Lipid Composition of High-Melting Seed Crystals Formed During Cocoa Butter Solidification

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High-melting seed crystals which form during the early stages of cocoa butter solidification possess a lipid composition different than the cocoa butter from which the seed crystals were grown. Significantly large quantities of glycolipids, 11.1%, and phospholipids, 6.6-8.1%, were found in the high-melting seed crystals along with a dramatic decrease in the simple lipid class. The fatty acids comprising the simple lipid fraction of the seed crystals were considerably more saturated than the fatty acids present in the same fraction of the original cocoa butter. The increase in the degree of saturation was reflected in the triacylglycerol composition. Cocoa butter samples were predominantly monounsaturated triacylglycerols while the seed crystal samples were mainly trisaturated triacylglycerols. The elevated melting point (60-70°C) of the seed crystals was due to the presence of higher melting complex lipids as well as to the increase in saturated triacylglycerol species. As a result of the evidence provided, the high-melting seed crystal is indeed a distinct crystalline entity and not an additional polymorphic form of cocoa butter.

The mechanism involved in the solidification of cocoa butter is an interesting but controversial area of confectionery science. Researchers have investigated the issue through numerous approaches involving different aspects of the crystallization phenomenon, including thorough studies on tempering, polymorphism, molecular or physical packing as well as studies aimed at the prevention and control of fat bloom formation. Limited studies (1) have addressed seed crystal formation on a compositional basis and involved the determination of the triacylglycerol distribution of the "bow-tie" crystal. The "bow-tie"crystals possess melting points of approximately 40°C and were rich in the triacylglycerol SOS in comparison to pure cocoa butter. The objective of this research is to determine the composition of the high melting seed crystals (2) formed during the early stages of cocoa butter solidification. Compositional analysis should provide information which may lead to a better understanding of the mechanism of cocoa butter solidification.

MATERIALS AND METHODS

Chemicals and reagents. Acetone, acetonitrile, chloroform, ethyl ether, hexane, and methanol were HPLC grade obtained form Fisher Scientific, while all other solvents were certified A.C.S. grade from Fisher Scientific. All other chemicals and reagents used in these studies were reagent grade from either Aldrich Chemical Company or Fisher Scientific unless otherwise noted. Water was distilled and deionized prior to use. Lipid standards were obtained in the purest available form from Supelco, Inc. (Bellefonte, PA) and/or Sigma Chemical Company (St. Louis, MO). Silicic acid was obtained from Mallinkrodt Analytical Reagents.

Samples. Seed crystals characterized in this study were obtained via static incubation of Ivory Coast cocoa butter melts at 26.5°C, and isolated via centrifugation as described previously (2). A 6:1 sample of Ivory Coast cocoa butter was divided into three equal aliquots. Each aliquot represented a sample (replicate) of the Ivory Coast cocoa butter. On consecutive days, seed crystals were grown from one of the three samples. The experimental sequence was performed to account for any daily variations which could have occurred in the solidification process. Hence, triplicate runs of seed crystal growth were conducted. For compositional analyses, each sample, whether seed crystal or cocoa butter, was divided into two subsamples and analyzed in duplicate. Melting point determinations of the isolated seed crystals were performed using differential scanning calorimetry (DSC) immediately following the isolation of the seed crystals. Scans were conducted from 0 to 110°C at a programmed heating rate of 20 deg/min.

Chromatographic separations. Pure cocoa butter samples and seed crystal samples were separated into simple lipid, glycolipid, and phospholipid fractions by silicic acid column chromatography (3). This chromatography was conducted so that compositional analysis of the individual lipid classes could be performed in addition to determining the quantitative distribution of lipid classes by gravimetric analysis.

Separation and qualitative identification of the lipid components present in cocoa butter and seed crystal samples were achieved by thin-layer chromatography (TLC) on Eastman Kodak silica gel analytical layers (100 μ thickness), or Alltech Associates Adsorbasil hard layers (200 μ thickness). Separation was achieved by developing the plates in a mixture of toluene/ethyl ether/ethyl acetate/acetic acid, 80/10/10/0.2 (v/v/v/v) (4). Plates were air dried and compounds visualized by staining with iodine vapor and/or by charring with a 50% sulfuric acid/0.5% potassium dichromate spray followed by heat treatment in a 160°C oven for 15 min. Phospholipids were positively identified by molybdenum blue spray (5).

Compositional assays. The phospholipid content of cocoa butter and seed crystals was quantitated by a phosphorus micromethod (6). Lipid samples, 0.5-5.5 mg, were analyzed for total phosphorus content following digestion, formation of a phosphomolybdic acid complex, and by measurement of the resulting color at 822 nm. The amount of inorganic phosphate present in each sample was determined by interpolation from a standard curve. The phosphorus value was multiplied by 25 to represent the concentration of phospholipid in the sample (7).

Fatty acid composition of pure cocoa butter, seed crystals, and their purified simple lipid, glycolipid and phospholipid fractions was determined by gas-liquid chroma-

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tography (GLC). Fatty acid methyl ester derivatives were prepared by acid-catalyzed esterification and transesterification of the free fatty acids and the O-acyl lipids, respectively (7). The methyl esters were separated by GLC on a 1.8 m x 2 mm i.d. glass column packed with GP 10% DEGS PS on 80/100 Supelcoport (Supelco, Inc., Bellefonte, PA). Separations were conducted isothermally at 180°C on a Hewlett Packard model 5730A gas chromatograph equipped with a flame ionization detector. Area integrations were performed by a Hewlett Packard model 3385A integrator. Standards were subjected to identical treatment and used for qualitative and quantitative purposes.

Triacylglycerol compositions of the simple lipid fractions of pure cocoa butter and seed crystals were determined by high performance liquid chromatography (HPLC) (1). Analyses were performed using a Perkin-Elmer model 410 LC pump and SEC-4 solvent environmental control chamber in conjunction with a Waters Associates model 401 differential refractometer detector. Acetonitrile/chloroform, 60/40, (v/v) was employed as the mobile phase and separation was achieved by pumping the solvent at 0.7 ml/min through an Alltech Associates C-18, 5 μ reversed phase column (25 cm x 0.46 cm i.d.). Internal spiking with standards was employed to identify the triaclyglycerols, POO, POP, SOO, POS, SOS, and SSS, in both cocoa butter and seed crystal chromatograms.

RESULTS

The melting points for three samples of seed crystals grown on successive days ranged from 58.82 to 59.91°C with a mean of 59.43°C ± 0.79°C SD. Similarities in melting point determinations and crystallization times indicate the solidification process for this particular cocoa butter was relatively uniform with respect to daily variation in solidification conditions. Seed crystals formed after six hours static incubation at 26.5°C displayed a melting point of slightly less than 60°C and a solids content greater than 95%, as determined by DSC. Thin-layer chromatographic analysis of the isolated seed crystal and cocoa butter samples indicate that the high-melting seed crystals possess a unique lipid composition (Fig. 1). Visually, the seed crystals appear to possess a substantial amount of complex lipid material as noted by the intense staining at the origin (A) in comparison to the cocoa butter from which the seeds were grown. Small amounts of free fatty acids (B) were observed (C) and diacylglycerols (D,E) were observed in the seed crystal samples when stained with iodine vapor. Based on the fact that saturated species do not readily stain with iodine vapors, the seed crystals possess highly saturated triacylglycerols. Unsaturated species such as those found in cocoa butter readily stain with iodine vapor. The noticeable difference in the intensity of iodine staining of the triacylglycerol fractions of the two samples establishes a relatively high degree of saturation for the seed crystal triacylglycerols.

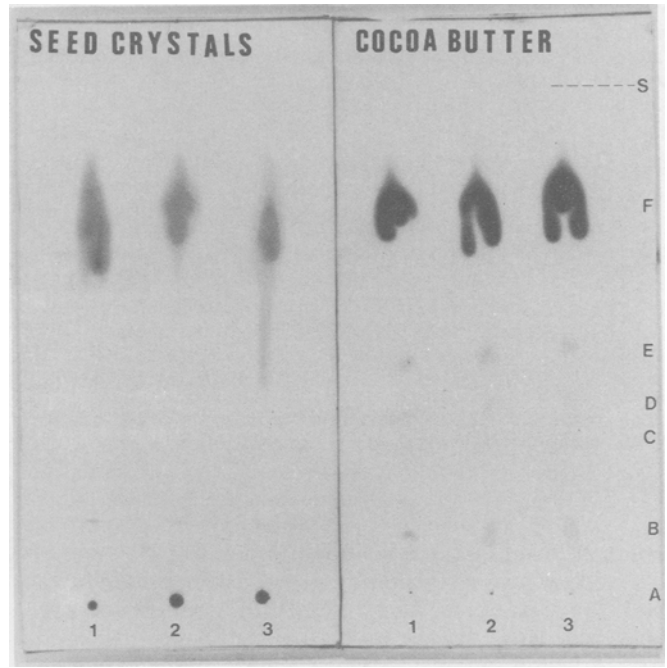

FIG. 1. Thin-layer chromatographic separation of lipid components present in seed crystals and Ivory Coast cocoa butter. Subscript numbers donate sample replicates. Letters denote lipid class as identified by standards. A) complex lipids, glyco- and phospholipids, B) free fatty acids, C) sterols, D) 1,2-diacylglycerolds, E) 1,3-diacylglycerols, F) triacylglycerols, S) solvent front. Compounds were visualized by staining with iodine vapor.

The quantitative distribution of the major lipid classes—simple lipids, glycolipids, and phospholipids—

TABLE 1

Distribution of Lipids by Class for Ivory Coast Cocoa Butter Seed Crystals

Sample	Simple lipids	Glycolipids	Phospholipids	Recovery
		(Mean wt % ± S.D.)[a]		(%)
Cocoa butter	98.75±36A[b]	0.89±0.32A	.037±0.06A	99.80±1.09A
Seed crystals	82.37±3.09B	11.08±2.26B	6.55±1.48B	95.06±6.65A

[a]Mean ± standard deviation (n=3).
[b]Means within a column with the same uppercase letter are not significantly different at $\alpha = 0.01$.

TABLE 2

Fatty Acid Composition[a] of Total and Simple Lipid, Glycolipid and Phospholipid Fractions of Ivory Coast Cocoa Butter and Seed Crystals

	Total lipid		Simple lipid		Glycolipid		Phospholipid	
Fatty acid	Cocoa butter	Seed crystal	Cocoa butter	Seed crystal	Cocoa butter	Seed crystal	Cocoa butter	Seed crystal
16:0	26.6±0.1A[b]	24.8±0.4B	26.5±0.2A	25.5±0.3B	27.3±0.8A	27.0±2.1A	29.8±2.1A	28.3±0.1A
18:0	36.9±0.1A	64.9±4.2B	36.8±0.2A	63.4±0.9B	35.9±0.9A	35.6±0.4A	28.4±1.7A	26.8±0.9A
18:1	33.0±0.1A	8.3±4.0B	33.3±0.2A	8.3±0.3B	30.3±2.1A	31.6±0.7A	30.6±1.3A	29.1±1.7A
18:2	2.6±0.2A	0.6±0.2B	3.2±0.1B	0.5±0.1B	4.0±0.4A	3.6±0.4A	7.0±1.1A	11.1±0.2B
18:3	trace	trace	trace	trace	1.6±0.4A	1.6±0.1A	3.2±0.8A	2.8±0.4A
20:0	1.0±0.0A	1.4±0.1B	0.3±0.1A	2.3±0.6B	0.8±0.2A	0.6±0.1A	1.4±0.0A	2.0±0.5A

[a]Mean weight % ± standard deviation of three samples with four replicates of each sample assayed.
[b]Means within row of each lipid fraction with same uppercase letter not significantly different at $\alpha = 0.01$.

present in each sample is shown in Table 1. The values obtained for pure cocoa butter—98.75% for simple lipids, 0.89% for glycolipids, and 0.37% for phospholipids—are consistent with values reported in the literature for pressed cocoa butter (8). On a weight percent basis, a significant difference was noted in all three lipid classes when comparisons were made between cocoa butter and the high-melting seed crystal samples. The phospholipid content of the seed crystals was 6.55%, representing an increase of nearly 18-fold, while glycolipids accounted for 11.08% of the sample, or a 12-fold increase. As expected, the quantity of simple lipids concurrently decreased from 98.75 to 82.37%.

Direct phophorus analysis of the seed crystal and cocoa butter samples also reveals a significant increase in the phospholipid content of the high-melting seed crystals. The values obtained for the phospholipid content as determined by direct phosphorus assay and as determined by gravimetric analysis were similar; 0.30% vs 0.37%, respectively, for pure cocoa butter, and 8.13% vs 6.55%, respectively, for seed crystal samples. Table 2 presents the fatty acid composition of the total lipid, simple lipid, glycolipid, and phospholipid fractions for Ivory Coast cocoa butter and the high-melting seed crystal samples. The total lipid fatty acid profile for Ivory Coast cocoa butter agrees with the fatty acid composition of cocoa butter reported by other researchers (9). Comparison of the total lipid fatty acid profiles for Ivory Coast cocoa butter and the high-melting seed crystal samples reveals that the concentration of all fatty acids were significantly different. The seed crystals displayed elevated stearic acid content, 64.9%, as compared to the stearic acid content of cocoa butter, 36.9%. Hence, a dramatic increase in the degree of saturation was evident. Conversely, the oleic, 8.3%, and linoleic, 0.6% acid contents of the seed crystals decreased considerably, from 33.0% and 2.6%, respectively. Greater then 90% of the seed crystal material was composed of saturated fatty acids. Typically, cocoa butter contains only 60-65% saturated fatty acids. Undoubtedly, this increase in degree of saturation has an impact on the melting point of the seed crystals.

Similar fatty acid profiles for the simple lipid fractions and the total lipid samples were expected for the cocoa butter samples, since it was previously established that simple lipids accounted for nearly 99% of the sample. In contrast, only slightly greater than 82% of the seed crystal sample was simple lipid materials. Therefore, differences in fatty acid content were more evident, as in the case of arachidic acid. Fatty acid profiles of the glycolipid fractions of cocoa butter and seed crystals were not significantly different. This similarity in fatty acid profiles indicates that glycolipids present in the cocoa butter sample and the seed crystal sample are similar despite a major concentration difference between cocoa butter (0.9%) and seed crystals (11.1%). Similar results were obtained for the phospholipid fatty acid profiles of cocoa butter and seed crystals with one exception. The linoleic acid content was statistically different. Therefore, as with the glycolipid fractions, the phospholipids in both samples were shown to be similar in terms of fatty acid composition despite concentration differences among the cocoa butter (0.4%) and seed crystal (6.6%) samples.

Both Ivory Coast cocoa butter samples and seed crystal samples were analyzed for triacylglycerol composition by HPLC. Thirteen peaks were resolved for cocoa butter and the seed crystal samples as illustrated in the representative chromatograms (Fig. 2). All triacylglycerol peaks were quantified. Triacylglycerol compositions for cocoa butter and the high- melting seed crystals are presented in Table 3. All triacylgylcerols, with the exception of SOA, were significantly different. Interestingly, with the exception of SOA, every triacylglycerol containing one or more unsaturated fatty acid showed a decrease in content in the seed crystals. Conversely, every triacylglycerol containing only saturated fatty acids, i.e., trisaturated triacylglycerols, showed dramatic increases in concentration in the seed crystals. The triacylglycerols, PPS, PSS, and SSS contributed significantly to the make-up of the seed crystals, greater than 65%, while the same triacylglycerols were only minor components in cocoa butter, less than 5%. An unidentified triacylglycerol, XXX (Fig. 2), suspected of being SSA, was found exclusively in the seed crystals. As a general trend, the trisaturated triacylglycerols were predominant in the seed crystal samples, while the monounsaturated triacylglycerols, namely POP, POS, and SOS, were predominant in the cocoa butter samples.

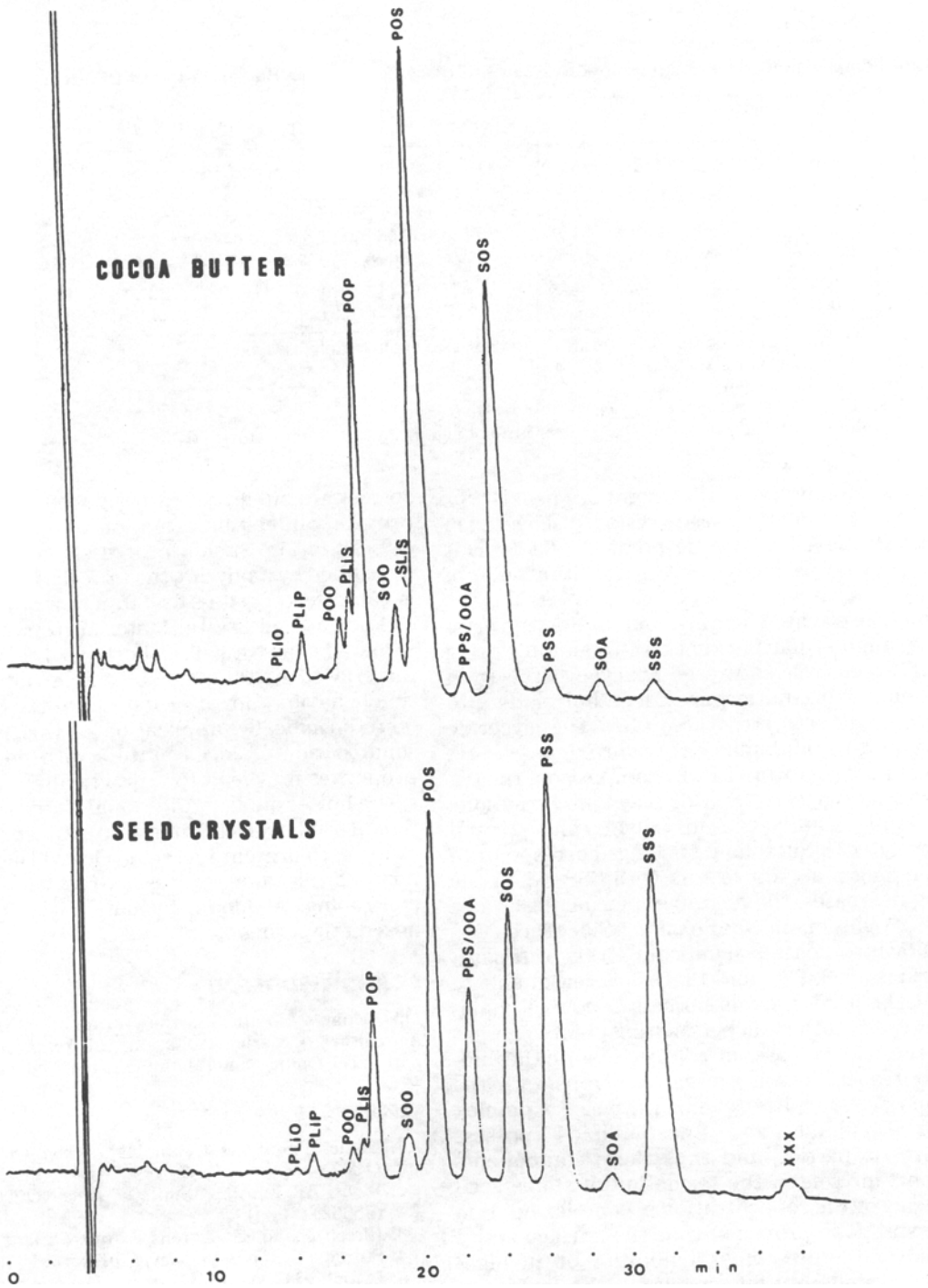

FIG. 2. High-pressure liquid chromatograms of triacylglycerol profile of the simple lipid fractions and seed crystals of Ivory Coast cocoa butter.

DISCUSSION

The elevated melting point of the seed crystals may be explained in two ways: a) by the presence of the relatively high-melting complex lipids, and/or b) by the presence of the highly saturated triacylglycerol species. Phosphatidyl choline is the major phospholipid component in cocoa butter (10). The melting point of dimyristoyl phosphatidyl choline is 236-237°C (11). Glycolipids also display melting points greater than cocoa butter. The abundance of trisaturated glycerides in the seed crystals may have even a greater impact on the melting point. One of the major triacylglycerols present in the seed crystal material, SSS, displays melting points of 54.0, 64.0, and 73.1°C for the three crystalline states, I (α), II (β)', and III (β), respectively (12). Seed crystals throughout these studies have routinely been determined to melt at approximately 60°C,

TABLE 3

Triacylglycerol Composition of the Simple Lipid Fractions of Ivory Coast Cocoa Butter and Seed Crystals

Sample	Triacylglycerol (Area %)[a]												
	PLiO[c]	PLiP[c]	POO[b]	PLiS[b]	POP[b]	SOO[b]	POS[b]	PPS[c] / OOA[c]	SOS[b]	PSS[c]	SOA[c]	SSS[b]	XXX[d]
Cocoa butter	0.57 ±0.08	2.19 ±0.08A[e]	2.86 ±0.08A	4.56 ±0.30A	14.98 ±0.29A	3.71 ±0.03A	37.98 ±1.00A	1.62 ±0.25A	26.92 ±0.61A	1.77 ±0.12A	1.75 ±0.16A	1.08 ±0.09A	—
Seed crystals	tr	0.75 ±0.13B	0.83 ±0.12B	1.38 ±0.28B	4.16 ±1.58B	1.96 ±0.22B	10.24 ±3.92B	11.04 ±1.38B	11.02 ±2.93B	28.33 ±4.99B	1.86 ±0.23A	26.29 ±3.12B	2.14 ±0.16

[a]Mean ± standard deviation of three samples with four replicates of each sample assayed.
[b]Triacylglycerol identified with internal standard.
[c]Triacylglycerol identified by retention data.
[d]Triacylglycerol not identified, but the triacylglycerol, SSA, is suspected.
[e]Means within a column with the same uppercase letter are not significantly different at $\alpha = .01$.

thus closely resembling the melting point of Form II SSS. Moreover, the highest melting seed crystals discovered in these studies displayed a melting point of 72.4°C. This melting point closely parallels the 73.1°C melting point of Form III SSS.

Considering the elevated melting point of the seed crystals in terms of molecular packing reveals an interesting relationship. Mixed oleic-saturated triacylglycerols such as those commonly found in pure cocoa butter tend to form triple chain-length structures (13). The glyceride SOS displays an X-ray long spacing of approximately 64 Å for its highest melting form (13). In comparison, the triunsaturated triacylglycerol SSS displays an X-ray long spacing of 45.0, 47.2, and 50.6 Å for polymorphs I, II, and III, respectively (12). Such long spacings correspond to double chain-length structures. As with SSS, when the long spacing decreases, the melting point increases as a direct result of tighter molecular packing. Since seed crystals were shown to possess significant levels of trisaturated glycerides (SSS, PSS, and PPS), it is reasonable to assume that the seed crystals possess a double chain-length structure and thus higher melting points.

Based on the evidence presented, as well as on the findings of other researchers, a general mechanism for the solidification of cocoa butter can be proposed. Complex lipids, both glycolipids and phospholipids, possess surface-active properties, and thus these components play an important role in the formation of stable seed crystals, judging by the concentration of complex lipids in the seed crystal. It is proposed that the surface-active complex lipids aid in directing the addition of the high-melting, trisaturated glyceride species to the surface of the complex lipid-rich nucleus. The triacylglycerols are incorporated into the growing seed crystal in order of decreasing melting point. The monounsaturated triacylglycerols then add to the growing seed (1); thus, SOS is likely to be the first major monounsaturated glyceride to add to the growing crystal.

Research efforts in our laboratory are continuing to more fully understand the mechanism of cocoa butter solidification. Determination of the phospholipid composition of the seed crystal may reveal the exact role that these complex lipids play in seed crystal formation. Analysis of the simple lipid composition is also necessary. In general, the progression in which the triacylglycerol species are integrated into the seed crystal structure will provide valuable information about the process of fat crystallization. Such a progression of incorporation can be studied by analyzing the triacylglycerol composition of isolated seed crystals as a function of time. The addition of known seed crystal material to molten cocoa butter should be investigated. Perhaps the addition of seed material to molten cocoa butter can alter the crystallization behavior and/or properties of the resulting crystals. More specifically, application of this knowledge to the solidification of cocoa butters with poor crystallization properties may lead to a practical means of up-grading cocoa butter quality. Additional studies relating the addition of seed crystal material to cocoa butter and its relationship to present temper cycles will be required to optimize the seeding process. Great potential exists for controlling fat bloom formation via seeding and optimized tempering.

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