ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

EFFECTS OF LIPOSOME ENCAPSULATION, SPRAY DRYING, AND ASCORBIC ACID PRESENCE ON COLOR STABILITY OF BLACK CARROT ANTHOCYANINS

Ph.D. THESIS

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

LİPOZOM ENKAPSÜLASYONU, PÜSKÜRTMELİ KURUTMA VE ASKORBİK ASİT VARLIĞININ KARA HAVUÇ ANTOSİYANİNLERİNİN RENK STABİLİTESİNE ETKİSİ

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KASIM 2017

Burcu Güldiken, a Ph.D. student of İTU Graduate School of Science Engineering and Technology student ID 506082504 successfully defended the thesis/dissertation entitled "EFFECTS OF LIPOSOME ENCAPSULATION, SPRAY DRYING, AND ASCORBIC ACID PRESENCE ON COLOR STABILITY OF BLACK CARROT ANTHOCYANINS", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family,

FOREWORD

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(Food Engineer)

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ABBREVIATIONS

BCE : Black Carrot Extract
E : Extract
LE : Lecithin
AA : Ascorbic Acid
RSM : Response Surface Methodology
DPPH : 1,1-diphenyl-2-picrylhydrazyl
Trolox : 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
HPLC : High Pressure Liquid Chromatography
TFA : Trifluoroacetic Acid
AOX : Antioxidant Capacity
TAC : Total Antioxidant Capacity
PDI : Polydispersity Index
gel : Gel filtered
AC : Anthocyanin Content

SYMBOLS

ζ	**:** Zeta
A	**:** Absorbance
a*	**:** Redness
b*	**:** Yellowness
L*	**:** Lightness
EE	**:** Encapsulation Efficiency
E	**:** Extract
LE	**:** Lecithin
DF	**:** Dilution Factor

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EFFECTS OF LIPOSOME ENCAPSULATION, SPRAY DRYING, AND ASCORBIC ACID PRESENCE ON COLOR STABILITY OF BLACK CARROT ANTHOCYANINS

SUMMARY

In the food industry, food colorants determine the food preferences of customers as color of a food is a function of freshness, type, ingredients and production method. In addition, it also affects customers' purchase behavior since an appealing color is highly desired by consumers. In today's world, food products are transported to far away places where they will be consumed. This may result in degradation and appearance loss. In addition, food producers desire to sell their products with an optimum color, and, therefore, food colorants are used extensively. In recent years, the increasing tendency of customers to consume healthy foods has led the food industry to seek natural colorants instead of stable synthetic colorants due to possible harmful effects of synthetic colorants.

Black carrot anthocyanins are water soluble and pH sensitive natural pigments. Black carrots are currently grown in the center part of Turkey as a highly nutritive crop. In addition, it is currently used in shalgam production and the canned food industry due to its high anthocyanin content. Furthermore, black carrot (*Daucus carota* var L.) is a potential source of acylated anthocyanins. Thus, black carrot anthocyanins are relatively stable. Nevertheless, they still suffer from degradation, that is they loose their color over time, especially in systems containing other reacting compounds such as vitamin C. In a food system, encapsulation is generally used to create a barrier between the component and environment. Encapsulation may be a suitable method to enhance their stability to the point where they may be used to replace synthetic colorants.

In this research a framework on characterization of black carrot anthocyanins and liposome encapsulation effect on stability of black carrot anthocyanins has been developed. The objectives of this Ph.D. thesis were (i) to optimize black carrot extraction using Response Surface Methodology (RSM) by evaluating anthocyanin concentration, phenolic content, antioxidant activity, and color attributes of black carrot; (ii) to compare the stability of black carrot anthocyanins, which are highly stable due to their large amount of acylated anthocyanins, with and without a liposome system in the presence of ascorbic acid; (iii) to understand the effects of both intramolecular copigmentation and liposome encapsulation on ascorbic acid related anthocyanin degradation; (iv) to investigate physical and chemical stability of liposomes with and without black carrot during storage; (v) to evaluate the stability of liposomes with black carrot extract during the spray drying process.

In first part of the thesis, optimum conditions for the extraction of black carrot anthocyanins were determined by response surface methodology. Central composite design of extraction factors (pH 2.5–6.5, temperature 4–72 °C, solvent/solid ratio 5:1–25:1 v/w, ethanol/water ratio 0:100–100:0 v/v) was generated as two replicates. Total phenolic content, total monomeric anthocyanin content, polymeric color, total

antioxidant activity, and anthocyanin composition determined by high performance liquid chromatography (HPLC) were used as responses. Except for color values, higher temperature, solvent/solid ratio, and ethanol concentration were observed to increase the extraction yield. However, polymeric color results were found to have minimum values at lower pH and solid/ solvent ratio, lower or moderate temperature, and higher ethanol concentration. Optimum extraction conditions were found as follows: 50 °C, pH 3.5, solvent solid ratio 10:1 (v/w), and ethanol/water ratio 75:25 (v/v) when all responses were considered. The validation of the optimum conditions for black carrot extraction was performed at specified values. Optimum extraction conditions were valuable to protect anthocyanins at the first step for further processes.

In the second part, stability of black carrot anthocyanins is evaluated in the presence of L-ascorbic acid. The degradation of vitamin C (L-ascorbic acid) on anthocyanins is a known fact in beverage systems. In this part, the effects of various liposomal systems, including black carrot extract (0.1%, 0.2%, 0.4% w/w) and lecithin (1%, 2%, 4% w/w), on the color and degradation of anthocyanin in different ascorbic acid (0.01%, 0.025%, 0.05%, 0.1% w/w) concentrations were examined via UV/VIS spectroscopy and visual control of the color. The physical characteristics of the liposomal systems resulted in particle diameters of 41–46 nm and zeta-potentials of (−23)–(−20) mV. The encapsulation efficiencies of the liposomal systems increased up to 50% with increasing lecithin concentrations. The encapsulation of black carrot extract in liposomes enhanced the color and stability of the anthocyanins during storage. This study showed that the degradation of anthocyanins due to ascorbic acid can be reduced by liposomes in aqueous solutions.

In the third part, the protective effect of the anthocyanin acylation and liposomal encapsulation of black carrot extract (BCE) on anthocyanin degradation was investigated in the presence of ascorbic acid. BCE (0.1%, 0.2%, 0.4% w/w) were encapsulated in liposomes using different lecithin concentrations (1%, 2%, 4% w/w). Physical characterization of liposomes was performed. Particle diameters were found to be less than 50 nm and ζ-potentials of (-23)-(-20) mV. Encapsulation efficiency determined by HPLC indicated that increasing lecithin concentrations raised the efficiency at the same BCE concentration up to 60%. Different concentrations of AA (0.01%, 0.025%, 0.05%, 0.1% w/w) were added to the BCE solutions and liposomes (1%, 2%, 4% lecithin) containing BCE (0.2% w/w). AA related color loss and individual anthocyanin degradation were examined by UV/VIS spectroscopy and HPLC, respectively. This study presented the potential positive effect of liposomal encapsulation on individual particularly acylated anthocyanins (sinapic, ferulic and p-coumaric acid derivates of cyanidin-3-xylosylglucosylgalactoside).

In the fourth part, the protection effect of black carrot anthocyanins on the stability of liposome was evaluated during storage. Physical and chemical stability of extracts (0.1%, 0.2%, and 0.4% w/w) including liposomes with various lecithin content (1%, 2%, and 4% w/w) were demonstrated. Z-average particle diameter and zeta potentials of liposomes were evaluated before and after 21 days of storage. Particle diameter of samples were found to be lower than 50 nm after storage and no statistical change was determined ($p<0.05$). Extract degradation, phenolic content, and antioxidant capacity of liposomes were also evaluated during storage to evaluate the biochemical stability. Hexanal analysis was performed to investigate the lipid oxidation in liposomes within 60 days. According to the results, oxidation of polyunsaturated fatty acids may be inhibited with the addition of extract to liposomes, however, lipid content should be limited according to the extract concentration. The study provided valuable data on the

contribution of anthocyanin addition to liposomes to overcome oxidation of unsaturated phospholipids.

In the final part, spray drying of black carrot anthocyanin were evaluated in different encapsulation systems (extract only, primary and secondary liposome systems and chitosan extract mixture). BCE included primary and secondary liposomes were successfully spray dried. All samples were found physically stable after spray drying processing. In addition, SEM images of liposomal systems showed powder without any crack that indicates stability of liposome systems to spray drying. However, liposome samples showed lower stability compared to BCE and BCE and chitosan mixtures according to the biochemical analysis of reconstituted powders.

To sum up, detailed analyses of colorant potential of black carrot anthocyanins in encapsulation and ascorbic acid conditions were evaluated. Short term storage results showed liposome encapsulation may provide enhancement of color attributes of black carrot in the presence of ascorbic acid. In addition, our liposomal system are clear and transparent and suitable for beverage industry. However, color loss in liposomal system was observed in long term storage. This may be prevented with copigmentation with other phenolics, second layer on liposomes, or using saturated lecithin source. On the other hand, results showed us anthocyanins can be used to reduce the oxidation rate of liposomes. Those studies lead us increase of copigmentation in liposomal system may result in mutual protection. Therefore, encapsulated black carrot anthocyanins can be used as natural colorants stable in process conditions.

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ÖZET

Gıda renklendiricileri, tüketici algısını etkilediği için gıda endüstrisinde önemli bir yere sahiptir. Gıdanın tazeliği, çeşidi, içeriği ve üretim metodu gıdanın rengini belirleyen önemli etmenlerdendir. Çekici bir renk tüketiciler tarafından tercih edilmekte ve tüketicilerin satın alma alışkanlıklarına etki etmektedir. Sentetik renklendiriciler, proses koşullarına dayanıklılıklarının yüksek olmasından ve düşük miktarlarda kullanımlarının iyi sonuç vermesinden dolayı yıllarca tercih edilmişlerdir. Fakat, son yıllarda sentetik renklendiricilerin sağlık üzerindeki olumsuz etkilerinin vurgulandığı çalışmaların çoğalması ve dünyada sağlıklı beslenme eğiliminin artması sonucu üreticiler doğal renklendiricilerin kullanımına yönelmişlerdir. Doğal renklendiricilerin kullanılmasında, üretim ve çevre koşullarına dayanıklılığın düşük olması problem oluşturmaktadır. Sentetik renklendiricilere getirilen yasal sınırlamalar ve kamuoyu etkisi doğal renklendiriciler ile ilgili çalışmaları arttırmıştır. Doğal renklendiriciler arasında kara havuç, antioksidan niteliği yüksek bir tarımsal ürün olarak sağlık açısından her geçen gün önem kazanmaktadır. Ana vatanı Türkiye ve Orta-Uzak Doğu bölgeleri olarak bilinen kara havucun Dünyada en az 3000 yıldan bu yana kültürü yapılmaktadır. Kara havuç (*Daucus carota* var L.) açillenmiş antosiyanin bakımından zengin bir tür olmasına rağmen halen özellikle reaktif bileşenlerin (örneğin, C vitamini) bulunduğu ortamlarda bozunmaktadır. Enkapsülasyon, gıda matrisi içerisinde korunmak istenen öz madde ve çevre koşulları arasında bariyer oluşturulması işlemidir. Bu nedenle, doğal renklendiricilerin dayanıklılığı enkapsülasyon ile arttırılarak sentetik renklendiricilerin kullanım ihtiyacının ortadan kaldırılması sağlanabilinmektedir.

Bu tez çalışması, yukarıda belirtilen bilgiler doğrultusunda kara havuç antosiyaninlerinin karakterizasyonunun gerçekleştirilmesi ve kara havuç antosiyaninlerinin lipozom ile enkapsülasyonunun bu bileşenlerin dayanıklılığına etkisinin araştırılması üzerine kurgulanmıştır. Bu doktora tezinin amaçları sırasıyla; (i) Kara havuç ekstraktının antosiyanin konsantrasyonu, fenolik içeriği, antioksidan aktivitesi ve renk özellikleri dikkate alınarak kara havuç ekstraksiyonu koşullarının Yanıt Yüzey Metodolojisi kullanılarak belirlenmesi, (ii) açillenmiş antosiyanin oranı fazla, dayanıklılığı yüksek kara havuç antosiyaninlerinin askorbik asit varlığında dayanıklılığının incelenmesi ve lipozom ile enkapsülasyonun aynı koşullarda kara havuç antosiyaninlerin dayanıklılığına etkisinin araştırılması, (iii) Yüksek basınçlı sıvı kromatografisi analizi ile lipozom enkapsülasyonu ve kara havuç antosiyaninlerinin molekül içi kopigmentasyonunun askorbik asit varlığında kara havuç antosiyaninlerinin dayanıklılığına etkisinin belirlenmesi, (iv) lipozomların kara havuç antosiyaninlerinin varlığında ve yokluğundaki fiziksel ve kimyasal dayanıklılığının depolama süresince belirlenmesi, (v) kara havuç ekstraktı içeren lipozomların püskürtmeli kurutma işlemi sırasındaki dayanıklılığının tespit edilmesidir.

Tezin ilk bölümünde kara havuç ekstraksiyonu için en uygun koşulların belirlenmesi yanıt yüzey yöntemi kullanılarak belirlenmeye çalışılmıştır. Antosiyaninlerin ekstraksiyonuna etki eden faktörlerden pH, sıcaklık, çözgen/katı madde oranı ve etanol/su oranı ekstraksiyon parametreleri olarak seçilmiştir. İki tekrarlı merkezi kompozit tasarım kullanılarak bu faktörlerden deneme deseni çıkarılmıştır. Faktörlerin minimum ve maksimum değerleri ön çalışmalar ile belirlenmiş olup pH, sıcaklık, çözgen/katı madde oranı ve etanol/su oranı için sırasıyla 2,5-6,5, 4-72°C, 5:1-25:1, ve 0:100-100:0 değerleri belirlenmiştir. Elde edilen ekstraktlara toplam fenolik madde miktarı, toplam monomerik antosiyanin miktarı, polimerik renk, toplam antioksidan aktivitesi ve yüksek basınçlı sıvı kromatografisi kullanılarak antosiyanin kompozisyon analizleri yapılmıştır. Renk analizleri haricinde, yüksek sıcaklık, çözgen/katı madde oranı ve etanol/ su oranının ekstraksiyon verimini arttırdığı tespit edilmiştir. Ancak, polimerik renk analizlerinin en düşük olduğu noktalar; düşük pH değerleri ve çözgen/katı madde oranı, düşük veya orta dereceli sıcaklıklar ve yüksek etanol oranı olarak belirlenmiştir. Elde edilen sonuçlara göre en uygun ekstraksiyon değerleri; pH 3,5, sıcaklık 50°C, çözgen/katı madde oranı 10:1 (v/w) ve etanol/su oranı 75:25 (v/v) olarak bulunmuştur. Yanıt yüzey yöntemiyle teorik olarak bulunan en uygun koşullar ile ekstraksiyon işlemi tekrarlanmış ve teorik model deneysel sonuçlarla desteklenmiştir. Böylece elde edilen modelin uygulanabilir olduğu belirtilmiştir.

Tezin ikinci bölümünde ise kara havuç antosiyaninlerinin farklı koşullarda dayanıklılığı incelenmiştir. Açillenmiş antosiyanin oranı yüksek kara havuç antosiyaninleri diğer antosiyanin kaynaklarına göre göreceli olarak farklı koşullarda daha dayanıklı olabilmektedir. Fakat C vitamini (L-askorbik asit) varlığında antosiyaninlerde zamanla bozunma gözlemlenebilmektedir. Bu nedenle, kara havuç ekstraktının farklı oranlarda askorbik asit varlığında dayanıklılığı UV/VIS spektroskopisiyle farklı zamanlarda alınan örneklerle incelenmiştir. Artan askorbik asit miktarının ekstraktın bozunmasını arttırdığı gözlenmiştir. Bu nedenle, lipozom ile enkapsülasyonun antosiyanin dayanıklılığına etkisi incelenmiştir. Farklı lesitin konsantrasyonları (%1, %2 ve %4) ve farklı antosiyanin miktarları (%0,1, %0,2 ve %0,4) kullanılarak yüksek basınçlı homojenizasyon yöntemi ile kara havuç ekstraktı içeren lipozom üretimi gerçekleştirilmiştir. Elde edilen lipozomların fiziksel karakterizasyonu için partikül boyutu (41-46 nm) ve zeta potansiyel analizleri [(-23)-(-20) mV] gerçekleştirilmiştir. Lipozomların enkapsülasyon etkinliği UV/VIS spektroskopisiyle belirlenmiş olup artan lesitin konsantrasyonlarının enkapsülasyon etkinliğini %50'ye kadar arttırdığı belirlenmiştir. Kara havuç ekstraktının lipozom ile enkapsülasyonu sonucunda, kara havuç ektraktlarının renk özelliklerinin askorbik asit varlığında iyileştiği gözlenmiştir. Bu çalışma ile askorbik asit varlığında antosiyaninlerin bozunmasının lipozom enkapsülasyonu ile azaltılabileceği gösterilmiştir.

Tezin üçüncü kısmında ise, çalışmanın daha önceki bölümlerinde lipozom ile enkapsülasyonun antosiyaninleri koruma etkisi gözlendiği için bu bilgiden yola çıkılarak açillenmiş antosiyaninlerin ve açillenmemiş antosiyaninlerin lipozom ile enkapsülasyonundan önce ve sonra askorbik asit varlığında antosiyanin miktarındaki değişim araştırılmıştır. Bu nedenle kara havuç ekstrasktlarının lipozom ile enkapsülasyonu gerçekleştirilmiş ve fiziksel karakterizasyonları yapılmıştır. Elde edilen lipozomların boyutu 50 nm'den az olarak ölçülmüştür. Zeta potansiyeli ise (-23)-(-20) mV olarak bulunmuştur. Enkapsülasyon etkinlikleri yüksek basınçlı sıvı kromatografisi kullanılarak antosiyanin miktarı üzerinden hesaplanmıştır. En yüksek enkapsülasyon etkinliği %60 olarak belirlenmiştir. Farklı miktarlardaki askorbik asit

(%0,01, %0,025, %0,05 ve %0,1 w/w), kara havuç ekstraktı (%0,2) içeren lipozom (%1, %2 ve %4 lesitin içeren) örneklerine ve sadece %0,2 ekstrakt çözeltisine uygulanmıştır. Depolama süresince alınan örneklerde yüksek basınçlı sıvı kromatografisi ile her bir antosiyaninin miktarındaki değişim gözlenmiş olup lipozom ile enkapsülasyonun sinapik, ferülik ve kumarik asit ile açillenmiş siyanidin türevlerine pozitif bir etkisi olduğu belirlenmiştir.

Tezin dördüncü aşamasında ise, kara havuç ekstraktı içeren ve içermeyen lipozomların fiziksel ve kimyasal dayanıklılığı depolama süresince ölçülmüştür. Bu çalışmada %0,1, %0,2 ve %0,4 w/w kara havuç ektraktı içeren farklı lesitin konsantrasyonlarında (%1, %2 ve %4) lipozomlar üretilmiştir. Ayrıca, hiç ekstrakt içermeyen %1, %2 ve %4 konsantrasyonlarında lesitin içeren lipozomlar üretilmiştir. Üretilen lipozomların fiziksel karakterizasyon analizleri yapılmıştır. Başlangıç örnekleri ve 21. güne ait örneklerde partikül boyutu ve zeta potansiyeli özellikleri karşılaştırılmıştır. Partikül boyutları 50 nm'nin altında bulunmuş olup depolama süresince istatistiksel olarak bir değişim gözlenmemiştir ($p<0,05$). Lipozomlara antosiyanin miktarı, fenolik madde miktarı ve antioksidan aktivitesi analizleri uygulanarak lipozomların biyokimyasal dayanıklılığı tespit edilmiştir. Lipozom üretiminde fosfolipidler kullanılmaktadır. Lipozomların kimyasal stabilitesi oksidasyon analizleri ile belirlenmiştir. Bu doğrultuda gaz kromatografisi kullanılarak ekstrakt içeren ve içermeyen lipozomlara hekzanal analizleri uygulanmıştır. Sonuçlar incelendiğinde, çoklu doymamış yağ asiti içeren lesitinlerin oksidasyonunun kara havuç antosiyaninleri ile önlenebileceği görülmüştür. Ayrıca, lipid konsantrasyonunun kara havuç antosiyaninleri ile oksidasyonun önlenmesine etkisi belirlenmiştir. Bu çalışma ile lipozomlara antosiyanin eklenmesinin doymamış yağ asitlerin oksidasyonunun önlenmesi üzerinde etkili olduğu belirlenmiş ve önemli veriler elde edilmiştir.

Tezin son kısmında ise kara havuç antosiyaninlerinin farklı sistemler (sadece ekstrakt, ekstrakt içeren lipozom, kitosan ile kaplanmış lipozom içerisinde ekstrakt ve kitosan ile ekstrakt karışımı) içerisinde püskürtmeli kurutucu ile enkapsülasyonu çalışılmıştır. Kara havuç antosiyaninlerini içeren birincil ve ikincil lipozom örnekleri fiziksel olarak başarılı bir şekilde püskürtmeli kurutma işlemi sonrasında elde edilmiştir. Taramalı elektron mikroskobu (SEM) analizleri sonucunda tozların morfolojik özellikleri belirlenmiştir. Lipozom içeren ürünlerde herhangi bir çatlak gözlenmemiştir. Fakat biyokimyasal analizlerde lipozom içeren örnekler, sadece kara havuç ekstraktı ve kara havuç-kitosan karışımı örneklerinden daha düşük dayanıklılık göstermiştir.

Bu tez çalışmasında kara havuç antosiyaninlerinin gıda renklendiricisi olma potansiyeli hem enkapsülasyon aşamasından sonra hem de askorbik asit varlığında değerlendirilmiştir. Kısa dönem depolama sonuçlarına göre, askorbik asitin kara havuç antosiyaninleri üzerindeki olumsuz etkisi lipozom enkapsülasyon metodu ile azaltılmıştır. Ayrıca, uygulanan lipozom sisteminin şeffaf ve berrak olmasından dolayı içecek sektörü için uygun olduğu görülmüştür. Fakat uzun dönem depolama sırasında lipozom ile enkapsülasyonu sağlanan antosiyaninlerde renk kaybı olduğu tespit edilmiştir. Tezin içinde de belirtildiği gibi kopigmentasyonun enkapsülasyona etkisi farklı fenolik bileşikler kullanılarak, lipozomlara ikincil bir kaplama yapılarak veya doymuş yağ asitleri içeren lesitin kaynağı kullanılarak ileriki çalışmalarda engellenebilir. Diğer yandan, uzun dönem depolama sonuçlarında öne çıkan bir başka sonuç ise antosiyaninlerin lipozomların oksidasyonunu engellediği veya ertelediğidir. Bu şekilde lipozomal sistemlerde kopigmentasyon arttırılarak iki yönlü koruma sağlanabilir. Böylece, enkapsüle edilmiş kara havuç antosiyaninleri zorlu proses koşullarında bile dayanıklılığını koruyabilir.

1. INTRODUCTION

In today's world, food products are transported to far away places where they will be consumed. This may result in degradation and appearance loss. Color of a food is a function of freshness, type, ingredients and production method of a food. In the food industry, food colorants determine the customers' perception. In addition, it also affects customers' purchase behavior since an appealing color is highly desired by consumers. For these reasons food producers have a desire to sell their products with an optimum color, and therefore food colorants are used in the manufacture.

There is an increasing market for natural food colorants. In this context, synthetic food colorants had been used for years to increase visual acceptance and improve color shades (Sadilova et al., 2009). In recent years, the increasing tendency of customers to consume healthy foods has led the food industry to seek natural colorants instead of stable synthetic colorants due to possible harmful effects of synthetic colorants (Wrolstad & Culver, 2012). In addition, due to increasing trend of vegan lifestyle and religious priorities also restricted the consumption of insect based food colorants such as carmine (E120) (Gras et al., 2015).

Nowadays, a natural anthocyanin source, black carrot, draws food industry's attention due to its nutritious value and colorant properties. There is a great need to better understand how their functionality is affected by the composition of the food matrix that is to be colored, process conditions used to generate the food matrix, and the structural organization of the matrix (e.g. liquid, emulsion, gel etc.) (Delgado-Vargas et al., 2000).

Black carrot (*Daucus carota* var L.) is a potential source of acylated anthocyanins. These compounds have shown to be more stable during pH change and (de-)hydration (Türker & Erdoğdu, 2006). Nevertheless, they still suffer from degradation, that is they loose their color over time, especially in systems containing other reacting compounds such as e.g. Vitamin C. Encapsulation may be a suitable method to

enhance their stability to the point where they may be used to replace synthetic colorants.

In the food systems, encapsulation is generally used to create a barrier between the component and environment. This barrier protects material against oxygen and light, and enhances the stability of the encapsulated compounds against heating and freezing. Moreover, such systems have increasingly been used to also control release of compounds (Fuchs et al., 2006). Phospholipids may be used as encapsulation (wall) material. When dispersed in an aqueous phase, phospholipids form core shell particles consisting of a bilamellar membrane and an aqueous core (Munin & Edwards-Lévy, 2011). These systems have been shown to be quite capable of carrying high concentrations of functional compounds but suffered previously from gradually leaking their content into the surrounding continuous (Mozafari et al., 2008). Recently, coating technologies were developed for liposomes that "seal" the particles thereby allowing their content to be maintained over prolonged periods. It is currently unknown though, how this coating technology may affect the functionality of colorants, since scattering and adsorption behavior may be changed.

1.1 Purpose of Thesis

The objectives of this thesis;

1. To optimize black carrot extraction using Response Surface Methodology (RSM) by evaluating anthocyanin concentration, phenolic content, antioxidant activity, and color attributes of black carrot.

2. To compare the stability of black carrot anthocyanins, which are highly stable due to their large amount of acylated anthocyanins, with and without a liposome system in the presence of ascorbic acid.

3. To investigate the effects of both intramolecular copigmentation and liposome encapsulation on ascorbic acid related anthocyanin degradation.

4. To investigate physical and chemical stability of liposomes with and without black carrot during storage.

5. To evaluate the stability of liposomes with black carrot extract during the spray drying process.

1.2 Literature Review

1.2.1 Black carrot polyphenols

In 2nd century, two words, *Daucus* and *carota*, were firstly used to differentiate carrot from parnship (*pastinaca*) by Galen (Stolarczyk & Janick, 2011). Purplish colored eastern carrots were originated in Afghanistan and red colored Japanese carrots were generated by crossing afghan and western orange carrots (Hanelt, 2001). It was reported that wild carrots were known as more diuretic and included higher medicinal value besides their higher cooking time (Grant, 2000). Colors of wild carrots are pale yellow or white, however, firstly domesticated carrots are purple or yellow (Stolarczyk & Janick, 2011). Cultivated carrots are mainly subdivided into two groups as anthocyanin rich *Daucus carota* ssp. *sativus* var. *atrorubens* Alef. and carotene rich *Daucus carota* ssp. *sativus* var. *sativus* (Hanelt, 2001). Black carrots are mostly cultivated in Afghanistan, Egypt, Pakistan, Turkey (in mostly Konya and Adana), and India (Türkyılmaz et al., 2012). Nowadays, black carrots are widely used as an alternative to synthetic colorants in food various food colorants as juices, candies, and soft drinks etc. (Montilla et al., 2011) due to its bright red shade in acidic pH values (Kırca et al., 2007b). In addition, black carrot dietary fibre concentrate may also be used in muffin production as an ingredient for functional food productions (Singh et al., 2016).

In general, carrots (*Daucus carota* L.) include phytochemicals as carotenoids, phenolics, polyacetylenes, isocoumarins, and sesquiterpenes (Metzger et al., 2008). In addition to these biocompounds black carrots are also rich source of anthocyanins. All types of carrots except white are an important source of bioavailable carotenoids (Surles et al., 2004). Contrary to common opinion, some varieties of purple carrots include α- and β-Carotene higher than orange carrots (Arscott & Tanumihardjo, 2010; Dosti et al., 2006). Carotenoids were identified using HPLC in purple carrot as α-carotene (4 mg/100g carrot), β-carotene (12 mg/100g carrot), and lutein (1 mg/100g carrot) (Surles et al., 2004).

Caffeic acid derivatives, caffeoylquinic acid derivatives, ferulic acid derivatives, cryptochlorogenic acid, chlorogenic acid, caffeoylquinic acid, 5-ferulolyquinic acid, di-caffeic acid derivative, and quercetin-3-*O*-galactoside were reported in black carrot concentrate as non-anthocyanin polyphenols (Gras et al., 2016). In addition, the

predominant phenolic acid in black carrot was found as 5-*O*-Caffeoylquinic acid (chlorogenic acid) (Figure 1.1) 657mg/kg in the roots and 5815 mg/kg in the concentrate (Kammerer et al., 2004a). Furthermore, the complex phenolic profile of black carrots may contribute to pigment stability (Kammerer et al., 2004a).

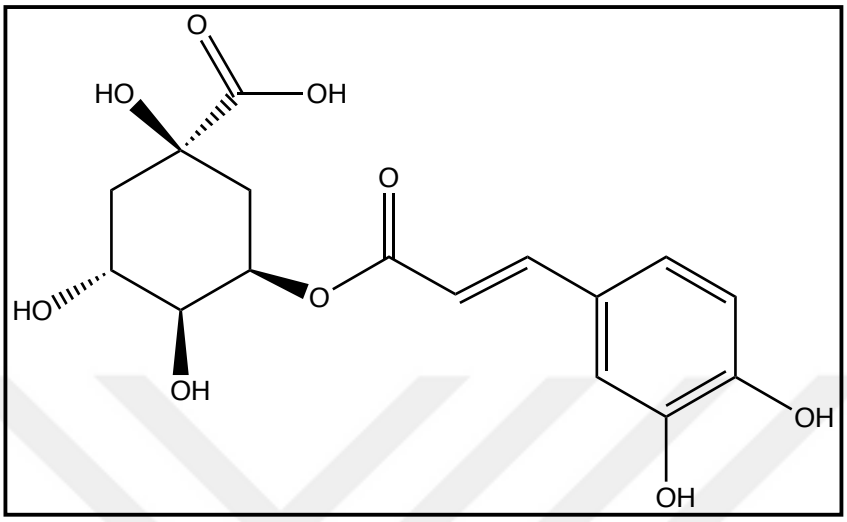
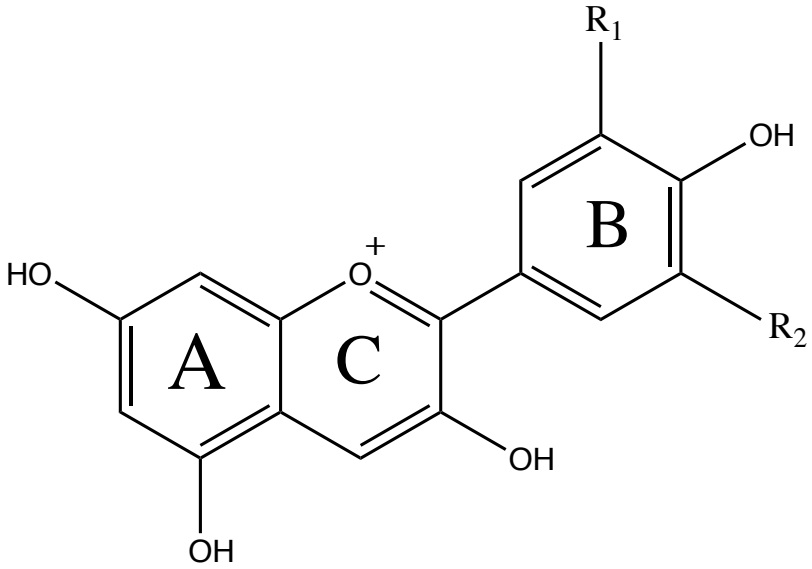


Figure 1.1 : Chemical structure of 5-*O*-Caffeoylquinic acid (chlorogenic acid).

Anthocyanins, categorized in flavonoids, are found in nature as glycosides of anthocyanidins acylated with/without aliphatic and aromatic acids (Türker & Erdoğdu, 2006). As seen in Figure2, the anthocyanidins (or aglycons) consist of an aromatic ring [A] bonded to an heterocyclic ring [C] that contains oxygen, which is also bonded by a carbon–carbon bond to a third aromatic ring [B] (Castañeda-Ovando et al., 2009). In addition, the number of hydroxyl groups, the degree of methylation of these groups, nature, number and position of the sugars bound to the anthocyanidin, and the physicochemical environment in which the anthocyanins are present, as well as the nature and number of aliphatic or aromatic acids attached to the sugar determine the color of anthocyanins (Mazza & Brouillard, 1990; Zozio et al., 2011). Common aglycones were presented in Figure 1.2.



Aglycone	R_1	R_2	Color
Cyanidin	OH	H	Orange-Red
Pelargonidin	H	H	Orange
Peonidin	OCH_3	H	Orange-Red
Delphinidin	OH	OH	Purple-Blue
Petunidin	OCH_3	OH	Purple
Malvidin	OCH_3	OCH_3	Purple

Figure 1.2 : Illustration of common aglycones.

Anthocyanin composition of black carrots mainly consist of cyanidin aglycones (cyanidin-3-xylosyl- glucosyl- galactoside, cyaniding -3- xylosyl- galactoside, cyaniding -3- xylosyl (sinapoylglucosyl) galactoside, cyaniding -3- xylosyl (feruloylglucosyl) galactoside, cyanidin-3-xylosyl (coumaroylglucosyl) galactoside) (Esatbeyoglu et al., 2016; Garcia-Herrera et al., 2016). Chemical structures of five major anthocyanins are presented in Figure 1.3. However, in some varieties of black carrots include perlargonidin xyl-glc-gal feruloyl and peonidin xyl-glc-gal feruloyl as minor components (See Figure 1.4) (Gras et al., 2016). This is firstly reported for aglycones other than cyanidin anthocyanins in black carrots according to comparison of the retention times and fragmentation patterns of peonidin and pelargonidin aglycones with standards after acid hydrolysis (Kammerer et al., 2003).

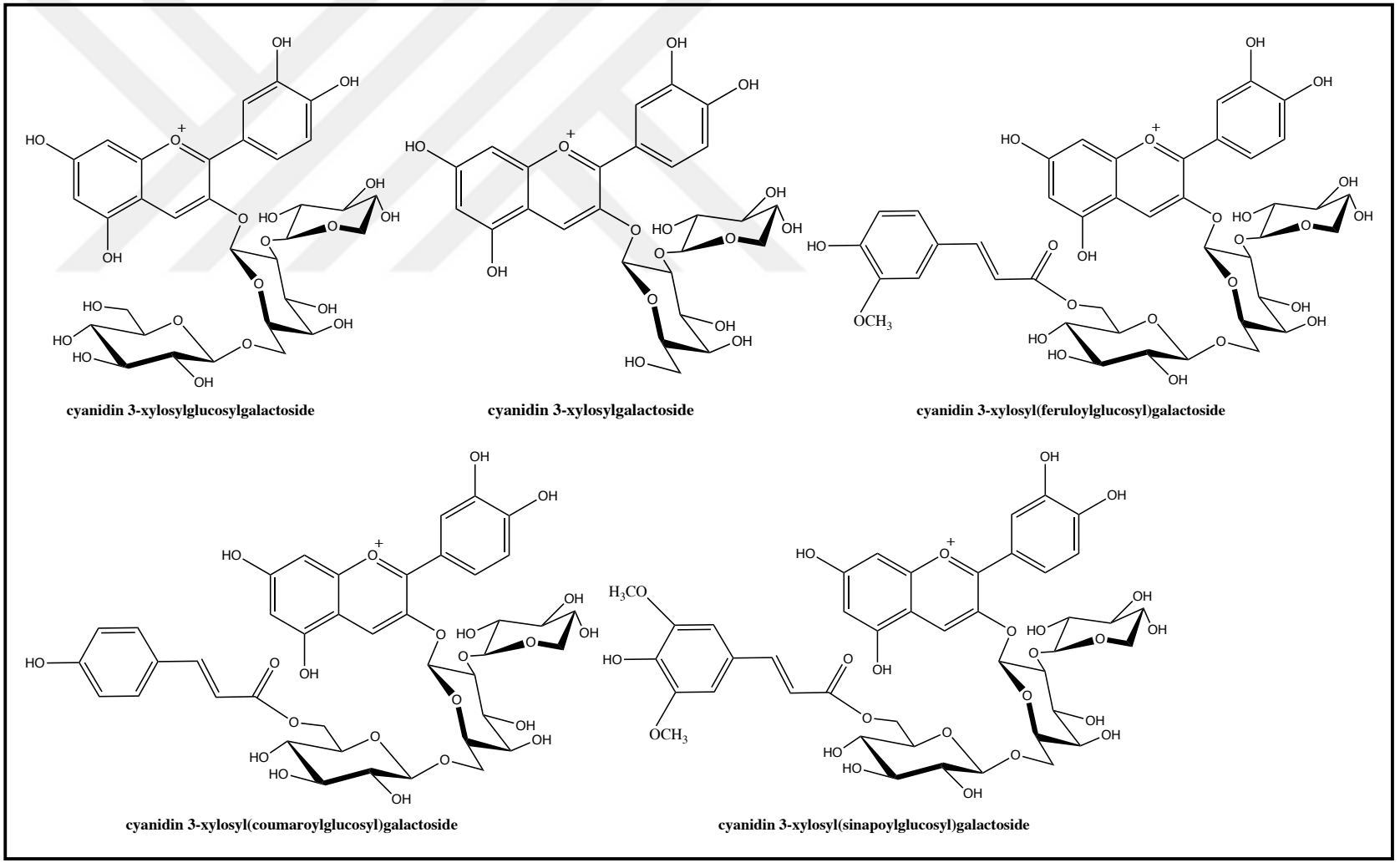


Figure 1.3 : Chemical structures of the five major anthocyanins i n black carrot.

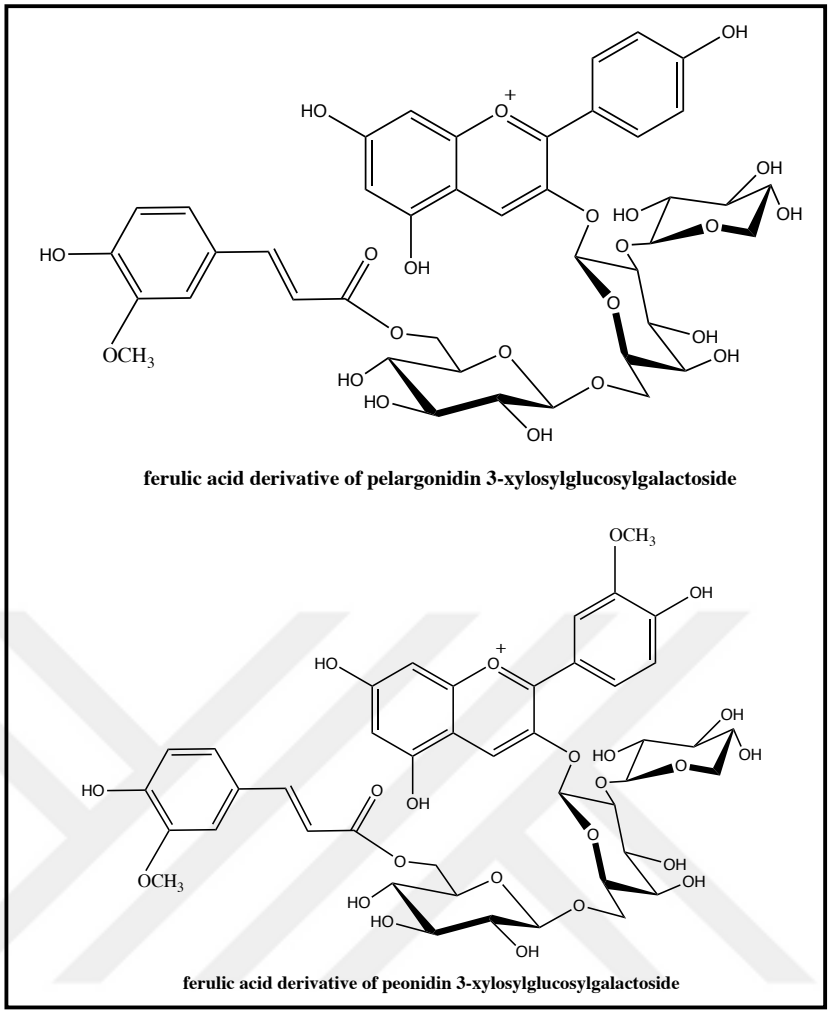


Figure 1.4 : Chemical structures of minor anthocyanins in black carrot.

It has been reported that anthocyanin content of black carrots was found up to 1750 mg kg^{-1} fresh weight (Kırca et al., 2006; Mazza & Miniati, 1993). Moreover, the major anthocyanin compound was determined as cyaniding -3- xylosyl (feruloylglucosyl) galactoside which is 72% of the total content of anthocyanin compounds (Olejnik et al., 2016). Furthermore, anthocyanin content of black carrot crops may have some differences due to the variations as seen in Figure 1.5.

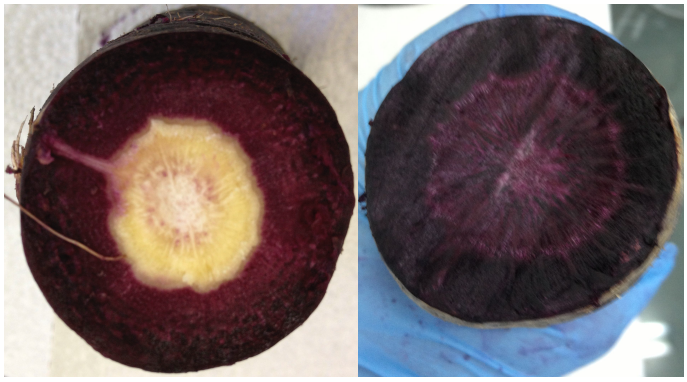


Figure 1.5 : Differences in black carrot crops.

1.2.2 Health effects of black carrot polyphenols

Researchers give special attention to black carrot (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) due to its phytochemicals and especially high anthocyanin content which play significant role in prevention of diseases, such as coronary hypertension, heart disease, obesity, diabetes, cancers and inflammatory bowel disease (Metzger et al., 2008; Olejnik et al., 2016; Tsuda, 2012). Many researchers have found significant results on health promotion effect of black carrot phytochemicals. In details, anti-diabetic activity of black carrot anthocyanins and copigment fractions caused inhibition on α-amylase and α-glucosidase activity and on cellular glucose uptake (Esatbeyoglu et al., 2016). Furthermore, specific attention of researches on anti-carcinogenic effect of black carrot was determined. In a study, extracts from black carrot cell culture were investigated and it was found that extracts may provide treatment against cancer especially brain cancer without deterioration of healthy cells (Sevimli-Gur et al., 2013). In addition, consuming black carrot may be more beneficial to health due to high antioxidant properties than other carrot crops (Sun et al., 2009). Inhibitory effects of black carrots on colon cancer were also investigated and the results supported health promotion effect of black carrots. Black carrot extracts showed positive effect on inhibition of human cancer cells in vitro (Netzel et al., 2007). Furthermore, colonic cells may be protected by black carrot anthocyanins against oxidative stress (Olejnik et al., 2016). On the other hand, a recent study reveals purple carrot showed higher anti-inflammatory properties by inhibition of COX-2 enzyme than red cabbage (Mizgier et al., 2016). Moreover, in another study investigated polyacetylenes from purple carrot extract showed that they possess anti-inflammatory bioactivity on macrophage and endothelial cells (Metzger et al., 2008).

1.2.3 Factors influencing anthocyanin stability

Anthocyanin usage as food colorant has some limitations due to their low stability against processing, formulation, and storage conditions (Giusti & Wrolstad, 2003). In detail, anthocyanin stability is affected by different factors such as pH, temperature, light, oxygen, its chemical structure and concentration, solvent type, the presence of other compounds (enzymes, proteins, flavonoids, ascorbic acid, sugars and metallic ions) (Markakis, 1982; Rein, 2005). However, most common problem on the

application of anthocyanin colorants in food products is their low stability to heat, light and pH changes (Kırca et al., 2006).

1.2.3.1 Chemical structure

Chemical structure of anthocyanins is one of the factors that affect stability and promote or inhibit the effects of other destabilizing factors. For instance, heat stability of monoacylated compounds are higher in acidic aqueous solution, however lower in neutral conditions than non-acylated compounds (Iliopoulou et al., 2015). Furthermore, anthocyanin bioavailability and stability may be affected from the presence of methoxyl or hydroxyl groups in the anthocyanin structure such as Delphinidin-3-glucoside which has three hydroxyl groups in its B ring is the one of the most unstable anthocyanins, and malvidin-3-glucoside is one of the most stable anthocyanins due to its methoxyl groups in its structure (Garcia-Herrera et al., 2016). On the other hand, sugar variety may also affect anthocyanin stability which stated according to stability difference between galactosidic, more stable, and arabinosidic anthocyanins (Markakis, 1982).

1.2.3.2 pH

In aqueous solutions, anthocyanin structure changes due to high reactivity of aglycone moiety in accordance with its chemical structure (Brouillard, 1982). There are four types of anthocyanin chemical structure (Figure 1.6) and concentration of these structures depends on their chemical properties and pH. At lower pH values concentration of flavylium cation is higher than other forms that reveals red colour. In general, it is known that flavylium cation is dominant at pH <2 and proton loss, occured at higher pH values (pH 6-7), generates quinoidal base and colorless carbinol base or chalcones are formed by hydration of flavylium cation at pH 4-5 (Cavalcanti et al., 2011; Davidović-Amić et al., 1994). On the other hand, acylated anthocyanin rich black carrot preserves its attractive red color up to pH 4 and bluish color reveals pH values higher than 6 (Cevallos-Casals & Cisneros-Zevallos, 2004). In addition, pH both affects anthocyanin color and stability (Markakis, 1982). For instance, during heating, anthocyanin loss of black carrots increased with higher pH values (from 4.3 to 6.0) (Kırca et al., 2007a).

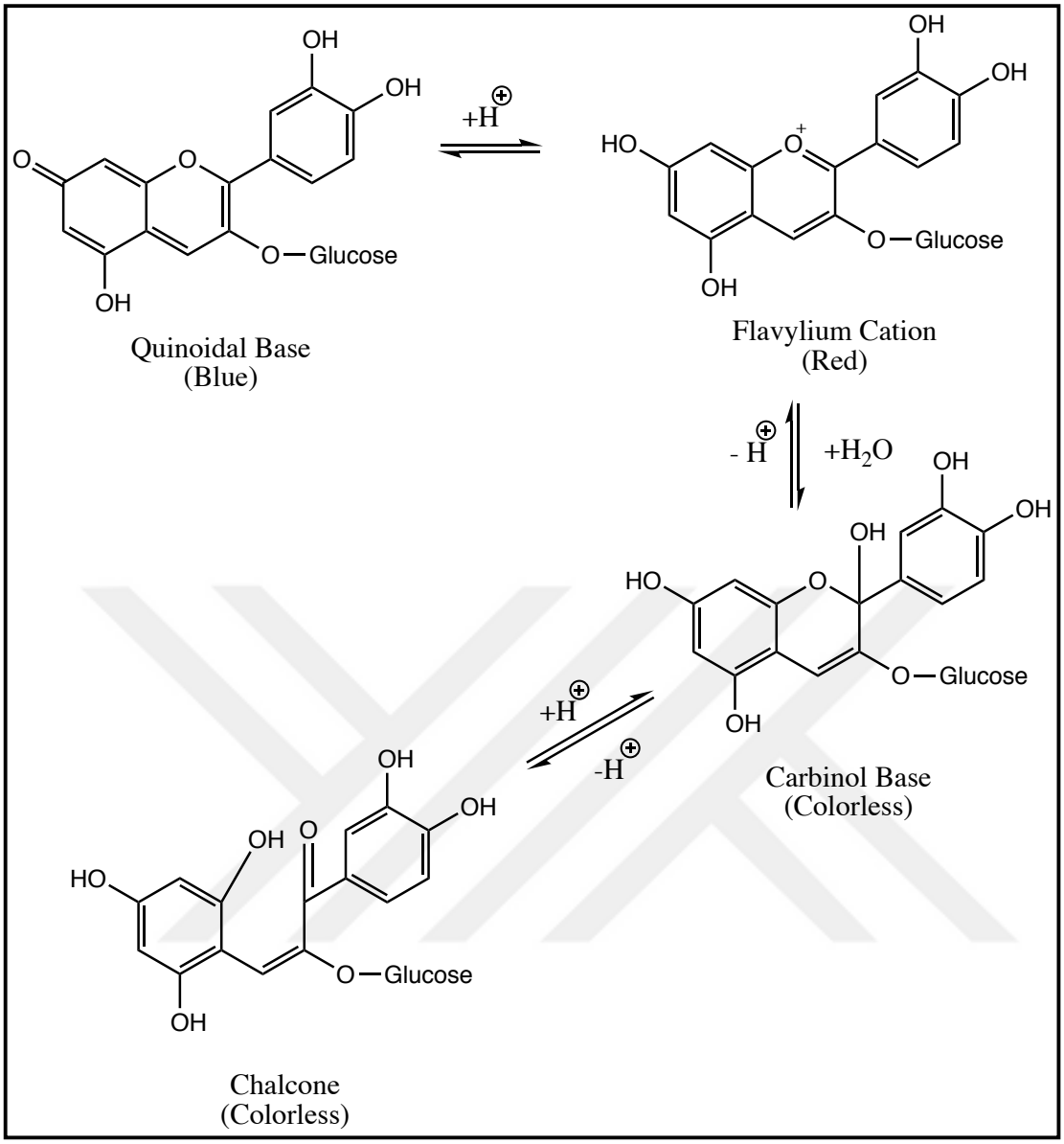


Figure 1.6 : Four equilibrium structures of cyanidin-3-glucoside.

1.2.3.3 Temperature

Storage temperatures and heating affect anthocyanin degradation in food products in previous studies. It is reported that the monomeric anthocyanin content and color density of black carrot reduced in time in accordance with storage temperature (4°C, 25°C, 40°C), however browning and polymeric color raised inversely (Turker et al., 2004). In addition, pasteurization process (90°C, 15 min) caused reduction in black carrot anthocyanins up to 16%, whereas heating of blackberry juices at 95°C for 3 min caused 48% anthocyanin loss and further loss (35%) were observed after pasteurization at 90°C (Türkyılmaz et al., 2012). Furthermore, effect of storage temperature on anthocyanin stability also depends on acylation. In a previous study,

acylated anthocyanins were found more stable than nonacylated ones irrelevant to storage temperatures or heating (Sadilova et al., 2007; Turker et al., 2004). In general, increasing temperature caused higher anthocyanin degradation during both heating and storage (Kırca et al., 2007a). Possible thermal degradation mechanism of cyaniding-3-glucoside is presented in Figure 1.7.

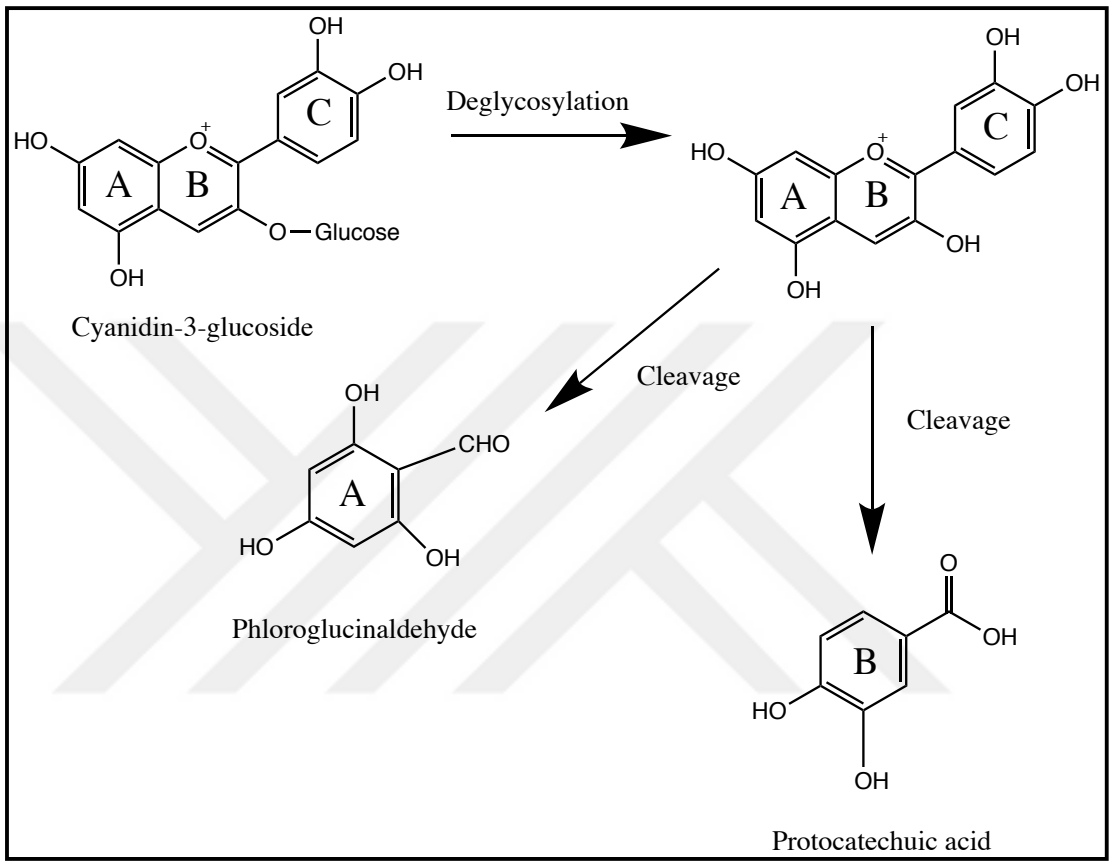


Figure 1.7 : Possible thermal degradation mechanism of cyanidin-3-glucoside (Sadilova et al., 2006).

1.2.3.4 Solid content

Solid content of anthocyanin including products is also a factor for anthocyanin stability. During heating, stability of black carrot anthocyanins reduced with increasing solid content that can be explained with increase in reaction rate due to higher meeting possibility of reactive molecules in concentrated media (Kırca et al., 2007a; Nielsen et al., 1993), however degradation rate of anthocyanins decreased with increasing solid content during storage (Kırca et al., 2007a). In addition, similar results were found for sour cherry (Cemeroglu et al., 1994) and blood orange anthocyanins (Kırca, 2003). However, another study reported that increasing solid content increased

anthocyanin degradation even at storage for pelargonidin based anthocyanins (Garzon, 2002).

1.2.3.5 Process conditions

In processing of food products many different steps are applied from raw to final product. Anthocyanins are susceptible to these process conditions. Even, black carrot anthocyanins are relatively stable, loss of black carrot anthocyanins increased at clarification step in juice processing with bentonite (up to 16%) or gelatin-kieselsol (up to 13%) after pasteurization (Türkyılmaz et al., 2012). In addition, jam and marmalade processing also reduced total phenolic content and antioxidant capacity of black carrots (Kamiloglu et al., 2015b)

On the other hand, processing may also have positive effect on anthocyanin content of food products. For instance, enzyme-assisted preparation in black carrot juice processing before pressing may increase total antioxidant activity, total phenolic and anthocyanin content (Khandare et al., 2011). Furthermore, black carrot jam and marmalade processing caused increase in bioaccessibility of bioactives in vitro (Kamiloglu et al., 2015b).

1.2.3.6 Ascorbic acid content

In food processing, ascorbic acid was utilized to stabilize nutrients (Brenes, 2005). In the United States, wide range of ascorbic acid fortification (0.04%- 11.4% w/w) to powdered beverage samples was reported (West & Mauer, 2013). Some fruits also have high ascorbic acid naturally which also affects anthocyanin stability. In a study, stability of black carrot anthocyanin are found higher in apple and grape juices than citrus juices at 70°C-80°C which have higher ascorbic acid content (Kırca et al., 2006). There are several suggested mechanisms on ascorbic acid related anthocyanin degradation. In 1951, the mechanism behind the oxidizing agent behavior of ascorbic acid explained that hydrogen peroxide formation during autooxidation process of ascorbic acid may be responsible for oxidation (Calcutt, 1951). On the other hand, direct condensation of ascorbic acid on anthocyanin molecule may also cause to color loss of anthocyanins (Poei-Langston & Wrolstad, 1981). It is reported that oxygen may have an important role on bleaching effect of ascorbic acid (Iacobucci & Sweeny, 1983). In addition, both autooxidation products of ascorbic acid (hydrogen peroxide, dehydroascorbic acid) and ascorbic acid may involve in oxidation of pyrylium ring

(Poei-Langston & Wrolstad, 1981; Wilska-Jeszka, 2002). Furthermore, mutual degradation of ascorbic and anthocyanins is proposed in previous studies (West & Mauer, 2013).

The proposed degradation mechanism by hydrogen peroxide, degradation product of ascorbic acid, is oxidative cleavage of the pyrylium ring (see Figure 1.8) according to Baeyer-Villiger type oxidation (Jurd, 1966).

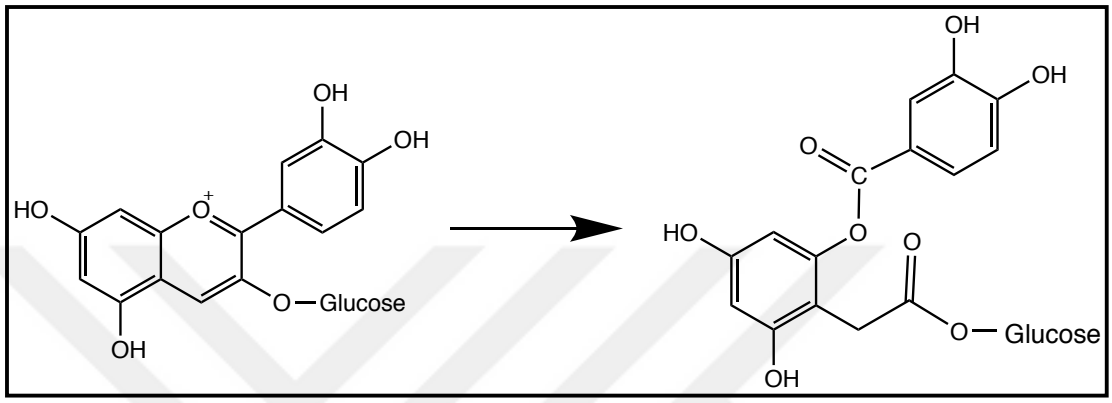


Figure 1.8 : Possible hydrogen peroxide oxidation mechanism of cyanidin-3-glucoside (Jurd, 1966).

Acerola has high content of ascorbic acid naturally and has low anthocyanin stability (De Rosso & Mercadante, 2007). Furthermore, ascorbic acid content of dried black carrots were reported as 9.3-11.5 mg/100g (Garba & Kaur, 2014). In addition, ascorbic acid related anthocyanin degradation was determined in previous studies (Brenes, 2005; Freedman & Francis, 1984; Hernandez-Herrero & Frutos, 2015; Li et al., 2013; Martí et al., 2002; Poei-Langston & Wrolstad, 1981).

1.2.4 Stabilization mechanisms of anthocyanins

Anthocyanin stabilization may enhance with different techniques including mainly encapsulation and association (Cavalcanti et al., 2011). Both methods are studied widely in previous studies.

1.2.4.1 Copigmentation

In consideration of only pH effect, anthocyanins should be in their colorless form, however, anthocyanins exist in their colored state in nature due to association of other compounds (Mazza & Brouillard, 1987). This naturally stabilization mechanism implies that copigmentation can stabilize anthocyanin color. There are four types of copigmentation; inter molecular copigmentation, intramolecular copigmentation,

metal complexation, and self-association (Gras et al., 2016). The illustrations of these mechanisms is presented in Figure 1.9. Copigmentation cause bathocromic shift, which is the shift of the maximum absorption wavelength, and increase color intensity (Rein, 2005). Backward of copigmentation is hydrophobic stacking interaction between anthocyanin and copigment (Brouillard & Dangles, 1994). It is driven generally with π-π with flavylium ions and copigments except metal ion interactions (Castañeda-Ovando et al., 2009). Copigments are colorless compounds including flavonoids, alkaloids, amino acids, nucleotides, other anthocyanins (Mazza & Brouillard, 1987). In addition, copigmentation is affected by both anthocyanin and copigment type and concentration, pH, temperature and metals (Gras et al., 2016; Osawa, 1982). It is reported that stabilization effect of copigmentation is higher in acylated anthocyanins than nonacyleted ones (Gras et al., 2016). Recent studies on anthocyanin association with other compounds were summarized in Table 1.1

When it comes to intermolecular copigmentation, there are weak hydrophobic forces between anthocyanins and colorless compounds including flavonoids, and other phenolic compounds (gallic acid, caffeic acid, ferulic acid, chlorogenic acid, etc.) (Eiro & Heinonen, 2002). Enhancing color stability with intermolecular co-pigmentation requires copigment concentration at least 9 times more than anthocyanins (Gras et al., 2016). Depending on pH, copigment can stabilize both flavylium cation and the quinonoidal base (Mazza & Brouillard, 1990). Furthermore, color enrichment of black carrot anthocyanins were performed using chlorogenic acid (Gras et al., 2016). In addition, stabilization effect of intermolecular copigmentation were also investigated among acylated and non acylated anthocyanins of black carrot in a study which indicated that stabilization effect reduced in acylated anthocyanins (Gras et al., 2017).

Intramolecular copigmentation is more effective than intermolecular copigmentation, and it can stabilize anthocyanin color at lower concentrations considering intermolecular effect (Dangles et al., 1993). Intramolecular copigmentation mechanism based on covalent bounds between copigments and anthocyanins (Gras et al., 2016). Interactions of anthocyanins with acyl groups are named as intramolecular copigmentation (Bloor & Falshaw, 2000).

In aqueous solutions, stability of acylated anthocyanins against thermal process is higher than non acylated counterparts due to sandwich-type stacking of aromatic acyl groups with pyrylium ring of the flavylium cation which prevents nucleophilic attack

of water to C-2 and C-4 positions (Iliopoulou et al., 2015; Malien-Aubert et al., 2001). In a similar manner, previous study concluded that acylated anthocyanins showed higher stability against heating at 90°C (Gras et al., 2016). Furthermore, intramolecular copigmentation may be used to explain the high stability of black carrot anthocyanins, acylated with hydroxycinnamic and hydroxybenzoic acid, at relatively high pH values (Kammerer et al., 2004b).

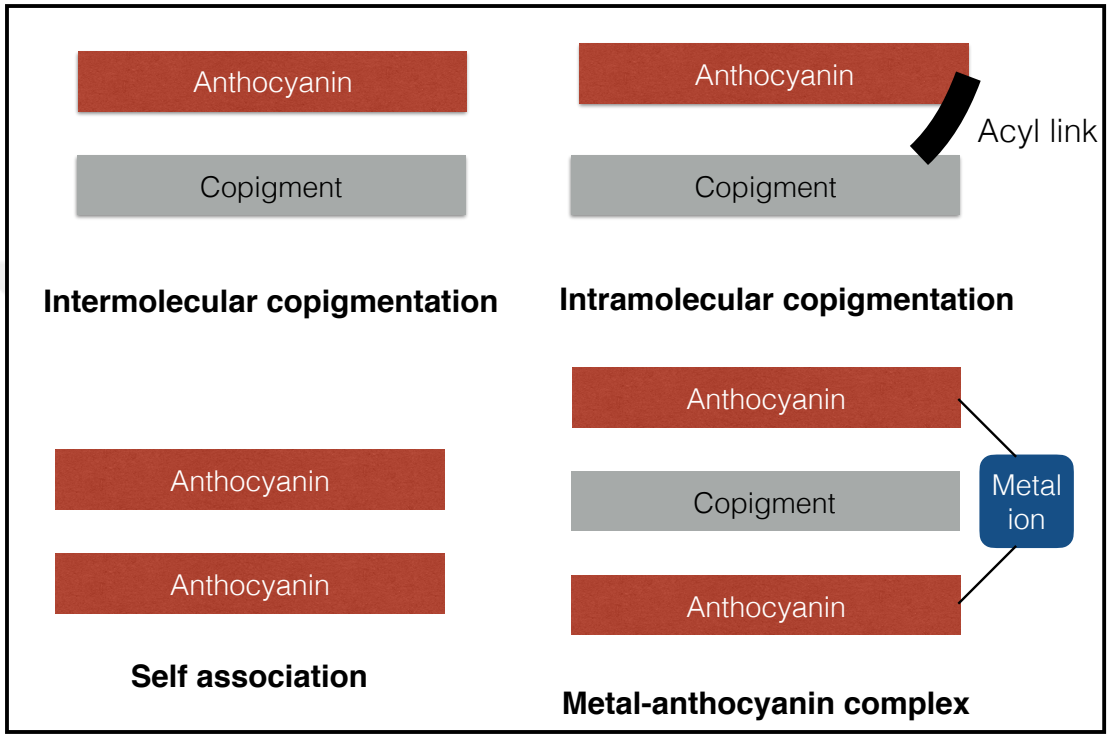


Figure 1.9 : Illustrations of copigmentation of anthocyanins.

1.2.4.2 Encapsulation

Encapsulation is utilized to prevent degradation effect of environmental or compositional on the component. Encapsulation may be used to increase stabilization of isolated anthocyanins (Yousuf et al., 2015). Encapsulation forms a barrier between core material and deteriorative effects that protects material against oxygen and light, and enhances the stability of the encapsulated compounds against heating and freezing. Furthermore, release control of compounds can be achieved with encapsulation (Fuchs et al., 2006).

Table 1.1 : Recent studies investigating copigmentation effect on various anthocyanin sources.

Anthocyanin source	Association type	Anthocyanin	Copigment	Stabilization Improvement	Ref
Purple carrot	Intermolecular copigmentation	Cyanidin-3-*O*-glucoside	Citrus pectin Beet pectin Whey protein	Biopolymers enhanced anthocyanin stability against ascorbic acid related degradation	(Chung et al., 2015)
Black carrot	Intermolecular copigmentation	Cyanidin-3-*O*-glucoside	Chlorogenic acid	pH dependent weak increase in thermal stability of anthocyanins	(Gras et al., 2017)
Black carrot	Intermolecular copigmentation	Cyanidin-3-*O*-glucoside	Chlorogenic acid	Increase in color stability against heating (90°C)	(Gras et al., 2016)
Blueberry	Intermolecular copigmentation	Cyanidin-3-glucoside	Blueberry pectin	Not defined	(Lin et al., 2016)
Black currant	Intermolecular copigmentation	Delphinidin- 3-glucoside, cyanidin-3-glucoside	Sugar beet pectin, Citrus pectin, Apple pectin	Pectin source dependent increase in anthocyanin stability during storage	(Buchweitz et al., 2013a)
Strawberry	Intermolecular copigmentation	Pelargonidin-3-glucoside	Sugar beet pectin, Citrus pectin, Apple pectin	Increase in anthocyanin retention with sugar beet and apple pectins	(Buchweitz et al., 2013b)
Red grape	Intermolecular copigmentation	Malvidin -3-*O*-glucoside Petunidin-3-*O*-glucoside	Rosemary extract	Increased anthocyanin stability against ascorbic acid related degradation	(Brenes, 2005)
Strawberry	Intramolecular copigmentation	Pelargonidin-3-glucoside	Black carrot juice concentrate	Increased color stability in jam processing	(Kırca et al., 2007b)

Encapsulation methods for water soluble biomolecules, spray drying, freeze drying, complex formation, gelation (thermal and ionic), liposomes, multilayer emulsions etc. (McClements, 2015), are convenient for anthocyanins. Recent studies on anthocyanin encapsulation is summarized in Table 1.2.

Spray drying is a widespread microencapsulation method for essential food materials. It ensures low cost and low humidity and good quality for food pigments (Burin et al., 2011). Wall material properties, inlet and outlet temperatures may affect final anthocyanin content in this method. Previous study reported that higher inlet temperatures resulted higher anthocyanin loses and 20-21DE maltodextrin increased final anthocyanin content (Ersus & Yurdagel, 2007). Spray drying may produce granular powder forms of anthocyanins with suitable wall materials and simply their transportation. In previous studies spray drying is favored due to its rapid and ease of the process. Cranberry (Main et al., 1978), Cabernet Sauvignon (Vitis vinifera L.) grapes (Burin et al., 2011), black carrot (Ersus & Yurdagel, 2007; Murali et al., 2015), pomegranate (Robert et al., 2010) were used as anthocyanin sources for spray drying encapsulation in previous studies.

Freze drying is a microencapsulation method which protects initial anthocyanin content without thermal degradation effect of other microencapsulation methods including high temperatures (spray drying, thermal gelation). However, due to its high cost and long process time, it is not the first choice of food industry. Similar to spray drying method wall materials are used prior to process. Anthocyanins are homogenized with wall materials and co-lyophilized (Khazaei et al., 2014). Several wall materials such as gum arabic, maltodextrin (Khazaei et al., 2014), and pullulan (Gradinaru et al., 2003) etc. can be used in freeze drying process.

Hydrophilic bioactives may form complexes with proteins, carbohydrates (cyclodextrins,) and lipids to increase their stability (McClements, 2015). Recent studies showed mannoproteins extracted from the yeast cell wall can be used for complex formation that enhances thermal stability of anthocyanins at neutral pH values (Guan & Zhong, 2015). Furthermore, cyclodextrins are commonly used in food industry and can be used to stabilize anthocyanins (McClements, 2015).

Thermal or ionic gelation are used in microencapsulation of hydrophilic biomaterials. Alginate and pectin are widely used polysaccharides, and also curdlan are used in

thermal gelation (Ferreira et al., 2009). On the other hand, ionic gelation of anthocyanins was performed with alginate, quitosan and cellulose acetate (Albarelli et al., 2009; Cavalcanti et al., 2011).

Encapsulation of hydrophilic, hydrophobic, and amphiphilic materials can be performed with liposomes (Fathi et al., 2012). Thus, liposome entrapment can be used to encapsulate anthocyanins efficiently. Phospholipids are used in liposome formation due to their core shell structure, consist of bilamellar membrane and aqueous core, forming capability (Munin & Edwards-Lévy, 2011). Illustration of liposome core shell structure is presented in Figure 1.10.

Liposome encapsulation method widely used for various polyphenol or anthocyanin rich plant extracts, including elderberry (Bryła et al., 2015), grape seed (Gibis et al., 2016; Gibis et al., 2014a; Gibis et al., 2012), black mulberry (Gültekin-Özgüven et al., 2016), hibiscus (Gibis et al., 2014b). Different lecithin sources were used in previous studies for liposome encapsulation such as egg yolk (Jaafar-Maalej et al., 2010), soy bean (Heinonen et al., 1998) and sunflower (Bryła et al., 2015) lecithins.

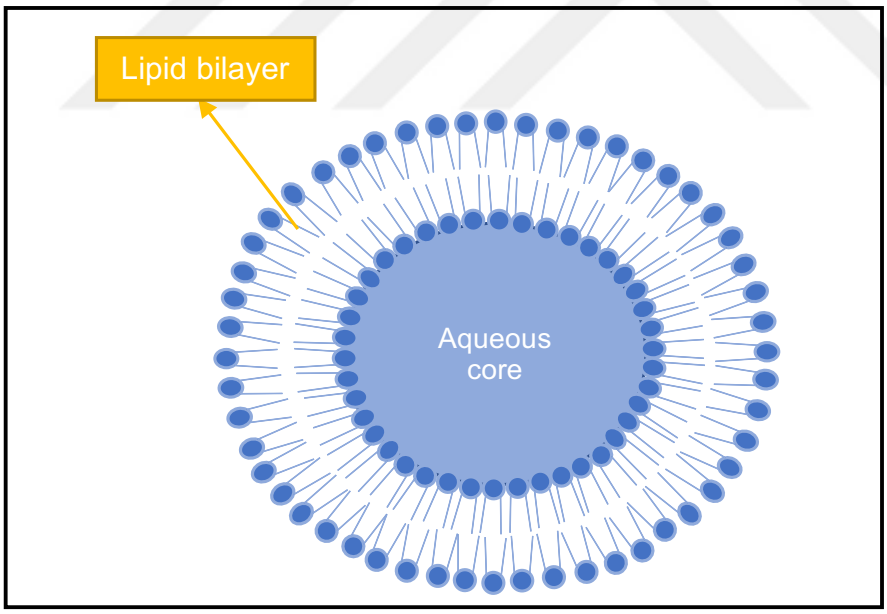


Figure 1.10 : Core-shell structure of liposome.

Liposomes may be formed with different methods including thin lipid hydration (Bangham et al., 1965), ethanol injection (Jaafar-Maalej et al., 2010), detergent removal (Yang et al., 2016), reverse phase evaporation (Szoka & Papahadjopoulos, 1978), sonication, extrusion, and high pressure homogenization (Gibis et al., 2013; Meure et al., 2008). An illustration of black carrot encapsulation with soy lecithin including liposomes using high pressure homogenization is presented in Figure 1.11.

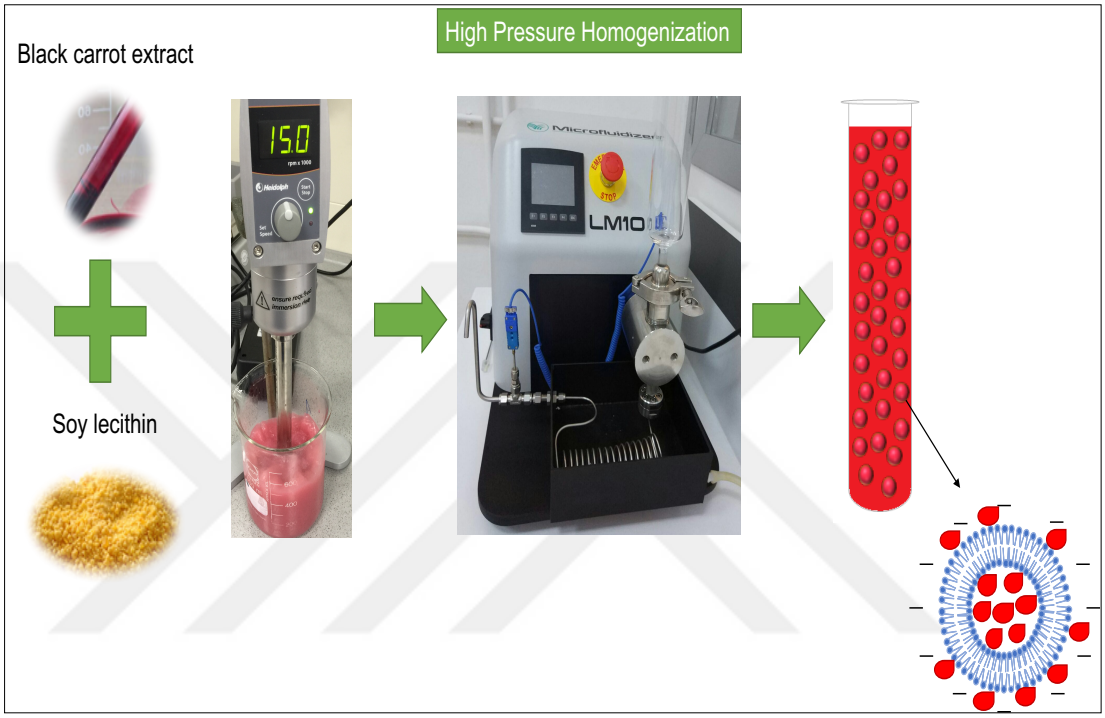


Figure 1.11 : Illustration of black carrot extract included liposomes.

Physical characterization of liposomes can be determined basically by particle size, zeta potential, and optical image analyses (Gibis et al., 2016). In addition, colloidal stability of liposomes can be measured with zeta potential which is higher when particle charge is sufficiently high to repel each other (Du Plessis et al., 1996). On the other hand, chemical stability of liposomes also determines shelf life of bioactive included liposomes. Hexanal formation, which is an oxidation product of unsaturated fatty acyl chain of phospholipids, can be used as an indicator for oxidative stability of liposomes (Gibis et al., 2014b).

Table 1.2 : Recent studies investigating cencapsulation effect on anthocyanin stability.

Anthocyanin source	Encapsulation technique	Encapsulation material	Stability study	Effect of encapsulation on anthocyanin stability	Ref
Cabernet Sauvignon (*Vitis vinifera* L.) grapes	Spray Drying	Maltodextrin, Maltodextrin/γ-cyclodextrin Maltodextrin/arabic gum	Light Temperature (4, 25°C)	Increase in anthocyanin stability	(Burin et al., 2011)
Blackberry	Thermal Gelation	Curdlan	Not defined	Not defined	(Ferreira et al., 2009)
Hibiscus sabdariffa L. anthocyanins	Freeze Drying	Pullulan	Light Temperature (40°C) Humidity	Slightly increase in anthocyanin stability	(Gradinaru et al., 2003)
Saffron petal's extract	Freeze Drying	Gum Arabic maltodextrin (M7 and M20)	Temperature (35°C)	Higher stability in encapsulated samples	(Khazaei et al., 2014)
Isabel grape bagasse (*Vitis labrusca*)	Spray Drying	Maltodextrin Gum arabic	Not defined	Not defined	(Valduga et al., 2008)

Table 1.2 (continued) : Recent studies investigating cencapsulation effect on anthocyanin stability.

Anthocyanin source	Encapsulation technique	Encapsulation material	Stability study	Effect of encapsulation on anthocyanin stability	Ref
Black currant	Thermal gelation	β-glucan	Not defined	Not defined	(Xiong et al., 2006)
Roselle calyces (*Hibiscus Sabdariffa L.*)	Microwave-assisted technique	Maltodextrin	Stability in water in oil emulsion	Increased stability in encapsulated samples	(Zaidel et al., 2014)
Red cabbage (*Brassica Oleracea*)	Microwave-assisted technique	Maltodextrin	Stability in water in oil emulsion	Increased thermal and phase stability with encapsulated samples	(Zaidel et al., 2014)
Cyanidin-3-*O*-glucoside	pH modification	Ferritin	Thermal and photostability	Increased in encapsulated samples	(Zhang et al., 2014)
Hibiscus sabdariffa	Liposome	Soy Lecithin	Not defined	Not defined	(Gibis et al., 2014b)

Table 1.2 (continued) : Recent studies investigating cencapsulation effect on anthocyanin stability.

Anthocyanin source	Encapsulation technique	Encapsulation material	Stability study	Effect of encapsulation on anthocyanin stability	Ref
Black Carrot	Spray Drying	Maltodextrin	Not defined	Not defined	(Ersus & Yurdagel, 2007)
Elderberry	Liposome	Soy Lecithin	Not defined	Not defined	(Bryła et al., 2015)
Pomegranate	Spray drying	Maltodextrin Soybean protein isolate	Thermal storage stability	Increased stability with maltodextrin included powders	(Robert et al., 2010)
Jabuticaba fruit	Ionic gelation	Sodium alginate	Not defined	Not defined	(Albarelli et al., 2009)

2. OPTIMIZATION OF EXTRACTION OF BIOACTIVE COMPOUNDS FROM BLACK CARROT USING RESPONSE SURFACE METHODOLOGY (RSM)

2.1 Introduction

Anthocyanins, which are glycosides of anthocyanidins, are natural colorants widely used in foods due to their non-toxic, bright colored and water soluble characteristics (Delgado-Vargas & Paredes-Lopez, 2003; Mortensen, 2006; Sari et al., 2012). In the US, daily intake of anthocyanins is reported to be approximately 180–215 mg/day as they are present in many fruits and vegetables (Mohdaly et al., 2009). Besides, high anthocyanin intake was emphasized last years due to their positive health effects. The previous reviews provide important information on the effects of phenolic compounds on human health covering both in vitro and in vivo studies (Chiva-Blanch & Visioli, 2012; Giampieri et al., 2014).

The sources of anthocyanins are mostly composed of grape and its products, but other sources such as red cabbage, elderberry, black currant, purple carrot, sweet potato, and red radish are also increasingly used as raw materials (Mortensen, 2006). Black carrot (*Daucus carota* var L.) is composed of both non- acylated (36.14 %) (cyanidin-3-xylosyl-galactiside and cyanidin-3-xylosyl-glucosyl-galactoside) and acylated anthocyanins (cyanidin-3-xylosyl-glucosyl-galoctoside with sinapic acid (3.64 %), ferulic acid (46.72 %), and coumaric acid (50.55 %) (Gizir et al., 2008). Especially, it is a potential source of acylated anthocyanins, which are more stable against pH change and hydration (Türker & Erdoğdu, 2006).

Extraction procedure is the vital step to obtain the optimum amount of anthocyanins and carry on the initial content into further processes. In a study, different anthocyanin extraction conditions (pH 2, 3, 4, temperature 25, 37.5, 50 °C) were studied, and the results indicated that pH 2 is the most stable pH value and increasing pH values reduce the pigment stability and extraction yield (Türker & Erdoğdu, 2006). In addition, temperature values higher than 45 °C reduced extraction yield severely, and optimum

temperature values were found to be in between 30-35 °C (Cacace & Mazza, 2003a). It was also indicated that extraction temperature might affect the storage stability of anthocyanin extracts (Cisse et al., 2012). Some of the parameters including acidity of the solvent, ethanol concentration, time, temperature, and solid/solvent ratio were partly studied in different studies (Cacace & Mazza, 2003b; Chen et al., 2007; Chirinos et al., 2007; Corrales et al., 2009; Fan et al., 2008; Ghafoor et al., 2009; Ku & Mun, 2008; Mané et al., 2007; Sun et al., 2007; Yang & Zhai, 2010), but in this study all important parameters for the extraction of anthocyanins from black carrot were evaluated together.

Evaluation of extraction optimization in most cases is performed with response surface methodology (RSM). RSM comprises of both mathematical and statistical techniques that are based on the fit of empirical models to the experimental data obtained in relation to experimental design (Bezerra et al., 2008). In several studies, RSM was applied to optimize anthocyanin extraction from various sources such as raspberry (Sun et al., 2007), mulberry (Zou et al., 2011), purple corn (*Zea mays* L.) cob (Yang & Zhai, 2010), Bokbunja (*Rubus coreanus* Miq.) marc (Ku & Mun, 2008), grape seeds (Ghafoor et al., 2009; Mané et al., 2007), purple sweet potato (Fan et al., 2008), and black currants (Cacace & Mazza, 2003b). The objective of this part is to perform RSM to optimize anthocyanin concentration, phenolic content, antioxidant activity, and color attributes of black carrot. By the help of this work, further studies pertaining black carrot anthocyanins may get use of these optimum extraction conditions to protect the content of bioactive compounds.

2.2 Materials and Methods

2.2.1 Plant material

Black carrots were transferred from Eroğlu Konsantre (Ereğli-Konya) to the Istanbul Technical University, Food Engineering Department, and stored at −80 °C immediately until analysis. All samples were ground to fine powder using a precooled grinder (IKA A11 Basic, IKA-Werke GmbH & Co. KG, Germany).

2.2.2 Chemicals

For extract preparation and analyses of total monomeric anthocyanin, total phenolic content, anthocyanin composition and antioxidant activity, gallic acid (≥98 %), ethanol

(≥99.8 %), Folin-Ciocalteu phenol reagent, 1,1-diphenyl-2- picrylhydrazyl (DPPH), methanol (≥99.9 %), sodium bicarbonate ($NaHCO_3$), potassium chloride (KCl), sodium acetate trihydrate ($CH_3COONa.3H_2O$), potassium metabisulfite ($K_2S_2O_5$), copper (II) chloride ($CuCl_2$) and ammonium acetate (NH_4Ac) 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and neocuproine were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). For all analysis, water was distilled and purified with the water purfication system (TKA GenPure; TKA, Niederelbert, Germany).

2.2.3 Extraction Procedure

2 ± 0.01 g frozen black carrot powder were placed in a 50-ml centrifuge tube. Ethanol-water solutions acidified with citric acid at different pH values (2.5–6.5) are shown in Table 1. The extraction solvent was added at different ratios (see Table 2.2), and the tubes were placed in a thermostatic water bath (Memmert WNB14, Nürnberg, Germany) and agitated at maximum speed for 3 h. After this extraction step, tubes were placed in a cooled ultrasonic bath for 15 min (VWR Ultrasonic Bath, Germany). The treated samples were centrifuged for 10 min at 5000 rpm, and the supernatants were collected for further analysis.

2.2.4 Experimental design

Five extraction parameters (pH, time, temperature, solvent/ solid ratio, and ethanol/solvent ratio), which were expected to have an effect on the anthocyanin and phenolic content of black carrot, were identified by preliminary experiments. The statistically significant variables were chosen using 2-level fractional factorial design. According to 2-level and five variable concept, (25 = 32) runs were not feasible for the preliminary experiments. Thus, one-half fraction was considered, and variables were selected as pH (2.5–4.0), time (1–24 h), temperature (4–30 °C), solvent/solid ratio (5–25 ml/g black carrot), and ethanol/solvent ratio (0–0.75 ml/ml. The runs were randomized, and all analyses were performed using MINITAB (version 16.0, MINITAB Inc.) statistical software (See Table 2.1 for the design matrix).

Table 2.1 : Design matrix of 2^{n-1} fractional factorial design.

Std Order	Run Order	Center Pt	Blocks	pH	Time	Temperature	Solvent/ solid	Ethanol/Solvent
14	1	1	1	4	1	30	25	0
8	2	1	1	4	24	30	5	0
9	3	1	1	2.5	1	4	25	0
11	4	1	1	2.5	24	4	25	0.75
10	5	1	1	4	1	4	25	0.75
1	6	1	1	2.5	1	4	5	0.75
4	7	1	1	4	24	4	5	0.75
6	8	1	1	4	1	30	5	0.75
3	9	1	1	2.5	24	4	5	0
5	10	1	1	2.5	1	30	5	0
13	11	1	1	2.5	1	30	25	0.75
15	12	1	1	2.5	24	30	25	0
16	13	1	1	4	24	30	25	0.75
7	14	1	1	2.5	24	30	5	0.75
2	15	1	1	4	1	4	5	0
12	16	1	1	4	24	4	25	0

After fractional factorial experiments, insignificant variable (time) was removed from the extraction variables. In addition, low and high values of parameters were found to change according to the preliminary experiments, as pH 2.5–6.5, temperature 4–72°C, solvent/ solid ratio 5–25 ml/g black carrot, and ethanol/solvent ratio 0–100 (ml/ml). Central composite design generated runs with four factors including pH, temperature (°C), solvent-solid ratio (ml/g black carrot), ethanol concentration (% v/v) and their five levels ($\alpha = 2$) were applied as two replicates. The matrix incorporated 16 cube points, five center points in cube, and eight axial points (See Table 2.2).

Table 2.2 : Central composite design of 4 variables for the extraction of black carrot.

Run	pH	Temperature (°C)	Solvent/ Solid Ratio (mL/g)	Ethanol Conc. (%)	Run	pH	Temperature (°C)	Solvent/ Solid Ratio (mL/g)	Ethanol Conc. (%)
1	5.5	55	20	75	32	5.5	21	20	25
2	3.5	55	10	25	33	4.5	38	15	0
3	4.5	38	15	50	34	3.5	21	10	75
4	5.5	21	10	75	35	4.5	38	25	50
5	5.5	21	20	75	36	2.5	38	15	50
6	3.5	21	20	75	37	4.5	38	15	50
7	6.5	38	15	50	38	4.5	38	15	50
8	4.5	38	15	50	39	5.5	21	20	75
9	3.5	55	10	25	40	3.5	21	10	25
10	4.5	38	15	50	41	5.5	21	20	25
11	5.5	55	10	75	42	5.5	21	10	25
12	4.5	38	15	100	43	5.5	55	20	25
13	3.5	55	10	75	44	4.5	72	15	50
14	4.5	38	15	50	45	4.5	38	15	50
15	4.5	38	5	50	46	3.5	55	20	75
16	3.5	21	10	25	47	4.5	38	15	50
17	3.5	55	20	25	48	4.5	38	15	0
18	3.5	21	20	75	49	4.5	38	15	50
19	4.5	38	15	50	50	4.5	4	15	50
20	4.5	38	15	50	51	4.5	38	15	100
21	3.5	55	20	25	52	3.5	21	20	25
22	5.5	55	10	25	53	4.5	38	5	50
23	4.5	38	15	50	54	5.5	21	10	25
24	5.5	55	10	75	55	3.5	55	20	75
25	4.5	4	15	50	56	6.5	38	15	50
26	4.5	38	15	50	57	5.5	55	10	25
27	3.5	55	10	75	58	4.5	72	15	50
28	3.5	21	10	75	59	2.5	38	15	50
29	4.5	38	15	50	60	5.5	55	20	75
30	3.5	21	20	25	61	5.5	21	10	75
31	5.5	55	20	25	62	4.5	38	25	50

2.2.5 Analysis of Total Phenolics

The total phenolics were determined using Folin-Ciocalteu reagent using the method of Velioglu et al. (1998) with slight modifications. 0.75 mL of daily prepared Folin-Ciocalteu reagent (diluted 10 times with distilled water) was added to 100 µl of the extract. The mixture was allowed to stand for 5 min at room temperature, and 0.75-mL sodium bicarbonate (6 % w/w) was added to the mixture. After 90 min of incuba-

tion, absorbance was measured at 725 nm (Shimadzu UV-1700, Japan). Results were reported as gallic acid equiv- alents (GAE)/100 g fresh weight.

The total monomeric anthocyanin content was determined by pH differential method (Giusti and Wrolstad 2001). Absorbance of samples diluted with pH 1.0 and pH 4.5 buffers was measured at 520 and 700 nm. The results were expressed as cyanidin-3-glucoside equivalents per 100-g fresh weight by using the following formula (2.1):

$$\text{Total monomeric anthocyanin } \left(\frac{\text{mg}}{\text{L}}\right) = \frac{\text{A}\times\text{MW}\times\text{DF}\times 1000}{\varepsilon\times \text{l}} \qquad (2.1)$$

Where $A=(A_{520nm}-A_{700nm})_{pH1.0}-(A_{520nm}-A_{700nm})_{pH4.5}$, MW is the molecular weight of cyanidin-3-glcside (449.2 g/mol), DF is the dilution factor, 1000 is the conversion factor from g to mg, ε is the molar extinction coefficient of cyanidin-3-glucoside (26900L/(mol.cm)), and l is the path length (cm).

2.2.6 Total antioxidant capacity assays

Total antioxidant activities were estimated by two different methods. In all assays, trolox was used as a reference compound and the results were expressed in terms of μmol TEAC/100g fresh weight.

The 2,2-diphenylpicrylhydrazyl (DPPH) assay was performed as described by Kumaran and Joel karunakaran (2006). 100 μL of each sample extract was mixed with 2 mL of 0.1 mM DPPH in methanol. After 30 min of incubation at room temperature, the absorbance of the mixture was measured at 517 nm against methanol.

The Cupric ion reducing antioxidant capacity (CUPRAC) assay developed by Apak et al. (2004); Apak et al. (2006) was used in this study. To a test tube 100 μl of extract, 1 ml each of 10 mM $CuCl_2$, 7.5 mM neocuproine and 1 M NH_4Ac buffer (pH: 7) solutions were added. Immediately, 1 mL of distilled water was added to the initial mixture so as to make the final volume 4.1 mL. After 60 min of incubation at room temperature, absorbance was read at 450 nm against blank.

2.2.7 Anthocyanin composition by HPLC

Freeze-dried extracts were dissolved in 75% aqueous methanol with 0,1% (v/v) HCl and filtered through a 0,45 μm membrane filter and injected into Waters 2695 HPLC system with PDA (Waters 2996) detector. Stationary phase was Supercosil® (Sigma-Aldrich, St. Louis, MO, USA) LC-18 column (25 × 4,6 mm, 5 μm) and the mobile

phase consisted of solvent A (Milli-Q water with 0,1% (v/v) trifluoroacetic acid (TFA), and solvent B (acetonitrile with 0,1% (v/v) TFA). The flow rate was 1 ml/min. A linear gradient was used as 95% solvent A and 5% solvent B at 0 min; 65% solvent A and 35% solvent B at 45 min; 25% solvent A and 75% solvent B at 47 min; and at 54 min returned to the initial conditions. The anthocyanin detection was performed at 520 nm. Identification of anthocyanins was performed based on the retention times and characteristic UV spectra, and quantification was determined by external standard curves.

2.2.8 Polymeric Color

Color density, polymeric color and percent polymeric color were determined using the method described by Giusti and Wrolstad (2001). Sample extracts were diluted with potassium chloride buffer (pH 1.0) at 512 nm until the absorbance readings become in the linear range of the UV-Vis spectrophotometer (Shimadzu UV-1700, Japan). Samples were diluted with distilled water according to previously determined dilution factor. For analysis, 0.2 mL 0.90 M potassium metabisulfite was added to 2.8 mL diluted sample (bleached sample) and 0.2 mL of distilled water was added to 2.8 mL diluted sample (control sample). After equilibrating for 15 min, absorbance of samples were read at 700, 512 and 420 nm, against distilled water.

Color density was calculated using the control sample with the following formula (2.2);

$$\text{Color density} = [(A_{420nm} - A_{700nm}) + (A_{512nm} - A_{700nm})] \times DF \qquad (2.2)$$

Polymeric color was calculated (2.3) using the bisulfite bleached sample as follows;

$$\text{Polymeric color} = [(A_{420nm} - A_{700nm}) + (A_{512nm} - A_{700nm})] \times DF \qquad (2.3)$$

Where DF is the dilution factor of the sample.

The percent of polymeric color was calculated with the following formula (2.4);

$$\text{Percent polymeric color} = (\text{polymeric color}/\text{color density}) \times 100 \qquad (2.4)$$

2.2.9 Hunter Color Values

The color attributes (Hunter L*, a*, and b* values) was measured with a CR-400 handheld chroma meter (Minolta, Tokyo, Japan). Chroma (C*) = $[(a^*)^2 + (b^*)^2]^{1/2}$ and

hue angle (h°) = $\tan^{-1}$(b*/a*) was calculated (Ahmed et al., 2010; Ersus & Yurdagel, 2007).

2.3 Results

2.3.1 Preliminary experiments

(2^{n-1}) Fractional factorial design results showed that ethanol/solvent ratio and solvent/solid ratio are statistically important parameters according to the low and high levels of parameters used within the study. After preliminary experiments, time was removed since it was found to be statistically insignificant. In addition, the differences of low and high values of parameters were expanded to establish a better central composite design by RSM.

2.3.2 Central composite design results

In order to optimize black carrot extraction process, results of total phenolic content analysis, total monomeric anthocyanin content analysis, antioxidant activity analysis, color analyses and anthocyanin component analysis by HPLC were used as model responses. ANOVA for the regressions was performed and statistically significant ($p<0.05$) terms for each experiment were determined. Only significant terms were included within the optimization model. In the model X_1, X_2, X_3, X_4 represents pH, temperature (°C), solvent/solid ratio (ml/g, v/w), and ethanol concentration (v/v), respectively.

2.3.3 Total phenolic content

According to the results (Table A.1), all extraction parameters were statistically significant ($p<0.05$) and lack of fit was found insignificant (Table A.2). Furthermore, regression coefficients was found as follows; R^2 = 82.57%, R^2 (predicted) = 72.54%, R^2 (adjacent) = 76.89%. Gallic acid equivalent total phenolic content was higher at low pH (pH<4) and ethanol concentration 50% (v/v). In addition, increase in temperature raised by 29% total phenolic content in black carrot extracts (See Figure 2.1). The empirical relationship between total phenolic content and extraction parameters in uncoded units is given in the following equation (2.5);

$$Y= 97.72 - 20.27X_1 - 1.31X_2 - 3.14X_3 + 2.61X_4 - 0.57X_1X_1 + 0.07X_3X_3 - 0.02X_4X_4 + 0.75X_1X_3 + 0.10X_1X_4 - 0.02X_2X_3 \quad (2.5)$$

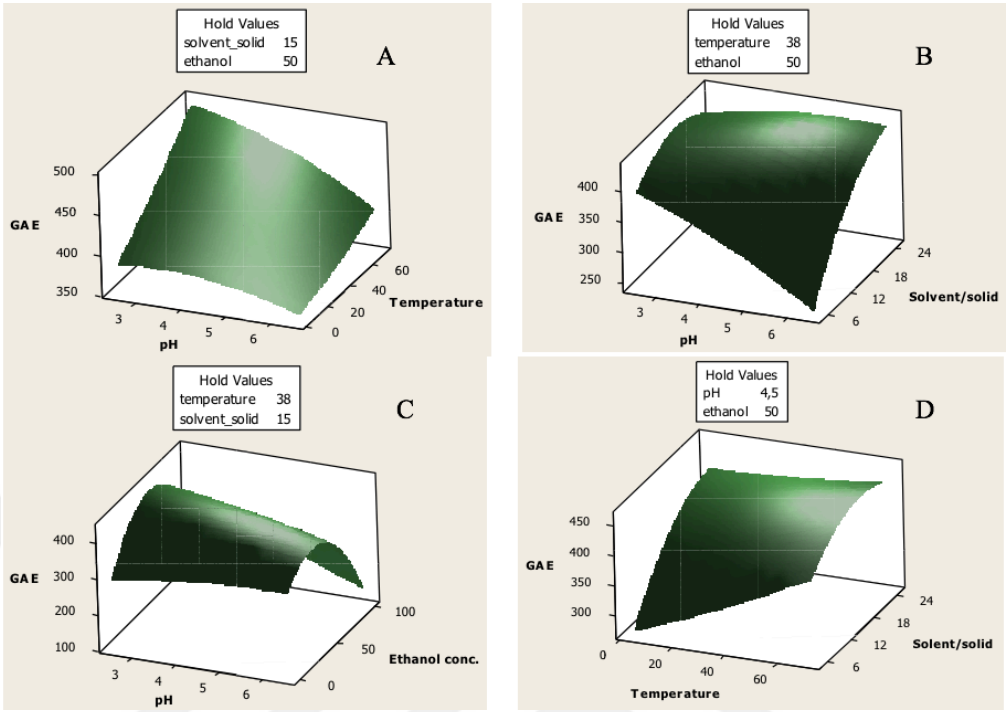


Figure 2.1 : Surface plots for total phenolic content of black carrot extracts. (A) Effect of pH and temperature on total phenolic content with solvent/solid ratio 15, ethanol concentration 50%; (B) Effect of pH and solvent/solid ratio on total phenolic content with temperature 38, ethanol concentration 50%; (C) Effect of pH and ethanol concentration on total phenolic content with temperature 38, solvent/solid ratio 15; (D) Effect of temperature and solvent/solid ratio on total phenolic content pH 4.5, ethanol concentration 50%.

2.3.4 Total monomeric anthocyanin content

Total monomeric anthocyanin content was calculated in terms of cyanidin-3-glycoside (Table A.1) . The result of fitting responses into the model, $R^2 = 93.13\%$, R^2(predicted) = 86.26%, R^2(adjacent) = 90.72% was found. All extraction parameters (Table A.3) were statistically significant ($p<0.05$) and lack of fit was found to be insignificant. Cyanidin-3-glycoside content was found directly proportional to the ethanol concentration, solid/solvent ratio and inversely proportional with pH (see Figure 2.2). Effect of temperature was determined as; low and high temperatures increase anthocyanin content in samples, whereas at ~30-35 °C, total monomeric anthocyanin content was at the lowest level. Model equation (2.6) for uncoded units is as follows;

$$Y= 39.90 - 1.18X_1 - 1.45X_2 - 1.42X_3 + 2.72X_4 - 1.87X_1X_1 + 0.03X_2X_2 - 0.02X_4X_4 + 0.11X_1X_4 - 0.01X_2X_4 - 0.03X_3X_4 \quad (2.6)$$

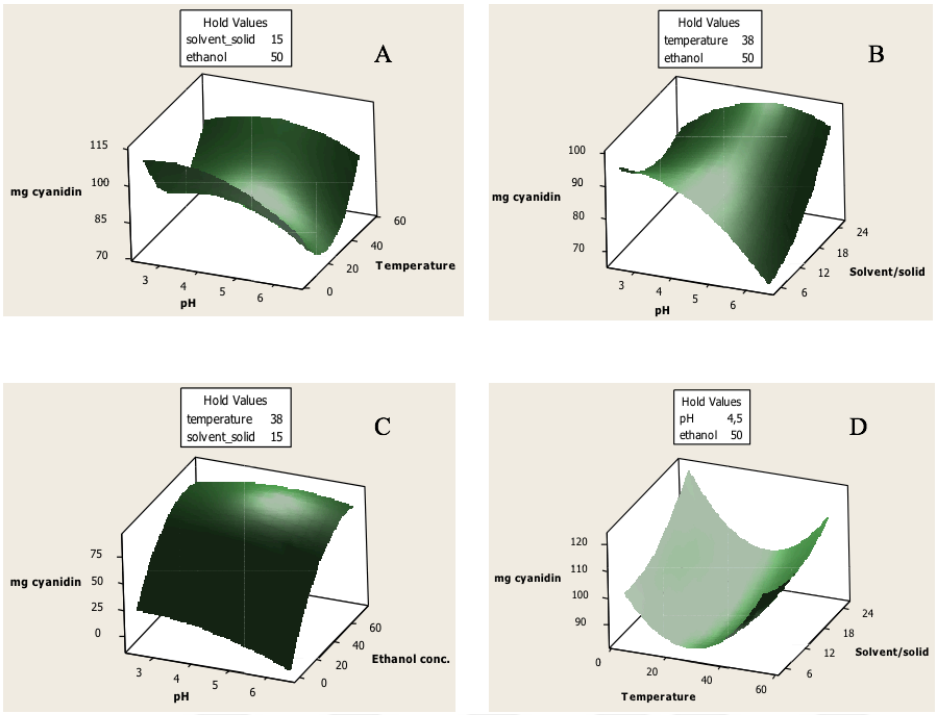


Figure 2.2 : Surface plots for total monomeric anthocyanins of black carrot extracts. (A) Effect of pH and temperature on total monomeric anthocyanins with solvent/solid ratio 15, ethanol concentration 50%; (B) Effect of pH and solvent/solid ratio on total monomeric anthocyanins with temperature 38, ethanol concentration 50%; (C) Effect of pH and ethanol concentration on total monomeric anthocyanins with temperature 38, solvent/solid ratio 15; (D) Effect of temperature and solvent/solid ratio on total monomeric anthocyanins pH 4.5, ethanol concentration 50%.

2.3.5 Total antioxidant capacity assays

The results of total antiocidant capacity assays are presented in Table A.1. Main effects of parameters were found to be similar in both antioxidant assays. According to the DPPH assay, antioxidant activity was at its highest level with an ethanol concentration around 50%, and increasing solvent/solid ratio and temperature (>40 °C) resulted with higher antioxidant activities (see Figure 2.3). In addition, in CUPRAC assay antioxidant activity was higher at low pH values (see Figure 2.4).

The empirical model (2.7) for DPPH analysis for uncoded parameter values is as follows;

$$Y=- 375.72 + 1.17X_2 + 36.66X_3 + 32.18X_4 - 43.31X_1X_1 + 0.29X_2X_2 - 0.35X_4X_4 \quad (2.7)$$

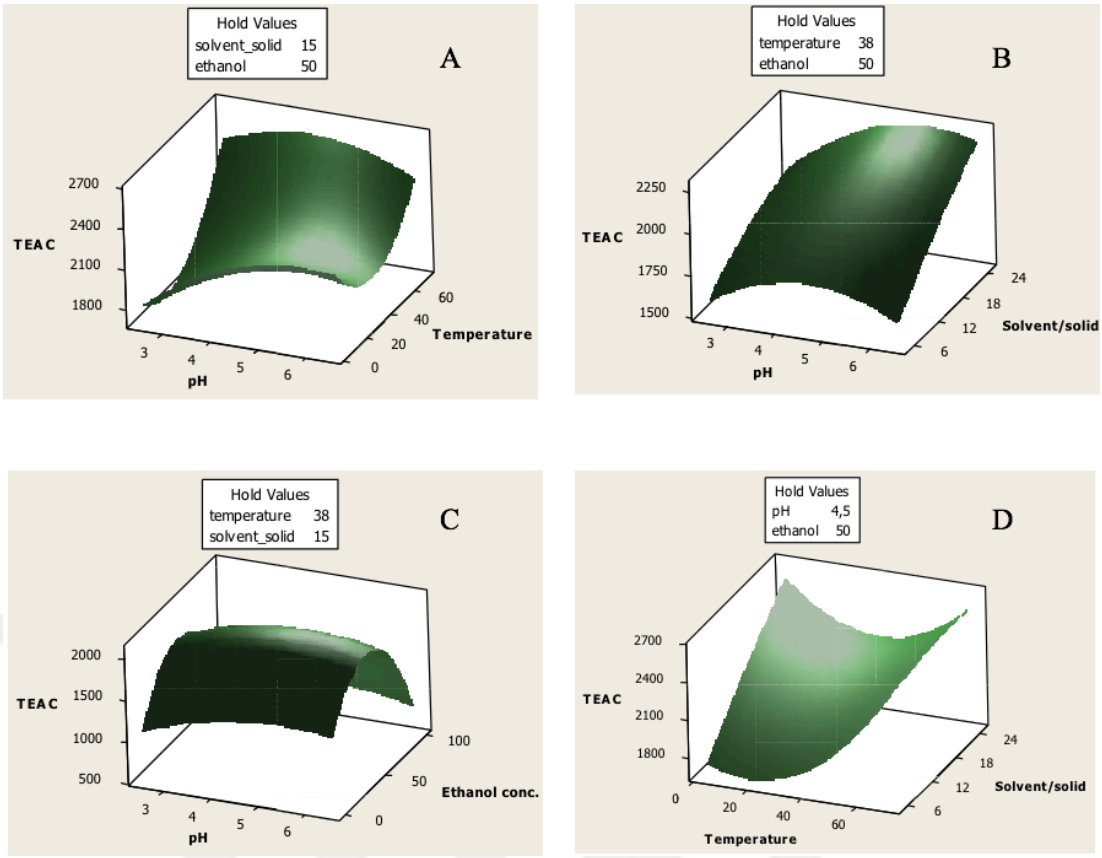


Figure 2.3 : Surface plots for AOX-DPPH of black carrot extracts. (A) Effect of pH and temperature on AOX-DPPH with solvent/solid ratio 15, ethanol concentration 50%; (B) Effect of pH and solvent/solid ratio AOX-DPPH with temperature 38, ethanol concentration 50%; (C) Effect of pH and ethanol concentration on AOX-DPPH with temperature 38, solvent/solid ratio 15; (D) Effect of temperature and solvent/solid ratio AOX-DPPH with pH 4.5, ethanol concentration 50%.

In DPPH assay**,** all extraction parameters except pH was statistically significant ($p<0.05$) and lack of fit was insignificant (Table A.4). According to the model, R^2 = 86.75%, R^2 (predicted) = 75.54%, and R^2 (adjacent) = 81.99% were found. However, in CUPRAC assay all parameters was found to be statistically significant ($p<0.05$) and lack of fit was insignificant (Table A.5). The coefficient of determination (R^2) was found as 90.68%, and R^2 (predicted) = 81.96%, R^2 (adjacent) = 86.31%.

RSM model equation (2.8) for CUPRAC analysis for uncoded extraction parameters can be seen as follows,

$$Y= 5933.87 - 864.14X_1 + 14.30X_2 + 103.96X_3 + 149.25X_4 + 0.35X_2X_2 - 4.47X_3X_3 - 0.84X_4X_4 - 15.20X_1X_4 \quad (2.8)$$

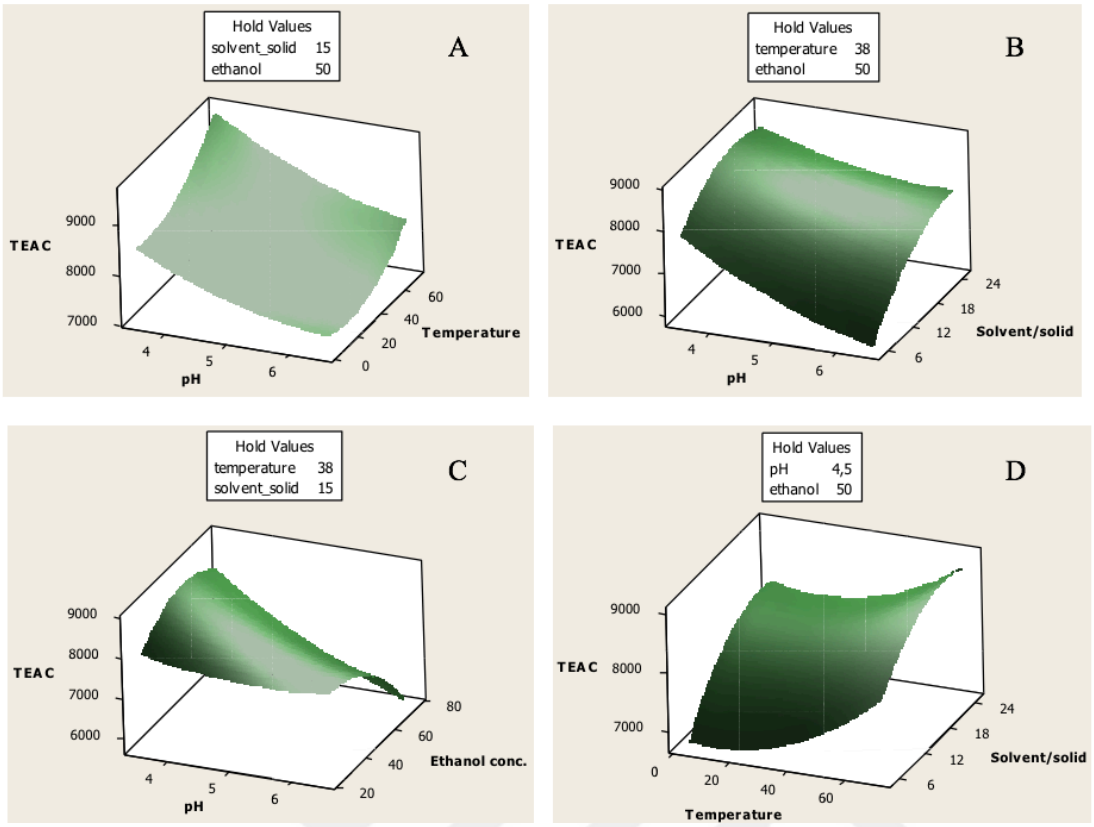


Figure 2.4 : Surface plots for AOX-CUPRAC of black carrot extracts. (A) Effect of pH and temperature on AOX- CUPRAC with solvent/solid ratio 15, ethanol concentration 50%; (B) Effect of pH and solvent/solid ratio AOX-CUPRAC with temperature 38, ethanol concentration 50%; (C) Effect of pH and ethanol concentration on AOX- CUPRAC with temperature 38, solvent/solid ratio 15; (D) Effect of temperature and solvent/solid ratio AOX- CUPRAC with pH 4.5, ethanol concentration 50%.

2.3.6 Anthocyanin composition with HPLC analysis

Anthocyanins were determined at 520 nm (Table A.6 and Figure A.1). According to model results, R^2 = 87.85%, R^2(predicted) = 75.31%, R^2(adjacent) = 82.69% was found. All parameters except solvent/solid ratio were found as statistically significant and lack of fit value of the model was insignificant (Table A.7).

The highest anthocyanin content was found at pH 3-4 and higher temperature and ethanol concentration increased anthocyanin content of extracts (Figure 2.5). The empirical relationship between total anthocyanin composition and extraction parameters in uncoded units is given in the following equation (2.9);

$$Y = -3.81 + 1.04X_1 - 0.03X_2 + 0.07X_4 - 0.13X_1X_1 - 0.004X_3X_3 - 0.003X_4X_4 + 0.001X_2X_3 - 0.002X_3X_4 \quad (2.9)$$

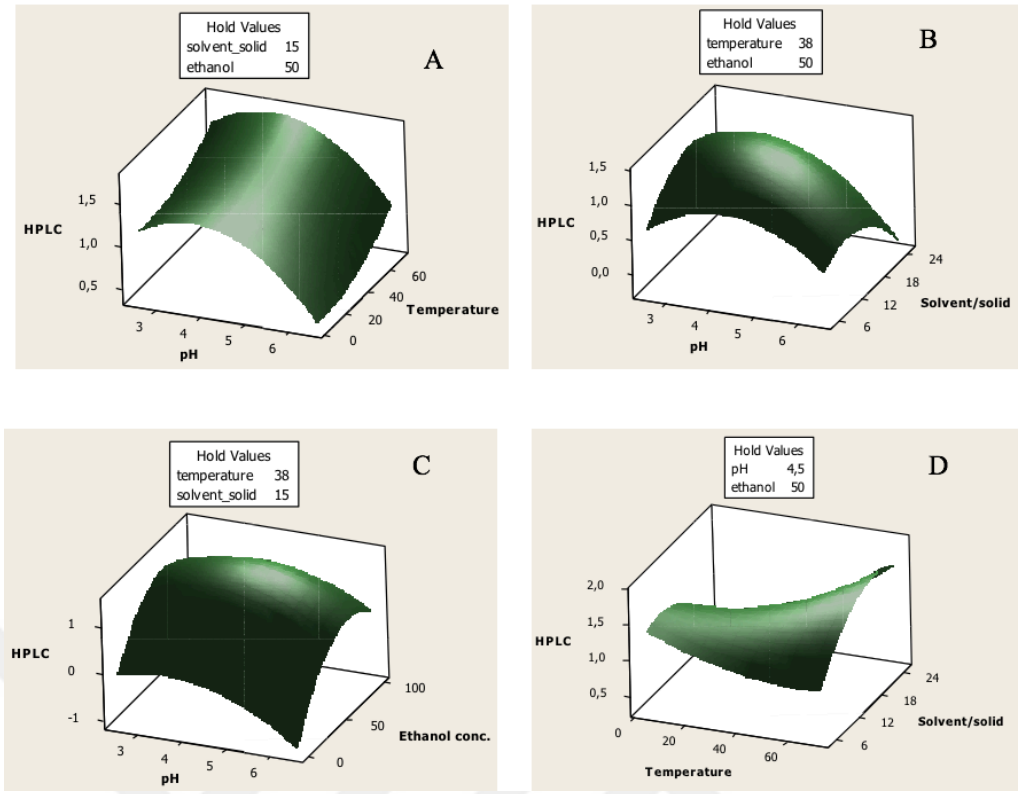


Figure 2.5 : Surface plots for anthocyanin composition with HPLC analysis of black carrot extracts. (A) Effect of pH and temperature on anthocyanin composition with solvent/solid ratio 15, ethanol concentration 50%; (B) Effect of pH and solvent/solid ratio on anthocyanin composition with temperature 38, ethanol concentration 50%; (C) Effect of pH and ethanol concentration on anthocyanin composition with temperature 38, solvent/solid ratio 15; (D) Effect of temperature and solvent/solid ratio on anthocyanin composition with pH 4.5, ethanol concentration 50%.

2.3.7 Color analysis

2.3.7.1 Polymeric color

The analysis results (see Appendix A, Table A.1) were found to fit to the model with the values R^2 = 97.29%, R^2(predicted) = 94.12%, R^2(adjacent) = 96.29%. According to the results, all extraction parameters were found to be significant ($p<0.05$) and lack of fit value was insignificant (Table A.8).

As it indicates loss of anthocyanins via polymerization, polymeric color is desired to be at its lowest value. The lowest value was obtained at low pH (pH<4), high ethanol concentration (>75%), low solvent/solid ratio (<15) and both low (<20°C) and moderate temperature values (40-55 °C) (see Figure 2.6). RSM model equation (2.10) is as follows;

$$Y= 16.78 + 19.11X_1 - 0.07X_2 + 0.93X_3 - 1.05X_4 - 1.36X_1X_1 - 0.01X_2X_2 \quad (2.10)$$
$$-0.04X_3X_3 + 0.003X_4X_4 + 0.01X_2X_4$$

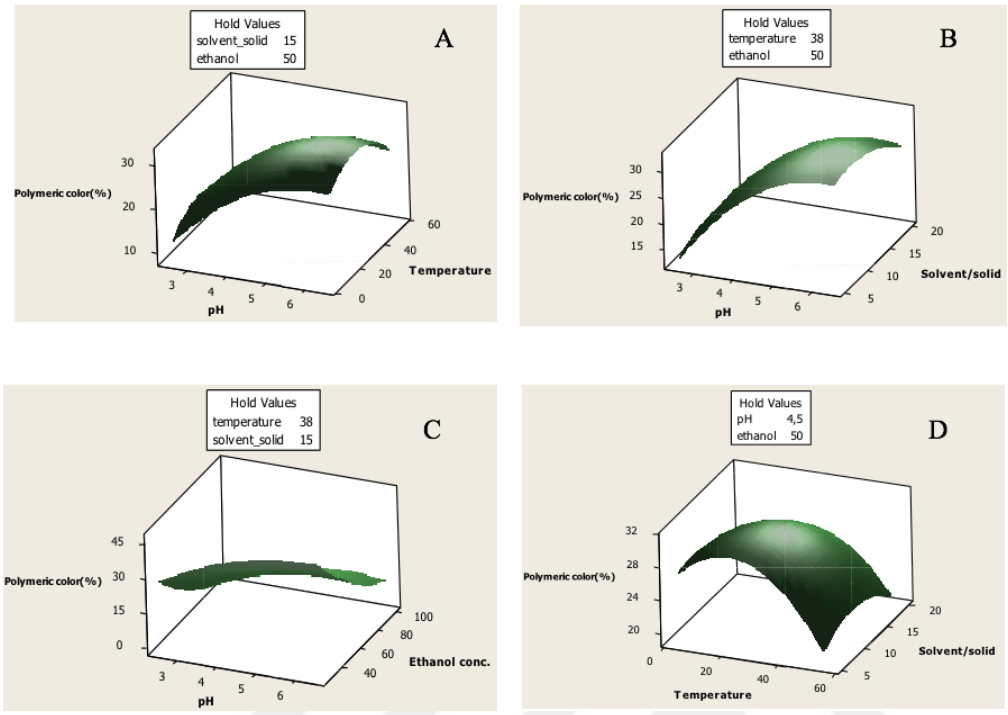


Figure 2.6 : Surface plots for polymeric color of black carrot extracts. (A) Effect of pH and temperature on polymeric color with solvent/solid ratio 15, ethanol concentration 50%; (B) Effect of pH and solvent/solid ratio on polymeric color with temperature 38, ethanol concentration 50%; (C) Effect of pH and ethanol concentration on polymeric color with temperature 38, solvent/solid ratio 15; (D) Effect of temperature and solvent/solid ratio on polymeric color with pH 4.5, ethanol concentration 50%.

2.3.7.2 Hunter Color Values

Hunter color parameters (L*, a*, b*) were identified with Minolta Chromameter-400 handheld colorimeter (see Appendix A, Table A.9). In addition Ponceau 4R was used as the reference color and L*(36.8 ± 0.03), a*(31.7 ± 0.10) and b*(16.3 ± 0.12) were obtained. The results for the reference material and the extracts were compared and the relationship between results and effects of extraction parameters could not be identified and fitted to the model.

2.3.8 Validation of the model

At the final step, for the evaluation of the optimum point, results of anthocyanin composition by HPLC and polymeric color were primarily selected due to the fact that these extracts will be used in color development. The points in which polymeric color

is at the lowest level, and the anthocyanin content at its highest level were used. As a result, optimum point for black carrot extraction was determined as pH 3.5, temperature 50 °C, solvent/solid ratio 10, ethanol concentration 75%. The comparison of experimental and theoretical results was found to be relatively close which indicated the high reliability of the model as reported in Table 2.3.

Table 2.3 : Comparison of theoratical and exprimental results of optimum extraction conditions.

Analyses	In Theory	Experimental Results
Total monomeric anthocyanin	97.7	85.7±1.9
Polymeric color	11.2	9.2±1.7
AOX-CUPRAC	8627.9	7276.3±210.2
AOX-DPPH	2021.6	1474.4±98.3
Total phenolic content	375.6	366.8±15.8
Anthocyanin composition-HPLC	1.6	1.1±0.2

2.4 Discussion

Negative health effects and legislative restrictions of synthetic additives promote use of their natural counterparts in food products (Cavalcanti et al., 2011; Herrero et al., 2006) which leads to scientific investigations on these natural components. Since, black carrot is a potential source of natural food color, it is important to find the parameters affecting the optimum color properties of black carrot extracts.

In previous studies, extraction optimization was performed with less parameters. Solvent/solid ratio (Cacace & Mazza, 2003b; Fan et al., 2008; Ku & Mun, 2008; Mané et al., 2007; Sun et al., 2007; Yang & Zhai, 2010; Zou et al., 2011), extraction time (Fan et al., 2008; Ghafoor et al., 2009; Ku & Mun, 2008; Mané et al., 2007; Sun et al., 2007; Yang & Zhai, 2010; Zou et al., 2011), temperature (Cacace & Mazza, 2003b; Fan et al., 2008; Ghafoor et al., 2009; Ku & Mun, 2008; Zou et al., 2011), and ethanol concentration (Cacace & Mazza, 2003b; Ghafoor et al., 2009) were selected as extraction parameters in different studies. In addition, Türker and Erdoğdu (2006) evaluated only the effect of temperature (25, 37.5 and 50 °C) and pH (2, 3, and 4) on the extraction efficiency of black carrot. Our research is a comprehensive study in which the effect of pH, temperature, solvent/solid ratio and ethanol: water ratio was evaluated simultaneously for black carrot anthocyanins.

Total anthocyanin content, phenolic content, and antioxidant activity results represented similar results (Table A.1). With higher temperatures and lower pH values, the amounts of bioactive compounds are observed to increase in the samples. In addition, ethanol concentration in the solvent played an important role. This result is in accordance with a previous report of Spigno et al. (2007) who proposed that increasing the water content in solvent from 40% to 60% reduced the yield of total phenolics by 28.7% in grape marc. Phenolic contents of berry samples were reported to be at its lowest values while extracting with 100% water at room temperature (Kähkönen et al., 2001). In addition, for maximum extraction of wheat bran phenolics, conditions were reported as ethanol concentration of 64% and extraction temperature of 60 °C (Wang et al., 2008). These reports support our results that ethanol concentration of 50% (v/v) and higher temperatures increases the total phenolic content of black carrot extracts.

In a previous study, stability of anthocyanins of black carrot was reported to be relevant with temperature, solid content, and pH (Kırca et al., 2007a). In addition, for the effect of temperature on the extraction yield of anthocyanins, it was observed that as the temperature increases from 30 °C to 100 °C, anthocyanin content increases by 30% (Cisse et al., 2012). Furthermore, temperature and pH of the extraction medium were found as effective parameters for anthocyanin extraction from black carrots and high temperatures and low pH values provided high extraction yields (Türker & Erdoğdu, 2006).

Antioxidant activity of black carrot was affected from ethanol concentration, temperature, solid/solvent ratio and pH. In this study, antioxidant activity was at the highest level at ethanol concentration around 50%, and higher solvent/solid ratio and temperature (>40 °C), lower pH values. Similarly, antioxidant capacity of red grape skin was found to increase during extraction with ethanol concentration of 50% and temperature of 70 °C (Corrales et al., 2009). On the other hand, Cacace and Mazza (2003b) reported that antioxidant index was mostly affected by solvent/solid ratio for black currant extracts.

In our study, anthocyanin composition of black carrot extracts was determined by HPLC and peaks were quantified as cyanidin derivatives (Table A.6 and Figure A.1). Major peaks were specified as cyanidin-3-xylosyl (glucosyl) galactoside, cyanidin-3-xylosyl galactoside, cyanidin-3-xylosyl (sinapoylglucosyl) galactoside, cyanidin-3-

xylosyl (feruloylglucosyl) galactoside, and cyanidin-3-xylosyl (coumaroylglucosyl) galactoside, respectively, which is in accordance with the previous reports (Kammerer et al., 2004b; Sadilova et al., 2006; Suzme et al., 2014). Similarly, Assous et al. (2014) reported that purple carrots contain 168.7 mg anthocyanin/100 g fresh weight and the major constituents was found as cyanidin-3-xylosyl-glucosyl-galactoside acylated with ferulic acid (33.65%) followed by cyanidin-3-xylosyl-glucosyl-galactoside acylated with coumaric acid (29.85%) and cyanidin-3-xylosyl-galactoside (28.70%) by HPLC.

Color of the extracts is related with total anthocyanin content as expected (Khandare et al., 2011). The percent polymeric color value for unstored fruit or vegetables is generally less than 10% (Türkyılmaz et al., 2012). For this reason, polymeric color may represent color quality of the extract which was aimed to be minimized within the model used in this study. Desired extract properties include higher color attributes. Lower pH and solid/solvent ratio, lower or moderate temperature, and higher ethanol concentration resulted with lower polymeric color. Thus, optimum conditions selected regarding all of these factors were pH 3.5, temperature 50°C, solvent/solid ratio 10, and ethanol concentration 75%.

2.5 Conclusions

During developing food colorant from black carrot, extraction is the important baseline that ensures higher total anthocyanin content, total phenolic content, antioxidant activity and color properties. In this study, after preliminary experiments pH, solid/solvent ratio, temperature, ethanol concentration was performed as extraction parameters, and RSM was used to evaluate the effects of these parameters. Except for color analysis, higher temperature, solid/solvent ratio and ethanol concentration were observed to increase the extraction yield. However, polymeric color results were found to have minimum values at lower pH and solid/solvent ratio, lower or moderate temperature, and higher ethanol concentration. Taking all these parameters into account, RSM results pointed out the optimum black carrot extraction conditions as pH 3.5, temperature 50 °C, solvent/solid ratio 10, and ethanol concentration 75% which leads to lower polymeric color, and higher anthocyanin content, antioxidant activity, and phenolic content.

3. IMPACT OF LIPOSOMAL ENCAPSULATION ON DEGRADATION OF ANTHOCYANINS OF BLACK CARROT EXTRACT BY ADDING ASCORBIC ACID

3.1 Introduction

In recent years, increasing tendency of customers to consume healthy food led food industry to seek natural colorants instead of stabile synthetic colorants. Anthocyanins, which are glycosides of anthocyanidins, are widely used as natural colorants in food industry due to their non-toxic, bright colored (orange, pink, red, violet, and blue) and water soluble characteristics (Castañeda-Ovando et al., 2009; Mortensen, 2006; Sari et al., 2012). The number of hydroxyl groups, the degree of methylation of these groups, the nature, number and position of the sugars bound to the anthocyanidin, and the physicochemical environment in which anthocyanins are present, and also the nature and number of aliphatic or aromatic acids attached to the sugar determine the color of anthocyanins (Mazza & Brouillard, 1990; Zozio et al., 2011).

Black carrots are rich source of anthocyanins mainly two non acylated cyanidin 3-xylosyl (glucosyl) galactoside and cyanidin 3-xylosylgalactoside and three acylated cyanidin 3-xylosyl (sinapoylglucosyl) galactoside, cyanidin 3-xylosyl (feruloylglucosyl) galactoside, and cyanidin 3-xylosyl (coumaroylglucosyl) galactoside (Gizir et al., 2008; Kammerer et al., 2004b; Montilla et al., 2011). In addition, amount of acylated anthocyanins was found mostly higher than 80% but ranged from 55-90% (Kammerer et al., 2004b). These high content of acylated anthocyanins may have slower degradation with light, pH, and temperature factors (Cevallos-Casals & Cisneros-Zevallos, 2004). On the other hand, there are some limitations on industrial usage of anthocyanins due to high raw material costs and changing stability according to pH, temperature, oxygen, light, their chemical structure, and presence of cofactors and/or ascorbic acid (AA) (Brenes, 2005).

Commonly fruit juices are enriched with ascorbic acid to prevent enzymatic browning reactions and enhance nutritional properties (Freedman & Francis, 1984; Pacheco-

palencia et al., 2007). Besides its antioxidant property, AA may reduce the stability of anthocyanins and increase the color and nutritional loss with mutual degradation of these compounds (Brenes, 2005; De Rosso & Mercadante, 2007; Poei-Langston & Wrolstad, 1981). In fruit juice systems, anthocyanin degradation due to the presence of AA should be prevented to popularize the usage of anthocyanins as natural colorants and prevent nutritional loss. In this concept, the stability of anthocyanin pigments was studied in the presence of AA considering copigmentation which improves color and stability of anthocyanins in low acidic environments (Brenes, 2005). Molecular association between pigments and other molecules resulted increased color properties expresses as copigmentation (Boulton, 2001). Relatively enhanced pigment stability were found through copigmented complexes formed between anthocyanin and biopolymers such as pectin and whey protein (Chung et al., 2015), and other phenolics as rutin (Hernandez-Herrero & Frutos, 2015), rosemary polyphenolics (Brenes, 2005), and quercetin (Shrikhande & Francis, 1974).

In addition to anthocyanin interactions with other compounds, encapsulation is also another fundamental stabilization mechanism for anthocyanins (Cavalcanti et al., 2011) due to its protection effect on anthocyanins against detrimental environment effects such as light, oxygen, humidity, and adverse effects from other compounds (Ferreira et al., 2009). In several studies, different encapsulation techniques including liophilization (Gradinaru et al., 2003), spray drying (Cai & Corke, 2000; Delgado-Vargas et al., 2000; Robert et al., 2010), thermal gelation (Xiong et al., 2006), and ionic gelation (Santos et al., 2013) provided positive effects on anthocyanin stabilization. In addition to these techniques, liposome entrapment can be used to encapsulate anthocyanins efficiently. Furthermore, both lipid soluble and water soluble bioactive compounds can be encapsulated in liposomes that enhances versatility of liposomal structure, which is composed of both lipid and phospholipids bilayers as closed systems (Mozafari et al., 2006). Previously, polyphenol- or anthocyanin-rich plant extracts from different sources including elderberry (Bryła et al., 2015), grape seed (Gibis et al., 2013; Gibis et al., 2014a; Gibis et al., 2012), hibiscus (Gibis et al., 2014b), black mulberry (Gültekin-Özgüven et al., 2016) were encapsulated in liposomes. Source of lecithin as a fundamental material for liposome is important for encapsulation stability and efficiency (McClements, 2015). Among egg yolk, soy bean and sunflower lecithins, liposomes produced from soy bean lecithin

were found more stable and uniform (Bryła et al., 2015). This study may prove the characteristics of anthocyanin encapsulated in liposomal system. On the other hand, there is a lack of knowledge about protection effect of liposomal encapsulation on anthocyanin degradation in the presence of AA.

The present study compares the stability of black carrot anthocyanins known as highly stable due to its high amount of acylated anthocyanins with and without liposome system in the presence of AA. We hypothesized that liposomal systems protects the extract against degradation effect of AA by creating a shell between extract and AA. Efficiency of encapsulation systems are important to present their possible effect. We therefore analysed the ascorbic acid effect on whole liposome system and gel filtered liposomes free from unencapsulated extract. Absorbance of samples was monitored over time to assess the pigment stabilizing effects of liposomes in the presence of AA.

3.2 Materials and Methods

3.2.1 Materials

Commercial black carrot extract was donated from Döhler GmbH (AG Darmstadt, Germany). The soy lecithin S75 (69.3% phosphatidylcholine, 9.8% phosphatidylethanolamine, and 2.1% lysophosphatidylcholine) was provided from Lipoid AG (Ludwigshafen, Germany).

L(+)-Ascorbic acid, sodium acetate, acetic acid glacial, acetonitril, citric acid monohydrate (purity ≥ 99.5%) were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Sodium citrate dihydrate (purity ≥99.0%) was purchased from SAFC (St. Louis, MO). Trifluoroacetic acid (302031-100 ml) and Sephadex G-50 (G50300-100g) were obtained from Sigma-Aldrich Co. (Steinheim, Germany).

3.2.2 Solution preparation

The black carrot extracts were dissolved in pH 3.5 acetate buffer (250 mM) as extract samples. Extract samples were prepared as three independent replicates for each 0.1%, 0.2%, and 0.4% w/w concentration, respectively. The lecithin solutions were prepared as dissolving lecithin at various concentrations (1%, 2%, and 4% w/w) in pH 3.5 acetate buffer (250 mM) and were stirred overnight to ensure dissolution and homogeneity. Lecithin solutions were prepared as three independent replicates for

each 1%, 2%, and 4% w/w concentration, respectively. Three replicates of each black carrot extracts (0.1%, 0.2%, and 0.4%) were dissolved in each lecithin solution samples (1%, 2%, and 4%) for extract encapsulated samples.

3.2.3 Preparation of liposomes

At pH 3.5 lecithin solutions were pre-homogenized with a high shear disperser (Heidolph Silent Crusher M, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 20000 rpm for 5 min. Thereafter, these pre-emulsions were homogenized with a high-pressure homogenizer (Microfluidizer Processor M-110EH, Microfluidiccs, Newton, MA, USA) to generate liposomes at 22500 psi, 5 times. During homogenization, homogenizer unit was cooled with ice to prevent heating of samples.

3.2.4 Liposome characterization

The particle diameter and ζ-potential were determined with a dynamic light scattering instrument (Nano ZS, Malvern Instruments, Worchestershire, U.K.). The refractive index for lecithin and aqueous part were set respectively at 1.44 and 1.33. The liposomal solutions were diluted to concentrations of 0.1% (w/w) lecithin before the measurement to avoid multiple scattering effects. The average and standard deviation of measurements were calculated from three freshly prepared samples with three measurements per sample.

3.2.5 Sephadex® gel filtration

Sephadex® gel were formed with the same method described previously by Gibis et al. (2014a). Liposome samples were passed through a cartridge (5 ml volume) of sephadex gel (fill height 3 cm). After centrifugation, gel filtered samples were collected for further analysis.

3.2.6 Encapsulation efficiency of liposomes

Absorbances of gel filtered and unfiltered liposomes were measured at 520 nm with UV/VIS-spectrophotometer (Perkin Elmer, LAMBDA 750 UV/Vis/NIR Spectrophotometer, MA, USA) according to modified method of Lohachoompol et al. against a reagent blank (Lohachoompol et al., 2004). Freshly prepared three samples for each liposomes including extracts (0.1%, 0.2%, and 0.4% w/w) were used for

encapsulation efficiency analyses of liposomes. Before analyses, gel filtered and unfiltered liposomes including extracts (0.1%, 0.2%, and 0.4%w/w) were diluted with ethanol (1:2 v/v) to settle down lecithin after centrifugation of diluted liposomes at 21380xg for 10 min (2 times). For calibration curve black carrot extract were diluted (0.01- 0.5% w/w). The ratio between of anthocyanin content before and after gel filtration as percent was determined as encapsulation efficiency (3.1) of liposomes. The concentration of free extract ($c_{free\ extract}$) is the difference between the total extract ($c_{total\ extract}$) and the gelfiltrated liposomal sample ($c_{gelfiltrated}$).

$$EE\ (\%) = \frac{C_{\ gelfiltrated}}{C_{total\ extract}} \cdot 100 = \frac{(1 - c_{free\ extract})}{c_{total\ extract}} \cdot 100 \qquad (3.1)$$

3.2.7 Degradation study

Preliminary studies were performed to determine the conditions for degradation study. Black carrot extracts were dissolved in acetate buffer at pH 3.0 (100 mM). Absorbance change of extracts mixed with 0.01%, 0.025%, 0.05%, and 0.1% ascorbic acid stored in both dark and light environments were measured during 24 hours using UV/VIS spectroscopy. Light conditions did not affect the results.

Ascorbic acid in various concentrations (0.01%, 0.025%, 0.05%, 0.1% w/w) were mixed with both 0.1% extract and 1%, 2%, 4% liposomal solutions including 0.1% extract. Three different samples were prepared with each ascorbic acid concentrations for extract and liposomal solutions and stored at room temperature in dark. Samples were taken during 24-hour storage and immediately analyzed with UV/VIS-spectroscopy.

Gel filtration were performed to liposome samples (4% lecithin & 0.2% extract w/w) as explained before to comprehend efficiency of liposome encapsulation method on protection of anthocyanins from ascorbic acid (0.1%) related to degradation. Flowchart of preparation gel-filtered liposome, diluted liposome solution, and diluted extract samples is presented in Figure 3.1. Anthocyanin amount of both extract and liposome solutions was tried setting equally at the beginning. For this reason, extract solution and unfiltered liposome samples were also diluted according to their initial absorbance. Each treatment was replicated three times.

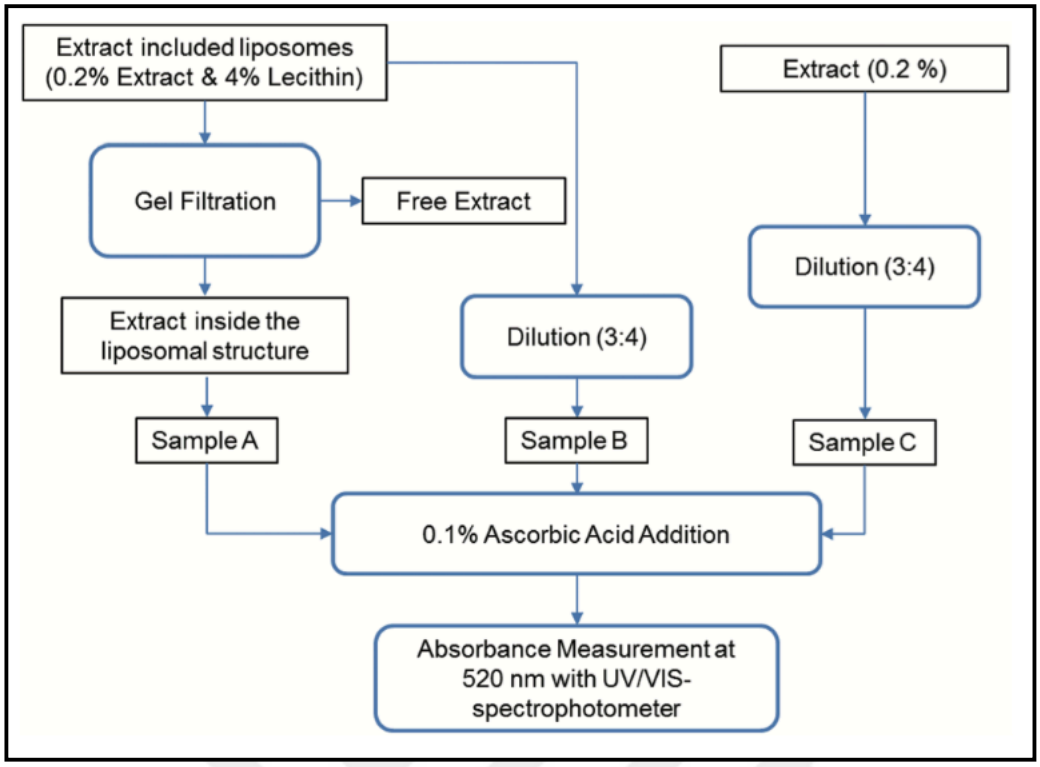


Figure 3.1 : Flowchart of the preparation three different samples (A) gel- filtered liposome (4% lecithin, 0.15% extract); (B) diluted liposome solution (3% lecithin, 0.15% extract); (C) diluted extract solution (0.15%).

3.2.8 Measurement of degradation

The absorbance of all samples were measured three times from 380 to 700 nm with UV/VIS-spectrophotometer (Perkin Elmer, LAMBDA 750 UV/Vis/NIR spectrophotometer, MA, USA). The degradation data were taken at 520 nm (maximum absorbance in the visible range) and average and standard deviation of samples were calculated for graphical presentations.

3.2.9 Data analysis

All experiments were performed at least 3 times for each triplicate samples. Average and standard deviation of all data were calculated using Microsoft Excel for Mac (version 15). Statistical analysis was carried out using SPSS software (version 21.0; SPSS, Chicago, IL, USA). Data of liposome characterization were compared using one-way analysis of variance (ANOVA) (Appendix B) followed by the Duncan *post hoc* test ($P<0.05$).

3.3 Results and Discussion

3.3.1 Absorbance change in black carrot extracts

The anthocyanin degradation of black carrot extracts (E) in 0.1% extract with various AA concentrations (0.01%, 0.025%, 0.05%, 0.1% w/w) were observed during 24 hours. The changes in absorbance of samples were presented in Figure 3.2 and Table C.1. As seen in Figure 3.2, for 0.1% extract solutions without AA are stable during the measurements. However, increasing concentrations of AA increased the anthocyanin degradation during time. The results are in accordance with previous study that found increasing rate of anthocyanin degradation while ascorbic acid content increased from 60 mg/L to 80 mg/L in sour cherry juices (Özkan, 2002). On the other hand, it was reported that degradation of acai anthocyanin did not depend on AA concentration (De Rosso & Mercadante, 2007).

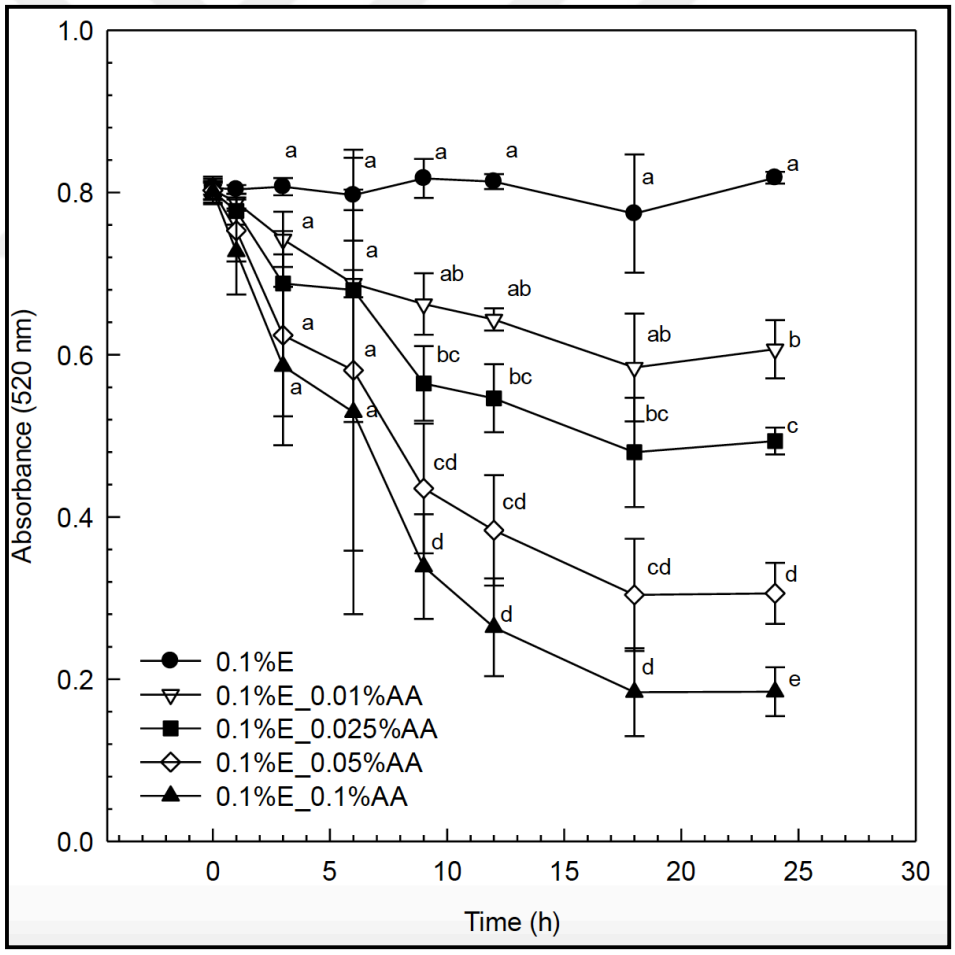


Figure 3.2 : The absorbance change of black carrot extract solutions (E) in the presence of different concentrations of AA during 24 hours.

According to previous theories the anthocyanin degradation due to ascorbic acid may be depend on hydrogen peroxide known as autoxidation product of ascorbic acid (Calcutt, 1951; Poei-Langston & Wrolstad, 1981) caused oxidation of flavylium salts to colorless reaction products (Jurd, 1966; Simpson, 1985). Nevertheless, it was reported by Garcia-Viguera (1999) that anthocyanin degradation may be caused by free radical mechanism (Iacobucci & Sweeny, 1983) unlikely previously stated as condensation reaction at position 4 of flavylium salt (Jurd, 1966) regarding unpreventable degradation of flavylium salts when position 4 is substituted. On the other hand, it is shown that instead of ascorbic acid itself, its degradation products are caused anthocyanin degradation such as dehydroascorbic acid, furfurals, and H_2O_2 (Özkan, 2002).

3.3.2 Encapsulation Effect of on anthocyanin degradation

3.3.2.1 Liposome characterization

Liposomes including black carrot extracts (0.1%, 0.2%, and 0.4% w/w) and free liposomes (without extract) were produced using microfluidization of soy lecithin at different concentrations (1%, 2%, and 4% w/w) in acetate buffer (250 mM, pH 3.5) at 22500 psi. The influence of lecithin and extract concentration on z-average particle diameter and zeta potential of the samples were presented in Table 3.1.

At the same extract level, altering lecithin concentration did not change the particle size significantly ($p<0.05$). In addition, free liposomes showed slightly lower particle size (41-43 nm) than liposomes containing black carrot extract (44-46 nm). Similarly, z-particle diameter of liposomes containing hibiscus extract or free liposomes were found smaller than 50 nm (Gibis et al., 2014b). The high pressure homogenization could be the reason of small particle diameter (Gibis et al., 2012). On the other hand, polydispersity indexes (PDI) of all the samples (~0.3) were not found to be different statistically ($p<0.05$). As shown before liposomes with PDI values close to 0.3 specified narrow size distribution (Yan et al., 2013). Furthermore, in previous studies, a reliable difference of the particle diameters between liposomes were reported for grape seed extract (87-120 nm) and free liposomes (40-50 nm) (Gibis et al., 2016; Gibis et al., 2012).

Table 3.1 : Particle diameter (z-average) and ζ-potential of the liposomal solutions.

Liposome				
Extract. (%)	Lecithin (%)	Particle size (nm)	PdI	ζ- Potential (mV)
0	1	41.2 ± 1.1^{c}	0.276 ± 0.020^{a}	-20.0 ± 1.1^{ab}
	2	42.0 ± 1.5^{bc}	0.276 ± 0.015^{a}	-20.3 ± 1.4^{ab}
	4	42.7 ± 0.6^{bc}	0.274 ± 0.007^{a}	-23.2 ± 2.3^{c}
0.1	1	44.2 ± 1.7^{ab}	0.338 ± 0.048^{a}	-21.9 ± 2.6^{bc}
	2	44.2 ± 0.6^{ab}	0.301 ± 0.024^{a}	-20.8 ± 1.6^{abc}
	4	44.5 ± 0.9^{a}	0.329 ± 0.033^{a}	-21.7 ± 0.8^{bc}
0.2	1	45.8 ± 2.0^{a}	0.338 ± 0.045^{a}	-20.7 ± 1.7^{abc}
	2	45.3 ± 1.7^{a}	0.330 ± 0.043^{a}	-21.7 ± 1.4^{bc}
	4	45.4 ± 0.7^{a}	0.303 ± 0.022^{a}	-21.7 ± 0.8^{bc}
0.4	1	43.7 ± 1.2^{ab}	0.342 ± 0.037^{a}	-20.9 ± 1.1^{abc}
	2	45.0 ± 1.5^{a}	0.321 ± 0.042^{a}	-18.9 ± 1.3^{a}
	4	45.2 ± 1.2^{a}	0.297± 0.027^{a}	-19.9 ± 1.0^{ab}

The data represent the average value ± standard deviation of three replicates from each sample. The different letters in the columns represent statistically significant differences ($p < 0.05$).

The ζ-potential of free liposomes only with 4% lecithin were measured as significantly lower (-23 mV) than all samples. It was found that a slight increase in ζ-potential of liposomes with highest lecithin (4%) and extract (0.4%) concentrations than liposomes with the same lecithin concentration. However, increasing extract concentrations had no appreciable influence on the ζ potential when considered all samples. Similarly, negative ζ-potential of gum arabic was not influenced from anthocyanin content as

explained by possible anthocyanin position would be in the core of complex (Guan & Zhong, 2015).

3.3.2.2 Encapsulation efficiency (EE) of liposomes

The encapsulation efficiency (EE) of black carrot extract including liposomes was measured by UV/Vis spectroscopy. Liposome solutions before and after sephadex gel filtration was analyzed after breaking down liposomal structure with ethanol similar to a recent study (Liu et al., 2008). Lecithin was removed from the solutions using centrifugation before the analysis. The effect of extract and lecithin concentration on EE of samples was presented in Table 3.2.

Table 3.2 : Encapsulation efficiency of the black carrot extract in liposomes with different lecithin concentrations (%).

Liposome		Encapsulation Efficiency (%)
Extract Conc. (%)	Lecithin Conc. (%)	
0.1	1	47 ±1.3 ^{ab}
	2	41 ± 6.4 ^{bc}
	4	39 ± 8.8 ^{cd}
0.2	1	32 ± 1.2 ^{d}
	2	50 ± 0.9 ^{a}
	4	48 ± 1.1 ^{ab}
0.4	1	32.9 ± 1.3 ^{d}
	2	39.0 ± 3.6 ^{cd}
	4	47.7 ± 0.5 ^{ab}

The data represent the average values ± standard deviation of three replicates from each sample. The different letters in the column represent statistically significant differences ($p < 0.05$).

According to results from UV/VIS spectroscopy, EE of liposomes were found between 32% and 50%. In addition, at the same extract concentration, increasing lecithin concentration enhanced EE of liposomes. As expected, increasing extract concentration lowered EE of liposomes with same lecithin concentration. In previous studies, EE of liposomes produced with soy lecithin was reported in wide range according to source of polyphenol-rich or anthocyanin-rich extracts as elderberry (25%) (Bryła et al., 2015), grape seed (88%) (Gibis et al., 2016), hibiscus (61-72%)

(Gibis et al., 2014b). It is depicted that complete liposome encapsulation may be obtained when encapsulated compound is soluble in the membrane (Bryła et al., 2015).

3.3.2.3 Effect of liposome formation on anthocyanin pigment degradation

Previous studies indicated that copigmentation of anthocyanins with biopolymers (Chung et al., 2015) and phenolic compounds (Brenes, 2005; Hernandez-Herrero & Frutos, 2015; Shrikhande & Francis, 1974) may enhance anthocyanin stability in solution systems including ascorbic acid. In this study, we examined the effect of liposome encapsulation method on anthocyanin and color stability in the presence of ascorbic acid. The absorbance change of liposomes including extract (0.1%) and lecithin (1%, 2%, and 4%) with various AA concentrations (0.01%, 0.025%, 0.05%, 0.1% w/w) were measured during 24 hours. For liposome samples, absorbance of free liposomes was subtracted from liposomes contained black carrot extract to prevent absorbance change due to lecithin concentration. In Figure 3.3, visual observations of 0.1% extract and 0.1% extract with 0.1% ascorbic acid during 24 hours were presented. Color loss due to ascorbic acid related degradation can be detected visually.

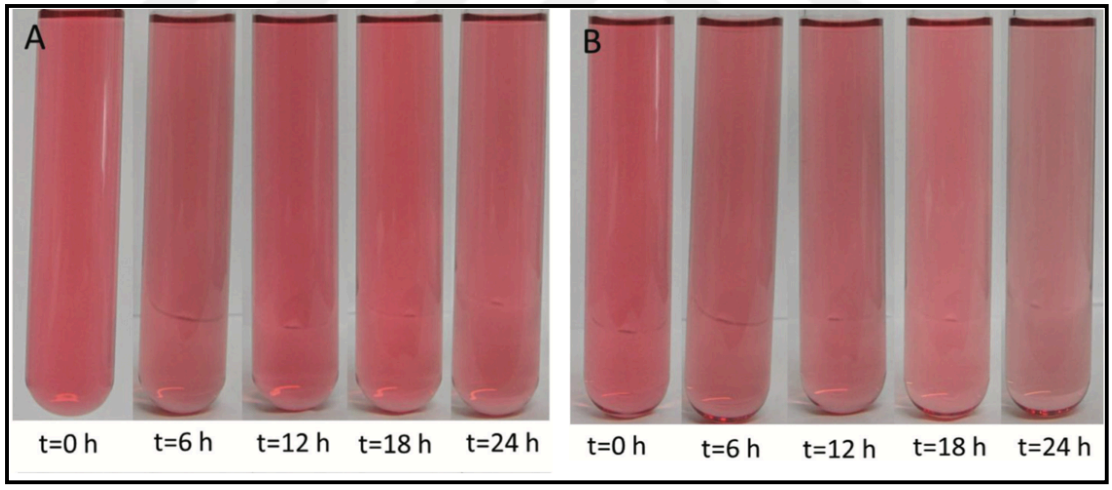


Figure 3.3 : (A) Visual observations of the extract (E – 0.1%), and (B) color fading after ascorbic acid (0.1%) addition to the extract (0.1%) during 24 hours.

Degradation effect of ascorbic acid on 0.1% black carrot extract encapsulated liposome samples with various lecithin contents were presented in Figure 3.4 and Table C.2. As seen in the Figure 3.4, change in absorbance values reduced when lecithin concentration increased in liposome samples. Although, the dose effect of ascorbic acid can be detected in both extract and liposomal solutions, degradation rate of anthocyanin pigments slowed down at higher lecithin concentrations.

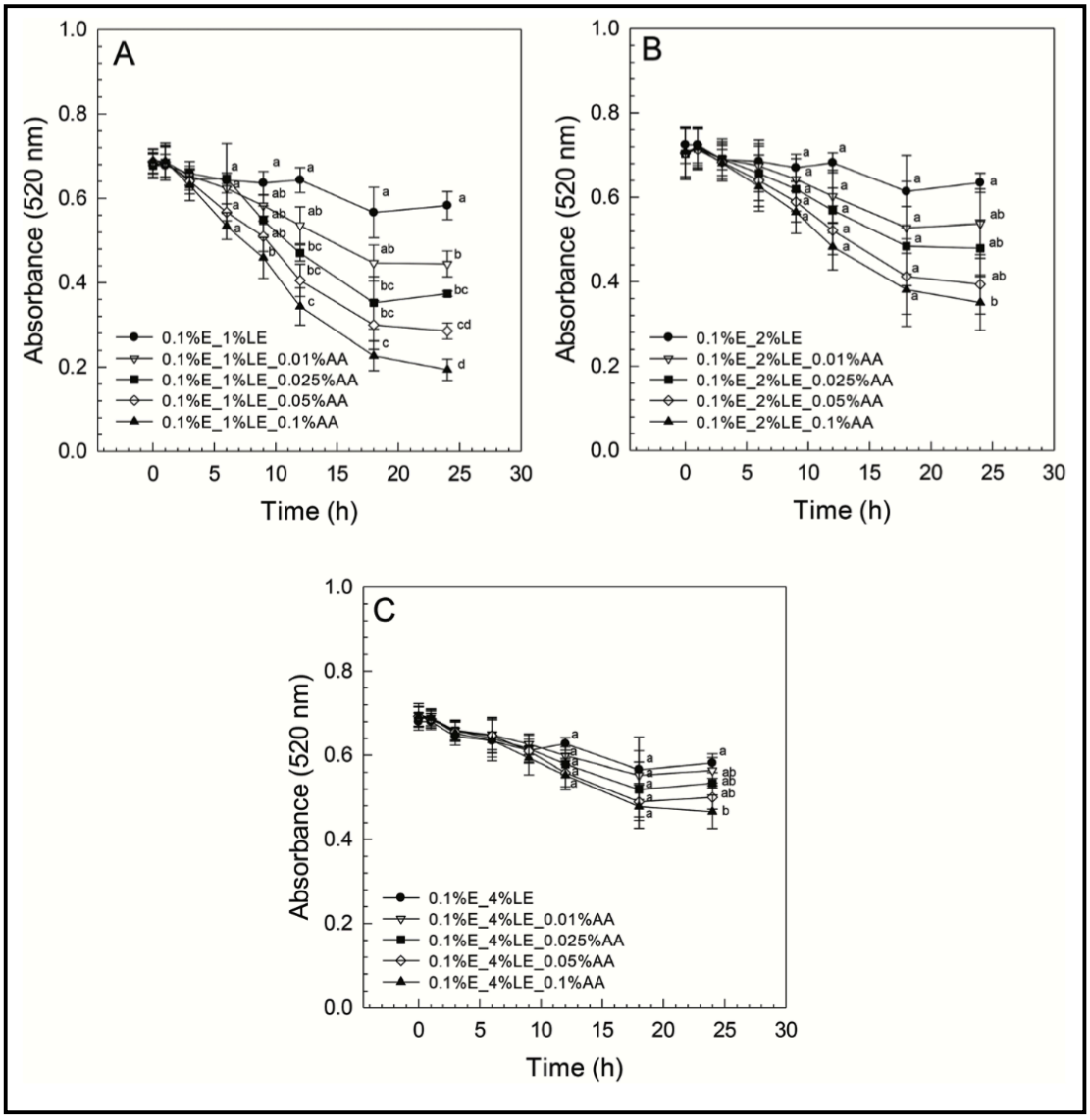


Figure 3.4 : Effects of ascorbic acid on the change in absorbance of 0.1% extract (E) encapsulated in liposomes with (A) 1% lecithin (LE), (B) 2% lecithin, and (C) 4% lecithin during 24 hours. The data represent the average values ± standard deviation of three replicates from each sample. The different letters represent statistically significant differences between treatments at each time ($p < 0.001$).

Collaterally, EE of these liposome samples increased with increasing lecithin concentrations (Table 3.2). This might be the explanation for increasing protection effect of liposomal system on anthocyanin degradation. Visual observations of 0.1% extract encapsulated liposomes including 1% lecithin and 4% lecithin with 0.1% ascorbic acid during 24 hours (see Figure 3.5) supported protection effect of liposomal system on anthocyanin color.

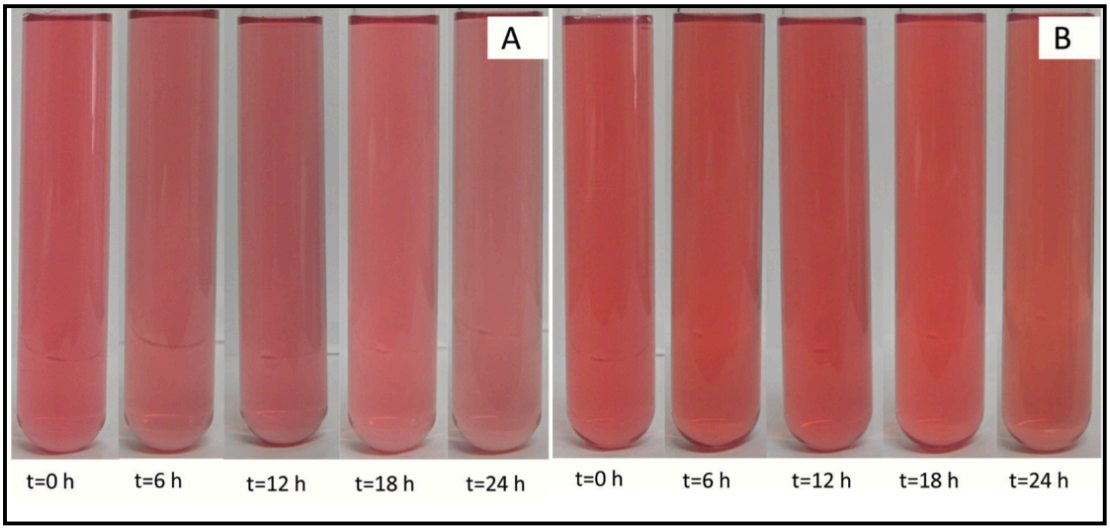


Figure 3.5 : Visual observations of color fading after ascorbic acid addition to the 0.1% extract entrapped in liposomal solutions with different lecithin concentrations (A) 1% lecithin, (B) 4% lecithin.

Proposed protection effect of liposomes were illustrated in Figure 3.6. We hypothesized that liposome formation prevents the ascorbic acid related degradation reaction of anthocyanins.

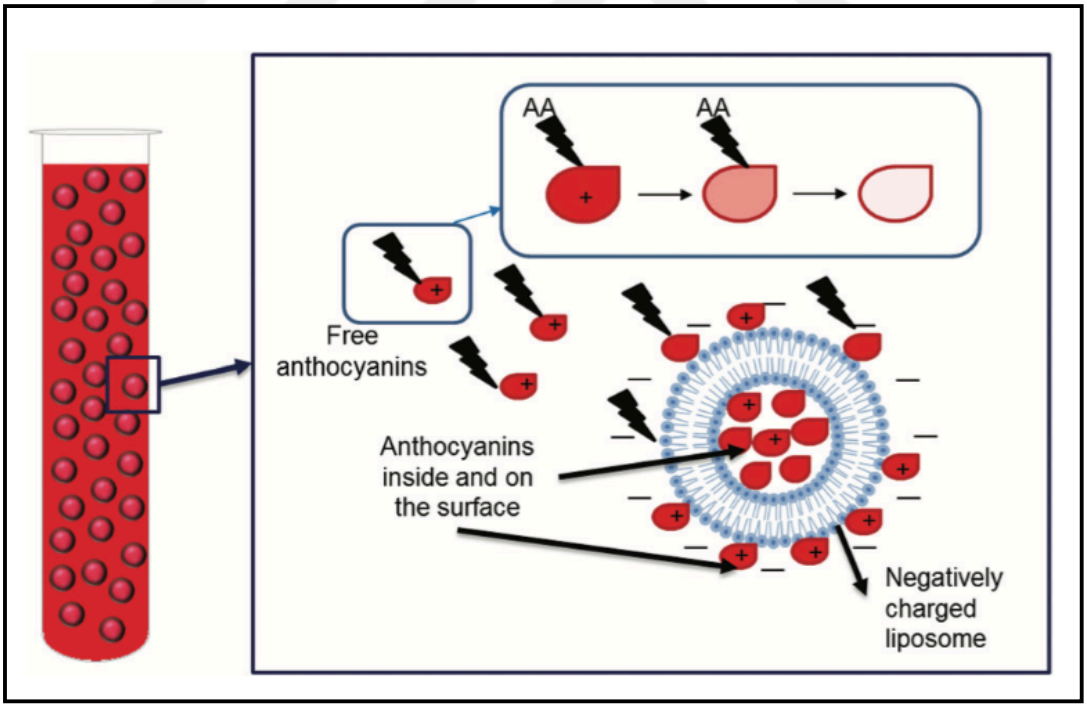


Figure 3.6 : Graphical illustration of the protection effect of liposomes on anthocyanin degradation in the presence of ascorbic acid (AA).

3.3.2.4 Structural protection effect of liposomal systems

Gel filtered liposome sample were mixed with 0.1% ascorbic acid to figure out the efficiency of liposome encapsulation method on protection of anthocyanins from ascorbic acid related degradation. Thus, anthocyanin amount of both extract and liposome solutions was set equally at the beginning. In Figure 3.6, flowchart of preparation of these samples was presented. The degradation effect of ascorbic acid (0.1% w/w) on the absorbance of extract was obvious comparing with gel-filtered liposomes (Figure 3.7 and Table C.3).

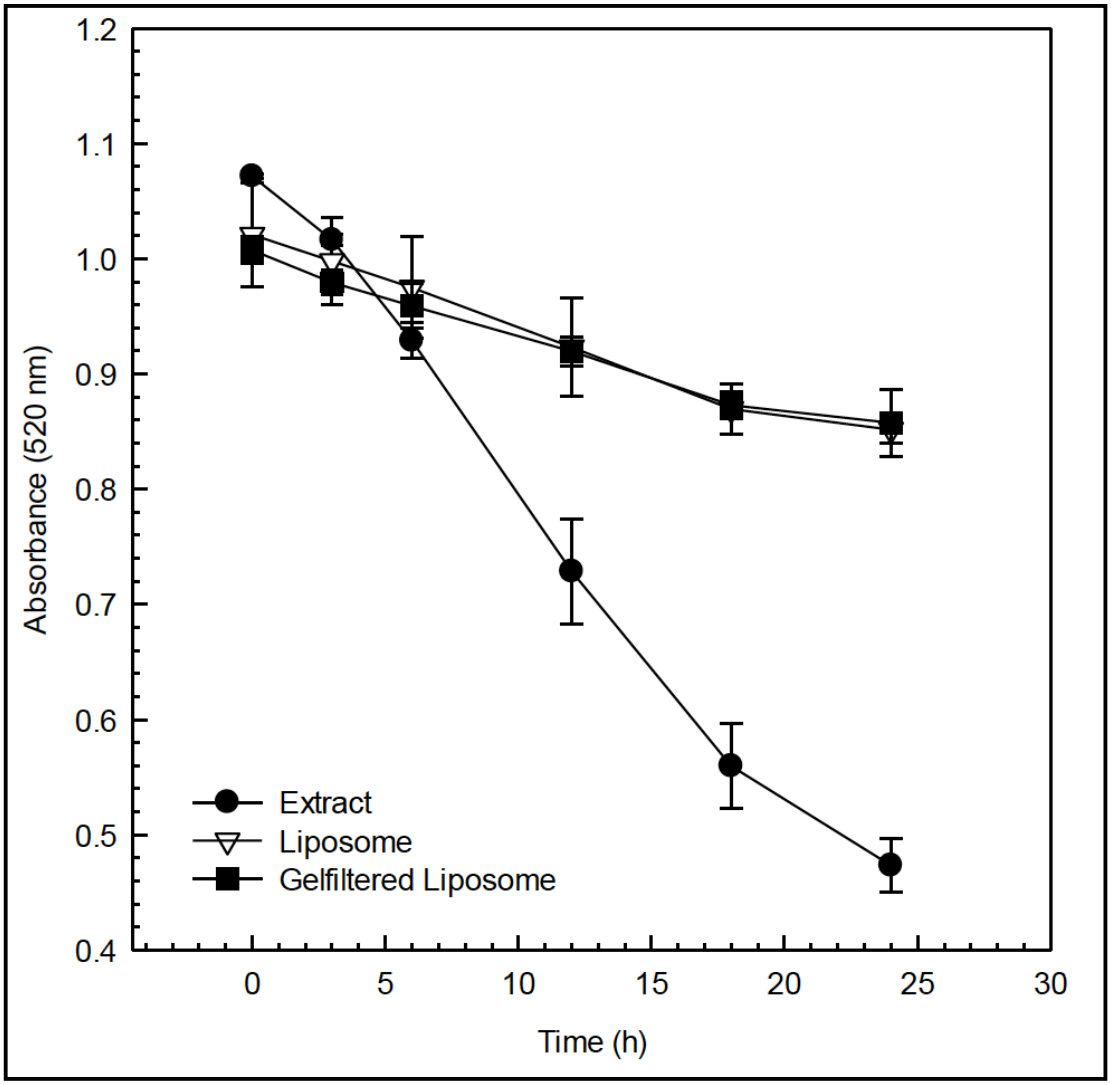


Figure 3.7 : Effects of 0.1% ascorbic acid on the absorbance change in the extract (0.15%), liposomes (0.15% extract, 3% lecithin) and gel-filtered liposomes (0.15% extract, 4% lecithin). The data represent the average values ± standard deviation of three replicates from each sample.

It is clear that gel filtered and unfiltered liposome system prevented absorbance change resulting color fading of anthocyanin extracts. In Figure 3.8, both color protected liposomal solutions and color fading of extract solution can be detected visually. These results strengthened previously found protection effect of liposomal encapsulation system on anthocyanin degradation.

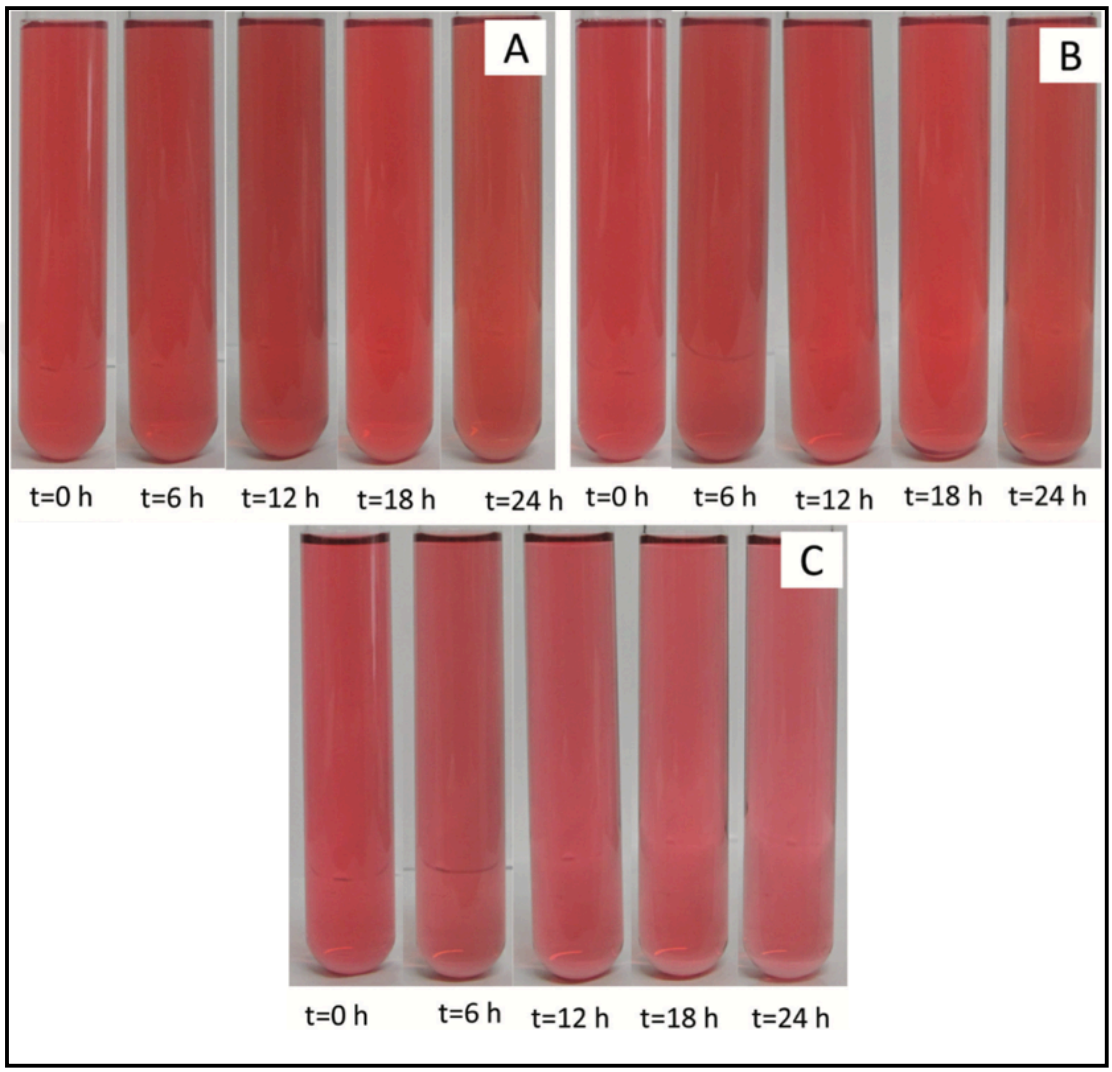


Figure 3.8 : Visual observations of color fading after ascorbic acid (0.1%) addition to (A) gel-filtered liposome (4% lecithin, 0.15% extract), (B) diluted liposome (3% lecithin, 0.15% extract), (C) extract (0.15%) solution during 24 hours.

3.4 Conclusions

Ascorbic acid can be found in food and beverage system naturally or added due to its preservation and nutritious effects. Many studies were reported to inhibit its degradation effect on anthocyanins. In this study, the effect of liposomal encapsulation on anthocyanin degradation and color stability study were demonstrated in the presence of ascorbic acid in accelerated storage conditions. Ascorbic acid related degradation of anthocyanins can be decreased while extract entrapped in liposomes. This study demonstrated the positive effect of liposome encapsulation on stability of anthocyanin known as natural colorants in the presence of ascorbic acid. The results of this study are important for food and beverage manufacturers, as liposomal encapsulation slowed down the fast degradation of anthocyanins and may contribute to developing applied ingredient system in future. Due to transparency of proposed liposomal system, it is convenient for clear beverages. However, further investigations is necessary to improve for the long-term stability of these liposomal capsules. One possibility may be the polymer coating of liposomes by layer-layer deposition or matrix encapsulation techniques.

4. ROLE OF ACYLATION AND LIPOSOMAL ENCAPSULATION ON ASCORBIC ACID RELATED ANTHOCYANIN DEGRADATION OF BLACK CARROT EXTRACT

4.1 Introduction

Anthocyanins, which are glycosylated polyhydroxy and methoxy derivatives of flavylium salts (Brouillard, 1982), are widely used as natural food colorants due to increasing concern on possibly negative effects of synthetic colorants on health. Anthocyanins are responsible for red to bluish color depending on pH and they are relatively stable in their original sources but after juice or extraction processes they lose their color stability. After extracting anthocyanins from their sources, additional protection is required for their stabilization. Encapsulation process protects anthocyanins against the degradation effect of light, oxygen, humidity, and other compounds (Ferreira et al., 2009). On the other hand, acylation with organic acids provide higher stability to anthocyanins. In neutral or slightly acidic conditions, anthocyanins without acyl groups are transformed to their colorless form due to hydration at the C-2 and/or C-4 position (Brouillard, 1982; Yonekura-Sakakibara et al., 2008)

Copigmentation is a phenomena that increase brightness and stability of anthocyanins as a result of interaction with organic substances even at relatively higher pH values (Osawa, 1982). Basically, copigments are colorless molecules which increases stability of anthocyanin chromophores at an adequate amount (Brouillard, 1988). Intermolecular copigmentation occurs due to a different molecule, however, covalent bonded acyl groups and flavylium cation define intramolecular copigmentation (Brouillard, 1982& 1988). Black carrot anthocyanins are examples of intramolecular copigmentation due to their mono-acylated anthocyanins (cyanidin 3-sinapoyl-xylosyl-glucosyl-galactoside, cyaniding 3-feruloyl-xylosyl-glucosyl-galactoside, and cyanidin 3-coumaroyl-xylosyl-glucosyl-galactoside) and they also have two non acylated anthocyanins (cyanidin 3-xylosyl-glucosyl-galactoside and cyanidin 3-xylosyl-galactoside) (Kammerer et al., 2006; Montilla et al., 2011).

Physical effects of acylation on anthocyanins are lower polarity, changed molecular geometry, and increasing molecular size (Zhao et al., 2017). Molecular sizes of anthocyanins are increased by acylation especially with aromatic acyl groups due to their structural properties that results steric barrier effect which prevents ions to attack the anthocyanins in aqueous media (Mazza & Brouillard, 1987; Zhao et al., 2017). Additionally, folded conformation may result due to long attached groups over the pyrylium ring (Figueiredo et al., 1996). These attached acyl groups create a sandwich type structure which protects C-2 and C-4 positions of the aglycone from the attack of water molecules (Figueiredo et al., 1999). Di-acylated anthocyanin stabilization is subjected to sandwich type protection, however mono-acylated anthocyanins are stabilized from one side protection that mechanisms can be visualized with stacking phenomenon (Giusti & Wrolstad, 2003).

In the United States, wide range of ascorbic acid fortification (0.04%- 11.4% w/w) to powdered beverage samples was reported (West & Mauer, 2013). In food processing, ascorbic acid was utilized to stabilize nutrients (Brenes, 2005). In a recent study, copigmentation effect of polysaccharide and proteins against ascorbic acid related degradation of anthocyanin color was presented by showing stronger hydrogen bonding between anthocyanin and copigment than ascorbic acid (Chung et al., 2015). In addition to copigmentation, encapsulation of anthocyanins is another way to increase the stability (Cavalcanti et al., 2011). Anthocyanins have been reported to be encapsulated with liposome systems in previous studies (Bryła et al., 2015; Gibis et al., 2013; Gibis et al., 2014a; Gibis et al., 2012; Gibis et al., 2014b; Gültekin-Özgüven et al., 2016).

In this study, black carrot extract was investigated for the effects of both intramolecular copigmentation and encapsulation on ascorbic acid related anthocyanin degradation. We hypothesized that protection effect of liposomal encapsulation may increase with the acylation of anthocyanins. For that matter, the difference in anthocyanin loss was discussed among acylated and non-acylated anthocyanins. We therefore analyzed the effect of ascorbic acid on free and liposomal encapsulated extracts over time by using HPLC.

4.2 Materials and Methods

4.2.1 Materials

Black carrot extract (*Daucus carota* ssp. *sativus*) was donated by Döhler GmbH (AG Darmstadt, Germany). The soy lecithin, containing 69.3% phosphatidylcholine, 9.8% phosphatidylethanolamine, and 2.1% lysophosphatidylcholine, was purchased from Lipoid AG (Lipoid S75, Ludwigshafen, Germany).

Trifluoroacetic acid, cyanidin 3-*O*-glucoside chloride, and Sephadex G-50 were purchased from Sigma-Aldrich Co. (Steinheim, Germany). Acetonitrile, acetic acid glacial, L(+)- ascorbic acid, anhydrous sodium acetate were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Deionized, double distilled water was used in all experiments.

4.2.2 Preparation of extract solutions

Extract solutions were prepared at different concentrations (0.1%, 0.2%, and 0.4%) in pH 3.5 acetate buffer (250 mM). Three independent replicates for each concentration were used.

4.2.3 Preparation of liposome solutions

Free liposomes were prepared from lecithin solutions by dissolving different lecithin concentrations (1%, 2%, and 4%) in pH 3.5 acetate buffer (250 mM) via two-stage homogenization. Firstly, solutions were homogenized with a high shear dispenser (Heidolph Silent Crusher M, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 20.000 rpm for 5 min. Afterwards, solutions were homogenized with a high-pressure homogenizer (Microfluidizer Processor M-110EH, Microfluidics, Newton, MA, USA) at 22500 psi in 5 passes. High pressure homogenizer was cooled with ice during homogenization to prevent sample heating. For extract encapsulated liposomes, each black carrot extract concentrations (0.1%, 0.2%, and 0.4%) were dissolved in three different lecithin solutions (1%, 2%, and 4%) and homogenized via two-stage homogenization. In addition, each liposome sample was prepared with three replicates.

4.2.4 Zeta (ζ) potential and particle size

Freshly prepared liposome samples were characterized with particle size and ζ-potential using a dynamic light scattering instrument (Nano ZS, Malvern Instruments, Worce stershire, UK). Liposomes were diluted to 0.1% (w/w) lecithin concentration to avoid multiple scattering effects. In the measurements, the refractive index values were used as 1.44 and 1.33 for the lecithin and aqueous part, respectively. The average and standard deviation of measurements were calculated from three independent samples using three measurements per sample.

4.2.5 Sephadex® gel filtration

Sephadex® gel filtration was used to remove unencapsulated extract part from liposome solution. Sephadex® gels were prepared using the same method as previously described (Gibis et al., 2014a). The liposome samples were passed through Sephadex® gel (height: 3cm) filled cartridge (volume: 5 ml). One ml of liposome samples was added to cartridges and centrifuged. The gel filtered samples were collected after centrifugation for further analysis.

4.2.6 Efficiency of liposome encapsulation

The encapsulation efficiency (EE) of black carrot extracts entrapped in liposomes was measured via HPLC as a ratio of anthocyanin content in the liposome solutions before and after Sephadex® gel filtration. Before HPLC analyses, gel filtered and unfiltered liposomes were broken down with ethanol using the similar method in a recent study (Liu et al., 2008). Extract including gel filtered and unfiltered liposomes diluted (1:2 v/v) with ethanol and centrifuged at 21380 g for 10 min (2 times). The lecithin free upper part was filtered through a 0.45 µm membrane filter and analyzed using an Agilent HPLC 1100 Series (Agilent, Waldbronn, Germany) equipped with ChemStation software. Phenomenex (Torrance, CA, USA) Hydro-RP 80 A-Synergi (250x4.6mm; 4µm) LC column with a SecurityGuard ™ guard column (4.0x2.0mm) were used at 40°C operating temperature. The mobile phase consisted of solvent A (Milli-Q water with 0.1% (v/v) trifluoroacetic acid (TFA), and solvent B (acetonitrile with 0.1% (v/v) TFA). A linear gradient was used as described in a previous study (Guldiken et al., 2016). The anthocyanin detection was performed at 520 nm as cyanidin 3-*O*-glucoside chloride. Identification was achieved based on the retention times and characteristic UV spectra, and quantification was done by external standard

curves. The ratio of total anthocyanin content before and after gel filtration as percent was determined for the encapsulation efficiency (4.1) of liposomes. The anthocyanin content of free extract ($AC_{free\ extract}$) is the difference between the unfiltered liposomal sample ($AC_{total\ extract}$) and gelfiltered liposomal sample ($AC_{gelfiltered}$).

$$EE(\%)=\frac{AC_{gelfiltered}}{AC_{total\ extract}} \times 100 = \frac{1-AC_{free\ extract}}{AC_{total\ extract}} \times 100 \quad (4.1)$$

4.2.7 Ascorbic acid related degradation

Extract (0.2%) and liposomal solutions (1%, 2%, and 4% of lecithin) entrapped 0.2% extract were mixed with various ascorbic acid concentrations (0.01%, 0.025%, 0.05%, and 0.1% w/w). Three independent replicates of solutions were prepared for each ascorbic acid concentration. All solutions were stored at room temperature in dark for 24 hours. During storage, samples were collected and analyzed immediately using UV/VIS-spectrophotometer (Perkin Elmer, LAMBDA 750 UV/Vis/NIR spectrophotometer, MA, USA).

The absorbance of all samples was measured at 520 nm (maximum absorbance in the visible range) three times. The average and standard deviation of measurements were calculated.

4.2.8 HPLC analysis for degradation

Liposome sample including 0.2% extract and 4% lecithin was chosen for the degradation study according to its high EE%. Before ascorbic acid (0.1%) addition to the extract and liposome solutions, liposome sample was gel-filtered to eliminate the contribution of unencapsulated extract to the anthocyanin content. Extract solution (0.2%) was also diluted with acetate buffer (3:5 v/v) according to EE% to fix initial anthocyanin content both in extract and liposomal solutions. Both extract and liposomal solutions were stored in dark at room temperature for 24 hours. Samples were taken and analyzed immediately via HPLC using similar procedure as explained before. Each treatment was replicated three times.

4.2.9 Data analysis

All experiments were carried out at least 3 times for each triplicate samples. The average and standard deviation of measurements were calculated using Microsoft Excel for Mac (version 15.32). Statistical analyses were performed using one-way

analysis of variance (ANOVA) (Appendix D), followed by Duncan post hoc test using SPSS software (version 21.0, SPSS, Chicago, IL, USA) and Tukey post hoc test using Minitab software (version 16.0, MINITAB Inc.).

4.3 Results and Discussion

4.3.1 Characterization of liposomes

Previously, it was reported that encapsulation may enhance the color stability of anthocyanins against ascorbic acid related degradation (Guldiken et al., 2017). In this study, degradation of black carrot extract by the effect of added ascorbic acid (AA) was investigated further by examining the anthocyanin stability capcules. For this purpose, free liposomes and black carrot extract included liposomes were prepared in various extracts (0.1%-0.4%) and lecithin concentrations (1%-4%). After two step homogenization, transparent liposomes were generated. Characterization of liposomes was performed using dynamic light scattering instrument. The results of z-average particle diameter and ζ-potential measurements were presented in Table 4.1. Particle size of both free liposomes and extract included liposomes were between 44-46 nm. Produced liposomes can be classified as small unilamellar vesicles (20-100 nm) (Fathi et al., 2012). In addition, it is reported that high pressure homogenization or high intensity sonication may generate small unilamellar vesicles (Laye et al., 2008). On the other hand, the particle diameter of liposomes containing elderberry extract produced by lipid film hydration was reported to be between 205-503 nm depending on lecithin type (Bryła et al., 2015). The ζ-potentials of liposomes were between -20 and -29 (see Table 4.1). In addition, change in lecithin concentration didn't significantly ($p>0.05$) alter the ζ-potential of the liposomes.

Table 4.1 : Particle diameter and ζ-potential of liposome samples.

Liposome		Particle Diameter (nm)	PdI	ζ-Potential (mV)
Extract Conc. (%)	Lecithin Conc. (%)			
0	1	45.6 ± 0.40^{ab}	0.397 ± 0.0093^{a}	-26.5 ± 0.37^{c}
	2	44.0 ± 0.28^{ab}	0.362 ± 0.0072^{abc}	-28.6 ± 1.62^{d}
	4	44.8 ± 1.32^{ab}	$0.371 \pm 0{,}0285^{ab}$	-27.9 ± 0.52^{cd}
0.1	1	44.3 ± 1.89^{ab}	0.337 ± 0.0500^{abcd}	-21.6 ± 2.24^{ab}
	2	44.4 ± 0.16^{ab}	0.300 ± 0.0223^{cd}	-21.7 ± 0.50^{ab}
	4	45.7 ± 0.51^{ab}	0.292 ± 0.0129^{d}	-21.8 ± 0.50^{ab}
0.2	1	45.7 ± 1.95^{ab}	0.322 ± 0.0539^{bcd}	-19.6 ± 1.59^{a}
	2	45.1 ± 0.98^{ab}	0.324 ± 0.0380^{bcd}	-21.5 ± 0.44^{ab}
	4	46.0 ± 0.50^{a}	0.336 ± 0.0443^{abcd}	-22.9 ± 1.27^{b}
0.4	1	43.7 ± 1.03^{b}	0.330 ± 0.0333^{bcd}	-21.6 ± 0.98^{ab}
	2	44.7 ± 1.95^{ab}	$0.310 \pm 0{,}0425^{bcd}$	-19.9 ± 0.71^{a}
	4	45.1 ± 0.55^{ab}	0.301 ± 0.0328^{cd}	-20.9 ± 0.62^{ab}

Data represent average values ± standard deviation of three replicates from each sample. Different letters in the columns represent statistically significant differences ($p < 0.05$).

4.3.2 Encapsulation Efficiency

Encapsulation efficiency (EE) was calculated to determine the ratio of extract entrapped inside the liposomes by using HPLC data. Before HPLC analyses, liposome solutions were diluted with ethanol to break down liposomes as explained in detail in our previous study (Guldiken et al., 2017). In this way, free extract and liposomes including extract were separated via Sephadex® gel separation method and lecithin was removed from the solutions by centrifugation before samples were injected to HPLC. According to EE of liposomal samples presented in Table 4.2, extract and lecithin concentration affect the entrapment efficiency of liposomal system,

significantly ($p < 0.05$). Increasing lecithin concentrations increased EE at the same extract concentration; however, increasing extract concentration reduced EE of the liposomes at the same lecithin concentration. As a result, 59% and 57% higher EE was measured in liposomes consisting of 4% lecithin and 0.1% or 0.2% extract, respectively. In several previous studies, EE of liposomes were detected by absorbance measurement in UV spectroscopy at 520-550 nm in elderberry extract as 25% (Bryła et al., 2015), hibiscus extract as 61% (2% lecithin and 0.2% extract)-72% (5% lecithin and 0.8% extract) (Gibis et al., 2014b), black carrot extract as 32% (1% lecithin and 0.2% extract)-48% (4% lecithin and 0.2% extract) (Guldiken et al., 2017) and by phenolic content measurements in grape seed extract as 88% (1% lecithin and 0.1% extract) (Gibis et al., 2016).

Table 4.2 : Encapsulation efficiency of black carrot extract in liposomes with different lecithin concentration (%) measured by HPLC.

Liposome		Encapsulation Efficiency (HPLC)
Extract Conc. (%)	Lecithin Conc. (%)	
0.1	1	33 ± 3.6 [d]
	2	51 ± 2.2 [b]
	4	59 ± 1.9 [a]
0.2	1	29 ± 2.0 [d]
	2	41 ± 5.1 [c]
	4	57 ± 4.9 [a]
0.4	1	30 ± 1.2 [d]
	2	40 ± 2.6 [c]
	4	51 ± 1.9 [b]

Data represent average values ± standard deviation of three replicates from each sample. Different letters in the columns represent statistically significant differences ($p < 0.05$).

4.3.3 Ascorbic acid effect on black carrot extract

Black carrot extract (0.2%) with different AA concentrations (0.01%, 0.025%, 0.05%, and 0.1%) were stored at room temperature for 24 hours. The degradation rate increased proportional to the ascorbic acid concentration in agreement with previous studies and the highest degradation in anthocyanins was detected at samples with 0.1%

AA (Guldiken et al., 2017; Özkan, 2002). Furthermore, as seen in Figure 4.1 and Table E.1, without AA addition, 0.2% extract solution was stable during the storage.

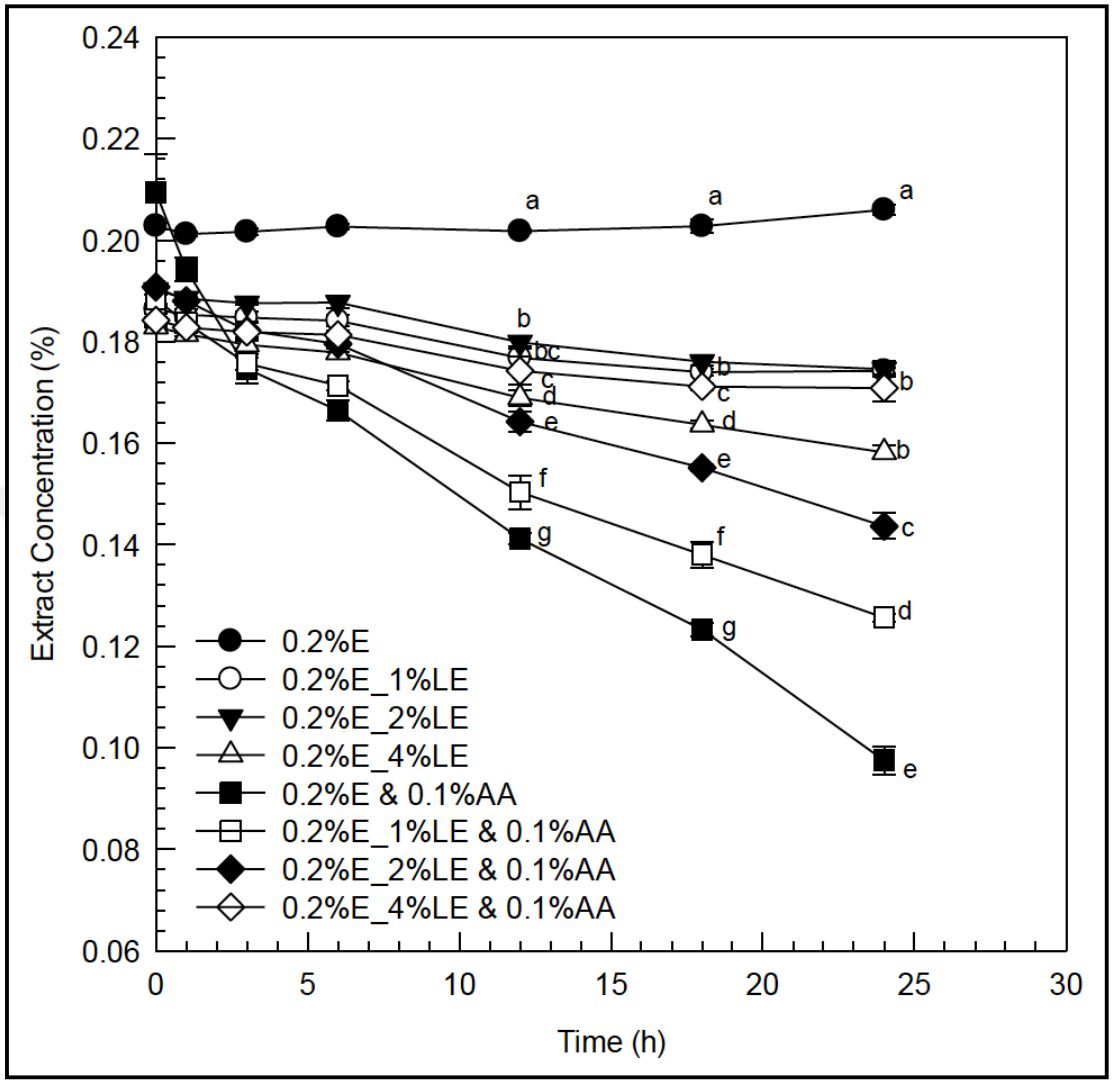


Figure 4.1 : Extract concentrations of black carrot extract and liposomal solutions with/without 0.1% ascorbic acid (AA) during 24 hours. The data represented in this figure includes average values ± standard deviation of three replicates from each sample. Different small letters represent statistically significant differences at that time ($p<0.05$).

Predictably, 0.1% AA addition causes the highest degradation of anthocyanins during storage. The effect of lecithin concentration on absorbance was eliminated by subtracting the absorbance of free liposomes from the extract including liposomes. In addition, liposomal samples with 0.1% AA showed lower degradation during this period. The protective effect of liposomal encapsulation was in accordance with a previous study which reported that degradation effect of digestive fluids on antioxidants was reduced in liposome samples (Takahashi et al., 2009). The difference

between samples with and without AA reduced while lecithin concentration rose among liposomal samples (Figure 4.1). Increasing encapsulation efficiency with increasing lecithin concentration (Table 4.2) may be the reason of higher protection ability of liposome encapsulation with higher lecithin concentrations. Basically, encapsulation methods may provide wall like protection on core material against outer undesired materials and environmental factors (Fang & Bhandari, 2010).

4.3.4 Effect of ascorbic acid on individual anthocyanins

In previous chapter, structural protection of liposome encapsulation on anthocyanin color degradation in the presence of ascorbic acid was determined. In this manner, further investigation was necessary to analyze the influence of liposomal encapsulation on individual anthocyanins in detail. For this purpose, the effect of ascorbic acid on degradation of anthocyanins was analyzed with HPLC. In Figure 4.2, both color protected liposome solution and color fading of extract solution can be detected visually.

Before HPLC analysis, lecithin was removed from gel filtrated liposome samples using ethanol as previously described. Furthermore, all samples were filtered with 0.45 μm membrane just before the HPLC analysis. Five major peaks were identified as cyanidin 3-xylosylglucosylgalactoside (cya 3-xylglcgal), cya 3-xylgal, sinapic acid derivative of cya 3-xylglcgal, ferulic acid derivative of cya 3-xylglcgal, and *p*-coumaric acid derivative of cya 3-xylglcgal, respectively (see Figure 4.3), which is in agreement with our previous results and findings of studies (Guldiken et al., 2016; Kammerer et al., 2003; Kammerer et al., 2004b). In addition, anthocyanin quantification of both liposome and extract samples were done using cyanidin 3-*O*-glucoside chloride as standard considering these previous studies.

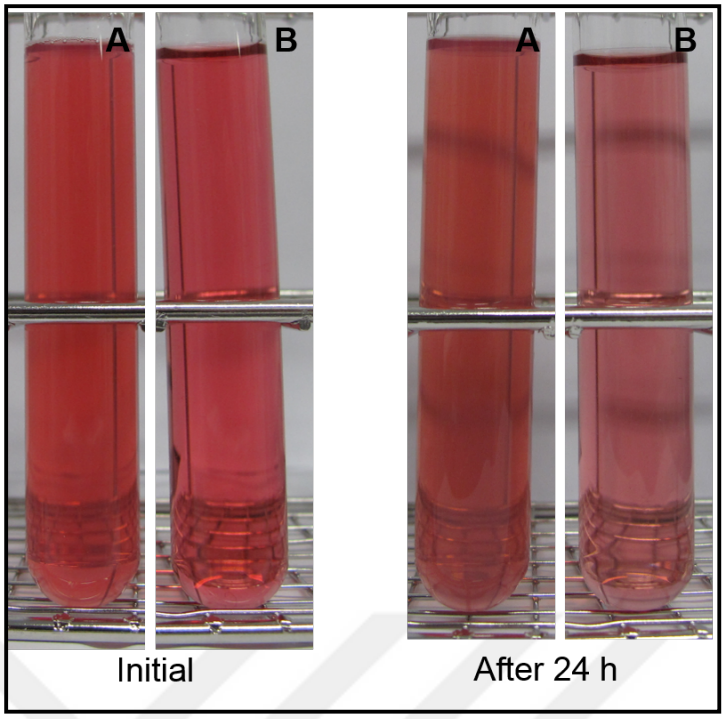


Figure 4.2 : Visual observations of color fading after addition of ascorbic acid (0.1%) to (A) gelfiltered liposome (0.2% extract & 4% lecithin) and (B) diluted 0.2% extract according to encapsulation efficiency during time.

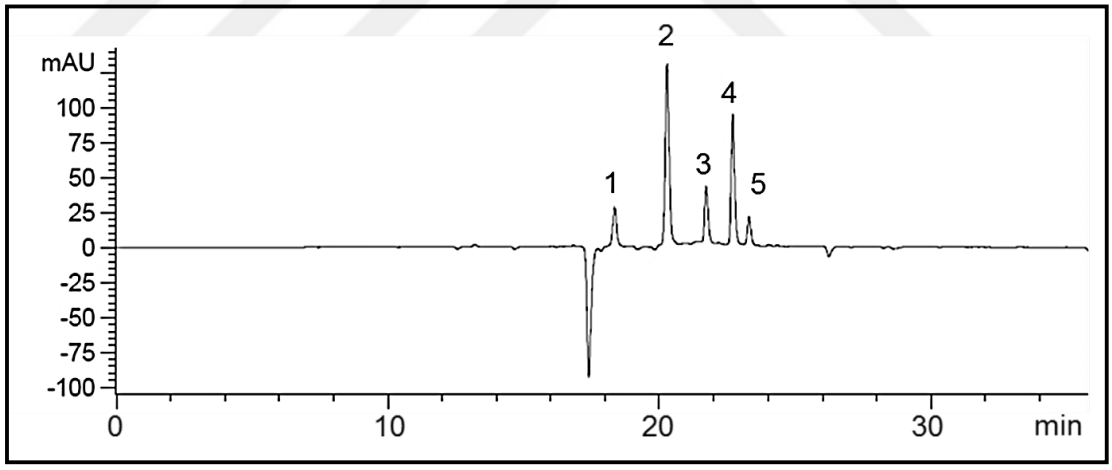


Figure 4.3 : The HPLC chromatogram obtained from 0.2% black carrot extract at 520 nm. Peaks: 1 = Cyanidin 3-xylosylglucosylgalactoside; 2 = Cyanidin 3-xylosylgalactoside; 3 = Cyanidin-3-xylosyl (sinapoylglucosyl) galactoside; 4 = Cyanidin-3-xylosyl (feruloylglucosyl) galactoside; 5 = Cyanidin-3-xylosyl (coumaroylglucosyl) galactoside.

As presented in Figure 4.4 and Table E.2, degradation effect of ascorbic acid on the contents of individual anthocyanins and total anthocyanins was determined. This data may provide information on the type of anthocyanins that are mostly encapsulated inside the liposomal system or incorporated in the liposomal membrane which provides its protection effect. In a previous study, it is reported that 8% of entrapped extract located in aqueous part inside the liposomes, however 92% of entrapped extract was incorporated in the liposomal membrane (Gibis et al., 2013). Therefore, encapsulated material may be located in aqueous part inside the liposome or integrated to liposomal membrane. Although acylated anthocyanins were reported to be stable against degradation factors such as pH, light, and temperature (Cevallos-Casals & Cisneros-Zevallos, 2004), the results of this analysis indicated that they degrade with the addition of ascorbic acid. In a similar manner, it was reported that in the presence of hydrogen peroxide, the stability of acylated anthocyanins was not higher than non-acylated ones (Del Pozo-Insfran et al., 2004). However, the prevention of ascorbic acid related degradation of anthocyanins using liposomes was found less efficient for non-acylated anthocyanins such as cya 3-xylglcgal and cya 3-xylgal in our study.

On the other hand, as shown in Figure 4.4, it was found that liposome encapsulation protected acylated anthocyanins such as sinapic acid derivative of cya 3-xylglcgal, ferulic acid derivative of cya 3-xylglcgal, and *p*-coumaric acid derivative of cya 3-xylglcgal from the influence of ascorbic acid. Total anthocyanin degradation of extract and liposome samples is shown in Figure 4.4. The degradation slope of total anthocyanin content of liposomal solution was found to be 69% lower than the extract solution. Similarly, the effect of encapsulation on reducing the degradation of anthocyanins was previously reported for pomegranate juice (Robert et al., 2010). Acylated anthocyanins are larger molecules than non-acylated anthocyanins because of the phenolic acids attached. Also, these results may indicate that encapsulation and intramolecular copigmentation can provide an additive or synergistic effect. On the other hand, further analyses are required to identify the capturing of individual anthocyanins in liposomal systems.

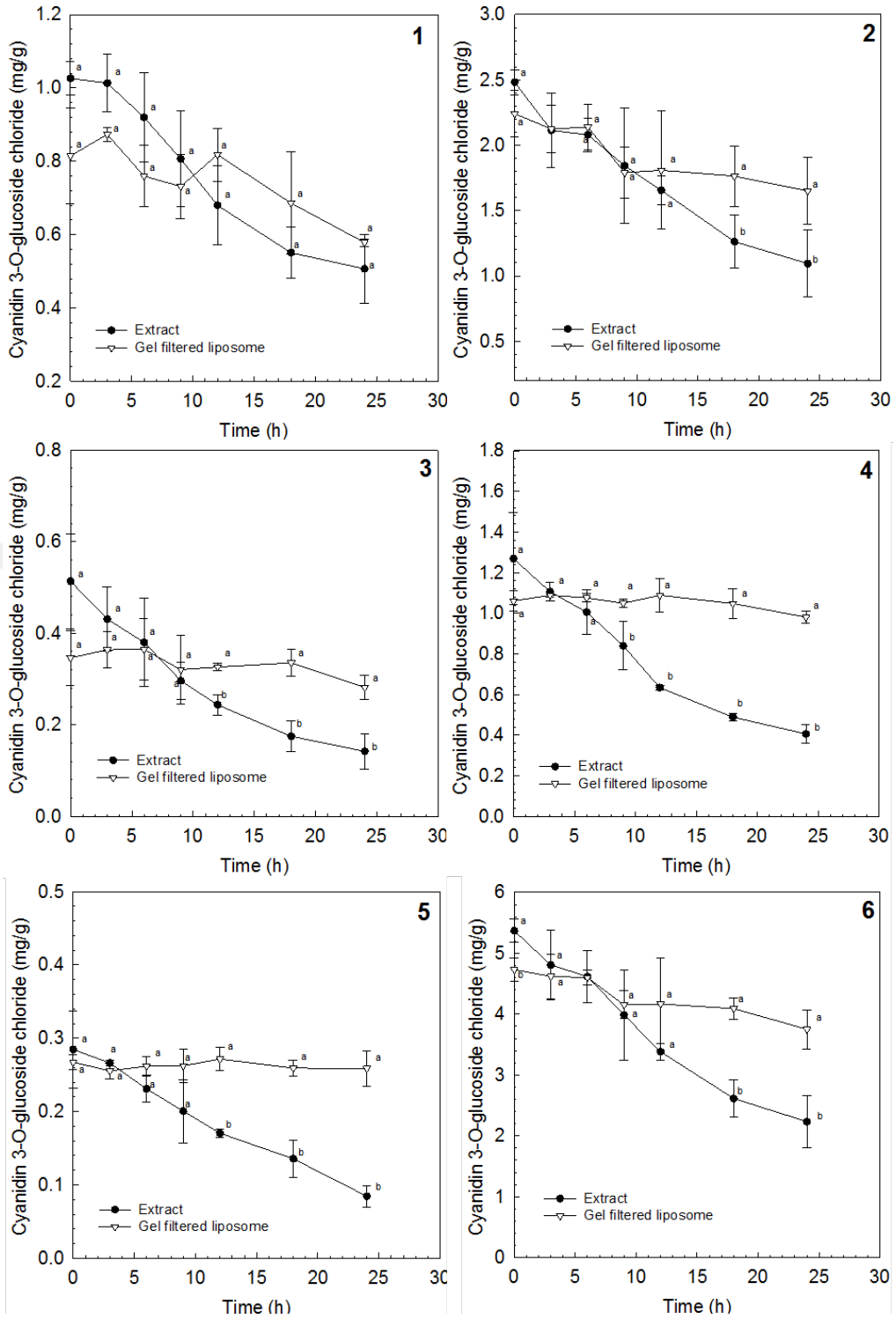


Figure 4.4 : The effect of 0.1% ascorbic acid on individual anthocyanins in both gel filtered liposome and black carrot extract solutions during 24 hours. (1) Cyanidin 3-xylosylglucosylgalactoside; (2) Cyanidin 3-xylosylgalactoside; (3) Cyanidin-3-xylosyl (sinapoylglucosyl) galactoside; (4) Cyanidin-3-xylosyl (feruloylglucosyl) galactoside; (5) Cyanidin-3-xylosyl (coumaroylglucosyl) galactoside; (6) Total of all peaks. Different small letters represent statistically significant differences at that time ($p<0.05$).

4.4 Conclusions

The results of the present study indicated that the presence of ascorbic acid results in color loss and degradation of anthocyanins. In this study, among other protection techniques, the effect of liposome encapsulation was discussed and the results showed that degradation of anthocyanins decreased in acylated anthocyanins as a result of liposome encapsulation. HPLC analysis of extract and liposomes indicated a possible relationship of acylation and encapsulation. However, further analyses are necessary to overcome possible concerns on the effect of acylation on entrapment efficiency in liposomes. This study presents the protective effect of liposome encapsulation during the storage time of 24 h on the stability of anthocyanins, known as unstable natural colorants, in the presence of ascorbic acid. To the best of our knowledge, the present study is the first study investigating the effect of liposomal encapsulation on individual anthocyanins. However, interaction of liposomes and individual anthocyanin standards might be of interest to investigate in future studies.

5. PHYSICAL AND CHEMICAL STABILITY OF ANTHOCYANIN-RICH BLACK CARROT EXTRACT LOADED LIPOSOMES DURING STORAGE

5.1 Introduction

Liposomes are widely used as delivery method of antimicrobials (Benech et al., 2002), vitamins (Banville et al., 2000), enzymes (Alkhalaf et al., 1988), and phenolic compounds (Coimbra et al., 2011) and preferred for their high biodegradability, and biocompatibility and nontoxic properties (Peschka et al., 1998). Liposomes which are composed of phospholipid bilayers are colloidal particles and formed by energy input (Mozafari et al., 2008). One of the most important properties of liposomes is the encapsulation capability of both hydrophilic and hydrophobic compounds at the same time (Mozafari et al., 2006). Conventional methods for liposome formation used in studies are multilamellar vesicles which were formed with thin lipid hydration method as the first preparation method (Bangham et al., 1965; Szoka Jr & Papahadjopoulos, 1980), followed with detergent removal (Yang et al., 2016), ether/ethanol injection (Jaafar-Maalej et al., 2010), reverse phase evaporation (Szoka & Papahadjopoulos, 1978), and emulsion methods (Bryła et al., 2015; Meure et al., 2008; Szoka Jr & Papahadjopoulos, 1980). Furthermore, high pressure homogenization, sonication, and extrusion are classified in post formation processing (Meure et al., 2008). In this study, high pressure homogenization method is used as previously used for phenolic compound encapsulation (Gibis et al., 2012). Important parameters in selecting the method for liposome formation are encapsulation efficiency, size distribution, storage stability, and protection efficiency (Meure et al., 2008).

Stability of liposomes can be examined by physical and chemical factors (Wu et al., 2011). Physical stability of liposomes is evaluated by size change (aggregation) and leakage of encapsulated material (Anderson & Omri, 2004). In addition, zeta potential is an indicator of colloidal stability of particles which is higher when particle charge is sufficiently high to repel each other (Du Plessis et al., 1996). In consideration of chemical stability of liposomes, there are oxidative and hydrolytic degradation

parameters that decrease the shelf life of liposomes (Grit & Crommelin, 1993). Liposome composition was also reported as an indicator of stability (Du Plessis et al., 1996). Oxidation of liposomes mainly occurs due to the oxidation of unsaturated fatty acyl chain of phospholipids which may be eliminated by antioxidant usage and controlled storage conditions (Grit & Crommelin, 1993).

Black carrot is a rich source of anthocyanins (1750 mg/kg), mostly composed of acylated anthocyanins (64-77%) (Gizir et al., 2008; Kamiloglu et al., 2015a). Five major anthocyanins in black carrot are cyanidin-3-xylosyl-galactiside, cyanidin-3-xylosyl-glucosyl-galactoside and cyanidin-3-xylosyl-glucosyl-galoctoside acylated with sinapic acid, ferulic acid and coumaric acid (Gizir et al., 2008; Kammerer et al., 2004b). For the chemical stability of liposomes, antioxidants such as α-tocopherol and butyl hydroxyl toluene can be used to prevent oxidation (Heurtault et al., 2003). On the other hand, anthocyanins are also powerful antioxidants (Gizir et al., 2008). Furthermore, besides their antioxidant power, black carrot provides bright red color at acidic conditions and is used as natural colorants (Kırca et al., 2006)

In this study, liposomes including black carrot extract were prepared with high pressure homogenization method and storage stability of extract including as well as free liposomes were investigated. Physical and chemical stability of liposomes were analyzed during storage. We hypothesized that black carrot extract as a rich anthocyanin source may increase the stability of liposomes. Current study evaluates the phenolic content, antioxidant activity, anthocyanin stability, and color properties of liposome samples together with lipid oxidation during the storage period.

5.2 Material and Methods

5.2.1 Materials

Black carrot extract (BCE) was provided by Döhler GmbH (AG Darmstadt, Germany). The soy lecithin (Lipoid S75) including 69.3% phosphatidylcholine, 9.8% phosphatidylethanolamine, and 2.1% lysophosphatidylcholine was obtained by Lipoid AG (Ludwigshafen, Germany). Sodium acetate, acetic acid glacial , ethanol were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Sodium carbonate anhydrous, Folin– Ciocalteu reagent, Sephadex G-50 (G50300-100 g), copper (II) chloride (CuCl2), ammonium acetate (NH_4Ac), 6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid (Trolox), sodium azide and neocuproine were obtained from Sigma- Aldrich (St. Louis, USA). All experiments were performed with double distilled water.

5.2.2 Preparation of liposomes

Black carrot extract (BCE) solutions were prepared by dissolving in acetate buffer (pH 3.5, 250 mM) as three independent replicates for each sample (0.1%, 0.2%, and 0.4% w/w). Sodium azide (0.02% w/w) was added during acetate buffer preparation to prevent microbial growth (Salminen et al., 2014). The lecithin solutions were prepared by dissolving lecithin at various concentrations (1%, 2%, and 4% w/w) as three independent replicates in acetate buffer for free liposomes or in BCE solutions for extract encapsulated liposome samples. Lecithin solutions were stirred overnight to ensure homogeneity. Two step homogenization were used to generate liposomes as described in our previous study. Firstly, lecithin solutions were homogenized with a high shear disperser (Heidolph Silent Crusher M, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 20 000 rpm for 5 min and immediately after liposomes were formed by high-pressure homogenizer (Microfluidizer Processor M-110EH, Microfluidics, Newton, MA, USA) at 22 500 psi and 5 times passes with a cooling unit.

5.2.3 Physical and chemical characterization of liposomes

The z-average particle diameter was determined using a dynamic light scattering instrument (Nano ZS, Malvern Instruments, Worcestershire, UK). Liposome samples were diluted up to lecithin concentration as 0.1% (w/w) with acetate buffer to prevent multiple scattering effects. The refractive index for the lecithin and aqueous part used as 1.44 and 1.33, respectively. The mean particle diameter (z-average) and polydispersity index (PDI) were reported. The average and standard deviation of results were calculated from three freshly prepared samples using three measurements per sample.

At the same dilution ratio, zeta (ζ) potential values of liposome samples were measured *via* dynamic light scattering instrument (Nano ZS, Malvern Instruments, Worcestershire, UK) using Smoluchowsky mathematical model.

5.2.4 Separation of unencapsulated extract

Sephadex® gel were used to remove unencapsulated extract from liposome samples. Formation of Sephadex® gel was performed as described in a previous study (Gibis et al., 2014a). Briefly, liposome samples were passed from 5 ml cartridges filled with 3 cm height of Sephadex gel® via centrifugation. Unencapsulated extract was trapped in cartridges and liposome part passed from cartridges. Gel filtered liposomes were used for further analyses. Before total phenolic content, antioxidant and anthocyanin analyses, liposome samples and gelfiltered liposomes withand without extract were diluted with ethanol (1:2 v/v) and centrifuged to remove lecithin.

5.2.5 Total phenolic content and encapsulation efficiency

Folin-Ciocalteu reagent used to determine total phenolic content of samples using the modified method of Velioglu et al. (1998). Folin-Ciocalteu reagent diluted with double distilled water (1:10 v/v) immediately before the experiments. 100 µl of sample was mixed with 0.75 mL of Folin-Ciocalteu reagent and vortexed. The mixture was left to stand for 5 min. 0.75-mL sodium bicarbonate (6 % w/w) was added to the mixture and vortexed. The mixture was incubated for 90 min and absorbance was measured at 725 nm *via* UV/VIS- spectrophotometer (Perkin Elmer, LAMBDA 750 UV/VIS/NIR spectrophotometer, MA, USA). Results were reported as mg gallic acid equivalents (GAE)/ml extract. Quantification of phenolic compounds was performed with external calibration curves using gallic acid as standard (stock solution 1000 mg/L in ethanol). Distilled water was used for the dilution of standard solutions to concentrations of 10 to 100 mg/L for calibration. Water is used for blank samples using the same method described above.

Encapsulation efficiency of liposomes was determined by phenolic content analysis. The amount ratio of phenolic compounds before and after gel filtration was specified as the encapsulation efficiency (5.1) in percentage. The amount of phenolic compounds of total extract ($\rho_{total\ extract}$) is the total of free extract ($\rho_{free\ extract}$) and the gel filtered liposome sample ($\rho_{gelfiltered}$).

$$EE(\%)=\frac{\rho_{gelfiltered}}{\rho_{total\ extract}}\times 100 = \frac{(1-\rho_{free\ extract})}{\rho_{total\ extract}}\times 100 \qquad (5.1)$$

5.2.6 Anthocyanin content

Anthocyanin amount in liposome samples were determined by spectrophotometrically at 530 nm using the modified method of (Lohachoompol et al., 2004) as described in (Gibis et al., 2014b). Black carrot extract was diluted to concentrations of 0.01- 0.6% w/v with 250mM, pH 3.5 acetate buffer for calibration curve. The absorbance difference before and after gel filtration were measured and calculated as extract concentration.

5.2.7 Total antioxidant capacity (TAC)

Total antioxidant capacity (TAC) was determined using cupric ion reducing antioxidant capacity (CUPRAC) assay. The antioxidant capacity was determined using the method of (Apak et al., 2004; Apak et al., 2006). 100 µl of sample was placed in a test tube and mixed with 1 ml each of 10 mM $CuCl_2$, 7.5 mM neocuproine, 1 M NH_4Ac buffer (pH 7) solutions and distilled water to set the final volume 4.1 mL. Mixtures were allowed to stand for 30 min and absorbance was read at 450 nm against a blank. Results were reported as mg trolox equivalents (TEAC)/100 ml extract. Before and after gel filtration total antioxidant capacity of liposome samples was determined.

5.2.8 Hexanal analysis

Hexanal, a secondary oxidation product, was determined using a Clarus 580 gas chromatograph with a Turbo Matrix autosampler (Perkin Elmer, Shelton, CT, USA) and a RTX-200 fused silica capillary column (30 m x 0.32 mm x 1 µm) (Restek GmbH, Bad Homburg, Germany) using the method of (Romeu-Nadal et al., 2004) with some modifications as described in Gibis et al. (2014b). Samples (1 mL) were placed in 20 mL of headspace vials. Sample temperature was equilibrated to 55°C for 5 min. Each sample run was performed for 15 min. The temperature of injector and flame ionization detector were set at 250°C. Hexanal concentration of samples was determined using external standard curve of hexanal.

5.2.9 Color analysis

Hunter color attributes (L*, a*, and b* values) of samples were measured with a CR-400 handheld chroma meter (Minolta, Tokyo, Japan).

5.2.10 Storage stability of liposomes

Free and extract included liposomes were stored during 21 days at room temperature in the dark. All samples were stored in air tight containers as full volume. All analyses were performed on stored samples during the storage period.

5.2.11 Data analysis

All experiments were performed at least 3 times for each triplicate sample. The average and standard deviation of all data were calculated using Microsoft Excel for Mac (version 15). Statistical analyses were performed using one-way analysis of variance (ANOVA) (Appendix F), followed by Duncan post hoc test using SPSS software (version 21.0, SPSS, Chicago, IL, USA) and Tukey post hoc test using Minitab software (version 16.0, MINITAB Inc.).

5.3 Results and Discussion

5.3.1 Physical stability of liposomes

Particle diameter and zeta potential of the liposomes specify physical stability of liposomes during storage. Particle diameters of all samples were found to be lower than 50 nm (Table 5.1). Liposome samples without extract showed significantly lower particle diameter ($p<0.05$). Gibis et al. (2013) proposed that free liposomes showed wider particle size distribution after the storage period due to the oxidative degradation of unsaturated fatty acids. However, particle diameters of liposomes did not change significantly after the storage ($p<0.05$) in our study.

Polydispersity index values (PdI) of 0.36 stated as narrow distribution of liposomes (Yan et al., 2013). Similarly, polydispersity index (PdI) values of liposomes were found as 0.269-0.352 and increased after the storage period (0.301-0.429) in our study. In another study, PdI values of soybean lecithin liposomes were reported to be around 0.490 (Bryła et al., 2015) being slightly higher that our values observed after 21 days storage. ζ-potential is one of the main indicators of physical stability of liposomes, and higher absolute value of ζ-potential indicate higher stability (Wu et al., 2011). Hence, our data presenting higher zeta potential (|-25 mV|- |-28 mV|) values with higher lecithin concentrations at the same extract concentration demonstrates a higher stability of liposomes

Table 5.1 : Encapsulation efficiency of of black carrot extract in liposomes after preparation according to total phenolics assay and change of particle diameters, ζ-potentials of liposome samples during storage.

Liposome Samples		Encapsulation Efficiency	z-Average Particle Diameter (nm)		PdI		ζ-Potential (mV)	
Extract (%)	Lecithin (%)	(%)	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21
0	1	-	42.2±0.55^{d,A}	43.0±0.84^{de,A}	0.331±0.00111^{a,B}	0.361±0.0098^{abc,A}	-25.3±1.75^{c,A}	-27.7±0.39^{bcd,A}
	2	-	42.4±0.29^{d,A}	41.0±0.83^{e,B}	0.269±0.0092^{b,A}	0.301±0.0367^{c,A}	-24.3±0.98^{bc,A}	-27.0±0.13^{b,B}
	4	-	42.9±0.92^{cd,A}	43.6±1.51^{cde,A}	0.273±0.0168^{b,B}	0.345±0.0389^{bc,A}	-26.0±1.09^{c,A}	-28.4±0.38^{ef,B}
0.1	1	57 ± 6.8^{b}	43.9±0.47^{bcd,A}	44.5±0.58^{cde,A}	0.340±0.0429^{a,A}	0.373±0.0070^{abc,A}	-21.3±0.98^{a,A}	-27.4±0.44^{bc,B}
	2	58 ± 2.3^{b}	45.4±0.26^{ab,A}	47.0±3.39^{abcd,A}	0.349±0.0079^{a,A}	0.374±0.0254^{abc,A}	-25.6±0.90^{c,A}	-27.8±0.16^{cde,B}
	4	66 ± 6.7^{a}	45.1±0.84^{abc,A}	48.8±4.32^{abc,A}	0.332±0.0090^{a,B}	0.374±0.0232^{abc,A}	-25.6±0.81^{c,A}	-28.6±0.22^{f,B}
0.2	1	55 ± 3.7^{b}	46.8±3.37^{a,A}	48.9±2.29^{abc,A}	0.352±0.0640^{a,A}	0.429±0.0601^{a,A}	-26.1±0.49^{c,A}	-27.4±0.27^{bc,B}
	2	58 ± 1.7^{b}	45.8±1.13^{ab,A}	50.8±5.36^{a,A}	0.351±0.0025^{a,B}	0.401±0.0253^{ab,A}	-26.3±0.46^{cd,A}	-27.9±0.44^{cdef,B}
	4	60 ± 3.3^{ab}	46.3±0.89^{ab,A}	45.1±2.17^{bcde,A}	0.337±0.0188^{a,A}	0.346±0.0306^{bc,A}	-28.0±0.42^{d,A}	-28.3±0.46^{def,A}
0.4	1	40 ± 1.0^{c}	44.7±0.86^{abc,A}	47.3±3.83^{abcd,A}	0.347±0.0126^{a,A}	0.420±0.0849^{ab,A}	-24.5±0.98^{bc,A}	-26.3±0.49^{a,B}
	2	45 ± 0.2^{c}	45.2±1.44^{abc,A}	50.7±3.89^{ab,A}	0.283±0.0197^{b,B}	0.400±0.0329^{ab,A}	-23.2±1.92^{b,A}	-27.5±0.34^{bc,B}
	4	46 ± 0.8^{c}	45.6±0.88^{ab,A}	46.9±1.26^{abcd,A}	0.306±0.0264^{ab,B}	0.365±0.0199^{abc,A}	-24.8±0.94^{bc,A}	-28.1±0.61^{cdef,B}

Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$). Different capital letters in the rows indicate statistically significant differences for each analyses ($p < 0.05$).

After storage, ζ-potential values of all samples increased to the range of |-26mV| - |-29 mV| (Table 5.1). Commonly, samples having higher zeta potential do not aggregate as a result of electric repulsion (Du Plessis et al., 1996; Fathi et al., 2012). Therefore, the results showed that physical condition of liposomes was adequately stable after 21 days of storage.

5.3.2 Encapsulation efficiency of liposomes

Encapsulation efficiency of extract including liposomes was determined using total phenolic analysis. Extract including liposome samples before and after gel filtration were analyzed. Lecithin was removed before analyses with ethanol dilution and centrifugation method to prevent interference to absorbance. Higher encapsulation of extract was achieved in liposomes at higher lecithin concentrations (see Table 5.2). However, increasing extract concentration reduced encapsulation of the extract in liposomes at the same lecithin concentration according to our previous data (Table 4.2). Black carrot phenolics were encapsulated in liposomes at a ratio of 40-66%. In previous chapter (3), black carrot anthocyanins could be encapsulated in liposomes between 32% and 50% efficiency. Some researchers reported encapsulation efficiency of grape seed phenolics to be 88% (Gibis et al., 2016) and hibiscus extract in a range of 61 to 72% (Gibis et al., 2014b). In addition, entrapped phenolics in the core of liposomes were reported as low as 7.6% (Gibis et al., 2013).

5.3.3 Biochemical stability of liposomes

Degradation of extract concentration, total phenolic content, and antioxidant capacity of liposome samples were evaluated for all parts of liposomes. Degradation of black carrot anthocyanins was presented at 0.4% extract concentration and different lecithin concentrations (1%, 2%, and 4%) during 21 days storage (Figure 5.1 and Table G.1). According to the results, the highest degradation was found in liposomes generated with 4% lecithin concentraion. After 21 days of storage, there was no statistically significant differences between gel filtered liposome part and liposome in 4% lecithin containing samples ($p<0.05$). That could be explained as degradation rate in aqueous part may be higher than inner part of liposomes. On the other hand, the reason of increased extract degradation in samples including 4% lecithin may also be caused by higher lipid oxidation of unsaturated fatty acids in soy lecithin mainly linoleic acid due to increasing lipid concentration (Gibis et al., 2013). Extract degradation in 0.1% and

0.2% extract including liposome samples were provided in Appendix D (Figures G.1 and G.2). In accordance with other results, degradation rate of extract was found to be higher in samples including higher lecithin concentrations. The change in the amount of bioactive materials inside the gel filtered liposomes might also be associated with random molecular movements causing diffusion of biomolecules (McClements, 2015).

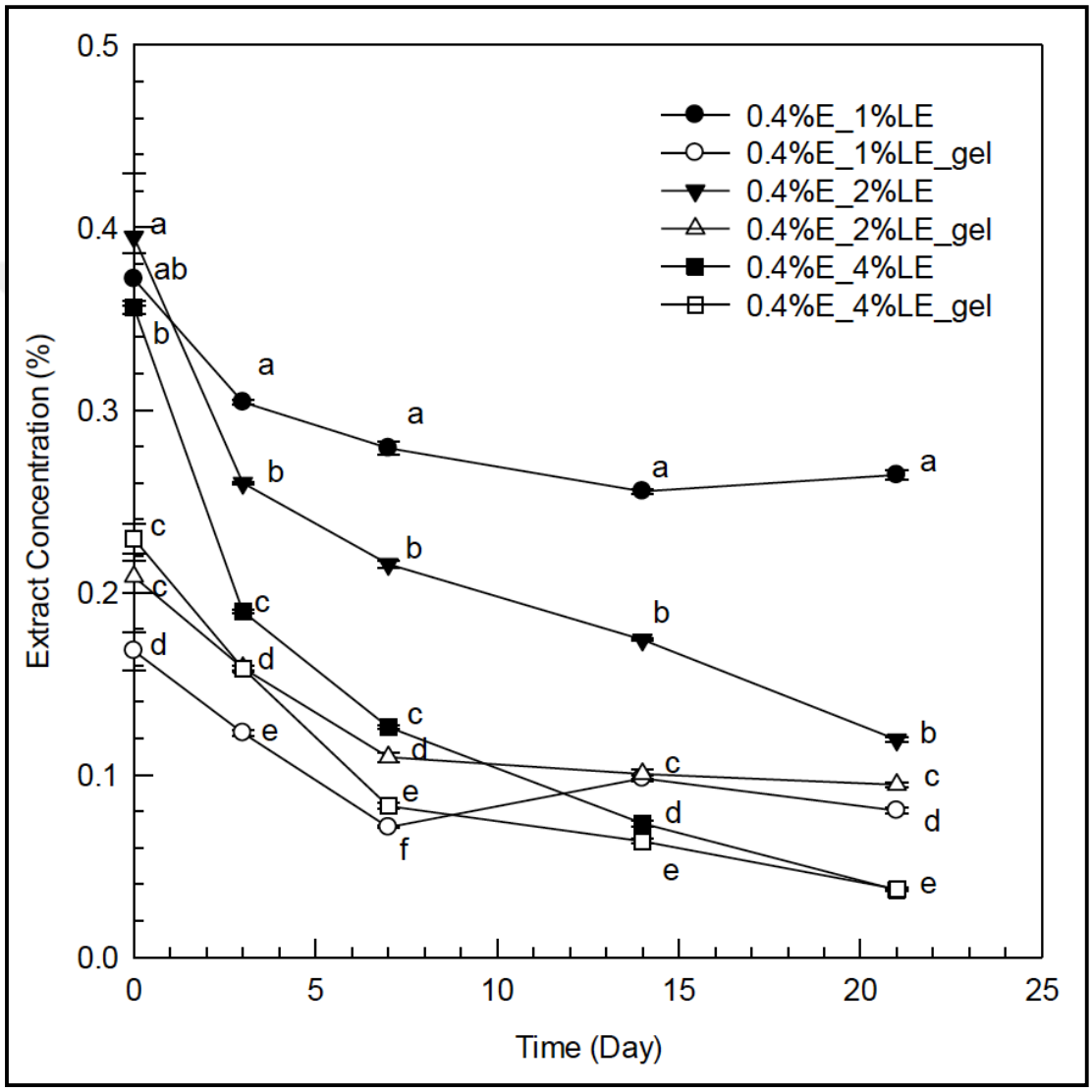


Figure 5.1 : Degradation of anthocyanin in liposome samples with 0.4% extract during storage period. Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

The content of total phenolics in liposome samples were reported as gallic acid equivalents. In Figure 5.2, the change in total phenolic content of liposome samples with 0.4% extract was shown. Different from extract degradation results, total phenolic contents of liposome samples for all lecithin concentrations were found similar after the storage period ($p<0.05$). Moreover, as presented in Figures G.3 and G.4, change in total phenolic content of liposomes with 0.1% and 0.2% extracts significantly differred according to the different lecithin concentrations after the storage period ($p<0.05$). In another study, 0.35 mg/ml gallic acid was reported for liposomes including 0.1% grape seed extract and 1% lecithin (Gibis et al., 2013). However, 0.20 mg/ml gallic acid was determined in 0.4% black carrot extract including liposomes in our study. In general, degradation in phenolic compounds were found to be lower than anthocyanin degradation (Figure 5.2 and Table G.1).

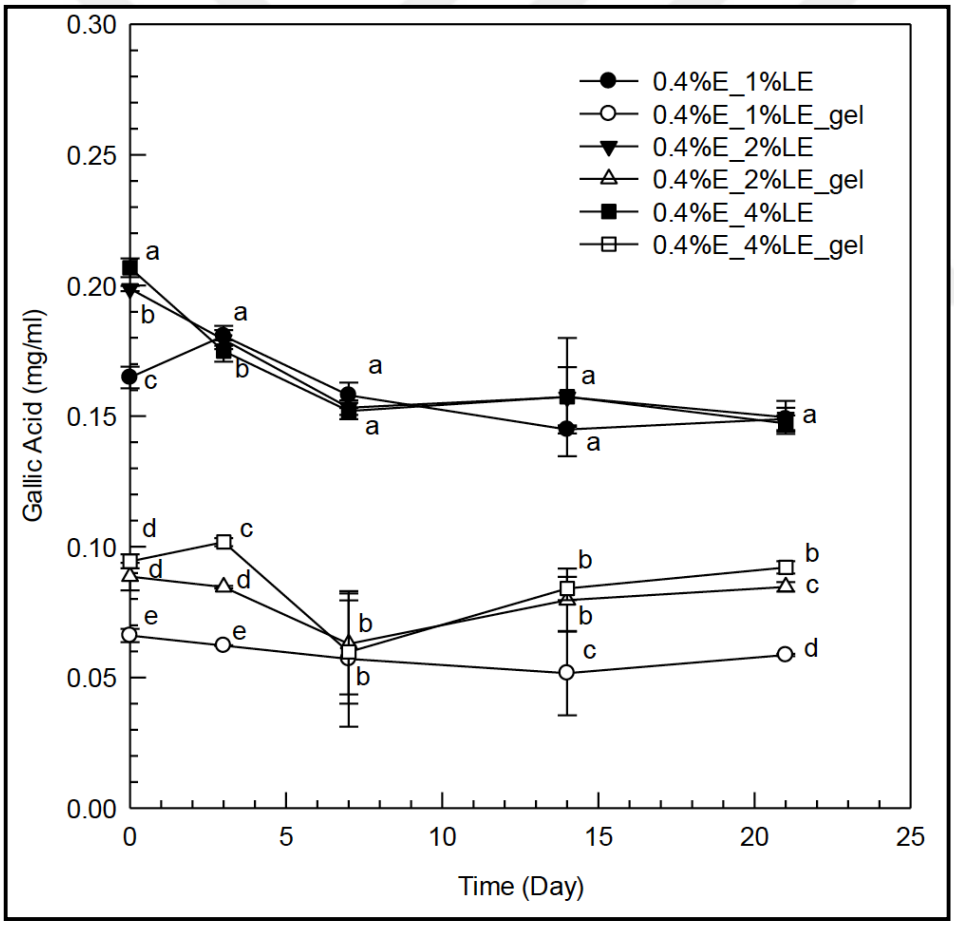


Figure 5.2 : The content of total phenolics in liposome samples with 0.4% extract during storage. Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

Various antioxidants were used to increase oxidation stability of phospholipids such as α-tocopherol and butyl hydroxyl toluene (Grit & Crommelin, 1993). In addition, liposomes can be used to encapsulate both lipid soluble and water soluble antioxidants to enhance the bioavailability of antioxidants (Mozafari et al., 2006). Therefore, antioxidant activity of liposome samples was also measured during the storage period. Antioxidant capacity was found to be the highest in 1% lecithin containing samples among liposome samples with 0.4% extract after 21 days of storage (Figure 5.3 and Table G.1).

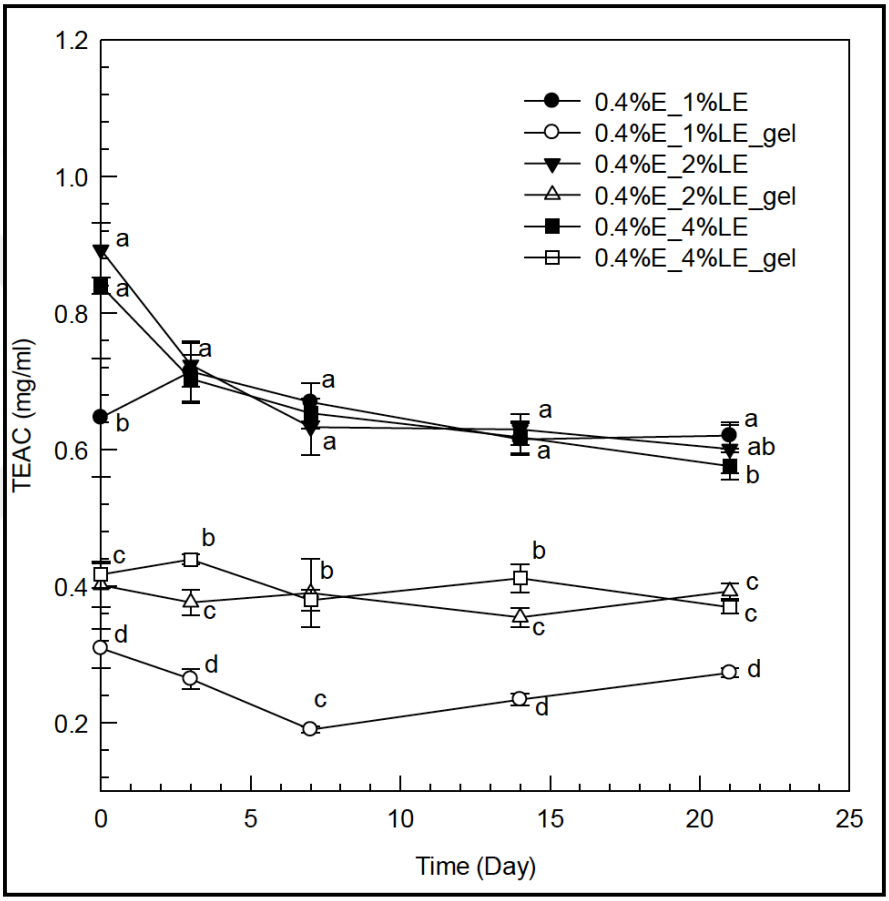


Figure 5.3 : Antioxidant capacity of liposome samples during storage. Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

In Figures G.5 and G.6, degradation in antioxidant activity of 0.1% and 0.2% extract including liposome samples was presented. The degradation trend is similar with 0.4% extract including liposomes. In addition, influence of encapsulation efficiency was determined on gel filtered liposome samples which resulted with higher values in samples with higher lecithin concentration.

5.3.4 Oxidative stability of liposomes

Liposomes composed of different lecithin concentrations (1%, 2%, and 4%) were analyzed for lipid oxidation for 60 days (Figure 5.4 and Table G.2). Hexanal was analyzed as a secondary oxidation product in liposomes. According to the results, extract containing liposomes showed lower hexanal concentrations during storage, while extract free liposomes gave a rapid increase in hexanal concentrations. It is known that anthocyanins may provide 7 times more protection against lipid peroxidation than α-tocopherol (Delgado-Vargas et al., 2000). In addition, the highest protection on oxidation was observed in 1% lecithin including liposomes. Similar to our findings, grape seed extract including liposomes produced with 1% lecithin showed higher stability against lipid oxidation during 140 days of storage (Gibis et al., 2013).

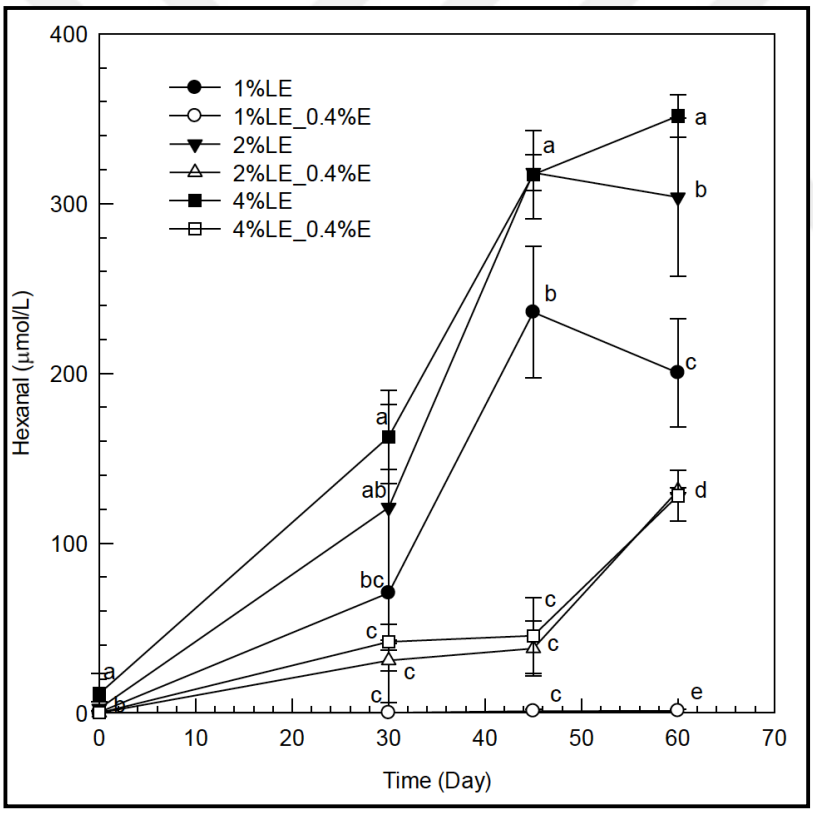


Figure 5.4 : Concentration of hexanal in liposomes with 0.4% extract during 60 days period. Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

The results indicated the inhibition effect of anthocyanins on lipid oxidation. On the other hand, increasing lecithin concentration during liposome formation may increase the encapsulation efficiency but then lipid oxidation may become the primary issue.

5.3.5 Color stability of liposomes

The Hunter color attributes (L*, a*, and b* values) of liposome samples were measured and results for 0.4% extract including liposomes were presented in Figure 5.5 and Table G.3. As known, when L* is close to "0", samples get darker and lighter when L* value is close to "100". As shown in Figure 5.5-A, L* values of 4% lecithin including liposome was found to be the highest. The color of liposomes showed that decrease in redness was mostly detected in 4% lecithin including liposomes (Figure 5.5-B). In addition, yellowness was increased in 4% lecithin including liposomes after 21 days of storage (Figure 5.5-C). These color results are in accordance with biochemical stability analysis results of liposomes. In Figures G.7 and G.8, color of 0.1% and 0.2% extract including liposome samples were also found to be in parallel with the results of 0.4% extract including liposomes.

Visual observations of liposomes including different lecithin concentrations (1%, 2%, and 4%) (Figure 5.6) supported the experimental results. As shown in Figure 5.6, increasing lecithin concentrations decrease the color stability during storage. The relationship between lecithin concentration and color loss can be explained with the degradation of flavylium cation by oxidation products of unsaturated fatty acids such as hydroperoxides (Panya et al., 2010). Similar to those findings, it was reported that hydrogen peroxide can cause decolorization of anthocyanins (Markakis, 1982). In addition, anthocyanins were found to be the most sensitive compounds to hydrogen peroxide bleaching followed by their phenolic levels and antioxidant capacities of pistachio shells (Seeram et al., 2006). Likewise, the loss in anthocyanin was higher than those of total phenolic content and antioxidant capacity of samples in this study. In addition, hexanal formation was inhibited with the extract addition that may indicate quenching of reactive oxygen species by anthocyanins. In another study, quenching ability of anthocyanins for singlet oxygen was correlated with total number of –OR substituents that increase the electron donating ability of anthocyanins (De Rosso et al., 2008). In addition, color bleaching as a result of anthocyanin oxidation may be explained by hydrogen peroxide like oxidation that results with the cleavage of pyrylium ring (Iacobucci & Sweeny, 1983). On the other hand, as a result of self association of anthocyanins higher concentration of extract including liposomes showed higher stability during the storage period (See Figures G.9 and G.10).

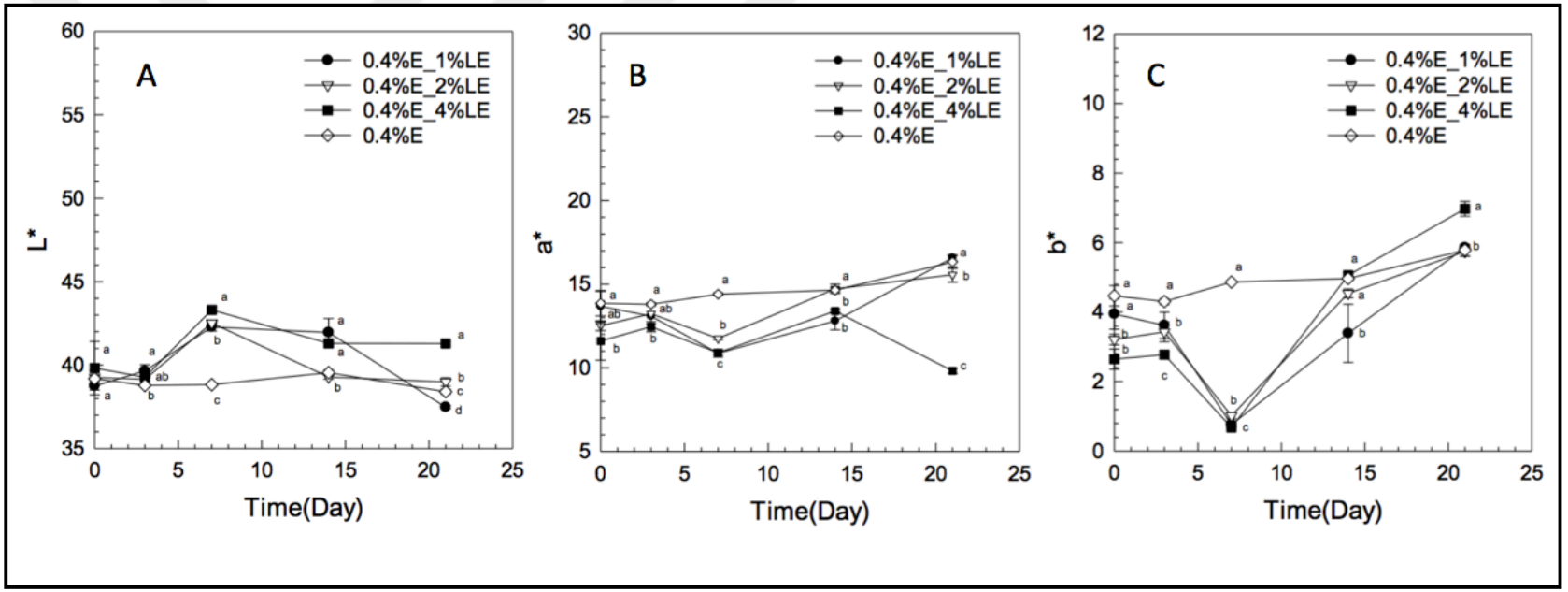


Figure 5.5 : Color attributes of 0.4% extract and liposome samples with 0.4% extract. (A) lightness; (B) redness; (C) yellowness. Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

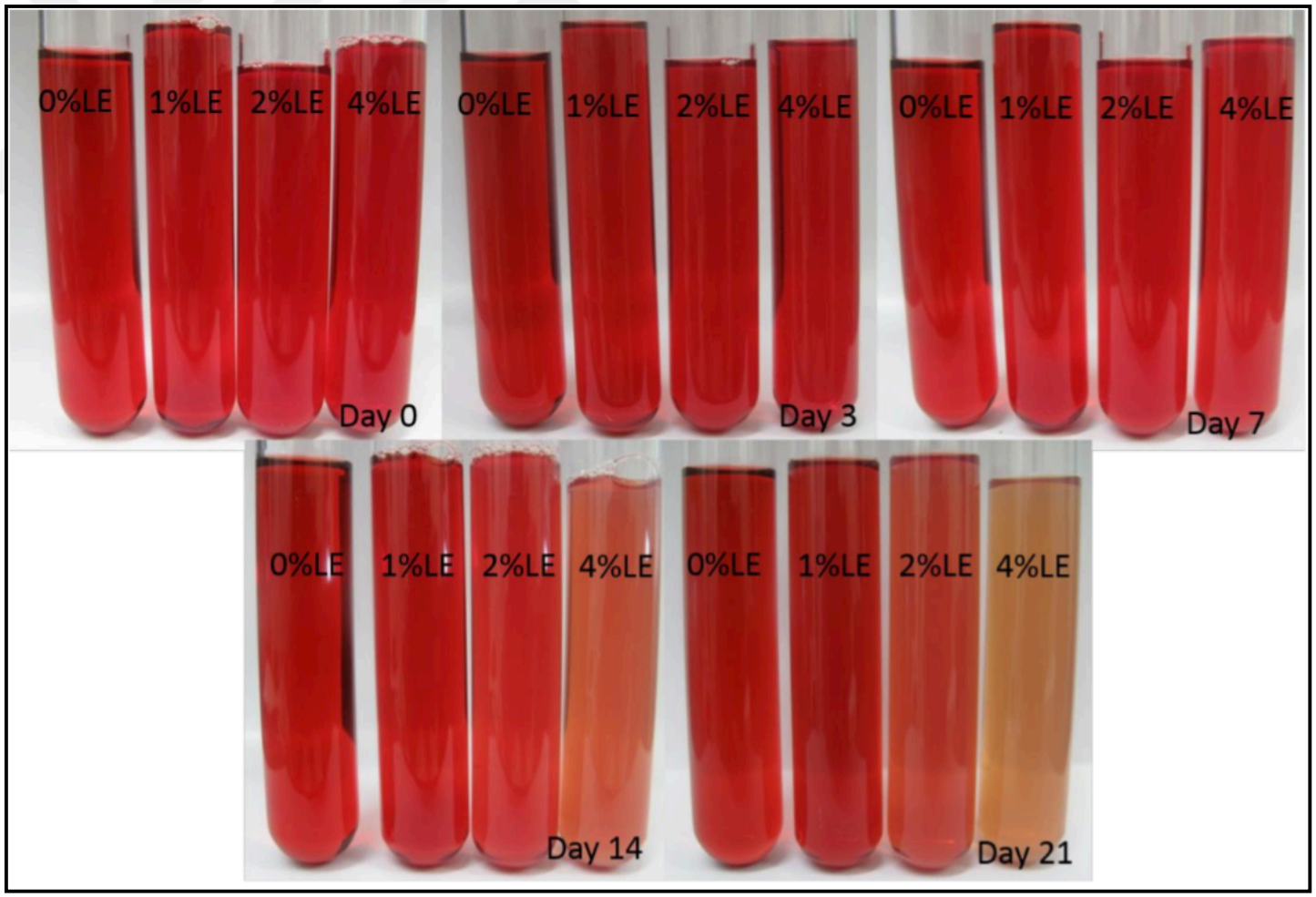


Figure 5.6 : Visual observations of 0.4% extract including liposomes during storage period.

5.4 Conclusions

Liposomes have been mostly used for encapsulation of bioactive compounds. In addition, storage stability of liposomes including unsaturated fatty acids was mostly affected by chemical degradation. In this study, physical and chemical stabilities of extracts containing liposomes with various lecithin contents were demonstrated. The oxidation of polyunsaturated fatty acids may be inhibited with the addition of extract to liposomes, however, lipid content should be limited according to the extract concentration. On the other hand, it should be noted that even though a protection effect on fatty acids is provided, bleaching of anthocyanins is observed due to the degradation of flavylium cation by oxidation products of unsaturated fatty acids. This study provided significant information on the potential limits of liposomes generated from unsaturated fatty acids. Further investigations will be necessary to increase storage stability of liposomes and it may be improved by a polymer coating as a layer on liposomes.

6. CHARACTERIZATION OF SPRAY DRIED LIPOSOMES WITH BLACK CARROT EXTRACT

6.1 Introduction

In food industry, transportation of raw materials is one of the important factors determining the cost of the product. Powder form ensures easy handling and shipping of products. Spray drying is a well-known cost effective process for reducing the storage and transport costs (Gharsallaoui et al., 2007) and for yielding products with higher reconstitution properties. Spray drying process is mostly preferred due to relatively short exposition time of heat sensitive bioactives and thereby prevention their degradation during encapsulation process (Waterhouse et al., 2017). Furthermore, spray drying is a microencapsulation method which provides protection of core material against deteriorative environmental conditions with wall material (Tonon et al., 2010). Different wall materials can be used in spray drying; maltodextrin (Robert et al., 2010; Silva et al., 2013), lactose (Chun et al., 2017; Koç et al., 2011), gelatin (Koç et al., 2011), pullulan (Koç et al., 2011), gum arabic (Silva et al., 2013), casein, β-cyclodextrin (Li et al., 2015; Sun-Waterhouse et al., 2013), alginate (Waterhouse et al., 2017), inulin (Sun-Waterhouse et al., 2013; Waterhouse et al., 2017), hydroxypropylmethycellulose (Sun-Waterhouse et al., 2013), yeast cells (Sultana et al., 2017), soybean protein isolates (Robert et al., 2010), and mesquite gum (Jiménez-Aguilar et al., 2011).

In previous studies, spray drying was performed for encapsulation of various core compounds such as; fish oil (Li et al., 2015), solid lipid nanoparticles (Freitas & Müller, 1998), d-limonene (Sultana et al., 2017), and anthocyanins from blackberry (Weber et al., 2017), blueberry (Jiménez-Aguilar et al., 2011; Waterhouse et al., 2017), jaboticaba (Silva et al., 2013), pomegranate (Robert et al., 2010; Vardin & Yasar, 2012; Yousefi et al., 2011), corozo (Osorio et al., 2010), acai (Tonon et al., 2010), kokum (Nayak & Rastogi, 2010), grape (De Souza et al., 2015; Moreno et al., 2016), black glutinous rice (Laokuldilok & Kanha, 2015), purple corn (Lao & Giusti, 2017), roselle (Idham et al., 2012), black mulberry (Fazaeli et al., 2012), and bayberry (Fang

& Bhandari, 2011). However, stickiness is a common phenomenon in spray-drying of fruit extracts due to intrinsic sugars and acids with low glass transition temperatures (Jaya & Das, 2004; Waterhouse et al., 2017).

Microencapculation of black carrot anthocyanins for producing a powdered form attracted many researchers. Ersus and Yurdagel (2007) studied microencapsulation on black carrot anthocyanins by spray drying and determined the highest anthocyanin retention at 160 °C with maltodextrin as a wall material. (Weber et al., 2017) investigated the stability of spray dried anthocyanin by increasing through copigmentation. As another encapsulation method, liposomes were also examined to encapsulate anthocyanins (Gibis et al., 2014b). Spray drying of liposome solutions was investigated in many studies with a wall material of lactose (Chun et al., 2017), maltodextrin (Gültekin-Özgüven et al., 2016; Karadag et al., 2013), dextran (Kukuchi et al., 1991), calcium alginate (Wang et al., 2015), xanthan gum (Toniazzo et al., 2014), glucose (Kukuchi et al., 1991), and guar gum (Toniazzo et al., 2014). The underlying mechanism for the formation of liposomes and nanoliposomes depend on basically the hydrophilic-hydrophobic interactions between phospholipids and water molecules (Mozafari et al., 2008). In addition, liposomes served primarily as model membrane systems because their bilayer structure could be manufactured to closely resemble the lipid fraction of cell membranes (Taylor et al., 2005).

Liposome methods offer the formation of non-toxic, biodegradable, and small sized capsules (Rashidinejad et al., 2014). In previous chapters (Chapters 3 and 4), protective effect of liposomes on BCE anthocyanins were examined against ascorbic acid related degradation. However, there is no information currently available on the degradation of BCE anthocyanins including liposomes under stress conditions occurring with spray drying process. The objective of this study is to test the hypothesis that liposomal encapsulation combined with a monolayer coating technique using an electrostatic depositioning approach may improve stability of BCE anthocyanins during spray drying.

6.2 Material and methods

6.2.1 Materials

Black carrot extract (BCE) (HF# 2.10592) was provided by Döhler GmbH (AG Darmstadt, Germany). The soy lecithin (Lipoid S75) including 69.3% phosphatidylcholine, 9.8% phosphatidylethanolamine, and 2.1% lysophosphatidylcholine was obtained by Lipoid AG (Ludwigshafen, Germany). Low molecular weight chitosan (purity >78%, degree of acylation 79%, viscocity 103 cP), sodium carbonate anhydrous, Folin– Ciocalteu reagent, Sephadex G-50 (G50300-100 g), copper (II) chloride (CuCl2), ammonium acetate (NH_4Ac), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), sodium azide and neocuproine were obtained from Sigma- Aldrich (St. Louis, USA). Sodium acetate (# 6773.3), acetic acid glacial (# 3738.5), ethanol (# 5054.1) were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Glucidex-21 was donated from Roquette (Lestrem, France). All experiments were performed with double-distilled water.

6.2.2 Preparation of buffer

A sodium acetate buffer solution (pH=3.5 ±0.1, 10mM) was prepared by dissolving 0.217 g sodium acetate and 2.845 g glacial acetic acid in 5L double-distilled water. Microbial growth was prevented by adding sodium azide (0.02% w/w) during acetate buffer preparation (Salminen, Aulbach, Leuenberger, Tedeschi, & Weiss, 2014).

6.2.3 Preparation of primary liposomes

0.1%, 0.2%, and 0.4% BCE solutions were prepared by dissolving in acetate buffer (pH 3.5, 10 mM) as three independent replicates.

The lecithin solutions were prepared by dissolving 2% lecithin as three independent replicates in acetate buffer for free liposomes or in BCE solutions for extract encapsulated liposome samples. Lecithin solutions were stirred overnight to ensure homogeneity. Liposomes were prepared in two step; firstly, lecithin solutions were homogenized with a high shear disperser (Heidolph Silent Crusher M, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 20 000 rpm for 5 min and immediately after liposomes were formed by high-pressure homogenizer (Microfluidizer Processor M-110EH, Microfluidics, Newton, MA, USA) at 22500 psi and 5 times passes with a cooling unit.

6.2.4 Sephadex® gel filtration

Before, antioxidant acitivity, total phenolic content, and anthocyanin retention analyses, Sephadex® gel filtration were used to remove unencapsulated extract from liposome samples. Formation of Sephadex® gel was described in a previous study (Gibis, Thellmann, Thongkaew, & Weiss, 2014). Briefly, 5 ml cartridges filled with 3 cm height of Sephadex gel® to form filter. 1ml liposome samples added to Sephadex gel® and lecithin part separated via centrifugation. Unencapsulated extract was trapped in cartridges and liposome part passed from cartridges. Before analyses, liposome samples and gel-filtered liposomes with and without extract were diluted with ethanol (1:2 v/v) and centrifuged to remove lecithin as described in Guldiken et al. (2017).

6.2.5 Encapsulation efficiency of primary liposomes

Encapsulation efficiency of liposomes was determined by total phenolic content analysis. The amount ratio of phenolic compounds before and after gel filtration was defined as the encapsulation efficiency in percentage.

6.2.6 Preparation of secondary liposomes

Primary liposomes were coated with low molecular weight chitosan. A chitosan stock solution (2%, w/w) was prepared by dissolving chitosan in pH 3.5 acetate buffer and pH of stock solution was adjusted with 1M HCl after overnight stirring at room temperature. Liposome solutions (including 2% lecithin) were coated with chitosan solutions (0.1%-2%) in a ratio 9:1 (v/v).

6.2.7 Spray drying

Maltodextrin (Glucidex dehydrated glucose syrup 21, Roquette, Lestrem, France) stock solution was prepared by dissolving in a pH 3.5 acetate buffer (40% w/w) and stirring overnight at room temperature. The pH of stock solution was adjusted to 3.5 with 1M HCl before use. Primary and secondary liposome solutions with and without BCE extract, BCE extract, BCE extract and 0.1% chitosan solutions were mixed with stock solution of maltodextrin in a ratio 1:1 (v/v) to prepare final solutions with 20% maltodextrin. The pre spray solutions were stirred for one hour prior to transferring to the feeder of a lab scale spray dryer (Mini Spray Dryer B-290, BÜCHI Labortechnik AG, Switzerland) while continously stirring. The spray dryer equipped with 1.5 mm

nozzle atomizer. The spray dryer was operated with inlet temperature at 160°C, outlet temperature at 90°C, feed rate of 2.5 cm^3/min and aspirator capacity of 100%.

6.2.8 Physical characterization of liposomes

The z-average particle diameter of liposome solutions was determined using a dynamic light scattering instrument (Nano ZS, Malvern Instruments, Worcestershire, UK). Liposome samples were diluted up to lecithin concentration as 0.1% (w/w) with acetate buffer to prevent multiple scattering effects. The refractive index for the lecithin and aqueous part were 1.44 and 1.33, respectively. The mean particle diameter (z-average) and polydispersity index (PDI) were reported for freshly prepared samples with three replicates.

At the same dilution ratio, zeta (ζ) potential values of liposome samples were measured *via* dynamic light scattering instrument (Nano ZS, Malvern Instruments, Worcestershire, UK) using Smoluchowsky mathematical model.

6.2.9 Powder characterization

6.2.9.1 Powder distribution

Laser diffraction technique was used to measure particle size distribution of powders. Mie theory was used to calculate size distribution. Mean particle size was measured with refractive index of 1.33, air pressure 2.2 bar, feed rate 60%, and air flow is at maximum by Mastersizer 2000 (Malvern Instruments Ltd, Malvern, UK). Each sample was measured at least 3 times with a measurement time of 10 s. Average and standard deviations were calculated.

6.2.9.2 Water activity

The water activity of the powders was determined at 25 °C using a water activity meter (AWSprint TH-500, Novasina, Lachen, Switzerland) as described by Sramek et al. (2016). Plastic sample dish was filled with powder and placed in the measuring chamber for measurement.

6.2.9.3 Moisture content determination

Karl Fischer titration method was used to determine residual moisture content of spray dried powders as described by Karadag et al. (2013). A Karl Fischer system of

Metrohm (Metrohm, Switzerland), equipped with a Titrino KF 841 and 20 mL burette was used. A two component system containing Hydranal-Solvent and Hydranal-Titrant 5 (both Sigma-Aldrich Laborchemikalien, Germany) was used for measurement.

6.2.9.4 Powder yield

The powder yield was calculated as the ratio of total dry weight of powder after subtraction of moisture content from total weight of powders at the end of spray drying process and the initial weight of solids that used in spray drying process (lecithin, BCE, chitosan, maltodextrin) as shown in following equation (6.1);

$$\text{Yield } (\%) = \frac{\text{total weight of powder after spray drying} * (1 - \text{moisture content of powder})}{\text{total theoretical dry weight of powder}} * 100 \qquad (6.1)$$

6.2.9.5 Scanning electron microscopy

Spray-dried powders were mounted onto separate, adhesive coated aluminium pin stubs and excess powder was removed. The stubs were sputter coated with a thin layer of gold in a Leica vacuum coating unit at 40 mA for 100 s 3 times, at a working distance of 50 mm by using an argon gas purge. The samples were examined using a NeoScope JCM-5000 SEM (Nikon Instruments, Tokyo, Japan) at high vacuum with an accelerating voltage of 10 kV. Images were taken at 500x, 1000x, and 4000x magnifications (Karadag et al., 2013).

6.2.10 Reconstitution of powders

1 g of spray dried samples were dissolved in 4 g acetate buffer (pH 3.5, 10mM) and solutions were stirred overnight to ensure homogeneity (Karadag et al., 2013). Resulted dispersions were used in further analyses.

6.2.11 Particle size distribution of reconstituted powders

Reconstituted samples were analyzed using a laser light diffraction particle size analyzer (LA-950, Horiba, Japan) to measure particle size distribution. This instrument uses Mie Theory to determine the particle size distribution. A refractive index for lecithin of 1.44 and 1.33 for the aqueous phase was used to calculate particle size distributions (Karadag et al., 2013).

6.2.12 Total phenolic retention

Folin-Ciocalteu reagent used to determine total phenolic content of samples using the modified method of Velioglu et al. (1998) as described in Guldiken et al. (2016). Results were reported as mg gallic acid equivalents (GAE)/ml extract (6.2).

$$Retention\ of\ TPC\ (\%) = \frac{Total\ phenolics\ in\ spray\ dried\ powder\ (mg\ GAE\ /L)}{Total\ phenolics\ in\ prespray\ drying\ solution\ (mg\ GAE\ /L)}\ x\ 100 \quad (6.2)$$

6.2.13 Anthocyanin content retention

Anthocyanin amount in liposome samples (6.3) were determined by pH differential method as described in Guldiken et al. (2016).

$$Retention\ of\ AC\ (\%) = \frac{Anthocyanin\ content\ in\ spray\ dried\ powder\ (mg\ GAE\ /L)}{Anthocyanin\ conten\ in\ prespray\ drying\ solution\ (mg\ GAE\ /L)}\ x\ 100 \quad (6.3)$$

6.2.14 Total antioxidant capacity (TAC) retention

Total antioxidant capacity (TAC) was determined using cupric ion reducing antioxidant capacity (CUPRAC) assay (6.4). The antioxidant capacity was determined using the method of Apak et al. (2004 & 2006). Results were reported as mg trolox equivalents (TEAC)/100 ml extract.

$$Retention\ of\ TAC\ (\%) = \frac{TAC\ in\ spray\ dried\ powder\ (mg\ GAE\ /L)}{TAC\ in\ prespray\ drying\ solution\ (mg\ GAE\ /L)}\ x\ 100 \quad (6.4)$$

6.2.15 Storage study

Spray dried powders were stored in sealed plastic cups in desiccators covered with aluminum foil to prevent light at room temperature for 21 days. Sampling of powders were performed in 1st, 7th, 14th, and 21st days of storage.

6.2.16 Data analysis

All experiments were performed at least 3 times for each duplicate sample. The average and standard deviation of all data were calculated using Microsoft Excel for Mac (version 15). Statistical analyses were performed using one-way analysis of variance (ANOVA) (Appendix H), followed by Duncan post hoc test using SPSS software (version 21.0, SPSS, Chicago, IL, USA)

6.3 Results and Discussion

6.3.1 Characterization of liposome dispersions

Visual appearance of primary liposomes with 2% lecithin is presented in Figure 6.1. Selection of the composition of primary liposome was performed according to encapsulation efficiency results of liposomes. The encapsulation efficiencies of liposomes including 2% lecithin with 0.1%, 0.2%, and 0.4% extract were found as 86.6±16.1, 82.2±9.7, and 46.9±6.3, respectively. The extract of 0.2% including liposomes was chosen for primary liposomes considering further dilutions. Similarly, (Gibis et al., 2016) used total phenolic analysis results to detect encapsulation efficiency of liposomes including 1% lecithin and 0.1% grape seed extract. The researchers reported an encapculation efficiency about 88% (Gibis et al., 2016).

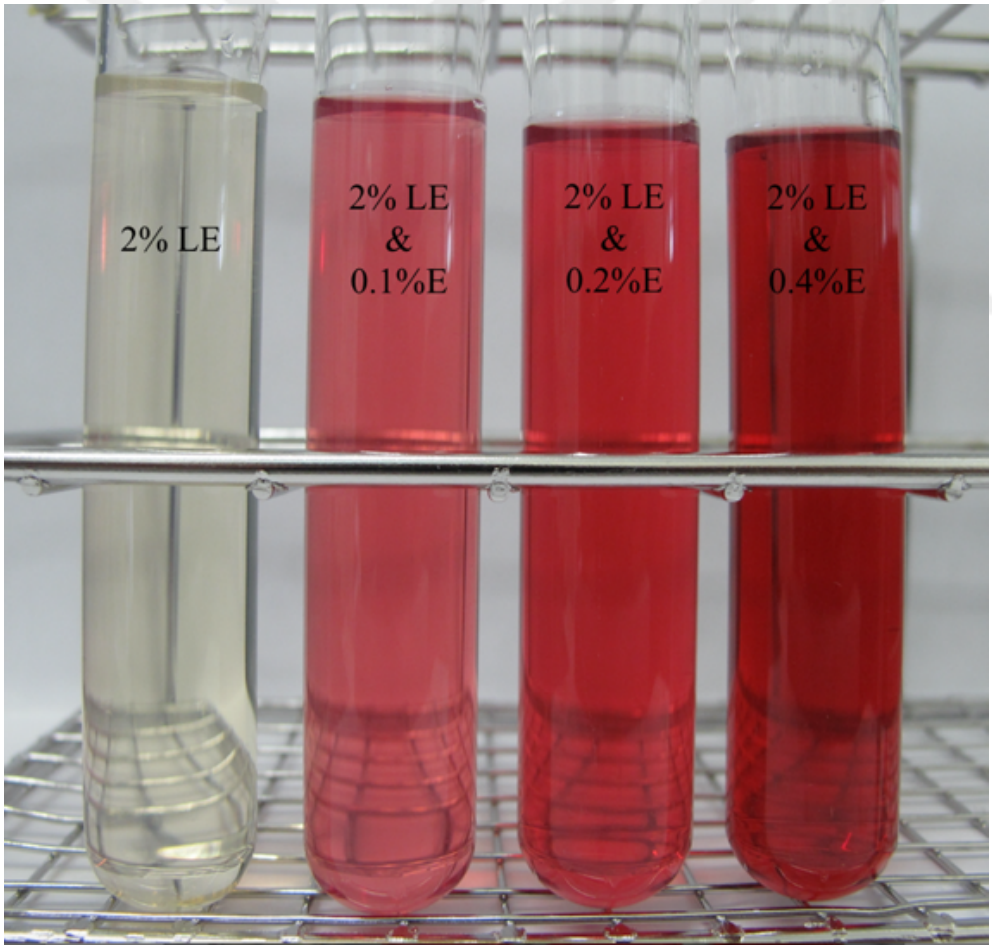


Figure 6.1 : Visual appearance of primary liposomes with 2% lecithin.

Preparation of secondary liposomes were performed with mixing primary liposomes (2% LE) with chitosan solution in different ratios (Figure 6.2). Below a critical chitosan concentration (<0.05%), both visual (Appendix Figure I.1) and numerical (Appendix Table I.1) data show the formation of bridging flocculation and aggregation due to the absence of sufficient polymer concentration to cover liposomes (Karadag et al., 2013). Zeta potential of liposomal solutions increased to 56.4 mV with chitosan concentration 0.2%. The particle diameter was found lowest at 0.1% chitosan concentration and zeta potential of the solution (51.4 mV) was in stable region according to (Wu et al., 2011) (Table I.1). Therefore, it is apparent that secondary liposomes were generated with 0.1% chitosan addition to primary liposomes.

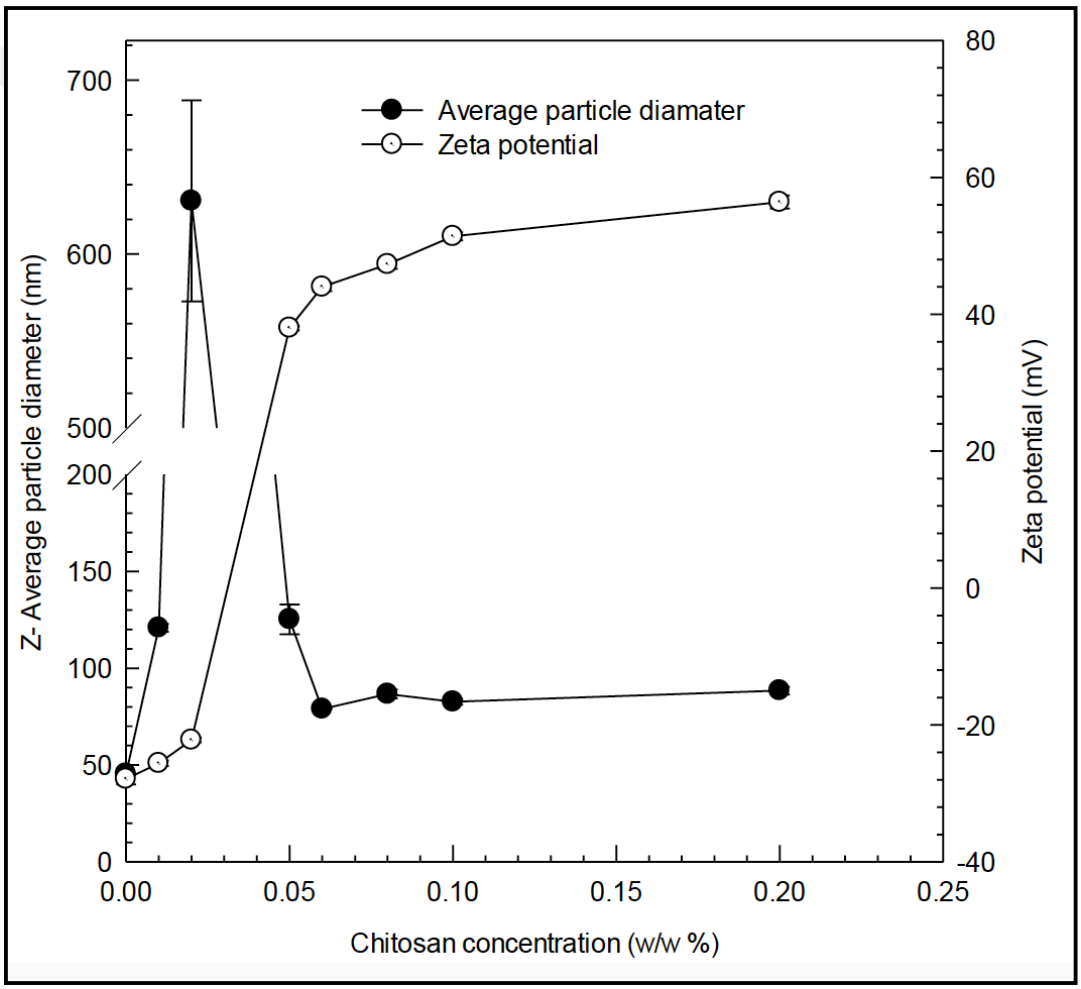


Figure 6.2 : Characterization of primary liposomes (2%LE) mixing with chitosan solution.

6.3.2 Characterization of powders

Drying temperature and concentration of wall material affects moisture content of spray dried powders (De Souza et al., 2015). Moisture contents of dried samples were found in the range of 3.8% and 5.0% (Table 6.1). Similar to our results, moisture content of spray dried Bordo grapes at outlet temperature of 90°C was reported to be between of 4.3% and 4.5% (De Souza et al., 2015). Highest powder yield (52.1%) was found with powders including extract and maltodextrin. In addition, coating of primary liposomes with chitosan resulted in formation of fine powders according to powder dispersion results in Table 6.1. According to Gharsallaoui et al. (2007), morphology of powders are related drying temperature and moisture content. Furthermore, visual appearance of powders of all samples is presented in Figure 6.3.

Table 6.1 : Characterization of powders.

Sample	**Yield**	**Powder Dispersion (µm)**	**Moisture Content (%)**	**Water activity**
BCE	52.1 ± 9.25^{a}	11.5 ± 0.5^{b}	4.5 ± 0.59^{ab}	0.189 ± 0.0113^{a}
Primary	43.8 ± 6.80^{ab}	19.1 ± 5.5^{b}	5.0 ± 0.51^{a}	0.133 ± 0.0025^{b}
BCE- Primary	44.7 ± 3.34^{ab}	30.6 ± 1.9^{a}	3.8 ± 0.88^{c}	0.132 ± 0.0014^{b}
Secondary	36.9 ± 3.36^{b}	17.0 ± 0.6^{b}	4.3 ± 0.11^{b}	0.154 ± 0.0021^{b}
BCE-Secondary	45.9 ± 4.26^{ab}	16.9 ± 0.3^{b}	4.6 ± 0.45^{ab}	0.179 ± 0.0046^{a}
BCE-Chitosan	34.1 ± 5.51^{b}	13.2 ± 0.4^{b}	4.7 ± 0.19^{ab}	0.181 ± 0.0004^{a}

Data represent average values ± standard deviation of two replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

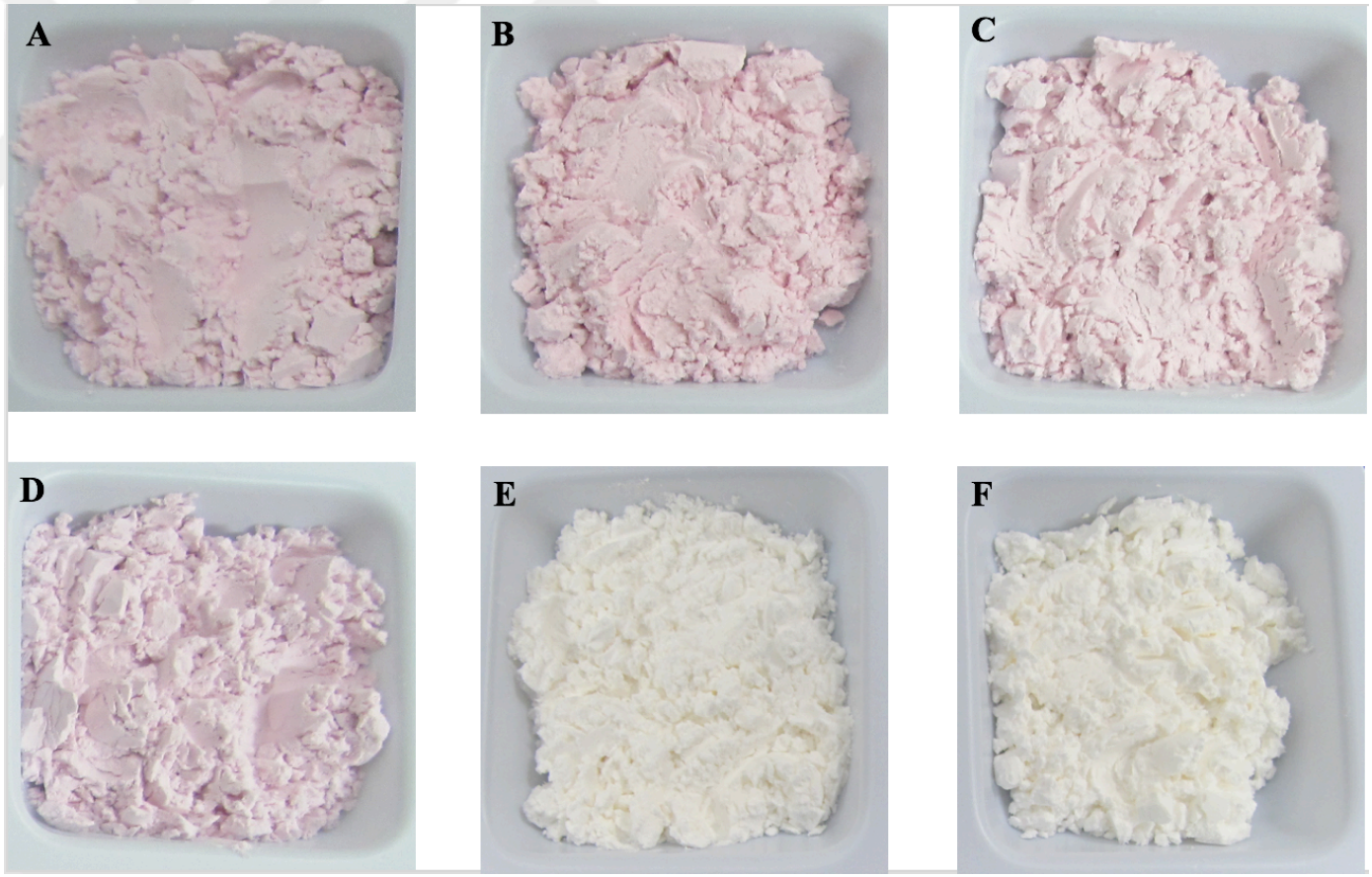


Figure 6.3 : Visual observations of powder samples; A:BCE, B:Primary-BCE; C:Secondary-BCE, D:BCE-Chitosan, E: Primary, F: Secondary liposomes.

All pre spray solutions spray dried under the same conditions. The morphology of spray dried powder samples was examined with their SEM images. According to Figure 6.4, all samples were successfully spray dried without occurrence of aggregation. However, wrinkled surfaces can be detected on SEM images as circled in the Figure 6.4. The presence of surface wrinkles may be associated with a rapid shrinkage of powders due to cooling of expanded particles during hot spray drying process (Waterhouse et al., 2017). The wall material is also an important factor on forming of wrinkles during spray drying of polyphenols, as (Sun-Waterhouse et al., 2013) showed wrinkled surface formation with alginate while smooth surfaces with inulin and β-cyclodextrin. Another wall material, maltodextrin DE24, yielded a smoother surface than tapioca starch (Loksuwan, 2007). On the other hand, it was reported that inlet temperature may also affect powder morphology; Lower inlet temperatures (140°C) generated wrinkled surface but high inlet temperature (180°C) resulted smooth surfaces during spray drying of blackberry powder (Ferrari et al., 2012). In our study, maltodextrin DE21 was used with an inlet temperature of 160°C which was found to be optimum spray drying inlet temperature by Ersus and Yurdagel (2007). As another factor, speed of film formation may also influence the surface characteristics. For instance, slow film formation process during drying and larger particles may lead to surface imperfections (Ré, 1998).

6.3.2.1 Characterization of reconstituted samples

Table 6.2 represents particle size measurements with dynamic light scattering method using Malvern Nano ZS (Malvern Instruments, Worcestershire, UK) to characterize liquid dispersions before and after reconstitution. Before spray drying, particle sizes of primary liposome solutions and maltodextrin containing pre spray solutions were close to each other (48-54 nm). The secondary liposomes were slightly bigger particles (71-83 nm) than primary liposomes (48-52 nm). Similar to our results, particle size of primary liposomes with grape seed extract was reported as 85.9, however 191.7 in secondary liposomes with grape seed extract (Gibis et al., 2016).

Spray dried powders reconstituted with acetate buffer at room temperature. After reconstitution, particle sizes were increased to 2-13 times. The highest particle size (666 nm) was found in BCE included primary liposomes (Table 6.2).

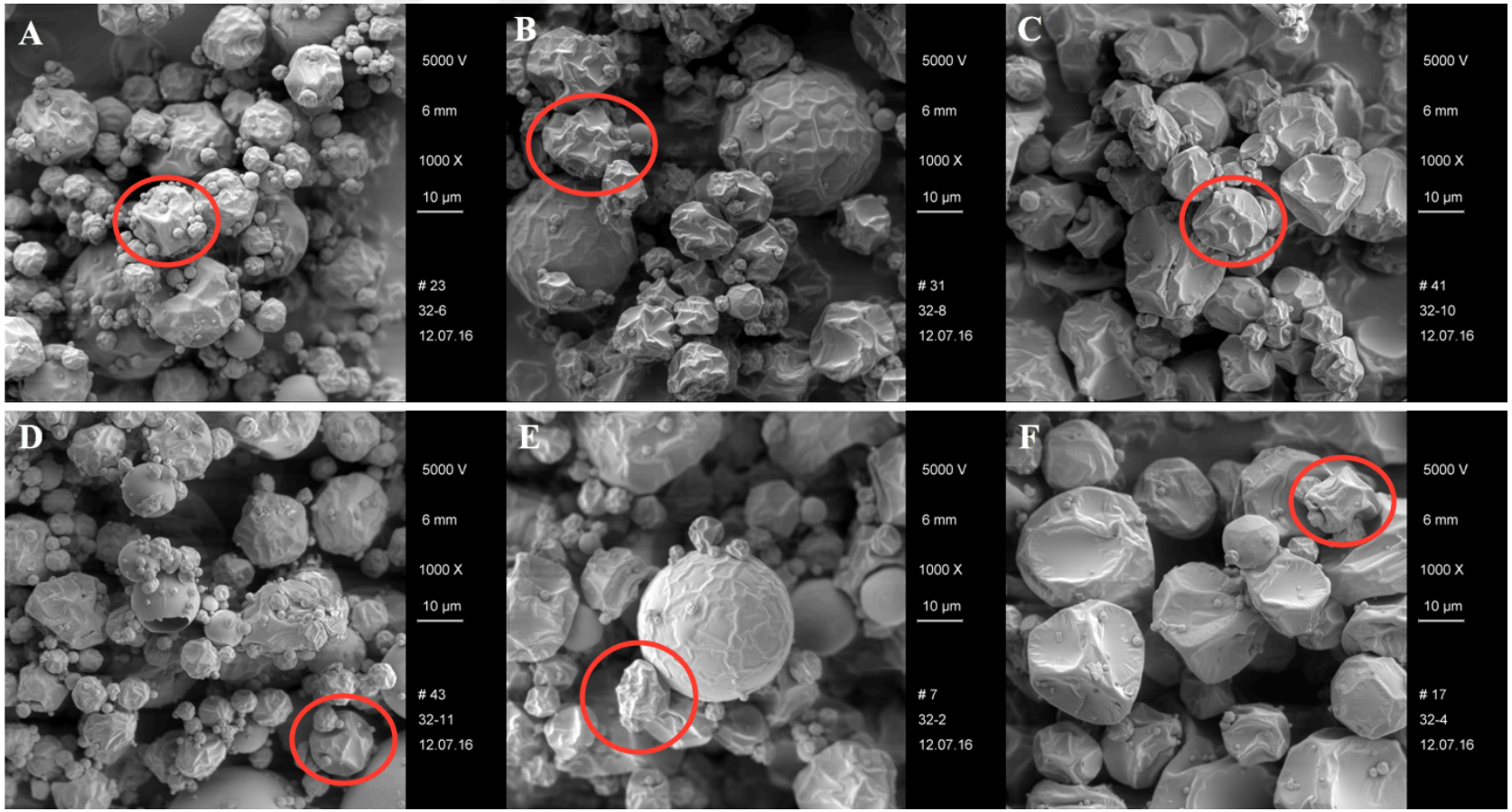


Figure 6.4 : SEM images of powders samples; A:BCE, B:Primary-BCE; C:Secondary-BCE, D:BCE-Chitosan, E: Primary, F: Secondary liposomes (circles show the wrinkled surface example).

Table 6.2 : Characterization of liquid dispersion before and after reconstitution.

Sample		Size (nm)*	PdI	Zeta Potential (mV)
Primary Liposome	Solution	47.7±5.0^{f}	0.282±0.013^{de}	-34.6±5.07^{e}
	Pre spray	52.5±1.2^{f}	0.260±0.006^{e}	-27.3±2.23^{cd}
	Reconstitution	199±16.0^{b}	0.557±0.094^{b}	-23.2±1.47^{c}
Primary Liposome & 0.2%Extract	Solution	51.5±0.58^{f}	0.289±0.007^{de}	-35.0±0.07^{e}
	Pre spray	54.3±0.490^{f}	0.247±0.002^{e}	-32.3±5.69^{de}
	Reconstitution	666.5±251.2	0.665±0.174^{a}	-24.6±0.05^{c}
Secondary Liposome	Solution	83.0±0.49^{d}	0.432±0.003^{bc}	52.5±1.64^{a}
	Pre spray	88.3±2.97^{d}	0.439±0.010^{bc}	50.5±1.04^{a}
	Reconstitution	154.6±8.78^{c}	0.405±0.017^{cd}	32.4±0.16^{b}
Secondary Liposome & 0.2%Extract	Solution	70.9±3.49^{e}	0.422±0.008^{bc}	47.2±1.86^{a}
	Pre spray	92.1±0.99^{d}	0.401±0.007^{cd}	49.4±0.65^{a}
	Reconstitution	209.3±2.93^{a}	0.330±0.009^{cde}	48.85±0.50^{a}

The data presented in this table consists of average values ± standard deviation of two independent batches. Different small letters in the rows represent statistically significant differences ($p<0.05$). *The value obtained by reconstitution of primary liposome with 0.2% extract was accepted as an outlier ($p<0.05$).

Table 6.2 also shows the presence of bigger particles in reconstituted samples due to higher PdI values. For example, the particle size of reconstituted liposome with 0.2% extract was found to be extraordinary which might be not suitable to be measured with dynamic light scattering instrument (Nano ZS, Malvern Instruments, Worcestershire, UK). Therefore, reconstituted samples were also measured using a laser light diffraction particle size analyzer (LA-950, Horiba, Japan). According to this analysis, particle size of primary liposomes with or without BCE was found 1.5±0.2 μm and 1.3±0.1 μm, respectively. On the other hand, particle sizes of secondary liposomes and the ones with BCE were bigger than primary liposomes (5.9±1.8μm and 9.0±2.7 μm, respectively). The particle size distribution results presented in Figures I.2 & I.3 (Appendix I) point out that bigger particles can easily be detected by a laser light diffraction particle size analyzer. Accordingly, primary liposomes with and without BCE showed an long-tailed size distribution. On the other hand, secondary liposomes with and without BCE had a bimodal size distribution revealing the presence of both small and large particles in the aqueous sample. Furthermore, low molecular weight maltodextrin (DE21) addition did not alter particle size and did not cause agglomeration similar to the results of Karadag et al. (2013).

6.3.2.2 Retention of bioactive compounds

Retention of anthocyanin contents, total phenolic contents and antioxidant capacities measured in reconstituted samples after storage period of powders are presented in Figure 6.5 and Table I.2. Retention of bioactive compounds in pre spray solutions were measured in reconstituted powder samples. Accordingly, the highest retention of anthocyanin content (Figure 6.5-A) was found in BCE and BCE-chitosan samples (98.1% and 95.1%) on day zero. However primary and secondary liposome samples gave significantly lower retention percentages by about 62.0-66.5% ($p<0.05$) at the beginning of storage period. During storage the retention of anthocyanin contents decreased in all samples (Appendix Table I.2). The biggest decline was observed with primary and secondary liposome samples approaching to almost half of the initial retention percentages. Similar to our results, high anthocyanin retention was found with maltodextrin as 88%-97% in Bordo grape powders (De Souza et al., 2015). In addition, gum arabic and modified starch mixture (1:1) caused 70% anthocyanin retention in pomegranate powder (De Araujo Santiago et al., 2016). On the other hand, without wall material spray drying of black carrot extract is not possible. Furthermore,

maltodextrin properties may also affect anthocyanin retention that is reported in a study performed by Ersus and Yurdagel (2007) as low DE (10) maltodextrin resulted higher anthocyanin retention after spray drying of black carrot anthocyanins regarding DE21 and DE29 maltodextrins.

Retention of phenolic content of all samples was presented in Figure 6.5-B and Table I.2 (Appendix I). Immediately after spray drying process, the highest retention of total phenolics was found in BCE samples as 88%. During spray drying, the degradation of phenolic compounds reached up to 35% for other samples. Phenolic content of BCE and BCE-chitosan samples did not change ($p<0.05$) during 21 days of storage. On the other hand, at the end of storage period there were no significant differences on retention of phenolic compounds among all samples ($p<0.05$).

After spray drying, the change in antioxidant acitivity of reconstituted samples was presented in Figure 6.5-C and Table I.2 (Appendix). Retention of antioxidant activity was found lowest in BCE-secondary liposome samples as 78%. During storage period, antioxidant acitivity retention of all samples lowered. After 21 days of storage, the highest retention was found in BCE samples (77%) and lowest retention was determined in BCE-secondary liposome samples (52%).

Retention of bayberry phenolics and anthocyanins were reported as 96% and 94%, respectively (Fang & Bhandari, 2011). Our total phenolic content retention results also indicate that maltodextrin yielded high phenolic retention (88%) in BCE samples. However, primary and secondary liposome samples showed lower anthocyanin, phenolic, and antioxidant activity retention after spray drying. This may be resulted due to oxidation of polyunsaturated phospholipids in liposome structure. In addition, chitosan layer on liposome structure did not provide a protection against degradation of bioactive materials. Similarly, lipid oxidation was reported after spray drying of polyunsaturated fatty acid enriched egg powders (Meynier et al., 2014).

Storage of spray dried powders at room temperature caused degradation of bioactive compounds in all samples. Similarly, highest retention of bioactive compounds after 21 days of storage was determined in BCE samples spray dried with maltodextrin. Storage of bayberry powders at room temperature for 6 months resulted decrease in retention of total phenolic and anthocyanin content as 6-9% and 9-37%, respectively (Fang & Bhandari, 2011). In another study, 120 days storage of pomegranate powder

resulted 60% retention of anthocyanins (De Araujo Santiago et al., 2016). Therefore, spray drying of anthocyanin sources can yield high retention without oxidative agents.

6.4 Conclusions

Spray drying of black carrot anthocyanin were evaluated in different encapsulation systems (extract only, primary and secondary liposome systems and chitosan extract mixture). BCE included primary and secondary liposomes were successfully spray dried. All samples were found physically stable after spray drying processing. In addition, SEM images of liposomal systems showed powder without any crack that indicates stability of liposome systems to spray drying. However, liposome samples showed lower stability compared to BCE and BCE and chitosan mixtures according to the biochemical analysis of reconstituted powders. Protection of bioactive compounds in liposome systems needs to be investigated through copigmentation of BCE with other phenolic compounds in a further study.

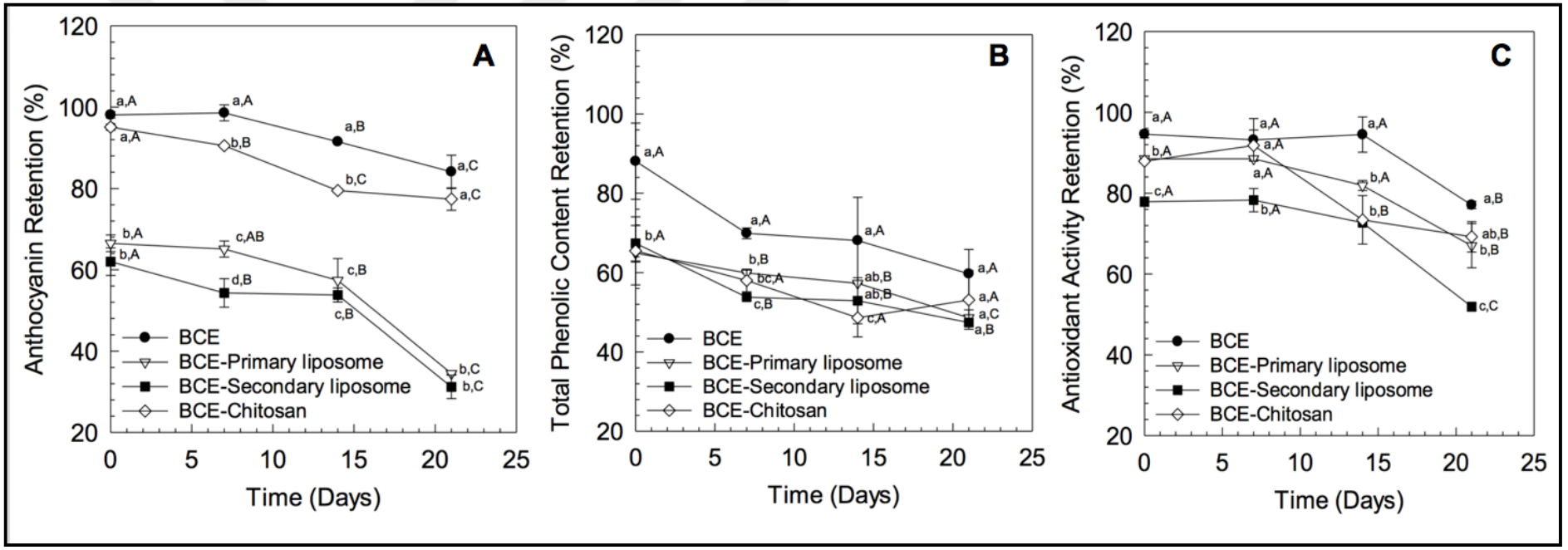


Figure 6.5 : Retention of bioactive compounds in reconstituted samples. The data presented in this table consists of average values ± standard deviation of two independent batches. Different small letters represent statistically significant differences ($p<0.05$) at the same time period. Different capital letters represent statistically significant differences ($p<0.05$) for each sample during storage.

7. CONCLUSIONS

For many years, black carrot crops have cultivated in our land. In functional food concept, black carrots play special role due to their polyphenol content. However, their role in our foods does not only depend on its nutritional value. One of the important nutritional value of black carrots is anthocyanins which can be in different color from red to blue depending on pH. Due to special anthocyanin profile and neutral taste, black carrots may be used as food colorants. Since, acylated anthocyanins are present at high concentration in all anthocyanins, stability of black carrot anthocyanins are high in most process conditions.

In the second chapter, extraction optimization of black carrot anthocyanins were performed. This was important to find optimal extraction conditions to prevent anthocyanins loss from extraction basement. After preliminary experiments pH, solid/solvent ratio, temperature, ethanol concentration was chosen as extraction parameters, and RSM was used to evaluate the effects of these parameters. The importance of this study is to evaluate all of these parameters together. In addition, results showed us; except for color analysis, higher temperature, solvent/solid ratio and ethanol concentration were observed to increase the extraction yield. However, polymeric color results were found to have minimum values at lower pH and solvent/solid ratio, lower or moderate temperature, and higher ethanol concentration . As known, polymeric color results should be kept at minimum and anthocyanin content, antioxidant acitivity, and phenolic content of extracts should be maximize to get high color and polyphenolic stability. Furthermore, taking all these parameters into account, RSM results pointed out the optimum black carrot extraction conditions as pH 3.5, temperature 50 °C, solvent/solid ratio 10, and ethanol concentration 75% which leads to lower polymeric color, and higher anthocyanin content, antioxidant activity, and phenolic content. These values can be also considered in novel extraction methods such as microwave assisted or ultrasound assisted extraction methods. Therefore, in future studies rapid extraction methods can be performed with this initial knowledge about extraction parameters.

In the third and fourth chapter, ascorbic acid related anthocyanin degradation were studied. Ascorbic acid can be found in food and beverage system naturally or added due to its preservation and nutritious effects. However, in previous studies the degradation effect of black carrot and ascorbic acid were not represented in details. Although various methods for preventing ascorbic acid related anthocyanin degradation were investigated in the available literature, no information is available on liposome encapsulation method. In this thesis, the effect of liposomal encapsulation on anthocyanin degradation and color stability study were demonstrated in the presence of ascorbic acid in accelerated storage conditions. According to third chapter results, ascorbic acid related degradation of anthocyanins can be reduced with liposomes. In the fourth chapter, the effect of liposome encapsulation was discussed and the results showed that degradation of anthocyanins decreased in acylated anthocyanins as a result of liposome encapsulation. HPLC analysis of extract and liposomes indicated the possible relationship of acylation and encapsulation. This study revealed the protective effect of liposome encapsulation during the storage time of 24 h on the stability of anthocyanins, known as unstable natural colorants, in the presence of ascorbic acid. To the best of our knowledge, the present study is the first study investigating the effect of liposomal encapsulation on individual anthocyanins. However, interaction of liposomes and individual anthocyanin standards might be of interest to investigate in future studies.

In the fifth chapter, storage stability of liposomes were investigated. According to results, liposomes including unsaturated fatty acids were mostly affected by chemical degradation. In this study, physical and chemical stabilities of extracts containing liposomes with various lecithin contents were demonstrated. On the other hand, the oxidation of polyunsaturated fatty acids may be inhibited with the addition of black carrot extract to liposomes. Unfortunately, it should be noted that even though a protection effect on fatty acids is provided, bleaching of anthocyanins is observed due to the degradation of flavylium cation by oxidation products of unsaturated fatty acids. This study provided significant information on the potential limits of liposomes generated from unsaturated fatty acids. Further investigations will be necessary to increase storage stability of liposomes and it may be improved by a polymer coating as a layer on liposomes or changing lecithin source to saturated phospholipids.

In the sixth chapter, spray drying of black carrot anthocyanin were evaluated in different encapsulation systems (extract only, primary and secondary liposome systems and chitosan extract mixture). Spray drying process was applied as a stress condition on samples. Liposomes were also coated with a layer of chitosan to present layering effect on stability. The results indicated high physical stability however decreased biochemical stability after spray drying of liposomal samples. Liposome samples showed lower stability compared to BCE and BCE and chitosan mixtures according to the biochemical analysis of reconstituted powders during storage of 21 days. Protection of bioactive compounds in liposome systems can be achieved by copigmentation with other phenolic compounds with BCE in a further study.

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APPENDICES

APPENDIX A: Supplementary Materials for Chapter 2.

APPENDIX B: Anova Tables of Chapter 3.

APPENDIX C: Supplementary Materials for Chapter 3.

APPENDIX D: Anova Tables of Chapter 4.

APPENDIX E: Supplementary Materials for Chapter 4.

APPENDIX F: Anova Tables of Chapter 5.

APPENDIX G: Supplementary Materials for Chapter 5.

APPENDIX H: Anova Tables of Chapter 6.

APPENDIX I: Supplementary Materials for Chapter 6.

APPENDIX A: Supplementary Materials for Chapter 2

Table A.1 : DPPH, CUPRAC, Total phenolic content, polymeric color and monomeric anthocyanin results of each run in RSM design.

	DPPH	CUPRAC	Total phenolic content	Polymeric color	Anthocyanin content
Run Order	µmol TEAC/100g fresh black carrot	µmol TEAC/100g fresh black carrot	GAE mg/100g	%	mg cyanidin-3-glycoside/100 g fresh weight
1	905.7	2475.0	247.5	55.7	24.7
2	1027.9	3094.7	327.8	61.8	24.9
3	805.6	2747.6	276.3	64.0	21.0
4	400.0	1128.8	137.5	51.5	12.0
5	431.3	1335.3	170.0	72.0	10.0
6	398.4	1290.6	170.2	70.6	9.1
7	799.4	2780.1	317.8	67.4	19.3
8	1121.0	3255.5	358.7	55.9	31.5
9	1074.1	3211.4	376.4	61.2	24.0
10	784.7	2755.3	253.3	65.7	22.9
11	732.8	2204.9	225.0	58.0	19.9
12	208.3	766.9	109.0	75.2	6.8
13	906.9	2834.6	277.5	62.0	22.6
14	979.9	3328.6	360.8	56.8	31.0
15	952.4	3083.7	328.1	62.6	23.2
16	856.9	2877.3	273.1	61.2	24.6
17	1288.2	4003.4	432.3	50.0	41.8
18	380.0	1172.8	159.6	60.8	9.2
19	1348.1	3026.8	539.9	45.2	46.5
20	1523.8	4207.6	525.0	46.8	45.8
21	1273.6	4092.8	458.7	47.3	45.1
22	1280.6	3983.4	451.6	49.9	38.9
23	1270.2	3575.3	458.0	48.0	44.9
24	769.5	2249.5	226.5	48.8	21.7
25	773.3	2141.2	227.8	56.7	22.3
26	1350.0	3681.9	457.3	40.2	43.5
27	699.5	2828.5	262.1	64.5	25.4
28	362.0	1138.0	135.2	51.3	9.5
29	1373.7	3895.3	458.8	41.9	50.4
30	965.8	3021.5	311.6	58.2	24.8
31	1223.0	3596.5	356.3	63.4	30.9
32	1020.3	2966.7	307.4	70.5	23.3
33	1103.9	3118.2	446.6	71.1	24.6

Table A.1 (continued) : DPPH, CUPRAC, Total phenolic content, polymeric color and monomeric anthocyanin results of each run in RSM design.

34	368.6	1182.7	137.6	50.6	11.5
35	1052.1	2900.8	319.4	61.6	27.4
36	869.7	3328.6	404.5	42.3	38.6
37	1343.3	3697.3	389.4	53.3	41.7
38	1229.3	3468.8	370.8	60.2	35.2
39	466.8	1197.2	168.8	56.4	14.5
40	702.4	2739.2	291.8	63.0	25.5
41	1056.4	3066.2	316.4	69.4	22.7
42	957.6	2987.0	290.4	65.6	22.4
43	1288.8	3633.0	347.8	65.3	27.8
44	1570.4	4481.9	507.2	62.5	51.2
45	1327.2	3880.0	411.2	57.8	36.2
46	943.0	3163.7	307.0	52.6	31.9
47	1263.5	3605.9	371.9	60.1	36.9
48	995.6	3298.1	347.2	74.0	24.3
49	1072.5	3246.3	353.6	59.3	36.1
50	725.8	1969.0	220.0	52.0	22.0
51	243.2	654.1	114.3	75.5	7.7
52	967.7	2997.2	322.6	55.3	33.4
53	1014.9	2715.4	310.4	61.3	23.7
54	1001.9	2367.4	309.0	71.2	22.0
55	973.4	3153.6	297.5	52.2	29.9
56	858.3	2633.4	274.6	70.1	22.9
57	1302.8	3658.3	446.7	57.3	37.6
58	1756.5	4725.7	511.3	44.4	54.6
59	881.1	3441.4	384.2	36.2	38.2
60	1070.0	3052.6	328.8	36.4	39.9
61	426.2	1270.0	133.2	51.1	11.7
62	824.2	2324.4	279.1	62.5	17.5

Table A.2 : ANOVA table of total phenolic content analysis.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	141478	141478	10105.6	14.55	0
Linear	4	63701	66428	16607.1	23.91	0
pH	1	5549	10663	10663.4	15.35	0
Temperature	1	15982	16328	16328.2	23.51	0
Solvent/Solid	1	19568	24432	24431.9	35.17	0
Ethanol	1	22601	24407	24407.5	35.14	0
Square	4	67914	69874	17468.5	25.15	0
pH*pH	1	160	524	523.7	0.75	0.39
Temperature*Temperature	1	2582	93	93	0.13	0.716
Solvent/Solid* Solvent/Solid	1	994	4629	4629	6.66	0.013
Ethanol*Ethanol	1	64178	66271	66271.2	95.4	0
Interaction	6	9864	9864	1644	2.37	0.046
pH*Temperature	1	1188	492	492.3	0.71	0.405
pH*Solvent/Solid	1	1674	3107	3106.8	4.47	0.04
pH*Ethanol	1	3121	4141	4141.5	5.96	0.019
Temperature* Solvent/Solid	1	928	1234	1233.5	1.78	0.19
Temperature*Ethanol	1	291	394	394.3	0.57	0.455
Solvent/Solid* Ethanol	1	2661	2661	2661.4	3.83	0.057
Residual Error	43	29871	29871	694.7		
Lack-of-fit	10	6685	6685	668.5	0.95	0.502
Pure Error	33	23186	23186	702.6		
Total	57	171349				

Table A.3 : ANOVA table of monomeric anthocyanin content analysis.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	20531.2	20531.2	1466.51	38.72	0
Linear	4	12806.4	8170.2	2042.56	53.93	0
pH	1	448.8	278.3	278.31	7.35	0.01
Temperature	1	875.1	2503.7	2503.66	66.1	0
Solvent/Solid	1	363.1	241.5	241.48	6.38	0.016
Ethanol	1	11119.4	4721.1	4721.1	124.65	0
Square	4	6252.6	6131.3	1532.83	40.47	0
pH*pH	1	209.4	188.9	188.94	4.99	0.031
Temperature*Temperature	1	1580.7	2455.7	2455.68	64.83	0
Solvent/Solid* Solvent/Solid	1	44.7	94.5	94.49	2.49	0.122
Ethanol*Ethanol	1	4417.7	4327.9	4327.86	114.26	0
Interaction	6	1472.2	1472.2	245.37	6.48	0
pH*Temperature	1	96.6	56.7	56.67	1.5	0.228
pH*Solvent/Solid	1	244.4	147.6	147.61	3.9	0.055
pH*Ethanol	1	254.6	233.5	233.49	6.16	0.017
Temperature* Solvent/Solid	1	57.4	57.6	57.59	1.52	0.225
Temperature*Ethanol	1	532.5	599.6	599.55	15.83	0
Solvent/Solid* Ethanol	1	286.7	286.7	286.74	7.57	0.009
Residual Error	40	1515.1	1515.1	37.88		
Lack-of-fit	8	321.1	321.1	40.14	1.08	0.404
Pure Error	32	1193.9	1193.9	37.31		
Total	54	22046.2				

Table A.4 : ANOVA table of DPPH analysis.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	5084769	5084769	363198	18.23	0
Linear	4	1517399	1421652	355413	17.84	0
pH	1	229568	78613	78613	3.95	0.054
Temperature	1	451838	328579	328579	16.5	0
Solvent/Solid	1	361404	434416	434416	21.81	0
Ethanol	1	474588	510681	510681	25.64	0
Square	4	3466693	3473747	868437	43.6	0
pH*pH	1	41173	102439	102439	5.14	0.029
Temperature*Temperature	1	739233	393622	393622	19.76	0
Solvent/Solid* Solvent/Solid	1	24602	6055	6055	0.3	0.585
Ethanol*Ethanol	1	2661685	2662695	2662695	133.67	0
Interaction	6	100677	100677	16780	0.84	0.545
pH*Temperature	1	30259	21232	21232	1.07	0.308
pH*Solvent/Solid	1	7457	6506	6506	0.33	0.571
pH*Ethanol	1	149	2472	2472	0.12	0.727
Temperature* Solvent/Solid	1	58469	53956	53956	2.71	0.108
Temperature*Ethanol	1	2365	2372	2372	0.12	0.732
Solvent/Solid* Ethanol	1	1977	1977	1977	0.1	0.754
Residual Error	39	776870	776870	19920		
Lack-of-fit	8	145361	145361	18170	0.89	0.535
Pure Error	31	631509	631509	20371		
Total	53	5861639				

Table A.5 : ANOVA table of CUPRAC analysis.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	15153551	15153551	1082397	20.81	0
Linear	4	10775823	11733126	2933282	56.4	0
pH	1	4543152	3343505	3343505	64.29	0
Temperature	1	3702300	1565146	1565146	30.1	0
Solvent/Solid	1	2519875	2322015	2322015	44.65	0
Ethanol	1	10496	669964	669964	12.88	0.001
Square	4	2257825	2997410	749353	14.41	0
pH*pH	1	218287	158339	158339	3.04	0.091
Temperature*Temperature	1	611622	498627	498627	9.59	0.004
Solvent/Solid* Solvent/Solid	1	892157	610241	610241	11.73	0.002
Ethanol*Ethanol	1	535759	1329888	1329888	25.57	0
Interaction	6	2119904	2119904	353317	6.79	0
pH*Temperature	1	56848	16465	16465	0.32	0.578
pH*Solvent/Solid	1	160164	144450	144450	2.78	0.106
pH*Ethanol	1	1684151	1640908	1640908	31.55	0
Temperature* Solvent/Solid	1	23123	14568	14568	0.28	0.601
Temperature*Ethanol	1	177991	147875	147875	2.84	0.102
Solvent/Solid* Ethanol	1	17626	17626	17626	0.34	0.565
Residual Error	30	1560169	1560169	52006		
Lack-of-fit	4	151210	151210	37802	0.7	0.601
Pure Error	26	1408960	1408960	54191		
Total	44	16713721				

Table A.6 : Concentration (mg/ml) of major anthocyanins by HPLC analysis.

RSM #	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	RSM #	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
1	0.11	0.26	0.08	0.66	0.17	32	0.00	0.00	0.00	0.07	0.01
2	0.09	0.19	0.05	0.56	0.10	33	0.00	0.00	0.00	0.01	0.00
3	0.05	0.10	0.04	0.45	0.11	34	0.01	0.03	0.02	0.24	0.06
4	0.02	0.04	0.03	0.29	0.06	35	0.00	0.00	0.00	0.01	0.00
5	0.02	0.05	0.03	0.34	0.07	36	0.02	0.04	0.01	0.11	0.02
6	0.03	0.06	0.04	0.40	0.09	37	0.04	0.11	0.04	0.43	0.11
7	0.03	0.06	0.02	0.21	0.04	38	0.04	0.08	0.05	0.48	0.10
8	0.05	0.11	0.06	0.53	0.13	39	0.01	0.01	0.01	0.16	0.04
9	0.08	0.18	0.05	0.56	0.12	40	0.00	0.00	0.00	0.04	0.00
10	0.04	0.09	0.05	0.45	0.11	41	0.00	0.00	0.00	0.03	0.01
11	0.12	0.29	0.06	0.69	0.16	42	0.00	0.00	0.00	0.04	0.00
12	0.01	0.03	0.01	0.10	0.02	43	0.03	0.08	0.04	0.40	0.08
13	0.15	0.32	0.08	0.81	0.19	44	0.13	0.25	0.05	0.55	0.13
14	0.04	0.08	0.05	0.46	0.11	45	0.05	0.13	0.06	0.55	0.17
15	0.02	0.05	0.02	0.18	0.04	46	0.05	0.12	0.04	0.52	0.11
16	0.00	0.00	0.01	0.11	0.02	47	0.03	0.06	0.04	0.43	0.09
17	0.09	0.16	0.06	0.58	0.13	48	0.00	0.00	0.00	0.01	0.00
18	0.01	0.02	0.02	0.28	0.06	49	0.02	0.04	0.02	0.31	0.06
19	0.05	0.09	0.05	0.46	0.10	50	0.07	0.17	0.04	0.37	0.10
20	0.05	0.10	0.04	0.44	0.10	51	0.01	0.02	0.01	0.20	0.05
21	0.10	0.22	0.06	0.65	0.15	52	0.00	0.00	0.00	0.06	0.01
22	0.04	0.08	0.04	0.39	0.08	53	0.01	0.03	0.02	0.25	0.06
23	0.04	0.08	0.05	0.47	0.10	54	0.00	0.00	0.00	0.03	0.00
24	0.09	0.19	0.07	0.69	0.14	55	0.03	0.07	0.03	0.43	0.10
25	0.05	0.11	0.04	0.33	0.08	56	0.02	0.04	0.03	0.29	0.04
26	0.05	0.09	0.05	0.51	0.13	57	0.01	0.03	0.02	0.24	0.06
27	0.09	0.19	0.05	0.60	0.13	58	0.13	0.24	0.05	0.54	0.13
28	0.02	0.05	0.03	0.37	0.08	59	0.04	0.09	0.04	0.43	0.08
29	0.03	0.05	0.03	0.34	0.08	60	0.03	0.08	0.03	0.47	0.10
30	0.00	0.00	0.01	0.11	0.02	61	0.00	0.00	0.01	0.07	0.01
31	0.03	0.05	0.03	0.35	0.08	62	0.03	0.05	0.03	0.30	0.07

Peak1: cyanidin-3-xylosyl (glucosyl) galactoside, Peak 2: cyanidin-3-xylosyl galactoside, Peak 3: cyanidin-3-xylosyl (sinapoylglucosyl) galactoside, Peak 4: cyanidin-3-xylosyl (feruloylglucosyl) galactoside, Peak 5: cyanidin-3-xylosyl (coumaroylglucosyl) galactoside.

Table A.7 : ANOVA table of HPLC analysis.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	9.3265	9.32647	0.66618	17.04	0
Linear	4	3.6221	4.08817	1.02204	26.15	0
pH	1	0.9645	0.89925	0.89925	23.01	0
Temperature	1	0.3587	0.40499	0.40499	10.36	0.003
Solvent/Solid	1	0.0006	0.06968	0.06968	1.78	0.191
Ethanol	1	2.2983	2.41794	2.41794	61.86	0
Square	4	3.342	3.16174	0.79043	20.22	0
pH*pH	1	0.3452	0.63674	0.63674	16.29	0
Temperature*Temperature	1	0.2607	0.05805	0.05805	1.49	0.232
Solvent/Solid* Solvent/Solid	1	0.2143	0.55708	0.55708	14.25	0.001
Ethanol*Ethanol	1	2.5218	2.39122	2.39122	61.17	0
Interaction	6	2.3624	2.36242	0.39374	10.07	0
pH*Temperature	1	0.0715	0.00078	0.00078	0.02	0.889
pH*Solvent/Solid	1	0.0019	0.07069	0.07069	1.81	0.188
pH*Ethanol	1	0.0143	0.02841	0.02841	0.73	0.4
Temperature* Solvent/Solid	1	0.1416	0.26991	0.26991	6.9	0.013
Temperature*Ethanol	1	0.2022	0.08841	0.08841	2.26	0.142
Solvent/Solid* Ethanol	1	1.931	1.93095	1.93095	49.4	0
Residual Error	33	1.2899	1.28993	0.03909		
Lack-of-fit	9	0.2868	0.28685	0.03187	0.76	0.651
Pure Error	24	1.0031	1.00308	0.04179		
Total	47	10.6164				

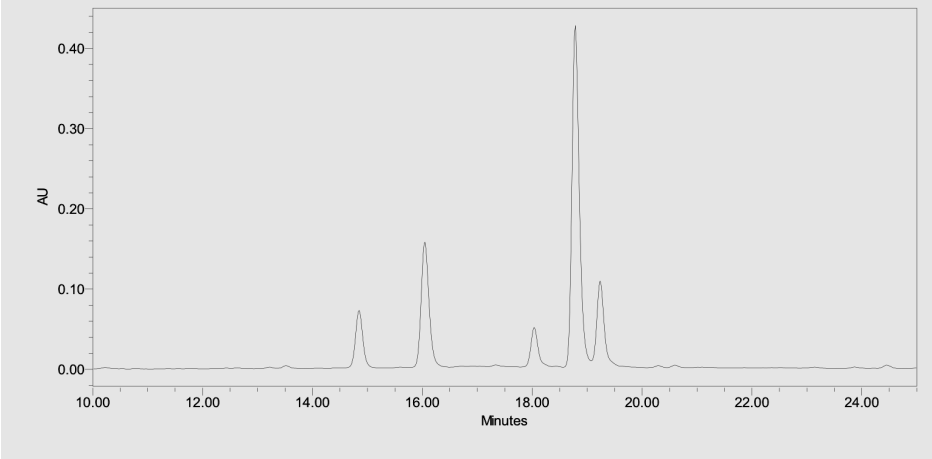

Figure A.1 : Concentration (mg/ml) of major anthocyanins by HPLC analysis.

Table A.8 : ANOVA table of polymeric color analysis.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	7666.06	7666.06	547.58	97.37	0
Linear	4	6784.31	6646.59	1661.65	295.46	0
pH	1	754.29	754.29	754.29	134.12	0
Temperature	1	383.91	486.42	486.42	86.49	0
Solvent/Solid	1	148.29	135.53	135.53	24.1	0
Ethanol	1	5497.81	5474.76	5474.76	973.47	0
Square	4	374.36	374.36	93.59	16.64	0
pH*pH	1	78.52	93.21	93.21	16.57	0
Temperature*Temperature	1	178.3	186.84	186.84	33.22	0
Solvent/Solid* Solvent/Solid	1	21.74	31.79	31.79	5.65	0.023
Ethanol*Ethanol	1	95.8	95.8	95.8	17.03	0
Interaction	6	507.39	507.39	84.57	15.04	0
pH*Temperature	1	0.14	0.14	0.14	0.03	0.874
pH*Solvent/Solid	1	5.96	5.96	5.96	1.06	0.31
pH*Ethanol	1	17.3	17.3	17.3	3.08	0.088
Temperature* Solvent/Solid	1	7.95	7.95	7.95	1.41	0.242
Temperature*Ethanol	1	473.7	473.7	473.7	84.23	0
Solvent/Solid* Ethanol	1	2.34	2.34	2.34	0.42	0.523
Residual Error	38	213.71	213.71	5.62		
Lack-of-fit	7	64.02	64.02	9.15	1.89	0.105
Pure Error	31	149.69	149.69	4.83		
Total	52	7879.77				

Table A.9 : Hunter color parameters L*, a*, b* of black carrot extracts according to RSM design.

Run #	L*	a*	b*	Run #	L*	a*	b*
1	27.9±0.04	14.3±0.02	-7.2±0.01	32	26.6±0.02	12.9±0.07	-3.8±0.05
2	25.9±0.05	9.6±0.02	-5.6±0.03	33	29.1±0.05	11.7±0.07	2.4±0.09
3	26.7±0.02	10.8±0.01	-6.0±0.01	34	26.6±0.02	14.2±0.02	-2.9±0.03
4	26.5±0.04	9.1±0.04	-6.1±0.03	35	27.1±0.02	15.0±0.04	-6.3±0.02
5	27.7±0.02	13.8±0.04	-7.4±0.02	36	29.7±0.05	22.0±0.03	2.4±0.02
6	29.5±0.05	18.4±0.02	-1.3±0.01	37	25.4±0.06	9.9±0.09	-5.3±0.01
7	26.6±0.02	9.5±0.08	-6.0±0.02	38	25.6±0.06	10.8±0.05	-5.2±0.01
8	26.7±0.18	10.4±0.06	-6.0±0.03	39	26.4±0.05	14.0±0.04	-6.5±0.05
9	24.9±0.01	10.4±0.06	-4.7±0.08	40	25.1±0.02	10.7±0.01	-4.5±0.05
10	25.6±0.02	11.2±0.04	-5.3±0.03	41	27.2±0.11	14.0±0.05	-4.0±0.02
11	25.5±0.01	9.9±0.01	-5.1±0.01	42	24.8±0.03	8.1±0.03	-5.1±0.02
12	29.0±0.01	19.7±0.04	-6.2±0.02	43	26.3±0.04	14.8±0.07	-5.0±0.03
13	26.5±0.04	13.7±0.04	-3.1±0.09	44	25.7±0.07	11.5±0.10	-5.1±0.03
14	25.5±0.05	11.0±0.03	-5.3±0.04	45	25.4±0.15	10.8±0.07	-5.2±0.05
15	24.3±0.03	4.9±0.02	-6.2±0.05	46	29.4±0.02	21.8±0.05	0.8±0.04
16	25.1±0.06	10.5±0.05	-4.5±0.04	47	25.6±0.02	11.7±0.04	-5.3±0.01
17	26.9±0.02	17.3±0.06	-3.5±0.03	48	29.0±0.05	11.9±0.03	2.4±0.05
18	29.2±0.04	21.3±0.10	0.7±0.05	49	25.5±0.03	11.2±0.01	-5.2±0.01
19	25.4±0.00	10.5±0.05	-5.3±0.02	50	25.5±0.01	11.2±0.02	-5.3±0.02
20	25.4±0.02	10.9±0.02	-5.3±0.03	51	29.9±0.05	20.6±0.05	-6.5±0.04
21	27.1±0.03	17.8±0.02	-3.4±0.02	52	27.5±0.07	17.9±0.10	-2.9±0.02
22	24.6±0.00	9.1±0.02	-5.3±0.05	53	24.1±0.03	5.1±0.04	-5.9±0.58
23	25.5±0.04	10.7±0.04	-5.3±0.02	54	24.9±0.00	8.8±0.05	-4.8±0.03
24	25.6±0.02	10.5±0.05	-5.1±0.02	55	29.4±0.03	21.7±0.07	0.8±0.04
25	25.5±0.03	10.6±0.03	-5.3±0.03	56	25.5±0.11	11.0±0.08	-5.4±0.05
26	25.6±0.06	10.9±0.04	-5.4±0.06	57	24.6±0.06	8.8±0.03	-5.4±0.06
27	26.5±0.03	14.0±0.07	-3.0±0.02	58	25.8±0.06	12.4±0.06	-5.1±0.04
28	26.9±0.08	15.2±0.11	-2.4±0.02	59	29.1±0.03	20.7±0.05	1.3±0.09
29	25.5±0.03	10.5±0.05	-5.3±0.02	60	26.7±0.04	14.5±0.11	-6.2±0.05
30	27.2±0.04	16.7±0.14	-2.9±0.06	61	25.4±0.06	9.6±0.07	-5.3±0.06
31	26.3±0.03	15.4±0.03	-4.6±0.01	62	27.0±0.10	15.0±0.10	-5.7±0.01

APPENDIX B: Anova Tables of Chapter 3

Table B.1 : Statistical analysis results of 0.1% extract samples with various ascorbic acid content for 24 hours.

Sample		Sum of Squares	df	Mean Square	F	Sig.
0.1%E	Between Groups	0.004	7	0.001	0.525	0.803
	Within Groups	0.019	16	0.001		
	Total	0.023	23			
0.1%E & 0.01%AA	Between Groups	0.139	7	0.020	17.297	0.000
	Within Groups	0.018	16	0.001		
	Total	0.157	23			
0.1%E & 0.025%AA	Between Groups	0.328	7	0.047	9.410	0.000
	Within Groups	0.080	16	0.005		
	Total	0.408	23			
0.1%E & 0.05%AA	Between Groups	0.802	7	0.115	11.681	0.000
	Within Groups	0.157	16	0.010		
	Total	0.958	23			
0.1% & 0.1%AA	Between Groups	1.240	7	0.177	16.426	0.000
	Within Groups	0.173	16	0.011		
	Total	1.413	23			
Time (hour)		Sum of Squares	df	Mean Square	F	Sig.
0	Between Groups	0.000	4	0.000	0.072	0.989
	Within Groups	0.002	10	0.000		
	Total	0.002	14			
1	Between Groups	0.011	4	0.003	2.908	0.078
	Within Groups	0.009	10	0.001		
	Total	0.020	14			
3	Between Groups	0.095	4	0.024	4.759	0.021
	Within Groups	0.050	10	0.005		
	Total	0.145	14			
6	Between Groups	0.129	4	0.032	1.141	0.392
	Within Groups	0.283	10	0.028		
	Total	0.413	14			
9	Between Groups	0.424	4	0.106	36.008	0.000
	Within Groups	0.029	10	0.003		
	Total	0.453	14			
12	Between Groups	0.558	4	0.139	67.598	0.000
	Within Groups	0.021	10	0.002		
	Total	0.578	14			
18	Between Groups	0.645	4	0.161	36.600	0.000
	Within Groups	0.044	10	0.004		
	Total	0.689	14			
24	Between Groups	0.745	4	0.186	236.872	0.000
	Within Groups	0.008	10	0.001		
	Total	0.753	14			

Table B.2 : Statistical analysis results of characterization of liposome samples.

Analyses		Sum of Squares	df	Mean Square	F	Sig.
Particle size	Between Groups	74.503	11	6.773	4.480	0.001
	Within Groups	36.284	24	1.512		
	Total	110.786	35			
PdI	Between Groups	0.021	11	0.002	1.671	0.142
	Within Groups	0.028	24	0.001		
	Total	0.049	35			
Zeta potential	Between Groups	41.607	11	3.782	2.161	0.056
	Within Groups	42.005	24	1.750		
	Total	83.612	35			
Encapsulation Efficiency	Between Groups	1046.494	8	130.812	8.476	0.000
	Within Groups	277.786	18	15.433		
	Total	1324.280	26			

Table B.3 : Statistical analysis results of 1% lecithin with 0.1% extract liposome samples with various ascorbic acid content for 24 hours.

Sample		Sum of Squares	df	Mean Square	F	Sig.
0.1%E -1%LE	Between Groups	0.035	7	0.005	4.574	0.006
	Within Groups	0.018	16	0.001		
	Total	0.053	23			
0.1%E -1%LE & 0.01%AA	Between Groups	0.199	7	0.028	25.733	0.000
	Within Groups	0.018	16	0.001		
	Total	0.217	23			
0.1%E -1%LE & 0.025%AA	Between Groups	0.387	7	0.055	28.650	0.000
	Within Groups	0.031	16	0.002		
	Total	0.418	23			
0.1%E -1%LE & 0.05%AA	Between Groups	0.519	7	0.074	67.628	0.000
	Within Groups	0.016	15	0.001		
	Total	0.535	22			
0.1%E -1%LE & 0.1%AA	Between Groups	0.830	7	0.119	88.056	0.000
	Within Groups	0.022	16	0.001		
	Total	0.852	23			
Time (hour)		Sum of Squares	df	Mean Square	F	Sig.
0	Between Groups	0.000	4	0.000	0.115	0.974
	Within Groups	0.008	10	0.001		
	Total	0.009	14			
1	Between Groups	0.000	4	0.000	0.036	0.997
	Within Groups	0.014	10	0.001		
	Total	0.014	14			
3	Between Groups	0.001	4	0.000	0.438	0.779
	Within Groups	0.008	10	0.001		
	Total	0.010	14			
6	Between Groups	0.030	4	0.007	4.299	0.028
	Within Groups	0.017	10	0.002		
	Total	0.047	14			
9	Between Groups	0.055	4	0.014	11.047	0.001
	Within Groups	0.012	10	0.001		
	Total	0.067	14			
12	Between Groups	0.163	4	0.041	31.042	0.000
	Within Groups	0.013	10	0.001		
	Total	0.176	14			
18	Between Groups	0.203	4	0.051	18.807	0.000
	Within Groups	0.024	9	0.003		
	Total	0.227	13			
24	Between Groups	0.267	4	0.067	107.675	0.000
	Within Groups	0.006	10	0.001		
	Total	0.273	14			

Table B.4 : Statistical analysis results of 2% lecithin with 0.1% extract liposome samples with various ascorbic acid content for 24 hours.

Sample		Sum of Squares	df	Mean Square	F	Sig.
0.1%E -2%LE	Between Groups	0.032	7	0.005	2.315	0.078
	Within Groups	0.031	16	0.002		
	Total	0.063	23			
0.1%E -2%LE & 0.01%AA	Between Groups	0.117	7	0.017	3.689	0.015
	Within Groups	0.072	16	0.005		
	Total	0.189	23			
0.1%E -2%LE & 0.025%AA	Between Groups	0.189	7	0.027	6.958	0.001
	Within Groups	0.062	16	0.004		
	Total	0.252	23			
0.1%E -2%LE & 0.05%AA	Between Groups	0.342	7	0.049	13.088	0.000
	Within Groups	0.060	16	0.004		
	Total	0.402	23			
0.1%E -2%LE & 0.1%AA	Between Groups	0.443	7	0.063	17.838	0.000
	Within Groups	0.057	16	0.004		
	Total	0.500	23			
Time (hour)		Sum of Squares	df	Mean Square	F	Sig.
0	Between Groups	0.001	4	0.000	0.069	0.990
	Within Groups	0.032	10	0.003		
	Total	0.033	14			
1	Between Groups	0.000	4	0.000	0.022	0.999
	Within Groups	0.021	10	0.002		
	Total	0.021	14			
3	Between Groups	0.000	4	0.000	0.043	0.996
	Within Groups	0.016	10	0.002		
	Total	0.017	14			
6	Between Groups	0.007	4	0.002	0.534	0.714
	Within Groups	0.034	10	0.003		
	Total	0.041	14			
9	Between Groups	0.021	4	0.005	2.414	0.118
	Within Groups	0.022	10	0.002		
	Total	0.043	14			
12	Between Groups	0.071	4	0.018	6.492	0.008
	Within Groups	0.027	10	0.003		
	Total	0.098	14			
18	Between Groups	0.104	4	0.026	2.970	0.074
	Within Groups	0.087	10	0.009		
	Total	0.191	14			
24	Between Groups	0.155	4	0.039	9.173	0.002
	Within Groups	0.042	10	0.004		
	Total	0.197	14			

Table B.5 : Statistical analysis results of 4% lecithin with 0.1% extract liposome samples with various ascorbic acid content for 24 hours.

Sample		Sum of Squares	df	Mean Square	F	Sig.
0.1%E -4%LE	Between Groups	0.036	7	0.005	5.131	0.003
	Within Groups	0.016	16	0.001		
	Total	0.053	23			
0.1%E -4%LE & 0.01%AA	Between Groups	0.061	7	0.009	8.002	0.000
	Within Groups	0.017	16	0.001		
	Total	0.078	23			
0.1%E -4%LE & 0.025%AA	Between Groups	0.091	7	0.013	10.773	0.000
	Within Groups	0.019	16	0.001		
	Total	0.110	23			
0.1%E -4%LE & 0.05%AA	Between Groups	0.136	7	0.019	19.770	0.000
	Within Groups	0.016	16	0.001		
	Total	0.152	23			
0.1%E -4%LE & 0.1%AA	Between Groups	0.166	7	0.024	16.886	0.000
	Within Groups	0.022	16	0.001		
	Total	0.189	23			
Time (hour)		Sum of Squares	df	Mean Square	F	Sig.
0	Between Groups	0.000	4	0.000	0.195	0.936
	Within Groups	0.006	10	0.001		
	Total	0.006	14			
1	Between Groups	0.000	4	0.000	0.086	0.985
	Within Groups	0.004	10	0.000		
	Total	0.004	14			
3	Between Groups	0.000	4	0.000	0.191	0.938
	Within Groups	0.005	10	0.001		
	Total	0.006	14			
6	Between Groups	0.000	4	0.000	0.073	0.989
	Within Groups	0.017	10	0.002		
	Total	0.017	14			
9	Between Groups	0.002	4	0.000	0.518	0.725
	Within Groups	0.008	10	0.001		
	Total	0.010	14			
12	Between Groups	0.011	4	0.003	3.950	0.036
	Within Groups	0.007	10	0.001		
	Total	0.018	14			
18	Between Groups	0.018	4	0.004	1.212	0.365
	Within Groups	0.036	10	0.004		
	Total	0.054	14			
24	Between Groups	0.026	4	0.007	8.484	0.003
	Within Groups	0.008	10	0.001		
	Total	0.034	14			

Table B.6 : Statistical analysis results of liposome samples with 0.1% ascorbic acid content for 24 hours.

Sample		Sum of Squares	df	Mean Square	F	Sig.
Extract	Between Groups	0.919	5	0.184	3993.553	0.000
	Within Groups	0.001	12	0.000		
	Total	0.920	17			
Liposome	Between Groups	0.072	5	0.014	1110.264	0.000
	Within Groups	0.000	12	0.000		
	Total	0.073	17			
Gel filtered liposome	Between Groups	0.054	5	0.011	501.336	0.000
	Within Groups	0.000	12	0.000		
	Total	0.054	17			
Time (hour)		Sum of Squares	df	Mean Square	F	Sig.
0	Between Groups	0.007	2	0.003	125.857	0.000
	Within Groups	0.000	6	0.000		
	Total	0.007	8			
3	Between Groups	0.002	2	0.001	45.348	0.000
	Within Groups	0.000	6	0.000		
	Total	0.002	8			
6	Between Groups	0.003	2	0.002	79.007	0.000
	Within Groups	0.000	6	0.000		
	Total	0.003	8			
12	Between Groups	0.075	2	0.037	1387.643	0.000
	Within Groups	0.000	6	0.000		
	Total	0.075	8			
18	Between Groups	0.194	2	0.097	4187.508	0.000
	Within Groups	0.000	6	0.000		
	Total	0.194	8			
24	Between Groups	0.291	2	0.145	3590.646	0.000
	Within Groups	0.000	6	0.000		
	Total	0.291	8			

APPENDIX C: Supplementary Materials for Chapter 3

Table C.1 : Absorbance change of 0.1% BCE with different concentrations of AA.

Time	0.1% BCE	0.1%BCE 0.01%AA	0.1%BCE 0.025%AA	0.1%BCE 0.05%AA	0.1%BCE 0.1%AA
0	0.806±0.0084	0.804±0.016	0.801±0.0104	0.803±0.0105	0.801±0.0157
1	0.804±0.0054	0.788±0.0104	0.777±0.0166	0.753±0.0381	0.727±0.053
3	0.807±0.0106	0.742±0.0342	0.688±0.0645	0.624±0.1	0.586±0.0976
6	0.797±0.0562	0.688±0.0168	0.68±0.163	0.581±0.2228	0.529±0.2491
9	0.818±0.024	0.663±0.0379	0.565±0.0461	0.435±0.08	0.339±0.0646
12	0.814±0.0091	0.644±0.0138	0.546±0.0421	0.383±0.0681	0.264±0.0602
18	0.774±0.0727	0.584±0.0667	0.48±0.0675	0.304±0.0692	0.184±0.0542
24	0.818±0.0072	0.607±0.0358	0.493±0.0166	0.306±0.0376	0.185±0.0301

Table C.2 : Absorbance change of 0.1% BCE encapsulated in liposomes with different concentrations of AA.

Time	0.1% BCE 1%LE	0.1%BCE 1%LE 0.01%AA	0.1%BCE 1%LE 0.025%AA	0.1%BCE 1%LE 0.05%AA	0.1%BCE 1%LE 0.1%AA
0	0.678±0.0274	0.678±0.0308	0.678±0.0295	0.687±0.0288	0.689±0.0287
1	0.678±0.0267	0.687±0.044	0.683±0.0404	0.686±0.0358	0.688±0.0379
3	0.659±0.028	0.653±0.0232	0.645±0.025	0.641±0.0302	0.631±0.0364
6	0.643±0.0171	0.624±0.0106	0.647±0.0831	0.567±0.0201	0.534±0.0316
9	0.636±0.0276	0.583±0.0249	0.548±0.034	0.51±0.0359	0.459±0.049
12	0.643±0.0296	0.536±0.0436	0.47±0.0189	0.405±0.0385	0.343±0.044
18	0.566±0.0596	0.447±0.0429	0.352±0.0622	0.3±0.0573	0.227±0.0351
24	0.583±0.0331	0.445±0.0307	0.374±0.0062	0.285±0.0195	0.194±0.0252
Time	0.1% BCE 2%LE	0.1%BCE 2%LE 0.01%AA	0.1%BCE 2%LE 0.025%AA	0.1%BCE 2%LE 0.05%AA	0.1%BCE 2%LE 0.1%AA
0	0.724±0.0439	0.705±0.0613	0.704±0.0611	0.705±0.0574	0.708±0.0587
1	0.724±0.043	0.72±0.0443	0.716±0.046	0.714±0.0482	0.718±0.049
3	0.69±0.024	0.692±0.0403	0.691±0.0472	0.683±0.0447	0.681±0.042
6	0.685±0.0423	0.675±0.0614	0.657±0.0644	0.639±0.061	0.626±0.0584
9	0.67±0.0318	0.643±0.0489	0.619±0.0521	0.589±0.0477	0.565±0.05
12	0.682±0.0236	0.603±0.062	0.569±0.0531	0.522±0.0577	0.483±0.0551
18	0.614±0.0857	0.528±0.1096	0.484±0.0939	0.412±0.0894	0.381±0.0866
24	0.635±0.0228	0.538±0.0822	0.48±0.0672	0.394±0.0702	0.35±0.0658
Time	0.1% BCE 4%LE	0.1%BCE 4%LE 0.01%AA	0.1%BCE 4%LE 0.025%AA	0.1%BCE 4%LE 0.05%AA	0.1%BCE 4%LE 0.1%AA
0	0.68±0.0205	0.696±0.0269	0.691±0.0242	0.693±0.0231	0.693±0.0235
1	0.681±0.0191	0.689±0.0187	0.685±0.0199	0.687±0.0212	0.688±0.0223
3	0.645±0.0097	0.659±0.0229	0.658±0.0257	0.656±0.023	0.653±0.0289
6	0.635±0.0214	0.648±0.0427	0.64±0.0449	0.647±0.041	0.635±0.049
9	0.611±0.0101	0.626±0.0244	0.616±0.0321	0.61±0.0288	0.593±0.0396
12	0.627±0.0148	0.599±0.0209	0.579±0.0242	0.559±0.0335	0.553±0.0342
18	0.566±0.0773	0.553±0.0578	0.519±0.0651	0.49±0.044	0.478±0.0514
24	0.581±0.0224	0.564±0.03	0.534±0.0117	0.5±0.0279	0.466±0.0398

Table C.3 : Absorbance change of extract, extract including liposome, and gelfiltered liposome with 0.1% AA during time.

Time(h)	Extract	Liposome	Gelfiltered liposome
0	1.072±0.002	1.021±0.045	1.008±0.012
3	1.016±0.005	0.998±0.038	0.98±0.011
6	0.929±0.016	0.975±0.044	0.959±0.02
12	0.729±0.046	0.923±0.042	0.92±0.013
18	0.56±0.037	0.87±0.022	0.873±0.011
24	0.474±0.023	0.852±0.011	0.858±0.029

APPENDIX D: Anova Tables of Chapter 4

Table D.1 : Statistical analysis results of characterization analyses of liposomes.

Characterization Analyses		Sum of Squares	df	Mean Square	F	Sig.
Particle size	Between Groups	18.011	11	1.637	1.227	0.322
	Within Groups	32.015	24	1.334		
	Total	50.026	35			
PdI	Between Groups	0.033	11	0.003	2.484	0.030
	Within Groups	0.029	24	0.001		
	Total	0.062	35			
Zeta Potential	Between Groups	304.569	11	27.688	22.507	0.000
	Within Groups	29.525	24	1.230		
	Total	334.094	35			
Encapsulation Efficiency	Between Groups	3161.376	8	395.172	40.739	0.000
	Within Groups	174.600	18	9.700		
	Total	3335.976	26			

Table D.2 : Statistical analysis results of extract concentrations of black carrot extract and liposomal solutions with/without 0.1% ascorbic acid (AA) during 24 hours.

Time (h)		Sum of Squares	df	Mean Square	F	Sig.
0	Between Groups	0.002	7	0.000	36.251	0.000
	Within Groups	0.000	16	0.000		
	Total	0.002	23			
1	Between Groups	0.001	7	0.000	125.030	0.000
	Within Groups	0.000	16	0.000		
	Total	0.001	23			
3	Between Groups	0.002	7	0.000	104.233	0.000
	Within Groups	0.000	16	0.000		
	Total	0.002	23			
6	Between Groups	0.003	7	0.000	248.065	0.000
	Within Groups	0.000	16	0.000		
	Total	0.003	23			
12	Between Groups	0.007	7	0.001	257.435	0.000
	Within Groups	0.000	16	0.000		
	Total	0.007	23			
18	Between Groups	0.013	7	0.002	1164.385	0.000
	Within Groups	0.000	16	0.000		
	Total	0.013	23			
24	Between Groups	0.024	7	0.003	139.754	0.000
	Within Groups	0.000	16	0.000		
	Total	0.024	23			

Table D.3 : Statistical analysis results of HPLC results of extract and liposome samples with 0.1% ascorbic acid (AA) during 24 hours for Cyanidin 3-xylosylglucosylgalactoside.

Time (h)		Sum of Squares	df	Mean Square	F	P
0	Sample	0.0394	1	0.0394	3.11	0.153
	Error	0.0508	4	0.0127		
	Total	0.0902	5			
3	Sample	0.0158	1	0.0158	0.45	0.540
	Error	0.1415	4	0.0354		
	Total	0.1573	5			
6	Sample	0.0384	1	0.0384	3.46	0.136
	Error	0.0443	4	0.0111		
	Total	0.0827	5			
9	Sample	0.0084	1	0.0084	0.68	0.457
	Error	0.0495	4	0.0124		
	Total	0.0578	5			
12	Sample	0.0001	1	0.0001	0.00	0.960
	Error	0.1582	4	0.0396		
	Total	0.1584	5			
18	Sample	0.0274	1	0.0274	2.26	0.207
	Error	0.0486	4	0.0121		
	Total	0.0760	5			
24	Sample	0.00770	1	0.00770	1.75	0.256
	Error	0.01760	4	0.00440		
	Total	0.02531	5			

Table D.4 : Statistical analysis results of HPLC results of extract and liposome samples with 0.1% ascorbic acid (AA) during 24 hours for Cyanidin 3-xylosylgalactoside.

Time (h)		Sum of Squares	df	Mean Square	F	P
0	Sample	0.0874	1	0.0874	4.84	0.093
	Error	0.0723	4	0.0181		
	Total	0.1597	5			
3	Sample	0.0002	1	0.0002	0.00	0.957
	Error	0.2280	4	0.0570		
	Total	0.2282	5			
6	Sample	0.0052	1	0.0052	0.22	0.660
	Error	0.0926	4	0.0231		
	Total	0.0978	5			
9	Sample	0.004	1	0.004	0.04	0.858
	Error	0.463	4	0.116		
	Total	0.468	5			
12	Sample	0.035	1	0.035	0.33	0.599
	Error	0.434	4	0.109		
	Total	0.470	5			
18	Sample	0.3771	1	0.3771	7.99	0.047
	Error	0.1887	4	0.0472		
	Total	0.5658	5			
24	Sample	0.5899	1	0.5899	13.20	0.036
	Error	0.1341	3	0.0447		
	Total	0.7240	4			

Table D.5 : Statistical analysis results of HPLC results of extract and liposome samples with 0.1% ascorbic acid (AA) during 24 hours for Cyanidin-3-xylosyl (sinapoylglucosyl) galactoside.

Time (h)		Sum of Squares	df	Mean Square	F	P
0	Sample	0.04199	1	0.04199	5.81	0.073
	Error	0.02889	4	0.00722		
	Total	0.07088	5			
3	Sample	0.00673	1	0.00673	2.06	0.225
	Error	0.01309	4	0.00327		
	Total	0.01982	5			
6	Sample	0.00035	1	0.00035	0.05	0.831
	Error	0.02749	4	0.00687		
	Total	0.02784	5			
9	Sample	0.00089	1	0.00089	0.24	0.647
	Error	0.01463	4	0.00366		
	Total	0.01553	5			
12	Sample	0.010185	1	0.010185	34.74	0.004
	Error	0.001172	4	0.000293		
	Total	0.011357	5			
18	Sample	0.038554	1	0.038554	40.02	0.003
	Error	0.003853	4	0.000963		
	Total	0.042408	5			
24	Sample	0.02924	1	0.02924	26.54	0.007
	Error	0.00441	4	0.00110		
	Total	0.03364	5			

Table D.6 : Statistical analysis results of HPLC results of extract and liposome samples with 0.1% ascorbic acid (AA) during 24 hours for Cyanidin-3-xylosyl (feruloylglucosyl) galactoside.

Time (h)		Sum of Squares	df	Mean Square	F	P
0	Sample	0.0647	1	0.0647	2.39	0.197
	Error	0.1080	4	0.0270		
	Total	0.1727	5			
3	Sample	0.00042	1	0.00042	0.38	0.573
	Error	0.00442	4	0.00111		
	Total	0.00484	5			
6	Sample	0.00737	1	0.00737	1.22	0.331
	Error	0.02417	4	0.00604		
	Total	0.03154	5			
9	Sample	0.06545	1	0.06545	9.17	0.039
	Error	0.02856	4	0.00714		
	Total	0.09401	5			
12	Sample	0.31067	1	0.31067	86.48	0.001
	Error	0.01437	4	0.00359		
	Total	0.32504	5			
18	Sample	0.46824	1	0.46824	169.54	0.000
	Error	0.01105	4	0.00276		
	Total	0.47929	5			
24	Sample	0.49572	1	0.49572	328.73	0.000
	Error	0.00603	4	0.00151		
	Total	0.50175	5			

Table D.7 : Statistical analysis results of HPLC results of extract and liposome samples with 0.1% ascorbic acid (AA) during 24 hours for Cyanidin-3-xylosyl (coumaroylglucosyl) galactoside.

Time (h)		Sum of Squares	df	Mean Square	F	P
0	Sample	0.00044	1	0.00044	0.31	0.606
	Error	0.00569	4	0.00142		
	Total	0.00613	5			
3	Sample	0.00017	1	0.00017	2.51	0.188
	Error	0.00028	4	0.00069		
	Total	0.00044	5			
6	Sample	0.001448	1	0.001448	5.95	0.071
	Error	0.000973	4	0.000243		
	Total	0.002421	5			
9	Sample	0.00573	1	0.00573	4.77	0.094
	Error	0.00481	4	0.00120		
	Total	0.01054	5			
12	Sample	0.015373	1	0.015373	112.26	0.000
	Error	0.000548	4	0.000137		
	Total	0.015921	5			
18	Sample	0.022911	1	0.022911	60.58	0.001
	Error	0.001513	4	0.000378		
	Total	0.024423	5			
24	Sample	0.045533	1	0.045533	114.02	0.00
	Error	0.001597	4	0.000399		
	Total	0.047130	5			

Table D.8 : Statistical analysis results of HPLC results of extract and liposome samples with 0.1% ascorbic acid (AA) during 24 hours for total of all peaks.

Time (h)		Sum of Squares	df	Mean Square	F	P
0	Sample	0.6130	1	0.6130	21.78	0.010
	Error	0.1126	4	0.0281		
	Total	0.7255	5			
3	Sample	0.052	1	0.052	0.22	0.661
	Error	0.926	4	0.232		
	Total	0.978	5			
6	Sample	0.000	1	0.000	0.00	0.956
	Error	0.407	4	0.102		
	Total	0.407	5			
9	Sample	0.042	1	0.042	0.14	0.729
	Error	1.213	4	0.303		
	Total	1.255	5			
12	Sample	0.921	1	0.921	3.13	0.152
	Error	1.178	4	0.294		
	Total	2.098	5			
18	Sample	3.2821	1	3.2821	52.35	0.002
	Error	0.2508	4	0.0627		
	Total	3.5329	5			
24	Sample	5.868	1	5.868	43.40	0.003
	Error	0.541	4	0.135		
	Total	6.408	5			

APPENDIX E: Supplementary Materials for Chapter 4

Table E.1 : Change in extract concentration (%) of samples with/without AA during storage.

Time (h)	0.2%E	0.2%E 1%LE	0.2%E 2%LE	0.2%E 4%LE	0.2%E 0.1%AA	0.2%E 1%LE 0.1%AA	0.2%E 2%LE 0.1%AA	0.2%E 4%LE 0.1%AA
0	0.203±0.0006	0.186±0.0008	0.19±0.0009	0.183±0.0001	0.209±0.0074	0.188±0.0009	0.191±0.0007	0.184±0.0007
1	0.201±0.0005	0.185±0.0011	0.188±0.0004	0.181±0.0004	0.194±0.0024	0.184±0.0007	0.188±0.0007	0.183±0.0005
3	0.202±0.0006	0.185±0.0011	0.188±0.0003	0.179±0.0008	0.175±0.0029	0.176±0.0012	0.182±0.0018	0.182±0.0009
6	0.203±0.0006	0.184±0.0011	0.188±0.0011	0.178±0.0009	0.166±0.002	0.171±0.0009	0.18±0.0016	0.181±0.0008
12	0.202±0.0003	0.177±0.0022	0.18±0.0013	0.169±0.0015	0.141±0.0012	0.15±0.0033	0.164±0.002	0.174±0.0027
18	0.203±0.0013	0.174±0.0006	0.176±0.0007	0.164±0.0008	0.123±0.0014	0.138±0.0025	0.155±0.0007	0.171±0.0007
24	0.206±0.0011	0.174±0.0006	0.175±0.0016	0.158±0.0014	0.097±0.0027	0.126±0.0008	0.144±0.0025	0.171±0.0027

Table E.2 : Change in anthocyanin concentration of samples with 0.1% AA during storage.

Sample	Time	Cyanidin-3-O-glucoside chloride (mg/g)					
		Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Total of all peaks
Extract	0	1.026±0.046	2.482±0.098	0.513±0.104	1.269±0.227	0.285±0.052	5.37±0.191
	3	1.013±0.078	2.113±0.284	0.431±0.07	1.106±0.046	0.266±0.004	4.807±0.574
	6	0.92±0.122	2.08±0.13	0.38±0.096	1.006±0.108	0.231±0.018	4.617±0.433
	9	0.806±0.13	1.843±0.439	0.296±0.04	0.841±0.118	0.201±0.043	3.987±0.744
	12	0.68±0.108	1.655±0.114	0.244±0.023	0.634±0.013	0.17±0.006	3.383±0.137
	18	0.551±0.069	1.262±0.2	0.175±0.033	0.489±0.017	0.136±0.025	2.613±0.309
	24	0.507±0.093	1.094±0.256	0.142±0.039	0.406±0.046	0.085±0.015	2.234±0.425
Gel filtered liposome	Time	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Total of all peaks
	0	0.815±0.131	2.241±0.177	0.346±0.06	1.061±0.049	0.267±0.01	4.731±0.195
	3	0.873±0.018	2.125±0.183	0.364±0.04	1.089±0.009	0.255±0.011	4.621±0.366
	6	0.76±0.085	2.139±0.171	0.365±0.068	1.076±0.021	0.262±0.013	4.602±0.127
	9	0.732±0.088	1.79±0.198	0.32±0.076	1.049±0.019	0.262±0.023	4.154±0.23
	12	0.818±0.072	1.808±0.452	0.326±0.009	1.089±0.084	0.272±0.015	4.167±0.755
	18	0.686±0.14	1.764±0.233	0.335±0.029	1.048±0.072	0.259±0.011	4.092±0.172
	24	0.578±0.01	1.65±0.255	0.282±0.026	0.981±0.03	0.259±0.024	3.751±0.323

(Peak 1) Cyanidin 3-xylosylglucosylgalactoside; (Peak 2) Cyanidin 3-xylosylgalactoside; (Peak 3) Cyanidin-3-xylosyl (sinapoylglucosyl) galactoside; (Peak 4) Cyanidin-3-xylosyl (feruloylglucosyl) galactoside; (Peak 5) Cyanidin-3-xylosyl (coumaroylglucosyl) galactoside.

APPENDIX F: Anova Tables of Chapter 5

Table F.1 : Statistical analysis results of characterization analyses of liposomes during storage.

Characterization Analyses		Sum of Squares	df	Mean Square	F	Sig.
Particle size	Between Groups	75.478	11	6.862	4.277	0.001
	Within Groups	38.502	24	1.604		
	Total	113.980	35			
PdI	Between Groups	0.032	11	0.003	4.296	0.001
	Within Groups	0.016	24	0.001		
	Total	0.049	35			
Zeta potential	Between Groups	93.456	11	8.496	7.366	0.000
	Within Groups	27.680	24	1.153		
	Total	121.136	35			
Particle size day 21	Between Groups	316.945	11	28.813	3.319	0.007
	Within Groups	208.360	24	8.682		
	Total	525.305	35			
PdI day 21	Between Groups	0.041	11	0.004	2.481	0.030
	Within Groups	0.036	24	0.002		
	Total	0.077	35			
Zeta potential day21	Between Groups	13.514	11	1.229	8.291	0.000
	Within Groups	3.556	24	0.148		
	Total	17.071	35			

Table F.2 : Statistical analysis results of extract concentration of liposomes during storage.

		Sum of Squares	df	Mean Square	F	Sig.
0.4%E-1%LE	Between Groups	0.026	4	0.007	145.190	0.000
	Within Groups	0.000	10	0.000		
	Total	0.027	14			
0.4%E-2%LE	Between Groups	0.131	4	0.033	133.208	0.000
	Within Groups	0.002	10	0.000		
	Total	0.134	14			
0.4%E-4%LE	Between Groups	0.190	4	0.047	12744.954	0.000
	Within Groups	0.000	10	0.000		
	Total	0.190	14			
0.4%E-1%LE-gelfiltration	Between Groups	0.018	4	0.005	189.058	0.000
	Within Groups	0.000	10	0.000		
	Total	0.018	14			
0.4%E-2%LE-gelfiltration	Between Groups	0.028	4	0.007	380.258	0.000
	Within Groups	0.000	10	0.000		
	Total	0.029	14			
0.4%E-4%LE-gelfiltration	Between Groups	0.074	4	0.018	1247.131	0.000
	Within Groups	0.000	10	0.000		
	Total	0.074	14			
Day 0	Between Groups	0.142	5	0.028	100.548	0.000
	Within Groups	0.003	12	0.000		
	Total	0.145	17			
Day 3	Between Groups	0.072	5	0.014	8487.800	0.000
	Within Groups	0.000	12	0.000		
	Total	0.072	17			
Day 7	Between Groups	0.101	5	0.020	4528.840	0.000
	Within Groups	0.000	12	0.000		
	Total	0.101	17			
Day 14	Between Groups	0.081	5	0.016	5503.764	0.000
	Within Groups	0.000	12	0.000		
	Total	0.081	17			
Day 21	Between Groups	0.107	5	0.021	10268.628	0.000
	Within Groups	0.000	12	0.000		
	Total	0.107	17			

Table F.3 : Statistical analysis results of total phenolic content of liposomes during storage.

Characterization Analyses		Sum of Squares	df	Mean Square	F	Sig.
0.4%E-1%LE	Between Groups	0.002	4	0.001	31.128	0.000
	Within Groups	0.000	8	0.000		
	Total	0.002	12			
0.4%E-2%LE	Between Groups	0.005	4	0.001	11.436	0.001
	Within Groups	0.001	10	0.000		
	Total	0.006	14			
0.4%E-4%LE	Between Groups	0.007	4	0.002	49.373	0.000
	Within Groups	0.000	10	0.000		
	Total	0.007	14			
0.4%E-1%LE-gelfiltration	Between Groups	0.000	4	0.000	.471	0.756
	Within Groups	0.002	10	0.000		
	Total	0.002	14			
0.4%E-2%LE-gelfiltration	Between Groups	0.001	4	0.000	2.796	0.085
	Within Groups	0.001	10	0.000		
	Total	0.002	14			
0.4%E-4%LE-gelfiltration	Between Groups	0.003	4	0.001	9.241	0.002
	Within Groups	0.001	10	0.000		
	Total	0.004	14			
Day 0	Between Groups	0.056	5	0.011	926.776	0.000
	Within Groups	0.000	12	0.000		
	Total	0.056	17			
Day 3	Between Groups	0.043	5	0.009	1108.327	0.000
	Within Groups	0.000	12	0.000		
	Total	0.044	17			
Day 7	Between Groups	0.040	5	0.008	32.677	0.000
	Within Groups	0.003	12	0.000		
	Total	0.043	17			
Day 14	Between Groups	0.031	5	0.006	31.720	0.000
	Within Groups	0.002	11	0.000		
	Total	0.033	16			
Day 21	Between Groups	0.023	5	0.005	367.759	0.000
	Within Groups	0.000	11	0.000		
	Total	0.023	16			

Table F.4 : Statistical analysis results of antioxidant capacity of liposomes during storage.

		Sum of Squares	df	Mean Square	F	Sig.
0.4%E-1%LE	Between Groups	0.019	4	0.005	1.896	0.195
	Within Groups	0.022	9	0.002		
	Total	0.041	13			
0.4%E-2%LE	Between Groups	0.171	4	0.043	34.960	0.000
	Within Groups	0.012	10	0.001		
	Total	0.183	14			
0.4%E-4%LE	Between Groups	0.125	4	0.031	54.423	0.000
	Within Groups	0.006	10	0.001		
	Total	0.131	14			
0.4%E-1%LE-gelfiltration	Between Groups	0.024	4	0.006	24.940	0.000
	Within Groups	0.002	10	0.000		
	Total	0.026	14			
0.4%E-2%LE-gelfiltration	Between Groups	0.004	4	0.001	1.195	0.371
	Within Groups	0.008	10	0.001		
	Total	0.012	14			
0.4%E-4%LE-gelfiltration	Between Groups	0.010	4	0.002	10.227	0.001
	Within Groups	0.002	10	0.000		
	Total	0.012	14			
Day 0	Between Groups	0.905	5	0.181	93.975	0.000
	Within Groups	0.023	12	0.002		
	Total	0.928	17			
Day 3	Between Groups	0.612	5	0.122	150.988	0.000
	Within Groups	0.010	12	0.001		
	Total	0.622	17			
Day 7	Between Groups	0.574	5	0.115	118.988	0.000
	Within Groups	0.012	12	0.001		
	Total	0.585	17			
Day 14	Between Groups	0.421	5	0.084	218.735	0.000
	Within Groups	0.005	12	0.000		
	Total	0.426	17			
Day 21	Between Groups	0.294	5	0.059	153.616	0.000
	Within Groups	0.004	11	0.000		
	Total	0.298	16			

Table F.5 : Statistical analysis results of hexanal concentration of liposomes during storage.

Hexanal Analysis		Sum of Squares	df	Mean Square	F	Sig.
1%LE	Between Groups	105976.669	3	35325.556	26.895	0.00
	Within Groups	9194.069	7	1313.438		
	Total	115170.739	10			
1%LE-0.4%E	Between Groups	3.102	3	1.034	2.822	0.117
	Within Groups	2.565	7	0.366		
	Total	5.667	10			
2%LE	Between Groups	207808.138	3	69269.379	39.316	0.00
	Within Groups	14094.765	8	1761.846		
	Total	221902.903	11			
2%LE-0.4%E	Between Groups	21526.720	3	7175.573	82.834	0.00
	Within Groups	606.379	7	86.626		
	Total	22133.099	10			
4%LE	Between Groups	219931.874	3	73310.625	218.225	0.00
	Within Groups	2687.524	8	335.941		
	Total	222619.398	11			
4%LE-0.4%E	Between Groups	25617.442	3	8539.147	41.523	0.00
	Within Groups	1439.536	7	205.648		
	Total	27056.977	10			
Day 0	Between Groups	297.253	5	59.451	2.268	0.114
	Within Groups	314.592	12	26.216		
	Total	611.845	17			
Day 30	Between Groups	53341.491	5	10688.298	7.380	0.004
	Within Groups	14456.545	10	1445.655		
	Total	67798.036	15			
Day 45	Between Groups	321289.072	5	64257.814	125.111	0.000
	Within Groups	6163.282	12	513.607		
	Total	327452.354	17			
Day 60	Between Groups	243166.637	5	48633.327	74.867	0.000
	Within Groups	7145.593	11	649.599		
	Total	250312.230	16			

Table F.6 : Statistical analysis results of hunter color analyses of liposomes during storage.

Hunter Analysis			Sum of Squares	df	Mean Square	F	Sig.
Day 0	L	Between Groups	1.740	3	0.580	0.769	0.543
		Within Groups	6.031	8	0.754		
		Total	7.770	11			
	a	Between Groups	10.057	3	3.352	4.819	0.033
		Within Groups	5.565	8	0.696		
		Total	15.621	11			
	b	Between Groups	5.820	3	1.940	20.071	0.000
		Within Groups	0.773	8	0.097		
		Total	6.594	11			
Day 3	L	Between Groups	1.130	3	0.377	3.583	0.066
		Within Groups	0.841	8	0.105		
		Total	1.971	11			
	a	Between Groups	2.526	3	0.842	3.362	0.076
		Within Groups	2.004	8	0.250		
		Total	4.530	11			
	b	Between Groups	3.568	3	1.189	19.431	0.000
		Within Groups	0.490	8	0.061		
		Total	4.058	11			
Day 7	L	Between Groups	35.519	3	11.840	468.048	0.000
		Within Groups	0.202	8	0.025		
		Total	35.721	11			
	a	Between Groups	24.973	3	8.324	390.190	0.000
		Within Groups	0.171	8	0.021		
		Total	25.144	11			
	b	Between Groups	36.939	3	12.313	4413.869	0.000
		Within Groups	0.022	8	0.003		
		Total	36.961	11			
Day 14	L	Between Groups	15.440	3	5.147	27.311	0.000
		Within Groups	1.508	8	0.188		
		Total	16.948	11			
	a	Between Groups	8.072	3	2.691	26.796	0.000
		Within Groups	0.803	8	0.100		
		Total	8.875	11			
	b	Between Groups	5.307	3	1.769	9.756	0.005
		Within Groups	1.451	8	0.181		
		Total	6.758	11			
Day 21	L	Between Groups	23.655	3	7.885	284.926	0.000
		Within Groups	0.221	8	0.028		
		Total	23.876	11			
	a	Between Groups	92.150	3	30.717	304.503	0.000
		Within Groups	0.807	8	0.101		
		Total	92.957	11			
	b	Between Groups	3.152	3	1.051	43.865	0.000
		Within Groups	0.192	8	0.024		
		Total	3.344	11			

APPENDIX G: Supplementary Materials for Chapter 5

Table G.1 : Change in biochemical stability of 0.4% extract including liposomes.

Time (day)		0.4%E 1%LE	0.4%E 1%LE_gel	0.4%E 2%LE	0.4%E 2%LE_gel	0.4%E 4%LE	0.4%E 4%LE_gel
0	Extract %	0.372±0.014	0.168±0.01	0.395±0.035	0.209±0.009	0.356±0.004	0.229±0.008
3		0.304±0.001	0.123±0.002	0.26±0.001	0.159±0.002	0.19±0.001	0.158±0.001
7		0.279±0.003	0.071±0.001	0.215±0.002	0.11±0.003	0.126±0.001	0.083±0.002
14		0.255±0.001	0.098±0.002	0.174±0.001	0.1±0.003	0.073±0.002	0.064±0.001
21		0.264±0.002	0.08±0.002	0.119±0.001	0.095±0.001	0.037±0.001	0.037±0.001
0	TEAC (mg/ml)	0.647±0.087	0.309±0.029	0.893±0.04	0.402±0.032	0.84±0.012	0.417±0.02
3		0.715±0.044	0.265±0.015	0.724±0.032	0.376±0.019	0.704±0.036	0.439±0.007
7		0.67±0.028	0.19±0.005	0.633±0.041	0.39±0.05	0.653±0.022	0.38±0.016
14		0.615±0.023	0.234±0.009	0.63±0.023	0.355±0.014	0.618±0.024	0.412±0.021
21		0.621±0.019	0.274±0.007	0.601±0.035	0.393±0.011	0.576±0.02	0.369±0.009
0	Gallic acid (mg/ml)	0.165±0.004	0.066±0.003	0.199±0.001	0.089±0.005	0.207±0.004	0.094±0.003
3		0.181±0.004	0.062±0	0.179±0.004	0.085±0.001	0.175±0.004	0.102±0.002
7		0.158±0.005	0.057±0.026	0.153±0.003	0.063±0.019	0.152±0.003	0.06±0.02
14		0.145±0.002	0.052±0.016	0.157±0.023	0.08±0.012	0.157±0.011	0.084±0.004
21		0.149±0.004	0.059±0	0.15±0.006	0.085±0.002	0.147±0.003	0.092±0.002

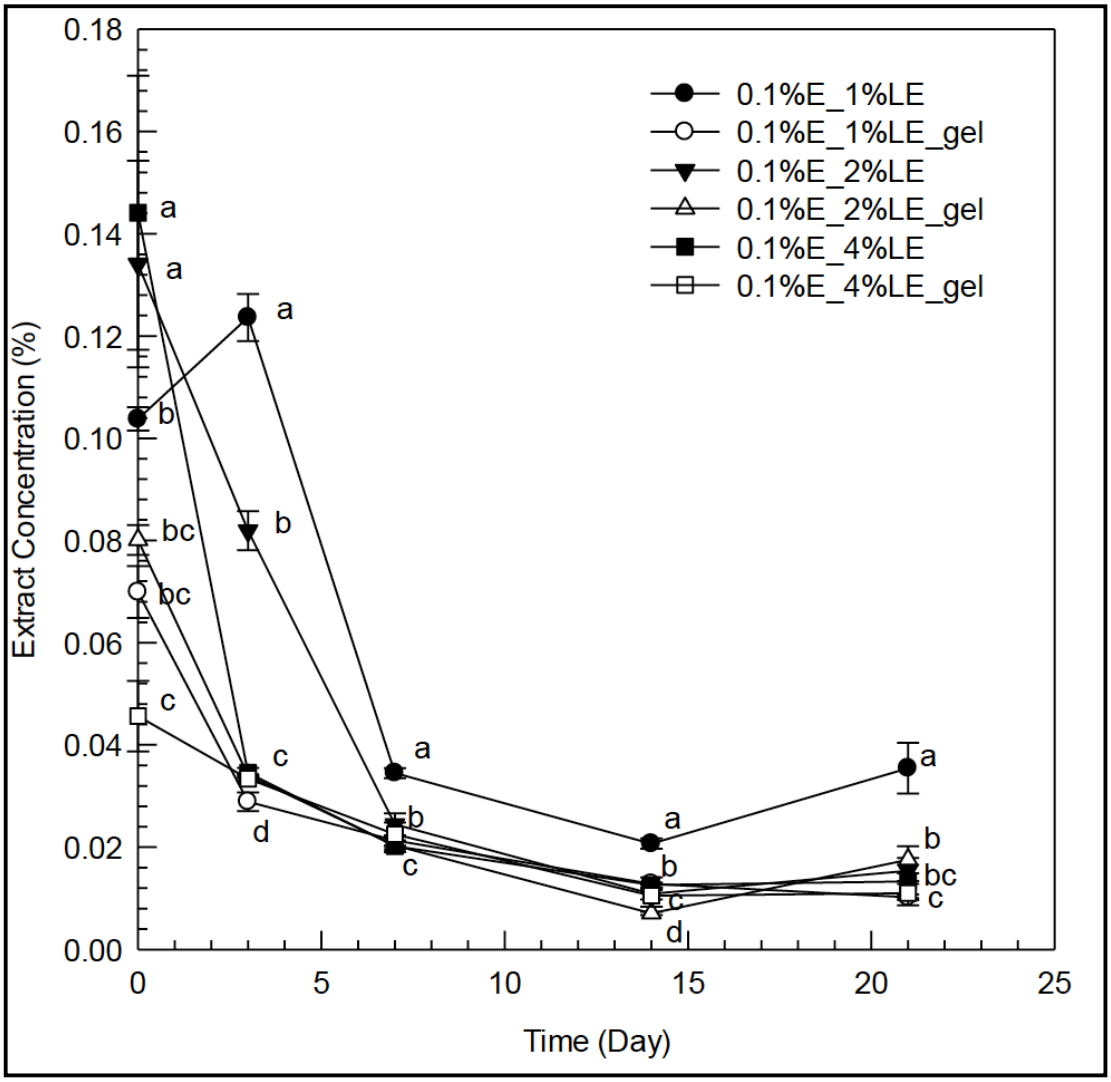

Figure G.1 : Degradation of anthocyanin in liposome samples with 0.1% extract during storage period. Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

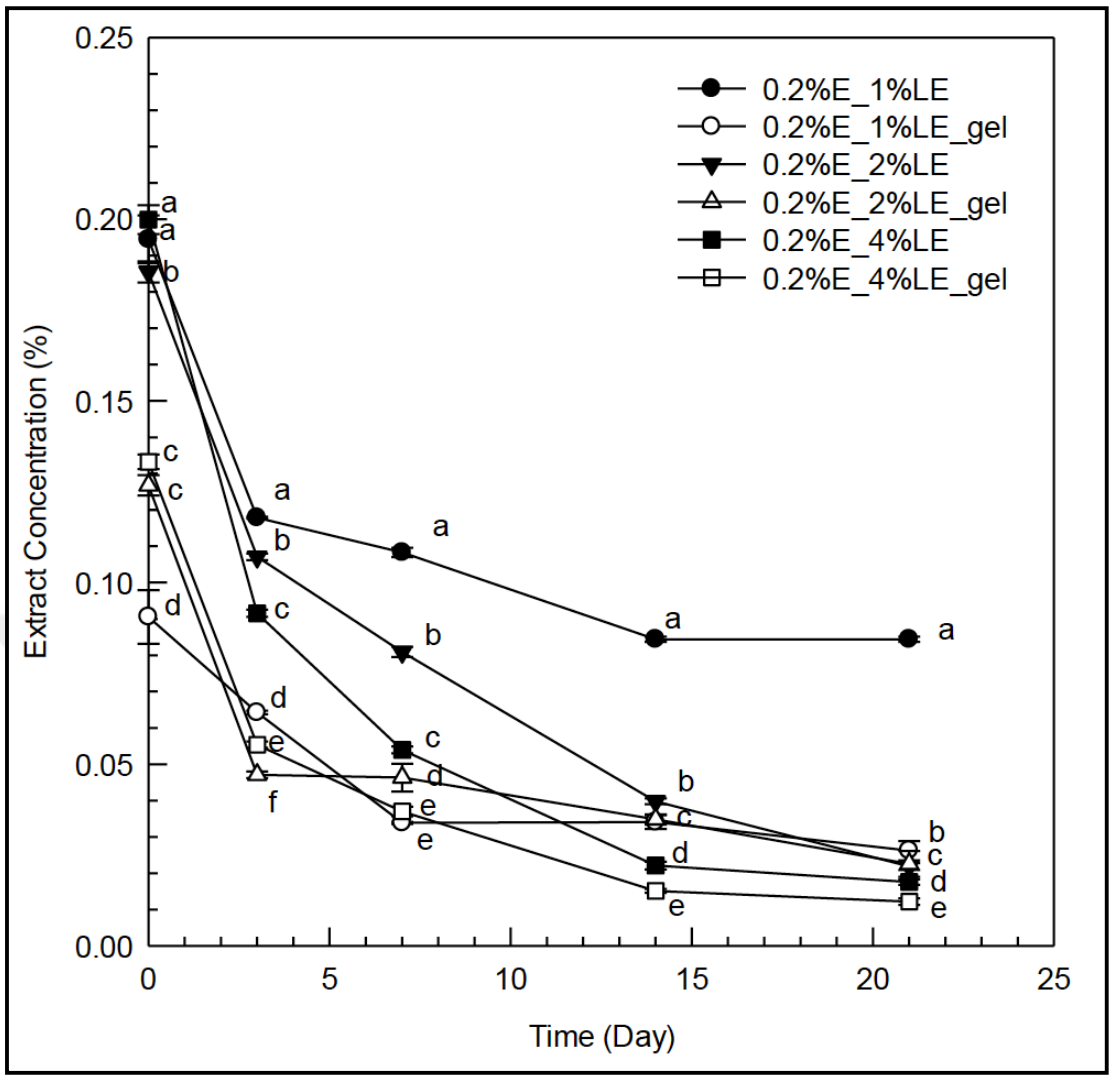

Figure G.2 : Degradation of anthocyanin in liposome samples with 0.2% extract during storage period. Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

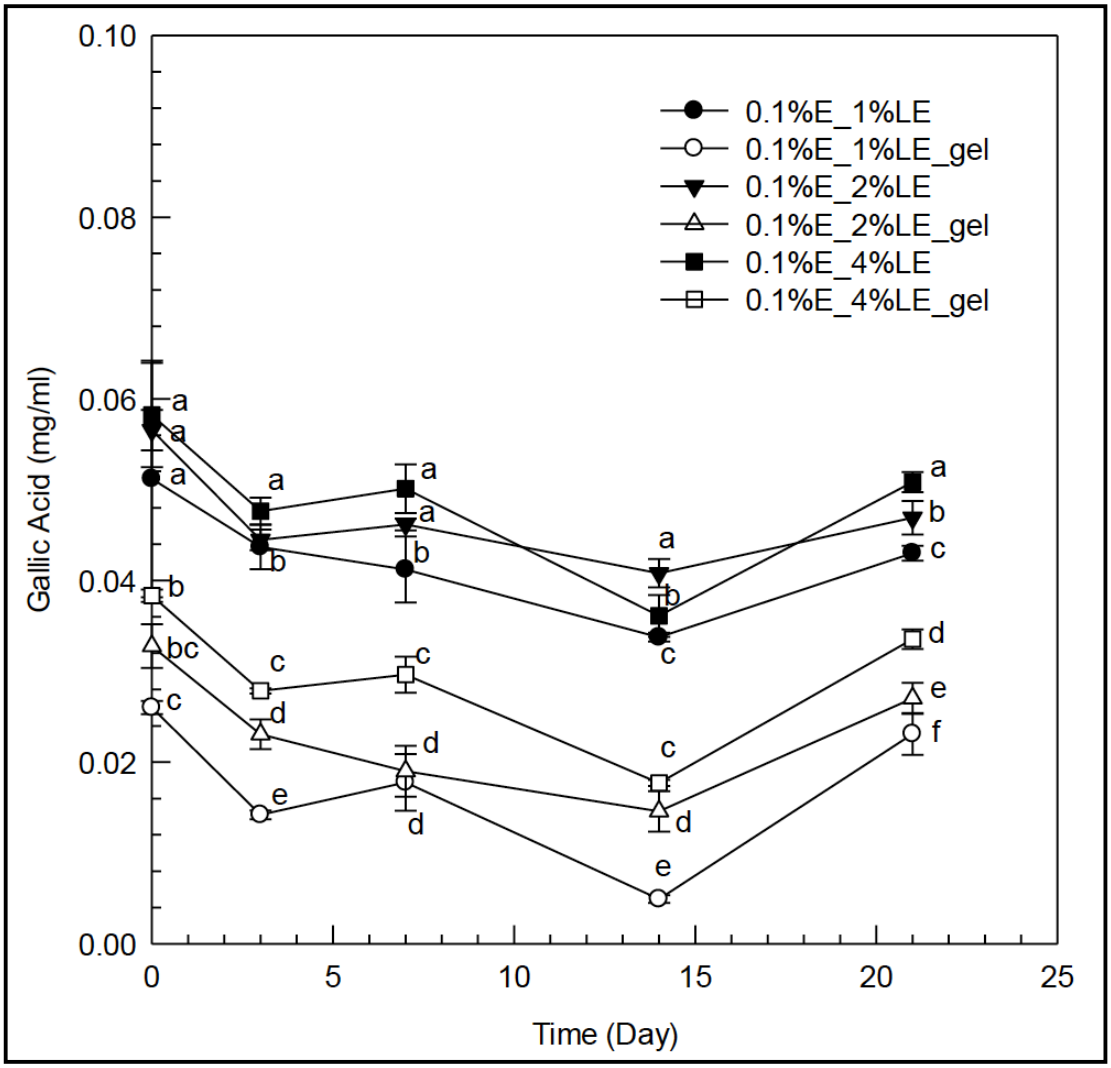

Figure G.3 : The content of total phenolics in liposome samples with 0.1% extract during storage. Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

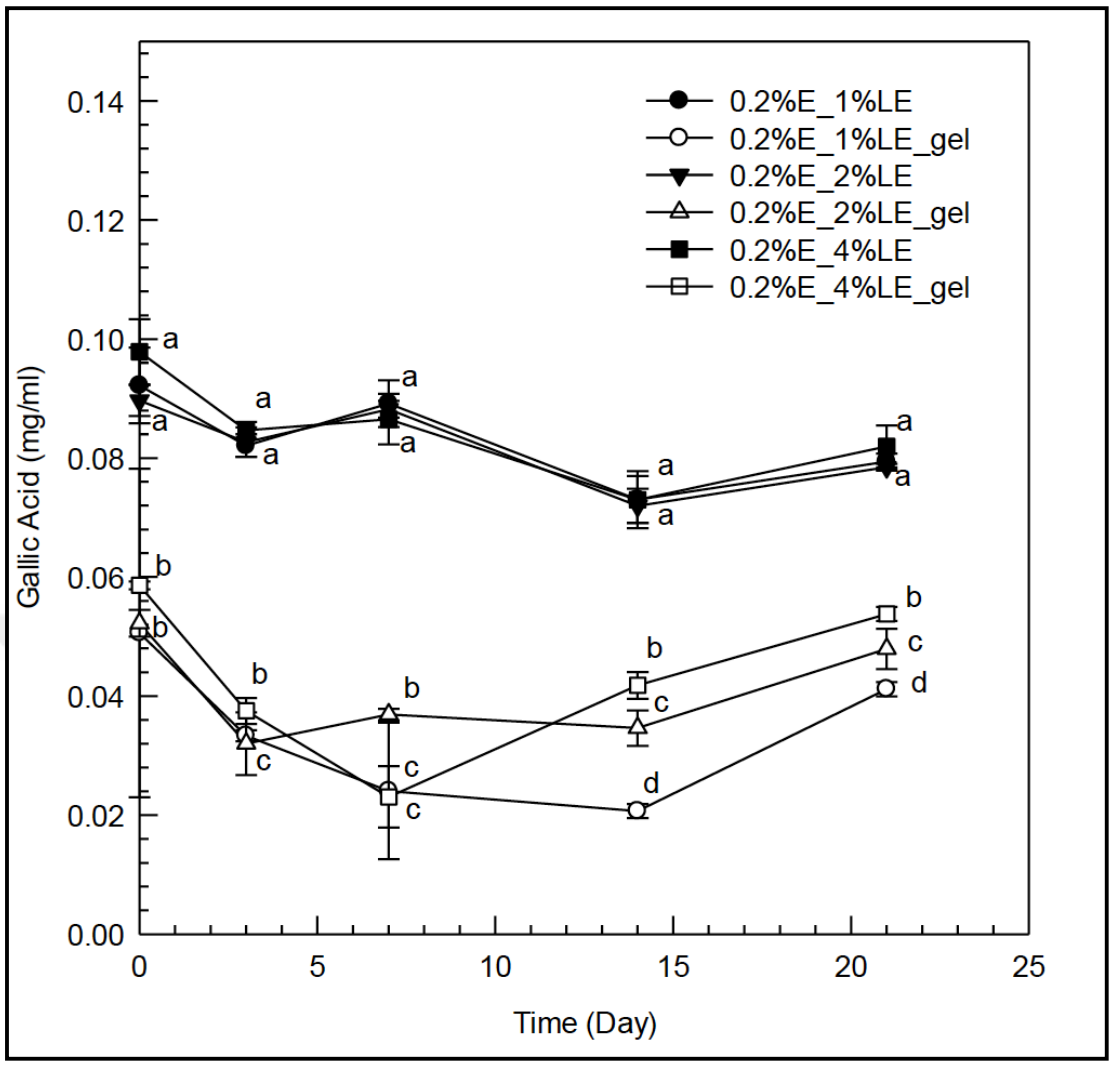

Figure G.4 : The content of total phenolics in liposome samples with 0.2% extract during storage. Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

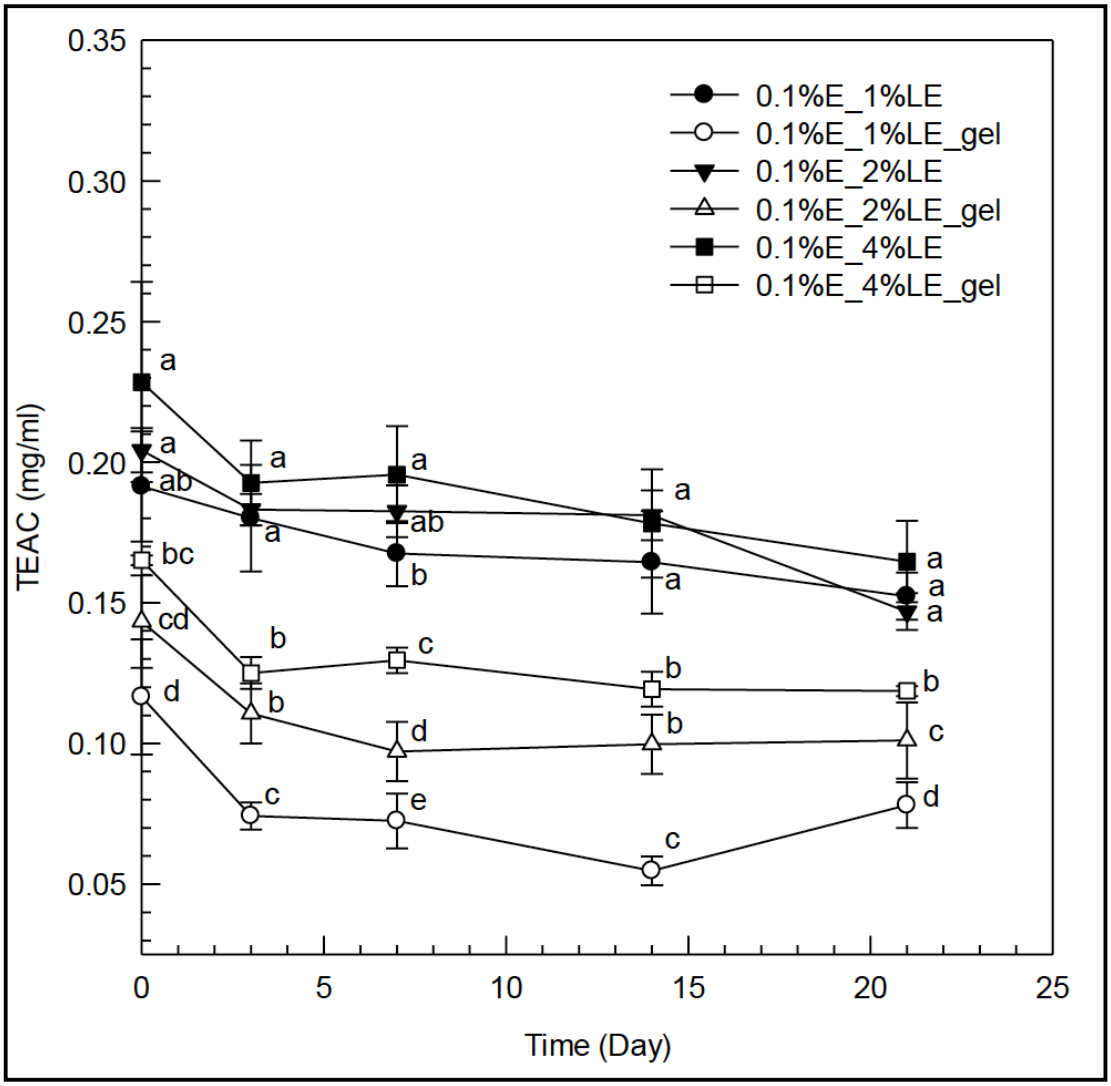

Figure G.5 : Antioxidant capacity of liposome samples with 0.1% extract during storage. Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

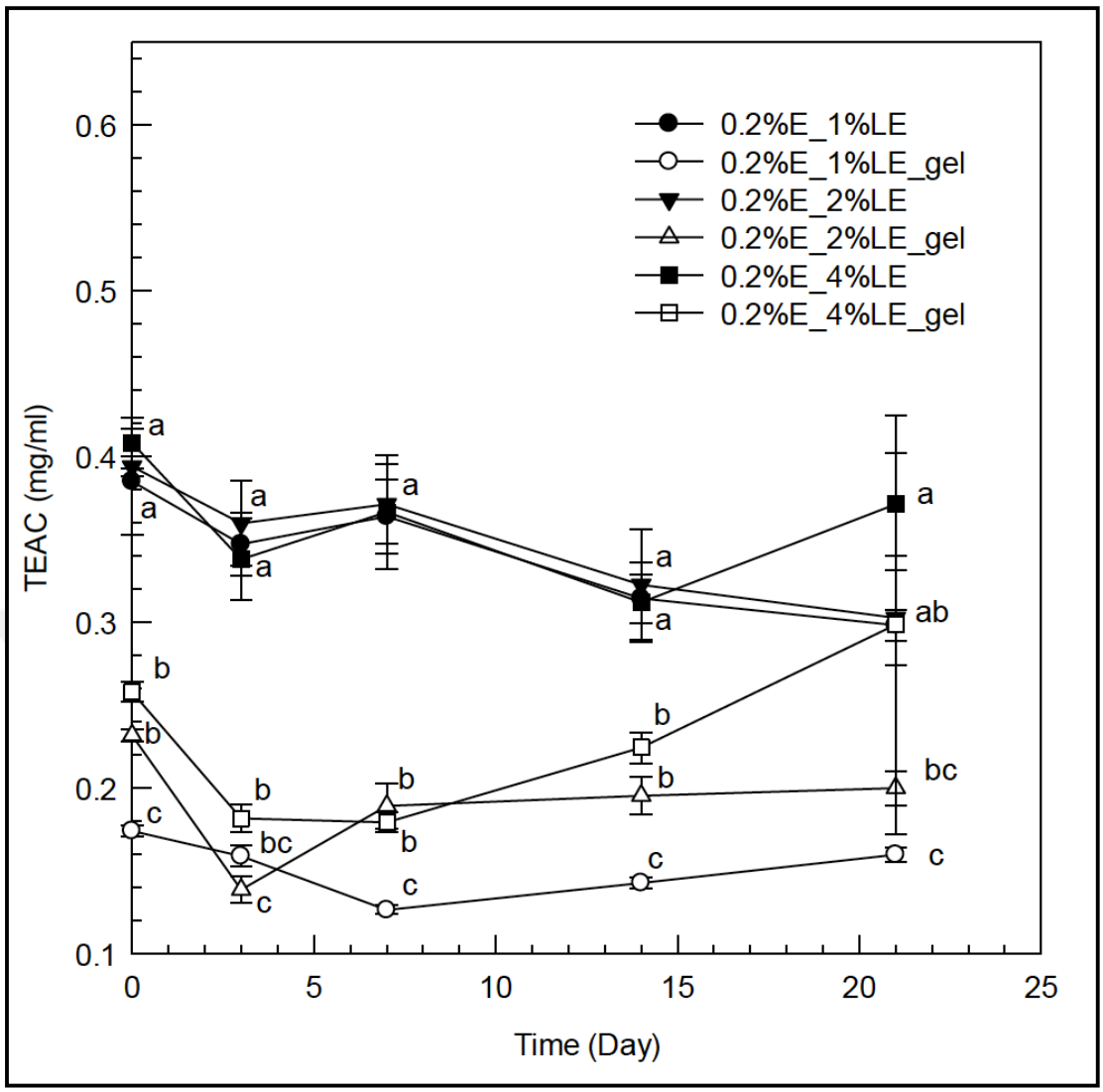

Figure G.6 : Antioxidant capacity of liposome samples with 0.2% extract during storage. Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

Table G.2 : Change in hexanal concentration in liposome samples during storage.

Sample	Hexanal concentration (μmol/L)			
	Day 0	Day 30	Day 45	Day 60
1%LE	0.3±0.6	70.6±64.4	236±38.7	200.3±32
1%LE 0.4%E	n.d.	n.d.	1±1.1	1.1±1.1
2%LE	2.5±3.8	120.9±68.9	318.3±10.5	303.8±46.6
2%LE 0.4%E	n.d.	30.8±6.2	37.9±16.2	130.9±1.5
4%LE	11.2±11.9	162.5±19	317.2±26.2	351.6±12.5
4%LE 0.4%E	n.d.	41.8±1.1	45.4±22.3	127.8±14.8

Table G.3 : Change in hunter color variables in liposome samples during storage.

Time (day)	Hunter variable	0.4%E 1%LE	0.4%E 2%LE	0.4%E 4%LE	0.4%E
0	L*	38.7±0.2	39.3±0.5	39.8±1.6	39.2±0.4
3		39.6±0.4	39.1±0.4	39.3±0.4	38.8±0.1
7		42.3±0.2	42.5±0.1	43.3±0.2	38.8±0.1
14		42±0.8	39.3±0.1	41.3±0.1	39.5±0.2
21		37.5±0.1	39±0.2	41.3±0.1	38.4±0.2
0	a*	13.7±0.9	12.5±0.3	11.6±1.1	13.9±0.8
3		13.1±0.7	13.2±0.6	12.5±0.3	13.8±0.1
7		10.9±0.2	11.8±0.1	10.9±0.1	14.4±0.1
14		12.8±0.5	14.7±0.3	13.4±0.1	14.6±0.1
21		16.6±0.2	15.6±0.4	9.8±0.2	16.3±0.4
0	b*	3.9±0.4	3.2±0.2	2.6±0.3	4.5±0.3
3		3.6±0.4	3.4±0.3	2.8±0.1	4.3±0.1
7		0.8±0.1	1±0.1	0.7±0	4.9±0
14		3.4±0.8	4.5±0.1	5.1±0.1	5±0.1
21		5.9±0.1	5.7±0.1	7±0.2	5.8±0.2

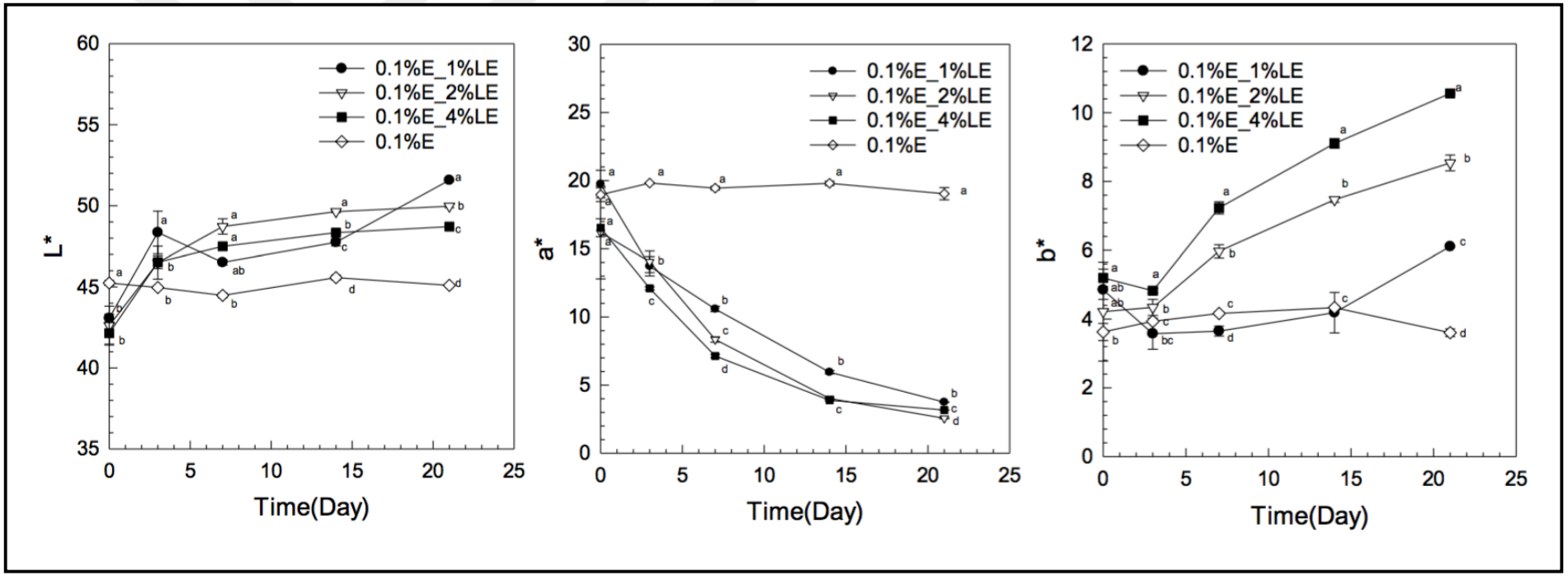

Figure G.7 : Color attributes of 0.1% extract and liposome samples with 0.1% extract. Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

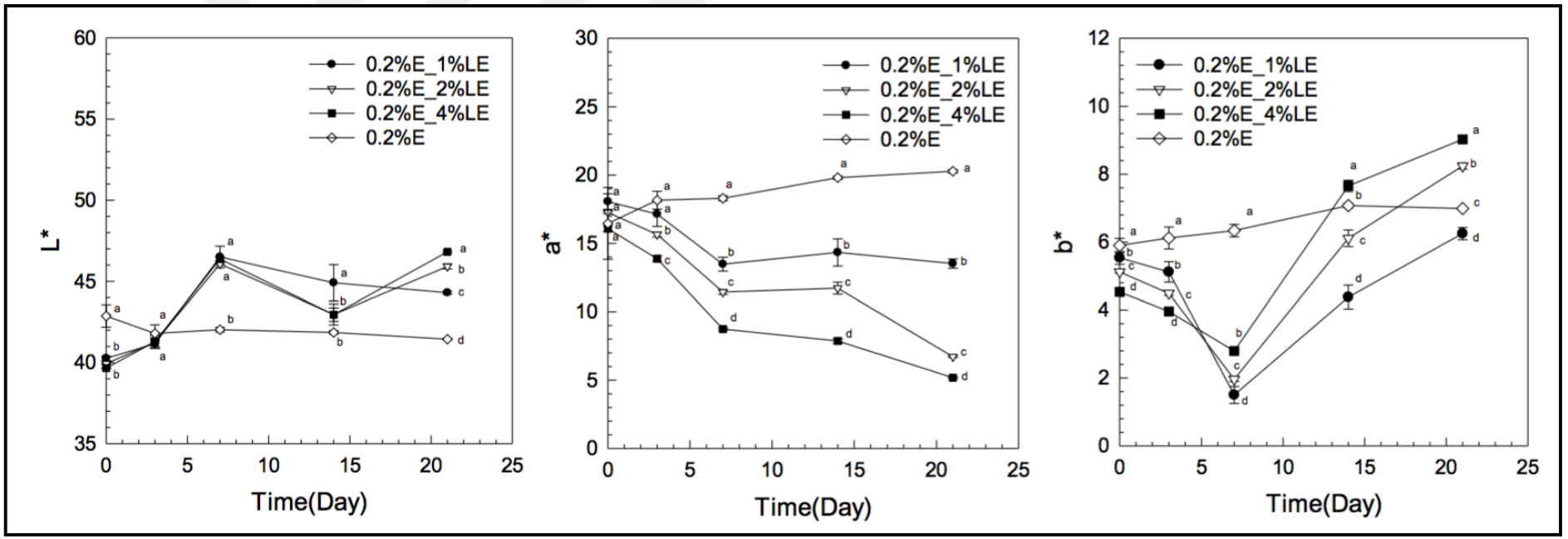

Figure G.8 : Color attributes of 0.2% extract and liposome samples with 0.2% extract. Data represent average values ± standard deviation of three replicates from each sample. Different small letters in the columns represent statistically significant differences ($p < 0.05$).

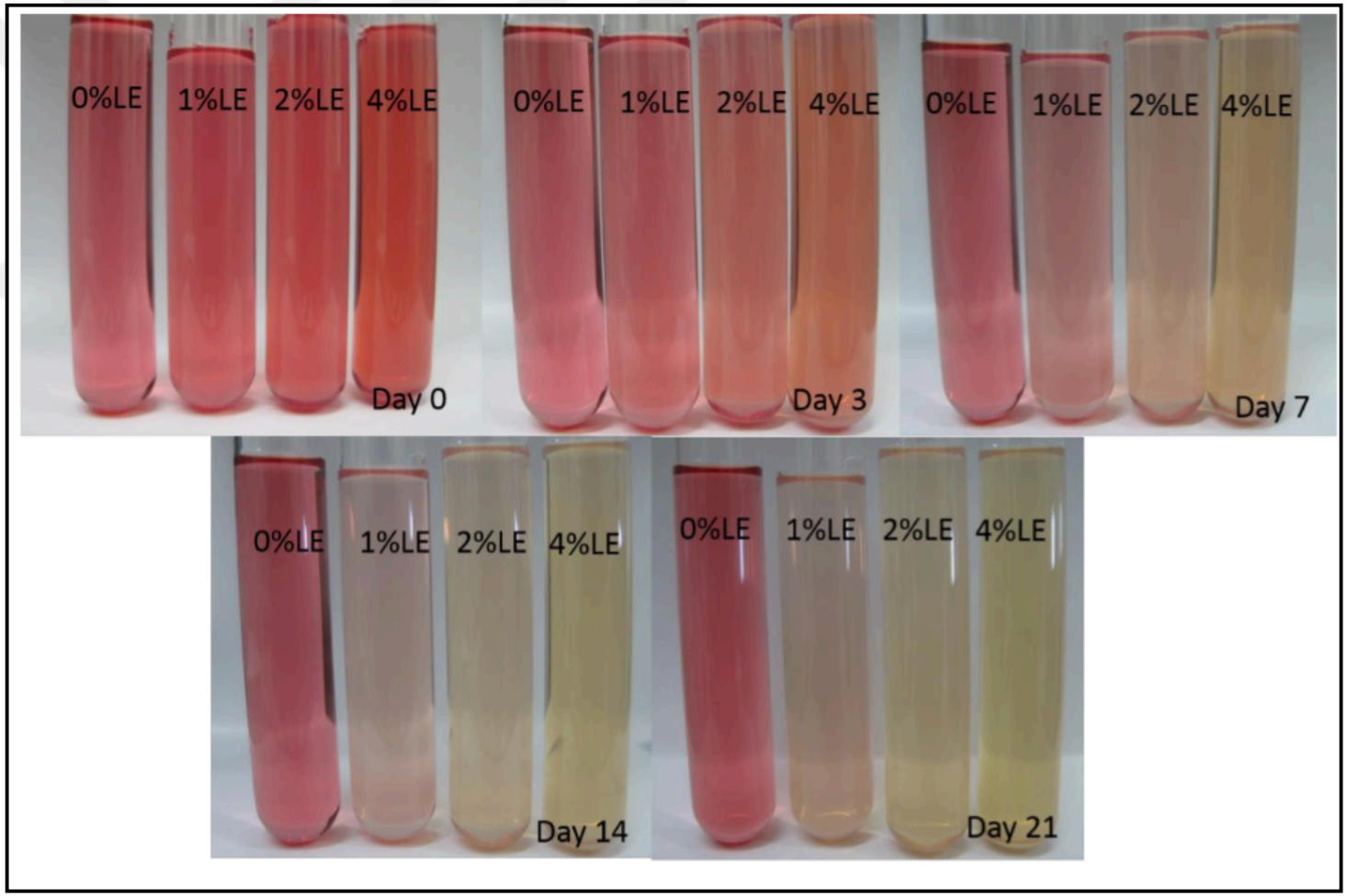

Figure G.9 : Visual observations of 0.1% extract including liposomes during storage period.

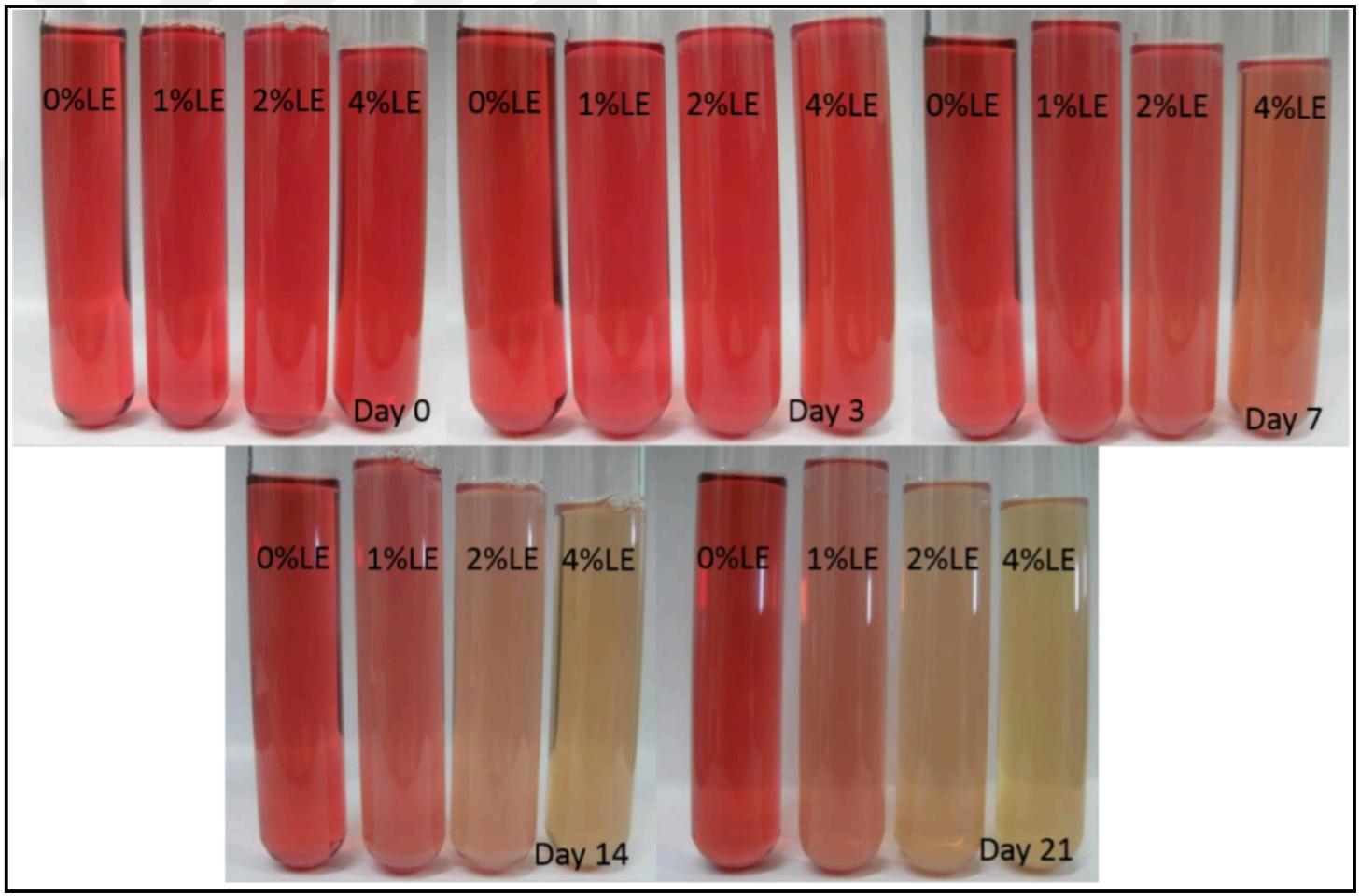

Figure G.10 : Visual observations of 0.2% extract including liposomes during storage period

APPENDIX H: Anova Tables of Chapter 6

Table H.1 : Statistical analysis results of characterization analyses of powders.

Powder characterization		Sum of Squares	df	Mean Square	F	P
Yield	Between Groups	425.446	5	85.089	2.517	0.146
	Within Groups	202.838	6	33.806		
	Total	628.285	11			
Powder dispersion	Between Groups	1362.283	5	272.457	7.697	0.000
	Within Groups	1061.968	30	35.399		
	Total	2424.251	35			
Moisture content	Between Groups	4.520	5	0.904	5.177	0.002
	Within Groups	5.238	30	0.175		
	Total	9.758	35			
Water activity	Between Groups	0.013	5	0.003	10.590	0.000
	Within Groups	0.004	18	0.000		
	Total	0.017	23			

Table H.2 : Statistical analysis results of characterization analyses of liquid dispersions.

Particle Characterization		Sum of Squares	df	Mean Square	F	P
Particle size	Between Groups	69945.516	10	6994.552	293.479	0.00
	Within Groups	262.165	11	23.833		
	Total	70207.681	21			
PdI	Between Groups	0.334	11	0.030	9.983	0.00
	Within Groups	0.036	12	0.003		
	Total	0.370	23			
Zeta potential	Between Groups	35740.879	11	3249.171	532.669	0.00
	Within Groups	73.198	12	6.100		
	Total	35814.076	23			

Table H.3 : Statistical analysis results of antioxidant activity retention of powders.

CUPRAC		Sum of Squares	df	Mean Square	F	Sig.
Day 0	Between Groups	286.173	3	95.391	171.865	0.000
	Within Groups	2.220	4	0.555		
	Total	288.393	7			
Day 7	Between Groups	273.251	3	91.084	7.116	0.044
	Within Groups	51.198	4	12.800		
	Total	324.449	7			
Day 14	Between Groups	617.508	3	205.836	14.356	0.013
	Within Groups	57.351	4	14.338		
	Total	674.860	7			
Day 21	Between Groups	667.428	3	222.476	19.318	0.008
	Within Groups	46.066	4	11.517		
	Total	713.494	7			
BCE	Between Groups	434.307	3	144.769	11.897	0.018
	Within Groups	48.674	4	12.169		
	Total	482.981	7			
BCE-Primary liposome	Between Groups	615.188	3	205.063	25.978	0.004
	Within Groups	31.574	4	7.894		
	Total	646.762	7			
BCE-Secondary liposome	Between Groups	932.631	3	310.877	111.068	0.000
	Within Groups	11.196	4	2.799		
	Total	943.827	7			
BCE-Chitosan	Between Groups	749.454	3	249.818	13.986	0.014
	Within Groups	71.446	4	17.862		
	Total	820.900	7			

Table H.4 : Statistical analysis results of anthocyanin content retention of powders.

Anthocyanin		Sum of Squares	df	Mean Square	F	Sig.
Day 0	Between Groups	1835.241	3	611.747	130.952	0.000
	Within Groups	18.686	4	4.672		
	Total	1853.927	7			
Day 7	Between Groups	2609.598	3	869.866	174.475	0.000
	Within Groups	19.942	4	4.986		
	Total	2629.541	7			
Day 14	Between Groups	1941.999	3	647.333	81.283	0.000
	Within Groups	31.856	4	7.964		
	Total	1973.855	7			
Day 21	Between Groups	4642.696	3	1547.565	190.876	0.000
	Within Groups	32.431	4	8.108		
	Total	4675.126	7			
BCE	Between Groups	277.755	3	92.585	17.686	0.009
	Within Groups	20.939	4	5.235		
	Total	298.694	7			
BCE-Primary liposome	Between Groups	1309.569	3	436.523	47.593	0.001
	Within Groups	36.688	4	9.172		
	Total	1346.257	7			
BCE-Secondary liposome	Between Groups	1326.602	3	442.201	47.705	0.001
	Within Groups	37.078	4	9.269		
	Total	1363.680	7			
BCE-Chitosan	Between Groups	462.202	3	154.067	75.365	0.001
	Within Groups	8.177	4	2.044		
	Total	470.379	7			

Table H.5 : Statistical analysis results of total phenolic content retention of powders.

Phenolic		Sum of Squares	df	Mean Square	F	Sig.
Day 0	Between Groups	746.419	3	248.806	6.323	0.053
	Within Groups	157.406	4	39.352		
	Total	903.825	7			
Day 7	Between Groups	277.776	3	92.592	36.445	0.002
	Within Groups	10.162	4	2.541		
	Total	287.938	7			
Day 14	Between Groups	422.266	3	140.755	3.192	0.146
	Within Groups	176.367	4	44.092		
	Total	598.633	7			
Day 21	Between Groups	185.859	3	61.953	2.571	0.192
	Within Groups	96.379	4	24.095		
	Total	282.238	7			
BCE	Between Groups	831.995	3	277.332	2.634	0.186
	Within Groups	421.114	4	105.279		
	Total	1253.109	7			
BCE-Primary liposome	Between Groups	280.075	3	93.358	40.686	0.002
	Within Groups	9.178	4	2.295		
	Total	289.253	7			
BCE-Secondary liposome	Between Groups	435.587	3	145.196	10.615	0.022
	Within Groups	54.712	4	13.678		
	Total	490.299	7			
BCE-Chitosan	Between Groups	314.220	3	104.740	2.623	0.187
	Within Groups	159.711	4	39.928		
	Total	473.931	7			

APPENDIX I: Supplementary Materials for Chapter 6

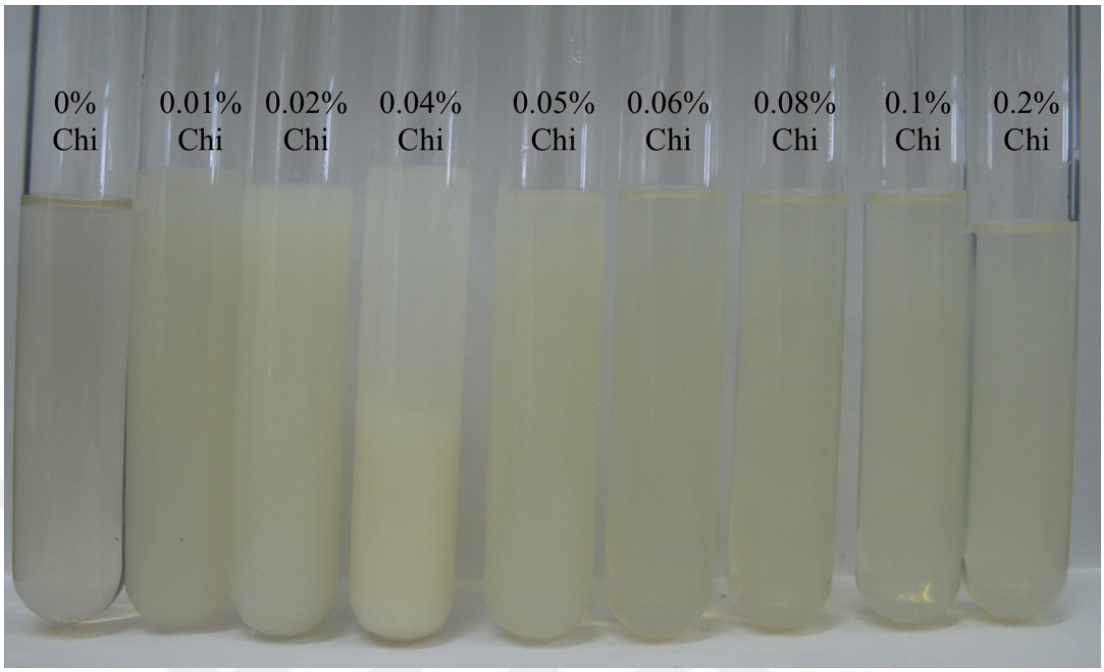

Figure I.1 : Liposome and chitosan mixtures.

Table I.1 : Characterization of primary liposomes mixed with different chitosan concentrations.

Chitosan concentration (%)	Particle diameter (nm)	PdI	Zeta Potential (mV)
0.01	120.80±2.005	0.420±0.0146	-25.6±0.40
0.02	630.53±57.863	0.593±0.0395	-22.2±0.34
0.04	5619.25±1843.838	1±0	30.8±0.51
0.05	125.20±7.647	0.632±0.0445	38.0±0.31
0.06	78.95±0.297	0.415±0.0064	44.0±0.61
0.08	86.66±2.266	0.422±0.0159	47.3±0.66
0.1	82.68±1.48	0.435±0.023	51.4±0.60
0.2	88.39±2.058	0.448±0.015	56.4±0.95

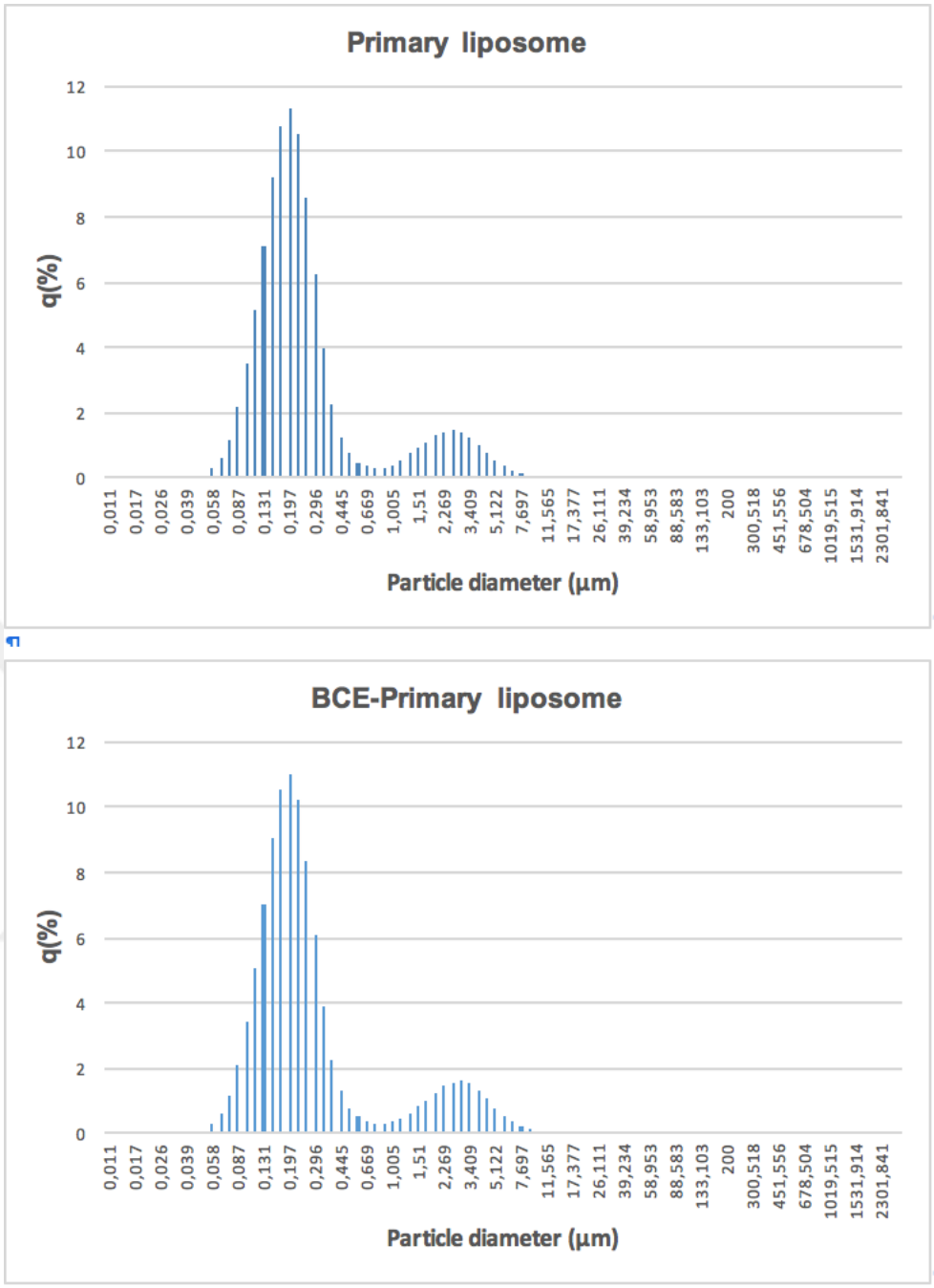

Figure I.2 : Particle diameter distribution of primary liposomes and BCE including primary liposomes.

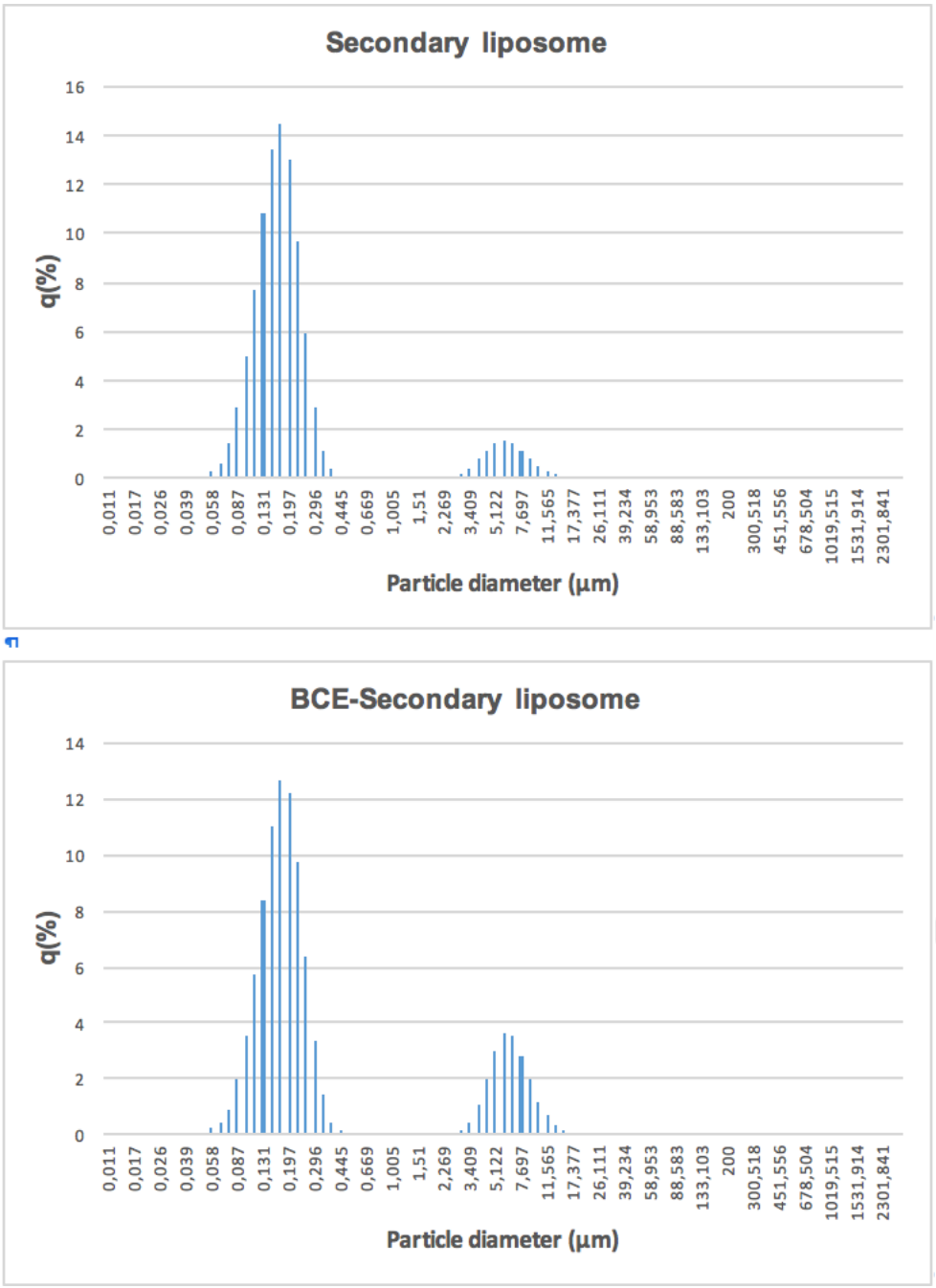

Figure I.3 : Particle diameter distribution of secondary liposome and BCE including secondary liposome.

Table I.2 : Retention of bioactive compounds in reconstituted samples.

Analysis	Sample	Day 0	1st Week	2nd Week	3rd Week
Anthocyanin retention (%)	BCE	$98.1\pm0.76^{a,A}$	$98.6\pm1.97^{a,A}$	$91.5\pm0.12^{a,B}$	$84.1\pm4.07^{a,C}$
	BCE-Primary liposome	$66.5\pm2.05^{b,A}$	$65.1\pm1.97^{c,AB}$	$57.4\pm5.35^{c,B}$	$34.5\pm0.2^{b,C}$
	BCE-Secondary liposome	$62.0\pm3.38^{b,A}$	$54.3\pm3.50^{d,AB}$	$53.8\pm1.73^{c,B}$	$31.2\pm2.87^{b,C}$
	BCE-Chitosan	$95.1\pm0.76^{a,A}$	$90.5\pm0.08^{b,B}$	$79.5\pm0.5^{b,C}$	$77.4\pm2.76^{a,C}$
Total phenolic content retention (%)	BCE	$88.1\pm9.6^{a,A}$	$69.9\pm1.34^{a,A}$	$68.1\pm10.91^{a,A}$	$59.7\pm6.12^{a,A}$
	BCE-Primary liposome	$64.9\pm2.25^{b,A}$	$59.9\pm0.17^{b,B}$	$57.3\pm0.17^{ab,B}$	$48.6\pm2.00^{a,C}$
	BCE-Secondary liposome	$67.4\pm4.5^{b,A}$	$53.8\pm0.38^{b,B}$	$52.9\pm5.8^{ab,B}$	$47.4\pm0.56^{a,B}$
	BCE-Chitosan	$65.5\pm8.6^{b,A}$	$58.0\pm2.86^{ab,A}$	$48.6\pm4.84^{c,A}$	$53.1\pm7.39^{a,A}$
Antioxidant activity retention (%)	BCE	$94.6\pm0.93^{a,A}$	$93.2\pm5.26^{a,A}$	$94.5\pm4.38^{a,A}$	$77.1\pm0.92^{a,B}$
	BCE-Primary liposome	$88.5\pm0.03^{b,A}$	$88.5\pm0.38^{a,A}$	$81.9\pm1.22^{b,A}$	$67.0\pm5.47^{b,B}$
	BCE-Secondary liposome	$77.9\pm1.15^{c,A}$	$78.3\pm2.89^{b,A}$	$72.7\pm0.62^{b,B}$	$51.9\pm1.06^{c,C}$
	BCE-Chitosan	$87.9\pm0.12^{b,A}$	$91.8\pm3.86^{a,A}$	$73.4\pm6.02^{b,B}$	$69.2\pm3.76^{ab,B}$

The data presented in this table consists of average values ± standard deviation of two independent batches. Different small letters in the columns represent statistically significant differences ($p<0.05$) for each analyses at the same time period. Different capital letters in the rows represent statistically significant differences ($p<0.05$) for each analyses and for each sample.

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PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Guldiken, B.,** Gibis, M., Boyacioglu, D., Capanoglu, E., & Weiss, J. (2017). Physical and Chemical Stability of Anthocyanin-rich Black Carrot Extract Loaded Liposomes During Storage. (Submitted to Food Research International).
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