UNIVERSITY OF GAZİANTEP
GRADUATE SCHOOL OF
NATURAL & APPLIED SCIENCES

PRODUCTION OF COCOA BUTTER EQUIVALENT BY ENZYMATIC INTERESTERIFICATION OF OLIVE-POMACE OIL

Ph.D. THESIS
IN
FOOD ENGINEERING

BY
OZAN NAZIM ÇİFTÇİ
JANUARY 2009

Production of Cocoa Butter Equivalent by Enzymatic Interesterification of Olive-Pomace Oil

Ph.D. Thesis
in
Food Engineering
University of Gaziantep

Supervisor
Prof.Dr. Sibel FADILOĞLU

by

Ozan Nazım ÇİFTÇİ

January 2009

T.C.
UNIVERSITY OF GAZİANTEP
GRADUATE SCHOOL OF
NATURAL & APPLIED SCIENCES
FOOD ENGINEERING

Name of the thesis: Production of cocoa butter equivalent by enzymatic interesterification of olive-pomace oil
Name of the student: Ozan Nazım ÇİFTÇİ
Exam date: 20.01.2009

Approval of the Graduate School of Natural and Applied Sciences

Prof. Dr. Ramazan KOÇ
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

Prof.Dr. Ahmet KAYA
Head of Department

This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

Prof.Dr. Sibel FADILOĞLU
Supervisor

Examining Committee Members

Prof.Dr. Dilek BOYACIOĞLU (Chairman) _____

Prof.Dr. Hasan KARAMAN _____

Prof.Dr. Fahrettin GÖĞÜŞ _____

Prof.Dr. Osman ERKMEN _____

Prof.Dr. Sibel FADILOĞLU _____

To my endless love Deniz

ABSTRACT

PRODUCTION OF COCOA BUTTER EQUIVALENT BY ENZYMATIC INTERESTERIFICATION OF OLIVE-POMACE OIL

ÇİFTÇİ, Ozan Nazım
Ph.D. in Food Engineering
Supervisor: Prof.Dr. Sibel FADILOĞLU
January 2009, 137 pages

It was aimed to add value to a cheap by-product, olive pomace oil, by converting it to a cocoa butter (CB)-like fat. For this purpose, immobilized *sn*-1,3 specific lipase-catalyzed acidolysis of refined olive pomace oil (ROPO) with palmitic (PA) and stearic (SA) acids was performed in model and natural system reactions to produce major triacylglycerols (TAGs) of CB.

Optimized reaction conditions in model system study were used in natural system reactions. Natural system reactions were carried out in laboratory scale batch stirred tank reactor (STR) and a packed bed reactor (PBR). The product of PBR system was selected as CB-like fat. The CB-like fat contained 10.9% 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), 19.7% 1(3)-palmitoyl-3(1)stearoyl-2-oleoyl-glycerol (POS) and 11.2% 1,3-distearoyl-2-oleoyl-glycerol (SOS), while commercial CB contains 18.9% POP, 33.1% POS and 24.7% SOS. The melting profile and SFC of the product were comparable to those of CB. Polarized light microscope (PLM) images showed no drastic changes in polymorphic behavior between CB and CB-like fat. In order to evaluate the performance of the product as a partial CB replacer, binary blends of CB and CB-like fat were prepared at different proportions and their physicochemical characteristics (TAG content, melting profile, solid fat content (SFC), microstructure, and oxidative stability) were investigated.

The results of the study suggested that produced CB-like fat is suitable for use in confectionery products as a CB replacer up to 30% without altering the physical and chemical properties of the product significantly.

Key words: Refined olive pomace oil, cocoa butter, cocoa butter-like fat, enzymatic acidolysis, *sn*-1,3 specific lipase packed bed reactor, physicochemical characteristics.

ÖZET

PRİNA YAĞINDAN ENZİMATİK İNTERESTERİFİKASYON İLE KAKAO YAĞI EŞDEĞERİ ÜRETİLMESİ

ÇİFTÇİ, Ozan Nazım
Doktora tezi, Gıda Mühendisliği
Tez Danışmanı: Prof.Dr. Sibel FADILOĞLU
Ocak 2009, 137 sayfa

Ucuz bir yan ürün olan prina yağı, kakao yağı benzeri yağa dönüştürülerek değerlendirilmek istenmiştir. Bu amaçla, kakao yağının ana triaçilgliserollerini (TAG) üretmek için rafine prina yağının palmitik ve stearik asitlerle asidoliz reaksiyonları *sn*-1,3 spesifik lipaz katalizörlüğünde model ve doğal sistemlerde gerçekleştirilmiştir.

Model sistemde optimize edilmiş reaksiyon şartları, doğal sistem reaksiyonlarında kullanıldı. Doğal sistem reaksiyonları labaratuar boyutu beç karıştırmalı tank reaktör ve dolgulu yatak reaktörde gerçekleştirildi. Dolgulu yatak reaktörden elde edilen ürün kakao yağı benzeri yağ olarak seçildi. Kakao yağı %18.9 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), %33.1 1(3)-palmitoyl-3(1)stearoyl-2-oleoyl-glycerol (POS) ve %24.7 1,3-distearoyl-2-oleoyl-glycerol (SOS) içerirken, kakao yağı benzeri yağ %10.9 POP, %19.7 POS and %11.2 SOS içerdi. Ürünün erime profili ve katı yağ oranı kakao yağınınkine benzerdi. Polarize ışık mikroskopu görüntüleri kakao yağı ve kakao yağı benzeri yağın polimorfik özellikleri arasında büyük farklar göstermemiştir. Ürünün kısmi kakao yağı ikamesi olarak performansını ölçmek için, kakao yağı ve kakao yağı benzeri yağın değişik oranlarda karışımları hazırlandı ve fizikokimyasal özellikleri (TAG içeriği, erime profili, katı yağ oranı, mikroyapı ve oksidatif stabilite) incelendi.

Çalışmanın sonuncu, üretilen kakao yağı benzeri yağın şekerleme ürünlerinde 30%' a kadar eklemelerinde son ürünün fiziksel ve kimyasal özelliklerini önemli bir ölçüde değiştirmediğini ve kullanıma uygun olduğunu göstermiştir.

Anahtar kelimeler: Rafine prina yağı, kakao yağı, kakao yağı benzeri yağ, enzimatik asidoliz, *sn*-1,3 spesifik lipaz, dolgulu yatak reaktör, fizikokimyasal özellikler.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor, Prof.Dr. Sibel Fadıloğlu, for her excellent guidance, caring, patience, and providing me with an excellent atmosphere for doing research.

I would also like to express my heartfelt gratitude to Prof.Dr. Fahrettin Göğüş for his guidance, persistent help, supporting me with his critical comments, and his suggestions regarding my work.

I am very grateful to Prof.Dr. Boleslaw Kowalski, who provided me with the opportunity to conduct research in his lab in Department of Food Sciences, Warsaw University of Life Sciences (SGGW), Warsaw, Poland. The fatherly guidance of Professor Kowalski has been very inspirational and his constant supports in my studies a great source of motivation. I also enjoyed the comfortable discussions with him about my research and other topics.

I am also grateful to Prof.Dr. Krygier Krzysztof, who introduced me to Professor Kowalski. Special thanks are extended to Dr. Eliza Gruczyńska, Dr. Joanna Bryś, Dr. Ewa Ostrowska-Ligęza, Dr. Magdalena Wirkowska, Dr. Katarzyna Tarnowska for their cheerful supports and friendships during my research in SGGW.

I would like to acknowledge the financial and technical support of University of Gaziantep, Scientific Research Projects Governing Unit, Turkey, and Ayhan Akpek, owner of Güvenal Sabun Prina Rafine Yağ San. ve Tic. A.Ş, Gaziantep, and Mehmet Gümüşer, manager of ZER Yağ San. ve Tic. A.Ş., Gaziantep.

I would also like to thank to my friends and colleagues Aylin Altan, Sibel Yağcı and Talip Kahyaoğlu for supporting me and encouraging me with their best wishes. I thank my parents, and brother Aytek, for their support.

Finally, a very special thank to my wife Deniz, with a deep sense of gratitude, for her untiring moral and loving support, encouragement and patience. I am pretty sure that without her everything would be very difficult. She is my inspiration.

CONTENTS

page

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABREVIATIONS

CB	Cocoa butter
CBE	Cocoa butter equivalent
DAG	Diacylglycerol
DSC	Differential Scanning Calorimeter
E_a	Activation energy (kJ/mol)
FAME	Fatty acid methyl ester
FFA	Free fatty acid
GC	Gas Chromatograph
HPLC	High Performance Liquid Chromatograph
k	Oxidation rate constant
MAG	Monoacylglycerol
OIT	Oxidation induction time
OOO	Triolein
PA	Palmitic acid
PBR	Packed bed reactor
PLM	Polarized Light Microscope
POP	1,3-dipalmitoyl-2-oleoyl-glycerol
POS	1(3)-palmitoyl-3(1)stearoyl-2-oleoyl-glycerol
PRP	Purified reaction product
ROPO	Refined olive pomace oil
SA	Stearic acid
SFC	Solid fat content
SL	Structured lipid
SOS	1,3-distearoyl-2-oleoyl-glycerol
STR	Stirred tank reactor
TAG	Triacylglycerol
TLC	Thin layer chromatography
UV	Ultraviolet
Z	Preexponential factor (min^{-1})
ε	Bed porosity

CHAPTER 1

INTRODUCTION

Improvement of the nutritional and functional properties of fats and oils is a popular topic of lipid biotechnology. For a long time, fats and oils have been modified enzymatically to produce structured lipids (SLs) (Macrae and Hammond, 1985). SLs can be manufactured to achieve regiospecific locations of fatty acids in the acylglycerols using specific lipases (Hoy and Xu, 2001). The commercial value and functional properties of fats and oils depend not only on fatty acid composition, but also on the distrubition of fatty acids on the glycerol backbone (Zhao et al., 2005).

Lipase-catalyzed acidolysis reactions are widely used for lipid modifications to improve functional properties of fats and oils, e.g. conversion of cheap commercial oils to high value added products (Forssell et al., 1992). Production of cocoa butter equivalent (CBE) fats (CB-like fats) by lipase catalyzed acidolysis of fats and oils is a good way of producing value added products.

Cocoa butter (CB) is a highly valued ingredient primarily used in the chocolate industry (Lipp et al., 2001). Of all the ingredients present in chocolate, none has more influence on its physical and chemical properties than CB. Due to its unique composition, CB gives desired physical properties to the manufactured product, e.g. gloss, snap, melting properties, etc (Lipp et al. 2001). But, because of the high cost and fluctuations in the supply and demand of CB, industry used alternatives with similar triacylglycerol (TAG) composition instead of CB. CB contains three main fatty acids: palmitic acid (C16), stearic acid (C18) and oleic acid (C18:1). CB is composed of three main symmetric TAGs: 21% POP (1,3-dipalmitoyl-2-oleoyl-glycerol); 40% POS(1(3)-palmitoyl-3(1)stearoyl-2-oleoyl-glycerol); 27% SOS (1,3-distearoyl-2-oleoyl-glycerol); with oleic acid at *sn*-2 position of glycerol backbone (Saldana et al. 2002).

Production of CBEs by enzymatic interesterification can be done by using *sn*-1,3 specific lipases that catalyze incorporation of stearic and palmitic acids to the *sn*-1,3 positions of a starting oil containing oleic acid at *sn*-2 position until a similar composition of CB is obtained. There are many studies reporting production of CBEs from different sources such as lard, tea seed oil, palm oil midfraction, sal fats, mango fat, illipe fat, kokum fat and shea oil (Undurraga et al. 2001; Wang et al. 2006; Sridhar et al. 1991).

Olive pomace oil can be considered as a good potential source for CB-like fat production because of its high *sn*-2 position oleic acid content and low cost. Olive pomace is a by product of virgin olive oil processing and is of varying importance to all of the countries of the Mediterranean basin where olives are grown (Gomes and Caponio, 1998). Olive pomace oil is obtained by solvent-extraction from the olive pomace which is left after mechanical press of olives. Because of long storage periods of pomace and high temperature drying applications, it is considered of a lower quality. So, it is used for soap making and industrial processes. Its chemical composition does not differ from refined olive oil (Kiritsakis et al., 1998).

The aim of this study was to valorize olive pomace oil by converting it to a value added product, CB-like fat. For this purpose enzymatic acidolysis reactions of refined olive pomace oil with palmitic and stearic acids were carried out under the catalysis of *sn*-1,3 specific lipase. Effects of reaction parameters on acidolysis were investigated in a model system where triolein was the source oil. Natural system productions, where refined olive pomace oil was the source oil, were carried out in batch stirrred tank reactor (STR) and packed bed reactor (PBR). The products (CB-like fat) were investigated by analysing their physicochemical properties (TAG content, melting properties, solid fat content (SFC), microstructure, oxidative stability). The compatibility limit of CB-like fat incorporation into CB was determined by investigating physicochemical characteristics of binary blends of CB and CB-like fat combined at different proportions.

CHAPTER 2

LITERATURE REVIEW

2.1 Structured Lipids

Fats and oils are one of the major food sources for humans. The major animal sources include butter, lard, tallow and fish (encounter ca 21% of the whole) and plant sources include sunflower, olive, corn, soy, cotton, palm, palmkernel, coconut, rape, groundnut and linseeed (encounter ca 79% of the whole). Most of these oil and fat sources are consumed as human food (81%, and the remainder used as animal feed or for the production of oleochemicals) (Gunstone, 2001).

The lipid produced, whether by plant or animal, is not necessarily ideal for its ultimate human use, and the natural product may have to be modified. This has been going on for at least a century, and most of the lipid that we use today has been modified in some way. Modifications can be made via technology or biology (Gunstone, 2001).

In a broad sense, structured lipids (SLs) are TAGs that have been modified by incorporation of new fatty acids, reconstructed to change the positions of fatty acids, or the fatty acid profile, from the natural state, or synthesized to yield novel TAGs. Their potency increases if each glycerol moiety contains both short and/or medium chain and long chain fatty acids. SLs combine the unique characteristics of component fatty acids such as melting behaviour, digestion, absorption, and metabolism to enhance their use in foods, nutrition, and therapeutics (Akoh, 2002). SLs may provide the most effective means of delivering fatty acids for nutritive or therapeutic purposes, and for targeting specific diseases and metabolic conditions (Lee and Akoh, 1998a).

SLs are often referred to as a new generation of fats and constituents of functional foods (Gunstone, 2001). They are also known as nutraceuticals: food or parts of food that provide medical of health benefits, including the potential for the prevention

and/or treatment of diseases (Kennedy, 1991). “Functional foods” is a term used to broadly describe foods that provide specific health benefits. Medical foods (medical lipids) are foods (lipids) developed for use under medical supervision to treat or manage particular disease or nutritional deficiency states. Other terms used to describe functional foods are physiologic functional foods, pharmafoods, and nutritional foods. The nomenclature is still confusing and needs to be worked out by scientists (Akoh, 2002).

2.1.2 What properties are desired from structured lipids?

The significant properties of a lipid depend on its final use. These are mainly nutritional or physical, but sometimes chemical. The nutritional significance of lipids attracts a lot of attraction. Lipids are an important component of the diet, and lipids in particular are important in the maintenance of good health and in the development and treatment of some diseases. Type of fatty acids and TAGs, and the balance between the various types make the difference. Properties of potential interest include:

- Total energy values of the produced fat or fat-like material with reduced energy value,
- Fatty acid composition with levels of saturated acids, cis and trans monoene acids, essential fatty acids, and long-chain polyunsaturated fatty acids,
- TAG composition, since bio-availability is linked to this factor. Fatty acid composition alone is insignificant,
- The presence and levels of nutritionally desirable minor constituents, such as carotenes, tocopherols, sterols, etc.
- Physical properties

The most important physical properties of fats and oils for the food industry are thermal properties associated with crystallization and melting, with the formation of solids and liquids and the behaviour of plastic fats that are mixtures of solid and liquid components. Physical properties are especially important in spreads, cooking baking fats, confectionery fats, frying oils, creams, as well as in cosmetic applications and lubricants (Gunstone, 2001).

2.1.3 Production of structured lipids

Fatty acid composition and also distribution on TAGs determine their physical and metabolic properties. It is possible to improve the physical, nutritional and pharmaceutical properties of fats and oils by giving them a definite structure (Macrae, 1985). Within this definition, structured TAGs are often produced by interesterification, either chemical or enzymatic. Lipase enzyme is widely used to produce such kind of fats and oils (Xu, 2000a). Interesterification is used to produce fats with desired functional and physical properties of food applications. Interest in interesterification from nutritional and functional standpoints is on the rise because of the possibility to produce trans-free margarines, CBEs, and reduced calorie foods (Osborn and Akoh, 2002a). Specific SLs can be produced by regiospecific lipases, and randomly SLs can be produced by nonspecific lipases (Iwasaki and Yamane, 2000; Hoy and Xu, 2001).

2.2 Lipases

Lipases (EC 3.1.1.3) are distributed throughout the living organisms which form two primary divisions of the phylogenic tree, namely the bacteria and a second division branching into both the eukorya, including animals, plants and fungi, and the archaea, with the former archaebacteria (Olson, 1994). Lipases are ubiquitous enzymes that catalyze the breakdown of fats and oils with subsequent release of free fatty acids (FFA), diacylglycerols (DAGs), monoacylglycerols (MAGs) and glycerol (Villeneuve et al., 2000). Their major substrates are long chain TAGs, and this property is the basis of an old definition of lipases as ‘long-chain fatty acid ester hydrolases’ or ‘esterases capable of hydrolyzing esters of oleic acid’ (Jaeger, 1994). These enzymes are fulfill a key role in the biological turnover of lipids. They are required as digestive enzymes to facilitate not only the transfer of lipids from one organism to another, but also the deposition and the mobilization of fat that is used as an energy reservior within the organism. They are also involved in the metabolism of intracellular lipids and therefore in the functionating of biological and physiological properties, and lately for their industrial applications (Villeneuve et al., 2000).

2.2.1 Lipases as applied catalysts

Lipases are capable of achieving esterification, interesterification (acidolysis, transesterification, alcoholysis), aminolysis, oximolysis and thiotransesterification in

anhydrous organic solvents, biphasic systems and in micellar solution with chiral specifity (Villeneuve et al., 2000). Lipases are currently used, or have the potential for use, in a wide range of applications: in the dairy industry for cheese flavor enhancement, acceleration of cheese ripening, and lipolysis of butterfat and cream; in the oleochemical industry for hydrolysis, glycerides, surfactants, ingredients of personal care products, pharmaceuticals, agrochemicals, and polymers (Bjorkling et al., 1991; Vulfson, 1994). Table 2.1 shows examples of industrial applications of lipases.

Table 2.1. Examples of industrial applications of lipases (Villeneuve et al., 2000)

Field of industry	Application	Product
Hydrolysis		
Food (dairy)	Hydrolysis of milk fat	Flavoring agents for dairy products
Chemical (oil processing)	Hydrolysis of oils and fats Analysis of fatty acid distribution in triacylglycerols	Fatty acids, MAGs, DAGs Reagents for lipid analysis
Chemical (detergent)	Removal of oil strains, spots and lipids	Detergents for laundry household uses
Medical	Blood TAG assay	Diagnostic kits
Esterification		
Chemical (fine chemical)	Synthesis of esters	Chiral intermediates Esters, emulsifiers
Food (chemical and pharmaceutical)	Interesterification of natural oils	Oils and fats (e.g., CBEs, human milk fat substitutes)

The increasing interest in lipase research over the past decades has likely occured for three reasons (Kazlauskas and Bornscheuer, 1998). The first is related to the molecular basis of the enzyme catalytic function or the lipase paradigm. Indeed, lipases, though water soluble, catalyze reactions involving insoluble lipid substrates at the lipid-water interface. This capability is due to the unique structural characteristic of lipases. These latter indeed contain a helical oligopeptide unit that covers the entrance to the active site. This so-called lid only moves upon access to a

hydrophobic interface such as a lipid droplet. Thus, it is not surprising that lipases as well as phospholipases have, for many years, served as models for studying the regulation of interfacial, enzyme catalyzed reactions. The second reason is linked to the enzyme's medical relevance, particularly to atherosclerosis and hyperlipidemia, and its importance in regulation and metabolism, since products of lipolysis such as FFAs and DAGs play many critical roles, especially as mediators in cell activation and signal transduction. Lastly, it was discovered that lipases are powerful tools for catalyzing not only hydrolysis, but also reverse reactions, such as esterification, transesterification and aminolysis in organic solvents (Villeneuve et al., 2000).

Recently, lipase-catalyzed glycerolysis and direct esterification for the biosynthesis of partial glycerides are increasingly being studied as possible alternatives to the classical method (Rosu, 1997). Accordingly, considerable attention has been paid lately to the commercial use of lipases. However, the current price of lipases is about an order magnitude higher than the necessary to match the energy costs associated with standard processes (Brady et al., 1988). Due to economical considerations their application on an industrial scale requires their immobilization and thus re-usability (Ivanov, 1997). Immobilized lipases are considered as lipases which are localized in a defined region of space, which is enclosed by an imaginary or material barrier which allows for physical separation of the enzyme from the bulk reaction medium, and which is at the same time permeable to reactant and product molecules (Balcao et al., 1996). An immobilized lipase preparation with high operational stability will reduce costs, making lipase-catalyzed reactions a feasible process option (Ferreria-Dias and Fonseca, 1995a), from an industrial point of view, immobilized lipases generally offer the economic incentives of enhanced thermal and chemical stability, and ease of handling, recovery, and reuse relative to nonimmobilized forms (Malcata et al., 1990; Yahya et al., 1998). Immobilization also facilitates dispersal of enzyme on a solid surface to provide for greater interfacial area and accessibility of substrates relative to the use of enzyme powders in low water reaction media (Bell et al., 1995).

2.2.2 Two major actions of lipase on lipids: Hydrolysis versus esterification

Enzymatic lipid modification and synthesis have been dramatically developed in the last 25 years. These progresses are largely attributed to the development of micro-aqueous enzymology. Traditionally, enzymes of such kind (hydrolases) are supposed

to work in water-abundant systems for hdrolysis. Under these conditions, hydrolysis is the only reaction that can be applied in lipid areas. In 1984, Zaks and Klibanov (1984) indicated that enzymes could work in a solvent micro-aqueous system. It means that enzymes can also be used for the catalysis of reverse reaction, i.e. esterification (Xu, 2003). It was an important development in lipase applications.

The hydrolysis of fats and oils (TAGs) is an equilibrium reaction and therefore it is possible to change the direction of the reaction to ester synthesis by modifying the reaction conditions (Figure 2.1).

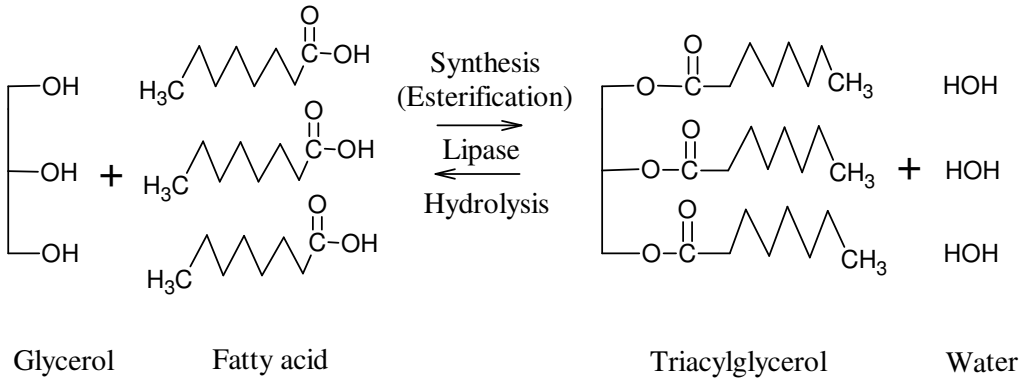


Figure 2.1. Enzymatic reaction of a lipase catalyzing hydrolysis or synthesis of a TAG substrate (Jaeger et al., 1994)

Lipases normally catalyze the hydrolysis of glycerides and the equilibrium of this process is far to the direction of hydrolysis products since water present is excess. When the concentration of water in the reaction medium is reduced, e.g., by working in an organic solvent containing a limited amount of water, a drastic shift of the chemical equilibrium can be observed, so that reagents and products are present in comparable concentrations. Mechanistically, this means that the reverse reaction, the esterification, takes place at a rate comparable to that of the hydrolysis reaction (Goderis et al., 1987). In esterification reactions, it is desirable to shift the position of thermodynamic equilibrium by removal of the water produced by the reaction (Arcos et al., 2000). As the amount of water present in the system increases, more TAGs are hydrolyzed without subsequent reesterification and equilibrium is more to the hydrolysis products. From this point of view, maximization of final TAG

concentration is obtained by working under circumstances as dry as possible (Goderis et al., 1987). The equilibrium between forward and reverse reactions in this case is controlled by the water content of the reaction mixture, so that in a non-aqueous environment lipases catalyze ester synthesis reactions (Jaeger et al., 1994). Different types of synthesis reactions can be distinguished: common ester synthesis from glycerol and fatty acids and the biotechnologically more important interesterification reactions, is the exchange of acyl groups, between an ester and an acid (acidolysis), an ester and an alcohol (alcoholysis), or an ester and an ester (transesterification) (Malcata et al., 1990). Interesterifications require a small amount of water, in addition to the amount needed for the enzyme to maintain an active hydrated state. As the presence of excess water will decrease the amount of ester synthesis products, the water content should be carefully adjusted to achieve accumulation of desired reaction products (Jaeger et al., 1994).

2.2.3 Lipases for the production of structured lipids

The use of lipases as biocatalysts for the production of SLs has many more potential benefits and advantages over chemical catalysts for future developments besides the specificity of lipases. The most important and obvious merits are (Xu, 2000a; Villeneuve, 2000; Babayan, 1987):

- Efficacy of lipases under mild reaction conditions (e.g. temperature and pressure)
- Utility in natural reaction systems and products
- Reduced side products and environmental pollution, so lowered waste costs
- Availability of lipases from a wide range of sources
- Ability to improve lipases by genetic engineering
- The use of lipases for the production of particular biomolecules
- Satification of consumer's demand for medical and nutritional products

For these reasons, many nutritional and functional SLs have been produced enzymatically.

2.2.4 Specificity of lipases

The TAG lipases have widely varying substrate specificities, preferring substrates with long and medium chain fatty acids over the short chain ones, and vice versa.

Morover, specifity of lipases for the fatty acids esterified at the *sn*-1, *sn*-2 and *sn*-3 positions of the glycerol backbone varies widely, ranging from nonspecificity for either of the three *sn*-1, *sn*-2 and *sn*-3 positions to strong *sn*-1,3 or specificity. The foregoing preperties of TAG lipases permit their use as biocatalysts for the preparation of SL products of definite composition and structure that often can not be obtained by reactions carried out using chemical catalysts. The main advantage of lipases that differentiates enzymatic interesterification from chemical interesterification is their specifity. Lipases can be grouped in four according to their specifity.

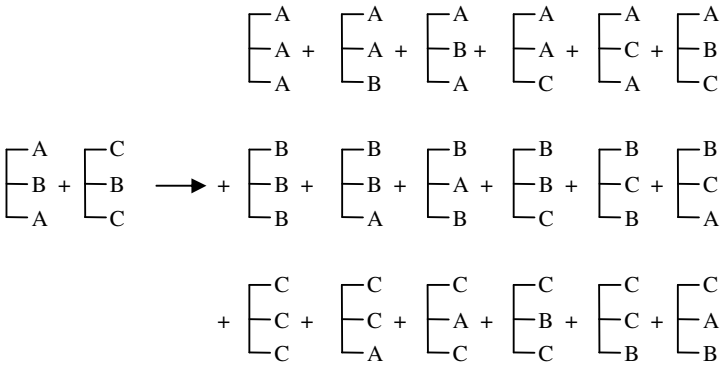
2.2.4.1 Nonspecific lipases

Certain lipases show no positional specificity during interesterification. These kind of lipases act randomly on all fatty acids in all positions and give products as chemical interesterification (Figure 2.2). These kind of lipases include *Candida cylindraceae*, *Corynebacterium acnes*, and *Staphylococus aureus* (Macrae, 1983, Gunstone, 1994).

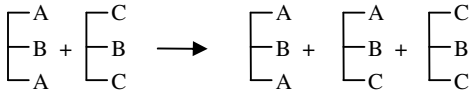
2.2.4.2 Positional specificity

Positional specificity, i.e. specific toward ester bonds in *sn*-1,3 of the TAG, results from an inability of lipases to act on position *sn*-2 on the TAG, due to steric hinderence (Figure 2.2). Steric hinderence prevents fatty acid in position *sn*-2 from entering the active site (Macrae, 1983; Macrae and How, 1988). An interesterification reaction using a *sn*-1,3 specific lipase will initially produce a mixture of TAGs, 1,2- and 2,3-DAGs, and FFAs (Macrae and How, 1988). After prolonged reaction periods, acyl migration can occur, with the formation of 1,3-diacylglycerols, which allows some randomization of the fatty acids existing at the middle position of the TAGs. This kind of lipases include *Rhizophus arrhizus*, *Aspergillus niger*, *Mucor miehei*, *Rhizophus delemar* (Macrae, 1983).

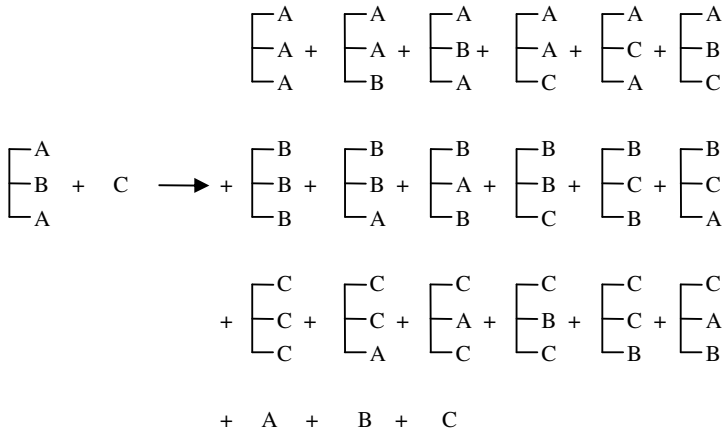
TAG mixtures: w*ith nonspecific lipases*



with sn-1,3 specific lipases



TAG + FFA mixture*: with nonspecific lipases*



with sn-1,3 specific lipases

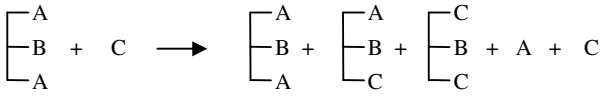


Figure 2.2. Specificity of TAG lipases: Products formed by interesterification of fat mixtures; A, B, C are fatty acids/acyl moieties (Macrae, 1983)

2.2.4.3 Stereospecificity

In TAGs, *sn*-1 and *sn*-3 positions are sterically distinct. Very few lipases differentiate between the two primary esters at the *sn*-1 and *sn*-3 positions, but when they do, the lipases possess stereospecificity. In reactions where the lipase is stereospecific, positions 1 and 3 are hydrolyzed at different rates. Stereospecificity is determined by the source of the lipase and the acyl groups, and can also depend on the lipid density at the interface, where an increase in the substrate concentration can decrease specificity due to steric hindrence (Willis and Marangoni, 2002). Lipase from *Pseudomonas* species and porcine pancreatic lipase have shown stereoselectivity when certain acyl groups are hydrolyzed (Uzawa et al., 1993).

2.2.4.4 Fatty acid specificity

Many lipases are specific toward particular fatty acid substrates. Most microbial lipases show little fatty acid specificity, with the exception of lipase from *Geotrichum candidum*, which is specific toward long chain fatty acids containing *cis*-9 double bonds (Macrae, 1983). Lipases can also demonstrate fatty acid chain length specificity, with some being specific toward long chain fatty acids and other being specific toward medium chain and short chain fatty acids. For example, porcine pancreatic lipase is specific toward shorter chain fatty acids, while lipase from *Penicillium cyclopium* is specific toward long chain fatty acids. As well, lipases from *Aspergillus niger* and *Aspergillus delemar* are specific toward both medium chain and short chain fatty acids (Desnuelle, 1972; Stamatis et al., 1993).

2.3 Enzymatic Reactions for Production of Structured Lipids

2.3.1 Interesterification

The development of methods to improve the nutritional and functional properties of fats and oils is great interest to food processors (Willis and Marangoni, 2002). One of the major areas of interest is in the use of lipase-catalyzed interesterification to improve the nutritional value, or alter the physical properties of fats and oils (Weete, 2002). The molecular weight, unsaturation and positional distribution of fatty acid residues on the glycerol backbone of TAGs are the principal factors determining the physical properties of fats and oils (Goderis et al., 1987). In natural fats, acyl groups are distributed in a nonrandom fashion. During chemical or enzymatic interesterification, acyl groups are redistributed first intramolecularly, then

intermolecularly, until a random distribution is achieved. Chemical interesterification produces a complete positional randomization of acyl groups in TAGs. With enzymatic interesterification, more control of final product composition is possible and glyceride mixtures that can not be obtained using chemical interesterification can be produced (Willis and Marangoni, 2002).

It is used in the manufacture of shortenings, margarines and spreads to improve their textural properties, modify melting behavior and enhance stability (Willis and Marangoni, 2002). Interesterification, whether chemical or enzymatic, is the exchange of acyl groups, between an ester and an acid (acidolysis), an ester and alcohol (alcoholysis) or an ester and an ester (transesterification) (Malcata et al., 1990).

a) Transesterification

Transesterification is the the exchange of acyl groups between two esters, namely two TAGs (Figure 2.3). It is used predominantly to alter the physical properties of individual fats and oils or fat-oil blends by altering the positional distribution of fatty acids in the TAGs (Willis and Marangoni, 2002).

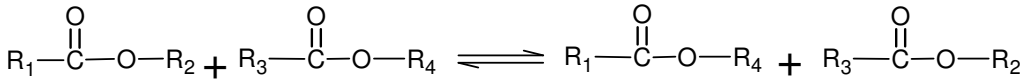


Figure 2.3. Lipase-catalyzed transesterification between two different TAGs (Willis and Marangoni, 2002)

Transesterification of butter using a nonspecific lipase has been reported to improve the plasticity of the fat. Willis and Marangoni (2002) reported that transesterification of butterfat with a positionally nonspecific lipase at 40°C increased the level of saturated C48 to C54 TAGs, monoene C38 and C46 to C52 TAGs and diene C40 to C54 TAGs. These authors reported that the DAG content increased by 45 % while the FFA content doubled. Overall, lipase-catayzed transesterification of butterfat at 40°C produced an increase in the SFC below 15°C and a decrease in the SFC above 15°C.

b) Alcoholysis

Alcoholysis is the reaction between an ester and alcohol, resulting in an alcohol exchange (Figure 2.4) (Xu et al., 2006; Willis and Marangoni, 2002).

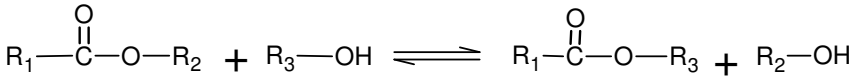


Figure 2.4. Lipase-catalyzed alcoholysis reaction between an acylglycerol and an alcohol (Willis and Marangoni, 2002)

The reaction of TAGs with monohydric alcohols generates the simple alcohol esters of fatty acids, which have found applications in the production of biodiesel, as well as alternative acyl donors of fatty acids, e.g. fatty acid methyl esters (Xu et al., 2006).

The main use of alcoholysis is in the performance of glycerolysis reactions. Glycerolysis is the exchange of acyl groups between glycerol and a TAG to produce MAGs, DAGs and TAGs. MAGs can be produced by ester exchange between TAGs and glycerols, or by FFAs and glycerol, although only the former reaction is termed glycerolysis (Figure 2.5) (Willis and Marangoni, 2002).

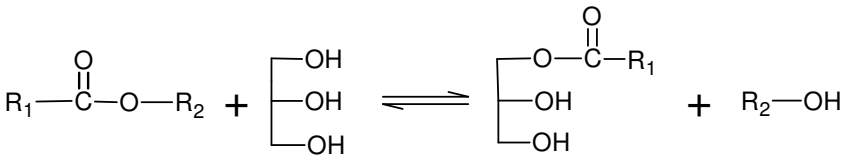


Figure 2.5. Lipase-catalyzed glycerolysis reaction between glycerol and a TAG to produce MAGs (Willis and Marangoni, 2002)

Glycerolysis of fats and oils produces industrially important MAG and DAG. Fatty acid MAG and their derivatives have many applications as surfactants and emulsifiers in a wide range of food, cosmetics and pharmaceutical products (Meffert, 1984). Lipase-catalyzed glycerolysis experiments, aimed at the production of MAG and/or DAG, have been carried out with free (McNeill et al., 1991) or immobilized lipases (Ferreira-Dias and Fonseca, 1993; Stevenson et al., 1993; Ferreira-Dias and Fonseca, 1995b), with lipases in reversed micelle systems (Chang and Rhee, 1990) or on liposomes in reversed micelles (Chang et al., 1991). Recently, Fadıloğlu et al.

(2003) decreased FFA content of olive pomace oil from 33% to 2% by lipase catalyzed glycerolysis.

From a wide range of microbial lipases, which were screened for glycerolysis, bacterial lipases were found to be the most suitable for this process (Bornscheuer and Yamane, 1994). The esterification of glycerol with FFA by lipases to produce primarily MAG was reported by several authors (Akoh et al., 1992; Berger and Schneider, 1992).

c) Acidolysis

Acidolysis is the acyl exchange reaction between TAGs and fatty acids (Figure 2.6). It is an effective means of incorporating novel fatty acids into TAGs (Willis and Marangoni, 2002).

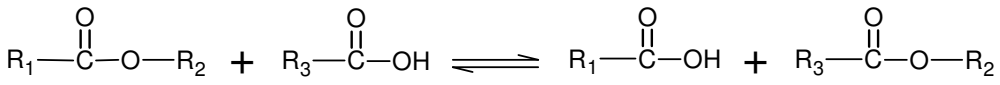


Figure 2.6. Lipase-catalyzed acidolysis reaction between an acylglycerol and an acid (Willis and Marangoni, 2002)

In principle the reaction can be conducted between any organic esters and acids. In most applications in the lipid field, esters can be TAGs, DAGs or MAGs, glycerophospholipids, alkyl fatty acid esters, etc. and acids can be fatty acids or other acids in the esterification section (Xu, 2003).

Enzymatic acidolysis is a reversible reaction. The reaction is commonly considered as a two-step reaction: hydrolysis and esterification. For this reason, DAGs are considered as the reaction intermediates. Through steps of hydrolysis and esterification, the new fatty acids in the system are incorporated into TAGs. The reaction finally reaches equilibrium. This procedure for the reaction between a TAG (LLL) and a fatty acid (M) is depicted in Figure 2.7.

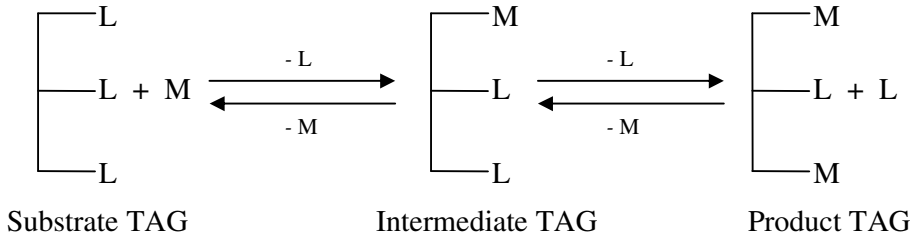


Figure 2.7. Reaction illustration of enzymatic acidolysis between LLL and M with *sn*-1,3 specific lipases. L and M are fatty acids (Xu, 2003)

Because of the thermodynamically unstable nature of DAGs, they are also the precursor of side reactions (Xu et al., 1999). The fatty acids migrate to the neighbor position in the glycerol backbone, which is called as acyl migration. This leads to a positional non-specificity of the lipases and the formation of by-products. The whole reaction between LLL and M is depicted in Figure 2.8.

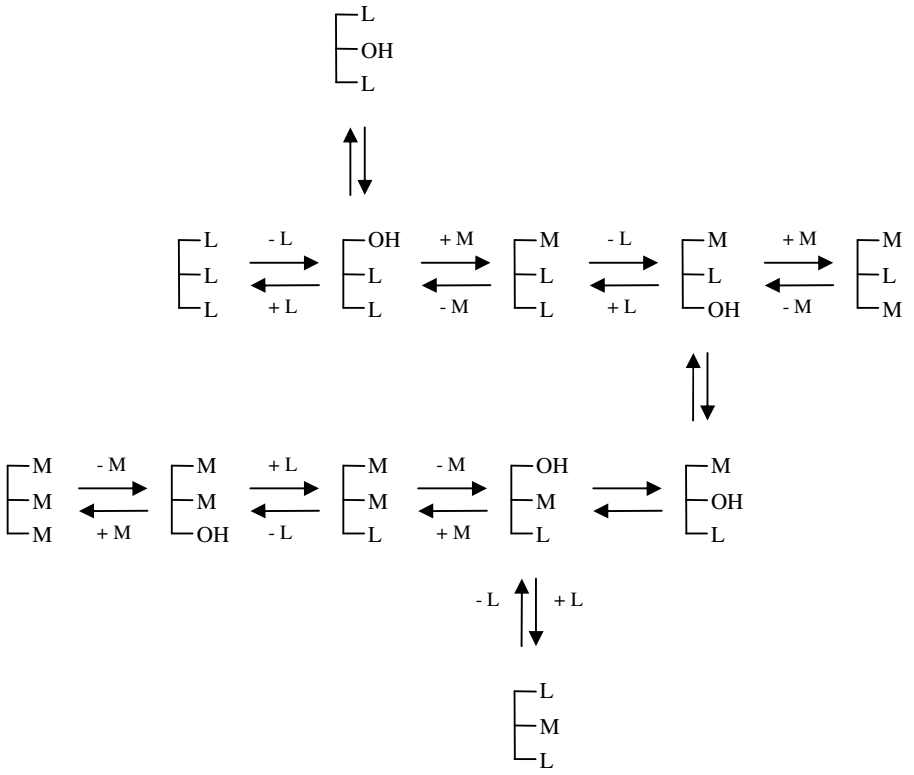


Figure 2.8. A step-by-step reaction pathway for the main reactions and side reactions during *sn*-1,3 specific lipase catalyzed acidolysis between LLL and M. are used. L and M are fatty acids (Xu, 2003)

A particular interest in this area is that *sn*-1,3 specific lipases can selectively catalyze the acyl exchange at the *sn*-1 and *sn*-3 positions whilst leaving the *sn*-2 acyl group unchanged. This provides an opportunity to tailor some functional lipids with special requirements for the fatty acid types located in *sn*-1,3- or 2-positions (Xu et al., 2006).

Acidolysis has been widely used for the production of structured TAGs such as human milk fat substitutes, anti-bloom agents and SLs containing medium or short chain fatty acids, and incorporation of free acid or ethyl ester forms of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) into vegetable and fish oils to improve their nutritional properties (Macrae, 1985; Balcao and Malcata, 1998; Gunstone, 1999; Xu, 2000a; Baljit et al., 2002; Xu, 2003).

An important application of *sn*-1,3 specific lipase-catalyzed acidolysis is the enzymatic production of CBEs (Sridhar et al., 1991; Undurraga et al., 2001; Wang et al., 2006; Liu et al., 2007, Abigor et al., 2003; Chang et al., 1990). The most common method is acidolysis of palm oil midfraction, which contains predominantly POP with stearic acid to increase the level of stearate in the lipid. Stearic acid was incorporated into palmolein to produce 25 % CB-like TAGs (Willis and Marangoni, 2002).

2.3.1.1 Factors affecting enzymatic interesterification

In considering all of the factors involved in enzymatic interesterification, all components of the system must be examined; namely water content, temperature, substrate composition, product composition and lipase load.

Temperature: In general, increasing the temperature increases the rate of interesterification, but very high temperatures can reduce the reaction rates due to irreversible denaturation of the enzyme (Willis and Marangoni, 2002). Animal and plant lipases are usually less thermostable than extracellular microbial lipases (Yamane, 1987). In a solvent-free reaction system temperature must be high enough to keep the substrates liquid (Ison et al., 1994). Temperatures do not to be as high in systems containing organic solvents since they easily solubilize hydrophobic substrates (Willis and Marangoni, 2002).

Water content: The water content in a reaction system is the determining factor as to whether the reaction equilibrium will be toward hydrolysis or ester synthesis. The activity of lipases at different water contents is dependent on the source of the enzyme. The optimal water content for interesterification by different lipases ranges from 0.04 to 11% (w/v), although most reactions require water content of less than 1% for effective interesterification (Malcata et al., 1992).

Enzyme purity and presence of other proteins: During immobilization, adsorption of proteins to surface-active supports is not limited soley to lipases. Other protein sources in the lipase solution can be adsorbed and this can have an effect on the loading and activity of the immobilized enzyme. Nonprotein sources of contamination during immobilization are usually not a problem because the lipase is preferentially adsorbed to the support (Willis and Marangoni, 2002).

Substrate composition and steric hindrance: The composition of the substrate can have a negative effect on the rate of hydrolysis and interesterification. Presence of hydroxyl group in the *sn*-2 position has a negative inductive effect, so that TAGs are hydrolyzed at a faster rate than DAGs, which are hydrolyzed at a faster rate than MAGs (Desnuelle, 1972). If the composition of the substrate is such that it impedes access of the substrate to the active site, any improvements in the nucleophilicity will not improve the activity (Bevinakatti, 1988). Oxidation of substrates, especially polyunsaturated fatty acids, is possible and can cause inhibition and decrease in lipase activity, especially reactions containing organic solvents (Willis and Marangoni, 2002).

Product accumulation: During interesterification of two TAGs, the production of MAGs and DAGs can lead to an increase in the rate of reaction, whereas the presence of high levels of FFAs can inhibit the initial hydrolysis of TAGs (Reyes and Hill, 1994). The loss of activity by lipase in the presence of high concentrations of FFAs has been attributed to production of high levels of free or ionized carboxylic acid groups, which would acidify the microaqueous phase surrounding the lipase or cause desorption of water from the interface (Kuo and Parkin, 1993).

Solvent type: Lipases differ in their selectivity to solvent type. An important solvent characteristic that determines the effect of solvent in enzymatic catalysis is the

polarity of the solvent. The catalytic activity of enzymes in solvents with log<2 is usually lower than that of enzymes in solvents with log>2. This is because hydrophilic or polar solvents can penetrate into the hydrophilic core of the protein and alter the functional structure (Rodionova et al., 1987). They also strip off the essential water of the enzyme (Zaks and Klibanov, 1988). Hydrophobic solvents are less able to remove or distort the enzyme-associated water and are less likely to cause inactivation of enzymes (Dordick, 1989).

2.3.2 Bioreactors for enzymatic interesterification

Enzyme reactors are central part of enzyme processes. An immobilized lipase reactor is a more cost-effective reactional system compared to a soluble lipase reactor. It may be considered as a portion of space in which the lipase macroporous movement is restricted to its boundaries. In general, contact of the reactants with the immobilized lipase is improved by convection relative to plain molecular transport. Various possibilities exist for reactors containing immobilized lipases, but most situations fall within one of the following cases: 1) a lipase in solid form precipitated within an organic liquid phase; 2) a lipase in soluble form in an aqueous phase and confined by a solid ultrafiltration membrane; 3) a lipase contained in an aqueous phase and confined by a surfactant lipid membrane within an organic liquid phase; 4) a lipase entrapped within a three-dimensional polymeric matrix dispersed within an aqueous pahse; and 5) a lipase attached to a solid support and dispersed within an organic liquid phase, or dispersed within an aqueous phase (Balcao et al., 1996).

Many different reactors have been studied and used for the enzymatic modification of lipids, such as STRs, PBRs, membrane reactors, gas-lifting reactors, fluidized reactors, simple circulating reactors, spraying reactors, foam reactors, etc. with/without internal or external extractive or separation steps or other enforcing steps (Xu, 2000b; Prazeres and Cabral, 1994; Paiva et al., 2000). The most common used bioreactors for enzymatic interesterification are batch STRs or PBRs. There are no universal criteria for the choice of a reactor. The following can be used for the evaluation of a suitable reactor for a particular system and process (Xu, 2003):

– reaction efficiency (homogeneity, dispersion, enzyme activity, etc.),
– product quality (yield and purity),
– enzyme reuses or stability,

– process feasibility and practicality,

– process productivity,

– process investment and availability,

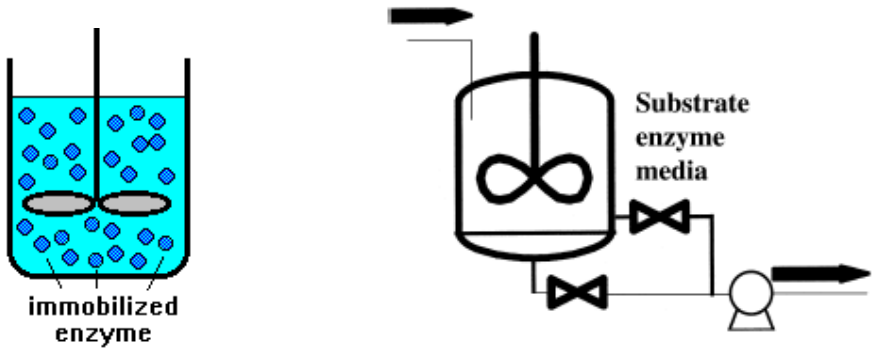
– process and product change flexibility,

– easiness of process operation, control, and monitoring.

Most reactions in the laboratory are carried in a batch STRs (Xu et al., 2006). These reactors operate batch-wise and consists of a vessel in which the reactant fluid mixture is stirred by some mechanical means (e.g., magnetic bars, submerged impellers, reciprocal oscillators, or end-over-end rotators) in a way that avoids the existance of temperature and concentration gradients.

2.3.2.1 Stirred tank reactors

A STR (Figure 2.9) is one in which all of the product is removed, as rapidly as is practically possible, after a fixed time by filtration or centrifugation. Generally this means that the enzyme and substrate molecules have identical residence times within the reactor, although in some circumstances there may be a need for further additions of enzyme and/or substrate (i.e. fed-batch operation).
(http://www.lsbu.ac.uk/biology/enztech/reactors.html)



a b

Figure 2.9. Simplified illustrations of (a) a batch STR, and (b) a continuous STR (http://www.lsbu.ac.uk/biology/enztech/reactors.html)

It requires less enzyme dosage and it is easy to apply vacuum or add molecular sieve to reduce the water content in the system. However it also has some drawbacks.

Longer reaction times are normally required due to the low enzyme dosage. It also gives rise to a high degree of acyl migration, which leads to nonspecificity of the interesterified products (Xu, 2003). The operating costs of batch reactors are higher than that of continuous processes due to the necessity for the reactors to be emptied and refilled both regularly and often. This procedure is not only expensive in itself but means that there are considerable periods when such reactors are not productive; it also makes uneven demands on both labour and services.
(http://www.lsbu.ac.uk/biology/enztech/reactors.html)

2.3.2.2 Packed bed reactors

An efficient reaction is the most essential requirement for a particular system. PBRs (Figure 2.10) are one of the most interesting reactor types that have currently received a lot of attention and applications in the lipid modification industry.

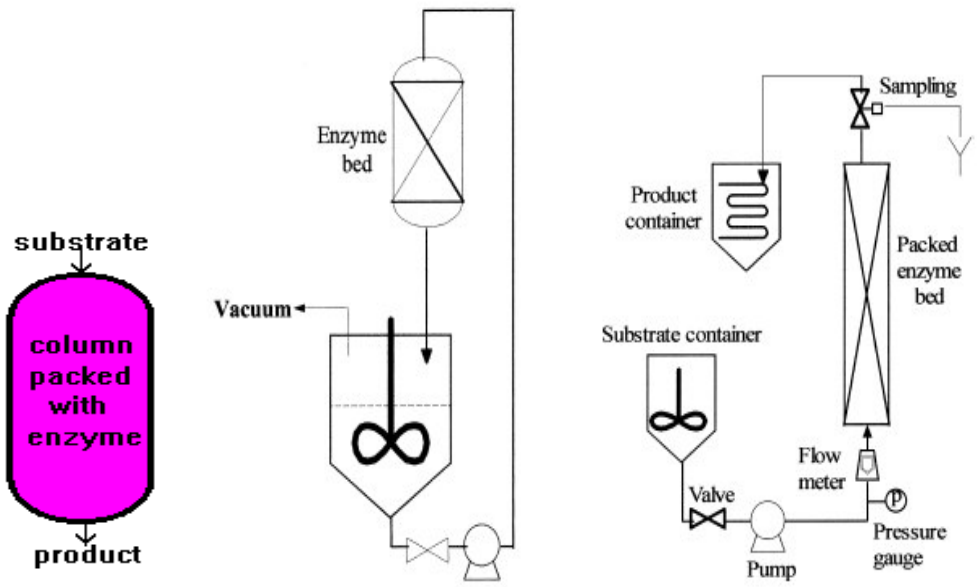


Figure 2.10. (a) Simple illustration of packed bed reactor, (b) A PBR system with product circulation and a storage tank with vacuum applied (c) A PBR system without product circulation (http://www.lsbu.ac.uk/biology/enztech/pbr.html; Xu, 2003)

The particular concerns for the application of PBRs in lipid modification can be (Xu, 2003):

- simple process and operation,
  - no filtration of enzyme necessary,
  - less damage for the enzyme particle size and structure,
  - continuous process easy and enzyme reuses simple,
  - low investment,
  - less reaction time and in turn leading to less side reactions
- better quality as a result of better reactions,
  - efficient reaction under optimal operation.

A few things on the other hand should be carefully controlled or avoided, such as:
– internal and external mass transfer limitations,
– channeling under either upward or downward flow,
– pressure drops under smaller size of enzyme particle
or higher flow rate,
– water content control and regulation,
– fouling of enzyme bed during the process running.

The most important characteristic of a PBR is that material flows through the reactor as a plug, so, they are also called plug flow reactors (PFR). Ideally, all of the substrate stream flows at the same velocity, parallel to the reactor axis with no back -mixing. All material present at any given reactor cross -section has an identical residence time. The longitudinal position within the PBR is, therefore, proportional to the time spent within the reactor; all product emerging with the same residence time and all substrate molecule having an equal opportunity for reaction. The conversion efficiency of a PBR, with respect to its length, behaves in a manner similar to that of a well -STR with respect to its reaction time. Each volume element behaves as a batch reactor as it passes through the PBR. Any required degree of reaction may be achieved by use of an idea PBR of suitable length (http://www.lsbu.ac.uk/biology/enztech/pbr.html).

There are three substrate flow possibilities in a packed bed and they are: Downward flow method, upward flow method, and recycling method. The recycling method (Figure 2.10b) is advantageous when the linear velocity of the substrate solution

affects the reaction flow rate (Xu, 2003). This is because the recycling method allows the substrate solution to be passed through the column at a desired velocity. For industrial applications, upward flow is generally preferred over downward flow because it does not compress the beds in enzyme columns as downward flow does. When gas is produced during an enzyme reaction, upward flow is preferred (http://www.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/packbed.htm).

Reduction in the enzyme activity in a single PBR should be considered and so, flow rates should be constantly adjusted and reduced to obtain a stable product quality and yield. However, this is often not practical in the industry plants. Since, stable process capacity can not be maintained even though the automatic adjustment of flow rates can be conducted. The way to maintain a stable production capacity for a practical process is to make a series PBR design in which a number of unit PBRs are set in series along the flow line and the ages of the enzyme in each PBR are different (Figure 2.11). The used-up PBR can be moved out of the series from the first step and the new one is then inserted into the series in the last step. In this way a stable production capacity can be maintained with little fluctuation if the number of unit PBRs is sufficient. (Xu, 2003).

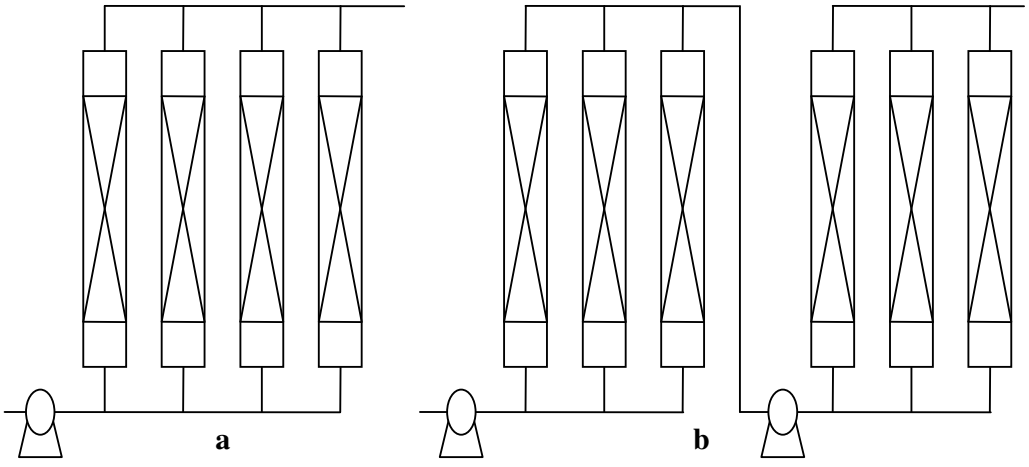


Figure 2.11. (a) Parallel setup of PBRs, (b) Series and parallel setup of PBRs (Xu, 2003)

2.4 Cocoa Butter Equivalents: A Promising Structured Lipid Produced Enzymatically

Before considering the CBEs, it is necessary to understand CB itself. Following sections give informations about CB and CBEs.

2.4.1 Cocoa butter

The properties of CB have drawn the attention of many investigators, owing to the significance of CB in the confectionary industry. CB is used on a very small scale in the pharmaceutical industry for the preparation of suppositories and in the cosmetics industry for the preparation of lipstick and body lotions. Some decennia ago CB was used quite a lot for the production of cosmetics, but now it has mostly been replaced by synthetic products (Pontillon, 1998b). The most important use of CB is, of course, the preparation of chocolate.

CB is derived from the cocoa bean, the seed of the cocoa tree (Theobroma cacao) (Smith, 2001). The cocoa tree is native to Central and South America. Today it is cultivated in a belt along the equator, the main producing countries are found in West Africa (Ivory Coast, Ghana, Nigeria), South-East Asia (Indonesia, Malaysia, Papua New Guinea) and Central and South America (Ecuador, Brazil) (Ulberth and Buchgraber, 2003). Typically, 100 g of cocoa beans produces 40 g of CB, 40 g of cocoa powder, and 20 g of waste material (shell, moisture, dirt, etc.) (Timms and Stewart, 1999). The different steps needed to produce CB from the cacao pod are summarized in Figure 2.12.

2.4.1.1 Composition of cocoa butter

For characterization of CB no attempt was made to include volatile compunds such as aromas. They can not be considered useful for the characterization of vegetable fats in mixtures with CB for two reasons: i) the foreign vegetable fats normally show no flavor at all; and ii) the flavor typical for CB alters significantly with climate, seasonal variations, country of origin, etc. Moreover, flavor constituents are easy to remove from CB in a process called deodorisation, widely applied in industry (Lipp and Anklam, 1998).

CB is composed principally of TAGs, although minor components include MAGs, DAGs, FFAs, phospholipids and other complex lipids (Smith, 2001).

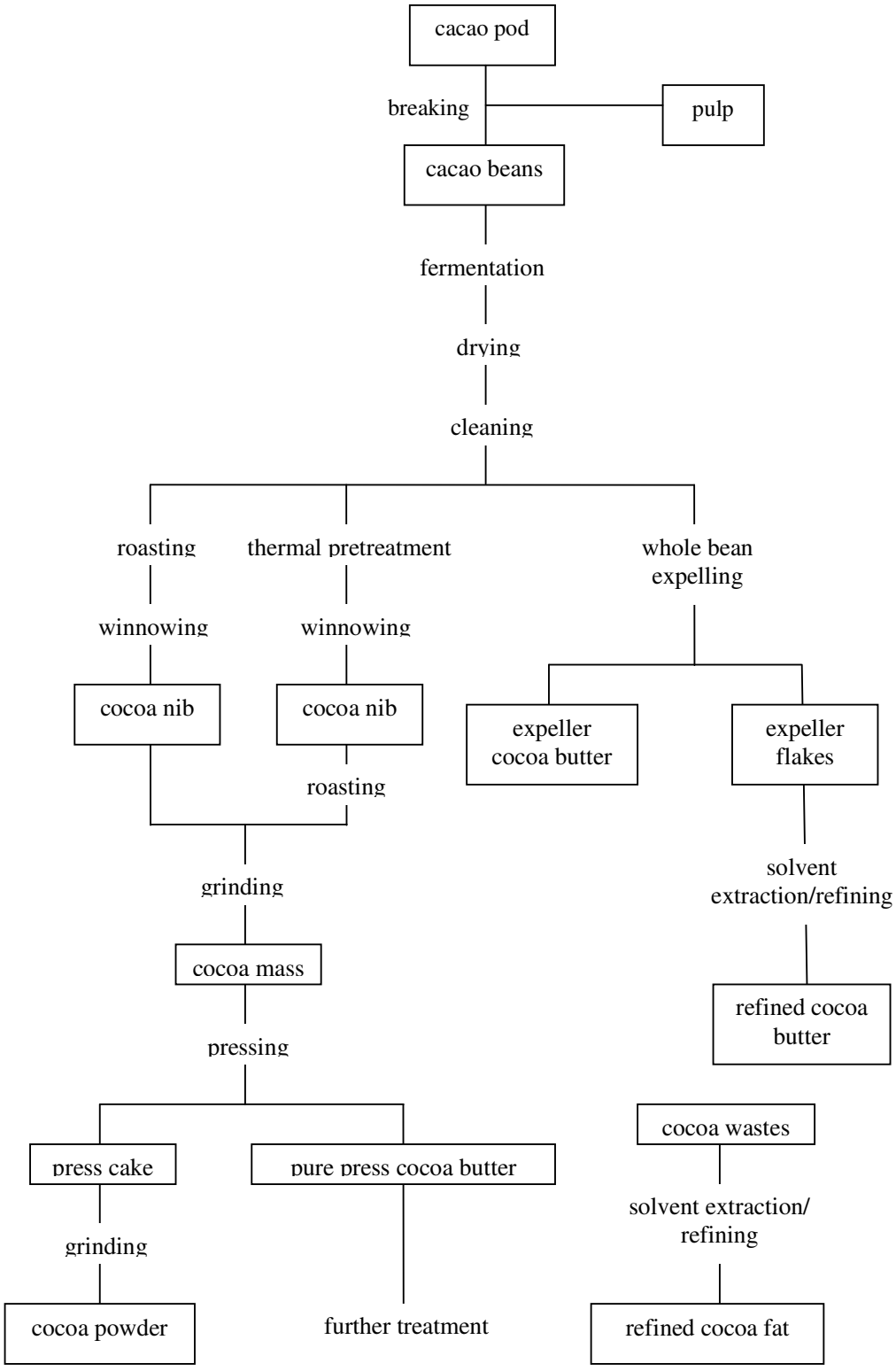


Figure 2.12. Overview of the different steps in the production of CB (Meursing, 1994)

2.4.1.1.1 Fatty acid composition

In terms of its fatty acid composition, CB is a relatively simple fat. Palmitic, stearic and oleic acid, generally account for over 95% of the fatty acids. Of the remaining acids, linoleic and arachidic acid are present at the highest level (Talbot, 1999). Table 2.2 gives an overview of the fatty acid composition of CB from different countries of origin (Kanematsu et al., 1978; Klagge and Sen Gupta, 1990, Van Malssen et al., 1996). The total amount of unsaturated fatty acids is higher in Brazilian CB. Values higher than 40% are reported, while for the other CBs values between 36 and 38% are found (Klagge and Sen Gupta, 1990 and Shukla, 1995).

Table 2.2. Fatty acid composition (area %) of CB for some countries of origin

Fatty acid	Kanematsu et al., 1978; Klagge and Sen Gupta, 1990						Van Malssen et al. (1996)
	Country of origin						
	Ecuador	Brazil	Ghana	Ivory Coast	Malaysia	Java	
Palmitic acid 16:0	25.6	25.1	25.3	25.8	24.9	24.1	20.0-23.8
Stearic acid 18:0	36.0	33.3	37.6	36.9	37.4	37.3	29.4-34.1
Oleic acid 18:1	34.6	36.5	32.7	32.9	33.5	34.3	29.4-34.7
Linoleic acid 18:2	2.6	3.5	2.8	2.8	2.6	2.7	2.2-3.7
Linolenic acid 18:3	0.1	0.2	0.2	0.2	0.2	0.2	0.1-0.3
Arachidic acid 20:0	1.0	1.2	1.2	1.2	1.2	1.2	0.8-1.1

2.4.1.1.2 Triacylglycerol composition

The TAG composition of typical CBs is given in Table 2.3. TAGs represent the major part of CB (97-98 %) with three main fatty acids: palmitic (C16); stearic (C18); and oleic fatty acis (C18:1). CB contains almost equal amounts of palmitic, stearic and oleic acids. However, these acyl groups are not distributed randomly across the glycerol positions (Smith, 2001). With the oleic fatty acid always esterified in the central position of the glycerol molecule while saturated fatty acids are in positions 1 and 3. CB presents three main symmetric TAGs: POP, POS,and SOS (Saldana et al., 2002).

Table 2.3. Typical TAG composition (%) of cocoa butter

TAG	Podlaha et al. (1984)							Van Malssen et al. (1996)
	Country of origin							
	Samoa	Ivory Coast	Ecuador	Malaysia	Ghana	Nigeria	Bahia	
PPL	1.6	1.9	1.9	1.5	1.9	1.9	1.7	0.6-1.5
SOL	0.5	0.9	0.8	0.7	0.4	0.8	1.0	0.4-2.4
POO	2.2	4.4	3.5	2.7	2.6	3.2	5.5	2.7-8.1
PSL	2.8	3.6	2.8	2.8	3.2	3.4	3.4	1.6-3.6
POP	16.4	15.9	15.3	13.8	15.2	14.8	14.0	11.0-15.4
SOO, PPP	3.7	6.0	4.8	3.8	4.5	5.1	8.4	3.6-10.9
SSL	2.1	1.8	1.5	2.0	2.1	1.9	2.1	1.0-2.3
POS	38.3	36.6	36.3	36.6	37.3	37.4	34.6	29.9-37.8
SOS	26.8	23.8	26.9	28.4	26.8	26.4	23.7	20.2-27.4
SOA	2.2	1.6	2.1	2.5	2.2	1.9	1.6	1.1-1.9
POL	0.8	0.6	0.7	0.6	0.6	0.8	1.1	
OOO	0.2	0.8	0.8	0.8	0.5	0.4	0.9	5.4-8.1
OOA	1.6	1.0	1.2	1.6	1.4	1.2	1.5	
PPS	0.4	0.4	0.3	0.6		0.7	0.3	
SSP	0.7	0.8	0.9	1.0	1.3	0.4	0.2	

(P: palmitic; O: oleic; S: stearic; L: linoleic)

Most recently Tagaki and Ando (1995) have confirmed that POS is present as a racemic mixture of POS and sn-1-stearoyl-2- oleyl-3-palmitoyl-glycerol (SOP). This Sat-O-Sat group comprises 70-88% of the CB TAGs. Variations arise due to the subspecies of the cocoa plant and the climatic conditions (higher ambient temperatures leading to higher Sat-O-Sat levels and lower Sat-O-O levels). These three major TAGs of CB are mainly responsible for providing the valuable crystallisation and melting characteristics (Lipp and Anklam, 1998).

Two other TAG classes present in CB are monounsaturated dioleoylglycerols (Sat-O-O) and the disaturated-2 linoleoyl-glycerols (Sat-L-Sat). These TAGs have lower melting points than Sat-O-Sat and contribute to the liquid phase. The main difference between CBs from different countries of origin is the ratio of mono-unsaturated (mainly POP, POS and SOS) to di-unsaturated (mainly 1-palmitoyl-2,3-dioleoylglycerol (POO) and 1-stearoyl-2,3-dioleoylglycerol (SOO)) TAGs. In general, especially Brazilian but also other South American CBs contain the highest amount of di-unsaturated TAGs (around 15%) while Asian CBs contain the lowest amount of these TAGs (around 5%). African CBs contain intermediate amounts (Chaiseri and Dimick, 1989).

2.4.1.1.3 Minor components

As well as TAGs, CB contains some other components in much smaller amounts. These so-called minor components include FFAs, MAGs, DAGs, phospho- and glycolipids and unsaponifiable matter.

Depending on the study FFA contents differ but are generally between 0.4 and 3%. Most of the obtained values are below the legal limit of 1.75%. Chaiseri and Dimick (1989) found three samples above this limit, originating from Ecuador, Malaysia and Peru, while Shukla (1995) found two samples above the limit, originating from Nigeria and Ivory Coast. These high values can be due to hydrolysis by lipase from mould contamination because of insufficient drying, extended fermentation or prolonged storage of fresh beans (Chaiseri and Dimick, 1989; Pontillon, 1998a).

Chaiseri and Dimick (1989) reported that South American, Asian and African CBs had content of FFA (mean values of 1.25, 1.44 and 1.27 % respectively) but that North and Central American CBs contained significantly lower amounts (average of

0.79%). The lower degree of fermentation in these regions is the probable explanation. Palmitic, stearic and oleic acids are the major FFAs found in CB (Chaiseri and Dimick, 1995a).

Shukla (1995) observed DAG contents between 1.1 and 2.8% for CBs from different countries of origin, but did not observe any correlation between the amount of DAGs and the production region. Pontillon (1998a) stated a mean value of 1%. Only trace amounts of MAGs are present (Pontillon, 1998a).

Reported phospholipid levels vary between 0.1-0.2% (Chaiseri and Dimick, 1995a), 0.34% (Arruda and Dimick, 1991), 0.37% (Davis and Dimick, 1989), 0.8-0.9% (Savage and Dimick, 1995) and 0.72-0.94% (Shukla, 1995). Parsons et al. (1969) found values between 0.28 and 0.45% in raw cacao-beans from different countries of origin. They also found a tendency for African beans to have a lower concentration of phospholipids than beans of American origin. For the amount of glycolipids, Pontillon (1998) reported a value of 0.3% while Chaiseri and Dimick (1995a) reported values between 0.3 and 0.8%.

The amount of unsaponifiable matter values range from 0.3 to 1.5% (Schlichter-Aronhime and Garti, 1988a).

2.4.1.2 Function of cocoa butter

CB is a highly valued ingredient primarily used in the confectionery, especially chocolate industry (Lipp et al., 2001). Due to its unique TAG composition, CB gives special physical properties to chocolate. Of all the ingredients present in chocolate, none has more influence on its physical and chemical properties than CB (Saldana et al., 2002). The crystal lattice of CB confers desired physical properties to the manufactured product, e.g. texture, gloss, snap, melting properties, etc (Lipp et al., 2001). The distribution of the ingredients of chocolate, i.e. the fine dispersion, is crucial for the taste of the product (Lipp and Anklam, 1998). CB acts as the continuous phase in chocolate, and supports the nonfat ingredient. Thus, being chocolate relatively hard and brittle at room temperature but melting rapidly in the mouth is greatly dependent on the properties of CB itself (Smith, 2001).

CB has a sharp melting range of 27-33°C and has ability to recrystallize during processing to a stable crystal mode (Smith, 2001). Figure 2.13 illusrates the melting behaviour of CB by showing the change in SFC as function of temperature.

This melting behaviour of CB is critical to its behaviour in chocolate formulations. If it were melt at a lower temperature, it would yield chocolate that was too soft. If it were melt at temperatures greater than 35°C, it would remain unmelted in the mouth and be perceived as waxy and chewy. The steepness of the melting profile (solid fat as a function of time) has a large impact on the flavor release. The flavor is present in the CB itself as well as in the nonfat components that are held by the CB melts. If the fat has a sharp melting profile, much of this flavor is released in a relatively short space of time, yielding an intense flavor. A reduction in steepness of the melting profile would slow flavor release and lessen the impact. This sharp melting will also give rise to a greater sensation of cooling in the mouth due to the heat absorbed by the fat during melting (Smith, 2001).

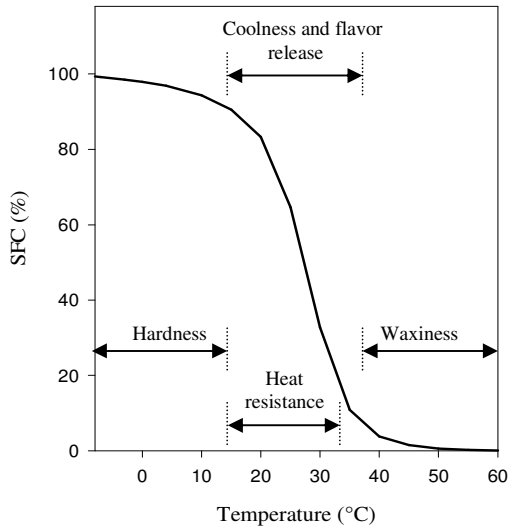


Figure 2.13. SFC of CB as function of temperature (Talbot, 1999)

2.4.2 Cocoa butter alternatives

World production of cocoa bean was ca. 3.4 million tons in 2006/2007 compared to ca. 1.5 million tons in 1970/1971. However during years the cocoa bean prices showed fluctuations. World production of cocoa beans dropped by almost 9% from

the previous season to 3.4 million tonnes in 2006/2007, mainly as a consequence of unfavourable weather conditions in many cocoa producing areas (ICCO Annual Report for 2006/2007). Triggered by this fluccuation in supply and demand, the volatility in cocoa bean prices, and poor quality of individual harvests, substitutes to cocoa products were sought, which led to the development of the so-called CB alternatives (CBAs) (Ulberth and Buchgraber, 2003).

The reasons of industry to use alternatives instead of CB are:

- CB is expensive.
- CB is a natural raw material, so changes occur in its quality.
- It needs tempering.
- Brightness of chocolates produced from CB has limited stability.
- Production cost of CB is high.
- Melting properties are not suitable for all applications and climates.
- Fluctuations in the supply and demand of CB.

In 1950's, studies on the chemical properties of CB showed that TAGs present in CB have a unique structure, and the oil blends that will be used in chocolate should have a similar TAG structure to those in CB. Unlike all animal fats, some vegetable oils contained TAGs of the appropriate structure. This made possible the production of specific fat fractions, which, when blended, had properties almost identical to those of CB itself (Talbot, 1999).

In order to perform satisfactorily in chocolate a CBE must meet certain requirements (Talbot, 1999) :

- Should show a similar melting profile to that of CB.
- Should have a similar TAG composition.
- Should have a similar crystallisation.
- It must be compatible with CB
- It must have a similar fat crystal microstructure

Vegetable oils which were used instead of CB in chocolate and its coatings and are called as CBAs. CBAs may be used for a partial or even total replacement of CB in confectionery and chocolate without impacting the functionality of the fat phase. It is even claimed that certain CBAs can improve some quality related features (resistance

to bloom, increased milk fat tolerance, etc.) of the finished product (Shukla, 1997). However, the main reason for the usage of CBAs is their economic advantage over good quality CB. Depending on their functional compatibility with CB, they are classified as (Lipp and Anklam, 1998):

a) Cocoa butter substitutes

CB substitutes (CBS) are lauric plant fats (containing lauric acid), chemically totaly different to CB, with some physical similarities; suitable only to substitute CB fully (Lipp and Anklam, 1998). Such fats are usually obtained from fats like palm kernel oil. They have a similar melting profile to CB but have quite different polymorphic behavior, being stable in the β′ polymorph. This is due to the fact that the TAG composition, incorporating 12:0 (lauric) acyl groups, is quite unlike the 16:0 and 18:0 TAGs found in CB. This dissimilarity in structure (molecular size and shape) gives rise to a phase separation in the solid between CB TAGs and those of the lauric substitute if more than a few percent (of fat phase) of CB is present in the substitute or vice versa. Figure 2.14 shows an isothermal binary diagram of CB /CBS blends.

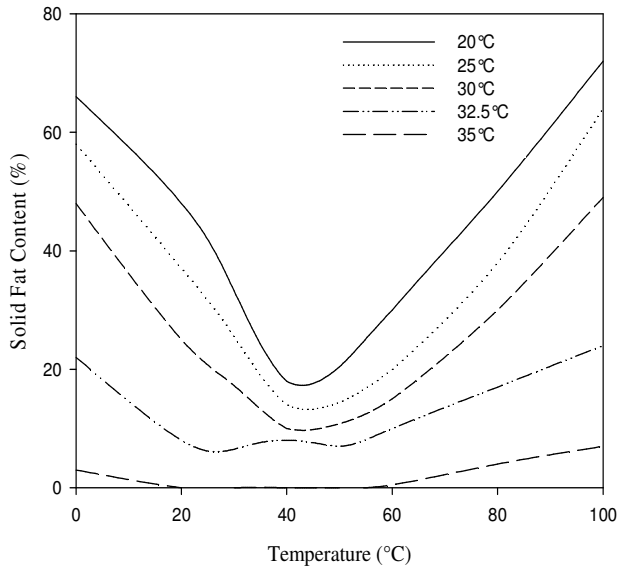


Figure 2.14. Isothermal diagram of CB/CBS blends (replotted from Smith, 2001)

b) Cocoa butter replacers

CB replacers (CBR) are non-lauric (not containing lauric acid) fats with a distribution of fatty acid similar to CB, but a completely different structure of the TAGs (Lipp and Anklam, 1998). Consequently, they have limited compatibility with CB; only in small ratios compatible to CB. They are generally produced from non-lauric oils such as palm oil and soyabean oil by hydrogenation and fractionation (Talbot, 1999). They are much more compatible with CB than are substitutes. Although they are different in TAG structure, the TAGs have similar acyl chain lengths, and hence the molecular size, to those found in CB. As a result, the two fats have greater tolerance for one another, perhaps up to 10-15% in the fat phase, dependent on the specific composition of the replacer. This can be seen in Figure 2.15 which shows the effect on melting properties of mixing CBR with CB (Smith, 2001).

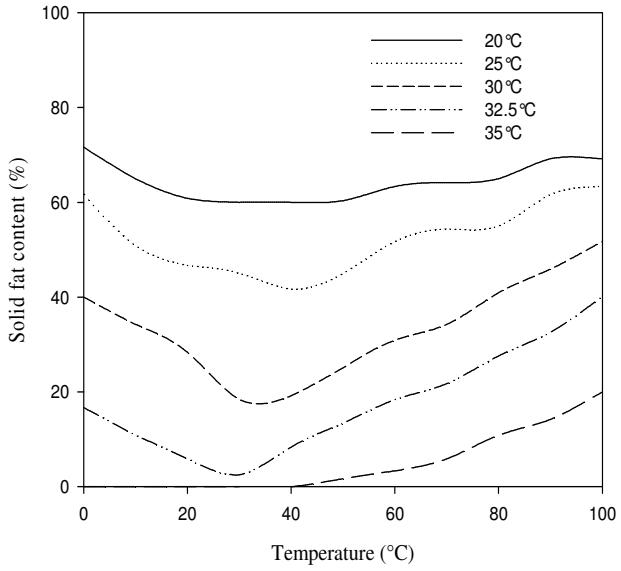


Figure 2.15. Isothermal diagram of CB/CBR blends (replotted from Smith, 2001)

c) Cocoa butter equivalents

CBEs are non-lauric plant fats, which are similar in their physical and chemical properties to CB and mixable with it in every amount without altering the properties of CB (Lipp and Anklam, 1998). To ensure complete compatibility (i.e., solid phase miscibility) with CB, a fat must have not only a similar melting profile but also a

similar molecular size and polymorphism. The best way to achive this is to mimic the molecular composition. Thus, CBEs are designed to have TAG compositions as close to CB as possible (Smith, 2001). CBEs are the closest to CB among alternatives and are totally compatible with CB (Akoh and Xu, 2002). They can be mixed in desired proportions, because they have a same composition as CB (Norberg, 2006). CBEs can replace CB partially or completely (Lipp and Anklam, 1998). Even it was proposed that they could improve some quality parameters (Shukla, 1997). They can give a product that is at least as good as CB with respect to bloom (Norberg, 2006). The isothermal binary diagram of CB and CBE in Figure 2.16 demonstrates the complete miscibility between the fats. There is a complete absence of eutectic behaviour (Smith, 2001).

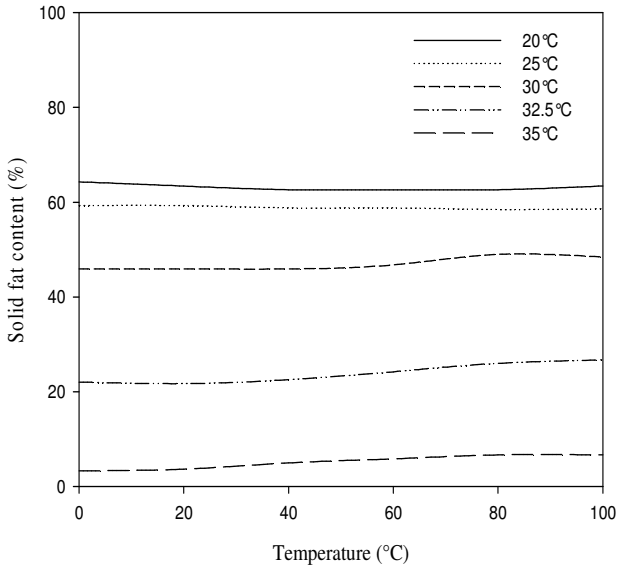


Figure 2.16. Isothermal diagram of CB/CBE blends (replotted from Smith, 2001)

2.4.3 Ways of designing cocoa butter equivalents

a) Plant sources

Variety of plant sources have been reported as an alternative source to CB. However none of those alternatives have found general acceptance (Ulberth and Buchgraber, 2003). The review of those plants are given briefly in Table 2.4.

Table 2.4. Alternative plant sources of CBEs

Plant source	Author
Aceituno (Simarouba glauca)	Jeyarani and Yella-Reddy, 2001
Cupuasu beans (Theobroma	Queiroz and Garcia, 1999
Dhupa (Vateria indica)	Sridhar et al., 1991
Mahua (Madhuca latifolia)	Jeyarani and Yella-Reddy, 1999
Phulwara (Madhuca butyracea)	Yella-Reddy and Prabhakar, 1994
Pili nut (Canarium ovatum)	Kakuda et al., 2000
Scindapsus officinalis	Daulatabad and Mirajkar, 1992
Shorea macrophylla, S. Mecistopteryx	Nesaretnam and Razak bin Modh Ali,
Theobroma subincanum	Bruni et al., 2000

b) Blending

Although no individual fats that have the same TAGs in precisely, there are commercial fats that contain some of the component glycerides, and the same proportions as CB. For example, palm oil contains POP, and this can be separated from the other glycerides in palm oil by fractionation. Shea and sal oils contain SOS, which can also be isolated by fractionation. Moreover, illipe butter contains mainly POS with some SOS. By careful fractionation and processing of the appropriate oils, and skilled blending of the obtained glyceride mixtures, it is possible to produce CBE fats with properties very close to those of CB (Smith, 2001). Figure 2.17 shows the production of a CBE blend. It is not possible to make a CBE from mixtures of natural oils that totally mimics the TAG composition of CB (Talbot, 1999). For example, by blending palm mid fraction with shea stearin only an approximate composition can be obtained. Subsequent addition of illipe brings the composition closer to but not identical to that of CB (Smith, 2001). However, as Padley et al. (1981) indicated it is not necessary to attain a completely identical composition. It is only necessary to mimic the behaviour.

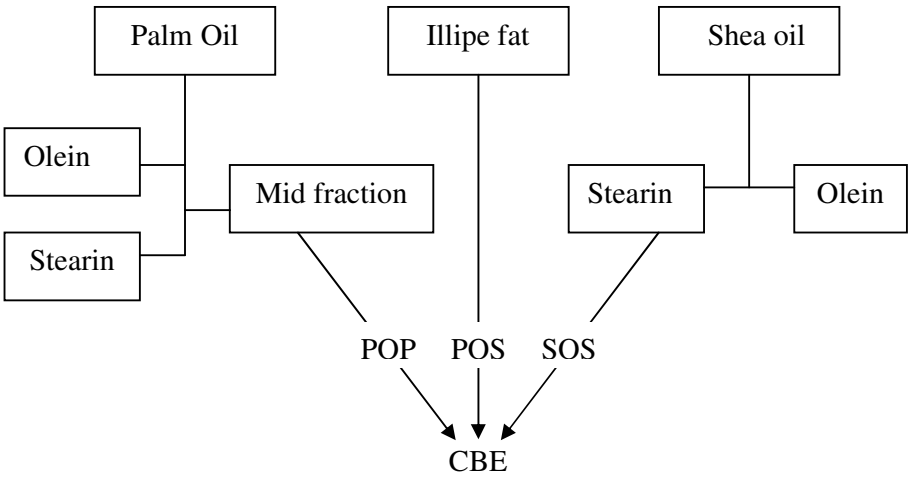


Figure 2.17. Process scheme for production of a CBE blend (Matissek, 2000)

c) Biotechnology

Fermentation was reported another way to produce CBEs. Hassan et al. (1993) and Hassan et al. (1995) used *Apiotrichum curvatum* and *Cryptococcus curvatus* yeasts, and Roux et al. (1994) used *Mucor circinelloides* mould to produce CBEs.

The developments of enzyme technology, especially in lipid modification and synthesis, make it possible to produce CBEs from other vegetable oils that mimic the composition and properties of CB. CBEs can be produced by modifiying the TAG composition of source fats and oils using regiospecific lipases in order to mimic the TAG composition of CB.

2.4.4 Processes for the production of CBEs by lipase-catalyzed acidolysis

Production of CBEs is done through introduction of palmitic and stearic acid to a starting oil that has high oleic acid content in the *sn*-2 position of the TAG (Figure 2.18). Thus, work on CBEs has focused on the use of TAG with oleic acid in the *sn*-2 position as starting materials (Khumalo et al., 2002). For that purpose, the enzyme used should be *sn*-1,3 regiospecific exchanging TAG residues only at positions *sn*-1 and *sn*-3, and preferably immobilized because in this form it is easily separated from the products and is also more thermostable than the free enzyme (Undurraga et al., 2001).

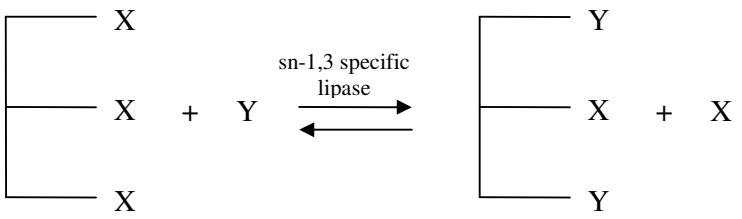


Figure 2.18. A principle illustration of enzymatic acidolysis between a TAG (XXX) and a fatty acid (Y)

To use the regiospecificity of lipases for specific modifications, we can choose different fats and oils that contain different major fatty acids at the *sn*-2 position of their TAGs or main specific TAG species. For the production of CBEs, generally palm oil midfraction has often been chosen as starting material. Oils and fats such as Chinese vegetable tallow (Xu et al., 1997), teaseed oil (Xu et al., 1994), olive oil, high-oleic-canola oil (Gitlesen et al., 1995), sal fats, mango fat, illipe fat, kokum fat and shea oil (Sridhar et al., 1991) have also been reported (Macrae and Hammond, 1985; Macrae, 1992).

Three types of acyl donors were used for the production of CBEs: TAGs, ethyl fatty acid esters, and FFAs. The choice of acyl donors depends on the product purity, reactivity, purification technology and price. No clear difference of reactivity between FFAs and their ethyl esters have been obtained.

STRs or simple glass vessels have used for the production of such SLs in most studies (Schmid et al., 1998; Xu et al., 1998; Soumanou et al., 1998; Shimada et al., 1996). PBRs seem to be more promising for industrial developments with immobilized lipases (Xu et al., 1999; Xu et al., 1998). Both batch STRs and PBRs can be used for this purpose from laboratory small scale to industrial plants. Since positional specificity is essential for this reaction in most applications, the reaction time should be as short as possible since acyl migration during the reaction process is proportional to the reaction time. For this consideration, PBRs have been considered the best reactors for this reaction system (Xu et al., 1998; Paez et al., 2002). Reaction in a PBR has shown to generate very low acyl migration compared to the reaction in a STR (Xu, 2003). Figures 2.19 and 2.20 show two different industrial production of

CBEs. For CBE production (Figure 2.19), high oleic sunflower oil is often used as the substrate oil and stearic acid as the acyl donor. The substrate passes the first packed enzyme bed and the first stage product is purified by distillation to separate the FFAs.

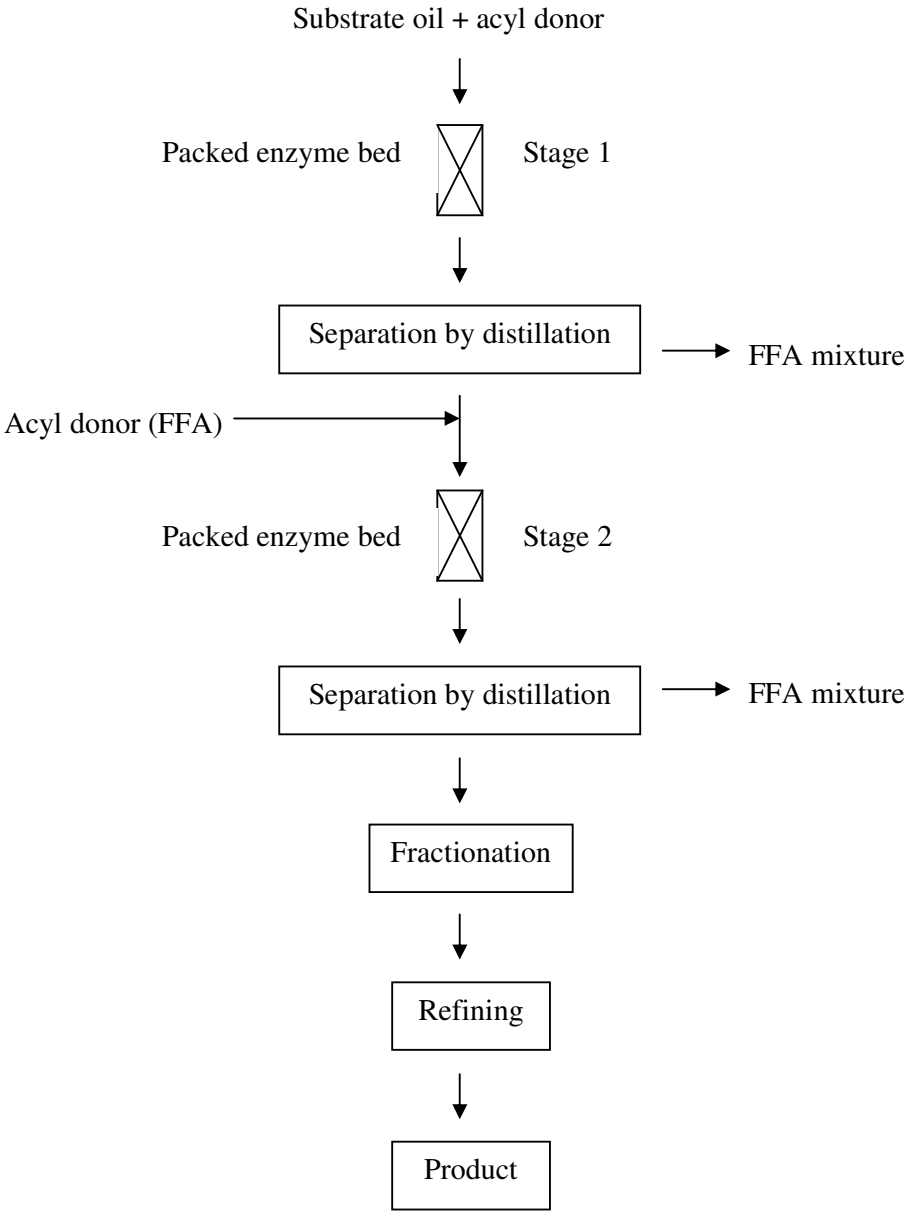


Figure 2.19. Industrial process scheme for the production of CBEs by lipase-catalyzed interesterification using PBR (Xu, 2003)

The purified product is further mixed with acyl donors and passes to the second-stage PBR. The second-stage product is purified by distillation. The purified product is

further fractionated to separate unreacted substrate oils and DAGs formed. A final refining, including bleaching and deodorization is necessary to make it suitable for edible purpose (Xu, 2000b).

In a different process as seen in Figure 2.20, hexane was used as the medium and ethyl stearate was used as the acyl donor to reduce the boiling point.

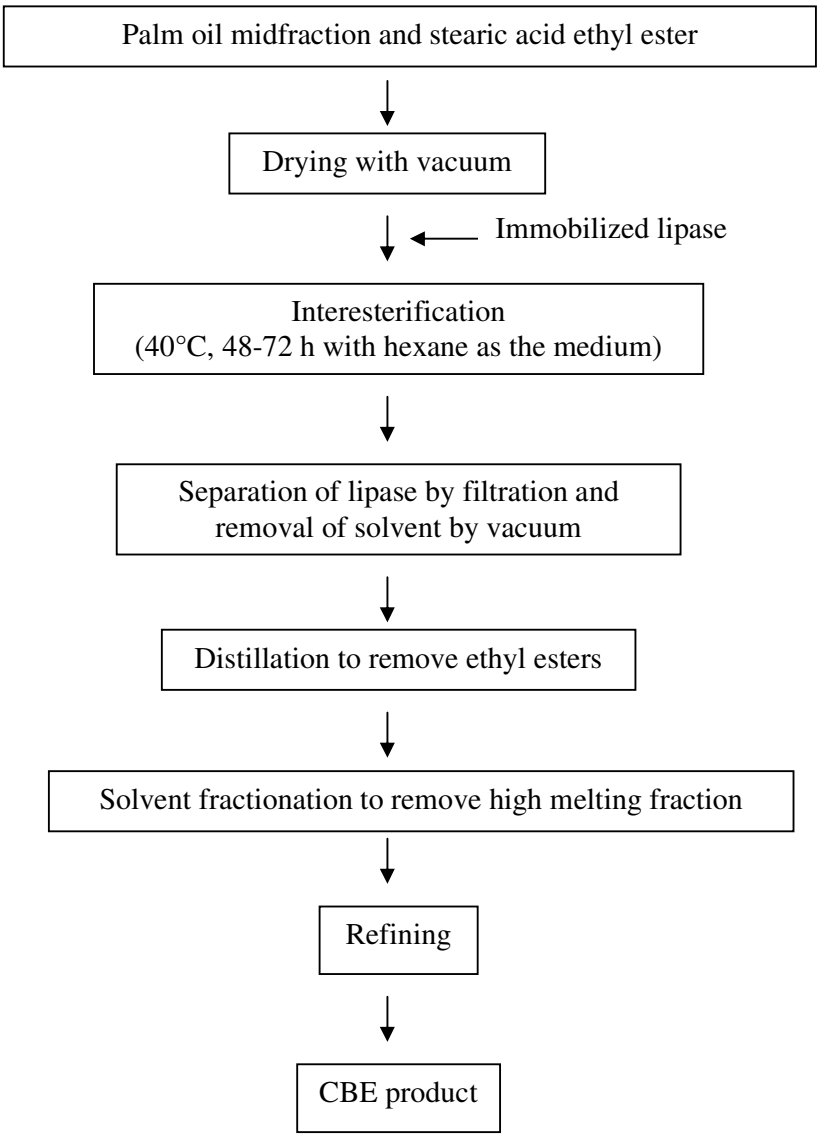


Figure 2.20. Batch process for the production of CBEs by lipase-catalysed interesterification in a STR (Xu, 2003)

This will increase the substrate solubility in hexane and reduce the viscosity of the system, and also reduce the distillation temperature afterwards (Xu, 2000b).

2.4.5 What properties are considered for a Cocoa Butter Equivalent evaluation?

2.4.5.1 Crystallization and melting

Crystallization of fats is important for oils and fats processors and for manufacturers using oils and fats in their end-products (Talbot et al., 2005). The cystallization process may be separated into two steps: nucleation and crystal growth. However, both steps often occur simultaneously, making it difficult to determine the kinetics of each process. The crystallization depends on the TAG composition, the level of minor components and processing conditions (temperature, cooling rate, agitation, etc.) (Herrera and Hartel, 2000). Ziegleder (1990) studied the isothermal CB crystallization via Differential Scanning Calorimeter (DSC) and established a strong temperature dependency in the range of 19-23°C. A plot of the Avrami rate constant versus temperature shows a decreasing straight line at temperature from 20°C onwards. This was attributed to the strong temperature dependence of the nucleation rate since the crystal growth rate is only very slight dependent on the temperature range studied. At temperatures below 20°C, the rate constant increases less than expected from the linear relationship. According to the author, this was caused by an incraese in the melt viscosity at these lower temperatures.

Melting and crystallization are reversible. The melting behaviour of fats in foods is important for both consumer perception and product stability. The melting of CB is an endothermic process where the release of heat from the mouth to the product melts the fat crystals, imparting a pleasant cooling sensation. The steeper the melting profile of a fat , the greater the cooling sensation. Complete melting of the fat phase will result in a rich mouth feel product, but a fat components that melt at or above mouth temperature will impart a waxy mouth feel (Martini et al., 2006).

2.4.5.2 Microstructure

Physical properties of fats are influenced by all levels of structure, but particularly by microstructure, since this is the level of structure closest to the macroscopic world. Microstructure includes the spatial distribution of mass, particle size, interparticle distance and particle shape. CB has a well-defined microstructural network. This

well-defined network is due to the strong and defined interactions between microstructural elements leading to a three-dimensional arrangement with similar intermicrostructural distances (Narine and Marangoni, 1999). Narine and Marangoni (1999) stated that highly ordered nature of TAGs in CB results in this regular network.

2.5 Olive Pomace Oil: A Potential Source for *sn*-1,3 Specific Lipase Catalyzed Cocoa Butter Equivalent Production

Before considering the olive pomace oil, it is necessary to understand olive oil itself. Because olive pomace oil is derived from olive oil and their chemical composition does not differ.

2.5.1 Composition of olive oil

Olive oil is composed of TAGs and contains small quantities of FFAs, glycerol, phosphatides, pigments, flavor compounds, sterols, unidentified resinous substances and other constituents (Kiritsakis, 1998).

2.5.1.1 The fatty acids of olive oil

The major fatty acids present as glycerides in olive oil are oleic (C18:1), linoleic (C18:2), palmitic (C16:0), and stearic acid (C18:0) (Codex Alimen. Comm. (CAC), 1970; IOOC, 1995; Europian Union (EU) Regulation No. 2568/1991). Oleic acid is present in higher concentrations (55-83 %) than other acids. Table 2.5 shows the fatty acid composition of olive oil (IOOC, 1995).

2.5.1.2 Triacylglycerols of olive oil

Most of the fatty acids of olive oil are present as TAGs (Kiritsakis, 1998). Theoretically, based on the fatty acid composition, more than 70 different TAGs should be present in olive oil. However, the number of TAGs actually encountered is much smaller because some TAGs are constantly absent and others occur in negligible amounts (Boskou, 1996). When DAGs are present, olive oil is of low quality (Kiritsakis, 1998). Fedeli (1977) reported the major TAGs as: POO (18.4 %), SOO (5.1 %), POL (5.9 %), OOO (43.5 %), OOL (6.8 %), (P: palmitic, O: oleic, S: stearic and L: linoleic acids). The three major TAGs of olive oil are OOL, OOO and POO. Table 2.6 shows the percentage distribution of individual TAGs of olive oil.

Table 2.5. Fatty acids of olive oil (Kiritsakis, 1998)

Acids	Content (%)
Oleic	55.0 - 83.0
Palmitic	7.5 – 20.0
Linoleic	3.5 – 21.0
Stearic	0.5 – 5.0
Palmitoleic	0.3 – 3.5
Linolenic	≤ 0.9
Myristic	≤ 0.1
Arachidic	≤ 0.6
Behenic	≤ 0.2*
Lignoceric	≤ 0.2
Heptadecanoic	≤ 0.3
Heptadecenoic	≤ 0.3
Eicosenoic	≤ 0.4

(* Limit is ≤ 0.3 for olive pomace oils)

2.5.1.3 Mono and diacylglycerols

The presence of partial glycerides in olive oil is due to either incomplete TAG biosynthesis or hydrolytic reactions. In virgin olive oil, concentrations of DAGs may range from 1 % to 2.8 % (Kiritsakis, 1998; Boskou, 1996). MAGs are present in much smaller quantities (less than 0.25 %) (Boskou, 1996).

In the DAG fraction C-34 and C-36 prevail (Boskou, 1996). Lampante and extracted olive oils have relatively higher amounts of C-36 (Kiritsakis, 1998). In the MAG fraction, glycerol oleate, glycerol linoleate and glycerol palmitate are the major constituents. Other MAGs found are; glycerol stearate, glycerol palmitoleate, glycerol linolenate and glycerol laurate (Boskou, 1996).

Table 2.6. Fatty acid distribution among the chief TAGs of olive oil (Boskou, 1996)

Number of double bonds per triacylglycerols	Fatty acid distribution	Content %
0	NA	0.0
1	POP	2.9
	PPO	0.6
	POS	0.5
	PSO	0.3
	SOS	0.2
	N.D	0.2
2	PPL	1.2
	POO	18.4
	POS	2.3
	PLS	0.1
	LPS	0.7
	SOO	5.1
	N.D	2.2
	PLO	0.2
3	POL	5.9
	OPL	0.9
	SLO	2.4
	LSO	0.7
	SOL	1.3
	OOO	43.5
	N.D	0.6
4	PLL	0.4
	LPL	0.2
	OOL	6.8
	OLO	3.5
	SLL	0.2
	LSL	0.3
	N.D	0.1

(P: palmitic; O: oleic; S: stearic; L: linoleic; N.D: non determined acid)

2.5.1.4 Minor nonglyceride constituents of olive oil

Several minor nonglyceride (unsaponifiable) constituents are present in olive oil. The nonglyceride fraction of olive oil contains nonglyceride fatty acid esters, hydrocarbons, sterols, triterpene alcohols, tocopherols, phenols, chlorophylls, flavor compounds and polar phenolic compounds such as hydroxytyrosol. Table 2.7 shows the minor nonglyceride constituents determined in virgin and refined olive oil.

Table 2.7. Nonglyceride constituents of virgin and refined olive oil (ppm) (Kiritsakis, 1998)

Nonglyceride constituents	Virgin olive oil	Refined olive oil
Hydrocarbons	2000	120
Squalene	1500	150
β-Carotene	300	120
Tocopherols	150	100
Phenols and related substances	350	80
Esters	100	30
Aldehydes and ketones	40	10
Fatty alcohol	200	100
Terpene alcohols	3500	2500
Sterols	2500	1500

2.5.2 Classification of olive oil

The IOOC (1985, 1995, 1997) proposed the following designations and definitions for olive oil and olive pomace oil, which are in general agreement with those of European Union (EU Commission Regulations, 1991, 1995).

Virgin Olive Oil, is the oil obtained from the fruit of the olive tree only by mechanical or other physical means under conditions, mainly thermal, that do not lead to alterations in the oil and which has not undergone treatment other than washing, decantation, centrifugation and filtration. The virgin olive oil which can be consumed as it is and referred to as natural, includes:

Extra Virgin Olive Oil, which has a maximum acidity, expressed as oleic acid, of no more than 1.0 g/100 g and meets the requirements for the sensory (organoleptic) characteristics and other quality criteria of this oil category.

Virgin Olive Oil, (the qualifier “fine” may be used at the production and the wholesale stage). Virgin olive oil which has a maximum acidity, expressed as oleic acid, of no more than 2.0 g/100 g meets the requirements for the sensory (organoleptic) characteristics and other quality criteria of this oil category.

Ordinary Virgin Olive Oil, which has a maximum acidity, expressed as oleic acid, of no more than 3.3 g/100 g meets the requirements for the sensory (organoleptic) characteristics and other quality criteria of this oil category.

Lampante Virgin Olive Oil, which has acidity expressed as oleic acid of no more than 3.3 g/100 g and/or sensory (organoleptic) characteristics and other quality criteria corresponding to this oil category. This oil can not be used for consumption as it is. It should undergo refining or must be used for technical purposes.

Refined Olive Oil, obtained from virgin olive oil by refining process, which does not lead to alterations in the initial glycerols structure.

Olive Oil, consisting of a blend of virgin olive oil (except lampante) and refined olive oil.

Olive Pomace Oil, extracted from olive pomace using solvent, to the exclusion of oils obtained by re-esterification processes and any mixture of other oils. It is marked as follows.

Crude olive pomace oil. Olive pomace oil intended for refining in order to be used for human consumption or used for technical purposes as it is.

Refined olive pomace oil. Oil obtained from crude olive pomace oil by refining process, which does not lead to alterations in the initial glyceride structure.

Olive pomace oil. A mixture of refined olive pomace oil and virgin olive oil (except lampante).

2.5.3 Olive pomace oil

Olive pomace is the by-product of olive fruit processing. Olive pomace is the pulpy material remaining after removing most of the oil from the olive paste (Kiritsakis, 1998). Olive pomace is of varying importance, mainly to the countries of the Mediterranean region where olive trees are grown (Gomes and Caponio, 2000). The commercial value of the olive pomace depends mainly on its oil and water content which in turn depends on the process applied (pressure, centrifugation, selective filtration) and on the operating conditions (Kiritsakis, 1998).

2.5.3.1 Olive pomace composition

Olive pomace contains fragments of skin, pulp, pieces of kernels and some oil. The main constituents are cellulose, proteins and water. The moisture of olive pomace obtained by the pressure process is low (Kiritsakis, 1998), while the centrifugation process results in high moisture content (Carola, 1975). Olive pomace also contains polyphenols and other constituents. Table 2.8 shows the composition of olive pomace obtained by pressure process.

Table 2.8. Composition of olive pomace obtained by a pressure-type olive oil mill (Kiritsakis, 1998)

Constituents	Content
Water	25.0
Nitrogen compounds	4.4
Non-nitrogen extractable compounds	20.0
Cellulose	40.0
Ash	6.6
Oil	4.0

2.5.3.2 Extraction of olive pomace oil

In most areas, olive pomace is further processed to extract olive pomace oil and other products. Furthermore, extraction of the oil from olive pomace reduces environmental contamination (IOOC, 1989). Figure 2.21 shows schematically the treatments and products obtained from olive pomace processing.

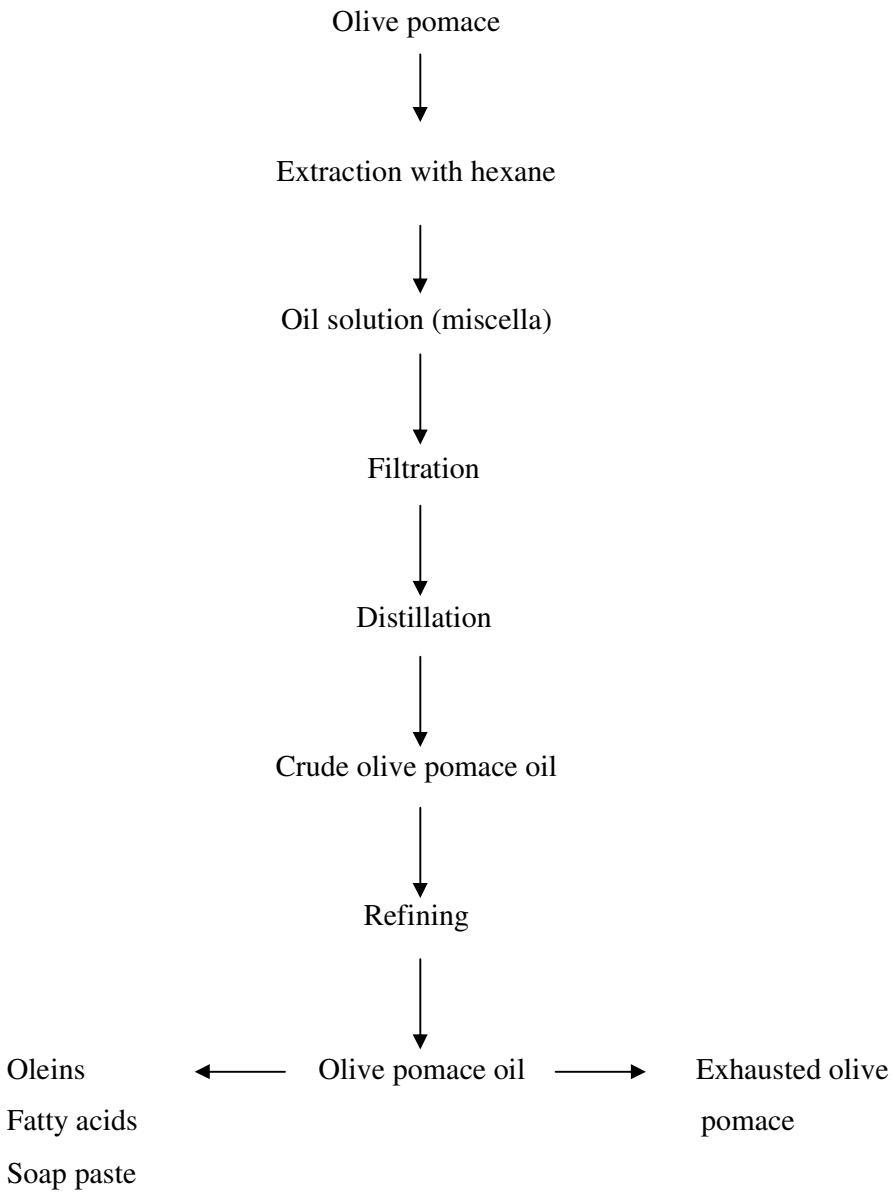


Figure 2.21. Treatments and by-products of olive pomace processing (Kiritsakis, 1998)

Pomace must be dried to a 10 % moisture content for extraction of olive pomace oil. Higher moisture content results in poorer recoveries. The driers consist of slowly rotating, large cylinders. They are equipped with an internal set of propellers or blades used to push the pomace to the opposite side in a current of hot air. A large mass of hot air blows in through a furnace. Olive pomace from which the oil has

been extracted (exhausted olive pomace) is used for heating the air. The olive pomace, hot and dry, is taken to the extractors where it is mixed with an ample amount of solvent. The extractors are arranged in a battery form. Thus, the solvent passes from one extractor to the next. The solvent/oil mixture is continuously collected at the outlet of the extractor. Once the oil in one extractor has been recovered, steam is injected to eliminate the solvent residue. The solvent passes through the load many times. Today, continuous extractors are available. The solvent used in the past for extraction of olive pomace oil was carbon disulfide. Most of the old plants had their own facilities for the production of carbon disulfide from sulfur and charcoal. The solvent carbon disulfide is not very selective. It pulls along resins and gums into the oil. Trichloroethylene was used as substitute for carbon disulfide. However, the cost of this solvent was high and the oil was inferior quality, so trichloroethylene was replaced with hexane, which is mainly used today. The amount of hexane used affects the quantity of oil recovered. Supercritical CO_2 can be used to extract olive pomace oil. It is a rapid and simple process. Supercritical CO_2 is inexpensive, nontoxic and does not contaminate the environment (Kiritsakis, 1998).

The extraction product (a mixture of oil and solvent) is called miscella. This product passes through the distillation unit, where it is indirectly heated by steam. The solvent vaporizes and is carried out to the condensers where it is recovered after eliminating the water by decantation. The oil obtained is crude olive pomace oil. It contains large amounts of FFA which increase its acidity and lower its quality. It also contains pigments (e.g. chlorophylls, anthocyanins) as well as other constituents contributing a characteristic flavor and sweet taste. Olive pomace oil has a fatty acid composition similar to virgin olive oil, but of inferior quality (Kiritsakis, 1998).

2.5.3.3 Deterioration of olive pomace oil

The oil present in olive pomace undergoes rapid deterioration due to the moisture content which favors TAG hydrolysis (Kiritsakis, 1998). Carola (1975) reported that the acidity of the olive pomace oil may increase from 5 % to 60 % in a short time. This is due to the presence of the enzyme lipase originating either from the olive fruit or from microorganisms (Gliomastix chartarum, Cephalosporium Sp., Aspergillus glaucus, etc.) growing on the olive pomace. These microorganisms are favored by high moisture and temperature during storage. Besides the increase in the acidity,

oxidation products (aldehydes and ketones) are also formed during storage of the olive (Kiritsakis, 1998). These products significantly affect the quality of the pomace oil (Carola 1975). In order to minimize the deterioration of the olive pomace oil, the olive pomace should be processed as soon as possible (Kiritsakis, 1998).

2.5.3.4 Refining of olive pomace oil

Refined olive pomace oil is produced by refining crude pomace oil, which is extracted by solvents from the olive pomace residue of olive oil processing (Gomes and Caponio, 2000). This includes neutralization, deodorization and decolorization. The products obtained from refining pomace oil are refined olive pomace oil, FFA, soap paste and exhausted olive pomace (Kiritsakis, 1998).

Refined olive pomace oil is an acceptable edible oil. Its chemical composition does not differ from refined olive oil (Kiritsakis, 1998). But, the quality of the oil recovered from the olive pomace is mainly depends on the process conditions. Long storage periods and high temperature drying applications makes the oil inferior grade (Fadıloğlu et al., 2003). High acidity olive pomace oils were mostly used for the production of household soaps, before detergents appeared on the market (Kiritsakis, 1998). In the last few years olive pomace oil has an increasing demand to be used as edible oils for frying purposes. Crude olive pomace oil is often very acid, colored and oxidized. Intensive refining is thus required to make it suitable for human consumption (Gomes and Caponio, 1998). However, its high free fatty acid content makes it very difficult to process by neutralization. The yield of product is also very low and it increases the cost of unit product produced. So it is necessary to reduce the free fatty acid content before refining process (Fadıloğlu et al., 2003).

2.6 Review of the Studies on Enzymatic Production of CB-Like Fats

Up to date several studies have been reported about enzymatic production of CB-like fat. Chang et al. (1990) preapared CB-like fat from completely hydrogenated cottonseed and olive oils by enzymatic interesterification. They conducted experiments batchwise at 70°C reaction temperature, 10% enzyme load under the catalysis of *sn*-1,3 specific *Mucor miehei* immobilized on an macroporous resin. They obtained a product with a melting point of 39°C in an optimum of 4 h reaction

time. Their product contained 23% POS and 28% SOS. Their yield of isolated CB-like fat was about 19% based on the weight of the original oils.

Mojovic et al. (1993) used celite-immobilized *Rhizophus arrhizus* to interesterify palm oil midfraction with stearic acid in *n*-hexane to produce a CBE. They carried out experiments in batch reactor. Their highest achieved stearic acid content in TAGs was 35.4%, corresponding to the addition of 120 IU of immobilized enzyme per gram of palm oil midfraction. They achieved the highest substrate conversion rate during the first 6h of the reaction. They estimated that the yield of the interesterified TAGs was 80.8%.

Undurraga et al. (2001) produced CBE through enzymatic interesterification of palm oil midfraction with stearic acid in a solvent-free system using Lipozyme as catalyst. They conducted studies both in batch and continuous PBRs.

Khumalo et al. (2002) evaluated some underutilised vegetable oils (*Strychnos madagascariensis* fruit shell, *Trichelia emetica* seed and *Ximenia caffra* seed) as source oil for CBE production. They used *sn*-1,3 specific lipase from *Rhizomucor miehei* to incorporate stearic and palmitic acids into 1 and 3 positions of the TAGs of these three source oils.

Osborn and Akoh (2002b) modified beef tallow to produce a substitute for CB. They incorporated stearic acid into beef tallow in a 1 L batch reactor using hexane as solvent and *Rhizomucor miehei* (IM60) as enzyme.

Abigor et al. (2003) prepared CB-like fats from refined, bleached, and deodorized palm oil and fully hydrogenated soybean oil by enzymatic interesterification. They reported an optimal weight ratio of deodorized palm oil to fully hydrogenated soybean oil as 1.6:1. they obtained a 45% yield based on the weight of the original substrates.

Liu et al. (2007) interesterified lard and tristearin to synthesize CB analog in supercritical carbon dioxide (SC-CO_2) by lipase. Their optimized reaction conditions was 1:4 mole ratio (lard to tristearin), 50°C, 17 Mpa SC-CO_2 system pressure, 3 h with an immobilized Lipozyme IM-20. they obtained a product with a yield and melting point of 63% and 34.5°C, respectively.

Gitlesen et al. (1995) studied lipase-catalyzed interesterification of high-oleic-acid rapeseed oil with stearic acid or methyl stearate was investigated. High yields of SOS (36%) and 1(3)-2-dioleyl-1(3)-monostearoyl glycerol (OOS) (27%) with small incorporation of stearic acid in the *sn*-2 position were obtained by using lipase from *Rhizopus arrhizus* immobilized on polypropylene powder and ethyl stearate as acyl donor.

However, some of the papers mentioned above characterized their products in terms of fatty acid content, and the yields were calculated on the basis of incorporated fatty acid. However, rather than fatty acid content the TAG content is important. Because the unique functional properties of CB is caused by the distribution of those fatty acids on glycerol backbone and producing POP, POS and SOS. Also, the fraction of those TAGs should be considered in CBE characterization

To the best knowledge of authors, there is no study on enzymatic production of CBEs from olive pomace oil.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Refined olive pomace oil (ROPO) was kindly provided by Bilginoğlu Marbil Yağ San. ve Tic. AŞ., İzmir, Turkey. Some properties of ROPO are given in Table 3.1.

Table 3.1. Some selected properties of ROPO

Property	Value
FFA (%)	0.2
Peroxide value (meq O_2/kg)	1.27
Color	L*: 57.2, a*: -1.78, b*: 48.1, YI: 87.1
TAG (%)	96
MAG +DAG (%)	2.5
Fatty acid composition (%)	Myristic acid, $C_{14:0}$: 0.03
	Palmitic acid, $C_{16:0}$: 13.00
	Palmitoleic acid $C_{16:1}$: 0.94
	Margaric acid, $C_{17:0}$: 0.07
	Heptadecenoic acid, $C_{17:1}$: 0.15
	Stearic acid, $C_{18:0}$: 3.00
	Oleic acid, $C_{18:1}$: 70.00
	Linoleic acid, $C_{18:2}$: 11.50
	Linolenic acid, $C_{18:3}$: 0.73
	Arachidic acid, $C_{20:0}$: 0.33
	Gadoleic acid, $C_{20:1}$: 0.08
	Behenic acid, $C_{22:0}$: 0.16

CB was donated by Şölen Çikolata Gıda San. ve Tic. A.Ş., Gaziantep, Turkey. TAG standards (OOO, POP, POS and SOS) and fatty acid standards were obtained from

Sigma Chemical Co. (St. Louis, MO). Palmitic acid (PA) (≥98% purity) and stearic acid (SA) (≥97% purity), porcine pancreatic lipase (type II, crude) (EC 3.1.1.3), silica gel (SG 60, 70-230 mesh), thin layer chromatography (TLC) plates (Kieselgel G) were obtained from Merck (Darmstadt, Germany). Immobilized *sn*-1,3 specific lipases (Lipozyme IM, immobilized from *Mucor miehei*, 42 U/g and 140 U/g) was purchased from Fluka Chemie GmbH. Acetone, acetonitrile and *n*-hexane were purchased from Sigma-Aldrich. All solvents used were of HPLC grade. All other reagents and solvents were of analytical or chromatographic grade.

3.2 Production of CB-like Fat

CB-like fat was produced by enzymatic acidolysis reactions. Firstly, TAGs of CB were produced in a model system to determine the optimum reaction conditions. Then, products were produced in natural system (using ROPO) via batch and continuous systems.

3.2.1 Synthesis of TAGs of CB in a model acidolysis system

Acidolysis reactions of OOO with PA and SA were performed at varying substrate mole ratios (OOO: PA: SA; 1:1:1, 1:1.5:1.5, 1:3:3, 1:4:4, 1:6:6, 1:8:8) enzyme loads (5, 10, 15, 20, 25, 30%, based on weight of substrates), temperature (40, 45, 50, 60°C), water contents (0, 1, 5, 10, 20, 30, 40%, based on weight of substrates) and time (0-72 h). 1:1:1 was chosen as the limiting substrate mole ratio considering the cleavage of oleic acids at *sn*-1 and 3 positions of OOO and binding 2 moles of PA/SA to the *sn*-1 and 3 positions of the glycerol backbone. Other substrate mole ratios were considered as excess. Initial weight of OOO in the reaction mixtures was 88.5 mg (0.1 mmol). Weights of PA and SA were adjusted according to the initial amount of OOO. Weight of substrates refers to the sum of the weights of OOO, PA and SA in a reaction mixture. The reaction mixtures were dissolved in 5 mL *n*-hexane in 50 mL erlenmayer flasks and incubated in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 200 rpm (Batch Stirred Tank Reactor) (Figure B.1). 50 µL aliquots were withdrawn at certain time intervals from the reaction mixtures into glass vials and stored at -20°C prior to analysis. The time course of the acidolysis reactions was followed by analyzing the reaction mixtures for their POP, POS and SOS contents by a reversed phase HPLC. All reactions were conducted in double.

3.2.2 Production in natural system: Batch system

Acidolysis reactions of ROPO with PA and SA were performed at varying substrate mole ratios (1:1:3, 1:2:3, 1:3:3, 1:3:1, 1:3:2, 1:2:6, 1:3:9, 1:4:12; ROPO: PA: SA; keeping mole ratio of ROPO constant), 20% enzyme load (based on weight of substrates), 45°C temperature and time up to 8 h. Selection of substrate mole ratios, enzyme load and reaction temperature were based on results obtained from model system study. Trials have shown that better results were obtained in the absence of water (data not shown). For this reason, the reactions were carried out in the absence of water. Weight of substrates refers to the sum of the weights of ROPO, PA and SA in a reaction mixture. Weight of PA and SA were adjusted according to the initial amount of ROPO. Molecular weight of ROPO was considered to be same as that of OOO (282 mol/g). The reaction mixtures were dissolved in 50 mL *n*-hexane in 100 mL erlenmayer flasks and incubated in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 200 rpm (Batch STR, Figure B.1). 50 µL aliquots were withdrawn at certain time intervals from the reaction mixtures into glass vials and stored at -20 °C prior to analysis. The time course of the acidolysis reactions was followed by analyzing the reaction mixtures for their POP, POS and SOS contents by a reversed phase HPLC. All reactions were performed duplicate.

3.2.3 Production in natural system: Continuous system

Continuous production of CB-like fat was done in a PBR. For this reason, a bioreactor was designed and set up in our laboratory. The details of the bioreactor and production in the bioreactor are given below.

3.2.3.1 Bioreactor set-up

Acidolysis reactions were carried out on a laboratory scale PBR similar to the system of Fomuso and Akoh (2002) (Figure B.2). Figure 3.1 shows the simple illustration of the bioreactor used in the study. The reactor had a jacketed glass column with dimensions of 1 cm (id) x 10 cm (l) (Figure B.3). Reaction mixture was pumped upward into the column using a peristaltic pump (Watson Marlow Bredel, model 505U, Falmouth, UK). The system temperature was maintained by a circulating water bath. Temperature of the reaction mixture in the substrate reservoir was preheated to the reaction temperature and maintained at that temperature during the reaction on a hot plate/stirrer. The column was packed with Lipozyme IM and the

upper and the lower ends of the column were layered with glass wool. The bed porosity (ε) of the enzyme packed bioreactor was calculated according to Xu et al. (1998) and details of the calculation are given in section 3.2.3.2. Characteristics of bioreactor and biocatalyst employed in the study are given in Table 3.2.

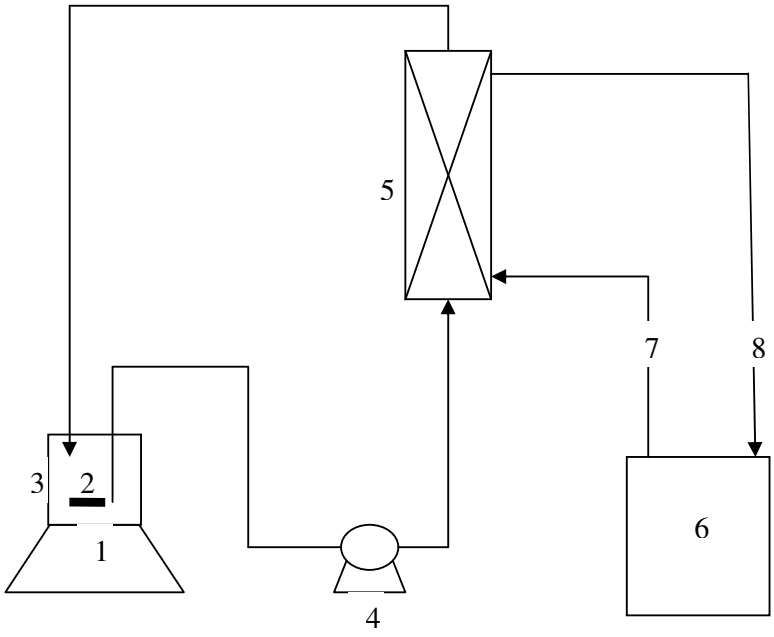


Figure 3.1. Simple illustration of the PBR used in the study. (1) Hot plate/stirrer, (2) magnetic bar, (3) substrate reservoir, (4) peristaltic pump, (5) packed enzyme bed, (6) circulating water bath, (7) enzyme bed heating water inlet, (8) enzyme bed heating water outlet

3.2.3.2 Calculation of the bed porosity (ε) of the bioreactor

Bed porosity (ε) was calculated based on the measured bulk and particle density values, by applying the following equation (Xu et al., 1998):

$$\varepsilon = 1 - \rho b/\rho ap \qquad (3.1)$$

where, ρb = bulk density (g/cm^3), ρap = particle density (apparent) (g/cm^3).

Measurement of particle density

Enzyme was weighed using an electronic balance (± 0.001g). The volume of the same enzyme was then determined by the method described under measurement of volume. ρap was calculated as mass divided by volume (g/cm^3). ρap is the average of four measurements.

Measurement of volume

The volume of the enzyme particles was measured by the liquid displacement method using liquid paraffin (SG = 0.8787 at 30°C) as the medium, similar to the method described by Zogzas et al. (1994), using a measuring cylinder of 22 mm inside diameter and 50 cm^3 capacity.

Measurement of bulk density

The weight of enzyme was measured using an electronic balance (± 0.001g). The pre-weighed enzyme was poured loosely into a measuring cylinder through a funnel. Without shaking, the horizontal plane representing average height of the product was estimated and volume was read from the scale. ρb was calculated as mass divided by volume (g/cm^3). ρb is the average of four measurements.

Table 3.2. Characteristics of the bioreactor and biocatalyst employed in the study

Reactor sizes	
Length	10.00 cm
Diameter	2.50 cm
Bed Diameter	1.00 cm
Total volume	7.80 cm^3
Cross section	0.78 cm^2
Biocatalyst characteristics	
Particle density (ρap)	0.70 g/cm^3
Bulk density (ρb)	0.38 g/cm^3
Total biocatalyst weight in reactor	5.50 g
Porosity (ε)	0.46

3.2.3.3 Determination of the residence time in the bioreactor

Residence time of the reaction mixture in the bioreactor was calculated according to the following calculations:

$$\text{Residence time} = \text{Bed void volume/flow rate} \quad (3.2)$$

$$\text{Bed void volume} = \varepsilon . V_{reactor} \quad (3.3)$$

3.2.3.4 Acidolysis reaction in the bioreactor

Reaction mixture typically comprised of ROPO, PA and SA in *n*-hexane. Substrate mole ratio (ROPO:PA:SA; 1:1:1, 1:1:3, 1:3:3, 1:2:6), enzyme load (10, 20, 40%), substrate flow rate (1.5, 4.5, 7.5, 15 mL/min) and solvent amount (150, 400 mL) were varied to study the effect of reaction conditions on product formation. Concentration of the substrates in reaction mixtures containing 150 mL and 400 mL of *n*-hexane were 0.063 g/mL and 0.023 g/mL, respectively. Reactions were carried out at 45°C. This optimized reaction temperature was based on the model system study. The bioreactor was operated at a mode which the mixture coming out of the reactor was recirculated to the substrate reservoir (product recirculation). A new enzyme bed was used for each parameter study. Samples were withdrawn from the substrate reservoir at different times and stored at -20°C until analysis. The time course of the acidolysis reactions was followed by analyzing the reaction mixtures for their POP, POS and SOS contents by a reversed phase High Performance Liquid Chromatograph (HPLC). All reactions were performed duplicate.

3.3 Isolation of TAGs

The produced TAGs (CB-like fat) were isolated in two steps. First, the mixture obtained from the reaction was neutralized to remove FFAs, and then it was purified by silica gel column chromatography. Neutralization was done in a similar manner as Lee and Akoh (1998b) with some modification. Reaction mixture (6-8 g) was mixed with 150 mL *n*-hexane and 1 mL phenolphthalein solution, and then required amount of 0.5 N KOH in 20% (vol) ethanol (B mL) was added until the pink color was observed (neutralized). Then, the mixture was transferred into a separatory funnel. The separatory funnel was shaken, and the upper phase was collected. Lower phase was washed again with 50 mL *n*-hexane and the upper phase was collected. Then, 2.66*B mL of 0.5 N KOH in 20% (vol) ethanol and 1.33*B mL of saturated NaCl solution were added to collected upper phase. After shaken, the upper phase was collected and *n*-hexane was evaporated by rotary vacuum evaporator to obtain the neutralized product containing TAGs, DAGs and MAGs. The neutralized product was centrifuged in case of presence of soap traces.

Then, the TAGs of the neutralized product were separated from MAGs and DAGs by column chromatography on silica gel (SG 60, 70-230 mesh, Merck)

(Polish Standard PN-ISO 8420, 1995). 1.6-2.0 g of neutralized product was dissolved in 20 mL petroleum ether:diethylether (87:13, vol/vol) and eluted through the silica column with petroleum ether:diethylether (87:13, vol/vol). Then, the solvent was evaporated, and the purified reaction product (PRP) was obtained. This PRP was selected as the product, and so called CB-like fat. PRP was used for the preparation of blends with CB, and for oxidative stability determinations.

3.4 Preparation of CB:CB-Like Fat Blends

CB and CB-like fat were melted at 80°C and blended at proportions of 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 30:70 and 0:100 (wt% CB:wt% CB-like fat).

3.5 Methods of Analysis

3.5.1 *sn*-2 Fatty acid analysis

Fatty acid composition at the *sn*-2 position of ROPO was determined using the method developed by Brockerhoff (1965). The oil was hydrolysed with porcine pancreatic lipase, a lipase selective for *sn*-1,3 positions of TAGs. 0.4 g of ROPO was weighed into a 100 mL flask, and 8 mL of Tris, 0.5 mL of $CaCl_2$, and 0.2 mL of Bile salt solutions were added. The mixture was incubated in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 40°C and 150 rpm. 0.2 g of *sn*-1,3 specific porcine pancreatic lipase was added at the end of 10 min. After 20 min, reaction was ended by adding 15 mL of ethanol and 5 mL of 6N HCl. The miture was taken into a separatory funnel and extracted by diethylether. The supernatant was taken and dried by a small amount of magnesium sulfate addition.

The products of lipolysis (supernatant) were separated by TLC plates that were developed with petroleum ether: diethyl ether: acetic acid (70:30:1, vol/vol/vol). The *sn*-2 MAG band was scraped off and was extracted with diethyl ether. The extracted lipid was analyzed by Gas Chromatograph (GC) as described below.

3.5.2 GC analysis

sn-2 MAGs of ROPO were converted to fatty acid methyl esters (FAME) and then analyzed by an Agilent 6890 series GC equipped with a HP88, 100 m x 0.250 mm x 0.25 µm capillary column (Agilent Technologies Inc., CA, USA). For FAME

preparation, 2 mL of *n*-hexane and 2 mL of KOH (1 M in ethanol) were added onto 1 drop of ROPO, and the mixture was shaked vigorously. The supernatant (FAME) was taken and injected into GC. One microliter of FAME mixture was injected into the GC system with split/splitless injector and flame ionization detector (FID). The inlet temperature was 250°C and the split ratio was 50:1. The carrier gas was hydrogen at 2.0 mL/min constant flow. The oven temperature was programmed at initial 120°C, held for 1 min, followed by increase of 10°C/min up to 175°C, held for 10 min, followed by increase of 5°C/min up to 210°C, held for 5 min, followed by increase of 5°C/min up to 230°C and held for 5 min. The detector was set at 280°C with 450 mL/min airflow, 40 mL/min hydrogen flow, and 30 mL/min helium makeup flow. The percent of each fatty acid was determined from retention times based on comparison with fatty acid standards.

3.5.3 Analysis of TAG content

TAG content of the samples were determined by HPLC. HPLC system consisted of quadratic pump (model LC-10ADVP; Shimadzu, Japan) equipped with a column (Sphereclone 5 µ ODS (2), 250 x 4.6 mm; Phenomenex, USA) with an accompanying guard column (40 x 3- mm id) of the same phase and an ultraviolet (UV) detector (Hewlett Packard Series 1100). Elution was monitored by UV absorbance at 215 nm. The mobile phase consisted of acetone and acetonitrile (50:50, vol/vol) with a flow rate of 1.0 mL/min. The column temperature was set at 50°C with a column heater (Eppendorf CH-30 column heater). 0.05 g of sample was diluted in 2 mL of acetone and filtered with syringe filter (0.2 µm, Minisart, Sartorius) prior to injection. Injection volume was 20 µL. A software integration system (ChemStation Rev. A.09.03 [1417], Agilent Technologies) was used for data collection and integration. All TAG contents were given in percentage area.

3.5.4 Thermal characteristics

3.5.4.1 Melting profile

Melting profile of the samples were analysed by a DSC (Perkin Elmer DSC-6, Norwalk, CN, USA). DSC procedure of Siew and Faridah (2000) was followed. The samples were completely melted at 80°C before being weighed. 10±0.5 mg of molten samples were hermetically sealed in an aluminium pan, with an empty pan as reference. Samples were initially heated to 80°C and held at this temperature for 10

min in the DSC instrument to erase the previous thermal history. The samples were then cooled to -60°C at 40°C/min. At the end of the cooling, the samples were heated from -60 to 80°C at 10°C/min. Onset, offset and peak temperatures were obtained directly from DSC software (Pyris version 7.0) calculation.

3.5.4.2 Solid fat content

Percentage of SFC of the samples were analysed by DSC using the same procedure given in section 3.5.4.1. The SFC at various temperatures were calculated from the data of the DSC heating thermograms by partial integration according to Tieko and Aparecida (1995). The partial areas were obtained directly from DSC software (Pyris version 7.0) calculation. The Figure B.4 shows an example for SFC calculation. The percentages corresponding each temperature represents the liquid fraction of the sample. Solid fat fraction for each temperature was obtained by substracting value of liquid fraction from 100.

3.5.5 Microstructure

Crystal network microstructure of the samples were examined by polarized light microscopy using a polarized light microscope (PLM) (Olympus BX51, Olympus Optical Co., Ltd., Tokyo, Japan) equipped with a Pixera color video camera (model PVC 100C, Los Gatos, CA, USA). A static crystallization method similar to the method of Narine and Marangoni (1999) was used. Samples were molten at 80°C for 15 min in order to erase the crystal memory and 20 µL of melt placed on a glass microscope slide which was heated to the same temperature. A glass coverslip at the same temperature of the sample was placed on top of the samples. Samples were then allowed to crystallize for 48 h at room temperature (21-23°C). A 40X lens was used to image the grayscale photograph of the samples.

3.5.6 Determination of the oxidative stability of CB:CB-like blends

The oxidative stability of the CB:CB-like fat blends were determined by a Perkin Elmer DSC (DSC-6 Norwalk, CN, USA). The samples were completely melted at 80°C before being weighed. 5.0±0.5 mg of sample was weighed into open aluminium pans, with an empty pan as reference, and placed in the sample chamber of DSC. The isothermal temperature program was programmed at four different temperatures (130, 140, 150, 160°C) and oxygen was passed through the sample

chamber at 100 mL/min flow rate. Similar to Tan et al. (2002), the oxidation induction time (OIT) of the oxidation reaction were determined by taking the time value corresponding closely to the intersection of the extrapolated baseline and the tangent line of the exotherm (Figure B.5).

3.5.7 Chemical analyses

FFA content and peroxide value of ROPO and CB were analyzed according to official methods AOCS Ca 5a-40, and AOCS Cd 8-53, respectively.

3.5.8 Color

HunterLab LabScan XE (Hunter Assocciates Laboratory, Inc., Reston, Virginia, USA) was used to determine color values of ROPO in terms of Hunter L*, a*, b* as measures of lightness, redness and yellowness, respectively. The measuring head was equipped with 51 mm diameter viewing port and used the system of diffuse illumination with 10° viewing geometry. The illuminant was D65. The colorimeter was calibrated against a standard white tile (L*=93.01, a*=-1.11, b*=1.30). The result is the average of four measurements.

3.5.9 Statistical analysis

The statistical analyses were done by the analysis of variance method (ANOVA) to determine the effect of each variable on the content of target TAGs (POP, POS, SOS). The ANOVA tests were performed using SPSS (version 10.0) package program (SPSS, 1999). Duncan and LSD tests were also applied to detect the differences of responses on the content of TAGs. Differences between samples were tested at 95% confidence interval.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Production of CB-Like Fat

4.1.1 Production in model system

There are many studies reporting production of CBEs from different sources such as lard, tea seed oil, palm oil midfraction, sal fats, mango fat, illipe fat, kokum fat and shea oil (Undurraga et al., 2001; Wang et al., 2006; Sridhar et al., 1991; Lipp and Anklam, 1998). However, there are limited studies regarding the production of CBEs in a model system. The efficiency of the acidolysis reaction for the production of CBEs depends on reaction parameters, namely, substrate ratio, reaction temperature, reaction time, enzyme concentration and water content (Fomuso and Akoh, 1998). Effect of each parameter on the system must be determined and optimized both for quality improvements of the products and for highest economical turnover.

Enzymatic interesterification of OOO with PA and SA was studied for the parameters: substrate ratio, reaction temperature, enzyme load, water content and reaction time. Lipozyme IM was reported as an efficient and suitable lipase for CBEs production (Liu and Shaw, 1997; Abigor et al., 2003). *n*-Hexane has been chosen as solvent because of its various advantages such as increasing the solubility of the substrates and prevention of acyl migration in the reactions (Zhao et al. 2005; Yang et al. 2005). Acyl migration is a problem for acidolysis which involves migration of acyls from *sn*-1,3 to *sn*-2 positions but also occurs with migration of acyls from the *sn*-2 into the *sn*-1,3 positions (Yang et al., 2005), especially in batch reactors where high ratio between enzyme and substrate demands long reaction times in order to reach equilibrium. It is very difficult to stop acyl migration fully in actual reactions. Temperature is another important parameter that have a profound effect on the acyl migration, since acyl migration is a thermodynamic process. The lower temperature

and the shorter time were reported to be advantageous both in terms of prevention of acyl migration (Xu, 2000).

The effect of mole ratio of OOO to PA and SA was studied from 1:1:1 to 1:8:8 at constant enzyme load (10%, based on weight of substrates) and at a temperature of 45°C to determine the upper limit of substrate mole ratio. 1:1:1 was chosen as the limiting substrate mole ratio. Limiting substrate mole ratio was selected considering the ability of binding of *sn*-1,3 specific lipase two fatty acids to the 1 and 3 positions of the glycerol backbone of the OOO. Hence, in order to complete the structure to a TAG, two moles of fatty acid is needed. Figure 4.1 shows the relationship between percent conversion and substrate ratio. The percent conversion of OOO to target TAGs was defined as the ratio of the content of target TAGs (POP+POS+SOS) to the content of initial OOO times hundred.

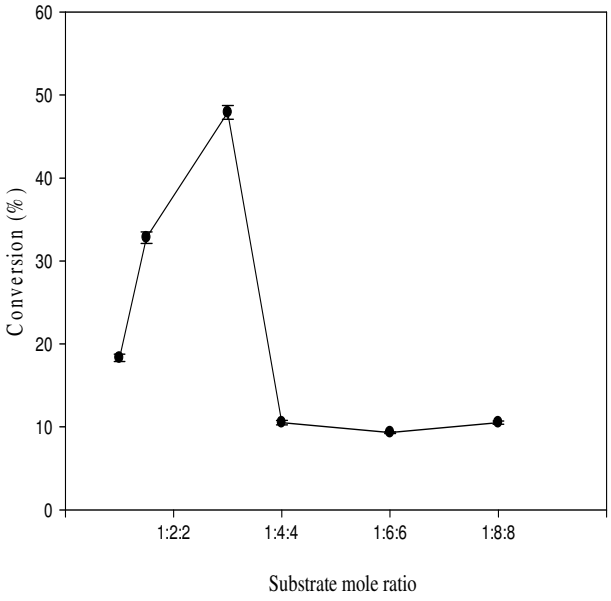


Figure 4.1. Effect of substrate ratio on percent conversion of OOO into POP, POS and SOS. Each mixture was incubated at 45°C for 10 h with 10% enzyme (based on weight of reactants, w/w)

Percent conversion increases with increasing substrate ratio and then declines when substrate ratio increases further. The highest product formation was obtained at 1:3:3 substrate ratio. Therefore, it seems that an excess of PA and SA above a molar ratio of 1:3:3 diminishes the reaction rate. This result is consistent with the findings of

Yankah and Akoh (2005), and Paez et al. (2003). Excess FFAs in the medium acidify the enzyme layer because of high levels of free or ionized carboxylic acid groups or cause desorption of water from the interface, and this causes a decrease in the activity of enzyme (Kuo and Parkin, 1993). In addition, high substrate ratios are economically not feasible, because purification of the products would require a cost-increasing extra separation step. Economy is one of the important parameters that should be considered. Therefore, 1:1:1, 1:1.5:1.5 and 1:3:3 substrate ratios were studied for further investigations.

Figures 4.2a, b, c and d show the effect of temperature on TAG formation. The reactions were carried out at different substrate ratios and constant enzyme load (10%). It is seen that there is a similar trend of product formation irrespective of temperature. The highest yield at 45°C was obtained at 24 h. However, the time needed for the highest yield was 48 h at 40 and 50°C, and 72 h at 60°C. There was no significant difference ($P>0.05$) between the highest TAG contents obtained from the reactions at 40, 45 and 50°C. Because, the lower temperature and the shorter time were reported to be advantageous both in terms of prevention of acyl migration (Xu, 2000) and being more economical, 45°C was chosen as the best temperature to study in the further experiments.

Contents of target TAGs increased with increasing substrate ratios (Figures 4.2a, b, c, d). The yields obtained at limiting amount of substrate ratio (1:1:1) are the lowest among all substrate ratios studied. This may be due to the need of an excess amount of fatty acid for better incorporation. Amounts of POP and SOS formed are very close to each other at all studied conditions. This results from incorporation of same amounts of PA and SA to 1 and 3 positions of OOO. So, it can be concluded that Lipozyme IM does not have higher reactivity and/or selectivity of PA over SA or vice versa. The production of highest amount of POS and equal amounts of POP and SOS make the composition of the product very similar to original CB. The best result was obtained at 1:3:3 substrate ratio, 45°C and 24 h with a product composition of 13.2% POP, 26.4% POS and 12.9% SOS. Therefore, 1:3:3 substrate ratio and 45°C reaction temperature were used for the subsequent experiments.

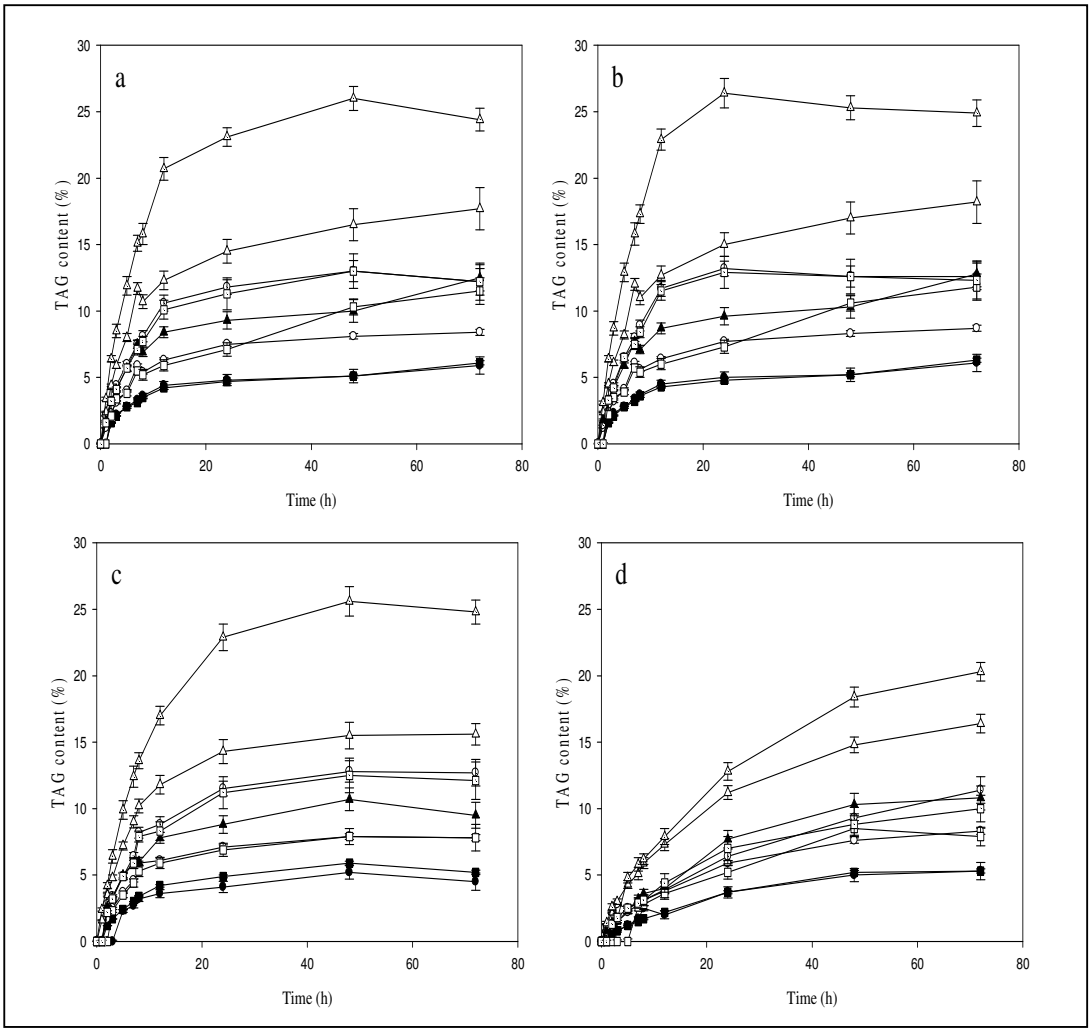


Figure 4.2. Effect of temperature on product formation at different substrate ratios. Each mixture was incubated with 10% enzyme (based on weight of reactants, w/w). POP% at 1:1:1 substrate ratio (●), POP% at 1:1,5:1,5 substrate ratio (○), POP% at 1:3:3 substrate ratio (⊙); POS% at 1:1:1 substrate ratio (▲),POS% at 1:1,5:1,5 substrate ratio (Δ), POS% at 1:3:3 substrate ratio (◬); SOS% at 1:1:1 (■), SOS% at 1:1,5:1,5 substrate ratio (□), SOS% at 1:3:3 substrate ratio (⊡). [(a) 40°C, (b) 45°C, (c) 50°C, (d) 60°C]

Effect of enzyme concentration on TAG formation was investigated for 5 h reaction time (Figure 4.3). Increase in enzyme load has no effect on the amount of product produced after a certain reaction time, therefore optimum enzyme load was determined at this early stage of the reaction. As shown in Figure 4.3, increased enzyme load accelerated the reaction rate and improved the incorporation of acyl donors under given conditions. Because, there was more active site per unit substrate

per unit time. The relationship between the acyl incorporation and the amount of added lipase was not linear. Increasing the amount of lipase above 20% (based on weight of substrates) had no significant effect (P> 0.05) on substrate conversion.

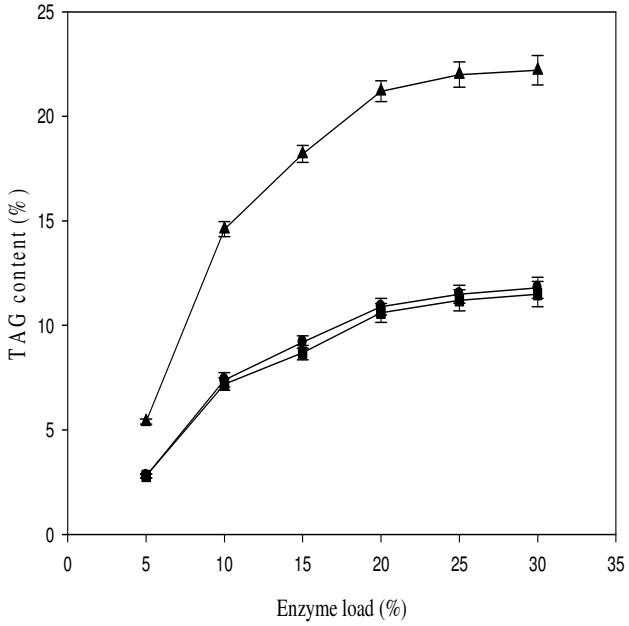


Figure 4.3. Effect of enzyme load on product formation. Each mixture was incubated at 1:3:3 substrate ratio at 45°C for 5 h. POP% (●), POS% (▲) and SOS% (■)

Figure 4.4 shows the change in TAG content with time at 20% enzyme concentration. POP, POS and SOS contents increased up to 10 h and remained constant after this time. TAG contents were 12.1, 24.5 and 12.1% for POP, POS and SOS, respectively, by the end of 10 h. However, minimum 24 h was required to obtain similar TAG contents if the enzyme concentration is 10%. It has been found that enzyme concentration of 20 % compared to that of 10 % decreased the reaction time significantly (P<0.05).

The activity of lipases at different water contents or water activity is dependent on the source of enzyme. Lipases from molds seem to be more tolerant to low water activity than bacterial lipases. The optimal water content for interesterification by different lipases ranges from 0.04 to 11% (wt/vol), although most reactions require water contents less than 1% for effective reaction (Bornaz et al., 1994; Malcata et al., 1992).

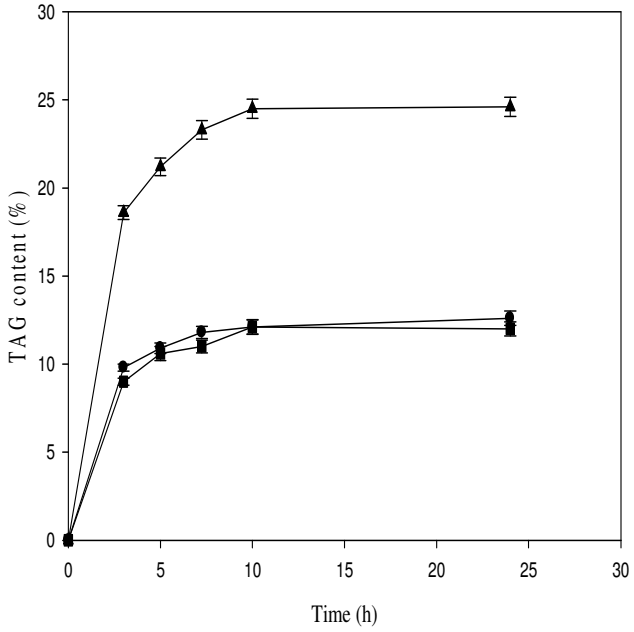


Figure 4.4. Change in TAG content (%) with time at 20% enzyme load. Each mixture was incubated at 1:3:3 substrate ratio at 45°C for 24 h. POP% (●), POS% (▲) and SOS% (■)

As shown in Figure 4.5, water content has a significant effect on acidolysis reaction. TAG content attained a peak at 5% and decreased with further increase in water content. The amount of water added was based on weight of substrates. Sellappan and Akoh (2000) have also reported that it would be better to calculate the amount of water based on weight of substrates instead of weight of enzyme. Lipases need a certain amount of water for activation. But an excess of water will shift the reaction to hydrolysis instead of synthesis. Too much water can inhibit interesterification, probably due to decreased access of hydrophobic substrates to the immobilized enzyme. The amount of water in the reaction system must be controlled for the orientation of the reaction process and for better results. Optimum water content depends on the reaction system, e.g., type of lipase, substrate, support and solvent (Mojovic et al., 1993). Each system (substrates, enzyme, etc.) may have different water content. So, it must be determined for each particular experiment design.

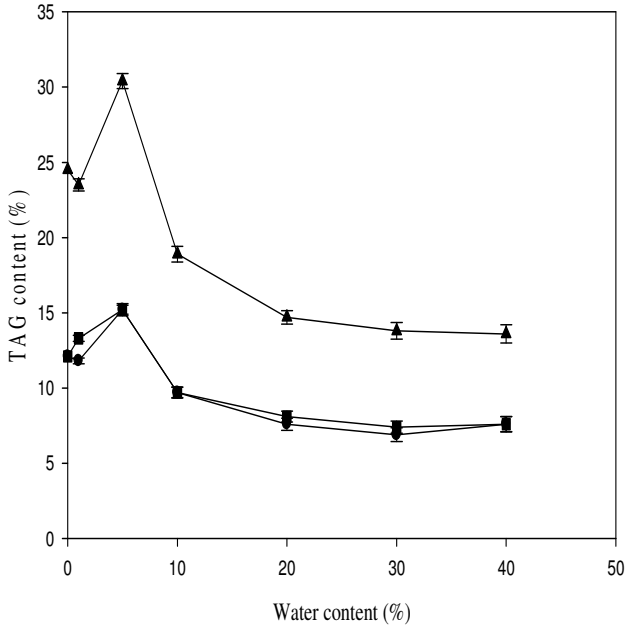


Figure 4.5. Effect of water content on product formation. Each mixture was incubated at 1:3:3 substrate ratio at 45°C for 10 h with 20% enzyme (based on weight of reactants, w/w). POP% (●), POS% (▲) and SOS% (■)

The best result (15.2% POP, 30.4 % POS. 15.2% SOS) was obtained at 1:3:3 (OOO:palmitic acid:stearic acid) substrate mole ratio and reaction parameters: time 10 h, temperature 45°C, enzyme load 20%, water content 5%. Under these conditions, triolein content decreased from an initial value of 35.3% to a residual amount of 9.1%.

4.1.2 Production in natural system

A source oil that would be used for CB-like fat production by *sn*-1,3 specific lipase should be rich in *sn*-2 oleic acid content. Palm oil midfraction (57% POP, 11% POS, 2% SOS) is one of the most common used source oil for CBE production both by blending and acidolysis. Although the content of major TAGs of CB (POP, POS, SOS) in ROPO was very low, *sn*-2 oleic acid content of TAGs found in ROPO was quite high (85 %). This makes ROPO a suitable source for CB-like fat production using Lipozyme IM, *sn*-1,3 specific lipase, which incorporates PA and SA to the 1 and 3 positions of the TAGs of ROPO to produce a fat rich in POP, POS and SOS.

4.1.2.1 Production in batch system

CB-like fat production

Batch system productions were achieved in a lab scale STR. A STR is a common system used in laboratory experiments with lipase-catalyzed interesterification due to its simplicity and low cost. No addition and removal of reactants and products is performed except at the initial and final stages of the reaction (Willis and Marangoni, 2002).

Firstly, acidolysis reactions were carried out at various substrate mole ratios to obtain the best emprical substrate mole ratio that would yield a fat with a TAG composition similar to CB. The commercial CB used in this study contained 18.9% POP, 33.1% POS and 24.7% SOS. Figures 4.6a,b,c show the change in POP, POS and SOS contents with time at different substrate mole ratios. It has been observed that there is a similar trend of product formation for all TAGs. Contents of target TAGs (POP, POS and SOS) increased with increasing substrate mole ratio and reaction time. POP, POS and SOS contents increased up to 6 h and remained constant after this time. The rate of conversion in stirred batch reactor decreases over time since there is a high initial level of substrate, which is reduced over time, with conversion to product. In order to maintain the same rate of conversion throughout the reaction, it would be necessary to add more immobilized enzyme to the reaction mixture (Cheetham, 1988).

Among the studied substrate mole ratios (1:1:3, 1:2:3, 1:3:3, 1:3:1, 1:3:2), 1:1:3 gave the most similar TAG content of CB. At 1:1:3 substrate mole ratio and 6 h, product contained 8.8% POP, 14.4% POS and 12.2% SOS. It has been observed that, SA is a poor acyl donor for this system compared to PA which was similar to the result of Liu et al. (2007).

As shown in Figure 4.7, doubling substrate ratio from 1:1:3 to 1:2:6 had a significant effect on the yield ($P<0.05$). The product contained 11% POP, 21.8% POS, 15.7% SOS at 1:2:6 substrate mole ratio. Further increase in substrate mole ratio (1:3:9, 1:4:12) did not cause any significant effect on yield ($P>0.05$). High substrate mole ratios are not feasible economically. Purification of products obtained from 1:3:9 and

1:4:12 caused high amount of neutral oil loss. This means an extra process and cost. Therefore, 1:2:6 was selected as the best substrate mole ratio.

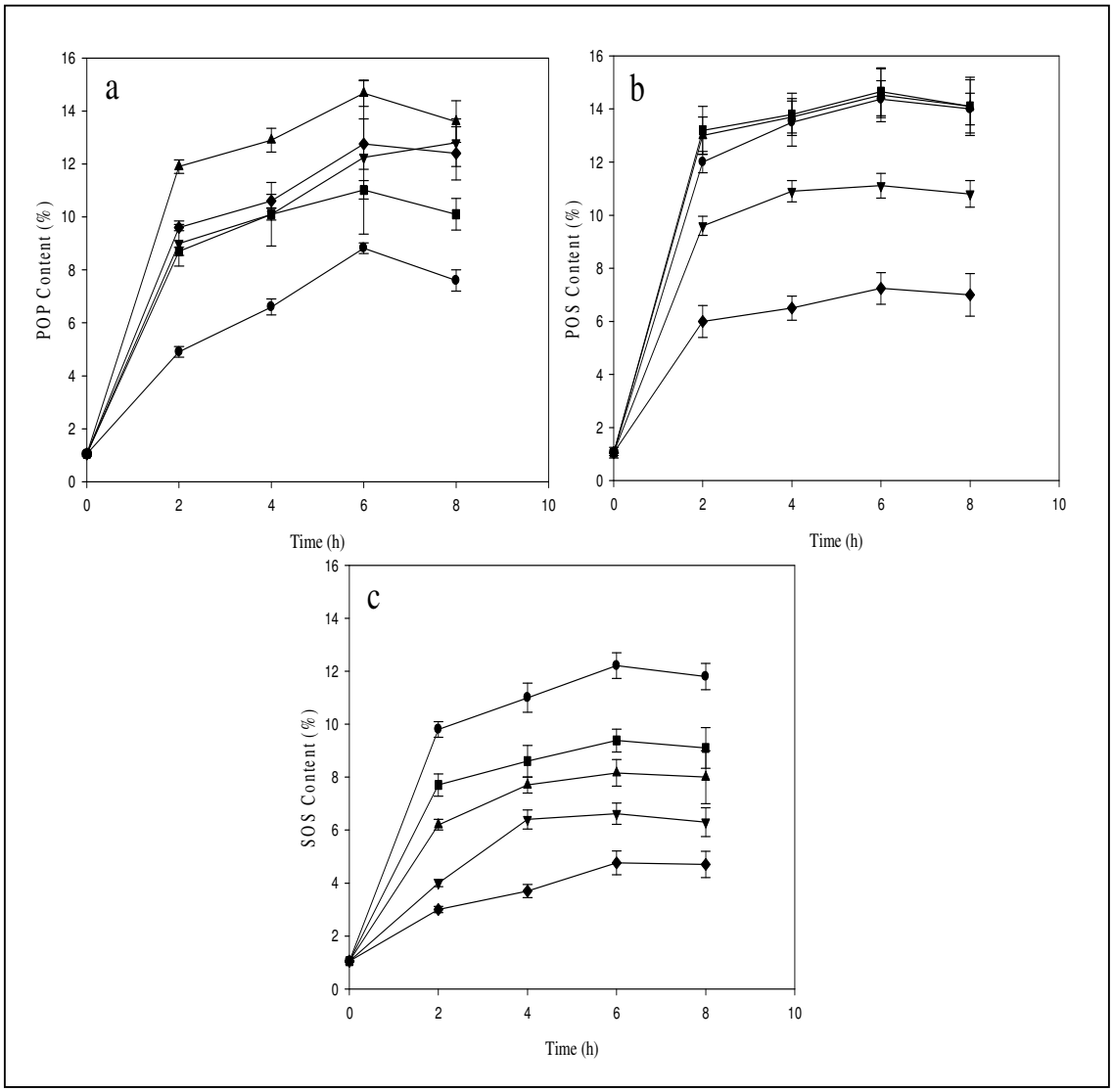


Figure 4.6. (a) Effect of substrate mole ratio on production of POP as a function of time. (●) 1:1:3, (■) 1:2:3, (▲) 1:3:3, (◆) 1:3:1 and (▼) 1:3:2; (b) Effect of substrate mole ratio on production of POS as a function of time. (●) 1:1:3, (■) 1:2:3, (▲) 1:3:3, (◆) 1:3:1 and (▼) 1:3:2; (c) Effect of substrate mole ratio on production of SOS as a function of time. (●) 1:1:3, (■) 1:2:3, (▲) 1:3:3, (◆) 1:3:1 and (▼) 1:3:2

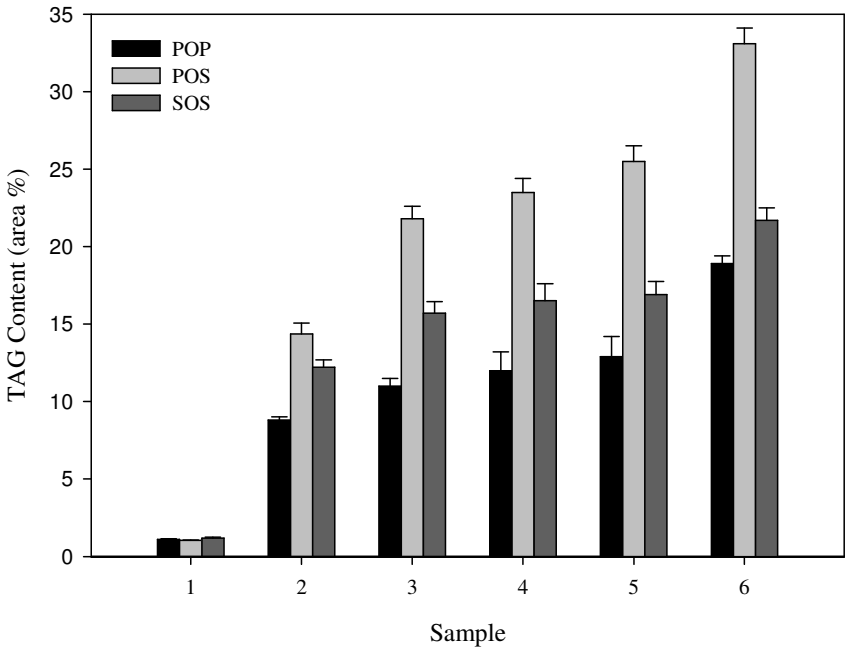


Figure 4.7. Content of target TAGs of ROPO, CB and folds of 1:1:3 substrate mole ratio. (1) ROPO, (2) 1:1:3, (3) 1:2:6, (4) 1:3:9, (5) 1:4:12 and (6) CB

Approximately 50% of the produced fat has major TAGs of CB. Abigor et al. (2003) obtained a yield of 45.6% for the production of CB-like fat from palm oil and hydrogenated soybean oil. Chang et al. (1990) reported a yield of CB-like fat of 19% when fully hydrogenated cottonseed oil was interesterified with olive oil.

Melting characteristics

In any fat, there is a direct relationship between the TAG composition and physical properties. The types of fatty acids dictate the melting behavior of a TAG. Also important is the FA positioning along the glycerol backbone, which affects structure and thereby melting. TAGs with a more asymmetrical distribution tend to have lower melting points (Rousseau and Marangoni, 2002). Figure 4.8 shows the melting profiles of the products from the mole ratio study. All samples exhibited more than one endothermic peak, indicating that the samples containing different melting components. The major melting peaks of all samples were labeled A. The peaks from the lower-melting polymorphs seem to change gradually toward higher melting ranges as the number of moles of PA and SA in the substrate ratio increases. Content of major TAGs is effective on increasing the melting peak. It is seen from Figure 4.8

that 1:1:3 gave the best melting property among all emprical substrate mole ratios (1:1:3, 1:2:3, 1:3:3, 1:3:1,1:3:2).

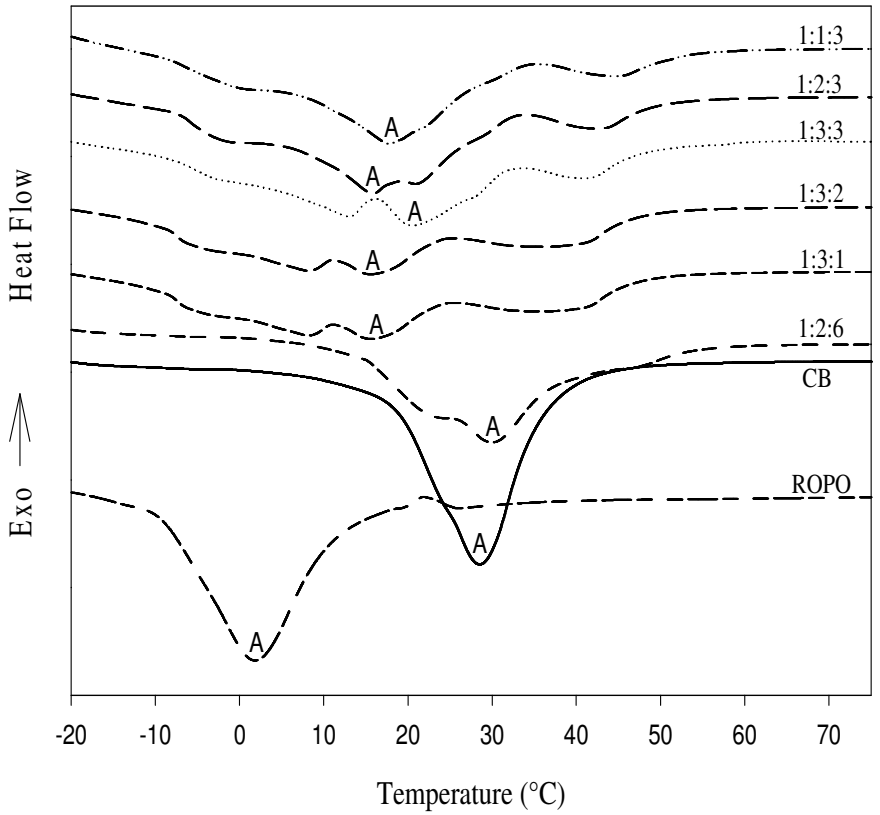


Figure 4.8. DSC melting profiles of CB and products of mole ratio study

Increasing the substrate mole ratio from 1:1:3 to 1:2:6 shifted the melting peak from 17.7 to 29.9°C, and made it sharper and more pronounced. Our product gave its major peak at 29.9°C while CB gave at 28.5°C. As seen on graph, a slight shoulder will be seen on the peak of CB, similar to the one on our product's major melting peak. The big shoulder and the broad peak of the product of 1:2:6 substrate mole ratio is due to the presence of some lower melting point TAGs of ROPO which were not converted to target TAGs. CB samples may have different compositions depending on the origin, so, they may have different melting behaviours. Solis-Fuentes and Duran-de-Bazua (2004) reported the thermogram of CB similar to our product with a low melting point fraction, with a maximum temperature of 11.64°C. Undurraga et al. (2001) reported a CB melting profile with two minor low melting point peaks between 5 and 15°C, and the other between 15 and 23°C.

Solid Fat Content

For the characterization of fats, rather than melting peak temperature, melting behaviour is important. Melting behaviour must have a sharp and narrow range of melting for a CB-like fat. Figure 4.9 shows the change in the SFC profiles of the fat samples from mole ratio study as function of temperature.

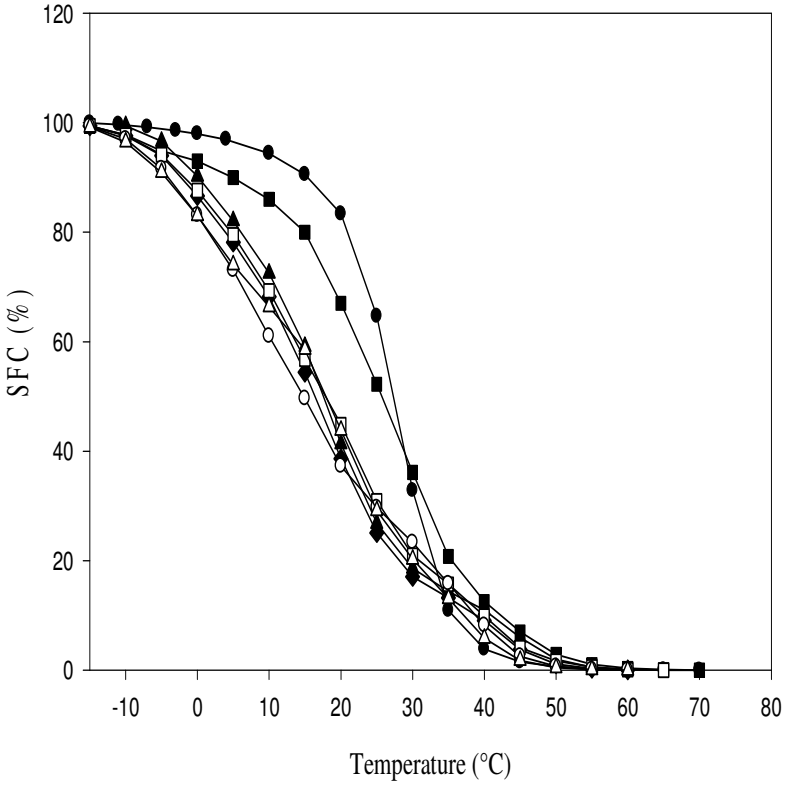


Figure 4.9. Change in SFC of CB and the products of mole ratio study as a function of temperature. (●) CB, (■) 1:2:6, (▲) 1:1:3, (◆) 1:2:3, (□) 1:3:3, (○) 1:3:1, (Δ) 1:3:2

Microstructure

The microstructure of fat crystal networks has a great effect on macroscopic properties (De Man and Beers, 1987). Fats having similar thermal properties may have different rheological properties if their microstructures are different. So, microstructure must also be considered to determine the macroscopic properties of a fat crystal network (Narine and Marangoni, 1999). Grayscale PLM photographs of CB and samples are shown in Figure 4.10. Solid phase appears as white or gray while the liquid phase as black. Because under polarization the anisotropic solid

phase of the network refracts light different than the isotropic liquid phase (Narine and Marangoni, 1999). As seen from Figure 4.10, product obtained from substrate mole ratio of 1:2:6 has the most similar crystal network characteristics to that of CB. As it is seen from the images, CB has well-defined microstructural network. This well-defined network is due to the strong and defined interactions between microstructural elements leading to a three-dimensional arrangement with similar intermicrostructural distances (Narine and Marangoni, 1999). Narine and Marangoni (1999) stated that highly ordered nature of TAGs in CB results in this regular network. We can conclude that the most similar TAG content to CB was obtained from mole ratio of 1:2.6 even only by visual inspection of the PLM images. Images obtained for other fats have an irregular, random spatial distribution of crystals showing the presence of assymetry in the molecular structure of TAGs. The irregular nature of the crystals shows the presence of weak interactions between them.

In summary, the highest yield (11% POP, 21.8% POS, 15.7% SOS) was obtained at 1:2:6 substrate mole ratio, 20% enzyme load, 6h reaction time and 45°C reaction temperature. All methods used for product characterization confirmed each other. A STR has the advantage of being relatively easy to build and free enzymes can be used, but it has the disadvantage that, unless immobilized, the enzyme can not be reused. As well, a larger system or longer reaction times are required to achieve equivalent degrees of conversionin comparision with other systems, and side reactions can be significant (Padt, 1990).

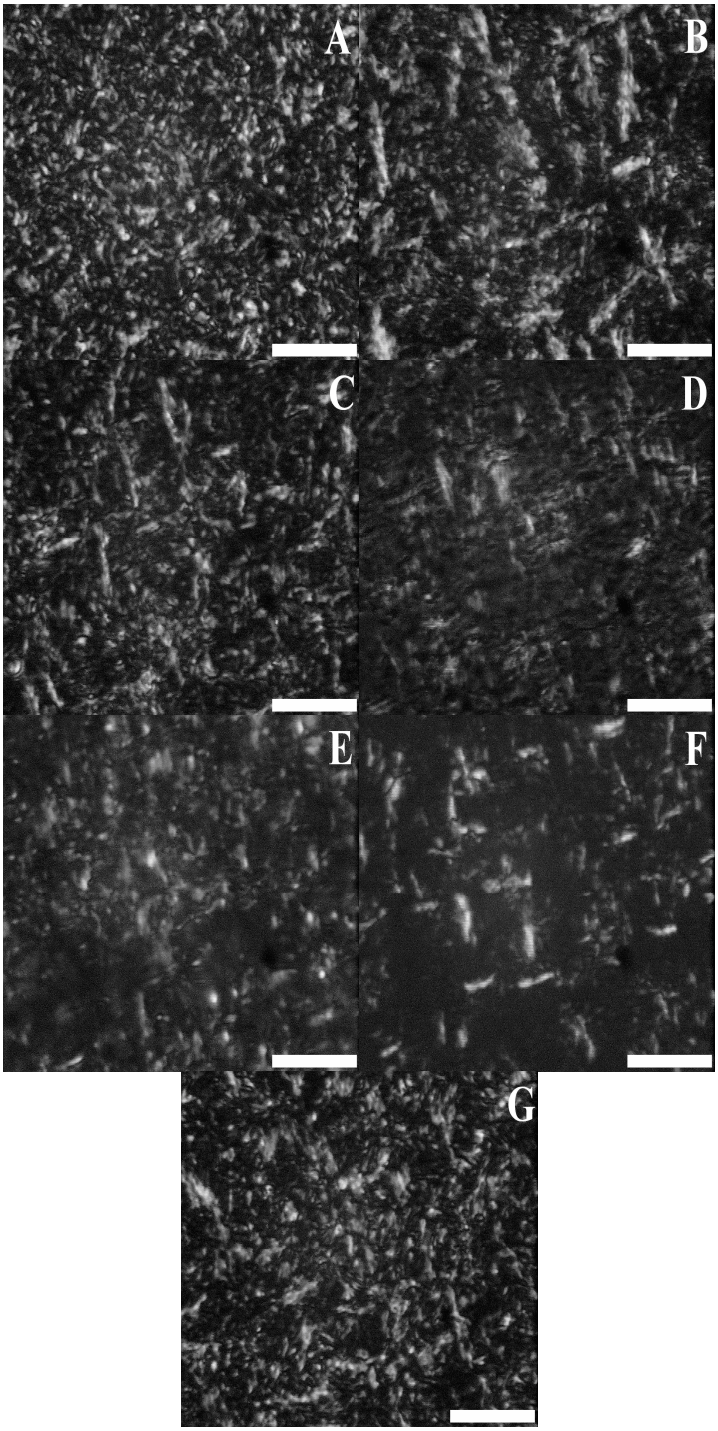


Figure 4.10. PLM grayscaled images of CB and products of mole ratio study. (A) CB, (B) 1:1:3, (C) 1:2:3, (D) 1:3:3, (E) 1:3:1, (F) 1:3:2 and (G) 1:2:6. The horizontal length of the inset bar represents 50μm

4.1.2.2 Production in continuous system

The optimized temperature (45°C) was based on the results of model system study. Certain amount of hexane was necessary in order to keep the reaction mixture, which contains excess amount of saturated fatty acids, liquid and circulate it throughout the bioreactor.

Effect of substrate ratio

Figures 4.11a,b,c show the effect of substrate mole ratio on formation of POP, POS and SOS as a function of time. The reactions were conducted at 20% enzyme load and 4.5 mL/min flow rate. It has been observed that there is a similar trend of product formation at all substrate mole ratios. Content of POP, POS and SOS increased up to 4h, and then stayed constant. As seen in Figure 4.11, among the substrate ratios studied (1:1:1, 1:1:3, 1:3:3), 1:1:3 gave the most similar TAG content of CB. At 1:1:3 substrate mole ratio and 4 h, product contained 7.6% POP, 14.1% POS and 11% SOS.

Doubling substrate mole ratio from 1:1:3 to 1:2:6, keeping mole ratio of ROPO constant, had a significant effect on the yield ($P<0.05$). At 1:2:6 substrate mole ratio, 20% enzyme load and 4.5 mL/min flow rate, the product contained 10.5% POP, 19% POS and 15.3% SOS. Further increase in substrate mole ratio would probably increase the yield. However, trials have showed that higher substrate mole ratios (1:3:9, 1:4:12) caused high level of neutral oil loss during purification. Economically thinking, higher substrate ratios are not feasible because they would require a cost-increasing extra separation step.

Effect of enzyme load

Effect of enzyme load on TAG formation was investigated at 4.5 mL/min flow rate, 1:2:6 mole ratio and varied ratios of enzyme loads packed into column. In general, the ratio between enzyme and substrates is much higher in a PBR than in conventional batch reactors, and it results in higher reaction performance (Laudani et al., 2007). As shown in Figure 4.12, increased enzyme load accelerated the reaction rate and improved the incorporation of PA and SA under given conditions. Because, at higher enzyme load there is more enzyme per unit substrate which in turn

increases the rate. The highest yield (12.3% POP, 21.3% POS and 16.6% SOS) was obtained at 40% enzyme load and 6 h reaction time.

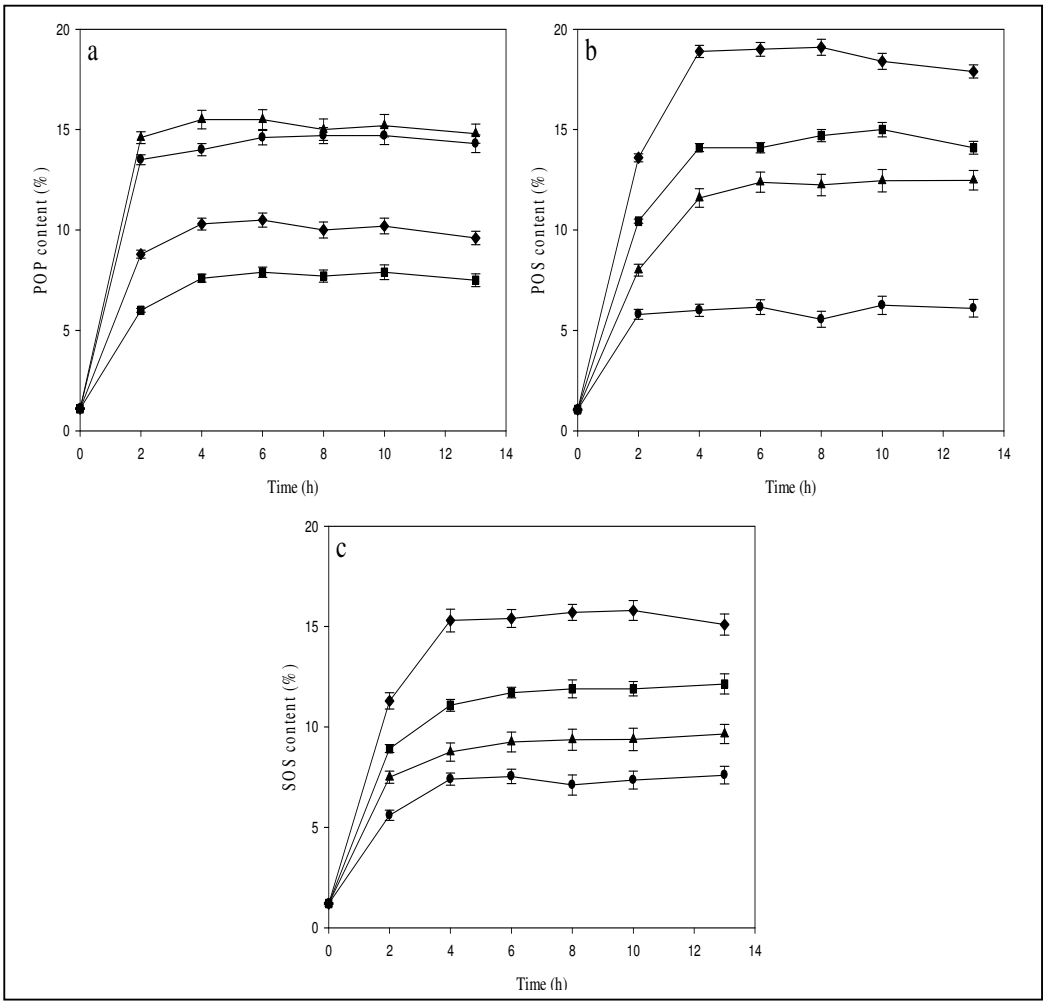


Figure 4.11. (a) Effect of substrate mole ratio on production of POP as a function of time. (●) 1:1:1, (■) 1:1:3, (▲) 1:3:3 and (◆) 1:2:6; (b) Effect of substrate mole ratio on production of POS as a function of time. (●) 1:1:1, (■) 1:1:3, (▲) 1:3:3 and (◆) 1:2:6; (c) Effect of substrate mole ratio on production of SOS as a function of time. (●) 1:1:1, (■) 1:1:3, (▲) 1:3:3 and (◆) 1:2:6

Effect of flow rate

The flow rate was varied from 1.5 to 15 mL/min, at 1:2:6 substrate mole ratio and 20% enzyme load. As shown in Figure 4.13, increasing flow rate up to 7.5 mL/min increased yield for all products. The content of TAGs reached equilibrium after 6 h

for all flow rates and no significant difference (P>0.05) was observed in the yield obtained at 4.5 and 7.5 mL/min. It has been reported that higher velocity improves mass transfer of the reactants from the bulk of the reactant mixture to the enzyme surface and hence increases reaction rate (Bailey and Ollis, 1977). Posorske et al. (1988) produced a CB substitute from palm stearin and coconut oil in a packed bed reactor. These authors found that decreasing the flow rates to increase the total product concentration caused a decrease in productivity. However, as seen in Figure 4.13, further increase in flow rate (from 7.5 to 15 mL/min) decreased product yield. This behavior also has been observed by other researchers (Laudani et al., 2007; Halim et al., 2009).

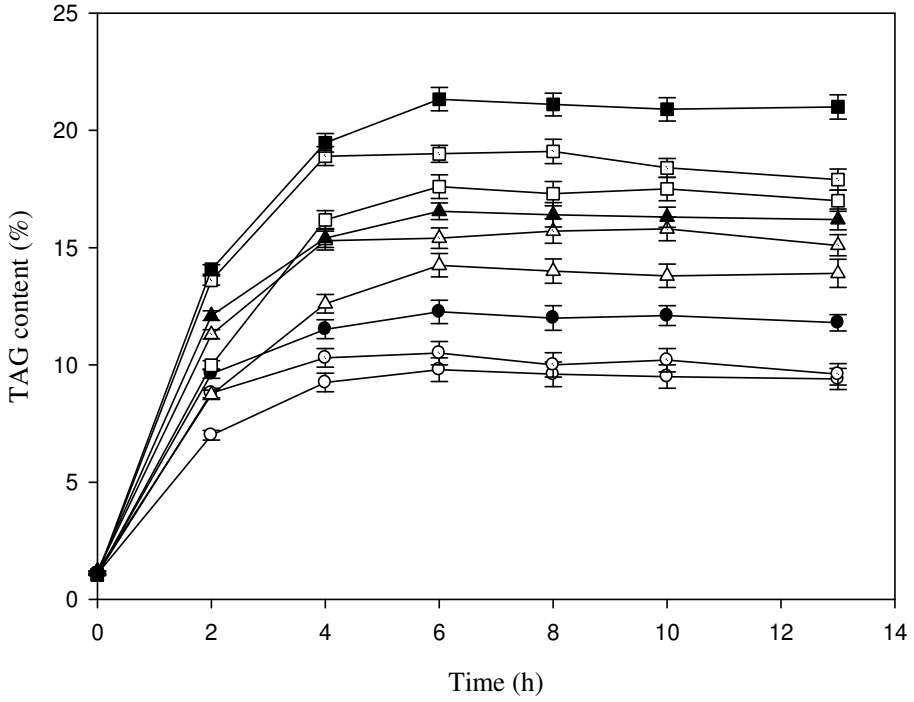


Figure 4.12. Effect of enzyme load on POP, POS and SOS formation. (○) POP at 10% enzyme load, (⊙) POP at 20% enzyme load, (●) POP at 40% enzyme load, (□) POS at 10% enzyme load, (⊡) POS at 20% enzyme load, (■) POS at 40% enzyme load, (Δ) SOS at 10% enzyme load, (◬) SOS at 20% enzyme load, (▲) SOS at 40% enzyme load

This decrease was probably due to the decreased contact time of enzyme and substrates. At low flow rates, low yields were obtained because of mass transfer resistance at liquid film layer. An increase in substrate flow rate caused reduction in

these mass transfer limitations so higher reaction rates were obtained resulting in increasing the yield (Tepe and Dursun, 2008). However, further increase in flow rate the substrate will only pass through the enzyme without interacting with the enzyme, consequently failing to bind at the enzyme active site. Hence, there is less contact between the substrate and enzyme active sites yielding lower yield (Halim et al., 2009).

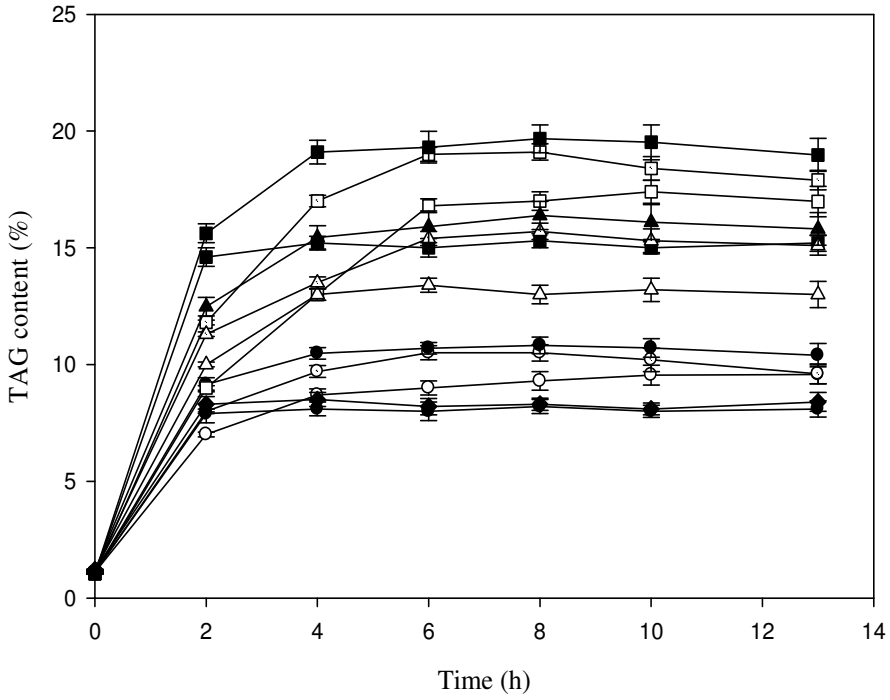


Figure 4.13. Effect of flow rate on POP, POS and SOS formation. (○) POP at 1.5 mL/min, (⊙) POP at 4.5 mL/min, (●) POP at 7.5 mL/min, (⦿) POP at 15 mL/min, (□) POS at 1.5 mL/min, (⊡) POS at 4.5 mL/min, (■) POS at 7.5 mL/min, (◘) POS at 15 mL/min, (Δ) SOS at 1.5 mL/min, (◬) SOS at 4.5 mL/min, (▲) SOS at 7.5 mL/min, (◆) SOS at 15 mL/min

The best result was obtained at 7.5 mL/min and 4 h with a yield of 10.7% POP, 19.3 % POS and 15.9% SOS.

Effect of solvent amount

Solvent was needed in this system in order to liquefy the substrates where saturated fatty acids (PA and SA) were used as acyl donors at a low reaction temperature (45°C). Use of less amount of solvent is advantageous in terms of economy and ease

of process, however, solubility of saturated fatty acids determines the minimum amount of solvent required. As seen in Figure 4.14, decreasing solvent amount increased yield significantly ($P<0.05$) within the first 6 h. Because, reaction mixture dissolved in lesser amount of solvent contains more substrate per unit volume of solvent. This means more substrate contacted with enzyme, and hence more substrate was converted to product per unit time.

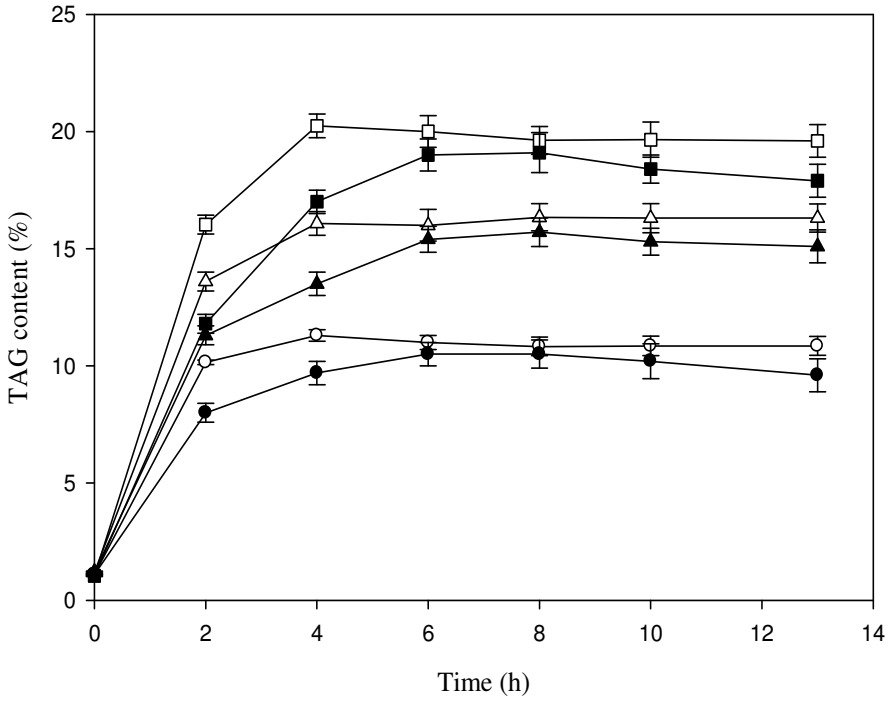


Figure 4.14. Effect of solvent amount on POP, POS and SOS formation. (○) POP at 150 mL *n*-hexane, (●) POP at 400 mL *n*-hexane, (□) POS at 150 mL *n*-hexane, (■) POS at 400 mL *n*-hexane, (Δ) SOS at 150 mL *n*-hexane, (▲) SOS at 400 mL *n*-hexane

According to the reaction parameter study, the highest yield (10.9% POP, 19.7% POS and 11.2% SOS) was obtained at 40% enzyme load, 1:2:6 substrate mole ratio, 45°C, 7.5 mL/min, 150 mL solvent and 3 h reaction time. This product was chosen as our product, so called CB-like fat, and used for blends preparation subsequent analyses. Characterization of this product was performed by DSC and PLM analyses. Total content of major TAGs in our product was approximately 43% while it was 78% in CB. This yield seems to be better when compared to the results of other CB-like fat production studies. Chang et al (1990) reported a yield of CB-like fat of 19%

when fully hydrogenated cottonseed oil was interesterified with olive oil. Abigor et al (2003) obtained a yield of 45.6% for the production of CB-like fat from lipase catalyzed interesterification of palm oil and hydrogenated soybean oil.

Operational stability

Operational stability of biocatalysts is an important characteristic of bioreactors. For practical application, a high operational stability is needed (Xu et al., 2002). Figure 4.15 shows the operational stability of the bioreactor. Acidolysis reaction was repeated for 50 times in the single packed reactor to determine the operational stability. Each run was conducted at the optimal conditions (40% enzyme load, 1:2:6 substrate mole ratio, 45°C, 7.5 mL/min, 150 mL solvent and 3 h). Degree of reaction was calculated according to the following equation:

$$\text{Degree of reaction} = (\text{POP\%+POS\%+SOS\%})_{\text{run x}} / (\text{POP\%+POS\%+SOS\%})_{\text{run 1}} * 100$$

where x is the number of run which operational stability was determined.

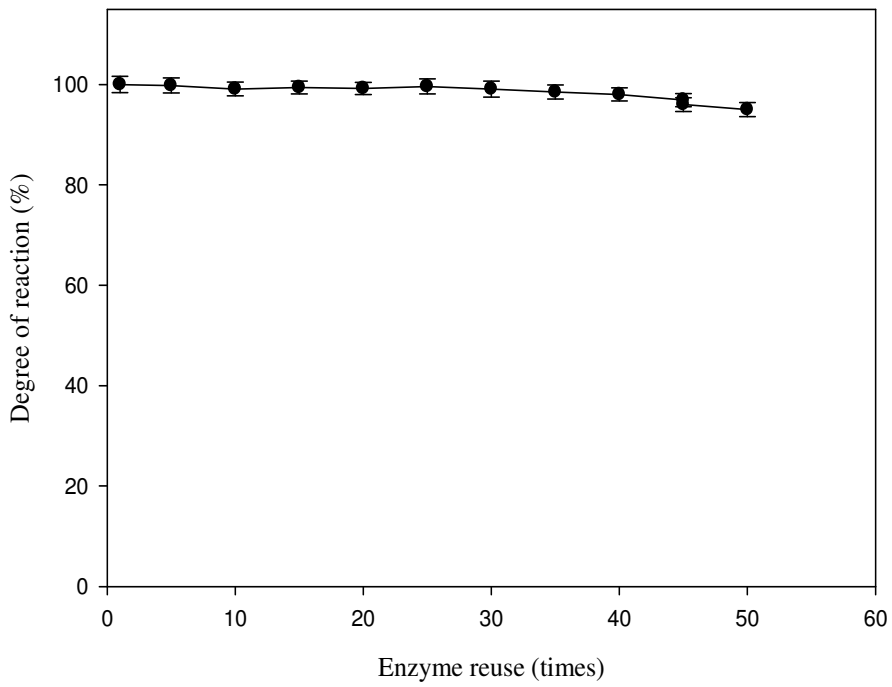


Figure 4.15. Operational stability of the bioreactor

Degree of the reaction could be maintained for up to 50 run without significant reduction ($P>0.05$). When calculated using Equations 3.2 and 3.3, this makes 150 h

of reaction, and 75 h of residence time in the bioreactor. This stability may be due to the presence of sufficient water to maintain enzyme activity in continuous operation and so preventing inactivation due to drying (Xu et al., 2002). The main advantages of PBRs are their easy application to large scale production, high efficiency, low cost, and ease of operation. They are more efficient than batch reactors but are prone to fouling and compression. The substrate has to be treated to remove any particulates and inhibitors that can build up over time and inactivate the lipase (Macrae, 1985). Macrae (1983) found that after treatment of palm oil midfraction and stearic acid to remove particulates and inhibitors, acidolysis reached completion after 400 h and there was not appreciable loss in lipase activity even after 600 h of operation. Wisdom et al. (1987) performed a pilot scale reaction using a 2.9 L packed bed reactor to esterify shea olein with stearic acid. It was found that with high quality substrates, only a small loss of activity was exhibited after 3 days with the production of 50 kg of product. However, when a lower grade shea oil was used, there was rapid inactivation of lipase.

Thermal characteristics

Figure 4.16 shows the melting profile of CB and CB-like fat. The onset and offset temperatures of melting peaks for CB were 18.5 and 39.5°C, while they were 17.5 and 43.0°C for CB-like fat, respectively. Both fats had a single melting peak at 28.5°C. For a CB-like fat production, rather than melting peak temperature, melting behaviour is more important. SFC is a good indicator of melting behaviour of fats. It must have a sharp and narrow range of melting. SFC curves of CB-like fat and CB are seen in Figure 4.17. Although both CB and CB-like fat had similar sigmoidal SFC curves, they differed in SFC as a function of temperature. CB-like fat had lower SFC than CB up to 35°C. This behaviour was probably due to unconverted TAGs of ROPO. Due to unsaturated nature of the TAGs of ROPO, unconverted TAGs lowered SFC. However, at the temperature range which influences mouth feel (33-38 °C) (De Man, 1992), CB-like fat and CB had similar SFC. CB-like fat and CB had an SFC of 10.3% and 10.9%, respectively, at 35°C. Both fats became completely liquid at 55°C.

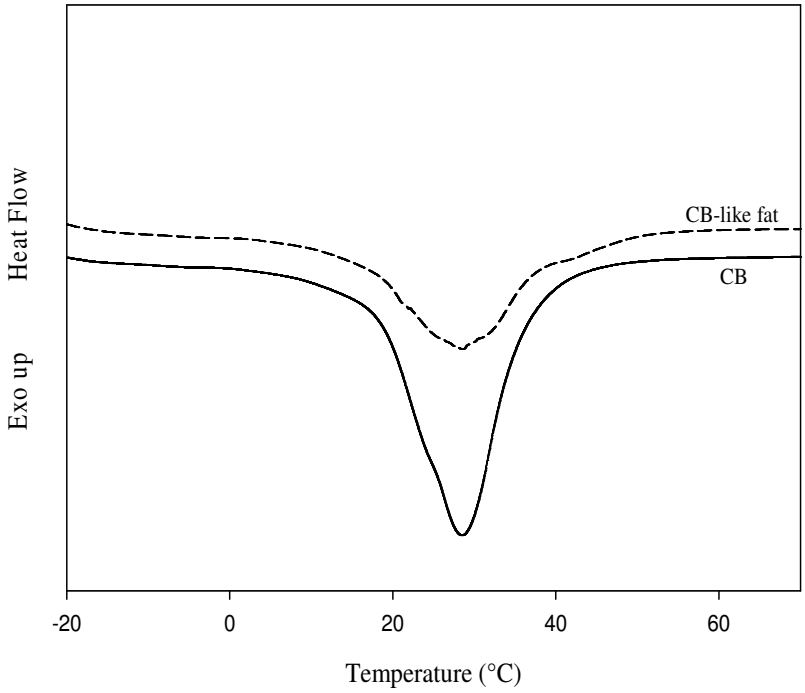


Figure 4.16. DSC melting profiles of CB and CB-like fat

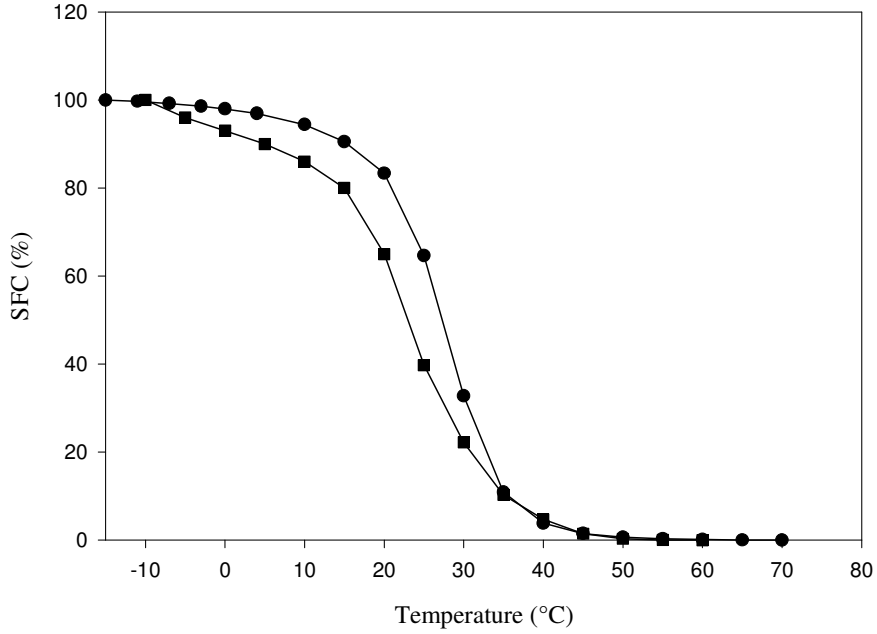


Figure 4.17. Change in SFC of CB and CB-like fat as a function of temperature. (●) CB, (■) CB-like fat

Microstructure

Investigations on the factors affecting the hardness and spreadability of chemically and enzymatically produced butterfat arose the interest in the microstructure of fat crystal networks (Rousseau et al., 1996; Marangoni and Rousseau, 1996; Rousseau and Marangoni, 1998). Fat containing materials of apparently the same physical properties (SFC, m.p., etc.) may have very different textural properties (Marangoni and Hartel, 1998). Differences in textural properties between fat-containing products might be attributed to differences in crystalline structure (size, distribution of size, shape, polymorph, surface characteristics, etc.). But, differences in crystalline structural characteristics also may be strongly influenced by the interactions between molecules in the crystalline state and the liquid oil in which the crystals are dispersed, leading to different crystalline structural characteristics at different structural levels (crystals, aggregates, and flocs or flocculates) (Yuping Shi et al., 2005).

PLM images of CB and CB-like fat are shown in Figure 4.18. No drastic changes in polymorphic behavior were observed between CB and CB-like fat. However, CB still had more densely and orderly packed crystals compared to CB-like fat. CB most likely had stronger intraparticle links. This was probably due to the highy ordered nature of TAGs of CB. The difference between microstructure of CB and CB-like fat was probably due to the unconverted TAGs of ROPO.

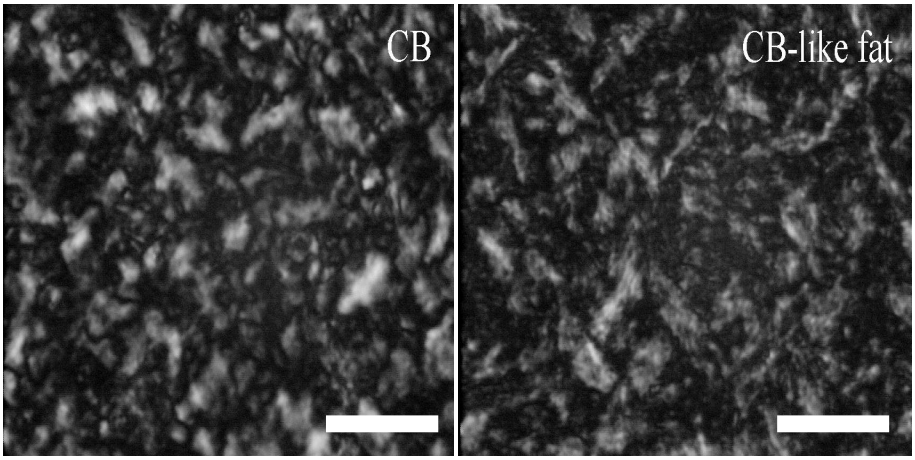


Figure 4.18. PLM grayscaled images of CB and CB-like fat. The horizontal length of the inset bar represents 50μm

In conclusion, the highest yield (10.9% POP, 19.7% POS and 11.2% SOS) was obtained at 40% enzyme load, 1:2:6 substrate mole ratio, 45°C, 7.5 mL/min, 150 mL solvent and 3 h.

4.2 Evaluation of the Performance of the CB-Like Fat

A CB-like fat is designed to replace CB partially. For this purpose blends of CB and CB-like fat are prepared. In Europe currently, 5% of CBE addition is permitted to chocolate. This would imply (given a typical fat content of about one third) 15% CBE in the fat phase. However, the most important thing deciding the amount of incorporation is the performance of the produced CB-like fat. The most important properties that should be checked fort he performance of a CB-like fat are TAG composition, thermal characteristics, and microstructure.

Hence, the performance of the product was determined by preperation of blends of CB and CB-like fat at different proportions. In order to determine the compatability limit of the product with CB, the crucial chemical and physical analyses of the blends were conducted.

Chemical composition

TAG composition is one of the most important chemical characteristics determining physical properties of fats and oils. The smallest characteristic structure present in fat crystal networks is at the molecular level. The major constituents of fats, including CB, are TAGs. TAGs are composed of a variety of fatty acids esterified to a glycerol backbone. The fatty acid chains may be saturated or unsaturated, branched or linear, short or long, and contain either odd or even numbers of carbons (Small, 1986). The physical properties of TAG molecules are dependent on fatty acid chain composition and distribution. The composition and distribution influence the polymorphic behavior of the various solid phases, which in turn influences various macroscopic properties of the material including melting behavior and hardness (de Man, 1982). Hence, determination of TAG content is crucial in order to investigate the compatibility of a fat blend. A CB replacer fat should not cause a significant change in TAG content when blended with CB.

Table 4.1 shows the TAG composition of the ROPO, CB, and CB-like fat. Figure 4.19 depicts the change in the content of major TAGs of the blends. CB-like fat

obtained from enzymatic conversion of ROPO contained 11% POP, 20% POS and 11.7% SOS, while commercial CB contained 18.9% POP, 33.1% POS and 24.7% SOS. Other two major TAGs present in the product were POO (13.7%) and SOO (9%). It has been reported that POO and SOO have caused longer induction times during isothermal crystallization at 26.5°C (Chaiseri et al., 1995).

Table 4.1. TAG composition of ROPO, CB-like fat and CB

TAG	%		
	ROPO	**CB-like fat**	**CB**
LLL	1.1 ± 0.10	1.2 ± 0.11	
OLLn	2.4 ± 0.12	2.3 ± 0.10	
PLLn	1.6 ± 0.10	1.4 ± 0.13	
LOL	8.0 ± 0.16	2.0 ± 0.10	
OLnO	3.2 ± 0.11	3.4 ± 0.16	
PLL	1.1 ± 0.1	1.2 ± 0.12	
PLP			1.3 ± 0.10
PLS			3.3 ± 0.12
SLS			2.0 ± 0.15
OLnO	10.0 ± 1.10	3.3 ± 0.13	
OOL	12.3 ± 0.9	10.0 ± 0.6	
PLO	7.7 ± 0.56	5.9 ± 0.40	
OOO	36.0 ± 0.17		
POO	7.4 ± 0.30	13.7 ± 0.25	5.4 ± 0.20
POP	1.1 ± 0.12	11.0 ± 0.10	18.9± 0.50
POS	1.1 ± 0.10	20.0 ± 1.70	33.1 ± 2.10
SOO	2.5 ± 0.20	9.0 ± 0.90	7.2 ± 0.35
SOS	1.2 ± 0.15	11.7 ± 1.00	24.7 ± 1.3

L: Linoleic acid, Ln: Linolenic acid, O: Oleic cid, P: Palmitic acid, S: Stearic acid.

It is important to control the content of MAGs and DAGs in the production stage. Because MAGs and DAGs have a strong effect on crystallization of fats. Total content of MAGs and DAGs in the product was 5.3%. It has been reported that MAGs have a retardation effect on crystallization. In general, micelles of MAGs are

thought to act as templates for crystallization and may induce heterogeneous nucleation (Gerson and Escher, 1966). Sambuc et al. (1980) showed that the addition of 4% of a mixture of monopalmitin and monostearin decreases the induction time of vegetable fats. But, MAGs in low concentrations will not affect the CB crystallization much (Chaiseri et al., 1995).

DAGs may either promote or delay crystallization, depending on composition and concentration. Shukla (1995) and Ziegleder (1988) noticed that CBs with a higher DAG level exhibit a slower crystallization. Cebula and Smith[18] reported that DAGs in Coberine (a CB equivalent fat) reduce the fat's crystallization induction time but subsequently slow down the velocity of growth. Siew and Ng (1996) have reported that dipalmitoylglycerol caused rapid palm olein crystallization, palmitoyloleoylglycerol retarded crystallization, and dioleoylglycerol had no significant effect.

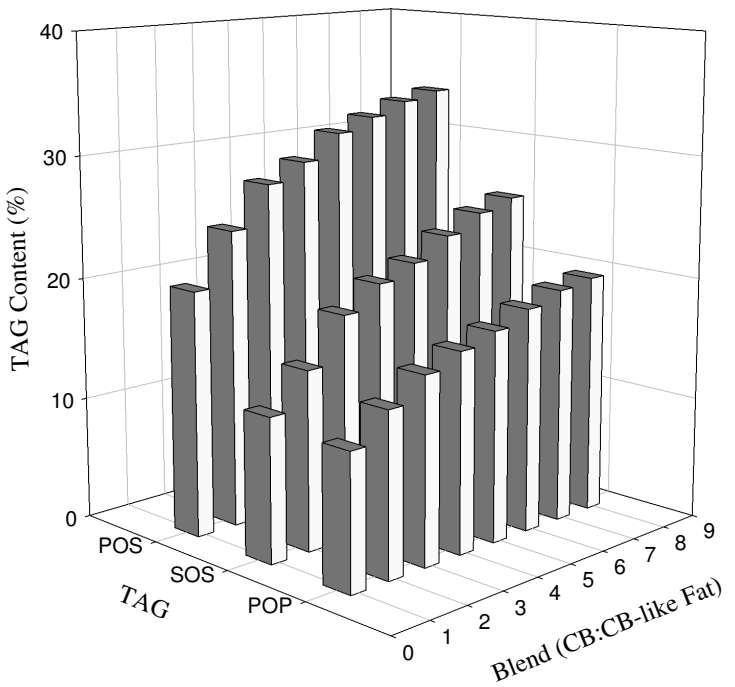


Figure 4.19. Effect of CB-like fat proportion on the content of major TAGs of CB. (1) 0:100, (2) 30:70, (3) 50:50, (4) 60:40, (5) 70:30, (6) 80:20, (7) 90:10, (8) 100:0

It has been observed that CB-like fat incorporation up to 30% did not cause a significant decrease ($P>0.05$) in TAGs of CB. Further increase in the proportion of CB-like fat in the blend caused a gradual decrease in the content of major TAGs.

Blend of 70:30 (17.2%POP, 31% POS and 21.3% SOS) was found to be the most compatible blend formulation in terms of TAG content.

Melting characteristics

Melting thermograms of the blends are depicted in Figure 4.20. CB (100:0) has a sharp and narrow melting peak at 28.5°C. Three major TAGs (POP, POS and SOS) and their content are responsible for this unique melting behaviour.

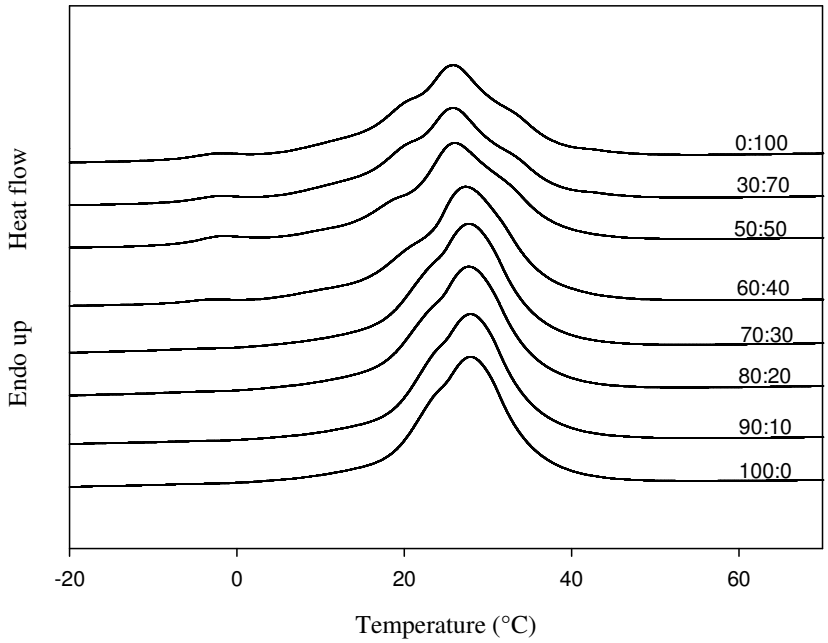


Figure 4.20. Melting thermograms of CB and CB-like fat blends

It has been observed that melting characteristic of the blend changed with increasing proportion of CB-like fat. Although the melting peak temperature did not change significantly, increasing proportions of CB-like fat in the blend made the shape and range of the melting peak wider and irregular. Also, small new melting peaks have appeared and became more pronounced with increasing CB-like fat proportion. This can be largely attributed to the decrease in the content of POP, POS and SOS, and also presence of unconverted TAGs of ROPO with low melting point. It has been observed that CB-like fat incorporation to CB should be limited to 30% in order to save the sharp and narrow melting range of CB.

SFC

The performance of confectionary fats in food products depends on melting behavior. SFC is a good indicator of melting behaviour of fats. SFC profile of the oils and fats or their blends is of considerable importance and is used as a guideline to judge whether a certain oil, fat or blend is suitable for a particular application (NorAini et al., 1995). Change in SFC of the blends as a function of temperature is given in Figure 4.21.

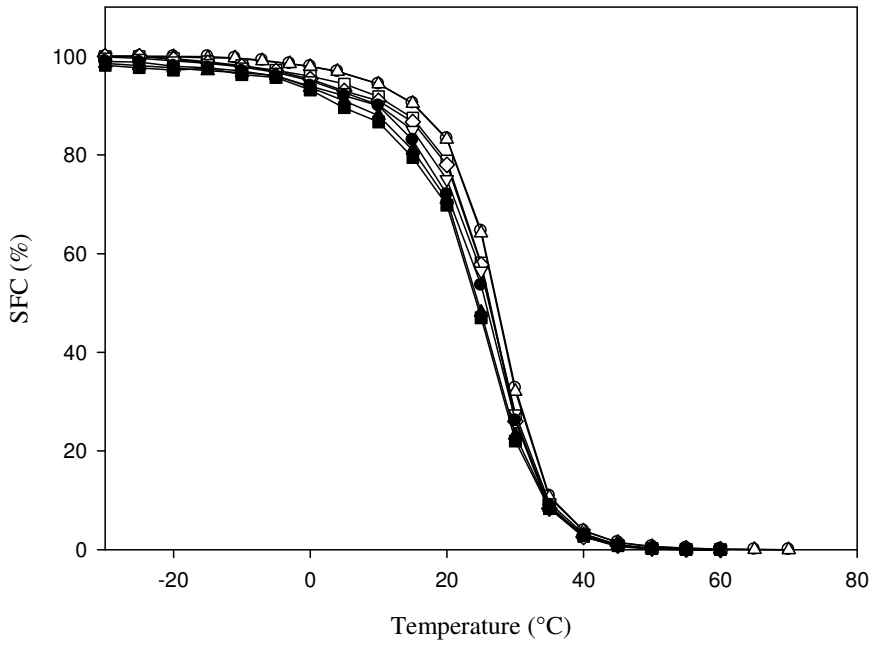


Figure 4.21. SFC of CB and CB-like fat blends as a function of temperature. (○) 100:0, (Δ) 90:10, (□) 80:20, (◇) 70:30, (▽) 60:40, (●) 50:50, (▲) 30:70, (■) 0:100

None of the blends changed the melting feature of CB. But, it has been observed that SFC decreased with increased ROPO proportion in the blend. Those variations of SFC are mainly due to differences in TAG contents of CB and CB-like fat.

Figure 4.22 shows the isothermal solid diagram of SFC for blends. For an ideal mixing compatibility, straight lines should be observed for each isothermal solid line of SFC. Significant decrease ($P<0.05$) in SFC was observed at all temperatures for blends which ROPO ratio was above 10%. Better compatibility between CB-like fat and CB was observed at temperature 35°C and over for all CB-like fat proportions. This means, in the mouth (at body temperature) the blends will not affect mouthfeel

significantly. According to Shukla (1995), softer CB would contain about 63% solid fat at 20°C and 20% solid fat at 30°C. In this study, these criteria were fulfilled at 20 and 30°C for all blends. In general, the interactions in the present blends were more noticeable at room temperature. No eutectic effect has been observed in the blends. Absence of eutectic effect shows the similarity between molecular volume, shape or polymorph of CB and CB-like fat (Noor Lida et al., 2006).

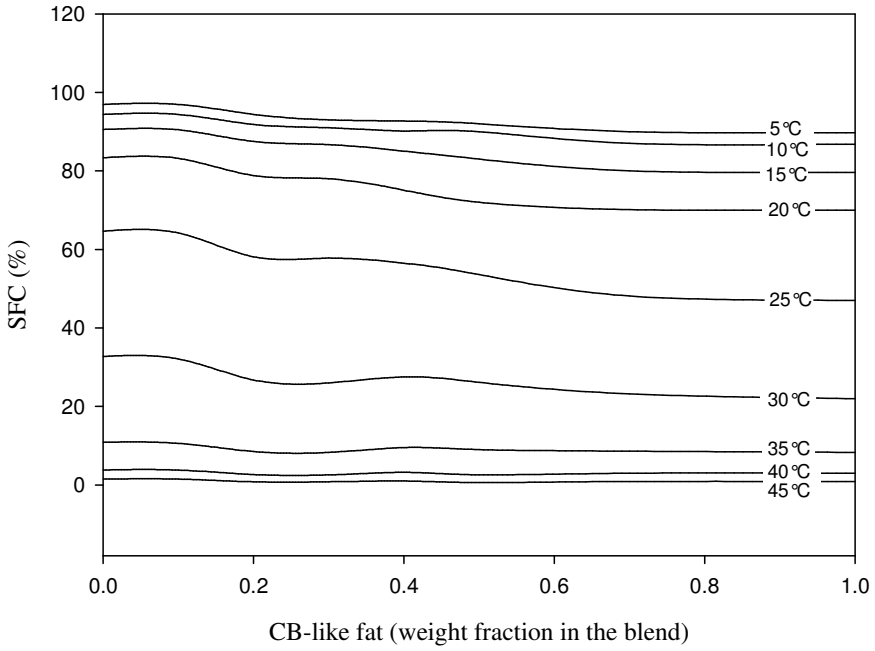


Figure 4.22. Isothermal solid diagram of SFC for CB and CB-like fat blends

Microstructure

The microstructure of fat crystal networks has a great effect on macroscopic properties (De Man and Beers, 1987). Hence, in order to determine the compatibility of fat blends, their microstructures should also be investigated in addition to chemical and physical characteristics. PLM images of the blends are depicted in Figure 4.23. It has been observed that the proportion of CB-like fat in the blend affected microstructure significantly over 30% incorporation. As seen from the images, incorporation of CB-like fat up to 30% had no effect on the crystal network. Further increase in CB-like fat proportion in the blend decreased the number of crystals, caused relatively irregular crystal shape and distribution in the network. This change in the microstructure is due to the decrease in major TAGs of CB and also presence of other TAGs coming from ROPO.

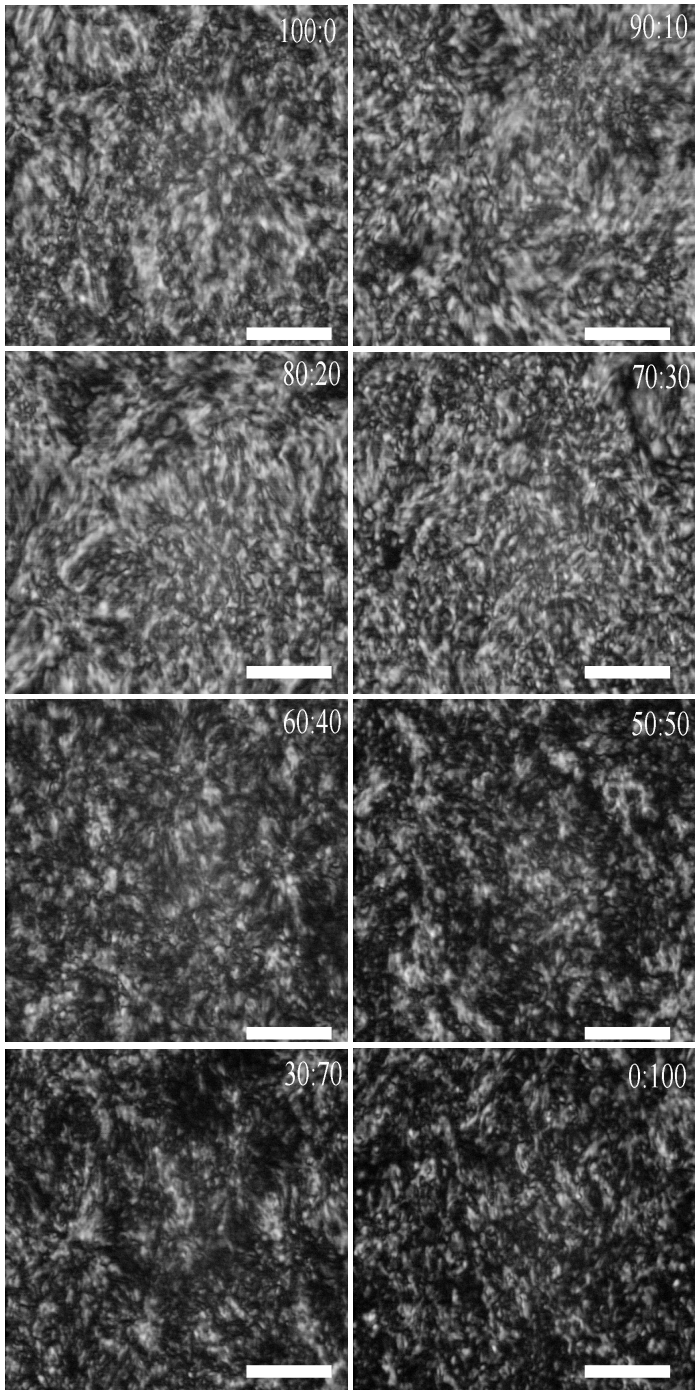


Figure 4.23. PLM grayscaled images of CB and CB-like fat blends. The horizontal length of the inset bar represents 50 µm

Highly ordered nature of TAGs in CB results in a regular network. A well-defined crystal network is due to the strong and defined interactions between microstructural

elements leading to a three-dimensional arrangement with similar intermicrostructural distances (Narine and Marangoni, 1999).

Oxidative stability

The kinetic parameters describing the induction period have been obtained as described by Kowalski et al. (2000). As an example, the exotherms obtained for the oxidation of CB:CB like fat blends at 140°C are shown in Figure 4.24.

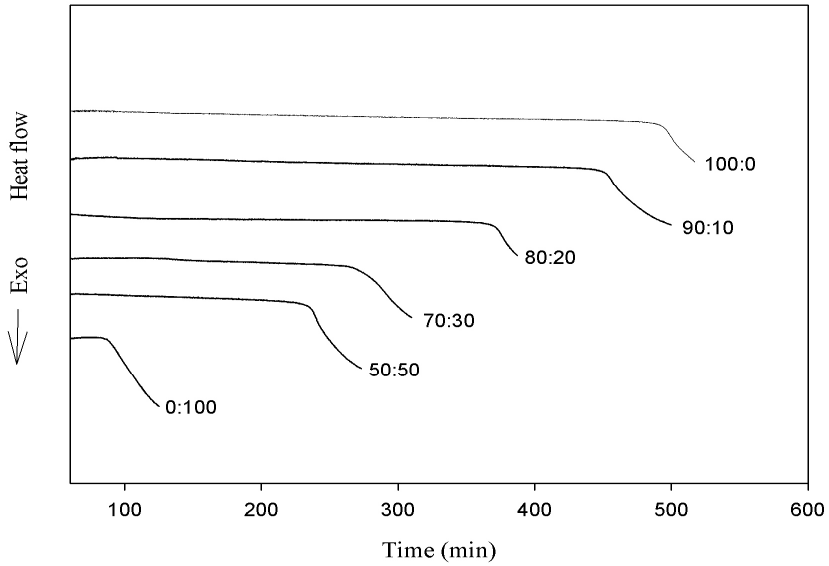


Figure 4.24. DSC exotherms for oxidation of the blends at 140°C

The logarithms of OIT values obtained from DSC measurements vs temperature were plotted for all samples (Figure 4.25). As seen from Figure 4.25, the log OIT show linear dependencies (correlation coefficients > 0.99) on the exotherm temperatures which can be described by the equation:

$$\log OIT = At + a \qquad (4.1)$$

A and a are adjustable coefficients and t is temperature in °C. The coefficints A and a were calculated by regression analysis for all samples and are given in Table 4.2. The OIT values for a given temperature can be calculated from equation 4.1. As seen from Figure 4.25, the relative resistance of blends on their thermal-oxidative degredation depend on temperature.

Table 4.2. Coefficients of equations (1) and (2), activation energies (E_a; kJ/mol), preexponential factors (Z; min^{-1}) of oxidation of the blends

Blend	-A	a	R^2	B	-b=logZ	R^2	E_a	k at 110°C	k at 130°C	k at 150°C	k at 160°C
100:0	0.0318	7.1470	0.9999	5549.7	10.757	0.9997	106.241	1.848e-4	9.683e-4	4.336e-3	8.712e-3
90:10	0.0315	7.0390	0.9936	5487.7	10.654	0.9944	105.054	2.117e-4	1.088e-3	4.794e-3	9.557e-3
80:20	0.0318	7.0070	0.9956	5531.1	10.839	0.9962	105.885	2.497e-4	1.300e-3	5.795e-3	1.161e-2
70:30	0.0285	6.4170	0.9973	4967.9	9.609	0.9977	95.103	4.345e-4	1.912e-3	7.320e-3	1.367e-2
60:40	0.0294	6.5810	0.9990	5197.9	10.191	0.9991	99.506	4.163e-4	1.963e-3	7.995e-3	1.536e-2
50:50	0.0313	6.7620	0.9999	5452.2	10.833	0.9995	104.375	3.958e-4	2.013e-3	8.782e-3	1.743e-2
0:100	0.0362	7.0310	0.9990	6295.6	13.287	0.9982	120.521	7.069e-4	4.625e-3	2.534e-2	5.591e-2

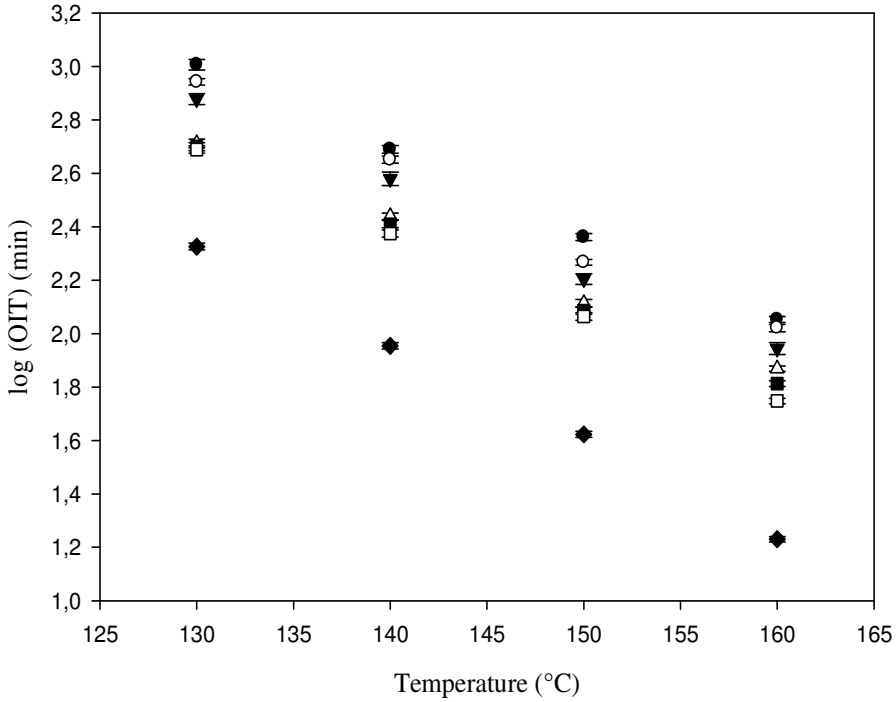


Figure 4.25. Logarithms of OIT versus DSC isothermal temperature. (●) 100:0, (○) 90:0, (▼) 80:20, (Δ) 70:30, (■) 60:40, (□) 50:50, (◆) 0:100

If the temperatures of DSC measurements are expressed in absolute scale , we can also obtain linear dependencies (correlation coefficients > 0.99) of log OIT on the reciprocal of exotherm temperatures. These dependencies can be described by the equation:

$$\log OIT = B(1/T) + b \qquad (4.2)$$

B and b are adjustable coeeficients and T is the absolute temperature. The coefficients were calculated by least squares fit procedure and are listed in Table 4.2. As for a given blend at OIT times the degree of conversion is the same independent of the temperature of oxidation. Using coeffcients B and b from equation 4.2, the Arrhenius activation energies and pre-exponential factors for an apparent reaction of blend oxidation can be calculated.

E_a, Z and k values at 110, 130, 150, and 160°C for the oxidation reaction for all blends are shown in Table 4.2. E_a values obtained varied from 95.103 to 120.521 kJ/mol. There was no apparent effect on either E_a and Z values with CB-like fat addition. For 0 and 100% CB-like, we observed that E_a values were 106.241 and

120.521, respectively; and the Z values were 10.757 and 13.287, respectively. Although, E_a and Z values were not affected by CB-like fat incorporation, a gradual increase on k values was observed as CB-like fat ratio in the blend increased. This suggests that k values seem to be more appropriate than E_a to compare the oxidative stability of fats and oils. These findings are consistent with the findings of Thurgood et al. (2007).

The effect of CB-like fat incorporation on the k values of blends as a function of temperature is shown in Figure 4.26. There was no significant difference in the k value obtained for different CB-like fat ratios at 110°C ($P>0.05$). At 130°C, there was no significant difference ($P>0.05$) between blends 70:30 and 60:40; and 60:40 and 50:50, the others were significantly different ($P<0.05$). At 150°C there was no significant difference ($P>0.05$) in k values between all blends. At 160°C, the difference between 100:0 and 90:10 insignificant ($P>0.05$), but the others were significantly different ($P<0.05$). In general, it has been observed that above 110°C, that increasing the ratio of CB-like fat in the blend increased the k value with increasing temperature. The effect of temperature on k values was also analyzed, and it has been observed that for all blends the increase in k value with temperature was significant ($P<0.05$).

The rate of oil oxidation depends on many factors such as fatty acid composition, TAG composition, catalysts, antioxidants, oil processing, etc. (Kowalski et al., 2000; Choe and Min 2006). CB-like fat obtained from enzymatic conversion of ROPO contained 11% POP, 20% POS and 11.7% SOS, while commercial CB contained 18.9% POP, 33.1% POS and 24.7% SOS (Figure 4.19). It has been observed that CB-like fat incorporation up to 30% (70:30) did not cause a significant decrease ($P>0.05$) in TAGs of CB. Further increase in the ratio of CB-like fat in the blend caused a gradual decrease in the content of major TAGs.

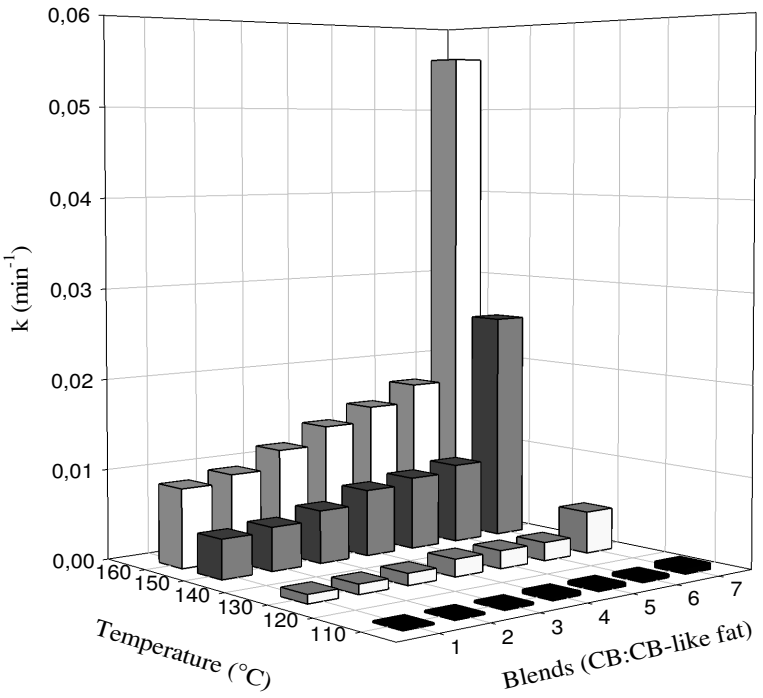


Figure 4.26. Effect of CB-like fat ratio and temperature on the oxidation rate constant (k). (1) 100:0, (2) 90:10, (3) 80:20, (4) 70:30, (5) 60:40, (6) 50:50, (7) 0:100

Figure 4.27 shows the relationship between effect of CB-like ratio on major TAGs (POP, POS and SOS), OIT and k value. It can be gathered from Figure 4.27 that there is a correlation between TAG content and oxidative stability. It has been observed that increasing the proportion of CB-like fat in the blend decreased the induction time. The decrease in oxidative stability with increasing CB-like fat ratio in the blend is largely attributed to the decrease in the content of POP, POS and SOS, and also presence of unsaturated TAGs of ROPO which were not converted to POP, POS and SOS during acidolysis. Because edible oils with higher degree of unsaturation are more susceptible to lipid oxidation.

DSC isothermal temperature had a significant effect ($P<0.05$) on the OIT. For all blends, OIT were significantly decreased as the isothermal temperature increased. Similar to the discussion of Tan et al. (2002), generally a 10°C increase in DSC temperature caused the OIT decrease approximately to the half of its previous value, with a good agreement with the Q_{10} law for the relationship between temperature and rate of chemical reaction.

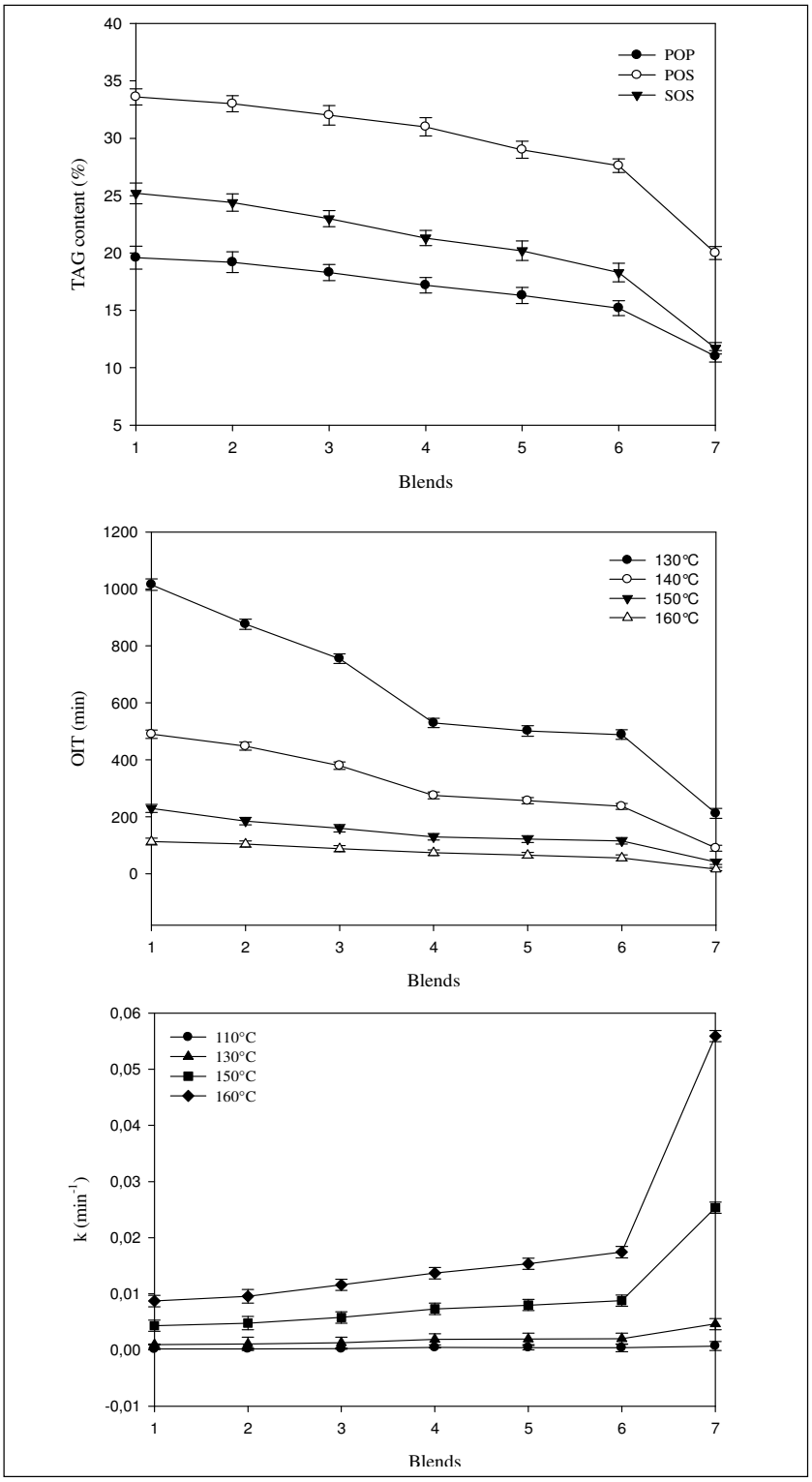


Figure 4.27. The relationship between the effect of CB-like ratio on major TAGs (POP, POS and SOS), OIT and k. (1) 100:0, (2) 90:10, (3) 80:20, (4) 70:30, (5) 60:40, (6) 50:50, (7) 0:100

Investigations on the compability limit of CB-like fat incorporation into CB, including melting behaviour, SFC and microstructure, showed that this CB-like fat could be incorporated into CB at 30% without altering the physical and chemical properties of the product significantly. However, in this study it has been observed that 30% CB-like fat incorporation caused the OIT to decrease to its half value for all DSC isothermal temperatures. However, even in this condition, the blend is still a stable fat.

DSC method was used successfully to determine the effect of the CB-like fat enzymatically produced from ROPO on the oxidative stability of CB-like fat-CB blends. The study provides kinetic parameters that can be used to predict the oxidation behaviour of different blends of CB-like fat and CB. It has been observed increasing CB-like fat ratio in the blend decreased the oxidative stability. Temperature and TAG content are effective on oxidative stability of the blends. Along with previous study, manufacturers will be able to select a CB:CB-like fat blend that better satisfies the functionality and the oxidative stability of their products. Oxidative stability must also be considered along with other chemical and physical properties during preparation of such food blends.

CONCLUSIONS

In the study, it was aimed to convert refined olive pomace oil to a cocoa butter-like fat. For an efficient production, firstly acidolysis reactions were investigated in the model system, where triolein was used as starting oil. It has been found that the best result (15.2% POP, 30.4 % POS, 15.2% SOS) was obtained at 1:3:3 (OOO:PA:SA) substrate mole ratio and reaction parameters: time 10 h, temperature 45^{o}C, enzyme load 20%, water content 5%. Natural system studies were conducted with the aid of the information obtained from model system study.

Trials on the determination of the optimum water content in the natural system, where refined olive pomace oil was used, have showed that the optimum water content depends on the reaction system, and it must be determined for each particular reaction system. Natural system studies were conducted in the absence of water due to the results of the trials on optimum water content determination. The highest yield (11% POP, 21.8% POS, 15.7% SOS) for the batch STR system was obtained at 1:2:6 substrate mole ratio, 20% enzyme load, 6h reaction time and 45°C reaction temperature. For the PBR system the highest yield (10.9% POP, 19.7% POS, 11.2% SOS) was obtained at 40% enzyme load, 1:2:6 substrate mole ratio, 45°C, 7.5 mL/min, 150 mL solvent and 3 h. It has been observed that PBR has some advantages over batch STR in terms of time, enzyme cost, and labor cost. Because, it has been seen that the enzyme packed bioreactor can retain its activity for at least 50 runs. This reduces the enzyme cost and makes the process easier when compared to STR. Because tank reactor needs replacement of fresh enzyme, or filtration, washing and drying of the enzyme for the next use.

Investigation of the physicochemical characteristics of the products have showed that the study succeeded. There was 19.6% POP, 33.6% POS and 25.2% SOS in CB, while our product contained approximately 11% POP, 20 POS and 11.7% POS. The melting profile and SFC of the product were comparable to those of CB. PLM images showed no drastic changes in polymorphic behavior between CB and product. It has been determined that the product can be incorporated into CB up

to 20% without altering the physical and chemical properties of the product significantly.

The results of the study suggested that the product produced here has the potential to add value to a by-product of olive oil industry. Enzymatically modified ROPO may be useful in the confectionary industry as a partial CB replacement. Replacement of CB up to a certain level with this product may reduce the costs of confectionary manufacturers.

Recommendations for Future Research

In the course of this study, we identified some points where further research was needed. These recommendations may serve to guide future research in this subject. First of all, a comprehensive project with an enough budget is needed.

Productions should be done in larger scale bioreactor with large volumes of substrates and enzyme. The progress of the reaction, and the possible problems in a large scale production, if there is, should be determined.

Purification of the product by short path distillation unit may only make this process feasable and applicable to industry. By this way, excess FFA may be recovered and re-entered to the reaction. This will reduce the cost of the process.

However, current enzyme prices are too high for the process to be feasible. So, a parallel study should be made to make enzymes available at a cost-effective price. This will include production of the enzyme from microbial sources, isolation and purification. Recent new technology achievements make us believe it will be possible to match the enzyme costs similar to costs for chemical catalysts.

REFERENCES

Abigor, R.D., Marmer, W.N., Foglia, T.A., Jones, K.C., Diciccio, R.J., Ashby, R. and Uadia, P.O. (2003). Production of Cocoa butter-like fats by the lipase-catalyzed interesterification of palm oil and hydrogenated soybean oil. *Journal of the American Oil Chemists' Society*, ***80***, 1193-1196.

Akoh, C. C. and Xu, X. (2002). *Enzymatic production of Betapol and other specialty fats*. In: T. M. Kuo, H. W. Gardner (Eds.), Lipid Biotechnology. New York: Marcel Dekker, pp. 461-478.

Akoh, C., Cooper, C. and Nwosu, C.V. (1992). Lipase G-catalyzed synthesis of monoglycerides in organic solvent and analysis by HPLC. *Journal of American Oil Chemists' Society*, ***69***, 257-260.

Akoh, C.C. (2002). Structured lipids. In: C.C. Akoh (Ed.), *Food Lipids Chemistry, nutrition, and biotechnology*. New York: Marcel Dekker, pp. 877-908.

AOCS Official Method Ca 5a-40 (1989). *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th edn., D. Firestone (Ed.), Champaign: American Oil Chemists' Society.

AOCS Official Method Cd 8-53. (1997). *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th edn., D. Firestone (Ed.), Champaign: American Oil Chemists' Society.

Arcos, J.A., Garcia, H.S. and Hill, C.G.Jr. (2000). Continuous enzymatic esterification of glycerol with (poly)unsaturated fatty acids in a packed-bed reactor. *Biotechnology and Bioengineering*, ***68***, 563-570.

Arruda, D.H. and Dimick, P.S. (1991). Phospholipid Composition of Lipid Seed Crystal Isolates from Ivory Coast Cocoa Butter. *Journal of the American Oil Chemists' Society*, ***68***, 385-390.

Babayan, V.K. (1987). Medium-chain triglycerides and structured lipids. *Lipids*, ***22***, 417-420.

Bailey, J.E. and Ollis, D.F. (1977). *Biochemical Engineering Fundamentals*. Tokyo: McGraw-Hill Kogakusha Ltd.

Balcao, V.M. and Malcata, F.X. (1998). Lipase catalyzed modification of milkfat. *Biotechnology Advances*, ***16***, 309-341.

Balcao, V.M., Paiva, A.L. and Malcata, F.X. (1996). Bioreactors with immobilized lipases: State of the art. *Enzyme and Microbial Technology*, ***18***, 392-416.

Baljit, S.G., Dyal, S.D. and Narine, S.S. (2002). Lipid shortenings: a review. *Food Research International*, ***35***, 1015-1048.

Bell, G., Halling, P.J., Moore, B.D., Patridge, J. and Rees, D.G. (1995). Biocatalyst behaviour in low-water systems. *Trends in Biotechnology*, ***13***, 468-473.

Berger, M. and Schneider, M.P. (1992). Enzymatic esterification of glycerol II. Lipase catalyzed synthesis of regioisomerically pure 1(3)-rac-monoacylglycerols. *Journal of the American Oil Chemists' Society*, ***69***, 961-965.

Bevinakatti, H.S. and Banerji, A.A. (1988). Lipase catalysis: Factors governing transesterification. *Biotechnology Letters*, ***10***, 397-398.

Bjorkling, F., Godtfredsen, S.E. and Kirl, O. (1991). The future impact of industrial lipases. *Trends in Biotechnology*, ***9***, 360-363.

Bornaz, S., Fani, J. and Parmentier, M. (1994). Limit of the solid fat content modification of butter. *Journal of the American Oil Chemists' Society*, ***71***, 1373-1380.

Bornscheuer, U.T. and Yamane, T. (1994). Activity and stability of the lipase in the solid-phase glycerolysis of triolein. *Enzyme and Microbiol Technology*, ***16***, 864-869.

Boskou, D. (1996). *Olive oil chemistry and technology*. Champaign, Illinois: AOCS Press.

Brady, C., Metcalfe, L., Slaboszewski, D. and Frank, D. (1988). Lipase immobilized on a hydrophobic, microporous support for the hydrolysis of fats. *Journal of American Oil Chemists' Society*, ***65***, 917-921.

Brockerhoff, H. (1965). A stereospecific analysis of triglycerides. *Journal of Lipid Research*, ***6***, 10-15.

Bruni, R., Bianchini, E., Bettarello, L. and Sacchetti, G. (2000). Lipid composition of wild Ecuadorian Theobroma subincanum Mart. seeds and comparison with two varieties of Theobroma cacaoL. *Journal of Agricultural and Food Chemistry*, ***48***, 691-694.

Carola, C. (1975). *Olive oil technology*. In: J.M. Moreno Martinez (Ed.), By products. Rome: FAO.

Cebula, D.J. and Smith, K.W. (1992). Differential scanning calorimetry of confectionery fats: Part II: Effects of blends and minor components. *Journal of the American Oil Chemists' Society*, ***69***, 992-998.

Chaiseri, S. and Dimick, P.S. (1989). Lipid and Hardners characteristics of Cocoa Butters from Different Geographic Regions. *Journal of the American Oil Chemists' Society*, ***66***, 1771-1780.

Chaiseri, S. and Dimick, P.S. (1995). Dynamic crystallization of cocoa butter.I.Characterization of simple lipids in rapid- and slow-nucleating cocoa butters and their seed crystals. *Journal of the American Oil Chemists' Society*, ***72***, 1491-1496.

Chang, M.K., Abraham, G., John, V.T. (1990). Production of Cocoa Butter-Like Fat from Interesterification of Vegetable Oils. *Journal of the American Oil Chemists' Society*, ***67***, 832-834.

Chang, P.S. and Rhee, J.S. (1990). Characterization of lipase-catalyzed glycerolysis of triglyceride in AOT-isooctane reversed micelles. *Biocatalysis*, ***3***, 343-355.

Chang, P.S., Rhee, J.S. and Kim, J-J. (1991). Continuous glycerolysis of olive oil by Chromobacterium viscoum lipase immobilized on liposome in reversed micelles. *Biotechnology and Bioengineering*, ***38***, 1159-1165.

Cheetham, P.S.J. (1988). *The application of immobilized enzymes and cells and biochemical reactors in biotechnology*. In: A. Wiseman (Ed.), Principles of enzyme engineering. Principles of Biotechnology, 2nd ed. New York: Chapman and Hall, pp. 164-201.

Choe, E. and Min, D.B. (2006). Mechanisms and Factors for Edible Oil Oxidation. *Comprehensive Review of Food Science and Safety*, ***5***, 1-18.

Codex Alimentarius commission. (1970). WHO/FAO, *Recommended international standards for olive oil, virgin and refined, and for refined olive residue oil*. CAC/RS-33.

Daulatabad, C. D. and Mirajkar, A. M. (1992). A new hydroxy fatty acid from Scindapsus officinalis seed oil. *International Journal of Food Science and Technology*, ***27***, 77-79.

Davis, T.R., and Dimick, P.S. (1989). Lipid Composition of High-Melting Seed Crystals Formed During Cocoa Butter Solidification. *Journal of the American Oil Chemists' Society*, ***66***, 1494-1498.

De Man, J.M. (1982). Microscopy in the study of fats and emulsions. *Food Microstructure*, ***1***, 209–222.

De Man, J.M. (1992). *Fats and oils: chemistry, physics and applications*, In: H.Y. Hui (Ed.), Encyclopedia of food science and technology. NewYork: Wiley, pp. 823-824.

De Man, J.M. and Beers, A.M. (1987). Fat crystal networks: Structure and rheological properties. *Journal of Texture Studies*, ***18***, 303-318.

Desnuelle, P. (1972). The Lipases. In: P.D. Boyer (Ed.), *The enzymes*, vol 7, 3rd edition New York: Academic Pres, pp. 575-616.

Dordick, J.S. (1989). Enzymatic catalysis in monophasic organic solvents. *Enzyme Microbiology and Technology*, ***11***, 194-211.

European Union (EU) Regulation. (1991). No. 2568. on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis. *Official Journal of the European Communities*, No. L 248.

European Union (EU) Regulation. (1995). No. 656 (Modification of No. 2568/91 Regulation). On the characteristics of olive oil and on the relevant methods of analysis. *Official Journal of the European Communities*, No. L 069.

Fadıloğlu, S., Çiftçi, O.N. and Göğüş, F. (2003). Reduction of free fatty acid content of olive-pomace oil by enzymatic glycerolysis. *Food Science and Technology International*, ***9***, 11-15.

Fedeli, E. (1977). *Lipids of olives in progress in the chemistry of fats and other lipids*. Oxford: Academic Press, pp. 57-74.

Ferreira-Dias, S. and da Fonseca, M.M.R. (1993). Enzymatic glycerolysis of olive oil: A reactional system with major analytical problems. *Biotechnology Techniques*, ***7***, 447-452.

Ferreira-Dias, S. and da Fonseca, M.M.R. (1995a). Production of monoglycerides by glycerolysis of olive oil with immobilized lipases: Effect of water activity. *Bioprocess Engineering*, ***12***, 327-337.

Ferreira-Dias, S. and da Fonseca, M.M.R. (1995b). Glycerolysis of olive oil: Batch operational stability of Candida rugosa lipase immobilized in hydrophilic polyurethane foams. *Bioprocess Engineering*, ***13***, 311-315.

Fomuso, L.B. and Akoh, C.C. (1998). Structured lipids: Lipase catalyzed interesterification of tricaproin and trilinolein. *Journal of the American Oil Chemists' Society*, ***75***, 405-410.

Fomuso, L.B. and Akoh, C.C. (2002). Lipase-catalyzed acidolysis of olive oil and caprylic acid in a bench-scale bed bioreactor. *Food Research International*, ***35***, 15-21.

Forssell, T.A., Kervinen, R., Lappi, M., Linko, P., Suortti, T. and Poutanen, K. (1992). Effect of enzymatic interesterification on the melting point of tallow rapeseed oil (LEAR) mixture. *Journal of the American Oil Chemists' Society*, ***69***, 126-129.

Gerson, T. and Escher, W.L. (1966). The effect of monoglycerides on the spreadability of butter. *New Zealand Journal of Science*, ***9***, 528-533.

Gitlesen, T., Svensson, I., Adlercreutz, P., Mattiason, B. and Nilsson, J. (1995). High-oleic acid rapeseed oil as starting material for the production of confectionery fats via lipase-catalyzed transesterification. *Industrial Crops and Products*, ***4***, 167-171.

Goderis, H.L., Ampe, G., Feyten, M.P., Fouwe, B.L., Guffens, W.M., Van Cauwenbergh, S.M. and Tobback, P.P. (1987). Lipase-catalyzed ester exchange reactions in organic media with controlled humidity. *Biotechnology and Bioengineering*, ***30***, 258-266.

Gomes, T. and Caponio, F. (1998). Evaluation of the state of oxidation of olive-pomace oils. Influence of the refining process. *Journal of Agricultural and Food Chemistry*, ***46***, 1137–1142.

Gomes, T. and Caponio, F. (2000). Possibility of improving the quality characteristics of olive-pomace oil and enhancing its differentiation from refined olive-pomace oil. *Journal of the Science of Food and Agriculture*, ***81***, 62-67.

Gunstone, F.D. (1994). Chemical properties. In: F.D. Gunstone, J.L. Harwood and F.D. Padley (Eds.), *The lipid handbook*, 2nd ed. London: Chapman and Hall, pp. 594-595.

Gunstone, F.D. (1999). Enzymes as biocatalysts in the modification of natural lipids. *Journal of the Science of Food and Agriculture*, ***79***, 1535-1549.

Gunstone, F.D. (2001). Why are structured lipids and new lipid sources required? In: F.D. Gunstone (Eds.), *Structured and Modified Lipids*. New York: Marcel Dekker Inc., pp. 1-422

Halim, S.F.A., Kamaruddin, A.H. and Fernando, W.J.N. (2009). Continuous biosynthesis of biodiesel from waste cooking palm oil in a packed bed reactor: Optimization using response surface methodology (RSM) and mass transfer studies. *Bioresource Technology*, ***100***, 710–716.

Hassan, M., Blanc, P. J., Granger, L. M., Pareilleux, A. and Goma, G. (1993). Lipid production by an unsaturated fatty acid auxotroph of the oleaginous yeast Apiotrichum curvatum grown in single- stage continuous culture. *Applied Microbiolology and Biotechnology*, ***40***, 483-488.

Hassan, M., Blanc, P. J., Pareilleu, A. and Goma, G. (1995). Production of cocoa butter equivalents from prickly-pear juice fermentation by an unsaturated fatty acid auxotroph of Cryptococcus curvatus grown in batch culture. *Process Biochemistry*, ***30***, 629-634.

Herrera, M.L. and Hartel, R.W. (2000). Effect of processing conditions on crystallization kinetics of a milk fat model system. *Journal of the American Oil Chemists' Society*, ***77***, 1177-1187.

Hoy, C.E. and Xu, X. (2001). *Structured Triacylglycerols*. In: F.D. Gunstone (Ed.), *Structured and Modified Lipids*. New York: Marcel Dekker Inc., pp. 209-239.

http://www.lsbu.ac.uk/biology/enztech/pbr.html

http://www.lsbu.ac.uk/biology/enztech/reactors.html

http://www.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/packbed.htm

ICCO Annual Report for 2006/2007. (2008). London: *International Cocoa Organization*.

International Olive Oil Council (IOOC). (1985). *International trade standard applying to olive oils and olive-residue oils*. COI/ T. 15/ No. 1., Madrid.

International Olive Oil Council (IOOC). (1989). *Olive pomace and olive-pomace oils*. CE/ R.31 /Doc. no 3.

International Olive Oil Council (IOOC). (1995). *Trade standard applying to olive oil and olive pomace oil*. COI/ T.15.NC no.2/ Rev.2.

International Olive Oil Council (IOOC). (1997). *Trade standard applying to olive oil and olive-pomace oil*. COI/ T. 15/ NC. No. 2 Rev.6., Madrid.

Ison, A.P., Macrae, A.R., Smith, C.G. and Bosley, J. (1994). Mass transfer effects in solvent-free fat interesterification reactions: Influence on catalyst design. *Biotechnology and Bioengineering*, ***43***, 122-130.

Ivanov, A.E. and Schneider, M.P. (1997). Methods for the immobilization of lipases and their use for ester synthesis. *Journal of Molecular Catalysis B: Enzymatic*, ***3***, 303-309.

Iwasaki, Y. and Yamane, T. (2000). Enzymatic synthesis of structured lipids. *Journal of Molecular Catalysis B: Enzymatic*, ***10***, 129-140.

Jaeger, K.E., Ransac, S., Dijkstra, B.W., Colson, C., van Heuvel, M. and Misset, O. (1994). Bacterial lipases. *FEMS Microbiology Reviews*, ***15***, 29-63.

Jeyarani, T. and Yella-Reddy, S. (1999). Heat-resistant cocoa butter extenders from mahua (Madhuca latifolia) and kokum (Garcinia indica) fats. *Journal of the American Oil Chemists' Society*, ***76***, 1431-1436.

Jeyarani, T. and Yella-Reddy, S. (2001). Cocoa butter extender from Simarouba glauca fat. *Journal of the American Oil Chemists' Society*, ***78***, 271-276.

Kakuda, Y., Jahaniaval, F., Marcone, M. F., Montevirgen, L., Montevirgen, Q. and Umali, J. (2000). Characterization of pili nut (Canarium ovatum) oil: fatty acid and triacylglycerol composition and physicochemical properties. *Journal of the American Oil Chemists' Society*, ***77***, 991-996.

Kanematsu, H., Marayana, T., Niiya, J., Imamura, M. and Matsumoto, T. (1978). Studies on the hard butter. I. On the components of cocoa butter. *Journal of Japanese Oil Chemists' Society*, ***27***, 179-183.

Kazlauskas, R.J. and Bornscheuer, U.T. (1998). *Biotransformation I. Biotechnology*, In: D.R. Kelly (Ed.), *Biotransformations with lipases*, Weinheim: Wiley-VCH.

Kennedy, J.P. (1991). Structured lipids: Fats of the future. *Food Technology*, ***45***, 76-83.

Khumalo, E.W., Majoko, L., Read, J.S. and Ncube, I. (2002). Characterization of some underutilized vegetable oils and their evaluation as starting materials for lipase-catalyzed production of cocoa butter equivalents. *Industrial Crops and Products*, ***16***, 237-244.

Kiritsakis, A. P. K., Lenart, E. B., Willet, W. C. and Hernandez, R. J. (1998). *Olive Oil from the tree to the table*. Connecticut, USA: Food and Nutrition Press Inc.

Kiritsakis, A.P.K. (1998). *Olive oil from the tree to the table*. USA: Food and Nutrition Press Inc.

Klagge, P. and Sen Gupta, A.K. (1990). Kakaobutter und ihre alternativen für schokoladenprodukte. *Fat Science and Technology*, ***92***, 485-492.

Kowalski, B., Gruczynska, E. and Maciaszek, K. (2000). Kinetics of rapeseed oil oxidation by pressure differential scanning calorimetry measurements. European *Journal of Lipid Science and Technology*, ***102***, 337-341.

Kuo, S.J. and Parkin, K.L. (1993). Substrate preferences for lipase mediated acyl-exchange reactions with butter oil are concentration-dependent. *Journal of the American Oil Chemists' Society*, ***70***, 393-399.

Laudani, C.G., Habulin, M., Knez, Z., Porta, C.D. and Reverchon, E. (2007). Immobilized lipase-mediated long-chain fatty acid esterification in dense carbon dioxide: bench-scale packed-bed reactor study. *The Journal of Supercritical Fluids*, ***41***, 74–81.

Lee, K.T. and Akoh, C.C. (1998a). Structured lipids: synthesis and applications. *Food Reviews International*, ***14***, 17-34.

Lee, K. T. and Akoh, C. (1998b). Characterization of enzymatically synthesized structured lipids containing eicosapentaenoic, docosahexaenoic, and caprylic Acids. *Journal of the American Oil Chemists' Society*, ***75***, 495–499.

Lipp, M. and Anklam, E. (1998). Review of cocoa butter and alternative fats for use in chocolate-Part A. Compositional data. *Food Che*mistry, ***62***, 73-97.

Lipp, M., Simoneau, C., Ulberth, F., Anklam, E., Crews, C., Prereton, P., Greyt, W., Schwack W. and Wiedmaier, C. (2001). Composition of genuine cocoa butter and cocoa butter equivalents. *Journal of Food Composition and Analysis*, ***14***, 399-408.

Liu, K.J. and Shaw, J.F. (1997). Synthesis of cocoa butter equivalent by lipase-catalyzed interesterification in supercritical carbon dioxide. *Journal of the American Oil Chemists' Society*, ***74***, 1477-1482.

Liu, K.J., Chang, H.M. and Liu, K.M. (2007). Enzymatic synthesis of cocoa butter analog through interesterification of lard and tristearin in supercritical carbon dioxide by lipase. *Food Chemistry*, ***100***, 1303-1311.

Macrae, A.R. (1983). Lipase-catalyzed interesterification of oils and fats. *Journal of the American Oil Chemists' Society*, ***60***, 291-294.

Macrae, A.R. (1985). *Interesterification of fats and oils*. In: J. Tramper, H.C. van der Plas, and P. Linko (Eds.), Biocatalysts in organic synthesis. Proceedings of an International Symposium held at Noorwijkerhout, The Netherlands (14-17 April 1985), Amsterdam: Elsevier Science Publishers, pp. 195-208.

Macrae, A.R. (1992). *Modifying oils-enzymatic methods*. In: U.K.S. Shukla and F.D. Gunstone (Eds.), *Proceeding of oils and fats in the nineties*. Frederica, Denmark: International Food Science Center A/S, pp. 199-208.

Macrae, A.R. and Hammond, R.C. (1985). Present and Future Application of Lipases. *Biotechnology and Genetic Engineering Reviews*, ***3***, 193-217.

Macrae, A.R. and How, P. (1988). Rearrangement process. U.S. patent no. 4,719,178.

Malcata, F.X., Reyes, H.R., Garcia, H.S., Hill, C.G.Jr. and Amundson, C.H.Jr. (1990). Immobilized lipase reactors for modification of fats and oils – a review. *Journal of American Oil Chemists' Society*, ***67***, 890-909.

Malcata, F.X., Reyes, H.R., Garcia, H.S., Hill, Jr. C.G. and Amundson, C.H. (1992). Kinetics and mechanisms catalyzed by immobilized lipases. *Enzyme Microbiology and Technology*, ***14***, 426-446.

Marangoni, A.G. and Hartel, R.W. (1998). Visualization and Structural Analysis of Fat Crystal Networks. *Food Technology*, ***52***, 46–51.

Martini, S., Award ,T. and Marangoni, A.G. (2006). *Structure and properties of fat crystal Networks*. In: F.D. Gunstone (Ed.), *Modifying lipids for use in food*. Boca Raton, FL: CRC Pres, pp. 142-169.

Matissek, R. (2000). Pflanzenfett in Schokolade – Rechtliche und analytische Aspekte. *Lebensmittelchemie*, ***54***, 25-30.

McNeill, G.P., Shimizu, S. and Yamane, T. (1991). High-yield enzymatic glycerolysis of fats and oils. *Journal of American Oil Chemists' Society*, ***68***, 1-5.

Meffert, A. (1984). Technical uses of fatty acid esters. *Journal of American Oil Chemists' Society*, ***61***, 255-258.

Meursing, E.H. (1994). *Cocoa mass, cocoa butter, cocoa powder*. In: S.T. Beckett (Ed.), *Industrial chocolate manufacture and use*. Glasgow: Chapman & Hall, pp. 70-82.

Mojovic, L., Siler-Marinkovic, S., Kukic, G. and Vunjak-Novakovic G. (1993). Rhizopus arrhizus lipase-catalyzed interesterification of the midfraction of palm oil to a cocoa butter equivalent fat. *Enzyme Microbiology and Technology*, ***15***, 438-443.

Narine, S.S. and Marangoni, A.G. (1999). The Difference Between Cocoa Butter and Salatrim Lies in the Microstructure of the Fat Crystal Network. *Journal of the American Oil Chemists' Society*, ***76***, 7-13.

Nesaretnam, K. and Razak bin Modh Ali A. (1992). Engkabang (illipe) – an excellent component for cocoa butter equivalent fat. *Journal of the Science of Food Agriculture*, ***60***, 15-20.

Noor Lida, H.M.D., Sundram, K., and Idris, N.A. (2006). DSC Study on the Melting Properties of Palm Oil, Sunflower Oil, and Palm Kernel Olein Blends Before and After Chemical Interesterification. *Journal of the American Oil Chemists' Society*, ***83***, 739-745.

NorAini, I., Embong, M.S., Aminah, A., Ali, A.R. and Che, Maimon, C.H. (1995). Physical Characteristics of Some Shortenings Based on Modified Palm Oil, Milkfat and Low Melting Milkfat Fractions. *Fat Science and Technology*, ***97***, 253–260.

Norberg, S. (2006). *Chocolate and confectionery fats*. In: F.D. Gunstone (Ed.), *Modifying lipids for use in food*. Boca Raton, FL:CRC Press, pp. 488-516.

Official Journal of European Communities (39). (1992). *Regulation CEE* 356/ 92.

Olson, G.J., Woese, C.R. and Overbeek, R. (1994). The winds of (evolutionary) change: Breathing new life into microbiology. *Journal of Bacteriology*, ***176***, 1-6.

Osborn, H.T. and Akoh, C.C. (2002a). Structured lipids-novel fats with medical, nutraceutical, and food applications. *Comprehensive Reviews in Food Science and Food Safety*, ***1***, 93-103.

Osborn, H.T. and Akoh, C.C. (2002b). Enzymatically modified beef tallow as a substitute for cocoa butter. *Journal of Food Science*, ***67***, 2480-2485.

Padley, F.B., Paulussen, C.N., Soeters, C. and Tresser, D. (1981). *Chocolate having defined hard fat* - US Patent 4276322.

Paez, B.C., Medina, A.R., Rubio, F.C., Cerdan, L.E. and Grima, E.M. (2003). Kinetics of lipase-catalyzed interesterification of triolein and caprylic acid to produce structured lipids. *Journal of Chemical Technology and Biotechnology*, ***78***, 461-470.

Paez, B.C., Medina, A.R., Rubio, F.C., Moreno, P.G., Grima, E.M. (2002). Production of structured triglycerides rich in n-3 polyunsaturated fatty acids by the acidolysis of cod liver oil and caprylic acid in packed-bed reactor: equilibrium and kinetics. *Chemical Engineering Science*, ***57***, 1237-1249.

Paiva, A. L., Balcao, V. B., Malcata, X. (2000). Kinetics and mechanisms reactions catalyzed by immobilized lipases. *Enzyme Microbiology and Technology*, ***27***, 187-204.

Podlaha, O., Toregard, B. and Puschl, B. (1984). TG-type composition of 28 cocoa butters and correlation between some of the TG-type components. *Lebensmittel-Wissenschaft und-Technologie,* ***17***, 77-82.

Polish Standard PN-ISO 8420. (1995). *Animal and vegetable fats and oils - Determination of polar compounds content*.

Pontillon, J. (1998). *Le beurre de cacao et les matières grasses en chocolaterie*. In: J. Pontillon (ed.), *Cacao et chocolat: production, utilization, caractéristiques*. Paris: Lavoisier. pp. 326–93.

Posorske, L.H., Lefebvre, G.K., Miller, C.A., Hansen, T.T. and Glenvig, B.L. (1988). Process consideration of continuous fat modification with immobilized lipase. *Journal of the American Oil Chemists' Society*, ***65***, 922-926.

Prazeres, D. M. F. and Cabral, J. M. S. (1994). Enzymatic membrane bioreactors and their applications. *Enzyme Microbiology and Technology*, ***16***, 738-751.

Queiroz, M. B. and Garcia, N. H. P. (1999). Evaluation of the roasting of cupuasu beans (Theobroma grandiflorum). *Brazilian Journal Food Technology*, ***2***, 167-173.

Reyes, H.R. and Hill, Jr. C.G. (1994). Kinetic modelling of interesterification reactions catalyzed by immobilized lipase. *Biotechnology and Bioengineering*, ***43***, 171-182.

Rodionova, M.V., Belova, A.B., Mozhaev, V.V., Martinek, K. and Berezin, I.V. (1987). Mechanism of denaturation of enzymes by organic solvents. *Chemical Abstracts*, ***106***, 18-23.

Rosu, R., Uozaki, Y., Iwasaki, Y. and Yamane, T. (1997). Repeated use of immobilized lipase for monoacylglycerol production by solid-phase glycerolysis of olive oil. *Journal of American Oil Chemists' Society*, ***74***, 445-450.

Rousseau, D. and Marangoni, A. G. (1998). Tailoring the textural attributes of butterfat/canola oil blends via Rhizopus arrhizus lipase-catalyzed interesterification. 1. Compositional modifications. *Journal of Agricultural and Food Chemistry*, ***46***, 2368–2374.

Rousseau, D. and Marangoni, A. (2002). *The Effects of Interesterification on the Physical Properties of Fats*. In: A.G. Marangoni and S.S. Narine (Eds.), *Physical properties of lipids*. New York: Marcel Dekker Inc.

Rousseau, D., Forestiere, K., Hill, A.R. and Marangoni, A.G. (1996). Restructuring butterfat through blending and chemical interesterification. 1.Melting behavior and triacylglicerol modifications. *Journal of the American Oil Chemists' Society*, ***73***, 963-972.

Roux, M. P., Kock, J. L. F., Botha, A., Preez, J. C., Wells, G. V., Botes, P. J. (1994). Mucor – a source of cocoa butter and gammalinolenic acid. *World Journal of Microbiology and Biotechnology*, ***10***, 417-422.

Saldana, M.D.A., Mohamed, R.S. and Mazzafera, P. (2002). Extraction of cocoa butter from Brazilian cocoa beans using supercritical CO2 and ethane. *Fluid Phase Equilibria*, ***194-197***, 885-894.

Sambuc, E., Dirik, Z., Reymond, G. and Naudet, M. (1980). Etude de la cristallisation de corps gras plastiques. VI. Influence des glycérides partiels et des phosphatides en absence et présence d' eau. A. Cas des monoglycérides stéaropalmitiques. *Revue Française des Corps Gras*, ***27***, 505-512.

Savage, C.M. and Dimick, P.S. (1995). Influence of Phospholipids During Crystallization of Hard and Soft Cocoa Butters. *Manufacturing Confectioner*, ***75***, 127-132.

Schlichter-Aronhime, J. and Garti, N. (1988). *Solidification and polymorphism in cocoa butter and the blooming problems*. In: N. Garti, K. Sato (Ed.), *Crystallization and polymorphism of fats and fatty acids*. New York: Marcel Dekker Inc., pp. 363–93.

Schmid, U., Bornscheuer, U.T., Soumanou, M.M., McNeill, G.P. and Schmid, R.D. (1998). Optimization of the reaction conditions in the lipase-catalyzed synthesis of structured triglycerides. *Journal of American Oil Chemists' Society*, ***75***, 1527-1531.

Sellappan, S. and Akoh, C.C. (2000). Enzymatic acidolysis of tristearin with lauric and oleic acids to produce coating lipids. *Journal of the American Oil Chemists' Society*, ***77***, 1127-11132.

Shimada, Y., Sugihara, A., Maruyama, K., Nagao, T., Nakayamo, S., Nakano, H., Tominaga, Y. (1996). Production of structured lipids containing essential fatty acids by immobilized Rhizopus delemar lipase. *Journal of the American Oil Chemists' Society*, ***73***, 1415-1420.

Shukla, V.K.S. (1995). Cocoa Butter Properties and Quality. *Lipid Technology*, ***5***, 54–57.

Shukla, V.K.S. (1997). Chocolate – the chemistry of pleasure. INFORM: *International News on Fats, Oils and Related Materials*, ***8***, 152–162.

Siew, W.L. and Faridah, M.D.J. (2000). Compositional and Differential Scanning Calorimetry (DSC) Studies of Crystals of Palm Olein. *Journal of Oil Palm Research*, ***12***, 1-13.

Siew, W.L. and Ng, W.L. (1996). Effect of diacylglycerols on the crystallization of palm oleins. *Journal of the Science of Food and Agriculture*, ***71***, 496-500.

Small, DM. (1986). *Glycerides*. In: D.J. Hanahan (Ed.), *The Physical Chemistry of Lipids, from Alkanes to Phospholipids*, Vol 4, New York: Plenum Press, pp. 475–522.

Smith, K.W. (2001). *Cocoa butter and cocoa butter equivalents*. In: F.D. Gunstone (Eds), Structured and Modified Lipids. New York: Marcel Dekker Inc., pp. 401-422 (2001).

Solis-Fuentes, J.A. and Duran-de-Bazua, M.C. (2004). Mango seed uses: thermal behaviour of mango seed almond fat and its mixtures with cocoa butter. *Bioresource Technology*, ***92***, 71-78.

Soumanou, M.M., Bornscheuer, U.T. and Schmid, R.D. (1998). Two-step enzymatic reaction for the synthesis of pure structured triacylglycerides. *Journal of American Oil Chemists' Society*, ***75***, 703-710.

SPSS 10.0 for Windows. (1999). *Statistical Software*, SPSS Inc., Chicago, IL.

Sridhar, R., Lakshminarayana, G. and Kaimal, T. (1991). Modification of selected Indian vegetable fats into cocoa butter substitutes by lipase-catalyzed ester interchange. *Journal of the American Oil Chemists' Society*, ***68***, 726-730.

Stamatis, H., Xenakis, A., Provelegiou, M. and Kolisi, F.N. (1993). Esterification reactions catalyzed by lipases in microemulsions: The role of enzyme location in relation to its selectivity. *Biotechnology and Bioengineering*, ***42***, 103-110.

Stevenson, D.E., Stanley, R.A. and Furneaux, R.H. (1993). Glycerolysis of tallow with immobilized lipase. Biotechnology Letters, ***15***, 1043-1048.

Takagi, T. and Ando, Y. (1995). Stereospecific analysis of monounsaturated triacylglycerols in cocoa butter. *Journal of the American Oil Chemists' Society*, ***72***, 1203-1206.

Talbot, G. (1999). *Vegetable fats*. In: S.T. Beckett (Ed.), *Industrial chocolate manufacture and use*. Blackwell Science, pp. 307-322.

Talbot, G., Smith, K. and Cain, F. (2005). Crystallization of 1,3-dipalmitoyl-2-oleoyl-glycerol (POP) studied using non-isothermal DSC. *Lipid Technology*, ***17***, 247-250.

Tan, C.P., Che Man, Y.B., Jinap, S. and Yusoff, M.S.A. (2002). Comparative studies of oxidative stability of edible oils by differential scanning calorimetry and oxidative stability index methods. *Food Chemistry*, ***76***, 385-389.

Tepe, O. and Dursun, A.Y. (2008). Combined effects of external mass transfer and biodegradation rates on removal of phenol by immobilized Ralstonia eutropha in a packed bed reactor. *Journal of Hazardous Materials*, ***151***, 9–16.

Thurgood, J., Ward, R. and Martini, S. (2007). Oxidation kinetics of soybean oil/anhydrous milk fat blends: A differential scanning calorimetry study. *Food Research International*, ***40***, 1030-1037.

Tieko, N. R. and Aparecida, L. (1995). Solid fat content determination by differential scanning calorimetry. *Grasas y Aceites*, ***46***, 337-343.

Timms, R.E. and Stewart, I.M. (1999). Cocoa butter, a unique vegetable fat. *Lipid Technology Newsletter*, ***10***, 101–107.

Ulberth, F. and Buchgraber, M. (2003). Analytical platforms to assess the authenticity of cocoa butter . *European Journal of the Lipid Science and Technology*, ***105***, 32-42.

Undurraga, D., Markovits, A. and Erazo, S. (2001). Cocoa butter equivalent through enzymic interesterification of palm oil midfraction. *Process Biochemistry*, ***36***, 933-939.

Uzawa, H., Noguchi, T., Nishida, Y., Ohrui, H. and Meguro, H. (1993). Determination of the lipase stereoselectivities using circular dichroism (DC); lipases produce chiral di-O-acylglycerols from tri-O-acylglycerols. *Biochimica Et Biophysica Acta*, ***1168***, 253-260.

Van der Padt, A., Edema, M.J., Sewalt, J.J.W. and van't Riet, K. (1990). Enzymatic acylglycerol synthesis in a membrane bioreactor. *Journal of the American Oil Chemists' Society*, ***67***, 347-352.

Van Malssen, K.F., Peschar, R. and Schenk, H. (1996). Real-time X-ray powder diffraction investigations on cocoa butter. II The relationship between melting behavior and composition of β-cocoa butter. *Journal of the American Oil Chemists' Society*, ***73***, 1217–1223.

Villeneuve, P., Muderhwa, J.M., Graille, J. and Haas, M.J. (2000). Customizing lipases for biocatalysis: A survey of chemical, physical and molecular biological approaches. *Journal of Molecular Catalysis B: Enzymatic*, ***9***, 113-148.

Vulfson, E.N. (1994). *Industrial applications of lipases*. In: P. Woolley and S.B. Petersen (Eds.), *Lipases*. Cambridge: Camridge University Pres, pp. 271.

Wang, H.X., Wu, H., Ho, C.T. and Weng, X.C. (2006). Cocoa butter equivalent from enzymatic interesterification of tea seed oil and fatty acid metyl esters. *Food Chemistry*, ***97***, 661-665.

Weete, J.D. *Microbial lipases*. (1998). In: C.C. Akoh and D.B. Min (Eds.), *Food Lipids Chemistry, nutrition, and biotechnology*. New York: Marcel Dekker, pp. 813-838.

Willis, M.W. and Marangoni, A.G. (2002). *Enzymatic interesterification*. In: C.C. Akoh and D.B. Min (Eds.), *Food Lipids Chemistry, nutrition, and biotechnology*. New York: Marcel Dekker, pp. 839-875.

Wisdom, R.A., Dunnil, P. and Lilly, M.D. (1987). Enzymic interesterification of. fats: laboratory and pilot-scale studies with immobilized lipase from Rhizophus arrhizus. *Biotechnology and Bioengineering*, ***29***, 1081-1085.

Xu, X. (2000a). *Enzyme bioreactors for lipid modifications*. INFORM, ***11***, 1004-1012.

Xu, X. (2000b). Production of specific structured triacylglycerols by lipase-catalyzed reactions: a review. *European Journal of Lipid Science and Technology*, ***102***, 287-303.

Xu, X. (2003). Engineering of enzymatic reactions and reactors for lipid modification and synthesis. *European Journal of Lipid Science and Technology*, ***105***, 289-304.

Xu, X., Balchen, S., Hoy, C.E., Adler-Nissen, J. (1998). Production of specific-structured lipids by enzymatic interesterification in a pilot continuous enzyme bed reactor. *Journal of the American Oil Chemists' Society*, ***75***, 1573-1579.

Xu, X., Guo, Z., Zhang, H., Vikbjerg, F. and Damstrup, M.L. (2006). *Chemical and enzymatic interesterification of lipids for use in food*. In: F.D. Gunstone (Ed.), *Modifying lipids for use in food*. Boca Raton, FL: CRC Press, pp. 235-272.

Xu, X., Hu, X. and Zhang, G. (1994). *Cocoa butter equivalents from teaseed oil by lipase-catalyzed modification*. In: Proceedings of International Symposium on New Approaches in the Production of Food Stuffs. Beijing: CCOA, pp. 808-811.

Xu, X., Hu, X., Balchen, S., Zhang, G. and Adler-Nissen, J. (1997). *Pilot batch production of cocoa butter-like fats from Chinese vegetable tallow by enzymatic interesterification*. In: Proceeding of International Symposium on the Approaches to Functional Cereals and Oils. Beijing: CCOA, pp. 104-112.

Xu, X., Mu, H., Hoy, C-E. and Adler-Nissen, J. (1999). Production of specifically structured lipids by enzymatic interesterification in a pilot enzyme bed reactor: Process optimization by responce surface methodology. *Fett Lipid*, ***101***, 207-214.

Xu, X., Porsgaard, T., Zhang, H., Adler-Nissen, J. and Hoy, C,E. (2002). Production of structured lipids in a packed-bed reactor with Thermomyces lanuginosa lipase. Journal of the American Oil Chemists' Society, ***79***, 561-565.

Yahya, A.R.M., Anderson, W.A. and Moo-Young, M. (1998). Ester synthesis in lipase-catalyzed reactions. *Enzyme and Microbial Technology*, ***23***, 438-450.

Yamane, T. (1987). Enzyme technology fort he lipids industry: An engineering overview. *Journal of the American Oil Chemists' Society*, ***4***, 1657-1662.

Yang, T., Fruekilde, M.B., Xu, X. (2005). Supression of acyl migration in enzymatic production of structured lipids through temperature programming. *Food Chemistry*, ***92***, 101-107.

Yankah, V.V. and Akoh, C.C. (2000). Lipase-catalyzed acidolysis of tristearin with oleic or caprylic acids to produce structured lipids. *Journal of the American Oil Chemists' Society*, ***74***, 495-500.

Yella-Reddy, S. and Prabhakar, J. V. (1994). Confectionery fat from phulwara (Madhuca butyracea) butter. *Fat Science and Technology*, ***96***, 387-390.

Yuping, S., Baomin, L. and Richard, W.H. (2005). Crystal Morphology, Microstructure, and Textural Properties of Model Lipid Systems. *Journal of the American Oil Chemists' Society*, ***82***, 399-408.

Zaks, A. and Klibanov, A. (1984). Enzymatic catalysis in organic media at 100 degrees C. *Science*, ***224***, 1249-1251.

Zhao, H., Lu, Z., Bie, X., Lu, F. and Liu, Z. (2005). Lipase catalyzed acidolysis of lard with capric acid in organic solvent. *Journal of Food Engineering*, ***78***, 41-46.

Ziegleder, G. (1988). Kristallisation von Kakaobutter unter statischen und dynamischen Bedingungen (DSC, Rheometer). *Suesswaren*, ***12***, 487-493.

Ziegleder, G. (1990). Thermoanalyse und kinetik der kristallisation von kakaobutter. *Fat Science and Technology*, ***92***, 481-485.

Zogzas, N.P., Maroulis, Z.B. and Marinos-Kouris, D. (1994). Densities, shrinkage and porosity of some vegetables during air drying. *Drying Technology*, ***12***, 1653–1666.

APPENDIX A

Table A.1 Statitical analysis of the effect of reaction parameters on the TAG content in model sytem acidolysis reactions

Dependent Variable: TAG content (%)						
	Reaction temperature		Mean Difference	Sig.	95% Confidence Interval	
	(I) VAR00002	(J) VAR00002	(I-J)		Upper Bound	Lower Bound
LSD	40℃	45℃	-.1833	.796	-1.6915	1.3249
		50℃	.4167	.558	-1.0915	1.9249
		60℃	4.1000(*)	.000	2.5918	5.6082
	45℃	40℃	.1833	.796	-1.3249	1.6915
		50℃	.6000	.403	-.9082	2.1082
		60℃	4.2833(*)	.000	2.7751	5.7915
	50℃	40℃	-.4167	.558	-1.9249	1.0915
		45℃	-.6000	.403	-2.1082	.9082
		60℃	3.6833(*)	.000	2.1751	5.1915
	60℃	40℃	-4.1000(*)	.000	-5.6082	-2.5918
		45℃	-4.2833(*)	.000	-5.7915	-2.7751
		50℃	-3.6833(*)	.000	-5.1915	-2.1751
Dependent Variable: TAG content (%)						
	Enzyme load		Mean Difference	Sig.	95% Confidence Interval	
	(I) VAR00002	(J) VAR00002	(I-J)		Upper Bound	Lower Bound
LSD	20	25	-.5833	.658	-.7373	-.4294
		30	-.9333	.785	-1.0873	-.7794
	25	20	.5833	.652	.4294	.7373
		30	-.3500	.585	-.5039	-.1961
	30	20	.9333	.725	.7794	1.0873
		25	.3500	.654	.1961	.5039
Dependent Variable: TAG content (%)						
	Water content		Mean Difference	Sig.	95% Confidence Interval	
	(I) VAR00002	(J) VAR00002	(I-J)		Upper Bound	Lower Bound
LSD	0%	1%	-.0667	.193	-.1698	.0365
		10%	3.3500(*)	.000	3.2468	3.4532
		20%	6.0000(*)	.000	5.8968	6.1032
		30%	6.7667(*)	.000	6.6635	6.8698
		40%	6.5167(*)	.000	6.4135	6.6198
		5%	-4.1333(*)	.000	-4.2365	-4.0302
	1%	0%	.0667	.193	-.0365	.1698
		10%	3.4167(*)	.000	3.3135	3.5198
		20%	6.0667(*)	.000	5.9635	6.1698
		30%	6.8333(*)	.000	6.7302	6.9365
		40%	6.5833(*)	.000	6.4802	6.6865
		5%	-4.0667(*)	.000	-4.1698	-3.9635
	10	0%	-3.3500(*)	.000	-3.4532	-3.2468
		1%	-3.4167(*)	.000	-3.5198	-3.3135
		20%	2.6500(*)	.000	2.5468	2.7532
		30%	3.4167(*)	.000	3.3135	3.5198
		40%	3.1667(*)	.000	3.0635	3.2698
		5%	-7.4833(*)	.000	-7.5865	-7.3802
	20	0%	-6.0000(*)	.000	-6.1032	-5.8968
		1%	-6.0667(*)	.000	-6.1698	-5.9635
		10%	-2.6500(*)	.000	-2.7532	-2.5468
		30%	.7667(*)	.000	.6635	.8698
		40%	.5167(*)	.000	.4135	.6198
		5%	-10.1333(*)	.000	-10.2365	-10.0302
	30	0%	-6.7667(*)	.000	-6.8698	-6.6635
		1%	-6.8333(*)	.000	-6.9365	-6.7302
		10%	-3.4167(*)	.000	-3.5198	-3.3135
		20%	-.7667(*)	.000	-.8698	-.6635
		40%	-.2500(*)	.000	-.3532	-.1468
		5%	-10.9000(*)	.000	-11.0032	-10.7968
	40	0%	-6.5167(*)	.000	-6.6198	-6.4135
		1%	-6.5833(*)	.000	-6.6865	-6.4802

Table A.1 continued

	10%	-3.1667(*)	.000	-3.2698	-3.0635
	20%	-.5167(*)	.000	-.6198	-.4135
	30%	.2500(*)	.000	.1468	.3532
	5%	-10.6500(*)	.000	-10.7532	-10.5468
5%	0%	4.1333(*)	.000	4.0302	4.2365
	1%	4.0667(*)	.000	3.9635	4.1698
	10%	7.4833(*)	.000	7.3802	7.5865
	20%	10.1333(*)	.000	10.0302	10.2365
	30%	10.9000(*)	.000	10.7968	11.0032
	40%	10.6500(*)	.000	10.5468	10.7532

Table A.2 Statistical analysis of the effcet of enzyme load on the reaction time

Dependent Variable: Reaction time					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	196.000(a)	1	196.000	392.000	.003
Intercept	1225.000	1	1225.000	2450.000	.000
Enzyme load	196.000	1	196.000	392.000	.003
Error	1.000	2	.500		
Total	1422.000	4			
Corrected Total	197.000	3			

a R Squared = .995 (Adjusted R Squared = .992)

Table A.3 Statistical analysis of the effect of substrate mole ratio on the TAG content in the batch system acidolysis reactions

Dependent Variable: TAG content (%)						
	Substrate mole ratio		Mean	Sig.	95% Confidence Interval	
	(I) VAR00002	(J) VAR00002	Difference (I-J)		Upper Bound	Lower Bound
LSD	1:1:3	1:2:6	-4.7983(*)	.000	-6.8059	-2.7908
		1:3:9	-6.4650(*)	.000	-8.4726	-4.4574
		1:4:12	-7.1817(*)	.000	-9.1892	-5.1741
		CB	-11.4983(*)	.000	-13.5059	-9.4908
		ROPO	10.5850(*)	.000	8.5774	12.5926
	1:2:6	1:1:3	4.7983(*)	.000	2.7908	6.8059
		1:3:9	-1.6667	.098	-3.6742	.3409
		1:4:12	-2.3833	.065	-4.3909	-.3758
		CB	-6.7000(*)	.000	-8.7076	-4.6924
		ROPO	15.3833(*)	.000	13.3758	17.3909
	1:3:9	1:1:3	6.4650(*)	.000	4.4574	8.4726
		1:2:6	1.6667	.098	-.3409	3.6742
		1:4:12	-.7167	.463	-2.7242	1.2909
		CB	-5.0333(*)	.000	-7.0409	-3.0258
		ROPO	17.0500(*)	.000	15.0424	19.0576
	1:4:12	1:1:3	7.1817(*)	.000	5.1741	9.1892
		1:2:6	2.3833	.051	.3758	4.3909
		1:3:9	.7167	.563	-1.2909	2.7242
		CB	-4.3167(*)	.000	-6.3242	-2.3091
		ROPO	17.7667(*)	.000	15.7591	19.7742
	CB	1:1:3	11.4983(*)	.000	9.4908	13.5059
		1:2:6	6.7000(*)	.000	4.6924	8.7076
		1:3:9	5.0333(*)	.000	3.0258	7.0409
		1:4:12	4.3167(*)	.000	2.3091	6.3242
		ROPO	22.0833(*)	.000	20.0758	24.0909
	ROPO	1:1:3	-10.5850(*)	.000	-12.5926	-8.5774
		1:2:6	-15.3833(*)	.000	-17.3909	-13.3758

Table A.3 continued

	1:3:9	-17.0500(*)	.000	-19.0576	-15.0424
	1:4:12	-17.7667(*)	.000	-19.7742	-15.7591
	CB	-22.0833(*)	.000	-24.0909	-20.0758

Table A.4 Statistical analysis of the effect of substrate mole ratio on the TAG content in the continuous system acidolysis reactions at 13th h

Dependent Variable: TAG content (%)						
	Substrate mole ratio		Mean	Sig.	95% Confidence Interval	
	(I) VAR00002	(J) VAR00002	Difference (I-J)		Upper Bound	Lower Bound
LSD	1:1:1	1:1:3	-1.9233(*)	.000	-2.4476	-1.3991
		1:2:6	-4.6167(*)	.000	-5.1409	-4.0924
		1:3:3	-2.9300(*)	.000	-3.4542	-2.4058
	1:1:3	1:1:1	1.9233(*)	.000	1.3991	2.4476
		1:2:6	-2.6933(*)	.000	-3.2176	-2.1691
		1:3:3	-1.0067(*)	.001	-1.5309	-.4824
	1:2:6	1:1:1	4.6167(*)	.000	4.0924	5.1409
		1:1:3	2.6933(*)	.000	2.1691	3.2176
		1:3:3	1.6867(*)	.000	1.1624	2.2109
	1:3:3	1:1:1	2.9300(*)	.000	2.4058	3.4542
		1:1:3	1.0067(*)	.001	.4824	1.5309
		1:2:6	-1.6867(*)	.000	-2.2109	-1.1624
Dependent Variable: TAG content (%)						
	Reaction time		Mean	Sig.	95% Confidence Interval	
	(I) VAR00002	(J) VAR00002	Difference (I-J)		Upper Bound	Lower Bound
LSD	0 h	10 h	-12.3354(*)	.000	-14.1595	-10.5114
		13 h	-12.1121(*)	.000	-13.9361	-10.2880
		2 h	-9.3117(*)	.000	-11.1357	-7.4876
		4 h	-11.5267(*)	.000	-13.3507	-9.7026
		6 h	-12.2633(*)	.000	-14.0874	-10.4393
		8 h	-12.4892(*)	.000	-14.3132	-10.6651
	10 h	0 h	12.3354(*)	.000	10.5114	14.1595
		13 h	.2233	.809	-1.6007	2.0474
		2 h	3.0238(*)	.001	1.1997	4.8478
		4 h	.8088	.382	-1.0153	2.6328
		6 h	.0721	.938	-1.7520	1.8961
		8 h	-.1537	.868	-1.9778	1.6703
	13 h	0 h	12.1121(*)	.000	10.2880	13.9361
		10 h	-.2233	.809	-2.0474	1.6007
		2 h	2.8004(*)	.003	.9764	4.6245
		4 h	.5854	.527	-1.2386	2.4095
		6 h	-.1512	.870	-1.9753	1.6728
		8 h	-.3771	.683	-2.2011	1.4470
	2 h	0 h	9.3117(*)	.000	7.4876	11.1357
		10 h	-3.0238(*)	.001	-4.8478	-1.1997
		13 h	-2.8004(*)	.003	-4.6245	-.9764
		4 h	-2.2150(*)	.018	-4.0391	-.3909
		6 h	-2.9517(*)	.002	-4.7757	-1.1276
		8 h	-3.1775(*)	.001	-5.0016	-1.3534
	4 h	0 h	11.5267(*)	.000	9.7026	13.3507
		10 h	-.8088	.382	-2.6328	1.0153
		13 h	-.5854	.527	-2.4095	1.2386
		2 h	2.2150(*)	.018	.3909	4.0391
		6 h	-.7367	.426	-2.5607	1.0874
		8 h	-.9625	.299	-2.7866	.8616
	6 h	0 h	12.2633(*)	.000	10.4393	14.0874
		10 h	-.0721	.938	-1.8961	1.7520
		13 h	.1512	.870	-1.6728	1.9753
		2 h	2.9517(*)	.002	1.1276	4.7757
		4 h	.7367	.426	-1.0874	2.5607
		8 h	-.2258	.807	-2.0499	1.5982
	8 h	0 h	12.4892(*)	.000	10.6651	14.3132

Table A.4 continued

		10 h	.1537	.868	-1.6703	1.9778
		13 h	.3771	.683	-1.4470	2.2011
		2 h	3.1775(*)	.001	1.3534	5.0016
		4 h	.9625	.299	-.8616	2.7866
		6 h	.2258	.807	-1.5982	2.0499

Dependent Variable: TAG content (%)

	Flow rate (I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Sig.	95% Confidence Interval Upper Bound	Lower Bound
LSD	1.5 mL/m	15 mL/mi	1.4386(*)	.041	.0597	2.8174
		4.5 mL/m	-1.3900(*)	.048	-2.7689	-.0111
		7.5 mL/m	-2.2590(*)	.001	-3.6379	-.8802
	15 mL/mi	1.5 mL/m	-1.4386(*)	.041	-2.8174	-.0597
		4.5 mL/m	-2.8286(*)	.000	-4.2074	-1.4497
		7.5 mL/m	-3.6976(*)	.000	-5.0765	-2.3188
	4.5 mL/m	1.5 mL/m	1.3900(*)	.048	.0111	2.7689
		15 mL/mi	2.8286(*)	.000	1.4497	4.2074
		7.5 mL/m	-.8690	.215	-2.2479	.5098
	7.5 mL/m	1.5 mL/m	2.2590(*)	.001	.8802	3.6379
		15 mL/mi	3.6976(*)	.000	2.3188	5.0765
		4.5 mL/m	.8690	.215	-.5098	2.2479

Dependent Variable: Degree of reaction

	Enzyme resuse (I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Sig.	95% Confidence Interval Upper Bound	Lower Bound
LSD	1.0000	10.0000	.95	.727	-4.81	6.71
		15.0000	1.30	.634	-4.46	7.06
		20.0000	-.70	.797	-6.46	5.06
		25.0000	.70	.797	-5.06	6.46
		30.0000	1.45	.596	-4.31	7.21
		35.0000	.75	.783	-5.01	6.51
		40.0000	1.50	.584	-4.26	7.26
		45.0000	4.53	.072	-.47	9.52
		5.0000	-.40	.883	-6.16	5.36
		50.0000	7.00	.521	1.24	12.76
	10.0000	1.0000	-.95	.727	-6.71	4.81
		15.0000	.35	.898	-5.41	6.11
		20.0000	-1.65	.547	-7.41	4.11
		25.0000	-.25	.927	-6.01	5.51
		30.0000	.50	.854	-5.26	6.26
		35.0000	-.20	.941	-5.96	5.56
		40.0000	.55	.840	-5.21	6.31
		45.0000	3.58	.146	-1.42	8.57
		5.0000	-1.35	.621	-7.11	4.41
		50.0000	6.05	.541	.29	11.81
	15.0000	1.0000	-1.30	.634	-7.06	4.46
		10.0000	-.35	.898	-6.11	5.41
		20.0000	-2.00	.467	-7.76	3.76
		25.0000	-.60	.826	-6.36	5.16
		30.0000	.15	.956	-5.61	5.91
		35.0000	-.55	.840	-6.31	5.21
		40.0000	.20	.941	-5.56	5.96
		45.0000	3.23	.186	-1.77	8.22
		5.0000	-1.70	.535	-7.46	4.06
		50.0000	5.70	.052	-.06	11.46
	20.0000	1.0000	.70	.797	-5.06	6.46
		10.0000	1.65	.547	-4.11	7.41
		15.0000	2.00	.467	-3.76	7.76
		25.0000	1.40	.609	-4.36	7.16
		30.0000	2.15	.435	-3.61	7.91
		35.0000	1.45	.596	-4.31	7.21
		40.0000	2.20	.424	-3.56	7.96
		45.0000	5.23	.542	.23	10.22
		5.0000	.30	.912	-5.46	6.06
		50.0000	7.70(*)	.013	1.94	13.46
	25.0000	1.0000	-.70	.797	-6.46	5.06

Table A.4 continued

	10.0000	.25	.927	-5.51	6.01
	15.0000	.60	.826	-5.16	6.36
	20.0000	-1.40	.609	-7.16	4.36
	30.0000	.75	.783	-5.01	6.51
	35.0000	.05	.985	-5.71	5.81
	40.0000	.80	.769	-4.96	6.56
	45.0000	3.83	.122	-1.17	8.82
	5.0000	-1.10	.687	-6.86	4.66
	50.0000	6.30	.534	.54	12.06
30.0000	1.0000	-1.45	.596	-7.21	4.31
	10.0000	-.50	.854	-6.26	5.26
	15.0000	-.15	.956	-5.91	5.61
	20.0000	-2.15	.435	-7.91	3.61
	25.0000	-.75	.783	-6.51	5.01
	35.0000	-.70	.797	-6.46	5.06
	40.0000	.05	.985	-5.71	5.81
	45.0000	3.08	.206	-1.92	8.07
	5.0000	-1.85	.500	-7.61	3.91
	50.0000	5.55	.058	-.21	11.31
35.0000	1.0000	-.75	.783	-6.51	5.01
	10.0000	.20	.941	-5.56	5.96
	15.0000	.55	.840	-5.21	6.31
	20.0000	-1.45	.596	-7.21	4.31
	25.0000	-.05	.985	-5.81	5.71
	30.0000	.70	.797	-5.06	6.46
	40.0000	.75	.783	-5.01	6.51
	45.0000	3.78	.126	-1.22	8.77
	5.0000	-1.15	.673	-6.91	4.61
	50.0000	6.25	.536	.49	12.01
40.0000	1.0000	-1.50	.584	-7.26	4.26
	10.0000	-.55	.840	-6.31	5.21
	15.0000	-.20	.941	-5.96	5.56
	20.0000	-2.20	.424	-7.96	3.56
	25.0000	-.80	.769	-6.56	4.96
	30.0000	-.05	.985	-5.81	5.71
	35.0000	-.75	.783	-6.51	5.01
	45.0000	3.03	.213	-1.97	8.02
	5.0000	-1.90	.489	-7.66	3.86
	50.0000	5.50	.060	-.26	11.26
45.0000	1.0000	-4.53	.072	-9.52	.47
	10.0000	-3.58	.146	-8.57	1.42
	15.0000	-3.23	.186	-8.22	1.77
	20.0000	-5.23	.542	-10.22	-.23
	25.0000	-3.83	.122	-8.82	1.17
	30.0000	-3.08	.206	-8.07	1.92
	35.0000	-3.78	.126	-8.77	1.22
	40.0000	-3.03	.213	-8.02	1.97
	5.0000	-4.93	.053	-9.92	.07
	50.0000	2.47	.304	-2.52	7.47
5.0000	1.0000	.40	.883	-5.36	6.16
	10.0000	1.35	.621	-4.41	7.11
	15.0000	1.70	.535	-4.06	7.46
	20.0000	-.30	.912	-6.06	5.46
	25.0000	1.10	.687	-4.66	6.86
	30.0000	1.85	.500	-3.91	7.61
	35.0000	1.15	.673	-4.61	6.91
	40.0000	1.90	.489	-3.86	7.66
	45.0000	4.93	.053	-.07	9.92
	50.0000	7.40	.516	1.64	13.16
50.0000	1.0000	-7.00	.521	-12.76	-1.24
	10.0000	-6.05	.541	-11.81	-.29
	15.0000	-5.70	.052	-11.46	.06
	20.0000	-7.70	.513	-13.46	-1.94
	25.0000	-6.30	.534	-12.06	-.54
	30.0000	-5.55	.058	-11.31	.21
	35.0000	-6.25	.536	-12.01	-.49

Table A.4 continued

	40.0000	-5.50	.060	-11.26	.26
	45.0000	-2.47	.304	-7.47	2.52
	5.0000	-7.40	.516	-13.16	-1.64

Table A.5 Statistical analysis of the effect of CB-like fat incorporation into CB on TAG content

Dependent Variable: POP Content (%)						
	CB-like fat incorporation (%)		Mean Difference	Sig.	95% Confidence Interval	
	(I) VAR00002	(J) VAR00002	(I-J)		Upper Bound	Lower Bound
LSD	.00	10.00	.4000	.741	-2.2906	3.0906
		20.00	1.3000	.298	-1.3906	3.9906
		30.00	2.4000	.074	-.2906	5.0906
		40.00	3.3000(*)	.022	.6094	5.9906
		50.00	4.4000(*)	.005	1.7094	7.0906
		70.00	6.3000(*)	.001	3.6094	8.9906
		100.00	8.6000(*)	.000	5.9094	11.2906
	10.00	.00	-.4000	.741	-3.0906	2.2906
		20.00	.9000	.463	-1.7906	3.5906
		30.00	2.0000	.125	-.6906	4.6906
		40.00	2.9000(*)	.038	.2094	5.5906
		50.00	4.0000(*)	.009	1.3094	6.6906
		70.00	5.9000(*)	.001	3.2094	8.5906
		100.00	8.2000(*)	.000	5.5094	10.8906
	20.00	.00	-1.3000	.298	-3.9906	1.3906
		10.00	-.9000	.463	-3.5906	1.7906
		30.00	1.1000	.373	-1.5906	3.7906
		40.00	2.0000	.125	-.6906	4.6906
		50.00	3.1000(*)	.029	.4094	5.7906
		70.00	5.0000(*)	.003	2.3094	7.6906
		100.00	7.3000(*)	.000	4.6094	9.9906
	30.00	.00	-2.4000	.074	-5.0906	.2906
		10.00	-2.0000	.125	-4.6906	.6906
		20.00	-1.1000	.373	-3.7906	1.5906
		40.00	.9000	.463	-1.7906	3.5906
		50.00	2.0000	.125	-.6906	4.6906
		70.00	3.9000(*)	.010	1.2094	6.5906
		100.00	6.2000(*)	.001	3.5094	8.8906
	40.00	.00	-3.3000(*)	.022	-5.9906	-.6094
		10.00	-2.9000(*)	.038	-5.5906	-.2094
		20.00	-2.0000	.125	-4.6906	.6906
		30.00	-.9000	.463	-3.5906	1.7906
		50.00	1.1000	.373	-1.5906	3.7906
		70.00	3.0000(*)	.033	.3094	5.6906
		100.00	5.3000(*)	.002	2.6094	7.9906
	50.00	.00	-4.4000(*)	.005	-7.0906	-1.7094
		10.00	-4.0000(*)	.009	-6.6906	-1.3094
		20.00	-3.1000(*)	.029	-5.7906	-.4094
		30.00	-2.0000	.125	-4.6906	.6906
		40.00	-1.1000	.373	-3.7906	1.5906
		70.00	1.9000	.142	-.7906	4.5906
		100.00	4.2000(*)	.007	1.5094	6.8906
	70.00	.00	-6.3000(*)	.001	-8.9906	-3.6094
		10.00	-5.9000(*)	.001	-8.5906	-3.2094
		20.00	-5.0000(*)	.003	-7.6906	-2.3094
		30.00	-3.9000(*)	.010	-6.5906	-1.2094
		40.00	-3.0000(*)	.033	-5.6906	-.3094
		50.00	-1.9000	.142	-4.5906	.7906
		100.00	2.3000	.084	-.3906	4.9906
	100.00	.00	-8.6000(*)	.000	-11.2906	-5.9094
		10.00	-8.2000(*)	.000	-10.8906	-5.5094
		20.00	-7.3000(*)	.000	-9.9906	-4.6094
		30.00	-6.2000(*)	.001	-8.8906	-3.5094

Table A.5 continued

		40.00	-5.3000(*)	.002	-7.9906	-2.6094
		50.00	-4.2000(*)	.007	-6.8906	-1.5094
		70.00	-2.3000	.084	-4.9906	.3906

Dependent Variable: POS Content (%)

	Enzyme resuse		Mean	Sig.	95% Confidence Interval	
	(I)	(J)	Difference		Upper	
	VAR00002	VAR00002	(I-J)		Bound	Lower Bound
LSD	0	10	.6000	.778	-4.1526	5.3526
		100	13.6000(*)	.000	8.8474	18.3526
		20	1.6000	.460	-3.1526	6.3526
		30	2.6000	.243	-2.1526	7.3526
		40	4.6000	.056	-.1526	9.3526
		50	6.0000(*)	.020	1.2474	10.7526
		70	9.3000(*)	.002	4.5474	14.0526
	10	0	-.6000	.778	-5.3526	4.1526
		100	13.0000(*)	.000	8.2474	17.7526
		20	1.0000	.641	-3.7526	5.7526
		30	2.0000	.360	-2.7526	6.7526
		40	4.0000	.088	-.7526	8.7526
		50	5.4000(*)	.031	.6474	10.1526
		70	8.7000(*)	.003	3.9474	13.4526
	100	0	-13.6000(*)	.000	-18.3526	-8.8474
		10	-13.0000(*)	.000	-17.7526	-8.2474
		20	-12.0000(*)	.000	-16.7526	-7.2474
		30	-11.0000(*)	.001	-15.7526	-6.2474
		40	-9.0000(*)	.002	-13.7526	-4.2474
		50	-7.6000(*)	.006	-12.3526	-2.8474
		70	-4.3000	.070	-9.0526	.4526
	20	0	-1.6000	.460	-6.3526	3.1526
		10	-1.0000	.641	-5.7526	3.7526
		100	12.0000(*)	.000	7.2474	16.7526
		30	1.0000	.641	-3.7526	5.7526
		40	3.0000	.184	-1.7526	7.7526
		50	4.4000	.065	-.3526	9.1526
		70	7.7000(*)	.006	2.9474	12.4526
	30	0	-2.6000	.243	-7.3526	2.1526
		10	-2.0000	.360	-6.7526	2.7526
		100	11.0000(*)	.001	6.2474	15.7526
		20	-1.0000	.641	-5.7526	3.7526
		40	2.0000	.360	-2.7526	6.7526
		50	3.4000	.138	-1.3526	8.1526
		70	6.7000(*)	.012	1.9474	11.4526
	40	0	-4.6000	.056	-9.3526	.1526
		10	-4.0000	.088	-8.7526	.7526
		100	9.0000(*)	.002	4.2474	13.7526
		20	-3.0000	.184	-7.7526	1.7526
		30	-2.0000	.360	-6.7526	2.7526
		50	1.4000	.516	-3.3526	6.1526
		70	4.7000	.052	-.0526	9.4526
	50	0	-6.0000(*)	.020	-10.7526	-1.2474
		10	-5.4000(*)	.031	-10.1526	-.6474
		100	7.6000(*)	.006	2.8474	12.3526
		20	-4.4000	.065	-9.1526	.3526
		30	-3.4000	.138	-8.1526	1.3526
		40	-1.4000	.516	-6.1526	3.3526
		70	3.3000	.148	-1.4526	8.0526
	70	0	-9.3000(*)	.002	-14.0526	-4.5474
		10	-8.7000(*)	.003	-13.4526	-3.9474
		100	4.3000	.070	-.4526	9.0526
		20	-7.7000(*)	.006	-12.4526	-2.9474
		30	-6.7000(*)	.012	-11.4526	-1.9474
		40	-4.7000	.052	-9.4526	.0526
		50	-3.3000	.148	-8.0526	1.4526

Table A.5 continued

Dependent Variable: SOS Content (%)

	Enzyme resuse (I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Sig.	95% Confidence Interval Upper Bound	Lower Bound
LSD	0	10	.8000	.593	-2.5148	4.1148
		100	13.5000(*)	.000	10.1852	16.8148
		20	2.2000	.164	-1.1148	5.5148
		30	3.9000(*)	.027	.5852	7.2148
		40	5.0000(*)	.008	1.6852	8.3148
		50	6.9000(*)	.001	3.5852	10.2148
		70	10.6000(*)	.000	7.2852	13.9148
	10	0	-.8000	.593	-4.1148	2.5148
		100	12.7000(*)	.000	9.3852	16.0148
		20	1.4000	.359	-1.9148	4.7148
		30	3.1000	.063	-.2148	6.4148
		40	4.2000(*)	.019	.8852	7.5148
		50	6.1000(*)	.003	2.7852	9.4148
		70	9.8000(*)	.000	6.4852	13.1148
	100	0	-13.5000(*)	.000	-16.8148	-10.1852
		10	-12.7000(*)	.000	-16.0148	-9.3852
		20	-11.3000(*)	.000	-14.6148	-7.9852
		30	-9.6000(*)	.000	-12.9148	-6.2852
		40	-8.5000(*)	.000	-11.8148	-5.1852
		50	-6.6000(*)	.002	-9.9148	-3.2852
		70	-2.9000	.078	-6.2148	.4148
	20	0	-2.2000	.164	-5.5148	1.1148
		10	-1.4000	.359	-4.7148	1.9148
		100	11.3000(*)	.000	7.9852	14.6148
		30	1.7000	.271	-1.6148	5.0148
		40	2.8000	.087	-.5148	6.1148
		50	4.7000(*)	.011	1.3852	8.0148
		70	8.4000(*)	.000	5.0852	11.7148
	30	0	-3.9000(*)	.027	-7.2148	-.5852
		10	-3.1000	.063	-6.4148	.2148
		100	9.6000(*)	.000	6.2852	12.9148
		20	-1.7000	.271	-5.0148	1.6148
		40	1.1000	.466	-2.2148	4.4148
		50	3.0000	.070	-.3148	6.3148
		70	6.7000(*)	.002	3.3852	10.0148
	40	0	-5.0000(*)	.008	-8.3148	-1.6852
		10	-4.2000(*)	.019	-7.5148	-.8852
		100	8.5000(*)	.000	5.1852	11.8148
		20	-2.8000	.087	-6.1148	.5148
		30	-1.1000	.466	-4.4148	2.2148
		50	1.9000	.223	-1.4148	5.2148
		70	5.6000(*)	.005	2.2852	8.9148
	50	0	-6.9000(*)	.001	-10.2148	-3.5852
		10	-6.1000(*)	.003	-9.4148	-2.7852
		100	6.6000(*)	.002	3.2852	9.9148
		20	-4.7000(*)	.011	-8.0148	-1.3852
		30	-3.0000	.070	-6.3148	.3148
		40	-1.9000	.223	-5.2148	1.4148
		70	3.7000(*)	.033	.3852	7.0148
	70	0	-10.6000(*)	.000	-13.9148	-7.2852
		10	-9.8000(*)	.000	-13.1148	-6.4852
		100	2.9000	.078	-.4148	6.2148
		20	-8.4000(*)	.000	-11.7148	-5.0852
		30	-6.7000(*)	.002	-10.0148	-3.3852
		40	-5.6000(*)	.005	-8.9148	-2.2852
		50	-3.7000(*)	.033	-7.0148	-.3852

Table A.6 Statistical analysis of the effect of CB-like fat incorporation into CB on solid fat content

	5℃	N	Subset				
		1	2	3	4	1	
	1	2	89.7000				
	0.7	2	90.0000	90.0000			
	0.5	2	92.0000	92.0000	92.0000		
	0.4	2	92.7000	92.7000	92.7000		
Duncan(a,b)	0.3	2		93.0000	93.0000		
	0.2	2			94.4000	94.4000	
	0	2				96.9400	
	0.1	2				96.9400	
	Sig.		.065	.065	.126	.102	
		N	Subset				
	10℃	1	2	3	1		
	1	2	86.8000				
	0.7	2	87.0000				
	0.5	2		90.0000			
	0.4	2		90.1500			
Duncan(a,b)	0.3	2		91.0000			
	0.2	2		91.8100	91.8100		
	0	2			94.3900		
	0.1	2			94.3900		
	Sig.		.880	.221	.090		
		N	Subset				
	15℃	1	2	3	4	1	
	1	2	79.6000				
	0.7	2	80.0000				
	0.5	2		83.0000			
	0.4	2		85.0200	85.0200		
Duncan(a,b)	0.3	2			86.7000		
	0.2	2			87.4900		
	0.1	2				90.4800	
	0	2				90.5300	
	Sig.		.749	.133	.086	.968	
		N	Subset				
	20℃	1	2	3	4	1	
	1	2	70.0000				
	0.7	2	70.1100				
	0.5	2	72.0000				
	0.4	2		75.0600			
Duncan(a,b)	0.3	2			78.0000		
	0.2	2			78.8100		
	0.1	2				83.2000	
	0	2				83.3400	
	Sig.		.114	1.000	.475	.900	
		N	Subset				
	25℃	1	2	3	4	1	
	1	2	47.0000				
	0.7	2	48.1000				
	0.5	2		53.5900			
	0.4	2			56.4900		
Duncan(a,b)	0.3	2			57.8000		
	0.2	2			58.1100		
	0.1	2				64.2000	
	0	2				64.6400	
	Sig.		.206	1.000	.088	.597	
		N	Subset				
	30℃	1	2	3	4	5	1
Duncan(a,b)	1	2	22.0000				

Table A.6 continued

	0.7	2		23.1800			
	0.3	2			26.0000		
	0.5	2			26.1300		
	0.2	2			26.7300	26.7300	
	0.4	2				27.5400	
	0.1	2					32.1000
	0	2					32.7500
	Sig.		1.000	1.000	.107	.069	.131

		N	Subset				
	35°C	1	2	3	4	1	
	0.3	2	8.3000				
	1	2	8.3000				
	0.2	2	8.5200				
	0.7	2	8.6000				
Duncan(a,b)	0.5	2		9.0100			
	0.4	2			9.5200		
	0.1	2				10.6000	
	0	2				10.8800	
	Sig.		.064	1.000	1.000	.065	

		N	Subset				
	40°C	1	2	3	4	1	
	0.3	2	2.5600				
	0.5	2	2.6000				
	0.2	2	2.6500				
	0.7	2		3.0000			
Duncan(a,b)	1	2		3.0000			
	0.4	2			3.2100		
	0.1	2				3.8000	
	0	2				3.8200	
	Sig.		.086	1.000	1.000	.662	

		N	Subset				
	45°C	1	2	3	4	5	1
	0.5	2	.5900				
	0.2	2		.8000			
	0.3	2		.8000			
	0.7	2			.8900		
Duncan(a,b)	1	2			.8900		
	0.4	2				1.0200	
	0	2					1.4900
	0.1	2					1.4900
	Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table A.7 Statistical analysis of the effect of CB-like fat incorporation on the oxidative stability of the final blend at 110°C

Dependent Variable: k value						
	CB-like fat incorporation (%)		Mean Difference (I-J)	Sig.	95% Confidence Interval	
	(I) VAR00002	(J) VAR00002			Upper Bound	Lower Bound
LSD	1,0000000	2,0000000	-,0018023000	,926	-,042570111	,038965511
		3,0000000	-,0043483000	,822	-,045116111	,036419511
		4,0000000	-,0167299000	,394	-,057497711	,024037911
		5,0000000	-,0155105000	,428	-,056278311	,025257311
		6,0000000	-,0141370000	,469	-,054904811	,026630811
		7,0000000	-,0349807000	,087	-,075748511	,005787111
	2,0000000	1,0000000	,0018023000	,926	-,038965511	,042570111
		3,0000000	-,0025460000	,895	-,043313811	,038221811
		4,0000000	-,0149276000	,445	-,055695411	,025840211
		5,0000000	-,0137082000	,483	-,054476011	,027059611

Table A.7 continued

	6,0000000	-,0123347000	,527	-,053102511	,028433111
	7,0000000	-,0331784000	,103	-,073946211	,007589411
3,0000000	1,0000000	,0043483000	,822	-,036419511	,045116111
	2,0000000	,0025460000	,895	-,038221811	,043313811
	4,0000000	-,0123816000	,525	-,053149411	,028386211
	5,0000000	-,0111622000	,566	-,051930011	,029605611
	6,0000000	-,0097887000	,615	-,050556511	,030979111
	7,0000000	-,0306324000	,129	-,071400211	,010135411
4,0000000	1,0000000	,0167299000	,394	-,024037911	,057497711
	2,0000000	,0149276000	,445	-,025840211	,055695411
	3,0000000	,0123816000	,525	-,028386211	,053149411
	5,0000000	,0012194000	,950	-,039548411	,041987211
	6,0000000	,0025929000	,893	-,038174911	,043360711
	7,0000000	-,0182508000	,353	-,059018611	,022517011
5,0000000	1,0000000	,0155105000	,428	-,025257311	,056278311
	2,0000000	,0137082000	,483	-,027059611	,054476011
	3,0000000	,0111622000	,566	-,029605611	,051930011
	4,0000000	-,0012194000	,950	-,041987211	,039548411
	6,0000000	,0013735000	,943	-,039394311	,042141311
	7,0000000	-,0194702000	,323	-,060238011	,021297611
6,0000000	1,0000000	,0141370000	,469	-,026630811	,054904811
	2,0000000	,0123347000	,527	-,028433111	,053102511
	3,0000000	,0097887000	,615	-,030979111	,050556511
	4,0000000	-,0025929000	,893	-,043360711	,038174911
	5,0000000	-,0013735000	,943	-,042141311	,039394311
	7,0000000	-,0208437000	,291	-,061611511	,019924111
7,0000000	1,0000000	,0349807000	,087	-,005787111	,075748511
	2,0000000	,0331784000	,103	-,007589411	,073946211
	3,0000000	,0306324000	,129	-,010135411	,071400211
	4,0000000	,0182508000	,353	-,022517011	,059018611
	5,0000000	,0194702000	,323	-,021297611	,060238011
	6,0000000	,0208437000	,291	-,019924111	,061611511

Table A.8 Statistical analysis of the effect of CB-like fat incorporation on the oxidative stability of the final blend at 130°C

Dependent Variable: k value						
	CB-like fat incorporation (%)		Mean Difference (I-J)	Sig.	95% Confidence Interval	
	(I) VAR00002	(J) VAR00002			Upper Bound	Lower Bound
LSD	1,0000000	2,0000000	-,0001197000(*)	,006	-,000199965	-,000039435
		3,0000000	-,0003317000(*)	,000	-,000411965	-,000251435
		4,0000000	-,0009437000(*)	,000	-,001023965	-,000863435
		5,0000000	-,0009947000(*)	,000	-,001074965	-,000914435
		6,0000000	-,0010447000(*)	,000	-,001124965	-,000964435
		7,0000000	-,0036567000(*)	,000	-,003736965	-,003576435
	2,0000000	1,0000000	,0001197000(*)	,006	,000039435	,000199965
		3,0000000	-,0002120000(*)	,000	-,000292265	-,000131735
		4,0000000	-,0008240000(*)	,000	-,000904265	-,000743735
		5,0000000	-,0008750000(*)	,000	-,000955265	-,000794735
		6,0000000	-,0009250000(*)	,000	-,001005265	-,000844735
		7,0000000	-,0035370000(*)	,000	-,003617265	-,003456735
	3,0000000	1,0000000	,0003317000(*)	,000	,000251435	,000411965
		2,0000000	,0002120000(*)	,000	,000131735	,000292265
		4,0000000	-,0006120000(*)	,000	-,000692265	-,000531735
		5,0000000	-,0006630000(*)	,000	-,000743265	-,000582735
		6,0000000	-,0007130000(*)	,000	-,000793265	-,000632735
		7,0000000	-,0033250000(*)	,000	-,003405265	-,003244735
	4,0000000	1,0000000	,0009437000(*)	,000	,000863435	,001023965
		2,0000000	,0008240000(*)	,000	,000743735	,000904265
		3,0000000	,0006120000(*)	,000	,000531735	,000692265
		5,0000000	-,0000510000	,194	-,000131265	,000029265
		6,0000000	-,0001010000(*)	,017	-,000181265	-,000020735

Table A.8 continued

	7,0000000	-,0027130000(*)	,000	-,002793265	-,002632735
5,0000000	1,0000000	,0009947000(*)	,000	,000914435	,001074965
	2,0000000	,0008750000(*)	,000	,000794735	,000955265
	3,0000000	,0006630000(*)	,000	,000582735	,000743265
	4,0000000	,0000510000	,194	-,000029265	,000131265
	6,0000000	-,0000500000	,203	-,000130265	,000030265
	7,0000000	-,0026620000(*)	,000	-,002742265	-,002581735
6,0000000	1,0000000	,0010447000(*)	,000	,000964435	,001124965
	2,0000000	,0009250000(*)	,000	,000844735	,001005265
	3,0000000	,0007130000(*)	,000	,000632735	,000793265
	4,0000000	,0001010000(*)	,017	,000020735	,000181265
	5,0000000	,0000500000	,203	-,000030265	,000130265
	7,0000000	-,0026120000(*)	,000	-,002692265	-,002531735
7,0000000	1,0000000	,0036567000(*)	,000	,003576435	,003736965
	2,0000000	,0035370000(*)	,000	,003456735	,003617265
	3,0000000	,0033250000(*)	,000	,003244735	,003405265
	4,0000000	,0027130000(*)	,000	,002632735	,002793265
	5,0000000	,0026620000(*)	,000	,002581735	,002742265
	6,0000000	,0026120000(*)	,000	,002531735	,002692265

Table A.9 Statistical analysis of the effect of CB-like fat incorporation on the oxidative stability of the final blend at 150°C

Dependent Variable: k value						
	CB-like fat incorporation (%)		Mean Difference (I-J)	Sig.	95% Confidence Interval	
	(I) VAR00002	(J) VAR00002			Upper Bound	Lower Bound
LSD	1,0000000	2,0000000	-,0004580000(*)	,028	-,000857800	-,000058200
		3,0000000	-,0014590000(*)	,000	-,001858800	-,001059200
		4,0000000	-,0029840000(*)	,000	-,003383800	-,002584200
		5,0000000	-,0036590000(*)	,000	-,004058800	-,003259200
		6,0000000	-,0044460000(*)	,000	-,004845800	-,004046200
		7,0000000	-,0210040000(*)	,000	-,021403800	-,020604200
	2,0000000	1,0000000	,0004580000(*)	,028	,000058200	,000857800
		3,0000000	-,0010010000(*)	,000	-,001400800	-,000601200
		4,0000000	-,0025260000(*)	,000	-,002925800	-,002126200
		5,0000000	-,0032010000(*)	,000	-,003600800	-,002801200
		6,0000000	-,0039880000(*)	,000	-,004387800	-,003588200
		7,0000000	-,0205460000(*)	,000	-,020945800	-,020146200
	3,0000000	1,0000000	,0014590000(*)	,000	,001059200	,001858800
		2,0000000	,0010010000(*)	,000	,000601200	,001400800
		4,0000000	-,0015250000(*)	,000	-,001924800	-,001125200
		5,0000000	-,0022000000(*)	,000	-,002599800	-,001800200
		6,0000000	-,0029870000(*)	,000	-,003386800	-,002587200
		7,0000000	-,0195450000(*)	,000	-,019944800	-,019145200
	4,0000000	1,0000000	,0029840000(*)	,000	,002584200	,003383800
		2,0000000	,0025260000(*)	,000	,002126200	,002925800
		3,0000000	,0015250000(*)	,000	,001125200	,001924800
		5,0000000	-,0006750000(*)	,003	-,001074800	-,000275200
		6,0000000	-,0014620000(*)	,000	-,001861800	-,001062200
		7,0000000	-,0180200000(*)	,000	-,018419800	-,017620200
	5,0000000	1,0000000	,0036590000(*)	,000	,003259200	,004058800
		2,0000000	,0032010000(*)	,000	,002801200	,003600800
		3,0000000	,0022000000(*)	,000	,001800200	,002599800
		4,0000000	,0006750000(*)	,003	,000275200	,001074800
		6,0000000	-,0007870000(*)	,001	-,001186800	-,000387200
		7,0000000	-,0173450000(*)	,000	-,017744800	-,016945200
	6,0000000	1,0000000	,0044460000(*)	,000	,004046200	,004845800
		2,0000000	,0039880000(*)	,000	,003588200	,004387800
		3,0000000	,0029870000(*)	,000	,002587200	,003386800
		4,0000000	,0014620000(*)	,000	,001062200	,001861800
		5,0000000	,0007870000(*)	,001	,000387200	,001186800
		7,0000000	-,0165580000(*)	,000	-,016957800	-,016158200

Table A.9 continued

	7,0000000	1,0000000	,0210040000(*)	,000	,020604200	,021403800
		2,0000000	,0205460000(*)	,000	,020146200	,020945800
		3,0000000	,0195450000(*)	,000	,019145200	,019944800
		4,0000000	,0180200000(*)	,000	,017620200	,018419800
		5,0000000	,0173450000(*)	,000	,016945200	,017744800
		6,0000000	,0165580000(*)	,000	,016158200	,016957800

Table A.10 Statistical analysis of the effect of CB-like fat incorporation on the oxidative stability of the final blend at 160°C

Dependent Variable: k value						
	CB-like fat incorporation (%)		Mean Difference (I-J)	Sig.	95% Confidence Interval	
	(I) VAR00002	(J) VAR00002			Upper Bound	Lower Bound
LSD	1,0000000	2,0000000	-,0008450000	,052	-,001698261	,000008261
		3,0000000	-,0028980000(*)	,000	-,003751261	-,002044739
		4,0000000	-,0049580000(*)	,000	-,005811261	-,004104739
		5,0000000	-,0066480000(*)	,000	-,007501261	-,005794739
		6,0000000	-,0087180000(*)	,000	-,009571261	-,007864739
		7,0000000	-,0471980000(*)	,000	-,048051261	-,046344739
	2,0000000	1,0000000	,0008450000	,052	-,000008261	,001698261
		3,0000000	-,0020530000(*)	,000	-,002906261	-,001199739
		4,0000000	-,0041130000(*)	,000	-,004966261	-,003259739
		5,0000000	-,0058030000(*)	,000	-,006656261	-,004949739
		6,0000000	-,0078730000(*)	,000	-,008726261	-,007019739
		7,0000000	-,0463530000(*)	,000	-,047206261	-,045499739
	3,0000000	1,0000000	,0028980000(*)	,000	,002044739	,003751261
		2,0000000	,0020530000(*)	,000	,001199739	,002906261
		4,0000000	-,0020600000(*)	,000	-,002913261	-,001206739
		5,0000000	-,0037500000(*)	,000	-,004603261	-,002896739
		6,0000000	-,0058200000(*)	,000	-,006673261	-,004966739
		7,0000000	-,0443000000(*)	,000	-,045153261	-,043446739
	4,0000000	1,0000000	,0049580000(*)	,000	,004104739	,005811261
		2,0000000	,0041130000(*)	,000	,003259739	,004966261
		3,0000000	,0020600000(*)	,000	,001206739	,002913261
		5,0000000	-,0016900000(*)	,001	-,002543261	-,000836739
		6,0000000	-,0037600000(*)	,000	-,004613261	-,002906739
		7,0000000	-,0422400000(*)	,000	-,043093261	-,041386739
	5,0000000	1,0000000	,0066480000(*)	,000	,005794739	,007501261
		2,0000000	,0058030000(*)	,000	,004949739	,006656261
		3,0000000	,0037500000(*)	,000	,002896739	,004603261
		4,0000000	,0016900000(*)	,001	,000836739	,002543261
		6,0000000	-,0020700000(*)	,000	-,002923261	-,001216739
		7,0000000	-,0405500000(*)	,000	-,041403261	-,039696739
	6,0000000	1,0000000	,0087180000(*)	,000	,007864739	,009571261
		2,0000000	,0078730000(*)	,000	,007019739	,008726261
		3,0000000	,0058200000(*)	,000	,004966739	,006673261
		4,0000000	,0037600000(*)	,000	,002906739	,004613261
		5,0000000	,0020700000(*)	,000	,001216739	,002923261
		7,0000000	-,0384800000(*)	,000	-,039333261	-,037626739
	7,0000000	1,0000000	,0471980000(*)	,000	,046344739	,048051261
		2,0000000	,0463530000(*)	,000	,045499739	,047206261
		3,0000000	,0443000000(*)	,000	,043446739	,045153261
		4,0000000	,0422400000(*)	,000	,041386739	,043093261
		5,0000000	,0405500000(*)	,000	,039696739	,041403261
		6,0000000	,0384800000(*)	,000	,037626739	,039333261

* The mean difference is significant at the .05 level.

(1) 100:0, (2) 90:10, (3) 80:20, (4) 70:30, (5) 60:40, (6) 50:50, (7) 0:100.

Table A.11 Statistical analysis of the effect of temperature on the oxidative stability of CB:CB-like fat blends

Dependent Variable: k value						
	CB-like fat incorporation (%)		Mean	Sig.	95% Confidence Interval	
	(I) VAR00002	(J) VAR00002	Difference (I-J)		Upper Bound	Lower Bound
LSD	110℃	130℃	-,0016099429	,743	-,011644972	,008425087
		150℃	-,0088231857	,082	-,018858215	,001211844
		160℃	-,018521328(*)	,001	-,028556358	-,008486299
	130℃	110℃	,0016099429	,743	-,008425087	,011644972
		150℃	-,0072132429	,151	-,017248272	,002821787
		160℃	-,016911385(*)	,002	-,026946415	-,006876356
	150℃	110℃	,0088231857	,082	-,001211844	,018858215
		130℃	,0072132429	,151	-,002821787	,017248272
		160℃	-,0096981429	,058	-,019733172	,000336887
	160℃	110℃	,0185213286(*)	,001	,008486299	,028556358
		130℃	,0169113857(*)	,002	,006876356	,026946415
		150℃	,0096981429	,058	-,000336887	,019733172

APPENDIX B

Figure B.1 Digital image of batch system set up used in the study

Figure B.2 Digital image of the packed bed reactor system set up used in the study

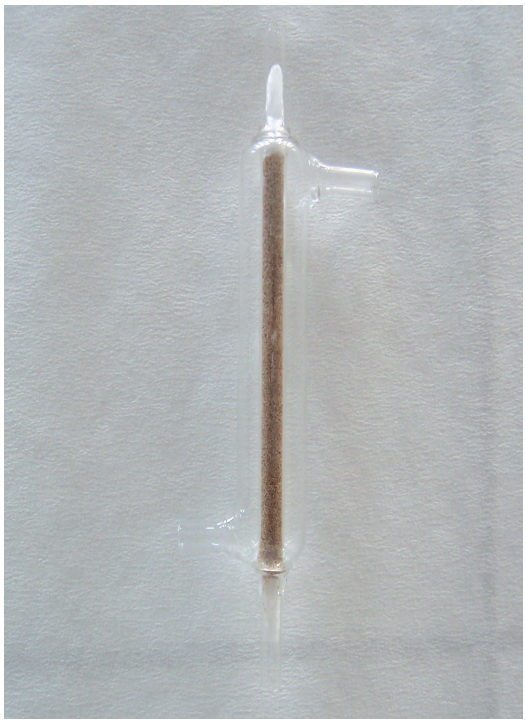


Figure B.3 Enzyme packed jacketed glass column

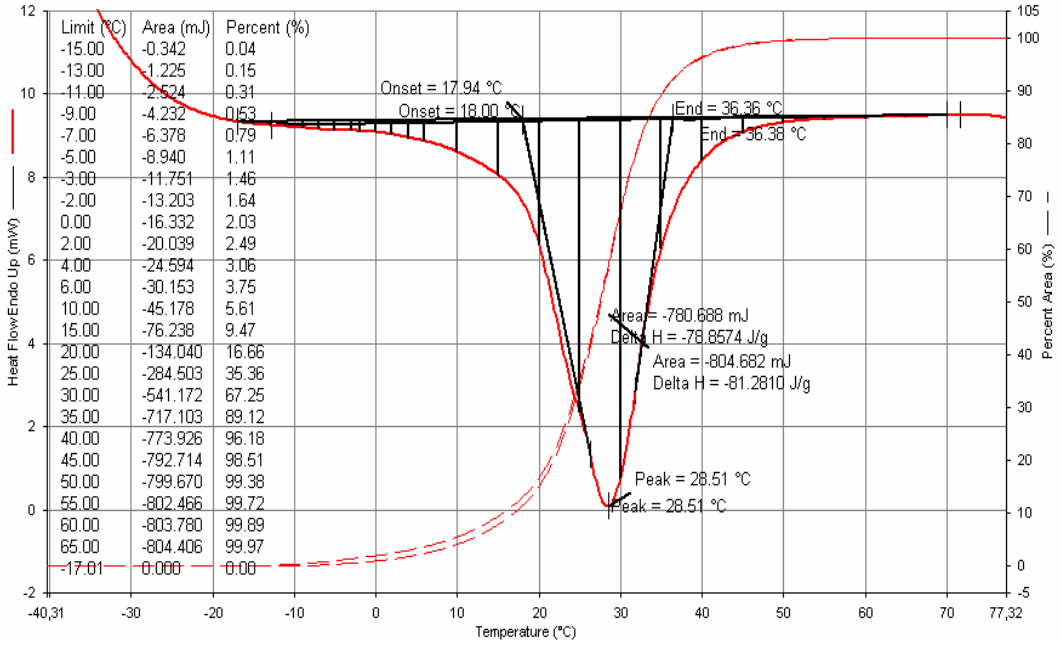


Figure B.4 Calculation of SFC using DSC

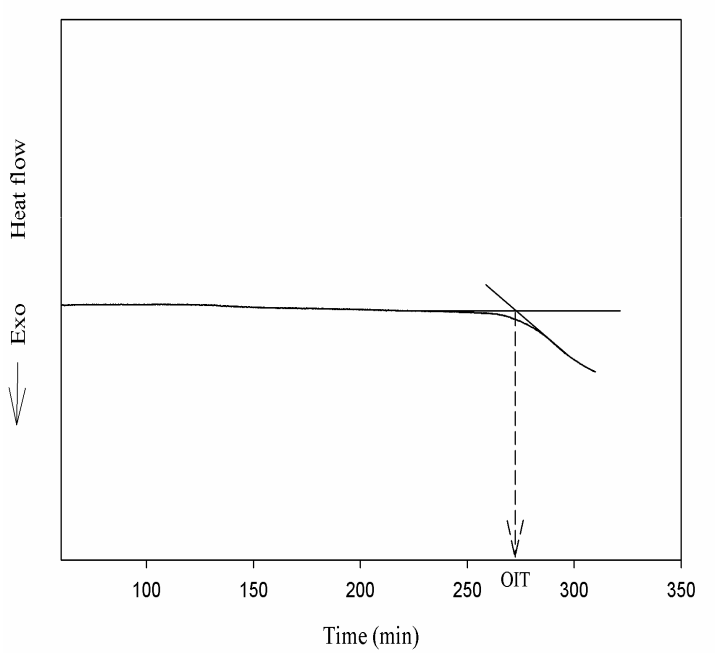


Figure B.5 Determination of the OIT from DSC thermogram

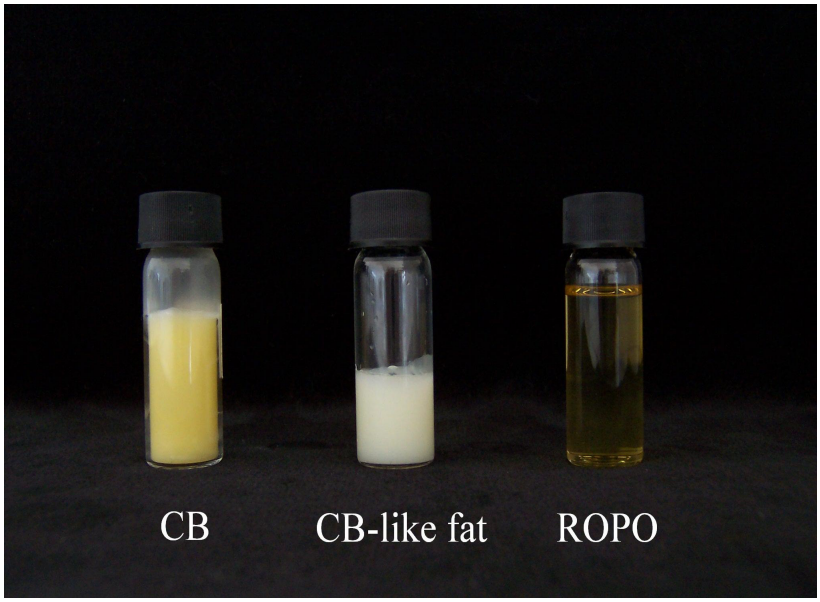


Figure B.6 Digital images of CB, ROPO, and produced CB-like fat at room temperature

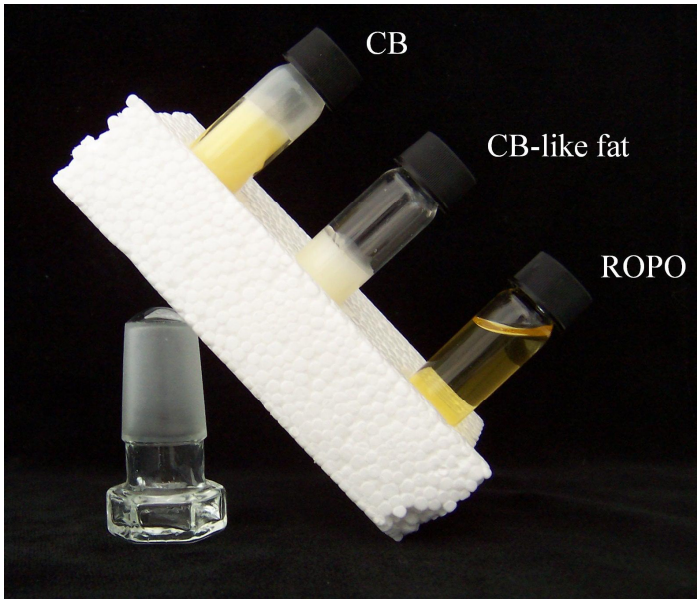


Figure B.7 Digital images of CB, ROPO, and produced CB-like fat on a ramp at room temperature

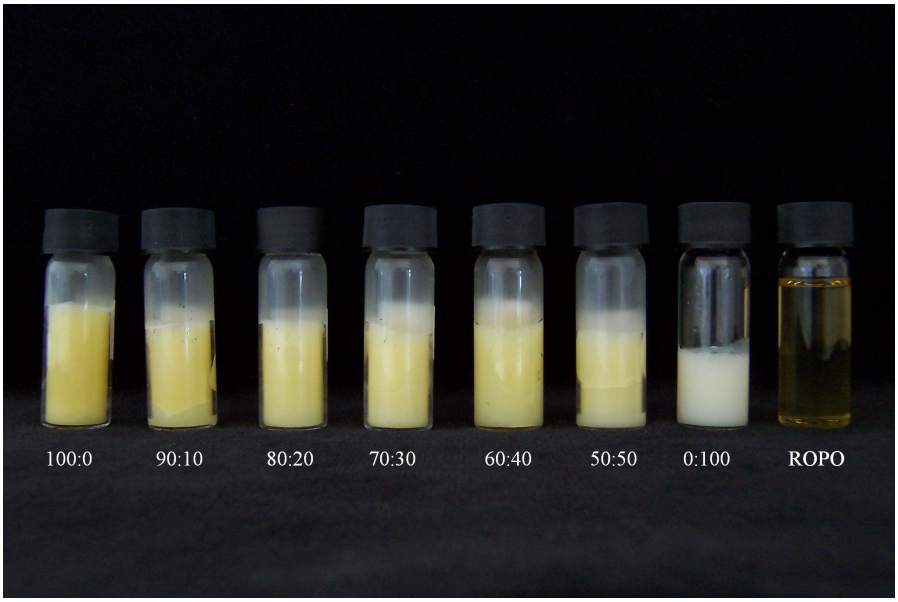


Figure B.8 Digital images of CB:CB-like fat blends, and ROPO at room temperature

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Çiftçi, Ozan Nazım

Nationality: Turkish (TC)

Date and Place of Birth: 12 September 1978 , Malazgirt

Marital Status: Married

Phone: +90 342 3172300

Fax: +90 342 3601105

email: ciftci@gantep.edu.tr

EDUCATION

Degree	Institution	Year of Graduation
Ph.D.	University of Gaziantep	January 2009
M.Sc.	University of Gaziantep	September 2003
B.Sc.	University of Gaziantep	June 2000

WORK EXPERIENCE

Year	Place	Enrollment
June 2007-June 2008	University of Gaziantep KOSGEB Technology Development Center , Gaziantep, Turkey	Expert (part time)
February 2006-July 2006	Warsaw University of Life Sciences, Department of Food Sciences, Warsaw, Poland	Visiting Scholar
January 2001- Present	University of Gaziantep, Faculty of Engineering, Department of Food Engineering, Gaziantep, Turkey	Research Assistant

FOREIGN LANGUAGES

English

PUBLICATIONS

Articles in Peer-Reviewed Journals

- Çiftçi O.N., Kowalski B., Göğüş F., Fadıloğlu F. Effect of the addition of a cocoa butter-like fat enzymatically produced from olive pomace oil on the oxidative stability of cocoa butter. *Journal of Food Science*. Revised and resubmitted.
- Çiftçi O.N., Fadıloğlu F., Göğüş F. and Güven A. Genetic programming approach to predict a model acidolysis system. *Engineering Applications of Artificial Intelligence*. In press.
- Çiftçi O.N., Fadıloğlu F., Göğüş F. Evaluation of the performance of a cocoa butter like fat enzymatically produced from olive pomace oil as a partial cocoa butter replacer. *Journal of the Science of Food and Agriculture*. Revised and resubmitted.
- Çiftçi O.N., Fadıloğlu F., Göğüş F. (2009). Utilization of olive pomace oil for enzymatic production of cocoa butter like fat. *Journal of the American Oil Chemists' Society*, 86, 119-125.
- Çiftçi O.N., Fadıloğlu F., Göğüş F. (2009). Conversion of olive pomace oil to cocoa butter-like fat in a packed-bed enzyme reactor. *Bioresource Technology*, 100, 324-329.
- Çiftçi O.N., Fadıloğlu F., Kowalski B., Göğüş F. (2008). Synthesis of cocoa butter triacylglycerols using a model acidolysis system. *Grasas y Aceites*, 59(4), 316-320.
- Çiftçi O.N., Fadıloğlu F., Göğüş F. and Güven A. (2008). Prediction of a model enzymatic acidolysis system using neural networks. *Grasas y Aceites*, 59(4), 375-382.
- Göğüş, F., Fadıloğlu, S., Çiftçi, O.N. (2004). Change in glyceride composition of olive-pomace oil during enzymatic esterification. *Nahrung/Food*, 48 (3), 205-208.

- Fadıloğlu, S., Çiftçi, O.N., Göğüş, F. (2003). Reduction of free fatty acid content of olive-pomace oil by enzymatic glycerolysis. *Food Science and Technology International*, 9 (1), 11-15.

Chapters in Books (In preparation)

- Çiftçi O.N. and Fadıloğlu S. Enzymes in fats, oils, flavors and fragrance industries. In: S.S. Marwaha and P.S. Panesar (Eds.), Enzymes in Food Processing: Fundamentals and Potential Applications. New Delhi, India: M/S I.K. International Publishing House Pvt Ltd.
- Çiftçi O.N. ve Fadıloğlu S. Lipidler. F. Göğüş and S. Fadıloğlu (Ed.), Gıda Kimyası.

PRESENTATIONS

- Ozan Nazım Çiftçi, Sibel Fadıloğlu, Fahrettin Göğüş, Boleslaw Kowalski. Enzymatic Production of Cocoa Butter-like Fat from Olive Pomace Oil in a Packed-bed Reactor. 6th Euro Fed Lipid Congress, 7-10 September 2008, Athens, Greece.
- Ozan Nazım Çiftçi, Sibel Fadıloğlu, Fahrettin Göğüş, Bolesław Kowalski. Utilization of olive-pomace oil for enzymatic production of cocoa butter-like fat. IFT Annual Meeting, June 28-July 1, 2008, New Orleans, USA.
- Ozan Nazım Çiftçi ve Sibel Fadıloğlu. Yapılandırılmış Yağların Enzimatik Sentezi. 5. Gıda Mühendisliği Kongresi, 8-10 Kasım 2007, Ankara, Türkiye.
- Ozan Nazım Çiftçi, Sibel Fadıloğlu, Fahrettin Göğüş, Bolesław Kowalski. Production of Triacylglycerols of Cocoa Butter in a Model Interesterification System. 2nd International Congress on Food and Nutrition, 24-26 October 2007, Istanbul, Turkey.
- Ozan Nazım Çiftçi and Sibel Fadıloğlu. Enzimatik interesterifikasyon ile biodizel üretimi. Türkiye 9. Gıda Kongresi, 24-26 Mayıs 2006, Bolu, Türkiye.

- Ozan Nazım Çiftçi, Sibel Fadıloğlu, Fahrettin Göğüş. Production of Cocoa Butter Equivalents by Enzymatic Interesterification: Review. İzmir 1st Food Congress, 19-21 April 2005, İzmir, Turkey.
- Ozan Nazım Çiftçi, Sibel Fadıloğlu, Fahrettin Göğüş. Prina yağının asitliğinin enzimatik yöntemle düşürülmesi. Türkiye 7. Gıda Kongresi, 22-24 Mayıs 2002, Ankara, Türkiye.