

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**GREEN EXTRACTION AND ENCAPSULATION OF BLACK ROSEHIP
POLYPHENOLS: IN VITRO BIOACCESSIBILITY, BIOAVAILABILITY,
AND BIOLOGICAL ACTIVITIES**



Ph.D. THESIS

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Department of Food Engineering

Food Engineering Programme

NOVEMBER 2022

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**SİYAH KUŞBURNU POLİFENOLLERİNİN YEŞİL EKSTRAKSİYONU VE
ENKAPSÜLASYONU: İN VİTRO BİYOERİŞİLEBİLİRLİK,
BİYOYARARLILIK VE BİYOLOJİK AKTİVİTELERİ**

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To my dad,



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(Food Engineer, M.Sc.)



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ABBREVIATIONS

ABTS	: 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt)
Aq	: Aqueous
ANOVA	: Analysis of variance
BBD	: Box-Behnken design
BRE	: Black rosehip extract
Caco-2	: Human colonic adenocarcinoma cell line
CC	: Catechin content
CCD	: Central Composite Design
CH	: Chitosan
CO₂	: Carbon dioxide
CUPRAC	: Cupric ion reducing antioxidant capacity
CY3C	: Cyanidin-3- <i>O</i> -glucoside
DCFH-DA	: Dichlorodihydrofluorescein diacetate
DE	: Dextrose equivalent
DMEM	: Dulbecco's Modified Eagle's Medium
DPPH	: 2,2-diphenyl-1-picryl-hydrazyl free-radical
DSC	: Differential Scanning Calorimetry
DW	: Dry weight
EE	: Encapsulation efficiency
EtOH	: Ethanol
GAE	: Gallic Acid Equivalent
HA	: Hydroalcoholic
H₂O	: Water
HBSS	: Hank's Balanced Salt Solution
HepG2	: Human liver carcinoma cell line
HPLC	: High Performance Liquid Chromatography
I	: Infusion
iROS	: Intracellular Reactive Oxygen Species
LC	: Loading capacity
LS180	: Human colon adenocarcinoma cell line
MD	: Maltodextrin
MeOH	: Methanol
MTT	: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
PBS	: Phosphate Buffered Saline
PDI	: Polydispersity index
PHWE	: Pressurized Hot Water Extraction
PLE	: Pressurized Liquid Extraction
RC	: <i>Rosa canina</i>
RE	: Retention efficiency
RPFE	: <i>Rosa pimpinellifolia</i> fruit extract
RSM	: Response surface methodology
SCO₂	: Supercritical carbon dioxide extraction

SEM	: Scanning Electron Microscopy
SLN	: Solid Lipid Nanoparticle
TAC	: Total Antioxidant Capacity
TFC	: Total Flavonoid Content
TE	: Trolox Equivalent
TEER	: Transepithelial electrical resistance
TMAC	: Total Monomeric Anthocyanin Content
TPC	: Total Phenolic Content
UAE	: Ultrasound Assisted Extraction
WI	: Whiteness Index
WP	: Whey protein



SYMBOLS

°C	: degree Celsius
a*	: red/green (color parameter)
b*	: blue/yellow (color parameter)
d[3,2]	: surface-weighted mean diameters (μm)
d[4,3]	: volume-weighted mean diameters (μm)
L	: liter, unit of volume
L*	: luminosity (color parameter)
m	: milli (10 ⁻³)
n	: nano (10 ⁻⁶)
pH	: power of hydrogen
α	: Angle of deviation from the direction of the principal stresses
T_g	: Glass transition temperature
T_{on}	: Onset temperature
T_{peak}	: Peak temperature
ζ	: Zeta (in zeta potential)
μ	: micro (10 ⁻⁶)



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GREEN EXTRACTION AND ENCAPSULATION OF BLACK ROSEHIP POLYPHENOLS: IN VITRO BIOACCESSIBILITY, BIOAVAILABILITY, AND BIOLOGICAL ACTIVITIES

SUMMARY

Due to the increased prevalence of degenerative diseases, there has been a high consumer demand for functional foods with added health benefits. The term “functional food” generally refers to products providing an additional health benefit, beyond basic nutrition. Consuming foods fortified with functional ingredients (e.g., vitamins, probiotics, omega n-3 fatty acids, minerals, and antioxidants) could help to mitigate the risk of chronic diseases and contribute to the physical and mental well-being. In that sense, (poly)phenols, phenolic compounds containing at least one phenolic hydroxyl group, are the most pronounced secondary metabolites found in plants possessing strong antioxidant power. However, to exploit the use of underutilized wild edible plant resources, convenient extraction methods need to be developed to separate and concentrate the (poly)phenols without using toxic hazardous chemical solvents. Hence, ‘green’ isolation of value-added ingredients for enrichment purposes from underutilized plant sources have importance for food industry. Moreover, (poly)phenols are susceptible to against harsh processing and environmental factors such as heat, oxygen, light, alkaline pH, or enzymes and therefore mostly have poor solubility and bioavailability. Moreover, their biological functions are significantly dependent on their bioavailability. In this regard, encapsulation applications enable increasing their stability by protecting from stresses mentioned above and/or improve their solubility, bioaccessibility and intestinal uptake which is especially desirable for manufacturing of functional foods/beverages and dietary supplements. Taking these facts into consideration, this thesis consist of the following objectives: (i) to characterize the (poly)phenols composition of black rosehip (*Rosa pimpinellifolia* L.) and its antioxidant as well as antiproliferative activity in comparison to common rosehip (*Rosa canina* L.); (ii) to optimize the extraction conditions of black rosehip (poly)phenols by means of green methods, in particular pressurized hot water extraction and ethanol modified supercritical carbon dioxide extraction; (iii) to evaluate the effects of extraction methods on bioaccessibility and bioavailability of (poly)phenols using combined *in vitro* gastrointestinal digestion/Caco-2 cell culture model; (iv) to improve processing and digestive stability of hydrophilic and lipophilic (poly)phenols with high antioxidant acitivity by converting them into edible and stable form for functional foods by means of encapsulation and to investigate the effects of liposomal encapsulation and spray drying processing on bioaccessibility and bioavailability of (poly)phenols using combined *in vitro* gastrointestinal digestion/Caco-2 cell culture model.

The first chapter was designed to characterize *Rosa pimpinellifolia* fruits collected from Gümüşhane Province in the Black Sea region of Turkey by means of (poly)phenol composition, *in vitro* antioxidant activity and other biological activities in comparison to commonly consumed rosehip specie. The angiotensin-converting

enzyme inhibition potential of black rosehip was also assessed by spectrophotometric assay. Since rosehip (*Rosa canina*) fruit has traditionally been widely consumed in the form of herbal tea, tea-type infusions of both *Rosa* species were prepared to reveal the consumption potential of black rosehip. In this context, tea-type infusions and aqueous methanol (80%, v/v) extracts were analyzed by LC-MS/MS in both rosehip species studying different parts of the fruits; whole fruit, flesh, and seed. Apart from other rosehip species, *Rosa pimpinellifolia* has its dark purplish-black color due to its rich anthocyanin content. Unsurprisingly, any anthocyanin was absent in *Rosa canina* fruit. Chromatographic analysis showed that (poly)phenol contents varied between rosehip species and drying method applied (freeze drying and air drying). Gallic acid, catechin, protocatechuic acid, procyanidin-B2, procyanidin oligomers and quercetin acid derivatives were principal in both rosehip species. For black rosehip, high level of anthocyanins, (poly)phenols content and high antiradical scavenging capacity were characteristic. Reactive oxygen species (ROS)-induced-DNA damage has been linked to the onset of many degenerative and cardiovascular diseases. Polyphenolic compounds protect cells against the harmful effects of ROS in various mechanisms. Flavonoids and phenolic acids fractions of *Rosa canina* have been reported to inhibit cell proliferation, however no study exist regarding the antiproliferative properties of black rosehip to the best of our knowlege. In that sense, cytotoxicity by MTT assay, ROS generation, and apoptotic effects by Acridine orange assay in breast cancer cells were evaluated in tea-type infusions and hydroalcoholic extracts from both species.

Response surface methodology was adopted to achieve an effective extraction procedure of (poly)phenols from black rosehip (*Rosa pimpinellifolia*) fruits. Pressurized hot water extraction and ethanol modified supercritical carbon dioxide extraction techniques were investigated by screening parameters including temperature, pressure, solvent composition, and solvent-to-solid ratio. Ultrasound assisted solvent extraction was also assessed. Simultaneous maximization in terms of extraction yield, total antioxidant capacity, total (poly)phenol content, catechin content, total monomeric anthocyanin content, and cyanidin-3-*O*-glucoside content was performed. Antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) radical scavenging activity and cupric ion reducing antioxidant capacity (CUPRAC) assays. The experimental data was subjected to the regression analysis to obtain second-order polynomial equations and the fitted polynomial equations are presented as contour plots also showing the linear and quadratic effects of the tested dependent variables. The optimum conditions for extraction was compared to the predicted values of RSM using an independent sample t-test. The optimized conditions were as follows: 75 °C with 10 mL g⁻¹ solvent-to-solid ratio under 100 bar during 60 mins, and 60 °C with 25 % aqueous ethanol under 280 bar during 60 mins for pressurized hot water extraction and ethanol modified supercritical carbon dioxide extraction, respectively. When these extracts were examined in terms of bioaccessibility and epithelial cells (Caco-2) uptake upon gastric and intestinal digestion *in vitro*, all extracts exhibited lower levels of total phenolic content compared to their undigested counterparts ($p < 0.05$).

Chapter 4 aimed to achieve the encapsulation of black rosehip extracts obtained via green techniques applied in previous chapter and to evaluate their antioxidant properties as well as processing and digestive stability. The black rosehip extract (BRE) which was obtained at optimized pressurized hot water extraction conditions, was further encapsulated in biopolymer-coated liposomes and spray dried using maltodextrin as carrier. Being readily available natural biopolymers, chitosan and

whey protein were used in coating of liposomes via electrostatic deposition method. In the literature, these biopolymers have been widely used for encapsulation purposes of various polyphenolic extracts. However, in the present thesis, for the first time a comparison between these materials for their efficiency to fabricate stable antioxidant powders. Nanosized particle diameters were achieved by 5 cycle microfluidization of the liposomal dispersions. During spray drying process, total phenolic content (TPC) in extracts decreased due to oxygen and heat exposure. However, the retention efficiency of TPC in biopolymer coated liposomal powders was found significantly higher than spray dried BRE. In a similar pattern as observed with retention of phenolics, retention of antioxidant capacity of the powders was confirmed with CUPRAC assay. In addition, encapsulation provided remarkable protection of the phenolics under *in vitro* gastrointestinal digestion conditions, resulting in up to a 5.6-fold more phenolics in the bioaccessible fraction, which also had 2.9–8.6-fold higher antioxidant activity compared to the non-encapsulated BRE. Similar results were obtained for encapsulated RPFE using ethanol injection method in preparation of liposomes. Besides, as an alternative colloidal delivery system to liposomes, solid lipid nanoparticles (SLN) were prepared using plant saponin glycyrrhizin to ensure stabilization at low concentrations which has great importance for the food, nutraceutical and pharmaceutical industries. For the first time a series of glycyrrhizin emulsified tristearin SLNs were successfully fabricated and physical stability was determined during 21 days of storage. The influence of glycyrrhizin on the physical stability and crystallization behavior of SLNs were evaluated by dynamic and static lights scattering, electrophoretic light scattering, optical microscopy, visual observations, and differential scanning calorimetry. The SLN formulations containing lower amount of surfactant revealed poor stability against aggregation as clearly observed by optical microscopy.

Beyond bioaccessibility, the absorption of (poly)phenols through the intestinal epithelial layer is an important factor in functional food development. Thus, the behavior of individual phenolic compounds loaded in dried liposomes should be investigated to gain insight regarding their bioavailability and resulting bioactivity. In the last chapter, the co-encapsulation of phenolic compounds into nanoliposomes in combination was explored in terms of particle characterization and bioavailability compared to their native (non-encapsulated) form. Phenolic compounds representing berry matrix (catechin, epicatechin, ferulic acid, and resveratrol) were loaded into chitosan- and whey protein-coated liposomal powders. For control purposes, the phenolic compounds were also spray dried without liposomes to observe the effect of liposomal encapsulation on protection of bioactive molecules against processing or digestive conditions. The digestive fate of the samples was determined using *in vitro* digestion coupled with Caco-2 cell monolayer model. The phenolic compounds, both in encapsulated or free form, decreased upon simulated gastrointestinal digestion, except cis-resveratrol possibly due to trans-to-cis isomerization that occurs during *in vitro* digestion. Significantly higher digestive stability, solubility, micellization efficiency, and bioaccessibility were found in encapsulated phenolic compounds ($p < 0.05$). On the contrary, whey protein coated liposomal formulation resulted in substantial increase in the cellular uptake of trans-resveratrol in comparison to other encapsulated formulations or in native form ($p < 0.05$). The epithelial permeation of the native and encapsulated micellar phenolics were further evaluated in Caco-2 transwell model system. Moreover, all digested formulations was analyzed in terms of fatty acid induced lipid accumulation in human hepatic cancer (HepG2) cells as an indicator of the effect of encapsulation on the potential biological activity.



SİYAH KUŞBURNU POLİFENOLLERİNİN YEŞİL EKSTRAKSİYONU VE ENKAPSÜLASYONU: İN VİTRO BİYOERİŞİLEBİLİRLİK, BİYOYARARLILIK VE BİYOLOJİK AKTİVİTELERİ

ÖZET

Dejeneratif hastalıkların yaygınlaşması ve yaşam tarzında meydana gelen değişiklikler nedeniyle, ilave sağlık yararları olan fonksiyonel gıdalara yönelik tüketici talebi artmaktadır. “Fonksiyonel gıda” terimi genellikle temel beslenmenin ötesinde ek bir sağlık yararı sağlayan ürünleri ifade etmektedir. Fonksiyonel bileşenlerle (örneğin vitaminler, probiyotikler, omega n-3 yağ asitleri, mineraller, lif ve antioksidanlar) zenginleştirilmiş gıdaların tüketimi kronik hastalıkların riskini azaltmanın yanı sıra fiziksel ve zihinsel sağlığı olumlu yönde etkilemektedir. Bitkilerde bulunan ikincil metabolitler arasında yer alan (poli)fenoller –en az bir fenolik hidroksil grubu içeren fenolik bileşikler, güçlü antioksidan aktiviteye sahiptir. Yaygın tüketimi olmayan yabani yenilebilir bitkiler bu bileşenlerin eldesi için uygun kaynaklar olmakla beraber, (poli)fenoller toksik kimyasal çözücüler kullanmadan izole etmek için uygun ekstraksiyon yöntemlerinin geliştirilmesi gerekmektedir. Bu nedenle, katma değerli gıda bileşenlerinin yeterince kullanılamayan bitki kaynaklarından 'yeşil' yöntemlerle izolasyonu gıda endüstrisi için önem taşımaktadır. (Poli)fenoller, ısı, oksijen, ışık, alkali pH veya enzimler gibi faktörlere karşı hassastır ve bu nedenle çoğunlukla zayıf çözünürlüğe ve biyoyararlanıma sahiptir. Bu bileşenlerin biyolojik işlevleri, önemli ölçüde biyoyararlanımlarına bağlıdır. Bu nedenle kapsülleme uygulamaları, yukarıda bahsedilen streslerden koruyarak stabiliteyi arttırmaya ve/veya özellikle fonksiyonel gıdaların/içeceklerin ve diyet takviyelerinin üretimi için gerekli olan çözünürlüklerini, biyoerişilebilirliklerini ve bağırsak emilimlerini iyileştirmeye olanak sağlamaktadır. Tüm bu unsurlar göz önünde bulundurulduğunda, bu tezin amaçları şu şekilde sıralanabilir: (i) siyah kuşburnu meyvesinin (*Rosa pimpinellifolia* L.) (poli)fenol bileşim, antioksidan ve antiproliferatif aktiviteleri bakımından karakterize etmek ve yaygın tüketimi olan *Rosa canina* L. türü ile karşılaştırmak; (ii) basınçlı sıcak su ekstraksiyonu ve etanol ile modifiye edilmiş süperkritik karbon dioksit ekstraksiyonu gibi yeşil yöntemlerle siyah kuşburnu (poli)fenollerinin verimli şekilde eldesi için ekstraksiyon koşullarının optimize edilmesi; (iii) *in vitro* mide-bağırsak sindirim/Caco-2 hücre kültürü modeli kullanılarak siyah kuşburnu (poli)fenollerinin biyoerişilebilirliği ve biyoyararlanımı üzerinde ekstraksiyon yöntemlerinin etkilerini değerlendirmek; (iv) yeşil yöntemlerle ekstrakte edilen yüksek antioksidan aktiviteye sahip hidrofilik ve lipofilik (poli)fenollerin kapsülleme yoluyla fonksiyonel gıdalarda kullanıma uygun yenilebilir ve stabil forma dönüştürülerek işlenme ve sindirim stabilitesini geliştirmek, diğer bir deyişle lipozomal kapsülleme ve püskürtmeli kurutma işleminin (poli)fenollerin biyoerişilebilirliği ve biyoyararlanımı üzerindeki etkilerini *in vitro* mide-bağırsak sindirim/Caco-2 hücre kültürü modeli kullanılarak araştırmaktır.

Tezin ilk bölümünde, Türkiye'nin Karadeniz bölgesindeki Gümüşhane ilinden toplanan siyah kuşburnu (*Rosa pimpinellifolia* L.) meyvesi içerdiği (poli)fenol

bileşiklerin karakterizasyonu ve antioksidan aktivite yönünden yaygın tüketimi olan kuşburnu (*Rosa canina* L.) türü ile karşılaştırılarak çalışılmıştır. Siyah kuşburnunun anjiyotensin dönüştürücü enzim inhibisyon potansiyeli de spektrofotometrik yöntem ile değerlendirilmiştir. Kuşburnu (*Rosa canina* L.) meyvesi geleneksel olarak bitki çayı formunda yaygın olarak tüketildiği için siyah kuşburnunun tüketim potansiyelini açığa çıkarmak üzere her iki türden çay tipi infüzyonlar hazırlanmıştır. Bu bağlamda, çay tipi infüzyon ve sulu metanol (%80, v/v) ekstraktları her iki kuşburnu türünde LC-MS/MS ile analizlenmiştir. Siyah kuşburnu, tüm meyve, etli, ve çekirdek kısımları ayrıca analizlenerek siyanidin-3-O-glukozit ve kateşin başta olmak üzere sırasıyla 5 antosiyanin ve 49 antosiyanin olmayan (poli)fenolik bileşik tespit edilmiştir. *Rosa pimpinellifolia* L., diğer kuşburnu türlerinden farklı olarak zengin antosiyanin içeriğinden dolayı koyu morumsu-siyah bir renge sahiptir. Beklendiği üzere *Rosa canina* L. meyvesinde antosiyanin izlenmemiştir. Kromatografi analizi sonucunda (poli)fenol içeriğinin kuşburnu türü ve uygulanan kurutma yöntemine (dondurarak kurutma ve havada kurutma) bağlı olarak önemli ölçüde farklılık gösterdiği bulunmuştur. Her iki kuşburnu türünde de gallik asit, kateşin, protokatekuik asit, prosiyanidin-B2, prosiyanidin oligomerleri ve kersetin türevleri tespit edilmiştir. Bitki kaynaklı antioksidanlar gıdaların özelliklerine veya muhafazası sürecine katkıda bulunmalarının yanı sıra oksidasyonla ilgili hastalıkların önlenmesinde de kullanılabilir. Reaktif oksijen türlerinin (ROS) neden olduğu DNA hasarı, birçok dejeneratif ve kardiyovasküler hastalık ile ilişkilendirilmektedir. (Poli)fenolik bileşikler, çeşitli mekanizmalarla hücreleri ROS'un zararlı etkilerine karşı korur. *Rosa canina* türünün flavonoid ve fenolik asit fraksiyonlarının hücre proliferasyonunu engellediği literatürde gösterilmiştir, ancak siyah kuşburnunun antiproliferatif özellikleri ile ilgili herhangi bir çalışma bilginiz dahilinde mevcut değildir. Siyah kuşburnu meyvesinin çay tipi infüzyonu ve sulu metanol ekstraktlarının sitotoksitesi MTT (3- (4,5-dimetiltiyazol-2-il) -2-5-difeniltetrazolyum bromür) testi ile, ROS üretimi ve apoptotik etkileri Akridin Oranj-Etidyum Bromit metodu ile insan meme kanseri hücrelerinde araştırılmıştır. Siyah kuşburnu meyvelerinin (poli)fenol içeriği ve antiradikal temizleme kapasitesinin doza bağımlı olduğu görülmüştür.

Siyah kuşburnu meyvesinden (poli)fenollerin yeşil yöntemlerle etkin şekilde eldesi için uygun ekstraksiyon koşulları yanıt yüzey metodolojisi ile araştırılmıştır. Bu kapsamda, basınçlı sıcak su ekstraksiyonu ve etanol ile modifiye edilmiş süperkritik karbon dioksit ekstraksiyonu yöntemleri sıcaklık, basınç, çözgen bileşimi ve çözgen-hammadde oranı gibi ekstraksiyon parametreleri çalışılarak optimize edilmiştir ve ultrason destekli çözgen ekstraksiyonu ile karşılaştırılmıştır. Sonuç olarak elde edilen ekstraktlarda ekstraksiyon verimi, toplam antioksidan kapasitesi, toplam (poli)fenol içeriği, kateşin içeriği, toplam monomerik antosiyanin içeriği ve siyanidin-3-O-glukozit içeriği analizlenerek bu parametrelerin eş zamanlı maksimizasyonu sağlanmıştır. Antioksidan aktivite, 2,2-difenil-1-pikrilhidrazil radikali (DPPH) radikal süpürme aktivitesi ve bakır iyonu azaltıcı antioksidan kapasite (CUPRAC) deneyleri kullanılarak değerlendirilmiştir. Deneysel veriler, regresyon analizine tabi tutularak uygun ikinci derece polinom denklemleri elde edilmiş, test edilen bağımlı değişkenlerin doğrusal ve ikinci dereceden etkilerini de gösteren kontur grafikleri ayrıca gösterilmiştir. Etkin ekstraksiyon işlemi için optimum koşullar, bağımsız örnek t-testi kullanılarak yanıt yüzey analizi ile tahmin edilen değerlerle karşılaştırılmıştır. Optimize edilen koşullar sırasıyla basınçlı sıcak su ekstraksiyonu için 10 mL g-1 çözücü-hammadde oranı ile 75 °C ve 100 bar 60 dakikada elde edilirken; etanol modifiye süper kritik karbondioksit ekstraksiyonu için aynı sürede 280 bar altında %25 sulu etanol ile 60 °C olarak bulunmuştur. Bu ekstraktların biyoyararlanımları *in vitro*

mide ve bağırsak sindirimi/Caco-2 epitel hücre modeli kullanılarak incelendiğinde, tüm ekstraktlar sindirim sonrasında daha düşük toplam fenolik içeriği kaydetmişlerdir ($p < 0,05$). Elde edilen sonuçlara göre, farklı yöntemlerle elde edilen ekstraktlardaki (poli)fenollerin biyoyararlanımları sözkonusu bileşiğe ve ekstrakttaki miktarına göre değişkenlik göstermiştir.

Oksidatif stresin azaltılmasında önemli bir potansiyele sahip olduğu anlaşılan siyah kuşburnu ekstraktlarında bulunan (poli)fenollerin fizikokimyasal faktörlere karşı stabilitelerinin artırılması bu bileşiklerin endüstriyel uygulamaları için büyük önem teşkil etmektedir. Bu sorunun üstesinden gelmek için, tezin önceki bölümünde yeşil yöntemlerle elde edilen siyah kuşburnu ekstraktların kapsüllenmesi ve antioksidan özelliklerinin işleme ve sindirim stabiliteleri değerlendirilmiştir. Optimize edilmiş basınçlı sıcak su ekstraksiyon koşullarında elde edilen siyah kuşburnu ekstraktı (BRE), biyopolimer kaplı lipozomlarda kapsüllenmiş ve taşıyıcı olarak maltodekstrin kullanılarak püskürtmeli kurutucuda toz hale getirilmiştir. Lipozomların elektrostatik depozisyon yöntemi ile kaplanmasında kolaylıkla bulunabilen doğal biyopolimerler olan kitosan ve peynir altı suyu proteini kullanılmıştır. Bu biyopolimerler, çeşitli (poli)fenolik ekstraktların kapsüllenmesi amacıyla yaygın olarak kullanılmaktadır. Bununla birlikte, bu biyopolimerler bu tez kapsamında ilk kez stabil formda antioksidan tozların üretimi için etkinlikleri açısından karşılaştırılmıştır. Enkapsülasyon etkinliği kaplanmamış BRE lipozomu, kitosan kaplı BRE lipozomu ve peynir altı suyu proteini kaplı BRE lipozomu için sırasıyla $67,2 \pm 0,7$, $76,7 \pm 0,8$ ve $87,2 \pm 4,0$ olarak bulunmuştur ($p < 0,05$). Hidrofilik karakterli ekstrakt yüklü lipozomal dispersiyonların ortalama partikül çapı ~ 114 nm olarak ölçülmüştür. Bununla beraber, optimize etanol ile modifiye edilmiş süperkritik karbondioksit yöntemiyle elde edilen ekstrakt (RPFE–*Rosa pimpinellifolia* meyve ekstrakt) ile etanol enjeksiyon yöntemi kullanılarak hazırlanan hidrofilik ve/veya lipofilik karakterli ekstrakt yüklü lipozomlarda partikül çapı ~ 95 nm olarak kaydedilmiştir. Lipozomal dispersiyonların 5 defa yüksek basınçlı homojenizatörden geçirilmesiyle nano boyutunda partikül çapları elde edilmiştir. Püskürtmeli kurutucuda kurutulmuş partiküllerin mikro yapısı, taramalı elektron mikroskobu ile gözlemlenmiştir. Püskürterek kurutma işlemi sırasında, ekstraktlardaki toplam fenolik içerik oksijen ve ısıya maruz kalma nedeniyle azalmıştır. Bununla birlikte, biyopolimer kaplı lipozomal tozlarda toplam fenolik içeriğin korunma etkinliği, yalnızca püskürtmeli kurutucuda kapsüllenene ekstraktan yüksek bulunmuştur ($p < 0,05$). Benzer şekilde, lipozomal tozların antioksidan kapasitesinin daha etkin şekilde korunduğu CUPRAC (Bakır(II) iyonu indirgeyici antioksidan kapasite) analizi ile doğrulanmıştır ($p < 0,05$). Bunlara ek olarak, kapsülleme simule mide-bağırsak sindirim koşullarında (poli)fenollerin önemli ölçüde koruyarak biyoerişilebilir fraksiyonda kapsüllenmemiş ekstrakta kıyasla sindirim sonrasında stabil kalan 5,6 kat daha fazla (poli)fenol ve 2,9 ila 8,6 kata kadar daha yüksek antioksidan aktivite sağlamıştır. Etanol enjeksiyon yöntemi kullanılarak hazırlanan RPFE yüklü lipozomlarda da benzer sonuçlar elde edilmiştir. Ayrıca, lipozomlara alternatif bir kolloidal taşıyıcı sistem olarak lipofilik karakterli bileşiklerin kapsüllenmesi için bitkisel kaynaklı saponin kullanılarak katı lipid nanopartiküller (SLN) hazırlanmıştır. Bu taşıyıcı sistemlerin düşük konsantrasyonlarda doğal emülgatör kullanılarak stabilizasyonu gıda ve ilaç endüstrilerinde büyük öneme sahiptir. Tez kapsamında, tristearin bazlı katı lipid nanopartiküller farklı glisirizin konsantrasyonları kullanılarak ilk kez üretilerek farklı pH koşullarında ve 21 günlük depolama sırasında fiziksel stabiliteleri araştırılmıştır. Hazırlanan katı lipid nanopartiküllerin fiziksel stabilitesi ve kristalizasyon davranışı dinamik ve statik ışık saçılımı, elektroforetik ışık saçılımı, optik mikroskopi, görsel

gözlemler ve diferansiyel tarama kalorimetri yöntemleri kullanılarak değerlendirilmiştir. Daha düşük miktarda saponin içeren formülasyonların agregasyona karşı stabilitelerinin düşük olduğu görüşmüş ve optik mikroskop ile de doğrulanmıştır. Sonuç olarak, emülgatör konsantrasyonu ve pH, katı lipid nanopartiküllerin partikül boyutunda, zeta potansiyelinde ve polimorfik stabilitesinde önemli rol oynadığı gösterilmiştir.

Biyoerişilebilirliğin yanı sıra, (poli)fenollerin bağırsak epitel hücrelerindeki emilimi potansiyel biyolojik aktivitelerini yerine getirebilmeleri bakımından fonksiyonel gıda formülasyonlarının geliştirilmesi için önemli bir faktördür. Bu nedenle, biyoyararlanımları ve buna bağlı olarak biyoaktiviteleri hakkında fikir edinmek için kurutulmuş lipozomlara yüklenen fenolik bileşiklerin stabilitelerinin bileşik bazında araştırılması gerekmektedir. Bu bağlamda tezin son bölümünde, seçilen fenolik bileşiklerin birlikte kapsüllenerek, yani birlikte nanolipozomlara yüklenen fenolik bileşiklerin doğal (kapsüllenmemiş) formlarına kıyasla stabiliteleri ve biyoyararlanımları araştırılmıştır. Kitosan ve peynir altı suyu proteini kaplı prolipozom tozlarını üretmek için kırmızı meyvelerdeki temel (poli)fenollerini temsil eden bileşikler (kateşin, epikateşin, ferulik asit ve resveratrol) incelenmiştir. Gıda ve takviye endüstrisinde genellikle kuru formdaki ürünler sulu çözeltiler veya dispersiyonlardan daha stabil ve uzun süreli depolama için daha uygun olmaları bakımından tercih edilmektedir. Lipozomal kapsüllemenin biyoaktif moleküllerin işleme veya sindirim koşullarına karşı korunması üzerindeki etkisi araştırılırken deney tasarımı kontrol amacıyla seçilen fenolik bileşikler püskürtmeli kurutucu ile de ayrıca kapsüllenmiştir. Püskürtmeli kurutma işleminin hazırlanan partiküllerin zeta potansiyeli üzerindeki etkisinin yeniden suda çözündürüldüklerinde istatistiksel olarak önemsiz olduğu görülmüştür ($p > 0,05$). Hazırlanan toz formülasyonların biyouyumluluğu insan bağırsak yolunun epitel hücrelerine karşılık gelen insan kolon adenokarsinom hücrelerinde (Caco-2) test edilmiş ve doğrulanmıştır. Örneklerin simüle mide ve bağırsak koşullarındaki stabiliteleri Caco-2 hücre modeli ile birleştirilmiş *in vitro* sindirim modeli kullanılarak belirlenmiştir. Hem kapsüllenmiş hem de serbest formdaki fenolik bileşikler sindirim sonrasında önemli ölçüde bozunmaya uğrarken yalnızca sindirim sırasında meydana gelen trans-cis izomerizasyonu nedeniyle cis-resveratrol artış göstermiştir. Enkapsülasyon işleminin etkinliği bu örneklerdeki fenolik bileşiklerde kaydedilen yüksek sindirim stabilitesi, çözünürlük, miselizasyon verimliliği ve biyoerişilebilirlik sonuçlarıyla kanıtlanmıştır ($p < 0,05$). Seçilen fenolik bileşiklerin kapsüllenmemiş formdaki stabiliteleri ayrı ve birlikte *in vitro* sindirim sonrası incelendiğinde biyoerişilebilirlikleri bakımından önemli bir fark gözlenmemiştir ($p > 0,05$). Diğer yandan, peynir altı suyu proteini kaplı lipozomal formülasyon, diğer kapsüllenmiş formülasyonlar veya doğal form ile karşılaştırıldığında trans-resveratrolün hücre alımında önemli bir artış bulunmuştur ($p < 0,05$). Serbest ve kapsüllenmiş fenoliklerin sindirim sonrası epitelyal emilimi Caco-2 transwell model sisteminde değerlendirilmiştir. Çalışılan Caco-2 hücrelerinin bütünlüğü ve geçirgenliği Trans-Epitel Elektrik Direnci (TEER) değerleri ile değerlendirmiştir. Fenolik bileşiklerin en düşük emilimi püskürtmeli kurutucuda kapsüllenen örnekte izlenmiştir. Nanolipozomal kapsülleme yöntemi, hücreler tarafından emilen veya geçirilen epikateşin ve ferulik asit oranlarını önemli ölçüde etkilemiştir ($p < 0,05$). Kapsülleme yoluyla bileşiklerin apikal geri kazanımında hiçbir fark gözlenmemiştir ($p > 0,05$). *In vitro* sindirim sonrasında tüm formülasyonlar, kapsüllemenin potansiyel biyolojik aktivitenin (anti-diyabetik aktivite) korunması üzerindeki etkisini incelemek için insan karaciğer kanseri (HepG2) hücrelerinde yağ birikimi açısından analiz edilmiştir. Sonuçlara göre, çalışılan fenolik bileşikler

(kateşin, epikateşin, ferulik asit ve resveratrol) hem doęal hem de kitosan kaplı lipozomal formda oleik ve palmitik asit kaynaklı hepatik steatozu baskıladıęı izlenmiştir ($p < 0,05$).





1. INTRODUCTION

Phytochemicals ('phyto' is from the Greek word meaning plant) present in plants have gained attention due to their physiological activities despite being the nonnutrient substances. These compounds can be present in root, stem, leaves, bark, seed, flower, and fruit that can yield a physiological response in the organism. More than 5000 phytochemicals have been identified, yet a large percentage is estimated as unknown (Harbourne et al., 2013). The potential of phytochemicals could even be more than it is recognized already as oxidative stress is correlated with a number of chronic diseases (Ames & Gold, 1991).

There is a diversity of phytochemical contents exist among genera of small fruits (Moyer et al., 2002). Moreover, there is mounting evidence that indicates genetic factors in berry fruits may have a significant impact on the quantity of bioactives (Anttonen & Karjalainen, 2005). According to Skrede, Wrolstad, and Durst (2010), berry fruits are commonly consumed in their raw or processed forms as components of diverse products such juices, juice concentrates, jams, and jellies. There are various dietary supplements that utilise their extracts or isolated components (Šavikin et al., 2014). The primary physiologically active components of berries, phenolics, which have significant antioxidant qualities, are typically linked to the health benefits of berries (Đorđević et al., 2010; Häkkinen et al., 1999). Antioxidants are key components in foods due to their ability to reduce free radical-mediated degradation of cells (Rice-Evans, Miller, & Paganga, 1996). Cells in humans and other organisms are constantly exposed to a variety of oxidizing agents. The damage in proteins, DNA, and lipids caused by oxidative stress lead to an augmented risk for cancer and cardiovascular disease (Ames et al., 1993). A adequate number of antioxidants must be taken in order to avoid or slow down the oxidative stress brought on by free radicals. Plant-based products contain a wide variety of phytochemicals such as (poly)phenols (see Frank et al. (2020) for the used terminology), such as phenolic acids, flavonoids, anthocyanins, proanthocyanidins, and carotenoids which can contribute to combat oxidative damage (Liu, 2003).

The influences of (poly)phenols on human health have been mainly attributed to their antioxidant properties (Machado, Pasquel-Reátegui, Barbero, & Martínez, 2015). The global polyphenol market was valued at USD 866.41 million in 2020 and it is projected to reach USD 1,423.41 million by 2026 according to the report of Mordor Intelligence (Anonymous, 2022). Traditional methods for isolating bioactive substances, such as (poly)phenols and anthocyanins, from vegetable matrices include stirring, heating, and/or extraction using organic solvents. Traditional methods, on the other hand, frequently take a long time and degrade the target compounds during extraction. They also require a lot of organic solvents, some of which can be harmful to human health and pollute the environment and must be properly separated from the extract before being disposed of (Caldas et al., 2018; Roseiro et al., 2013). The hydroxyl groups of (poly)phenols can neutralize free radicals and reactive oxygen species (ROS). For the majority of living things, the creation of energy in biological processes depends on oxygen. However, a small portion of the oxygen consumed by the body—between 2 and 3 percent—is transformed into ROS and free radicals, which hasten the oxidative damage to different biomolecules (Cabiscol et al., 2000). Numerous pathophysiological illnesses, such as inflammation, cancer, and neurodegenerative diseases, have been linked to this oxidative damage (Thanan et al., 2015). Moreover, *R. canina* wine showed substantial antimutagenic effect *in vitro* (Czyzowska et al., 2015).

2. LITERATURE REVIEW

2.1 Berry Polyhenols

Several fruits and berries, including sour cherries, blackberries, strawberries, and raspberries, have lower total antioxidant capacities than rose hips (Halvorsen et al., 2002). Consumption of *Rosa canina* fruits in European nations through traditional herbal teas, marmalades, jams, nectars, wine, soups, food supplements, etc (Yildiz & Alpaslan, 2012). The abundance of phenolic compounds in Rosaceae fruits, which are well established for their antioxidant, antimutagenic, and anticarcinogenic properties, may be the reason for their significant physiological functions (Tapiero, Tew, Ba, & Mathe, 2002). Nonetheless, depending on the species and plant's geographical origin, the amount of these phytochemicals may vary. (Poly)phenols including flavonoids, phenolic acids, tannins, and anthocyanins were shown to be significant biologically active constituents present in rosehips (Ogah et al., 2014). Several authors compared the chemical composition of various wild grown and cultivated *Rosa* species in which *Rosa canina* L. is studied extensively among others (Ercisli, 2007; Adamczak et al. 2012; Demir et al., 2014; Elmastas et al. 2017).

The genus *Rosa* contains around 200 species (Jiménez et al., 2017; Nadpal et al., 2016). Rose hips, the fruits of rose plants, are used in ethnomedicine for the treatment of colds, flu, infections, influenza, inflammations, and diabetes (Barros et al. 2010; Cakilcioglu et al., 2011). Rosehip berries can be consumed fresh, but they are mostly used fresh or dry to be used in forms of teas, juices, jams, and marmalades (Nybom & Werlemark, 2017; Patel, 2017). Being very rich in vitamin C content, the use of rosehip extracts as antioxidant additives has been suggested in food industry (Vossen et al., 2012) and extracts commercialised as dietary supplements (Nybom & Werlemark, 2017). The consumption of dried fruit-based herbal teas is common due to special aroma, fruity flavor, and lack of astringency (Erasmus et al., 2017).



Figure 2.1 : Morphology of hips from (A) *R. canina* and (B) *R. pimpinellifolia*.

2.1.1 Black rosehip

One of the key regions for rosehip species' genetic diversity is Anatolia. The interest in rose hips has increased after its remedial potential for a number of diseases has been reported (Mármol et al., 2017). However, to the best of our knowledge no data exist regarding black rosehips from our country. (Poly)phenols including flavonoids, phenolic acids, tannins, and anthocyanins are shown to be significant biologically active constituents present in rosehips (Ogah et al., 2014). Several authors compared the chemical composition of various wild grown and cultivated *Rosa* species in which *R. canina* is the most abundant and studied species among all (Ercisli, 2007; Adamczak et al. 2012; Demir et al., 2014; Elmastas et al. 2017). Significant differences in total phenolic content among different rosehips originating from different geographical zones have been reported (Jimenez et al., 2017). On the other hand, *R. pimpinellifolia* L. syn. *R. spinosissima* L. is endemic to Europe and Western Asia with small and black or dark purple color hips due to rich anthocyanin content (Koczka, Stefanovits-Bányai, & Ombódi, 2018). Recently, Koczka et al. (2018) compared water and ethanolic extracts from *R. canina*, *R. gallica*, *R. rugosa*, *R. spinosissima*. It was shown that *Rosa pimpinellifolia* hips has a much highest phenolic level and antioxidant activity among the tested ones. Similar observations were obtained from hydroethanolic extracts of Lithuanian *R. pimpinellifolia* L. cultivar “Single Cherry” recently (Liaudanskas et al., 2021). To the best of our knowledge, no data has yet to be reported in cytotoxic properties of *R. pimpinellifolia* L. species using *in vitro*.



Figure 2.2 : Hips from *R. pimpinellifolia* after milling and freeze drying.

Anthocyanins are plant secondary metabolites known as functional pigments with various health benefits. Among them, the most prevalent natural anthocyanidin in the plant kingdom is cyanidin-3-*O*-glucoside (Rupasinghe, Arumuggam, Amararathna, & De Silva, 2018) including black rosehip. Cyanidin-3-*O*-glucoside possesses a good radical scavenging capacity against superoxide but not hydroxyl radicals. Strong evidence backs up cyanidin-3-*O*-glucoside's bioactivities as a radical scavenger, gastroprotective, and chemopreventive that works to prevent diseases like *Helicobacter pylori* infection, age-related illnesses, metabolic syndrome, cardiovascular diseases, and cancer. The most pertinent mechanisms are enzyme inhibition, competitive protein-binding, epigenetic activity, and radical scavenging capacity (Olivas-Aguirre et al., 2016).

2.2 Biological activities of polyphenols

In addition to the well-known health advantages of eating a diet high in fruits and vegetables, studies on the protective properties of phenolic compounds obtained from plants have also been conducted, (poly)phenols in other words, has gained considerable attention (Wang et al., 2020). However, the interactions between (poly)phenols and food matrix plays a crucial role during the release in the gastrointestinal tract to exert health benefits (González-Aguilar et al., 2017). Furthermore, the biological activity of (poly)phenols depends on its structure, size, type, and the number of functional groups (López-Martínez et al., 2015). Rosehip fruits are also very rich in terms of vitamin C content (Vossen et al., 2012). Ascorbic acid has been indicated to act as pro-oxidants for the induction of apoptotic cell death (Sakagami & Satoh, 1997).

2.3 Digestive Fate of Polyphenols

2.3.1 Bioaccessibility

Bioaccessibility is described as the amount of a biocomponent that is released from the food matrix in the gastrointestinal tract, so that it can be ready to be absorbed. Bioaccessibility strongly affects bioavailability (Palafox-Carlos et al., 2011). Releasing from the food matrix and then solubilization in association micelles is the first step of absorption by the epithelial cells (Dima et al., 2020a). Bioaccessibility is described as the percentage ratio between the amount of a compound solubilized in the micellar phase and the amount of compound in raw material (Dima et al., 2020a). Experimental determination of bioaccessibility is performed using *in vitro* assays. Due to their numerous benefits, such as being more rapid, having no ethical restrictions, being able to control the conditions, and producing repeatable results, *in vitro* digestion experiments are conducted to predict bioaccessibility (Minekus et al., 2014). The most realistic simulation of the gastrointestinal tract conditions are based on chemical, biochemical, and mechanical conditions of digestive tract (Alminger et al., 2014).

2.3.2 Bioavailability

Beyond digestion, bioavailability is a complicated phenomenon which also includes absorption, metabolism, tissue distribution, and finally bioactivity. In this regard, *in vitro* digestion protocols are preferred over *in vivo* studies which are expensive, slow and uneasy to perform (Wojtunik-Kulesza et al., 2020). The structure and physicochemical characteristics of bioactive compounds together with the type of food matrix have a complex influence on their release and bioavailability (Dima et al., 2020b). The food matrix is a complex system with a microstructure in which the biocomponents interact with each other (Aguilera, 2019).

After intake, cyanidin-3-*O*-glucoside can be absorbed by the gastric epithelium (1%–10%) and then transformed further by gut. It reaches the bloodstream (<1%). Protocatechuic acid and phloroglucinaldehyde were reported as the major metabolites of cyanidin-3-*O*-glucoside (Olivas-Aguirre et al., 2016).

2.4 Effect of Extraction Methods on Polyphenols

Due to the advantages such as the use of alternative solvents (such as ethanol and carbon dioxide), promising high quality for the final extract, and lower energy consumption, new and "greener" extraction techniques for the isolation of biocomponents from natural sources have recently drawn more attention.

2.4.1 Conventional solvent extraction

The scientific literature contains a vast of information on conventional extraction techniques for biologically active substances. However, there are restrictions when trying to get extracts without solvents. Organic solvent extraction not only possesses health concerns, but also harms the environment. In addition, degradation or loss of the compounds of interest, the use of large volumes of hazardous solvents, and long extraction times are other disadvantages. Therefore, alternative extraction methods called as 'green technologies' that can significantly decrease the need for organic solvents (Tsao & Deng, 2004).

2.4.2 Green extraction techniques

2.4.2.1 Ultrasound assisted extraction

Ultrasound has been defined as the frequency exceed the threshold for human auditory detection that is 20 kHz. The output source of the ultrasound makes the surrounding medium vibrate and then ultrasound wave transfers energy to other neighboring particles (Wen et al., 2018). Power, frequency and amplitude are the crucial parameters in ultrasound process (Pico, 2013).

2.4.2.2 Pressurized liquid extraction

The term "pressurized liquid" refers to pressurized solvents at high temperatures but below their critical points, which can remain in a liquid state throughout the extraction process (Herrero et al., 2015). Applying pressurized water and a high temperature in the subcritical phase can change the solvent's polarity and dielectric constant, improving the extraction process. The process increases mass transfer rate while maintaining the biological potency of the extracts (Zakaria & Kamal, 2016). Pressurized hot water extraction (PHWE) has advantages for industrial applications, such as being cost and time effective, and the use of water as solvent being non-toxic,

recyclable, and readily available (Plaza & Turner, 2015). In PHWE, the role of the pressure is to keep water in liquid state during extraction (Saleh et al., 2011). Previous RSM studies for pressurized liquid extraction–PLE and PHWE of plant phenolics demonstrated that both temperature and solvent-to-solid ratio were important factors affecting yield (Luthria, 2008; Xu et al., 2015; Saravana et al., 2016).

2.4.2.3 Supercritical fluid extraction

High selectivity, low viscosity, high diffusivity, and high solvent power are only a few of the benefits of supercritical and subcritical fluid extraction. In worldwide markets, supercritical fluid extraction has become more significant, leading to technological advancements mostly in areas related to the food and pharmaceutical industries (Veggi, Cavalcanti, & Meireles, 2014). One of the most established supercritical fluid technologies is the supercritical carbon dioxide extraction (SCO₂) (Esquivel-Hernández et al., 2017). SCO₂ extraction processes are advantageous for being greener and faster than traditional solvent extraction. Carbon dioxide is the most preferred solvent as it is odorless, environmentally benign, and simple to separate from the extract. Additionally, because of its low critical temperature, it can be utilized to extract reactive and thermally sensitive substances. This method has advantages over conventional ones, including the ability to continuously modify the solvent power/selectivity, elimination of harmful substances, and a decrease in post-processing expenses because solvent removal from the extracts is no longer necessary (Piantino et al., 2008).

Various natural extracts can be collected by supercritical extraction processes (Jacques, Santos, Dariva, Oliveira, Caramao, 2006; Cavero et al., 2007). Depending on the specific temperature, pressure, and molar fractions of the component solvents, a homogenous solvent mixture can be created by mixing varied amounts of the polar solvents carbon dioxide, ethanol, and water. The most important parameter—the percentage of co-solvent—provides a rise in the total phytochemical content when the amount of co-solvent is higher. However, the overall phytochemical content and antioxidant capacity did not significantly vary when temperature and pressure were changed (Pimentel-Moral et al., 2019).

2.5 Encapsulation Strategies to Improve Stability of Polyphenols

Bioactive compounds such as (poly)phenols, vitamins, and omega-3-fatty acids are often susceptible to oxygen, light, and heat (Champagne & Fustier, 2007). Colloidal delivery systems provide a powerful tool to ensure the stability of these compounds to obtain a prolonged shelf life and enhanced bioavailability by embedding them in a protective matrix (McClements et al., 2009; Dordevic et al., 2015).

2.5.1 Liposomes

Incorporation of labile (poly)phenols in liposomal delivery systems are used to improve the aqueous solubility and to achieve targeted and/or sustained release of bioactives (Amri et al., 2012). Liposomes are microscopic bilayer vesicles resulted from dispersing phospholipids in aqueous medium possessing several advantages such as being biodegradable, biocompatible, and ability to encapsulate hydrophilic and lipophilic molecules simultaneously. Thus, liposomes are used to enhance stability, solubility, bioaccessibility, and/or bioavailability of many bioactives including phenolics for their utilization in food, pharmaceutical, and cosmetic industries (Tsai & Rizvi, 2016; Has & Sunthar, 2020). Nonetheless, liposomes are labile structures and thermodynamically instable leading the leakage of the encapsulated compounds upon storage (Liu, Ye, & Singh, 2015). Coating is an efficient way to improve the stability of liposomal membrane using chitosan (Laye, McClements, & Weiss, 2008; Hao et al., 2017; Esposto et al., 2021), whey protein (Frenzel & Steffen-Heins, 2015; Frenzel & Steffen-Heins, 2015a; Dag, Guner, & Oztop, 2019), alginate (Liu et al., 2016; Ghaleshahi & Rajabzadeh, 2020), PEG (Dadashzadeh et al., 2010), & polylysine (Alemán et al., 2016), and other biopolymers (Dag, Guner, & Oztop, 2019).

2.5.2 Solid lipid nanoparticles

An alternative to liposomes and other colloidal carriers is solid lipid nanoparticles (SLN) (Ekambaram et al., 2012). Among the lipid-based delivery systems, solid lipid nanoparticles (SLN) are fabricated using lipids which are solid at room temperature. A major advantage of these colloidal systems is that they are made of physiological and biodegradable lipids which eliminates their toxicity (Pardeike, Hommoss, & Müller, 2009). compared to fat emulsions and liposomes (Dudhipala & Veerabrahma, 2016). Moreover, SLNs are also feasible for large scale production (Aditya et al.,

2014). Nevertheless, SLN suspensions undergo a polymorphic transition from α - to β -subcell crystals, which is often accompanied by particle aggregation and gel formation (Awad et al., 2008). Molecules comprising both hydrophilic and hydrophobic areas with high water solubility are able to cover the new surface area formed during conversion of α - to β -subcell crystal (Aditya & Ko, 2015). Thus, surfactants have been indicated to play a key role in the stability of SLN (Helgason et al., 2009; Salminen et al., 2013; 2014).

Ensuring physical stability is critical for new carriers. Additionally, utilization of natural surfactants which are efficient at low concentrations has great importance in food industry. Plant saponins appear to be highly active at emulsification and stabilization of colloidal particles (Ozturk & McClements, 2016; Böttcher & Drusch, 2017). In colloidal particles, steric stabilization is accomplished by adsorbing surfactants on the surface (Sozer & Kokini, 2009; Weiss et al., 2008). Plant-based emulsifiers are of interest due to the possibility to replace artificial ones. Plant saponins have a high potential to stabilize dispersed systems in various industrial applications owing to their interfacial property (Böttcher, & Drusch, 2017).

2.5.3 Spray drying

Drying of liposomal dispersions enhances physicochemical stability during storage and enable their incorporation into food formulations (Gültekin-Özgüven et al., 2016). Being a convenient, time and cost-efficient process, spray-drying is the mostly preferred dehydration technology enabling liposomal dispersions to be converted into powders. It is well established that coating liposomes with biopolymers before drying increases stability and preserves vesicle shape (Yu et al., 2021). Increasing the bilayer membrane stiffness during powder reconstitution prevents a significant loss of the chemicals that are contained (Sarabandi & Jafari, 2020).

3. POLYPHENOLS COMPOSITION AND BIOLOGICAL ACTIVITY OF BLACK (*ROSA PIMPINELLIFOLIA* L.) AND ORANGE (*ROSA CANINA* L.) ROSEHIP BERRIES

3.1 Introduction

Rosehip fruits are generally used fresh or dried in forms of teas (Nybom & Werlemark, 2017; Patel, 2017). Consumption of plant-based beverages was demonstrated as one of the most common way of supplying biologically active secondary metabolites (Pulido et al., 2003). Herbal and tea infusions are on demand in daily diet thanks to their fragrance, fruity flavor, lower amounts of caffeine (Savikin et al., 2014). Hence, the aim of this study was to determine and compare the phenolic composition and bioactivities of *R. pimpinellifolia* and *R. canina* hips in tea-type infusions and hydroalcoholic extracts in terms of antioxidant, ACE inhibitory, and antiproliferative properties. To the best of our knowledge, no data has yet to be reported in cytotoxic properties of *R. pimpinellifolia* species using *in vitro* cell lines.



Figure 3.1 : Fresh fragmented hips from *R. pimpinellifolia* and *R. canina*.

3.2 Materials and Methods

3.2.1 Plant material

R. pimpinellifolia and *R. canina* fruits were collected from Gümüşhane province in Blacksea region of Turkey. The debris and spoiled fruits were separated. After

washing, fruits, with and without seeds, and seeds dried in oven (Memmert UN55, Germany) to a constant weight at 40 °C during 72 h with air circulation. Prior to infusion, air-dried rosehips were milled into a powder. For hydroalcoholic extracts, fresh rosehips milled using liquid nitrogen and then lyophilized overnight. All samples stored at -80°C and prior the experimentation.

3.2.2 Preparation of methanolic extracts and tea-type infusions

For hydroalcoholic extracts, 1 g of lyophilized powder was extracted using 10 mL methanol:water (80:20, v/v) for 30 min in an ultrasonic bath, centrifuged, and filtered. The extracts then concentrated under vacuum and lyophilized overnight.

For tea-type infusions, 10 g powdered oven-dried samples (with air circulation at 40 °C for 72 h) were added to 100 mL of boiling distilled water, kept at room temperature for 30 min, filtered, and lyophilized.

3.2.3 Sample preparation for chromatography

For anthocyanin analysis; 4 mL of acidified (5% formic acid, v/v) aqueous methanol (80%, v/v) were added to 50 mg lyophilized sample and sonicated with probe at 60% amplitude for 20 s. After centrifugation, the supernatant was collected and the solid residues were re-extracted thrice. A total of 20 mL combined methanolic extracts were concentrated in rotary (bath temp. <40 °C). The dried extract was dissolved in acidified 1 mL of 50% aqueous methanol (1%,v/v), then membrane-filtered (0.45 µm) into amber vials prior to HPLC analysis.

For non-anthocyanin phenolic analysis; same extraction procedure was followed using 500 mg lyophilized samples. Non-anthocyanin phenolics separated from anthocyanins using SPE cartridge (Chromabond® C18ec) as described previously elsewhere (Kammerer, Carle, & Schieber, 2004).

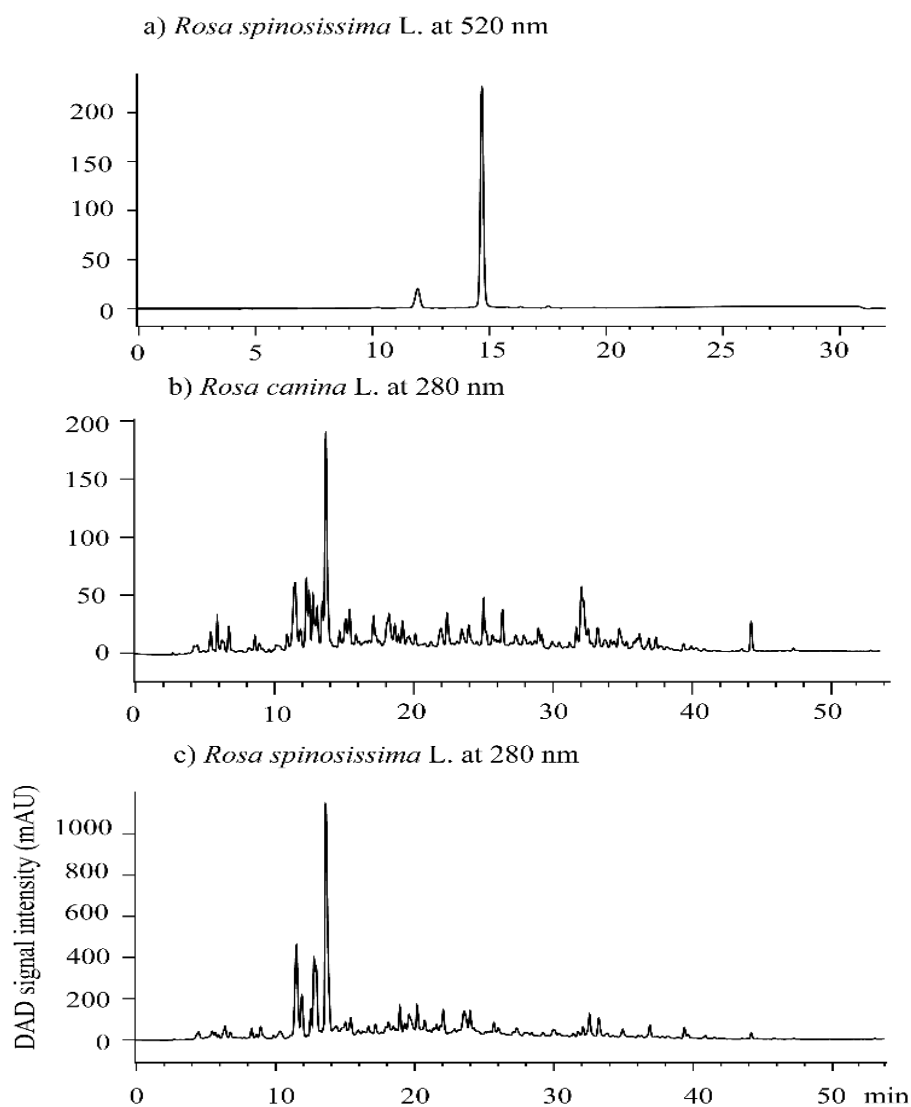


Figure 3.2 : Chromatographic separation of anthocyanins at 520 nm (a), and non-anthocyanin phenolics in aqueous methanolic extracts of *Rosa canina* (b) and *Rosa pimpinellifolia* (c) at 280 nm.

3.2.4 HPLC-DAD-ESI/MSⁿ analysis

The chromatographic analysis was accomplished using an 1100 series HPLC system (Agilent, Waldbronn, Germany) as described Erşan et al. (2016). The mobile phases were water for eluent A methanol for eluent B both contained 1 % (v/v) and 5 % (v/v) formic acid for the separation of non-anthocyanin phenolics and anthocyanins, respectively. For the multistage mass spectrometry, the system was interfaced with an Esquire 3000+ ion-trap mass spectrometer (Bruker Daltonics, Germany). For anthocyanins, the ESI source operated in positive ion mode whereas it operated in negative ion mode for others.

3.2.5 Total phenolic content

Total phenolic content was measured by Folin-Ciocalteu assay, as described by Altin et al. (2018). The absorbance was measured at 765 nm and results were expressed as mg gallic acid equivalents (GAE) per gram of sample in DW.

3.2.6 Total flavonoid content

Total flavonoid content was tested using aluminium chloride calorimetric assay as described by Altin et al. (2018).

3.2.7 Total antioxidant capacity

The hydrogen-donating or radical-scavenging ability of samples measured using the stable radical DPPH radical according to Altin et al. (2018). The percent DPPH decolorisation of sample at 517 nm was calculated using Equation 3.1.

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100 \quad (3.1)$$

where A0 is the absorbance of negative control; A1 is the absorbance of reaction mixture. Results expressed in terms of IC₅₀ value, the sample concentration necessary to decrease the absorbance of DPPH by 50%.

ABTS radical scavenging assay was conducted according to Kocyigit et al. (2019). The resulting color change was measured at 734 nm and results were represented as IC₅₀ values (µg/mL).

3.2.8 Angiotension-I converting enzyme (ACE) inhibitory activity

Angiotensin converting enzyme (ACE) is a key enzyme in the renin angiotensin system responsible for conversion of angiotensin (Ang) I into Ang II, a vasoconstrictor causing to elevated blood pressure (Wu, Liao, & Udenigwe, 2017). The *in vitro* ACE inhibition activity was evaluated using hippuryl-L-histidyl-L-leucine (HHL) as a substrate (Chang et al., 2001). Briefly, A1 consists of 100 µL sample, 100 µL ACE solution (40 mU/mL), and 100 µL HHL (15 mM) solutions. A2 consists of 100 µL sample and 200 µL borate buffer (20 mM, 0.3 M NaCl, pH 8.3). A3 consists of 100 µL borate buffer, 100 µL ACE solution, and 100 µL HHL solution. A4 contains 300 µL borate buffer. After 30 min at 37 °C, 3 mL of alkaline solution of OPA solution (pH 12.0) was added to cease the enzymatic reaction. The absorbance of each reaction

was measured at 390 nm. Inhibition by sample solutions was computed using equation 3.2.

$$\text{ACE inhibitory activity (\%)} = \left[1 - \frac{A1 - A2}{A3 - A4}\right] \times 100 \quad (3.2)$$

The IC₅₀ value corresponding to the dry extract concentration necessary to inhibit ACE by 50%, was determined for each sample by plotting inhibition for the five different concentrations.

3.2.9 Cytotoxicity

The breast cancer cells were seeded into 96-well plates at a density of 1.5×10^4 cells per well. After a day, the cell lines were treated with various concentrations of different dry extracts. MTT assay was performed according to Kocyigit et al. (2019). The IC₅₀ was computed by an inhibition curve.

3.2.10 Reactive oxygen species generation

Intracellular ROS content was measured by flow cytometry using DCFH-DA. Briefly, cells were plated at 2×10^5 density in 6-well plates and treated with various concentrations of different dry extract of black and orange rosehip for 24 h. The positive control group and dry-extract treated groups were then exposed to H₂O₂ (500 μ M) for 4h. Then, the cells were stained with 10 μ M DCFH-DA at 37°C for 30 min away from the light. The dihydrodichlorofluorescein fluorescence was analyzed in the cells by a FACs calibur flow cytometer and results were expressed as the percent negative control group fluorescence intensity.

3.2.11 Acridine orange assay

Acridine orange, a fluorescent dye is taken up by both viable as well as non-viable cells. Cells were incubated for adherence followed by incubation with or without dry rosehip extracts for 24 h as described in Kocyigit et al. (2019). MCF-7 cells stained using DAPI at a concentration of 3 nM for 10 min and observed under the fluorescence microscope to imaging nuclei of cells (Zeiss CellDiscoverer 7-Automated Live Cell Imaging).

3.2.12 Statistical analysis

Each experiment was conducted in triplicate unless stated otherwise and the data are expressed as the mean $\pm$ standard deviation. One-way analysis of variance with Tukey's post-hoc test ($P < 0.05$) was used to test for significant differences between individual means (Minitab Inc. State College, USA).

3.3 Results and Discussion

3.3.1 Total phenolic content and free radical scavenging activity

Total phenolic content (TPC), total flavonoid content (TFC) and free radical scavenging activity levels of different rosehip extracts were measured by photometric methods (Table 3.1). Total phenolic content was expressed as mg gallic acid equivalent per gram dry extract. Results showed that TPC of different rosehip extracts decreased in the following order: black rosehip methanolic (80%) extract > black roseship infusion > rosehip methanolic (80%) extract > rosehip infusion. This trend was also applied for the antioxidant activity, namely ABTS and DPPH radical scavenging assays which suggests that there is a correlation between TPC and antioxidant activity in accordance with the literature (Nobossé, Fombang, & Mbofung, 2018; Ma et al., 2021).

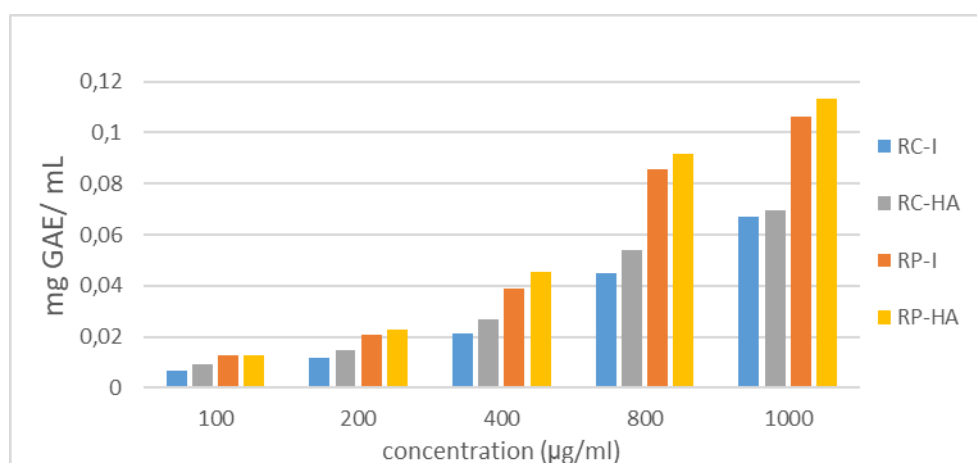


Figure 3.3 : Total phenolic contents of different rosehip extracts at various concentrations. RC-I, *Rosa Canina* infusion. RC-HA, *Rosa Canina* hydroalcoholic extract. RP-I, *Rosa Pimpinellifolia* infusion. RP-HA, *Rosa Pimpinellifolia* hydroalcoholic extract.

In general, 80% methanol is better solvent for the efficient extraction of total phenolic compounds than pure water (Sepahpour et al., 2018; Ismail, Pu, Guo, Ma, & Liu,

2019). Higher TPC and antioxidant activities were found for the black rosehip (*Rosa pimpinellifolia*), possibly due to anthocyanins in comparison to common rosehip (*Rosa canina*) (Figure 3.3).

Table 3.1 : Total phenolic content and IC₅₀ values for ACE inhibitory, DPPH and ABTS radical scavenging activity.

Sample	TPC (mg GAE/ g)	TFC (mg CE/g)	ACE inhibition (%)	DPPH radical scavenging activity	ABTS radical scavenging activity
RC-I	30.33 ± 0.15 ^d	13.79 ± 2.03 ^d	10.16 ± 0.32 ^d	162.0 ± 2.5 ^d	251.4 ± 1.91 ^d
RC-HA	43.66 ± 0.72 ^c	17.30 ± 1.75 ^c	8.51 ± 0.20 ^b	104.7 ± 3.0 ^c	232.1 ± 1.83 ^c
RP-I	52.72 ± 0.54 ^b	54.56 ± 3.12 ^b	9.81 ± 0.17 ^c	77.8 ± 4.2 ^b	150.05 ± 1.73 ^b
RP-HA	78.01 ± 0.91 ^a	77.23 ± 4.31 ^a	6.73 ± 0.18 ^a	54.7 ± 2.1 ^a	105.8 ± 1.89 ^a

Abbreviations: RP-I, *Rosa pimpinellifolia* infusion; RP-HA, *Rosa pimpinellifolia* hydroalcoholic extract; RC-I, *Rosa canina* infusion; RC-HA, *Rosa canina* hydroalcoholic extract
TPC and TFC values represented per g dry extract. IC₅₀ values for ACE inhibition given as mg extract/ml whereas as µg/ml for DPPH and ABTS assay. All in dry basis.

In addition, there is a correlation between antioxidant capacity and proliferation effects of extracts. Proliferation in cancer cells was observed at concentrations where rosehip extracts inhibited ABTS radical by approximately 50%. In particular, the anticancer properties of black rosehip extracts on cancer cells were most probably related to their strong pro-oxidant activities rather than their antioxidant effect (Kocyigit & Selek, 2016). When liqueurs made from different berries of 10 species, the liqueur from black rosehip was found to contain the most of substances which react with the Folin–Ciocalteu reagent and had the highest antioxidant activity among other berries (Sokół-Łętowska et al., 2014). Plant (poly)phenols are well known to exhibit antioxidative, anticarcinogenic and antimutagenic activities (Yen, Duh, & Tsai, 2002).

3.3.2 Polyphenols composition

For the qualitative analysis of the (poly)phenols, the HPLC system was coupled to mass spectrometry was used to analyze the phenolic composition of *R. canina* and *R. pimpinellifolia*. Five anthocyanins and forty nine non-anthocyanin phenolics were determined in black rosehip infusion and hydroalcoholic extracts (Table 3.2). No anthocyanin pigment was present in common rosehip, *Rosa canina* as expected. To the best of our knowledge, this is the first detailed report regarding identification of phenolic compounds in black rosehip, although a few studies has been published recently. The drying of *R. pimpinellifolia* fruit has been investigated recently and

Table 3.2 : HPLC-DAD-ESI-HR-MSn data of phenolic compounds from rosehip (*R. canina* and *R. pimpinellifolia*) samples.

no.	t _R (min)	λ _{max} (nm)	[M] ⁺ / [M-H] ⁻ m/z exptl. (theor.) a	ESI-MSn m/z	proposed structure
<i>anthocyanins</i>					
1	11,9	516, 274	611.1605 (611.1612)	[611]: 449, 287	cyanidin dihexoside
2	14,8	519, 281	449.1078 (449.1084)	[449]: 287	cyanidin 3- <i>O</i> -glucoside ^b
3	15,8	505, 279	433.1129 (433.1135)	[433]: 271	pelargonidin 3- <i>O</i> -glucoside ^b
4	16,4	520, 277	463.1233 (463.1240)	[463]: 301 [463→301]: 286	peonidin 3- <i>O</i> -glucoside ^b
5	17,5	524, 282	419.0972 (419.0978)	[419]: 287 [419→287]: 175, 111, 81	cyanidin pentoside
<i>non-anthocyanin phenolics</i>					
6	5,5	283	169.0133 (169.0137)	[169]: 125	gallic acid ^b
7	6,0	316	182.9926	[183]: 139, 111	unknown 1
8	6,4	256, 292	783.0696 (783.0681)	[783]: 765, 481, 421, 301, 283, 275, 257, 231, [783→301]: 229, 184	dihexahydroxydiphenic acid hexoside
9	6,8	274	343.0668 (343.0665)	[343]: 191, 169, 125 [343→191]: 173, 111	galloyl quinic acid
10	8,3	271	305.0663 (305.0661)	[305]: 261, 219, 179, 137, 125 [305→219]: 191, 177, 139, 123	galocatechin ^b
11	8,7	257	153.0182 (153.0188)	[153]: 109	protocatechuic acid ^b
12	11,6	278	577.1348 (577.1346)	[577]: 559, 451, 425, 407, 331, 289, 245 [577→407]: 387, 363, 321, 297, 281, 251, 243, 227, 161	procyanidin dimer 1
13	12,0	280	865.1996 (865.1980)	[865]: 847, 739, 715, 695, 577, 543, 525, 407, 327, 287 [865→695]: 677, 659, 637, 543, 525, 451, 391, 299, 245	procyanidin trimer 1

Table 3.2: (continued) HPLC-DAD-ESI-HR-MSn data of phenolic compounds from rosehip (*R. canina* and *R. pimpinellifolia*) samples.

no.	t _R (min)	λ _{max} (nm)	[M] ⁺ / [M-H] ⁻ m/z exptl. (theor.) a	ESI-MSn m/z	proposed structure
14	12,4	270	345.0823 (345.0822)	[345]: 297, 183, 167, 124 [345→183]: 168	methyl gallate hexoside
15	12,9	278	577.1348 (577.1346)	[577]: 559, 531, 491, 451, 425, 407, 391, 365, 289 [577→407]: 389, 345, 285, 253, 243, 225, 199, 161	procyanidin dimer 2 (procyanidin B1) ^b
16	13,0	280	865.1997 (865.1980)	[865]: 847, 739, 713, 695, 677, 577, 543, 525, 405, 297 [865→695]: 677, 651, 567, 543, 525, 451, 405, 363, 289, 243, 199	procyanidin trimer 2
17	13,2	280	451.1242 (451.1240)	[451]: 341, 271, 253, 203, 137 [451→137]: 109	catechin hexoside
18	13,5	280	865.1981 (865.1980)	[865]: 847, 739, 713, 695, 577, 543, 525, 449, 295 [865→695]: 677, 651, 587, 543, 491, 451, 405, 363, 299, 243	procyanidin trimer 3
19	13,8	278	289.0714 (289.0712)	[289]: 271, 245, 205, 163, 123 [289→245]: 229, 203, 175, 161	(+)-catechin ^b
20	14,3	280	577.1354 (577.1346)	[557]: 559, 541, 451, 425, 407, 339, 289, 245, 175 [557→407]: 389, 363, 281, 269, 253, 227, 199, 125	procyanidin dimer 3
21	14,7	280	577.1353 (577.1346)	[557]: 559, 533, 491, 451, 425, 407, 381, 349, 299, 245, 201 [557→407]: 389, 343, 299, 281, 255	procyanidin dimer 4
22	15,0	279	865.2000 (865.1980)	[865]: 847, 739, 713, 695, 575, 407, 289 [865→695]: 677, 651, 587, 543, 525, 451, 407, 387, 243	procyanidin trimer 4
23	15,5	317, 283	325.0927 (325.0923)	[325]: 265, 235, 205, 187, 163, 145, 117 [326→163]: 119	coumaryl hexoside
24	16,7	280	577.1353 (577.1346)	[557]: 559, 541, 451, 425, 407, 339, 289, 245, 175 [557→407]: 389, 363, 281, 269, 253, 227, 199, 125	procyanidin dimer 5
25	17,5	328, 294	353.0877 (353.0873)	[353]: 191	3-O-caffeoyl quinic acid (chlorogenic acid) ^b
26	17,8	330, 298	355.1034 (355.1029)	[355]: 295, 217, 193, 175, 133 [355→193]: 177, 149, 133	feruloyl hexoside

Table 3.2: (continued) HPLC-DAD-ESI-HR-MSn data of phenolic compounds from rosehip (*R. canina* and *R. pimpinellifolia*) samples.

no.	t _R (min)	λ _{max} (nm)	[M] ⁺ / [M-H] ⁻ m/z exptl. (theor.) a	ESI-MSn m/z	proposed structure
27	19,4	278	729.1468 (729.1456)	[729]: 712, 683, 603, 577, 559, 441, 407, 381, 289, 243 [729→407]: 389, 297, 285, 256, 243	procyanidin dimer gallate
28	19,5	338, 280	385.1140 (385.1135)	326, 295, 277, 247, 223, 205, 149	sinapoyl hexoside
29	19,8	278	865.1992 (865.1980)	[865]: 847, 739, 713, 695, 575, 407, 287 [865→695]: 677, 651, 587, 543, 525, 451, 405, 365, 243	procyanidin trimer 5
30	20,3	280	577.1352 (577.1346)	[577]: 559, 451, 425, 407, 329, 289, 255 [577→407]: 387, 363, 281, 255, 213	procyanidin dimer 6
31	22,1	276	497.0933 (497.0931)	[497]: 434, 345, 183 [497→345]: 183, 167	methyl digallate hexoside
32	22,4	281	337.0923 (337.0923)	[325]: 301, 280, 163, 143, 119	coumaryl quinic acid
33	25,1	282	449.1087 (449.1084)	[449]: 287, 151 [449→287]: 151, 135, 125	eriodictyol hexoside 1
34	26,5	293	449.1088 (449.1084)	[449]: 401, 341, 314, 269, 184, 151 [449→287]: 225	eriodictyol hexoside 2
35	30,1	350, 250	615.0990 (615.0986)	[615]: 593, 463, 301, 271 [615→463]: 343, 301, 271	quercetin galloyl hexoside 1
36	32,1	357, 256	463.0880 (463.0877)	[463]: 301, 255, 151 [463→301]: 271, 179, 152	quercetin 3-O-galactoside ^b
37	32,3	353, 261	477.0671 (477.0669)	[477]: 301 [477→301]: 179	quercetin 3-O-glucuronide ^b
38	32,6	355, 254	463.0880 (463.0877)	[463]: 435, 301, 271, 179, 151 [463→301]: 193, 151	quercetin 3-O-glucoside ^b
39	33,3	367, 351sh, 254	300.9987 (300.9984)	[301]: 257, 215, 173, 165, 147	ellagic acid ^b
40	33,9	357, 255	599.1041 (599.1037)	[599]: 463, 301, 255 [599→463]: 301, 179	quercetin galloyl deoxyhexoside
41	34,2	355, 257	433.0774 (433.0771)	[433]: 301, 255 [433→301]: 271, 255, 179, 151, 137	quercetin pentoside 1

Table 3.2: (continued) HPLC-DAD-ESI-HR-MSⁿ data of phenolic compounds from rosehip (*R. canina* and *R. pimpinellifolia*) samples.

no.	t _R (min)	λ _{max} (nm)	[M] ⁺ / [M-H] ⁻ m/z exptl. (theor.) ^a	ESI-MS ⁿ m/z	proposed structure
42	34,8	356, 257	433.0773 (433.0771)	[433]: 301, 151 [433→301]: 179, 151	quercetin pentoside 2
43	35,0	365, 330sh, 251	463.0880 (463.0877)	[463]: 301 [463→301]: 273, 179, 151, 107	quercetin 4-O-glucoside ^b
44	35,9	350, 257	447.0931 (447.0927)	[447]: 301, 179, 151 [447→301]: 179, 151	quercitrin (quercetin 3-O-rhamnoside) ^b
45	36,1	364, 250	447.0567 (447.0564)	[447]: 315, 255, 227 [447→315]: 301	ellagic acid deoxy-hexoside 1
46	36,3	364, 250	447.0568 (447.0564)	[447]: 315, 255, 227 [447→315]: 301	ellagic acid deoxy-hexoside 2
47	36,8	341	491.1191	[491]: 463, 345, 183, 168 [491→345]: 183, 168	unknown 2
48	37,2	320	429.1764	[429]: 385, 249, 205, 179, 161, 143 [429→249]: 205	unknown 3
49	39,4	296	471.1293	[471]: 429, 307, 163 [471→307]: 247, 203, 119	unknown 4
50	39,6	370, 260	615.0988 (615.0986)	[615]: 581, 549, 465, 314, 301 [615→301]: 179, 151	quercetin galloyl hexoside 2
51	39,9	370, 253	301.0351 (301.0348)	[301]: 273, 211, 179, 151, 107	quercetin ^b
52	41,1	316	711.3962	[711]: 665, 503, 441 [711→503]: 485, 453, 441, 421, 409, 355	unknown 5
53	41,6	361sh, 315, 268	609.1251 (609.1244)	[609]: 463, 301 [609→463]: 354, 301, 271, 255, 179	quercetin coumaroyl hexoside
54	44,2	355sh, 315, 265	593.1296 (593.1295)	[593]: 519, 447, 307, 285, 255, 177 [593→285]: 241, 153	luteolin/kaempferol coumaroyl hexoside

t_R: retention time; λ_{max}: UV/vis absorption maxima, sh: shoulder.

^a Position mode were used for anthocyanins and negative for non-anthocyanin phenolics.

^b Verified by an authentic standard.

3.3.3 ACE inhibitory activity

Angiotensin-converting enzyme (ACE) inhibitory activities on different extracts of two rosehip varieties were determined (Table 3.1). The use of ACE inhibitor biomolecules are of interest due to the relaxing effects of the veins and arteries, thereby lowering the blood pressure (El-Sohaimy et al., 2022). Plant based phenolic compounds are considered as good sources of ACE inhibitors (Khan & Kumar, 2019). IC₅₀ values represents the concentration that inhibits 50% of ACE activity. IC₅₀ values were found within the range of 7.85 to 10.48 mg/mL. Among all, the best inhibitory activity, lowest IC₅₀ value, was recorded for black rosehip hydromethanolic extract followed by black rosehip infusion. The phenolic and anthocyanin content may be responsible for the inhibition. The ACE inhibitory activities was corroborated with the results of Chaudhary, Sabikhi, Hussain, & Kumar (2020), who reported similar concentrations for roselle (*Hibiscus sabdriffa*) and ashwagandha (*Withania somnifera*) and attributed the exhibited inhibition to the presence of (poly)phenols, protein and ascorbic acid in the herbal extracts (Chaudhary, Sabikhi, Hussain, & Kumar, 2020). The hydroxyl (OH) groups in (+)-catechin, (-)-epicatechin and (-)-epigallocatechin were conceived to contribute on the procyanidin-protein interaction, yielding the formation of a strong bond between the procyanidins and ACE (Fernandez & Labra, 2013).

3.3.4 Cytotoxicity

The anticancer effects of different rosehip varieties were investigated and compared *in vitro*. The MCF-7 breast cancer cells were treated with different doses of rosehip extracts in dry basis. Their effects on cell viabilities were recorded after 24 hours by using MTT cell viability assay and the IC₅₀ values were calculated accordingly. The results exhibited that MCF-7 cells respond to extracts with dose dependent concentration. As shown in Figure 3.4, cell viability increased at lower doses of rosehip extracts, whereas at higher doses it was found that cell viability decreased significantly especially in black rosehip extracts. The IC₅₀ doses for breast cancer cell were calculated by the concentration-response curve. The IC₅₀ levels were found as 512,7 µg/ml, 731,3 µg/ml and > 800 µg/ml for black rosehip methanolic extract, black rosehip infusion, rosehip infusion and methanolic extracts, respectively in accordance with the data obtained by means of phenolic content and antioxidant activity.

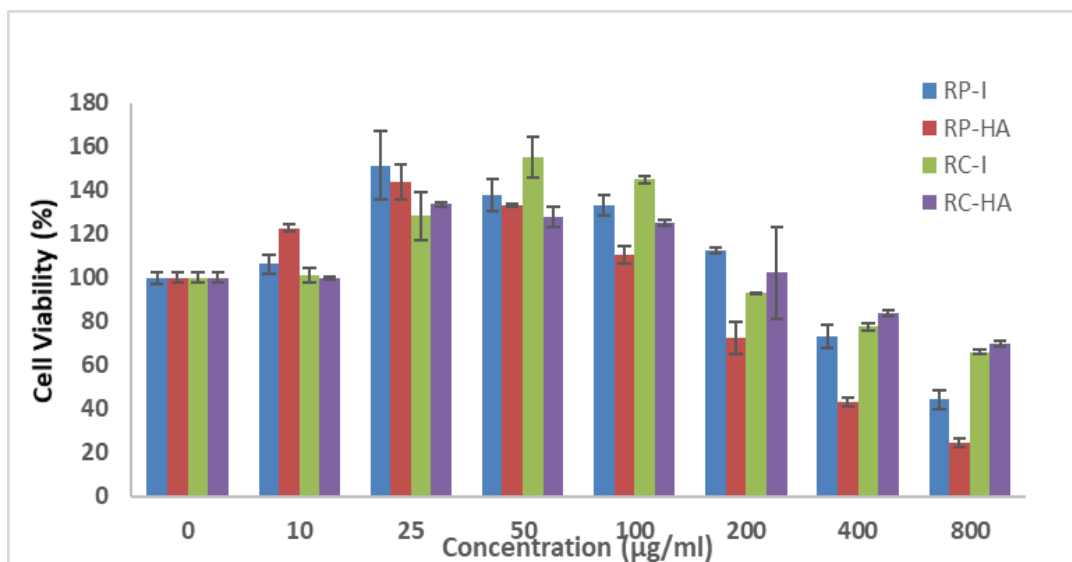


Figure 3.4 : Cytotoxicity of different rosehip extracts in MCF-7 cells by MTT assay. RC-I, *Rosa Canina* infusion. RC-HA, *Rosa Canina* hydroalcoholic extract. RP-I, *Rosa Pimpinellifolia* infusion. RP-HA, *Rosa Pimpinellifolia* hydroalcoholic extract.

3.3.5 Reactive oxygen species generation

The intracellular ROS levels generated in MCF-7 cells after exposure to the rosehip extracts were analyzed. The results indicated that low doses of rosehip extracts reduced intracellular ROS content in cancer cells. However, intracellular ROS content significantly increased at higher levels of rosehip samples in a dose-dependent manner. There were negative correlations between viable cells and intracellular ROS levels in breast cancer cells. Bioactive ingredients present in rosehip varieties generally exhibited powerful antioxidant, free radical scavenging and proliferative properties. Cell growth effects of common rosehip fractions were assessed in HeLa, MCF7 and HT-29 cell lines earlier (Tumbas et al., 2012). The authors reported the lowest IC₅₀ values were obtained for rosehip flavonoids as 80.63, 248.03 and 363.95 mg L⁻¹, respectively. In this study, we found that especially high doses of black rosehip samples exhibited anti-proliferative activity on MCF-7 cancer cells in a dose-dependent manner. It has been also known that anti-proliferative effect of rosehip is related with the presence of potent antioxidant activity. Therefore, ROS levels in cancer cells treated with different doses of four rosehip extracts were analyzed in order to clarify the mechanism of their anti-proliferative activity.

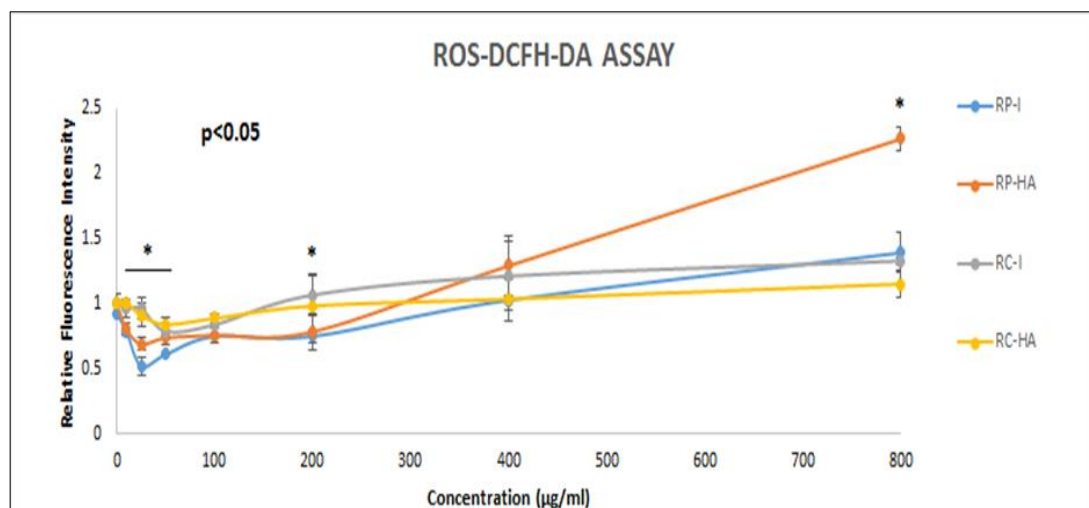


Figure 3.5 : Intracellular ROS levels generated in MCF-7 cells after exposure to different rosehip extracts. RC-I, *Rosa Canina* infusion. RC-HA, *Rosa Canina* hydroalcoholic extract. RP-I, *Rosa Pimpinellifolia* infusion. RP-HA, *Rosa Pimpinellifolia* hydroalcoholic extract.

According to the results, increasing rosehip extract until ~200 µg/ml concentration exerted a proportionally decreased iROS production and, there was a close relationship between iROS levels and cell proliferation (Figure 3.5). However, at higher doses, ROS levels were found to be higher for all rosehip extracts. High doses of natural antioxidants are known to possess pro-oxidant activity and generate ROS through Fenton reaction (León-González, Auger, & Schini-Kerth, 2015). Especially in cancer cells, higher concentrations of antioxidant molecules cause DNA damage and apoptosis through the pro-oxidant activity (Forester & Lambert, 2011). At concentrations below 10 µM, green tea (poly)phenols did not appear to induce ROS-mediated DNA damage, however, they prevented hydrogen peroxide-mediated DNA damage (Sugisawa, Kimura, Fenech, & Umegaki, 2004). It has been explained that concentrations of green tea (poly)phenols higher than 10 µM affect redox balance of the cells which creates a pro-oxidant environment. Oxidative DNA damage is a good marker of cancer (Ames & Gold, 1991). In this thesis, it has been clarified that the apoptotic and anti-proliferative effect of rosehip extracts on breast cancer cells does not depend on the antioxidant activities of the phenolic compounds present in rosehip extracts, rather on their pro-oxidant activities.

3.3.6 Apoptosis

Many cellular structures and organelles are damaged or destroyed as a consequence of apoptosis (D'Arcy, 2019). In this study, the apoptotic effects of different rosehip

extracts were evaluated using acridine orange (AO) and ethidium bromide (EB) - AO/EB double staining method. According to the results, the viable cell ratios decreased and the apoptotic cell ratios significantly increased with the increasing doses of black rosehip extract ($p < 0.05$). On the contrary, no statistically significant increase was found in apoptosis for common rosehip extracts (Figure 3.6). In other words, it has been shown that black rosehip extracts cause cell death via apoptosis. However, common rosehip extracts, which already had weak cell death effects, did not show any significant apoptotic activity on breast cancer cells. The black rosehip extract seems to activate the apoptosis pathway through generation of reactive oxygen species and leads to cell death. Further mechanism-based studies are required to demonstrate these apoptotic effects. Cell images after treatment of different rosehip extracts are shown in Figure 3.7.

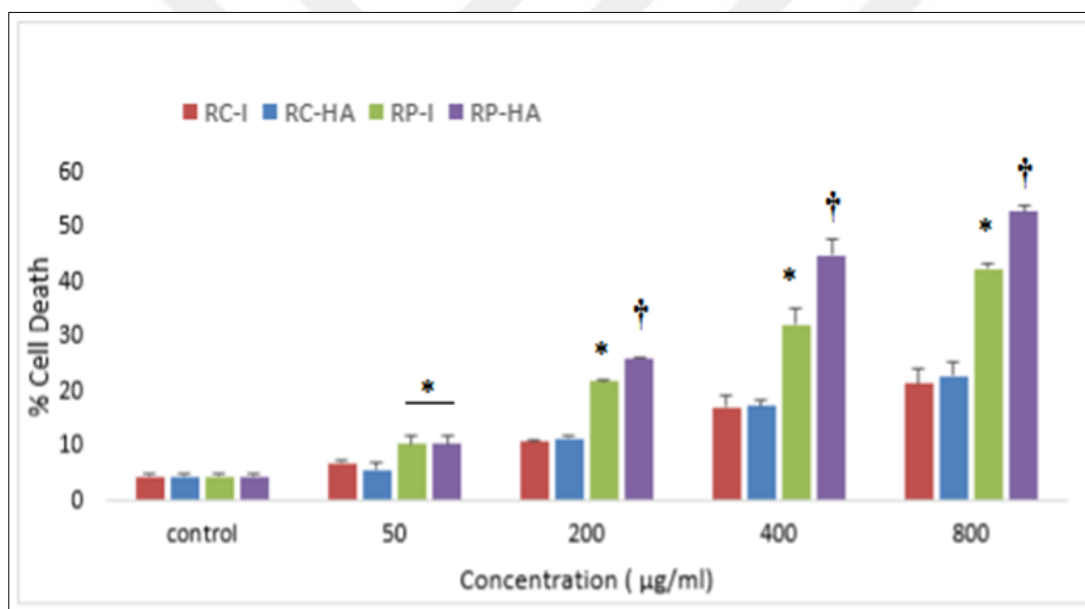


Figure 3.6 : Results for the apoptosis by AO/EB Double Staining. RC-I, *Rosa Canina* infusion. RC-HA, *Rosa Canina* hydroalcoholic extract. RP-I, *Rosa Pimpinellifolia* infusion. RP-HA, *Rosa Pimpinellifolia* hydroalcoholic extract.

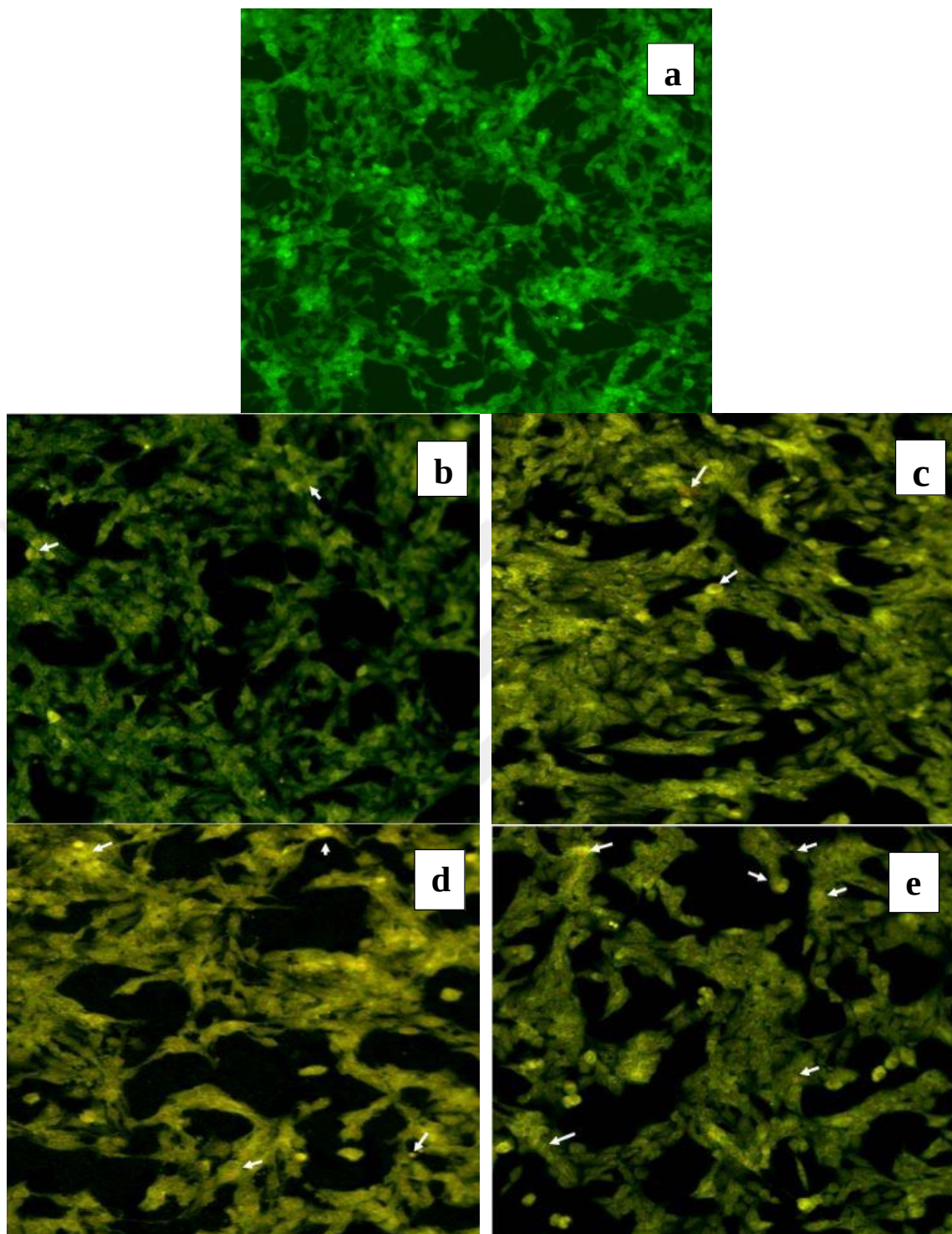


Figure 3.7 : Membrane integrity and morphological changes in the MCF-7 cells after treatment of (a) control, (b) *Rosa Canina* infusion, (c) *Rosa Canina* hydroalcoholic extract, (d) *Rosa Pimpinellifolia* infusion (e) *Rosa Pimpinellifolia* hydroalcoholic extract, 800 µg/mL.

3.4 Conclusion

When black rosehip was compared with common orange rosehip, the hydromethanolic *R. pimpinellifolia* L. extract induced DNA damage, apoptosis, and cytotoxicity in

breast cancer cell line followed by *R. pimpinellifolia* L. infusion. Taking the antioxidant and ACE inhibitory activity of these extracts into account, the obtained results can be related to the quantity of the phenolic compounds that were estimated during the research. In addition to the presence of anthocyanins, the amounts of catechin, gallocatechin, procatechuic acid, procyanidin B1, procyanidin dimer, procyanidin trimer, isoquercetin, and isoquercitrin in *Rosa pimpinellifolia* were higher than *Rosa canina*.



4. GREEN EXTRACTION OF PHENOLIC COMPOUNDS FROM BLACK ROSEHIP: OPTIMIZATION AND *IN VITRO* BIOAVAILABILITY

4.1 Introduction

There has been a consumer demand for functional foods with added health beneficial properties due to the increased prevalence of degenerative diseases and change in lifestyle (Daliri & Lee, 2015; Nwosu & Ubaoji, 2020). Foods derived bioactive compounds from plant origin are rich and cheap sources for developing value-added ingredients for enrichment purposes. However, to exploit the use of underutilized wild edible plant resources, suitable and convenient extraction methods need to be proposed for separation of bioactive compounds (Galanakis, 2012). Green extraction methods overcome disadvantages of conventional organic solvent extraction for utilization of edible plant materials.

However, to the best of our knowledge, no studies have been carried out for the extraction of black rosehip (poly)phenols without using toxic organic solvents by means of supercritical fluid technology and pressurized hot water extraction. In this study, different extraction techniques have been applied including supercritical supercritical fluid and pressurized liquid extraction in comparison to solvent extraction. Black rosehip berries were studied as raw material due to its high antioxidant (poly)phenols content and to the best of our knowledge, an underutilized berry is presented to the literature. Response surface methodology was applied to obtain (poly)phenol rich extracts at optimum extraction conditions. For the supercritical fluid extraction, carbon dioxide was employed as being nontoxic, and having the mild conditions of temperature (31°C) and pressure (74 bar) required to reach the supercritical state (Zoccali, Donato, & Mondello, 2019). Within supercritical carbondioxide extraction study, parameters including pressure, temperature, and co-solvent composition (EtOH:H₂O) were investigated. For pressurized liquid extraction, hot water has been used as solvent and temperature, solid-to-solvent ratio were the parameters evaluated.

4.2 Materials and Methods

Sample preparation was done as described in section 3.2.2.

4.2.1 Pressurized hot water extraction

Optimizing the extraction of hydrophilic phenolic compounds from black rosehip by PHWE using RSM was conducted on a lab-scale pressurized solvent extraction system (Separex, France). The sample was placed in 500 mL A40 steel extraction vessel and deionized and degassed water was pumped using P210 pump maintaining a pressure of 100 bar with no agitation for 60 min. An extraction period of 60 min was recognized enough to obtain most of the valuable constituents from plants. The extracts were stored in glass bottles at 4 °C until analysis.

4.2.1.1 Experimental design

A central composite design (CCD) was used to optimize the best extraction conditions to obtain the highest phenolic and anthocyanin content and antioxidant capacity. The CCD variables under analysis were temperature (X1, 40-60-80 °C) and solvent-to-solid ratio (X2, 10:1-25:1-40:1) (Table 4.1). The range of values for extraction temperature and solvent-to-solid ratio were selected according to the literature. The maximum temperature was set to 80 °C as thermal degradation of anthocyanins occur significantly at higher temperatures. A total of 13 experiments with five replicates at a central point were randomly employed where the investigated responses were extraction yield (Y; Y1), total phenolic content (TPC; Y2), catechin content (CC; Y3), total monomeric anthocyanin content (TMAC; Y4), cyanidin-3-glucoside content (CYN3C; Y5), and cupric ion reducing antioxidant capacity (TAC; Y6). Dependent variable values were determined in triplicate, and the CCD data was analyzed using a response surface analysis to fit a second-order polynomial model. Statistical analysis of response surface methodology (RSM) design results was performed with the use of Minitab 16 (Minitab Inc. State College, USA). 3D and contour response surface graphs were constructed using Matlab R2020b (Math-Works Inc., USA).

Table 4.1 : Independent variables used for optimization in PHW extraction.

Independent variable	Symbol	Coded levels		
		-1	0	1
Temperature, °C	X1	40	60	80
Solvent-to-solid ratio, mL/g	X2	10	25	40

4.2.2 Supercritical CO₂ extraction

To screen out the optimum conditions of supercritical carbon dioxide extraction (SCO₂), ethanol added as modifier and the effects of process parameters including pressure, temperature, co-solvent (ethanol) concentration for the extraction of polar phenolic compounds were investigated. RSM is considered as a useful tool in optimizing experimental conditions enabling the maximization of various responses simultaneously (Hajj et al., 2012). The supercritical carbon dioxide extraction process was performed using a supercritical fluid extraction system (Waters SFE 1000, USA). Extraction parameters including pressure, temperature, and co-solvent concentration were optimized for a better extraction yield of phenolics with high antioxidant capacity. Extraction procedure was carried out in dynamic mode at a constant mass flow rate of 50 g min⁻¹ of CO₂ and co-solvent as ethanol mixtures (10% with regard to carbon dioxide mass feed) during 60 min. Twelve grams of lyophilized whole fruits used for each run. Moreover, to evaluate the effect of extraction method on (poly)phenols bioaccessibility and Caco-2 cellular uptake in black rosehip extracts, the extract obtained at optimized conditions of supercritical CO₂ was compared with extracts obtained by other green technologies such as PHWE and UA-ethanol extraction.

4.2.2.1 Experimental design

A Box-Behnken experimental design considering three factors and three levels was selected, in order to optimize the extraction conditions, being pressure (X1; 150–350 bar), temperature (X2; 40–60 °C), and co-solvent concentration (X3; 20–100 % EtOH:H₂O) were evaluated (Table 4.2). To evaluate the effect of these factors, extraction yield (%), total phenolic content (TPC) and total antioxidant capacity (TAC) by two different assays, CUPRAC (Y1), and DPPH (Y2) were determined. A total of 17 experimental runs were conducted in a randomized order to minimize unexpected variability in the responses due to extraneous factors. The results were statistically analyzed using Minitab 16 software to evaluate the suitability of the proposed model and determine the optimum extraction conditions. Treatment of multiple responses and selection of optimum conditions were based on desirability function D. The model fitting was employed by evaluating coefficient of determination (R²) and lack of fit. The significance was determined for all independent variables, their interactions and

their quadratic effects, considering $p < 0.05$ as significant. The extract obtained via optimal conditions is described as SCO₂-aqEtOH.

Table 4.2 : Independent variables used for optimization in SCO₂ extraction.

Independent variable	Symbol	Coded levels		
		-1	0	1
Pressure, bar	X1	150	250	350
Temperature, °C	X2	40	50	60
Co-solvent concentration, % EtOH (v/v)	X3	20	60	100

4.2.3 Solvent extraction

Methanol is the most widely used solvent for phenolic extraction. However, taking the final potential uses of the crude extracts and its toxicity into account, ethanol is preferred over methanol (Oancea, Stoia, & Coman, 2012). Solvent extraction was applied as the control and was compared with the ethanol modified SCO₂ and PHWE. Conventional maceration extraction (using a magnetic stirrer) has also been performed to represent conventional solvent extraction.

Absolute ethanol used as solvent in ultrasound assisted (UA) extraction which was proceeded in an ultrasonic bath (Sonorex Digitec DT 255 H, Germany) for 30 min before being centrifuged for 10 min at 5000 rpm (Hettich Rotanta 460R). A total of two static extraction cycles were performed and the supernatants were combined.

4.2.4 Global yield

The collected extracts were put in glass flasks and weighed in analytical balance and they dried in oven. Global yield (% w/w) was calculated by the mass ratio between extract and feed in dry basis.

4.2.5 Spectrophotometric assays

4.2.5.1 Total phenolics

Total phenolic content was measure as described earlier in section 3.2.6.

4.2.5.2 Total anthocyanins

Total monomeric anthocyanin content (TMAC) was estimated using the pH differential method (AOAC, 2005).

4.2.5.3 Antioxidant activity: DPPH assay

The hydrogen-donating or radical-scavenging ability of samples analyzed by DPPH (2,2-diphenyl-1-picryl-hydrazyl) method according to Altin et al. (2018). Results were expressed as mg of Trolox equivalents (TE) per gram of fruit on dry weight (mg TE/g dw).

4.2.5.4 Antioxidant activity: CUPRAC assay

Cupric ion reducing antioxidant capacity (CUPRAC) assay has been applied as described earlier (Apak, Güçlü, Özyürek, Karademir, & Altun, 2005). Results were expressed as mmol of Trolox equivalents (TE) per gram of fruit on dry weight (mg TE/g dw).

4.2.6 Analysis of hydrophilic compounds

Individual phenolics and anthocyanins in the extracts were detected using an high performance liquid chromatography (HPLC) with a PDA detector (SPD M20A, Shimadzu) as described previously in Şensu et al. (2021). A gradient of mobile phase A (MQ-water/formic acid, 99.9/0.1 v/v) and mobile phase B (acetonitrile) was used for phenolics whereas MQ-water/formic acid, 95:5 v/v was used as mobile phase A for the separation of anthocyanins.

4.2.7 Analysis of lipophilic compounds

For the chromatographic analysis of liposoluble compounds, the extraction and quantification protocols were conducted as described by Montoya-Arroyo et al. (2022). For the analysis of carotenoids, ethanolic suspensions were diluted in acetonitrile–methanol–water (85:10:5, v/v) and injected into Shimadzu HPLC system. For the analysis of vitamin E, samples suspended in methanol: ethanol (80:20, v/v) suspension was injected into a Jasco HPLC system.

4.2.8 *In vitro* digestion

The gastrointestinal stability of non-encapsulated extract and encapsulated forms was assessed (n = 6) according to a modified INFOGEST method (Rodrigues et al., 2017). For the gastric phase, pepsin was added into final concentration of 5 mg/ml to extracts (pH 2, final volume 8.5 ml), incubated in the dark at 37 °C for 1 h with continuous shaking (120 rpm). In the intestinal phase, final concentrations of 0.6 mg/ml lipase,

1.2 mg/ml pancreatin and 4.8 mg/ml bile extract prepared in 100 mM NaHCO₃ were added (15 ml final volume, pH 6.5, using 1 N NaOH). After 2 h incubation, the final digests were centrifuged (4,700 ×g, 60 min, 4 °C) and filtered through 0.2 µm membrane filter (Filtropur S, Sarstedt, Germany) to separate the soluble and bioaccessible fraction, respectively. The bioaccessibility (%) calculated as the percentage ratio of the amount of TPC or TAC recuperated after simulated gastrointestinal digestion to represent the potential *in vitro* bioaccessibility of (poly)phenols.

4.2.9 Cytotoxicity

Cytotoxicity of extracts (to evaluate possible toxic effects of chemical compounds from extracts) and their digests (to determine the appropriate dilutions before uptake studies) were investigated using neutral red uptake (NRU) assay as described earlier (Flory et al., 2022). Differentiated cells in 48-well plates were incubated with all encapsulated formulations and their native form, diluted in phosphate buffered saline (PBS), a positive control (0.1% Triton X-100), and a negative control (PBS) for 3 h at 37 °C and 5% CO₂. The added samples were then removed and the cells were further incubated with 140 µM neutral red dye (Sigma-Aldrich, Germany) for 2 h. Then, the dye was discarded, and neutral red destain solution was added and incubated for 15 min while shaking. Absorbance was quantified at 540 nm and results.

4.2.10 Caco-2 uptake of phenolics

The intestinal uptake of phenolics was performed using differentiated Caco-2 cells (Kruger, Sus, & Frank, 2020). Differentiated Caco-2 cells were washed twice with 1 ml of 37°C phosphate-buffered saline (PBS). The diluted bioaccessible fraction were added and incubated for 3 h. The supernatant was removed, the cells rinsed and then detached using a cell scraper. All samples collected were stored at -80°C.

The Caco-2 cells were deproteinised and phenolics extracted and further subjected to glucoronidase hydrolysis in order to quantify free and conjugated catechin and epicatechin. „Cellular uptake“ is the amount of the compound taken up by the Caco-2 cells (µg/well). The HPLC analysis for the phenolic compounds in digests and cell lysates were performed using a JASCO HPLC system. The chromatographic separation was achieved as described earlier (Kruger, Sus, and Frank, 2020).

4.2.11 Statistical analysis

Statistical analysis was done as described in section 3.2.13.

4.3 Results and Discussion

4.3.1 PHW extraction

4.3.1.1 Model fitting

A three-level and two factor central composite design (CCD) was applied to make the response surface optimization for PHWE of hydrophilic bioactive compounds from BR. The extraction temperature (X1) and solvent-to-solid ratio (X2) were chosen as the two main independent variables and measured independent variables in 13 experiments is given in Table 4.3. Other factors were kept constant throughout all experiments that are pressure of 100 bar and extraction time of 60 min. Dried whole fruit consisting of flesh and seed parts (5 % of residual moisture) were used as solid raw material for the extraction. In static extraction mode, solvent-to-solid ratio and moisture content of feed should be taken into account to obtain better extraction yields (Gilbert-López, Plaza, Mendiola, Ibáñez, & Herrero, 2017).

Table 4.3 : Experimental conditions and results of extraction yield, TAC, TPC, TMAC, CC, and CYN3C for black rosehip in dry basis.

Factors			Experimental values					
Run	Temperature (°C)	Solvent-Solid Ratio (mL/g)	Yield	TAC	TPC	CC	TMAC	CY3C
1	40 (-1)	40 (1)	17.6	381.41	40.51	1.1	1.19	0.96
2	60 (0)	25 (0)	25.1	514.69	53.45	1.63	3.13	3.26
3	60 (0)	10 (-1)	28.3	892.83	65.61	3.38	4.74	4.1
4	60 (0)	40 (1)	22.3	514.65	54.08	1.85	3.35	3.38
5	60 (0)	25 (0)	24.8	464.29	55.33	2.15	3.77	3.55
6	80 (1)	10 (-1)	29.6	936.98	72.92	4.32	4.98	4.44
7	60 (0)	25 (0)	23.3	513.83	50.91	1.26	3.25	2.97
8	60 (0)	25 (0)	25.2	509.57	52.55	1.73	3.41	3.42
9	80 (1)	25 (0)	24.2	708.84	58.48	2.19	4.25	3.73
10	60 (0)	25 (0)	25.8	606.96	54.31	1.94	3.48	3.18
11	40 (-1)	10 (-1)	26.2	604.02	48.64	2.01	2.3	3.05
12	80 (1)	40 (1)	21.4	514.43	55.46	2.25	4.44	3.51
13	40 (-1)	25 (0)	20.3	411.92	43.68	1.5	1.65	1.22

Abbreviations: Global Yield (%); TPC (mg GAE/g); CC (mg/g); TMAC (mg cyn3glu/g); CY3C (mg/g); TAC (mmol TE/g). All in dry basis.

The RSM model was used to generate response surface plots in order to represent graphically the relationship between independent (temperature and solvent-to-solid ratio) and dependent variables (yield, TAC, TPC, CC, TMAC, and CYN3C). As illustrated in Figure 4.1a-f, temperature and solvent-to-solid ratio significantly contribute to all the responses analyzed. The efficiency of extraction process is quantitatively related to global yield. Global yields in the range of 17.6-29.6 % were achieved under described operation conditions suggesting that a good amount of soluble components can be acquired using PHWE (Table 4.3). Solvent choice affects the extraction efficiency as well as extract properties. Similar yields (29.7%) has been reported in water extracts in previous studies (Ammar, Ennouri, & Attia, 2015).

Phenolic and anthocyanin constituents of BRE obtained via different conditions of PHWE were in the same range as those reported previously in literature (Pashazadeh, Zannou, & Koca, 2020) with slight quantitative differences. TMAC values were lower than those found by Odabas & Koca (2021), using microwave-assisted aqueous two-phase extraction with ethanol/ammonium sulfate. The PHW extracts obtained optimized conditions yielded remarkable TAC values via CUPRAC assay. Pashazadeh, Zannou, Galanakis, Aldawoud, Ibrahim, & Koca (2021b) reported DPPH radical scavenging and ferric reducing antioxidant power results for BR using aqueous methanol after convective drying process. A number of antioxidant capacity assays is present in literature as they do not correlate well due to differences in test procedures as well as surrounding chemistry of antioxidant compounds (Apak et al., 2005). Greater antioxidant potential in our extracts might also be attributed to a possible higher presence of other hydrophilic antioxidant constituents since PHWE is also an efficient method for vitamin C extraction (Nuapia, Cukrowska, Tutu, & Chimuka, 2020). Spectrophotometric analysis of PHW extracts revealed total monomeric anthocyanin contents in the range of 1.19–4.98 mg per g of dry fruit.

The regression analysis of experimental data was subjected to a regression coefficient to obtain second order polynomial equations in terms of coded variables are given as follows:

$$\begin{aligned} \text{Global Yield} = & 9.536040 + 0.721555X_1 - 0.486312X_2 \\ & - 0.005326X_1^2 + 0.004170X_2^2 + 0.008775X_1X_2 \end{aligned} \quad (4.1)$$

$$\begin{aligned}
&\text{Total antioxidant capacity} \\
&= 286.755000 + 18.096500X_1 - 27.274800X_2 \\
&\quad - 0.058252X_1^2 + 0.533582X_2^2 - 0.183282X_1X_2
\end{aligned} \tag{4.2}$$

$$\begin{aligned}
&\text{Total (poly)phenols} \\
&= 5.102240 + 1.781410X_1 - 1.051170X_2 \\
&\quad - 0.009473X_1^2 + 0.022104X_2^2 - 0.0078775X_1X_2
\end{aligned} \tag{4.3}$$

$$\begin{aligned}
&\text{Catechin} = 1.929970 + 0.046974X_1 - 0.147372X_2 + 0.002750X_2^2 \\
&\quad - 0.000632X_1X_2
\end{aligned} \tag{4.4}$$

$$\begin{aligned}
&\text{Total monomeric anthocyanins} \\
&= -3.642710 + 0.248289X_1 - 0.164783X_2 \\
&\quad - 0.001574X_1^2 + 0.002062X_2^2 + 0.000465X_1X_2
\end{aligned} \tag{4.5}$$

$$\begin{aligned}
&\text{Cyanidin - 3 - O - glucoside} \\
&= -3.0962710 + 0.262347X_1 - 0.207847X_2 \\
&\quad - 0.001939X_1^2 + 0.002168X_2^2 + 0.000963X_1X_2
\end{aligned} \tag{4.6}$$

The fitted polynomial equations are further presented as contour plots. The results of ANOVA, the goodness of fit, and the adequacy of the models are summarized in Table A.1. Considering all response surface models, the “lack of fit” was nonsignificant ($p > 0.05$). The adjusted determination coefficients (R^2_{adj}) were also close to R^2 . These findings confirm the adequacy of the model terms to represent the experimental data and to predict the six analyzed parameters. Extraction temperature and solvent-to-solid ratio had significant effects ($p < 0.05$) on the analyzed responses.

4.3.1.2 Effects of process conditions on phenolics, anthocyanins and antioxidant capacity

In the case of phenolic and anthocyanin compounds, optimization of the temperature is necessary in PHWE for enhanced solubility and mass transfer but also to minimize the thermal degradation (Plaza & Turner, 2015). All responses were substantial when the temperature was raised from 40 °C to 80 °C, probably due to greater extraction efficiency with better mass transfer due to the increase in solubility ($p < 0.05$). Figure 4.1d-f depict a slight plateau at temperatures above 75 °C in the response surfaces of TAC, TMAC, and CYN3C. One explanation for this might be a partial thermal degradation of anthocyanins major antioxidant compounds in agreement with the previous report of Liqid et al. (2014). Our preliminary experiments confirmed a 2.2 fold anthocyanin yield decrease when PHWE process was performed at 100 °C (data not shown). A similar trend has been reported by Koyu et al. (2017) when the temperature augmented from 60 °C to 80 °C during optimization of subcritical water

extraction of *Morus nigra* L. fruits at 150 bar for 60 min. Hence, an extraction temperature of 75 °C was set as the optimum condition that maximizes the desirability function of RSM tool for production of the extract rich in bioactives to be encapsulated as higher temperatures did not confer any rise in yield. As can be seen from Table A.1, the model exhibited a significant ($p < 0.01$) relationship between the TPC and independent variables, having predicted R^2 (0.8227) in reasonable agreement with the adjusted R^2 (0.9454). Considerable interaction effects ($p < 0.05$) among the extraction temperature and solvent-to-solid ratio were also observed on all dependent variables analyzed except global yield according to ANOVA analysis.

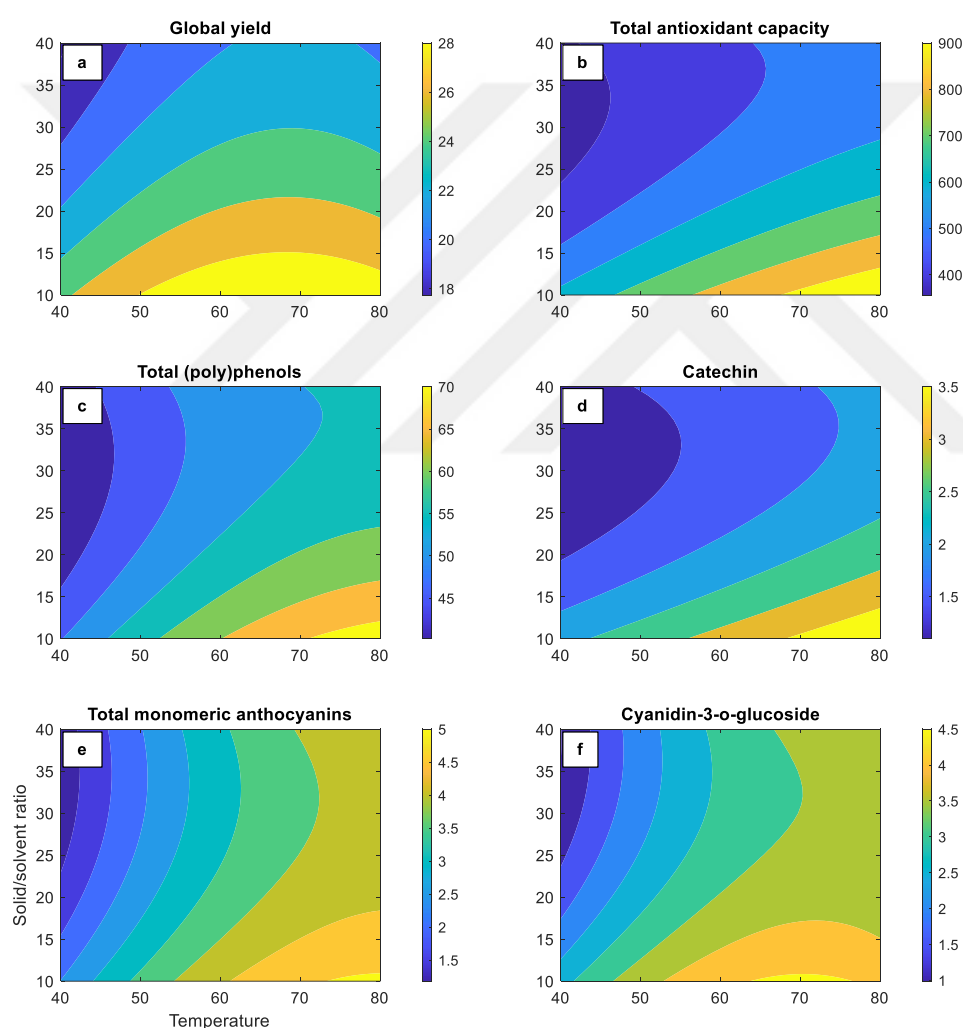


Figure 4.1 : Response surface contour plots for PHWE of black rosehip showing effects of interactions of temperature and solvent-to-solid ratio on (a) global yield (%), (b) total antioxidant capacity (mmol TE/g), (c) total (poly)phenols (mg GAE/g), (d) catechin content (mg/g), (e) total monomeric anthocyanins (mg cyn3glu/g), and (f) cyanidin-3-O-glucoside content (mg/g), in dry basis.

Another important parameter for the recovery of bioactive compounds is solvent-to-solid ratio. Minimal consumption of solvent is the second crucial aspect after maximization of the yield in an industrial scale extraction (Spigno & De Faveri, 2009). The higher solvent-to-solid ratio (v/w), the lower amounts of extracted TPC and TMAC were, as can be inferred from the negative significant linear coefficients ($p < 0.05$) in Table A.1. The extraction yield is expected to gradually raise with increasing solid-liquid ratio according to mass transfer principles (Cacace & Mazza, 2003). However, the results implied that solute diffusion did not enhance with increasing the solvent-to-solid ratio in our static extraction setting. Previous authors suggested that a large solvent volume may result to insufficient energy to facilitate cell wall breakage for an efficient extraction of plant secondary metabolites (Drevelegka & Goula, 2020). The extraction yield is also dependent on how the ratio between solvent and solid is regulated that is keeping the solvent volume or the solid mass constant (Spigno & Faveri, 2009). In our experimental setting, solvent-to-solid ratio was adjusted by changing the solid mass while the solvent volume was kept constant. A similar pattern in yield was observed by Clodoveo, Crupi, Muraglia, & Corbo (2022) when solid-solvent ratio increased in ultrasound assisted extraction of (poly)phenols from carob pods using aqueous ethanol as solvent.

Current trend strongly favors scaling-up of non-conventional green extraction techniques for recovering phenolic compounds that consumes less organic solvent(s) and assures highest yield at lower costs. Santos and coworkers (2012) reported a 40-fold lower cost of manufacturing and 2.15 and 1.66-fold more anthocyanins and phenolics obtained from jabuticaba skins by pressurized liquid extraction using ethanol as a solvent compared to conventional low-pressure solvent extraction (Santos, Veggi, & Meireles, 2012). Using pressurized hot water as solvent of extraction, PHWE technique is even a greener version of pressurized liquid extraction as water is perhaps the most available naturally occurring liquid on Earth (Plaza & Turner, 2015; Vazquez-Roig & Picó, 2015). We did not perform an economic evaluation for the feasibility of large-scale PHWE operation within the scope of this study. However, future studies should be focus on economic feasibility of the global process.

4.3.1.3 Validation of optimal conditions

The main advantage of RSM is simultaneous maximization of multiple responses in the investigated experimental domain. Based on above findings, PHWE conditions giving the best levels of several factors were attained and they showed that a temperature of 75°C and solvent-to-solid ratio 10:1 mL g⁻¹ were optimal working under 100 bar. The adequacy of the model for predicting optimum yields was experimentally tested using determined optimized conditions.

Table 4.4 : Experimental data of the validation of predicted value at optimal PHWE conditions.

	Global Yield	TAC	TPC	CC	TMAC	CYN3C
Experimental value ^a	27.90 ± 1.60	930.48 ± 24.37	68.90 ± 3.94	3.90 ± 0.21	5.28 ± 0.41	4.08 ± 0.51
Predicted value	29.5501	946.8044	71.2877	3.9836	5.0344	4.5350
Desirability	0.97750	0.99624	0.94977	0.96245	1.0000	1.0000
p*	0.216	0.366	0.403	0.957	0.414	0.669

bbreviations: Global Yield (%); TPC (mg GAE/g); CC (mg/g); TMAC (mg cyn3glu/g); CYN3C (mg/g); TAC (mmol TE/g). All in dry basis.

* Indicate significant differences (p < 0.05).

The optimal extract, BRE revealed mean values of 68.90 ± 3.94, 3.90 ± 0.21, 5.28 ± 0.41, 4.08 ± 0.51 mg and 930.48 ± 24.37 mmol per g of dry fruit for TPC, CC, TMAC, CYN3C, and TAC, respectively, in verification experiments. Being similar (p > 0.05) to the respective predicted ones, these findings allowed validation of the experimental design and demonstrating the effectiveness and good adequacy of the RSM (Table 4.4). The optimum conditions for extraction were compared to the predicted values of RSM using an independent sample t-test. PHWE process provided good (poly)phenols yield with high antioxidant capacity for a notably shorter extraction time of 60 min, comparing to conventional extraction process. HPLC analysis showed that cyn3glu was the most abundant anthocyanin in all PHWE extracts followed by peonidin-3-glucoside, cyanidin-3-galactoside, and delphinidin-3-glucoside (data not shown) in agreement with the literature data (Odabaş & Koca, 2021).

4.3.2 SCO₂ extraction

4.3.2.1 Model fitting

Using ethanol as co-solvent, the optimization of supercritical carbon dioxide extraction conditions was carried out to improve the extraction efficiency of black rosehip (poly)phenols. A three-level and three factor Box-Behnken design (BBD) was

implemented for the response surface optimization for SCO₂ extraction process. The operating pressure (150–350 bar), temperature (40–60 °C), and co-solvent concentration (20–100 %) were considered as the operating variables and measured responses in 17 experiments is given in Table 4.5. The range of values used (low, medium and high) for each factor and the experimental responses are presented in Table 4.2. Regarding the concentration of solvent modifier, different mixtures of ethanol/water were tested. Other factors were kept constant throughout all experiments that are extraction time of 60 min and solvent to solid ratio of 25 mL/g. Dried whole fruit consisting of flesh and seed parts (5 % of residual moisture) were used as solid raw material during dynamic extraction.

The RSM model was used to generate response surface plots in order to represent graphically the relationship between independent (pressure, temperature and co-solvent concentration) and dependent variables (yield, TPC, CC, TMAC, Cyn3glu, TAC_{DPPH} and TAC_{CUPRAC}). As shown in Figure 4.5, extraction yield increases with increasing pressure and temperature while decreasing the co-solvent concentration (that is the concentration of ethanol in aqueous ethanol). The quadratic model was not found to be significant ($p < 0.05$) in terms of all investigated responses at the levels tested in this study (Table A.2).

The efficiency of extraction process is quantitatively related to global yield. Global yields in the range of 2.4–25.1 % were achieved under described operation conditions (Table 4.5). Solvent choice affects the extraction efficiency, in other words, co-solvent concentration was the most effective parameter on global yield ($p < 0.05$). Being green solvents with the possibility of direct use in edible formulations, water and ethanol are commonly used solvents at low cost. As co-solvents, water-ethanol mixtures has been demonstrated to be more efficient for recovery of phenolic compounds (Campone et al., 2018; Monroy et al., 2016; Da Porto, Natolino, & Decorti, 2014a).

Table 4.5 : Experimental conditions and results of extraction yield, TPC, CC, TMAC, Cyn3gl and TAC in black rosehip in dry basis.

Run	Factors			Experimental values						
	X ₁ (bar)	X ₂ (°C)	X ₃ (%)	Yield (%)	TPC (mg GAE/g)	CC (mg/g)	TMAC (mg cyn3glu/ g)	Cyn3glu (mg/g)	TAC _{DPPH} (mmol TE/g)	TAC _{CUPRAC} (mmol TE/g)
1	250	50	60	21.6	67.74	3.36	9.04	7.91	136.2	573.9
2	250	50	60	17.8	56.73	3.12	9.15	6.77	122.9	558.2
3	250	40	100	2.4	12.89	0.97	2.03	2.45	15.8	84.0
4	250	40	20	13.7	44.29	3.53	7.98	7.41	112.0	491.4
5	350	50	100	3.1	1.10	0.44	0.13	1.26	8.6	20.3
6	350	40	60	15	45.82	2.83	3.35	7.31	107.1	434.0
7	250	50	60	21.8	55.69	3.06	7.92	6.70	113.3	593.5
8	350	60	60	19.5	58.97	2.99	6.65	7.00	121.1	629.0
9	150	60	60	14.9	48.07	2.74	6.01	5.90	104.4	553.1
10	250	60	20	19.8	72.87	4.59	9.95	9.03	192.7	754.8
11	250	50	60	17.4	54.05	2.95	9.16	7.98	118.7	554.5
12	150	50	100	3.7	4.93	0.46	1.25	1.01	16.7	42.7
13	250	50	60	18.2	60.10	2.86	7.84	7.26	138.6	519.8
14	150	40	60	12.9	32.23	3.16	7.03	6.62	55.4	426.6
15	150	50	20	19.4	50.92	3.83	9.82	8.31	167.9	582.0
16	350	50	20	25.1	63.59	4.17	10.34	7.71	172.0	646.2
17	250	60	100	4.5	5.08	0.76	1.30	1.50	14.8	57.8

Abbreviations: TPC, total phenolic content. CC, catechin content by HPLC analysis. TMAC, total monomeric anthocyanin content. Cyn3glu, cyanidin-3-O-glucoside content by HPLC analysis. TAC_{DPPH}, total antioxidant capacity by DPPH assay. TAC_{CUPRAC}, total antioxidant capacity by CUPRAC assay.

4.3.2.2 Effects of process conditions on phenolics, anthocyanins and antioxidant capacity

Table A.3 shows that the co-solvent concentration (X₃) affected positive and significantly all the response variables except total monomeric anthocyanins. These results were in agreement with the study performed by Monroy et al. (2016) which suggest as composition of solvent modifier (ethanol) have an important influence on phenolic compound recovery. The highest extraction of phenols are obtained at lowest co-solvent percentages used, when the molar fractions of water and ethanol are the lowest and those of carbon dioxide the highest. Da Porto, Natolino, & Decorti (2014b) investigated different co-solvent percentages (15, 10, 7.5%) for the extraction of proanthocyanidins. They reported that low concentration of ethanol–water co-solvent (7.5 and 10%) favored better extraction at constant CO₂ flow rate (4 and 6 kg/h). In a similar work, factors including ethanol concentration (5–15%), extraction time (30–60 min), temperature (40–70 °C), and pressure (150–300 bar) was studied for supercritical CO₂ extraction of (poly)phenols and antioxidant activities from peel of custard apple fruit (Phan, Hong, Huu, & Nguyen Thi, 2022). At an ethanol

concentration of 12%, the optimum conditions were reported as: the, 54 min, 52°C, 261 bar. In addition, the use of ethanol as co-solvent has been reported to enhance the phenolic fraction yield in comparison to ethyl acetate. Authors found the highest yield as 19.033%, w/w total extract under SCO₂ extraction conditions of 30 MPa and 40 °C (Castro-Vargas et al., 2010).

Table A.3 shows these regression coefficients while the absolute t-value and p-value determined the importance of each coefficient in the proposed model. In this study, model terms with $p < 0.001$ are highly significant, with $0.001 \leq p < 0.05$ are significant and with $p \geq 0.05$ are insignificant. In order to develop the RSM models, quadratic regression fitting on the experimental values was applied (Table A.3). The nonsignificant factors ($p > 0.05$) are excluded from the polynomial equation. The mathematical equations relating the experimental data to the process parameters can be written in terms of the coded factors as follows:

$$\begin{aligned} \text{Global Yield} = & -83.0598 + 0.0066X_1 - 3.4649X_2 - 0.4755X_3 - 0.0329X_2^2 \\ & - 0.0038X_3^2 + 0.0006X_1X_2 - 0.0004X_1X_3 - 0.0025X_2X_3 \end{aligned} \quad (4.7)$$

$$\begin{aligned} \text{TPC} = & -236.295 + 0.509X_1 + 6.458X_2 + 2.291X_3 - 0.001X_1^2 \\ & - 0.045X_2^2 - 0.013X_3^2 - 0.001X_1X_3 - 0.023X_2X_3 \end{aligned} \quad (4.8)$$

$$\begin{aligned} \text{Catechin} = & 2.00015 + 0.00226X_1 + 0.01817X_2 + 0.04632X_3 \\ & - 0.00002X_1^2 - 0.00041X_3^2 + 0.00015X_1X_2 - 0.00079X_2X_3 \end{aligned} \quad (4.9)$$

$$\begin{aligned} \text{TMAC} = & -28.8284 + 0.0174X_1 + 1.3425X_2 + 0.1438X_3 - 0.0001X_1^2 \\ & - 0.0147X_2^2 - 0.0012X_3^2 + 0.0011X_1X_2 - 0.0001X_1X_3 \\ & - 0.0017X_2X_3 \end{aligned} \quad (4.10)$$

$$\begin{aligned} \text{Cyn3glu} = & -1.31336 + 0.03046X_1 - 0.09188X_2 - 0.16205X_3 \\ & - 0.00006X_1^2 - 0.00136X_3^2 - 0.00161X_2X_3 \end{aligned} \quad (4.11)$$

$$\begin{aligned} \text{TACdpph} = & -602.678 + 0.616X_1 + 23.075X_2 + 2.511X_3 - 0.001X_1^2 \\ & - 0.182X_2^2 - 0.015X_3^2 - 0.051X_2X_3 \end{aligned} \quad (4.12)$$

$$\begin{aligned} \text{TACuprac} = & -718.300 + 2.351X_1 + 17.842X_2 + 18.389X_3 - 0.004X_1^2 \\ & - 0.126X_3^2 - 0.005X_1X_3 - 0.181X_2X_3 \end{aligned} \quad (4.13)$$

Response surface contour plots for SCO₂ extract of black rosehip showing effects of interactions of pressure, temperature and co-solvent concentration on (a) TPC and (b) CC are given in Figure 4.3 and on (c) TMAC and (d) Cyn3glu are given in Figure 4.4. As the temperature increased from 40 to 60°C, phenolic content and anthocyanin content were increased. This can be explained that rising the temperature in SCO₂ extraction using ethanol as modifier enhances the solubility of (poly)phenols, eventually increasing their extraction. Similar observations were reported in previous studies (Castro-Vargas et al., 2010). The phenolic content increased from 50.92 to 63.59 mg GAE/g when the pressure risen from 150 to 250 bar (at 50 °C with co-solvent concentration 20 %). However, when the co-solvent concentration is 100 %, total phenolic content and total monomeric anthocyanin content decreased from 4.93 to 1.10 mg GAE/g and from 1.25 to 0.13 mg Cyn3gluE/g, respectively. Plots showed that total phenolic and anthocyanin yield increased with increasing pressure until a specific point, and then decreased if the pressure was raised further. Pressure raise can increase the SCO₂ fluid density, hence can strengthen interactions between the supercritical fluid and the raw material (Maran et al., 2014). Table A.3 shows a highly significant positive linear effect of co-solvent concentration ($p < 0.001$) on the recoveries of total phenolic content, catechin content and cyanidin-3-*O*-glucoside content.

Figure 4.5 e and f shows the effects of SCO₂ extraction conditions on antioxidant activity by DPPH and CUPRAC assays, respectively. The highest antioxidant activity results were obtained when 250 bar pressure applied at 60 °C with co-solvent concentration 20 % that are 192.7 and 754.8 mmol TE/ g by DPPH and CUPRAC assays, respectively. But with further increase in the pressure yielded negative effects in terms of antioxidant activity as well as (poly)phenol contents. At a high pressure, the condition may be more favored for complex substances which are not responsible for the DPPH or CUPRAC assays. These results are similar to those reported by de Souza et al. (2018), whom reached the highest phenolic content at 25 MPa from *Arctium Lappa* leaves. In another work, maximum (poly)phenols yield was extracted at 250 bar from guayusa leaves (Cadena-Carrera et al., 2019).

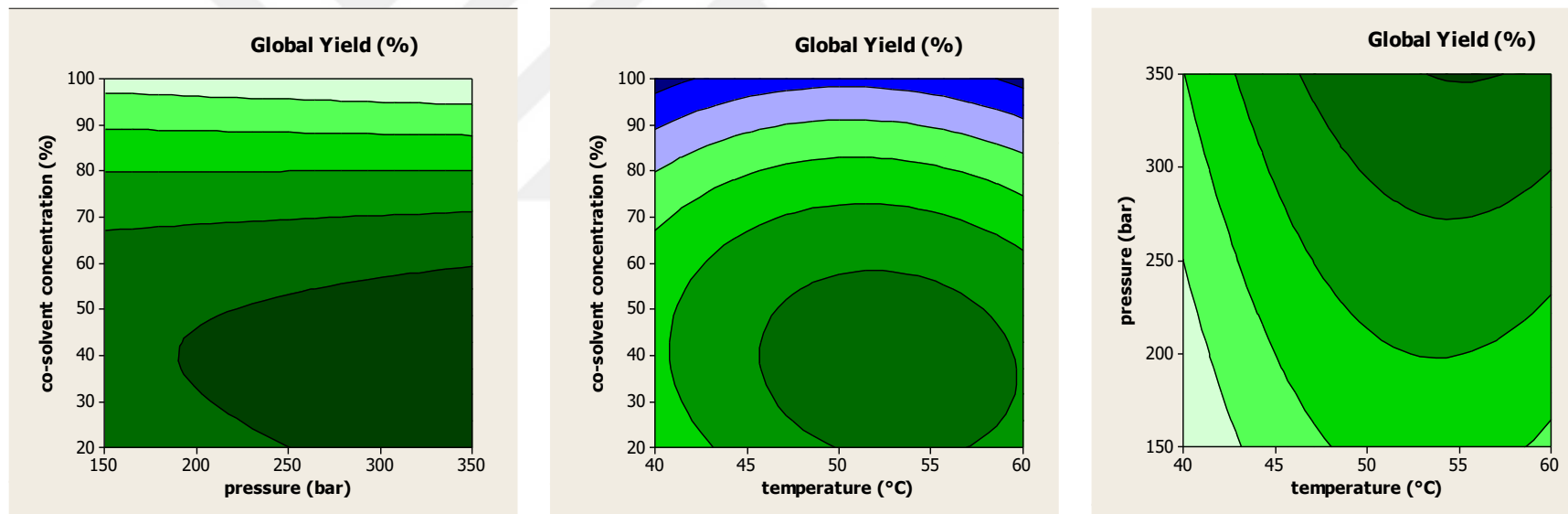


Figure 4.2 : Response surface contour plots for SCO_2 extract of black rosehip showing effects of interactions of pressure, temperature and co-solvent concentration on global yield.

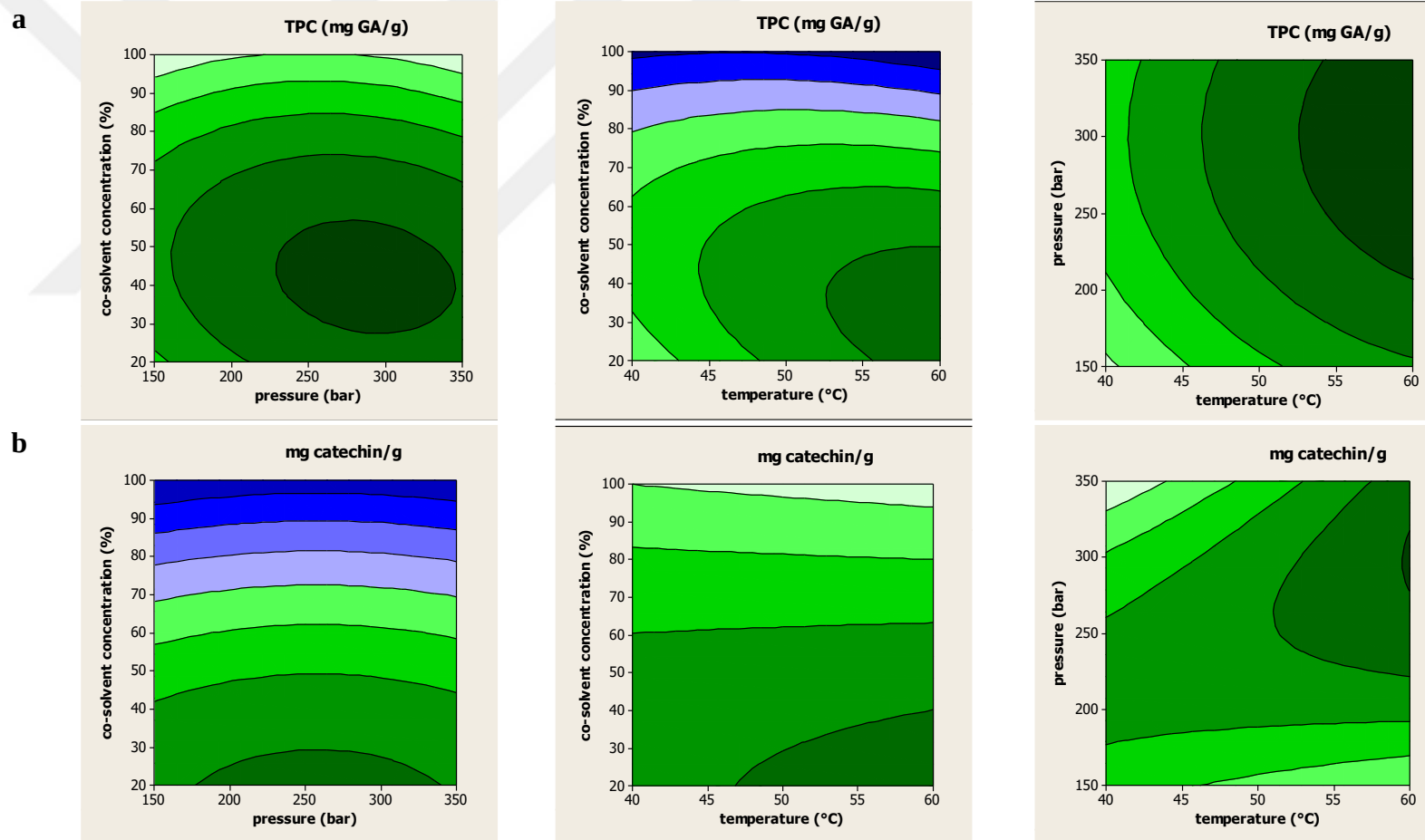
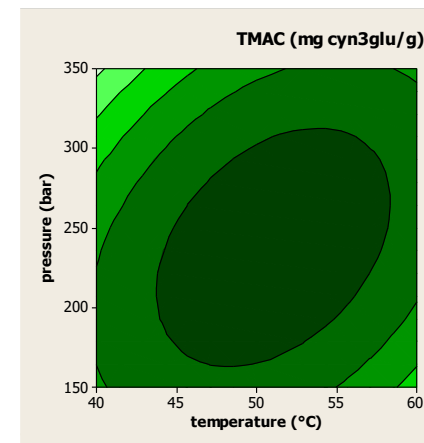
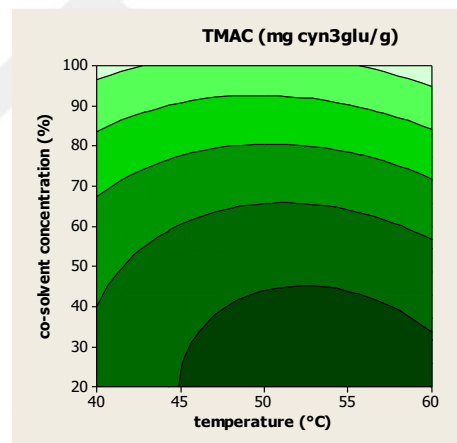
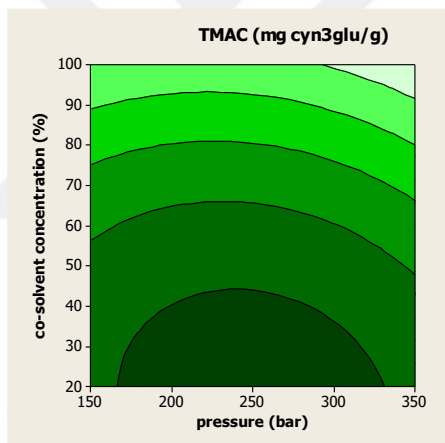


Figure 4.3 : Response surface contour plots for SCO_2 extract of black rosehip showing effects of interactions of pressure, temperature and co-solvent concentration on (a) TPC and (b) CC.

C



d

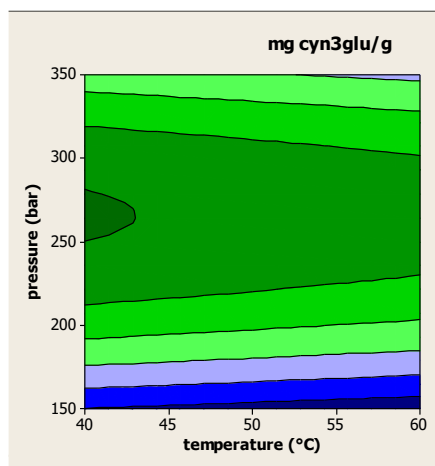
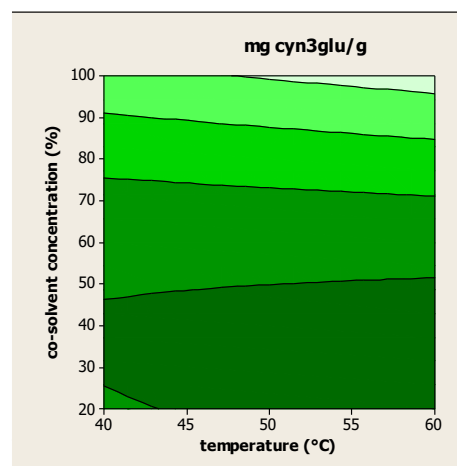
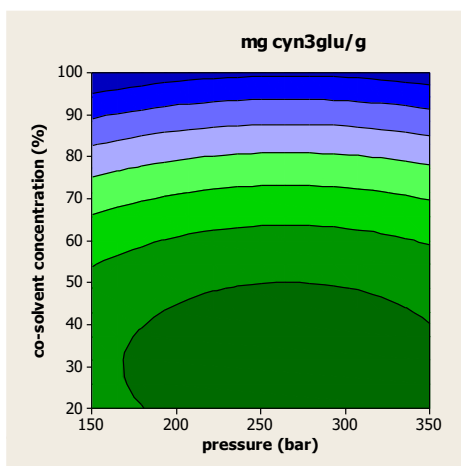
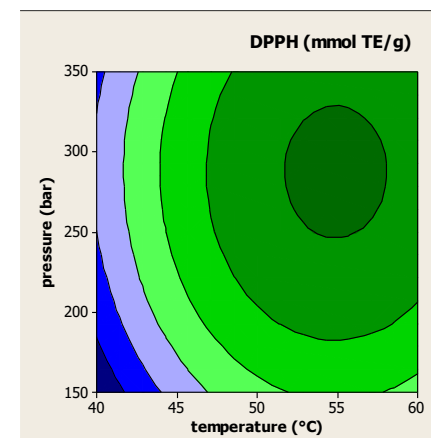
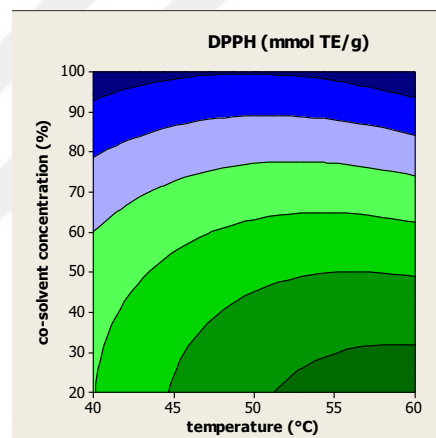
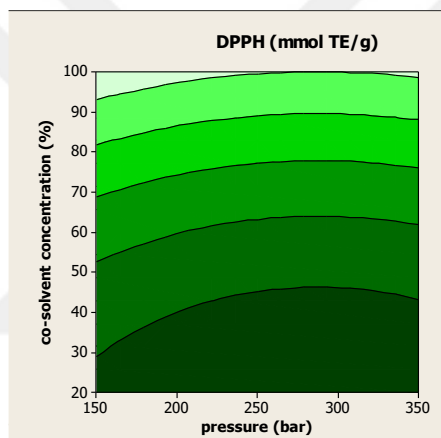


Figure 4.4 : Response surface contour plots for SCO₂ extract of black rosehip showing effects of interactions of pressure, temperature and co-solvent concentration on (c) TMAC and (d) cyn3glu content.

e



f

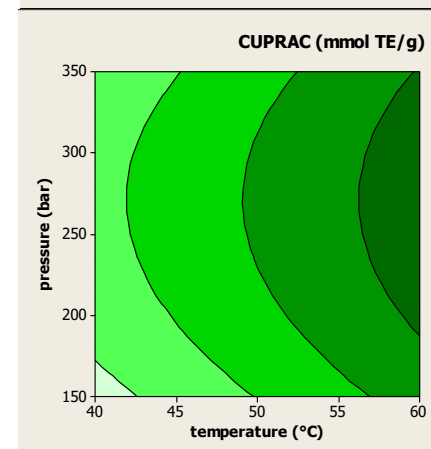
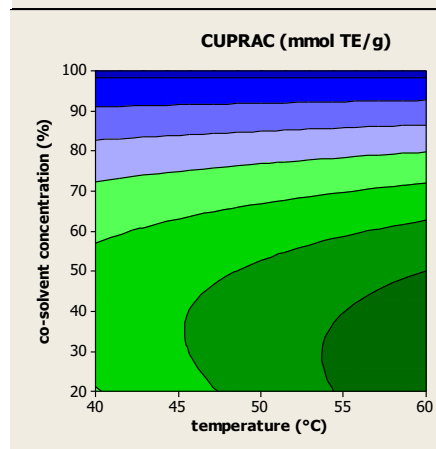
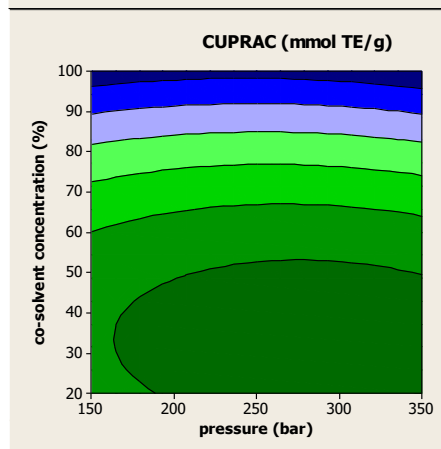


Figure 4.5 : Response surface contour plots for SCO₂ extract of black rosehip showing effects of interactions of pressure, temperature and co-solvent concentration on antioxidant capacity (e) DPPH and (f) CUPRAC.

4.3.2.3 Validation of optimal conditions

Optimized SCO₂ extraction conditions provided by statistical software (pressure 280 bar; temperature 60 °C; and EtOH concentration 25%) showed a desirability levels of 100 % with an extraction yield of 25.3 %. As can be depicted from Table 4.6, high desirability values for the responses (TPC, CC, TMAC, Cyn3glu, DPPH and CUPRAC) were obtained under optimal extraction conditions. Results are expressed as per gram lyophilized fruit.

Table 4.6 : Experimental data of the validation of predicted value at optimal SCO₂ conditions.

	Yield	TPC	CC	TMAC	Cyn3glu	TAC _{DPPH}	TAC _{CUPRAC}
Experimental value	25.05 ± 1.54	76.58 ± 4.25	4.65 ± 0.39	10.89 ± 0.56	9.05 ± 0.22	183.2 ± 7.4	789.2 ± 12.8
Predicted value	25.2985	77.3792	4.55665	11.1924	9.03575	190.9545	767.1667
Desirability	1.0000	1.0000	0.987958	1.0000	0.990951	1.0000	1.0000
p*	0.809	0.777	0.306	0.449	0.939	0.211	0.097

Abbreviations: Global Yield (%); TPC (mg GAE/g); CC (mg/g); TMAC (mg cyn3glu/g); Cyn3glu (mg/g); TAC (mmol TE/g). All in dry basis.

* Indicate significant differences (p < 0.05).

The extracts of spearmint leaves obtained by SCO₂ extraction (optimal conditions: 60 °C, 200 bar, 60 min) and conventional solvent extraction (Soxhlet extraction using 70% ethanol) yielded comparable results (Bimakr et al., 2011). Taking the obtained values by PHWE and UAE into consideration, the capability of the applied extraction procedure to yield a good extraction efficiency is comparable with those obtained by UAE using ethanol. This result confirms that SCO₂ can be used as valid alternative to the conventional solvent extraction for the recovery of (poly)phenols from black rosehip fruit.

4.3.3 Ultrasound assisted solvent extraction

To compare PHWE and SCO₂ using aqueous ethanol as co-solvent, another extraction of black rosehip performed using absolute ethanol as solvent that enables recovery of both lipophilic and hydrophilic (poly)phenols. Although the quantities for the individual phenolics differ based the nature of the extraction method and used solvent, total phenolic content in the ultrasound assisted absolute ethanol extract (UA-EtOH) was the highest among others (Table 4.8.).

4.3.4 Individual compounds in black rosehip

Rosehips are commonly considered as sources of various health-promoting constituents including carotenoids, differences in content and composition may exist in rosehips owing to genetic variation, variations within and between years, and used analytical method as well as the degree of maturation (Andersson, Rumpunen, Johansson, & Olsson, 2011). Individual lipophilic compound contents in black rosehip extracts are given in Table 4.7.

Table 4.7 : Carotenoid contents ($\mu\text{g g}^{-1}$ DW) in different extracts of black rosehip as determined by HPLC (n = 4).

Carotenoids	UA-EtOH extract	SCO ₂ -aqEtOH extract	Fruit	Flesh
Lutein	1.04 ± 0.03	0.18 ± 0.01	1.08 ± 0.16	1.68 ± 0.01
Zeaxanthin	5.46 ± 0.36	0.77 ± 0.03	4.44 ± 0.67	6.77 ± 0.07
β -cryptoxanthin	1.28 ± 0.03	0.23 ± 0.01	0.80 ± 0.11	0.78 ± 0.24
β -carotene	106.90 ± 2.67	4.36 ± 0.18	10.75 ± 0.91	14.73 ± 2.15
Vitamin E				
Gamma-Tocopherol	3.57 ± 0.09	15.09 ± 0.61	9.72 ± 1.30	17.21 ± 3.03
Alpha-Tocopherol	10.62 ± 0.27	83.90 ± 3.39	72.52 ± 6.78	136.93 ± 1.09

β -carotene was the major carotenoid in all black rosehip samples, followed by zeaxanthin, with 24.5–7.0 times lower concentrations in SCO₂-aqEtOH extract than the UA-EtOH extract (Table 4.7). α -tocopherol content of black rosehip flesh was measured as $136.93 \pm 1.09 \mu\text{g g}^{-1}$ DW. Andersson, Olsson, Gustavsson, Johansson, & Rumpunen (2012) found mean annual tocopherol content $187.6 \pm 16.9 \mu\text{g g}^{-1}$ DW in seedless black rosehip that sampled on different harvesting times also higher than other Rosa species; *R. Rubiginosa* and *R. Dumalis*. (Poly)phenols were demonstrated to possess higher *in vitro* antioxidant capacity in comparison to carotenoids and vitamins making them the main dietary antioxidants (Richelle, Tavazzi, & Offord, 2001).

On the other hand, contents of hydrophilic compounds in black rosehip extracts, that is PHW and SCO₂ extraction of anthocyanins and phenolics are summarized in Table 4.3 and 4.5, respectively. Polar solvents are needed for the effective extraction of anthocyanins as they occur as glycosides. Of the common organic solvents, methanol indeed performs better for anthocyanin extraction indeed (Trikas et al., 2016). However, ethanol is much safer than methanol in regards to food and environment (Xiao, Han, & Shi, 2008).

4.3.5 Changes in phenolic compounds after *in vitro* digestion

Table 4.8 shows the effect of the *in vitro* digestion on the antioxidant activity black rosehip extracts obtained via these methods: pressurized hot water extraction (PH-H₂O), aqueous ethanol modified supercritical carbon dioxide extraction (SCO₂-aqEtOH), and ultrasound assisted ethanol extraction (UA-EtOH). After simulated digestion in the stomach and intestine, all extracts had reduced antioxidant activity compared to their undigested forms ($p < 0.05$).

Table 4.8 : Changes in the antioxidant capacity of black rosehip extracts obtained via different methods during *in vitro* digestion.

Extract	Digestive Recovery (%)	
	DPPH assay	CUPRAC assay
SCO ₂ -aqEtOH	10.7 ± 2.9 ^A	6.9 ± 0.8 ^B
PH-H ₂ O	11.2 ± 1.9 ^A	9.0 ± 0.9 ^A
UA-EtOH	8.9 ± 3.4 ^A	6.7 ± 1.2 ^B

According to the cupric ion reducing activity results upon *in vitro* digestion, the retention of antioxidant activity was higher in PH-H₂O extract. Table 4.9 shows the effect of the *in vitro* digestion on the phenolic compounds of different black rosehip extracts. The changes in phenolic compound profile after the *in vitro* digestion was due to the solubility of phenolic compounds and their interaction with other substances in the environment (Czubinski et al., 2019). It is noteworthy that a new compound, quercetin (data not shown) was observed in the samples after digestion. This could be attributed to the hydrolysis of glycosidic bonds from quercetin 3-*O*-glucoside that occur during digestion (Valentová, Vrba, Bancířová, Ulrichová, & Křen, 2014).

Table 4.9 : Changes in phenolic compounds from of black rosehip extracts obtained via different methods during *in vitro* digestion. Results are expressed as µg/ mL digesta.

Extract	Undigested (UD)	Digested fraction (DG)	Aqueous fraction (AQ)	Micellar fraction (MF)
Catechin				
SCO ₂ -aqEtOH	1070.4 ± 23.9 ^{Ba}	459.9 ± 54.0 ^{Bb}	401.9 ± 88.1 ^{Bbc}	323.2 ± 36.5 ^{Bc}
PH-H ₂ O	961.1 ± 37.4 ^{Ba}	418.5 ± 77.6 ^{Bb}	365.9 ± 90.2 ^{Bb}	238.2 ± 22.3 ^{Cc}
UA-EtOH	1642.5 ± 262.2 ^{Aa}	721.5 ± 109.2 ^{Ab}	638.9 ± 114.1 ^{Ab}	495.4 ± 60.5 ^{Ab}
Epicatechin				
SCO ₂ -aqEtOH	406.6 ± 52.5 ^{Aa}	157.4 ± 19.5 ^{Ab}	125.4 ± 35.1 ^{Ab}	119.7 ± 12.8 ^{Ab}
PH-H ₂ O	255.0 ± 31.1 ^{Ba}	114.2 ± 30.2 ^{Bb}	87.5 ± 24.6 ^{Abc}	61.8 ± 6.4 ^{Bc}
UA-EtOH	388.3 ± 98.1 ^{Aa}	140.8 ± 30.6 ^{ABb}	116.5 ± 30.6 ^{Ab}	101.5 ± 32.4 ^{Ab}
Isoquercetin				
SCO ₂ -aqEtOH	510.7 ± 10.7 ^{Aa}	146.9 ± 10.0 ^{Ab}	136.4 ± 4.0 ^{Ab}	127.7 ± 9.7 ^{Ab}
PH-H ₂ O	252.9 ± 12.1 ^{Ca}	61.5 ± 5.3 ^{Cb}	52.8 ± 3.6 ^{Bb}	46.3 ± 4.4 ^{Cb}
UA-EtOH	479.3 ± 4.3 ^{Ba}	137.1 ± 16.3 ^{Bb}	123.8 ± 14.9 ^{Abc}	98.1 ± 11.0 ^{Bc}
Vanillin				
SCO ₂ -aqEtOH	37.6 ± 2.0 ^{Aa}	14.8 ± 1.4 ^{Ab}	9.8 ± 1.3 ^{Ac}	9.0 ± 0.5 ^{Ac}
PH-H ₂ O	21.8 ± 0.4 ^{Ca}	7.9 ± 1.3 ^{Bb}	6.6 ± 0.1 ^{Bc}	6.0 ± 0.6 ^{Bc}
UA-EtOH	28.3 ± 3.1 ^{Ba}	8.4 ± 1.7 ^{ABb}	7.2 ± 1.4 ^{Bb}	6.4 ± 0.5 ^{Bb}

Means with significant differences ($p < 0.05$) in each extracts indicated by different capital letters (same column) and among digestion phase by different lowercase letters.

The bioaccessibility levels of individual compounds in various extracts were differed depending on the phenolic compound although they have statistically insignificant difference among their digestive stability ($p > 0.05$) (Table 4.10). The instability of catechin, epicatechin, in other words monomeric flavan-3-ols, and isoquercetin (flavonoids) were known under alkaline intestinal conditions due to chemical changes including oxidation, polymerisation and transformation (Rodríguez & Tironi, 2020). Oliveira, A., & Pintado, M. (2015). Up to 80 % loss in the catechin content was reported upon simulated digestion (Table 4.10). A higher decrease (90 %) was reported for pure (+)-catechin by Oliveira & Pintado (2015). In addition, the authors concluded that the loss of antioxidant activity of catechin could be result of oxidation reactions at near-neutral pH (pH 7) in the intestine. The *in vitro* antioxidant power of flavonoids and their metabolites depends upon the arrangement of functional groups in the molecular structure (Heim, Tagliaferro, & Bobilya, 2002).

Table 4.10 : Solubilization and micellization of phenolic compounds during *in vitro* gastrointestinal digestion.

Extract	Digestive Stability ¹ (%)	Solubility ² (%)	Micellization Efficiency ³ (%)	Bioaccessibility ⁴ (%)
Catechin				
SCO ₂ -aqEtOH	42.94 ± 4.60	37.48 ± 7.72	70.33 ± 2.37 ^A	30.17 ± 3.12 ^A
PH-H ₂ O	43.50 ± 7.51	38.15 ± 9.72	57.43 ± 9.20 ^B	20.89 ± 4.37 ^B
UA-EtOH	44.16 ± 4.90	39.24 ± 6.01	69.13 ± 5.86 ^A	30.32 ± 1.39 ^A
Epicatechin				
SCO ₂ -aqEtOH	39.27 ± 7.15	31.28 ± 9.54	76.22 ± 4.08 ^A	29.85 ± 5.09 ^A
PH-H ₂ O	44.47 ± 7.81	34.17 ± 8.42	58.76 ± 9.21 ^B	24.35 ± 2.31 ^B
UA-EtOH	36.53 ± 1.90	30.29 ± 4.44	74.81 ± 9.38 ^A	25.81 ± 1.79 ^{AB}
Isoquercetin				
SCO ₂ -aqEtOH	28.75 ± 1.52	26.72 ± 0.83 ^A	86.89 ± 2.20	24.99 ± 1.65 ^A
PH-H ₂ O	24.33 ± 2.34	20.96 ± 2.37 ^B	75.63 ± 8.95	18.38 ± 2.56 ^B
UA-EtOH	28.60 ± 3.23	25.82 ± 2.99 ^{AB}	71.83 ± 6.82	20.46 ± 2.09 ^{AB}
Vanillin				
SCO ₂ -aqEtOH	39.44 ± 1.11 ^A	26.03 ± 3.70	60.39 ± 1.36 ^B	23.82 ± 1.06 ^{AB}
PH-H ₂ O	36.14 ± 1.63 ^A	30.19 ± 0.95	76.62 ± 10.93 ^{AB}	27.57 ± 2.63 ^A
UA-EtOH	28.74 ± 2.29 ^B	25.45 ± 3.11	79.13 ± 3.67 ^A	22.73 ± 1.14 ^B

4.3.6 Cellular uptake of phenolics by Caco-2 cells

The major phenolics present in black rosehip are flavan-3-ols, namely catechin and epicatechin as monomers, as well as oligomers of these two monomers (dimers and trimers) as mentioned in section 3.3.2 previously. The cellular uptake of micellar (digested) catechin and epicatechin from black rosehip extracts obtained via different methods are given in Table 4.11. The uptake results of the Caco-2 cell model experiments are calculated in two methods: as percentage of the amount in the cells and remaining phenolic compounds stable after the 3 h incubation period (Cell lysate + Supernatant, C+S) or as percentage of the amount of the bioaccessible compounds added initially (Raw material, RM). Taking this stability issue into consideration, higher values for absorption of catechin and epicatechin were measured when results were calculated as percentage of the C+S. The results indicated that the extracts have different degrees of uptake for each phenolic compound in Caco-2 cells.

The results also demonstrated that higher stability of the compounds caused higher degree of cellular absorption for instance in case of PH-H₂O extract gave considerably

lower phenolic absorption rate compared to other two extracts ($p < 0.05$). This could be attributed to the stability of phenolics correlated with the initial quantity present in micellar fraction after *in vitro* digestion (Table 4.9). Unsurprisingly, different extracts contain various amounts of polyphenol classes or individual polyphenolic compounds (Romani et al., 2004). A similar trend was observed in the micellization efficiency and bioaccessibility of catechin and epicatechin from black rosehip extracts obtained via different methods (Table 4.10).

Table 4.11 : Caco-2 cellular uptake of micellar phenolics from black rosehip extracts obtained via different methods.

	SCO ₂ -aqEtOH	PH-H ₂ O	UA-EtOH
<i>Catechin</i>			
Uptake as %, RM ¹	0.51 ± 0.18^a	0.21 ± 0.06^b	0.60 ± 0.29^a
Uptake as %, (C+S) ²	19.89 ± 2.54^a	9.62 ± 2.56^b	16.27 ± 9.64^a
Stability in DMEM after 180 min (%)	33.57 ± 0.21^b	18.06 ± 2.05^c	45.01 ± 3.02^a
<i>Epicatechin</i>			
Uptake as %, RM	0.68 ± 0.77^b	0.35 ± 0.09^b	1.47 ± 0.76^a
Uptake as %, (C+S)	11.44 ± 8.73^{ab}	6.35 ± 1.59^b	14.26 ± 3.42^a
Stability in DMEM after 180 min (%)	26.67 ± 2.80^b	8.13 ± 1.27^c	59.93 ± 0.82^a

Human epithelial cells are widely used in intestinal absorption and transport experiments, however the changes (poly)phenols undergo during incubation, due to their chemical instability under the cell culture conditions might lead to inaccurate conclusions (Gómez-Juaristi et al., 2020). In this study, the stability of catechin and epicatechin in Dulbecco's Modified Eagle's Medium (DMEM) under the cell culture conditions are shown in Figure 4.6. The culture medium has been reported to affect phenol isomerization to a higher extent than cells leading scarce absorption of catechin monomers and minimal metabolism by the intestinal epithelium cells (Gómez-Juaristi et al., 2020).

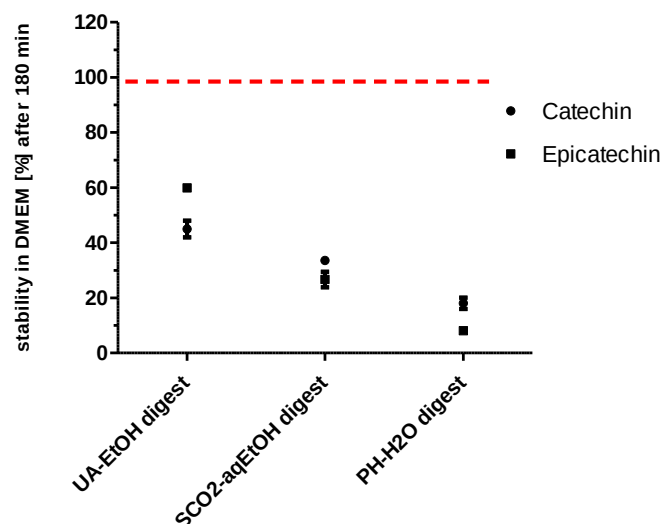


Figure 4.6 : Stability of phenolics from different extracts in DMEM after 180 min incubation without cells.

4.3.7 Cytotoxicity of extracts

The cytotoxicity of the extracts were determined on intestinal cells *in vitro* using NRU assay. The histogram reported in Figure 4.7 represent the viability of cells after 3 h of incubation with black rosehip extracts obtained via different methods and grape pomace extract obtained using ultrasound assisted ethanol extraction, included in the medium in different amounts expressed as mg/mL. The extracts substantially reduced the viability of Caco-2 cells after incubation for 3 hours ($p < 0.05$) with no significant difference among extraction method in black rosehip ($p > 0.05$). Indeed, given 80% viability as the lowest acceptable limit, all extracts showed to be safe up to a concentration of 15 mg/mL. In the literature, cytotoxic potential of plant extracts usually studied at lower concentrations and longer time periods like 24 hours. In this study, higher concentrations were applied in a shorter time period. In hydromethanolic extracts of grape pomace, a concentration higher than 400 $\mu\text{g/mL}$ was indicated to achieve 50% of growth inhibition in human tumor cell lines in 24 hours (Peixoto et al., 2018). Salau, Yakubu, & Oladiji (2013) investigated cytotoxic activity of aqueous extracts of two traditional herbs: *Anogeissus leiocarpus* and *Terminalia avicennioides* root barks in Ehrlich Ascites Carcinoma (EAC) cells for 3 hours and 24 hours. They reported 80% viability for approximately 10 and 5 $\mu\text{g/mL}$ of plant extract in 3 hours and 24 hours incubation, respectively.

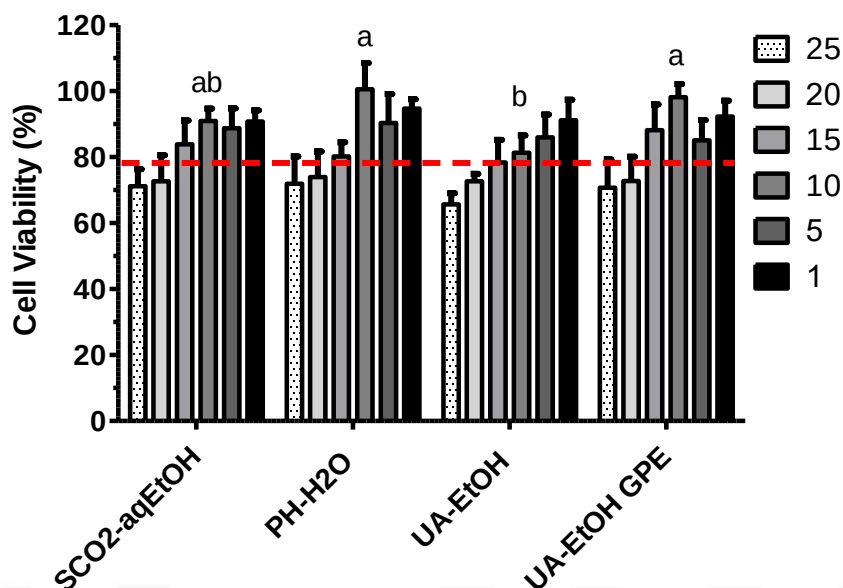


Figure 4.7 : Cytotoxicity of black rosehip extracts (mg/mL) obtained via different methods, and grape pomace extract obtained via ultrasound assisted extraction using ethanol as solvent in Caco-2 cells.

4.4 Conclusion

In the context of (poly)phenols, pressure, temperature, time and co-solvent to biomass ratio influence the extraction efficiency that enables the process optimization (Jiao, 2018). In the PHWE study, a good extraction yield (27.9 %) was obtained within 60 min when PHWE was performed with a solvent-to-solid ratio of 10:1 mL/g at 75 °C and 100 bar. The obtained optimized black rosehip extract had a high antioxidant activity (930.5 mmol TE/g dry fruit), as well as (poly)phenols (68.9 mg GAE/g dry fruit) and anthocyanins, including cyanidin-3-*O*-glucoside (4.08 mg/g dry fruit). In the SCO₂ extraction, results highlight that the composition of solvent modifier as well as temperature are the main experimental factors that influence the phenolic yield in supercritical fluid process.

Extracts recovered using supercritical carbon dioxide modified with aqueous ethanol exhibited higher antiradical activity and (poly)phenolic content. HPLC-DAD analysis revealed that the extracts contained high amounts of anthocyanins mainly cyanidin-3-*O*-glucoside as well as catechin. The observed rates of individual phenolic compound recovery and cellular absorption were similar to those observed for initial phenolic content of extracts and the stability level of phenolics in micellar fraction after in vitro digestion, respectively. To sum up, the obtained results demonstrated the potential of

carbon dioxide modified with aqueous ethanol and pressurized hot water as green solvents for the extraction of phenolics and anthocyanins. The extraction procedure and solvent used affects the efficiency of the process. The results of the optimized models provides important knowledge about the supercritical extraction of black rosehip using pressurized hot water and carbon dioxide + ethanol/water mixture as solvent for future work in terms of process scale-up.





5. ENHANCING THE STABILITY AND SOLUBILITY OF ANTIOXIDATIVE BLACK ROSEHIP EXTRACTS BY NANO/ MICRO-ENCAPSULATION TECHNIQUES

Chosen optimized extracts were further used for liposomal encapsulation in order to enhance the stability during shelf life in fortified food products and digestive conditions in the body for improved absorption. Phenolic compounds are extensively metabolized in the digestive tract after consumption, hence their bioavailability in food systems should be taken into consideration regarding the potential health benefits (Xie et al., 2016). Within this scope, liposomal formulations were prepared using different methods based on the characteristics of the obtained extracts.

5.1 Introduction

To convert black rosehip into a high value-added product, several processes proposed recently by different authors such as aromatic vinegar production (Pashazadeh et al., 2021a), tea (Pashazadeh et al., 2020) and yogurt (Szołtysik et al., 2020) production with good antioxidant activity, and ice cream production as a natural colorant due to their color stability during storage (Zor & Sengul, 2022). In this regard, Zor and co-authors (2022) prepared extracts containing phenolics and anthocyanins from fruit flesh and seeds using water and 7% acetic acid-added ethanol: water (1:4, v/v) as solvents in classical solvent extraction and ultrasonic extraction methods. Microwave assisted aqueous two-phase extraction has been studied for better anthocyanin recovery from black rosehip (Odabaş & Koca, 2021). In another study, the authors also fabricated freeze- and spray-dried microencapsulated anthocyanin rich extract obtained by aqueous two-phase extraction using 30% ethanol and 19% ammonium sulfate (Odabaş & Koca, 2020). (Poly)phenolic compounds recovered via applied green methods from black rosehip were encapsulated and converted into dried form in order to obtain a high-value ingredient for the functional food industry. Thus, non-encapsulated and encapsulated black rosehip extracts were subjected to *in vitro* digestion to evaluate the bioaccessibility of (poly)phenols.

5.2 Materials and Methods

5.2.1 Materials

Lecithin, 70% phosphatidylcholine from non-GMO soybean (Lipoid P75®) was kindly gifted from Lipoid GmbH (Ludwigshafen, Germany). Medium molecular weight chitosan was donated by Primex (Siglufjörður, Iceland). Whey protein isolate (WP) with 89% protein content was purchased from Isopure Company. Maltodextrin with a dextrose equivalent of 16 (Glucidex®) was donated (Roquette, France). Triton X-100 was obtained from Merck (Darmstadt, Germany). Porcine bile extract (B8631), pancreatic lipase (L3126), pancreatin (P3292), pepsin (P7000), were all from Sigma-Aldrich.

5.2.2 Encapsulation of hydrophilic compounds

5.2.2.1 Liposome preparation

Liposomes were prepared as described earlier by Gültekin-Özgüven et al. (2016). Lecithin (Lipoid® P75) and lyophilized black rosehip extract (BRE) were mixed in a mass ratio of 10:1 and dissolved in acetate buffer (pH 3.5; 0.1 M) at room temperature. Liposomal dispersions with and without BRE were homogenized by a high shear disperser and then passed five times via a high-pressure homogenizer (25.000 psi).

5.2.3 Encapsulation of hydrophilic-lipophilic compounds

5.2.3.1 Liposome preparation by ethanol injection

Liposomes were prepared using ethanol injection and high-pressure microfluidization method as described previously by Zou et al. (2014) with minor modifications. Lecithin (Lipoid® P75), cholesterol, Tween 80, and *Rosa pimpinellifolia* fruit extract (RPFE) were mixed in a mass ratio of 8:2:4:1. The mixture was dissolved in ethanol and injected into phosphate buffer (pH 4) while stirring. After one hour, ethanol was removed under vacuum at 60 °C. The dispersions were subjected to microfluidization, as described in section 5.2.2.1.

5.2.4 Biopolymer coating of liposomes by electrostatic deposition

Negatively charged liposomes were coated by electrostatic deposition of oppositely charged biopolymer layer to maintain stability. The deposited biopolymer layer

enables the template structures to withstand a number of environmental stresses better than uncoated ones (Laye, McClements, & Weiss, 2008; Chun, Choi, Min, & Weiss, 2013). Chitosan and whey protein were utilized as different coating materials. To decide the optimum biopolymer concentrations for coating, all liposome dispersions with and without extract were added to chitosan (0.2–1.0%, w/v) (Gültekin-Özgüven et al., 2016) and whey protein solutions (2.0–10.0%, w/v) (Frenzel & Steffen-Heins, 2015) that dissolved in distilled water containing acetic acid (pH 3.0) with a ratio of 1:1 (w/v) and stirred overnight at room temperature. Chitosan stock solution was prepared and filtered with a sintered glass filter.

5.2.5 Encapsulation of lipophilic compounds

5.2.5.1 Solid lipid nanoparticle preparation

A 10 mM sodium phosphate buffer solution (pH 7) was prepared and the pH of the solution was adjusted by 0.1 M sodium hydroxide or hydrochloric acid. In order to inhibit microbial growth, sodium azide (0.02% w/w) was added. Aqueous surfactant solutions were prepared by dispersing glycyrrhizin at five different concentrations: 0.25, 0.5, 1, 1.5, and 2% in phosphate buffer and then heated up to 85°C in a water bath. Lipid nanoparticles were fabricated using the hot high-pressure homogenization method as described previously (Salminen et al., 2016). Tristearin (10%, w/w) as the lipid phase was melted at 85°C in a water bath and then mixed with the hot surfactant solution (90% w/w), and pre-emulsified using an ultra-turrax and further dispersed under high pressure (500 bar) for five times. Pre-heating was done by cycling hot (90 °C) water to prevent crystallization during the homogenization. The final emulsion was cooled down in an ice bath to induce crystallization, and then stored at 20°C.

5.2.6 Spray drying

Dehydrated liposomal powders were produced via spray drying in order to make them industrially applicable. It provides better protection and retention of the labile compounds like (poly)phenols. Spray drying encapsulation of the black rosehip extract was performed by dissolving black rosehip extract (0.05%, w/v) in maltodextrin (20%, w/v) solution. Similarly, coated liposomal dispersions with and without black rosehip extract were also mixed with pre-hydrated maltodextrin solution while stirring for spray drying. The resulting liposomal feed consisted of 20% (w/v) maltodextrin,

0.05% (w/v) black rosehip extract, 0.5% (w/v) lecithin, and 0.2% (w/v) chitosan or 2.0 % (w/v) whey protein and dried at a feed rate of 2.5 cm³/min and 0.67 m³/min air flow using Mini spray dryer B-290. The inlet air temperature was set at 150 ± 5 °C resulting in an outlet temperature of 90 ± 5 °C. The spray-dried powders were collected, packed in airtight bags, and placed in a desiccator at 4 °C.

5.2.7 Characterization of capsule formulations

5.2.7.1 Encapsulation and Retention Efficiency

Encapsulation efficiency (EE) of liposomal dispersions was determined using the dialysis technique according to Zou et al. (2014) with few modifications. To remove the non-entrapped BRE phenolics from liposomes, a 5 mL aliquot of liposomal dispersion was placed into 10 kDa dialysis tubing cellulose membrane (Sigma Aldrich, Germany) and immersed in 100 mL of acetate buffer (pH 3.5) with mild stirring, and buffer was refreshed every 2 h during 6 h. Destabilization of liposomes was achieved by the addition of Triton X-100 (0.15%, w/v) to enable the reagents to reach the entrapped phenolics. The ratio of the content of phenolic compounds before and after dialysis was defined as the encapsulation efficiency in percentage, as given in equation 5.1.

$$\text{Encapsulation efficiency} = \frac{\text{TPC or TAC in dialyzed fraction}}{\text{TPC or TAC in before dialysis}} \times 100 \quad (5.1)$$

Unloaded liposomes without extract were used as blank in the calculations. In order to determine the effect of the spray drying process on total phenolic content (TPC) and total antioxidant capacity (TAC) of the samples, retention efficiency (RE) was calculated as the ratio of the TPC or TAC in spray dried powder after reconstitution in acetate buffer (1:10, w/w) to TPC or TAC in pre-dried feed solution in percentage, as described in equation 5.2.

$$\text{Retention efficiency} = \frac{\text{TPC or TAC in spray dried powder}}{\text{TPC or TAC in pre-dried feed solution}} \times 100 \quad (5.2)$$

5.2.7.2 Particle size and ζ-Potential

The average particle size, polydispersity index, and ζ-potential of liposomes was measured by a static light scattering instrument (Mastersizer, 2000; Malvern, UK).

Liposome dispersions were diluted to 1:5 with acetate buffer solution (pH 3.5) prior to all measurements. The refractive index used for lecithin and aqueous phase was 1.44 and 1.33, respectively. The spray-dried samples were reconstituted in buffer with a ratio of 1:5 (w/v). The particle size and ζ -potential were also determined after reconstitution.

The particle size distribution of the lipid nanoparticles was assessed by a laser diffraction instrument (Horiba LA-950, Germany). Particle size distributions are determined according to Mie's light scattering theory. Results are expressed as volume-surface area mean diameter $d_{32} = \sum n_i^3 / \sum n_i^2$, and volume mean diameter $d_{43} = \sum n_i^4 / \sum n_i^3$ where n_i is the number of droplets of diameter d_i in the population.

5.2.7.3 Morphology

The particle morphology of spray-dried powders was observed using a scanning electron microscope (SEM; ZEISS EVO LS 10).

5.2.7.4 Physicochemical properties

Production yield for the spray drying process was computed by the mass ratio of the resultant powder to the total solid content in the feed. The moisture content and solubility were analyzed using the methods described earlier by Rehman et al. (2021). The color characteristics (L^* , a^* , b^* values) were measured using a Minolta colorimeter (CR 400, Japan). Whiteness Index (WI), hue and chroma were calculated accordingly (Vargas et al., 2008).

5.2.7.5 Differential scanning calorimetry

The thermal behavior of the nanoparticles was carried out using a DSC (Perkin Elmer Life and Analytical Sciences, USA). For the analysis, an amount of approximately 10 mg of sample was heated in hermetically sealed flat-bottom aluminum pans and scanned from 20 °C to 85 °C at 20 °C/min, and then cooled down under nitrogen environment. Empty aluminum pans were used as reference.

5.2.8 *In vitro* digestion

The simulated gastrointestinal digestion was performed using the *in vitro* digestion protocol as described previously in section 4.2.8.

5.2.9 Statistical analysis

Statistical analysis was done as described in section 3.2.13.

5.3 Results and Discussion

5.3.1 Particle size and ζ -potential

5.3.1.1 Effect of biopolymer concentration on coating efficiency of liposomes

The change from negative to positive values in the zeta potential of nanosized liposomes show that they are coated successfully and due to the sufficient polymer concentration to cover the liposomes. The changes in zeta (ζ) potential, polydispersity index, and mean particle diameter after addition of 0–0.5, w/v% chitosan and whey protein 0–5.0, w/w% in Table 5.1 and Table 5.2, respectively. The uncoated primary liposomes was anionic and addition of cationic biopolymers changed the surface charge of liposomes from negative to positive. The ζ –potential of liposomes was dependent on the biopolymer concentration, and it was increased from -6.8 mV up to 16.8 with the addition of chitosan indicating that chitosan fully covered the surfaces of vesicles. The mean diameter of was also increased with increasing chitosan and whey protein concentration. Addition of 4.0 % w/v whey protein yielded 207.3 ± 5.0 nm particle size with 16.8 ± 1.4 mV ζ –potential. However, further increase in whey protein concentration (5.0 % w/v) resulted in significant increase in mean particle size (341.7 ± 8.1 nm) and decrease in the ζ –potential of the vesicles 11.7 ± 0.5 mV ($p < 0.05$).

Table 5.1 : Changes in mean particle diameter, polydispersity index (PDI), and ζ - potential after addition of 0–0.5, w/v% chitosan on black rosehip extract loaded liposomes prepared by ethanol injection method.

Sample	Particle size (nm)	PDI	ζ –potential (mV)
0.0% Lip Blank	86.0 ± 1.1^f	0.267 ± 0.009	-4.3 ± 0.7^d
0.0% Lip	95.4 ± 0.8^{ef}	0.268 ± 0.006	-6.8 ± 1.8^d
0.1% Ch-Lip Blank	102.3 ± 2.3^{de}	0.280 ± 0.004	13.7 ± 0.5^{bc}
0.1% Ch-Lip	101.3 ± 2.1^{de}	0.298 ± 0.001	13.4 ± 1.0^{bc}
0.2% Ch-Lip Blank	118.1 ± 2.2^c	0.273 ± 0.007	13.1 ± 0.4^{bc}
0.2% Ch-Lip	122.4 ± 1.7^{bc}	0.287 ± 0.016	15.3 ± 0.9^{ab}
0.3% Ch-Lip Blank	141.4 ± 16.3^a	0.291 ± 0.038	15.4 ± 0.6^{ab}
0.3% Ch-Lip	141.5 ± 0.9^a	0.300 ± 0.062	12.6 ± 1.1^c
0.4% Ch-Lip Blank	111.2 ± 1.4^{cd}	0.283 ± 0.008	11.4 ± 0.4^c
0.4% Ch-Lip	133.1 ± 1.6^{ab}	0.250 ± 0.007	16.8 ± 1.4^a
0.5% Ch-Lip Blank	119.2 ± 3.6^{bc}	0.302 ± 0.044	12.5 ± 0.2^c
0.5% Ch-Lip	124.7 ± 1.6^{bc}	0.319 ± 0.060	11.7 ± 0.5^c

Table 5.2 : Changes in mean particle diameter, polydispersity index (PDI), and ζ - potential after addition of 0–5.0, w/v% whey protein on black rosehip extract loaded liposomes prepared by ethanol injection.

Sample	Particle size (nm)	PDI	ζ -potential (mV)
0.0% Lip Blank	86.0 $\pm$ 1.1 ^h	0.267 $\pm$ 0.009 ^d	-4.3 $\pm$ 0.7 ^e
0.0% Lip	95.4 $\pm$ 0.8 ^g	0.268 $\pm$ 0.006 ^d	-6.8 $\pm$ 1.8 ^e
1.0% Wp-Lip Blank	92.2 $\pm$ 0.4 ^{gh}	0.271 $\pm$ 0.009 ^d	-0.7 $\pm$ 0.8 ^d
1.0% Wp -Lip	94.7 $\pm$ 0.3 ^{gh}	0.363 $\pm$ 0.010 ^{bc}	-5.0 $\pm$ 0.9 ^e
2.0% Wp -Lip Blank	134.1 $\pm$ 1.3 ^f	0.298 $\pm$ 0.007 ^{cd}	1.9 $\pm$ 0.1 ^c
2.0% Wp -Lip	136.3 $\pm$ 2.3 ^f	0.298 $\pm$ 0.002 ^{cd}	2.7 $\pm$ 0.9 ^{bc}
3.0% Wp -Lip Blank	150.8 $\pm$ 1.4 ^e	0.276 $\pm$ 0.009 ^d	2.7 $\pm$ 0.5 ^{bc}
3.0% Wp -Lip	147.8 $\pm$ 1.1 ^e	0.275 $\pm$ 0.010 ^d	3.6 $\pm$ 0.9 ^{bc}
4.0% Wp -Lip Blank	169.3 $\pm$ 1.0 ^d	0.284 $\pm$ 0.009 ^d	4.2 $\pm$ 0.4 ^{bc}
4.0% Wp -Lip	207.3 $\pm$ 5.0 ^c	0.469 $\pm$ 0.023 ^a	5.2 $\pm$ 1.0 ^{ab}
5.0% Wp -Lip Blank	322.4 $\pm$ 3.0 ^b	0.309 $\pm$ 0.013 ^{cd}	6.8 $\pm$ 0.8 ^a
5.0% Wp -Lip	341.7 $\pm$ 8.1 ^a	0.411 $\pm$ 0.079 ^{ab}	6.9 $\pm$ 0.7 ^a

Table 5.3 shows particle size measurements using dynamic light scattering technique to characterize the uncoated and coated liposomes in liquid dispersion form before and after spray drying. Nano scale mean vesicle size of the liposomes achieved by the use of microfluidization. Biopolymer coating of liposomes slightly increased the particle size and loading BRE into liposomes resulted in bigger particles ($p < 0.05$). Polydispersity index (PDI) values of between 0.1 and 0.25 proves a narrow size distribution while values above 0.5 illustrates a very broad size distribution (Tamjidi, Shahedi, Varshosaz, Nasirpour, 2014). The relatively high PDI measurements for chitosan coated samples could be resulted from intrinsic factors of chitosan including PDI, degree of acetylation, and molecular weight (Tsai & Chen, 2017).

The addition of maltodextrin prior to spray drying resulted no alteration in particle size as maltodextrin is an osmotically inactive substance ($p > 0.05$) (Frenzel & Steffen-Heins, 2015), but it did cause an appreciable increment in the diameter after reconstitution (Table 5.3). Being a neutral polysaccharide, the effect of maltodextrin addition on ζ -potential of the liposomal dispersions was also insignificant as expected ($p > 0.05$). By contrast, ζ -potential of anionic liposomal dispersions (-25.77 mV) drastically raised to 52.97 mV and 21.97 mV by coating oppositely charged chitosan and whey protein, respectively ($p < 0.05$). On the contrary, spray drying process caused a marginal reduction in ζ -potential as can be depicted from values after reconstitution ($p < 0.05$). A high ζ -potential and low PDI value is taken as an indicator for physical stability of nanoparticles (Othman, et al., 2018).

Table 5.3 : Average particle diameter, polydispersity index and ζ -Potential of unloaded (blank) liposomes and black rosehip extract (BRE) obtained via PHW loaded liposomes coated with chitosan and whey protein before and after spray drying.

Sample	Particle size (nm)	PDI	ζ -potential (mV)
Liposome dispersions			
Blank liposome	98.57 $\pm$ 2.24 ^{gh}	0.205 $\pm$ 0.013 ^{bc}	-14.50 $\pm$ 0.92 ^h
BRE Liposome	114.30 $\pm$ 1.61 ^{fgh}	0.240 $\pm$ 0.007 ^c	-25.77 $\pm$ 1.12 ⁱ
Blank Ch-coated liposomal dispersion	158.00 $\pm$ 2.27 ^{fg}	0.431 $\pm$ 0.035 ^{abc}	48.97 $\pm$ 0.75 ^{ab}
Ch-coated liposomal dispersion	194.47 $\pm$ 0.60 ^{def}	0.480 $\pm$ 0.004 ^{ab}	52.97 $\pm$ 2.45 ^a
Blank Wp-coated liposomal dispersion	148.07 $\pm$ 1.58 ^{fg}	0.223 $\pm$ 0.004 ^c	22.17 $\pm$ 0.35 ^d
Wp-coated liposomal dispersion	142.37 $\pm$ 2.48 ^{fg}	0.241 $\pm$ 0.018 ^{bc}	21.97 $\pm$ 0.85 ^d
Pre-dried dispersions			
Blank Ch-coated liposome mixed with MD	148.93 $\pm$ 3.45 ^{fg}	0.479 $\pm$ 0.024 ^{ab}	50.97 $\pm$ 0.75 ^a
Ch-coated liposome mixed with MD	164.60 $\pm$ 2.16 ^{efg}	0.553 $\pm$ 0.010 ^a	51.40 $\pm$ 1.91 ^a
Blank Wp-coated liposome mixed with MD	137.83 $\pm$ 1.56 ^{fgh}	0.237 $\pm$ 0.011 ^{bc}	18.67 $\pm$ 0.81 ^{de}
Wp-coated liposome mixed with MD	123.27 $\pm$ 0.71 ^{fgh}	0.228 $\pm$ 0.008 ^c	21.63 $\pm$ 1.17 ^d
BRE mixed with MD	52.85 $\pm$ 20.23 ^h	0.561 $\pm$ 0.309 ^a	12.40 $\pm$ 1.93 ^f
After reconstitution			
Blank Ch-coated liposomal powder	370.07 $\pm$ 45.32 ^{ab}	0.569 $\pm$ 0.027 ^a	42.57 $\pm$ 2.81 ^c
Ch-coated liposomal powder	384.33 $\pm$ 25.22 ^a	0.560 $\pm$ 0.049 ^a	45.70 $\pm$ 1.70 ^{bc}
Blank Wp-coated liposomal powder	254.84 $\pm$ 57.12 ^{cd}	0.411 $\pm$ 0.034 ^{abc}	15.43 $\pm$ 1.02 ^{ef}
Wp-coated liposomal powder	247.33 $\pm$ 52.42 ^{cde}	0.247 $\pm$ 0.013 ^{bc}	16.93 $\pm$ 0.67 ^e
BRE powder	284.73 $\pm$ 64.12 ^{bc}	0.250 $\pm$ 0.058 ^{bc}	7.05 $\pm$ 2.00 ^g

Samples mixed with maltodextrin-MD (20 w/v%) prior to spray drying.

Table 5.4 : Particle size, polydispersity index (PDI) and ζ -Potential of liposome dispersions coated with optimum concentrations of chitosan and whey protein before and after spray drying.

Sample	Particle size (nm)	PDI	ζ -potential (mV)
Liposome dispersions			
Blank liposome	85.97 $\pm$ 1.09 ^e	0.256 $\pm$ 0.005 ^{cd}	-5.91 $\pm$ 0.45 ^f
Liposome	95.39 $\pm$ 0.81 ^e	0.293 $\pm$ 0.036 ^{cd}	-6.76 $\pm$ 0.48 ^f
Chitosan-coated blank liposome	106.70 $\pm$ 1.44 ^{de}	0.458 $\pm$ 0.008 ^{abcd}	21.03 $\pm$ 0.67 ^{ab}
Chitosan-coated liposome	107.77 $\pm$ 2.23 ^{de}	0.470 $\pm$ 0.026 ^{abcd}	23.97 $\pm$ 0.47 ^a
Whey protein-coated blank liposome	191.08 $\pm$ 1.44 ^c	0.414 $\pm$ 0.041 ^{abcd}	6.16 $\pm$ 0.76 ^c
Whey protein-coated liposome	183.72 $\pm$ 0.67 ^c	0.499 $\pm$ 0.019 ^{abc}	5.84 $\pm$ 0.18 ^{cd}
Pre-dried dispersions			
Chitosan-coated blank liposome	101.18 $\pm$ 1.25 ^{de}	0.526 $\pm$ 0.042 ^{ab}	22.40 $\pm$ 0.40 ^{ab}
Chitosan-coated liposome	108.10 $\pm$ 2.59 ^{de}	0.548 $\pm$ 0.047 ^{ab}	22.33 $\pm$ 0.55 ^{ab}
Whey protein-coated blank liposome	197.74 $\pm$ 0.82 ^c	0.409 $\pm$ 0.021 ^{abcd}	3.05 $\pm$ 0.23 ^{cde}
Whey protein-coated liposome	188.35 $\pm$ 2.65 ^c	0.439 $\pm$ 0.032 ^{abcd}	5.02 $\pm$ 0.17 ^{cd}
RPFE mixed	47.04 $\pm$ 21.64 ^e	0.531 $\pm$ 0.256 ^{ab}	-3.71 $\pm$ 0.80 ^f
After reconstitution			
Chitosan-coated blank liposome powder	345.13 $\pm$ 37.88 ^{ab}	0.599 $\pm$ 0.041 ^a	21.23 $\pm$ 3.84 ^{ab}
Chitosan-coated liposome powder	406.73 $\pm$ 47.55 ^a	0.588 $\pm$ 0.015 ^a	19.50 $\pm$ 1.15 ^b
Whey protein-coated blank liposome powder	356.77 $\pm$ 22.24 ^{ab}	0.410 $\pm$ 0.101 ^{abcd}	1.54 $\pm$ 0.09 ^e
Whey protein-coated liposome powder	339.63 $\pm$ 38.26 ^b	0.341 $\pm$ 0.040 ^{bcd}	2.84 $\pm$ 0.19 ^{de}
RPFE powder	162.63 $\pm$ 25.89 ^{cd}	0.253 $\pm$ 0.090 ^d	0.54 $\pm$ 0.44 ^e

Samples mixed with maltodextrin-MD (20 w/v%) prior to spray drying.

5.3.1.2 Effect of emulsifier on physical stability of SLN

First, the impact of glycyrrhizin concentration (0.25-2% w/w) on the physical stability of 10% (w/w) tristearin SLN at pH 3 and 7 was evaluated during 21-days of storage at 20°C. The SLN produced with glycyrrhizin at pH 3 and 7 were nanosized ($dH = 176-224$ nm; $d_{32} = 148-212$ nm) with monomodal ($PDI < 0.2$) particle size distributions directly after preparation (Table B.1). This was also verified by the static light scattering data showing monomodally distributed particle sizes. All the SLN at pH 3 and 7 were also homogenous and did not display any visible aggregation on the day of preparation.

At pH 7, SLN emulsified with the lowest concentration (0.25%) of glycyrrhizin had a mean hydrodynamic particle diameter of 187 nm with a PDI of 0.21 on the day of the preparation (Table B2). The d_{32} -value was similar to this, however, a slightly higher d_{43} -value of 260 ± 63 nm was measured already on day 1, indicating that some larger particles (Figure 5.1) were present as detected also by the optical microscopy (Figure 5.2). Nevertheless, the SLN were still monomodally distributed and no phase separation nor aggregation was visually detected in the samples. Over the 21-day storage time, however, the d_{43} -values of SLN stabilized with 0.25% glycyrrhizin increased slightly (Table B.2). This corroborated with the particle size distribution data showing that some larger particles around 20-40 μm were present. On the other hand, increasing the concentration to 0.5 to 2.0% glycyrrhizin resulted in the formation of SLN that remained stable over the 21-day storage time.

The visual observation revealed that all the SLN at pH 3 were liquid on the day of the preparation, and no aggregation was detected (Figure 5.1). During storage, however, the SLN emulsified with 0.25, 0.5, 1.5 and 2.0% glycyrrhizin gelled, whereas the SLN stabilized by 1.0% glycyrrhizin remained liquid. SLN stabilized with 0.25 and 0.5% glycyrrhizin showed the presence of large particles after 14 days of storage, that was also detected in the optical microscopy images. When the concentration of the surfactant to cover the hydrophobic surfaces generated upon solidification in SLN are insufficient, instability is observed during storage due to inadequate repulsion between particles (Helgason et al., 2009).

At pH 3, the physical stability of the SLN emulsified with glycyrrhizin was different from pH 7. Similar to pH 7, the SLN emulsified with 0.25% glycyrrhizin showed the

formation of the largest particles ($dH = 224$ nm, $d32 = 212$ nm), whereas increasing the glycyrrhizin concentration up to 2.0% decreased the initial particle sizes. The visual observation revealed that all the SLN at pH 3 were liquid on the day of the preparation (Figure 5.2), and no aggregation was detected. During storage, however, the SLN emulsified with 0.25, 0.5, 1.5 and 2.0% glycyrrhizin gelled, whereas the SLN stabilized by 1.0% glycyrrhizin remained liquid. SLN stabilized with 0.25 and 0.5% glycyrrhizin showed the presence of large particles after 14 days of storage, that was also detected in the optical microscopy images. When the concentration of the surfactant to cover the hydrophobic surfaces generated upon solidification in SLN are insufficient, instability is observed during storage due to inadequate repulsion between particles (Helgason et al., 2009). However, the ζ -potential measurements revealed that the all the SLN stabilized by glycyrrhizin at pH 3 were highly negatively charged ($\zeta \approx -50$ to -70 mV), and should have therefore provided enough repulsive electrostatic forces between the particles. Similarly, all the SLN at pH 7 had negative charges ($\zeta \approx -25$ to -38 mV) (Table B.1). This indicates that the instability was not based on electrostatic interactions, but hydrophobic interactions.

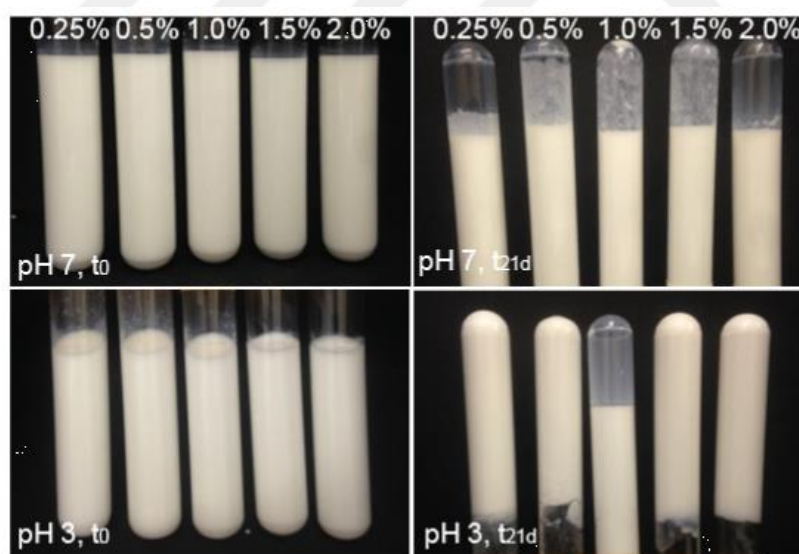


Figure 5.1 : Photographic images of tristearin (10% w/w/) solid lipid nanoparticles emulsified by glycyrrhizin (0.25-2.0%) on day 0 (A) and day 22 (B).

SLN dispersions (10 mL) were placed in test tubes and photographic images were taken for visual observation. The microstructure was observed using an optical microscope (Carl Zeiss MicroImaging GmbH, Germany).

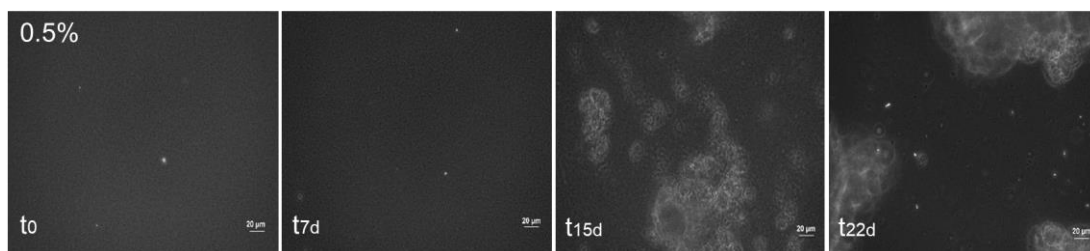


Figure 5.2 : Optical microscopy images of 10% (w/w) tristearin solid lipid nanoparticles emulsified with 0.5% (w/w) glycyrrhizin at pH 3 after preparation (t0) and during storage at 20°C. The scale bar is 20 μm.

Interestingly, SLN stabilized with 1.0 % glycyrrhizin at pH 3 was the only system that remained liquid and nanosized over the 21-day storage test. On the other hand, SLN stabilized with 1.5 and 2.0% glycyrrhizin gelled after 14 days. Nevertheless, the particle size and PDI data indicated that the particle sizes did not change during 21 days, and no aggregation was detected in the microstructure. The solubility of glycyrrhizin in phosphate buffered solution is correlated with pH and it can easily form a gel at room temperature (Koga et al., 2003).

5.3.2 Encapsulation and retention efficiency

The encapsulation efficiency was at satisfactory level in all samples in terms of TPC as shown in Table 5.5. Uncoated BRE liposome, chitosan coated BRE liposome, and whey protein coated BRE liposome, were found to be 67.2 ± 0.7 , 76.7 ± 0.8 , 87.2 ± 4.0 , respectively ($p < 0.05$). Better encapsulation efficiencies were favored in biopolymer coated liposomes in agreement with earlier studies (Davidov-Pardo, Joye, & McClements, 2015; Altin et al., 2018a).

Unlike freeze drying process which is disadvantageous in terms of cost and time, spray drying is the most often used process in drug delivery systems for converting aqueous materials into a dried and powder form (Yu, Chuesiang, Shin, & Park, 2021). However, the retention of bioactive compounds during processing is required to preserve the functional properties. High temperatures during spray-drying process may have a detrimental effect on thermo-labile (poly)phenols (Kuck & Noreña, 2016). Maltodextrin addition to uncoated liposomes has been reported to lead to break down and creaming before spray drying, that could be resulted from the exclusion of vesicles by maltodextrin (Karadag et al., 2013).

Table 5.5 : Encapsulation efficiency (EE) and retention efficiency (RE) of BRE loaded liposomal dispersions and their dried form.

Formulation	TPC	EE (%)	CUPRAC	EE (%)
Liposomal dispersion	329.71 ± 0.92 ^a	67.2 ± 0.7 ^c	827.41 ± 16.53 ^a	60.4 ± 1.1 ^b
Ch-coated liposomal dispersion	161.80 ± 1.39 ^b	76.7 ± 0.8 ^b	293.50 ± 25.5 ^c	69.0 ± 4.6 ^{ab}
Wp-coated liposomal dispersion	124.98 ± 7.48 ^c	87.2 ± 4.0 ^a	346.61 ± 34.17 ^b	70.4 ± 4.4 ^a
		RE (%)		RE (%)
Ch-coated liposomal powder	59.22 ± 2.10 ^d	62.9 ± 1.5 ^a	114.15 ± 18.95 ^f	69.8 ± 4.1 ^a
Wp-coated liposomal powder	61.79 ± 2.70 ^d	65.6 ± 2.1 ^a	124.89 ± 8.93 ^d	72.2 ± 2.4 ^a
BRE powder	55.62 ± 1.54 ^d	56.4 ± 1.8 ^b	118.62 ± 1.09 ^e	63.9 ± 4.9 ^a

Abbreviations: BRE: Black rosehip extract. Ch: Chitosan; Wp: Whey protein; TPC: Total phenolic content (mg GAE/L); CUPRAC: Cupric ion reducing antioxidant capacity (mg TE/L).

TPC of the fabricated powders ranged from 55.62 to 61.79 mg per L after reconstitution (Table 5.5). The amount of phenolics in samples decreased owing to oxygen and heat exposure during spray drying. The loss of phenolic compounds is attributed to the exposure of oxygen and high temperatures during spray drying, which may result in breakdown and polymerization reactions (Kuck & Noreña, 2016). The retention efficiency of TPC in biopolymer coated liposomal powders was found significantly higher than spray dried BRE ($p < 0.05$). These results were in agreement with our previous research in which chitosan coated mulberry extract nanoliposomes spray dried with 20% MD using same process conditions yielded total phenolic and anthocyanin retention efficiencies of 69% and 56%, respectively (Gültekin-Özgüven et al., 2016). In a similar pattern as observed with retention of phenolics, retention of antioxidant capacity of the powders was confirmed with CUPRAC assay. Chitosan coated nanoliposomes loaded flaxseed-peptide fractions retained about 90% and 86% of the free radical scavenging activity by DPPH and ABTS assays after spray drying (Sarabandi & Jafari, 2020). A similar trend was observed according to the encapsulation efficiency and retention efficiency results of the liposomes prepared by ethanol injection method as can be depicted from Table 5.6.

Table 5.6 : Encapsulation efficiency (EE) and retention efficiency (RE) of RPFE loaded liposomal dispersions and their dried form.

	TPC (mg GAE/L)	EE (%)	TAC (mg TE/L)	EE (%)
Lip (0.2%, w/v)	230.86 ± 1.32 ^a	74.2 ± 2.8 ^a	380.11 ± 27.67 ^a	64.9 ± 3.2 ^a
Ch-Lip (0.1%, w/v)	124.98 ± 7.48 ^c	77.8 ± 0.2 ^a	211.08 ± 29.86 ^c	65.1 ± 2.1 ^a
Wp-Lip (0.1%, w/v)	154.61 ± 20.98 ^b	79.1 ± 7.8 ^a	279.96 ± 27.39 ^b	69.5 ± 0.6 ^a
		RE (%)		RE (%)
Ch-Lip powder (0.05%, w/v)	50.04 ± 0.57 ^d	64.7 ± 8.5 ^a	94.36 ± 10.02 ^d	76.0 ± 0.9 ^a
Wp-Lip powder (0.05%, w/v)	65.93 ± 0.78 ^d	62.5 ± 2.8 ^a	86.79 ± 9.76 ^d	65.1 ± 4.8 ^b
RPFE powder (0.05%, w/v)	52.20 ± 0.49 ^d	53.5 ± 1.0 ^b	78.78 ± 5.55 ^d	47.6 ± 2.3 ^c

Abbreviations: RPFE: Rosa pimpinellifolia fruit extract. CH: Chitosan WP: Whey protein. Lip: Liposome. TPC: Total phenolic content. CUPRAC: Cupric ion reducing antioxidant capacity.

5.3.3 Morphology of spray dried particles

The SEM micrographs of spray-dried particles of different formulations were shown in Figure 5.3. Dried particles exhibited spherical structures with dents and wrinkles on particle surfaces. Either rapid evaporation of water within the particles due to high inlet temperature or the slow water diffusion rate due to low inlet temperature cause to particle shrinkage during spray drying (Gandia-Herrero et al., 2010).

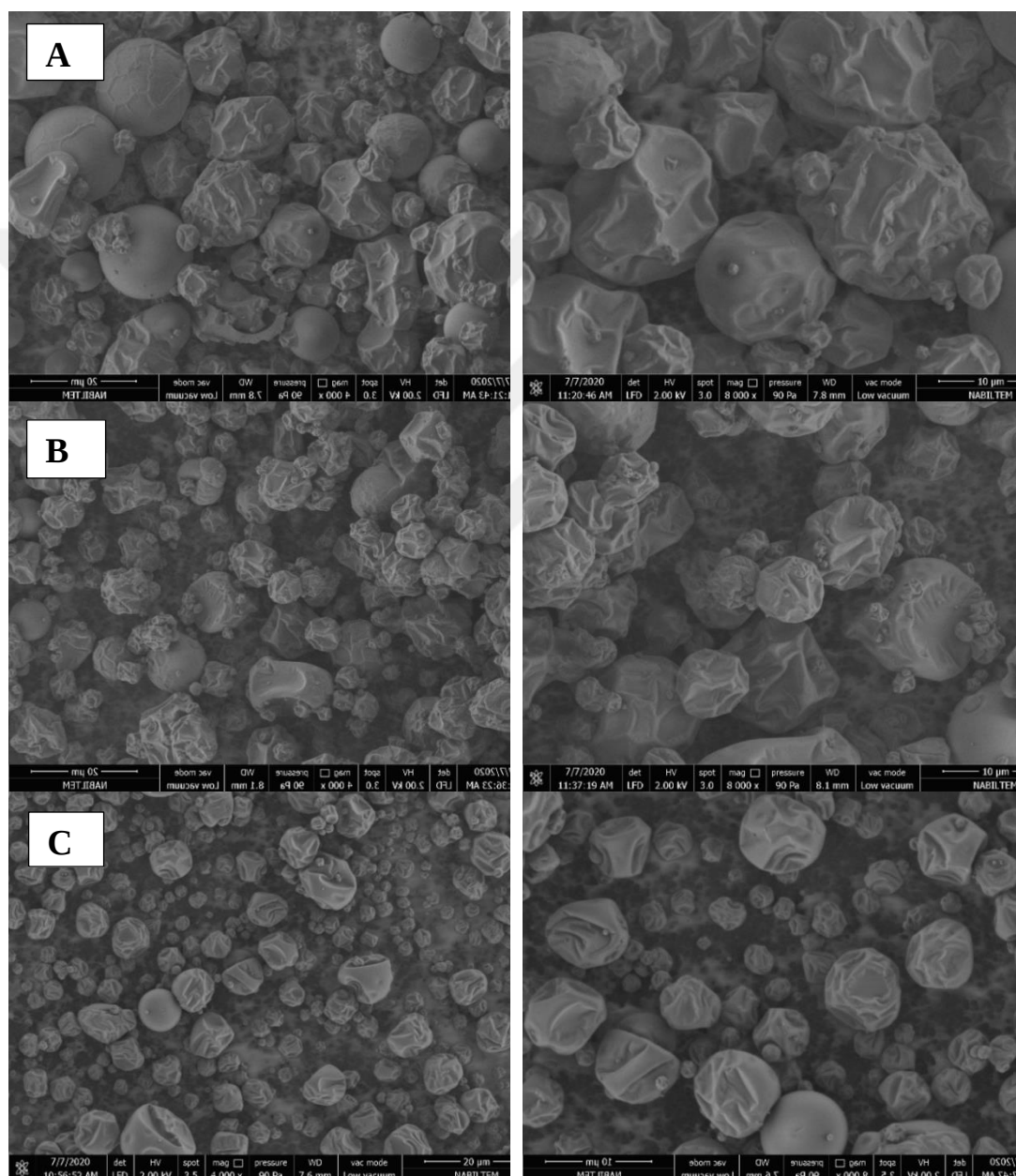


Figure 5.3 : SEM micrographs of loaded chitosan coated liposome powder (A), loaded whey protein coated liposome powder (B) and RPFE powder (C).

Lack of apparent cracks or fissures on the particles in all formulations demonstrates good protection of internal material encapsulated in liposomes. Small particles

attached to the surface of the capsules might indicate incomplete encapsulation of the internal material (Fig. 5.4).

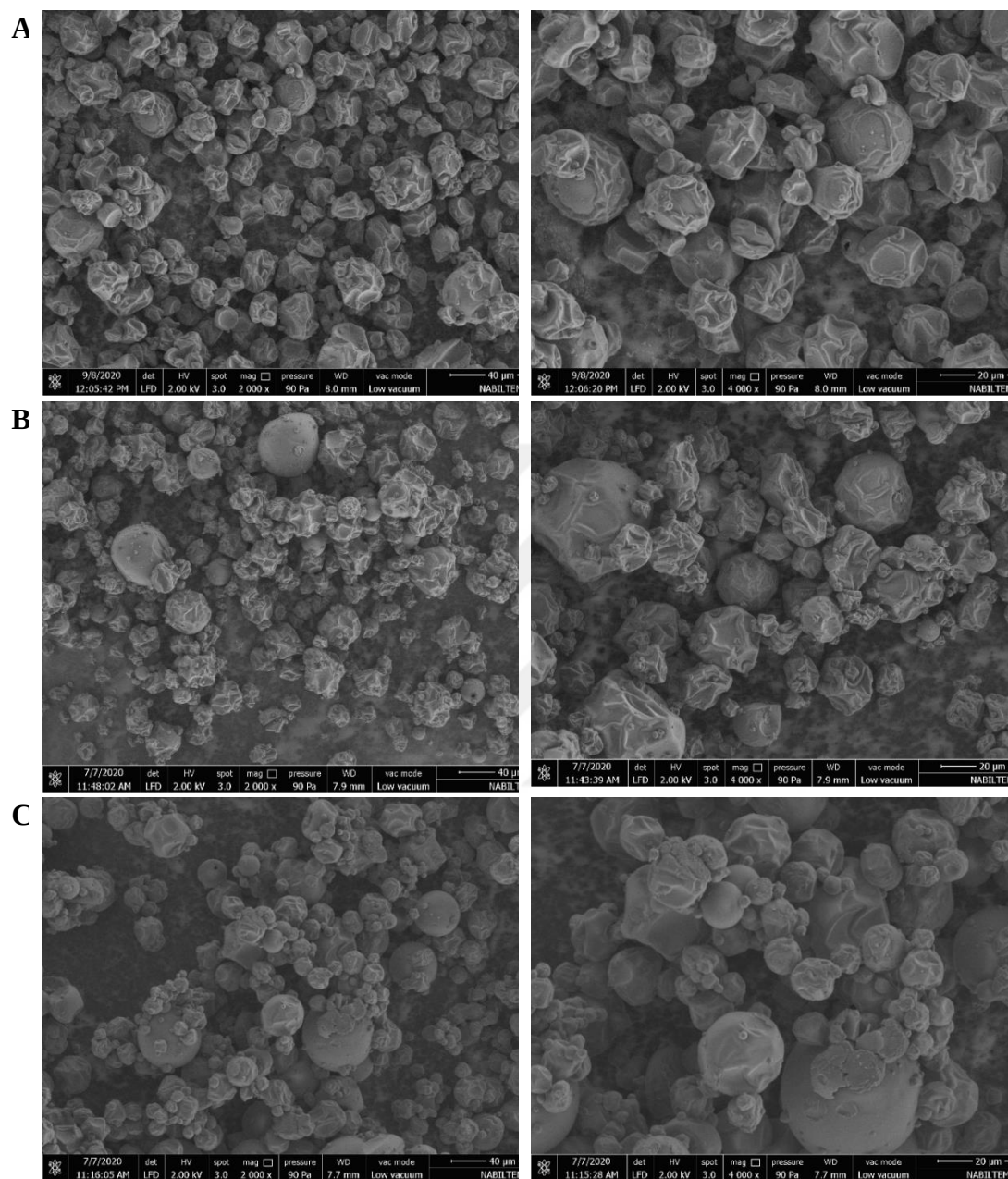


Figure 5.4 : Scanning electron microscopy images of loaded chitosan coated liposome powder (A), loaded whey protein coated liposome powder (B) and BRE powder (C).

5.3.4 Physicochemical properties

5.3.4.1 Effect of emulsifier on thermal behavior of SLN

Upon heating, two endothermic peaks at ~55-56 °C and ~68-70 °C were observed for SLN emulsified by glycyrrhizin regardless of the used surfactant concentration, pH, or

the storage time (Figure 5.5). The first and second peaks of the DSC heating curve correspond to the melting of the α - and β -subcell crystals of bulk lipid tristearin, respectively (Salminen et al., 2014). In the beginning of the storage time, the majority of the detected crystals in the SLN with varying concentrations of glycyrrhizin comprised α -subcell crystals with minor β -subcell crystals present. The β -subcell crystals of tristearin are the most thermodynamically stable polymorph with a high melting point at 73°C (Elisabettini, Desmedt, & Durant, 1996). DSC thermographs revealed that the SLN stabilized by glycyrrhizin melted about 3-5°C lower than the corresponding bulk tristearin as also reported in literature (Heurtault et al., 2003; Salminen et al., 2016), and is a result of their small particle size and supercooling effects. In other words, confinement of tristearin within the small volume of the interior of nanoparticles induces higher disorder of the lipid phase (Sandri et al., 2017). In all SLN samples, an exothermic peak at around ~ 60 -62 °C present represents the polymorphic transition from thermodynamically unstable α - form to stable β - form in agreement with previous works (Oh and others, 2002; Salminen et al., 2013).

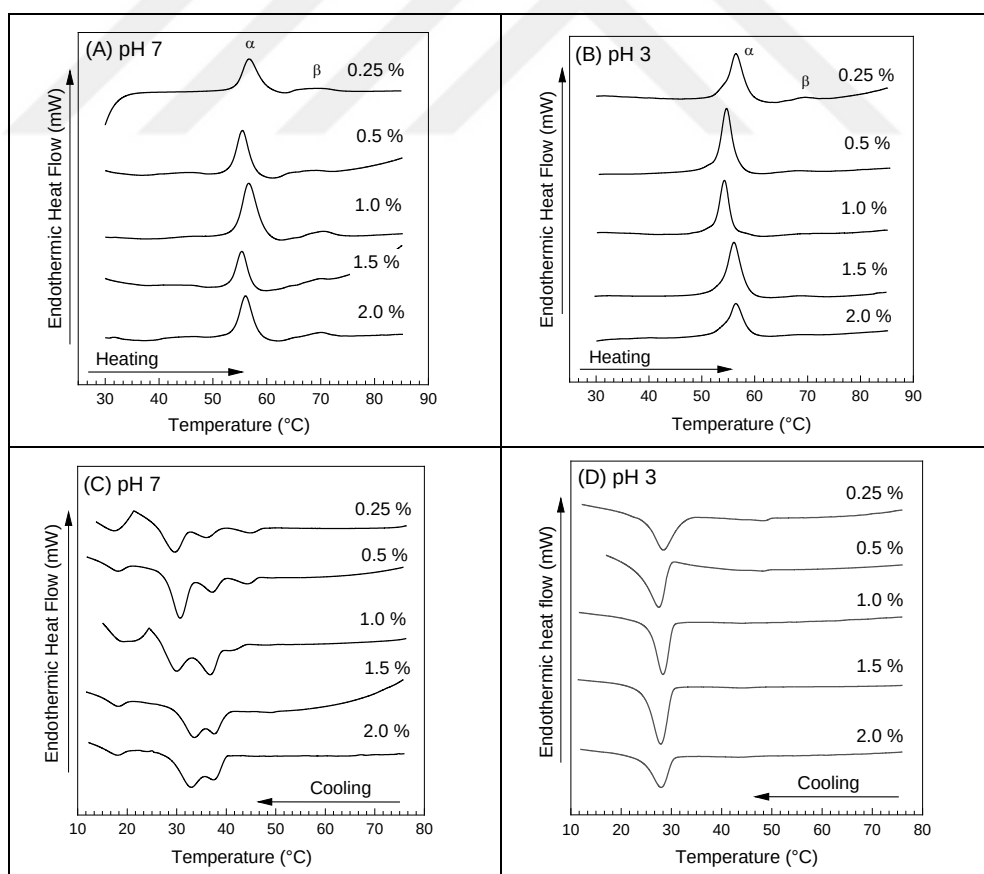


Figure 5.5 : DSC heating (A-B) and cooling (B-C) thermographs of 10% (w/w) tristearin solid lipid nanoparticles emulsified with glycyrrhizin (0.25-2.0% w/w) at pH 7 and 3 stored at 20°C

During polymorphic transition, the shape of the particle alters from spherical to platelet-like resulting in growth of the surface area of the particle. Therefore, lipid droplets crystallize close or slightly below the $T_{conset} = 52\text{ }^{\circ}\text{C}$ of bulk tristearin when they are prone to coalescence (Helgason et al., 2008). Several crystallization peaks observed in the DSC cooling thermographs for SLN samples stabilized with low surfactant concentrations may be attributed to the crystallization of the coalesced and intact emulsion particles, respectively. This illustrates the existence of stable particles in the dispersion although some had coalesced (Helgason et al., 2011).

5.3.4.2 Physical properties of liposomal powders

Physicochemical properties of the fabricated powders are given in Table 5.7. Moisture content is a critical property of powder products as it indicates the residual water in powders. A range of 1–6% is pursued in industry for storage stability of powder (Zhang, Zhang, Chen, & Quek, 2020). In current study, the moisture content of all spray dried samples was below 6 % ($p > 0.05$). Meanwhile, yield of the powders was changed between 60.5% and 72.3% and high solubility which all of them above 90% ($p > 0.05$). Odabaş & Koca (2020) reported similar values for powder yield and solubility that were $52.39 \pm 0.65\%$ and $91.14 \pm 1.79\%$, respectively, for spray dried BRE microcapsules in maltodextrin-gum arabic (3:1). Color measurements indicated entrapment of anthocyanins within biopolymer coated liposomes, resulting lower a^* values than spray dried BRE ($p < 0.05$) (Table 5.7). a^* coordinate is related to the anthocyanin content in BR fruit that is the pigment responsible for the redness of the fabricated powders. Compared to blank formulations, chitosan coating also attenuated whiteness index in liposomal samples ($p > 0.05$) whereas whey protein coating yielded a significant decrease ($p < 0.05$). The resultant spray dried powders had light pink color meanwhile the blank powders were white, which is also demonstrated by the luminosity (L^*) values. Visual appearance of prepared powders is given in Appendix Figure B.5.

Table 5.7 : Characterization of spray dried PH-H₂O extract (BRE) and liposomal powders.

Formulation	Moisture content (%)	Process yield (%)	Solubility (%)	WI (%)	L*	a*	b*	Chroma	Hue angle
Blank Ch-coated liposomal powder	5.0 ± 0.6	72.3 ± 1.2a	94.3 ± 1.2	95.85 ± 0.76ab	96.14 ± 1.34c	2.94 ± 0.11b	0.28 ± 0.18c	2.97 ± 0.10b	5.48 ± 3.59d
Ch-coated liposomal powder	5.2 ± 0.9	60.5 ± 2.7b	95.4 ± 0.3	95.33 ± 0.24b	98.33 ± 0.78ab	-0.49 ± 0.08c	3.76 ± 0.83b	3.79 ± 0.83b	97.47 ± 0.38a
Blank Wp-coated liposomal powder	4.6 ± 1.0	70.9 ± 1.1a	93.9 ± 4.7	97.12 ± 0.04a	96.57 ± 0.16bc	2.33 ± 0.12d	2.14 ± 0.16a	3.17 ± 0.19b	42.21 ± 1.07b
Wp-coated liposomal powder	5.9 ± 0.8	62.0 ± 5.1b	96.3 ± 4.0	92.10 ± 0.59c	99.62 ± 0.04a	-0.30 ± 0.01d	2.84 ± 0.07b	2.86 ± 0.06b	95.87 ± 0.28a
BRE powder	5.2 ± 0.8	61.5 ± 2.4b	98.6 ± 5.3	95.09 ± 1.06 b	95.32 ± 0.52c	5.94 ± 0.58a	2.26 ± 0.34b	6.36 ± 0.66a	20.79 ± 1.07c

Table 5.8 : Characterization of spray dried SCO₂-aqEtOH extract (RPFE) and liposomal powders.

Formulation	Moisture content (%)	Process yield (%)	Solubility (%)	WI (%)	L*	a*	b*	Chroma	Hue angle
Blank Ch-Lip powder	4.8 ± 1.1 ^a	75.2 ± 0.9 ^a	94.5 ± 0.6 ^b	98.00 ± 0.04 ^a	99.43 ± 0.08 ^a	-0.27 ± 0.01 ^d	1.9 ± 0.06 ^{ab}	1.92 ± 0.06 ^d	98.01 ± 0.27 ^a
Ch-Lip powder	5.5 ± 0.9 ^a	74.7 ± 0.7 ^a	97.1 ± 2.1 ^a	96.66 ± 0.15 ^b	97.70 ± 0.23 ^b	1.69 ± 0.01 ^c	1.73 ± 0.03 ^b	2.42 ± 0.02 ^c	45.71 ± 0.54 ^b
Blank Wp-Lip powder	5.2 ± 1.0 ^{ab}	73.2 ± 1.5 ^a	94.5 ± 0.4 ^b	97.92 ± 0.02 ^a	99.67 ± 0.06 ^c	-0.26 ± 0.01 ^d	2.04 ± 0.03 ^a	2.06 ± 0.03 ^d	97.27 ± 0.29 ^a
Wp-Lip powder	4.8 ± 1.7 ^{ab}	73.1 ± 1.4 ^a	94.2 ± 0.9 ^b	94.42 ± 0.13 ^d	96.86 ± 0.06 ^a	4.28 ± 0.15 ^a	1.70 ± 0.16 ^b	4.61 ± 0.20 ^a	21.64 ± 1.04 ^c
RPFE powder	5.1 ± 0.4 ^b	71.5 ± 1.1 ^a	97.4 ± 1.3 ^a	95.52 ± 0.34 ^c	97.03 ± 0.58 ^{bc}	3.31 ± 0.17 ^b	0.38 ± 0.18 ^c	3.33 ± 0.15 ^b	6.71 ± 3.38 ^d

Results expressed as mean SD (n = 3). Ch: Chitosan; Wp: Whey protein; WI: Whiteness Index; L*: Luminosity; a*: Red/Green Value; b*: Blue/Yellow Value. tween samples.

5.3.5 Bioaccessibility

To determine total (poly)phenols and total antioxidant capacity in the bioaccessible fraction of the encapsulated black rosehip extract, they were incubated under simulated gastro-intestinal conditions. (Poly)phenols are considered bioaccessible when they are released from the food matrix during digestion and solubilised in a form that the enterocytes can absorb (Fernández-García et al., 2009). The percentage total (poly)phenols in the bioaccessible fraction of the chitosan-coated (69.3 ± 2.2 %) and whey protein-coated (65.3 ± 9.7 %) liposomal extracts were approximately 2.3-fold higher compared to the spray dried extract (30.3 ± 1.3 %) and 5.6-fold higher compared to the non-encapsulated extract (12.4 ± 1.0 %) (Figure 5.6).

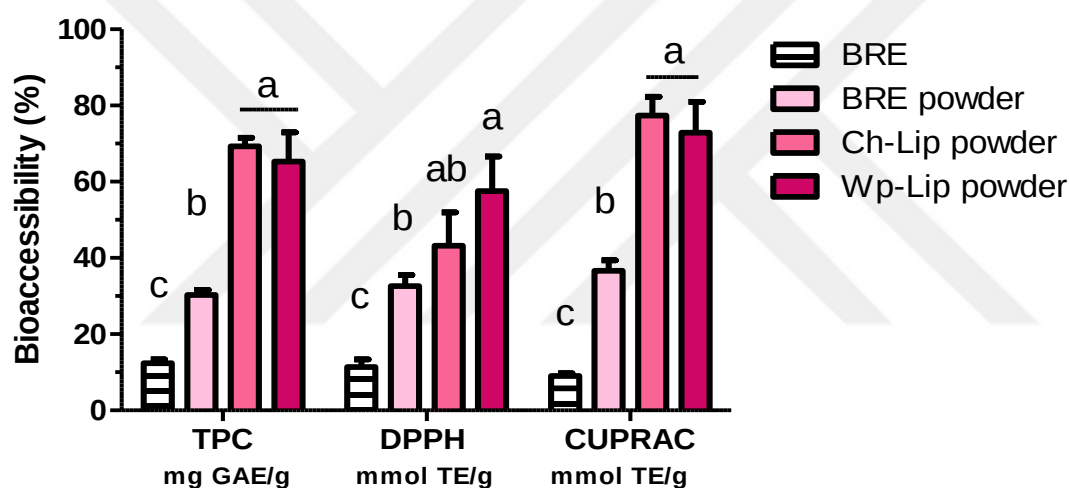


Figure 5.6 : Total (poly)phenolic content (TPC), DPPH radical scavenging activity, and Cupric ion reducing antioxidant capacity (CUPRAC) in the bioaccessible fraction of freeze dried (BRE), spray dried (BRE powder), and chitosan- and whey protein coated liposome powdered (Ch-Lip and Wp-Lip) BRE after *in vitro* gastrointestinal digestion.

Total antioxidant capacity in the bioaccessible fraction followed the same trend as the total (poly)phenols for spray dried BRE, demonstrating approximately 2.9- and 4.1-fold increase in radical scavenging activity and cupric ion reducing antioxidant capacity, respectively. Similarly, chitosan- and whey protein-coated liposomal powders had 3.8- and 8.6-fold higher radical scavenging activity while 5.0- and 8.1-fold higher in cupric ion reducing antioxidant capacity than the non-encapsulated extract after *in vitro* digestion. These results suggest that the encapsulation process and biopolymer coating greatly enhanced the amount phenolics and antioxidant activity in

the bioaccessible fraction ($p < 0.05$). A similar trend has been reported for liposomal encapsulated mulberry waste extract by our research group previously (Gültekin-Özgüven et al., 2016). The application of such delivery systems in dry form yielded better results than in dispersion form (Altin, Gültekin-Özgüven, & Özçelik, 2018b). Also, when liposomal encapsulated cocoa phenolics in a spray dried form were incorporated into an ayran beverage, the bioaccessibility improved about 2.5 folds in comparison to their aqueous dispersion form.

Chitosan coating has been well established to increase in vitro bioaccessibility of (poly)phenols in liposomal delivery systems (Gültekin-Özgüven et al., 2016; Altin et al., 2018a; Sarabandi & Jafari, 2020; Hızır-Kadı et al., 2020). Recent studies have also reported significant improvements in the astaxanthin (Pan, Li, Hou, Chang, Li, & Li, 2022) and curcumin (Gómez-Mascaraque et al., 2017) bioaccessibility after whey protein coating of liposomes. In our study, while both coatings increased the total (poly)phenols and total antioxidant capacity in the bioaccessible fraction, no significant difference was observed between the liposomes coated with chitosan and whey protein ($p > 0.05$). It is noteworthy that this is the first study in which the effect of chitosan and whey protein coating on the bioaccessibility of (poly)phenols was compared. Whey proteins, as a byproduct from cheese or the casein manufacturing process, are well-utilized in the food industry as they are nutritious, widely available, and cheap (Wu et al., 2021). In comparison, chitosan, which is obtained through the deacetylation of chitin from crustacean exoskeletons, is a more costly biopolymer (more than one thousand euros per kilogram at Aldrich) despite being a source from abundant marine wastes (Kadouche et al., 2017). However, in our study, the amount of chitosan used for the liposome coating was 10 times less than the needed amount of whey protein indicating no substantial economical difference between both the biopolymers in the coating process. A similar trend was obtained for encapsulated RPFE using ethanol injection method (Figure 5.7).

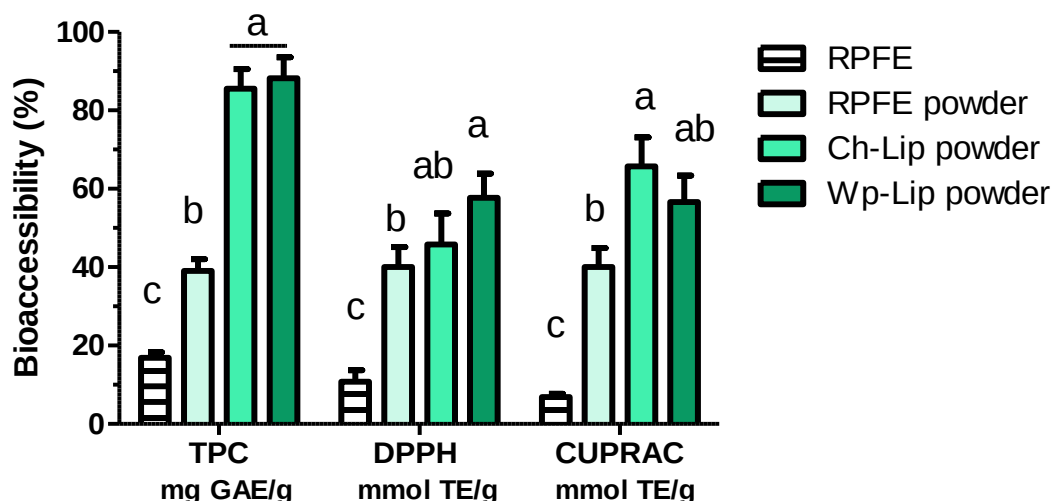


Figure 5.7 : Total (poly)phenolic content (TPC), DPPH radical scavenging activity, and Cupric ion reducing antioxidant capacity (CUPRAC) in the bioaccessible fraction of freeze dried of *Rosa pimpinellifolia* fruit extract (RPFE), spray dried (RPFE powder), and chitosan- and whey protein coated liposome (Ch-Lip and Wp-Lip powder) after *in vitro* gastrointestinal digestion.

5.4 Conclusion

In order to preserve the potentially bioactive compounds during food processing and digestion, BRE-loaded stable dehydrated coated liposomal formulations were fabricated successfully. The *in vitro* bioaccessibility of BRE (poly)phenols was augmented approximately 5.6 folds by loading them into coated liposomes with ensured antioxidant activity ($p < 0.05$). Utilizing the most widely used and readily available natural biopolymers, chitosan and whey protein as coating, liposomes were dried via spray dryer as a feasible approach for the industrial scaled production. In conclusion, the developed edible black rosehip extract formulations provide stability to the contained (poly)phenols and preserve their antioxidant activity during digestion and can thus be used to fortify food products with potentially bioactive compounds. Further investigations will be required to explore the effects on individual (poly)phenols in the encapsulated extract during digestion and storage.

Utilization of natural surfactants which are efficient in ensuring physical stability at low concentrations has great importance in the food and pharmaceutical industries. Plant saponins appear to be highly active at emulsification and stabilization of colloidal particles. However, no previous literature exists on the effect of saponin glycyrrhizin on the stability of SLNs. In this study, for the first time naturally-derived saponin

glycyrrhizin stabilized tristearin SLNs are successfully fabricated. In this study, a series of SLN dispersions were successfully fabricated emulsified with glycyrrhizin and physical stability was determined during 21 days of storage. The SLN formulations containing lower amount of surfactant revealed poor stability against aggregation as clearly observed by optical microscopy. Emulsifier concentration and pH played important roles in shaping the particle size, ζ potential, and polymorphic stability of lipid nanoparticles. Nevertheless, further studies are required to investigate additional parameters for an efficient delivery of bioactives via stable SLN formulations.





6. CACO-2 CELL MONOLAYER PERMEATION AND HEPATIC LIPID ACCUMULATION OF BERRY PHENOLICS IN FREE AND SPRAY DRIED LIPOSOMAL FORM

Encapsulation using pure analytical phenolic compounds that were also performed to represent a mixture of common berry phenolics in order to obtain detectable results from the Caco-2 monolayer studies by HPLC.

6.1 Introduction

Being the most pronounced plant secondary metabolites, phenolic compounds are known to exhibit inhibitory activities against type 2 diabetes in addition to antioxidant activity due to their structural diversity (Lin et al., 2016; Siddiqui et al., 2022). These compounds mostly have poor solubility and bioavailability (Altin et al., 2018). However, their biological functions are significantly dependent on their bioavailability. In this regard, encapsulation applications enable increasing their stability by protecting from stresses mentioned above and/or improve their solubility, bioaccessibility and cellular uptake which is especially desirable for manufacturing of functional foods/beverages and dietary supplements (Grgić et al., 2020).

Studies on modification and dehydration of liposomes ensure the stability of phenolics for nutraceutical delivery purposes; however, information on cellular uptake of phenolics through dried liposomal formulations have not been reported. The absorption through the intestinal epithelial layer is important factor in the design of nutraceuticals or fortified foods (McClements & Xiao, 2014). To the best of our knowledge, the co-encapsulation of dried nanoliposomes for more than two phenolic compounds and enhancement of their bioavailability in comparison to native forms has not been explored. Thus, among the biopolymers extensively used for coating of liposomes, chitosan- and whey protein-coated nanoliposomes powders loaded with phenolic compounds with different polarity degree (catechin, epicatechin, ferulic acid, and resveratrol) were investigated to study the effects of the form of phenolics on their bioavailability and thus bioactivity. The *in vitro* digestion/Caco-2 cell model used to

to assess the absorption and transport of phenolics within different formulations. The bioaccessibility and bioavailability of non-encapsulated phenolics were also investigated in native form. After the digestion, all phenolics in mixed micelles was further evaluated in terms of hepatic lipid accumulation.

6.2 Materials and Methods

6.2.1 Chemicals

Lecithin, 70% phosphatidylcholine from non-GMO soybean (Lipoid P75®) was kindly gifted from Lipoid GmbH (Ludwigshafen, Germany). Medium molecular weight chitosan was donated by Primex (Siglufjörður, Iceland). Whey protein isolate (WP) with 89% protein content was purchased from Isopure Company. Commercial maltodextrin (MD) (DE=18–20) was purchased Tunckaya Kimyevi Maddeler Inc., Turkey. Catechin (purity $\geq 98\%$), epicatechin ($\geq 90\%$), ferulic acid ($\geq 98\%$), resveratrol ($\geq 99\%$), porcine bile extract (B8631), pancreatic lipase (L3126), pancreatin (P3292), pepsin (P7000), Dulbecco's Modified Eagle Medium (DMEM), β -glucuronidase (type H-1 solution; Helix pomatia), and Tween®80 (polysorbate 80) were all from Sigma-Aldrich (Steinheim, Germany). All the reagents were of analytical grade throughout the study.

6.2.2 Preparation of proliposomes

2% (w/v) lecithin and a drop (0.02 g) of Tween-80 were dissolved were stirred in distilled water at room temperature. Phenolic compounds (0.05%, w/v) were dissolved in 1 mL ethanol and added into lecithin solution. Loaded liposomes with phenolics and blank (unloaded) liposomes were prepared by homogenizing solutions followed by microfluidization as described in section 5.2.2.1.

Negatively charged nanoliposomes were coated by positively charged chitosan and whey protein biopolymers as described in section 5.2.4.

6.2.3 Spray drying

The coated nanoliposomal dispersions containing phenolics, their blank formulations and native phenolic compounds (0.0125% w/v, dissolved in 1 mL ethanol) were mixed with pre-hydrated maltodextrin solution in distilled water (20% w/v). Spray drying procedure was performed as described in section 5.2.6.

6.2.4 Characterization of capsule formulations

6.2.4.1 Particle size and ζ -potential

The characteristics of nanoliposomal dispersions and reconstituted spray-dried powders was performed as described in section 5.2.7.2.

6.2.4.2 Encapsulation efficiency and loading capacity

As an indicator for spray-dried particles to hold the core phenolic compounds inside, encapsulation efficiency (%) was measured by comparing the quantities of phenolics before and after spray drying. Loading capacity was calculated as the amount of phenolic compound per gram powder, respectively.

6.2.4.3 Physicochemical properties

Physicochemical properties was analyzed as described in section 5.2.7.4.

Morphology. The particle morphology of spray-dried powders were observed using a scanning electron microscope (SEM; ZEISS EVO LS 10).

Differential scanning calorimetry (DSC). The phase transition of the spray-dried formulations were evaluated by a Q10 model DSC (TA Instruments Inc., USA). A few milligrams of sample were hermetically sealed in aluminum pans and the measurements were conducted in a temperature range between -10 °C and 300 °C at a constant rate of 10 °C/min under nitrogen atmosphere. The glass transition temperature (T_g), as an indicator of stability during storage, was calculated as the midpoint of the curve inflection using the TA-2000 software.

6.2.5 In vitro digestion

The simulated digestion protocol was applied as described in section 4.2.8.

6.2.6 HPLC analysis

The HPLC analysis for the phenolic compounds in undigested and digested samples were conducted using a JASCO HPLC system as described in section 4.2.11.

6.2.7 Cytotoxicity

Cell viabilities in the undigested and digested samples were tested as described in section 4.2.10.

6.2.8 Cellular uptake by Caco-2

Caco-2 uptake of digested micellar phenolics were evaluated as given in section 4.2.11.

6.2.9 Transepithelial permeability

The cells seeded as 10^5 cells per insert on 0.4 μm pore size membrane 12 trans-well plates (Corning COSTAR, USA) and grown for 21 days to obtain a differentiated intestinal cell monolayer. The monolayer integrity was monitored using an epithelial voltohmmeter, EVOM2. The transepithelial electrical resistance (TEER) for each insert was calculated: $\text{TEER} = (R_{\text{Monolayer}} - R_{\text{blank}}) \times 1.12 \text{ cm}^2$. To compare the transport efficiencies of digested fractions, cells were incubated with the diluted digests in HBSS (pH 6.5) for 180 minutes at 37 °C and 5% CO₂. The TEER was measured at different time point intervals (30, 60, 90, 120, and 180). After incubation of 180 min, the cells were detached from the membrane with ethanol and samples taken from the apical chamber were stored at -80 °C. The samples were collected from basolateral chamber, which was immediately refilled with HBSS pH 7.4 + 4% bovine serum albumin (w/v). The integrity of the cell monolayers was also confirmed by quantifying the transport of fluorescein Lucifer yellow from the apical to the basolateral chamber, with a 3% Lucifer yellow transport was accepted. The stability of digested phenolics in diluted in HBSS pH 6.5 for 180 minutes at 37 °C and 5% CO₂ was confirmed by HPLC. As described in section 2.6.3, glucoronidase hydrolysis was employed for all samples from the permeability study, but not for the stability of micellar phenolics in culture medium without cells (Figure C.1).

6.2.10 Rhodamine123 assay

P-glycoprotein efflux (P-gp) activity by measuring the accumulation of the fluorescent substrate rhodamine 123, as described by Harmsen et al. (2013). To determine the appropriate concentrations of micellar formulations that did not show cytotoxic effects, NRU assays were conducted as mentioned earlier (2.6.2). The human colon carcinoma-derived cell line LS18, between passages 46 and 51 were seeded at a density 3×10^5 in a 48 well plate. After 48 h, the cells were washed with HBSS and incubated at 37 °C for 1 h with 10 μM rhodamine 123 in the presence or absence of the P-gp Inhibitor (elacridar). After washing thrice with ice-cold HBSS, the cells were lysed with lysis buffer for 10 min at room temperature while shaking. Then, they were

incubated for 30 min at 37 °C. Fluorescence intensity was read by a plate reader at excitation and emission wavelengths of 485 nm and 529 nm, respectively and was normalized to protein content using BCA protein assay.

6.2.11 ORO assay

Oil-red-O staining (ORO) assay was used for measuring intracellular lipid content in the human hepatocellular carcinoma cell line (HepG2) (Ricci et al., 2009). Prior to ORO experiments, the cell viability of all micellar formulations with and without free fatty acids (FFA) was tested to determine non-cytotoxic working concentrations using MTT assay.

HepG2 cells were subcultured at an initial density of 3×10^5 cells per well in 12-well plates in DMEM high glucose at 37°C in an incubator containing 5% CO₂. After 24 h the high glucose medium was changed to the starvation medium, more precisely to DMEM containing low glucose substituted with 1% BSA. After another 24 h the medium was discarded and treatment solutions were added for 24 h. For FFA, 0.3 mM stock solution was used containing oleic acid (OA) and palmitic acid (PA) in a ratio of 2:1 OA:PA. After the 24 h of incubation, the cells were washed twice with 600 µl cold PBS- and fixed with 300 µl of 4% paraformaldehyde (PFA) per well, shaking with 30 rpm at room temperature for 45 minutes. After fixation, PFA was removed and the cells were washed with 400 µl of 70% EtOH and stained with 350 µl ORO solution. The ORO solution was prepared with 60 vol% ORO (0.5% in isopropanol) and then filtered 3 times with filter paper to remove remaining solid particles. After 15 minutes shaking on a rotation shaker with 30 rpm at room temperature, the ORO solution was removed and the cells were washed carefully with 400 µl of 70% EtOH to remove unbound staining. Plates were dried at 40°C to evaporate the remaining EtOH. To quantify the lipid deposition stained by ORO, 350 µl isopropanol were added to each well. After shaking at 200 rpm at room temperature for 15 minutes, the samples were transferred in a 96-well plate in duplicates. The absorbance was measured at 490 nm.

6.2.12 Statistical analysis

Statistical analysis was done as described in section 3.2.13.

6.3 Results and Discussion

6.3.1 Characterization of particles

Table 6.1 shows the particle size measurements using the dynamic light scattering technique to characterize the uncoated and coated liposomes in liquid dispersion form before and after spray drying. Due to microfluidization process, nano scale vesicle sizes of the liposomes were obtained. The sizes presented are z-averages of the liposome hydrodynamic diameter based on scattering intensity. Loading phenolics into the liposomes resulted in bigger particles ($p < 0.05$). Uncoated loaded liposomes showed a mean diameter of 122.1 ± 1.6 nm. Comparing the coated towards the uncoated samples, and biopolymer coating of the liposomes slightly increased the particle size and the polydispersity index (PDI). PDI is an indicator of the width of the particle size distribution, values of between 0.1 and 0.25 prove a narrow size distribution, while values above 0.5 illustrate a very broad size distribution (Tamjidi et al., 2014). The relatively high PDI measurements for chitosan-coated samples could be the result of the intrinsic factors of chitosan including PDI, the degree of acetylation, and molecular weight (Tsai & Chen, 2017).

Table 6.1 : Particle size (nm), polydispersity index (PDI), and ζ -potential (mV) of nanoliposome dispersions coated with chitosan (0.4 w/w%) and (4.0 w/w%) whey protein, and reconstituted powders spray dried with maltodextrin (20 w/w%).

Sample	PDI	Particle size (nm)	ζ -potential (mV)
<i>Liposome dispersions</i>			
Blank NL	0.210 ± 0.009^d	108.9 ± 0.9^d	-15.8 ± 1.2^d
NL	0.180 ± 0.009^d	122.1 ± 1.6^d	-18.7 ± 0.2^d
Blank CH-NL	0.568 ± 0.007^a	286.4 ± 74.6^{ab}	46.7 ± 2.9^b
CH-NL	0.367 ± 0.063^c	326.7 ± 29.2^a	51.1 ± 0.9^a
Blank WP-NL	0.396 ± 0.072^c	190.7 ± 1.2^{bcd}	12.0 ± 0.5^c
WP-NL	0.222 ± 0.013^d	167.0 ± 2.2^{cd}	10.8 ± 0.6^c
<i>Reconstitution after spray drying</i>			
Blank CH-NL	0.537 ± 0.006^{ab}	282.6 ± 3.4^{ab}	46.5 ± 0.4^b
CH-NL	0.564 ± 0.009^a	361.7 ± 41.0^a	53.1 ± 1.7^a
Blank WP-NL	0.473 ± 0.078^{abc}	276.7 ± 32.7^{ab}	11.9 ± 0.6^c
WP-NL	0.482 ± 0.053^{abc}	324.8 ± 37.3^a	14.0 ± 1.1^c
SD	0.423 ± 0.039^{bc}	275.0 ± 69.8^{abc}	-26.6 ± 2.4^e

Blanks represents non-loaded control samples for each formulations. Abbreviations: Blank NL: Non-loaded blank nanoliposomes, NL: Phenolics loaded nanoliposomes.

The effect of the maltodextrin addition on the ζ potential of the liposomal dispersions was insignificant as it is a neutral polysaccharide ($p > 0.05$). On the contrary, the ζ potential of the anionic liposomal dispersions (-18.7 ± 0.2 mV) drastically increased to 51.1 ± 0.9 mV and 10.8 ± 0.6 mV by coating oppositely charged chitosan and whey

protein, respectively, which proved a successful coating in accordance with the previous literature (Park, Jo, & Jeon, 2014; Tan, Feng, Zhang, Xia, & Xia, 2016).

Liposomes are often comprised of phospholipids containing relatively high amounts of unsaturated fatty acids (Thompson et al., 2006) making them prone to destabilization against harsh process conditions. It is generally accepted that solid-state products are more stable than aqueous solutions or dispersions, as dry powder formulations are more stable and thus more appropriate for long-term storage (Ingvarsson et al., 2011). Therefore, prepared liposomal formulations were dehydrated using a spray dryer. As can be depicted from Table 6.1, the effect of spray-drying process on the ζ potential was nonsignificant after reconstitution ($p>0.05$).

Table 6.2 : The effect of encapsulation method on the loading capacity and encapsulation efficiency of phenolic compounds.

Compound	Loading Capacity (mg/ g powder)			Encapsulation Efficiency (%)		
	SD	CH-NL	WP-NL	SD	CH-NL	WP-NL
Catechin	1.14±0.02 ^a	0.78±0.01 ^b	0.65±0.02 ^c	90.87±1.99 ^a	66.48±1.63 ^b	64.71±1.11 ^b
Epicatechin	1.28±0.05 ^a	0.89±0.02 ^b	0.79±0.01 ^c	95.04±3.01 ^a	76.37±1.14 ^b	79.28±0.90 ^b
Ferulic acid	1.39±0.04 ^a	1.01±0.02 ^b	0.88±0.02 ^c	95.15±3.84 ^a	86.04±1.20 ^b	88.19±1.36 ^b
trans-Resveratrol	1.15±0.04 ^a	0.85±0.03 ^b	0.68±0.02 ^c	91.64±3.25 ^a	72.95±2.41 ^b	67.97±1.60 ^c
cis-Resveratrol	0.06±0.01 ^a	0.03±0.00 ^b	0.02±0.00 ^b	91.70±6.99 ^a	38.51±2.24 ^b	36.43±2.77 ^b

All formulations were dried using spray dryer and maltodextrin used as a carrier. Abbreviations: SD: Spray-dried maltodextrin encapsulated phenolic liposomes. CH-NL: chitosan-coated phenolic nanoliposomes. WP-NL: whey protein-coated phenolic nanoliposomes. Total bulk weights are 40, 40.2, and 50 g for SD, CH-NL, and WP-NL formulations, respectively.

During the production of liposomes, the different handling steps implemented lead to losses of encapsulated core materials resulting in a decrease of the encapsulation efficiency (Figure 6.1). The results for the encapsulation efficiency of liposomes are lower than spray dried phenolics possibly due to the loss in filtration and/or microfluidization steps (Table 6.2). Moreover, the chitosan coated liposomes showed a significantly higher degree of encapsulation efficiency compared with whey protein liposomes possibly due to the total bulk weight of the formulation as the values for encapsulation efficiency was calculated by comparing the quantities of phenolics before and after spray drying. Similarly, loading capacity of samples differ due to different total bulk weights of the prepared formulations. Total bulk weights are 40, 40.2, and 50 g for spray dried phenolics, chitosan coated liposomal phenolics, and whey protein coated liposomal phenolics, respectively.

The dextrose equivalent (DE) of glucose polymers represents the degree of hydrolysis of the maltodextrin. DE also shows the molecular weight and T_g –glass transition

temperature (Morene et al., 2016). However, when different DE maltodextrins were tested, Çam et al. (2014) reported nonsignificant differences on yield and encapsulation efficiency (Çam et al., 2014). In a similar trend, no statistically significant differences were observed among our samples in terms of yield, moisture content, water activity, and solubility (Table 6.3). All fabricated powdered formulations had high water solubility, yield and low moisture contents (as well as a_w) which are in line with existing literature (Vonghirundecha, Chusri, Meunprasertdee, & Kaewmanee, 2022). The moisture content is recognized as an indication for the storage stability because of its effect on glass transition and crystallization behavior (Liao et al., 2021). The moisture contents of all samples were $<6.0\%$. In addition, T_g of spray-dried powders is an indicator of powder stability during storage and storage conditions below its T_g to hinder mobility of molecules has been recommended. Results for all samples are in the same range as those reported for other publications (Pan et al., 2022). The retention percentage of phenolic compounds within different formulations after 6 months storage at $4\text{ }^{\circ}\text{C}$ is given in Table C.1. In addition, the microimages of the prepared formulations by scanning electron microscopy is given in Figure C.3.



Figure 6.1 : Filtration step before microfluidization of liposomes.

Table 6.3 : Physicochemical characterization of powdered samples.

Formulation	Moisture content (%)	Water activity (a_w)	Process yield (%)	Solubility (%)	T_g ($^{\circ}\text{C}$)
SD	5.9 ± 1.3	0.275 ± 0.017	64.0 ± 0.5^d	95.0 ± 1.6	59.5 ± 2.7^a
Blank CH-NL	4.0 ± 0.6	0.211 ± 0.030	70.7 ± 0.2^b	94.0 ± 0.8	48.9 ± 3.3^b
CH-NL	4.9 ± 0.4	0.219 ± 0.016	75.2 ± 0.1^a	95.4 ± 1.7	50.4 ± 1.2^b
Blank WP-NL	5.2 ± 0.5	0.198 ± 0.018	71.5 ± 0.4^b	97.3 ± 3.4	50.6 ± 4.6^b
WP-NL	5.3 ± 0.5	0.235 ± 0.022	75.1 ± 0.4^a	95.2 ± 2.5	53.6 ± 2.9^{ab}

6.3.2 Cytotoxicity

The biocompatibility of the formulations was tested in Caco-2 cells corresponding to the epithelial cells of the human intestinal tract. The tested concentrations are determined according to the amounts used for the purpose of food fortification, 2.5 or 5 % in general (Ni, Sun, Zhao, & Xia, 2015; Rezvani et al., 2019; Šeregelj et al., 2021). In the performed toxicity assay, 50 mg/ml refers to 5 % powder fortification and 50 μ g powder approximately contains 50 μ g individual phenolic compound. Hence, neither blank coated liposomes nor phenolics loaded coated liposomes exhibited any cytotoxic effects at the concentrations that are used in food or beverage fortification (Figure 6.2). The cytotoxicity test results revealed the first evidence that the chitosan/whey protein-coated phenolics loaded powders may not cause harmful effects.

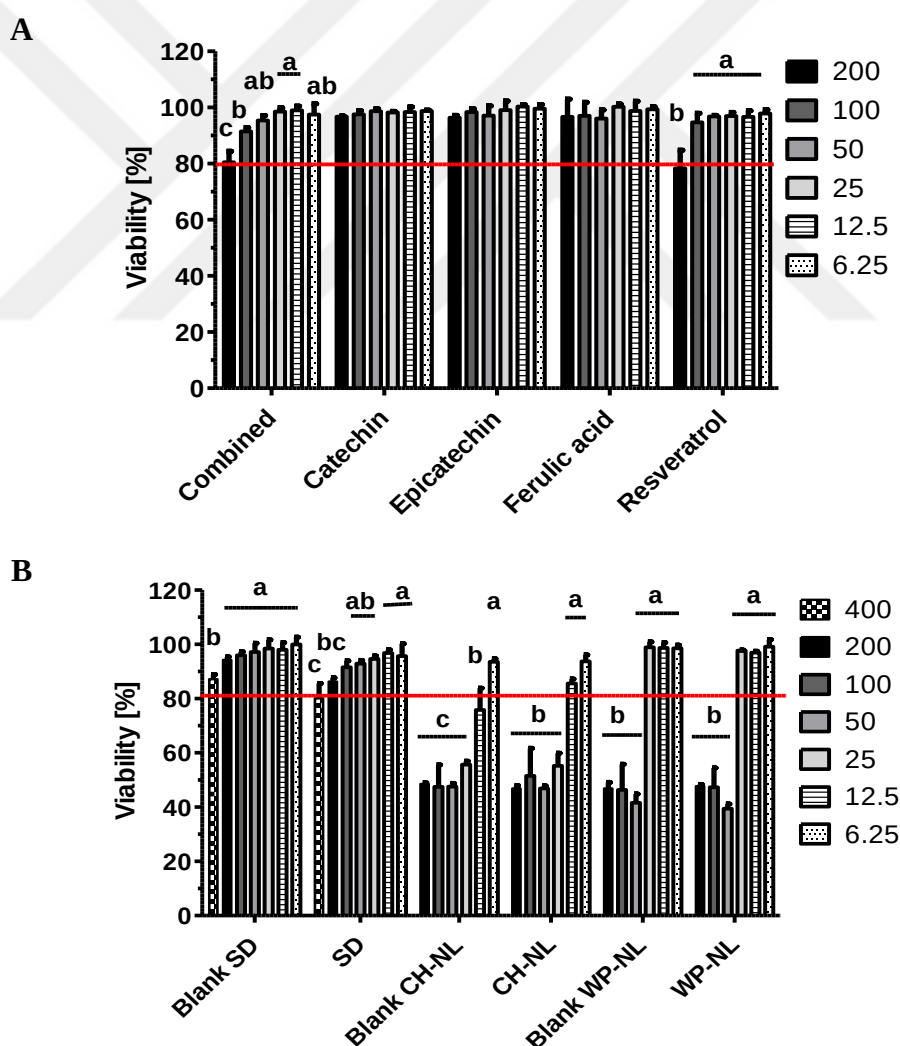


Figure 6.2 : The cytotoxicity (% of control - PBS) of different concentrations of the native phenolic compounds in μ g/mL (A) and their encapsulated forms in mg/mL.

6.3.3 Bioaccessibility

The measurement of phenolics present in the *in vitro* intestinal digesta represents the total amount of phenolics released. The results of the digestive stability, solubility, micellization efficiency and bioaccessibility of phenolics in various forms after *in vitro* gastrointestinal digestion are given in Table 6.4. The digestive stability (%) refers to the amount of compound not degraded during digestion (amount in the whole digest). The aqueous solubility (%) refers to the amount of compound solubilised after digestion (amount in supernatant after centrifuging). The micellization efficiency (%) refers to the amount of compound in the filtered fraction divided by as percentage of the amount of compound in the whole digest. Bioaccessibility (%) refers to the amount of compound incorporated into soluble micelles, smaller than 0.2 μm .

Table 6.4 : The digestive stability (%), solubility (%), micellization efficiency (%) and bioaccessibility (%) of native and encapsulated phenolics *in vitro*.

Formulation	Catechin	Epicatechin	Ferulic acid	trans-Resveratrol	cis-Resveratrol
Digestive Stability ¹ (%)					
Individual native	45.8 $\pm$ 3.7 ^d	49.0 $\pm$ 6.0 ^b	57.9 $\pm$ 7.9 ^c	42.0 $\pm$ 10.2 ^b	143.1 $\pm$ 12.2 ^c
Native	54.0 $\pm$ 3.9 ^c	56.9 $\pm$ 4.5 ^b	69.3 $\pm$ 5.2 ^b	39.0 $\pm$ 8.8 ^b	124.8 $\pm$ 14.5 ^c
SD	79.6 $\pm$ 2.0 ^b	78.7 $\pm$ 4.4 ^a	94.2 $\pm$ 4.0 ^a	84.5 $\pm$ 5.4 ^a	159.6 $\pm$ 29.4 ^{bc}
CH-NL	88.1 $\pm$ 4.2 ^a	87.9 $\pm$ 8.7 ^a	95.9 $\pm$ 2.8 ^a	89.7 $\pm$ 1.9 ^a	230.0 $\pm$ 86.5 ^a
WP-NL	81.7 $\pm$ 2.1 ^b	77.5 $\pm$ 4.1 ^a	93.9 $\pm$ 2.1 ^a	90.9 $\pm$ 5.2 ^a	259.1 $\pm$ 13.3 ^a
Solubility ² (%)					
Individual native	41.7 $\pm$ 3.8 ^d	46.6 $\pm$ 5.3 ^b	51.0 $\pm$ 7.4 ^b	37.6 $\pm$ 5.6 ^c	76.6 $\pm$ 4.2 ^c
Native	49.1 $\pm$ 2.0 ^c	50.7 $\pm$ 6.5 ^b	57.8 $\pm$ 10.7 ^b	36.9 $\pm$ 6.2 ^c	64.8 $\pm$ 11.9 ^c
SD	77.0 $\pm$ 0.6 ^a	75.0 $\pm$ 1.6 ^a	90.4 $\pm$ 2.0 ^a	80.8 $\pm$ 4.4 ^b	144.6 $\pm$ 38.9 ^b
CH-NL	71.1 $\pm$ 6.1 ^b	49.8 $\pm$ 3.2 ^b	92.3 $\pm$ 2.2 ^a	13.7 $\pm$ 1.9 ^d	70.5 $\pm$ 19.1 ^c
WP-NL	78.8 $\pm$ 1.5 ^a	74.3 $\pm$ 1.3 ^a	89.2 $\pm$ 1.5 ^a	89.7 $\pm$ 6.0 ^a	250.1 $\pm$ 11.9 ^a
Micellization Efficiency ³ (%)					
Individual native	86.1 $\pm$ 4.0 ^{ab}	87.4 $\pm$ 7.1 ^a	78.5 $\pm$ 13.6 ^{ab}	69.6 $\pm$ 9.1 ^b	38.7 $\pm$ 12.2 ^b
Native	74.9 $\pm$ 10.6 ^b	72.5 $\pm$ 9.5 ^b	66.9 $\pm$ 12.9 ^b	80.8 $\pm$ 8.4 ^{ab}	45.3 $\pm$ 5.7 ^b
SD	91.2 $\pm$ 8.5 ^a	88.7 $\pm$ 7.0 ^a	88.3 $\pm$ 9.7 ^a	81.6 $\pm$ 11.9 ^{ab}	86.5 $\pm$ 8.0 ^a
CH-NL	72.8 $\pm$ 7.9 ^b	55.8 $\pm$ 8.6 ^c	90.9 $\pm$ 5.5 ^a	16.2 $\pm$ 5.9 ^c	21.6 $\pm$ 1.8 ^c
WP-NL	91.4 $\pm$ 3.3 ^a	91.6 $\pm$ 4.7 ^a	88.5 $\pm$ 1.9 ^a	85.3 $\pm$ 5.5 ^a	87.4 $\pm$ 10.0 ^a
Bioaccessibility ⁴ (%)					
Individual native	41.0 $\pm$ 1.4 ^b	42.6 $\pm$ 3.2 ^b	45.2 $\pm$ 8.3 ^b	31.5 $\pm$ 3.0 ^c	54.2 $\pm$ 12.8 ^c
Native	40.2 $\pm$ 4.1 ^b	42.4 $\pm$ 9.4 ^b	45.1 $\pm$ 7.3 ^b	32.8 $\pm$ 4.9 ^c	58.0 $\pm$ 7.6 ^c
SD	72.4 $\pm$ 5.7 ^a	69.6 $\pm$ 3.6 ^a	82.9 $\pm$ 6.7 ^a	68.4 $\pm$ 6.2 ^b	138.7 $\pm$ 32.2 ^b
CH-NL	69.5 $\pm$ 4.2 ^a	48.4 $\pm$ 3.1 ^b	89.4 $\pm$ 2.5 ^a	15.2 $\pm$ 4.4 ^d	74.8 $\pm$ 13.8 ^c
WP-NL	74.7 $\pm$ 2.8 ^a	70.8 $\pm$ 2.0 ^a	83.7 $\pm$ 2.1 ^a	87.8 $\pm$ 3.8 ^a	243.0 $\pm$ 27.9 ^a

Abbreviations: Individual native; individual phenolic compounds in free native form. Combined Native; a mix of all phenolic compounds in free native form. CH-NL; chitosan-coated phenolic nanoliposomes spray-dried with maltodextrin. WP-NL; whey protein-coated phenolic nanoliposomes spray-dried with maltodextrin. SD. Spray-dried maltodextrin encapsulated phenolics. ¹ Defined as the amount in digesta divided by as percentage of the amount in the initial raw material. ² Defined as the amount soluble in aqueous fraction divided by as percentage of the amount in the initial raw material. ³ Defined as the amount in the filtered fraction divided by as percentage of the amount in the digesta. ⁴ Defined as the amount in the filtered fraction divided by as percentage of the amount in the initial raw material.

The phenolics in all samples decreased upon simulated gastrointestinal digestion, except cis-Resveratrol as can be depicted from Table 6.4. This is possibly due to trans-to-cis isomerization that occurs during *in vitro* digestion which also resulted very high bioaccessibility which is in line with previous findings by de Moraes and co-workers (2020) who studied the changes in the phenolic compounds from edible flowers through a simulated intestinal barrier (de Moraes et al., 2020). The high bioaccessibility of the form cis- compared to trans-resveratrol was interpreted by the authors through the isomerization of trans-resveratrol in digestive conditions (Koga, Andrade, Ferruzzi, & Lee, 2016).

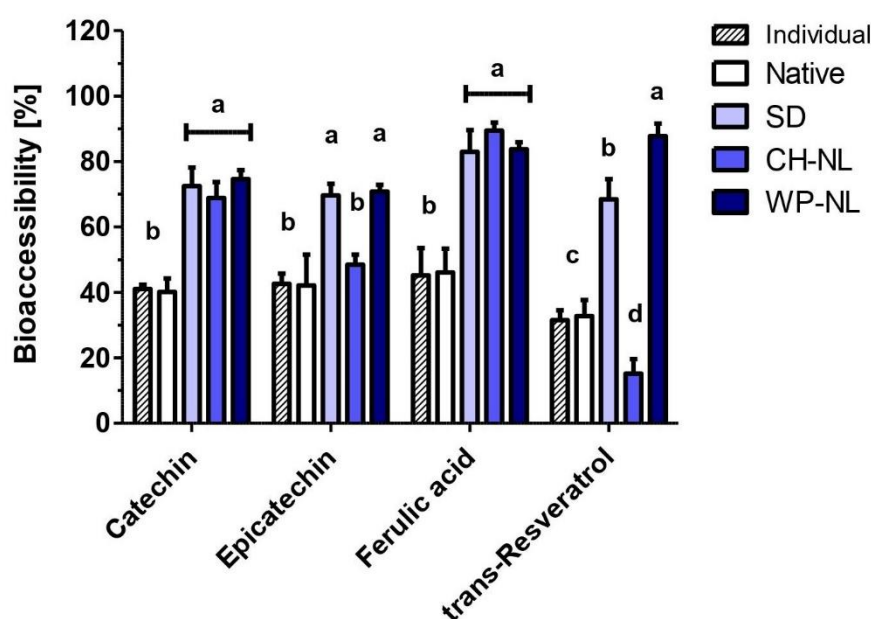


Figure 6.3 : The bioaccessibility of micellar phenolics in different formulations. Native represents phenolic compounds in free form. Values represent means $\pm$ SD of n=6.

The digestive stability, solubility, micellization efficiency, and bioaccessibility are significantly higher in encapsulated samples ($p < 0.05$) (Figure 6.3). No significant difference was observed between individual and in combination of phenolics in native form by means of bioaccessibility ($p > 0.05$). Bioaccessibility, solubility, micellization efficiency of trans-Resveratrol were low in CH-NL although digestive stability was high ($p < 0.05$). Because the amount of trans-Resveratrol greatly reduced after centrifugation step, precipitation observed. This implies a possible interaction between chitosan and trans-Resveratrol. Most of the phenolic compounds being released from chitosan coated liposome at gastric conditions because of the higher solubility of chitosan at acidic pH. The release behavior of chitosan encapsulation has been shown

to be pH dependent that is a 9 times higher release of phenolic compounds was obtained at pH 3 than pH 7 (da Silva et al., 2022). The centrifugation force has been demonstrated to have a significant effect on the amount of encapsulated (poly)phenols, as insoluble non-encapsulated phenols are centrifuged away with the waxes debris force or time (Šturm et al., 2019). Previous papers reported chitosan can significantly decrease the bioaccessibility of vitamin D and β - carotene which was attributed to the binding of mixed micelles causing sedimentation (Tan et al., 2020; Zhang et al., 2015).

6.3.4 Caco-2 cell uptake

Caco-2 cells are preferred for *in vitro* uptake studies because of their similarity to intestinal enterocytes (Sambuy et al., 2005). The results of the experiments were calculated in two ways. Firstly, as cellular uptake as percentage of the amount in the cells and remaining phenolic compounds stable after the 3 h incubation period (Cell lysate + Supernatant, C+S) and secondly as percentage of the amount of the bioaccessible phenolics added initially (Raw material, RM), displayed in Figure 6.4. Oxidative degradation may occur during incubation period as cells were incubated in the presence oxygen. Hence, taking this stability issue into consideration, as expected, higher values for absorption of phenolics were found when results were calculated as percentage of the C+S.

The cellular uptake of bioaccessible catechin, epicatechin and ferulic acid were poor from the bioaccessible fraction with no statistical significance between native individual or in combination ($p>0.05$). This indicates that combination of the studied native phenolic compounds did not play a major role on the absorption in comparison to their individual form ($p>0.05$) which implies no synergism or antagonism. On the other hand, a good level of the uptake of the bioaccessible resveratrol (Figure 6.4). Moreover, whey protein coated liposomal encapsulated formulation resulted substantial increase in the cellular uptake of trans-Resveratrol in comparison to other encapsulated and native forms ($p<0.05$). Besides, great uptake of cis-Resveratrol could be attributed to trans-cis isomerization occurred during incubation period.

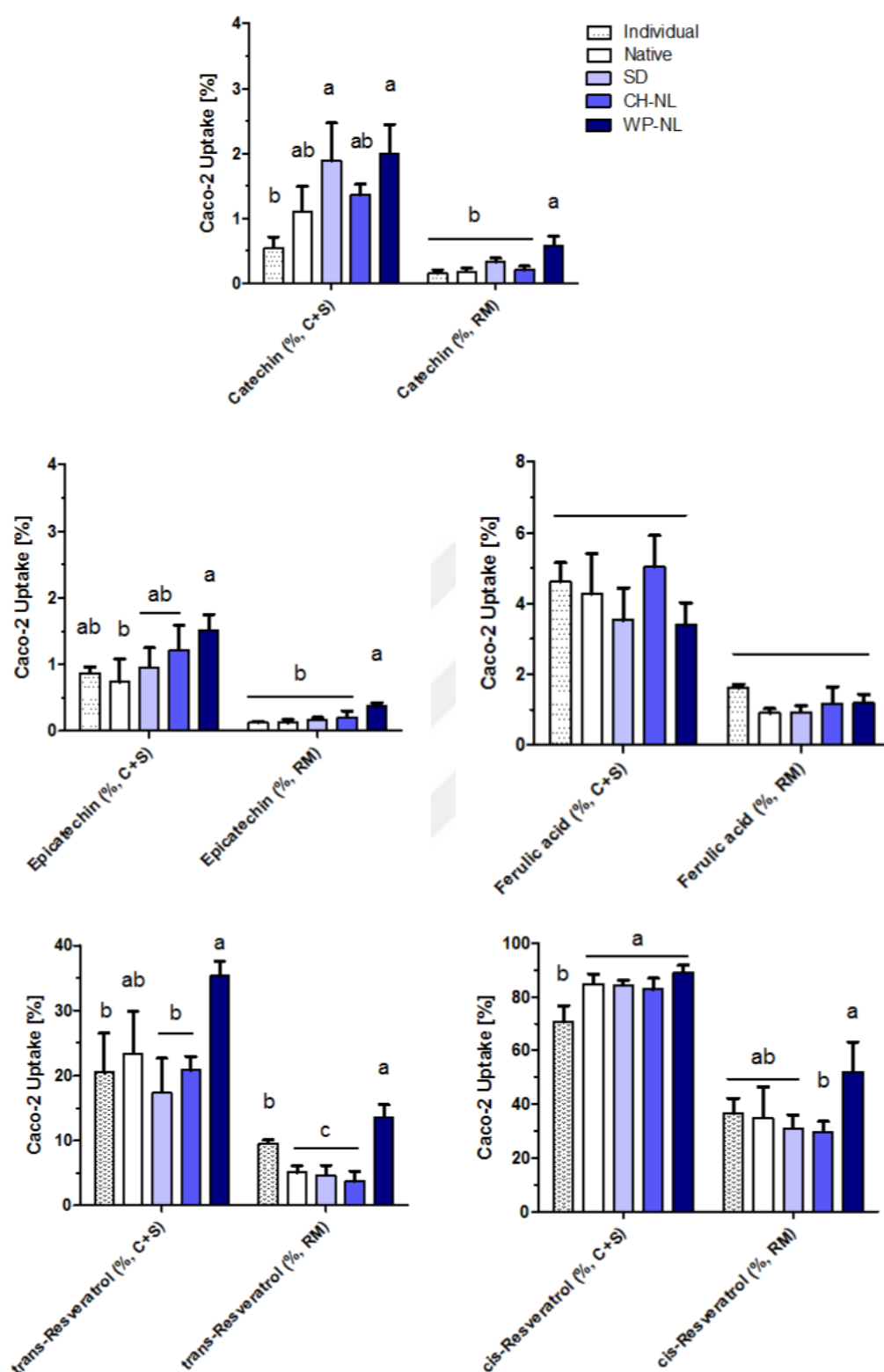


Figure 6.4 : The Caco-2 cellular uptake of micellar phenolics in different formulations as percentage of cell lysate + supernatant (C+S) and raw material (RM).

6.3.5 Caco-2 monolayer permeability

Table 6.5 shows the transport of the native and encapsulated micellar phenolics through the Caco-2 cell monolayers. In particular, Figure 6.5 shows the recoveries of

phenolics transported from the apical to the cells (B) and basolateral side (C) across Caco-2 cell monolayers. The transport of phenolics encapsulated into nanoliposomes was compared with the native (non-encapsulated) compounds following their *in vitro* digestion. The cytotoxicity of the prepared formulations on Caco-2 cells was assessed using NRU assay. More than 85% cells were viable after treating with 1:8 dilution of micellar phenolics. Based on the cell viability data, the concentrations for transport assay was determined.

Table 6.5 : Trans-epithelial transport of the micellar phenolics by Caco-2 cells.

Formulation	Catechin	Epicatechin	Ferulic acid	trans-Resveratrol	cis-Resveratrol
Cell monolayer absorption ¹ (ng/mg protein)					
Native	799.0 ± 297.0 ^a	580.9 ± 236.6 ^b	4509.0 ± 1102.2 ^{bc}	8724.1 ± 2004.7 ^a	631.4 ± 228.4 ^{ab}
SD	316.7 ± 99.5 ^b	318.1 ± 37.7 ^{ab}	5735.4 ± 1098.5 ^a	8190.5 ± 2343.5 ^a	728.4 ± 279.7 ^a
CH-NL	422.5 ± 107.4 ^b	297.9 ± 68.6 ^b	3399.3 ± 764.1 ^{bc}	2744.5 ± 357.4 ^b	224.2 ± 69.9 ^c
WP-NL	388.5 ± 114.9 ^b	327.6 ± 95.4 ^{ab}	2336.8 ± 604.2 ^c	7799.9 ± 2256.3 ^a	554.5 ± 174.8 ^{ab}
Basolateral chamber ² (ng/mL)					
Native	4.3 ± 1.3	4.7 ± 1.5	275.3 ± 76.3 ^b	101.9 ± 57.9 ^{bc}	3.2 ± 1.1
SD	4.5 ± 2.4	5.2 ± 2.7	512.6 ± 196.8 ^{ab}	231.4 ± 73.3 ^a	9.2 ± 6.3
CH-NL	6.6 ± 3.3	8.4 ± 3.8	652.6 ± 97.9 ^a	46.7 ± 9.8 ^c	3.1 ± 1.1
WP-NL	6.4 ± 3.3	6.1 ± 2.0	312.3 ± 58.0 ^b	175.3 ± 68.7 ^{ab}	4.3 ± 1.5
Overall absorption ³ (%)					
Native	0.30 ± 0.16 ^{ab}	0.28 ± 0.11 ^{bc}	1.16 ± 0.21 ^b	5.11 ± 2.14 ^{ab}	39.23 ± 19.30
SD	0.16 ± 0.07 ^b	0.22 ± 0.10 ^c	1.59 ± 0.28 ^{ab}	2.89 ± 0.25 ^b	27.94 ± 7.23
CH-NL	0.41 ± 0.07 ^a	0.58 ± 0.09 ^a	1.81 ± 0.16 ^a	6.51 ± 1.78 ^a	40.91 ± 7.54
WP-NL	0.38 ± 0.11 ^{ab}	0.45 ± 0.05 ^{ab}	1.61 ± 0.17 ^a	5.27 ± 1.31 ^a	35.28 ± 9.40

Abbreviations: Individual native; individual phenolic compounds in free native form. Native; all phenolic compounds in free native form. CH-NL; chitosan-coated phenolic nanoliposomes spray-dried with maltodextrin. WP-NL; whey protein-coated phenolic nanoliposomes spray-dried with maltodextrin. SD. Spray-dried maltodextrin encapsulated phenolics. ¹ Defined as the amount of micellar phenolics taken up by Caco-2 cell transport model. ² Defined as the amount of micellar phenolics permeated into basolateral chamber in Caco-2 cell transport model. ³ Defined as the overall absorbed amount of micellar phenolics that is sum of uptaken and permeated phenolics divided by the amount added to apical chamber initially.

The overall absorption of phenolics was defined as the sum of the uptake plus the secretion of compounds into the basolateral side (Table 6.5). The lowest overall absorption was observed in spray dried phenolics among other vehicles. Nanoliposomal encapsulation affected considerably the ratios of epicatechin and ferulic acid absorbed or permeated by the cells ($p < 0.05$). No difference was observed in apical recovery of compounds by means of encapsulation ($p > 0.05$). Very high apical recovery ratio of cis-resveratrol implies trans-to-cis isomerization. In the case of cell monolayer recovery, encapsulation yielded nonsignificant results in comparison to

native phenolics, except for trans-resveratrol (Figure 6.5B). The basolateral recovery of catechin and epicatechin was very low (Figure 6.5C). Gómez-Juaristi and co-workers (2020) have found epicatechin recoveries of 96 % and 1.5 % for apical and basolateral compartments, respectively. However, they reported no intracellular accumulation in the Caco-2 cell monolayer after 4 h of incubation period (Gómez-Juaristi et al., 2020). A theoretically possible explanation for the observed uptake rates would be the differences in degradation of phenolics through native or encapsulated forms in cell culture medium.

The apparent permeability coefficients (Papp) of native and encapsulated phenolics through the Caco-2 monolayer are given in Figure 6.6. In the present study, the low Papp values of catechin and epicatechin ($< 2 \times 10^{-7}$) indicated its limited transepithelial absorption. However, the transport of epicatechin and ferulic acid loaded in chitosan coated liposome was significantly ($p < 0.05$) higher than the native (control) as indicated by higher Papp value of the former. The results suggested that the chitosan coated liposome formulation was suitable for enhancing the permeability of these compounds. A medium range of *in vivo* absorption is predicted for ferulic acid and trans-resveratrol according to Papp values. On the other hand, the high Papp for cis-resveratrol is probably due to conversion of trans-resveratrol to cis-resveratrol that has also been indicated while studying the metabolism of grape trans-resveratrol on Caco-2 cells (Toaldo et al., 2015). Trans-resveratrol was observed as effective as the cis isomer on Caco-2 cell proliferation inhibition (Storniolo & Moreno, 2018).

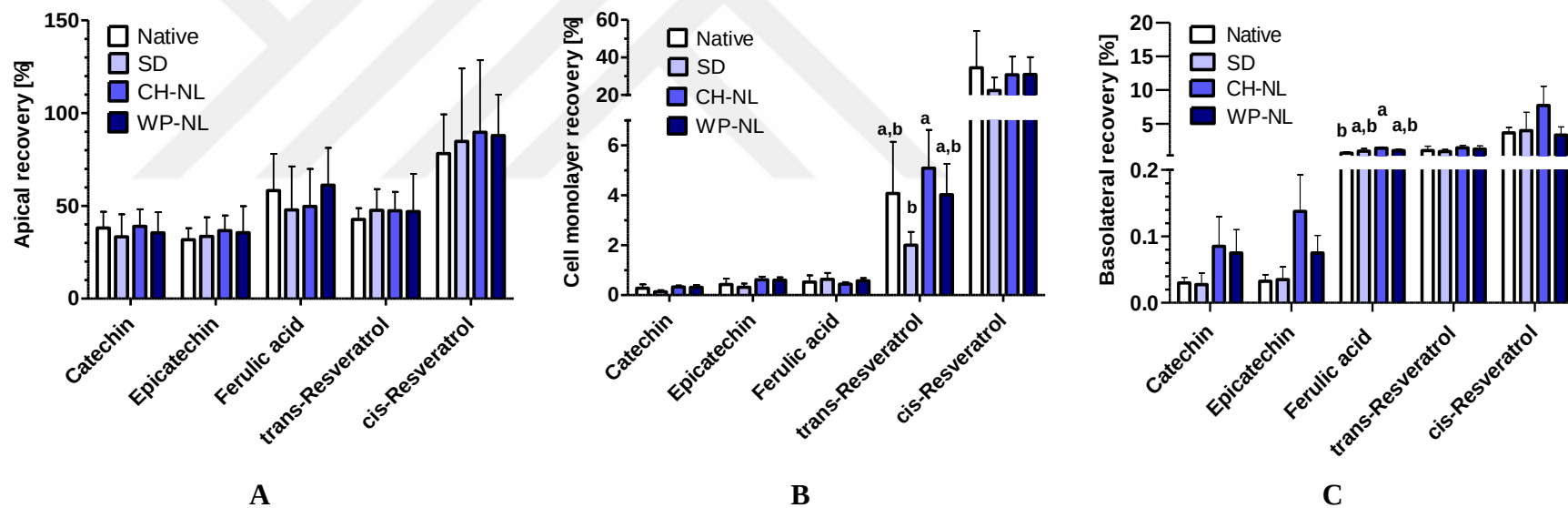


Figure 6.5 : Recoveries of micellar phenolic compounds during epithelial transport as percentage of initial concentrations in (A) apical chamber, (B) cell monolayer, and (C) basolateral chamber after 180 min incubation in 37 °C.

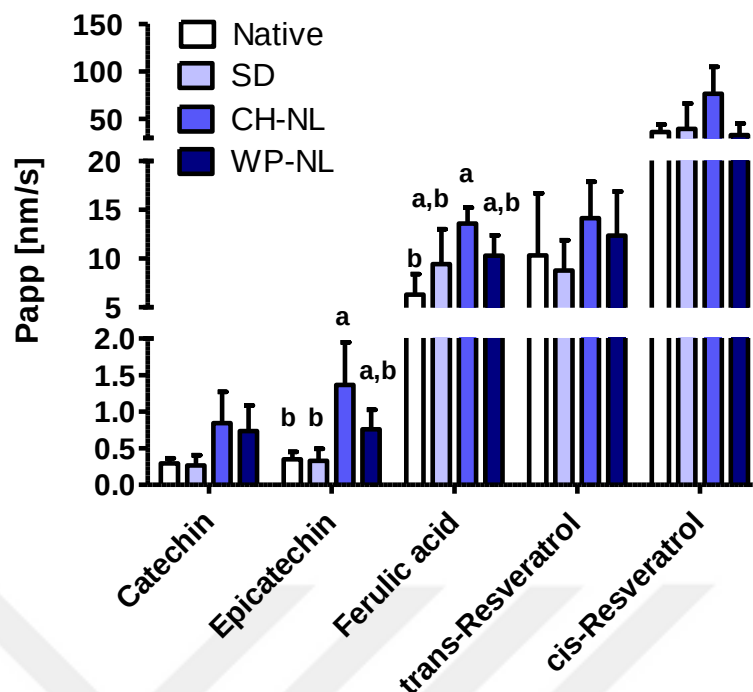


Figure 6.6 : Apparent permeability coefficients (P_{app} , 10^{-7} nm/s) of micellar phenolics in the Caco-2 cell transport model.

P_{app} values are correlated with the recovery values obtained for the cell and basolateral side (Figure 6.5). No significant difference was exhibited in terms of encapsulation or trans-resveratrol. These results could imply the role of encapsulation was mainly to improve the solubility, stability and hence bioaccessibility of the tested phenolic compounds rather than their bioavailability according to the experimental setting used in study. Further metabolism of phenolic compounds in the body include methylation, glucuronidation, and sulfation. The enzymes responsible for (poly)phenol glucuronidation in the small intestine are known as uridine diphosphate glucuronosyltransferases -UGT (Corona et al., 2014). In ideal conditions, to quantify all types (aglycone, sulfate, glucuronide, methyl or even double conjugations) of parent compound and its metabolites present in plasma will be the best approach. However, this could easily end up by monitoring more than 50 compounds and there are no currently proper standards (Guy et al., 2009). In this study, β -glucuronidase treatment was applied to achieve hydrolysis of glucuronide conjugates of phenolic compounds to be able to include them in the measurements.

Intracellular homeostasis of xenobiotic substances such as phenolic compounds are known to be controlled by their influx and efflux. P-glycoprotein (P-gp) is among the most investigated efflux transporters in the intestine (Domínguez-Avila et al., 2017).

It transports amphipathic, hydrophobic, and cationic molecules in the basal-to-apical and blood-to-lumen directions (Estudante et al., 2013). Orally ingested phenolic compounds can be internalized by enterocytes may reach circulation, be effluxed back into the lumen by P-glycoprotein (P-gp) or be metabolized by phase I and phase II enzymes (Domínguez-Avila et al., 2017). The efflux transporter P-gp activity and/or P-gp expression in cells are important in terms of pharmacokinetic parameters of many drugs as modulation of their bioavailability is possible by means of P-gp inhibition (Nguyen, Duong, & Maeng, 2021). Thus, in this chapter, the effects of native and nanoliposomal phenolics on P-gp activity was also evaluated in LS180 human colon epithelial cells. The fluorescent P-gp substrate rhodamine123 was measured after 3 h incubation by means of intracellular accumulation in the presence of phenolics as individual native, in combination, and encapsulated forms as well as P-gp inducer Rifampicin 25 (Figure 6.7).

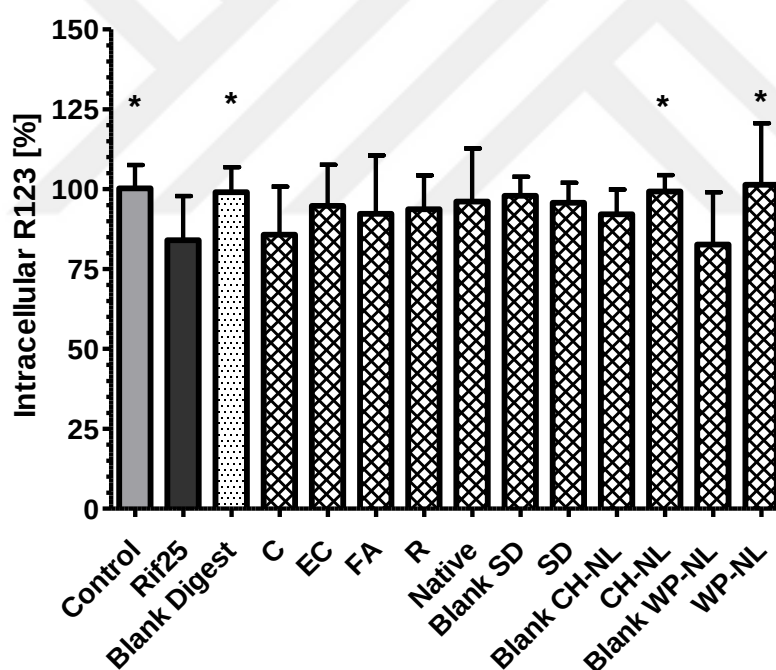
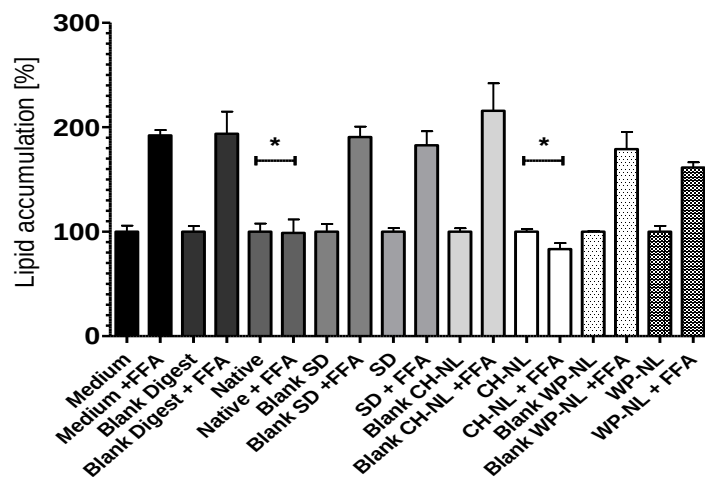


Figure 6.7 : Effects of micellar phenolics in different forms on intracellular accumulation of Rhodamine 123 in LS 180 cells. * indicates significance according to Tukey's test ($p \leq 0.01$) ($n=6$). C: Catechin, EC: Epicatechin, FA: Ferulic acid, R: trans-Resveratrol.

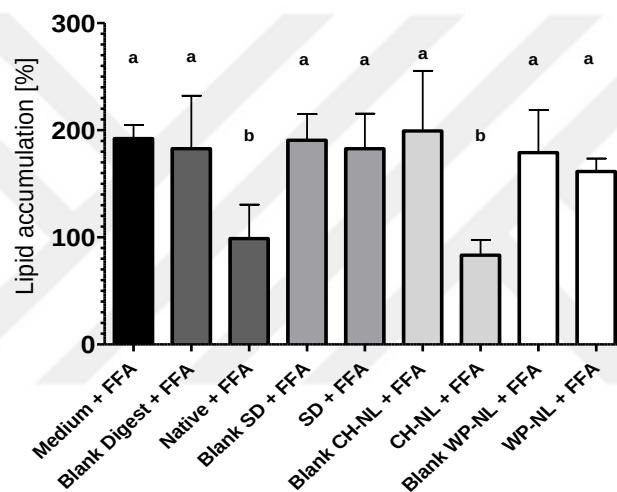
6.3.6 Lipid accumulation in HepG2 cells

The prevalence of type 2 diabetes is expanding worldwide along with obesity. Reducing lipid accumulation in insulin target tissues is an important anti-diabetic approach (Zeng et al., 2012). In this respect, HepG2, a human hepatic cancer cell line was used as a well-established *in vitro* model for lipid metabolism studies. Cell viability analysis was done prior to the lipid accumulation using MTT assay for 24 h in order to detect non-toxic concentrations of the studied samples together with free fatty acids (data not shown). Lipid accumulation after incubation with different micellar phenolic formulations is presented in Figure 6.8. According to the results, the added micellized phenolics (catechin, epicatechin, ferulic acid, and resveratrol) suppressed oleic and palmitic acid induced hepatic steatosis both in native and chitosan coated liposomal form (Figure 6.8A). The reason why whey protein coated liposomal form did not alleviate lipid accumulation could be attributed to the lower content of phenolics due to the dilution applied in the experiment (2.5 fold more dilution was used in WP-NL). The loss of biological activity during spray drying may occur as a result of the increased temperatures during the process, and/or the shear stress during atomization in the nozzle (Schutyser et al., 2019). The inlet/outlet temperature of the spray drying did not seem to affect when maltodextrin used in spray drying encapsulation of olive mill wastewater phenolic extract (Kreatsouli et al., 2019). However, the extract spray dried at 100 °C (57 °C outlet temperature) yielded reducing power of 689 ± 55 µg/mL and 2 fold higher protective activity against ROO-induced DNA damage than the extract spray dried at 160 °C (86 °C outlet temperature) resulted in non-detectable reducing power.

A



B



C

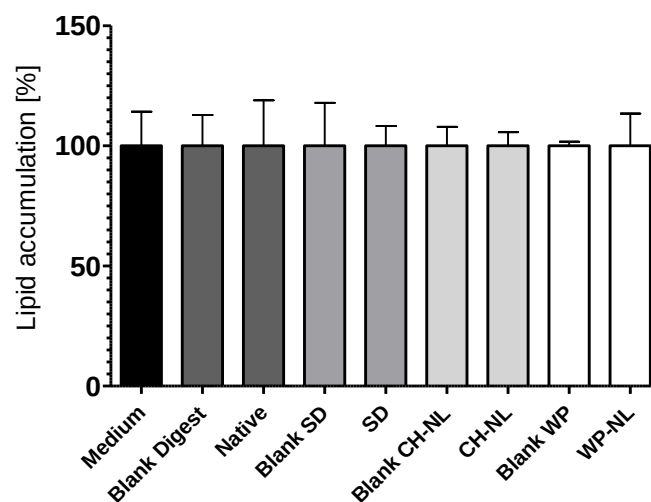


Figure 6.8 : Lipid accumulation after incubation with different micellar phenolic formulations, was determined by ORO assay and is expressed in % of treatment blank control. FFA = free fatty acids (oleate and palmitate in a 2:1 ratio, concentration=0.3 mM).

6.4 Conclusion

In the present study, spray dried biocompatible liposomes coated with chitosan or whey protein were successfully developed, characterized and applied for oral administration of selected phenolic compounds. The present work gives some insights into the simulated gastrointestinal digestion fate of free and nanoencapsulated selected phenolic compounds as well as mechanisms of their transport through Caco-2 cell monolayers. Nanoliposomal encapsulation significantly improved bioaccessibility of catechin, epicatechin, ferulic acid, and trans-resveratrol by protecting them from degradation in gastrointestinal tract. Further research is required to explore the effects of macronutrients on the bioaccessibility and Caco-2 permeability of phenolics and also to validate the obtained results *in vivo*.



7. CONCLUSIONS

There is special interest in the isolation of (poly)phenols from natural sources without a market value in order to find value-added ingredients for food industry recently. The consumption of (poly)phenols are linked to various health beneficial effects relying on protection against many diseases due to their antioxidant power. The main driving forces of the present thesis were to obtain practical data for the recovery of potentially health beneficial (poly)phenols from an underutilized berry sources by means of 'green' –edible, environmental friendly, and effective extraction methods and convert them into a dry stable form by means of encapsulation form in order to produce an ingredient suitable for use in functional foods and beverages by preserving their stability against processing and digestion conditions. Black rosehip (*Rosa pimpinellifolia* L.) was chosen as the raw material to obtain good antioxidative (poly)phenols. Black rosehip has considerable contents of vitamin C, vitamin E and (poly)phenols like phenolic acids, flavonoids, and anthocyanins. Chromatographic characterization of black rosehip (poly)phenols and their antiproliferative activity was investigated and compared with common rosehip, *Rosa canina* L. In this respect, both infusion (to represent the potential of tea when consumed as beverage) and hydromethanolic extracts were prepared and analyzed (Chapter 3). Within this scope, detailed identification of *Rosa pimpinellifolia* L. (poly)phenols was conducted for the first time using HPLC system was coupled to mass spectrometry in freeze dried and air dried samples together with well known and widely consuming *Rosa canina* L. variety. Five anthocyanins and forty nine non-anthocyanin phenolics were determined in black rosehip infusion and hydroalcoholic extracts. When the individual phenolic compounds in two rosehip varieties were compared, the levels of catechin, gallic acid, procatechuic acid, procyanidin B1, procyanidin dimer, procyanidin trimer, isoquercetin, and isoquercitrin levels of *Rosa pimpinellifolia* were higher than *Rosa canina* ($p < 0.05$). High concentrations of black rosehip hydromethanolic extract and infusion exhibited anti-proliferative activity on breast cancer cells in a dose-dependent manner in addition to activating the apoptosis pathway through generation of ROS.

Despite the great antioxidative potential of black rosehip (poly)phenols, it is an underutilized berry in the field of food even pharmaceutic and cosmetic. Thus, in Chapter 4, optimization of extraction conditions of 'green' techniques were attempted to produce novel antioxidant polyphenol-rich extracts from black rosehip. In this regard, two novel downstream processing using pressurized hot water extraction and supercritical carbon dioxide extraction using aqueous ethanol as modifier for the efficient recovery of phenolic compounds from *Rosa pimpinellifolia* L. fruits were performed. Response surface methodology was applied to study the effects of extraction parameters including temperature, pressure, solvent composition, and solvent-to-solid ratio in order to maximize the yield of extraction and antioxidant activity of the obtained extracts. These extracts were further compared in terms of bioaccessibility upon simulated digestion and uptake in the human epithelial cell line Caco-2 also with the ultrasound assisted ethanol extract. Optimization of ultrasound assisted extraction was not performed to save time and resources as there was already a vast amount of literature regarding extraction of (poly)phenols from plant matrices with ultrasound technology.

In Chapter 5, the processing and digestive stability of antioxidant black rosehip extracts obtained via green methods were attempted to be improved by means of nano/micro-encapsulation techniques. With this aim, liposome encapsulation and spray drying was performed as a promising strategy for the incorporation of hydrophilic and lipophilic bioactive compounds into functional foods. To increase the membrane stability of fragile liposomes, they were coated by electrostatic deposition of another biopolymers layer to maintain stability. The deposited biopolymer layer was chosen as chitosan and whey protein enabling the liposomal membrane to withstand harsh processing and digestive conditions. The encapsulation efficiency was at satisfactory level in terms of total phenolic contents, that were 67.2 ± 0.7 , 76.7 ± 0.8 , and 87.2 ± 4.0 for uncoated black rosehip extract loaded liposome, chitosan coated black rosehip extract loaded liposome, and whey protein coated black rosehip extract loaded liposome, respectively. Moreover, the loss of phenolic compounds during spray drying is due to oxygen exposure and high temperatures was proved by means of the retention efficiency which indicated significantly higher total phenolic content in biopolymer coated liposomal powders than only spray dried black rosehip extract. Also, for the encapsulation of lipophilic compounds, the influence of saponin

glycyrrhizin has been investigated to prepare stable solid lipid nanoparticle delivery systems. In this respect, tristearin solid lipid nanoparticles emulsified with glycyrrhizin at varying concentrations at pH 7 and pH 3 were produced. The influence of glycyrrhizin on the physical stability and crystallization behavior of solid lipid nanoparticles was evaluated by dynamic and static lights scattering, electrophoretic light scattering, optical microscopy, visual observations, and differential scanning calorimetry. Overall, glycyrrhizin exhibited a remarkable ability to stabilize solid lipid nanoparticles against polymorphic transition during 21 days storage period.

In order to represent a mixture of common berry phenolics, liposomal encapsulation of pure phenolic compounds yielding detectable results from the cellular uptake studies by HPLC were investigated further in Chapter 6. Thus, the selected phenolic compounds were liposomal encapsulated and spray dried to investigate their digestive fate using *in vitro* gastrointestinal digestion/Caco-2 cell culture model in comparison to their free native form. The fabricated nanoliposomal formulations had high water solubility, yield and low moisture contents. When liposomal dispersions were reconstituted after spray drying, particles yielded zeta potential values of -26.6 ± 2.4 , 14.0 ± 1.1 , and 53.1 ± 1.7 mV for spray-dried phenolics, whey protein-coated phenolic loaded nanoliposomes, and chitosan-coated phenolic loaded nanoliposomes, respectively. The biocompatibility of the formulations was also tested and no cytotoxic effects was indicated at the concentrations that are used in food or beverage fortification. Nanoliposomal encapsulation significantly improved bioaccessibility of catechin, epicatechin, ferulic acid, and trans-resveratrol by protecting them from degradation in gastrointestinal tract. Encapsulation of phenolics yielded up to 2.1 and 3 fold increase in the bioaccessibility upon *in vitro* digestion. Moreover, trans-epithelial permeability of the micellar phenolics indicated the highest overall absorption for trans-resveratrol followed by ferulic acid in Caco-2 cells. Furthermore, human hepatic cancer cell line–HepG2 was used as an *in vitro* model for lipid metabolism of the selected (poly)phenols to investigate the influence of encapsulation in retention of the anti-diabetic activity. Results showed that micellarized phenolics alleviated fatty acid induced hepatic lipid accumulation in chitosan coated liposomal form as well as their native while only spray dried (poly)phenols did not show any significant effect ($p > 0.05$).

Overall, the results of this thesis proved that the black rosehip can be utilized as a source of functional food ingredients. Findings of thesis sensitize to the preservation of the wild and local black rosehip specie in addition to its valorization. The application of green extraction methods are effective solutions to obtain and valorize black rosehip (poly)phenols which can contribute sustainability for the industry where promising antioxidant ingredients derived from natural sources are in need by the manufacturers. Furthermore, extraction studies can be extended to obtain anthocyanin-rich extracts by modifying the extraction parameters like acidity of the solvents. In order to extend the scope of this work, future research can be performed to fabricate dried, stable and encapsulated multifunctional formulations for desired dietary preferences. The use of other biopolymers should be considered by means of coating liposomes and carrier agents in spray drying either dietary fiber based or protein based ones boosting the promised health benefits beyond (poly)phenols and storage stability. Although an initial evaluation of the release of black rosehip (poly)phenols in the digestive system was conducted by photometry and liquid chromatography, further research should be conducted focusing on the changes in individual phenolic compounds in black rosehip upon simulated digestion using more sophisticated techniques such as liquid chromatography coupled with mass spectroscopy and interactions of phenolic compounds with other macronutrients in different food and beverage matrices affecting their bioaccessibility and bioavailability *in vitro*. On a longer-term perspective, animal studies for bioavailability are needed to validate the obtained results and to gain better insight *in vivo*.

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APPENDICES

APPENDIX A: Chapter 4–Supplementary materials

APPENDIX B: Chapter 5–Supplementary materials

APPENDIX C: Chapter 6–Supplementary materials



APPENDIX A : Chapter 4–Supplementary materials

Table A.1 : Model summary and analysis of variance (ANOVA) of the effects of PHWE conditions on the analyzed responses.

Source	Sum of squares						Mean of squares					
	Y1	Y2	Y3	Y4	Y5	Y6	Y1	Y2	Y3	Y4	Y5	Y6
Model	119.91	337.108	817.85	6.80	15.00	11.40	23.98	67.422	163.57	1.70	3.00	2.28
X1 (°C)	20.55	102.155	486.49	2.33	12.13	6.94	20.55	102.155	486.49	2.33	12.13	6.94
X2 (mL/g)	86.71	181.425	229.69	3.09	1.54	2.35	86.71	181.425	229.69	3.09	1.54	2.35
X1 ²	10.16	1.626	11.60	-	0.66	1.12	12.54	1.500	39.66	-	1.09	1.66
X2 ²	2.43	39.809	68.32	1.24	0.59	0.66	2.43	39.809	68.32	1.24	0.59	0.66
X1·X2	0.06	12.093	21.76	0.14	0.08	0.33	0.06	12.093	21.76	0.14	0.08	0.33
Residual	6.37	23.793	26.89	1.10	0.47	0.51	0.91	3.399	3.84	0.14	0.07	0.07
Lack-of-Fit	2.71	12.970	15.46	0.32	0.23	0.31	0.90	4.323	5.15	0.08	0.08	0.10
Pure Error	3.65	10.823	11.43	0.78	0.23	0.20	0.91	2.706	2.86	0.19	0.06	0.05
Total	126.27	360.901	844.74	7.90	15.47	11.91						
Model	26.37	19.84	42.58	12.36	44.83	31.48	<0.0001*	0.001*	<0.0001*	0.002*	<0.0001*	<0.0001*
X1 (°C)	22.60	30.05	126.65	16.97	181.19	95.82	0.002*	0.001*	<0.0001*	0.003*	<0.0001*	<0.0001*
X2 (mL/g)	95.34	53.38	59.80	22.45	23.02	32.39	<0.0001*	0.000*	<0.0001*	0.001*	0.002*	0.001*
X12	13.79	0.44	10.32	-	16.35	22.93	0.008*	0.528	0.015**	-	0.005*	0.002*
X22	2.67	11.71	17.79	8.99	8.88	9.07	0.146	0.011**	0.004**	0.017**	0.021**	0.020**
X1·X2	0.07	3.56	5.67	1.05	1.17	4.61	0.806	0.101	0.049**	0.336	0.316	0.069
Lack-of-Fit	0.99	1.60	1.80	0.41	1.33	2.07	0.482	0.323	0.286	0.793	0.381	0.247
	Y1	Y2	Y3	Y4	Y5	Y6						
R2-Sq	0.9496	0.9341	0.9682	0.8607	0.9697	0.9574						
R2-Sq (pred)	0.7596	0.6958	0.8227	0.5906	0.8432	0.7126						
R2-Sq (adj)	0.9136	0.8870	0.9454	0.7911	0.9481	0.9270						

Independent variables: X1, temperature (°C); X2, solvent-to-solid ratio (mg/mL). Y1, global yield (%); Y2, total antioxidant capacity (mmol TE/g); Y3, total (poly)phenols (mg GAE/g); Y4, catechin (mg/g); Y5, total monomeric anthocyanins (mg cyanidin-3-O-glucoside/g); and Y6, cyanidin-3-O-glucoside (mg/g) in dry basis. DF: Degree of freedom; F-Value: Fisher distribution value; p-Value: * significance at p < 0.01; ** significance at p < 0.05.

Table A.2 : Model summary and analysis of variance (ANOVA) of the effects of SCO₂ conditions on the analyzed responses.

Source	Sum of squares							Mean of squares						
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y1	Y2	Y3	Y4	Y5	Y6	Y7
Model	784.548	8540.11	25.2820	184.795	110.160	52775.5	888417	28.069	1067.51	3.61172	20.5328	18.3600	7539.4	126917
X1 (bar)	17.955	138.89	0.0076	1.657	0.259	518.6	1955	0.062	391.72	0.00417	0.2340	1.5344	625.9	8367
X2 (°C)	27.401	309.60	0.0455	1.557	0.016	2542.5	39020	46.293	170.65	0.01467	6.9320	1.2278	2178.8	46303
X3 (%)	514.707	5390.94	22.7342	139.246	86.067	43306.7	643890	35.930	767.23	0.40355	3.2821	4.9392	1184.8	53732
X1 ²		383.15	0.2097	10.557	2.042	721.5	9998		277.49	0.14650	8.1937	1.3870	483.0	5910
X2 ²	55.655	130.33		10.334		1603.2		45.716	84.17		9.0683		1398.7	
X3 ²	153.092	1788.20	1.7988	14.277	20.124	2411.9	170726	153.092	1788.20	1.79885	14.2771	20.1239	2411.9	170726
X1·X2	1.659		0.0866	4.671				1.659		0.08660	4.6711			
X1.X3	10.059	68.02		0.681			1871	10.059	68.02		0.6814			1871
X2.X3	4.020	330.99	0.3996	1.813	1.651	1671.1	20959	4.020	330.99	0.39962	1.8132	1.6512	1671.1	20959
Residual	36.473	169.87	0.4282	4.911	2.838	1724.6	6366	4.559	21.23	0.04758	0.7015	0.2838	191.6	707
Lack-of-Fit	17.449	51.70	0.2783	3.066	1.364	1238.0	3407	4.362	12.93	0.05565	1.0219	0.2273	247.6	681
Pure Error	19.025	118.16	0.1499	1.845	1.474	486.6	2959	4.756	29.54	0.03748	0.4612	0.3685	121.6	740
Total	821.021	8709.98	25.7102	189.705	112.998	54500.1	894783							
	Y1	Y2	Y3	Y4	Y5	Y6	Y7							
R ² -Sq	0.9556	0.9805	0.9833	0.9741	0.9749	0.9684	0.9929							
R ² -Sq (pred)	0.7569	0.9132	0.9040	0.7262	0.9267	0.8688	0.9732							
R ² -Sq (adj)	0.9112	0.9610	0.9704	0.9408	0.9598	0.9437	0.9873							

Table A.3 : Model summary and analysis of variance (ANOVA) of the effects of SCO₂ conditions on the analyzed responses.

Source	F value							p-value						
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y1	Y2	Y3	Y4	Y5	Y6	Y7
Model	21.51	50.27	75.92	29.27	64.70	35.35	179.43	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
X1 (bar)	0.01	18.45	0.09	0.33	5.41	3.27	11.83	0.910	0.000*	0.774	0.582	0.042**	0.104	0.007*
X2 (°C)	10.15	8.04	0.31	9.88	4.33	11.37	65.46	0.013**	0.003*	0.592	0.016**	0.064	0.008*	0.000*
X3 (%)	7.88	36.13	8.48	4.68	17.41	6.18	75.96	0.023**	0.022**	0.017**	0.067	0.002**	0.035**	0.000*
X1 ²		13.07	3.08	11.68	4.89	2.52	8.35		0.007*	0.113	0.011**	0.051	0.147	0.018**
X2 ²	10.03	3.96		12.93		7.30		0.013**	0.082		0.09		0.024**	
X3 ²	33.58	84.22	37.81	20.35	70.92	12.59	241.36	0.000*	0.000*	0.000*	0.03**	0.000*	0.006*	0.000*
X1·X2	0.36		1.82	6.66				0.563	0.111	0.210	0.036**			
X1.X3	2.21	3.20		0.97			2.64	0.176	0.004*		0.357			
X2.X3	0.88	15.59	8.40	2.58	5.82	8.72	29.63	0.375		0.018**	0.152	0.037**	0.016**	0.000*
Residual														
Lack-of-Fit	0.92	0.44	1.48	2.22	0.62	2.04	0.92	0.532	0.778	0.362	0.229	0.716	0.255	0.547

Independent variables: X1, pressure (bar); X2, temperature (°C); X3, co-solvent concentration (%). Y1, global yield (%); Y2, total (poly)phenols (mg GAE/g); Y3, catechin content (mg/g); Y4, total monomeric anthocyanins (mg cyanidin-3-O-glucoside/g); Y5, cyanidin-3-O-glucoside (mg/g); Y6, total antioxidant capacity by DPPH assay (mg TE/g); and Y7, total antioxidant capacity by CUPRAC assay (mg TE/g) all in dry basis. DF: Degree of freedom; F-Value: Fisher distribution value; p-Value: * significance at p < 0.01; ** significance at p < 0.05.

APPENDIX B : Chapter 5–Supplementary materials

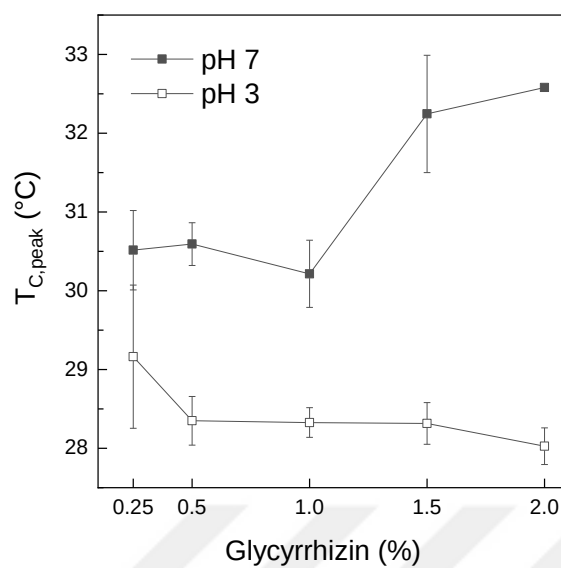


Figure B.1 : Mean crystallization temperatures (T_C , peak) of 10% (w/w) tristearin solid lipid nanoparticles emulsified with glycyrrhizin (0.25-2.0% w/w) at pH 7 and pH 3 stored at 20°C

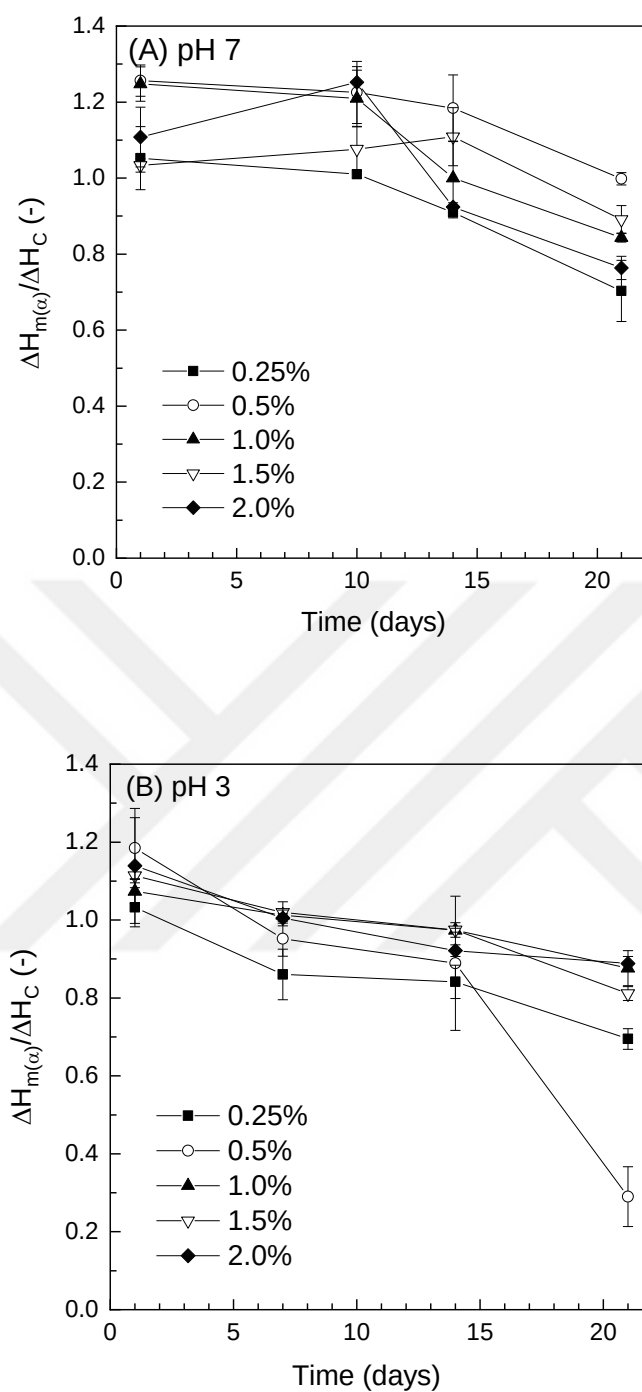


Figure B.2 : Ratio of α -subcell crystal melting enthalpy to crystallization enthalpy ($\Delta H_{m(\alpha)}/\Delta H_C$) in 10% (w/w) tristearin solid lipid nanoparticles emulsified with glycyrrhizin (0.25-2.0% w/w) at pH 7 (A) and pH 3 (B) during storage at 20°C.

Table B.1 : Mean hydrodynamic particle diameter d_H (z-average) and polydispersity index (PDI) of tristearin (10% w/w/) solid lipid nanoparticles stabilized with glycyrrhizin (0.25-2.0%) at pH 3 and 7 during storage at 20°C at determined by dynamic light scattering.

pH	$C_{\text{glycyrrhizin}}$ (%)	Day 1		Day 7		Day 14		Day 21	
		d_H (nm)	PDI (-)	d_H (nm)	PDI (-)	d_H (nm)	PDI (-)	d_H (nm)	PDI (-)
7	0.25	187 ± 6	0.21 ± 0.01	215 ± 6	0.25 ± 0.02	232 ± 11	0.28 ± 0.05	241 ± 7	0.26 ± 0.02
	0.5	180 ± 3	0.20 ± 0.01	188 ± 4	0.20 ± 0.01	195 ± 5	0.22 ± 0.03	212 ± 23	0.23 ± 0.02
	1.0	180 ± 3	0.19 ± 0.01	180 ± 3	0.21 ± 0.01	179 ± 2	0.20 ± 0.01	189 ± 4	0.22 ± 0.02
	1.5	172 ± 3	0.20 ± 0.02	185 ± 5	0.19 ± 0.01	183 ± 9	0.21 ± 0.02	186 ± 3	0.22 ± 0.02
	2.0	177 ± 7	0.20 ± 0.01	180 ± 3	0.20 ± 0.01	188 ± 8	0.24 ± 0.02	184 ± 4	0.22 ± 0.02
3	0.25	224 ± 6	0.17 ± 0.02	227 ± 2	0.18 ± 0.02	241 ± 7	0.20 ± 0.02	278 ± 5	0.23 ± 0.01
	0.5	192 ± 4	0.14 ± 0.02	197 ± 3	0.15 ± 0.02	403 ± 204	0.49 ± 0.15	434 ± 203	0.54 ± 0.12
	1.0	182 ± 2	0.14 ± 0.02	182 ± 2	0.15 ± 0.02	184 ± 3	0.15 ± 0.02	185 ± 6	0.19 ± 0.03
	1.5	179 ± 2	0.14 ± 0.01	179 ± 3	0.16 ± 0.01	182 ± 3	0.15 ± 0.01	177 ± 2	0.16 ± 0.01
	2.0	176 ± 2	0.14 ± 0.01	178 ± 3	0.16 ± 0.02	177 ± 2	0.15 ± 0.01	174 ± 2	0.15 ± 0.01

Table B.2 : Mean particle diameters (d_{32} , d_{43}) (in nm) of tristearin (10% w/w) solid lipid nanoparticles stabilized with glycyrrhizin (0.25-2.0%) at pH 3 and 7 during storage at 20°C determined by static light scattering.

pH	$C_{\text{glycyrrhizin}}$ (%)	Day 1		Day 7		Day 14		Day 21	
		d_{32}	d_{43}	d_{32}	d_{43}	d_{32}	d_{43}	d_{32}	d_{43}
7	0.25	175 ± 1	260 ± 63	183 ± 2	229 ± 3	185 ± 0	365 ± 185	186 ± 2	356 ± 80
	0.5	170 ± 1	201 ± 1	173 ± 1	205 ± 2	174 ± 1	207 ± 1	174 ± 2	209 ± 3
	1.0	165 ± 1	193 ± 1	169 ± 4	198 ± 5	170 ± 3	200 ± 3	167 ± 2	198 ± 1
	1.5	167 ± 2	205 ± 13	170 ± 2	197 ± 3	171 ± 1	200 ± 1	167 ± 1	195 ± 1
	2.0	167 ± 0	196 ± 1	165 ± 2	192 ± 0	169 ± 2	197 ± 3	167 ± 1	195 ± 2
3	0.25	212 ± 8	265 ± 7	206 ± 9	302 ± 60	199 ± 1	262 ± 1	203 ± 8	1501 ± 319
	0.5	161 ± 0	182 ± 0	160 ± 3	216 ± 42	35688 ± 9529	65702 ± 3305	49578 ± 3832	92927 ± 14072
	1.0	156 ± 3	177 ± 3	157 ± 2	177 ± 2	152 ± 1	171 ± 1	156 ± 1	176 ± 1
	1.5	150 ± 1	169 ± 1	154 ± 3	169 ± 4	159 ± 12	173 ± 6	155 ± 2	175 ± 2
	2.0	148 ± 2	163 ± 7	148 ± 2	162 ± 4	150 ± 2	168 ± 3	152 ± 1	162 ± 6

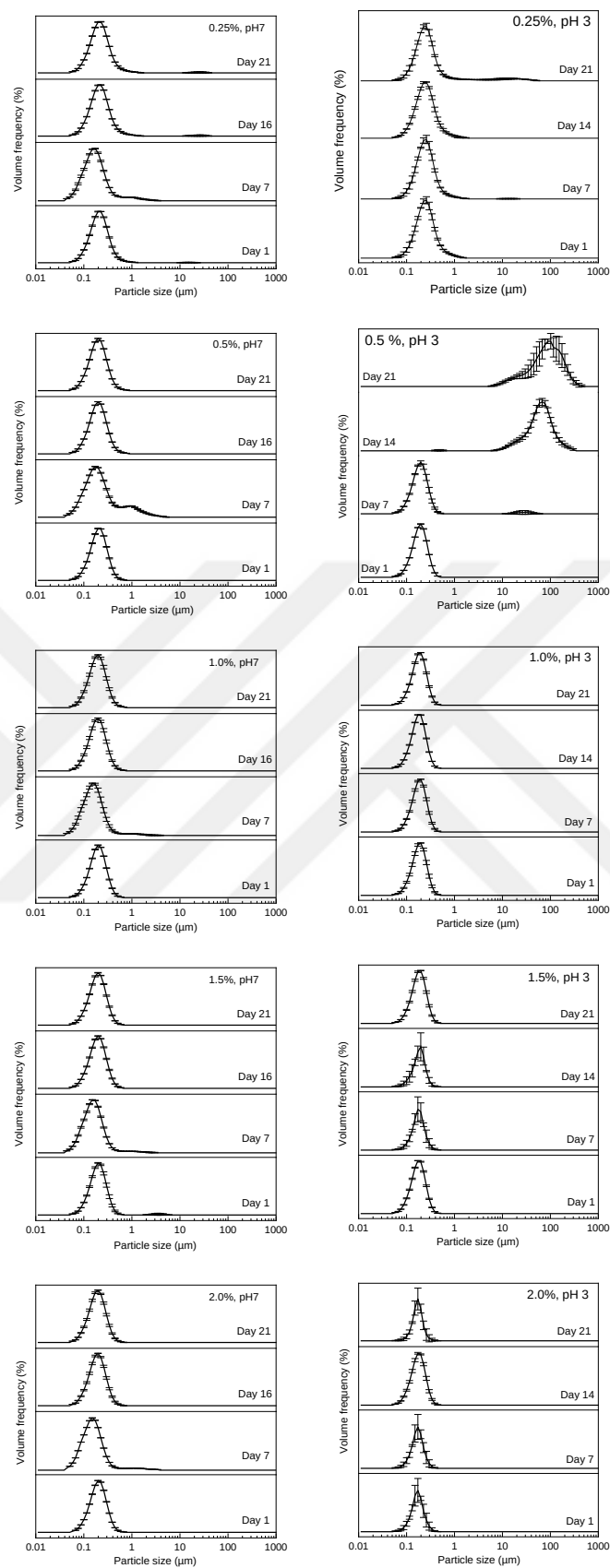


Figure B.3 : Particle size (d43) distributions of tristearin (10% w/w) solid lipid nanoparticles stabilized with glycyrrhizin (0.25-2.0%) at pH 3 and 7 during storage at 20°C.

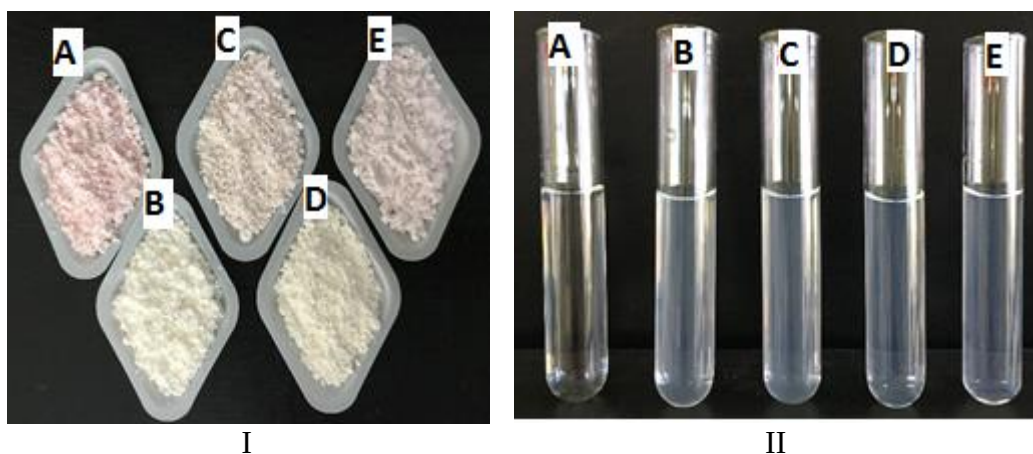


Figure B.4 : Visual appearance of powders (I): extract loaded chitosan-coated liposome (A), unloaded chitosan-coated liposome (B), extract loaded whey protein-coated liposome (C), unloaded whey protein-coated liposome (D), extract powder (E) and reconstituted samples (II): extract powder (A), chitosan-coated unloaded liposome (B), extract loaded chitosan-coated liposome (C), whey protein-coated unloaded liposome (D), extract loaded whey protein-coated liposome (E).

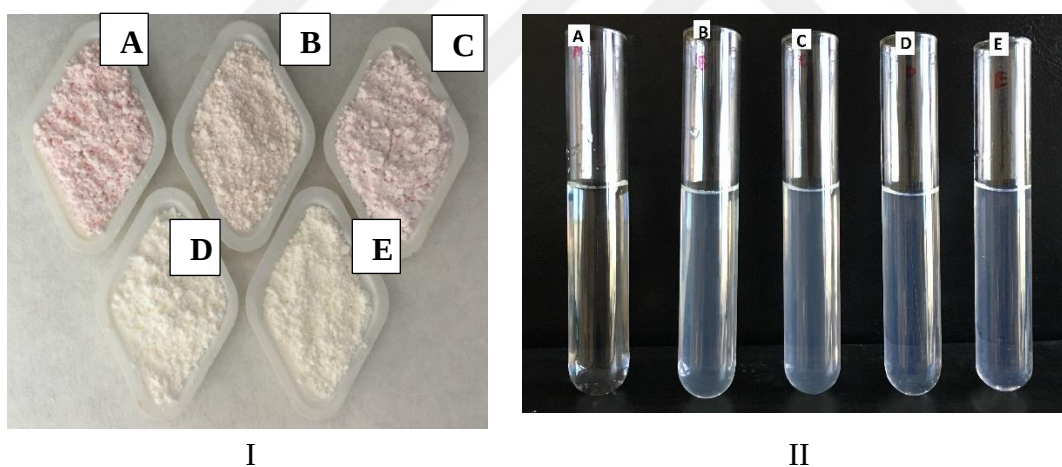


Figure B.5 : Visual appearance of powders (I): loaded chitosan-coated liposome (A), loaded whey protein-coated liposome (B), extract powder (C), chitosan-coated control liposome (D), whey protein-coated control liposome (E) and after reconstitution in distilled water (II): extract powder (A), chitosan-coated control liposome (B), loaded chitosan-coated liposome (C), whey protein-coated control liposome (D), loaded whey protein-coated liposome (E).

APPENDIX C : Chapter 6–Supplementary materials

Table C.1 : Retention percentage of phenolic compounds within different formulations after 6 months storage at 4 °C.

Compound	SD	CH-NL	WP-NL
Catechin	93.0 ± 4.1	91.8 ± 2.1	90.1 ± 2.5
Epicatechin	94.6 ± 3.2	92.8 ± 3.6	91.1 ± 6.9
Ferulic acid	98.7 ± 0.7	97.7 ± 0.7	98.2 ± 1.1
Resveratrol	91.0 ± 5.0	91.1 ± 4.4	93.6 ± 2.5

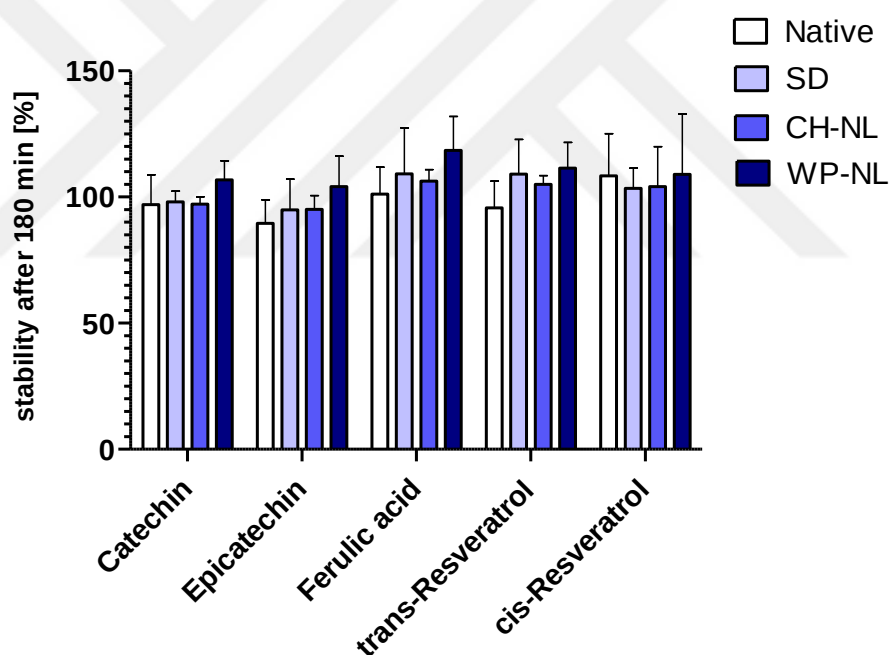


Figure C.1 : Stability of micellar phenolics in culture medium (HBSS) without cells incubated for 180 min.

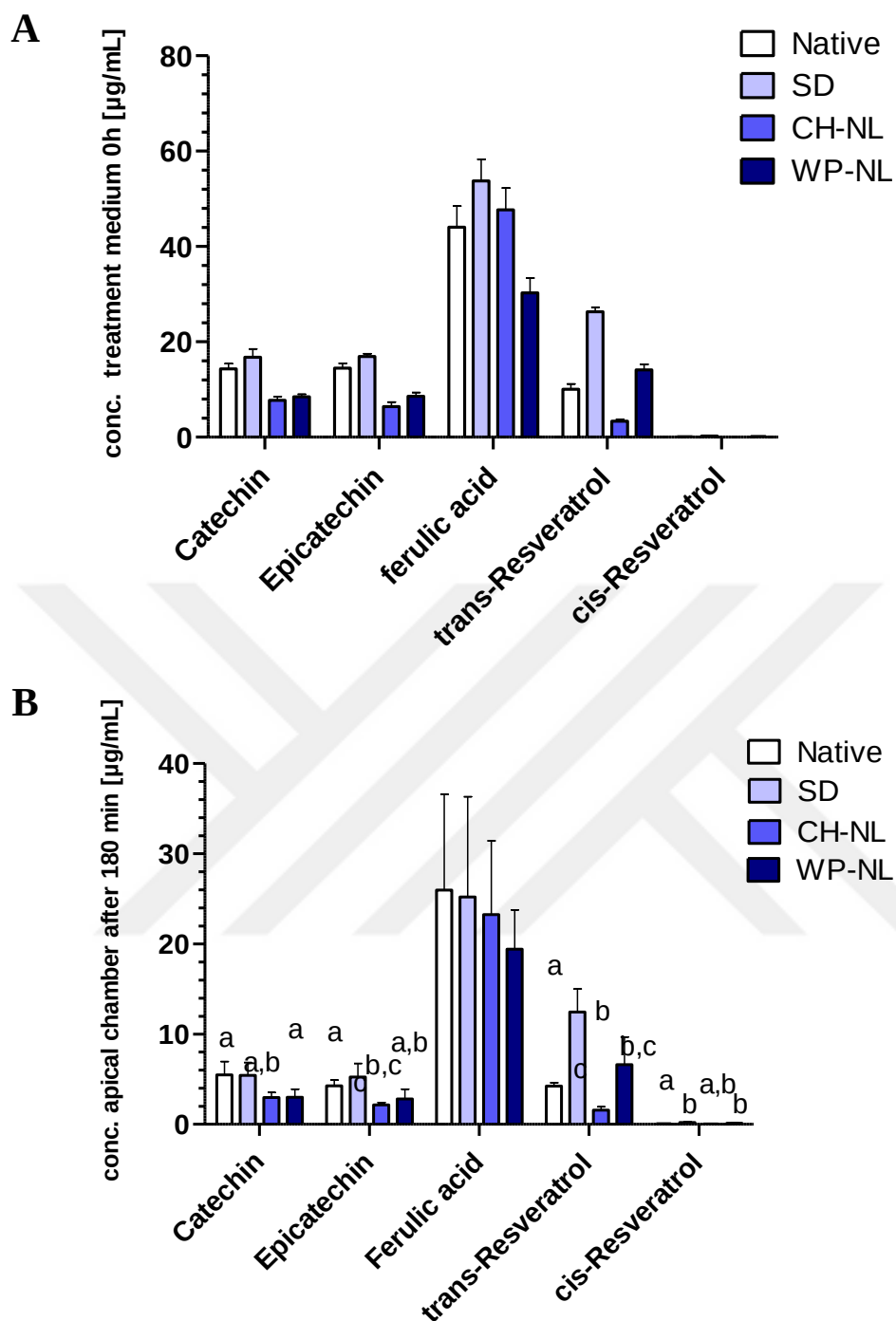


Figure C.2 : Concentration of micellar phenolics initially (A) and after 180 min of incubation (B) in the apical chamber.

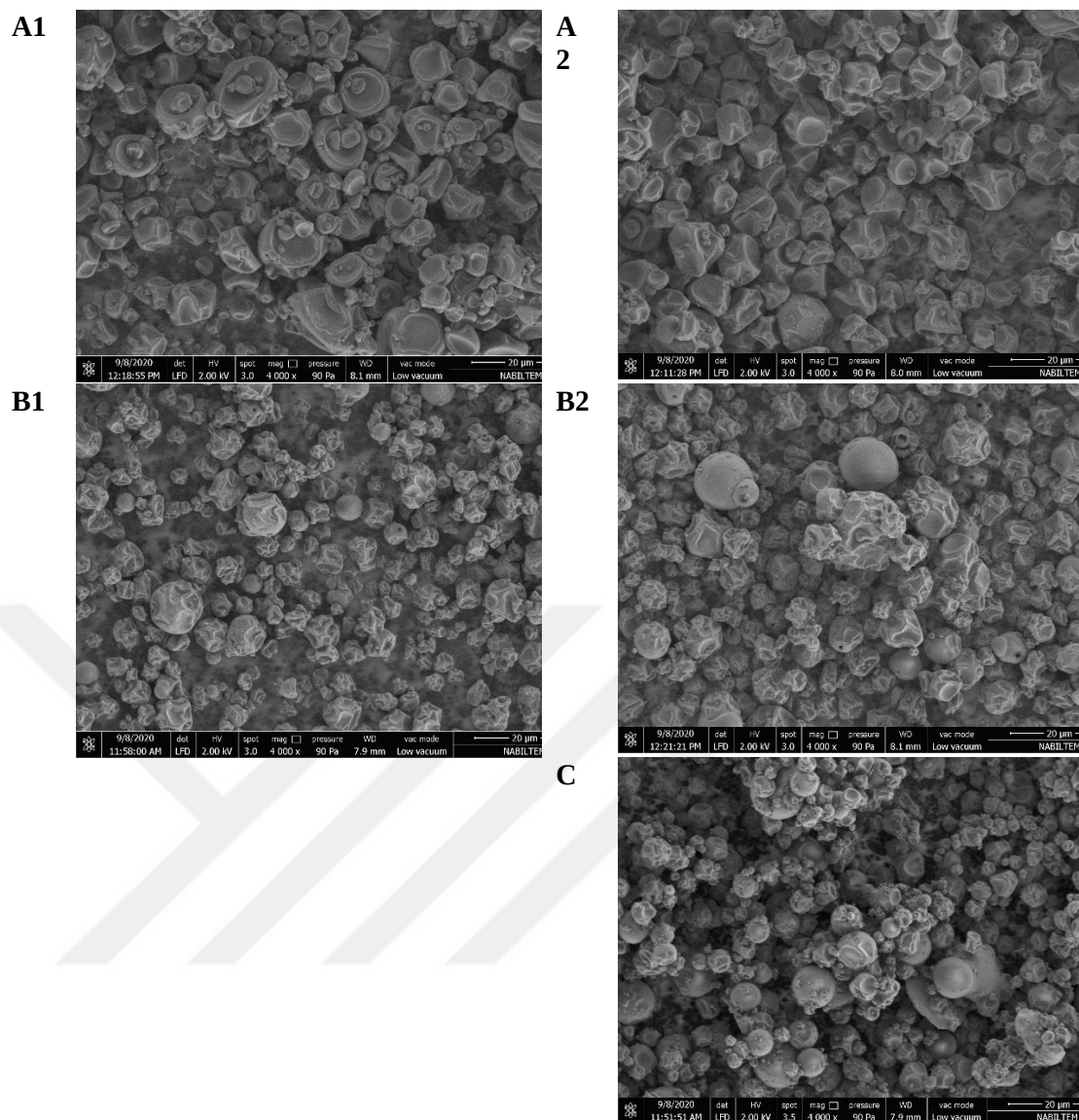


Figure C.3 : Scanning electron microscopy images of unloaded blank (A1) and loaded chitosan coated liposome powder (A2), unloaded blank (B1) and loaded whey protein coated liposome powder (B2) and spray dried phenolics powder (C). Pictures were taken at 4,000x magnifications.



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Projects:

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