ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

PRODUCTION AND ENCAPSULATION OF MLM-TYPE STRUCTURED LIPIDS AND APPLICATION IN KEFIR PRODUCT

Ph.D. THESIS

Alev YÜKSEL BİLSEL

Department of Food Engineering

Food Engineering Programme

JULY 2019

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

PRODUCTION AND ENCAPSULATION OF MLM-TYPE STRUCTURED LIPIDS AND APPLICATION IN KEFIR PRODUCT

Ph.D. THESIS

Alev YÜKSEL BİLSEL
(506112501)

Department of Food Engineering

Food Engineering Programme

Thesis Advisor: Assoc. Prof. Dr. Neşe ŞAHİN YEŞİLÇUBUK

JULY 2019

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

MLM-TİPTE YAPILANDIRILMIŞ YAĞLARIN ÜRETİMİ, ENKAPSÜLASYONU VE KEFİR ÜRÜNÜNE UYGULANMASI

DOKTORA TEZİ

Alev YÜKSEL BİLSEL
(506112501)

Gıda Mühendisliği Anabilim Dalı

Gıda Mühendisliği Programı

Tez Danışmanı: Doç. Dr. Neşe ŞAHİN YEŞİLÇUBUK

TEMMUZ 2019

Alev YÜKSEL BİLSEL, a Ph.D. student of ITU Graduate School of Science Engineering and Technology student ID 506112501, successfully defended the thesis/dissertation entitled "PRODUCTION AND ENCAPSULATION OF MLM-TYPE STRUCTURED LIPIDS AND APPLICATION IN KEFIR PRODUCT", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Assoc. Prof. Dr. Neşe ŞAHİN YEŞİLÇUBUK**
İstanbul Technical University

Jury Members : **Assist. Prof. Dr. Dilara NİLÜFER ERDİL**
Istanbul Technical University

Assist. Prof. Dr. Aslı CAN KARAÇA
Istanbul Technical University

Assist. Prof. Dr. Birsen DEMİREL
Bilgi University

Assist. Prof. Dr. Fatma CEBECİ
Bayburt University

Date of Submission : 28 June 2019
Date of Defense : 24 July 2019

To my family,

FOREWORD

I would like to express my thanks and sincere gratitude to my dear supervisor Assoc. Prof. Dr. Neşe Şahin Yeşilçubuk for her patience, guidance and support during my study. I would like to offer my special thanks to Prof. Dr. Selma ŞAHİN and Assist. Prof. Dr. Dilara NİLÜFER ERDİL for their contributions during my thesis.

I also would like to thank Prof. Dr. Hale GÜRBÜZ, Prof. Dr. Fatma Neşe KÖK, Dr. Abdulhalim KILIÇ and Dr. Ayşe SAYGÜN for their insight and help on my experiments.

I appreciate all research assistants and administrative staff especially Nalan DEMİR at Food Engineering Department for their help during my laboratory studies.

I would like to thank my parents Zübeyde and Muhittin YÜKSEL, my brothers Sadık and Nurettin YÜKSEL for their endless love and every kind of support throughout my life. Lastly, I would like to present my special thanks to my beloved husband Ongun BİLSEL who did not leave me alone during my thesis study. I feel so lucky to have you in my life.

June 2019 Alev YÜKSEL BİLSEL

TABLE OF CONTENTS

Page

ABBREVIATIONS

2-MAG : 2-Monoacylglycerol
AA : Arachidonic Acid
ALA : Alpha (or α-) Linolenic Acid
ANOVA : Analysis of Variance
AOCS : American Oil Chemists' Society
BHT : Butylated Hydroxytoluene
CCC : Central Composite Circumscribed Design
CFU : Colony Forming Unit
CY : Coacervate Yield
DF : Degree of Freedom
DGLA : Dihomo-Gamma-Linolenic Acid
DSC : Differential Scanning Calorimetry
DHA : Docosahexaenoic Acid
EE : Encapsulation Efficiency
EPA : Eicosapentaenoic Acid
FAME : Fatty Acid Methyl Esters
FA : Fatty Acid
FFA : Free Fatty Acid
FID : Flame-Ionization Detector
GA : Gum Arabic
GE : Gelatin
GFS : Gastric Fluid Solution
GLA : Gamma- (or γ-) Linolenic Acid
GLC : Gas-Liquid Chromatography
IFS : Intestinal Fluid Solution
LA : Linoleic Acid
LAB : Lactic Acid Bacteria
LCFAs : Long Chain Fatty Acids
MAG : Monoacylglycerol
MCFAs : Medium Chain Fatty Acids

MS : Mean Squares
n-3 : Omega-3
n-6 : Omega-6
n-9 : Omega-9
OA : Oleic Acid
***p*-AV** : *p*-anisidine Value
PDI : Polydispersity Index
PV : Peroxide Value
PUFA : Polyunsaturated Fatty Acids
RSM : Response Surface Methodology
SDA : Stearidonic Acid
SDS : Sodium Dodecyl Sulphate
Sr : Substrate Molar Ratio
SS : Sum of Squares
SEM : Scanning Electron Microscope
SL : Structured Lipid
TAG : Triacylglycerol
TG : Transglutaminase
TLC : Thin Layer Chromatography
TOTOX : Total Oxidation Value

SYMBOLS

a*	: Red/green (color parameter)
b*	: Blue/yellow (color parameter)
β_0	: Intercept
β_i	: Linear Term
β_{ii}	: Quadratic Term
β_{ij}	: Interaction Regression Coefficients
ΔE	: Total Color Difference
L*	: Lightness/darkness (color parameter)
m1, m2, m3	: Mass of the Total Oil
M_{lipid}	: Molecular Weight of SL
p	: Probability (statistical analysis)
R^2	: Coefficient of Determination
Y	: Response
X_i , X_j	: Independent variables
Σ	: **S**um

LIST OF TABLES

Page

LIST OF FIGURES

Page

PRODUCTION AND ENCAPSULATION OF MLM-TYPE STRUCTURED LIPIDS AND APPLICATION IN KEFIR PRODUCT

SUMMARY

Lately, it is possible to produce structured lipids (SLs) with improved functional properties and some health benefits. Structured lipids are generally defined as triacylglycerols (TAGs) that the fatty acid composition and/or their positional distribution in glycerol molecules have been changed via chemical and/or enzymatic reactions and/or by genetic modifications. Today's scientific improvements enable SLs to be suitable for medical and nutritional applications. Also, they can be designed to meet the growing need for healthier foods and to prevent obesity, cancer and cardiovascular disease.

Enzymatic production of structured lipids (SLs) including both medium chain fatty acids (MCFAs) and long chain fatty acids (LCFAs) in one triacyglycerol molecule (MLM-type SLs) has gained attention of researchers since they can be designed for special health requirements of patients and meet the specification of consumers who desire functional food products with health-enhancing and disease-preventing properties. In addition, MLM-type SLs is also an attractive issue of the researchers since they have been shown tumor suppressing effects according to the findings of some studies. Bioactive food components such as lipids are sensitive to food processing or storage conditions, also during digestion process, their bioavailibity and bioaccessibility may be decreased. To overcome such problems, encapsulation of these compounds is important in order to protect their functionality and also food quality.

Considering the above issue, the aim of this thesis is (i) to produce MLM-type SLs from tricaprylin and Echium oil fatty acids (FAs) (ii) to evaluate anti-tumorigenic effects of produced SLs and Echium oil via cell culture studies (iii) to encapsulate synthesized SLs by complex coacervation and to charactarize the capsules (iv) to encapsulate synthesized SLs by complex coacervation with/without transglutaminase, assess oxidative stability of the capsules, conduct an in vitro gastrointestinal digestion in order to evaluate release profiles of the encapsulated SLs and use them in a food application.

In order to accomplish these aims, four different experimental studies (Chapters 3-6) were conducted during the thesis study. First of all, MLM-type SL were enzymatically produced and the reaction conditions were optimized in Chapter 3. Then, in Chapter 4, possible anti-tumorigenic activity of the SL and *Echium* oil on MCF-7 human breast cancer cell line was investigated. In the next step, SLs were encapsulated by complex coacervation of gelatin and gum arabic. Also, particle size, melting behaviour and structural characteristics of the capsules were determined (Chapter 5). Lastly, oxidative stability of the encapsulated SLs were assessed. Besides, the capsules were added into a probiotic kefir product and an *in vitro*

digestion model was conducted to identify oil release behaviour of the fortified kefir and freeze-dired capsules in Chapter 6.

In this concept, first step of this study (Chapter 3) aimed at production of MLM-type SLs consisting of MCFA (caprylic acid, C8:0) from tricaprylin at *sn*-1,3 positions, and long chain FAs originated from *Echium* oil at *sn*-2 position, via enzymatic acidolysis reactions with the use of commercially immobilized Lipozyme®RM IM lipase enzymes. In the study, effects of reaction temperature (50-60 ºC), reaction time (6-12 h) and substrate molar ratio (3-6 mol/mol; total free fatty acids/tricaprylin) on incorporation of SDA were investigated. Optimum reaction conditions were determined by response surface methodology (RSM) by using central composite circumscribed design (CCC) with five levels. Optimum temperature, time and substrate molar ratio obtained from the models were 60 ºC; 6 h, 6 mol/mol, respectively. Furthermore, SLs with 6.2% SDA content at *sn*-2 position were produced by scaling up process. SL was obtained with nearly 78-79% of long-chain fatty acids at *sn*-2 position. In addition, SLs were also investigated for the melting characteristics by using differential scanning calorimetry (DSC). According to melting profile analysis, the melting peaks of tricaprylin and *Echium* oil were sharper and narrower while the SL had more broadened peaks.

In the second part of the study (Chapter 4), anti-tumorigenic activity of synthesized SLs (Chapter 3) was evaluated by human breast cancer cell lines (MCF-7) to unravel the possible effects of long chain fatty acids on breast cancer. This part of the study is important since cancer preventive/suppressive activity of EPA and DHA have been shown in numerous studies. But, effects of SLs produced from *Echium* oil FAs on breast cancer cells need to be investigated. For this reason, we aimed at determining the anti-tumorigenic activity of SL containing both n-3 and n-6 FAs via cell viability analysis of MCF-7 breast cancer cells. The cells were treated with SL and *Echium* oil at 30 and 120 µg/ml concentrations for 24 h. Cell viability test results showed that oil treatment reduce the growth of MCF-7 cancer cells. It can be said that extent of cell growth is nearly the same for both of SL and *Echium* oil. 30 µg/ml of *Echium* oil inhibits MCF-7 cell growth about 17%, on the other hand, SL at the same concentration suppressed cell proliferation about 10% at the same concentration.

In Chapter 5, structured lipids containing 78% of long-chain fatty acids (FAs) at *sn*-2 position and medium chain FAs at *sn*-1,3 positions were encapsulated by complex coacervation method using gelatin and gum arabic. This study was aimed to assess the effects of different wall material concentration (1-2%), core:wall ratio (1:1 and 2:1) and homogenization rate (7400, 10000 and 15000 rpm) on encapsulation of the SL. According to the results, 84.11 ± 0.77% was found as the highest encapsulation efficiency that was obtained in gelatin 2% (w/v), gum arabic 2% (w/v), 1:1 core:wall ratio (w/w) and at 15000 rpm homogenization rate. In addition, particle size for the coacervates ranged from 19 to 263 nm. Moreover, the samples showed homogeneous size distribution pattern. Furthermore, electron microscope (SEM) and differential scanning calorimetry (DSC) were used to unravel morphological characteristics and thermal behavior of the capsules, respectively.

In the next part of the study (Chapter 6), the objective of the study was to encapsulate structured lipids (SLs) by complex coacervation of gelatin and gum arabic with or without using transglutaminase enzymes and to develop a functional kefir product via the addition of encapsulated SLs in the form of suspension and freeze-dried coacervates. In order to evaluate the oxidative stability of the encapsulation products,

the samples were stored for 30 days. According to oxidative stability tests, coacervate solutions were found more liable to lipid oxidation compared to freeze-dried capsules. In addition, enzyme treated samples had higher oxidation levels when compared to non-enzyme treated ones. Moreover, kefir was produced via traditional methods and then fortified with complex coacervation products. During the storage of the fortified kefir product for 10 days at 4 °C, the pH value was decreased, and titratable acidity slightly increased during the storage period. Also, fortified kefir sample and the freeze-dried capsules were subjected to an *in vitro* controlled release study. The findings of the release study showed that kefir had no significant matrix effect on oil release from the freeze-dried capsules ($p > 0.05$).

In the final part of the study, the general discussions and concluding remarks are given in Chapter 7 along with future prospects and challenges.

MLM-TİPTE YAPILANDIRILMIŞ YAĞLARIN ÜRETİMİ, ENKAPSÜLASYONU VE KEFİR ÜRÜNÜNE UYGULANMASI

ÖZET

Son zamanlarda, fonksiyonel özellikleri geliştirilmiş ve sağlık açısından faydalı bazı özelliklere sahip olan yapılandırılmış yağların (YY'ler) üretimi mümkün olmaktadır. Yapılandırılmış yağlar, genel olarak, yağ asidi bileşimi ve/veya gliserol moleküllerindeki poziyonel dağılımları kimyasal ve/veya enzimatik reaksiyonlar ve/veya genetik modifikasyonlar yoluyla değiştirilen triaçilgliseroller (TAG'lar) olarak tanımlanmaktadır. Günümüzdeki bilimsel gelişmeler YY'lerin tıbbi ve beslenme alanları için uygun olmasını sağlamaktadır. Ayrıca, YY'ler daha sağlıklı gıdalara karşı giderek artan ihtiyacı karşılamak ve obezite, kanser, kardiyovasküler vb.hastalıkları önlemek için de tasarlanabilmektedir.

Hastaların özel sağlık ihtiyaçlarına göre tasarlanabilmesi ve hastalık önleyici ve sağlığa faydalı fonksiyonel gıda ürünleri talep eden tüketicilerin beklentilerini karşılayabilmesi açısından bir triaçilgliserol molekülünde hem orta zincirli yağ asitleri (OZYA) hem de uzun zincirli yağ asitleri (UZYA) içeren yapılandırılmış yağların (YY) enzimatik sentez ile üretimi araştırmacıların dikkatini çekmiştir. Ek olarak, bu tipteki YY'lerin tümör baskılayıcı etkileri bazı çalışmalar ile kanıtlandığı için araştırmacılar için çekici bir konu haline gelmiştir. Yağlar gibi biyoaktif gıda bileşenleri, gıda işleme ve depolama koşullarına karşı hassastırlar. Ayrıca, bu süreçte biyoyararlık ve biyoerişilebilirlik değerleri azalabilir. Bu gibi sorunların üstesinden gelmek için, bu tip bileşenlerin enkapsüle edilmesi, onların işlevselliklerinin ayrıca gıda kalitesinin koruması açısından önemlidir.

Yukarıda bahsedilen konuyu dikkate alarak, bu tez çalışmasında (i) trikaprilin ve *Echium* yağında bulunan yağ asitleri kullanarak MLM-tipteki YY'lerin üretimi (ii) üretilen YY ve *Echium* yağının hücre kültürü çalışmaları ile anti-tümörijenik etkilerinin değerlendirilmesi (iii) sentezlenen YY'lerin kompleks koaservasyon yöntemiyle enkapsülasyonu ve elde edilen kapsüllerin karakterize edilmesi (iv) YY'lerin kompleks koaservasyon yönetimi ile transglutaminaz enzimi kullanılarak veya kullanılmadan enkapsüle edilmesi, kapsüllerin oksidatif stabilitesinin değerlendirilmesi, enkapsüle edilen YY'lerin salım profilini değerlendirmek için *in vitro* gastrointestinal sindirimin gerçekleştirilmesi ve gıda uygulamasında kullanımı amaçlanmaktadır.

Bu amaçları gerçekleştirmek için dört farklı deneysel çalışma (Bölüm 3-6) gerçekleştirilmiştir. İlk olarak, 3. Bölümde, MLM- tipteki YY enzimatik olarak üretilmiş ve reaksiyon koşulları optimize edilmiştir. Daha sonra, 4. Bölümde, üretilen YY ve *Echium* yağının MCF-7 insan meme kanseri hücre hattında olası anti-tümörijenik aktivitesi araştırılmıştır. Bir sonraki adımda, YY'ler jelatin ve arap zamkının kompleks koaservasyonu ile enkapsüle edilmiştir. Ayrıca, partikül boyutu, erime davranışı ve kapsüllerin yapısal özellikleri belirlenmiştir (Bölüm 5). Son olarak, 6. Bölüm'de, enkapsüle edilen YY'lerin oksidatif stabilitesi

değerlendirilmiştir. Ayrıca, kapsüller probiyotik bir kefir ürününe eklenmiş ve bu kefir ürünü ve dondurularak kurutulmuş kapsüllerin yağ salım davranışını belirlemek için *in vitro* sindirim modeli uygulanmıştır.

Bu çalışmanın ilk basamağı (Bölüm 3), trikaprilin ve *Echium* yağının yağ kaynağı olarak kullandıldığı, Lipozyme® RM IM lipaz enzimlerince katalizlenen, *sn*-1,3 pozisyonlarında OZYA ve *sn*-2 pozisyonunda UZYA içeren YY'lerin üretimi amaçlamaktadır. Çalışmada, reaksiyon sıcaklığının (50-60 ºC), reaksiyon süresinin (6-12 saat) ve substrat molar oranının (3-6 mol / mol; toplam serbest yağ asitleri / trikaprilinin) SDA katılımı üzerindeki etkileri araştırılmıştır. Optimum reaksiyon koşulları, beş seviyeli Merkezil Bileşik Deney Tasarım (CCC) kullanılarak tepki-yüzey yöntemi (RSM) ile belirlenmiştir. Modellerden elde edilen optimum sıcaklık, zaman ve substrat molar oranı sırasıyla 60 °C; 6 saat, 6 mol / mol'dür. Ayrıca, büyük ölçekte üretilen YY'ler *sn*-2 pozisyonunda %6.2 SDA içeriğine sahiptir. Çalışmada elde edilen YY'ler *sn*-2 pozisyonunda yaklaşık %78-79 UZYA içermektedir. Bunlara ek olarak, diferansiyel tarama kalorimetrisi kullanılarak YY'lerin erime davranışları incelenmiştir. Erime profili analizine göre, trikaprilin ve *Echium* yağının erime noktaları daha keskin ve daha dar olmakla birlikte, YY'nin ise daha geniş erime davranışına sahip olduğu gözlenmiştir.

Çalışmanın ikinci aşamasında (Bölüm 4), sentezlenen YY'lerin (Bölüm 3) anti-tümörijenik aktivitesi ve uzun zincirli yağ asitlerinin meme kanseri üzerindeki olası etkileri insan meme kanseri hücre hattı kullanılarak değerlendirilmiştir. EPA ve DHA'nın kanser önleyici/baskılayıcı aktivitesi birçok çalışmada gösterildiği için çalışmanın bu kısmında önemlidir. Fakat, *Echium* yağından üretilen YY'lerin meme kanseri üzerindeki etkilerinin araştırılması gerekmektedir. Bu nedenle, hem n-3 hem de n-6 YA'ları içeren YY'nin anti-tümörijenik aktivitesi MCF-7 hücre hattı kullanılanarak araştırılmıştır. Hücreler, 24 saat boyunca 30 ve 120 µg/ml konsantrasyona sahip YY ve *Echium* yağı ile muamele edilmiştir. Hücre canlılık analizine göre kullanılan yağların MCF-7 kanser hücrelerinin büyümesini azalttığı gösterilmiştir. Kanserli hücrelerdeki büyüme oranının hem YY hem de *Echium* yağı muamelesi için neredeyse aynı olduğu söylenebilir. 30 µg/ml *Echium* yağı, MCF-7 hücre büyümesini yaklaşık % 17 azaltırken, aynı konsantrasyondaki YY ise hücre proliferasyonunu yaklaşık % 10 baskılamıştır.

Bölüm 5'te, *sn*-2 pozisyonunda %78 oranında UZYA ve *sn*-1,3 pozisyonlarında OZYA içeren YY'ler, duvar materyali olarak jelatin ve arap zamkının kullanıldığı kompleks koaservasyon yöntemi ile enkapsüle edilmiştr. Bu çalışmanın amacı, duvar materyali konsantrasyonu (% 1-2), çekirdek:duvar oranı (1:1 ve 2:1) ve homojenizasyon hızı (7400, 10000 ve 15000 rpm) gibi deneysel parametrelerinin enkapsülasyon işlemi üzerindeki etkilerinin araştırılmasıdır.. En yüksek enkapsülasyon verimi (% 84,11 ± 0,77) % 2 jelatin (w/v), % 2 arap zamkı (w/v), 1:1 çekirdek: duvar oranı (w/w) ve 15000 rpm homojenizasyon hızına sahip deney koşullarında elde edilmiştir . Buna ek olarak, koaservatların partikül büyüklüğü 19 ile 263 nm arasında değişmektedir. Ayrıca, elde edilen numunelerin küçük polidispersite indeksi (PDI) değerlerine sahip olduğu, yani homojen bir dağılım gösterdikleri açığa çıkmıştır. Kapsüllerin morfolojik özellikleri taramalı elektron mikroskobu (SEM) ve ısıl davranışları ise diferansiyel taramalı kalorimetri (DSC) ile belirlenmiştir.

Çalışmanın son kısmında (Bölüm 6), jelatin ve arap zamkı kullanılarak YY'lerin enkapsüle edilmesi ve bu enkapsüle edilen YY'lerin süspansiyon veya dondurularak

kurutulmuş halde kefire eklenerek fonksiyonel bir kefir ürününün geliştirilmesi amaçlanmıştır. Enkapsüle edilen YY'lerin oksidatif stabiliteleri 30 günlük soğuk depolama süresince değerlendirilmiştir. Elde edilen veriler, koaservat süspansiyonlarının dondurularak kurutulmuş kapsüllere kıyasla yağ oksidasyonuna daha duyarlı olduğunu göstermiştir. Geleneksel yöntemle üretilen kefir örnekleri kompleks koaservasyon ürünleri zenginleştirilmiş ve ardından 4 °C'de 10 gün boyunca depolanmıştır. Depolama süresince numunelerin pH değerleri azalırken, titre edilebilir asitlik miktarı artış göstermiştir. Ayrıca, dondurularak kurutulmuş kapsülleri içeren takviye edilmiş kefir numunesiyle *in vitro* kontrollü salım çalışması gerçekleştirilmiştir. Elde edilen sonuçlara göre, istatistiksel açıdan dondurularak kurutulmuş kapsüllerden yağ salınım oranı üzerinde kefirin bir matriks etkisi olmadığı tespit edilmiştir ($p > 0.05$).

Çalışmanın son bölümünde ise (Bölüm 7), genel tartışmalar ve sonuçlar, gelecekteki beklentiler ve zorluklar belirtilmiştir.

1. INTRODUCTION

Dietary fatty acids are important macronutrients for humans. Especially, omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have many beneficial effects on human health from cardiovascular disease to cancer. Thus, health authorities recommend intake of omega-3 fatty acids for a healthy living. However, main dietary sources of EPA and DHA are fish oils that may also contain environmental pollutants. Besides, fish sources are getting decreased in the last decades due to over-fishing and environmental problems. This situation causes to research for alternative sources of omega-3 fatty acids. At this point, stearidonic acid (SDA) originated from plants has the potential of being sustainable and vegetarian EPA source since it is an intermediate metabolite in the conversion pathway of α-linolenic acid (ALA) to EPA and DHA. Besides, among the fatty acids, medium chain fatty acids (MCFAs) have many health benefits such as being rapid energy source and being not accumulated as body fat. MCFAs are important components of parenteral enteral nutrition for those who need special lipid formulations to overcome their lipid malabsorption disorders such as Crohn's disease, cystic fibrosis, colitis and premature birth.

Health benefits of medium chain fatty acids (MCFAs) and long chain fatty acids (LCFAs) are well documented and today's biotechnological improvements allow the production of structured lipids (SLs) including both of these fatty acids in one TAG molecule. Enzymatic production of MLM-type SLs has been studied by using different substrate sources. But among them, there is no study considering the production, encapsulation and food application of MLM-type SL with long chain fatty acids derived from plant oil at *sn*-2 position of the TAG molecule as proposed in this study.

The main objectives of this thesis can be listed as:

- To produce MLM type SL containing SDA as well as long chain fatty acids found in *Echium* oil for specific functional and health purposes (Chapter 3),

- To determine anti-proliferative effects of SLs on human breast cancer cell line (Chapter 4),
- To encapsulate SLs by complex coacervation of gelatin and gum arabic and to perform their characterization studies (Chapter 5),
- To determine oxidative stability of the capsules produced with/without use of transglutaminase, to identify *in vitro* release profile of the capsules and their usage in the food application (Chapter 6).

The schematic outline of this Ph.D. research is given in Figure 1.1.

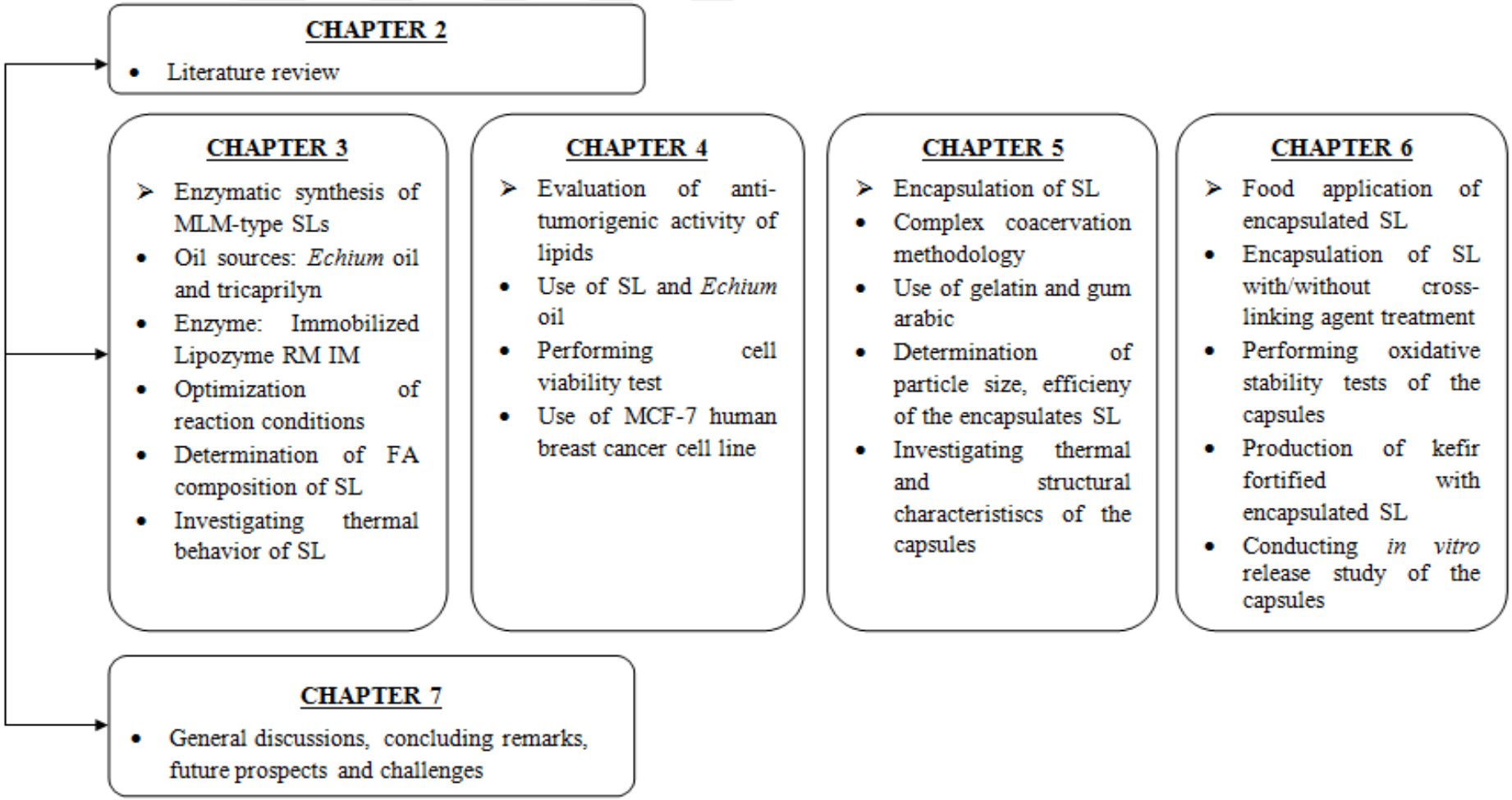


Figure 1.1 : The schematic outline of this Ph.D. research.

2. LITERATURE REVIEW

2.1 Structured Lipids

Fats and oils originated from plant and animals are very important nutrient sources for human diet since it provides energy, essential fatty acids and fat-soluble vitamins. Besides, lipids in diets are crucial to humans for optimal growth and development and to maintain healthy being (Zam, 2015). Triacylglycerols (TAGs) are the main parts (~95%) of the fats and oils that are specific for each fat and oil. The structure of a TAG consists of three fatty acid esterified at different stereospecific positions (*sn*-1, *sn*-2 and *sn*-3) of a glycerol molecule as shown in Figure 2.1 (Gurr *et al*., 2016).

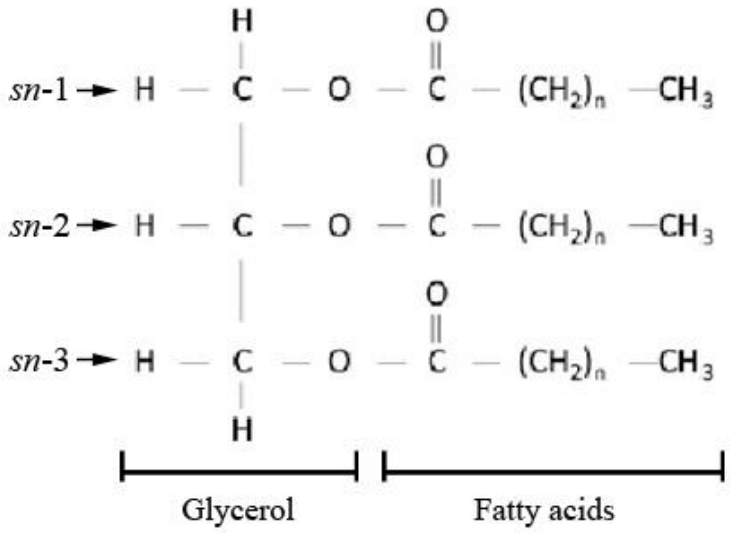


Figure 2.1 : Structure of a triacylglycerol molecule (Adapted from Gurr *et al*., 2016).

TAGs in fats and oils have different positional distribution of fatty acids which enable the lipids to have their own characteristic physical properties. In addition to physical properties, health benefits and also metabolic fate of TAG molecule are also determined by fatty acid type and position at TAG molecule (Jala and Kumar, 2018; Willis and Marangoni, 2007). Lately, researchers pay more attention to improve physical properties of lipids (e.g melting behavior) and its nutritional value by changing fatty acid composition, and also to obtain structured lipids (SLs) designed for new applications. Structured lipids also referred as new generation of lipids,

functional foods, designer foods, engineered foods, nutritional foods, that are produced via chemical and/or enzymatic synthesis, genetic engineering, partial hydrogenation and fractionation by changing the fatty acid composition and/or its positional distribution in the glycerol molecule (Osborn and Akoh, 2002; Zam, 2015).

2.1.1 Enzymatic synthesis of structured lipids

Current consumer demands on natural products, and also environmental and health concerns make more preferable green chemistry and natural processes that are using natural ingredients. Recently, synthesis of structured lipids via enzymatic reactions is becoming a popular topic of lipid modification in which lipases are predominantly used as reaction catalysts. Lipases are biocatalysts, so the processes considered as natural within the use of these enzymes. For this reason, these biocatalysts are more preferable than chemical catalysts in the modification of lipid processes which consider the consumer demands on natural food products (Akoh *et al*., 2002; Jala and Kumar, 2018).

Although, chemical synthesis is a relatively cheap methodology. Due to the its non-selectivity of the reaction, positional distribution of fatty acids in the final product cannot be controlled. Besides, since chemical reaction conditions such as temperature, pH and pressure are extremely high, many problems may be occurred (e.g. environmental pollution, detrimental changes in products, etc.) during the process. However, enzymatic interesterification operates under mild reaction conditions which results with less environmental pollution in comparison with chemical interesterification (Jala and Kumar, 2018; Kim and Akoh, 2015). In addition, enzymatic process has many advantages over chemical process owing the use of lipases to catalyze the reactions as shown in Table 2.1.

Table 2.1: Comparison of enzymatic and chemical methods for the synthesis of sTAG* (Bornscheuer *et al*., 2003).

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

*sTAG:Structured triacylglycerols, PUFA: Polyunsaturated fatty acids

Among the interesterification reactions, transesterification, acidolysis and alcoholysis reactions are of commercial interest. Figure 2.2. shows representative lipase catalysed transesterification, acidolysis and alcoholysis reactions (Willis and Marangoni, 2007).

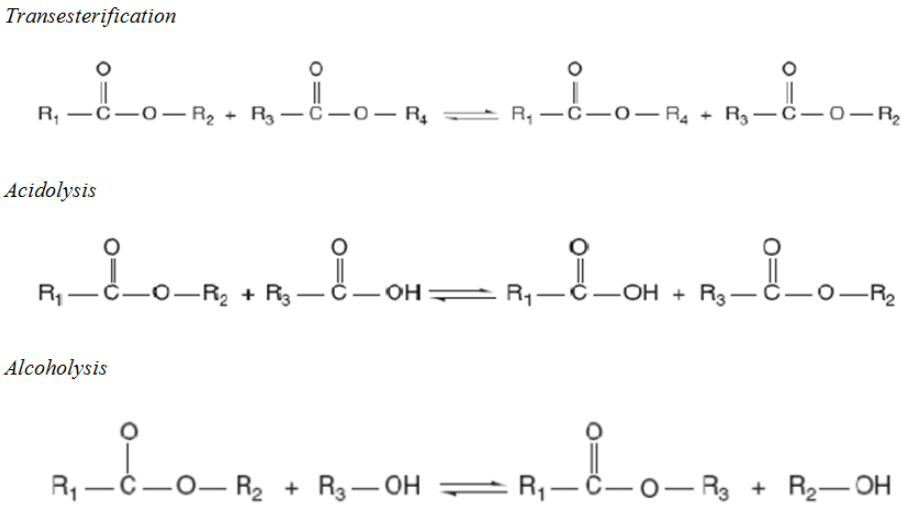


Figure 2.2 : Lipase catalysed transesterification, acidolysis and alcoholysis reactions (Adapted from Willis and Marangoni, 2007).

Transesterification occurs by the exchange of acyl groups between the two esters, while acidolysis occurs as the transfer of an acyl group between an acid and an ester as represented. In other words, the incorporation of new fatty acids into the TAG structure. Alcoholysis is an esterification reaction between an alcohol and an ester (Willis and Marangoni, 2007).

2.1.2 Lipase enzymes

Lipases (EC 3.1.1.3, triacylglycerol acylhydrolase) are enzymes that catalyze the hydrolysis of lipids and are naturally found in plants, animals and microorganisms. Lipases originated from bacteria and molds have many advantages such as having high activity and stability, being available on a large scale and catalyzing various reactions (Ferreira-Dias *et al*., 2013; Goswami and Stewart, 2016).

Lipase enzymes catalyze the reactions, such as transesterification (ester interchange), acidolysis and alcoholysis that are used in the production of structured lipids. In these reactions, positional distribution of fatty acids of the final products can be controlled due to the positional selectivity of lipases (Ferreira-Dias *et al*., 2013; Osborn and Akoh, 2002). Moreover, lipases have mainly three types of specificity: positional specifity, stereospecificity and fatty acid specificity (Goswami and Stewart, 2016). Substrate specificity and regiospecificity in the hydrolysis of TAGs, catalyzed by some lipases is shown in Table 2.2 (Weber and Mukherjee, 2008).

Table 2.2: 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 fatty acids.

Specificity of the lipases are the main advantages of enzymatic interesterification reactions over chemical interesterification reactions since the final products of enzymatic interesterification are the targeted specific products with less formation of by-products (Ferreira-Dias *et al*., 2013; Willis and Marangoni, 2007). Besides, properties of the lipases can be modified via genetic engineering, directed evolution or protein engineering applications. In addition, lipases can be used many times by immobilization techniques (Goswami and Stewart, 2016). Many lipid products, such as cocoa butter alternatives, reduced calorie fats, human milk fat analogues, and margarines can be produced via lipase catalyzed reactions (Şahin-Yeşilçubuk and Akoh, 2017).

2.1.3 Action mechanism of lipase enzymes

When the structures of lipases from different sources were examined, it was observed that all of them tended to have similar three-dimensional structure. As a result of comparing the amino acid sequences of many lipases, it was observed that although they differ greatly, they fold similarly and contain similar catalytic regions.

While in the solution, the active site of the lipase is covered by a helical part. However, in the presence of oils or organic solvents, there is a conformational change in which this helix lid is opened and the hydrophobic center containing the active site is exposed (Khan *et al*., 2017; Willis and Marangoni, 2007). Figure 2.3 shows the open and closed form of the lipase enzyme of *Thermomyces lanuginosus*.

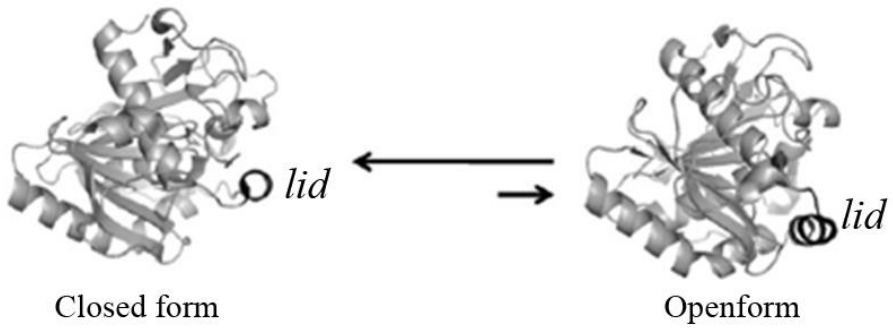


Figure 2.3 : Open and closed structure of *Thermomyces lanuginosus* lipase (Fernandez-Lafuente, 2010).

As a result of this conformational change in the structure of lipase, the release of the Asp / Glu-His-Ser (Asparagine / Glutamine-Histidine-Serine) catalytic triple group in the active site triggers lipase activation and makes the nucleophilic attack on the

hydroxyl group of the serine amino acid bonds. Thus, the interesterification reaction is initiated by hydrolysis of an ester bond and the formation of an acyl-enzyme intermediate, followed by acyl exchange and formation of new ester bonds to form a new TAG molecule (Akoh *et al*., 1998, Camp *et al*., 1998; Goswami and Stewart, 2016; Stergiou *et al*., 2013). The reactions involved in the catalytic cycle of lipases are shown in Figure 2.2.

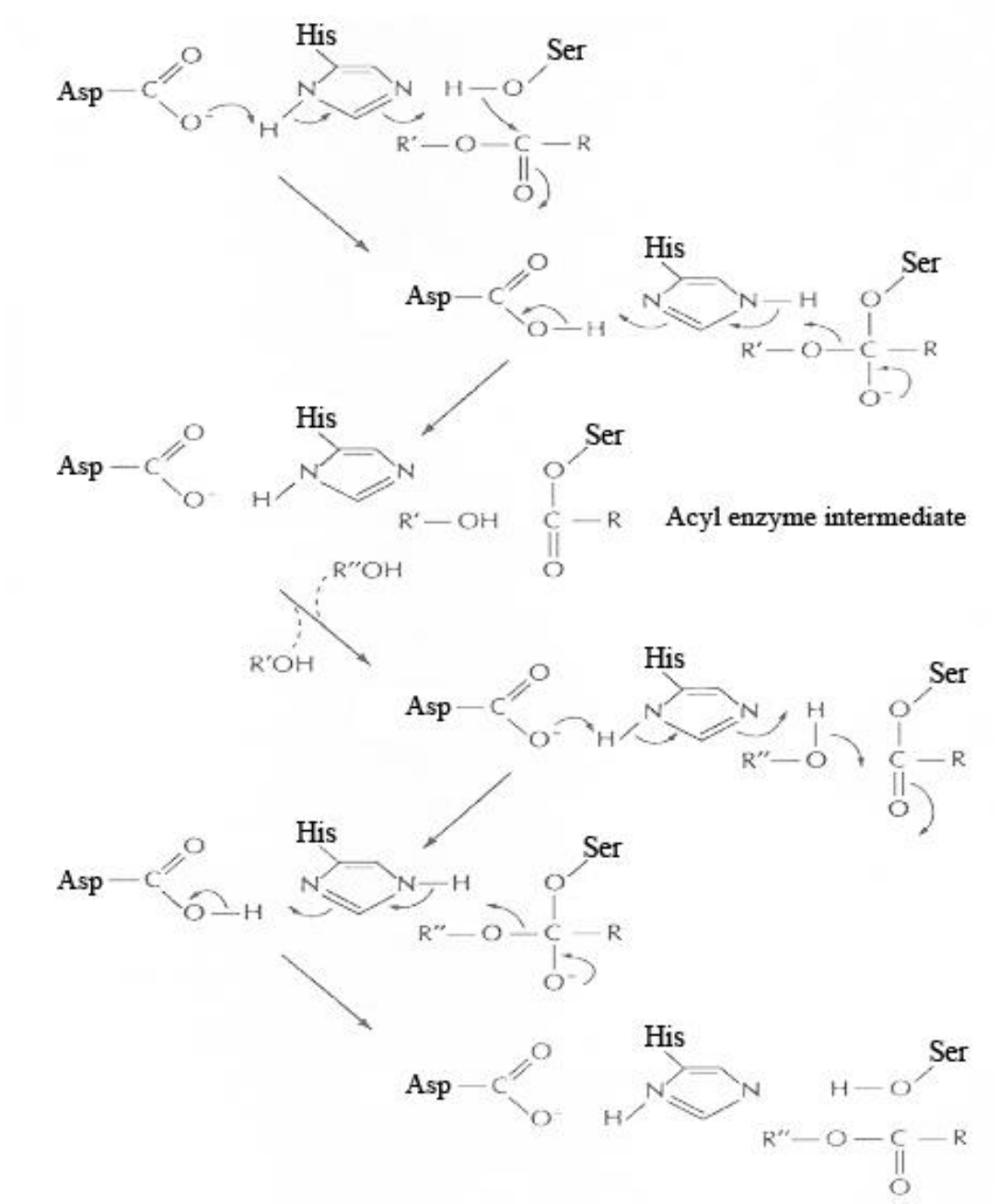


Figure 2.4 : Catalytic mechanism of enzymatic interesterification reactions catalyzed by lipase enzymes (Marangoni, 2002).

2.1.4 Factors affecting lipase activity during interesterification reactions

2.1.4.1 Temperature

Temperature is one of the important factors affecting lipase activity. Generally, increase in temperature also increases the rate of interesterification; however, at very high temperatures, the reaction rate decreases due to enzyme denaturation. Plant and animal lipases have less heat resistance than extracellular microbial lipases. The optimum temperature for the enzymes varies according to the enzyme source. The optimum temperature range for most immobilized enzymes is between 30 °C and 62 °C, but for free enzymes this temperature range tends to be lower. Immobilized lipases are more resistant to thermal degradation because the immobilization process inhibits enzyme denaturation by restricting enzyme movement (Camp *et al*., 1998; Willis and Marangoni; 2007).

Increasing the reaction temperature may increase acyl incorporation, but at the same time, it may lead more acyl migration (Yang *et al*., 2005). Acyl migration is defined as the inevitable side reactions of acidolysis reactions catalyzed by lipases and it mainly refers to the migration of fatty acids in the *sn*-1 and *sn*-3 positions to the *sn*-2 position (which can also be seen as the acyls in the *sn*-2 position to the *sn*-1 and *sn*-3 positions). It is stated that acyl migration is suppressed by lowering the reaction temperature (Foresti and Ferreira, 2010; Yang *et al*., 2003a).

2.1.4.2 Time

It is important to determine the shortest reaction time required to achieve higher incorporation levels and to minimize production costs (Senanayake and Shahidi, 1999). Because, long reaction times are resulted in more acyl migration (Jennings and Akoh, 1999). Therefore, reaction times are generally shortened to minimize acyl migration (Marangoni, 2002; Schmid *et al*., 1998).

2.1.4.3 Substrate molar ratio

The TAG composition of the products obtained by enzymatic reactions depends on the ratio of the substrate after the reaction has reached equilibrium. The high substrate molar ratio will increase acyl incorporation by advancing the reaction equilibrium in the product direction (Yang *et al*., 2003a).

For some lipases, it has been reported that high concentrations of free fatty acids, which are acyl donors, may cause a decrease in lipase activity in acidolysis reactions. Because, in acidolysis reactions, opening of the lid in the catalytic region of lipase enzymes can be prevented by excess free fatty acids in the environment. This explains the low catalytic activity of some lipases in acidolysis reactions (Yang *et al*., 2003b).

The excess amount of the substrate increases the process cost and also may lead to substrate inhibition of lipases. If the high molar ratio is selected, then shorter reaction time should be chosen to prevent acyl migration (Jennings and Akoh, 1999; Senanayake and Shahidi, 2004). The choice of the substrate molar ratio is also related to the cost of purification and the separation of free fatty acids or acyl donors by evaporation and/or distillation. Therefore, it is necessary to select a suitable substrate molar ratio (Yang *et al*., 2003a).

2.1.4.4 Other factors

Other than time, temperature and substrate molar ratio, lipase activity also affected by pH, water content and substrate composition, enzyme load, solvent type (solvent-free media or in the presence of an organic solvent) and reaction system (in batches or in continuous mode) (Ferreira-Dias *et al*., 2013; Kleiner and Akoh, 2018). Lipases are only active at certain pH values. In general, most of them are active between the pH values of 7.0 to 9.0. on the other hand, they can be also active between pH 4 to 10. Water is essential for enzymatic activity, hence enzymatic reactions can be inhibited in the reaction environment with low water activity. Besides, mold lipases are more stable in low water activity levels when compared to bacterial lipases (Willis and Marangoni, 2007).

2.1.5 Unsaturated fatty acids used in the production of structured lipids

Various fatty acids are used in the synthesis of structured lipids. Fatty acid type and their positions in the TAG molecule determine the functional and physical properties, metabolism, and health benefits of SL (Akoh and Kim, 2007). All unsaturated fatty acids taken on a normal diet are almost omega-3, omega-6, and omega-9 (also called n-3, n-6, n-9 or ω-3, ω-6, ω-9) fatty acids (Watkins and German, 2007). N-3 and n-6 fatty acids are polyunsaturated fatty acids (PUFA) whereas n-9 fatty acids are mostly monounsaturated fatty acids (Lee and Lee, 2006).

2.1.5.1 Omega-9 fatty acids

Omega-9 fatty acids or monounsaturated fatty acids are found as oleic acid (OA, C18:1n-9) in vegetable oil sources such as canola, olive, peanut and high-oleic sunflower. Oleic acid can be synthesized in human body, therefore it is not assumed to be an essential fatty acid. However, it has a balanced role in lowering plasma cholesterol in the body (Akoh and Kim, 2007).

2.1.5.2 Omega-6 fatty acids

Linoleic acid (LA, C18: 2n-6), the most common n-6 fatty acid, is found mainly in the oil of many vegetables and in the seeds of many plants. LA is essential fatty acids since humans and mammals are unable to synthesis them in the body. For this reason, absence of LA in the diet causes many health problems such as dermatitis, excessive water loss from the skin, growth and reproductive disorders, and late healing of wounds (Akoh and Kim, 2007). LA is converted to n-6 fatty acids in the body such as γ-linolenic acid (GLA, C18:3n-6), dihomo-gamma-linolenic acid (DGLA, C20:3n-6) and arachidonic acid (AA, C20:4n-6) (Borsonelo and Galduróz, 2008). Figure 2.5 shows the metabolic pathway of n-6 PUFAs.

GLA is an important component of phospholipids present in the cell membrane and is the precursor of prostaglandin E1. GLA have a positive effect on many diseases for instance diabetes, high blood pressure, cancer, sclerosis, schizophrenia, osteoporosis, cardiovascular diseases, skin diseases, allergies, inflammatory diseases (Ahmed *et al*., 2009; Huang and Huang, 2006). Evening primrose, borage oil, blackcurrant oil and hemp seed oil are the oil sources rich in GLA (Guil-Guerrero *et al*., 2010).

2.1.5.3 Omega-3 fatty acids

Omega-3 fatty acids have a structure consisting of 18 or more carbon atoms containing two or more double bonds. The most important n-3 fatty acids include α-linolenic acid (ALA, C18:3n-3), stearidonic acid (SDA, C18:4n-3), eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:5n-3) (Gebauer *et al*., 2005). Figure 2.5 represents the metabolic pathway of n-3 PUFAs.

Both n-3 and n-6 PUFAs are precursor molecules of eicosanoids such as prostaglandins, thromboxanes and leukotrienes. These bioactive molecules have

important functions in inflammation and immune responses, platelet aggregation, cell growth, and cell differentiation (Lone and Taskén, 2013).

N-3 fatty acids are obtained by biosynthesis from the precursor molecules ALA or directly from their nutritional sources. However, human beings need the enzymes necessary for the synthesis of ALA. Therefore, ALA is considered essential fatty acid, (Agastoni, 2010). ALA is mostly found in soybean, flaxseed, nuts, chloroplast of green leafy plants (Akoh and Kim, 2007).

SDA is the intermediate molecule that takes place in the biosynthesis of EPA and DHA (Walker *et a*l., 2013). Oil from the plant source of *Echium plantagineum L.*, *Boraginaceae,* black currant, the families of *Primulaceae* and *Grossulariaceae* have high concentrations of SDA, and also some microbial oils, fish oils, and genetically engineered SDA-soybean are the sources of SDA (Tahvonen *et al*., 2005; Miller *et al*., 2007; Guil-Guerrero *et al*., 2010; Teichert and Akoh, 2011).

Almost all of the SDA is converted to EPA, thus SDA amount of tissues is negligible in human body (Whelan, 2009). EPA is the precursor of some prostaglandins and leukotrienes in the body, also it contributes to immune system functions (Koletzko *et al*., 2001; Lora and Lewis, 2007). In addition, EPA has beneficial effects on the treatment of cardiovascular diseases, schizophrenia and some cancer diseases (Ward and Singh, 2005). DHA is mostly found in retina and brain cells and has important effects on cell membrane functions, development of nervous system, photoreceptor differentiation, activation of rhodopsin, protection against oxidative stress, activation of various enzymes and function of ion channels. (Lapillonne and Jensen, 2009; Brenna *et al*., 2009). Furthermore, SDA potentially have similar positive effects of EPA and DHA on some diseases such as cardiovascular diseases, inflammation, cancer and neurologic disorders. In addition, SDA is the substrate of cyclooxigenase enyzme which produces prostaglandin E2 having inflammation and aggregation preservative effect (Coupland, 2008; Whelan, 2009). SDA originated from plants has the potential of being sustainable and vegetarian EPA source since EPA is mainly derived from fish oils. Fish stocks are decreased day by day due to over fishing activities and negative effects of climate change. Also, fish oils may have some environmental pollutions (dioxsins, polychlorated biphenyls, toxic heavy metals such as mercury) as well as have undesired smell and taste (Chávez-Servín *et al*., 2009; Whelan, 2009).

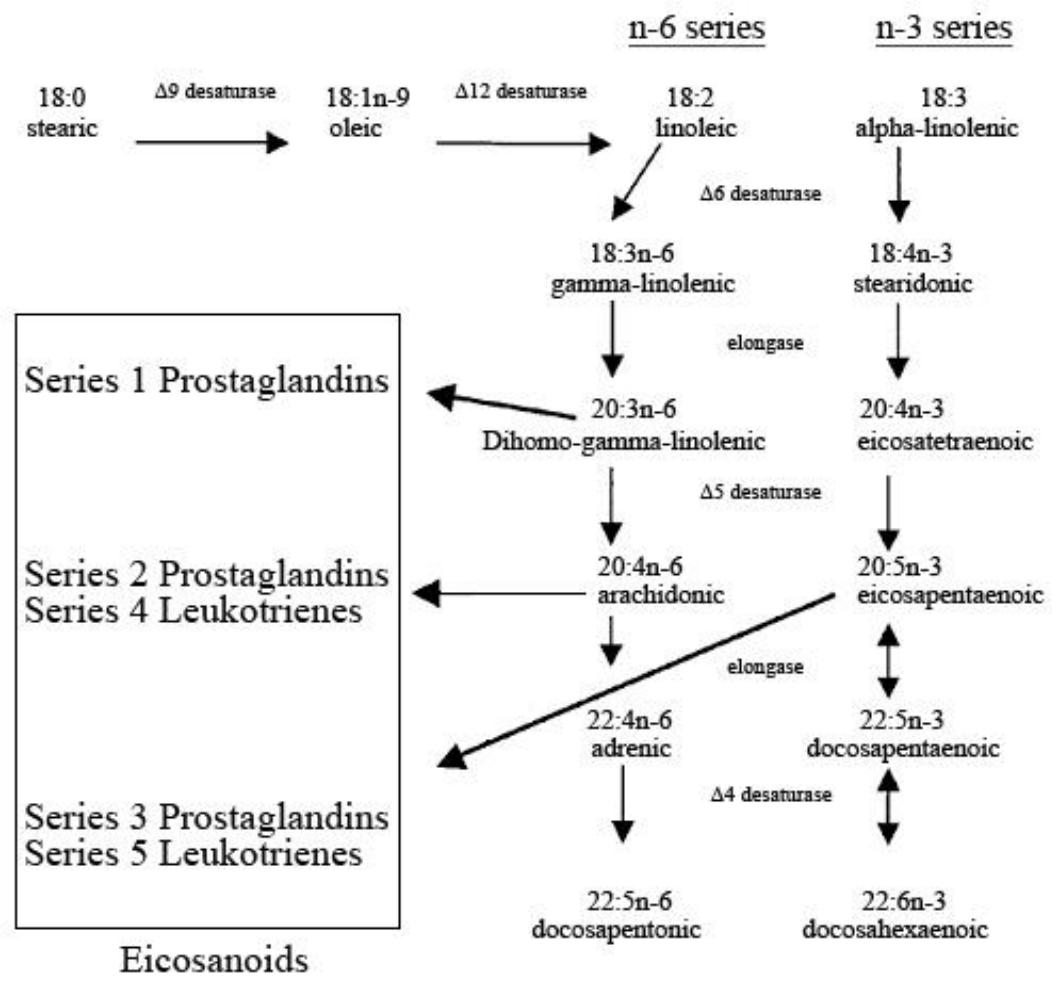


Figure 2.5 : Metabolic pathways for n-3 and n-6 PUFAs (Adapted from Gebauer *et al*., 2005).

2.1.6 *Echium* oil

E. plantagineum is a plant of *Boraginaceae* family and is originated from Mediterranean and Macaronesia (Berti *et al*., 2007). This plant is also known as purple viper's bugloss in our country and the flowering period is between March and September, and grows in the fields, coastal areas and barren places (Karaca, 2008). Figure 2.6 shows the images of *Echium plantagineum* plant.

Figure 2.6 : *Echium plantagineum* plant (Url-1; Url-2).

Echium seeds contain 9-16% SDA and also GLA and ALA. Fatty acid composition of *E. plantagineum* from different sources is given in Table 2.3. *Echium* oil has many potential uses in the pharmaceutical industry for the treatment of eczema, acne and other skin diseases, and in the cosmetics and personal care products industry. Although *Echium* is known as invasive herb in Eastern Australia, it is grown commercially in the United Kingdom (UK) and Europe (Berti *et al*., 2007).

Table 2.3 : Fatty acid composition (%) of *Echium plantagineum* oil.

Fatty Acid	*Echium* oil[1]	*Echium* oil (seed oil)[2]	*Echium* oil (oil from leaves)[3]	*Echium* oil[4]
C14:0	-	-	-	0.1
C16:0	6.7	6.4	20.3	7.7
C18:0	3.4	2.8	3.0	2.7
C18:1n-9	14.5	12.9	5.8	13.4
C18:2n-6	14.1	13.7	6.9	15.2
C18:3n-6	10.7	9.1	2.2	11.3
C18:3n-3	31.7	36.6	29.4	33.3
C18:4n-3	13.7	12.9	5.3	13.4
C20:0	0.1	0.1	0.9	0.3

[1]*Echium* oil used in this study

[2]Guil-Guerrero *et al.,* 2000a

[3]Guil-Guerrero *et al.*, 2000b

[4]Özcan, 2008

2.1.7 MLM-type structured lipids

Recently, production of MLM-type SLs with medium chain fatty acids (MCFAs, C6-C10) at *sn*-1 and *sn*-3 position, and long chain fatty acids (LCFAs, C12-C24) at *sn*-2 position has gained attention for clinical and nutritional purposes. During digestion, MCFAs are cleaved from the TAG molecule via activity of *sn*-1,3 specific pancreatic lipase and then transported to liver through portal vein where it is rapidly turn into glucose. In addition, MCFAs do not have carnitine dependence and do not require chylomicrons. Therefore, they can easily be oxidized to produce energy as well. Furthermore, MCFAs do not further esterified as a newly synthesized TAG molecule thus they have low tendency to be deposited as body fat which is beneficial for the control of body weight (Kim and Akoh, 2015; Yao *et al*., 2017; Ye *et al*., 2019). On the other side, LCFAs are absorbed as *sn*-2 monoacylglycerol (2-MAG) through

lymphatic system and are mainly used in biosynthetic processes (Mu and Høy, 2004). Digestion of triglicerides are represented in Figure 2.7.

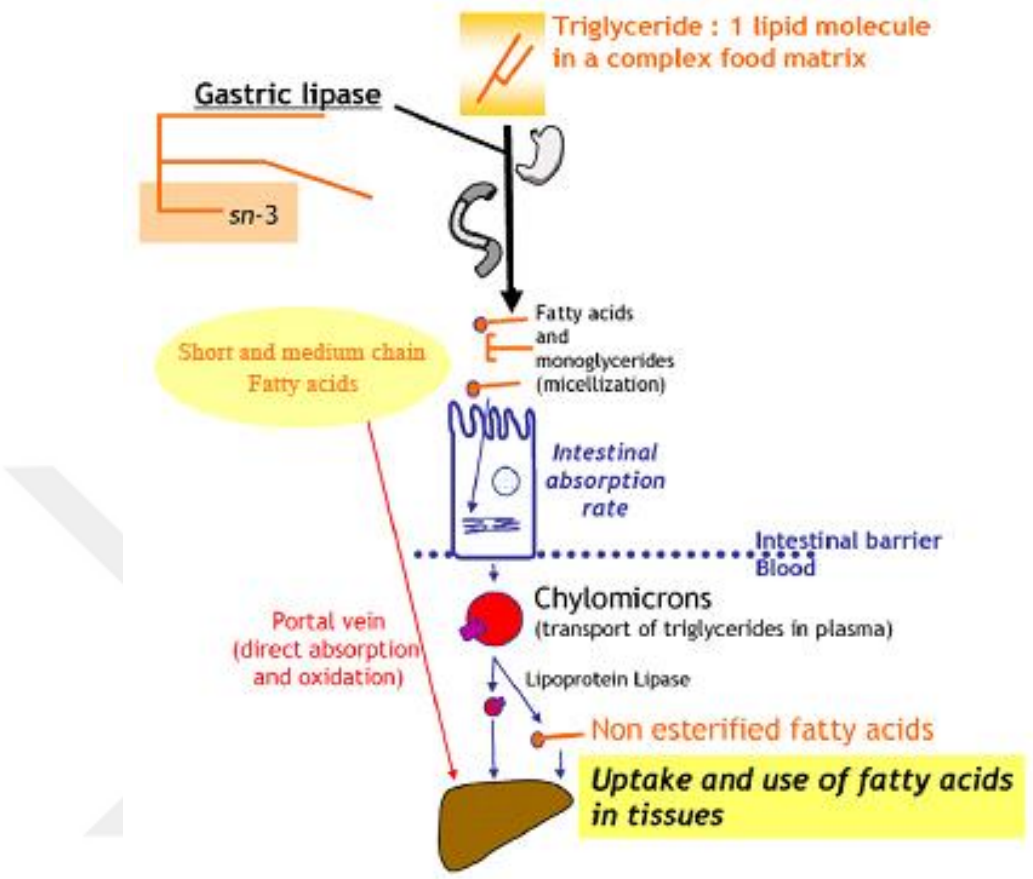


Figure 2.7 : Digestion of triglicerides (Adapted from Michalski *et al*., 2013).

MCFAs have been used for years to satisfy the nutritional needs of patients with lipid malabsorption such as Crohn's disease, cystic fibrosis, colitis and premature birth (Akoh *et al*., 2002; Jala and Kumar, 2018). However, MCFAs do not supply the essential fatty acid requirement of the body, so they cannot be used as lipid source alone (Nunes *et al.*, 2011). For this reason, MLM-type SLs are designed as one TAG molecule representing both MCFAs and LCFAs which permit more controlled release of fatty acids into bloodstream (Osborn and Akoh, 2002). MLM-type SLs are mostly designed for nutritional purposes and for the most efficient delivery of the desired fatty acid by targeting specific diseases and metabolic conditions (Jala and Kumar, 2018). The production of such SLs is an important food source for patients suffering from pancreatic insufficiency or other absorption disorder (Iwasaki and Yamane, 2000). Studies unraveled that MLM-type emulsions are the safer and effective way of energy delivery for patients who needs long term parenteral

nutrition (Matulka *et al*., 2006; Rubin *et al*., 2000). Many MLM- type SL products have been commercialized for medical and food applications. For example, as reduced calorie fat, Benefat® (Cultor Food Science Inc.) is used in products, chocolate coating and nutrition bars and Caprenin® (Procter & Gamble) has an application as confectionary coating fat (Kim and Akoh, 2015). Laurical® (Calgene Inc.) is used in medical nutrition products and confectionary coating, coffee whiteners, whipped toppings and filling fats; Neobee® (Stepan Corp.) in nutritional bar processing; Structolipid® (Fresenius Kabi) and Captex® (Abitec Corp.) as a rapid source of energy for patients and parenteral nutrition (Kim and Akoh, 2015; Lee and Lee, 2006).

SLs have many advantages over naturally occurring fats or physical fat mixtures: improving immune function, reducing cancer risk, preventing thrombosis, and improving nitrogen balance. Anti-tumorigenic effect of chemically synthesized MLM-type SLs has been shown in an *in vivo* study conducted with mice having Yoshida sarcoma tumor (Ling *et al.*,1991). This study showed that tumor protein synthesis was decreased; tumor genesis was retarded; body weight was decreased and nitrogen balance was improved in mice fed with SL made of fish oil and MCFAs.

In the study of Straaup and Høy (2000), SL produced enzymatically from rapeseed oil and decanoic acid (C10:0), chemically synthesized SL from rapeseed oil and tridecanoin and physical mixture of rapeseed oil and tridecanoin used in the feeding of three different groups of mice. Although these three lipids used in the study have the same fatty acid content, the positional distributions of the fatty acids in the TAG molecule are different. At the end of the study, MLM-type SL obtained by enzymatic synthesis was found to be better hydrolyzed and absorption rate was higher compared to others.

In another study, MLM-type SL produced by enzymatic synthesis using soybean oil and caprylic acid and pysical mixture of soybean oil and tricaprylin (PHY) was used for animal feeding study. After 21 days of feeding, it was showen that total cholesterol and plasma TAG levels were higher in mice fed with SL and PHY than in the control group (soybean oil-fed). This situation was explained by higher content of unsaturated fatty acids in soybean oil that reduces blood cholesterol and TAG levels in the body. In addition, caprylic acid was determined in the liver of the SL-fed

group, but not found in PHY-fed group. This study proved that positional distribution of fatty acids in TAG molecules lead to different metabolic fates (Lee *et al*., 2000).

In the study of Hita *et al*. (2009), MLM-type SL with caprylic acid at *sn*-1,3 positions and docosohexaenoic acid (DHA) at *sn*-2 position was produced via enzymatic reactions. Researchers concluded that designed SL has the potential of being an important nutrition component for the development of central nervous system of premature babies.

Kim *et al*. (2010) designed a MLM-type SL consisting of caprylic acid and gamma-linolenic acid (GLA) derived from borage oil by enzymatic synthesis method for premature babies and for those who have lipid malabsorption such as cystic fibrosis. In another study, corn oil and caprylic acid were the substrates of enzymatic process yielding with MLM-type SL purposed for the patients with special nutrition requirements (Öztürk, Ustun and Aksoy, 2010). In the study of Nunes *et al*. (2011), MLM-type SL aimed for clinical nutrition was produced via enzymatic reactions between olive oil and capric acid.

2.2 Encapsulation Technology

Encapsulation is the process that bioactive component (core material) is entrapped in wall material in order to protect the core material from unwanted environmental changes. Food applications of encapsulation technology is widely used since stability of encapsulated substances is improved, and also changes in their functionality resulted from extreme processing or storage conditions such as high temperature and moisture levels is kept minimal (Nedović *et al*., 2013). In addition, encapsulation technology enables bioactive food components to be preserved from destructive conditions of upper gastrointestinal tract hence the controlled release of bioactive material is became possible (de Vos *et al*., 2010).

Encapsulation technology has many applications in food, chemistry, agriculture, feed, medicine, pharmacy, veterinary, biotechnology (Narang, Delmarre and Gao, 2007; Nedovic *et al*., 2011). It is possible to categorize encapsulation technologies according to the particle size of the product: nanoencapsulation (10-1000 nm); microencapsulation (0.1-5000 μm) and macroencapsulation (> 5000 μm). Microencapsulation and macroencapsulation are generally used in food industry for

many purposes: to extent shelf life, increase nutritional value, provide digestibility and shorten ripening time. Nanoencapsulation technology is preferred in medicine, pharmacy and biotechnology for controlled release systems, organ-tissue targeted drug design, etc. (Gökmen, Palamutoğlu, Sarıçoban, 2012; Danhier *et al*., 2012).

Encapsulation technology enables many active substances to be encapsulated and protected. Most commonly used active components in encapsulation are vitamins, minerals, enzymes, proteins, organic acids, probiotics, prebiotics, essential oils, sweeteners, preservatives, colorants, flavors, fatty acids (n-3, conjugated linoleic acid), carotenoids (β-carotene, lycopene), antioxidants (tocopherol) , flavonoids, polyphenols) and drug active substances (Nedovic *et al*., 2011, Narang *et al.,* 2007, Danhier *et al*., 2012).

Wall materials used in the encapsulation process affect the encapsulation efficiency and also capsule stability. It is important to determine the wall material considering the size, shape, stability, permeability of the capsules to be produced.The properties of an ideal wall material can be listed as to show good rheological behavior at high concentration, dissolve the core material and stabilize the encapsulation emulsion, not interact chemically with the core material, keep active material within its structure during processing/storage, protect the core material from environmental conditions and dissolve in suitable solvent for food industry (Ray, Raychaudhuri and Chakraborty, 2016). Many different materials such as carbohydrates, proteins and lipids derived from plant, marine, microbial/animal sources are suited for encapsulation technology in food industry as shown in Table 2.4 (Wandrey, Bartkowiak and Harding, 2010).

Table 2.4 : Wall materials for encapsulation technology in food industry (Wandrey, Bartkowiak and Harding, 2010).

Origin	Carbohydrate polymer	Protein	Lipid
Plant	Starch	Gluten (corn)	Fatty acids/alcohols
	Cellulose	Isolates (pea, soy)	Glycerides
	Plant exudates		Waxes
	Gum arabic		Phospholipids
	Gum karaya		
	Mesquite gum		
	Plant extracts		
	Galactomannans		
	Soluble soybean Polysaccharide		
Marine	Carrageenan		
	Alginate		
Microbial/animal	Xanthan	Caseins	Fatty acids/alcohols
	Gellan	Whey proteins	Glycerides
	Dextran	Gelatin	Waxes
	Chitosan		Phospholipids (Shellac)

There are various types of encapsulation technologies; among them coacervation, spray drying, spray cooling, freeze drying, fluid bed coating and extrusion procedures are the most widely used technologies (de Vos *et al*., 2010). Schematic representation of encapsulation techniques used in food industry is given in Figure 2.8.

Spray drying is commonly used in food industry in order to encapsulate bioactive materials with a particle size range between 10-400 μm. This method is based on the dispersion of the core material into a liquefied wall material and spraying through nozzles into a controlled environment. It enables to produce uniform capsules in a continous operation mode. The spray drying process is easy to apply and economical. On the other hand, there are some disadvantages of spray drying such as installation costs, need of high temperatures for drying, presence of micro-cracks on the surface as a result of high temperatures (Celli, Ghanem and Brooks, 2015).

Spray cooling and chilling is not often used in food industry but it is an economical encapsulation technique to produce capsules with a size range from 20 to 200 μm. This technique is used for some organic and inorganic salts, enzymes, flavours, textural ingredients. The principle of the method is same with spray drying excluding

evoparation of water. It is possible to construct a secondary coating for controlled release, also it can be operated continuously and scaled up. However, because of the having lipophlic wall material, the products of this method may not be suitable for their addition into foods. Also, leakage of core material was observed during the storage of spray cooled/chilled capsules (Celli, Ghanem and Brooks, 2015; Gouin, 2004).

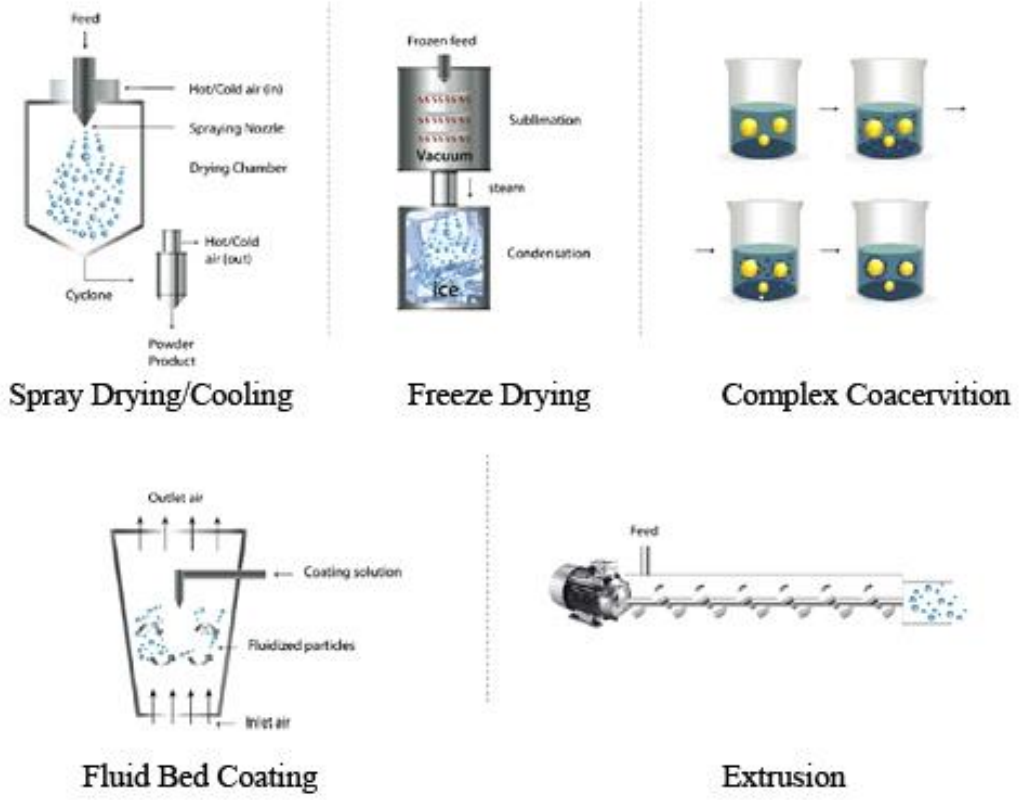


Figure 2.8 : Encapsulation technologies used in food industry (de Souza Simões *et al*., 2017).

Freeze-drying is a suitable method for heat sensitive materials and aromas. In this technique, an aqueous solution of active material is prepared to obtain an amorphous powder. Firstly, the sample is frozen and then sublimation is occurred at low pressure and temperature yieding with dry sample. Freeze-drying can be applied alone or in combination with other methods such as coacervation. It is not cost-effective method since process time is very long (> 20 h). Besides, porous structure of the final product may resulted with instable active material (Celli, Ghanem and Brooks, 2015).

In fluid bed coating method, the powder sample that is suspended by an air stream is encapsulated with coating solution (usually lipids). This method is not economical

due to the long process time. This process has a narrow application because the coating material is lipophilic and the initial material should be in powder form (Coronel-Aguilera and Martín-González, 2015).

Extrusion is based on dissolving active material in sodium alginate solution and then forcing the suspension through a pipette, syringe, a coaxial needle or a spraying nozzle into gelling bath. The particle size of the capsules is ranged between 200-5000 μm (Zuidam and Shimoni, 2010). It is used to encapsulate seed oils, antioxidants and probiotics (de Souza Simões *et al*., 2017).

2.2.1 Complex coacervation method

Coacervation technology is based on a simple principle that the oppositely charged bioactive component and matrix molecule in the solution are mixed together to form a complex (colloidal phase) which is then separated from aqueous phase (de Vos *et al*., 2010; Poshadri and Kuna, 2010).

Coacervation technology consists of two methods: simple coacervation with one wall material, and complex coacervation with two or more wall materials (Poshadri and Kuna, 2010). In complex coacervation method, many different negatively charged polysaccharides (e.g. gum arabic, pectin, alginate and carboxy methyl cellulose) can be combined with positively charged protein molecules (e.g. gelatin and albumin or plant based proteins) to construct the wall material (Zhang *et al*., 2012). Figure 2.9 represents a schematic diagram of encapsulation process via complex coacervation technique.

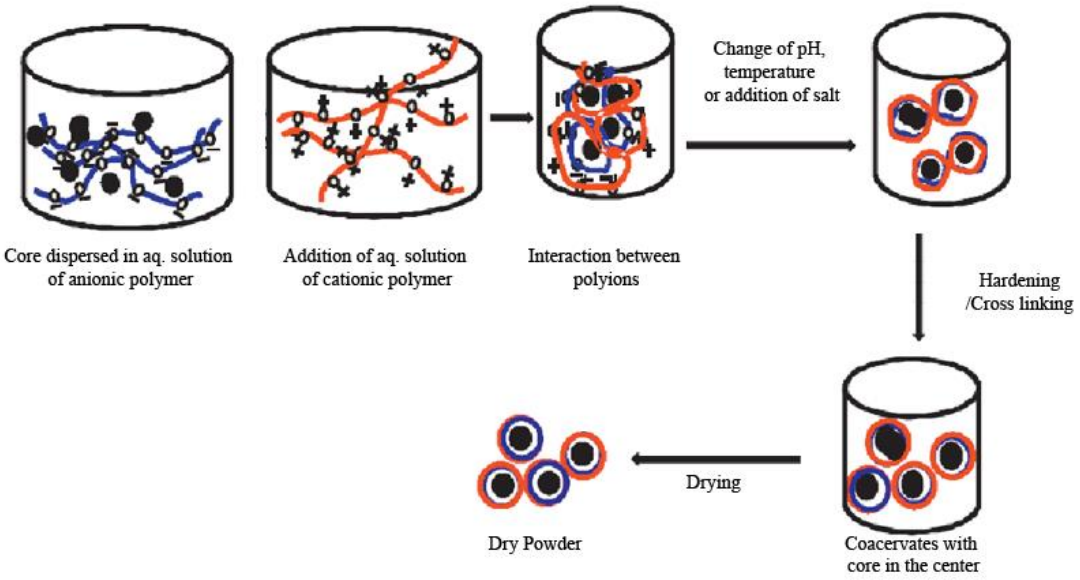


Figure 2.9 : Encapsulation of active ingredients by complex coacervation technology (Timilsena *et al*., 2019).

This technique generally includes four steps (Timilsena *et al*., 2019):

- Preparation of wall materials' solutions
- Stirring up wall materials and core material and their further homogenization
- Adjustment of temperature and pH to obtain coavervates
- Hardening of coacervates with cross-linking agents

Parameters such as pH, temperature, stirring rate, ion concentration, type of wall material, and the ratio of matrix molecule to bioactive component affect the characteristics of capsules (de Vos *et al.,* 2010; Timilsena *et al.,* 2019). Also, hardening step is important to obtain solidified products with improved thermal and mechanical stabilization characteristics since weak ionic interactions between polymers form coacervates. Hardening of coacervates can be achieved by enzymatic cross-linking or chemical cross-linking. Formaldehyde, glutaraldehyde, glyoxal, diisocyanate, epichlorohydrin and polyamines are the cross-linking agent that are used traditionally in chemical cross-linking process. Aldehyde residues of cross-linker and amino groups of the protein are linked together to produce coacervates with improved drying properties and storage stability. Despite its higher linking yields and effectivenes in stabilization of coacervates, traditional cross-linkers such as glutaraldehyde has a limited application in food industry because of its toxicity

(Eghbala and Choudhary, 2018; Timilsena *et al*., 2019). For this reason, lately, novel materials such as genipin, sodium tripolyphosphate and tannic acid have been gained attention as an alternative cross-linking agent due to their non-toxic property (Silva *et al*., 2008; Wei and Huang, 2019).

In enzymatic cross-linking process, transglutaminase and oxidase enzymes such as tyrosinase and laccase can be used in order to construct protein−polysaccharide complexes. This process has mild reaction conditions and higher specifity. In this method, oxidase enzyme acts on tyrosine residues of proteins to produce highly reactive quinone residues. Then, these quinone residues react with nucleophilic substituents of polysaccharides.

Transglutaminase (TGase; protein-glutamine gamma-glutamyltransferase- EC 2.3.2.13) enzyme is derived from plants, animals and microorganisms and are used in various indsutrial applications (Fatima and Khare, 2018). Microbial transglutaminases are economical, enviromentally friendly, stabile in higher reaction temperatures and also they do not require Ca^{+2} ions which prevents the formation of unvanted calcium-protein complexes (Kieliszek and Misiewicz, 2014). This enzyme covalently bonds γ-carboxamide groups of glutamine residues and ε-amino group of lysine residues (Wei and Huang, 2019) as shown in Figure 2.10.

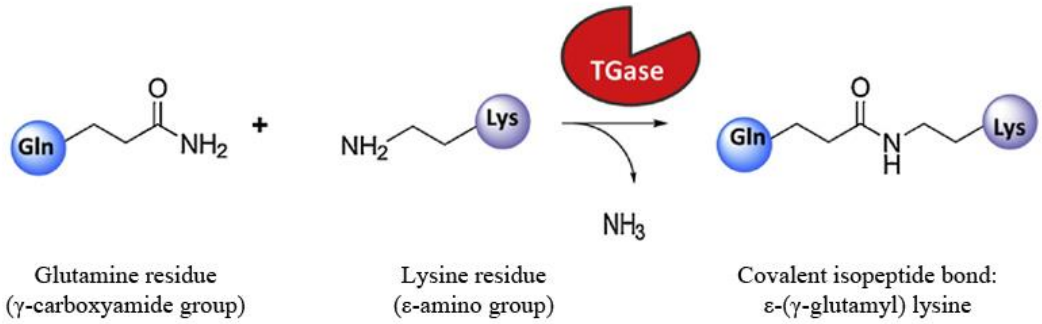


Figure 2.10 : Action mechanism of transglutaminase enzyme (Adapted from Fatima and Khare, 2018).

2.2.2 Encapsulation of oils by complex coacervation

Bioactive agents such as lipids are sensitive to food processing and storage conditions for instance heat, light and high moisture levels, and mechanical stress as well. Unwanted taste and smell in the food may be occurred due to the possible interaction between lipids and other food components during food processing and storage period. Also, lipids are very sensitive to unfavorable environment of food

matrix that is resulted with loss of their functionality. Encapsulation technology get rid of such problems hence it provides safer delivery of lipids (Aloys *et al*., 2016; Nedović *et al*., 2013).

Complex coacervation supply encapsulated powder products with high oil loading content (> 60%) and less surface oil amounts (< 0.1%) whereas spray dried powders have relatively low oil loading capacity and high surface oil levels (30% and 0.2-1.0%, respectively) (Barrow, Nolan and Jin, 2007; Kralovec *et al*., 2012; Zuidam and Shimoni, 2010). Surface oil of the capsules is a handicap since they have not only negative effect on wettability and dispersability of the powder but also oxidation risk and possibility of rancid flavor development (Kralovec *et al*., 2012; Millqvist-Fureby, Elofsson and Bergenstahl, 2001; Vega *et al*., 2005). So, the capsules with less surface oil are beneficial during food processing and storage periods.

2.2.3 Wall materials

2.2.3.1 Gelatin

Gelatin is a high molecular weight polypeptide found in animals and can be derived from skin, bones and tendon of pigs, cow and fish (Karim and Bhat, 2008). Gelatin structure (Figure 2.11) contains several amino acid residue such as alanine, glycine, proline, arginine, glutamine and and 4-hydroxyproline and it is positively charged below its isoelectric point (IEP) (Devi *et al*., 2017).

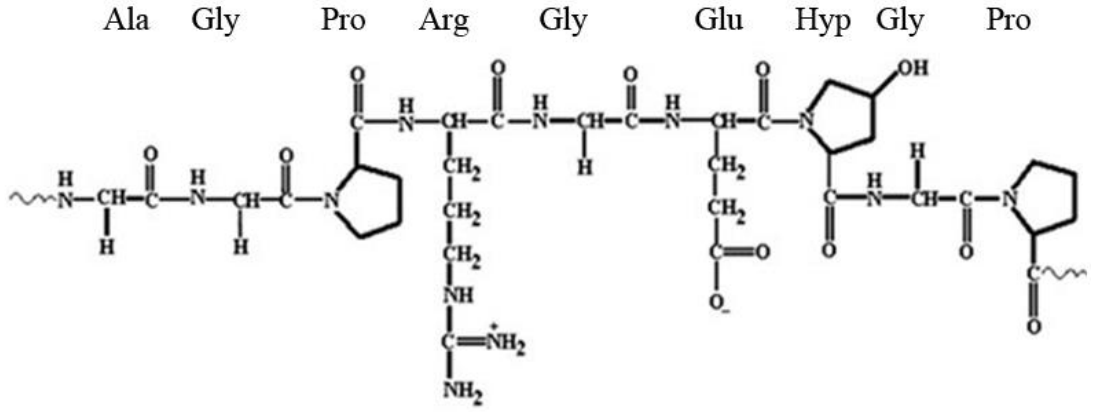


Figure 2.11 : The structure of gelatin (Devi *et al*., 2017).

The partial acidic/basic hydrolysis of collagen in which the cross-links of collagen is denatured, is used to produce two types of gelatin. Type A gelatin is the product of

acid treated pig skin, and the basic hydrolysis of bones or cowhide is resulted in type B gelatin (Harrington and Morris, 2009; Karim and Bhat, 2008).

Gelatin is widely used in food industry as a hydrocolloid and also commonly used as wall material in complex coacervation method (Comunian and Favaro-Trindade, 2016). Because of its high protein and amino acid content, gelatin has a common use as food additive in food industry. Hydrocolloid structure of gelatin allows diverse use in food industry such as confectionery (mainly chewability, texture and foam stabilization), jelly desserts (fat reduction and to provide the mouth feel), dairy products (to provide stabilization and texture) and meat products (to bind water) (Nishimoto *et al*,. 2005).

2.2.3.2 Gum arabic

Gum arabic is a water-soluble polysaccharide obtained from secretion of acacia tree and its structure consists of polysaccharide chain of L-arabinose, D-galactose, L-rhamnose and D-glucuronic acid, and 2% protein content that enables to behave as an emulsifier (Comunian and Favaro-Trindade, 2016). Food industry widely use gum arabic because it is natural, non-toxic, odorless, tasteless, colorless, completely dissolves in water, biocompatitable and biodegradable. Gum arabic is mostly used in the food industry for stabilization and encapsulation, control of texture, color, retention and protection of chemically reactive and volatile oils and flavors . It is also used as glaze in candy products and it prevents sugar crystallization (Long and Ramsburg, 2011). Gum arabic is a negatively charged molecule and in combination with gelatin and gum arabic, complex coacervates can be produced in appropriate enviromental conditions such as pH, temperature, polymer concentration or ratio (Comunian and Favaro-Trindade, 2016).

2.2.4 Studies on complex coacervation of lipids

Researchers have conducted several studies regarding encapsulation of oils via complex coacervation method with different wall materials. Dong *et al.* (2011) produced coacervates with good storage stability. In the study, gelatin and gum arabic were used as wall material in order to encapsulate peppermint oil with the help of transglutaminase as the cross-linking agent.

Bustraen and Salaün (2014) applied complex coacervation technology for the encapsulation of a commercial triglycerides blend. In the study, gum arabic and

chitosan were chosen as wall polymers and sodium tripolyphosphate (NaTPP) was used as hardening agent. According to results, highest encapsulation yield and loading content were found as 97% and 67.4%, respectively. It was also unraveled that pH value of 3.6 and a ratio of chitosan to gum arabic mixtures of 0.25 (w/w) were the optimal levels for the production of these capsules.

Although gelatin is commonly used as wall material in complex coacervation method, it has some disadvantages from the point of consumers who demand Kosher and Halal foods/food ingredients (Zuidam and Shimoni, 2010). For this reason, in recent years, researchers pay attention to alternative wall materials free from gelatin such as β-lactoglobulin, bovine serum albumin, egg albumin, soy proteins, pea proteins and whey proteins combined with gum arabic, carrageenan and pectin (de Kruif, Weinbreck and De Vries, 2004).

Among the protein based wall materials, soy proteins are also being investigated for their effects on capsule characteristics and encapsulation efficiency. Researchers have been used soy glycinin- sodium dodecyl sulphate (SDS) wall combination to encapsulate hexadecane (Lazko *et al*, 2004a), soy protein isolate-gum arabic complex to entrap the orange oil (Jun-xia *et al*., 2011), soy protein isolate-gum arabic combination for the encapsulation of omega-3 fatty acids (de Conto *et al*., 2013).

It has been indicated that core concentration must be kept below 50% (w/w) in order to obtain high process yield (Lazko *et al*., 2004b; Mendanha *et al*., 2009; Rusli *et al*., 2006; Jun-xia *et al*., 2011). In a study, it has been exhibited that change of wall/core ratio from 1/1 to 1/3 has resulted with decrease in encapsulation efficiency from 92% to 79% (Mendanha *et al*., 2009). This issue is explained as higher amounts of oil will lead an incomplete emulsification which causes production of unstable emulsions (Jun-xia *et al*., 2011).

In the study of Zhang *et al*. (2012), gelatin and gum arabic were the used wall materials for entrapping microalgal oils. The study aimed to investigate the effects of hardening parameters such as time, temperature, pH and concentration on the release rate of complex cocaervation microcapsules. According to results, optimum parameters for hardening were found for 6 h at 15 ˚C and pH 6.0 with transglutaminase concentration of 15 U/g gelatin, (Zhang *et al*., 2012).

Complex coacervation is also used to produce a commercial product named Ocean Nutrition Canada MEG-3 which is used as an ingredient with improved stability for food and beverages. This product includes gelatin and polyphosphate as wall materials of the encapsulated omega-3 fatty acids from fish oil with a shelf life of 18 month at 4 °C (Barrow, Nolan and Jin, 2007).

Complex coacervation methodology also allows the formation of coacervates at nano scale. In the study of Lv *et al*. (2014) jasemine oil was encapsulated at nano size by using complex coacervation of gelatine and gum arabic. The resulted capsules had a particle size ranged from 51 to 384 nm which were determined by Zetasizer Nano. The formation of nanoparticles as a result of complex coacervation was explained by the weakness of electrostatic forces between polymers used as wall materials, and formation of soluble complexes.

In another study, Habibi *et al.* (2017) encapsulated fish oil by complex coacervation of gelatin and gum arabic for their further use in fortification of pomegranate juice. Maximum encapsulation efficiency (~76%) was achieved at 1.5% encapsulant and 3% fish oil concentration.

Ifeduba and Akoh (2015) successfully encapsulated SDA rich soybean oil via complex coacervation of gelatin and gum arabic at different homogenization rates. Apart from the classical gelatin-gum arabic system, Maillard reaction products were also added into coacervation emulsion. As a result of the study, over 99% of encapsulation efficiency was obtained in all coacervation conditions.

Echium plantaginemum seed oil was also encapsulated in complex coacervation system of gelatin, gum arabic and cashew gum in the study of Comunian *et al.* (2017). The coacervates with enhanced oxidative stability (~9.6 hours) was provided at gelatin:cashew gum (1:2.5 ratio) combination and the average particle size was about 13 μm.

2.3 *In vitro* Release Studies

2.3.1 Delivery of bioactive molecules

Recently, bioactive ingredients, such as polyphenols, fatty acids proteins, vitamins, and probiotics are added into food products in order to obtain functional food products with improved health benefits. However, these bioactive molecules are

affected by the food processing or destructive conditions of digestion system in the body that causes the unwanted changes in food products and decrease in bioaccessibility and bioavailability of the bioactive components (de Vos *et al*., 2010; Nedović *et al*., 2013). To overcome such problems, food-grade delivery systems, such as lipid based, protein based, carbohydrate based and mixed delivery systems have been adapted from the pharmaceutical science (Benshitrit *et al*., 2012). Lipid based delivery systems are suitable for molecules have lipophilic property. This method is based on the encapsulation of bioactive molecules by oil droplets in order to improve the core material stability and also their release properties during digestion (McClements; 2010, McClements and Li, 2010). Protein based delivery systems use animal (casein, gelatin, etc) or plant (soy, glycinin and gliadin) originated proteins in encapsulation processes such as coacervation, spray drying, extrusion. This system have the advantages of proteins, for instance the highness of nutrion value, having diverse physicochemical characteristics and being safe, on the other side allergenity and odor smell of proteins are the handicaps of this method (Benshitrit *et al*., 2012). Food carbohydrates derived form plant, animal and marine sources are also used to deliver bioactive agents and permits controlled release of bioactive molecule through the digestion system (Augistin and Hemar, 2009). In addition, protein-polysaccharide interactions are used in mixed delivery systems to improve physical and chemical stability of emulsion and their deliver properties (Dickinson, 2008). The bioavailability, stability, absorption and retention time in human digestive system of bioactive molecules are affected by the size of colloids. Also, type of the nutrient has a key role in the determination of absorption sites (Benshitrit *et al*., 2012).

2.3.2 Gastrointestinal digestion of foods

It is important to take into account the fate of the food grade delivery systems after their digestion in the body by means of their bioavailability and intestinal permeability. For this reason, understading the human digestion system is required to improve controlled release of bioactive molecules (Benshitrit *et al*., 2012). Gastrointestinal tract is consists of many different organs and tissues connected to each other throug which foods are digested into small molecules to join the bloodstream. Figure 2.12 represents the structure and main physicochemical conditions of gastrointestinal tract. Digestion of food products begins at the mouth in

a neutral pH environment with the contribution of saliva, teeth and tongue to mechnanical and enzymatical process. Then, processed food is subjected to gastric digestion via peristaltic movements of stomach in acidic conditions caused by HCl secretion. Also, enzymatic activity of pepsin and gastric lipase occurs to obtain basic constituents of food, so they can easily absorbed in the small intestine. Gastric enzymatic activity is inhibited in the small intestine due to higher pH levels (pH 6-7.5) of environment. Enzymes such as pancreatic lipase and trypsin, bile salts and inorganic salts take place in the digestion process at this stage. Some of the food fractions are absorbed in the small intestine and the others are transferred into large intestine (colon) for their final digestion. Microbial fermentation, absorption of water and minerals occur at this part of the system (Benshitrit *et al*., 2012; Mao and Miao, 2015; Singh and Sarkar, 2011).

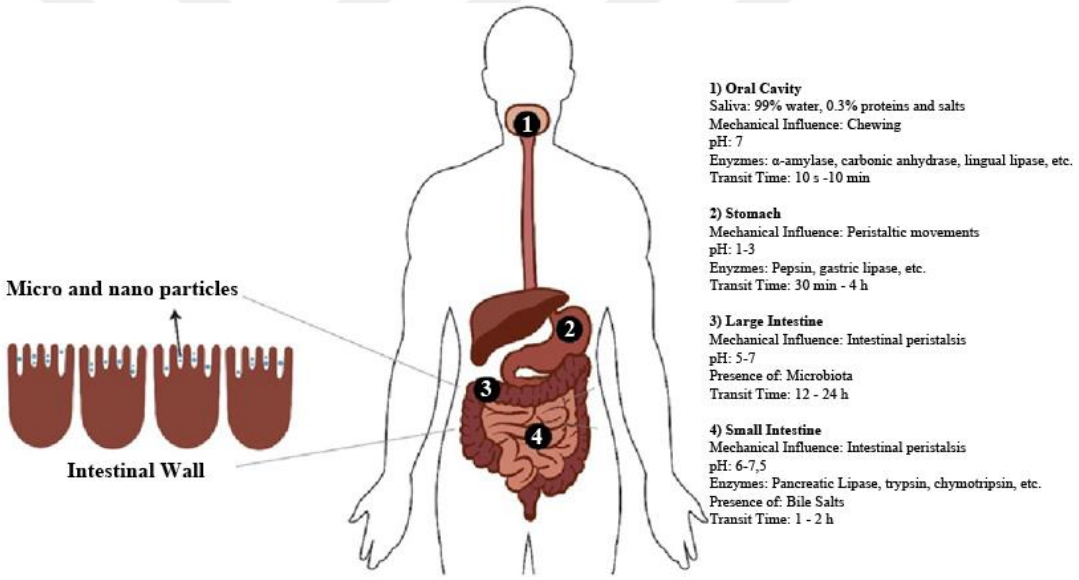


Figure 2.12 : The structure and main physicochemical conditions of human gastrointestinal tract (de Souza Simões *et al*., 2017).

2.3.3 Gastrointestinal digestion models

Since the gatrointestinal system is complicated, *in vivo*, *in vitro* and Caco-2 cell models have been constructed in order to understand the digestion process deeply.

2.3.3.1 *In vivo* digestion models

In vivo digestion models measure the degree and rate of bioactive molecules'absorption after their consumption by humans or animals such as rodents, rabbits, pigs or calves. Generally, the concentration of the nutrient digested in pre-

determined dose is tracked by changes in the blood plasma leves within the measurement times. Also, radioisotope labeling, stable isotopes, metabolic balance and rate of repletion are the other methods used to assess bioavailability of the nutrients in this model. The handicaps of this model are complexity of the live organisms, being expensive and time consuming, and also ethical concerns (Benshitrit *et al*., 2012; de Souza Simões *et al*., 2017).

2.3.3.2 *In vitro* digestion models

In vitro digestion models mimic the digestion conditions and processes that are occured in human gastrointestinal tract and they are categorized as static and dynamic models. Both models are economical, faster, free of ethical concerns and have a simple application (Alminger *et al*., 2014; Li *et al*., 2013).

In vitro static digestion models have common use in which oral, gastric and small intestine conditions are mimicked via use of artificial digestion fluids. The digestion of isolated or purified food components is performed at certain temperature for a certain time in a buffer environment where pH is kept constant (Lucas-González *et al*., 2018).

The dynamic *in vitro* digestion models have been developed to closely mimic the conditions of gastointestinal tract. TIM was generated by Minekus *et al*. (1995) at TNO Nutrition and Food Research (Zeist, the Netherlands) in which TIM-1 mimics the the conditions of stomach, duodenum, jejunum, and ileum and TIM2 simulates the large intestine. TIM-1 is performed in four chambers at constant temperature and it enables peristaltic movements, pH changes, secretion addition. The simulated human intestinal microbial ecosystem (SHIME) was developed by Molly *et al*. (1993) in order to simulate the intestinal conditions. This model consists of five chambers and coupled with pH probes and a pump system. When compared to static models, dynamic models are highly complex and costly.

2.3.3.3 Caco-2 cell models

Caco-2 cells are human epithelial cell models that are isolated from colon carcinoma and can be co-cultured to obtain cell lines in order to have better simulation of human intestinal mucosa. The bioaccumulation and toxicity level, the adhesion to or the absorption rate through intestinal epithelial cells can be evaluated by using this model (Gonzales *et al*., 2015).

2.3.4 Studies on *in vitro* release of encapsulated lipids

For fod applications, control of lipid digestion is cruial to create novel food products, therefore, in vitro controlled release studies considering digestion lipids delivered by different food grade models gained much attentiton by researchers. In the study of Li and McClements (2011), medium chain trigliceride are encapsulated within hydrogel beads and in vitro controlled release study was performed by simulating the conditions of small intestine. Lipid digestion was retarded more in large hydrogel beads than small hydrogel beads. In addition, the total amount of free fatty acids released from the encapsulated lipids was less than that from the free lipids.

Siow and Ong (2013) encapsulated garlic oil by complex coacervation of gelatin and gum arabic with/without cross-linking agent (formaldehyde) treatment. In the study, *in vitro* controlled relase study was performed for 5 h of incubation in pepsin solution with a pH of 2. According to the results, the cross-linked coacervates showed significantly slower release than non-cross-linked ones ($p \leq 0.05$).

In an another study, Ifeduba and Akoh (2015) successfully entraped SDA soybean oil within the gelatin and gum arabic coacervates to conduct *in vitro* controlled release study with the use of simulated gastric and intestinal fluids set at 37 °C and 120 rpm for 2 h. The maximum amount of free fatty acid released from the coacervates were about 53% and 56% for gastric and intestinal phases.

Soybean oil was also encapsulated by complex coacervation using gelatin and gum arabic in order to perform controlled release study at at 37 °C for 2 h in constant pH (pH = 1.2). Transglutaminase and glutaraldehyde were used to determine the effect of differents types of cross-linking agents on release from coacervates. The maxiumum oil release was recorded as 32.3% and 10.3% for transglutaminase and glutaraldehyde treatments, respectively (Tello *et al*, 2016).

In the study of Comunian *et al*. (2017), *E. plantagineum* seed oil was encapsulated by complex coacervation of gelatin-arabic gum and gelatin-cashew gum for food application. Also, sinapic acid (cross-linker/antioxidant) was used in the microencapsulation process. The release study of oil was performed by *in vitro* gastrointestinal digestion model using simulated gastric and intestinal fluids for 180 min at 37 °C. After 180 min, 45% and 76% of the oil was released in simulated gastric and intestinal fluids, respectively.

2.4 Anti-tumorigenic Activity and Cell Culture Study

2.4.1 Cancer

Cancer is a large group of diseases caused by the uncontrolled growth of healthy cells in any tissue of the body. Cancer cells vary according to their tissue, cell type and mutations. It is possible to divide the cancer into five groups according to the tissue type: carcinoma, sarcoma, leukemia and lymphoma, germ cell tumor and blastoma (Nabavi *et al*., 2015).

According to worldwide mortality rates, cancer is the second reason of death and breast cancer is the most common type among women. Carcinogenesis is caused by genetic and epigenetic factors such as inherited mutations, hormonal changes, smoking, alcohol consumption, pollution, exposure to ultraviolet rays and carcinogenic agents and viral infections (Hyndman, 2016; Nabavi *et al*., 2015).

2.4.2 Role of fatty acids in cancer

Eating habits of people is an effective parameter of cancer epigenetics. A fat-rich diet is linked to higher incidence of cancer. Besides, in the metabolic pathway of n-6 and n-3 (Figure 2.5), LA competes with ALA for Δ-6 desaturase enzyme and therefore restricts the conversion of ALA to EPA in the body. The western diet with higher ratio of n-6/n-3 fatty acids ratio is associated with cancer, because LA and ALA play crucial role in the biosynthesis of pro- and anti-inflammatory molecules, respectively. On the other hand, epidemiological studies indicated that diet with higher content of n-3 FAs decrease the risk of certain types of cancer including breast cancer (Xue *et al*., 2014; Zhang *et al*., 2008). N-3 FAs inhibits the formation of n-6 FA derived prostaglandin E2 that are the molecules contributes the inflammatory response, cell growth, apoptosis, angiogenesis and metastasis (Horia and Watkins, 2005).

However, studies on n-3 FAs still unadequate to make clear the molecular mechanisms of cancer inhibition but it is proposed that n-3 FAs may alter the lipid composition and fluidity of the cell membrane (Corsetto *et al*., 2011). In addition, in the presence of n-3 FAs, membrane of cancer cells that contain plenty of unsaturated FAs are more liable to free radicals (Nabavi *et al*., 2015). Besides, tumor suppression

of n-3 FAs can also occurred via interfering cell replication or leading to cell death by apoptosis or necrosis (Corsetto *et al*., 2011).

2.4.3 MCF-7 cell lines

Breast cancer is the most common type of cancer among women in the world and it affects about 2.1 million women per year (Url-3). The main risk factors for breast cancer listed as inherited genes (BRCA1 and BRCA2), obesity, alcohol consumption, high estrogen levels and age. BRCA 1 and BRCA2 are tumor suppressor genes that play a role in DNA repair mechanism. It is observed that 10% of breast cancer cases caused by mutations of these genes (Klevos *et al*., 2017).

MCF-7 is the most studied human breast cancer cell line in the world (Lee, Oesterreich and Davidson, 2015). MCF-7 cell line was first isolated from the pleural effusion of a female patient by Dr. Soule and co-workers in 1973 at the Michigan Cancer Foundation (MCF) where the cell line takes its name. MCF-7 cells exhibit a similar morphology to epithelial cells and proliferate in monolayer in vitro. These cells have low metastatic potential (Comşa, Cîmpean and Raica, 2015).

2.4.4 Studies on cancer cell lines

Cancer preventive/suppressive activity of EPA and DHA has been shown in numerous studies. Furthermore, studies has unraveled that EPA and DHA have synergistic effect when used in combination with chemo- and radio therapy of cancer cell lines besides, studies have indicated that the apoptosis mechanism is triggered by n-3 FAs (EPA and DHA) in cancer cell lines (Nabavi *et al*., 2015).

N-3 polyunsaturated fatty acids have also been associated with a reduced risk of colon cancer (Terry *et al*., 2003). Colon cancer is one of the major causes of cancer death in the worldwide (Jemal *et al*., 2010). Besides, many patients with colon cancer develop a resistance to chemotherapy (Coleman *et al*., 2008). However, some studies have showed that n-3 PUFAs increases the sensitivity to chemotherapy (Calviello *et al*., 2005) and radiotherapy (Cai *et al*., 2014) of different colon cancer cell lines.

DHA was also investigated for its possible cytotoxic effect on neuroblastoma cells. In the study of Lindskog *et al*. (2006), the human neuroblastoma cell lines were incubated with drugs and also fatty acids (DHA and OA) at a concentration ranged 25-150 μM for 24, 48, 72, or 96 h. It was found that DHA was cyctotoxic to the

neuroblastoma cells and it increased the drug sensitivity of the cancer cells. On the contrary, OA had no effect on cell viability of neuroblastoma cell lines.

Fish oils were also studied by researcers for their anti-tumorigenic activity. Rehman *et al*. (2016) treated human non-melanoma carcinoma cell lines with imiquimod (IMQ), combination of IMQ- fish oil (42% EPA and 21% DHA), EPA and DHA for 24 h. The results showed that IMQ- fish oil treatment was effective in inhibition of cancer cell growth.

In an *in vitro* study, SDA and ALA were compared for their application on breast cancer cell lines. The researchers investigated the levels of cyclooxygenase-2 (COX-2) and prostaglandin E2 levels since they are associated with increased tumorigenic and metastatic potentials of human breast cancer cells. According to the findings of the study, SDA was found to be more effective than ALA on suppression of COX-2 gene expression (Horia and Watkins, 2005).

Corsetto *et al*. (2011) have investigated the effects of AA, EPA and DHA on MDA-MB- 231 and MCF-7 human breast cancer cell lines by cell viability, lipid composition and Western blot analysis. In the study, the cancer cells were treated with 200 μM AA, 230 μM EPA and 200 μM DHA during incubation at 37 °C for 72 h. According to the results, EPA and DHA leads to an increase in PUFA content of cancer cell membrane, also a decrease in number of viable cancer cells by triggrering cell apoptosis.

In an another study, Bonatto *et al*. (2015) have demonstrated that there has been a reduction in the cell viability for breast tumor derived from surgical resections that were treated with 1.5 and 1.10 (v/v) dilutions of fish oil. In addition, according to apoptosis and necrosis analyses, fish oil treated cells showed higher apoptosis and lower necrosis.

Moreover, researchers have also indicated that DHA treatment altered the signalling pathway in MCF-7 cancer cell line which leads to apoptosis of cancer cells. In the study, cancer cell lines were incubated with both n-6 and n-3 series of FAs at a concentration of 100 μM (Tsai *et al.*, 2017).

Stearidonic acid-enriched flax seed oil has also been used in the MCF-7 cancer cell line study. In the research of Subedi *et al*. (2015), the cells were incubated with 150 μM mixture of fatty acids that represented the SDA-enriched flax oil at 37 °C for 48

h. The researchers observed about 19% inhibition in the proliferation of cancer cells after the oil treatment procedure when compared to the control cells. Also, animal feeding experiments showed that a diet containing SDA-enriched flax seed oil suppressed the tumor growth.

3. USE OF *ECHIUM* OIL FATTY ACIDS AND TRICAPRYLIN AS SUBSTRATES OF ENZYMATIC INTERESTERIFICATION FOR THE PRODUCTION OF STRUCTURED LIPIDS[1]

3.1 Abstract

Structured lipids (SLs) were produced from free fatty acids of *Echium* oil and tricaprylin by enzymatic acidolysis reactions. Lipozyme® RM IM, immobilized *sn*-1,3 specific lipase was used in the enzymatic reactions. In order to optimize incorporation of stearidonic acid (SDA), three factors were chosen [Reaction temperature (50-60 ºC), reaction time (6-12 hour) and substrate molar ratio (3-6 mol/mol (total free fatty acids/tricaprylin)] for application of response surface methodology (RSM) by using central composite circumscribed design (CCC) with five levels. Optimum temperature, time and substrate molar ratio obtained from the models were 60 ºC; 6 h, 6 mol/mol, respectively. Furthermore, SLs with 6.2% SDA content at *sn*-2 position were produced by scaling up process. SL was obtained with nearly 78-79% of long-chain fatty acids at *sn*-2 position. According to melting profile analysis, the melting peaks of tricaprylin and *Echium* oil were sharper and narrower while the SL had more broadened peaks.

Keywords: *Echium oil; Enzymatic acidolysis; Lipozyme® RM IM; Stearidonic acid; Response surface methodology; Tricaprylin*

3.2 Introduction

Health benefits of medium chain fatty acids (MCFAs) and long chain fatty acids (LCFAs) are well documented and today's biotechnological improvements allow the production of SLs including both of these fatty acids in one triacylglycerol (TAG)

[1] This chapter is based on the paper "Yüksel, A. and Şahin-Yeşilçubuk, N. (2018). Use of *Echium* oil fatty acids and tricaprylin as substrates of enzymatic interesterification for the production of structured lipids, *Grasas y Aceites*, *69* (1), e236."

molecule. Recently, production of MLM-type SLs with medium chain fatty acids (MCFAs, C6-C10) at *sn*-1 and *sn*-3 positions, and long chain saturated and unsaturated fatty acids (LCFAs, C12-C24) at *sn*-2 position has gained attention for clinical and nutritional purposes (Osborn and Akoh, 2002). During digestion, MCFAs are cleaved from the TAG molecule via activity of *sn*-1,3 specific pancreatic lipase and then transported to liver through portal vein where it is rapidly turn into glucose. In addition, MCFAs do not have carnitine dependence and they don't need chylomicrons; and can easily be oxidized to produce energy as well. Furthermore, MCFAs do not further esterified as a newly synthesized TAG molecule thus they have low tendency to be deposited as body fat which is beneficial for the control of body weight (Akoh *et al*., 2002; Osborn and Akoh, 2002). On the other side, LCFAs are absorbed as *sn*-2 monoacylglycerol (MAG) through lymphatic system and are mainly used in biosynthetic processes (Mu and Hoy, 2004).

MCFAs have been used for years to satisfy the nutritional needs of patients with lipid malabsorption such as Crohn's disease, cystic fibrosis, colitis and premature birth (Akoh *et al*., 2002). However, MCFAs do not supply the essential fatty acid requirement of the body, so they cannot be used as lipid source alone (Nunes *et al*., 2011). For this reason, MLM-type SLs are designed as one TAG molecule represents both MCFAs and LCFAs which permit more controlled release of fatty acids into bloodstream (Osborn and Akoh, 2002). Studies unraveled that MLM-type emulsions are the safer and effective way of energy delivery for patients who needs long term parenteral nutrition (Rubin *et al*., 2000; Matulka, Noguchi and Nosaka, 2006). Enzymatic synthesis of MLM-type SLs has gained importance in recent years. Enzymatic production of MLM-type SLs has been studied by using different substrate sources. In the study of Hita *et al.* (2009), MLM-type SL with caprylic acid at *sn*-1,3 positions and docosohexaenoic acid (DHA) at *sn*-2 position was produced via enzymatic reactions. Researchers concluded that designed SL has the potential of being an important nutrition component for the development of central nervous system of premature babies. Kim *et al.* (2010) designed a MLM-type SL consisting of caprylic acid and gamma-linolenic acid (GLA) derived from borage oil by enzymatic synthesis method for premature babies and for those who have lipid malabsorption such as cystic fibrosis. In another study, corn oil and caprylic acid were the substrates of enzymatic process yielding with MLM-type SL purposed for

the patients with special nutrition requirements (Öztürk, Ustun and Aksoy, 2010). In the study of Nunes *et al.* (2011), MLM-type SL aimed for clinical nutrition was produced via enzymatic reactions between olive oil and capric acid.

SDA is an important fatty acid since it is an intermediate metabolite of omega-3 pathway in which alpha-linolenic acid (ALA) is converted to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and shows similar health effects like EPA and DHA. SDA is more efficiently converted into EPA and DHA when compared to ALA (Whelan, 2009). Oil from the plant source *Echium plantagineum L., Boraginaceae,* black currant, the families *Primulaceae* and *Grossulariaceae* have high concentrations of SDA, and also some microbial oils, fish oils, and genetically engineered soybean and canola are the sources of SDA (Whelan, 2009; Surette, 2013). SDA originated from plants has the potential of being sustainable and vegetarian EPA source since EPA is mainly derived from fish oils. Fish stocks are decreased day by day due to over fishing activities and negative effects of climate change. Also, fish oils may have some environmental pollution (dioxins, polychlorated biphenyls, toxic heavy metals such as mercury) as well as have undesired smell and taste (Chávez-Servín *et al*., 2009). Furthermore, PUFA content of the oil reaches up to nearly 60%. Thus, due to the high presence of SDA content of *Echium* oil, it was chosen as one of the substrate oil. Since the aim of the study was to incorporate long-chain fatty acids into the *sn*-2 position of the SLs, *Echium* oil served to fulfill the desired oil composition and distribution of the fatty acids in the newly synthesized TAG structure.

In the literature, there are several studies about enzymatically synthesized SLs with the use of different SDA plant sources as substrate oil. In the study of Kleiner *et al*. (2012), firstly, low temperature crystallization (LTC) was applied in order to increase SDA content of modified soy bean oil from 25% to 48.72% (TAG) and 60.78% (FFA). In the next step, SL with the highest SDA content (53.46 ± 1.85% SDA with 36.37 ± 3.14% at *sn*-2 position) was produced via acidolysis reaction between TAG and FFA of LTC catalyzed by Lipozyme® TL IM in solvent free conditions. Ifeduba and Akoh (2014) used genetically modified SDA soybean oil and caprylic acid to produce SLs via enzymatical reactions in their study. In an another study, Gökçe *et al*. (2013) obtained low calorie SL with 64.4% of PUFA content at *sn*-2 position via enzymatic acidolysis reaction catalyzed by Lipozyme® RM IM where *Echium* oil and

lauric acid were used as oil source. Bilgiç *et al*. (2012) used *Echium* oil and olive oil as substrates of enzymatic acidolysis reactions catalyzed by Lipozyme® TL IM in order to incorporate SDA into olive oil. As a result of the study, SLs were produced 4.9% SDA and 43% PUFA content. To sum up, previous studies used SDA rich oil sources or *Echium* oil to enrich oils in terms of omega-3 and omega-6 PUFAs (Bilgiç and Yeşilçucuk, 2012; Kleiner *et al*., 2012; Gökçe, Yeşilçubuk and Üstün, 2013; Ifeduba and Akoh, 2014). However, no previous study investigated the influence of reaction conditions for obtaining MLM-type SL from *Echium* oil fatty acids and tricaprylin. Therefore, in this study, production and optimization of MLM-type SLs containing SDA via enzymatic acidolysis reactions was aimed. In order to characterize the products, fatty acid composition, *sn*-2 positional composition and melting profiles of the SLs were determined.

3.3 Materials and Methods

3.3.1 Materials

Echium oil (*E. plantagineum*) used in this study was purchased from Harke Group, GmbH (Germany). Tricaprylin was purchased from Sigma Chemical Co. (St. Louis, MO). Lipozyme® RM IM commercially immobilized *sn*-1,3 specific lipase from Rhizomucor miehei was donated by Novo Nordisk A/S branch (İstanbul, Turkey). The enzyme activity of Lipozyme® RM IM was 275 UIN/g. TLC plates were purchased from Merck (Whitehouse, NJ). All other reagents and solvents which were purchased either from Sigma Chemical Co. (St. Louis, MO) and Merck (Whitehouse, NJ) were of analytical or chromatographic grade.

3.3.2 Preparation of free fatty acids from *Echium* oil

Echium oil was saponified to obtain free fatty acids according to Yüksel and Yeşilçubuk (2012). For the saponification process, 25 g of *Echium* oil and 5.75 g of KOH were weighed into a flask and 11 mL of distilled water and 66 mL of aqueous ethanol [95% (v/v)] was added into the oil. The mixture was heated in a water bath at 60 °C for 1 hour. After 1 h, the reaction was stopped by adding 60 mL of distilled water. The unsaponifiable matter was extracted twice into hexane layer (100 mL) and they were discarded. Then, the mixture was acidified (pH = 1.0) with 3 mol/L of HCl. After acidification, free fatty acids were extracted into 50 mL of hexane which

was further passed through anhydrous sodium sulfate column. Hexane was evaporated at 40 °C by using rotary evaporator. The free fatty acids were stored at -18°C for further use.

3.3.3 Acidolysis reactions

Acidolysis reactions with different molar ratios of free fatty acids and tricaprylin were performed in screw-capped amber glass bottles (Table 1). Lipozyme® RM IM (10% weight of total substrates) was added to the reaction medium together with 3 mL of n-hexane. The reaction mixture was placed in an orbital shaker (IKA, KS4000i, Germany) rotating at 200 rpm at different conditions determined by RSM design generated by Modde 11.0 (Umetrics, Umeå, Sweden). After the enzymatic reactions, reaction mixture was passed through a glass column packed with anhydrous sodium sulfate as described in Sahin *et al*. (2005a). All reactions were carried out in duplicate and the results are reported as the average data.

3.3.4 Experimental design for RSM study

A three-factor, 5-level central composite circumscribed design (CCC) was applied by the use of Modde 11.0 (Umetrics, Umeå, Sweden) software to investigate how chosen parameters affect incorporation of SDA during the enzymatic reactions. Reaction temperature (T, °C) (50-60 °C), reaction time (t, h) (6-12 h) and substrate molar ratio (Sr, total free fatty acids/tricaprylin (3-6 mol/mol) were the parameters of the response surface methodology (RSM) study. The factor ranges were selected according to the preliminary studies (data not given here) and literature survey. Table 2.1 shows the independent variables together with the experimental design of this study. Experiments were carried out randomly and average of two parallel results of each experiment points are reported as the mol% of the fatty acids.

3.3.5 Analysis of reaction products

Reaction products (50 µL) were separated as TAG band on a silica gel G TLC plate (20 x 20 cm) by using petroleum ether, diethyl ether, and acetic acid (80:20:0.5, v/v/v) mixture as solvent system. 0.2% 2,7-dichlorofluorescein in methanol was sprayed on TLC plate to make the TAG bands visible under UV light. Then, the TAG bands were incubated with 3 mL of 6% HCl in methanol and 40 µL of C17:0 internal standard (10 mg/mL) in test tubes and they were placed in an oven at 75 °C.

After 2 hours, reaction products were mixed with 2 mL of *n*-hexane and 1 mL of 0.1 M KCl and were centrifugated at 1000 rpm for 3 minutes to obtain upper layer which was then passed through anhydrous sodium sulfate column. Finally, fatty acid methyl esters (FAME) obtained were then analyzed by gas chromatography to evaluate the fatty acid composition of substrate oils and reaction products (Jennings and Akoh, 1999).

3.3.6 Fatty acid composition analysis

Shimadzu GC 2010 Plus gas-liquid chromatography (GLC) (Milan, Italy) equipped with a flame-ionization detector (FID) and SP-2380 capillary column (60m x 0.32mm ID x 0.20 μm film thickness) (Supelco Inc., Bellefonte, PA, USA) was used in order to evaluate substrate oils and reaction products for their fatty acid composition. Both the injector and detector temperatures were held at 250 °C. The oven temperature was initially held at 60 °C for 1 min, and was then programmed to 165 °C for 30 min at a rate of 20 °C/min (first ramp); to 190°C for 35 min at a rate of 10 °C/min (second ramp); to 210 °C for 10 min at a rate of 20 °C/min (third ramp). The hydrogen was used as the carrier gas and flow rate was 1.02 mL/min. Sample volume was 1 μL and relative amounts of FAME were calculated as mol% fatty acid (FA) by computer with reference to heptadecanoic acid as the internal standard. Reported results were the average values of duplicate analyses.

3.3.7 *Sn*-2 positional fatty acid analysis

TAG bands scrapped off from TLC plates were incubated with 1.0 M Tris-HCl buffer (2 mL), 0.05% bile salt solution (0.5 mL), 2.2 g/100 g calcium chloride solution (0.2 mL) and pancreatic lipase (40 mg) in the test tubes placed in a water bath at 40 °C for 3 min. 6 mol/L HCl (1 mL) and diethyl ether (4 mL) were added and then centrifuged. An anhydrous sodium sulfate column was used to filter the upper phase. Thereafter, a 200 μL aliquot was spotted on TLC plates coated with silica gel G, and TLC plates were placed into the tank and hexane, diethyl ether, and formic acid (60:40:1.6, v/v/v) were used as developing solvents. After the band seperation, 2,7-dichlorofluorescein in methanol (0.2 g/100 mL) was sprayed on TLC plate in order to visualise the 2-monoacylglycerol (2-MAG) band under UV light. For identification, 2-monoolein standard (Sigma) was used as marker. The 2-MAG band corresponding to the marker bang was scrapped off into a screw-capped test

tube, methylated and analyzed by GLC as previously described (Pina-Rodriguez and Akoh, 2009a).

3.3.8 Scale-up process

For scale-up synthesis of SLs, optimum reaction conditions generated by RSM was employed. Levels of tricaprylin and total free fatty acids were increased approximately 40 times. Also, enzyme and hexane levels were scaled-up to levels those agreed with the increased amount of substrate oils. The reaction mixture containing substrates at a 6 mol/mol substrate molar ratio was incubated at 60 ºC and agitated in an orbital shaking air-bath at 200 rpm for 6 hours. In the end, the mixture was passed through anhydrous sodium sulfate column in order to stop the enzymatic reaction.

3.3.9 Removal of free fatty acids from the reaction mixture

The reaction products of the scale-up process were purified according to the procedure outlined by (Lee *et al*., 2004). After the enzymatic interesterification reaction, excess hexane was removed by a rotary evaporator, and 60 mL of 0.5 mol/L KOH solution prepared with ethanol (20 mL/100 mL) and 110 mL hexane were added to the product subsequently transferred to a separatory funnel. The upper layer was collected, mixed with 3-4 drops of phenolphthalein solution and titrated with 0.5 mol/L KOH solution prepared with ethanol (20 mL/100 mL). 30 mL of saturated NaCl solution was added to the mixture when the color of the mixture turned to pink. Then, the mixture was shaken vigorously and passed through anhydrous sodium sulfate column. Collected hexane phase was removed by rotary evaporator and kept at -18 °C until used.

3.3.10 Melting profile determination

The melting profiles of tricaprylin, *Echium* oil and the SL product of the scale-up process were determined by differential scanning calorimetry (DSC) Q10 model (TA Instruments, New Castle, DA) according to the AOCS Official Method Cj 1-94 (1989).

3.3.11 Statistical analysis

The regression analysis, statistical significance, analysis of variance (ANOVA), and response surface applications were carried out by using Modde 11.0 (Umetrics, Umeå, Sweden) software. Second-order coefficients were generated by regression analysis. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and ANOVA data.

A second-order polynomial model was used to fit the data obtained from the experimental design as shown in Table 3.1:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^{\,2} + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad (3.1)$$

where Y is the response (incorporation of SDA at *sn*-1, 2, 3 positions), β_0 is the intercept; β_i is the linear term (first-order model); β_{ii} is the quadratic term (second-order model), β_{ij} is the interaction regression coefficients, and X_i and X_j are the independent variables.

3.4 Results and Discussion

3.4.1 Fatty acid profile of the substrate oils

Fatty acid profile of substrate oils (tricaprylin and *Echium* oil) was determined by GLC-FID. The samples were analyzed in duplicate and average results were calculated. According to the results, tricaprylin consisted of 99.5% caprylic acid. Besides, *Echium* oil fatty acids contained predominantly α-linolenic acid (31.7 %), oleic acid (14.5 %), linoleic acid (14.1 %), stearidonic acid (13.7 %) and γ-linolenic acid (10.7 %). Fatty acid profile of *Echium* oil fatty acids was found to be close to the *Echium* oil fatty acid composition (Guil-Guerrero *et al*., 2000a; Özcan, 2008; Bilgiç and Yeşilçubuk, 2012).

3.4.2 Model fitting for the optimization study

Production of MLM-type SLs containing stearidonic acid at *sn*-2 position between tricaprylin and free fatty acids of *Echium* oil by enzymatic acidolysis reactions was aimed in this current study. Experimental design with three-factor and five-level CCC was applied in order to optimize the reaction conditions. Table 3.1 showed each

design point and observed responses in terms of SDA incorporation at *sn*-1, 2, 3 positions (mol%). Regression coefficients (β) and significance (P) values are given in Table 3.2.

As it can be seen from Table 3.2, first-order parameters such as time (t) and substrate molar ratio (Sr) were significant and they both had positive effects on the incorporation of SDA. Since the other terms such as temperature, quadratic terms and interaction terms were not found to be significant, they were not presented in Table 3.2. Therefore, the model equation including the significant terms for SDA incorporation at *sn*-1, 2, 3 positions can be written as:

$$\text{SDA incorporation (mol\%)} = 5.85 + 0.41t + 0.84Sr \quad (3.2)$$

Table 3.1: Experimental design and observed responses for incorporation of SDA(mol%)[a,b]

Experiment No	T (°C)	t (h)	S_r (mol/mol)	SDA incorporation (mol %)
1	50	6	3	4.28
2	50	12	3	5.07
3	60	6	3	3.91
4	60	12	3	5.09
5	50	6	6	5.46
6	50	12	6	6.19
7	60	6	6	6.06
8	60	12	6	6.13
9	55	3.95	4.5	4.50
10	55	14.05	4.5	6.21
11	46.59	9	4.5	4.80
12	63.41	9	4.5	6.18
13	55	9	1.98	3.55
14	55	9	7.02	7.08
15	55	9	4.5	5.65
16	55	9	4.5	6.10
17	55	9	4.5	5.78

[a]Mean, *n=2*. [b]Abbreviations: T, reaction temperature (°C); t, reaction time (h); Sr: substrate molar ratio (mole of total free fatty acids/mole of tricaprylin).

Table 3.2 : Regression coefficients (β) and significance levels (*P*-Values) for incorporation of SDA[a].

Variables	Coefficients (β)	*P*-value [a]
Constant	5.85	< 0.0001
t	0.41	0.0092
Sr	0.84	0.002

[a]*P* value, level of significance. See Table 3.1 for other abbreviations.

Fitness of the model was evaluated by analysis of variance, and the results are given in Table 3.3. According to the ANOVA analysis presented in Table 3.3, since F_{model} (8.07) is very high compared to $F_{9,7}$ value (3.69) (α=0.05), obtained model is regarded as suitable for prediction (Sahin *et al*., 2005a; Sahin *et al*., 2005b; Yüksel and Yeşilçubuk, 2012). Besides, model error is also used to determine if the model is fit to the experiment results. Since *p* value of the model error for SDA incorporation at *sn*-1, 2, 3 positions was 0.195, there were no significant ($p > 0.05$) lack of fit in the model (Rao *et al*., 2002; Lumor and Akoh; 2005; Sahin *et al*., 2005a; Chopra *et al*., 2011).

Table 3.3 : ANOVA table for SDA incorporation[a].

	SDA Incorporation				
	DF	**SS**	**MS**	***F* value**	***P*- value**
Total	17	512.96	30.17		
Constant	1	498.32	498.32		
Total correcred	16	14.65	0.92		
Regression	9	13.36	1.48	8.07	0.006
Residual	7	1.29	0.18		
Lack of fit (model error)	5	1.18	0.24	4.40	0.195
Pure error (replicate error)	2	0.11	0.05		

[a]Abbreviations: DF, Degree of freedom; SS, Sum of squares; MS, Mean squares.

The coefficient of determination (R^2) for SDA incorporation at *sn*-1, 2, 3 positions was found as 0.91. Also, relationship between observed values and predicted values from the model is given in Figure 3.1. According to the graph given in Figure 3.1, the observed vs. predicted plot of SDA showed a linear distribution. It also means that, the model generally represents the real relationship between the reaction parameters and the response (Yang *et al*., 2003).

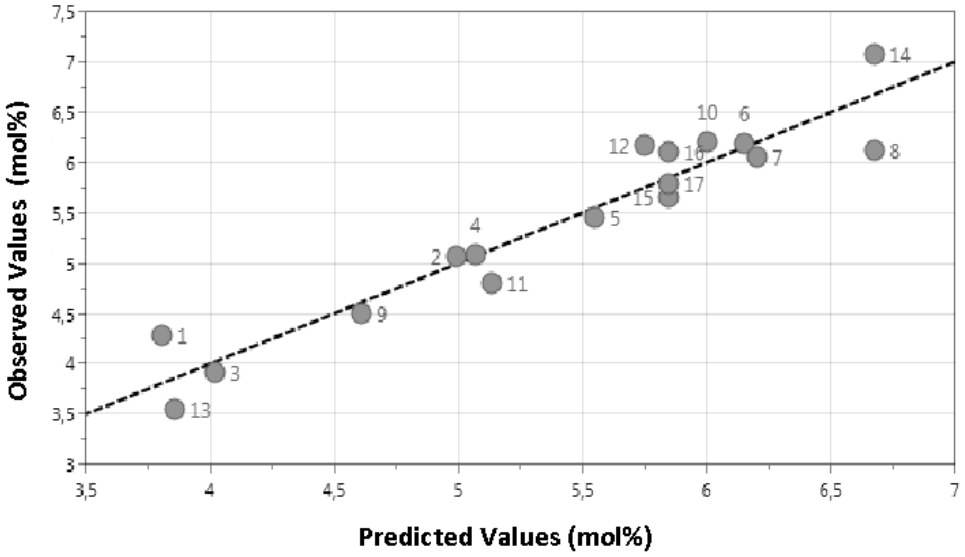


Figure 3.1 : The observed vs. predicted plot of SDA incorporation.

3.4.3 Optimization study

Response surface methodology was used to evaluate and also to predict/optimize relationship between reaction parameters and response. The contour plots obtained by interaction of temperature, time and substrate molar ratio on enzymatic incorporation of SDA into tricaprylin are given in Figures 3.2, 3.3 and 3.4. While drawing the contour plots, the third variables were kept at medium values.

As can be seen from Figure 3.2, SDA incorporation at *sn*-1, 2, 3 positions increases with the increase in both time and substrate molar ratio within the observed experimental ranges (Temperature: 60 ˚C).

Effect of substrate molar ratio and temperature on incorporation of SDA at *sn*-1, 2, 3 positions is shown in Figure 3.3. It can be revealed that SDA incorporation increases with increasing substrate molar ratio almost independently from temperature within the observed experimental ranges (Time: 9 h).

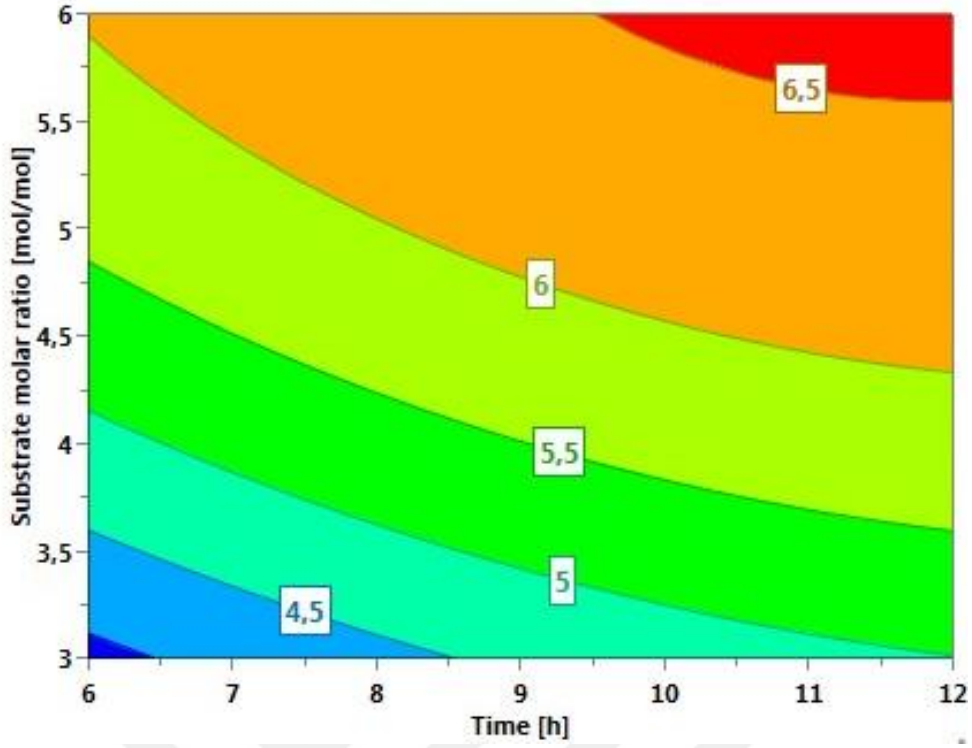


Figure 3.2 : Response contour plots between substrate molar ratio and time for SDA incorporation.

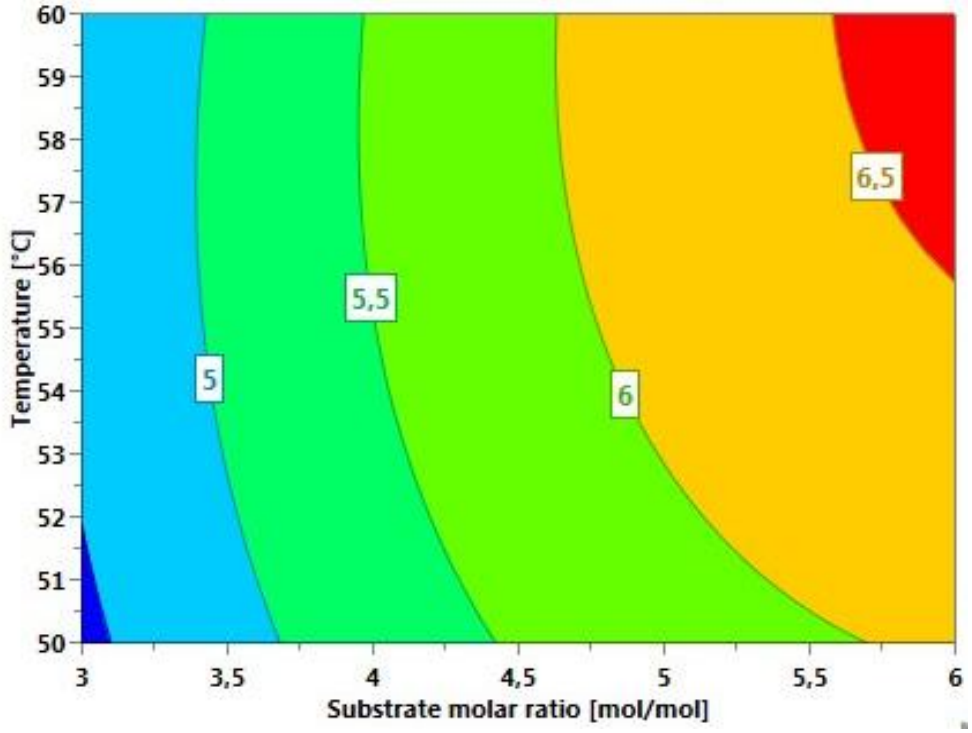


Figure 3.3 : Response contour plots between temperature and substrate molar ratio for SDA incorporation.

Effects of time and temperature on incorporation of SDA at *sn*-1, 2, 3 positions are shown in Figure 3.4. According to this figure, SDA incorporation increases with the increase in time within the observed experimental ranges (Substrate molar ratio: 6 mol/mol).

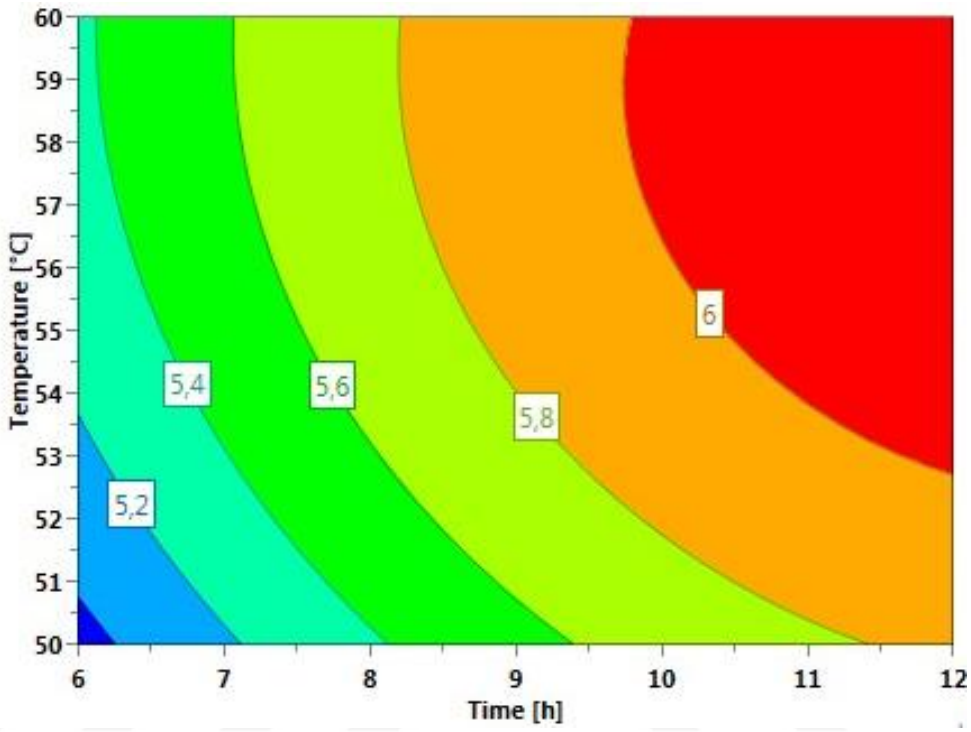


Figure 3.4 : Response contour plots between temperature and for SDA incorporation.

In this work, it was aimed to maximize the SDA incorporation at *sn*-1, 2, 3 positions, thus according to optimizer function of Modde 11.0, the optimal conditions for the maximum incorporation of SDA (6.8 mol%) was found as 59.9 °C, 10.7 hours and 6.6 mol/mol for temperature, time and substrate molar ratio, respectively. In order to reduce the cost of enzymatic process, lower substrate molar ratio and a shorter reaction time were selected as optimal conditions for SDA incorporation which were 60 °C reaction temperature, 6 h reaction time and 6 mol/mol substrate molar ratio. At these reaction conditions, 6.1% SDA incorporation was predicted from the generated model.

3.4.4 Model verification and scale-up production at optimal reaction conditions

Optimal conditions for SDA incorporation at *sn*-1, 2, 3 positions were determined as 60 °C, 6 h and 6 mol/mol substrate molar ratio and SDA incorportion was predicted as 6.1% at these conditions. In order to confirm the model prediction power,

enzymatic reactions at these conditions were performed in small scale. Moreover, scale-up process was also carried out at these optimum conditions. The results of both small scale and scale-up processes are given in Table 3.4.

Table 3.4 : Fatty acid composition and *sn*-2 positional distributions of fatty acids (mol%) of SLs produced under optimal conditions

	Small-Scale Experiments		**Scale-up Process**	
Fatty Acid	***sn*-1, 2, 3**	***sn*-2**	***sn*-1, 2, 3**	***sn*-2**
C8:0	50.08	21.00	53.94	21.64
C16:0	4.17	22.45	5.23	24.62
C18:0	1.78	21.67	1,94	13.92
C18:1n-9	7.85	20.47	7.24	17.71
C18:2n-6	7.74	nd[a]	6.31	nd
C18:3n-6	4.60	nd	4.41	nd
C18:3n-3	17.42	7.93	14.33	15.91
C18:4n-3	6.06	6.48	6.61	6.20

[a] nd, not detected.

As can be seen in Table 3.4, the experimental incorporation value for SDA obtained from both small scale (6.1%) and scale-up (6.6%) production was satisfactorily close to each other and to the predicted value (6.1%) from the generated model. *Sn*-2 position of SLs were mostly (78-79%) occupied by long chain fatty acids including palmitic, stearic, oleic, α-linolenic and stearidonic acids. In addition to this, the yield of the reaction product after the removal of FFAs was 20.5%.

3.4.5 Melting behavior of substrate oils and SL

Melting behavior of substrate oils (tricaprylin and *Echium* oil) and SL obtained in scale-up production were evaluated by the DSC melting thermograms shown in Figure 3.5. Main endothermic/exothermic peaks are consecutively numbered in each thermograph. Figure 3.5 shows the melting profile of tricaprylin with one sharpen peak (peak 3 at 10 °C) since it contains saturated fatty acids at about 99.5%. Besides, tricaprylin has another two peaks at -50 °C and -15 °C. *Echium* oil has four melting points (-49 °C, -34 °C, -27 °C and -11 °C, respectively) as it can be seen in Figure 3.5. If we compare melting behavior of tricaprylin and *Echium* oil, *Echium* oil has narrower melting point range (-50 to -10 °C). Figure 3.5 also shows the melting thermogram of SL produced in scale-up process. This thermogram reveals that the incorporation of unsaturated fatty acids into tricaprylin was resulted with the

presence of new melting points at lower temperatures (peaks 1, 2, 3 and 4 indicated the temperatures -47 °C, -37 °C, -22 °C and -6 °C, respectively). Moreover, characteristic melting peak of tricaprylin (peak 3 of tricaprylin thermogram) was disappeared. SL has no characteristic melting peak when compared to DSC thermograms of tripcaprylin and *Echium* oil.

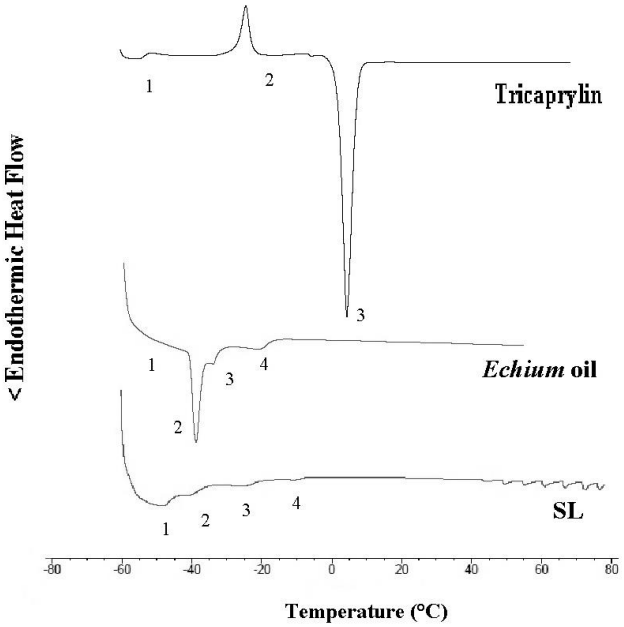


Figure 3.5 : DSC melting thermograms of tricaprylin, *Echium* oil and SL.

3.5 Conclusions

MLM-type structured lipid obtained as a result of this study contained SDA as well as other omega-3 fatty acid ALA together with medium chain caprylic acid. We believe that MLM-type SLs produced within the concept of this study will satisfy the needs of patients with special nutrition requirements as well as consumers who increasingly demand functional foods having health benefits. These SLs can be used for patients having lipid malabsorption, for premature babies, hospitalized patients for supplying quick energy and more. Moreover, construction of novel or designer structured lipid molecules from *Echium* oil substrates will be promising for food, therapeutic, and nutritional uses in the near future.

4. EVALUATION OF ANTI-TUMORIGENIC ACTIVITY BY CELL CULTURE STUDIES

4.1 Introduction

Today, it is well known that diet plays a key role for being healthy. Lipids supply energy, essential fatty acids and fat-soluble vitamins requirements of humans (Akoh *et al*., 2002). N-3 and n-6 series of PUFAs have beneficial effects on many diseases from diabetes to cancer. Recently, increase in fat consumption and the high n-6/n-3 ratio in diet are major problems of today's lifestyle. It has been shown that fat-rich diets and high n-6/n-3 ratio in diet has been linked to increase the incidence of certain type of cancer (Nabavi *et al*., 2015). Contrary, cancer preventive/suppressive activity of n-3 PUFAs has been shown in numerous studies.

Breast cancer is the most common type of cancer among women worldwide and it affects about 2.1 million women per year (Url-3). MCF-7 is the most studied human breast cancer cell line in the world (Lee, Oesterreich and Davidson, 2015). In the study of Horia and Watkins (2005), human breast cancer cells were treated with SDA and ALA for their possible anti-tumorigenic activity. According to the results, it was concluded that SDA was more effective than ALA in suppression of cancer related genes.

MCF-7 cancer cell line was treated with SDA-rich flax seed oil in the study of Subedi *et al*. (2015). After 48 h incubation, the cell viability of cancer cells were inhibited about 19%. Besides, according to animal studies, SDA-enriched flax seed oil- feeding suppressed the tumor growth.

In an another study, human breast cancer cell lines were incubated with n-3 FAs at different concentrations for 72 h. The data gathered from the cell viability test showed that EPA and DHA inhibited the cancer cell growth via cell apoptosis (Corsetto *et al*., 2011).

Tsai *et al.* (2017) used MCF-7 cancer cell line in order to understand possible anti-tumorigenic effects of n-6 and n-3 series of FAs. The study revealed that DHA caused to apoptosis of cancer cells.

In this study, our aim is to evaluate the anti-tumorigenic activity of SL containing both n-3 and n-6 FAs via cell viability analysis of MCF-7 human breast cancer cells.

4.2 Materials and Methods

4.2.1 Materials

Echium plantagineum seed oil was purchased from Harke Group, GmbH (Germany). Structured lipid used in the cell culture study was produced via enzymatic reactions between tricaprylin and *Echium* oil fatty acids and it mainly consists of 78-79% long-chain FAs. MCF-7 human breast cancer cell line was provided from Yeditepe University Genetics and Bioengineering Department. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Lab. (Kumamoto, Japan). All other reagents and solvents which were purchased from Sigma Chemical Co. and Merck (Whitehouse, NJ) were of analytical or chromatographic grade.

4.2.2 Cell viability assay

MCF-7 human breast cancer cells were grown in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin–streptomycin (Sigma-Aldrich) in an incubator at 37˚C with 5% CO_2.

Cells were seeded in 96-well plates at a density of 1.2 x 10^4 cells per well for 24 h. SL and *Echium* oil were dissolved in absolute ethanol, and then mixed with culture medium. The culture medium in plates were changed with the medium containing 30 and 120 μg/mL SL and *Echium* oil. The oil concentrations were determined according to the results of our preliminary studies (data not given here) and literature survey. Control cells were grown in the medium containing the same concentration of absolute ethanol (v/v) as the oil containing medium and not treated with SL or *Echium* oil. The final ethanol concentration was less than 0.1% (v/v). After 24 h incubation, cells were treated with 10 μL CCK-8, and incubated for another 2 h. Optical densities were measured at 450 nm using Elisa Plate Reader. The viability

percentage of proliferating cells was calculated according to the control well containing ethanol.

4.2.3 Statistical analysis

For the statistical data analysis, SPSS Statistics 22.0 (IBM Corporation, NY, USA) was used for analysis of independent samples *t*-test at the significance level of 5%.

4.3 Results and Discussion

According to cell viability test results shown in Figure 4.1, 24 h of oil treatment decreased the viability of MCF-7 cells for all oil treatments. It can be said that extent of cell growth is nearly the same for both SL and *Echium* oil. Besides, according to the *t*-test results, type of oil treatment or oil concentration had no significant effect on the cell viability ($p > 0.05$).Among the treatments, 30 µg/mL of *Echium* oil treatment was found to be the most effective one to inhibit MCF-7 cell growth (about 17% inhibition compared to control). Besides, SL suppressed MCF-7 cell proliferation about 10% and 8% at 30 and 120 µg/ml oil concentrations, respectively.

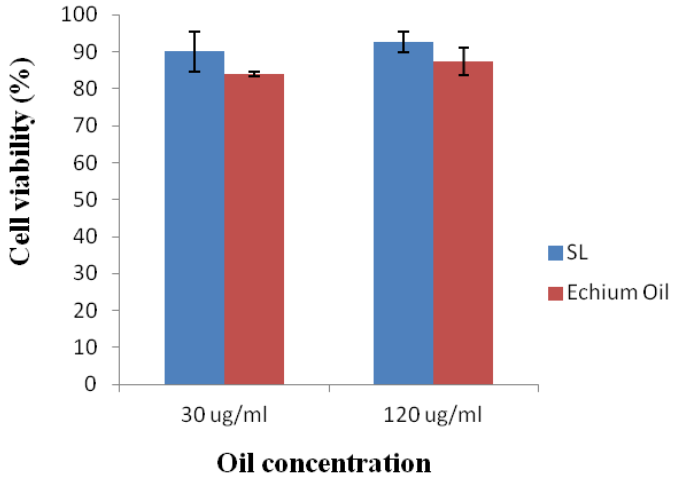


Figure 4.1 : Cell viability of MCF-7 cells treated with SL and *Echium* oil.

Similarly, about 19% cell inhibition was achieved in the study of Subedi *et al*. (2015) in which MCF-7 cell line was treated with a fatty acid mixture (predominantly consists of 15 % GLA, 31.2% ALA and 26.1 % SDA) representing SDA-rich flax seed oil composition at 150 µM concentration for 48 h. *Echium* oil and SDA-rich flax seed oil have higher n-3 and n-6 fatty acid composition than SL. For this reason,

SL may show less anti-tumorigenic activity compared to *Echium* oil and SDA-rich flax seed oil.

4.4 Conclusions

Potential anti-cancer effects of SL containing both n-3 and n-6 series of fatty acids need to be explored since there exist limited *in vitro* study assesing the effects of n-3 and n-6 fatty acids on breast cancer cell lines in the literature. In this study, we demonstrated the possible anti-tumorigenic effects of SL on MCF-7 cell line. Despite the results are promising, additional assays should be performed in order to identify the cell growth inhibition mechanisim of SL. MLM-type SLs brings the health effects of both medium- and long chain fatty acids. Also, positional distribution of fatty acids in MLM-type SL enable the controlled delivery of desired fatty acids targeting specific diseases. The data gathered from this study unraveled that there is no significant different between MLM-type SL and *Echium* oil to inhibit the growth of MCF-7 cells. For this reason, MLM-type SL may be a functional ingredient of lipid emulsions for nutrition of patients or added into food products to respond the consumers' demand on functional and healthier foods.

5. ENCAPSULATION OF STRUCTURED LIPIDS CONTAINING MEDIUM- AND LONG CHAIN FATTY ACIDS BY COMPLEX COACERVATION OF GELATIN AND GUM ARABIC[2]

5.1 Abstract

In this research, structured lipids (SLs) containing 78% of long-chain fatty acids (FAs) at *sn*-2 position and medium chain FAs at *sn*-1,3 positions were encapsulated by complex coacervation method using gelatin and gum arabic as wall materials in order to obtain protein-carbohydrate complexes. The objective of this study was to investigate the effects of experimental parameters such as wall material concentration (1-2%), core:wall ratio (1:1 and 2:1) and homogenization rate (7400, 10000 and 15000 rpm) on encapsulation process of the SL. The highest encapsulation efficiency (84.11 ± 0.77%) was obtained in gelatin 2% (w/v), gum arabic 2% (w/v), 1:1 core:wall ratio (w/w) and at 15000 rpm homogenization rate. In addition, particle size for the coacervates ranged from 19 to 263 nm. Moreover, the samples had smaller polydispersity index (PDI) values, which mean that they showed homogeneous size distribution pattern. Besides, morphological characteristics and thermal behavior of the capsules were determined via scanning electron microscope (SEM) and differential scanning calorimetry (DSC), respectively.

Keywords: Encapsulation; Complex coacervation; Gelatin; Gum arabic; Structured lipids; Omega-3 and omega-6 fatty acids

5.2 Practical Applications

Structured lipids (SLs) with medium chain fatty acids (MCFAs, C6-C10) at *sn*-1 and *sn*-3 positions, and long chain fatty acids (LCFAs, C12-C24) at *sn*-2 position has

[2] This chapter is based on the paper "Yüksel, A. and Şahin-Yeşilçubuk, N. (2018). Encapsulation of structured lipids containing medium- and long chain fatty acids by complex coacervation of gelatin and gum Arabic, *Journal of Food Process Engineering, 41* (8), e12907."

gained attention for food, nutritional and clinical purposes. These SLs are generally designed for special health requirements and meet the daily requirements of fatty acids, especially the long-chain polyunsaturated fatty acids (LCPUFAs) for their health-enhancing and disease-preventing properties. The addition of LCPUFAs into the product formulation may lead an increase in the stability problems of these fatty acids, thus appropriate technologies such as encapsulation of lipids should be implemented. Encapsulation technology enables SLs to be protected from possible detrimental effects of food processing and storage conditions. The use of encapsulation technology in conjuction with SL production are evolving and ongoing issues, hence there is only few studies in the literature that considered encapsulation of SLs.

5.3 Introduction

Recently, synthesis of SLs via enzymatic reactions is becoming a popular topic of lipid modification, which uses lipases as reaction catalysts. Lipid modifications enable to combine advantages of both medium chain FAs at *sn*-1,3 positions and long chain FAs at *sn*-2 position of one triacyglycerol molecule (MLM-type SLs) that makes lipids functional as they can be designed for special health requirements of patients and meet the demands of consumers who desire functional food products with health-promoting and disease-preventing properties (Akoh *et al*, 2002). Since MLM-type SLs are low in calories, they can be useful for the treatment of lipid malabsorption and obesity (Nunes *et al.*, 2011). Besides, studies unraveled that MLM-type emulsions are the safer and effective way of energy delivery for patients who needs long term parenteral nutrition (Rubin *et al*., 2000; Matulka *et al*., 2006). Moreover, MLM-type SLs have many food applications such as cooking oil, energy bar, butter fat, margarine and shortenings, beverages, nutrient admixtures and coating lipid (Lee *et al*., 2012).

Since bioactive agents such as lipids are sensitive to food processing and storage conditions, they have high tendency to lose their functionality. Encapsulation technology overcomes such problems hence it enables protected delivery of bioactive components (de Vos *et al*., 2010). Among various types of encapsulation technologies, complex coacervation technology is highly preferred for encapsulation of lipids since encapsulated products have high oil loading capacity and less surface

oil which provide better dispersion of the powder in food products and reduced risk of rancid flavor development (Kralovec *et al*., 2012). In complex coacervation method, many different negatively charged polysaccharides (e.g. gum arabic, pectin, alginate and carboxy methyl cellulose) can be combined with positively charged protein molecules (e.g. gelatin and albumin) to construct the wall material (Zhang *et al*., 2012). Parameters such as pH, ion concentration, type of wall material, and the ratio of matrix molecule to bioactive component affect the characteristics of the capsules (de Vos *et al*., 2010).

Researchers have conducted many studies regarding encapsulation of oils extracted from different sources via complex coacervation method using gelatin and gum arabic. Dong *et al*. (2011) produced coacervates with good storage stability using gelatin and gum arabic as wall materials in order to encapsulate peppermint oil with the help of transglutaminase as the cross-linking agent. In the study of Zhang *et al*. (2012), gelatin and gum arabic were the used wall materials for entrapping microalgal oils. Furthermore, jasmine essential oil was encapsulated successfully at nanoscale by complex coacervation of gelatin and gum arabic (Lv *et al*., 2014). Moreover, stearidonic acid soybean oil (Ifeduba and Akoh, 2015) and *Echium* oil (Comunian *et al*., 2017) were also encapsulated by complex coacervation methodology in which gelatin and gum arabic were used as wall materials.

Encapsulation of SLs is a new issue of scientific studies hence there are only two studies in the literature that considered microencapsulation of SLs via spray drying technology (Kikuchi *et al*., 2006; Nagachinta and Akoh, 2013). From this point, our study will be unique since it is aimed to encapsulate MLM-type SLs by complex coacervation technology. The aim of this study was to investigate the effects of wall material composition, core:wall ratio and homogenization rate on complex coacervation of SLs containing high amounts of LCPUFAs at *sn*-2 position that were produced in our previous study (Yüksel and Şahin-Yeşilçubuk, 2018a) by enzymatic acidolysis reactions catalyzed via commercially immobilized *sn*-1,3 specific lipases. Since the SL is containing 78-79% of long-chain FAs such as α-linolenic acid (ALA), γ-linolenic acid (GLA) and stearidonic acid (SDA), encapsulation of the SL has been performed to protect the lipids from surrounding environment in order to increase the stability of the coated material. Considering the above, encapsulation efficiency and coacervate yield of the lyophilized products were evaluated to reveal

the effects of experimental parameters on encapsulation process. In addition, particle size, polydisperty index, morphological characteristics and thermal behavior of the capsules were also investigated.

5.4 Materials and Methods

5.4.1 Materials

Gum arabic from acacia tree was purchased from Sigma Chemical Co. (St. Louis, MO). Edible bovine gelatin (100 Bloom) was purchased from a local grocery. The SL used as core material in this study was enzymatically produced from tricaprylin and *Echium* oil fatty acids and it predominantly contains 78-79% of long-chain FAs. All other reagents and solvents which were purchased from Sigma Chemical Co. (St. Louis, MO) and Merck (Whitehouse, NJ) were of analytical or chromatographic grade.

5.4.2 Encapsulation of SLs

Encapsulation of SLs via complex coacervation methodology was conducted according to the study of Yeo *et al.* (2005) with slight modifications. The design of experimental factors were based on the data (not given in this work) gathered from our preliminary experiments and intensive literature search (Yeo *et al*., 2005; Zuanon *et al*., 2013; Ifeduba and Akoh, 2015; Yari *et al*., 2016; Habibi *et al*., 2017). Gelatin solutions (25 mL) and gum arabic solutions (25 mL) were prepared by using distilled water with the concentrations of 1% and 2%, w/v; and the gelatin:gum arabic ratio was used as 1:1. In order to dissolve the gelatin solution, hot plate with magnetic stirrer (IKA-Werke RT5, Germany) was used to set the temperature to 50 ºC with a continuous stirring at 300 rpm. SL (1:1 or 2:1; core:total wall material, w/w) was added to the gelatin solution and then homogenization was obtained at different speed rates (7400, 10000 and 15000 rpm for 3 min) by using a homogenizer (IKA, T18 Ultra-Turrax, Germany). Then, 25 mL of gum arabic solution was added dropwise into the emulsion where the temperature kept at 50 ºC with a continuous stirring. The pH was adjusted to 4.0 ± 0.01 using 0.1 M acetic acid. After pH adjustment step, the mixture was cooled in an ice bath keeping magnetic stirring during 20 min. Samples were stored at 4 ºC for 24 h to improve the formation of microcapsules. Vacuum filtration was applied in order to separate the microcapsules

and the recovered samples were frozen at -18 ºC. Frozen samples were lyophilized by a freeze-drier (Christ Alpha 1-2 LD Plus, Germany) at -52 ºC and 0,001 mbar for 16 h. In order to obtain a fine powder, lyophilized samples were ground by an analytical mill (IKA A11, Germany) and kept at -18 ºC until further analysis.

5.4.3 Coacervate yield

Coacervate yield was calculated as the ratio between final mass of the dried microcapsules (dried at 70 ºC until a constant weight was reached) and the mass of initial core and wall materials, according to the equation given below (5.1) (Zuanon *et al.*, 2013):

$$CY\% = \frac{\text{Mass of the dried microcapsules}}{\text{Initial mass of the core and wall materials}} \quad (5.1)$$

5.4.4 Encapsulation efficiency

Encapsulation efficiency was calculated according to the equation given below (5.2) (Habibi *et al.*, 2017):

$$EE\% = \frac{m1 - m2}{m3} \quad (5.2)$$

where m1, m2 and m3 are the mass of the total oil of the capsule, surface oil of the capsule, and total oil added into the initial emulsion, respectively. In order to obtain mass of the surface oil of the capsules, 0.5 g of sample was mixed with 5 mL of *n*-hexane and were centrifuged at 5000 g for 3 min. Then, the samples were passed through a filter paper followed by washing the capsules with *n*-hexane. After that, hexane was completely evaporated in an oven at 70 ºC until a constant weight was obtained. Remained oil was weighed as the surface oil. Total oil of the capsules were determined the method used by Ifeduba and Akoh (2015). Briefly, 0.5 g of dried capsule was mixed with 1 mL of 10 M HCl at 60 ºC for 3 hours. The sample was then placed into a separatory funnel in order to extract the oil with 10 mL diethyl ether and 10 mL petroleum ether for twice. The solvent layer was transferred into a pre-weighed flask for the evaporation of the solvent by using a rotary evaporator at 60 ºC. Finally, after evaporation step, the flask was weighed and the total oil of the capsule was calculated.

5.4.5 Particle size analysis

Particle size (Z-average) and polydispersity index (PDI) of the samples were determined with dynamic light scattering (DLS) by Zetasizer Nano ZS (Malvern Instruments Ltd, Waorcestershire, UK). The Z-ave can be defined as the average mean particle diameter assuming spherical particles (Strasdat and Bunjes, 2013). Lyophilized samples were dissolved in distilled water and then dispersed in glycerin. Refractive indexes of water and glycerin were 1.332 and 1.471, respectively. All measurements were conducted in triplicate.

5.4.6 Morphology

Morphological assessment of lyophilized coacervates was performed using a SEM (scanning electron microscope) (Zeiss Supra 55VP-FEG, Zurich, Switzerland). The samples were fixed on a metal support and a carbon double-sided tape, coated with a fine gold layer, and then visualized under various magnifications at an excitation voltage of 10 kV. The sample sizes were measured using the calibrated scale bar on the micrograph.

5.4.7 Thermal behavior

Thermal behavior of SL, gelatin, gum arabic and lyophilized microcapsules were determined by differential scanning calorimetry (DSC) Q10 model (TA Instruments, New Castle, DA). 5 mg of samples were placed in aluminium pan and a sealed empty pan was used as the reference. DSC analysis was conducted at a heating rate of 10 ºC/min between a temperature range of -50 ºC and 90 ºC (Zuanon *et al*., 2013).

5.4.8 Statistical analysis

For the statistical data analysis, SPSS Statistics 24.0 (IBM Corporation, NY, USA) was used for analysis of variance (ANOVA) and independent samples *t*-test at the significance level of 5%.

5.5 Results And Discussion

5.5.1 Encapsulation yield

Encapsulation conditions and encapsulation process yield values are given in Table 5.1. According to results, higher coacervate yields (80-87%) were obtained at 2%

wall material concentration and a core:wall ratio of 2:1. In addition, according to *t*-test results, there was a significant difference between wall materials concentration and coacervate yield ($p < 0.05$). Furthermore, there is not a significat effect of experimental parameters on encapsulation efficiency since $p > 0.05$. Moreover, as can be seen in Table 5.1, encapsulation efficiency was independent of particle size of the samples. Besides, the highest encapsulation efficiency (84.11 ± 0.77%) was obtained in gelatin 2% (w/v), gum arabic 2% (w/v), 1:1 core:wall ratio (w/w) and 15000 rpm homogenization rate. In the study of Habibi *et al.* (2017) olive oil was encapsulated by complex coacervation where gelatin and gum arabic were used as wall materials. According to results, higher encapsulation values were achieved at 1:1 core:wall ratio. In another study, olive oil coacervates were produced via complex coacervation of gelatin and gum arabic in which maximum encapsulation efficiency was obtained with a wall material concentraion of 2% (Yari *et al*., 2016).

5.5.2 Particle size distribution

Microcapsules were evaluated by their structural characteristics such as size and PDI values and the results are given in Table 5.1. According to results, average particle sizes ranged from 19 to 263 nm. It can be seen from Table 5.1 that higher wall material concentrations resulted in higher mean particle size. Besides, according to *t*-test results, there was a statistically significant difference between wall material concentrations and Z-average sizes ($p<0.05$). In the study of Lv *et al.*(2014), they encapsulated jasmine oil by complex coacervation of gelatin and gum arabic. Similary, they produced coacervates with no hardening agent at nanoscale ranging from 74 to 384 nm. The formation of nanoparticles as a result of complex coacervation was explained by the weakness of electrostatic forces between polymers used as wall materials, and formation of soluble complexes (Lv *et al.* 2014). PDI is expressed as the level of homogenity of particle size distribution ranged between 0 and 1. Particles with smaller PDI values (< 0.5) have more homogeneous size distribution pattern. According to Table 5.1, higher PDI values

Table 5.1 : Encapsulation conditions, coacervate yield, encapsulation efficiency and mean particle size of the microcapsules.

Concentraitons of gelatin and gum arabic solutions (%)	Core:Wall Ratio	Homogenization rate (rpm)	Coacervate Yield (%)	Encapsulation Efficiency (%)	Particle Size (Z-ave, r.nm)	Polydispertiy Index
1	1	7400	76.54 ± 5.20	69.07 ± 1.43	19.89 ± 1.15	0.172 ± 0.140
		10000	76.51 ± 1.53	72.50 ± 0.15	79.92 ± 11.03	0.405 ± 0.154
		15000	79.35 ± 5.42	76.05 ± 1.22	25.12 ± 9.54	0.958 ± 0.073
	2	7400	76.39 ± 3.73	78.31 ± 1.11	141.5 ± 12.56	0.201 ± 0.094
		10000	75.50 ± 0.84	75.97 ± 1.51	55.57 ± 5.27	0.935 ± 0.113
		15000	76.79 ± 4.61	70.15 ± 1.63	161.00 ± 15.94	0.196 ± 0.165
2	1	7400	86.80 ± 265	75.56 ± 1.19	263.70 ± 31.03	0.737 ± 0.228
		10000	86.03 ± 2.58	78.63 ± 0.65	78.97 ± 19.52	0.505 ± 0.335
		15000	76.95 ± 2.20	84.11 ± 0.77	200.90 ± 16.42	0.213 ± 0.182
	2	7400	86.69 ± 4.03	70.23 ± 0.60	118.60 ± 14.81	0.457 ± 0.094
		10000	87.45 ± 6.56	72.51 ± 0.27	172.20 ± 28.53	0.306 ± 0.175
		15000	80.97 ± 0.33	76.95 ± 0.41	153.00 ± 15.49	0.286 ± 0.182

(0.505, 0.737, 0.935 and 0.958) indicated that particles had nonhomogeneous, very broad size distribution and may contain large particles, whereas the particles with smaller PDI values showed homogeneous and narrower distribution pattern. Furthermore, there was a significant difference between wall materials concentration and PDI values ($p < 0.05$). Also, PDI values was independent from core:wall ratio and homogenization rate ($p > 0.05$).

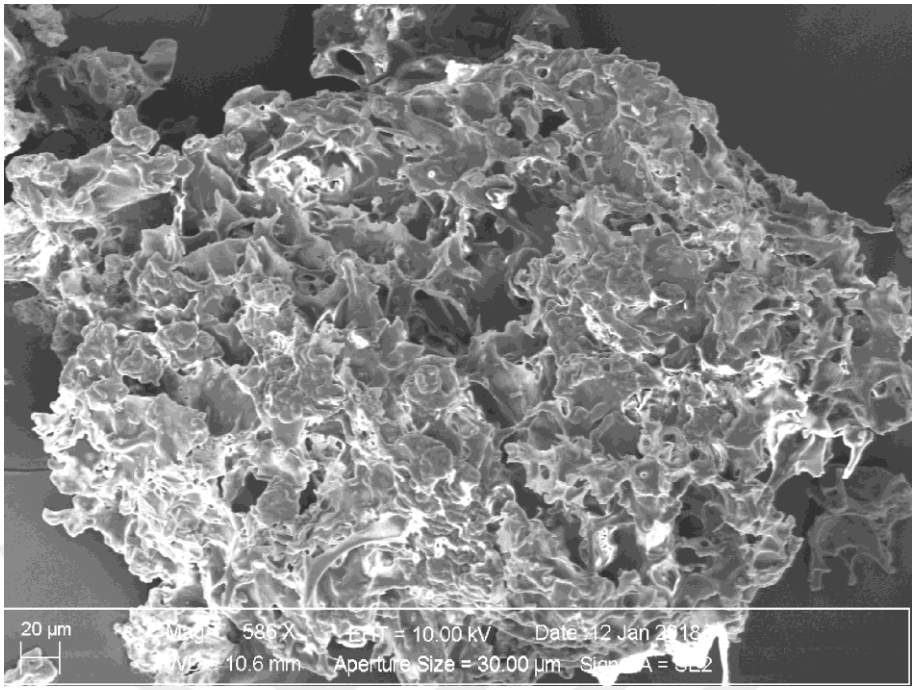
5.5.3 Morphology

Surface morphology of encapsulated SLs were examined by using a SEM at various magnifications and Figure 5.1 represents micrographs of the samples obtained in gelatin 2% (w/v), gum arabic 2% (w/v), 1:1 core:wall ratio (w/w) and 15000 rpm homogenization rate. As shown in Figure 5.1A, lyophilized samples showed porous morphology with non-characteristic shape that is the typical result of sublimination of frozen water in freeze-drying process. Similar morphological characteristics have been reported in the literature for encapsulated oils in which lyophilization was chosen as the drying method and also it has been concluded that type of drying method affects the surface morphology of encapsulated samples (Huang *et al*., 2012; Zuanon *et al*., 2013; Rutz *et al*., 2017). Figure 5.1B revealed that relatively spherical and irregular formations were occurred on the surface however, the particles were agglomerated and adhered to each other which is resulted in porous structure. This phenomenon can be explained by the absence of cryoprotectant during the freeze-drying process of the encapsulated SLs since they protect the surface of capsules from aggregation, deformation and formation of collapsed structures (Morais *et al*., 2016). Such samples with non-characteristic and irregular ,non-spherical shapes were resulted via electrostatic bond breaking during freezing or lyophilization process (Silva *et al*., 2012). The dried samples with cracks and pores on its surface were formed due to removal of water during lyophilization (Yari *et al*., 2016). Also, samples are collapsed as a result of structural transformations caused by temperature and water content changes occured during lyophilization process (Zuanon *et al*., 2013).

5.5.4 Thermal behavior

Thermal behavior of SL, gelatin (GE), gum arabic (GA) and lyophilized microcapsules (obtained in gelatin 2% (w/v), gum arabic 2% (w/v), 1:1 core:wall

(A)



(B)

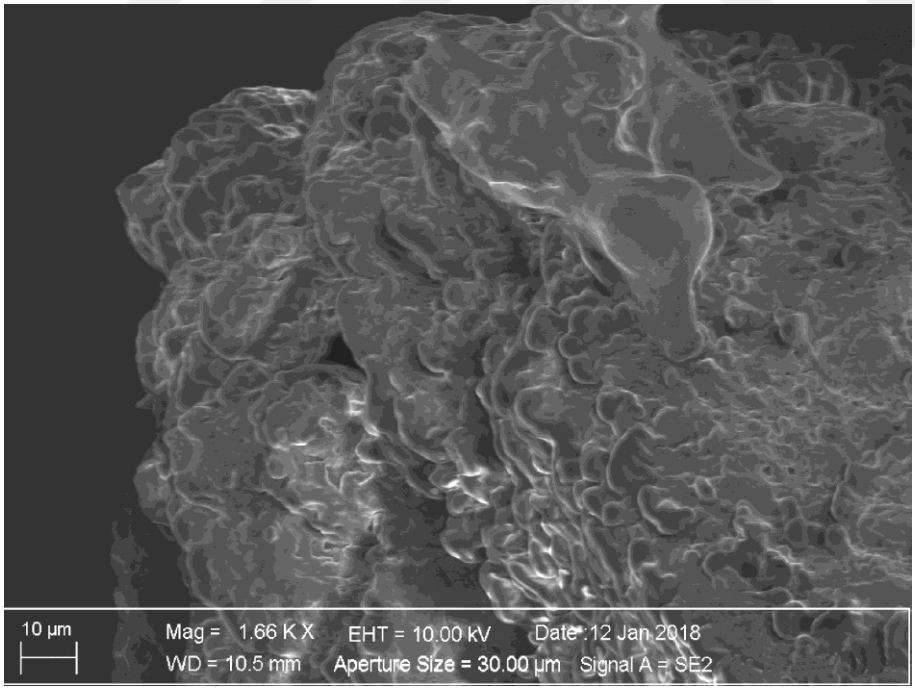


Figure 5.1 : SEM micrographs of the encapsulated SLs obtained in gelatin 2% (w/v), gum arabic 2% (w/v), 1:1 core:wall ratio and 15000 rpm homogenization rate at (A) x586 and (B) x1660 magnifications.

ratio (w/w) and 15000 rpm homogenization rate) were evaluated by the DSC thermograms as shown in Figure 5.2. Thermogram of SL had a single broadened endotherm peak (melting temperature) at -12 ºC. Thermograms of GE and GA had gelation temperature at 62 ºC and 65 ºC, respectively. However, gelation at 83 ºC was observed for the encapsulated SLs which means the formation of a new structure with improved thermal stability. A similar result was also reported by Huang *et al* that the coacervates produced in their study were more thermally stable than both of the wall materials used (Huang *et al*., 2012). Furtermore, as can be easily seen from Figure 5.2, endothermic and exothermic events of coacervate thermogram were similar to those of core and wall materials. For example, thermogram of microcapsule had an endothermic event around 62 ºC which was observed in DSC thermograms of GE and GA.

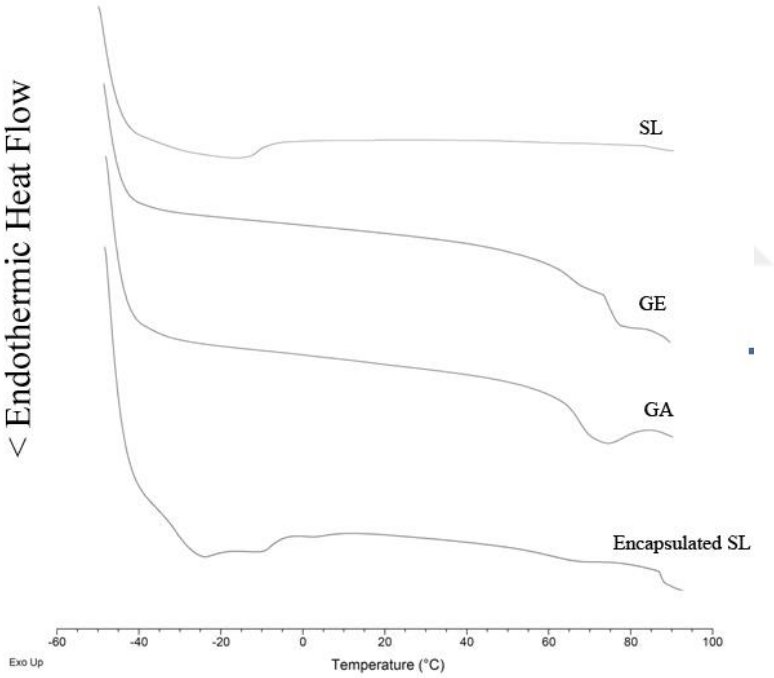


Figure 5.2 : DSC thermograms of SL, gelatin (GE), gum arabic (GA) and encapsulated SL.

5.6 Conclusions

MLM-type SLs containing omega-3/6 fatty acids and caprylic acid was successfully encapsulated via complex coacervation methodology using gelatin and gum arabic as wall materials. The coacervates produced relatively had higher EE and the highest EE (~84%) was achieved in gelatin 2% (w/v), gum arabic 2% (w/v), 1:1 core:wall

ratio and at 15000 rpm homogenization rate. Furthermore, lower PDI values of the coacervates were associated with their homogeneous and narrower distribution pattern. We believe that, encapsulation process will protect SLs from undesirable changes during food processing and storage. The applied methodology may therefore open perspectives for the development of functional foods, nutraceuticals and specific health products containing encapsulated MLM-type SLs.

6. PRODUCTION OF PROBIOTIC KEFIR FORTIFIED WITH ENCAPSULATED STRUCTURED LIPIDS AND INVESTIGATION OF MATRIX EFFECTS BY MEANS OF OXIDATION AND *IN VITRO* DIGESTION STUDIES[3]

6.1 Abstract

The aim of the present study is to encapsulate structured lipids (SLs) by complex coacervation of gelatin and gum arabic with or without using transglutaminase enzymes and to develop a functional kefir product via the addition of encapsulated SLs in the form of suspension and freeze-dried coacervates. Encapsulated SLs were evaluated for their oxidative stability during 30 days of cold storage. The data showed that coacervate solutions were more sensitive to lipid oxidation compared to freeze-dried capsules. Traditionally produced kefir samples that were fortified with complex coacervation products were stored for 10 days at 4 °C. The pH values of the samples decreased, whereas titratable acidity consistently increased during the storage period. Moreover, an *in vitro* controlled release study was conducted with a fortified kefir sample containing freeze-dried capsules. According to the results, kefir had no significant matrix effect on oil release from the freeze-dried capsules ($p > 0.05$).

Keywords: Encapsulation, Structured lipid, Complex coacervation, Kefir, Functional Food, *In vitro* release

6.2 Introduction

Structured lipids assembled with different fatty acids bear the potential to supply functional foods with improved health effects. The production of structured lipids

[3] This chapter is based on the paper "Yüksel-Bilsel, A. and Şahin-Yeşilçubuk, N. (2019). Production of probiotic kefir fortified with encapsulated structured lipids and investigation of matrix effects by means of oxidation and *in vitro* digestion studies, *Food Chemistry, 296*, 17-22."

(SLs), which predominantly involve lipase driven enzymatic reactions, has gained increased attention within the scientific community. A single triacylglycerol (TAG) molecule can be modified by lipases, incorporating a variety of functional fatty acid units, such as omega-3 fatty acids, medium and long chain fatty acids (Ward and Singh, 2005).

Enzymatic production of structured lipids including both medium chain fatty acids (MCFAs) and long chain fatty acids (FAs) in one triacyglycerol molecule (MLM-type SLs) is an attractive issue to researchers because MLM-type SLs can satisfy the consumer demand for foods products with beneficial health effects (Akoh, Sellappan, Fomuso and Yankah, 2002; Mu and Høy, 2004; Osborn and Akoh, 2002).

During the processing and storage period, bioactive agents such as lipids can be adversely affected by unfavorable conditions such as high temperature and moisture levels as well as mechanical stress. In addition, the interaction between lipids and other food components during food processing and storage may lead to the production of unwanted tastes and smells in the food products. To prevent such problems, encapsulation technology is widely used in food industry to overcome such undesired problems (Nedović, Kalušević, Manojlović, Petrović and Bugarski, 2013).

Kefir is a traditional dairy beverage originating from Caucasus and widely consumed in Russia and Eastern Europe (Turkmen, 2017). As a probiotic drink, kefir has antibacterial (Cevikbas, Yemni, Ezzedenn, Yardimici, Cevikbas and Stohs, 1994; Rodrigues, Caputo, Carvalho, Evangelista and Schneedorf, 2005; Yüksekdağ, Beyatli, & Aslim, 2004), antitumor, anticarcinogenic, (Cevikbas *et al*., 1994; Liu, Wang, Lin and Lin, 2002) and cholesterol-lowering (Rattray and O'Connell, 2011) properties, and it also improves the immune system (Matar, Valdez, Medina, Rachid and Perdigon, 2001; Perdigón, Fuller and Raya, 2001) and lactose tolerance (Rattray *et al*., 2011).

To the best of our knowledge, this is the first study to develop a kefir product that is fortified with encapsulated SLs. In the present study, SLs produced from tricaprylin and *Echium* oil fatty acids were encapsulated via complex coacervation of gelatin and gum arabic according to reaction parameters from our previous study (Yüksel and Şahin-Yeşilçubuk, 2018b). Additionally, apart from our previous study,

transglutaminase enzyme was used as a cross-linking agent during the encapsulation process. Oxidative stability measurements of encapsulated SLs in the form of aqueous suspension and freeze-dried capsules were conducted, and the results were compared with those gathered from oxidative stability experiments of free SLs and free *Echium* oil. The application of complex coacervation products in kefir was studied and lipid oxidation was measured during 10 days of storage at 4 °C. Fortified kefir samples were assessed for their pH, titratable acidity, color and oxidative stability within the storage period at 4 °C for 10 days. Furthermore, *in vitro* digestion was applied to the freeze-dried capsules and fortified kefir samples in order to observe any possible matrix effect during the digestion of the SLs. The schematic outline of the study is presented in Figure 6.1.

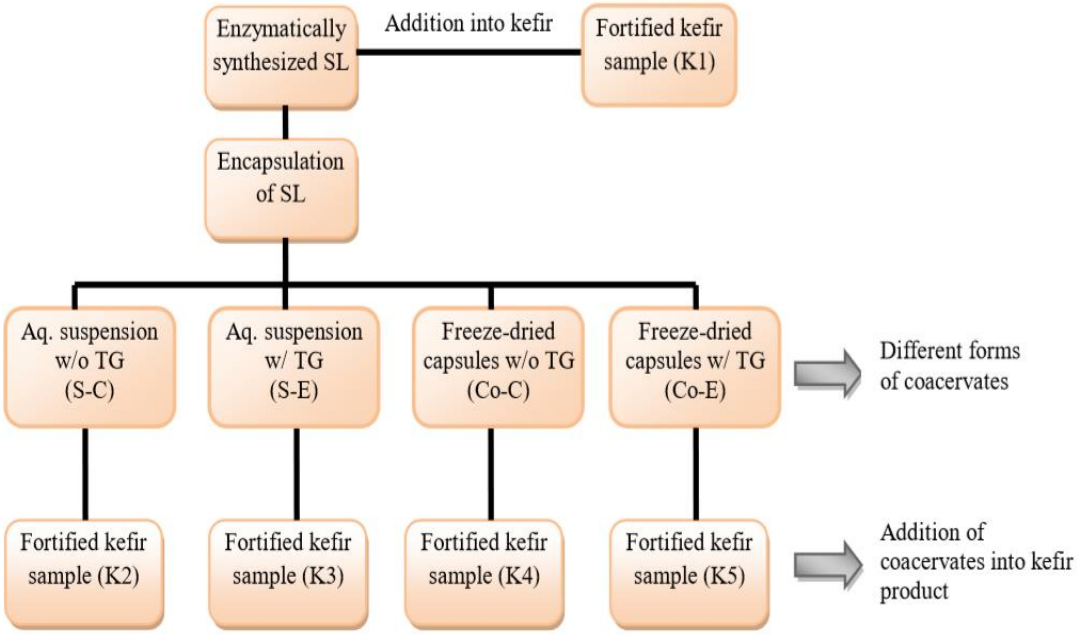


Figure 6.1 : A schematic outline of the study.

6.3 Materials and Methods

6.3.1 Materials

The MLM-type SLs used in this study include 78-79% long-chain FAs that were produced by enzymatic reactions between tricaprylin and *Echium* oil fatty acids (Yüksel and Şahin-Yeşilçubuk, 2018a). Gum arabic from the acacia tree was obtained from Sigma Chemical Co. (St. Louis, MO), and edible bovine gelatin (gel strength 100 Bloom) from a local grocery in İstanbul, Turkey. Kefir grains were

obtained from a private farm located in Istanbul, Turkey. The lipase from porcine pancreas (Type II, 100-400 units/mg protein, using olive oil), butylated hydroxytoluene (BHT), and sodium chloride were provided from Sigma-Aldrich (St. Louis, MO). Transglutaminase (120 U/g) was purchased from Benosen Food Additives (Istanbul, Turkey); pepsin from porcine gastric mucosa (0.7 FIP-U/mg) from Merck (Whitehouse, NJ); and bile salts from Fluka™. All other reagents and solvents were purchased from Sigma Chemical Co. and Merck (Whitehouse, NJ) were of analytical or chromatographic grade.

6.3.2 Encapsulation of SLs by complex coacervation

The SLs were encapsulated by using complex coacervation of gelatin and gum arabic as stated by Yeo, Bellas, Firestone, Langer and Kohane (2005) with some modifications. Gelatin solution (2%, w/v, 50 mL) and gum arabic solution (2%, w/v, 50 mL) were prepared. The gelatin solution was heated to 50 °C under constant stirring speed at 300 rpm by using an IKA RTC Standard. Then, SL with a ratio of 1:1 (core:total wall material, w/w) was transferred into the gelatin solution followed by homogenization at 15,000 rpm for 3 min by using a Heidolph SilentCrusher M. In the next step, 50 mL the gum arabic solution was added drop by drop into the gelatin/SL emulsion at 50 °C with continuous stirring. After that, 0.1 M acetic acid was used to adjust pH of the solution (4.0 ± 0.01). Thereafter, the solution was placed into an ice bath for 20 min and after cooling samples were kept in a refrigerator (4 °C) overnight to obtain coacervates. To produce samples hardened with transglutaminase (TG), 0.5% (w/w) TG was added to the suspension after the pH adjustment step and the suspension was maintained under magnetic stirring (100 rpm) for 12 h to complete the cross-linking reaction (Ifeduba and Akoh, 2015). The samples were filtered and frozen at -18 ºC. Lyophilized samples were obtained using a Christ Alpha 1-2 LD Plus where lyophilization temperature, pressure and time were -52 ºC, 0.001 mbar and 16 h, respectively. Finally, IKA A11 basic was used to grind freeze-dried samples that were stored at 4 °C for further use.

6.3.3 Oxidative stability studies

Peroxide (PV) and *p*-anisidine (*p*-AV) values of *Echium* oil, SL, freeze-dried capsules and coacervate suspensions were determined according to AOCS Cd 8-53

and AOCS Cd 18–90 methods, respectively. The total oxidation value (TOTOX) was calculated according to the formula given below (6.1):

$$TOTOX = 2(PV) + p\text{-}AV \quad (6.1)$$

The lyophilized samples were stored at room temperature (~25 °C); the coacervate suspensions and oils were kept at 4 °C (Ifeduba *et al*., 2016). To measure the PV and *p*-AV, lipids in the capsules were extracted according to Ifeduba *et al*. (2016). Briefly, 10 g coacervate suspension (made by weighing 2.5 g lyophilized sample and mixing with 10 mL distilled water), 8 mL ethanol (96%, v/v) and 2 mL NaOH (7 M) were transferred into a separatory funnel and mixed thoroughly. Diethyl ether (10 mL) and BHT (0.01, w/v) were added to the mixture and shaken well. Following the formation of two layers, hexane (10 mL) was added and the separatory funnel was shaken gently. Next, 1 M HCl (10 mL) and 0.4 M sodium sulfate solution (10 mL) were used to wash the organic phase. Then, organic phase was transferred into a preweighed flask and the solvent was evaporated to determine the amount of the oil extracted.

6.3.4 *In vitro* digestion

An *in vitro* controlled release study of encapsulated SL was designed by mimicking the gastrointestinal environment. The aqueous gastric and the intestinal fluid solutions used in the release study were prepared according to Comunian *et al.* (2017). The gastric fluid solution (GFS) was comprised of 0.3% pepsin and 0.5% sodium chloride, and the intestinal fluid solution (IFS) was comprised of 0.3% bile salts, 0.9% sodium chloride, and 1% pancreatin. To simulate the gastric phase, 0.4 mg freeze-dried capsules and 20 mL GFS were mixed in a glass flask, and the pH was adjusted to 1.8 with 1 M HCl. Subsequently, the sample mixture was placed in an orbital shaker (IKA, KS4000i, Germany) rotating at 120 rpm and 37 °C for 2 h. Next, the sample solution was extracted with 25 mL petroleum ether and 25 mL diethyl ether in a separatory funnel. After phase separation, organic phase was collected, and the extraction step was repeated again. The organic phase was transferred to a pre-weighed flask and the solvent was evaporated at 40 °C using a rotary evaporator. Finally, the flask was weighed and the amount of oil released from the capsule was calculated (Comunian *et al.*, 2017; Ifeduba and Akoh, 2015).

To simulate the intestinal phase, the sample solution treated with GFS as described above was mixed with 20 mL IFS and 4.8 mg/mL pancreatic lipase in a glass flask, and the pH was adjusted to 7.8 with 1 M NaOH. Flasks containing sample mixtures were placed in an orbital shaker (IKA, KS4000i, Germany) rotating at 120 rpm and 37 ˚C for 30, 60, 90, 120, 150, and 180 min. (Comunian *et al.*, 2017; Ifeduba *et al*., 2015; Li and McClements, 2011). After each incubation period, 10 mL the digested sample solution was mixed with 20 mL 96% (v/v) ethanol and 2 drops of 1% (w/v) phenolphthalein to titrate with 0.1 N NaOH. The amount of released free fatty acid in the sample solution was calculated using the following equation (6.2) (Li *et al.*, 2011):

$$\mathrm{FFA}\,(\%) = 100 \times \frac{\mathrm{Vx\ N\ x\ M_{lipid}}}{\mathrm{m_{lipid}\ x\ 2}} \qquad (6.2)$$

where V is the volume of NaOH used for titration, N is the normality of the NaOH, M_{lipid} is molecular weight of SL (667.58 g/mol based on the fatty acid composition) and m_{lipid} indicates the total oil content of the test sample (0.168 g). For the determination of FFA%, we assumed that 2 fatty acid molecules would cleave off one triacylglycerol molecule.

6.3.5 Production of kefir fortified with SL

First, pasteurized cow milk (3.1% milk fat) was heated to 20-25 °C and inoculated with kefir grains (2%) followed by fermentation for 24 h at 20-25 °C. Then, the grains were collected by filtering milk through a sieve, and the kefir samples were stored at 4 °C (Karagözlü and Kavas, 2001). Kefir samples were fortified with SL (K1), freeze-dried capsules (K2: coacervates with non-TG treatment; K3: coacervates with TG treatment) and coacervate suspensions (K4: suspensions with non-TG treatment; K5: suspensions with TG treatment). Kefir samples (K0) not subjected to any fortification were used as control samples. The oil fortifications were performed considering that SL concentration provides 10% of the minimum daily intake of eicosapentaenoic acid (EPA) per 150 g portion of fortified kefir product.

6.3.6 Characterization of kefir products

Enumeration of the lactic acid bacteria (LAB) found in the control kefir sample was performed by incubation of kefir sample in MRS (de Man, Rogosa and Sharpe) agar

at 37 °C for 48 h. White and opaque colonies grown on petri dishes were counted after incubation.

Kefir products were evaluated for pH, titratable acidity, color and oxidative stability for 0, 5 and 10 day of storage at 4 °C. The pH of all kefir samples was measured using a pH meter (HI 2211-02, Hanna Instruments). For the determination of titratable acidity (% of lactic acid), 10 g kefir sample and 1 mL phenolphthalein (1%, w/v), was mixed, and then titrated with 0.1 N NaOH until obtaining a light pink end point (James, 1999).

The color analysis of kefir samples were performed using a Chroma Meter CR-400 colorimeter (Konica Minolta Sensing Inc, Japan), and the results were expressed as the total color difference (ΔE) calculated from the L^* a^* b^* values recorded at the first and last day of storage according to equation (6.3) (Comunian *et al*., 2017) below:

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (6.3)$$

The oxidative stability of fortified kefir samples was performed as described in section 6.3.3.

6.3.7 *In vitro* release profile of encapsulated SL in kefir samples

Release profiles of the fortified kefir samples were also evaluated to determine if the food matrix has any significant effect on the *in vitro* release properties of encapsulated SL. The samples were treated with both gastric and intestinal fluid solutions as described in section 6.3.4. Unfortified kefir sample was used as a control sample.

6.3.8 Sensory analysis

The sensory analysis of fortified and nonfortified kefir products after 0, 5 and 10 days of storage was conducted by a consumer acceptance test with 10 trained panelists. Approximately 50 g of each sample was served into transparent plastic glasses coded with three-digit random numbers. The consumers were asked to assess the appearance, smell, texture, and overall quality using a 6-point (0: not present; 5: very intense) scale at room temperature in daylight (Irigoyen, Arana, Castiella, Torre and Ibáñez, 2005).

6.3.9 Statistical analysis

For the statistical data analysis, SPSS Statistics 24.0 (IBM Corporation, NY, USA) was used for analysis of variance (ANOVA) and independent samples *t*-tests at a significance level of 5%.

6.4 Results and Discussion

6.4.1 Oxidative stability of encapsulated SL

The peroxide value (PV), *p*-anisidine value (*p*-AV), and TOTOX values were measured for the freeze-dried samples after 30 days of storage at room temperature (~25 °C) and for the coacervate suspensions after 30 days of storage at 4 °C. The results are given in Figure 6.2. As seen in Figure 6.2, peroxide values increased during the storage period. Among the encapsulated SL samples, the freeze-dried control sample (Co-C) had the minimum peroxide level (21.1; 41.8 meq/kg), and the coacervate suspension treated with enzyme (S-E) had the maximum (29.4; 57.5 meq/kg) peroxide level after 0 and 30 days of storage, respectively. The peroxide values of suspension samples were higher than those of freeze-dried samples. This can be explained by the decrease in the particle size of oil droplets during the homogenization step which results in an increase in the surface area of the oil. This means that oil droplets were more subject to oxygen exposure in the coacervate suspension (Ifeduba *et al*., 2016). Additionally, this phenomenon explains why SL and *Echium oil* had lower peroxide values than encapsulated SL.

The PV indicates the formation of primary oxidation products, and the *p*-AV method is used for the assessment of the secondary oxidation products of oils. Among the different encapsulation treatments, the highest *p*-AV (16.9; 51.5) and the lowest *p*-AV (12.9; 29.4) values were of the S-E and Co-C samples, respectively (Figure 6.2). Similarly, Ifeduba *et al.* (2016), obtained PV of 41.5 and 36.7 meq/kg and *p*-AV of 12.8 and 12.1 for suspensions and freeze dried powders of encapsulated stearidonic acid soybean oil (SDASO) by complex coacervation of gelatin and gum arabic stored at 4 °C and 25 °C for 30 days, respectively.

The TOTOX value is used to determine the total oxidative stability of the oil. According to Figure 6.2, the S-E sample had the highest TOTOX values (75.8; 166.6) during the storage period. The samples without transglutaminase treatment (-

C) had lower TOTOX values than those treated with TG. This may be due to the hardening step of encapsulation process where coacervate suspensions were subject to oxygen at room temperature resulting in increased lipid oxidation. When *Echium*

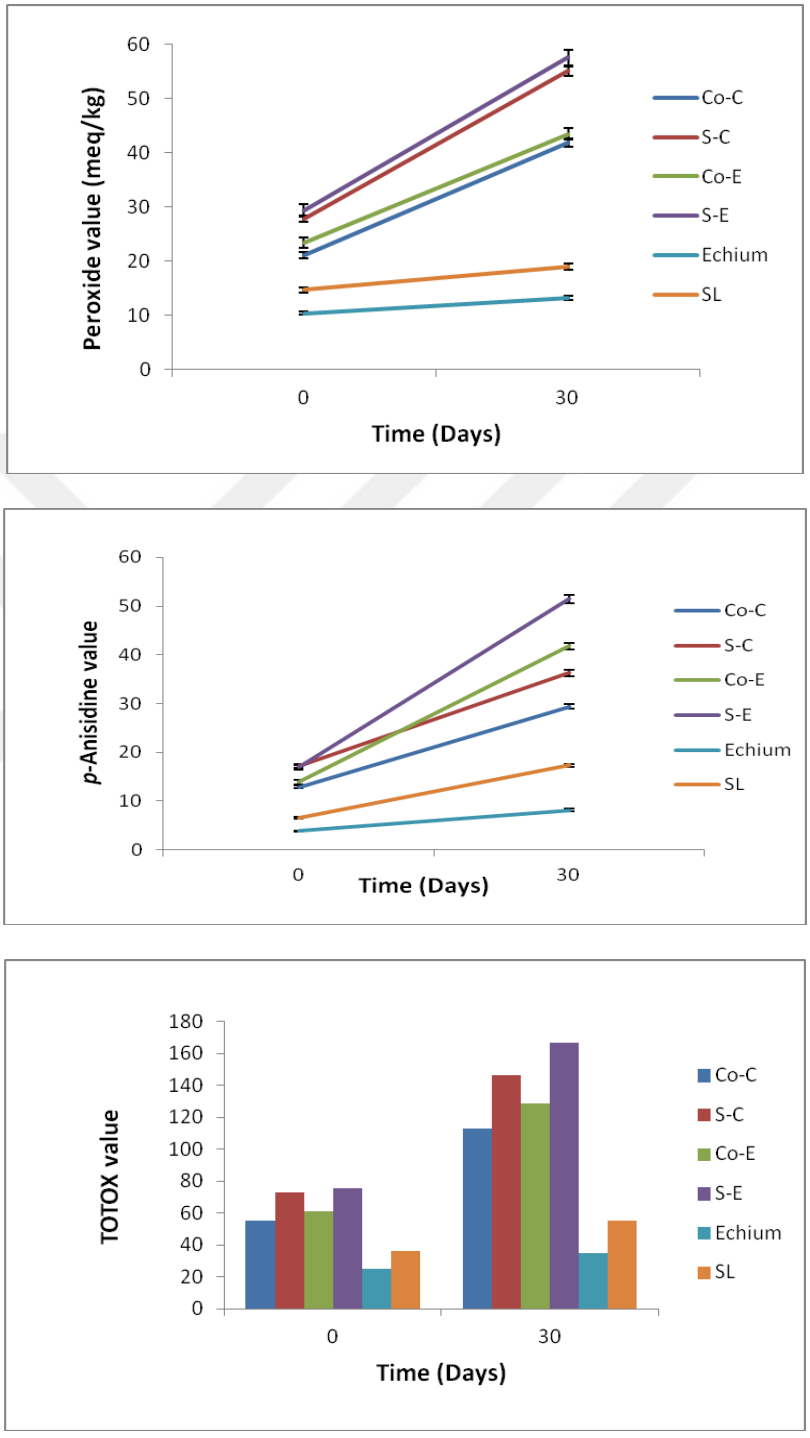


Figure 6.2 : Peroxide, *p*-Anisidine and TOTOX values of freeze-dried capsules (Co), capsule suspensions (S), SL and *Echium* oil during 10 days of cold storage. E is for samples treated with transglutaminase; and C is for control samples not treated with transglutaminase.

oil was compared to SL, it can be concluded that *Echium* oil was more resistant to lipid oxidation. This is explained by the removal of natural antioxidants (e.g., tocopherols and phospholipids) found in the initial oil source during the production of structured lipids making SL more susceptible to lipid oxidation (Jennings and Akoh, 2009; Maduko, Park and Akoh, 2008; Pina-Rodriguez and Akoh, 2009b).

6.4.2 *In vitro* digestibility of encapsulated SL

The oil release properties of the capsules were determined by mimicking gastrointestinal digestion and the results are given in Figure 6.3. According to the *in vitro* release study results, approximately 60% of the SLs were released from the freeze-dried capsules in the gastric phase. During 180 min of intestinal digestion, ~76% of SLs were subject to lipolysis. Additionally, there was an increase in the oil release within the intestinal phase.

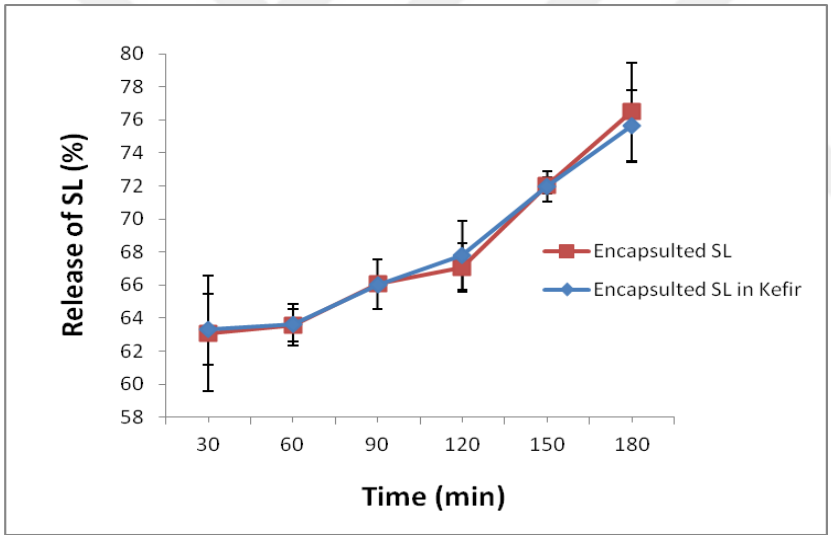


Figure 6.3 : The *in vitro* oil release kinetics of encapsulated SL and encapsulated SL in kefir samples during the intestinal phase.

6.4.3 Properties of fortified kefir products

LAB counts of the kefir used in this study were 6.81 log CFU/mL, which meets the recommended level (≥ 6 log CFU/mL) for fermented products in order to be considered a probiotic food with health benefits (Ramchandran and Shah 2010; Saadi, Zaidi, Oomah, Haros, Yebra and Hosseinian, 2017; Shah, 2007).

The pH, titratable acidity and color change of the kefir products during cold storage are presented in Table 6.1. The initial pH values of control and fortified kefir samples ranged from 4.26 to 4.29. During the storage, pH decreased slowly and the

lowest pH value recorded was 4.12. Similarly, in their studies, Boudjou, Zaidi, Hosseinian and Oomah (2014) and Kim, Jeong, Song and Seo (2018) showed that there was a slight decrease in the pH values of the control kefir samples. In the study by Comunian *et al*. (2017), decreasing pH values were observed for both the control yogurt sample and the encapsulated/nonencapsulated *Echium* oil-added yogurt samples during 30 days of storage at 4 °C. Among the different treatments, there is not a significant difference for pH values ($p > 0.05$) for the same storage time. However, it can be concluded that different storage times have a statistically significant effect on pH values ($p < 0.05$).

As a measure of lactic acid concentration, the initial titratable acidity was between 0.65% and 0.84%. As seen from Table 1, titratable acidity values increased during the storage period. The lowest value was approximately 1.1%, in which coacervate suspension (K4 and K5 treatments) were added. On the other hand, the control kefir sample has the highest value (1.514%) for titratable acidity after 10 days of storage. According to the *t*-test results, we can conclude that the coacervate addition inhibited the increase of titratable acidity when compared to control kefir sample ($p < 0.05$). In contrast, Comunian *et al*. (2017) reported that addition of encapsulated *Echium* oil slightly increased the titratable acidity of fortified yogurt samples when compared to nonfortified yogurt during cold storage. For titratable acidity values, the first day of storage is significantly different after 5 and 10 days of storage ($p < 0.05$), whereas treatments have no significant effect on titratable acidity values ($p > 0.05$).

Fortified kefir samples were also evaluated for color changes during their storage for 10 days at 4 °C. L*, a* and b* parameters were measured, where L* indicates lightness, a* is the red/green coordinate, and b* is the yellow/blue coordinate. Total color differences (ΔE) were calculated from the values measured at 0 and 10 days of storage and the results are given in Table 6.1. According to the results, K1 treatment has the lowest color change (0.42) whereas K2 and K4 treatments showed the most change in color (0.54 and 0.53, respectively) when compared to the control kefir sample. Additionally, coacervate-added samples had higher color change, which was also observed in the study by Comunian *et al*. (2017). This is associated with the addition of microcapsules including gelatin and gum arabic. Our results were lower than the results (0.66 - 2.98) found by Ifeduba *et al*. (2015); on the other hand, Comunian *et al*. (2017) obtained ΔE values ranging from 0.26 to 0.47.

Table 6.1: pH, titratable acidity and color difference (ΔE) values of fortified and non-fortified kefir samples during 10 days of storage at 4 °C.

Treatments[a]/ Days	K0	K1	K2	K3	K4	K5
Storage Day:0						
pH	4.26 ± 0.02c	4.27 ± 0.01c	4.28 ± 0.01c	4.29 ± 0.03c	4.28 ± 0.02c	4.27 ± 0.02c
Titratable acidity	0.836 ± 0.01a	0.845 ± 0.01a	0.793 ± 0.00a	0.780 ± 0.01a	0.658 ± 0.00a	0.652 ± 0.01a
Storage Day:5						
pH	4.21 ± 0.01b	4.20 ± 0.01b	4.21 ± 0.02b	4.20 ± 0.02b	4.23 ± 0.03b	4.19 ± 0.02b
Titratable acidity	1.269 ± 0.00b	1.290 ± 0.00b	1.074 ± 0.01b	1.069 ± 0.01b	0.920 ± 0.00b	0.912 ± 0.02b
Storage Day:10						
pH	4.13 ± 0.02a	4.12 ± 0.02a	4.13 ± 0.01a	4.12 ± 0.02a	4.14 ± 0.02a	4.14 ± 0.03a
Titratable acidity	1.514 ± 0.01b	1.433 ± 0.00b	1.260 ± 0.01b	1.234 ± 0.00b	1.103 ± 0.01b	1.089 ± 0.00b
ΔE (Color difference)	Ref.	0.42	0.54	0.51	0.53	0.52

According to Tukey test, means followed by same letters in a column are not significantly different at 95% confidence interval.

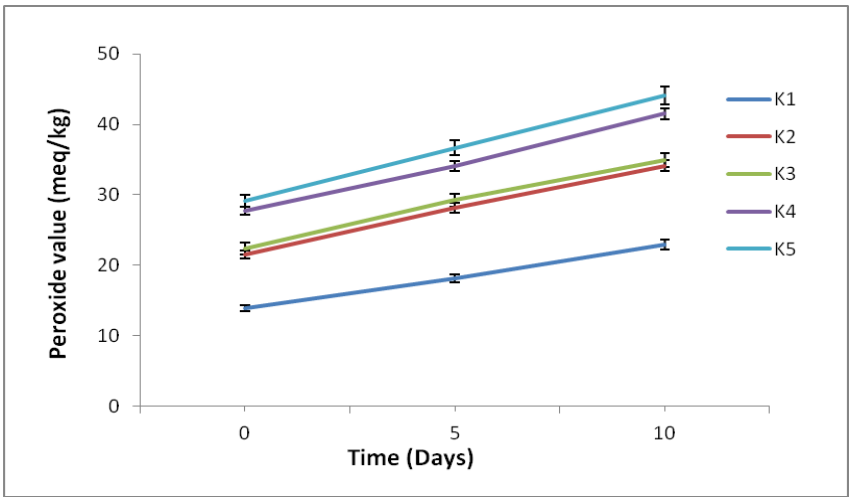
[a]Abbreviations: K0: control kefir sample; K1: kefir added SL; K2: kefir added coacervates with non TG treatment; K3: kefir added cocaervates with TG treatment; K4: kefir added suspensions with non TG treatment; K5: kefir added suspensions with TG treatment; Ref: reference.

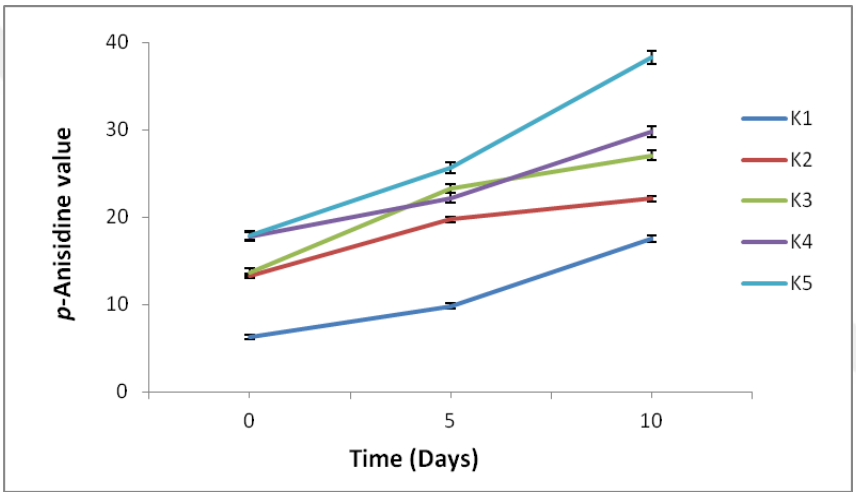
6.4.4 Oxidative stability of fortified kefir samples

To estimate the primary and secondary lipid oxidation of fortified kefir samples during 10 days of storage at 4 °C, PV and *p*-AV were measured (Figure 6.4). As seen from Figure 5.4, K2 and K3 samples had almost the same PV values (~28, 29, 34 meq/kg for 0, 5 and 10 days of storage, respectively). K1 and K5 had sharper increases in *p*-AV compared to other samples. According to Figure 6.4, the TOTOX values of K1 and K5 increased by ~29 and ~50 units, respectively, whereas the other samples increased by approximately 38 units between 0 and 10 days of storage. Additionally, during the storage period, changes in PV and *p*-AV were recorded from 9 to 14.9 meq/kg and from 8.8 to 20, respectively. Based on the findings, freeze-dried capsules without transglutaminase had the highest stability. Furthermore, the kefir product that was fortified with freeze-dried capsules also showed a higher oxidative stability. For this reason, freeze-dried capsules with nontransglutaminase treatment are more suitable for encapsulation of SLs. In a similar study by Ifeduba *et al.* (2015) in which encapsulated SDASO fortified yogurt samples were stored at 4 °C for 14 days, PV and *p*-AV changes ranged from 10.1 to 11.1 and from 4.1 to 16.1, respectively. The kefir sample fortified with free SL (K1) had the lowest oxidation value whereas coacervate and suspension fortification of kefir resulted in higher oxidation levels throughout the 10 days of cold storage. Moreover, transglutaminase-treated coacervates and suspensions (K2 and K4) were more vulnerable to lipid oxidation than nonenzyme treated coacervates and suspensions (K3 and K5). This is probably due to the homogenization step of the complex coacervation method, in which oil droplets are reduced in size and have a larger surface area. As a result of this physical change, oil droplets are more prone to lipid oxidation as described before in section 6.4.1.

6.4.5 *In vitro* digestibility of fortified kefir samples

To determine the matrix effect on oil release from the capsules during digestion, the fortified kefir sample was subjected to *in vitro* digestion, and the unfortified sample was used as a blank sample. Since freeze-dried capsules were more resistant to lipid oxidation and kefir with freeze-dried capsules had a higher consumer acceptance in the sensory analysis, we selected the lyophilized capsule added kefir for the simulated digestion study. According to the results, nearly 59% of the SL was





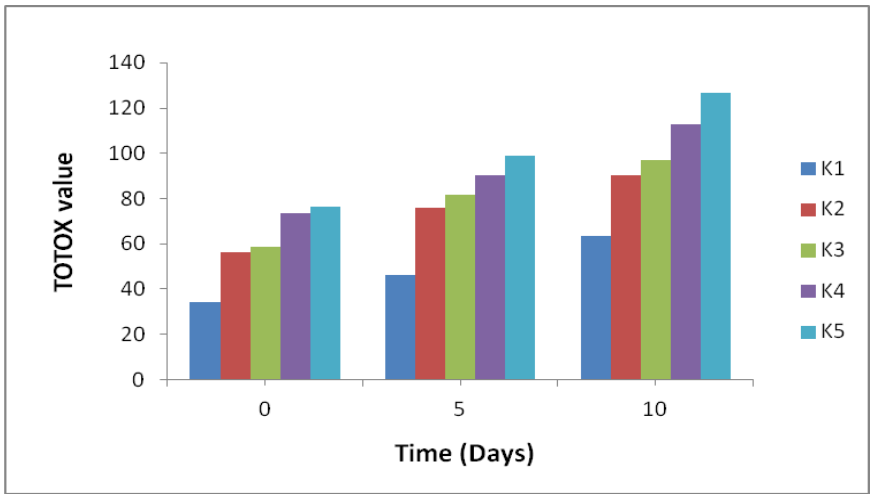


Figure 6.4 : Peroxide, *p*-Anisidine and TOTOX values of fortified and nonfortified kefir samples within the 10 day-storage period at 4 °C. See Table 5.1 for abbreviations.

digested during the gastric phase and throughout the intestinal phase lipolysis, approximately 75% of SL was released. The oil release diagram showed an increase during the intestinal digestion (Figure 6.3). We can conclude that as a food matrix, kefir has no meaningful inhibitory effect on oil release from the capsule during the *in vitro* gastric and intestinal digestion experiments ($p > 0.05$).

6.4.6 Sensory analysis

In this study, according to the sensory analysis of the kefir samples during 10 days of storage, the characteristic odor of kefir and sour smell persisted. The participants did not observe any mold formation throughout the storage. A slight serum separation was detected after 0 and 10 days of storage. The total scores of gas formation were the highest at 5 days of storage, and high gas formation was observed in the K0 and K2 samples. There was a 3-fold increase in the brightness of kefir samples between 0 and 10 days of cold storage. Additionally, with regard to the sensory evaluation, there were significant differences between 5 and 10 days of storage in the color and brightness of samples ($p < 0.05$). Among the fortified samples, K2 (freeze-dried capsules with nontransglutaminase treatment) had the highest score for overall acceptance of samples.

6.5 Conclusions

We produced encapsulated SLs by complex coacervation using gelatin and gum arabic as wall materials with the use of transglutaminase enzyme. Additionally, we demonstrated the possibility of fortification of kefir with encapsulated SLs. The data suggested that kefir exhibits no inhibitory effect on oil release during an *in vitro* digestion simulation. Moreover, fortified kefir samples remained acceptable after up to 10 days of cold storage according to their pH, titratable acidity and total color difference values. Although oxidative stability tests indicated that both the coacervate suspension and the freeze-dried capsules were liable to lipid oxidation, application of antioxidants or similar materials can improve the oxidative stability of encapsulated SLs. In conclusion, we successfully developed a functional kefir product that was fortified with medium and long chain FAs. Being a probiotic product, this modified kefir can also deliver health benefits associated with medium and long chain fatty acids that can meet the consumers' demand for functional dairy products.

7. CONCLUSIONS AND RECOMMENDATIONS

Recently, enzymatic production of structured lipids including both MCFAs and long chain FAs in one TAG molecule (MLM-type SLs) is interest of researchers because, they can deliver the intended fatty acids targeting special health requirements of patients and alos satisfy the increasing demands of consumers on functional food products with health beneficial properties. Bioactive food components such as lipids are easily affected by the deteriorative conditions of food processing and storage. Also, conditions of human gastrointestinal tract may alter their activity and bioavailability during the digestion process. Encapsulation technology has been developed to overcome such problems of food components and this technology enables bioactive components to be applied in food formulas.

In the first step of this study, the aim was to produce MLM-type SLs consisting of caprylic acid from tricaprylin and SDA derived from *Echium* oil by enzymatic acidolysis reactions catalyzed by Lipozyme® RM IM enzymes. Effects of reaction temperature, reaction time and substrate molar ratio on incorporation of SDA were investigated. Optimum temperature, time and substrate molar ratio were found 60 ºC, 6 h and 6 mol/mol, respectively, by response surface methodology. In addition, SDA content at *sn*-2 position of SLs produced at scale-up process was 6.2%. Also,the amount of long-chain fatty acids located at *sn*-2 position of SL was found about78-79%. Furthermore, according to melting profile analysis performed by using DSC, the melting peaks of tricaprylin and *Echium* oil were sharper and narrower while the SL had more broadened peaks. SL have different fatty acid composition and distrubition compared to tricaprylin and *Echium* oil. There is a direct relationship between the TAG composition of lipids and their physical properties. The type of fatty acids determines the melting behavior of TAG. In addition, the position of the SL in the glycerol molecule affects the structure. Therefore, a change in TAG composition affects physical behavior, particularly melting behavior.

In the second part of the study, SLs were evaluated for their possible anti-tumorigenic activity on MCF-7 human breast cancer cell lines. Although, the cancer

preventive/suppressive activity of EPA and DHA has been shown in numerous studies, available research to date has not assessed the effects of SLs produced from *Echium* oil FAs on breast cancer cell lines. Accordingly, this part of the study was aimed at determining the anti-tumorigenic activity of MLM-type SL containing both n-3 and n-6 series FAs by conducting MCF-7 cell viability tests. The MCF-7 cells treated with 30 and 120 μg/mL of SL and *Echium* oil for 24 h. The data gathered from the cell viability test showed that both oil treatments reduce the growth of MCF-7 cancer cells. According to the statistical analysis, there is not a significant difference between SL and *Echium* oil treatment for the inhibition of cell growth ($p > 0.05$). 30 μg/ml of *Echium* oil inhibited MCF-7 cell growth about 17%, on the other hand, MLM-type SL suppressed cell proliferation about 10% at the same concentration. However, statistical analysis showed that the viability of MCF-7 cells are independent of different oil concentrations ($p > 0.05$).

Lipids are sensitive to food processing and storage conditions, and also interaction between lipids and other food components during food processing and storage may resulted in production of unwanted taste and off-odor in the food products. Encapsulation technology overcomes such problems hence it enables protected delivery of bioactive components. For this reason, in the third step of the study, MLM-type SLs obtained at optimum conditions were encapsulated by complex coacervation of gelatin and gum arabic. Experimental parameters such as wall material concentration (1-2%), core:wall ratio (1:1 and 2:1) and homogenization rate (7400, 10000 and 15000 rpm) were investigated. The highest encapsulation efficiency was about 84 % obtained with gelatin 2% (w/v) and gum arabic 2% (w/v), 1:1 core:wall ratio (w/w) and at 15000 rpm homogenization rate. The particle size of the coacervates ranged between 19-263 nm and the coacervates showed homogeneous size distribution pattern. According to the results of morphology analysis performed by SEM, freeze-dried coacervates had porous structure with non-characterictic shape. Also, thermal behavior of the capsules were determined via DSC. The DSC thermogram of encapsulated SLhave similarity with those of core and wall materials.

Finally, complex coacervation of SLs with/without transglutaminase enzyme treatment by using gelatin and gum arabic was performed at the last step of this study. The products of encapsulation process in the form of suspension or freeze-

dried coacervates was added into kefir product in order to develop a functional food product. Oxidative stability tests conducted after 30 days of storage identified that the coacervate solutions were more sensitive to lipid oxidation compared to freeze-dried capsules. Moreover, the samples treated with transglutaminase had higher lipid oxidation levels compared to non-enzyme treated samples. *In vitro* release study was conducted with the freeze-dried capsules and fortified kefir product. The results showed that kefir had no significant matrix effect on oil release from the freeze-dried capsules ($p > 0.05$).

Encapsulation of SLs is a new issue of scientific studies hence there is only two studies in the literature that considering encapsulation of SLs (not MLM-type) via spray drying technology. From this point, our study will be unique since it is aimed to encapsulate MLM-type SLs by complex coacervation technology. This study is also unique in the sense that the release profiles of encapsulated SLs will be investigated for the first time. In addition, possible food matrix effects on release from the encapsulated MLM-type SL was also determined for the first time.

Alternative n-3 fatty acids sources are needed due to the several reasons such as depletion of fish stocks, climate change, presence of environmental pollutants in fish oils. Lately, plant derived n-3 fatty acid sources are interest of researchers to substitute fish oils. Also, plant oils are suitable for vegetarian and vegan nutrition. Consedering the above reasons, our study showed the possiblity of production SL by use of plant derived oil sources. *Echium* plant is not commercialized in our country. But, it is believed that *Echium* plant may gain an economic value by the commercial production of these MLM-type SLs in the future.

Encapsulation of MLM-type SLs enable them to be protected from possible detrimental effects of food processing and storage conditions. Furthermore, SLs will be delivered without losing their bioactivity; and also their release will be controlled by the application of encapsulation technology. However, enzymatic synthesis lead to lose of oxidative stability of newly produced SL. Also, many studies have reported that encapsulation of SLs has lower oxidative stability compared to their natural oil sources. To obtain more stabile coacervates as well as functional food products in which these coacervates are added, oxidative stability of encapsulated SLs must be improved with use of antioxidants or other substances.

The other challenge of the study is high cost of immobilized lipase enzymes. The search for low-cost lipases is important to increase SL production in food industry because of its benefits in terms of nutritional and environmental aspects.

Besides, anti-proliferative activity of MLM-type SL on human breast cancer cell line was indicated in this study. On the other hand, cell viability test is not the only indication of tumor suppressing property. Hence, additional assays should be performed in order to identify the tumor suppressing mechanism of MLM-type SL.

We believe that findings of this thesis study will be beneficial by means of public health and industrial production. MLM-type SLs produced within the concept of this study have potential to satisfy the needs of patients with special nutrition requirements as well as consumers who increasingly demand functional foods having health benefits produced by processes that are less toxic to the environment.

REFERENCES

Agostoni, C. (2010). Docosahexaenoic acid (DHA): From the maternal–foetal dyad to the complementary feeding period, *Early Human Development, 86 (Suppl.),* S3–S6.

Ahmed, S. U., Reddy, K. K., Swathy, S. L., Singh, S. K., Kanjilal, S., Prasad, R. B. N. and Pandey, A. (2009). Enrichment of γ-linolenic acid in the lipid extracted from Mucor zychae MTCC 5420, *Food Research International, 42*, 449-453.

Akoh, C. C. and Kim, B. H. (2007). Structured lipids. In C. C. Akoh, and D. Min, B. (Eds.), *Food Lipids: Chemistry, Nutrition, and Biotechnology*, (pp. 841-873). Boca Raton : CRC Press,.

Akoh, C. C., Sellappan, S., Fomuso, L.B. and Yankah, V.V. (2002). Enzymatic Synthesis of Structured Lipids. In T.M. Kuo, H.W. Gardner (Eds.), *Lipid Biotechnology* (pp.432-460). New York : Marcel Dekker Inc.

Alminger, M., Aura, A. M., Bohn, T., Dufour, C., El, S. N., Gomes A, Karakaya, S., Martínez-Cuesta, M. C., McDougall, G. J., Requena, T. and Santos, C. N. (2014). In vitro models for studying secondary plant metabolite digestion and bioaccessibility. Compr Rev, *Food Sci Food Saf, 13*, 413–36.

Aloys, H., Korma, S. A., Alice, T. M., Chantal, N., Ali, A. H., Abed, S. M. and Ildephonse, H. (2016). Microencapsulation by Complex Coacervation: Methods, Techniques, Benefits, and Applications - A Review, *American Journal of Food Science and Nutrition Research*, *3* (6), 188-192.

AOCS Official Method Cd 8-53 and 18-90 (2009). In D. Firestone (Ed.), *Official methods and recommended practices of the American Oil Chemists' Society* (6th ed.). Champaign, IL: American Oil Chemists' Society

AOCS Official method Cd 12b-92 (1989). In D. Firestone (Ed.), *Official methods and recommended practices of the American Oil Chemists' Society* (4th ed.). Champaign, IL: American Oil Chemists' Society

AOCS Official Method Cj 1-94 (1989). In D. Firestone (Ed.), *Official methods and recommended practices of the American Oil Chemists' Society* (4th ed.). Champaign, IL: American Oil Chemists' Society

Augustin, M. A. and Hemar, Y. (2009). Nano- and micro-structured assemblies for encapsulation of food ingredients, *Chem. Soc. Rev.*, *38* (4), 902–912.

Barrow, C. J., Nolan, C. and Jin, Y. L. (2007). Stabilization of highly unsaturated fatty acids and delivery into foods, *Lipid Technology*, *19*, 108–111.

Benshitrit, R. C., Levi, C. S., Tal, S. L., Shimoni, E. and Lesmes, U. (2012). Development of oral food-grade delivery systems: Current knowledge and future challenges, *Food Funct., 3* (1), 10-21.

Berti, M., Johnson, B.L., Dash, S., Fischer, S., Wilckens, R. and Hevia, F. (2007). *Echium*: a source of stearidonic acid adapted to the northern great plains in the US. In J. Janick and A. Whipkey (Eds.), *Issues in New Crops and New Uses*, (pp. 120-125). Virginia: ASHP.

Bilgiç S. and Yeşilçubuk N. Ş. (2012). Lipase-Catalyzed Acidolysis of Olive Oil with *Echium* Oil Stearidonic Acid: Optimization by Response Surface Methodology, *J Am Oil Chem Soc., 89*, 1971-1980.

Bonatto, S. J. R., Yamaguchi, A. A., Coelho, I., Nunes, E. A., Iagher, F., Fukushima, J. I., Saboia, L. V. and Fernandes, L. C. (2015). Fish oil reduces the proliferation of cells cultured from human breast and colorectal tumours: An in vitro study, *Journal of Functional Foods, 19*, 941–948.

Borsonelo, E. C. and Gaulduróz, J. C. F. (2008). The role of polyunsaturated fatty acids (PUFAs) in development, aging and substance abuse disorders: review and propositions, *Prostaglandins, Leukotrienes and Essential Fatty Acids, 78*, 237–245.

Boudjou, S., Zaidi, F., Hosseinian, F. and Oomah, B. D. (2014). Effects of faba bean (*Vicia faba* L.) flour on viability of probiotic bacteria during kefir storage, *Journal of Food Research, 3* (6), 13-22.

Brenna, J. T., Salem, N., Sinclair, A. J. and Cunnane, S. C. (2009). A-Linolenic acid supplementation and conversion to n-3 long chain polyunsaturated fatty acids in humans, *Prostaglandins, Leukotrienes and Essential Fatty Acids, 80* (2-3), 85-91.

Butstraen, C. and Salaün, F. (2014). Preparation of microcapsules by complex coacervation of gum Arabic and chitosan, *Carbohydrate Polymers, 99*, 608– 616.

Cai, F., Sorg, O., Granci, V., Lecumberri, E., Miralbell, R., Dupertuis, Y. M., and Pichard, C. (2014). Interaction of ω-3 polyunsaturated fatty acids with radiation therapy in two different colorectal cancer cell lines, *Clinical Nutrition, 33* (1), 164-170.

Calviello, G., Di Nicuolo, F., Serini, S., Piccioni, E., Boninsegna, A.,Maggiano, N., Ranelletti, F.O. and Palozza, P. (2005). Docosahexaenoic acid enhances the susceptibility of human colorectal cancer cells to 5-fluorouracil, *Cancer Chemother. Pharmacol., 55*, 12–20.

Camp, J. V., Huyghebaert, A. and Goeman, P. (1998). Enzymatic synthesis of structured modified fats. In A. B. Christophe, (Ed.), *Structural Modified Food Fats:Synthesis, Biochemistry and Use.* (pp. 20-45). Champaign, Illinois : AOCS Press

Celli, G. B., Ghanem, A. and Brooks, M. S. L. (2015). Bioactive Encapsulated Powders for Functional Foods — a Review of Methods and Current Limitations, *Food Bioprocess Technol, 8*, 1825–1837.

Cevikbas, A., Yemni, E., Ezzedenn, F. W., Yardimici, T., Cevikbas, U. and Stohs, S. J. (1994). Antitumoural antibacterial and antifungal activities of kefir and kefir grain, *Phytotherapy Research, 8* (2), 78–82.

Chávez-Servín J. L., Castellote A. I., Martín M., Chifré R. and López-Sabater M. C. (2009). Stability during storage of LC-PUFA-supplemented infant formula containing single cell oil or egg yolk, *Food Chem. 113*, 484-492.

Chopra R., Rastogi N. K. and Sambaiah K. (2011). Enrichment of rice bran oil with α-linolenic acid by enzymatic acidolysis: Optimization of parameters by response surface methodology, *Food Bioproc. Tech., 4*, 1153-1163.

Coleman, M. P., Quaresma, M., Berrino, F., Lutz, J. M., De Angelis, R., et al. (2008). Cancer survival in five continents: a worldwide population-based study (CONCORD), *Lancet Oncol., 9*, 730–756.

Comşa, Ş., Cîmpean, A. M. and Raica, M. (2015). The Story of MCF-7 Breast Cancer CellLine: 40 years of Experience in Research, *Anticancer Research, 35* (6), 3147-3154.

Comunian, T. A., Chaves, E. I., Thomazini, M., Moraes, I. C.F., Ferro-Furtado, R., de Castro, I. A. and Favaro-Trindade, C. S. (2017). Development of functional yogurt containing free and encapsulated *Echium* oil, phytosterol and sinapic acid, *Food Chemistry, 237*, 948–956.

Comunian T. A. and Favaro-Trindade, C. S. (2016). Microencapsulation using biopolymers as an alternative to produce food enhanced with phytosterols and omega-3 fatty acids: A review, *Food Hydrocolloids, 61***,** 442-457.

Coronel-Aguilera, C. P. and Martín-González, M. F. S. (2015). Encapsulation of spray dried β-carotene emulsion by fluidized bed coating technology, *LWT - Food Science and Technology, 62*, 187–193.

Corsetto, P. A., Montorfano, G., Zava, S., Jovenitti, I. E., Cremona, A., Berra, B. and Rizzo, A. M. (2011). Effects of n-3 PUFAs on breast cancer cells through their incorporation in plasma membrane, *Lipids Health Dis.*, 10:73.

Coupland, K. (2008). Stearidonic acid: a plant produced omega-3 PUFA and a potential alternative for marine oil fatty acids, *Lipid Technology, 20* (7), 152-154.

Danhier, F., Ansorena, E., Silva, M. J., Coco, R., Breton, A. and Preat, V. (2012). PLGA-based nanoparticles: An overview of biomedical applications, *Journal of Controlled Release, 161*, 505–522.

Devi, N., Sarmah, M., Khatun, B. and Maji, T. (2017). Encapsulation of active ingredients in polysaccharide–protein complex coacervates, *Advances in Colloid and Interface Science, 239*, 136-145.

de Conto, L. C., Grosso, C. R. F. and Gonçalves, L. A. G. (2013). Chemometry as applied to the production of omega-3 microcapsules by complex coacervation with soy protein isolate and gum Arabic, *LWT - Food Science and Technology, 53*, 218-224.

de Kruif, C. G., Weinbreck, F. and De Vries, R. (2004). Complex coacervation of proteins and anionic polysaccharides, *Current Opinion in Colloid and Interface Science, 9*, 340–349.

de Souza Simões, L., Madalena, D. A., Pinheiro, A. C., Teixeira, J.A., Vicente, A. A. and Ramos, Ó. L. (2017). Micro- and nano bio-based delivery systems for food applications: In vitro behavior, *Adv Colloid Interface Sci., 243*, 23-45.

de Vos, P., Faas, M. M., Spasojevic, M. and Sikkema, J. (2010). Encapsulation for preservation of functionality and targeted delivery of bioactive food components, *International Dairy Journal*, *20*, 292–302.

Dickinson, E. (2008). Interfacial structure and stability of food emulsions as affected by protein-polysaccharide interactions, *Soft Matter, 4* (5), 932–942.

Dong, Z., Maa, Y., Hayat, K., Jia, C., Xia, S. and Zhang, X. (2011). Morphology and release profile of microcapsules encapsulating peppermint oil by complex coacervation, *Journal of Food Engineering*, *104*, 455–460.

Eghbala, N. and Choudhary, R. (2018). Complex coacervation: Encapsulation and controlled release of active agents in food systems, *LWT - Food Science and Technology, 90*, 254–264.

Fatima, S. W. and Khare, S. K. (2018). Current insight and futuristic vistas of microbial transglutaminase in nutraceutical industry, *Microbiological Research 215*, 7–14.

Fernandez-Lafuente, R. (2010). Lipase from *Thermomyces lanuginosus*: uses and prospects as an industrial biocatalyst, *Journal of Molecular Catalysis B: Enzymatic*, (Vol. 62, No. 3-4, pp. 197–212).

Ferreira-Dias, S., Sandoval, G., Plou, F. and Valero, F. (2013). The potential use of lipases in the production of fatty acid derivatives for the food and nutraceutical industries, *Electronic Journal of Biotechnology, 16(3):1.*

Foresti, M.L. and Ferreira, M.L. (2010), Lipase-catalyzed acidolysis of tripalmitin with capric acid in organic solvent medium: analysis of the effect of experimental conditions through factorial design and analysis of multiple responses, *Enzyme and Microbial Technology, 46*, 419–429.

Gebauer, S., Harris, W.S., Kris-Etherton, P.M. and Etherton, T.D. (2005). Dietary n-6:n-3 fatty acid ratio and health. In C. C. Akoh and O. Lai (Eds.), *Healthful Lipids*. (pp. 221-248). Illınois : AOCS Press.

Gonzales, G. B., Van Camp, J., Vissenaekens, H., Raes, K., Smagghe, G. and Grootaert, C. (2015). Review on the use of cell cultures to study metabolism, transport, and accumulation of flavonoids: from mono-cultures to co-culture systems, *Compr Rev Food Sci Food Saf, 14*, 741–54.

Goswami, A. and Stewart, J. D. (2016). Introduction, Types of Reactions, and Sources of Biocatalysts. In A. Goswami, J. D. Stewart (Eds.), *Organic Synthesis Using Biocatalysis*, (pp. 1-38). J. D, Elsevier Inc.

Gouin, S. (2004). Microencapsulation: industrial appraisal of existing technologies and trends, *Trends in Food Science & Technology*, *15*, 330–47.

Gökmen, S., Palamutoğlu, R. and Sarıçoban, C. (2012). Gıda endüstrisinde enkapsülasyon uygulamaları, *Gıda Teknolojileri Elektronik Dergisi, 7*, 36-50.

Guil-Guerrero, J. L., Gómez-Mercado, F., García-Maroto, F. and Campra-Madrid, P. (2000a). Occurrence and characterization of oils rich in γ-linolenic acid Part I: *Echium* seeds from Macaronesia, *Phytochemistry*, *53*, 451-456.

Guil-Guerrero, J. L., Gómez-Mercado, F., García-Maroto, F. and Campra-Madrid, P. (2000b). Occurrence and characterization of oils rich in γ-linolenic acid Part II: fatty acids and squalene from Macaronesian *Echium* leaves, *Phytochemistry*, *54*, 525-529.

Guil-Guerrero, J. L., Rincón-Cervera, M. A. and Venegas-Venegas, E. (2010). Gamma-linolenic and stearidonic acids: purification and upgrading of C18-PUFA oils, *Eur. J. Lipid Sci. Technol*, *112*, 1068–1081.

Gurr, M. I., Harwood, J. L., Frayn, K. N., Murphy, D. J. and Michell, R. H. (2016). *Lipids: Biochemistry, Biotechnology and Health*, Chichester : John Wiley & Sons

Habibi, A., Keramat, J., Hojjatoleslamy, M. and Tamjidi, F. (2017). Preparation of fish oil microcapsules by complex coacervation of gelatin–gum Arabic and their utilization for fortification of pomegranate juice, *Journal of Food Process Engineering*, *40*, e12385.

Harrington, J. C. and Morris, E. R. (2009). Conformational ordering and gelation of gelatin in mixtures with soluble polysaccharides, *Food Hydrocolloids, 23* (2), 327-336.

Hita, E., Robles, A., Camacho, B., Gonzalez, P. A., Esteban, L., Jimenez, M. J., Manio, M. M. and Molina, E. (2009). Production of structured triacylglycerols by acidolysis catalyzed by lipases immobilized in a packed bed reactor, *Biochem. Eng. J. 46*, 257–264.

Horia, E. and Watkins, B. A. (2005). Comparison of stearidonic acid and α-linolenic acid on PGE2 production and COX-2 protein levels in MDA-MB-231 breast cancer cell cultures, *Journal of Nutritional Biochemistry, 16***,** 184– 192.

Huang, G. Q., Sun, Y. T., Xiao, J. X. and Yang, J. (2012). Complex coacervation of soybean protein isolate and chitosan, *Food Chemistry*, *135*, 534–539.

Huang, Y. W. and Huang, C. Y. (2006). Gamma-Linolenic Acid (GLA). In F. Shahidi (Ed.), *Nutraceutical and Specialty Lipids and their Co-Products*, (pp.169-181). Boca Raton : CRC Press.

Hyndman, I. J. (2016). Review: the contribution of both nature and nurture to carcinogenesis and progression in solid tumours, *Cancer Microenvironment, 9* (1), 63–69.

Ifeduba, E. A. and Akoh, C. C. (2015). Microencapsulation of stearidonic acid soybean oil in complex coacervates modified for enhanced stability, *Food Hydrocolloids, 51*, 136-145.

Ifeduba, E. A. and Akoh, C. C. (2016). Microencapsulation of stearidonic acid soybean oil in Maillard reaction-modified complex coacervates, *Food Chemistry, 199*, 524–532.

Irigoyen, A., Arana, I., Castiella, M., Torre, P. and Ibáñez, F.C. (2005). Microbiological, physicochemical, and sensory characteristics of kefir during storage, *Food Chemistry, 90*, 613–620.

Iwasaki, Y. and Yamane, T. (2004). Enzymatic synthesis of structured lipids, *Adv Biochem Eng Biotechnol., 90*, 151-71.

Jala, R. C. R., and Kumar, C. G. (2018). Designer and Functional Food Lipids in Dietary Regimes: Current Trends and Future Prospects. In A. M. Holban and Alexandru Mihai Grumezescu in Alternative and Replacement Foods, Pages 283-316, Eds , Academic Press.

James, C. S. (1999). *Analytical chemistry of foods* (2nd ed.). London, UK: Chapman & Hall.

Jemal, A., Siegel, R., Xu, J., and Ward, E. (2010). Cancer statistics, 2010., *CA Cancer J Clin., 60* (5), 277-300.

Jennings, B. H. and Akoh, C. C. (1999). Enzymatic modification of triacylglycerols of high eicosapentaenoic and docosahexaenoic acids content to produce structured lipids, *J Am Oil Chem Soc. 76*, 1133-1137.

Jennings, B. H. and Akoh, C. C. (2009). Effectiveness of natural versus synthetic antioxidants in a rice bran oil-based structured lipid, *Food Chemistry*, *114*, 1456-1461.

Jun-xia, X., Hai-yan, Y. and Jian, Y. (2011). Microencapsulation of sweet orange oil by complex coacervation with soybean protein isolate/gum Arabic, *Food Chem., 125*, 1267–1272.

Karaca, A. (2008) Aydın yöresinde bal arılarının (*Apis mellifera* L.) yararlanabileceği bitkiler ve bazı özellikleri, *ADÜ Ziraat Fakültesi Dergisi*, *5* (2), 39-66.

Karagozlu, C. and Kavas, G. (2000). Alkollü fermente süt içecekleri: Kefir ve kimizin özellikleri ve insan beslenmesindeki önemi, *Dünya Gıda, 6* (7), 86-93.

Karim, A. A. and Bhat, R. (2008). Gelatin alternatives for the food industry: recent developments, challenges and prospects, *Trends in Food Science & Technology*, *19* (12), 644-656.

Khan, F. I., Lan, D., Durrani, R., Huan, W., Zhao, Z. and Wang, Y. (2017). The Lid Domain in Lipases: Structural and Functional Determinant of enzymatic Properties, *Front Bioeng Biotechnol, 5:16.*

Kieliszek, M. and Misiewicz, A., (2014). Microbial transglutaminase and its application in the food industry. A review, *Folia Microbiol., 59*, 241–250.

Kikuchi, S., Fang, X., Shima, M., Katano, K., Fukami, H. and Adachi, S. (2006). Oxidation of Arachidonoyl Glycerols Encapsulated with Saccharides, *Food Science and Technology Research, 12* (4), 247-251.

Kim, B. H. and Akoh, C. C. (2015) Recent Research Trends on the Enzymatic Synthesis of Structured Lipids, *J Food Sci., 80* (8), C1713-C1724.

Kim, D. H., Jeong, D., Song, K. Y. and Seo, K. H. (2018). Comparison of traditional and backslopping methods for kefir fermentation based on physicochemical and microbiological characteristics, *LWT - Food Science and Technology, 97*, 503–507.

Kim, H. R, Hou, C. T., Lee, K. T., Kim, B. H. and Kim, I. H. (2010). Enzymatic synthesis of structured lipids using a novel cold-active lipase from *Pichia lynferdii* NRRL Y-7723, *Food Chem. 122*, 846–849.

Kleiner, L. and Akoh, C. C. (2018). Applications of Structured Lipids in Selected Food Market Segments and their Evolving Consumer Demands. In U. T. Bornscheuer (Ed.), *Lipid Modification by Enzymes and Engineered Microbes*, (pp. 179-202), AOCS Press

Klevos, G. A., Ezuddin, N. S., Vinyard, A., Ghaddar, T., Gort, T., Almuna, A., Abisch, A. and Welsh, C. F. (2017). A Breast Cancer Review: Through the Eyes of the Doctor, Nurse, and Patient, *Journal of Radiology Nursing, 36* (3), 158–165.

Koletzko, B., Rodriguez-Palmero, M., Demmelmair, H., Fidler, N., Jensen, R. and Sauerwald, T. (2001). Physiological aspects of human milk lipids, *Early Human Development, 65 (Suppl.)*, S3-S18.

Kralovec, J. A., Zhang, S., Zhang, W. and Barrow, C. J. (2012). A review of the progress in enzymatic concentration and microencapsulation of omega-3 rich oil from fish and microbial sources, *Food Chemistry, 131*, 639–644.

Lapillonne, A. and Jensen, C. L. (2009). Reevaluation of the DHA requirement for the premature infant, *Prostaglandins, Leukotrienes and Essential Fatty Acids, 81*, 143–150.

Lazko, J., Popineau, Y., Renard, D. and Legrand, J. (2004a). Microcapsules based on glycinin-sodium dodecyl sulfate complex coacervation, *J. Microencapsul., 21*, 59–70.

Lazko, J., Popineau, Y. and Legrand, J. (2004b). Soy glycinin microcapsules by simple coacervation method, *Colloid Surf., 37***,** 1–8.

Lee, A. V., Oesterreich, S. and Davidson, N. E. (2015). MCF-7 cells-changing the course of breast cancer research and care for 45 years, *Journal of the National Cancer Institute, 107* (7), 1-4.

Siow, L. F. and Ong, C. S. (2013). Effect of pH on Garlic Oil Encapsulation by Complex Coacervation, *J Food Process Technol, 4:1*.

Lee, J. H. and Lee, K. T. (2006). Structured lipids production. In C. C. Akoh (Ed.), *Handbook of Functional Lipids*, (pp. 489-511), Boca Raton: CRC Press.

Lee, J. H., Shin, J. A., Lee, J. H. and Lee, K. T. (2004). Production of lipase-catalyzed structured lipids from safflower oil with conjugated linoleic acid and oxidation studies with rosemary extracts, *Food Res. Int., 37*, 967-974.

Lee, K. T., Akoh, C. C., Flatt, W. P. and Lee, J. L. (2000). Nutritional effects of enzymatically modified soybean oil with caprylic acid versus physical mixture analogue in obese zucker rats, *Journal of Agricaltural Food Chemistry, 48*, 5696-5701.

Lee, Y. Y., Tang, T. K. and Lai, O. M. (2012). Health Benefits, Enzymatic production, and application of medium- and long-chain triacylglycerol (MLCT) in food industries: a review, *Journal of Food Science, 77* (8), 137-144.

Li, J., Ye, A., Lee, S. J. and Singh, H. (2013). Physicochemical behaviour of WPI-stabilized emulsions in *in vitro* gastric and intestinal conditions. *Colloids Surf B: Biointerfaces, 111*, 80–7.

Li, Y. and McClements, D. J. (2011). Controlling lipid digestion by encapsulation of protein-stabilized lipid droplets within alginate-chitosan complex coacervates, *Food Hydrocolloids, 25*, 1025-1033.

Lindskog, M., Gleissman, H., Ponthan, F., Castro, J., Kogner, P. and Johnsen, J. I. (2006). Neuroblastoma cell death in response to docosahexaenoic acid: Sensitization to chemotherapy and arsenic-induced oxidative stress, *Int J Cancer, 118* (10), 2584-93.

Ling, P. R., Istfan, N. W., Lopes, S. M., Babayan, V. K., Blackburn, G. L. and Bistrian, B. R. (1991). Structured lipid made from fish oil and medium-chain triglycerides alters tumor and host metabolism in Yoshida sarcoma-bearing rats, *Am. J. Clin. Nutr, 53*, 1177–1184.

Liu, J. R., Wang, S. Y., Lin, Y. Y. and Lin, C. W. (2002). Antitumor activity of milk kefir and soy milk kefir in tumor-bearing mice, *Nutrition and Cancer 44* (2), 183–187.

Lone, A. M. and Taskén, K. (2013). Proinflammatory and Immunoregulatory Roles of Eicosanoids in T Cells, *Frontiers in Immunology*, *4*, 130.

Long, T. and Ramsburg, C. A. (2011). Encapsulation of nZVI particles using a Gum Arabic stabilized oil-in-water emulsion, *Journal of Hazardous Materials, 189* (3), 801-8.

Lora, K. R. and Lewis, N. M. (2007). Omega-3 and omega-6 fatty acids. In J. A. Driskell (Ed.), *Sports Nutrition: Fats and Proteins*, (pp. 63-89), Boca Raton : CRC Press.

Lucas-González, R., Viuda-Martos, M., Pérez-Alvarez, J. A. and Fernández-López, J. (2018). In vitro digestion models suitable for foods: Opportunities for new fields of application and challenges, *Food Research International 107*, 423–436

Lumor, S. E and Akoh, C. C. (2005). Enzymatic incorporation of stearic acid into a blend of palm olein and palm kernel oil: Optimization by response surface methodology, *J Am Oil Chem Soc*., *82*, 412-426.

Lv, Y., Yang, F., Li, X., Zhang, X. and Abbas, S. (2014). Formation of heat-resistant nanocapsules of jasmine essential oil via gelatin/gum arabic based complex coacervation, *Food Hydrocolloids, 35*, 305-314.

Maduko, C. O., Park, Y. W. and Akoh, C. C. (2008). Characterization and oxidative stability of structured lipids: infant milk fat analog, *Journal of American Oil Chemists' Society*, *85*, 197-204.

Mao, L. and Miao, S. (2015). Structuring food emulsions to improve nutrient delivery during digestion. *Food Eng Rev, 7*, 439–51.

Marangoni, A. G. (2002). Lipases: structure, function, and properties. In T. M. Kuo and H. W. Gardner (Eds.), *Lipid Biotechnology*, (pp. 357-387). New York : Marcel Dekker, Inc.

Martín, Á., Varona, S., Navarrete, A. and Cocero, M. J. (2010). Encapsulation and Co-Precipitation Processes with Supercritical Fluids: Applications with Essential Oils, *The Open Chemical Engineering Journal, 4*, 31-41.

Matar, C., Valdez, J. C., Medina, M., Rachid, M. and Perdigon, G. (2001). Immunomodulating effects of milks fermented by *Lactobacillus helveticus* and its non-proteolytic variant, *Journal of Dairy Research, 68* (4), 601–609.

Matulka, R. A., Noguchi, O. and Nosaka, N. (2006). Safety evaluation of a medium- and long-chain triacylglycerol oil produced from medium-chain triacylglycerols and edible vegetable oil, *Food Chem. Toxicol***.** *44*, 1530–1538.

McClements, D. J. (2010). Design of Nano-Laminated Coatings to Control Bioavailability of Lipophilic Food Components, *J. Food Sci.,75(1)*, R30–R42.

McClements, D. J. and Li Y. (2010). Structured emulsion-based delivery systems: Controlling the digestion and release of lipophilic food components, *Adv. Colloid Interface Sci., 159* (2), 213–228.

Mendanha, D.V., Ortiz, S.E.M., Favaro-Trindade, C.S., Mauri, A., Monterrey-Quintero, E.S. and Thomazini, M. (2009). Microencapsulation of casein hydrolysate by complex coacervation with SPI/pectin, *Food Res. Int., 42*, 1099–1104.

Michalski, M. C., Genot, C., Gayet, C., Lopez, C., Fine, F., Joffre, F., Vendeuvre, J. L., Bouvier, J., Chardigny, J. M. and Raynal-Ljutovac, K. (2013). Multiscale structures of lipids in foods as parameters affecting fatty acidbioavailability and lipid metabolism, *Progress in Lipid Research, 52*, 354–373

Miller, M. R., Nichols, P. D. and Carter, C. G. (2007). Replacement of dietary fish oil for Atlantic salmon parr (Salmo salar L.) with a stearidonic acid containing oil has no effect on omega-3 long-chain polyunsaturated fatty acid concentrations, *Comparative Biochemistry and Physiology, Part B, 146*, 197–206.

Millqvist-Fureby, A., Elofsson, U. and Bergenstahl, B. (2001). Surface composition of spray-dried milk protein-stabilized emulsions in relation to pre-heat treatment of proteins, *Colloids and Surfaces B:Biointerfaces, 21***,** 47–58.

Minekus, M., Marteau, P., Havenaar, R. and Huisintveld, J. H. J. (1995). A Multicompartmental Dynamic Computer-Controlled Model Simulating the Stomach and Small-Intestine, *ATLA, Altern. Lab. Anim., 23* (2), 197–209.

Molly, K., Vande Woestyne, M. and Verstraete, W. (1993). Development of a 5-step multichamber reactor as a simulation of the human intestinal microbial ecosystem. *Appl Microbiol Biotechnol, 39*, 254–8.

Morais, A. R., Alencar Édo, N., Xavier Júnior, F. H., de Oliveira, C. M., Marcelino, H. R., Barratt, G., Fessi, H., do Egito, E. S. and Elaissari, A. (2016). Freeze-drying of emulsified systems: a review, *International Journal of Pharmaceutics, 503 (1–2)*, 102-114.

Mu, H. and Høy, C. E. (2004). The digestion of dietary triacylglycerols, *Journal of Progress in Lipid Research, 43*, 105–133.

Nabavi, S. F., Bilotto, S., Russo, G. L., Orhan, I. E., Habtemariam, S., Daglia, M., Devi, K. P, Loizzo, M. R., Tundis, R. and Nabavi, S. M. (2015). Omega-3 polyunsaturated fatty acids and cancer: lessons learned from clinical trials, *Cancer Metastasis Rev., 34*, 359–380.

Nagachinta, S. and Akoh, C. C. (2013). Spray-dried structured lipid containing long-chain polyunsaturated fatty acids for use in infant formulas, *Journal of Food Science, 78* (10), 1523–1528.

Narang, S. A., Delmarre, D. and Gao, D. (2007). Stable drug encapsulation in micelles and microemulsions, *International Journal of Pharmaceutics, 345*, 9–25.

Nedovic, V., Kalusevic, A., Manojlovic, V., Levic, S. and Bugarski, B. (2011). An overview of encapsulation technologies for food applications, *Procedia Food Science, 1*, 1806 – 1815.

Nedović, V., Kalušević, A., Manojlović, V., Petrović, T. and Bugarski, B. (2013). Encapsulation systems in the food industry. In S. Yanniotis, P. Taoukis, N. G. Stoforos, & V. T. Karathanos (Eds.), *Advances in food process engineering research and applications* (pp. 229-253). New York: Springer.

Nishimoto, M., Sakamoto, R., Mizuta, S. and Yoshinaka, R. (2005). Identification and characterization of molecular species of collagen in ordinary muscle and skin of the Japanese flounder (Paralichthys olivaceus), *J. FoodChem., 90*, 151-156.

Nunes, P. A., Pires-Cabral, P. and Ferreira-Dias, S. (2011). Production of olive oil enriched with medium chain fatty acids catalysed by commercial immobilised lipases, *Food Chem.*, 127, 993–998.

Nunes, P. A., Pires-Cabral, P. C., Guillen, M., Valero, F., Luna, D. and Ferreira-Dias, S. (2011). Production of MLM-type structured lipids catalyzed by immobilized heterologous *Rhizopus oryzae* lipase, *Journal of the American Oil Chemist' Society, 88* (4), 473–480.

Osborn, H. T. and Akoh, C. C. (2002). Structured lipids-novel fats with medical, nutraceutical, and food applications, *Comprehensive Reviews in Food Science and Food Safety*, *3*, 110-120.

Özcan, T. (2008). Analysis of the total oil and fatty acid composition of seeds of some *Boraginaceae* taxa from Turkey, *Plant Syst. Evol*., *274*, 143–153.

Öztürk, T., Ustun, G. and Aksoy, H. A. (2010). Production of medium-chain triacylglycerols from corn oil: Optimization by response surface methodology, *Bioresour. Technol., 101*, 7456–7461.

Perdigón, G., Fuller, R. and Raya, R. (2001). Lactic acid bacteria and their effect on the immune system, *Current Issues in Intestestinal Microbiology*, *2* (1), 27–42.

Pina-Rodriguez, A. M. and Akoh, C. C. (2009a). Enrichment of amaranth oil with ethyl palmitate at the sn-2 position by chemical and enzymatic synthesis, *J Agric Food Chem, 57*, 4657-4662.

Pina-Rodriguez, A. M. and Akoh, C. C. (2009b). Synthesis and characterization of a structured lipid from amaranth oil as a partial fat substitute in milk-based infant formula, *Journal of Agricultural and Food Chemistry*, *57*, 6748-6756.

Poshadri, A. and Kuna, A. (2010). Microencapsulation technology: a review, *J. Res. ANGRAU, 38* (1), 86-201.

Ramchandran, L. and Shah, N. P. (2010). Characterization of functional, biochemical and textural properties of symbiotic low-fat yogurts during refrigerated storage, *LWT-Food Science and Technology, 43*, 819-827.

Rao, R., Manohar, B., Sambaiah, K. and Lokesh, B. R. (2002). Enzymatic acidolysis in hexane to produce n-3 or n-6 FA-enriched structured lipids from coconut oil: Optimization of reactions by response surface methodology, *J Am Oil Chem Soc., 79*, 885-890.

Rattray, F. P. and O'Connell, M. J. (2011). Kefir. In J.W. Fuquay, P. F. Fox, and P. L. H. McSweeney (Eds.), *Encyclopedia of dairy sciences* (2nd ed., Vol. 2, pp. 518–524). Oxford: Elsevier Ltd.

Ray, S., Raychaudhuri, U. and Chakraborty, R. (2016). An overview of encapsulation of active compounds used in food products by drying technology, *Food Bioscience, 13*, 76–83

Rehman, K., Mohd Amin, M. C., Yuen, N. P. and Zulfakar, M. H. (2016). Immunomodulatory Effectiveness of Fish Oil and omega-3 Fatty Acids in Human Non-melanoma Skin Carcinoma Cells, *J Oleo Sci., 65* (3), 217-24.

Rodrigues, K. L., Caputo, L. R. G., Carvalho, J. C. T., Evangelista, J. and Schneedorf, J. M. (2005). Antimicrobial and healing activity of kefir and kefiran extract, *International Journal of Antimicrobial Agents, 25,* 404–408.

Rubin, M., Moser, A., Vaserberg, N., Greig, F., Levy, Y., Spivak, H., Ziv, Y. and Lelcuk, S. (2000). Structured triacylglycerol emulsion, containing both medium- and long-chain fatty acids, in long-term home parenteral nutrition: a double-blind randomized cross-over study, *Nutrition, 16*, 95–100.

Rusli, J. K., Sanguansri, L. and Augustin, M. A. (2006). Stabilization of oils by microencapsulation with heated protein–glucose syrup mixtures, *J. Am. Oil Chem. Soc., 83*, 965–971.

Rutz, J. K., Borges, C. D., Zambiazi, R. C., Crizel-Cardozo, M. M., Kuck, L. S. and Noreña, C. P. Z. (2017). Microencapsulation of palm oil by complex coacervation for application in food systems, *Food Chemistry, 220*, 59–66.

Saadi, L. O., Zaidi, F., Oomah, B. D., Haros, M., Yebra, M. J. and Hosseinian, F. (2017). Pulse ingredients supplementation affects kefir quality and antioxidant capacity during storage, *LWT - Food Science and Technology, 86*, 619-626.

Sahin, N., Akoh, C. C. and Karaali, A. (2005a). Lipase-catalyzed acidolysis of tripalmitin with hazelnut oil fatty acids and stearic acid to produce human milk fat substitutes, *J Agric Food Chem, 53*, 5779-5783.

Sahin, N., Akoh, C. C. and Karaali A. (2005b). Enzymatic production of human milk fat substitutes containing γ-linolenic acid: Optimization of reactions by response surface methodology, *J. Am. Oil Chem. Soc*., *82*, 549–557.

Schmid, U., Bornscheuer, U. T., Soumanou, M. M., Mcneil, G. P. and Schimid, R. D. (1998). Optimization of the reaction conditions in the lipase catalyzed synthesis of structured triacylglycerides, *J. Am. Oil Chem.Soc., 75*, 1527-1531.

Senanayake, S.P.J.N. and Shahidi, F. (2004). Incorporation of docosahexaenoic acid (DHA) into evening primrose (*Oenothera biennis* L.) oil via lipase-catalyzed transesterification, *Food Chemistry, 85*, 489–496.

Shah, N. P. (2007). Functional cultures and health benefits, *International Dairy Journal, 17*, 1262-1277.

Silva, D. F., Favaro-Trindade, C. S., Rocha, G. A. and Thomazini, M. (2012). Microencapsulation of lycopene by gelatin-pectin complex coacervation, *Journal of Food Processing and Preservation, 36*, 185–190.

Silva, S. S., Motta, A., Rodrigues, M. T., Pinheiro, A. F. M., Gomes, M. E., Mano, J. F., Reis, R. L. and Migliaresi, C. (2008). Novel genipin-cross-linked chitosan/silk fibroin sponges for cartilage engineering strategies, *Biomacromolecules, 9* (10), 2764-74.

Singh, H. and Sarkar, A. (2011). Behaviour of protein-stabilised emulsions under various physiological conditions, *Adv. Colloid. Interf. Sci., 165*, 47–57.

Straaup, E. M. and Høy, C-E. (2000). Structured lipids improve fat absorption in normal and malabsorbing rats, *J. Nutr., 130*, 2802–2808.

Strasdat, B. and Bunjes, H. (2013). Incorporation of lipid nanoparticles into calcium alginate beads and characterization of the encapsulated particles by differential scanning calorimetry, *Food Hydrocolloids, 30*, 567-575.

Subedi, K., Yu, H. M., Newell, M., Weselake, R. J., Meesapyodsuk, D., Qiu, X., Shah, S. and Field, C. J. (2015). Stearidonic acid-enriched flax oil reduces the growth of human breast cancer in vitro and in vivo, *Breast Cancer Res Treat, 149*, 17–29.

Surette, M. E. (2013). Dietary omega-3 PUFA and health: Stearidonic acid-containing seed oils as effective and sustainable alternatives to traditional marine oils, *Mol. Nutr. Food Res., 57* (5), 748–759.

Şahin-Yeşilçubuk, N. and Akoh, C. C. (2017). Biotechnological and Novel Approaches for Designing Structured Lipids Intended for Infant Nutrition, *J. Am. Oil Chem. Soc., 94*, 1005-1034.

Stergiou, P. Y., Foukis, A., Filippou, M., Koukouritaki, M., Parapouli M., Theodorou, L. G., Hatziloukas, E., Afendra, A., Pandey, A. and Papamichael, E.M. (2013). Advances in lipase-catalyzed esterification reactions, *Biotechnology Advances, 31*, 1846–1859.

Tahvonen, R. L., Schwab, U. S., Linderborg, K. M., Mykkänen, H. M. and Kallio, H. P. (2005). Black currant seed oil and fish oil supplements differ in their effects on fatty acid profiles of plasma lipids, and concentrations of serum total and lipoprotein lipids, plasma glucose and insulin, *Journal of Nutritional Biochemistry, 16*, 353–359.

Teichert, S. A. and Akoh, C. C. (2011). Stearidonic acid soybean oil enriched with palmitic acid at the sn-2 position by enzymatic interesterification for use as human milk fat analogues, *Journal of Agricultural and Food Chemistry, 59* (10), 5692-5701.

Tello, F., Prata, A. S., Rodrigues, R. A. F., Sartoratto, A. and Grosso, C. R. F. (2016). Improving the performance of transglutaminase-crosslinked microparticles for enteric delivery. *Food Research International, Vol. 88, Part A*, 153-158.

Terry, P. D., Rohan, T. E., and Wolk, A. (2003). Intakes of fish and marine fatty acids and the risks of cancers of the breast and prostate and of other hormonerelated cancers: a review of the epidemiologic evidence, *Am. J. Clin. Nutr., 77*, 532–543.

Timilsena, Y. P., Akanbi, T. O., Khalid, N., Adhikari, B. and Barrow, C. J. (2019). Complex coacervation: Principles, mechanisms and applications in microencapsulation, *International Journal of Biological Macromolecules, 121*, 1276–1286.

Tsai, C. H., Shen, Y. C., Chen, H. W., Liu, K. L., Chang, J. W., Chen, P. Y., Lin, C. Y., Yao, H. T., Li, C. C. (2017). Docosahexaenoic acid increases the expression of oxidative stressinduced growth inhibitor 1 through the PI3K/Akt/Nrf2 signaling pathway in breast cancer cells, *Food and Chemical Toxicology, 108,* 276–288.

Turkmen, N. (2017). Kefir as a functional dairy product. In R. R.Watson, R. J. Collier, & V. R. Preedy (Eds.), *Dairy In Human Health and Disease Across The Lifespan* (1st ed., pp. 373-383). London: Elsevier Inc.

Walker, C. G., Jebb, A. A., Calder, P. C. and Phil, D. (2013). Stearidonic acid as a supplemental source of ω-3 polyunsaturated fatty acids to enhance status for improved human health, *Nutrition, 29*, 363–369.

Wandrey, C., Bartkowiak, A. and Harding, S. E. (2010). Materials for encapsulation. In N. J. Zuidam and V. A. Nedović (Eds.), *Encapsulation Technologies for Active Food Ingredients and Food Processing*, (pp. 31-100). NY, USA : Springer.

Ward, O. P. and Singh, A. (2005). Omega-3/6 fatty acids: alternative sources of production, *Process Biochemistry*, *40*, 3627–3652.

Weber, N. and Mukherjee, K. D. (2008). Lipid biotechnology. In C. C. Akoh, and D. B. Min (Eds.), *Food Lipids: Chemistry, Nutrition, and Biotechnology*, (pp. 708-748). Boca Raton : CRC Press.

Vega, C., Kim, E. H. J., Chen, X. D. and Roos, Y. H. (2005). Solid-state characterization of spray-dried ice cream mixes, *Colloids and Surfaces B: Biointerfaces, 45*, 66–75.

Watkins, S. M. and German, J. B. (2007). Unsaturated fatty acids. In C. C. Akoh and D. B. Min (Eds.), *Food Lipids: Chemistry, Nutrition, and Biotechnology*, (pp. 514-538). Boca Raton : CRC Press.

Wei, Z. and Huang, Q. (2019). Assembly of Protein−Polysaccharide Complexes for Delivery of Bioactive Ingredients: A Perspective Paper, J. Agric. *Food Chem., 67,* 1344−1352.

Whelan, J. (2009). Dietary stearidonic acid is a long chain (n-3) polyunsaturated fatty acid with potential health benefits, *J. Nutr., 139*, 5–10.

Xue, M., Wang, Q., Zhao, J., Dong, L., Ge, Y., Hou, L., Liu, Y., Zheng, Z. (2014). Docosahexaenoic acid inhibited the Wnt/β-Catenin pathway and suppressed breast cancer cells in vitro and in vivo, *J Nutr Biochem., 25* (2), 104-110.

Yang, T., Xu, X., He, C. and Li, L. (2003a). Lipase-catalyzed modification of lard to produce human milk fat substitutes, *Food Chem. 80*, 473-481.

Yang, T., Fruekilde, M-B. and Xu, X. (2003b). Applications of immobilized *Thermomyces lanuginosa* lipase in interesterification, *J. Am. Oil Chem. Soc., 80*, 881–887.

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

Yao, M., McClements, D. J., Zhao, F., Craig, R. W. and Xiao, H. (2017). Controlling the gastrointestinal fate of nutraceutical and pharmaceutical-enriched lipid nanoparticles: From mixed micelles to chylomicrons, *NanoImpact, 5*, 13–21.

Yari, S., Nasirpour, A. and Fathi, M. (2016). Effect of polymer concentration and acidification time on olive oil microcapsules obtained by complex coacervation, *Applied Food Biotechnology*, *3* (1), 53-58.

Ye, Z., Li, R., Cao, C., Xu, Y. J., Cao, P., Li, Q. and Liu, Y. (2019). Fatty acid profiles of typical dietary lipids after gastrointestinal digestion and absorbtion: A combination study between in-vitro and in-vivo, *Food Chemistry, 280*, 34–44.

Yeo, Y., Bellas, E., Firestone, W., Langer, R. and Kohane, D. S. (2005). Complex coacervates for thermally sensitive controlled release of flavor compounds, *Journal of Agricultural and Food Chemistry, 53*, 7518-7525.

Yüksekdağ, Z. N., Beyatli, Y. and Aslim, B. (2004). Determination of some characteristics coccoid forms of lactic acid bacteria isolated from Turkish kefirs with natural probiotic, *LWT- Food Science and Technology, 37* (6), 663–667.

Yüksel, A. and Şahin-Yeşilçubuk, N. (2018a). Use of *Echium* oil fatty acids and tricaprylin as substrates of enzymatic interesterification for the production of structured lipids, *Grasas y Aceites*, *69* (1), e236.

Yüksel, A. and Şahin-Yeşilçubuk, N. (2018b). Encapsulation of structured lipids containing medium- and long chain fatty acids by complex coacervation of gelatin and gum arabic, *Journal of Food Process Engineering, 41* (8), e12907.

Yüksel, A. and Yeşilçubuk, N. Ş. (2012). Enzymatic production of human milk fat analogues containing stearidonic acid and optimization of reactions by response surface methodology, *LWT-Food Sci. Technol., 46*, 210-216.

Zam, W. (2015). Structured lipids: methods of production, commercial products and nutraceutical characteristics, *Progress in Nutrition, 17* (3), 198-213.

Zhang, K., Zhang, H., Hu, X., Bao, S. and Huang, H. (2012). Synthesis and release studies of microalgal oil-containing microcapsules prepared by complex coacervation, *Colloids and Surfaces B: Biointerfaces, 89*, 61– 66.

Zhang, P., Boudyguina, E., Wilson, M. D., Gebre, A. K. and Parks, J. S. (2008). *Echium* oil reduces plasma lipids and hepatic lipogenic gene expression in Apob100-only LDL receptor knockout mice, *Journal of Nutritional Biochemistry, 19* (10), 655–663.

Zuanon, L. A. C., Malacrida, C. R. and Telis, V. R. N. (2013). Production of turmeric oleoresin microcapsules by complex coacervation with gelatin-gum arabic, *Journal of Food Process Engineering*, *36*, 364–373.

Zuidam, N.J. and Shimoni, E., (2010). Overview of Microencapsulates for Use in Food Products or Processes and Methods to Make Them. In N.J. Zuidam and V.A. Nedović (Eds.), *Encapsulation Technologies for Active Food Ingredients and Food Processing*, (pp. 3-29). NY, USA : Springer.

Url-1<*https://florabase.dpaw.wa.gov.au/browse/profile/6681*>, accessed on 20.06.2019.

Url-2<*https://kocaelibitkileri.com/echium-plantagineum/#*>, accessed on 20.06.2019.

Url-3<*https://www.who.int/cancer/prevention/diagnosis-screening/breast-cancer/en/* >, accessed on 24.06.2019.

CURRICULUM VITAE

Name Surname **:** Alev YÜKSEL BİLSEL

Place and Date of Birth **:** Amasya, 1982

E-Mail **:** alev_yuksel@yahoo.com

EDUCATION **:**

- **B.Sc.** **:** 2006, İstanbul Technical University, Faculty of Arts and Sciences, Molecular Biology and Genetics
- **M.Sc.** **:** 2011, İstanbul Technical University, Faculty of Chemical and Metallurgical Engineering, Food Engineering

PROFESSIONAL EXPERIENCE AND REWARDS:

- 2016 -2017 Food Analyst, *Yeditepe University Research and Development Center Laboratories*, İstanbul
- 2014-2016 Food Analyst, *Intertek Testing Services*, İstanbul

- 2019 Promotion Awards for International Publications, TÜBİTAK
- 2018 Promotion Awards for International Publications, TÜBİTAK
- 2012 Promotion Awards for International Publications, TÜBİTAK
- 2012-2014 Doctoral Scholarship, Çamlıca Kültür ve Yardım Vakfı
- 2010 Grant for Msc Students, ITU Scientific Research Projects Unit (BAP) Project No: 33579.

PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Yüksel-Bilsel, A.** and Şahin-Yeşilçubuk, N. (2019). Production of probiotic kefir fortified with encapsulated structured lipids and investigation of matrix effects by means of oxidation and in vitro digestion studies, *Food Chemistry*, *296*, 17-22.

- **Yüksel, A.** and Şahin-Yeşilçubuk, N. (2018). Use of *Echium* oil fatty acids and tricaprylin as substrates of enzymatic interesterification for the production of structured lipids, *Grasas y Aceites, 69* (1), e236.
- **Yüksel, A.** and Şahin-Yeşilçubuk, N. (2018). Encapsulation of structured lipids containing medium and long chain fatty acids by complex coacervation of gelatin and gum arabic, *Journal of Food Process Engineering*, *41* (8), e12907.
- **Yüksel-Bilsel,** A., Kılıç, A., Kök, F. N., Şahin-Yeşilçubuk, N. (2019). "Yapılandırılmış Yağların MCF-7 Kanser Hücresi Üzerindeki Antiproliferatif Etkisi", *YABİTED IV. Bitkisel Yağ Kongresi*, 17-19 April, Istanbul, Turkey. (Abstract)

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

- Taş, M., Balcı, M., **Yüksel, A.** and Şahin-Yeşilçubuk, N. (2015). Consumer awareness, perception and attitudes towards genetically modified foods in Turkey, *British Food Journal, 117* (5), 1426-1439.
- **Yüksel, A.** and Şahin-Yeşilçubuk, N. (2012). Enzymatic production of human milk fat analogues containing stearidonic acid and optimization of reactions by response surface methodology, *LWT-Food Science and Technology, 46* (1), 210-216.
- Bilgiç, S., **Yüksel, A.** (2012). "University Students' Perception and Attitudes towards Functional Foods in Istanbul", 3rd International Conference on Industrial Engineering and Operations Management (IEOM), 3-6 July, Istanbul, Turkey (Full Text)
- **Yüksel, A.,** Özdal, T., Daşkaya, C., Özçelik, B. (2012). "Gurme Yağlar: Üretim Teknikleri, Özellikleri ve Sağlık Açısından Faydaları", Türkiye 11. Gıda Kongresi, 10-12 October, Hatay, Turkey (Abstract).
- Yeşilçubuk, N. Ş., Bilgiç, S., **Yüksel, A.** (2011). "Enzimatik Yöntemler İle Sağlıklı Yağların Üretimi", 2. Zeytinyağı ve Sofralık Zeytin Sempozyumu, 7-8 December, İzmir, Turkey. (Full Text-Oral Presentation)
- Yeşilçubuk, N. Ş., **Yüksel, A. (**2011**).** "Lipase- Catalysed Production of Human Milk Fat Substitutes Enriched with stearidonic Acid", *9th Euro Fed Lipid Congress,* 18-21 September, Rotterdam, Holland. (Abstract-Oral Presentation)
- **Yüksel, A.,** Yeşilçubuk, N. Ş. (2010). "Production of Human Milk Fat Substitutes Containing DHA-Rich Algal Oil", *8th Euro Fed Lipid Congress*, 21-24 November, Munich, Germany. (Abstract)
- **Yüksel, A.,** Turan, D., Yeşilçubuk, N. Ş. (2010). "Structured Lipids Designed for Infant Nutrition", *VII. International Nutrition and Dietetics Congress*, 14-18 April, Istanbul, Turkey. (Abstract)
- Turan, D., **Yüksel, A.,** Yeşilçubuk, N. Ş. (2010). "The Importance Of Docosahexaenoic Acid (DHA) and Arachidonic Acid (ARA) in Infant Nutrition and Their Use in Human Milk Fat Analogs", *VII. International Nutrition and Dietetics Congress*, 14-18 April, Istanbul, Turkey. (Abstract)