EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Food Safety and Quality Unit
B-2440 GEEL (Belgium)

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

Report on the Validation Study

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2003 **EUR 20777 EN**

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Luxembourg: Office for Official Publications of the European Communities, 2003
ISBN 92-894-6035-0
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Abstract

A European collaborative study has been conducted to validate an analytical procedure for the quantification of cocoa butter equivalents in cocoa butter and plain chocolate. In principle, the fat obtained from plain chocolate according to the Soxhlet principle is separated by high-resolution gas chromatography into triglyceride fractions according to their acyl-C-numbers, and within a given number, also according to unsaturation. The amount of cocoa butter equivalents in cocoa butter is estimated by Partial Least Squares regression analysis applied to the relative proportions of the five main triglycerides present in cocoa butter. Fifteen laboratories participated in the validation study. For all test samples the predicted cocoa butter equivalent amounts were in close agreement with the true values. The results of the ring test clearly demonstrated that the applied method performs well at the level of the statutory limit of 5% cocoa butter equivalent addition to chocolate with a prediction error of 0.4% to 0.6%, assuming a chocolate fat content of 20% to 30%.

Keywords: Cocoa butter, Cocoa butter equivalents, HR-GC, Triglyceride profile, Multilinear regression analysis, Quantification, Inter-laboratory validation study

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LIST OF ABBREVIATIONS

CB	cocoa butter
CBE	cocoa butter equivalent
CRM	certified reference material
HR-GC	high-resolution gas chromatography
PLS	Partial Least Squares
PMF	palm mid fraction
POO	1,2-dioleoyl-3-palmitoyl-rac-glycerol
POP	1,3-dipalmitoyl-2-oleoylglycerol
POS	1-palmitoyl-2-oleoyl-3-stearoylglycerol
r	repeatability
R	reproducibility
RSD_r	repeatability relative standard deviation
RSD_R	reproducibility relative standard deviation
RMSE	root mean square error
SOO	1,2-dioleoyl-3-stearoyl-rac-glycerol
SOS	1,3-distearoyl-2-oleoylglycerol
s_r	repeatability standard deviation
s_R	reproducibility standard deviation
TG	triglyceride

1 RATIONALE – BACKGROUND

The new European Chocolate Directive 2000/36/EC [1] allows the addition of up to 5% of vegetable fats other than cocoa butter (CB), the so-called cocoa butter equivalents (CBEs), to chocolate. Permitted fats derive from palm oil, illipé (borneo tallow or tengkawang), sal, shea, kokum gurgi, and mango kernel.

Member States' laws, regulations and administrative provisions need to comply with the new Chocolate Directive before August 2003. The product label has to provide a correct, neutral and objective indication of the presence of such substances, and must not mislead the consumer. On the other hand, the Directive does not preclude the labelling of chocolate products to indicate that CBEs have not been added (so called 'negative labelling'). Unfortunately, the Directive does not cover aspects regarding methods of analysis for law enforcement. Due to their similarity to genuine CB, CBEs are difficult to detect. For detection purpose the Institute of Reference Materials and Measurements (IRMM) of the Joint Research Centre (JRC) has recently elaborated and validated a suitable method [2]. However, the monitoring of wrongful labelling and the protection against fraud requires not only detection methods for the presence of added CBEs, but also methods that can achieve a reliable quantification of these fats.

The JRC developed an analytical approach using chemometrics for the prediction of the CBE amount in cocoa butter and plain chocolate [3]. To this end, the triglyceride composition of a range of genuine cocoa butter samples as well as raw materials for CBE production and formulated CBEs was determined by using a gas chromatography capillary column coated with a medium polarity stationary phase. In order to ensure high accuracy a reference material certified for its triglyceride composition (IRMM-801) was used for calibration purpose. By using this standardised data base, CB/CBE blends with a known composition were arithmetically prepared in order to model a relation between the individual triglycerides measured and the amount of CBEs present in CB. Seven different cocoa butters, representing the whole range of the biological variability of analysed cocoa butter samples, were selected to compute mixtures containing 10%, 15% and 20% CBEs. This would translate to levels of CBEs in chocolate of about 2% to 3%, 3% to 4.5% and 4% to 6%, respectively, assuming a fat content of chocolate of 20% to 30%.

2 OBJECTIVE

- To conduct an international collaborative study in order to validate a gas chromatography-based method combined with PLS regression analysis to quantify the added amount of CBEs in cocoa butter and plain chocolate.
- To demonstrate high comparability of the results between laboratories and commutability of the elaborated approach by using a matrix material of genuine cocoa butter with a certified triglyceride profile.

3 METHOD DESCRIPTION

Cocoa butter, or the fat obtained from plain chocolate according to the Soxhlet principle, is separated by high-resolution gas chromatography (HR-GC) into triglyceride fractions according to their molecular weight and degree of unsaturation using a thermostable polarizable capillary column. The content of the five main triglycerides is normalised so that the sum of POP, POS, POO, SOS, SOO equals 100% (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). PLS regression analysis applied to the relative proportions of the five main triglycerides is used to estimate the CBE content on the basis of cocoa butter. The regression model is given in Equation 1. The amount of CBE present in cocoa butter is approximated by a linear combination of the predictors, i.e. the individual triglycerides.

Equation 1:

%CBE in CB = 37.439 + 1.175 x POP – 1.939 x POS – 0.121 x POO + 0.982 x SOS – 0.097 x SOO

PLS, a well-known method for multivariate statistical data evaluation proved to be the method of choice to model a relation between the relative proportions of the five main triglycerides measured and the amount of CBEs present in cocoa butter. The statistical analysis was performed using the Unscrambler® 7.6 version (CAMO ASA, Oslo, Norway). Arithmetic mixtures, prepared on the basis of a carefully elaborated data base of the triglyceride profile of individual genuine cocoa butter and CBE samples, were used in order set up a calibration set of 882 CB/CBE blends.

The effectiveness of the elaborated regression model for future predictions was checked by means of various validation data sets and the resulting standard error for prediction, which is given by the root mean square error (RMSE). This is a measurement of the average difference between predicted and actual CBE content in cocoa butter. It is interpreted as the average modelling error, expressed in the same units as the original values (g CBE/100 g CB). The computed RMSE for the determination of all possible CBE/CB blends was 1.1%. For ca 73% of the analysed CBE/CB blends the resulting prediction error was covered by the computed average RMSE of 1.1%. Assuming a fat content of chocolate of 20% to 30% this figure translates to an average prediction error of 0.22% to 0.33% CBE. Eighty-five % of all CBE proportions in CB were predicted with errors ranging from ±2% corresponding to cocoa butter, or ±0.4% related to the final product chocolate (fat content 20%) or ±0.6% (fat content 30%). By restricting the model to commercially available CBEs, 85% of the blends were predicted with errors ranging from ±1% CBE in cocoa butter, or ±0.2% CBE related to the final product chocolate (fat content 20%) to ±0.3% (fat content 30%). For 99% of all analysed commercial CBEs in mixture with CB the resulting prediction error was within ±2%. Assuming a fat content of chocolate of 20% to 30%, this figure translates to an uncertainty of ±0.4% to ±0.6% CBE.

Furthermore, the efficiency of the regression models to assess compliance with the statutory limit was scrutinised by checking the number of correctly, false positively and false negatively classified samples for different action levels. For an action level of 5.4% CBE (=statutory limit of 5% plus a safety margin of 0.4%) no false positive result, i.e. an unjustified legal action, was observed. This indicates that samples with a predicted CBE amount of 5.4% of any unknown CBE could be identified as non-compliant with the label declaration.

The CBE content on the basis of the final product, expressed in grams of CBE per 100 grams of chocolate, is calculated by determining the fat content of the chocolate according to the AOAC Official Method 963.15 [4].

4 PARTICIPANTS

4.1 Co-ordination of the validation study

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Geel (B)

4.2 Preparation and distributing of the test samples

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4.3 Homogeneity tests

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4.5 Collation and statistical evaluation of results

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Geel (B)

5 TEST SAMPLES

Cocoa butter and CBE samples (Table 1) were obtained from the cocoa processing industry and other vegetable fat producers. The chocolate sample was purchased in a retail store in the UK. Nine mixtures containing 15%, 25% and 30% of CBE in pure cocoa butter were gravimetrically prepared (Table 2), dissolved in iso-octane and sent to the participating laboratories.

The CBEs included in the study consisted of a variety of commercially available fats. Illipé fat, which resembles CB very closely and poses therefore the biggest problem regarding detection and quantification, was used in combination with other CBEs.

Furthermore, one chocolate sample, which was labelled to contain vegetable, had to be measured. In order to obtain the CBE amount on the basis of the final product, the fat content of the chocolate sample was determined by the JRC according to the AOAC Official Method 963.15 [4] by triplicate determination resulting in a fat content of 26.34%.

All samples were randomly coded (Table A 1, Annex A). One ampoule of the cocoa butter CRM (IRMM 801) was provided for calibration purposes and system suitability check.

Table 1: Genuine CBs and CBEs used for the preparation of CB/CBE mixtures

CB No.	*Country Origin*	*CBE No.*	*Type [composition; %]*
I	Grenada	I	PMF/Sal/Mango [50/25/25]
II	Ghana	II	PMF/Shea [50/50]
III	Ivory Coast/Nigeria/Cameroon	III	PMF/(Shea + Illipé) [35/65]

Table 2: Samples used for the study

Sample No.	*CB used*	*CBE used*	*CB [%]*	*CBE [%]*
14	CB I	CBE III	85.21	14.79
15	CB I	CBE III	75.08	24.92
16	CB I	CBE III	69.99	30.01
17	CB II	CBE II	84.91	15.09
18	CB II	CBE II	75.10	24.90
19	CB II	CBE II	69.99	30.01
20	CB III	CBE I	85.03	14.97
21	CB III	CBE I	75.03	24.97
22	CB III	CBE I	70.04	29.96
12	Cadbury's Bournville: The original plain chocolate [ingredients: vegetable fat]			

5.1 Homogeneity study

The CB/CBE blends dissolved in iso-octane were considered to be homogeneous. Homogeneity of the chocolate sample was mandatory in order to make matrix data from various laboratories comparable too. The homogeneity test was carried out as described in the preceding European report on the validation study for detection purpose [2]. All tests confirmed that the between-units inhomogeneity was insignificant ($P>0.05$). Consequently, homogeneity of the chocolate sample was considered to be sufficient and fit for purpose to be used as test material for the validation study.

6 THE VALIDATION STUDY

6.1 Design of the validation study

Since the analytical part of the detection and quantification methods are based on the same measuring principle, i.e. triglyceride profiling by HR-GC, the samples were analysed within the same ring test that has been conducted to validate the analytical procedure for the detection of CBEs in cocoa butter and plain chocolate [2]. Differences in the standard operation procedure of the two methods exist in the number of individual triglycerides used for data treatment and in the mathematical evaluation of the data. The participants were requested to submit the raw data of the individual triglycerides. Using the submitted raw data the mathematical calculations for the prediction of the present CBE amounts were performed later on by the JRC.

6.2 Analysis of test samples

Before analysing the test samples the participants had to check the system suitability, i.e. a sufficient resolution of the critical pairs POS/POO and SOS/SOO and the determination of reliable response factors of the five main triglycerides. The test samples had to be analysed in random order. Response factors for the five main triglycerides (POP, POO, POS, SOS, SOO) had to be determined before analysing the first test sample and after every 10^{th} analyses by using the cocoa butter CRM (IRMM 801). The sample number, retention time and the area counts of individual triglyceride fractions had to be reported using an electronic spreadsheet provided by the JRC. In order to avoid any transcription errors, the raw data were later on automatically applied to the proposed algorithm (Equation 1) by using the electronic spreadsheet.

6.3 Technical evaluation of the results submitted

The results of the individual laboratories were examined along with the submitted raw data and chromatograms. Based on the technical evaluation of the results, 13 data sets out of the 15 submitted were accepted; laboratory 14 did not comply with the requirements of the requested chromatographic system and laboratory 15 failed to determine proper and repeatable response factors demonstrating a sound functioning of the chromatographic system. Details on the employed chromatographic systems and the requested performance criteria are given in the report on the validation study for the detection of CBEs in cocoa butter and plain chocolate [2].

6.4 Statistical evaluation of the results submitted

The individual results accepted on technical grounds as submitted by the participants are given in form of tables in Annex A (Tables A 2 to A 11). For each data set, identified by a laboratory code, the five major triglycerides i.e. POP, POS, POO, SOS and SOO, normalised to 100%, are listed. Furthermore, the estimated CBE amounts in the fat blends determined by applying Equation 1, are given in the same Tables.

The resulting laboratory means of the quantified CBE amounts are plotted in increasing order with the corresponding range (Figures A 1 to A 10, Annex A).

The data sets accepted on technical grounds were subjected to statistical tests as described in ISO 5725:

- Cochran test to identify outlying variances
- Single Grubbs and double Grubbs test to detect outlying data set averages.

In Tables A 12 to A 14 all data accepted on technical grounds were included in the computation of precision figures, while Tables A 15 to A 17 contain the results of the statistical evaluation performed after removal of the detected outliers (Annex A). By removing statistical outliers the relative standard deviation of reproducibility (RSD_R) for the quantification of the CBE amount was reduced from 2.8% to 2.4% for CB/CBE blends and from 5.6% to 4.4% for the chocolate sample.

The calculated HORRAT values that can be used as a guide to determine the acceptability of the precision of a method ranged from 0.5 to 1.6, demonstrating a good performance of the method.

7 RESULTS

Table 3 gives a brief outline of the resulting range of laboratory mean values and the respective standard deviation of reproducibility after removal of statistical outliers. The data relate to the proportional CBE in CB/CBE blends. Tables 4 and 5 list the same results based on the final product chocolate corresponding to fat contents of 20% and 30%, respectively.

Table 3: Comparison of predicted and actual values including the range of laboratory mean values and respective standard deviation of reproducibility and RSD_R (results reported in g CBE/100 g CB) obtained by the PLS model

Sample Number	*actual value*	*predicted value (range of means)*	*bias*	s_R	RSD_R
14	14.79	15.14 (14.57 - 15.61)	-0.4	0.36	2.4
15	24.92	24.80 (24.01 - 25.41)	0.1	0.41	1.7
16	30.01	29.56 (28.58 - 30.03)	0.4	0.54	1.8
17	15.09	16.63 (16.31 - 16.88)	-1.6	0.23	1.4
18	24.90	25.87 (25.14 - 26.44)	-1.0	0.43	1.7
19	30.01	30.69 (30.03 - 31.39)	-0.7	0.45	1.5
20	14.97	15.65 (15.18 - 16.12)	-0.7	0.31	2.0
21	24.97	24.17 (23.46 - 24.62)	0.8	0.36	1.5
22	29.96	28.53 (27.75 - 28.94)	1.4	0.41	1.4
12	unknown	13.99 (12.70 - 15.04)	-	0.61	4.4

The obtained over-all mean values were in close agreement with the known values. The differences between the predicted and true values were well within the expected range of ±2%. Assuming a fat content of chocolate of 20% or 30% this translates to ±0.4% or ±0.6%, respectively. The efficiency test of the method based on arithmetic mixtures has shown that for 99% of the commercial CBEs when blended with CB and 85% of all CBEs in mixture with CB the resulting error for predicting the CBE percentage in the blends was ±2%. Hence, the results of the ring test clearly demonstrated conformity with the results obtained by the elaborated model based on arithmetically simulated mixtures.

The predicted amount of vegetable fat of the real chocolate sample was 13.99 g/100 g on fat basis. The determined fat content of the chocolate was 26.34%, resulting in 3.68 g CBE/100 g chocolate. Therefore, this sample was considered to be compliant with the statutory limit of 5%.

Table 4: Comparison of predicted and actual values on the basis of the final product chocolate, assuming a chocolate fat content of 20 % (results reported in g CBE/100 g chocolate) obtained by PLS model

Sample Number	*actual value*	*predicted value (range of means)*	*bias*
14 [1)]	2.96	3.03 (2.91 – 3.12)	-0.07
15 [1)]	4.98	4.96 (4.80 – 5.08)	0.02
16 [1)]	6.00	5.91 (5.72 – 6.01)	0.09
17 [1)]	3.02	3.33 (3.26 – 3.38)	-0.31
18 [1)]	4.98	5.17 (5.03 – 5.29)	-0.19
19 [1)]	6.00	6.14 (6.01 – 6.28)	-0.14
20 [1)]	2.99	3.13 (3.04 – 3.22)	-0.14
21 [1)]	4.99	4.83 (4.69 – 4.92)	0.16
22 [1)]	5.99	5.71 (5.55 – 5.79)	0.28
12 [2)]	unknown	3.68 (3.35 – 3.96)	

[1)] assumed fat content of chocolate is 20%
[2)] determined fat content of chocolate is 26.34%

Table 5: Comparison of predicted and actual values on the basis of the final product chocolate, assuming a chocolate fat content of 30 % (results reported in g CBE/100 g chocolate) obtained by PLS model

Sample Number	*actual value*	*predicted value (range of means)*	*bias*
14 [1)]	4.44	4.54 (4.37 – 4.68)	-0.10
15 [1)]	7.48	7.44 (7.20 – 7.62)	0.04
16 [1)]	9.00	8.87 (8.57 – 9.01)	0.13
17 [1)]	4.53	4.99 (4.89 – 5.06)	0.46
18 [1)]	7.47	7.76 (7.54 – 7.93)	-0.29
19 [1)]	9.00	9.21 (9.01 – 9.42)	-0.21
20 [1)]	4.49	4.70 (4.55 – 4.84)	-0.21
21 [1)]	7.49	7.25 (7.04 – 7.39)	0.24
22 [1)]	8.99	8.56 (8.33 – 8.68)	0.43
12 [2)]	unknown	3.68 (3.35 – 3.96)	

[1)] assumed fat content of chocolate is 30%
[2)] determined fat content of chocolate is 26.34%

The method was validated for CBE levels close to the statutory addition limit of 5% CBE in the final product chocolate, i.e. admixtures of 15, 25 and 30 g CBE/100 g cocoa butter corresponding to 3, 5 and 6 g (assumed fat content of chocolate 20%) or 4.5, 7.5 and 9 g on chocolate basis (assumed fat content of chocolate 30%).

8 CONCLUSIONS

The results of the collaborative study showed that the analytical approach (triglyceride profiling by HR-GC and data treatment by PLS regression analysis) is a reliable method for the quantification of CBEs in cocoa butter and plain chocolate. The outcome of the ring test proved that the model elaborated in-house by the JRC on the basis of arithmetically simulated mixtures can be successfully used by individual testing laboratories. Comparability of the results between individual laboratories and commutability with the elaborated model is maintained by using the cocoa butter CRM (IRMM 801).

The chocolate Directive allows the addition of up to 5% vegetable fats in chocolate. By applying the validated method samples containing more than 5.4% of any unknown CBE can be identified as being non-compliant with label declaration (fat content of chocolate assumed to be 20%). By using an action level of 5.4%, no false positive result was obtained. The set of samples used to derive the above mentioned figures represented the whole range of CB samples (soft, hard and commercial mixtures thereof) and a large range of various CBEs, including CBEs containing illipé fat. The only restriction to the sample set was the exclusion of pure illipé fat, since it is irrelevant in practical terms as admixture to chocolate.

The main advantage of the tested methodological approach is that the end user has just to calibrate the gas chromatographic separation system using the certified reference material (IRMM 801), separate the sample in question and use the mathematical equation for subsequent data treatment in order to quantify the CBE amount present in cocoa butter or plain chocolate. In combination with the preceding method for detection an important measure to assess compliance with labelling provisions is offered.

9 LITERATURE

[1] Directive 2000/36/EC of the European Parliament and the Council of 23 June 2000 relating to cocoa and chocolate products intended for human consumption. OJ L197, 19-25.

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

[3] Buchgraber, M., Anklam, E. (2003) publication in preparation.

[4] Official Methods of Analysis of AOAC International (1995) 16th Edition. Volume II. Chapter 31. AOAC Official Method 963.15.

ANNEX A

Table A 1: Sample codes for individual laboratories

Tables A 2 – A 11: Results accepted on technical grounds

Figures A 1 – A 10: Bar-charts of results accepted on technical grounds

Tables A 12 – A 14: Statistical evaluation of results accepted on technical grounds

Tables A 15 – A 17: Statistical evaluation of results accepted on technical and statistical grounds

Table A 1: Sample codes for individual laboratories

	Sample number									
	12	14	15	16	17	18	19	20	21	22
Lab code	Labelled Sample Code									
1	137	77	98	240	152	190	268	205	257	56
2	134	71	87	233	145	188	265	215	246	57
3	122	62	93	230	153	199	278	207	253	54
4	140	73	97	234	156	184	264	217	249	59
5	135	69	83	221	142	193	276	210	241	60
6	124	66	94	239	154	186	277	214	247	43
7	132	65	89	225	144	189	279	218	248	41
8	139	74	99	229	141	197	266	206	254	42
9	133	67	81	222	157	196	274	212	245	55
10	125	72	96	226	149	191	273	208	256	50
11	136	75	88	231	158	183	275	219	250	53
12	138	70	84	236	148	195	270	202	242	58
13	129	78	100	237	155	187	272	220	255	51
14	131	68	92	235	146	194	263	201	260	45
15	121	61	95	232	143	192	262	203	243	44

Table A 2: Results accepted on technical grounds for sample 14 (two replicates)

	POP [1]		POS [1]		POO [1]		SOS [1]		SOO [1]		CBE addition [2]	
Lab	A	B	A	B	A	B	A	B	A	B	A	B
1	21.18	21.15	41.06	41.07	1.76	1.77	33.30	33.27	2.70	2.74	14.93	14.84
2	21.08	21.04	41.09	41.10	1.86	1.86	33.16	33.20	2.81	2.81	14.59	14.55
3	20.93	20.76	40.90	40.85	1.65	1.68	33.69	33.81	2.83	2.90	15.32	15.34
4	20.77	20.67	40.74	40.79	1.74	1.75	33.92	33.93	2.83	2.85	15.66	15.46
5	21.06	21.01	41.30	41.16	1.60	1.59	33.52	33.54	2.52	2.70	14.57	14.79
6	20.70	20.45	41.14	41.33	1.66	1.84	34.00	33.71	2.50	2.67	14.92	13.95
7	20.45	20.56	41.21	41.25	1.54	1.54	34.33	34.17	2.47	2.48	14.84	14.72
8	20.83	21.14	40.84	40.93	1.73	1.71	33.81	33.47	2.80	2.75	15.43	15.29
9	20.82	20.82	40.85	40.90	1.64	1.63	33.61	33.64	2.47	2.66	15.25	15.16
10	20.98	20.93	40.80	40.98	1.80	1.69	33.60	33.58	2.81	2.81	15.47	15.05
11	20.96	20.78	40.59	40.83	2.05	1.82	33.69	33.74	2.71	2.82	15.91	15.31
12	20.71	20.69	40.88	40.88	1.66	1.67	33.86	33.92	2.88	2.84	15.27	15.30
13	20.85	21.00	40.86	40.88	1.79	1.82	33.60	33.46	2.90	2.83	15.19	15.19

Table A 3: Results accepted on technical grounds for sample 15 (two replicates)

	POP [1]		POS [1]		POO [1]		SOS [1]		SOO [1]		CBE addition [2]	
Lab	A	B	A	B	A	B	A	B	A	B	A	B
1	22.11	22.10	37.73	37.72	1.76	1.77	35.43	35.45	2.98	2.97	24.54	24.55
2	21.93	21.89	37.82	37.71	1.72	1.80	35.58	35.56	2.95	3.03	24.30	24.44
3	21.95	21.83	37.68	37.60	1.67	1.89	35.86	35.68	2.85	3.00	24.89	24.70
4	21.47	21.29	37.46	37.54	1.67	1.65	36.44	36.56	2.96	2.96	25.31	25.07
5	21.89	21.94	37.85	37.61	1.62	1.65	35.72	35.77	2.93	3.03	24.35	24.93
6	21.73	21.73	37.63	37.90	1.88	1.70	35.92	36.01	2.84	2.66	24.77	24.37
7	21.54	21.64	38.08	38.01	1.56	1.59	36.14	36.03	2.69	2.73	23.94	24.09
8	21.68	21.98	37.44	37.54	1.67	1.73	36.22	35.79	2.99	2.97	25.38	25.11
9	21.89	21.87	37.56	37.53	1.66	1.69	35.82	35.88	2.88	2.98	25.01	25.11
10	21.72	21.82	37.67	37.77	1.57	1.67	35.99	35.81	3.05	2.93	24.77	24.51
11	22.00	21.97	37.56	37.47	1.53	1.66	35.99	36.04	2.91	2.87	25.32	25.50
12	21.84	21.83	37.55	37.63	1.73	1.68	35.81	35.88	3.07	2.97	24.93	24.85
13	22.20	21.96	37.47	37.54	1.81	1.73	35.36	35.67	3.16	3.10	25.06	24.95

Table A 4: Results accepted on technical grounds for sample 16 (two replicates)

	POP [1]		POS [1]		POO [1]		SOS [1]		SOO [1]		CBE addition [2]	
Lab	A	B	A	B	A	B	A	B	A	B	A	B
1	22.35	22.33	35.94	35.94	1.73	1.74	36.90	36.92	3.08	3.07	29.72	29.73
2	22.45	22.51	36.12	36.21	1.98	1.96	36.14	35.99	3.31	3.33	28.71	28.45
3	22.20	22.25	35.86	35.89	1.73	1.69	37.12	37.05	3.09	3.12	29.92	29.86
4	21.98	22.10	35.80	35.81	1.72	1.65	37.18	37.36	3.32	3.08	29.81	30.16
5	22.51	22.32	35.96	35.92	1.70	1.64	36.72	36.91	3.11	3.21	29.71	29.75
6	22.22	22.09	35.92	36.17	1.53	1.57	37.34	37.23	2.98	2.94	30.08	29.35
7	22.34	22.42	36.38	36.40	1.66	1.66	36.72	36.61	2.90	2.91	28.70	28.66
8	22.65	22.50	35.84	35.82	1.72	1.70	36.64	36.83	3.15	3.15	30.01	30.06
9	22.35	22.33	35.89	35.88	1.71	1.65	36.82	37.01	3.11	3.21	29.76	29.93
10	22.35	22.51	35.79	36.02	1.80	1.71	36.97	36.94	3.09	2.82	30.08	29.84
11	22.42	22.41	35.99	35.88	1.52	1.70	37.13	36.99	2.94	3.02	29.99	30.01
12	22.21	22.22	36.13	36.18	1.72	1.76	36.83	36.75	3.11	3.10	29.14	28.96
13	22.25	22.43	36.17	36.31	1.60	1.82	37.14	36.56	2.84	2.89	29.45	28.79

(1) g TG / 100 g total TGs (total TGs = POP+POS+POO+SOS+SOO = 100 %)
(2) g CBE / 100 g CB

Table A 5: Results accepted on technical grounds for sample 17 (two replicates)

	POP [1]		POS [1]		POO [1]		SOS [1]		SOO [1]		CBE addition [2]	
Lab	A	B	A	B	A	B	A	B	A	B	A	B
1	21.47	21.48	39.78	39.80	2.63	2.64	32.32	32.29	3.80	3.79	16.58	16.53
2	21.54	21.39	39.99	39.81	2.72	2.60	32.01	32.56	3.74	3.65	15.94	16.67
3	21.50	21.44	39.81	39.74	2.61	2.63	32.33	32.39	3.75	3.80	16.56	16.69
4	21.22	21.28	39.79	39.77	2.47	2.47	32.67	32.63	3.85	3.84	16.62	16.70
5	21.43	21.48	39.40	39.62	2.61	2.61	32.58	32.42	3.98	3.88	17.52	16.98
6	21.32	21.60	39.87	39.85	2.61	2.65	32.43	32.42	3.77	3.48	16.35	16.72
7	21.45	21.38	40.37	40.20	2.32	2.45	32.47	32.45	3.39	3.53	15.64	15.83
8	21.64	21.63	39.66	39.67	2.68	2.72	32.18	32.14	3.85	3.84	16.85	16.79
9	21.44	21.45	39.78	39.77	2.63	2.63	32.68	32.51	3.98	3.88	16.88	16.74
10	21.58	21.58	39.89	39.71	2.62	2.69	32.25	32.43	3.67	3.59	16.44	16.96
11	21.33	21.33	39.66	39.57	2.68	2.80	32.52	32.47	3.81	3.83	16.83	16.93
12	21.42	21.34	39.89	39.84	2.51	2.49	32.43	32.52	3.75	3.80	16.43	16.52
13	21.44	21.55	39.81	39.85	2.59	2.58	32.38	32.28	3.79	3.74	16.55	16.50

Table A 6: Results accepted on technical grounds for sample 18 (two replicates)

	POP [1]		POS [1]		POO [1]		SOS [1]		SOO [1]		CBE addition [2]	
Lab	A	B	A	B	A	B	A	B	A	B	A	B
1	23.62	23.62	36.70	36.71	2.72	2.71	33.17	33.16	3.80	3.80	25.89	25.87
2	23.61	23.65	36.90	36.82	2.68	2.65	33.12	33.27	3.68	3.60	25.47	25.82
3	23.59	23.72	36.75	36.70	2.70	2.67	33.14	33.19	3.82	3.72	25.72	26.04
4	23.42	23.48	36.70	36.87	2.51	2.36	33.57	33.61	3.80	3.68	26.09	25.90
5	23.91	23.53	36.60	36.69	2.66	2.64	33.06	33.17	3.77	3.97	26.34	25.81
6	23.36	23.69	37.48	36.80	2.66	2.68	32.98	33.16	3.52	3.67	23.93	25.79
7	23.70	23.70	37.19	37.18	2.45	2.46	33.20	33.18	3.46	3.48	25.15	25.13
8	23.86	23.66	36.58	36.53	2.76	2.76	32.93	33.21	3.87	3.85	26.16	26.30
9	23.53	23.59	36.70	36.66	2.69	2.66	33.15	33.26	3.77	3.98	25.78	26.02
10	23.83	23.77	36.68	36.53	2.44	2.72	33.35	33.24	3.70	3.74	26.40	26.48
11	23.49	23.60	36.72	36.66	2.64	2.60	33.35	33.50	3.79	3.63	25.89	26.32
12	23.57	23.80	36.82	36.75	2.48	2.49	33.43	33.24	3.70	3.72	25.89	26.11
13	23.59	23.68	37.20	36.92	2.65	2.54	32.98	33.20	3.58	3.66	24.75	25.60

Table A 7: Results accepted on technical grounds for sample 19 (two replicates)

	POP [1]		POS [1]		POO [1]		SOS [1]		SOO [1]		CBE addition [2]	
Lab	A	B	A	B	A	B	A	B	A	B	A	B
1	24.71	24.77	35.03	35.05	2.82	2.75	33.55	33.61	3.89	3.83	30.76	30.88
2	24.69	24.78	35.25	35.13	2.65	2.72	33.79	33.75	3.62	3.62	30.59	30.90
3	24.65	24.70	34.95	35.00	2.68	2.74	33.89	33.76	3.83	3.80	31.21	31.03
4	24.51	24.65	35.16	35.00	2.48	2.54	34.09	33.89	3.76	3.92	30.86	31.13
5	24.72	24.77	35.10	35.22	3.02	2.94	33.20	32.98	3.96	4.08	30.28	29.87
6	24.71	24.88	35.27	35.37	2.48	2.40	34.10	33.94	3.45	3.41	30.94	30.78
7	24.77	24.77	35.36	35.38	2.73	2.72	33.37	33.36	3.77	3.77	30.06	30.00
8	25.16	24.08	35.00	34.95	2.73	2.68	33.30	34.40	3.81	3.89	31.12	31.03
9	24.79	24.80	35.13	35.13	3.05	2.97	33.29	33.07	3.96	4.08	30.38	30.17
10	24.69	24.72	34.73	34.94	3.05	2.91	33.76	33.85	3.77	3.58	31.53	31.25
11	24.79	24.98	34.90	35.12	3.14	2.98	33.45	33.38	3.72	3.54	30.99	30.76
12	24.68	24.73	35.12	35.16	2.74	2.74	33.49	33.46	3.97	3.90	30.49	30.45
13	24.75	24.62	35.28	35.36	2.65	2.63	33.50	33.55	3.82	3.84	30.31	30.05

(1) g TG / 100 g total TGs (total TGs = POP+POS+POO+SOS+SOO = 100 %)
(2) g CBE / 100 g CB

Table A 8: Results accepted on technical grounds for sample 20 (two replicates)

	POP [1]		POS [1]		POO [1]		SOS [1]		SOO [1]		CBE addition [2]	
Lab	A	B	A	B	A	B	A	B	A	B	A	B
1	22.75	22.75	40.12	40.12	2.70	2.71	30.58	30.58	3.85	3.84	15.71	15.69
2	22.75	22.85	40.17	40.24	2.64	2.60	30.69	30.54	3.76	3.76	15.72	15.56
3	22.64	22.62	40.33	40.24	2.63	2.66	30.62	30.66	3.78	3.83	15.23	15.41
4	22.40	22.40	40.22	39.98	2.43	2.94	31.21	30.75	3.75	3.94	15.76	15.68
5	22.60	22.81	40.05	40.02	2.67	2.64	30.81	30.61	3.86	3.91	15.89	15.99
6	22.47	22.48	40.21	40.68	2.77	2.48	30.93	31.01	3.62	3.36	15.56	14.79
7	22.86	22.80	40.31	40.28	2.71	2.71	30.36	30.43	3.76	3.78	15.24	15.30
8	22.82	22.19	40.06	39.83	2.70	2.70	30.57	31.34	3.85	3.94	15.89	16.34
9	22.74	22.77	40.16	40.21	2.69	2.67	30.90	30.70	3.87	3.91	15.92	15.66
10	22.95	22.92	40.19	40.20	2.81	2.77	30.46	30.56	3.59	3.55	15.69	15.75
11	22.70	22.61	40.04	40.12	2.66	2.65	30.78	30.67	3.81	3.94	16.00	15.63
12	22.65	22.74	40.21	40.12	2.60	2.62	30.62	30.58	3.92	3.94	15.44	15.69
13	21.88	22.16	41.07	40.93	1.67	1.73	33.06	32.86	2.31	2.32	15.55	15.95

Table A 9: Results accepted on technical grounds for sample 21 (two replicates)

	POP [1]		POS [1]		POO [1]		SOS [1]		SOO [1]		CBE addition [2]	
Lab	A	B	A	B	A	B	A	B	A	B	A	B
1	26.08	26.08	37.26	37.29	2.83	2.83	29.89	29.89	3.93	3.92	24.45	24.39
2	26.13	26.00	37.52	37.54	2.88	2.76	29.71	29.98	3.78	3.72	23.84	23.94
3	25.97	26.27	37.46	37.57	2.76	2.73	29.89	29.55	3.91	3.89	23.95	23.76
4	25.71	26.07	37.43	37.29	2.37	2.70	30.61	30.09	3.89	3.85	24.46	24.60
5	26.23	26.39	37.39	37.61	2.81	2.80	29.75	29.55	3.82	3.64	24.25	23.84
6	26.07	26.29	37.93	37.45	2.55	2.39	30.12	30.28	3.32	3.59	23.46	24.80
7	25.22	25.37	37.93	37.90	2.39	2.40	31.07	30.93	3.39	3.40	23.43	23.50
8	26.38	26.52	37.27	37.27	2.80	2.83	29.69	29.54	3.86	3.86	24.61	24.62
9	26.19	26.20	37.32	37.30	2.80	2.79	29.77	29.57	3.94	3.76	24.35	24.24
10	26.33	26.47	37.41	37.45	2.82	2.87	29.94	29.67	3.50	3.54	24.55	24.36
11	26.24	26.43	37.26	37.50	2.93	2.76	29.80	29.41	3.78	3.90	24.56	23.93
12	26.04	25.99	37.50	37.42	2.61	2.66	29.90	30.01	3.95	3.91	23.98	24.18
13	26.01	26.13	37.40	37.48	2.73	2.85	30.11	29.75	3.75	3.78	24.34	23.97

Table A 10: Results accepted on technical grounds for sample 22 (two replicates)

	POP [1]		POS [1]		POO [1]		SOS [1]		SOO [1]		CBE addition [2]	
Lab	A	B	A	B	A	B	A	B	A	B	A	B
1	27.85	27.86	35.85	35.85	2.93	2.92	29.39	29.37	3.99	3.99	28.76	28.76
2	27.74	27.86	36.04	36.07	3.09	3.02	29.16	29.07	3.97	3.98	28.02	28.01
3	27.46	27.80	36.15	36.05	2.78	2.81	29.69	29.50	3.92	3.84	28.04	28.43
4	27.70	27.78	35.77	35.90	2.86	2.75	29.74	29.58	3.93	3.99	29.09	28.78
5	28.27	28.12	36.10	36.21	2.78	2.92	29.51	29.32	3.34	3.42	28.98	28.36
6	27.76	28.04	36.21	36.22	2.51	2.78	29.80	29.32	3.72	3.64	28.43	28.24
7	27.79	27.81	36.48	36.43	2.65	2.63	29.53	29.56	3.56	3.57	27.68	27.82
8	28.01	28.39	35.87	35.89	2.88	2.88	29.32	29.01	3.92	3.83	28.86	28.96
9	27.93	27.92	35.96	35.94	2.85	3.00	29.62	29.42	3.28	3.37	28.95	28.74
10	27.83	27.98	36.12	35.98	3.21	2.99	29.31	29.38	3.54	3.66	28.14	28.68
11	27.82	27.84	35.82	36.01	3.21	2.98	29.70	29.58	3.44	3.59	29.11	28.65
12	27.82	27.84	35.99	36.04	2.77	2.75	29.45	29.35	3.97	4.02	28.53	28.35
13	27.63	27.95	35.85	35.90	2.79	2.78	29.65	29.39	4.09	3.98	28.76	28.79

(1) g TG / 100 g total TGs (total TGs = POP+POS+POO+SOS+SOO = 100 %)
(2) g CBE / 100 g CB

Table A 11: Results accepted on technical grounds for sample 12 (two replicates)

	POP [1]		POS [1]		POO [1]		SOS [1]		SOO [1]		CBE addition [2]	
Lab	A	B	A	B	A	B	A	B	A	B	A	B
1	22.81	22.69	39.48	39.36	6.59	6.58	27.87	28.03	3.25	3.34	13.94	14.19
2	22.91	22.98	39.67	39.49	6.29	6.15	27.82	28.08	3.31	3.30	13.68	14.38
3	22.85	22.82	39.51	39.35	6.41	6.53	28.05	28.07	3.18	3.23	14.13	14.40
4	23.13	23.00	39.69	39.65	6.01	6.04	27.86	28.06	3.31	3.25	13.96	14.10
5	22.84	22.80	39.34	39.34	6.59	6.49	27.98	28.11	3.25	3.26	14.35	14.45
6	22.93	23.08	41.13	39.96	5.07	5.95	28.25	27.98	2.61	3.03	11.50	13.52
7	22.65	22.42	40.71	40.57	4.33	4.31	29.71	30.11	2.60	2.58	13.51	13.91
8	23.05	23.39	39.30	39.34	6.65	6.65	27.74	27.38	3.25	3.24	14.42	14.39
9	23.04	23.03	39.73	39.76	6.55	6.45	28.01	28.13	3.36	3.37	13.84	13.90
10	22.81	22.61	38.30	38.42	8.95	8.94	27.17	27.13	2.77	2.89	15.31	14.78
11	22.62	22.81	39.67	39.84	6.69	7.01	28.13	27.60	2.89	2.74	13.64	12.97
12	22.61	22.57	40.15	40.00	5.93	6.01	27.97	27.98	3.34	3.44	12.58	12.81
13	23.00	23.22	39.55	39.52	6.27	6.41	27.91	27.58	3.28	3.27	14.11	14.08

(1) g TG / 100 g total TGs (total TGs = POP+POS+POO+SOS+SOO = 100 %)
(2) g CBE / 100 g CB

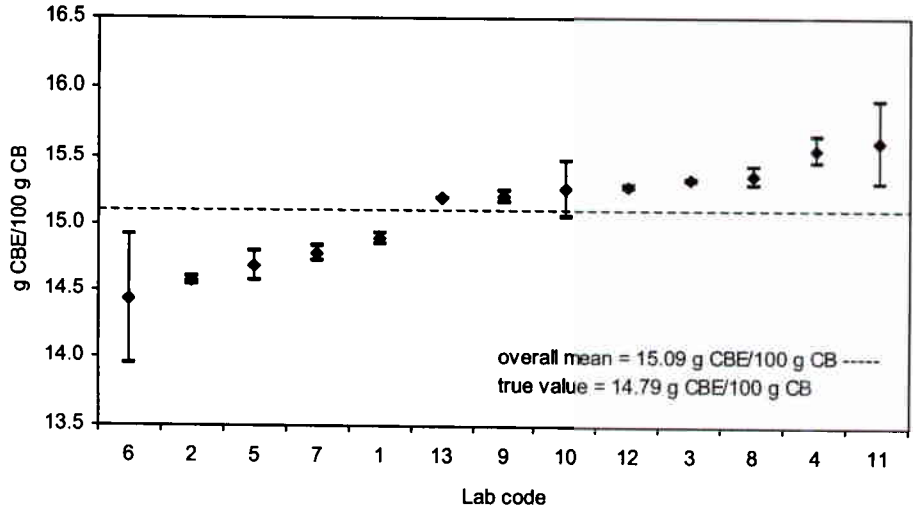

Figure A 1: Bar charts of results accepted on technical grounds for sample 14 (Laboratory means + range)

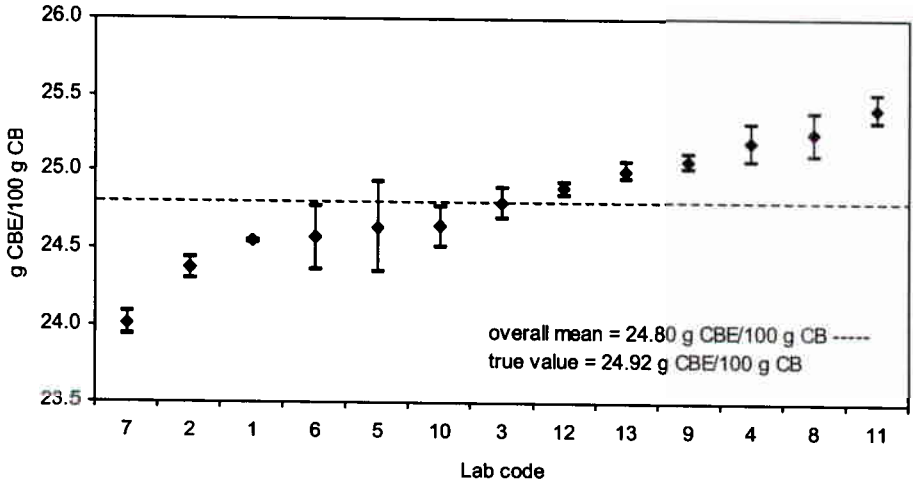

Figure A 2: Bar charts of results accepted on technical grounds for sample 15 (Laboratory means + range)

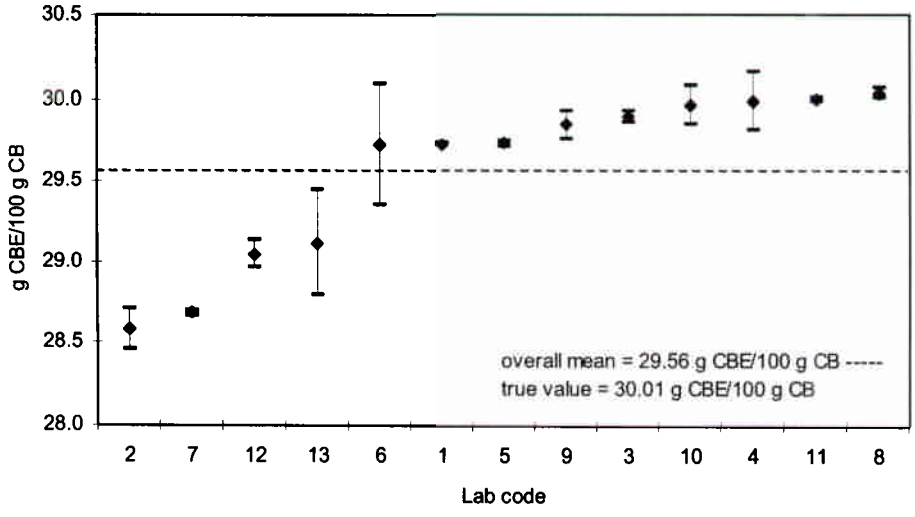

Figure A 3: Bar charts of results accepted on technical grounds for sample 16 (Laboratory means + range)

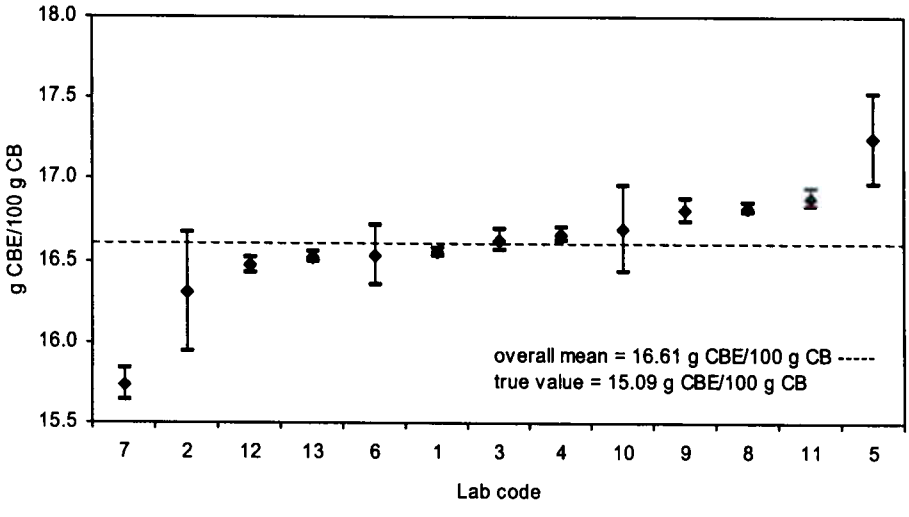

Figure A 4: Bar charts of results accepted on technical grounds for sample 17 (Laboratory means + range)

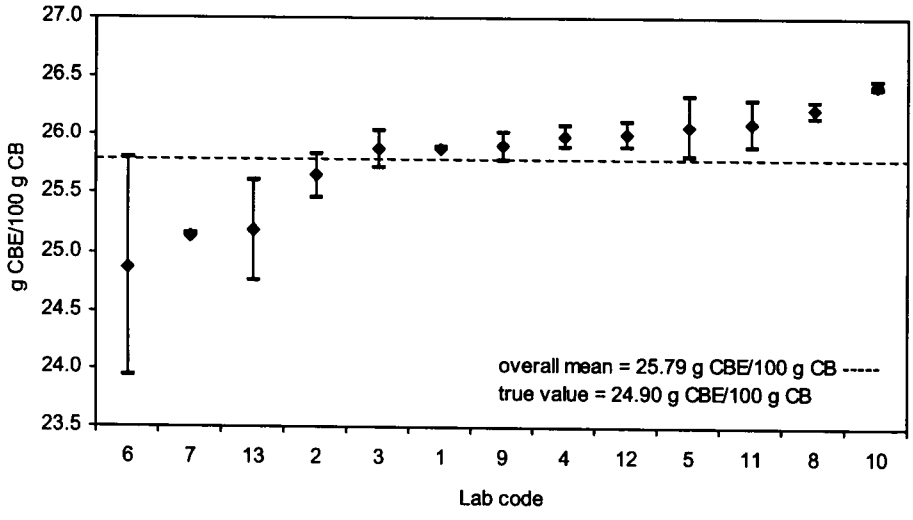

Figure A 5: Bar charts of results accepted on technical grounds for sample 18 (Laboratory means + range)

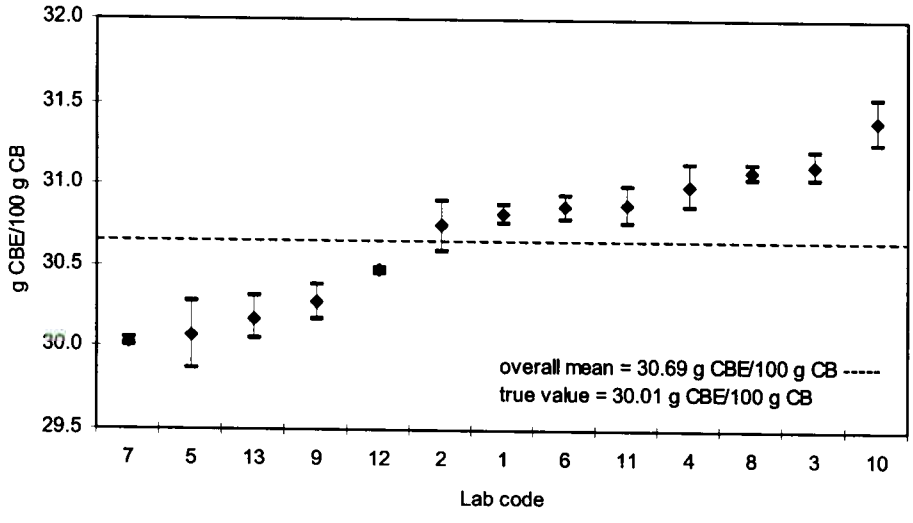

Figure A 6: Bar charts of results accepted on technical grounds for sample 19 (Laboratory means + range)

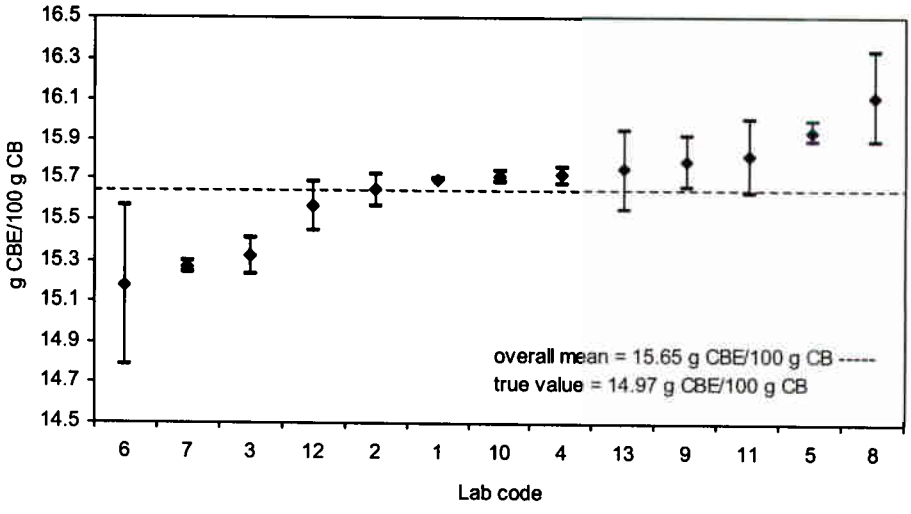

Figure A 7: Bar charts of results accepted on technical grounds for sample 20 (Laboratory means + range)

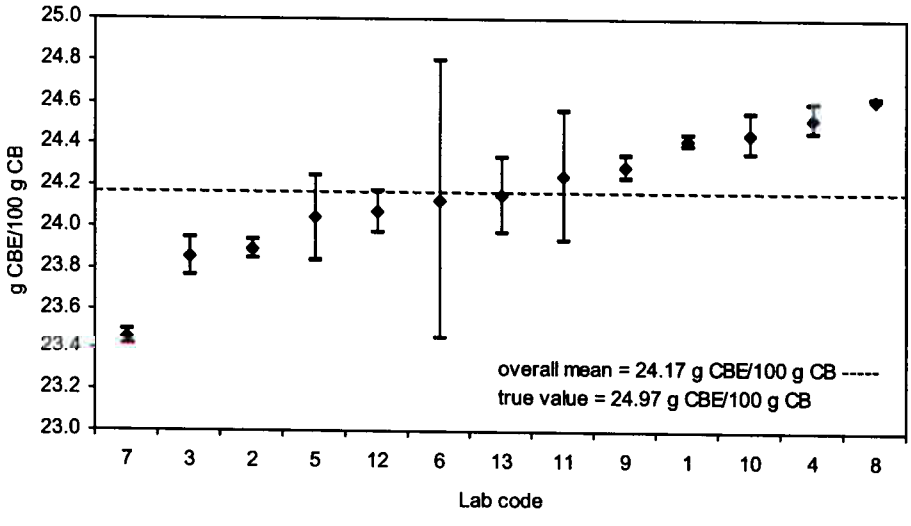

Figure A 8: Bar charts of results accepted on technical grounds for sample 21 (Laboratory means + range)

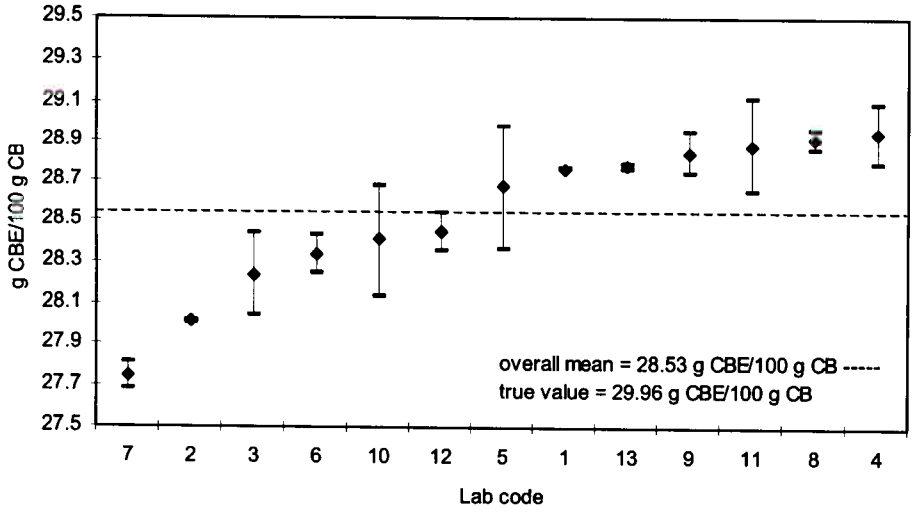

Figure A 9: Bar charts of results accepted on technical grounds for sample 22 (Laboratory means + range)

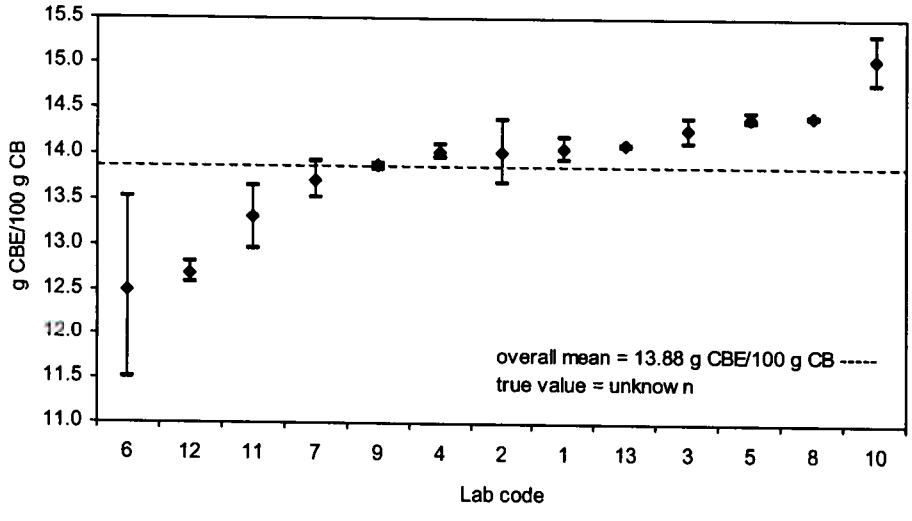

Figure A 10: Bar charts of results accepted on technical grounds for sample 12 (Laboratory means + range)

Table A 12: Statistical evaluation of the results accepted on technical grounds

Sample number	*12*	*14*	*15*	*16*
Year of collaborative study	2002			
Number of laboratories	13	13	13	13
Mean value, g CBE/100 g CB	13.88	15.09	24.80	29.56
True value, g CBE/100 g CB	-	14.79	24.92	30.01
Bias, g CBE/100 g CB	-	-0.3	0.12	0.45
Repeatability limit r [r = 2.8 x s_r], g/100 g	1.31	0.70	0.51	0.62
Repeatability standard deviation s_r, g/100 g	0.47	0.25	0.18	0.22
Repeatability relative standard deviation RSD_r, %	3.4	1.6	0.7	0.8
Reproducibility limit R [R = 2.8 x s_R], g/100 g	2.16	1.17	1.15	1.51
Reproducibility standard deviation s_R, g/100 g	0.77	0.42	0.41	0.54
Reproducibility relative standard deviation RSD_R, %	5.6	2.8	1.7	1.8
HORRAT value = RSD_R/predicted RSD_R (1)	2.1	1.0	0.7	0.8

(1) predicted $RSD_R = 2C^{-0.1505}$; C = estimated mean concentration

Table A 13: Statistical evaluation of the results accepted on technical grounds

Sample number	*17*	*18*	*19*
Year of collaborative study	2002		
Number of laboratories	13	13	13
Mean value, g CBE/100 g CB	16.61	25.79	30.69
True value, g CBE/100 g CB	15.07	24.90	30.01
Bias, g CBE/100 g CB	-1.54	-0.89	-0.68
Repeatability limit r [r = 2.8 x s_r], g/100 g	0.64	1.24	0.44
Repeatability standard deviation s_r, g/100 g	0.23	0.44	0.16
Repeatability relative standard deviation RSD_r, %	1.4	1.7	0.5
Reproducibility limit R [R = 2.8 x s_R], g/100 g	1.08	1.57	1.26
Reproducibility standard deviation s_R, g/100 g	0.39	0.56	0.45
Reproducibility relative standard deviation RSD_R, %	2.3	2.2	1.5
HORRAT value = RSD_R/predicted RSD_R (1)	0.9	0.9	0.6

(1) predicted $RSD_R = 2C^{-0.1505}$; C = estimated mean concentration

Table A 14: Statistical evaluation of the results accepted on technical grounds

Sample number	*20*	*21*	*22*
Year of collaborative study	2002		
Number of laboratories	13	13	13
Mean value, g CBE/100 g CB	15.65	24.17	28.53
True value, g CBE/100 g CB	14.97	24.97	29.96
Bias, g CBE/100 g CB	-0.68	0.8	1.03
Repeatability limit r [r = 2.8 x s_r], g/100 g	0.63	0.90	0.62
Repeatability standard deviation s_r, g/100 g	0.22	0.32	0.22
Repeatability relative standard deviation RSD_r, %	1.4	1.3	0.8
Reproducibility limit R [R = 2.8 x s_R], g/100 g	0.87	1.09	1.14
Reproducibility standard deviation s_R, g/100 g	0.31	0.39	0.41
Reproducibility relative standard deviation RSD_R, %	2.0	1.6	1.4
HORRAT value = RSD_R/predicted RSD_R (1)	0.7	0.6	0.6

(1) predicted $RSD_R = 2C^{-0.1505}$; C = estimated mean concentration

Table A 15: Statistical evaluation of the results accepted on technical and statistical grounds

Sample number	*12*	*14*	*15*	*16*
Year of collaborative study	2002			
Number of laboratories	13	13	13	13
Number of outliers	1	1	0	0
Identity of outlying laboratories	6	6	-	-
Reason for removal (C=Cochran; DG=Double Grubbs)	C	C	-	-
Number of accepted results	12	12	13	13
Mean value, g CBE/100 g CB	13.99	15.14	24.80	29.56
True value, g CBE/100 g CB	-	14.79	24.92	30.01
Bias, g CBE/100 g CB	-	-0.35	0.12	0.45
Repeatability limit r [$r = 2.8 \times s_r$], g/100 g	0.72	0.47	0.51	0.62
Repeatability standard deviation s_r, g/100 g	0.26	0.17	0.18	0.22
Repeatability relative standard deviation RSD_r, %	1.8	1.1	0.7	0.8
Reproducibility limit R [$R = 2.8 \times s_R$], g/100 g	1.72	1.00	1.15	1.51
Reproducibility standard deviation s_R, g/100 g	0.61	0.36	0.41	0.54
Reproducibility relative standard deviation RSD_R, %	4.4	2.4	1.7	1.8
HORRAT value = RSD_R/predicted RSD_R (1)	1.6	0.9	0.7	0.8

(1) predicted $RSD_R = 2C^{-0.1505}$; C = estimated mean concentration

Table A 16: Statistical evaluation of the results accepted on technical and statistical grounds

Sample number	*17*	*18*	*19*
Year of collaborative study	2002		
Number of laboratories	13	13	13
Number of outliers	2	1	0
Identity of outlying laboratories	5, 7	6	-
Reason for removal (C=Cochran; DG=Double Grubbs)	DG	C	-
Number of accepted results	11	12	13
Mean value, g CBE/100 g CB	16.63	25.87	30.69
True value, g CBE/100 g CB	15.07	24.90	30.01
Bias, g CBE/100 g CB	-1.56	-0.97	-0.68
Repeatability limit r [$r = 2.8 \times s_r$], g/100 g	0.60	0.72	0.44
Repeatability standard deviation s_r, g/100 g	0.21	0.26	0.16
Repeatability relative standard deviation RSD_r, %	1.3	1.0	0.5
Reproducibility limit R [$R = 2.8 \times s_R$], g/100 g	0.64	1.20	1.26
Reproducibility standard deviation s_R, g/100 g	0.23	0.43	0.45
Reproducibility relative standard deviation RSD_R, %	1.4	1.7	1.5
HORRAT value = RSD_R/predicted RSD_R (1)	0.5	0.7	0.6

(1) predicted $RSD_R = 2C^{-0.1505}$; C = estimated mean concentration

Table A 17: Statistical evaluation of the results accepted on technical and statistical grounds

Sample number	*20*	*21*	*22*
Year of collaborative study		2002	
Number of laboratories	13	13	13
Number of outliers	0	1	0
Identity of outlying laboratories	-	6	-
Reason for removal (C=Cochran; DG=Double Grubbs)	-	C	-
Number of accepted results	13	12	13
Mean value, g CBE/100 g CB	15.65	24.17	28.53
True value, g CBE/100 g CB	14.97	24.97	29.96
Bias, g CBE/100 g CB	-0.68	0.8	1.03
Repeatability limit r [$r = 2.8 \times s_r$], g/100 g	0.63	0.53	0.62
Repeatability standard deviation s_r, g/100 g	0.22	0.19	0.22
Repeatability relative standard deviation RSD_r, %	1.4	0.8	0.8
Reproducibility limit R [$R = 2.8 \times s_R$], g/100 g	0.87	1.00	1.14
Reproducibility standard deviation s_R, g/100 g	0.31	0.36	0.41
Reproducibility relative standard deviation RSD_R, %	2.0	1.5	1.4
HORRAT value = RSD_R/predicted RSD_R (1)	0.7	0.6	0.6

(1) predicted $RSD_R = 2C^{-0.1505}$; C = estimated mean concentration

LB-NA-20-777-EN-C

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