POS and SOS in the test sample with respect to all triacylglycerols present in the test sample by

Equation 8: $$M_{i;\,total} = \frac{F_i \times A_i}{\sum A_{all\ TGs}} \times 100\,\%$$

where

A_i	is the peak area corresponding to the triacylglycerol i in the test sample
$\sum A_{all\ TGs}$	is the sum of the peak areas attributed to all triacylglycerols in the test sample (excluding alpha-cholestane)
F_i	is the response factor for the triacylglycerol i (as determined in Equation 7)
$M_{i;\,total}$	is the mass fraction in percent of triacylglycerol i in the test sample

Report the results to two decimal places.

7.2.3 Correction for milk fat contribution

Calculate the contribution of the mass percentages of the triacylglycerols POP, POS and SOS deriving from milk fat by

Equation 9: $$M_{i;mf} = \frac{M_{MF;\,choc}\ \mathrm{x}\ M_{i;ref}}{100\,\%}$$

where

$M_{i;\,ref}$	is the average mass fraction in percent of triacylglycerol i in a milk fat, i.e. POP=3.99 %, POS=2.19 %, SOS=0.45 % (values are obtained from a standardised database)
$M_{MF;\,choc}$	is the mass fraction in percent of milk fat in the test sample (as determined in Equation 5)
$M_{i;\,mf}$	is the mass fraction in percent of triacylglycerol i derived from milk fat in the test sample

Subtract the obtained mass percentages of the three triacylglycerols derived from milk fat (Equation 9) from the mass percentages for the three triacylglycerols obtained for the test sample (Equation 8).

Equation 10: $$M_{i;\,corr.} = M_{i;\,total} - M_{i;mf}$$

Normalise the obtained mass percentages of the three triacylglycerols (Equation 10) to 100 %:

Equation 11: $POP_{corr.} + POS_{corr.} + SOS_{corr.} = 100\,\%$

Report the results to two decimal places.

7.2.4 Decision if sample is pure cocoa butter

The variability of the triacylglycerol composition of cocoa butter is expressed by Equation 12 using the normalised triacylglycerols, i.e. $POP_{corr.}$ + $POS_{corr.}$ + $SOS_{corr.}$ = 100 % as determined in Equation 11.

Equation 12: $POP_{corr.} = 43.734 - 0.733 \times SOS_{corr.}$ (residual $SD = 0.125$)

The principle of the method is that for pure cocoa butter samples POS is practically constant for wide variations of POP and SOS, resulting in a linear relationship (so-called “CB-line”, Equation 12) between POP and SOS. CBE admixtures 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.
For 99% of all analyses, pure cocoa butter complies with:

Equation 13: $POP_{corr.} < 44.025 - 0.733 \times SOS_{corr.}$

A greater value of $POP_{corr.}$, as given by Equation 13, means that the sample is not pure cocoa butter. The advantage of the elaborated approach is that by using the cocoa butter CRM for calibration purpose, 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 13).

7.3 Quantification of CBEs in test sample

7.3.1 Determination of response factors for five main triacylglycerols

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:

Equation 14: $$P_{i;ref} = \frac{A_{i;ref}}{\sum A_{i;ref}} \times 100\,\%$$

Equation 15: $F_i = \frac{M_{i;ref}}{P_{i;ref}}$

where

$A_{i;\,ref}$	is the peak area of the triacylglycerol i in the cocoa butter CRM
$\Sigma A_{i;\,ref}$	is the sum of the peak areas attributed to POP, POS, POO, SOS, SOO in the cocoa butter CRM
$P_{i;\,ref}$	is the percentage of triacylglycerol i in the cocoa butter CRM (from peak areas)
$M_{i;\,ref}$	is the mass fraction in percent of triacylglycerol i in the cocoa butter CRM as given in the certificate [1], i.e. POP=18.14 %, POS=44.68 %, POO=2.26 %, SOS=31.63 % and SOO=3.29 %
F_i	is the detector response factor of triacylglycerol i in the cocoa butter CRM

Report the results to two decimal places.

7.3.2 Calculation of mass percentages of triacylglycerols

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

Equation 16: $M_{i;\,choc} = \frac{F_i \times A_i}{\sum (F_i \times A_i)} \times 100\,\%$

where

F_i	is the response factor of the triacylglycerol i (as determined in Equation 15)
A_i	is the peak area corresponding to the triacylglycerol i in the test sample
$M_{i;\,choc}$	is the mass fraction in percent of triacylglycerol i in the test sample

Report the results to two decimal places.

7.3.3 Calculation of CBE content in cocoa butter

The mass fraction in percent of CBE in the cocoa butter, $M_{CBE;\,cocoa}$, is calculated by using a multiple linear regression analysis (Equation 17) of the relative proportions of the five main triacylglycerols; i.e. POP_{choc} + POS_{choc} + POO_{choc} + SOS_{choc} + SOO_{choc} = 100 % as determined in Equation 16 and the milk fat content of the sample, i.e. $M_{MF;\,choc}$ as determined in Equation 5.

Equation 17: $M_{CBE;\,cocoa} = -4.247 - (0.232 \times M_{MF;\,choc}) + (1.522 \times POP_{choc}) - (1.469 \times POS_{choc}) + (1.097 \times POO_{choc}) + (1.287 \times SOS_{choc}) + (0.261 \times SOO_{choc})$

Report the result to two decimal places.

7.3.4 Calculation of CBE content in chocolate

The mass fraction in percent of CBE in the final product chocolate, $M_{CBE;\,choc}$, is calculated by applying Equation 18:

Equation 18: $$M_{CBE;\,choc} = \frac{M_{fat;\,choc} \times M_{CBE;\,cocoa}}{100\,\%}$$

where

$M_{fat;\,choc}$	is the mass fraction in percent of fat in chocolate (Equation 1)
$M_{CBE;\,cocoa}$	is the mass fraction in percent of CBE in cocoa butter (Equation 17)
$M_{CBE;\,choc}$	is the mass fraction in percent of CBE in chocolate

Report the result to two decimal places.

8 Procedural requirements

8.1 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 (8.2) are met.

8.2 System suitability

8.2.1 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. This can be done with the cocoa butter CRM as shown in Figure A 1.

- The HR-GC separation system shall be capable of separating PSB from neighbouring peaks within CN 38 group. This can be tested with a pure milk fat sample as shown in Figure A 3.

- The HR-GC separation system shall be capable of showing no co-elution for the internal standard alpha-cholestane. This can be tested with a pure milk fat sample using alpha-cholestane as internal standard as shown in Figure A 4.

NOTE: In the case of failure, the chromatographic conditions (e.g. sample size, column temperature, carrier gas flow, etc.) must be optimized.

8.2.2 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 HR-GC conditions. Experience has shown that for a properly functioning chromatographic system the response factors for the five triacylglycerols (POP, POS, POO, SOS, SOO) vary within a range of 0.80 to 1.20. The stability of the system has to be verified by repeating analysis (at least triplicates). The obtained relative standard deviations of the determined detector response factors shall be less than 5 %.

- To check the stability of the separation system a calibration curve for PSB wit alpha-cholestane as internal standard shall be established (at least duplicate injection of each calibration solution). The average detector response factor for PSB shall be calculated. The relative error of the minimum obtained response factor and the relative error of the maximum obtained response factor shall be less than 5 % with respect to the average response factor.

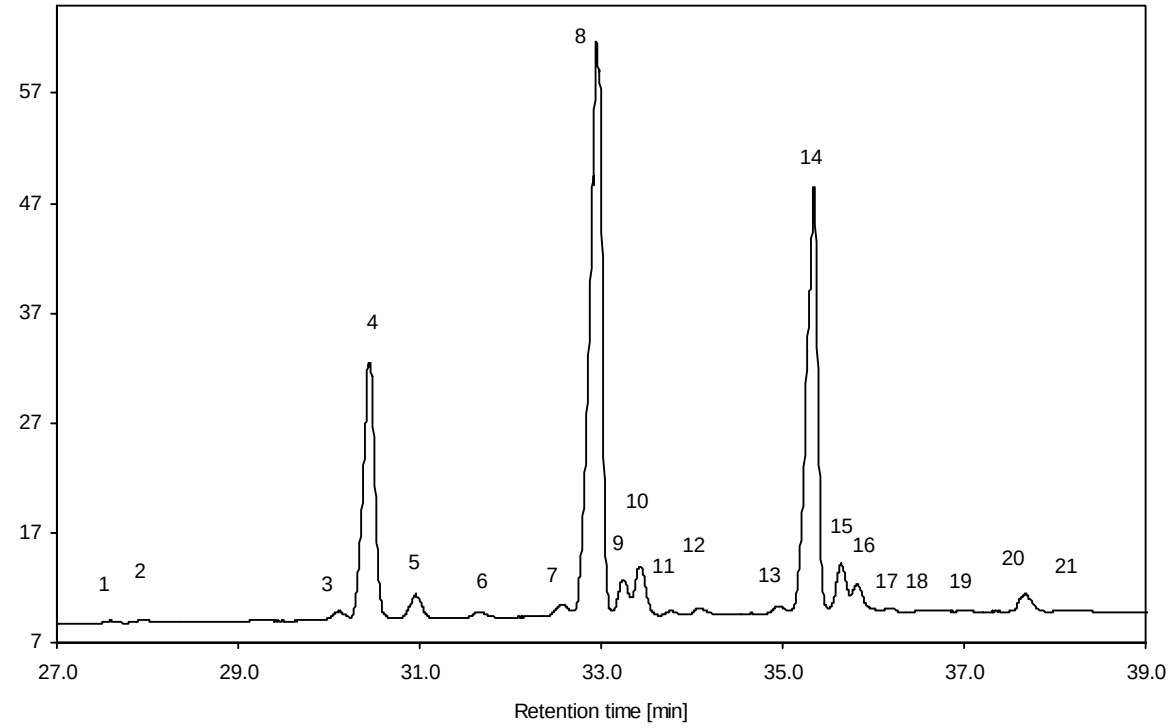
NOTE: In the case of failure, the chromatographic conditions (e.g. sample size, column temperature, carrier gas flow, etc.) must be optimized.

Bibliography

[1] Koeber, R., Buchgraber, M., Ulberth, F., Bacarolo, R., Bernreuther, A., Schimmel, H., Anklam, E. and Pauwels, J. *The certification of the content of five triglycerides in cocoa butter*, 2003, EUR 20781 EN, ISBN 92-894-6036-9

[2] Official Methods of Analysis of AOAC International, AOAC Official Method 963.15: *Fat in Cacao Products*, 1995

ANNEX



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

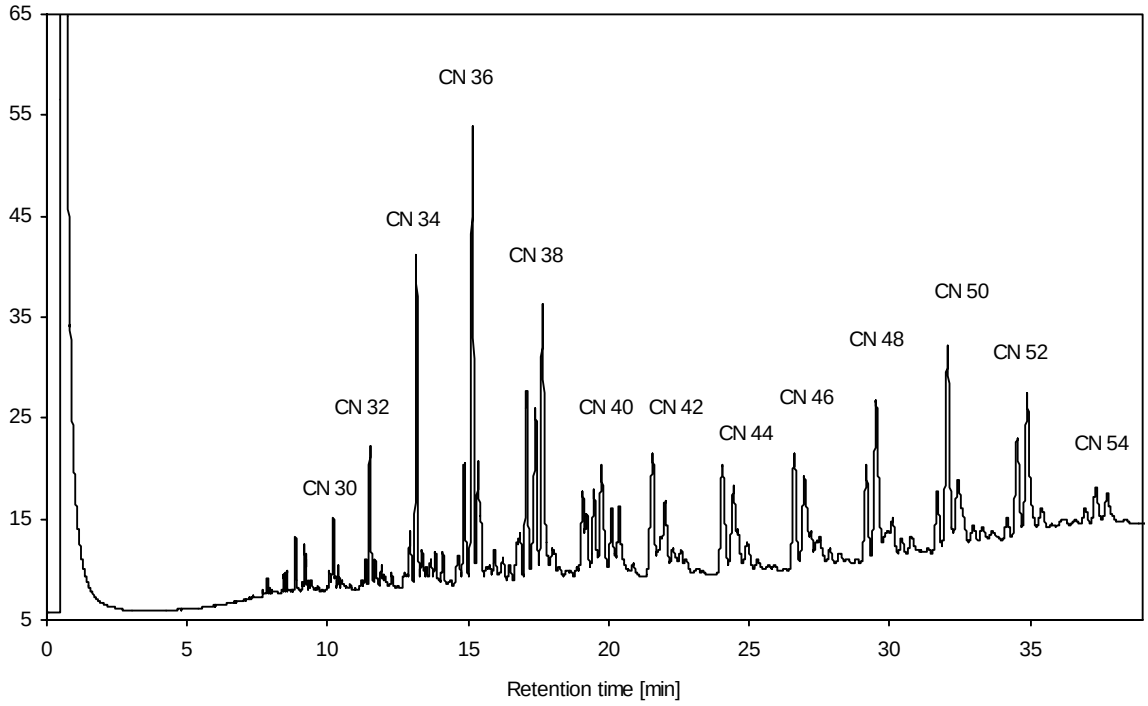
Experimental conditions

GC column:	25 m × 0,25 mm fused silica capillary column coated with 0,1 µm Chrompack TAP
Temperature programme:	100 °C held for 2 min; 30 °C/min to 270 °C held for 1 min; 2.5 °C/min to 340 °C held for 7 min
Injector:	Cold on-column
Detector (FID):	360 °C
Carrier gas:	H_2 with a constant flow rate of 3.5 ml/min
Amount injected	0.5 µl of a 0.2 mg/mL solution in iso-octane

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 cocoa butter CRM



Experimental conditions

GC column:	25 m × 0,25 mm fused silica capillary column coated with 0,1 µm Chrompack TAP
Temperature programme:	100 °C held for 2 min; 30 °C/min to 270 °C held for 1 min; 2.5 °C/min to 340 °C held for 7 min
Injector:	Cold on-column
Detector (FID):	360 °C
Carrier gas:	H_2 with a constant flow rate of 3.5 mL/min
Amount injected	0.5 µl of a 0.5 mg/mL solution in iso-octane

Figure A 2: Triacylglycerol profile of pure milk fat

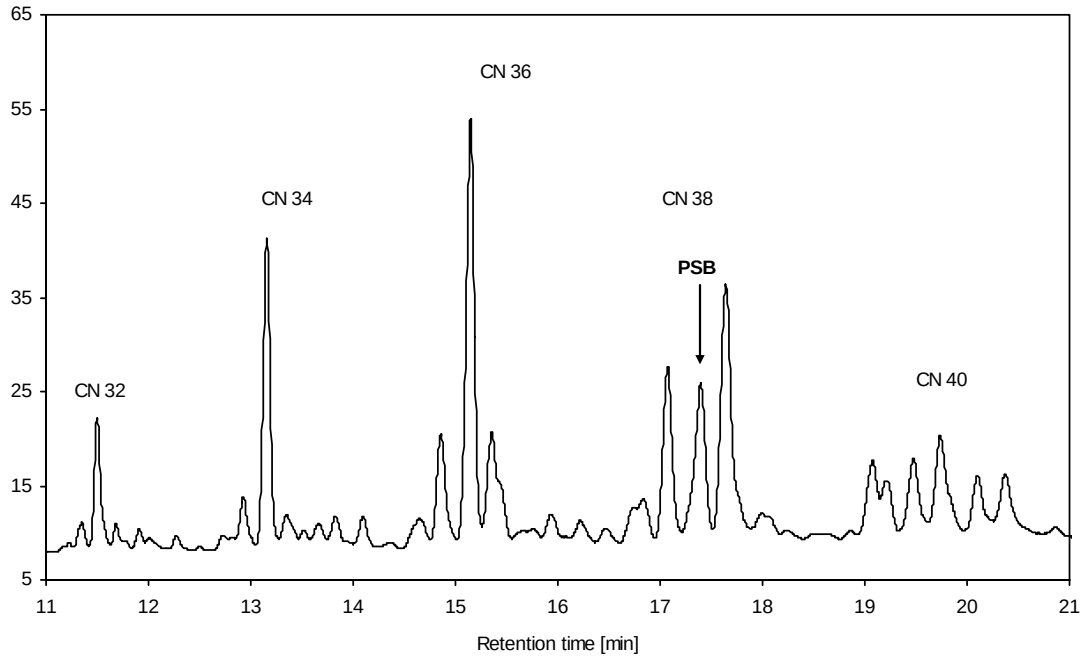


Figure A 3: Triacylglycerol profile of pure milk fat: Chromatogram window for the part were PSB (1-Palmitin-2-stearin-3-butyrin) elutes

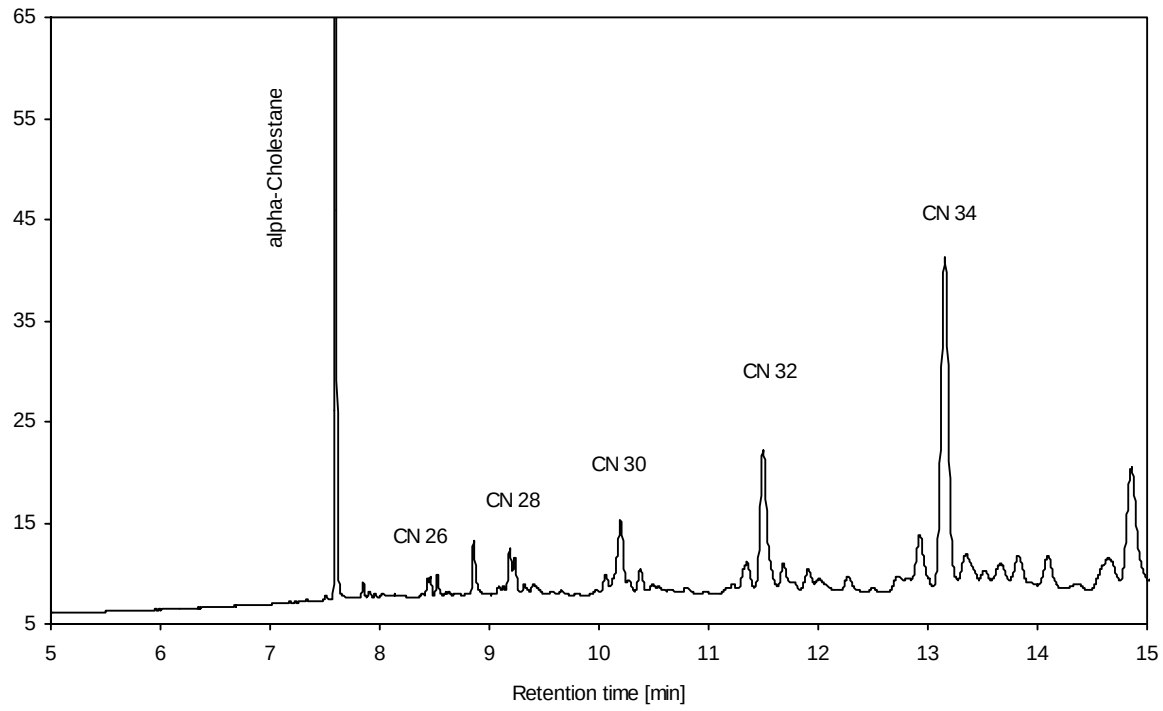


Figure A 4: Triacylglycerol profile of pure milk fat with the addition of alpha-cholestane: Chromatogram window for the part were alpha-cholestane elutes

ANNEX B – HOMOGENEITY DATA

Table B 1. Individual TAG data obtained for homogeneity study for selected units of chocolate sample 1

Unit	Replicate	Chocolate sample 1					
		PSB [1]	%-POP [2]	%-POS [2]	%-POO [2]	%-SOS [2]	%-SOO [2]
1	**A**	0.27	18.43	44.47	2.39	31.66	3.05
	B	0.26	18.37	44.49	2.38	31.68	3.08
2	**A**	0.28	18.54	44.38	2.43	31.60	3.05
	B	0.26	18.39	44.44	2.41	31.69	3.07
3	**A**	0.28	18.49	44.41	2.43	31.65	3.02
	B	0.26	18.32	44.48	2.41	31.69	3.10
4	**A**	0.26	18.43	44.40	2.39	31.68	3.10
	B	0.26	18.47	44.41	2.42	31.62	3.08
5	**A**	0.26	18.47	44.41	2.40	31.65	3.07
	B	0.25	18.37	44.49	2.37	31.67	3.09
6	**A**	0.27	18.52	44.38	2.44	31.61	3.05
	B	0.26	18.44	44.45	2.40	31.67	3.05
7	**A**	0.27	18.43	44.45	2.43	31.66	3.04
	B	0.27	18.54	44.36	2.41	31.65	3.05
8	**A**	0.26	18.45	44.39	2.42	31.64	3.08
	B	0.27	18.45	44.44	2.41	31.62	3.09
9	**A**	0.25	18.45	44.43	2.39	31.66	3.07
	B	0.27	18.45	44.45	2.39	31.64	3.07
10	**A**	0.27	18.52	44.39	2.41	31.64	3.04
	B	0.27	18.50	44.37	2.41	31.66	3.06

[1] g PSB/100 g chocolate fat; [2] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table B 2. Individual TAG data obtained for homogeneity study for selected units of chocolate sample 2

Unit	Replicate	Chocolate sample 2					
		PSB [1]	%-POP [2]	%-POS [2]	%-POO [2]	%-SOS [2]	%-SOO [2]
1	**A**	0.29	18.69	44.01	2.59	31.56	3.15
	B	0.29	18.68	44.02	2.53	31.55	3.21
2	**A**	0.29	18.68	44.01	2.55	31.55	3.21
	B	0.29	18.74	44.03	2.52	31.53	3.17
3	**A**	0.29	18.68	44.02	2.60	31.56	3.14
	B	0.29	18.73	43.96	2.58	31.52	3.20
4	**A**	0.29	18.69	44.02	2.58	31.56	3.16
	B	0.30	18.66	44.00	2.53	31.62	3.18
5	**A**	0.29	18.69	44.00	2.57	31.56	3.18
	B	0.30	18.59	43.98	2.48	31.80	3.14
6	**A**	0.29	18.75	44.00	2.55	31.54	3.17
	B	0.30	18.69	44.00	2.60	31.51	3.20
7	**A**	0.30	18.69	44.04	2.54	31.57	3.15
	B	0.30	18.67	44.01	2.50	31.63	3.18
8	**A**	0.27	18.69	43.99	2.58	31.53	3.20
	B	0.29	18.69	44.01	2.55	31.57	3.18
9	**A**	0.30	18.68	43.99	2.54	31.59	3.19
	B	0.29	18.71	43.98	2.53	31.57	3.21
10	**A**	0.29	18.67	44.00	2.53	31.59	3.21
	B	0.29	18.69	44.01	2.56	31.57	3.18

[1] g PSB/100 g chocolate fat; [2] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table B 3. Individual TAG data obtained for homogeneity study for selected units of chocolate sample 3

Unit	Replicate	Chocolate sample 3					
		PSB [1]	%-POP [2]	%-POS [2]	%-POO [2]	%-SOS [2]	%-SOO [2]
1	**A**	0.40	18.57	44.31	2.85	31.16	3.11
	B	0.40	18.56	44.33	2.84	31.19	3.07
2	**A**	0.40	18.56	44.25	2.84	31.19	3.17
	B	0.41	18.55	44.33	2.84	31.19	3.09
3	**A**	0.42	18.58	44.33	2.84	31.13	3.12
	B	0.41	18.56	44.34	2.82	31.18	3.10
4	**A**	0.41	18.56	44.33	2.81	31.17	3.12
	B	0.41	18.55	44.32	2.84	31.20	3.09
5	**A**	0.40	18.56	44.33	2.84	31.13	3.14
	B	0.41	18.56	44.30	2.84	31.20	3.10
6	**A**	0.40	18.56	44.33	2.80	31.18	3.13
	B	0.41	18.58	44.27	2.85	31.13	3.17
7	**A**	0.41	18.59	44.28	2.86	31.13	3.15
	B	0.40	18.61	44.32	2.80	31.16	3.12
8	**A**	0.41	18.58	44.31	2.80	31.19	3.13
	B	0.42	18.48	44.24	2.81	31.28	3.18
9	**A**	0.42	18.50	44.30	2.85	31.23	3.11
	B	0.40	18.56	44.36	2.80	31.20	3.07
10	**A**	0.39	18.56	44.32	2.81	31.22	3.10
	B	0.41	18.57	44.28	2.84	31.17	3.14

[1] g PSB/100 g chocolate fat; [2] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table B 4. Individual TAG data obtained for homogeneity study for selected units of chocolate sample 4

Unit	Replicate	Chocolate sample 4					
		PSB [1]	%-POP [2]	%-POS [2]	%-POO [2]	%-SOS [2]	%-SOO [2]
1	**A**	0.39	19.99	41.63	3.43	31.43	3.58
	B	0.40	20.01	41.73	3.30	31.42	3.54
2	**A**	0.40	20.04	41.69	3.30	31.43	3.55
	B	0.39	20.05	41.68	3.33	31.42	3.52
3	**A**	0.40	20.05	41.72	3.29	31.42	3.51
	B	0.38	20.04	41.72	3.22	31.46	3.55
4	**A**	0.39	20.04	41.73	3.29	31.42	3.52
	B	0.41	20.05	41.72	3.26	31.45	3.51
5	**A**	0.39	20.05	41.73	3.25	31.43	3.54
	B	0.40	20.05	41.73	3.28	31.42	3.52
6	**A**	0.39	20.08	41.69	3.26	31.48	3.49
	B	0.39	20.02	41.64	3.37	31.45	3.53
7	**A**	0.40	20.05	41.69	3.26	31.46	3.54
	B	0.39	20.04	41.70	3.26	31.46	3.54
8	**A**	0.38	20.04	41.70	3.24	31.47	3.50
	B	0.39	20.05	41.67	3.27	31.47	3.54
9	**A**	0.39	20.02	41.70	3.25	31.46	3.57
	B	0.39	20.08	41.70	3.20	31.42	3.50
10	**A**	0.39	20.04	41.66	3.32	31.45	3.53
	B	0.39	20.02	41.69	3.31	31.44	3.54

[1] g PSB/100 g chocolate fat; [2] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table B 5. Individual TAG data obtained for homogeneity study for selected units of chocolate sample 5

Unit	Replicate	Chocolate sample 5					
		PSB [1]	%-POP [2]	%-POS [2]	%-POO [2]	%-SOS [2]	%-SOO [2]
1	**A**	0.44	23.96	36.50	3.31	32.49	3.74
	B	0.45	23.96	36.46	3.31	32.52	3.76
2	**A**	0.46	23.97	36.51	3.29	32.51	3.72
	B	0.45	23.96	36.50	3.33	32.49	3.72
3	**A**	0.44	23.97	36.49	3.32	32.48	3.74
	B	0.45	23.96	36.48	3.34	32.48	3.75
4	**A**	0.45	23.99	36.51	3.28	32.50	3.71
	B	0.45	23.96	36.51	3.24	32.54	3.75
5	**A**	0.46	23.97	36.47	3.33	32.45	3.77
	B	0.46	23.98	36.38	3.26	32.56	3.83
6	**A**	0.45	23.96	36.49	3.32	32.50	3.74
	B	0.45	23.98	36.44	3.31	32.53	3.74
7	**A**	0.45	23.96	36.45	3.29	32.54	3.75
	B	0.45	23.98	36.50	3.29	32.51	3.71
8	**A**	0.45	23.95	36.46	3.35	32.47	3.77
	B	0.46	23.97	36.45	3.41	32.48	3.69
9	**A**	0.44	23.92	36.50	3.29	32.53	3.75
	B	0.45	23.97	36.47	3.31	32.48	3.76
10	**A**	0.45	23.94	36.48	3.29	32.52	3.78
	B	0.44	23.94	36.49	3.31	32.51	3.75

[1] g PSB/100 g chocolate fat; [2] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table B 6. Individual TAG data obtained for homogeneity study for selected units of chocolate sample 6

Unit	Replicate	Chocolate sample 6					
		PSB [1]	%-POP [2]	%-POS [2]	%-POO [2]	%-SOS [2]	%-SOO [2]
1	**A**	0.30	21.57	40.05	2.75	32.34	3.30
	B	0.30	21.57	40.07	2.77	32.24	3.35
2	**A**	0.31	21.56	40.08	2.77	32.27	3.31
	B	0.31	21.53	40.08	2.77	32.32	3.30
3	**A**	0.30	21.53	40.04	2.79	32.27	3.36
	B	0.31	21.54	40.07	2.78	32.29	3.31
4	**A**	0.31	21.53	40.08	2.79	32.30	3.30
	B	0.30	21.53	40.06	2.79	32.28	3.34
5	**A**	0.30	21.57	40.08	2.82	32.25	3.28
	B	0.31	21.55	40.08	2.75	32.29	3.32
6	**A**	0.31	21.54	40.09	2.77	32.30	3.30
	B	0.31	21.56	40.05	2.75	32.34	3.30
7	**A**	0.30	21.54	40.05	2.82	32.25	3.34
	B	0.29	21.54	40.01	2.76	32.29	3.40
8	**A**	0.30	21.56	40.06	2.80	32.26	3.33
	B	0.29	21.57	40.05	2.79	32.27	3.32
9	**A**	0.30	21.58	40.05	2.77	32.26	3.34
	B	0.29	21.54	40.08	2.80	32.25	3.33
10	**A**	0.30	21.52	40.06	2.85	32.21	3.36
	B	0.29	21.55	40.05	2.79	32.27	3.34

[1] g PSB/100 g chocolate fat; [2] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

ANNEX C – COLLABORATIVE STUDY GUIDELINES

1 Objective

To validate an analytical method for the detection and quantification of cocoa butter equivalents (CBEs) in cocoa butter-milk fat (CB-MF) mixtures and milk chocolate by triacylglycerol (TG) profiling using high-resolution capillary gas chromatography (HR-GC), and subsequent data evaluation by simple and multiple linear regression analysis.

2 Samples

The shipment contains

- 7 fat samples, consisting of pure CB, CB-MF and CB-CBE-MF mixtures, dissolved in 5 mL iso-octane (ca c = 10 mg/ml; the accurate concentrations of the fat solutions are given on the sample label)
- 6 grated chocolate samples (ca 20 g), from which the fat has to be extracted.

Blind duplicates are provided from each sample. The samples are labelled randomly.

In total there are 26 test samples.

Additionally,

- 1 ampoule of the cocoa butter certified reference material (CRM) IRMM-801 (5 g),
- 1 ampoule of a pure milk fat (ca 1 g),
- 1 ampoule of 1-Palmitin-2-stearin-3-butyrin (PSB) dissolved in 5 mL iso-octane (c = 5.0169 mg/mL),
- 1 ampoule of alpha-cholestane dissolved in 5 mL iso-octane (c = 9.9744 mg/mL)

is provided for calibration purposes and system suitability check.

3 Methods

Participants have to apply the attached Standard Operation Procedure (SOP) for the detection and quantification of cocoa butter equivalents in milk chocolate (***20060613 SOP.pdf***).

4 Sample work-up

4.1 System suitability check

- Use a mixture of pure milk fat with alpha-cholestane as internal standard to check the resolution power of the applied method.
- Use the cocoa butter CRM to check the resolution power of the applied method.
- Use the cocoa butter CRM to determine detector response factors of the five triacylglycerols (POP, POO, POS, SOS, SOO) (at least three replicates).

– Use the PSB and the alpha-cholestane solutions to establish a calibration curve for PSB, to determine the detector response factor and to check the stability of the system (at least duplicate injection of each calibration solution).

NOTE: Proceed with the analysis of the test samples only if the system suitability criteria are fulfilled as laid down in the SOP.

4.2 Analysis of test samples

The 14 fat test solutions have to be analysed once (**in total 14 analysis**).

NOTE: The accurate concentration of the solutions is given on the sample label. The SOP does not explain how to handle fat solutions. However, the solutions have to be diluted just to the concentration levels normally used in the laboratory.

From the 12 grated chocolate samples (20 g portions) the fat shall be obtained according to the SOP (1 rapid fat extraction and 1 Soxhlet fat extraction for each sample). The obtained fat has to be dissolved in a proper solvent and each fat solution analysed once (**in total 24 analysis**).

The samples have to be analysed in random order.

A calibration curve for PSB using alpha-cholestane as internal standard has to be determined as described in the SOP. This has to be done before analysing the first sample, after the 19th analysis and after the last test sample (in total three calibration curves).

NOTE: The concentration of the PSB solution is 5.0169 mg/mL iso-octane and the concentration of the alpha-cholestane solution is 9.9744 mg/mL iso-ocatne. Both solutions have to be diluted to the concentration levels normally used in the laboratory.

Response factors for the five triacylglycerols (POP, POO, POS, SOS, SOO) have to be determined before analysing the first sample, after the 19th analysis and after the last test sample by using the cocoa butter CRM (in total three determinations).

A flow-scheme detailing the handling of the samples is given below:

Design of the system suitability check

- Optimise resolution using cocoa butter CRM
- Optimise resolution using pure milk fat with alpha-cholestane (internal standard)
- Determine response factors for the five triacylglycerols (POP, POS, POO, SOS, SOO) using cocoa butter CRM (at least three replicates)
- Establish calibration curve for PSB using alpha-cholestane as internal standard (at least two replicates of each calibration solution)

Proceed with analysis of test samples only if system suitability criteria are met!

Analysis of test samples

- Establish calibration curve 1 for PSB to determine average response factor (6 injections)
- Determine response factors for the five triacylglycerols (POP, POS, POO, SOS, SOO) using cocoa butter CRM (1 injection)
- **Analyse the first 19 test samples (randomly chosen) (19 injections)**
- Establish calibration curve 2 for PSB to determine average response factor (6 injections)
- Determine response factors for the five triacylglycerols (POP, POS, POO, SOS, SOO) using cocoa butter CRM (1 injection)
- **Analyse the second 19 samples (randomly chosen) (19 injections)**
- Establish calibration curve 3 for PSB to determine average response factor (6 injections)
- Determine response factors for the five triacylglycerols (POP, POS, POO, SOS, SOO) using cocoa butter CRM (1 injection)
- Report and calculate results using the electronic reporting sheet

4.3 Reporting results

Identify peaks by comparison of the retention times of the test samples with those of the cocoa butter CRM and the reference standards (PSB and alpha-cholestane). The elution orders are given in the Standard Operation Procedure.

Use the attached electronic reporting sheet (MS Excel; ***20060613 Electronic reporting.xls***):

- Report **chromatographic conditions** (column type, instrument, injection technique, etc.)

- Report the outcome of the system suitability check (**System suitability Resolution**; **System suitability CRM**; **System suitability PSB**)

- Report the data for the PSB calibration curves (**Calibration curve mean 1**; **Calibration curve mean 2**)

- Report the obtained data for the test samples as follows (**Analysis 1-19**; **Analysis 20-38**):

  - Report the ***sample name*** given on the sample label.
  - Report the final ***Sample concentration*** (mg/mL) of the test samples and the ***Cholestane concentration*** of the added alpha-cholestane (mg/mL).
  - Report the obtained ***fat content of milk chocolate*** determined by Soxhlet extraction.
  - Report the ***average response factor*** determined for ***PSB*** and the obtained ***intercept*** and ***slope*** for the calibration curve.
  - Report the ***raw area counts*** of ***alpha-cholestane, PSB, POP, POS, POO, SOS, SOO***.
  - Report the ***raw area sum*** of ***all triacylglycerols*** (Exclude peaks only if they appear also in the blank run and don't include area counts of alpha-cholestane).

NOTE: The electronic reporting sheet has been password protected in order to avoid any modifications of its structure. You are only allowed to put in data in the yellow-marked and green-marked cells. All necessary calculations will be done automatically.

Submit the electronic reporting sheet by e-mail to the following address:

manuela.buchgraber@ec.europa.eu

Additionally, send hard copies of all chromatograms and integrator print outs to the following address:

Dr Manuela Buchgraber
Food Safety and Quality Unit
European Commission; DG Joint Research Centre
Institute for Reference Materials and Measurements (IRMM)
Retieseweg 111
B-2440 Geel (Belgium)

Deadline for submission of results: 23 August 2006

General remarks

Read all the instructions completely and carefully; if you have any questions, check them with the co-ordinating laboratory (**manuela.buchgraber@ec.europa.eu**) before you begin the analysis.

The solutions of the samples have to be completely liquefied before the preparation of the final concentration. If there are still solids present after thoroughly mixing the submitted solutions, allow the test samples to warm up to 30 °C.

Before opening the cocoa butter CRM, the ampoule has to be warmed by immersion in a drying oven set to 50 °C until the content has melted. When a clear solution is obtained, wipe off the adhering water, 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. The sample has to be completely liquefied before the preparation of the final test solution. Pipettes (or similar equipment) used for transferring the sample during weighing operations should be brought to a temperature of ca. 55 °C in order to avoid partial fractionation.

The pure milk fat has to be completely liquefied before the preparation of the final test solution. Pipettes (or similar equipment) used for transferring the sample during weighing operations should be brought to a temperature of ca. 55 °C in order to avoid partial fractionation.

Make at least one practice run on your own samples to familiarise yourself with the procedure so that you can avoid errors in manipulations.

On receipt of the samples store them in the fridge until analysis.

Follow the method you have chosen in detail; do not insert minor modifications.

ANNEX D – APPLIED METHODS

Table D 1. HR-GLC methods for triacylglycerol analysis of test samples used by individual laboratories

Laboratory	**1**	**2**	**3**	**4**	**5**	**6**
GC apparatus						
- brand name	Varian 3400	Fisons 8000	Agilent 6890	Agilent 6890N	Agilent 6890	HP 5890 II
Carrier gas						
- type	He	He	He	H_2	H_2	H_2
- if constant pressure (kPa)	100	180	-	-	-	-
- if constant flow (mL/min)	-	-	2.2	2	2	1.5
Column characteristics						
- stationary phase	ULTIMETAL	CB-TAP	CB-TAP	CB-TAP	CB-TAP	CB-TAP
- length [m]	25	25	25	25	25	25
- i.d. [mm]	0.25	0.25	0.25	0.25	0.25	0.25
- film thickness [µm]	0.05	0.1	0.1	0.1	0.1	0.1
Temperature mode						
- Oven						
- injection temperature [°C], hold time [min]	200 / 2	200 / 1	100 / 0.5	200 / 1	200 / 0	200 / 1
- programme rate 1 [°C/min]	20	14	40	14	20	24
- temperature [°C], hold time [min]	320 / 0	270 / 0	280 / 1	270 / 0	270 / 0	270 / 0
- programme rate 2 [°C/min]	1	2.5	2.5	2.5	5	2.5
- temperature, hold time	360 / 10	340 / 30	340 / 17	340 / 10	340 / 15	340 / 13
- programme rate 3 [°C/min]	-	10	-	-	25	-
- final temperature [°C], hold time [min]	-	350 / 9	-	-	200	-
- Injector temperature [°C]	65-370	360	oven track	365	370	350
- Detector temperature [°C]	370	360	360	365	360	370
Injection mode						
- manual/automatic	manual	automatic	-	automatic	automatic	automatic
- split/on-column/PTV	PTV	split	OCI	split	split	split
- if split [split ratio]	-	1:20	-	1:10	1:10	1:10
Test sample						
- Test sample conc. [mg/mL solvent]	0.5	2.5	0.5	1.0	2.5	0.5
- alpha-cholestane in test sample (mg/mL solvent)	0.01	0.02	0.002	0.02	0.02	0.01
- Volume injected [µl]	0.1	1.0	0.5	1.0	1.0	1.0
Integrator/Computer software						
- brand name	ChemStation	ChromCard	ChemStation	ChemStation	Chromeleon	ChemStation

Table D 2. HR-GLC methods for triacylglycerol analysis of test samples used by individual laboratories (cont.)

Laboratory	**7**	**8**	**9**	**10**	**11**	**12**
GC apparatus						
- brand name	Fisons 8000	HP 5890	Perkin Elmer	HP 5890 II	Agilent 6890N	Agilent 6890N
Carrier gas						
- type	H_2	He	H_2	H_2	H_2	H_2
- if constant pressure (kPa)	150	135	130	140	-	-
- if constant flow (mL/min)	-	-	-	-	3.5	2
Column characteristics						
- stationary phase	CB-TAP	CB-TAP	RTx-65TG	CB-TAP	CB-TAP	CB-TAP
- length [m]	25	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	0.1	0.1	0.1	0.1
Temperature mode						
- Oven						
- injection temperature [°C], hold time [min]	100 / 2	200 / 1	200 / 1	200 / 1	100 / 2	200 / 1
- programme rate 1 [°C/min]	30	14	15	30	30	14
- temperature [°C], hold time [min]	270 / 1	270	360 / 0	270 / 0	270 / 1	270 / 0
- programme rate 2 [°C/min]	3	2	1	2.5	2.5	2.5
- temperature, hold time	340 / 10	340 / 30	370	355 / 2	340 / 7	340 / 10
- programme rate 3 [°C/min]	-	-	-	-	-	-
- final temperature [°C], hold time [min]	-	-	-	-	-	-
- Injector temperature [°C]		340	380	140-340	oven track	360
- Detector temperature [°C]	350	360	380	350	360	360
Injection mode						
- manual/automatic	manual	automatic	automatic	automatic	automatic	automatic
- split/on-column/PTV	OCI	split	split	PTV	OCI	split
- if split [split ratio]	-	1:7	-	-	-	1:10
Test sample						
- Test sample conc. [mg/mL solvent]	0.2	5.0	2.5	0.3	0.5	5.0
- alpha-cholestane in test sample (mg/mL solvent)	0.00	0.10	0.010	0.00	0.00	0.02
- Volume injected [µl]	0.5	1.0	1.0	1.0	0.5	1.0
Integrator/Computer software						
- brand name	ChromCard	Empower®	Turbochrom	ChemStation	ChemStation	ChemStation

ANNEX E – SUBMITTED DATA

Table E 1. Results accepted on technical grounds for sample 1 (chocolate fat for GLC analysis obtained by rapid fat extraction)

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.25	0.25	19.21	18.73	44.73	44.41	2.40	2.50	30.72	31.31	2.94	3.04
2	0.25	0.28	18.99	18.70	44.32	44.18	2.40	2.27	30.96	31.36	3.33	3.48
3	0.27	0.24	18.43	18.54	44.34	44.27	2.48	2.44	31.70	31.69	3.05	3.06
4	0.26	0.26	18.71	18.80	44.23	44.24	2.50	2.51	31.46	31.33	3.09	3.11
5	0.27	0.28	18.53	18.51	44.33	44.34	2.43	2.45	31.63	31.63	3.08	3.07
6	0.28	0.28	18.68	18.61	44.51	44.35	2.41	2.43	31.41	31.52	2.99	3.09
7	0.22	0.25	18.54	18.55	44.32	44.19	2.52	2.56	31.46	31.57	3.15	3.13
8	0.27	0.27	18.46	18.51	44.41	44.30	2.43	2.43	31.67	31.75	3.02	3.00
9	0.27	0.27	18.31	18.33	44.14	44.09	2.81	2.85	31.67	31.61	3.07	3.11
10	0.25	0.20	18.80	18.64	44.48	44.49	2.42	2.33	32.35	31.48	1.95	3.07
11	0.26	0.26	18.46	18.36	44.27	44.32	2.41	2.45	31.87	31.77	2.99	3.10
12	0.26	0.27	18.38	18.39	44.17	44.18	2.41	2.41	31.94	31.92	3.10	3.10

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 2. Results accepted on technical grounds for sample 1 (chocolate fat for GLC analysis obtained by rapid fat extraction)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g total fat/100 g chocolate		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B	A	B
1	yes	yes	11.08	11.03	-	-	34.85	34.85	3.88	3.83	-	-
2	yes	yes	11.13	12.62	-	-	35.31	35.31	3.93	4.46	-	-
3	yes	yes	12.00	10.57	-	-	36.65	36.65	4.27	3.98	-	-
4	yes	yes	11.80	11.48	-	-	35.43	35.43	4.17	4.07	-	-
5	yes	yes	12.12	12.44	-	-	34.65	34.65	4.21	4.31	-	-
6	yes	yes	12.64	12.55	-	-	34.75	34.75	4.22	4.32	-	-
7	yes	yes	10.03	11.18	-	-	34.84	34.84	3.50	3.89	-	-
8	yes	yes	12.38	12.34	-	-	34.75	34.75	4.30	4.30	-	-
9	yes	yes	12.01	12.00	-	-	34.77	34.77	4.16	4.18	-	-
10	yes	yes	11.08	9.06	-	-	34.42	34.42	3.82	3.99	-	-
11	yes	yes	11.47	11.69	-	-	34.75	34.75	3.99	4.05	-	-
12	yes	yes	11.56	11.95	-	-	34.65	34.65	4.00	4.15	-	-

[(1)] if sample is pure CB or not

Table E 3. Results accepted on technical grounds for sample 2 (chocolate fat for GLC analysis obtained by rapid fat extraction)

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.29	0.30	18.85	18.38	43.89	43.68	2.62	2.48	31.26	32.06	3.38	3.40
2	0.29	0.30	18.32	18.33	43.09	42.62	2.84	2.40	31.67	31.28	4.08	5.36
3	0.27	0.31	18.85	18.78	43.69	43.69	2.66	2.62	31.66	31.73	3.14	3.18
4	0.29	0.29	19.07	19.09	43.73	43.65	2.67	2.69	31.28	31.34	3.24	3.23
5	0.31	0.31	18.85	18.82	43.77	43.81	2.58	2.63	31.61	31.57	3.21	3.16
6	0.29	0.30	19.14	19.02	43.98	43.78	2.48	2.53	31.26	31.45	3.14	3.22
7	0.28	0.26	18.82	18.70	43.63	43.60	2.74	2.83	31.55	31.50	3.26	3.37
8	0.30	0.31	18.78	18.80	43.79	43.76	2.58	2.57	31.69	31.66	3.18	3.20
9	0.29	0.30	18.78	18.76	43.32	43.29	2.74	2.73	31.87	31.86	3.30	3.36
10	0.27	0.28	18.86	18.87	43.88	43.99	2.52	2.52	31.52	31.43	3.22	3.19
11	0.30	0.30	18.80	18.83	43.77	43.66	2.65	2.62	31.52	31.68	3.27	3.21
12	0.31	0.31	18.09	18.09	43.24	43.31	2.71	2.74	32.59	32.52	3.38	3.34

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 4. Results accepted on technical grounds for sample 2 (chocolate fat for GLC analysis obtained by rapid fat extraction)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g total fat/100 g chocolate		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B	A	B
1	no	no	13.02	13.25	0.90	1.34	35.70	35.70	4.66	4.72	0.32	0.48
2	no	no	12.87	13.31	2.26	2.23	35.69	35.69	4.61	4.73	0.81	0.79
3	no	no	12.25	13.62	1.87	1.50	37.16	37.16	4.38	5.25	0.67	0.58
4	no	no	12.78	12.81	1.59	1.81	36.05	36.05	4.61	4.61	0.57	0.65
5	no	no	13.63	13.70	1.30	1.18	35.55	35.55	4.82	4.89	0.46	0.42
6	no	no	13.24	13.58	0.94	1.30	35.40	35.40	4.85	4.82	0.34	0.46
7	no	no	12.44	11.46	1.85	2.01	35.48	35.48	4.42	4.06	0.66	0.71
8	no	no	13.80	14.08	1.22	1.19	35.35	35.35	4.88	4.97	0.43	0.42
9	no	no	12.88	13.40	2.56	2.46	35.69	35.69	4.59	4.79	0.91	0.88
10	no	no	12.10	12.60	1.34	0.95	35.18	35.18	4.44	4.25	0.33	0.47
11	no	no	13.52	13.21	1.22	1.67	35.93	35.93	4.86	4.75	0.44	0.60
12	no	no	13.82	13.93	2.33	2.14	35.75	35.75	4.94	4.98	0.83	0.76

[(1)] if sample is pure CB or not

Table E 5. Results accepted on technical grounds for sample 3 (chocolate fat for GLC analysis obtained by rapid fat extraction)

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.48	0.48	18.22	18.23	43.84	44.04	2.82	2.71	31.64	31.74	3.48	3.28
2	0.45	0.43	18.63	18.33	44.01	43.61	2.74	2.93	31.10	31.61	3.52	3.52
3	0.44	0.45	18.51	18.34	44.03	43.84	2.87	2.85	31.32	31.73	3.27	3.24
4	0.43	0.44	18.88	18.72	44.04	43.93	2.88	2.88	30.94	31.21	3.26	3.26
5	0.45	0.45	18.63	18.55	44.05	44.11	2.84	2.86	31.23	31.23	3.25	3.25
6	0.42	0.46	18.90	18.67	44.23	44.06	2.67	2.81	30.94	31.13	3.27	3.32
7	0.43	0.44	18.49	18.56	44.10	43.81	3.04	2.96	31.08	31.26	3.29	3.40
8	0.44	0.45	18.57	18.48	44.08	44.03	2.79	2.82	31.41	31.38	3.15	3.29
9	0.44	0.43	18.34	18.34	43.80	43.72	3.29	3.34	31.24	31.29	3.34	3.31
10	0.39	0.41	18.65	19.96	44.29	44.74	2.76	2.47	31.06	30.96	3.23	1.87
11	0.45	0.45	18.57	18.51	43.99	44.06	2.82	2.85	31.44	31.26	3.18	3.32
12	0.45	0.45	17.78	17.82	43.51	43.53	2.96	2.96	32.31	32.28	3.45	3.41

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 6. Results accepted on technical grounds for sample 3 (chocolate fat for GLC analysis obtained by rapid fat extraction)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g total fat/100 g chocolate		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B	A	B
1	yes	yes	21.50	21.28	-	-	32.10	32.10	6.92	6.81	-	-
2	yes	yes	20.05	19.21	-	-	31.86	31.86	6.32	6.18	-	-
3	yes	yes	19.74	19.89	-	-	32.22	32.22	6.37	6.39	-	-
4	yes	yes	19.10	19.37	-	-	32.28	32.28	6.17	6.25	-	-
5	yes	yes	20.01	20.00	-	-	31.60	31.60	6.28	6.36	-	-
6	yes	yes	18.75	20.66	-	-	31.80	31.80	6.28	6.44	-	-
7	yes	yes	19.08	19.64	-	-	31.53	31.53	5.97	6.24	-	-
8	yes	yes	19.86	20.26	-	-	31.95	31.95	6.33	6.48	-	-
9	yes	yes	19.63	19.17	-	-	31.84	31.84	6.26	6.10	-	-
10	yes	yes	17.54	18.43	-	-	31.88	31.88	5.89	5.58	-	-
11	yes	yes	20.11	19.91	-	-	32.09	32.09	6.46	6.39	-	-
12	yes	yes	19.95	20.20	-	-	32.05	32.05	6.40	6.47	-	-

[(1)] if sample is pure CB or not

Table E 7. Results accepted on technical grounds for sample 4 (chocolate fat for GLC analysis obtained by rapid fat extraction)

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.45	0.45	20.16	19.94	41.72	41.64	3.03	3.01	31.56	31.78	3.53	3.63
2	0.42	0.43	20.07	19.89	41.03	41.54	3.22	2.97	31.72	31.94	3.95	3.67
3	0.40	0.44	20.06	19.79	41.49	41.44	3.17	3.15	31.64	31.96	3.63	3.65
4	0.42	0.43	20.27	20.16	41.49	41.45	3.20	3.18	31.45	31.59	3.59	3.62
5	0.44	0.44	20.16	20.04	41.68	41.60	3.16	3.16	31.39	31.57	3.61	3.63
6	0.44	0.45	20.09	20.21	41.73	41.72	3.04	3.07	31.40	31.27	3.75	3.73
7	0.42	0.45	19.94	19.83	41.44	41.31	3.40	3.28	31.49	31.66	3.72	3.92
8	0.44	0.44	20.04	20.05	41.69	41.67	3.13	3.12	31.57	31.57	3.57	3.59
9	0.43	0.42	20.00	20.04	41.09	41.15	3.37	3.29	31.77	31.74	3.77	3.78
10	0.40	0.39	20.15	20.00	41.74	41.92	3.02	3.03	31.50	31.49	3.60	3.56
11	0.44	0.43	20.00	20.04	41.57	41.60	3.18	3.12	31.54	31.56	3.71	3.67
12	0.43	0.44	19.23	19.22	41.07	41.12	3.27	3.27	32.59	32.57	3.83	3.81

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 8. Results accepted on technical grounds for sample 4 (chocolate fat for GLC analysis obtained by rapid fat extraction)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g total fat/100 g chocolate		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B	A	B
1	no	no	20.14	20.20	5.31	5.38	32.15	32.15	6.46	6.51	1.71	1.73
2	no	no	18.63	18.99	7.06	5.90	32.31	32.31	6.01	6.14	2.28	1.91
3	no	no	17.73	19.44	6.36	6.01	32.32	32.32	5.72	6.30	2.05	1.95
4	no	no	18.66	19.03	6.22	6.21	32.44	32.44	6.05	6.18	2.02	2.02
5	no	no	19.75	19.35	5.41	5.68	31.85	31.85	6.32	6.13	1.73	1.80
6	no	no	19.84	20.28	5.12	5.08	32.00	32.00	6.34	6.17	1.64	1.62
7	no	no	18.66	20.06	6.10	5.95	31.80	31.80	5.90	6.41	1.93	1.90
8	no	no	19.74	19.76	5.41	5.45	31.80	31.80	6.28	6.28	1.72	1.73
9	no	no	18.97	18.90	6.97	6.84	32.02	32.02	6.09	6.03	2.24	2.18
10	no	no	17.78	17.43	5.74	5.32	32.02	32.02	5.60	5.67	1.71	1.83
11	no	no	19.54	19.16	5.61	5.67	32.37	32.37	6.31	6.21	1.81	1.84
12	no	no	19.26	19.37	6.73	6.58	32.32	32.32	6.23	6.25	2.18	2.12

[(1)] if sample is pure CB or not

Table E 9. Results accepted on technical grounds for sample 5 (chocolate fat for GLC analysis obtained by rapid fat extraction)

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.53	0.52	23.89	23.83	36.11	36.64	3.18	3.19	32.90	32.63	3.91	3.71
2	0.50	0.49	23.88	23.77	36.19	35.79	3.45	3.21	32.58	32.65	3.90	4.57
3	0.48	0.46	23.89	23.93	36.18	36.19	3.40	3.37	32.72	32.69	3.81	3.82
4	0.48	0.47	24.03	24.18	36.19	36.23	3.38	3.38	32.59	32.41	3.81	3.80
5	0.51	0.47	23.91	23.95	36.26	36.30	3.38	3.37	32.56	32.56	3.89	3.83
6	0.49	0.50	24.12	23.98	36.69	36.76	3.22	3.21	32.10	32.26	3.87	3.80
7	0.47	0.49	23.86	23.91	36.15	36.24	3.41	3.44	32.58	32.46	3.99	3.95
8	0.50	0.51	23.91	23.89	36.35	36.37	3.30	3.30	32.67	32.66	3.78	3.78
9	0.49	0.49	23.90	23.83	35.80	35.79	3.61	3.67	32.71	32.73	3.99	3.98
10	0.46	0.46	24.01	24.04	36.62	36.49	3.21	3.15	32.38	32.51	3.78	3.82
11	0.48	0.48	23.89	23.89	36.26	36.24	3.40	3.39	32.61	32.56	3.83	3.93
12	0.51	0.51	22.87	22.92	35.86	35.88	3.51	3.54	33.69	33.61	4.06	4.05

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 10. Results accepted on technical grounds for sample 5 (chocolate fat for GLC analysis obtained by rapid fat extraction)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g total fat/100 g chocolate		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B	A	B
1	no	no	23.40	22.98	20.48	19.30	25.40	25.40	5.94	5.84	5.20	4.90
2	no	no	22.01	21.58	20.55	21.07	25.57	25.57	5.62	5.52	5.25	5.39
3	no	no	21.52	20.33	20.78	21.04	26.35	26.35	5.54	5.48	5.35	5.67
4	no	no	21.32	20.96	20.83	20.86	25.64	25.64	5.47	5.37	5.34	5.34
5	no	no	22.45	21.04	20.27	20.58	25.30	25.30	5.68	5.32	5.13	5.21
6	no	no	21.73	22.45	19.37	19.04	25.00	25.00	5.52	5.34	4.76	4.84
7	no	no	20.91	21.93	20.80	20.39	24.87	24.87	5.16	5.50	5.13	5.11
8	no	no	22.16	22.54	20.24	20.07	25.00	25.00	5.54	5.64	5.06	5.02
9	no	no	21.91	21.66	21.51	21.59	25.21	25.21	5.52	5.48	5.42	5.46
10	no	no	20.24	20.28	19.97	20.30	25.11	25.11	5.08	5.10	5.01	5.11
11	no	no	21.19	21.50	20.61	20.52	25.41	25.41	5.38	5.49	5.24	5.24
12	no	no	22.50	22.70	20.92	20.83	25.36	25.36	5.71	5.77	5.31	5.29

[(1)] if sample is pure CB or not

Table E 11. Results accepted on technical grounds for sample 6 (chocolate fat for GLC analysis obtained by rapid fat extraction)

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.30	0.28	21.28	21.36	40.02	40.06	2.61	2.78	32.49	32.15	3.60	3.64
2	0.36	0.34	21.50	21.34	39.78	39.68	2.53	2.64	32.63	32.59	3.57	3.76
3	0.31	0.31	21.54	21.53	39.89	39.89	2.77	2.75	32.45	32.49	3.34	3.35
4	0.32	0.31	21.78	21.62	39.90	39.80	2.76	2.75	32.22	32.47	3.34	3.35
5	0.33	0.34	21.59	21.55	39.98	39.98	2.72	2.74	32.36	32.36	3.35	3.37
6	0.33	0.33	21.69	21.72	40.25	40.23	2.69	2.66	32.01	32.02	3.37	3.37
7	0.34	0.33	21.75	21.52	39.86	39.93	2.91	2.85	32.01	32.30	3.47	3.40
8	0.34	0.34	21.47	21.53	40.01	39.96	2.69	2.68	32.51	32.47	3.32	3.36
9	0.33	0.33	21.48	21.50	39.55	39.57	2.86	2.88	32.60	32.58	3.51	3.47
10	0.29	0.30	21.59	21.59	40.24	40.19	2.59	2.61	32.23	32.27	3.34	3.35
11	0.34	0.34	21.57	21.53	39.83	39.98	2.70	2.75	32.50	32.28	3.39	3.46
12	0.35	0.35	20.72	20.67	39.57	39.47	2.84	2.87	33.35	33.43	3.52	3.56

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 12. Results accepted on technical grounds for sample 6 (chocolate fat for GLC analysis obtained by rapid fat extraction)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g total fat/100 g chocolate		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B	A	B
1	no	no	13.26	12.70	11.86	11.82	33.85	33.85	4.51	4.28	4.03	3.98
2	no	no	15.86	15.24	12.04	12.20	34.05	34.05	5.40	5.19	4.10	4.15
3	no	no	14.04	14.03	12.34	12.34	32.43	32.43	4.32	4.78	3.80	4.21
4	no	no	14.20	13.93	12.35	12.61	34.19	34.19	4.85	4.77	4.22	4.31
5	no	no	14.90	15.35	11.92	11.76	33.90	33.90	5.04	5.22	4.03	4.00
6	no	no	14.94	15.01	11.18	11.21	33.65	33.65	4.98	5.20	3.73	3.80
7	no	no	15.02	14.60	12.10	12.02	33.99	33.99	5.11	4.95	4.12	4.08
8	no	no	15.52	15.60	11.69	11.79	33.25	33.25	5.18	5.16	3.90	3.90
9	no	no	14.93	14.73	12.87	12.91	33.75	33.75	5.03	4.98	4.34	4.36
10	no	no	13.11	13.20	11.64	11.75	33.23	33.23	4.34	4.40	3.85	3.92
11	no	no	15.21	15.01	12.21	11.76	34.32	34.32	5.23	5.14	4.20	4.03
12	no	no	15.48	15.55	12.49	12.71	34.34	34.34	5.30	5.35	4.28	4.37

[(1)] if sample is pure CB or not

Table E 13. Results accepted on technical grounds for sample 1 (chocolate fat for GLC analysis obtained by Soxhlet fat extraction)

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.32	0.31	19.05	18.22	44.47	44.22	2.37	2.44	31.13	31.87	2.99	3.25
2	0.28	0.28	18.59	18.65	44.28	44.24	2.51	2.50	31.37	31.47	3.24	3.14
3	0.29	0.27	18.52	18.52	44.37	44.31	2.43	2.40	31.74	31.82	2.94	2.95
4	0.28	0.27	18.72	18.63	44.26	44.19	2.42	2.43	31.55	31.68	3.06	3.06
5	0.31	0.31	18.62	18.63	44.35	44.30	2.42	2.49	31.64	31.54	2.97	3.04
6	0.30	0.28	18.66	18.87	44.16	44.28	2.55	2.50	31.34	31.20	3.29	3.15
7	0.23	0.23	18.28	18.51	44.67	44.61	2.42	2.31	31.52	31.47	3.12	3.10
8	0.30	0.30	18.61	18.59	44.39	44.41	2.40	2.39	31.56	31.53	3.04	3.08
9	0.28	0.28	18.44	18.33	44.32	44.10	2.58	2.85	31.62	31.67	3.04	3.04
10	0.15	0.25	18.59	18.85	44.74	44.73	2.21	1.87	32.23	32.02	2.24	2.53
11	0.28	0.27	18.45	18.41	44.27	44.30	2.37	2.46	31.91	31.70	2.99	3.14
12	0.28	0.28	17.73	17.71	43.77	43.79	2.52	2.53	32.80	32.78	3.18	3.19

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 14. Results accepted on technical grounds for sample 1 (chocolate fat for GLC analysis obtained by Soxhlet fat extraction)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g total fat/100 g chocolate		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B	A	B
1	yes	yes	14.26	14.03	-	-	35.00	34.70	4.99	4.87	-	-
2	yes	yes	12.31	12.63	-	-	35.30	35.32	4.35	4.46	-	-
3	yes	yes	12.73	12.26	-	-	35.61	37.69	4.53	4.62	-	-
4	yes	yes	12.33	12.10	-	-	35.36	35.49	4.36	4.29	-	-
5	yes	yes	13.65	13.88	-	-	34.70	34.60	4.74	4.80	-	-
6	yes	yes	13.54	12.68	-	-	34.80	34.70	4.75	4.81	-	-
7	yes	yes	10.14	10.23	-	-	34.87	34.81	3.54	3.56	-	-
8	yes	yes	13.68	13.76	-	-	34.70	34.80	4.75	4.79	-	-
9	yes	yes	12.31	12.41	-	-	34.69	34.85	4.27	4.32	-	-
10	yes	yes	6.91	11.16	-	-	34.47	34.37	2.38	3.84	-	-
11	yes	yes	12.36	11.88	-	-	34.83	34.66	4.31	4.12	-	-
12	yes	yes	12.69	12.46	-	-	34.56	34.73	4.38	4.33	-	-

[(1)] if sample is pure CB or not

Table E 15. Results accepted on technical grounds for sample 2 (chocolate fat for GLC analysis obtained by Soxhlet fat extraction)

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.36	0.35	18.52	18.54	43.63	43.58	2.54	2.48	32.02	31.98	3.30	3.42
2	0.29	0.31	18.97	18.97	43.83	43.47	2.68	2.61	31.32	31.57	3.21	3.38
3	0.30	0.29	18.88	18.91	43.67	43.71	2.61	2.62	31.72	31.70	3.12	3.06
4	0.30	0.30	18.79	18.95	43.62	43.68	2.57	2.62	31.84	31.58	3.17	3.17
5	0.34	0.34	18.89	18.86	43.76	43.63	2.63	2.66	31.58	31.63	3.14	3.23
6	0.31	0.31	19.25	19.26	43.84	43.86	2.63	2.65	31.02	31.01	3.26	3.22
7	0.28	0.28	18.84	18.81	44.04	43.89	2.45	2.58	31.49	31.39	3.18	3.33
8	0.33	0.33	18.83	18.91	43.86	43.81	2.57	2.57	31.60	31.53	3.15	3.18
9	0.31	0.32	18.87	18.82	43.34	43.26	2.81	2.77	31.69	31.80	3.29	3.35
10	0.22	0.23	18.92	18.90	44.19	44.33	2.35	2.27	32.51	32.31	2.04	2.20
11	0.31	0.31	18.91	18.24	43.69	42.19	2.61	5.91	31.62	30.63	3.18	3.02
12	0.32	0.32	18.10	18.08	43.27	43.26	2.71	2.71	32.59	32.59	3.34	3.36

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 16. Results accepted on technical grounds for sample 2 (chocolate fat for GLC analysis obtained by Soxhlet fat extraction)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g total fat/100 g chocolate		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B	A	B
1	no	no	15.83	15.73	1.01	1.04	35.80	35.60	5.67	5.60	0.36	0.37
2	no	no	13.17	13.78	1.24	1.91	35.80	35.57	4.71	4.90	0.44	0.68
3	no	no	13.30	13.15	1.73	1.72	35.78	38.53	4.76	5.07	0.62	0.66
4	no	no	13.36	13.48	1.77	1.62	36.09	36.01	4.82	4.85	0.64	0.59
5	no	no	15.23	15.03	1.02	1.32	35.40	35.70	5.39	5.37	0.36	0.47
6	no	no	14.01	13.94	1.04	1.05	35.60	35.20	5.42	5.29	0.37	0.37
7	no	no	12.69	12.39	0.81	1.10	35.54	35.42	4.51	4.39	0.29	0.39
8	no	no	14.97	15.02	0.80	0.89	35.40	35.30	5.30	5.30	0.28	0.31
9	no	no	13.78	14.11	2.32	2.40	35.64	35.73	4.91	5.04	0.83	0.86
10	no	no	9.83	10.46	2.29	1.60	35.23	35.12	3.69	3.45	0.56	0.80
11	no	no	13.68	13.67	1.52	5.06	35.92	35.95	4.91	4.91	0.55	1.82
12	no	no	14.15	14.14	2.20	2.20	35.73	35.76	5.06	5.06	0.79	0.79

[(1)] if sample is pure CB or not

Table E 17. Results accepted on technical grounds for sample 3 (chocolate fat for GLC analysis obtained by Soxhlet fat extraction)

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.53	0.53	18.47	18.79	43.93	44.00	2.77	2.93	31.68	31.10	3.14	3.19
2	0.41	0.43	18.47	18.64	43.65	43.88	2.75	2.83	31.23	31.22	3.90	3.43
3	0.45	0.43	18.33	18.31	43.97	43.85	2.87	2.84	31.62	31.81	3.21	3.20
4	0.41	0.41	18.50	18.75	44.00	44.05	2.71	2.83	31.64	31.16	3.15	3.21
5	0.48	0.47	18.50	18.56	43.94	44.02	2.87	2.82	31.42	31.40	3.27	3.20
6	0.42	0.44	18.81	18.68	44.01	44.03	2.79	2.83	31.01	31.11	3.38	3.34
7	0.36	0.37	18.45	18.49	44.33	44.41	2.62	2.67	31.24	31.19	3.37	3.25
8	0.46	0.47	18.56	18.49	44.25	44.23	2.78	2.73	31.21	31.37	3.19	3.17
9	0.42	0.41	18.39	18.38	44.05	44.02	2.97	2.91	31.33	31.40	3.25	3.29
10	0.32	0.32	18.58	18.53	44.60	44.68	2.34	2.43	32.10	32.22	2.38	2.14
11	0.42	0.42	18.51	18.59	44.10	44.01	2.78	2.77	31.35	31.43	3.26	3.19
12	0.45	0.46	17.80	17.80	43.51	43.53	2.95	2.96	32.31	32.30	3.43	3.40

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 18. Results accepted on technical grounds for sample 3 (chocolate fat for GLC analysis obtained by Soxhlet fat extraction)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g total fat/100 g chocolate		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B	A	B
1	yes	yes	23.63	23.68	-	-	32.20	32.00	7.61	7.58	-	-
2	yes	yes	18.28	18.93	-	-	31.54	32.17	5.76	6.09	-	-
3	yes	yes	19.97	19.30	-	-	32.29	32.15	6.45	6.20	-	-
4	yes	yes	18.35	18.08	-	-	32.29	32.26	5.93	5.83	-	-
5	yes	yes	21.48	20.91	-	-	31.40	31.80	6.74	6.65	-	-
6	yes	yes	18.71	19.68	-	-	31.40	32.20	6.74	6.73	-	-
7	yes	yes	16.12	16.58	-	-	31.27	31.78	5.04	5.27	-	-
8	yes	yes	20.70	21.02	-	-	31.90	32.00	6.60	6.73	-	-
9	yes	yes	18.59	18.39	-	-	31.89	31.79	5.93	5.85	-	-
10	yes	yes	14.31	14.38	-	-	31.95	31.81	4.60	4.55	-	-
11	yes	yes	18.64	18.84	-	-	32.11	32.08	5.99	6.05	-	-
12	yes	yes	20.17	20.39	-	-	32.08	32.01	6.47	6.53	-	-

[(1)] if sample is pure CB or not

Table E 19. Results accepted on technical grounds for sample 4 (chocolate fat for GLC analysis obtained by Soxhlet fat extraction)

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.49	0.52	20.11	19.98	41.76	41.24	3.01	2.93	31.52	32.42	3.60	3.43
2	0.39	0.40	20.08	20.28	41.20	41.31	2.98	3.14	31.38	31.42	4.36	3.85
3	0.43	0.43	19.97	19.77	41.31	41.45	3.21	3.16	31.74	32.02	3.77	3.60
4	0.41	0.41	20.02	20.00	41.52	41.51	3.06	3.09	31.82	31.84	3.58	3.55
5	0.46	0.48	20.05	20.08	41.62	41.57	3.10	3.17	31.71	31.59	3.51	3.60
6	0.45	0.45	20.22	20.33	41.78	41.84	3.12	3.07	31.18	31.09	3.70	3.67
7	0.39	0.35	19.79	19.87	41.78	41.56	3.01	3.12	31.64	31.67	3.78	3.78
8	0.45	0.46	19.98	20.02	41.81	41.76	3.05	3.03	31.64	31.68	3.51	3.51
9	0.42	0.41	20.01	19.97	41.10	41.55	3.37	3.42	31.76	31.46	3.77	3.60
10	0.27	0.31	20.00	20.00	42.08	42.19	2.54	2.65	32.18	32.43	3.19	2.73
11	0.41	0.41	20.04	20.06	41.60	41.45	3.11	3.29	31.59	31.68	3.66	3.52
12	0.45	0.44	19.20	19.24	41.06	41.12	3.31	3.29	32.59	32.53	3.84	3.81

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 20. Results accepted on technical grounds for sample 4 (chocolate fat for GLC analysis obtained by Soxhlet fat extraction)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g total fat/100 g chocolate		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B	A	B
1	no	no	21.96	23.21	4.71	6.02	32.10	32.20	7.05	7.47	1.51	1.94
2	no	no	17.27	17.83	6.55	6.65	32.26	32.35	5.57	5.77	2.11	2.15
3	no	no	19.04	19.30	6.38	6.06	32.24	32.39	6.14	6.25	2.06	1.96
4	no	no	18.16	18.42	6.24	6.22	32.41	32.47	5.89	5.98	2.02	2.02
5	no	no	20.61	21.26	5.47	5.35	32.00	31.70	6.59	6.74	1.75	1.70
6	no	no	19.99	19.99	5.02	4.90	32.10	31.90	6.62	6.78	1.61	1.56
7	no	no	17.32	15.78	5.46	6.41	31.64	31.95	5.48	5.04	1.73	2.05
8	no	no	20.31	20.65	4.99	5.08	31.80	31.80	6.46	6.57	1.59	1.62
9	no	no	18.76	18.19	7.01	6.04	32.10	31.93	6.02	5.81	2.25	1.93
10	no	no	12.15	13.89	6.59	6.33	32.11	31.92	4.46	3.88	2.03	2.10
11	no	no	18.08	18.38	5.94	6.41	32.32	32.43	5.84	5.96	1.92	2.08
12	no	no	20.02	19.78	6.56	6.49	32.37	32.26	6.48	6.38	2.12	2.09

[(1)] if sample is pure CB or not

Table E 21. Results accepted on technical grounds for sample 5 (chocolate fat for GLC analysis obtained by Soxhlet fat extraction)

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.59	0.57	23.37	23.31	36.40	36.23	3.18	3.20	33.20	33.20	3.86	4.07
2	0.44	0.47	24.18	23.95	36.28	36.23	3.06	3.39	32.40	32.19	4.07	4.25
3	0.48	0.46	23.95	24.06	36.24	36.14	3.35	3.36	32.74	32.70	3.72	3.74
4	0.47	0.45	24.07	23.88	36.31	36.27	3.29	3.25	32.53	32.89	3.79	3.71
5	0.51	0.52	24.03	23.99	36.35	36.39	3.29	3.30	32.62	32.60	3.71	3.72
6	0.48	0.50	24.09	24.03	36.75	36.76	3.29	3.29	32.04	32.06	3.83	3.85
7	0.43	0.48	23.66	23.94	36.47	36.76	3.35	3.19	32.63	32.39	3.90	3.71
8	0.52	0.51	23.92	23.87	36.57	36.68	3.23	3.21	32.52	32.53	3.76	3.70
9	0.46	0.48	23.77	23.87	36.15	35.81	3.61	3.64	32.68	32.70	3.79	3.98
10	0.40	0.33	24.35	24.47	36.80	37.22	2.55	2.82	33.11	32.20	3.19	3.30
11	0.45	0.45	23.98	23.93	36.24	36.16	3.31	3.36	32.69	32.71	3.78	3.85
12	0.50	0.50	23.01	22.96	35.94	35.92	3.53	3.51	33.52	33.61	3.99	4.00

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 22. Results accepted on technical grounds for sample 5 (chocolate fat for GLC analysis obtained by Soxhlet fat extraction)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g total fat/100 g chocolate		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B	A	B
1	no	no	26.17	25.25	18.99	19.41	25.40	25.40	6.65	6.41	4.82	4.93
2	no	no	19.66	21.05	20.80	20.33	25.55	25.58	5.02	5.38	5.31	5.20
3	no	no	21.30	20.55	20.79	21.23	25.76	26.94	5.49	5.54	5.36	5.72
4	no	no	20.67	20.19	20.70	20.96	25.65	25.62	5.30	5.17	5.31	5.37
5	no	no	22.65	23.28	20.20	19.93	25.30	25.30	5.73	5.89	5.11	5.04
6	no	no	21.42	22.42	19.29	18.98	24.60	25.40	5.57	5.91	4.74	4.82
7	no	no	19.03	21.19	20.43	19.40	24.67	25.07	4.70	5.31	5.04	4.86
8	no	no	23.10	22.83	19.43	19.24	25.00	25.00	5.78	5.71	4.86	4.81
9	no	no	20.27	21.26	21.11	21.63	25.11	25.30	5.09	5.38	5.30	5.47
10	no	no	17.69	14.73	20.87	20.28	25.05	25.16	4.43	3.71	5.23	5.10
11	no	no	19.81	20.04	21.08	21.17	25.30	25.52	5.01	5.11	5.33	5.40
12	no	no	22.06	22.21	20.89	20.89	25.30	25.42	5.58	5.65	5.29	5.31

[(1)] if sample is pure CB or not

Table E 23. Results accepted on technical grounds for sample 6 (chocolate fat for GLC analysis obtained by Soxhlet fat extraction)

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.41	0.38	21.78	21.54	40.18	40.01	2.73	2.80	31.98	32.32	3.34	3.34
2	0.33	0.38	21.80	22.20	39.54	39.63	2.84	2.62	32.03	32.10	3.79	3.45
3	0.36	0.36	21.57	21.71	39.82	39.88	2.88	2.81	32.33	32.25	3.40	3.35
4	0.34	0.35	21.75	21.65	39.90	39.87	2.72	2.74	32.27	32.37	3.36	3.38
5	0.38	0.39	21.56	21.59	39.88	39.93	2.78	2.79	32.39	32.32	3.40	3.37
6	0.35	0.35	21.81	21.94	40.14	40.25	2.77	2.73	31.78	31.71	3.50	3.37
7	0.30	0.32	21.64	21.60	40.28	40.15	2.71	2.71	32.00	32.20	3.37	3.34
8	0.38	0.38	21.60	21.55	40.14	40.15	2.66	2.71	32.32	32.25	3.29	3.34
9	0.36	0.36	21.54	21.61	39.42	39.47	2.97	2.96	32.54	32.41	3.53	3.55
10	0.27	0.24	21.53	21.58	40.58	40.54	2.36	2.29	32.84	32.98	2.69	2.61
11	0.33	0.34	21.58	21.65	39.87	39.81	2.78	2.78	32.29	32.41	3.47	3.35
12	0.36	0.35	20.73	20.70	39.50	39.53	2.85	2.85	33.38	33.40	3.55	3.52

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 24. Results accepted on technical grounds for sample 6 (chocolate fat for GLC analysis obtained by Soxhlet fat extraction)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g total fat/100 g chocolate		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B	A	B
1	no	no	18.29	17.00	10.64	11.32	34.00	33.70	6.22	5.73	3.62	3.82
2	no	no	14.91	16.96	12.69	12.44	34.07	34.02	5.08	5.77	4.32	4.23
3	no	no	15.82	15.90	12.06	11.96	30.76	34.10	4.87	5.42	3.71	4.08
4	no	no	15.30	15.63	12.07	12.02	34.16	34.21	5.23	5.35	4.12	4.11
5	no	no	17.05	17.20	11.63	11.47	33.80	34.00	5.76	5.85	3.93	3.90
6	no	no	15.90	15.93	11.14	10.99	33.40	33.90	5.69	5.83	3.72	3.72
7	no	no	13.29	14.20	11.45	11.61	34.04	33.94	4.52	4.82	3.90	3.94
8	no	no	17.12	17.20	11.04	10.91	33.40	33.10	5.72	5.69	3.69	3.61
9	no	no	15.85	16.00	12.99	12.81	33.69	33.80	5.34	5.41	4.38	4.33
10	no	no	11.93	10.71	11.66	12.17	33.10	33.35	3.95	3.57	3.86	4.06
11	no	no	14.90	15.09	12.08	12.35	34.41	34.24	5.13	5.17	4.16	4.23
12	no	no	15.88	15.76	12.59	12.53	34.27	34.40	5.44	5.42	4.31	4.31

[(1)] if sample is pure CB or not

Table E 25. Results accepted on technical grounds for sample 7

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.00	0.00	17.70	17.91	44.35	44.49	2.31	2.31	32.05	31.80	3.59	3.48
2	0.00	0.00	18.25	18.04	44.47	44.72	2.40	2.30	31.58	31.44	3.30	3.50
3	0.00	0.00	18.10	18.05	44.54	44.49	2.38	2.35	31.59	31.72	3.40	3.39
4	0.00	0.00	18.39	18.55	44.43	44.49	2.42	2.45	31.32	31.04	3.43	3.47
5	0.00	0.00	18.17	18.12	44.56	44.59	2.34	2.36	31.54	31.55	3.40	3.38
6	0.00	0.00	18.36	18.62	44.74	44.86	2.29	2.34	31.24	30.87	3.38	3.31
7	0.00	0.00	18.10	18.02	44.22	44.52	2.50	2.43	31.70	31.60	3.47	3.43
8	0.00	0.00	18.10	18.07	44.53	44.54	2.32	2.29	31.66	31.73	3.38	3.37
9	0.00	0.00	18.04	18.05	44.83	44.97	2.30	2.31	31.49	31.36	3.34	3.31
10	0.00	0.00	18.44	18.51	45.11	45.02	2.06	2.10	31.32	31.30	3.07	3.08
11	0.00	0.00	18.08	18.11	44.55	44.60	2.37	2.32	31.48	31.57	3.53	3.40
12	0.00	0.00	17.39	17.36	43.98	44.05	2.45	2.47	32.61	32.55	3.57	3.57

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 26. Results accepted on technical grounds for sample 7 (assumed total fat content of chocolate= 30 %)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B
1	yes	yes	0.00	0.00	-	-	0.00	0.00	-	-
2	yes	yes	0.00	0.00	-	-	0.00	0.00	-	-
3	yes	yes	0.00	0.00	-	-	0.00	0.00	-	-
4	yes	yes	0.00	0.00	-	-	0.00	0.00	-	-
5	yes	yes	0.00	0.00	-	-	0.00	0.00	-	-
6	yes	yes	0.00	0.00	-	-	0.00	0.00	-	-
7	yes	yes	0.00	0.00	-	-	0.00	0.00	-	-
8	yes	yes	0.00	0.00	-	-	0.00	0.00	-	-
9	yes	yes	0.00	0.00	-	-	0.00	0.00	-	-
10	yes	yes	0.00	0.00	-	-	0.00	0.00	-	-
11	yes	yes	0.00	0.00	-	-	0.00	0.00	-	-
12	yes	yes	0.00	0.00	-	-	0.00	0.00	-	-

[(1)] if sample is pure CB or not

Table E 27. Results accepted on technical grounds for sample 8

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.36	0.37	18.86	18.25	43.88	43.94	2.78	2.83	30.96	31.29	3.52	3.69
2	0.35	0.34	18.68	18.26	43.90	43.44	2.88	2.87	30.88	31.40	3.65	4.03
3	0.35	0.33	18.47	18.54	43.92	44.03	2.89	2.86	31.17	31.05	3.55	3.52
4	0.34	0.34	18.67	18.85	44.00	43.95	2.83	2.97	30.99	30.75	3.51	3.48
5	0.38	0.37	18.66	18.64	44.05	44.06	2.87	2.89	30.91	30.91	3.51	3.50
6	0.34	0.35	19.50	19.24	44.62	44.50	2.85	2.73	29.60	30.07	3.43	3.45
7	0.30	0.33	18.52	18.57	44.61	43.86	2.73	2.86	30.74	31.12	3.40	3.59
8	0.38	0.37	18.51	18.59	44.10	44.13	2.79	2.78	31.10	31.00	3.49	3.50
9	0.36	0.36	18.56	18.52	43.99	44.04	2.97	2.99	30.92	30.92	3.56	3.52
10	0.33	0.32	19.07	19.08	44.60	44.57	2.48	2.48	30.69	30.73	3.16	3.14
11	0.37	0.35	18.58	18.53	43.99	44.09	2.82	2.91	31.08	30.92	3.53	3.54
12	0.40	0.38	17.83	17.81	43.54	43.48	2.99	2.96	31.95	32.06	3.69	3.69

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 28. Results accepted on technical grounds for sample 8 (assumed total fat content of chocolate= 30 %)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B
1	yes	yes	15.95	16.33	-	-	4.79	4.90	-	-
2	yes	yes	15.47	15.35	-	-	4.64	4.61	-	-
3	yes	yes	15.54	14.73	-	-	4.66	4.42	-	-
4	yes	yes	15.07	15.03	-	-	4.52	4.51	-	-
5	yes	yes	17.00	16.43	-	-	5.10	4.93	-	-
6	yes	yes	15.50	15.76	-	-	4.65	4.73	-	-
7	yes	yes	13.43	14.66	-	-	4.03	4.40	-	-
8	yes	yes	16.80	16.59	-	-	5.04	4.98	-	-
9	yes	yes	16.00	15.98	-	-	4.80	4.79	-	-
10	yes	yes	14.60	14.43	-	-	4.38	4.33	-	-
11	yes	yes	16.47	15.69	-	-	4.94	4.71	-	-
12	yes	yes	17.60	16.97	-	-	5.28	5.09	-	-

[(1)] if sample is pure CB or not

Table E 29. Results accepted on technical grounds for sample 9

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.40	0.39	19.18	19.20	43.30	43.34	2.90	2.79	31.13	31.14	3.49	3.53
2	0.36	0.35	19.19	19.27	42.94	43.22	2.96	2.87	31.17	31.25	3.73	3.39
3	0.35	0.34	19.11	19.05	43.28	43.24	2.92	2.90	31.11	31.23	3.58	3.58
4	0.34	0.35	19.41	19.44	43.20	43.36	2.96	2.93	30.87	30.71	3.57	3.56
5	0.36	0.36	19.24	19.25	43.32	43.36	2.90	2.91	30.95	30.93	3.59	3.55
6	0.33	0.32	19.86	19.73	43.80	43.68	2.73	2.78	30.24	30.36	3.37	3.45
7	0.31	0.34	19.17	19.40	43.28	43.57	2.99	2.85	31.04	30.65	3.53	3.53
8	0.37	0.38	19.30	19.18	43.51	43.43	2.82	2.84	30.88	31.07	3.49	3.49
9	0.35	0.35	19.19	19.25	43.35	43.41	3.01	3.02	30.90	30.80	3.55	3.52
10	0.32	0.32	19.88	19.77	43.83	43.96	2.52	2.55	30.60	30.57	3.17	3.16
11	0.37	0.37	19.23	19.28	43.37	43.35	2.99	2.89	30.84	31.02	3.58	3.46
12	0.40	0.40	18.46	18.49	42.82	42.85	3.02	3.01	31.96	31.92	3.74	3.72

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 30. Results accepted on technical grounds for sample 9 (assumed total fat content of chocolate= 30 %)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B
1	no	no	17.75	17.19	1.35	1.36	5.33	5.16	0.41	0.41
2	no	no	15.89	15.52	2.51	2.22	4.77	4.66	0.75	0.66
3	no	no	15.48	15.12	1.83	2.01	4.64	4.54	0.55	0.60
4	no	no	15.26	15.50	2.17	1.68	4.58	4.65	0.65	0.50
5	no	no	16.08	16.06	1.59	1.54	4.82	4.82	0.48	0.46
6	no	no	14.81	14.68	0.96	1.21	4.44	4.40	0.29	0.36
7	no	no	13.71	15.23	2.28	1.21	4.11	4.57	0.68	0.36
8	no	no	16.64	16.93	1.08	1.20	4.99	5.08	0.32	0.36
9	no	no	15.60	15.45	1.63	1.56	4.68	4.63	0.49	0.47
10	no	no	14.40	14.25	1.24	0.89	4.32	4.27	0.37	0.27
11	no	no	16.56	16.66	1.35	1.52	4.97	5.00	0.40	0.46
12	no	no	17.87	18.02	2.19	2.10	5.36	5.41	0.66	0.63

[(1)] if sample is pure CB or not

Table E 31. Results accepted on technical grounds for sample 10

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.38	0.40	24.18	24.13	38.75	38.89	3.05	3.13	30.38	30.01	3.64	3.84
2	0.32	0.31	24.29	24.44	38.63	38.27	3.18	3.31	30.10	29.57	3.80	4.41
3	0.34	0.34	24.19	24.15	38.44	38.58	3.35	3.25	30.34	30.36	3.67	3.66
4	0.33	0.34	24.28	24.35	38.65	38.52	3.16	3.26	30.24	30.19	3.66	3.68
5	0.37	0.36	24.32	24.23	38.74	38.71	3.23	3.22	30.09	30.17	3.62	3.67
6	0.33	0.36	24.77	24.25	39.20	39.04	2.98	3.14	29.58	29.92	3.48	3.65
7	0.33	0.32	24.14	24.26	38.62	39.06	3.27	3.12	30.24	29.97	3.72	3.58
8	0.37	0.36	24.17	24.32	38.85	38.92	3.12	3.14	30.27	30.02	3.59	3.61
9	0.37	0.35	24.31	24.28	38.59	38.59	3.28	3.34	30.14	30.13	3.68	3.66
10	0.32	0.32	24.86	24.97	39.28	39.27	2.74	2.79	29.89	29.73	3.23	3.24
11	0.36	0.35	24.21	24.28	38.71	38.58	3.25	3.23	30.15	30.25	3.68	3.67
12	0.40	0.39	23.28	23.32	38.26	38.33	3.34	3.28	31.23	31.23	3.89	3.85

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 32. Results accepted on technical grounds for sample 10 (assumed total fat content of chocolate= 30 %)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B
1	no	no	17.10	17.83	15.05	14.26	5.13	5.35	4.51	4.28
2	no	no	14.24	13.90	15.87	16.32	4.27	4.17	4.76	4.90
3	no	no	15.21	15.29	16.24	15.85	4.56	4.59	4.87	4.76
4	no	no	14.83	15.08	15.81	16.09	4.45	4.52	4.74	4.83
5	no	no	16.40	16.17	15.25	15.32	4.92	4.85	4.57	4.60
6	no	no	15.07	16.14	14.60	14.44	4.52	4.84	4.38	4.33
7	no	no	14.76	14.30	15.80	14.88	4.43	4.29	4.74	4.47
8	no	no	16.66	16.21	14.89	14.83	5.00	4.86	4.47	4.45
9	no	no	16.29	15.51	15.61	15.79	4.89	4.65	4.68	4.74
10	no	no	14.11	14.22	14.90	14.91	4.23	4.26	4.47	4.47
11	no	no	16.20	15.39	15.29	15.87	4.86	4.62	4.59	4.76
12	no	no	17.67	17.20	15.73	15.72	5.30	5.16	4.72	4.72

[(1)] if sample is pure CB or not

Table E 33. Results accepted on technical grounds for sample 11

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.34	0.38	25.41	25.51	37.38	37.27	3.23	3.30	30.07	30.02	3.91	3.91
2	0.37	0.37	25.74	25.89	37.01	37.33	3.49	3.19	29.95	29.99	3.80	3.60
3	0.34	0.35	25.72	25.47	37.04	37.25	3.25	3.29	30.27	30.26	3.72	3.72
4	0.34	0.34	25.75	25.66	37.20	37.31	3.34	3.23	29.97	30.16	3.74	3.64
5	0.37	0.36	25.68	25.69	37.32	37.25	3.32	3.30	29.97	30.02	3.71	3.74
6	0.32	0.35	26.46	26.27	38.03	37.98	3.11	3.19	28.99	29.05	3.41	3.51
7	0.31	0.32	25.72	25.75	37.72	37.70	3.17	3.13	29.76	29.79	3.63	3.63
8	0.37	0.38	25.73	25.55	37.55	37.49	3.23	3.20	29.88	30.14	3.61	3.63
9	0.34	0.37	25.61	25.65	37.25	37.24	3.44	3.43	29.99	29.96	3.71	3.71
10	0.32	0.29	26.26	26.21	37.87	37.88	2.80	2.79	29.78	29.84	3.29	3.27
11	0.37	0.37	25.67	25.70	37.22	37.28	3.35	3.30	30.03	30.11	3.74	3.62
12	0.39	0.39	24.68	24.66	36.99	36.98	3.41	3.42	31.02	31.03	3.91	3.91

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 34. Results accepted on technical grounds for sample 11 (assumed total fat content of chocolate= 30 %)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B
1	no	no	15.35	16.71	19.19	19.20	4.60	5.01	5.76	5.76
2	no	no	16.34	16.62	20.12	19.48	4.90	4.99	6.04	5.84
3	no	no	15.27	15.38	20.42	19.73	4.58	4.61	6.13	5.92
4	no	no	15.10	15.19	19.98	19.77	4.53	4.56	5.99	5.93
5	no	no	16.28	16.11	19.40	19.61	4.88	4.83	5.82	5.88
6	no	no	14.78	15.83	18.32	18.06	4.43	4.75	5.50	5.42
7	no	no	13.72	14.19	19.01	18.97	4.12	4.26	5.70	5.69
8	no	no	16.56	16.92	18.83	18.87	4.97	5.08	5.65	5.66
9	no	no	15.24	16.30	19.79	19.58	4.57	4.89	5.94	5.87
10	no	no	14.37	12.89	18.98	19.30	4.31	3.87	5.69	5.79
11	no	no	16.45	16.36	19.60	19.60	4.93	4.91	5.88	5.88
12	no	no	17.40	17.18	19.60	19.66	5.22	5.15	5.88	5.90

[(1)] if sample is pure CB or not

Table E 35. Results accepted on technical grounds for sample 12

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.39	0.37	25.31	25.50	37.89	37.86	7.53	7.41	25.73	25.65	3.54	3.59
2	0.33	0.31	25.67	25.68	37.84	37.64	8.18	8.32	25.13	25.27	3.18	3.09
3	0.34	0.34	25.25	25.22	37.51	37.60	8.11	8.10	25.55	25.56	3.59	3.52
4	0.34	0.34	25.68	25.92	37.69	37.60	7.84	8.06	25.27	24.92	3.52	3.50
5	0.36	0.36	25.48	25.46	37.71	37.64	8.02	8.03	25.26	25.33	3.53	3.54
6	0.32	0.35	25.97	26.77	37.63	38.61	9.22	7.01	23.85	24.24	3.33	3.38
7	0.32	0.32	25.38	25.34	37.52	37.43	8.25	8.09	25.24	25.54	3.61	3.61
8	0.38	0.37	25.43	25.58	37.93	37.99	7.67	7.67	25.50	25.32	3.47	3.45
9	0.37	0.35	24.99	25.00	37.51	37.52	9.10	8.98	24.91	25.01	3.49	3.49
10	0.32	0.32	26.32	26.37	38.44	38.52	6.83	6.63	25.22	25.32	3.20	3.16
11	0.35	0.34	25.25	25.53	37.55	37.60	8.21	8.04	25.33	25.40	3.66	3.43
12	0.37	0.36	24.46	24.48	37.35	37.42	8.25	8.25	26.21	26.15	3.73	3.69

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 36. Results accepted on technical grounds for sample 12 (assumed total fat content of chocolate= 30 %)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B
1	no	no	17.42	16.62	16.85	17.14	5.23	4.99	5.05	5.14
2	no	no	14.63	13.79	17.96	18.77	4.39	4.14	5.39	5.63
3	no	no	15.10	15.28	18.26	18.05	4.53	4.58	5.48	5.42
4	no	no	15.23	15.01	17.96	18.28	4.57	4.50	5.39	5.48
5	no	no	15.98	16.25	17.64	17.76	4.79	4.88	5.29	5.33
6	no	no	14.36	15.58	18.33	15.91	4.31	4.41	5.50	5.50
7	no	no	14.45	14.08	18.38	18.72	4.33	4.22	5.51	5.62
8	no	no	16.99	16.51	16.91	16.92	5.10	4.95	5.07	5.08
9	no	no	16.28	15.65	17.83	18.00	4.89	4.69	5.35	5.40
10	no	no	14.10	14.21	16.84	16.67	4.23	4.26	5.05	5.00
11	no	no	15.67	15.17	17.94	18.24	4.70	4.55	5.38	5.47
12	no	no	16.36	16.24	18.04	17.92	4.91	4.87	5.41	5.38

[(1)] if sample is pure CB or not

Table E 37. Results accepted on technical grounds for sample 13

Lab	g PSB/100 g chocolate fat		%-POP [(1)]		%-POS [(1)]		%-POO [(1)]		%-SOS [(1)]		%-SOO [(1)]	
	A	B	A	B	A	B	A	B	A	B	A	B
1	0.38	0.36	28.69	28.65	34.65	34.66	3.34	3.29	29.25	29.60	4.06	3.79
2	0.35	0.34	29.28	28.61	34.61	34.17	3.27	3.61	29.39	29.59	3.45	4.02
3	0.34	0.35	28.24	28.29	34.51	34.47	3.49	3.46	29.96	29.96	3.80	3.82
4	0.34	0.33	28.74	28.80	34.61	34.62	3.41	3.43	29.52	29.37	3.72	3.76
5	0.36	0.39	28.60	28.62	34.56	34.70	3.50	3.51	29.55	29.44	3.79	3.73
6	0.33	0.36	29.11	28.89	35.28	35.24	3.24	3.24	28.85	29.06	3.52	3.57
7	0.29	0.30	28.77	28.66	34.92	34.98	3.34	3.36	29.34	29.35	3.64	3.65
8	0.38	0.37	28.41	28.39	34.81	34.82	3.36	3.38	29.69	29.73	3.72	3.68
9	0.35	0.37	28.55	28.31	34.41	34.62	3.67	3.90	29.56	29.38	3.81	3.79
10	0.33	0.31	29.24	29.13	35.14	35.30	2.94	2.96	29.32	29.27	3.35	3.33
11	0.34	0.34	28.57	28.60	34.57	34.50	3.57	3.44	29.49	29.72	3.80	3.73
12	0.37	0.37	27.50	27.49	34.30	34.30	3.59	3.60	30.61	30.64	4.00	3.98

[(1)] %-POP+%-POS+%-POO+%-SOS+%-SOO = 100 %

Table E 38. Results accepted on technical grounds for sample 13 (assumed total fat content of chocolate= 30 %)

Lab	Qualitative Decision [(1)]		g MF/100 g chocolate fat		g CBE/100 g chocolate fat		g MF/100 g chocolate		g CBE/100 g chocolate	
	A	B	A	B	A	B	A	B	A	B
1	no	no	16.95	16.07	26.93	27.40	5.09	4.82	8.08	8.22
2	no	no	15.63	15.33	28.13	28.61	4.69	4.60	8.44	8.58
3	no	no	15.22	15.63	27.87	27.88	4.57	4.69	8.36	8.36
4	no	no	15.07	14.94	27.84	27.80	4.52	4.48	8.35	8.34
5	no	no	16.11	17.21	27.61	27.04	4.83	5.16	8.28	8.11
6	no	no	14.80	16.09	26.39	26.10	4.44	4.83	7.92	7.83
7	no	no	13.12	13.50	27.54	27.25	3.93	4.05	8.26	8.17
8	no	no	17.05	16.67	26.75	26.85	5.11	5.00	8.03	8.06
9	no	no	15.48	16.30	28.10	27.25	4.64	4.89	8.43	8.17
10	no	no	14.57	13.75	27.08	26.82	4.37	4.12	8.12	8.04
11	no	no	15.21	15.33	27.78	28.03	4.56	4.60	8.33	8.41
12	no	no	16.43	16.32	27.77	27.82	4.93	4.90	8.33	8.35

[(1)] if sample is pure CB or not

ANNEX F – STATISTICAL EVALUATION OF RESULTS ACCEPTED ON TECHNICAL GROUNDS

Table F 1. Statistical evaluation of determined PSB amounts in chocolate fat accepted on technical grounds (samples 1-6; chocolate fat for GLC analysis obtained by rapid fat extraction)

Sample number	1	2	3	4	5	6	8	9	10	11	12	13
Year of collaborative trial	2006											
Number of laboratories	12	12	12	12	12	12	12	12	12	12	12	12
Mean value, g PSB/100 g chocolate fat	0.26	0.29	0.44	0.43	0.49	0.33	0.35	0.36	0.35	0.35	0.35	0.35
Repeatability limit r [r=2.8 x s_r], g/100 g	0.04	0.03	0.03	0.03	0.03	0.01	0.03	0.02	0.03	0.03	0.03	0.03
Repeatability standard deviation s_r, g/100 g	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Repeatability relative standard deviation RSD_r, %	5.7	3.1	2.6	2.6	2.3	1.6	2.6	2.3	2.7	3.5	2.8	3.3
Reproducibility limit R [r=2.8 x s_R], g/100 g	0.05	0.04	0.05	0.05	0.05	0.06	0.06	0.08	0.07	0.07	0.07	0.07
Reproducibility standard deviation s_R, g/100 g	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.03	0.03	0.03	0.02	0.03
Reproducibility relative standard deviation RSD_R, %	7.3	4.8	4.4	4.1	4.0	6.1	6.4	7.7	7.6	7.5	6.8	7.2
Horrat value = RSD_R/predicted RSD_R [(1)]	1.49	0.99	0.97	0.89	0.90	1.29	1.36	1.65	1.62	1.60	1.44	1.53

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

Table F 2. Statistical evaluation of determined PSB amounts in chocolate fat accepted on technical grounds (chocolate fat for GLC analysis obtained by Soxhlet extraction)

Sample number	1	2	3	4	5	6
Year of collaborative trial	2006					
Number of laboratories	12	12	12	12	12	12
Mean value, g PSB/100 g chocolate fat	0.28	0.31	0.43	0.42	0.48	0.35
Repeatability limit r [r=2.8 x s_r], g/100 g	0.06	0.01	0.02	0.04	0.06	0.04
Repeatability standard deviation s_r, g/100 g	0.02	0.00	0.01	0.01	0.02	0.01
Repeatability relative standard deviation RSD_r, %	7.5	1.6	1.8	3.2	4.3	3.9
Reproducibility limit R [r=2.8 x s_R], g/100 g	0.10	0.09	0.15	0.16	0.15	0.11
Reproducibility standard deviation s_R, g/100 g	0.04	0.03	0.05	0.06	0.05	0.04
Reproducibility relative standard deviation RSD_R, %	12.9	10.6	12.5	13.2	11.3	11.2
Horrat value = RSD_R/predicted RSD_R [(1)]	2.66	2.21	2.76	2.91	2.52	2.40

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

Table F 3. Statistical evaluation of determined MF amounts in chocolate fat accepted on technical grounds (samples 1-6; chocolate fat for GLC analysis obtained by rapid fat extraction)

Sample number	1	2	3	4	5	6	8	9	10	11	12	13
Year of collaborative trial	2006											
Number of laboratories	12	12	12	12	12	12	12	12	12	12	12	12
Mean value, g MF/100 g chocolate fat	11.59	13.14	19.72	19.19	21.72	14.64	15.72	15.86	15.66	15.69	15.46	15.53
True value, g MF/100 g chocolate fat	16.56	16.56	19.56	19.56	21.51	18.81	14.99	14.98	15.02	15.04	14.99	15.05
Bias, g MF/100 g chocolate fat	4.97	3.43	-0.16	0.37	-0.22	4.17	-0.73	-0.88	-0.64	-0.65	-0.47	-0.48
Repeatability limit r [r=2.8 x s_r], g/100 g	1.81	1.13	1.41	1.38	1.37	0.64	1.11	1.01	1.12	1.49	1.15	1.35
Repeatability standard deviation s_r, g/100 g	0.65	0.40	0.50	0.49	0.49	0.23	0.40	0.36	0.40	0.53	0.41	0.48
Repeatability relative standard deviation RSD_r, %	5.6	3.1	2.6	2.6	2.3	1.6	2.5	2.3	2.6	3.4	2.7	3.1
Reproducibility limit R [r=2.8 x s_R], g/100 g	2.44	1.83	2.40	2.20	2.41	2.54	2.77	3.31	3.28	3.22	2.87	3.08
Reproducibility standard deviation s_R, g/100 g	0.87	0.65	0.86	0.79	0.86	0.91	0.99	1.18	1.17	1.15	1.02	1.10
Reproducibility relative standard deviation RSD_R, %	7.5	5.0	4.3	4.1	4.0	6.2	6.3	7.4	7.5	7.3	6.6	7.1
Horrat value = RSD_R/predicted RSD_R [(1)]	2.71	1.83	1.70	1.60	1.57	2.32	2.38	2.82	2.83	2.77	2.50	2.68

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

Table F 4. Statistical evaluation of determined CBE amounts in chocolate fat accepted on technical grounds (samples 2-6; chocolate fat for GLC analysis obtained by rapid fat extraction)

Sample number	2	4	5	6	9	10	11	12	13
Year of collaborative trial	2006								
Number of laboratories	12	12	12	12	12	12	12	12	12
Mean value, g CBE/100 g chocolate fat	1.63	5.92	20.50	12.07	1.61	15.39	19.38	17.72	27.44
True value, g CBE/100 g chocolate fat	1.27	6.35	20.35	11.68	2.00	16.03	19.98	20.08	28.04
Bias, g CBE/100 g chocolate fat	-0.36	0.43	-0.15	-0.38	0.38	0.64	0.60	2.35	0.60
Repeatability limit r [$r=2.8 \times s_r$], g/100 g	0.56	0.77	0.86	0.36	0.76	0.87	0.63	1.52	0.77
Repeatability standard deviation s_r, g/100 g	0.20	0.27	0.31	0.13	0.27	0.31	0.22	0.54	0.27
Repeatability relative standard deviation RSD_r, %	12.2	4.6	1.5	1.1	16.8	2.0	1.2	3.1	1.0
Reproducibility limit R [$r=2.8 \times s_R$], g/100 g	1.45	1.69	1.78	1.31	1.30	1.66	1.54	2.03	1.73
Reproducibility standard deviation s_R, g/100 g	0.52	0.61	0.64	0.47	0.46	0.59	0.55	0.72	0.62
Reproducibility relative standard deviation RSD_R, %	31.7	10.2	3.1	3.9	28.7	3.8	2.8	4.1	2.2
Horrat value = RSD_R/predicted RSD_R [(1)]	8.54	3.34	1.22	1.42	7.72	1.45	1.11	1.58	0.93

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

Table F 5. Statistical evaluation of determined CBE amounts in chocolate fat accepted on technical grounds (chocolate fat for GLC analysis obtained by Soxhlet extraction)

Sample number	**2**	**4**	**5**	**6**
Year of collaborative trial	2006			
Number of laboratories	12	12	12	12
Mean value, g CBE/100 g chocolate fat	1.65	5.95	20.33	11.86
True value, g CBE/100 g chocolate fat	1.27	6.35	20.35	11.68
Bias, g CBE/100 g chocolate fat	-0.38	0.40	0.02	-0.18
Repeatability limit r [r=2.8 x s_r], g/100 g	2.11	1.14	0.91	0.57
Repeatability standard deviation s_r, g/100 g	0.75	0.41	0.33	0.20
Repeatability relative standard deviation RSD_r, %	45.6	6.8	1.6	1.7
Reproducibility limit R [r=2.8 x s_R], g/100 g	2.51	1.87	2.30	1.87
Reproducibility standard deviation s_R, g/100 g	0.90	0.67	0.82	0.67
Reproducibility relative standard deviation RSD_R, %	54.3	11.2	4.0	5.6
Horrat value = RSD_R/predicted RSD_R [(1)]	14.63	3.67	1.59	2.04

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

Table F 6. Statistical evaluation of determined total fat contents of chocolate samples accepted on technical grounds (total fat content determined by Soxhlet extraction)

Sample number	1	2	3	4	5	6
Year of collaborative trial	2006					
Number of laboratories	12	12	12	12	12	12
Mean value, g total fat/100 g chocolate	34.98	35.70	31.93	32.11	25.35	33.74
True value, g total fat/100 g chocolate	35.56	35.56	31.95	31.95	25.11	33.81
Bias, g total fat/100 g chocolate	0.58	-0.14	0.02	-0.16	-0.24	0.07
Repeatability limit r [$r=2.8 \times s_r$], g/100 g	1.22	0.81	0.71	0.34	0.87	1.96
Repeatability standard deviation s_r, g/100 g	0.44	0.29	0.26	0.12	0.31	0.70
Repeatability relative standard deviation RSD_r, %	1.2	0.8	0.8	0.4	1.2	2.1
Reproducibility limit R [$r=2.8 \times s_R$], g/100 g	1.87	1.11	0.82	0.70	1.26	2.07
Reproducibility standard deviation s_R, g/100 g	0.67	0.40	0.29	0.25	0.45	0.74
Reproducibility relative standard deviation RSD_R, %	1.9	1.1	0.9	0.8	1.8	2.2
Horrat value = RSD_R/predicted RSD_R [(1)]	0.82	0.47	0.38	0.33	0.72	0.93

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

Table F 7. Statistical evaluation of determined MF amounts in chocolate accepted on technical grounds (samples 2-6, chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet; samples 9-13, assumed fat content of chocolate = 30 %)

Sample number	1	2	3	4	5	6	8	9	10	11	12	13
Year of collaborative trial	2006											
Number of laboratories	12	12	12	12	12	12	12	12	12	12	12	12
Mean value, g MF/100 g chocolate	4.08	4.70	6.31	6.15	5.50	4.95	4.72	4.76	4.70	4.71	4.63	4.66
True value, g MF/100 g chocolate	5.89	5.89	6.25	6.25	5.40	6.36	4.50	4.49	4.51	4.51	4.50	4.52
Bias, g MF/100 g chocolate	1.81	1.19	-0.06	0.10	-0.10	1.41	-0.22	-0.27	-0.19	-0.20	-0.13	-0.14
Repeatability limit r [r=2.8 x s_r], g/100 g	0.45	0.57	0.32	0.48	0.33	0.38	0.33	0.30	0.34	0.45	0.28	0.41
Repeatability standard deviation s_r, g/100 g	0.16	0.20	0.11	0.17	0.12	0.14	0.12	0.11	0.12	0.16	0.10	0.14
Repeatability relative standard deviation RSD_r, %	3.9	4.3	1.8	2.8	2.1	2.8	2.5	2.3	2.6	3.4	2.2	3.1
Reproducibility limit R [r=2.8 x s_R], g/100 g	0.60	0.74	0.77	0.67	0.60	0.98	0.83	0.99	0.98	0.97	0.87	0.92
Reproducibility standard deviation s_R, g/100 g	0.22	0.26	0.27	0.24	0.22	0.35	0.30	0.35	0.35	0.35	0.31	0.33
Reproducibility relative standard deviation RSD_R, %	5.3	5.6	4.4	3.9	3.9	7.1	6.3	7.4	7.5	7.3	6.7	7.1
Horrat value = RSD_R/predicted RSD_R [(1)]	1.63	1.77	1.44	1.28	1.27	2.26	1.99	2.35	2.36	2.31	2.12	2.23

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

Table F 8. Statistical evaluation of determined CBE amounts in chocolate accepted on technical grounds (samples 2-6, chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet; samples 9-13, assumed fat content of chocolate = 30 %)

Sample number	2	4	5	6	9	10	11	12	13
Year of collaborative trial	2006								
Number of laboratories	12	12	12	12	12	12	12	12	12
Mean value, g CBE/100 g chocolate	0.58	1.90	5.20	4.07	0.48	4.62	5.81	5.35	8.23
True value, g CBE/100 g chocolate	0.45	2.03	5.11	3.95	0.60	4.81	5.99	6.02	8.41
Bias, g CBE/100 g chocolate	-0.13	0.13	-0.09	-0.12	0.12	0.19	0.18	0.67	0.18
Repeatability limit r [r=2.8 x s_r], g/100 g	0.19	0.24	0.28	0.28	0.23	0.26	0.19	0.19	0.23
Repeatability standard deviation s_r, g/100 g	0.07	0.09	0.10	0.10	0.08	0.09	0.07	0.07	0.08
Repeatability relative standard deviation RSD_r, %	11.6	4.5	1.9	2.4	16.8	2.0	1.2	1.2	1.0
Reproducibility limit R [r=2.8 x s_R], g/100 g	0.52	0.56	0.59	0.54	0.39	0.50	0.46	0.53	0.52
Reproducibility standard deviation s_R, g/100 g	0.19	0.20	0.21	0.19	0.14	0.18	0.17	0.19	0.19
Reproducibility relative standard deviation RSD_R, %	31.8	10.5	4.1	4.7	28.7	3.8	2.8	3.5	2.2
Horrat value = RSD_R/predicted RSD_R [(1)]	7.34	2.89	1.30	1.46	6.44	1.21	0.93	1.13	0.77

[(1)] predicted $RSD_R = 2C^{-0.15}$; C = estimated mean concentration

ANNEX G – MEAN & RANGE PLOTS

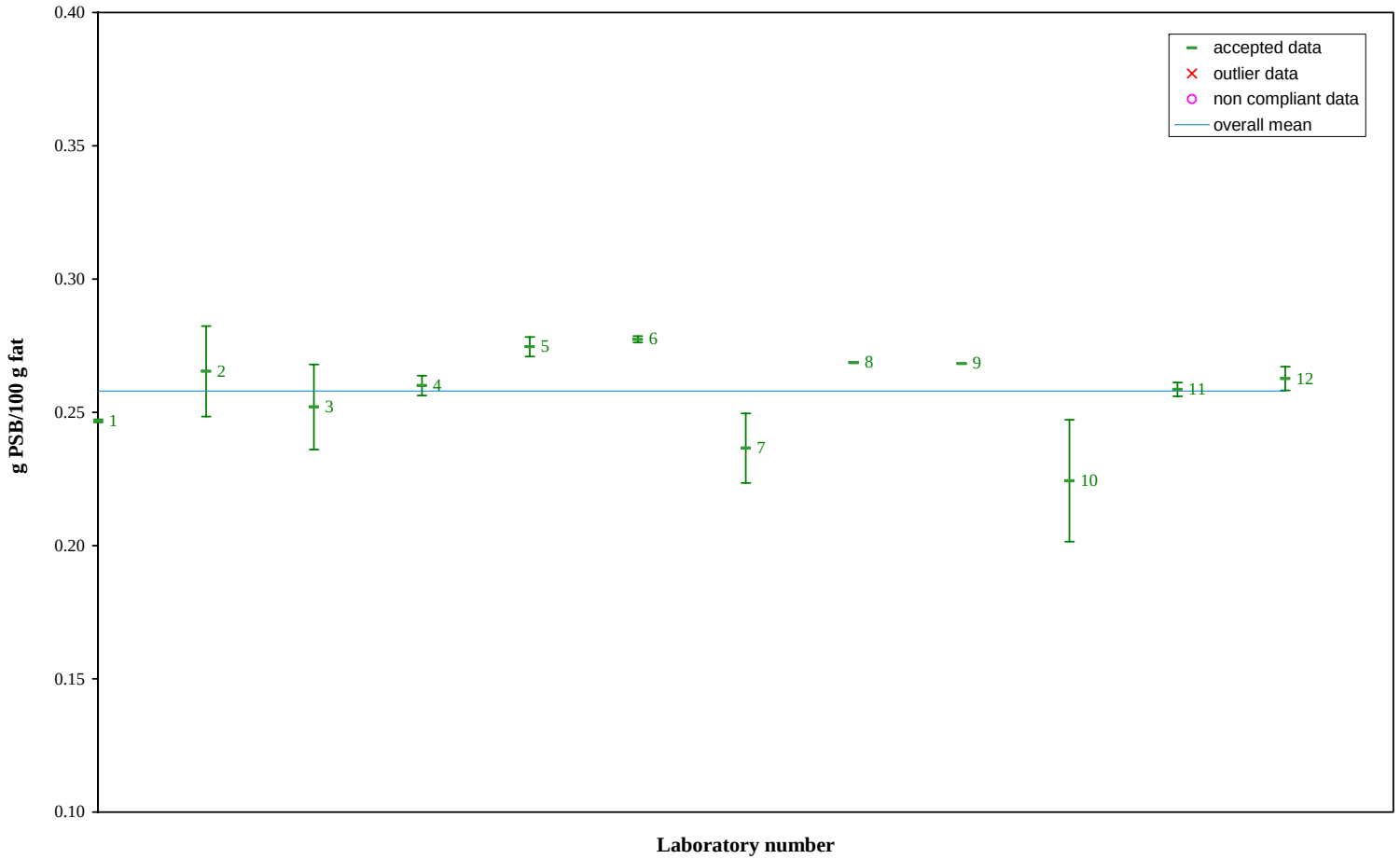


Figure G 1. Sample 1: Laboratory means and ranges of determined PSB amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

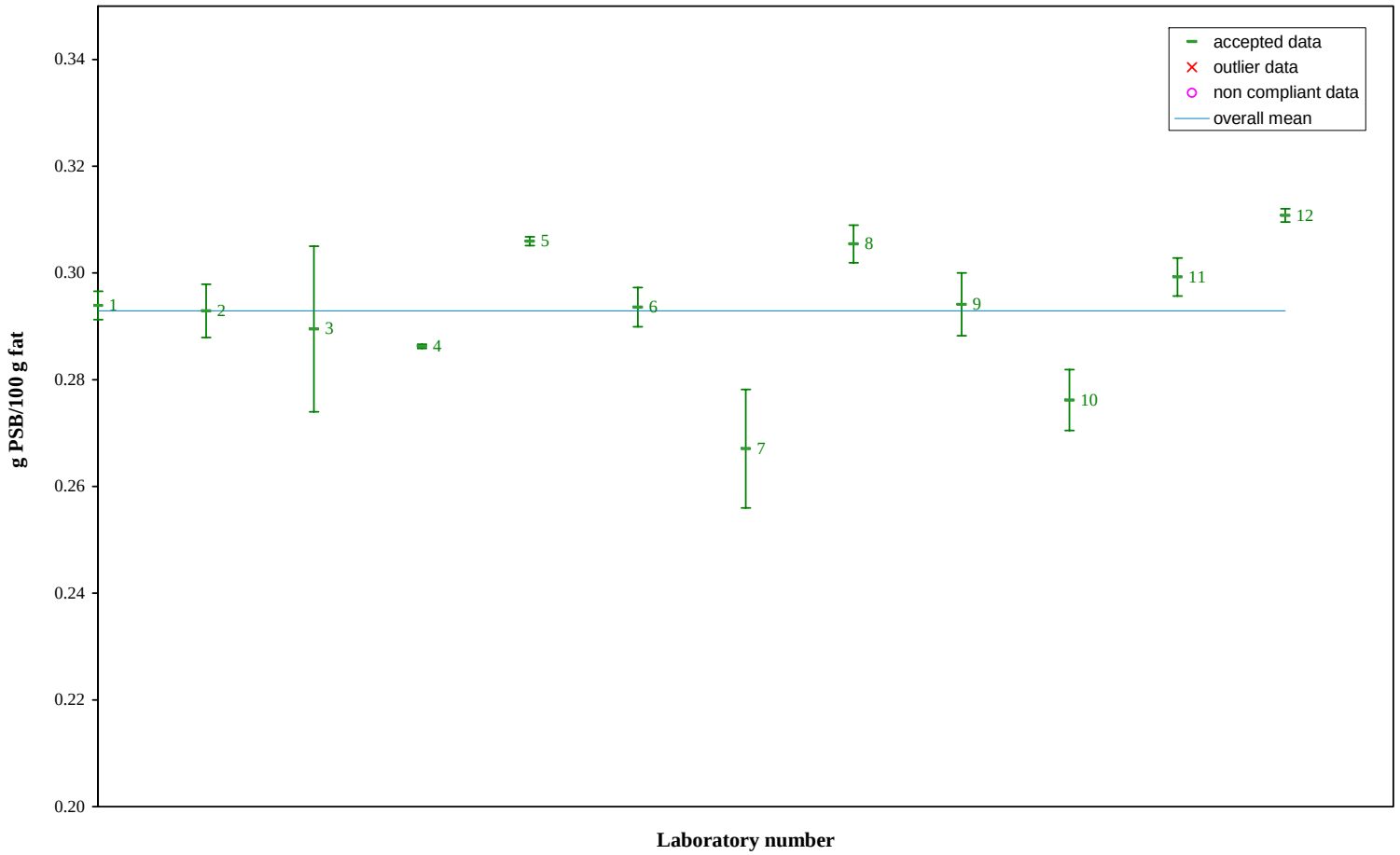


Figure G 2. Sample 2: Laboratory means and ranges of determined PSB amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

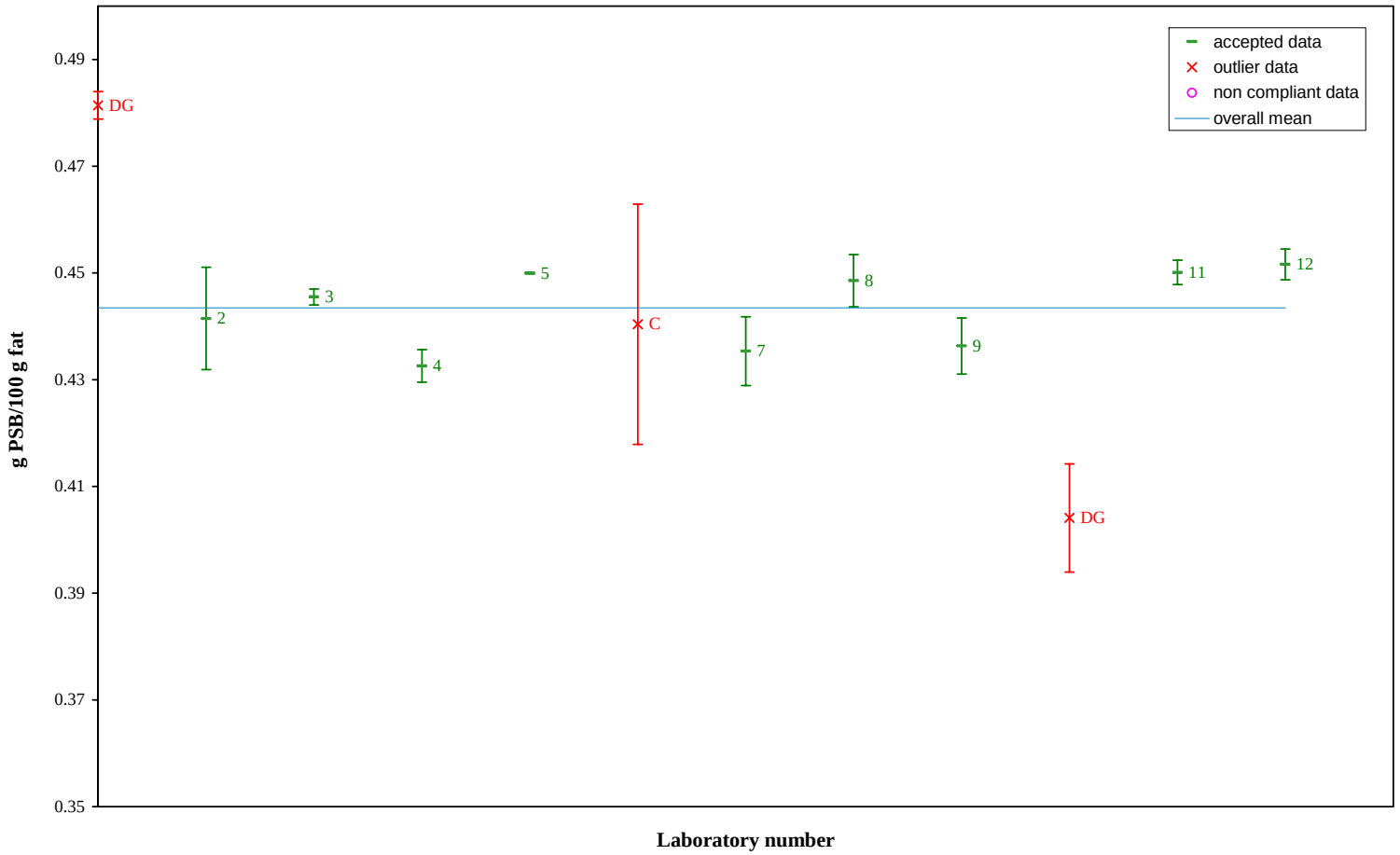


Figure G 3. Sample 3: Laboratory means and ranges of determined PSB amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

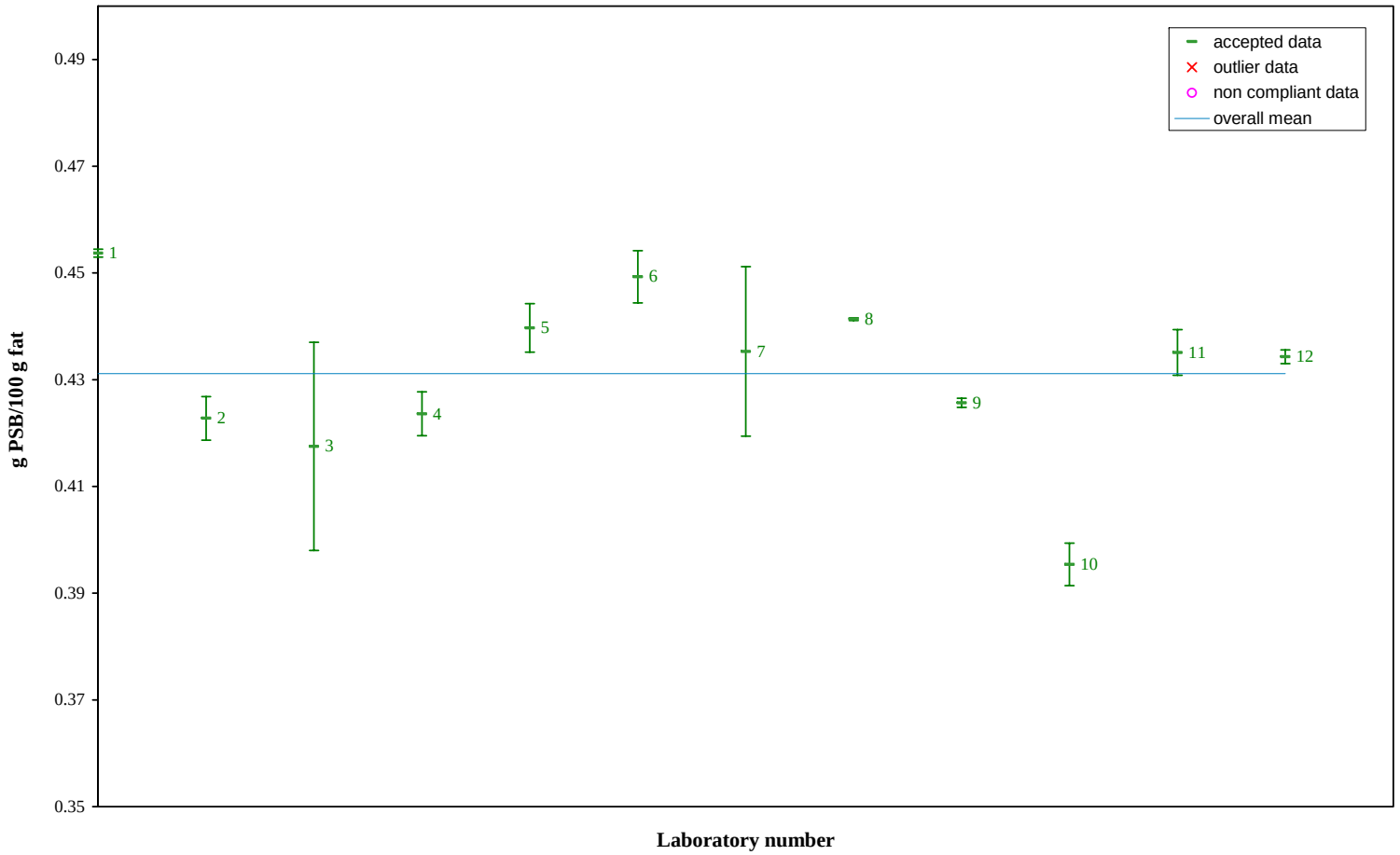


Figure G 4. Sample 4: Laboratory means and ranges of determined PSB amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

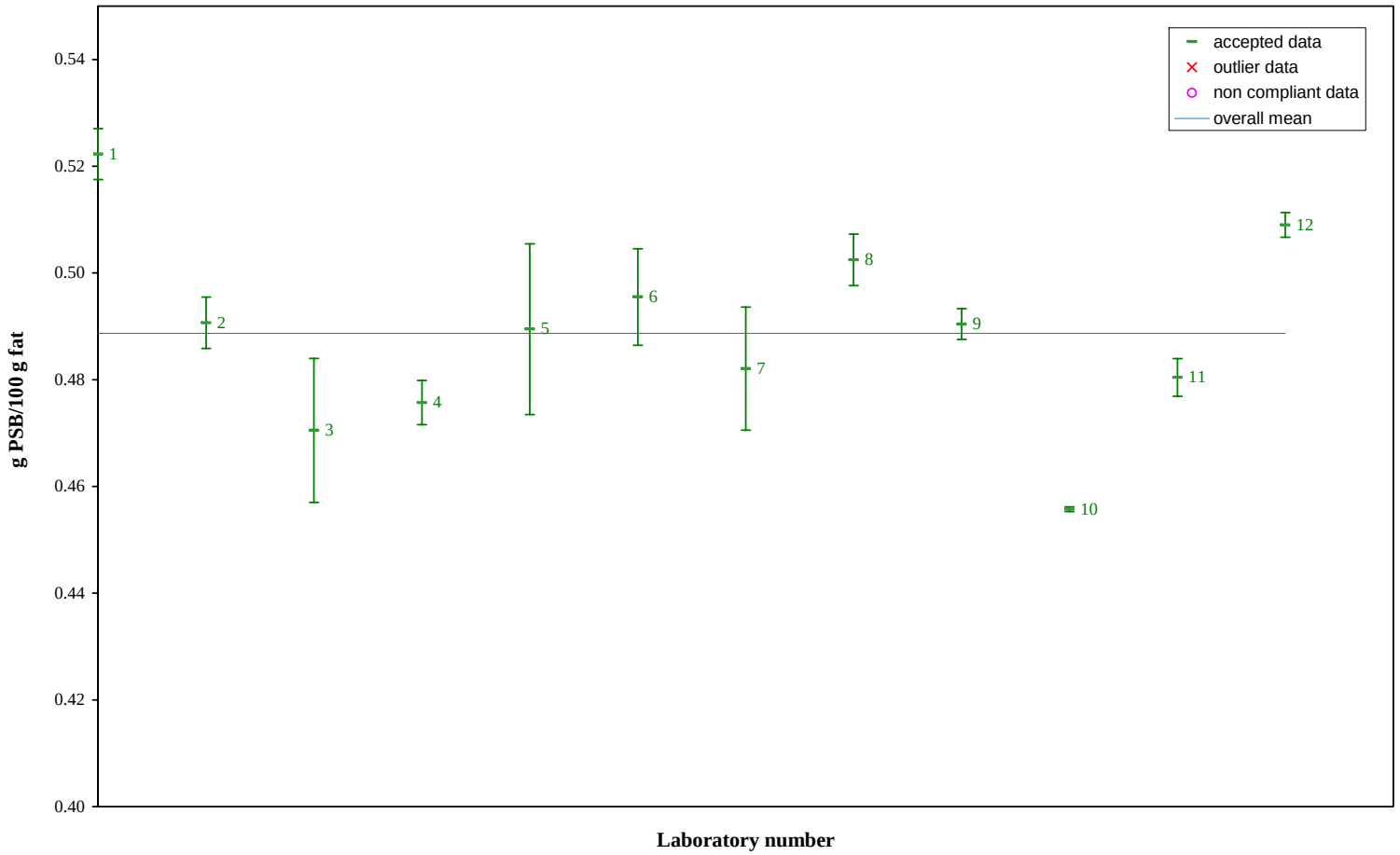


Figure G 5. Sample 5: Laboratory means and ranges of determined PSB amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

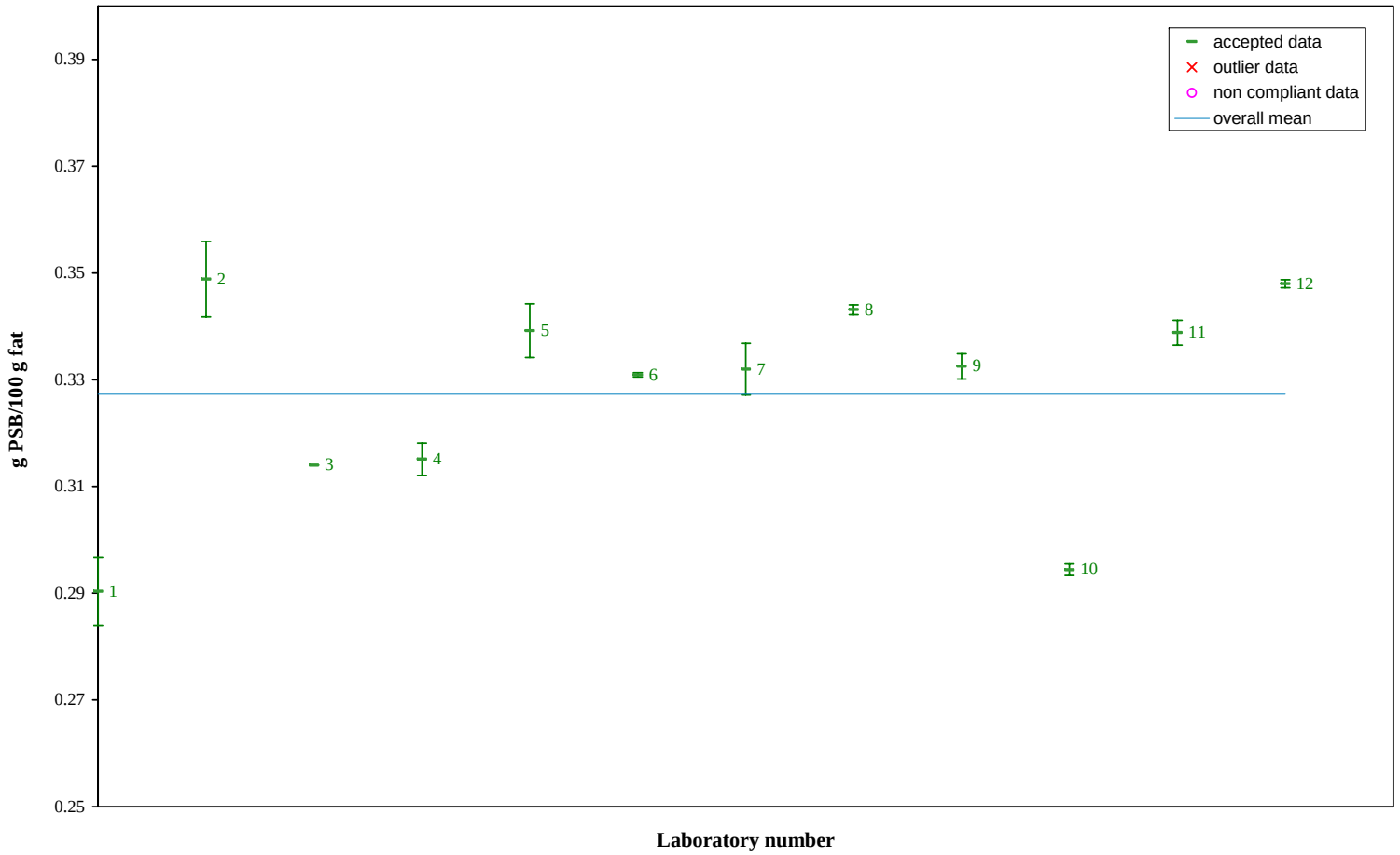


Figure G 6. Sample 6: Laboratory means and ranges of determined PSB amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

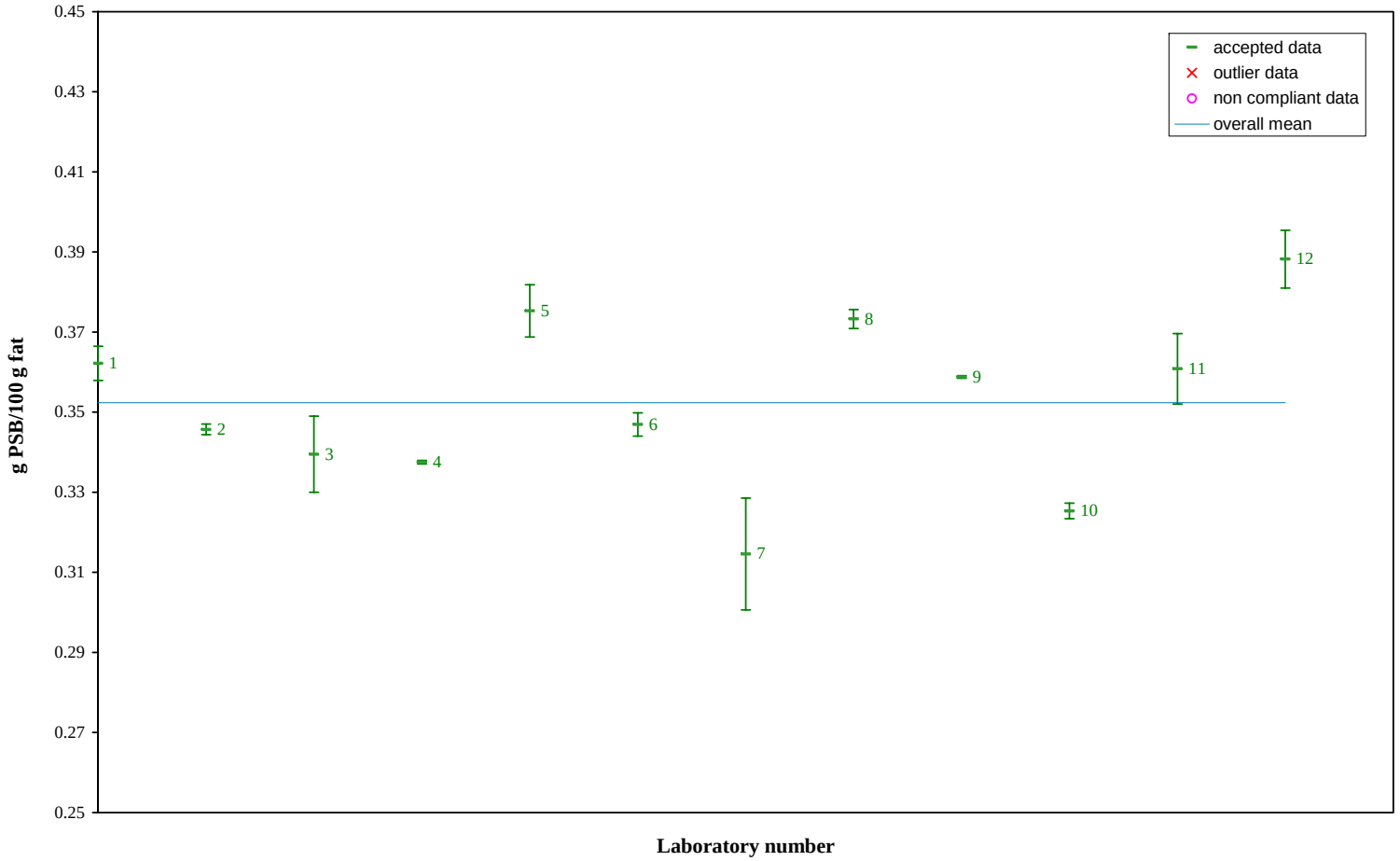


Figure G 7. Sample 8: Laboratory means and ranges of determined PSB amounts in chocolate fat (blind duplicates)

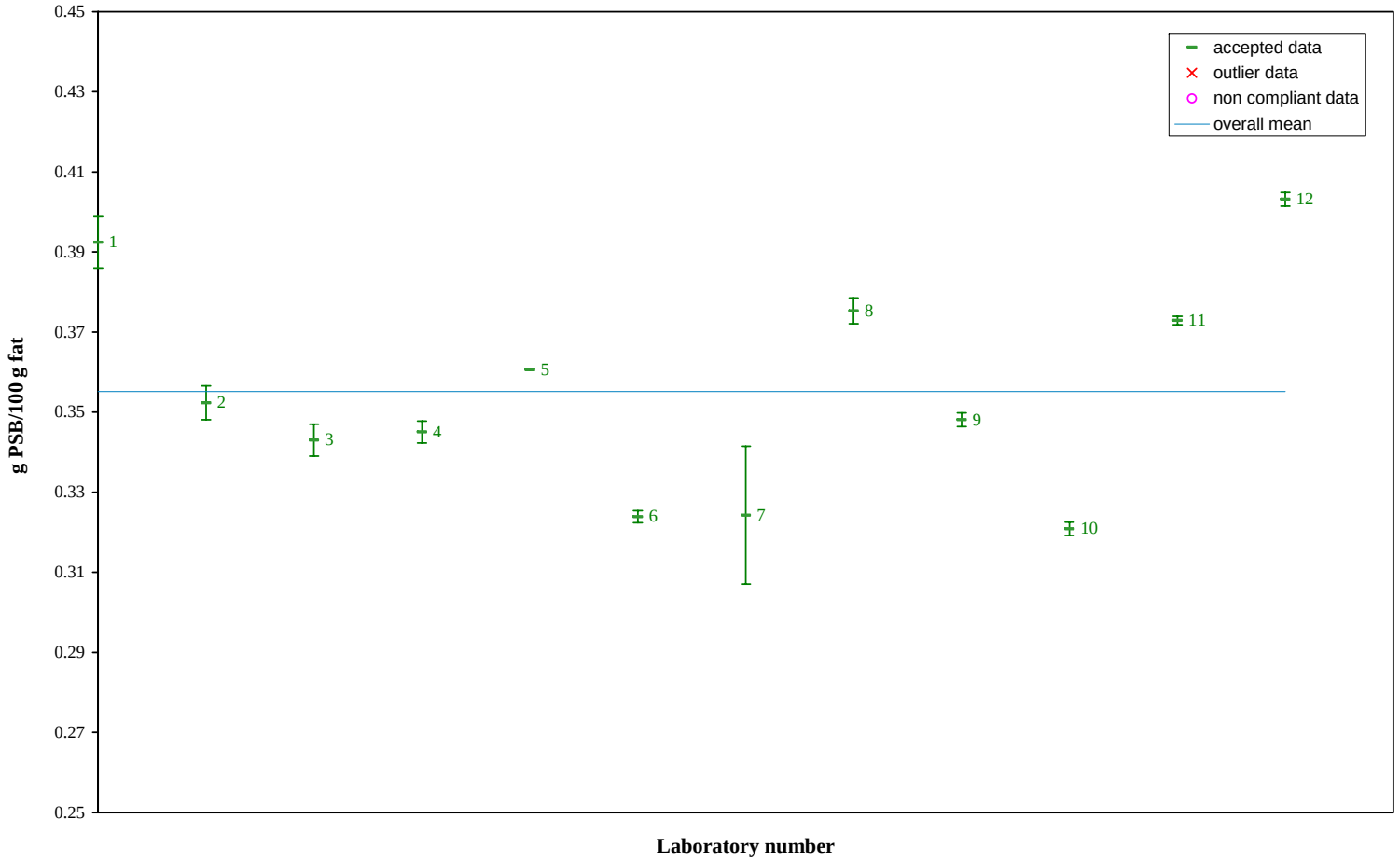


Figure G 8. Sample 9: Laboratory means and ranges of determined PSB amounts in chocolate fat (blind duplicates)

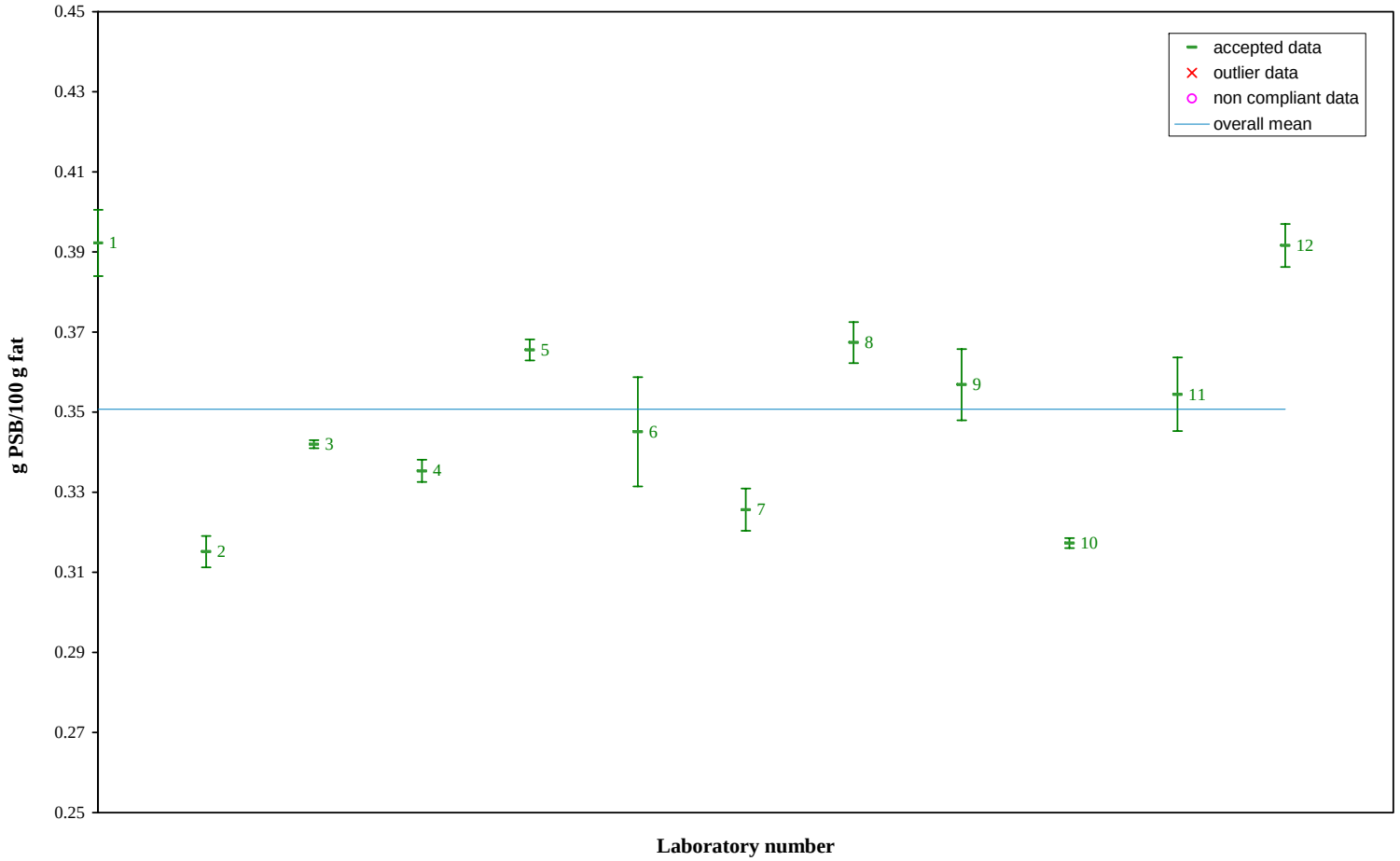


Figure G 9. Sample 10: Laboratory means and ranges of determined PSB amounts in chocolate fat (blind duplicates)

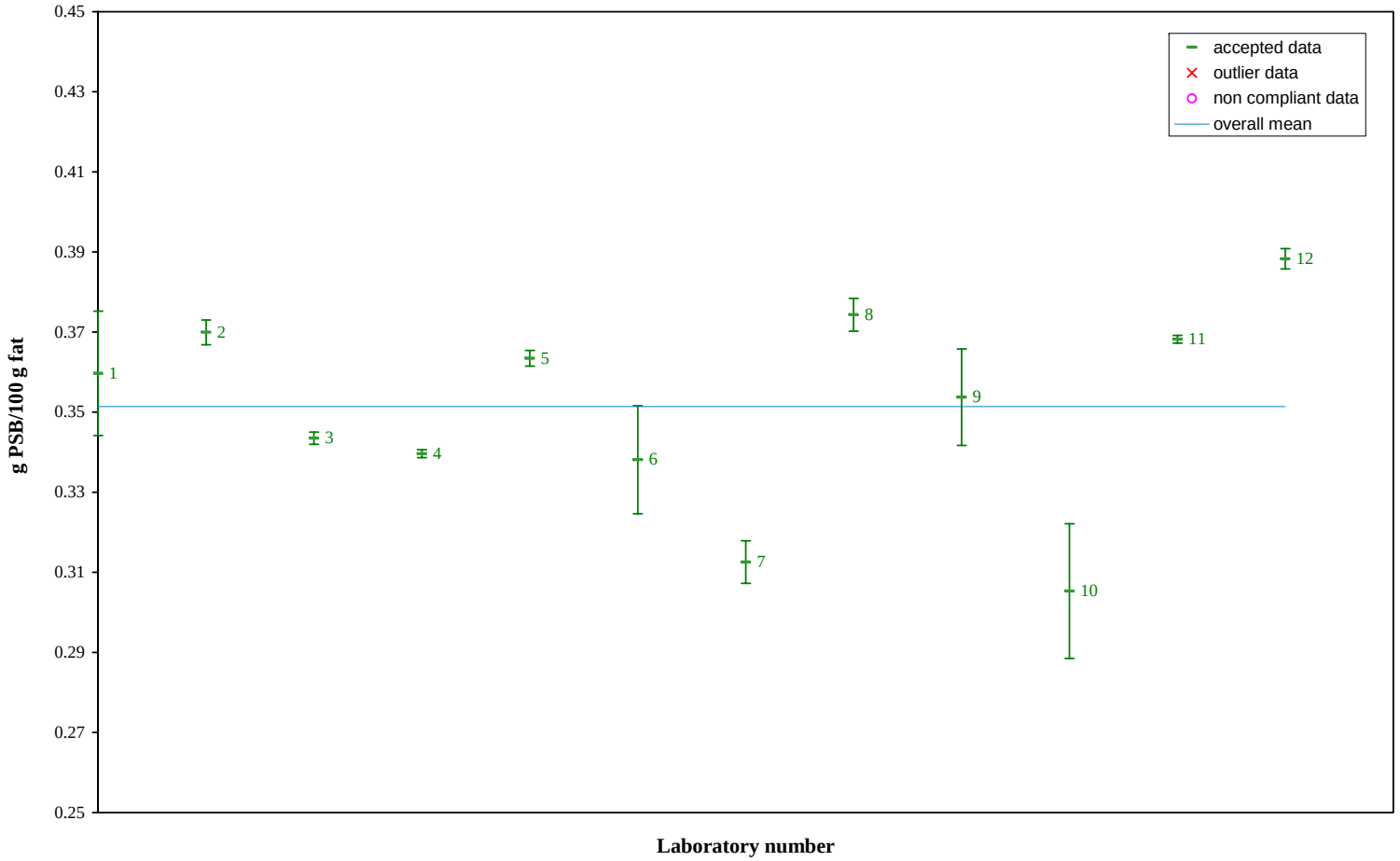


Figure G 10. Sample 11: Laboratory means and ranges of determined PSB amounts in chocolate fat (blind duplicates)

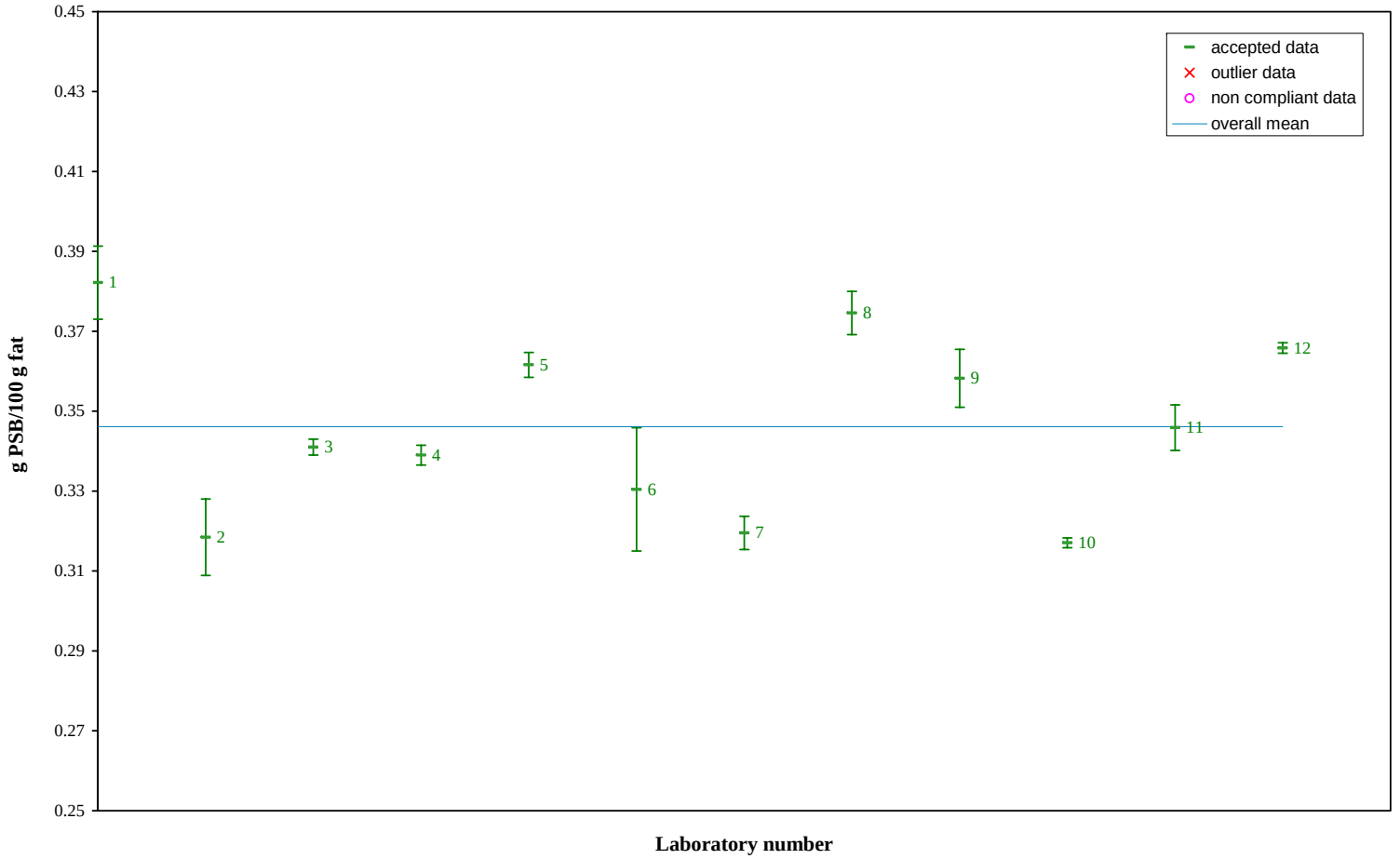


Figure G 11. Sample 12: Laboratory means and ranges of determined PSB amounts in chocolate fat (blind duplicates)

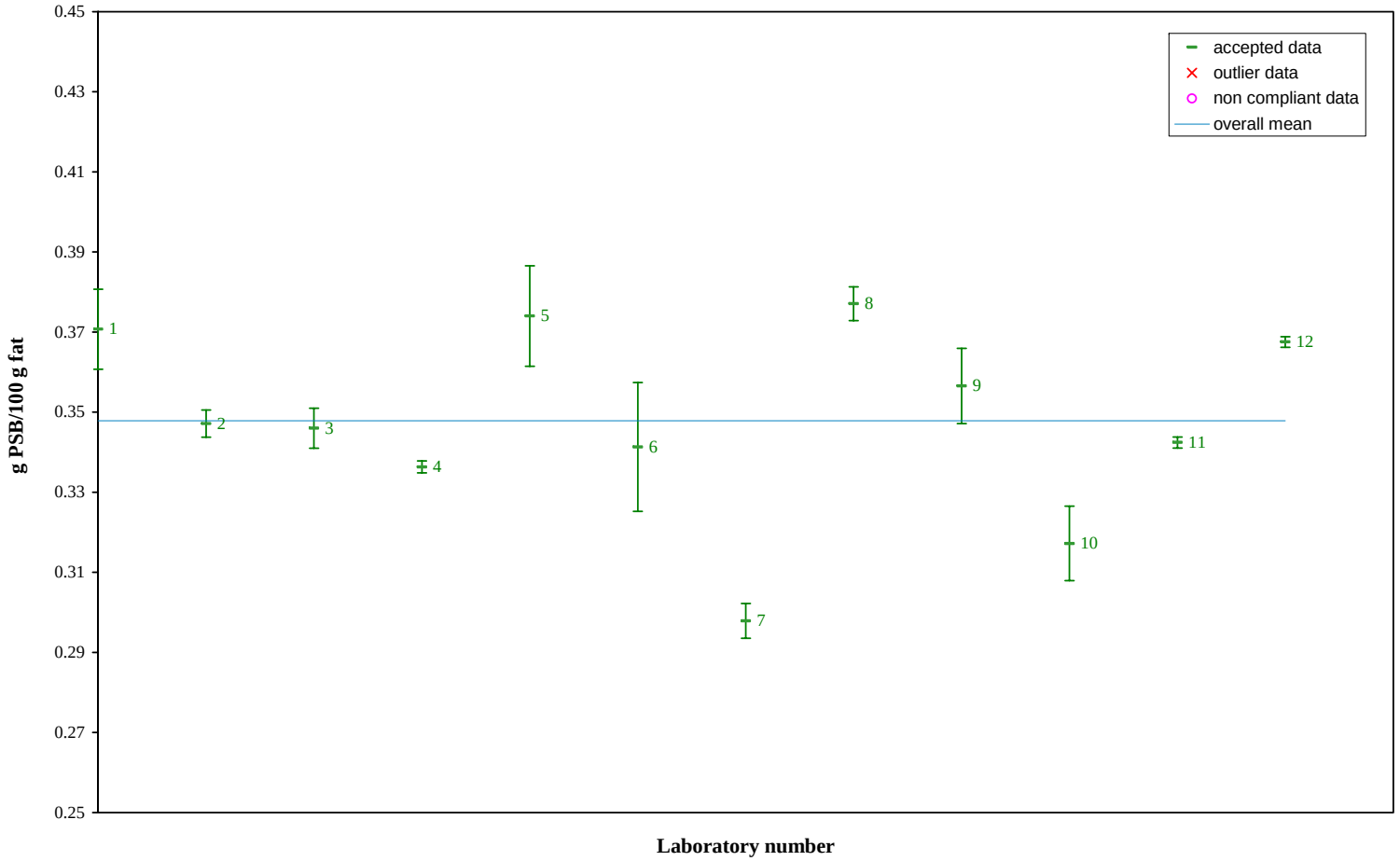


Figure G 12. Sample 13: Laboratory means and ranges of determined PSB amounts in chocolate fat (blind duplicates)

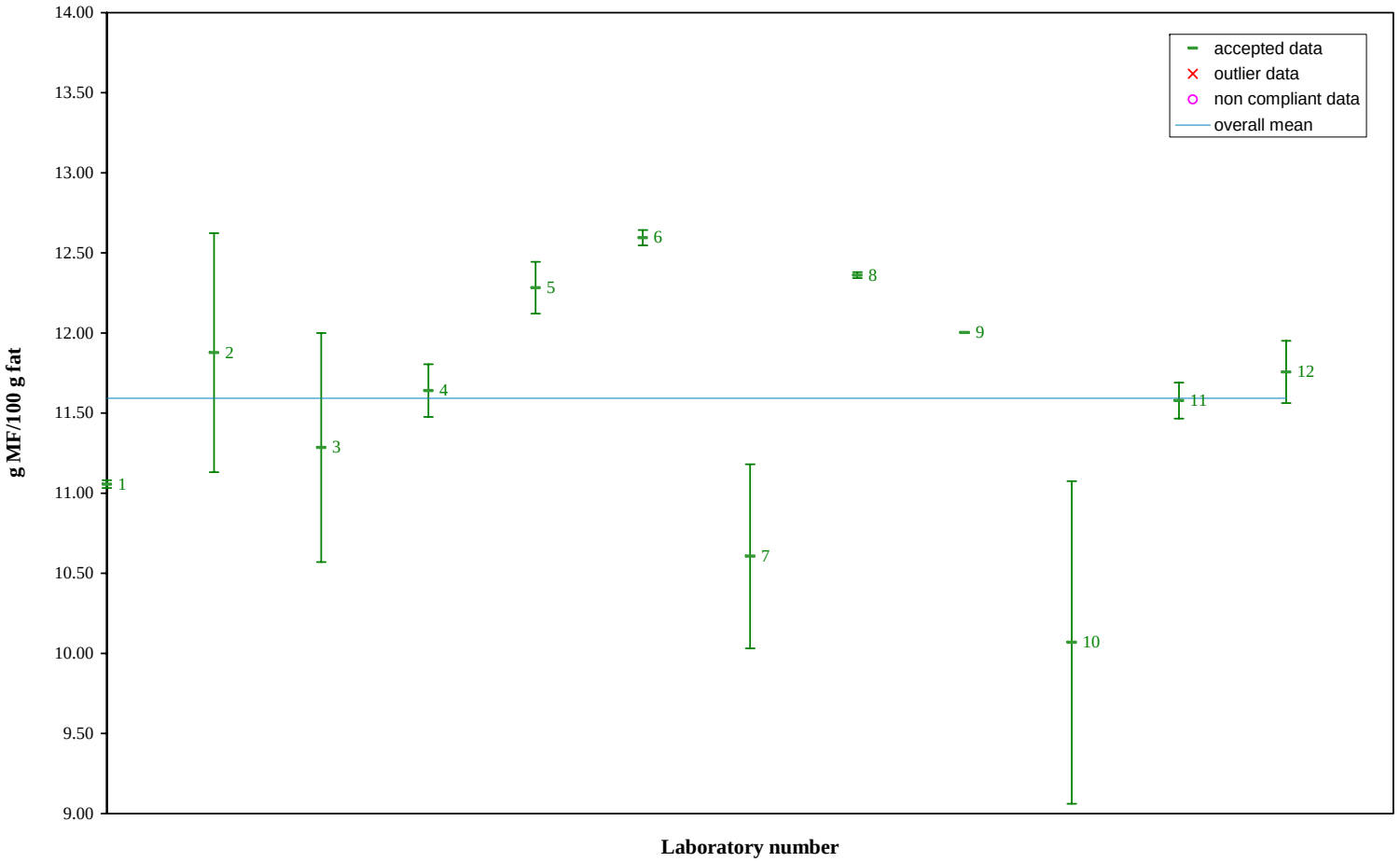


Figure G 13. Sample 1: Laboratory means and ranges of determined MF amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

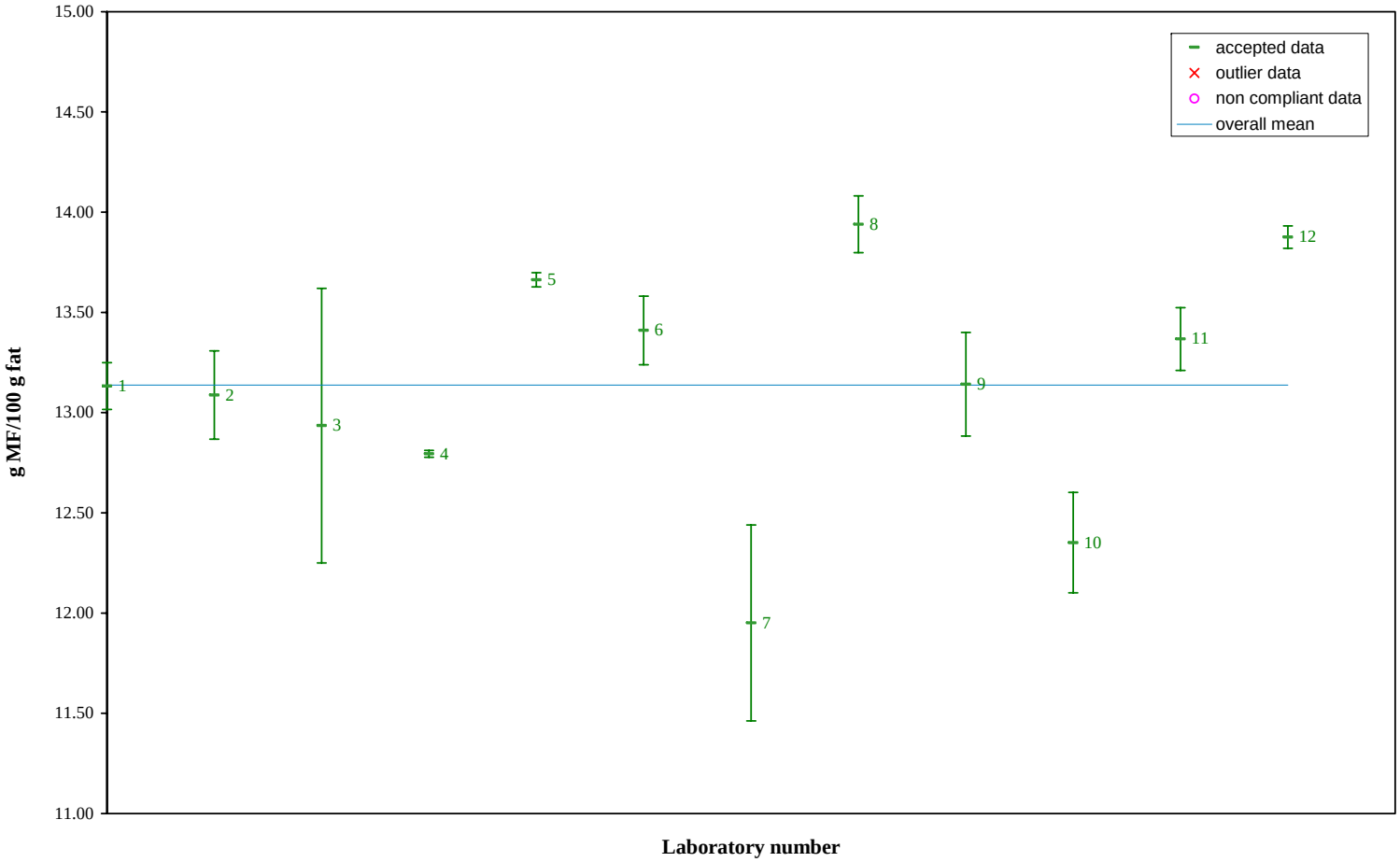


Figure G 14. Sample 2: Laboratory means and ranges of determined MF amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

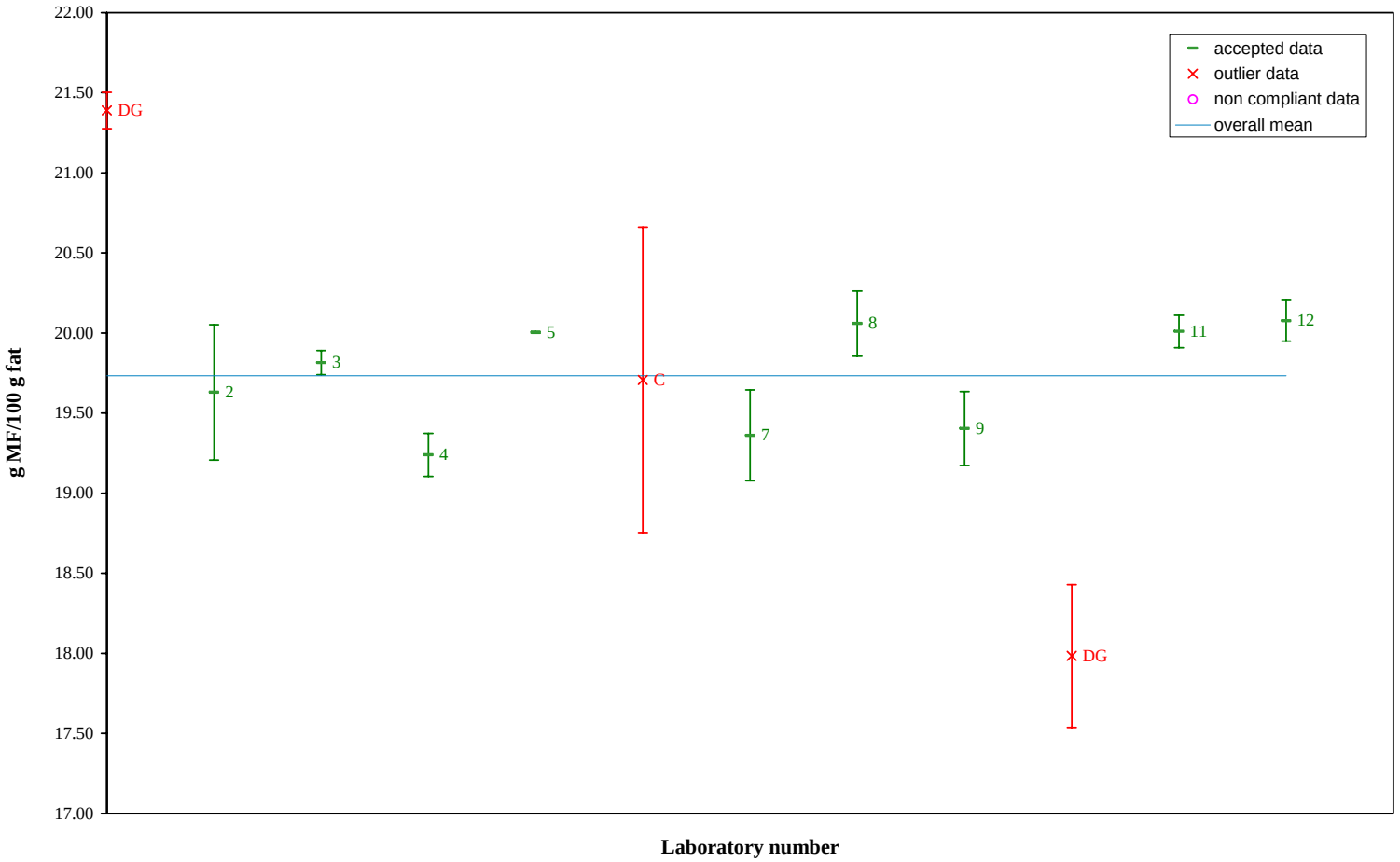


Figure G 15. Sample 3: Laboratory means and ranges of determined MF amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

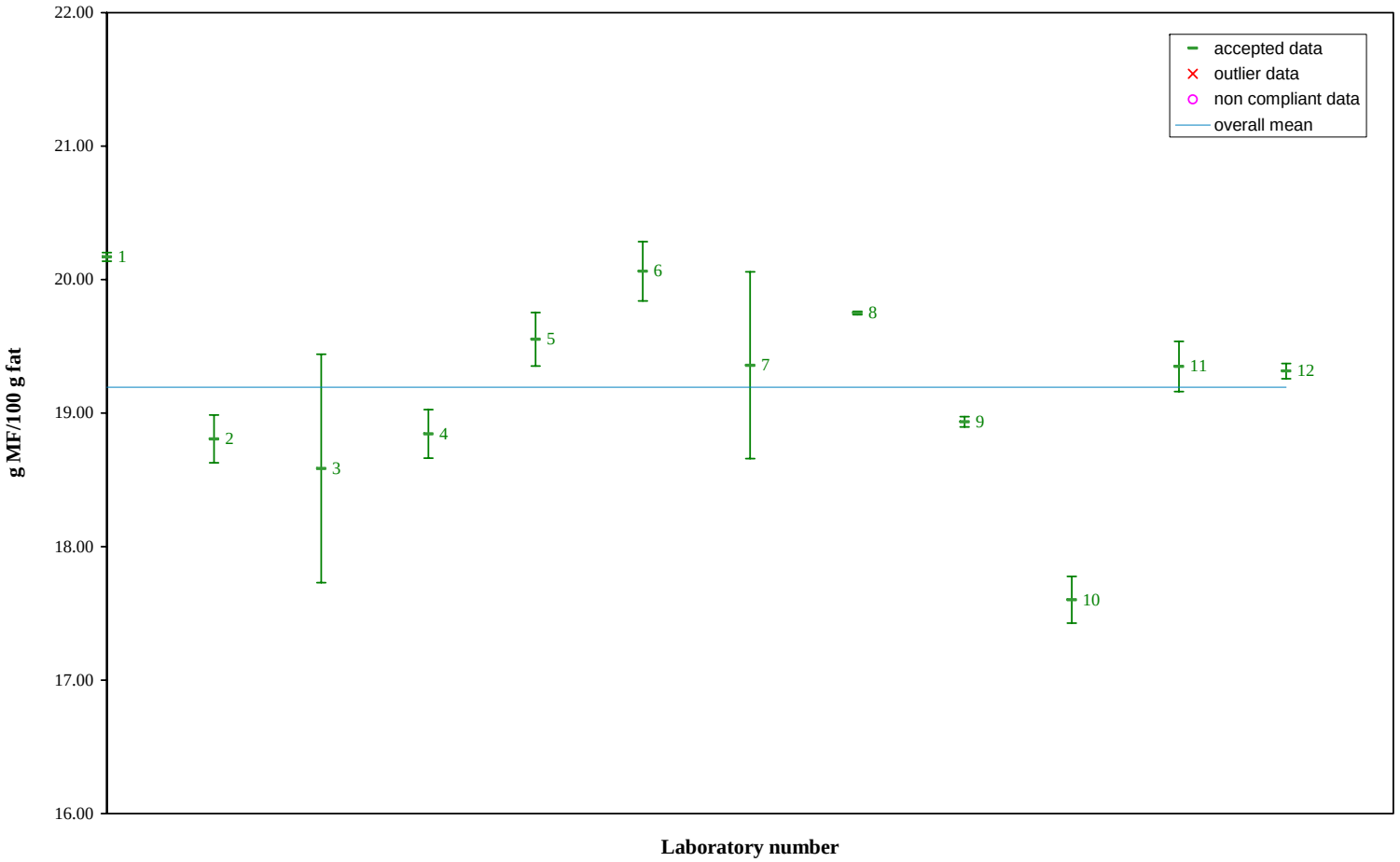


Figure G 16. Sample 4: Laboratory means and ranges of determined MF amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

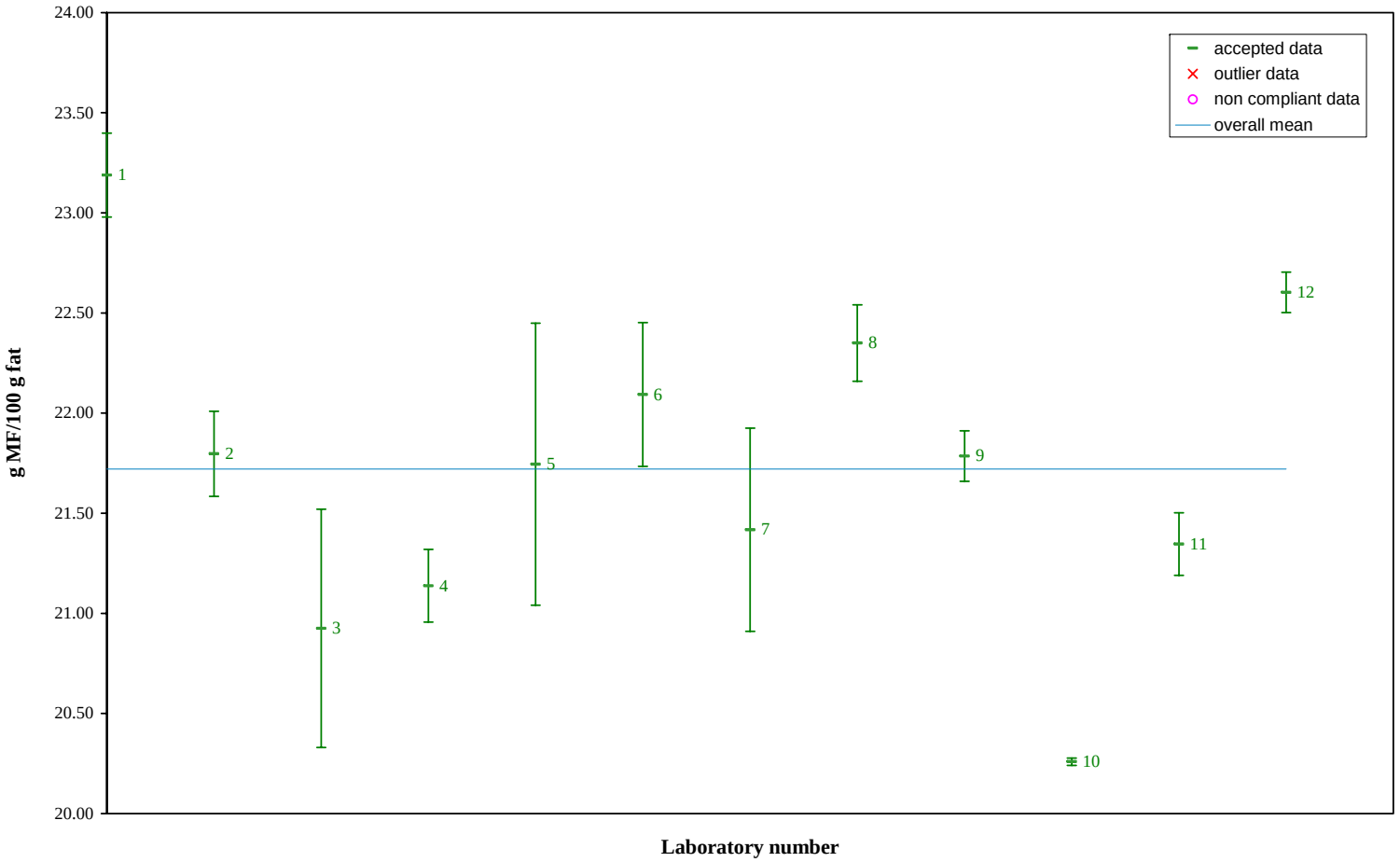


Figure G 17. Sample 5: Laboratory means and ranges of determined MF amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

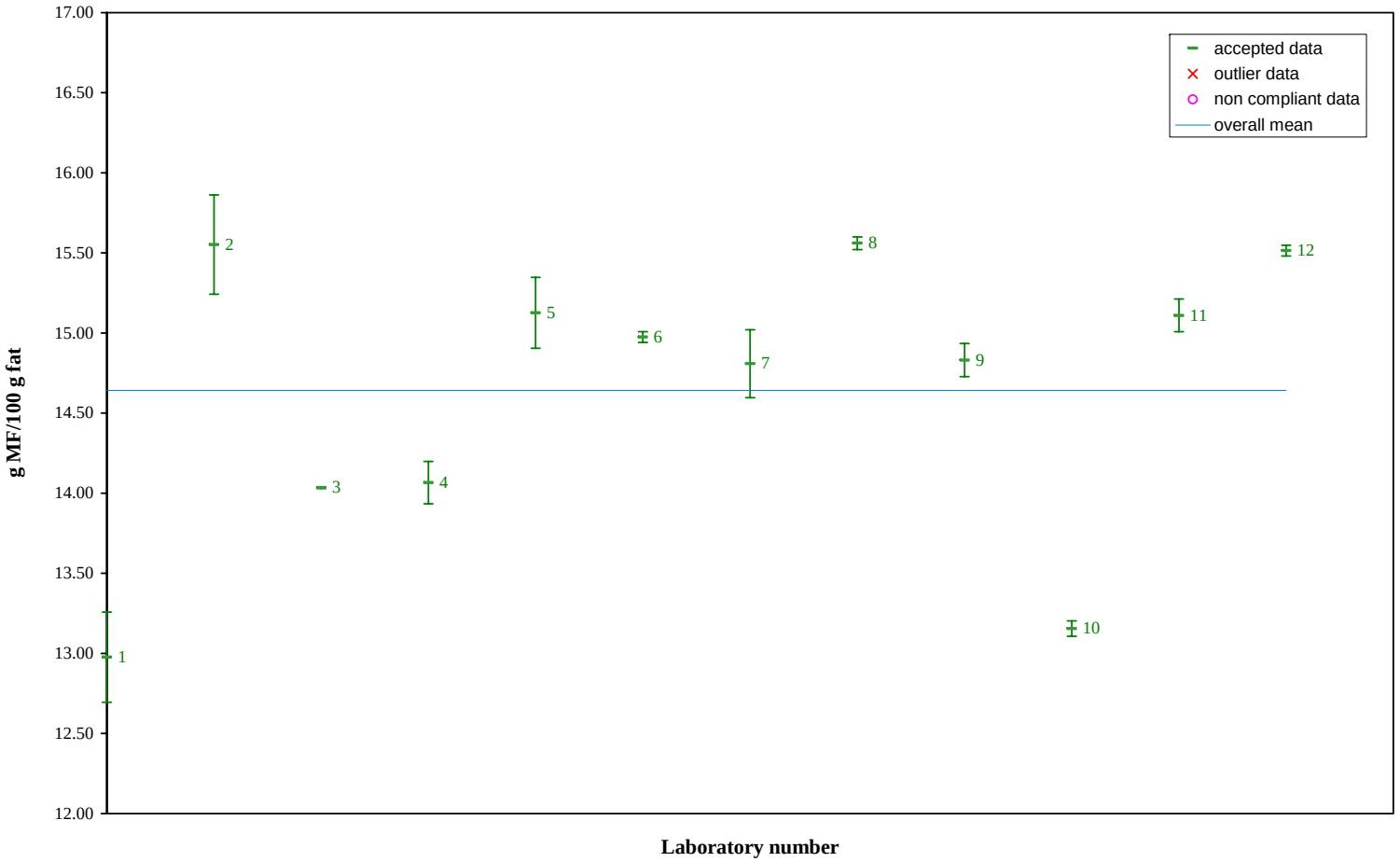


Figure G 18. Sample 6: Laboratory means and ranges of determined MF amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

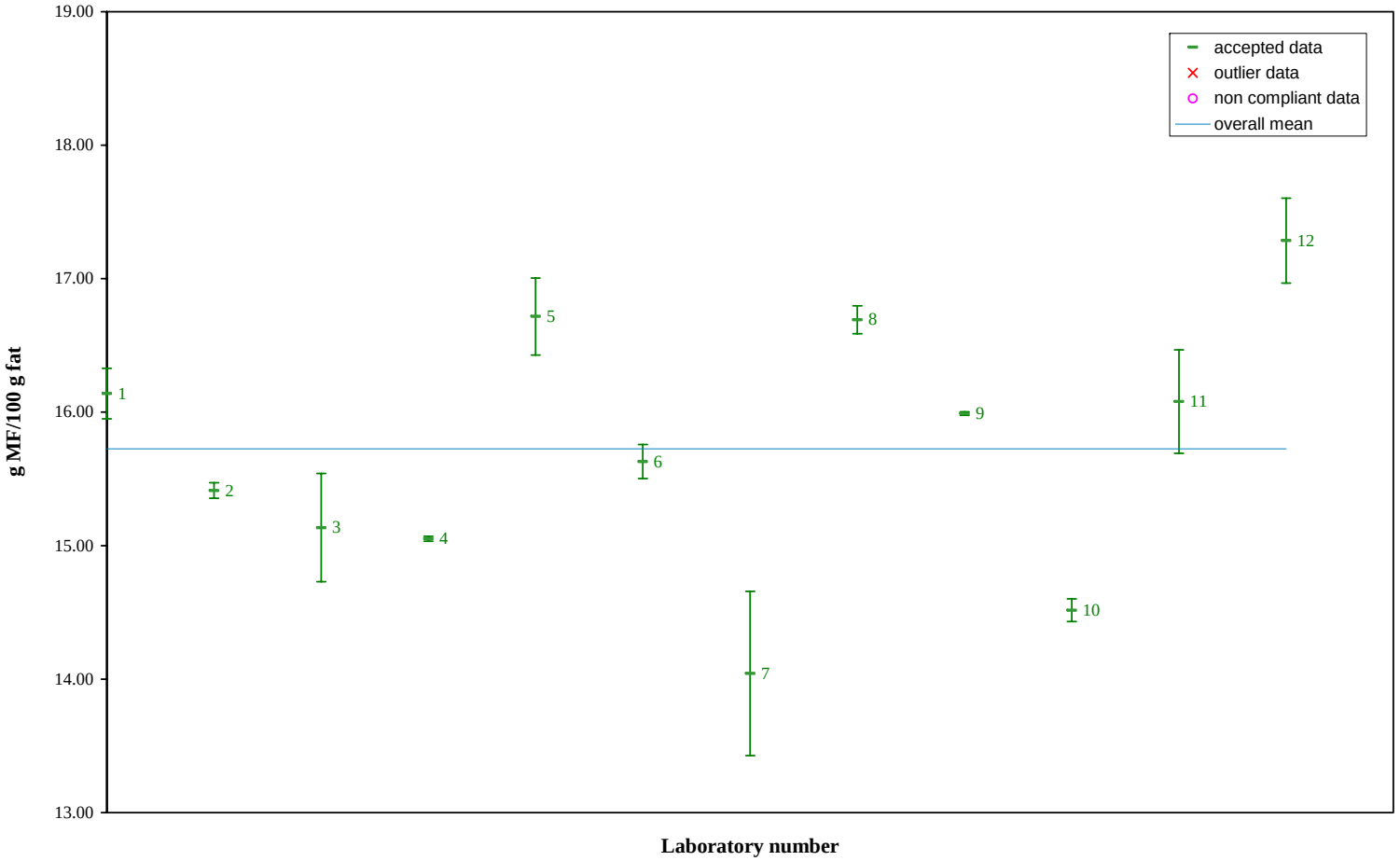


Figure G 19. Sample 8: Laboratory means and ranges of determined MF amounts in chocolate fat (blind duplicates)

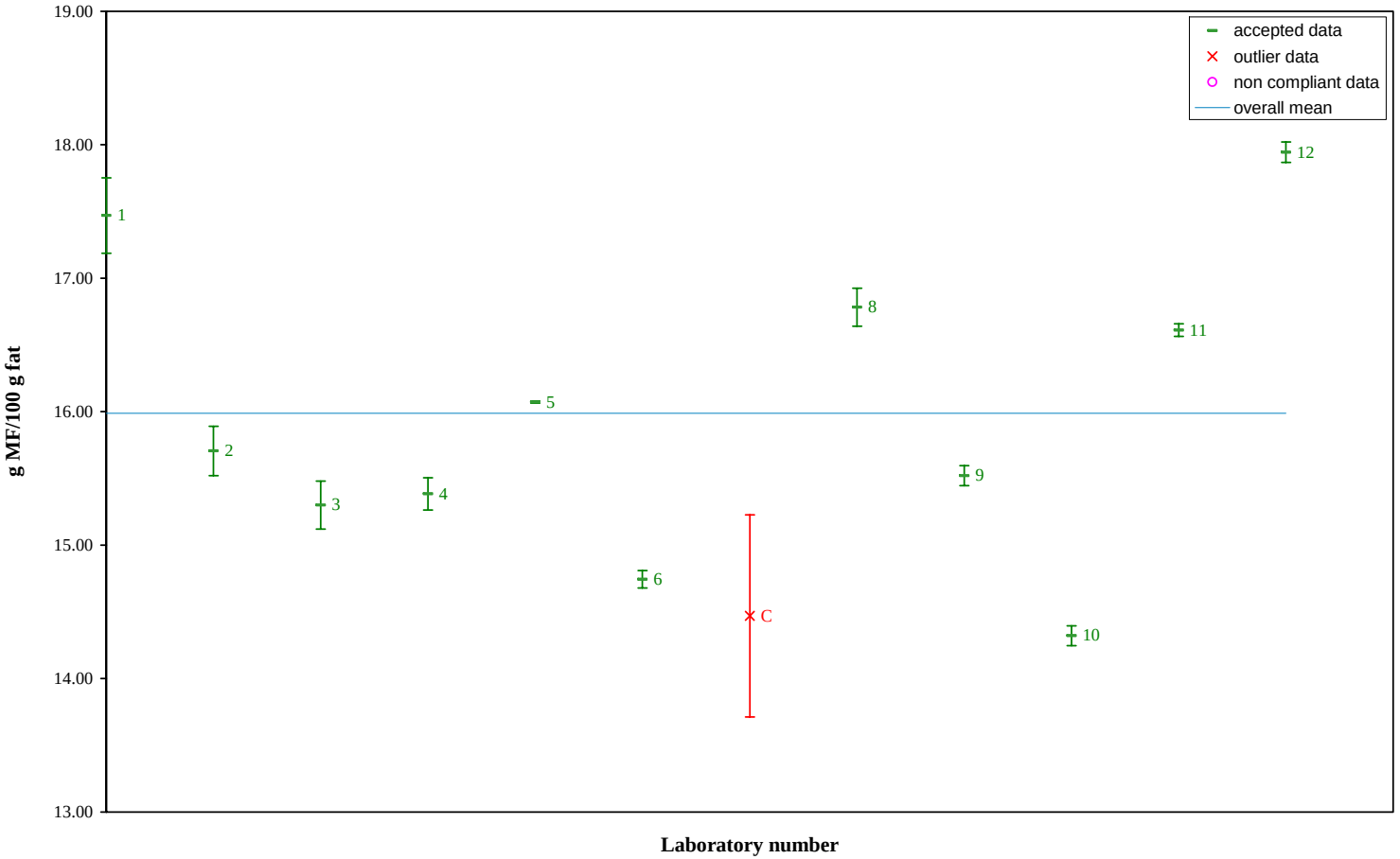


Figure G 20. Sample 9: Laboratory means and ranges of determined MF amounts in chocolate fat (blind duplicates)

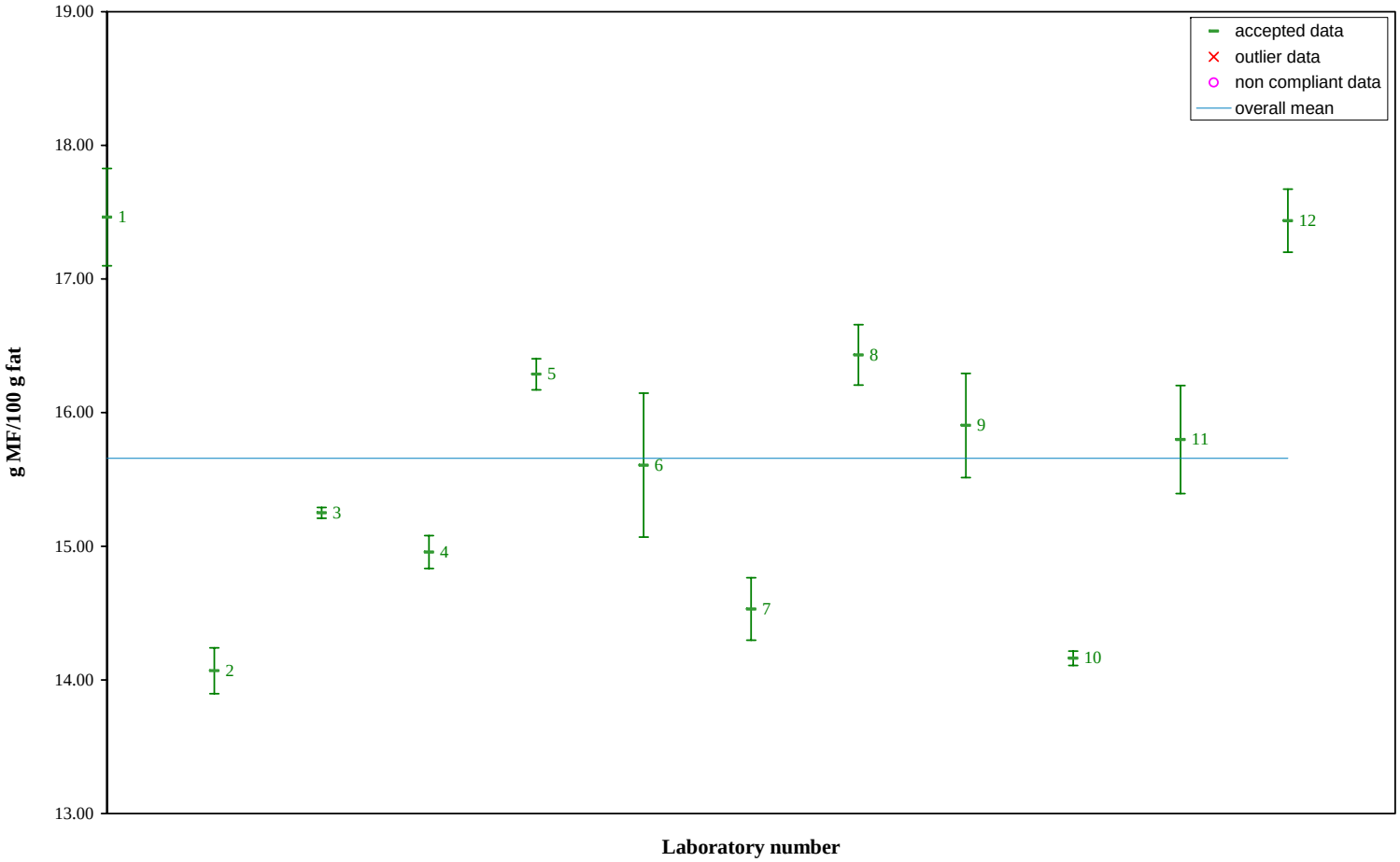


Figure G 21. Sample 10: Laboratory means and ranges of determined MF amounts in chocolate fat (blind duplicates)

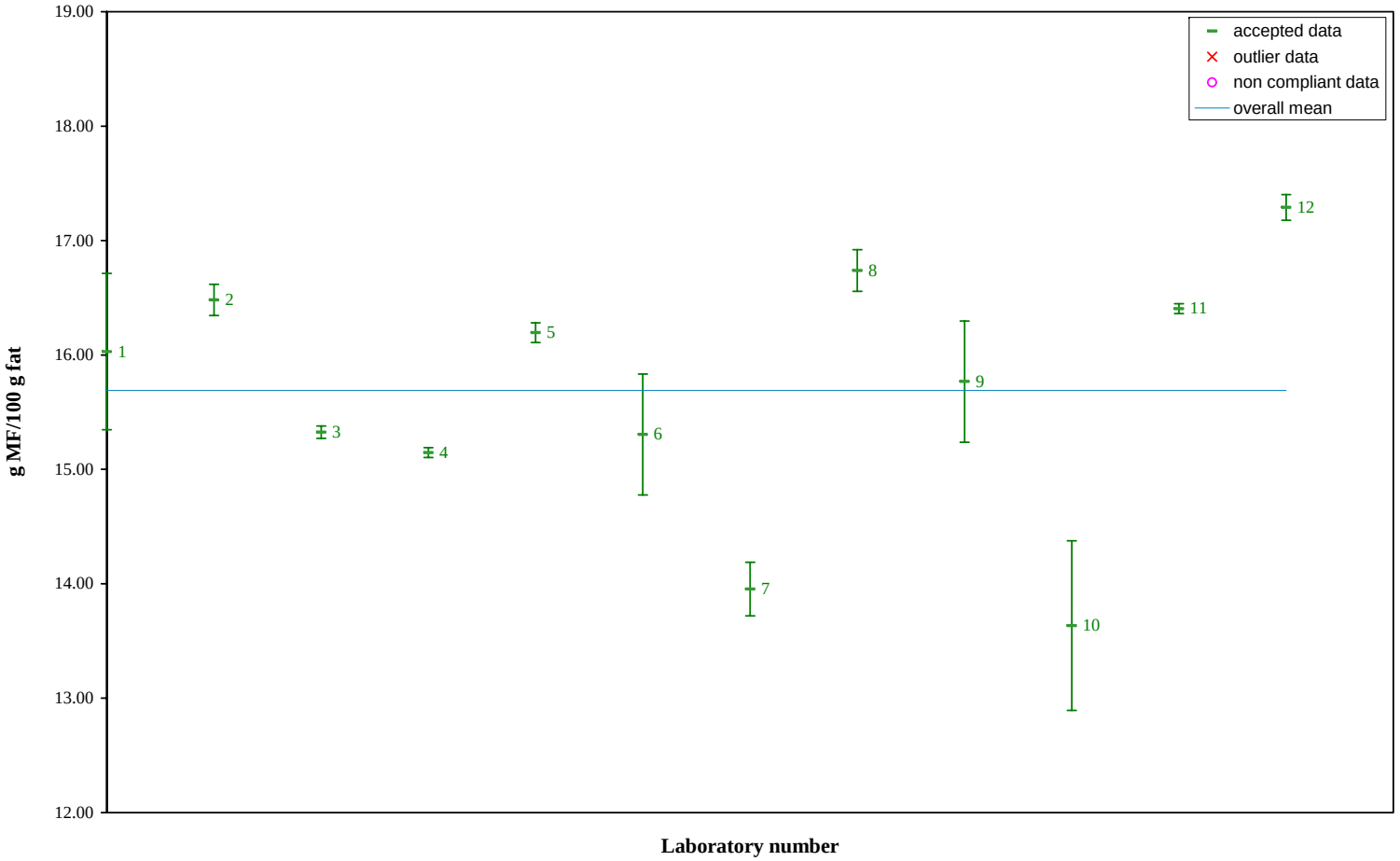


Figure G 22: Sample 11: Laboratory means and ranges of determined MF amounts in chocolate fat (blind duplicates)

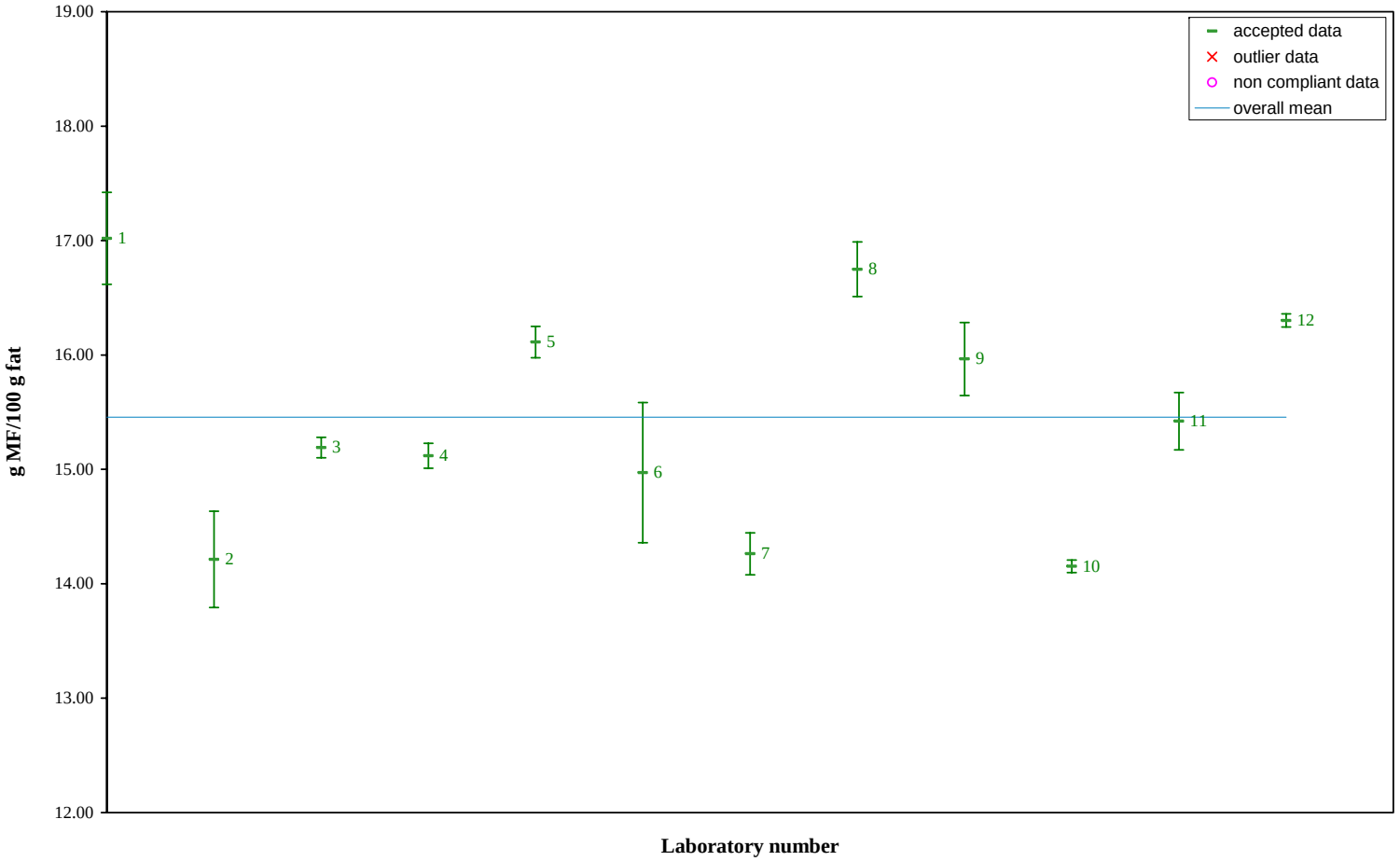


Figure G 23. Sample 12: Laboratory means and ranges of determined MF amounts in chocolate fat (blind duplicates)

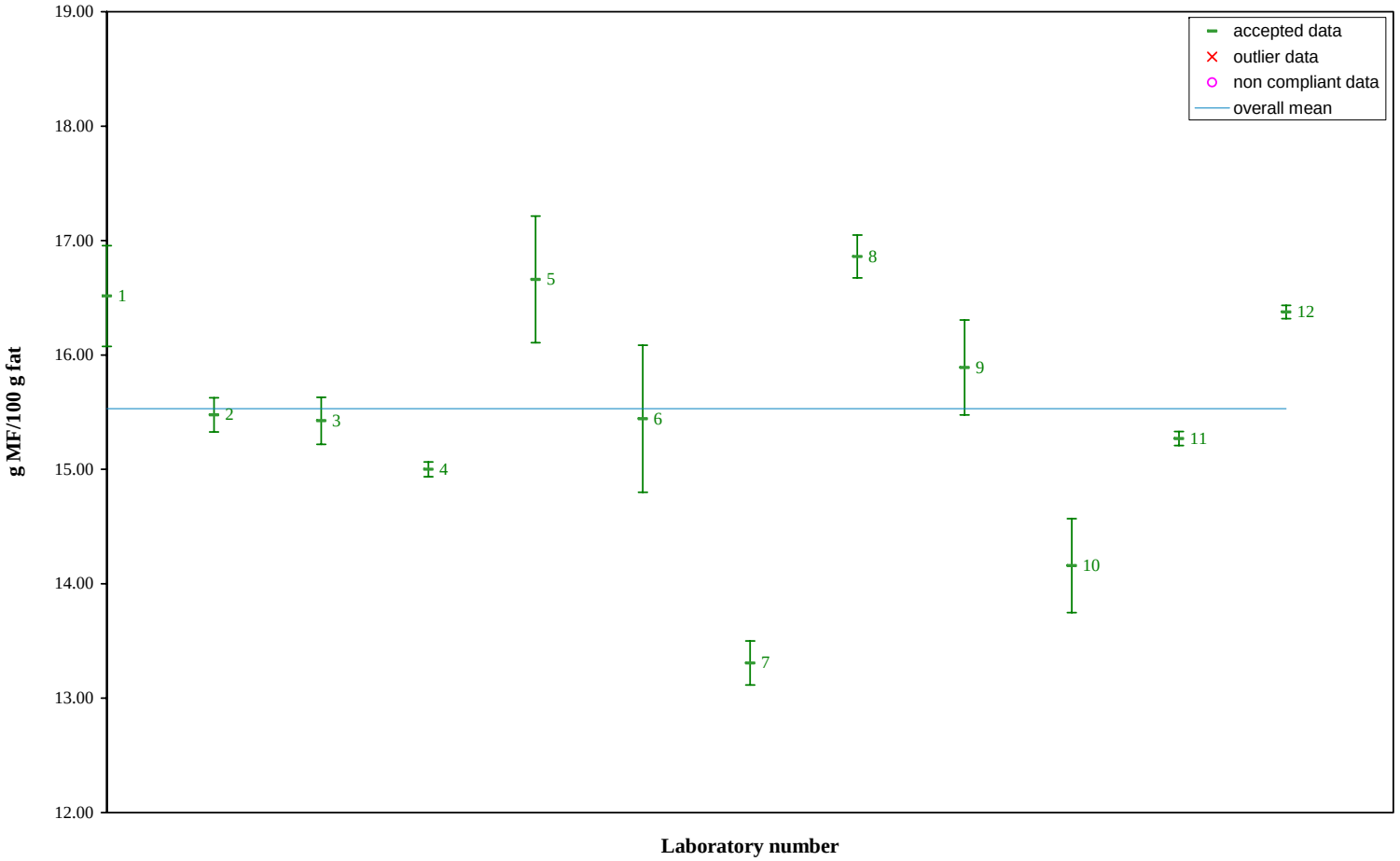


Figure G 24. Sample 13: Laboratory means and ranges of determined MF amounts in chocolate fat (blind duplicates)

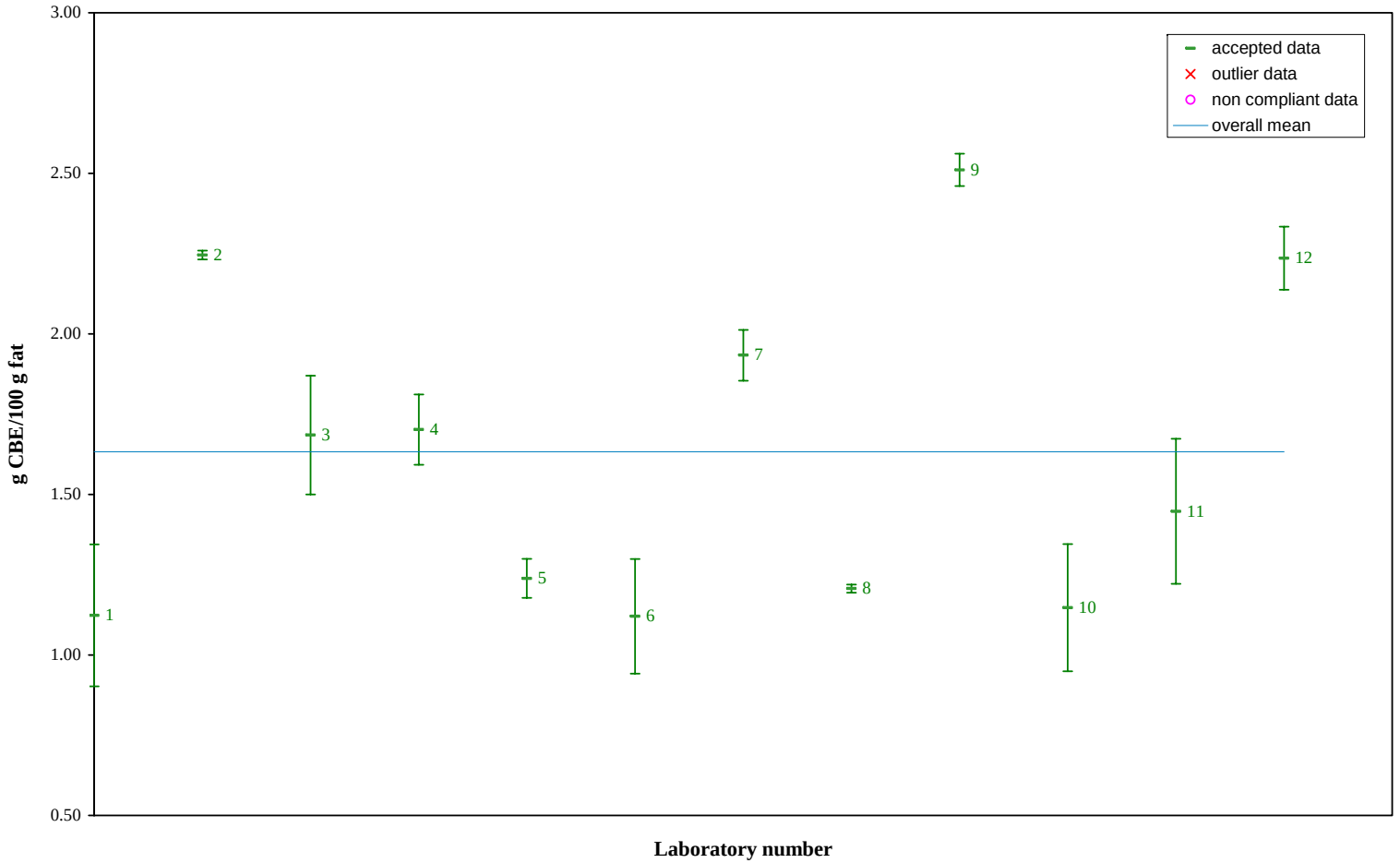


Figure G 25. Sample 2: Laboratory means and ranges of determined CBE amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

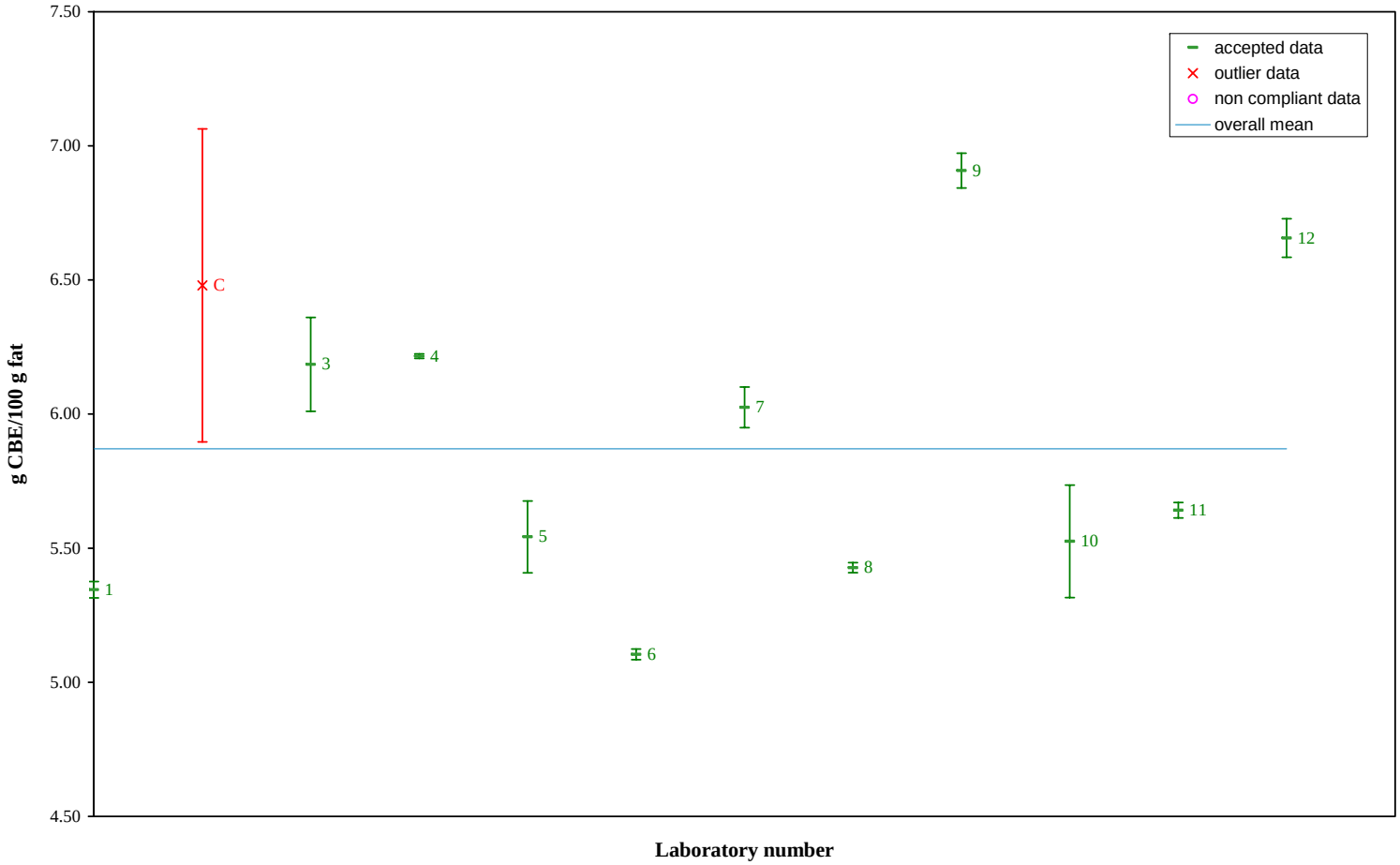


Figure G 26. Sample 4: Laboratory means and ranges of determined CBE amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

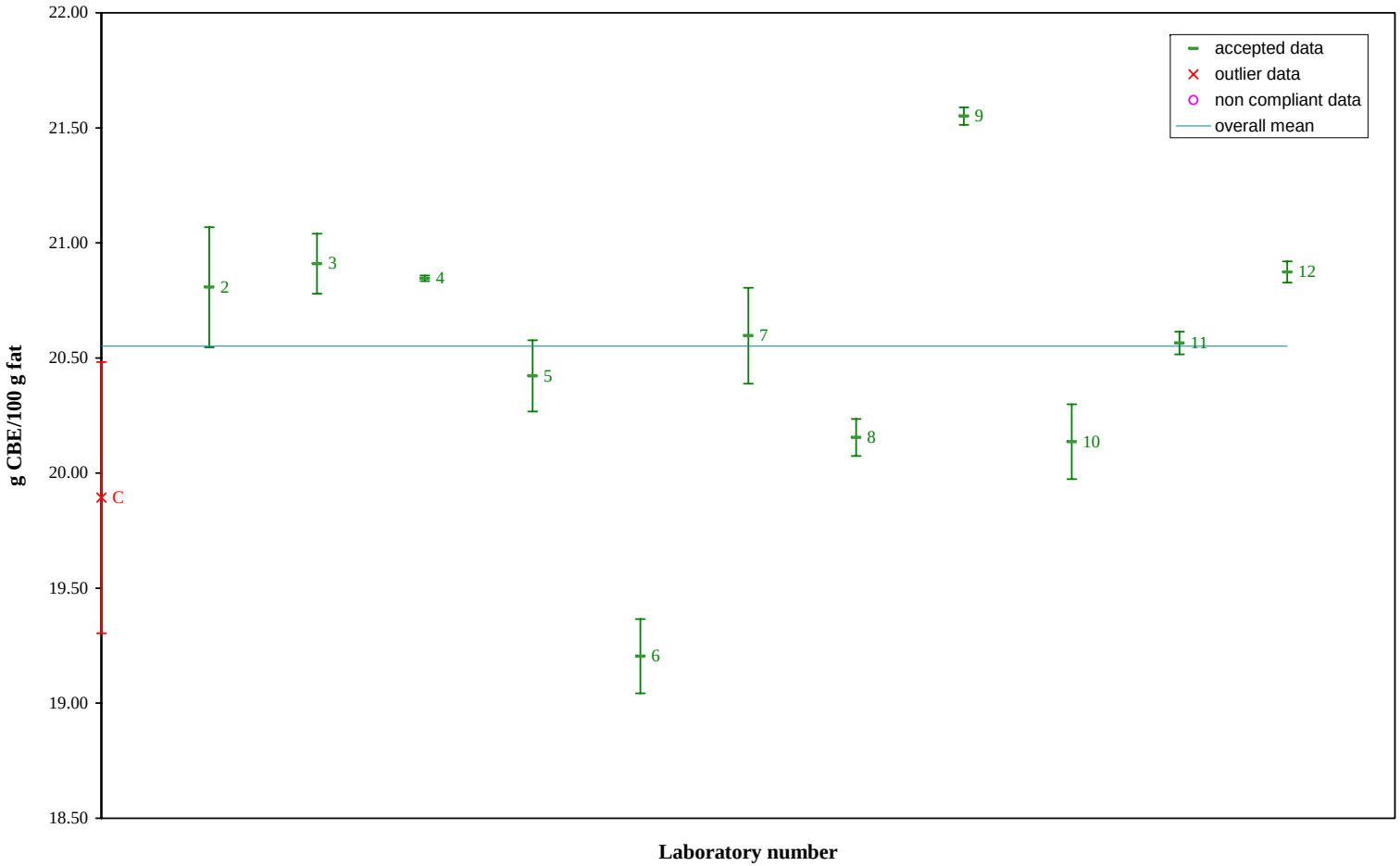


Figure G 27. Sample 5: Laboratory means and ranges of determined CBE amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

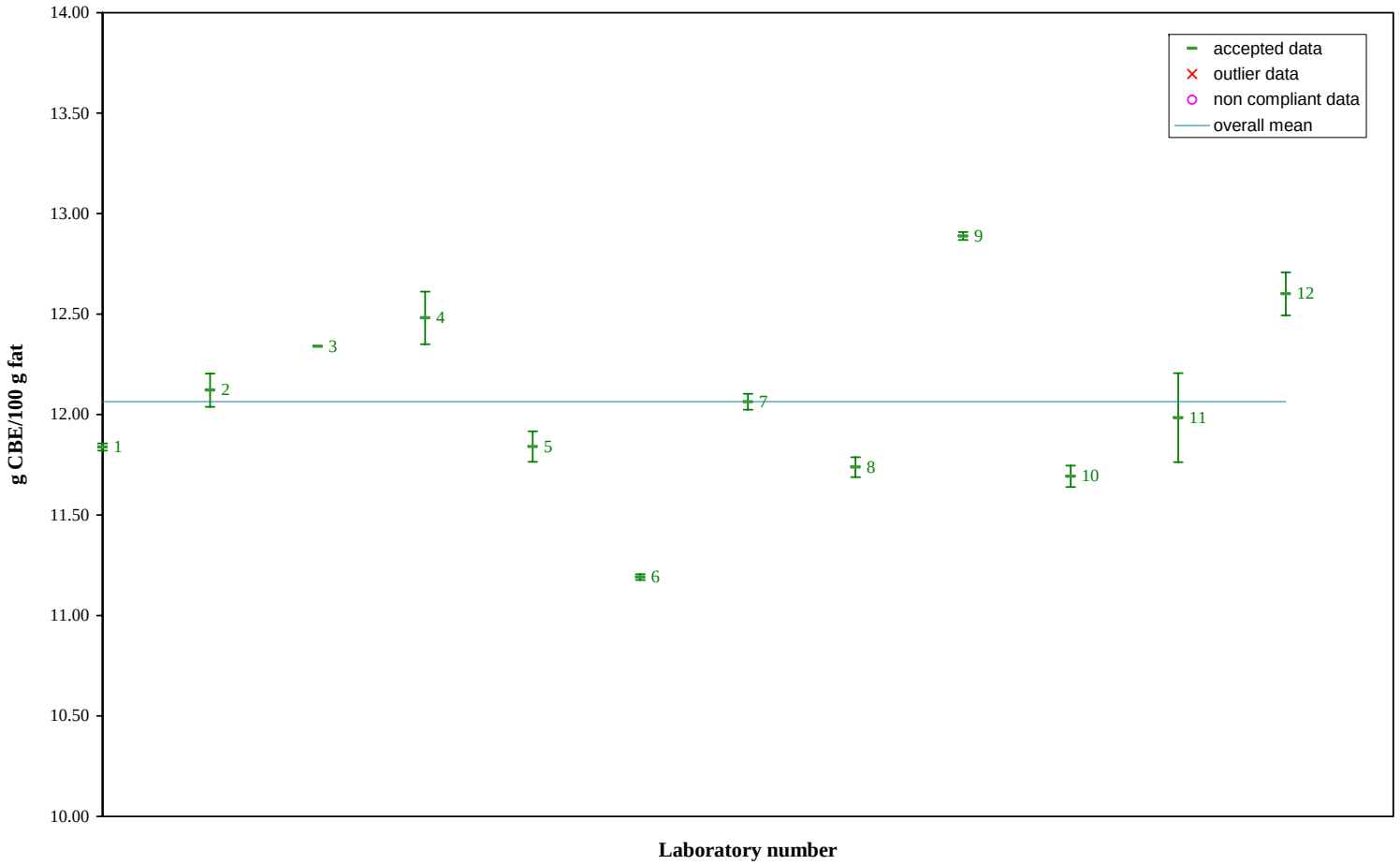


Figure G 28. Sample 6: Laboratory means and ranges of determined CBE amounts in chocolate fat (chocolate fat for GLC analysis obtained by rapid fat extraction; blind duplicates)

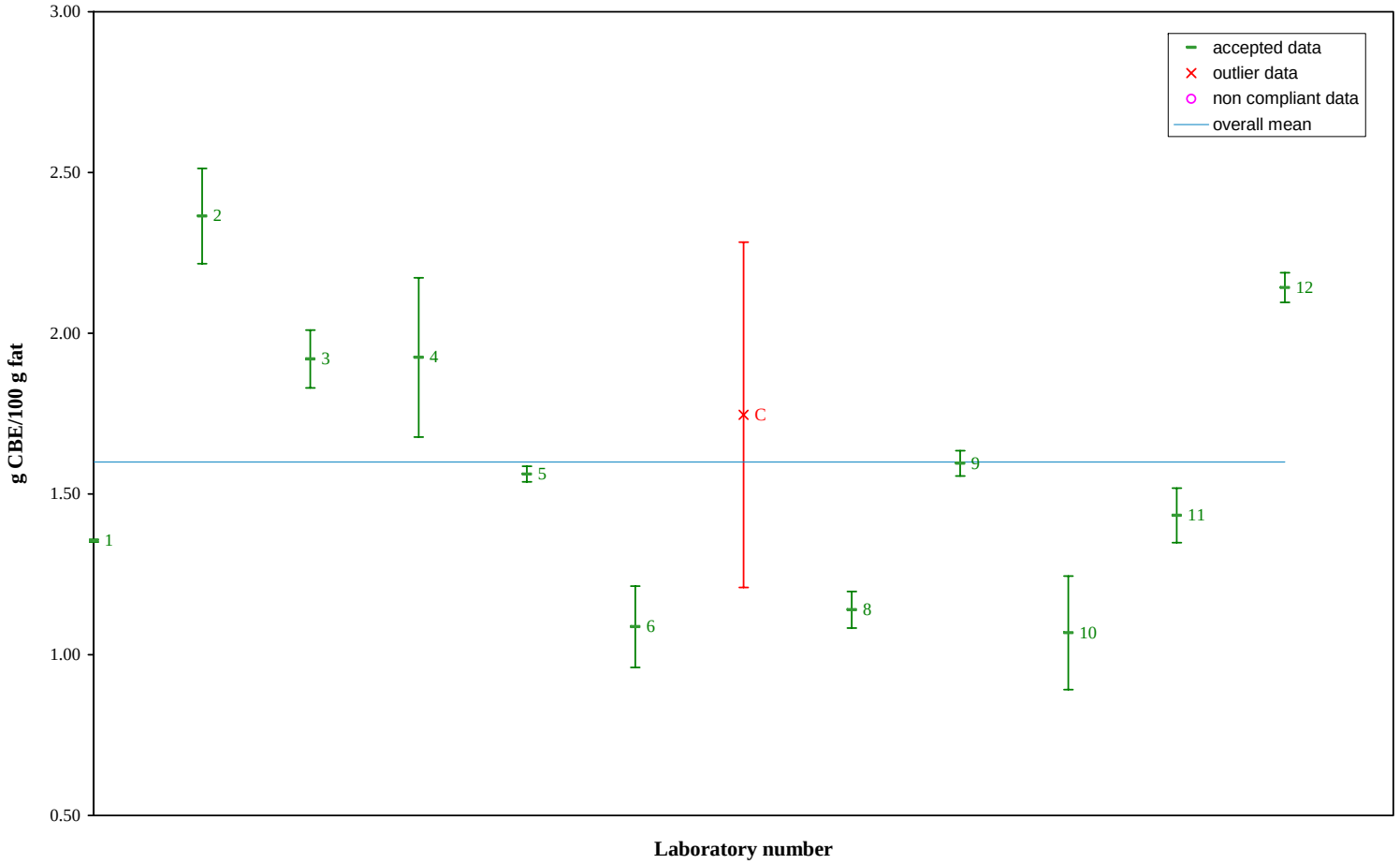


Figure G 29. Sample 9: Laboratory means and ranges of determined CBE amounts in chocolate fat (blind duplicates)

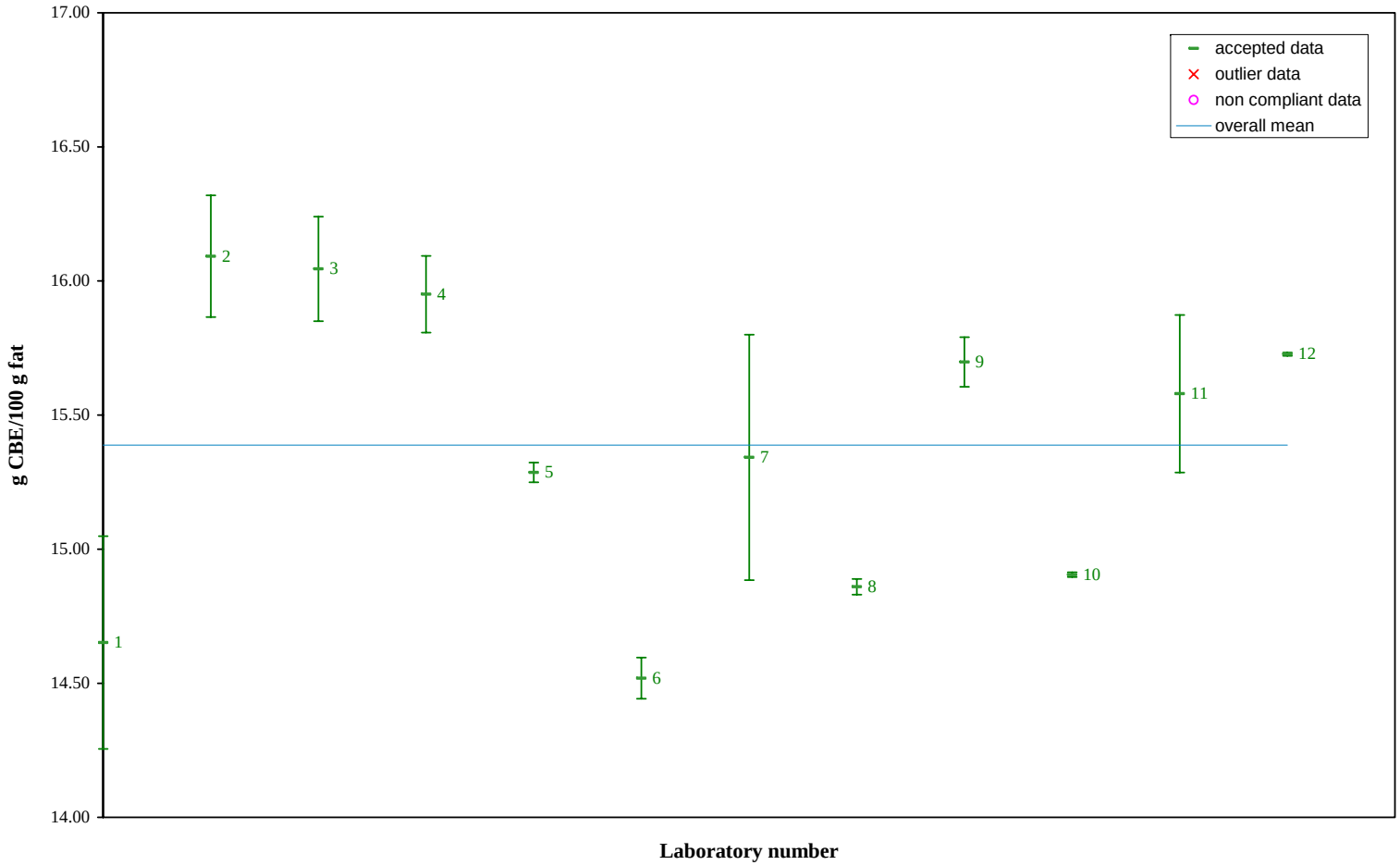


Figure G 30. Sample 10: Laboratory means and ranges of determined CBE amounts in chocolate fat (blind duplicates)

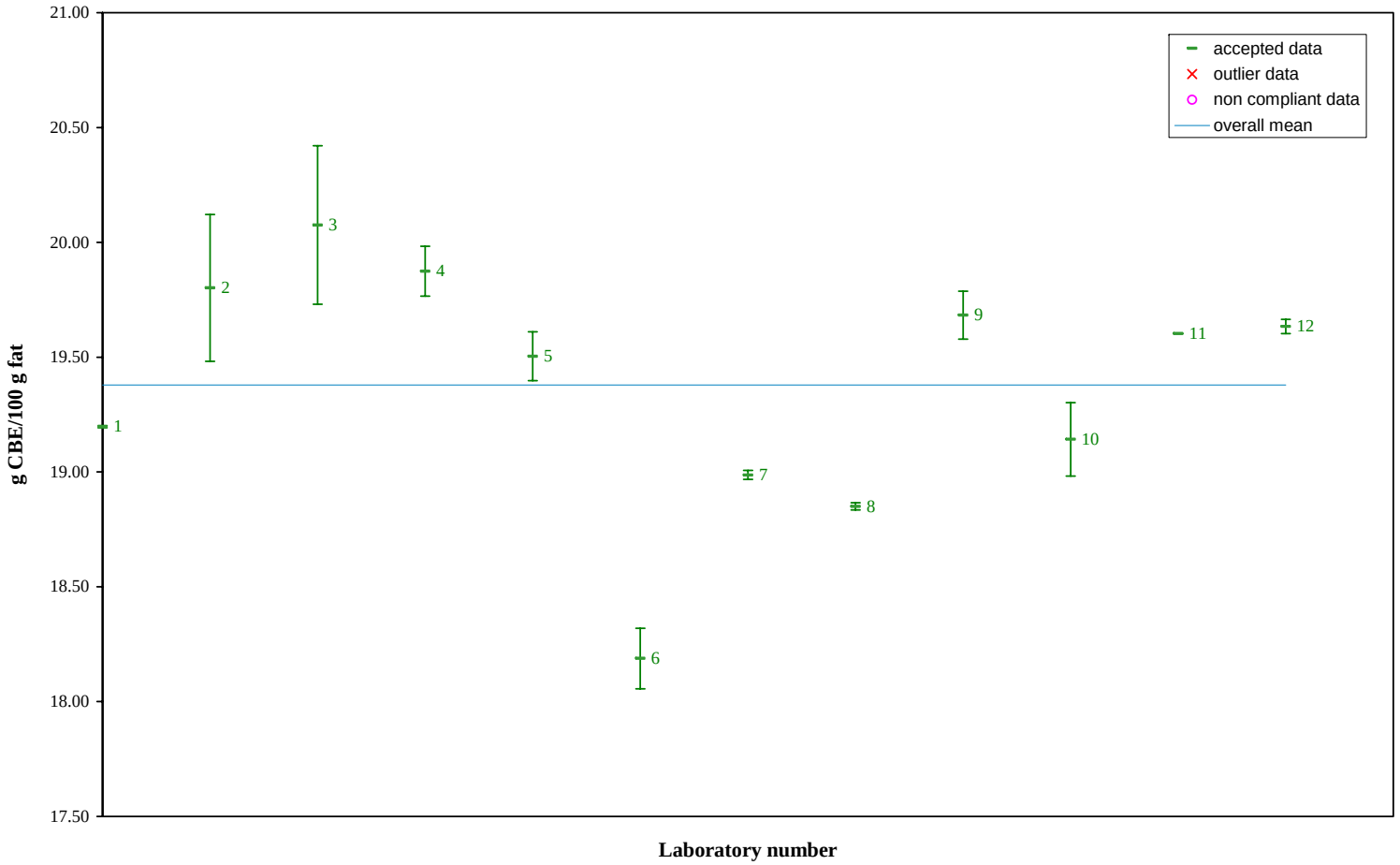


Figure G 31. Sample 11: Laboratory means and ranges of determined CBE amounts in chocolate fat (blind duplicates)

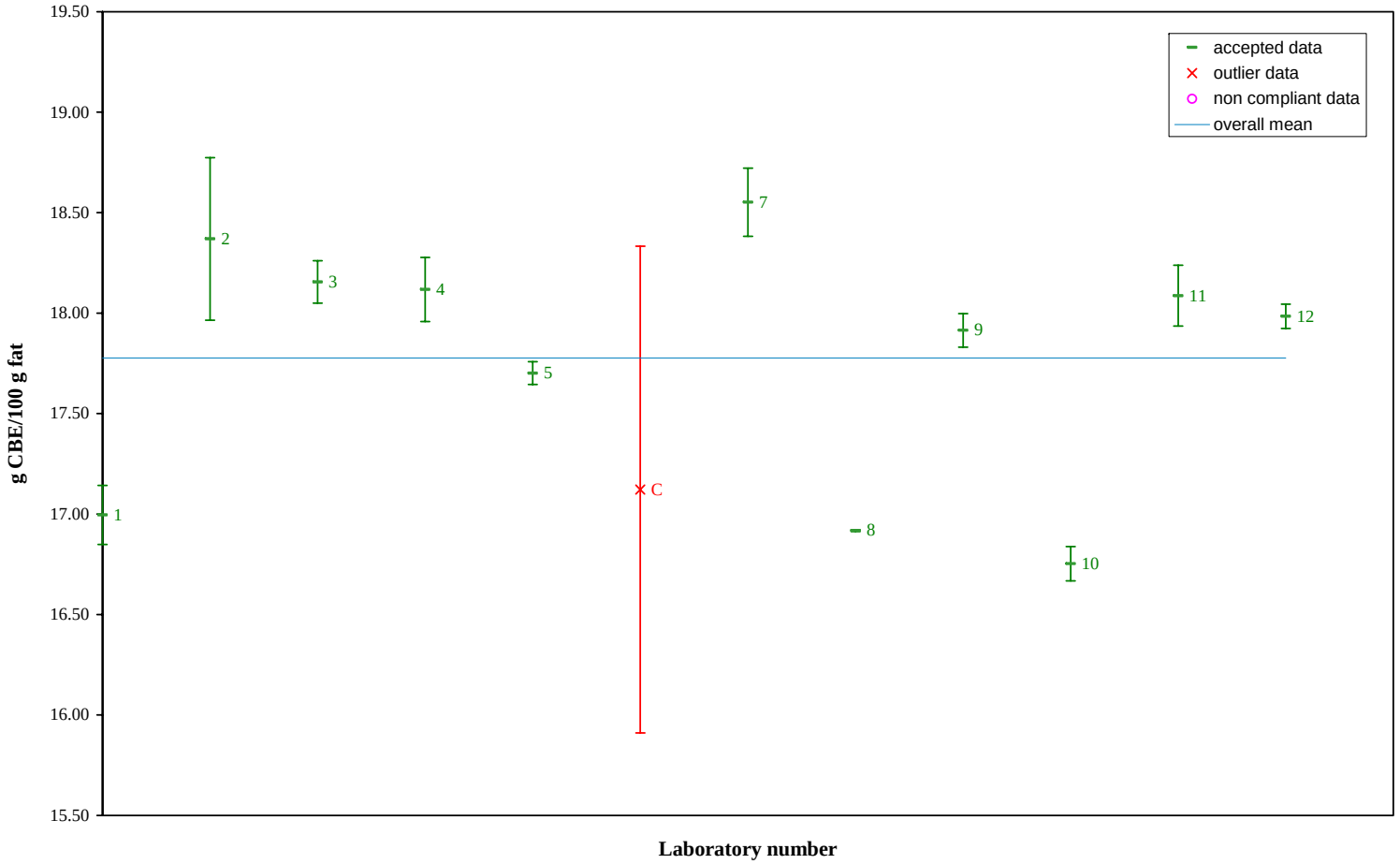


Figure G 32. Sample 12: Laboratory means and ranges of determined CBE amounts in chocolate fat (blind duplicates)

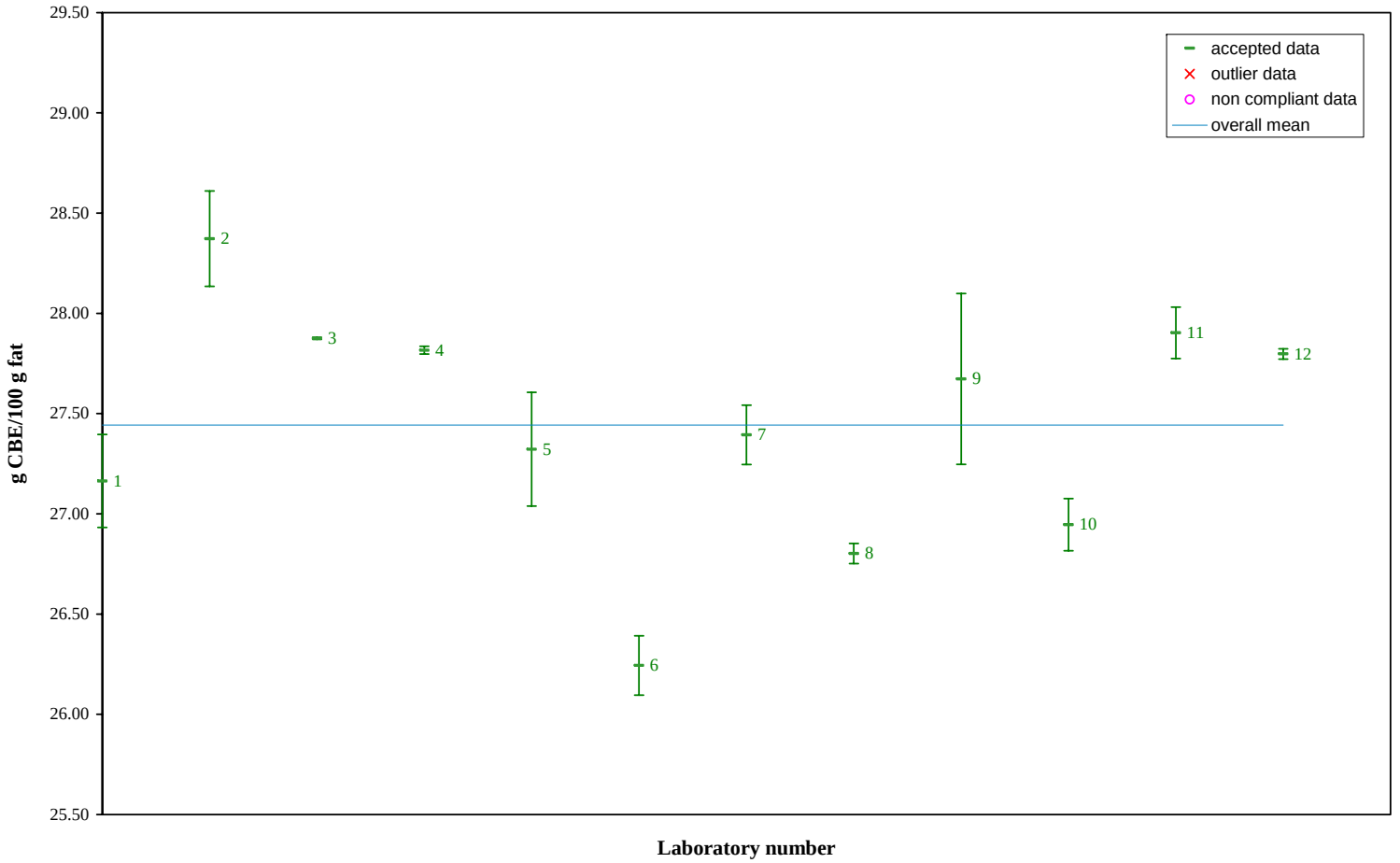


Figure G 33. Sample 13: Laboratory means and ranges of determined CBE amounts in chocolate fat (blind duplicates)

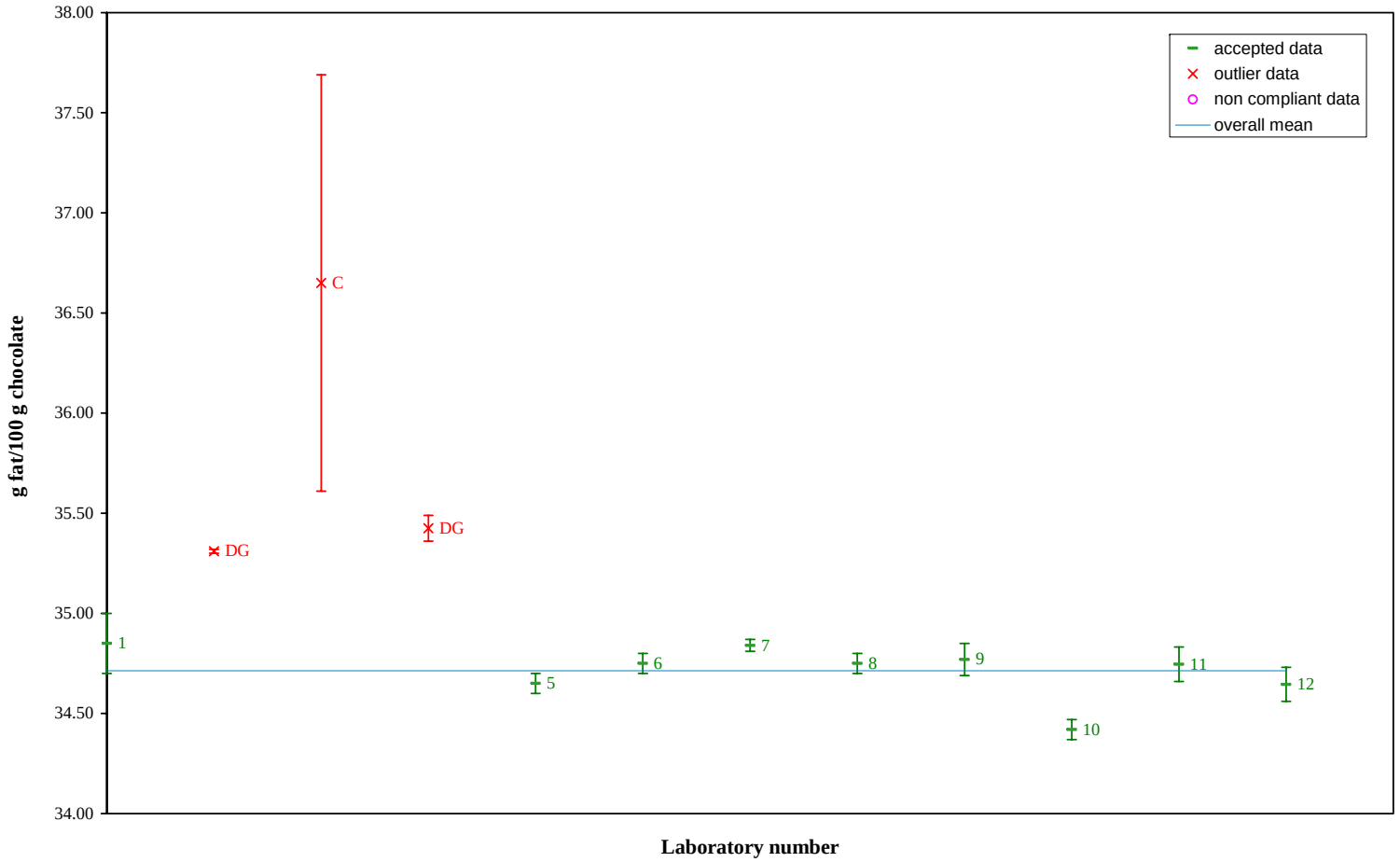


Figure G 34. Sample 1: Laboratory means and ranges of determined total fat amounts of chocolate using Soxhlet extraction (blind duplicates)

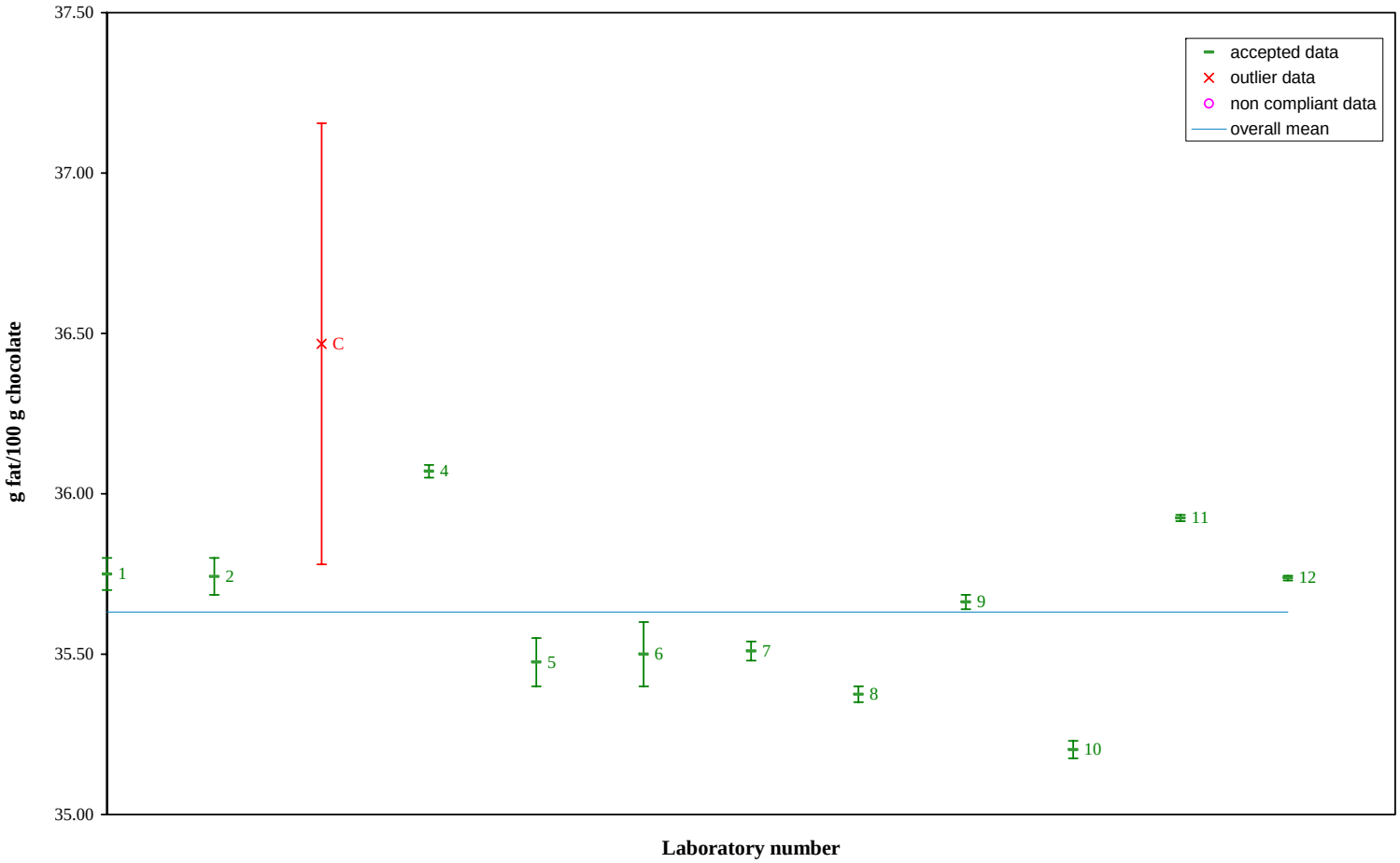


Figure G 35. Sample 2: Laboratory means and ranges of determined total fat amounts of chocolate using Soxhlet extraction (blind duplicates)

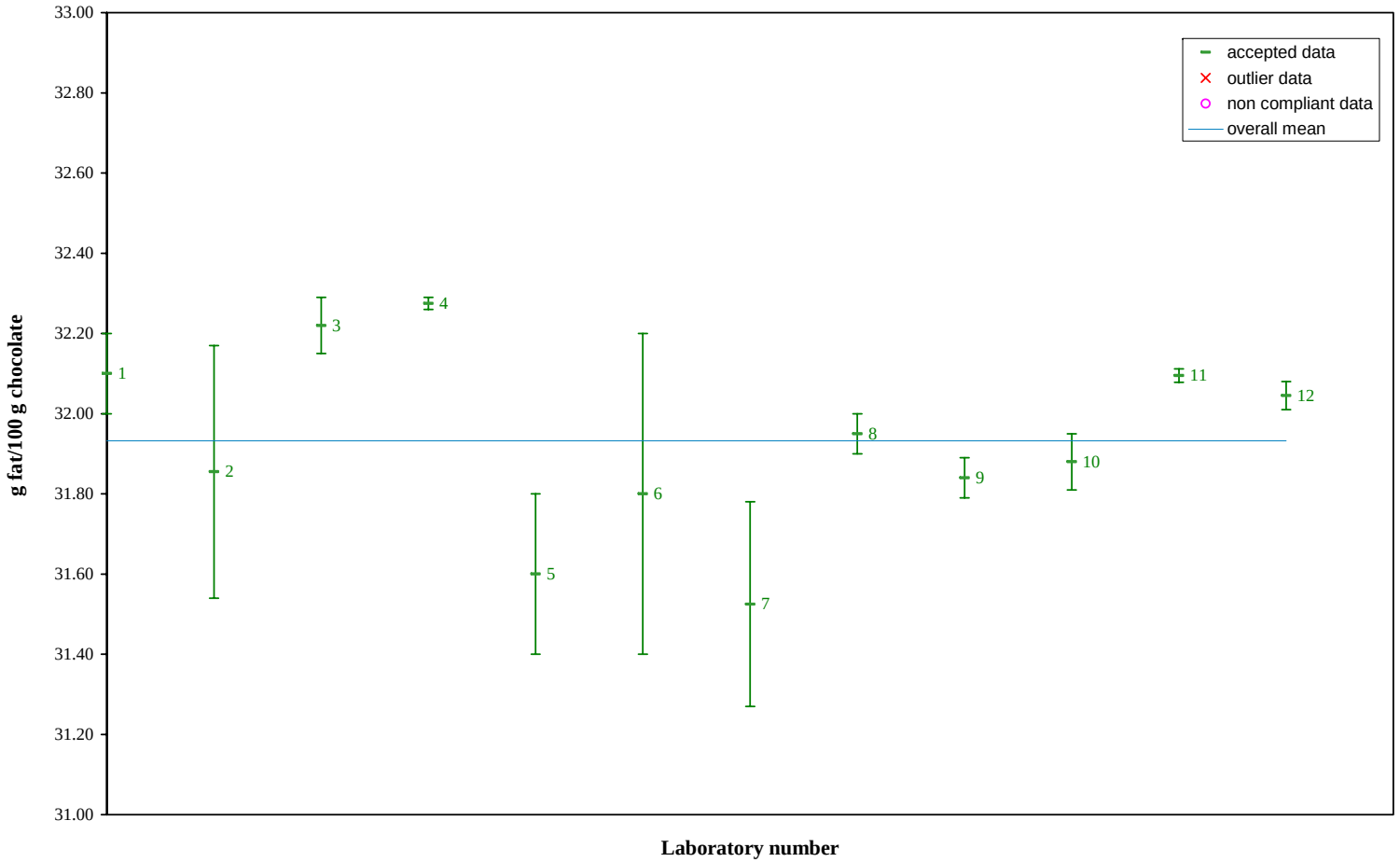


Figure G 36. Sample 3: Laboratory means and ranges of determined total fat amounts of chocolate using Soxhlet extraction (blind duplicates)

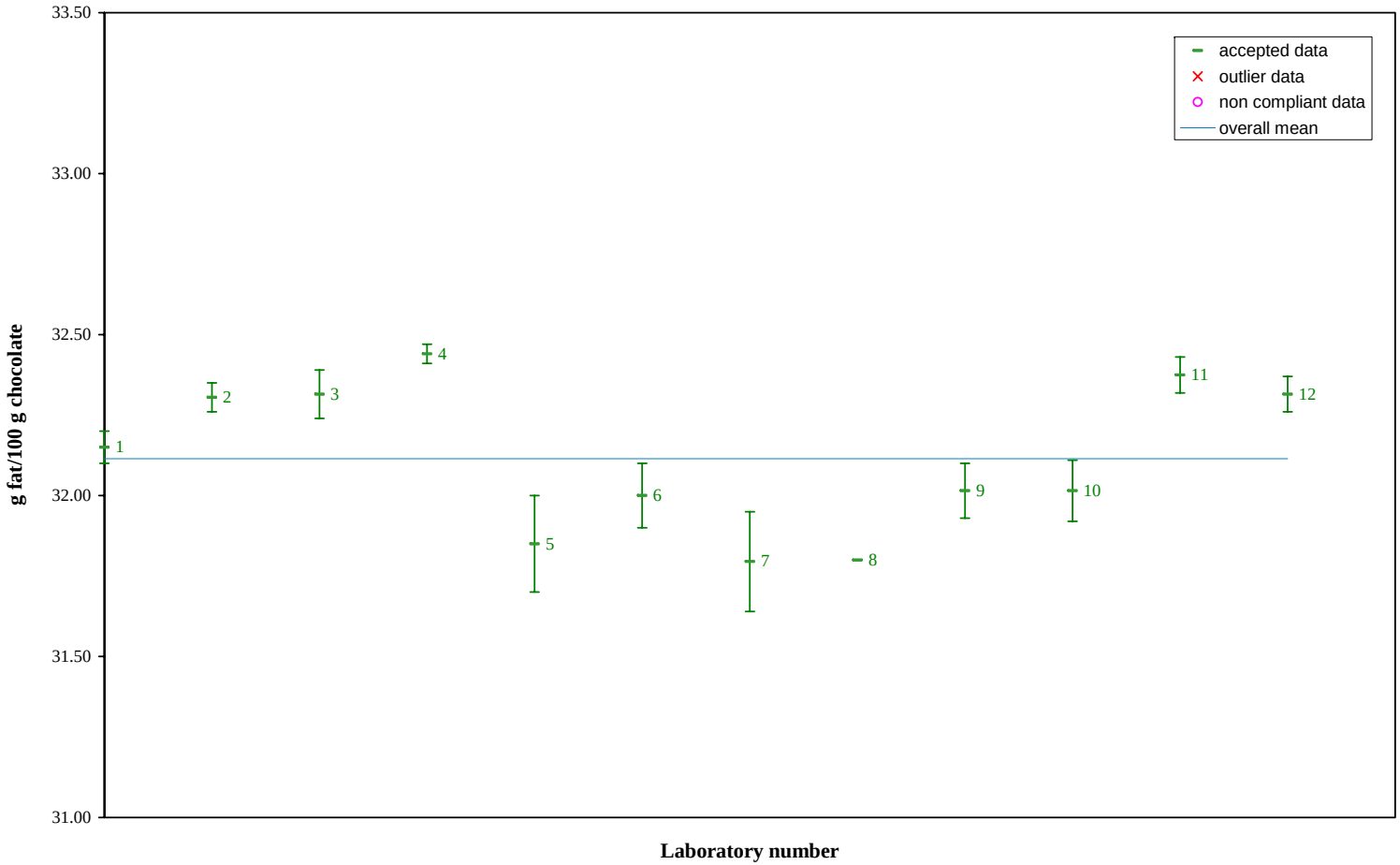


Figure G 37. Sample 4: Laboratory means and ranges of determined total fat amounts of chocolate using Soxhlet extraction (blind duplicates)

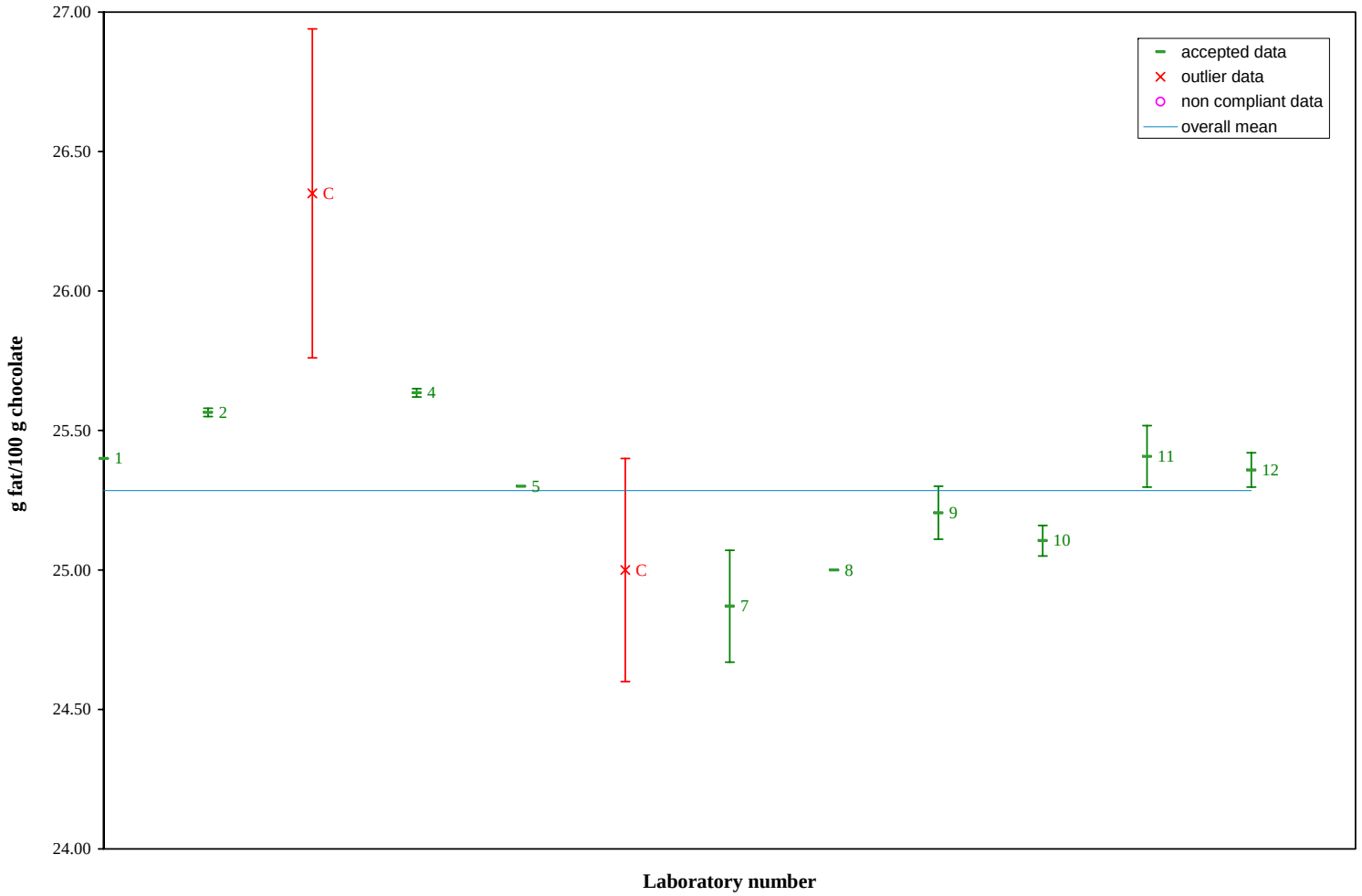


Figure G 38. Sample 5: Laboratory means and ranges of determined total fat amounts of chocolate using Soxhlet extraction (blind duplicates)

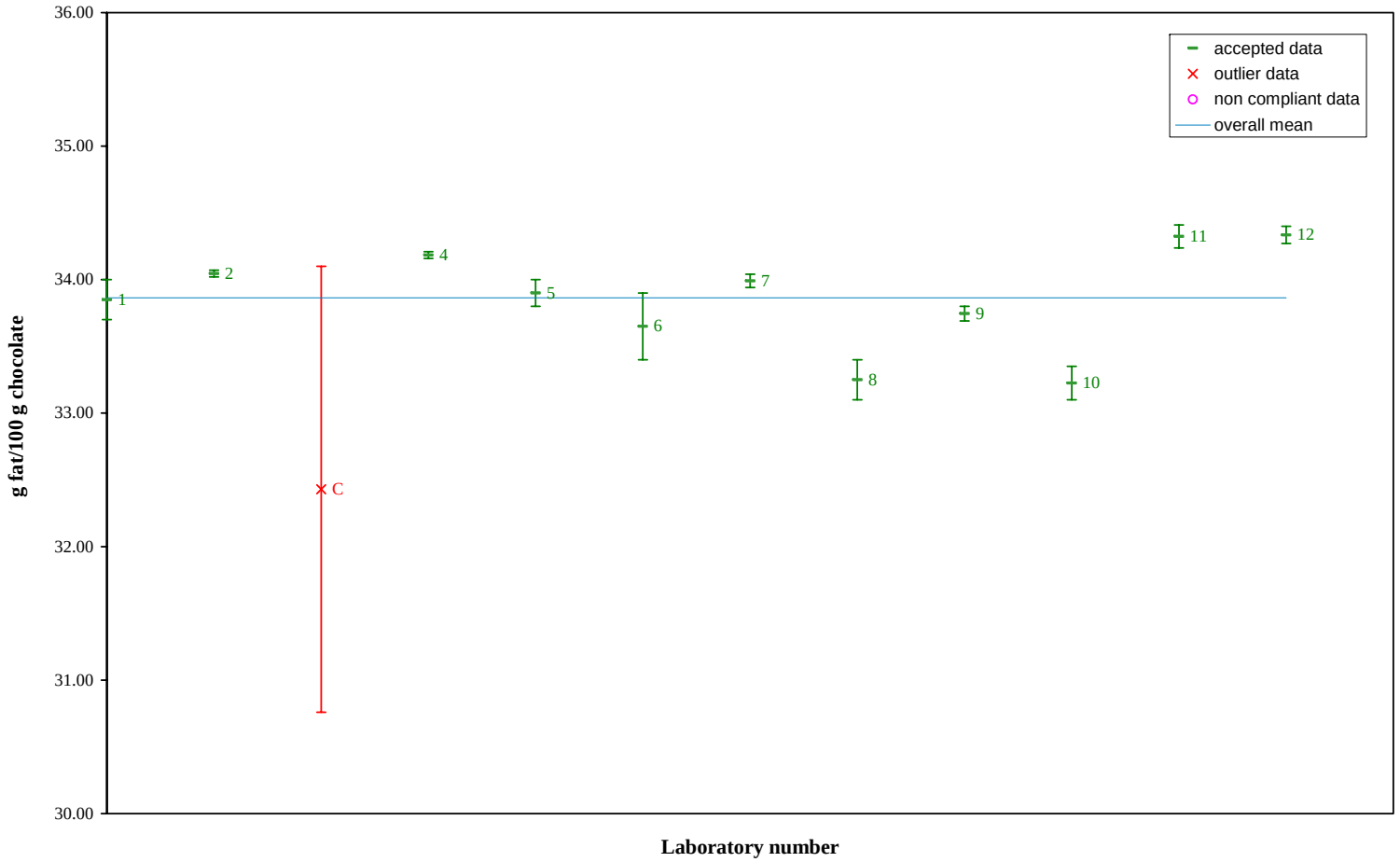


Figure G 39. Sample 6: Laboratory means and ranges of determined total fat amounts of chocolate using Soxhlet extraction (blind duplicates)

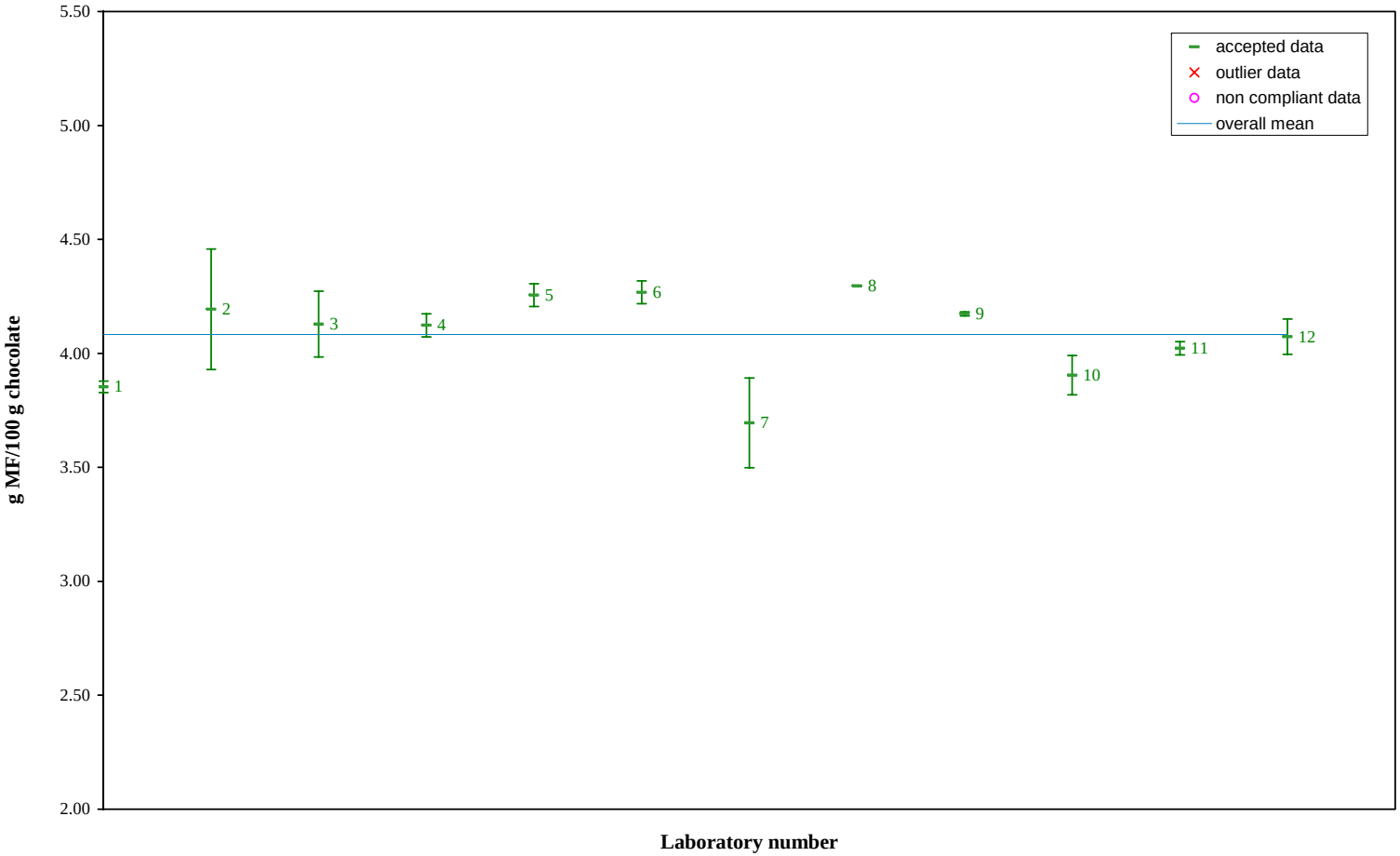


Figure G 40. Sample 1: Laboratory means and ranges of determined MF amounts in chocolate (chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet fat extraction; blind duplicates)

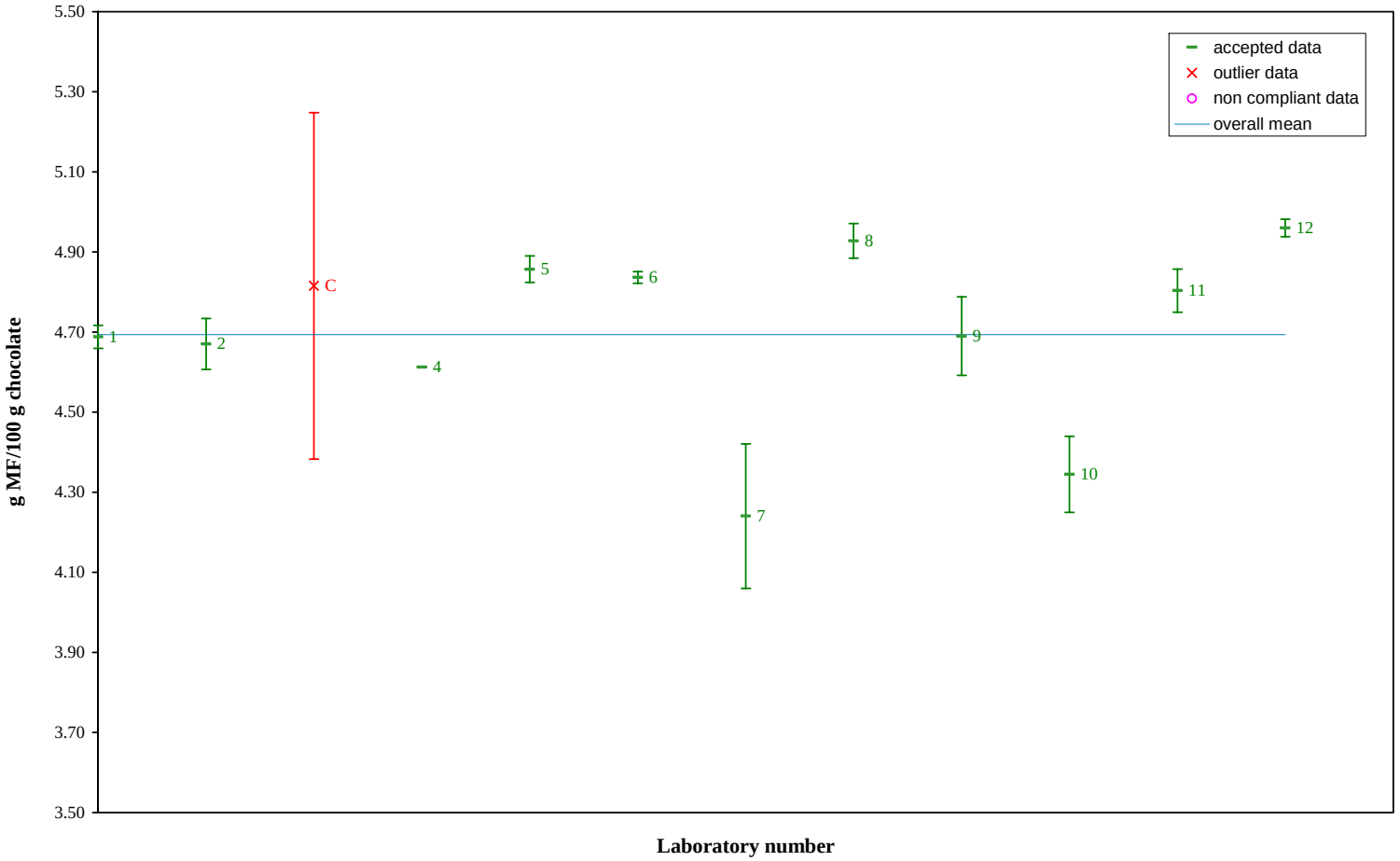


Figure G 41. Sample 2: Laboratory means and ranges of determined MF amounts in chocolate (chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet fat extraction; blind duplicates)

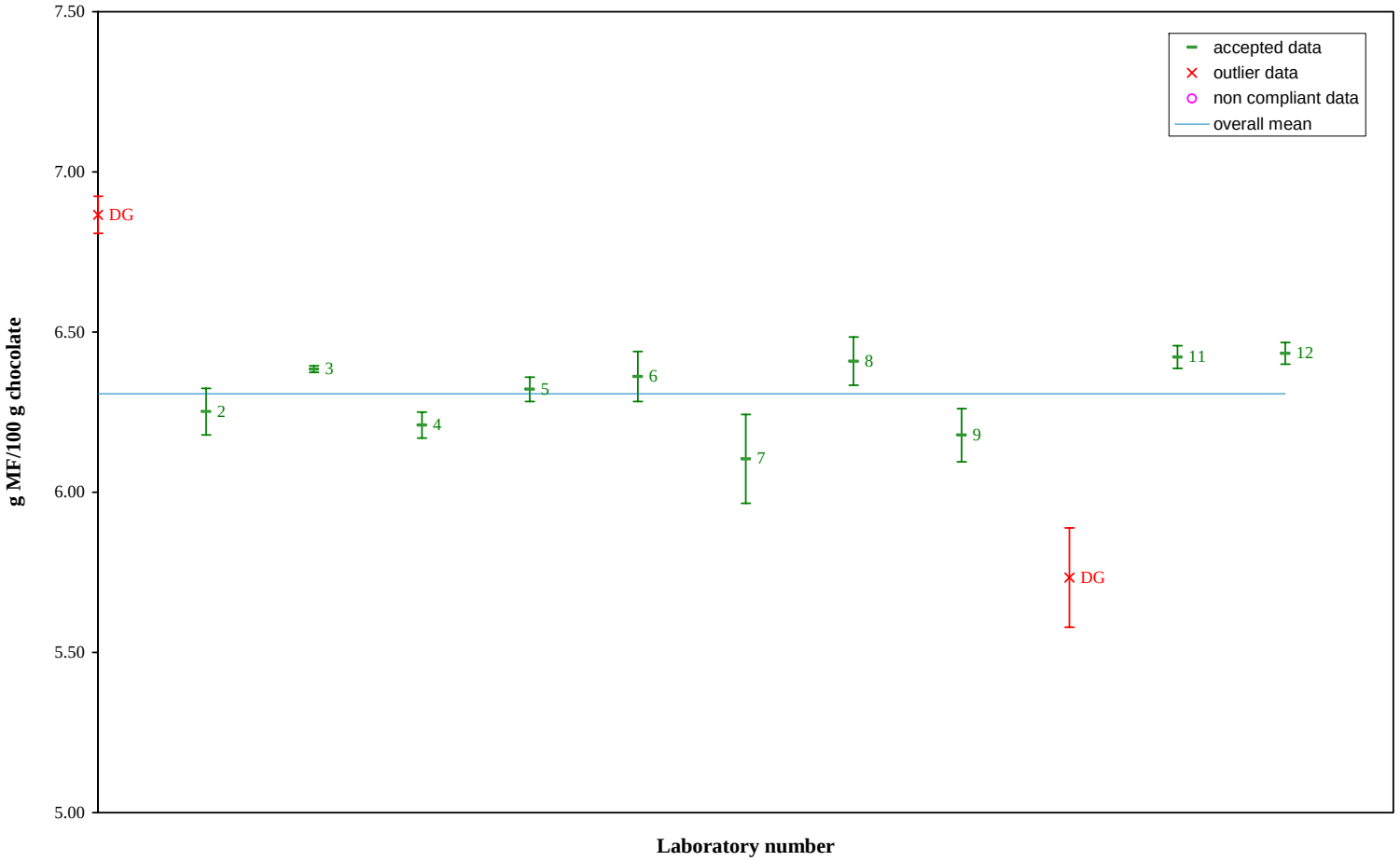


Figure G 42. Sample 3: Laboratory means and ranges of determined MF amounts in chocolate (chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet fat extraction; blind duplicates)

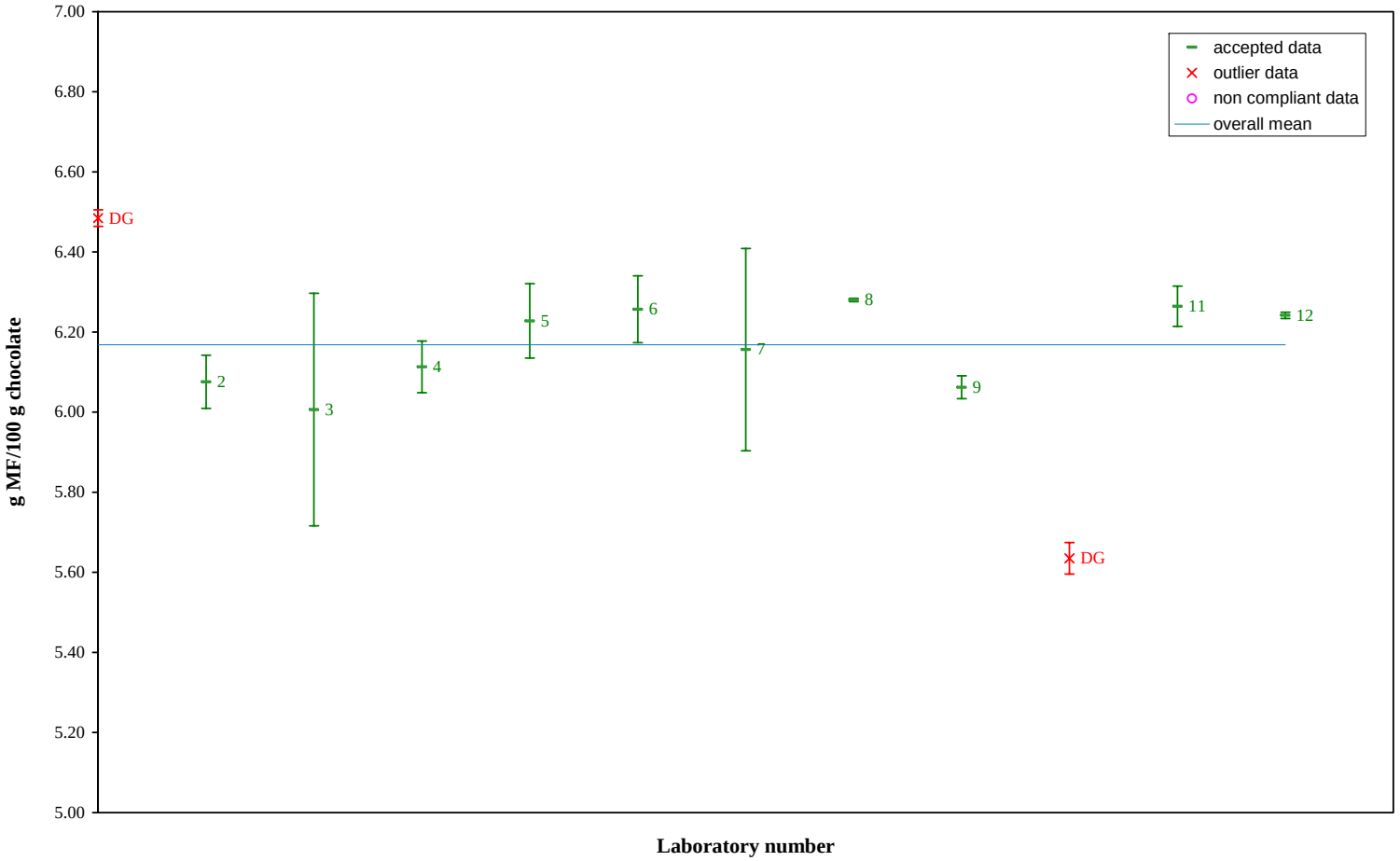


Figure G 43. Sample 4: Laboratory means and ranges of determined MF amounts in chocolate (chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet fat extraction; blind duplicates)

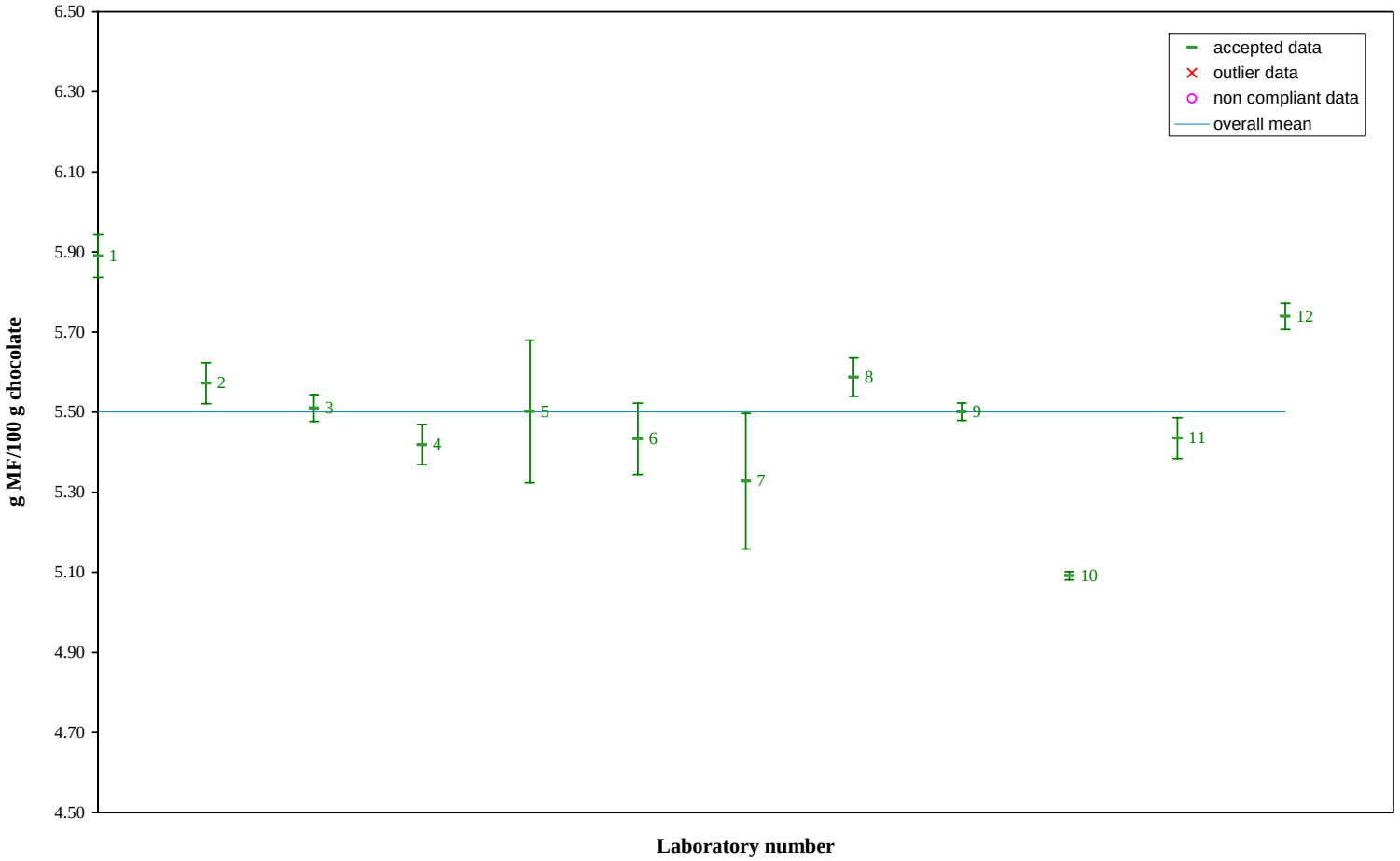


Figure G 44. Sample 5: Laboratory means and ranges of determined MF amounts in chocolate (chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet fat extraction; blind duplicates)

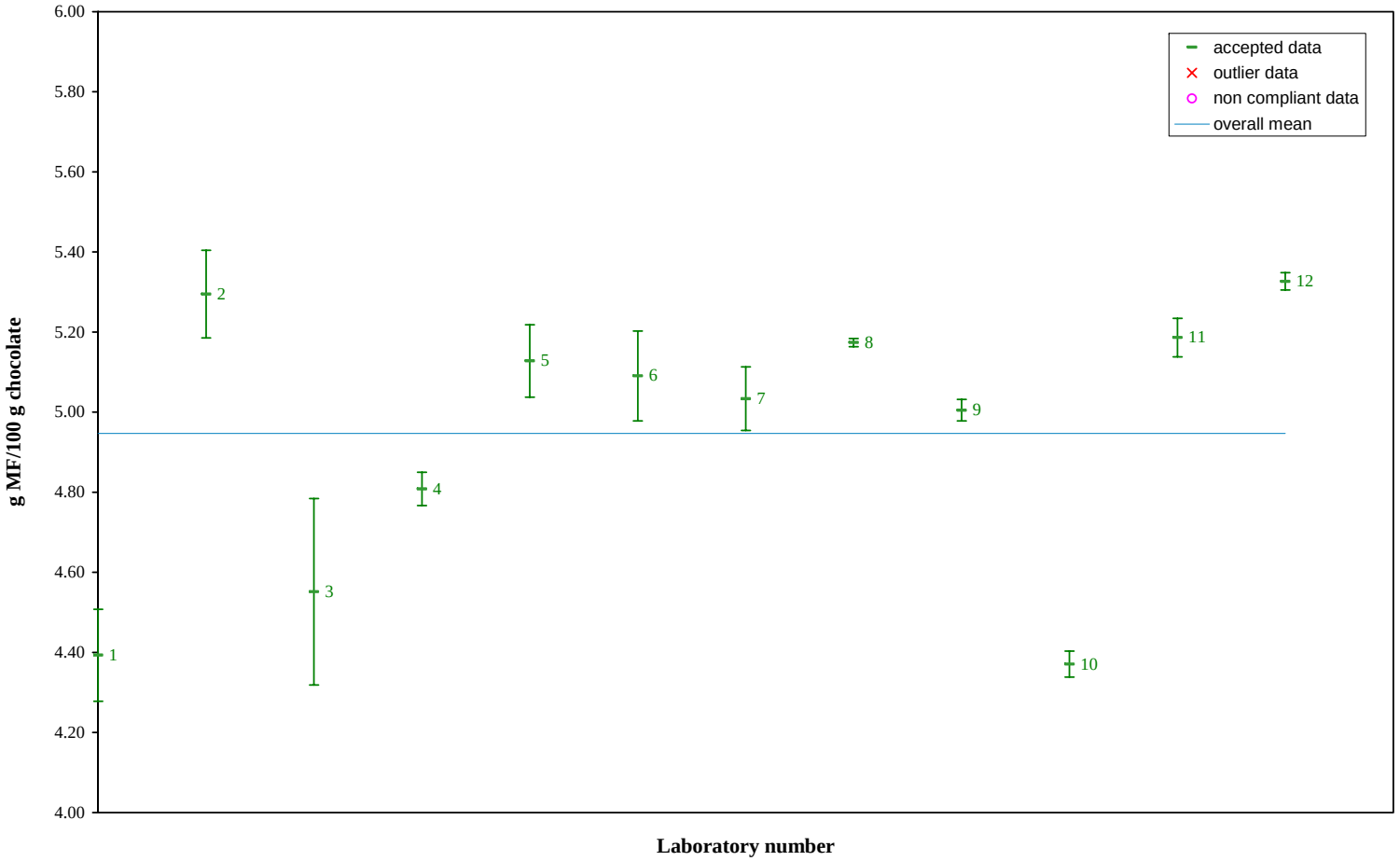


Figure G 45. Sample 6: Laboratory means and ranges of determined MF amounts in chocolate (chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet fat extraction; blind duplicates)

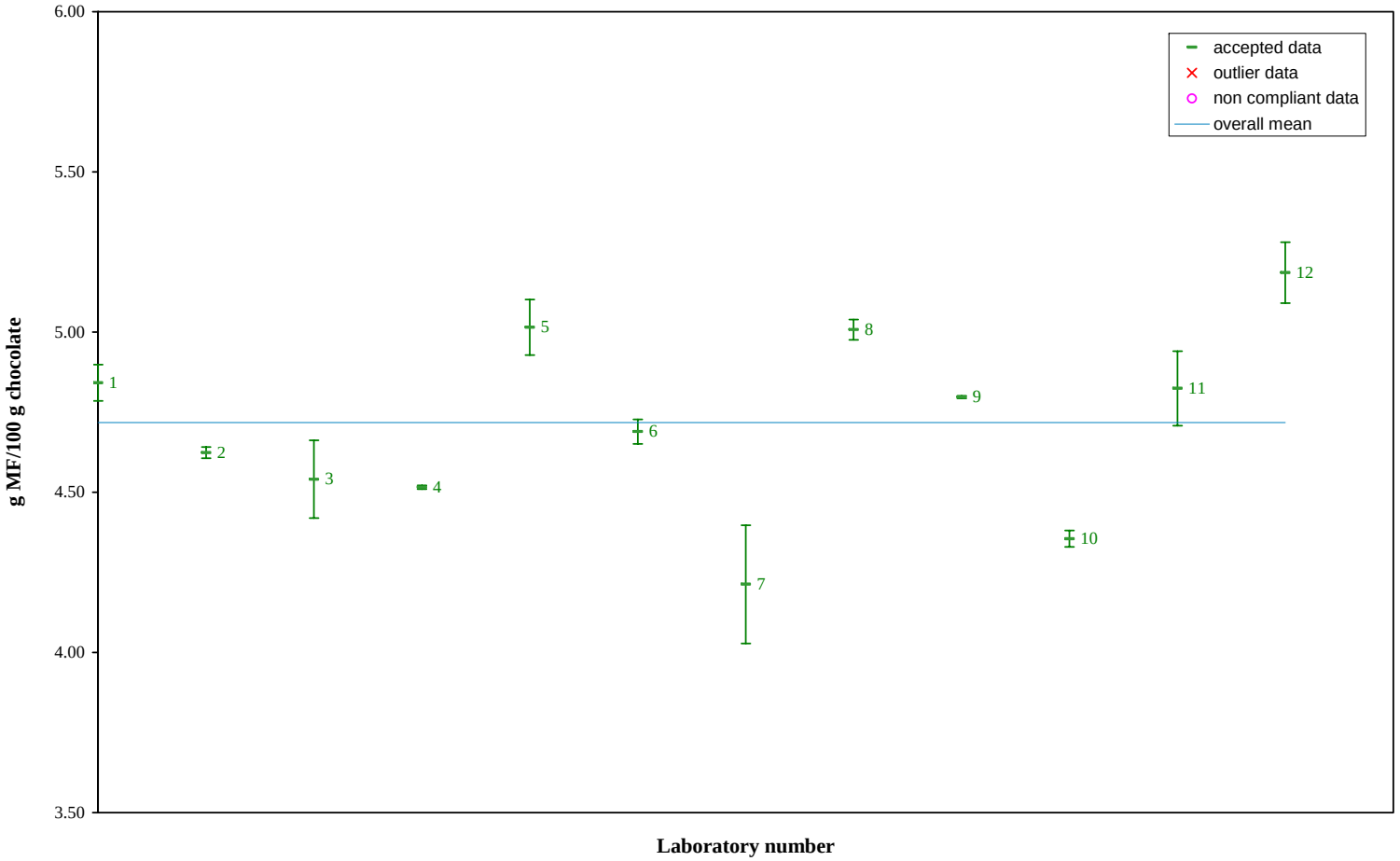


Figure G 46. Sample 8: Laboratory means and ranges of determined MF amounts in chocolate (blind duplicates; assumed fat content of chocolate= 30 %)

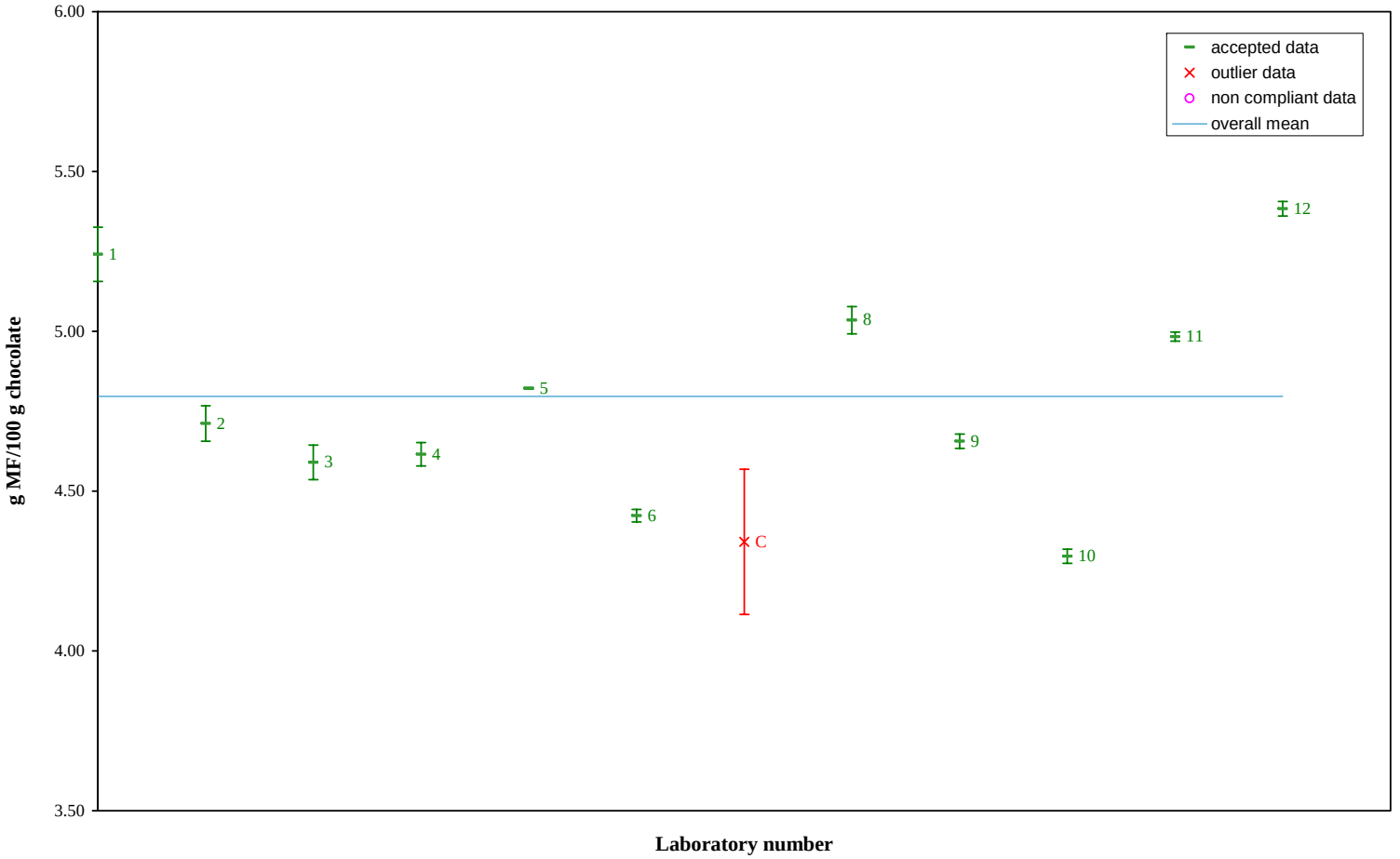


Figure G 47. Sample 9: Laboratory means and ranges of determined MF amounts in chocolate (blind duplicates; assumed fat content of chocolate= 30 %)

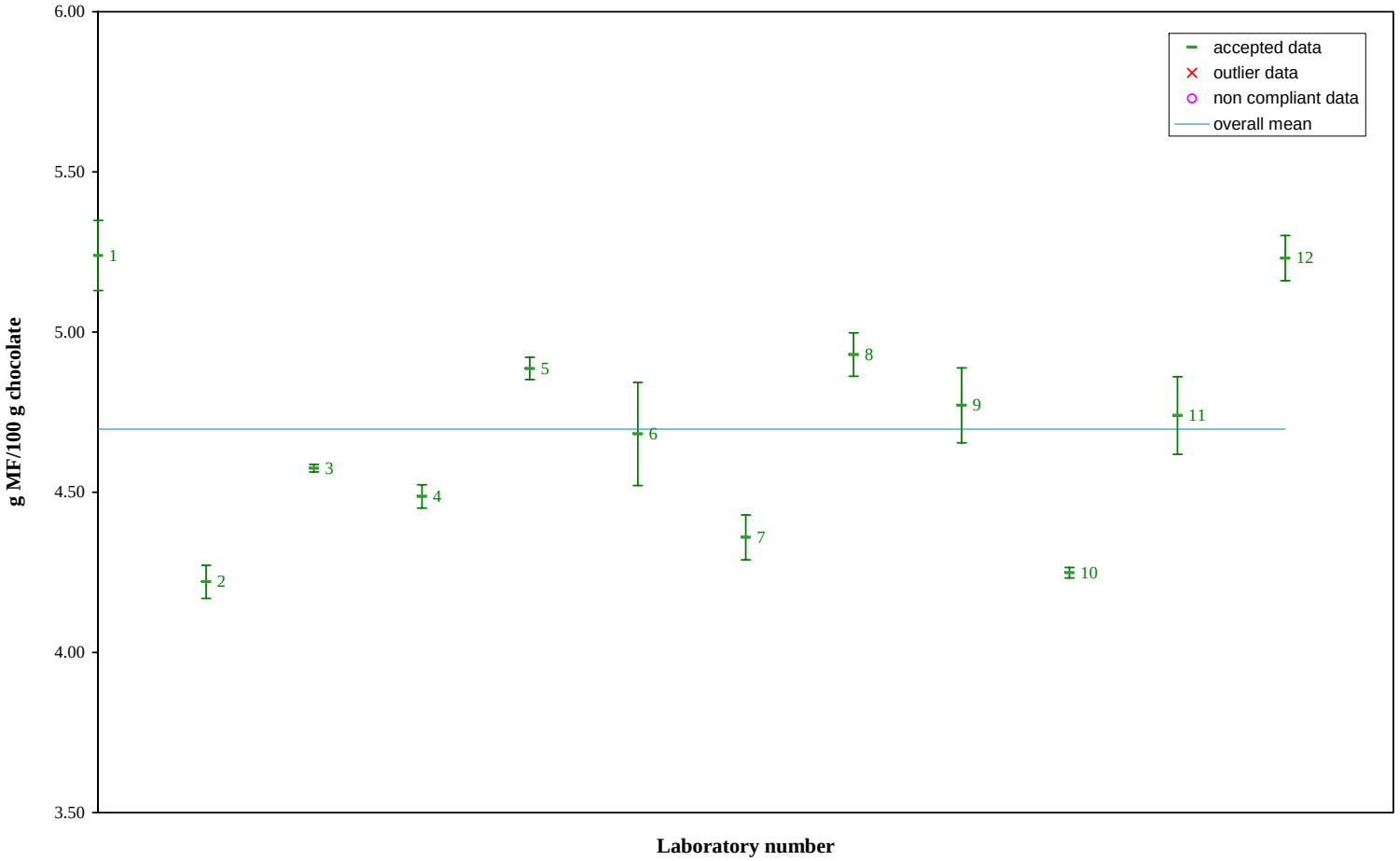


Figure G 48. Sample 10: Laboratory means and ranges of determined MF amounts in chocolate (blind duplicates; assumed fat content of chocolate= 30 %)

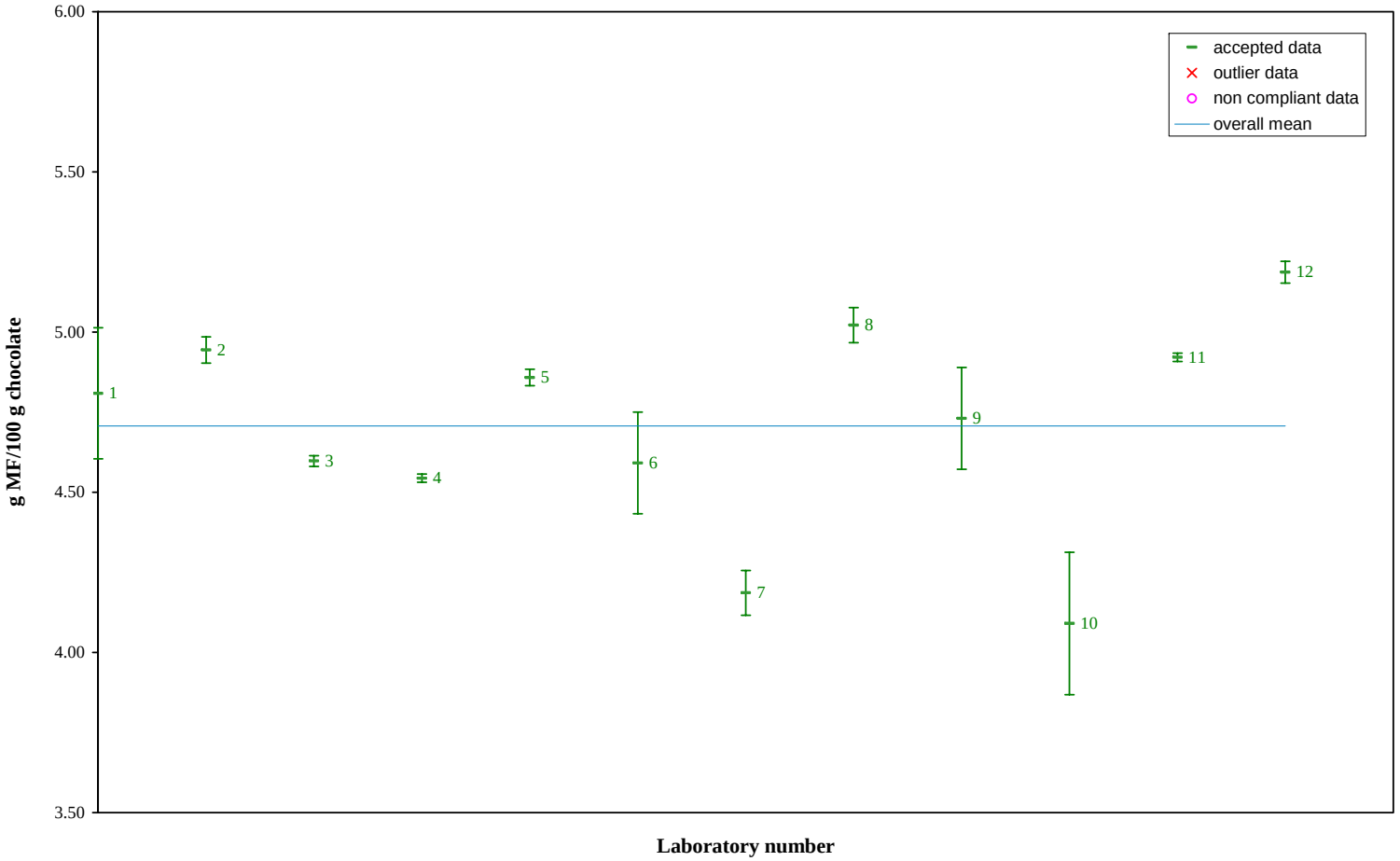


Figure G 49. Sample 11: Laboratory means and ranges of determined MF amounts in chocolate (blind duplicates; assumed fat content of chocolate= 30 %)

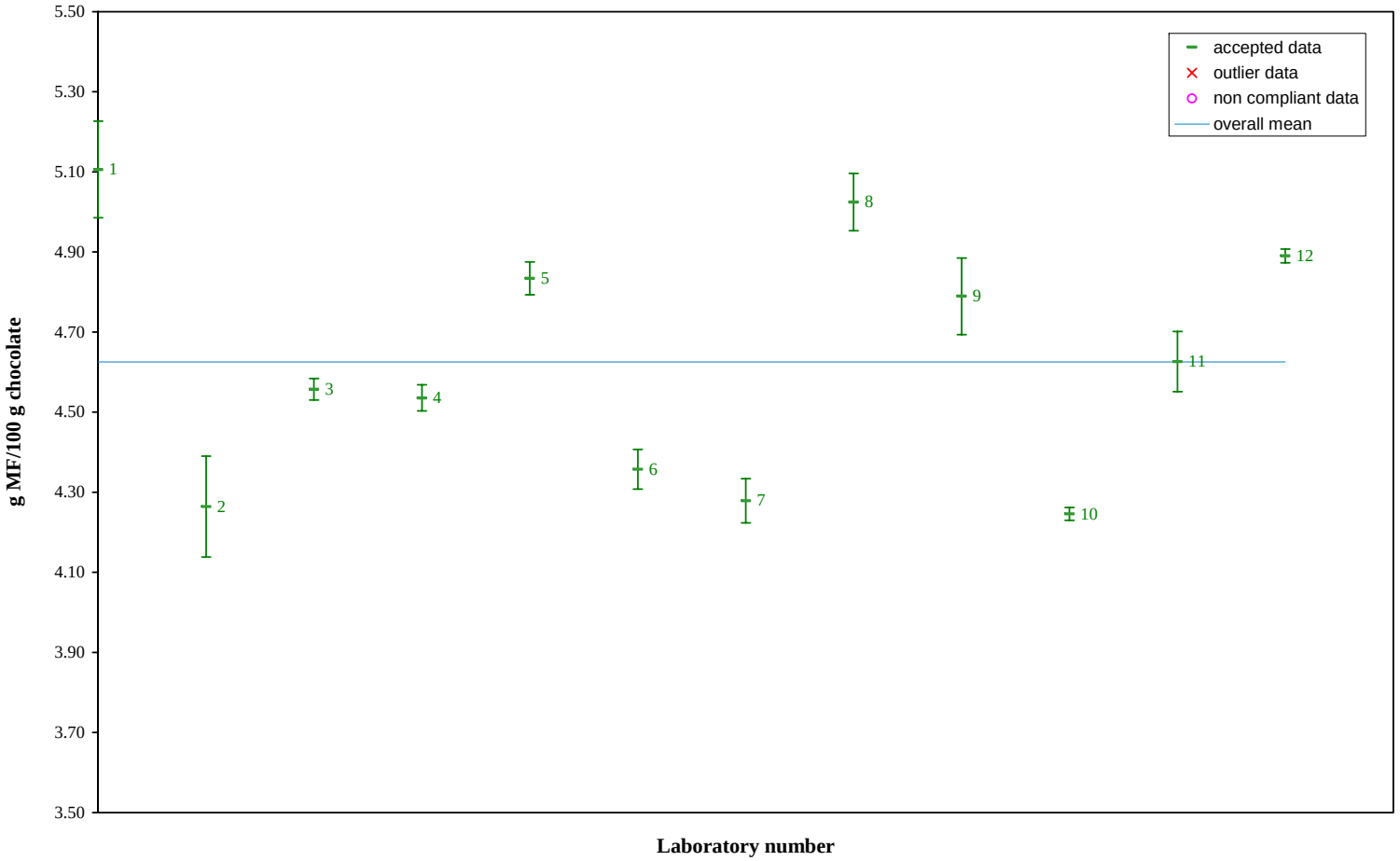


Figure G 50. Sample 12: Laboratory means and ranges of determined MF amounts in chocolate (blind duplicates; assumed fat content of chocolate= 30 %)

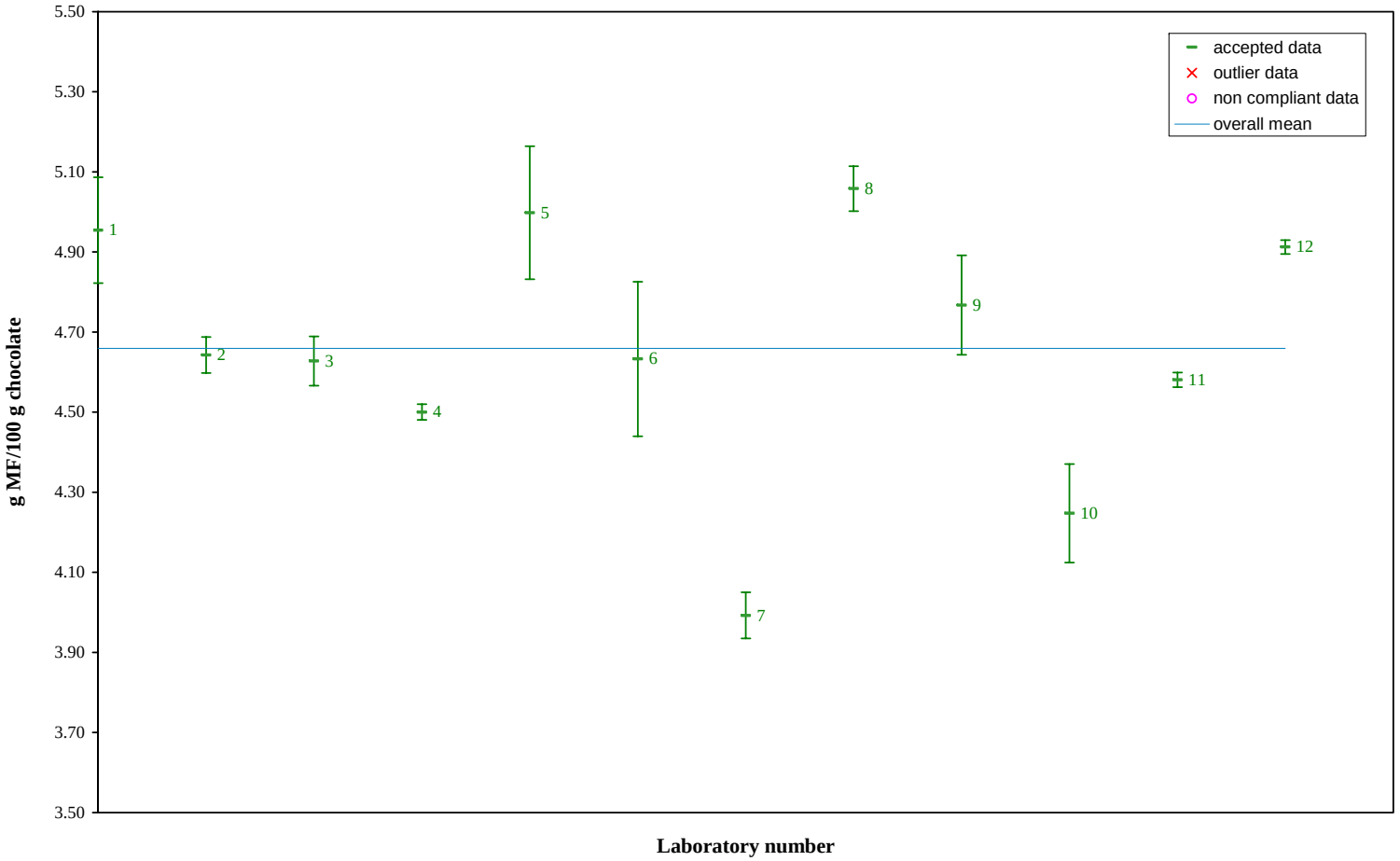


Figure G 51. Sample 13: Laboratory means and ranges of determined MF amounts in chocolate (blind duplicates; assumed fat content of chocolate= 30 %)

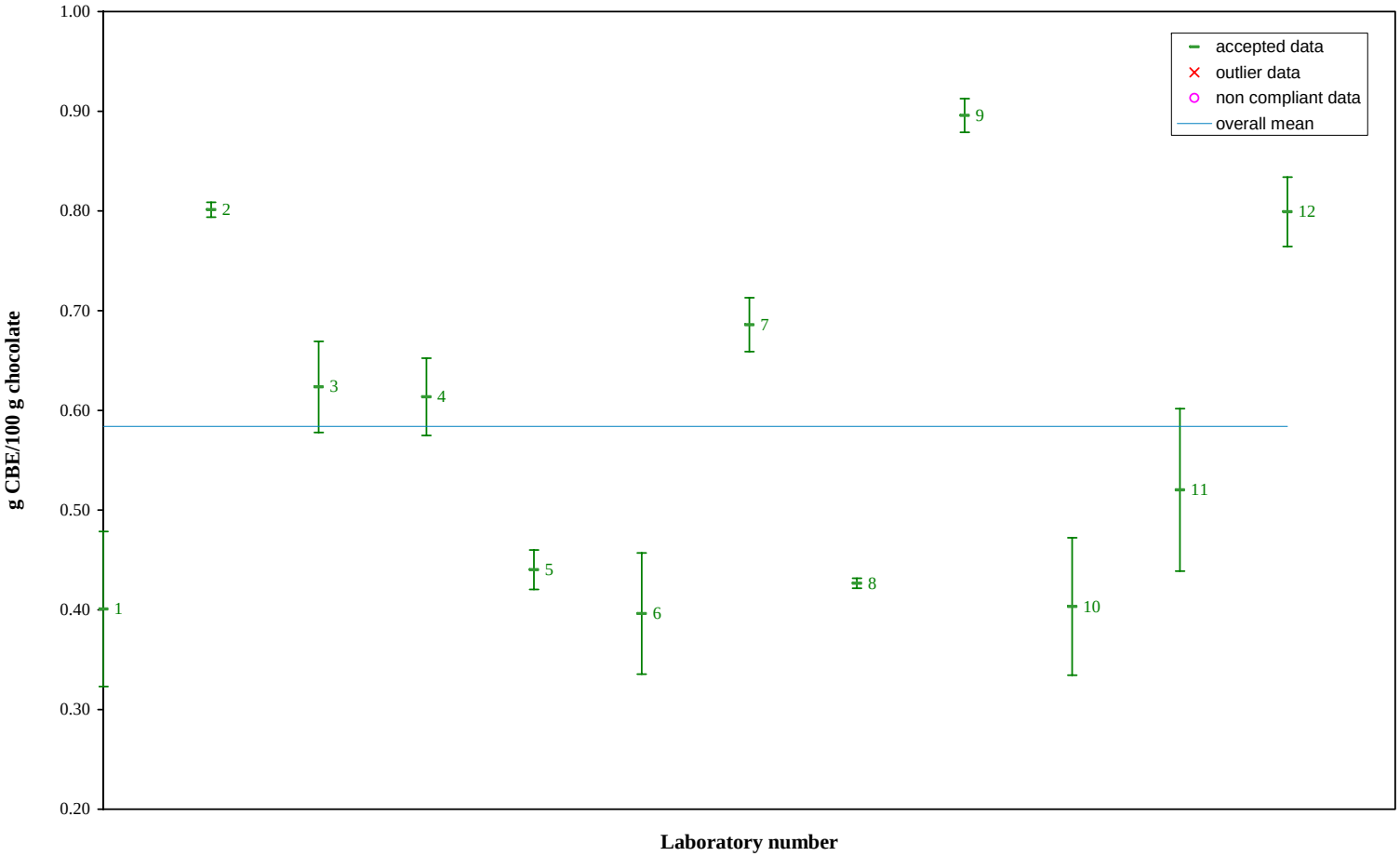


Figure G 52. Sample 2: Laboratory means and ranges of determined CBE amounts in chocolate (chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet fat extraction; blind duplicates)

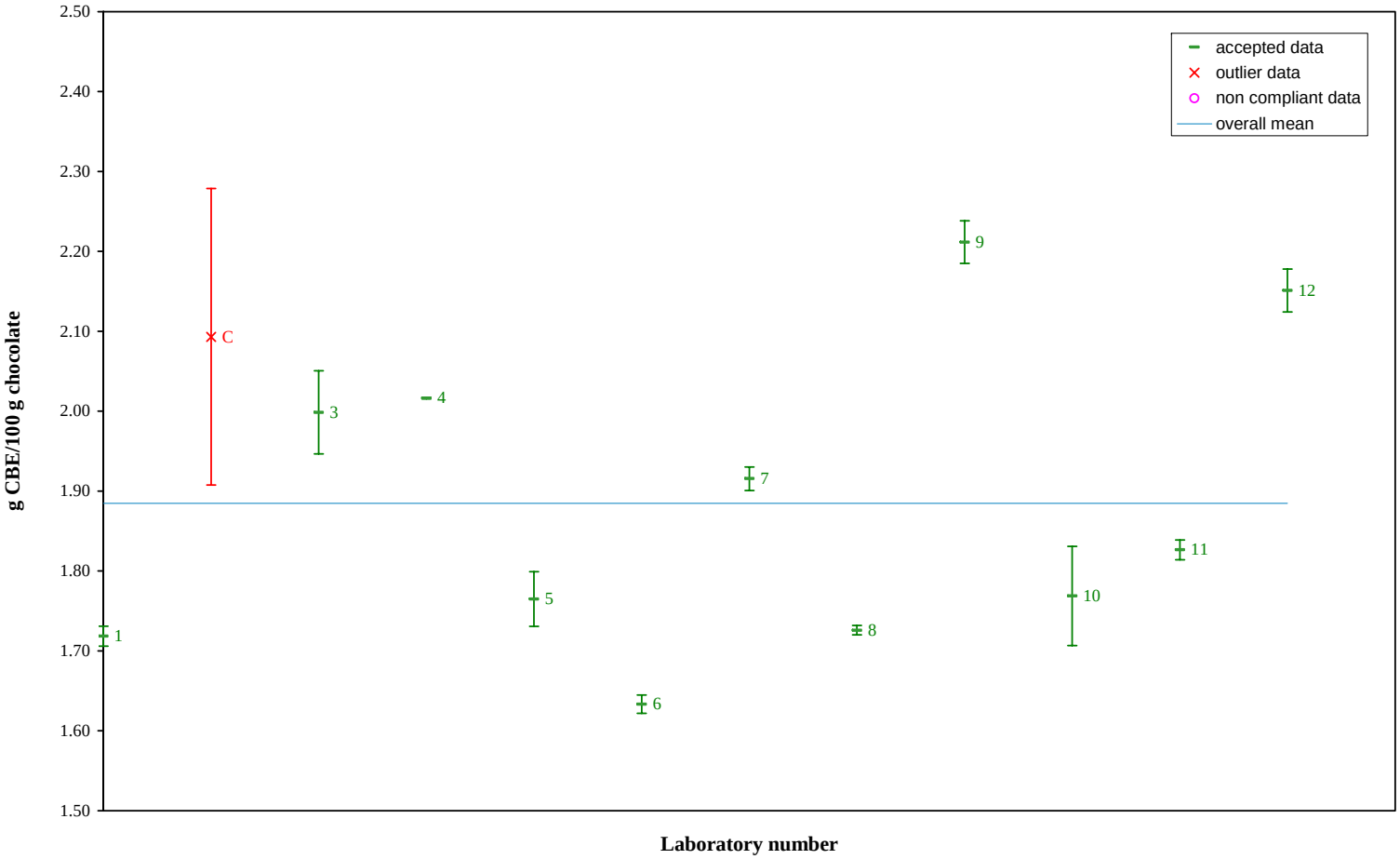


Figure G 53. Sample 4: Laboratory means and ranges of determined CBE amounts in chocolate (chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet fat extraction; blind duplicates)

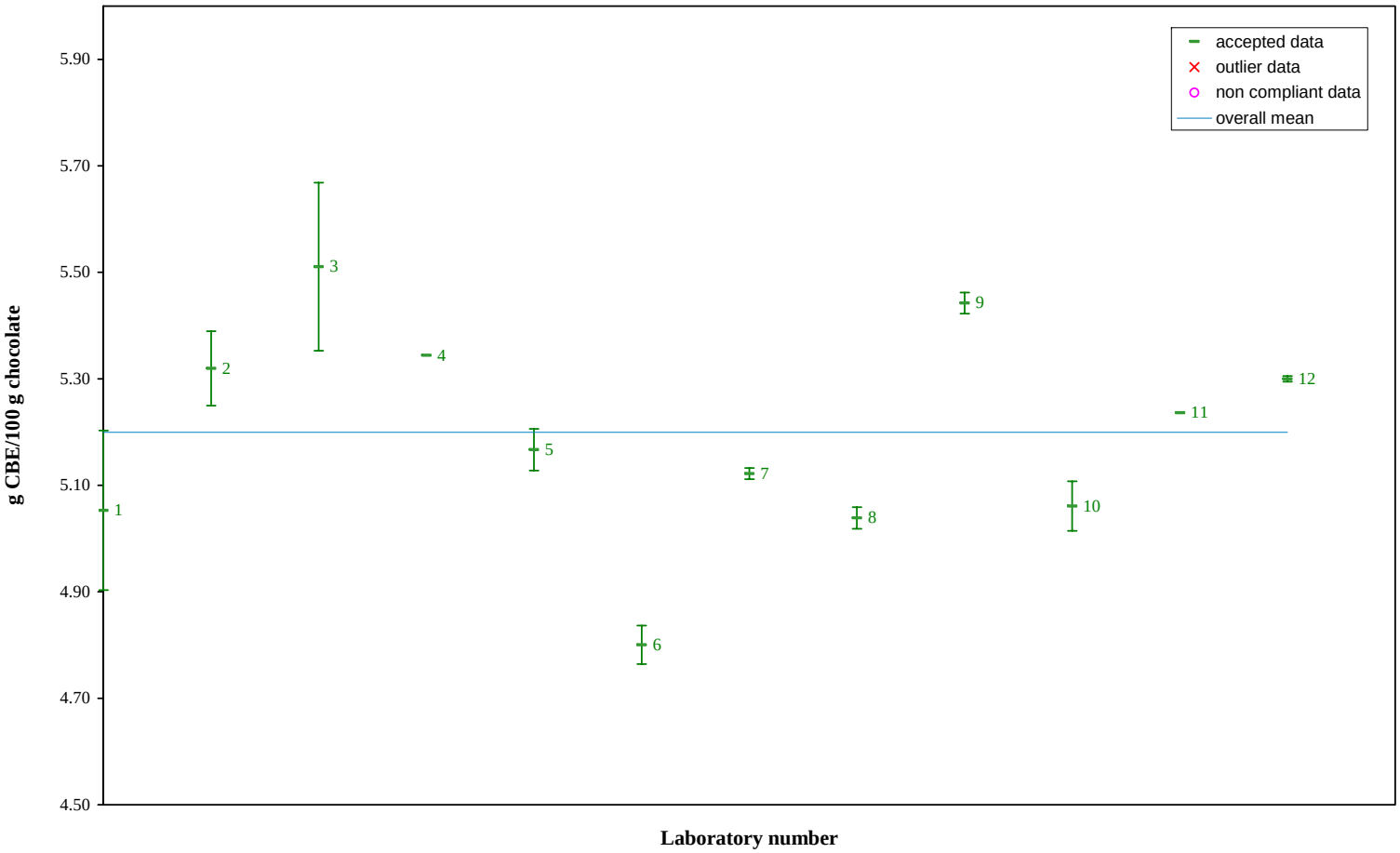


Figure G 54. Sample 5: Laboratory means and ranges of determined CBE amounts in chocolate (chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet fat extraction; blind duplicates)

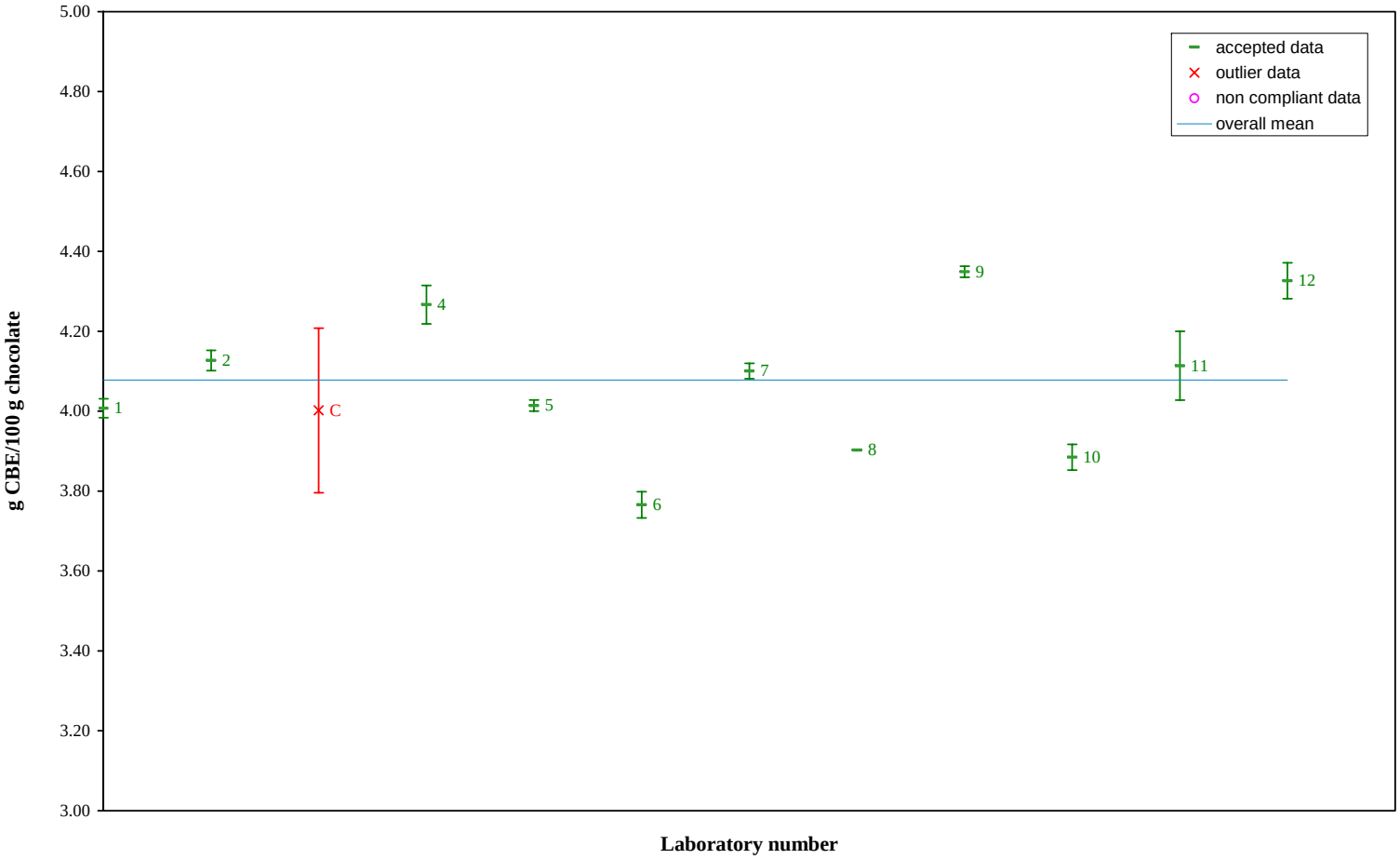


Figure G 55. Sample 6: Laboratory means and ranges of determined CBE amounts in chocolate (chocolate fat for GLC analysis obtained by rapid fat extraction; total fat content determined by Soxhlet fat extraction; blind duplicates)

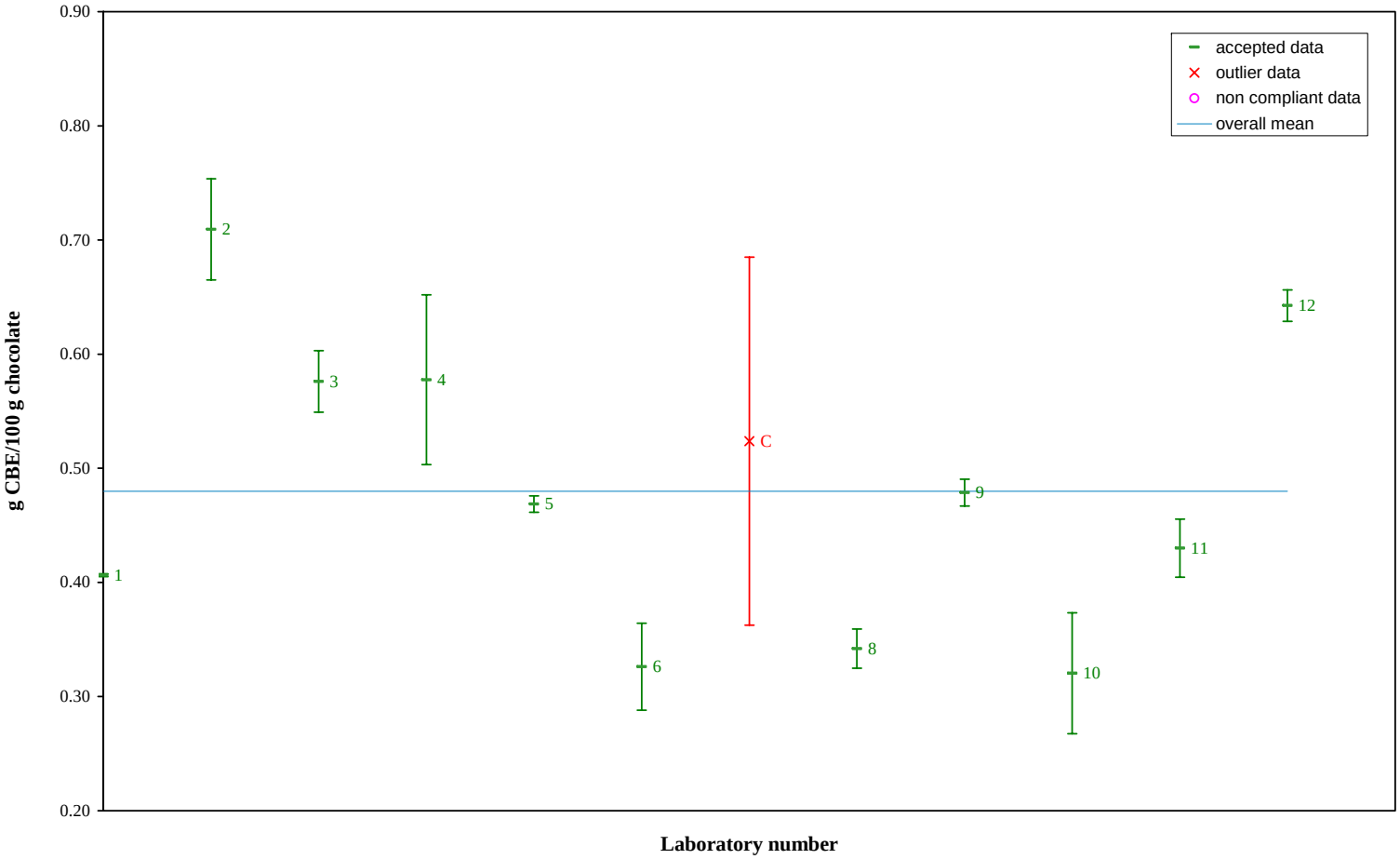


Figure G 56. Sample 9: Laboratory means and ranges of determined CBE amounts in chocolate (blind duplicates; assumed fat content of chocolate= 30 %)

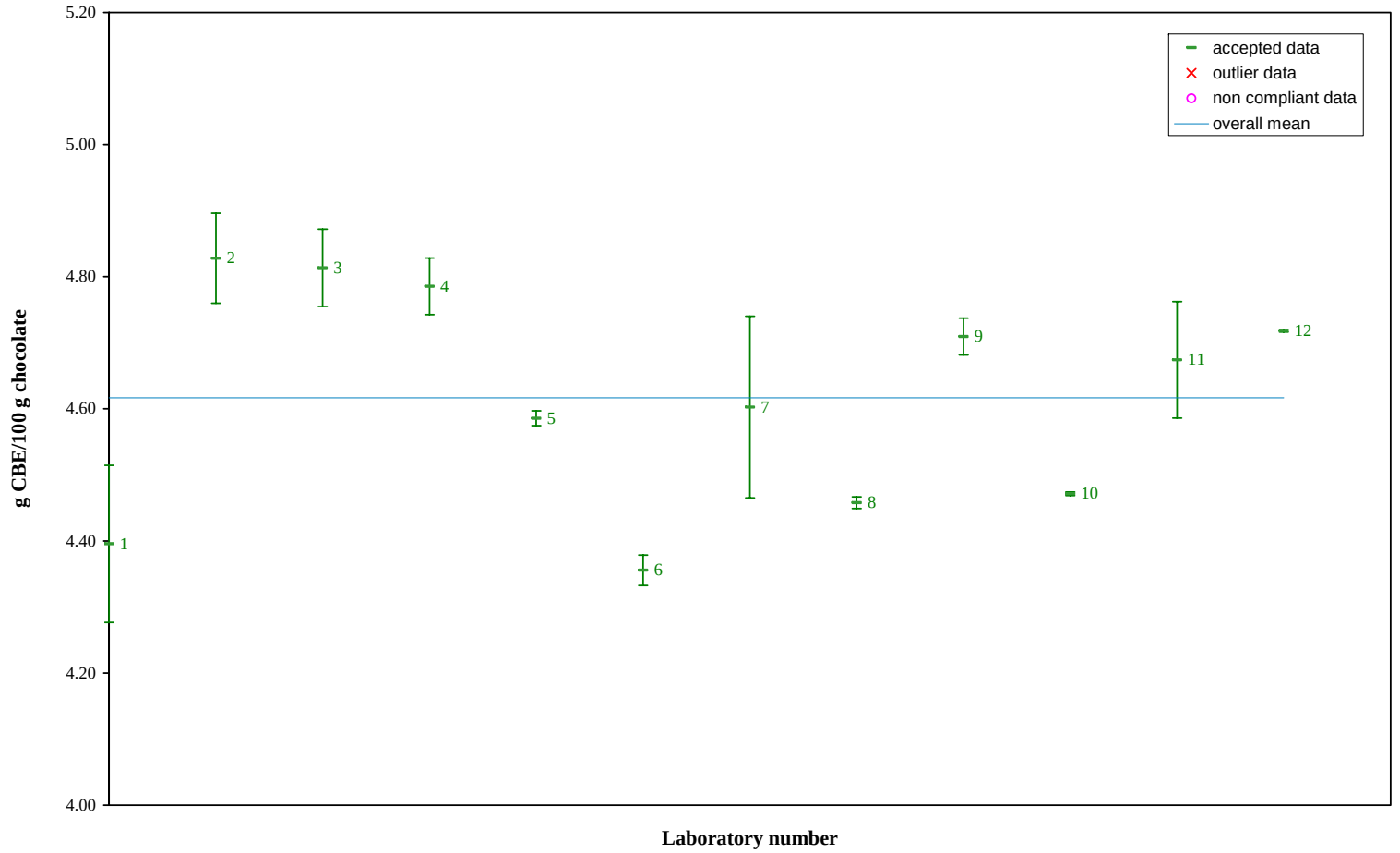


Figure G 57. Sample 10: Laboratory means and ranges of determined CBE amounts in chocolate (blind duplicates; assumed fat content of chocolate= 30 %)

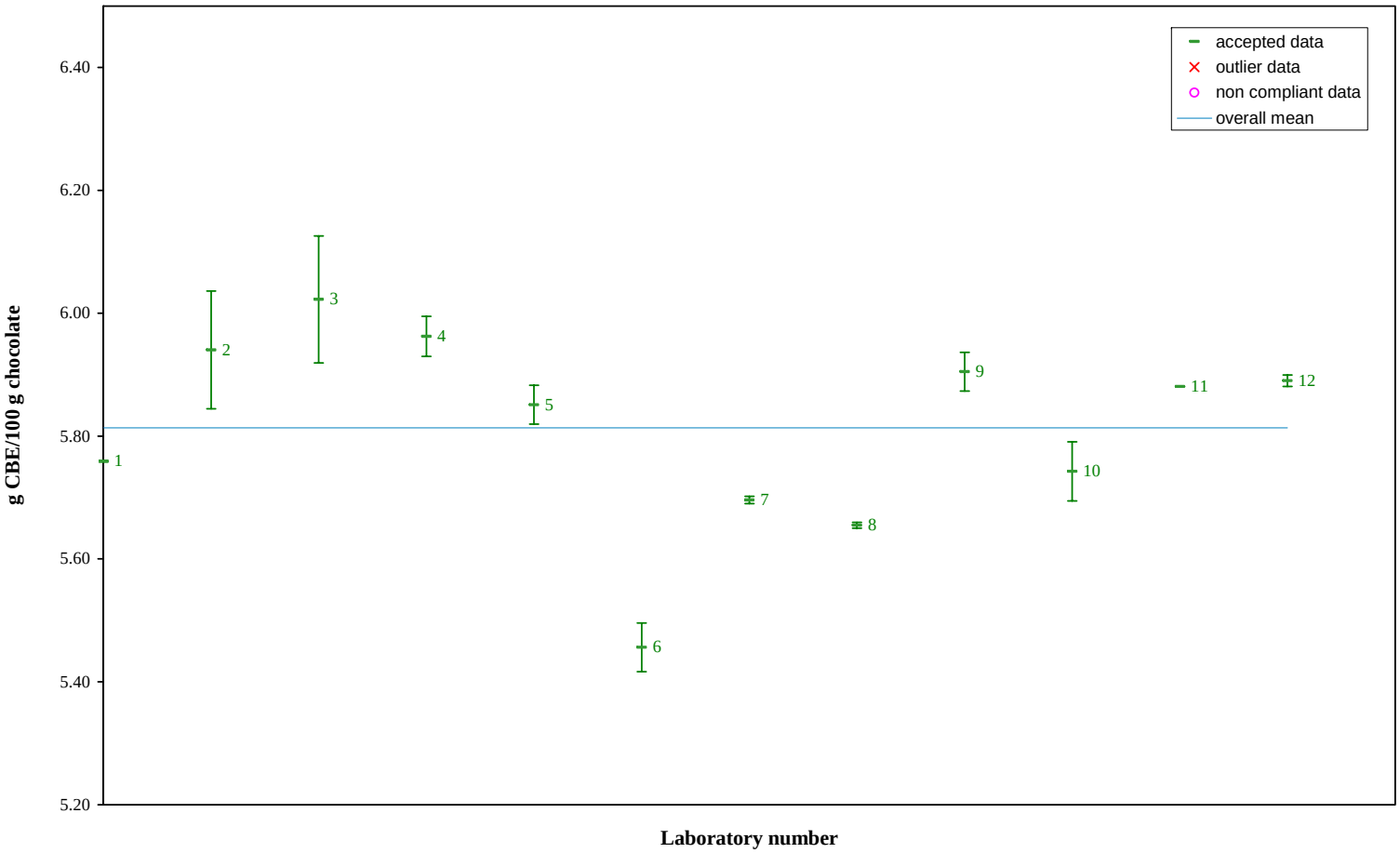


Figure G 58. Sample 11: Laboratory means and ranges of determined CBE amounts in chocolate (blind duplicates; assumed fat content of chocolate= 30 %)

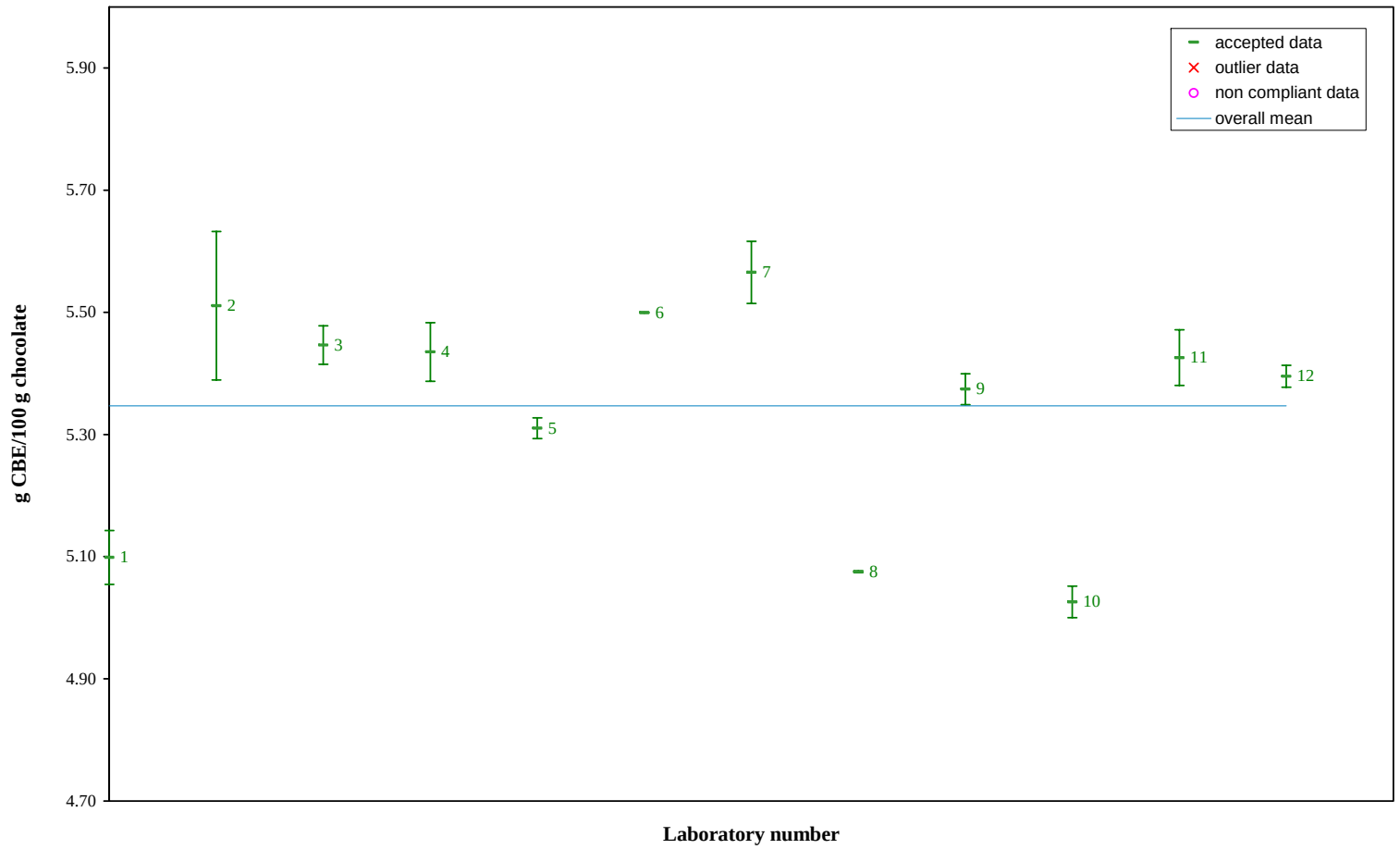


Figure G 59. Sample 12: Laboratory means and ranges of determined CBE amounts in chocolate (blind duplicates; assumed fat content of chocolate= 30 %)

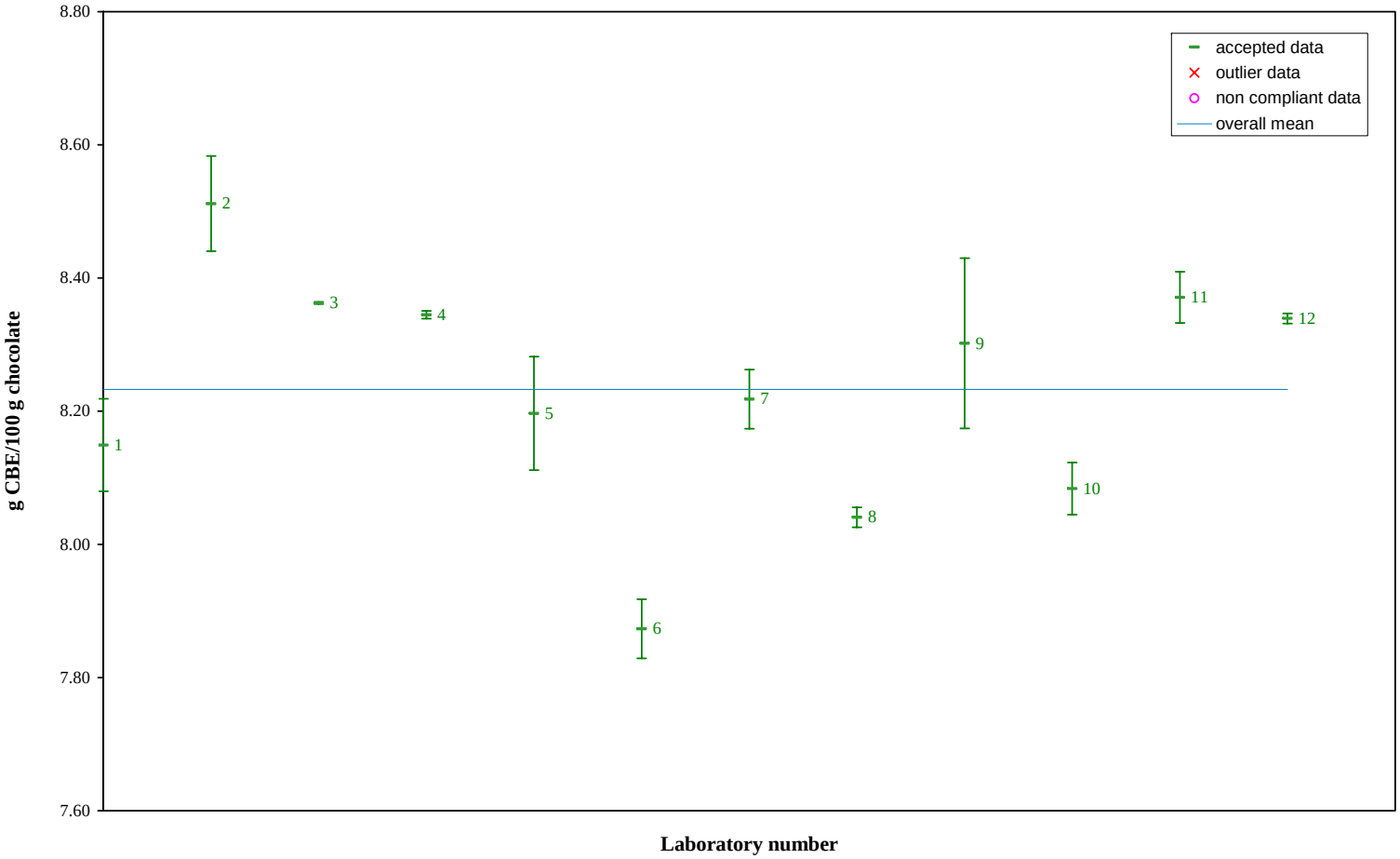


Figure G 60. Sample 13: Laboratory means and ranges of determined CBE amounts in chocolate (blind duplicates; assumed fat content of chocolate= 30 %)

European Commission

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Abstract

A European collaborative trial has been conducted to validate an analytical approach comprising method procedures for the determination of milk fat and the detection and the quantification of cocoa butter equivalents (CBEs) in milk chocolate. The whole approach is based on (i) comprehensive standardized databases covering the triacylglycerol composition of a wide range of authentic milk fat, cocoa butter as well as CBE samples and 947 gravimetrically prepared mixtures thereof, (ii) the availability of a certified cocoa butter reference material (IRMM-801) for calibration, (iii) an evaluation algorithm, which allows a reliable quantification of the milk fat content in chocolate using a simple linear regression model, (iv) a subsequent correction of triacylglycerols deriving from milk fat, (v) mathematical expressions to detect the presence of CBEs in milk chocolate, and (vi) a multivariate statistical formula to quantify the amount of CBEs in milk chocolate. The elaborated approach has the advantage that by performing a single triacylglycerol analysis using high resolution gas liquid chromatography several questions, needed to control correct labelling of milk chocolate, can be answered. Twelve laboratories participated in the validation study. CBE admixtures were detected down to a level of 0.5 g CBE/100 g chocolate, without false-positive or false-negative results. The applied model performed well at the statutory limit of 5 % CBE addition to chocolate with a prediction error of 0.7 %. The relative standard deviation for reproducibility (< 5 %) for quantification of CBEs did not show a difference for real chocolate samples and for chocolate fat solutions, demonstrating that the whole approach is applicable to real milk chocolate samples. The objective of the performed collaborative trial, i.e., to demonstrate that the defined method protocol is fit-for-purpose, was accomplished.

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