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Detection of Cocoa Butter Equivalents in Chocolate

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ABSTRACT

The fatty acids at the sn-2 position and the sterol composition of cocoa butter and three common cocoa butter equivalents (CBE), namely Coberine, Choclin and Calvetta, were studied comparatively, in order to develop a sensitive method for detecting CBE in chocolate. Differences observed in the composition of saturated fatty acids at position-sn-2 present some interest in detecting CBE in chocolate. Differences found in 4-desmethyl and 4-methylsterol compositions, although quite significant, did not present any practical interest because of the relatively small amounts present in CBE. The 4,4'-dimethylsterol or triterpene alcohol fraction was found to have a potential for determining CBE in chocolate. Thus, the triterpene alcohols of Coberine were further fractionated on argentation thin layer chromatography (TLC) and analyzed by gas liquid chromatography (GLC) and gas chromatography-mass spectrometry (GC-MS). α-Amyrin was found in 48.2% of the triterpene alcohols of Coberine and was absent from cocoa butter. Cycloartenol, the main 4,4'-dimethylsterol of cocoa butter, and α-amyrin were well resolved on an OV-17 glass capillary column.

INTRODUCTION

Cocoa butter equivalents (CBE) are produced from vegetable oils by hydrogenation, fractionation and interesterification and possess chemical and physical properties similar to cocoa butter. The difficulty in determining CBE in cocoa butter is not only their similar composition, but also the natural variation of the composition of cocoa butter itself. Moreover, chocolate fats usually contain, in addition to cocoa butter, milk fat or other permitted fats (e.g. hazelnut oil or other seed oils), the presence of which hinders the determination of the nonpermitted CBE in most European countries. In the United Kingdom, Denmark and Ireland the addition of 5% non-cocoa butter fats, apart from milk fat, is permitted (1).

Among the methods proposed for the determination of CBE or other cocoa butter substitutes in chocolate, the most discussed is the GLC analysis of chocolate fat triglycerides, which are separated according to their carbon number (1,2). However, the sensitivity of the method is 15% in cocoa butter (5% in chocolate), and in the case of milk chocolate additional test procedures must be used to establish the presence of foreign fats, independently of the single triglyceride analysis (3).

The objective of the present study was to develop a sensitive method for the detection of CBE which would be suitable for plain (not containing milk) and milk chocolate. Thus, the triglyceride structure and the sterol composition of cocoa butter and three common CBE's, Coberine, Choclin and Calvetta, were studied comparatively (4).

EXPERIMENTAL

Cocoa butter was extracted from powdered cocoa beans (roasted) deprived of shells and seed buds, in a Soxhlet apparatus with petroleum ether, for 24 hr. The samples of cocoa beans were obtained from chocolate manufacturers in Greece. The samples of prime pressed cocoa butter, Coberine, Choclin and Calvetta were provided by Croklaan b.v., Holland, and the milk powder sample by Ion Co., Greece.

Fatty acid methyl esters were prepared according to AOCS method (5). Determination of fatty acids at position-sn-2 of glycerides was performed according to the modified IUPAC method 2.210 (6). GLC: Tracor 550, stainless steel column EGGS 10% on gas chrom Q 100-120 mesh, 1.8 m × 3 mm i.d.

The fat (100g) in 1000 ml alcoholic 1.0 N potassium hydroxide was refluxed for 1 hr under nitrogen. The mixture was diluted with 2000 ml distilled water and the unsaponifiable matter extracted with three 1000-ml portions of diethyl ether, freshly distilled. The combined extracts were washed five times with 800-ml portions of distilled water, dried over anhydrous sodium sulphate, and the solvent was removed by a rotary evaporator.

Unsaponifiable matter was fractionated on preparative TLC silica G plates with petroleum ether:diethyl ether: formic acid (50:50:0.5, v/v/v). The plate was sprayed with a 0.1% w/v 2',7'-dichlorofluorescein solution in ethanol and observed under UV light. Diethyl ether was used to recover the three sterolic fractions (4-desmethyl, 4-methyl and 4,4'-dimethyl sterols or triterpene alcohols) from the plates. The solvent was removed and the fractions were weighed and analyzed by GLC.

Consequently, the three sterolic fractions were acetylated for 24 hr at room temperature with pyridine + acetic anhydride (1+5ml); an excess of water was poured into the mixture and sterol acetates were extracted with n-hexane.

Sterols and triterpene alcohols and their acetates were analyzed with a Perkin Elmer-Sigma 2 gas chromatograph equipped with FID. The chromatograph was fitted with a 1.8 m glass column, 2mm i.d., packed with 3% OV-17 on gas chrom Z, 80-100 mesh, or a 25 m WCOT glass capillary column OV-17. The temperature and the flow rate of the carrier gas were such that the retention time of sitosterol was 30 min.

The triterpene acetates of Coberine were further fractionated on preparative TLC silica gel G plates impregnated with silver nitrate: the plate was plunged into a solution (about 1000 ml) of 1:1 ethanol:20% w/v silver nitrate in water; after about half a minute the plate was removed, allowed to drain and dried at about 70 C for 15-20 min. The developing solvent was methylene chloride (in the absence of light). The plate was then sprayed with a 2',7'-dichloro-fluorescein solution and observed under UV light. The bands were cut off, extracted with diethyl ether and their components analyzed by GLC and GC-MS. RR_f and RRT were given relative to cholesteryl acetate.

GC-MS-analysis was performed with a Finnigan Mat 44 apparatus. The chromatograph was fitted with a 25m WCOT glass capillary column, SE-54. Operating conditions: column 200 C, helium carrier gas at 2ml/min, emission current 0.7mA ionizing voltage 78eV, and accelerating high voltage 1750V, scanning speed 1 spectrum/sec.

RESULTS AND DISCUSSION

Table I presents the composition % of cocoa butter and CBE fatty acids and saturated fatty acids at position sn-2, i.e, palmitic + stearic acid. The fatty acid composition may be of use for detecting CBE in cocoa butter only in the case of Calvetta, where the ratio of palmitic to stearic acid is 9 times higher than in cocoa butter (7). Saturated fatty acids are esterified almost exclusively at the combined sn-1, 3-positions of cocoa butter triglycerides (8), as shown with many other triglycerides of vegetable origin. In the interesterified CBE, the percentage of saturated fatty acids at position sn-2 in their triglycerides is much higher. Thus, determination of fatty acids at position sn-2 may be of use in detecting CBE in plain chocolate.

Fractionation of the unsaponifiable matter of fats on silica gel layer is usually performed with petroleum ether: diethylether (1:1) by which 4-desmethyl and 4-methylsterols are well separated. However, the 4,4'-dimethylsterol or triterpene alcohol fraction contains aliphatic alcohols and needs further purification before GLC analysis. In this investigation we found that with petroleum ether:diethyl ether:formic acid (50:50:0.5) all the three sterolic fractions were separated satisfactorily.

The tentative assignment shown in Tables II, III and IV was based on the RRT values of the free sterols or triterpene alcohols and of their acetates (relative to cholesteryl acetate) (9).

The 4-desmethylsterol composition of cocoa butter and some CBE was studied previously (7,9). As shown in Table II, the main difference between the desmethylsterol composition concerns the higher percentage of Δ^7 stigmastenol in CBE desmethylsterols. However, the amount of total desmethylsterols in CBE is much smaller in relation to cocoa butter so that GLC analysis of a mixture of 5% Choclin in cocoa butter gives results similar to pure cocoa butter.

The 4-methylsterol composition of CBE was investigated for the first time in this study (Table III). Differences of the 4-methylsterol composition between CBE and cocoa butter were more impressive than those of desmethyl-

TABLE I

Composition % of Fatty Acids and Saturated Fatty Acids at Position sn-2 of Cocoa Butter and CBE

Methylester	Cocoa butter	Coberine	Choclin	Calvetta
Lauric	tr	0.1	0.2	0.1
Myristic	tr	0.1	0.5	0.8
Palmitic	26.5	35.8	39.2	58.5
Palmitoleic	0.3	0.1	0.2	0.1
Stearic	33.5	31.2	22.1	8.5
Oleic	35.4	30.0	33.8	29.1
Linoleic	3.3	1.6	3.4	1.9
Linolenic	0.8	1.0	0.5	0.8
Arachidic	0.2	0.1	0.1	0.2
Saturated fatty acids at position sn-2 (%)[a]	1.7	15.9	16.4	16.7

[a]Palmitic + stearic methylester.

TABLE II

GLC Analysis of Cocoa Butter and CBE 4-Desmethylsterols (%)[a]

Sterol	RRT[b]	Cocoa butter	Coberine	Choclin
Cholesterol	0.75	1.4	–	1.9
Brassicasterol	0.88	tr	–	–
Campesterol	0.99	8.5	15.4	9.5
Stigmasterol	1.09	25.8	7.2	8.7
Sitosterol	1.25	58.4	65.5	49.3
Δ^5-Avenasterol	1.39	4.8	tr	–
Δ^7-Stigmastenol	1.46	1.1	11.9	29.0
Δ^7-Avenasterol	1.65	tr	tr	1.6

[a]OV-17 packed column.
[b]Relative to cholesteryl acetate.

TABLE III

GLC Analysis of Cocoa Butter and CBE 4-Methylsterols (%)[a]

Sterol	RRT[b]	Cocoa butter	Coberine	Choclin
31-Norlanostenol	0.85	4.0	–	0.6
Lophenol	1.03	15.2	1.0	1.2
Obtusifoliol	1.15	13.9	38.7	11.3
31-Norcycloartenol	1.20	3.9	4.7	23.1
Gramisterol and /or cycloeucalenol	1.36	16.9	29.2	54.0
Ethyllophenol and/or unidentified	1.65	8.2	1.8	–
Citrostadienol	1.86	37.9	17.6	9.8
Unidentified	2.00	–	7.0	–

[a]OV-17 packed column.
[b]Relative to cholesteryl acetate.

TABLE IV

GLC Analysis of Cocoa Butter and CBE Triterpene Alcohols (%)[a]

Alcohol	RRT[b]	Cocoa butter n=5, variation	$\bar{X}$	Coberine n=3, $\bar{X}$	Choclin n=3, $\bar{X}$	Calvetta n=1	Milk fat n=1
Lanostenol	1.09	1.6- 2.4	1.9	0.4	0.5	0.8	7.1
Unidentified	1.17	–	–	–	–	–	2.0
Lanosterol	1.29	9.1-11.4	10.4	29.9	30.8	31.9	74.7
β-Amyrin and/or butyrospermol	1.40						10.1
Unidentified	1.47	–	–	–	–	–	6.1
Cycloartenol and/or α-amyrin	1.54	63.2-74.1	69.3	53.4	52.6	51.1	–
Lupeol	1.61	–	–	11.0	11.2	10.4	–
24-Methylene-cycloartanol	1.71	9.0-11.8	10.3	2.4	1.9	3.1	–
Unidentified	1.80	4.2- 6.9	5.7	–	–	–	–
Unidentified	1.90	–	–	2.9	3.0	2.7	–
Cyclobranol and/or unidentified	2.08	1.8- 3.4	2.4	–	–	–	–
Mg of triterpene alcohols/100 g of fat		35		1560	760	41	16

[a]OV-17 packed column.

[b]Relative to cholesteryl acetate.

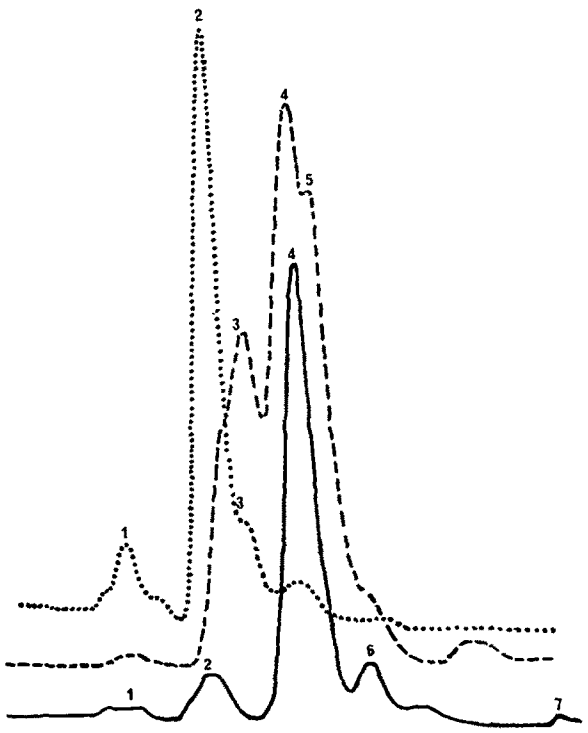

FIG. 1. GLC analysis of triterpene acetates on an OV-17 glass packed column; cocoa butter (—), CBE (Coberine, Choclin and Calvetta) (-----) and milk fat (. . . .). Tentative assignment: lanostenyl 1, lanosteryl 2, butyrospermyl and/or β-amyrin 3, cycloartenyl and/or α-amyrin 4, lupeyl 5, 24-methylene-cycloartanyl 6, and cyclobranyl acetate 7.

sterols. Cocoa butter 4-methylsterol fraction contains a higher percentage of citrostastadienol than the fractions of CBE, which in turn contain higher percentages of obtusifoliol (Coberine) and gramisterol and/or cycloeucalenol (Choclin). However, GLC analysis of a mixture of 5% Choclin in cocoa butter showed the differences are not of practical interest, as they also occur in the case of desmethylsterols.

GLC analysis of the 4-4′-dimethylsterols or triterpene alcohols of a mixture of 5% Choclin in cocoa butter showed, without any doubt, the presence of Choclin. According to these results the triterpene alcohols of the following samples were analyzed: a) four samples of cocoa butter extracted from cocoa beans and one sample of prime pressed cocoa butter; b) three samples of Coberine, three samples of Choclin and one sample of Calvetta, and c) one sample of milk powder fat. The results are shown in Table IV and representative gas chromatograms of the triterpene acetates on an OV-17 packed column in Figure 1.

The differences between the triterpene alcohol composition found in cocoa butter and CBE are significant. The CBE triterpene alcohols have similar composition and differ from those of cocoa butter in that they contain lupeol, higher percentages of β-amyrin and butyrospermol and lower percentages of 24-methylenecycloartanol. The much higher amounts of triterpene alcohols in 100 g of fat mainly in Coberine and Choclin in comparison to cocoa butter indicate the high sensitivity with which these CBE may be detected in cocoa butter (ca 1%). Analysis of both prime pressed cocoa butter and cocoa butter extracted from cocoa beans showed no significant differences in the triterpene alcohol composition. Thus, pressed cocoa butter added to chocolate does not create any problem. The same is true with the triterpene alcohols of milk fat which consist mainly of lanosterol, as is expected in a non-photosynthetic system.

The triterpene alcohols of Coberine (as acetates) were further fractionated by argentation TLC into eight major bands, the components of which were analyzed by GLC and GC-MS. The tentative assignment of the triterpene acetates was based on the RR_f, RRT, mass spectra and literature data (9).

The fraction from band-1 (RR_f 1.36) afforded two component peaks on GLC with RRT 1.66, Ms m/e: 468 (M^+, rel. int. 2%), 453 (M^+-CH_3, 1%), 408 (M^+-HOAc, 1%), 393 (M^+-CH_3-HOAc, 1%), 219 (42%), 218 (100%), 203 (48%), 189 (36%), 175 (12%), and RRT 1.87 Ms m/e: 468 (M^+, 4%), 453 (5%), 408 (1%), 393 (3%), 219 (45%), 218 (100%), 203 (24%), 189 (37%), 175 (18%), which were identified as β- and α-amyrin, respectively (11-13).

Band-2 (RR_f 0.83) gave one triterpenyl acetate with RRT 2.23, Ms m/e: 468 (M^+, rel. int. 51%), 408 (51%), 393 (22%), 326 (5%), 249 (100%), 229 (63%), 218 (46%) which was assigned to ψ-taraxasteryl acetate (13,14).

Band-3 (RR_f 0.75) exhibited four major peaks on GLC with RRT 1.32, Ms m/e: 468 (M^+, rel. int. 21%), 453 (56%), 408 (8%), 393 (100%), 357 (M^+-side chain, 6%), 315 (5%), 301 (12%), 297 (10%), 255 (11%), 241 (25%), RRT 1.56, Ms m/e: 468 (M^+, 30%), 453 (48%), 408 (11%), 393 (100%), 301 (9%), 297 (7%), 295 (5%), 255 (16%), 241 (33%), RRT 1.88, Ms m/e: 468 (M^+, 32%), 453 (20%), 408 (98%), 393 (100%), 365 (50%), 357 (5%), 355 (6%), 297 (48%), 295 (25%), 286 (68%), 270 (48%), 255 (23%), 241 (20%), and RRT 2.29, Ms m/e: 468 (M^+, 39%), 408 (30%), 393 (15%), 326 (10%), 249 (100%), 229 (72%), 218 (63%), which were assigned to euphyl, lanosteryl, cycloartenyl and taraxasteryl acetate, respectively (12,14,15).

Band-4 (RR_f 0.63) gave only one major component with RRT 1.94, Ms m/e: 468 (M^+, rel. int. 6%), 408 (6%), 365 (8%), 281 (6%), 250 (7%), 229 (10%), 219 (8%), 218 (18%), 203 (30%), 190 (60%), 189 (100%), 175 (25%), 173 (20%), which was assigned to lupeyl acetate (13,14).

Band-5 (RR_f 0.43) exhibited two major components with RRT 1.70, Ms m/e: 468 (M^+, rel. int. 11%), 453 (70%), 393 (100%), 355 (19%), 301 (20%), 297 (15%), 295 (19%), 283 (20%), 255 (31%), 241 (28%), and RRT 2.09, Ms m/e: 482 (M^+, 10%), 467 (10%), 422 (45%), 407 (50%), 379 (45%), 357 (12%), 355 (16%), 315 (10%), 301 (45%), 300 (100%), 297 (63%), 295 (25%), 270 (45%), 255 (50%), 241 (47%), which were identified as butyrospermyl and 24-methylene-cycloartanyl acetate respectively (16).

Band-6 (RR_f 0.24) afforded one major component with RRT 1.80, Ms m/e: 468 (M^+, rel. int. 8%), 453 (24%), 408 (5%), 393 (52%), 357 (8%), 355 (100%), 315 (17%), 301 (39%), 297 (23%), 295 (40%), 255 (44%), 241 (59%), which was assigned to parkeyl acetate (16).

Band-7 (RR_f 0.14) also afforded one major component with RRT 1.90 Ms m/e: 482 (M^+, rel. int. 5%), 467 (14%), 439 (12%), 407 (24%), 398 (41%), 355 (100%), 315 (10%), 301 (19%), 295 (22%), 255 (30%), 241 (28%), which was assigned to 4,4',14-trimethyl-24-methylene-5a-cholest-7-en-3β-yl acetate.

Finally, band-8 (RR_f 0.05) afforded two major peaks on GLC with RRT 1.65 Ms m/e: 468 (M^+, rel. int. 5%), 425 (5%), 408 (14%), 393 (24%), 365 (22%), 357 (20%), 299 (100%), 297 (50%), 272 (14%), 257 (45%), 243 (17%), and RRT 1.79, Ms m/e: 482 (M^+, 27%), 467 (12%), 439 (7%), 422 (26%), 407 (13%), 379 (27%), 357 (95%), 299 (100%), 297 (80%), 272 (30%), 257 (48%), 243 (75%), which were assigned to dammaradienyl and 24-methylene-dammarenyl acetate respectively (17).

The results of the triterpene alcohol analysis of Coberine are shown in Table V and compared to similar results obtained with cocoa butter (18). The triterpene alcohols of

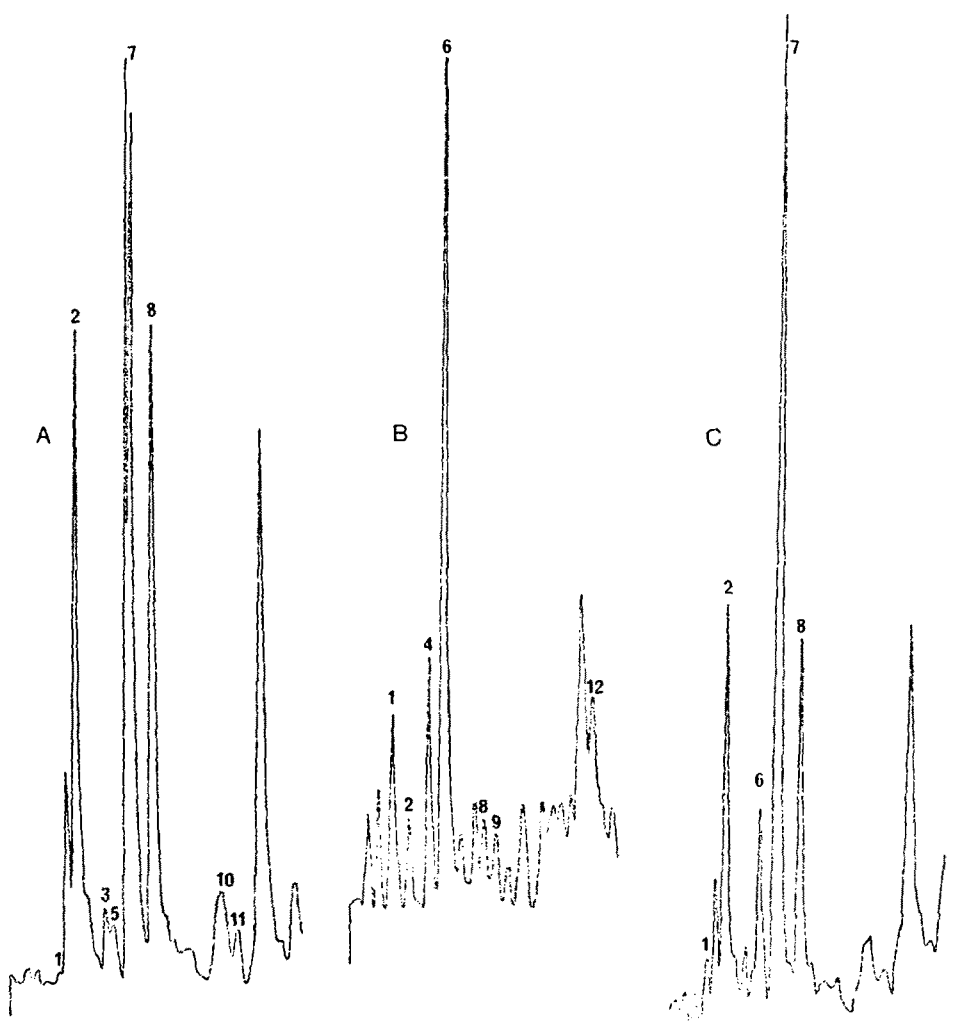

FIG. 2. GLC analysis of triterpene alcohols on an OV-17 glass capillary column; A, Coberine; B, cocoa butter; C, 5% Coberine in cocoa butter. Tentative assignment: lanosterol 1, β-amyrin 2, butyrospermol 3, 24-methylene-lanostenol 4, parkeol 5, cycloartenol 6, α-amyrin 7, lupeol 8, 24-methylene-cycloartanol 9, ψ-taraxasterol 10, taraxasterol 11, and cyclobranol 12.

TABLE V

GLC and GC-MS Analysis of Cocoa Butter and CBE Triterpene Alcohols After Fractionation by Argentation TLC

RRT[a]	Acetate	Percentage (approx.) Cocoa butter	Coberine
1.31	Lanostenyl	2.1	–
1.32	Euphyl	–	0.4
1.57	Lanosteryl	1.5	0.4
1.66	β-Amyrin	1.6	14.9
1.67	Dammaradienyl	–	0.6
1.69	24-Methyl-lanostenyl	0.1	–
1.70	4,4',14-Trimethyl--cholest-7,24(25)-dien-3β-yl	0.6	–
	Butyrospermyl	–	5.0
	Unidentified	0.9	0.4
1.76	24-Methylene-lanostenyl	11.4	–
1.77	Unidentified	3.4	–
1.80	Parkeyl	–	2.3
1.81	24-Methylene-dammarenyl	–	0.7
1.84	4,4'-Dimethyl-24-methylene--cholest-7en-3β-yl	2.0	–
1.87	α-Amyrin	–	48.2
1.88	Cycloartenyl	53.9	0.5
1.90	4,4',14-Trimethyl-24-methylene--cholest-7en-3β-yl	2.8	3.1
1.94	Lupeyl	0.4	19.2
2.08	24-Methylene cycloartanyl Cycloaudenyl	10.5	1.4
2.18	Unidentified	6.0	–
2.28	ψ-Taraxasteryl	–	2.4
2.29	Taraxasteryl	–	0.5
2.34	4,4'-Dimethyl-24-ethylidene--cholest-7en-3β-yl	1.6	–
2.56	Cyclobranyl	1.2	–

[a]Relative to cholesteryl acetate.

Coberine differ substantially from those of cocoa butter: they consist of ca 75% of pentacyclic triterpene alcohols, ca 17% of tetracyclic triterpene alcohols of non-sterolic nature, and ca 8% of 4,4'-dimethylsterols. On the contrary, ca 95% of the triterpene alcohols of cocoa butter are 4,4'-dimethylsterols.

As already noted, detection of CBE in chocolate by using packed columns can be based on the presence of β-amyrin, butyrospermol and lupeol. However, a better characteristic is α-amyrin which makes up 48% of the triterpene alcohols of Coberine and is completely absent from cocoa butter. α-Amyrin and cycloartenol, the latter being present in high proportions in cocoa butter triterpene alcohols, are not resolved on packed columns. Thus, the free 4,4'-dimethylsterols or triterpene alcohols of cocoa butter, Coberine and a mixture of 5% Coberine in cocoa butter were analyzed on an OV-17 glass capillary column. The three chromatograms obtained are shown in Figure 2. α-Amyrin and cycloartenol are quite satisfactorily resolved on the capillary column, and the presence of Coberine is obvious.

Work is in progress with other inadmissible fats used as cocoa butter substitutes and other permitted fats (e.g. hazelnut oil) for the final formulation of the method.

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Erratum

An incorrect address was given in the April 1985 *JAOCS* (62:745) for a co-author of the paper "Determination of Ascorbyl Palmitate by High Performance Liquid Chromatography." The proper current address for W. M. Cort is: Cort Consultants, 4395 Brandywine Drive, Sarasota, FL 33583.