PHYSICOCHEMICAL PROPERTIES OF A COCOA BUTTER EQUIVALENT SYNTHESIZED FROM ILLIPE BUTTER AND PALM MID-FRACTION AND ITS APPLICATION IN 'CHOCOLATES'

by

ADIGUNA BAHARI

(Under the Direction of Casimir C. Akoh)

ABSTRACT

Cocoa butter equivalents (CBEs) are fats that can mimic the properties of cocoa butter by having similar triacylglycerols profile to cocoa butter. The purpose of this study was to produce a CBE from illipe butter and palm mid-fraction by enzymatic interesterification. The major TAGs of the interesterified product (IP) were 1-palmitoyl-2-oleoyl-3-stearoylglycerol (POS) at 42.7 ± 1.0%, 1,3-distearoyl-2-oleoylglycerol (SOS) at 29.9 ± 0.3%, and 1,3-dipalmitoyl-2-oleoylglycerol (POP) at 19.1 ± 1.0% which were very similar to CB. Thermal behavior, polymorphism, solid fat content, and crystal microstructure of IP were analyzed and was shown to match CB as well. IP was formulated into dark and white 'chocolates' and their textural, rheological, particle size distribution and fat bloom properties analyzed. The analysis results showed that properties of the 'chocolates' made from IP and CB were similar and therefore suggested that IP is compatible with CB and can be used in chocolate as a CBE.

INDEX WORDS: Cocoa butter equivalent, Structured lipids, Illipe butter, Palm mid-fraction, Enzymatic interesterification, Thermal behavior, Solid fat content, Polymorphism, Crystal microstructure, Particle size, Texture, Rheology, Fat bloom

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ADIGUNA BAHARI

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ADIGUNA BAHARI

Major Professor:	Casimir C. Akoh
Committee:	Jose I. Reyes De Corcuera
	William L. Kerr

Electronic Version Approved:

Suzanne Barbour
Interim Dean of the Graduate School
The University of Georgia
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DEDICATION

To my mother and father for their eternal love and support, and my brothers for the discussions and inspirations.

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CHAPTER 1

INTRODUCTION

Cocoa butter (CB) is a core ingredient in chocolates as it is the major contributor to its unique properties, such as melting just below body temperature (34-36 ^{o}C), smooth mouthfeel and texture, as well as hard snap. Due to the specific ratio of triacylglycerols (TAGs) it contains, CB is probably the only natural source of fat that can melt just below body temperature. There are three types of TAGs that are predominant in CB, they are 1-palmitoyl-2-oleoyl-3-stearoylglycerol (POS; S = stearic) 38.0-42.1%, 1,3-distearoyl-2-oleoylglycerol (SOS) 22.8-30.0%, and 1,3-dipalmitoyl-2-oleoylglycerol (POP; P = palmitic, O = oleic) 16.8-19% (Lipp et al., 2001). Since physicochemical properties of TAGs basically depend on chain length and saturation degree of the fatty acids, and the position where they are esterified at the glycerol backbone molecule, these disaturated triacylglycerols at the respective ratios give cocoa butter the melting ranges from 34-38 ^{o}C (Rohm, Schäper, & Zahn, 2018). From these TAGs, it is seen that CB contains only three major fatty acids that make up over 95% of all fatty acids. The major fatty acids are oleic acid (C18:1) at around 35%, stearic acid (C18:0) at around 34%, and palmitic acid (C16:0) at around 26%. This relatively simple fatty acid make-up contributes to the rapid melting of fat over a small temperature range (Beckett, 2008). The different TAG molecules in CB can also pack in different ways, such that there are the α, β' and β polymorphic forms of CB with increasing densities and melting stabilities (Wagh & Martini, 2017). The most stable polymorphic form (β form), gives chocolate the shiny surface gloss and hard snap. All these properties of CB make chocolate a popular and unique confectionary product.

Due to fluctuations in cocoa prices in the last three decades and high demand, researchers have tried to find alternative fats and oils to cocoa butter that may reduce the production cost of

chocolates (Kim & Akoh, 2015). In general, there are two types of cocoa butter alternatives (CBA) based on how compatible they are to CB. Cocoa butter equivalents (CBEs) are fats/oils (mostly from tropical plant sources) that are totally compatible with CB without altering the product properties. CBEs are usually very similar in fat makeup with CB. Cocoa butter substitutes (CBSs) on the other hand are fats/oils that can be used with CB at a limited ratio as it will alter the chocolate properties at a higher ratio (Lipp et al., 2001). CBSs are usually different in terms of fat composition compared to CB. It is relatively easier to produce a CBS than a CBE. CBS are usually produced from different blends of tropical fat such as the different palm oil fractions, palm kernel oil, illipe butter (IB), shea butter, mango kernel fat, etc. However, in order to produce CBEs that have a similar fat profile with CB, lipases are often required to restructure the fat profile of the substrates. In the process, a structured lipid (SL) is produced. SLs are fats or oils that have been either enzymatically or chemically restructured in order to obtain a specific desired fat property (Lee & Akoh, 1998). In this case, the structured lipid CBE needs to have similar TAG profile to CB. CBEs can also serve as the base of a non-chocolate confectionary product and is often used in confections where flavors are added. However, in many countries, the addition of foreign fats in chocolates are regulated. In the EU, 5% of several foreign fats are permitted to be used and allowed to be still labelled as 'chocolate'. In the US however, no foreign fats are permitted in a product that is labelled as 'chocolate'.

CBEs need to be tested in chocolate applications to validate the compatibility of the CBE with CB. Several properties that are important for a chocolate product include melting point, texture, particle size distribution, rheological and fat bloom properties. Melting point is strongly related to proper tempering and polymorphism, as unstable polymorphs have a low stability and undesired melting point. Particle size distribution affects the mouthfeel of the chocolate. Large particle size (>30μm) imparts a gritty mouthfeel in chocolate compared to silky mouthfeel for smaller particle size. The texture of the chocolate determines whether chocolate would have a clean hard snap. Rheological properties relate to processing consideration of a chocolate such as

pumping and is also an important parameter for chocolates used as coating. Fat bloom resistance relates to the storage capacity of chocolates, as improper storage conditions leads to dulling surface color, a cosmetic flaw in chocolate products (Afoakwa, 2010; Afoakwa, Paterson, & Fowler, 2007).

This thesis includes five chapters. The first chapter introduces the content of the thesis as well as the research objectives. The second chapter is a literature review of topics related to the thesis including CB, CBA, IB, palm mid-fraction (PMF), SL, polymorphism, crystal microstructure, tempering and fat bloom. The third chapter includes the synthesis of cocoa butter equivalent by enzymatic interesterification of IB and PMF. The physicochemical properties of the interesterified product (IP) were compared to its non-interesterified physical blend (PB), IB, and CB. The physicochemical properties include relative TAG profile, fatty acid profile, thermal behavior (melting and crystallizing points), solid fat content, polymorphism, and crystal morphology. The fourth chapter presented the texture, rheology, and fat bloom study of 'chocolates' made from CBE synthesized in the previous chapter. The four different fats (IP, PB, IB, and CB) were applied to make dark and white 'chocolate' products. White 'chocolate' was made to test the use of the CBEs in a non-chocolate confectionary product. The physical properties important to chocolate properties were tested. These properties include melting point, particle size distribution, texture (hardness), rheology (plastic viscosity and plastic yield stress) and fat bloom resistance. The fifth chapter presents the conclusion of the whole research along with possible future work.

The objectives of this research were:

1. To synthesize a CBE by enzymatic interesterification using IB and PMF in order to obtain an IP with fat profile similar to CB. The product was analyzed and compared to its non-interesterified PB, substrate (IB) and standard (CB) with respect to differences in TAG and fatty acid profile, thermal behavior, solid fat content, polymorphism, and crystal morphology.
2. To determine the particle size distribution, textural, rheological, and fat bloom properties of 'chocolates' made from the IP compared to its non-interesterified PB, substrate (IB) and standard (CB). Dark 'chocolates' were made to determine the compatibility of the CBE in a chocolate complex, whereas white 'chocolate' was made to determine the properties of the interesterified product as a non-chocolate confectionary product.

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CHAPTER 2

LITERATURE REVIEW

Cocoa Butter

Cocoa butter (CB) is the pale-yellow edible fat that is extracted from cocoa beans of the tree *Theobroma cacao*. Historical records showed that cocoa beans were probably first used amongst the Olmec, Maya and Aztec cultures as a beverage containing divine power as it was believed to be able to cure medical illnesses. Three consistent medicinal roles identified were 1) to gain weight from extremely thin and weak patients; 2) to recover the nervous system of these patients; and 3) to improve digestion and bowel function (Dillinger et al., 2000). In 1544, chocolate beverage first travelled across the Atlantic ocean when Mayans were brought to Spain by Dominica friars (Coe & Coe, 1996). Within the 17th century, the medicinal and beverage use of cacao drinks have spread across England, France and other Western European countries, which then prompted the start of cacao plantations in Spanish colonies such as the Philippines (Bloom, 1998; Knapp, 1930).

Over time, people began to learn that a mixture of ground cocoa beans and sugar yields a hard substance that did not melt in the mouth. For this solid chocolate to melt, additional fat need to be added. In 1828, Van Houten from Netherlands developed the technology to extract cocoa fat/butter. Cocoa bean was pressed and had some of its fat removed. This resulted in CB that can be added to make solid chocolate, and reduced fat cocoa powder that is still incorporated in cocoa drinks (Beckett, 1999).

Today, the chocolate industry is a worldwide business with cacao plantations all over the world (Table 2.1). In a 2015 production data from International Cocoa Organization (ICCO, 2017), Africa contributes to the highest cocoa beans production at 72.3%, South America at 18.3%, and Asia at 9.4%. Cote d′Ivoire produced the most cocoa beans in 2014 (1.796 million

tons). It is worth mentioning that even though Asia contributes to the least area of production, Indonesia is the third largest producer of cocoa beans in the world. In 2017, the global chocolate industry was worth more than US $80 billion, with Mars Inc. as the top producer of chocolate, contributing to US $18 billion (International Cocoa Organization, 2018). The top 10 confectionary companies are shown in Table 2.2.

Chocolate is a popular and unique confectionary product due to its ability to melt at just below body temperature (34-38 ^{o}C), giving a cool melting sensation as it touches the inside of the mouth. A good chocolate is also associated with a glossy surface and having a clean hard snap. These qualities are contributed from the properties of CB. CB accounts for 50-57% of cocoa beans on a dry weight (Steinberg, Bearden, & Keen, 2003). Aside from the neutral lipids, cocoa is also known to contain a rich source of phenolic phytochemicals (611 mg of gallic acid equivalents), flavonoids (564 mg of epicatechin equivalents) as well as natural sources of antioxidants (tocopherols) higher than tea and red wine (Erickson, Weissberger, & Keeney, 1973; Lee, Kim, Lee, & Lee, 2003). As a fat, cocoa butter is composed of different kinds of triacylglycerols (TAGs). The fatty acid composition of CB is high in saturated (palmitic, stearic acid) and monounsaturated fatty acids (oleic acid) (Table 2.3). It has been long known that there are three major TAGs in cocoa butter. They are 1,3-dipalmitoyl-2-oleoylglycerol (POP; P = palmitic, O = oleic), 1-palmitoyl-2-oleoyl-3-stearoylglycerol (POS; S = stearic), and of 1,3-distearoyl-2-oleoylglycerol (SOS). Lipp et al. (2001) analyzed 42 various cocoa butter samples from around the world and found that the major TAGs lie within the following ranges: POS 38.0-42.1%; SOS 22.8-30.0%; POP 16.8 - 19.0% (Table 2.4) (Lipp et al., 2001). Since TAGs melt depending on their fatty acid composition, the combination of these three major disaturated TAGs at this specific range defines the melting temperature of cocoa butter at near body temperature (Afoakwa, 2010).

Cocoa Butter Alternatives

World cocoa production has been steadily increasing from 1.5 million tons in 1978, 2.7 million tons in 1979, and up to 4.3 million tons in 2014 (Table 2.1). Although CB is a core ingredient for the physical and organoleptic properties of chocolate, there has been a number of factors that drove the development of cocoa butter alternatives (Gunstone, 2001). First, as mentioned earlier, more fat is needed in proportion to the nonfat cocoa solids found in the bean to produce a suitable chocolate. Second, CB supply has been shown to be uncertain and variable which leads to the final factor, that is the fluctuation in CB prices, which has seen an increasing trend over time (Figure 2.1) (Trading Economics, 2018). Cocoa prices have reached a historical high in July 1977 at US $4361.48/ton. Due to the fluctuation of prices of CB over the years, cheaper alternatives to CB have been developed. Because different fats impart different functionalities in chocolate, labelling classifications are defined as follows (Lipp & Anklam, 1998):

Cocoa butter alternatives (CBA): general name for additional fats that fulfill the function of cocoa butter partially or as a whole;

a) Cocoa butter equivalent (CBE): non-lauric plant fats, which have similar physical and chemical properties to cocoa butter and compatible with cocoa butter in any amount without changing the properties of cocoa butter.
    a. Cocoa butter extender (CBEX): a subgroup of CBEs, but not compatible in every ratio with cocoa butter.
    b. Cocoa butter improver (CBI): high content in solid triacylglycerols, used for improving soft cocoa butters.
b) Cocoa butter replacer (CBRs): non-lauric plant fats with similar fatty acid distribution with cocoa butter but having different triacylglycerol range. Only compatible with cocoa butter in small ratio, altering the properties of the blend in bigger ratio.

c) Cocoa butter substitutes (CBS): lauric and myristic plant fats with totally different chemical and physical characteristics with cocoa butter, therefore only suitable for whole substitution of cocoa butter.

It is important for CBEs not to contain lauric acid fat, as lauric acid (usually from palm kernel oil) will produce a eutectic effect with CB, resulting in softening and phase separation of chocolate products, eventually forming fat bloom (Lonchampt & Hartel, 2004). Due to addition of foreign fats into chocolate, and to be transparent to customers and maintain quality, many of the labelling terms are regulated. The European Union decreed a directive (EU Directive 2000/36/EC) that only six tropical fats are allowed up to 5% of the chocolate mass for the product to still be labelled as chocolate. The permissible fats are illipe butter, palm oil, sal, shea, kokum gurgi, and mango kernel (Table 2.5). United States are stricter however, not allowing any additional non-cocoa butter fats in order for the product to be labelled as chocolate.

Illipe Butter

Illipe butter (IB) refers to the kernel fat that is produced from the rainforest tree genera of *Shorea,* from the family *Dipterocarpaceae*. Endemic to the island of Borneo and Sumatera, *Shorea* spp are dipterocarps and are characterized by their production of two winged fruits (*Di* = two, *ptero* = wing, *karpus* = fruit) (Figure 2.2). The term "illipe" was taken from the Tamil name for nuts of *Madhuca* spp (Sapotaceae) from India, however it was later applied to other nuts with similar properties that were exploited from Southeast Asia. Illipe is more well known as tengkawang in Indonesia, and engkabang in Malaysia. Other names include Borneo tallow, or vegetable tallow (Blicher-Mathiesen, 1994).

In the past, illipe seed production have reached 3600 tons/year in Indonesia alone. During 1985-1989 the production reached up to 10,677 tons (Winarni, Sumadiwangsa, & Setyawan, 2004). The increase of CB prices from 1970 resulted in the interest to use CBE, and the demand for IB increased accordingly. Unlike palm oil and shea butter, IB do not need to be fractionated and is therefore simpler and cheaper to process. Aside from its well-known CBE properties, IB

has been utilized in cosmetics for lipstick, body butter, hair conditioner, etc. It is known to be a good moisturizer, can recover elasticity in dry skins, as well as a good UV protector (Barwa, 2016). However, due to erratic good and bad harvest seasons, as well as land competition with palm oil plantations, the use of commercial IB have dwindled (Heidhues, 2003).

The fat contents of illipe nuts ranges from 45 to 70% varying on the species of the nuts. Table 2.6 and Table 2.7 show the fatty acid profile of several *Shorea* species and the TAG profile of IB. From Table 2.6, we can see that the fatty acid makeup of IB is very similar to CB (Nesaretnam & Ali, 1992). Like CB, the major fatty acid in IB is stearic acid (C18:0) at 39.4-46.7%, oleic acid (C18:1) at 33.2-35.8%, and palmitic acid (C16:0) at 16.0-23.1%. IB also contains traces of myristic acid (C14:0), linoleic acid (C18:2), linolenic acid (C18:3), as well as arachidonic acid (C20:0). In terms of its TAG composition, the major TAG species in illipe butter is also similar to cocoa butter: SOS at 53.1%, POS at 36%, and POP at 6.6%. Aside from CBEs and cosmetics, another interesting study has tried to mimic lard from blending IB and canola oil (Illiyin, Marikkar, Shuhaimi, Mahiran, & Miskandar, 2013)

Palm Mid-Fraction

Palm mid-fraction (PMF) is a specialty fat dominant in POP TAG (40-50%). Due to its high POP value, PMF has a sharp melting point and slip melting point at 35-36 ^{o}C.

Two major forms of oil can be harvested from the palm fruit. Crude palm oil (CPO) is reddish brown in color and are derived from the mesocarp or flesh of the fruit. CPO is then refined, bleached, and deodorized to produce palm oil that meets safety standards for food. Palm kernel oil (PKO) is extracted from the kernel (seed) of the palm fruit. While PKO is used for non-food product and oleochemical industry (laundry detergent, soap, shampoo, cosmetics, etc), palm oil has a wide array of usage within the food industry. The key to its wide function is locked within the different higher melting point phases that exist in palm oil (Koushki, Nahidi, & Cheraghali, 2015).

Through controlled cooling, palm oil can be first fractionated into two major fractions: liquid oil/palm olein (~85%) and solid fraction/palm stearin (~15%). The trisaturated TAGs in palm stearin is around 35% compared to 7% in the liquid palm oil and 2% in palm olein (Kellens, Gibon, Hendrix, & De Greyt, 2007). Palm olein is then fractionated to obtain soft palm mid-fraction and super olein. Soft palm mid fraction is again fractionated to obtain hard palm mid-fraction. Hard palm mid-fraction is the fraction that is the core substance of all CBEs in the market. The fractionation process and food application of each fractionated product can be seen in Figure 2.3 (Calliauw, 2011)

Structured Lipids

Structured lipids (SLs) are lipids that have been modified from their natural biosynthetic form (Akoh & Kim, 2017). The classification of lipids could be in the form of acylglycerols such as TAGs, diacylglycerols (DAGs), and monoacylglycerols (MAGs) or in the form of phospholipids. Modification includes any form of changes that occurred in the positional or composition of fatty acids from the native state. SLs can be synthesized through chemical or enzymatic reaction; however, enzymatic reaction is often favored due to its ability to specifically catalyze reactions at the *sn*-1,3 positions. This way, enzymatic reaction has less formation of byproducts than the randomized characteristic of chemical reaction. Enzymatic reaction is conducted in a milder temperature than chemical reaction, which reduces the loss of quality of the temperature sensitive substrates and products. Furthermore, using enzymes replaces the use of harmful reagents and is easier to recover the product, which results in a more environmentally friendly and safer process (Kim & Akoh, 2015).

Each fatty acid has a unique physical and chemical properties, and the position of fatty acids in a TAG also dictate the properties of that TAG. This way, the major attraction in synthesizing SL is that the product could be tailor-made to achieve fats with specific properties. Several studies that have synthesized SL for possible use in the food industry include applications like medium chain triacylglycerols (MCTs), human milk fat (HMFs), *trans*-free fats, low calorie

oils, omega-3 rich fats, emulsifiers (MAGs/DAGs), phosopholipids, and CBEs (Table 2.8) (Kim & Akoh, 2015; Sproston, 2016).

Generally, there are three types of reaction to produce SLs. 1) Acidolysis reaction incorporates fatty acyl groups from free fatty acids (FFAs) to TAG molecules. 2) interesterification reaction exchanges fatty acyl groups between two or more TAG molecules. 3) Alcoholysis reaction substitutes fatty acyl groups from TAG molecules to an alcohol such as glycerol (glycerolysis) or ethanol (ethanolysis).

CBE SLs are usually synthesized through acidolysis reaction by a TAG and a specific fatty acid. This way the incorporation of the fatty acid could be tracked, and the desired TAG level could be selected. Several studies have conducted this type of reaction to synthesize CBE. One study conducted acidolysis of palm mid-fraction and stearic acid (Undurraga, Markovits, & Erazo, 2001). In the second study, tea seed oil, methyl palmitate, and methyl stearate was reacted by acidolysis as well (Wang, Wu, Ho, & Weng, 2006). In the former, the TAG composition of the product was similar to CB. In the latter, the fatty acid composition of the CBE was similar to that of CB. It is difficult to achieve CBEs with only blending of two or more fat substrates. Often, CBS are made in the process. Previous study have blended PMF, palm kernel oil and palm stearin to produce CBS containing 37.5% POP, 4.2% POS, and 0% SOS (Nirupam Biswas, Cheow, Tan, Kanagaratnam, & Siow, 2017). Another study used mango kernel fat and PMF to produce CBS containing 12.7% POP, 6.7% POS, and 26.9% SOS (Sonwai, 2014). As shown in the previous two examples, CBS do not contain similar TAG properties to CB, and will alter the properties of the chocolate when blended in a higher ratio.

Polymorphism

Polymorphism is the ability of fat molecules to crystallize to a number of different packing formations. Depending on cooling conditions, TAGs can crystallize into several polymorphic forms. Different polymorphs show different physical characteristics, most notably in its melting point and hardness. In products such as chocolate, where the snap melting point at

body temperature is a major feature of the product, understanding the polymorphism of CB is of utmost importance. In fact, as early as 1945, researchers have been studying the polymorphism of different TAGs such as desaturated TAGs (Lutton, 1951) and tristearin (Lutton, 1945). The method to analyze crystal polymorphism was through x-ray diffraction. Different polymorphic form will diffract x-ray at different angles, which is then translated to short spacing length.

The two major type of classification and of cocoa butter polymorphism are shown in Table 2.9 (d′Souza, Deman, & Deman, 1990). The first is the classification by Vaeck (1960) that includes the γ, α, β′, β and the second is the Roman numeral I to VI classification by Wille and Lutton (1966). For both cases, the order of classification is in increasing stability. It is easy to discriminate the γ and α polymorph. γ has a strong short spacing at 4.19 and 3.7 Å. α polymorph has only one short spacing at 4.24 Å. There are a lot of mixed reports when it comes to the β′ and β polymorph. Wille and Lutton divided the β′ and β phase into two different phases each (β′ into III and IV, β into V and VI). Even though III & IV and V & VI shared the same β′ and β polymorph respectively, their melting point was all different from each other.

Studies would continue to find inconsistent medium and weak additional short spacing peaks in several β′ and β polymorph. To start, Wille and Lutton identified the β′ (III and IV) to have a strong short spacing at 4.2, 3.8 Å and various other weak and medium peaks in the range of 3.8 to 4.9 Å. For β polymorph (V and VI) the strong peak was found at 4.6 Å. Over time, other reports classifying versions of CB polymorphism showed up. Witzel & Becker (1969) classified β′1, β′2 and pre β polymorph based on missing and additional peaks. Riiner (1970) defined β′2 as having a very strong peak at 4.19 Å and a medium peak at 3.72 Å. Chapman, Akehurst, & Wright (1971) and Hicklin, Jewell, & Heathcock (1985) also published their finding on the I to VI polymorph, with several missing or additional short spacing peaks. Generally, it is agreeable that β′ polymorph is identified by a strong short spacing of 4.2 Å and 3.8 Å, while β polymorph is indicated by a strong peak at 4.6 Å. Medium and weak peaks from 3.3-5.4 Å have been found to accompany these two phases.

Van Malssen et al. (1999) showed that γ, α, β′ phases can crystallize directly from a liquid melt while two β phase, V and VI are obtained via a phase transformation from the β′ phase only. Van Malssen proposed the idea that β′ is more likely to be a phase range rather than as a set of individual phases.

γ is the least stable phase in CB. It is stable for only 10 days at -10 ^{o}C. At higher temperature, γ phase transforms rapidly into α. Due to the weak stability, it is hard to determine the exact melting range of γ. The melting / disappearing range was established to be approximately -8 to +5 ^{o}C. α phase can be formed easily either by the unstable γ phase, or directly from the liquid melt. α phase is more stable than γ, but not stable enough to prevent transformation to β′ within an hour above 6 ^{o}C. There are many classes of β′ in literature. The physical basis of β′ as a phase range can be based on CB being a mixture of TAG, and solid CB contains different groups of crystallites with its own different TAG composition. Different cooling rate affects the various possibility of crystal formation, thus influencing distribution. Eventually, this results in a different distribution of melting characteristics and diffraction patterns. β phase is the most stable phase and forms via phase transformation from β′. β crystallization directly from melt can only occur if the melt has a previous β memory (van Malssen et al., 1999).

Crystal Microstructure

Hicklin et al. (1985) used transmission electron microscopy to visualize the crystal structures in different polymorphs. Class I CB polymorph lacks any distinct morphology, form II showed order lamellae, and form III showed protruding tubular crystals. Dimick & Manning (1987) used scanning electron microscopy and polarized light microscopy to elucidate crystal structures and associated "feather-like" big microstructures to β polymorph. In several cases, different microstructures have been associated with particular polymorphic forms. Identifying microstructure in fats are becoming more important, as the macro properties depend on its microscopic crystal networks. Due to ease of access and function, polarized light microscopy has

been the popular method to study crystal structure in recent years. Using polarized light microscopy, Marangoni & McGauley (2003) presented an extensive study on relationship between crystallization behavior, polymorphism and the resulting microstructure in a CB.

γ and α polymorphs had granular appearance despite the temperature – time combination (Figure 2.5). In β′ polymorph, different observations occur depending on the time and temperature conditions. β′ polymorph formed at 15 ^{o}C via the α form showed granular structures with some evidence of crystal clustering. More aggregation of crystallites was observed in β′ polymorph formed at higher temperatures (20 ^{o}C and 22 ^{o}C). β′ polymorph formed at 24 ^{o}C also showed crystallites with needle-like structures (Figure 2.6).

β polymorph displayed a wider range of variety of microstructures. In a 4-5-week crystallization period at 20 and 22 ^{o}C, the microstructure was no longer uniform as was seen in α and β′ polymorph (Figure 2.7). Morphology in the earlier stages of crystallization was predominant; granular structures composes most of the continuous phase, while various sizes of microstructures was visible (600 μm to 2 mm). The large microstructures had featherlike appearance. β polymorph at various combination of time and temperature showed this featherlike large microstructure. The β polymorph in the study was formed from β′, therefore Marangoni concluded that the phase transition of β′ to β will usually lead to these large microstructures.

At 26 ^{o}C crystallization temperature, just after one day of incubation, small crystallites and large needle-like structures were already observed (Marangoni & McGauley, 2003) The microstructure of β′ at 26^{o}C was characterized by needlelike crystals at around ~50 μm. After β′ to β transition has taken place, large microstructure distinctive of β polymorph was visible; large microstructure with granular center, needle-like periphery and feather-like structures (Figure 2.8).

The same polymorph of CB may have different composition of microstructure when cooled at different temperatures. At low crystallization temperatures, there were not many changes in microstructure, whereas as temperature become higher, more differentiation of microstructures occured as a result of phase separation / fractionation.

Tempering

In order for chocolate to be satisfactorily processed into a solid product, it needs to undergo tempering process. Tempering is a process of slow crystallization to make sure chocolate solidifies into the most stable and desired form of CB (β form). Less stable polymorphic forms tend to transform into higher melting and more stable forms. This process resulted in the changes in packing of the crystal fats, which manifests in the properties of chocolate in a several ways, such as changes in the physical appearance of chocolate surface (smooth becomes rough) or visible recrystallization (fat bloom). Therefore, the goal of tempering is to develop a sufficient number of seed crystal to ensure that the total fat phase could crystallize in a balanced manner into a more stable polymorphic form.

What factors make a good tempering process? Fessas et al. (2005) conducted an extensive study to elucidate the polymorphous transition in cocoa butter using a quantitative DSC study. Cocoa butter samples were cooled at various cooling rates from 50 ^{o}C down to different annealing temperatures (10, 15, 20 or 25 ^{o}C), where they are rested for various time periods (5, 10, 15, 15, 30, 60 min, 3, 5, 10, 20 h) (Figure 2.9). There were two major factors that affects the resulting polymorph in a cooling process. The first is the temperature of the annealing process, and the second is the annealing duration (time). At an annealing temperature of 10 ^{o}C, only α can be achieved (indicated by a low melting onset). Raising the annealing temperature to 15, 20 or 25 ^{o}C, a β′ polymorph can be formed with a longer annealing time.

In common practice, tempering a chocolate follows 4 steps. Chocolates are first melted at high temperature (~50 ^{o}C) to make sure all fats are melted. Chocolate is then cooled to 27 ^{o}C, to allow packing of β′ and β crystals. The temperature is then raised to 32 ^{o}C to melt out β′ crystals, as the melting point of β′ crystals are 27-29 ^{o}C (Wille & Lutton, 1966). Chocolate is also stirred while cooling to induce crystallization. Another method that chocolatier often use to temper chocolate is called tabling. Chocolate is first poured on a marble slab, and a spatula is used to spread the chocolate on the marble slab until the chocolate thickens. The chocolate is then

transferred into a bowl of warm chocolate to melt out the unstable chocolate (Beckett, 1999). However, the two tempering methods explained above are often unclear regarding the time duration of each step. The IUPAC method of tempering fat is easier and more specific. Fat is first melted at 60 ^{o}C for 30 min, and then cooled at 0 ^{o}C for 90 min. Fat is then kept at 26.5 ^{o}C for 48 h, and then cooled again at 0 ^{o}C for 90 min (Torbica, Jovanovic, & Pajin, 2006).

Fat Bloom

Fat bloom manifests as white specks on the surface of chocolate, dulling the initial gloss and eventually spreading all over the whole chocolate. Many factors can contribute to fat bloom, including improper processing conditions, abuse of storage temperature, as well as the composition of the chocolate. Fat bloom can be characterized based on its crystal shape, composition and polymorphism (Lonchampt & Hartel, 2004).

In terms of crystal morphology, small fat crystals were found to be growing not only at the surface of the fat bloom but in the whole chocolate mass. The crystals were 2-10 μm in length and irregular in shape (Jewell, 1972). Whymper & Bradley (1925) first found that fat bloom crystals have a higher melting point (1-4 ^{o}C) than the well-tempered chocolate. This spurred the first theory of fat bloom formation, which is fractionation of higher melting fats. They explained that the higher melting point is due to fractionation and recrystallization of higher melting CB. More recently however, a contending theory states that the increase of melting point may be due to the result of polymorphic transition into the most stable β form. This polymorphic transition theory is more widely accepted as most studies correlate the fat bloom finding with a βVI polymorph (Lonchampt & Hartel, 2004). Although fat bloom was always found to have βVI polymorph, chocolate could still have a polymorphic transition from βV to βVI without a visual bloom (Bricknell & Hartel, 1998).

Fat bloom could be inhibited through changes in the composition or processing methods. In general, higher solid fat content in chocolate is able to inhibit fat bloom. The addition of

stearin of CB (a higher melting fraction) has been found to reduce bloom formation in chocolate (Buscato et al., 2018). Specific fractions of other vegetable fats that have a high solid fat content such as trilaurin was also shown to delay the development of fat bloom (Kawada, Suzuki, Kamata, & Matsui, 1971). Milk fat has also been known to have an anti-bloom effect. Hendricx found that substitution of cocoa butter with hydrogenated milk fat was able to delay or eliminate fat bloom (Hendrickx, De Moor, Huyghebaert, & Janssen, 1971).

Emulsifiers are also well known for their function to inhibit fat bloom. The mechanism of emulsifiers in delaying fat bloom is quite complicated. However, there seemed to be three major effects that emulsifiers have on fat bloom. First, they improve crystallization by increasing the number of seeds that are formed, while reducing the crystal size (Arruda & Dimick, 1991). Second, emulsifiers increase the melting point of the fat, therefore the fat product has better thermal resistance (Wilson, 1999). Third, they are able to prevent transition of fat crystals into βVI polymorph which are associated with fat bloom (Nakae, Kometani, Nishimura, Takii, & Okada, 2000).

In terms of processing, as stated above, tempering is a critical process in making sure fat bloom is contained. Chocolate that are under tempered (cooled rapidly) would cause fat bloom in under 24 hours. Rapid cooling may promote formation of unstable polymorphs. Proper cooling is important to prevent early fat bloom development. Storage conditions of chocolate is also important to control fat bloom. Buscato et al. (2018) found that fat bloom did not occur in a dark chocolate that is stored at 20 ^{o}C, whereas chocolate that undergoes temperature abuse and oscillation up to 32 ^{o}C will experience an acceleration of fat bloom.

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Table 2.1 Global production of cocoa beans (thousand tons)[a]

	2014/2015		Estimates 2015/16		Forecasts 2016/17	
Africa	3074	72.3%	2918	73.3%	3565	75.8%
Cameroon	232		211		240	
Cote d′Ivoire	1796		1581		2010	
Ghana	740		778		950	
Nigeria	195		200		225	
Others	111		148		140	
America	777	18.3%	666	16.7%	757	16.1%
Brazil	230		140		180	
Ecuador	261		232		270	
Others	286		294		307	
Asia & Oceania	400	9.4%	397	10%	379	8.1%
Indonesia	325		320		290	
Papua New Guinea	36		36		40	
Others	39		41		49	
World total	4251	100%	3981	100%	4700	100%

[a]Data obtained from ICCO Quarterly Bulletin of Cocoa Statistics, Vol XLIII, No. 3, Cocoa year 2016/17. Retrieved from https://www.icco.org/statistics/production-and-grindings/production.html

Table 2.2 Global production of chocolate products[a]

Company	Net Sales (2017) (US$ millions)
Mars Wrigley Confectionary, div of Mars Inc (USA	18000
Ferrero Group (Luxembourg / Italy)	12000
Mondelez International (USA)	11560
Meiji Co Ltd (Japan)	9652
Nestle SA (Switzerland)	8818
Hershey Co (USA)	7533
Chocoladenfabriken Lindt & Sprungli AG (Switzerland)	4106
Ezaki Glico Ltd (Japan)	3242
Arcor (Argentina)	3100
Pladis (UK)	2816

[a]Data obtained from International Cocoa Organization. Includes production of non-confectionary items. Retrieved from https://www.icco.org/about-cocoa/chocolate-industry.html

Table 2.3 Fatty acid composition (g individual fatty acid methyl ester/100 g of total fatty acid methyl esters) of cocoa butter (CB)[a]

	C14:0	C16:0	C18:0	t-C18:1	c-C18:1	C18:2	C20:0
CB (n=42)							
Mean	0.00	26.23	35.76	-	33.60	2.68	0.93
Median	0.00	26.31	35.79	-	33.43	2.78	0.92
Minimum	0.00	24.78	32.86	-	32.70	1.09	0.82
Maximum	0.09	26.91	37.68	-	37.08	3.36	1.10
S.D.	0.018	0.371	0.867	-	0.758	0.340	0.057

[a]Data obtained from Lipp et al. (2001). (With permission).

Table 2.4 Triacylglycerol composition (g individual triacylglycerol/100g of total triacylglycerols) of cocoa butter (CB)[a]

	POP[b]	PLP	POS	POO+PLS	SOS	SOO+SLS	OOO
CB (n=42)							
Mean	18.27	1.82	42.08	5.58	26.39	4.64	1.23
Median	18.37	1.89	42.12	5.60	26.26	4.52	1.21
Minimum	16.80	0.78	38.03	3.09	22.83	3.27	1.02
Maximum	19.03	2.08	43.76	9.45	30.02	9.79	1.52
S.D.	0.483	0.236	0.828	0.919	1.260	1.002	0.102

[a]Data obtained from Lipp et al. (2001). (With permission).
[b]Abbreviations are as described; L, lauric acid (C12:0); P, palmitic acid (C16:0); S, stearic acid (C18:0); O, oleic acid (C18:1).

Table 2.5 Vegetable fats allowed for use as cocoa butter equivalent (CBE) in chocolate following EU Directive 2000/36/EC[a]

Name of vegetable fat	Scientific name of fat source
Illipe	*Shorea* spp.
Kokum gurgi	*Garcinia indica*
Mango kernela	*Mangifera indica*
Palm oil	*Elais guineensis, Elaeis olifera*
Sal	*Shorea robusta*
Shea	*Butyrospermum parkii*

[a]Data was recreated and readapted from Deporteere (2011).

Table 2.6 Physicochemical characteristics of the fat of the illipe species in comparison with cocoa butter and palm mid-fraction (PMF)[a]

	Sample				
	Shorea singkawang	Shorea mecistopteryx	Shorea macrophylla	Cocoa butter	PMF
SMP[b] (°C)	34.5	34.9	36.8	34.0	33.0
Iodine value	33.7	36.3	30.0	36.0	35.9
FFA	0.38	0.41	0.27	-	-
FAC (%)					
C12	-	-	0.1	-	0.1
C14	0.1	0.1	0.1	0.1	0.8
C16:0	23.1	22.1	16.0	26.1	42.8
C16:1	-	1.1	0.2	0.2	-
C18:0	40.9	39.4	46.7	35.9	6.7
C18:1	33.4	35.8	33.2	33.2	34.7
C18:2	0.5	0.6	0.9	2.9	3.9
C18:3	1.6	1.8	0.2	0.9	0.1
C20:0	-	0.1	0.2	0.2	0.5

[a]Data obtained from Nesaretnam & Ali (1992). (With permission).
[b]Abbreviations are as described; SMP, slip melting point; FFA, free fatty acid; FAC, fatty acid content.

Table 2.7 Triacylglycerol (TAG) composition of illipe fat[a]

Lipid type	Illipe fat
Monounsaturated TAGs[b]	
PLLn	-
POL/SLL	-
POO/SOL	0.2 ± 0.1
SOO	1.0 ± 0.0
PLL	-
Disaturated	
PSLn	-
SPO	36.0 ± 0.2
SOS	53.1 ± 0.8
PPL	-
PPO	6.6 ± 0.1
SOA	-
Trisaturated	
Unknown	3.1 ± 0.3

[a]Data obtained from Illiyin et al. (2013). (With permission).
[b]Fatty acid abbreviations are as described; L, lauric; P, palmitic; S; stearic, O; oleic, L, linoleic; Ln, linolenic; A, arachidonic.

Table 2.8 Food applications and desired composition of various structured lipids[a,b]

Product type	Applications	Desired composition
Cocoa butter alternatives	• Bakery products • Chocolate • Confectionary products	sn-POP TAGs sn-POS TAGs sn-SOS TAGs
Diaclyglycerols (DAGs)	• Anti-obesity effects • DAG oils • Emulsifiers	sn-1,3 DAG
Human milkfat (HMF) analogues	• Increased lipid absorption for infants • Infant formula • Infant formula enriched with ARA and DHA	sn-OPO TAGs and other TAGs containing ARA, DHA, and MCFAs
Medium and long chain triacylglycerols (MLCTs)	• Parental and enteral feeding • Rapid energy source • Treatment of lipid malabsorption and metabolic syndrome	sn-MLM TAGs
Monoacylglycerols (MAGs)	• Emulsifiers	2-MAG
Reduced calorie fats	• Snack foods • *trans*-Free margarines • Shortening • Sauces • Baked chips • Baked goods • Reduces health implications of *trans*-fatty acids	TAGs containing SCFAs
Structured phospholipids (PLs)	• Emulsifiers • Phospholipids enriched with n-3 and MCFAs • More bioavailable carrier of fatty acids	PLs with 1 acyl group attached PLs enriched with various fatty acids

[a]Data was recreated and adapted from Akoh and Kim (2008) and Sproston (2016).
[b] Abbreviations are as described; TAG, triacylglycerol; M, medium chain fatty acid; L, long chain fatty acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; P, palmitic acid; O, oleic acid; S, stearic acid; SCFA, short chain fatty acid; MCFA, medium chain fatty acid.

Table 2.9 Reported x-ray diffraction data for cocoa butter[a]

Willie & Lutton (1966)		Witzel & Becker (1969)		Riiner (1970)		Chapman et al. (1971)		Hicklin et al. (1986)	
Short spacing[b]	Poly-morph	Short spacing	Poly-morph	Short spacing	Poly-morph	Short spacing	Poly-morph	Short spacing	Poly-morph
4.19 VS, 3.70 S, 4.24 VS	I	-	-	4.19 VS, 3.72 M,	β′2	4.17 S, 3.87 M,	I	4.19 VS, 3.70 S, 4.25 S	I
4.24 VS	II	4.21	α	4.25 (d) S	α	4.24 VS	II	4.63 M, 4.25 S, 3.87 M	II
4.92 VW, 4.62 W, 4.25 VS, 3.86 S	III	4.66, 4.33, 4.22, 3.86	β′1	-	-	4.20 VS, 3.87 W	III	4.35 VS, 4.17 W	III
4.35 VS, 4.15 W, 3.97 M, 3.81 M	IV	4.58,4.33, 4.16	β′2	4.61 S, 3.90 W, 3.77 W	β′1	4.32 S, 4.13 S 3.88 W, 3.75 M	IV	5.43 M, 4.60 VS, 3.99 M, 3.88 W, 3.76 M, 3.68 W	IV
5.40 M, 5.15 W, 4.58 VS, 4.23 VW, 3.87 M, 3.75 W, 3.67 W, 3.39 VW	V	5.42, 4.59, 3.98, 3.85, 3.76, 3.67	Pre β	4.61 S, 3.90 W, 3.77 W	β	4.58 VS, 4.22 W, 3.98 S, 3.87 M, 3.73 M, 3.65 S	V		V
5.43 M, 5.15 W, 4.59 VS, 4.27 VW, 4.04 W, 3.86 M, 3.70 S, 3.36 VW	VI	5.44, 4.59, 4.00, 3.86, 3.70	β			4.53 VS, 4.21 W, 4.01 W, 3.84 M, 3.67 S	VI	5.47 M, 5.16 W, 4.60 VS, 4.28 W, 4.04 M, 3.88 S, 3.71 S	VI

[a]Data obtained from d′Souza, Deman and Deman (1990). (With permission).
[b]Abbreviations are as described; VS, very strong; S, strong; M, medium; W, weak; VW, very weak;

Figure 2.1 Fluctuation of global cocoa prices from 1959-2018. Image obtained from https://tradingeconomics.com/commodity/cocoa

Figure 2.2 Illipe nuts. Image obtained from Workshop Jaringan Tengkawang Report (2016).

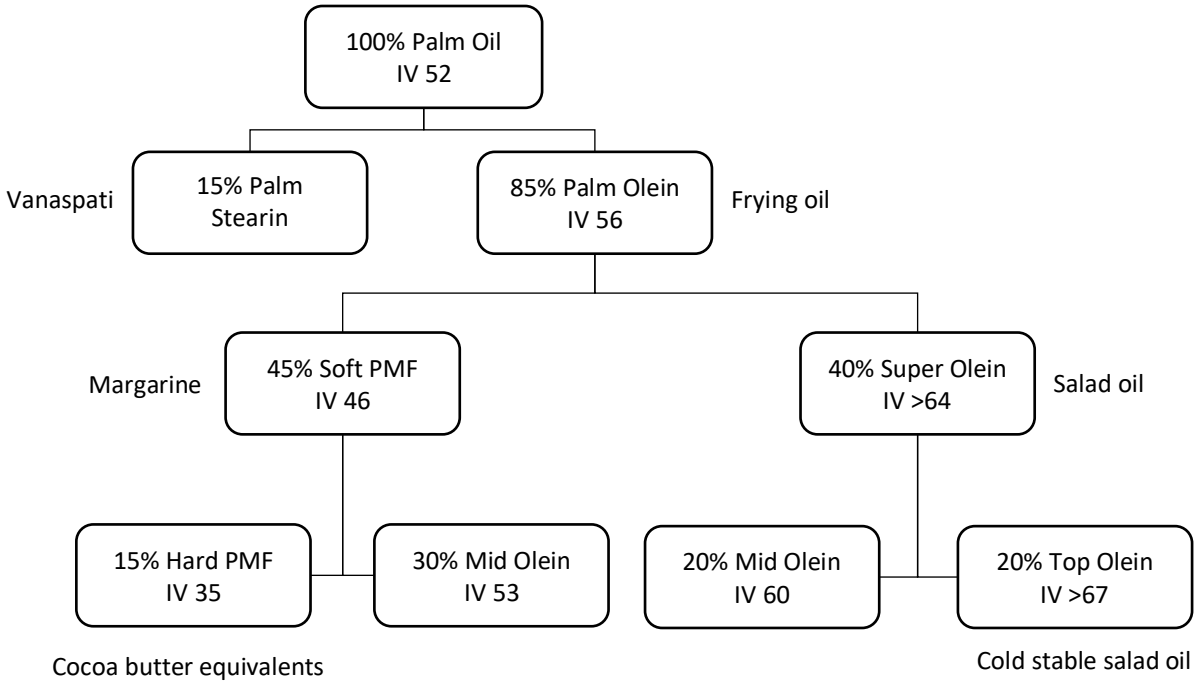

Figure 2.3 Multistage fractionation of palm oil with possible food applications for the various fractions. Image was recreated and adapted from Calliauw (2011).

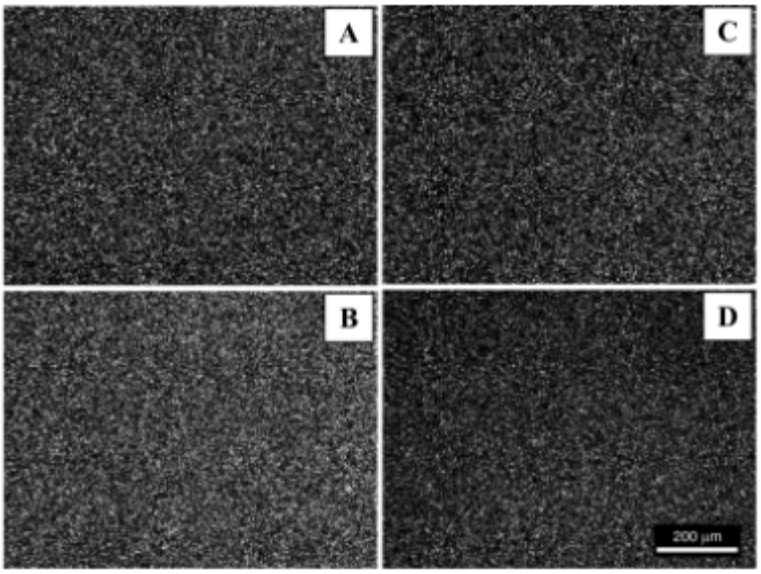

Figure 2.4 Images obtained by PLM of the α form of cocoa butter crystallized at -20 ºC for 1 day (A), -20 ºC for 7 days (B), -15 ºC for 7 days (C), and 0 ºC for 1 day. Image as reported by Marangoni & McGauley (2003). (With permission).

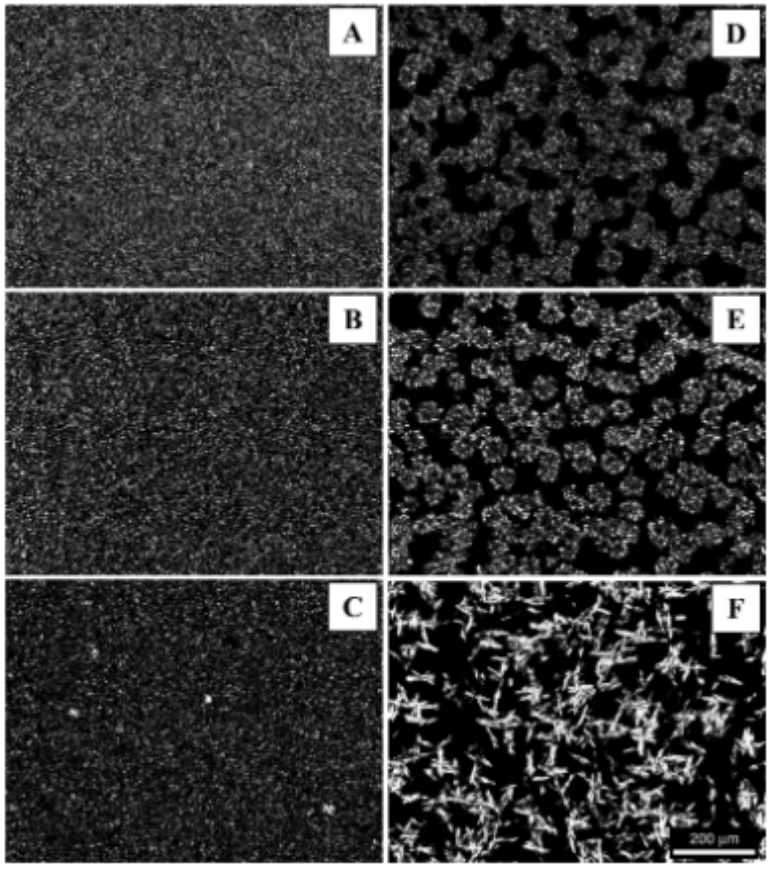

Figure 2.5 Micrographs of the β′ form obtained by static crystallization at 0 ºC for 14 days (A), 10 ºC for 5 days (B), 15 ºC for 14 days (C), 20 ºC for 1 day (D), 22 ºC for 1 day (E), and 24 ºC for 3 days. Image as reported by Marangoni & McGauley (2003). (With permission).

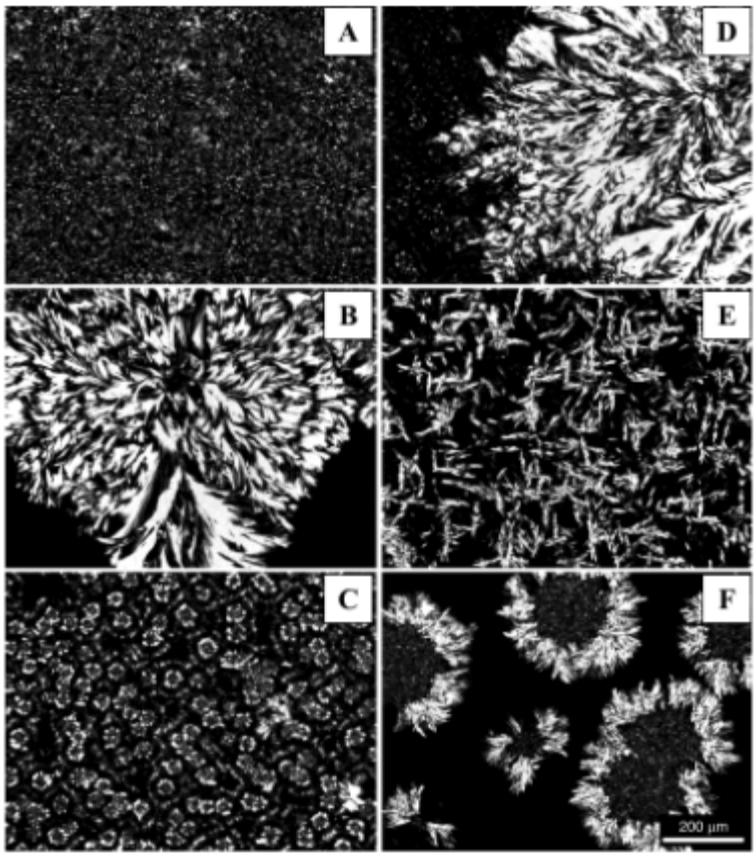

Figure 2.6 Micrographs of the β form obtained by static crystallization at 20 ^{o}C for 28 days (A), 22 ^{o}C for 28 days (C and D), 26 ^{o}C for 28 days (E and F). Image as reported by Marangoni & McGauley (2003). (With permission).

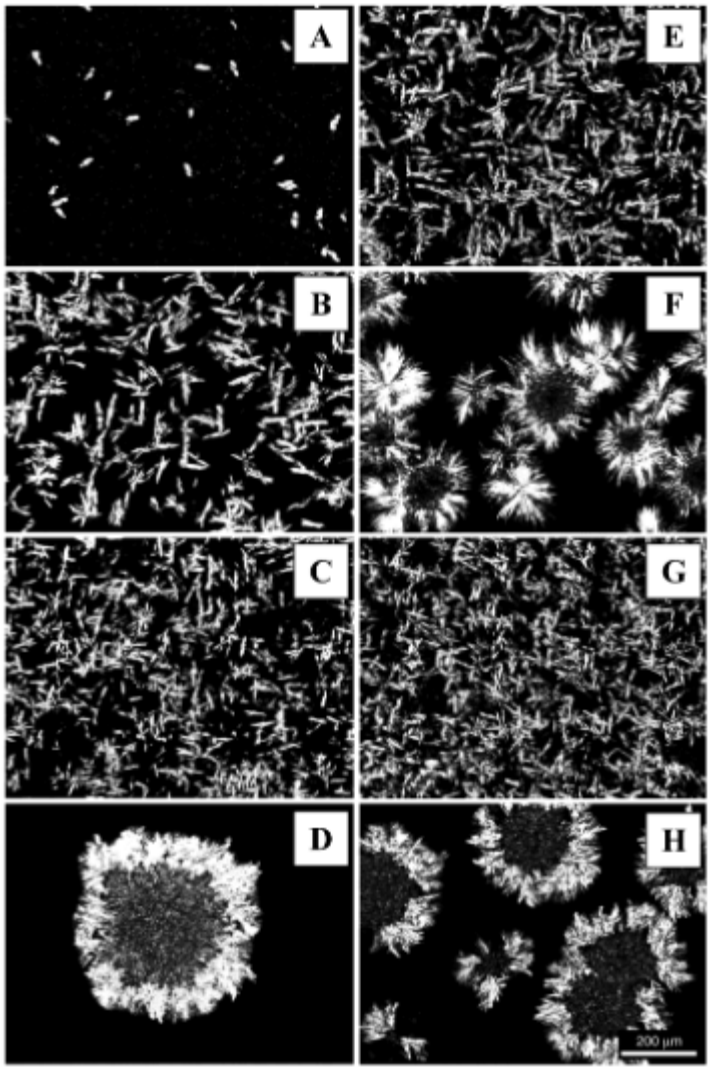

Figure 2.7 PLM images of cocoa butter statically crystallized at 26°C for 1 (A), 5 (B), 7 (C), 14 (D), 21 (E) and 28 days (F). Image as reported by Marangoni & McGauley (2003). (With permission).

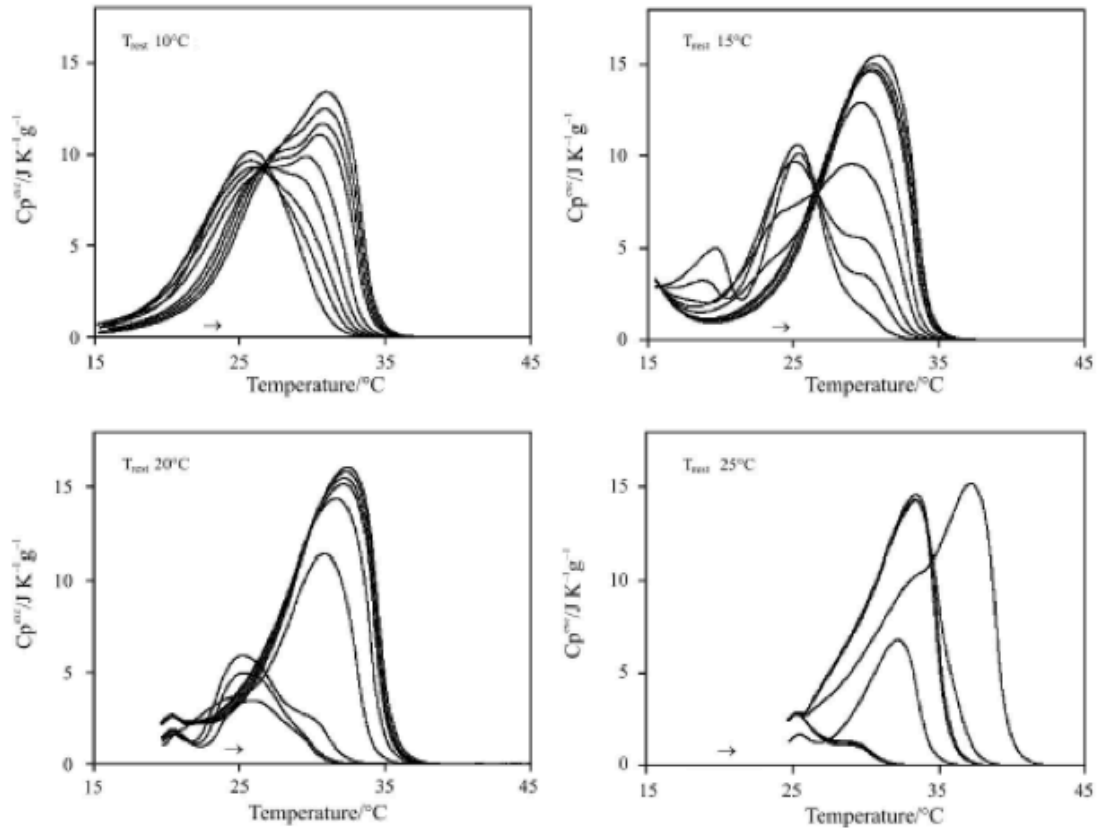

Figure 2.8 DSC traces (heating run at 2 K min^{-1}) of cocoa butter samples cooled from the molten state (50°C) at 2 K min-1 and kept at rest for various lapses of time (5, 10, 15, 30, 60 min, 3, 5, 10, 20 h) at four different resting temperatures T_{rest} (10, 15, 20 and 25°C). Image as reported by Fessas et al. (2005). (With permission).

CHAPTER 3

SYNTHESIS OF A COCOA BUTTER EQUIVALENT BY ENZYMATIC INTERESTERIFICATION OF ILLIPE BUTTER AND PALM MID-FRACTION

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Abstract

This study aims to synthesize a cocoa butter equivalent (CBE) structured lipid from a blend of illipe butter (IB) and palm mid-fraction (PMF) by means of enzymatic interesterification using *Rhizomucor miehei sn*-1,3 specific lipase, Lipozyme® RM IM (Novozymes North America, Inc., Franklinton, NC, USA) as the biocatalyst. Physical and chemical attributes of the CBE and cocoa butter (CB) were analyzed. The synthesized CBE matched triacylglycerol (TAG) profile range of a commercial CB and is therefore hypothesized to show similar physical and chemical characteristics with CB. TAG profile, fatty acid constituents, melting and cooling behavior, polymorphism and crystal morphology were determined using high-performance liquid chromatography (HPLC), gas chromatography (GC), differential scanning calorimetry (DSC), X-ray diffraction (XRD), and polarized light microscopy (PLM), respectively. Four enzymatically interesterified blends of IB:PMF at different weight ratios were analyzed for their TAG profiles, and a ratio of IB:PMF 10:3 (%, w/w) at 5% enzyme load and 30 minutes reaction time gave similar TAG results with CB. The TAG values of the IB:PMF 10:3 interesterified product (IP) were: 1,3-dipalmitoyl-2-oleoylglycerol (POP) at 19.1 ± 1.0%, 1-palmitoyl-2-oleoyl-3-stearoylglycerol (POS) at 42.7 ± 1.0% and 1,3-distearoyl-2-oleoylglycerol (SOS) at 29.9% ± 0.3%. The melting and cooling profile of IP and CB showed no significant difference. X-ray diffraction of IP and CB displayed similar dominant peaks at 4.6 Å, representing a β polymorph. Both CB and IP have similar granular spherulitic crystals.

Keywords: Cocoa butter equivalent, illipe butter, palm mid-fraction, enzymatic interesterification, structured lipid, Lipozyme® RM IM

Abbreviation List

CB	:	Cocoa butter
CBE	:	Cocoa butter equivalent
CBI	:	Cocoa butter improver
IB	:	Illipe butter
IP	:	Interesterified product
PB	:	Physical blend
PMF	:	Palm mid-fraction
POP	:	1,3-Dipalmitoyl-2-oleoylglycerol
POS	:	1-Palmitoyl-2-oleoyl-3-stearoylglycerol
SOS	:	1,3-Distearoyl-2-oleoylglycerol
SFC	:	Solid fat content
TAG	:	Triacylglycerol

Introduction

Cocoa butter (CB) is used in confectionery products to make chocolate. It is characterized by a sharp melting temperature at around body temperature (35-37 ^{o}C). The reason for its sharp melting temperature is due to the unique triacylglycerol (TAG) composition of CB. CB consists of three main TAG molecular species: 15-19% of 1,3-dipalmitoyl-2-oleoylglycerol (POP; P = palmitic, O = oleic), 36-41% of 1-palmitoyl-2-oleoyl-3-stearoylglycerol (POS; S = stearic), 24-31% of 1,3-distearoyl-2-oleoylglycerol (SOS) (Lipp et al., 2001). This specific range of TAG species composition contributes to the physical and sensory features of CB.

Due to the variation in crop yield, limited supply and high demand for cacao beans, the price of CB often fluctuates. CB is the most expensive ingredient in the production of chocolate. To address this issue, cocoa butter equivalents (CBEs) and cocoa butter substitutes (CBSs) are often used to reduce cost and to improve functionality. CBEs are non-lauric plant fats which have similar physical and chemical properties to CB and mixable without altering the properties of cocoa butter (Lipp et al., 2001). CBSs are lauric plant fats that are chemically different from CB but have some of the same physical properties when blended with CB. Illipe, sal, shea, kokum, mango kernel fat and palm oil are the vegetable fats that are permitted to be used as a CBE in the European Union Directive 2000/36/EC (Stewart & Kristott, 2004).

Structured lipids (SLs) are lipids that have been modified from their natural biosynthetic forms by chemical or enzymatic methods (Kim & Akoh, 2015). Without enzymatic interesterification, it is difficult to obtain a CBE that resembles the TAG profile of CB. There are many studies that have produced CBS and cocoa butter improvers (CBI) that can increase hardness and melting point of the cocoa butter blend (Nirupam Biswas et al., 2017; Maheshwari & Yella Reddy, 2005). However, CBS and CBI can only be added in a certain amount before altering the properties of the chocolate product. There have only been a few published studies that produced CBEs with similar TAG profile with CB. Undurraga, Markovits, & Erazo (Undurraga et

al., 2001) produced CBE through enzymatic reaction of palm mid-fraction (PMF) and stearic acid with Novo lipase Lipozyme® and was able to achieve CBE TAG profile close to CB.

Illipe (*Shorea sp*) is an endemic plant originating from the forest of Borneo Island. Other names of illipe include tengkawang, engkabang or Borneo tallow. *Shorea sp* belongs to the Dipterocarpaceae family, a group of plants that produces two-winged fruits. One of the major species utilized for butter production is the *Shorea stenoptera,* mainly found in the tropical lowland rainforest of Borneo island, emerging as a large forest species that reach heights of 40-70 meters. *S. stenoptera* contains 41- 48% fat, with 98% being TAG (Blicher-Mathiesen, 1994). Illipe butter (IB) has a low POP (6.6%), high POS (36.0%) and high SOS (53.1%) (Illiyin et al., 2013). This characteristic makes IB an excellent substrate with potential for CBE synthesis. In most CBE production, PMF is used as a POP donor as PMF has a high POP level (50 – 70%). Due to a high SOS level, IB may serve as a CBI as well.

There have been only a few reports using IB to enhance its properties as a CBE. Nasaretnam & Ali (Nesaretnam & Ali, 1992) characterized the physicochemical properties of several species of Shorea and made CBE blend from IB:PMF that has a soft consistency. In this project, IB and PMF were enzymatically interesterified to produce a CBE that closely resembled the TAG profile of CB. PMF was used as POP donor to increase the POP level in IP. To distinguish the effect of enzymatic interesterification, a physical blend of the chosen ratio was used for comparison. Fatty acid composition, TAG profile, melting and crystallizing profile, solid fat content, polymorphism, and crystal morphology were analyzed.

Materials and Methods

Materials

CB and IB were purchased from JEdwards International (Braintree, MA, USA). Palm mid-fraction was bought from Fuji Vegetable Oil (Savannah, GA, USA). Lipozyme® RM IM (*Rhizomucour miehei* lipase immobilized on an acrylic resin carrier) with a specific activity of 442 IUN g^{-1} (interesterification unit per gram) as specified by manufacturer) was donated by

Novozymes North America, Inc (Franklinton, NC, USA). One IUN is the amount of enzyme activity that liberates 1 μmol of butyric acid from tributyrin per minute under defined standard conditions. TAG reference standard mixtures (GLC-437 and GLC 570) and GLC-461 FAME mix were purchased from Nu-check Prep, Inc. (Elysian, MN, USA). All other reagents and solvents were of analytical or HPLC grade and purchased from Fisher Chemical (Norcross, GA, USA), Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), and J. T. Baker Chemical Co. (Phillipsburg, NJ, USA).

Preparation of Fat Mixture

The TAG profile of IB and PMF were first analyzed and based on the results, a gram-scale reaction scheme was planned. 5 g total mixture of IB and PMF at different ratios were mixed. The mixture of IB:PMF (w/w) were: A (10:2), B (10:2.5), C (10:3), and D (10:3.5). Each mixture was reacted by addition of 10% (based on the total weight of substrates) Lipozyme® RM IM in a 60 ^{o}C shaking water bath at 200 rpm. These reaction parameters were chosen as it was shown to be the best conditions for interesterification reaction using Lipozyme® RM IM while taking into consideration reaction time and enzyme cost (Zou et al., 2014). Samples were pulled at 2, 4, 6, and 8 hours. Reactions were stopped by filtering the sample through anhydrous sodium sulfate column. Free fatty acids (FFA) were removed through alkaline deacidification by modification of AOCS Official Method Ca 5a-40 (AOCS, 2011).

TAG Molecular Species

TAG molecular species of samples were analyzed using Agilent 1100 HPLC system (Agilent Technologies Inc, Santa Clara, CA, USA) equipped with a Sedex 55 evaporative light scattering detector (ELSD) and a 250 mm x 4 mm, 5 μm particle size, Ultrasphere C18 reverse-phase analytical column. Acetonitrile (A) and acetone:methyl tert-butyl ether (90:10, v/v) (B) was used to create a linear gradient elution as shown in Table 3.1 The flow rate was set at 0.7 ml/min. The column temperature was set at 25 ^{o}C, ELSD drift tube was set at 50^{o}C, nebulizer gas pressure was set at 350000 Pa (3.5 bar) and gain at 12. 20 μL of sample was injected at a concentration of

2 mg/mL in acetone. The retention times of TAG molecular species were determined by comparing with the equivalent carbon number (ECN) of TAG standards and also with previously published results (Buchgraber, Ulberth, & Anklam, 2000; Dionisi et al., 2004). TAG molecular species percentage was then determined from the peak area of the chromatogram.

Liter-Scale Synthesis

Liter-scale synthesis was carried out in a 1-L stir batch reactor under vacuum with a circulating water bath at 60 ^{o}C. Substrate and enzyme were mixed at 200 rpm using an SL 2400 StedFast stirrer (Fischer Scientific Co., Fair Lawn, NJ, USA) fitted with a 4-blade propeller. The reaction was stopped by filtering through a Buchner funnel containing filter paper and anhydrous sodium sulfate. The reaction product was passed through short path distillation to remove FFA as described by Moore & Akoh (Moore & Akoh, 2017). Percent yield was calculated based upon product weight before and after short path distillation. FFA content as oleic acid equivalents was determined according to AOCS Official Method Ca 5a-40 (AOCS, 2011).

Fatty Acid Composition and Positional Analysis

Reaction products were first converted to fatty acid methyl esters (FAME) following AOAC official method 996.01 with modifications described by Alvarez & Akoh (Álvarez & Akoh, 2015). *Sn*-2 positional fatty acid was prepared by using pancreatic lipase, following a method described by Ifeduba & Akoh (2013). Fatty acid profile was analyzed with Agilent 6890 N GC System (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and Supelco SO-2560 capillary column (100 m x 0.25 mm ID 0.2 μm film) (Sigma-Aldrich Co. St. Louis, MO, USA). C17:0 was the internal standard and GLC-461 FAME mix was the external standard. Helium was the carrier gas at a flow rate of 1.1 mL/min. The average mol percentage and standard deviations were calculated.

Thermal Behavior

Thermal behavior of the products was analyzed with 204 F1 Phoenix DSC (Netszch Instruments North America, Burlington, MA, USA) with Proteus thermal analysis software.

Nitrogen gas was used at a flow rate of 20 mL/min. Instrument was calibrated with indium. 5-9 mg of samples were hermetically sealed in an aluminum pan. All of the pans were then heated at 50 ^{o}C for 10 min and then stabilized following IUPAC method (Paquot & Hauntfenne, 1987) with slight modification. All samples were cooled to 4 ^{o}C for 2 h, 24 ^{o}C for 48 h, and finally 4 ^{o}C for 2 h. An empty hermetically sealed aluminum pan was used as the reference blank. Heating run started at 22 ^{o}C, cooled down to -50 ^{o}C at 10 ^{o}C/min, held at -50 ^{o}C for 5 min, and heated up to 50 ^{o}C/min at 10 ^{o}C /min. Crystallizing run started at 22 ^{o}C, heated up to 80 ^{o}C at 20 ^{o}C /min, held at 80 ^{o}C for 10 min, and then cooled down to -75 ^{o}C at -5 ^{o}C /min, and held for 30 min.

Polymorphism

Samples were first melted at 50 ^{o}C and poured into an XRD sample well. Samples were then stabilized following IUPAC method with slight modification as described above. Polymorphic form of the fat crystal was determined with a Bruker D8 Advance X-ray Diffractometer (Billerica, MA, USA) fitted with a Co-Kα radiation (k = 1.7889 Å), voltage 35 kV and current 40 mA. Samples were analyzed at 2θ angles from 15^{o} – 35^{o} with a scan rate of 0.05 sec/step. Short (d) spacing was determined using EVA-diffraction software (Billerica, MA, USA).

Solid Fat Content

Solid fat content was analyzed using a Benchtop NMR MQC Analyzer (Oxford Instruments, Oxfordshire, UK) following the AOCS Official Method Cd 16b-93 (Society & Firestone, 1994) for stabilizing confectionary fats. Samples were melted at 80 ^{o}C for 15 min and filled in NMR tube to 1.5 cm height. Samples in NMR tube were heated at 60 ^{o}C for 5 min, and then tempered using the following profile: 4 ^{o}C for 2 h, 26 ^{o}C for 48 h, 4 ^{o}C for 2 h. Samples in the NMR tube were then held for 60 min from 10-40 ^{o}C at 5 ^{o}C intervals before analysis.

Polarized Light Microscopy

Crystal morphology was analyzed using a Leica DMLB upright compound microscope (Wetzlar, Germany) with a polarizing filter. Spot Idea camera and software (Sterling Heights, MI,

USA) was used to obtain the images. Glass slide and samples were first melted at 60 °C for 10 min to erase crystal memory. 15 µL from each sample was then pipetted to the center of the glass slide and covered with a coverslip. The slides were allowed to cool and crystallize at 20 °C for 5 days and then the crystal structure was analyzed.

Statistical Analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) using JMP Pro 13 software (Cary, NC, USA). Tukey′s test was used to determine the significant differences at a level of $P < 0.05$. All experimental data were obtained by true treatment replication in triplicates.

Results and Discussion

Gram-Scale Time Course Reaction

A time course reaction at gram-scale was conducted to determine the best blend ratio and reaction time to synthesize CBE. At all ratios, the starting blend had a low POP (7.4 ± 0.5%) and high SOS (46.6 ± 0.4%) value, corresponding to the TAG values in IB (Figure. 3.1). POP percentage increased over the course of the reaction and stabilized at 4 h. POP stabilized at different percentages based on the ratio of PMF in the blend. POP levels of A (10:2), B (10:2.5), C (10:3), and D (10:3.5) stabilized at 14.7 ± 0.5%, 17.4 ± 1.2%, 19.0 ± 0.6%, and 24.0 ± 0.7%, respectively (Figure 3.1a). Blends with higher PMF ratio showed POP stabilization at higher percentage due to a greater amount of POP contributed by PMF in the blend. POS value did not show a clear trend over the reaction times (Figure 3.1b). However, the difference between final and initial POS values of all the blends were less than 5%. SOS value showed a decreasing trend over the course of the reaction and stabilized at 3 h. Blends with lower PMF ratio have SOS stabilization at a higher percentage. SOS levels of A (10:2), B (10:2.5), C (10:3), and D (10:3.5) stabilized at 40.7 ± 0.9%, 35.5 ± 0.9%, 32.6 ± 1.1%, and 30.9 ± 0.9%, respectively (Figure 3.1c). This can be explained because high PMF ratio blends have lower illipe butter mass, hence lower SOS values. From this small-scale experiment, the blend C (10:3) at 2 h reaction time resulted in

the POP, POS, and SOS values of 21.4 ± 0.7, 43.6 ± 0.5 and 35.0 ± 0.1%, respectively. This blend and reaction time was chosen to be scaled up.

Liter-Scale TAG Molecular Species

Liter-scale synthesis of the selected blend C (10:3) was performed for 2 h. However, the resulting TAG ratio from the liter-scale synthesis did not match the small-scale reaction results. POS level was above 50% (Table 3.2). This result was probably due to a more effective mixing of a stirrer in the liter-scale reaction compared to a shaking water bath in the gram-scale test tube reaction. After several adjustments, it was determined that using IB:PMF ratio of 10:3 for a reaction time of 30 min and with 5% enzyme load instead of 10% was able to give an interesterified product (IP) with TAG closely resembling cocoa butter (Table 3.2). A physical blend (without enzymatic interesterification) of IB:PMF at 10:3 (PB) was also prepared to compare the effect of enzymatic interesterification on physicochemical properties of the CBE. The TAG molecular species of IP, PB, IB, PMF and commercial CB can be seen in Table 3.3 The TAG molecular species of commercial CB and IB were consistent with previous studies (Illiyin et al., 2013; Lipp et al., 2001) . The major TAG molecular species of IP were POP (18.3 ± 1.5%), POS (41.6 ± 0.9%) and SOS (29.8 ± 3.4%). The percentage of these three major TAG in IP closely resembled CB. In PB, the major TAG molecular species were POP (16.8 ± 0.4%), POS (33.6 ± 0.9%) and SOS (37.9 ± 0.7%). Compared to IP and CB, the POS value of PB was slightly lower while the SOS values was higher. The POS and SOS values of PB resembled IB, which shows the absence of enzymatic interesterification. From comparing the TAG result of IP and PB, it is likely that stearic acids from SOS were cleaved by the lipase to form other TAG moieties, most notably POS.

Fatty Acid Composition

Total and *sn-2* positional fatty acid composition of substrates and products are shown in Table 3.4 Palmitic, oleic and stearic acids were the major constituent fatty acids in IP, PB, and CB. Both products (IP and PB) do not have noticeable differences in fatty acid composition

compared with CB. IB had a high percentage of total stearic acid (40.6 ± 0.2%) while palmitic acid was the major fatty acid in PMF (44.5 ± 1.2%). After blending IB and PMF at 10:3 ratio, the total fatty acid profile of IP and PB were comparable to that of CB. Enzymatic interesterification does not change the total fatty acid profile, therefore the fatty acid profile of IP and PB were very similar. The fatty acid values of IB, PMF, and CB were consistent with previous results (Lipp et al., 2001; Nesaretnam & Ali, 1992). Naturally, oleic acid is the main component at the *sn*-2 position across all samples. PB has a higher percentage of *sn*-2 stearic acid than its substrates (6% more than IB). This might occur due to acyl migration of stearic acid from the *sn*-1,3 position to the *sn*-2 position. The extent of acyl migration is influenced by reaction parameters such as time and temperature. Previous study has shown that acyl migration of stearic acid (7-10%) from *sn*-1,3 position to the *sn*-2 position in a physical blend using a similar reaction parameter occurred (Ifeduba Martini, & Akoh, 2016).

Thermal Behavior

The onset melting temperature for IP, PB, IB, and CB were 32.7, 34.2, 34.3 and 30.9 ^{o}C, respectively (Table 3.5). Only IP had an onset melting temperature that is not significantly different from CB. This shows that IP can be substituted for CB without changing melting properties. PB and IB have a higher temperature stability, with melting onset starting at 34^{o}C. The crystallizing completion temperature for IP, PB, IB, and CB were 6.3, 8.2, 10.7 and 6.3 ^{o}C, respectively (Table 3.5). IB completed crystallization at 10.7 ^{o}C, which is the highest temperature among the other samples. IP and CB completely crystallized at 6.3 with no significant difference. This further showed that the thermal behavior of IP and CB were alike and therefore IP can be used for CB substitution without altering melting and crystallizing behaviors significantly. PB on the other hand showed a significantly higher melting onset and crystallization completion temperatures than IP and CB. The higher thermal stability and faster crystallization of IB and PB may be due to a higher percentage of SOS triacylglycerol. Triacylglycerol with more stearic fatty acid is shown to have higher melting point (Lutton, 1951).

Polymorphism

XRD patterns and polymorphic structures of IP, PB, IB, and CB are shown in Figure 3.2. Assignments of polymorphs were based on published short spacing characteristics of CB (van Malssen et al., 1999) : α = 4.24 Å, β' = 3.80, 4.20 Å, and β = 4.60 Å. All samples displayed a very strong peak at 4.60 Å, medium peaks from 3.67-4.00 Å and a very weak peak at 4.20 Å, indicating a dominant crystallization of a β polymorph. For CB and IB, this result is in agreement with previous studies (Illiyin et al., 2013; van Malssen et al., 1999) as CB and IB have been shown to be able to crystallize in β polymorph. PMF was reported to crystallize as a β' polymorph (Biswas, Cheow, Tan, & Siow, 2016), but in IP and PB, the peak at 4.20 Å which indicated a β' polymorph was very weak and almost unnoticeable. This might be due to the very low PMF ratio being used in the IP and PB blend, compared to the high ratio of IB used in the blend. It is important for CBE to be able to crystallize in a β polymorphic structure in order for the resulting chocolate to have a hard texture and yet be able to melt at the desired melting point near body temperature.

Solid Fat Content

SFC profile of each sample is shown in Figure 3.3 Naturally, IB had the most solid fat content due to its higher POS and SOS content. The SFC in CB was consistent with previous results (Torbica et al., 2006). At 25 ^{o}C, IP had the lowest SFC at 62.2 ± 1.0%. PB and CB have similar SFC at 72.6 ± 0.7% and 73.6 ± 0.4%, respectively. However, at 30 ^{o}C, CB has the lowest SFC at 12.6 ± 2.4%. The SFC of IP and PB were 22.3 ± 2.2% and 30.3 ± 1.4%, respectively. At 35 ^{o}C, the SFC of IB was 14.4 ± 2.1%, while all the other samples were at 1%. At 40 ^{o}C, all the samples have completely melted. According to Nilson (Nillson, 1986) SFC values measured below 25 ^{o}C indicate hardness, values calculated in the range of 25 and 30 ^{o}C indicate resistance to heat, values calculated between of 27 - 33 ^{o}C present intense melting of cocoa butter while bringing cooling sensation and flavor release in the mouth, while SFC values at temperatures above 35 ^{o}C involve the presence of waxy fat fraction. In this case, PB had better heat resistance

than CB and IP, while IB had a fat fraction that may induce a waxy taste. One desirable property of a cocoa butter is to be able to melt rapidly in the mouth. Although PB has a higher thermal resistance, the SFC data suggest that IP would be able to display a faster mouthfeel melt, as IP has a lower SFC than PB in the range of 27-33^{o}C. On the other hand, IB was shown to be a good candidate as a CBI as it has the highest SFC level at all temperature points.

Crystal Morphology

Crystal microstructures of samples were observed using PLM at 24 ^{o}C after 5 days. According to Marangoni & McGauley (2003) β polymorph could display different microstructures in which the morphology are no longer uniform. The presence of large microstructure also indicates that β polymorph was obtained by a phase transition from β′ (Marangoni & McGauley, 2003). All samples showed large microstructures indicating a β morphology which might have been obtained by a phase transition from β′. IP crystal microstructures were shown to have predominantly granular crystals. These small granulites are located in the center, while larger featherlike crystallites (50 μm) appear in the periphery (Figure 3.4a). A similar pattern can be seen in PB, where the center of the crystals was predominantly granular, with featherlike crystallites in the periphery. However, in PB, the featherlike periphery crystals were much larger (100-200 μm) and dominated more area inwards (Figure 3.4b). IB showed predominant small spherulites, which were individually larger and denser than the granule crystals in IP and PB (Figure 3.4c). CB showed a mixture of granular crystals and small spherulites, without the presence of large microstructures (Figure 3.4d). Crystal formation is a chronological process, and from these images, we can observe that IB crystals formed the earliest, indicated by the predominant dense spherulites. This is consistent with the finding of crystallization completion data discussed in ′Thermal Behaviour′. In terms of crystal morphology, PB had a more similar structure with IB, while IP was more similar to CB. This supports the functional properties of IP as a CBE.

Conclusion

A CBE was synthesized from IB and PMF through a lipase-catalyzed enzymatic interesterification at a ratio of 10:3. The product (IP) had similar fatty acids profile and TAG molecular species as with CB. IP did not show a significant difference in terms of melting onset and crystalization completion temperatures with CB, meaning that IP could be used as a CBE without altering the thermal behavior of CB. This was further supported by the ability of IP to crystallize as β polymorph. SFC and crystal morphology of IP were also shown to be similar to CB. The physical blend of IB:PMF at 10:3 ratio was shown to be thermally more stable than both IP and CB, and therefore may potentially be utilized as a CBI.

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Table 3.1 Mobile phase elution gradient. Solvent flow is set at 0.7 mL/min

Time (min)	Acetonitrile (%, v/v)	Acetone: MTBE (9:1) (%, v/v)
0	35	65
5	35	65
10	30	70
25	24	76
30	5	95
35	35	65

MTBE = methyl tert-butyl ether

Table 3.2 Relative TAG composition (area%[a]) of blend C (10:3) in a liter-scale time course reaction[b] using 10 and 5% enzyme load

		TAG[c]		
Enzyme (%)	Time (min)	POP	POS	SOS
10	0	21.1 ± 1.4	40.2 ± 1.8	37.7 ± 2.6
	30	20.0 ± 0.7	41.2 ± 1.3	29.8 ± 1.4
	60	18.9 ± 1.1	47.1 ± 1.4	26.2 ± 1.3
	120	16.0 ± 1.9	59.5 ± 2.1	24.7 ± 1.2
5	0	19.8 ± 0.4	42.0 ± 1.5	37.4 ± 1.3
	30	19.1 ± 1.0	42.7 ± 1.0	29.9 ± 0.3
	60	22.1± 1.3	45.0 ± 0.8	26.2 ± 0.2
	120	20.2 ± 0.5	50.4 ± 0.3	24.0 ± 0.3

Mean ± SD, n=3

[a]Area% were calculated from the HPLC chromatogram result

[b]Reaction condition: substrate molar ratio 10:3 (IB:PMF), temperature of 60 ^{o}C, stir rate of 200 rpm in a liter-scale reactor

IB = illipe butter, PMF = palm mid-fraction

[c]Letters correspond to the following FA: P = palmitic, S = stearic, O = oleic

Table 3.3 Relative triaclglycerol (TAG0 composition (area%[a]) of interesterified product (IP), physical blend (PB), illipe butter (IB), palm mid-fraction (PMF), and cocoa butter (CB)

TAG[b]	ECN (DB)[c]	IP[d]	PB	IB	PMF	CB
PLO	46 (1)	0.3 ± 0.1	0.7 ± 0.1	ND	6.0 ± 0.1	ND
PLP	46	0.6 ± 0.2	0.8 ± 0.1	ND	7.4 ± 0.0	1.1 ± 0.0
OOO	48 (3)	ND	ND	0.4 ± 0.0	ND	ND
POO+PLS	48 (2), 48	3.8 ± 2.2	4.0 ± 0.9	ND	22.0 ± 0.7	5.0 ± 1.0
POP	48 (1)	18.3 ± 1.5	16.8 ± 0.4	7.3 ± 0.2	50.7 ± 0.7	17.6 ± 1.9
PPP	48	ND	ND	ND	1.1 ± 0.0	ND
SOO+SLS	50 (2), 50	2.3 ± 1.5	4.6 ± 0.7	7.1 ± 0.9	ND	9.3 ± 1.1
POS	50 (1)	41.6 ± 0.9	33.6 ± 0.9	35.2 ± 0.7	5.8 ± 0.0	40.2 ± 0.6
PPS	50	ND	ND	ND	ND	0.2 ± 0.1
SOS	52 (1)	29.8 ± 3.4	37.9 ± 0.7	47.0 ± 0.2	0.3 ± 0.0	25.7 ± 0.8
PSS	52	ND	ND	0.1 ± 0.0	ND	0.1 ±0.1
SOA+SSS	54 (1), 54	0.4 ± 0.2	0.7 ± 0.1	1.4 ± 0.1	ND	0.6 ± 0.2

Mean ± SD, n=3, ND = not detected.

[a]Area% were calculated from the HPLC chromatogram result

[b]Letters correspond to the following FA: P = palmitic, S = stearic, O = oleic, L = linoleic, and A = arachidonic acid.

[c]Equivalent carbon number (ECN) = TC-2 x DB; TC is the total carbon number of acyl group; DB is the total number of double bonds in TAG.

[d]Reaction condition for IP: substrate molar ratio 10:3 (IB:PMF), enzyme load of 5% w/w, temperature of 60 ^{o}C, and time of 30 min, stir rate of 200 rpm in a liter-scale reactor

Table 3.4 Relative total and *sn*-2 positional fatty acid composition (mol%) of interesterified product (IP), physical blend (PB), illipe butter (IB), palm mid-fraction (PMF), and cocoa butter (CB)

Fatty acid	IP total	*sn*-2	PB total	*sn*-2	IB total	*sn*-2	PMF total	*sn*-2	CB total	*sn*-2
C12:0	0.1 ± 0.0	0.5 ± 0.3	0.1 ± 0.1	1.3 ± 0.6	Trace	Trace	0.2 ± 0.0	1.0 ± 0.3	ND	ND
C14:0	0.4 ± 0.1	0.7 ± 0.4	0.3 ± 0.1	1.2 ± 0.0	0.1 ± 0.0	Trace	1.0 ± 0.0	0.9 ± 0.1	0.1 ± 0.0	ND
C16:0	25.9 ± 0.1	12.5 ± 2.9	26.4 ± 0.2	13.9 ± 0.8	20.2 ± 0.2	5.8 ± 0.3	44.5 ± 1.2	17.4 ± 3.3	28.9 ± 0.0	4.2 ± 0.7
C18:0	33.0 ± 0.1	7.2 ± 3.3	34.2 ± 0.2	11.6 ± 0.6	40.6 ± 0.2	6.1 ± 0.7	5.5 ± 0.7	2.2 ± 1.3	33.3 ± 0.0	4.4 ± 1.0
C18:1	32.5 ± 0.6	76.3 ± 5.3	32.7 ± 0.4	70.2 ± 0.5	35.3 ± 0.0	83.1 ± 1.8	36.2 ± 0.3	55.7 ± 2.6	32.7 ± 0.1	82.4 ± 1.6
C18:2	5.1 ± 0.6	2.8 ± 1.6	3.3 ± 0.6	1.8 ± 0.0	1.7 ± 0.1	3.1 ± 3.0	10.9 ± 1.2	21.7 ± 2.2	0.8 ± 0.1	ND
C20:0	3.1 ± 0.2	ND	2.2 ± 0.0	ND	1.8 ± 0.0	1.9 ± 0.7	0.7 ± 0.2	ND	3.3 ± 0.0	8.9 ± 0.2

Mean ± SD, n=3, ND = not detected, trace = < 0.1 mol%

Table 3.5 Melting and crystallizing characteristics of interesterified product (IP), physical blend (PB), illipe butter (IB) and cocoa butter (CB)

Sample	Melting profile			Crystallizing profile		
	Onset (°C)	Completion (°C)	ΔH (J/g)	Onset (°C)	Completion (°C)	ΔH (J/g)
IP	32.7 ± 0.3[ab]	37.9 ± 0.6[a]	122.4 ± 11.6[a]	16.1 ± 0.1[a]	6.3 ± 0.3[a]	-78.6 ± 3.0[a]
PB	34.2 ± 0.3[a]	39.7 ± 0.6[a]	110.2 ± 5.9[a]	17.4 ± 0.4[b]	8.2 ± 0.2[b]	-77.1 ± 5.7[a]
IB	34.3 ± 0.5[a]	40.0 ± 0.6[a]	116.5 ± 6.0[a]	18.2 ± 0.2[d]	10.7 ± 0.2[c]	-86.4 ± 10.1[a]
CB	30.9 ± 1.4[b]	38.6 ± 1.3[a]	134.3 ± 12.6[a]	15.5 ± 0.1[c]	6.3 ± 1.1[a]	-83.8 ± 3.1[a]

Values are mean ± SD, n = 3
[a-d] Means in the same column with different subscripts are significantly different.

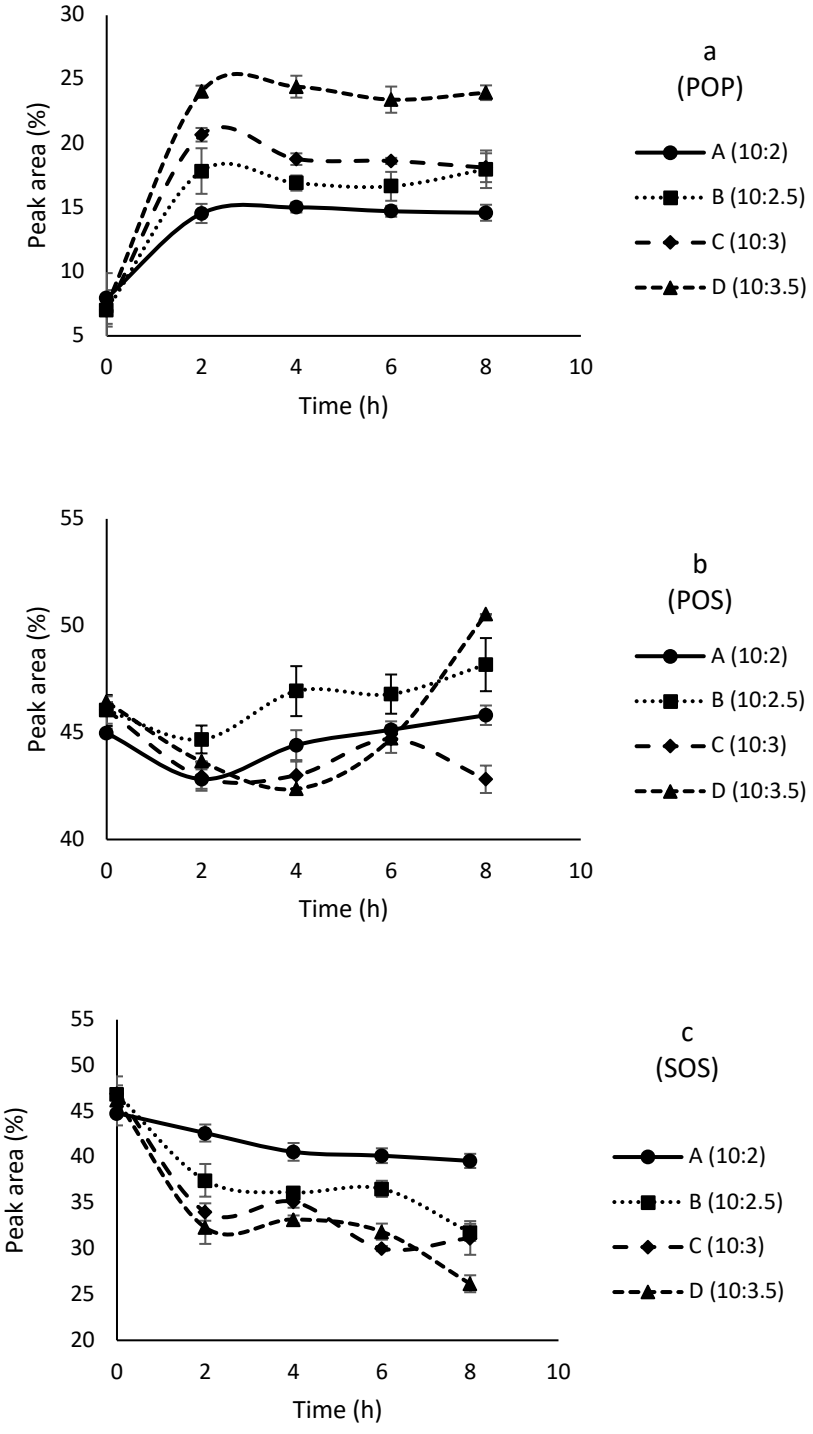

Figure 3.1 Time course showing changes in (a) POP, (b) POS and (c) SOS levels during e interesterification of illipe butter (IB) and palm mid-fraction (PMF) using four different bl (IB:PMF) in a gram-scale reaction

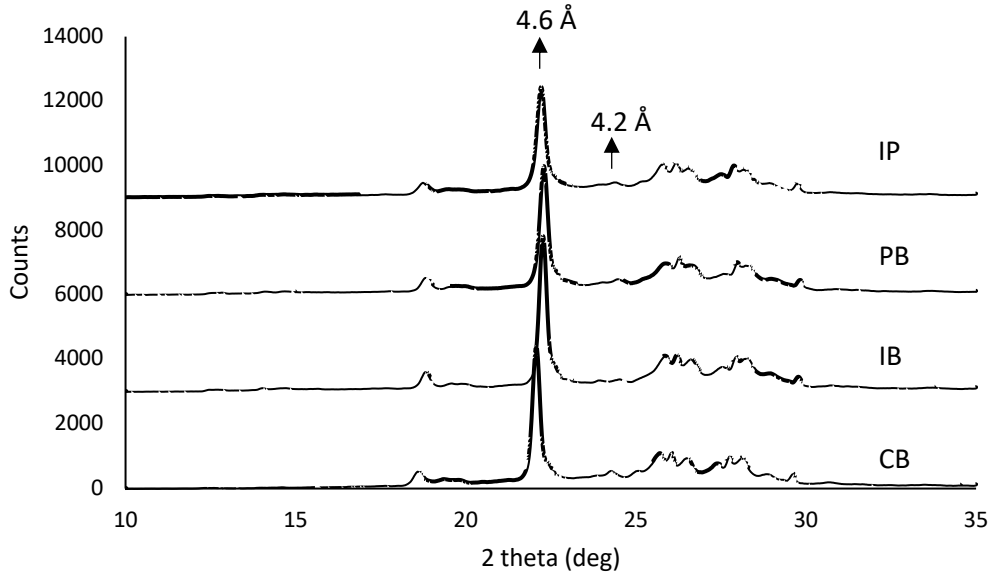

Figure 3.2 X-ray diffraction patterns of interesterified product (IP), physical blend (PB), illipe butter (IB) and cocoa butter (CB)

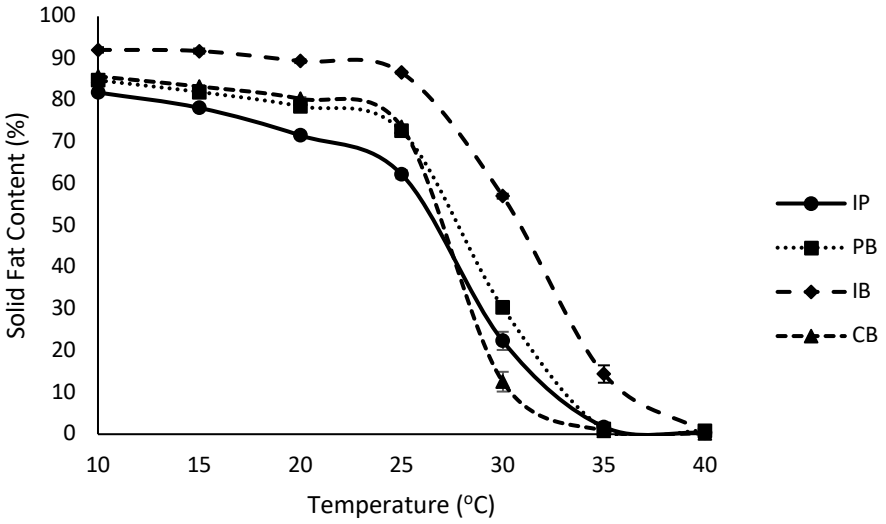

Figure 3.3 Solid fat content of interesterified product (IP), physical blend (PB), illipe butter (IB) and cocoa butter (CB)

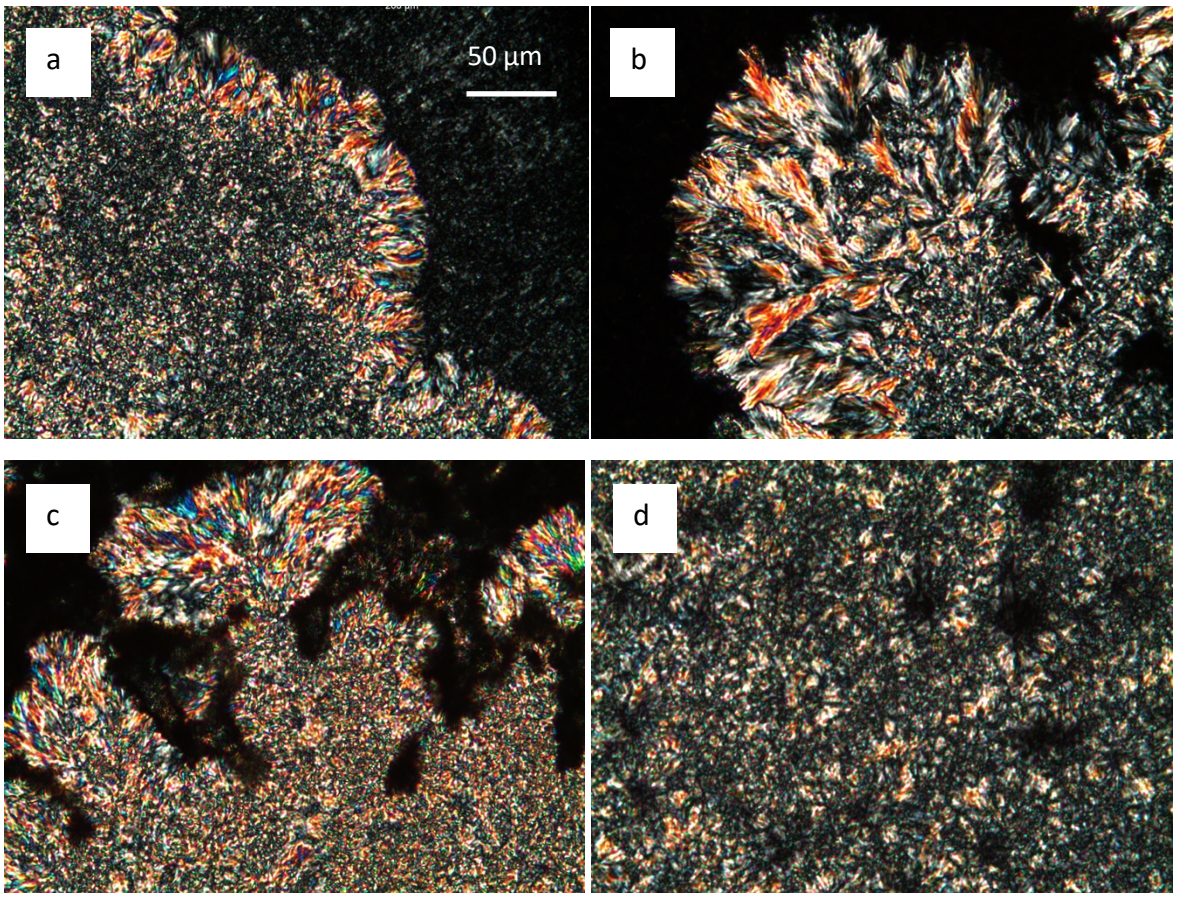

Figure 3.4 Polarized light microscopy images (x20) of a interesterified product (IP), b physical blend (PB), c illipe butter (IB), and d cocoa butter (CB) after 5 days at 20 ^{o}C

CHAPTER 4

TEXTURE, RHEOLOGY AND FAT BLOOM STUDY OF 'CHOCOLATES' MADE FROM COCOA BUTTER EQUIVALENT SYNTHESIZED FROM ILLIPE BUTTER AND PALM MID-FRACTION

Bahari, A., & Akoh, C. C. (2018). *LWT-Food Science and Technology*, doi:10.106/j.lwt.2018.07.013. Reprinted with permission from Elsevier.

Abstract

Cocoa butter equivalent (CBE) enzymatically synthesized from illipe butter and palm mid fraction (IP), its non-interesterified physical blend (PB), illipe butter (IB), and cocoa butter (CB) were used to make dark (D) and white (W) 'chocolates'. Their particle size distribution, texture, and rheology were compared. White 'chocolate' made with illipe butter (WIB) had the highest melting completion temperature of 39.4 ^{o}C. D_{90} and $D_{(4,3)}$ values in both dark and white 'chocolate' from CBE and CB were not significantly different. Hardness was measured in terms of breaking strength and both dark and white 'chocolates' made from IP and CB displayed similar breaking strength while PB had the lowest breaking strength. Only dark 'chocolate' was used to measure fat bloom. Fat bloom was delayed in dark 'chocolate' made with illipe butter (DIB), and it was further delayed in dark 'chocolate' made with cocoa butter (DCB) and dark 'chocolate' made with interesterified product (DIP) when 0.5% sugar ester was added. This study suggests that CBE from illipe butter and palm mid-fraction can be used to make dark and white 'chocolate' with similar textural, particle size, rheological and fat bloom properties with CB. DIB and the addition of 0.5% sugar ester in chocolates may also be used to delay fat bloom development.

Keywords: Rheology, fat bloom, dark chocolate, cocoa butter equivalent, illipe butter

Abbreviation List:

CB	:	Cocoa butter
CBE	:	Cocoa butter equivalent
DCB	:	Dark cocoa butter chocolate
DIP	:	Dark interesterified product ‘chocolate’
DIB	:	Dark illipe butter ‘chocolate’
DPB	:	Dark physical blend ‘chocolate’
IP	:	Interesterified product
IB	:	Illipe butter
PB	:	Physical blend
PSD	:	Particle size distribution
WCB	:	White cocoa butter chocolate
WIB	:	White illipe butter ‘chocolate’
WIP	:	White interesterified product ‘chocolate’
WPB	:	White physical blend ‘chocolate’

Introduction

Cocoa butter equivalents (CBEs) are alternative fats and oils sources that are fully compatible with the physical and sensory properties of cocoa butter (CB) (Lipp et al., 2001). CBEs are mostly similar in triacylglycerols (TAGs) composition to that of CB, but often not identical. They can be made by mixing certain ratios of special tropical fats such as shea, illipe, or palm oil fraction. However, in order to obtain a CBE that has a closely similar TAG composition with CB, *sn*-1,3 specific lipase is often used to modify the blend mixture. In a previous study (Bahari & Akoh, 2018), a CBE from illipe butter and palm mid-fraction was synthesized by enzymatic interesterification reaction using immobilized Lipozyme® RM IM lipase. The TAG profile of the interesterified product were very similar to CB (Table 4.1). Since the fat profile of CBEs may not be identical to CB, it is important to test whether 'chocolate' made with CBEs will alter the physical properties of chocolates made with CB. It should be noted that 'chocolate' containing non-cocoa butter are regulated in their labelling of the term chocolate. 'Chocolate' mentioned in this study is a chocolate-like confectionary product as it would not be classified as chocolate in the US or EU because it contained or exceeded the 5% limit for permitted foreign fat.

One property that determines the mouthfeel and texture of chocolate is its particle size distribution. Particle size of the final chocolate product directly impacts the mouthfeel and texture after chocolate has melted in the mouth. Chocolate with significant number of particle diameter larger than 30 μm often impart a gritty feel to the tongue. To produce chocolate with a creamy and silky texture, a particle size of 20 to 30 μm is desired (Beckett, 2008).

Another property to be taken into consideration is its rheological properties, such as viscosity and yield stress. Viscosity can be considered as an internal friction to movement. Like a Bingham fluid, chocolate has a yield stress, where a considerable amount of force is needed to start the flow of chocolate. Once chocolate starts flowing, it becomes thinner (less viscous) as

more force is used. To simplify the rheological data for factory use, the Casson mathematical method is widely used to describe chocolate rheological properties. A viscometer measures a few point on the curve, and two flow parameters: casson yield value and casson plastic viscosity were obtained through an equation (Beckett, 2008). The Casson yield stress is the energy required to start the chocolate to flow. High yield stress means the chocolate is able to stand by itself, whereas low yield stress is more favored as a chocolate coating. Casson plastic viscosity signifies the energy that is needed to keep the chocolate moving after it has started to flow.

Another desirable property of a chocolate is a glossy hard texture with a distinct snap. The breaking strength of chocolate bar can be determined using a three-point bend rig. Force is applied to the center of the chocolate while the two ends are fixed at 2 points. The amount of force needed to snap the chocolate is defined as the breaking strength (force / unit width).

The surface gloss of the chocolate depends on how well the chocolate was tempered. A well-tempered chocolate could keep its gloss for months at a proper storage temperature. If a chocolate is not well tempered, grey-white specks would gradually appear and take over the surface of the chocolate. This white manifestation is called fat bloom. Although fat bloom is not harmful to health, it is a cosmetic flaw to chocolate products and is the number one quality problem in the chocolate industry. Two major theories revolve around fat bloom formation. The phase separation theory explains that higher melting TAGs could separate out of the matrix and form the bloom. On the other hand, the polymorphic transition theory is based upon transition of βV into a more stable βVI polymorph. This theory came about because studies found the presence of βVI polymorph on chocolates with fat bloom (Lonchampt & Hartel, 2004).

Emulsifiers have been known for their function to inhibit fat bloom. First, they increase the melting point of the fat, producing a better thermal resistance of the product (Wilson, 1999). Second, they improve crystallization by increasing the number of seeds crystals (Arruda & Dimick, 1991). Third, they are able to prevent transition of fat crystals into βVI polymorph which are associated with fat bloom (Nakae, Kometani, Nishimura, Takii, & Okada, 2000).

This research elucidates the particle size distribution, rheological data, hardness of dark and white 'chocolates' and fat bloom formation of dark 'chocolate' made from a cocoa butter equivalent (CBE) synthesized by the enzymatic interesterification of illipe butter and palm-mid fraction. The impact of sucrose fatty acid ester as an emulsifier on chocolate bloom inhibition was also tested.

Materials and Methods

Materials

Interesterified product (IP) and physical blend (PB) from a previous study (Bahari & Akoh, 2018) were used. Their TAG profile is shown in Table 4.1. Cocoa liquor was donated by Cargill (Milwaukee, WI, USA). Cocoa butter (CB) and illipe butter (IB) was purchased from JEdwards (Braintree, MA, USA). Confectioners 10-X powdered sugar and powdered skim milk were purchased from local Kroger supermarket (Athens, GA, USA). Liquid lecithin was purchased from Fearn Natural Products (Mequon, WI, USA). Ryoto sugar ester S-170 sucrose fatty acid ester as emulsifier or antibloom agent was donated by Mitsubishi Chemical America (White Plains, NY, USA). S-170 sugar ester is lipophilic, having an HLB value of 1. The stearic acid composition of S-170 sugar ester is approximately 70%, while the di, tri, and polyester composition is approximately 100% (Corporation, 1999). All other reagents and solvents used were of analytical or HPLC grade.

Preparation of Chocolate

Dark and white 'chocolates' were prepared according to the recipe of Beckett (Beckett, 1999) (Table 4.2). Added fat was either IP, PB, CB or IB. It should be noted that the 'chocolate' containing IP, PB, and IB are regulatorily not classified as chocolate in the US or EU because it contained or exceeded the 5% limit for permitted foreign fat. Since dark 'chocolate' was made using cocoa liquor which contains 50% CB, white 'chocolate' was also prepared in order to explore the full properties of IP, PB, and IB in a confectionary product (0% CB). Dark 'chocolate' contains a total of 32% fat (50% of chocolate liquor and 12% from added fat). All the

ingredients were put into a CocoaT Melanger ECGC-12SL (Cocoatown, Roswell, GA, USA) and mixed for 15 min after the last ingredient was added. 'Chocolate' was then tempered using Revolation 2 tempering machine (Chocovision, Poughkeepsie, NY). 'Chocolates' were then analyzed or set into molds. Molds were shaken to minimize air bubbles in the chocolate. Chocolate in the molds were left overnight at 20 ^{o}C and sealed into a plastic bag and stored at 4 ^{o}C for hardness analysis or wrapped in aluminum foil and stored for 3 days at 20 ^{o}C for fat bloom analysis. A 1-cm thick mold was used for hardness analysis and a commercial mold was used for fat bloom analysis.

Thermal Behavior

Melting temperature range of the 'chocolates' were analyzed with 204 F1 Phoenix DSC (Netszch Instruments North America, Burlington, MA, USA) with Proteus thermal analysis software. Nitrogen gas was used at a flow rate of 20 mL/min. The instrument was calibrated with indium. Five to nine mg of samples were hermetically sealed in an aluminum pan. An empty hermetically sealed aluminum pan was used as the reference blank. The heating run started at 22 ^{o}C, cooled down to -50 ^{o}C at 10 ^{o}C/min, held at -50 ^{o}C for 5 min, and heated up to 50 ^{o}C/min at 10 ^{o}C /min. Crystallizing run started at 22 ^{o}C, heated up to 80 ^{o}C at 20 ^{o}C /min, held at 80 ^{o}C for 10 min, and then cooled down to -75 ^{o}C at -5 ^{o}C /min, and held for 30 min.

Particle Size Distribution

Particle size distribution of the dark and white 'chocolates' were analyzed using a Beckman Coulter Laser Diffraction Particle Analyzer LS 13 320 (Brea, CA, USA). Two grams of 'chocolate' was diluted in 50mL isopropyl alcohol (RI 1.3776) and vortexed for 2 min. Sample was loaded into the particle analyzer until an obscuration of 0.2 was achieved. The size distribution was measured as relative volume of particles. The data that was reported were distribution below 90% (D_{90}), median (D_{50}), distribution below 10% (D_{10}), and volumetric area mean ($D_{(4,3)}$).

Texture Analysis

Hardness in terms of breaking strength was measured using a TA-XT2i Texture Analyzer (Stable Microsystems, Godalming, UK) three-point bend rig. Samples were conditioned by holding at 20 ^{o}C for 1 h before measuring. The following perimeters were used: pre-test speed was set at 1 mm/s, test speed at 1.1 mm/s, post-test speed at 10 mm/s, rupture test distance at 4 mm, distance at 10 mm, force at 100 g, time at 5 s, count at 5, load cell at 25 kg, and trigger force at 20 g. The breaking strength (kg/cm) was reported.

Rheology

Rheological properties of the dark and white 'chocolates' were measured using a Model Discovery HR-2 hybrid rheometer (TA Instrument Inc., New Castle, DE, USA) with a bob and cup geometry. Bob and cup diameter were 31.09 and 37.00 mm, respectively. Samples were first melted at 50 ^{o}C for 60 min and transferred to the cup. Rheological measurements were taken at 40 ^{o}C. Temperature was maintained by using a recirculating water bath. The measurement cycle started with preconditioning at 40 ^{o}C for 60 s. Sample was then pre-sheared at 5 s^{-1} for 10 min. Shear stress was analyzed with increasing shear rate from 5 to 50 s^{-1} within 2 min, and then decreasing from 50 to 5 s^{-1}. Fifty measurements were taken within each ramp. TRIOS software (TA Instrument Inc., New Castle, DE, USA) was used to calculate the Casson plastic viscosity and and Casson yield stress.

Fat Bloom

Dark 'chocolates' were subjected to temperature cycling, alternating at 20 ^{o}C for 16 h and at 30 ^{o}C for 8 h. After 15 days, the temperature was changed to alternate at 20 ^{o}C for 12 h and at 30 ^{o}C for 12 h to accelerate the fat bloom process. Temperature cycling was programmed in using Intellus Model E41HO (Percival, Perry, IA, USA). L*, a*, and b* color space was analyzed by measuring random surface of the chocolate every three days for a month, and the 52nd day during the 20 ^{o}C incubation period using Konica Minolta CR-300 Colorimeter (Konica Minolta, Tokyo, Japan). Whiteness index (WI) of the 'chocolate' was measured using the following equation:

$$WI = 100 - \sqrt{(100 - L)^2 + a^2 + b^2}$$

L^* = lightness value

a^* = green-red color value

b^* = blue-yellow color value

Statistical Analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) using JMP Pro 13 software (Cary, NC, USA). Tukey′s test was used to determine the significant differences at a level of $P < 0.05$. All experimental data were obtained in triplicates except for fat bloom analysis, where it was done in duplicates.

Results and Discussion

Thermal Behavior

Thermal analysis by differential scanning calorimetry for dark and white 'chocolate' of all fat samples can be seen in Table 4.3. In 'dark chocolate', there were no significant differences in melting onset and completion temperatures between all fat samples. The melting onset range for dark 'chocolate' samples was from 29.3 to 31.2 ^oC, while the melting completion range was from 36.3-38.0 ^oC. Cocoa liquor was used for making dark 'chocolate', and from the manufacturer′s data, the cocoa liquor contained 50% CB. All dark 'chocolate' samples have similar melting range, and this could be due to the mutual composition of 50% cocoa butter from the cocoa liquor. The melting range of DCB was similar to published results (Ali, Selamat, Man, & Suria, 2001; Loisel, Lecq, Keller, & Ollivon, 1998). For white 'chocolate', the melting onset for all samples were not significantly different and ranges from 29.6-31.7 ^oC. The melting completion for WIP, WPB, and WCB were 36.8 ^oC, 37.6 ^oC, and 36.2 ^oC, respectively. WIB had a higher melting completion than the other white 'chocolates' at 39.4 ^oC. This was expected as IB contains higher stearic acid content in its triacylglycerols compared to CB. (Bahari & Akoh,

2018; Illiyin et al., 2013). Thermal behavior data suggested that all dark and white 'chocolate' samples were well tempered.

Particle Size Distribution

Particle size distribution histograms for dark and white 'chocolate' samples are presented in Figure 4.1. Dark 'chocolates' showed unimodal distribution with mode at around 10 µm (Figure 4.1a). This data corresponds to previous studies that showed a similar unimodal distribution for dark 'chocolates' at 10 µm (Afoakwa, Paterson, & Fowler, 2008). In white 'chocolate', the histogram showed a bimodal distribution, with modes at around 0.9 and 10.5 µm (Figure 4.1b). The mode at 10.5 µm suggests similar particle size to dark 'chocolate', while the mode at 0.9 µm correspond to particles in skim milk (Jhanwar & Ward, 2014). The particle size distribution (PSD) value for various parameters can be seen in Table 4.4. In order for chocolates to have a smooth silky texture, particle size distribution should be below 30 µm. In dark 'chocolate', D_{10} was around 1.2 to 1.5 µm with the largest D_{10} value in DCB. D_{50} values range from 7.9 to 10.1 µm, with the largest D_{50} value in DIP. D_{90} values range from 24.8 to 30.4 µm. Other studies have shown dark chocolate at similar particle size having similar D_{10}, D_{50}, D_{90} and $D_{(4,3)}$ values (Afoakwa et al., 2007; Do, Hargreaves, Wolf, Hort, & Mitchell, 2007).

White 'chocolates' have a smaller particle size distribution, possibly due to the absence of cocoa solids. D_{10} values ranges from 0.7 to 0.8 µm. D_{50} values ranges from 4.6 to 4.7 µm. D_{90} values range from 21.5 to 28.3 µm. $D_{(4,3)}$ values range from 8.3 to 10.8 µm, with the lowest value in WPB. These data did not show much difference on the effect of replacing CB with CBE in terms of particle size distribution, therefore similar mouthfeel experience could be achieved using the CBE.

Texture Analysis

The hardness of 'chocolates' can be seen in Figure 4.2. White chocolates have a lower breaking strength than dark 'chocolates'. However, the trend for each 'chocolate' types was similar. The breaking strength for DIP, DPB, DIB, and DCB were 15.0, 11.9, 16.6, and 15.7

kg/cm, respectively. DIB had the highest breaking strength, while DPB had the lowest breaking strength. Hardness of DIP and DPB were not significantly different to DCB. The breaking strength for WIP, WPB, WIB, and WCB were 9.9, 8.8, 12.8, and 11.4 kg/cm, respectively. WIB had the highest breaking strength, while WPB had the lowest breaking strength. When comparing the white 'chocolate' made from CBE, only WIP had a similar hardness to WCB, whereas WPB showed significantly lower hardness than WCB.

Although other studies have shown that structured lipid as cocoa butter alternatives usually decrease the hardness of chocolate possibly due to eutectic softening effect (Osborn & Akoh, 2002; Zarringhalami, Sahari, Barzegar, & Hamidi-Esfehani, 2010), our data shows that the incorporation of IP into 'chocolate' did not affect the hardness of the chocolate. However, PB might have some eutectic effect with CB as DPB displayed softer texture.

Rheology

Casson plastic viscosity and yield stress are shown in Table 4.5. DIB had the highest viscosity (6.1 Pa.s), DCB had an intermediate viscosity (5.5 Pa.s), while DIP and DPB had similar viscosity values (5.0 Pa.s). One plausible explanation why DIB had the highest viscosity might be due to the high stearic acid-containing TAG in DIB. Noureddini, Teoh, & Clements (Noureddini, Teoh, & Clements, 1992) showed that stearic acid has the highest viscosity among other saturated fatty acids at the same temperature. The viscosity values in white 'chocolate' were lower than that of dark chocolate, possibly due to the absence of cocoa solids. In white 'chocolate', WCB had the highest plastic viscosity (3.2 Pa.s), whereas WIP, WPB, and WIB were 2.0, 2.5, and 2.4 Pa.s, respectively. For Casson yield stress, there was no significant differences between samples of dark and white 'chocolates'. The yield stress error was quite high because the first experimental trial gave a higher result than the next two replicates. Since chocolate is a thixotropic compound, it took time after the first trial to go back to the original viscosity. Although IB resulted in a higher viscosity when incorporated into 'chocolate', DIP did not show

significant difference with DCB and could act as a CBE without impacting the chocolate flow property.

Fat Bloom

Figure 4.3 shows the level of fat bloom in terms of WI in dark 'chocolate' with different fat types. DIP, DPB, and DCB were the first to develop distinct fat bloom at 18 days of storage and showed similar occurrence patterns. All of these three samples maintained a stable high WI values over the course of the experiment. DIB developed distinct fat bloom formation after 30 days. However, the WI was still significantly less than the first occurrence of fat bloom in DIP, DPB, or DCB. Fat bloom kept on increasing in DIB until it reached similar values to DIP, DPB, and DCB after 52 days. Both chocolates that contained sugar ester as emulsifier or bloom inhibitor (CBSE and IPSE) showed a longer delay of fat bloom formation (Figure 4.3). IPSE was shown to be the most resistant to fat bloom development, with WI of only 29.8 after 52 days. In comparison, CBSE had a WI of 42.1 after 52 days. Photograph of fat bloom development in all the chocolate samples can be seen in Figure 4.4.

The fat bloom development might be accelerated due to the change in temperature program at the 15th day, where the exposure to 30 ^{o}C was increased from 8 h to 12 h. Among the dark chocolates without addition of sugar ester, DIB was the most resistant to fat bloom. Studies has shown that increasing melting point of the fat (Buscato et al., 2018; Weyland, 1992) was able to delay fat bloom formation. IB had a higher stearic acid content than CB, therefore a higher melting point and stronger resistance to fat bloom was expected and indeed observed. Emulsifiers have also been known to resist the formation of fat bloom. Previous literature showed that incorporating sorbitan monostearate was successful in delaying fat bloom formation in dark chocolate (Buscato et al., 2018; Easton, Kelly, Bartron, Cross, & Griffin, 1952; Garti, Schlichter, & Sarig, 1986). Emulsifiers have been reported to slow down crystallization (Smith & Povey, 1997) and have an inhibitory effect on βV-βVI transition (Siew & Ng, 1996). Emulsifiers are generally permissible in chocolate under a level of 1.5% total mass, however each emulsifier′s

maximal concentration is also regulated through the Codex Alimentarius (Lonchampt & Hartel, 2004).

Conclusion

A good cocoa butter equivalent must be compatible with cocoa butter and should not alter the physical properties when used in chocolate formulation. Previously, the physicochemical properties of IP were elucidated and shown to have similar properties with CB. This study translated those similar physicochemical properties from the fat and applied it in a chocolate product. Our results showed that the incorporation of IP into both dark and white 'chocolate' gave similar texture, particle size distribution, and hardness with CB. On the other hand, IP could also be used as a non-chocolate confectionary product with similar physical properties to chocolate. The difference between IP and its non-interesterified version PB was only found in the textural property, where PB 'chocolates' were found to have lower hardness. In addition, we found that illipe butter was able to delay fat bloom in 'chocolates'. It was shown that the addition of 0.5% sugar ester can strongly resist fat bloom formation in dark chocolate.

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Table 4.1 Relative TAG composition (area%[a]) of interesterified product (IP), physical blend (PB), illipe butter (IB), palm mid-fraction (PMF), and cocoa butter (CB) (as reported by Bahari & Akoh, 2018)

TAG[b]	ECN (DB)[c]	IP[d]	PB	IB	PMF	CB
PLO	46 (1)	0.3 ± 0.1	0.7 ± 0.1	ND	6.0 ± 0.1	ND
PLP	46	0.6 ± 0.2	0.8 ± 0.1	ND	7.4 ± 0.0	1.1 ± 0.0
OOO	48 (3)	ND	ND	0.4 ± 0.0	ND	ND
POO+PLS	48 (2), 48	3.8 ± 2.2	4.0 ± 0.9	ND	22.0 ± 0.7	5.0 ± 1.0
POP	48 (1)	18.3 ± 1.5	16.8 ± 0.4	7.3 ± 0.2	50.7 ± 0.7	17.6 ± 1.9
PPP	48	ND	ND	ND	1.1 ± 0.0	ND
SOO+SLS	50 (2), 50	2.3 ± 1.5	4.6 ± 0.7	7.1 ± 0.9	ND	9.3 ± 1.1
POS	50 (1)	41.6 ± 0.9	33.6 ± 0.9	35.2 ± 0.7	5.8 ± 0.0	40.2 ± 0.6
PPS	50	ND	ND	ND	ND	0.2 ± 0.1
SOS	52 (1)	29.8 ± 3.4	37.9 ± 0.7	47.0 ± 0.2	0.3 ± 0.0	25.7 ± 0.8
PSS	52	ND	ND	0.1 ± 0.0	ND	0.1 ±0.1
SOA+SSS	54 (1), 54	0.4 ± 0.2	0.7 ± 0.1	1.4 ± 0.1	ND	0.6 ± 0.2

Mean ± SD, n=3, ND = not detected.

[a]Area% were calculated from the HPLC chromatogram result.

[b]Letters correspond to the following FA: P = palmitic, S = stearic, O = oleic, L = linoleic, and A = arachidonic acid.

[c]Equivalent carbon number (ECN) = TC-2 x DB; TC is the total carbon number of acyl group; DB is the total number of double bonds in TAG.

[d]Reaction condition for IP: substrate molar ratio 10:3 (IB:PMF), enzyme load of 5% w/w, temperature of 60 ^{o}C, and time of 30 min, stir rate of 200 rpm in a liter-scale reactor.

Table 4.2 Formulation of dark and white 'chocolates'. Additional fat used was either interesterified product (IP), physical blend (PB), illipe butter (IB), or cocoa butter (CB)

	Composition (g)					
	Cocoa liquor	Skim milk	Additional fat	Sugar	Soy lecithin	Sugar ester
Dark 'chocolate'	40		12	47.5	0.5	
White 'chocolate'		20	32	47.5	0.5	
Dark 'chocolate' for fat bloom analysis	40		12	47.5	0.5	0.5

Table 4.3 Melting characteristics of dark (D) and white (W) 'chocolate' with different fat sources: interesterified product (IP), physical blend (PB), illipe butter (IB) and cocoa butter (CB)

Sample	Melting profile		
	Onset (°C)	Completion (°C)	ΔH (J/g)
DIP	29.8 ± 0.1a	37.8 ± 0.6a	36.8 ± 4.2a
DPB	29.3 ± 0.3a	37.0 ± 0.6a	30.8 ± 6.7a
DIB	31.2 ± 0.4a	38.0 ± 2.1a	39.2 ± 15.7a
DCB	30.7 ± 1.8a	36.3 ± 0.6a	39.0 ± 3.3a
WIP	30.2 ± 0.3a	36.8 ± 0.5ab	30.1 ± 13.8a
WPB	31.0 ± 0.4a	37.6 ± 0.6ab	30.5 ± 4.8a
WIB	31.7 ± 0.5a	39.4 ± 1.9b	36.2 ± 3.8a
WCB	29.6 ± 1.5a	36.2 ± 0.8a	38.6 ± 15.3a

Values are mean ± SD (n = 3).
[a-d] Means in the same column with different subscripts are significantly different ($p < 0.05$).

Table 4.4 Particle size distribution (PSD) of dark (D) and white (W) 'chocolate' with different fat sources: interesterified product (IP), physical blend (PB), illipe butter (IB) and cocoa butter (CB)

	Particle Size Distribution (μm)			
Sample	D_{10}	D_{50}	D_{90}	$D_{(4,3)}$
DIP	1.3 ± 0.1a	10.1 ± 0.2a	30.4 ± 1.9a	14.6 ± 1.4a
DPB	1.3 ± 0.1a	9.4 ± 0.2ab	26.6 ± 0.6a	12.0 ± 0.2ab
DIB	1.2 ± 0.0a	8.3 ± 1.3b	24.8 ± 2.8a	11.3 ± 1.0b
DCB	1.5 ± 0.1b	7.9 ± 0.4b	29.8 ± 4.5a	12.1 ± 1.4ab
WIP	0.7 ± 0.1c	4.7 ± 0.2c	23.5 ± 1.9b	9.0 ± 1.4c
WPB	0.8 ± 0.0c	4.6 ± 0.1c	21.5 ± 0.9b	8.3 ± 0.4d
WIB	0.8 ± 0.0c	4.7 ± 1.0c	20.4 ± 4.4b	10.4 ± 1.5c
WC	0.8 ± 0.1c	5.7 ± 0.5c	28.3 ± 2.6b	10.8 ± 1.0c

Values are mean ± SD (n = 3).
[a-d] Means in the same column with different subscripts are significantly different ($p < 0.05$).

Table 4.5 Rheological values of dark (D) and white (W) 'chocolate' with different fat sources: interesterified product (IP), physical blend (PB), illipe butter (IB) and cocoa butter (CB)

Sample	Casson plastic viscosity (Pa.s)	Casson yield stress (Pa)
DIP	5.0 ± 0.2a	15.0 ± 5.7a
DPB	5.0 ± 0.1a	15.4 ± 4.3a
DIB	6.1 ± 0.6b	23.8 ± 14.0a
DCB	5.5 ± 0.2ab	18.4 ± 6.5a
WIP	2.0 ± 0.0c	2.6 ± 0.1b
WPB	2.5 ± 0.0c	2.9 ± 0.1b
WIB	2.4 ± 0.2c	2.7 ± 0.3b
WCB	2.3 ± 0.0c	4.4 ± 0.2b

Values are mean ± SD (n = 3).
[a-d] Means in the same column with different subscripts are significantly different ($p < 0.05$).

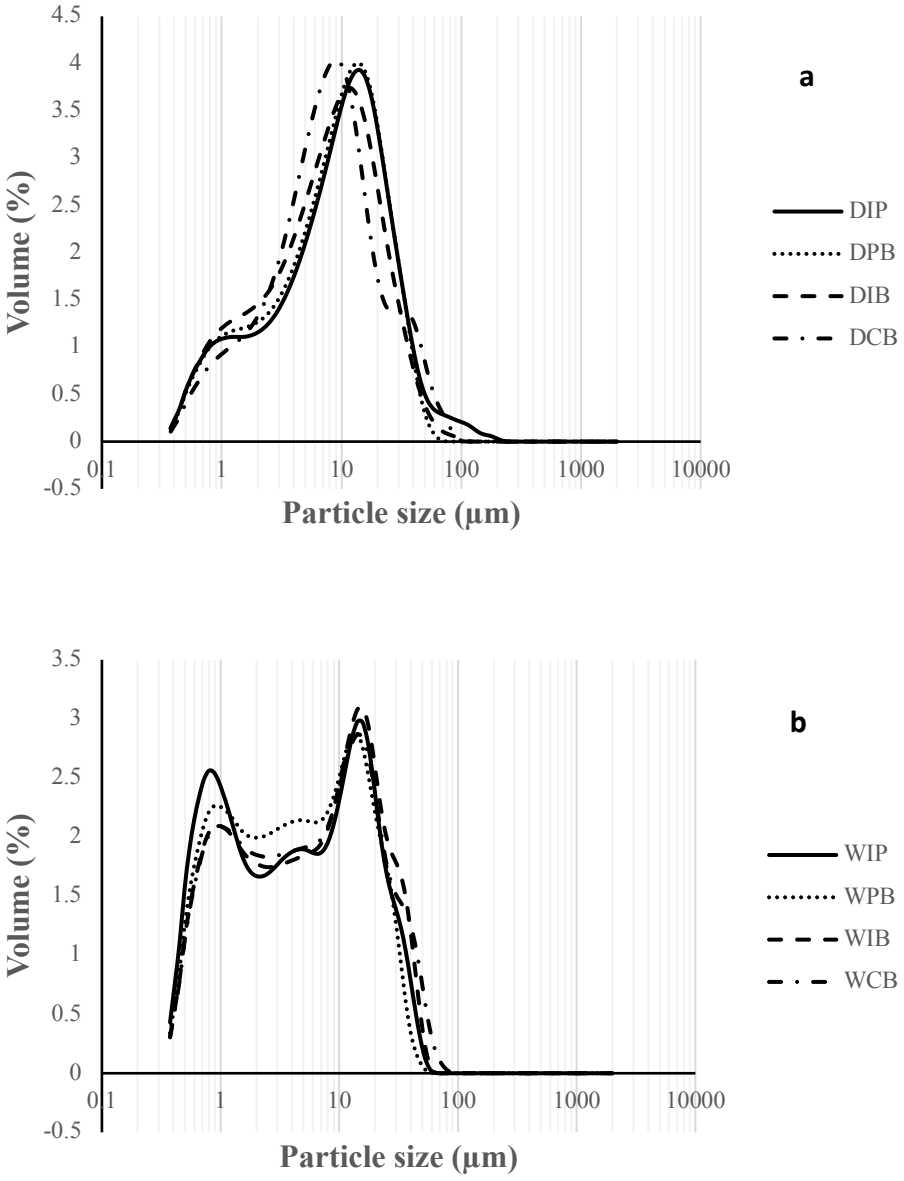

Figure 4.1 Particle size distribution histogram of dark (D) and white (W) 'chocolates' with different fat sources: interesterified product (IP), physical blend (PB), illipe butter (IB) and cocoa butter (CB). (a) dark 'chocolate', (b) white 'chocolate'

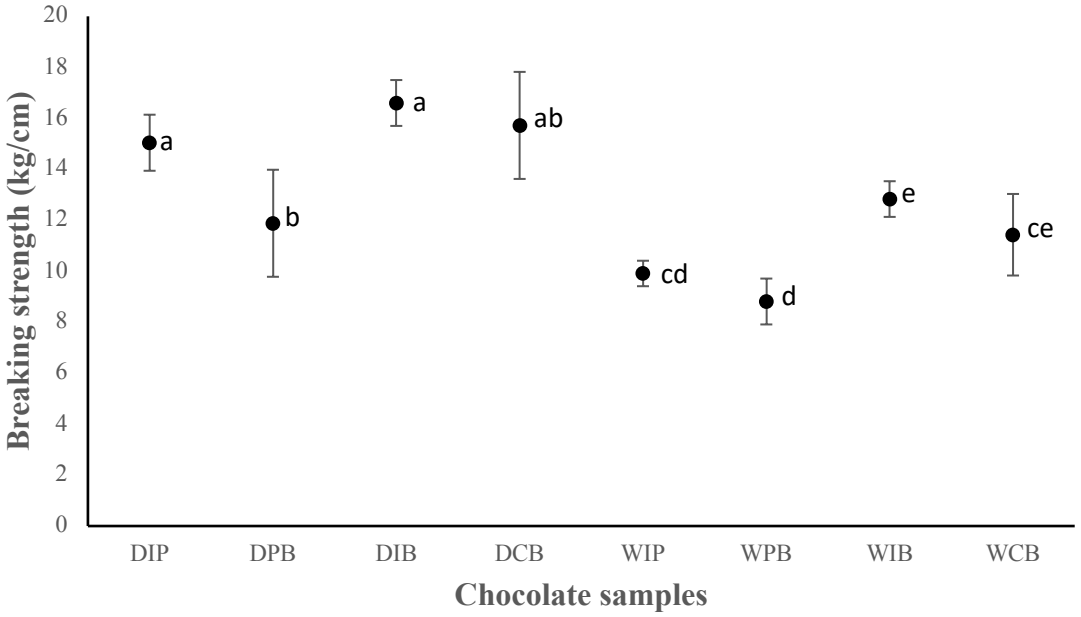

Figure 4.2 Breaking strength (hardness) values of (D) and white (W) 'chocolates' with different fat sources: interesterified product (IP), physical blend (PB), illipe butter (IB) and cocoa butter (CB)

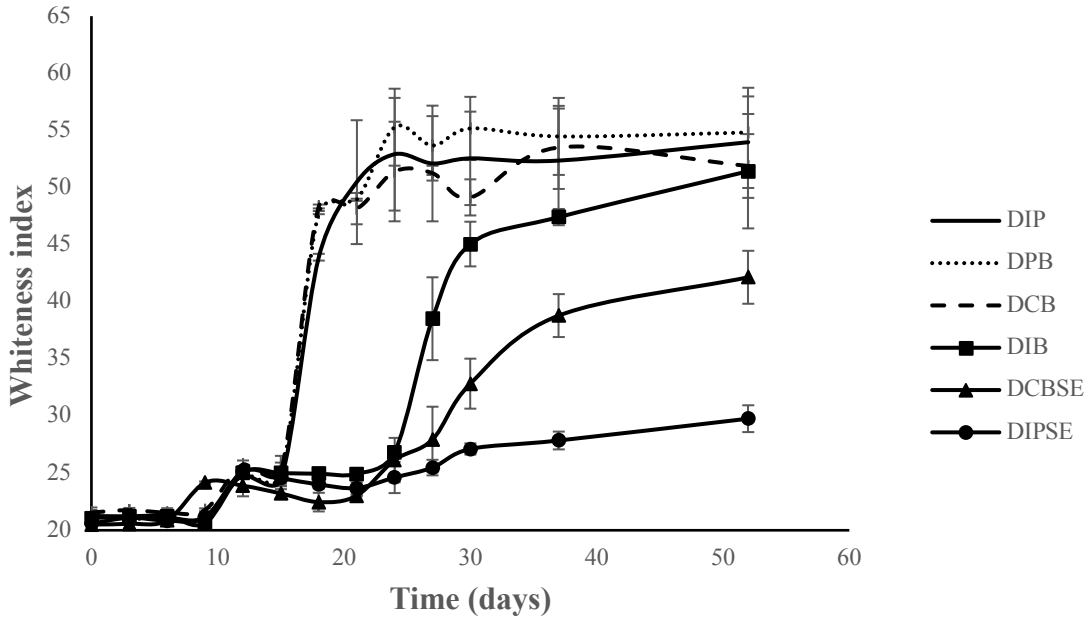

Figure 4.3 Whiteness index (WI) of dark 'chocolate' (D) samples over storage time under oscillating temperature. Oscillating temperature was changed on the 15th day. (IP) interesterified product, (PB) physical blend, (CB) cocoa butter, (IB) illipe butter, (CBSE) cocoa butter with sugar ester, (IPSE) interesterified product with sugar ester

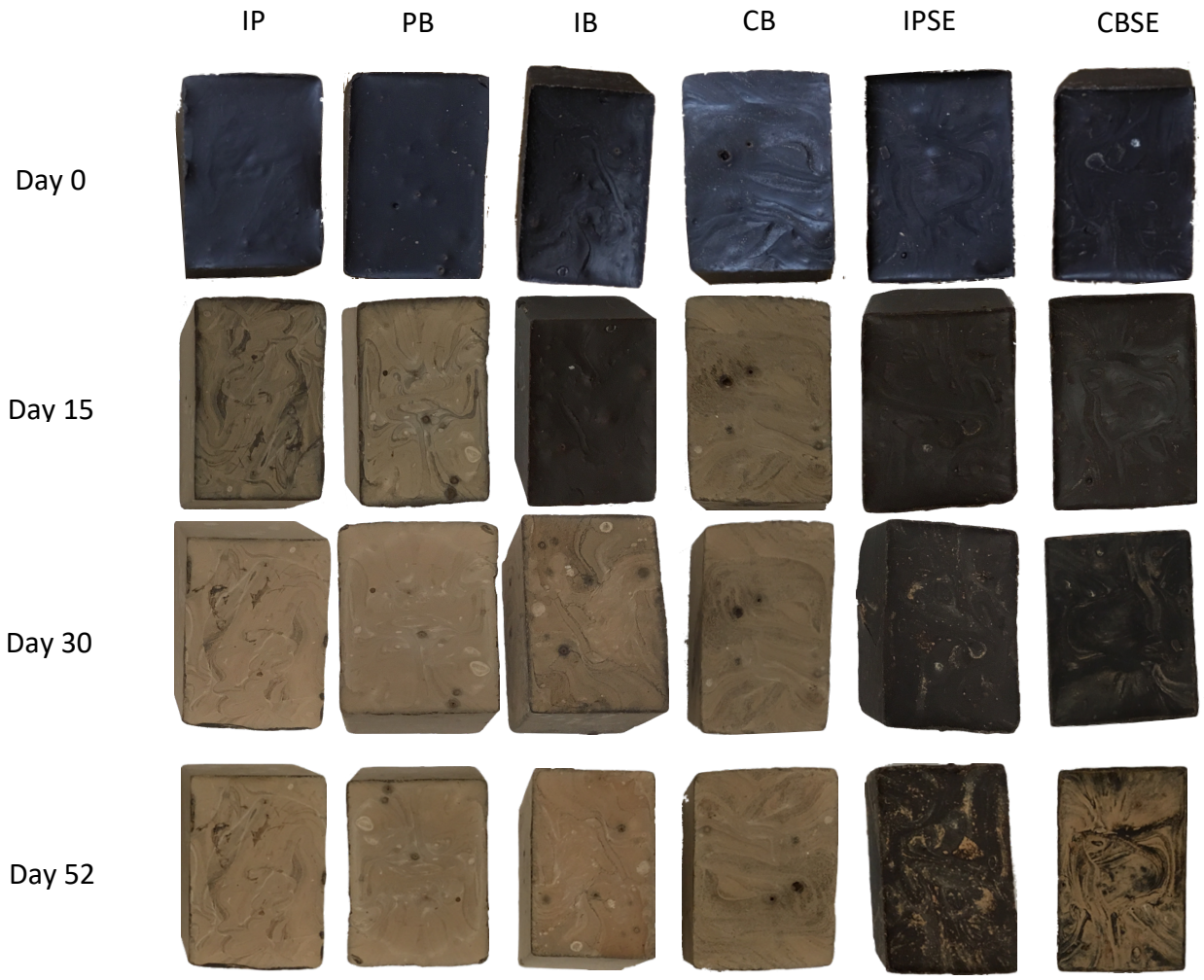

Figure 4.4 Photograph of fat bloom formation in dark 'chocolate' samples under oscillating temperature at the 1st, 15th, 30th, and 52nd day of storage

CHAPTER 5

CONCLUSIONS

There are many studies that have produced cocoa butter alternatives from cheaper and a more readily available options than cocoa butter (CB). These alternatives might only be cocoa butter substitutes (CBSs) to replace 5% fat from the total chocolate mass or it could also be a cocoa butter equivalent (CBE) to create another confectionary product with chocolate-like properties. For a CBE, it is important to make sure that it has a similar fat profile to CB so that it does not alter physicochemical properties of the final chocolate product.

In our first study, a CBE was synthesized from illipe butter (IB) and palm mid-fraction (PMF) through a lipase-catalyzed interesterification. Four ratios were tested, and the ratio of 10:3 for 30 min reaction using 5% lipozyme® RM IM resulted in a very similar fat profile to CB. The interesterified product (IP) had similar fatty acids profile and triacylglycerol (TAG) molecular species as CB and it was within the range of CB fat profile as reported in previous studies. IP did not show a significant difference in terms of melting onset and crystallization completion temperatures with CB, meaning that IP could be used as a CBE without altering the thermal behavior of CB. The thermal stability result was later supported by the ability of IP that crystallized as β polymorph. The solid fat content values of IP were closest to CB from 27-35 ^{o}C, indicating a good melt near body temperature. The crystal morphology of IP showed similar small spherulites compared to the big featherlike structures of IB and physical blend (PB).

A good CBE must be compatible with CB and should not alter the physical properties when used in chocolate formulation. In the second study, the various fat samples produced from the first study was applied in a chocolate product. Dark 'chocolate' was made to see the compatibility of the fats with CB, and white 'chocolate' was made to see the full properties of the fat without inclusion of cocoa butter. Furthermore, fat bloom resistance effects from the addition

of various fats, and sugar ester were analyzed in dark 'chocolate'. Our results showed that the incorporation of IP into both dark and white 'chocolate' gave similar texture, particle size distribution, and hardness with CB. IP could also be used as a non-chocolate confectionary product with similar physical properties to chocolate. The difference between IP and PB was only found in the textural property, where PB 'chocolates' were found to have lower hardness, probably due to eutectic effects from the physical blending. In addition, we found that IB was able to delay fat bloom in dark 'chocolates'. It was also shown that the addition of 0.5% sugar ester can strongly resist fat bloom formation in dark chocolate.

A future study that should be conducted from this research is a sensory evaluation of the dark and white chocolates made from these various fat samples. IB has a unique flavor that is quite different from CB and would compete with the flavor of CB. A descriptive analysis in sensory evaluation of dark and white chocolates made from the CBE should be conducted to determine the consumers acceptance of this new flavor. Sensory evaluation of the product would also be able to validate the physicochemical similarities or differences between CBE and CB that were determined from the experimental results.