İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

LIPASE-CATALYZED INCORPORATION OF CONJUGATED LINOLEIC ACID INTO CORN OIL: OPTIMIZATION USING RESPONSE SURFACE METHODOLOGY

M.Sc. Thesis by
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Department : **Advanced Technologies**

Programme : **Molecular Biology-Genetics and Biotechnology**

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MISIR YAĞI İLE KONJUGE LİNOLEİK ASİT KARIŞIMININ ENZİMATİK ASİDOLİZİ İLE YAPILANDIRILMIŞ YAĞ ÜRETİMİ VE OPTİMİZASYONU

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FOREWORD

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ABBREVIATIONS

AA	**:** Arachidonic acid
ALA	**:** α-Linoleic acid
ANOVA	**:** Analysis of the variance
AOCS	**:** American Oil Chemists' Society
CLA	**:** Conjugated linoleic acid
DAG	**:** Diacylglycerol
DHA	**:** Docosahexaenoic acid
EFA	**:** Essential fatty acid
EPA	**:** Eicosapentaenoic acid
FAME	**:** Fatty acid methyl ester
FDA	**:** U.S Food and Drug Administration
FFA	**:** Free fatty acid
GC	**:** Gas chromatography
GRAS	**:** Generally regarded as safe
HPLC	**:** High performance liquid chromatography
LA	**:** Linoleic acid
LCFA	**:** Long-chain fatty acid
MAG	**:** Monoacylglycerol
MCFA	**:** Medium-chain fatty acid
PUFA	**:** Polyunsaturated fatty acid
R^2	**:** Coefficient of determination
RSM	**:** Response surface methodology
TAG	**:** Triacylglycerol
TL	**:** *Thermomyces lanuginosa*

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LIPASE-CATALYZED INCORPORATION OF CONJUGATED LINOLEIC ACID INTO CORN OIL: OPTIMIZATION USING RESPONSE SURFACE METHODOLOGY

SUMMARY

Lipids are a wide variety of hydrocarbon molecules that are not soluble in water but in organic solvents. They take over important structural and functional roles in human body therefore must be supplied with diet. The naturally occurring lipids in animal tissues or plants such as fats and oils are the main sources of lipids taken with human diet. Dietary lipids are not always nutritionally ideal, they may require modifications.

Structured lipids are lipids that are modified with chemical or enzymatic methods for various purposes. Among these purposes, restructuring oils or fats for obtaining enhanced nutritional properties is of great importance. The positional distribution of fatty acids in the glycerol backbone or the composition of the fatty acids in a triacylglycerol molecule is altered to produce structured lipids. Structured lipids are produced from natural oils or fats with chemical or enzymatical methods. Enzymatical methods using lipases, the enzymes catalyzing hydrolysis of lipid molecules, have several advantages over chemical methods such as, milder reaction conditions, cleaner products, reduced waste materials and most importantly, the specificity feature of lipases that allows the production of specific reaction products. Enzymatic methods for producing structured lipids utilize the reactions catalyzed by lipases which are transesterification, acidolysis and alcoholysis. In these reactions, the factors that affect the activity of lipases and process yield can be listed as water, solvent type, pH, temperature, substrates' molar ratio, time, cofactors and enzyme immobilization. These reaction parameters can be optimized in producing structured lipids.

In this study, a corn oil enhanced with conjugated linoleic acid (CLA) is produced. The aim to produce this structured lipid is to alter the nutritional quality of corn oil by incorporating CLA, a polyunsaturated fatty acid with many health benefits. The resulting structured lipid is expected to be a healthier corn oil due to its new CLA content. To incorporate CLA into corn oil, enzymatic acidolysis reactions utilizing a *sn*-1,3-specific lipase, Lipozyme TL IM, were conducted. The effects of reaction parameters on reaction yield were studied. Optimization of the reaction parameters was done via Response Surface Methodology and data obtained from experiments were computed with software Statictica 6.0. The resulting structured lipid consisted of ~60 (weight %) of CLA. Future studies investigating the effects of reaction parameters on process using different enzymes, or the effect of the structured lipid on animals, or methods for producing the structured lipid in large scale can be done.

MISIR YAĞI İLE KONJUGE LİNOLEİK ASİT KARIŞIMININ ENZİMATİK ASİDOLİZİ İLE YAPILANDIRILMIŞ YAĞ ÜRETİMİ VE OPTİMİZASYONU

ÖZET

Lipidler, suda çözünmeyen ancak organik çözücülerde çözünen hidrokarbonlardır. Vücudumuzda önemli yapısal ve fonksiyonel görevleri bulunan lipidlerin besinler ile alınmaları şarttır. Gıdalarla alınan lipidler, hayvansal ya da bitkisel kaynaklı olabilir. Ancak diyetle alınan lipidler her zaman besleyici yönden ideal olmadıklarından modifikasyonlar gerektirirler.

Yapılandırılmış yağlar, lipid moleküllerinin yapılarının değişik amaçlar doğrultusunda, kimyasal veya enzimatik olarak değiştirilmesi ile oluşturulmuş moleküllerdir. Bu amaçlar arasında, yağların besleyici etkilerini güçlendirmek için yeniden yapılandırılmaları önemli bir yer tutar. Yapılandırılmış yağlardan günümüzde sağlık ve beslenme amaçları ile yararlanılmakta, bu amaçlar doğrultusunda lipidler, yapılarında bulunan bazı yapıtaşlarınca zenginleştirilmekte, trigliserol molekülündeki yağ asitlerinin kompozisyonu ya da dağılımı değiştirilmektedir. Yapılandırılmış yağların üretimi için kimyasal veya enzimatik yöntemler kullanılmaktadır. Lipid moleküllerinin hidroliz reaksiyonlarını katalizleyen enzimler olan lipazların kullanıldığı enzimatik yöntemin kimyasal yönteme göre avantajları oldukça çoktur. Bu avantajlara örnek olarak, daha ılımlı reaksiyon koşulları ile çalışma, daha saf ürünler elde etme, daha az atık madde oluşumu ve en önemli olarak da sadece istenilen ürünü elde etmeye olanak tanıyan ve lipazlara özgü biz özellik olan spesifiklik verilebilir. Enzimatik yöntemde, lipazların katalizlediği transesterifikasyon, asidoliz ve alkoliz reaksiyonları ile yapılandırılmış yağlar üretilir. Bu reaksiyonlarda, enzim aktivitesini ve ürün oluşumunu etkileyen faktörler arasında su, çözücü türü, sıcaklık, pH, substratların mol oranı, kofaktörler, reaksiyon süresi ve enzim immobilizasyonu bulunur. Üretim amaçlanırken, bu reaksiyon parametreleri optimize edilebilir.

Bu çalışmada konjuge linoleik asitten zenginleştirilmiş bir mısır yağı üretilmiştir. Bu yapılandırılmış yağı üretmekteki amaç, mısır yağı içerisine sağlığa pek çok yararı bilinen bir çoklu doymamış yağ asidi olan konjuge linolelik asit katarak, mısır yağını daha sağlıklı bir yağ haline getirmektir. Çalışmada triaçilgliserol yapısının 1 ve 3. pozisyonlarına spesifisite gösteren bir lipaz enzimi, Lipozyme TL IM, seçilerek, enzimatik asidoliz reaksiyonları yürütülmüştür. Reaksiyon ürününe etki eden parametrelerin etkileri incelenmiş, etkili olduğu görülen parametreler, substratların mol oranı ve sıcaklık olarak belirlenmiş, bir deney tasarımı oluşturulmuş ve mısır yağına konjuge linoleik asit katılımı optimize edilmiştir. Optimizasyon için Tepki Yüzey Metodolojisi ve Statistica 6.0 programı kullanılmıştır.

Optimum koşullarda mısır yağından ~60(% ağırlık) konjuge linoleik asit içeren yapılandırılmış yağ üretilmiştir. Mısır yağı ile konjuge linoleik asidin enzimatik asidoliz reaksiyonu sonucunda elde edilen yapılandırılmış yağın canlı metabolizması üzerindeki etkilerinin görülmesi için hayvan deneyleri yapılması önerilmektedir. Ayrıca bir başka lipaz ya da çözücü sistemi kullanılarak deney yürütülebilir. Bunlara ek olarak, elde edilen yapılandırılmış yağın büyük ölçekli üretimi için yöntemler araştırılabilir.

1. INTRODUCTION

The annual production of oils and fats is reported to be around 113 million tons. They are produced majorly from 4 animal fats including; butter, lard, tallow, and fish (supplying ~21% of the whole) and 11 vegetable oils which are, soy, cotton, corn, palm, palmkernel, coconut, olive, rape, sunflower, groundnut, and linseed. (~79%). 81% of the production is consumed as food, while the rest is used either as animal feed (5%) or for the production of oleochemicals (14%). The products, whether plant or animal originated, are not necessarily ideal for our diet, thus they may require modifications.

Structured lipids are lipids that have been reconstructed from natural fats and oils to change the positions of fatty acids, or the fatty acid profile having special functionality or nutritional properties. Structured lipids can be produced chemicaly or enzymatically. Enzymatic interesterification creates designed structured lipids, thus results in specific placement of the fatty acids on the glycerol backbone, while chemical processing produce randomized structured lipids.

Lipases, enzymes that primarily catalyze lipid hydrolysis of lipids are powerful tools that also function in acidolysis, alcoholysis and transesterification reactions. Among these reactions, enzymatic acidolysis in particular, is important for creating structured triacylglycerols. Specificity of lipases is a feature that renders enzymatic reactions advantageous from chemical reactions. Producing structured lipids via enzymatic interesterification reactions using 1-3 specific lipases is one of the major application of lipases.

The aim of this study is to produce a structured lipid; conjugated linoleic acid incorporated corn oil, and to optimize the enzymatic reaction parameters via Response Surface Methodology. The many properties of conjugated linoleic acid that are beneficial to health provide the motivation for producing this structured corn oil.

2. LITERATURE SURVEY

2.1 Lipids

Even though there is no exact definition, lipids have been described as a wide variety of natural products including fatty acids and their derivatives, steroids, terpenes, carotenoids, and bile acids, which have in common a ready solubility in organic solvents such as diethyl ether, hexane, benzene, chloroform, or methanol. Lipids are also defined as substances that are insoluble in water; soluble in organic solvents such as chloroform, ether or benzene; containing long-chain hydrocarbon groups in their molecules; and being present in or derived from living organisms [1].

Lipids can be classified based on physical properties at room temperature (oils are liquid and fats are solid), their polarity (polar and neutral lipids), their essentiality for humans (essential and nonessential fatty acids), or their structure (simple or complex). Neutral lipids comprise of fatty acids, alcohols, glycerides, and sterols, while polar lipids include glycerophospholipids and glyceroglycolipids. But, since some short chain fatty acids are very polar, a structure -based classification is preferable. A classification based on structure subdivides lipids into derived, simple and complex groups. Derived lipids are fatty acids and alcohols which are the building blocks of simple and complex lipids. Simple lipids group, in which the hydrolysis reaction yields at most two types of products, comprise of mainly acylglycerols, etheracylgylcerols, sterols, and their esters and wax esters that are composed of fatty acids and alcohol components whereas complex lipids like glycerophospholipids and sphingolipids yield three or more products on hydrolysis [1, 2].

In a chemical classification driven by the distinct hydrophobic and hydrophilic elements constituting the lipid backbone, lipids are assigned to categories listed in Table 2.1.

Table 2.1: Categories and examples of lipids [2].

Category	Abbreviation	Example
Fatty acyls	FA	Arachidonic acid
Glycerolipids	GL	1-hexadecanoyl-2-(9*Z*-octadecenoyl)-s*n*-glycerol
Glycerophopholipids	GP	1-myristoyl-2-palmitoyl-*sn*-glycerophosphocholine
Sphingolipids	SP	*N*-(tetradecanoyl)-sphing-4-enine
Sterol lipids	ST	cholest-5-en-3β-ol
Prenol lipids	PR	Retinol
Saccharolipids	SL	UDP-3-*O*-(3*R*-hydroxy-tetradecanoyl)αd-*N*-acetylglucosamine
Polyketides	PK	aflatoxin B_1

2.1.1 Lipids and nutrition

Lipids in foods, besides providing energy, have important functions, such as affecting the texture and flavor and therefore the palatability of the food. The dietary lipids and the lipids in the body comprise of the free fatty acids (FFAs); the esters of glycerol and fatty acids (triacylglycerolas, diacylglycerols, monoacylglycerols); the esters of glycerol containing phosphate groups (glycerophospholipids); lipids that contain sugar groups (glycolipids); cholesterol and its esters.

Lipids take over very important roles in the body; triacylglycerols provide insulation by being stored in the adipose tissue and can be utilized to meet energy requirements when needed. The dietary fat is taken mostly as triglyceride, providing 9.0 kcal/g. That is a bigger amount of energy compared to the energy taken up as proteins or carbohydrates. Phospholipids, glycolipids and cholesterol have structural functions such as being integral components of cell membranes; In addition, triacylglycerols, diacylglycerols, monoacylglycerol, sterols , steryl esters, free fatty acids, wax esters, and hydrocarbons act as protective agents for the skin. Lipids function in the cellular metabolism; they do not only provide energy by being oxidized, but they are also used as precursors in synthesizing biologically active substances such as prostaglandins and steroid hormones and bile acids (cholesterol). Fat-soluble vitamins, A, D, E, and K, which are necessary for vision (vitamin A), regulating

calcium metabolism (vitamin D), preventing the autoxidation of unsaturated lipids (vitamin E), and normal clotting of blood (vitamin K) , are not synthesized in the body and have to be taken up with the diet. [3,4]. Lipids also function in brain development, inflammatory processes, atherosclerosis, carcinogenesis, aging and cell renewal[5].

2.1.2 Essential fatty acids

Fatty acids are hydrocarbons possessing a carboxyl group (–COOH) at one end of the hydrocarbon chain and a methyl group (CH_3) at the other end. They differ in length of the hydrocarbon chain, (short-chain, medium-chain, long-chain fatty acids) in the degree of saturation, and also in location of double bonds. According to the degree of saturation, fatty acids can be classified as saturated (containing no double bonds), monounsaturated (containing one double bond), and polyunsaturated (containing several double bonds).

Two different systems are used in classifying unsaturated fatty acids: the delta (Δ) and the omega (ω) numbering system. The carboxyl carbon is the first carbon in the delta system, whereas the first carbon is denoted as the methyl carbon in the omega system. For numbering, the double bonds in the fatty acid chain are counted from either the carboxyl or the methyl end, differing according to the system used. For example, the polyunsaturated fatty acid, α-linolenic acid is named as $18{:}3\Delta^{9c,12c,15c}$ or 18 carbons in the delta system , with three double bonds at the carbons 9, 12, and 15. However, in the omega system it is 18:3ω3 or 18 carbons, containing three double bonds with the first one located at carbon 3. Fatty acids that contain the first double bond three carbons ahead from the methyl end are called omega-3 fatty acids and are shown as ω-3 (or n-3) fatty acids. Fatty acids with the first double bond located six carbons away from the methyl end are called omega-6 fatty acids with the symbol of ω -6 (or n-6) fatty acids [6].

In 1929, it was suggested for the first time that some fats were essential for life. It was thought that, although most lipids could be synthesized in body *de novo*, some fatty acids in particular were being utilized as precursors of other important functional polyunsaturated fatty acids. Later, it was realized that two representatives of the ω-3 and ω-6 fatty acids; linoleic acid (LA) and alpha-linolenic acid (ALA) could not be synthesized *de novo* [3]. Mammals cannot synthesize these two fatty

acids because in fatty acid synthesis, they are not able to to desaturate further that nine carbons from the carboxyl end of the hydrocarbons with 16- or 18-carbons. Thus, Linoleic acid and α-linolenic acid are considered as essential fatty acids that must be taken with the diet[6]. Linoleic acid, produced mainly by plants and mostly enriched in their seeds, is a precursor for polyunsaturated fatty acid arachidonic acid and eicosanoids [6,7]. Arachidonic acid is an important polyunsaturated fatty acid that is abundant in brain and has a functional role in membranes.

α-Linolenic acid is a precursor of EPA and DHA. DHA, just like arachidonic acid, is the abundant polyunsaturated fatty acid in the brain and is crucial for brain development and function [6]. Table 2.2 shows the essential fatty acids belonging to n-3 and n-6 families.

Table 2.2: Essential fatty acids and their derivatives [3].

Common Name	Scientific Name	Chemical Notation
Omega-6 family		
linoleic acid (LA)	octadecadienoic acid	C18:2 (Δ9,12)
gammalinolenic acid (GLA)	octadecatrienoic acid	C18: 3 (Δ6,9,12)
dihomogammalinolenic acid	eicosatetraenoic acid	C20: 3 (Δ8,11,14)
arachidonic acid	eicosatetraenoic acid	C20: 4 (Δ5,8,11,14)
osbond acid	docosapentaenoic acid	C22: 5 (Δ4,7,10,13,16)
Omega-3 family		
linolenic acid	octadecatrienoic acid	C18:3 (Δ9,12,15)
steriodonic acid	octadecatetraenoic acid	C18:4 (Δ6,9,12,15)
timnodonic acid	eicosapentaenoic acid	C20:5 (Δ5,8,11,14,17)
cervonic acid	docosahexaenoic acid	C22:6 (Δ4,7,10,13,16,19)

Polyunsaturated fatty acids (PUFAS) are required by humans and animals mostly for synthesis of lipid mediators and production of membranes that have the optimum lipid bilayer structure and functional properties[6]. It has been observed that in the absence of essential fatty acids, animals develop symptoms such as dry and scaly

skin, excessive water consumption,reduced growth, infertility, etc. Lately, it has been concluded that the symptoms pointing out deficiency are resulting mostly from the deficiency of long-chain polyunsaturated fatty acids, rather than their precursors, LA and ALA [3].

Dietary sources of omega-6 fatty acids are vegetable oils such as soybean, corn, sunflower, safflower oil, cotton seed oils, while oils like linseed and canola are rich in omega-3 fatty acids. Eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) are main components of oils in fish and shellfish. Fish like salmon, trout and herring are high in EPA and DHA [8]. Table 2.3 shows dietary sources of EFAs and PUFAs.

Table 2.3: Dietary sources of essential fatty acids and amounts of fatty acids contained [8].

Product	LA	ALA	AA	EPA+DHA
n-6 FA rich foods				
Corn Oil	50000	900		
Cotton Seed Oil	47800	1000		
Peanut Oil	23900			
Soybean Oil	53400	7600		
Sunflower Oil	60200	500		
Safflower Oil	74000	470		
Margarine	17600	1900		
Lard	8600	1000	1070	
Chicken Egg	3800	220		
Bacon	6080	250	250	
Ham	2480	160	130	
Soya Bean	8650	1000		
Maize	1630	40		
Almond	9860	260		

Table 2.3 (contd): Dietary sources of essential fatty acids and amounts of fatty acids contained [8].

Brazil Nut	24900			
Peanut	13900	530		
Walnut	34100	6800	590	
n-3 FA rich foods				
Canola Oil	19100	8600		
Linseed Oil	13400	55300		
Herring	150	61.66	36.66	1700
Salmon	440	550	300	1200
Trout	74		30	500
Tuna	260	270	280	400
Cod	4	2	3	300
* Data reported as mg/ 100 g				

2.1.3 Digestion and adsorption of lipids

In human diet, nearly 95% of lipids taken with diet comprise of triacylglycerols mainly composed of long-chain fatty acids [10]. Long chain fatty acids are fatty acids that have a chain length composed of at least 14 carbons. Other dietary lipids include phospholipids, cholesterol and its esters [4].The digestion of TAG consists of hydrolysis into partial acylglycerols, and free fatty acids and is followed by absorption [9]. Actually, the first step of digestion, a predigestion occurs via lingual lipase, secreted from the serous glands (von Ebner's glands) of the tongue. Later on, in the stomach, proteolysis via gastric lipase, which is secreted from the chief cells of the gastric mucosa, liberates lipoproteins from the lipids. Despite the action of gastric lipase, lipolysis in the stomach of adults is a small amount. Together, lingual and gastric lipases digest approximately one third of dietary lipids. Both lingual and gastric lipases rather cleave the *sn*-3 position of the TAG molecule instead of the *sn*-1 position. Dietary TAGs are majorly digested by a colipase dependent pancreatic lipase; the essential enzyme for the efficient digestion in the small intestine [9].

Intestinal absorption is a complex process and chain length is an important factor in absorption of fatty acids. Absorption involves several steps including solubilization of lipid, diffusion across the unstirred water layer, mediated and non-mediated transport across the brush border membrane, diffusion across the cytosol, metabolism in the cytosol, binding to lipoproteins and exit across the basolateral membrane into the lymph or portal blood [5]. Triacylglycerols cannot cross cellular membranes, they need to be hydrolyzed before they are metabolized. Because long chain fatty acids, in contrast to other energetic nutrients, are poorly soluble in aqueous solution and because they exhibit detergent properties that are potentially harmful for cellular integrity, they must be dispersed into mixed micelles in the intestinal lumen, bound to soluble lipid-binding proteins (LBP) in intestinal absorptive cells. In the enterocytes, triacylglycerols will be re-synthesized and secreted into lymph as TAG-rich lipoproteins, chylomicrons [10]. Chylomicrons are large TAG-rich lipoprotein particles composed of triacylglycerols along with phospholipids, cholesterol, and glycerol, with the function of providing a mechanism for dietary fat and other fat soluble compounds to be carried from the site of absorption to other parts of the body for uptake and metabolism. Chylomicrons are released into the lymph and then into the blood circulation. In the circulation, the enzyme lipoprotein lipase hydrolyzes the triacylglycerol component of the chylomicrons[6]. Following release from chylomicrons, LCFA are to be metabolized in mitochondria or peroxisomes or stored in the adipocytes [11]. Fatty acids are oxidized by enzyme systems of mitochondrea or peroxisomes via β-oxidation which produces CO_2 and water by enzyme systems. The β-oxidation system is dependent on carnitine as a cofactor [12]. The entrance of long chain fatty acids into mitochondria for utilization in energy generating processes is facilitated by carnitine [13].

Despite the properties of being slowly metabolised and stored as body fat, long chain fatty acids have many great beneficial properties. Dietary long-chain saturated fatty acids are not only of great importance as nutrients used as energy sources but also are the main constituents of adipose tissue [11]. Essential fatty acids, a sub class of long chain fatty acids, are of great importance in human nutrition. Unsaturated fatty acids like omega-3 fatty acids, is known for their effects of lowering the risk of coronary heart disease (CHD) and protecting against sudden cardiac death through antiarrhythmic, antiatherogenic, antithrombotic, and vasoprotective mechanisms.

Research shows that mediterranean diet based on olive oil that is rich in polyunsaturated fatty acids and phenols is protective against cancer and coronary heart disease [14]. Conjugated linoleic acid, another long chain fatty acid, has many health benefits such as, reduction in body fat upon use, regulatory effects on immune system, and anti-carcinogenic effects.

2.2 Lipases

Because lipids mostly contain fatty acids or their esters, one of the best studied enzymes for lipid synthesis is the lipase family (triacylglycerol acylhydrolase EC 3.1.1.3) [15]. Lipases are water-soluble enzymes that act on water-insoluble substrates, catalyzing the hydolysis of triacylgycerols (triacylglyceride) to give mixtures of free fatty acids, diacylglycerols (diglycerides), monoacylglycerols (monoglycerides) and glycerol. Sources of lipases are plants, animals and microorganisms [15, 16].

Lipases are proteins having molecular weights ranging from 9000 to 70 000 Daltons. They do not need a cofactor for catalysis. Even though they are water soluble, their substrates are water-insoluble, thus they hydrolyze at the interface between the aqueous and lipid phases. The reaction rate is extremely low when water-soluble substrates are utilized, but it increases dramatically at the presence of a second hydrophobic phase. So, having the ability to act both in bulk solution and at interfaces, the activity of lipases is greatly enhanced at interfaces; a phenomenon referred to as 'interfacial activation' [15]. Crystallograpy studies have pointed out that interfacial activation is a result of a conformational change in the enzyme due to interfacial binding. This conformational change occurs because of a lid movement: when a helical fragment about 20 amino acids, called the "lid" or "flap", closes the active site, it prevents substrate molecules from entering .But with interfacial binding, however, this lid is displaced by a conformational change exposing the active site to solvent and substrate [16]. The substrate binding sites of lipases have two types; one type has a hydrophobic cleft at the surface of the protein as in the example of lipase from *Rhizomucor miehei* and related lipases, and the other one has an L-shaped, approximately 20 carbons long, hydrophobic tunnel, leading from active site at the surface through the center of the molecule to the other side. Examples with this type of binding sites are lipases from *Candida rugosa* and

Geotrichum candidum. The structural differences between the types of binding sites have important effects in the selectivities of these enzymes [15].

2.2.1 Specificity of lipases

The main property of lipases that renders enzymatic interesterification different and advantageous from chemical interesterification is their specificity [16]. Specificity of lipases falls into three main categories: positional or regio specificity, substrate specificity and stereospecificity. Using the lipase with the most appropriate selectivity is an important factor in the production of commercially interesting products [17].

2.2.1.1 Non-specific lipases

Some lipases have no positional or fatty acid specificity. Interesterification using these lipases results in randomly distributed fatty acids in all positions, yielding the same products with chemical interesterification. Some examples of non specific lipases are lipases obtained from microorganisms *Candida cylindraceae*, *Corynebacterium acnes* and *Staphylococcus aureus* [16].

2.2.1.2 Positional specificity

The inability of lipases to act on position *sn*-2 on the triacylglycerol, due to steric hindrance appears as an important property; positional specificity (i.e.,specificity toward ester bonds in positions *sn*-1,3 of the triacylglycerol).The failure of fatty acid in position *sn*-2 to enter the active site is achieved by steric hindrance. An interesterification reaction utilizing a 1,3-specific lipase firstly yields a mixture of triacylglycerols, 1,2- and 2,3-diacylglycerols, and free fatty acids. Extended reaction periods may result in acyl migration, with the formation of 1,3-diacylglycerols, allowing some randomization of the fatty acids existing at the middle position of the triacylglycerols. Compared to chemical interesterification, using 1,3-specific lipases, interesterification of oils with a high degree of unsaturation in the *sn*-2 position of triacylglycerols will decrease the saturated to unsaturated fatty acid level. Some 1,3-specific lipases are those obtained from *Aspergillus niger, Mucor miehei, R.arrhizus*, and *Rhizopus delemar*. The enzyme specificity of lipases can change individually, due to the microenvironmental factors affecting the reactivity of the functional groups or substrate molecules. For example, *Pseudomonas fragi* lipase is known as

1,3-specific, but most probably due to a microemulsion environment, it has also been observed to have yielded random interesterification products . At present, there are difficulties in identifying lipases that are specific toward fatty acids in the *sn*-2 position. An example to such type is the lipase from *Candida parapsilosis* that hydrolyzes the *sn*-2 position more rapidly than either of the *sn*-1 and *sn*-3 positions under aqueous conditions and that is also specific to long-chain polyunsaturated fatty acids. The positional specificity of some lipases can cause nutritional differences between chemically interesterified fats and oils and enzymatically interesterified samples. For example in fish and some vegetable oils containing the essential polyunsaturated fatty acids in high degrees, these fatty acids are mostly found in the *sn*-2 position. 2-monoacylglycerols are more easily absorbed than sn-1 or sn-3-monoacylglycerols in intestines. By using a lipase that is 1,3-specific, the fatty acid composition of the positions 1 and 3 can be changed to meet the desired structural requirements as the nutritionally beneficial essential fatty acids in position 2 are retained. This cannot be achieved with random chemical interesterification [16].

2.2.1.3 Stereospecificity

In triacylglycerols, the positions *sn*-1 and *sn*-3 are sterically separate. The lipases that can differentiate between the two primary esters at the *sn*-1 and *sn*-3 positions possess stereospecificity. They are quite rare. In the presence of a stereospecific lipase, the positions 1 and 3 are hydrolyzed at different rates. The source of the lipase and acyl groups determines stereospecificity. The lipid density at the interface and the differences in chain length also affect stereospecificity. Lipase derived from *Pseudomonas* species and porcine pancreatic lipase have shown stereoselectivity when acyl groups are hydrolyzed [16].

2.2.1.4 Fatty acid specificity

Most lipases show specificity towards certain fatty acids. Generally, lipases obtained from microbial sources have little fatty acid specificity, except for the lipase of *G.candidum* , a lipase that is specific toward long-chain fatty acids with cis-9-double bonds. Fatty-acid-chain-length specificity is also observed in some lipases; some are specific toward long-chain fatty acids while others are specific toward medium-chain and short-chain fatty acids. For example, *Penicillium cyclopium* lipase shows specificity toward long-chain fatty acid *as* porcine pancreatic lipase is specific to

shorter-chain fatty acids. Also, lipases from the organisms *A.niger* and *Aspergillus delemar* are known to show specificity towards both medium-chain and short-chain fatty acids [16].

2.2.2 Lipase catalyzed reactions

Lipases, depending on the water content of the system, can catalyze fully reversible reactions like hydrolysis as well as glycerolysis and interesterification. In figure 2.1, an example with glycerides, TAG represents triacylglycerols, DAG represents diaclyglycerols, MAG represents monoacylglycerols and FFA represents free fatty acids. When water content is high in the system, hydrolysis is enhanced. On the contrary, in water-limiting conditions, esterification is enhanced [16].

$$TAG + H_2O \leftrightarrow DAG + FFA$$
$$DAG + H_2O \leftrightarrow MAG + FFA$$
$$MAG + H_2O \leftrightarrow Glycerol + FFA$$

Figure 2.1 Lipase catalyzed hydrolysis and esterification [16].

Interesterification refers to the exchange of fatty acids between different esters[15]. Transesterification, acidolysis and alcoholysis are among interesterification reactions of commercial interest [16].

2.2.2.1 Transesterification

Transesterification is the exchange of acyl groups between two esters, between a triacylglycerol and a fatty acid or a methyl ester / ethyl ester of a fatty acid [16]. Transesterification is mostly used for altering the physical properties of fats and oils or fat–oil blends by changing the positional distribution of fatty acids in the triacylglycerols. For example, it has been reported that transesterification of butter using a nonspecific lipase improved the plasticity of the fat. It was presented that transesterification of butterfat using a positionally nonspecific lipase increased the level of saturated C_{48} to C_{54} triacylglycerols, monoene C_{38} and C_{46} to C_{52} triacylglycerols, and diene C_{40} to C_{54} triacylglycerols. Generally, lipase-catalyzed transesterification reactions, compared to chemical interesterifications, produce fats with a slightly lower solid fat content. Another application of transesterification

reactions with lipases includes improving the textural properties of tallow and rapeseed oil mixtures and developing of cocoa butter equivalents [18]. Figure 2.2 shows the transesterification reaction between two acylglycerols.

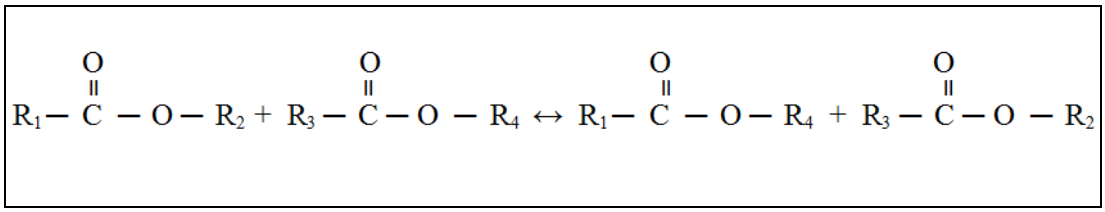

Figure 2.2 Transesterification between two acylglycerols [18].

2.2.2.2 Acidolysis

Acidolysis, as shown in figure 2.3, refers to the transfer of an acyl group between an acid and an ester [18].

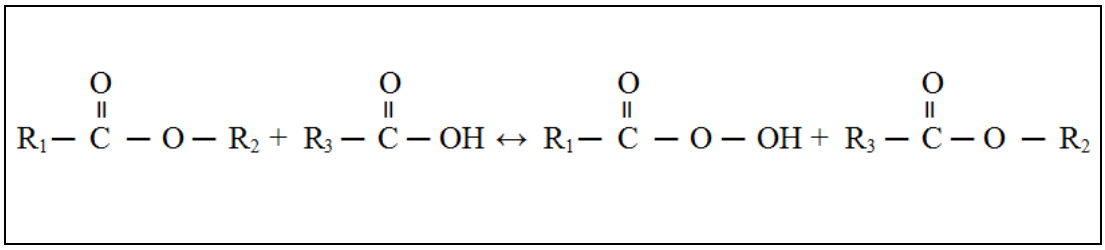

Figure 2.3 Lipase catalyzed acidolysis reaction between an acylglycerol and an acid [18] .

Acidolysis is an effective method to incorporate novel free fatty acids into triacylglycerols. Applications of acidolysis include incorporating free acid or ethyl ester forms of eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) into vegetable and fish oils with the aim of improving their nutritional properties, producing structured lipids and cocoa butter substitutes[18].

2.2.2.3 Alcoholysis

Alcoholysis reaction occurs between an ester and an alcohol [16]. In Figure 2.4, an alcoholysis reaction is presented.

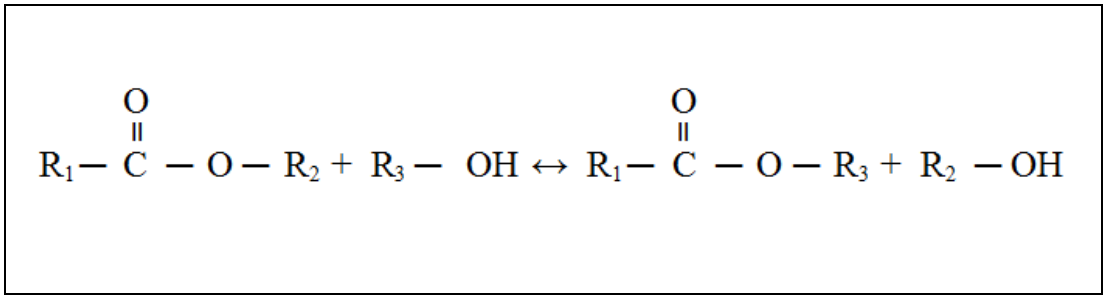

Figure 2.4 Lipase- catalyzed alcoholysis between an an acylglycerol and an alcohol [18].

Alcoholysis has been used to produce methyl esters via esterification of triacylglycerols and methanol.Alcoholysis is mainly used in performing glycerolysis reactions. Glycerolysis is exchanging of acyl groups between glycerol and triacylglycerol to yield monoacylglycerols, diacylglyc erols, and triacylglycerols. To catalyze glycerolysis, often non-specific lipases are used to obtain a wide range of products [18].

2.2.3 Factors affecting activity of lipases and process yield

2.2.3.1 Water

It is a known fact that water is essential for almost all enzymatic catalysis. Water that plays a role in all non-covalent reactions, maintains the active conformation of proteins, facilitates diffusion of reagents and maintains enzyme dynamics. It is shown in a study that for enzymes and solvents, enzymatic activity that is tested strongly increases with an increase in the water content of the solvent. Another study of the same researchers points out that the absolute amount of water needed for catalysis for different enzymes varies significantly between solvents. Hydration levels which correspond to one monolayer of water can result in active enzymes. Even though many enzymes are able to keep their active states in many organic solvents, the best non-aqueous reaction media for enzymatic reactions were found to be the hydrophobic, water-immiscible solvents. Enzymes in these solvents mostly keep the layer of essential water and so do their native configuration, and therefore their catalytic activity [19].

2.2.3.2 Solvent type

The reaction kinetics and catalytic efficiency of an enzyme is dramatically affected by the type of organic solvent used. For this reason, choosing the appropriate solvent

is important. Two major factors that are effective in choosing are; the extent to which the solvent affects the activity or stability of the enzyme and the effect of the solvent on the equilibrium position of the desired reaction. The equilibrium position in an organic phase usually differs from the one in water because of the differential solution of the reactants. For example, because the product is less polar than the starting material, the hydrolytic equilibrium is usually shifted in favor of the synthetic product. The solvent, due to its nature, can also inhibate or inactivate enzymes by directly interacting with them. The solvent may also disrupt hydrogen bonding and hydrophobic interactions, altering the native conformation of the protein thus leading to reduced activity and stability. Lipases differ according to their sensitivities to solvent types. An important characteristic of solvent in determining the effect of solvent in enzymatic catalysis is its polarity, which is measured by means of the partition coefficient (P) of a solvent between octanol and water. This is considered as a quantitative measure of polarity, also known as log P value . The enzymes in solvents with log P<2 have lower catalytic activities than that of enzymes in solvents with log P>2. This can be explained with hydrophilic or polar solvents being able to penetrate into the hydrophilic core of the protein and change the functional structure .What they also do is stripping off the essential water of the enzyme. But solvents that are hydrophobic are less likely to remove the enzyme associated water and to cause enzyme inactivation Two other important factors that must be taken into account while choosing a solvent for a given reaction are ; the solubility of the reactants in the solvent and the need for the chosen solvent to be inert to the reaction. Along with these, solvent density, viscosity, surface tension, toxicity, flammability, waste disposal, and cost must also be considered in choosing the appropriate solvent for a reaction [19].

2.2.3.3 pH

Enzymatic reactions in aqueous solutions depend strongly on pH. Studies investigating the effect of pH on enzyme activity in organic solvents point out that enzymes have an ability to remember the pH of the ultimate aqueous solution to which they were exposed. This means that the optimum pH of the enzyme in an organic solvent matches with the pH optimum of the last aqueous solution to which it was exposed. This phenomenon is referred to as pH memory. The nature of the enzyme, the substrate concentration, the stability of the enzyme, the temperature, and

the length of the reaction are factors determining a favorable pH range for an enzyme [19]. Lipases are highly dependent on pH and temperature. An extreme increase or decrease in pH value will result in partial or complete denaturation of the lipase. The optimum pH for lipases is between 6 and 9. This optimum point tends to shift towards the acidic range in the presence of salts and emulsifiers and also in the presence of fatty acids produced during hydrolysis. On the contrary, esterification will result in more alkaline pH unless buffers are used [20].

2.2.3.4 Temperature

Temperature is an important parameter for all chemical reactions. For non enzymatic reactions, reaction rates increase as temperature increases, but in the case of enzyme catalyzed reactions, a temperature optimum is observed, resulting from the opposite effects of increasing temperature on reaction rate and on the rate of enzyme thermal inactivation [21].

Change of temperature has effects on parameters such as; enzyme stability, affinity of enzyme for substrate, and preponderance of competing reactions. Since an enzyme is a protein, integrity of three dimensional structure of its active site is essential for maintaining its activity. Any factor that affects the integrity of the enzyme structure, will naturally affect its activity and stability. Stability of an enzyme not only depends on temperature, but also on pH, ionic strength, nature of buffer, presence or absence of substrate, concentration of enzyme as well as other proteins in the system, time of incubation, and the presence or absence of activators and inhibitors[19,22].

Thermostability of lipases is influenced considerably with enzyme source. For example, animal and plant lipases are found to be less thermostable compared to microbial extracellular lipases. Another factor influencing the thermostability of a lipase is the presence of a substrate. Since substrates remove excess water from the vicinity of the enzyme, the overall conformational mobility of the enzyme is restricted. Thermostability of lipases for higher process temperatures during industrial applications is throughly investigated .As some fats remain solid at a low temperature (e.g.,butterfat at 20°C) , others like hardened coconut oil start melting at 40 °C. Moreover, higher temperature has a reducing effect in viscosity of the mixture, therefore formation of microreactors is avoided. Microreactors are places at where water can be enriched during interesterification. As a result, hydrolysis occurs.

Thermostability of an enzyme is a factor that needs to be taken into account by the before commercialization of any enzymatic process, considering the potential for saving energy and minimizing thermal degradation [19, 20].

2.2.3.5 Substrates' molar ratio

Determining the mole ratio of substrates is of great importance. A higher substrate ratio is not considered as cost effective because it results in increased downstream processes as the concentrations of free fatty acids utilized increases. On the contrary, insufficient mole ratio leads to noticeable decreases in reaction yields. Reaction yields do not always increase proportionally with substrate mole ratio. It is a known fact that excessive amounts of substrate concentrations cause inhibition, a phenomenon referred to as substrate inhibition [23].

2.2.3.6 Cofactors

Lipase activity is influenced by salts such as calcium salts, bile salts and sodium chloride. They may be effective in improving the lipolytic enzyme activity by counteracting the inhibitory effects of soaps [20].

2.2.3.7 Time

In enzyme catalyzed reactions, just as in many processes, obtaining the highest reaction yields in minimum time is desired. Since there will be a decrease in cost as the reaction time is minimized, reaction time is a parameter that requires optimization [23].

2.2.3.8 Enzyme immobilization

Lipases are generally immobilized for purposes of ease of physical separation and reuse of the lipase after reaction, reducing the costs for expensive lipases. Immobilization allows termination of the reaction at a desired degree of esterification. Moreover, immobilization, compared to free enzymes, confers additional stability to lipases [19, 20].

2.2.4 Applications of lipases

The industrial enzyme market of today is estimated to be over $600 million. Lipases are represented with a percentage of 4% in the worldwide market. The three major

worldwide industrial companies working with enzyme are; Novo Nordisk, Genencor, and Solvay. Lipases find a wide range of applications; they are used for cheese flavor enhancement, acceleration of cheese ripening, lipolysis of butterfat and cream in the dairy industry. They are very broadly used in the oleochemical industry for hydrolysis, glycerolysis, and alcoholysis of fats and oils, and for the synthesis of structured triglycerides, surfactants, ingredients of personal care products, pharmaceuticals, agrochemicals, and polymers [24]. Major applications for lipases in industry are shown in Table 2.4.

Even though sources of lipases are animals, plants and microorganisms, with the increasing biotechnological advances, lipases derived from microbial sources are used widely. Microorganisms that produce lipases exist in great number of species and they are still increasing in numbers. Some of the lipases were selected to be industrialized due to their possible application in various fields. Many microbial lipases have been cloned and expressed in industrial yields using hosts that are suitable for industrial scale fermentations. For some applications, lipases are required to be immobilized. The potential for enhancement of enzyme stability is one of the major reasons for immobilizing enzymes used in industry along with the cost effectiveness due to the ability of repeated reuse. The immobilized lipase derived from the microorganism *Mucor miehei*, marketed with the trade name Lipozyme®, is an example for immobilized lipases used on an industrial scale [25].

Table 2.4: Application areas of microbial lipases in industry [25].

Industry	Effect	Product
Dairy	Milk fat hydrolysis Cheese ripening Modification of butter fat	Flavoring agents Cheese Butter
Bakery	Flavor improvement and shelf life prolongation	Bread and similar bakery products
Beverage	Improvement of aroma	Beverages
Food dressing	Improvement of quality	Mayonnaise, salad dressings, whipped creams

Table 2.4 (contd): Application areas of microbial lipases in industry [25].

Health Food	Interesterification	Nutritional products containing structured lipids
Meat and Fish	Flavor development and removal of fat	Meat and fish
Fat and Oil	Interesterification Hydrolysis	Cocoa butter, margarine Fatty acids, glycerol, mono- and diacylglycerols
Chemical	Enantioselectivity Synthesis	Chiral building blocks and chemicals Chemicals
Pharmaceutical	Interesterification Hydrolysis	Structured lipids Digestive aids
Cosmetic	Synthesis	Emulsifiers Moisturizing agents
Leather	Hydrolysis	Leather industry products
Paper	Hydrolysis	Paper industry products
Cleaning	Hydrolysis	Surfactants

Lipase-catalyzed reactions have several benefits compared to chemical reactions. Some of these include; stereospecificity, milder reaction conditions (room temperature, atmospheric pressure), cleaner products, and reduced waste materials [24].

One of the major areas these enzymes draw attention for is lipase-catalyzed interesterification to improve the nutritional value, or change the physical properties of vegetable or fish oils, or to make structured oils.

2.3 Modifying Lipids

Most of the lipid that is consumed today has been modified in some way. They can be modified via applications of technology or biology. To modify lipids, one must define the desired properties and express them in appropriate compositional terms.

The purpose to of use defines the properties of a lipid. These properties that are seeked to design lipids can be nutritional, physical, or chemical. Many products , such as; structured lipids containing essential fatty acids, long-chain polyunsaturated fatty acids, fats or fat-like materials with reduced energy value, functional foods, infant formulas, dietary supplements, are obtained by altering the nutritional or chemical properties. Changing physical properties is important in production of spreads, cooking and baking fats, frying oils, creams, as well as in cosmetic applications and lubricants. Physical properties to be altered are usually associated with crystallization, crystal form and melting behavior. For example, frying oils (and lubricants) should be crystal-free and should not contain triacylglycerols because they promote crystallization. For spreads, solids need to be in β' -crystal form and remain in that way [26].

2.4 Structured Lipids

Structured triacylglycerols are lipids that are modified or restructured from natural oils and fats, having special functionality or nutritional properties for nutritional, edible, and pharmaceutical purposes [27]. In producing structured lipids, triacylglycerols are modified chemically or enzymatically in order to change the fatty acid composition or positional distribution in the glycerol backbone [28].

Structured lipids are a large variety of products including fat-based fat substitutes (cocoa butter equivalents , breast milk fat substitutes, and some low-calorie fats), oils enriched in essential fatty acids or long chain n-3 polyunsaturated fatty acids (EPA, DHA), margarines or other plastic fats, or triacylglycerols containing both long-chain and medium / short-chain fatty acids for specific purposes [27,29].

Fat substitutes, a class of fat replacers are molecules that physically, chemically, and functionally mimic triacylglycerols in fats and oils. Fat substitutes can replace conventional fats and oils with several advantages like being lower or zero in calories, being partially absorbed, having better functionality and nutritional properties. They find application mostly in functional foods used in nutrition. A quality fat substitute or replacer must have good taste and mouthfeel, and stability for frying and baking, must be able to reduce calories, or provide health benefits to the consumer [30]. Zero and reduced calorie fat-based substitutes are important fat-based

substitutes. For constructing structured triacylglycerols, techniques like hydrogenation, blending, interesterification, directed esterification, fatty acid enrichment, genetic engineering of plants, can be used [31].

2.4.1 Sources of fatty acids for structured lipid synthesis

Many fatty acids with different functions and properties are used in the synthesis of structured lipids. Using these advantages, maximum benefit is aimed to be obtained from the structured oils. The fatty acid sources are; short-chain fatty acids, medium-chain fatty acids, polyunsaturated fatty acids, saturated long-chain fatty acids, and monounsaturated fatty acids. Table 2.5 shows the levels of some of these fatty acids used for production of structured lipids for clinical applications. The fatty acid component and the position of fatty acids in the triacylglycerol molecule determine the functional and physical properties, the metabolism, and the health benefits of the oil [19].

Table 2.5: Suggested optimum levels of fatty acids for structured lipids in clinical nutrition [19].

Fatty acid	Levels and Function
n-3	2%–5% to enhance immune function, reduce blood clotting, lower serum triacylglycerols, and reduce risk of coronary heart disease
n-6	3%–4% to satisfy essential fatty acid requirement in the diet
n-9	Monounsaturated fatty acid (18:ln-9) for the balance of long-chain fatty acid
short and medium-chain fatty acids	30%–65% for quick energy and rapid absorption, especially for immature neonates, hospitalized patients, and individuals with lipid malabsorption disorders

2.4.2 Production of structured lipids

Structured triacylglycerols can be produced by enzymatic or chemical methods. Lipases are required for production of structured TAG, as they offer the fatty acid selectivity, positional selectivity, and stereoselectivity [31]. Lipases obtained from

microorganisms; *Aspergillus niger, Mucor javanicus, M. miehei, Rhizopus arrhizus, R. delemar*, and *R. niveus* are utilized in particular for enzymic acidolysis [32]. Chemical methods, unless very sophisticated, yield only mixtures of randomized triacylglycerols [31].

Table 2.6 summarizes the advantages of lipase-catalyzed reactions over chemical synthesis.

Table 2.6: Comparison of enzymatical and chemical methods for synthesis of structured TAG [31].

Enzymatic process	Chemical process
Selective	Non-selective
Mild reaction conditions	High temperature, pressure and drastic pH conditions
Biocompatible	
Reduced environmental pollution	Problem of environmental pollution
Availability of lipases with different properties	
Many possibilities for reaction engineering	Some possibilities to change reaction conditions
Ability to improve lipase properties (e.g. by genetic engineering and/ or directed evolution and/or rational protein design and/or mutagenesis)	
Immobilized lipases can be used many times	
Compatible with PUFA	PUFA can be destroyed

Structured lipids produced with fatty acids from different oils is given in Table 2.7.

Table 2.7: Studies on the synthesis of structured lipids with specific oils and fatty acids [32].

Oil	Fatty Acid
Canola Oil	Caprylic acid Stearic acid
Coconut Oil	Lauric acid
Corn Oil	CLA
Cottonseed Oil	Lauric acid
Menhaden Oil	CLA γ-Linolenic acid
Olive Oil	Caprylic acid
Palm Oil	CLA n-3 fatty acids
Palm oleil	Lauric acid
Perilla Oil	Caprylic acid
Rice bran Oil	Capric acid
Soybean Oil	CLA Lauric acid
Sunflower Oil	CLA
Tuna Oil	Caprylic acid
MCT (trilaurin and tricaprylin)	EPA
	Oil
Olive oil	Partially hydrogenated palm oil
Palm Oil	Fully hydrogenated soybean oil

2.4.3 Commercial examples of structured lipids

2.4.3.1 Olestra / Olean

Sucrose fatty acid polyester, olestra or olean, is composed of six to eight fatty acids chemically esterified to sucrose molecule. Olestra was discovered by Procter & Gamble (Cincinati, OH), getting the original patent in 1971. U.S. Food and Drug Administration (FDA) approved the use of Olestra in January 1996 for replacing the conventional fat in savory snacks like potato chips cheese puffs and crackers. Olestra is a completely non-absorbable zero-calorie fat substitute. This fat substitute non only mimics the culinary and gastronomic characteristics of fats and oils but also cuts calories. Because Olestra passes through the gastrointestinal track without being digested or absorbed, it may cause gastrointestinal effects such as abdominal cramping and stool softening. Olestra has reducing effects in the absorption of fat-soluble vitamins and nutrients such as carotenoids. It was required by FDA that, foods containing Olestra must be labeled to inform consumers about the potential gastrointestinal effects and the addition of fat-soluble vitamins to compensate for the effects of olestra on their malabsorption [30, 33].

2.4.3.2 Sorbestrin

Sorbestrin belongs to the family of carbohydrate-based fatty acid polyesters that also contains sucrose polyesters. Sorbitol and sorbitol anhydrides construct the backbone of Sorbestrin. Developed by Pfizer Inc., Sorbestrin is a mixture of tri-, tetra-, and pentaesters of sorbitol and sorbitol anhydrides with fatty acids. Sorbestrin has a caloric content of 1.5 Kcal / g, thus serves as a reduced-calorie fat based fat substitute. It is used in salad dressings, mayonnaise, baked goods, and fried foods [30-33].

2.4.3.4 Betapol

Palmitic acid, the constituent of human milk fat triacylglycerols, occurs predominantly at the *sn*-2 position of the triacylglycerol molecule. Unilever markets the product Betapol, a human milk substitute. Betapol is produced via a 1,3-specific lipase-catalyzed process and is composed primarily 1,3-dioleoyl-2-palmitoyl-sn-glycerol [15, 31].

Table 2.8 presents some other structured lipids and their market names.

Table 2.8: Commercial examples of structured lipids [31, 32].

Product	Manufacturer	Composition	Properties/ Applications	Synthesis
Captex®	Cultor Food Science Inc. USA	C8:0/ C10:0 (60%) and C12:0 (30%) or C8:0 /C10:0(40%) and C18:2(40%)	pharmaceuticals for parenteral and enteric hyperalimentation, cosmetic industry	Details not available
Caprenin®	Procter & Gamble , USA	C8:0 , C10:0 and C22:0	GRAS product, low-calorie fat (5 kcal/g), ingredient of soft candies, candy bars and confectionery coatings for nuts, cookies, etc.	Interesterification between coconut, palm kernel and rapeseed oils
Benefat®	Cultor Food Science Inc, USA	C18:0 (high content) and C2:0, C4:0, or C6:0	Low-calorie fat (5 kcal/g) intended for use in oven-baked French fries, baked and dairy products , dressings, dips, sauces, cocoa butter substitutes, chocolate-flavored coatings	Interesterification of hydrogenated vegetable oils with triacylglycerols containing acetic and/or propionic and/or butyric acids

Table 2.8 (contd): Commercial examples of structured lipids [31, 32].

Neobee®	Stepan Co, USA	C8:0, C10:0,and long chain fatty acids(n-6 , n-3)	Incorporated into nutritional and medical beverages or snack bars	Details not available
Impact®	Novartis Nutrition, USA	Randomized high-lauric acid oil and high-linoleic acid oil	For patients who suffered trauma, surgery, sepsis or cancer	Interesterification of oils rich in lauric and linoleic acids

2.5 Corn Oil

Corn, following wheat and rice, is one of the major cereal grains. In the western world, more than 90% of the corn grain is processed and fed to animals, while in Africa and Asia almost all of the production, done by traditional processing, without separating the germ, is used for human consumption. Currently industrial processing of corn is done either by wet or dry milling, obtaining starch, sweeteners, products ranging from large grits to fine flour , corn oil and feed products. Corn, containing 34 to 52 % oil, is considered as an important source of edible oil [34].

2.5.1 Origin and classification

Corn, (*Zea mays*) belongs to the Gramineae family and is a South America originated plant. Nowadays, corn can be cultivated around the world where the summer is sufficiently long and warm, allowing the grain to ripen.

Based on the physical properties (color, size, shape and hardness), and chemical properties (content of chemical constituents) of the grain, the cultivated types can be categorized as dent corn, flint corn, sweet corn, flour corn, pop corn, waxy corn, pod corn and high-oil corn. The dent and flint corns, also named as field corn, are the abundantly cultivated types for feed and food purposes [34].

2.5.2 Kernel structure and chemical composition

The major parts of kernel are the pericarp, the endosperm, and the germ. The pericarp is composed of insoluble non-starch carbohydrates. The endosperm contains a large amount of starch, less proteins, sugars, minerals and no oil. The germ is rich in oil, but it also contains most of the sugars and minerals in the kernel [34]. The composition of dent corn is given in Table 2.9.

Table 2.9: Nutritional composition of dent corn [34].

Kernel Part	% dry weight of whole kernel	Composition of Kernel parts (%, dry basis)				
		Starch	Fat	Protein	Ash	Sugar
Whole kernel	100	72.4	4.7	9.6	1.43	1.94
Endosperm	82.3	86.6	0.86	8.6	0.31	0.61
Germ	11.5	8.3	34.4	18.5	10.3	11.0
Pericarp	5.3	7.3	0.98	3.5	0.67	0.34
Tip-cap	0.8	5.3	3.8	9.7	1.7	1.5

As presented in Table 2.10, the oil content of germ in high-oil is higher, which reduces the proportion of endosperm in the kernel. The germ of regular corn contains about 34 % oil, which is a lower amount compared to the oil in germ of high-oil types that contain over 50% oil.

Table 2.10: Nutritional composition of high-oil corn [34].

Constituent (%)	Whole kernel	Endosperm	*Germ*	*Hull*
Fraction	100	67.2	24.7	7.4
Water	8.7	16.9	8.3	6.1
Protein	8.8	7.0	11.3	4.9
Oil	19.5	1.0	52.1	5.5
Crude Fiber	2.0	0.5	3.7	15.8
Ash	1.6	0.4	5.6	1.9

2.5.2.1 Lipids

Most of the oil is found in the germ. The germ of regular corn varieties contains about 35 % oil. The germ of these varieties is separated by wet or dry milling processes to be used for commercial oil extraction. The high-oil corn, containing up to 50 % oil in germ, can be directly utilized for commercial oil extraction. Lipids in corn are mostly free, but the bound ones make up of 0.3 to 0.9 % of the kernel, mostly being bound to starch. 80 % of the total lipids are triglycerides and the rest is composed of phospholipids (8 %), diglycerides (1- 2.9 %), sterols (1.3-5.5 %), sterylesters (1.1-2.9%) glycolipids (2-5.4 %), free fatty acids (0.3-0.9 %) and waxes, pigments and odor-active compounds. Careteoid pigments such as xanthophylls (0.2-5.0 mg/kg) and carotenes (0.2-7 mg/ kg) are also present in corn oil in minor amounts. For Corn oil production, all the components except for triglycerides need to be removed with refining. The final product is composed of 98 % triglycerides [34].

2.5.2.2 Fatty acid composition of corn triaclyglycerols

The level of saturated fatty acids in corn oil contains is relatively low (< 15 %) and the level of linolenic acid (18:3), which is susceptible to oxidation is very low [35]. When compared, it can be observed that the fatty acid composition of kernel oil and fiber oil are very similar to that of corn germ oil as shown in Table 2.11. The fatty acid composition of corn oil, like most other oils, will vary depending upon the seed type, climatic conditions, and growing season. Corn oil from the United States cornbelt is the highest in polyunsaturated fatty acids because of the climate and growing conditions. Corn oil produced in other countries is generally lower in linoleic fatty acid content and higher in oleic fatty acid [34].

Table 2.11: The fatty acid composition of Corn (germ) Oil and Corn Fiber Oil [35].

Oil	mol % of Total Fatty Acids					
	16:0	18:0	20:0	18:1	18:2	18:3
Germ oil (RBD) US	11.0± 0.5	1.8± 0.3	0.2± 0.2	25.3± 0.6	60.1± 1.0	1.1± 0.3
Germ oil (RBD) US	9.2 - 16.5	0- 3.3	0.3- 0.7	20- 42.2	39.4- 65.6	0.5- 1.5
Germ oil (RBD) US	10.90	1.80	nr	24.2	58.0	0.70
Germ oil (RBD) US	11.0± 0.6	1.7± 0.3	nr	25.8± 0.9	59.8± 1.2	1.1± 0.4
Germ oil (RBD) Int	12.9± 1.4	2.6± 0.6	nr	33.1± 2.5	48.8± 2.4	1.4± 0.4
Kernel oil (crude) Int	9.2 - 11.8	1.1- 1.7	0.3- 0.5	19.5- 30.4	53.0- 65.3	1.2- 2.1
Corn fiber oil (crude)	13.8 ± 0	1.7± 0	0.3- 0	23.8± 0.1	56.4± 0.1	2.6± 0

Abbreviations: nr-not reported; US- US hybrids; Int-international hybrids; 16:0-palmitic acid, 18:0-stearic acid; 20:0-arachidic acid; 18:1-oleic acid; 18:2-linoleic acid; 18:3-linolenic acid; RBD-refined, bleached, and deodorized oil.

2.5.2.3 Triacylglycerol molecular species

For quantitative analysis of triacyglycerols' molecular species of fats and oils from different plants and animals, Reversed-phase HPLC techniques have been developed .In investigations of the triacylglycerols' molecular species of refined corn oil, oleate- linoleate-linoleate and linoleate-linoleate-linoleate were the two most abundant molecular species throughout identified species. Quantitative analysis of the molecular TAG species of refined corn oil are given in Table 2.12.

Table 2.12: Quantitative analysis of triaclyglycerol molecular species in refined corn germ oil [35].

TAG Molecular Species	Area %	Area %[a]	Area %[b]
LLO	19.98	21.5	23.0
LLL	17.79	25.4	22.6
LLP	13.71	14.7	15.2
OOL	11.82	10.7	10.6
PLO	10.85	10.0	10.4

Table 2.12 (contd): Quantitative analysis of triaclyglycerol molecular species in refined corn germ oil [35].

PPL	2.48	2.5	1.7
OOP	3.48	2.9	2.4
LLS	2.64	2.2	1.8
LOS	1.77	1.8	1.3
OOO	4.35	2.8	3.2
PPO	1.55	0.9	0.4
PLS	0.78	0.8	0.4
LLLn	0.91	1.2	0.8
LnLO	2.20	0.9	2.3
OOS	0.56	0.6	0.5
POS	0.20	0.3	0.3
PLnL	0.43	0.5	0.5
PPP	0.0	0.0	0.1
OOLn	1.09	0.1	1.0
PLnO	0.0	0.1	0.5
PPS	0.36	0.0	0.1
SSL	0.0	0.1	0.3
LnLS	0.0	0.1	0.0
SSO	0.0	0.0	0.0
PPLn	0.0	0.0	0.2
SSP	0.0	0.0	0.1
SSS	0.0	0.0	0.1

[a] HPLC- Mass Spec values [b] HPLD-Flame Ionization Detector Values

Abbreviations; Ln- Linolenic acid; L- Linoleic acid; O- Oleic acid; S- Stearic acid; P- Palmitic acid

Silver ion HPLC was also used for quantitative analysis of corn oil triacylglycerols. With the method, the triacylglycerols were separated into 11 fractions, with the largest 2 fractions having five and six double bonds. This method identified the two most abundant molecular species as oleate-linoleate-linoleate and linoleate-linoleate-linoleate, confirming the results obtained from reversed-phase HPLC [35].

2.5.2.4 Other components of corn oil

Corn kernel has a protein amount of 8 to 18.2 %. Corn proteins are albumin, globulin, prolamin and glutelin. Starch, with a content of 64-78 % on a dry basis, is the predominant carbohydrate of the corn. Other carbohydrates are: total fiber, crude fiber, acid detergent fiber, pentosans, water soluble polysaccharides, and other sugars like sucrose, glucose, fructose and raffinose in minor amounts. The high-oil corn, compared to normal corn, contains less starch. The mineral content of corn kernel ranges between 1.1 to 3.9 %, with the major minerals of phosphorus, potassium, magnesium, sulfur and calcium while corn vitamins include; vitamin E, thiamin, riboflavin, pantothenic acid, biotin, folic acid, choline, niacin, and pyridoxine .

In addition, of all commercial vegetable oils, corn oil has the highest levels of unsaponifiables (1.3-2.3 %). Phytosterols, tocopherols and squalene are the most abundant chemical components in the unsaponifiable fraction of corn oil [35].

2.5.3 Processing of corn for oil

Corn oil is a byproduct of commercial corn process. Even though it is possible to obtain oil directly from kernels, due to the high cost of extracting the low levels of oil in the kernels, it is not preferred. A process called "wet milling" was developed to efficiently isolate the high levels of starch in the kernels [35].

Through dry and wet-milling processes about 8 to 10 % of the corn is produced industrially. The germ is separated to be used for commercial oil extraction with these processes. Wet milling process is the predominating technique in which the kernel is separated into five fractions, which are starch, steep-water solubles, course and fine fiber, corn gluten meal, and germ. Corn germ which is rich in oil, is the source of commercial corn oil. Corn oil could more accurately be called "corn germ oil" [34, 35].

2.5.4 Extraction and refining

2.5.4.1 Conventional and alternative corn germ extraction processes

Solvent extraction is the only step to obtain oils from oilseed. On the contrary, after flaking of wet milled corn germ, a substantial amount of "fines" that interfere with the efficiency of the extraction process are produced. Therefore, direct extraction from germ cannot be possible for corn oil production. In traditional process, first the oil is removed from the wet milled germ with a heating process, then mechanically expelled, followed by hexane extraction. Hexane is removed from the oil-rich material via evoporation, heat, and vacuuming. In dry milling, oil is usually obtained from germ by full press via an expeller.

Ethanol is also used as an organic solvent for the extraction of corn oil.with the advantages of having a higher flash point, being a "food-grade" solvent, readily producable from corn via fermentation. On the other hand, higher boiling point and increased polarity of ethanol are the main disadvantages of this method.

In addition, many aqueous and enzyme-assisted methods utilizing commercial cellulases, hemicellulases, polygalcaturonases, galactomannases, and pectolytic enzymes for extraction have been reported [35].

2.5.4.2 Refining

Aside from its major components, the triacylglycerols, crude oil also contains minor nonpolar and polar lipid components. Free fatty acids, pigments, volatiles, phospholipids, and waxes consist of the major undesirable components in crude corn oil that need to be removed by several refining steps .Most companies prefer alkali refining method for fatty acids removal. In alkali refining method, bases are added to neutralize the free fatty acid soaps into byproducts called "soapstocks". An alternative to this method is the removal of free fatty acids by "physical refining" or "steam refining". This method involves treating the oil at high temperature and vacuuming to volatilize the free fatty acids [35].

2.5.5 Physiochemical properties of corn oil

The physical and chemical properties of corn oil are presented in Table 2.13. The physical characters of low cloud point, low melting point, low smoke point, pale yellow color, and bland flavor make it a favorable oil for food purposes [34].

Table 2.13: Corn oil physical characters [36].

Characteristic	**Typical**	**Range**
Specific gravity, 25/25°C	0.91875	0.915 to 0.920
Refractive index, 25°C	—	1.470 to 1.474
Iodine value	124.0	118.0 to 128.0
Saponification number	—	187 to 193
Unsaponifiable number	—	1.3 to 2.3
Titer (°C)	—	14.0 to 20.0
Melting point (°C)	—	–12 to –10
Solidification point (°C)	—	–1.0 to –20.0
Cloud point (°C)	–9.5	—
Cold test (hours)	20 dewaxed	—
Wax (%)	—	0.15 to 0.5
AOM (active oxygen method) stability (hours)	19	16 to 19
Tocopherol content (ppm): α-Tocopherol β-Tocopherol γ-Tocopherol δ-Tocopherol	 152 12 1276 61	 116 to 172 0 to 22 1119 to 1401 59 to 65

2.6 Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) comprises of positional and geometric isomers of linoleic acid (C18:2). CLA contains conjugated double bonds at carbons 10 and 12, or 9 and 11 with possible *cis* and *trans* combination, differing from linoleic acid which contains double bonds between carbons 9 and 10, and 12 and 13 [32].17 isomers of CLA in food products were identified with analytical methods. These isomers include, *t*12,*t*14; *t*11,*t*13; *t*10,*t*12;*t*9,*t*11; *t*8,*t*10; *t*7,*t*9; *t*7,*c*9; *t*6,*t*8; *c*12,*t*14; *t*11,*c*13; *c*11,*t*13; *c*10,*t*12; *c*8,*t*10; *c*9,t11; *c*7,*t*9 ; *c*9,*c*11 and *c*11,*c*13 isomers.(c refers to *cis*-, and t refers to *trans*- isomer of CLA). Two most abundant isomers, *c*9, *t*11 and *t*10, *c*12, are the chosen for research in particular [37].

2.6.1 Synthesis of CLA

We have a limited capacity of producing CLA from vaccenic acid (11-trans 18:1) in our body. Most of the CLA, we take with diet. Ruminant animals, such as cows, goats, and sheep can produce CLA naturally in their rumen via biohydrogenation of unsaturated fatty acids. CLA is synhesized by the catalytic activity of linoleic acid isomerase enzyme of the bacteria *Butyrivibrio fibrisolvens* in rumen. The synthesized products accumulate in milk and meat of these animals. CLA, for this reason, is obtained via consuming many dairy products and meat. Little amount of CLA is formed in other mammalian tissues, via action of enzyme Δ^9-stearoyl coenzyme A desaturase, which uses vaccenic acid as substrate [38].

The major CLA isomer in milk (90% of total CLA) and beef (75% of total CLA), is *c* 9, *t* 11-octadecadienoic acid, in other words, rumenic acid. Rumenic acid is the biologically most active form of CLA. CLA content of some products is given in Table 2.14.

Table 2.14: CLA content of some products [37].

Food products	CLA content (mg/g of fat)
Lamb and beef	4.0- 5.0
Butter	12.3-14.2
Yoghurt	3.3- 4.6
Cheese	1.5-19.9
Milk	5.0-30.0
Ghee	10.0-25.0
Safflower oil	0.7

2.6.2 Commercial production of CLA

Methods for commercial production of CLA are; alkali isomerization of linoleic acid, dehydration of ricinoleic acid methyl ester, and microbial synthesis from linoleic acid using cultures of different microorganisms. Alkali isomerization is the used most commonly in industrial production because it is the cheapest method above all. Commercial CLA products to be used as dietary supplements are in oil, soft gel, capsule, and powder forms [39].

2.6.3 Potential health benefits of CLA

When the anticarcinogenic properties of CLA isolated from grilled ground beef were first recognized in the 1970s, many different biological effects of CLA, such as inhibiting tumor growth, reducing atherosclerotic risk, reducing body fat, and immunomodulation have been reported. It has been shown that a small amount of CLA consumption (0.5% of calorie intake) changes gene expression having beneficial effects on carcinogenesis, obesity, diabetes, and atherosclerosis in experimental animals [39]. In animal studies, the reducing effect of CLA on atherogenesis and on fat deposit was shown. CLA was found to have an increasing effect on lean body mass [40].

2.7 Literature search on structured lipids with CLA and/or corn oil

In a study conducted by Martinez *et al.* lipase-catalyzed acidolysis of corn oil with conjugated linoleic acid in hexane was studied. They obtained the greatest incorporation when they used a CLA:corn oil weight ratio of 1:7 (1:2.22 mole ratio). The employed enzymes were two commercial immobilized lipases from *Rhizomucor miehei* and *Candida antarctica.* They catalyzed the interesterifications effectively. More than 90% of the total products were found to be triacylglycerols. It was observed that for both immobilized enzymes, CLA residues were esterified mainly at the sn-1,3 positions of the triacylglycerols [41].

A study was done by Vu *et al.* to produce diacylglycerol-enriched structured lipid that contained mainly c9, t11 and t10, c12 isomers of conjugated linoleic acids. A two-step process was employed in the study. First step consisted of synthesizing the structured TAG by lipid-mediated acidolysis of corn oil with CLA. The acidolysis resulted in a structured triacylglycerol that contained 30.4 mol% CLA. 45.5% of the CLA incorporated was located at sn-1,3 positions of the glycerol backbone. In the following step, a lipase-catalyzed glycerolysis was conducted between structured triacylglycerol and glycerol with the aim of producing a diacylglycerol-enriched structured lipid. After 48 hours or reaction time, a product containing 6.8% monoacylglycerol, 31.5% diacylglycerol and 61.1% was obtained [42].

A comparison of linoleic and conjugated linoleic acid incorporations into tristerain was done in a study conducted by Yang *et al.* In the study, the acyl incorporation and migration of linoleic and conjugated linoleic acids in enzymatic acidolysis were compared in a solvent-free system. In both of the systems Lipozyme RM IM-catalyzed acidolysis at 60° C temperature and 5% by weight enzyme load (based on substrates) was conducted. One system was composed of tristearin (SSS) and linoleic (L) or conjugated linoleic (cL) acids (1:6, mol/mol) while the other was between tristearin and the mixture of linoleic and conjugated linoleic acids (1:3:3, mol/mol/mol). The triacylglycerol composition of the products were monitored with gas chromatography, pancreatic lipase hydrolysis, and high performance liquid chromatography. Both acyl incorporation and migration of linoleic acid was found to

be faster compared to those of conjugated linoleic acid. As a result of 5 hour of reaction, there were 13.0% LLL, 46.5% LSL, 27.7% LSS, and 5.6% SSS in the product for a system between tristearin and linoleic acid. On the other hand, there were 2.4% cLcLcL, 10.4% cLScL, 50.9% cLSS, and 36.2% SSS in the product for a system between tristearin and conjugated linoleic acid. Looking at the results, it was suggested that linoleic acid was more reactive than conjugated linoleic acid in the enzymatic acidolysis probably due to its rigid structure [43].

In another study done by Kawashima *et al.* it was aimed to prepare the regioisomers of the structured lipid that was created using one mole and two moles of caprylic acid. It was found that the TAG consisting of medium-chain fatty acid (MCFA) at the 1,3-positions and long-chain fatty acid (LCFA) at the 2-position, and the TAG with LCFA at the 1(3)-position and MCFA at 2,3(1)-positions were regioisomers. A large-scale production of the two TAG regioisomers was attempted. As LCFA, a commercially available FFA mixture (FFA-CLA) containing 9-*cis*, 11-*trans* (9*c*, 11*t*)- and 10*t*,12*c*-CLA , and for MCFA caprylic acid was selected. Consequently, regioisomers were purified from reaction mixtures by stepwise short-path distillation. The results point out that a process employing immobilized lipase *R.miehei* and short-path distillation is effective for the large-scale preparation of regiospecific TAG isomers [44].

2.8 Optimization

The relationship between the system input and output characterizes any system or process. If the engineering principle or mechanism of a process or system is not fully understood, an empirical modeling of the process is used to study it. The output from the system is optimized as soon as the system or process is known. Optimization, defined as getting the possible best from a given situation, is done by conducting experiments considering the carefully calculated input values. These input values are referred to as, independent variables, or process variables or the factors, whereas the output is called the response or the dependent variable.

Any optimization problem is composed of three parts;

-Set of variables that can be controlled with optimization method.

-Set of requirements that must be achieved with optimization method

-The objective function; performance measure to compare one alternative to another.

Objective function refers to the function to be optimized (minimization, maximization) [45].

2.8.1 Regression analysis

Regression is used for predicting the value of a response. Regression analysis alone does not give causal relationships. Regression analysis can be used to test the validity of the hypothesized relationships in case a hypothesis about the dynamics of a system is proposed. For a successful regression analysis, plotting data is important. Without examining plots of the dependent variable(s) vs. the independent variable(s) first, blindly applying computer programs to perform regression computations can be misleading for researchers [46].

2.8.1.1 Linear regression

This basic regression model uses one independent variable in which the statistical relation is linear.

$$Y_i = \beta_0 + \beta_1 X_i + \varepsilon_i \quad i = 1,2.....,n \qquad \textbf{(2.1)}$$

Y_i : the response in the *i*th case

X_i : the value of the independent variable in the *i*th case

β_0 and β_1 : parameters / coefficients of the regression equation to be estimated in analysis

ε_i : the unexplained deviation between the observed value of *Y* and its predicted value, called a "residual" [46, 47].

The regression function, also called the response function, shows the relationship between the expected (mean) value of Y and the value of the independent variable X. For linear regression model, the regression function is:

$$E\{Y\} = \beta_0 + \beta_1 X_i \qquad \textbf{(2.2)}$$

The graph of this function is referred to as the *regression line*. Parameter β_0 is the *intercept* of the line, while β_1 is the *slope* [47].

The regression parameters are to be estimated from sample data. As n shows number of sample cases with each one consisting of an observation on the dependent variable

and an observation on the independent variable. The observations are represented as (X_i ,Y_i) for the *i*th case.

It is possible to obtain the point estimates of β_0 and β_1 with the *method of least squares*. In this method, the goodness of a fit of a straight line is measured by the sum of the squared deviations (Q). For the least squares line, the sum of the squared vertical distances of the Y observations from the fitted line is minimum. We end up with the equations 2.3 and 2.4 when we want to obtain the least squares estimators of β_0 and β_1 (b_0 and b_1, they need to minimize Q) [47].

$$b_1 = \frac{\sum\left[\left(X_i - \overline{X}\right)\left(Y_i - \overline{Y}\right)\right]}{\sum\left(X_i - \overline{X}\right)^2} \quad \textbf{(2.3)}$$

$$b_0 = \overline{Y} - b_1\overline{X} \quad \textbf{(2.4)}$$

Based on estimated regression coefficients, the *estimated regression function* is as follows:

$$\hat{Y} = b_0 + b_1X \quad \textbf{(2.5)}$$

In equations 2.3 and 2.4, the estimates b_1 and b_0 minimize the sum of squared differences between the observed and the predicted values of y. In other words, they minimize the sum of squared residuals:

$$SS_{RES} = \sum (y_i - \hat{y})^2 \quad \textbf{(2.6)}$$

This is how the regression equation is fit to the data using the *least squares method*. [46] ANOVA (analysis of the variance) can be used to test the affectivity of the obtained regression. The confidence interval, however, cannot be obtained from ANOVA directly [46]. To assess the quality of regression equation, another criteria, the coefficient of determination (R^2), given in 2.7, can be used.

$$R^2 = 1 - \frac{SS_{Res}}{SS_{Tot}} \quad \textbf{(2.7)}$$

The R^2 value higher than 0.75 is acceptable, as a value of 1 indicates a completely linear relationship between the results and the regression [46].

2.8.1.2 Multiple linear regression

Sometimes, for obtaining an acceptable prediction of a response, *y* , more than one independent variable may be required. Multiple regression allows multiple independent variables to be included in the regression equation for *y*

In one situation, because the relationship between *x* and *y* is not a straight line, a polynomial in a single variable, *x,* is needed, as shown in 2.8

$$y = \beta_0 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 \quad \textbf{(2.8)}$$

In another case, more than one independent variable may be influencing the response, as given in 2.9.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 \quad \textbf{(2.9)}$$

Sometimes, combination of these two cases may exist [46].

In multicolinearity, two or more independent variables are highly correlated with each other. This correlation, because it would influence the values of the estimated coefficients, *b*i*'s,* may lead to incorrect conclusions about the importance of each term in the model. An approach called response surface methodology (RSM), helps avoiding the problems of multicolinearity [46].

2.8.2 Response surface methodology

Response surface methodology (RSM) is an empirical modeling approach that uses polynomials as local approximations to the system input/output relationship to understand the quantitative relationships between multiple input values and one output response. RSM, often focusing on optimizing the response, can be extended to multiple responses [45].

An RSM experiment must be composed of quantitative factors. First, a set of samples must be prepared according to the conditions specified by the selected RSM treatment structure. After the analysis of samples by a sensory panel, a stepwise regression analysis is conducted with the obtained average responses. As a result of the analysis, a predictive equation relating the value of the response(s) to the values of the independent variables is obtained. This equation can be depicted graphically in a response surface plot .The predicted relationship, can also be displayed in an easily

interpretable "contour plot".

Several types of designed experiments can be used in RSM. In one part of the plan all possible combinations of the low and high levels of independent variables are shown. This part in a two-factor RSM experiment is composed of four points: (low, low), (low, high), (high, low), and (high, high).In the RSM experiment, the factorial part is increased by a center point .The center point is replicated at least three times for providing an independent estimate of experimental error. The RSM is designed in a way is that the low levels of all the factors are assigned to the coded value of –1; the high levels are all assigned the coded value of +1; and the center point is assigned the coded value of 0 [46].

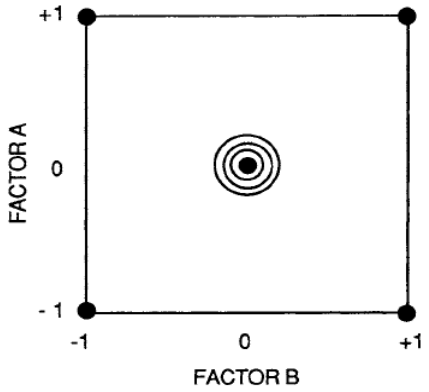

Figure 2.5: A two factor, first-order RSM experiment [46].

In Figure 2.5, the arrangement of factorial and the center points in an RSM experiment with two independent variables is shown. This type of RSM experiment is called a first-order RSM experiment. The regression equation is as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_k X_k \qquad \textbf{(2.10)}$$

In equation, β_1 is the coefficient of the regression equation to be estimated, X_1 is the coded level of the k-factors in the experiment. These first-order RSM experiments are used in identifying general trends and in determining whether the correct ranges have been selected for the independent variables or not.

First-order models are usually inadequate in predicting the response in the presence of a complex relationship between the dependent variable and the independent variables. In this case, a second-order RSM structure is required. The regression

model that can be fit to a second-order RSM structure is as shown below:

$$Y=\beta_0 + \beta_1 X_1 + \beta_2 X_2 + ...\beta_k X_k \quad \textbf{(2.11)}$$
$$+ \beta_{11} X_1^2 + \beta_{22} X_2^2 + ...\beta_{kk} X_k^2$$
$$+ \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + ...\beta_{k-1,k} X_{k-1} X_k$$

By adding squared and cross-product terms in the model, the predicted response surface is allowed to "bend" and "flex,"which results in an improved prediction of complex relationships. A widely used type of second-order RSM experiments are the central-composite structures. To develop central-composite experiments, a set of axial or points are added to a first-order RSM [46].

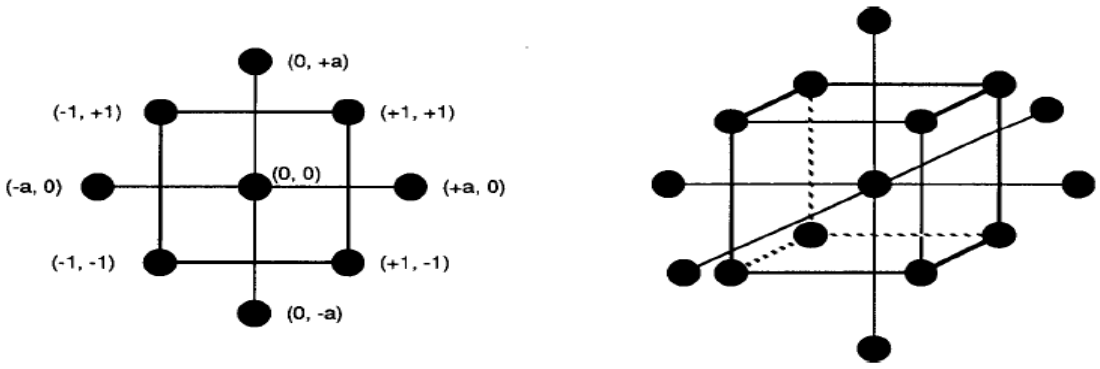

Figure 2.6: Central composite RSM experiment [45].

In Figure 2.6, the arrangement of the factorial, axial, and center points in an RSM experiment with two and three independent variables is illustrated.

A face centered central composite design is a type of composite RSM experiment with its independent variables confined within upper and lower bounds. It has a cuboidal design [45].

3. MATERIALS AND METHODS

3.1 Materials

The corn oil used in the study was a commercially available food grade corn oil, "Bizim Yağ", and it was obtained from a local store. It was a product of Besler Gıda, İstanbul and was distributed by Ülker. CLA was obtained as gel capsules from GNC under the market name TONALIN-CLA. One CLA capsule containment was noted as 1000 mg. A sample of commercially available lipase, Lipozyme TL IM was supplied by Nova-Nordisk A/S (Copenhagen, Denmark). Immobilized on silica, Lipozyme TL IM is a sn-1,3 spesific lipase obtained from the microorganism *Thermomyces lanuginosa.* All other chemicals used as solvent or used in the purification of reaction products and their analysis were obtained from Merck (Darmstadt, Germany).

3.2 Methods

3.2.1 Characterization of materials

All substrates and TAG products of the acidolysis reactions, in order to be analyzed, were converted to corresponding methyl esters by BF_3 esterification method. Methyl esters were analyzed by a Hewlett-Packard 5890 II gas chromatograph (Hewlett-Packard, Waldron, Germany). The conditions for analysis are listed on Table 3.1.
Peaks were identified by comparing the retention times with those of a mixture of standart methyl esters analyzed at same conditions.

Table 3.1 : Analysis conditions for GC [23].

Detector Type	FID [1]
Detector Temperature (°C)	280
Injection Temperature (°C)	250
Gas flow rates (mL / min)	
Nitrogen	1.6
Hydrogen	33
Air	460
Split ratio	88:1
Oven Temperature	150 °C (5 min) 150-275 °C (5°C/min) 275°C (5min)
Column Type	Capillary Column TRB-5HT (2)

(1) Flame ionization detector (2) 30m x 0.25 mm x 0.10 µm film thickness of %5 diphenyl and %95 dimethyl polysiloxane

3.2.2 Acidolysis reactions

The acidolysis reactions were carried out in dark-colored, heat-resistent 25mL screw-capped flasks containing 1 g of substrate mixture consisting of CLA and corn oil at different molar ratios, different amounts of Lipozyme TL IM and 5mL of *n*-hexane. The mixtures were incubated for different periods in an orbital shaker (Edmund Bühler, KS-15, Germany) at 200 rpm at various temperatures ranging from 30ºC to 60ºC. The flasks were taken out of the orbital shaker when the desired reaction time came to an end and samples of 2 mL were withdrawn from the reaction mixtures to an erlenmeyer flask for analysis. All reactions were duplicated.

3.2.3 Analysis of products following acidolysis

Following withdrawal from reaction flask, 5mL of ethanol and 5mL of n-hexane was added to each sample in the erlenmeyer flask to inactivate the reaction and then immediately titrated against 0.02 mol L^{-1} NaOH to neutralise the released and unused FFAs. Phenolphtalein was used as indicator in observing the conversion of fatty acids into their sodium salts. The conversion was observed by the change of color from white to pink. The mixture was placed in a separatory funnel and mixed

with 25 mL of distilled water and 50 mL of n- hexane to perform liquid-liquid extraction. TAGs were soluble in organic phase thus separated from the water phase in which sodium salts were soluble. The extraction procedure was repeated several times by adding water to organic phase until a neutral pH was reached. The water phase was discarded. The pH was controlled with a pH paper. The hexane was subsequently evaporated in a rotary evaporator at 80°C and the remaining TAGs were recovered for analysis. To determine the fatty acid composition of the TAGs, they were first converted to their corresponding fatty acid methyl esters (FAMEs) by the BF_3/methanol esterification procedure (AOCS method Ce-2-66). The fatty acid composition of the FAMEs were then determined by capillary gas chromatography.

3.2.4 Selecting the independent variables for experimental design

Substrate molar ratio, reaction temperature, enzyme amount, reaction time and solvent type are known to be the important factors in acidolysis reactions . Based on knowledge from literature and previously conducted experiments, two variables were selected to be optimized using response surface methodology. The two factors selected were temperature (X_1, ºC) and substrate molar ratio (X_2, CLA/Corn Oil. The response (dependent variable) was CLA content of the product (weight %).

3.2.5 Experimental design and optimization of selected parameters

The reaction independent variables were optimized by response surface methodology .A three-level, two-factor face centered cube design was selected. To determine the maximum (+1), minimum(-1) and mean(0) values for the selected independent variables, several experiments were done, studying the effect of temperature and substrates mole ratio as well as the effects of enzyme amount and reaction time. Then an experimental design consisting of 11 experiments was created. All the experiments were duplicated. In the experiments, every combination of minimum, maximum and mean values for the two independent variables plus three more experiments in which only mean values for both independent variables were investigated were conducted. The data from experiments were analyzed by response surface using software Statistica 6.0.

4. RESULTS AND DISCUSSION

This study was done to produce a structured corn oil in which conjugated linoleic acid was incorporated and to optimize the reaction parameters via response surface methodology. Selecting the parameters to be optimized was decided by conducting pre-optimization experiments and literature research data. Following determination of selected independent variables and their corresponding levels, +1, 0 and -1, a central composite design with 2 variables and 3 levels were computed, resulting in 11 experiments. The data obtained from experiments were computed by Statistica 6.0 software, to optimize the selected parameters.

4.1 Fatty acid composition of substrates

At the beginning of the study, the fatty acid composition of corn oil was determined by GC under the analysis conditions described in Section 3.2.1. Table 4.1 shows the fatty acid compositions of corn oil previously determined by GC.

Table 4.1: Fatty acid composition of corn oil

Fatty acid	Corn Oil (weight %)
C 16:0	10.4
C 18:2	55.5
C 18:1	30.9
C 18:0	2.2

4.2 Determining the independent variables and their levels

Pre-optimization experiments investigated the effects of substrates molar ratio, temperature, time and enzyme amount on incorporation of CLA into corn oil. It was seen that at constant temperature (50°C) and enzyme amount (10 w/w %), increased amount of CLA resulted in increased incorporation , while increased reaction time (1 , 3 and 5 hours) had no effect. Table 4.2 presents the results of these experiments.

Table 4.2 : Incorporation of CLA into corn oil after enzymatical acidolysis of substrates at molar ratios of 1:3, 1: 5, 1:7 and 1:9 for 1, 3 and 5 hours.

	Incorporation of CLA into corn oil (weight %)			
Reaction time (h)	Substrates molar ratio (mole / mole)			
	1:3	1:5	1:7	1:9
1	39.4	49.5	55.7	59.1
3	39.9	48.7	56.3	59.0
5	40.0	50.1	56.2	60.0

Consequently, it was decided that it would be necessary to study the effects of enzyme amount and temperature as well.

The effect of enzyme amount was investigated at constant substrates molar ratio (1:7), temperature (50°C) and reaction time (3 h). As presented in Table 4.3, the increasing amount of enzyme had no considerable effect on incorporation of conjugated linoleic acid into corn oil.

Table 4.3: The effect of enzyme amount used in enzymatic acidolysis for incorporation of CLA into corn oil

Enzyme amount (w / w)	CLA incorporated into corn oil (weight %)
10	56.3
15	56.2
20	55.7

Temperature was the other parameter expected to be effective in enzymatic acidolysis of CLA and corn oil, therefore the effect of temperature was observed via experiments at which other parameters like molar ratio (1:7), enzyme amount (10 w/w) and reaction time (3 h) were kept constant. The results for these experiments are given in Table 4.4.

Table 4.4: The effect of temperature on enzymatic acidolysis of CLA and corn oil.

Temperature (°C)	CLA incorporated into corn oil (weight %)
30	55.5
45	56.4
60	55.5

4.3 Experimental Design for Response Surface Methodology

According to the pre-optimization experiments' data, the ranges of settings for the two independent variables were chosen as follows: reaction temperature, 30-60ºC; CLA/Corn oil molar ratio, 3-7. In experimental design, a two-variable, three level central composite face centered cube design was used. It consisted of 11 experiments. The values corresponding levels for the selected independent variable levels are shown in Table 4.5.

Table 4.5: Independent variables and their coded values used at experimental design.

Independent Variables	Coded variable levels		
	-1	0	+1
Corn oil: Conjugated linoleic acid molar ratio	3	5	7
Temperature (°C)	30	45	60

Table 4.6 presents the experimental design consisting of 11 experimental points and their coded values

Table 4.6: Selected independent variables for experimental design of 11 experiments.

Number of Experiment	Independent Variables			
	Temperature (°C)		Corn Oil: CLA molar ratio (mole / mole)	
	Code	Value	Code	Value
1	-1	30	-1	1:3
2	-1	30	0	1:5
3	0	45	-1	1:3
4	+1	60	+1	1:7
5	+1	60	0	1:5
6	0	45	+1	1:7
7	- 1	30	+1	1:7
8	+1	60	-1	1:3
9	0	45	0	1:5
10	0	45	0	1:5
11	0	45	0	1:5

The results of the optimization experiments are given in Table 4.7

Table 4.7: Responses obtained for the selected independent variables.

Number of experiment	Temperature (°C)	Corn oil: CLA Molar ratio (mole / mole)	Response (CLA weight %)
1	30	1:3	39.8
2	30	1:5	48.9
3	45	1:3	40.6
4	60	1:7	55.9
5	60	1:5	49.4
6	45	1:7	56.5
7	30	1:7	55.2
8	60	1:3	40.1
9	45	1:5	50.0
10	45	1:5	49.8
11	45	1:5	50.1

4.4 Statistical evaluation of reaction parameters

The results given in Table 4.7 were evaluated using Statistica 6.0 (Stat Soft ® Inc.) software. The effects of independent variables on the response were calculated. Variance analysis were done. For the central composite design with 2 variables and 3 levels, the following quadratic polynomial was used.

$$Y=\beta_0 + \beta_1 X_1 +\beta_2 X_2+...\beta_k X_k + \beta_{11} X_1^2 + \beta_{22} X_2^2 +...\beta_{kk} X_k^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + ...\beta_{k-1,k} X_{k-1} X_k \quad \textbf{(4.1)}$$

In the equation, Y is the response, β_0 is the mean effect, and β_k , β_{kk} , and $\beta_{k-1,k}$ are representing the linear regression coefficient, quadratic regression coefficient and the linear regression coefficent of the interaction of independent variables respectively.

The linear and quadratic effects and coefficients were determined , according to the

interactions between independent variables and responses.

The regression analysis results are shown in Table 4.8.

Table 4.8: Regression coefficients of the second-order polynomials for response

	Effect	Standart error	t(5)	p	Coefficient
Mean/Intercept	49.96316	0.063595	785.6463	0.000000	49.96316
CLA:Corn Oil(L)	15.70000	0.101221	155.1067	0.000000	7.85000
CLA: Corn Oil(Q)	- 2.81579	0.155775	-18.0760	0.000010	-1.40789
Temperature (L)	0.50000	0.101221	4.9397	0.004323	0.25000
Temperature (Q)	- 1.61579	0.155775	-10.3726	0.000143	- 0.80789
Molar ratio(L) by temperature (L)	0.20000	0.123969	1.6133	0.167598	0.10000

Considering the data in Table 4.8, the following quadratic equation for the response is obtained.

$$CLA\ \% = 50 + 7.9\ M - 1.4\ M^2 + 0.2\ T - 0.8\ T^2 + 0.1\ MT \qquad \textbf{(4.2)}$$

In the equation, M represents the substrate molar ratio and T represents temperature. Table 4.9 gives the variance analysis. Evaluating the p values, it can be concluded that the interaction between substrate molar ratio and temperature cannot be trusted, for it has a p value of 0.67598. A p value below 0.01 indicates a confidence interval of 99% while the values that are bigger are not to be trusted. The correlation coefficient, R^2, was calculated as 0.99, pointing out to the good quality of regression equation. The sum of residuals was an acceptable value of 0.15.

Table 4.9: Variance Analysis

	SS	df	MS	F	p
CLA: Corn Oil molar ratio (L)	369.7350	1	369.7350	24058.10	0.000000
CLA: Corn Oil molar ratio (Q)	5.0215	1	5.0215	326.74	0.000010
Temperature (L)	0.3750	1	0.3750	24.40	0.004323
Temperature (Q)	1.6535	1	1.6535	107.59	0.000143
Molar ratio * Temperature (L)	0.0400	1	0.0400	2.60	0.167598
Error	0.0768	5	0.0154		
Total SS	379.0673	10			

In Table 4.10, the observed minimal, the observed maximal and the critical values for the independent variables are given.

Table 4.10: Observed minimal, maximal and critical values

	Observed minima	Critical value	Observed maxima
CLA: Corn Oil (molar ratio)	3	10,59900	7
Temperature (°C)	30	49,91973	60

Based on equation (4.2), the CLA incorporation into corn oil at these critical values was calculated as 61.2 % (weight %).

Figure 4.1 shows the plot of the observed values vs the predicted values

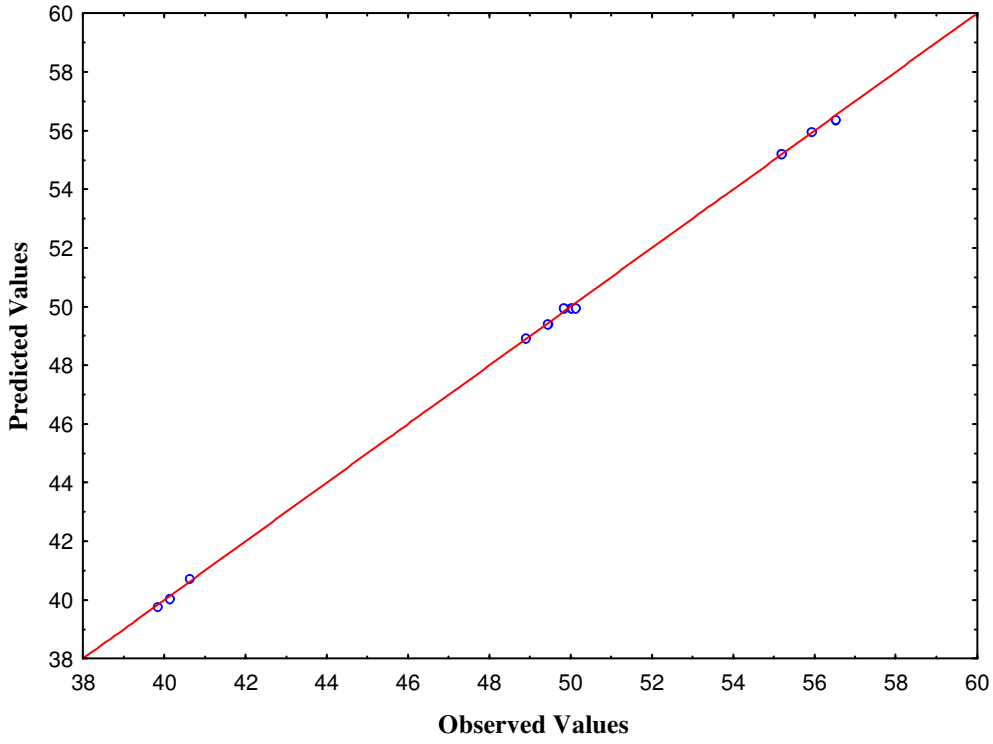

Figure 4.1: The predicted and experimental values of CLA incorporation into corn oil

4.5 Interpreting the Response Surface and Contour Plots

The response surface and contour plots in Figures 4.2 and 4.3 show the effects of temperature and CLA: corn oil molar ratio on the enzymatic incorporation of CLA into corn oil.

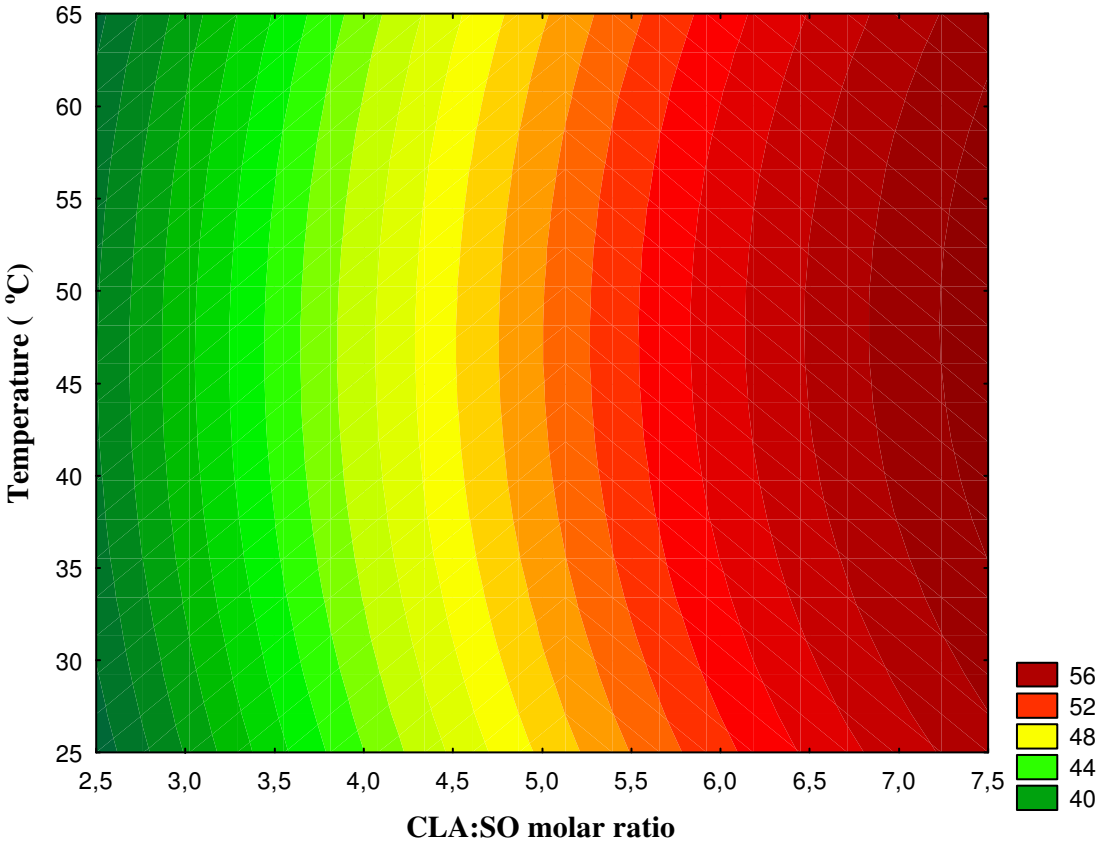

Figure 4.2: The contour plot demonstrating the effect of temperature and CLA: CO (corn oil) molar ratio on CLA incorporation (weight %).

The independent variables, temperature and substrates (CLA and corn oil) molar ratio, are shown on x and y axis on the contour plot. The response, CLA incorporation into corn oil is shown with different color levels of related areas. According to the contour plot, the high incorporation of CLA into corn oil was observed at a temperature range of 30-60°C and at a substrates mole ratio between 7.0 and 7.5. The effect of temperature and CLA: corn oil molar ratio on the enzymatic incorporation of CLA into corn oil is demonstrated on three dimensional response surface plot on figure 4.3.

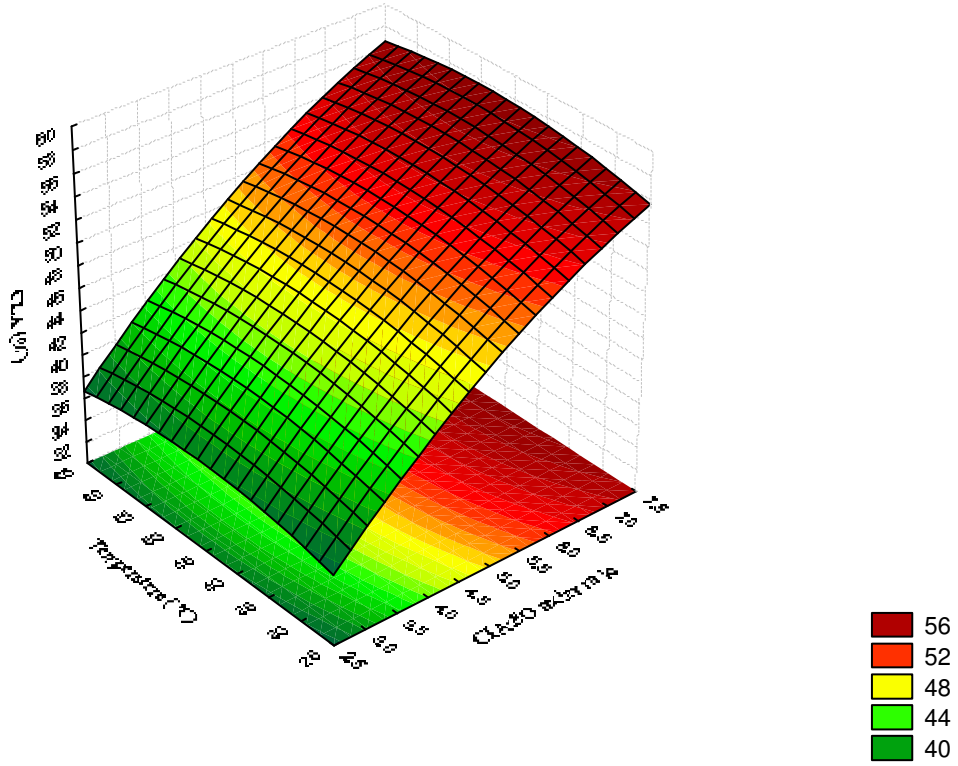

Figure 4.3: The response surface plot illustrating the effects of temperature and CLA: corn oil molar ratio on CLA incorporation

At the response surface plot shown at Figure 4.3, the levels of variables are illustrated on three dimensions.

Looking at the plots, it is possible to briefly say that substrates molar ratio has the greatest effect on enzymatic incorporation of CLA into corn oil, whereas the other independent variable, temperature, has little, almost no impact on CLA incorporation, when compared to the that of substrates molar ratio.

5. CONCLUSIONS

Corn oil is widely consumed as an edible oil around the world. Since the physical properties allow it , corn oil is generally used in frying processes, which do not result in foods that are considered as beneficial to health. Therefore, corn oil can be restructured in a way that it gains properties making it healthier.

Searching through literature, it was concluded that it would be appropriate to enrich corn oil with CLA, a polyunsaturated fatty acid with various health benefits. With the aim of rendering corn oil a healthier edible oil in many aspects, enzymatic acidolysis reactions of CLA and corn oil in n-hexane were conducted. A *sn*-1,3 specific commercial lipase, Lipozyme TL IM was used for catalysis. Experimental data were computed using the software Statistica 6.0 and the reaction parameters were optimized with response surface methodology, using a two-variable and three-level face centered cube design. In the resulting structured lipid, it was observed that CLA incorporation into corn oil was quite successful, with a percentage of 60 (weight %).

For future studies, it can be useful to investigate the effects of other reaction parameters. For example, the effects of solvents other than hexane can be studied. A study in which another enzyme other than Lipozyme TL IM is used would be useful for observing the effects of temperature, enzyme amount, time and substrates molar ratio on process yield. For investigating the effects of the structured lipid on health, conducting animal trials is suggested. Methods for large-scale production of the structured lipid is another promising area in research of structured lipids.

REFERENCES

[1] **O'Keefe, S. F**., 2002. Nomenclature and Classification of Lipids. In: *Food Lipids: Chemistry, Nutrition, and Biotechnology. (* C. C. Akoh and D. B. Min, eds.) M. Dekker, New York. pp. 1-2.

[2] **Fahy, E., Subramaniam, S., Brown, H., Glass .C.K., Merrill, A.H., Murphy, R.C., Raetz, C.R.H., Russell, D.W., Seyama , Y., Shaw, W. Shimizu, T., Spener, F., van Meer, G., VanNieuwenhze, M.S., White, S.H., Witztum, J.L., Dennis, E.A**. A comprehensive classification system for lipids. *J. Lipid Res.* 46:839–861 (20:05).

[3] **Kyle, D.J.** 2002. Essential Fatty Acids as Food Additives. In: *Food Additives.* (A.L. Branen, P.M. Davidson, S. Salminen, and J.H. Thorngate III, eds.) M. Dekker, New York, NY. pp. 227-28.

[4] **Welch, A.V., Borlak, J.T.** 2000. Absorption and Transport of Dietary Lipid. In *Fatty Acids in Foods and Their Health Implications.*(C.K. Chow, ed.) M. Dekker, New York, NY. pp. 451-452.

[5] **Clandinin, M.T., Thomson, A.B.R.** 2000. Intestinal Absorption of Lipids: A View Toward the Millennium. In: *Fat Digestion and Absorption*. (A. B.Christophe and S. De Vriese, eds.) AOCS Press,Champaign, Illinois. pp. 298-305.

[6] **Lora, K.R., Lewis, N.M.** 2007. Omega 3 and Omega 6 Fatty Acids. In: *Sports Nutrition: Fats and Proteins*. (J. A. Driskall, ed.) CRC Press.pp.63-69.

[7] **Watkins, S.M., German, J.B**. 2002. Unsaturated Fatty Acids. In: *Food Lipids: Chemistry, Nutrition and Biotechnology* (C. C. Akoh and D. B. Min, eds.) M. Dekker, New York. pp. 559-582.

[8] **Russo, C.L.** 2009. Dietary *n*-6 and *n*-3 polyunsaturated fatty acids: From biochemistry to clinical implications in cardiovascular prevention. *Biochemical Pharmacology.* 77:937-946.

[9] **Hoy, C-E., Mu, H.** 2000. Effects of Triacylglycerol Structure on Fat Absorption. In: *Fat Digestion and Absorption*. (A.B.Christophe and S. De Vriese, eds.) AOCS Press, Champaign, Illinois. pp. 220, 224.

[10] **Niot, I., Poirier, H., Tran, T.T.T., Besnard, P.** 2009. Intestinal Absorption of long-chain fatty acids : Evidence and Uncertainties. *Progress in Lipid Research.* 48 :101–115.

[11] **Beermann, C., Jelinek, J., Reinecker, T., Hauenschild, A., Boehm, G., Klör, H-U.** 2003. Short term effects of dietary medium-chain fatty acids and n-3 long-chain polyunsaturated fatty acids on the fat metabolism of healthy volunteers. *Lipids in Health and Disease*. 2:10.

[12] **Nelson, G.J**. 2000. Effects of Dietary Fatty Acids on Lipid Metabolism. In: *Fatty Acids in Foods and Their Health Implications.*(C.K. Chow, ed.) M. Dekker, New York, NY. pp. 490-491.

[13] **Rebouche, C.J.** 1992**.** Carnitine function and requirements during the life cycle. *FASEB J*. 6:3379-3386.

[14] **Giacosa, A., Hull ,W.E., Haubner, R., Owen, R.W., Spiegelhalder, B., Bartsch, H.** 2000. Olive-oil consumption and health: the possible role of antioxidants. *Lancet Oncol*. 1:107-12.

[15] **McNeill, G.P**. 1998. Enzymatic processes. In: *Lipid Synthesis and Manufacture*. (F.D. Gunstone, ed.) Boca Raton: Sheffield Academic Press. pp. 289-296.

[16] **Marangoni, A.G.** 2002.Lipases : Structure, Function, and Properties. In: *Lipid Biotechnology.* (T.M. Kuo and H.W. Gardner, eds.) M. Dekker, New York. pp. 357-369.

[17] **Diks, R.M.M., Bosley, J.A**. 2000. The Exploitation of Lipase Selectivities for the Production of Acylglycerols. In : *Enzymes in Lipid Modification*. (U.T. Bornscheuer, ed.)Wiley-VCH, Weinheim, New York. pp.3-4.

[18] **Willis, W.M., Marangoni, A.G.** 2002**.** Enzymatic Interesterification. In: *Food Lipids: Chemistry, Nutrition and Biotechnology.(*C. C. Akoh and D. B. Min, eds.)M. Dekker, New York. pp. 839-844.

[19**] Akoh, C.C., Kim, B.H.** 2002. Structured Lipids. In: *Food Lipids: Chemistry, Nutrition and Biotechnology. (*C.C.Akoh and D.B.Min, eds.) M. Dekker, New York. pp. 841-862.

[20] **Camp, J.V., Huyghebaert, A., Goeman, P.** 1998. Enzymatic Synthesis of Structured Modified Fats. In: *Structural Modified Food Fats: Synthesis , Biochemistry, and Use*. (A.B. Christophe, ed.)AOCS Press, Champaign, Illinois. pp. 25-29.

[21] **Perkin, K.L**. 1993. Environmental Effects on Enzyme Activity. In: *Enzymes in Food Processing.* (T. Nagodawithana and G. Reed, eds.).Academic Press Inc. pp. 57-64.

[22] **Whitaker, J.R.** 1994. Principles of Enzymology for the Food Sciences. Dekker, New York. page:167.

[23] **Öztürk, T**. 2007. Optimization of Caprylic Acid Incorporation into Corn Oil via Enzymatic Acidolysis using Response Surface Methodology, M.Sc Thesis, Istanbul Technical University, Institute of Science and Technology. pp: 22-23.

[24] **Weete, J.D., Lai, O-M., Akoh, C.C.** 2002. Microbial Lipases. In: *Food Lipids: Chemistry, Nutrition and Biotechnology (*C. C. Akoh and D. B. Min, eds.) M.Dekker, New York. pp. 813-830.

[25] **Godtfredsen, S.E.** 1993. Lipases. In: E*nzymes in Food Processing*. (T. Nagodawithana and G. Reed, eds.) Academic Press Inc. pp. 205-214.

[26] **Gunstone, F.** 2001.Why Are Structured Lipids and New Lipid Sources Required? In: *Structured and Modified Lipids.* (F.D. Gunstone, ed.) M.Dekker, New York. pp. 1-3.

[27] **Hoy, C-E., Xu,X.** 2001.Structured Triacylglycerols. In: *Structured and Modified Lipids.* (F.D. Gunstone, ed.) M.Dekker, New York. pp. 209-210.

[28] **Iwasaki, Y., Yamane, T.** 2000. Enzymatic syntesis of structured lipids. *Journal of Molecular Catalysis B: Enzymatic*. 10: 129-140.

[29] **Porsgaard, T.** 2006. Clinical Studies with Structured Triacylglycerol. In : *Handbook of Functional Lipids*. (C. Akoh, ed.) Boca Raton: CRC Press, pp. 419-420.

[30] **Akoh, C**. 2000. Fat-Based Fat Substitutes. In: *Fatty Acids in Foods and Their Health Implications* .(C.K.Chow, ed.) M. Dekker, New York. pp.375-382.

[31] **Bornscheuer, U.T., Adamczak, M., Soumanou, M.M.** 2003. Lipase-catalyzed synthesis of modified lipids. In: *Lipids for Functional Foods and Nutraceuticals*. (F.D.Gunstone, ed.) The Oily Press. pp. 149-153.

[32] **Lee, J-H., Lee, K-T.** 2006. Structured Lipids Production. In: *Handbook of Functional Lipids*. (C. Akoh, ed.) Boca Raton: CRC Press. pp. 497-505.

[33] **Finley, J.W., McDonald, A., Klemann, L.P.** 1996. Low Calorie Fats. In: Lipid Technologies and Applications.(F. D. Gunstone, F. B. Padley, eds.) M. Dekker, New York. pp.508-509.

[34] **Salunkhe, D.K., Chavan, J.K., Adsule, R.N.** 1992. Corn (Maize). In: *World Oil Seeds: Chemistry, Technology and Utilization.* (S.S. Kadam, ed.)Van Nostrand Reinhold, New York. pp. 403-414.

[35] **Moreau, R.A.** 2005. Corn Oil. In: Bailey's Industrial Oil and Fat Products. (F.Shahidi, ed.) Wiley, New York; Chichester. pp. 149-158.

[36] **O'Brien, R.D.** 2004. Fats and Oils: Formulationg and Processing for Applications. Boca Raton, Fla: CRC Press. pp. 21-22.

[37] **Adamczak, M., Bornscheuer, U.T., Bednarski, W.** 2008. Properties and biotechnological methods to produce lipids containing conjugated linoleic acid. *Eur. J. Lipid Sci. Technol.* 110: 491–504.

[38] **Fernie, C.E.** 2003. Conjugated linoleic acid. In: *Lipids for Functional Foods and Nutraceuticals*. (F.D.Gunstone, ed.) The Oily Press. pp. 291-311.

[39] **Dunford, N.T.** 2004. Oil and Oilseed-based Bioactive Compounds and their Health Effects. In: *Nutritionally Enhanced Edible Oil and Oilseed Processing.* (N.T. Dunford, H.B. Dunford, eds.) AOCS Press, Champaign, Illinois. pp. 6-7.

[40] **Watkins, B.A., Li, Y.** 2002. Conjugated Linoleic Acids: Nutrition and Biology. In: *Food Lipids: Chemistry, Nutrition and Technology.* (C. C. Akoh and D. B. Min, eds.) M. Dekker, New York. pp. 582-586.

[41] **Martinez, C.E., Vinay, J.C., Brieva, R., Hill, C.G., Garcia, H.S.** 2003. Lipase-catalyzed Acidolysis of Corn Oil with Conjugated Linoleic Acid in Hexane. *Journal of Food Lipids.* 10: 11-24.

[42] **Vu, P-L., Park, R-K., Lee, Y-J., Kim, Y-M., Nam, H-Y., Akoh, C.C., Lee, K-T.** 2007. Two-Step Production of Oil Enriched in Conjugated Linoleic Acids and Diacylglycerol. *J. Am. Oil Chem. Soc.* 84:123-128.

[43] **Yang, T., Xu, X., Li, L.** 2007. Comparison of Linoleic and Conjugated Linoleic Acids in Enzymatic Acidolysis of Tristearin. *Journal of Food Lipids.* 8:149 -161 .

[44] **Kawashima, A., Nagao, T., Watanabe, Y., Kobayashi, T., Ikuo, I., Tominaga Y., Shimada, Y.** 2004. Preparation of regioisomers of structured TAG consisting of one mole of CLA and two moles of caprylic acid. *J. Am. Oil Chem. Soc.* 81: 1013-1020.

[45] **Erdoğdu, F.** 2009. Optimization: An Introduction. In: Optimization in Food Engineering. (F. Erdoğdu, ed.) Boca Raton: CRR Press, New York.pp.111-113

[46] **Meilgaard, M., Civille, G.V., Carr, B.T.** 1999. Sensory Evaluation Techniques, Boca Raton: CRC Press. pp. 314-334.

[47] **Neter, J., Wasserman, W., Whitmore, G.A.** 1993. Applied Statistics. Allyn and Bacon. pp 531-555.

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