JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Alternative Method for the Quantification by Gas Chromatography Triacylglycerol Class Analysis of Cocoa Butter Equivalent Added to Chocolate Bars

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Directive 2000/36/EC allows chocolate makers to add up to 5% of only six specific cocoa butter equivalents (CBEs) to cocoa butter (CB). A quantification method based on triacylglycerol (TAG) class analysis by gas chromatography with an unpolar column was set up for routine control purposes of chocolate bars. Mixtures of CBEs/CB were elaborated according to a Placket−Burman experiment design and analyzed by gas chromatography. A matrix was built with the normalized values of TAG classes (C50, C52, C54, and C56) of pure CBs of various origins, homemade CB/CBE mixtures (1 CB type), and mixtures containing CBE with CBs of various origins. A multivariate calibration equation was computed from this matrix using a partial least-squares regression technique. CBE addition can be detected at a minimum level of 2%, and the mathematical model allows its quantification with an uncertainty of 2% with respect to the cocoa butter fats. The model has also been applied for deconvolution and quantification of each CBE of a CBE mixture in chocolate bars.

KEYWORDS: Cocoa butter equivalent; cocoa butter; TAG; gas chromatography; chemometric

INTRODUCTION

Three years ago, all of the European Union member states adopted a new directive relative to chocolate elaboration (*1*). Since August 3, 2003, directive 2000/36/EC has allowed chocolate makers to add six cocoa butter equivalents (CBEs) to chocolate, requiring the addition be specified on the packaging. These six CBEs (illipé, karite or shea, sal, mango, palm, and kokum) can be added up to 5% of total product weight (after deduction of edible garniture). Thus, for a chocolate that contains 30% cocoa butter (CB), the added CBE amount can be as high as 15% with respect to CB fats. *In the following text, the percentage values will be expressed with respect to cocoa butter fats*. For routine control purposes, an easy, rapid, and robust method is needed as no quantitative method has been available until now. The method should allow two points of control. The first is whether added CBEs can be detected or not, that is, defining a detection limit. The second objective is to evaluate the amount of CBE added, that is, building a quantification equation to control whether the admixture goes over the authorized limit. The difficulty of quantifying added CBE comes from the small amounts to be added and the relative similarities of CBE (*2*, *3*) and cocoa butter fat compositions. With the six fats defined, many research groups are now working on this problem. Several means of investigation have been envisaged: sterol and triterpene alcohol degradation, fatty acids, and triacylglycerols (TAGs).

Analyzing sterol and triterpene alcohol degradation products provides useful information for detecting added CBEs. The main drawback of this method is that it is not quantitative by itself (*4*, *5*). Carbon isotope composition in fatty acids was determined using gas chromatography−isotopic ratio mass spectrometry, detecting a minimum of 15% of added CBEs. This technique is therefore not suitable for low-level CBE addition detection (*6*). A third means of investigation is based on gas chromatographic determination of TAGs, the main components of cocoa butter. A TAG is based on a glycerol backbone reacted with three fatty acids. Oleic (O), palmitic (P), and stearic (S) acids are the three main fatty acids found in cocoa butter. An unpolar gas chromatography (GC) column detects triacylglycerol classes (C*n*, where *n* indicates the number of carbons) as all of the individual TAGs with the same carbon skeleton are eluted simultaneously. Padley et al. plotted %C50 versus %C54 after normalizing C50, C52, and C54 peak areas: all of the cocoa butter points are plotted along a straight line, and the given equation provides a limit. Any points over the limit imply CBE addition to cocoa butter (*7*). Young envisaged a similar approach with a slightly modified data treatment (*8*), but if their method allows CBE addition detection, quantifying the added amount relies upon knowing the added CBE TAG profile. Moreover, the quantification was difficult when a CBE mixture was used. After tentatively exploring the TAG class pathway (*9*), Anklam et al. very recently developed a model based on each TAG response (*10*−*12*), achieving such resolution by using high-resolution gas chromatography (HR-GC) (*13*). Their method has been validated for detecting CBE admixture at a level of 2%.

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10.1021/jf0351523 CCC: $27.50
Published on Web 04/20/2004

In addition, they established an equation that is a function of the percentage of five TAGs, allowing added CBE quantification (*14*).

During this period, we developed a method based on TAG class response by gas chromatography for control purposes as from our point of view the signal treatment is easier for routine control application and as the study of TAG classes is less sensitive to experimental conditions. Thus, in this study, TAG class data were used to determine, first, the minimum level of CBE admixture by using the Padley model with slight modifications. Second, a partial least-squares (PLS) regression was applied to the matrix elaborated with these TAG class data. A mathematical equation was built, containing the four %C*n* TAG class values, allowing quantification of the CBE admixture. The limits of this model were tested by tentatively deconvoluting a CBE complex mixture in each of its CBE components.

MATERIALS AND METHODS

Reagents. Bulk cacao butter fat (CB_M) used for CBE/CB_M mixture came from Barry Cacao, France, without specified geographic origin. Different manufacturers provided various origin chocolate bars that were analyzed. The six pure cocoa butter equivalents were provided by the Institute for Health and Consumer Protection (European Commission, Ispra, Italy). Extraction and GC solvents were isohexane (Suprasolv quality, VWR) and *n*-heptane (HPLC grade, VWR), respectively.

TAG Extraction. TAG extraction from chocolate was carried out with a Soxhlet apparatus according to the French official method (*15*). A Soxhlet cartridge is filled with ∼5 g of grated chocolate to extract a sufficient amount of fat and left under isohexane reflux for 4 h. After solvent evaporation under vacuum, the fat is dried in an oven (60 °C, under air) during 3 h. The TAG amount recovered depends on the initial cocoa butter percentage in the chocolate, that is, for a 30% CB chocolate, 1.5 g of fat is recovered; the yield is close to 100% with respect to the expected fat amount contained in the chocolate sample. All of the fats were stored at 4 °C.

CBE/CB Mixtures. Eight mixtures containing six CBEs were elaborated. After the CBE had been melted, 80 or 40 mg of each CBE was added using a micropipet (∼10 μL) in a vial. A micropipet was used for commodity as the fats are in their liquid state and it adds an amount close to the one expected. Anyway, the exact amount of added fat is controlled after each addition by weight. This weight is used to compute the exact percentage of CBE added to the cocoa butter. These eight mixtures were stored at 4 °C before use. Then, these CBE mixtures were incorporated to cocoa butter fat at three percentage levels, that is, 5, 15, and 20%: CBE mixtures and CB_M were melted separately; then, in different vials, they were added to get close to the desired percentage. To compute the real percentage CBE/CB_M, the amounts of CB_M and CBE were controlled by weight after the addition of each component. These final 24 mixtures were stored at 4 °C before GC analysis.

GC Analysis. TAG analysis was performed using a Carlo Erba (HRGC 5160 Mega series), equipped with an "on-column" injector and a DB1 model column (10 m × 0.32 mm × 0.1 μm, J&W Scientific). Nitrogen (c grade), first deoxygenated, was used as carrier gas at a flow rate of 3.2 mL/min. The fats were first melted and vortexed to ensure homogeneity. Then ∼10 μL of fats was dissolved in 5 mL of *n*-heptane, and 0.5 μL of the solution was injected "on-column" at 80 °C. The oven temperature was increased from 80 to 320 °C at a rate of 30 °C/min, then to 380 °C at 10 °C/min, and held at this temperature for 10 min. Data treatment was performed using Star Varian software.

Data Processing. PLS regression was performed using the software Pirouette (Infometrix, version 3.02). The matrix was elaborated with the GC values of various origin pure cocoa butters, homemade mixtures, and mixtures prepared with authorized CBEs and various origin CBs. The data matrix was made of rows, each of them corresponding to one sample analyzed and five columns. The first four columns were fed with the TAG class value, that is, %C50, %C52, %C54, and %C56, the fifth one containing the real percentage of the CBE admixture to cocoa butter. A "mean-center" treatment was applied to the data before statistical computation that used a maximum factor of 3 and a cross-validation method. To test the model and to determine the prediction uncertainty, many intermediate models were elaborated after the exclusion of three to four different samples each time. Then these excluded samples were tested with the intermediate model, and an added CBE predicted value was obtained so as to compute the average difference between predicted and real CBE amounts using the root-mean-square error prediction (RMSEP) according to (*16*−*18*)

$$\mathrm{RMSEP} = \sqrt{\frac{\sum_{i=1}^{n}(\%_i^{\mathrm{pred}} - \%_i^{\mathrm{real}})^2}{n-1}}$$

where $\%_i^{\mathrm{real}}$ corresponds to the real percentage of CBE admixture to the cocoa butter and $\%_i^{\mathrm{pred}}$ to the value predicted by the established model.

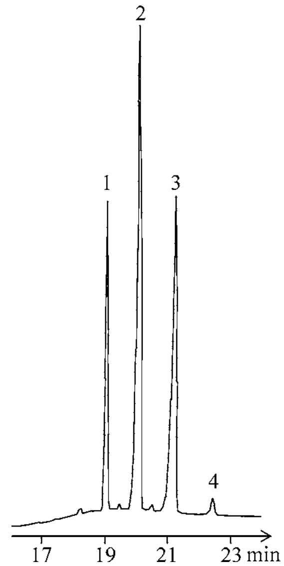

Figure 1. Typical gas chromatogram of the cocoa butter (CB_M) used to elaborate CBE/CB_M obtained with a DB 10 m × 0.32 mm × 0.1 μm capillary column with nitrogen-c as the carrier gas. Triacylglycerol classes: C50 (1), C52 (2), C54 (3), and C56 (4).

RESULTS AND DISCUSSION

The detection limit of CBEs added to cocoa butter fat was the first step of this work. The elaborated equation is based on Padley's work (*7*). A cocoa butter gas chromatogram is characterized by the four signals corresponding to the different TAG classes C50, C52, C54, and C56. To build his model (*7*), Padley used the peak areas of only three TAG classes—C50, C52, and C54, considering the C56 value to be negligible—and normalized them. After plotting %C50 versus %C54, he built a "cocoa butter equation", which gave the pure CB limit values; that is, all of the samples over this line are adulterated by CBE. In some cases, Padley specified that C56 values should be used for accurate determination as some CBEs contain a high percentage of C56. Thus, from our point of view, it seemed that the %C56 value should be considered for a global control of an unknown sample. For this reason, Padley's equation could not be used as described (*7*). TAGs of chocolate bars from various geographical origins were studied by GC after TAG extraction according to the described experimental protocol. A typical GC chromatogram is presented in **Figure 1**. The four peaks can be detected between 18 and 23 min and correspond to the TAG classes (C*n*) C50, C52, C54, and C56. Then their areas were summed and normalized; some examples are listed

Table 1. TAG Class Relative Percentages (%C*n*) Determined for Various Cocoa Butters and Mixtures CBE/CB Determined by Gas Chromatography

sample[a]	%C50	%C52	%C54	%C56
CB_M	18.02	46.25	33.98	1.76
IRMM 801	18.54	46.20	33.41	1.85
illipé	8.80	37.70	49.72	3.78
mix 1 (5.06%)	17.86	44.72	35.27	2.15
mix 3 (14.74%)	16.79	41.89	38.76	2.55
mix 8 (19.94%)	16.50	40.63	40.16	2.64

[a] Percentage in parentheses corresponds to the amount of CBE added to the cocoa butter CB_M.

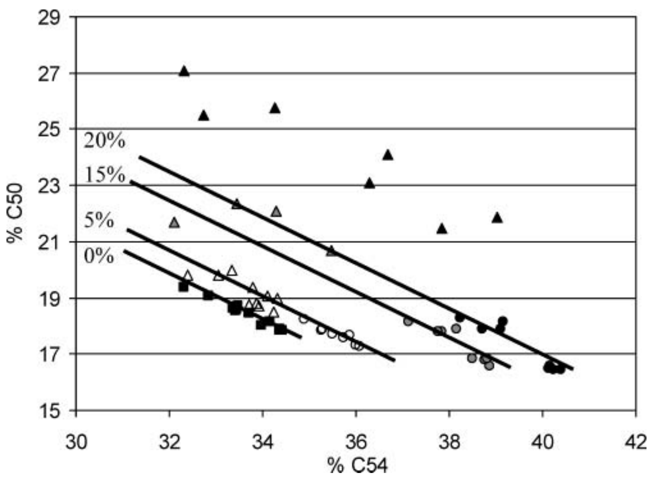

Figure 2. Cocoa butter variability and effect of CBE added to CB_M as the relationship between %C50 and %C54: (■) pure cocoa butter; (○) homemade mixtures containing a mixture of six CBEs at different percentages in CB_M; (△) tested samples. Shading of the two latter symbols: none, CBE content ≈5%; gray, CBE content ≈15%; black, CBE content ≥20%.

in **Table 1**. In **Figure 2**, %C50 versus %C54 is plotted for the studied chocolates showing the TAG ratio variability related to the geographical origin of the chocolates. To avoid false positives, in control cases, only the highest values were considered to establish the following cocoa butter equation

$$\%C50 = -0.82\%C54 + 46.1 \qquad (1)$$

where %C50 and %C54 are the experimental values obtained after normalization using the four TAG class areas. The equation constants (−0.82 and 46.1) differ slightly from Padley's, reflecting the effect of taking into account the C56 value. This equation has been tested using samples from a collaborative trial (*10*−*14*). A set of test mixtures, elaborated with various origin CBs that differ from the one used for this study, was prepared with <5% of CBE. In all cases, the admixture of CBE to cocoa butter was detected, using eq 1, at a level as low as 2%. IRMM 801 is a cocoa butter certified material for one TAG (POP) but not for TAG class analysis (*19*). Nevertheless, due to its homogeneity and stability, it was used during this study as an internal quality control for the GC apparatus but not for the method. For this application, this material, the TAG class values of which are listed in **Table 1**, could be used as a calibration value to determine the apparatus response factor. Thus, experimental values could be corrected, if necessary, to apply this model.

Once the pure cocoa butter equation was established, the second topic was to quantify the added CBE. To solve this type of problem, multivariate chemometric techniques need to be applied to a matrix built with %C*n* values of various fats. To build the data matrix, two strategies could be considered to obtain %C*n* values.

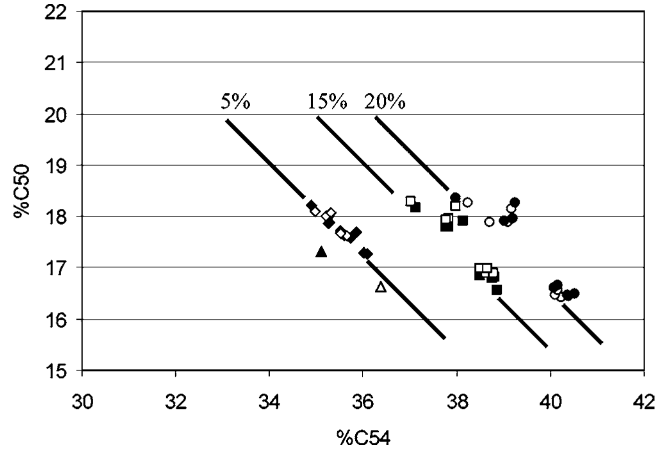

Figure 3. Comparison between experimental and simulated data for homemade CBE/CB_M mixtures for various amounts of CBE admixtures: (□, ○, ◇) CBE mixtures listed in **Table 2** and incorporated to CB at various percentages; (△) admixture of pure illipé fat to CB_M at 15% level; (black symbols) experimental data; (white symbols) simulated data.

First, eq 2, obtained by considering mass balance, could be used to compute theoretical data

$$\%Cn^{\text{mix}} = (1-a)\%Cn^{\text{CB}} + a\sum_{x=1}^{6} z_x \%Cn^{x(\text{CBE})} \qquad (2)$$

where $\%Cn^{\text{mix}}$ is the computed TAG class percentage of the mixture CB/CBE. $\%Cn^{\text{CB}}$ and $\%Cn^{x\text{CB}}$ correspond to the experimental value of TAG class percentage of the pure cocoa butter and pure CBE, respectively; *x* varies from one to six as the mixtures can be elaborated with one or more CBEs, *z* is the mass fraction of each fat in the CBE mixture, and *a* is the mass fraction of CBE added to cocoa butter. One plot in **Figure 3** corresponds to a real mixture containing 15.15% of illipé alone. To simulate this plot, eq 2 was used considering the z_1 factor to be equal to 1, *a* to be equal to 0.1515, $\%C50^{1,\text{CBE}}$ to be 8.80, and $\%C50^{\text{CB}}$ to be 18.02 (see **Table 1**). Similar computation was performed with %C54, and for more complex mixtures the $\%Cn^{x,\text{CBE}}$ of each CBE added to the cocoa butter must be considered.

The second option was to elaborate real mixtures with known added CBE percentages and to study these mixtures' response by characterization techniques. Comparison between simulated and experimental results is plotted in **Figure 3**. Even if there is a good agreement between values, one can notice some differences that may come from experimental conditions. For this reason, elaborating real mixtures was chosen, as the results would consider mixture preparation effects and experimental errors. However, directive 2000/36/EC does not specify if the CBE admixture must be made with only one CBE or if it can be made with a CBE mixture. Thus, as an infinity of CBE/CB mixtures could not be tested, a Placket−Burman (PB) design (*20*) was the starting point to elaborate a calibration set as it minimized the number of experiments with optimal efficiency. This PB experiment design indicates that, for six variable parameters, only eight experiments are sufficient to have a precise evaluation of all the possible combinations. Thus, eight pure CBE mixtures were prepared, and the exact mass of each added fat was recorded after each addition to compute the real

Table 2. Percentages of the Six Authorized CBEs Used To Elaborate the Eight Mixtures According to the Pseudo Packet and Burman Design

mix	illipé (%)	karite (%)	sal (%)	mango (%)	kokum (%)	palm (%)
1	20.10	20.12	19.93	9.75	20.17	9.93
2	9.94	20.45	19.30	20.20	9.72	20.38
3	10.83	11.18	23.14	22.31	21.50	11.05
4	19.64	9.98	9.54	19.98	21.53	19.33
5	11.22	21.83	11.03	10.89	22.60	22.44
6	22.24	11.19	22.46	11.46	10.72	21.91
7	21.84	21.82	11.79	21.47	12.51	10.57
8	19.30	19.44	20.32	10.54	19.27	11.13

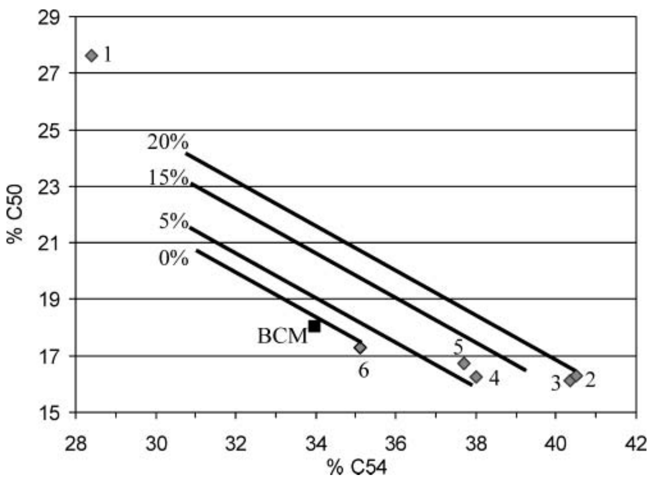

Figure 4. Effect of one pure CBE admixture to cocoa butter CB_M at a level of 15%: (■) cocoa butter used for the mixtures CBE/CB_M; (◇) CBE/CB_M mixture with (1) palm, (2) kokum, (3) karité, (4) sal, (5) mango, and (6) illipé.

pure CBE mixture composition listed in **Table 2**. Even if the mixtures' compositions are quite arbitrary, it is not far from reality as some manufacturers told us that, if they use CBE in their chocolate, it will be a CBE mixture so as not to be dependent on any CBE supplier. These eight pure CBE mixtures were incorporated to cocoa butter fat at three different percentage levels, that is, 5, 15, and 20%, in order to determine limit equations for these added CBE ranges. Thus, the control result could be given as a percentage range of adulteration. The GC results are plotted in **Figure 2**. The values obtained are mostly aligned when %C50 versus %C54 is plotted. By CB line extrapolation, straight lines were drawn delimiting a clear range of CBE added to CB. In **Figure 2** the test batch values are also plotted. Most of the sample values are within the limits defined by the model mixture. Some of the test batch values are out of range. This could be explained by the TAG ratio variability due to the chocolate origin, by a content of 50% palm oil in the CBE mixture, and as palm oil is mainly made of C50 TAG class, it leads to a strong increase of the %C50 value compared to %C54. Moreover, the mixtures prepared with only one pure CBE (**Figure 4**) are under- or overestimated (the illipé problem will be discussed later). It seems that a representation in two dimensions can provide some qualitative data but is not sufficient for a good quantification. For this reason, multivariate calibration techniques were used. A matrix containing added CBE percentages and TAG class values of various geographical origin pure CBs, homemade mixtures, and mixtures containing various authorized CBEs with various origin cocoa butters. TAG class values of various origin pure CBs were included in the matrix in order to take into account the TAG class variability.

Table 3. Comparative Data between the Real Amount of CBEs Added to Cocoa Butter and the Values Predicted by Equation 3

type of fat[a]	CBE[b] (%)	real (%)	predicted (%)
pure CB from equator	0	0.0	0.8
PMF[c]/shea + illipé	35/65	4.05	3.8
PMF/shea	50/50	15.09	16.3
PMF/sal/mango	50/25/25	24.97	24.8
PMF/shea + illipé	35/65	30.01	33.4
palm	100	15.4	14.4
kokum	100	15.2	17.9
karite	100	15.4	17.1
sal	100	15.1	13.1
mango	100	14.8	10.4
illipé	100	15.1	2.3

[a] Type of CBE used in the CB admixture. [b] Composition of the CBEs mixture. [c] Palm midfraction

A PLS regression was applied to the elaborated matrix as described under Materials and Methods. The software allowed the possibility to extract the regression coefficient affected to each %C*n* value. The linear combination is given in the equation

$$A = 53.460 + (1.197 \times \%C50) - (2.464 \times \%C52) + (1.129 \times \%C54) + (0.138 \times \%C56) \quad (3)$$

where A is the computed percentage of CBE added to CB and the various %C*n* need to be replaced by the experimental values obtained by GC from an unknown composition chocolate. The coefficient values of %C50 to %C54, in eq 3, are much greater than that of %C56, approximately reflecting the relative importance of each TAG class. Nevertheless a model was built considering only the %C50 to %C54 values, and even though the predicted values were not far from the real ones, the RMSEP (see below) value was slightly higher (2.5%). "Intermediate" models were built after two to three samples had been excluded. Then the model tested these samples and predicted a value used to determine an average error between predicted and real CBE amount according to the equation presented in the experimental part. Some examples of predicted values are listed in **Table 3**. A RMSEP value of 1.6% is obtained in 98% of the tested values. Thus, the quantification uncertainty for this model can be estimated to be 2%, which is a reasonable value.

The established model was applied to CBE/CB mixtures containing only one type of CBE, even though this is the most unlikely case as discussed previously. In **Figure 4** are plotted the experimental values for 15% added CBE, and the values predicted by the model are listed in **Table 3**. Except for illipé, eq 1 indicates cocoa butter adulteration. Most of the predicted values were in good agreement with the real added CBE amount. In the case of admixture of pure mango fat, the predicted value is underestimated by 4%, which is greater than the overall uncertainty of the model. Even though it is not fully satisfactory, at least there is no overestimation of the amount that would lead to a false positive conclusion. The main difficulties, as already pointed out (*7*, *10*), come from detecting and quantifying illipé alone as when this fat is mixed with other CBEs, the total added amount is easily predicted. Effectively, with respect to the five other CBEs (*2*, *21*), illipé fat is made with nearly the same C*n* TAG class ratio as cocoa butter (**Table 1**). Thus, added illipé only slightly modified the various C*n* percentages of cocoa butter, and the predicted percentage was so underestimated (real, 15%; predicted, 3%) that the value was not useful for quantification. Thus, an alternative method, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, was explored to tentatively detect and quantify illipé addition more

Table 4. Real and Computed Percentage Comparison for the Six Authorized Cocoa Butter Equivalents (Model Obtained by Applying PLS Regression to the Matrix Based on GC Data)[a]

sample		illipé (%)	karite (%)	sal (%)	mango (%)	kokum (%)	palm (%)	total CBE (%)
pure BC equator	real	0	0	0	0	0	0	
	predicted	0.1			0.3	0.4		0.8
mix 1 (14.64%)	real	2.9	2.9	2.9	1.4	2.4	1.4	
	predicted	2.2	3.2	3.2	2.3	3.0	0.9	14.8
PMF/sal/mango (24.97%)	real	0	0	6.24	6.24	0	12.49	
	predicted	0.9	4.6	1.5	1.3		13.9	22.2

[a] Values in total CBE column correspond to the sum of the individual CBE percentages.

precisely (*21*), but until now, despite its selectivity, this technique did not increase the accuracy of this CBE detection. As shown in **Figure 4**, 15% added illipé leads to an increase of %C54 and a discrepancy of %C50 with respect to the cocoa butter. Thus, it seems that determining cocoa butter geographical origin, through metal analysis, for example, and the TAG class related with the origin (*22*) could be another track to control illipé admixture. If illipé detection is still a real problem, the importance of this fat in chocolate bar preparation is minor, compared to palm fat, for example, as worldwide production is very low.

The latter discussion is more prospective work in that it is not really useful for control purposes. Thus, to determine how far the model could provide information on CBE admixture, a CBE admixture of a CBE/CB mixture was tentatively deconvoluted in its individual CBE components. For this topic, the data matrix was slightly modified: the fifth column containing the global amount of added CBE (see data treatment section) was replaced by six columns containing the exact percentage of individual added CBEs. Then the new model was applied to several test samples; some results are listed in **Table 4**. The first observation is the good agreement between the real total amount of added CBEs and the predicted value of the sum of all the individual CBE predicted values, within the RMSEP value. This result indicates the accuracy of eq 3 to quantify the global amount of CBE admixture. The pure CB prediction is good as the model predicted only a negligible amount of CBE. For a homemade mixture, containing each of the six CBEs, the predicted results are also correct even for illipé admixture. In the third case, if the computation is good for palm admixture, there is some discrepancy between predicted and real CBE amount for sal and mango fats. Moreover, the model predicted an admixture of karité that was not the case. This result can be explained by the TAG class composition that is very close for these three fats (*2*, *21*), karité, sal, and mango, showing the model cannot clearly differentiate these three fats on the basis of the TAG class analysis only.

CONCLUSION

To control the application of directive 2000/36/EC that allows the addition of six CBEs to cocoa butter at a maximum level of 5%, a quantification method has been developed. Gas chromatography with an unpolar column was used to detect and quantify the CBE admixture. All of the results are based on TAG class analysis, that is, %C50, %C52, %C54, and %C56. A pure "cocoa butter equation" has been established allowing admixture detection as low as 2%, that is, 0.6% with respect to a chocolate bar containing 30% of cocoa butter, for example. Then a matrix was elaborated with the experimental TAG class data of various CBE/CB mixtures and various geographical origin pure cocoa butters. A PLS data treatment has been applied to this matrix, providing a quantification equation of global CBE admixture. This equation allows the quantification of CBE admixture with an uncertainty of 2% (0.6% with respect to a chocolate bar containing 30% of CB, for example). As the study of TAG class is highly reproducible by GC, these results can be used for routine control of CBE admixture to chocolate bars. In the particular case of a pure illipé admixture, the detection is extremely sensitive and could be overcome by knowing the geographical origin of the cacao beans used for the chocolate bar elaboration. This work is underway.

ACKNOWLEDGMENT

We thank Dr. E. Anklam of the IHCP for kindly supplying the cocoa butter equivalent fats and inviting our laboratory to participate in the collaborative ring-test.

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Received for review October 8, 2003. Revised manuscript received February 12, 2004. Accepted March 10, 2004.

JF0351523