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Triacylglycerol Analysis for the Quantification of Cocoa Butter Equivalents (CBE) in Chocolate: Feasibility Study and Validation

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A new European legislation (2000/36/CE) has allowed the use of vegetable fats other than cocoa butter (CB) in chocolate up to a maximum value of 5% in the product. The vegetable fats used in chocolate are designated as cocoa butter replacements and are called cocoa butter equivalents (CBE). The feasibility of CBE quantification in chocolate using triacylglycerol (TAG) profiles was conducted by analyzing 55 samples of CBs and 31 samples of CBEs using a liquid chromatograph equipped with an evaporative light scattering detector (HPLC-ELSD). Statistical evaluation of the data obtained has been performed, and a simulation study has been carried out to assess the viability to use this method for quantifying the amount of CBE in real mixtures and in chocolates. The TAGs POP, POS, PLS, and the ratios POP/PLS, POS/PLP (P, palmityl; O, oleyl; S, stearyl; L, linoleyl) are particularly significant to discriminate between CB and CBE. Analysis of 50 mixtures between 5 different CBEs and 10 different CBs at 2 different concentration levels is presented. The data are visualized and interpreted. A mathematical model has been developed to assess the amount of CBE in real mixtures. This predictive model has been successfully applied and validated on dark chocolates including authorized CBE. The results are affected by ±2.1% absolute average error. In particular, estimations between 10 and 20% of CBE show a very good match. On the other hand, values equal to or smaller than 5% show a larger prediction error (detection limit of the method). For the main purpose of this method (i.e., quantification of CBE at 5% max in chocolate, which represents about 15% of the total fat) this model shows very good results. For milk chocolate, the mathematical model can also be used if TAG are integrated from partition number (PN) 46 to 54. Consequently, the model proposed provides sufficient information to verify the real application of the European legislation.

KEYWORDS: Adulteration; cocoa butter; cocoa butter equivalent; chocolate; triacylglycerol; data visualization; high-performance liquid chromatography; vegetable fat

INTRODUCTION

A recent European legislation (2000/36/CE) has admitted the use of vegetable fats at a maximum level of 5% of the product for chocolate manufacturing (*1*). The vegetable fats used in chocolate are designated as cocoa butter replacements and are called cocoa butter equivalents (CBE). These are tropical fats or mixtures of tropical fats (*1*) such as palm (*Elaeis guineensis and Elaeis olifera*), illipe (*Shorea spp.*), shea (*Butyrospermum parkii*), kokum gurgi (*Garcinia indica*), mango kernel (*Mangifera indica*) and sal (*Shorea robusta*). They are fully compatible with cocoa butter (CB) and may be used in chocolate both for technological and economical reasons (*2*−*3*). CBE quantification in chocolate is interesting both to guarantee fair economic competition and to assess authenticity of chocolate products. However, at this moment, no reliable method is available for CBE quantification in chocolate (*4*).

The quantification of vegetable fat in chocolate was extensively reviewed by Lipp and Anklam (*3*−*4*). The authors came to the conclusion that there is no unique approach for the identification and quantification of foreign fats in chocolate. The most promising results could be obtained by using several markers and applying statistical analysis to the results. In a recent study, Lipp et al. (*9*) demonstrated that minor components, such as tocopherols, tocotrienols, and sterenes, are not useful for the quantification of CBE. Macarthur et al. (*8*) suggested a pre-identification of the type of added fat using steradiene analysis, to improve the precision of the TAG method. Nevertheless, the analysis of triacylglycerols (TAGs) is considered as giving the most valuable information. One of the first quantitative methods based on TAG analysis was proposed in 1980 by Padley et al. (*5*). CBE quantification was carried out by gas−liquid chromatography (GLC) of TAGs making use

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Table 1. Samples, Names, and Suppliers of the CBs Analyzed in the Present Study

samples	supplier	origin	samples	supplier	origin
CB-01	Nestlé	Africa/Asia	CB-29	Gerkens	Nigeria
CB-02	Aarhus Olie	African origin	CB-30	Nord Cacao	100% West Africa
CB-03	Gerkens	African origin	CB-31	Nord Cacao	70% West Africa/30% Far East
CB-04	Gerkens	African origin	CB-32	Nord Cacao	50% West Africa/50% Far East
CB-05	Gerkens	Africa/Asia	CB-33	Nord Cacao	Peru
CB-06	ADM Hull	Africa/Asia	CB-34	Nord Cacao	Nigeria
CB-07	Bensdorp	Africa/Asia	CB-35	Nord Cacao	Indonesia
CB-08	Nestlé	Ivory Coast	CB-36	Société Africaine de Cacao (SACO)	Ivory Coast
CB-09	Nestlé	Ivory Coast	CB-37	Dutch Cocoa BV	West African
CB-10	Nestlé	Malyasia	CB-38	Dutch Cocoa BV	West African
CB-11	Ispra European Community	Mixture of cocoa beans from different origins	CB-39	Dutch Cocoa BV	West African
CB-12	Nestlé	Brazil	CB-40	Dutch Cocoa BV	West African
CB-13	Nestlé	Ghana/Ivory Coast	CB-41	Dutch Cocoa BV	West African
CB-14	Nestlé	Malaysia	CB-42	Dutch Cocoa BV	West African
CB-15	Nestlé	Ghana/Ivory Coast	CB-43	Dutch Cocoa BV	West African
CB-16	ADM Hull	Africa/Asia	CB-44	Dutch Cocoa BV	West African
CB-17	ADM Hull	Africa/Asia	CB-45	Dutch Cocoa BV	West African
CB-18	ADM Hull	Africa/Asia	CB-46	Dutch Cocoa BV	West African
CB-19	Barry Callebaut	Africa/Asia	CB-47	Dutch Cocoa BV	West African
CB-20	Barry Callebaut	Africa/Asia	CB-48	Dutch Cocoa BV	West African
CB-21	Barry Callebaut	Africa/Asia	CB-49	Dutch Cocoa BV	Dominican Republic
CB-22	Barry Callebaut	Africa/Asia	CB-50	Dutch Cocoa BV	Dominican Republic
CB-23	Barry Callebaut	Africa/Asia	CB-51	Dutch Cocoa BV	Dominican Republic
CB-24	Barry Callebaut	Africa/Asia	CB-52	Dutch Cocoa BV	Dominican Republic
CB-25	Barry Callebaut	Africa/Asia	CB-53	Dutch Cocoa BV	Dominican Republic
CB-26	Barry Callebaut	Africa/Asia	CB-54	Dutch Cocoa BV	Dominican Republic
CB-27	Gerkens	Ghana	CB-55	Dutch Cocoa BV	Dominican Republic
CB-28	Gerkens	Malaysia			

of the linear relationship existing between TAGs containing 50 carbons and TAGs containing 54 carbons.

Buchgraber et al. (*7*) compared the results obtained by GLC and by HPLC coming to the conclusion that the two methods provide equivalent results. In a recent study, Simoneau et al. (*6*) proposed a quantitative method based on the TAG analysis and subsequent application of a mathematical model. In the cited article, the description of the statistical methods used is accurate and clear.

Our present study takes into account a larger number of different cocoa butter origins and aims to use not only TAG contents but also TAG ratios. TAG profiles of 55 samples of cocoa butters and 31 samples of commercially available CBE were statistically analyzed to predict the feasibility of quantification of the amount of CBE in real mixtures and chocolate. In a second time, 50 mixtures of CBE in CB were made, and the TAG profiles were analyzed using HPLC-ELSD. Combining the data, a mathematical model was established to quantify CBE in chocolate samples. This mathematical model was then validated using chocolate samples.

MATERIALS AND METHODS

Sampling. Analyzed CB and CBE from different suppliers and geographical origins are listed in **Tables 1** and **2**, respectively. Mixtures of CBs and CBEs used for mathematical modelization are given in **Table 3**. Chocolate without added CBE, and having a fat content of 30.75 g/100 g was manufactured. This chocolate was used as base to produce nine chocolate samples, with final total fat of 36.5%, containing different level (5, 10, and 20%) of three different CBE (CBE-11, CBE-8, and CBE-26). The 0% CBE chocolate was obtained by addition of cocoa butter to reach the fat content of 36.5%. Two milk chocolate samples, which do not contain CBE, were also analyzed.

Fat Extraction. Fat extraction from chocolate was carried out according to Mojonnier method (*10*). Briefly, the chocolate sample was homogenized and dissolved in water at 60 °C before adding an ammonia-ethyl alcohol solution. The fat was extracted with diethyl ether and petroleum ether. Because of the high fat content of the samples (up to 30%), the extraction was repeated three times. The solvents were removed and the extracted fat was determined by gravimetry. All the solvents were obtained from Merck (Darmstadt, Germany).

Table 2. Samples, Suppliers, and Origins of the CBEs Analyzed in the Present Study

sample	name	supplier
CBE-1	Choclin batch 135557 (13.3.99)	Loders Croklaan
CBE-2	Choclin	Loders Croklaan
CBE-3	Coberine 154655 (16.9.99)	Loders Croklaan
CBE-4	Illexao 30-71	Aarhus Olie
CBE-5	Illexao 30-96	Aarhus Olie
CBE-6	Illexao 30-69	Anglia Oils
CBE-7	Illexao 30-61(October 99)	Aarhus Olie
CBE-8	Illexao 30-61 (Batch 36550)	Aarhus Olie
CBE-9	Shokao 95	Aarhus Olie
CBE-10	Palm Mid Fraction (Charge 561)	Nutriswiss
CBE-11	Akomax R (1999-10-18)	Karlshamns
CBE-12	Akomax R (1999-03-08)	Karlshamns
CBE-13	Akomax R (Prod 990615)	Karlshamns
CBE-14	Akomax R (Prod 990824)	Karlshamns
CBE-15	Akomax R (Prod 990910)	Karlshamns
CBE-16	Akomax R (Prod 990601)	Karlshamns
CBE-17	Akomax R (Prod 990826)	Karlshamns
CBE-18	Akomax R (Prod 990915)	Karlshamns
CBE-19	Akomax R (Prod 990917)	Karlshamns
CBE-20	Akomax E (1999-03-08)	Karlshamns
CBE-21	Akonord XS	Karlshamns
CBE-22	Akonord XT (18.10.99)	Karlshamns
CBE-23	Akonord E (1999-03-08)	Karlshamns
CBE-24	Ertina 20 NUK	Vamo Fuji
CBE-25	Ertina NUK1	Vamo Fuji
CBE-26	Ertina 20	Vamo Fuji
CBE-27	Ertina E3R	Vamo Fuji
CBE-28	Ertina 20	Vamo Fuji
CBE-29	CBE-B (October 99)	Loders Croklaan
CBE-30	Couva 700	Loders Croklaan
CBE-31	Chocosine Illipe based	Walter Rau
CBE-32	Chocosine Shea based	Walter Rau

Table 3. Matrix Used for the Mathematical Modelization

	CB-12	CB-33	CB-36	CB-34	CB-27	CB-51	CB-9	CB-28	CB-10	CB-35
CBE-10	10[a]	20	10	20	10	20	10	20	10	20
CBE-29	20	10	20	10	20	10	20	10	20	10
CBE-11	10	20	10	20	10	20	10	20	10	20
CBE-26	20	10	20	10	20	10	20	10	20	10
CBE-8	10	20	10	20	10	20	10	20	10	20

[a] Relative % of CBE added in CB.

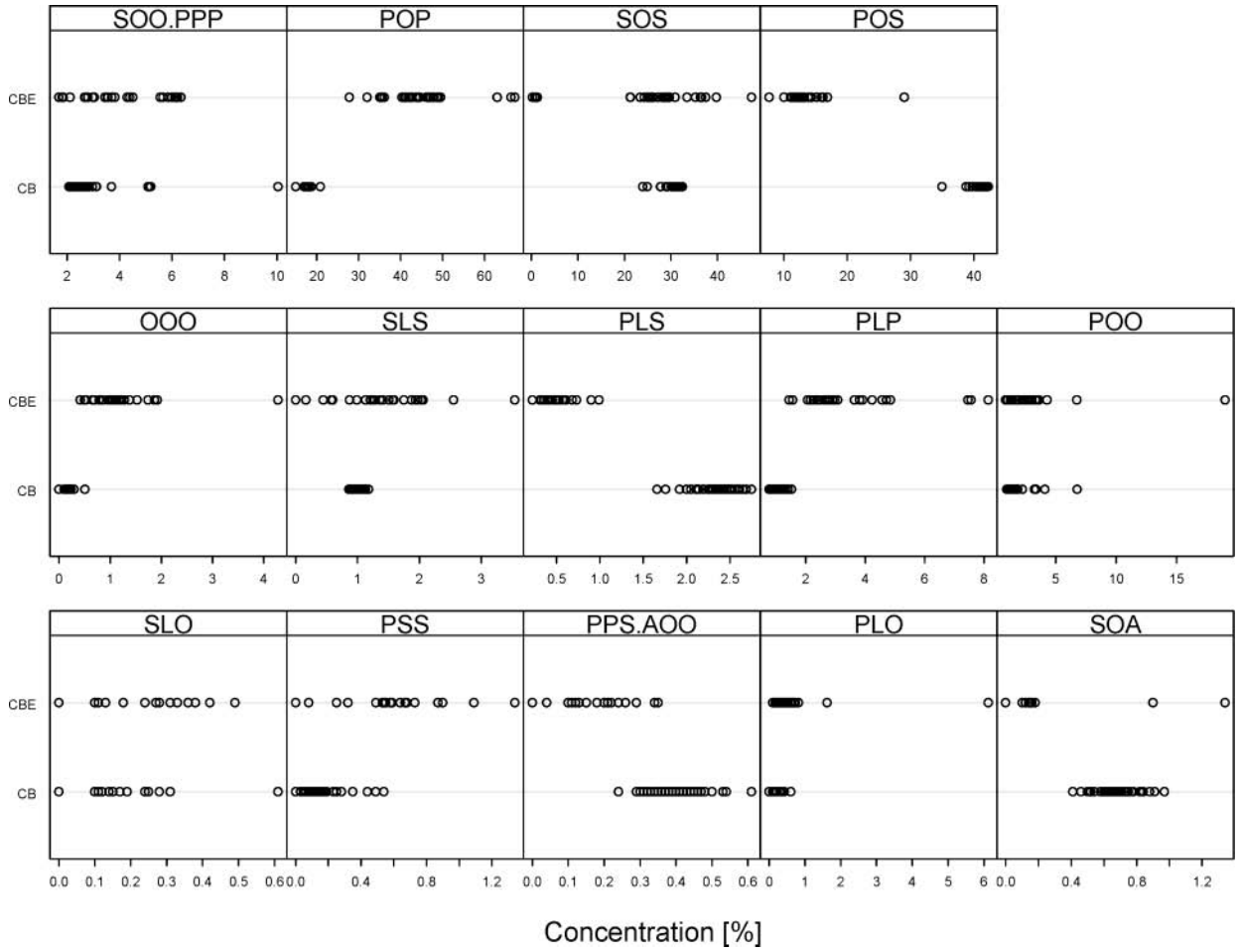

Figure 1. TAG content in 55 CBs and 31 CBEs.

TAG Profiles Analysis. TAGs were analyzed by reversed-phase high performance liquid-chromatography (HPLC) in isocratic mode, with a Lichrospher 100-5-RP18 ce, (500-mm length × 4-mm ID, 5-μm particle size, Macherey−Nagel, Oensingen, Switzerland). The HPLC system consisted to a Liquid chromatograph LC 625 Waters (Montreux-Chailly, Switzerland). The mobile phase was acetonitrile/chloroform (40:60 v/v). All solvents used were HPLC grade and were obtained from Merck (Darmstadt, Germany). TAGs were detected with an evaporative light scattering detector (ELSD), Ercatech, Bern, Switzerland). The gas used in ELSD was nitrogen at 1.4 bar, and the neubulization was conducted at 50 °C.

Statistics. The following softwares: S-Plus 6.1 (Insightful Corp., Seattle, WA), NCSS (NCSS, Kaysville, UT), SAS (SAS Institute Inc., Cary, NC), and Excel (Microsoft Corp) were used for statistical analyses.

RESULTS AND DISCUSSION

Feasibility Study. The TAG compositions of 55 CBs and 31 CBEs samples (**Tables 1** and **2**) were performed by HPLC. The following TAGs have been measured: PLO, PLP, OOO, SLO, POO, PLS, POP, SOO + PPP, SLS, POS, PPS + AOO, SOS, PSS, and SOA. The peaks were identified in comparison with a CB reference sample (CB-11) from the European Commission, (DG JRC, Ispra, Italy). Percentages of the TAG measured for CBs and CBEs are presented in **Figure 1**. Note that the scales on the horizontal axis differ from one TAG to another. The molecules are ordered from bottom to top and left to right by increasing average concentration. We see that PLS, POP, and POS perfectly discriminate CBs and CBEs. Without the two visible outliers, this would also be the case for SOA. Some ratios of TAG are also interesting (**Figure 2**). Indeed, the ratios POS/PLP and POP/PLS perfectly discriminate CBs and CBEs. The goal of this feasibility study is to determine whether such data might be used to quantify the amount of CBE in real mixtures.

We simulate the mixtures on the computer, assuming that the concentrations C of the molecules simply add up, without any interaction ($c_{\text{molecule in mix}} = (p_{\text{CBE}} \times c_{\text{molecule in pure CBE}}) + (p_{\text{CB}} \times c_{\text{molecule in pure CB}})$, where $p_{\text{CBE}} + p_{\text{CB}} = 1$). For this instance, we assume that p_{CBE} varies between 0 (pure CB) and 0.2 (CBE = 20%), and we simulate mixes for $p_{\text{CBE}} = 0.01$,

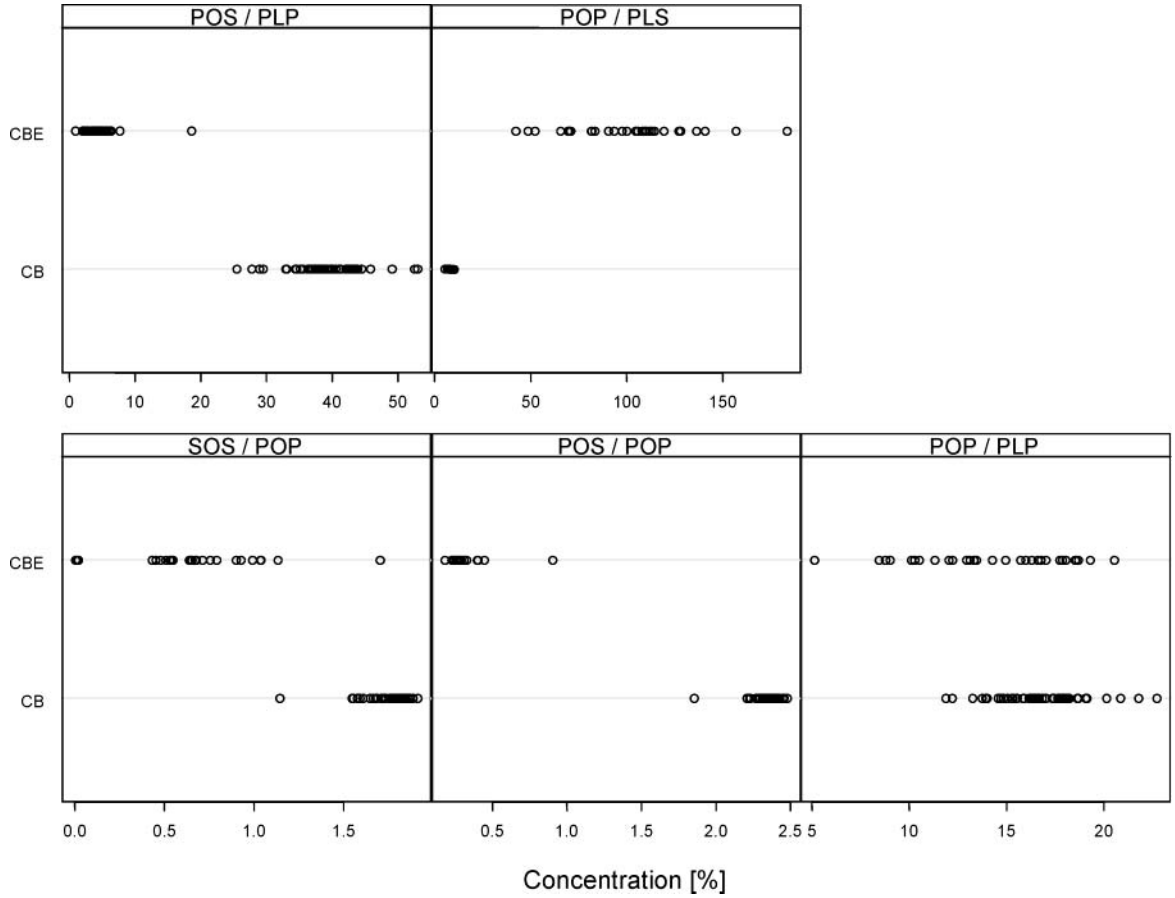

Figure 2. Selected TAG ratios values in 55 CBs and 31 CBEs.

0.02, ... up to 0.2. We proceed as follows for each value of p_{CBE} and for a specific molecule or a ratio of two molecules: choose 1000 values randomly among the CB samples, with replacement; choose 1000 values randomly among the CBE samples, with replacement; compute the concentration of these 1000 pairs of values, using the given value for p_{CBE}. A total of 1000 possible mixes were generated, and both mean concentration and standard deviation of these 1000 virtual mixtures were evaluated.

These values are illustrated for POP in **Figure 3**. We could see how the concentration of POP raises increasing quantity of CBE. The values at 0 are not simulated; these are the actual measured concentrations of POP of the 55 pure CB samples. To determine at which proportion of CBE we might be significantly different from the average concentration of pure CB samples, we summarize the above data in the following way: for CB, we compute a classic 95% confidence interval (CI): $Mean_{CB} \pm t_{0.975;n-1} \cdot (SD_{CB}/\sqrt{n})$, where $t_{0.975;n-1}$ is the 0.975 quantile of a *t*-distribution with $n-1$ degrees of freedom, and n is the sample size (here $n = 55$); for the simulated mixes, we compute also a 95% CI, but instead of using $n = 1000$, we use $n = 55$, or else the CIs would not be comparable.

For POP (**Figure 3**), a proportion of roughly 2% or more could be potentially detected. After this level, the CIs do not overlap anymore compared with the one at 0% CBE. In addition, CIs become wider when the proportion of CBE increases due to the higher variability in the pure CBE samples. We repeated these computations for several other molecules and ratios. Results indicate that 6, 3, 6, 3, and 8% of CBE might be potentially detected by the relative content of PLS, POS, SOS/POP, POS/POP, and POS/PLP, respectively. Furthermore, the ratio POP/PLS (**Figure 4**) might be useful to detect less than 1% of CBE.

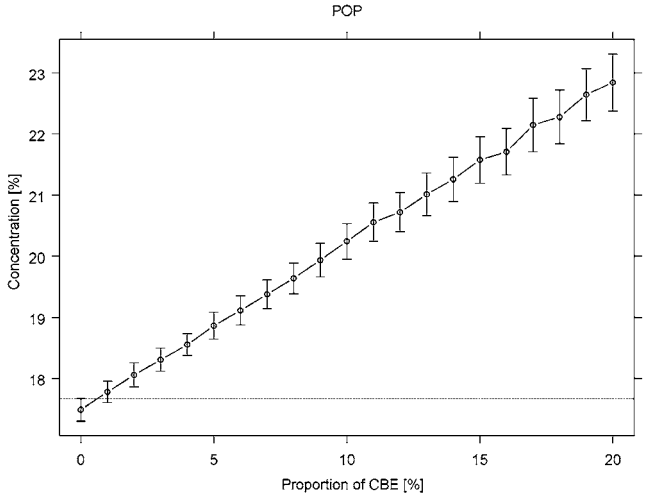

Figure 3. Concentration of POP in 1000 CB/CBE mixtures obtained by computational simulation. The line joins the mean values for each proportion. The values at 0 are not simulated: These are the actual measured concentrations of POP of the 55 pure CB samples.

The performed feasibility study has shown very promising results. If the strong hypothesis about simple addition of CB and CBE concentrations in real mixtures is valid, the simulation study shows that it might be feasible to establish a statistical model for predicting the amount of CBE in real mixtures. Three TAGs (POP, PLS, and POS) were found particularly significant to discriminate between CB and CBE. In particular, because

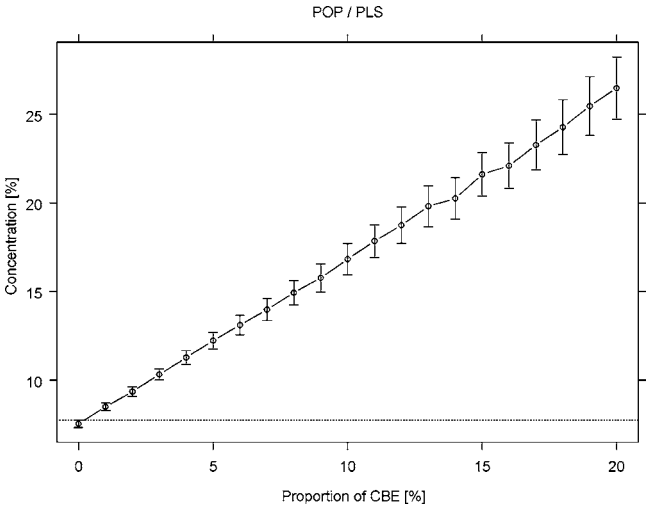

Figure 4. Evolution of the POP/PLS ratio in 1000 CB/CBE mixtures obtained by computational simulation; less than 1% of CBE might be detected by using this ratio.

we try to quantify CBE in CB, the TAG POP would be the most appropriate for this purpose. In addition, two ratios between TAGs (POS/PLP and POP/PLS) are able to distinguish between CB and CBE. According to our simulation, the ratio would allow an estimation of a very low quantity of CBE in CB (>1%). Nevertheless, the above findings must be interpreted very carefully. They rely on the strong assumptions that mixtures of pure CBs and CBEs can be simulated by just adding the concentrations, multiplied by the proportion of each type of cocoa butters. If this assumption is valid, it is not surprising that the above simulation is rather promising; this is due to the fact that several molecules and ratios of molecules discriminate CB and CBE samples perfectly, at least for the 86 samples available. An approach similar to the one used by Simoneau et al. (*5*) would be needed to validate these results. We would need the concentrations of these triglycerides for a wide range of mixtures. An experimental design might be used to generate a representative sample of mixtures based on the available samples.

Mathematical Modelization. The modelization procedure implied the analysis of TAG composition of 50 mixtures between 5 different CBE and 10 different CB, at addition levels of 10 and 20% of CBE in CB. To analyze a minimum of mixtures while having sufficient relevant information for further statistical analyses, we have selected 10 relevant CBs of different geographical origins and showing different ratios POS/PLP (three with low ratios (CB1-12, CB-33, CB-36), four with intermediate ratios (CB-9, CB-34), and three with high ratios (CB-10, CB-27, CB-51)) and 5 relevant CBEs showing different ratios POP/PLS and POS/PLP (one with low POP/PLS ratio (CBE-10), one with intermediate POP/PLS ratio (CBE-29), one with high POP/PLS ratio (CBE-26), two with intermediate POS/PLP ratio, one with high POP/PLS ratio (CBE-11), and one with low POP/PLS ratio (CBE-8)).

In the matrix given in **Table 3**, we ranked CBs and CBEs according to their POS contents (from left to right and top to bottom). There are 50 possible combinations, and we assigned to each combination a level of concentration: 10 or 20%. In fact, the European legislation requires that vegetable fats other than CB may not exceed 5% of the finished product. This means that fat extracted from chocolates could contain about 15% CBE. Therefore, the proposed mixtures with 10 and 20% include this critical value. Finally, nine samples of chocolate containing

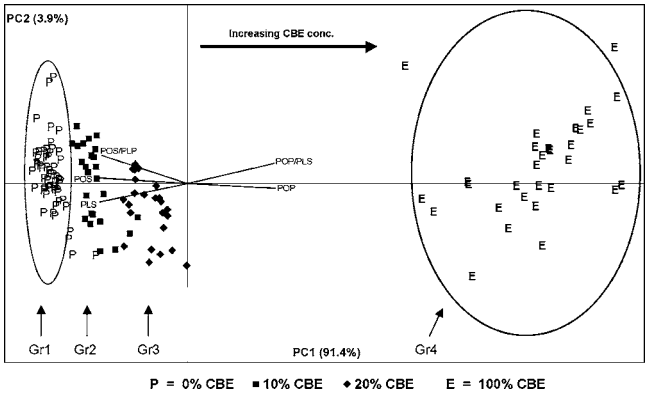

Figure 5. Graphical representation of the 136 samples using principal component analysis (PCA).

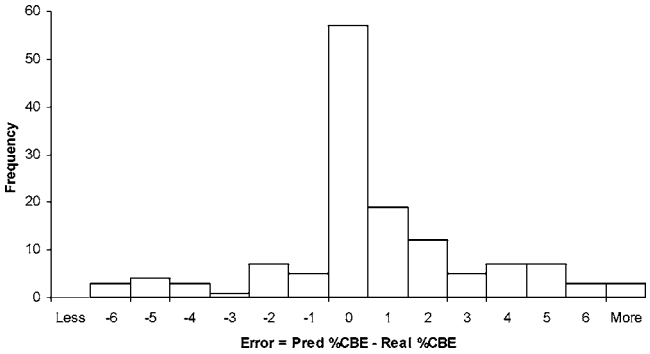

Figure 6. Verification of the model: distribution of model errors %.

different known percentages (approximately 5, 10, and 20%) of three different CBEs allowed by the EU (CBE-7, CBE-11 and CBE-26, respectively) were produced. The fat was extracted and TAG compositions were analyzed to further validate the proposed model for CBE quantification in chocolate.

The statistical analysis of the TAG contents of the 86 already available pure samples (55 pure CB and 31 pure CBE) and of the 50 mixtures allowed us to establish a mathematical model for predicting the amount of CBE in CB. In total, 136 samples were available and were described by means of 20 initial variables (i.e., TAGs). Some combinations of these 20 variables were computed (such as ratios or sums), leading to more than 30 variables of characterization. All of these variables were included in models (linear and non linear ones, mainly involving quadratic terms = variable^2, or cross product terms = $var_i \times var_j$), which have been compared in terms of predictive capabilities and R^2-adjusted. We finally kept the three best potential models that came out of this model comparison analysis based on our two quality criteria quoted above. After evaluation of both analytical and mathematical considerations, one unique final model was selected.

This final model involves three initial variables (PLS, POP, and POS) and two ratios of these variables (POP/PLS and POS/PLP). These 5 variables appeared to be very promising to discriminate the 136 samples, which is confirmed by a 2-dimensional graphical representation using principal component analysis (PCA, **Figure 5**). On this map, four groups are already clearly distinguished: from left to right, Group 1 = 0% of CBE, Group 2 = 10% of CBE, Group 3 = 20% of CBE and Group 4 = 100% of CBE samples. These 5 variables were also selected to predict the percentage of CBE giving the following mathematical equation: predicted % CBE= 118.8 − (20.4 × PLS) + (0.3 x POP) − (1.50 × POS) − [0.02 × (POP/PLS)] − [0.40 × (POS/PLP)].

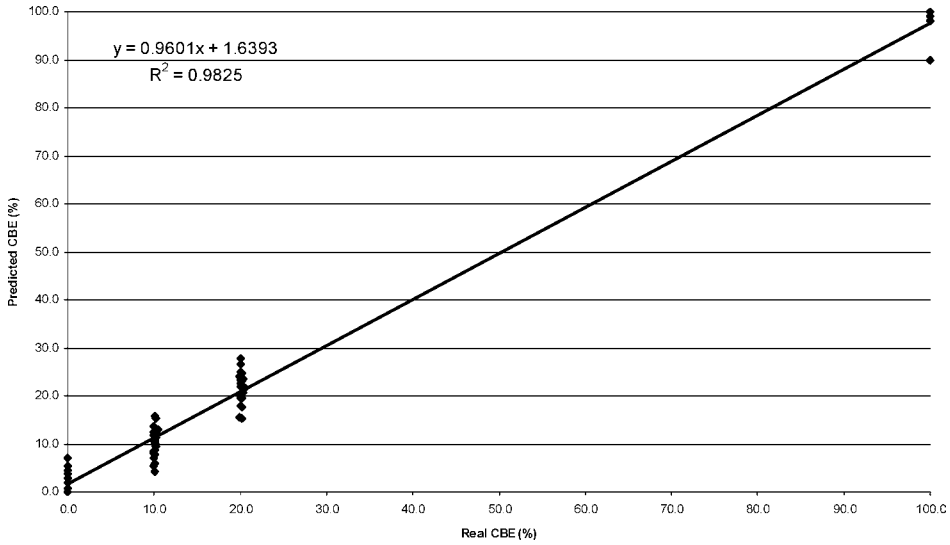

Figure 7. Verification of the model: real CBE% vs predicted CBE%.

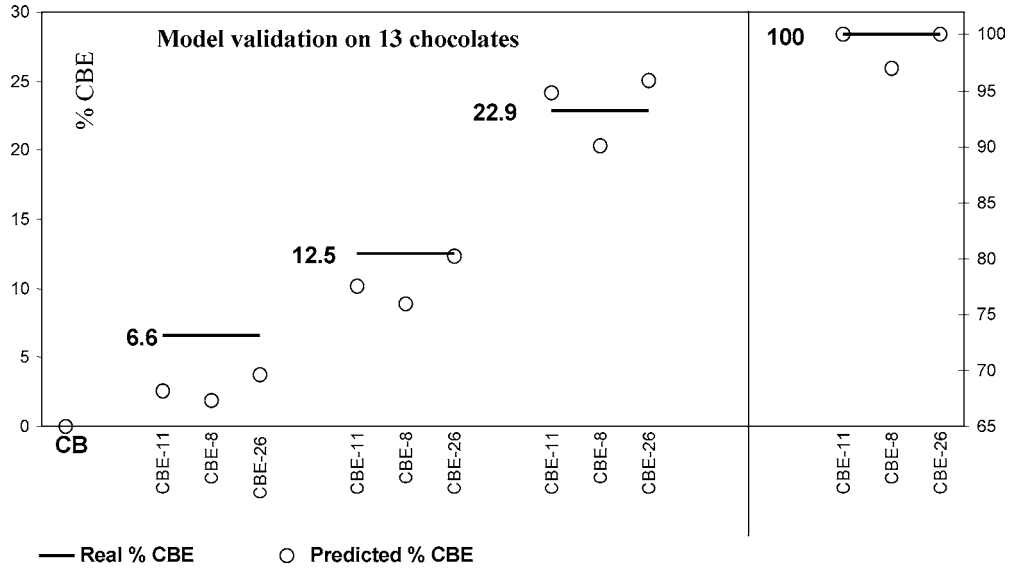

Figure 8. Validation of the model on chocolate samples: real CBE% vs predicted CBE% (13 points).

Model Quality and Validation. *Quality Estimation.* Prediction quality on the experimental samples was calculated (136 points used). On the 136 available samples, the proposed model predicted the percentage of CBE with an average absolute error of ±1.85%, which represents a very good result (see **Figure 6** for the distribution of the errors).The model has shown a very good multiple R^2 coefficient (R^2= 0.990), which corresponds to the correlation between the real CBE percentage and the predicted CBE percentage (see **Figure 7**). Moreover, the linear regression of these two variables (real vs predicted) is very close to the diagonal line $Y = X$, which is another very good indication. In conclusion, this model presented the "best adjusted R^2" (Adj $R^2 = 0.989$), which made it the most appropriate one, against all the other potential predictive models.

Validation Step. The fat was extracted from chocolates, and TAG profiles were analyzed. The mathematical model developed was tried on these chocolate profiles (**Figure 8**). The average absolute error (AAError) in the prediction of CBE content in the 13 samples used for the validation was ±2.1%. In particular, estimations between 10 and 20% of CBE show a very good match. Indeed, AAError for the three samples around 10% (12.5% in fact), for the three samples around 20% (22.9% in fact), and for the three samples around 5% (6.6% in fact) are ±2.0, ±2.0, and ±3.9%, respectively. However, values around 5% show a larger prediction error. It can be considered that 5% and total fat corresponds to the detection limit of the method. Therefore, the developed method provides good results for the quantification of CBE at a maximum level of 5% in chocolate that corresponds to about 15% of total fat.

To complete the validation, two chocolates containing milk with no CBE addition were also manufactured and analyzed. Many minor peaks were present in the chromatograms due to the milk fat presence (**Figure 9**). The application of the prediction model brings to CBE estimation of 0.5 and 4.5%. Both these values are below the detection limit. The presence of milk triglycerides may interfere and lead to a wrong estimation of the presence of CBE. However, if only TAG ranging from PN 46 to 54 are considered for the normalization of the profile, the application of the prediction model brings to CBE an estimation of 0%. Therefore, in theses conditions, the contribution of TAG originated from milk fat seems to be negligible.

Three TAGs (POP, PLS, and POS) and two TAG ratios (POS/PLP and POP/PLS) were found particularly significant to discriminate between CB and CBE. These observations were validated using mixtures between CBs and CBEs and real

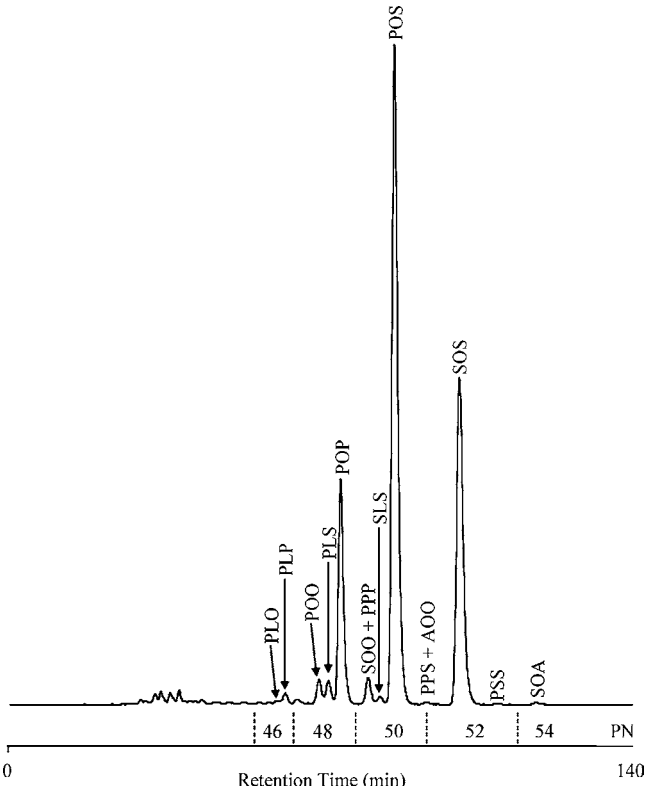

Figure 9. TAG distribution of milk chocolate lipid extract by reversed-phase high-performance liquid chromatography with an evaporative light scattering detector.

chocolate samples. A mathematical model was determined and applied to quantify CBE content in chocolates. The main purpose of this method is the quantification of CBE at 5% max in chocolate, which correspond to about 15% of the total fat. Moreover, estimations between 10 and 20% of CBE show a very good match, and results obtained are affected by ±2.1% AAError. Therefore, the model provides sufficient information to verify the real application of the European legislation. However, values equal to or smaller than 5% show a larger prediction error, which corresponds to the detection limit of the method (i.e., less than 2% of CBE in chocolate). For milk chocolate, the mathematical model could also give evaluation on the presence of CBE in chocolate, but only TAG ranging from PN 46 and 54 should be considered.

In conclusion, the determination of the TAG profile is a key parameter both for authenticity assessment of CB and for the estimation of the CBE content in chocolate. The proposed model could probably be used with TAG data obtained using other analytical techniques such as capillary GLC or reverse-phase HPLC with refractive index detector. However, the presented analytical platform is not dedicated to the identification of CBE source. For this instance, minor lipid components such as sterol or sterol esters should be considered in further studies.

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