**İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY**

# OPTIMIZATION of CAPRYLIC ACID INCORPORATION into CORN OIL via ENZYMATIC ACIDOLYSIS USSING RESPONSE SURFACE METHODOLOGY

**M.Sc. Thesis by**
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**JUNE 2007**

**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ**

# MISIR YAĞI İLE KAPRİLİK ASİTİN ENZİMATİK ASİDOLİZİNİN TEPKİ YÜZEY METODOLOJİSİYLE OPTİMİZASYONU

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May 2007 TARIK ÖZTÜRK

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**ABBREVATIONS**

**ALA** : α-Linoleic acid
**ANOVA** : Analysis of the variance
**AOCS** : American Oil Chemists' Society
**Asp** : Aspartic acid
**CA** : Caprylic acid
**CBS** : Cacao butter substitutes
**CLA** : Conjugated linoleic acid
**DAG** : Diacylglycerols
**DHA** : Docosahexaenoic acid
**DPA** : Docosapentaenoic acid
**EC** : Caprylic acid ethyl esters
**EFA** : Essential fatty acids
**EPA** : Eicosapentaenoic acid
**EPG** : Esterified propoxylated glycerols
**FA** : Fatty acids
**FDA** : U.S. food and drug administration
**GLC** : Gas liquid chromatography
**Glu** : Glutamic acid
**HDL** : High density lipoprotein
**His** : Histidine
**HPLC** : High performance liquid chromatography
**L** : Linoleic acid
**LCFAs** : Long Chain fatty acids
**LDL** : Low density lipoprotein
**Ln** : Linolenic acid
**MAG** : Monoacylglycerols
**MCFAs** : Medium chain fatty acids
**O** : Oleic acid
**P** : Palmitic acid
**$R^2$** : Coefficient of determination
**RSM** : Response surface methodology
**S** : Stearic acid
**SCFAs** : Short chain fatty acids
**SCO** : Single-cell oil
**Ser** : Serine
**SFC** : Supercritical fluid chromatography
**$SS_{res}$** : Sum of squares of residuals
**TAG** : Triacylglycerols
**TL** : *Thermomyces lanuginosa*
**VLDL** : Very low density lipoprotein
**PUFA** : Poly unsaturated fatty acid
**MLM** : Medium long medium

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**OPTIMIZATION of CAPRYLIC ACID INCORPORATION into CORN OIL via ENZYMATIC ACIDOLYSIS USING RESPONSE SURFACE METHODOLOGY**

## SUMMARY

The aims of the study were to produce a MLM type structured lipid by acidolysis and optimize the reaction conditions of acidolysis. Therefore optimization of caprylic acid incorporation into corn oil via enzymatic acidolysis using response surface methodology was studied. Where corn oil was used as a long chain fatty acid (L) source and caprylic acid was used as a medium chain fatty acid (M). The acidolysis reaction of corn oil with caprylic acid was catalyzed by a *Thermomyces lanuginosa* originated sn-1,3 specific lipase, Lipozyme TL IM in hexane. For the optimization of the reaction, caprylic acid content (response) in the resulting structured lipids was determined. The independent variables of the reaction were chosen as enzyme amount (weight % of substrates), substrates mole ratio, and reaction time (h).

Response surface methodology (RSM) based on three-variable (factor), three-level face centered-cube design was used to optimize the reaction. The experimental data were analyzed by response surface procedure using Statistica 6.0 to fit the second-order polynomial model predicted for optimization of corn oil acidolysis. Temperature and agitation rate were kept constant at 50 ºC and 220 rpm, respectively.

The maximum caprylic acid incorporation into corn oil was 29.8% (mol %). The critical values of independent variables for the response at which caprylic acid content of the modified oil was 21.5±0.8 % (mol%) were determined to be as 13.2% enzyme amount based on substrates weight, 1: 3.9 corn oil/caprylic acid mole ratio and 3.1 hours reaction time.

The obtained quadratic polynomial had a regression coefficient of 0.99 which indicated that the predicted response values obtained from the empirical model are in agreement with the observed values in the range of the operating variables. The experimental validation gave a good correlation of % 22 % (mol%) with the computed value of 21.5±0.8 (mol%) at the optimum point.

The resulting structured lipid has many benefits such as; a faster absorption rate, a decreased tendency to be deposited as body fat, a lower calorie value, the ability of being digested in the lack of pancreatic lipase than corn oil and high linoleic acid (C18:2 n-6) at sn-2 position. Linoleic acid which is an essential fatty acid is important in human nutrition. Due to this properties the resulting structured lipid can be used both for a high quality fat for daily use and medical purposes such as, pancreatic insufficiencies and fat malabsorption problems.

## MISIR YAĞI İLE KAPRİLİK ASİTİN ENZİMATİK ASİDOLİZİNİN TEPKİ YÜZEY METODOLOJİSİYLE OPTİMİZASYONU


## ÖZET

Bu çalışmada mısır yağından sn-1,3 pozisyonlarında orta uzunlukta yağ asidi ve sn-2 pozisyonunda da uzun zincirli yağ asidi bulunan yapılandırılmış yağların üretimi amaçlanmıştır. Bu yağların üretiminde orta uzunlukta yağ asidi olarak kaprilik asit (C 8:0) seçilmiştir. Enzimatik asidoliz reaksiyonu tepki yüzey metodolojisine uygun olarak yürütülmüş ve reaksiyon parametreleri optimize edilmiştir. Asidoliz reaksiyonları Lipozyme TL IM varlığında hekzan ortamında 50 ºC ve 220 rpm'de gerçekleştirilmiştir. Enzim miktarı, substratların mol oranı ve reaksiyon süresi bağımsız değişken, mısır yağına katılan kaprilik asitte bağımlı değişken (tepki) olarak seçilmiştir.

Yapılan deneyler sonucu en çok %29,8 mol kaprilik asitin mısır yağına katılması sağlanmıştır. Yüzey tepki metodu ile belirlenen optimum noktalar %13,2 enzim, 1:3,2 mısıryağı:kaprilik asit mol oranı ve 3,15 saat reaksiyon süresidir. Bu koşullarda mısır yağına % 22 kaprilik asit katılımı gerçekleştirilebilir.

Reaksiyon parametreleri ile tepki arasındaki ilişkiyi veren ikinci dereceden polinom olan bir model denklem oluşturulmuştur. Model denklemden elde edilen tahmin tepkileri ile gözlenen tepki değerleri arasındaki lineer bağlantının korelasyon katsayısı 0,99 dur. Optimum noktada yürütülen deneyler sonucunda teorik olarak beklenen % 22,0 lık kaprilik asit yüzdesine karşılık % 21,5 ± 0,8 lik bir kaprilik asit katılımı bulunmuştur. Bu veri de model denkleminin güvenilirliğini desteklemiştir.

Bu çalışma sonucu elde edilen yapılandırılmış yağ, çabuk absorbe edilme özelliğine sahiptir ve vücut yağı olarak depolanma eğilimi de düşüktür. Düşük kalorili olan bu yağın sindirimi için pankreatik lipaza ihtiyaç duyulmaz ve sn-2 pozisyonunda yüksek oranda esansiyel bir yağ asidi olan linoleik asit (C 18:2 n6) içermesi de bu yağın değerini büyük ölçüde arttırmaktadır.

Elde edilen bu yağ gıda sanayinde bir yağ ikame maddesi olarak değerlendirilebilir. Tıbbi olarak da bu yağ, pankreatik enzim yetersizliği ve/veya yağ emiliminde sorunları olan hastalar için bir esansiyel yağ asidi kaynağı olarak kullanılabilir.

## 1. INTRODUCTION

Science can roughly be defined as the sum of organized knowledge on a specific subject obtained by scientific method. Technology can be defined as the application of this systematic knowledge to produce values in terms of goods or services. Thus scientific improvements have usually been followed by technological developments. Expectations and realities are changed by technologic implementation. People are expecting healthier and longer lives in a more realistic sense due to the developments in nutritional and biological sciences. In order to satisfy these expectations there is a gap for new nutrients. Nutrients that provide such advantageous health benefits ahead of their actual nutritional effects are called nutraceuticals or functional foods if these nutraceuticals are in forms of ordinary foods or added to them.

The market of non fat low calorie fat substitutes and low calorie fat substitutes is increasing due to the increased awareness of obesity and cardio vascular diseases related to high fat intake. Although the nonfat low calorie fat substitutes have many uses they are mostly heat unstable or have other adverse effects such as decreased absorption of fat soluble vitamins and digestion problems. Therefore, fat based fat substitutes are more beneficial in many cases as their enhanced physical properties ability to replace a higher variety of fats and less adverse effects.

Structured lipids are modified triacylglycerols. These modifications can be related to the fatty acid composition and specific positioning of these fatty acids in more advanced cases. These modifications devote advantageous properties to the modified lipid. These properties can be physical or nutritional properties making it more desirable for the consumer. These properties give structured lipids a wide range of use for medical or nutritional purposes.

Structured lipids were first used by patients suffering fat malabsorption problems as an alternative for simple physical blends of medium chain triglycerides and long chain ones. The medium chain fatty triglycerides were rapidly absorbed at these patients resulting with no improved absorption of long chain triglycerides. Therefore,

structured lipids containing both medium chain length fatty acids and long chain fatty acids on the same triglyceride were used with success. These successful results leaded to increased research in specially designed structured lipids for specific medical cases. Structured lipids are beginning to be used in some specific medical cases with success.

There are several strategies for structured lipid production the most commonly used strategy is enzymatic or chemical acidolysis or ester interchange. While lipases are employed at enzymatic process chemical catalysts are used in chemical process. A less common strategy is crop improvements by selective cross breading or genetic manipulation. Nowadays inter esterification is the most commonly used strategy for lipid modification. This technology finds applications at the production of trans acid free margarines and cocoa butter substitutes.

At the literature research many lipase catalyzed structured lipid applications were found. The main medium chain length fatty acids used were Caprylic acid (C8:0) and Capric acid (C10:0). Various kinds of fats were used for structured lipid production as well. But at any study enzymatic acidolysis of caprylic acid into corn oil was reported. Therefore the optimization of caprylic acid incorporation into corn oil via enzymatic acidolysis using response surface methodology was studied at this study.

## 2. LITERATURE SURVAY

### 2.1 Lipids and Their Properties

Lipids are a major source of storage energy, are important precursors in the body's metabolic processes and are essential components of cell membranes and other biological structures. Lipids come mainly from ingested foods or are synthesized in the body, primarily in the liver. The lipids of our diet are known as fats or oils, depending on their physical state at room temperature. Properties of lipids are mainly related to their fatty acids (FA) or triacylglycerols (TAG) composition. Depending on their properties lipids have significant nutritional physical and rarely chemical uses. At structural lipids, functional foods, infant formula, dietary supplements, and pharmaceutical uses the nutritional properties of lipids are important. Where as at cooking and backing fats, frying oils, spreads, and creams physical properties of lipids are important [1].

#### 2.1.1 Nutritional properties of lipids

Lipids in human nutrition are mainly originated from plant and animal sources. Lipids occur in "visible" forms such as, adipose tissue, dairy products, and seed oils, and "hidden" forms such as, in fried foods, snacks and chocolates in daily diet [1].

Fats and oils are an important component of our daily diet as they provide energy, contain essential nutrients and add palatability and flavor to cooked food. Essential nutrients in fats and oils are essential fatty acids and fat soluble vitamins as vitamin A, vitamin D, vitamin E, vitamin K[1]. Although the energy value of fats changes according to the present of less absorbed high melting temperature fatty acids or less energy containing short or medium chain fatty acids it is generally accepted as 9 kcal/g. Fats have higher energy value than proteins and carbohydrates. Fats are not only valuable for their high energy but also especially required during pregnancy, lactation and growth at childhood. Fat left after these requirements are met is stored in adipose tissue as body fat [1].

### 2.1.2 Essential fatty acids

Fatty acids which can not be synthesized by human body because of the lack of desaturase enzyme are called essential fatty acids (EFA). EFA have to be taken from dietary plant sources. α-Linolenic acid (ALA, 18:3) and linoleic acid (LA, 18:2) are EFA. α-linolenic (18:3) and γ-linoleic acid (18:2) are important omega-3 acids (ω-3 or n-3). Linoleic acid (18:2) is an important omega-6 acid (ω-6 or n-6). ALA and LA form long chain polyunsaturated fatty acids in the body which are essential for human. Long chain polyunsaturated fatty acids formed from the EFA are ω-3 and ω-6 acids. Arachidonic acid (AA, 20:4 ω-6) can be synthesized from linoleic acid (LA, 18:2 ω-6). Eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3) can be synthesized from α-Linolenic acid (ALA, 18:3 ω-3).

Long chain polyunsaturated fatty acids of omega-3 and omega-6 families are important for different tissues. The n-6 family acids are important constituents of membranes in adipose tissue, liver, muscle, kidney, and reproductive organs. The n-3 family acids are important constituents of nerve tissue, retina, and sperm. More over 20:3 (n-6), 20:4 (n-6) and 20:5(n-3) acids originating from n-6 and n-3 family are precursors of eicosanoids. Eicosanoids are physiologically valuable molecules. These are important by maintaining homeostasis and show physiological effects at concentrations down to $10^{-9}$g/g tissue levels [1].

### 2.1.3 Effect of fatty acid chain length on absorption and nutrition

The chain length of fatty acids is important by determining the faith of fatty acids in human body. Since short and medium chain length fatty acids are not transported in form of lipoproteins they do neither increase cholesterol levels nor are stored in adipose tissue [1].

Short chain fatty acids (SCFA, $C_2$ - $C_6$) which are also called volatile fatty acids are mainly produced by anaerobic fermentation of carbohydrates and proteins in bowel. (4,5) SCFA can be taken from the daily diet as well. The major dietary sources of SCFA are milk, and butter. Since SCFA are water soluble small molecules they are faster absorbed than other fatty acids. SCFA have lower energy values than other fatty acids. SCFA, (2:0), (3:0), (4:0), and (6:0) have 3.5 , 5.0, 6.0, and 7.5 kcal/g,

respectively.(6,7) SCFA are not only used as energy sources but are also important for maintaining colon morphology, and water electrolyte balance in colon [4].

Medium chain fatty acids (MCFA) are $C_8$ - $C_{12}$ fatty acids. Palm kernel and coconut oils are the main sources for MCFA. Medium chain fatty acids are shorter and more soluble than long chain fatty acids. Hence MCFA are selectively transported by the hepatic portal vein to the liver. MCFA are metabolized as rapid as glucose in human body. MCFA do not need a specific protein, carnitine to enter the mitochondria [6,8]. As free MCFA are not able to esterify to TAG they tend not to be deposited as body fats [4]. Although MCFA are useful by dealing with obesity they can not provide all needed fatty acids. Therefore MCFA are recommended to be used with healthier unsaturated fatty acids [4, 6, 8].

Long Chain fatty acids (LCFA) are fatty acids with a carbon chain length ranging from 14-24. Most fats and oils obtained from our daily diet are mainly composed of LCFA. LCFA are bigger molecules and more hydrophobic than MCFA. Therefore LCFA are insoluble and can not be transported in blood or been absorbed. Hence LCFA have to be packed. LCFA are packed as chylomicrons [8]. Chylomicrons are spherical 750 to 6000 Å triglyceride particles formed triglycerides. Triglycerides are formed by reesterification of enterocytes absorbed sn2 monoglycerides and exogenous fatty acids [4, 9 10]. Chylomicrons are transported by lymph system and enter the blood flow. LCFA are finally bound by carnitine proteins and metabolized in mitochondria. Or they are modified to body fats and stored in adipose tissues. As a result of these more complex absorption and metabolic circumstances LCFA are metabolized less rapidly [8].

Although LCFA tend to be stored as body fat and are less rapidly metabolized they have many beneficial properties too. For instance essential fatty acids are a sub class of them. LCFA such as n-3 LCFA, poly unsaturated fatty acids and oleic acid (18:1 n-9) have beneficial effects on cholesterol levels. They decrease low density lipoprotein (LDL) and very low density lipoprotein (VLDL) cholesterol levels where as they increase the high density lipoprotein (HDL) cholesterol levels [8].

But not all LCFA have a beneficial effect on cholesterol levels. Saturated LCFA increase LDL and VLDL cholesterol levels. The tendency to increase cholesterol

levels is related with their chain lengths. While myristic acid (14:0) has the highest influence on cholesterol levels stearic acid (18:0) seams to affect it marginally [1].

Conjugated linoleic acid (CLA) is an important LCFA. CLA is found in ruminants tissues. CLA has shown good results at anti cancer studies and hence began to be used in therapy [11].

### 2.1.4 Physical properties of fats and oils

Crystallization, crystal form, and melting behavior are the main physical properties of fats and oils. For instance frying oils and lubricants are desired to be crystal free. Therefore it is important that they do not contain crystallized TAG or encourage crystallization. Where as, at spreads certain structure of crystallization is desired. TAG form β-Crystals and β'-Crystals. B'-Crystals are desired at spreads. Since they are relatively small and incorporate much liquid B'-Crystals give rise to better products. While β-Crystals tend to form needle like agglomerates and cause a grainy texture and undesired products. β'-Crystals are likely to be formed by mixed chain length TAG while β-Crystals tend to be formed by same chain length TAG [1].

## 2.2 Corn Oil

Corn oil is the $10^{th}$ most produced oil in the world with 2.04 Million tons per year. The major producers of corn oil are USA (57%), EU-15 (10%), and Japan (5%) (12,13). Corn kernels contain just 5% oil. Therefore Corn oil is not produced directly from kernels. Since it would be costly be unfavorable. Corn oil is obtained from corn germ. Corn germ is a side product of corn milling. Two main corn milling processes are in use: The corn wet milling and dry milling. The first corn wet mill in USA was opened in 1842 to isolate effectively cornstarch. Germ amounts and their oil contents obtained by dry milling and wet milling are not the same. At dry milling 11.24 % (w/w) germ is produced while 4.9 % (w/w) is produced at wet milling, which are contain 20-25% (w/w) and 44-50% (w/w) oil**,** respectively. Germs obtained by wet milling are more popular in corn oil production. In 1996 it was estimated that approximately 90% of corn germ oil was produced from wet milling originated germs [12, 14].

### 2.2.1 Extraction and refining of corn oil

Traditionally Oil from oil seeds is extracted by a single solvent extraction step. But in case of corn oil extraction there are problems interfering with the extraction process. Therefore corn oil is produced in two steps. First crude oil is obtained by corn germ extraction. Secondly crude corn oil is refined by various steps [12,15].

#### 2.2.1.1 Corn germ extraction

Firstly oil is obtained from the 44 to 55% oil containing wet milled germs by preheating and expelling. An expeller is a high pressure mechanical press used for oil extraction. After the mechanic process the removing 6 to 11 % oil is removed by partial pressing and hexane extraction. The removing cake is subjected to extrusion and hexane extraction resulting in 1 to 2% oil containing collets. These collets are usually used as animal feed due to their high protein content [12,15,16].

Oil from dry milled germs is usually extracted by expelling. The resulting oil has lower free fatty acid levels, lower phosphorus contends, and increased concentration of tocopherols than wet milled germ oil [12,17].

#### 2.2.1.2 Refining of crude corn oil

By refining steps it is aimed to remove free fatty acids, pigments, volatiles, phospholipids, and waxes from crude corn oil. By refining firstly the phospholipids and fatty acids are removed. Degumming is a treatment used to remove phospholipids from crude oil using water and centrifugal force. Although degumming is usually the first step by oil refining it is not employed at corn oil if alkali refining is employed. Alkali refining is a process which uses bases to neutralize fatty acids and phospholipids and removes them as a by product called soapstock [12,18]. An alternative method for alkali treatment is physical refining which is performed after water degumming to remove free fatty acids. Physical refining uses high temperatures at vacuum to volatize free fatty acids. After these refining steps the resulting oil is bleached by acid activated clay filtration in order to remove pigments and oxidation products. Oil subjected to bleaching is winterized. Winterizing or dewaxing is to cool the resulting oil down between 5-10 ºC in order to precipitate waxes and saturated TAG and remove them. Finally the winterized oil is deodorized by refining at vacuum and temperatures over 200 ºC. This process

removes undesirable odors, flavor components and some actually desired phytosterols and tocopherols. Owing to the fact that phytosterols and tocopherols are removed the deodorizer makes distillate a source for phytosterol containing functional foods, and precursor for some steroid pharmaceuticals [12, 19-21].

### 2.2.2 Composition of corn oil

#### 2.2.2.1 Fatty acid composition of corn oil

Edible oils are often compared according to their fatty acid composition. Corn oil has some characteristic properties. Corn oil has high polyunsaturated fatty acid content. The polyunsaturated fatty acid content is almost completely occupied by linoleic acid (18:2) and almost no linolenic acid (18:3). Linoleic acid is an essential fatty acid and usually found between 50 to 60% in corn oil. Where as linolenic acid is undesirable due to its high tendency to oxidation and found bellow 2% in corn oil. More over corn oil has a low saturated fatty acid concentration. Saturated fatty acid concentration of corn oil is lower than 15%.

The fatty acid composition of corn oil depends on the corn hybrids used and climate and location where they are grown. It has been observed that U.S. corn oil has higher linoleic acid concentrations than international one. It has also been observed that the same corn hybrid grown in colder regions had higher linoleic acid content. Table 2.1 summarizes the fatty acid composition of U.S corn oil and international (Int) corn oil [12,18,22].

**Table 2.1:** Corn oil fatty acid composition of various sources.

| Oil | Fatty acid composition (mol% ) | | | | |
|---|---|---|---|---|---|
| | Palmitic (P) (16:0) | Stearic (S) (18:0) | Oleic (O) (18:1) | Linoleic (L) (18:2) | Linolenic (Ln) (18:3) |
| U.S. oil | 11.0 ± 0.6 | 1.7 ± 0.3 | 25.8 ± 0.9 | 59.8 ± 1.2 | 1.1 ± 0.4 |
| Int. oil | 12.9 ± 1.4 | 2.6 ± 0.6 | 33.1 ± 2.5 | 48.8 ± 2.4 | 1.4 ± 0.4 |

#### 2.2.2.2 Triacylglycerol composition of corn oil

Fats and oils obtained from plants and animals are complex mixtures of TAG. TAG compositions of oils and fats have been investigated by capillary gas liquid chromatography (GLC), reverse-phase high performance liquid chromatography

(HPLC) and supercritical fluid chromatography (SFC). Investigations made by reverse-phase HPLC found 19 to 27 individual TAG species in corn oil and determined that oleate-linoleat-linoleat (OLL) and linoleat-linoleat-linoleat (LLL) were the most abundant TAG species in corn oil [12, 23-25].

Silver ion HPLC was also used to investigate corn oil TAG species. Silver ions form complexes with double bounds at organic molecules. Silver ion HPLC uses this principle for separation. Silver ion HPLC separated Corn oil in 11 fractions. The results confirmed that oleate-linoleat-linoleat (OLL) and linoleat-linoleat-linoleat (LLL) were the most abundant TAG species according to their number of double bounds [26]. Table 2.2 shows the quantitative analysis of corn oil TAG species obtained by different methods [24,25].

## 2.3 Low Calorie Fat Substitutes

Although fats and oils have multiple advantageous properties and are essential nutrient they have undesirable properties as well. The main undesirable properties of lipids are related to their high calorie values and possible effects on LDL cholesterol. It has been tried to over come these problems using fat substitutes. Fat substitutes can be investigated in two main groups which are lipid based and non lipid based fat substitutes. Non lipid based fat substitutes aim to use non fat substances to replace fats by imitating its effect while lipid based substances aim to increase the SCFA and MCFA concentration in fats which have fewer calories and are less possible to be deposited as body fat [27,28, 1].

### 2.3.1 Non fat low calorie fat substitutes

#### 2.3.1.1 Olestra (Sucrose polyester)

Olestra is a commercial available fat substitute of Procter and Gamble Company (Cincinnati, OH). Olestra is produced by esterifying disaccharide sucrose with six to eight fatty acids. The resulting polymer mimics TAG by taste and mouth feel. Olestra was approved by United States food and drug administration (FDA) after several studies in 1996. It has been used to replace lipids in chips and crackers successfully and is aimed to be used in salad dressings, table spreads, and diary products as well in the future. Olestra has a calorie value of zero as it is indigestible by lipolytic

enzymes and so not absorbed in digestive tract. A main disadvantage of olestra is decreased vitamin A,D,E, and K absorption which is tried to be over come by adding these vitamins to olestra. It is also reported that olestra might reduce cholesterol absorption and cause carotenoid depletion. It possibly could also lead to flatulence, abdominal cramping, diarrhea, and increased bowl movement [29-31].

**Table 2.2:** Quantitative analysis of TAG species found to be in corn oil using different methods.

| TAG Species | Analytical results from Strecker et. al. (Area %) | HPLC- mass spectroscopy (Area %) | HPLD- Flame ionization Detector (Area %) |
|---|---|---|---|
| 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 |
| 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 |

#### 2.3.1.2 Simplesse

Simplesse is a commercially available cholesterol-free fat substitute. It was developed by the NutraSweet Co. (Chicago, IL) in 1988. Simplesse consists of micro particulate milk and egg white protein. It is produced by heating these, protein solution and homogenizing it at higher temperatures to let them coagulate in 0.1 to 2.0 μm particles. Although a rich, creamy mouth feel is achieved which could be used to replace fat in dairy products, and oil-based foods it is unsuitable for backing or cooking applications since the protein micro particles get gelatinized at elevated temperatures. Simplesse is digested as ordinary proteins in digestive tract yielding between 1 to 2 kcal/g [29,32-34].

#### 2.3.1.3 Sorbestrin (Sorbitol Polyester)

Sorbestrin is a liquid low calorie heat stable fat substitute consisting of fatty acid esters of sorbitol and sorbitol anhydrides. Although sorbestrin was first discovered by Pfizer Inc. (New York, NY) in the late 1980s it is still under development of Danisco Cultor America Inc. (Ardsley, NY) and not commercially available. Sorbestrin's most amazing property is that it is suitable for all vegetable oil applications such as, salad dressing, mayonnaise, and fired or baked foods. Sorbestrin has 1.5 kcal/g energy and a mild oil-like taste [29].

#### 2.3.1.4 Esterified propoxylated glycerols (EPG)

EPG are commercially available fat substitutes manufactured by ARCO Chemical Co. (Newton Square, PA). EPG are analogs of TAG in which the ester linkage is replaced by an ether linkage. This has been achieved by locating a propoxyl group between the glycerol backbone and the fatty acids. EPG are produced by synthesizing polyether glycols using glycerol and propylene oxide and than synthesizing EPG using these polyether glycols and fatty acids from various sources. They are applicable to replace fat in formulated products and baking or frying applications [29].

#### 2.3.1.5 Paselli

Paselli is a commercially available fat substitutes manufactured by Avebe America Inc. (Princeton, NJ). It is based on potato starch and gives rise to a thermostable gel at right thermal conditions. Paselli is taste neutral and has an energy value of

approximately 3.8kcal/g. However, Paselli has a wide range of application it is commercially used in ice cream, puddings, and meat products [29, 30, 33].

#### 2.3.1.6 N-Oil

N-Oil is a since 1984 commercially available fat substitute manufactured and marketed by National Starch and Chemical Corp. (Bridgewater, NJ). N-Oil consists of tapioca dextrin and has an approximately calorie value of 1kcal/g. This product is resistant to thermal, acidic, and shear stresses. N-Oil gives a feeling of high fat contend. It is applicable to even totally replace fat at frozen desserts, salad dressings, puddings, spreads, dairy products, and soups [29,30,33,34].

### 2.3.2 Lipid low calorie fat substitutes

At lipid based low calorie fat substitutes the total calorie is typically reduced by increasing the MCFA or SCFA contents of fats. At some examples also a saturated LCFA was introduced which is poorly absorbable. Generally structured lipids are used for these purpose rather than just blending TAG. Structural lipids can roughly be defined as lipids with manipulated fatty acid composition or position. Structured lipids can be produced using chemical or enzymatic processes. These fat substitutes have generally a calorie value about 5 to 7 Kcal/g. This value is relatively low when compared to 9 Kcal/g which is generally assumed for fats and oils. Lipid low calorie fat substitutes are produced to be applicable in coatings, dips, chips, and production of diary and bakery products. Depending on their fatty acid composition some kinds of these fat substitutes are also applicable as cocoa butter substitutes [29,32].

## 2.4 Structured Lipids

Structured lipids (SL) are modified TAG. These modifications can be related to the fatty acid composition of specific TAG species. While in more advanced cases both the fatty acid composition and the position of these fatty acids are modified. SL are produced by chemical or enzymatic processes. SL are made for increasing physical, chemical, or nutritional properties of TAG. Many SL have successfully been synthesized for nutritional, pharmaceutical and medical purposes. Using these SL valuable results were achieved on many metabolic parameters such as, improvements in nitrogen balance, immune function, and cholesterol levels [29,35].

Foods having advantageous health benefits ahead of their actual nutritional effects are called nutraceuticals. If these nutraceuticals are in form of ordinary foods or added to them they are called functional foods. SL can be designed as nutraceuticals to be used in functional foods or medical applications. SL are named functional lipids when they are used in functional foods [29,36].

SL can be synthesized using various strategies. Random reesterification of fatty acids on a glycerol back bone is one of those. These fatty acids can be obtained by hydrolyzing TAG prior the reesterification or various other sources. Various kinds of fatty acids such as SCFA, MCFA, LCFA, EFA and mono or poly unsaturated fatty acids and their triglycerides can be used for SL synthesis. The resulting TAG species have more advanced properties than simple blends of them. SL have metabolic advantages on simple blends of TAG such as being less toxic or being faster absorbed. Synthesizing side specific SL having specific fatty acids at sn-1,3 and sn-2 positions devotes them even more advanced metabolic unique properties. For instance SL having MCFA at sn-1,3 and EFA at the sn-2 position would be fast absorbed, serve as a faster energy source and serve as an EFA source all together [29,37,38].

### 2.4.1 Esterification

Where hydrolysis is the separation of two molecules with the incorporation of one water molecule esterification is the combination of two molecules with the separation of one water molecule. The reaction between fatty acids and hydroxyl groups on the glycerol back bone is an esterification reaction. Esterification and hydrolysis reactions are reversible and the reverse reactions of each other. The direction of such reactions is controlled by the amount of water present. If water is bellow a certain concentration esterification will occur, if water concentration increases the reaction will turn to hydrolysis. Therefore water accumulation is undesired at esterification reactions and has to be removed from the environment. But on the subject of enzymatic reactions certain amounts of water are essential for the reaction because enzymes are bio-molecules and need some water to protect their active conformation [1,6,27].

The products of an esterification reaction between glycerol and free fatty acids are monoacylglycerols (MAG), diacylglycerols (DAG), TAG and unreacted glycerol and

fatty acid molecules. These products have many applications such as pharmaceutical, food, and cosmetic applications [1,6,27]. Figure 2.1 demonstrates the esterification reaction[27].

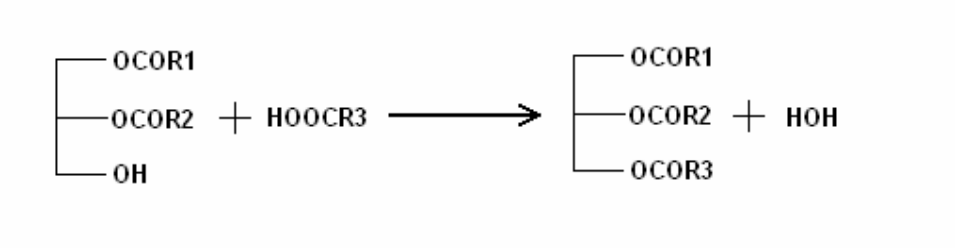


**Figure 2.1:** Esterification reaction

### 2.4.2 Chemical synthesis of SL

At chemical synthesis of SL, usually a mixture of medium chain TAG and long chain TAG is used. This process of exchanging the fatty acid species of different TAG molecules is generally called ester interchange or transesterification. This reaction takes place at anhydrous reaction conditions and presence of a chemical catalyst. At the first strategy high temperatures and alkali metals or alkali metal alkylates as catalysts are needed [1,39,40]. But at the second strategy the reaction temperature is highly flexible and inversely proportional to time. The catalyst and its amount are important reaction parameters as well. The most widely used catalyst is sodium methoxide. Less than 0.1% (wt/wt) sodium methoxide is sufficient to catalyze ester exchange. But it has to be supported by other catalysts because it is highly sensitive to water, peroxides and free fatty acids. Undesired TAG are also produced due to the random nature of chemical transesterification [1,41-44].

To sum up, the synthesis of specific SL is not possible by chemical processes. Chemical interesterification is a useful and commercial technology for production of trans fatty acids free margarines and a good alternative for hydrogenation technology. But it is not suitable for production of SL for more advanced cases. As for SL used as nutraceuticals not only the fatty acid composition but also the position of fatty acids is important due to metabolic reasons [39].

### 2.4.3 Enzymatic synthesis of SL

SL can be produced using lipases as catalyst. TAG lipases are scientifically defined as triacylglycerol acylhydrolases and universally coded EC 3.1.1.3. These enzymes

are able to catalyze various reactions. These reactions include hydrolysis and esterification of fatty acids. In the hydrolysis reactions TAG, DAG, and MAG are hydrolyzed. Where esterification reactions catalyze esterification of fatty acids to a glycerol backbone (esterification), fatty acids substitution between free fatty acids and an existing TAG (acidolysis), and ester substitution between two existing TAG (ester interchange) [27,39].

The major weakness of enzymatic synthesis is the high cost of enzymes. Their costs will decrease with improvements in genetic and process developments. Enzymes used for such applications have to be immobilized due to economical reasons. Using immobilized enzymes is important for continuous process development and enabling re-usage of enzymes [39,45].

#### 2.4.3.1 Enzymatic acidolysis

At enzymatic acidolysis the fatty acid composition is altered by substituting fatty acids in the TAG. The main advantage of acidolysis is easy downstream processing by distillation or other appropriate technique. The enzyme specificity used in acidolysis determines the fatty acids being substituted. The most commercially used lipases are sn-1,3 specific lipases. Figure 2.2 shows the acidolysis reaction between triolein OOO (oleic acid, 18:1) and caprylic acid (8:0) [47].

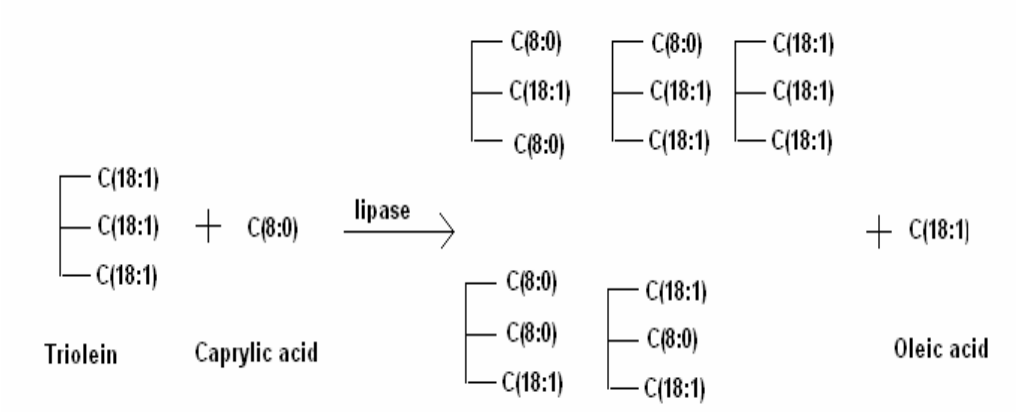


**Figure 2.2:** Acidolysis reaction of triolein and caprylic acid

Acidolysis reactions catalyzed by sn-1,3 specific lipases are commercially used in production of cocoa butter substitutes (CBS) from palm midfraction oil. This application has been patented by Unilever. The reaction substitutes palmitic acid (P,

16:0) with free stearic acid (S 18:0) to obtain SOS and POS. This reaction can be summarized as following;

$$POP + S \rightarrow POS + SOS$$

Another commercial example is Betapol® which is also produced by Unilever using a sn-1,3 specific lipase. Betapol is infant formulas, and consists of unsaturated acids at sn-1,3 positions and palmitic acid at sn-2 position [1,48,49].

A Japanese company produces also modified TAG using a sn-1,3 specific enzyme named Bohenin ®. Bohenin consists of behenic acid (22:0) at sn-1,3 and oleic acid at sn-2 positions. Bohenin is used in chocolates to prevent fat blooms to occur [1].

### 2.4.3.2 Ester interchange

At these reactions, the acyl groups interchange between two TAG and TAG and fatty acid esters. The products obtained by ester interchange can vary according to the catalyst. Specific lipases and non specific lipases could be as biocatalysts [1]. Figure 2.3 and Figure 2.4 are demonstrating the ester interchange reactions between two TAG and TAG and ethyl ester, respectively [50,51].

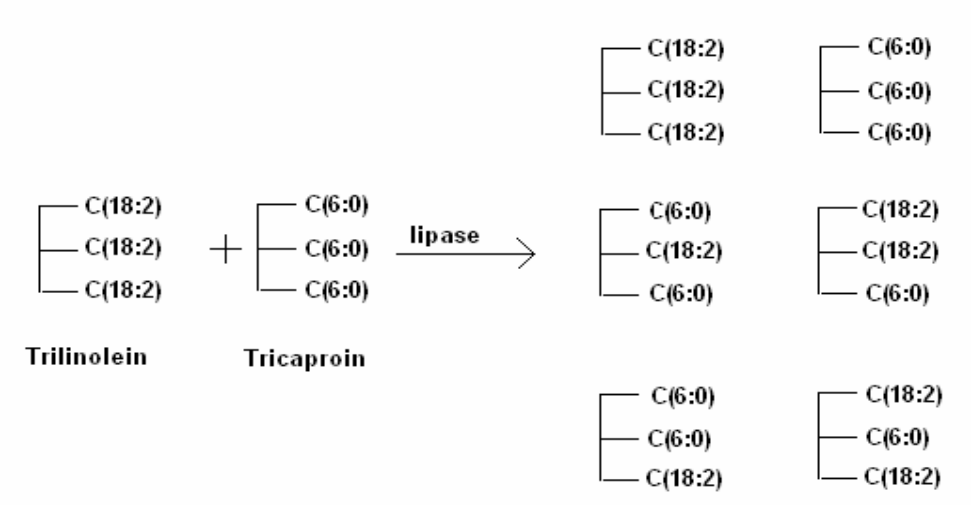


**Figure 2.3:** Ester interchange reaction between trilinolein and tricaproin [50].

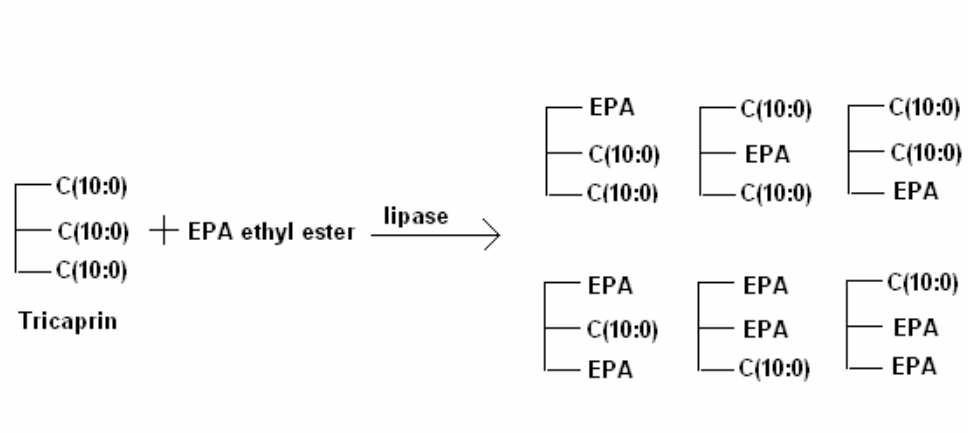


**Figure 2.4:** The ester interchange reaction between tricaprin and Eicosapentaenoic acid ethyl ester [51].

Ester interchange reactions between two TAG have some weaknesses. Firstly if fatty acids at sn-2 and sn-1,3 are not different, the resulting TAG will be similar for specific or non specific lipases. Secondly is taking place of acyl migration. Acyl migration is proportional to reaction temperature and, DAG content, and it is catalyzed by acids, resins, and silica.

Although considering the advantages of chemical ester interchange technology, enzyme catalyzed reactions are appear to be not feasible nowadays, they could have potential applications in the future[1].

### 2.4.3.3 Alcoholysis

At these reactions the alcohols are substituted with ester groups on glycerol molecules. Figure 2.5 demonstrates a general alcoholysis reaction [6].

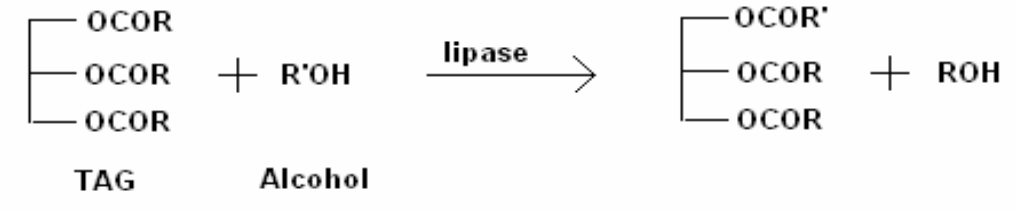


**Figure 2.5:** Alcoholysis reaction[6]

Not all lipases are available for this reaction. PUFA rich TAG were reported to be produced from PUFA rich partial glycerides using alcoholysis [27,52].

Glycerolysis reactions are also alcoholysis reactions. Glycerolysis reactions can be used for the production of specific MAG and DAG. MAG have a wide range of application in food industry [53].

## 2.5 Lipases

### 2.5.1 Types of lipases

Lipases are classified into 5 types according to their substrate and position specificity. Type 1 lipases are neither position nor fatty acid specific. Type 2 lipases are sn-1,3 specific and prefer sn-1 and sn-3 positions at similar rates. Type 3 lipases have a different affinity against MAG, DAG and TAG. Type 4 lipases are fatty acid specific and have especially higher affinities against specific fatty acids. Type 5 lipases are special enzymes, which are stereospecific having higher affinities for sn-1 positions than sn-3 positions or other specificities [27, 54,55].

### 2.5.2 Lipase structure

The relation ship between lipase structure and catalytic activity, substrate selectivity, and stereospecificity has been studied with substrate-analogous inhibitors since 1990 [53,56]. All microbial lipases are not highly similar but members of the α/β hydrolase fold family, which have a hydrophobic eight stranded β-sheet core surrounded by two layers of amphiphilic α-helices [53 ,57].

The catalytic sides of studied lipases were found to quite similar. Whereas, the substrate binding side were not as alike as the catalytic sides. The substrate binding sides had found to have varying size and shape [53,57]. At a considerable amount of lipases a mobile component called lid was found that covered the catalytic side. This lid was found to be consisting of two short α-helices. At appropriate conditions the lid was found to be repositioning giving access to the catalytic side and activating the lipase. No other major conformational changes were found to occur on lipase between active and inactive states [53].

The substrate binding side is a deep elliptical pocket on the central β-sheet. Lipases are divided in 3 different kinds of binding sides. They could be hydrophobic "crevice-like" and located close to the protein surface as it is the case at *Rhizomucor* and *Rhizopus* lipases. They could be "funnel-like" such as it is the case at *Candida antarctica*, and *Pseudomonas* lipases. Third they could be "tunnel-like" like *Candida rugosa* and *Geotrichum candidum* lipases [53,58]. Studies made on *Pseudomonas* lipases with TAG analogue inhibitors showed that substrate binding was dominated by Van der Waals interactions and an additional hydrogen bound between the carbonyl oxygen of the sn-2 fatty acid helped for fixation [53,59].

### 2.5.3 Catalytic mechanism of lipases

When water amount is restricted in the environment lipase activity can be shifted from hydrolysis to interesterification. The partially positive charged carbonyl atom of the fatty acid esters is primarily suspected for nucleophilic attack. The active side of lipases contains a highly conserved amino acid trio. This trio consists of one Aspartic or glutamic acid (Asp or Glu), one histidine (His) and one Serine (ser) amino acid. They serve as a charge relay system where Asp or Glu and His corporate with the as nucleophile acting Ser to increases the catalytic activity [27, 60].

At appropriate conditions at which the lipase is active the interesterification takes place in fours steps. First a positive charged carbonyl atom of esters is attacked by serine's hydroxyl group. At the second step a tetrahedral intermediate is formed between them. Afterwards the carbon oxygen bound is broken and water or alcohol released at the third step. Finally the intermediate reacts with an alcohol such as a DAG and regenerates serine. Figure 2.6. demonstrates the catalytic activity of the amino acid trio [27, 60].

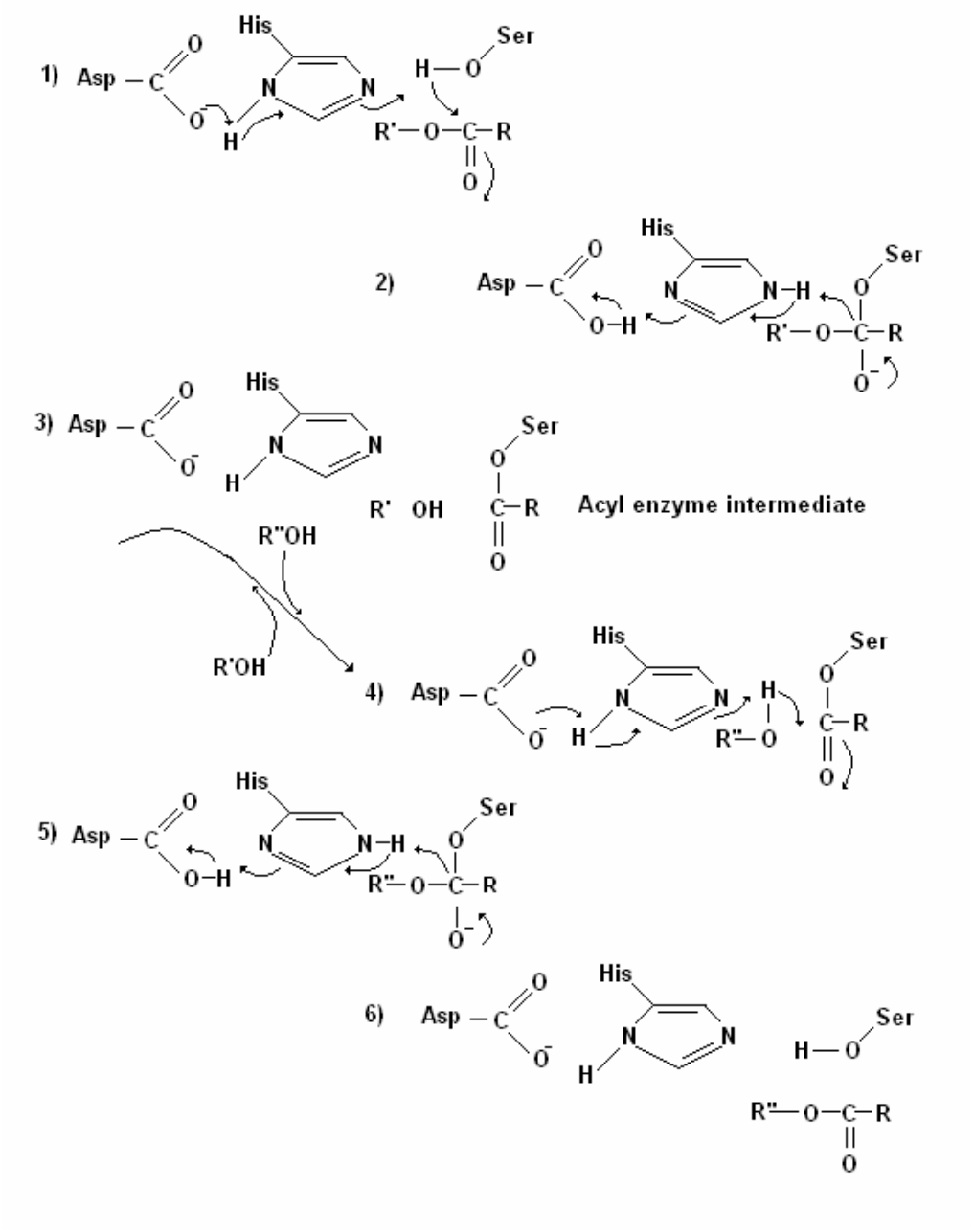


**Figure 2.6:** The catalytic mechanism of the amino acid trio by interesterification [60]

### 2.5.4 Factors effecting enzymatic reaction and process yield

#### 2.5.4.1 Water

Water is essential for almost all enzymes. Water is responsible for maintaining the active conformation of proteins, facilitating reagent diffusion, and maintaining enzyme dynamics [27,61].

Some studies have also concluded that increased water content in solvents resulted in higher enzyme activities. But the optimum water content is highly variable depending on the enzyme and solvent type [39,62].

#### 2.5.4.2 Hydration levels

One mono layer of water was found to be enough for enzyme activity. To maintain enzymes activity in solvents the protection of this mono layer is essential. It has been found that this mono layer was less susceptible to be damaged at hydrophobic water immiscible organic solvents [39, 63-67].

#### 2.5.4.3 Solvent type

Organic solvents used can dramatically affect the reaction kinetics and catalytic activity. Thus, two important factors have to be considered by this choice. These are the enzyme activity and the equilibrium at the presence of this selected solvent. For instance the effect of a solvent on hydrolysis or esterification reactions will be different. Enzymes could be inactive in various solvents as these solvents effect destructively on hydrogen bounds or hydrophobic interactions on these enzymes [39,67,68].

Variable sensitivities against similar solvents were observed among different lipases. Polarity of a solvent was determined to be an important property by solvent selection. The polarity of a solvent is measured by its partition coefficient between octanol and water. For solvents these partition coefficient is expressed in means of log P. If this value is less than 2 for a solvent the enzyme activity was generally found to be altered at this solvent. It is probable that the enzymes are dehydrated by such solvents or that enzymes are inactivated as a result of solvent penetration into their hydrophobic cores [39, 63, 69-71]. Hydrophobic solvents are less likely to show such effects. Solubility of reactants in the solvent, and that the solvent is inert to the reaction are two other critical factors has to be considered when choosing the most appropriate solvent for a reaction. More over the solvent cost, viscosity, density, surface tension and toxicity of solvent, waste disposal and flammability of solvent have also be considered by selecting the most appropriate solvent for a reaction. Although there is much literal information about solvent selection, experimental optimization is necessary for new process as they are highly variable according to the reaction type and enzyme used [39,72].

#### 2.5.4.4 Effect of pH

The effect of pH on enzyme activity at aqueous solutions is a well known phenomenon. Enzymes in organic solvents are affected by pH as well. The pH optimum for a lipase employed in organic solvent is in rule accepted to be the same pH optimum of its last exposed aqueous solution. This tendency to "remember" the optimum pH of its last exposed aqueous solution is called "pH memory". Although the optimum pH for enzymes is variable most lipases have pH optimum about 6 to 9. But it has also to be considered that the pH will change during the reaction. It will become more basic in the case of esterification and more acidic at hydrolysis reactions as the reaction takes place [27, 39, 73].

#### 2.5.4.5 Effect of temperature

Temperature is an important parameter for an enzymatic reaction. Not only the stability and affinity of an enzyme but also the dominant reaction can be affected noticeably by temperature [39, 74]. The thermostability of an enzyme strongly depends on the reaction environment and enzyme source. Extracellular microbial lipases were found generally to be more thermostable than animal or plant lipases [39, 75]. It has also been discovered that enzymes could be much more stable in organic solvents and presence of substrates. Since water is critical for unfolding and inactivation of enzymes. Presence of substrates also occupies the enzyme and restricts its dynamics which results in increased extended thermostability. It has also been found that immobilization could have positive effects on enzyme stability [39 76].

Higher reaction temperatures are essential for some reactions as fats are still solid at lower temperatures. Higher reaction temperatures have more advantages like decreasing viscosity and, preventing "microreactors" formation. Microreactors are defined as microenvironment formations where antagonist reactions begin to take place [27]. Although these advantage the enzyme having the highest temperature optimum is not necessarily the most suitable enzyme for the reactions. High temperatures mean increased energy consumption and thermal degradation. In conclusion, there are no certain rules applicable for all experimental processes. Optimization of most suitable enzyme and temperature has to be done regarding all these factors [27, 39].

#### 2.5.4.6 Effect of substrates mole ratio

Determination of substrates mole ratio is of critical importance. A higher substrate ratio will lead to increased downstream processing costs as the concentration of acyl donors or free fatty acids will increase. Insufficient mole ratio will lead to noticeably decrease reaction yields. Reaction yield does not always increase proportionally to substrate ratio it is not unusual that excessive substrate concentrations cause inhibition. This phenomenon is called substrate inhibition [1,27,39,77,53].

#### 2.5.4.7 Effect of time

It is desired to obtain the highest reaction yields in minimum time. Minimum reaction time will decrease the process costs. Therefore the reaction time also has to be optimized regarding to the substrates and enzymes [78, 79].

### 2.6 Metabolism of Structured Lipids

SL has been found to make use of four possible phenomena on lipid metabolism. The first phenomenon is that the rate and extend of different fatty acids digestion is different. The second phenomenon is the sn-1,3 specificity of gastric and pancreatic lipases which preserves the fatty acid on sn-2 position unhydrolyzed. The third phenomenon is that palmitic and stearic acids are absorbed with relatively low rates due to their high melting points, and tendency to form hydrated acid soaps with divalent cations. The fourth phenomenon is that the absorption of the fatty acid at sn-2 position of a sn-2 MAG is independent of the melting points of this sn-2 fatty acid. One or more of these phenomena can employed designing specific SL to improve lipid metabolism in desired manner [4].

#### 2.6.1 Absorption of MCFA and LCFA containing structured lipids

Lingual, gastric and pancreatic lipases are employed in digestive tract. While a considerable amount of long chain TAG are digested by pancreatic lipases, medium chain TAG are almost completely digested by lingual and gastric lipases. Medium chain TAG and long chain TAG hydrolysis are different and they are also differently transported to liver. While medium chain TAG are transported by portal vein to liver without undergoing reesterification, long chain TAG are reesterified in intestinal cells and carried by lymph system. This property makes medium chain TAG

especially attractive for individuals suffering pancreatic insufficiencies or fat malabsorptions and preterm infants. But medium chain TAG can not full fill the need for EFA of the body. Therefore EFA must also be included in their diets. Therefore MCFA and LCFA containing TAG are essential [4,80,81].

Experiment done with SL containing MCFA and LCFA gave satisfactory results. First promising studies were done by Jandacek *et al.* using 2-linoleoyl-1,3-dicapryloyl glycerol (CaLCa). At these studies they concluded that the CaLCa was almost as fast hydrolysed as medium chain TAG in digestive tract. They also made comparative studies showed that CaLCa was not only faster absorbed but also higher levels of Linoleic acid were absorbed at the end. Since Linoleic acid levels of CaLCa feed mouse were found to be higher that OLO feed ones [4,82].

After these promising results from Jandacek *et al.* [88] more studies were done to determine the effect of positions of MCFA and LCFA on the glycerol back bone. In order to determine these absorption patterns of MLM TAG containing SL and randomly synthesized SL were observed by Jensen *et al.* [83]. They used a almost pure mixture of 1,3 dicaproyl 2-linoleoyl glycerol [CLC] as a MLM type structured lipid source. While they used random SL consisting of 66% Capric[10:0] and 33% linoleic acid at sn-1, 2, 3 positions as random SL at their comparative study. They concluded that more linoleic acid was absorbed by the MLM feed mouse. They demonstrated that not only the fatty acid composition but also their positions were important at their studies. Finally studies made by Christensen *et al*. [84]in 1995 on rats showed that MLM type SL were a good source of essential fatty acids for patient with fat absorption or pancreatic problems. The also concluded that MLM type fatty acids are absorbed almost as fast as medium chain TAG, they act as a FAt absorbable EFA if these EFA are at sn-2 position, they are a good source of EFA for several patients, and that they are less suspected to be stored as body fat as they contain MCFA[4,84].

### 2.7 Applications of Structured Lipids

Structured lipids have many applications while they can be used for specific metabolic cases they can be used to improve physical description of fats as well. A study made on Yoshida sarcoma-bearing rats, indicated that a SL containing fish oil

and MCFA had effects on decreasing tumor protein synthesis, reducing tumor growth and decreased body weight and nitrogen balance as well. More over it was reported in this study that the effect of this structured lipid showed synergy with tumor necrosis factor [85]. At a related study the effects of such an SL, containing fish oil and MCFA, was compared with a physical blend of fish oil and MCT. The results indicated an improved nitrogen balance at structured lipid consumption [86]. But other studies made indicated that the structured lipids were not enough were alone responsible for this effect but also the ratio of MCT to LCT at their synthesis could be critical [87].

Other studies focused on the absorption of SL. It was found that SL containing caprylic acid at sn-1,3 positions and a LCFA at sn-2 position were more rapidly absorbed and metabolized in booth health and patients suffering pancreatic insufficiencies [88]. More over it was observed that these kinds of structured lipids had metabolic advantages over physical blends at various studies [89]. By some studies it was also found that such an MLM type structured lipid could be used as a core material in fat emulsion-based drug delivery systems [90].

Injured rats were feed with SL and a physical blend of these fats to investigate their effects. It was found that the SL consisting of Safflower oil and MCFA induced enhanced increase in body weight, enhanced nitrogen balance, and elevated serum albumin levels [89]. More over a increased linoleic acid uptake was reported at cystic fibrosis patients feed with such linoleic acid and MCFA containing SL [91]. Such an SL has also been reported to increase the immune functions [92].

Studies made using radio-labeled *Pseudomonas* showed that structured lipids preserved the reticuloendothelian function as they improve the nitrogen balance [93]. It reported by an other study that when a SL composing of fish oil and MSFA was feed for a long time the metabolic effects persisted after this regular SL uptake was stopped[94]. It was also reported that the fatty acid composition of human milk cold be mimicked by reacting tripalmitin with unsaturated fatty acids using a 1,3 specific lipase as catalyst. Such an SL was developed and commercialized as Betapol [95].

## 2.8 Literature Research on Structured Lipids Containing Caprylic Acid

At a study made by Xu *et al.* commercially immobilized 1,3-specific lipase, Lipozyme IM, from *Rhizomucor miehei* was used to catalyze the enzymatic acidolysis reaction between canola oil and caprylic acid in a packed bed bioreactor. At this study they examined the effects of substrate flow rate, substrate molar ratio, reaction temperature, and substrate water contends. After this study they concluded that all investigated factors had an effect on reaction yield [96].

At a study made by Kawashima *et al.* immobilized *R. miehei* lipase was used for enzymatic incorporation of caprylic acid in to tri conjugated linoleate for MLM synthesis, and enzymatic incorporation of CLA in to tricaprylate for LMM synthesis. They obtained 51.1% (w/w) MLM type structured lipids and 51.8 % (w/w) LMM type structured lipids. They further obtained 92.3% pure MLM with 49.1% recovery, and 93.2% pure LMM with 52.3% recovery after purification by short-path distillation.[97]

At a study made by Huang and Akoh eight commercially-available lipases were used at the same conditions to investigate their effects. They used 10% w/w of these enzymes to catalyze the transesterification reaction between triolein and caprylic acid. Using 100 mg triolein, 78.0 mg caprylic acid ethyl ester, and 3 mL hexane at 45 ºC for 24 h they converted most triolein into structured lipids using Immobilized lipase IM60 from *Rhizomucor miehei*. They obtained 41.7% dicaprylоolein, 46.0% monocaprylоolein, and 12.3% unreacted triolein for these lipase [98].

At a study made by Fomuso and Akoh A 1,3-specific lipase, IM 60 from Rhizomucor miehei was used as the biocatalyst. They investigated the effect of solvent, temperature, substrate mol ratio, and flow rate/residence on the caprylic acid incorporation. They found flow rate of 1 ml/min, residence time of 2.7 hours and molar ratio of 1:5 (olive oil/caprylic acid) at 60 ºC, to be optimum. They investigated the sn-2 position of obtained structured lipids at these optimum point. They found that the structured lipid had 7.2% caprylic acid, 69.6% oleic acid, 21.7% linoleic acid and 1.5% palmitic acid at the sn-2 position for this optimum point [99].

At a study made by Lai *et al.* a sn-1,3 specific lipase, Lipozyme ® IM 60 from *Rhizomucor miehei*, was used to catalyze the enzymatic acidolysis between palm

olein and caprylic acid was used. They obtained 30.5% of caprylic acid incorporation into the palm olein at 24 hours in a packed bed bioreactor [100].

At a study made by Lee *et al.* a sn-1,3 specific lipase, Lipozyme ® IM 60 from *Rhizomucor miehei* was used to catalyze the transesterification reaction between peanut oil and caprylic acid in a stirred-batch reactor. They investigated the effects of different substrate ratios, agitation speeds and loss of enzyme activity at repeated re use. They found that at 2 % of enzyme the agitation speed and substrate ratio were critical but agitation speed loses its importance at increased enzyme percentages (4%). They yielded 14.3 mol % caprylic acid incorporation at 50 ºC, 640 rpm, molar ratio of 1:2 for peanut oil: caprylic and 2 % of enzyme, and results between 24.2-24.9 % at 4% enzyme 1:1 molar ratio and agitation speeds between 200 and 750 rpm. They also found that the caprylic acid incorporation decreased from 15%mol to 9.7% mol after the enzyme was re used 5 times [101].

At a study made by Soumanou *et al.* 1,3-regiospecific lipases [from *Rhizomucor miehei, Rhizopus delemar*, and *Rhizopus javanicus*] was used to synthesize MLM type structured lipids. They used a two step strategy. At the first step they obtained sn-2 MAG by enzymatic alcoholysis of triolein, trilinolein, or peanut oil and yielded 71.8% sn-2 MAG after purification by crystallization. At the second step they obtained 90 % caprylic acid at sn-1,3 and 98.5% unsaturated long-chain fatty acids at sn-2 positions after enzymatic esterification of these MAG with caprylic acid [102].

At a study made by Rimescu *et al*. a three step strategy was used for SL synthesis. At the first step they obtained free EPA by hydrolysis. At the second step they synthesized EEE from EPA and glycerol. At the third step they synthesized CEC by interesterification of EEE with caprylic acid ethyl esters under optimum conditions. They used Novozym TM at the first two steps and Lipozyme TM lipase at the last one. They obtained 88% of regiospecific CEC type TAG which needed no additional purification [103].

At a study made by Lee and Foglia *Carica papaya* latex was used as a lipase to catalyze enzymatic acidolysis between caprylic acid and chicken fat. They optimized the mol ratio and obtained 23.4 mol % caprylic incorporation at 1:2 mol ratio of chicken fat: caprylic acid. They further concluded that *Carica papaya* lipase was 1,3 specific as a result of the NMR studies they made [104].

At a study made by Iwasaki *et al.* *Rhizomucor miehei* lipase and *Pseudomanas sp.* KWI-56 lipase were used at the enzymatic acidolysis of caprylic acid into SCO. They obtained more than 60 % mol incorporation of caprylic acid into corn oil when *Pseudomonas* lipase was used and only 23 % mol at *Rhizomucor* lipase. They also investigated the resulting TAG species. They found that 36 % of all TAG contained two CA and 77-78 % of them contained a unsaturated FA at sn-2 position when pseudomonas lipase was employed. Whereas, only 22 % of all TAG contained two caprylic acids but all had unsaturated fatty acids at sn-2 positions [105].

At a study made by Yang *et al.* although synthesis of human milk fat substitutes were studied they also investigated the enzymatic incorporation of caprylic acid into sunflower oil using Lipozyme RM IM and TL lipase. They found that TL lipase had a lower yield at these enzymatic acidolysis but only a slightly lower yield at ester-ester interchange reactions [106].

At a study made by Huang, and Akoh Enzymatic trans esterification was used for SL synthesis. They used caprylic acid ethyl esters and soybean oil and high oleic acid sunflower oil. Than they optimized the Incubation time, molar ratio of caprylic acid ethyl esters to total triglycerides, percentage of soybean oil and TAG concentration via response surface methodology. After these optimization 26.4 hours 1:8 mole ratio 75 % of soy oil and 0.58 M TAG concentration was found to be optimum respectively. At these point a statistical caprylic acid contend of 67.6 (mol%) and linoleic acid percent of 14.5% was calculated. They also verified their optimizations experimentally [107].

At a study made by Ko *et al.* Lipozyme IM from *Rhizomucor miehei* and Lipozyme TL IM were used to catalyze the transesterification between perilla oil and caprylic acid. They investigated the effects of reaction time, reaction temperature, enzyme load, and added water content on transesterification reaction. They determined that 24 hr reaction time, 3% added water, 10% enzyme load at 55ºC were the optimum conditions. They obtained 61.9 mol % at these optimum points. They also determined that Lipozyme TL IM had a higher affinity at lower temperatures where as Lipozyme IM were more heat stable [108].

At a study made by Wongsakul *et al.* a two step strategy were used for SL synthesis. At the first step they obtained sn-1,3 dilaurin or dicaprylin using various enzymatic

methods. At the consequent step they esterified oleic acid or oleic acid vinyl esters at the sn-2 position. They used immobilized lipases from *Burkholderia cepacia* (Amano PS-D) in n-hexane at 60ºC as catalyst to synthesize two different MLM type TAG containing caprylic acid or lauric acid at sn-1,3 positions and oleic acid at sn-2 position. They obtained 87 mol % and 78 mol % yields respectively. But regiospecific analyses indicated that 25.7% caprylic acid and 11.1 % lauric acid were at sn-2 positions respectively at these 2cases [109].

At a study made by Vu *et al.* a two-step production strategy was employed to enriching corn oil in conjugated linoleic acids and diacylglycerol. they obtained a reaction yield of 30.4 mol % at optimum conditions and 45.5 % of CLA was found to be at sn-1,3 positions. The final product they obtained after 48 h contained 6.8% monoacylglycerol, 31.5% diacylglycerol and 61.1% TAG [110].

At a study made by Vu *et al.* two different blends were used to compare their effects. The first blend was corn oil and corn oil diglycerides and the second was, diglyceride enriched structured lipids obtained from capric acid and CLA incorporation into corn oil. They found that the second blend had positive effects on increasing TAG metabolism and decreasing the abdominal adipose tissue contend they more over concluded that these mixture had positive effects on cholesterol metabolism in the liver [111].

At a study made by Nagata *et al.* a four week diet containing structured lipids or corn oil were feed to rats to investigate their effects metabolic effects. They concluded at the end of these study that at sn-2 position linoleic acid and at sn-1,3 positions MCFA containing structured lipids have a positive effect on serum and liver lipid profiles and that they are preferable substrates contributing to the energy supply in rat [112].

### 2.9 Regression Analysis

Regression analysis can be used to statistically estimate the value of a response or the relationship between two variables. But a simple regression analysis is of poor meaning it is much more valuable when it is used to check if the outputs of a system are really like the hypothetically predicted ones. This comes since it does not proofs

that the dependent variable is determined by the independent variable it only states that there is a highly possible solely numerically relationship [113].

It is also important to plot the data obtained by numerical analysis. Because it can easily cause errors or lead to inaccurate results to blindly use the computed results without checking the plot between independent and dependent variables [113].

### 2.9.1 Linear regression

In linear regression analysis the value of a single dependent variable is predicted in means of a single independent variable. This equation resembles the equation of a linear plot;

$$y = ax + b + a\ residual \qquad (2.1)$$

In the case of a linear regression a and b would be called regression coefficients and the residual would be taken as an unexplained experimental variation [113].

In such a case the co efficient a and b can be predictable using;

$$a = \frac{\sum (x_i - \bar{x})\ (y_i - \bar{y})}{\sum (x_i - \bar{x})^2} \qquad (2.2)$$

$$b = \bar{y} - a\,\bar{x} \qquad (2.3)$$

The value of y at a certain point is found by such a regression analysis using the equation

$$\acute{y} = ax + b \qquad (2.4)$$

Where a and b are the coefficients and x is the independent variable at this selected point. At a regression analysis it is aimed to minimize the difference between predicted ý and observed y value. In order to do this the sum of squares of these residues ($SS_{res}$) is tried to be minimized. The method used at regression analysis is called least square method [113].

$$SS_{res} = \Sigma\ (y_{i-}\ \acute{y}_i)^2 \qquad (2.5)$$

The affectivity of these obtained regression using least square method can be tested by ANOVA (analysis of the variance) which tests if the variance is significantly

reduced or this regression is a better "summary" of the obtained data but the confidence interval can not be obtained from ANOVA directly the obtained f values have to be compared with the f value for the desired confidence interval to state these [113].

Another method for examining the meaning of our regression analysis is calculating its coefficient of determination ($R^2$). This is calculated by;

$R^2 = 1- (SS_{res} / \text{Sum of total squares})$ (2.6)

If the calculated $R^2$ value is higher than 0.75 it is usually acceptable while a value of 1 indicates a completely linear relationship between the regression and results [113].

### 2.9.2 Multiple linear regression

Although in some cases simple linear regression is sufficient to predict the response there are many cases where this method is insufficient. In such cases a polynomial of a single variable or more than one independent variable is needed. Multiple regression analysis is a modification of simple linear regression that enables to make regression analysis for such cases as well [113].

One of the biggest problems with multiple linear regression is "multicolinearity." Multicolinearity can be defined as the cases in which two or more independent variables highly correlate. These phenomena can cause to misevaluations of the effects of related independent variables. Therefore studying the correlations between independent variables is important for obtaining better results when using multiple linear regression analysis. Another possibility to avoid the multicolinearity problem is using a specially designed multiple regression, known as response surface methodology [113].

### 2.9.3 Response surface methodology

Response surface methodology (RSM) is a specially designed regression analysis. RSM is different than fractional treatment structures as it does not solely aims to determine if and how the factors effects the response but also aims to find out the value of the response, dependent variable, in terms of independent variables. Because of this aim all variables has to be quantitative at RSM [113].The main advantage of RSM is to decrease the number of experiments which are have to be done to determine responses at various dependent variable levels. The results or responses at

these experimental points are used for a step wise regression analysis to predict a graphical equation of the response in means of independent variables. These graphical expression can be three dimensional or a two dimensional counter plot of these three dimensional graph can be used. Such graphs make it possible for a researcher to predict the response in means of independent variables at every desired point on these graph or surface.[113,114]

Different types of experimental designs can be used for RSM experiments. The most common used experimental design is similar to fractional design. It consists of all possible combinations of low and high levels for selected variables and center points. At a center point the average values, (low + high) /2, of the variables are taken. In many cases these center points are replicated several times for an independent experimental error approximation. These experimental levels are commonly coded as +1 -1 and 0 for maximum minimum and center points respectively [113, 114].

A first order two factor RSM experiment is shown in Figure 2.7. The points are demonstrating the positions of selected experimental points and central points [113].

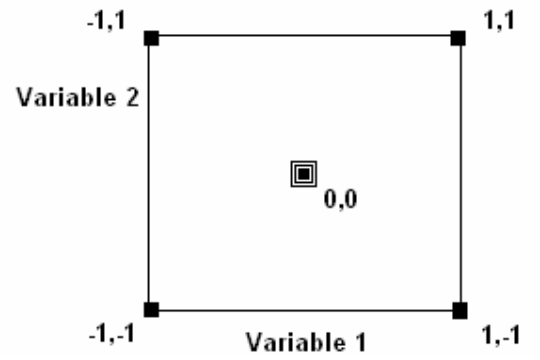


**Figure 2.7:** An experimental design for a two factors first order RSM experiment

The regression equation for such a first order RSM design would be like

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_k x_k \qquad (2.6)$$

At this equation the regression coefficient is symbolized with $\beta_1$ and $x_1$ is used to define the coded variable levels such as, +1, -1 or 0. Such experimental designs are usually employed at the early stages of experimental research to roughly predict the appropriate variable levels. Although a first order model has ability to give some

kind of knowledge about the relationship between independent variables and the related response it is insufficient to show complex relationships [113,114]

At a second order RSM experiment the regression equation includes additionally the squares and cross products of the factors. The regression equation for a second order RSM design would be like;

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_k x_k + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \ldots + \beta_{kk} x_k^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 \ldots + \beta_{k-1\,k}\, x_{k-1}\, x_k \quad (2.7)$$

The demonstration of selected experimental design points for such a second order two factor RSM experiment would be like shown on Figure 2.8 [113].

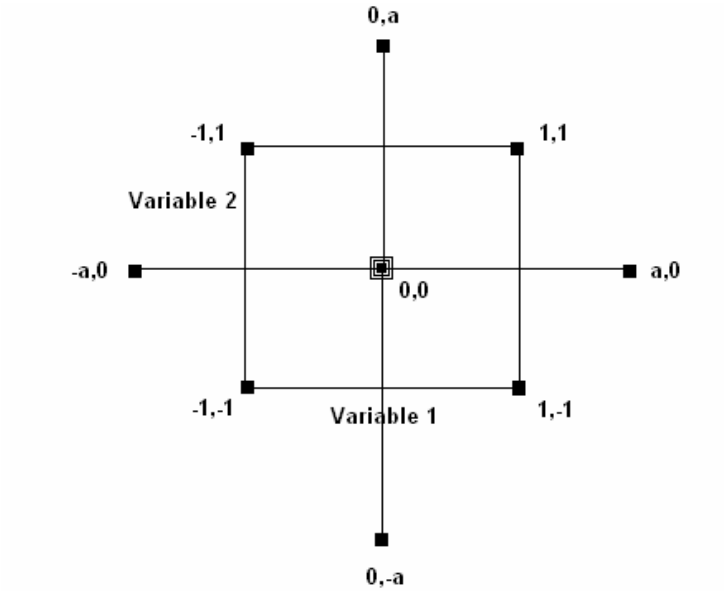


**Figure 2.8:** The graphical representation of experimental design points for a two variable second factor RSM experimental design.

The extra square and cross products in a second order design give the resulting response surface the ability to "bend" and "flex" making it more effective by predicting complex relationships between response and independent variables [113,114].

A widely used second order RSM design is the central composite rotatable design. In this design "axial" or "star" points are added to a first order design. These points are generally coded with α. This α value is the distance between the center of experimental design and the selected variable. This value is calculated by the 0.25th

power of noncentral first order experimental design points. For example these α value for the first order experimental design on Figure 2.8 would be the 0.25th power of 4 resulting in 1.414213 [113,114].

An other commonly used design is the face centered cube design which is quite similar to central composite rotatable design with the only difference that the α value is replaced with 1 resulting that all noncentral experimental points are on the same cube surface. Figure 2.9 demonstrates central composite rotatable design and face centered cube design for a three variable second order experimental RSM design [6,113,115].

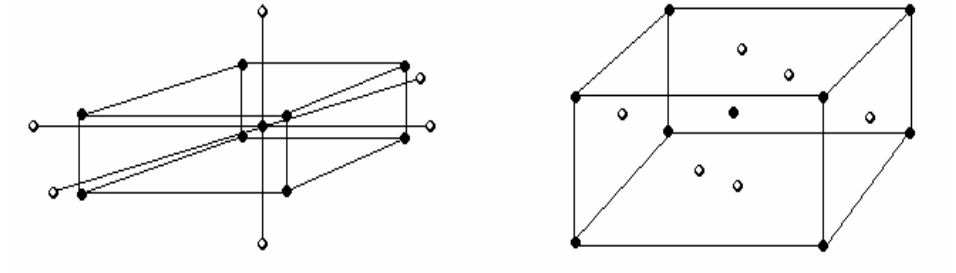

**Figure 2.9:** Demonstration of experimental design points at a three variable second order central composite rotatable and face centered cube RSM design.

# 3. MATERIALS AND METHODS

## 3.1. Materials

A commercially available food grade corn oil "Bizim yağ" used in the study was obtained from a local store. It was a product of Besler Gıda, Istanbul and was distributed by Ülker. The carprylic acid was obtained from Sigma-Aldrich (Buchs, Switzerland). A sample of a commercially available lipase, Lipozyme TL IM was supplied by Nova-Nordisk A/S (Copenhagen, Denmark). Lipozyme TL IM is an on silica immobilized sn-1,3 specific *Thermomyces lanuginosa* lipase. All other chemicals used in the purification of the reaction products, their analysis, and as solvent were obtained from Merck (Darmstadt, Germany).

## 3.2 Methods

### 3.2.1 Characterization of materials

In order to analyze the fatty acid compositions of all substrates and TAG products of the reactions they were converted to their corresponding methyl esters by $BF_3$ esterification. These methyl esters were analyzed by a Hewlett-Packard 5890 II gas chromatograph (Hewlett-Packard, Waldron, Germany). The analysis conditions are summarized on Table 3.1. Peaks were identified by comparing the retention times with those of a mixture of standard methyl esters analyzed at the same conditions.

**Table 3.1:** Analysis conditions for GC

| | |
|---|---|
| Detector Type | FID[(1)] |
| Detector temperature ($^{o}$C) | 280 |
| Injection temperature ($^{o}$C) | 250 |
| Gas flow rates (mL/ min) | |
| Nitrogen | 1.6 |
| Hydrogen | 33 |
| Air | 460 |
| Split ratio | 88:1 |
| Oven temperature | 150 $^{o}$C (5 min)<br>150-275 $^{o}$C (5 $^{o}$C/min)<br>275 $^{o}$C (5 min) |
| Column type | Capillary Column<br>TRB-5ht[(2)] |

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

### 3.2.2 Acidolysis of corn oil and caprylic acid

Acidolysis reactions were conducted in the dark-colored, heat-resistant 30mL reaction flasks. Firstly the needed substrate amounts for the desired substrate mole ratio were calculated. The reaction flasks having substrate mixture consisted of corn oil and caprylic acid (1g) and 5 mL hexane were placed in a temperature controlled orbital shaker (Edmund Bühler, KS-15, Germany) at 200 rpm and 50°C. The reactions were started by adding the proper amount of enzyme, Lipozyme TL IM to the reaction mixtures. After a certain time the flasks were taken out of the orbital shaker and samples were taken by a glass pipette into an erlenmeyer flask for the analysis.

### 3.2.3 Analysis of acidolysis products

For the inactivation of enzyme 2.5 mL ethanol was added for each mL of sample and shacked vigorously. Having added ethanol as much hexane as ethanol was added. The reaction samples consisted of TAG and free fatty acids were titrated against 0.02 M NaOH in order to neutralize free fatty acids. Phenolphthalein was used as an indicator. When the color of the mixture was shifted from white to pink it was assumed that all fatty acids were converted into their sodium salts. Then the mixture was poured into a separatory funnel and thoroughly mixed with 50 mL of hexane, 25 mL of water and 10 mL of saturated NaCl solution to perform liquid-liquid

extraction. TAG species were soluble in the organic phase while the sodium salts were soluble in water. NaCl was used to break the water/oil emulsion. Organic phase was separated from water phase which was discarded. Liquid-liquid extraction was performed several times by adding fresh water to the organic phase till a neutral pH was obtained. The pH was controlled with a pH paper. The neutral hexane phase containing acylglyceroles were dehydrated using anhydrous $Na_2SO_4$. Then the acylglycerol fraction was recovered, following hexane removal at 45 °C using a rotary evaporator.

For the determination of fatty acid composition, the acylglycerol fraction (TAG) was converted into corresponding methyl esters according to the related American oil Chemists' Society (AOCS) method and analyzed by capillary gas chromatography

The compositions of TAG species of corn oil and synthesized TAG obtained at the critical conditions were determined using a reverse phase high performance liquid chromatography (HPLC) method. For the HPLC analysis the samples were dissolved in an appropriate solvent, methanol, iso-propanol, acetonitrile, and analyzed. A mobile phase of a isopropanol-methanol gradient was used in the analysis conducted with SPD-10A UV-vis detector. An isocratic mobile phase of a mixture of methanol, iso-propanol and acetonitrile was used for the analysis conducted with RID-10A refractive index detector. Shimadzu HPLC apparatus equipped with a 250 mm 5 μm C18 column, Shimadzu SCL-10A vp system controller, DGU-14A degasser, LC-10A pump, SIL-10AD vp auto injector, CTO-10AC vp oven and Class VP software was used for the analysis.

### 3.2.4 Selection of independent variables and their levels for experimental design

For the optimization of caprylic acid incorporation into corn oil via response surface methodology, 3 variables (factors) were optimized using face centered cube design. The selected variables were the enzyme amount (%, based on substrate weight), mole ratio of substrates, caprylic acid: corn oil, and the reaction time. These variables were selected according to the literature research and previous experiences. It was known that reaction temperature, solvent type and water amount were important variables for the acidolysis reaction. But it was already known that the optimum temperature of the enzyme used in the study, Lipozyme TL IM was 50 ºC. At higher temperatures, the half live of the enzyme would decrease and the reaction would be

uneconomical even if the reaction rate would possibly increase. It was also known that the proper solvent would be hexane as it is a commonly used solvent at fat and oil extraction which can easily be recycled. Hexane is a hydrophobic solvent which increases the lipase activity in acidolysis reactions. More over it was known that the commercial immobilized enzyme Lipozyme TL IM contained one single layer of water which was enough for acidolysis reactions. Therefore, it was decided to conduct the reactions using hexane as solvent at 50 ºC.

To determine the maximum (+1), minimum (-1) and mean (0) values for our selected independent variables (enzyme amount, substrates mole ratio, and the reaction time) several pre-optimization experiments were done. First of all the levels of enzyme amount were investigated. Five experiments were designed for this purpose. In these experiments the mole ratio of the substrates (corn oil/caprylic acid) and reaction time were kept constant at 1:3, and 6 h, respectively, where as the enzyme amounts were selected as 3-15 % based on substrates weight. The levels of substrate mole ratio were determined performing the reactions using 12% enzyme at 6 h. In these reactions used substrate mole ratios (corn oil/caprylic acid) were 1:1- 1:7. Finally levels of the reaction time were investigated changing the time 2-10h at the constant substrate mole ratio (1:3) and enzyme amount (12%). All of the reactions were performed at least in duplicate.

### 3.2.5. Experimental Design and Optimization of Selected Parameters

The reaction independent variables were optimized via response surface methodology. A three-level, three-factor face centered cube design were selected. Total 17 experiments with three center points (0,0,0) was adopted in this study. The experimental data were analyzed by the response surface using Statistica 6.0. software. All reactions were performed at least in duplicate.

## 4. RESULTS AND DISCUSION

The aim of this study was to produce MLM type structured lipids by enzymatic acidolysis of caprylic acid into corn oil and optimize the reaction parameters. The resulting MLM type structured lipids contained mainly EFA (linoleic C18:2, n6) at sn-2 position and easily metabolized MCFA (caprylic C 8:0) at sn-1,3 positions. These kind of structured lipids have many advantages at once. The main advantages are; lower energy values, decreased susceptibility to be deposited as body fat, to be a faster absorbable essential fatty acid source, and a good essential acid source for patients suffering pancreatic insufficiencies or fat malabsorption problems.

Experiments were performed in order to optimize these reaction parameters via response surface methodology. After the pre-optimization experiments and literature research parameters which are needed to be optimized and their corresponding levels as +1, 0, and -1 were determined. A central composite design with 3 variables and 3 levels were computed. This design resulted in 17 experiments. These experiments were performed at least in duplicate. The obtained values were computed by Statistica 6.0 software in order to optimize these 3 parameters via response surface methodology.

### 4.1 Fatty acid Composition of Used Substrates

At this study first the fatty acid composition of caprylic acid and corn oil was determined by GC under the analysis conditions described in Section 3.2.1. Table 4.1 gives the fatty acid composition of corn oil and caprylic acid.

**Table 4.1:** Fatty acid composition of caprylic acid (CA) and corn oil

| | Caprylic acid (mol %) | Corn Oil (mol %) |
|---|---|---|
| C 8:0 | 99 | - |
| C 16:0 | <1 | 12.0±0.1 |
| C 18:2 | <1 | 54.6±2.2 |
| C 18:1 | <1 | 30.5±0.4 |
| C 18:0 | <1 | 2.3±0.7 |

## 4.2 Determination of Independent Variables and Their Levels for Experimental Design

Experiments were done to determine the corresponding amounts for the +1, 0 and -1 levels of the related independent variables. Table 4.2 shows the fatty acid composition of the obtained TAG after 6 hours in reaction mixtures containing 6, 9, 12, 15 and 18 % enzyme at a mole ratio of 1:3 corn oil to caprylic acid.

**Table 4.2:** Fatty acid composition of obtained TAG after enzymatic acidolysis reaction to determine the enzyme amount corresponds to 1, 0,-1 (Corn oil: Caprylic acid, 1:3, 6 hours reaction time)

| | **Fatty acid composition (mol %)** | | | | |
|---|---|---|---|---|---|
| **Fatty acids** | **6% Enzyme** | **9% Enzyme** | **12% Enzyme** | **15% Enzyme** | **18% Enzyme** |
| 16:0 | 7.7 | 8.5 | 7.0 | 9.6 | 9.3 |
| 18:2 | 50.9 | 43.4 | 45.4 | 45.6 | 50.2 |
| 18:1 | 20.1 | 23.0 | 20.9 | 22.4 | 25.1 |
| 18:0 | 1.6 | 1.6 | 1.3 | 1.9 | 1.7 |
| **Caprylic acid** | **19.0** | **22.8** | **25.1** | **19.7** | **13.5** |

After these pre-optimization experiments the enzyme levels were selected. 12 % enzyme was selected as 0 as the highest yield were obtained at these point. +1 and -1 coded values were selected as 15% and 9 %, respectively.

The second experiments were carried out to select the substrate mole ratio levels, varying mole ratios of 1:1, 1:3, 1:5, 1:7, 1:9 corn oil to caprylic acid at 12% enzyme and 6h. Table 4.3 shows the fatty acid composition of the obtained TAG after 6 hours in reaction mixtures.

**Table 4.3:** Fatty acid composition of obtained TAG after enzymatic acidolysis reaction to determine the corn oil: caprylic acid mole ratios corresponding to +1, 0,-1 (enzyme amount:12%, 6 hours reaction time)

| | Fatty acid composition (mol %) | | | | |
|---|---|---|---|---|---|
| **Fatty acids** | **1:1** | **1:3** | **1:5** | **1:7** | **1:9** |
| 16:0 | 8.9 | 7.0 | 6.6 | 7.2 | 8.3 |
| 18:2 | 55.6 | 45.4 | 49.3 | 47.8 | 48.5 |
| 18:1 | 20.8 | 20.9 | 16.1 | 16.3 | 16.5 |
| 18:0 | 1.7 | 1.3 | 1.2 | 1.4 | 1.5 |
| **Caprylic acid** | **12.3** | **25.1** | **26.2** | **26.8** | **24.7** |

According to these results 1:3 corn oil: caprylic acid mole ratio was selected as 0. +1 and -1 levels of the substrate mole ratio were selected as 1:1 and 1:5, respectively.

Table 4.4 demonstrates the fatty acid composition of the obtained TAG after 2, 4, 6, 8, 10 hours of reaction time in reaction mixtures containing 12 % enzyme and 1:3 corn oil to caprylic acid mole ratio.

**Table 4.4:** Fatty acid composition of obtained TAG after enzymatic acidolysis reaction to determine the reaction time corresponding to +1, 0, -1 (12% enzyme,1:3 substrate mole ratio)

| | Fatty acid composition (mol %) | | | | |
|---|---|---|---|---|---|
| **Fatty acids** | **2 (h)** | **4 (h)** | **6 (h)** | **8 (h)** | **10 (h)** |
| 16:0 | 8.6 | 6.9 | 7.0 | 7.6 | 6.6 |
| 18:2 | 53.2 | 52.6 | 45.4 | 46.7 | 46.0 |
| 18:1 | 19.1 | 17.1 | 20.9 | 20.6 | 17.8 |
| 18:0 | 1.6 | 1.3 | 1.3 | 1.5 | 1.2 |
| **Caprylic acid** | 16.8 | 21.3 | 25.1 | 23.0 | 27.8 |

After these pre-optimization experiments the levels of reaction times were selected. Reaction times of 6, 8 and 10 h were selected as +1, 0 and -1, respectively.

### 4.3 Experimental Design for Response Surface Methodology and Optimization of Reaction Parameters

The reaction parameters for enzymatic acidolysis of caprylic acid and corn oil were optimized via response surface methodology (RSM). At these study a three variable three level central composite face centered cube design was used. 17 experiments

were conducted according to this design. Corresponding values for the three selected independent parameter levels are shown in Table 4.5.

**Table 4.5:** Independent variables and their coded values used at face centered cubic central composite experimental design.

| **Independent variables** | **Coded variable levels** | | |
|---|---|---|---|
| | -1 | 0 | 1 |
| **Enzyme amount (w/w %)** | 9 | 12 | 15 |
| **Corn oil: Caprylic acid mole ratio** | 1:1 | 1:3 | 1:5 |
| **Reaction time (h)** | 6 | 8 | 10 |

The17 designed experimental points and their actual and coded values are shown at Table 4.6.

**Table 4.6**: Selected experimental points for the cubic central composite experimental design

| **Number of Experiment** | **Enzyme amount (%)** | | **Corn oil: Caprylic acid Mole ratio** | | **Time (h)** | |
|---|---|---|---|---|---|---|
| | **Code** | **Value** | **Code** | **Value** | **Code** | **Value** |
| 1 | -1 | 9 | -1 | 1:1 | -1 | 6 |
| 2 | -1 | 9 | -1 | 1:1 | 1 | 10 |
| 3 | -1 | 9 | 1 | 1:5 | -1 | 6 |
| 4 | -1 | 9 | 1 | 1:5 | 1 | 10 |
| 5 | 1 | 15 | -1 | 1:1 | -1 | 6 |
| 6 | 1 | 15 | -1 | 1:1 | 1 | 10 |
| 7 | 1 | 15 | 1 | 1:5 | -1 | 6 |
| 8 | 1 | 15 | 1 | 1:5 | 1 | 10 |
| 9 | -1 | 9 | 0 | 1:3 | 0 | 8 |
| 10 | 1 | 15 | 0 | 1:3 | 0 | 8 |
| 11 | 0 | 12 | -1 | 1:1 | 0 | 8 |
| 12 | 0 | 12 | 1 | 1:5 | 0 | 8 |
| 13 | 0 | 12 | 0 | 1:3 | -1 | 6 |
| 14 | 0 | 12 | 0 | 1:3 | 1 | 10 |
| 15 | 0 | 12 | 0 | 1:3 | 0 | 8 |
| 16 | 0 | 12 | 0 | 1:3 | 0 | 8 |
| 17 | 0 | 12 | 0 | 1:3 | 0 | 8 |

Tables from 4.7 to 4.10 show the fatty acid compositions of the obtained TAG at the selected 17 experimental design points.

**Table 4.7:** Fatty acid composition of obtained TAG after enzymatic acidolysis reaction (Enzyme amount: 9%) (Experiments numbered from 1 to 4)

| **Fatty acids** | **Fatty acid composition (mol %)** | | | |
|---|---|---|---|---|
| | **Corn oil: Caprylic acid, 1:1** | | **Corn oil: Caprylic acid, 1:5** | |
| | **2 h** | **10 h** | **2 h** | **10 h** |
| 16:0 | 8.9 | 9.1 | 9.2 | 7.1 |
| 18:2 | 56.0 | 56.6 | 54.1 | 46.0 |
| 18:1 | 23.0 | 21.6 | 16.7 | 19.8 |
| 18:0 | 1.7 | 1.7 | 1.6 | 1.3 |
| **Caprylic acid** | **9.6** | **10.2** | **17.8** | **25.2** |

**Table 4.8:** Fatty acid composition of obtained TAG after enzymatic acidolysis reaction (Enzyme amount: 15%) (Experiments numbered from 5 to 8)

| **Fatty acids** | **Fatty acid composition (mol %)** | | | |
|---|---|---|---|---|
| | **Corn oil: Caprylic acid, 1:1** | | **Corn oil: Caprylic acid, 1:5** | |
| | **2 h** | **10 h** | **2 h** | **10 h** |
| 16:0 | 9.0 | 9.0 | 7.5 | 7.1 |
| 18:2 | 55.1 | 53.4 | 49.4 | 42.3 |
| 18:1 | 22.0 | 22.9 | 18.5 | 18.8 |
| 18:0 | 1.7 | 1.7 | 1.4 | 1.3 |
| **Caprylic acid** | **11.4** | **12.2** | **22.6** | **29.8** |

**Table 4.9:** Fatty acid composition of obtained TAG after Enzymatic acidolysis reaction (Time: 6 hours) (Experiments numbered from 9 to 12)

| **Fatty acids** | **Fatty acid composition (%)** | | | |
|---|---|---|---|---|
| | **Corn oil: Caprylic acid, 1:1** | | **Corn oil: Caprylic acid, 1:3** | **Corn oil: Caprylic acid, 1:5** |
| | **9% Enzyme** | **9% Enzyme** | **12% Enzyme** | **15% Enzyme** |
| 16:0 | 8.6 | 7.7 | 9.1 | 7.1 |
| 18:2 | 51.0 | 48.6 | 53.8 | 46.2 |
| 18:1 | 20.5 | 19.6 | 22.4 | 18.6 |
| 18:0 | 1.6 | 1.4 | 1.7 | 1.3 |
| **Caprylic acid** | **17.7** | **22.1** | **12.4** | **26.2** |

**Table 4.10:** Fatty acid composition of obtained TAG after enzymatic acidolysis reaction (Corn oil: Caprylic acid mole ratio: 1:3; Enzyme: 12%) (Experiments numbered from 13 to 17)

| Fatty acids | Fatty acids composition (%) | | | | |
|---|---|---|---|---|---|
| | **2 h** | **10 h** | **6 h** | **6 h** | **6 h** |
| 16:0 | 8.1 | 7.2 | 7.2 | 7.6 | 7.6 |
| 18:2 | 47.4 | 48.1 | 51.1 | 46.8 | 46.7 |
| 18:1 | 20.8 | 18.2 | 19.0 | 19.8 | 20.6 |
| 18:0 | 1.5 | 1.4 | 1.4 | 1.3 | 1.5 |
| **8:0** | **21.5** | **24.5** | **20.7** | **24.0** | **23.0** |

In table 4.11 the responses as incorporated caprylic acid (mol %) are shown.

**Table 4.11:** Responses obtained for the selected experimental points for a cubic central composite experimental design

| **Number of Experiment** | **Enzyme amount (%)** | **Corn oil: Caprylic acid Mole ratio** | **Time (hour)** | **Response (Caprylic acid mol %)** | |
|---|---|---|---|---|---|
| | | | | **Observed** | **Predicted** |
| 1 | 9 | 1:1 | 6 | **9.6** | 9.4 |
| 2 | 9 | 1:1 | 10 | **10.2** | 9.9 |
| 3 | 9 | 1:5 | 6 | **17.8** | 17.9 |
| 4 | 9 | 1:5 | 10 | **25.2** | 25.0 |
| 5 | 15 | 1:1 | 6 | **11.4** | 11.6 |
| 6 | 15 | 1:1 | 10 | **12.2** | 12.1 |
| 7 | 15 | 1:5 | 6 | **22.6** | 22.8 |
| 8 | 15 | 1:5 | 10 | **29.8** | 29.9 |
| 9 | 9 | 1:3 | 8 | **17.7** | 18.2 |
| 10 | 15 | 1:3 | 8 | **22.1** | 21.7 |
| 11 | 12 | 1:1 | 8 | **12.4** | 12.8 |
| 12 | 12 | 1:5 | 8 | **26.2** | 25.9 |
| 13 | 12 | 1:3 | 6 | **21.5** | 21.1 |
| 14 | 12 | 1:3 | 10 | **24.5** | 24.9 |
| 15 | 12 | 1:3 | 8 | **20.7** | 22.5 |
| 16 | 12 | 1:3 | 8 | **24.0** | 22.5 |
| 17 | 12 | 1:3 | 8 | **23.0** | 22.5 |

### 4.4 Statistical Evaluation of the Reaction Parameters

Statistica 6.0 (Stat Soft® Inc.) software was used to evaluate the results given in Table 4.11. The effects of independent variables on the response (dependent variable) were computed. Firstly variance analysis was done. A quadratic polynomial was used by the evaluation of the central composite design with 3 variables and 3 levels.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{j=1}^{k} \sum_{i>j}^{k} \beta_{ij} X_i X_i \qquad (4.1)$$

Where Y represents the response, $\beta_0$ the mean effect, $\beta_i$, $\beta_{ii}$, $\beta_{ij}$, are representing the linear, quadratic, and interactions respectively. $X_i$ and $X_j$ are representing the independent variables.

Depending on the interactions between dependent and independent variables their linear and quadratic effects and coefficients were determined. These evaluations were represented in Table 4.12.

**Table 4.12:** Regression coefficients of the second-order polynomials for response

| | **Effect** | **Standard error** | **t** | **P** | **Coefficient** |
|---|---|---|---|---|---|
| Mean/Intercept | 22.50282 | 0.425257 | 52.91574 | 0.000000 | **22.50282** |
| (1)Enzyme amount (L) | 3.52000 | 0.628549 | 5.60020 | 0.000816 | **1.76000** |
| Enzyme amount (Q) | -5.10986 | 1.214320 | -4.20800 | 0.003996 | **-2.55493** |
| (2) Caprylic acid :Corn oil mole ratio (L) | 13.16000 | 0.628549 | 20.93709 | 0.000000 | **6.58000** |
| Caprylic acid :Corn oil mole ratio (Q) | -6.30986 | 1.214320 | -5.19621 | 0.001258 | **-3.15493** |
| (3)Time (L) | 3.80000 | 0.628549 | 6.04567 | 0.000518 | **1.90000** |
| Time (Q) | 1.09014 | 1.214320 | 0.89774 | 0.399149 | **0.54507** |
| Enzyme amount*Mole ratio (L) | 1.40000 | 0.702740 | 1.99220 | 0.086609 | **0.70000** |
| Enzyme amount*Time (L) | 0.00000 | 0.702740 | 0.00000 | 1.000000 | **0.00000** |
| Mole ratio*Time (L) | 3.30000 | 0.702740 | 4.69591 | 0.002219 | **1.65000** |

According to the statistical evaluations showed in Table 4.12, the obtained quadratic equation for the dependent variable, response, in the means of independent variables is given as follows:

Caprylic acid % = 22.50+ 1.76*E + 6.58M + 1.90T – 2.55*$E^2$ – 3.15$M^2$

+0,55$T^2$ + 0,70*E*M + 0,00*E*T + 1,65 M*T (4.2)

Where, E represents the enzyme amount, M the substrate mole ratio, and T time at this equation.

p values calculated by Statistica 6.0 software are given in Table 4.13. These values indicate the confidence of the calculated values. They are computed by comparing the calculated F values with the F values from the Tables. A p value below 0.01 indicates a confidence interval of 99%. If the p values in Table 4.13 are evaluated a poorer confidence interval than 99 % is obtained only for quadratic effect of time (T*T) and, interaction between enzyme amount and molar ratio (E*M). The interaction between enzyme amount and time (E*T) had a p value of 1 and coefficient of 0. This indicates that it had no effect at all. The other p values were all below 0.01 indicating a higher confidence interval than 99%.

**Table 4.13:** Variance analysis

| | SS | df | MS | F | p |
|---|---|---|---|---|---|
| (1)Enzyme amount (L) | 30.9760 | 1 | 30.9760 | 31.3622 | 0.000816 |
| Enzyme amount (Q) | 17.4892 | 1 | 17.4892 | 17.7073 | 0.003996 |
| (2) Caprylic acid :Corn oil molar ratio (L) | 432.9640 | 1 | 432.9640 | 438.3619 | 0.000000 |
| Caprylic acid :Corn oil molar ratio (Q) | 26.6681 | 1 | 26.6681 | 27.0006 | 0.001258 |
| (3)Time (L) | 36.1000 | 1 | 36.1000 | 36.5501 | 0.000518 |
| Time (Q) | 0.7960 | 1 | 0.7960 | 0.8059 | 0.399149 |
| Enzyme amount*Molar ratio (L) | 3.9200 | 1 | 3.9200 | 3.9689 | 0.086609 |
| Enzyme amount*Time (L) | 0.0000 | 1 | 0.0000 | 0.0000 | 1.000000 |
| Molar ratio*Time (L) | 21.7800 | 1 | 21.7800 | 22.0515 | 0.002219 |
| Error | 6.9138 | 7 | 0.9877 | | |
| Total SS | 622.6988 | 16 | | | |

The correlation coefficient, $R^2$, for the computed quadratic equation was found 0.99. Also the sum of residuals was found 0.2. These are indicating the conformity of the computed statistical model.

Table 4.14 shows the computed, observed minimal, observed maximal and critical values for the three independent variables according to the response surface model. Caprylic acid incorporation calculated at these critical points was 22 % (mol%).

**Table 4.14:** Observed maxima, minima and critical values.

| | Observed minima | Critical value | Observed maxima |
|---|---|---|---|
| **Enzyme (%)** | 9 | 13.21942 | 15 |
| **Caprylic acid :Corn oil (mol)** | 1 | 3.90579 | 5 |
| **Reaction time (hours)** | 6 | 3.14324 | 10 |

Figure 4.1 plots the observed values against the corresponding predicted values which were calculated according to the computed model.

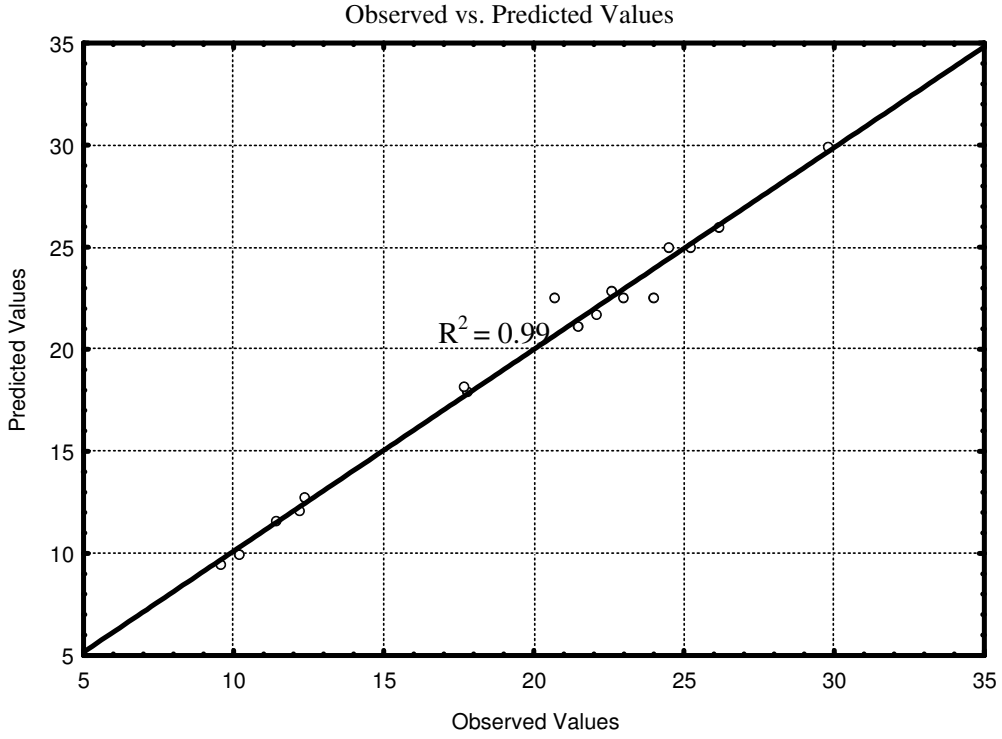


**Figure 4.1:** Correlation of experimental and predicted caprylic acid incorporation (mol%)

### 4.5 Interpretation of Response Surface and Contour Plots

The effects of enzyme amount and corn oil: caprylic acid mole ratio on the enzymatic caprylic acid incorporation into corn oil are demonstrated on the response surface plots and their contour plots are given in Figure 4.2.a - Figure 4.4.b. At

response surface plots the levels of variables are shown on three axis and at the contour plots the independent variables are shown on x and y axis and the caprylic acid incorporation is shown by the color levels of the related areas. At each graph the third variable whose effect was not shown on the graph was kept constant at its critical level.

The effect of enzyme amount (wt %) and caprylic acid: corn oil mole ratio on caprylic acid incorporation is illustrated in Figure 4.2.a

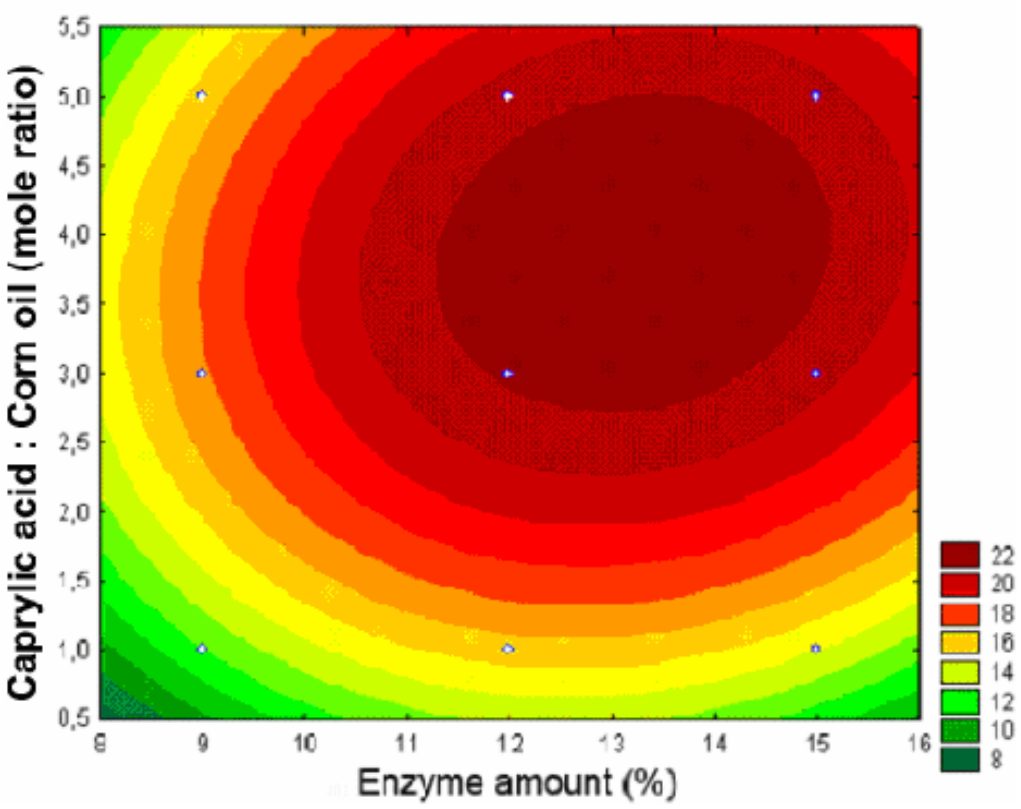


**Figure 4.2.a:** The contour plot showing the effect of enzyme amount (%) and caprylic acid: corn oil mole ratio on caprylic acid incorporation (mol%)

According to this contour plot the maximum caprylic acid incorporation was estimated between 11-15 % enzyme amount (%) and a corn oil: caprylic acid mole ratio between 1:3 and 1:5.

The effect of enzyme amount (wt %) and caprylic acid: corn oil mole ratio on caprylic acid incorporation is illustrated on the three dimensional response surface plot on figure 4.2.b

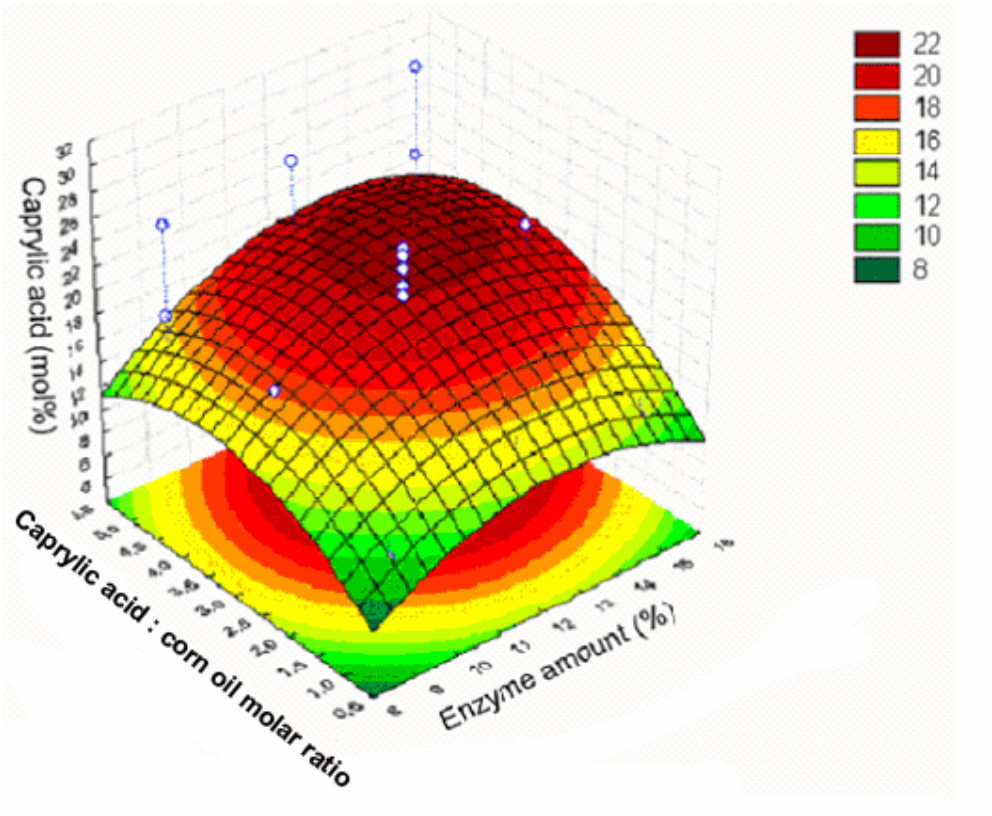


**Figure 4.2.b:** The response surface plot showing the effect of enzyme amount (%) and caprylic acid: corn oil mole ratio on caprylic acid incorporation (mol%)

As shown in Figure 4.2.b, enzyme amount (%) and mole ratio of the substrates had a similar effect on caprylic acid incorporation. It can also be observed that the slope decreases at points closer to maximum caprylic acid incorporation.

The effect of enzyme amount (wt %) and time (h) on caprylic acid incorporation is illustrated in Figure 4.3.a

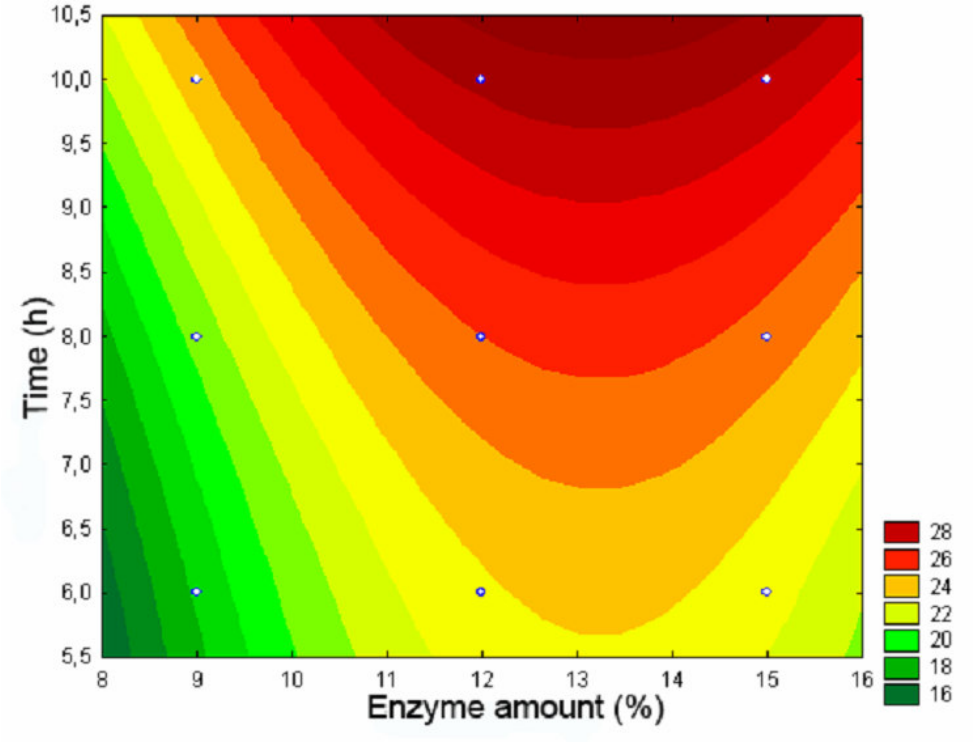


**Figure 4.3.a:** The contour plot showing the effect of enzyme amount (%) and time (h) on caprylic acid incorporation (mol%)

According to this contour plot the maximum caprylic acid incorporation was estimated between 12-15 % enzyme amount (%) and time between 10-10.5 h. It can also be observed that enzyme amount were more critical for caprylic acid incorporation than time.

The effect of enzyme amount (wt %) and reaction time on caprylic acid incorporation is illustrated on the three dimensional response surface plot in Figure 4.3.b

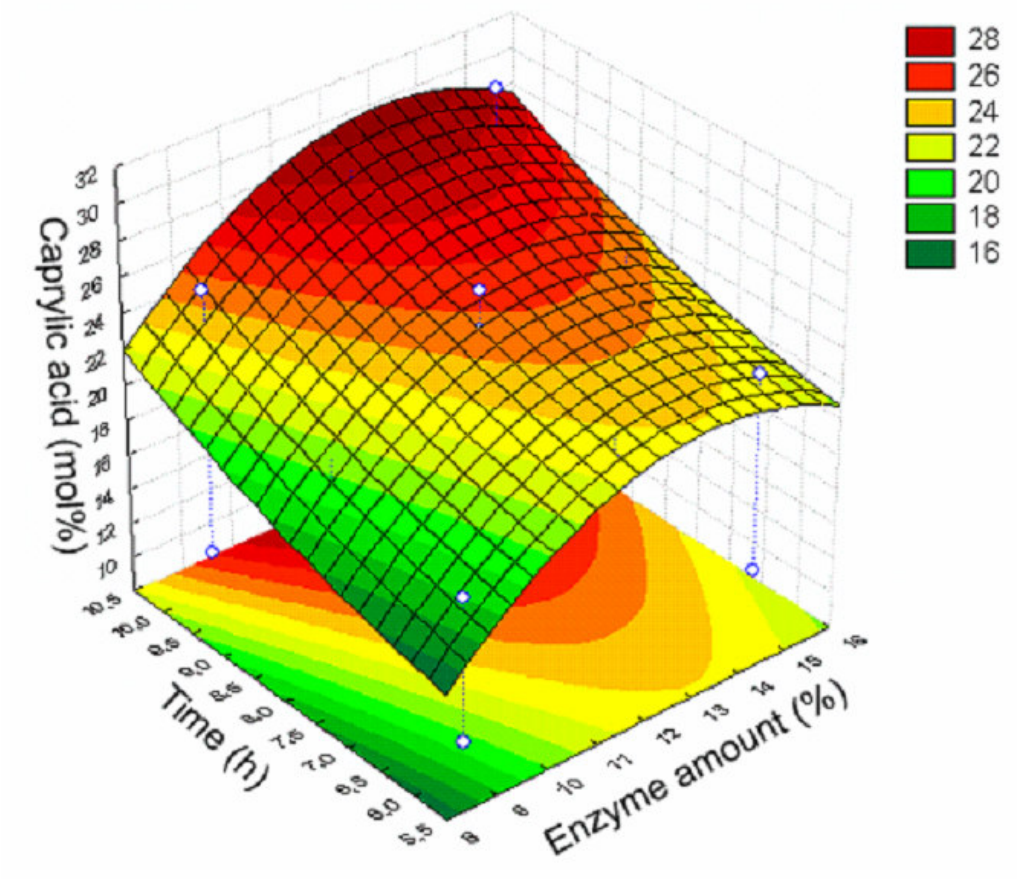


**Figure 4.3.b:** The response surface plot showing the effect of enzyme amount (%) and time (h) on caprylic acid incorporation (mol%)

At this response surface plot it is clearly observed that caprylic acid incorporation is maximum between enzyme amounts of 12% to 15 %.

The effect of caprylic acid: corn oil mole ratio and time (h) on caprylic acid incorporation is illustrated on the two dimensional contour plot of the three dimensional response surface plot on Figure 4.4.a

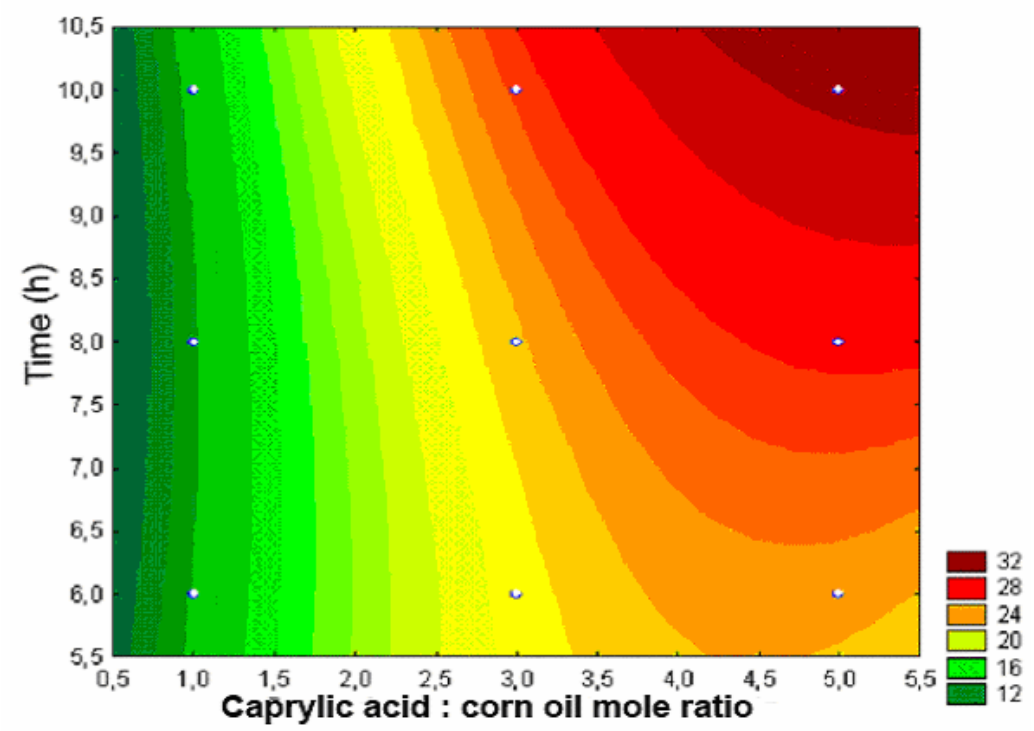


**Figure 4.4.a:** The contour plot showing the effect of substrate mole ratio and time (h) on caprylic acid incorporation (mol%)

According to this contour plot the maximum caprylic acid incorporation was estimated between 1:5 and 1:5.5 corn oil: caprylic acid mole ratio and time between 10-10.5 h. It can also be observed that mole ratio were more critical for caprylic acid incorporation than time.

The effect of enzyme amount (wt %) and caprylic acid: corn oil mole ratio on caprylic acid incorporation is illustrated on the three dimensional response surface plot on figure 4.4.b

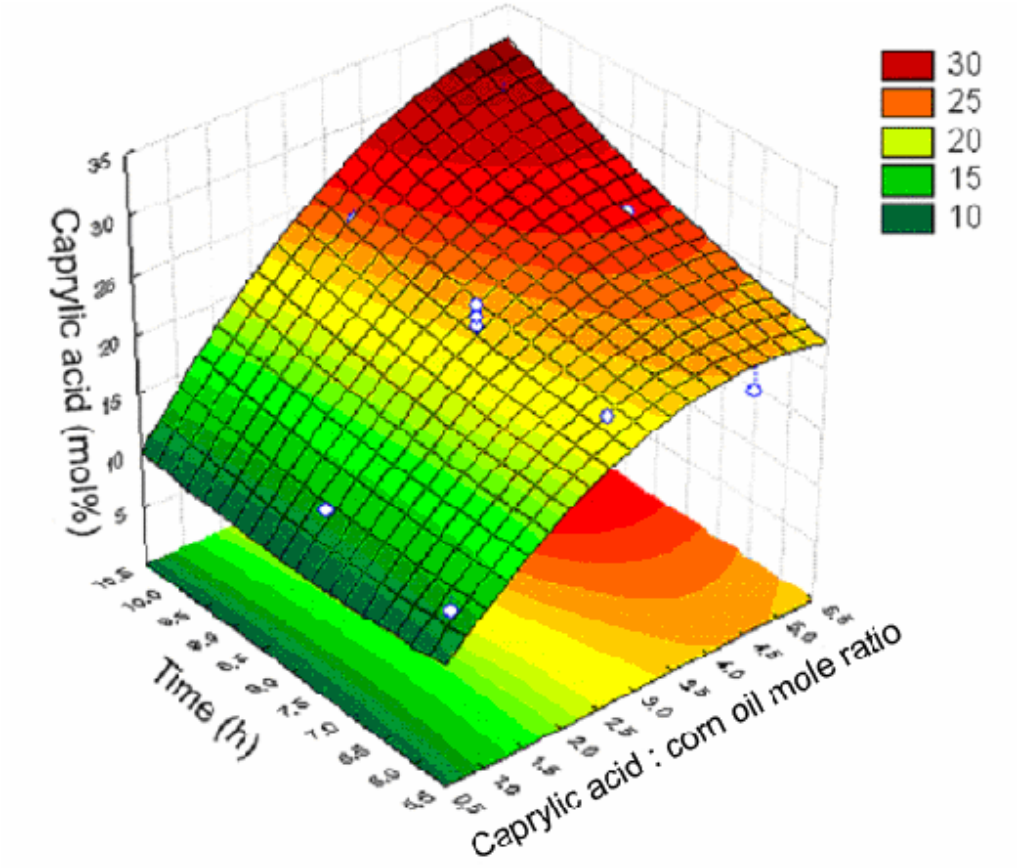


**Figure 4.4.b:** The response surface plot showing the effect of caprylic acid: corn oil mole ratio and time (h) on caprylic acid incorporation (mol%)

At this response surface plot it is observed that substrate mole ratio is more critical in caprylic acid incorporation than time. Reaction time has almost no effect till a mole ratio of 1:3 (corn oil: caprylic acid).

### 4.6 Analysis of TAG species

The chromatogram obtained by the HPLC analysis of corn oil using a refractive index detector is given in Figure 4.5

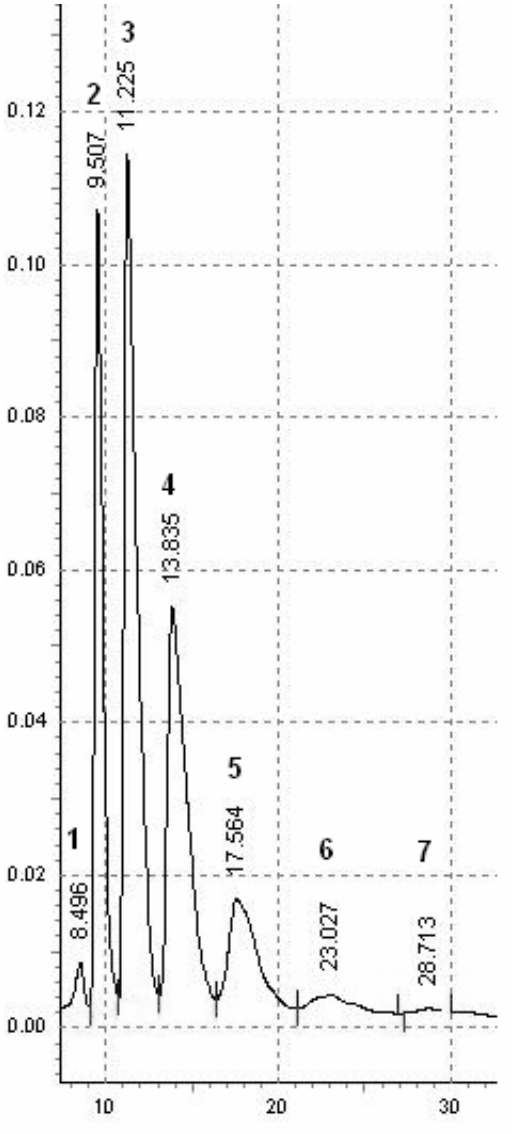


**Figure 4.5:** Chromatogram obtained by the HPLC analysis of corn oil using a refractive index detector( using a 50% methanol and 50% acetone mixture and a flow rate of 1ml/min as mobile phase)

The retention times, the area percentages of the peaks and identified TAG species are given in Table 4.15.

**Table 4.15:** The retention times, the area percentages of the peaks and identified TAG species of corn oil (Detector: Refractive index ).

| Peak number | Retention time (min) | Predicted TAG species | Area (%) |
|---|---|---|---|
| 1 | 8.4 | LLL | 16 |
| 2 | 9.5 | LLO | 16 |
| 3 | 11.2 | OOL | 21 |
| 4 | 13.8 | LLP | 21 |
| 5 | 17.6 | LOP | 18 |
| 6 | 23.0 | PPL | 8 |
| 7 | 28.7 | n.i | <1 |

The chromatogram obtained by the HPLC analysis of corn oil using an ultra violet detector is given in Figure 4.6.

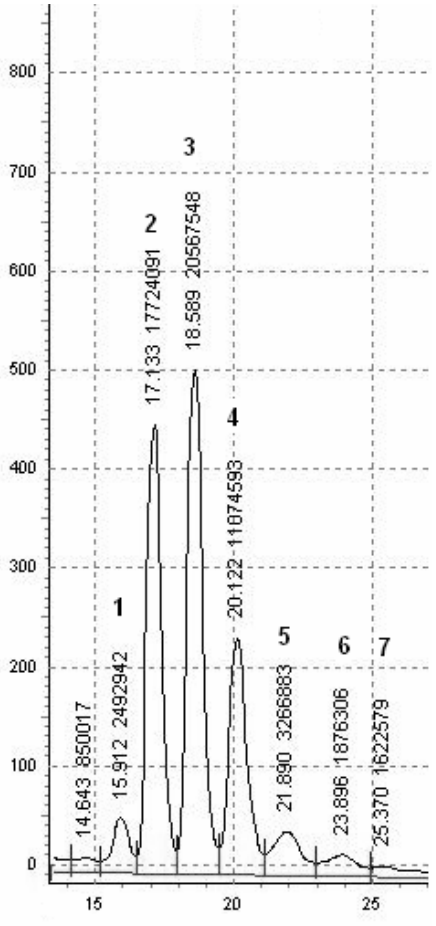


**Figure 4.6:** The chromatogram obtained by the HPLC analysis of corn oil using an ultra violet detector( using a 100% methanol to 100% isopropanol gradient and a flow rate of 1ml/min as mobile phase)

The retention times, the area percentages of the peaks and identified TAG species are given in Table 4.16.

**Table 4.16:** The retention times, the area percentages of the peaks and identified TAG species of corn oil (Detector: UV)

| Peak number | Retention time (min) | Predicted TAG specie | Area (%) |
|---|---|---|---|
| 1 | 16.0 | - | 5 |
| 2 | 17.1 | LLL | 22 |
| 3 | 18.6 | LLO | 39 |
| 4 | 20.1 | OOL | 21 |
| 5 | 21.9 | LLP | 6 |
| 6 | 23.9 | LOP | 4 |
| 7 | 25.4 | PPL | 3 |

The chromatogram obtained by the HPLC analysis of structured TAG species prepared at critical conditions using a refractive index detector is given in Figure 4.7.

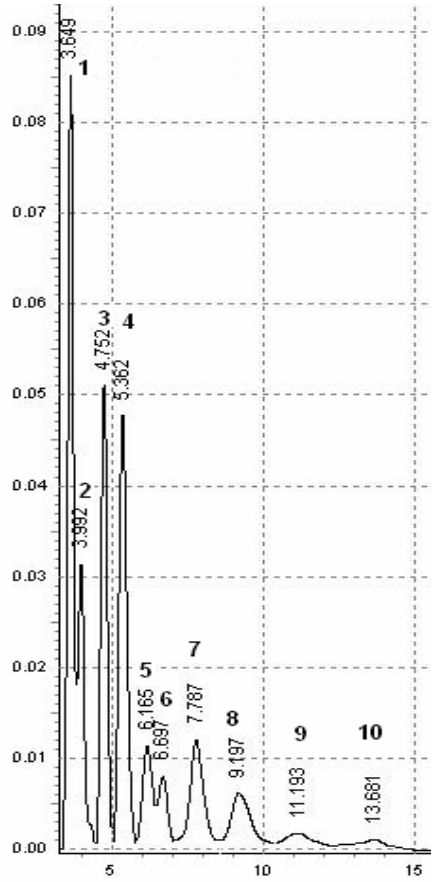


**Figure 4.7:** HPLC chromatogram of structured TAG species using a refractive index detector( using a 40% methanol 40% acetone and 20% acetonitrile gradient and a flow rate of 1.5ml/min as mobile phase)

The retention times, the area percentages of the peaks and identified TAG species of acidolysis product are given on Table 4.17.

**Table 4.17:** The retention times, the area percentages of the peaks and identified TAG species of acidolysis product (Detector: Refractive index)

| Peak number | Retention time (min) | Predicted TAG species | Area (%) |
|---|---|---|---|
| 1 | 3.6 | C8:0 L C8:0 | 10 |
| 2 | 4.0 | C8:0 O C8:0 | 10 |
| 3 | 4.8 | C8:0 L L | 10 |
| 4 | 5.4 | C8:0 L O and C 8:0 O L | 12 |
| 5 | 6.2 | C8:0 L P | 8 |
| 6 | 6.7 | LLL | 7 |
| 7 | 7.8 | LLO | 14 |
| 8 | 9.2 | OOL | 14 |
| 9 | 11.2 | LLP | 11 |
| 10 | 13.7 | LOP | 3 |

According to the composition shown in Table 4.17 the caprylic acid content of the product was calculated as 23 % (mol%). This result is in good agreement with the fatty acid composition of the products determined by GC at the critical points.

The chromatogram obtained by the HPLC analysis of structured TAG species prepared at critical conditions using an UV detector is given in Figure 4.8.

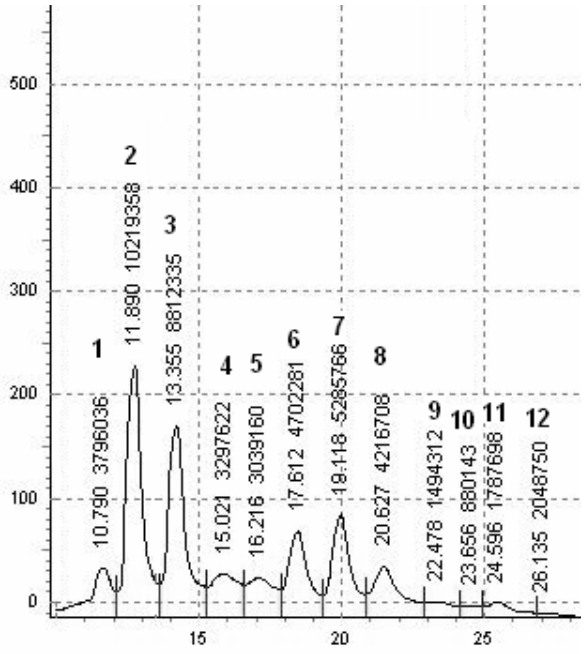


**Figure 4.8:** HPLC chromatogram of structured TAG species using an ultraviolet detector ( using a 100% methanol to 100% isopropanol gradient and a flow rate of 1.5 ml/min as mobile phase)

The retention times, the area percentages of the peaks and identified TAG species of acidolysis product are given in Table 4.18.

**Table 4.18:** The retention times, the area percentages of the peaks and identified TAG species of acidolysis product (Detector: UV)

| Peak number | Retention time (min) | Predicted TAG species | Area (%) |
|---|---|---|---|
| 1 | 10.8 | C8:0 L C8:0+ C8:0 O C8:0 | 8 |
| 2 | 11.9 | C8:0 L L | 21 |
| 3 | 13,4 | C8:0 L O and C 8:0 O L | 18 |
| 4 | 15.0 | C8:0 L P | 7 |
| 5 | 16.2 | n.i | 6 |
| 6 | 17.6 | LLL | 9 |
| 7 | 19.2 | LLO | 11 |
| 8 | 20.6 | OOL | 9 |
| 9 | 22.5 | LLP | 3 |
| 10 | 23.7 | PPL | 2 |
| 11 | 24.6 | n.i | 4 |
| 12 | 26.1 | n.i | 4 |

As can be seen in the chromatograms, TAG species containing saturated fatty acids could not be observed by UV detector. The composition of TAG species in corn oil determined by HPLC equipped with refractive index detector was in good agreement with the literature [24,25].

## 5. CONCLUSION

Although fat consumption is related with obesity and cardiovascular problems they have multiple advantageous properties and are essential nutrients as well. Therefore it is not recommended to completely exclude lipids from our diet. The permanent solution to decrease negative effects of fats on health is to use modified TAG having low calorie and containing essential fatty acids which are called structured lipids as fat substitutes instead of fats.

In this study it was aimed to produce a structured lipid having not only a decreased calorie value but also fat absorbable essential fatty acids. For this purpose, enzymatic acidolysis of corn oil with caprylic acid in n-hexane was conducted and optimization of caprylic acid incorporation into corn oil using response surface methodology was studied. At this study a sn-1,3 specific commercial lipase, Lipozyme TL IM was used. The fatty acids composition of the resulting TAG were determined by capillary gas chromatography and caprylic acid incorporation into corn oil (mole %) were calculated. The caprylic acid contents of the modified TAG were taken as responses.

Using three-variable and three-level face centered cube design a total of 17 design points with three center points was adopted for the optimization of corn oil acidolysis. The independent variables studied were enzyme amount, substrate mole ratio and reaction time. The experimental data were computed using Statistica 6.0 software to fit the second order polynomial model predicted for optimization of caprylic acid incorporation into corn oil.

According to this optimization it was determined that the critical values of the independent variables were as follows: enzyme amount,13.2 %; 1:3.9 corn oil/ caprylic acid mole ratio and 3.1 hours reaction time. At this critical point, 22% (mol%) caprylic acid incorporation into corn oil was observed. The predicted critical values were experimentally verified and a caprylic acid incorporation of 21.5±0.8 %

(mol %) was achieved. These experimental results were in good agreement with the predicted mathematical model. The resulting SL can be used as a high quality low calorie fat substitute, or for medical purposes such as for patients suffering pancreatic insufficiencies or fat malabsorption problems.

It is suggested to investigate the effect of different enzymes and reaction parameters on caprylic acid incorporation into corn oil for further studies. It is more over suggested to study this acidolysis reaction in a continuous bioreactor.

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