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Determination of Cocoa Butter Equivalents in Milk Chocolate by Triacylglycerol Profiling

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An analytical approach for the detection and quantification of cocoa butter equivalents (CBEs) in milk chocolate is presented. It is based on (i) a comprehensive standardized database covering the triacylglycerol composition of a wide range of authentic milk fat ($n = 310$), cocoa butter ($n = 75$), and 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 milk fat content in chocolate fats 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 detection limit was 1% CBE in chocolate fat (0.3% CBE in milk chocolate, having a fat content of 30%). For quantification, the average error for prediction was 1.2% CBE in chocolate fat, corresponding to 0.4% in milk chocolate (fat content, 30%).

KEYWORDS: Milk fat; cocoa butter equivalents; milk chocolate; triacylglycerols; GLC

INTRODUCTION

Chocolate products, governed in the European Union by Directive 2000/36/EC (*1*), may contain vegetable fats other than cocoa butter (CB) up to 5% of the total weight, provided that their labeling is supplemented by the statement, "Contains vegetable fats in addition to cocoa butter". Only six vegetable fats (so-called cocoa butter equivalents, CBEs), clearly specified in Annex II of the Directive, can be used singly or in blends, that is, Illipé (*Shorea* spp.), palm oil (*Elaeis guineensis* and *Elaeis olifera*), sal (*Shorea robusta*), shea (*Butyrospermum parkii*), kokum gurgi (*Garcinia indica*), and mango kernel (*Mangifera indica*). Because of the similar chemical composition and physical properties of CB and CBEs, it is extremely difficult to quantify them, and in some cases, it is even difficult to detect them. A need has been recognized within official control laboratories for reliable analytical methods to prove label compliance to protect consumers from fraudulent malpractice.

To allow implementation and enforcement of the Directive 2000/36/EC (*1*), an integrated approach for determining CBEs in plain chocolate using triacylglycerol (TAG) profiling by high-resolution gas−liquid chromatography (HR-GLC) has been developed (*2*) and validated in international collaborative trials (*3*). To facilitate the usage of the approach, an analytical toolbox named "CoCal-1 (= cocoa butter calculation toolbox)" has been established, consisting of a validated method for the detection of CBEs in dark chocolate (*4*) and a validated method for the quantification of CBEs in dark chocolate (*5*), both of them adopted as official methods by the International Organization for Standardization (ISO) (*6*, *7*); a certified CB reference material 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 has been only applicable to dark chocolate and not to milk chocolate since TAGs originating from milk fat (MF) had an impact on the final evaluation logarithms established for dark chocolate. The analysis of milk chocolate requires knowledge about the level of MF, allowing one to correct the observed TAG analysis for the presence of MF TAGs. A new analytical approach for the determination of MF in chocolate fats using TAG profiling by HR-GLC is presented in ref *10*.

This part addresses the other two questions left to control correct labeling of milk chocolate, that is, (i) is there any other fat in addition to CB present and, if yes, (ii) how much? A modification of the original approach for analysis of CBEs in dark chocolate is presented, having the advantage of allowing a reliable quantification of the MF content in chocolate fats and the correction of TAGs deriving from MF using a single TAG analysis.

MATERIALS AND METHODS

Materials. Sampling of genuine CB and CBE samples took place from 1992 to 2005. Emphasis was put on the fact that the sample collection contained (i) CBs from individual harvests comprising samples of South American, Asian, and African origins and (ii) CB blends as used for the production of chocolate. The set of CBE samples comprised (i) raw materials (as specified in Annex II of Directive 2000/

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36/EC) for CBE production and (ii) a broad variety of commercially available CBE blends. A detailed description of the MF database, calibration standards, and chemicals used is given in ref *10*. On special request, real milk chocolate samples, reflecting the range of commercially available chocolates, varying in composition and with known levels of CBEs, were produced by Barry Callebaut (Lebbeke-Wieze, Belgium).

Sample Preparation. CB-MF (222) and 725 CB-CBE-MF mixtures, simulating chocolate fats obtained from real milk chocolates, were gravimetrically blended, as described in ref *10*. A representative amount of chocolate fat from chocolate samples for TAG analysis was obtained by extraction with two 10 mL portions of *n*-hexane using 5 g of grated chocolate, vortexing, centrifuging, decanting, and combining the extracts. The solvent was then evaporated and finally dried under a stream of nitrogen. The determination of the accurate amount of chocolate fat in chocolate was done according to AOAC Official Method 936.15 (*11*).

Preparation of Chocolate Fats for TAG Analysis. Chocolate fats (0.1 g), that is, CB-MF and CB-CBE-MF blends and fats obtained from chocolate samples, were dissolved in 10 mL of *iso*-octane. These stock solutions were treated for split and cold on-column injection (OCI) in the same way as described in ref *10*.

Gas Chromatography. All analyses were performed on a 6890N GC (Agilent Technologies, Diegem, Belgium) equipped with a 7683 autoinjector, a split/splitless injection port, a cold OCI, flame ionization detector, and the GC ChemStation software Rev. A.10.02 for chromatogram processing.

Analysis of TAGs. All analyses of chocolate fats and MF, CB-MF, and CB-CBE-MF blends were performed as outlined in ref *10*.

Calibration of TAGs for CBE Detection. Identification and quantification of the three main TAGs used for the detection of CBEs, that is, 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (POS), and 1,3-distearoyl-2-oleoyl-glycerol (SOS), was done by using the CB-certified reference material (IRMM-801) (*8*). To this end, response factors for the three TAGs (POP, POS, and SOS) were determined by analyzing IRMM-801 using experimental conditions identical to those used for the test sample:

$$P_{i,\mathrm{ref}} = \frac{A_{i,\mathrm{ref}}}{\sum A_{\mathrm{allTGs,ref}}} \times 100\% \quad (1)$$

$$F_i = \frac{M_{i,\mathrm{ref}}}{P_{i,\mathrm{ref}}} \quad (2)$$

where $A_{i,\mathrm{ref}}$ is the peak area of the TAG i in the IRMM-801, $\sum A_{\mathrm{allTGs,ref}}$ is the sum of the peak areas attributed to all TAGs in the IRMM-801, $P_{i,\mathrm{ref}}$ is the percentage of TAG i in the IRMM-801 (from peak areas), $M_{i,\mathrm{ref}}$ is the mass fraction in percent of TAG i in the IRMM as given in the certificate (*8*), that is, POP = 16.00%, POS = 39.40%, and SOS = 27.90%, and F_i is the detector response factor of TAG i in the IRMM-801.

The mass percentages of the TAGs POP, POS, and SOS with respect to all TAGs in a test sample were determined by:

$$M_{i,\mathrm{total}} = \frac{F_i \times A_i}{\sum A_{\mathrm{allTGs}}} \times 100\% \quad (3)$$

where A_i is the peak area corresponding to the TAG i in the test sample, $\sum A_{\mathrm{allTAGs}}$ is the sum of the peak areas attributed to all TAGs in the test sample, F_i is the response factor for the TAG i (as determined by eqs 1 and 2), and $M_{i,\mathrm{total}}$ 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 deriving from MF was calculated by:

$$M_{i,\mathrm{mf}} = \frac{M_{\mathrm{MF,sample}} \times M_{i,\mathrm{ref}}}{100\%} \quad (4)$$

where $M_{\mathrm{MF,sample}}$ is the mass fraction in percent of MF in the test sample, as determined via 1-palmitoyl-2-stearoyl-3-butyroyl-glycerol (PSB) in ref *10*, $M_{i,\mathrm{ref}}$ is the average mass fraction in percent of TAG i in a MF, that is, POP = 3.99%, POS = 2.19%, and SOS = 0.45%, and $M_{i,\mathrm{mf}}$ is the mass fraction in percent of TAG i derived from MF in the test sample.

The obtained mass percentages of the three TAGs originating from MF (eq 4) were subtracted from the mass percentages of the three TAGs from the test sample (eq 3) by:

$$M_{i,\mathrm{corr.}} = M_{i,\mathrm{total}} - M_{i,\mathrm{mf}} \quad (5)$$

The MF-corrected mass percentages of the three TAGs, that is, $M_{i,\mathrm{corr.}}$ (eq 5), were normalized to 100% (eq 6):

$$\mathrm{POP}_{\mathrm{corr.}}\% + \mathrm{POS}_{\mathrm{corr.}}\% + \mathrm{SOS}_{\mathrm{corr.}}\% = 100\% \quad (6)$$

Calibration of TAGs for CBE Quantification. Identification and quantification of the five main TAGs used for the quantification of CBEs, that is, POP, POS, 1,2-dioleoyl-3-palmitoyl-glycerol (POO), SOS, and 1,2-dioleoyl-3-stearoyl-glycerol (SOO), was done by using IRMM-801 as described in refs *7* and *12*.

Statistical Analysis. Statistical analyses were carried out with the STAGRAPHICS Version Plus 5.1 computer package (Manugistics Inc., United States) and Statistica (StatSoft Inc., United States). Partial least-squares (PLS) regression analysis was carried out with the Unscrambler version 7.6 software program (CAMO ASA, Oslo, Norway).

RESULTS AND DISCUSSION

Much research has been targeted at detecting and quantifying the addition of CBEs to dark and milk chocolate, necessary to allow enforcement of Directive 2000/36/EC. In the late 1970s, Padley and Timms (*13*, *14*) and Fincke (*15*, *16*) jointly developed a method based on packed column GLC of CB TAGs, which has found wide application for purity control purposes in industry and food control. An extension of this approach, based on the same detection principle but a new quantification algorithm, was developed and validated for dark chocolate. For analysis, a fused silica capillary column coated with medium polarity phenylmethylsilicone stationary phases allows a more detailed insight into the complexity of chocolate fats (*6*, *7*). Up to now, the existing standardized approach for dark chocolate was not implementable for milk chocolate. As shown in **Figure 1**, MF contains the same TAGs (POP, POS, POO, SOS, and SOO) as used for the detection and quantification of CBEs in dark chocolate. The principle of the detection method for dark chocolate is that for pure CBs there is a linear relationship between POP and SOS. An addition of any CBE will cause a deviation from this "CB line". By applying this approach to genuine CB containing 15% MF, without any correction for MF TAGs, the same result would be generated as for a CB sample containing 4% CBE (**Figure 2**). The same outcome was observed for the quantification algorithm, which is based on a PLS regression analysis model using the five main TAGs present in CB (*5*). Dionisi et al. (*17*) established a mathematical formula to quantify CBEs in chocolate based on TAG profiling using liquid chromatography equipped with an evaporative light-scattering detector. To discriminate between CB and CBEs, the TAGs POP, POS, PLS and the ratios POP/PLS and POS/PLP were used. The model was validated for dark chocolate and showed an absolute average error of ±2.1%, which is comparable to the official methods from ISO (*6*, *7*). However, Dionisi et al. (*17*) suggest that their model could be applied to milk chocolate without undergoing a correction for MF TAGs. From their point of view, the interferences from MF were negligible when quantitative chromatogram evaluation was restricted to peaks with carbon number groups 46−54. This assumption, which was based on the analysis of just two milk chocolate samples, is contrary to our findings. In our survey of 310

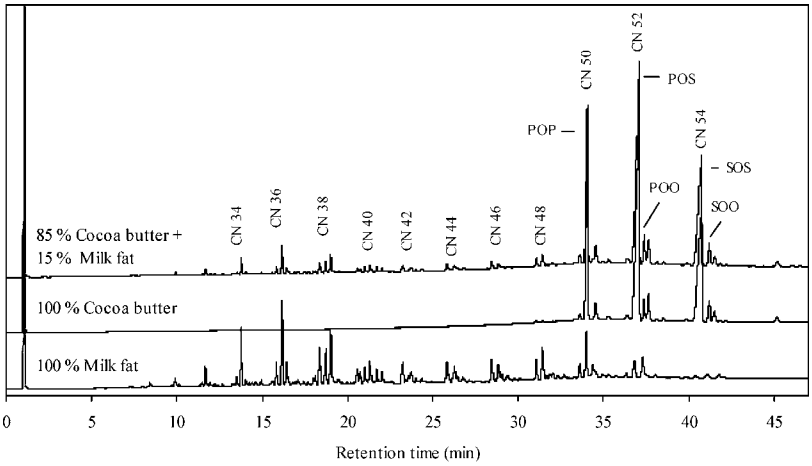

Figure 1. TAG profiles of pure CB, pure MF, and a mixture thereof obtained by capillary column GLC.

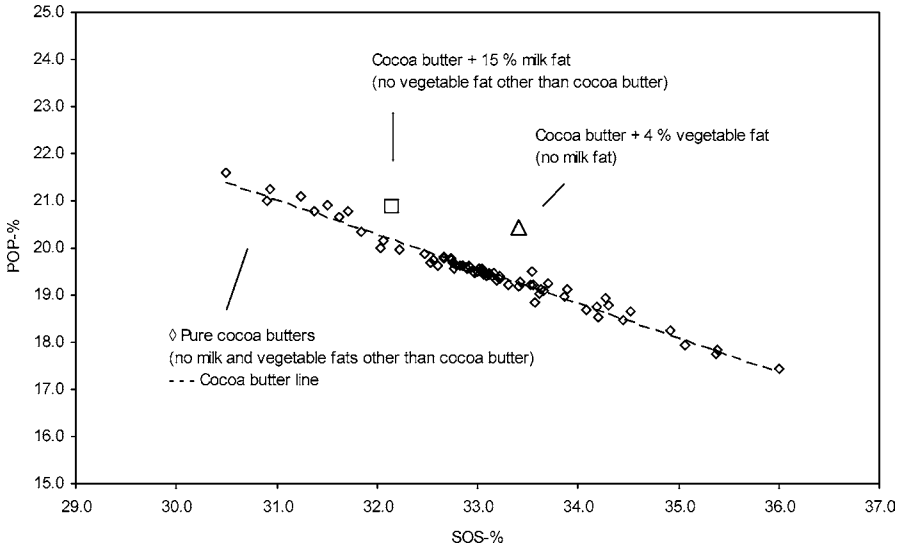

Figure 2. Relationship between normalized content of POP and SOS of pure CB samples and two chocolate fats.

Table 1. Descriptive Statistics of the Five Main TAGs, Originating from MF, Which Interfere with the CBE Evaluation Algorithms

	valid N	mean	standard deviation	standard error	minimum	maximum
g POP/100 g MF	310	3.99	0.28	0.02	3.34	5.03
g POS/100 g MF	310	2.19	0.31	0.02	1.51	3.39
g POO/100 g MF	310	3.46	0.47	0.03	2.43	4.80
g SOS/100 g MF	310	0.45	0.10	0.01	0.21	0.75
g SOO/100 g MF	310	0.97	0.22	0.01	0.47	1.63

	confidence −95%	confidence 95%	lower quartile	upper quartile	10th percentile	90th percentile
g POP/100 g MF	3.95	4.02	3.84	4.12	3.62	4.33
g POS/100 g MF	2.16	2.23	1.93	2.41	1.83	2.56
g POO/100 g MF	3.40	3.51	3.10	3.77	2.87	4.07
g SOS/100 g MF	0.44	0.46	0.37	0.51	0.32	0.59
g SOO/100 g MF	0.94	0.99	0.79	1.11	0.70	1.26

genuine MF samples (*10*), the average concentration of the five TAGs, that is, POP, POS, POO, SOS, and SOO, in MF was determined (**Table 1**). The highest contribution came from POP and POS, both used in the model established by Dionisi et al. (*17*), and POO. POP had an average value of 3.99 g/100 g MF, ranging from 3.34 to 5.03 g/100 g MF. The input from SOS and SOO as compared to the other three TAGs was rather low, with values of 0.45 and 0.97 g/100 g MF, respectively. Hence, when chocolate fat is analyzed for compliance with label declaration, it will be necessary to correct the observed TAG profile for the presence of MF TAGs; otherwise, the final results will be misleading.

Detection of CBEs in Milk Chocolate. In principle, the presence of CBEs in dark chocolate is detected by linear regression analysis applied to the relative proportions of the three TAG fractions, that is, POP, POS, and SOS. The variability of the TAG composition of pure CB is expressed by eq 7 using the normalized TAGs, that is, POP% + POS% + SOS% = 100% (*6*).

$$\text{POP\%} = 43.734 - 0.733 \times \text{SOS\%} \quad \text{(residual standard deviation} = 0.125) \quad (7)$$

This equation was established by using the TAG profile of 75 individual genuine CBs (*4*, *6*). The fit of the data to the CB line is shown in **Figure 2**. If the data are normally distributed, then 99% of all analyses are beneath the average value plus 2.326 × residual standard deviation. Therefore, for 99% of all analyses, pure CB complies with

$$\text{POP\%} < 43.734 - 0.733 \times \text{SOS\%} + 2.326 \times 0.125 \quad (8)$$

that is, POP% < 44.025 − 0.733 × SOS%.

A greater value of POP, as given by eq 8, means that the sample is not pure CB. In the case of a milk chocolate, the obtained TAG chromatograms have to be corrected for the

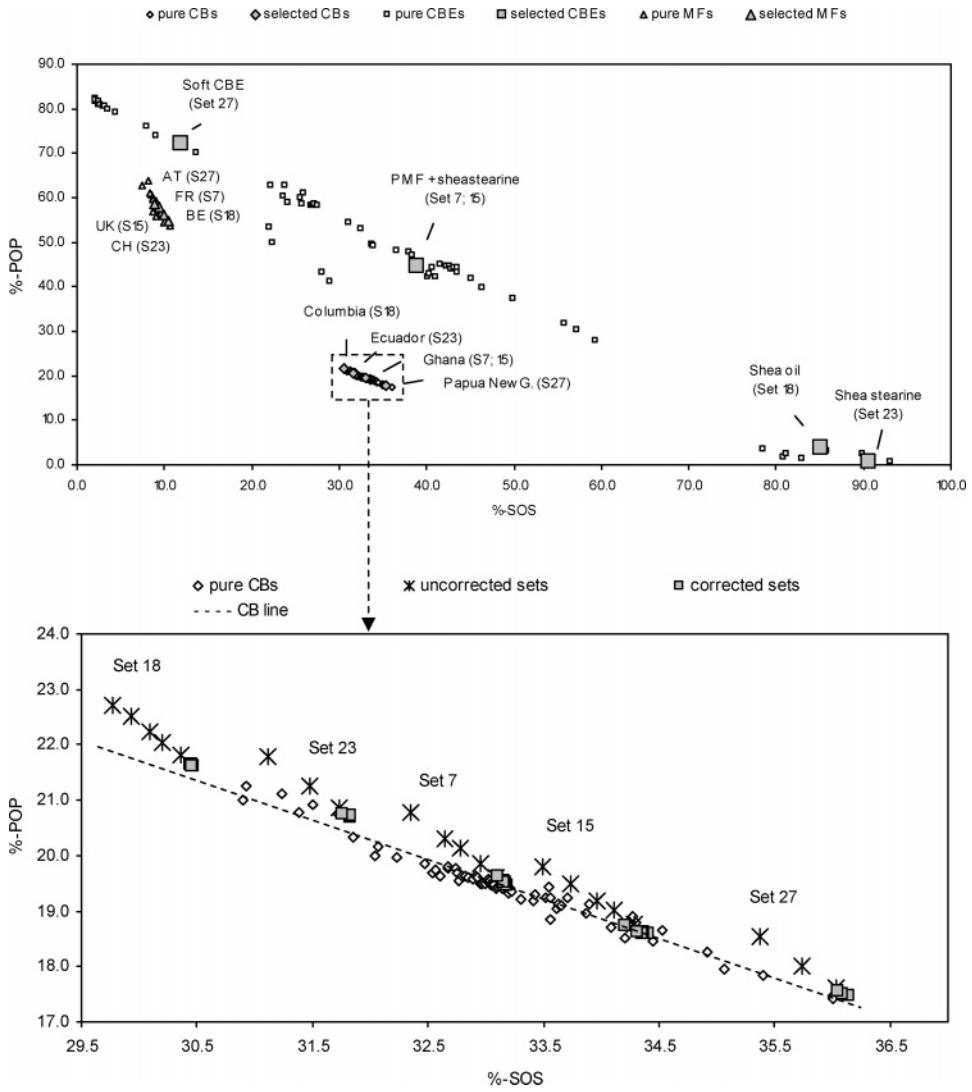

Figure 3. Relationship between normalized content of POP and SOS of CB, CBE, and MF samples and mixtures thereof.

contribution of the mass percentages of the TAGs POP, POS, and SOS originating from MF. To this end, the average content of these TAGs in MF can be used (**Table 1**). After determining the MF content via PSB as described in ref *10*, the mass percentages of the TAGs POP, POS, and SOS in the test sample with respect to all TAGs present in the test sample are determined (eq 3). The second step is to calculate the contribution of the mass percentages of the TAGs POP, POS, and SOS deriving from MF (eq 4), followed by the subtraction (eq 5) of the mass percentages of the three TAGs derived from MF (eq 4) from the mass percentages of the three TAGs obtained for the test sample (eq 3). After normalizing the obtained mass percentages of the MF-corrected TAGs to 100% (eq 6), the same decision rules as for dark chocolate (eq 8) to detect if there are any CBEs present in the chocolate fat can be applied.

The performance and validity of the established evaluation principle were tested by analyzing and applying the whole approach to gravimetrically prepared CB-MF ($n = 222$) and CB-CBE-MF ($n = 725$) mixtures. To demonstrate to what extent TAGs originating from MF can bias the final evaluation algorithm, the results were treated once using data corrected for MF contribution and once using uncorrected TAG data. In most of the CB-MF blends (181 out of 222 samples), uncorrected TAG data resulted in a false-positive decision, that is, a genuine CB would be recognized as CB having an admixture of CBE, which stresses the need to perform a correction for the presence of MF in chocolate fats. A few examples are shown in **Figure 3**. In the case of using uncorrected data sets, the samples deviated from the CB line, whereas by applying corrected data sets the samples were positioned correctly. By using the newly established cascade of formulas, none of the 222 CB-MF gave a false-positive result; each genuine CB sample was registered as being genuine CB. All CB-CBE-MF mixtures were correctly classified down to a level of 1% CBE in the fat mixtures, translating to a level of 0.3% CBE in the final products assuming a fat content of 30% in chocolate. The advantage of the approach is that by using IRMM-801 for calibration purposes, the mathematical expressions can be used by individual testing laboratories for verifying the purity of CB in mixtures with MF, without tackling the problem of establishing a CB line or determining the average contribution of the five main TAGs originating from MF. Calibration by IRMM-801 automatically links the results obtained in a laboratory to the CB and newly established MF database and the associated elaborated decision rules. In principle, the end user has only to obtain a TAG profile of the sample in question by HR-GLC and determine seven peaks, that is, α-cholestane (internal standard) and PSB, which are used to quantify the MF proportion in chocolate fat (*10*), POP, POS, POO, SOS, and SOO, which are used to detect and quantify CBEs in chocolate fat. By using these TAG peaks, several useful pieces of

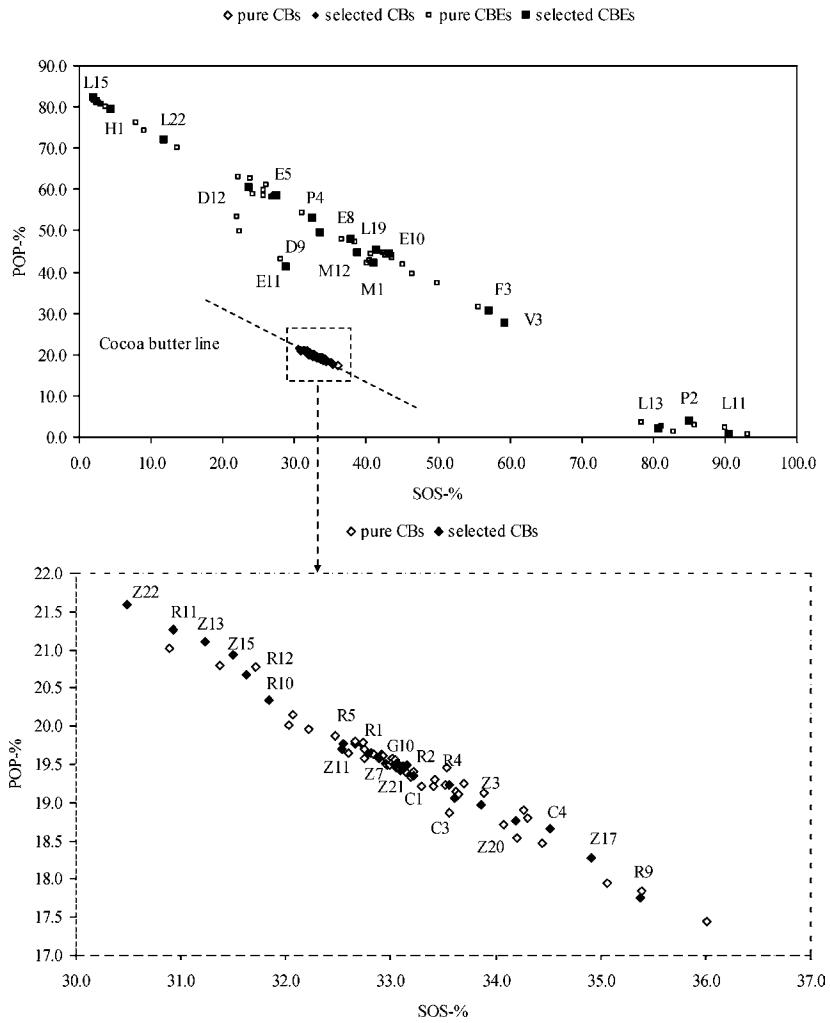

Figure 4. Relationship between normalized content of POP and SOS of selected CB and CBE samples. The codes of the individual CBs and CBEs are described in ref *10*.

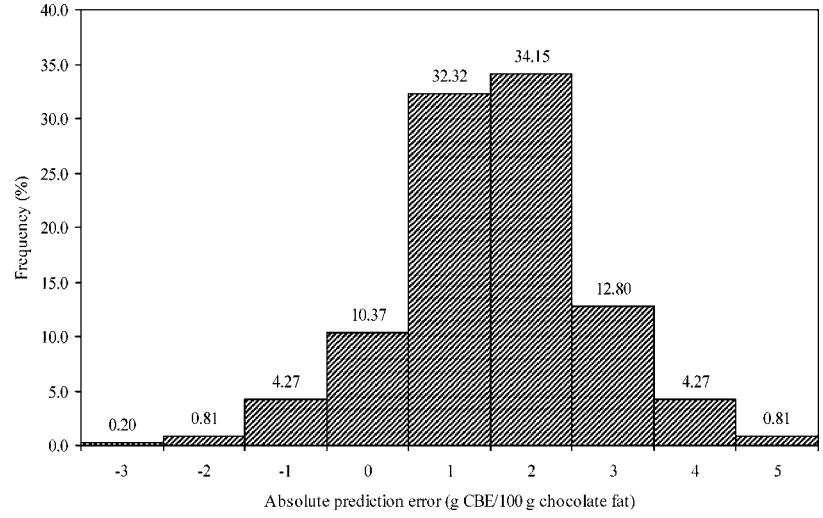

Figure 5. Distribution of absolute prediction errors as obtained for the whole data set ($n = 725$ samples).

information can be obtained, that is, (i) the MF 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 has to be answered, that is, how much CBE has been added?

Quantification of CBEs in Milk Chocolate. For dark chocolate, a reliable quantification of CBEs, to monitor correct labeling at the statutory limit of 5%, is obtained by applying a PLS regression model (eq 9) to the relative proportions of the five main TAGs (*5*, *7*):

$$\text{g CBE/100 g CB} = 37.44 + 1.18 \times \text{POP\%} - 1.94 \times \text{POS\%} - 0.12 \times \text{POO\%} + 0.98 \times \text{SOS\%} - 0.10 \times \text{SOO\%} \quad (9)$$

Table 2. Agreement between Actual and Experimentally Determined MF and CBE Contents of Various CB-CBE-MF Mixtures[a]

MF Contents

	% MF			absolute error		relative error	
sample	known	predicted SP	predicted OCI	SP	OCI	SP	OCI
			calculation on fat basis				
1	6.0	6.2	6.2	–0.1	–0.1	–2.2	–2.3
2	14.4	14.4	14.2	0.0	0.2	0.0	1.4
3	6.3	6.4	6.4	–0.1	–0.1	–1.4	–2.1
4	24.2	24.9	24.7	–0.7	–0.5	–2.9	–2.0
5	16.2	16.5	16.0	–0.3	0.2	–1.7	0.9
6	5.6	5.7	5.8	–0.1	–0.2	–2.2	–3.6
7	11.3	11.6	11.0	–0.3	0.3	–3.0	2.4
8	19.7	19.8	19.6	–0.1	0.1	–0.5	0.5
9	26.1	26.2	26.1	–0.1	0.0	–0.5	–0.1
10	14.9	15.0	14.7	0.0	0.3	–0.2	1.8
11	9.3	9.3	9.4	0.1	–0.1	0.8	–0.9
12	15.4	15.8	15.5	–0.3	–0.1	–2.1	–0.6
13	14.9	15.3	15.1	–0.4	–0.2	–2.9	–1.6
14	24.5	24.7	24.4	–0.2	0.2	–0.8	0.7
			calculation on chocolate basis (assuming a fat content of 30%)				
1	1.8	1.9	1.9	0.0	0.0	–2.2	–2.3
2	4.3	4.3	4.3	0.0	0.1	0.0	1.4
3	1.9	1.9	1.9	0.0	0.0	–1.4	–2.1
4	7.3	7.5	7.4	–0.2	–0.1	–2.9	–2.0
5	4.9	4.9	4.8	–0.1	0.0	–1.7	0.9
6	1.7	1.7	1.7	0.0	–0.1	–2.2	–3.6
7	3.4	3.5	3.3	–0.1	0.1	–3.0	2.4
8	5.9	5.9	5.9	0.0	0.0	–0.5	0.5
9	7.8	7.9	7.8	0.0	0.0	–0.5	–0.1
10	4.5	4.5	4.4	0.0	0.1	–0.2	1.8
11	2.8	2.8	2.8	0.0	0.0	0.8	–0.9
12	4.6	4.7	4.7	–0.1	0.0	–2.1	–0.6
13	4.5	4.6	4.5	–0.1	–0.1	–2.9	–1.6
14	7.4	7.4	7.3	–0.1	0.1	–0.8	0.7

CBE Contents

	% CBE			absolute error		relative error	
sample	known	predicted SP	predicted OCI	SP	OCI	SP	OCI
			calculation on fat basis				
1	8.7	8.0	7.9	0.7	0.8	8.2	9.4
2	9.0	8.9	8.9	0.1	0.2	1.4	1.8
3	10.3	10.6	10.3	–0.2	0.0	–2.1	0.1
4	10.5	9.9	10.0	0.7	0.6	6.2	5.4
5	15.0	13.9	13.6	1.1	1.3	7.5	9.0
6	15.5	14.2	14.0	1.3	1.5	8.5	10.0
7	18.9	18.4	18.4	0.5	0.5	2.9	2.6
8	19.0	18.9	19.1	0.1	–0.1	0.7	–0.4
9	24.7	24.7	24.7	0.0	0.0	0.0	0.1
10	25.6	24.4	24.0	1.2	1.6	4.7	6.3
11	29.6	28.7	28.7	1.0	0.9	3.3	3.1
12	30.3	28.8	28.7	1.5	1.6	4.8	5.2
13	30.9	30.7	31.1	0.2	–0.2	0.5	–0.7
14	31.1	31.6	31.6	–0.5	–0.5	–1.8	–1.7
			calculation on chocolate basis (assuming a fat content of 30%)				
1	2.6	2.4	2.4	0.2	0.2	8.2	9.4
2	2.7	2.7	2.7	0.0	0.0	1.4	1.8
3	3.1	3.2	3.1	–0.1	0.0	–2.1	0.1
4	3.2	3.0	3.0	0.2	0.2	6.2	5.4
5	4.5	4.2	4.1	0.3	0.4	7.5	9.0
6	4.7	4.3	4.2	0.4	0.5	8.5	10.0
7	5.7	5.5	5.5	0.2	0.1	2.9	2.6
8	5.7	5.7	5.7	0.0	0.0	0.7	–0.4
9	7.4	7.4	7.4	0.0	0.0	0.0	0.1
10	7.7	7.3	7.2	0.4	0.5	4.7	6.3
11	8.9	8.6	8.6	0.3	0.3	3.3	3.1
12	9.1	8.6	8.6	0.4	0.5	4.8	5.2
13	9.3	9.2	9.3	0.1	–0.1	0.5	–0.7
14	9.3	9.5	9.5	–0.2	–0.2	–1.8	–1.7

[a] SP, split injection; OCI, cold OCI.

The initial strategy to reliably quantify CBEs in milk chocolate was to use the same correction principle as for the detection part, that is, subtract the amount of the five TAGs deriving from MF from the whole TAG profile and apply subsequently the same PLS model (eq 9) as established for dark chocolate. However, the final outcome in terms of prediction error was not satisfactory as compared to the results obtained for dark chocolate. A regression model using the amount of MF present in a sample as an additional variable on top of the five main TAGs proved to be more successful. To this end, TAG data of 725 CB-CBE-MF blends varying in type and amount of MF, CB, and CBEs (**Figure 4**) were used for building the quantification model. The contents of the five main TAGs obtained were normalized so that the sum of POP% + POS% + POO% + SOS% + SOO% equaled 100%. In addition, the PSB amount of the samples was used to calculate the MF contents of the samples, as described earlier (*10*). These six variables were finally used to model a relation between the individual TAGs measured and the amount of CBE present in the chocolate fat. Interpretation of the data was done by means of chemometric tools, already successfully applied by others (*18*−*21*) for the detection of adulterated MFs.

To develop the model, the data set ($n = 725$) was randomly split into two parts, that is, a calibration data set ($n = 363$) and a validation data set ($n = 362$). By using the six variables (MF content, POP, POS, POO, SOS, and SOO), a PLS regression analysis was computed to predict the concentration of CBEs in chocolate fats. The validation data set was used to check the effectiveness of the elaborated calibration function, resulting in no significant differences between the two data sets. The obtained mean average deviation (MAD) was 0.94 g/100 g fat chocolate fat for the calibration set and 0.90 g/100 g for the validation data set and the root-mean-square error of prediction (RMSEP) 1.20 g/100 g and 1.16, respectively. Hence, the two data sets were merged to calculate the final prediction model based on all data sets available ($n = 725$):

$$\text{g CBE/100 g chocolate fat} = -4.25 - 0.23 \times \text{MF\%} + 1.52 \times \text{POP\%} - 1.47 \times \text{POS\%} + 1.09 \times \text{POO\%} + 1.29 \times \text{SOS\%} + 0.26 \times \text{SOO\%} \quad (10)$$

The PLS regression analysis resulted in an MAD of 0.93 g/100 g chocolate fat and a RSMEP of around 1.19 g/100 g, corresponding to 0.30 g/100 g and 0.35 g/100 g in chocolate (assuming a fat content of 30%), respectively, which is comparable to the results obtained for dark chocolate (*2*, *7*). The distribution of prediction errors is given in **Figure 5**, showing a spread ranging from −3 to 5%. For more than 95% of the samples, the prediction error was within ±3%, translating to ±0.9% in a chocolate with a fat content of 30%. The multiple R^2 coefficient of the regression model was 0.978, which corresponds to the correlation between the known CBE amount and the predicted CBE level.

For a representative set of CB-CBE-MF mixtures, split injection as well as cold OCI was applied to demonstrate the robustness of the approach. The good agreement between the actual and the experimentally determined MF and CBE contents, irrespective of the sample introduction technique applied, is shown in **Table 2**. Moreover, the experimentally obtained values were once determined using split injection and once cold OCI. For both injection techniques, the same results were obtained, allowing the final end user to choose between different operating conditions.

Application to Chocolate Samples. To prove that the established approach, which was developed on CB-CBE-MF

Table 3. Agreement between Actual and Experimentally Determined MF and CBE Contents of Various Tailor-Made Chocolate Samples

sample	sample description	% total fat chocolate	% MF known	% MF predicted	absolute error	% CBE known	detection of CBEs	% CBE predicted	absolute error
			calculation on fat basis						
chocolate 1	milk chocolate, full cream milk powder, no CBE	35.9	16.3	15.1	1.2	0.0	no		
chocolate 2	milk chocolate, full cream milk powder, CBE addition low level	35.9	16.3	15.1	1.1	1.4	yes	1.8	–0.4
chocolate 3	milk chocolate, skimmed milk powder + MF, no CBE	27.8	22.6	24.6	–2.0	0.0	no		
chocolate 4	milk chocolate, skimmed milk powder + MF, CBE low level	27.8	22.6	24.6	–2.1	7.2	yes	5.8	1.4
chocolate 5	milk chocolate, crumb + MF + full cream milk powder + skimmed milk powder + whey powder, CBE addition at statutory level	28.8	17.5	18.9	–1.4	17.4	yes	15.9	1.5
chocolate 6	white chocolate, CBE addition at statutory level	36.8	16.4	15.6	0.8	13.6	yes	13.1	0.5
			calculation on chocolate basis						
chocolate 1	milk chocolate, full cream milk powder, no CBE	35.9	5.8	5.4	0.4	0.0	no		
chocolate 2	milk chocolate, full cream milk powder, CBE addition low level	35.9	5.8	5.4	0.4	0.5	yes	0.6	–0.1
chocolate 3	milk chocolate, skimmed milk powder + MF, no CBE	27.8	6.3	6.8	–0.6	0.0	no		
chocolate 4	milk chocolate, skimmed milk powder + MF, CBE low level	27.8	6.3	6.9	–0.6	2.0	yes	1.6	0.4
chocolate 5	milk chocolate, crumb + milk fat + full cream milk powder + skimmed milk powder + whey powder, CBE addition at statutory level	28.8	5.0	5.4	–0.4	5.0	yes	4.8	0.2
chocolate 6	white chocolate, CBE addition at statutory level	36.8	6.0	5.8	0.3	5.0	yes	4.9	0.1

blends, is applicable to real chocolate samples, the fat extracted from tailor-made chocolate samples with known MF and CBE levels was separated by HR-GLC. The obtained TAG profile was used (i) to determine the MF content (*10*), (ii) to calculate and correct for the contribution of TAGs deriving from MF, (iii) to determine the presence/absence of CBEs, and, if present, (iv) to quantify the amount of the CBE admixture in the samples (**Table 3**). For all six chocolate samples, the determined MF content was close to the actual MF content. Furthermore, all samples were classified correctly, that is, no false-positive and false-negative results occurred. Finally, for samples recognized as containing CBE besides CB, the actual CBE content was determined. For all samples, the absolute prediction error was less than 0.4%.

In conclusion, the elaborated approach, which can be easily transferred to routine testing laboratories, has the advantage that by performing a single TAG analysis using HR-GLC several questions, enabling the enforcement of Directive 2000/36/EC for milk chocolates, can be answered. It is based on (i) a comprehensive standardized database covering the TAG composition of a wide range of authentic MF, CB, and CBE samples and more than 900 blends thereof; (ii) the availability of a certified CB reference material (*8*) for calibration; (iii) an evaluation algorithm, which allows a reliable quantification of the MF content in chocolate fats and the correction of TAGs deriving from MF; (iv) mathematical expressions to detect the presence of CBEs in milk chocolate; and (v) a multivariate statistical formula to quantify the accurate amount of CBEs in the final product. On the basis of the in-house validated procedure, full method validation by a collaborative trial was carried out. The results will be reported elsewhere.

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LITERATURE CITED

(1) Directive 2000/36/EC of the European Parliament and of the Council of 23 June 2000 relating to cocoa and chocolate products intended for human consumption. *Off. J. Commission Eur. Commun.* **2000**, *L197*, 19−25.

(2) Buchgraber, M.; Senaldi, Ch.; Ulberth, F.; Anklam, E. Detection and quantification of cocoa butter equivalents in cocoa butter and plain chocolate by gas liquid chromatography of triacylglycerols. *J. AOAC Int.* **2004**, *87*, 1153−1163.

(3) Buchgraber, M.; Ulberth, F.; Anklam, E. Method validation for detection and quantification of cocoa butter equivalents in cocoa butter and plain chocolate. *J. AOAC Int.* **2004**, *87*, 1164−1172.
(4) Buchgraber, M.; Anklam, E. Validated method: Method description for the detection of cocoa butter equivalents in cocoa butter and plain chocolate. EUR 20742 EN, 2003.
(5) Buchgraber, M.; Anklam, E. Validated method: Method description for the quantification of cocoa butter equivalents in cocoa butter and plain chocolate. EUR 20831 EN, 2003.
(6) ISO 23275-1. Animal and vegetable fats and oils—Cocoa butter equivalents in cocoa butter and plain chocolate—Part 1: Determination of the presence of cocoa butter equivalents. 2006.
(7) ISO 23275-2. Animal and vegetable fats and oils—Cocoa butter equivalents in cocoa butter and plain chocolate—Part 2: Quantification of cocoa butter equivalents. 2006.
(8) Koeber, R.; Buchgraber, M.; Ulberth, F.; Bacarolo, R.; Bernreuther, A.; Schimmel, H.; Anklam, E.; Pauwels, J. The certification of the content of five triglycerides in cocoa butter. EUR 20781 EN, 2003.
(9) http://www.irmm.jrc.be/html/activities/cocoa_butter_calculation_toolbox/index.htm.
(10) Buchgraber, M.; Androni, S.; Anklam, E. Quantification of milk fat in chocolate fats by triacylglycerol analysis using gas-liquid chromatography. *J. Agric. Food Chem.* **2007**, *55*, 3275−3283.
(11) *Official Methods of Analysis of AOAC International*; AOAC Official Method 963.15: Fat in Cacao Products; AOAC International: Gaithersberg, MD, 1995.
(12) Buchgraber, M.; Ulberth, F.; Anklam, E. Cluster analysis for the systematic grouping of genuine cocoa butter and cocoa butter equivalent samples based on triglyceride patterns. *J. Agric. Food Chem.* **2004**, *52*, 3855−3860.
(13) Padley, F. B.; Timms, R. E. Determination of cocoa butter equivalents in chocolate. *J. Am. Oil Chem. Soc.* **1980**, *57*, 286−293.
(14) Padley, F. B.; Timms, R. E. Analysis of confectionery fats II. Gas−liquid chromatography of triglycerides. *Lebensm.-Wiss. Technol.* **1978**, *11*, 319−322.
(15) Fincke, A. Möglichkeiten und Grenzen einfacher gaschromatographischer Triglyceridanalysen zum Nachweis fremder Fette in Kakaobutter und Schokoladenfetten. 1. Mitteilung: Verteilung der nach C-Zahlen Klassifizierten Triglyceride in Kakaobutter. *Dtsch. Lebensm. Rdsch.* **1980**, *76*, 162−167.
(16) Fincke, A. Möglichkeiten und Grenzen einfacher gaschromatographischer Triglyceridanalysen zum Nachweis fremder Fette in Kakaobutter und Schokoladenfetten. 4. Mitteilung: Auswertung gaschromatographischer Triglyceridanalysen von Milchschokoladenfetten. *Dtsch. Lebensm. Rdsch.* **1980**, *76*, 389−396.
(17) Dionisi, F.; Golay, P. A.; Hug, B.; Baumgartner, M.; Callier, P.; Destaillats, F. Triacylglycerol analysis for the quantification of cocoa butter equivalents (CBE) in chocolate: Feasibility study and validation. *J. Agric. Food Chem.* **2004**, *52*, 1835−1841.
(18) Ulberth, F. Detection of milk fat adulteration by linear discriminant analysis of fatty acid data. *J. AOAC Int.* **1994**, *77*, 1326−1334.
(19) Ulberth, F. Quantitation of foreign fat in foreign fat/milkfat mixtures by multivariate regression analysis of fatty acid data. *J. Agric. Food Chem.* **1995**, *43*, 1556−1560.
(20) Lipp, M. Comparison of PLS, PCR and MLR for the quantitative determination of foreign oils and fats in butter fats of several European countries by their triglyceride composition. *Z. Lebensm. Unters. Forsch.* **1996**, *202*, 193−198.
(21) Lipp, M. Determination of the adulteration of butter fat by its triglyceride composition obtained by GC. A comparison of the suitability of PLS and neural networks. *Food Chem.* **1996**, *55*, 389−395.

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