ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

FORTIFICATION OF AYRAN (DRINKING YOGHURT) WITH ENCAPSULATED COCOA HULL WASTE EXTRACT BY NANO-LIPOSOMAL SYTEMS, SHELF LIFE AND BIOACCESSIBILITY STUDIES

M.Sc. THESIS

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Thesis Advisor: Prof. Dr. Beraat ÖZÇELİK

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

AYRAN (İÇİLEBİLİR YOĞURT'UN) NANO-LİPOZOMAL SİSTEMLER İLE ENKAPSÜLE ENDİLEN KAKAO KABUĞU ATIĞI EKSTRAKTI İLE ZENGİNLEŞTİRİLMESİ: RAF ÖMRÜ VE BİYOYARARLILIK ÇALIŞMASI

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KASIM, 2016

Gokce Altin, a **M.Sc.** student of ITU **Graduate School of** Science Engineering and Technology student ID **506141514**, successfully defended the **thesis/dissertation** entitled "**FORTIFICATION OF AYRAN (DRINKING YOGHURT) WITH ENCAPSULATED COCOA HULL WASTE EXTRACT BY NANO-LIPOSOMAL SYTEMS, SHELF LIFE AND BIOACCESSIBILITY STUDIES**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 10 October 2016
Date of Defense : 04 November 2016

To my Professor and my family,

FOREWORD

Fortification of foods with bioactive compounds and develop new functional foods are one of the hot topics for food reseach area. The aim of this thesis was enriched the ayran (drinking yoghurt) with bioactive compounds of cocoa hull waste extract which was encapsulated with chitosan coated and spray dried nano-liposomes. In this study, the bioavailability and protection level of spray dried and chitosan liposomes with cocoa hull waste extract in ayran (drinking yoghurt) during the shelf life period was investigated. I hope the results of this study will be guide the further reseachs about food and pharmaceutical area.

Firstly, I would like to express my appreciation and special gratitude to my supervisors, Prof. Dr. Beraat ÖZÇELİK for her guidance and support throughout this study. It is my pleasure to be a student of her.

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October 2016

Gokce Altin

Food Engineer

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ABBREVIATIONS

CHWE : Cocoa Hull Waste Extract
CUPRAC : Cupric Ion Reducing Antioxidant Capacity
DDA : Degreeof Deacylation
DPPH : 2,2-diphenly-1-picrylhydrazyl
EE : Encapsulation Efficiency
EN : Engineered Nanoparticle
FAOSTAT : Food and Agricultural Organization of the United Nations
LbL : Layer-by-Layer
MD : Maltodexrind
PDA : Photodiode Array Detector
SEM : Scanning Electron Microscopy
SGF : Simulated Gastric Fluid
SIF : Simulated Intestinal Fluid
SPE : Solid Phase Extraction
TAC : Total Antioxidant Content
TFC : Total Flavonoid Content
TPC : Total Phenolic Content
UHPLC : Ultra High Performance Liquid Chromotography

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FORTIFICATION OF AYRAN (DRINKING YOGHURT) WITH ENCAPSULATED COCOA HULL WASTE EXTRACT BY NANO-LIPOSOMAL SYTEMS, SHELF LIFE AND BIOACCESSIBILITY STUDIES

SUMMARY

In this thesis, ayran (drinking yoghurt) was fortificated (1:10 w/w) with encapsultated cocoa hull phenolics which extracted from cocoa hull waste. Nano-liposomal systems were used for encapsulation that were chitosan coated (secondary) liposomes and spray dried liposomes. The protection level of cocoa hull phenolics and their biovailability in these systems were investigated in fortificated ayran (drinking yoghurt) during shelf life period.

Cocoa hull waste was a phenolic rich source and encapsulation of these bioactive compounds in nano-liposomal system was an intelligent way both to protect them negative environment conditions and to increase their bioavaibility. In these study we encapsulated cocoa hull waste extract (CHWE) by fine-disperse anionic liposomes with different concentration (0.1-1.0 %). Encapsulation efficiency of liposomes was depended on extract concentration which also effect the particle size of liposomes.

The maximum encapsulation efficiency was found 73.6% in concentration 0.2% w/v and the particle size is 157 nm, the ζ –potential is – 25 mV at this concentration. To enhance the liposome structure, their surface was coated with chitosan and then were solidificated by spray dryer. Layer-by-layer deposition method was used for coated the primary anionic liposomes with cationic chitosan (0.4 w/v%) and before the spray drying they mixed with maltodextrin (MD) (20 w/v%). Change of encapsulation efficiency (EE%) of bioactive compounds in primary, chitosan coated (secondary) and spray dried liposome was investigated, the spray dried liposome had the higher EE%. The protection of cocoa hull phenolics by liposomal systems during in-vitro digestion was investigated by comparing freeze dried CHWE, spray dried CHWE, secondary liposome with CHWE and spray dried liposome with CHWE. The better protection of bioactive compounds in gastro-intestinal digestion (in-vitro) was supported by spray dried liposomes. It is showed that covering the liposomes with chitosan layer provided the liposome stability and getting them to dry powder forms enhanced both stability and bioaccessibility. The bioavailability of these compounds in spray dried liposome at least 4 times higher than freeze dried forms.

The phenolic acids profile and their bioavailability of CHWE and the CHWE in nano-liposomal systems was determined by in ultra high performance liquid chromatography (UHPLC). There were detected 10 major phenolic compounds. Their stability in gastro-intestinal digestion showed differences which based on molecular structure of phenolic acids and their location in the liposome. The bioactive compounds of cocoa hull were protected better in spray dried liposomes compared with secondary liposome, spray dried CHWE and freeze dried CHWE. The cocoa hull waste contained both flavon-3-ol monomers, flavan (quercetin) and

phenolic acids. The interaction of these compounds with carbohydrate and oil bases molecules was an important parameter of their degradation level and bioavailability. In addition, the content and location of them in liposome structure (interior or surface) is another factor to determine the protection degree.

According to these datas, the ayran (drinking yoghurt) was enriched with both encapsulated and unencapsulated cocoa phenolics which extracted from cocoa hull waste. To determine the protection level of liposomes and their effect of bioaccessibility; freeze dried cocoa hull waste extract (CHWE), spray dried CHWE, chitosan coated liposome (secondary liposome) with CHWE and spray dried chitosan liposome with CHWE were used for fortification. Protection of bioactive compounds which were total phenolic compound (TPC), total antioxidant compound (TAC) and total flavonoid compound (TFC) in fortificated ayran (drinking yoghurt) samples was investigated before and after in-vito digestion during the shelf-life period (1^{st}, 5^{th} and 10^{th} and 15^{th} day) at 4^{o}C storage temperature. Spray dried liposome showed the highest protection level of phenolic compounds both before and after in-vitro digestion which were at least 2 times and 5 times higher than ayran (drinking yoghurt) with freeze dried CHWE before and after in-vitro digestion, respectively. The bioavailabilty of CHWE phenolics in ayran (drinking yoghurt) were increased at least 7 folds when they are added with spay dried liposomes, instead of freeze dried CHWE.

The CHWE phenolic profile of ayran samples were identified by UHPLC, and determined these compounds in ayran samples during shelf-life period. The spray dried liposomes showed the better protection of all phenolic compounds in ayran during shelf life period.

In addition, liposomal systems showed the better protection level of phenolics both before and after in-vitro digestion. The surface charge of liposomes and their interaction of other food components, especially ayran proteins was an important parameter for liposome stability, as spray dried liposomal systems provided higher protection of phenolics compared with secondary liposomes during shelf life.

There are several studies about fortification of yoghurt and yoghurt products with polyphenol extracts, but using the liposomal encapsulation of polyphenols is the first study for enrichment the ayran (drinking yoghurt). In additon these is the first study about fortification of ayran, which is a traditional fermented milk beverage in Turkey and contain salts, with polyphenol extracts.

AYRANIN NANO-LİPOZOMAL SİSTEMLER İLE ENKAPSÜLE ENDİLEN KAKAO KABUĞU ATIĞI EKSTRAKTI İLE ZENGİNLEŞTİRİLMESİ: RAF ÖMRÜ VE BİYOYARARLILIK ÇALIŞMAS

ÖZET

Bu tezde, ayran enkapsüle edilen kakao kabuğu fenolikleri ile zenginleştirilmiştir (1:10 w/w). Enkapsülasyonda nano-lipozomal sistemler olan kitosanla kaplı ikincil ve püskürtmeli kurutulmuş lipozomlar kullanılmıştır. Kakao kabuğu fenoliklerinin bu sistemler ile korunma seviyeleri ve biyoyararlılıkları ayran formülasyonunda raf ömrü süresince incelenmiştir.

Zengin fenolik içeriğine sahip kakao kabuğu atığı ekstraktının içerdiği biyoaktif bileşenlerin nano-lipozomal sistemler ile enkapsüle edilmesi ile bu aktif bileşenler olumsuz çevre koşullarıdan korunmuş olmakta ve bu bileşenlerin biyoyararlılıkları arttırılmaktadır. Bu çalışmada kakao kabuğu atığı ekstraktı iyi-dağılımlı aniyonik lipozomlar ile farklı konsantrasyonlarda (0,1-0,9 %) enkapsüle edilmiştir. Lipozomların enkapsülasyon veriminin ve partikül büyüklüklerinin ekstrakt konsantrasyonuna bağlı olduğu gözlemlenmiştir.

Enkapsülasyon veriminin tespiti için toplam fenolik madde miktarı, toplam antioksidan miktarı ve toplam flavonoid madde miktarı spektrofotometrik yöntemler kullanılarak tespit edilmistir. Toplam fenolik madde miktarı tayininde Folin–Ciocalteu, toplam antioksidan madde miktarı tayininde CUPRAC (cupric ion reducing antioxidant capacity) ve DPPH (2,2-diphenyl-1-picrylhydrazyl) metotları kullanılmıştır. 0,2% ağırlık/hacim konsantrasyonda maksimum enkapsülasyon verimi elde edilmiştir. Bu konsantrasyonda partikül büyüklüğü 157 nm ve ζ –potansiyali – 25 mV olarak ölçülmüştür.

Lipozomun yapısal dayanıklılığını arttırmak için lipozom yüzeyi kitosan ile kaplanmış ve daha sonra püskürtmeli kurutucu kullanılarak yüzeyleri kitosan ile kaplanan lipozomlar toz hale getirilmiştir. Kitosan ile kaplanmamış olan birincil lipozomların yüzeyleri tabaka tabaka depozisyon yöntemi kullanılarak katyonik kitosan (0,4 ağırlık/hacim %) ile kaplanmış ve maltodekstrin (20 ağırlık/hacim %) ile karıştırıldıktan sonra püskürtmeli kurutucu ile katı forma getirilmiştir. Birincil lipozomların kitosan ile kaplanmış ikincil lipozomların ve toz forma getirilen lipozomların enkapsülasyon verimleri karşılaştırılmış, sonuç olarak; toz formdaki lipozomların en yüksek enkapsülasyon verimine sahip olduğu tespit edilmiştir.

Bunların yanı sıra dondurularak kurutulmus kakao kabuğu atığı exkstraktının, püskürtmeli kurutucuda kurutularak toz forma getirilmiş kakao kabuğu atığı exkstraktının, ikincil lipozomlardaki kakao kabuğu atığı exkstraktının ve toz formdaki lipozomlardaki kakao kabuğu atığı ekstraktının içerdiği biyoaktif bileşenlerin in vitro sindirimi öncesi ve sonrası korunumları spektrofotometrik yöntemler (toplam fenolik madde miktarı, toplam antioksidan miktarı ve toplam

flavonoid madde miktarı tespiti) kullanılarak karşılaştırılmış sonuç olarak toz formdaki lipozomların en iyi korumayı sağladığı tespit edilmştir. Buna ilaveten lipozom ile enkapsüle edilen kakao kabuğu atığı ekstarktlarındaki biyoaktif bileşenler in vitro sindirim sırasında enkapsüle edilmeyenlere göre daha az degradasyona uğramıştır. Lipozom yüzeyinin kitosan ile kaplanması lipozom yapısını sağlamlaştırmış toz forma getirmek yapıyı daha da dayanıklı hale getirmiş ve biyoyararlılığı arttırmıştır. Kakao kabuğu fenoliklerinin biyoyararlılıkları diğer örneklere göre püskürtmeli kurutulmuş lipozomlarda en az 4 kat daha fazla bulunmuştur.

Ultra yüksek performanslı sıvı kromotografisi kullanılarak enkapsüle edilmemiş (dondurarak kurutulmuş ve püskürtmeli kurutucuda toz forma getirilmiş) ve enkapsüle edilmiş (kitosan ile kaplı olan ikincil lipozomlar ve püskürtmeli kurutucu ile toz forma getirilen lipozomlar) olan kakao kabuğu atığı ekstraktının biyoyararlılıkları ve in vitro sindirim öncesi ve sonrası fenolik asit profilleri araştırılmış sonuç olarak 10 temel fenolik bileşen tespit edilmiştir bu bilesenler flavon-3-ol monomerleri, flavan (quercetin) and phenolic asitlerden meydana gelmektedir.

Tespit edilen fenolik bileşenlerin mide-bağırsak sistemindeki in-vitro sindirimi fenolik bileşenlerin molekül yapısına ve lipozomdaki miktarlarına ve lipozomda bulundukarı yere (lipozomun iç tabakası olarak adlandırılan korda ya da lipozomun yüzeyinde) göre farklılıklar göstermektedir. Bunların yanı sıra fenolik bileşenlerin karbonhidrat ve yağ molekülleri ile etkileşimi bu bileşenlerin degradasyon seviyelerini ve biyoyararlılıklarını etkileyen bir diğer önemli parametredir. Yaptığımız çalışmalar göstermiştir ki; lipozom ile enkapsülasyon, biyoaktif bileşenlerin daha iyi korunmasını sağlayarak biyoyararlılıklarını arttırmıştır. Bununla beraber ikincil lipozomlar ile toz forma getirilen lipozomlar karşılaştırıldığında toz forma getirilen lipozomların biyoyararlıklılarının ve koruma seviyelerinin daha iyi olduğu tespit edilmiştir

Tüm bu veriler ışığında ayran toz formdaki kitosan ile kaplanan lipozom ile enkapsüle edilmiş kakao kabuğu atığı ekstraktı ve sıvı formdaki kitosan ile kaplanan lipozom (ikincil) ile enkapsüle edilmiş kakao kabuğu atığı ekstraktı ile zenginleştirilmiştir.

Sıvı bir ürün olan içilebilir yoğurda toz forma getirililmiş lipozomal yapıların katılmasının biyoaktif bileşenin korunması ve bu biyoaktif bileşenlerin biyoyararlılığına olan etkisi incelenmiştir. Bunun yanı sıra enkapsülsyonun kakao kabuğu atığı ekstraktındaki biyoaktif bileşenlerin korunumu ve biyoyararlılığı üzerine olan etkilerini tespit edebilmek için içilebilir yoğurt aynı zamanda; dondurularak kurutulmus kakao kabuğu atığı ekstraktı ve püskürtmeli kurutucuda toz forma getirilmiş kakao kabuğu atığı ekstraktı ile de zenginleştirilmiştir. Kontrol grubu olarak zenginleştirilme yapılamamış olan ayran örnekleri kullanılmıştır. Tüm ayran örnekleri 4°C depolanmış ve raf ömrü boyunca (15 gün) biyoaktif bileşenlerin korunumu ve biyoyararlılıkları incelenmiştir.

Toz formdaki lipozom ile enkapsüle edilmiş kakao kabuğu atığı ekstraktı, ikincil lipozom ile enkapsüle edilmiş kakao kabuğu atığı ekstraktı, dondurularak kurutulmuş kakao kabuğu atığı ekstraktı ve püskürtmeli kurutucuda toz forma getirilmiş kakao kabuğu atığı ekstraktı ile de zenginleştirilen ayran örneklerindeki biyoaktif bileşen miktarları; toplam fenolik madde, toplam antioksidan madde ve toplam flavonoid madde miktarlarının spektrofotometrik yöntemler ile analizlenmesi ile tespit

edilmiştir. Analizler 1. 5. 10. ve 15. günlerde in vitro sindirim öncesi ve sonrası yapılmış, raf ömrüne bağlı olarak zenginleştirilmiş içilebilir yoğurt örneklerinin biyoyararlılıkları ve kakao kabuğu ekstraktlarındaki biyoaltif madde miktarlarındaki korunum incelenmiştir. Ayran, püskürtmeli lipozomlar ile zenginleştirildiğinde; kakao kabuğu fenoliklerinin korunumu dondurularak kurutulmuş kakao kabuğu fenolikleri ile zenginleştirmeye göre raf ömrü süresince in-vitro sindirim öncesi 2 kat sonrası, 5 kat artmıştır. Bu iki ayran örneği karşılaştırıldığında; püskürtmlei kurutulmuş lipozomlardaki kakao fenoliliklerinin raf ömrü boyunca biyoyararlılıklarının en az 7 kat arttığı saptanmıştır.

Raf ömrü süresince ayran örneklerindeki kakao kabuğu atığı ekstraktlarının biyoaktif madde profili; in vitro sindirim öncesi ve sonrası ultra yüksek performanslı sıvı kromatografisi ile tanımlanmıştır. Tespit edilen fenolik maddeler, ayran ürününe katmadan önce dondurulmuş kakao kabuğu ekstraktındaki, püskürtmeli kurutucuda toz forma getirilmiş kakao kabuğu ekstraktındaki, ikincil lipozomdaki kakao kabuğu ekstraktındaki ve toz forma getirilmiş lipozomdaki kakao kabuğu ekstraktındaki biyoaktif bileşenler ile aynı olup; bozunma seviyelerine bağlı olarak bu biyoaktif bileşenlerin miktarları içilebilir ayran örnekleri arasında farklılık göstermektedir.

Lipozomların yüzey yükleri lipozomun stabilitesini belirleyen önemli faktörlerden biridir. Aktif yüzey yüklerine sahip olan lipozomların bulundukları ortamdaki diğer moleküller ile etkileşime girmeleri ile lipozomların yapıları dağılabilir. Ayranın lipozomal yapılar kullanılarak biyoaktif bileşenlerce zenginleştirildiği bu çalışmada, lipozomlar ve ayran proteinlerinin etkileşimi lipozomların raf ömrü süresince dayanıklılığını etkileyen önemli faktörlerden biridir. Raf ömrü süresince in-vitro sindirim öncesi ve sonrası yapılan ultra yüksek performanslı sıvı kromotografisi analizlerinde ayrana katılmış olan toz forma getirilmiş lipozomların; ikincil lipozomlara göre kakao kabuğu atığı ekstraktlarındaki biyoaktif bileşenleri daha iyi koruduğu ve bu bileşenlerin biyoyararlılıklarını arttırdığı tespit edilmiştir.

Bunlara ek olarak, lipozom ile enkapsüle edilmiş kakao kabuğu atığı ekstraktlarındaki biyoaktif bileşenler; enkapsüle edilmeyenlere göre ayranda raf ömrü süresince in-vitro sindirim öncesi ve sonrası daha az degrade olmuştur.

Litaretürde yoğurt ve yoğurt ürünlerinin polifenolik ve fenolik maddelerce zenginleştirilmesi ile ilgili mevcut çalışmalar bulunmaktadır. Bununla beraber, lipozom ile enkapsüle edilerek korunumu ve biyoyararlılığı arttırılmış olan biyoaktif maddelerin ayrana katılması ve lipozomların bir gıda matrisi içerisindeyken biyoyararlılık çalışmasının yapılması bir ilktir. Bunların yanı sıra, bu çalışma tuz içeren geleneksel bir Türk içeceği olan ayranın biyoaktif maddelerce zenginleştirildiği ve raf ömrü süresince biyoyararlılık çalışmalarının yapıldığı ilk çalışmadır.

1. INTRODUCTION

Cocoa (*Theobroma cacao* L.) is a popular food ingredient especially chocolate and bakery industry. Several studies highlighted that consumption of cocoa containing foods provide positive health effects which are associated with the phytochemical contents of cocoa (Tomas-Barberan, Borges & Crozier, 2011).

Catechins, flavonol glycoside, anthocyanins and procynanidins are the main cocoa polyphenols and these bioactive compounds reduce a lower risk of cardiovascular disease and cancer as they have antioxidant, antiradical and anti-carcogenic properties (Brava, 1998; Wollgast & Ankham, 2000, Buijsse et al., 2010).

Polyphenols are highly instable antioxidative bioactive compound, they intearct with other food components such as proteins and carbohydrates and they are degraded by light, soluble oxygen and enzyems.

Encapsulation technology is an effective way to protect polyphenols from negative environment conditions, in addition this technology inhibit the inteaction beween polyphenols and other molecues and it can allow masking of odour and colour of polyphenols. Moreover, bioavaibility of polyphenols increased when they are encapsulated (Fhang & Bhandari, 2010; Kanouni et al., 2002; Muschiolik, 2007).

Liposomes are enclosed spherical vesicles formed by dispersion of certain polar lipids in an aqua solvent. Therefore, they are organized in one or several concentric phospholipidic bilayers with an internal aqua phase, which makes them as carrier systems for both water and oil soluble functional compound. They are; biodegradable, biocompatible, nontoxic and non-immunogenic. They have a great potential for applications pharmaceutical, food and agricultural industries (Anweker, Patel, & Singhai, 2011; McClements & Li, 2010; Laye et al., 2008; Taylor et al., 2005).

The modification of liposome surfaces provides the liposomes stabilization. Layer-by-Layer (LbL) technology is a simple and versatile method which supports stabilization of liposomes (Ciobanu et al., 2007). In addition, solidification of

liposomal deliery systems enhance their stability, shelf life, bioavailability and increase their usage area (Moraes, 2012).

In this study, we investigated the possible usage of dry liposomal delivery systems containing cocoa hull waste phenolic extract in ayran (drinking yoghurt) to improved new functional beverage. For these aim, first, primary liposomes were produced by using high pressure homogenization method and they coated with cationic chitosan (secondary liposome) with respect to the layer by layer deposition method. For getting liposomal dry powders, the spray drying method is used, hence the chitosan coated liposomes mixed with the maltodextrin which is simplify spray drying. After the solidification, liposomal dry powders were added into industrially produced ayran (drinking yoghurt). The liposome stability and the level of encapsulation protection of the cocoa phenolics was observed compared with the differenceses between the ayran (drinking yoghurt) with freeze dried extract, extract mixed with maltodextrin (dry powder forms), chitosan coated liposomes and spray dried liposomes during the self life period.

This thesis showed that covering the liposomes with chitosan layer provided the liposome stability and getting them to dry powder forms enhanced both stability and bioaccessibility. The cocoa hull phenolics was protected better in spray dried liposomes compared with secondary liposome, spray dried CHWE and freeze dried CHWE. The cocoa hull waste contained both flavon-3-ol monomers, flavan (quercetin) and phenolic acids. According to these findings ayran (drinking yoghurt) was fortificated with both unencapsulated and encapsulated cocoa phenolics. It is showed that liposomal systems had the better protection level of phenolics both before and after in-vito digestion. The surface charge of liposomes and their interaction of other food components, especially ayran (drinking yoghurt) proteins was an important parameter to liposome stability, as spray dried liposomal systems provided higher protection of phenolics compared with secondary liposomes during shelf life. In addition, the content and location of them in liposome structure (interior or surface) is another factor to determine the protection degree. On the other hand, interaction between phenolics-proteins and phenolics-carbohydrates cause difficulties to determine the exact phenolic amount before in-vitro digestion, hence more research is need to this issue. Our results are guide for studies on functional food industry.

2. LITERATUR SUMMERY

2.1 Cocoa and Bioactive Compound of Cocoa

Cocoa beans are the seeds of the fruits which are harvesting from the cocoa trees (*Theobroma cacao*). After harvesting, beans are extracted from pods, fermented and dried to produce the cocoa products (Dias, 2014). At the end of the cocoa production %92 of the original fruit is being waste (Ntiamoah & Afrane, 2008). In 2012, Food and Agricultural Organization of the United Nations (FAOSTAT) is reported (2014) that approximately 55 million tons of cocoa fruits are produced by the main cocoa producing countries (Ivory Coast, Indonesia, Ghana, Nigeria, Cameroon, and Brazil) and only 4.4 million tons of cocoa beans are used for cocoa goods and the 50.6 million tons being waste.

Cocoa hulls are the part of the cocoa bean which being waste at the end of the cocoa production. The composition of cocoa hulls approximately (g/kg) proteins (181); nitrogen (29); fiber (606); fat (68); moisture (101); ash (81); phytic acid (5.9); theobromine (12); phenolics (18.2) (Arlorio et al., 2001).

Phenolic acids and polyphenols are natural polyphenols that are secondary metabolites of plants. There has been classified more than 8000 phenolic structures in cocoa plant in addition; flavonol glycoside, anthocyanins and procyanidins are the main cocoa polyphenols and these bioactive compounds have antioxidant, antiradical and anti-carcogenic properties (Brava, 1998; Wollgast & Ankham, 2000). Due to their potential health benefits, there are several studies about using these bioactive compounds as a food additive for improving functional foods (Fang & Bhandari, 2010). Phenolics are contributing to flavor, color, astringency and bitterness of fruits and vegetables. Thus, they could show some negative sensory effects when they added directly to the food products such as altering the original taste and color of the original food. In addition, they are effected environmental conditions in negatively,

they can be oxidate by light, moisture and oxygen or degrade by enzyems (Muschiolik, 2007).

2.2 Encapsulation of Bioactive Compound

Encapsulation technology is an effective way to protect bioactive compound from negative environment conditions, moreover it can allow masking of odour and colour of bioactive compound and also bioavailability of these compounds is increased when they are encapsulated (Fhang & Bhandari, 2010; Kanouni et al., 2002; Muschiolik, 2007). Microemulsions, liposomes, emulsions, multiple emulsions, solid-lipid nanoparticles, biopolimer nanoparticles and microgels, which are shown in Figure 2.1, are commonly used for encapsulation of bioactive compound (McClements, 2014). There are different encapsulation methods; emulsification, coacervation, fluid bed coating and extrusion technologies, liposome encapsulation, and cyclodextrin encapsulation are the most commonly used (Fang & Bhandari, 2010).

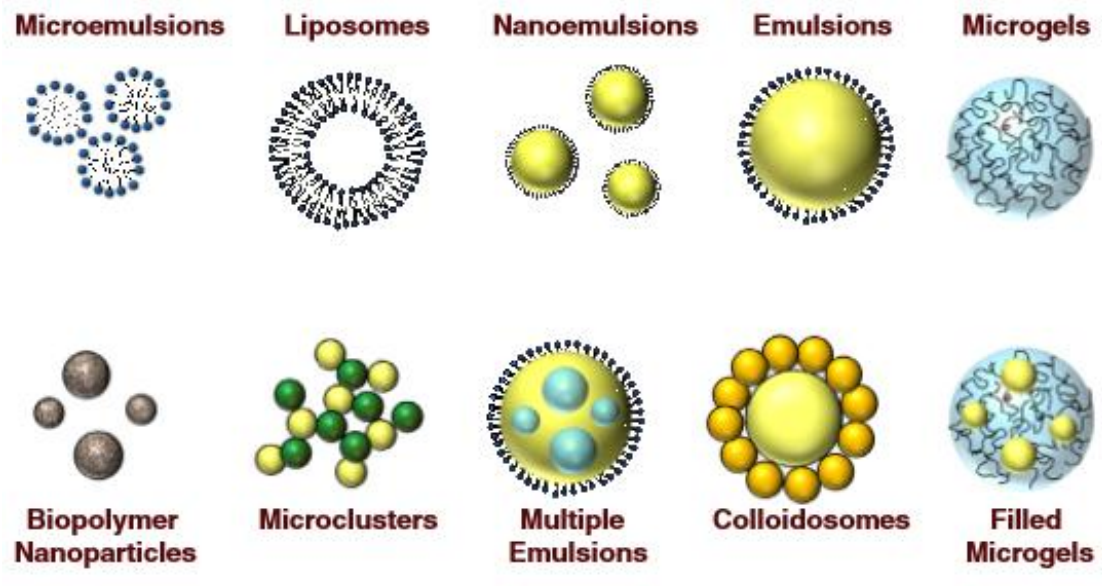


Figure 2.1 : Particle-based delivery systems that can be used to encapsulation of bioactive compound.

Engineered nanoparticles are new area for encapsulation of food componenets and bioactive compounds. Basicly they divided two main group; non-lipid and lipid based ENs (Yao et al., 2015). In Table 2.1 ENs based delivery systems are shown

according to their ingredients and functional food componenes which are encapsulated by them.

Table 2.1 : Engineered nanoparticles based delivery systems.

	Delivery systems	**Functional food components**	**Ingredients**
Non-lipid-based ENs	Biopolymeric nanogels, antisolvent precipitation	Lipophilic compound (omega-3 fatty acids, CLA, oil soluble vitamins, etc.)	Miscible solvents, biopolymers, surfactants
	Biopolymeric nanoparticles	Omega-3/omega-6 polyunsaturated fatty acids	Water, dextrin
	Biopolymeric nanoparticles	Curcumin	Water, b-cyclodextrin, modified starch, surfactant
	Organogel based nanoemulsion	Curcumin	Water, organogel, surfactant
	Nanocomplex	Omega-3 fatty acids	Water, b-lactoglobulin, protein
	Protein-based micelles	Vitamin D	Water, surfactant (casein)
	Micelles	Curcumin	Water, surfactant
	Nanoformulation	Co Q10	Water, surfactant, glycerol
Lipid-based ENs	Nanoemulsion	Lipophilic compound (vitamin E, b-carotene)	Water, surfactant, oil
	Nanoemulsion	Co Q10	Water, surfactant, oil
	Liposome	Polyphenols (curcumin, resveratrol)	Water, cholest, phospholipids, erol
	Nanoliposome	EGCG	Water, phospholipids, cholesterol
	Nanoemulsion	Curcumin	Water, MCT, surfactant
	Solid lipid nanoparticles	Lipophilic compound (carotenoids, omega-3 fatty acids, phytosterols)	Oil, surfactant
	Microemulsion	Curcumin	Water, surfactants, oil
	Micelles	Lycopene	Oil, water, lecithin, surfactant

2.2.1 Liposomes

Liposomes are enclosed spherical vesicles formed by dispersion of certain polar lipids in an aqua solvent which means they are organized in one or several concentric phospholipidic bilayers with an internal aqua phase. Liposomes are nontoxic, non-immunogenic, biodegradable and biocompatible moreover, they are carrier systems for both water and oil soluble functional compounds such as, antioxidants, flavors, antimicrobials and bioactive compounds and also they can be carry these compounds to the designated target and release them on demand. Therefore, they have great

potential for applications for pharmaceutical, food and agricultural industries (Anweker et al., 2011; McClements & Li, 2010; Laye et al., 2008; Taylor et al., 2005). Thin layer dispersion, freeze-thawing, reverse-phase evaporation, homogenization, microfluidization and heating method are the most commonly used methodologies for liposome preparation (Liu et al., 2015).

Liposomes can protect the carried compound, which are in the aqueous interior core, from negative external conditions such as pH, oxygen, light or enzymes (Shehata et al., 2008). However, liposomes are highly flexible and fragile bilayer membranes so they have some restrictions due to their insufficient chemical and physical stabilities (Park et al., 2014; Panya et al., 2010). One of the major problem is the aggregation of vesicles as liposomes tend to attach to each other, so particle size distribution is changed and the other major limitation is tendency to leak in liposome structure, both of these situations are causing to destruction the encapsulated material and the encapsulation conditions (Park et al., 2014; Taylor et al., 2005). In addition, liposome membrane structures consist the unsaturated fatty acids and there might be occur oxidation reactions and these reactions are leading to formation of hydroperoxides, malondialdehyde, hexanal and other very rancid olfactory substances (Panya et al., 2010). The modification of surface of liposomes provides the liposomes stabilization.

Layer-by-Layer (LbL) technology is a simple and versatile method which supports stabilization of liposomes (Ciobanu et al., 2007). In these method, multilayer films are developed by alternating the deposition of oppositely charged polyelectrolytes with electrostatic attraction (Jeon et al., 2015). Figure 2.2 shows the schematic diagram of a polymer coated liposome. Pectin, chitosan or combined pectin/chitosan are the polymers that can be used for coating the liposome surface (Gibis et al., 2014).

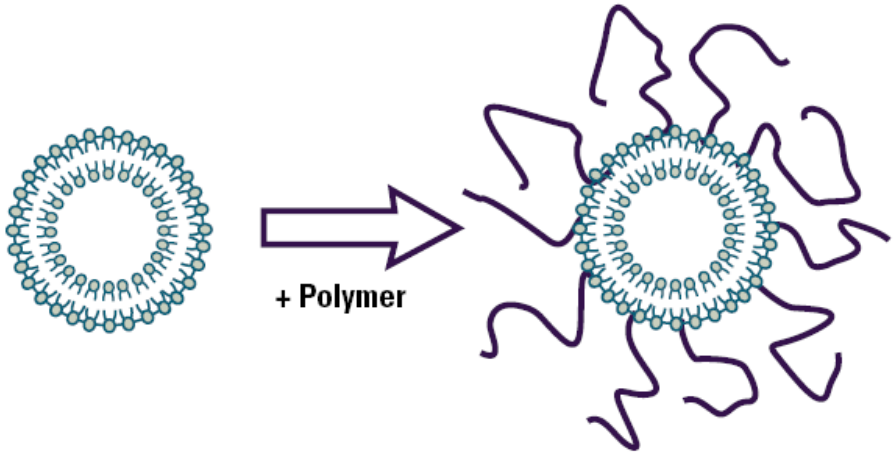


Figure 2.2 : The schematic diagram of a polymer coated liposome.

Liposome systems are being in an aqueous environment which allows much physical and chemical decomposition. Converting the liposomes to dry forms is an intelligent way to improve their stability. In addition, they are being more applicable for industrial usage. Power bed grinding, freeze-drying, fluidized bed drying, or spray drying are some solidification methods. Spray drying is a suitable technique for production of dry liposomal powders as it is energy and time saving, more economic and wide range of usage in food industry (Moraes et al., 2012).

2.3 Ayran (Drinking Yoghurt)

In 21th centruy consumers prefer to consume functional foods as they known reduce the risk of disease and more healty than other industrial foods. As these preference lead to growth of the global functional beverage market sector in the food industry (Marsh et al., 2014). During 2003 to 2010 the global functional food and drink market increased 1.5 folds, (Leatherhead, 2011).

Fermented milks especially yoghurt style products are the most popular functional beverages (Marsh et al., 2014). Ayran (drinking yoghurt) is the most preferable fermented beverage in Turkey especially in summer season. Aproximatelly 1 million-ton ayran (drinking yoghurt) is consumed annually in Turkey (Kocak & Avsar, 2009).

Ayran (drinking yoghurt) is a fermented dairy product prepared by the addition of water to yoghurt (homemade) or addition of yoghurt culture; *Streptococcus thermophilus* and *L. delbrueckii* subsp. *Bulgaricus* to standarized milk (industrially

produced) (TFC, 2009). Several authors are reported the composition of ayran (drinking yoghurt); the total dry matter (1.07–11%), protein (1.44–3.48%), salt (0.17– 1.75%) and fat (0.1–3%). In adition the pH varies between 3.44 and 4.44 and titratable acidity is 0.4–1.73% (Gulmez et al., 2003; Kocak et al., 2006; Sanli et al., 2011; Sen & Kuplulu, 2004; Patir et al., 2006; Tamucay-Ozunlu & Kocak, 2010).

Ayran (drinking yoghurt) has a high nutrition value as it is including high content of vitamin and calcium and also it is easily digestible dairy product (Kok-Tas & Guzel-Seydim, 2010). In homemade ayran (drinking yoghurt) preparation, yoghurt is dillueted by water and mixed until the desired concentration that is generally between 30 % and 50% and table salt is added nearly 1% for improving the taste. In industrial ayran (drinking yoghurt) production, the dry matter content of milk is standardized, yoghurt culture (*Streptococcus thermophilus* and *L. delbrueckii* subsp. *Bulgaricus*) is added to the standarized milk and fermented, during the fermantation the exopolysaccharide is produced by the culture and at the end of the fermantation the product is diluted with water and added salt. Finally, ayran (drinking yoghurt) is quickly cooled to stop fermentation (Erkaya et al., 2015).

Fortification of yoghurt and yoghurt products (ayran) with antioxidant-rich extracts appears to be convenient functional food format (Howard et al., 2000). There are several studies about development functional yoghurt products by adding them to antioxidant-rich sources such as enrichment of yoghurts with grape (*Vitis vinifera*) seed extracts (Chouchouli et al., 2013), green, white and black tea (Muniandy et al., 2016), apple polyphenols (Sun-Waterhouse et al., 2012), berry polyphenols (Sun-Waterhouse, Zhou & Wadhwa, 2013), pomgranate peel extracts (El-Said et al., 2014).

2.4 Bioaccessibility of Bioactive Compounds in Nano-Liposomes

Encapsulation of bioactive compounds by engineered nanoparticles (ENs)-based delivery system is an effective way to improve their bioavailability (Yao et al.,2014). Gastro-intestinal track condition shows some negative effect in bioactive compounds, which may change in the location, chemistry and physical state of them. Hence, their bioaccesibility are decreasing.

Liposomes are one of the lipid-based ENs. During the gastro-intestinal digestion, liposomes hydrolyzed by lipases to the free fatty acids and monoacylglycerols. These digestion products interact with bile salts and phospholipids in the lumen of the small intestine to form “mixed micelles” with complex structures (Yao et al., 2014). After the liposome digestion, encapsulated bioactive compounds are transferred to the mixed micelles which enhance their bioacccesibility (Porter & Charman, 2001; Sun et al., 2015).

In nano-scale generation of mixed micelles more rapidly than larger particle, which accelerate transfer of the BC from the particles to the mixed micelles (Yao et al., 2014).

3. EXPERIMENTAL PROCEDURE

3.1 Materials and Methods

3.1.1 Materials

Cocoa hull wastes were local chocolate factory and ayran was provided from local supermarket. Lecithin (Soybean phospholipids, 97%- Ultralec® P) was provided by Rotel, Turkey, chitosan with 80% DDA (degree of deacylation) was donated from Primex (Siglufjordur, Iceland) and maltodextrin was a gift from Tunckaya Kimyevi Maddeler Ticaret ve Sanayi Inc., Turkey. Sodium acetate tri-hydrate, acetic acid, sodium hydroxide, hydrochloric acid, potassium chloride, gallic acid, neocuproine, trolox, DPPH reagent, copper (II) chloride, catechin, pancreatin and bile extract were purchased from Sigma-Aldrich Co. (St. Louis, USA). Sephadex G50 was purchased from GE Healthcare Life Sciences (Uppsala, Sweeden). Folin Ciocalteu's phenol reagent, ammonium acetate, sodium hydroxide, sodium chloride, sodium nitrite, aluminium chloride, potassium dihydrogen phosphate, sodium monohydrogen phosphate heptahydrate and pepsin was purchased from Merck KGaA (Darmstadt, Germany). Triton X100 was purchased from Carl Roth GmbH (Karlsruhe, Germany). Methanol was purchased from J.T. Baker (Netherlands). Potassium chloride was purchased from Carlo Erba Reagent SpA, Rodano (MI). Acetone was purchased from Emboy (Turkey) and SPE cartridges was purchased from Macherey-Nager (Duren, Germany).

3.2 Preparation of Cocoa Hull Waste Extract

The phenolic compounds extraction from cocoa hull waste extract was made by according to the method (Azizah et al., 1999) with slight modifications**.** First, the cocoa butter was removed from cocoa hull waste**.** Cocoa hull waste was mixed with hexane 1:10 w/w, stirred with a vortexer for 60 seconds, placed in ultrasonic water bath at 30 °C for 10 minutes and centrifugated at 30 °C, 5000 rpm for 10 minutes.

The residues were collected and the residual hexane was removed in hood. After removing the cocoa butter; the phenolic compounds were extracted by methanol/MQ water (80% v/v) with 1:10 (w/v), stirred with a vortexer for 60 seconds, placed in ultrasonic water bath at 30 °C for 10 minutes and centrifugated at 4°C, 5000 rpm for 10 minutes. After centrifugation, the top-methanolic phase containing phenolic compounds was collected, methanol was removed in a rotary evaporator at 40 °C. The remained extract was freeze dried and storage -20°C.

3.3 Preparation of Uncoated and Chitosan Coated Liposomes

2 % (w/v) lecithin powder in acetate buffer (pH = 3.5 ± 0.1; 0.1 M) was stirred overnight at room temperature and CHWE (0.1-1.0%, w/v) were added to lecithin solution and dissolved. Liposomes with and without CHWE were prepared by homogenizing solutions with a high shear disperser (DI-25 Yellowline, IKA) for 10 min at 9.500 rpm and then it passed five times through high pressure homogenizer (Microfluidizer Processor M-110L, Microfluidics, Newton, USA) at homogenization pressure of 25.000 psi. For coated liposomes with chitosan; chitosan was dissolved in acetate buffer (pH = 3.5 ± 0.1; 0.1 M) (%0.8 w/w) was stirred overnight at room temperature and filtrated. Liposomes without and with CHWE (0.2% w/v which was an optimum concentration for encapsulation) were added to chitosan solutions (1:1 w/w) and stirred overnight at room temperature hence negatively charged liposomes were coated by electrostatic deposition of positively charged chitosan layer.

3.4 Measurements of Zeta (ζ) Potential and Particle Size Distribution

ζ –potential was measured by a Zeta-sizer (Zeta-sizer 2000, Malvern Instruments), before the measurement liposomal dispersions were diluted to a particle concentration of approximately 0.005% (w/v) with acetate buffer. Results are reported as the average and standard deviation of measurements made from two freshly prepared samples. A static light scattering instrument (Master sizer 2000, Malvern Instruments) was used for measuring the particle size distribution of samples. For calculating the particle size distributions; a refractive index was used that is 1.44 and 1.33 for lecithin for the aqueous phase. Average particle diameters were reported by using the volume mean diameter ($d_{4,3}$). All particle size measurements were made on at least two freshly prepared samples with three

readings made per sample. Powder samples was dissolved in acetate buffer (pH=3.5) 1:5 (w/v) for reconstruction. The ζ –potential and particle diameter were measured after reconstitution using the same methods that explained before.

3.5 Removal of Unencapsulated Extract by Gel Filtration

Both the extract which had not been encapsulated in liposomes, and chitosan which had not bound to liposomal surfaces were removed by Sephadex gel filtration method (Gibis et al., 2012). Firstly, sephadex G50 solution (5 wt % in deionized water) was prepared and syringes (6 mL) were filled with that solution until a layer of about 3 cm of gel had been formed. 1.5 mL of acetate buffer was added on top of the gels. Then syringes were placed into falcon tubes and the Sephadex G50 column then centrifuged at 3000 rpm for 10 min. After that, 1.5 mL of sample was added on top of the gels and the centrifugation repeated. Gel filtered samples collected in the falcon tubes were then further used.

3.6 Determination of Encapsulation Efficiency

The encapsulation efficiency was determined by the method (Gibis et al., 2012), that consist determination by spectrophotometric assays (determination of total phenolic content, total antioxidant activity and total flavonoid content). Encapsulation efficiency (EE%) of primary liposomes, chitosan coated liposomes and the liposomal powder were determined.

TPC, TAC and TFC were determined both liposomes in the aquaeous phase and gel filtrated liposomes and also destabilized liposomes. Triton X-100 was added for liposomes destabilization due to make the phenolics in the interior of liposomes accessible to reagent. Gel-filtered liposomes were deliberately destabilized by addition of 3 mL of 0.15 w/v% Triton X-100 followed by vortexed and centrifugation at 6000 rpm for 10 minutes.

3.7 Spectrophotometric Assays

3.7.1 Determination of total phenolic content (TPC)

Total phenolic content was measured according to Folin–Ciocalteu reagent test by Gibis et al. (2012) with slight modifications. 200 µL of the diluted sample was mixed with 1.5 mL of the diluted Folin–Ciocalteu reagent (1:10 with MQ water), stirred with a vortexer, 1.2 mL of the sodium carbonate solution (7.5%, w/v) was added and again stirred with a vortexer. The sample was left to stand for 48 min in dark, centrifuged at 300 rpm for 10 minutes and extinction of top phase was measured at 765 nm. The results were expressed as mg gallic acid per L sample.

3.7.2 Determination of total antioxidant content (TAC)

CUPRAC (cupric ion reducing antioxidant capacity) method (Apak et al., 2005) and DPPH (2,2-diphenyl-1-picrylhydrazyl) method (Kumaran et al., 2006) was used to determine the total antioxidant capacity with slight modifications. In CUPRAC method, 100 µL sample was placed in test tube and then, 1 mL of ammonium acetate (pH:7), 1 mL of neocuproine solution ($7{,}5x10^{-3}$M), 1 mL of copper (II) chloride solution (10^{-2} mM), 1 mL of MQ water were added respectively. The sample was left to stand for 25 min in dark and centrifuge 600 rpm for 5 minutes its extinction was measured at 450 nm. In DPPH method, 100 µL sample was placed in test tube and then 2 mL of DPPH solution (10^{-1} mM) were added and shake for 10 seconds. The sample was left to stand for 25 min in dark and centrifuged 600 rpm for 5 minutes and its extinction was measured at 517 nm. The results were expressed as mg trolox per L sample for both CUPRAC and DPPH methods.

3.7.3 Determination of total flavanoid content (TFC)

250 µL sample was diluted with 1.25 mL of MQ water. 75 µL of sodium nitrite solution (5% w/v) was added, after 6 minutes 150 µL of aluminum chloride solution (10 % w/v) was added. After 5 minute 500 µL of sodium hydoxide (1 M) was added, then 2.5 mL of MQ water was added and shake for 10 seconds. The sample was centrifuged 600 rpm for 1 minutes and its extinction was measured at 510 nm. The results were expressed as mg catechin per L sample (Dewanto *et.al*., 2002).

3.8 Spray Drying

Before the spray drying application, CHWE (0.05% w/v), chitosan coated liposomal dispersion with and without CHWE were mixed with maltodextrin solutions (20% w/v) and stirred overnight at room temperature. The feeding mixture was included 20 % (w/v) MD, 0.5 % (w/v) lecithin and 0.2 % (w/v) chitosan for chitosan coated liposomal dispersion with and without CHWE. A laboratory scale spray-drier whis is shown in Fifure 3.1 (Mini spray dryer B-290, BUCHI, Switzerland) was used for solidification which equipped with a 1.5-mm nozzle atomizer operated at an atomizing air flow of 5 cm^3/min a feed rate of 2.5 cm^3/min at inlet temperature of 170 °C resulting in outlet temperature of 75-80 °C and 0.67 m^3/min air flow. Dried

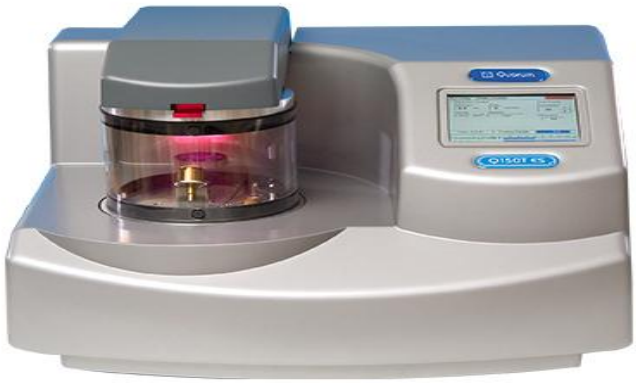
powders were stored in airtight containers and placed in a desiccator at room temperature.

Figure 3.1 : Spray Dryer which used for solidification of freeze dried CHWE and chitosan coated liposomes with CHWE.

3.9 Scanning Electron Microscopy (SEM)

Spray dried liposomes with and without CHWE and spray dried CHWE were mounted onto adhesive-coated aluminum pin stubs. Excess powder was removed by blowing dry air across. The stubs were sputter coated with a thin layer of gold in a Leica vacuum coating unit at 40 mA for 100 seconds 3 times, at a working distance

of 50 mm by using an argon gas purge (Quorum SC7620 Sputter Coater). The samples were examined using a Quanta FEG 250 SEM which is shown in Figure 3.2.



The SEM was operated at high vacuum with an accelerating voltage of 10 kV. Images were taken at 4000 and 12000 magnifications.

Figure 3.2 : Scanning electron microscopy.

3.10 Fortification of Ayran (Drinking Yoghurt) Formulation

Secondary and spray dried liposomes with and without CHWE, spray dried and freeze dried CHWE were added to industrially produced ayran (drinking yoghurt) (10% w/w). The level of liposomal encapsulation protection of the phenolic compounds of the CHWE and their bioaccessibility was observed during in shelf life period (1^{st}, 5^{th} and 10^{th} and 15^{th} day) at 4^{o}C storage temperature. Fresh ayran (drinking yoghurt) was extracted in defined days to determine the phenolic concervation in ayran (drinking yoghurt) formulation. In vitro studies fresh ayran (drinking yoghurt) samples was used. Gultekin-Ozguven et al. (2016) was enriched the dark chocolate with spray dried black mulberry waste extract. As presence of cocoa butter and cooca mass, they had to be used spray dried liposmes for enrichment. However, both aqueus liposome dispersion (secondary liposomes) and dry powders (spray dried powders) could be used for fortification of ayran. As spray drying inceased the liposome stability (Karadag et al., 2013; Gultekin-Ozguven et al., 2016) we added both secondary and spray dried liposome to compare the stabilities between these liposomes in food product.

3.11 Preparation of Ayran (Drinking Yoghurt) Extracts

20 g of ayran (drinking yoghurt) sample was mixed with 30 ml of acetone solution (30 ml acetone; 0.1 ml HCL was complated 100 ml with distilated water) was vortexed and keep at + 4^{o}C overnight, sample was filtrated with Whatman No:2 filter paper and the aqueous phase was collected. Aceton was removed by rotary evaporator at 40^{o}C, samples were centrifugated at 10 000 rpm, 2 min. 10 ^{o}C. The top-aqueos phase was collected and the water was removed by freze dryer. Extracts were storaged at -20 °C.

3.12 In-vitro Digestion

Bioaccesibility studies were done according to the method which developed by Tan et.al. (2014). All steps made in mixing water bath at 37 °C, 100 rpm. First, 13.5 mL of basal saline (140 mM NaCl, 5 mM KCl) was placed in mixing water bath and the 1.5 mL of sample was added and they mixed for 10 min and the mixture was treated with 4.5 mL simulated gastric fluid (SGF) (containing 3.2 g/L pepsin 1 M HCI), followed by adjusting pH to 2.0 using 1.0 M NaOH for initiated the gastric digestion. After 1 h incubation at 37 °C, the pH of the sample was adjusted to 7.5 with 1.0 M NaOH. Then 4.5 mL simulated intestinal fluid (SIF) (containing 4.76 mg/mL pancreatin and 5.16 mg/mL porcine bile extract in PBS, pH 7.5) was added. During 2 h of the intestinal digestion process the pH of the solution was maintained at 7.5 by adding 0.1 M NaOH manually hence during the liposome digestion the phenolic acids released and they were dropped the pH. The pH must be 7.5 for simulating the intestinal environment. After the digestion the sample ware centrifuged at 6000 rpm for 15 minutes. The top phases were collected and storged at -20 °C for further analyses. To determine the % bioaccessibility equation 3.1 was used.

$$Bioaccessibility\ \% = \frac{Amount\ of\ CHWE\ in\ the\ micelle\ phase}{Amount\ of\ CHWE\ in\ the\ formulation} x100 \qquad (3.1)$$

3.13 Quantification and Identification of Phenolic Compound in CHWE, CHWE in Liposomal Systems and Fortified Ayran (Drinking Yoghurt) Samples During Shelf Life Period by Using UHPLC

A solid phase extraction (SPE) method was used to purified the digested samples before the UHPLC analyses. 1.5 mL of samples were acidified with 30 µL of formic acid and centrifuged (Labnet Spectrafuge 16M, Labnet International Inc., Woodbridge, NJ, USA) at 16000g for 10 minutes. 500 mg/6 mL C18 SPE cartridges (MACHEREY-NAGEL GmbH & Co.KG, Germany) were conditioned by rinsing with 6 mL of formic acid/methanol (1:100, v/v) and then 4 mL of formic acid/MQ water (1:100 v/v). 1.5 mL of sample which acidified with formic acid and centrifuged was passed into the activated cartridges and then the cartridges washed with 5 mL of formic acid/MQ water (1:100 v/v). Samples were eluted with 1.5 mL of formic acid/methanol (1:100, v/v) and filtered through 0.45 µL membrane filters. Before the UHPLC analyses, primary, secondary and spray dried samples were destabilized by tritonX100, mixed with methanol (1:1 v/v) and centrifuged at 10000 rpm for 2 minutes after that filtered through 0.45 µL membrane filters. The CHWE extract was mixed with methanol (1:1 w/v) and centrifuged at 10000 rpm for 2 minutes after that filtered through 0.45 µL membrane filters. A Shimadzu LC -10A apparatus (Kyoto, Japan) equipped with a SPD-M10A photodiode array detector (PDA) was used for analytical UHPLC separations. Reversed-phase chromatography was performed with 250 x 4.6 mm Kromasil 100 C-18 column packed with 5µm particles (Teknokroma, Barcelona, Spain), fitted with a security guard C18 ODS (4 x3.0 mm i.d). Gradient were formed with He-degassed solvent. Solvent A was H_2O containing 0.1 % formic acid, and solvent B was MeCN by applying different elution conditions. Separation was accomplished starting with 5% A for 2 min at pressure of 115 bar, followed by a linear gradient was performed for 10 min from 5% B to 95 % A and subsequent linear gradient from 20 to 95% A in 5 min. The flow rate was 0.5 $mL.min^{-1}$ and the operating temperature was 40 ^0C. The injection volume was 10µL. The chromatogram was recorded at 286 nm (phenolic acids).

3.14 Statistical analysis

All analyses were repeated at least three times using triplicate samples. Data were subjected to statistical analysis using IBM SPSS software (version 24.0, Chicago, IL, USA). Differences were analyzed by Tukey post hock test. p value of <0.05 was chosen to determine significant differences.

4. RESULTS & DISCUSSION

In this study, cocoa hull waste extract (CHWE) was encapsulated chitosan coated nano-liposomes and then these liposomes and freeze dried CHWE was converting the dry forms by using spray dryer. The stability and bioaccessibility of unencapsulated and encapsulated CHWE investigated and the spray dried lipsomes with CHWE showed the highest protection level both before and after digestion. Moreover, for comparing the unencapsulated and encapsulated CHWE performance in food system, ayran (drinking yoghurt) was fortified with freeze dried CHWE, spray dried CHWE, secondary lipsomes with CHWE and spray dried liposomes with CHWE. The stability and bioaccessibilty of them was determined during the shelf life period (15 days at 4 °C). According the results the ayran (drinking yoghurt) which fortified by spray dried lipsomes with CHWE had a higher protection level dring storage and showed higher protection and accumulation results in bioavaibility study during the shelf life period.

4.1 Characterization of Primary (Uncoated), Secondary (Chitosan Coated) and Spray Dried Liposomes with and without Cocao Hull Waste Extract

To characterize primary (uncoated) liposomes, secondary (chitosan coated) and spray dried liposomes with and without CHWE ζ –potential and the mean particle diameter of samples were measured. Mean particle diameter of primary liposomes without CHWE was measured approximately 156 nm at 25.000 psi homogenization pressure. The particle size of primary liposomes with CHWE had higher value as it depends on the extract concentration. We studied in primary liposomes which have % 0.0 to % 0.9 (w/v) concentration of CHWE to determine the optimum concentration for encapsulation. The ζ –potential and the mean particle diameter of primary liposomes was given Figure 4.1.

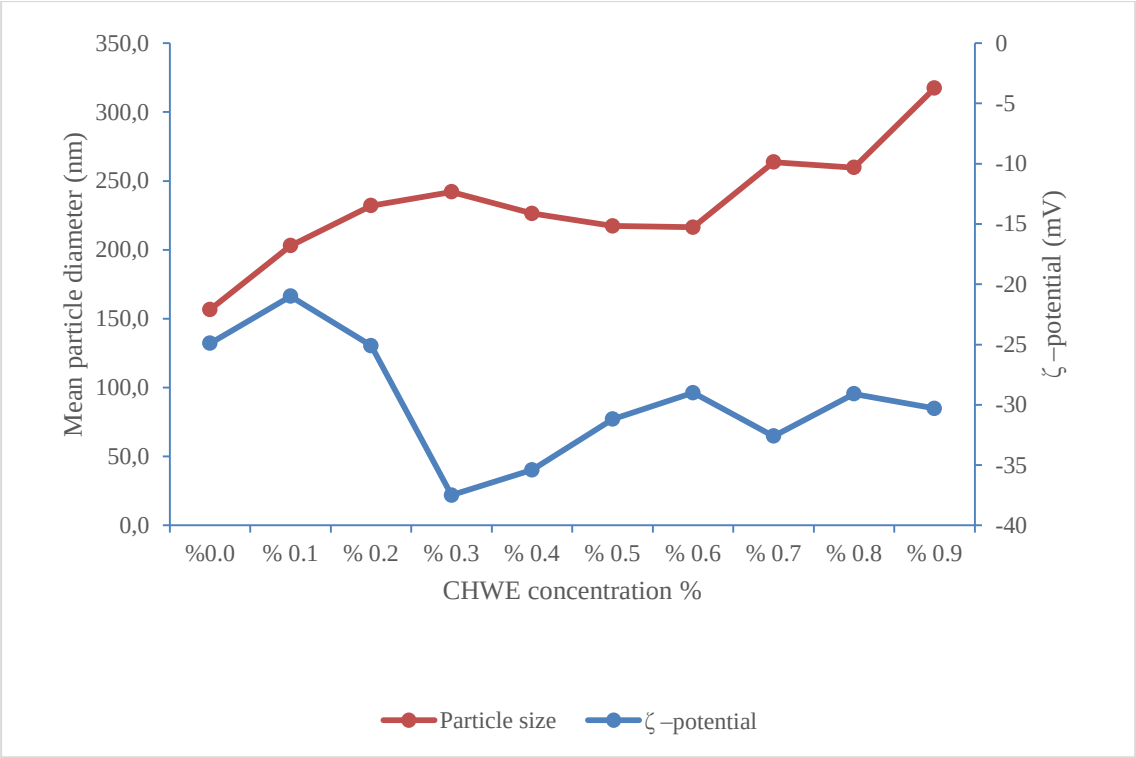


Figure 4.1 : Charge in ζ –potential and mean particle diameter (nm) in primary liposomes with different concentration of CHWE (0.0% - 0.9%).

Chitosan was used enhanced the liposome stability. As the size of chitosan coated liposomes depends on chitosan concentration, there might occurred flocculation if the chitosan concentration was lower than 0.2 (w/v %) (Karadag et al., 2013, Chun et al., 2013). On the other hand, flocculation was reduced by increasing chitosan concentration and the mean diameter of aggregates became minimum at ~0.4 chitosan concentration (w/v %) (Gultekin-Ozguven et al., 2016). We used this level to cover the liposome surface with chitosan and the mean particle diameter of chitosan coated liposomes without and with % 0.1 (w/v) concentration of CHWE was measured approximately 151 and 360 nm respectively. Primary liposomes ζ –potential values, ranging between -21.0 and -37.5 mV which shows that positively charged phenolics was encapsulated by negatively charged liposomes (Figure 1). After addition of the chitosan the surface charge of the liposomes change from negative from positive (~ + 38 mV) which shows the primary liposomes were coated with chitosan. Particle size and zeta potential of the primary liposomes, chitosan coated liposomes, spray dried liposomes without CHWE and with CHWE and spray dried CHWE are shown in Table 4.1.

Table 4.1 : Particle size and ζ –potential of primary (0.2 w/v%), secondary (0.1w/v%), spray dried liposomes (0.05 w/v%) with and without CHWE and spray dried CHWE (0.05 w/w%).

	Primary liposome without CHWE	**Primary liposome with CHWE**	**Secondary liposome without CHWE**	**Secondary liposome with CHWE**	**Spray dried liposome without CHWE**	**Spray dried liposome with CHWE**	**CHWE mixed with MD**
Particle size (nm)	156.67 ± 2.1	232 ± 2.8	145.22 ± 4.9	399.5 ± 2.3	174 ± 4.5	231 ± 3.2	-
ζ –potential (mV)	-24.85 ± 2.33	-25.05 ± 3.32	35.3 ± 5.02	35.7 ± 0.92	-33.8 ± 0.51	-33.9 ± 0.42	-13.1 ± 0.23

*Values are presented as mean values ± standard deviation (n=3).

4.2 Encapsulation Efficiency, Content and Location of Cocoa Hull Phenolics in Primary (Uncoated), Secondary (Chitosan Coated) and Spray Dried Liposomes

Gibis et al., (2012) reported that liposomes were not able to encapsulate the all extract. They encapsulated grape seed extract by liposomes and their results showed that the extract located in not only interior of the liposome, but also aqueous phase and liposome surface. Figure 4.2 shows the CHWE concentrations of primary liposomes and their encapsulation efficiency.

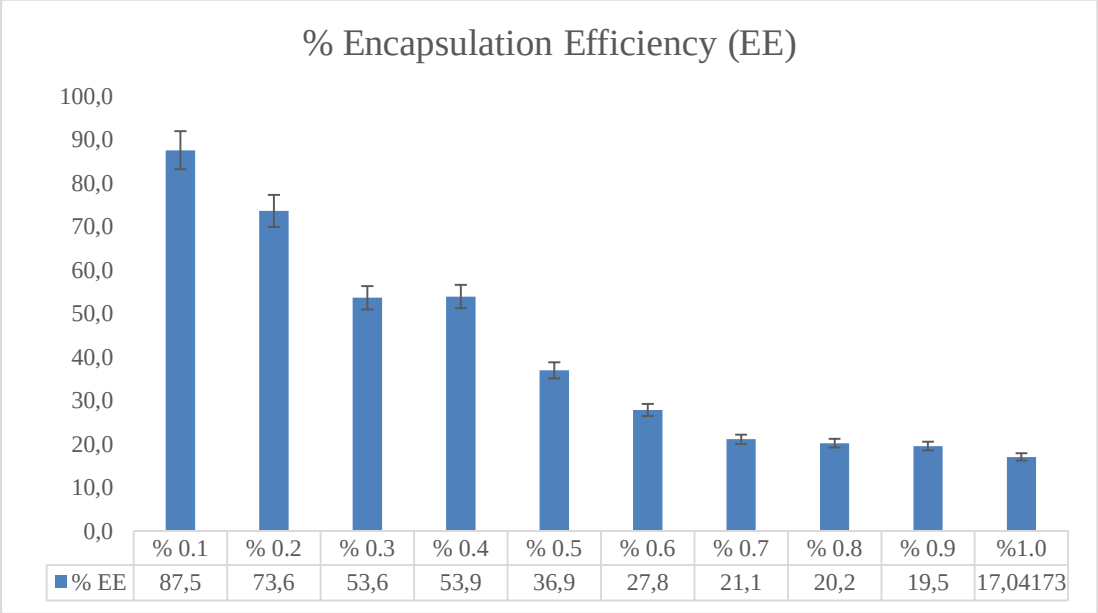


Figure 4.2 : Encapsulation efficiency (%) in primary liposomes in different cocoa hull waste extract concentration (0.1-1.0 %).

*Error bars represent the standard deviation.

Encapsulation efficiency, content and location of CHWE in primary (uncoated), secondary (chitosan coated) and spray dried liposomes were determined by according to the method of (Gibis et al., 2012). To find the optimum encapsulation efficiency in primary liposomes, CHWE concentrations of 0.1-1 % (w/v) were studied. Total phenolic content (TPC) were investigated to find the optimum encapsulation efficiency. TPC was determined in the aqueous phase containing liposomes before and after gel filtration. After removing unencapsulated phenolics by gel filtration, Triton X-100 were used to destabilized the liposomes by the way phenolics in the interior of liposomes accessible to reagent. The value of TPC in this step consists of phenolics on the surface or in the membrane plus the amount located in the interior of liposomes. The amount of phenolics inside the liposomes was

calculated by subtraction of the measured amount on intact liposomes and the measured amount on destabilized liposomes. Whether the highest efficiency % was found in 0.1% (w/v) concentration of CHWE, 0.2% (w/v) concentration of CHWE with 73.6 % was selected. Hence in the following steps the multiple dilution cause difficulties during the spectrophotometric measurements for 0.1 % (w/v) concentration of CHWE. Our findings are comparable with the results reported by Gibis et al. (2012) and Gultekin-Ozguven et al. (2016). They reported that 83.5% of the added grape seed extract 0.1% (w/v) and 76.8% of the added black mulberry waste extract 0.2% (w/v) was incorporated on the surface and to the interior of the liposomes, respectively.

Encapsulation efficiency of primary, secondary and spray dried liposomes was investigated on TPC, TAC and TFC and % EE capacity of spray dried liposomes had better results for all cases are shown in Figure 4.3.

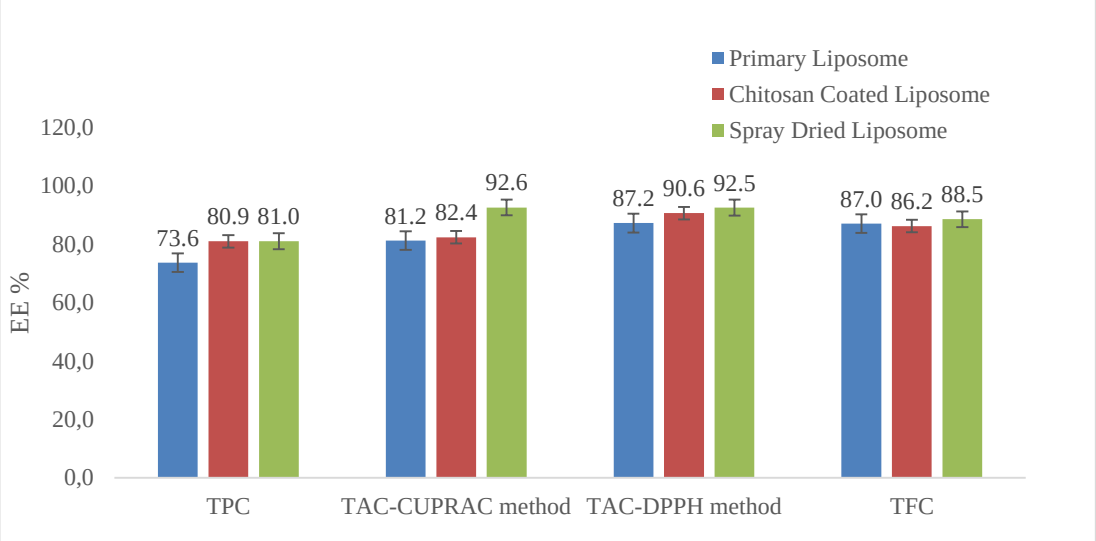


Figure 4.3 : Encapsulation efficiency (%) of primary, secondary and spray dried liposomes.

*Error bars represent the standard deviation.

0.2% (w/v) concentration of CHWE with 73.6 % was used for further steps which were coating with chitosan and spray drying. Total phenolic, antioxidant and flavonoid substance encapsulation efficiency on primary, chitosan coated and spray dried liposomes with 0.2 (w/v) concentration of CHWE were investigated. There was an increment of EE% after covering liposomes with chitosan. Chitosan was covering both liposome, the CHWE was on the surface of liposome and free CHWE which

was located in near the liposome surface, that could be increase the encapsulation yield. After the solidification of liposomes there were not a significant increment in encapsulation efficiency.

The TPC, TAC and TFC on surface or membrane of the liposomes was calculated after gel filtration. In primary liposome, TPC was found 71.68 ± 8.14 mg/L on liposome surface and 205.58 ± 29.13 mg/L was determined interior of the liposome. TAC was determined two different methods. According to the CUPRAC method TAC was found 96.33 ± 15.39 mg/L on surface and 113.37 ± 17.66 mg/L was inside, while with DPPH method TAC was calculated 96.82 ± 3.26 mg/L on surface and 243.05 ± 9.39 mg/L in the interior of liposome. TFC on the surface was 28.23 ± 6.48 mg/L and 81.65 ± 13.13 mg/L inside of the primary liposome. TPC, TAC and TFC on surface of secondary liposome was increased. Chitosan and its derivatives have an effective antioxidant capacity (Yen et.al., 2008).

Presence of chitosan was effected the increasing of the TPC, TAC and TFC on the surface of secondary liposomes, but their content in the interior of liposome was not change.

On the other hand, there was a decline in TPC, TAC and TFC values in surface or membrane and interior of spray dried liposome. Secondary liposomes were mixed with maltodextrin before spray drying. During analyzed the TPC, TAC and TFC of spray dried liposomes in spectrophotometric assays, turbidity was observed the samples after adding the reagents, hence the samples was centrifuged for clarification.

Maltodextrin could be responsible the turbidity and after the centrifugation, residues might include not only maltodextrin but also, phenolic, antioxidant and flavonoid compounds which were bounded to chitosan and the chitosan collapsed with maltodextrin.

In addition, there was no significant different ($p < 0.05$) between CHWE amount which was interior in primary, secondary and spray dried liposome. The content and location of phenolic, antioxidant and flavonoid amounts in primary, secondary and spray dried liposomes with concentration of CHWE 0.2 w/v%, 0.1 w/v%, 0.05 w/v%, respectively are shown in Table 4.2.

Table 4.2 : Content and location of TPC, TAC and TFC (mg/L) in primary, secondary and spray dried liposomes with 0.2% (w/v) 0.1% (w/v) and 0.05% (w/v) concentration of CHWE, respectively.

	TPC (mg/L)		TAC-CUPRAC (mg/L)		TAC-DPPH (mg/L)		TFC (mg/L)	
	Surface of liposome	Interior of liposome	Surface of liposome	Interior of liposome	Surface of liposome	Interior of liposome	Surface of liposome	Interior of liposome
Primaray Liposomes	71.68 ± 8.14[a]	205.58 ± 29.13[a]	96.33 ± 7.65[a]	113.37 ± 17.66[a]	96.82 ± 3.26[a]	243.05 ± 9.39[a]	28.23 ± 6.48[a]	81.65 ± 13.13[a]
Secondary Liposomes	151.88 ± 15.39[b]	182.1 ± 25.96[a]	143.73 ± 21.62[a]	184 ± 35.31[a]	157.82 ± 21.84[b]	247.76 ± 14.73[b]	50.58 ± 17.05[a]	101.19 ± 61.56[ab]
Spray Dried Liposomes	24.98 ± 7.3[a]	110.85 ± 28.36[a]	20.58 ± 11.67[b]	107,24 ± 39.23[a]	26.79 ± 13.77[b]	140.23 ± 14.23[b]	39.45 ± 13.81[a]	22.23 ± 3.22[b]

*Values are presented as mean values ± standard deviation (n=6). Different small letters in the columns represnet statistically significant differences ($p<0.05$) in each section.

DPPH method was giving better results compared with CUPRAC method to detect the TAC in liposomal systems. CUPRAC method based on reduction of antioxidant compounds (especially polyphenols) by copper (II) reagent. The number and position of the hydroxyl groups of polyphenols as well as the degree of conjugation of the whole molecule are important parameters for the correct measurement of these method (Apak et al., 2004). In contrast to CUPRAC method, the 2,2-diphenyl-1-picrylhydrazyl (DPPH reagent) is reduced by antioxidant compounds (hydrogen-donating antioxidants) in DPPH method (Brand-Williams, Cuvelier, & Berset, 1995). The bioactive composition of CHWE has showed variety. In CUPRAC method some antioxidant compounds might not detected. In addition, existence of chitosan and maltodextrin might restrict the accessibility of reagent to antioxidant compounds.

4.3 Spray Drying of Liposomes

Secondary liposomes with and without CHWE and the CHWE was mixed with maltodextrin (MD) 20% (w/v) before the spar drying. Karadag et al. (2013) reported that MD incerased the the thickness of the interfacial layer of the secondary liposomes and altering the surface charge of them. Due to the osmotic driving force, the mean particle diameter of spray dried liposomes decreased (Karadag et al., 2013: Gultekin-Ozguven et al., 2016) as we found the same decrease which are shown in Table 4.1. The moisture content of spray-dried liposome with and without CHWE and spray dried CHWE was found < 5%.

4.4 Powder Morphology

The structure of spray dried liposome with and without CHWE and spray dried CHWE mostly spherical structure with some small wrinkles and dents (Figure 4.4). In spray drying process, high evaporation rates were responsible to formation of skin around the droplet as solidification of wall material (Shen & Quek, 2014). Thermal expansion of air and vaporaziation of water inside the drying particles lead to dents and wrinkles on the surface which depends on drying rate and viscoelastic properties of wall matrix (Peres et al., 2011). The wrinkles and dents on the surface of spray drying particles using maltodextrin during spray drying was reported before Peres et al. (2011) and Gultekin-Ozguven et al. (2016). We calculated the most of the particles had an average diameter of less than 3 μm where the particule size of the

spray dried lipoosmes without CHWE was smaller than spray dried liposomes with CHWE. These results were correlated to results in Table 4.1.

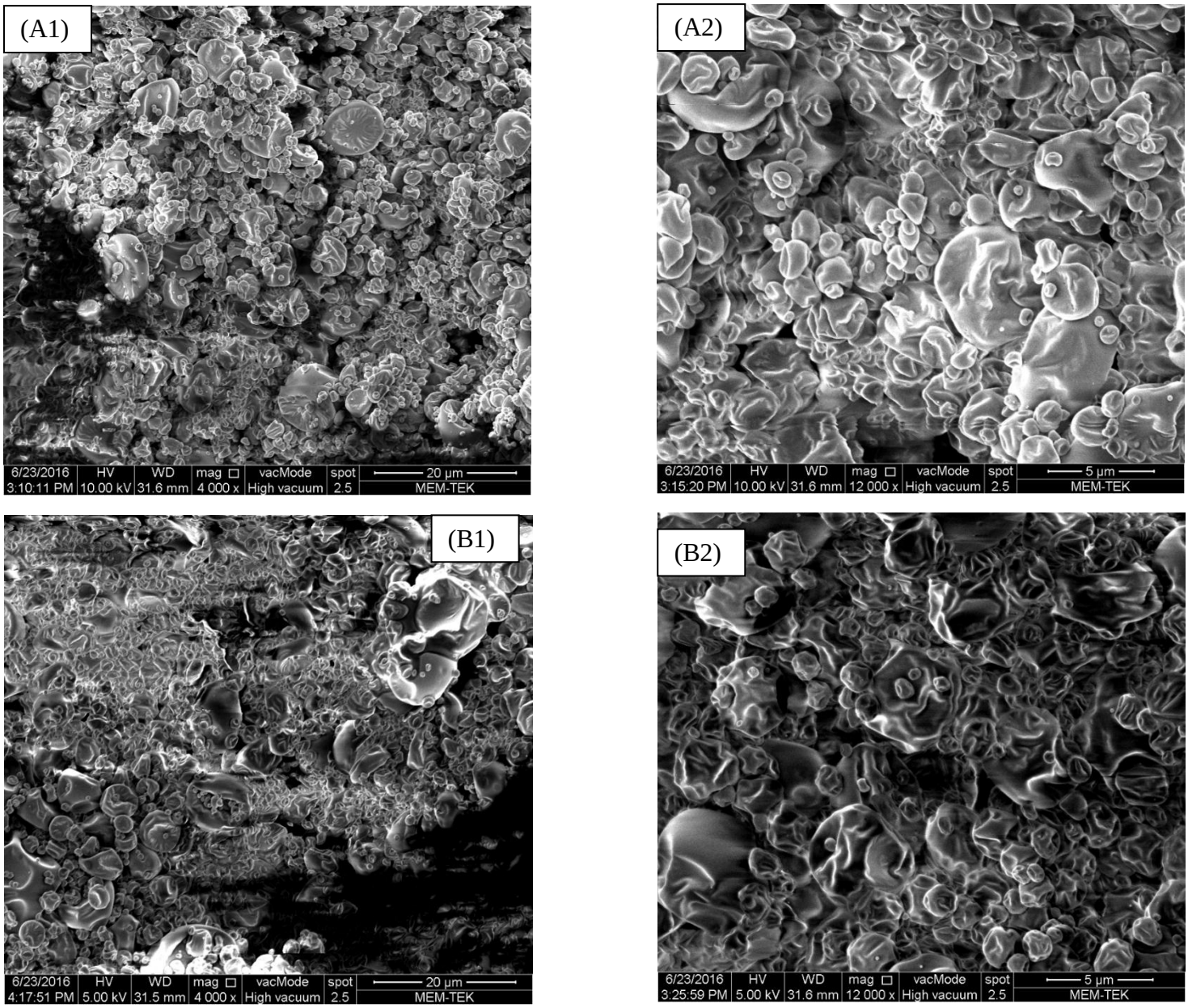


Figure 4.4 : SEM images of spray dried liposome without CHWE (A), spray dried liposome with CHWE (B), spray dried CHWE (C).

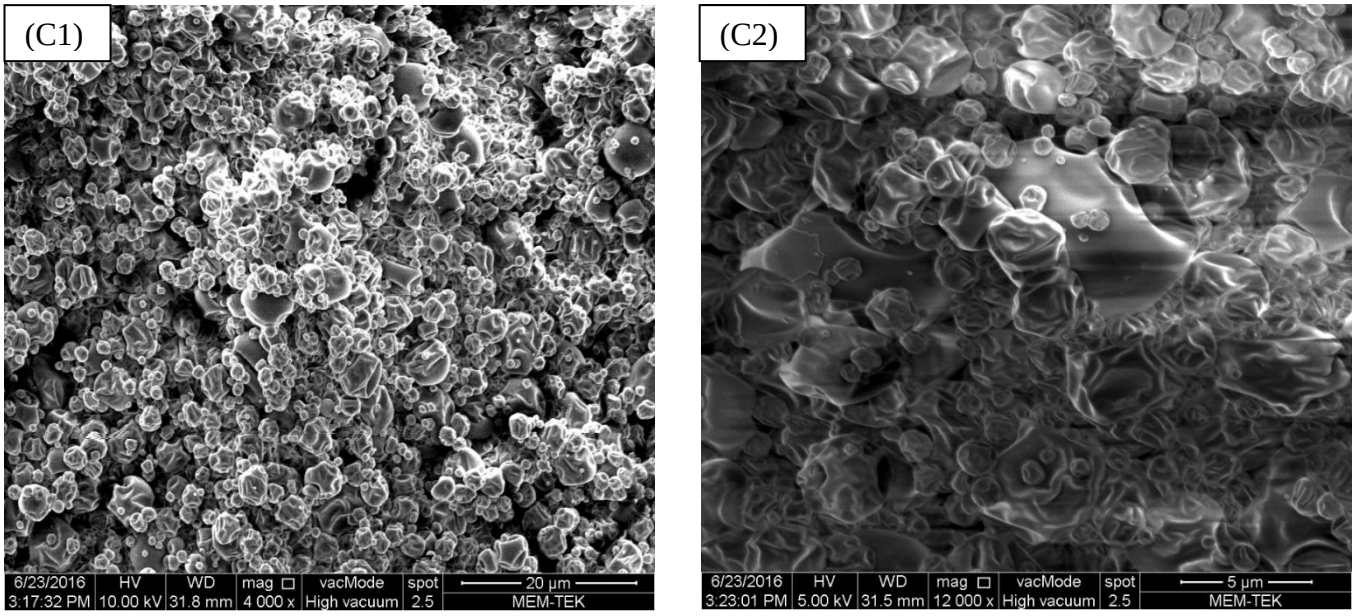


Figure 4.4 (Continued): SEM images of spray dried liposome without CHWE (A), spray dried liposome with CHWE (B), spray dried CHWE (C).

4.5 Effect of Spray Drying on Stability of Cocoa Hull Waste Extract

Retentions of TPC, TAC and TFC were calculated with to the equation 4.1 (Fang & Bhandari, 2011).

$$BC\ retention\% = \frac{BC\ in\ spray\ dried\ powder}{BC\ in\ feed\ solution} * 100 \qquad (4.1)$$

TPC, TAC-CUPRAC, TAC-DPPH, TFC retention of spray dried liposomes was 60.7%, 16.3%, 55.7% and 38.8%, respectively. As the DPPH method might suitable than CUPRAC in liposomal systems the TAC % was showed same which was mentioned before. Our results are comparable with the results reported by Gultekin-Ozguven et al. (2016) who found that %69.2 TPC retention of spray dried liposomes with concentration of 0.05% black mulberry waste extract

4.6 Protection of Bioactive Compound in Fortificated Ayrans (Drinking Yoghurt) During Shelf-Life Period

TPC, TAC and TFC was determined in fortificated ayran samples in 1st, 5th, 10th and 15th days. Table 4.3 showed the TPC, TAC and TFC (mg/mL) in ayran (drinking yoghurt) samples which were fortificated with with freeze dried extract (S1), spray

dried extract (S2), secondary liposome (S3), spray dried liposome (S4) during shelf life period, control was non-fortifcated ayran (drinking yoghurt).

The TPC, TAC and TFC of CHWE in ayran (drinking yoghurt) with spray dried liposome at least two times higher than ayran (drinking yoghurt) with secondary liposome. Our results showed that encapsulation of CHWE decreased the degradation of phenolic compound of CHWE in ayran (drinking yoghurt) formulation during shelf life period.

The phenolic, antioxidant and flavonoid activity of ayran (drinking yoghurt) with spray dried liposome provided the highest level as the results was significantly different ($p < 0.05$) from other samples in 1st, 5th, 10th and 15th days. Our findings showed that the TPC, TAC and TFC in ayran (drinking yoghurt) with spray dried liposome at least five times higher than control groups (Table 4.3).

Moreover, there were no significant difference ($p > 0.05$) in TAC (determined by DPPH method) between ayran (drinking yoghurt) with freeze dried extract and ayran (drinking yoghurt) with secondary liposome in 1st, 5th, 10th and 15th days. In addition, the flavonoid and antioxidant activity of spray dried CHWE was found higher than CHWE in secondary liposomes in ayran (drinking yoghurt) samples (Table 4.3).

The unstability of secondary liposomes might explained by surface charge phenomenon. The pH level of ayran (drinking yoghurt) varied from 4.17-4.10, and at these value the zeta potential of yoghurt proteins ~ -20 mV (Erkaya et al., 2015; Sejersena et al., 2007). Interaction between the negativly charged ayran (drinking yoghurt) proteins and positively charged chitosan destroyed the structre of secondary liposome. Distribution of coated surface of secondary liposome allowed to released of unstabile primary liposome and also which might enhance the degradation of CHWE phenolics that were located on surface of the lipsomes, so the detected amount of TAC and TFC in ayran (drinking yoghurts) might belong only the interior of the secondary lipsome. On the other hand, existence of maltodextrin altered the surface charge of secondary liposomes and freeze dried extract from positive to negative (Karadag et al.,2013; Gultekin-Ozguven et al., 2016).

Table 4.3 : TPC, TAC and TFC (mg/mL) in ayran (drinking yoghurt) with freeze dried extract (S1), with spray dried extract (S2), with secondary liposome (S3), with spray dried liposome (S4) during shelf life period, control was non-fortifcated ayran (drinking yoghurt).

	TPC			
	1st Day	5th Day	10th Day	15th Day
Control	0.27 ± 0.021^{Aa}	0.22 ± 0.03^{Ba}	0.27 ± 0.04^{Aa}	0.23 ± 0.01^{Ba}
S1	0.43 ± 0.02^{Ab}	0.37 ± 0.01^{Bb}	0.36 ± 0.01^{Bb}	0.28 ± 0.02^{Cb}
S2	0.58 ± 0.03^{Abc}	0.68 ± 0.05^{Bc}	0.51 ± 0.03^{Ac}	0.59 ± 0.02^{Ac}
S3	0.60 ± 0.04^{Ac}	0.52 ± 0.01^{Ad}	0.57 ± 0.06^{Ac}	0.48 ± 0.02^{Ad}
S4	1.31 ± 0.04^{Ad}	1.23 ± 0.05^{Ae}	1.25 ± 0.05^{Ad}	1.29 ± 0.03^{Ae}
	TAC-CUPRAC			
	1st Day	5th Day	10th Day	15th Day
Control	0.10 ± 0.01^{Aa}	0.09 ± 0.01^{Ba}	0.10 ± 0.01^{Aa}	0.08 ± 0.01^{Ba}
S1	0.17 ± 0.01^{Ab}	0.17 ± 0.02^{Ab}	0.12 ± 0.01^{Bb}	0.11 ± 0.01^{Bb}
S2	0.17 ± 0.03^{Ab}	0.10 ± 0.03^{Bbc}	0.14 ± 0.03^{ABbc}	0.11 ± 0.02^{Bb}
S3	0.30 ± 0.01^{Ac}	0.21 ± 0.02^{Bd}	0.17 ± 0.02^{Bc}	0.22 ± 0.01^{Bd}
S4	0.44 ± 0.04^{Ad}	0.22 ± 0.04^{Be}	0.24 ± 0.02^{Bd}	0.27 ± 0.02^{Be}
	TAC-DPPH			
	1st Day	5th Day	10th Day	15th Day
Control	0.03 ± 0.005^{Aa}	0.02 ± 0.01^{Aa}	0.02 ± 0.005^{Aa}	0.01 ± 0.005^{Ba}
S1	0.05 ± 0.02^{Aab}	0.04 ± 0.005^{Aa}	0.03 ± 0.005^{Aa}	0.01 ± 0.005^{Ba}
S2	0.34 ± 0.02^{Ac}	0.23 ± 0.02^{Ab}	0.14 ± 0.03^{Bb}	0.08 ± 0.01^{Cb}
S3	0.09 ± 0.007^{Ab}	0.02 ± 0.001^{Ba}	0.02 ± 0.005^{Ba}	0.01 ± 0.003^{Ba}

*Values are presented as mean values ± standard deviation (n=6). Different small letters in the columns or different capital letters in the rows represent statistically significant differences ($p<0.05$) in each section.

Table 4.3 (Continued): TPC, TAC and TFC (mg/mL) in ayran (drinking yoghurt) with freeze dried extract (S1), with spray dried extract (S2), with secondary liposome (S3), with spray dried liposome (S4) during shelf life period, control was non-fortifcated ayran (drinking yoghurt).

S4	0.43 ± 0.04^{Ad}	0.39 ± 0.05^{Ac}	0.48 ± 0.01^{Ac}	0.25 ± 0.01^{Bc}
	TFC			
	1st Day	5th Day	10th Day	15th Day
Control	0.03 ± 0.003^{Aa}	0.02 ± 0.001^{Ba}	0.02 ± 0.003^{Ba}	0.02 ± 0.001^{Ba}
S1	0.04 ± 0.004^{Aa}	0.03 ± 0.005^{Ba}	0.03 ± 0.002^{Ba}	0.03 ± 0.004^{Ca}
S2	0.12 ± 0.005^{Ab}	0.12 ± 0.005^{Ab}	0.10 ± 0.02^{Bb}	0.03 ± 0.003^{Ca}
S3	0.06 ± 0.005^{Ac}	0.05 ± 0.004^{Ac}	0.06 ± 0.003^{Ac}	0.05 ± 0.002^{Ab}
S4	0.20 ± 0.02^{Ad}	0.14 ± 0.01^{Bd}	0.14 ± 0.001^{Bd}	0.09 ± 0.001^{Cc}

*Values are presented as mean values ± standard deviation (n=6). Different small letters in the columns or different capital letters in the rows represent statistically significant differences ($p<0.05$) in each section.

Thus, phenolic compound detected higher amount in ayran (drinking yoghurt) with spray dried liposome and ayran (drinking yoghurt) with spray dried CHWE rather than ayran (drinking yoghurt) with secondary liposome and ayran (drinking yoghurt) with freeze dried CHWE as existance of maltodextrin might inhibits the interaction between yoghurt proteins and spray dried particles.

The phenolic compound in ayran (drinking yoghurt) which fortificated with unencapsulated freeze dried CHWE showed the highest degredation ratio at the end of shelf-life which are shown in Figure 4.5. In addition, we found that the retention (%) of phenolic compound during shelf-life period was higher in liposomal systems both secondary and spray dried liposomes. In particular TPC retention in drinking yoghurt with spray dried liposomes was almost totally protected. When we compare the protection level of flavonoid and antioxidant compound in ayran (drinking yoghurt) with spray dried liposome and secondary liposome, we found that in spray dried liposome showed higher degradation level at the end of shelf-life (Figure 4.5). And also, the total amount of these compound were different between these two samples since, the first day of shelf-life.

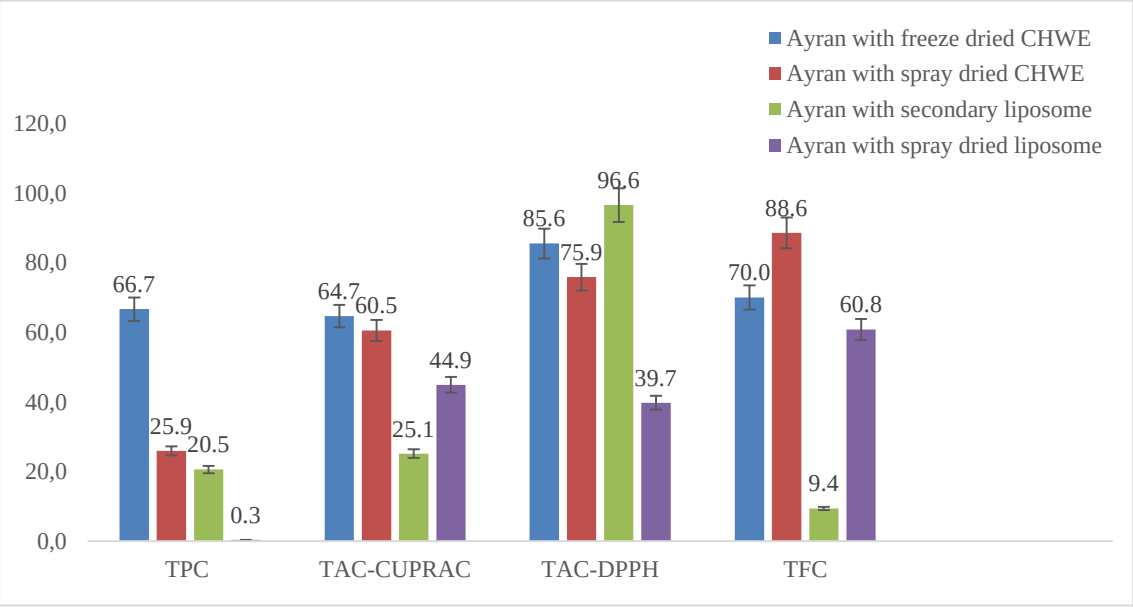


Figure 4.5 : Degradation ratio (%) of cocao hull waste phenolics in ayran (drinking yoghurt) with freeze dried extract, ayran with spray dried extract, with secondary liposome and with spray dried liposome at the end of shelf life period (15th day).

*Error bars represent the standard deviation.

As chitosan surfaces of secondary liposomes were not stabile in ayran formulation, flavonoids and antioxidant were protected by spray dried lipsomos better than secondary lipsomes. In secondary lipsome, distribution of coating surface enhanced the degredation of bioactive compound where located on surface of the lipsomes, so the detected amount of TAC and TFC might belong the interior of the lipsome. The amount of TPC, TAC and TFC in ayran (drinking yoghurt) with spray dried liposome which provided the highest bioactive compound protection which was significantly different ($p < 0.05$) from ayran (drinking yoghurt) with freeze dried extract, ayran (drinking yoghurt) with spray dried extract, ayran (drinking yoghurt) with secondary liposome and control ayran in 1^{st}, 5^{th}, 10^{th} and 15^{th} days. Moreover, there were no significant difference ($p > 0.05$) in TAC (determined by DPPH method) between ayran (drinking yoghurt) with freeze dried extract and ayran (drinking yoghurt) with secondary liposome in 5^{th}, 10^{th} and 15^{th} days. On the other hand, TAC (determined by CUPRAC method) was not significantly different in ayran (drinking yoghurt) with freeze dried extract and ayran (drinking yoghurt) with spray dried extract in 1^{st}, 5^{th}, 10^{th} days (Table 4.3). In all cases, there was a significant difference ($p < 0.05$) on bioactive compounds between the first and last day of shelf life in control ayran (drinking yoghurt), ayran (drinking yoghurt) with freeze dried extract and ayran (drinking yoghurt) with spray dried extract. The content of bioactive compound in control samples is probably due to the presence of polyphenols in milk (Besle et al., 2010). TPC amount of ayran (drinking yoghurt) with secondary and spray dried liposomes shows no significant differences ($p > 0.05$), each their own shelf life, in first and last day. Which showed the liposomal systems provide the protection of TPC during shelf life. TAC was determined two different methods which were, CUPRAC (cupric ion reducing antioxidant capacity) method (Apak et al., 2005) and the DPPH (2,2-diphenyl-1-picrylhydrazyl) method (Kumaran et al., 2006). Diffireinceis between the methods, caused the differencies on detected amounts.

4.7 In-vitro Digestion

Bioavailability of bioactive compound of CHWE was investigated both liposome systems (chitosan coated liposome and spray dried liposome) and unencapsulated forms (freeze-dried and spray-dried CHWE) and also the bioavailability of them were defined in fortificated samples.

Our results showed that CHWE in spray dried liposome had the highest bioavaibility, in additon after fortification of ayran (drinking yoghurt) with uencapsulated and encapsulated CHWE, same observation was occurred which is the spray dried liposome with CHWE had the highest protection and accumulation level during in-vitro digestion during the shelf life period.

4.7.1 Bioaccesibility of unencapsulated and encapsulated cocoa hull phenolics before fortification of ayran (drinking yoghurt)

Bioaccesibility of freeze dried CHWE (0.05% w/v dissolved in acetate buffer pH 3.5), spray dried CHWE (0.05% w/v), secondary (0.1% w/v) and spray dried liposomes with (0.05% w/v) concentration of CHWE were investigated. The amount of TPC, TAC and TFC of digested samples and bioaccessibility % of these nutraceuticals were calculated. The results showed that, the pure CHWE has a lowest bioavailability. According to these findings, the bioavailability of CHWE phenolics were increased 5 folds when they were encapsulated with secondary and spray dried liposomes, moreover the bioaccessibility of antioxidant compounds of CHWE at least 4 times in secondary and 8 times in spray dried liposomes were higher than freeze dried CHWE. In addition, the flavonoid bioaccessibility in secondary and spray dried liposomes also 7 and 10 times were higher than freeze-dried CHWE, respectively. On the other hand, the spray dried CHWE also increased the CHWE bioavailability but not higher than liposomal systems. Figure 4.6 showed the bioavailability of phenolic, antioxidant and flavonoid compounds in CHWE where in secondary liposome (0.1% CHWE), in spray dried liposome (0.05% CHWE), freeze dried (0.05 %) and spray dried (0.05 %) forms.

When the CHWE encapsulated by nano-liposomes, there was a great increase in its bioaccessibility and conservation. Liposomes protect the CHWE from negative environmental gastro-intestinal conditions (Yao et al., 2014), especially pH and dissolved oxygen. We observed that chitosan (0.4 w/v%) was start dissolving in pH

> ~ 5. According to that observation we assumed that secondary and spray dried liposome structure may protect until the digestion in intestine where the pH is 7.5. Thus possible degradation of CHWE in oral and gastric environment may protected. The CHWE concentration of secondary liposomes (0.1% w/v) was reduced to 0.05% w/v after spray drying as the secondary liposomes mixed with maltodextrin solution (20% w/v) 1:1 ratio (w/w) before the solidification process. Despite the concentration of CHWE in spray dried liposomes was lower than secondary liposomes, higher bioactive compounds were detected in spray dried liposomes rather than secondary liposomes after in vitro digestion. The stability of secondary liposomes was lower than spray dried liposomes, as they were in the aqueous solution (Moraes et al., 2013). Not only the lower moisture content of spray dried liposomes but also presence of maltodextrin was supported secondary protected layer on liposomes, in which the higher stability in gastro-intestinal track was enhanced. In addition, the lower particle size of spray dried liposomes was increased the bioaccesibility of them, which was explained by formation of the mixed micelle rapidly in small particle size, so the transfer of bioactive compounds to the mixed micelles was also increased (Porter & Charman, 2001; Yao et al., 2014; Sun et al., 2015). We compared the pure CHWE, spray dried CHWE and spray dried liposomes with CHWE in same concentration. Although the spray dried CHWE was increased the phenolic retention, the spray dried liposomes provided better protection of CHWE in gastro-intestinal track. As they have lower particule size and stronger structure.

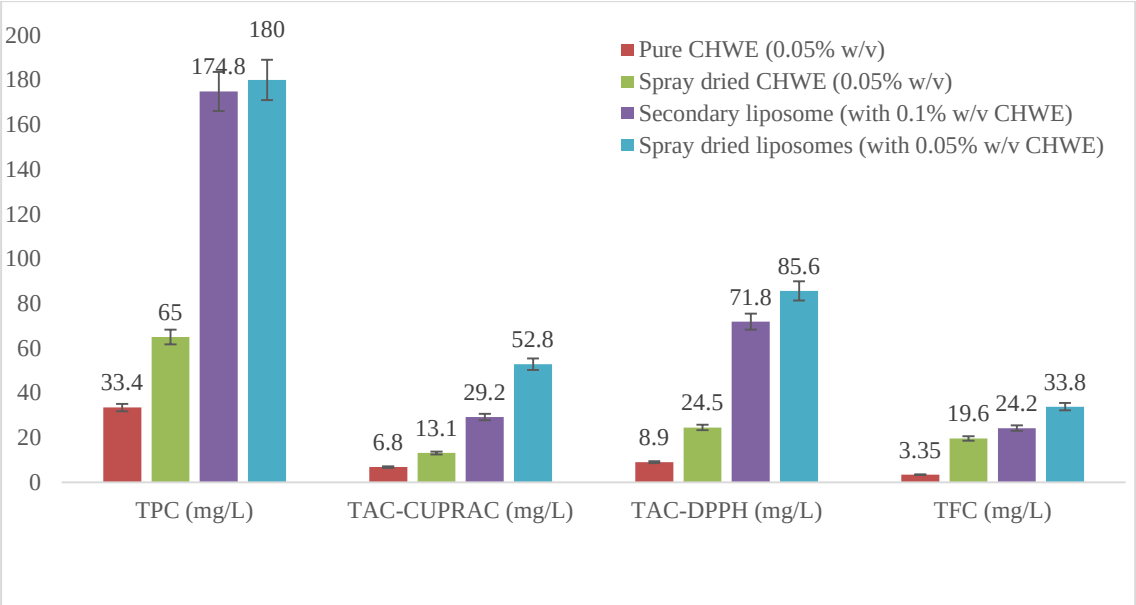


Figure 4.6 : The bioavailability of phenolic, antioxidant and flavonoid compound in cocoa hulls where in secondary liposome (0.1% CHWE), in spray dried liposome (0.05% CHWE), freeze dried (0.05 %) and spray dried (0.05 %) forms.

*Error bars represent the standard deviation.

4.7.2 Bioaccessibility of cocoa hull waste extract phenolics in fortificated ayran (drinking yoghurt) during shelf-life period

TPC, TAC and TFC of fortificated ayran (drinking yoghurt) samples was determined after in vitro digestion in 1st, 5th, 10th and 15th days. Table 4.4 shows the TPC, TAC and TFC (mg/mL) in digested fortificated ayran (drinking yoghurt) samples during shelf life period, control was non-fortifcated ayran (drinking yoghurt).

The phenolic, antioxidant and flavonoid activity of CHWE in ayran (drinking yoghurt) with spray dried liposomes was detected higher than other samples after in-vitro digestion which was significantly different ($p < 0.05$) from other samples in all cases (Table 4.4). In addition, there was no significant difference ($p > 0.05$) between the phenolic, antioxidant and flavonoid activity of control ayran (drinking yoghurt) and ayran (drinking yoghurt) with freeze dried CHWE after in-vitro digestion during shelf life period (Table 4.4) which showed that the unencapsulted CHWE was highly degraded.

The results showed that, the bioavailability of CHWE phenolics in ayran (drinking yoghurt) were increased when they are encapsulated with spray dried liposome instead of added them freeze dried forms. Digestibility of bioactive compounds was

affected the exixtence of enzyemes, bile salts and the change of pH (2.0 in gastric fluid; 7.50 in intestinal fluid) in gastro-intestinal track which lead to degradation of them, in additon the complexity of the food matrix is an another important paremeter bioavailability of phenolics, as they interacted with other food components such as proteins and carbohydrates (Argyri et al., 2005; Saura-Calixto et al., 2007). The interaction between the yoghurt proteins and polyphenols in CHWE leads to the formation of soluble or insoluble protein-polyphenol complexes (Papadopoulou & Frazier, 2004; Rawel, Kroll, & Hohland, 2001).

The detected amounts of phenolics before in vitro digestion in ayran (drinking yoghurt) with freeze dried extract and with spray dried extract were lower than the exact amount because, the interaction between the protein-phenolics and maltodextrin (carbohydrate)-phenolics. During in vitro digestion, the digestive enzymes leading to break these link and there occurred significant increase in phenol concentration (Saura-Calixto et al., 2007).

Table 4.4 : TPC, TAC and TFC (mg/mL) in digested ayran (drinking yoghurt) with freeze dried extract (S1), ayran with spray dried extract (S2), ayran with secondary liposome (S3), ayran with spray dried liposome (S4) during shelf life period, control was non-fortifcated ayran (drinking yoghurt).

	TPC			
	1st Day	5th Day	10th Day	15th Day
Control	$0.325 \pm 0{,}0097^{aA}$	$0{,}326 \pm 0.008^{aA}$	0.31 ± 0.036^{aA}	0.32 ± 0.018^{aA}
S1	$0.366 \pm 0{,}0064^{aA}$	$0{,}347 \pm 0.005^{aA}$	0.324 ± 0.029^{aA}	0.333 ± 0.024^{aA}
S2	$0.936 \pm 0{,}0886^{bA}$	$0{,}777 \pm 0.039^{bA}$	0.76 ± 0.027^{bA}	0.802 ± 0.033^{bB}
S3	$0.805 \pm 0{,}0212^{bA}$	$0{,}691 \pm 0.019^{cB}$	0.72 ± 0.038^{bB}	0.731 ± 0.04^{cB}
S4	$2.014 \pm 0{,}1165^{cA}$	$1{,}632 \pm 0.064^{dB}$	1.71 ± 0.15^{cB}	1.56 ± 0.022^{dB}
	TAC-CUPRAC			
	1st Day	5th Day	10th Day	15th Day
Control	0.011 ± 0.0073^{aA}	0.035 ± 0.019^{aAB}	0.046 ± 0.023^{aB}	0.119 ± 0.012^{aC}
S1	0.027 ± 0.0062^{aA}	0.041 ± 0.016^{aA}	0.066 ± 0.032^{aA}	0.12 ± 0.018^{aB}
S2	0.334 ± 0.016^{bA}	0.27 ± 0.0441^{bA}	0.296 ± 0.015^{bA}	0.382 ± 0.034^{bB}
S3	0.182 ± 0.04^{cA}	0.131 ± 0.028^{cA}	0.285 ± 0.116^{bB}	0.238 ± 0.045^{cB}
S4	0.767 ± 0.08^{dA}	0.435 ± 0.149^{dB}	0.6 ± 0.114^{cAB}	0.407 ± 0.047^{bB}
	TAC-DPPH			
	1st Day	5th Day	10th Day	15th Day
Control	0.029 ± 0.005^{aA}	0.01 ± 0.006^{aB}	0.015 ± 0.01^{aB}	0.006 ± 0.004^{aB}
S1	0.035 ± 0.002^{aA}	0.013 ± 0.004^{aB}	0.02 ± 0.007^{aB}	0.0108 ± 0.005^{aB}
S2	0.046 ± 0.014^{aA}	0.021 ± 0.01^{aA}	0.083 ± 0.051^{aA}	0.035 ± 0.023^{aA}
S3	0.077 ± 0.014^{aA}	0.041 ± 0.016^{aB}	0.034 ± 0.024^{aB}	0.021 ± 0.018^{aB}
S4	0.303 ± 0.08^{bA}	0.326 ± 0.045^{bA}	0.313 ± 0.042^{bA}	0.081 ± 0.043^{bB}

*Values are presented as mean values ± standard deviation (n=6). Different small letters in the columns or different capital letters in the rows represent statistically significant differences ($p<0.05$) in each section.

Table 4.4 (Continued) : TPC, TAC and TFC (mg/mL) in digested ayran (drinking yoghurt) with freeze dried extract (S1), ayran with spray dried extract (S2), ayran with secondary liposome (S3), ayran with spray dried liposome (S4) during shelf life period, control was non-fortifcated ayran (drinking yoghurt).

	TFC			
	1st Day	5th Day	10th Day	15th Day
Control	0.013 ± 0.003^{aA}	$0.013 \pm 0,003^{aA}$	0.006 ± 0.003^{aB}	0.004 ± 0.002^{aB}
S1	0.025 ± 0.003^{aA}	0.015 ± 0.003^{aB}	0.013 ± 0.004^{aB}	0.007 ± 0.003^{aC}
S2	0.148 ± 0.006^{bA}	0.073 ± 0.006^{bB}	0.026 ± 0.003^{bB}	0.024 ± 0.004^{bC}
S3	0.035 ± 0.006^{aA}	0.029 ± 0.002^{aAB}	0.071 ± 0.005^{aB}	0.015 ± 0.005^{abC}
S4	0.294 ± 0.031^{cA}	0.226 ± 0.021^{cB}	0.2 ± 0.012^{cB}	0.148 ± 0.015^{cC}

*Values are presented as mean values ± standard deviation (n=6). Different small letters in the columns or different capital letters in the rows represent statistically significant differences ($p<0.05$) in each section.

On the other hand, existance of chitosan and maltodextrin layer on secondary and spray dried liposomes might inhibit the detectable amounts of phenolic compounds. The fluctuatuion on % bioavaibilites of samples explanied the different interaction level of phenol-protein and phenol-carbohydrate complexes. Bioaccessibility (% retention) of TPC, TAC and TFC in fortificated ayran (drinking yoghurt) samples during shelf life period is shown in Figure 4.7.

Acoording to the Figure 4.7 phenolic activity of CHWE in fortificated ayran (drinking yoghurt) with spray dried liposomes were 7, 9, 9, and 7 times higher than ayran (drinking yoghurt) with freeze dried CHWE at 1st, 5th, 10th and 15th days, respectively.

Ortega et al. (2009) reported that, the fat content in the digesta allowed the formation of the lipid emulsion droplets and the incorporation of the cocoa phenols into the lipid phase, which provided protective effect during in-vito digestion.

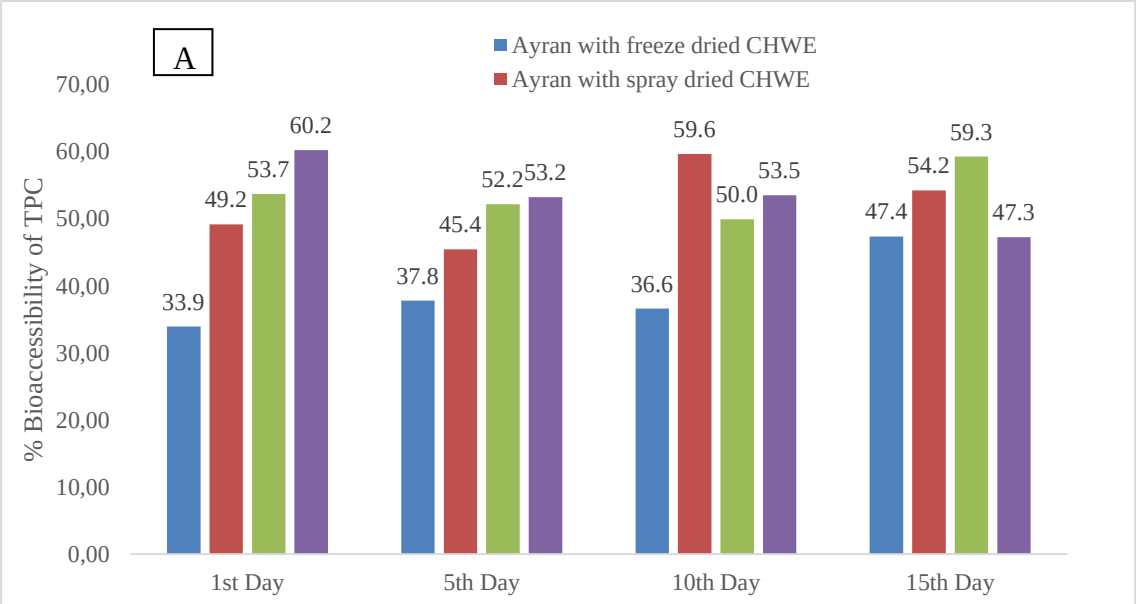
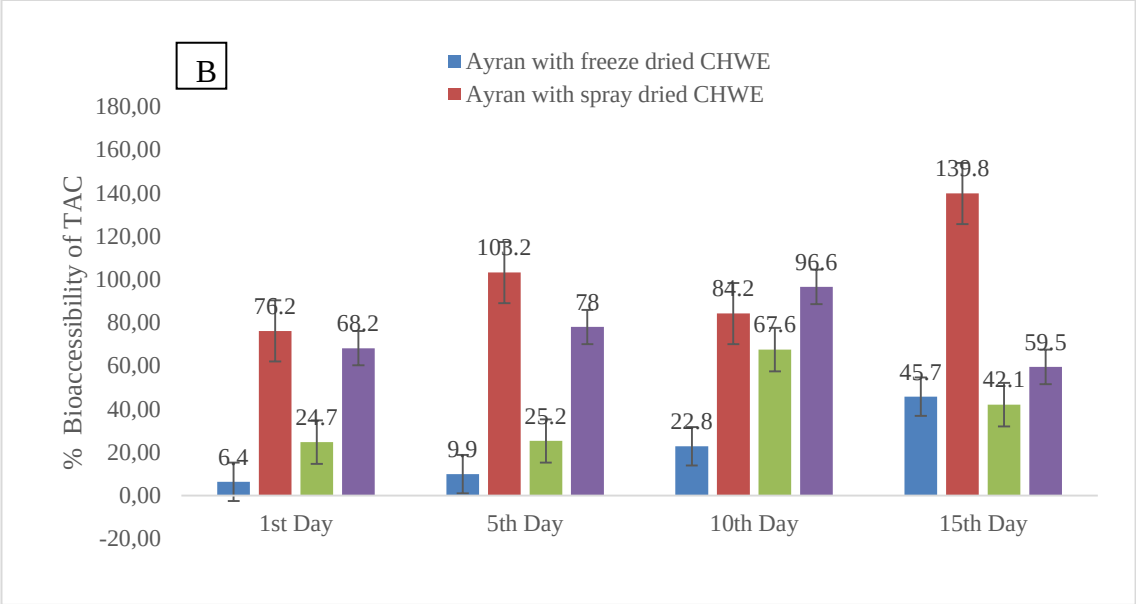


Figure 4.7 : Bioaccessibility (% retention) of TPC (A), TAC which determined by CUPRAC method (B), TAC which determined by DPPH method (C) and TFC (D) in fortificated ayran (drinking yoghurt) samples during shelf life period.

*Error bars represent standard deviation.



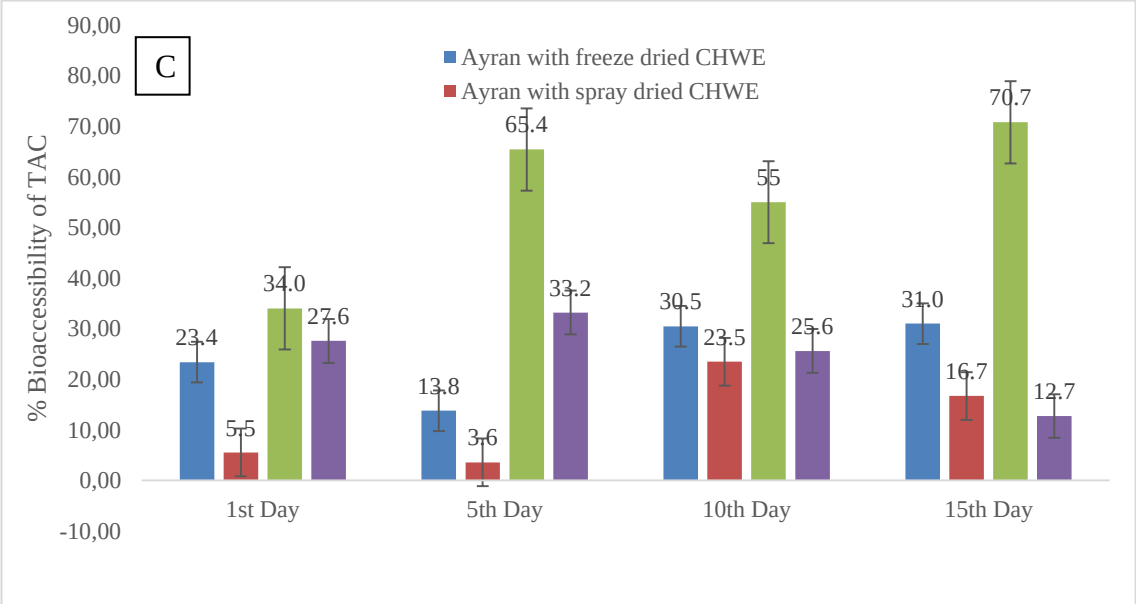


Figure 4.7 (Continued) : Bioaccessibility (% retention) of TPC (A), TAC which determined by CUPRAC method (B), TAC which determined by DPPH method (C) and TFC (D) in fortificated ayran (drinking yoghurt) samples during shelf life period.

*Error bars represent standard deviation.

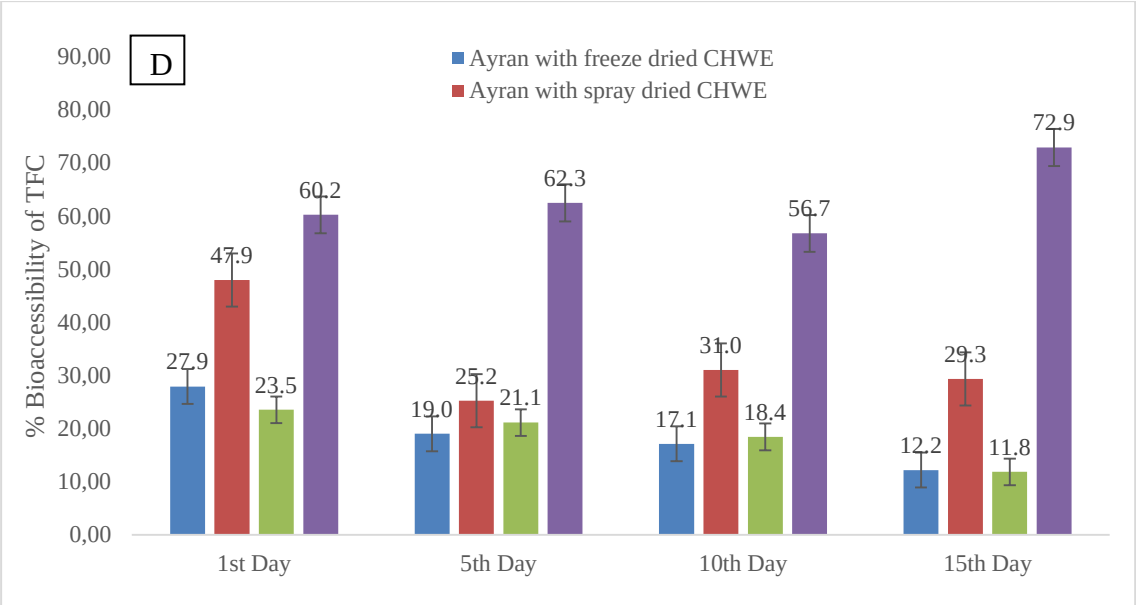


Figure 4.7 (Continued): Bioaccessibility (% retention) of TPC (A), TAC which determined by CUPRAC method (B), TAC which determined by DPPH method (C) and TFC (D) in fortificated ayran (drinking yoghurt) samples during shelf life period.

*Error bars represent standard deviation.

Liposomes are one of the lipid-based nano particles. During in-vitro digestion, they hydrolyzed by lipases to the free fatty acids and monoacylglycerols. The digestion products interact with bile salts and phospholipids in the lumen of the small intestine to form “mixed micelles” with complex structures (Yao et al., 2014). After the liposome digestion, encapsulated bioactive compounds are transferred to the mixed micelles which enhance their bioacccesibility (Porter & Charman, 2001; Sun et al., 2015).

It is found that, bioactive compounds were detected higher amount in ayran (drinking yoghurt) with spray dried liposomes and significantly different ($p < 0.05$) from other samples in all cases.

4.8 Quantification and Identification of Phenolic Compound in Cocoa Hull Waste Extract Using UHPLC

Phenolic compound in CHWE was determined before and after in-vitro digestion and effect of drying and encapsulation process was investigated by comparing the results of freeze dried CHWE. In addtion the phenolic compounds of unencapsulated and encapsulated CHWE was determined after addion of them in drinking yoghurt during shelf-life period before and after in-vitro digestion.

4.8.1 Phenolic compound in freeze-dried, spray-dried cocoa hull waste extract, secondary and spray dried liposome with cocoa hull waste extract before and after *in-vitro* digestion

Flavan-3-ols, anthocyanins and proanthocyanins are the main 3 groups of cocoa polyphenols and also condensed tannis, flavonoids, phenolic acids and some minor compounds comprised the phenolic compounds of cocoa (Sanchez-Rabaneda et al., 2000; Wollgast & Anklam, 2000). Ten major phenolic compound, namely, catechin, epicatechin (flavan-3-ols), quercetin (flavon) ferulic acid, gallic acid, p-coumaric acid, syringic acid, t-cinnamic acid, vanilic acid and vanillin (phenolic acids) were detected before and after in vitro digestion in freeze dried CHWE, spray dried CHWE, secondary and spray dried liposomes with CHWE using UHPLC, which these compounds detected in cocoa powder by Ortega et al. (2009). Table 4.5 shows the phenolic profile (mg/g) of freeze dried CHWE (1% w/v dissolved in acetate buffer pH 3.5), spray dried CHWE (0.05% w/v), secondary (0.1% w/v) and spray dried liposomes with (0.05% w/v) before and after in vitro digestion.

(+)-Catechin and (-)-epicatechin which compose of procyanidins are the main form of cocoa flavanol monomers (Ortega et al. 2008). We detected 1.2415 ± 0.252 mg/g epicatechin and 1.817 ± 0.413 mg/g catechin in freeze dried CHWE. Our results are comparable with the results reported Ioannone et al. (2015) and Miller et al. (2009) who found that 1.12 mg/g epicatechin and 1.34 mg/g catechin in cocoa beans before roasting process and 1.263-2.827 mg/g epicatechin and 0.347-0.896 mg/g of catechin in non-fat cocoa powder, respectively.

It has been reported that heat and pressure might degrade the procyanidin to catechin and epicatechin, moreover (-)-epicatechin epimerized to (-)-catechin (Wang & Helliwell, 2000). High pressure application (25.000 psi) during the liposomal encapsulation of bioactive compounds in CHWE might cause the degredation of procyanidin, thus the detected amount of catechin in secondary liposomes was higher than freeze dried CHWE and epicatechin in spray dried liposome was higher than freeze dried CHWE which the results are shown in Table 4.5.

Table 4.5 : The phenolic profile (mg/g) of freeze dried and spray dried cocoa hull waste extract, secondary and spray dried liposomes with cocoa hull waste extract before and after in vitro digestion. (nd refers not defined).

		Freeze Dried CHWE	**Spray Dried CHWE**	**Secondary Liposome with CHWE**	**Spray Dried Liposome with CHWE**
Before in vitro digestion					
Flavon-3-ol monomers	Catechin	1.817 ± 0.413	0.377 ± 0.0317	2.026 ± 0.312	1.549 ± 1.349
	Epicatechin	1.2415 ± 0.252	0.506 ± 0.0951	0.45 ± 0.054	2.3911 ± 1.158
Flavone	Quercetin	10.746 ± 0.356	2.011 ± 0.194	1.533 ± <0.005	7.715 ± 0.376
Phenolic Acids	Ferulic Acid	1.6 ± 0.074	0.547 ± 0.084	0.463 ± <0.005	1.872 ± 0.053
	Gallic Acid	5.105 ± 2.228	2.005 ± 0.091	1.498 ± 0.546	6.754 ± 0.183
	p-Coumaric Acid	5.908 ± 1.608	6.031 ± 0.296	4.958 ± <0.005	25.049 ± 0.834
	Syringic Acid	32.687 ± 5.052	1.857 ± 0.105	1.767 ± 0.01	9.068 ± 0.307
	t-Cinnamic Acid	1.554 ± 0.184	0.837 ± 0.057	0.634 ± <0.005	3.088 ± 0.281
	Vanilic Acid	6.577 ± 1.717	1.493 ± 0.325	Nd	2.244 ± 0.011
	Vanilin	0.037 ± <0.005	0.007 ± <0.005	0.008 ± <0.005	0.051 ± <0.005

*Values are presented as mean values ± standard deviation (n=3).

Table 4.5 (**Continued):** The phenolic profile (mg/g) of freeze dried and spray dried cocoa hull waste extract, secondary and spray dried liposomes with cocoa hull waste extract before and after in vitro digestion. (nd refers not defined).

After in vitro digestion sd <0,005					
Flavon-3-ol monomers	Catechin	0.058	0.580	0.089	0.328
	Epicatechin	0.534	4.323	1.043	3.05
Flavone	Quercetin	0.289	1.583	0.5222	1.623
Phenolic Acids	Ferulic Acid	0.14	0.321	0.125	0.265
	Gallic Acid	0.158	0.937	0.333	1.222
	p-Coumaric Acid	0.223	0.848	0.353	1.025
	Syringic Acid	0.034	0.846	0.182	0.67
	t-Cinnamic Acid	0.107	0.314	0.165	0.494
	Vanilic Acid	0.233	0.619	0.326	0.951
	Vanilin	0.0005	0.005	0.002	0.007

*Values are presented as mean values ± standard deviation (n=3).

The spray dried CHWE in which concentration of 0.05 (w/v) had the highest catechin+epicatechin, quercetin and total phenolic acids concentration (TPA). Chemical structure of polyphenols is the main parameter of absorption and metabolism of these compounds (Bravo, 1998). They tent to interact with the other compound which were located in environment such as protein and carbohydrates (Ortega et al., 2009).

The major phenolic acid of the freeze dried extract was found syringic acid (49.8%), whereas the p-coumaric acid was the major one for spray dried extract (38.5%), secondary liposome (35.1%) and spray dried liposome (43%). The lechitin structure of liposome and existing of chitosan and maltodextrin layer in secondary liposome, spray dried liposome and spray dried CHWE might responsible these difference.

According to the bioavailability results which are shown in Figure 4.8, the maximum phenolic compound degradation after in-vitro digestion was calculated in freeze dried CHWE, which the result was similar to spectrophotometric results. The pH condition of gastro-intestinal track is responsible to cathechin digestion rather than digestive enzymes, moreover residual dissolved oxygen in intestine allows epimerization of catechins as well as auto-oxidation (Green et al., 2007; Shim et al., 2012). It is reported that, on average 44.4% and 91.8% of catechin were degrade gastric and intestional digestion, respectively (Tenore et al., 2015) and we found on average 61% of phenolic and antioxidant compound and also 89% of flavonoid compound of pure CHWE was degrade in vitro digestion.

The bioavailability of all phenolic derivatives of CHWE in freeze dried CHWE was lower than 4% and the flavon-3-ol monomers (catechin+epicatechin) bioavailability were 115%, 8% and 16% in spray dried CHWE, secondary liposome and spray dried liposome, respectively. Moreover, in spray dried CHWE quercetin bioavailability was 79%, while it was 34% in secondary and 21% in spray dried liposome. And the bioavailability of total phenolic acids was 26% in spray dried CHWE, 22% in secondary liposome and 8% in spray dried liposome.

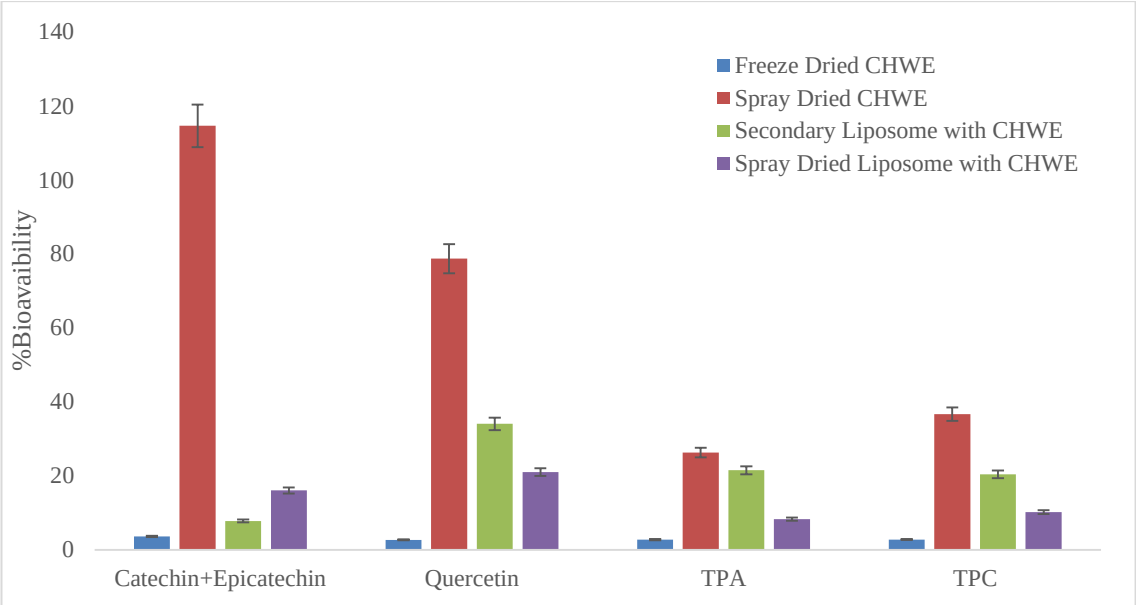


Figure 4.8 : The catechin+epicatechin, quercetin, total phenolic acids (TPA) and total phenolic compound (TPC) bioavaibility (%) of freeze dried CHWE, spray dried CHWE (0.05% w/v), secondary liposome with CHWE (0.1% w/v) and spray dried liposome with CHWE (0.05 w/v).

*Error bars represent standard deviation.

The alkaline pH in SIF lead to oxidation and polymerization of phenlic compounds and other phenolic derivatives such as chalcones which are not bioavailable was generated (Rodriguez-Roque et al., 2014). Quercetin, TPA and TPC was detected higher amount in spray dried CHWE (0.05% w/v) rather that spray dried CHWE (0.05% w/v) and secondary liposome (0.1% v/w) after in vitro digestion. On the other hand, in spray dried CHWE had the highest catechin+epicatechin concentration. Several studies reported the flavanol bioaccessibility increased in carbohydrate matrix (Schramm et al., 2003; Neilson et al., 2009; Rodriguez-Mateos et al., 2012). The spray dried CHWE showed the higher phenolic bioaccessibility for all detected phenolic compounds which is shown in Figure 4.8. Because the phenols linked to carbohydrates the detected amount of phenols was lower before in vitro digestion, in addition during in vitro digestion the digestive enzymes leading to break these link and there occurred significant increase in phenol concentration (Saura-Calixto et al., 2007). Moreover, liposome structure inhibits the interaction of phenolic compounds which located interior of liposome and carbohydrates in secondary and spray dried liposome with CHWE. The degradation level of them in liposomal structure might be related with location of these compounds in liposome structure.

When the spectrophometric assays and UHPLC assay results were compared, there were a difference in detectable amount of TPC, as structure of liposomes caused some restrictions on spectrophometric measurements which mentioned before. In addition, we could not detect all phenolic derivatives and flavons in UHPLC.

4.8.2 Quantification and identification of phenolic compound in ayran (drinking yoghurt) samples during shelf-life period by UHPLC

We detected ten major phenolic compound, namely, catechin, epicatechin (flavon-3-ols), quercetin (flavan) ferulic acid, gallic acid, p-coumaric acid, syringic acid, t-cinnamic acid, vanilic acid and vanillin (phenolic acids) before and after in vitro digestion in fortificated ayran (drinking yoghurt) samples which were determined in unencapsulated and encapsulated CHWE. The phenolic profile (mg/g) of ayran (drinking yoghurt) samples during shelf life period which were fortificated with freeze dried extract (S1), ayran (drinking yoghurt) with spray dried extract (S2), ayran (drinking yoghurt) with secondary liposome (S3), ayran (drinking yoghurt) with spray dried liposome (S4) was shown in Table 4.6. In addition, Table 4.7 shows the the phenolic profile (mg/g) of ayran (drinking yoghurt) samples after in vitro digestion during the shelf life period. We did not identify any cocoa phenolics in control ayran (drinking yoghurt) similar to other studies which were about fortification of yoghurt and yoghurt product by phenolic extracts (Chouchouli et a., 2013; Sun-Waterhouse et al., 2012). Ayran (drinking yoghurt) with spray dried liposomes shows the higher phenolic compound protection both before and after in-vitro digestion. Morever both ayran (drinking yoghurt) with spray dried liposome and ayran (drinking yoghurt) with secondary liposome had a higher flavon-3-ol compounds at least two folds from ayran with freeze and spary dried CHWE which the results were given in Table 4.6 and Table 4.7. This results show that the liposomal systems inhibit the interaction between phenolic compounds and other food ingredients and reduce the risk of degradation of phenolics, which was explained before. The bioavailability of flavan-3-ol (catechin and epicatechin) was % 102.30, % 74.85, % 82.76 and % 96.48; flavon (quercetin) was % 155.87, % 71.89, % 89.70 and %53.56; total phenolic acid was % 85.28, % 107.32, % 51.42 and % 64.18 in ayran (drinking yoghurt) with spray dried liposome at 1^{st}, 5^{th}, 10^{th} and 15^{th} days, respectively).

Chemical structure of phenolic compounds and their location on the liposomal systems were effect their degradation level before and after in-vitro digestion during shelf-life period.

Table 4.6 : The phenolic profile (mg/g) of ayran (drinking yoghurt) samples during shelf life period which were fortificated with freeze dried extract (S1), with spray dried extract (S2), with secondary liposome (S3), with spray dried liposome (S4) and control was non-fortifcated ayran (drinking yoghurt).

		Flavon-3-ols		**Flavan**	**Phenolic Acids**						
		Catechin	Epicatechin	Quercetin	Ferulic Acid	Gallic Acid	p-Coumaric Acid	Syringic Acid	t-Cinnamic Acid	Vanilic Acid	Vanilin
DAY 1											
	Control	-	-	-	-	-	-	-	-	-	-
	S1	0.118 ± 0.003	0.064 ± 0.101	0.257 ± 0.273	0.078 ± 0.013	0.272 ± 0.023	0.573 ± 0.02	0.278 ± 0.042	0.138 ± 0.018	0.148 ± 0.004	0.002 ± <0.001
	S2	0.082 ± 0.013	0.081 ± 0.118	0.343 ± 0.001	0.066 ± 0.012	0.408 ± 0.041	1.053 ± 0.191	0.214 ± 0.24	0.140 ± 0.017	0.210 ± 0.08	0.001 ± <0.001
	S3	0.147 ± 0.004	1.043 ± 0.283	0.371 ± 0.053	0.101 ± 0.006	0.406 ± 0.030	1.205 ± 0.054	0.273 ± 0.015	0.170 ± 0.014	0.224 ± 0.002	0.002 ± <0.001
	S4	0.177 ± 0.108	3.339 ± <0.001	1.238 ± 0.009	0.636 ± 0.18	1.318 ± 0.447	3.958 ± 0.386	0.953 ± 0.026	0.527 ± 0.054	0.666 ± 0.015	0.004 ± 0.003
DAY 5											
	Control	-	-	-	-	-	-	-	-	-	-
	S1	0.046 ± 0.024	0.552 ± 0.11	0.293 ± 0.047	0.065 ± 0.020	0.186 ± 0.038	0.810 ± 0.065	0.508 ± 0.026	0.116 ± 0.006	0.138 ± 0.022	0.001 ± <0.001
	S2	0.008 ± 0.006	0.105 ± <0.001	0.906 ± 0.081	0.092 ± <0.001	0.393 ± 0.024	1.244 ± 0.056	-	0.156 ± 0.018	0.178 ± 0.068	0.003 ± <0.001
	S3	0.059 ± 0.041	1.183 ± 0.205	0.503 ± 0.076	0.098 ± 0.005	0.415 ± 0.036	1.070 ± 0.082	0.828 ± 0.054	0.139 ± 0.007	0.214 ± 0.016	0.002 ± <0.001

*Values are presented as mean values ± standard deviation (n=3).

Table 4.6 (Continued): The phenolic profile (mg/g) of ayran (drinking yoghurt) samples during shelf life period which were fortificated with freeze dried extract (S1), with spray dried extract (S2), with secondary liposome (S3), with spray dried liposome (S4) and control was non-fortifcated ayran (drinking yoghurt).

	S4	0.597 ± 0.19	3.383 ± 1.09	2.366 ± 0.402	0.415 ± 0.001	1.703 ± 0.273	1.389 ± <0.001	0.848 ± 0.226	0.564 ± 0.025	0.923 ± 0.061	0.006 ± 0.001
DAY 10											
	Control	-	-	-	-	-	-	-	-	-	-
	S1	0.047 ± 0.004	0.389 ± <0.001	0.243 ± 0.083	0.055 ± 0.014	0.298 ± 0.014	0.750 ± 0.035	0.460 ± <0.01	0.129 ± 0.024	0.185 ± 0.019	0.001 ± <0.001
	S2	0.070 ± 0.002	0.648 ± <0.001	0.939 ± <0.001	0.092 ± 0.02	0.317 ± <0.101	1.179 ± 0.101	0.316 ± <0.001	0.159 ± 0.01	0.209 ± 0.052	0.002 ± <0.001
	S3	0.093 ± 0.028	1.148 ± 0.13	0.596 ± 0.04	0.126 ± 0.005	0.431 ± <0.001	1.051 ± <0.001	0.936 ± 0.048	0.216 ± 0.028	0.310 ± 0.017	0.003 ± <0.001
	S4	0.490 ± <0.001	4.189 ± 0.27	1.945 ± 0.047	0.491 ± 0.082	1.833 ± <0.001	4.600 ± 0.06	1.506 ± 0.023	0.613 ± 0.044	0.890 ± 0.03	0.017 ± 0.008
DAY 15											
	Control	-	-	-	-	-	-	-	-	-	-
	S1	0.048 ±0.01	0.381 ± <0.001	0.179 ± 0.001	0.022 ± 0.021	0.148 ± <0.001	0.474 ± 0.042	0.225 ± <0.001	0.072 ± 0.004	0.086 ± 0.017	0.001 ± <0.001
	S2	0.141 ± 0.037	0.468 ± 0.197	0.541 ± 0.164	0.102 ± 0.007	0.339 ± <0.001	1.099 ± <0.001	0.344 ± <0.001	0.220 ± 0.033	0.218 ± 0.027	0.003 ± <0.001
	S3	0.127 ± <0.001	1.186 ± 0.063	0.605 ± 0.053	0.202 ± 0.004	0.488 ± 0.041	1.021 ± 0.075	0.854 ± 0.002	0.173 ± 0.01	0.286 ± 0.005	0.002 ± <0.001
	S4	0.360 ± 0.019	1.037 ± <0.001	3.079 ± 0.574	0.616 ± <0.001	1.873 ± 0.264	3.883 ± 0.393	1.318 ± 0.306	0.658 ± 0.142	1.053 ± 0.066	0.047± 0.029

*Values are presented as mean values ± standard deviation (n=3).

Table 4.7 : The phenolic profile (mg/g) of ayran (drinking yoghurt) samples after in vitro digestion which were fortificated with freeze dried extract (S1), with spray dried extract (S2), with secondary liposome (S3), with spray dried liposome (S4) and control was non-fortifcated ayran (drinking yoghurt).

		Flavaon-3-ols		**Flavan**	**Phenolic Acids**						
		Catechin	Epicatechin	Quercetin	Ferulic Acid	Gallic Acid	p-Coumaric Acid	Syringic Acid	t-Cinnamic Acid	Vanilic Acid	Vanilin
DAY 1											
	Conrol	-	-	-	-	-	-	-	-	-	-
	S1	0.030	0.249	0.188	0.044	0.145	0.144	0.1	0.069	0.119	<0.001
	S2	0.262	3.023	1.799	0.509	1.369	1.259	1.057	0.642	1.333	0.008
	S3	0.052	0.918	0.391	0.128	0.363	0.373	0.205	0.118	0.326	0.001
	S4	0.331	3.265	1.93	0.529	1.77	1.69	0.961	0.757	1.154	0.0131
DAY 5											
	Conrol	-	-	-	-	-	-	-	-	-	-
	S1	0.029	0.418	0.172	0.044	0.113	0.127	0.089	0.063	0.077	<0.001
	S2	0.275	3.339	1.667	0.421	1.383	1.278	0.698	0.62	0.994	0.009
	S3	0.093	0.759	0.317	0.1	0.201	0.209	0.181	0.1	0.206	0.001
	S4	0.291	2.668	1.701	0.367	1.463	1.576	0.859	0.673	1.335	0.005
DAY 10											
	Conrol	-	-	-	-	-	-	-	-	-	-
	S1	0.03	0.23	0.159	0.036	0.107	0.125	0.066	0.062	0.094	<0.001

*Values are presented as mean values ± standard deviation (n=3) ± <0.001.

Table 4.7 (Continued): The phenolic profile (mg/g) of ayran (drinking yoghurt) samples after in vitro digestion which were fortificated with freeze dried extract (S1), with spray dried extract (S2), with secondary liposome (S3), with spray dried liposome (S4) and control was non-fortifcated ayran (drinking yoghurt).

	S2	0.448	2.603	1.654	0.429	1.061	1.04	0.916	0.495	1.087	0.003
	S3	0.083	0.698	0.319	0.133	0.232	0.245	0.185	0.132	0.2	0.001
	S4	0.217	3.655	1.745	0.522	0.862	0.839	1.161	0.566	1.162	0.004
DAY 15											
	Conrol	-	-	-	-	-	-	-	-	-	-
	S1	0.041	0.298	0.168	0.043	0.143	0.127	0.088	0.057	0.117	<0.001
	S2	0.29	2.998	1.588	0.432	1.33	0.825	1.11	0.677	0.932	0.005
	S3	0.041	0.881	0.32	0.115	0.204	-	0.142	0.135	0.114	0.001
	S4	0.382	3.034	1.649	0.507	1.372	1.41	0.983	0.476	1.307	0.009

*Values are presented as mean values ± standard deviation (n=3) ± <0.001.

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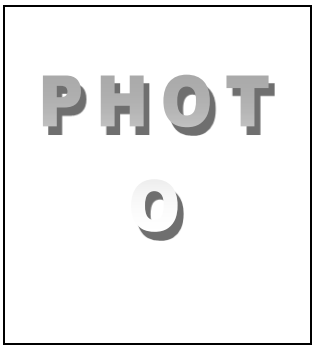
CURRICULUM VITAE



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