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Quantification of Milk Fat in Chocolate Fats by Triacylglycerol Analysis Using Gas−Liquid Chromatography

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The development and in-house testing of a method for the quantification of milk fat in chocolate fats is described. A database consisting of the triacylglycerol profiles of 310 genuine milk fat samples from 21 European countries and 947 mixtures thereof with chocolate fats was created under a strict quality control scheme using 26 triacylglycerol reference standards for calibration purposes. Out of the individual triacylglycerol fractions obtained, 1-palmitoyl-2-stearoyl-3-butyroyl-glycerol (PSB) was selected as suitable marker compound for the determination of the proportion of milk fat in chocolate fats. By using PSB values from the standardized database, a calibration function using simple linear regression analysis was calculated to be used for future estimations of the milk fat content. A comparison with the widely used butyric acid method, which is currently used to determine the milk fat content in nonmilk fat mixtures, showed that both methods were equivalent in terms of accuracy. The advantage of the presented approach is that for further applications, i.e., determination of foreign fats in chocolate fats, just a single analysis is necessary, whereas for the same purpose, the C4 method requires two different analytical methods.

KEYWORDS: Milk fat; chocolate fats; triacylglycerol analysis; gas−liquid chromatography

INTRODUCTION

The European Parliament and Council adopted Directive 2000/36/EC (*1*), authorizing the replacement of cocoa butter (CB) by vegetable fats other than CB (so-called cocoa butter equivalents, CBEs), on 23 June 2000. The objective of the Directive was to simplify community provisions concerning chocolate with a view to allowing the free movement of chocolate products within the internal market. Member States' laws, regulations, and administrative provisions have to comply with the Directive since August 2003. For implementation 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) was developed (*2*), validated in international collaborative trials (*3*−*5*), and finally adopted by the International Organization for Standardization (ISO) (*6*, *7*). So far, this standardized analytical approach established for plain chocolate has not been applicable to milk chocolate since TAGs deriving from milk or milk fat (MF) interfered with the detection and quantification of CBEs in the chocolate. When milk chocolate is analyzed, it will be necessary to correct the observed TAG analysis for the presence of MF TAGs, requiring knowledge of the amount of MF present in the product.

The problem of estimating the MF content in mixtures of fats or chocolates has already prompted a great deal of research. Traditionally, wet chemistry methods such as the Reichert Meissl value and the Polenske value have been used to estimate the MF in chocolate (*8*, *9*). Padley and Timms (*10*, *11*), along with Fincke (*12*), attempted to calculate the proportion of MF content in CB and chocolate fats by means of TAG analysis using packed column GLC, where TAGs are separated according to their carbon number (CN). To determine the MF content in chocolate fats, a formula based on the sum of the TAGs of CN40, CN42, and CN44 was established. This proved to be satisfactory for milk chocolate fats where MF is the minor component of the mixture and the main interest lies in determining whether or not the chocolate fat is pure CB (*13*). Variants of the procedure, based on the same evaluation principle, were described later by others (*14*). A drawback of the approach is that the formulas no longer apply if CB is mixed with coconut or palm kernel fats. In the case of lauric fat addition, the content of TAGs with CN40, CN42, and CN44 represents the sum of those coming from milk and lauric fats. Pontillion (*15*) proposed a way of calculating the contents of lauric fat and pure MF. The MF content is calculated from TAGs CN40 to CN44; so, in the presence of lauric fat, a correction has to be applied to these TAGs as well.

Several authors have proposed methods for the determination of MF, based on the content of fatty acids typical for ruminant MF such as butyric acid or the ratio between myristic and palmitic acids (*16*, *17*). Other strategies to detect adulterated

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MF are based on the concentration ranges of even more than two fatty acids using multivariate statistical evaluation tools (*18*, *19*). Currently, the analysis of butyric acid (C4) in a mixed fat is a widely applied method (*20*−*22*) and has, for instance, already been applied to quantitate small amounts of MF in CB or chocolate fats (*23*−*25*). These methods are based on the fact that C4 is not found in CB, coconut oil (CNO), or any of the vegetable fats used in the chocolate-manufacturing industry. Nevertheless, depending on feeding conditions and lactation, the amount of C4 varies considerably. If a sample of the pure MF is not available, an average C4 content may be used instead, which could assist in accurately determining the MF content. Such a value has been determined for 136 representative European MF samples (*26*). However, with respect to the correct labeling of chocolate, this method can only provide an answer to one out of three questions; that is, what is the content of MF in the chocolate fat? The method is not satisfactory for the other two questions; that is, (i) is there any other fat in addition to CB present and, if yes, (ii) how much?

This study presents an improved analytical approach for the determination of MF in chocolate fats, which is based on a standardized database consisting of the TAG profile of genuine MF samples and mixtures thereof with chocolate fats. The TAG database, obtained by HR-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 requirements (i) of being present in reasonable amounts, allowing a reliable quantification of even low MF proportions in chocolate fats, (ii) of having an acceptable natural variability, and (iii) of being present only in MF and no other fats. Results of a comparison with the traditional C4 method are presented. The advantage of the presented approach is that for further applications, that is, determination of foreign fats in chocolate fats, a single analysis is performed, whereas for the same purpose, the C4 method requires two different analytical methods.

MATERIALS AND METHODS

Materials. Butter samples (257) from 21 different European countries were collected in retail stores over the period 2001−2005. MF for analysis was obtained by melting the butter in an oven at 50 °C, and the oil layer was dried with anhydrous Na_2SO_4 and filtered. Additionally, 53 anhydrous MF samples from six different European countries were provided by Kraft Foods (Väsby, Sweden) over the period of February 2005 to October 2005. The resulting 310 anhydrous MF samples were stored in amber glass bottles at −20 °C prior to analysis. CB samples and CBE samples were donated by industry sources over the period 1992−2005.

Methyl butyrate, methyl valerate, tricapryloyl-glycerol, tricaprinoyl-glycerol, trilauroyl-glycerol, trimyristoyl-glycerol (MMM), 1,2-dimyristoyl-3-palmitoyl-glycerol (MMP), 1,3-dimyristoyl-2-oleoyl-glycerol, 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), tristearoyl-glycerol, and α-cholestane were purchased from Sigma Aldrich (Bornem, Belgium). 1-Myristoyl-2-palmitoyl-3-butyroyl-glycerol (MPB), 1-myristoyl-2-oleoyl-3-butyroyl-glycerol (MOB), PSB, 1,2-distearoyl-3-butyroyl-glycerol (SSB), 1,3-dipalmitoyl-2-myristoyl-glycerol (PMP), tripalmitoyl-glycerol, 1-myristoyl-2-oleoyl-3-palmitoyl-glycerol (MOP), 1,2-dioleoyl-3-myristoyl-glycerol, 1,3-distearoyl-2-myristoyl-glycerol, 1,2-dipalmitoyl-3-stearoyl-glycerol, 1,2-dipalmitoyl-3-linoleoyl-glycerol, 1,2-distearoyl-3-palmitoyl-glycerol, 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (POS), 1,2-dioleoyl-3-palmitoyl-glycerol (OOP), 1,3-distearoyl-2-oleoyl-glycerol (SOS), 1,2-dioleoyl-3-stearoyl-glycerol (OOS), 1,3-stearoyl-2-linoleoyl-glycerol, and 1,2-dioleoyl-3-arachidoyl-glycerol were purchased from Larodan fine chemicals (Malmo, Sweden). All other chemicals were of analytical grade and obtained from VWR International (Leuven, Belgium).

Sample Preparation. The samples (MFs, CBs, CBEs, and the CB-certified reference material) were warmed in an oven (50−55 °C) until completely melted. For each of the 21 European countries, an average MF mixture was prepared by gravimetrically blending equal proportions of the available individual MF samples. These "MF country mixtures" were used to prepare gravimetrically 222 CB-MF and 725 CB-MF-CBE mixtures (in total, 947 blends). To this end, 23 different CBs, 16 different MF country mixtures, and 10 CBEs were selected representing the whole range of samples (**Table 1**). The MF concentrations ranged from 5 to 30%, whereas the admixture of the individual CBEs was between 1 and 30%.

Preparation of Samples for TAG Analysis. For analysis of the pure MF samples ($n = 310$), 10 mg of fat was dissolved in 10 mL of *iso*-octane. To 1 mL of this solution, 1 mL of α-cholestane standard solution (0.02 mg/mL α-cholestane in *iso*-octane) was added. A 0.5 μL amount of this solution was injected using cold on-column injection (OCI) for gas chromatography (GC) analysis. For analysis of the CB-MF ($n = 222$) and CB-MF-CBE samples ($n = 725$), stock solutions were prepared by dissolving the fat sample (100 mg) in 10 mL of *iso*-octane. For split injection, 1 mL of the stock solution was mixed with 1 mL of α-cholestane standard solution (0.04 mg/mL α-cholestane in *iso*-octane). One microliter of this solution was injected into the GC. For OCI, the stock solution was diluted 1:10, and to 1 mL of the resulting solution 1 mL of α-cholestane standard solution (0.004 mg/mL α-cholestane in *iso*-octane) was added. A 0.5 μL amount of this solution was injected for GC analysis.

Preparation of Samples for Butyric Acid Methyl Ester Analysis. For butyric acid methyl ester analysis, the fat sample (100 mg) was dissolved in 5 mL of *n*-hexane and 1 mL of methyl valerate standard was added (0.387 mg/mL methyl valerate in *n*-hexane). The solution was mixed with 200 μL of sodium methylate solution (2 M in methanol) and thoroughly mixed for 1 min. After 5 min, 0.5 g of sodium hydrogen sulfate-monohydrate was added to the solution and centrifuged for 3 min (2000 rpm). One microliter of the supernatant was injected for GC analysis.

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, a flame ionization detector (FID), and the GC ChemStation software Rev. A.10.02 for chromatogram processing.

Analysis of TAGs. TAGs were separated on a fused silica capillary column (25 m × 0.25 mm i.d.) coated with 0.1 μm CB-TAP phase (Varian, Inc., Middelburg, The Netherlands). The FID temperature was set at 360 °C. Hydrogen was used as the carrier gas. For split injection, the injection port was set at 360 °C using a constant flow of 2 mL/min at a split ratio of 1:10. The oven was programmed from 200 (held for 1 min) to 270 °C at 14 °C/min, raised to 340 °C at 2.5 °C/min, and then held isothermally for 10 min. The sample volume injected was 1 μL. For OCI injection, the temperature of the OCI was set to the "oven-track" mode; that is, it was maintained 3 °C above the oven temperature during the temperature program. The flow rate of the carrier gas was held constant at 3.5 mL/min by the electronic pressure control system of the 6890N GC. The oven was programmed from 100 (held for 1 min) to 300 °C (held for 2 min) at 50 °C/min, raised to 340 °C at 30 °C/min, and then held isothermally for 7 min. The sample volume injected was 0.5 μL.

Calibration and Quantification of TAGs. Calibration and quantification of the MF samples were done by preparing a TAG stock calibration solution (3.66 mg/mL *iso*-octane) that consisted of equal proportions of 26 TAG reference standards. An average response factor for each TAG was determined by analyzing six different dilutions of the stock solution (1.83, 0.92, 0.46, 0.23, 0.11, and 0.057 mg/mL) containing different concentrations of the individual TAGs (ca 0.065, 0.033, 0.016, 0.008, 0.004, and 0.002 mg/mL of each TG) but always the same concentration of α-cholestane (0.01 mg/mL). For each calibration solution, response factors for every TAG *i*, that is, $F_{TAG,i}$, were calculated by

$$F_{TAG,i} = \frac{C_{TAG,i} \times A_{cholestane,i}}{C_{cholestane,i} \times A_{TAG,i}} \quad (1)$$

Table 1. Selected Samples Used To Prepare CB-MF and CB-CBE-MF Blends; in Total 947 Samples

set	CB type	CB code	MF (country mix)	CBE type	CBE code
1	West Africa	SW	all countries	no CBE added	
2	Ecuador Saci	Z15	France	no CBE added	
3	West Africa	SW	Ireland	no CBE added	
4	West Africa	SW	Estonia	no CBE added	
5	West Africa	SW	Spain	no CBE added	
6	West Africa	SW	Denmark	no CBE added	
7	Ghana Taksi	Z21	France	PMF + shea stearine	M12
8	Ghana Taksi	Z21	Germany	PMF + shea stearine	M12
9	Indonesia	Z17	Germany	Coberine plus	F3
10	Columbia Almacena	Z22	Belgium	soft CBE	L22
11	Ecuador	Z13	Latvia	PMF (from Malaysia)	L15
12	IRMM-801	IRMM801	Ireland	Akonord E	E5
13	Brazil	Z11	United Kingdom	PMF + shea stearine	M12
14	Brazil	Z11	Hungary	PMF + shea stearine	M12
15	Ghana Taksi	Z20	United Kingdom	PMF + shea stearine	M12
16	Grenada	R10	Hungary	soft CBE	L22
17	Papua New Guinea	R9	Latvia	PMF (from Malaysia)	L15
18	Columbia Almacena	Z22	Belgium	shea oil	P2
19	Indonesia	Z17	Italy	soft CBE	E11
20	Cameroon	G10	Italy	shea oil	P2
21	Indonesia	Z17	Estonia	Coberine plus	F3
22	Ghana Taksi	Z20	Belgium	Illexao 30–66	P4
23	Ecuador	Z13	Switzerland	shea stearine (from West Africa)	L11
24	Brazil	Z11	Spain	PMF + shea stearine	M12
25	Ghana Taksi	Z20	Czech Republic	Illexao 30–67	P4
26	Grenada	R10	Netherlands	Akonord E	E5
27	Papua New Guinea	R9	Austria	soft CBE	L22
28	Ghana Taksi	Z21	Denmark	PMF (from Malaysia)	L15
29	Brazil	Z11	Hungary	PMF + shea stearine	M12
30	Brazil	Z11	Denmark	PMF + shea stearine	M12
31	Indonesia	R12	Switzerland	shea stearine (from West Africa)	L11
32	Indonesia	R12	Latvia	PMF (from Malaysia)	L15
33	Ecuador	R11	Belgium	soft CBE	L22
34	West Africa	R5	all countries	PMF (50%) + illipé (25%) + shea (25%)	E8
35	Cameroon	Z7	Poland	PMF (50%) + exotic fats (50%)	M1
36	Ivory Coast	C1	Lithuania	traditional CBE, fully equivalent to CB	L19
37	Papua New Guinea	Z3	Ireland	Kokum	E10
38	West Africa	R1	all countries	harder palm midfraction	H1
39	West Africa	R2	Poland	Sal fat (from India)	L13
40	West Africa	R4	Slovenia	Illexao 30–96	V3
41	Malaysia	C4	France	PMF + shea stearine	D12
42	Nigeria	C3	all countries	Sal	D9
43	West Africa	R4	Poland	traditional CBE, fully equivalent to CB	L19
44	Indonesia	Z17	France	soft CBE	L22
45	Indonesia	Z17	Ireland	Coberine plus	F3

where $A_{TAG,i}$ is the peak area of the TAG *i* in the calibration solution *i*, $A_{cholestane,i}$ is the peak area of the internal standard α-cholestane in the calibration solution *i*, $C_{TAG,i}$ is the concentration (mg/mL) of the TAG *i* in the calibration solution *i*, $C_{cholestane,i}$ is the concentration (mg/mL) of the internal standard α-cholestane in the calibration solution *i*, and $F_{TAG,i}$ is the detector response factor of TAG *i* in the calibration solution *i*.

An average response factor for TAG *i*, that is, $F_{TAG,i;mean}$, obtained from the six calibration solutions, was calculated and used for further calculations. The mass fraction in percent of an individual TAG *i* in a MF sample, that is, $M_{TAG,i;sample}$, was calculated as follows:

$$M_{TAG,i;sample} = \frac{A_{TAG,i} \times C_{cholestane} \times F_{TAG,i;mean} \times 100\%}{A_{cholestane} \times C_{sample}} \quad (2)$$

where $A_{TAG,i}$ is the peak area of TAG *i* in a test sample, $A_{cholestane}$ is the peak area of the internal standard α-cholestane in a test sample, $F_{TAG,i;mean}$ is the average response factor for TAG *i*, $C_{cholestane}$ is the concentration (mg/mL) of the internal standard α-cholestane in a test sample, and C_{sample} is the concentration (mg/mL) of a test sample.

Calibration and quantification of CB-MF and CB-MF-CBE mixtures was as follows: For split injection, an average response factor for PSB was determined by analyzing six different calibration solutions containing different concentrations of PSB (0.04, 0.03, 0.02, 0.01, 0.005, and 0.0025 mg/mL) but always the same concentration of α-cholestane (0.02 mg/mL) using the same calculation principle as mentioned before (eq 1). For OCI injection, the same procedure was applied using the same calibration solutions, only diluted with the factor of 10. The mass fraction in percent of PSB in a CB-MF or CB-MF-CBE sample, that is, $M_{PSB,sample}$, was calculated as follows:

$$M_{PSB,sample} = \frac{A_{PSB} \times C_{cholestane} \times F_{PSB,mean} \times 100\%}{A_{cholestane} \times C_{sample}} \quad (3)$$

where A_{PSB} is the peak area of PSB in a test sample, $A_{cholestane}$ is the peak area of the internal standard α-cholestane in a test sample, $F_{PSB,mean}$ is the average response factor for PSB, $C_{cholestane}$ is the concentration (mg/mL) of the internal standard α-cholestane in a test sample, and C_{sample} is the concentration (mg/mL) of a test sample.

Analysis of Butyric Acid. Butyric acid (in form of its methyl ester) was separated from other fatty acid methyl esters on a 25 m fused silica capillary column (internal diameter, 0.25 mm) coated with 0.2 μm of CP-Wax 58 (Varian, Middelburg, The Netherlands). Hydrogen was used as the carrier gas with a flow rate of 1.1 mL/min at a split ratio of 1:50. The temperature of both the injector and the detector was 265 °C. The oven was programmed from 45 (held for 1 min) to 75 °C at 5 °C/min, raised to 260 °C at 30 °C/min, and then held isothermally for 5 min. The sample volume injected was 1 μL.

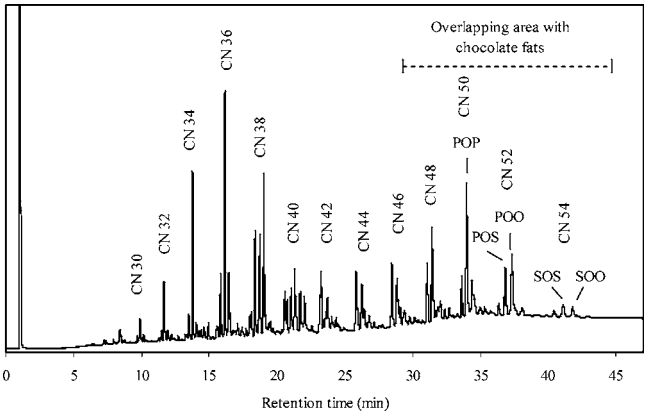

Figure 1. TAG profile of pure MF sample obtained by capillary column GLC using a medium polar stationary phase.

Calibration and Quantification of Butyric Acid. Calibration and quantification of CB-MF samples were, except for some modifications, according to Molkentin and Precht (*27*). An average response factor for methyl butyrate was determined by analyzing six different calibration solutions containing different concentrations of methyl butyrate (0.80, 0.60, 0.40, 0.20, 0.10, and 0.05 mg/mL) but always the same concentration of methyl valerate (0.20 mg/mL). The mass fraction in percent of methyl butyrate in a test sample was determined using the same calculation principle as for PSB (eqs 1 and 2). The final results were expressed as g C4/100 g fat using a conversion factor of 1.16 to convert methyl butyrate into butyric acid.

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).

RESULTS AND DISCUSSION

To determine the MF content in milk chocolate by means of specific TAGs, it is necessary to take into consideration the natural variations in MF, mainly a function of feeding and lactation. This can complicate the quantitative determination of MF in chocolate fats. The main aspect of the first part of the work was to collect a representative number of authentic and genuine MF samples and to set up a suitable specimen data bank. The sampling plan was set up to take into account regional and seasonal variations in the MF composition. In a measurement campaign, the TAG profile of 310 MF samples was analyzed by HR-GLC using a medium polarity stationary phase. Besides the CN separation, the TAGs are separated according to their degree of unsaturation, giving a much more detailed picture of the TAG composition in comparison to the profile obtained on a nonpolar column. The chromatograms recorded for the samples contained more than 200 individual TAG peaks (**Figure 1**). Compositional data were calibrated using 26 different TAG reference standards varying in their CN group. The established standardized TAG database was employed (i) for the selection of a potential TAG that could be used to calculate the MF content in CB-MF and CB-CBE-MF mixtures and milk chocolate and (ii) for determining the average contribution of the five TAGs deriving from MF that have an impact on the evaluation algorithms to detect and quantify CBEs in chocolate, that is, POP, POS, POO, SOS, and SOO (**Figure 1**). The latter part is communicated and described in detail in ref *28*.

In **Table 2**, the compositions of a few TAGs, which were considered as potential markers (CN 34-46), are given. Out of the individual TAG fractions obtained, PSB proved to be the most suitable marker compound for the determination of the proportion of MF in chocolate fats by fulfilling the following requirements, that is, (i) present in reasonable amounts allowing a reliable quantification of even low MF proportions in chocolate fats, (ii) acceptable natural variability, and (iii) present only in MF and no other fats. The first two criteria were best fulfilled by PSB. MOB showed the same natural spread as PSB, but the average amount of the 310 MF samples was 25% less than for PSB. MPB was present in higher amounts than PSB, but the natural variability was higher as well. The rest of the TAGs, which did not contain butyric acid, were present in lower amounts and had a higher variability than PSB.

Table 2. Descriptive Statistics of Individual TAGs Tested as Potential Markers for MF Content Predictions ($n = 310$)

CN group	CN 34	CN 36	CN 38	CN 40	CN 42	CN 44	CN 46
TAG	MPB	MOB	PSB	SSB	MMM	MMP	PMP
mean	2.45	1.60	2.15	1.71	1.80	1.70	1.58
minimum	1.92	1.19	1.57	1.37	1.23	1.19	1.04
maximum	3.02	1.83	2.73	2.05	2.32	2.18	1.99
SD	0.21	0.09	0.13	0.14	0.23	0.20	0.18
RSD	8.66	5.91	5.89	7.97	12.85	11.73	11.59

To prove the latter hypothesis, numerous fats that can be present in chocolate fats such as lauric fats, CBEs made from tropical fats, hazelnut oil, almond oil, pistachio oil, etc., were analyzed to test possible interferences. MFs differ from most non-MFs in containing substantial amounts of TAGs with CN numbers below 46. Non-MFs can contain substantial amounts, sometimes up to 10%, of DGs with CN 32−38, which overlap with TAGs of the same CN when the whole fat is analyzed by GLC using unpolar stationary phases, where the separation is only according to CN. Therefore, in former methods, where mainly unpolar stationary phases were used, only TAGs with CN 40−44 remained to discriminate between MF and non-MFs (*10*−*14*). However, two types of oils contain significant amounts of TAGs with CN 40−44, too, namely, palm kernel oils and CNOs (*10*−*12*). With unpolar columns, no differentiation can be made between CN 40−44 either originating from lauric fats or MFs, resulting in an incorrect MF quantification when those fats are present in a fat mixture. Mainly due to the development of efficient columns having high thermal stability, analysis of MF by GLC using capillary columns has improved in recent years. The use of temperature resistant medium polarity capillary columns enhances the resolution power largely and allows determination of individual TAG species. **Figure 2** shows the TAG profiles obtained for a pure MF, palm kernel olein (PKO), palm kernel stearine (PKS), and CNO (typical lauric fats) by HR-GLC using medium polar stationary phases, capable of resolving TAGs according to CN and degree of unsaturation. The marker substance PSB was completely resolved from any interfering components, even in the presence of lauric fats (**Figure 2**). The advantage over former approaches is that the MF content can be calculated by determining the PSB content in the presence of lauric fats, without applying correction factors (*15*).

Nevertheless, the results calculated from the PSB content are subjected to a degree of uncertainty because the PSB content of MF varies between certain limits. To obtain a representative mean value for the PSB content in MF, the 310 MF samples collected from 21 European countries were evaluated for their PSB content (**Figure 3**). The overall mean content of PSB in these samples was determined to be 2.15 g/100 g MF. The amount was considered to be sufficient for future MF predictions in chocolate fats having a MF content of less than 5%. Assuming a fat content of chocolate of 30%, this would translate to 1.5% MF in the final product, whereas a normal milk chocolate needs

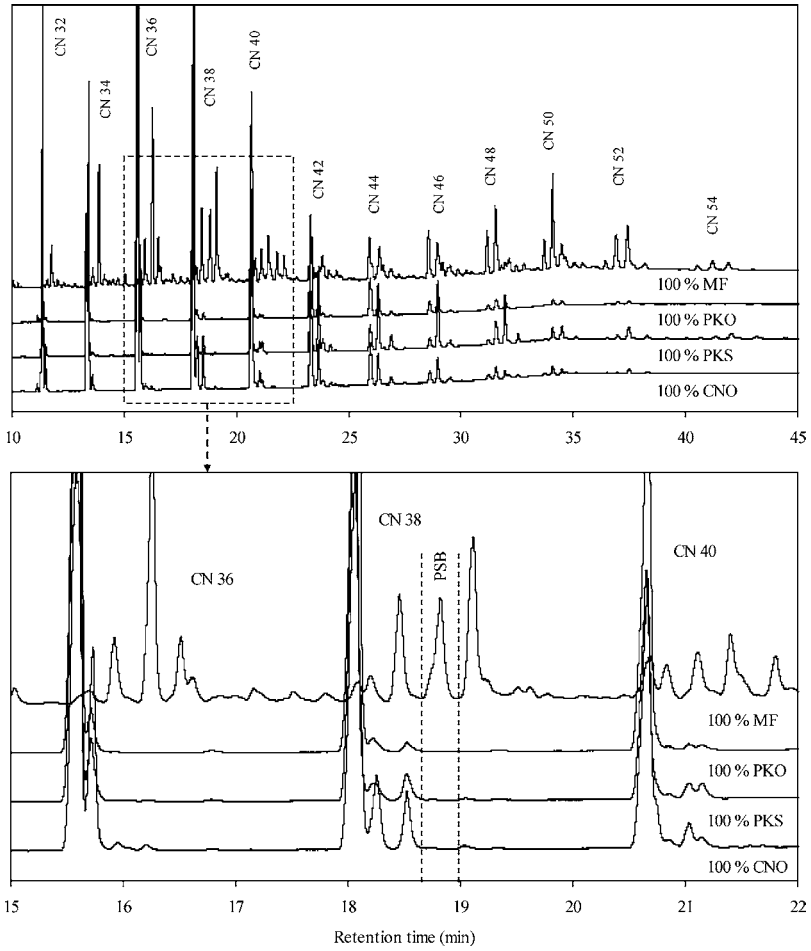

Figure 2. TAG profiles of pure MF, PKS, PKO, and CNO obtained by capillary column GLC.

to have at least 3.5%. **Figure 4**, showing the frequency distribution of the PSB contents, exhibits that the PSB content is normally distributed, ranging from 1.57 to 2.73 g/100 g MF. Eighty-five percent of the MF samples had PSB values between 1.9 and 2.4 g/100 g MF.

In the second part of the study, more than 900 gravimetrically prepared CB-MF and CB-CBE-MF mixtures with known MF contents, simulating the composition of real chocolate fats, were analyzed for their TAG profile using HR-GLC. The total data set ($n = 947$) was randomized and divided into two parts, that is, a calibration set ($n = 474$) and a validation set ($n = 473$). First, the experimentally determined PSB content and the known MF content of the calibration data set were used to calculate a calibration function by least-squares for future estimations of the amount of MF present in an unknown sample (% MF $=$ $0.202 + 44.016 \times$ PSB), resulting in a R^2 value of 0.976. The resulting mean average deviation (MAD) was 0.89 g/100 g fat chocolate fat and the root-mean-square error of prediction (RMSEP) 1.29 g/100 g. MAD is a general estimator of bias and RMSEP expresses the average uncertainty that can be expected when predicting values for new samples. The results of future predictions can then be presented as "predicted values $\pm$ 2 $\times$ RMSEP". In a second step, the established calibration function was applied to the validation data set to test the suitability of the statistical model. No significant differences between the two data sets were found. The obtained MAD was 0.88 g/100 g chocolate fat. In a last step, the two data sets were amalgamated, to establish a final mathematical model based on as many data sets as available. By using all of the data sets ($n = 947$), the following simple linear regression model was computed (eq 4):

$$M_{\mathrm{MF,sample}} = 0.190 + (44.036 \times M_{\mathrm{PSB,sample}}) \quad (n = 947 \text{ samples}) \quad (4)$$

where $M_{\mathrm{PSB,sample}}$ is the mass fraction in percent of PSB in the test samples (as determined in eq 3) and $M_{\mathrm{MF,sample}}$ is the mass fraction in percent of MF in the test sample.

The obtained MAD, a measure of bias, was used to test the performance of the model. The resulting MAD was 0.90 g/100 g chocolate fat, ranging from -6 to 5 g/100 g chocolate fat. Assuming a fat content of chocolate of 30%, this uncertainty of prediction amounted to 0.3 g MF/100 g chocolate, ranging from -1.8 to 1.5 g MF/100 g chocolate. The distribution of the relative prediction errors for the data set is given in **Figure 5**. Ninety-five percent of the values are within a range of $\pm$10%, which is comparable to results obtained by the classical C4 method (*27*, *29*). Finally, the results obtained by the linear regression model were compared to the results obtained by calculating the MF percentages using the average PSB content 2.15 g/100 g from the MF database (a) and the determined PSB content in the chocolate fat (b) via the equation $x = b/a \times 100$. Both the MAD (1.05 g/100 g chocolate fat) and the RSMEP (1.5 g/100 g) were slightly higher as compared to the results obtained by the linear regression model. This is most probably due to the incorporation of the most extreme PSB values from the database.

To prove that the currently used C4 method (*29*) does not give superior results to the established PSB method, several CB-

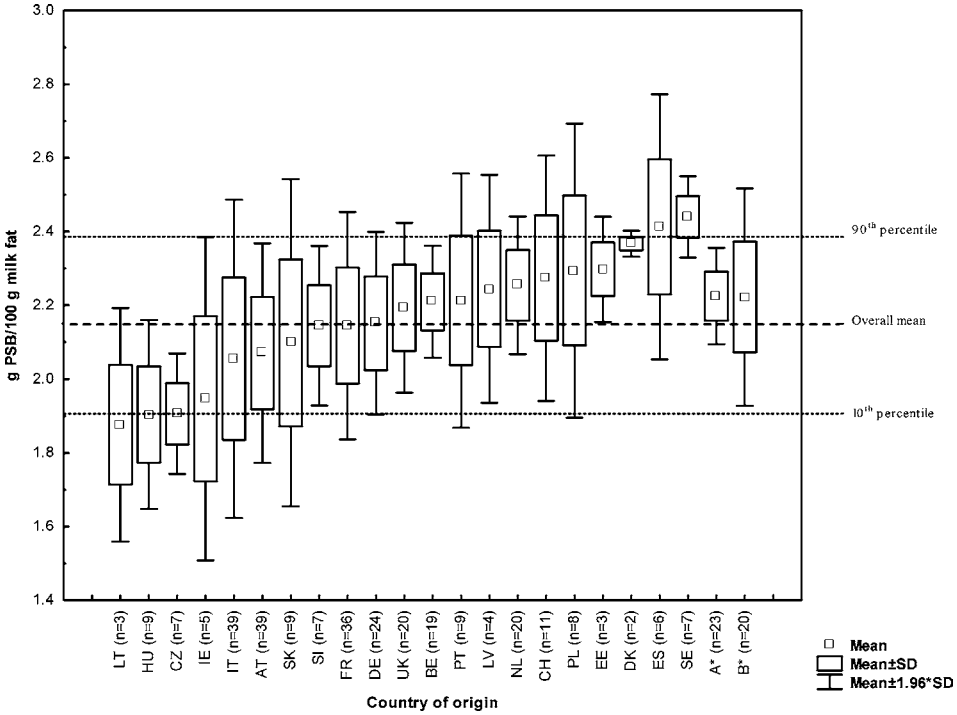

Figure 3. Distribution of PSB contents in MF samples from 21 different countries collected from 2001 to 2005: LT, Lithuania; HU, Hungary; CZ, Czech Republic; IE, Ireland; IT, Italy; AT, Austria; SK, Slovakia; SI, Slovenia; FR, France; DE, Germany; UK, United Kingdom; BE, Belgium; PT, Portugal; LV, Latvia; NL, Netherlands; CH, Switzerland; PL, Poland; EE, Estonia; DK, Denmark; ES, Spain; SE, Sweden; A*, samples obtained from February to October 2005 from six different countries; and B*, MF country mixtures.

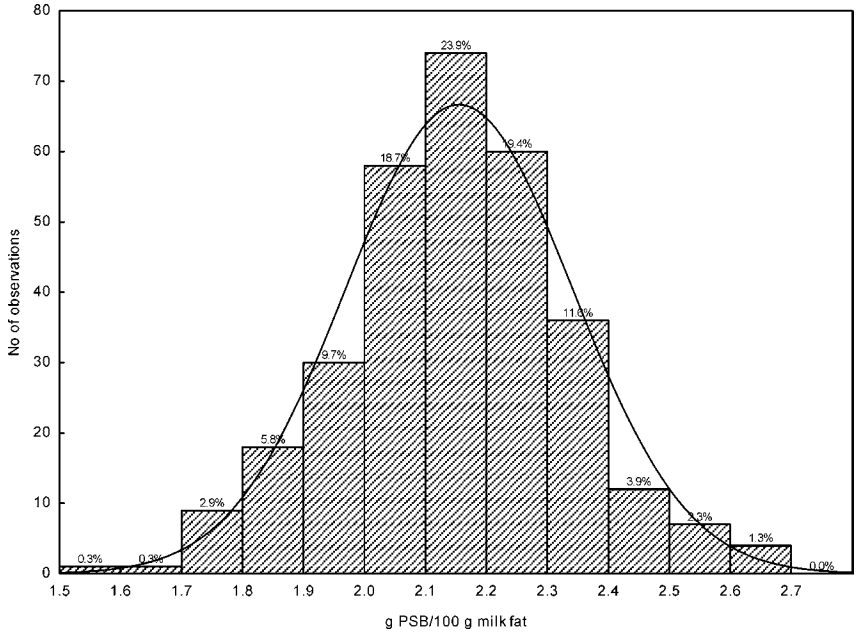

Figure 4. Frequency distribution of the PSB contents (g/100 g MF) in 310 MF samples with indication of the standard normal distribution.

MF mixtures were analyzed by applying both methods and the obtained MF contents were compared to the actual MF concentrations. The C4 contents were analyzed, with slight modifications, by a GLC method involving analysis of fatty acid methyl esters with an internal standard (*30*). In **Tables 3** and **4**, two variants of MF quantitation (with and without availability of the C4 and PSB value of the MF samples used for blend formulation) are compared with respect to the relative deviation between actual and determined MF content in mixtures with CB within a wide range of proportions. In the first calculation variant, the MF percentages were calculated from the analyzed C4 and PSB contents in the pure MF (a) and in the mixed fat (b) using the equation $x = b/a \times 100$ (**Table 3**). In the second calculation, variant (a) was replaced by an average C4 content of 3.42 g/100 g (*26*) and an average PSB value of 2.15 g/100 g derived from the newly established PSB MF database (**Table 4**). Calculation variant one shows that there is no significant difference between the two methods used. The obtained and the actual MF contents are in close agreement independent of the applied method. The accuracy of the absolute C4 and PSB content is of importance for the determination of the proportion of MF in chocolate. This applies in particular when the C4 (*27*) and the PSB content of the pure MF cannot be determined and empirical values are used for calculating the proportion of MF

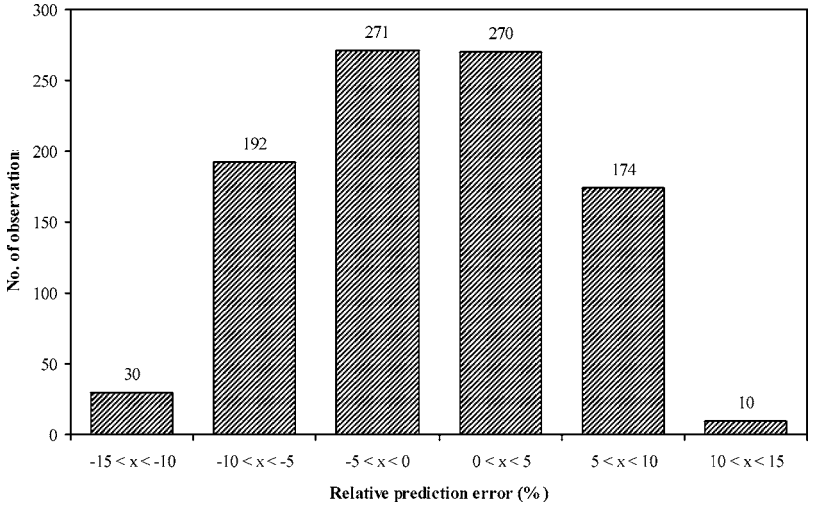

Figure 5. Distribution of relative prediction errors as obtained for the data set ($n = 947$) by the final mathematical model.

Table 3. Comparison of Actual and Experimentally Determined Values Using Different Methods[a]

		calculation based on known MF					
		% MF predicted			relative error		
set	% MF known	FAME-C4	SP-PSB	OCI-PSB	FAME-C4	SP-PSB	OCI-PSB
1	5.0	5.1	4.8	4.7	–2.2	2.3	4.5
	10.0	9.9	9.7	9.8	1.0	3.2	2.3
	15.1	14.9	14.9	14.8	1.0	1.2	1.9
	20.2	20.0	20.1	20.3	0.9	0.2	–0.5
	25.2	25.0	25.1	25.3	0.8	0.2	–0.5
	30.2	29.7	30.3	30.4	1.8	–0.4	–0.6
2	5.1	5.1	4.8	4.8	–1.3	5.7	5.0
	10.1	10.2	9.8	9.8	–1.8	2.7	2.5
	15.1	15.1	14.8	14.9	–0.2	1.9	1.5
	20.1	20.1	20.0	20.1	0.3	0.8	0.1
	25.2	25.1	25.1	25.3	0.8	0.7	–0.4
	29.8	30.1	30.1	30.3	–0.9	–1.0	–1.4
3	5.0	5.2	4.6	4.6	–4.9	7.0	6.4
	10.1	10.0	10.0	10.0	1.0	1.5	1.1
	15.2	15.4	15.1	15.1	–1.1	0.7	0.9
	20.5	20.7	20.4	20.5	–0.6	0.7	0.2
	25.3	25.0	25.3	25.5	1.2	–0.1	–0.8
	30.1	29.9	30.3	30.4	0.7	–0.6	–0.9
4	5.0	5.0	4.6	4.6	–1.6	8.2	7.1
	10.1	10.0	9.8	9.8	0.6	2.3	2.9
	15.1	15.1	15.0	14.9	0.4	0.8	1.4
	20.2	20.0	20.1	19.9	0.6	0.3	1.3
	25.2	25.0	25.1	25.2	0.8	0.3	0.2
	30.2	29.8	30.2	30.2	1.2	–0.2	–0.1

[a] FAME-C4, butyric acid value; SP-PSB, PSB by split injection; OCI-PSB, PSB by cold OCI.

Table 4. Comparison of Actual and Experimentally Determined Values Using Different Methods[a]

		calculation based on average database values					
		% MF predicted			relative error		
set	% MF known	FAME-C4	SP-PSB	OCI-PSB	FAME-C4	SP-PSB	OCI-PSB
1	5.0	4.8	5.2	5.1	3.1	–4.1	–2.2
	10.0	9.4	10.0	10.0	6.1	0.3	–0.2
	15.1	14.2	15.2	15.0	6.1	–0.6	0.7
	20.2	19.0	20.4	20.4	5.9	–1.0	–1.0
	25.2	23.7	25.3	25.3	5.9	–0.7	–0.5
	30.2	28.2	30.5	30.3	6.8	–1.0	–0.4
2	5.1	4.9	5.2	5.3	2.8	–3.3	–4.1
	10.1	10.3	10.4	10.3	–2.6	–3.0	–2.7
	15.1	14.7	15.5	15.4	2.8	–2.7	–2.4
	20.1	19.9	20.8	20.8	1.2	–3.1	–3.1
	25.2	24.0	26.0	26.1	4.8	–2.9	–3.2
	29.8	28.9	31.2	31.0	3.0	–4.5	–4.0
3	5.0	5.1	5.1	5.2	–2.8	–3.6	–3.9
	10.1	10.1	10.7	10.7	0.2	–5.8	–5.2
	15.2	15.2	16.0	15.8	0.4	–5.5	–4.1
	20.5	20.7	21.5	21.4	–0.8	–4.9	–4.2
	25.3	25.0	26.6	26.5	1.2	–5.4	–4.8
	30.1	31.0	30.8	30.9	–3.1	–2.3	–2.6
4	5.0	5.2	5.3	5.4	–4.4	–6.5	–8.9
	10.1	10.3	11.0	11.0	–2.1	–9.3	–9.4
	15.1	15.5	16.6	16.6	–2.4	–9.8	–9.7
	20.2	20.6	22.1	22.0	–2.1	–9.8	–9.1
	25.2	25.7	27.6	27.7	–1.9	–9.5	–9.9
	30.2	30.6	33.1	33.2	–1.5	–9.8	–9.9

[a] FAME-C4, butyric acid value; SP-PSB, PSB by split injection; OCI-PSB, PSB by cold OCI.

instead. Results using the mean C4 or PSB values from the databases instead of the C4 or PSB content of the actual MF samples for calculation are shown in **Table 4**. The deviations from the actual MF contents are higher than in **Table 3**. The greater deviations are caused by using the average C4 content of 3.42 g/100 g instead of the real contents of the four base MFs, ranging from 3.24 g to 3.51 g/100 g. The same holds true for values determined using the average PSB content of 2.15 g/100 g instead of the real base MF values ranging from 2.17 g to 2.36 g/100 g. Set 4 shows a greater relative deviation obtained by using the PSB method as compared to the C4 since the randomly chosen base MF had a PSB value of 2.36 g/100 g, that is, almost 10% higher than the average PSB value (2.15 g/100 g) from the database. In the case of the C4 method, the actual value was 3.51 g/100 g, that is, only 2.5% higher than the average C4 content (3.42 g/100 g). The PSB content of this particular MF was rather near to the upper limit of the variation range than to the mean, whereas the C4 value was close to the database mean value. The contrary picture is given by set 1. Relative errors of 6% for the C4 method are due to the greater deviation of the actual (3.24 g/100 g) to the average C4 database content (3.42 g/100 g). In comparison, the PSB method results in relative deviations of around 1% having an actual PSB content of 2.17 g/100 g, which is very close to the PSB database value of 2.15 g/100 g. Thus, both methods can be seen as acceptable ways of carrying out a quantitative determination of MF in chocolate fats. Because of the natural variation of the PSB content in European MFs, relative deviations from the actual MF content may amount up to ±10% when calculating with a mean PSB content instead of the PSB content of the component sample. Using base MFs with more extreme PSB contents not included in the 90 percentile range of the database (**Figure 3**),

the errors could even be higher, which was observed for C4 contents of European MFs as well (*26*). The PSB method is not meant to replace the widely used C4 method for the determination of MF in non-MF mixtures. However, in the special case of chocolate fats, it can be considered as a good alternative.

Because the sample introduction technique can play a crucial role in TAG analysis using HR-GLC, the same study was performed using once classical split injection and once cold OCI. Classical hot split injection is considered by far the least suitable technique, causing strong discrimination and decomposition (*31*, *32*). Programmed temperature vaporizer split injection (*32*, *33*) and above all cold OCI (*34*, *35*) are the most frequently used approaches to reduce or eliminate thermal decomposition, discrimination, and other effects associated with classical hot split injection. In the present study, the results obtained by different sample introduction techniques did not show any difference. For both injection techniques, a good optimization of operating conditions allows a nearly nondiscriminatory sample introduction, which can reduce losses of individual TAGs but generally cannot eliminate them, requiring a careful determination of response factors for the respective TAGs.

The advantage of the elaborated approach is that either the mathematical expression (eq 4) or the average PSB value from the MF database can be used by individual testing laboratories for determining the MF amount in chocolate fats, provided a careful calibration for PSB is performed. Calibration automatically links the results obtained in a laboratory to the MF database and the elaborated mathematical equation. Moreover, provided that certain performance criteria are fulfilled, the method can differ in the type of capillary column, the sample injection technique, and chromatographic conditions, offering an increased amount of procedural flexibility to the analyst.

In conclusion, from an analytical point of view, the described approach is very similar to the official butyric acid method, which is traditionally used for the quantification of MF in fat mixtures with non-MFs (*22*, *25*−*27*, *29*). Both methods have the advantage that butyric acid, part of PSB as well, is specific for MF and does not occur in any other fat. In applications where simply the MF content is needed, the analyst might choose the traditional butyric acid method. However, to control correct labeling of milk chocolates, the MF amount has to be determined (i) to prove compliance of the MF content itself and (ii) as a prerequisite for carrying out a reliable detection and quantification of CBEs in milk chocolate, usually based on TAG profiling by GLC. Thus, for the latter purpose, a determination of the MF using the butyric acid method would result in the application of two different analytical methods, that is, (i) fatty acid analysis and (ii) TAG analysis, whereas the PSB method proposed here offers the advantage of applying just one single analytical method, TAG analysis, leading to several useful pieces of information (*28*). The method described here offers an important measure to assess compliance with labeling provisions and is suitable for a rapid screening of large numbers of samples to determine the MF content in chocolate fats.

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