Rapid MS Method for Analysis of Cocoa Butter TAG

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ABSTRACT: Ammonia negative ion CI-MS was applied to analyze the M.W. distribution and regioisomeric structure of TAG in cocoa butter and in cocoa butter equivalents. The M.W. distribution results obtained for a reference cocoa butter were consistent with corresponding results obtained in an intercomparison study by chromatographic methods. Minor but statistically significant differences were observed when proportions of the three major M.W. species (52:1, 54:1, and 50:1; acyl carbon number/number of double bonds) in a mixture of nine cocoa butters and in mixtures containing 10 or 20% (w/w) of specific cocoa butter equivalents were compared. Tandem MS was used to determine the regioisomeric structure of the three major TAG M.W. species in cocoa butter and in cocoa butter equivalents. The regioisomeric structure in cocoa butter and in all the equivalents analyzed were nearly identical, oleic acid being located primarily in the *sn*-2 position. These results cannot be exploited in detecting added foreign fats in this case. However, the present study shows that useful TAG composition data, which may be used to detect foreign fats in cocoa butter by applying chemometric data evaluation, can be obtained by MS in a significantly shorter time compared to chromatographic methods.

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Cocoa butter (CB) is responsible for the characteristic melting properties of chocolate as well as the dispersion of other components in chocolate formulations. The price of this high-value product often fluctuates, and availability of the best-quality CB on the market may be uncertain. For these reasons replacing part of it with alternative fats in chocolate is of great interest for economical reasons. The addition of cocoa butter equivalents (CBE) may also be desirable to produce harder CB to increase the stability of chocolate, especially in warm climates. The fats used as alternatives to CB in chocolate products have been reviewed recently (3). These fats are often mixtures and can consist of palm oil fractions, illipé butter, shea butter, sal butter, and kokum butter or a variety of other exotic fats and their fractions (3). Modification of vegetable oils by modern enzymatic interesterification may also be used to produce CBE that closely mimic the composition and properties of CB (4). The new European Chocolate Directive 2000/36/EEC (5) allows the addition of up to 5% CBE in chocolate products. The fats to be used have been specified in the directive and are the following: palm oil, illipé (borneo tallow or tengkawang), sal, shea, kokum gurgi, and mango kernel. In addition to the mandatory labeling of the addition of CBE, labeling to indicate that those fats have not been added is also allowed. Therefore, a perceived need exists within official control laboratories for the availability of precise methods for the quantification of such vegetable fats in chocolate in order to implement the new directive. A comprehensive review of analytical methods applied to the identification and quantification of CB and alternative fats has been published recently (6). Most methods applied are based on analysis of FA, TAG, or unsaponifiable fractions using GC or HPLC (7,8). Most recently, authentication methods for CB have been based mainly on analysis of the TAG composition. HPLC and GC have both been shown to be suitable for analysis of TAG molecular species of CB. By applying HPLC or GC for molecular species analysis of CB TAG and GC for FA composition analysis and by using multiple linear regression data analysis, it was possible to detect 2–3% of foreign fat in CB (corresponding to about 0.7–1% in chocolate) in cases when the origin of the CB and CBE was unknown (7,8). These results could be confirmed by applying a simple and rapid GC method for TAG analysis and by using a robust mathematical approach (11). It has been demonstrated that, in the case of having information on the type of CB and CBE used, the threshold could be as low as 0.25–0.5% (corresponding to 0.1–0.2% in chocolate). Silver ion HPLC has also been applied to separate TAG subfractions of CB, and GC has been used to analyze the FA composition of each fraction, which has enabled 5% added CBE to be identified in CB (12,13).

Negative ion CI-MS utilizing a direct insertion technique has been applied to M.W. distribution analysis of various TAG mixtures (14–19). The method results in comparable separation of molecular species with chromatographic techniques but in a significantly shorter analysis time. Tandem MS may be further applied to determine the FA combinations of TAG and their regioisomeric structures within the M.W. species (14,16,20–22). When used in combination, these techniques produce detailed information about TAG molecular structures. Since application of MS to CB analyses has been limited mainly to pyrolysis MS (23,24), and because of the potential of the present method, we analyzed TAG in CB and

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in three CBE to assess the suitability of the method for identifying foreign fats in CB.

MATERIALS AND METHODS

Materials. 1,3-Dipalmitoyl-2-oleoyl-*sn*-glycerol (50:1); 1,2-dioleoyl-3-palmitoyl-*sn*-glycerol (52:2); 1-palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol (52:1); 1,2-dioleoyl-3-stearoyl-*sn*-glycerol (54:2), and 1,3-stearoyl-2-oleoyl-*sn*-glycerol (54:1) were purchased from Larodan Fine Chemicals AB (Malmö, Sweden) and were used to prepare a reference mixture consisting of 50:1 (18 mol%), 52:2 (5 mol%), 52:1 (44 mol%), 54:2 (5 mol%), and 54:1 (28 mol%). The reference CB was a sample that had been analyzed in an intercomparison study of 15 laboratories in 1999 as part of a precertification study (25). To evaluate the suitability of the MS method for detecting foreign fats in CB, three CBE (Illexao 30-71, Coberine, and illipé) and the reference CB were used to prepare mixtures containing 10 and 20% (w/w) of Illexao 30-71, Coberine, or illipé, respectively. Illexao 30-71 was provided by Aarhus Oliefabrik A/S (Aarhus, Denmark), Coberine was from Loders Croklaan (Wormerveer, The Netherlands), and illipé from Walter Rau Neusser Öl und Fett AG (Neuss, Germany). Solutions of standard TAG and CB samples were prepared in *n*-hexane (HPLC grade, Rathburn Chemicals Ltd., Walkerburn, Scotland) at concentrations of approximately 1 mg/mL for MS analyses.

M.W. distribution of TAG. M.W. distribution of TAG was analyzed by ammonia (≥99.998%, Prax Air, Oevel, Belgium) negative ion CI-MS utilizing a direct exposure probe for sample introduction (15). A Finnigan MAT TSQ-700 triple quadrupole mass specrometer (Finnigan MAT, San Jose, CA) equipped with a combined EI/CI ion source was used to perform the MS analyses. The pressure of ammonia (8500 mtorr), the ion source temperature (200°C), the electron energy (70 eV), the filament current (400 μA), and the probe heating rate (40 mA/s) were selected according to a method optimization study published previously (15). The range of *m/z* 700–1000 was scanned to monitor the $[M - H]^-$ ions of TAG. The reference mixture mimicking CB was analyzed to ensure that no response correction factors were needed for TAG molecular species existing in CB, as reported previously for other TAG mixtures (15). An average of four replicate analyses of each sample was presented. Recently developed automatic software was applied to spectra interpretation and processing of the results (16).

Regioisomeric structure of TAG. The same instrumentation and analysis parameters as in the determination of M.W. distribution were used in tandem MS analyses. In addition, argon (≥99.998%; AGA, Lidingö, Sweden) in a pressure of 1.4 mtorr with 15 eV collision energy was used to produce collision-induced dissociation fragment ion spectra of selected TAG acyl carbon number/number of double bonds (ACN/DB) molecular species. The fragment ions were detected by scanning *m/z* values from 100 to 1000. The regioisomeric structure of TAG molecular species was determined on the basis of the formation of $[RCO_2]^-$ and $[M - H - RCO_2H - 100]^-$ fragment ions, as reported previously (14,20–22). Fatty acyl constituents comprising the TAG were calculated using abundances of $[RCO_2]^-$ fragment ions produced in collision-induced dissociation. The easier formation of $[M - H - RCO_2H - 100]^-$ ions by cleavage of the fatty acyl moiety from primary *sn*-positions, enabled the distribution of fatty acyl constituents between *sn*-1/3 and *sn*-2 positions in the TAG to be calculated (14,20–22). Each sample was analyzed four times and averaged results were presented. Automatic software was applied for fragment ion spectra interpretation and regioisomeric structure calculation (16).

Statistical analyses. Results of the analysis of TAG in CB and CBE were evaluated using a one-way ANOVA with a significance level of 0.05 (*P*-values).

RESULTS AND DISCUSSION

The CB sample that had been analyzed in an intercomparison study, in which 13 laboratories had analyzed the TAG composition by GC and 2 laboratories had used HPLC (25), served as a reference. The TAG distribution of the same reference CB was analyzed by ammonia negative ion CI-MS. The results of chromatographic and MS analyses of reference CB TAG are presented in Table 1. The averages of mean values of TAG compositions obtained by the 15 laboratories

TABLE 1
TAG Composition of Reference Cocoa Butter as Analyzed by Ammonia Negative Ion CI-MS and by Chromatographic Methods in an Intercomparison Study of 15 Laboratories[a]

MS analysis[b]		Chromatographic analysis[c]	
TAG ACN/DB	Mol%	TAG	Mol%
48:1	0.2 ± 0.02	MOP	0.2 ± 0.1
48:0	0.1 ± 0.04	PPP + SOO[d]	—
50:2	2.1 ± 0.1	PLP	1.8 ± 0.3
50:1	17.4 ± 0.6	POP	16.4 ± 0.5
50:0	—	PPS	0.6 ± 0.2
52:3	0.6 ± 0.1	PLO	0.3 ± 0.1
52:2	5.9 ± 0.9	POO + PLS[e]	5.2 ± 0.6
52:1	39.8 ± 1.7	POS	40.4 ± 1.3
52:0	—	PSS	0.8 ± 0.3
54:3	0.5 ± 0.4	SLO	0.2 ± 0.2
54:2	4.8 ± 0.9	PPP + SOO[d] SLS + OOO	4.9 ± 0.7
54:1	26.8 ± 1.8	SOS	27.8 ± 1.1
54:0	—	SSS	0.4 ± 0.2
56:2	0.4 ± 0.1	AOO	0.1 ± 0.1
56:1	1.5 ± 0.5	SOA	1.0 ± 0.2

[a]ACN/DB, acyl carbon number/number of double bonds; M, myristic acid (14:0); P, palmitic acid (16:0); L, linoleic acid (18:2); O, oleic acid (18:1); S, stearic acid (18:0); A, arachidic acid (20:0).
[b]Mean ± SD of four replicate analyses.
[c]Overall mean ± SD in an intercomparison study of 15 laboratories in which 13 laboratories analyzed TAG by GC and 2 laboratories used HPLC.
[d]PPP (48:0) and SOO (54:2) co-eluted in chromatographic analyses as did SLS (54:2) and OOO (54:3). For comparison with MS results, the abundances were summed and treated as 54:2.
[e]POO (52:2) and PLS (52:2) were separated in chromatographic analyses. The results were summed for comparison with MS results.

were used to compare the results with M.W. distributions obtained by MS. In MS analysis, TAG are separated according to different MW of molecular species with different ACN/DB ratios, whereas in chromatographic analyses, TAG are separated on the basis of different polarities of molecules possessing different FA combinations. Owing to different analysis methods and different separations of TAG molecular species, a direct comparison of the results was not possible. In chromatographic analyses (Table 1) the TAG PPP (48:0) and SOO (54:2) co-eluted, as did SLS (54:2) and OOO (54:3) [P, palmitic acid (16:0); S, stearic acid (18:0); O, oleic acid (18:1); L, linoleic acid (18:2); SOO denotes a FA combination in TAG, not a stereospecific structure; the same is true for other TAG]. According to MS analysis, 54:2 was the most abundant of these molecular species, whereas 48:0 and 54:3 were minor species. Thus, the abundances determined for these molecular species were added together and treated as a 54:2 species when compared to MS results (Table 1). In addition POO and PLS, both possessing an ACN/DB ratio of 52:2, were separated in chromatographic analyses, whereas in the MS analysis they appeared with the same *m/z* value. Although the proportions of POO and PLS could have been resolved by tandem MS (14,20–22), the proportions of POO and PLS in chromatographic results were added together to enable a direct comparison with MS M.W. distribution data (Table 1). The proportions of the main M.W. species (>2 mol%) in reference CB obtained by MS and the M.W. species compositions calculated using chromatographic results from the intercomparison study (25) are compared in Figure 1. According to our statistical evaluation of the results obtained by different methods, only the proportion of 50:1 differed significantly ($P < 0.05$). Overall, the results corresponded very well, and both methods showed comparable variation in proportions of molecular species between replicate analyses.

Table 2 shows the TAG M.W. distribution of CB and the three CBE that were used to evaluate the suitability of the MS method for identifying foreign fats in CB. The three main M.W. species in CB were 52:1 (39.3 mol%), 54:1 (24.0 mol%), and 50:1 (18.4 mol%). The M.W. distribution of TAG in CBE clearly differed from that in CB, but the same three M.W. species as in CB also formed the majority in the CBE (Table 2). Illexao 30-71 (12.1 mol%) and Coberine (15.0 mol%) had lower proportions of 52:1 than did CB (39.3 mol%), whereas the proportion in illipé fat (39.4 mol%) in this respect was almost identical with CB. The proportion of 54:1 was clearly lower in Illexao 30-71 (17.7 mol%) and higher in illipé (41.5 mol%), whereas in Coberine (27.4 mol%) the proportion was only slightly higher compared to CB (24.0 mol%). Correspondingly, the proportions of 50:1 in Illexao 30-71 (49.1 mol%) and Coberine (37.1 mol%) were clearly higher than in CB (18.4 mol%), whereas in illipé (10.3 mol%) the proportion was lower compared to CB (Table 2). Based on observed differences between CB and CBE in proportions of M.W. species, it was expected that addition of the CBE being investigated could be detected by analyzing the M.W. distribution of TAG. Figure 2 presents the main M.W.

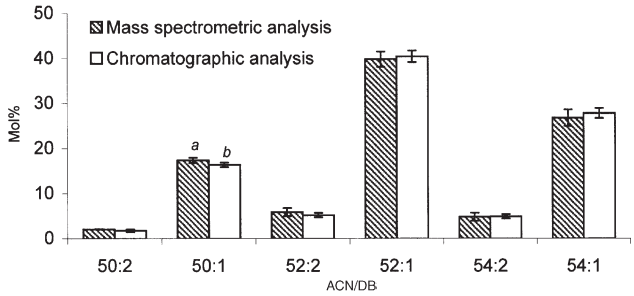

FIG. 1. The proportions of the main M.W. species (>2 mol%) in the reference cocoa butter. Results obtained by ammonia negative ion CI-MS are compared to results obtained by chromatographic methods from an intercomparison study of 15 laboratories. Significant differences ($P < 0.05$) are marked with different letters. See Table 1 for standardization of results for comparison. ACN/DB, acyl carbon number/number of double bonds.

species in mixtures in which 10 or 20% (w/w) of each CBE was added to CB. Because chocolate typically contains around 20–30% CB, these additions corresponded to 2–3% and 4–6% of foreign fat in chocolate. As expected, on the basis of the results presented in Table 2, the addition of Illexao 30-71 caused the proportion of 50:1 in CB to increase and that of 52:1 to decrease (Fig. 2A). The three main M.W. species were analyzed statistically to evaluate the significance of changes in composition ($P < 0.05$). The proportion of 52:1 was significantly different in CB and in CB containing 10 and 20% Illexao 30-71. In the case of 50:1, only the mixture containing 20% Illexao 30–71 was significantly different from CB (Fig. 2A). Although the proportion of 54:1 in Illexao 30–71 was slightly lower than in CB, the proportion of 54:1 was not significantly altered by addition, according to MS analysis (Fig. 2A). When Coberine was added to CB, the proportion of 50:1 and 54:1 was expected to increase and that of

TABLE 2
M.W. Distribution of TAG in Cocoa Butter and in Cocoa Butter Equivalents as Determined by Ammonia Negative Ion CI-MS[a]

	Mol%[b]			
ACN/DB	Cocoa butter	Illexao 30-71	Coberine	Illipé
46:0	—	0.5 ± 0.3	—	—
48:1	—	1.3 ± 0.2	1.0 ± 0.4	—
48:0	—	3.2 ± 0.9	1.3 ± 0.3	—
50:2	2.2 ± 0.2	5.6 ± 0.3	3.2 ± 0.2	—
50:1	18.4 ± 1.2	49.1 ± 1.8	37.1 ± 1.4	10.3 ± 0.6
52:6	—	0.9 ± 0.1	0.6 ± 0.2	—
52:3	0.5 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	—
52:2	5.8 ± 0.5	3.7 ± 0.2	5.0 ± 0.6	1.9 ± 0.2
52:1	39.3 ± 0.7	12.1 ± 1.8	15.0 ± 2.4	39.4 ± 1.1
54:6	1.4 ± 0.2	—	—	0.9 ± 0.2
54:3	0.8 ± 0.1	0.7 ± 0.1	1.5 ± 0.5	—
54:2	5.1 ± 0.2	3.4 ± 0.3	5.3 ± 0.5	2.6 ± 0.7
54:1	24.0 ± 0.7	17.7 ± 1.5	27.4 ± 1.3	41.5 ± 1.0
56:6	0.8 ± 0.04	—	0.6 ± 0.1	0.8 ± 0.1
56:1	1.3 ± 0.2	1.1 ± 0.2	1.3 ± 0.1	2.7 ± 0.3

[a]See Table 1 for abbreviations.
[b]Mean ± SD of four replicate analyses.

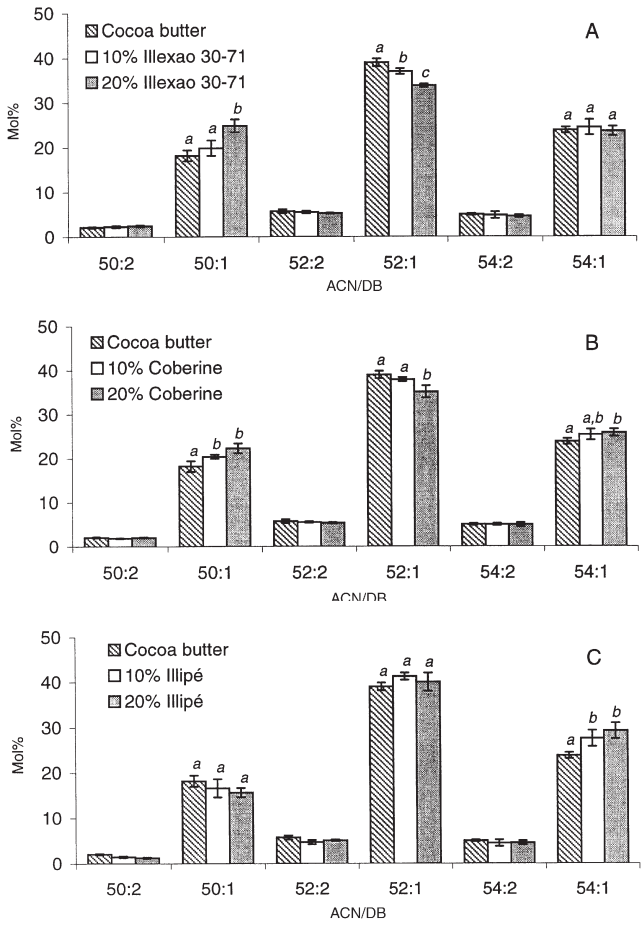

FIG. 2. The main TAG M.W. species (>2 mol%) in cocoa butter and mixtures in which 10 or 20% (w/w) of Illexao 30-71 (A), Coberine (B), or illipé (C) was added to cocoa butter, as analyzed by ammonia negative ion CI-MS. The proportions of the three M.W. species were statistically analyzed; and significant differences ($P < 0.05$) are marked with different letters. See Figure 1 for abbreviations.

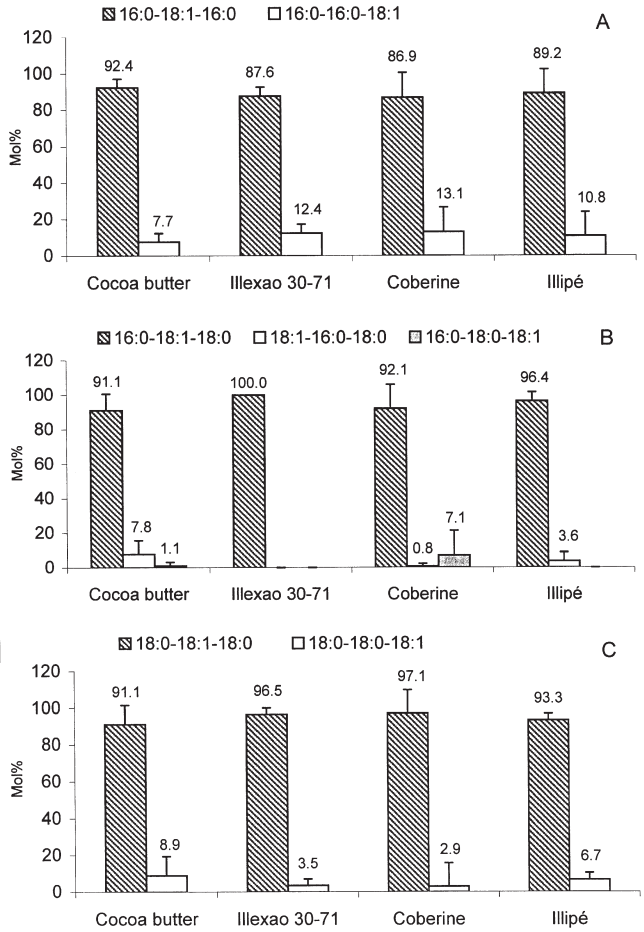

FIG. 3. Regioisomeric structures of ACN/DB 50:1 (A), 52:1 (B), and 54:1 (C) M.W. species in cocoa butter, Illexao 30-71, Coberine, and illipé as analyzed by ammonia negative ion chemical ionization tandem MS. 16:0-16:0-18:1 is used to denote *sn*-16:0-16:0-18:1 + *sn*-18:1-16:0-16:0 and the same is true for other FA combinations. See Figure 1 for other abbreviations.

52:1 to decrease (Table 2). The addition of 10% Coberine caused a significant difference in the proportion of 50:1 but not in proportions of 52:1 and 54:1 (Fig. 2B). The proportions of all three major M.W. species differed significantly from CB in the mixture where 20% Coberine was added, whereas only the proportion of 52:1 differed significantly with the addition of 10% Coberine (Fig. 2B). The composition of illipé fat most closely resembled the composition of CB (Table 2). Addition of 10 or 20% illipé resulted in a significant difference only in the proportion of 54:1, and the two levels of addition did not differ significantly (Fig. 2C).

To further characterize the TAG molecular structures in CB and in the CBE investigated, the regioisomeric structures of the three main M.W. species were determined by ammonia negative ion CI tandem MS. Figure 3 presents the regioisomeric structures obtained for M.W. species 50:1 (A), 52:1 (B), and 54:1 (C) in CB, Illexao 30-71, Coberine, and illipé fat. The results obtained for CB clearly showed the preferential location of oleic acid in the *sn*-2 position in the M.W. species investigated (Fig. 3). This corresponds well with previously published results concerning the overall stereospecific structure of CB TAG (12,13,26,27). In a previous study (26), three fractions from CB TAG were separated by argentation TLC and by HPLC. These fractions were mainly composed of (i) *sn*-16:0-18:1-16:0, (ii) *sn*-18:0-18:1-16:0 + *sn*-16:0-18:1-18:0, and (iii) *sn*-18:0-18:1-18:0 (26). According to stereospecific analysis of the fractions, the proportion of oleic acid in the *sn*-2 position of TAG in each fraction was 92.3, 93.4, and 92.7 mol%, respectively. In the present study, the results obtained by tandem M.S. analysis of corresponding TAG M.W. species were 92.4, 91.1, and 91.1 mol% (Fig. 3), showing strong agreement between the results obtained by the two different methods.

The regioisomeric structure of the M.W. species in the three CBE investigated closely resembled that of CB, with oleic acid forming the clear majority of FA in the *sn*-2 position of TAG in these fats as well (Fig. 3). The regioisomeric

structures of TAG in mixtures of CB and CBE were not investigated, since the regioisomeric structures in the CBE investigated were nearly identical with CB (Fig. 3). Owing to the similarity of regioisomerism in CB and CBE, significant differences between the mixtures and CB were not anticipated. Nevertheless, the tandem MS method proved to be fully applicable for determining the regioisomeric structure of CB TAG and may be used to distinguish foreign fats if differences in regioisomerism occur.

The corresponding separation and quantification of molecular species of reference CB TAG were achieved by ammonia negative ion CI-MS and by chromatographic methods, as shown by comparing the MS results of the present study with chromatographic results obtained in the intercomparison study of 15 laboratories (25). Furthermore, the tandem MS method enabled a fast and reliable determination of regioisomeric structure of TAG M.W. species in CB. Determination of the stereospecific or enantiomeric composition of CB TAG is also possible by chromatographic and enzymatic or chemical degradation methods (26,28). Although these methods enable the *sn*-1/3 positions of TAG to be differentiated, which is not possible by the MS method, analyses often include demanding analysis steps and are time consuming. In contrast, the MS method requires only minimal sample preparation. This may be considered an advantage if a fast and simple method is desired for implementation of the new chocolate directive. As shown in Figure 2, the differences caused by the addition of CBE were minor, leading to the conclusion that MS cannot be used alone to quantify added CBE. In addition, it must be stated that the number of CBE in this study was limited to three, and only one CB reference was used for analysis. CB can differ significantly in their composition depending on the geographical origin. The most promising approach yet to quantify foreign fats in CB is a chemometric evaluation of TAG composition data and compositional data of the unsaponifiable fraction of CB (6). The fast and simple method to produce such TAG compositional data by GC has recently been developed (11). Parallel data may also be produced by the present MS method. The equipment required for a MS analysis is more expensive than that required for chromatographic techniques, and MS also requires expertise of the operators. Nevertheless, MS could be a method of choice if fast analysis of a large number of samples is required. The short analysis time of this method offers a superior advantage over the existing chromatographic techniques presently employed for analyzing TAG. Also, the recent development of fully automated software (16), enabling fast and reliable mass spectral data processing, offers an additional benefit of this methodology.

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