Stereospecific Analysis of Monounsaturated Triacylglycerols in Cocoa Butter

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ABSTRACT: The enantiomeric composition of the monounsaturated triacylglycerols (TG) from cocoa butter was estimated. The monounsaturated TG were separated into three fractions by reversed-phase high-performance liquid chromatography (HPLC), and each fraction was subjected to the stereospecific analysis with chiral-phase HPLC. The results indicated that the major TG consisted of equal amounts of 1-stearoyl-2-oleoyl-3-palmitoyl-*sn*-glycerol (SOP-*sn*-TG) and POS-*sn*-TG (47 mol%), 1,3-distearoyl-2-oleoyl-glycerol (SOS-TG) (33 mol%), and POP-TG (19 mol%). The contents of SOP-*sn*-TG and POS-*sn*-TG are 1.30 times that of the POP-TG content, and the SOS-TG content is 1.30^2 times that of the POP-TG content. The term "priority factor" is proposed for the ratio of the stearoyl group/palmitoyl group, 1:30 at the *sn*-1 and *sn*-3 or 1(3)-position. It shows a distinct specificity for particular fatty acids or their Coenzyme A esters in random esterification at each position of the glycerol moiety in the biosynthesis of cocoa butter TG.

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KEY WORDS: Chiral-phase high-performance liquid chromatography, cocoa butter, priority factor, stereospecific analysis, triacylglycerols.

The enantiomeric composition of the molecular species in the triacylglycerols (TG) of natural fats usually cannot be calculated from the stereospecific distribution data of the acyl groups in the TG. Stereospecific analysis of the acyl groups in natural TG gives only indirect information on the enantiomeric structure of TG molecules because the TG in natural fats have complex compositions based on the distribution of acyl groups on the glycerol backbone. In the present study, the enantiomeric composition of monounsaturated TG in cocoa butter was estimated by stereospecific analysis of three TG fractions that were separated from the monounsaturated TG by reversed-phase high-performance liquid chromatography (HPLC). The stereospecific analysis of TG was carried out by a chiral-phase HPLC method (1,2) with minor amounts of TG (3). Stereospecific analysis of the major TG species in the monounsaturated fraction of cocoa butter by partial hydrolysis at a specific position or specific acyl group by pancreatic lipase, *Geotrichum candidum* lipase, and phospholipase A_2 was reported by Sampugna and Jensen (4). In the method used in this paper, the procedures are simpler and easier to conduct, and the compositions obtained are comparable with the previous methods. Moreover, the data obtained by this method can be used to relate the lipid composition and hardness characteristics of cocoa butter.

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MATERIALS AND METHODS

Materials. Raw cocoa butter, yellowish solid at 20°C, was obtained by extraction of unroasted cocoa beans with chloroform in a homogenizer. The TG fraction was separated from other lipids by column chromatography on Kiesel Gel 60 (Merck, Darmstadt, Germany) with chloroform for elution and, subsequently, by preparative thin-layer chromatography (TLC) on Kiesel Gel 60G plates (20 × 20 cm, 0.5 mm thickness; Merck) with *n*-hexane/diethyl ether (85:15, vol/vol) for development.

Argentation TLC. Silicic-acid plates (20 × 20 cm, 0.25 mm thickness) were immersed in 10% $AgNO_3$–acetonitrile solution for 20 min. After air-drying, the plates were activated by heating at 110°C for 1 h (5). The refined cocoa butter TG (80 mg) was spotted onto eight plates as chloroform solution (0.5 mL) and developed with benzene/chloroform/acetic acid (90:10:1, vol/vol/vol). Bands were detected by spraying with 2,7-dichlorofluorescein solution, exposed to ultraviolet (UV) light, and the TG were extracted with diethyl ether.

Argentation HPLC. The silver-ion column for HPLC was prepared by the method of Christie (6). HPLC was done with a Shimadzu LC 6A isocratic pump (Shimadzu Co., Kyoto, Japan) and a Hitachi L-4200 UV detector (Hitachi Co., Tokyo, Japan). A column of Nucleosil 5SA (25 cm × 4 mm i.d.; Macherey-Nagel, Duern, Germany) was flushed with distilled water at a flow rate of 0.5 mL/min for 1 h, then with 1% w/w aqueous ammonium nitrate solution at 0.5 mL/min for 1 h, and distilled water at a flow rate of 1.0 mL/min for 1 h. Silver nitrate (0.2 g) in distilled water (1.0 mL) was injected onto the column *via* a Rheodyne Model 7125 loop (20 μL) valve (Rheodyne, Inc., Cotai, CA) in 20-μL aliquots at 30-s intervals. The column was washed with methanol for 1 h, then with 1,2-dichloroethane/dichloromethane (1:1, vol/vol) at a

flow rate of 0.3 mL/min for 1 h prior to analyses. HPLC of the refined cocoa butter TG (15 mg) was done with *n*-hexane/diethyl ether (350:1, vol/vol) as mobile phase at a flow rate of 0.5 mL/min at ambient temperature. Detection was at 220 nm. The separation was repeated 15 times, and the monounsaturated fraction was collected and pooled.

Reversed-phase HPLC. HPLC was done isocratically with the same instrument used in the argentation HPLC and a Supelcosil LC-18 column (25 cm × 4.6 mm i.d.; Supelco, Bellefonte, PA) with acetonitrile/tetrahydrofuran (7:3, vol/vol) (7) as mobile phase at a flow rate of 1.0 mL/min at ambient temperature. Detection was at 220 nm. Aliquots of 4 mg monounsaturated TG fraction in 20 μL acetonitrile/tetrahydrofuran (1:1, vol/vol) were injected 15 times on the column. Three peaks emerged and each peak was collected and used for the stereospecific analysis of TG.

Partial hydrolysis of TG. Stereospecific analysis of the acyl groups in the TG fractions was carried out by the method of Takagi and Ando (2) with some modifications for the analysis of minor amounts of TG (3). The first step, partial hydrolysis of TG, was carried out by the modified Brockerhoff procedure (2,3). The products obtained by the partial hydrolysis of the TG (10–20 mg) with ethyl magnesium bromide were immediately dissolved in dry toluene (1 mL) and then reacted with 50 mg 3,5-dinitrophenyl isocyanate in the presence of dry pyridine (0.1 mL) overnight at ambient temperature. Fractionation of the products by preparative TLC on Kiesel Gel 60G plates (0.25 mm thickness) with chloroform/acetone (96:4, vol/vol) gave di-3,5-dinitrophenylurethane (3,5-DNPU) derivatives of the 1- and 2-monoacylglycerols (MG). Their complete separation was confirmed by HPLC on silicic acid (3).

Chiral-phase HPLC. Chiral-phase HPLC of the 1-MG derivatives was conducted isocratically in the same instrument used in the argentation HPLC and two Sumichiral OA-4100 (25 cm × 4 mm i.d. columns; Sumika Chemical Analysis Service, Osaka, Japan), connected in series, with *n*-hexane/1,2-dichloroethane/ethanol (40:12:3, vol/vol/vol) as mobile phase at a flow rate of 0.5 mL/min at −10°C. Detection was at 254 nm (2).

Gas–liquid chromatography (GLC). The 1-MG 3,5-DNPU were converted to fatty acid methyl esters by the procedures described in a previous paper (2). GLC analyses of the methyl esters were done with a Shimadzu GC 9A or 6AM instrument and a fused-silica column coated with Silar 5CP (50 m × 0.25 mm i.d., 0.2 μm film thickness). Detection was done by flame-ionization detection. Peak area integration was done with a Shimadzu C-R6A integrator. Other conditions are as described in a previous paper (2).

RESULTS AND DISCUSSION

Analysis of total and monounsaturated TG. The stereospecific distribution of fatty acids in the total TG of cocoa butter is given in Table 1. Palmitic and stearic acids are located mainly at the *sn*-1 and *sn*-3 positions. Oleic and linoleic acids are located primarily at the *sn*-2 position. A small amount of arachidic acid was found at the *sn*-3 position. Thus, the composition shown in Table 1 is in accord with the acyl distribution pattern of cocoa butter TG reported by Brockerhoff (8). The TG of ordinary seed oils contain the polyunsaturated fatty acids, such as linoleic acid in the *sn*-2 position, but saturated fatty acids are enriched at the *sn*-1 and *sn*-3 positions, and oleic acid is evenly distributed at the *sn*-1, *sn*-2, and *sn*-3 positions. In contrast, oleic acid is found largely at the *sn*-2 position of cocoa butter TG (9). A similar tendency was found in this study (Table 1). In the early stages of cocoa seed maturation, 105 d postanthesis (dpa), the total fatty acids contained 3 mol% of linolenic acid, but it decreased to trace amounts (<0.5 mol%) at 130 dpa (10). In this study, the *sn*-2 position contained 0.4 mol% linolenic acid. The monounsaturated TG were separated from the total TG by argentation TLC and then argentation HPLC, and the stereospecific analysis of the monounsaturated TG fraction was carried out by chiral-phase HPLC (2,3). The results are given in Table 1. Palmitic and stearic acids are located predominantly at the *sn*-1 and *sn*-3 positions, oleic acid exclusively at the *sn*-2 position, and a minor amount of arachidic acid is at the *sn*-3 position. Table 1 shows that the fatty acid distribution is simple, and it suggests that 1,3-dipalmitoyl-2-oleoyl-glycerol (POP-TG), 1,3-distearoyl-2-oleoyl-glycerol (SOS-TG), 1-stearoyl-2-oleoyl-3-palmitoyl-*sn*-glycerol (SOP-*sn*-TG), and 1-palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol (POS-*sn*-TG) are the major components. However, it is still difficult to calculate the percentage of each component from the data of Table 1. Therefore, the monounsaturated TG fraction was further fractionated by reversed-phase HPLC, and the fractions obtained were subjected to stereospecific analyses.

TABLE 1
Stereospecific Analysis of Cocoa Butter Triacylglycerols and Its Monounsaturated Fraction

		Composition (mol%)[a]		
Acyl group	Total	*sn*-1	*sn*-2	*sn*-3
Total triacylglycerols				
16:0	26.0	36.9	2.5	40.2
16:1n-9	0.2	0.2	0.2	0.3
18:0	35.3	52.8	1.9	52.4
18:1n-9	34.3	9.1	87.5	3.9
18:2n-6	3.0	0.7	7.4	0.6
18:3n-3	0.2	ND[b]	0.4	ND
20:0	1.0	0.4	ND	2.7
Monounsaturated fraction[c]				
16:0	28.7	42.1	3.8	41.7
18:0	37.6	54.8	3.3	54.4
18:1n-9	33.3	3.2	92.9	2.8
20:0	0.4	ND	ND	1.1

[a]Wt% was calculated from peak area % by using the calibration factors obtained from gas–liquid chromatography of reference mixtures. Mol% was calculated from wt% and the molecular weight of each component.
[b]ND, not detected.
[c]Separated from total triacylglycerols by argentation thin-layer chromatography and high-performance liquid chromatography (yield 81.38).

Enantiomeric composition of monounsaturated TG. The monounsaturated TG were clearly resolved to the three peaks.

The three peaks had retention times of 31.7, 39.1, and 49.3 min. Peak resolution was 6.3 between peaks 1 and 2 and 6.1 between peaks 2 and 3. Table 2 lists the fatty acid distribution for each fraction obtained by the stereospecific analysis method. The abbreviation A is used for the eicosanoyl group. In reversed-phase HPLC, the relative retention time of a lipid component has been shown in terms of an equivalent carbon number (ECN), defined as the number of carbon atoms in the aliphatic residues (*CN*) less twice the number of olefinic bonds (*n*) per molecule, i.e.:

$$ECN = CN - 2n \quad [1]$$

where the carbons of the glycerol moiety are not counted for *CN*. TG components with the same ECN tend to elute together in HPLC (11). The position of the fatty acids within the TG molecules has no effect on the separation in this instance. The retention times of peaks 1, 2, and 3 agreed with those of the TG reference materials, tripalmitin (ECN 48), dipalmitomonostearin (ECN 50), and monopalmito-distearin (ECN 52), respectively. It indicated that the ECN of peaks 1, 2, and 3 are 48, 50, and 52, respectively. These ECN and the acyl distribution in TG shown in Table 2 indicate that peak 1 mainly contains POP-TG, peak 2 contains nearly equal amounts of POS-*sn*-TG and SOP-*sn*-TG, and peak 3 contains SOS-TG as the major component, with 1-palmitoyl-2-oleoyl-3-eicosanoyl-*sn*-glycerol (POA-*sn*-TG) as a minor component.

Table 3 shows the enantiomeric composition of the monounsaturated TG as estimated from the data in Table 2. When the TG percents are calculated from the data of Table 2, the values whose RN values were less than 4% in Table 2 were excluded. In this paper, RN percent is defined as the relative percent of the minor desired acyl group combined with the predominant neighboring acyl group. For example, the RN percent of 16:0 at *sn*-2 in peak-1 fraction in Table 2 is 3.8,

TABLE 2
Stereospecific Analysis of Each Peak Fraction Separated from Total Monounsaturated Fraction[a]

Acyl group	Total	Acyl group mol% and SD		
		sn-1	*sn*-2	*sn*-3
Peak 1 (18.2%)[b]				
16:0	66.4	95.8 ± 1.4[c]	7.3 ± 0.2	96.1 ± 1.8
18:0	0.4	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
18:1n-9	33.2	3.2 ± 0.3	92.3 ± 1.5	3.4 ± 0.1
Peak 2 (46.8%)				
16:0	33.1	48.7 ± 1.0	3.2 ± 0.1	47.9 ± 0.8
18:0	33.5	47.6 ± 0.9	3.4 ± 0.1	48.9 ± 1.2
18:1n-9	33.4	3.6 ± 0.1	93.4 ± 1.7	3.1 ± 0.1
Peak 3 (35.0%)				
16:0	1.6	4.0 ± 0.2	0.4 ± 0.0	0.3 ± 0.0
18:0	64.5	93.5 ± 1.2	6.9 ± 0.2	92.9 ± 1.5
18:1n-9	32.4	2.5 ± 0.1	92.7 ± 1.8	3.0 ± 0.1
20:0	1.5	ND[d]	ND	3.8 ± 0.2

[a]Fractions 1–3 were separated from the monounsaturated fraction in Table 1 by reversed-phase high-performance liquid chromatography.
[b]Yields (wt%) of each fraction by weighing.
[c]Mean value and SD in triplicate analyses.
[d]ND, not detected.

TABLE 3
Estimated Composition of the Monounsaturated Triacylglycerols

Triacylglycerol	Peak[a]		Composition (mol%)	
	Number	mol%	Each peak	Total
POP-TG	1	18.9	100	19
POS-*sn*-TG[b]	2	47.1	51	24
SOP-*sn*-TG			49	23
SOS-TG	3	34.0	96.2	32.7
POA-*sn*-TG[c]			3.8	1.3

[a]Peaks separated by reversed-phase high-performance liquid chromatography.
[b]POP-TG, 1,3-dipalmitoyl-2-oleoyl-glycerol; POS-*sn*-TG, 1-palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol; SOP-*sn*-TG, 1-stearoyl-2-oleoyl-3-palmitoyl-*sn*-glycerol; SOS-TG, 1,3-distearoyl-2-oleoyl-glycerol; POA-*sn*-TG, 1-palmitoyl-2-oleoyl-3-eicosanoyl-*sn*-glycerol.
[c]A, eicosanoyl.

which is obtained by the following calculation: [7.3/(95.8 + 96.1)] × 100. The RN percent of 18:1n-9 at *sn*-1 in the peak-1 fraction is 3.5, and is obtained by the following calculation: [3.2/92.3] × 100. The selection of less than 4 RN% for excluding a given acyl group is based on the discussion of acyl migration during the course of the analysis. The mole percent of POS-*sn*-TG 51 in Table 3 was obtained from 48.7 mol% for P at *sn*-1 and 48.9 mol% for S at *sn*-3 in Table 2 as the averaged values, and the mol% of SOP-*sn*-TG 49 in Table 3 was similarly obtained from 47.6 mol% for S at *sn*-1 and 47.9 mol% for P at *sn*-3 in Table 2. The proportional allotment was used to make the sum 100 mol% in the last step of the calculation.

Acyl migration in the course of analysis. Acyl migration in the course of analysis can affect the accuracy of the method. The accuracy of our method was examined by analysis of two synthetic TG, 1,3-dioleoyl-2-stearoyl-glycerol and 1,3-dipalmitoyl-2-linolenoyl-glycerol, in our previous paper (2). The results showed that the *sn*-1 and *sn*-3 positions can be contaminated with 2.5–3.0 mol% (the same for RN%) of acyl groups that migrated from the *sn*-2 position. On the other hand, the *sn*-2 position can be contaminated with 4–6 mol% (2.3 RN%) of acyl groups that migrated from the *sn*-1 or *sn*-3 position. These results demonstrate the low extent of isomerization of MG under the conditions of analysis (2). The stereospecific analysis of a few natural TG also gave other evidence for the low extent of isomerization of MG. Yew seed oil TG have *cis,cis*-5,9-18:2 (50.0 mol%) at the *sn*-3 position, but only 1.7 mol% (3.4 RN%) of 5,9-18:2 at the *sn*-2 position. Rapeseed oil TG showed linoleic and linolenic acids, 39.3 and 21.3 mol%, respectively, at the *sn*-2 position, but each was found only 1.2 mol% at the *sn*-1 and 1.3 mol% at the *sn*-3 position (2). Taylor and co-workers (12,13) used our method (2) in the stereospecific analysis of the seed TG from high-erucic acid (22:1n-9) Brassicaceae. The TG from three genotypes of *Brassica napus* seeds showed 22:1n-9 in concentrations of 42–58 mol% and 53–64 mol% in the *sn*-1 and the *sn*-3 positions, respectively, but 22:1n-9 was only 2.1–4.4 mol% (2.0–3.7 RN%) at the *sn*-2 position. The same oils showed linolenic acid at a concentration of 20–22 mol% at the *sn*-2 position, but linolenic acid was not found at the *sn*-3 position. These data support our assumption that acyl migra-

tion is minimal in our method of analyses. From that data, the minor percents (<4 RN%) in Table 2 are not representative for the percent of acyl groups in the original TG. To obtain a reliable measure of the original TG that contain acyl groups with minor RN%, a more accurate analytical method with less acyl migration must be developed.

Major biosynthetic pathway for TG. Plant TG biosynthesis proceeds through the glycerol 3-phosphate pathway (14). The *sn*-3-glycerol-phosphate, produced by the action of the enzyme glycerol kinase on free glycerol, is acylated sequentially by specific acyltransferases in these steps: (i) at position *sn*-1 by glycerol phosphate acyltransferase; (ii) at *sn*-2 by lysophosphatidate acyltransferase to form phosphatidic acid; and (iii) the phosphate group is removed by phosphatidate phosphatase, and the resultant diacylglycerol is acylated to TG by diacylglycerol acyltransferase. Each of the enzymes that catalyze steps i–iii can have preferences for the coenzyme A esters of particular fatty acid combinations in the intermediates. The acylation at *sn*-1 occurs mainly by the priority factor S/P 1.30, that at *sn*-2 mainly by oleic acid, and that at *sn*-3 nearly by the same S/P (Table 4). The data in Table 4 show that the saturated acyl group that occupied the *sn*-1 position does not influence the selection of the saturated acyl group in its introduction to the *sn*-3 position. In steps i and iii in the biosynthesis of TG, total introduction of stearoyl–CoA is 1.30 times that of palmitoyl–CoA, depending upon the priority factor. Because acylation of the stearoyl group occurs twice in the formation of SOS-TG, its content is expected to be 1.30^2 times that of the POP-TG content. The agreement between measured and calculated values in Table 4 supports the idea of the priority factor, which can be used for comparing TG compositions.

TABLE 4
Calculation of the Monounsaturated Triacylglycerol mol% by the Priority Factor

	Esterified acyl groups and products[a]			
sn-1	P[b]		S	
sn-2	+O		+O	
sn-DG[c]	PO-*sn*-DG		SO-*sn*-DG	
Ratio[d]	1.00		1.30	
sn-3	+P	+S	+P	+S
sn-TG[e]	POP-TG	POS-*sn*-TG	SOP-*sn*-TG	SOS-TG
Ratio[f]	1.00	1.30	1.30	1.30^2
mol% Calcd[g]	19	25	25	32
Found[h]	19	24	23	33

[a]Esterified acyl groups, products and their ratios, and the comparison of triacylglycerol compositions calculated and found.
[b]Esterification at the *sn*-1 position.
[c]Esterified *sn*-1,2-diacyl intermediates.
[d]Ratio of *sn*-1,2-DG expected by the priority factor.
[e]Esterified triacylglycerol products.
[f]Ratios of triacylglycerol expected by the priority factor.
[g]Obtained with multiplication, 19 (POP-TG mol%) by 1.30 for POS-*sn*-TG and SOP-*sn*-TG, and by 1.30^2 for SOS-TG. Abbreviations as in Table 3.
[h]Data as shown in Table 3.

Racemic structures of POS in cocoa butters. Shlenk (15) described that POS-TG from cocoa beans is racemic because its X-ray diffraction data are identical with those of synthetic POS-TG and grossly different from those of a POS-TG enantiomer. Sampugna and Jensen (4) reported that the major triacid monounsaturated TG in cocoa butter is racemic POS-TG by stereospecific analysis of the TG by the enzymatic method. In this paper, the racemic structure of POS-TG in a sample of cocoa butter was concluded by analytical techniques.

Chaiseri and Dimick (16) recently reported lipids and hardness characteristics of cocoa butters from different geographic regions. In general, cocoa butters from Asia and Oceania were hardest, those from South America were softest, and those from North and Central America and from Africa were intermediate. Soft cocoa butters were characterized by high 1-palmitoyl-2,3-dioleoyl-glycerol, high 1-stearoyl-2,3-dioleoyl-glycerol, and low SOS-TG contents. Hard cocoa butters have opposite TG compositions (16). Further investigations with the methods presented by this paper must be applied to other samples of cocoa butters to reach a general conclusion whether POS-*sn*-TG and SOP-TG in cocoa butters are racemic or not.

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