FOOD COMPOSITION AND ADDITIVES

Gas-Liquid Chromatographic Determination of Milk Fat and Cocoa Butter Equivalents in Milk Chocolate: Interlaboratory Study

MANUELA BUCHGRABER and SIMONA ANDRONI
European Commission, Directorate-General Joint Research Centre, Institute for Reference Materials and Measurements, Food Safety and Quality Unit, Retieseweg 111, B-2440 Geel, Belgium

Collaborators: E.-M. Berndt; I. Kaufmann-Horlacher; A. Lomnitz; R. Matissek; R. Maurer; G. Neuberger; M.T. Rodriguez-Estrada; M. Schreiner; S. Schulokat; M. Schut; N. Wachter

A collaborative trial was conducted to validate an analytical approach comprising method procedures for determination of milk fat and the detection and quantification of cocoa butter equivalents (CBEs) in milk chocolate. The whole approach is based on (*1*) comprehensive databases covering the triacylglycerol composition of a wide range of authentic milk fat, cocoa butter, and CBE samples and 947 gravimetrically prepared mixtures thereof; (*2*) the availability of a certified cocoa butter reference material for calibration; (*3*) an evaluation algorithm, which allows reliable quantitation of the milk fat content in chocolate; (*4*) a subsequent correction to take account of the triacylglycerols derived from milk fat; (*5*) mathematical expressions to detect the presence of CBEs in milk chocolate; and (*6*) a multivariate statistical formula to quantitate the amount of CBEs in milk chocolate. Twelve laboratories participated in the validation study. CBE admixtures were detected down to a level of 0.5 g CBE/100 g milk chocolate, without false-positive or -negative results. The applied quantitation model performed well at the statutory limit of 5% CBE addition to milk chocolate, with a prediction error of 0.7%, and HorRat values ranging from 0.8 to 1.5. The relative standard deviation for reproducibility (RSD_R) values for quantitation of CBEs in analyses of chocolate fat solutions ranged from 2.2 to 3.8% and for analyses of real chocolate samples, from 4.1 to 4.7%, demonstrating that the whole approach, based solely on chocolate fat blends, is applicable to real milk chocolate samples.

An integrated approach for the detection and quantitation of cocoa butter equivalents (CBEs) in dark chocolate by using triacylglycerol (TAG) profiling by gas-liquid chromatography (GLC) was developed (1) and validated by an international collaborative trial (2); this approach is important because it can be used to assess compliance with labeling provisions. It allowed the implementation and enforcement of Directive 2000/36/EC (3), which authorizes the replacement of cocoa butter (CB) by vegetable fats other than CB (so-called CBEs), at least for dark chocolate. Member States' laws, regulations, and administrative provisions have had to comply with Directive 2000/36/EC (3) since August 2003. To facilitate the use of the approach, an analytical toolbox named "CoCal-1" (cocoa butter calculation toolbox) has been established, consisting of a validated method for detection of CBEs in dark chocolate (4), a validated method for quantitation of CBEs in dark chocolate (5), both methods after standardization by the International Organization for Standardization (ISO; 6, 7), a certified CB 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). This analytical toolbox has been used by many control laboratories since its publication.

However, until now, this standardized analytical approach for dark chocolate could not be used for milk chocolate because TAGs derived from milk or milk fat (MF) interfered with the detection and quantitation of CBEs in the chocolate. When milk chocolate is analyzed, it will be necessary to correct the observed TAG pattern for the presence of MF TAGs, and the amount of MF present in the product must be known. The problem of estimating the MF content in mixtures of fats or chocolates has already prompted a great deal of research (10–20). Currently, determination of butyric acid in a mixed fat is a widely applied method (21–23) and has already been used to measure small amounts of MF in chocolate fats (24–26). However, with respect to the correct labeling of chocolate, this method can provide only one of the answers to 3 possible questions, the content of MF in the chocolate fat. The method is not satisfactory for addressing the other 2 possibilities, i.e., the presence of any other fat in addition to CB and the amount.

Received December 18, 2006. Accepted by SG January 30, 2007.
Corresponding author's e-mail: manuela.buchgraber@ec.europa.eu

Table 1. Samples used for the study[a]

Sample	Sample description	CBE type	CB, %	CBE, %	MF, %
	Chocolate samples[b]				
1	Milk chocolate, FCMP, no CBE	—	29.67	0.00	Unknown
2	Milk chocolate, FCMP, CBE addition at low level	50% PMF + 50% SOS-rich fat	29.22	0.45	Unknown
3	Milk chocolate, SKMP + MF, no CBE	—	25.70	0.00	Unknown
4	Milk chocolate, SKMP + MF, CBE addition at low level	50% PMF + 50% SOS-rich fat	23.67	2.03	Unknown
5	Milk chocolate, crumb + MF + FCMP + SKMP + WP, CBE addition at statutory level	50% PMF + 50% SOS-rich fat	14.60	5.11	Unknown
6	White chocolate, CBE addition at statutory level	50% PMF + 50% SOS-rich fat	23.50	3.95	Unknown
	Chocolate fat solutions				
7	West African CB, no CBE	—	100.00	0.00	0.00
8	West African CB + mixture of 310 MF samples, no CBE	—	85.01	0.00	14.99
9	West African CB + mixture of 310 MF samples, CBE addition at low level	70% PMF + 30% SOS-rich fat	83.03	2.00	14.98
10	West African CB + mixture of 310 MF samples, CBE addition at statutory level	70% PMF + 30% SOS-rich fat	68.95	16.03	15.02
11	West African CB + mixture of 310 MF samples, CBE addition at statutory level	70% PMF + 30% SOS-rich fat	64.99	19.98	15.04
12	West African CB + mixture of 310 MF samples, CBE addition at statutory level	100% soft PMF	64.94	20.08	14.99
13	West African CB + mixture of 310 MF samples, CBE addition at statutory level	70% PMF + 30% SOS-rich fat	56.91	28.04	15.05

[a] CB = Cocoa butter; CBE = cocoa butter equivalent; FCMP = full cream milk powder; MF = milk fat; PMF = palm midfraction; SKMP = skimmed milk powder; SOS = 1,3-distearoyl-2-oleoylglycerol; WP = whey powder.

[b] Samples were prepared in 40 kg quantities.

Therefore, an alternative to the classical butyric acid method was developed for determination of MF in chocolate fats, based on a database consisting of the TAG profile of genuine MF samples and mixtures thereof with other chocolate fats. The MF TAG database, obtained by 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 necessary requirements: (*1*) to be present in reasonable amounts, i.e., an average PSB value of 2.15 g/100 g MF, which allowed a reliable quantitation of even low MF proportions in chocolate fats, (*2*) to have an acceptably low natural variability, and (*3*) to be present only in MF and in no other fats. The advantage of the developed method is that for further applications, i.e., determination of CBEs in chocolate fats, just a single analysis is performed, whereas for the same purpose the butyric acid method requires 2 different analytical methods (27).

By using the information obtained from the MF quantitation, a modification of the existing approach for detection and quantitation of CBEs in dark chocolate (CoCal-1) was developed for milk chocolate (CoCal-2; 27). CoCal-2 is based on (*1*) comprehensive standardized databases covering the TAG composition of a wide range of authentic MF, CB as well as CBE samples and 947 gravimetrically prepared mixtures thereof, (*2*) the availability of a certified CB reference material (IRMM-801) for calibration, (*3*) an evaluation algorithm, which allows reliable quantitation of the MF content in chocolate fats by using a simple linear regression model, (*4*) a subsequent correction of TAGs originating from MF, (*5*) mathematical expressions to detect the presence of CBEs in milk chocolate, and (*6*) a multivariate statistical formula to quantitate the amount of CBEs in milk chocolate. The advantage of the developed approach is that by performing a single TAG analysis using GLC, several useful pieces of information can be determined, i.e., (*1*) the MF content in the sample, (*2*) the contribution of TAGs derived from MF, (*3*) the presence/absence of CBEs, and, when the detection approach indicates that the CB is not pure, (*4*) the quantity of CBE admixture.

The aim of the work described in this paper was to fully validate the analytical approach consisting of procedures for the determination of MF and the detection and quantitation of CBEs in milk chocolate. The collaboratively tested approach described in this paper, which was developed on the basis of extensive in-house testing of the method (27), proved its validity to be used by control laboratories to assess the correct

Table 2. Predefined performance criteria for GLC methods

GLC	Performance criterion	Tested with
Resolution	Separation of critical pairs POS/POO and SOS/SOO with a chromatographic resolution of ≥1.0	CB CRM (IRMM-801)
Resolution	Separation of PSB from neighboring peaks of compounds within carbon number group 38	Pure MF sample
Resolution	No coelution of the internal standard α-cholestane with other TAGs	Pure MF sample + α-cholestane
Detector response	Flame-ionization detector RFs[a] of TAGs (POP, POS, POO, SOS, and SOO) shall not differ significantly from unity; RSD of determined detector RFs shall be <5%	IRMM-801 (3 replicates)
Detector response	relative error of the minimum RF obtained for PSB and the relative error of the maximum RF obtained for PSB shall be <5% with respect to the average RF for PSB	Calibration solutions: mixture of PSB + α-cholestane for cold on-column injection or CB + PSB + α-cholestane for split injection

[a] RF = Response factor.

labeling of milk chocolate according to Directive 2000/36/EC (3).

Validation Study

Test Samples

Seven chocolate fat samples dissolved in isooctane were prepared by gravimetrically blending CB, CBE, and MF samples in different proportions (Table 1). A representative MF sample was obtained by mixing equal amounts of 310 individual MF samples collected in 21 European countries over the period 2001–2005. CB and some of the MF samples were provided by Kraft Foods (Väsby, Sweden), and the CBE samples were obtained from Britannia Food Ingredients (Goole, UK). Six real milk chocolate samples varying in composition and with known levels of CBEs were produced by Barry Callebaut N.V. (Lebbeke-Wieze, Belgium; Table 1).

Homogeneity

Homogeneity of the milk chocolate samples (samples 1–6) was assessed by internationally agreed procedures (28). From each chocolate sample, the contents of 10 sample containers (units) were taken randomly from the sequence, and the contents of each container were split into 2 equal parts (unit subsample). The fat from each unit subsample was extracted according to AOAC Official Method **963.15** (29) and randomly analyzed for TAG composition by GLC by using a Chrompack column (0.25 mm × 25 m, 0.1 μm CB-TAP; Varian, Inc., Middelburg, The Netherlands). The homogeneity of the milk chocolate samples was checked by determining 6 TAGs, i.e., PSB, 1,3-dipalmitoyl-2-oleoylglycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (POS), 12-dioleoyl-3-palmitoylglycerol (POO), 1,3-distearoyl-2-oleoylglycerol (SOS), and 1,2-dioleoyl-3-stearoylglycerol (SOO), which are used for determination of the MF content and the detection and quantitation of CBEs in milk chocolate. The within- and between-unit standard deviations for the PSB, POP, POS, POO, SOS, and SOO contents were calculated by using 1-way analysis of variance (ANOVA) and applying the *F*-test at the 95% confidence level. All tests confirmed that the between-unit inhomogeneity was insignificant ($P > 0.05$). Therefore, the homogeneity of the chocolate samples was considered sufficient for them to be used as test materials for the validation study. The chocolate fat samples dissolved in isooctane (samples 7–13) were considered to be homogeneous.

Design of the Validation Study

Fifteen laboratories from 8 Member States of the European Union (EU) with experience in TAG analysis were contacted to participate in the study. Of these, 12 laboratories submitted results.

The participants received a shipment containing blind duplicates of the 6 grated milk chocolate samples, from which the fat had to be extracted, and blind duplicates of the 7 chocolate fat samples dissolved in isooctane (in total 26 test samples), coded by the coordinating laboratory. In addition, 1 ampoule of the CB certified reference material (CRM), 1 ampoule of an average pure MF, 1 ampoule of PSB dissolved in isooctane, 6 ampoules of a mixture of CB with different levels of PSB dissolved in isooctane, and 1 ampoule of α-cholestane dissolved in isooctane were provided for calibration purposes and the system suitability check. Furthermore, participants received a method protocol, collaborative study guidelines, and an electronic evaluation and reporting sheet (MS Excel® format). The participants were requested to follow the method protocol exactly. However, the GLC method gave some freedom to choose procedural parameters within certain limits. Therefore, in order to demonstrate that the GLC methods applied were fit-for-purpose the participants had to meet predefined performance criteria (Table 2).

Table 3. GLC methods used in the study by the participants

	Laboratory code											
Parameter	1	2	3	4	5	6	7	8	9	10	11	12
Carrier gas	He	He	He	H_2	H_2	H_2	H_2	He	H_2	H_2	H_2	H_2
Constant pressure, kPa	100	180	—	—	—	—	150	135	130	140	—	—
Constant flow, mL/min	—	—	2.2	2	2	1.5	—	—	—	—	3.5	2
Column												
Stationary phase	Ultimetal	CB-TAP	CB-TAP	CB-TAP	CB-TAP	CB-TAP	CB-TAP	CB-TAP	RTx-65TG	CB-TAP	CB-TAP	CB-TAP
Length, m	25	25	25	25	25	25	25	25	30	25	25	25
Internal diameter, mm	0.25	0.25	0.25	0.25	0.25	0.25	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	0.1	0.1	0.1	0.1	0.1	0.1
Oven												
Injection temp., °C/hold time, min	200/2	200/1	100/0.5	200/1	200/0	200/1	100/2	200/1	200/1	200/1	100/2	200/1
Program rate 1, °C/min	20	14	40	14	20	24	30	14	15	30	30	14
Final temp., °C/hold time, min	320/0	270/0	280/1	270/0	270/0	270/0	270/1	270/0	360/0	270/0	270/1	270/0
Program rate 2, °C/min	1	2.5	2.5	2.5	5	2.5	3	2	1	2.5	2.5	2.5
Final temp., °C/hold time, min	360/10	340/30	340/17	340/10	340/15	340/13	340/10	340/30	370/0	355/2	340/7	340/10
Program rate 3, °C/min	—	10	—	—	25	—	—	—	—	—	—	—
Final temp., °C/hold time, min	—	350/9	—	—	200	—	—	—	—	—	—	—
Injector temp., °C	65–370	360	Oven track	365	370	350		340	380	140–340	Oven track	360
Detector temp., °C	370	360	360	365	360	370	350	360	380	350	360	360
Injection mode												
Manual (M)/automatic (A)	M	A	—	A	A	A	M	A	A	A	A	A
Split/OCI/PTV[a]	PTV	Split	OCI	Split	Split	Split	OCI	Split	Split	PTV	OCI	Split
Split ratio	—	1:20	—	1:10	1:10	1:10	—	1:7	—	—	—	1:10

[a] OCI = Cold on-column injection; PTV = programmed temperature vaporizer.

The 7 chocolate fat samples (provided as blind duplicates) had to be analyzed once (in total 14 analyses). From each grated milk chocolate sample (provided as blind duplicates, in total 12 samples), the fat had to be obtained once by rapid extraction (extraction of fat from 5 g grated chocolate with two to three 10 mL portions of a suitable fat solvent, e.g., *n*-heptane, isooctane; 12 extractions) and once by Soxhlet extraction (12 extractions; 29). The chocolate fats obtained had to be dissolved in isooctane, and each fat solution had to be analyzed in randomized order by GLC (in total 24 analyses).

An average response factor (RF) for PSB obtained by using α-cholestane as the internal standard had to be determined before analysis of the first sample, after the 19th analysis, and after the analysis of the last test sample. Laboratories employing a split injection technique had to use a mixture of CB and PSB dissolved in isooctane, allowing a calibration in the matrix to protect the PSB, in order to obtain suitable RF values, whereas for cold on-column injection techniques PSB dissolved in isooctane was sufficient.

RFs for the 5 TAGs (POP, POO, POS, SOS, and SOO) had to be determined before analysis of the first sample, after the 19th analysis, and after the last test sample by using the CB CRM (IRMM-801).

Experimental

Principle

The test sample, i.e., the chocolate fat obtained from milk chocolate by using a rapid fat extraction procedure, is separated by GLC into TAG fractions according to the molecular weight and degree of unsaturation of the TAGs. Individual TAG fractions (PSB, POP, POS, POO, SOS, and SOO) are used to (*1*) calculate the MF content in the chocolate fat (g MF/100 g chocolate fat), (*2*) determine the presence/absence of CBEs in chocolate fat by using a simple linear regression model based on the 3 TAGs (POP, POS, and SOS) corrected for the TAG contribution originating from MF, and in case the detection approach indicates that the sample is not pure CB, (*3*) quantitate the amount of the CBE admixture in chocolate fat (g CBE/100 g chocolate fat) by using a partial least-squares (PLS) regression model with 6 input variables, i.e., the 5 TAGs (POP, POS, POO, SOS, and SOO) normalized to 100% and the determined MF content of the chocolate fat.

Finally, to control the correct labeling of milk chocolate, the results related to chocolate fat are converted into g MF/100 g chocolate and g CBE/100 g chocolate, which requires the accurate determination of the total fat content of the chocolate using a Soxhlet extraction procedure (29). If the detection approach demonstrates the absence of CBEs in the chocolate fat, the quantitation of CBEs and the determination of the total fat content of the chocolate are not necessary. A detailed description of the whole approach is given by Buchgraber and Androni (27).

Calculations

(**a**) *Quantitation of PSB and MF in chocolate fat*.—The RF of PSB was determined by injection of 6 calibration solutions under experimental conditions identical to those used for the test sample. For each calibration solution, an RF for PSB, F_{PSB}, had to be calculated by the following equation:

$$F_{PSB;\,i} = \frac{C_{PSB;\,i} \times A_{Cholestane;\,i}}{C_{Cholestane;\,i} \times A_{PSB;\,i}} \qquad (1)$$

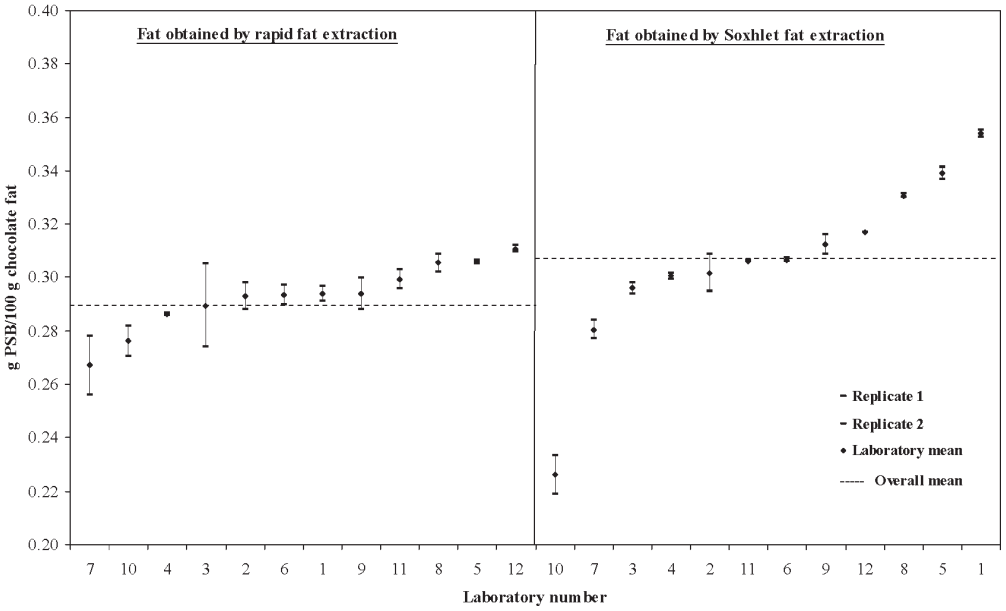

Figure 1. Comparison of laboratory means of PSB content in chocolate fat of sample 2, obtained by different extraction procedures (error bars represent range of blind duplicates).

Table 4. Method performance data[a] for determination of PSB in chocolate fats[b]

Parameter	Sample No.											
	1	2	3	4	5	6	8	9	10	11	12	13
No. of laboratories	12	12	12	12	12	12	12	12	12	12	12	12
No. of outliers	0	0	3	0	0	0	0	1	0	0	0	0
Identity of outlying laboratories			6, 1, 10					7				
Reason for removal			C, DG[c]					C				
No. of accepted laboratories	12	12	9	12	12	12	12	11	12	12	12	12
Mean value, g PSB/100 g chocolate	0.26	0.29	0.44	0.43	0.49	0.33	0.35	0.36	0.35	0.35	0.35	0.35
r, g/100 g[d]	0.04	0.03	0.02	0.03	0.03	0.01	0.03	0.01	0.03	0.03	0.03	0.03
s_r, g/100 g	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01
RSD_r, %	5.7	3.1	1.5	2.6	2.3	1.6	2.6	1.2	2.7	3.5	2.8	3.3
R, g/100 g[e]	0.05	0.04	0.02	0.05	0.05	0.06	0.06	0.07	0.07	0.07	0.07	0.07
s_R, g/100 g	0.02	0.01	0.01	0.02	0.02	0.02	0.02	0.03	0.03	0.03	0.02	0.03
RSD_R, %	7.3	4.8	2.0	4.1	4.0	6.1	6.4	7.3	7.6	7.5	6.8	7.2
HorRat[f]	1.49	0.99	0.43	0.89	0.90	1.29	1.36	1.57	1.62	1.60	1.44	1.53

[a] Based on results accepted on technical and statistical grounds.

[b] r = Repeatability limit; s_r = repeatability standard deviation; RSD_r = repeatability relative standard deviation; R = reproducibility limit; s_R = reproducibility standard deviation; RSD_R = reproducibility relative standard deviation; HorRat = RSD_R/predicted RSD_R.

[c] C = Cochran's test; DG = double Grubbs' test.

[d] $r = 2.8 \times s_r$.

[e] $R = 2.8 \times s_R$.

[f] Predicted $RSD_R = 2C^{-0.15}$, where C = estimated mean concentration.

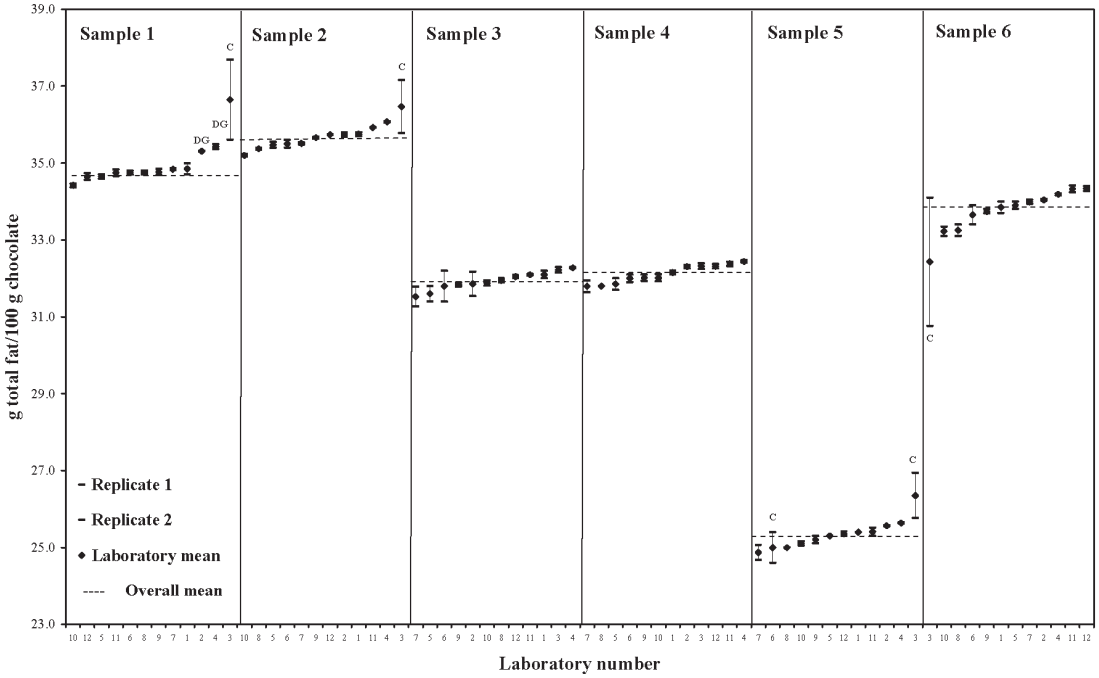

Figure 2. Laboratory means of total fat amounts in all chocolate samples, obtained by using Soxhlet extraction (error bars represent range of blind duplicates; C = Cochran's test; DG = double Grubbs' test).

where $A_{PSB;\ i}$ is the peak area of PSB in calibration solution i, $A_{Cholestane;\ i}$ is the peak area of the internal standard α-cholestane in calibration solution i, $C_{PSB;\ i}$ is the concentration (mg/mL) of PSB used in calibration solution i, $C_{Cholestane;\ i}$ is the concentration (mg/mL) of the internal standard α-cholestane used in calibration solution i, and $F_{PSB;\ i}$ is the detector RF of PSB in calibration solution i.

An average RF for PSB, $F_{PSB;\ mean}$, obtained from the 6 calibration solutions had to be calculated and used for further calculations. Laboratories employing a split injection technique used a mixture of CB and PSB dissolved in isooctane to obtain suitable RF values, whereas for cold on-column injection techniques, PSB dissolved in isooctane was sufficient. The mass fraction in percent of PSB in the test sample (chocolate fat), $M_{PSB;\ choc\ fat}$, was calculated as follows:

$$M_{PSB;\ choc\ fat},\ \% = \frac{A_{PSB} \times C_{Cholestane} \times F_{PSB;\ mean} \times 100}{A_{Cholestane} \times C_{Sample}} \quad (2)$$

where A_{PSB} is the peak area of PSB in the test sample, $A_{Cholestane}$ is the peak area of the internal standard α-cholestane in the test sample, $F_{PSB;\ mean}$ is the average RF for PSB (*see* Equation 1), $C_{Cholestane}$ is the concentration (mg/mL) of the internal standard α-cholestane in the test sample, C_{Sample} is the concentration (mg/mL) of the test sample, and $M_{PSB;\ choc\ fat}$ is the mass fraction in percent of PSB in the test sample.

The mass fraction in percent of MF in the chocolate fat, $M_{MF;\ choc\ fat}$, was calculated as follows:

$$M_{MF;\ choc\ fat} = 0.19 + (44.04 \times M_{PSB;\ choc\ fat}) \quad (3)$$

where $M_{PSB;\ choc\ fat}$ is the mass fraction in percent of PSB in chocolate fat (*see* Equation 2), and $M_{MF;\ choc\ fat}$ is the mass fraction in percent of MF in chocolate fat.

This calibration function was established by using data from a database extensively tested in-house and holding information on the TAG profile of >900 gravimetrically prepared CB-MF and CB-CBE-MF mixtures with known MF contents, simulating the composition of real chocolate fats (27).

(**b**) *Detection of CBE in chocolate fat.*—The RFs of the TAGs POP, POS, and SOS were determined by injection of a CB CRM solution under experimental conditions identical to those used for the test sample. The percentage of each of the 3 TAGs with respect to all TAGs present in the CB CRM was calculated by the following equations:

$$P_{i;\ ref},\ \% = \frac{A_{i;\ ref}}{\sum A_{all\ TGS;\ ref}} \times 100 \quad (4)$$

and

$$F_i = \frac{M_{i;\ ref}}{P_{i;\ ref}} \quad (5)$$

where $A_{i;\ ref}$ is the peak area of the TAG i in the CB CRM, $\Sigma A_{all\ TGs;\ ref}$ is the sum of the peak areas attributed to all TAGs in the CB CRM, $P_{i;\ ref}$ is the percentage of TAG i in the CB CRM, $M_{i;\ ref}$ is the mass fraction in percent of TAG i in the CB CRM as given in the certificate (POP = 16.00%, POS = 39.40%, and SOS = 27.90%; 8), and F_i is the detector RF of TAG i in the CB CRM.

Table 5. Method performance data[a] for determination of MF in chocolate

Parameter	Sample No.											
	1	2	3	4	5	6	8	9	10	11	12	13
No. of laboratories	12	12	12	12	12	12	12	12	12	12	12	12
No. of outliers	0	1	2	2	0	0	0	1	0	0	0	0
Identity of outlying laboratories		3	1, 10	1, 10				7				
Reason for removal		C	DG	DG				C				
No. of accepted laboratories	12	11	10	10	12	12	12	11	12	12	12	12
Mean value, g MF/100 g chocolate	4.08	4.69	6.31	6.17	5.50	4.95	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 MF/100 g chocolate	—	—	—	—	—	—	–0.22	–0.31	–0.19	–0.20	–0.13	–0.14
r, g/100 g	0.45	0.30	0.28	0.52	0.33	0.38	0.33	0.16	0.34	0.45	0.28	0.41
s_r, g/100 g	0.16	0.11	0.10	0.19	0.12	0.14	0.12	0.06	0.12	0.16	0.10	0.14
RSD_r, %	3.9	2.3	1.6	3.0	2.1	2.8	2.5	1.2	2.6	3.4	2.2	3.1
R, g/100 g	0.60	0.67	0.38	0.52	0.60	0.98	0.83	0.95	0.98	0.97	0.87	0.92
s_R, g/100 g	0.22	0.24	0.13	0.19	0.22	0.35	0.30	0.34	0.35	0.35	0.31	0.33
RSD_R, %	5.3	5.1	2.1	3.0	3.9	7.1	6.3	7.0	7.5	7.3	6.7	7.1
HorRat	1.63	1.61	0.70	1.00	1.27	2.26	1.99	2.23	2.36	2.31	2.12	2.23

[a] Based on results accepted on technical and statistical grounds (for samples 1–6, chocolate fat for GLC analysis was obtained by rapid fat extraction, and total fat content was determined by Soxhlet extraction; for samples 8–13, the assumed fat content of the chocolate was 30%). *See* also footnotes b–f in Table 4.

The mass percentage of the TAGs POP, POS, and SOS in the test sample with respect to all TAGs present in the test sample was calculated by the following equation:

$$M_{i;\,total},\ \% = \frac{F_i \times A_i}{\sum A_{all\ TGs}} \times 100 \qquad (6)$$

where A_i is the peak area corresponding to the TAG i in the test sample, $\Sigma A_{all\ TGs}$ is the sum of the peak areas attributed to all TAGs in the test sample, F_i is the RF of TAG i (*see* Equation 5), and $M_{i;\,total}$ is the mass fraction in percent of TAG i in the test sample.

The contribution of the mass percentages of the TAGs POP, POS, and SOS originating from MF was calculated by the following equation:

$$M_{i;\,mf} = \frac{M_{MF;\,choc\ fat} \times M_{i;\,ref}}{100} \qquad (7)$$

where $M_{i;\,ref}$ is the average mass fraction in percent of TAG i in an MF, i.e., POP = 3.99%, POS = 2.19%, and SOS = 0.45% [values obtained from database (27)], $M_{MF;\,choc\ fat}$ is the mass fraction in percent of MF in the test sample (see Equation 3), and $M_{i;\,mf}$ is the mass fraction in percent of TAG i derived from MF in the test sample.

The mass percentages obtained for the 3 TAGs derived from MF (Equation 7) were subtracted from the mass percentages of the 3 TAGs obtained for the test sample (Equation 6).

$$M_{i;corr.} = M_{i;total} - M_{i;mf} \qquad (8)$$

The mass percentages obtained for the 3 TAGs (Equation 8) were normalized to 100%:

$$POP_{corr.} + POS_{corr.} + SOS_{corr.} = 100\% \qquad (9)$$

The variability of the TAG composition of CB is expressed by Equation 10 (2, 3):

$$POP - \% = 43.73 - 0.73 \times SOS - \%$$
$$(\text{residual standard deviation} = 0.125) \qquad (10)$$

The principle of the method is that for pure CB samples POS is practically constant for wide variations of POP and SOS; this results in a linear relationship (so-called "CB-line," Equation 10) between POP and SOS. CBE and other fat admixtures will cause the TAG analysis to deviate from the "CB-line" to the extent that their POS value differs from the POS value of CB. For 99% of all analyses, pure CB complies with the following equation:

$$POP_{corr.} < 44.03 - 0.73 \times SOS_{CORR.} \qquad (11)$$

A greater value of $POP_{corr.}$, as given by Equation 11, means that the sample is not pure CB. The advantage of the elaborated approach is that by using the CB CRM for calibration, the mathematical expression can be used by individual testing laboratories for verifying the purity of CB, without tackling the problem of establishing a "CB-line" as a prerequisite. Calibration by the CB CRM automatically links the results obtained in a laboratory to the CB and MF TAG databases and the elaborated mathematical formulas (Equations 4–11).

(**c**) *Quantitation of total fat in chocolate.*—The mass fraction in percent of total fat in the test sample (milk chocolate), $M_{fat;\,choc}$, was calculated as follows:

$$M_{fat;\,choc},\ \% = \frac{w_{fat}}{w} \times 100 \qquad (12)$$

where w is the mass of the test sample taken, in grams, w_{fat} is the mass of the total fat obtained from the test sample by Soxhlet extraction (29), in grams, and $M_{fat;\,choc}$ is the mass fraction in percent of total fat in the test sample.

(**d**) *Quantitation of MF in chocolate.*—The mass fraction in percent of MF in the final product chocolate, $M_{MF;\,choc}$, was calculated by applying Equation 13:

$$M_{MF;\,choc} = \frac{M_{fat;\,choc} \times M_{MF;\,choc\ fat}}{100} \qquad (13)$$

where $M_{fat;\,choc}$ is the mass fraction in percent of total fat in chocolate (*see* Equation 12), $M_{MF;\,choc\ fat}$ is the mass fraction in percent of MF in chocolate fat (*see* Equation 3), and $M_{MF;\,choc}$ is the mass fraction in percent of MF in chocolate.

(**e**) *Quantitation of CBE in chocolate.*—The RFs of the TAGs POP, POS, POO, SOS, and SOO were determined by injection of the CB CRM solution under experimental conditions identical to those used for the samples. The percentage of each of the 5 TAG fractions was calculated by the following equations:

$$P_{i;\,ref},\ \% = \frac{A_{i;\,ref}}{\sum A_{i;\,ref}} \times 100 \qquad (14)$$

and

$$F_i = \frac{M_{i;\,ref}}{P_{i;\,ref}} \qquad (15)$$

where $A_{i;\,ref}$ is the peak area of the TAG i in the CB CRM, $\Sigma A_{i;\,ref}$ is the sum of the peak areas attributed to POP, POS, POO, SOS, and SOO in the CB CRM, $P_{i;\,ref}$ is the percentage of TAG i in the CB CRM, $M_{i;\,ref}$ is the mass fraction in percent of TAG i in the CB CRM as given in the certificate (POP = 18.14%, POS = 44.68%, POO = 2.26%, SOS = 31.63%, and SOO = 3.29%, i.e., normalized to 100%; 8), and F_i is the detector RF of TAG i in the CB CRM.

The mass percentages of the TAGs POP, POS, POO, SOS, and SOO in the test sample were calculated by the following equation:

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

Table 6. Method performance data[a] for determination of CBE in chocolate

	Sample No.								
Parameter	2	4	5	6	9	10	11	12	13
No. of laboratories	12	12	12	12	12	12	12	12	12
No. of outliers	0	1	0	1	1	0	0	0	0
Identity of outlying laboratories		2		3	6				
Reason for removal		C		C	C				
No. of accepted laboratories	12	11	12	11	11	12	12	12	12
Mean value, g CBE/100 g chocolate	0.58	1.88	5.20	0.48	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	0.15	–0.09	–0.13	0.12	0.19	0.18	0.67	0.18
r, g/100 g	0.19	0.12	0.28	0.15	0.14	0.26	0.19	0.19	0.23
s_r, g/100 g	0.07	0.04	0.10	0.05	0.05	0.09	0.07	0.07	0.08
RSD_r, %	11.6	2.2	1.9	1.3	10.4	2.0	1.2	1.2	1.0
R, g/100 g	0.52	0.54	0.59	0.53	0.38	0.50	0.46	0.53	0.52
s_R, g/100 g	0.19	0.19	0.21	0.19	0.14	0.18	0.17	0.19	0.19
RSD_R, %	31.8	10.1	4.1	4.7	28.5	3.8	2.8	3.5	2.2
HorRat	7.34	2.79	1.30	1.45	6.37	1.21	0.93	1.13	0.77

[a] Based on results accepted on technical and statistical grounds (for samples 2–6, chocolate fat for GLC analysis was obtained by rapid fat extraction, and total fat content was determined by Soxhlet extraction; for samples 9–13, the assumed fat content of the chocolate was 30%). *See* also footnotes b–f in Table 4.

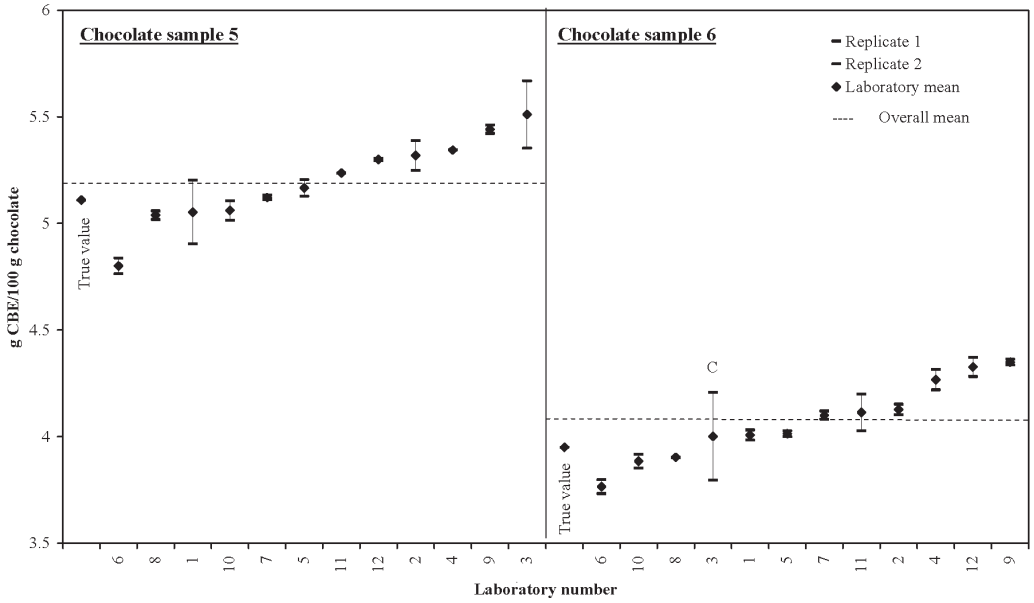

Figure 3. Laboratory means of CBE amounts in chocolate samples 5 and 6 (error bars represent range of blind duplicates; C = Cochran's test).

where F_i is the RF of the TAG i, i.e., POP, POS, POO, SOS, and SOO (*see* Equation 15), A_i is the peak area corresponding to the TAG i in the test sample, and $M_{i;\ choc\ fat}$ is the mass fraction in percent of TAG i in the test sample.

The mass fraction in percent of CBE in chocolate fat, $M_{CBE;\ choc\ fat}$, was calculated by using a PLS regression analysis (Equation 17) of the relative proportions of the 5 main TAGs, i.e., $POP_{choc\ fat}$, $POS_{choc\ fat}$, $POO_{choc\ fat}$, $SOS_{choc\ fat}$, and $SOO_{choc\ fat}$ as determined in Equation 16 and the MF content in chocolate fat, i.e., $M_{MF;\ choc\ fat}$, as determined in Equation 3.

$$M_{CBE;\ choc\ fat} = -4.24 - (0.23 \times M_{MF;\ choc\ fat}) + (1.52 \times POP_{choc\ fat}) - (1.47 \times POS_{choc\ fat}) + (1.09 \times POO_{choc\ fat}) + (1.29 \times SOS_{choc\ fat}) + (0.26 \times SOO_{choc\ fat}) \quad (17)$$

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

$$M_{CBE;\ choc} = \frac{M_{fat;\ choc} \times M_{CBE;\ choc\ fat}}{100} \quad (18)$$

where $M_{fat;\ choc}$ is the mass fraction in percent of total fat in chocolate (*see* Equation 12), $M_{CBE;\ choc\ fat}$ is the mass fraction in percent of CBE in chocolate fat (*see* Equation 17), and $M_{CBE;\ choc}$ is the mass fraction in percent of CBE in chocolate.

Results and Discussion

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 an appropriately functioning chromatographic system by fulfilling the required performance criteria (Table 2). Details of the submitted data are summarized in a comprehensive report (27). On the basis of the technical evaluation of the submitted results, all data sets from the 12 laboratories were accepted for the validation.

A brief outline of the GLC methods used by the participants and accepted on technical grounds is given in Table 3. All collaborators used a flame-ionization detection (FID) and narrow-bore fused-silica columns coated with medium-polarity stationary phases containing 50–65% phenyl methyl polysiloxane groups. The columns used in the ring trial were either from Varian-Chrompack (0.25 mm × 25 m, 0.1 μm CB-TAP, or 0.25 mm × 25 m, 0.05 μm Ultimetal) or from Restek (0.25 mm × 30 m, 0.1 μm Rtx-65TG). Different types of sample injection techniques, i.e., cold on-column injection (OCI; 3 laboratories), split (7 laboratories), and programmed temperature vaporizer (PTV; 2 laboratories) injection, were used. Further controllable parameters, different in the individual methods, were type of carrier gas, carrier-gas flow rate and/or inlet pressure, and temperature programming. The data sets accepted on technical grounds were subjected to statistical tests by procedures described by Horwitz (30).

Quantitation of PSB and MF in Chocolate Fat

A comparison of results obtained for the PSB content in chocolate fat by one analysis of the fat from rapid fat extraction and one analysis of the fat from Soxhlet extraction showed that the comparability of PSB data obtained in different laboratories is significantly better when the fat from the rapid fat extraction procedure for GLC analysis is used. For example, for chocolate sample 2, the results obtained by

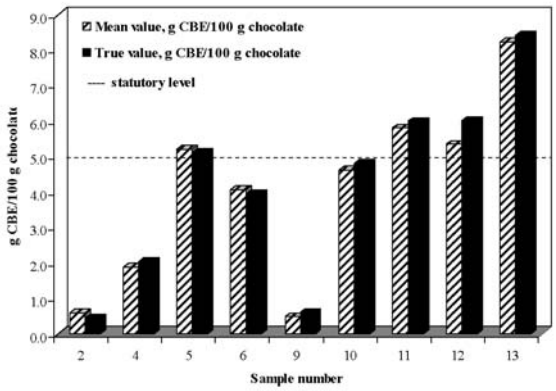

Figure 4. Comparison of true and experimentally determined values for CBE in chocolate (samples 2–6, total fat content determined by Soxhlet extraction; samples 9–13, assumed fat content of chocolate = 30%).

Soxhlet extraction ranged from 0.22 to 0.36 g PSB/100 g chocolate fat, whereas the data obtained by rapid fat extraction ranged only from 0.26 to 0.31 g PSB/100 g chocolate fat (Figure 1). The relative standard deviation for reproducibility (RSD_R) with no removal of any outliers was <7.3% for all 6 chocolate samples in the case of rapid fat extraction. Moreover, the results were in the same range as those obtained for the pure chocolate fat solutions. By analyzing the fat obtained from Soxhlet extraction, the RSD_R for all chocolate samples was >10.6%. Therefore, to calculate the final precision figures for the PSB content in chocolate fat, the results from the rapid fat extraction method were used (Table 4). The RSD_R values ranged from 2 to 4.6%. The calculated HorRat values, which can be used to indicate the acceptability of the precision of a method, ranged from 0.43 to 1.60, demonstrating acceptable performance of the method.

The MF content in chocolate fat was determined via the experimentally determined PSB content (Equation 2) by using a simple linear regression model (Equation 3). Actually, the resulting precision figures, relative standard deviation for repeatability (RSD_r) and RSD_R, were the same as those obtained for the PSB content, because the MF content is determined from the PSB amount.

Detection of CBE in Chocolate Fat

The outcome of the study was summarized as a number of "correct," "false positive" (CB-CBE-MF mixtures recognized as genuine CB), and "false negative" (genuine CB or CB-MF mixture recognized as CB-CBE or CB-CBE-MF mixture) results. The efficiency of the detection approach (percentage of correctly classified samples) was 100%. The correct classification of all samples suggested a detection limit of 0.5 g CBE/100 g chocolate.

Quantitation of Total Fat in Chocolate

To check label compliance of chocolate products, the results must be expressed in g MF/100 g chocolate and g CBE/100 g chocolate. Thus, it was necessary to determine the accurate amount of chocolate fat present in the chocolate samples. The recommended procedure in the method protocol for quantitating the amount of fat in chocolate was AOAC Official Method **936.15** (29). However, alternative extraction procedures were allowed to be used (e.g., accelerated solvent extraction, supercritical carbon dioxide extraction, or microwave extraction), provided that the same results were obtained. Figure 2 shows the plotted laboratory means and the corresponding laboratory ranges (analyses of blind duplicates) obtained for the 6 chocolate samples. In addition, the graph highlights the data sets from individual laboratories that were rejected for statistical reasons (C = Cochran's test; DG = Double Grubb's test). By removing statistical outliers, the RSD_R obtained was <1.2%, and the HorRat values were <0.5, indicating that most of the laboratories had excellent experience with the applied methods.

Quantitation of MF in Chocolate

By using the determined total fat contents of the chocolate samples (Equation 12) and an average assumed total fat content for the chocolate fat solutions (samples 8–13) of 30%, the results obtained for the MF content based on chocolate fat (g MF/100 g chocolate fat) were converted to g MF/100 g chocolate (Equation 13).

The RSD_R values for the chocolate samples (samples 1–6) ranged from 2.1 to 7.1%, whereas the RSD_R values for the chocolate fat solutions (samples 8–13) ranged from 6.3 to 7.5% (Table 5), demonstrating that the whole approach, which is based solely on chocolate fat blends, is applicable to real chocolate samples. Moreover, the results suggest that the additional analytical steps that must be applied in the case of real chocolate samples, i.e., (*1*) the extraction of the chocolate fat from the chocolate samples by rapid fat extraction to be used for the TAG profiling and (*2*) the determination of the total fat content of chocolate samples by Soxhlet extraction, do not alter the final outcome.

The overall mean MF values obtained for the chocolate fat solutions (samples 8–13) were in close agreement with the true MF values. The relative prediction errors, which ranged from –3.1 to –6.7%, were well within the expected range of ±10% (27). In the case of the chocolate samples, the true MF content was unknown. Nevertheless, the approximate MF contents of the chocolates given by the producer, based on estimations of the fat content of the individual sample ingredients, also showed good agreement with the experimentally determined values.

Quantitation of CBE in Chocolate

In cases where the detection approach indicated the presence of vegetable fats other than CB, the added CBE amount was quantitated. The 5 TAGs POP, POS, POO, SOS, and SOO (normalized to 100%) and the determined MF amount of the chocolate fat (Equation 3) were subjected to a PLS regression model (Equation 17) to calculate the final CBE content in chocolate fat. By using the determined total fat contents of the chocolate samples (Equation 12) and an average assumed total fat content for the chocolate fat

solutions of 30%, the results obtained for the CBE content based on chocolate fat (g CBE/100 g chocolate fat) were converted to g CBE/100 g chocolate (Equation 18).

Precision data regarding the performance of the quantitation method are summarized in Table 6. The RSD_R for quantitation of CBEs around the statutory limit of 5% did not show a difference for real chocolate samples (samples 5 and 6) and for chocolate fat solutions (samples 10–13). The calculated HorRat values ranged from 0.77 to 1.45, demonstrating a good performance of the method. The results for samples 2, 4, and 9 are given just as an example to show that the RSD_R in the case of very low CBE additions, i.e., <2 g CBE/100 g chocolate, is increasing. For samples with a CBE addition of >2 g CBE/100 g chocolate, the RSD_R was in all cases <5%. This result is due to the fact that the established PLS model (Equation 17) for calculating the final CBE addition was fitted to CBE amounts around the statutory level of 5% of the final chocolate product to fulfill the requirements of Directive 2000/36/EC (3).

Figure 3 shows the range of laboratory mean values and the conformity of the true and predicted values for chocolate samples 5 and 6. The overall mean values obtained 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 not larger than ±0.6% (Figure 4). With the assumption of a 30% fat content for chocolate, this translates to ±0.2% relative to the final product. In the case of sample 12, which contained a soft palm midfraction, a somewhat higher bias was obtained, i.e., 2.3% (which translates to 0.7%, assuming a fat content of 30% for chocolate). Nevertheless, the error was still within the expected range of ±0.9% (27).

Conclusions

The results of this collaborative trial show that the proposed approach produces acceptably accurate, repeatable, and reproducible results and offer an important means to enforce the correct labeling of milk chocolate. It has the advantage that by performing a single TAG analysis using GLC, several useful pieces of information can be determined, i.e., (*1*) the MF content of the sample, (*2*) the contribution of TAGs derived from MF, (*3*) the presence/absence of CBEs in the sample, and (*4*) the CBE content of the sample. The HorRat values ranged from 0.77 to 1.45, demonstrating good performance of the whole approach. The method performed well at the statutory limit of 5% CBE addition to milk chocolate with a prediction error of 0.7%. CBE admixtures were detected down to a level of 0.5 g CBE/100 g chocolate, without false-positive or -negative results.

High comparability of data between individual laboratories was demonstrated, resulting in excellent precision data. No differences were observed for real chocolate samples and for chocolate fat solutions, demonstrating that the whole approach, which was at first developed for chocolate fat blends (27), is applicable to real chocolate samples. The compulsory use of the CB CRM (IRMM-801) for calibration and the system suitability check ensures high comparability of the results between individual testing laboratories. Moreover, the commutability of the elaborated approach, which is based on reliable databases created under strict quality control schemes that reflect as much as possible the natural variability of CBs, MFs, and CBEs, is guaranteed.

Acknowledgments

We thank Olivier Nuytten (Barry Callebaut N.V.) for providing tailor-made chocolate samples and Marianne Lindblom (Kraft Foods) and Ralph Timms (Britannia Food Ingredients, Goole, UK) for providing MF, CB, and CBE samples. In particular, we express our appreciation to the following collaborators for their participation in the study:

Annlouise Lomnitz, Karlshamns Sweden AB, Karlshamn, Sweden

Eva-Maria Berndt, Kraft Foods, Munich, Germany

Gerhard Neuberger, Ferrero oHGmbH, Stadtallendorf, Germany

Ingrid Kaufmann-Horlacher, Chemisches und Veterinäruntersuchungsamt Stuttgart, Fellbach, Germany

Marcel Schut, Gerkens Kakao B.V., Wormer, The Netherlands

Maria Teresa Rodriguez-Estrada, University of Bologna, Bologna, Italy

Matthias Schreiner, University of Natural and Applied Life Sciences (BOKU), Vienna, Austria

Norbert Wachter, ADM Noble & Thörl GmbH, Hamburg, Germany

Reinhard Matissek and Robert Maurer, Lebensmittelchemisches Institut des Bundesverbandes der Deutschen Süßwarenindustrie, Köln, Germany

Sabine Schulokat, Eurofins, Wiertz-Eggert-Jörissen GmbH, Hamburg, Germany

References

(1) Buchgraber, M., Senaldi, C., Ulberth, F., & Anklam, E. (2004) *J. AOAC Int.* **87**, 1153–1163

(2) Buchgraber, M., Ulberth, F., & Anklam, E. (2004) *J. AOAC Int.* **87**, 1164–1172

(3) EC Council Directive 2000/36 (June 23, 2000) in *Off. J. Eur. Comm.* **L 197**, 19–25

(4) Buchgraber, M., & Anklam, E. (2003) EUR 20742 EN, European Communities

(5) Buchgraber, M., & Anklam, E. (2003) EUR 20831 EN, European Communities

(6) International Organization for Standardization (2006) Method 23275-1

(7) International Organization for Standardization (2006) Method 23275-2

(8) Koeber, R., Buchgraber, M., Ulberth, F., Bacarolo, R., Bernreuther, A., Schimmel, H., Anklam, E., & Pauwels, J. (2003) EUR 20781 EN, European Communities

(9) http://www.irmm.jrc.be/html/activities/cocoa_butter_calculation_toolbox/index.htm

(10) Hadorn, H., & Zuericher, K. (1972) *Rev. Int. Choc.* **27**, 82–92

(11) Padley, F.B., & Timms, R.E. (1980) *J. Am. Oil Chem. Soc.* **57**, 286–293
(12) Timms, R.E. (1980) *J. Dairy Res.* **47**, 295–303
(13) Fincke, A. (1980) *Dtsch. Lebensm. Rundsch.* **76**, 162–167
(14) Padley, F.B., & Timms, R.E. (1978) *Lebensm. Wiss. Technol.* **11**, 319–322
(15) Fincke, A. (1980) *Dtsch. Lebensm. Rundsch.* **76**, 389–396
(16) Pontillon, J. (1995) *J. Am. Oil Chem. Soc.* **72**, 861–866
(17) Phillips, A.R., & Sanders, B.J. (1968) *J. Assoc. Public Anal.* **6**, 89–95
(18) Muuse, B., & Martens, R. (1993) *Int. Dairy Fed. Bull.* **285**, 65–69
(19) Ulberth, F. (1994) *J. AOAC Int.* **77**, 1326–1334
(20) Ulberth, F. (1995) *J. Agric. Food Chem.* **43**, 1556–1560
(21) Precht, D. (1992) *Z. Lebensm. Unters. Forsch.* **194**, 1–8
(22) Precht, D. (1992) *Z. Lebensm. Unters. Forsch*. **194**, 107–114
(23) Ulberth, F. (1998) *Z. Lebensm. Unters. Forsch. A* **206**, 305–307
(24) Precht, D. (1990) *Fat Sci. Technol.* **92**, 153–161
(25) Precht, D. (1990) *Fat Sci. Technol.* **92**, 275–281
(26) Young, C.C. (1984) *J. Am. Oil Chem. Soc.* **61**, 576–581
(27) Buchgraber, M., & Androni, S. (2006) EUR 22553 EN, European Communities
(28) Thompson, M., & Wood, R. (1993) *J. AOAC Int.* **76**, 926–940
(29) *Official Methods of Analysis* (2000) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **963.15**
(30) Horwitz, W. (1995) *Pure Appl. Chem.* **67**, 331–343