ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

ENCAPSULATION OF AQUEOUS *HIBISCUS SABDARIFFA* L. EXTRACT IN FOOD-GRADE HIGH INTERNAL PHASE PICKERING EMULSIONS STABILIZED BY SOY PROTEIN ISOLATE

M.Sc. THESIS

Hümeyra ÇAVDAR

Department of Food Engineering

Food Engineering Programme

JUNE 2023

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

ENCAPSULATION OF AQUEOUS *HIBISCUS SABDARIFFA* L. EXTRACT IN FOOD-GRADE HIGH INTERNAL PHASE PICKERING EMULSIONS STABILIZED BY SOY PROTEIN ISOLATE

M.Sc. THESIS

Hümeyra ÇAVDAR
(506211516)

Department of Food Engineering

Food Engineering Programme

Thesis Advisor: Esra ÇAPANOĞLU GÜVEN

JUNE 2023

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

SULU *HIBISCUS SABDARIFFA* L. EKSTRAKTININ SOYA PROTEİNİ İLE STABİLİZE EDİLEN GIDA SINIFI YÜKSEK DAHİLİ FAZLI PICKERING EMÜLSİYONLARDA ENKAPSÜLASYONU

YÜKSEK LİSANS TEZİ

Hümeyra ÇAVDAR
(506211516)

Gıda Mühendisliği Anabilim Dalı

Gıda Mühendisliği Programı

Tez Danışmanı: Prof. Dr. Esra ÇAPANOĞLU GÜVEN

HAZİRAN 2023

Hümeyra ÇAVDAR, a M.Sc. student of ITU Graduate School student ID 506211516, successfully defended the thesis/dissertation entitled "ENCAPSULATION OF AQUEOUS *HIBISCUS SABDARIFFA* L. EXTRACT IN FOOD-GRADE HIGH INTERNAL PHASE PICKERING EMULSIONS STABILIZED BY SOY PROTEIN ISOLATE", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor: **Prof. Dr. Esra Çapanoğlu GÜVEN** **.....**
Istanbul Technical University

Jury Members : **Assoc. Prof. Dr. Aslı Can KARAÇA** **……..**
Istanbul Technical University

Assoc. Prof. Dr. Zeynep Tacer CABA **……**
Bahcesehir University

Teslim Tarihi **:16.06.2023**
Savunma Tarihi **:15.06.2023**

To my beloved family,

FOREWORD

I would like to extend my heartfelt thanks and appreciation to my supervisor and mentor, Prof. Dr. Esra ÇAPANOĞLU GÜVEN, for their invaluable guidance, support, and education throughout this journey. Her expertise and mentorship have played a crucial role in shaping my scientific and academic development.

I am also grateful to Asst. Prof. Dr. Gülay ÖZKAN, who helped me gain self-confidence in the laboratory since the beginning of my graduate education, greatly enriched my research experience and taught me many of the analyses I performed.

I would like to express my sincere thanks to my office-mates, Elif Feyza AYDAR, Zehra MERTDİNÇ, and Fatma Duygu CEYLAN ADRAR, for their support and friendship during this time. Their presence has made the journey more enjoyable and fulfilling.

Lastly, I would like to convey a special note of appreciation and gratitude to my parents, Ayşe and Ali ÇAVDAR, my sister Rümeysa ÇAVDAR, and my husband Mehmet DİNÇTÜRK. Their unwavering love, patience, and support have been the cornerstone of my education and life. I am deeply grateful for their constant encouragement and belief in me.

June 2023

Hümeyra ÇAVDAR
(Research Assistant)

TABLE OF CONTENTS

Pages

FOREWORD ..... **vii**
ABBREVIATIONS ..... **xi**
SYMBOLS ..... **xiii**
LIST OF TABLE ..... **xv**
LIST OF FIGURE ..... **xvii**
SUMMARY ..... **xix**
ÖZET ..... **xxi**
1. INTRODUCTION **1**
1.1 Purpose of Thesis **2**
1.2 Literature Review **2**
2. MATERIALS AND METHODS **13**
2.1 Materials **13**
2.2 Preparation of Aqueous *Hibiscus* Extract (AHE) **13**
2.3 Preparation of Soy Protein Isolate (SPI) Gel **13**
2.4 Preparation of Emulsions **14**
2.4.1 High internal phase pickering emulsions (HIPPEs) **14**
2.4.2 High internal phase pickering-double emulsions (HIPP-DEs) **15**
2.5 Storage Stability **15**
2.6 Encapsulation Efficiency **16**
2.7 Determination of Emulsifying Properties **16**
2.8 Particle size, PDI and Zeta Potential **17**
2.9 Creaming Stability **17**
2.10 Centrifugation Stability **17**
2.11 *In Vitro* Gastrointestinal Digestion **17**
2.12 The Quantification of Polyphenols with HPLC-DAD **18**
2.13 Spectrophotometric Assays **19**
2.13.1 Total anthocyanin content (TAC) **19**
2.13.2 Total phenolic content (TPC) **19**
2.13.3 Antioxidant activity **19**
2.13.4 Bioaccessibility calculations **19**
2.14 Statistical Analysis **20**
3. RESULT AND DISCUSSION **21**
3.1 Storage Stability **21**

3.2 Creaming Stability **24**
3.3 Centrifugation Stability **25**
3.4 Determination of emulsifying properties **26**
3.5 Particle size, zeta potential and PDI **29**
3.5.1 Particle size **29**
3.5.2 Zeta potential **31**
3.5.3 PDI **33**
3.6 Encapsulation Efficiency **34**
3.7 Effect of *In Vitro* Gastrointestinal Digestion on TAC, TPC, DPPH and ABTS of HIPPEs and HIPP-DEs **36**
3.7.1 Total anthocyanin content **36**
3.7.2 Total phenolic content **38**
3.7.3 Bioaccessibility of phenolics and anthocyanins **41**
3.7.4 Antioxidant activity **43**
3.7.5 Quantification of Polyphenols with HPLC-DAD **45**
4. CONCLUSION AND RECOMMENDATIONS 49
REFERENCES 51
CURRICULUM VITAE 63

ABBREVIATIONS

ABTS : 2,2’ Azinobis (3-Ethylbenzothiazoline-6-Sulfonic Acid) Diammonium Salt

C3-GE: Cyanidin 3-Glucoside Equivalent

DPPH : 2,2-Diphenyl-1-picrylhydrazyl

EAI : Emulsion Activity Index

ESI : Emulsion Stability Index

GAE : Gallic Acid Equivalent

SPI : Soy Protein Isolate

TAC : Total Anthocyanin Content

TE : Trolox Equivalent

TFC: Total Flavonoid Content

TPC : Total Phenolic Content

SYMBOLS

g : Gram

mg : Miligram

mL : Mililiter

p : Confidence Level

pH : Potential of Hydrogen

LIST OF TABLES

Page

Table 1.1: Nutritional and bioactive compounds in Hibiscus Sabdariffa..... **3**

Table 1.2: Encapsulation of bioactives within HIPPEs and DEs stabilized SPI..... **10**

Table 2.1: HIPPEs with different formulations..... **14**

Table 2.2: HIPP-DEs with different formulations. **15**

Table 3.1: EAI and ESI values of HIPPEs. **28**

Table 3.2: EAI and ESI values of HIPP-DEs..... **29**

Table 3.3: Total anthocyanin content (mg C-3-GE/ 100 g) of HE and HIPPEs. **38**

Table 3.4: Total anthocyanin content (mg C-3-GE/ 100 g) of HE and HIPP-DEs... **38**

Table 3.5: Effect of *in vitro* digestion on TPC (mg GAE/100 g) of HE and HIPPEs. **40**

Table 3.6: Effect of *in vitro* digestion on TPC (mg GAE/g) of HE and HIPP-DEs. **41**

Table 3.7: Effects of in vitro digestion on DPPH (mg TE/100 g dw) and ABTS (mg TE/100 g dw) of *Hibiscus* extracts (HE) and HIPPEs. **44**

Table 3.8: Effects of in vitro digestion on DPPH (mg TE/100 g dw) and ABTS (mg TE/100 g dw) of *Hibiscus* extracts(HE) and HIPP-DEs. **45**

Table 3.9: Anthocyanin and phenolic profile of HIPPEs**.**..... **47**

LIST OF FIGURES

Page

Figure 3.1: HIPPEs stabilized by only different SPI gel concentrations. **21**
Figure 3.2: HIPPEs stabilized by only different lecithin concentrations. **22**
Figure 3.3: HIPPEs stabilized by different SPI gel and lecithin concentrations..... **23**
Figure 3.4: HIPP-DEs stabilized by different SPI gel and lecithin concentrations .. **23**
Figure 3.5: Changes in creaming index (CI) of HIPPEs during storage..... **25**
Figure 3.6: Changes in creaming index (CI) of HIPP-DEs during storage..... **25**
Figure 3.7: Degree of phase separation of L4 and L6 after centrifugation..... **26**
Figure 3.8: HIPP-DEs after centrifugation **26**
Figure 3.9: Change in the particle size and zeta potential between different HIPPEs. **32**
Figure 3.10: Change in the particle size and zeta potential between different HIPP-DEs. **33**
Figure 3.11: PDI values of HIPPEs. **34**
Figure 3.12: PDI values of HIPP-DEs. **34**
Figure 3.13: EEA and EEP values of HIPPEs **35**
Figure 3.14: EEA and EEP values of HIPPE-DEs..... **36**
Figure 3.15: Bioavailability of phenolics and anthocyanins of HIPPEs. **42**
Figure 3.16: Bioavailability of phenolics and anthocyanins of HIPP-DEs. **43**
Figure 3.17: Change in anthocyanin and phenolic profile of L6S6 during in vitro digestion **48**
Figure 3.18: Change in anthocyanin and phenolic profile of L6S6-D during in vitro digestion **48**

ENCAPSULATION OF AQUEOUS *HIBISCUS SABDARIFFA* L. EXTRACT IN FOOD-GRADE HIGH INTERNAL PHASE PICKERING EMULSIONS STABILIZED BY SOY PROTEIN ISOLATE

SUMMARY

Hibiscus Sabdariffa L. is a highly versatile plant that finds applications in various industries due to its abundant nutrients, potent bioactive compounds such as phenolics and anthocyanins, and natural colour pigments. In addition to its use as a colouring agent, its positive health effects, such as antibacterial, antioxidant, anticholesterol and prevention of gastrointestinal problems, spread the consumption of the product for health purposes. However, the fact that bioactive ingredients are easily affected and damaged by many factors limits this product's use in many industries. Therefore, integrating *Hibiscus Sabdariffa* into food products and various formulations becomes more complex and limits the methods used. Encapsulation, a preferred method for much bioactive protection, appears at this point. Encapsulation comes to fields as a technique that allows us to apply in different areas, such as preserving these components and controlled release and integrating them into product formulations. Emulsification by encapsulation method has gained a solid place in industry and literature. High Internal Phase Emulsions are among the leading emulsions with high volume encapsulation and high stability against external factors. Similarly, double emulsion systems can enhance bioactive components' protection with their nested phase structure. The debate over the many adverse effects of preferred emulsifiers and stabilizers for emulsion stability, such as human health and environmental pollution, has accelerated scientific research into discovering alternative products. Plant proteins have gained tremendous interest in recent years as they have the potential to be an alternative to conventional emulsifiers and stabilizers. The various functional properties and amphiphilic characteristics indicate that these proteins are significantly effective in the stability of the emulsion system. Like many other types of plant proteins, soy protein is a preferred product due to its easy availability and processability.

In this study, analyses were conducted to identify and evaluate the encapsulation of aqueous *Hibiscus* extract in the High Internal Phase Pickering Emulsions (HIPPE) and High Internal Phase Double Emulsions (HIPP-DE) systems stabilized by soy lecithin and soy protein isolate. The stability of the emulsions obtained, characteristics and the effects of in vitro gastrointestinal digestion on the phenolics and anthocyanins in the extract with the presence of soy protein and soy lecithin were investigated.

The extraction of phenolics and anthocyanins from powdered Hibiscus calyces was realized by ultrasonic method, and water was used as a solvent. Emulsions with an internal volume of 80% have been created using soy protein isolate (SPI) gel and lecithin. A HIPPE resistant to phase separation could not be obtained using soya protein isolation gel alone, while emulsions (L4 and L6) containing 4% and 6% lecithin and phase separation resistance could be achieved. Combinations of different concentrations of lecithin and soy protein isolated gel stabilized other HIPPEs. After 24 hours of storage, HIPPEs stable against phase separation were obtained by homogenizing HIPP-DEs at a volume of 50% with a 6% SPI gel. When emulsions stored for 24 hours were observed, SPI gels and lecithin at varying concentrations acted as a synergistic mechanism that effectively prevented phase segregation and ensured stability in the emulsion system. All HIPP-DEs also showed superior

resistance to phase separation. According to the CI results, an increase in the concentration of soy lecithin from 2% to 4% in HIPPE containing 6% SPI gel resulted, indicating increased stability, leading to a decrease in the CI value on day 1; however, no significant difference in CI was observed between HIPP-DEs. EAI and ESI values show a statistically significant increase when the SPI concentration increases from 2% to 4% in HIPPEs and HIPP-DEs with 4% and 6% lecithin concentrations. Still, surprisingly, no significant increase was observed with further increases in SPI. In contrast, the particle size of HIPPEs with 4% and 6% lecithin concentrations showed a significant decrease as the SPI gel concentration increased from 2% to 6%. In HIPPEs, the zeta potential was negative, and the absolute values increased by increasing the SPI gel concentration from 2% to 4%, while in L4S4, it was observed that the HIPPE had the highest absolute zeta potential (-41.21 ± 1.23 mV). However, an increase in SPI gel concentration from 4% to 6% decreased the absolute zeta potential value. The increase in the concentration of soy protein isolate (SPI) from 2% to 6% resulted in a significant decrease in the PDI values of HIPPE containing 4% and 6% lecithin. The lowest PDI values were obtained in L4S6 and L6S6 HIPPE with an SPI concentration of 6%. The same decline has been observed in HIPP-DEs.

Different changes have been observed in the stability and characteristics of emulsions, as well as in the study of bioactive components and their properties. Encapsulation Efficiency (EE) determinations stated that HIPP-DEs produced higher EE than HIPPEs due to high SPI gel concentrations. The total anthocyanin content (TAC), total phenolic content (TPC), DPPH and ABTS of the resulting aqueous Hibiscus extract were analyzed. The TAC (mg Cy-3-GC equivalent/100 g), TPC (mg GAE/100 g), DPPH (mg TE/100g) and ABTS (mg TE/100g) values were found as 31.13±1.23, 2619.01±17.31, 335.12±1.21 and 223.21±2.56 respectively. Significant decreases in TPC in Hibiscus extract were observed with in vitro gastrointestinal digestion. In HIPPEs prepared with a concentration of 4 % lecithin (L4, L4S2, L4 S4, L4S6), the SPI gel concentration increased from 0 % to 4 %, while anthocyanins in the stomach environment showed more excellent stability compared to the intestinal environment. When the TPC of the digested phenolics of *Hibiscus* extract was compared to those of digestive HIPPEs and HIPP-DEs, higher TPC values were observed in all emulsions. Maximum TPC values in the stomach and intestinal digestion reached 709.82 ±2.06 mg/100g and 1160.71 ±21.01 mg/100g, respectively. In the stomach and intestinal phases of in vitro digestion, the rate of phenolic release has been significantly influenced by the pH of the environment. According to HPLC results, aqueous *Hibiscus* extract has detected anthocyanins of delphinidin-3-glucoside, cyanidin-3-glucoside, and cyanidin-3-rutinoside. Four phenolic acids have also been detected in the extract and all emulsions, including gallic acid, syringic acid, ferulic acid and chlorogenic acid. After gastric digestion, a decrease in the content of chlorogenic acid, ferulic acid and gallic acid was observed, while syringic acid increased, showing a different tendency. The highest concentrations of each anthocyanins and phenolics concentrations were obtained in L6S6-D.

As a result, changes in SPI gel and lecithin concentrations have been effective in many conditions, such as the stability of emulsions, their properties, and the effects of in vitro digestion on anthocyanins and phenolics. In addition, according to the results of encapsulation efficiency, it can be stated that HIPPEs and HIPP-DEs are effective emulsion systems in the encapsulation of *Hibiscus* extract.

SULU HIBISCUS SABDARIFFA L. EKSTRAKTININ SOYA PROTEİNİ İLE STABİLİZE EDİLEN GIDA SINIFI YÜKSEK DAHİLİ FAZLI PICKERING EMÜLSİYONLARDA ENKAPSÜLASYONU

ÖZET

Hibiscus Sabdariffa L., zengin besin içeriği, fenolik ve antosiyaninler açısından güçlü biyoaktif bileşenleri, doğal renk pigmentleri sayesinde birçok endüstride geniş kullanım alanı bulmaktadır. Renklendirici olarak kullanımının yanı sıra antibakteriyel antioksidan, antikolesterol ve gastrointestinal problemler gibi sağlık üzerindeki olumlu etkileri ürünün sağlık amacıyla tüketimini yaygınlaştırmaktadır. Ancak biyoaktif bileşenlerin birçok farklı faktörden kolaylıkla etkilenmesi ve zarar görmesi bu ürünün birçok endüstride kullanımını sınırlandırmaktadır. Bu nedenle *Hibiscus Sabdariffa*'nın gıda ürünü formülasyonlarına entegrasyonu zorlaşmakta ve kullanılacak yöntemleri sınırlandırmaktadır. Birçok biyoaktif koruma önlemi için tercih edilen bir yöntem olan enkapsülasyon bu noktada ortaya çıkmaktadır. Enkapsülasyon, yalnızca bu bileşenlerin korunması değil, aynı zamanda kontrollü salınım ve ürün tüketimine dahil edilmesi gibi farklı alanlarda uygulama sağlayan bir teknik olarak karşımıza çıkmaktadır. Emülsifikasyon ile enkapsülasyon yöntemi, hem endüstride hem de literatürde sağlam bir yer edinmiştir. Yüksek Dahili Fazlı Emülsiyonlar, yüksek hacimli enkapsülasyon, yüksek stabilite ve biyoaktif bileşenleri farklı etkenlere karşı koruma özellikleri ile ön plana çıkan emülsiyon türleri arasındadır. Benzer şekilde Çift Katlı Emülsiyon sistemleri de iç içe fazlardan oluşan yapıları ile biyoaktif bileşenler için yüksek koruma sağlayabilmektedir. Emülsiyon stabilitesi için tercih edilen emülgatörler ve stabilizatörlerin insan sağlığı ve çevre kirliliği gibi birçok olumsuz etkileri üzerine yapılan tartışmalar alternatif ürünlerin keşfedilmesi üzerine gerçekleştirilen bilimsel çalışmaları hızlandırmıştır. Bitkisel proteinler konvansiyonel emülgatör ve stabilizatörlere alternatif olma potansiyeline sahip oldukları için son yıllarda yoğun ilgi görmektedir. Çeşitli fonksiyonel özellikleri ve amfifilik karakterleri, bu proteinlerin emülsiyon sisteminin stabilitesini korumada önemli ölçüde etkili olduğunu göstermektedir. Diğer birçok bitkisel protein türü gibi soya proteinleri de kolay ulaşılabilirliği ve işlenebilirliği nedeniyle tercih edilen bir üründür. Bu çalışmada sulu *Hibiskus* extraktının soya lesitin ve soya protein izolatı içeren Yüksek Dahili Fazlı Pickering Emülsiyonlar (HIPPE) ve Yüksek Dahili Fazlı Pickering Çift Emülsiyon (HIPP-DE) sistemlerinde enkapsülasyonu, elde edilen emülsiyonların stabilitesi, emülsiyon özellikleri ve *in vitro* gastrointestinal sindirimin soya proteini ve lesitin varlığında ekstrakttaki fenolikler ve antosiyaninler üzerindeki etkilerinin ortaya çıkarılması ve değerlendirilmesi üzerine analizler gerçekleştirilmiştir.

Toz haline getirilen *Hibiscus* çanaklarından fenolik ve antosiyaninlerin ekstraksiyonu ultrasonik yöntemle gerçeleştirilmiş ve çözgen olarak su kullanılmıştır. Soya proteini izolat (SPI) jeli ve lesitin kullanılarak %80 iç hacme sahip emülsiyonlar oluşturulmuştur. Yalnızca soya proteini izolat jeli kullanılarak faz ayrımına karşı dirençli bir HIPPE elde edilemezken, %4 ve %6 lecithin içeren ve faz ayrımına karşı dirençli emülsiyonlar (L4 ve L6) elde edilebilmiştir. Diğer HIPPE'ler ise farklı lecithin ve soy protein izolat jeli konsantrasyonlarının kombinasyonları ile stabil hale getirilmiştir. 24 saat depolama sonrasında faz ayrımı gözlemlenmeyen ve stabil halde bulunan HIPPE'ler, %6 SPI jel ile hacimce %50 oranında homojenize edilerek HIPP-DE'ler elde edilmiştir. 24 saat boyunca depolanan emülsiyonlar gözlemlendiğinde,

HIPPE'lerde değişen konsantrasyonlarda SPI jeli ve lesitinin kullanılması, faz ayrımını etkili bir şekilde önleyen ve emülsiyon sisteminde stabilite sağlayan sinerjistik bir mekanizma görevi üstlenmiştir. Ayrıca elde edilen tüm HIPP-DE'ler faz ayrımına karşı üstün bir direnç göstermiştir. CI sonuçlarına göre, %6 SPI jeli içeren HIPPE'lerde soya lesitin konsantrasyonunun %2'den %4'e çıkarılması, 1. günde CI değerinin düşmesine yol açarak, stabilitenin arttığını gösterirken, %6 lesitin konsantrasyonu artmasına neden olmuştur; ancak, HIPP-DE'ler arasında CI açısından önemli bir fark gözlemlenmemiştir. Hem EAI hem de ESI değerleri, %4 ve %6 lesitin konsantrasyonlarına sahip HIPPE'lerde ve HIPP-DE'lerde SPI konsantrasyonu %2'den %4'e yükseldiğinde istatistiksel olarak anlamlı bir artış sergilerken, şaşırtıcı bir şekilde, SPI konsantrasyonu daha da arttığında anlamlı bir artış gözlenmemiştir. Bu durumun aksine, %4 ve %6 lesitin konsantrasyonlarına sahip HIPPE'lerin parçacık boyutu, SPI jel konsantrasyonu %2'den %6'ya çıktıkça önemli bir azalma sergilemiştir. HIPPE'lerde zeta potansiyelinin negatif olduğu ve SPI jel konsantrasyonunun %2'den %4'e arttırılmasıyla mutlak değerlerinin arttığı gözlemlenirken, L4S4 ne yüksek mutlak zeta potansiyeline (-41,21 ± 1,23 mV) sahip HIPPE olmuştur. Ancak, %4'ten %6'ya artan SPI jel konsantrasyonu, mutlak zeta potansiyel değerinde azalmaya sebebiyet vermiştir. Soya proteini izolatının (SPI) konsantrasyonunun %2'den %6'ya çıkarılması, %4 ve %6 lesitin içeren HIPPE'lerin PDI değerlerinde anlamlı bir azalmaya yol açmıştır. En düşük PDI değerleri, SPI konsantrasyonu %6 olduğu olduğu L4S6 ve L6S6 HIPPE'lerinde elde edilmiştir. Aynı düşüş HIPP-DE'ler içinde geçerli olmuştur.

Emülsiyon stabilitesi ve karakteristiklerinin yanı sıra emülsiyon sistemlerinin biyoaktif bileşenler ve özellikleri açısından incelenmesi ile farklı değişimler ortaya konmuştur. Enkapsülayon verimliliği (EE) araştırıldığında görülmüştür ki HIPP-DE'ler yüksek SPI jel konsantrasyonu sayesinde HIPPE'lere göre daha yüksek EE sağlamıştır. Elde edilen sulu *Hibiscus* ekstraktının toplam antosiyanin miktarı (TAC), toplam fenolik miktarı (TPC), DPPH ve ABTS yönünden analiz edilmiştir. TAC (mg Cy-3-GC eşdeğeri/100 g), TPC (mg GAE/100 g) , DPPH (mg TE/100 g) and ABTS (mg TE/100g) değerleri sırasıyla 31.13±1.23, 2619.01±17.31, 335.12±1.21 ve 223.21±2.56 olarak bulunmuştur. *In vitro* gastrointestinal sindirim ile birlikte *Hibiskus* ekstraktında TPC'de önemli düşüşler gözlemlenmiştir. %4 lesitin konsantrasyonu ile hazırlanan HIPPE'lerde (L4, L4S2, L4S4, L4S6) SPI jel konsantrasyonu %0'dan %4'e yükselirken, mide sindiriminde antosiyaninler bağırsak ortamına göre daha yüksek stabilite göstermiştir. *Hibiskus* ekstraktının sindirilmiş fenoliklerinin TPC'si sindirilmiş HIPPE'lerin ve HIPP-DE'lerinkiyle karşılaştırıldığında, tüm emülsiyonlarda daha yüksek TPC değerleri gözlemlenmiştir. Mide ve bağırsak sindirimlerinde maksimum TPC değerleri sırasıyla 709.82±2.06 mg / 100g ve 1160.71±21.01 mg/100 g'a ulaşmıştır. *In vitro* sindirimin mide ve bağırsak fazları enasında fenoliklerin salınım hızı, ortam pH'ından önemli ölçüde etkilenmiştir. HPLC sonuçlarına göre, sulu *Hibiskus* ekstraktında delfinidin-3-glukozit, siyanidin-3-glukozit, ve siyanidin-3-rutinozit antosiyaninleri tespit edilmiştir. Ayrıca ekstrakt ve tüm emülsiyonlarda gallik asit, sirincik asit, ferulik asit, klorojenik asit olmak üzere dört fenolik asit tespit edilmiştir. Gastrik sindirimden sonra, klorojenik asit, ferulik asit ve gallik asit içeriğinde bir azalma gözlemlenirken, sirincik asit farklı bir eğilim göstererek artmıştır. En yüksek konsantrasyon antosiyanin ve fenolik konsantrasyonları L6S6-D'de elde edilmiştir.

Sonuç olarak, SPI jel ve lesitin konsantrasyonlarındaki değişimler, emülsiyonların kararlılığı, özellikleri, in vitro sindirimin antosiyaninler ve fenolikler üzerindeki etkileri gibi birçok koşulda etkili olmuştur. Ayrıca enkapsülasyon verimliliği

sonuçlarına göre *Hibiskus* ekstraktının enkapsülasyonunda HIPPE ve HIPP-DE'nin etkili emülsiyon sistemler olduğu söylenebilir.

1. INTRODUCTION

Hibiscus Sabdariffa L. finds a wide range of uses in many industries thanks to its rich nutritional content and bioactive ingredients rich in phenolic and anthocyanins and the natural color pigments obtained. In addition to its use as a colorant, its positive health effects including antibacterial antioxidant, anticholesterol and gastrointestinal problems make the consumption of the product widespread for this purpose. However, the fact that bioactive components are easily affected and damaged by many different factors limits the use of this product in many industries. For this reason, the integration of Hibiscus Sabdariffa into food product formulations becomes difficult and limits the methods to be used. Encapsulation, which is a preferred method for many bioactive protection measures, is the sovereignty that emerges at this point. Encapsulation is not only the preservation of these measures, but also containment provisions as a technique applied in areas such as controlled release and incorporation into product consumption. There are many types such as spray drying, freeze drying, coacervation, liposome and emulsification. The method of encapsulation by emulsification is technically conservative provisions that have gained a solid place in both industry and literature. In this method, bioacti production is protected and transported by being trapped in water or oil phase, depending on the characteristics of its properties. High Internal Phase Emulsions are among the emulsion types that come to the fore thanks to their high volume encapsulation, high stability and ability to protect the contents against different factors. Similarly, Double Emulsion systems can provide high protection for bioactive components with their structure consisting of nested phases.However, emulsifiers and stabilizers are used to homogenize these two phases, which do not mix with each other as they have a normal emulsion appearance. Lecithin, Tween, Span and PGPR are conventionally preferred emulsifiers and stabilizers. However, the ongoing discussions on the existence of many negative results such as human health and environmental pollution, as well as the use of them, and the studies of scientific studies on alternative substances have been accelerated. Plant-based proteins have attracted intense interest in recent years because they have the potential to be an

alternative to conventional emulsifiers and stabilizers. The various functional properties and amphiphilic characters feel that these proteins are significantly effective in maintaining the stability of the emulsion system. Soy proteins, like many other vegetable protein types, are a preferred product due to their easy accessibility and processability.

1.1 Purpose of Thesis

The aim of this study is to encapsulate aqueous *Hibiscus Sabdariffa* L. extract in lecithin and soy protein based food-grade High Internal Phase Pickering Emulsions (HIPPE) and High Internal Phase Pickering Double Emulsions (HIPP-DE) systems, to examine the emulsions obtained in terms of emulsion stability and emulsion properties, and to reveal the effects of *in vitro* gastrointestinal digestion on phenolics and anthocyanins in the context of bioavailability. Although various studies researched the encapsulation of bioactive compounds, no aqueous *Hibiscus* extract encapsulation within food grade HIPPE and HIPP-DE systems have been studies to the best of our knowledge.

1.2 Literature Review

Hibiscus Sabdariffa L., belonging to the Malvaceae family, has garnered global recognition as a medicinal plant in the field of human health (Ismail et al., 2008). Commonly known as Roselle, it is referred to by various names such as Kenaf, Karkade, and Asam paya, varying across different countries or regions (Ismail et al., 2008). Widely distributed and cultivated in tropical and subtropical regions, including Africa, India, and Sudan, *H.Sabdariffa* exhibits distinctive characteristics such as smooth, cylindrical red stems and green leaves (Da-Costa-Rocha et al., 2014). *H.Sabdariffa* as a valuable ingredient in the food industry, particularly in the development of smoothies, beverages, jellies, and confectionery products. Its natural reddish color is attributed to the presence of water-soluble color pigments, primarily anthocyanins, offering a preferable alternative to synthetic colorants (Amer et al., 2022). In response to increasing consumer demand for healthier and more natural food options, the replacement of artificial colorants with *H.Sabdariffa*-derived pigments has gained significant attention (Ko et al., 2017). From a food science perspective, *H.Sabdariffa* possesses notable nutritional and bioactive compounds that contribute to

its medicinal properties. The calyces of the plant exhibit a rich composition of carbohydrates, proteins, fats, and various phytochemicals (Jabeur et al., 2017). These phytochemical constituents, including anthocyanins, flavonoids, and phenolics, have been extensively studied for their therapeutic effects in treating diverse diseases (Formagio et al., 2015). Notably, *H.Sabdariffa* demonstrates a wide range of biological activities, encompassing antibacterial and antioxidant properties, cancer chemoprevention, appetite suppression, lipid metabolism modulation, and beneficial effects on hypertension and gastrointestinal issues (Da-Costa-Rocha et al., 2014; Zhen et al., 2016). These therapeutic effects are attributed to bioactive compounds present in *H.Sabdariffa*, such as phenolic acids, flavonoids, anthocyanins, and organic acids (Herranz-López et al., 2018; Riaz & Chopra, 2018). Table 1.1 represents the nutritional and bioactive compounds in *Hibiscus Sabdariffa*.

Table 1.1: Nutritional and bioactive compounds in Hibiscus Sabdariffa.

Compound	Bioactive compound	Amount	Reference
Carbohydrates		87 ± 1 g/100 g dw	Jabeur et al. (2017)
Fibers	Dietary fiber (total)	33.9 ± 3.56 g/100 g dw	
Proteins		5.5 ± 0.4 g/100 g dw	
Fats		0.59 ± 0.06 g/100 g dw	
Phenolic acid & Flavonoids	Chlorogenic acid	7.55 ± 2.75 g/100 g dw	Zhen et al. (2016)
	Quercetin	3.2 g/ 100 g dw	Alarcón-Alonso et al. (2012)
	Rutin	2.1 g/ 100 g dw	
	5-O-Caffeoyl-shikimic acid	11.43 ± 0.87 g/100 g dw	Piovesana et al. (2019)
	Quercetin-3-rutinoside	7.77 ± 0.28 g/100 g dw	
Anthocyanins	Delphinidin-3-sambubioside	218.17 ± 12.69 g/100 g dw	
	Cyanidine-3-sambubioside	70.42 ± 5.26 g/100 g dw	

The identification and quantification of bioactive compounds play a crucial role in understanding the intricate chemistry of plant tissues, which are influenced by a diverse array of primary and secondary metabolites. In order to perform accurate analytical procedures, it is imperative to prepare a representative sample that reflects

the real product matrix. Particularly when dealing with complex sample matrices containing multiple components, it becomes essential to purify or separate non-target compounds from the sample before analysis to prevent interference with the targeted compounds (Hapsari & Setyaningsih, 2021). Extraction is commonly employed as a sample preparation step to isolate the desired compounds from the sample matrix (Bylda et al., 2014). However, the complexity of phenolic compounds and their interactions with other constituents in the matrix present challenges in developing an optimal extraction method capable of extracting multiple analytes from such a complex matrix. Thus, the selection of an appropriate extraction method is pivotal in the analytical procedure. Traditional extraction methods like Soxhlet, maceration, percolation, and infusion have traditionally been favored for various samples due to their simplicity (Hidayat & Wulandari, 2021; López-Bascón & De Castro, 2020). Nonetheless, novel extraction methods such as microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and ultrasound-assisted extraction (UAE) have emerged in recent years, specifically targeting the extraction of phenolic compounds (Mena-García et al., 2019). These advanced techniques have gained popularity due to their ability to reduce extraction time and solvent consumption (Jiang et al., 2021). Regarding the extraction of bioactive compounds from *H. Sabdariffa*, UAE, MAE, and SFE are the preferred methods (Cassol et al., 2019; Paraíso et al., 2019; Pimentel-Moral et al., 2019).

Among the alternative novel extraction techniques, microwave-assisted extraction (MAE) operates through a unique heating mechanism that utilizes radiation within the frequency range of 300 MHz to 300 GHz (Hapsari & Setyaningsih, 2021). This method enhances heat energy transfer via dielectric heating and frictional resistance, resulting in efficient extraction (Routray & Orsat, 2012). Extraction time, temperature, solvent properties, and the ratio of product to solvent also influence the effectiveness of this method. Supercritical fluid extraction (SFE) employs a supercritical solvent above its critical point, which exhibits liquid-like properties while maintaining gas-like characteristics (Idham et al., 2021). The low viscosity and high diffusivity coefficient of supercritical solvents enhance extraction rates by facilitating solvent flow and rapid penetration into the matrix, respectively (Da Silva et al., 2016). Consequently, the extraction process is accelerated, enabling efficient extraction of targeted compounds from the product matrix. ultrasound-assisted extraction (UAE) is the most commonly employed method for extracting phenolic compounds from H. Sabdariffa (Hapsari &

Setyaningsih, 2021). It relies on the application of ultrasonic waves at specific frequencies and amplitudes to disrupt cell walls and facilitate the release of compounds into the solvent. This phenomenon is achieved through high shear stress generated by cavitation bubbles (Lefebvre et al., 2021). Optimal extraction parameters such as pulse cycle, ultrasonic power, time, temperature, and solvent characteristics can be adjusted based on the specific product to enhance the extraction efficiency (Pollini et al., 2020). Each extraction method follows a specific procedure tailored to the type of phenolics and sample matrices involved. Typically, the extraction of bioactive compounds from H. Sabdariffa utilizes solvents such as methanol, ethanol and distilled water. The calyces of Hibiscus sabdariffa are readily available in the commercial market and have versatile applications in both medicinal and non-medicinal domains. One notable medicinal application involves their use in the preparation of hot and cold beverages. Research conducted by Alzweiri et al. (2011) indicates that hot H. sabdariffa tea is particularly beneficial for individuals with hypertension, whereas the cold form of the beverage is more suitable for those with hypotension.

In a separate investigation conducted by Halim et al. (2022), the production of jelly candy rich in anthocyanins was examined, utilizing red *Hibiscus Sabdariffa* extract as a natural colorant. The jelly candy formulation involved the incorporation of the extract alongside corn syrup and gelling ingredients. The addition of *Hibiscus Sabdariffa* extract enhanced the candy's visual appeal due to the presence of anthocyanins, which imparted a vibrant coloration. Furthermore, Kim et al. (2019) conducted a study involving the production of functional yogurt incorporating *Hibiscus Sabdariffa*. This research revealed the potential use of *Hibiscus Sabdariffa* as a significant ingredient in dairy products, contributing as a coloring agent and enhancing the functional properties. The sensory analysis results indicated a positive reception. Notably, the authors found it necessary to supplement the yogurt with artificial *Hibiscus* flavor, as the natural flavor from *Hibiscus* calyces was not adequately perceived. Additionally, various studies have investigated the utilization of *Hibiscus Sabdariffa* in diverse food products such as ice cream, pudding, wine, chocolates, and fermented beverages (Adadi et al., 2020; Ai et al., 2021; Homayuni Rad et al., 2021).

Although *Hibiscus* has the potential to be used in different food products, the direct utilization of it into food formulations can be challenging due to three main factors. Firstly, the high concentrations of bioactives may negatively affect the taste and odor

of food, affecting its sensory properties (Nejatian et al., 2022). Secondly, the low solubility of bioactives in aqueous media and their tendency to bind with other components of the food formulation may result in low bioaccessibility in the human body (Choi & McClements, 2020). Finally, bioactives may be sensitive to environmental and operational conditions, such as light exposure, oxidation, thermal processing, and UV irradiation, which can reduce their effectiveness (Ranadheera et al., 2016). Encapsulation techniques have been developed as a solution to the sensitivity of bioactives when incorporated directly into food formulations. Encapsulation involves entrapping bioactives within a carrier or wall material, which can be a protein, polysaccharide, fat, or a combination of different materials (Castro-Enríquez et al., 2020).

Encapsulation methods play a crucial role in preserving and stabilizing bioactive compounds, and several techniques are commonly employed, including spray-drying, freeze-drying, coacervation, liposome formation, and emulsification. Each method possesses distinct advantages and limitations, and the selection of an appropriate technique depends on the specific properties of the bioactive compound and the intended application.

Freeze-drying and spray-drying techniques are particularly effective for solidifying and stabilizing heat-sensitive bioactive components such as polyphenols, anthocyanins, and probiotics (Jouki et al., 2021; Kanha et al., 2021; Rishabh et al., 2021; Šturm et al., 2019). Freeze-drying, being a low-temperature process, is widely regarded as a reliable method for preserving the bioactive constituents. However, it suffers from drawbacks such as being time-consuming and requiring high energy input, thereby posing challenges for large-scale production systems (Kandasamy & Naveen, 2022). Consequently, there is an increasing demand for alternative methods like spray drying, which can produce encapsulated powders with higher efficiency and lower cost on an industrial scale (Piñón-Balderrama et al., 2020).

Coacervation is a process involving the separation of colloidal systems into two distinct phases: one phase consists of hydrocolloids or coacervates, while the other phase constitutes a dilute continuous phase. Two types of coacervation methods are commonly utilized: simple coacervation and complex coacervation. Simple coacervates can be generated by gradually introducing ethanol or sodium sulfate to a protein solution (Aloys et al., 2016). On the other hand, complex coacervates can be formed by associating two oppositely charged phases in an aqueous environment,

under controlled parameters such as pH, polymer concentration, and ionic strength. Notable examples of these distinct phases include combinations of proteins, chitosan and anionic polysaccharides, and proteins and polysaccharides (Baiocco et al., 2021; Muhoza et al., 2022; Warnakulasuriya & Nickerson, 2018). Emulsions have gained widespread recognition as highly effective encapsulation and delivery systems for a wide range of bioactive molecules, including lipophilic, hydrophilic, and amphiphilic compounds (McClements & Li, 2010). These emulsion systems offer efficient encapsulation, contribute to the preservation of the chemical stability of bioactive compounds, and enable controlled release (Karim et al., 2022). Notably, emulsion-encapsulated polyphenols have demonstrated enhanced biological activities compared to their free form, particularly during gastrointestinal digestion (Sun et al., 2018).

The structure of an emulsion consists of two immiscible liquids, typically oil and water, with one serving as the dispersed phase and the other as the continuous phase (Tadros, 2013). Emulsions can be further classified based on the distribution of the oil and water phases. An oil-in-water (O/W) emulsion refers to a system in which oil droplets are dispersed within a continuous water phase, as found in milk or mayonnaise (Clark, 2013). Conversely, a water-in-oil (W/O) emulsion is formed by dispersing water droplets within the oil phase, akin to the composition of butter. The unique characteristics of these different phases directly influence the selection of bioactive substances suitable for encapsulation.

Significant advancements in scientific research have resulted in the development of diverse emulsion systems specifically designed for the encapsulation and delivery of bioactive compounds, thereby unlocking their remarkable health benefits. These emulsion systems play a pivotal role in the food industry by serving as encapsulation platforms for incorporating bioactives into various food formulations. One prominent emulsion system employed for bioactive encapsulation is High Internal Phase Emulsion (HIPEs).

HIPEs, also known as high internal phase emulsions, are characterized by a substantial volume of the internal phase, constituting at least 74% (Shi et al., 2020). This higher internal volume fraction distinguishes HIPEs from traditional emulsions. Notably, HIPEs offer several advantages in terms of encapsulating and preserving bioactive compounds. The elevated volume fraction of the internal phase allows for a greater loading capacity of bioactives, enabling the encapsulation of higher concentrations of active compounds compared to conventional emulsion systems (Díaz-Ruiz et al.,

2021). Depending on the nature of the bioactives to be encapsulated, the internal phase of HIPEs can be composed of either water or oil (McClements et al., 2007). Controlled release is another crucial attribute of HIPEs, enabling the gradual and sustained release of encapsulated bioactives over time (Jin et al., 2017). This controlled release mechanism offers potential for targeted and prolonged delivery of bioactive compounds (Katouzian & Jafari, 2017).

HIPEs find extensive applications in various industries, including food, pharmaceuticals, and cosmetics (Gao et al., 2021). They have been effectively utilized for encapsulating a wide range of bioactive compounds, such as vitamins, essential oils, and plant extracts (Chen et al., 2020; Díaz-Ruiz et al., 2021; Feng et al., 2023). In addition to HIPEs, double emulsions (DEs) have gained widespread usage for bioactive encapsulation. Double emulsions, also known as multiple emulsions or W/O/W emulsions, consist of nested emulsion systems (Clegg et al., 2016). The active compound is typically enclosed within the internal aqueous phase, which is surrounded by an oil phase and an external aqueous phase (Kaimainen et al., 2015). This layered structure provides an additional barrier and a controlled environment for the encapsulated compounds. By adjusting the properties of the internal and external aqueous phases, controlled release of bioactives can be achieved (Giroux et al., 2016). Lecithin, Tween, Span, and PGPR are commonly preferred emulsifiers employed to stabilize emulsion systems. Lecithin, derived from sources such as soybeans and eggs, is a natural emulsifier with both lipophilic and hydrophilic properties (Wang et al., 2021). Tween and Span are frequently used together as emulsifiers, with Tweens being hydrophilic and Spans being lipophilic (Mosca et al., 2013). Polyglycerol polyricinoleate (PGPR), a hydrophobic semi-synthetic emulsifier, is commonly utilized for emulsion stabilization (Márquez et al., 2010). However, due to increasing concerns regarding health and the environment, particularly in the food industry, there is a growing reluctance to use synthetic surfactants as emulsifiers. Synthetic surfactants have been found to be detrimental to beneficial microorganisms, contribute to environmental pollution, and pose risks to human health (Rasheed et al., 2020). Consequently, there is a need to explore eco-friendly alternatives that promote good health to replace these synthetic emulsifiers. These emulsifiers and stabilizers, which are preferred in food emulsion systems, can cause many side effects such as abdominal pain, cramps and diarrhea from a certain dose. In addition, consumers may have negative attitudes towards these ingredients, which are in the food additives class. One

promising alternative is the use of solid particles as stabilizers in emulsions, known as Pickering emulsifiers (PE). PEs offer several advantages over surfactant-stabilized emulsions. They can prevent droplet or particle coalescence in the dispersed phase and reduce toxicity (Chevalier & Bolzinger, 2013; Wu & Ma, 2016). PEs have the potential for increased utilization in the food and pharmaceutical industries (Shi et al., 2016). The use of proteins as an emulsifier can both prevent these side effects and consumer concerns and enrich the nutritional content of the products. Various solid particles, including nanoparticles and microparticles, can be employed to stabilize PEs. Pickering particles derived from polysaccharides, proteins, and polyphenols have vast areas of application. Among these, proteins, particularly plant-based proteins, have gained significant attention in recent years. Proteins offer a wide range of options for creating emulsions with diverse characteristics. Protein-based nanoparticles provide advantages such as improved emulsion stability, controlled release of bioactive compounds, and enhanced texture and mouthfeel in food products. Their amphoteric nature, exhibiting both hydrophilic and hydrophobic properties, makes proteins particularly suitable for producing stable food-grade Pickering emulsions (Lam et al., 2014). In summary, proteins serve as versatile and nutritious emulsifiers, enabling the development of various emulsion-based food products with enhanced stability and functionality. Plant-based proteins such as soy, pea, kafirin, and zein offer a wide range of options for Pickering particles and emulsion stabilization (De Folter et al., 2012; Liu & Teng, 2016; Shao & Tang, 2016; Xiao & Huang, 2015). Soy protein isolate (SPI), a prominent soy protein product commercially available, is produced through a conventional alkali extraction-acid precipitation process (Verfaiilie et al., 2023). These proteins possess an amphipathic nature, exhibiting both hydrophilic and hydrophobic properties, which enables them to diffuse, adsorb, and stabilize the interfaces of oil droplets during emulsification (Djuardi et al., 2020; Yan et al., 2021). Table 2.2 presents the different studies about the encapsulation of bioactives within High Internal Phase Pickering Emulsions (HIPPEs) and Double Emulsions (DEs) stabilized by soy protein isolate (SPI).

Table 1.2: Encapsulation of bioactives within HIPPEs and DEs stabilized SPI.

Emulsion	Emulsifiers/Stabilizers	Bioactive compound	Effect of encapsulation	Reference
HIPPE	Soy protein isolate	β-Carotene	The retention of β-carotene in HIPPEs was found to be 74.1%, which is significantly higher compared to that in corn oil.	Wang et al., 2022
HIPPE	Soy protein isolate – epigallocatechin-3-gallate covalent composite microgel particles	β-carotene	After 42 days of storage, the HIPPEs exhibited a higher retention rate and bioaccessibility of β-carotene	Geng et al., 2022
HIPPE	Soy protein isolate– dextran complexes	Quercetin	High encapsulation efficiency for (98.19 %)	Du et al., 2022
DE	Bacterial cellulose nanofibers/soy protein isolate/chitosan complex	Curcumin	Higher bioaccessibility of curcumin (77.4%) compared to extract (32.8 %)	Shen et al., 2023
DE	Soybean protein-polyglutamic acid complex	Nattokinase	Increased bioaccessibility up to 80.69%.	Li et al., 2023

Several studies in the literature have provided evidence that the addition of lecithin can enhance the stability of emulsions containing soybean protein and whey protein, even when the conditions are close to the proteins' isoelectric point (Mantovani et al., 2013; Scuriatti et al., 2003). Additionally, Comas et al. (2006) observed improved stability in emulsions composed of native or denatured soybean protein isolates, sunflower oil, and water when lecithin was included. The interaction between proteins and lecithin is attributed to hydrophobic interactions, which play a crucial role in maintaining the protein-phospholipid interactions. Recent research conducted by Li et al. (2014) further supports the notion that lecithin and soybean protein can interact through electrostatic and hydrophobic interactions, leading to favorable changes in protein conformation and improved emulsifying properties.

2. MATERIALS AND METHODS

2.1 Materials

Soy protein isolate (90% protein content) was supplied by Vegrano Co., Ltd. (Istanbul, Türkiye). Corn oil was purchased from a local supermarket (Istanbul, Türkiye). *Hibiscus Sabdariffa* L. calyces were supplied by TOS-The Organic Spices Co., Ltd. (Antalya, Türkiye). Soybean lecithin was purchased from Sigma-Aldrich. All the chemicals utilized in this study were analytical grade.

2.2 Preparation of Aqueous *Hibiscus* Extract (AHE)

Extraction procedure was conducted based on the study of Pinela et al. (2019) with some modifications. Dried *Hibiscus* calyces were ground to obtain powder with a blender (Kiwi Classic 575, Bad Homburg, Germany) for 30 s at 22,000 g and stored at 4°C temperature in the dark, until use. After weighing 1.5 g of powder into falcon tubes (50 ml), 50 ml of distilled water was added (Pinela et al., 2019). The prepared mixtures were vortexed for 10 s and kept in an ultrasonic bath (USC900TH; VWR, Radnor, PA, USA) for 30 minutes. All samples were centrifuged at 2700 x g (4000 RPM) for 10 minutes at 4 °C (Universal 32R; HettichZentrifugen, Tuttlingen, Germany), and the supernatants were collected. Finally, the resulting supernatant was filtered using Whatman filter paper No. 4 to obtain the desired extract.

2.3 Preparation of Soy Protein Isolate (SPI) Gel

Soy protein isolate (SPI) solutions with protein concentrations of 2%, 4%, and 6% were prepared. The solutions were left overnight with stirring using a magnetic stirrer and centrifuged to remove non-hydrated proteins (Urbonaite et al., 2015). After complete hydration, SPI solutions were heated in a water bath at 95 °C for 15 minutes, followed by rapid cooling in an ice bath to reach room temperature (Huang et al., 2022).

2.4 Preparation of Emulsions

2.4.1 High internal phase pickering emulsions (HIPPEs)

Different emulsions with varying SPI gel and lecithin concentrations were prepared. These include only corn oil and pure aqueous Hibiscus extract (control), SPI (2%, 4% and 6%) and Hibiscus extract mixture and corn oil (without lecithin), containing Hibiscus extract and corn oil (2%, 4% and 4% lecithin concentration) and finally SPI (2%, 4% and 6%)-Hibiscus extract and corn oil (with 2%, 4% and 4% lecithin concentrations) emulsions (Table 2.1). Initially, 20 mL corn oil mixture was prepared at different lecithin concentrations (0%, 2%, 4%, 6%). To ensure efficient mixing of the lecithin with the corn oil, the mixture was stirred on a magnetic stirrer at 5000 rpm for 10 minutes. 80 mL of SPI gel solutions were mixed with aqueous Hibiscus extract using a magnetic stirrer (5000 rpm, 3 min). Mixing continued until a homogeneous SPI-Hibiscus mixture is obtained. First, SPI-Hibiscus extract was mixed with lecithin-free corn oil. Then, SPI-Hibiscus mixture was added to corn oil samples containing lecithin (2%, 4%, 6%). Finally, pure *Hibiscus* extract (without SPI gel) was mixed with corn oil phase containing lecithin. After homogenization, the presence/absence of W/O emulsions with 80% internal volume fraction was checked. In each homogenization process, Ultraturrax (IKA, T18) was run at 12000 rpm for 3 minutes.

Table 2.1: HIPPEs with different formulations.

Phase	**Corn oil (without lecithin)**	**Corn oil (2% lecithin)**	**Corn oil (4% lecithin)**	**Corn oil (6% lecithin)**
Hibiscus extract	Control	L2	L4	L6
Hibiscus extract + 2 % SPI gel	S2	L2S2	L4S2	L6S2
Hibiscus extract + 4 % SPI gel	S4	L2S4	L4S4	L6S4
Hibiscus extract + 6 % SPI gel	S6	L2S6	L4S6	L6S6

2.4.2 High internal phase pickering-double emulsions (HIPP-DEs)

Each 50 mL of W/O HIPPE, which did not show phase separation after 24 hours, was homogenized with 50 mL 6% SPI gels with the help of Ultraturrax (12000 rpm, 3 min) and it was examined whether W/O/W was formed or not. Table 2.2 presents HIPP-Des with different formulations

Table 2.2: HIPP-DEs with different formulations.

W/O/W HIPP-DEs	Formulation
L4-D	L4+6% SPI gel
L6-D	L6+6% SPI gel
L2S6-D	L2S6+6% SPI gel
L4S2-D	L4S2+6% SPI gel
L4S4-D	L4S4+6% SPI gel
L4S6-D	L4S6+6% SPI gel
L6S2-D	L6S2+6% SPI gel
L6S4-D	L6S4+6% SPI gel
L6S6-D	L6S6+6% SPI gel

2.5 Storage Stability

Following the preparation of the emulsions, they were promptly stored at a temperature of 4 °C for a duration of 24 hours. During this storage period, stability observation was conducted to determine whether any phase separation occurred within the emulsions.

2.6 Encapsulation Efficiency

The encapsulation efficiency (EE) of the HIPPEs and HIPP-DEs stabilized by lecithin and SPI gel was determined based on the study of Du et al. (2022) with slight modifications. 1 mL of each emulsions and 5.0 mL of ethanol was vortexed for 30 seconds. Then centrifugation was applied at 8000 x g and for 10 minutes. The Total Anthocyanin Content (TAC) and Total Phenolic Content (TPC) of the supernatant was analyzed. The encapsulation efficiency of each emulsion was calculated using Eqs. (2.1) and (2.2).

$$\text{EEA (\%)} = 1 - \frac{TAC\ of\ supernatant}{TAC\ of\ emulsion} \tag{2.1}$$

$$\text{EEP (\%)} = 1 - \frac{TPC\ of\ supernatant}{TPC\ of\ emulsion} \tag{2.2}$$

2.7 Determination of Emulsifying Properties

The emulsifying properties of HIPPEs and HIPP-DEs were analyzed according to the method of Li et al. (2014). 50 mL of emulsions were sampled immediately after preparation and after 10 min. All samples were diluted 100 times using 0.1% sodium dodecyl sulfate (SDS). The absorbance of the samples immediately after preparation (A_0) and after 10 min (A_{10}) using a spectrophotometer (VWR UV-3100PC, VWR International, San Francisco, CA). The emulsifying activity index (EAI) and emulsion stability index (ESI) were calculated using Eqs. (2.3) and (2.4), respectively.

$$\text{ESI (min)} = \frac{A_0}{A_0 - A_{10}} \text{ x } (T_{10} - T_0) \tag{2.3}$$

$$\text{EAI } (m^2/g) = 2 \text{ x T x } \frac{A_0 \text{ x N}}{10000 \text{ X } \theta \text{ X L X C}} \tag{2.4}$$

Where A_0 is the absorbance at 0 min; A_{10} is the absorbance at 10 min; T_{10} is the time at the 10 min; T_0 is the time at the 0 min; T equals to 2.303; N is the dilution factor (100); θ is the proportion of the oil phase (0.2); L is the thickness of the cuvette (1 cm); C is the concentration of the SPI (g/ml).

2.8 Particle size, PDI and Zeta Potential

The particle size, PDI and zeta potential of the each emulsions were measured by dynamic light scattering device (Nano-ZS, Malvern Instruments, Malvern, UK) based on the study of Karaca et al. (2011).

2.9 Creaming Stability

The creaming stability analysis was carried out according to the method of Aslan & Dogan. (2018) with slight modifications. Creaming index calculation was used to determine the tendency of emulsions to separate into two phases, and it involves filling a falcon tube with 30 ml of emulsion sample and monitoring it for 14 days at 4°C. The height of the clear liquid layer on top of the emulsion (serum) and the total height of the emulsion were measured on days 1, 7, and 14. To calculate the creaming index value, the height of the serum (H_s) was divided by the total height of the emulsion (H_t) and multiplied by 100. The resulting creaming index value can range from 0 to 100 and indicates the degree of creaming tendency of the emulsion, with higher values indicating a greater tendency to separate (Aslan & Dogan, 2018).
The following formula (2.5) was used to calculate the creaming index value:

$$\text{Creaming index} = \frac{H_S}{H_t} * 100 \qquad (2.5)$$

2.10 Centrifugation Stability

The HIPPEs and HIPP-DEs (30 mL) were transferred into 50 mL falcon tubes and subjected to centrifugation at 5000 rpm for a duration of 20 minutes (Li et al., 2022). The visual appearance of the emulsions after centrifugation was evaluated.

2.11 *In Vitro* Gastrointestinal Digestion

The *in vitro* gastrointestinal digestion protocol was conducted, making certain modifications based on the research conducted by Minekus et al. in 2014. This protocol aimed to simulate the process of digestion in the oral, gastric and intestinal digestions. During the simulation of oral digestion, each emulsion (5 mL) was mixed with salivary juice (4 mL), 0.3 mol/L $CaCl_2$ (25 µL), and distilled water (975 µL). The mixture was then incubated for two minutes at 37 °C in a shaking water bath (Memmert SV 1422,

Memmert GmbH & Co. Nürnberg, Germany). Next, the simulation of gastric digestion took place by adding gastric juice (7.5 mL), pepsin (1.6 mL), and $CaCl_2$ (5 µL) to the solution. The pH of the mixture was adjusted to 3.0 using 1 mol/L HCl, and distilled water was added for volume adjustment. The mixture was incubated once again for two hours at 37 °C in a shaking water bath. After simulating gastric digestion, 5 mL aliquots were taken from each mixture. The gastric mixture was then combined with intestinal juice (8.25 mL), pancreatin (3.75 mL), bile (1.875 mL), and $CaCl_2$ (30 µL). The pH of the mixture was adjusted to 7.0 using NaOH, and the total volume of the mixture was completed to 30 mL using distilled water. The mixture was incubated for two hours in a shaking water bath at 37 °C. Simultaneously, a blank sample containing only water instead of emulsion was prepared. Following the incubation period, the simulated gastric and intestinal samples were centrifuged for five minutes at 4000 rpm and 4 °C (Hettich, Tuttlingen, Germany). The resulting supernatants were stored at -20 °C until further analysis.

2.12 The Quantification of Polyphenols with HPLC-DAD

Polyphenols in the samples were quantified using the method described by Capanoglu et al. (2008). Prior to analysis, the samples were passed through a 0.45 µm membrane filter (Waters 2996) and then injected into a Waters 2695 HPLC system equipped with a PDA detector. The column used was a Supelcosil LC-18 (25 cm x 4.60 mm, 5 µm) from Sigma-Aldrich in Steinheim, Germany. Spectral measurements were performed at wavelengths of 280 nm, 312 nm, and 360 nm. The eluents used were TFA/MQ water (1 mL/L; eluent A) and TFA/acetonitrile (1 mL/L; eluent B). The injection volume was 10 mL or the flow rate was set at 1 mL per minute. The gradient elution profile was as follows: at 0 minutes, 95% solvent A and 5% solvent B; at 45 minutes, 65% solvent A and 35% solvent B; at 47 minutes, 50% solvent A and 50% solvent B; and at 54 minutes, the system returned to its initial state. Phenolic acids were quantified using authentic standards. The results of each analysis were reported as mg/100 g dry sample weight, and each analysis was performed in triplicate.

2.13 Spectrophotometric Assays

2.13.1 Total anthocyanin content (TAC)

The total anthocyanin content (TAC) was determined using the pH-differential method as described by Giusti & Wrolstad. (2001). To perform the analysis, 1 mL of the extracted solution was transferred into a 10 mL volumetric flask to prepare two dilutions of the sample. One dilution was adjusted to volume with potassium chloride buffer at pH 1.0, and the other dilution was adjusted to volume with sodium acetate buffer at pH 4.5. Both dilutions were allowed to equilibrate for 15 minutes. The absorbance of each dilution was then measured at 510 nm and 700 nm, using a blank cell filled with distilled water as the reference (Giusti & Wrolstad, 2001).

2.13.2 Total phenolic content (TPC)

Total phenolic content (TPC) assay was carried out according to the method of Singleton and Rossi (1965). The experiment with Folin–Ciocalteu reagent was implemented at 765 nm. Calibration curve was plotted using gallic acid in the range of 0.01–0.4 mg/ml and TPC results were reported as mg gallic acid equivalents (GAE) per 100 g dw sample.

2.13.3 Antioxidant activity

The antioxidant capacities of the infusions were determined by both 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Molyneux, 2004) and 2,2‘-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Nenadis et al., 2004) assays. Measurements were performed at 450 and 517 nm, respectively. Calibration curves were plotted using Trolox in the range of 0.01–0.4 mg/ml and results were reported as Trolox equivalents (TE) per 100 g of dw sample.

2.13.4 Bioaccessibility calculations

The bioaccessibility was calculated using following formula;

Bioaccesibility (%) = $(BC_{digested}/BC_{non\text{-}digested})$ x100

Where $BC_{digested}$ was the amount of bioactive compounds (TAC, TPC) recovered in the supernatants of the centrifuged final digesta. $BC_{non\text{-}digested}$ was the amount of bioactive compounds in non-digested rosehip infusion.

2.14 Statistical Analysis

All experiments were conducted with a minimum of three replicates to ensure statistical robustness. The error bars displayed on the figures represent the standard deviations of the data. The results are presented as the mean value ± the standard deviation. For statistical analysis, the SPSS software (version 20.0, SPSS Inc., Chicago, IL, USA) was utilized. The processing conditions were evaluated using a one-way analysis of variance (ANOVA), followed by a Tukey post hoc test. A significance level of $p < 0.05$ was considered statistically significant.

3. RESULT AND DISCUSSION

3.1 Storage Stability

Storage stability of emulsions were determined based on whether any phase separation occurred during 24 h storage at 4 °C. Visual examination revealed phase separation in the control emulsion after approximately 2 min at room temperature. Figure 3.1 exhibits products stabilized by only SPI gel. As can be observed, these products could not provide stability for 24 h and even phase separation occurred in about 5 minutes. For this reason, it has been observed that a stable emulsion system cannot be obtained by using only SPI gel.

Figure 3.1: HIPPEs stabilized by only different SPI gel concentrations.

Figure 3.2 represents products stabilized by only lecithin. As can be observed, only L2 could not provide stability for 24 h and even phase separation occurred in about 8

minutes. However, L4 and L6 presented 24 h stability against phase separation. For this reason, it can be stated that increase in lecithin concentration provided enhanced stability for emulsions.

Figure 3.2: HIPPEs stabilized by only different lecithin concentrations.

In the case HIPPEs containing both SPI gel and lecithin, variations in storage stability against phase separation were observed. Figure 3.3 shows highly stable HIPPEs against phase separation for 24 h and even phase separation could not occured for 45 h. As it can be stated from here, a synergistic mechanism of action became dominant in HIPPEs where SPI gel and lecithin were used at different concentrations and significantly prevented phase separation.

Figure 3.3: HIPPEs stabilized by different SPI gel and lecithin concentrations.

Each 50 mL of stable W/O HIPPE, which did not show phase separation after 24 hours, was homogenized with 50 mL 6% SPI gels. Thus, HIPP-DEs can be termed as HIPPEs with double emulsions morphology. All HIPP-DEs showed a excellent resistance to phase separation. Figure 3.4 demonstrates highly stable HIPP-DEs against phase separation for 24 h.

Figure 3.4: HIPP-DEs stabilized by different SPI gel and lecithin concentrations.

According to these observed results, it can be said that lecithin and soy protein isolate present a synergistic mechanism of action. Different studies have indicated that lecithin can interact with zein nanoparticles and exhibit synergistic effects to enhance emulsion stability (Chuacharoen & Sabliov, 2016; Dai et al., 2016). Taking this into consideration, lecithin was incorporated to improve the stability of SPI-stabilized High Internal Phase Pickering Emulsions (HIPPEs). Additionally, a two-step emulsification

process can generate a double emulsion morphology. The findings suggest that lecithin plays a role also in forming the HIPP-DE structure, potentially because lecithin is an lipophilic surfactant that can stabilize water-in-oil emulsions on its own (Knoth et al., 2005).

3.2 Creaming Stability

The creaming in emulsions is caused by instabilities resulting from the differences in densities between the oil and water phases, as well as the gravitational force acting on them (Tadros, 2004). Therefore, measuring the amount of cream that separates over a given time period is a useful method for evaluating the stability of the emulsion. The creaming stability of the emulsions was evaluated by both CI calculation and visual observation during storage for 12 h, 1, 5 and 9 days, and the results were presented in Figs. 3.5 and 3.6. While the CI value did not show significant difference between HIPPEs on the 12th, differences became observable on day 1 ($p<.05$). The findings of this study also indicate that in HIPPEs with 6 % SPI gel concentration, the increase in soybean lecithin concentration from 2% to 4 % resulted in a decrease in the creaming rate from 24.12 ± 0.45% to 17.79 ± 0.32% (day 1). These results suggest that the addition of lecithin enhances the stability of the system, particularly at a concentration of 4%. However, further increases in the lecithin concentration up to 6% in HIPPEs result in a significant increase in CI. Similar results were also obtained for day 5.). A different result was obtained for HIPP-DEs. Here, the creaming index value did not show a significant difference between emulsions ($p<.05$). The findings of Wang et al. (2017) indicate that as the lecithin concentration increases from a certain concentration, the creaming index (CI) value also increases, indicating a higher degree of instability in the emulsion. The increase in CI is attributed to the sensitivity of the adsorbed layer to external forces, as the instability observed can be explained by the inability of the interfacial protein layer to withstand surface pressure, resulting in the collapse of protein molecules into the serum phase and noticeable creaming of the emulsions (Wang et al., 2017). Thus, it can be concluded that the stability of the emulsion was improved to a certain extent by the addition of lecithin, and that a concentration of 4% was the most effective concentration for enhancing the creaming stability of the emulsion. In HIPP-DEs, on day 5 and day 9, CI values did not show a statistical difference between emulsions ($p<.05$).

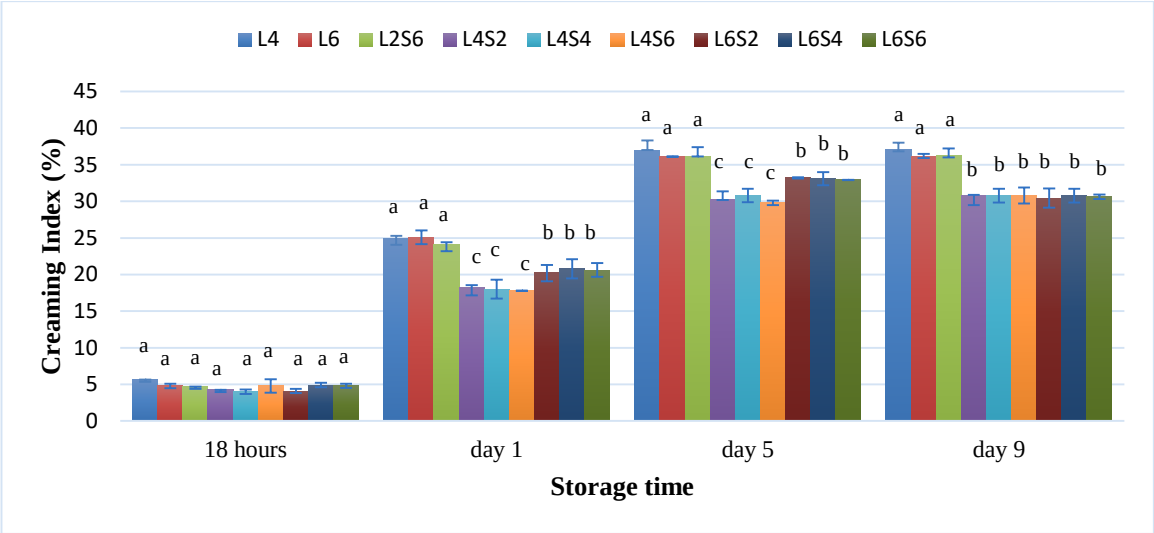

Figure 3.5: Changes in creaming index (CI) of HIPPEs during storage.

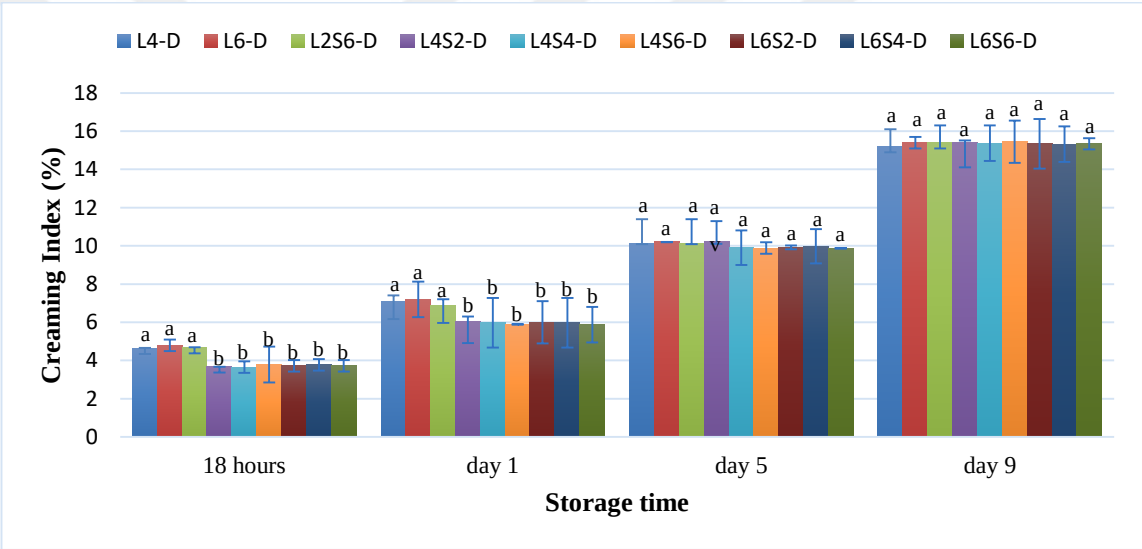

Figure 3.6: Changes in creaming index (CI) of HIPP-DEs during storage.

3.3 Centrifugation Stability

The HIPPEs and HIPP-DEs (30 mL) were transferred into 50 mL falcon tubes and subjected to centrifugation at 5000 rpm for a duration of 20 minutes (Li et al., 2022). The visual appearance of the emulsions after centrifugation was evaluated. As can be clearly observed from Figure 3.7 and Figure 3.8, phase separation was observed only in L4 and L6 in HIPPEs. No phase separation was observed in HIPP-DEs. It can be said that the reason for this situation is that emulsions using only lecithin cannot resist centrifugation, and SPI gel plays an important role in providing emulsion stability.

Figure 3.7: Degree of phase separation of L4 and L6 after centrifugation.

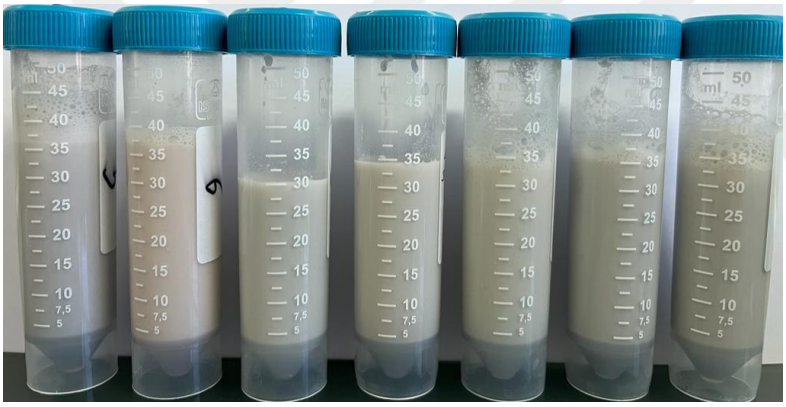

Figure 3.8: HIPP-DEs after centrifugation.

3.4 Determination of emulsifying properties

The large interfacial area between the water and oil phases results in poor thermodynamic stability (Abdulredha et al., 2022). Therefore, flocculation, coalescence, and phase separation can occur in the structure of emulsions (Jin et al., 2021). The ability of a protein to adhere to the oil/water interface is determined by its emulsifying property (Liu & Wang, 2021). Determining the emulsifying properties of proteins that localize themselves at the oil/water interface in order to reduce the interfacial tension (Baldino et al., 2023) is necessary to achieve the desired emulsion stability. Also, the effects of commonly used emulsifiers on the specific emulsion systems is a subject that needs to be examined. In this study, the emulsification activity

index (EAI) and emulsification stability index (ESI) were measured to determine emulsifying property. The ESI measures the turbidity of an emulsion, whereas the EAI indicates the interfacial area (m^2) that is stabilized for each weight (g) unit of a protein (Cha et al., 2019). Soy protein isolate (SPI), an important plant-based protein, possesses amphiphilic properties that allow its use as an emulsifier in food products (Deng, 2021; Liao et al., 2021). Lecithin, mixture of phospholipids, phosphatidylcholine, ethanolamine, and inositol, is one of the emulsifiers that actively reduces the surface tension in emulsion production (Wiyani et al., 2016).

This study aimed to evaluate the EAI and ESI of HIPPEs and HIPP-DEs stabilized by SPI and soy lecithin. The emulsions were shown to have different EAI and ESI values were depicted in Table 3.1. In HIPPEs containing 4% and 6% lecithin, EAI and ESI values increased statistically when the SPI concentration increased from 2% to 4% ($p<.05$). The same observation was also achieved for HIPP-DEs (Table 3.2). In the study of Pirestani et al. (2017), an increase in EAI and ESI values with increasing SPI concentration was attributed to the adsorption of SPI on the o/w interface can stabilize the system through viscoelastic film formation around oil droplets. Similarly, Guan et al. (2020) showed that the increase in EAI and ESI values was related to the emulsifying capabilities of SPI, which makes oil droplets well dispersed in the emulsion system. Interestingly, no statistically significant increase occurred when the SPI concentration increased from 4% to 6% ($p<.05$). This phenomenon may be due to the protein concentration at the interface exceeding the maximum load and the excess protein left from the interface flocculating by forming hydrophobic interaction, which results in a constant EAI value (Guan et al., 2020). In addition, each HIPP-DE has higher EAI and ESI values than its HIPPEs. The lowest EAI and ESI values were observed for L2S6 and L4 emulsions in HIPPEs and for L2S6-D and L4 emulsions in HIPPDEs. However, L6 and L6-D showed higher EAI and ESI values than these emulsions ($p<.05$).

The alterations in EAI and ESI as a function of different lecithin concentrations are presented. The findings revealed that as the concentration of lecithin increased from 2% to 4%, both EAI and ESI increased gradually and attained their peak values at a lecithin concentration of 4%. However, both EAI and ESI decreased slightly at higher concentration of lecithin (6%) ($p<.05$). This indicated that an intermediate level of lecithin addition led to significant improvement in emulsifying properties. In the study of Zou et al. (2020), this change was attributed to the localization of the majority

of phospholipid molecules on the aqueous side of the oil-water interface, resulting in the formation of protein-lecithin complexes that exhibit enhanced emulsifying properties. The elevation in EAI might be due to the increased exposure of hydrophobic groups which strengthened the interactions between proteins and lipids (Zou et al., 2020). In addition, the decrease in EAI and ESI values with the increase in lecithin concentration from 4% to 6% was explained by the decrease in surface hydrophobicity.

Table 3.1: EAI and ESI values of HIPPEs.

HIPPEs	EAI (m^2/g)	ESI (min)
L4	49.21 ± 0.56^{f}	39.12 ± 0.45^{e}
L6	70.19 ± 0.27^{e}	30.29 ± 1.09^{e}
L2S6	77.12 ± 0.65^{d}	36.46 ± 1.34^{d}
L4S2	85.21 ± 0.34^{b}	45.11 ± 3.67^{b}
L4S4	89.92 ± 0.97^{a}	49.83 ± 0.34^{a}
L4S6	89.96 ± 1.17^{a}	49.96 ± 0.34^{a}
L6S2	80.12 ± 1.21^{c}	39.12 ± 2.34^{c}
L6S4	86.12 ± 0.10^{b}	46.14 ± 1.34^{b}
L6S6	86.18 ± 0.03^{b}	46.10 ± 1.18^{b}

*The results are represented as mean ± standard deviation (n=3).
*Different lowercase letters indicate significant differences between different emulsions ($p < 0.05$)

Table 3.2: EAI and ESI values of HIPP-DEs.

HIPP-DEs	EAI (m^2/g)	ESI (min)
L4-D	79.11±0.12[e]	45.21±0.64[e]
L6-D	80.29±0.78[e]	45.68±1.34[e]
L2S6-D	85.22±0.64[d]	57.12±1.24[d]
L4S2-D	95.01±1.14[b]	60.09±0.04[b]
L4S4-D	99.12±1.21[a]	69.28±2.13[a]
L4S6-D	99.16±3.45[a]	69.92±3.21[a]
L6S2-D	90.09±9.98[c]	60.62±1.67[c]
L6S4-D	95.45±1.24[b]	66.69±1.64[b]
L6S6-D	95.56±1.35[b]	67.09±0.03[b]

*The results are represented as mean ± standard deviation (n=3).
*Different lowercase letters indicate significant differences between different emulsions ($p < 0.05$)

3.5 Particle size, zeta potential and PDI

3.5.1 Particle size

The particle size characteristics of multiple emulsions are depicted in Figure 3.9 and Figure 3.10. In emulsions containing 6% SPI gel, the particle size value exhibited a statistically significant decrease as the lecithin concentration increased from 2% to 4%.

The smallest aggregates were observed at the lecithin concentration of 4%. However, when the lecithin concentration was increased from 4% to 6%, the particle size value increased at 2% and 4% SPI gel concentrations ($p<.05$). According to Sun et al. (2018), as the lecithin concentration increased, the size of whey protein aggregates also increased. According to the study by Teng et al. (2020), the particle size of the emulsion initially decreased with increasing lecithin concentration up to a certain point. However, further increase in lecithin concentration resulted in an increase in particle size. This can be explained by the displacement solubilisation principle, where excessive lecithin concentration caused the whey protein at the oil-water interface to be replaced by lecithin, leading to an increase in the lecithin content in the aqueous phase (Martinez et al., 2011). This reduced the amount of protein at the interface, resulting in larger particle sizes in the emulsion. The interaction between lecithin and whey protein, causing partial unfolding of the protein, was suggested as one possible reason for the observed increase in particle size (Teng et al., 2020). Additionally, the formation of complexes between lecithin and protein could also contribute to the increase in particle size. On the other hand, the decrease in particle size with increasing lecithin concentrations was attributed to the formation of a reverse micelle or vesicle-like structure between protein and lecithin, which led to a reduction in particle size (Dai et al., 2016). Similar trends in particle size changes with increasing lecithin concentrations were observed in studies conducted by Wang et al. (2017) and Zou et al. (2020).

The particle size of HIPPEs with 4% and 6% lecithin concentrations exhibited a significant reduction as the SPI gel concentration increased from 2% to 6%. Specifically, the HIPPE with the lowest particle size (400.03±2.23 nm) was observed in the L6S6 (6% lecithin and 6% SPI gel) emulsion. This trend was also observed in HIPP-DEs, where the lowest particle size(352±3.32 nm) was obtained in L6S6-D (6% lecithin and 6% SPI gel). In the study by Sirovec et al. (2022), it was observed that the addition of pea protein isolate in the emulsification process of oregano and rosemary extracts caused a significant decrease in particle size, with increasing concentrations of pea protein isolate showing a stronger effect. Sohn et al. (2017) explained that increasing the emulsifier concentration leads to improved coverage of oil droplets at the interface, which ultimately leads to a reduction in the size of emulsion droplets.

3.5.2 Zeta potential

Figure 3.9 and 3.10 illustrate the variation in zeta potential among different emulsions. In general, emulsions with higher zeta potential values exhibit enhanced stability due to the increased repulsion between droplets, which is a result of electrostatic forces. This repulsion prevents droplet coalescence (Zu et al., 2019). Zeta potential serves as a quantitative measure of the surface charge density of proteins and acts as an indicator of potential stability of the emulsion system (Li et al., 2020). A higher absolute value of negative zeta potential indicates stronger electrostatic repulsion between droplets, leading to improved emulsion stability (Tamnak et al., 2016). In HIPPEs, the zeta potential was found to be negative and their absolute values increased with increasing SPI gel concentration from 2 % to 4 % (at the 4 % lecithin concentration). The highest absolute value of the zeta potential (-41.21 ± 1.23 mV) was observed for L4S4. In the study of Kwaamba et al. (2015), the increase in the absolute value of zeta potential was attributed to changes in the secondary structure of whey protein induced by soy lecithin, causing the protein structure to become more extended. Conformational changes in protein, resulting in the exposure of negatively charged whey protein residues and a decrease in zeta potential (Kwaambwa, Maikokera, & Nermark, 2015). As a result, this could reduce the exposure of positively charged groups and increase the negative charge of the protein. However, from 4 % to 6 % SPI gel concentration, absolute zeta potential value started to decrease. It was attributed that, an increase in protein content from a certain limit can lead to the formation of protein aggregates that replaced the protein at the oil-water interface, resulting in decreased emulsion stability (Li et al., 2018). Other studies have also shown that high protein concentrations can cause protein molecules to aggregate, making it difficult for water molecules to penetrate them and leading to decreased stability of the emulsions (Berton-Carabin et al., 2014). Also, when the lecithin concentration increased from 4 % to 6 %, decrease in absolute zeta potential values was observed for L6S2, L6S4 and L6S6. From a certain concentration, the interaction between lecithin and SPI results in electrostatic and hydrophobic interactions, which significantly affects the functional properties of SPI. The results of the research also suggest that in HIPPEs that contain 6% SPI gel the absolute ζ-potential increased from 30.88 ± 1.12 to 35.7 ± 0.98 as the lecithin concentration increased from 2% to 4%, with this change being significant ($P < 0.05$) at a concentration of 4%. This decrease can be attributed to the unfolding of proteins

caused by lecithin binding, which facilitates the exposure of negatively charged groups in SPI, thus reducing the zeta-potential (Zou et al., 2020). In the literature, there are some studies which states that the lecithin addition increases the absolute zeta-potential of casein and whey protein (García-Moreno et al., 2017; Wang et al., 2017). Additionally, the electrostatic attraction between negatively charged phosphate groups in lecithin and positively charged amine groups in SPI also contributes to the reduction in zeta-potential (Surh et al., 2006). HIPP-DEs, as shown in Figure ??, exhibited higher absolute zeta potential values compared to HIPPEs. Among the HIPP-DEs, L4-D and L6-D had lower absolute zeta potential values compared to the other samples. However, there were no statistically significant difference observed among the remaining HIPP-DEs. It should be noted that HIPP-DEs have a higher concentration of soy protein isolates compared to HIPPEs. This higher concentration of soy protein isolates may have contributed to the increase in negative surface charge, thus resulting in the significant difference in zeta potential values.

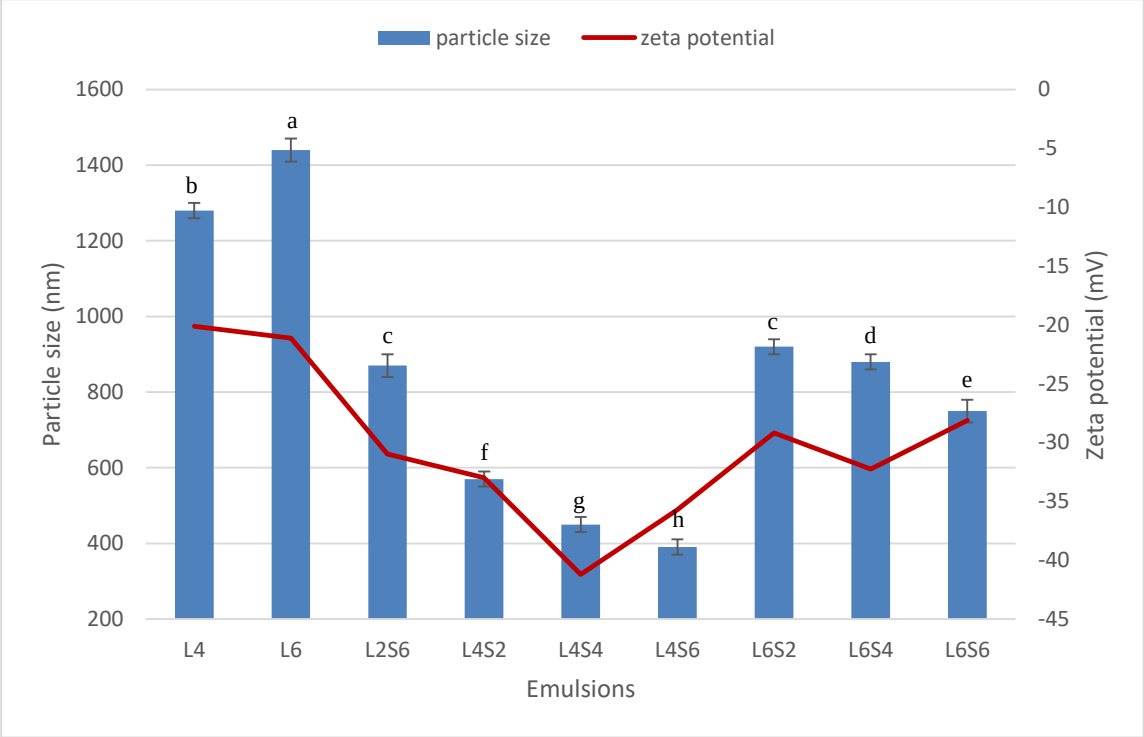

Figure 3.9: Change in the particle size and zeta potential between different HIPPEs.

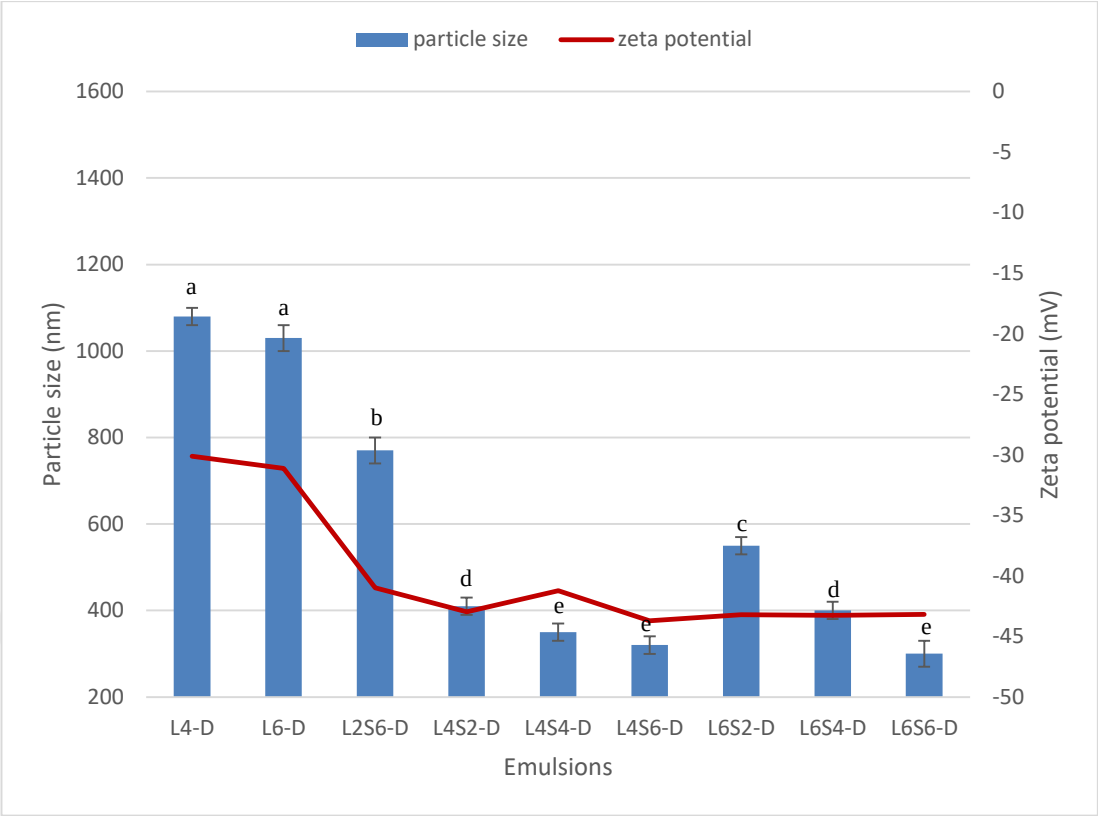

Figure 3.10: Change in the particle size and zeta potential between different HIPP-DEs.

3.5.3 PDI

The graph in Figure 3.11 illustrates how the PDI varies among emulsions. Increasing the concentration of soy protein isolate (SPI) from 2% to 6% led to significant decreases in the polydispersity index (PDI) of HIPPEs containing 4 % and 6 % lecithin. Lowest PDI values were observed when the SPI concentration was 6%. Same decrease was also observed HIPP-DEs (Figure 3.12). In the study of Teng et al. (2020), results were consistent. SPI functions as an emulsifier by reducing tension at the interface between oil and water, preventing oil droplets from aggregating, and increasing the thickness of the interfacial layer (Hebishy et al., 2015). This leads to increased emulsion stability. At a certain concentration, the interaction between lecithin and SPI induces electrostatic and hydrophobic interactions, which significantly affect both the emulsion properties and functional properties of proteins (Anal et al., 2019).

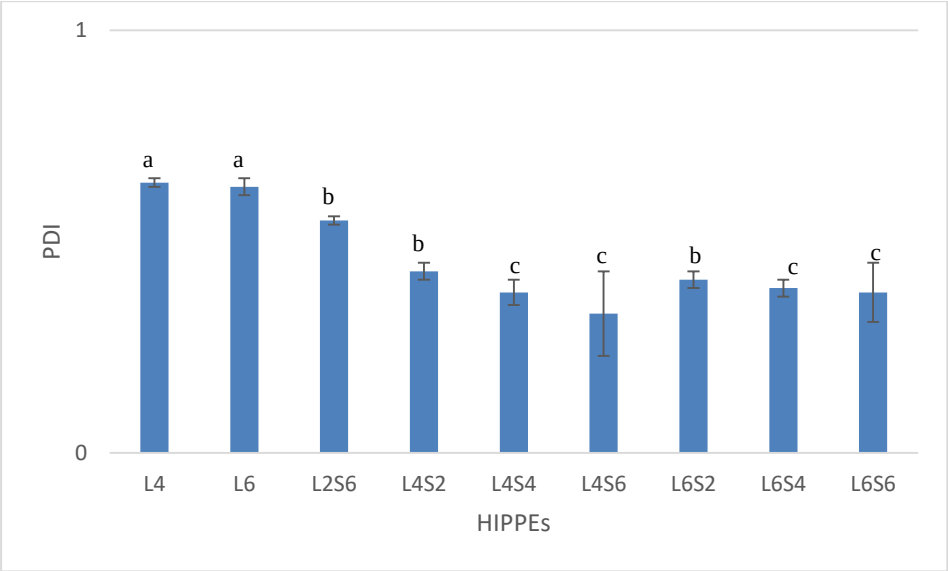

Figure 3.11: PDI values of HIPPEs.

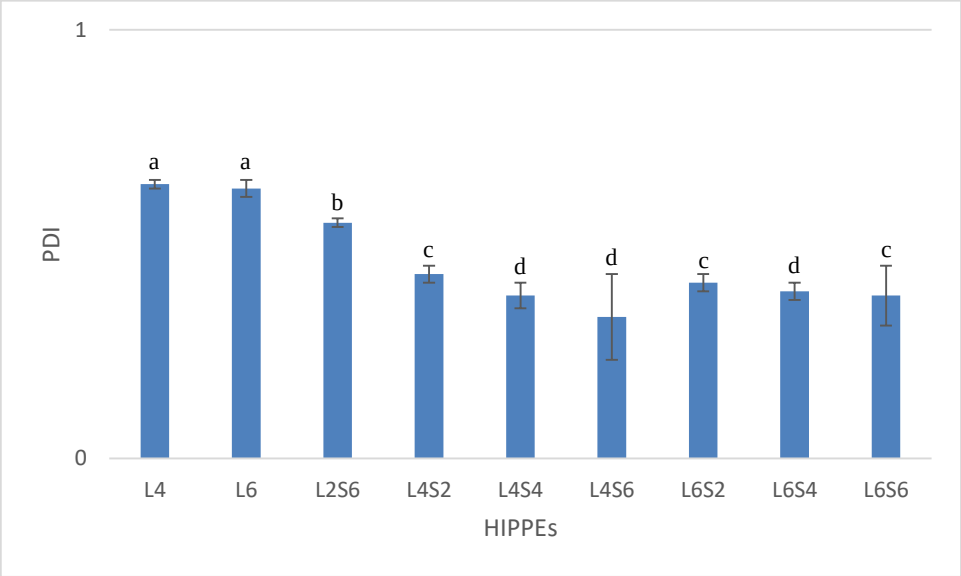

Figure 3.12: PDI values of HIPP-DEs.

3.6 Encapsulation Efficiency

The Figure 3.13 shows EEA and EEP values of different HIPPEs. As it is shown, L4, L6 and L2S6 could not present significant difference between EEA and EEP values ($p<.05$). The L6S6 had the highest (88.9, 83.1) EEA (%) and EEP (%) values, respectively. In HIPPEs containing 4 % lecithin, a statistically significant increase is observed in the EEA and EEP values of with increasing SPI gel concentration from L4S2 to L4S6. The same situation was observed in emulsions containing 6% lecithin. In emulsions containing 4% and 6 % SPI gel concentration, lecithin concentration

increased from 2% to 4%, while EEA and EEP values showed a statistically significant increase ($p<.05$). No significant increase was observed as the concentration of lecithin increased from 4% to 6%. Additionally, there is a obvious difference between the encapsulation efficiencies of phenolics and anthocyanins. EEA values are higher than EEP values. Similar observations were achieved in the case of HIPP-DEs. In the study of Zahed et al. (2023) similarly, EEA values were higher than EEP values in the system containing maltodextrin and soy protein isolate. Higher EEA values were associated with the difference in electrical charge between anthocyanins and phenolic compounds (Zahed et al., 2023).

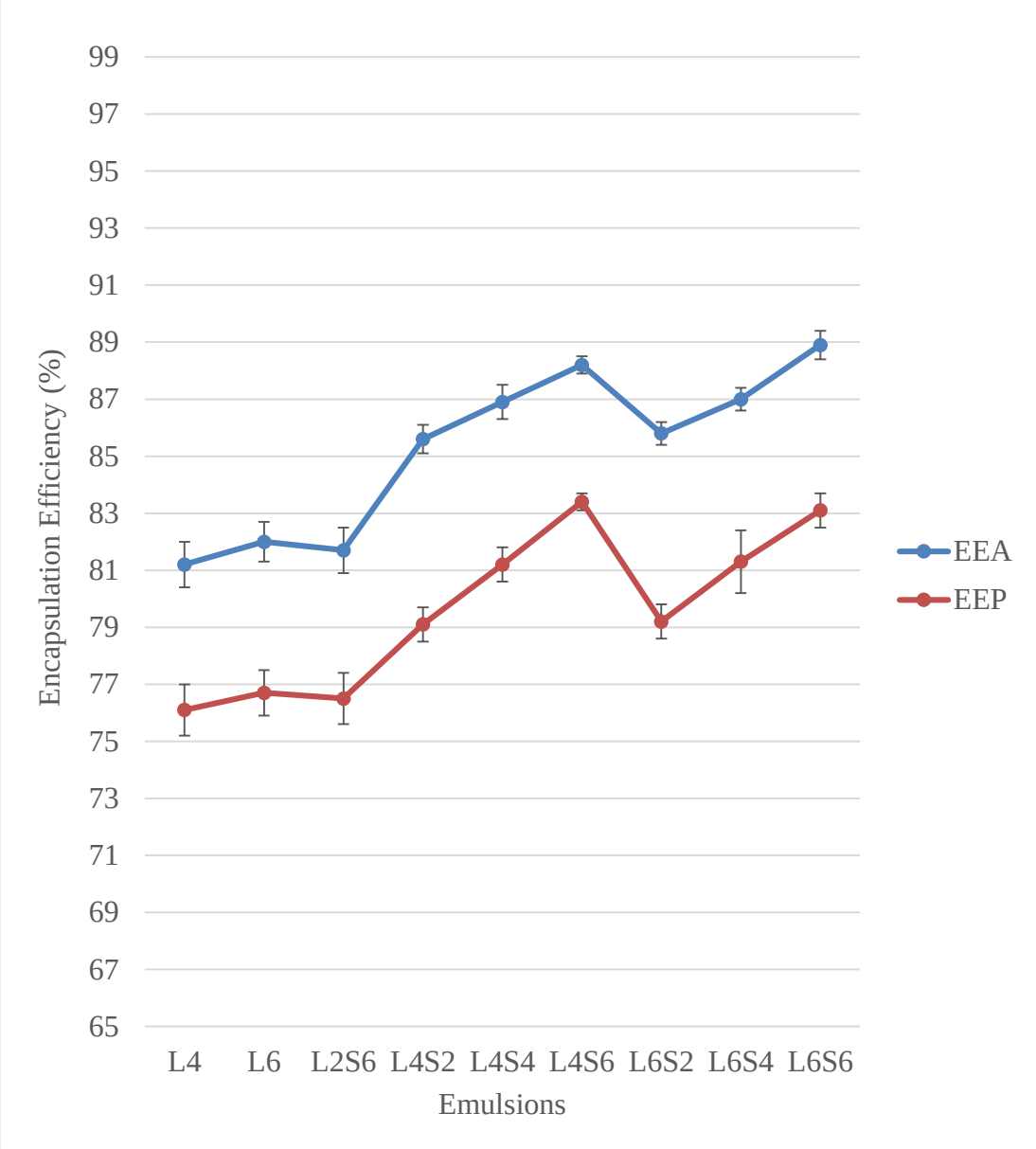

Figure 3.13: EEA and EEP values of HIPPEs.

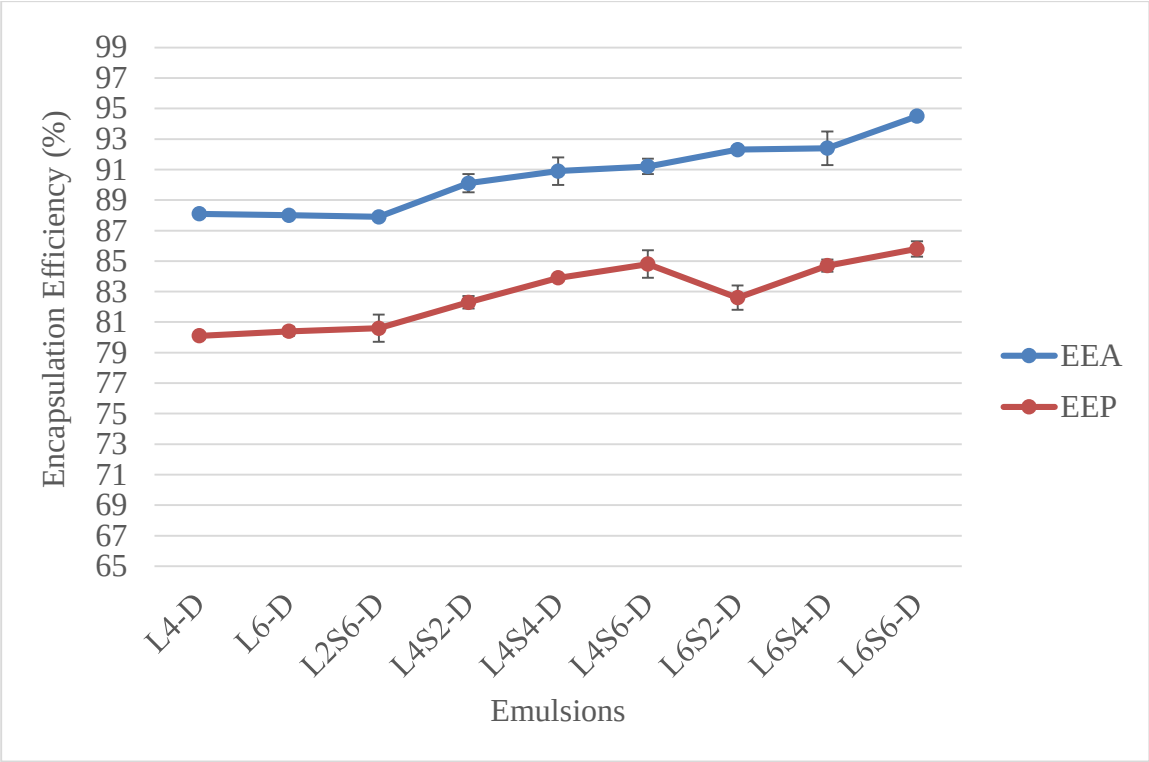

Figure 3.14: EEA and EEP values of HIPPE-DEs.

3.7 Effect of *In Vitro* Gastrointestinal Digestion on TAC, TPC, DPPH and ABTS of HIPPEs and HIPP-DEs

The potential application of aqueous *Hibiscus* extract in functional foods holds significant promise due to biological activity of anthocyanins and phenolics. Enhancing the stability of anthocyanins and phenolics in the digestive system is crucial to enable efficient utilization of them. Therefore, *in vitro* simulated digestion was applied to assess the protective effect of HIPPEs and HIPP-DEs on bioactive compounds. As a control group, aqueous *Hibiscus* extract was used.

3.7.1 Total anthocyanin content

Table 3.3 illustrates the variations in TAC of aqueous *Hibiscus* extract (AHE) and various HIPPEs throughout the process of *in vitro* digestion. To compare the stabilities, *in vitro* digestion protocol was conducted. For undigested products, all HIPPEs presented lower TAC values compared to the AHE. This decrease can be related with the entrapment of the anthocyanins in emulsion system and possible interactions between soy proteins and anthocyanins. The hydroxyl groups of anthocyanins, particularly in *Hibiscus*, enable them to engage with proteins and peptides in the

emulsion (Chickhoune et al., 2017). In a study by Yuksel et al. (2010), it was observed that flavonoids, including anthocyanins, possess a strong affinity towards the hydrophobic regions located on the surface of proteins. These flavonoids have a propensity to bind with these hydrophobic regions on proteins, which can be attributed to the hydrophobic nature of flavonoids and the exposed hydrophobic areas on proteins (Yuksel et al., 2010). For nonencapsulated AHE, the anthocyanins remained stable and TAC remained nearly constant during gastric digestion. In HIPPEs containing 4% lecithin (L4, L4S2, L4S4, L4S6), the SPI gel concentration increased from 0% to 4%, while the amount of increase in TAC value in gastric digestion decreased in percent. However, when the SPI gel concentration increased from 4% to 6%, no statistically significant difference was observed in the TAC value during gastric digestion ($p<.05$). In HIPPEs containing 6% lecithin (L4, L4S2, L4S4, L4S6) also provided similar changes during gastric digestion. In the study of Betz & Kulozik. (2011), similar observation was achieved during encapsulation of bilberry extract within whey protein gel. The results showed that increasing the protein concentration from 15% to 20% decreased the release rate of anthocyanin during gastric digestion (Betz & Kulozik, 2011). However, increasing the protein concentration from 20% to 25% (w/w) did not significantly affect the release rate. It was suggested that changing the protein concentration from a certain concentration do not have a substantial effect on the gel network's internal structure to form a complete diffusion barrier (Betz & Kulozik, 2011). It can be observed that the non-encapsulated aqueous *Hibiscus* extract remained quite stable when exposed to the simulated gastric environment, with no significant changes observed in its TAC ($p>0.05$). Anthocyanins, when present in an acidic environment, exist in the flavylium cation form, which imparts a red color (Cooper-Driver, 2001). These compounds are known to exhibit relatively good stability under such conditions (Jackman et al., 1987). Also, TAC values decreased significantly during intestinal digestion. According to Lang et al. (2020), anthocyanins are relatively stable during simulated gastric digestion. Therefore, the preservation and protection of anthocyanins should be focused on the intestinal digestion process. The alkaline environment of the intestine is the main factor causing anthocyanin degradation and limiting their bioaccessibility (Lang et al., 2020).

Table 3.3: Total anthocyanin content (mg C-3-GE/ 100 g) of HE and HIPPEs.

Emulsion	Undigested	Gastric digestion	Intestinal digestion
HE	31.13±1.23^{aA}	30.09±0.78^{aA}	12.03±0.47^{cB}
L4	21.07±0.29^{bA}	19.37±0,64^{dA}	15.21±0.24^{aB}
L6	20.06±0.05^{bcA}	19.47±2,0^{cdA}	15.22±0.17^{abB}
L2S6	19.21±0.23^{cA}	19.04±4,26^{dA}	13.06±1.31^{bB}
L4S2	16.12±0.12^{dA}	15.19±0.27^{abcA}	12.96±0.05^{bB}
L4S4	15.43±0.42^{dA}	15.24±0.04^{abA}	12.72±0.27^{bcB}
L4S6	15.01±0.01^{dA}	15.88±0.01^{abA}	12.73±0.19^{bB}
L6S2	16.03±0.05^{dA}	15.15±0.04^{bcA}	12.94±0.04^{bB}
L6S4	15.43±0.21^{dA}	15.65±0.04^{deA}	12.75±0.04^{bA}
L6S6	15.13±0.15^{dA}	15.92±0.05^{aA}	13±0.28^{bcB}

*The results are represented as mean ± standard deviation (n=3).
*Different lowercase letters indicate significant differences between different emulsions ($p < 0.05$)
*Different uppercase letters indicate significant differences between digestion phases.

Table 3.4: Total anthocyanin content (mg C-3-GE/ 100 g) of HE and HIPP-DEs.

Emulsion	Undigested	Gastric digestion	Intestinal digestion
HE	31.13±1.21^{aA}	30.92±0.76^{aA}	12.03±0.47^{aB}
L4	11.23±0.25^{bA}	10.23±0.05^{dB}	9.54±0.46^{bC}
L6	11.04±0.11^{bA}	10.62±0.26^{cdB}	9.55±0.04^{bC}
L2S6	10.32±0.28^{bA}	9.91±0.03^{bcdB}	9.13±0.23^{bC}
L4S2	9.88±0.09^{cA}	9.06±0.05^{bcB}	9.01±0.38^{cC}
L4S4	9.01±0.12^{cdA}	9.17±0.02^{bcB}	8.35±0.28^{cC}
L4S6	8.76±0.05^{cdeA}	8.46±0.43^{bB}	8.14±0.54^{cC}
L6S2	9.72±0.17^{cdeA}	9.08±0.03^{bcB}	8.94±0.37^{cC}
L6S4	9.04±0.04^{deA}	9.17±0.02^{bB}	8.41±0.12^{cC}
L6S6	8.51±0.49^{eA}	8.49±0.11^{bB}	8.12±0.25^{cC}

*The results are represented as mean ± standard deviation (n=3).
*Different lowercase letters indicate significant differences between different emulsions ($p < 0.05$)
*Different uppercase letters indicate significant differences between digestion phases.

3.7.2 Total phenolic content

H. Sabdariffa polyphenols have potential use in functional foods due to their biological activity. However, in the case of bioaccessibility, improving their stability in the

gastrointestinal system is crucial. For this reason, *in vitro* gastrointestinal digestion was used to assess the protective effect of lecithin and SPI gel based HIPPEs and HIPP-DEs on polyphenols. Aqueous *H. Sabdariffa* was used as a control group for comparison. Aqueous *Hibiscus* extracts had higher TPC values, concerning their emulsions, before *in vitro* gastrointestinal digestion. Sirovec et al. (2022) conducted a study where they compared the total phenolic content (TPC) values of rosemary and oregano extracts with protein-based emulsion systems. The findings of this study indicated that differences in TPC values between emulsions were appeared. These variations in TPC values of emulsified extracts could be attributed to the presence of certain compounds like ascorbic acid and proteins, which can react with the Folin-Ciocalteu reagent and potentially interfere with the accurate determination of TPC using the Folin-Ciocalteu assay (Bastola et al., 2017). In the context of HIPPEs with the same lecithin concentration, it was observed that an increase in the concentration of SPI gel resulted in higher TPC values. This finding aligns with the results obtained in the study conducted by Sirovec et al. (2022), where a similar observation was made. Specifically, an increase in the concentration of commercial pea protein was found to be associated with an increase in the TPC value for emulsified rosemary extracts. From Table 3.5, significant decreases in TPC were observed in the Hibiscus extract after *in vitro* digestion. 407.59±6.48 and 927.6±19.3 of TPC of Hibiscus extract remained in the gastric and intestinal phase, respectively, representing approximately 15% and 35% of the initial TPC. Similar significant losses in TPC during *in vitro* gastrointestinal digestion have been reported in other studies, such as elderberry (approximately 80% loss), grape pomace (over 50% reduction), Goji berries (only 30% TPC remaining in digests and berry pomace (approximately 72% reduction) (Jara-Palacios et al., 2018; Pinto et al., 2017; Rocchetti et al., 2018; Xiong et al., 2020). Comparing the TPC of the digested phenolics of Hibiscus extract with that of the digested HIPPEs and HIPP-DEs, significantly higher TPC values were observed in the latter. The maximum TPC value reached up to 703.36±3.32 mg and 1110.7±21.01 mg in the gastric and intestinal digestions, respectively. In the study of Xiong et al. (2020), berry pomace phenolics interacted with rice-pea protein isolate also provided similar increase in TPC values of blueberry (from 23% to 69%) and muscadine grape (from 31% to 62%).

The release rate of phenolics during the gastric and intestinal phases of in vitro digestion was influenced by pH of the environment. Generally, pH changes have an

impact on the availability of protein sites for interactions with both proteins and phenolic compounds (Schefer et al., 2021). It was stated that at the beginning of the gastric phase, when the pH was below the pKa of the carboxyl groups of phenolic acids, there was a rapid reduction in the release of phenolic compounds. This indicates that the phenolic acids were in a protonated state and less accessible for release (Marinea et al., 2023). However, in the intestinal phase, the bioaccessibility levels significantly increases ($p<.01$) compared to the gastric phase. The increase in pH affects the electrostatic interactions between proteins and phenolic acids. At pH 7, where the carboxyl groups of both phenolic acids and amino acids like glutamic acid and aspartic acid are deprotonated electrostatic repulsion occurs, leading to a higher release of phenolic compunds during intestinal digestion. Additionally, TPC values of *Hibiscus* extract and emulsions after gastric digestion are lower than those of intestinal digestion. According to the study, total phenolic content and antioxidant activity increases in the intestinal digestion due to pH changes and effects of digestive enzymes, and suggested that the decomposition of phenolics could significantly increase antioxidant activity (Tarko & Duda-Chodak, 2020).

Table 3.5: Effect of *in vitro* digestion on TPC (mg GAE/100 g) of HE and HIPPEs.

Emulsion	Undigested	Gastric digestion	Intestinal digestion
HE	2619.01 ± 17.31^{aA}	407.59 ± 6.48^{aC}	1224.66 ± 19.33^{aB}
L4	1222.11 ± 6.18^{bA}	700.73 ± 0.64^{aC}	795.15 ± 3.40^{eB}
L6	1217.67 ± 6.81^{bA}	709.82 ± 2.06^{aC}	796.72 ± 1.46^{eB}
L2S6	1211.03 ± 2.00^{bA}	$700,22\pm4.26^{aC}$	999.41 ± 2.42^{dB}
L4S2	1282.83 ± 11.00^{cA}	701.14 ± 3.92^{cdC}	1091.14 ± 0.99^{cB}
L4S4	1288.37 ± 1.46^{cd}A	690.19 ± 3.11^{eC}	1094.43 ± 4.33^{cB}
L4S6	1300.22 ± 8.59^{cdeA}	666.92 ± 2.28^{fC}	1148 ± 1.73^{bB}
L6S2	1292.93 ± 0.95^{cdeA}	703.36 ± 13.32^{cC}	1082.67 ± 1.53^{cB}
L6S4	1288.37 ± 5.98^{deA}	693.04 ± 2.60^{deC}	1095.25 ± 4.31^{cB}
L6S6	1307.75 ± 1.22^{eA}	$669,77\pm13.11^{fC}$	1160.71 ± 21.01^{bB}

*The results are represented as mean ± standard deviation (n=3).
*Different lowercase letters indicate significant differences between different emulsions ($p<0.05$)
*Different uppercase letters indicate significant differences between digestion phases.

Table 3.6: Effect of *in vitro* digestion on TPC (mg GAE/g) of HE and HIPP-DEs.

Emulsion	Undigested	Gastric digestion	Intestinal digestion
HE	2619.01±17.31^{aA}	407.59±6.48^{aC}	1224.66±19.33^{aB}
L4-D	799.41±1.49^{bA}	328.57±4.33^{dC}	538.67±3.47^{fB}
L6-D	797.73±0.69^{bA}	328.79±3.56^{dC}	559.30±1.42^{eB}
L2S6-D	858.00±4.37^{bA}	331.34±1.57^{dC}	642.78±2.43^{dB}
L4S2-D	886.22±2.51^{cA}	464.14±16.98^{cC}	791.14±0.94^{cB}
L4S4-D	890.79±3.02^{cA}	560.39±4.28^{bC}	794.43±4.39^{cB}
L4S6-D	893.09±1.42^{cA}	695.52±2.47^{aC}	798±1.77^{cB}
L6S2-D	889.74±2.77^{cA}	465.38±20.29^{cC}	822.67±1.57^{bB}
L6S4-D	893.27±2.01^{cA}	564.94±4.80^{bC}	823.25±4.39^{bB}
L6S6-D	902.66±0.99^{cA}	692.33±19.42^{aC}	825.77±21.11^{bB}

*The results are represented as mean ± standard deviation (n=3).
*Different lowercase letters indicate significant differences between different emulsions ($p < 0.05$)
*Different uppercase letters indicate significant differences between digestion phases.

In the field of food-grade emulsions, the most commonly encountered types are oil-in-water or water-in-oil emulsions. However, a challenge arises during the digestion process, as oil-in-water emulsions tend to become unstable in gastric juice, resulting in leakage and potential loss of the nutritional benefits of encapsulated active ingredients. On the other hand, while water-in-oil emulsions effectively protect the encapsulated actives with the oily layer, they may have an unpleasant taste due to the continuous phase being an oily liquid. To address this challenge, the use of a water-in-oil-in-water HIPP-DE system. By introducing an outermost aqueous phase, the taste and stability problems can be achieved. When the HIPP-DE is delivered to the stomach, although the outermost aqueous phase may be disrupted by gastric juice, the remaining w/o emulsion can still protect the aqueous *Hibiscus* extract, ensuring successful delivery to the intestine.

3.7.3 Bioaccessibility of phenolics and anthocyanins

Figure 3.15 outlines the variations in bioaccessibility measurements of Hibiscus extract and diverse emulsions throughout in vitro digestion. The bioaccessibility of Hibiscus anthocyanins was measured to be 38.64 ± 1.01% in aqueous extract form, 72.14 ± 0.56% when encapsulated in L4 and reached to 85.92 ± 1.13% in L6S6.

Similarly, the bioaccessibility of phenolics was also measured to be 46.76 ± 1.12% in aqueous extract and 69.97 ± 0.93% when encapsulated in L4 and reached to 86.58 ± 1.22% in L6S6. The stability and bioaccessibility of anthocyanins and phenolics were found to be significantly improved when they were encapsulated by HIPPEs compared to aqueous extract. On the other hand, HIPP-DEs provided much more protection than HIPPEs and significantly increased anthocyanin and phenolic bioaccessibility. While the anthocyanin bioaccessibility of aqueous extract was 38.64 ± 2.32%, it increased 85.59 ± 2.32% with L4. The highest anthocyanin bioavailability was obtained with L6S6-D with 93.17± 1.12%. Such kind of a increase was also observed for phenolic bioaccessibility. The results indicate that both types of emulsions are effective in resisting the digestive process in the gastrointestinal tract. However, HIPP-DEs exhibited a higher level of protection compared to HIPPEs. It can be concluded that HIPPE based emulsion systems exhibit a superior protective effect on anthocyanins and phenolics during the digestion process in the gastrointestinal tract (Shi et al., 2020). According to the results, the bioaccessibility of polyphenols in HIPPEs was higher than in aqueous *Hibiscus* extract solution. This indicates that emulsion system resisted digestion in the gastrointestinal tract, providing a better protective effect on polyphenols.

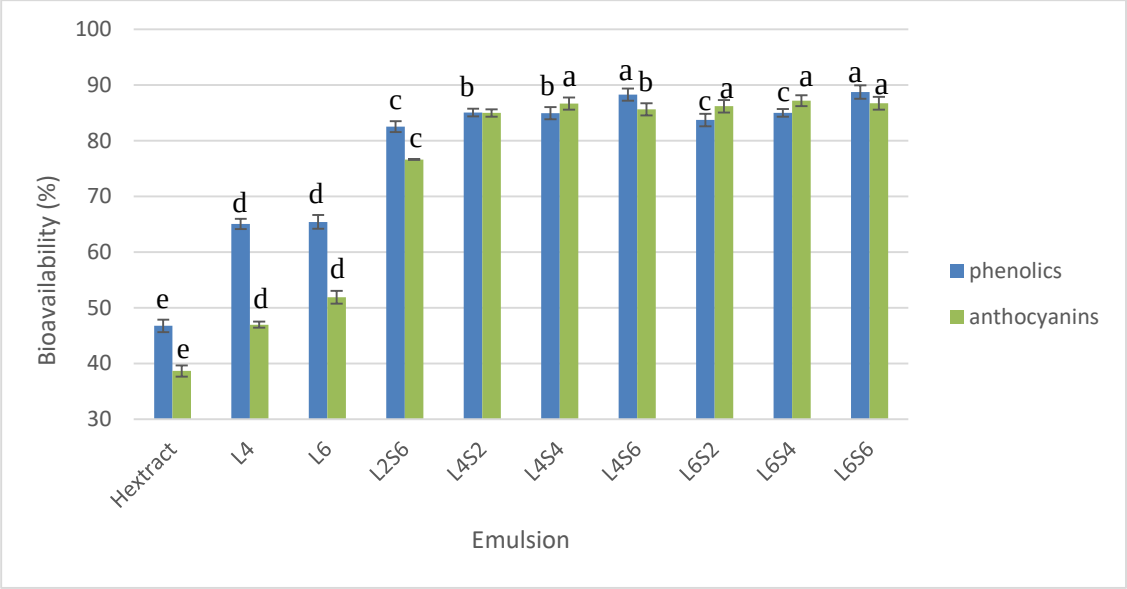

Figure 3.15: Bioaccessibility of phenolics and anthocyanins of HIPPEs.

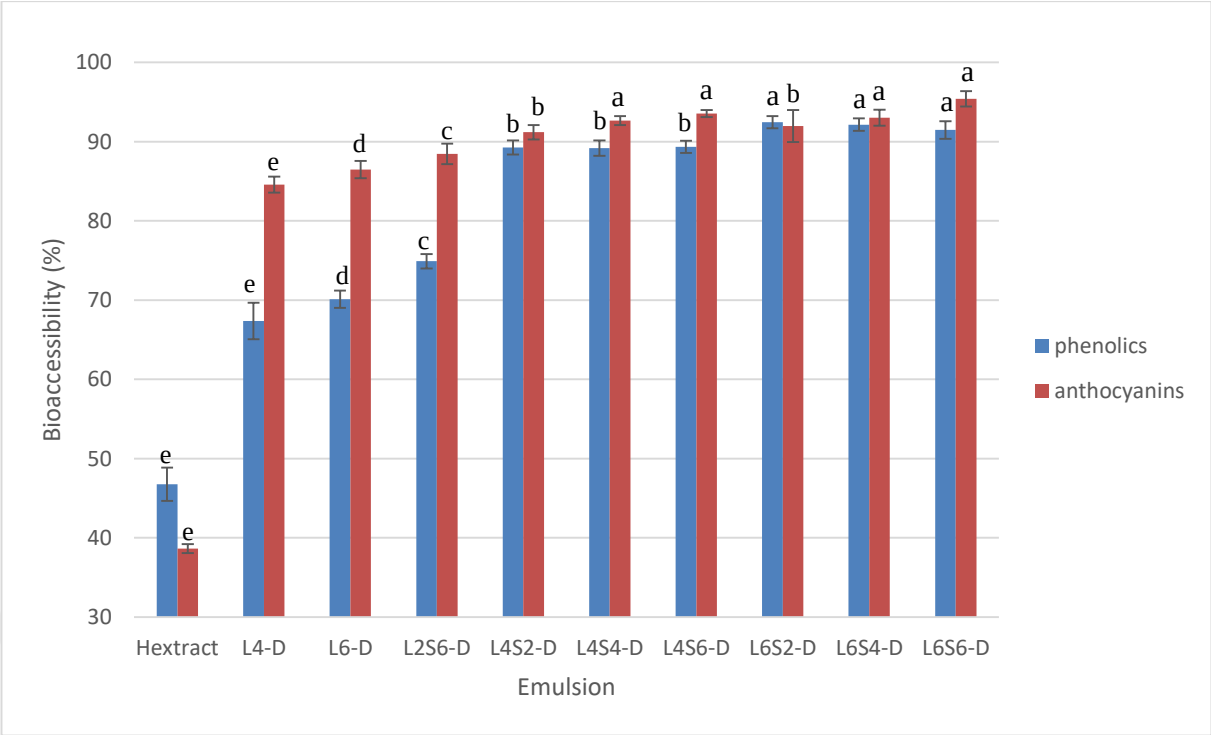

Figure 3.16: Bioaccessibility of phenolics and anthocyanins of HIPP-DEs.

3.7.4 Antioxidant activity

To assess the antioxidant activity of *Hibiscus* extract and emulsion, DPPH and ABTS assays were conducted. During *in vitro* digestion, the DPPH and ABTS values of *Hibiscus* extract and various emulsions experience changes. The results, as depicted in Table 3.7, there is a significant difference in antioxidant activity between undigested *Hibiscus* extract and all emulsions ($p < .05$). In HIPPEs with the same lecithin concentration (4% and 6%), the DPPH and ABTS value increases as the SPI gel concentration increases ($p < .05$). In the study of Sirovec et al. (2022), in line with the present study, the DPPH value of emulsified oregano extract increased as the amount of pea protein isolate increased. During in vitro digestion, the DPPH and ABTS values of *Hibiscus* extract decreased from 335.12±1.21 to 259.12±3.22 and from 223.21±2.56 to 159.12±1.23, respectively. The situation that occurs similarly in extracts and emulsions is that DPPH and ABTS values go more than intestinal digestion as a result of gastric digestion. However, DPPH and ABTS values of emulsion systems after intestinal digestion are higher than Hibiscus extract. Similar changes in DPPH and ABTS values were obtained for HIPP-DEs. It was suggested that the protein carrier systems protect the polyphenols from degradation and preserved their antioxidant activities during *in vitro* digestion (Xiong et al., 2020). In the study of Lang et al.

(2020), the addition of soybean 7S and 11S globulins on cyanidin-3-glucoside significantly increased the DPPH value of anthocyanin during intestinal digestion. Similarly, the ABTS of the compounds were higher throughout the simulated digestion. These findings indicate that soy protein may enhance the antioxidant activity of anthocyanins during gastrointestinal digestion.

Table 3.7: Effects of in vitro digestion on DPPH (mg TE/100 g dw) and ABTS (mg TE/100 g dw) of *Hibiscus* extracts (HE) and HIPPEs.

Emulsion	Antioxidant activity	Undigested	Gastric digestion	Intestinal digestion
HE	DPPH	335.12 ± 1.21^{fA}	225.22 ± 8.18^{fC}	259.12 ± 3.22^{eB}
	ABTS	223.21 ± 2.56^{fA}	129.12 ± 1.23^{fC}	159.12 ± 1.23^{gB}
L4	DPPH	337.14 ± 2.14^{eA}	231.54 ± 2.89^{fA}	261.65 ± 4.11^{eB}
	ABTS	225.12 ± 1.87^{eA}	128.45 ± 2.06^{eC}	160.78 ± 3.11^{fC}
L6	DPPH	336.23 ± 2.09^{eA}	245.45 ± 3.12^{eC}	255.11 ± 1.89^{eC}
	ABTS	237.98 ± 1.23^{eA}	135.66 ± 4.12^{eC}	155.21 ± 2.16^{eB}
L2S6	DPPH	441.12 ± 1.03^{dA}	251.24 ± 3.45^{dC}	378.12 ± 5.09^{dC}
	ABTS	341.77 ± 1.93^{dA}	151.99 ± 2.23^{dC}	277.69 ± 1.23^{dB}
L4S2	DPPH	446.27 ± 0.99^{cA}	264.15 ± 1.03^{cC}	385.08 ± 2.09^{cB}
	ABTS	345.99 ± 2.33^{cA}	163.34 ± 4.12^{cC}	287.23 ± 3.23^{cB}
L4S4	DPPH	467.89 ± 3.09^{bA}	289.17 ± 4.15^{bC}	418.73 ± 3.99^{bB}
	ABTS	359.87 ± 1.06^{bA}	188.34 ± 4.21^{bC}	319.09 ± 3.87^{bB}
L4S6	DPPH	478.21 ± 2.56^{aA}	299.87 ± 2.96^{aC}	430.01 ± 4.01^{aB}
	ABTS	373.87 ± 3.01^{aA}	199.98 ± 2.79^{aC}	331.23 ± 3.06^{aB}
L6S2	DPPH	446.27 ± 2.06^{cA}	263.12 ± 1.78^{cC}	390.08 ± 0.99^{cB}
	ABTS	349.98 ± 1.68^{cA}	165.76 ± 1.11^{cC}	298.66 ± 1.78^{cB}
L6S4	DPPH	467.89 ± 0.99^{bA}	279.14 ± 5.21^{bC}	412.67 ± 0.94^{bB}
	ABTS	367.98 ± 2.33^{bA}	179.44 ± 2.33^{bC}	311.09 ± 2.33^{bB}
L6S6	DPPH	478.21 ± 5.11^{aA}	289.67 ± 2.34^{aC}	426.76 ± 3.21^{aB}
	ABTS	371.90 ± 1.79^{aA}	190.34 ± 0.33^{aC}	338.09 ± 4.13^{aB}

*The results are represented as mean ± standard deviation (n=3).
*Different lowercase letters indicate significant differences between different emulsions ($p < 0.05$)
*Different uppercase letters indicate significant differences between digestion phases.

Table 3.8: Effects of in vitro digestion on DPPH (mg TE/100 g dw) and ABTS (mg TE/100 g dw) of *Hibiscus* extracts(HE) and HIPP-DEs.

Emulsion	Antioxidant activity	Undigested	Gastric digestion	Intestinal digestion
HE	DPPH	335.12±1.21^{fA}	225.22±1.18^{fC}	259.12±3.22^{fB}
	ABTS	223.21±2.56^{fA}	129.12±1.23^{fC}	159.12±1.23^{eB}
L4	DPPH	337.14±2.14^{eA}	227.54±2.89^{eC}	261.65±4.11^{eB}
	ABTS	225.12±1.87^{eA}	128.45±2.06^{eC}	160.78±3.11^{eB}
L6	DPPH	336.23±2.09^{eA}	235.45±3.12^{eC}	255.11±1.89^{eB}
	ABTS	237.98±1.23^{eA}	135.66±4.12^{eC}	155.21±2.16^{eB}
L2S6	DPPH	441.12±1.03^{dA}	251.24±3.45^{dC}	378.12±5.09^{dB}
	ABTS	341.77±1.93^{dA}	151.99±2.23^{dC}	277.69±1.23^{dB}
L4S2	DPPH	446.27±0.99^{cA}	264.15±1.03^{cC}	385.08±2.09^{cB}
	ABTS	345.99±2.33^{cA}	163.34±4.12^{cC}	287.23±3.23^{cB}
L4S4	DPPH	467.89±3.09^{bA}	289.17±4.15^{bC}	418.73±3.99^{bB}
	ABTS	359.87±1.06^{bA}	188.34±4.21^{bC}	319.09±3.87^{bB}
L4S6	DPPH	478.21±2.56^{aA}	299.87±2.96^{aC}	430.01±4.01^{aB}
	ABTS	373.87±3.01^{aA}	199.98±2.79^{aC}	331.23±3.06^{aB}
L6S2	DPPH	446.27±2.06^{cA}	263.12±1.78^{cC}	390.08±0.99^{cB}
	ABTS	349.98±1.68^{cA}	165.76±1.11^{cC}	298.66±1.78^{cB}
L6S4	DPPH	467.89±0.99^{bA}	279.14±5.21^{bC}	412.67±0.94^{bB}
	ABTS	367.98±2.33^{bA}	179.44±2.33^{bC}	311.09±2.33^{bB}
L6S6	DPPH	478.21±5.11^{aA}	289.67±2.34^{aC}	426.76±3.21^{aB}
	ABTS	371.90±1.79^{aA}	190.34±0.33^{aC}	338.09±4.13^{aB}

*The results are represented as mean ± standard deviation (n=3).
*Different lowercase letters indicate significant differences between different emulsions ($p < 0.05$)
*Different uppercase letters indicate significant differences between digestion phases.

3.7.5 Quantification of Polyphenols with HPLC-DAD

Based on the HPLC analysis delphinidin-3-glucoside, cyanidin-3-glucoside and cyanidin-3-rutinoside were detected in aqueous *Hibiscus* extract. The changes in concentration of each anthocyanins during in vitro digestion of aqueous extract and L6S6 and L6S6-D were presented in the Figure 3.17 and Figure 3.18. During gastric digestion, the concentrations of all anthocyanins were almost stable and then they decreased after intestinal digestion. Comparing *Hibiscus* extract and HIPPEs, it was observed that amount of each anthocyanin found in emulsions was higher than than

pure extract ($p<.05$). After intestinal digestion, delphinidin-3-glucoside was undetectable in *Hibiscus* extract and all emulsions. These findings indicate that SPI can protect anthocyanins by delaying their release during *in vitro* digesiton. The variations in the release properties of anthocyanins can be attributed to the interactions between the wall materials and anthocyanins, such as hydrophobic interactions and static quenching facilitated by soy protein isolate, which effectively enhance the stability of anthocyanins (Chen et al., 2019). As can be seen, while anthocyanins showed high stability during gastric digestion, they decreased with intestinal digestion. Lang et al. (2020) stated that incorporating soybean 7S and 11S globulins with cyanidin-3-glucoside exhibit relative stability during simulated gastric digestion.

Four phenolic acids, namely gallic acid, syringic acid, ferulic acid, chlorogenic acid, were identified in *Hibiscus* extract and all emulsions. After gastric digestion, chlorogenic acid content decreased. Previous studies have demonstrated the transformation of phenolic compounds by gut microbiota, supporting the notion that encapsulated anthocyanins by soy protein isolate provides more convertible phenolic compounds (Wu et al., 2020). However, decreasing trend for these phenolics during gastric digestion was not valid for syringic acid. This suggests that gallic acid and ferulic acid were produced and immediately utilized by gut microbiota. After gastric digestion the highest concentration of syringic acid was obtained for extracts encapsulated L6S6-D. Previous studies have shown that gut microbiota can degrade cyanidin-3-glucoside and delphinidin-3-glucoside into syringic acid during *in vitro* digestion (Chen et al., 2017). Hence, the increase in syringic acid may be attributed to the metabolization of anthocyanins. Given the notable amount of syringic acid, it can be concluded that SPI-based HIPPEs and HIPP-DEs can be used to encapsulate anthocyanin extract to obtain higher amounts of syringic acid after gastric digestion.

Table 3.9: Anthocyanin and phenolic profile of HIPPEs**.**

Emulsion	Delphinidin-3-glucoside	Cyanidin-3-glucoside	Cyanidin-3-rutinoside	Chlorogenic acid	Ferulic acid	Gallic acid	Syringic acid
HE	220.00 ±2.31^{e}	218.12 ±1.03^{g}	110.21±1.23^{e}	30.12±1.99^{e}	12.23± 1.12^{d}	10.01± 0.99^{e}	5.21± 0.09^{e}
L4	231.22 ±1.67^{d}	229.11 ±1.13^{f}	109.23±2.01^{e}	30.28±1.12^{e}	12.78± 1.98^{d}	10.22± 0.45^{e}	5.12± 0.71^{e}
L6	232.40 ±2.12^{d}	230.01 ±1.67^{e}	110.12±1.22^{e}	30.44±1.09^{e}	12.98± 0.43^{d}	10.04± 0.56^{e}	5.16± 0.06^{e}
L2S6	246.10 ±1.34^{c}	243.12 ±1.08^{c}	115.45±1.01^{d}	32.12±3.23^{d}	13.45± 1.21^{c}	12.34± 0.22^{d}	6.65± 0.04^{d}
L4S2	248.90 ±2.34^{b}	246.78 ±1.56^{b}	120.12±1.34^{c}	35.78±3.22^{c}	14.51± 1.98^{c}	14.98± 1.09^{c}	6.98± 0.09^{c}
L4S4	250.12 ±2.33^{a}	248.12 ±1.23^{a}	124.54±4.21^{b}	38.91±1.01^{b}	16.93± 1.23^{b}	15.21± 1.01^{b}	7.23± 0.13^{b}
L4S6	250.18 ±2.09^{a}	250.11 ±2.34^{a}	129.34±2.33^{a}	41.23±1.02^{a}	17.98± 1.23^{a}	15.9± 1.91^{b}	7.45± 0.66^{a}
L6S2	249.01 ±2.13^{a}	237.78 ±2.31^{d}	120.23±2.67^{c}	35.89±2.08^{c}	14.69± 0.67^{c}	14.99± 0.56^{c}	7.01± 0.43^{c}
L6S4	251.21 ±2.19^{a}	250.09 ±3.01^{a}	125.50±2.89^{b}	38.99±4.05^{b}	16.99± 0.45^{b}	15.15± 0.44^{b}	7.26± 0.13^{b}
L6S6	250.17 ±3.01^{a}	248.98 ±2.02^{a}	130.20±3.44^{a}	41.98±2.06^{a}	18.01± 0.45^{a}	16.22± 0.67^{a}	7.49± 0.09^{a}

*The results are represented as mean ± standard deviation (n=3).
*Different lowercase letters indicate significant differences between different emulsions ($p < 0.05$)

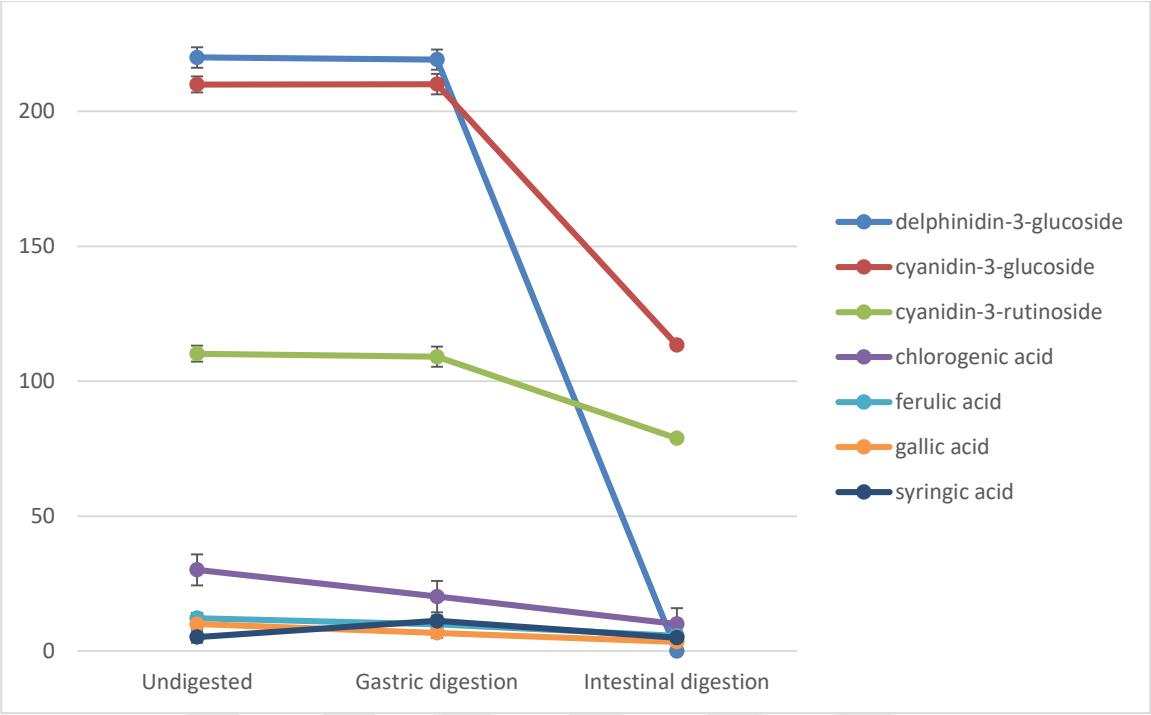

Figure 3.17: Change in anthocyanin and phenolic profile of L6S6 during in vitro digestion.

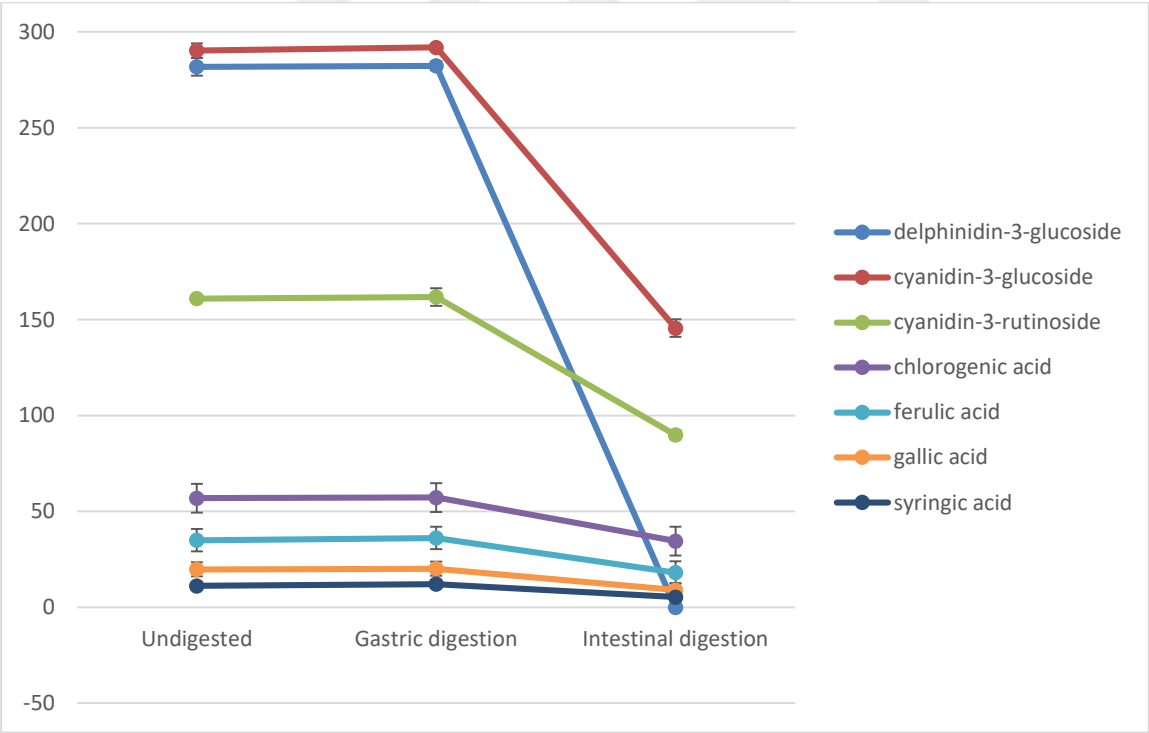

Figure 3.18: Change in anthocyanin and phenolic profile of L6S6-D during in vitro digestion.

4. CONCLUSION AND RECOMMENDATIONS

Hibiscus Sabdariffa L. is widely utilized across multiple industries due to its abundant nutritional content and powerful bioactive components, including phenolics, anthocyanins, and natural color pigments. Besides serving as a colorant, it is also highly valued for its positive health effects, such as antibacterial and antioxidant properties, as well as its potential benefits for cholesterol and gastrointestinal issues, leading to its widespread consumption. However, the susceptibility of its bioactive components to damage from various factors poses limitations on its utilization in numerous industries. Encapsulation has emerged as the dominant and preferred technique for protecting bioactive components, not only ensuring their preservation but also enabling controlled release and consumption within various applications. Emulsification-based encapsulation, widely recognized in both industry and literature, stands as a well-established approach. High Internal Phase Emulsions (HIPEs) have gained prominence due to their exceptional capacity for encapsulation, high stability, and efficient protection of bioactive components against diverse factors. Similarly, Double Layer Emulsion systems, characterized by their nested phase structures, offer significant protection for bioactive components. Concerns surrounding the negative effects of emulsifiers and stabilizers typically used to enhance emulsion stability, such as impacts on human health and environmental pollution, have prompted intensified scientific research towards discovering alternative products. In recent years, vegetable proteins have garnered significant interest as potential substitutes for conventional emulsifiers and stabilizers. These proteins possess various functional properties and amphiphilic characteristics that contribute significantly to maintaining emulsion stability. Among different types of plant-based proteins, soy protein has emerged as a preferred option due to its easy accessibility and processability. This study focused on encapsulating aqueous *Hibiscus* extract in High Internal Phase Pickering Emulsions (HIPPE) and High Internal Phase Pickering Double Emulsions (HIPP-DE) systems using lecithin and soy protein. Throughout the study the emusion stability, emulsion characteristics, and the effects of soy protein and lecithin during in vitro

gastrointestinal digestion on phenolics and anthocyanins were investigated. The SPI gel emulsions obtained by the method used alone could not demonstrate the ability to stabilize 80% internal-volume HIPPEs. Instead, emulsion stability has been achieved by combining with lecithin in different proportions. The lecithin and SPI rates used influenced emulsion properties such as creaming index, ESI/EAI, zeta potential and particle size. It was also concluded that increased protein concentrations were effective in points such as encapsulation efficiency of phenolic and anthocyanins and in vitro stability protection against digestion. In addition, HIPP-DEs have demonstrated more effective properties in many aspects than HIPPEs. With its nutritional and textural characteristics, HIPPEs are also an important milestone in the development of functional foods. However, food-grade emulsions restrict the processability of the ingredients used to design to a certain extent. For example, the use of solvents that allows higher phenolic or anthocyanin extraction and the necessary pH adjustments to ensure the enhancement and modification of the functional properties of proteins prevents the design of food-grade emulsions of these products. In this context, there is very little work in literature in this direction and it has an open potential for improvement.

REFERENCES

Abdel-SalamOmar, M. E., YounessEman, R., MohammedNadia, A., Youssef, M. M., OmaraEnayat, A., & SleemAmany, A. (2014). Citric acid effects on brain and liver oxidative stress in lipopolysaccharide-treated mice. *Journal of medicinal food*, *17*(5).

Abdulredha, M. M., Hussain, S. A., Abdullah, L. C., & Hong, T. L. (2022). Water-in-oil emulsion stability and demulsification via surface-active compounds: a review. *Journal of Petroleum Science and Engineering*, 209, 109848.

Adadi, P., & Kanwugu, O. N. (2020). Potential application of tetrapleura tetraptera and hibiscus sabdariffa (malvaceae) in designing highly flavoured and bioactive pito with functional properties. Beverages, *6*(2), 22.

Ai, J., Wu, Q., Battino, M., Bai, W., & Tian, L. (2021). Using untargeted metabolomics to profile the changes in roselle (Hibiscus sabdariffa L.) anthocyanins during wine fermentation. Food Chemistry, 364, 130425.

Alam, G., Sartini, & Alfath, A. (2019). Comparison of microwave assisted extraction (MAE) with variations of power and infusion extraction method on antibacterial activity of rosella calyx extract (Hibiscus sabdariffa). *Journal of Physics: Conference Series*, *1341*(7), 072002.

Alara, O. R., Abdurahman, N. H., Obanijesu, E. O., Alara, J. A., & Abdul Mudalip, S. K. (2020). Extract-rich in flavonoids from Hibiscus sabdariffa calyces: Optimizing microwave-assisted extraction method and characterization through LC-Q-TOF-MS analysis. *Journal of Food Process Engineering*, *43*(2), e13339.

Alarcón-Alonso, J., Zamilpa, A., Aguilar, F. A., Herrera-Ruiz, M., Tortoriello, J., & Jimenez-Ferrer, E. (2012). Pharmacological characterization of the diuretic effect of Hibiscus sabdariffa Linn (Malvaceae) extract. *Journal of ethnopharmacology*, *139*(3), 751-756.

Aloys, H., Korma, S. A., Alice, T. M., Chantal, N., Ali, A. H., Abed, S. M., & Ildephonse, H. (2016). Microencapsulation by complex coacervation: Methods, techniques, benefits, and applications-A review. American *Journal of Food Science and Nutrition Research*, *3*(6), 188-192.

Alzweiri, M., Al Sarhan, A., Mansi, K., Hudaib, M., & Aburjai, T. (2011). Ethnopharmacological survey of medicinal herbs in Jordan, the Northern Badia region. *Journal of Ethnopharmacology*, *137*(1), 27-35.

Amaya-Cruz, D. M., Perez-Ramirez, I. F., Ortega-Diaz, D., Rodriguez-Garcia, M. E., & Reynoso-Camacho, R. (2018). Roselle (Hibiscus sabdariffa) by-product as functional ingredient: effect of thermal processing and particle size reduction on bioactive constituents and functional, morphological, and structural properties. *Journal of Food Measurement and Characterization*, *12*, 135-144.

Amer, S. A., Al-Khalaifah, H. S., Gouda, A., Osman, A., Goda, N. I., Mohammed, H. A., ... & Mohamed, S. K. A. (2022). Potential effects of

anthocyanin-rich roselle (Hibiscus sabdariffa L.) extract on the growth, intestinal histomorphology, blood biochemical parameters, and the immune status of broiler chickens. *Antioxidants*, *11*(3), 544.

Anal, A. K., Shrestha, S., & Sadiq, M. B. (2019). Biopolymeric-based emulsions and their effects during processing, digestibility and bioaccessibility of bioactive compounds in food systems. *Food hydrocolloids*, *87*, 691-702.

Aslan, D., & Dogan, M. (2018). The influence of ultrasound on the stability of dairy-based, emulsifier-free emulsions: rheological and morphological aspect. *European Food Research and Technology*, *244*, 409-421.

Baiocco, D., Preece, J. A., & Zhang, Z. (2021). Encapsulation of hexylsalicylate in an animal-free chitosan-gum Arabic shell by complex coacervation. Colloids and Surfaces A*: Physicochemical and Engineering Aspects*, *625*, 126861.

Baldino, N., Mileti, O., Lupi, F., & Gabriele, D. (2023). Interfacial rheology of food: protein as a model food. *In Advances in Food Rheology and Its Applications* (pp. 3-26). Woodhead Publishing.

Bastola, K. P., Guragain, Y. N., Bhadriraju, V., & Vadlani, P. V. (2017). Evaluation of standards and interfering compounds in the determination of phenolics by Folin-Ciocalteu assay method for effective bioprocessing of biomass. *American Journal of Analytical Chemistry*, *8*(6), 416-431.

Berton-Carabin, C. C., Ropers, M. H., & Genot, C. (2014). Lipid oxidation in oil-in-water emulsions: Involvement of the interfacial layer. *Comprehensive Reviews in Food Science and Food Safety*, *13*(5), 945-977.

Betz, M., & Kulozik, U. (2011). Whey protein gels for the entrapment of bioactive anthocyanins from bilberry extract. *International Dairy Journal*, *21*(9), 703-710.

Bylda, C., Thiele, R., Kobold, U., & Volmer, D. A. (2014). Recent advances in sample preparation techniques to overcome difficulties encountered during quantitative analysis of small molecules from biofluids using LC-MS/MS. *Analyst*, *139*(10), 2265-2276.

Carvajal-Zarrabal, O., Barradas-Dermitz, D. M., Orta-Flores, Z., Hayward-Jones, P. M., Nolasco-Hipólito, C., Aguilar-Uscanga, M. G., (2012). Hibiscus sabdariffa L., roselle calyx, from ethnobotany to pharmacology. *Journal of experimental pharmacology*, 25-39.

Cassol, L., Rodrigues, E., & Noreña, C. P. Z. (2019). Extracting phenolic compounds from Hibiscus sabdariffa L. calyx using microwave assisted extraction. Industrial crops and products, *133*, 168-177.

Castro-Enríquez, D. D., Montaño-Leyva, B., Del Toro-Sánchez, C. L., Juaréz-Onofre, J. E., Carvajal-Millan, E., Burruel-Ibarra, S. E., (2020). Stabilization of betalains by encapsulation—a review. *Journal of food science and technology*, *57*, 1587-1600.

Cha, Y., Shi, X., Wu, F., Zou, H., Chang, C., Guo, Y., ... & Yu, C. (2019). Improving the stability of oil-in-water emulsions by using mussel myofibrillar proteins and lecithin as emulsifiers and high-pressure homogenization. *Journal of Food Engineering*, *258*, 1-8.

Chen, K., Lei, L., Lou, H., Niu, J., Yang, D., Qiu, X., & Qian, Y. (2020). High internal phase emulsions stabilized with carboxymethylated lignin for

encapsulation and protection of environmental sensitive natural extract. *International journal of biological macromolecules*, *158*, 430-442.

Chevalier, Y., & Bolzinger, M. A. (2013). Emulsions stabilized with solid nanoparticles: Pickering emulsions. Colloids and Surfaces A: *Physicochemical and Engineering Aspects*, *439*, 23-34.

Chikhoune, A., Gagaoua, M., Nanema, K. D., Souleymane, A. S., Hafid, K., Aliane, K., ... & Vovk, I. (2017). Antioxidant activity of Hibiscus sabdariffa extracts incorporated in an emulsion system containing whey proteins: Oxidative stability and polyphenol–whey proteins interactions. *Arabian Journal for Science and Engineering*, *42*, 2247-2260.

Choi, S. J., & McClements, D. J. (2020). Nanoemulsions as delivery systems for lipophilic nutraceuticals: Strategies for improving their formulation, stability, functionality and bioavailability. *Food science and biotechnology*, *29*, 149-168.

Chuacharoen, T., & Sabliov, C. M. (2016). Stability and controlled release of lutein loaded in zein nanoparticles with and without lecithin and pluronic F127 surfactants. Colloids and surfaces A: *Physicochemical and engineering aspects*, *503*, 11-18.

Cid-Ortega, S., & Guerrero-Beltrán, J. A. (2015). Roselle calyces (Hibiscus sabdariffa), an alternative to the food and beverages industries: a review. *Journal of Food Science and Technology*, 52, 6859-6869.

Clark, J. P. (2013). Emulsions: when oil and water do mix. *Food Technology*, *67*(8), 1-9.

Clegg, P. S., Tavacoli, J. W., & Wilde, P. J. (2016). One-step production of multiple emulsions: microfluidic, polymer-stabilized and particle-stabilized approaches. *Soft Matter*, *12*(4), 998-1008.

Comas, D. I., Wagner, J. R., & Tomás, M. C. (2006). Creaming stability of oil in water (O/W) emulsions: Influence of pH on soybean protein–lecithin interaction. *Food Hydrocolloids*, *20*(7), 990-996.

Cooper-Driver, G. A. (2001). Contributions of Jeffrey Harborne and co-workers to the study of anthocyanins. *Phytochemistry*, *56*(3), 229-236.

Dai, L., Sun, C., Wang, D., & Gao, Y. (2016). The interaction between zein and lecithin in ethanol-water solution and characterization of zein–lecithin composite colloidal nanoparticles. *PLoS One*, *11*(11), e0167172.

Da-Costa-Rocha, I., Bonnlaender, B., Sievers, H., Pischel, I., & Heinrich, M. (2014). Hibiscus sabdariffa L.–A phytochemical and pharmacological review. *Food chemistry*, *165*, 424-443.

Da Silva, R. P., Rocha-Santos, T. A., & Duarte, A. C. (2016). Supercritical fluid extraction of bioactive compounds. *Trends in Analytical Chemistry*, *76*, 40-51.

De Folter, J. W., Van Ruijven, M. W., & Velikov, K. P. (2012). Oil-in-water Pickering emulsions stabilized by colloidal particles from the water-insoluble protein zein. *Soft Matter*, *8*(25), 6807-6815.

Deng, L. (2021). Current progress in the utilization of soy-based emulsifiers in food applications—A Review. *Foods*, 10(6), 1354.

Díaz-Ruiz, R., Valdeón, I., Álvarez, J. R., Matos, M., & Gutiérrez, G. (2021). Simultaneous encapsulation of trans-resveratrol and vitamin D3 in highly concentrated double emulsions. *Journal of the Science of Food and Agriculture*, *101*(9), 3654-3664.

Du, X., Hu, M., Liu, G., Yan, S., Qi, B., Zhang, S., ... & Zhu, X. (2022). Development of high-internal-phase emulsions stabilized by soy protein isolate–dextran complex for the delivery of quercetin. *Journal of the Science of Food and Agriculture*, *102*(14), 6273-6284.

Duy, N. Q., Thoai, H., & Lam, T. (2019). Effects of Different Extraction Solvent Systems on Total Phenolic, Total Flavonoid, Total Anthocyanin Contents and Antioxidant Activities of Roselle (Hibiscus sabdariffa L.) Extracts. ASIAN J. Chem, *31*, 2517-2521.

Djuardi, A. U. P., Yuliana, N. D., Ogawa, M., Akazawa, T., & Suhartono, M. T. (2020). Emulsifying properties and antioxidant activity of soy protein isolate conjugated with tea polyphenol extracts. *Journal of Food Science and Technology*, *57*, 3591-3600.

Feng, T., Wang, X., Fan, C., Wang, X., Wang, X., Cui, H., ... & Huang, Q. (2023). The selective encapsulation and stabilization of cinnamaldehyde and eugenol in high internal phase Pickering emulsions: Regulating the interfacial properties. *Food Chemistry*, *401*, 134139.

Formagio, A. S. N., Ramos, D. D., Vieira, M. C., Ramalho, S. R., Silva, M. M., Zárate, N. A. H., ... & Carvalho, J. E. (2015). Phenolic compounds of Hibiscus sabdariffa and influence of organic residues on its antioxidant and antitumoral properties. *Brazilian Journal of Biology*, *75*, 69-76.

Gao, H., Ma, L., Cheng, C., Liu, J., Liang, R., Zou, L., ... & McClements, D. J. (2021). Review of recent advances in the preparation, properties, and applications of high internal phase emulsions. *Trends in Food Science & Technology*, *112*, 36-49.

García-Moreno, P. J., Horn, A. F., & Jacobsen, C. (2014). Influence of casein–phospholipid combinations as emulsifier on the physical and oxidative stability of fish oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, *62*(5), 1142-1152.

Geng, M., Li, L., Feng, X., Xu, J., Huang, Y., Teng, F., & Li, Y. (2022). Encapsulation of β-carotene in high internal phase Pickering emulsions stabilized by soy protein isolate–epigallocatechin-3-gallate covalent composite microgel particles. *Journal of Molecular Liquids*, *360*, 119511.

Giusti, M. M., & Wrolstad, R. E. (2001). Characterization and measurement of anthocyanins by UV-visible spectroscopy. Current protocols in food analytical chemistry, (1), F1-2.

Giroux, H. J., Robitaille, G., & Britten, M. (2016). Controlled release of casein-derived peptides in the gastrointestinal environment by encapsulation in water-in-oil-in-water double emulsions. *Food Science and Technology*, *69*, 225-232.

Guan, H., Diao, X., Liu, D., Han, J., Kong, B., Liu, D., ... & Zhang, L. (2020). Effect of high-pressure processing enzymatic hydrolysates of soy protein isolate on the emulsifying and oxidative stability of myofibrillar protein-prepared oil-in-water emulsions. *Journal of the Science of Food and Agriculture*, *100*(10), 3910-3919.

Halim, Y., Evelyne, C., Rosa, D., & Ramli, S. (2022). Development of Roselle (Hibiscus sabdariffa L.) Calyx Jelly Candy. Caraka Tani: *Journal of Sustainable Agriculture*, *37*(2), 357-372.

Hapsari, B. W., & Setyaningsih, W. (2021). Methodologies in the analysis of phenolic compounds in roselle (Hibiscus sabdariffa L.): composition,

biological activity, and beneficial effects on human health. *Horticulturae*, *7*(2), 35.

Hebishy, E., Buffa, M., Guamis, B., Blasco-Moreno, A., & Trujillo, A. J. (2015). Physical and oxidative stability of whey protein oil-in-water emulsions produced by conventional and ultra high-pressure homogenization: Effects of pressure and protein concentration on emulsion characteristics. *Innovative Food Science & Emerging Technologies*, *32*, 79-90.

Herranz-López, M., Olivares-Vicente, M., Encinar, J. A., Barrajón-Catalán, E., Segura-Carretero, A., Joven, J., & Micol, V. (2017). Multi-targeted molecular effects of Hibiscus sabdariffa polyphenols: an opportunity for a global approach to obesity. *Nutrients*, *9*(8), 907.

Hidayat, R., & Wulandari, P. (2021). Methods of extraction: Maceration, percolation and decoction. *Eureka Herba Indonesia*, *2*(1), 68-74.

Huang, Z. X., Lin, W. F., Zhang, Y., & Tang, C. H. (2022). Outstanding Freeze-Thaw Stability of Mayonnaise Stabilized Solely by a Heated Soy Protein Isolate. *Food Biophysics*, *17*(3), 335-343.

Homayouni Rad, A., Karbalaei, F., Torbati, M. A., Moslemi, M., Shahraz, F., Babadi, R., & Javadi, M. (2021). Effect of Hibiscus sabdariffa and Camellia synensis extracts on microbial, antioxidant and sensory properties of ice cream. *Journal of Food Science and Technology*, 1-10.

Hsiao, Y. P., Lai, W. W., Wu, S. B., Tsai, C. H., Tang, S. C., Chung, J. G., & Yang, J. H. (2015). Triggering apoptotic death of human epidermal keratinocytes by malic Acid: Involvement of endoplasmic reticulum stress-and mitochondria-dependent signaling pathways. *Toxins*, *7*(1), 81-96.

Idham, Z., Putra, N. R., Nasir, H., Yian, L. N., Mohd Idrus, N. F., & Che Yunus, M. A. (2021). Extraction and Solubility Modeling of Anthocyanins Rich Extract from Hibiscus sabdariffa L. using Supercritical Carbon Dioxide. *Malaysian Journal of Fundamental and Applied Sciences*, *17*(6), 720–730.

Islam, M. (2019). Food and medicinal values of Roselle (Hibiscus sabdariffa L. Linne Malvaceae) plant parts: A review. Open J Nutr Food Sci, *1*(1), 1003.

Ismail, A., Ikram, E. H. K., & Nazri, H. S. M. (2008). Roselle (Hibiscus sabdariffa L.) seeds nutritional composition protein quality and health benefits. *Food*, *2*(1), 1-16.

Jouki, M., Khazaei, N., Rashidi-Alavijeh, S., & Ahmadi, S. (2021). Encapsulation of Lactobacillus casei in quince seed gum-alginate beads to produce a functional synbiotic drink powder by agro-industrial by-products and freeze-drying. *Food Hydrocolloids*, *120*, 106895.

Katouzian, I., & Jafari, S. M. (2016). Nano-encapsulation as a promising approach for targeted delivery and controlled release of vitamins. *Trends in Food Science & Technology*, *53*, 34-48.

Kandasamy, S., & Naveen, R. (2022). A review on the encapsulation of bioactive components using spray-drying and freeze-drying techniques. *Journal of Food Process Engineering*, *45*(8), e14059.

Karim, A., Rehman, A., Feng, J., Noreen, A., Assadpour, E., Kharazmi, M. S., ... & Jafari, S. M. (2022). Alginate-based nanocarriers for the delivery and controlled-release of bioactive compounds. *Advances in Colloid and Interface Science*, 102744.

Knoth, A., Scherze, I., & Muschiolik, G. (2005). Stability of water-in-oil-emulsions containing phosphatidylcholine-depleted lecithin. *Food Hydrocolloids*, *19*(3), 635-640.

Ko, A., Lee, J. S., Sop Nam, H., & Gyu Lee, H. (2017). Stabilization of black soybean anthocyanin by chitosan nanoencapsulation and copigmentation. *Journal of food biochemistry*, *41*(2), e12316.

Lang, Y., Li, B., Gong, E., Shu, C., Si, X., Gao, N., ... & Meng, X. (2021). Effects of α-casein and β-casein on the stability, antioxidant activity and bioaccessibility of blueberry anthocyanins with an in vitro simulated digestion. *Food Chemistry*, *334*, 127526.

Lefebvre, T., Destandau, E., & Lesellier, E. (2021). Selective extraction of bioactive compounds from plants using recent extraction techniques: A review. *Journal of Chromatography* A, *1635*, 461770.

Li, D., Hu, M., Hou, L., Gao, Y., Tian, Z., Wen, W., ... & Wang, F. (2023). The structural and functional properties of soybean protein-polyglutamic acid complex effected the stability of W/O/W emulsion encapsulated Nattokinase. *Food Chemistry*, *414*, 135724.

Li, J., Li, Y., & Guo, S. (2014). The binding mechanism of lecithin to soybean 11S and 7S globulins using fluorescence spectroscopy. *Food Science and Biotechnology*, *23*, 1785-1791.

Li, X. L., Liu, W. J., Xu, B. C., & Zhang, B. (2022). Simple method for fabrication of high internal phase emulsions solely using novel pea protein isolate nanoparticles: Stability of ionic strength and temperature. *Food Chemistry*, *370*, 130899.

Liao, P., Yang, S., Dai, S., Lian, Z., Yang, J., Zhang, Q., ... & Jiang, L. (2022). The multilayered emulsion-filled gel microparticles: Regulated the release behavior of β-carotene. *Journal of Food Engineering*, *331*, 111119.

Liu, F., & Tang, C. H. (2016). Soy glycinin as food-grade Pickering stabilizers: Part. III. Fabrication of gel-like emulsions and their potential as sustained-release delivery systems for β-carotene. *Food Hydrocolloids*, *56*, 434-444.

López-Bascón, M. A., & De Castro, M. L. (2020). Soxhlet extraction. In Liquid-phase extraction (pp. 327-354). *Elsevier*.

Mantovani, R. A., Cavallieri, Â. L. F., Netto, F. M., & Cunha, R. L. (2013). Stability and in vitro digestibility of emulsions containing lecithin and whey proteins. *Food & Function*, *4*(9), 1322-1331.

Marinea, M., Ellis, A., Golding, M., & Loveday, S. M. (2023). Delivering Phenolic Acids in Soy Protein Gels: Noncovalent Interactions Control Gastrointestinal Bioaccessibility. *Food Biophysics*, *18*(2), 218-227.

Martínez, K. D., Ganesan, V., Pilosof, A. M., & Harte, F. M. (2011). Effect of dynamic high-pressure treatment on the interfacial and foaming properties of soy protein isolate–hydroxypropylmethylcelluloses systems. *Food Hydrocolloids*, *25*(6), 1640-1645.

McClements, D. J., Decker, E. A., & Weiss, D. J. (2007). Emulsion-based delivery systems for lipophilic bioactive components. *Journal of food science*, *72*(8), R109-R124.

Mena-García, A., Ruiz-Matute, A. I., Soria, A. C., & Sanz, M. L. (2019). Green techniques for extraction of bioactive carbohydrates. *Trends in Analytical Chemistry*, *119*, 115612.

Mosca, M., Cuomo, F., Lopez, F., & Ceglie, A. (2013). Role of emulsifier layer, antioxidants and radical initiators in the oxidation of olive oil-in-water emulsions. *Food Research International*, *50*(1), 377-383.

Muhoza, B., Qi, B., Harindintwali, J. D., Koko, M. Y. F., Zhang, S., & Li, Y. (2022). Combined plant protein modification and complex coacervation as a sustainable strategy to produce coacervates encapsulating bioactives. *Food Hydrocolloids*, *124*, 107239.

Nenadis, N., Wang, L. F., Tsimidou, M., & Zhang, H. Y. (2004). Estimation of scavenging activity of phenolic compounds using the ABTS•+ assay. *Journal of agricultural and food chemistry*, *52*(15), 4669-4674.

Nejatian, M., Darabzadeh, N., Bodbodak, S., Saberian, H., Rafiee, Z., Kharazmi, M. S., & Jafari, S. M. (2022). Practical application of nanoencapsulated nutraceuticals in real food products; a systematic review. *Advances in Colloid and Interface Science*, 102690.

Nguyen, M. P. (2021). Physicochemical characteristics, viability of starters, total phenolics and antioxidant activities of functional yoghurt supplemented with extracts from Hylocereus polyrhizus, Hibiscus sabdariffa and Peristrophe bivalvis. *Plant Science Today*, *8*(1), 149-154.

Jabeur, I., Pereira, E., Barros, L., Calhelha, R. C., Soković, M., Oliveira, M. B. P., & Ferreira, I. C. (2017). Hibiscus sabdariffa L. as a source of nutrients, bioactive compounds and colouring agents. *Food Research International*, *100*, 717-723.

Jara-Palacios, M. J., Gonçalves, S., Hernanz, D., Heredia, F. J., & Romano, A. (2018). Effects of in vitro gastrointestinal digestion on phenolic compounds and antioxidant activity of different white winemaking byproducts extracts. *Food Research International*, *109*, 433-439.

Jiang, H., Zhang, T., Smits, J., Huang, X., Maas, M., Yin, S., & Ngai, T. (2021). Edible high internal phase Pickering emulsion with double-emulsion morphology. *Food Hydrocolloids*, *111*, 106405.

Jin, Y., Liu, D., & Hu, J. (2021). Effect of surfactant molecular structure on emulsion stability investigated by interfacial dilatational rheology. *Polymers*, *13*(7), 1127.

Jin, W., Zhu, J., Jiang, Y., Shao, P., Li, B., & Huang, Q. (2017). Gelatin-based nanocomplex-stabilized Pickering emulsions: Regulating droplet size and wettability through assembly with glucomannan. *Journal of agricultural and food chemistry*, *65*(7), 1401-1409.

Kaimainen, M., Marze, S., Järvenpää, E., Anton, M., & Huopalahti, R. (2015). Encapsulation of betalain into w/o/w double emulsion and release during in vitro intestinal lipid digestion. *Food Science and Technology*, *60*(2), 899-904.

Kim, S. H., Lim, H. W., Chon, J. W., Song, K. Y., & Seo, K. H. (2019). Sensory profiles of dairy products supplemented with Hibiscus sabdariffa Linnaeus (Roselle) powder: A preliminary study. *Journal of Dairy Science and Biotechnology*, *37*(4), 247-255.

Lam, S., Velikov, K. P., & Velev, O. D. (2014). Pickering stabilization of foams and emulsions with particles of biological origin. *Current Opinion in Colloid & Interface Science*, *19*(5), 490-500.

Li, Y., Wu, C. L., Liu, J., Zhu, Y., Zhang, X. Y., Jiang, L. Z., ... & Teng, F. (2018). Soy protein isolate-phosphatidylcholine nanoemulsions prepared using high-pressure homogenization. *Nanomaterials*, *8*(5), 307.

Liu, C., & Wang, X. (2021). The physicochemical properties and stability of flaxseed oil emulsions: effects of emulsification methods and the ratio of soybean protein isolate to soy lecithin. *Journal of the Science of Food and Agriculture*, *101*(15), 6407-6416.

Paraíso, C. M., Dos Santos, S. S., Correa, V. G., Magon, T., Peralta, R. M., Visentainer, J. V., & Madrona, G. S. (2019). Ultrasound assisted extraction of hibiscus (Hibiscus sabdariffa L.) bioactive compounds for application as potential functional ingredient. *Journal of food science and technology*, *56*, 4667-4677.

Pimentel-Moral, S., Borrás-Linares, I., Lozano-Sánchez, J., Arráez-Román, D., Martínez-Férez, A., & Segura-Carretero, A. (2019). Supercritical CO2 extraction of bioactive compounds from Hibiscus sabdariffa. *The Journal of Supercritical Fluids*, *147*, 213-221.

Pinela, J., Prieto, M. A., Pereira, E., Jabeur, I., Barreiro, M. F., Barros, L., & Ferreira, I. C. (2019). Optimization of heat-and ultrasound-assisted extraction of anthocyanins from Hibiscus sabdariffa calyces for natural food colorants. *Food chemistry*, *275*, 309-321.

Pinto, J., Spínola, V., Llorent-Martínez, E. J., Fernández-de Córdova, M. L., Molina-García, L., & Castilho, P. C. (2017). Polyphenolic profile and antioxidant activities of Madeiran elderberry (Sambucus lanceolata) as affected by simulated in vitro digestion. *Food Research International*, *100*, 404-410.

Piñón-Balderrama, C. I., Leyva-Porras, C., Terán-Figueroa, Y., Espinosa-Solís, V., Álvarez-Salas, C., & Saavedra-Leos, M. Z. (2020). Encapsulation of active ingredients in food industry by spray-drying and nano spray-drying technologies. *Processes*, *8*(8), 889.

Piovesana, A., Rodrigues, E., & Noreña, C. P. Z. (2019). Composition analysis of carotenoids and phenolic compounds and antioxidant activity from hibiscus calyces (Hibiscus sabdariffa L.) by HPLC-DAD-MS/MS. *Phytochemical Analysis*, *30*(2), 208-217.

Pirestani, S., Nasirpour, A., Keramat, J., Desobry, S., & Jasniewski, J. (2017). Effect of glycosylation with gum Arabic by Maillard reaction in a liquid system on the emulsifying properties of canola protein isolate. *Carbohydrate Polymers*, *157*, 1620-1627.

Pollini, L., Tringaniello, C., Ianni, F., Blasi, F., Manes, J., & Cossignani, L. (2020). Impact of ultrasound extraction parameters on the antioxidant properties of Moringa oleifera leaves. *Antioxidants*, *9*(4), 277.

Rasheed, T., Shafi, S., Bilal, M., Hussain, T., Sher, F., & Rizwan, K. (2020). Surfactants-based remediation as an effective approach for removal of environmental pollutants—A review. *Journal of Molecular Liquids*, *318*, 113960.

Riaz, G., & Chopra, R. (2018). A review on phytochemistry and therapeutic uses of Hibiscus sabdariffa L. Biomedicine & Pharmacotherapy, *102*, 575-586.

Rishabh, D., Athira, A., Preetha, R., & Nagamaniammai, G. (2021). Freeze dried probiotic carrot juice powder for better storage stability of probiotic. *Journal of Food Science and Technology*, 1-9.

Rocchetti, G., Chiodelli, G., Giuberti, G., Ghisoni, S., Baccolo, G., Blasi, F., ... & Lucini, L. (2018). UHPLC-ESI-QTOF-MS profile of polyphenols in Goji berries (Lycium barbarum L.) and its dynamics during in vitro

gastrointestinal digestion and fermentation. *Journal of Functional Foods*, *40*, 564-572.

Schefer, S., Oest, M., & Rohn, S. (2021). Interactions between phenolic acids, proteins, and carbohydrates—Influence on dough and bread properties. *Foods*, *10*(11), 2798.

Scuriatti, M. P., Tomás, M. C., & Wagner, J. R. (2003). Influence of soybean protein isolates-phosphatidycholine interaction on the stability on oil-in-water emulsions. *Journal of the American Oil Chemists' Society*, *80*(11), 1093-1100.

Shao, Y., & Tang, C. H. (2016). Gel-like pea protein Pickering emulsions at pH 3.0 as a potential intestine-targeted and sustained-release delivery system for β-carotene. *Food research international*, *79*, 64-72.

Shen, R., Yang, X., & Lin, D. (2023). PH sensitive double-layered emulsions stabilized by bacterial cellulose nanofibers/soy protein isolate/chitosan complex enhanced the bioaccessibility of curcumin: In vitro study. *Food Chemistry*, *402*, 134262.

Shi, A., Feng, X., Wang, Q., & Adhikari, B. (2020). Pickering and high internal phase Pickering emulsions stabilized by protein-based particles: A review of synthesis, application and prospective. *Food Hydrocolloids*, *109*, 106117.

Sohn, B., Olenskyj, A., & Feng, Y. (2017). Correlation between emulsifier concentration and emulsion droplet size in oil-in-water emulsion stabilized by zein nanoparticles. *i-ACES*, *3*(1), 1-5.

Sinela, A., Rawat, N., Mertz, C., Achir, N., Fulcrand, H., & Dornier, M. (2017). Anthocyanins degradation during storage of Hibiscus sabdariffa extract and evolution of its degradation products. *Food Chemistry*, *214*, 234-241.

Sirovec, S., Tušek, A. J., Benković, M., Valinger, D., Cvetnić, T. S., Kljusurić, J. G., & Jurina, T. (2022). Emulsification of Rosemary and Oregano Aqueous Extracts and Their In Vitro Bioavailability. *Plants*, *11*(23), 3372.

Suhaili, N. I. M., & Manshoor, N. (2022). Ethnomedicine, phytochemistry, and bioactivities of Hibiscus sabdariffa L.(Malvaceae). *Journal of Herbmed Pharmacology*, *11*(4), 451-460.

Sun, B., Wang, W., He, Z., Zhang, M., Kong, F., Sain, M., & Ni, Y. (2018). Improvement of stability of tea polyphenols: A review. *Current pharmaceutical design*, *24*(29), 3410-3423.

Šturm, L., Črnivec, I. G. O., Istenič, K., Ota, A., Megušar, P., Slukan, A., ... & Ulrih, N. P. (2019). Encapsulation of non-dewaxed propolis by freeze-drying and spray-drying using gum Arabic, maltodextrin and inulin as coating materials. *Food and Bioproducts Processing*, *116*, 196-211.

Tadros, T. (2004). Application of rheology for assessment and prediction of the long-term physical stability of emulsions. *Advances in colloid and interface science*, *108*, 227-258.

Tadros, T. F. (2013). Emulsion formation, stability, and rheology. Emulsion formation and stability, 1-75.

Tamnak, S., Mirhosseini, H., Tan, C. P., Ghazali, H. M., & Muhammad, K. (2016). Physicochemical properties, rheological behavior and morphology of pectin-pea protein isolate mixtures and conjugates in

aqueous system and oil in water emulsion. *Food Hydrocolloids*, *56*, 405-416.

Tarko, T., & Duda-Chodak, A. (2020). Influence of food matrix on the bioaccessibility of fruit polyphenolic compounds. *Journal of agricultural and food chemistry*, *68*(5), 1315-1325.

Urbonaite, V., De Jongh, H. H. J., Van Der Linden, E., & Pouvreau, L. (2015). Water holding of soy protein gels is set by coarseness, modulated by calcium binding, rather than gel stiffness. *Food hydrocolloids*, *46*, 103-111.

Wang, Y., Fan, B., Tong, L. T., Lu, C., Li, S., Sun, J., ... & Wang, F. (2022). High internal phase emulsions stabilized solely by soy protein isolate. *Journal of Food Engineering*, *318*, 110905.

Wang, S., Shi, Y., Tu, Z., Zhang, L., Wang, H., Tian, M., & Zhang, N. (2017). Influence of soy lecithin concentration on the physical properties of whey protein isolate-stabilized emulsion and microcapsule formation. *Journal of Food Engineering*, *207*, 73-80.

Wang, M., Yan, W., Zhou, Y., Fan, L., Liu, Y., & Li, J. (2021). Progress in the application of lecithins in water-in-oil emulsions. *Trends in Food Science & Technology*, *118*, 388-398.

Warnakulasuriya, S. N., & Nickerson, M. T. (2018). Review on plant protein–polysaccharide complex coacervation, and the functionality and applicability of formed complexes. *Journal of the Science of Food and Agriculture*, *98*(15), 5559-5571.

Wu, Y., Han, Y., Tao, Y., Li, D., Xie, G., Show, P. L., & Lee, S. Y. (2020). In vitro gastrointestinal digestion and fecal fermentation reveal the effect of different encapsulation materials on the release, degradation and modulation of gut microbiota of blueberry anthocyanin extract. *Food research international*, *132*, 109098.

Wu, J., & Ma, G. H. (2016). Recent studies of Pickering emulsions: particles make the difference. *Small*, *12*(34), 4633-4648.

Xiao, J., Li, C., & Huang, Q. (2015). Kafirin nanoparticle-stabilized Pickering emulsions as oral delivery vehicles: Physicochemical stability and in vitro digestion profile. *Journal of agricultural and food chemistry*, *63*(47), 10263-10270.

Xiong, J., Chan, Y. H., Rathinasabapathy, T., Grace, M. H., Komarnytsky, S., & Lila, M. A. (2020). Enhanced stability of berry pomace polyphenols delivered in protein-polyphenol aggregate particles to an in vitro gastrointestinal digestion model. *Food chemistry*, *331*, 127279.

Xiong, J., Grace, M. H., Esposito, D., Wang, F., & Lila, M. A. (2016). Phytochemical characterization and anti-inflammatory properties of Acacia mearnsii leaves. Natural product communications, *11*(5), 1934578X1601100524.

Verfaillie, D., Janssen, F., Van Royen, G., & Wouters, A. G. (2023). A systematic study of the impact of the isoelectric precipitation process on the physical properties and protein composition of soy protein isolates. *Food Research International*, *163*, 112177.

Yan, S., Xie, F., Zhang, S., Jiang, L., Qi, B., & Li, Y. (2021). Effects of soybean protein isolate− polyphenol conjugate formation on the protein structure and emulsifying properties: Protein− polyphenol

emulsification performance in the presence of chitosan. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *609*, 125641.

Yuksel, Z., Avci, E., & Erdem, Y. K. (2010). Characterization of binding interactions between green tea flavanoids and milk proteins. *Food Chemistry*, *121*(2), 450-456.

Zahed, N., Esmaeilzadeh Kenari, R., & Farahmandfar, R. (2023). Effect of different extraction methods on antioxidant properties and encapsulation efficiency of anthocyanin of pomegranate peel. *Food Science & Nutrition*. *11*(6), 356-362.

Zheoat, A. M., Gray, A. I., Igoli, J. O., Ferro, V. A., & Drummond, R. M. (2019). Hibiscus acid from Hibiscus sabdariffa (Malvaceae) has a vasorelaxant effect on the rat aorta. *Fitoterapia*, *134*, 5-13.

Zhu, Z., Wen, Y., Yi, J., Cao, Y., Liu, F., & McClements, D. J. (2019). Comparison of natural and synthetic surfactants at forming and stabilizing nanoemulsions: Tea saponin, Quillaja saponin, and Tween 80. *Journal of colloid and interface science*, *536*, 80-87.

CURRICULUM VITAE

Name Surname **:** Hümeyra ÇAVDAR

EDUCATION:

BSc **:** 2016-2021 Konya Food and Agriculture University, Food Engineering

MSc **:** 2021-2023 Istanbul Technical University, Faculty of Chemical and Metallurgical Engineering, Department of Food Engineering

PROFESSIONAL EXPERIENCE:

2022- … Research assistant, Istanbul Technical University