İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

PRODUCTION OF HUMAN MILK FAT SUBSTITUTES ENRICHED WITH DOCOSAHEXAENOIC ACID AND ARACHIDONIC ACIDS

M.Sc. Thesis by
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Department : Food Engineering

Food Engineering

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ABBREVIATIONS

2-MAG	**:** 2-monoacylglycerol
ADHD	**:** Attention Deficit Hyperactivity Disorder
AA	**:** Araşidonik Asit
ALA	**:** α-Linolenic Acid
ARA	**:** Arachidonic acid
ANOVA	**:** Analysis of Variance
CCD	**:** Central Composite Design
CCF	**:** Central Composite Face-Centered Design
ÇDYA	**:** Çoklu Doymamış Yağ Asidi
DAG	**:** Diacylglycerol
DHA	**:** Docosahexaenoic Acid
DSC	**:** Differential Scanning Calorimeter
EFA	**:** Essential Fatty Acids
EPA	**:** Eicosapentaenoic Acid
ESPGHAN	**:** European Society for Pediatric Gastroenterology, Hepatology and Nutrition
FAME	**:** Fatty Acid Methyl Ester
FAO	**:** Food and Agriculture Organization
FFA	**:** Free Fatty Acid
FSANZ	**:** Food Standards Australia New Zealand
GLA	**:** γ-Linolenic Acid
GLC	**:** Capillary Gas-Liquid Chromatograpy
HMF	**:** Human Milk Fat
HMFS	**:** Human Milk Fat Substitutes
ISSFAL	**:** International Society for the Study of Fatty Acids
IOM	**:** The Institute of Medicine
LA	**:** Linoleic Acid
LC-PUFA	**:** Long Chain Polyunsaturated Fatty Acids
MAG	**:** Monoacylglycerol
MCFA	**:** Medium Chain Fatty Acid
OSI	**:** Oil Stability Index
PUFA	**:** Polyunsaturated Fatty Acid
RSM	**:** Response Surface Methodology
SCFA	**:** Short Chain Fatty Acid
SCO	**:** Single-Cell Oil
SL	**:** Structured Lipid
TAG	**:** Triacylglycerol
TLC	**:** Thin-Layer Chromatography
TYY	**:** Tepki Yüzey Yöntemi
YY	**:** Yapılandırılmış Yağ
WHO	**:** World Health Organization

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PRODUCTION OF HUMAN MILK FAT SUBSTITUTES ENRICHED WITH DOCOSAHEXAENOIC ACID AND ARACHIDONIC ACIDS

SUMMARY

Human milk fat (HMF) has been accepted as a "gold standard" to feed infants as it includes all the necessary nutrients in significant amounts including long chain polyunsaturated fatty acids (LC-PUFAs) like docosahexaenoic acid (DHA) and arachidonic acid (ARA). Unlike other animal milk fat or animal and plant based fats and oils; it includes approximately 25% palmitic acid of which 70% is located at *sn*-2 position of triacylglycerols (TAG). This unique structure of HMF provides improved absorption of fatty acids and calcium. Because of these advantages of HMF, scientists have been trying to synthesize structured lipids (SL) resembling and providing the advantages of HMF.

In this study, our aim was to produce human milk fat substitutes (HMFS) enriched with DHA and ARA in two steps. In the first part of the study, hazelnut oil which had less than 1% of palmitic acid at the *sn*-2 position was enriched with palmitic acid and ethyl palmitate as the acyl donor in the presence of a nonspecific enzyme, Novozym® 435 (lipase B from *C. antarctica*). The reaction conditions were optimized with Response Surface Methodology (RSM). "Central composite face-centered design" (CCF) was applied to obtain the best fitting model. The independent variabels of the model were substrate molar ratio (Sr: 4-6 mol/mol) and reaction time (t: 6-18 h). The reaction temperature was fixed at 65ºC. Total palmitic acid amount in the TAG structure and at the *sn*-2 position (mol%) were the responses.

According to contour plots generated by Modde 5.0 software, optimal conditions for the reactions were determined as 17 h with substrate molar ratio of 6. Model verification was performed in gram-scale using these parameters and palmitic acid content of enriched hazelnut oil was measured as 63.48% as total palmitic acid and as 71.13% palmitic acid at the *sn*-2 position.

In the second part of the study, DHA and ARA were incorporated into enriched hazelnut oil in the presence of an *sn*-1,3 specific lipase, Lipozyme® RM IM obtained from *Rhizomucor miehei*. Reaction conditions for this part were selected as follows: 50 and 60ºC as reaction temperature, 2:1 and 3:2 as ARA/DHA ratio, 1:0.1 and 1:0.05 as substrate molar ratio. Reaction time was held constant as 3 h. Considering both ARA and DHA incorporations, gram-scale production for this part was performed at 60ºC with substrate molar ratio of 1:0.1 and ARA/DHA ratio of 3:2. SL, obtained as a result of this reaction included 57.26% total palmitic acid, 2.73% ARA and 2.38%DHA contents. Palmitic acid amount of SL at the *sn*-2 position was determined as 66%.

Characterization of the products was also performed in scope of this study. Tocopherol content, melting profile and oxidative stability of substrates, enriched hazelnut oil, and SL were investigated. Tocopherol content of SL was found to be lower than hazelnut oil but higher than enriched hazelnut oil. Enriched hazelnut oil

and SL showed wider melting and crystallization range when compared to hazelnut oil. Oxidative stability of structured lipid was found to have the lowest value whereas oxidative stability of hazelnut oil had the highest value. As a result of this study, HMFS, containing high amount of palmitic acid at the *sn*-2 position and also DHA and ARA at *sn*-1,3 positions was obtained. It is believed that this kind of product can be used in infant formulas providing similar advantages as HMF.

DOKOSAHEKZAENOİK ASİT VE ARAŞİDONİK ASİT İLE ZENGİNLEŞTİRİLMİŞ ANNE SÜTÜ YAĞI BENZERLERİNİN ÜRETİMİ

ÖZET

Anne sütü yağı , dokosahekzaenoik asit (DHA) ve araşidonik asit (AA) gibi çoklu doymamış yağ asitleri (ÇDYA) de dahil olmak üzere bebek için gerekli tüm bileşenleri içermesi bakımından "altın standart" olarak kabul edilmektedir. Diğer canlıların sütleri, hayvansal ve bitkisel kaynaklı yağların aksine, anne sütü yağı yaklaşık %25 oranında palmitik asit içermektedir. Bu oranın yaklaşık %70'i triaçilglierolün (TAG) *sn*-2 pozisyonunda yer almaktadır. Anne sütü yağının bu benzersiz özelliği yağ asitlerinin ve kalsiyum emiliminin yüksek olmasını sağlamaktadır. Anne sütü yağının kazandırdığı bu avantajlar nedeniyle, araştırmacılar anne sütünün avantajlarını sağlayan ve anne sütüne benzer özellikler gösteren yapılandırılmış yağ (YY) üretme çalışmalarına başlamışlardır.

Bu çalışmada, iki aşamada DHA ve AA ile zenginleştirilmiş anne sütü yağı benzerlerinin üretimi amaçlanmıştır. Çalışmanın ilk aşamasında, *sn*-2 pozisyonunda %1'den daha az palmitik asit içeren fındık yağı palmitik asit ve etil palmitat ile spesifik olmayan Novozym® 435 enzimi (*C. Antarctica*'dan elde edilen) kullanılarak zenginleştirilmiştir. Reaksiyon koşulları Tepki Yüzey Yöntemi (TYY) ile optimize edilmiştir, Merkezil Bileşik Deney Tasarımı ile en uygun model elde edilmeye çalışılmıştır. Modelin bağımsız değişkenleri substrat mol oranı (S: 4-6 mol/mol) ve zaman (t: 6-18 sa) olarak belirlenmiş, reaksiyon sıcaklığı 65ºC'de sabit tutulmuştur. Toplam palmitik asit ve *sn*-2 pozisyonundaki palmitik asit miktarları (%mol) tepki olarak ölçülmüştür.

Modde 5.0 yazılımı ile oluşturulan tepki yüzey izdüşüm grafiklerine göre reaksiyon için gerekli optimum koşullar 17 sa ve 6 substrat mol oranı olarak belirlenmiştir. Model doğrulaması bu değerler kullanılarak gerçekleştirilmiş, zenginleştirilmiş fındık yağındaki palmitik asit miktarı %63,48 olarak, *sn*-2 pozisyonundaki palmitik asit miktarı ise %71,13 olarak tespit edilmiştir.

Çalışmanın ikinci aşamasında, zenginleştirilmiş fındık yağına, *sn*-1,3 spesifik lipaz enzim olan ve *Rhizomucor miehei*'den elde edilen Lipozyme® RM IM yardımıyla DHA ve AA inkorpore edilmeye çalışılmıştır. Çalışmanın bu kısmında reaksiyon koşulları şu şekilde seçilmiştir: reaksiyon sıcaklığı olarak 50 ve 60ºC, AA/DHA oranı olarak 2:1 ve 3:2, substrat mol oranı olarak 1:0,1 ve 1:0,05. Reaksiyon için gerekli zaman 3 saatte sabit tutulmuştur. Düşük substrat mol oranında, DHA ve AA inkorporasyonu düşük gözlenmiştir ve 50ºC'de elde edilen sonuçlar 60ºC'de elde edilen sonuçlara göre daha düşük tespit edilmiştir. AA ve DHA inkorporasyonu sonuçlarına göre, bu aşamanın gram ölçekte üretimi 60ºC'de, 1:0,1 substrat mol oranında ve 3:2 AA/DHA oranında gerçekleştirilmiştir. Bu reaksiyon sonucunda elde edilen YY'nin %57,26 oranında palmitik asit, %2,71 oranında AA ve %2,38 oranında DHA içerdiği tespit edilmiştir. YY'nin *sn*-2 pozisyonundaki palmitik asit miktarı ise %66,05 olarak belirlenmiştir.

Bu çalışma kapsamında ürünlerin karakterizasyonu çalışması da gerçekleştirilmiştir. Substratların, zenginleştirilmiş fındık yağının ve YY'nin tokoferol içeriği, erime ve donma profili ve oksidatif stabilitesi incelenmiştir. YY'nin tokoferol içeriği zenginleştirilmiş fındık yağından daha fazla, fındık yağından ise daha az bulunmuştur. En yüksek oksidatif stabilite fındık yağında, en düşük oksidatif stabilite ise YY'de tespit edilmiştir. Bu çalışma sonunda *sn*-2 pozisyonu palmitik asit ile, *sn*-1,3 pozisyonları da DHA ve AA ile zenginleştirilmiş anne sütü yağına benzer YY üretilmiştir. Elde edilen bu ürünün, anne sütü yağının avantajlarını da sağlayabilmesi açısından bebek mamalarında kullanılabileceği düşünülmektedir.

1. INTRODUCTION

A wide variety of natural products such as fatty acids, derivatives of fatty acids, steroids, terpenes, carotenoids, bile acids are grouped under lipids which are soluble in organic solvents but insoluble in water (O'Keefe, 2008). Lipids provide concentrated energy for humans and take place in important metabolic activities. Furthermore they are sources of essential fatty acids (EFA) which cannot be synthesized by humans, in addition fat-soluble vitamins and carotenoids (Bhaskar and Miyashita, 2007; Sikorska-Wiśniewska and Szumera, 2007).

"Structured lipids" (SL) can be defined as chemically or enzymatically modified form of naturally occurring lipids. SLs can be produced in the form of monoacylglycerols (MAG), diacylglycerols (DAG), triacylglycerols (TAG) and phospholipids. With the production of SLs, products that have altered health benefits or nutritional value can be obtained (Akoh and Kim, 2008). As well as individuals, for newborn infants, the main energy source is lipids (Jumpsen and Clandinin, 1995; Sikorska-Wiśniewska and Szumera, 2007). Although breast milk is the most valuable source for infant nutrition as it includes all the necessary nutrients for normal growth and development, due to some metabolic, economic (because of the increase in the number of working women) or health related reasons it is not always possible to feed the infant with breast milk (Jumpsen and Clandinin, 1995). It has the proper temperature, anti-bacterial effect, and it is fresh and does not need any additional step for preparation (Sardesai, 2003). Unfortunately, breast-feeding has been decreasing day-by-day in developing and industrial countries. Less than 50% of infants are being fed with human milk of which 60% are fed less than 6 months (Das, 2006). This situation results in feeding infants with infant formulas (Jumpsen and Clandinin, 1995). In both cases, the half of the total required energy is provided by fat (Jumpsen and Clandinin, 1995; Sikorska-Wiśniewska and Szumera, 2007).

EFAs are omega-3 (n-3) and omega-6 (n-6) series fatty acids that include two or more cis double bonds. They should be obtained through diet because animal tissues cannot synthesize them (Klurfeld, 2008).

Energy requirements of infants depend on several factors such as basal metabolic rate, cost of growth, and losses with stool or urine (Bhatia *et al*., 2002). Total fat intake amount should be 30-35% of the total energy intake. For preterm infants, daily energy requirement is 460-502 kJ/kg (100-120 kcal/kg); whereas it should be increased to 480-520kJ/kg (115-125 kcal/kg) if insufficient growth is observed (Sikorska-Wiśniewska and Szumera, 2007). Similarly, premature infants need daily 460-545 kj/kg (110-130 kcal/kg) of energy intake (Bhatia *et al*., 2002).

The aim of this study was to produce SLs having similar palmitic acid amount at *sn*-2 position as found in the human milk fat (HMF), which provides specific advantages to babies. In order to produce that kind of product; two steps were followed. In the first part, hazelnut oil, palmitic acid and ethyl palmitate were used as raw materials to incorporate palmitic acid and ethyl palmitate into *sn*-2 position of hazelnut oil. The reaction conditions were optimized by using Response Surface Methodology (RSM) and gram-scale production was performed at optimal conditions. In the second part, it was aimed to incorporate docosahexaenoic acid (DHA) and arachidonic acid (ARA) to the enriched hazelnut oil to provide the health benefits associated with these long-chain polyunsaturated fatty acids (LC-PUFAs).

2. LITERATURE REVIEW

2.1 Hazelnut Oil

Hazelnut (*Corylus avellana* L.), which belongs to *Betulaceae* family, is distributed on the coasts of Black Sea region in Turkey. Hazelnut is also cultivated in Italy, Spain, Portugal, France, USA, New Zealand, China, Azerbaijan and some other countries (Alasalvar *et* al., 2009). Total hazelnut production in the world is approximately 770,000 MT. Nearly 70% of hazelnuts are cultivated in Turkey which is the largest producer in the world (Sathe et al., 2009). Turkey is followed by Italy (Shahidi and Miraliakbari, 2006). According to the statistical data adapted from Url-1 and Url-2, hazelnut production of Turkey was approximately 500000 MT in 2009 of which economical value was more than 2 million US dollars the previous year (2011). Generally, hazelnuts include approximately 60% of lipids (Shahidi and Miraliakbari, 2006). Therefore, hazelnut is considered as an excellent source of energy. In addition, hazelnuts are rich sources of vitamin E, biotin, thiamin, pyridoxine, panthotenic acid, and folate. Oleic acid is the predominant fatty acid in hazelnuts. Hazelnuts include small amount of saturated fatty acids (SFA) whereas they are generally made up monounsaturated fatty acids (MUFA) (Alasalvar *et* al., 2009). As for hazelnuts, the predominant fatty acid of hazelnut oil is oleic acid followed by linoleic, palmitic and stearic acids. In addition, hazelnut oil includes α-tocopherol and phytosterols in the form of β-sitosterol (Shahidi and Miraliakbari, 2006). As hazelnut oil includes high amount of MUFAs and SFAs, it promotes health benefits such as decrease in the risk of heart disease, stroke, and certain types of cancer (Alasalvar *et* al., 2009).

2.2 Human Milk

2.2.1 Properties and composition of human milk

Human milk contains proper amounts of fatty acids, protein, carbohydrate, lactose, water, and amino acids for growth and development (Das, 2006).

It is rich in PUFAs, especially DHA and ARA (Das, 2006). The fatty acid profile of human milk is generally accepted as the "gold standard" when designing the composition of infant formulas (Hamosh, 2008).

Although the amount of fat and fatty acid composition of breast milk varies between countries because of geographical locations or eating habits, it can be assumed that breast milk is rich in LC-PUFAs. It includes both n-6 and n-3 fatty acids. Fatty acid composition of breast milk obtained by different researchers is given in Table 2.1 (Jumpsen and Clandinin, 1995).

DHA content varies between 0.1-1.0 percent whereas ARA content varies between 0.5-1.0% in breast milk. DHA and ARA contents of breast milk are not very high but they are in significant amount for infant nutrition and growth (Huang *et al.*, 2003). ARA concentration of human milk does not vary with maternal nutrition while DHA concentration has high variability according to the maternal diet. In the populations, consuming high amount of meats has low levels of DHA in human milk; on the other hand, populations consuming high amounts of fish have high levels of DHA. Table 2.2 shows ARA and DHA contents of human milk according to the populations having different eating habits (Hamosh, 2008).

Table 2.1: Fatty acid composition data of mature human milks analyzed by different researchers.

Fatty acid	Milk 1[a]	Milk 2[b]	Milk 3[c]
Capric acid (10:0)	-	-	0.97 ± 0.28
Lauric acid (12:0)	4.4	-	4.46 ± 1.17
Myristic acid (14:0)	5.2	-	5.68 ± 1.36
Palmitic acid (16:0)	22.5	-	22.20 ± 2.28
Stearic acid (18:0)	8.7	-	7.68 ± 1.85
Oleic acid (18:1n-9)	35.5	-	35.51 ± 2.73
Linoleic acid (18:2n-6)	14.5	10.76	15.58 ± 1.99
Linolenic acid (18:3n-3)	1.5	0.81	1.03 ± 0.21
γ-Linolenic acid (18:3n-6)	-	0.16	-
Eicosatrienoic acid (20:3n-6)	-	0.26	0.53 ± 0.15
Arachidonic acid (20:4n-6)	0.5	0.36	0.60 ± 0.29
Eicosapentaenoic acid (20:5n-3)	-	0.04	-
Clupanodonic acid (22:5n-3)	-	0.17	0.11 ± 0.15
Docosahexaenoic acid (22:6n-3)	0.15	0.22	0.23 ± 0.14

[a] Jumpsen and Clandinin, 1995.
[b] % wt/wt; Das, 2006.
[c] %; Hamosh, 2008.

Table 2.2: ARA and DHA contents of mature human milk according to different populations (Hamosh, 2008).

Country	ARA content, %	DHA content, %
United States	0.71	0.21
Germany	0.36	0.22
Hungary	0.50	0.10
Sweden	0.40	0.30
England	0.19	0.29
Spain	0.50	0.34
South Africa	0.60	0.20
Tanzania	0.60	0.27
Gambia	0.31	0.39
Nigeria	0.82	0.93
St. Lucia	0.58	0.56
China	0.64	0.71
Malay	0.47	0.90
India	0.57	0.90
Canadian Unit	0.60	1.40

Replacing human milk with milks from several animals had been tried but it did not provide features of human milk because animal milk varieties include protein and electrolytes in large amounts, which is not good and healthy for the infants (Sardesai, 2003). Human milk is unique in nature because of its TAG structure (Jandacek, 2008). HMF contains 25% of palmitic acid, of which nearly 70% is located at *sn*-2 position of the TAG and *sn*-1 and *sn*-3 positions are being occupied by LC-PUFAs. This unique structure provides advantages to infant such as increase in digestion and absorption of the fatty acids thus improving the calcium absorption (Hamosh, 2008; Takeuchi, 2010). Free palmitic acid can be precipitated as calcium soap in the intestine but 2-monoacylglycerol (2-MAG) which has palmitic acid at the *sn*-2 position, does not precipitate. Consequently, both calcium and 2-MAG become bioavailable for the infants (Jandacek, 2008).

2.2.2 Importance of human milk for term and preterm infants

Breast milk has been proposed to have protective effects for several diseases such as sudden infant deaths, type I diabetes, Crohn's disease, ulcerative colitis, lymphoma, allergic diseases, and other chronic digestive diseases and it is related to cognitive development (Das, 2006).

It also has a future protective effect on development of obesity, insulin resistance, high blood pressure, and occurrence of type I diabetes and coronary heart diseases. It is found that breast-fed infants had better IQ scores than formula fed infants (Das, 2006).

2.3 Long-Chain Polyunsaturated Fatty Acids (LC-PUFAs)

Polyunsaturated fatty acids (PUFAs) include 18 or more carbon atoms and have at least two double bonds (Gebauer et al., 2005). They are composed of three main classes as Δ-3, Δ-6, and Δ-9 fatty acids (Gebauer et al., 2005; Fernandes, 2006).

Nomenclature is based on the number of the carbon that first double bond is positioned when counted from the methyl end of the fatty acid (Jumpsen and Clandinin, 1995; Fernandes, 2006). According to shorthand nomenclature, they can be mentioned as omega-3 (ω-3), omega-6 (ω-6), and omega-9 (ω-9) or n-3, n-6 and n-9 fatty acids. This nomenclature cannot be used for trans fatty acids and the fatty acids with double bonds. In order to prevent misunderstanding, shorthand names of fatty acids should be used with ω (O'Keefe, 2008).

n-3 and n-6 fatty acids are essential fatty acids since animals cannot synthesize them either by elongation or desaturation because lacking the required enzyme but n-9 fatty acids are considered to be non-essential as they can be synthesized by animal tissues (Jumpsen and Clandinin, 1995; Hamosh, 2008).

Also during desaturation of n-3 and n-6 fatty acids, competition for the required enzymes occurs since they use the same enzyme for the reactions. Because of this fact, the tissue levels of each other are affected in a counterproductive way (Gebauer *et al*., 2005). Due to this fact, these fatty acids should be obtained from diet (Jumpsen and Clandinin, 1995; Weber and Mukherjee, 2005). N-6 and n-3 series PUFAs are of great interest as they are precursors of eicosanoids. They can also be used in specialty products such as nutracueticals (Weber and Mukherjee, 2008). Figure 2.1 shows the metabolic pathways for n-3 and n-6 fatty acids (Hamosh, 2008).

Accretion of LC-PUFAs in tissues of infants begins with the last trimester of pregnancy. As a result, preterm infants suffer from LC-PUFA deficiency. Human milk does not show consistency in terms of LC-PUFA content during pregnancy and after birth (Hamosh, 2008).

As DHA and ARA have beneficial effects on biological functions, they have been added to infant formulas physically (Hamosh, 2008).

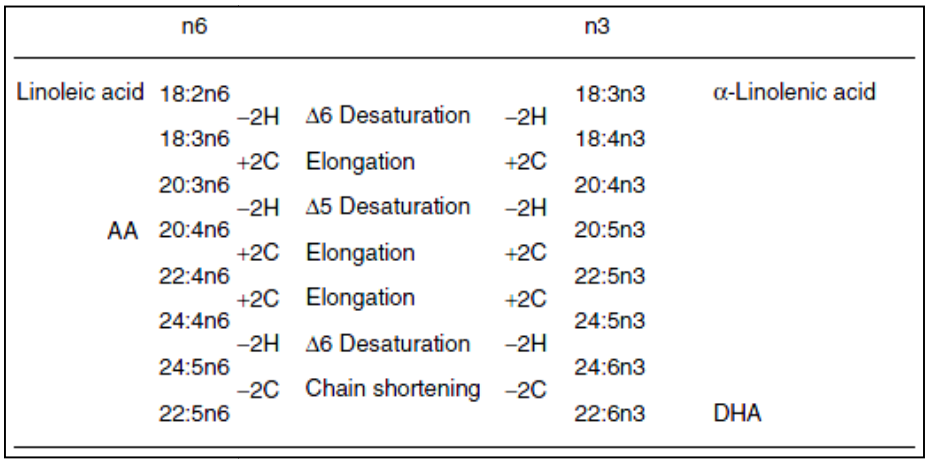

Figure 2.1: Metabolic pathways for n-3 and n-6 fatty acids (Hamosh, 2008).

Premature infants have higher requirement for LC-PUFAs because they have limited EFA stores and limited activity of elongase and desaturase enzymes that are responsible for producing PUFAs. Since fast growing organs like brain need LC-PUFAs, premature infants need supplemented formulas to gain adequate amounts of these EFAs (Sikorska-Wiśniewska and Szumera, 2007). Physical addition of DHA and ARA to infant formulas said to be beneficial for visual acuity, normal growth and development (Das, 2006).

N-3 and n-6 PUFAs are important for humans during development as they play important roles in the body such as being a component of the central nervous system and developing membranes, taking part in platelet aggregation, transporting membrane-bound enzymes and functioning of immune system (Jumpsen and Clandinin, 1995).

Marine oils, egg phospholipids, and single-cell oils (SCOs) can be used as sources of PUFAs but it should be kept in mind that marine oils contain both eicosapentaenoic acid (EPA) and DHA (Huang, *et al.*, 2003). It results in imbalanced n-6/n-3 fatty acid ratio as EPA and DHA have a reduction effect on ARA level. In addition, α-linolenic acid (ALA) reduces the level of ARA (Jumpsen and Clandinin, 1995).

The first two years of life is critical in terms of brain growth and development. N-6 and n-3 PUFAs have positive effects on memory, visual acuity, visual recognition and mental development (Weber and Mukherjee, 2005).

Especially, n-3 fatty acids are important for cognition. In later life, they have inhibitory effect on attention deficit hyperactivity disorder (ADHD), dyslexia, dyspraxia, and autistic behaviors. They are constituents of central nervous system and retina (Weber and Mukherjee, 2005; Sikorska-Wiśniewska and Szumera, 2007). Because of these reasons, addition of DHA and ARA to formulas is recommended (Huang, *et al.*, 2003). As can be seen in Figure 2.1, ARA and DHA can be synthesized through their dietary precursors, which are linoleic acid (LA) and ALA respectively or obtained directly from dietary sources (Wainwright and Huang, 2003).

2.3.1 N-3 series fatty acids and DHA

N-3 fatty acids are essential for specific organs and several systems (Fernandes, 2006; Bhaskar and Miyashita, 2007). They can lower blood lipids and they play specific roles in biochemical and physiological responses (Bhaskar and Miyashita, 2007). n-3 series fatty acids take place in normal development of nervous systems of infants, prevent coronary heart disease, inhibit some cancer types, prevent inflammatory diseases, prevent skin disorders, diabetes and depression (Fernandes, 2006). The deficiency of these fatty acids leads to brain dysfunction, learning and sleep disorders, and abnormal visual function (Sikorska-Wiśniewska and Szumera, 2007).

DHA is found highly in tissues of the central nervous system but rarely present in other tissues (Wainwright and Huang, 2003). It is a structural component of brain and retina and is very important in terms of biological functions (Hamosh, 2008; Fernandes, 2006; Akoh and Kim, 2008; Bhaskar and Miyashita, 2007; Sikorska-Wiśniewska and Szumera, 2007). DHA is more important for infants because EFAs are required in the period of fatal development and completion of producing important cells in the brain and retina after birth (Bhaskar and Miyashita, 2007). As infants' ability or capacity to convert ALA to DHA may be insufficient during early development, addition of DHA to diet is a requirement for them (Wainwright and Huang, 2003; Hamosh, 2008). It is mentioned that when compared to human milk-fed infants, formula-fed infants have lower brain DHA concentrations resulting in lower intelligence levels (Bhaskar and Miyashita, 2007). In addition to that, breast milk fed and DHA-enriched formula fed infants have better visual acuity with respect to regular commercial formula fed infants (Saito and Nishizawa, 2006).

The highest level of DHA is present in retina and the cerebral cortex of the brain so the lack or excess of this fatty acid has an impact on retinal functions and visual acuity. A balanced diet in terms of n-6/n-3 fatty acid ratio is essential for infants. It was shown is various studies that diets containing n-3 fatty acids provide improved visual acuity for infants. Especially incorporation of n-3 fatty acids can be more important for proper development and visual acuity of preterm and low-birth weight infants (Jumpsen and Clandinin, 1995). Major source and biological functions of DHA is summarized in Table 2.3 (Wynn and Ratledge, 2006).

DHA also has a significant effect on blood pressure and heart rate. It has been supported with studies that increased blood pressure occurs because of n-3 PUFA deficiency in perinatal period so an adequate intake of n-3 fatty acids is required during the early stage of life (Bhaskar and Miyashita, 2007). If DHA is lacking, brain membrane fatty acid composition changes that results in decrease in brain DHA content (Wainwright and Huang, 2003).

Table 2.3: Major sources and biological functions of DHA (Wynn and Ratledge, 2006).

Sources (Plants and Animals)	Fish: *Brevoortia, Engraulis, Sardina, Scomber spp.*
Sources (Microorganisms)	Fungi: *Thraustochytrium, Entomophthora spp.* Algae: *Gonyaulax, Gyrodinium, Crypthecondium* Bacteria: *Colwellia, Moritella (Vibrio) marinus*
Major Dietary Sources	Cold water fish, shellfish, algae
Nutritional Values and Applications	Beneficial effects on blood lipid profiles, to reduce the risk of coronary heart disease, arthritis, inflammation, hypertension, psoriasis, other autoimmune disorders and cancer, nutraceutical additives for processed food, DHA incorporated into infant formulae for improvement of vision and memory

2.3.2 N-6 series fatty acids and ARA

LC-PUFA's are the precursors of eicosanoids, which are derived from EFAs via enzymatic reactions (Sikorska-Wiśniewska and Szumera, 2007). They are cyclized version of EFAs having very short half-lives and always containing 20 carbon atoms (Klurfeld, 2008). ARA is the main precursor of eicosanoids and it is found at high levels in many tissues (Huang, *et al.*, 2003; Wainwright and Huang, 2003).

Eicosanoids are composed of prostaglandins, tromboxanes, prostacyclins, leukotrienes, and lipoxins (Sardesai, 2003). Prostaglandin family includes two major series which are E and F. Leukotrienes are not cyclized compounds. They are critical mediators of inflammatory responses such as anaphylaxis, increased vascular permeability, attraction of leukocytes and their activation (Klurfeld, 2008). Tromboxanes and prostaglandins can take part in vasoconstriction and platelet aggregation while leukotrienes play a role in constriction of bronchial airway musculature, in vascular permeability, and in interactions between the endothelium and white cell population (Jumpsen and Clandinin, 1995). 1-, 2-, and 3- series prostaglandins and 3-, 4-, and 5- series leukotrienes are produced via C20:3n-6, C20:4n-6, and C20:5n-3, respectively. Figure 2.2 and 2.3 show the leukotriene and prostaglandin metabolites of ARA (O'Keefe, 2008).

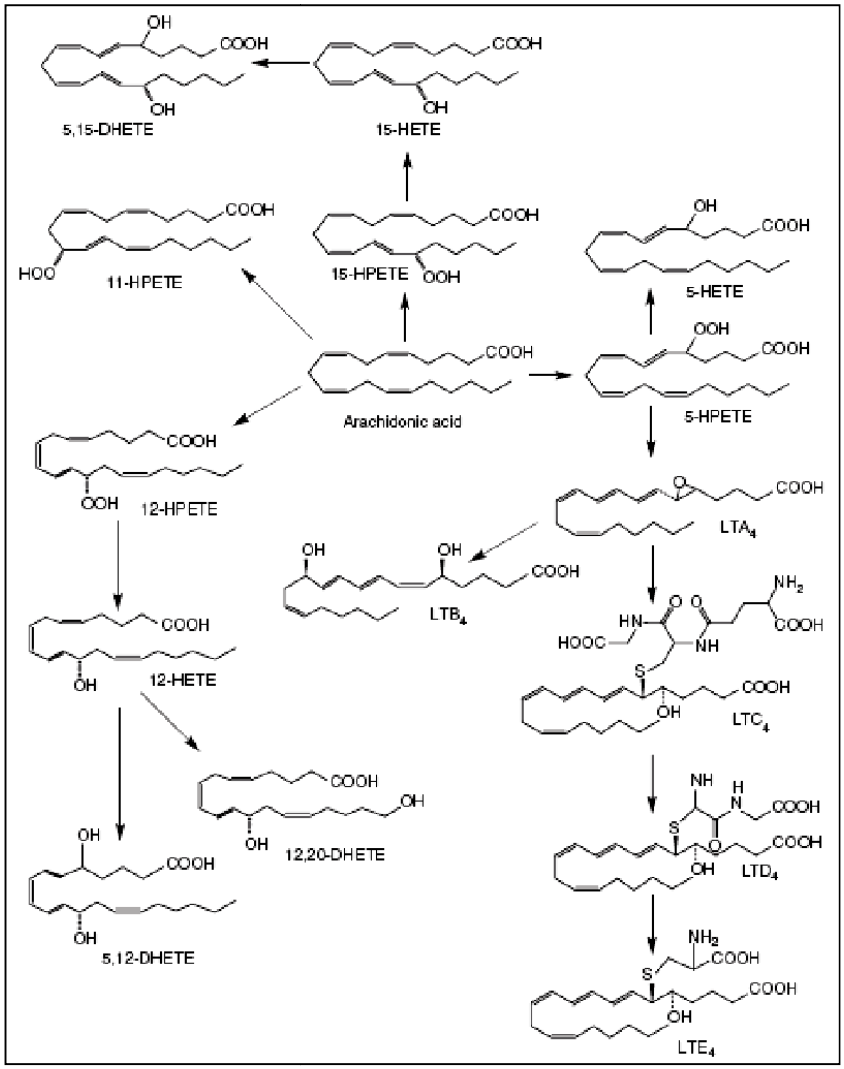

Figure 2.2: Leukotriene metabolites of ARA (O'Keefe, 2008).

Thromboxanes are synthesized by platelets, cause platelet aggregation, and vascular constriction. Prostacyclins are produced by blood vessels and inhibit platelet aggregation. Thromboxanes and prostacyclins do not take part in immune responses. As fatty acid molecule becomes more unsaturated, it provides more fluidity to the cell membrane. N-3 and n-6 fatty acids can modulate immunological reactions (Klurfeld, 2008).

Eicosanoids regulate many inflammatory and hypersensitive reactions. The balance between dietary n-6 and n-3 fatty acids alters the profile of eicosanoids formed and is therefore important to the control of vasoconstrictive, thrombogenic and immunogenic activities (Jumpsen and Clandinin, 1995). Eicosanoids, dietary fat and the immune system have a close relationship as dietary fats can modulate immune responses (Klurfeld, 2008).

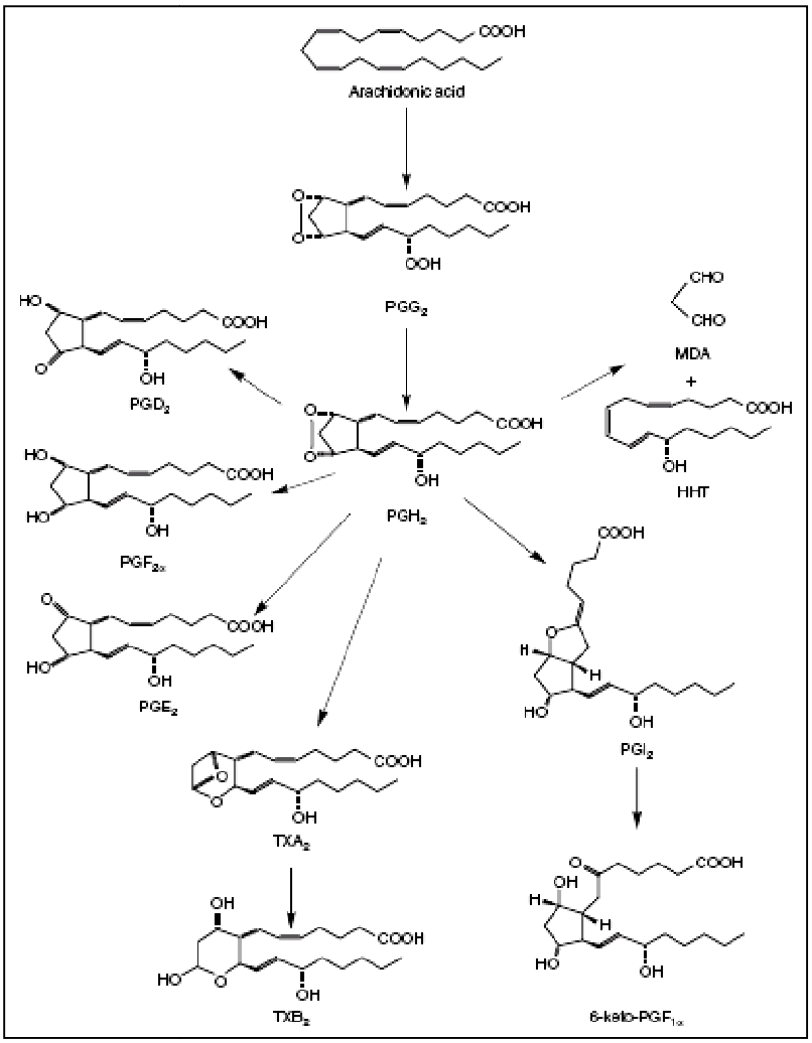

Figure 2.3: Prostaglandin metabolites of ARA (O'Keefe, 2008).

N-6 series fatty acid deficiency results in growth retardation, thickening of the skin, decreased skin pigmentation, muscular contraction disorders, and susceptibility to infections (Sikorska-Wiśniewska and Szumera, 2007).

ARA is important for fetal development and growth after birth (Huang, *et al.*, 2003; Das, 2006). ARA status is an important factor in the growth of fetus and premature infant. Plasma triglyceride content of ARA is correlated with the birth weight of the newborn infant (Jumpsen and Clandinin, 1995). Major sources and biological functions of ARA are shown in Table 2.4 as a summary (Wynn and Ratledge, 2006).

Table 2.4: Major sources and biological functions of ARA (Wynn and Ratledge, 2006).

Sources (Plants and Animals)	Fish: *Brevoortia, Clupea, Sardina spp.,* animal tissues
Sources (Microorganisms)	Fungi: *Pythium, Mortierella spp.* Algae: *Porphyridium* spp. Mosses: *Rhytidiadelphus*, *Brachythecium, Erthynchium spp.*
Major Dietary Sources	Liver, brain, egg yolk lecithin
Biological Functions	Major component of most membrane phospholipids, precursor of prostaglandin PGE2
Nutritional Values and Applications	Ingredient in various infant formulae along with DHA

2.3.3 n-6/n-3 fatty acid ratio and importance for human health

In mature human milk ARA:DHA ratio is around 1.5 (Das, 2006). Not only the deficiency of both n-3 and n-6 fatty acids are important for infants and can cause physiological disorders, but also excess of these fatty acids also affects the metabolic reactions, so the ratio of those fatty acid families should be optimum (Jumpsen and Clandinin, 1995). In addition, competition of these fatty acids for the same enzyme makes n-6/n-3 ratio more important in the diet (Wainwright and Huang, 2003). In many countries, recommendation for n-6/n-3 fatty acid ratio has been made or has been in discuss (Christophe, 2003).

Table 2.5 shows the recommendations for n-6/n-3 fatty acid ratio. European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) suggests adding DHA and ARA to infant formulas for low-birth-weight babies by different organizations because of the reason that they are not able to synthesize them (Sikorska-Wiśniewska and Szumera, 2007).

Table 2.5: Recommendation for n-6/n-3 fatty acid ratio by some organizations (Wynn and Ratledge, 2006).

Name of organization	Recommendation for n-6/n-3 ratio
The Institute of Medicine (IOM)	4.2:1 – 16,7:1[a]
International Society for the Study of Fatty Acids (ISSFAL)	~2:1[a]
Japanese Society for Lipid Nutrition	2:1[a]
Food Standards Australia New Zealand (FSANZ)	2:1[b]
European Society for Paedicatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)	5:1-15:1[c]
Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Fats and Oils in Human Nutrition	5:1-10:1[a]

[a] Gebauer *et al.*, 2005.
[b] FSANZ, 2007.
[c] ESPGHAN, 2005.

2.3.4 DHA and ARA deficiency

It has been shown in a studies that prolonged EFA deficiency or total fat deficiency causes reduction of DHA and increase of docosapentaenoic acid (C22:5n-6) in the brain of rats and mice. In another study, it has been observed that infants fed with skim milk can suffer from EFA deficiency (Jumpsen and Clandinin, 1995).

N-3 fatty acid deficiency causes reduction in the levels of ALA (C18:3n-3) and DHA while increase in the levels of adrenic acid (C22:4n-6) and docosapentaenoic acid (C22:5n-6). It causes difficulties in learning and visual functions and abnormal electroretinogram may occur (Jumpsen and Clandinin, 1995).

N-6 fatty acid deficiency can cause reduction in growth, failure in reproduction, changes in skin, hair and liver pathology. In addition, LA and ARA levels can decrease (Jumpsen and Clandinin, 1995).

As it is mentioned earlier fatty acids compete for the same enzyme, so increase of the amount of this fatty acid in diet is becoming a requirement (Jumpsen and Clandinin, 1995).

LC-PUFAs are very important for infants as they are essential for a lot of biological functions as well as proper development. In addition, neonates are more at risk than infants in terms of EFA deficiency are as they have low fat reserves (Jumpsen and Clandinin, 1995).

2.3.5 Excess amount of DHA and ARA

Excess amount of n-3 fatty acids can cause some adverse effects such as increased requirement of antioxidants and vitamin E, reduction of platelet aggregation, and inhibition of ARA metabolism for prostaglandin formation (Jumpsen and Clandinin, 1995).

Excessive amount of n-6 series fatty acids can cause insulin resistance and hyperinsulinemia whereas excessive amount of n-3 fatty acids especially DHA is related to prolonged bleeding time (Gebauer *et al*., 2005).

2.4 Single-Cell Oils (SCOs)

SCOs are produced via single-cell organisms in order to be used as dietary supplements (Huang, *et al.*, 2003). Usage of SCOs as a lipid source has been popular because of the opportunity for large-scale production and capability of producing unusual fatty acids or unusual amount of specific fatty acids (Watkins and German, 2008).

DHASCO® and ARASCO® are two examples of single-cell oils produced by *Crypthecodinium cohnii* and *Mortierella alpina* respectively (Huang, *et al.*, 2003; FSANZ, 2003).

For infant formulas, physical addition of these two products are suitable, even they can be mixed with other fatty acids. As they do not interfere with other fatty acids, intended ARA/DHA ratio can be obtained similarly to the amount found in breast milk (Huang, *et al.*, 2003).

2.4.1 DHA rich single cell oil (DHASCO®)

Marine oils are primary sources of DHA but it was not possible to add marine oils to infant formulas because of their disadvantages. Firstly, they do not include specifically DHA, besides it includes EPA. EPA has specific benefits for individuals but for infant nutrition it is not suitable as it causes growth retardation (Weber and Mukherjee, 2008).

Secondly, fish oils gives specific fish odor to the product. As a result, investigation of SCOs for producing specifically DHA has occurred (Weber and Mukherjee, 2008).

Two different microalgae species, *Crypthecodinium cohnii* and *Schizochytrium*, are selected to produce DHA (Wynn and Ratledge, 2006). *Crypthecodinium cohnii* is heterotrophic, nonphotosynthetic marine algae. It contains about 40% of DHA (Weber and Mukherjee, 2008). DHASCO® also is in triglyceride oil form which is standardized with high oleic sunflower oil. Table 2.6 shows fatty acid composition of DHASCO® (FSANZ, 2003).

It is mentioned in FSANZ report that in 2001 DHASCO® extracted oil is added to the infant formula up to a maximum level of 1.25% which corresponds to 0.5% of DHA (2003).

Table 2.6: Fatty acid composition of DHASCO® (FSANZ, 2003).

Fatty acid	DHASCO®, mol %
Myristic acid (14:0)	10-20
Palmitic acid (16:0)	10-20
Palmitoleic acid (16:1n-7)	0-2
Stearic acid (18:0)	0-2
Oleic acid (18:1n-9)	10-30
Linoleic acid (18:2n-6)	0-5
Arachidic acid (20:0)	0-2
Behenic acid (22:0)	0-2
Docosapentaenoic acid (22:5n-3)	0-1
Docosahexaenoic acid (22:6n-3)	40-45
Nervonic acid (24:0)	0-2
Others	0-3

2.4.2 ARA rich single cell oil (ARASCO®)

ARA-rich Single Cell Oil was first investigated in the mid 1960s. There were many microorganisms examined for ARA production but only two fungi were capable of producing ARA. (Wynn and Ratledge, 2006).

Mortierella alpina and *Pythium* sp. are two fungi that are used for commercial production. *Mortierella alpina* is more productive than *Pythium* sp. (Wynn and Ratledge, 2006).

Physically it is a free flowing triglyceride oil containing undetectable level of cyclic or trans fatty acids. Fatty acid composition can be seen in Table 2.7 (FSANZ, 2003).

It includes 40% of ARA after standardization with high oleic sunflower oil. ARASCO® has been added to infant formula up to 1.25% in 2001 in New Zealand and Australia equal to the maximum level of 0.5% of ARA (FSANZ, 2003).

Table 2.7: Fatty acid composition of ARASCO® (FSANZ, 2003).

Fatty acid	ARASCO®, mol %
Myristic acid (14:0)	0-2
Palmitic acid (16:0)	3-15
Palmitoleic acid (16:1n-7)	0-2
Stearic acid (18:0)	5-20
Oleic acid (18:1n-9)	5-38
Linoleic acid (18:2n-6)	4-15
Linolenic acid (18:3n-3)	1-5
Arachidic acid (20:0)	0-1
Eicosatrienoic acid (20:3n-6)	1-5
Arachidonic acid (20:4n-6)	38-44
Behenic acid (22:0)	0-3
Docosapentaenoic acid (22:5n-3)	0-3
Lignoseric acid (24:0)	0-3

2.5 Structured Lipids

In order to synthesize SLs short chain fatty acids (SCFAs), medium chain fatty acids (MCFAs), PUFAs, saturated LCFAs, and monounsaturated fatty acids can be used. The fatty acids and their positions on TAG determine the physical and functional properties of the SL (Akoh and Kim, 2008).

2.5.1 Production methods of structured lipids

There are several reaction types for obtaining SLs. They can be produced via partial hydrogenation, transesterification, fractionation, genetic engineering, and so on (Christophe, 2003).

2.5.1.1 Transesterification

Production of new triacylglycerols through transesterification reactions is carried out by changing the positions of fatty acid groups of TAGs between or within the molecule (Willis and Marangoni, 2008).

It may occur by exchanging of ester groups between oil-oil, oil-fatty acid (acidolysis), or oil-alcohol (alcoholysis) compounds. It can be performed either chemically or enzymatically. Chemical method requires sodium methoxide or sodium hydroxide as a chemical agent while in enzymatic methods lipases are used In enzymatic reactions two types of lipases are used: *sn*-1,3 specific lipases and lipases which have no position specificity (Takeuchi, 2010).

With the use of *sn*-1,3 specific lipases, production of SLs that have specific fatty acids at specific positions is possible (Takeuchi, 2010). Figure 2.4 shows a representative transesterification reaction between two TAG molecules (Willis and Marangoni, 2008).

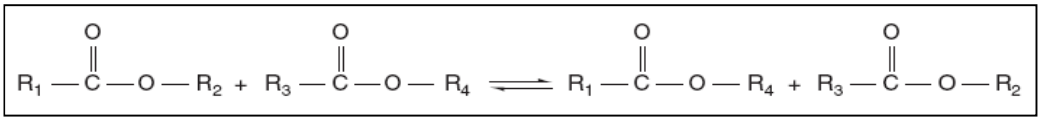

Figure 2.4: Lipase catalyzed transesterification reaction between two different TAGs (Willis and Marangoni, 2008).

2.5.1.2 Acidolysis

Acidoysis reaction is the exchange of acyl groups between an acid and an ester. Incorporation of novel free fatty acids or ethyl ester forms of them into TAGs is carried out by acidolysis reactions. Figure 2.5 shows a representative acidolysis reaction between an acylglycerol and an acid (Willis and Marangoni, 2008).

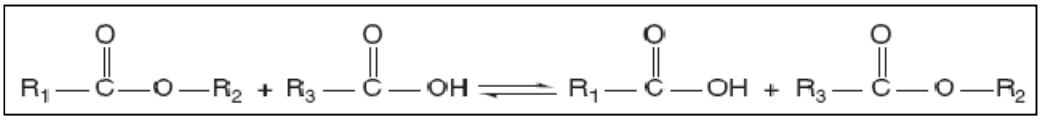

Figure 2.5: Lipase catalyzed acidolysis reaction between an acylglycerol and an acid (Willis and Marangoni, 2008).

2.5.1.3 Alcoholysis

Alcoholysis is the esterification reaction between an alcohol and an ester. Figure 2.6 shows the reaction. Alcoholysis is generally used because of its high yield in glycerolysis reactions. Glycerolysis is the exchange of acyl groups between glycerol and a TAG to produce MAGs, DAGs and TAGs (Willis and Marangoni, 2008).

Glycerolysis is generally carried out in the presence of non-specific lipases giving a wide range of reaction products. In Figure 2.6 a representative glycerolysis reaction between a glycerol and a TAG is shown (Willis and Marangoni, 2008).

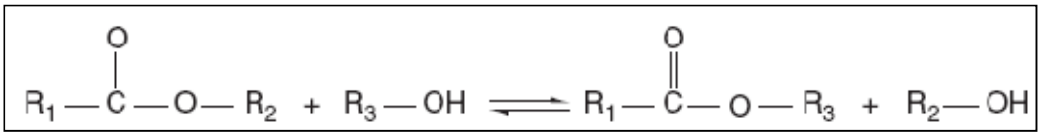

Figure 2.6: Lipase catalyzed alcoholysis reaction between an acylglycerol and an alcohol (Willis and Marangoni, 2008).

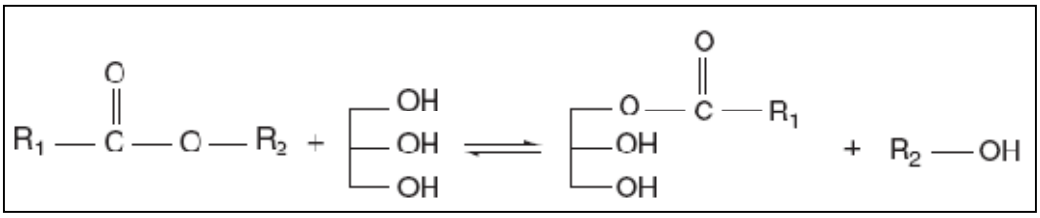

Figure 2.7: Lipase catalyzed glycerolysis reaction between glycerol and a TAG to produce MAG (Willis and Marangoni, 2008).

2.5.2 Enzymatic method for production of SLs

TAG lipases can catalyze hydrolysis as well as reverse reaction of lipolysis. In addition, they can catalyze interesterification and transesterification reactions, which occur between TAG-fatty acid (acidolysis), TAG-alcohol (alcoholysis) and TAG-glycerol (glycrolysis) in low water media. Substrate specificity and regiospecificity in the hydrolysis of TAGs, catalyzed by some lipases is shown in Table 2.8 (Weber and Mukherjee, 2008).

Enzyme-catalyzed reactions have the advantage of operating effectively under relatively mild conditions when compared to chemical methods. When a reaction occurs with enzymes, it can be carried out 10^6-10^{15} times faster than chemical methods even at room temperatures (Willis and Marangoni, 2008).

Table 2.8: Specificity of TAG lipases (Weber and Mukherjee, 2008).

Source of Lipase	Fatty Acid Specificity	Positional Specificity
Microorganisms		
Aspergillus niger	S, M, L	*sn*-1,3 >> *sn*-2
Candida antarctica	S > M, L	*sn*-3
*Candida rugosa (*syn. *C. cylindracea)*	S, L > M	*sn*-1,2,3
Chromobacterium viscosum	S, M, L	*sn*-1,2,3
Rhizomucor miehei	S > M, L	*sn*-1,3 >> *sn*-2
Penicillium roquefortii	S, M >> L	*sn*-1,3
Pseudomonas aeruginosa	S, M, L	*sn*-1
Pseudomonas fluorescens	S, L > M	*sn*-1,2,3
Rhizopus delemar	S, M, L	*sn*-1,2,3
Rhizopus oryzae	M, L > S	*sn*-1,3 >> *sn*-2
Plants		
Rapeseed (*Brassica napus*)	S > M, L	*sn*-1,3 > *sn*-2
Papaya (*Carica papaya*) latex		*sn*-3
Animal tissues		
Porcine pancreatic	S > M, L	*sn*-1,3
Rabbit gastric	S, M, L	*sn*-3

S: short chain, M: medium chain, L: long chain.

Lipases can be obtained from different sources such as animal (pancreatic lipase, lingual lipase, pharyngeal lipase, gastric lipase, etc.), plant (oilseed lipases, lipases that present at germ and the bran part of cereals, etc.) and microbial (fungal lipases, bacterial lipases, yeast lipases, etc.) sources. Among these lipases, animal and plant lipases are generally less thermostable than microbial lipases. In addition, microbial lipases can be produced less expensively than mammalian enzymes (Weete *et al.,* 2008).

2.5.3 Immobilization of enzymes

Industrial lipases have limited use in the industry, as they are expensive. In order to solve this problem, immobilization of the enzymes on an inert support material is developed (Weete *et al.,* 2008). Immobilized enzymes can be used in hydrolytic, synthetic, interesterification and transesterification reactions (Weete *et al.,* 2008).

Ion exchange resins, absorbents like silica gel, micro porous polypropylene and nylon can be selected for inert support material. Also naturally immobilized enzymes can be used. When using naturally immobilized enzymes, there is no need for extraction, purification, and immobilization. They can be directly used after physical separation from the growth medium. Immobilized enzymes have the advantage to be used for several times, to operate actively for longer time, and to be removed easily from the reaction environment (Weete *et al.,* 2008).

In hydrolysis and synthesis reactions, using immobilized enzymes is becoming popular. As they are reusable, rapid, cheap, they have advantages over free enzyme systems. In addition, they can control product formation; can be removed from the reaction medium easily with minimal inactivation even if the medium has impurities (Willis and Marangoni, 2008).

Enzymes can be immobilized by covalent bonding (chemical form) and adsorption or entrapment in a gel matrix or microcapsules (physical form). Adsorption is the generally used method for immobilization. In this method lipase is contacted an aqueous solution with an organic or inorganic surface-active adsorbent. The objective of immobilization is to maximize the level of enzyme loading per unit volume of support. The degree of immobilization depends on several conditions, including pH, temperature, solvent type, ionic strength, and protein and adsorbent concentrations (Willis and Marangoni, 2008).

2.5.4 Factors affecting lipase activity

2.5.4.1 pH

Lipases are only active at certain pH values. They can contain basic, neutral, and acidic residues. Most lipases are active generally between the pH values of 7.0 to 9.0 but there can be lipases active between pH 4 to 10. For example, the optimum pH for lipase from *Pseudomonas* species is around 8.5, whereas fungal lipases obtained from *A. niger* and *R. delemar* are acidic lipases (Willis and Marangoni, 2008).

2.5.4.2 Temperature

Enzymes can be denaturized irreversibly at very high temperatures. Due to this fact, increasing temperature generally increases the rate of reaction, at very high temperatures reaction rate decreases (Willis and Marangoni, 2008).

Animal and plant lipases are more vulnerable to heat compared to microbial lipases. In addition, immobilized lipases are more heat stable than the other enzymes as immobilization fixes the enzyme in one conformation, which reduces the susceptibility of the enzyme to denaturation (Willis and Marangoni, 2008).

2.5.4.3 Water content and water activity

The source of the enzyme determines the activity of enzymes at different water contents and water activity levels. Bacterial lipases are less stable than mold lipases in low water activity levels. The water content in a reaction system is the determining factor as to whether the reaction equilibrium will be toward hydrolysis or ester synthesis. Ester synthesis depends on low water activity. Too low water activity levels prevents all reactions from occurring because lipases need a certain amount of water to remain hydrated which is essential for enzymatic activity (Willis and Marangoni, 2008).

2.5.4.4 Substrate composition and steric hindrance

The rate of interesterification reaction can be affected by the composition and conformation of the substrate. For PUFAs, oxidation is possible and it causes inhibition of the activity of lipases especially in organic solvents. Due to this factor, in flow-through systems such as fixed bed reactors, highly refined oils should be used (Willis and Marangoni, 2008).

2.5.5 Specificity of lipases

As mentioned before, lipase-catalyzed reactions have the advantage of specificity over chemical reactions (Willis and Marangoni, 2008). Lipases are divided into three groups according to their specificity (Weete *et al.,* 2008). They can show positional specificity, stereo specificity, or substrate specificity (Willis and Marangoni, 2008).

There is another group of lipase that shows no specificity with respect to the position of the acyl group on the glycerol molecule, or to the specific nature of the fatty acid component of the substrate. Complete breakdown of the substrate to glycerol and fatty acids occurs with nonspecific lipases (Weete *et al.,* 2008).

Those lipases provide complete randomization of all fatty acids in all position after the reaction giving the same products with chemical reaction. *Candida cylindraceae, Corynebacterium acnes,* and *Staphylococcus aureus* lipases are the examples for non-specific lipases (Willis and Marangoni, 2008).

2.5.5.1 Positional specificity

The positional specificity of lipases on *sn*-1 and *sn*-3 positions of TAGs is due to the steric hindrance conflict that prevents the fatty acid located at the *sn*-2 from binding to the active sites (Willis and Marangoni, 2008; Weete *et al.,* 2008). Most microbial lipases show this feature (Weete *et al.,* 2008). As a result of interesterification with *sn*-1,3 specific lipases mixture of TAGs, 1,2- and 2,3-DAGs, and free fatty acids occur. *Aspergillus niger, M. miehei, Rhizopus arrhizus,* and *Rhizopus delemar* lipases are examples showing positional specificity (Willis and Marangoni, 2008).

2.5.5.2 Stereospecificity

Very few lipases differentiate between two primary esters at the *sn*-1 and *sn*-3 positions, but when they do, the lipases possess stereospecificity. In reactions where the lipase is stereospecific, positions 1 and 3 are hydrolyzed at different rates. Stereospecificity is determined by the source of the lipase and the acyl groups, and can also depend on the lipid density at the interface, where an increase in substrate concentration can decrease specificity due to steric hindrance. Differences in chain length can also affect the specificity of the lipase. Lipase from *Pseudomonas* species and porcine pancreatic lipase have shown stereoselectivity when certain acyl groups are hydrolyzed (Willis and Marangoni, 2008).

2.5.5.3 Substrate specificity

The last group of lipases show preference for a specific fatty acid or chain length range (Weete *et al.,* 2008). Most lipases from microbial sources show little fatty acid specificity. Some of lipases may show fatty acid chain length specificity such as porcine pancreatic lipase and *Penicillium cyclopium* lipases. Porcine pancreatic lipase is specific toward short-chain fatty acids while *Penicillium cyclopium* lipase is specific towards long-chain fatty acids. If interesterification reaction is being carried out in organic media, lipases can be specific towards certain alcohol species (Willis and Marangoni, 2008).

2.6 Human Milk Fat Substitutes (HMFS) Studied by Other Researchers

SLs can be synthesized by enzymatic modification reactions in order to produce specific products such as HMFSs. As mentioned earlier, scientists have been paying attention for production of HMFS as they are gaining importance.

Sahin *et al* (2005a), studied the production of HMFSs containing GLA. Concentrated γ-linolenic acid from borage oil and fatty acids from hazelnut oil were incorporated to tripalmitin in the presence of two different *sn*-1,3 specific lipases, Lipozyme® RM IM and Lipozyme® TL IM. The reaction conditions were optimized using Response Surface Methodology with rotatable five-level central composite design (CCD). Three factors as independent variables were selected as substrate molar ratio (Sr: 12-16 mol of total FA/mol of tripalmitin), temperature (T: 55-65ºC), and reaction time (t: 12-24 h). GLA and oleic acid incorporation were measured as responses. SLs obtained at the end of that study included 74.9% and 73.9% of palmitic acid by Lipozyme® RM IM and Lipozyme® TL IM, respectively. As HMF includes approximately 70% of palmitic acid at the *sn*-2 position, it was mentioned that, SL containing GLA can be used as HMFS (Sahin et al., 2005a).

In another study performed by Sahin *et al.*, fatty acids from hazelnut oil and stearic acid were incorporated to tripalmitin using different substrate molar ratio, reaction time and reaction temperature, selected as independent variables. Lipozyme® RM IM was used as the biocatalyst. According to the study, oleic acid incorporation increased with an increase in substrate molar ratio and reaction time, while stearic acid incorporation increased with reaction time generally for all substrate molar

ratios. Oleic acid/palmitic acid ratio is positively affected from both reaction time and substrate molar ratio (2005b).

Srivastava *et al.* (2006) produced SLs containing palmitic and oleic acids using oleic acid or methyl oleate as acyl donors. Lipase LIP1 from *Candida rugosa* and Lipozyme® RM IM were used as biocatalysts. The effects of reaction time (6, 12, and 24 h), reaction temperature (35, 45, and 55ºC), and substrate molar ratio (1:1-1:4) on oleic acid incorporation were examined. Oleic acid incorporation was observed to be higher with methyl oleate than oleic acid. Highest incorporation was observed at 45ºC with methyl oleate (Srivastava *et al.*, 2006).

In 2006 Sahin *et al.*, produced HMFS containing n-3 fatty acids. Tripalmitin, hazelnut oil fatty acids, and n-3 fatty acid concentrate were used as substrates for acidolysis reactions with the biocatalyst, Lipozyme® RM IM. Response surface methodology was used to optimize reaction conditions. The independent variables selected were substrate molar ratio, reaction time, and temperature. EPA plus DHA incorporation and oleic acid incorporation were the related responses. At optimal conditions generated by the model (12.4 mol/mol, 55ºC, and 24 h), oleic acid incorporation was measured as 40% and EPA+DHA incorporation was determined as 5%. Palmitic acid content of the structured lipid was 76.6%.

Karabulut *et al* (2007)., used vegetable oil mix to produce a SL resembling HMF in the presence of *sn*-1,3 specific lipase, Lipozyme® TL IM. Reaction temperature was set to 60ºC and reaction time was selected as 2, 4, 6, 8, 12, and 24 h. At the end of the study, HMFS containing unsaturated fatty acids located predominantly at *sn*-1,3 positions and palmitic acid at the *sn*-2 position but not with a desired level (2007).

Maduko *et al.*, also studied with vegetable oil blends to incorporate them into skim caprine milk to produce goat milk based HMFS. The vegetable oil blend was composed of coconut, safflower, and soybean oils with the ratio of 2.5:1.1:0.8 respectively. Lipozyme® RM IM was selected as the enzymatic catalyst of the reaction and substrate molar ratio and reaction time were used as reaction parameters. In the scope of this study, palmitic, oleic and linoleic acid incorporations at *sn*-2 position were investigated. Among the products, the SLs obtained at 12 h with substrate molar ratio of 1:1 showed better similarity to HMF (2007).

Pina-Rodriguez and Akoh (2009a) studied with amaranth oil to produce HMFS. Amaranth oil was firstly enriched with palmitic acid at the *sn*-2 position in the presence of Novozym 435.Later on, customized amaranth oil obtained from this reaction step, was used as a substrate to incorporate DHA. For the incorporation of DHA, *sn*-1,3 specific lipase Lipozyme® RM IM was used as biocatalyst. Response surface methodology was used to optimize the reaction conditions.

At the end of these two interesterification reactions, the SL had palmitic, stearic, oleic, linoleic, linolenic, and DHA contents of 33.9%, 2.8%, 23.3%, 37.3%, 0.7%, and 1.9%, respectively. Original amaranth oil and SL was analyzed in terms of characterization and fatty acid composition, melting profile, chemical characteristics, oxidative stability, phytosterol, tocopherol, and squalene analysis were performed (Pina-Rodriguez and Akoh, 2009b).

3. MATERIALS AND METHODS

3.1 Materials

Refined hazelnut oil used in this project was purchased from a grocery store in Istanbul, Turkey. DHASCO® and ARASCO®, which are SCOs rich in DHA and ARA, respectively were donated by Martek Biosciences Company, Columbia.

External (Supelco 37 Component FAME Mix) and internal (C15:0-pentadecanoic acid and C17:0-heptadecanoic acid) standards for fatty acid compositional analysis and tocopherol (α-, β-, γ-, and δ-) standards were purchased from Sigma-Aldrich Corporation (St. Louis, MO).

Trilinolein and 2-oleoylglycerol standards for product (TAG and 2-MAG) identification were purchased from Sigma-Aldrich Corporation (St. Louis, MO) and Avanti Polar Lipids, Inc. (Alabaster, AL) respectively.

Novozym® 435 lipase and immobilized enzyme Lipozyme® RM IM from *R. miehei* for transesterification reaction were purchased from Novo Nordisk, Denmark.

All the other chemicals and solvents (analytical and/or chromatographic grade) were purchased from Sigma-Aldrich Corporation (St. Louis, MO), Avantor Performance Materials (Phillipsburg, NJ), or Fisher Scientific (Pittsburgh, PA).

3.2 Methodology

This study consisted of two parts. In the first part, the *sn*-2 position of hazelnut oil was enriched with palmitic acid and ethyl palmitate in the presence of a nonspecific enzyme, Novozym® 435 (lipase B from *C. antarctica*).

In the second part of the study, DHA and ARA were incorporated into enriched hazelnut oil in the presence of *sn*-1,3 specific lipase, Lipozyme® RM IM obtained from *Rhizomucor miehei*. The methods used in the study are summarized in Figure 3.1.

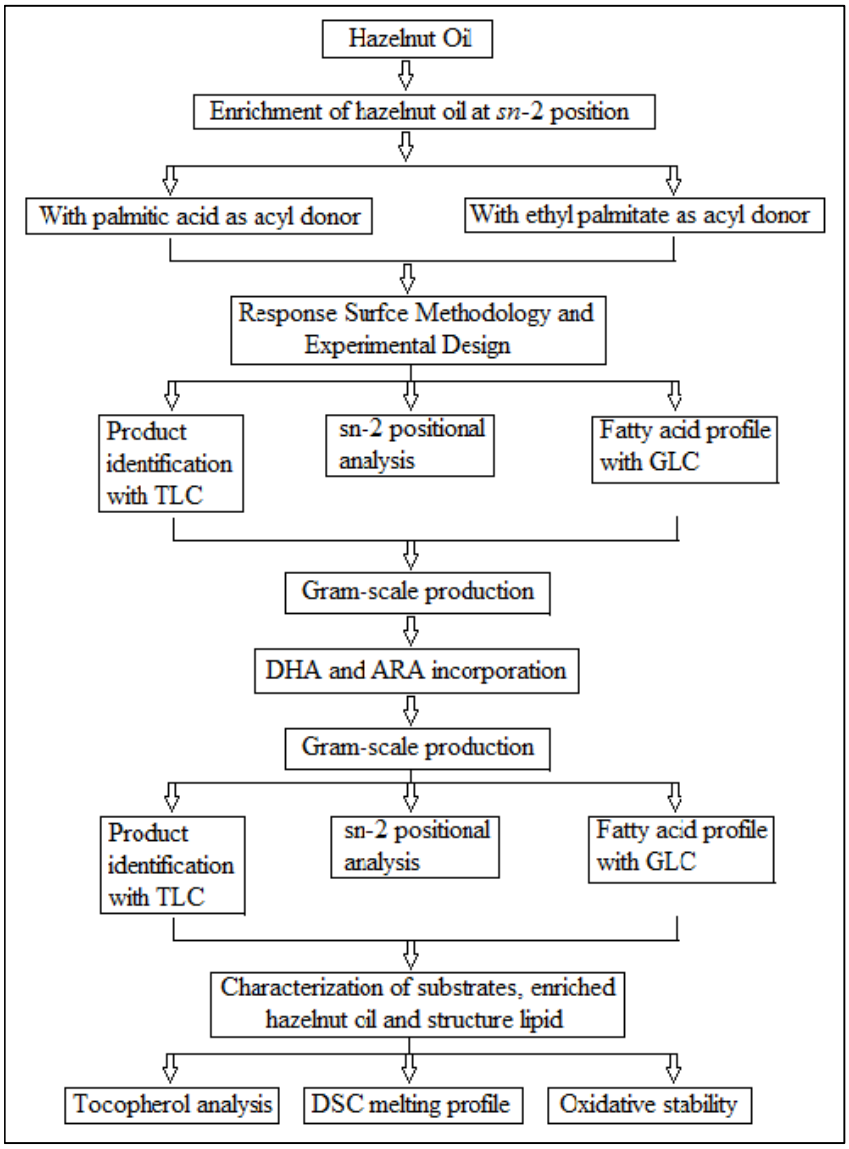

Figure 3.1: Flow chart of the study and methods used

3.2.1 Substrate characterization

3.2.1.1 Preparation of Fatty Acid Methyl Esters (FAME)

Methylation of hazelnut oil, DHASCO®, and ARASCO® was performed according to AOAC Official Method 996.01, Section E. For preparation of FAMEs of hazelnut oil, 100 mg of oil was weighed into screw-capped test tubes and 1 mL of C17:0 internal standard (20 mg/mL) was added.

For reaction products analysis, 50 µL of internal standard was added to the test tubes. To both hazelnut oil and TAG band tubes, 2 mL 0.5 N NaOH was added and vortexed. Tubes were incubated in oven at 100 ºC for 5 min for saponification.

Then, 2 mL of 14% BF_3 in methanol was added, vortexed for 1 min and incubated at 100 ºC for 5 min again. Tubes were removed from oven and cooled under cold water. Later on, 2 mL of hexane and 2 mL of saturated NaCl solutions were added, vortexed for exactly 2 min and centrifuged at 1000 rpm for 3 min. After cenrtifugation, upper hexane layer was passed through sodium sulfate column. Furthermore, 0.5-1 mL of this layer was taken into capillary gas-liquid chromatograpy (GLC) vials and analyzed with capillary GLC.

3.2.1.2 Fatty acid profile analysis by using capillary gas-liquid chromathography (GLC)

Materials used for the project and reaction products were analyzed in terms of fatty acid profile and *sn*-2 position by using capillary GLC, Agilent Technologies 6890N. Methylated samples were run with internal standard C17:0 (20 mg/mL). Also Supelco FAME37 mix was used as an external standard. The results were calculated as mol % using a programme. Table 3.1 expresses the analysis conditions of the instrument.

Table 3.1: Analysis conditions of capillary gas-liquid chromatograph (GLC).

Instrument part	Condition
Column	Supelco SP-2560, 100 m length and 0.25 mm inner daimeter, 0.20 μm film thickness
Detector	Flame ionization detector (FID), 260ºC
Helium flow	1.1 mL/min
Oven program	Hold at 150ºC for 3 min, ramp temperature to 215ºC at 10ºC/min, hold at 215ºC for 40 min
Injection volume	1 μL
Split ratio	5:1

3.2.2 Experimental design for RSM study

In order to determine the effects of parameters chosen for incorporation of total palmitic acid and palmitic acid at the sn-2 position of hazelnut oil, RSM was used. CCF design resulting in eight possible combinations of both independent factors and three center points were used in the model.

According to preliminary experiments and literature review, the independent variables were chosen as substrate molar ratio (Sr: 1/4-1/6 hazelnut oil/FA) and time (t: 6-18 h). The reaction temperature (T) was set at 65ºC.

Total palmitic acid amount in the TAG structure and at the sn-2 position were the measured responses. Experimental design was generated using Modde 5.0 software (Umetrics, Umea, Sweden) according to CCF design. Independent and coded variables and their symbols are given in Table 3.2.

Table 3.2: Independent and coded variables and their symbols.

Independent variables	Symbol	Coded variables		
		-1	0	+1
Substrate molar ratio (mol/mol)	Sr	4	5	6
Reaction time (h)	t	6	12	18

3.2.3 Acidolysis reactions

Acidolysis reactions were performed for the first part of the study. In this part of the study, hazelnut oil was enriched with palmitic acid as acyl donor to increase the palmitic acid amount of the oil at the *sn*-2 position in the presence of a nonspecific enzyme, Novozym® 435.

Milligram-scale production was performed with addition of palmitic acid as acyl donor to hazelnut oil, and then enzyme (10% of total reactants) and 3 mL of hexane was added as a solvent. Materials were weighed in screw-cap test tubes, coated with teflon tape and incubated in a water bath at 65ºC with 200 rpm orbital shaking. After the reaction, the products were passed through sodium sulfate column into small teflon taped vials to remove the enzyme and the moisture. The vials were kept at -18ºC for further analysis. The reactions were run in triplicates. Gram-scale production was performed in a stirred-batch bioreactor containing a four-blade mixer in a solvent-free environment. The length and width of each blade was 2 cm and 1 cm, respectively. The inner diameter of the bioreactor was 10.5 cm and the height was 15 cm (Akoh and Lee, 1998). After the reaction, the resulting product was passed through sodium sulfate column and vacuum filtered. Product was kept at -18ºC for further analysis.

3.2.4 Transesterification reactions

For the first part of the study, hazelnut oil was also enriched with ethyl palmitate as acyl donor in the presence of Novozym® 435. The reactions were performed as in acidolysis reactions and the product (enriched hazelnut oil) which has higher palmitic acid amount at the *sn*-2 position was chosen.

Furthermore, gram-scale production was performed and final product was used as a substrate for incorporation of DHA and ARA through enzymatic transesterification reactions. In the second part of the study, incorporation of DHA and ARA into enriched hazelnut oil was performed via transesterification reactions in the presence of *sn*-1,3 specific lipase, Lipozyme® RM IM. ARASCO® and DHASCO® were physically mixed in 3:2 and 2:1 ratios. Reaction time, temperature and substrate molar ratio were chosen as 3 h, 50 and 60 ºC, 0.1 and 0.05, respectively. Miligram-scale reactions were carried out in a shaking water bath with 200 rpm orbital shaking and experiments were run in triplicates. The resulting products were passed through sodium sulfate column into teflon taped small vials and they were kept at -18ºC for further analysis. Gram-scale production was also carried out in the water bath using round-bottom flask. After the reaction, the product was passed through sodium sulfate column and vacuum filtered.

3.2.5 Free fatty acid (FFA) removal with short-path distillation unit

After gram-scale production of acidolysis and transesterification reactions, excess FFA were removed with short-path distillation unit (UIC Inc., Joliet, IL) to the level of <1%. Feed vessel temperature was kept at 55 ºC, temperature of wiper blade vessel where the oil was turning around was 155 ºC, and condensation temperature was 20 º C. Feeding rate of the sample was 100 mL/h and the pump vacuum was kept at <135 mTorr.

3.2.6 Free fatty acid (FFA) determination

After removal of FFAs with short path distillation unit, FFAs were analyzed by using AOCS Official Method Ac 5-41. Approximately 7.05±0.005 g of oil was weighed into a suitable flask. 75 mL isopropanol and 1 mL of phenolphthalein indicator solution were added and titrated with 0.25 M NaOH until a faint pink color was obtained and persisted for about a minute. FFAs of the oil were calculated as oleic acid.

3.2.7 Product identification with thin-layer chromatography (TLC)

Product identification with TLC was performed according to the method already applied by Jennings and Akoh (1999).

After the reaction, products were passed through sodium sulfate column, which were then spotted on TLC plates coated with silica gel G. It was recommended to dry the plates in the oven at 100 ºC and developing solvent should be in eqilibrium before spotting. 100 µL product and a proper TAG standard was spotted on TLC plates and placed in equilibrated tank.

Petroleum ether:diethyl ether:acetic acid (80:20:0.5 v/v/v) solvent system was used to develope the plates. TLC plates were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under UV light. TAG bands were determined according to TAG standard. Corresponding TAG bands were scraped off into screw-capped test tubes and analyzed with GLC in terms of fatty acid profile of TAG and *sn*-2 position.

3.2.8 Lipase catalyzed sn-2 positional analysis

After the recovery TAG bands into screw-capped test tubes, extraction step with diethyl ether was carried out to prevent the inhibition of the enzyme with silica gel G. To the TAG bands, 2 mL of diethyl ether was added, vortexed for 1 min and centrifuged at 1000 rpm for 3 min. Diethyl ether layer was passed through sodium sulfate column. This step was repeated twice and diethyl ether layers were collected in the test tubes. Diethyl ether layer was removed completely under nitrogen gas to get purified TAG. Then, 2 mL of Tris-HCl buffer (1M, pH 8.0), 0.5 mL 0.05% sodium cholate solution, and 0.2 mL 2.2% calcium chloride solution were added. In order to emulsify the sample, tubes were vortexed for 2 min.

Later on, 40 mg of pancreatic lipase was added and vortexed for an additional minute. Samples were incubated in a water bath at 40 ºC for 3 min. In order to stop the reaction 1 mL of 6 N HCl and 4 mL of diethyl ether were added, vortexed for 2 min and centrifuged at 1000 rpm for 3 min. Diethyl ether layer was passed through sodium sulfate column and the volume was decreased to one third under nitrogen gas.

Samples were spotted on TLC plates coated with silica gel G and developed with hexane:diethyl ether:formic acid (60:40:1.6 v/v/v) solvent system. Plates were sprayed with 0.2% 2,7-dichlorofluorescein in methanol, visualized under UV light and 2-MAG bands were determined according to 2-oleoylglycerol standard. Corresponding 2-MAG bands were scraped off into screw-capped test tubes and after methylation, they were analyzed by GLC (Jennings and Akoh, 1999).

3.2.9 Tocopherol content analysis

Standard preparation and sample analysis for tocopherol content determination was performed according to the method of Shin et al. (2009). In order to prepare calibration curve, stock solution of standards were prepared by weighing approximately

50 mg of α-, β-, and δ-T and 100 mg of γ-T in 25 mL of hexane, respectively. From the stock solutions, different concentrations of standard solutions were prepared. 10 μL of sample extracts and tocopherol standard solutions were injected per run.

Different concentrations of stock solutions of the standards were analyzed with liquid chromatograph (Shimadzu LC-6A pump equipped with an RF-10AXL fluorescence detector (Shimadzu Corp., Columbia, MD), a SpectraSERIES AS 100 autosampler (Thermo Separation Products, Inc., San Jose, CA)). A normal-phase LiChrosorb Si 60 column (4 mm x250 mm, 5 μm particle size; Hibar Fertigsäule RT, Merck, Darmstadt, Germany), and an isocratic mobile phase comprised of 0.85% (v/v) isopropanol in hexanes at a flow rate of 1.0 mL/min were used. Before use, mobile phase was vacuum filtered through a 0.45 μm nylon membrane filter and degassed by stirring under vacuum.

The excitation and emission wavelengths for the fluorescent determination of tocopherol isomers were 290 and 330 nm, respectively.

3.2.10 Melting profile analysis by differential scanning calorimeter (DSC)

AOCS Official Method Cj 1-94 is followed in order to determine melting and cristallization profile of materials and products after gram-scale production with DSC. Dry ice in acetone was used as coolant. Purge gas was nitrogen with the pressure of 10 psi. Indium was used for the calibration of the instrument. Melting point of indium is at 156.6 ºC and entalpy value (ΔH) is 28.45 J/g. An empty and hermetically sealed aluminum pan was used as the reference.

Approximately 8-12 mg of products were weighed into aluminum pans and hermetically sealed again for sample analysis. Samples were cooled from 80 ºC to -55 ºC with a rate of 10 ºC/min and heated to 80 ºC with a rate of 5 ºC/min. Thermograms generated by the instrument were analyzed with Syris (Perkin-Elmer, Shelton, CT) software.

3.2.11 Oxidative stability experiment

AOCS Cd 12b-92 is the reference method for oxidative stability experiments (1997). Oxidative stability of substrate oils and reaction products after gram-scale production was determined by using OSI instrument. It is an accelerated method to determine the stability of the products. At set temperature, the time required for the product to reach induction time was measured. For this prupose, 5 gr of each sample was weighed into glass tubes of the instrument. Liquid samples were directly weighed while solid samples were liquified before weighing. Calibration of OSI instrument was performed with deionized water. Analysis temperature was kept at 110 ºC.

3.2.12 Statistical analysis

The regression analysis, statistical significance, analysis of variance (ANOVA), and response surface applications were carried out by using Modde 5.0 (Umetrics, Umeå, Sweden) software. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and ANOVA.

A second-order polynomial model for two variables was used to fit the data obtained from the experimental design as shown in Equation 3.1.

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i=1}^{1} \sum_{j=i+1}^{2} \beta_{ij} X_i X_j \qquad \textbf{(3.1)}$$

Where;

Y is dependent variable or response (response 1, incorporation of total palmitic acid; response 2, incorporation of palmitic acid at *sn*-2 position),

β_0 is a constant,

β_i is the linear term coefficient,

β_{ii} is the quadratic term coefficient,

β_{ij} is the interaction term coefficient,

X_i and X_j are the independent variables (Myers and Montgomery, 2002).

The advantages of a second-order model is that it is flexible, it can take a wide variety of functional forms, so it will often work well as an approximation to the true response surface (Myers and Montgomery, 2002).

In addition it is easy to estimate the parameters and also second-order model works well in solving real response surface problems (Myers and Montgomery, 2002).

For the second part of the study, the effect of different temperature, substrate molar ratio, and ARA/DHA ratio were analyzed using ANOVA and Tukey simultaneous tests. All data was analyzed by MINITAB 16 statistical software (PA, USA).

4. RESULTS AND DISCUSSION

4.1 Fatty Acid Compositions of Materials

Fatty acid profiles were determined with Agilent Technologies 6890N capillary gas-liquid chromatograph for hazelnut oil, palmitic acid, ethyl palmitate, DHASCO® and ARASCO®. The results are given in Table 4.1.

Table 4.1: Fatty acid profiles of hazelnut oil, palmitic acid, ethyl palmitate, DHASCO®, and ARASCO®.

Fatty acid	Hazelnut oil	Palmitic acid	Ethyl palmitate	DHASCO®	ARASCO®
Caprylic acid (8:0)	-	-	-	0.20±0.00	-
Capric acid (10:0)	-	-	-	0.83±0.00	-
Lauric acid (12:0)	-	-	-	3.35±0.02	-
Myristic acid (14:0)	-	0.52±0.25	0.15±0.00	8.96±0.02	0.55±0.10
Myristoleic acid (14:1)	-	-	-	0.13±0.00	-
Palmitic acid (16:0)	5.74±0.01	99.01±0.25	99.74±0.01	6.89±0.00	8.59±0.25
Palmitoleic acid (16:1n-7)	0.17±0.00	-	-	2.11±0.00	-
Heptadecanoic acid (17:0)	-	-	-	0.08±0.03	0.40±0.03
Heptadecenoic acid (17:1)	-	-	-	0.09±0.02	-
Stearic acid (18:0)	2.87±0.01	0.46±0.01	-	0.83±0.01	8.61±0.21
Oleic acid (18:1n-9)	82.30±0.03		0.11±0.01	24.73±0.05	22.33±0.57
Linoleic acid (18:2n-6)	8.54±0.02	-	-	1.21±0.00	7.05±0.20
Linolenic acid (18:3n-6)	0.12±0.00	-	-	-	2.90±0.08
Arachidic acid (20:0)	0.13±0.00	-	-	0.11±0.00	0.79±0.02
Eicosenoic acid (20:1n-9)	0.12±0.00	-	-	0.15±0.00	0.38±0.01
Eicosadienoic acid (20:2)	-	-	-	-	0.66±0.01
Arachidonic acid (20:4n-6)	-	-	-	-	42.93±1.14
Behenic acid (22:0)	-	-	-	0.26±0.00	1.75±0.04
Lignoceric acid (24:0)	-	-	-	0.05±0.00	1.33±0.03
Docosahexaenoic acid (22:6n-3)	-	-	-	50.1±0.01	-

The samples were analyzed in triplicates; average and standard deviation of the results were calculated. According to the results, molecular weight of hazelnut oil, DHASCO®, and ARASCO® were determined as 880.61±0.01 g, 921.42±0.00 g, and 915.02±0.85 g, respectively.

The fatty acid composition of hazelnut oil used in this study contained oleic acid (82.30%), linoleic acid (8.54%), and palmitic acid (5.74%).

The purity of ethyl palmitate and palmitic acid used for the enrichment of hazelnut oil in terms of palmitic acid at *sn*-2 position were found as higher than 99%. DHA content of DHASCO® was determined as 50.1 % whereas ARA content of ARASCO® was found as 42.93%. These values were in conformity with the product data sheets supplied by the manufacturer.

Fatty acid profile of hazelnut oil which had been studied by different researchers are also summarized in Table 4.2. Palmitic acid, palmitoleic acid, stearic acid, and linolenic acid amounts of hazelnut oil used in this study were found to be similar to the amounts mentioned in Table 4.2.

Table 4.2: Fatty acid profile of hazelnut oil analyzed by different researchers.

Fatty acid	HO[a]	HO[b]	HO[c]	HO[d]
Palmitic acid (16:0)	5.61	4.08-5.94	5.23	4.4-6.8
Palmitoleic acid (16:1n-7)	0.15	0.12-0.24	0.20	0.1-0.3
Stearic acid (18:0)	3.44	1.62-2.03	2.53	1.9-2.6
Oleic acid (18:1n-9)	77.23	73.80-80.07	81.06	73.6-81.6
Linoleic acid (18:2n-6)	11.39	11.96-16.53	10.65	9.8-16.6
Linolenic acid (18:3n-6)	0.30	0.12-0.16	0.09	0.1
Arachidic acid (20:0)	-	0.08-0.11	0.11	0.1
Eicosenoic acid (20:1)	-	0.12-0.15	0.15	0.2

[a] Chiavaro *et al.*, 2008.
[b] Savage *et al.*, 1997.
[c] Karabulut *et al.*, 2005.
[d] Benitez-Sànchez *et al.*, 2003.

4.2 Sn-2 Positional Analysis of Hazelnut Oil

Fatty acids located at *sn*-2 position of hazelnut oil are given in Table 4.3. As it can be seen from table, hazelnut oil was rich in oleic acid at the *sn*-2 position. Palmitic acid amount was found as 0.84%. The other major fatty acid at the *sn*-2 position was linoleic acid and also it included a very small amounts of stearic acid.

Table 4.3: *Sn*-2 positional analysis of hazelnut oil.

Fatty acid	Mol %
Palmitic acid (16:0)	0.84±0.36
Stearic acid (18:0)	0.61±0.67
Oleic acid (18:1n-9)	86.02±0.20
Linoleic acid (18:2n-6)	11.65±0.29

4.3 Enrichment of Hazelnut Oil with Palmitic Acid at Sn-2 Position

As a primary step, hazelnut oil was enriched with both palmitic acid and ethyl palmitate in order to increase the palmitic acid amount at *sn*-2 position.

As it can be seen from Table 4.1 and Table 4.2 hazelnut oil contains 5.74% palmitic acid of which 0.84% was located at *sn*-2 position. To increase the palmitic acid content at *sn*-2 position close to the values of human milk, enzymatic acidolysis were carried out.

4.3.1 Enrichment with palmitic acid as acyl donor

The experimental design created by Modde 5.0 software and the observed responses in terms of total palmitic acid and palmitic acid at *sn*-2 position are given in Table 4.4.

Table 4.4: Experimental design for RSM and observed responses for enrichment of hazelnut oil with palmitic acid.

Exp. #	Independent variables		Responses (Incorporation, % weight)	
	Substrate molar ratio (Sr; mol/mol)	Time (t; h)	Total palmitic acid amount	Palmitic acid amount at *sn*-2 position
1	4	6	12.97	7.85
2	4	18	23.49	11.97
3	6	6	11.54	8.86
4	6	18	24.58	18.36
5	5	6	12.66	8.25
6	5	18	32.54	17.02
7	4	12	18.39	9.94
8	6	12	17.45	10.11
9	5	12	17.13	8.80
10	5	12	18.31	9.11
11	5	12	19.06	10.50

Regression coefficients (β) and *P*-values for the enrichment of hazelnut oil with palmitic acid in terms of total palmitic acid and palmitic acid at the *sn*-2 position are given in Table 4.5 and 4.6, respectively. According to Table 4.5, among the first order parameters, "time" had statistical importance (α=0.05) for enrichment of hazelnut oil with palmitic acid. "Substrate molar ratio" was not statistically significant for palmitic acid incorporation. In addition, second order parameteres were not statistically significant (α=0.05).

Table 4.5: Regression coefficients and *P*-value for enrichment of hazelnut oil with palmitic acid (total palmitic acid).

Independent variables	Regression coefficients (β)	*P*-value[a]
Constant	19.05	<0.0001
t	7.24	0.001
Sr	-0.21	0.85
t*t	2.22	0.24
Sr*Sr	-2.46	0.20
t*Sr	0.63	0.66

[a] *P*-value, level of significance
t: time, Sr: substrate molar ratio

The equation for total palmitic acid incorporation to hazelnut oil using palmitic acid as acyl donor can be written as follows:

$$\textit{Incorporation \% (total palmitic acid)} = 19.05 + 7.24t - 0.21Sr + 2.22t*t - 2.46Sr*Sr + 0.63t*Sr \quad \textbf{(4.1)}$$

Table 4.6: Regression coefficients and *P*-value for enrichment of hazelnut oil with palmitic acid (palmitic acid at *sn*-2 position).

Independent variables	Regression coefficients (β)	*P*-value
Constant	9.77	<0.0001
t	3.73	0.001
Sr	1.26	0.06
t*t	2.41	0.03
Sr*Sr	-0.20	0.82
t*Sr	1.35	0.09

[a] *P*-value, level of significance
t: time, Sr: substrate molar ratio

As it can be seen from Table 4.6; among the first order parameters, "time" was found to be statistically significant (α=0.05). Among second order parameters, "time*time" had also statistical importance (α=0.05) for enrichment of hazelnut oil with palmitic acid (palmitic acid at *sn*-2 position). Other parameters did not have any statistical importance. The equation for this reaction can be written as follows (with palmitic acid):

$$\textit{Incorporation \% (palmitic acid at sn-2 position)} = 9.77 + 3.73t + 1.26Sr + 2.41t*t - 0.20Sr*Sr + 1.35t*Sr \quad \textbf{(4.2)}$$

ANOVA table for total palmitic acid and palmitic acid at *sn*-2 position can be seen in Table 4.7.

Table 4.7: ANOVA table for total palmitic acid and palmitic acid at *sn*-2 position in the enrichment reaction with palmitic acid.

Total palmitic acid	DF	SS	MS	F	P[a]
Total	11	4311.15	391.92		
Constant	1	3937.63	3937.63		
Total Corrected	10	373.52	37.35		
Regression	5	338.37	67.67	9.63	0.013
Residual	5	35.15	703		
Lack of Fit	3	33.25	11.08	11.71	0.080
Pure Error	2	1.89	0.95		
Palmitic acid at *sn*-2 position	DF	SS	MS	F	P
Total	11	1449.93	131.81		
Constant	1	1325.94	1325.94		
Total Corrected	10	123.99	12.40		
Regression	5	115.62	23.13	13.82	0.006
Residual	5	8.37	1.67		
Lack of Fit	3	6.73	2.24	2.74	0.279
Pure Error	2	1.64	0.82		

[a] P, level of significance
DF: degree of freedom, SS: sum of squares, MS: mean of squares, F: F value, P: P value

The coefficients of determination (R^2) for palmitic acid and palmitic acid at *sn*-2 position were found as 0.91 and 0.93, respectively. According to the ANOVA results presented in Table 4.7, the models generated also represented a satisfactory prediction since F values of models (9.63, 13.82) are very high compared with $F_{5,5}$ value (5.05) (α=0.05). Since *P*-values of the model errors for palmitic acid and palmitic acid at *sn*-2 position were 0.013 and 0.006, respectively, the model had lack of fit (P>0.05), and the *P*-values for the regression probability was <0.001 (Table 4.7). The contour plots were drawn to investigate the relationship between the responses and the reaction parameters. The contour plots obtained by interaction of time and substrate molar ratio on incorporation of palmitic acid catalyzed by Novozym® 435 are given in Figures 4.1 and 4.2, respectively.

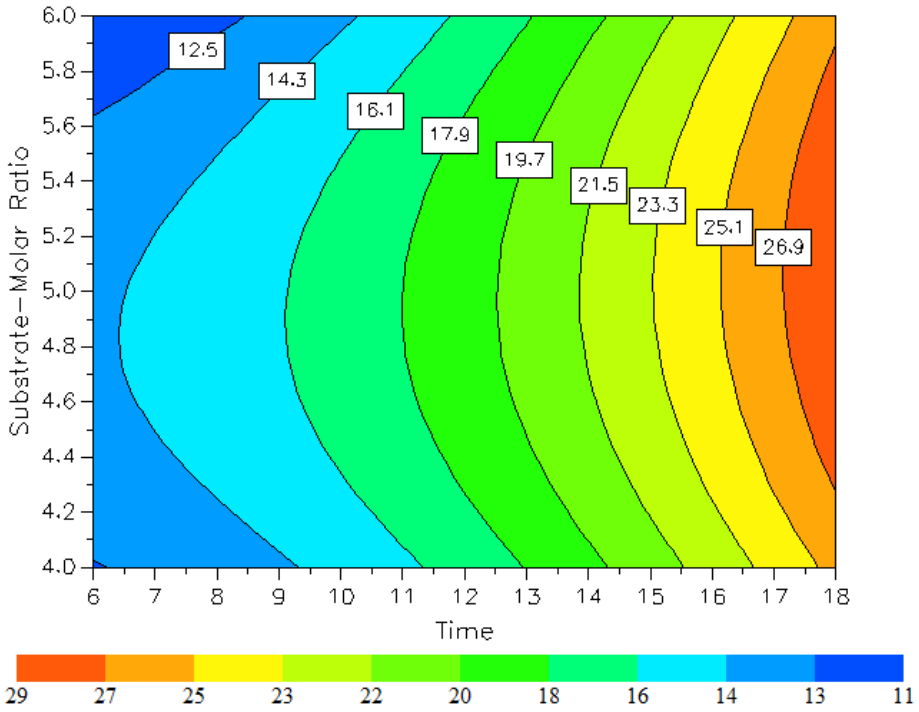

Figure 4.1: Response contour plot for total palmitic acid for enrichment with palmitic acid.

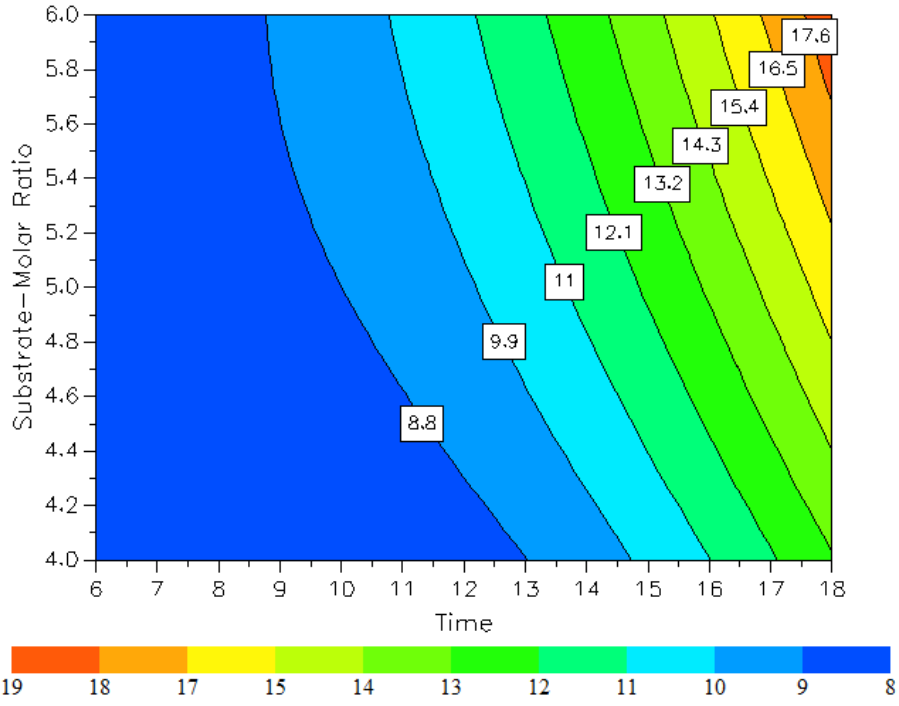

Figure 4.2: Response contour plot for palmitic acid at *sn*-2 position for enrichment with palmitic acid.

According to Figure 4.1, it can be seen that increase in time resulted in higher incorporation of total palmitic acid while increase in substrate molar ratio did not have a significant effect in incorporation. Maximum incorporation was observed since 17th h of the reaction within the experimental range of this study. In Figure 4.2 it can be observed that time had a positive effect on palmitic acid incorporation to sn-2 position of hazelnut oil at a certain point. Before 13 h it did not have a significant change in incorporation. After 13 h, incorporation increased as reaction time was increased. Again, substrate molar ratio did not have effect on the incorporation significantly.

4.3.2 Enrichment with ethyl palmitate as acyl donor

The experimental design created by Modde 5.0 software and the observed responses in terms of total palmitic acid and palmitic acid at *sn*-2 position are given in Table 4.8 excluding the outliers because deleting unimportant variables from the model improves the precision of estimation for the parameter estimates of the retained variables (Myers and Montgomery, 2002).

Table 4.8: Experimental design for RSM and observed responses for enrichment of hazelnut oil with ethyl palmitate.

Exp. #	Independent variables		Responses (Incorporation, % weight)	
	Substrate molar ratio (Sr; mol/mol)	Time (t; h)	Total palmitic acid amount	Palmitic acid amount at *sn*-2 position
1	4	6	16.78	14.90
2	4	18	29.44	22.83
3	6	6	18.97	15.54
4	6	18	66.40	34.10
5	5	6	22.73	13.15
6	5	18	41.75	36.06
7	4	12	27.44	23.41
8	6	12	41.08	33.09
9	5	12	33.43	26.53
10	5	12	26.50	20.17
11	5	12	32.42	27.64

Insignificant linear, quadratic, and interaction terms at significance level (α=0.05) were eliminated. Regression coefficients (β) and *P*-values for the enrichment of hazelnut oil with ethyl palmitate in terms of total palmitic acid and palmitic acid at *sn*-2 position are given in Table 4.9 and 4.10.

Table 4.9: Regression coefficients (β) and *P*-values for enrichment of hazelnut oil with ethyl palmitate (total palmitic acid).

Independent variables	Regression coefficients (β)	*P*-value[a]
Constant	34.08	0.0001
t	10.94	0.003
Sr	6.55	0.015
t*t	-3.00	0.17
Sr*Sr	-0.98	0.60
t*Sr	5.32	0.055

[a] *P*-value, level of significance
t: time, Sr: substrate molar ratio

Table 4.9 shows that; amoung the first order parameters "time" and "substrate molar ratio" had statistical importance (α=0.05) for enrichment of hazelnut oil with ethyl palmitate. Second order parameters were not statistically important. The equation for this reaction can be written as follows (with ethyl palmitate):

$$\textit{Incorporation \% (total palmitic acid)} = 34.08 + 10.94t + 6.55Sr - 3.00t*t - 0.98Sr*Sr + 5.32t*Sr \quad \textbf{(4.3)}$$

Table 4.10: Scaled and centered coefficients and *P* values for enrichment of hazelnut oil with ethyl palmitate (palmitic acid at *sn*-2 position).

Independent variables	Scaled and centered coefficients (β)	*P*-value[a]
Constant	27.22	<0.0001
t	10.53	0.001
Sr	5.90	0.006
t*t	-2.75	0.085
Sr*Sr	0.89	0.47
t*Sr	6.11	0.01

[a] *P*-value, level of significance
t: time, Sr: substrate molar ratio

As it can be seen from Table 4.10; among first order parameters; "time" and "substrate molar ratio" had statistical importance (α=0.05).

Among the second order parameters; "time*substrate molar ratio" was statistically significant for enrichment of hazelnut oil with ethyl palmitate (palmitic acid at *sn*-2 position) (α=0.05). The equation for this reaction can be written as follows (with ethyl palmitate):

$$Incorporation\ \%\ (palmitic\ acid\ at\ sn\text{-}2\ position) = 27.22 + 10.53t + 5.90Sr - 2.75t*t + 0.89Sr*Sr + 6.11t*Sr \quad \textbf{(4.4)}$$

ANOVA table for total palmitic acid and palmitic acid at *sn*-2 position can be seen in Table 4.11.

Table 4.11: ANOVA table for total palmitic acid and palmitic acid at *sn*-2 position in the enrichment reaction with ethyl palmitate.

Total palmitic acid	DF	SS	MS	F	P
Total	9	8377	930.78		
Constant	1	7746.35	7746.35		
Total Corrected	8	630.65	78.83		
Regression	5	614.45	122.89	22.75	0.014
Residual	3	16.21	5.40		
Lack of Fit	2	15.70	7.85	15.39	0.177
Pure Error	1	0.51	0.51		
Palmitic acid at *sn*-2 position	DF	SS	MS	F	P
Total	9	5568.74	618.75		
Constant	1	5048.1	5048.1		
Total Corrected	8	520.64	65.08		
Regression	5	513.99	102.80	46.34	0.005
Residual	3	6.66	2.22		
Lack of Fit	2	6.04	3.02	4.90	0.304
Pure Error	1	0.62	0.62		

[a] P, level of significance
DF: degree of freedom, SS: sum of squares, MS: mean of squares, F: F value, P: P value

The coefficients of determination (R^2) for palmitic acid and palmitic acid at *sn*-2 position were found as 0.97 and 0.99, respectively. According to the ANOVA results presented in Table 4.11, the models generated also represented a satisfactory prediction since F values of models (22.75, 46.34) are very high compared with $F_{5,3}$ value (9.01) (α=0.05). Since *P*-values of the model errors for palmitic acid and palmitic acid at *sn*-2 position were 0.177 and 0.304, respectively, there were no significant (P>0.05) lack of fit in the models.

The contour plots were drawn to investigate the relationship between the responses and the reaction parameters. The contour plots obtained by interaction of time and substrate molar ratio on incorporation of palmitic acid catalyzed by Novozym® 435 are given in Figures 4.3 and 4.4, respectively.

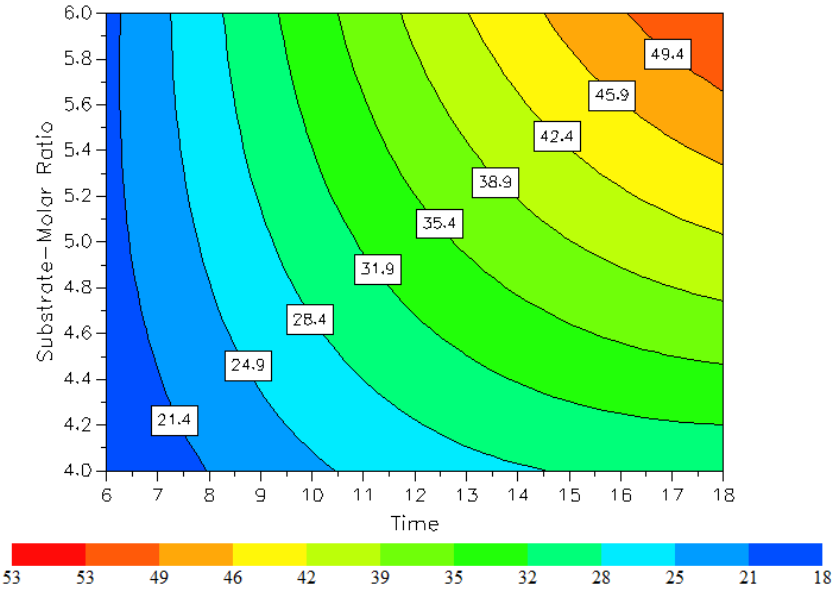

Figure 4.3: Response contour plot for total palmitic acid for enrichment with ethyl palmitate.

According to Figure 4.3 it can be said that increase in time and substrate molar ratio resulted in higher in incorporation of total palmitic acid. Very low reaction time did not have an effect on palmitic acid incorporation. Maximum incorporation was observed in 17th h of the reaction with substrate molar ratio of 5.5-6.0 within reaction parameters.

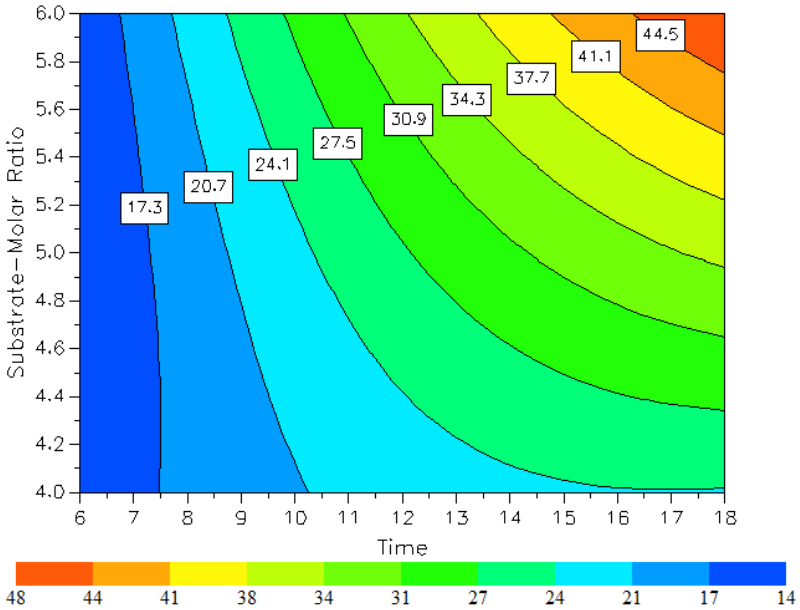

Figure 4.4: Response contour plot for palmitic acid at *sn*-2 position for enrichment with ethyl palmitate.

Figure 4.4 shows that time and substrate molar ratio had positive effect on palmitic acid incorporation to sn-2 position of hazelnut oil at a certain point. But at low levels of susbtrate molar ratio and reaction time, incorporation was independent from these factors. Maximum incorporation was observed at 16-18 h with substrate molar ratio of 5.5-6.0 within the experimental range.

In 2006, Srivastava et al. produced HMFS with LIP1 enzyme using oleic acid or methyl oleate as acyl donor. Substrate molar ratio, time and temperature were the independent variables for this study. Oleic acid incorporation was observed higher when using methyl oleate. With methyl oleate as acyl donor, incorporation of oleic acid was increased with an icrease in reaction time. Up to 1:3 substrate molar ratio positively affected incorporation, above this ratio incorporation began to decrease.

According to the study of Pina-Rodriquez and Akoh (2009a; 2009b) another vegetable oil, amaranth oil, was used for enrichment with palmitic acid at sn-2 position through chemical and enzymatic interesterification reactions. Although the susbtrate molar ratio of amaranth oil to ethyl palmitate and reaction time were selected the same with this study, incoporation of palmitic acid into amaranth oil observed to be higher than incorporation into hazelnut oil. It may be due to the reaction temperature chosen or the nature of the vegetable oil itself. In this study the reaction temperature was selected as 65ºC, while it was 60ºC in their study.

Sahin et al., investigated the effect of substrate molar ratio, time and temperature on GLA and oleic acid incorporation. They observed increase both in GLA and oleic acids with the increase in susbtrate molar ratio and reaction time (2005a). In another study they aimed to incorporate hazelnut oil fatty acids and stearic acid into tripalmitin with different substrate molar ratio, time and temperature. Oleic acid incorporated more as reaction time and substrate molar ratio increased at all temperatures. Also stearic acid incorporation generally increased for all substrate molar ratios when reaction time increased (2005b). In 2006, in another study they produced HMFS containing n-3 fatty acids. Considering contour plots, it was observed that substrate molar ratio did not have a strong effect at shorter reaction times but between 18-30 h incorporation increased with an increase in susbtrate molar ratio.

Pina-Rodriguez and Akoh investigated the effect of reaction time and substrate molar ratio on palmitic acid incorporation keeping reaction temperature constant. According to contour plots, it was observed that reaction time had a positive effect on total palmitic acid incorporation while substrate molar ratio did not have a significant effect at lower and negative effect at higher values (2009a). In another study, they investigated the effect of substrate molar ratio, reaction time and temperature on the incorporation of DHA. Substrate molar ratio was found to affect DHA incorporation positively while time affected the reaciton in a negative way (2009b).

4.4 Model Verification and Gram-Scale Production of Enriched Hazelnut Oil at Optimal Reaction Conditions

According to contour plots, optimal conditions for the enrichment reactions were determined as 17 h with 6 substrate molar ratio, using ethyl palmitate as the raw material. Ethyl palmitate was chosen for the enrichment of hazelnut oil because palmitic acid incoporation to *sn*-2 position of hazelnut oil was observed higher with ethyl palmitate when compared with palmitic acid.

At these optimal conditions gram-scale production was performed. After gram-scale production, FFAs from reaction product were removed by short-path distillation unit. After the removal, FFA value was measured as 0.1%. The results of gram-scale production are mentioned in Table 4.12.

Table 4.12: Fatty acid profiles and *sn*-2 positional analysis of g-scale production of enriched hazelnut oil.

Fatty acid	TAG mol %	*sn*-2 mol %
Palmitic acid (16:0)	63.48±0.03	71.13±6.25
Palmitoleic acid (16:1n-7)	0.12±0.03	-
Stearic acid (18:0)	1.33±0.00	3.55±0.74
Oleic acid (18:1n-9)	31.56±0.02	22.96±5.76
Linoleic acid (18:2n-6)	3.33±0.01	2.37±0.36
Linolenic acid (18:3n-6)	0.12±0.01	-
Arachidic acid (20:0)	0.06±0.00	-

As can be seen in Table 4.12, palmitic acid was enriched in hazelnut oil and the *sn*-2 position was mostly occupied by palmitic acid at 71.13%. The products were analyzed for their palmitic acid in total and at *sn*-2 position. According to these

results, the molecular weight of enriched hazelnut oil was calculated as 835.58±0.02 g.

4.5 Production of Human Milk Fat Substitutes Enriched With DHA and ARA

As a second part of this study, hazelnut oil which was enriched with palmitic acid at *sn*-2 position was further enriched with DHA and ARA at *sn*-1,3 positions. The product having high amounts of palmitic acid at *sn*-2 position was chosen for the second part of the study. The purified product obtained from large scale production was used as the substrate for inclusion of DHA and ARA through enzymatic transesterification reactions. Two different temperatures (50 and 60 ºC), ARA/DHA ratio (2:1 and 3:2), and substrate molar ratio (1:0.1 and 1:0.05) were chosen as variables for transesterification reactions. *Sn*-1,3 specific lipase Lipozyme® RM IM was chosen as the biocatalyst for the reactions. Reactions were run for 3 h in triplicates and average results with standard deviations were calculated. Total palmitic acid at *sn*-2 position and palmitic acid, ARA, DHA amounts of TAG of miligram-scale production are given in Table 4.13 and Table 4.14, respectively.

Table 4.13: Fatty acid profile of miligram-scale production of enriched TAGs.

Temperature (T)	ARA/DHA ratio	Substrate molar ratio (Sr)	Palmitic acid, mol %	ARA, mol %	DHA, mol %
50 ºC	2:1	1:0.1	59.10±0.03	2.96±0.22	1.00±0.19
		1:0.05	61.24±0.35	1.55±0.21	0.48±0.15
50 ºC	3:2	1:0.1	60.20±0.65	2.29±0.08	0.89±0.09
		1:0.05	61.23±0.42	1.38±0.10	0.63±0.05
60 ºC	2:1	1:0.1	58.17±0.41	3.40±0.30	1.46±0.15
		1:0.05	61.37±0.44	1.58±0.24	0.49±0.13
60 ºC	3:2	1:0.1	58.88±0.27	2.72±0.06	1.46±0.10
		1:0.05	61.61±0.42	1.24±0.06	0.51±0.04

Table 4.14: Palmitic acid content at *sn*-2 position of enriched TAGs.

Temperature (T)	ARA/DHA ratio	Substrate molar ratio (Sr)	Palmitic acid, mol %
50 ºC	2:1	1:0.1	60.62±4.04
		1:0.05	58.68±1.09
50 ºC	3:2	1:0.1	56.07±0.96
		1:0.05	58.74±0.31
60 ºC	2:1	1:0.1	65.10±5.49
		1:0.05	62.50±1.01
60 ºC	3:2	1:0.1	62.47±0.51
		1:0.05	64.98±0.87

Statistical analysis was performed using ANOVA and Tukey Simultaneous Tests to analyze the effects of temperature, substrate molar ratio, and ARA/DHA ratio on total palmitic acid, DHA and ARA incorporations. The results of ANOVA analysis are given in Table 4.15, Table 4.16, and Table 4.17, respectively. In addition, ANOVA table for palmitic acid incorporation at *sn*-2 position is given in Table 4.17.

Table 4.15: ANOVA table for total palmitic acid incorporation.

Source	DF	Seq SS	Adj SS	Adj MS	F	*P*
T	1	1.1180	1.1180	1.1180	6.21	0.024
Sr	1	30.9628	30.9628	30.9628	171.93	0.000
DHA/ARA	1	1.5708	1.5708	1.5708	8.72	0.009
T*Sr	1	2.8566	2.8566	2.8566	15.86	0.001
T*DHA/ARA	1	0.0067	0.0067	0.0067	0.04	0.850
Sr*DHA/ARA	1	0.9283	0.9283	0.9283	5.15	0.037
T*Sr*DHA/ARA	1	0.1568	0.1568	0.1568	0.87	0.365
Error	16	2.8814	2.8814	1.1801		
Total	23	40.4814				

[a] P, level of significance
DF: degree of freedom, Seq SS: sequential sum of squares, Adj MS: adjusted mean of squares, F: *F*-value, P: *P*-value, T: temperature, Sr: substrate molar ratio, DHA/ARA: DHA/ARA ratio

According to ANOVA table for total palmitic acid incorporation, temperature, substrate molar ratio and DHA/ARA ratio had significant effect on total palmitic acid incorporation. In addition, "temperature*substrate molar ratio" and "substrate molar ratio*DHA/ARA ratio" were statistically significant interaction terms. Moreover "temperature*DHA/ARA ratio" and "temperature*substrate molar ratio*DHA/ARA ratio" terms were not statistically significant on the incorporation of palmitic acid (α=0.05).

Table 4.16: ANOVA table for DHA incorporation.

Source	DF	Seq SS	Adj SS	Adj MS	F	*P*
T	1	0.31282	0.31282	0.31282	21.45	0.000
Sr	1	2.72027	2.72027	2.72027	186.53	0.000
DHA/ARA	1	0.00135	0.00135	0.00135	0.09	0.765
T*Sr	1	0.49307	0.49307	0.49307	33.81	0.000
T*DHA/ARA	1	0.00015	0.00015	0.00015	0.01	0.920
Sr*DHA/ARA	1	0.02940	0.02940	0.02940	2.02	0.175
T*Sr*DHA/ARA	1	0.02160	0.02160	0.02160	1.48	0.241
Error	16	0.23333	0.23333	0.01458		
Total	23	3.81198				

[a] P, level of significance
DF: degree of freedom, Seq SS: sequential sum of squares, Adj MS: adjusted mean of squares, F: *F*-value, P: *P*-value, T: temperature, Sr: substrate molar ratio, DHA/ARA: DHA/ARA ratio

ANOVA table for DHA incorporation is given in Table 4.16. Temperature, substrate molar ratio and "temperature*substrate molar ratio" were statistically significant whereas DHA/ARA ratio, "temperature*DHA/ARA ratio", "substrate molar ratio*DHA/ARA ratio", and "temperature*substrate molar ratio*DHA/ARA ratio" were not statistically significant (α=0.05).

Table 4.17: ANOVA table for ARA incorporation.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
T	1	0.2128	0.2128	0.2128	6.51	0.021
Sr	1	11.8161	11.8161	11.8161	361.49	0.000
DHA/ARA	1	1.2974	1.2974	1.2974	39.69	0.000
T*Sr	1	0.3601	0.3601	0.3601	11.02	0.004
T*DHA/ARA	1	0.0131	0.0131	0.0131	0.40	0.536
Sr*DHA/ARA	1	0.2688	0.2688	0.2688	8.22	0.011
T*Sr*DHA/ARA	1	0.0081	0.0081	0.0081	0.25	0.626
Error	16	0.5230	0.5230	0.0327		
Total	23	14.4993				

[a] P, level of significance
DF: degree of freedom, Seq SS: sequential sum of squares, Adj MS: adjusted mean of squares, F: *F*-value, P: *P*-value, T: temperature, Sr: substrate molar ratio, DHA/ARA: DHA/ARA ratio

ANOVA table for ARA incorporation given in Table 4.17 shows that, temperature, substrate molar ratio, DHA/ARA ratio, "temperature*substrate molar ratio", and "substrate molar ratio*DHA/ARA ratio" were statistically significant whereas "temperature*DHA/ARA ratio" and "temperature*substrate molar ratio*DHA/ARA ratio" were not statistically significant (α=0.05).

According to Tukey Simultaneous Tests, palmitic acid incorporation decreased with an increase in temperature, substrate molar ratio, and DHA/ARA. DHA incorporation was positively affected by the increase in temperature and substrate molar ratio whereas DHA/ARA ratio did not have significant effect. ARA incorporation was positively affected by an increase in temperature, substrate molar ratio and DHA/ARA ratio.

ANOVA table for palmitic acid incorporation at *sn*-2 position given in Table 4.18 shows that temperature and "substrate molar ratio*DHA/ARA ratio" were statistically significant terms whereas substrate molar ratio, DHA/ARA ratio, "temperature*substrate molar ratio", "temperature*DHA/ARA ratio", and "temperature*substrate molar ratio*DHA/ARA ratio" were not statistically significant.

Table 4.18: ANOVA table for palmitic acid incorporation at *sn*-2 position.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
T	1	163.115	146.324	146.324	24.09	0.000
Sr	1	0.667	0.142	0.142	0.02	0.881
DHA/ARA	1	6.255	7.161	7.161	1.18	0.296
T*Sr	1	0.570	0.223	0.223	0.04	0.851
T*DHA/ARA	1	6.264	6.264	6.264	1.03	0.327
Sr*DHA/ARA	1	32.311	31.547	31.547	5.19	0.039
T*Sr*DHA/ARA	1	0.085	0.085	0.085	0.01	0.908
Error	14	85.020	85.020	6.073		
Total	21	294.287				

[a] P, level of significance

DF: degree of freedom, Seq SS: sequential sum of squares, Adj MS: adjusted mean of squares, F: *F*-value, P: *P*-value, T: temperature, Sr: substrate molar ratio, DHA/ARA: DHA/ARA ratio

According to Tukey Simultaneous Tests, palmitic acid incorporation at *sn*-2 position was decreased with an increase in substrate molar ratio while incorporation was increased with an increase in temperature and DHA/ARA ratio.

4.6 Gram-Scale Production of SLs Enriched with DHA and ARA

In order to perform gram-scale production, 60 ºC reaction temperature, 3:2 ARA/DHA ratio, and 1:0.1 substrate molar ratio were chosen, since total palmitic, DHA and ARA incorporations were higher at these conditions.

Since the targeted aim of this study was to include the product in infant formulae, reaction was performed in a solvent free medium. At the end of the reaction, fatty acid profile and fatty acids at *sn*-2 position were determined.

Palmitic acid, ARA and DHA contents of the product were found as 57.26±0.36%, 2.73±0.04%, and 2.38±0.06%, respectively. Palmitic acid content of the SL at *sn*-2 position was analysed as 66.05±2.23%.

At gram-scale production DHA incorporation was observed to be higher when compared with miligram-scale production. This may be due the reaction condition in which no solvent was used. Even DHA content was increased at gram-scale production, the amount was still lower than the level of ARA content which is recommended.

4.7 Characterization of the Products

4.7.1 Tocopherol contents

Concentrations of the tocopherols were calculated from each peak area determined by the Chemstation software based on the fluorescence response of each tocopherol in a 10 μL injection of the working standard stock solution.

Tocopherol contents of DHASCO®, ARASCO®, hazelnut oil, enriched hazelnut oil, and SL are given in Table 4.19. Also tocopherol contents of hazelnut oil analyzed by different researchers are mentioned in Table 4.20.

Table 4.19: Tocopherol contents of substrates and products.

Product	α-Tocopherol (μg/g)	β-Tocopherol (μg/g)	γ-Tocopherol (μg/g)	δ-Tocopherol (μg/g)
DHASCO®	270	27	345	169
ARASCO®	243	27	429	212
Hazelnut oil	673	30	140	13
Enriched hazelnut oil	46	19	61	12
Structured lipid	84	19	85	23

Table 4.20: Tocopherol contents of hazelnut oil analyzed by different researchers.

Tocopherol (μg/g)	HO[a]	HO[b]	HO[c]
α-Tocopherol	199.1-409.0	398.9	329-448
β-Tocopherol	6.2-16.7	10.1	2-6
γ-Tocopherol	18.6-148.9	106.3	5-47
δ-Tocopherol	1.0-6.6	3.7	0.3-4.5

[a] Savage *et al.*, 1997.
[b] Karabulut *et al.*, 2005.
[c] Benitez-Sànchez *et al.*, 2003.

Hazelnut oil, used in this project had higher tocopherol contents than the hazelnut oils analyzed by different researchers as shown in Table 4.20. As can be seen in Table 4.19, tocopherol content of hazelnut oil decreased during enrichment reactions. The reason for this situation can be related to the high reaction temperature, exposure of the samples to light, and the time applied during reactions (65 ºC and 17 h). In addition during removal of FFAs with short path distillation unit, tocopherols can also be removed. The SL in this study had higher tocopherol content than enriched hazelnut oil. It may be related to the tocopherols present in DHASCO® and ARASCO® substrate oils used in enzymatic reactions.

Pina-Rodriguez and Akoh also observed similar results with the original oil they used and the structured lipid that they produced. In their study the original oil lost 94.5% of tocopherols during reactions and removal of FFAs with short-path distillation unit (2009b). Another report on this issue was mentioned by Maduko *et al.*. they observed approximately 54% tocopherol loss during production of structured lipid from vegetable oil blends with short-path distillation process. They also resported heat and light as responsible factors of tocopherol loss (2008).

4.7.2 Melting profile analysis

Melting profiles of hazelnut oil, DHASCO®, ARASCO®, enriched hazelnut oil and the final SL were determined by DSC instrument (Perkin-Elmer Co., Norwalk, CT). The samples were analyzed in duplicate and average results which are mentioned in Table 4.21 as melting behavior and in Table 4.22 as crystallization behavior were calculated.

Table 4.21: Melting behavior of products.

Product	Onset Point (ºC)	Peak T_{P1} (ºC)	Peak T_{P2} (ºC)	Peak T_{P3} (ºC)	Peak T_{P4} (ºC)	Peak T_{P5} (ºC)	End Point (ºC)	Entalpy (J/g)
DHASCO®	-25.5	~ -24	~ -17	-	-	-	-19.5	3.9
ARASCO®	-31.4	~ -30	~ -27	-	-	-	-27.1	7.5
Hazelnut oil	-31.5	~ -29	~ -13	~ -8	-	-	-0.6	76.6
Enriched hazelnut oil	5.65	~ 6	~ 13	~ 29	~ 40	-	43.0	49.7
Structured lipid	-31.9	~ -30	~ -5	~ 3	~ 24	~ 42	42.1	48.4

Table 4.22: Crystallization behavior of products.

Product	Onset Point (ºC)	Peak T_{P1} (ºC)	Peak T_{P2} (ºC)	Peak T_{P3} (ºC)	Peak T_{P4} (ºC)	Peak T_{P5} (ºC)	End Point (ºC)	Entalpy (J/g)
DHASCO®	19.5	~ 17	-	-	-	-	15.1	-0.5
ARASCO®	-7.0	~ -7	-	-	-	-	-7.1	-0.1
Hazelnut oil	-44.1	~ -47	-	-	-	-	-51.4	-42.5
Enriched hazelnut oil	34.4	~ -32	~ -27	~ 12	~ 4	~ -9	9.3	-56.2
Structured lipid	28.5	~ 26	~ 23	~ 8	~ 3	~ -9	-10.0	-38.3

Chiavaro *et al.* (2008), also analyzed heating and cooling behaviors of hazelnut oil with DSC instrument. According to heating thermograms, onset point and enthalpy values were found as -20.6 ºC and 85.7 J/g respectively. Cooling thermograms show that onset point is -15.8 ºC and enthalpy is 70.3 J/g.

In another study; onset point, peak temperature, end point, and enthalpy were measured as -16.87 ºC, 7.14 ºC, -1.13 ºC, and 78.62 J/g respectively when they are heated from -60 to 25 ºC (Fasina *et al.*, 2006).

Pina-Rodriguez and Akoh, who studied a vegetable oil (amaranth oil) to produce SL containing high amount of palmitic acid at *sn*-2 position, also observed similar DSC thermograms with this study. The original amaranth oil had only one endothermic peak resulting in a narrow melting range. Customized amaranth oil (modified vegetable oil containing high amount of palmitic acid at *sn*-2 position after acidolysis) and SL (DHA incorporated) had a wider melting range compared to original oil (2009b).

Ths SL synthesized had a higher melting point than the original hazelnut oil. A similar result was obtained by Maduko *et al.*. The SL that they studied had higher melting temperature than the original vegetable oils that they used as raw material for their study (2008).

4.7.3 Oxidative stability experiment

The oxidative stabilities of hazelnut oil, DHASCO®, ARASCO®, enriched hazelnut oil and structured lipids were measured with OSI instrument (Omnion Inc., IL) in duplicates and average results of OSI values are mentioned in Table 4.23.

Table 4.23: Oxidative stability index of substrates and products.

Oils	OSI value (h)
DHASCO®	2.73
ARASCO®	2.03
Hazelnut oil	19.63
Enriched hazelnut oil	0.88
Structured lipid	0.80

As can be seen from Table 4.23, hazelnut oil had the higher oil stability index when compared with other oils. Since DHASCO® and ARASCO® have high amounts of PUFAs which cause an increase in susceptibility to oxidation, their oxidative stability values were found to be lower.

Enriched hazelnut oil had even less OSI value than those products that have highly polyunsaturated fatty acids.

OSI value of hazelnut oil is found to be 19.63 h. According to the study of Savage *et al.,* oils extracted from different hazelnut species grown in New Zealand were found to vary between 15.9-25.3 h which are similar to the results obtained in this study (1997).

Although the enriched hazelnut oil obtained by scale-up process includes high amounts of saturated fatty acid, it has a lower stability relative to hazelnut oil, since the rearrangement of lipid structure via lipase catalyzed reactions caused the SL to be more unstable to oxidation than its starting raw material.

In addition, the decrease of oxidative stability after the enzymatic reactions and purification is linked to the loss of tocopherols and phospholipids during the removal of free fatty acids from the reaction mixture. The same situation was observed for the final product enriched with DHA ans ARA.

According to the study of Pina-Rodriguez and Akoh, the similar results were observed with amaranth oil and the SL which was amaranth oil enriched with palmitic acid at *sn*-2 position and also DHA. The OSI value of SL was much more lower than the original oil probably because of interesterification reactions and short-path distillation process (Pina-Rodriguez and Akoh, 2010).

5. CONCLUSIONS

In scope of this study, production of HMFS enriched with palmitic acid at *sn*-2 position and DHA and ARA at *sn*-1,3 positions was performed in two steps.

According to responses, the highest incorporation of palmitic acid was observed when using ethyl palmitate at 18 h reaction time with substrate molar ratio of 5. Incorporation of palmitic acid at these conditions was found as 41.75% and 36.06% of it was located at sn-2 position of the TAG.

At optimal conditions generated by the software (17 h with substrate molar ratio of 6) gram-scale production of enriched hazelnut oil was performed using stirred-batch bioreactor. The results were observed as 63.48% and 71.13% for total palmitic acid and palmitic acid at sn-2 position, respectively.

Incorporation at gram-scale production was higher than expected values. The reason for this may be due to the solvent free reaction environment used in this production.

In the second part of the study, enriched hazelnut oil obtained from the first part was used as one of the substrates. In the presence of Lipozyme® RM IM, DHASCO® and ARASCO® were used to incorporate DHA and ARA, respectively. Reaction time was selected as 50 ºC and 60ºC, ARA/DHA ratio was selected as 2:1 and 3:2, substrate molar ratio was selected as 1:0.1 and 1:0.05. Reaction time was set constant to 3 h. For lower substrate molar ratios, incorporation of DHA and ARA were lower. In addition, results obtained at 50ºC were lower than the results obtained at 60ºC.

Lowest incorporation of DHA was observed at 50ºC with substrate molar ratio of 1:0.1 and ARA/DHA ratio of 2:1 while highest incorporation was measured at 60ºC with substrate molar ratio of 1:0.1 and ARA/DHA ratio of both 2:1 and 3:2.

Lowest incorporation of ARA was observed at 60ºC with substrate molar ratio of 1:0.05 and ARA/DHA ratio of 3:2 whereas highest incorporation was measured at 60ºC with substrate molar ratio of 1:0.1 and ARA/DHA ratio of 2:1.

Based on these findings, by considering both ARA and DHA incorporation, gram-scale production for this part was performed at 60ºC with substrate molar ratio of 1:0.1 and ARA/DHA ratio of 3:2. Structured lipid, obtained as a result of this reaction included 57.26% total palmitic acid, 2.73% ARA and 2.38%DHA contents.

Characterization studies included analysis of tocopherol content, melting profile, and oxidative stability.

Tocopherol content of structured lipid was found lower than substrates but higher than enriched hazelnut oil. Melting and crystallization graphs of DSC instrument showed that enriched hazelnut oil and structured lipid had a wider melting and crystallization range. The oxidative stability index for enriched hazelnut oil was close to the value of structured lipid while hazelnut oil was the most stable oil.

As a result of this study, human milk fat substitute containing high amounts of palmitic acid at *sn*-2 position and also DHA and ARA at *sn*-1,3 positions was obtained. It is believed that this SL can be used in infant formulas providing similar advantages as human milk fat and these SLs could deliver the health benefits associated with DHA and ARA.

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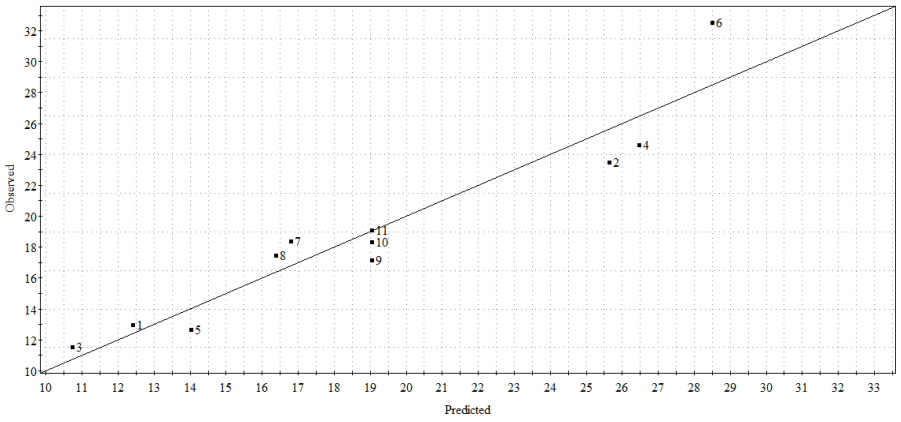
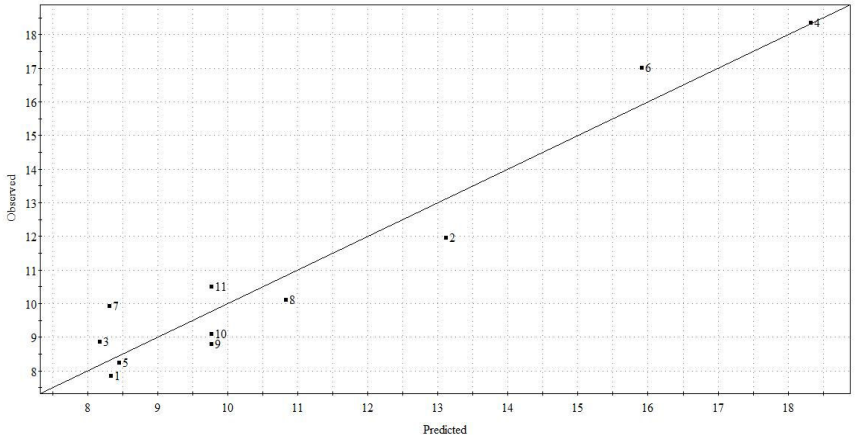
APPENDICES

APPENDIX A.1 : Observed vs. Predicted Plots

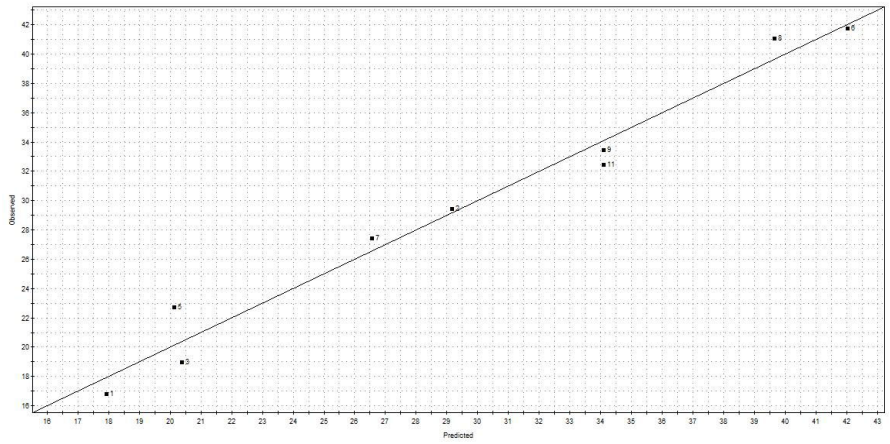
APPENDIX A.1



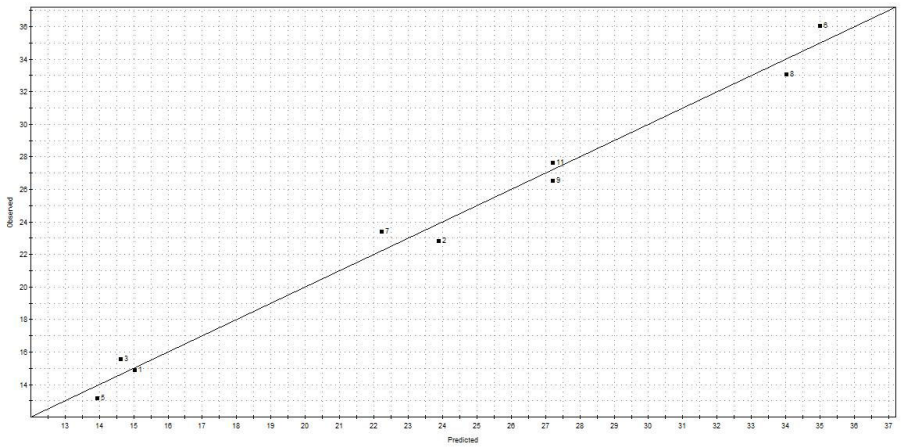
(a)



(b)



(c)



(d)

Figure A.1: Observed vs. predicted plots (a) for total palmitic acid incorporation with palmitic acid as acyl donor, (b) palmitic acid incorporation at *sn*-2 position with palmitic acid as acyl donor (c) for total palmitic acid incorporation with ethyl palmitate as acyl donor, (d) palmitic acid incorporation at *sn*-2 position with ethyl palmitate acid as acyl donor

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Publications:

- **Turan, D.,** Yuksel, A., Sahin, N., 2010. "The Importance of Docosahexaenoic Acid (DHA) and Arachidonic Acid (ARA) in Infant Nutrition and Their Use in Human Milk Fat Analogs", The 7th International Nutrition and Dietetics Congress, 14-18 April, Istanbul, Turkey.
- Yuksel, A., **Turan, D.,** Sahin, N., 2010. "Structured Lipids Designed for Infant Nutrition", The 7th International Nutrition and Dietetics Congress, 14-18 April, Istanbul, Turkey.