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Cluster Analysis for the Systematic Grouping of Genuine Cocoa Butter and Cocoa Butter Equivalent Samples Based on Triglyceride Patterns

MANUELA BUCHGRABER, FRANZ ULBERTH,* AND ELKE ANKLAM

European Commission, DG Joint Research Centre, Institute for Reference Materials and Measurements, Retieseweg, 2440 Geel, Belgium

The triglyceride profile of cocoa butters (CBs) from different geographical origins, varieties, growing seasons, and a number of cocoa butter equivalents (CBEs) was determined by capillary gas liquid chromatography. Hierarchical cluster analysis was applied to the five main triglycerides of the samples for the ability to find natural groupings among (a) CBs of various provenance and (b) CBE samples of different types. The samples were clustered using Ward's method, and the similarity values of the linkages were represented by dendrograms. The five triglycerides contained adequate information to obtain a meaningful sample differentiation. This information can be used to assess the purity and the origin of the CB sample examined.

KEYWORDS: Cocoa butter; cocoa butter equivalents; triglycerides; hierarchical cluster analysis

INTRODUCTION

Chocolate has been enjoyed around the entire world for centuries and is one of the most popular and widespread treats of modern day. Cocoa butter (CB), the fat extracted from cocoa beans, is one of the most expensive ingredients in chocolate formulations. There are three main cocoa producing regions, i.e., (a) Central and South America, (b) West Africa, and (c) South East Asia/Oceania. Natural variations in the physicochemical properties of CB are well-known, i.e., South East Asia/Oceania butters are harder than those from Africa, and Central and South America butters are the softest (*1*).

The Directive 2000/36/EC of the European Parliament and of the Council authorizes the use of up to 5% of six selected vegetable fats (termed cocoa butter equivalents (CBEs)) to replace CB in chocolate in EU Member States (*2*). The reasons to find alternatives to CB and to replace parts of CB in chocolate were economic and technological ones.

A lot of work has been carried out to determine the natural variations of the principle constituents of CB, the triglycerides (TGs) (*3*−*5*). The main motivation has been to find appropriate analytical approaches often entailing multivariate statistical data analysis for determining an adulteration of CB or chocolate with foreign fats (*6*, *7*). Nevertheless, an extensive collection of compositional data covering all potential sources of variation is of paramount importance for elaborating statistical decision making rules. A general question facing researchers in many areas of inquiry is how to organize observed data into meaningful structures. The search for natural groupings among samples is one preliminary way to study data sets. Because of its unsupervised character, cluster analysis can be used to perform a preliminary data scan and to uncover the structure residing in a data set. Objects are grouped in clusters in terms of their nearness or similarity (*8*).

The objective of this study was to cluster CBs and CBEs on the basis of their TG composition. While similarities among or differences between CBs are quite apparent (e.g., soft South American CBs vs hard East Asian CBs) such natural groupings are not so evident for commercial CBEs as well as raw materials used for blend formulation. The insight gained into the commonalties in composition of CBEs should in turn be beneficial for devising efficient strategies to detect and quantify CBEs in CB or chocolate confectionery.

MATERIALS AND METHODS

Materials. CB samples ($n = 74$) and CBE samples ($n = 75$) were donated by industry sources. Samples were collected over the period 1992 to 2001. The set of genuine CB samples represented (a) CBs from individual crops comprising samples of South American, Asian, and African origin, which are in general not used in pure form by the chocolate industry, and (b) CB blends as used for the production of chocolate by food manufacturers. The samples were chosen to reflect as close as possible the true variability of CB produced in the last 10 years.

The set of CBE samples represented (a) raw materials for CBE production as specified in Directive 2000/36/EC (2) and (b) a broad variety of commercially available CBEs. All of the samples were stored at 4 °C in the dark prior to analysis. The cocoa butter certified reference material IRMM-801 (*9*) was from the European Commission, Institute for Reference Materials and Measurements, Geel, Belgium.

Sample Preparation. The test samples (CBs, CBEs, and the CB certified reference material) were warmed in a drying oven (50−55

* Corresponding author: Franz Ulberth, European Commission, DG Joint Research Centre, Institute for Reference Materials and Measurements, Retieseweg, 2440 Geel, Belgium. Tel: +32(0)14-571 600. Fax: +32(0)14-590 406. E-mail: franz.ulberth@cec.eu.int.

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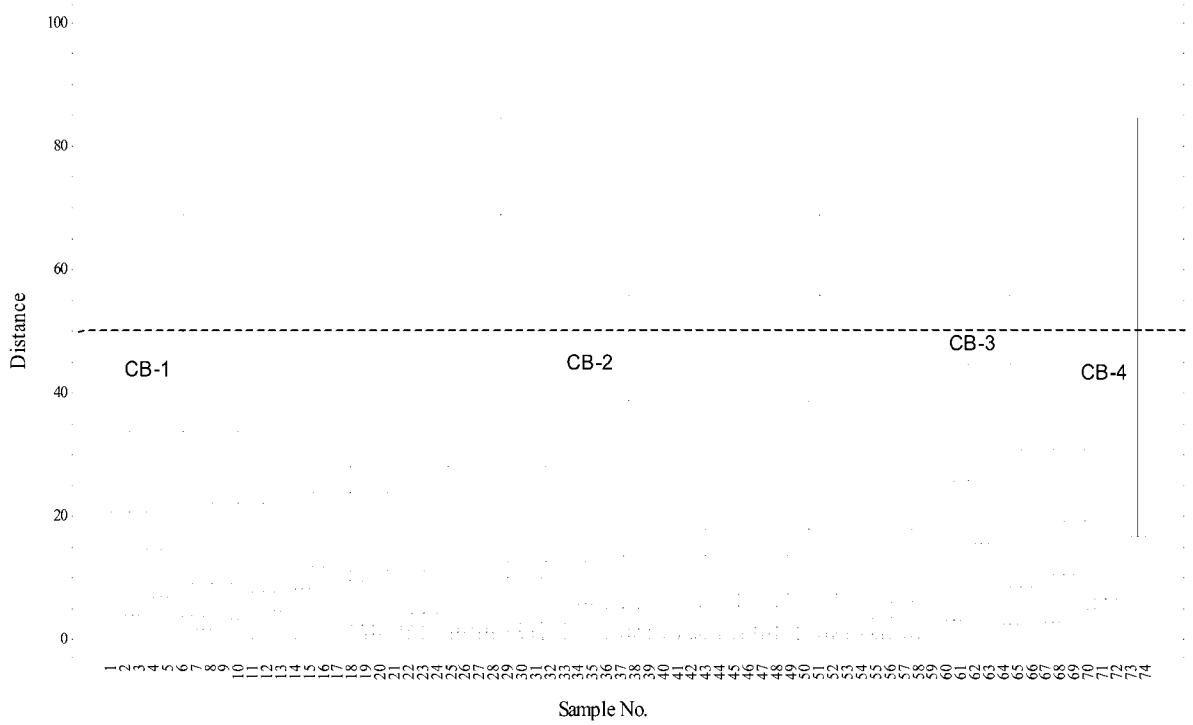

Figure 1. Hierarchical cluster analysis of the major triglycerides of CB samples.

°C) until completely melted. Pipets used for transferring the sample during weighing operations were brought to the same temperature to avoid partial fat fractionation. Solutions of CB and CBE in *iso*-octane (0.05%) were prepared for gas chromatographic analysis.

Gas Chromatography. Triglycerides 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), which was operated in a Carlo Erba HRGC 5300 gas chromatograph (Thermo Finnigan, Rodano, Italy) equipped with a cold on-column injector and a flame ionization detector (FID) in combination with the Chrom Card software (Thermo Finnigan, Rodano, Italy). The FID temperature was set at 360 °C. Hydrogen was used as the carrier gas, with the column pressure being 150 kPa. For TG separation, 0.5 μL of 0.05% sample solutions in *iso*-octane was on-column injected at a column temperature of 100 °C. The oven temperature was programmed from 100 °C (held 2 min) to 340 °C at 30 °C/min and then held isothermally for 25 min.

Identification was performed by retention time matching to standard substances. The five main TGs, i.e., 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (POS), 1,2-dioleoyl-3-palmitoyl-glycerol (POO), 1,3-distearoyl-2-oleoyl-glycerol (SOS), and 1,2-dioleoyl-3-stearoyl-glycerol (SOO) were quantified by area normalization. Area-% were converted to mass-% using experimentally determined response factors.

Determination of Response Factors. Response factors of the five main TGs were determined by injection of the CB certified reference material (IRMM-801) solution, certified for the five major TGs, using experimental conditions identical to those used for the samples. Response factors had to be calculated using the following equations:

$$AR_i\,[\%] = \frac{AR_i}{\sum AR_i} \times 100 \qquad (1)$$

$$RF_i = \frac{MR_i\,[\%]}{AR_i\,[\%]} \qquad (2)$$

in which AR_i is the area under the peak corresponding to TG_i in IRMM-801; ΣAR_i is the sum of the areas under the peaks attributed to POP, POS, POO, SOS, SOO in IRMM-801; MR_i [%] is the mass-% of TG_i in IRMM-801 as given in the Certificate (*9*); AR_i [%] is the area-% of TG_i in IRMM-801; and RF_i is the detector response factor of TG_i in IRMM-801.

The certified mass fraction (g/100 g of total TG) of TGs in IRMM-801 is POP 18.14%, POS 44.68%, POO 2.26%, SOS 31.63%, and SOO 3.29%.

Statistical Analysis. Statistical analyses were carried out with the STAGRAPHICS Ver. 3.0 computer package (Manugistics Inc., USA). Euclidean distance and Ward algorithms were used to compute hierarchical clusters.

RESULTS AND DISCUSSION

A triglyceride database representative of genuine CB and CBEs was setup as the basis for the authenticity assessment of genuine CB. The TG profiles of 74 CBs and 75 CBEs were determined by high temperature GC. Although the chromatograms recorded for the samples contained 21 peaks, only the five main TG fractions, i.e., POP, POS, POS, SOS, and SOO were used for databanking. The main reason for using theses five substances was that they could be calibrated by experimentally evaluated response factors using the CB reference material (IRMM 801) certified for these TGs; for the remaining TGs standard substances are not easily available. Together those five TGs make up approximately 85 to 90% of the TG profile of CB and CBEs (*10*). For further statistical analyses, they were normalized to give 100%.

Multivariate analysis of compositional data (TG, fatty acids, unsaponifiables, volatile compounds, etc.) has been used extensively to cluster olive oils according to attributes such as geographical origin, variety, and olfactorial properties (*11*−*16*). Cluster analysis has also been applied for chemotaxonomy of almond oil (*17*, *18*), but not so much for other edible oil commodities (*19*, *20*). CBs and CBEs have been characterized by bulk and molecular isotope analyses, FTIR spectroscopy, and pyrolysis mass spectrometry in combination with chemometry (*21*−*23*). Sample similarities on the basis of hierarchical clustering of the TG composition of CB or CBEs has not been investigated so far.

In this work, the Eucledian distance was taken as a measure of the proximity between samples, and a hierarchical agglom-

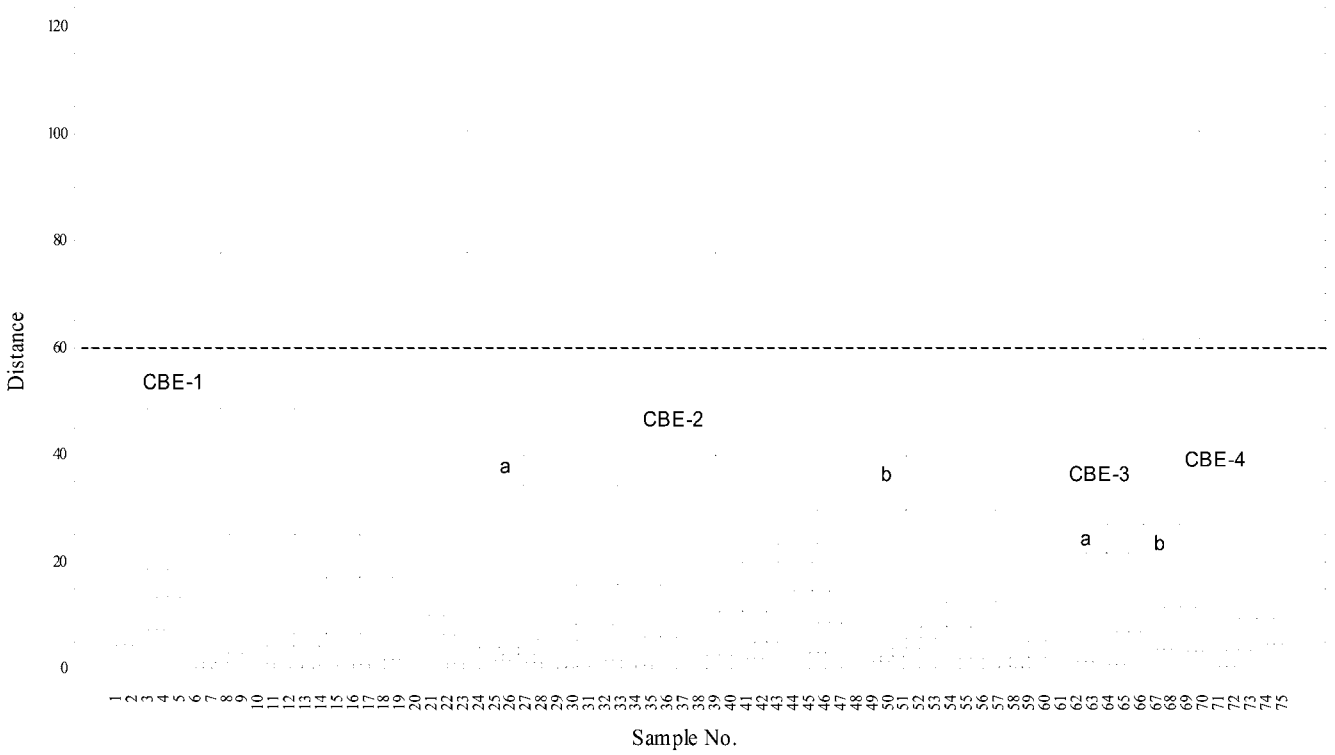

Figure 2. Hierarchical cluster analysis of the major triglycerides of CBE samples.

Table 1. Sample Information on CB Samples Provided by Suppliers[a]

group	sample description
CB-1	Malaysia, Tawau Crop 1992 (1); Papoua New Guinea (2, 4); Indonesia Crop 1991 (3); Indonesia 10 (5); Malaysia, 1. Eastern (6); Malaysia (7, 9, 12); Malaysia, 3. Tiara (8); Malaysia, 2. Majulah (10); Papua New Guinea Crop 1994 (11); Malaysia, Tawau Crop 1991 (13)
CB-2	Togo (14); Ghana Crop 1994 (15); Ivory Coast/Nigeria/Cameroon (16–18, 54); cocoa butter, no more information available (19, 24, 31, 33, 34, 35); CB African (20, 27); Mix deodorized (21, 40, 43, 44, 48, 51, 53, 56, 58); Mix nondeodorized (22, 30, 32, 41, 45–47, 55); Tanzania (23); Ghana Taksi (25, 36); West Africa, Crop 1995, deodorized (26, 28, 49); Nigeria, Apapa Crop 1993 (29); West Africa, Crop 1995, raw product (37–39, 42, 50, 52, 57); Madagascar (59)
CB-3	Brazil (60, 62); Equador Crop 1994 (61); Columbia Almacena (63); Guadeloupe (64, 67); Sao Tome (65); Grenada (66); Sao Tome Crop 1994 (68); Ecuador (69, 71); Ecuador Ecua cacao (70); Ecuador Saci Quality Cacao Products (72)
CB-4	Bahai (73); Brazil Ilheus Crop 1993 (74)

[a]Numbers in bracket correspond to the sample no. in Figure 1.

erative procedure (Ward's method) was employed to establish clusters. The hierarchical clustering method uses the distances between variables when forming the clusters. The Euclidean distance is the geometric distance in multidimensional space, and is probably the most commonly chosen type of distance. Ward's method uses an analysis of variance approach to evaluate the distance between clusters (*24*). The results obtained for the CB and CBE samples are shown as dendrograms in **Figure 1** and **Figure 2**. In principle, the higher the level of aggregation, the less similar are the members in the respective class.

Cocoa Butter Samples. Hierarchical cluster analysis grouped the CB samples into four clusters at a linkage level of 50 (**Figure 1**). Further information about the origin of the samples reported by the CB suppliers is given in **Table 1**. Results of descriptive statistics of the TG profiles of the individual groups are reported in **Table 2**.

The first cluster (CB-1) was composed of 13 samples including CBs from South East Asia/Oceania. The samples had a lower amount of POP and SOS in relation to groups 2 and 3. The POP concentration increased almost linearly going from group CB-1 to group CB-3, while the SOS content decreased. The difference in POS between the three groups was marginal. Samples statistically assigned to group CB-1 exhibited the lowest content of di-unsaturated triglycerides (POO + SOO < 5%). For the remaining groups an increase in the amount of di-unsaturated TGs up to 17% was observed. The second cluster (CB-2) was made up of 46 samples. They were identified as samples originating mainly from Africa, and CB blends on commercial offer. The third cluster (CB-3) included 13 samples, mostly from South America. Additionally, samples from Sao Tome and Grenada were allocated to group CB-3. The last cluster (CB-4) was only formed by two samples (Bahai and Brazil Illheus Crop). Those two samples had a much lower POP, POS, and SOS content than the rest of the samples, whereas the amounts of POO and SOO were much higher in comparison to the other three groups (POO + SOO > 16%). Since di-unsaturated TGs are low melting substances, such butters tend to be softer. Bahain CBs are known for their softness (*25*) resulting in unsatisfactory crystallizing properties (*4*).

For comparative purposes the Padley and Timms (*26*) approach to detect CBEs in CB is depicted in **Figure 3**. To this end, the content of POP, POS, and SOS were normalized to

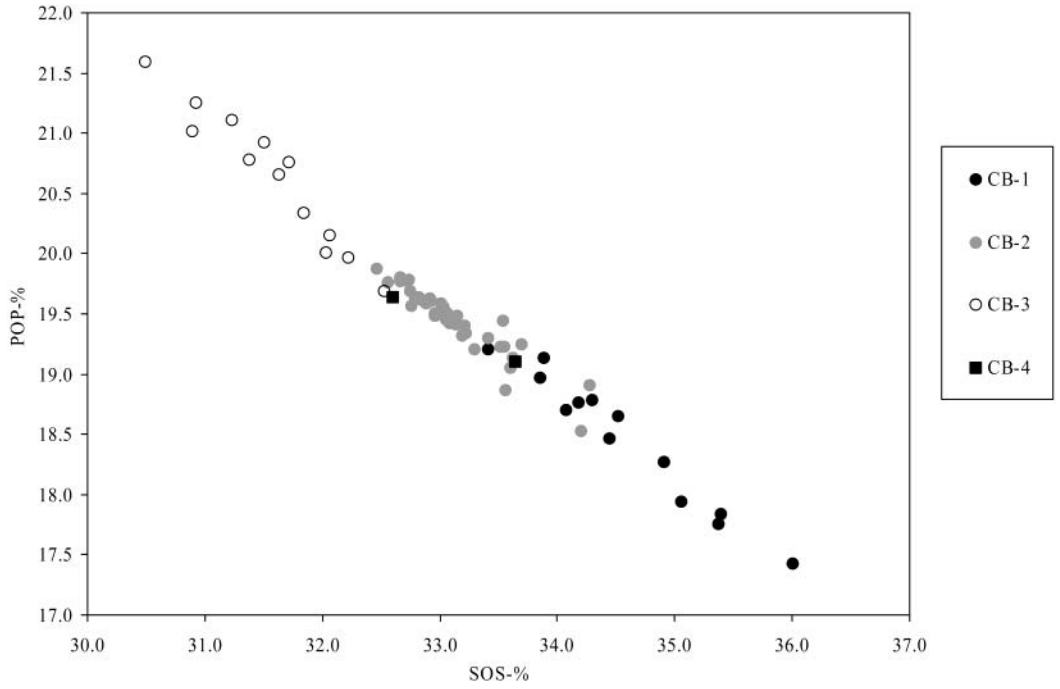

Figure 3. Correlation between SOS and POP of CB samples.

Table 2. Triglyceride Composition of CB Samples[a]

	POP	POS	POO	SOS	SOO
		CB-1			
mean	17.60	44.82	1.84	32.99	2.76
median	17.65	44.86	1.91	32.82	2.79
minimum	16.90	44.08	0.88	31.94	1.71
maximum	18.35	45.43	2.30	35.08	3.56
SD	0.52	0.39	0.38	0.85	0.48
		CB-2			
mean	18.25	44.51	2.50	31.10	3.66
median	18.31	44.60	2.42	31.11	3.61
minimum	17.18	43.53	2.12	30.38	3.07
maximum	18.77	45.03	3.30	32.19	4.94
SD	0.29	0.32	0.23	0.36	0.34
		CB-3			
mean	19.17	44.42	2.97	29.35	4.08
median	19.22	44.39	2.95	29.43	4.11
minimum	17.95	42.71	1.75	27.44	2.53
maximum	19.88	45.78	4.74	30.51	6.46
SD	0.59	0.92	0.89	0.97	1.17
		CB-4			
mean	16.24	39.84	6.65	27.78	9.51
median	16.24	39.84	6.65	27.78	9.51
minimum	15.85	39.22	6.35	27.62	8.95
maximum	16.63	40.45	6.95	27.93	10.06
SD	0.55	0.87	0.42	0.22	0.79

[a] g of individual TG/100 g of total TGs.

100% and the POP content was plotted vs. SOS. Three main groupings were apparent, which corresponded to clusters CB-1 to CB-3. The Bahai and Brazil Illheus Crop did not form a separate group, but were spread out in the space occupied by CB-2.

Cocoa Butter Equivalents. CBE samples consist of the same TGs as CB but quantitative percentages differ from those of CB. The variation of the TG profile of CBEs can be quite large.

CBE samples mainly from palm oil sources were clearly grouped together in cluster CBE-1 (**Figure 2**). The sample description submitted by the suppliers is given in **Table 3**. Palm oil is obtained from the flesh of the fruit of the oil palm *Elaeis guineensis* (*27*). As a raw material for CBE formulation it is mostly fractionated to produce a middle-melting fraction rich in POP, i.e., palm-mid fraction (PMF) (*28*). Characteristic of CBE-1 was a high POP content (**Table 4**). The maximum amount recorded within this group was 78.6% POP (mean 71.5%), while the SOS content was quite low with a mean value of 4.8%. Hierarchical cluster analysis discriminated commercial CBEs (CBE-2) from shea, kokum, mango and sal fat (CBE-3), and illipé fat (CBE-4). Samples from group CBE-4 exhibited a relatively high level of POS (>35%), significantly higher than any other of the groups, as well as a high level of SOS (>47 %). All samples of this group were illipé fat types. Illipé's chemical composition closely resembles that of CB, yet with a slightly higher melting point, because of its higher concentration of SOS.

During the early 1970s, a combination of growing CBE markets and the uncertainty associated with illipé crops of varying sizes led CBE manufacturers to explore alternative sources of so-called SOS fats. Kokum butter is the fat extracted from the seed kernels of the kokum tree, *Garcinia indica*. Its TG composition consists of up to 80% of SOS. Mango kernel fat is obtained from the seed kernels of the fruit of the mango tree (*Mangifera indica*). Solvent fractionation produces a stearin with a high concentration of SOS. Sal fat comes from the seeds of the sal tree, *Shorea robusta*. The fat has acceptable properties for use as a raw material, but can be improved by fractionation to give a more useful stearin with a high concentration of SOS. Shea butter is produced from the nuts of the shea tree, *Butyrospermum parkii*. The content of SOS is too low for its effective use as a raw material for CBE formulation. Solvent fractionation gives a useful stearin (*29*). Because of their chemical composition, those samples were clearly assigned to group CBE-3. The maximum SOS concentration was reached with more than 79%. All the samples contained a marginal amount of POP (POP < 3.6%). In comparison to groups CBE-1 and CBE-2, the POS concentration was significantly lower (mean = 10.8%). Furthermore, there was a decrease from group CBE-1 to CBE-3 in the POO amount, while the SOO increased. A further subclassification showed a grouping of (a) shea fat/

Table 3. Sample Information on CBE Samples Provided by Suppliers[a]

group	sample description
CBE-1	Palm-mid fractions (1, 6, 8–10, 12–17); CBE (2); soft CBE (3); RN 614 (4); Muster 2 (5); Muster 3 (7); Chovetta (11); Illexao 40–55 (18, 19)
CBE-2	Mix PMF/Illipe (20–23); Illexao 40–15 (24); Mix PMF/Shea stearine (25, 49, 60); Choclin (26, 28); CBE (mix PMF/exotic fat) (27, 29, 30, 55); Illexao 30–71 (31, 32); Illexao 30–66 (33); RN 231 (34); traditional CBE (35, 36, 41, 44); Akonord E (37); CBE (sal type) (38); CBE v15 (39); BS 1146 (40); RN 201 (42); Akomax E (43); Illexao 30–96 (45, 46); Coberine (47, 52, 53, 57); BS1437/N (48); Illexao 30–61 (50, 56); Muster 1 (51); Chocosine (53); Mix PMF/Illipe/Shea stearin (54); CBE (shea type) (58, 59); CBE, no more information available (61)
CBE-3	Shea fat (62); Shea stearin (63, 65); Kokum (64, 66); Mango kernal stearin (67); Sal stearin, India (68, 70); Sal fat, India (69)
CBE-4	Illipé fat (71–74)

[a]Numbers in bracket correspond to the sample no. in Figure 2.

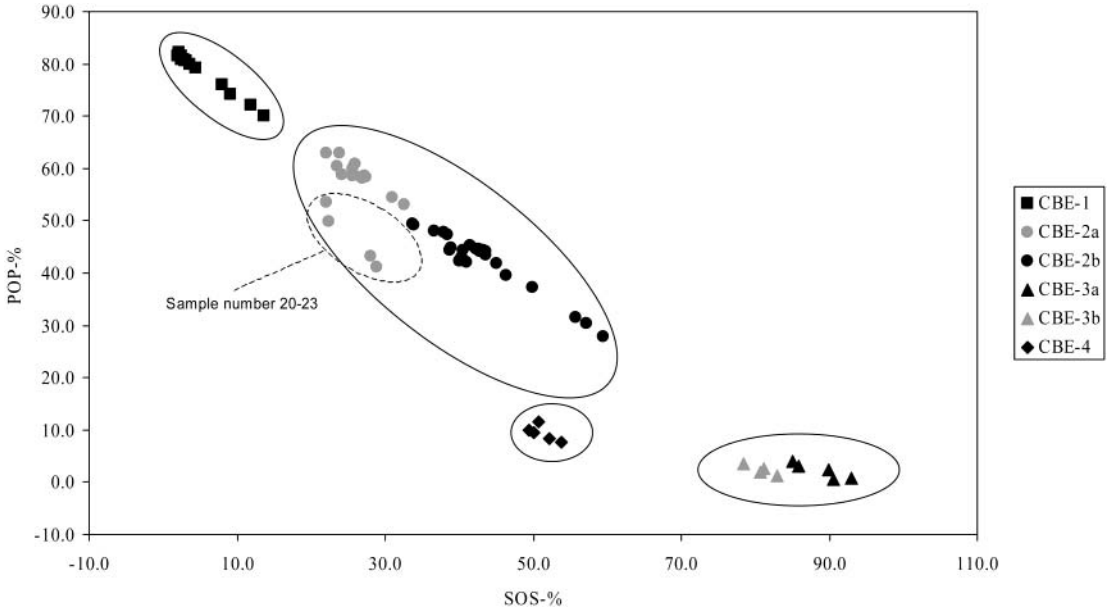

Figure 4. Correlation between SOS and POP of CBE samples.

Table 4. Triglyceride Composition of CBE Samples[a]

	POP	POS	POO	SOS	SOO
		CBE-1			
mean	71.54	15.33	6.84	4.85	1.44
median	75.61	15.30	4.36	2.79	0.75
minimum	42.86	12.59	2.38	1.67	0.43
maximum	78.64	21.76	17.56	18.75	4.12
SD	9.09	1.85	4.92	4.51	1.25
		CBE-2			
mean	45.31	14.80	2.85	33.43	3.61
median	43.36	13.83	2.61	34.72	3.27
minimum	25.70	11.32	1.63	20.79	1.31
maximum	59.98	28.83	9.46	55.03	10.04
SD	8.63	4.19	1.21	9.05	1.77
		CBE-3			
mean	1.91	10.79	1.55	74.00	11.75
median	2.00	10.31	0.97	77.85	11.88
minimum	0.47	5.24	0.69	65.88	7.17
maximum	3.60	15.50	2.68	79.45	15.72
SD	1.08	3.67	0.82	5.79	2.85
		CBE-4			
mean	9.03	37.74	1.30	49.15	2.78
median	9.04	38.55	1.05	48.21	2.74
minimum	7.40	35.36	0.68	47.49	1.55
maximum	10.77	39.15	2.35	51.23	4.02
SD	1.30	1.66	0.64	1.81	0.95

[a]g of individual TG/100 g of total TGs.

stearin and kokum fat and (b) of sal fat/stearin and mango kernel stearin. This is also evident on the Padley and Timms plot (POP vs. SOS) of the CBE samples (**Figure 4**).

The main group was represented by cluster CBE-2, which was made up of 42 mainly commercially offered CBE blends consisting of various ratios of the former mentioned raw materials for CBE production (groups CBE-1, -3, and -4). In commercial blends, several fats or fractions of the fats are mixed to resemble CB as closely as possible (*29*). A subclassification of the group CBE-2 discriminated between (a) softer CBE blends, e.g., consisting of 70% PMF and 30% exotic fats, and (b) harder CBE blends, e.g., composed of 35% PMF and 65% exotic fats. Within CBE-2a samples with number 20−23 were mixtures of PMF and illipé. In **Figure 4**, the same distinction could be observed. Samples belonging to the CBE-2 cluster clearly reflect that commercial CBEs contain substantially more POP and less POS than CB, but similar amounts of SOS (*5*). Since POS is not contained in substantial amounts in palm oil or fractions thereof, nor in shea, sal, or kokum fat, commercial CBEs contain less POS than genuine CB.

In conclusion, the TG profile of a comprehensive set of authenticated products (74 CB and 75 CBE of various origins) was determined, and these data can serve as basis for detecting and quantitating CBEs in CB. Hierarchical cluster analysis allowed the detection of commonalties among different products according to their geographical origin and/or the manufacturing process. This information can be used to either identify the origin of the CB or to predict the technological properties and the field of application of CBEs.

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