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ENCAPSULATION OF OLIVE LEAF EXTRACT BY DOUBLE EMULSION METHOD

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

DEMET SÖNMEZLER

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FOR
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FOOD ENGINEERING

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Approval of the thesis:

ENCAPSULATION OF OLIVE LEAF EXTRACT BY DOUBLE EMULSION METHOD

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ABSTRACT

ENCAPSULATION OF OLIVE LEAF EXTRACT BY DOUBLE EMULSION METHOD

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January 2023, 125 pages

Olive leaves are obtained as a waste product in olive and olive oil production. Biophenols, abundantly found in olive leaves, has well-known health benefits and can be used in dietary supplements, nutraceuticals, or functional food ingredients. However, the sensitivity of biophenols to heat, light and oxidizing agents necessitates the development of various methods, such as encapsulation, to increase their bioavailability. Undesired sensorial properties of the extract can also be masked by encapsulation.

In this study, double emulsions were preferred as an encapsulation method owing to the protective effect it provides to the active substance and the control over its release. W/O/W type of double emulsions was prepared considering the water solubility of olive leaf extract. The effect of different pea flour concentrations (15%, 20%, 25%) and different homogenization methods (high-speed homogenization, ultrasonication) on emulsion properties was experimented. The prepared emulsions were characterized with respect to their particle size, rheology, encapsulation efficiency, stability, optical images and release behavior.

As hypothesized, flours acted as emulsifiers in the outer aqueous phase to increase the stability of emulsions. It was observed that both the long-term and instant stability of emulsions were correlated with the viscosity and particle size. Increasing pea flour concentration from 15% to 25% resulted in a 25% increase in the stability of double emulsions prepared with high-speed homogenization (HSH) and a 30% increase in emulsions prepared with ultrasonication (US). The higher stability of emulsions prepared with 25% was due to their higher viscosity and smaller particle size. Storage temperature also affected the stability of emulsions as emulsions stored at 20°C showed faster degradation compared to 4°C. Moreover, due to the lower viscosity, this effect was more prominent in emulsions in which 15% pea flour was employed. US treatment did not decrease the average particle size of emulsions. Average encapsulation efficiency values for double emulsions prepared with HSH and US were 88.3% and 85.9%, respectively. As a result, pea flours could be used to encapsulate olive leaf extract successfully with high encapsulation efficiencies by using double emulsion method.

Keywords: Olive Leaf Extract, Double Emulsion, Encapsulation, Pea flour, Ultrasonication

ÖZ

ZEYTİN YAPRAĞI ÖZÜTÜNÜN İKİLİ EMULSİYON METODUYLA ENKAPSÜLASYONU

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Ocak 2023, 125 sayfa

Zeytin yaprağı, zeytin ve zeytinyağı üretiminde atık ürün olarak elde edilmektedir. Zeytin yaprağında bol miktarda bulunan biyofenollerin sağlık açısından faydalı oldukları bir çok çalışmada gösterilmiştir ve bunlar diyet takviyelerinde, nutrasötiklerde veya fonksiyonel gıda bileşenlerinde kullanılabilirler. Ancak biyofenollerin ısıya, ışığa, okside edici maddelere karşı olan duyarlılığı, biyoyararlılıklarının arttırılması için enkapsülasyon gibi çeşitli yöntemlerin geliştirilmesini gerekli kılmıştır. Bunun yanısıra enkapsülasyon, zeytin yaprağı özütünün sahip olduğu istenmeyen duyusal özellikleri maskeleme potansiyeline sahiptir.

Bu çalışmada ikili emülsiyon yöntemi aktif maddeye sağladığı koruyucu etki ve salınım üzerinde sağladığı kontrol dolayısıyla tercih edilmiştir. Farklı bezelye unu konsantrasyonlarının (%15, %20, %25) ve homojenizasyon yöntemlerinin (yüksek hızlı homojenizasyon, ultrasonikasyon) emülsiyon özellikleri üzerine olan etkisi incelenmiştir. Zeytin yaprağı özütünün suda çözünebilir olduğu göz önüne alınarak S/Y/S tipi ikili emülsiyonlar kullanılmıştır. Hazırlanan emülsiyonların

karakterizasyonu için partikül boyutu ve dağılımı, reolojileri, enkapsülasyon verimleri, stabiliteleri, mikroskop görüntüleri ve salım hızları incelenmiştir.

Çalışma sonucunda, bezelye unlarının beklenildiği üzere dış fazda emülgatör görevi gördüğü ve emülsiyonların stabilitesine katkıda bulunduğu gözlemlenmiştir. Emülsiyonların hem anlık stabilitesinin hem de depolama sırasında stabilitelerinin viskozite ve partikül boyutlarıyla ile ilişkili olduğu görülmüştür. Bezelye unu konsantrasyonunun %15'ten %25'e yükseltilmesi, yüksek hızlı homojenizasyon yöntemi ile hazırlanan ikili emülsiyonların stabilitesinde %25, ultrasonikasyon yöntemi ile hazırlanan emülsiyonlarda ise %30'luk bir artışa sebep olmuştur. %25 un konsantrasyonu ile hazırlanan emülsiyonların, yüksek viskoziteye ve daha küçük parçacık boyutlarına sahip olmaları nedeniyle daha yüksek stabiliteye sahip oldukları görülmüştür. Aynı zamanda depolama sıcaklığının da stabiliteye etkisi olmuştur. Emülsiyonlar 20°C'de depolandıklarında, 4°C'ye kıyasla daha hızlı bozulma göstermişlerdir. %15 bezelye unu kullanılan emülsiyonlarda viskozitenin daha düşük olmasından dolayı bu etki daha belirgin bir şekilde görülmüştür. Ultrasonikasyon uygulanması emülsiyonların ortalama parçacık büyüklüğünü düşürmemiştir. Yüksek hızlı homojenizasyon ve ultrasonikasyon ile hazırlanan ikili emülsiyonlar için ortalama enkapsülasyon verimliliği %88.3 ve %85.9 olarak bulunmuştur. Sonuç olarak, unların zeytin yaprağı özütünün ikili emülsiyon yöntemiyle enkapsülasyonunda başarılı bir şekilde kullanılabileceği gösterilmiştir.

Anahtar Kelimeler: Zeytin Yaprağı Özütü, İkili Emulsiyon, Enkapsülasyon, Bezelye Unu, Ultrasonikasyon

To my beloved family and friends,

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LIST OF ABBREVIATIONS

ABBREVIATIONS

$D_{3,2}$: Surface moment mean diameter (μm)

$D_{4,3}$: Volume moment mean diameter (μm)

DE: Double emulsion

EE: Encapsulation efficiency

HSH: High-speed homogenization

IS: Instant stability

K: Consistency coefficient (Pa.s)

n: Flow behavior index

O/W: Oil in water emulsion

O/W/O: Oil in water in oil emulsion

OLE TPC: TPC content of OLE (mg GAE/g)

OLE: Olive Leaf Extract

PE: Primary emulsion

PGPR: Polyglycerol polyricinoleate

Rpm: Revolution per minute

SE: Standard error

SS: Storage stability

TPC: Total phenolic content

US: Ultrasonication

W/O: Water in oil emulsion

W1: Inner water phase of the emulsion

W1/O/W2: Water in oil in water emulsion

W2: Outer water phase of the emulsion

CHAPTER 1

INTRODUCTION

The olive tree, or Olea europaea L., is a small tree indigenous to tropical and mild temperate zones. It is a member of the family Oleaceae (Ghanbari et al., 2012). Since olive trees are accommodated in the Mediterranean climate, olives and their products have an important place in the diet. By 2015, nearly 98% of the olive trees were on the Mediterranean coasts (Peralbo-Molina & Luque deCastro, 2013). Thus, countries that are in the Mediterranean region produce and consume these products extensively. As stated by the Turkish Ministry of Food and Agriculture, in 2021, 1,738,680 tonnes of olives were produced (2022). In addition, Turkey is among the top 5 in the world in olive oil production (TEPGE, 2020).

Production of most crops has traditionally been focused on producing a single high-value product while disposing of the by-products. In some cases, these products can also be used to produce lower-quality products (Peralbo-Molina & Luque deCastro, 2013). Every year, a significant number of byproducts and wastes are obtained from both olive tree agriculture and the olive processing sector while the majority of these products have no practical usage (Talhaoui et al., 2015).

Olive leaves are one of the by-products of table olive and olive oil production together with olive cake, olive mill wastewater, and olive wood. These by-products are also transported to the factory together with the olives and cannot be consumed or converted into a marketable product. Olive leaves make up about 10% of the weight that is gathered and delivered to the factory after the trees are harvested (Difonzo et al., 2017; Herrero et al., 2011).

Leaves are also obtained through the pruning of olive trees. According to Talhaoui et al. (2015), even though it changes with respect to pruning conditions, approximately 5 kg of olive leaves are produced per tree annually. As in the case of oil industry, these undesired products can also be polluting for the environment (Peralbo-Molina & Luque deCastro, 2013). Thus, it is both important and crucial to find innovative ways for the valorization of these by-products, which will also support sustainable agriculture.

1.1 Olive Leaf Extract

Olive leaves vary between 5-6 cm in length and 1-1.5 cms in width. Even though their features vary depending on the environmental conditions, these leaves are characterized by a grayish-green color, fine edges, and a short stem, as seen in Figure 1.1 (Borjan et al., 2020).

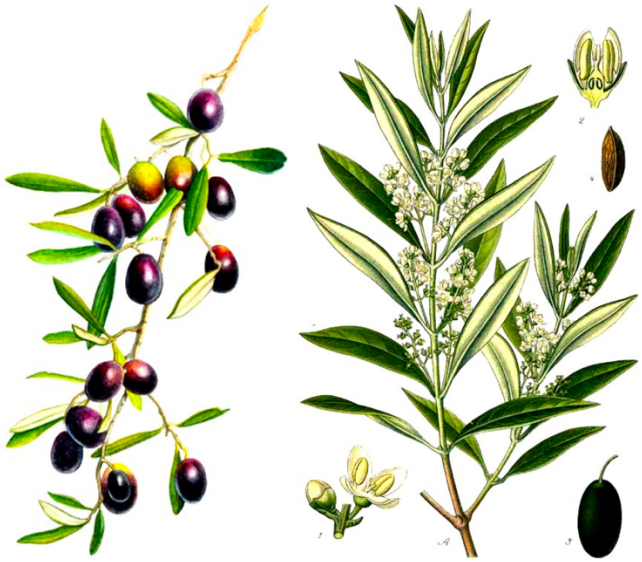

Figure 1.1 An illustration of olive branch and leaves (Debib & Boukhatem, 2017)

Leaves are one of the richest sources of phenolic compounds among the various parts of the olive tree (Rafiee et al., 2011). Since it possesses polyphenolic compounds that are valuable in natural medicine due to a wide variety of health-promoting effects, olive leaf extract has historically been referred to as an herbal supplement (Borjan et al., 2020).

Researchers employed a variety of methods for the extraction of phenolics from olive leaves. These methods include solvent extraction, ultrasonic and microwave-assisted extraction, supercritical fluid extraction, and pressurized liquid extraction using different solvents (Topuz & Bayram, 2022). The primary biophenol in olive leaf extract is Oleuropein which can reach a concentration of 60–90 mg/g in dry leaves (Ghanbari et al., 2012; Gharehbeglou et al., 2019). Olive leaves are also recognized as rich sources of hydroxytyrosol, tyrosol, tocopherol, elenolic acid derivatives, luteolin, diosmetin, rutin, oleuropein aglycone, ligstroside aglycone along with caffeic acid, p-coumaric acid, and vanillic acid (Debib & Boukhatem, 2017; Ghanbari et al., 2012; Martín-García et al., 2022). The chemical structure of some of the phenolic compounds in olive leaves is given in Figure 1.2.

Oleuropein possesses effects that include antiviral, cardio-protective, and anti-hypertensive (Borjan et al., 2020; Debib & Boukhatem, 2017). Other phenolic compounds found in olive leaf extract are also found to have anti-inflammatory, anti-diabetic as well as anti-tumorigenic properties (Borjan et al., 2020; Martín-García et al., 2022). Moreover, as a result of the synergistic interactions between the components of polyphenol-rich extracts, which often contain a variety of phenolic compounds, the potential health benefits of the extract may be enhanced (Bamba et al., 2018; Borjan et al., 2020).

Since olive leaves have a diverse polyphenol profile, olive leaf extract is promising as a natural antioxidant (Robert et al., 2019). Han & Baik (2008) emphasized that in tea, fruits, and supplements derived from natural products with antioxidant properties, total antioxidant activity was substantially proportional to the total phenolic content. Olive leaf extract can be added to products with high oil

content for reduction of oxidation which will, in return, reduce the formation of off-flavors (Mohammadi, Jafari, Assadpour, et al., 2016).

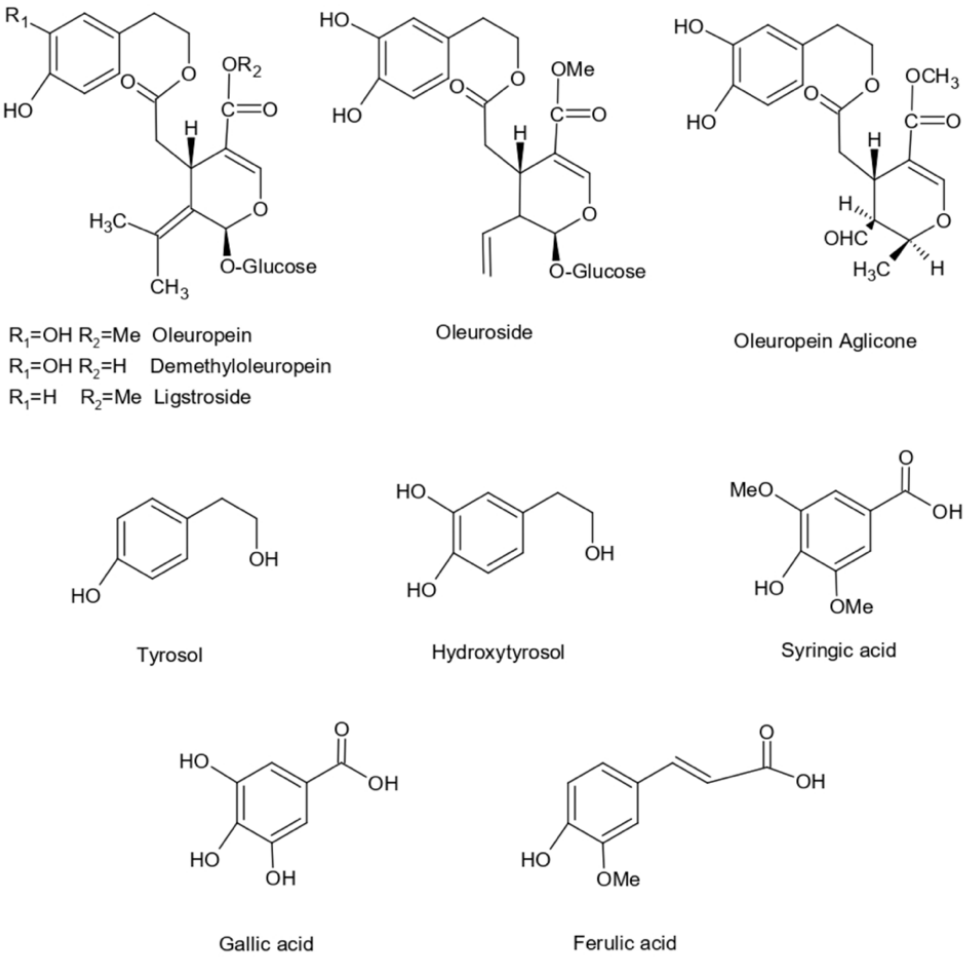

Figure 1.2 Chemical structures of phenolic compounds found in olive leaf extract (Borjan et al., 2020)

Additionally, the leaves have antibacterial capabilities that can eliminate microorganisms, including bacteria, fungi, and mycoplasma (Ghanbari et al., 2012). Oleuropein is thought to exert its antimicrobial effect by denaturation of proteins, damaging bacterial membranes, and disrupting the peptidoglycan layer or enhancing its permeability, similar to other phenolic compounds (Bayram et al., 2020; Martín-García et al., 2022). Sudjana et al. (2009) observed that commercial olive leaf extract is effective against *Campylobacter jejuni*, *Staphylococcus aureus and Helicobacter pylori* whereas Borjan et al. (2020) stated that phenolic compounds from the extract are also effective on *Bacillus cereus, Escherichia coli, Candida albicans* and *Pseudomonas aeruginosa.*

However, as polyphenols are highly sensitive to environmental stresses after being extracted from plant sources, delivering phenolic compounds through dietary supplements represents significant difficulties (Bamba et al., 2018; González-Ortega et al., 2021). Even during digestion, oleuropein is susceptible to oxidative and enzymatic reactions, which may limit its health benefits (Gharehbeglou et al., 2019). On the other hand, when oleuropein enters the colon, it is fermented by the microbiota to produce a wide variety of bioactive constituents, which results in greater bioavailability (González-Ortega et al., 2021; Kanha et al., 2021).

In addition, olive leaf extract is characterized by its bitter flavor and dark brown color (Borjan et al., 2020). In olive production, in the presence of acidic or alkaline pH, oleuropein is degraded into more bland-tasting phenolics such as hydroxytyrosol and elenolic acid, which improves the final taste of the product (González-Ortega et al., 2021). When oleuropein is incorporated into foods as an extract, it can produce off-flavor and aromas (Sanhueza et al., 2022). Thus, difficulty in adding to other food can be encountered (Velderrain-Rodríguez et al., 2019).

1.2 Encapsulation

Encapsulation of compounds can aid selective delivery and controlled release of compounds in various environments. It can also mask the undesired sensorial properties and function as a protective barrier for the active compound (Giroux et al., 2016). Using encapsulation, new delivery systems for bioactive compounds with enhanced water solubility, physicochemical stability, bioaccessibility, and bioavailability can be created (Gharibzahedi & Smith, 2021). The surrounded compound is named as the active or core material, and the polymer that protects the active ingredient and controls its release is referred to as the coating (Quintero et al., 2018). The average size of particles produced with encapsulation can range from a few nanometers to a few millimeters (Wandrey et al., 2010).

Commonly, three steps are involved in the encapsulation process: Forming a wall around the material, preventing leakage, and keeping unwanted materials out (Gibbs et al., 1999). A variety of methods are utilized in encapsulation. Different methods can offer distinct advantages, and the best method will depend on a variety of factors. Some extensively used physical methods for capsule formation are spray drying, freeze-drying, emulsification, and extrusion, while physicochemical methods include coacervation, ionic gelation, and solvent evaporation-extraction. On the other hand, interfacial cross-linking and interfacial polymerization are examples of chemical processes (Shahidi & Han, 1993).

Kosaraju et al. (2006) prepared chitosan microspheres using spray drying for delivery of olive leaf extract with a loading efficiency of 27%. Spray drying of olive leaf phenolics was also studied by Kiritsakis et al. (2018) and maltodextrin was used as the carrier agent. Furthermore, liposomes were produced for the encapsulation of oleuropein. Capsules were then incorporated into acidic model drinks and storage under refrigeration conditions was investigated. The average efficiency for encapsulation of oleuropein was found to be 34% (González-Ortega et al., 2021).

A similar approach was implemented by Ganje et al. (2016) in which encapsulated and unencapsulated olive leaf extracts were added to tomato paste. Jolayemi et al. (2021), also carried out research on encapsulation of olive leaf extract by using xanthan gum as a stabilizer. In their research, the effect of olive leaf extract on the physical and oxidative stability of salad dressings was evaluated.

Emulsion-based encapsulation methods are becoming increasingly popular since they may be used in functional food items to encapsulate either or both hydrophilic and hydrophobic bioactive chemicals (Aditya et al., 2015; Gharibzahedi & Smith, 2021). High-efficiency encapsulation, maintenance of chemical stability, and controlled release further make emulsions a promising method for preserving and delivering polyphenols (W. Lu et al., 2016).

Understanding the principles behind the formation of emulsions and manipulating the bulk properties of emulsion systems is already a high priority to the food industry since they are essential for the processing, storage, and handling of foodstuff (Dapueto et al., 2019). Emulsification is especially favorable when the encapsulated material is added to liquid functional products such as beverages. In this case, the absorption and bioavailability of the core material are directly affected by the chemical and physical stability of emulsions (R. Zhang et al., 2020).

Mohammadi, Jafari, Assadpour, et al. (2016) reported that when olive leaf phenolics were encapsulated using whey proteins, 22% of the core materials were released after 20 days of storage compared to 8.1% when pectin and whey proteins were both employed in double emulsions. When whey protein-pectin nano-emulsions containing olive leaf extract were incorporated into soybean oil, oxidative stability of olive leaf extract was enhanced (Mohammadi, Jafari, Esfanjani, et al., 2016). Niknam et al. (2020) similarly prepared W/O/W emulsions using olive mill wastewater as the core compound. After three high-energy methods (microfluidization, ultrasonication and high-speed mixing) were compared for optimization of the process, a phenolics retention of 68.6% and antioxidant activity of 89.5% was obtained.

1.3 Double Emulsions

Double emulsions are multi-compartment systems composed of an emulsion dispersed as droplets in a continuous outer phase (McClements, 2012). Owing to the structure of double emulsions, there is a potential to encapsulate compounds with different solubility and antioxidant mechanisms. In this way, a synergistic effect can be obtained between two or more core compounds (W. Silva et al., 2018).

Mainly, there are two types of double emulsions; W/O/W and O/W/O. Both of these can be used for different purposes. Double emulsions, first of all, are used in controlling the release rate of functional ingredients or generating a modulated release. These constituents are then incorporated into foodstuff, and an increase in bioavailability can be achieved (Jimenez-Colmenaro, 2013). In some cases, it is possible to trigger destabilization of the interface and cause an intentional release of the encapsulated compound in response to specific triggers (Leister & Karbstein, 2020).

Other uses for double emulsions include, isolation of functional materials from other ingredients, which may slow down or prevent their chemical degradation, stabilization of labile components, and protection of these ingredients from adverse environmental conditions such as changes in pH, presence of extreme temperatures, and oxidative compounds (Gharibzahedi & Smith, 2021; Jimenez-Colmenaro, 2013; Velderrain-Rodríguez et al., 2019). It can also slow the evaporation of volatile active ingredients from the core to the surface (Quintero et al., 2018).

Moreover, they can increase consumer acceptance by masking the undesired sensory attributes such as bitter, astringent, or oxidative flavors. Formulation of healthier and more functional foods through the production of low-calorie or reduced-fat products, as well as providing healthier fatty acid profiles, can also be accomplished (Bou et al., 2014).

A variety of core materials have been encapsulated using emulsions and double emulsions. Many of these studies concentrated on the protection and delivery of phenolic compounds. A summary of these studies is given in Table 1.1. There are also studies which focused on encapsulation of microorganisms (Flores-Andrade et al., 2017; J. L. Silva et al., 2022), vitamins (Benichou et al., 2007; Bou et al., 2014; Guo et al., 2021; Matos et al., 2015), minerals (Ilyasoglu Buyukkestelli & El, 2019; Kabakci et al., 2021) and amino acids (Kocaman et al., 2020).

Table 1.1 Double emulsion studies on encapsulation of phenolic compounds

Encapsulated Compound	**Reference**
Blueberry Pomace Extract	(Bamba et al., 2018)
Olive Leaf Extract	(Jolayemi et al., 2021)
Olive Leaf Extract	(Mohammadi, Jafari, Assadpour, et al., 2016)
Olive Leaf Extract	(Mohammadi, Jafari, Esfanjani, et al., 2016)
Pomegranate Peel Extract	(Sanhueza et al., 2022)
Sour Cherry Pomace Extract	(Tumbas Šaponjac et al., 2016)
Gallic Acid and Quercetin	(W. Silva et al., 2018)
Mango Peel Extract	(Velderrain-Rodríguez et al., 2019)

Due to the presence of extra layers, double or multiple emulsions are superior with respect to encapsulation, protection, and release of encapsulated compounds (Jimenez-Colmenaro, 2013). Kiokias & Varzakas (2017) emphasized that when successfully prepared, double emulsion processing can produce systems that are quite stable and well-defined with reproducible particle sizes.

Water-in-oil-in-water (W/O/W) emulsions are widely utilized in the encapsulation of hydrophilic compounds by loading them inside the internal water phase (Kanha et al., 2021). Inner and outer aqueous phases of water-in-oil-in-water emulsions frequently differ from each other with respect to concentration. Thus, W1 and W2 abbreviations are used (Leister & Karbstein, 2020). A representation of W/O/W emulsions is given in Figure 1.3.

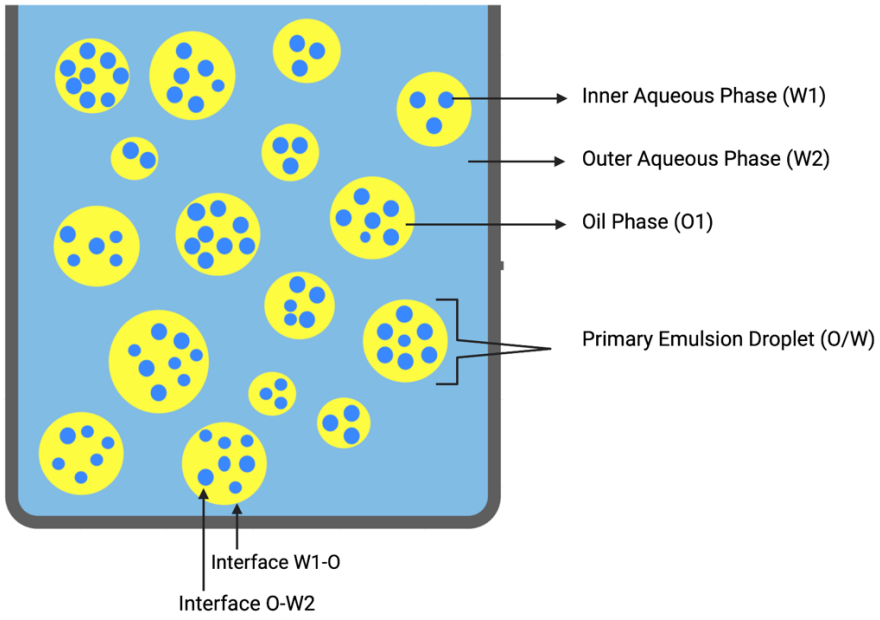

Figure 1.3 Schematic representation of W/O/W Emulsions

Two different methods can be adopted in the preparation of double emulsions. In one step method, an emulsion containing a non-ionic emulsifier or multiple emulsifiers is heated, causing phase inversion and as a result, a multiple emulsion is formed (Lamba et al., 2015). In the two-step emulsification method, on the other hand, the primary emulsion is prepared using high shear under more energetic conditions, and the multiple emulsion is prepared using gentle stirring (Faridi Esfanjani et al., 2017; R. Zhang et al., 2020).

Conditions employed in the preparation of the emulsion will determine the nature of the emulsion. Application of high shear during second emulsification will result in a two-phased emulsion rather than three, while lower shear will cause emulsions with large particle size and high polydispersity, which results in lower stability and efficiency (Jimenez-Colmenaro, 2013; R. Zhang et al., 2020).

The mobility of the materials between the inner and outer phase is also influenced by the oil phase, which acts as a membrane between the two aqueous phases (Bou et al., 2014). Different vegetable and mineral oils can be used as the oil phase of double emulsions. The nature of oil is also found to be effective on particle size, stability, and interfacial tension, which will have an impact on the diffusion rate of the core compound (Bonnet et al., 2009).

The main drawbacks of utilizing double emulsions in the food industry include difficulties in preparation and characterization, together with controlling the release and stability of the emulsions (Benichou et al., 2007; Dickinson, 2011; Hemar et al., 2010). Double emulsions are complex systems consisting of phases with inverse characteristics, which makes understanding the system challenging.

Furthermore, as molecules from the two emulsion phases are in direct contact with each other at the interface, repelling interfacial forces may lead to a strong tendency for the emulsion to separate into several phases (Fasinu et al., 2015). This phenomenon is especially applicable to double emulsions due to the presence of multiple thermodynamically unstable interfaces (Estrada-Fernández et al., 2018).

The ability of an emulsion to maintain its properties over time, such as size distribution, state of aggregation, or spatial arrangement of droplets, is known as stability. The stability of emulsions influences the quality and shelf life of many food items significantly (Can Karaca, 2020). The method of production, the composition of the emulsion, the nature of the oil phase, the kind of emulsifiers, and the character of the entrapped components are all factors that impact stability (Pitchaon et al., 2013).

Instability mechanisms typically present in emulsions are coalescence, gravitational separation such as creaming or sedimentation, flocculation, diffusion, Ostwald ripening, phase inversion, or phase separation (Espinosa-Álvarez et al., 2019; McClements, 2007). Frequently, these processes occur simultaneously and add to the double emulsion's total rate of instability (Dickinson, 2011). Instability due to coalescence and diffusion is generally irreversible, conversely, other mechanisms, such as creaming and agglomeration, can be reversible depending on the conditions present (Leister & Karbstein, 2020). Common instability mechanisms for emulsions are given in Figure 1.4.

Investigation and quantification of the instability can be difficult due to the complex nature of emulsions. Optical microscopy, measurement of particle size, rheology, and encapsulation efficiency are some of the methods that are being used to determine the loss of stability of emulsions during storage. Differential scanning calorimetry and observation of physical separation can also be helpful in the examination of instability (Leister & Karbstein, 2020).

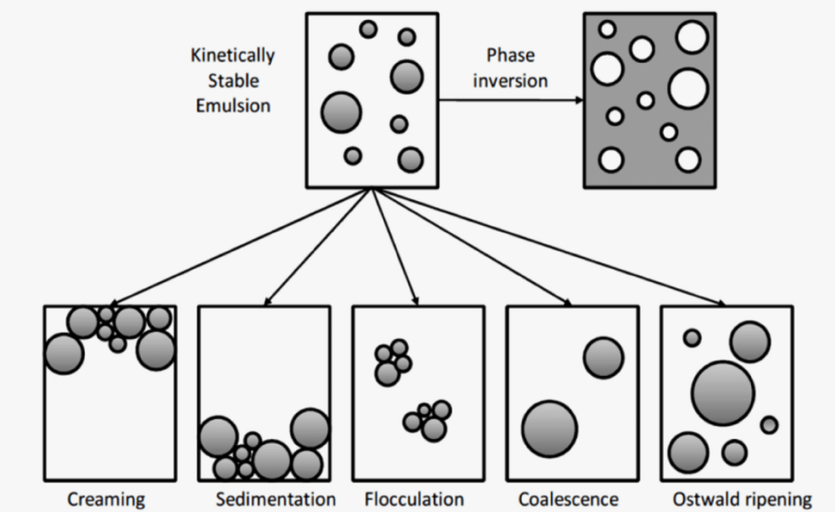

Figure 1.4 Instability mechanisms in emulsions (McClements, 2015)

Thermodynamic instability caused by the concentration difference of two contrasting phases can be avoided by addition of sugars, polysaccharides, or electrolytes such as sodium chloride. By balancing the osmotic pressure between the inner and outer aqueous phases, water transfer between the two phases is minimized and emulsion coalescence and Ostwald ripening can be reduced. (Dickinson, 2011; Fasinu et al., 2015; Hemar et al., 2010). Addition of emulsifiers, stabilizers, or antioxidants and controlling the pH can also increase emulsifying capacity (J. Yang et al., 2021).

1.4 Double Emulsion Preparation Methods

High-pressure homogenizers, rotor-stator systems, ultrasonic emulsification, membrane emulsification, and microfluidics are all common methods frequently used in the preparation of double emulsions (Leister & Karbstein, 2020). As for the machinery being used, mixing instruments, homogenizers, and ultrasonic equipment are commonly employed in the industry. However, in these methods, it is difficult to regulate the mean particle size (Mudrić et al., 2019).

Droplet properties of the systems are significantly affected by the homogenization method used in primary emulsion production (Altuntas et al., 2017). Additionally, high shear stress present in these methods can be problematic for heat-labile compounds as a result of the increase in temperature (Mudrić et al., 2019).

Another critical problem with high-energy homogenization treatments like micro fludization is "over-processing", which refers to the re-coalescence of small droplets as a result of a low rate of surfactant adsorption, a short resistance time with higher frequency droplet collisions, and a lack of active surfactant to cover the large surface area of newly produced droplets (Altuntas et al., 2017).

Low-energy methods such as phase inversion and spontaneous emulsification can produce emulsions with more homogeneous characteristics and the cost of equipment is lower. However, they can only be applied using a limited number of

oil/surfactant combinations and require high surfactant-to-oil ratios, which reduces the industrial applicability (Y. Yang et al., 2012).

Y. Yang et al. (2012) compared the application of a high-energy method (micro fluidization) with a low-energy method (spontaneous emulsification). They commented that the amount of emulsifier needed to produce smaller droplets in the low-energy method was the main disadvantage due to safety concerns and the high cost of emulsifiers. In their research, Ravanfar et al. (2018) found that microfluidics was also promising in emulsions formation. With layer-by-layer deposition, researchers were able to monitor the process closely during micro fluidization. Researchers commented that having control over and being able to regulate the process was an advantage of this method.

In conclusion, to prepare emulsions with the desired characteristics, homogenization conditions must be carefully controlled, which is hard to achieve in double emulsions due to a large number of parameters.

1.4.1 High-Speed Homogenization

A method frequently employed in the processing of emulsions is high-speed shear homogenization. In high-speed homogenization, a high rotating speed is applied by the agitator, which flows the suspensions and creates a significant shearing force resulting in samples with small particle sizes (Zhou et al., 2019). Guerra-Rosas et al. (2016) recognized that it was critical to have smaller droplets in the W1/O emulsion for increased stability due to decreased impact of gravity and Brownian motion. In some cases, researchers observed that high-speed homogenization can alter physiochemical characteristics by increasing the solubility of biopolymers in the solution and decreasing average particle size (Muhoza et al., 2022).

Conventional methods such as high-speed mixing or high-pressure homogenization generally produce emulsions that are more polydisperse and heterogeneous. Altuntas et al. (2017) observed that high-speed homogenization produced

emulsions with smaller particle size compared to ultrasonication and micro fluidization. Researchers also observed higher stability in emulsions with smaller particle size. However, the polydispersity of emulsions increased significantly when high-speed homogenization was used as compared to both methods. Dickinson (2011) underlined that the amount of polydispersity affects both the inner and outer droplets causing an increase in the degree of uncertainty in the characterization of the particle size distribution.

Moreover, Trujillo-Cayado et al. (2017) investigated the effect of using different rotor-stator equipment and a high-pressure homogenizer in preparation of green emulsifiers. Larger droplets were obtained with the high-pressure device, which resulted in lower stability. Emulsions obtained with rotor stator devices were more stable than the high-pressure samples, and both exhibited a similar destabilization mechanism. In contrast, Zhou et al. (2022) observed that emulsions prepared using high-speed homogenization had less stability than those prepared with ultrasonication using the same amount of power. It was demonstrated that performance is related to not only the method being used but also the parameters of homogenization.

1.4.2 Ultrasonication

Ultrasonication is a straightforward and inexpensive unit operation that can create small droplets with a narrow particle size distribution (Jafari et al., 2007). In ultrasound emulsification, the sonicator probe produces mechanical vibrations and leads to cavitation. Cavitation is the reason behind the production of emulsions using ultrasonication (Altuntas et al., 2017). As the ultrasonic vibrations pass through the medium, cavitation bubbles are produced. The sonication bubbles expand until they reach a critical size and then collapse violently, which can generate extreme pressure and temperatures as well as alter the physical, chemical, and thermal properties of the emulsion (Zhou et al., 2021).

In addition, application of ultrasound creates interfacial waves. These waves are unstable and cause the oil phase to disperse into the continuous aqueous phase. Besides, proteins can partially unfold and denature as a result of the high shear and temperatures produced by the ultrasonic process. As a result, the stabilization of interfaces can be enhanced (Gharehbeglou et al., 2019).

Ultrasonication is advantageous since it uses lower energy with shorter processing times. Shorter processing also limits the contact of the oil with the environment and limits oxidation to a certain extent. Kinetically stable emulsions with smaller particle sizes could be obtained. Due to the high efficiency of ultrasonication, it is labeled as a 'green technology' (Nishad et al., 2021). Cleaning of the equipment is also easier compared to other types of homogenization methods (Vélez-Erazo et al., 2018).

Nevertheless, in some cases, an increase in ultrasonication duration may lead to an increase in span values and diameter of the particles. This is due to 'overprocessing' of emulsions. As a result of excessive homogenization, globules of the dispersed phase are disrupted, and re-coalescence of droplets may occur (Kentish et al., 2008). As acoustic cavitation increases the emulsion temperature, proteins may be denatured during processing (Vélez-Erazo et al., 2018). Another reason for this phenomenon is that the rate of formation for droplets may be greater than the rate of protein adsorption, causing inadequate coverage of the interface (Jafari et al., 2008). Likewise, the amount of emulsifier might not be enough to cover the surface of newly forming droplets, causing coalescence and expansion of droplets (Vélez-Erazo et al., 2018).

Ultrasonication was used as a method to encapsulate oleuropein using nano-emulsions. Using pectin-whey protein concentrate complexes as stabilizers, researchers obtained an encapsulation efficiency of 91% for optimum conditions for encapsulation (Gharehbeglou et al., 2019). Ye et al. (2016) used ultrasonication as an approach to increase the solubility of the insoluble fraction of pea proteins. Tetradecane was chosen as the core material, and microspheres were fabricated by

applying high-intensity ultrasound irradiation. The intensity and duration of the ultrasonication step were effective on properties such as solubility and surface activity of pea proteins which in return influenced shell thickness. Researchers debated that tetradecane can be easily replaced with other functional liquids in future studies.

Al-Maqtari et al. (2021) and Nishad et al. (2021) demonstrated ultrasonication treatment for encapsulation of phenolic-rich extracts, while Pattnaik & Mishra (2022) produced double emulsions for encapsulation of A, D, B_9, and B_{12} vitamins. Other applications include fabrication of cheddar cheese analogs (Leong et al., 2020) and skimmed milk (Leong et al., 2018) using double emulsion method with canola and sunflower oil as the middle phase.

1.5 Emulsifiers

The term "emulsifier" refers to amphiphilic surface-active molecules that reduce surface tension, which are naturally present attractive forces. They interact with both dispersed and continuous phases of the emulsion and minimize the attraction between the molecules of the same liquid as well as the repelling force between the liquids (Kale & Deore, 2017). The physical properties of emulsion, as well as its encapsulation efficiency and stability throughout storage, are affected by emulsifiers being used.

The choice of surfactant relies on the type of emulsion that is being fabricated. Oil-in-water emulsions are typically made using hydrophilic surfactants and water-in-oil emulsions with lipophilic surfactants (Velderrain-Rodríguez et al., 2019). Hydrophilic-lipophilic balance (HLB) value is crucial for choosing a suitable emulsifier. Hydrophilic surfactants have high HLB values (8-18), while lipophilic surfactants tend to have lower HLB values (4-6) (Ohadi et al., 2020).

In double emulsions, two different interfaces are present. Consequently, two separate emulsifying agents with different natures, one to stabilize the inner

droplets and another to stabilize the droplets of the secondary emulsion, will be needed.

Current knowledge about emulsifiers for conventional simple emulsions cannot be directly adapted to double emulsions. In double emulsions, two separate water phases are linked to the same oil phase; hence, water molecules can diffuse from one aqueous phase to the other. Likewise, in these systems, water molecules, emulsifiers of two different types, and active core material interact with each other making the system more complex to characterize (Leister & Karbstein, 2020).

Theoretically, in double emulsions, lipophilic emulsifier is found in the inner interface, while hydrophilic one is present in the outer aqueous layer and these compounds do not interact or affect each other's efficiency. However, emulsifiers are amphiphilic and they tend to interact with both phases of the emulsions at varying degrees (Leister & Karbstein, 2020). Thus, the actual stability of the emulsion differs from the theoretical one.

Some examples of the materials used as surfactants in foods are acyl lactylates, dioctyl sodium sulfosuccinate, propylene glycol monosucrose and sorbitan esters (Adeyi et al., 2019). Polyglycerol polyricinoleate (PGPR) is one of the emulsifiers used in this study. Due to its efficiency and stability, PGPR is one of the most prominent lipophilic surfactants utilized in W1/O emulsions (Andrade & Corredig, 2016; Kocaman et al., 2020). It is classified as GRAS (generally recognized as safe) by US Food and Drug Administration (Polyglycerol Polyricinoleate, 2009). However, addition of PGPR in food products can cause an unpleasant taste when its concentration exceeds 5% (Jimenez-Colmenaro, 2013; Zhang et al., 2020). Lecithin, on the other hand, is a natural emulsifier that has a wide range of applications in the industry. Its usage is not regulated since good manufacturing practices are used to determine the maximum level (General Standard For Food Additives, 1995). Lecithin cannot produce W/O type emulsion when it is used individually. However, when combined with a strong lipophilic surfactant, it enhances rather than limits the lipophilic surfactant's functionality (Altuntas et al.,

2017). It is therefore used together with PGPR as a lipophilic surfactant in our study.

Estrada-Fernández et al. (2018) accentuated that, due to regulations and customer demands, there is a limited selection of emulsifiers that can be used in the formulation of double emulsions that are intended for direct human consumption, making their development challenging. There is growing interest in natural emulsifiers that can substitute synthetic ones entirely or partly. Natural emulsifiers are preferred over their safety, low cost, and high availability (Fasinu et al., 2015).

Dickinson (2011) emphasized that using food-grade emulsifiers instead of synthetic polymeric stabilizing agents and small-molecule emulsifiers will facilitate the development of stable double emulsions for use in food. Inherently, many researchers recognized the possibility of using legumes as natural emulsifiers for the production of stable emulsions.

1.5.1 Use of Legumes as Emulsifiers

Legumes belong to the family Fabaceae or Leguminosae and are seeds of these plants (Felix et al., 2018; R. Yang et al., 2021). Well-known legumes include soybeans, peas, chickpeas, lentils, and other various types of beans. They provide a diverse nutritional profile as they are abundant in carbohydrates, protein, dietary fiber, vitamins, and minerals, along with phytochemicals (Han & Baik, 2008; Ren et al., 2021). Legumes provide almost 20% protein by weight, which is comparable to meat in terms of protein content (Felix et al., 2018). Besides, legumes are known to have a low glycemic index. Consequently, another motivation to include legume flour as an ingredient in newly formulated products includes a decrease in fluctuations in blood sugar due to slower glucose release (Z. H. Lu et al., 2018).

It is becoming more important to create healthful foods from underutilized, sustainable sources as a result of expected demographic and environmental changes (Raikos et al., 2014). Main motivation for the use of plant proteins over animal

proteins is due to the agricultural aspects and biodiversity preservation (Vasilean et al., 2018).

As production of plant proteins entails less consumption of natural resources, and they are considered "environmentally economic" (Quintero et al., 2018). Moreover, designing and developing foods with added health benefits necessitates strategies for optimizing the presence of such substances, either by increasing the proportion of those with beneficial effects or by limiting the content of others with negative health implications (Jimenez-Colmenaro, 2013). Legume proteins are typically added to the formulation of various food products with significant customer acceptance (Gharibzahedi & Smith, 2021).

Physical chemistry characteristics, referred to as functional qualities, have an impact on how proteins behave during preparation, processing, and consumption (Dulger Altıner & Hallaç, 2017). The growing interest in using legumes in the industry is also a result of their functional properties, such as water holding, fat absorption, gelling, swelling, pasting, and foaming abilities, among others, which play an important role in new product development (Ashraf et al., 2012; Fasinu et al., 2015; Gharibzahedi & Smith, 2021).

Since proteins bind both water and oil at the same time, their function as emulsifiers in food processing is also well known (Ashraf et al., 2012). Proteins in legume flours prevent coagulation of particles as they form a flexible film layer and decrease interfacial tension, subsequently increasing stability (Can Karaca, 2020; Mohammadi, Jafari, Assadpour, et al., 2016). Thus, research has increasingly focused on the use of unique structures of these proteins as carriers or vehicles for the binding, encapsulation, and delivery of bioactive substances (R. Yang et al., 2021).

The production and stability of emulsions depend on the denaturation and partial unfolding of protein molecules and their adsorption at the interface with the proper hydrophobic and hydrophilic orientation (Raikos et al., 2014). As a result, the degree of denaturation has an impact on the microstructure of the emulsions.

Dapueto et al. (2019) investigated the effect of denaturation of whey proteins on the physical characteristics of emulsions.

Researchers commented that the adsorption process at the oil-water interface is dominated by the native protein fraction, and the denatured/aggregated fraction provides the appropriate apparent viscosity for stabilizing emulsions. Bigger particle size was obtained when native protein ratio was less than 70%.

According to Pereira Souza et al. (2017), good water solubility, low viscosity, and a strong ability to maintain active solutions are the most crucial inherent properties of carriers for water-soluble bio-actives. Although 100% solubility is not a definite requirement, protein solubility plays a significant impact in the emulsifying characteristics (Liang & Tang, 2013). In some cases, their poor water solubility and increased emulsion viscosity at high solid concentrations may limit the application of legume flours (Gharibzahedi & Smith, 2021).

Furthermore, due to their hydrophilic nature, polysaccharides are rarely absorbed in the interface (McClements, 2012; Porfiri et al., 2017). This drawback can be addressed by using them together with other biopolymers (R. Zhang et al., 2020). Nonetheless, for rheological regulation of the continuous aqueous phase in O/W emulsions, it is usual practice to incorporate polysaccharides at low concentrations as thickening and gelling agents, which is also a common practice for double emulsions (Dickinson, 2011).

Polysaccharides may increase the stability of the emulsion through reducing gravitational separation due to increased emulsion viscosity (Dapueto et al., 2019). They can also offer increased resistance to the protease and gastric juice environment compared to proteins and other surfactants. It was observed that, compared to emulsions stabilized by protein alone, those stabilized by both protein and polysaccharides exhibit greater resistance to environmental effects through simultaneous stabilization provided by different compounds (Estrada-Fernández et al., 2018; R. Zhang et al., 2020).

Modification treatments such as heating, germination or soaking can also modify the protein and starches, increasing their hydrophobicity which affects their functionality. These treatments can also improve the nutritional properties of legume flours by eliminating anti-nutritional substances (Dapueto et al., 2019; R. Zhang et al., 2020). Food processing technologies such as high-pressure homogenization and hydrolysis can also change the structure and enhance the properties of legume proteins (R. Yang et al., 2021).

Moreover, lipid peroxidation is one of the main issues with emulsions; therefore, emulsifying the protein sources in their natural matrices with bioactive substances is an interesting substitute for synthetic antioxidants in overcoming free radicals by acting as scavengers (Vasilean et al., 2018). Furthermore, metal ions are chelated by phenolic compounds present in the flours, which reduces oxidation and promotes the use of legume flours as whole (Han & Baik, 2008).

Sridharan et al. (2020) investigated the necessity of legume protein purification for use as an emulsifier. The similarity between the interfacial tension profiles of concentrated pea protein and pea flour suggested that proteins are the main factors stabilizing the interface. Pea protein and pea flour emulsions also had similar rheological behavior, aggregate size, and particle size. The elastic nature of the interface is not affected by the absence or presence of starch. It was also demonstrated that the inclusion of non-protein material had no effect on the emulsification ability of proteins. Their findings indicated that legume flours, such as pea flour, may be employed in their natural form to create stable emulsions and that non-protein components have no detrimental effects on functionality.

Separation of proteins from the biopolymers that they coexist with requires complex purification techniques as well as careful adjustment of extraction conditions and solvents (Sridharan et al., 2020). The procedures involved in protein purification also cost energy and result in mass losses due to inefficient use of raw materials by only using part of the plant. As a result, they contradict the sustainability value of plant-based materials (X. Li et al., 2021).

The complexity of plant materials is also a drawback for the preparation of protein isolates and concentrates (Sridharan et al., 2020). Solubility, molecular weight and structure, charge density, and emulsifying properties of proteins are all altered by the extraction process (Muhoza et al., 2022). Li et al. (2021) also explained that the low solubility and functionality of commercial protein isolates from major plant protein sources, is due, at least in part, to current plant protein purification methods, which result in a high degree of protein denaturation and aggregation.

W/O/W emulsions were prepared for the encapsulation of magnesium using different concentrations of unpurified pea and lentil flours. Using high-speed homogenization and ultrasonication, encapsulation efficiencies as high as 99% were achieved. Ultrasonication treatment was effective in reducing the particle size of emulsions and increasing stability. Double emulsions were then used for the fortification of cakes and the stability of magnesium increased compared to the primary emulsion when both types of legume flours were used (Kabakci et al., 2021).

Furthermore, Fasinu et al. (2015) found that Bambara groundnut flour has demonstrated emulsifying potential and contains a balanced amount of protein and carbohydrates. When Bambara groundnut flour and starch-stabilized emulsions were compared, flours provided superior stability. Moreover, additional nutrients were included in the emulsion as a result of its use in emulsification.

Adeyi et al. (2014) likewise observed that Bambara groundnut flour can be successfully used as the outer phase of oil in water emulsions thanks to its thixotropic, pseudoplastic and viscoelastic structure after gelatinization. Furthermore, soy and pea flours were used in the production of core-shell microcapsules. Researchers concluded that advanced microstructures can be made sustainably starting with unmodified materials and resource-intensive purification of plant biopolymers is not necessary (X. Li et al., 2021).

1.5.2 Pea flour

Pea proteins are the second most sought-after legume protein after soy. According to Han & Baik (2008), yellow peas have 24.5 g of protein, 1.1 g of lipid, 53.3 g of starch, and 2.9 g of ash per 100 g dry weight. Lysine content in peas is also significant for addressing the necessary amino acid deficit in diets (Kurt & Ceylan, 2018). Pea proteins are favored over other proteins as they are more digestible and provide a clean label for the product (Ge et al., 2020). They are less allergenic and can be consumed by people with gluten sensitivity (Z. H. Lu et al., 2018). Being widely available, economically feasible, biodegradable, and biocompatible with a variety of active substances, pea flours are good substitutes for synthetic emulsifiers (Raikos et al., 2014; R. Yang et al., 2021).

Protein components of foods, in most cases, are the main factors influencing functional qualities (Boye et al., 2010). Proteins found in peas can be generally classified as vicillin (7S), legumin (11S), and albumins (2S), with 7S and 11S being the most prevalent (Kurt & Ceylan, 2018). Water-insoluble globulin fraction makes up 70-90% of the total proteins, while albumin fraction, which is water-soluble, makes up 10-30% (Muhoza et al., 2022; Ye et al., 2016).

The hydrophobic surface stabilized by the trimeric 7S globulin is linked to the low solubility of globulins in water. On the other hand, hydrophilic surface of albumin, which is made up of two polypeptides joined together by disulfide bonds, has high solubility in water. One of the critical factors that determine how well proteins function as emulsifiers is thought to be the ratio of polar to non-polar groups within the protein structure (Vasilean et al., 2018). This ratio depends mainly on the type of legume and the extraction process (Muhoza et al., 2022). Concisely, the physical qualities of the flour are substantially affected by the ratio and type of proteins.

There is plenty of research on pea protein and flours regarding their emulsifying ability using different homogenization methods. The change in their emulsifying ability after application of different treatments such as pH, heat application, and

pressure was also investigated. The ability of pea proteins to stabilize emulsions was demonstrated by Can Karaca (2020). Varying concentrations of maltodextrin, gum Arabic, and pea protein were used as stabilizers in oil-in-water emulsions. As a result, smaller droplets with increasing stability were obtained with low oil concentrations.

Additionally, pea proteins combined with other polysaccharides were used in the encapsulation of essential oils obtained from hyssop and hemp (Hadidi et al., 2022; Lan et al., 2021). Encapsulation of hydrophilic compounds such as lutein and cinnamaldehyde was also accomplished using wall materials derived from pea (Caballero & Davidov-Pardo, 2021; Feng et al., 2022).

The functionality of food compounds is affected by a variety of factors including interactions with other food components, composition, pH, ionic strength, heat treatment, and other external conditions, in addition to intrinsic features such as molecule size and structure (Felix et al., 2018; Raikos et al., 2014).

Production of emulsions is facilitated by either an acidic or an alkaline pH, whereas in the presence of neutral pH, a very low emulsifying capacity is observed. This clearly indicates the importance isoelectric point of the protein in emulsion formation (Ashraf et al., 2012). Acevedo et al. (2017) and Felix et al. (2018) also observed higher protein solubility at alkaline pH values, which is further away from the isoelectric point. Such improved emulsifying abilities at an alkaline pH may result from dissociation and partial unfolding of globular proteins. As a result, hydrophobic amino acid residues are exposed, which changes the surface activity and adsorption behavior (Raikos et al., 2014).

1.6 Objective of the Study

Olive leaf extract is one of the by-products of table olive and olive oil production. Ordinarily, the leaves of the olive tree are carried to the factory and do not possess any economic importance to the producer. These leaves are known to possess antimicrobial and antioxidant activity in addition to their many health-promoting properties. Thus, phenolic compounds can be extracted from the leaves and used in the production of functional products. On the other hand, phenolic compounds are prone to degradation in the presence of adverse environmental conditions. They can also produce off tastes and flavors when they are directly added to the food products or consumed directly.

Encapsulation of these compounds can increase the stability of these health-promoting substances while decreasing the formation of unwanted sensorial properties. The main objective of this study is to encapsulate the olive leaf extract using double emulsion method. One of the desired results was to provide controlled release whilst protecting the coated olive leaf extract against adverse environmental conditions. Since they have an extra layer, double emulsions provide better protection to the encapsulated compound.

Pea flour was used for the stabilization of the outer aqueous phase of the emulsions. Double emulsions are expected to show superior protective behavior compared to primary emulsions and higher stability compared to unencapsulated olive leaf extract. The effect of increasing flour concentration and ultrasonication process on the stability and physical properties of emulsions were also investigated in this thesis. There is a very limited number of publications in the literature on encapsulation of olive leaf phenolics, but none of these research papers employed legume flours as emulsifiers for double emulsions.

CHAPTER 2

MATERIALS & METHODS

2.1 Materials

Olive leaf extract, the core material for this study, was supplied by Immunat Herbal Pharmaceuticals Inc. (Muğla, Turkey). Sunflower oil (Yudum Food Ind. Trade Inc., Balıkesir, Turkey) for the oil phase of double emulsions was purchased from a local market. Pea flour was obtained from Molar Chemical Trading Co. Inc. (Turkey). The composition of the flour was 55.3% carbohydrate, 21% protein, and 1.6% fat. Lipophilic surfactants, PGPR and lecithin, were supplied from Eti Food Ind. Trade Inc (Eskişehir, Turkey) and Lipoid Gmbh (Ludwigshafen, Germany), respectively.

Other reagents used in the study (ethanol, gallic acid, Folin-Ciaocalteu reagent, sodium carbonate, NaCl) were analytical grade and purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Lastly, sodium azide, which was used as an antimicrobial agent, was purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

2.2 Preparation of Double Emulsions

Two-step emulsification, the most frequently used method for fabricating double emulsions, was used in the preparation of double emulsions. In accordance with this method, first, the W/O primary emulsion is produced using higher shear forces. Then, the primary emulsion is combined with the other aqueous phase for the

preparation of W/O/W emulsion. The inner aqueous phase of the emulsion is named as W1, while the outer aqueous phase is named W2.

An illustration of the two-step emulsion technique followed during double emulsion preparation is given in Figure 2.1.

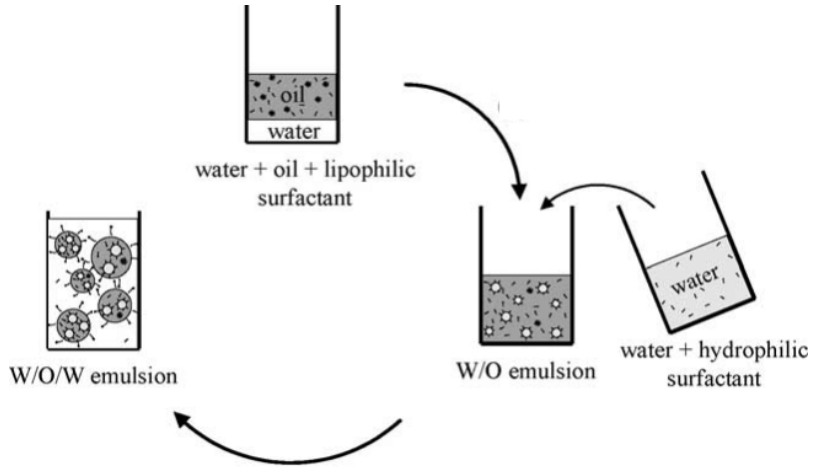

Figure 2.1 Two-step emulsification technique (van der Graaf et al., 2005)

2.2.1 W1/O (Primary) Emulsion Preparation

For the preparation of the inner aqueous phase, OLE (30 g/100 g W1), sodium azide (0.05 g NaN3/100 g W1), and sodium chloride (0.6 g/100 g W1) were mixed with distilled water using a magnetic stirrer for 15 min at 300 rpm (Daihan Scientific, South Korea). NaCl was used to maintain the osmotic balance between the two aqueous phases. NaCl is effective in decreasing the interfacial tension together with the emulsifiers and enhancing the stability of emulsions.

Chemical antimicrobial preservatives are added to many types of food emulsions to prevent spoilage during storage and assure consumer safety. In preliminary experiments, it was determined that an antimicrobial agent, sodium azide, was needed for long-term stability experiments to prevent microbial spoilage of the emulsions during storage at higher temperatures.

PGPR and lecithin were used as lipophilic emulsifiers. For the preparation of oil phase (O); PGPR (2.5 g/100 g O), lecithin (2.5 g/100 g O), and sunflower oil (95 g/100 g O) were first homogenized using a high-speed homogenizer at 10000 rpm for 1 min (IKA T25 Ultra-turrax, IKA Works Co., Malaysia).

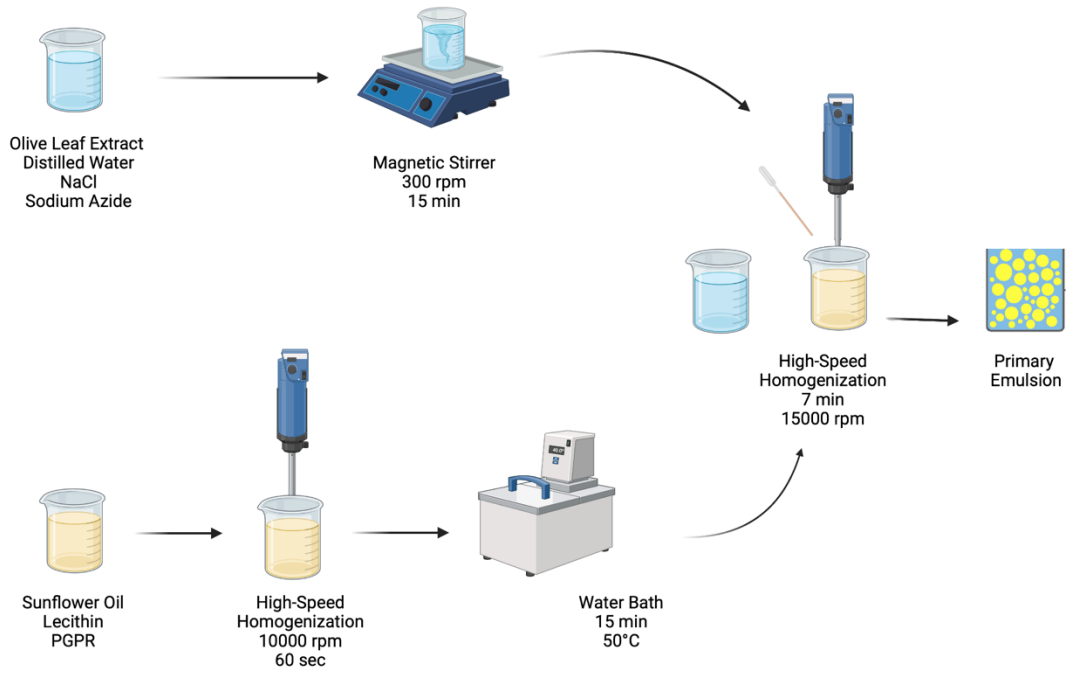

Figure 2.2 Preparation of Primary Emulsion Using High-Speed Homogenization (Prepared with BioRender.com)

After the mixing of emulsifiers with sunflower oil, the mixture was left in a 50°C water bath (Mikrotest MCS 30, Ankara, Turkey) for 15 min. While heating, the mixture was also stirred at 50 rpm. Heating of the oil phase and stirring during the heating procedure was performed to increase the solubility of the emulsifiers inside the oil phase.

Primary emulsion was then prepared using a high-speed homogenizer. The ratio of the W1 phase to the oil phase for the primary emulsion was selected as 40% to 60%, as it was determined as the optimum ratio (Altuntas et al., 2017). First, the oil phase was mixed at 15 000 rpm for 30 s; then the W1 phase was added into the oil phase while stirring for 6 min. Lastly, the mixture was stirred for 30 s at the same stirring speed. The method used for the preparation of primary emulsions with high-speed homogenization is given in Figure 2.2.

Samples with ultrasonication application were prepared by first forming the emulsion using high-speed homogenization using the same parameters; then, samples were treated with an ultrasonic homogenizer (OMNI, Sonic Ruptor 400, GA, US) at 80 W power, 20 kHz frequency, and 50% pulse for 20 min. Ultrasonication parameters were selected based on the preliminary experiments. During the application of ultrasonication, emulsions were kept inside an ice bath to prevent emulsions from being heated.

2.2.2 W1/O/W2 (Double) Emulsion Preparation

To prepare the outer aqueous phase of the emulsions, different amount of pea flour was added to distilled water. For the preparation of samples with 15% flour, 15 g of pea flour was weighed, and distilled water was added until the weight of the beaker reached 100 g. Likewise, 20 g and 25 g of flour were mixed with distilled water to obtain 100 g of W2 when preparing samples with 20% and 25% flour. NaCl (0.6

g/100 g W2) was also added to balance the osmotic pressure with the inner phase. After dissolving the pea flour and NaCl in water, the solution was mixed using a high-speed homogenizer at 9500 rpm for 30 s.

The pH of the solutions was adjusted to 12 by adding 2 M NaOH to ensure higher solubility of pea flour in water and to obtain solutions with higher viscosity. It was demonstrated that pea proteins showed better solubility and thus emulsifying ability in pH values further from their isoelectric point (Liang & Tang, 2013). While adjusting the pH, solutions were mixed using a magnetic stirrer to measure the pH correctly.

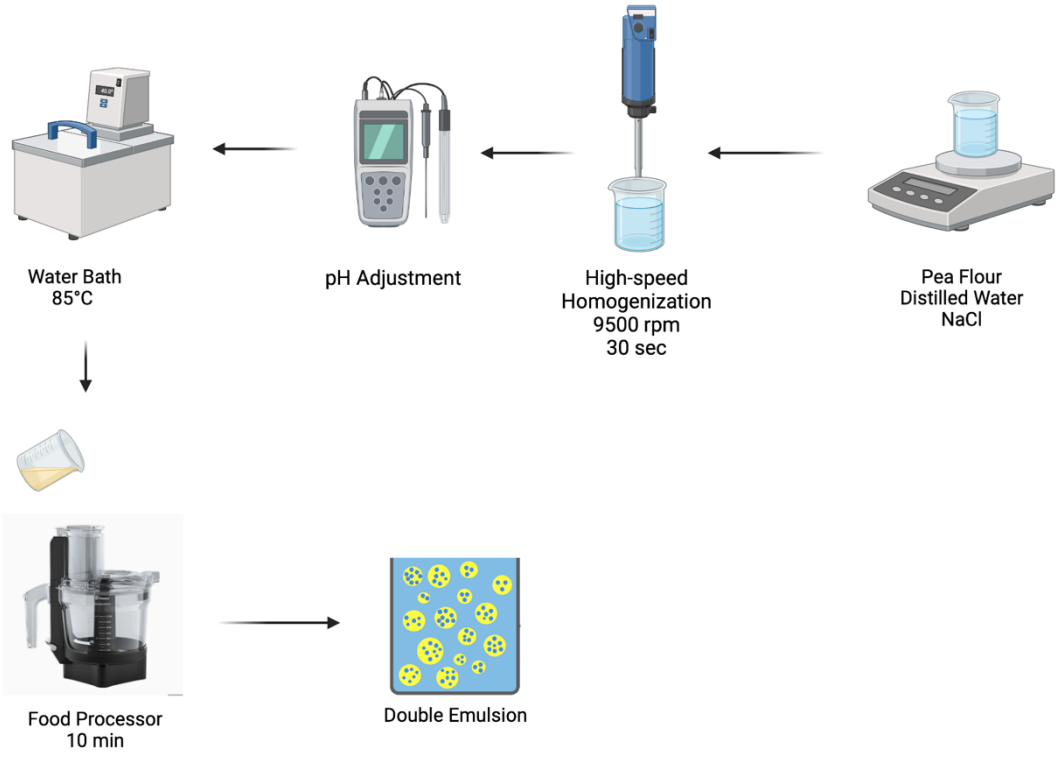

Figure 2.3 Preparation of Double W1/O/W2 Emulsion (Prepared with BioRender)

After the pH adjustment, flour solutions were heated to 75°C using a water bath. The water bath was adjusted to 85°C, and continuous shaking at 50 rpm was applied during the heating procedure. Solutions were cooled to 45-50°C at room temperature before the preparation of double emulsion.

Subsequently, flour mixtures were added inside the mixing bowl of the home-type food processor (Arçelik K-1190, Turkey). The ratio of primary emulsion to the outer aqueous phase (W2) phase was again selected as 40% to 60%. Flour solutions were mixed for 1 min at 600 rpm. Then, primary emulsion was added drop by drop while mixing of the outer phase took place for 7 min at 800 rpm. Newly prepared W/O/W emulsions were mixed for an additional 2 min at 600 rpm using the food processor. After 5 min, emulsions were filled into 50 mL falcon tubes and stored at 20°C for further analyses. The method used to prepare W/O/W double emulsions is given in Figure 2.3.

2.3 Characterization of Emulsions

Characterization of the emulsions requires the determination of rheological and morphological properties besides stability, release behavior and encapsulation efficiency (R. Zhang et al., 2020). In this study, particle size, rheology, encapsulation efficiency, stability, and optical images of the prepared emulsions were examined.

2.3.1 Dilution Test

The dilution test is a simple test to evaluate the type of the outer phase of the emulsion (Saikia & Sultan, 2020). In this test, two beakers were filled with water

and sunflower oil. Then, a few drops of the prepared emulsion was dropped inside these two beakers. Based on the miscibility of the dropped emulsion, it is possible to determine the characteristics of the emulsion.

If the emulsion is W/O as expected, the emulsion will dissolve in oil and will remain insoluble when dropped into water. On the other hand, final double emulsions were expected to be insoluble in oil and soluble in water due to the outer aqueous phase. Dilution test was used, particularly in the earlier part of the experiments, to confirm if the correct type of emulsion was being prepared after the treatments.

2.3.2 Stability of Emulsions

Stability is the capacity of emulsion to withstand changes in its characteristics. It is one of the most significant emulsion characteristics and often determines the shelf life of the emulsion (Adeyi et al., 2019). In this study, two types of stability were tested; instant stability and long-term stability.

2.3.2.1 Instant Stability

As long-term stability tests are typically time-consuming, accelerated stability or instant stability tests can be performed to gain insight into the stability of emulsions prior to long-term storage (Kale & Deore, 2017). Centrifugation is commonly used as a way for testing accelerated phase separation (Mudrić et al., 2019). For the instant stability test, 15 mL centrifuge tubes were filled with the newly formed emulsion and sealed with plastic caps. The height of the emulsions was measured prior to testing. Tubes were placed in the centrifuge (Hanil Scientific Inc., MF-80, Korea) and centrifuged for 10 min at 3000 rpm.

The height of the supernatant was measured after centrifugation for the calculation of instant stability. Experiments were performed as duplicates.

$$Instant\ Stability\ (\%) = \left(\frac{h_s}{h_o}\right)\ x\ 100 \qquad \text{(Equation 1)}$$

In Equation (1), h_s is the height (cm) of the upper part of the centrifuged emulsion (supernatant) after centrifugation and h_o is the initial height (cm) of the sample in the tube.

2.3.2.2 Long-Term Stability

For quantification of long-term stability, glass tubes were filled with emulsions and stored for longer periods of time. Two tubes were kept at room temperature (20°C) and two were placed in the refrigerator (4°C). The height of the emulsions was measured before storage. After preparation, the separated part of the emulsions was recorded after 3 days and once every week after the first measurement. The long-term stability of the emulsions at the predefined day of storage was found using Equation (2);

$$Storage\ Stability\ (\%) = \left(\frac{h_c}{h_o}\right) x\ 100 \qquad \text{(Equation 2)}$$

where h_c is the height of the total separated phase (cm), and h_o is the initial height of the emulsion (cm) before the storage period.

2.3.3 Particle Size Analysis

A laser diffraction particle size analyzer (Mastersizer 3000, Malvern Instruments, UK) was used in evaluation of mean particle size and size distribution of oil droplets of double emulsions. Laser diffraction measures the change in intensity of light scattered as the laser beam passes inside the sample. Then scattering patterns were analyzed for the calculation of particle sizes which is based on Mie theory of light scattering. Particle size analyzer was equipped with a wet dispersion unit (Hydro EV, Malvern Instruments, UK).

Beaker was filled with water which is the continuous phase of emulsions. Refractive index for the dispersant and the dispersed phase were selected as 1.33 and 1.46, respectively, considering the dispersant is water and the dispersed phase is sunflower oil. Globule absorbance was taken as 0.01. Obscuration limits for the instrument was in the range of 8% to 20%. Moreover, stirring speed was kept below 2000 rpm to prevent the emulsion droplets from disintegrating.

The surface moment mean diameter ($D_{3,2}$), volume moment mean diameter ($D_{4,3}$) and span of the particles were calculated by the software of the particle size analyzer. Two separate measurements were taken from each sample. Samples were thoroughly mixed before sampling.

$$D_{3,2} = \frac{\sum n_i {d_i}^3}{\sum n_i {d_i}^2} \qquad \text{(Equation 3)}$$

$$D_{4,3} = \frac{\sum n_i {d_i}^4}{\sum n_i {d_i}^3} \qquad \text{(Equation 4)}$$

Where, n_i is the number of related particles per unit volume and d_i is the diameter of the particles.

Span values were also calculated by the software using Equation (5),

$$Span = \frac{d(90)-d(10)}{d(50)} \quad \text{(Equation 5)}$$

Where, d(10), d(50) and d(90) corresponds to the diameter of particles that are inside the range of 10, 50, and 90%, respectively.

2.3.4 Rheology

Rheological measurements were done using Brookfield RST viscometer (Ametek Inc., Massachusetts, USA) fit with a bob and cup spindle (CCT-25). Approximately 15 g of double emulsion sample was filled inside the chamber of the rheometer. Rheological measurements were carried out 2 h after the preparation of emulsions at 20°C. Measurements were taken as duplicates. Shear stress data were measured as the shear rate was increased from 0.1 to 150 s^{-1}. Rheo3000 software (Ametek Inc., Massachusetts, USA) was used for the calculation of apparent viscosity from the shear rate data.

The flow behavior of the samples were evaluated by fitting shear stress (τ) data and shear rate ($\dot{\gamma}$) data to Power law model:

$$\tau = K \dot{\gamma}^{n} \quad \text{(Equation 6)}$$

Where, $\dot{\gamma}$ is the shear rate (1/s), τ is the shear stress (Pa), n is the flow behavior index (dimensionless) and K is the consistency coefficient (Pa.s). Apparent viscosity of samples was evaluated at 150 s^{-1}.

2.3.5 Optical Imaging

A light microscope was employed for the inspection of the morphological characteristics of double emulsions. A preliminary examination showed that the emulsions needed dilution in prior to obtain a clear image from the microscope. Therefore, samples were diluted (1:5) with distilled water and vortexed (ZX3, VELP Scientifica, Italy). Diluted solutions were spread as a thin layer on the glass slide and covered with a cover slip. Slide was then fixed on the inverted light microscope (PrimoVert, Zeiss, Germany) and examined.

TopView software (SPECwise, Inc., FL, USA) was utilized to analyze the images taken using Sony CCD Digital Video Microscope Camera (Tokyo, Japan). Images were obtained using lenses providing 10× and 40× magnification.

2.3.6 Encapsulation Efficiency

Encapsulation efficiency is one of the main parameters in defining the success of emulsions. In double emulsions, it is defined as the amount of the core material that is still contained inside the primary part of the emulsion after the second emulsification step (Dickinson, 2011).

$$EE\ (\%) = \frac{TPC_{DE} - TPC_{W2,c}}{TPC_{DE}}\ x\ 100 \qquad \text{(Equation 7)}$$

Where, EE (%) is the encapsulation efficiency, TPC_{DE} is the total phenolic content of the extract added to the inner phase, TPC_{W2} is the phenolic content of outer aqueous phase which was separated through centrifugation and filtration. The method followed for the separation of the outer phase is given in 2.3.6.1.

$$TPC_{W2,c} = TPC_{W2} - TPC_{W2,0} \quad \text{(Equation 8)}$$

Phenolic content of the pea flour also needs to be considered when calculating the total phenolic content of the W2 phase. Double emulsions were prepared without the core material were produced and their phenolic content ($TPC_{W2,0}$) was subtracted from the phenolic content of samples. In Equation (8), the resulting phenolic content was named as corrected TPC ($TPC_{W2,c}$).

2.3.6.1 Recovery of the W2 Phase/Sample Preparation

For the TPC analysis, W2 phase of the emulsions needs to be separated. Two hours after preparation, emulsions were diluted with distilled water with a dilution rate of 1:2 w/w. Dilution procedure was performed while mixing with a magnetic stirrer at 200 rpm for 5 min. Samples were then put into 50 ml centrifuge tubes and centrifuged (Nuve NF-1200R, Turkey) for 15 min at 2000 rpm. The first step of the centrifugation was done to gently separate the W2 phase without disrupting the primary emulsion.

After the first centrifugation, W2 phase, which is the infranatant part, it is drawn into a syringe and transferred to a 15 mL centrifuge tube. Subsequently, another centrifugation step was carried out to remove the remaining oil particles and flour particles. In the second step, samples were centrifuged for 15 min at 8000 rpm. The clear part of the infranatant was passed through filter paper (Whatman, No 4) and

then through PTFE 0.45 µm syringe filter. Filtered samples were kept at -18 until measurement of TPC.

TPC was analyzed using the method in *2.3.6.2.* The process followed for separation of W2 phase is given in Figure 2.4.

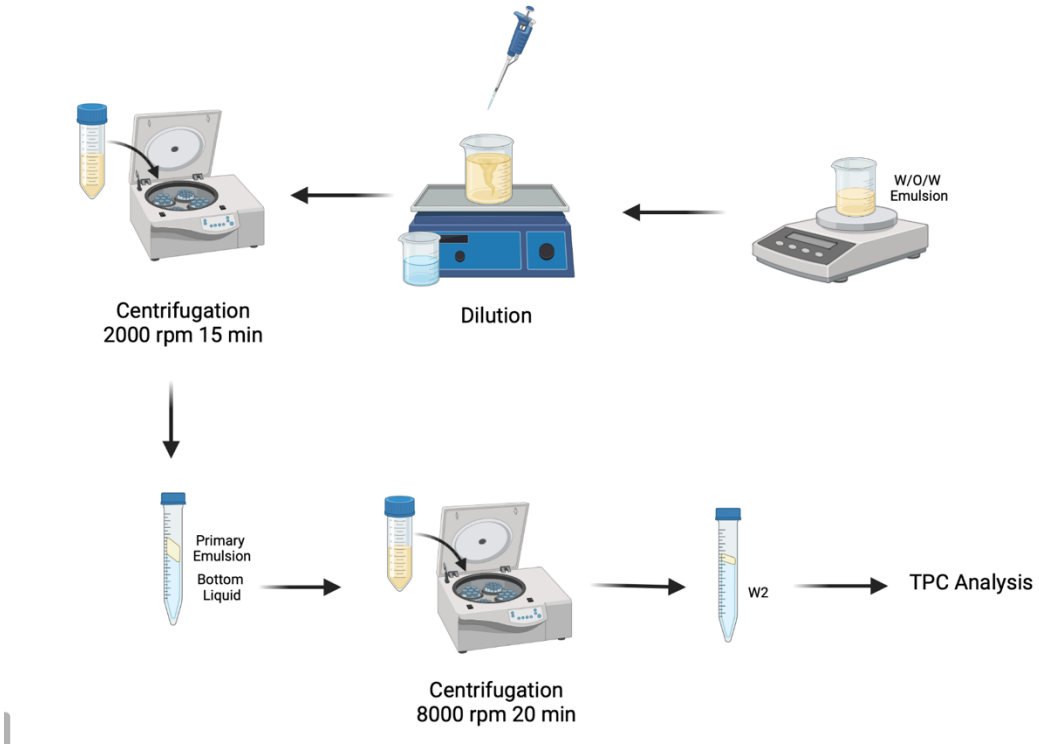

Figure 2.4 Separation of W2 phase for TPC analysis (Prepared with BioRender.com)

2.3.6.2 Phenolic Content Analysis

Total phenolic content (TPC) analysis was performed using modified version of Folin-Ciacalteou method (Cilek et al., 2012). Samples were diluted using ethanol:water mixture (50:50 v/v) as the dilutent and 500 µL of sample was added to a test tube. Then, 2.5 mL of Folin-Ciocalteuau reagent (0.2 N) was added and the mixture was vortexed. After it is kept in a dark place for 5 min, 2 mL of sodium carbonate (75 g/L) was added which was followed by vortexing.

Samples were left in the dark at room temperature for 1 hour. Then, absorbance of the samples was read at 760 nm using UV/VIS spectrometer (Mapada Instruments Co. Ltd., V1800, Shanghai).

Gallic acid was dissolved in the same solvent with the samples (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mg/L) and was used as a standard for the preparation of the calibration curve. Phenolic content of samples was expressed as Gallic acid equivalents using the following linear equation;

$$A = 0.1\,x\,C + 0.0231\,,\ R^2 = 0.9979 \qquad \text{(Equation 9)}$$

In Equation (9), A is the absorbance of the sample at 760 nm and C is the concentration of Gallic Acid. The calibration curve was given in Appendix A. TPC results of the samples were expressed as gallic acid equivalents in mg per g.

2.3.7 Release of Olive Leaf Extract

After completing the analyses for the characterization of samples, the release of olive leaf extract in emulsions prepared with high-speed homogenization and 25% pea flour was investigated as these conditions were found as the optimum parameters. Emulsions were prepared using the same method explained in 2.2.1 and 2.2.2. Two replicates of the emulsion were prepared and stored at 37°C for 13 days in plastic vials.

The first sample was taken 24 h after the preparation of emulsions. After the first day, sampling was done on day 4, day 7, day 10, and day 13. Before emulsion samples were taken, vials were first taken outside the heater for the temperature of the sample to reach room temperature as it might affect the diffusion of phenolic compounds to the solvent used in TPC analysis.

The double emulsion inside the vial was mixed thoroughly to ensure the homogenization of the sample. Samples were then diluted with distilled water with a dilution rate of 1:2 w/w. Recovery of the W2 phase from the emulsions was performed using the method in 2.3.6.1.

Recovered samples were stored at -18 until the end of the storage period. Samples were then thawed at room temperature in the dark, and TPC analysis was conducted using the same method in 2.3.6.2. The cumulative release (%) of the emulsions was calculated using Equation (10).

$$Release\ (\%) = \frac{TPC_{W2,c}}{TPC_{DE}}\ x\ 100 \qquad \text{(Equation 10)}$$

where, $TPC_{W2,c}$ is the corrected phenolic content of the outer aqueous phase and TPC_{DE} is the total phenolic content of the double emulsion.

Moreover, as Çilek Tatar (2018) explained, first-order kinetics can be used to describe the release kinetics of OLE from double emulsions. Equation for the first order kinetics is given in Equation (11).

$$c_a = c_{a0}\, x\, e^{-kt} \qquad \text{(Equation 11)}$$

In Equation (11), k is the release rate constant (day^{-1}), t is the duration of the storage (days), c_{a0} is the c_a is the TPC content of the W2 phase. Natural logarithm of the data was fitted linearly with the storage duration of the samples to find the release rate constant, k.

2.4 Statistical Analysis

The presence of a significant difference between different samples was analyzed using analysis of variance (ANOVA). Minitab software (Minitab Inc., UK) was used to perform ANOVA. Providing a significant difference, Tukey's Comparison Test ($p \leq 0.05$) was applied to compare samples concerning their difference. Results were expressed as mean ± standard error. Results represent the mean of two replicates. Statistical tests were also conducted using two replications.

CHAPTER 3

RESULTS & DISCUSSION

3.1 Particle Size and Distribution

To investigate the particle size and distribution of emulsions, samples were first dispersed inside water, which was an appropriate solvent since the outer phase was water-based. Then, measurements were taken using a laser diffraction particle size analyzer. During particle size measurements, droplets of the inner aqueous phase are not considered to contribute significantly to the scattering since they were thought to be enclosed within the oil globules (Sapei et al., 2012).

As emphasized by Garti & Aserin (1996), it is crucial to produce emulsions with smaller particle size in the primary emulsion to obtain samples with superior stability. However, the processing parameters, the type of emulsion, the viscosity of the phases, composition, the nature of the emulsifier and the concentration of the dispersed phase are all parameters that have a significant impact on particle size (Bou et al., 2014; Sanhueza et al., 2022). As these parameters vary considerably between studies, comparing the particle size with other studies is challenging.

All the emulsions, irrespective of the homogenization method and flour concentration, showed multimodal particle size distribution (Figure 3.1, Figure 3.2). According to our results, the type of distribution changed with respect to the emulsifiers used during the emulsification rather than the homogenization parameters or concentration of the emulsifier.

Kabakci (2022) also observed multimodal particle size distributions for double emulsions prepared with pea flour. Similar behavior was also observed in the study of Sridharan et al. (2020), in which pea protein concentration and pea flour were

used as emulsifiers for O/W type of emulsions. Both studies supported the multimodal type of distribution observed in our study.

Moreover, Hemar et al. (2010) stated that bimodal distribution was seen in emulsions with PGPR which might have also caused the primary emulsion to display multimodal particle size distribution. Bimodal distribution of droplets might signify that the different emulsifiers in the solution could affect the particles separately and that no certain interaction was present between the different biopolymers (Choi et al., 2020).

Surface moment mean diameters ($D_{3,2}$) of double emulsions prepared with different homogenization methods are shown in Figure 3.3. D_{50}, $D_{4,3}$ and span values for double emulsions were summarized in Table 3.1. The average diameter of the samples decreased significantly as the flour concentration was increased ($p<0.05$) (Figure 3.1, Figure 3.2).

For emulsions prepared using HSH, surface mean diameter was found as 12.76 μm, 8.88 μm and 6.84 μm for samples prepared with 15%, 20%, and 25% flour, respectively. Whereas for US, surface mean diameter values were found as 12.15 μm, 6.88 μm and 6.79 μm (Figure 3.3). Mehrnia et al. (2017) also observed similar behavior when biopolymer concentration was increased for Angum/Arabic gum and whey protein double emulsions, which was explained by better coverage of droplets.

Moreover, Pimentel-Moral et al. (2018) demonstrated that the emulsifier concentration might be one of the causes of the final emulsion's greater particle size and decreased stability during storage, which resulted from the majority of the individual droplets generated during emulsification not being preserved in the mixture. As a result, with greater surfactant concentrations, the size of the droplets during emulsification would be smaller.

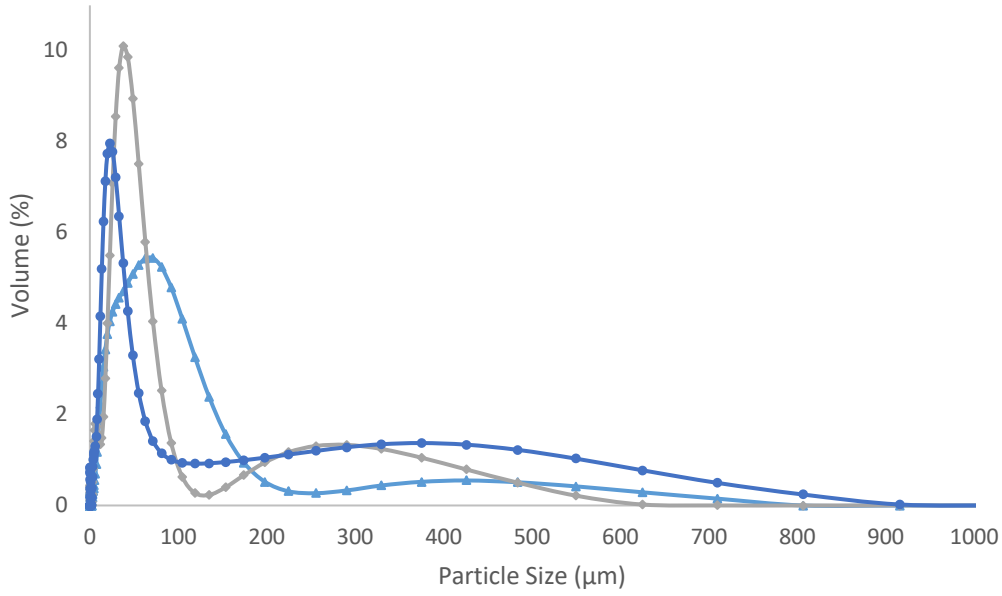

Figure 3.1 Particle size distribution of double emulsions prepared with high-speed homogenization using different concentrations of pea flour (▲):15%, (♦):20%, (●):25%

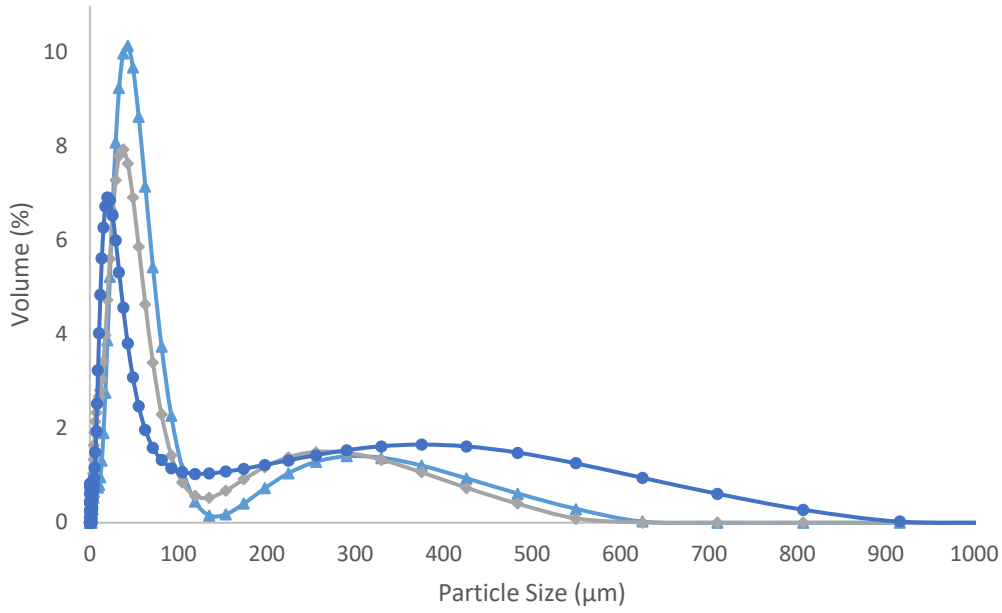

Figure 3.2 Particle size distribution of double emulsions prepared with ultrasonication using different concentrations of pea flour (▲):15%, (♦):20%, (●):25%

Increasing the protein concentration reduces the interfacial tension, increases the surface area, and causes particle size to decrease (Can Karaca, 2020). Likewise, increasing biopolymer concentration from 2.5 to 5% were shown to decrease droplets diameter due to the higher viscosity of 5% biopolymer solution (Jafari et al., 2012). Similar behavior was observed by Assadpour et al. (2016) for nanoemulsions prepared with whey protein and maltodextrin. Researchers stated that the increase in particle size was expected because when surfactant content was low, the number of dispersed water droplets and their surface area increased dramatically. As the lower amount of emulsifier molecules cannot stabilize these droplets, they flocculate and merge together, resulting in coalescence and larger droplets.

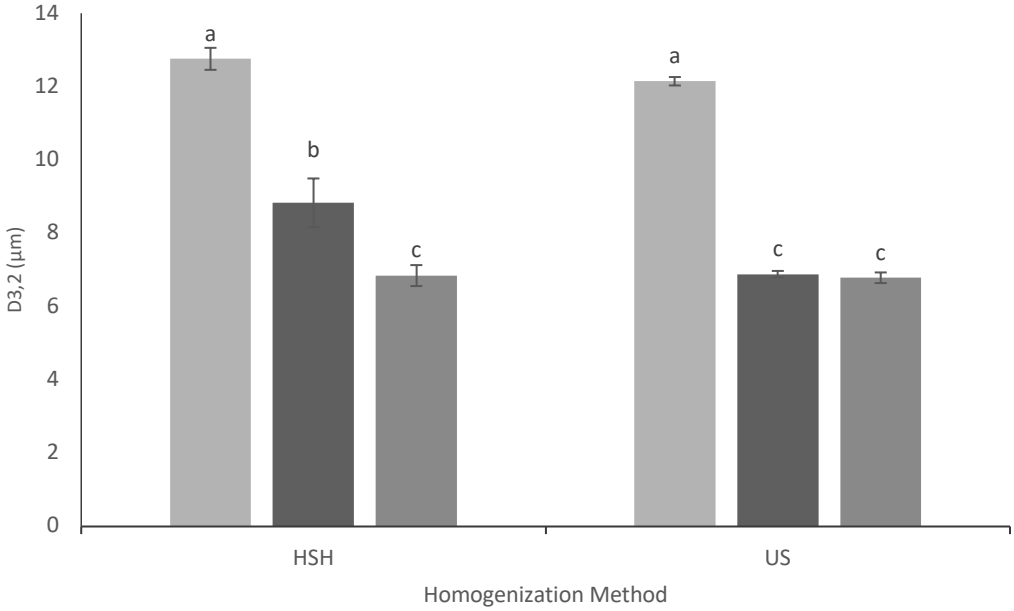

Figure 3.3 Surface moment mean diameter ($D_{3,2}$) of double emulsions prepared with different homogenization methods (HSH, US) and different concentrations of pea flour (15%: ■, 20%: ■, 25%: ■) *Different letters in the bars represent a significant difference ($p \leq 0.05$)

In addition, Sridharan et al. (2020) observed that protein concentration was effective in the particle size of emulsions stabilized with pea protein. Samples with 0.1% and 0.15% protein by weight had larger oil droplets (around 10 μm), while a decrease to 2.5 μm was seen in average particle size when the protein content was increased to 0.2%wt. Researchers also emphasized that in systems with low protein content, the particle size of the emulsions was highly dependent on the protein amount; however as the protein content increased, the interface becomes saturated with sufficient protein and thus, protein content was not the primary parameter affecting the particle size after a critical biopolymer content (Sridharan et al., 2020).

Theoretically, high energy input combined with the cavitation produced by sound waves decreases the particle size of the emulsions (Zhou et al., 2022). Yildirim et al. (2017) prepared double emulsions using sodium caseinate, xanthan gum and lecithin-whey protein concentrate as emulsifiers for the outer aqueous phase. Two high-energy methods (high-speed homogenization, ultrasonication) and different ratios for outer and inner phases was employed in the preparation of emulsions. Ultrasonicated samples prepared with xanthan gum had lower mean particle sizes.

In addition, Mahdi Jafari et al. (2007) stated that when treatment duration at 100 μm of amplitude was extended, the particle size and span of O/W emulsions decreased. Acoustic cavitation has therefore fractured water droplets under these ultrasonic treatment parameters (100 μm of amplitude and 3 min), causing them to accumulate enough surfactant to produce smaller particles and lower span value.

However, the results of Velderrain-Rodríguez et al. (2019) demonstrated that the ultrasonic treatment only had an impact on the emulsions' particle size when the amplitude was above 60 μm. Yildirim et al. (2017) also reported that emulsions with sodium caseinate and lecithin-whey protein concentrate had higher mean particle sizes after application of ultrasonication. Researchers stated that when the particle size of the emulsion was already in the micron range, the droplet

characteristic may be affected by the over-processing phenomenon, and an increase in the particle size was observed.

In addition, ultrasonication efficiency is dependent on the position of the probe inside the sample. Oil droplets closer to the ultrasonication probe were subjected to more disruption, resulting in a more polydisperse emulsion. K. Zhang et al. (2020) observed that the position of the probe is effective in the mean droplet diameter and polydispersity index of water-in paraffin oil emulsions. The optimum position for the probe was determined as 2 centimeters from the top of the liquid with increasing mean diameter and polydispersity in both shallow and deeper positions.

Particle size is also dependent on the pH of the pea flour solutions. The particle size of the emulsions decreased significantly in emulsions prepared with pH values distant to the isoelectric point of the pea proteins, which shows a similar behavior with the solubility of proteins. $D_{4,3}$ values decreased 5-fold when the pH of the sample was increased from pH 5 to 7. Another 5-fold decrease was observed when the solution pH was further decreased to pH 9 (Liang & Tang, 2013). This shows that the net charge of biopolymers, as well as the interactions among them, are strongly influenced by pH, which was also observed by Assadpour et al. (2016). As a result, pH affects the ability of biopolymers to act as emulsifiers and thus the particle size of the emulsions. In the isoelectric point, proteins precipitate and form large aggregates. Since these aggregates are poorly absorbed in the interface, the repulsive forces between the emulsion droplets are low, which results in lower stability and larger particle size.

Sridharan et al. (2020) commented that higher protein content might result in emulsions with narrower particle size distributions and thus less polydispersity. This contradicts with the findings of our study due to the significant differences in viscosity between the samples and the high amount of polysaccharides in the solutions. Higher span values were found for emulsions with higher legume flour concentrations (Table 3.1).

On the other hand, span values for emulsions prepared with ultrasonication did not differ significantly from high-speed homogenization ($p>0.05$). Higher span values imply that the emulsion is more polydisperse. In a similar study, Matos et al. (2018) obtained double emulsions with high polydispersity and bimodal particle size distribution for emulsions for encapsulating resveratrol using similar homogenization equipment and parameters to our study. Their results also agree with Hemar et al. (2010) and Matos et al. (2015), in which the mean diameter of double emulsions were found to be in the 10-30 μm range. The increase in the span values following ultrasonic homogenization also demonstrated that ultrasonic homogenization created emulsions with a larger range of various particle sizes considering span is a measure of polydispersity.

D_{90} value in particle size testing expresses the particle size of 90% of the material. Even though ultrasonication treatment did not affect particle size significantly for various concentrations, a lower trend in D_{90} values was observed for samples treated with ultrasonication ($p<0.05$).

Since they are thermodynamically unstable, the particle size of the emulsions continues to increase over time and eventually, separation occurs under gravitational force (Kale & Deore, 2017). Chemical aging of the emulsifier also contributes to the increasing particle size during storage. In the study of K. Zhang et al. (2020), after a day of storage at 4°C, the mean diameter of W/O emulsions for encapsulating chitosan increased from 198.5 nm to 204.6 nm while the emulsions stored at 25°C increased from 198.5 nm to 225.7 nm. The polydispersity index was also affected, and an increase from 0.326 to 0.331 was noted for storage at 4°C and 0.326 to 0.343 for emulsions at 25°. Contrarily, in some studies the particle size of the emulsions decreased during storage which was explained by the shrinking of the particles in W1 phase (Sanhueza et al., 2022).

Table 3.1 D_{50}, $D_{3,2}$ and $D_{4,3}$ and span values for double emulsions prepared with different homogenization methods and pea flour concentrations

Homogenization Method	Flour Conc. (%)	D_{50} (μm)	$D_{4,3}$ (μm)	Span
HSH	15	43.75 ± 0.865^{a}	68.86 ± 2.71^{a}	2.82 ± 0.135
	20	31.03 ± 0.685^{bc}	57.53 ± 1.17^{ab}	4.89 ± 0.470
	25	24.66 ± 1.012^{c}	51.53 ± 4.28^{bc}	5.25 ± 1.365
US	15	36.59 ± 3.685^{ab}	66.30 ± 0.81^{a}	2.33 ± 0.205
	20	24.98 ± 2.167^{c}	53.15 ± 1.33^{bc}	4.92 ± 0.210
	25	22.81 ± 0.205^{c}	42.99 ± 1.35^{c}	8.69 ± 0.160

*Different letters in the same column represents significant difference ($p \leq 0.05$)

3.2 Rheology

Our study revealed that the rheological properties correlate with the stability of emulsions. The rheological properties of the emulsions will be effective in the applicability of the final product in food products in the future.

A high correlation of determination (R^2) was obtained when flow data obtained from the rheometer was fitted with power model. Yildirim et al. (2017) indicated that double emulsions generally have R^2 values higher than 0.997. In our study, R^2 values for all the samples were indeed higher than 0.997 except for samples prepared with 15% pea flour and using ultrasonication which had an R^2 value of 0.985 (Table 3.2).

As S. bin Choi et al. (2018) pointed out, reliance of viscosity on the shear rate is an important relationship in the rheology of double emulsions. The flow behavior of the emulsions was identified as shear-thinning based on the flow behavior index (n) values since the apparent viscosity of the double emulsions decreased as the shear rate increased (Figure 3.4, Figure 3.5, Figure 3.6).

Table 3.2 Consistency index and flow behavior index of double emulsions prepared with different methods (HSH, US) and pea flour concentrations (15%, 20%, 25%)

Flour Conc. (%)	Homogenization Method	k (Pa.s^n)	n	R^2
15	HSH	0.94 ± 0.072^{c}	0.722 ± 0.0117^{a}	0,9971
	US	0.38 ± 0.054^{c}	0.769 ± 0.0035^{a}	0,9858
20	HSH	3.24 ± 0.406^{b}	0.613 ± 0.0213^{b}	0,9992
	US	1.52 ± 0.240^{bc}	0.701 ± 0.0140^{a}	0,9983
25	HSH	10.39 ± 0.646^{a}	0.526 ± 0.0027^{c}	0,9995
	US	12.03 ± 0.347^{a}	0.502 ± 0.0191^{c}	0,9992

*Different letters in the same column represents a significant difference ($p \leq 0.05$)

Double emulsions generally exhibit shear thinning behavior (Choi et al., 2018; Yildirim et al., 2017). Shear thinning behavior is a property of emulsions that have loosely bound droplet aggregates (McClements, 2009). The aggregates display higher viscosity rates at lower shear rates before they begin to flow. However, as the shear rate rises, the weak connections between droplets may disintegrate, and the aggregates may align in the direction of the shear (Choi et al., 2018; Sridharan et al., 2020).

Yildirim et al. (2017) also stated that the deformation of the network that formed in the equilibrium state is the cause of the shear-thinning behavior of double emulsions. As the shear force was applied, network rupture occurred, and the droplets of the primary emulsion were deformed due to increased shear stress. Shear-thinning behavior can also be interpreted as a smooth transition between a hard phase, in which droplets are rigid, and a soft phase, in which they are deformable (Foglino et al., 2017). These mechanisms contribute to the decrease in viscosity.

The flow behavior index (n) varied from 0.526 to 0.722 for emulsions prepared with HSH, while it changed between 0.502 and 0.769 for ultrasonicated samples (Table 3.2). The application of ultrasonication did not change the flow behavior of the emulsions significantly ($p<0.05$).

Moreover, higher values for the flow behavior index were observed for the samples stabilized by 15% flour solution, which indicated that the behavior of emulsions was more closer to Newtonian (Table 3.2.). In general, the flow behavior of double emulsions became closer to Newtonian fluids in higher shear rates as droplet deformation grew more steady (S. bin Choi et al., 2018).

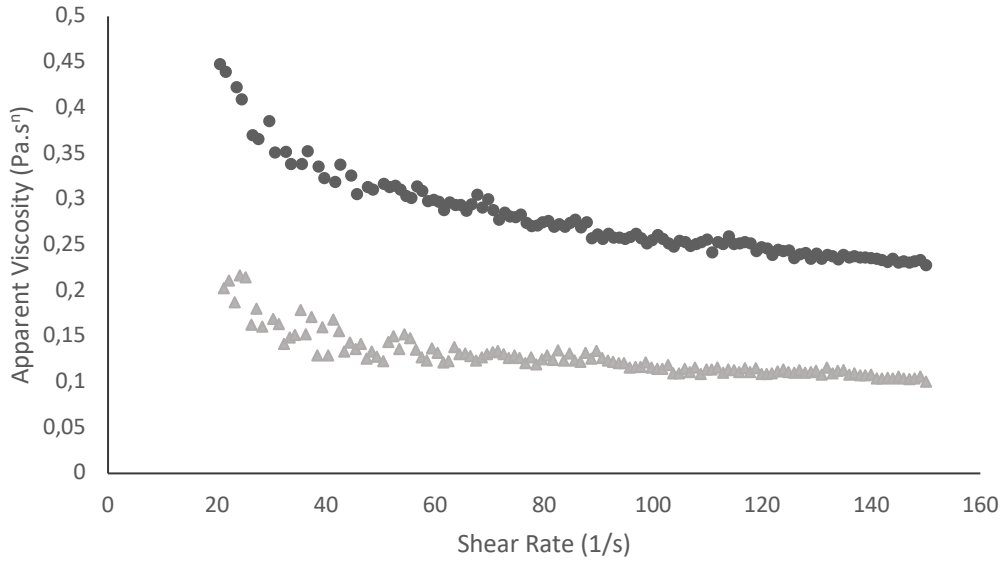

Figure 3.4 Apparent viscosity of double emulsions prepared with different methods using 15% pea flour (●):HSH, (▲):US

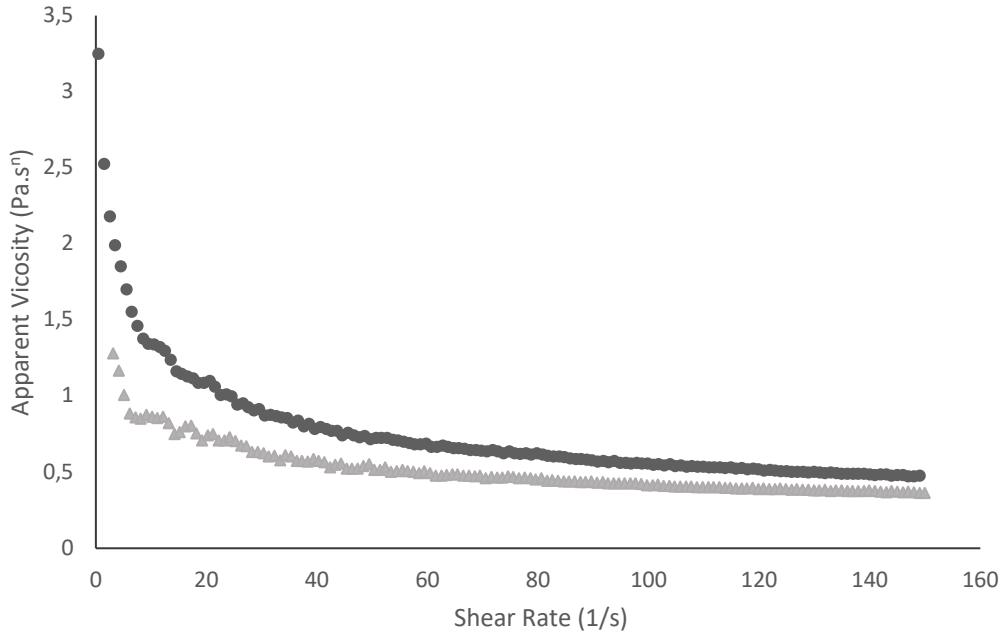

Figure 3.5 Apparent viscosity of double emulsions prepared with different methods using 20% pea flour (●):HSH, (▲):US

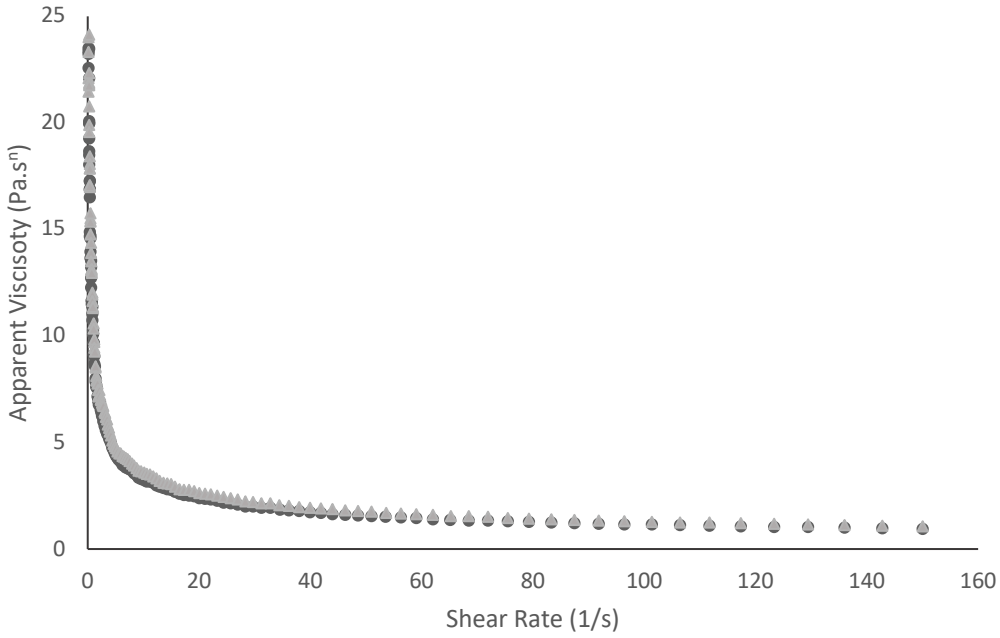

Figure 3.6 Apparent viscosity of double emulsions prepared with different methods using 25% pea flour (●):HSH, (▲):US

Zhou et al. (2022) reported that emulsions prepared with HSH and US were non-newtonian and shear thinning. In addition, when US and HSH treatments were compared, HSH showed higher apparent viscosity and shear stress values compared to the other treatments. Emulsions with smaller particle sizes ordinarily have higher viscosities and lower flow indices, and they exhibit strong shear-thinning characteristics (Zhou et al., 2022). Matos et al. (2018) also commented that their double emulsions behaved as pseudoplastic fluids with greater shear thinning in lower flow behavior index (n) values. Moreover, Assadpour et al. (2016) observed that for double emulsions stabilized by whey protein and maltodextrin, emulsifier content was the most critical parameter determining the viscosity of emulsions which was commented to be a result of the higher viscosity of the emulsifier itself. The concentration of the core compound and volume fraction of the dispersed phase was less effective in the final viscosity.

Figure 3.7 summarizes the apparent viscosity of emulsions taken at a shear rate value of 150 s^{-1}. It was seen that the apparent viscosity of emulsions with 25% flour was significantly higher ($p<0.05$) than the other samples, which contributes to the increased stability of emulsions significantly. Thicker emulsions are obtained as the solid content of the outer phase increases. (Sridharan et al., 2020)

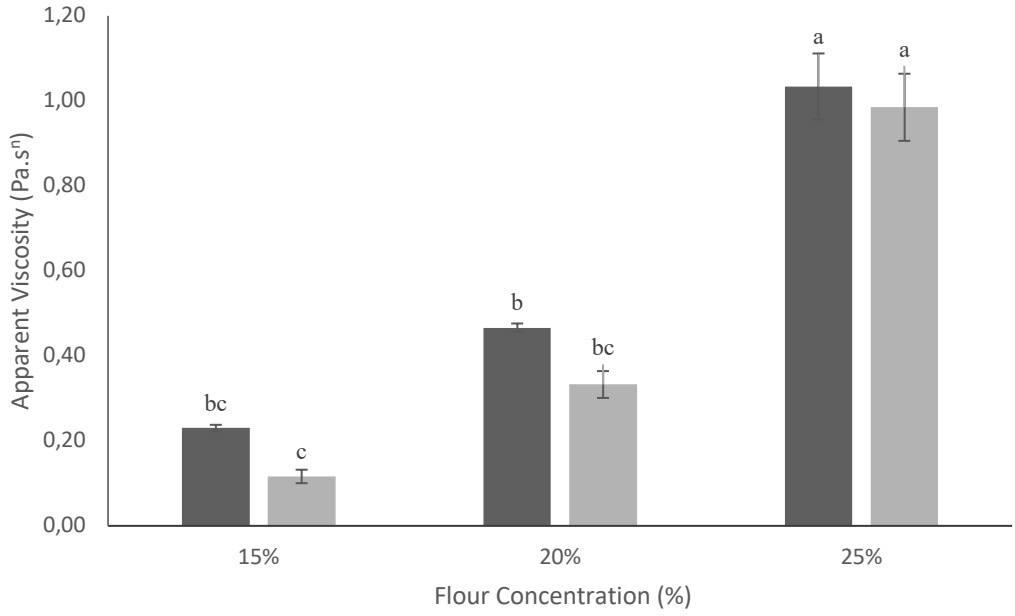

Figure 3.7 Apparent viscosity of double emulsions prepared with different pea flour concentrations (15%, 20%, 25%) and homogenization methods (HSH: ■, US: ■) *Different letters in the bars represent a significant difference ($p\leq0.05$)

Furthermore, a decrease was observed in the apparent viscosity of emulsions when ultrasonication was applied (Figure 3.7). This effect was more prominent in emulsions prepared with 15% pea flour (Figure 3.4).

Decreasing viscosity as a result of high-intensity ultrasonic treatment was also observed by Kaltsa et al. (2013) in emulsions prepared with whey protein isolate and olive oil. The decreasing trend of viscosity was consistent with emulsions produced with all types of gums (xanthan gum, guar gum, locust bean gum). As previously mentioned, cavitation is the main principle of ultrasonication. Formation and collapse of bubbles inside the solution cause formation of high temperature, pressure and shear rates. As a result, the bond and linkages between the molecules in the solution can break and structure may change (Tiwari et al., 2010).

Decrease in emulsion viscosity was similarly observed by Li & Xiang (2019) for emulsions prepared with coconut oil, xanthan gum, and propylene glycol alginate. Pre-emulsions were first using HSH and then US was applied. Researchers found that emulsions prepared with both emulsifier ratios (3:7 and 4:6) showed a significant decrease after US treatment at 270 W power for 7 min. Similarly, when different treatments (HSH, US and HPH) were compared, samples prepared through HSH showed the highest apparent viscosity, while US homogenized emulsions had the least viscosity among the treatments (Zhou et al., 2022).

Consistency index (k) of emulsions exhibited the same trend with the apparent viscosities of emulsions. In general, a higher consistency index is observed for more viscous emulsions due to increasing solid content as well as the thickening properties of polysaccharides inside the pea flour. Thus, increasing the concentration of pea flour caused a significant increase in the k value of emulsions ($p<0.05$).

Furthermore, the viscosity of the emulsions is expected to increase during storage. Possible reasons for this are the interaction of compounds in the outer phase, the formation of surfactant micelles, and the rise in particle size (Mohammadi, Jafari, Assadpour, et al., 2016; Rafiee et al., 2011).

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3.3 Instant Stability

The ability of an emulsion to preserve its physicochemical qualities over time is referred to as emulsion stability (Dickinson, 2009). Due to the extended time needed to achieve results from long-term stability tests, samples were subjected to centrifugation for accelerated stability testing. Normally, particles inside the emulsions are subjected to gravitational forces, and subsequently, separation occurs. Through the forces applied during centrifugation, it is possible to accelerate the phase separation and to compare the stability of emulsions right after preparation.

Correlation coefficients were calculated to assess the correlation of instant stability results with long-term stability. For emulsions stored at 4°C, the correlation coefficient was 0.945 ($p=0.000$), whereas for storage at 20°C, it was found as 0.767 ($p=0.004$). Hence, the instant stability of emulsions can provide guidance to the stability of emulsions during prolonged storage at 4°C.

As stated by Matos et al. (2018), the viscosity and densities of both continuous and dispersed phases along with the mean particle size of the dispersed droplets are the main factors affecting the stability of emulsions. When instant stability results were analyzed, it was seen that emulsions prepared with 25% flour had the highest stability, with 67.3% and 69.4% for HSH and US, respectively. The instant stability of emulsions prepared with different methods (HSH, US) and concentrations (15%, 20%, 25%) is given in Figure 3.8.

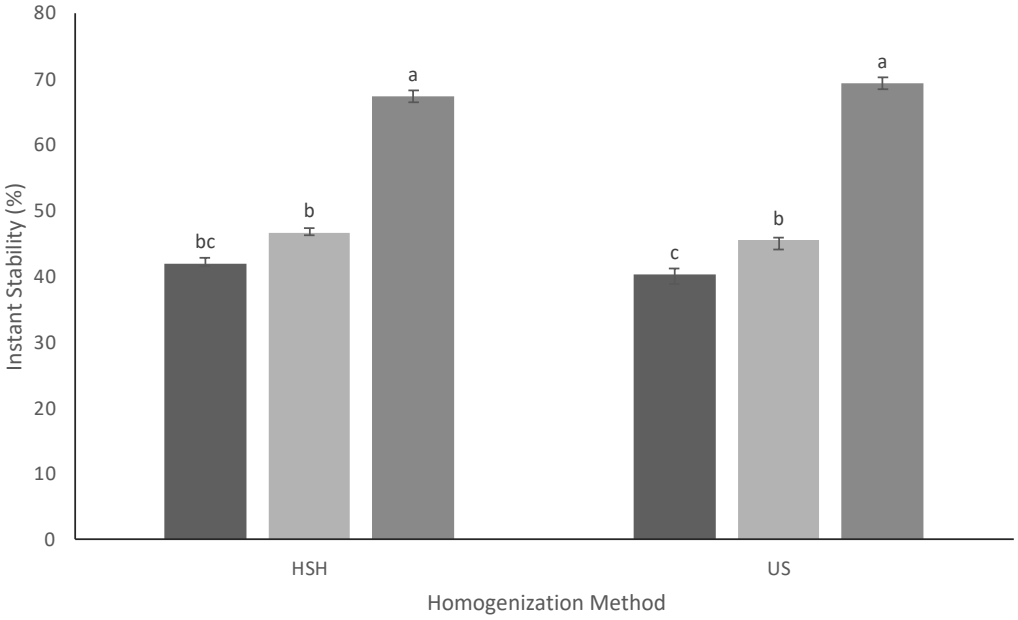

Figure 3.8 Instant stability of double emulsions prepared with different homogenization methods (HSH, US) and different concentrations of pea flour (15%: ■, 20%: ■, 25%: ■) *Different letters in the bars represent a significant difference ($p \leq 0.05$)

The concentration of flour in the outer phase had a significant effect on the stability of emulsions ($p<0.05$). As the flour concentration increased, the total amount of biopolymers in the solution increased accordingly. With more compounds to stabilize the interface, higher stability was observed. Furthermore, biopolymers with a high molecular weight and concentration could form a thick gel around the droplets, which had a positive effect on stability (Yildirim et al., 2017).

When Sridharan et al. (2020) investigated the use of unpurified pea flours as emulsifiers, they observed that the protein portion of flour maintained the interface between phases of the emulsion.

Additionally, the absence of starch did not alter the interface's elastic properties, suggesting that non-protein molecules had no impact on the elastic network of the interfacial proteins. This supports the idea that proteins and polysaccharides can contribute to stability through different mechanisms (Sridharan et al., 2020).

The higher apparent viscosity (consistency index) of 25% emulsions also contributed to the stability, which was previously explained in 2.3.4. Instant stability results had a positive correlation with consistency index ($r=0.995$, $p=0.000$) and a negative correlation with mean particle size ($r= -0.823$, $p=0.044$), as expected.

3.4 Long-Term Stability

Instability in emulsions during storage occurs through various mechanisms such as creaming, flocculation, sedimentation, coalescence, and diffusion. Ostwald ripening could also take place in emulsions especially when the particle size of the droplets greatly (McClements, 2015). pH and pressure variations, temperature variability, relative emulsion composition, particle size, microstructures, as well as microbiological and environmental stressors such as agitation and light are physicochemical factors that might impact emulsion instability. These effects can appear at any point of preparation, storage, or consumption (Fasinu et al., 2015).

Instability through gravitational separation may be observed as the separation of the emulsion into phases. For instance, particles of the inner dispersed phase (in our case, the W/O emulsion) tend to move to the upper part of the glass tube due to the lower viscosity of oil, which results in creaming (Matos et al., 2018). Instability through diffusion, on the other hand, is a result of the concentration gradient between phases and causes the core compound to transport from the inner phase to the outer phase. These instability mechanisms governing the emulsions need to be carefully considered when designing emulsion systems.

Different instability mechanisms affect emulsions simultaneously, and the total instability of an emulsion is cumulative of these effects. However, the dominant mechanism may depend on a variety of factors. For instance, Liang & Tang (2013) stated according to their research that droplet coalescence was the main cause of emulsion instability for pea protein isolate emulsions under acidic pH values, whereas bridging flocculation dominated under neutral or alkaline pH values. The addition of polysaccharides to the outer phase can also change the release mechanism of the core compound from emulsions. Mohammadi, Jafari, Assadpour, et al., (2016) stated that phenolic compounds transported from the inner droplets to the outer phase in whey protein-pectin stabilized emulsions while the main instability mechanism for whey protein emulsions was rupturing of the interface.

Long-term stability was determined by visual inspection of the emulsions, similar to the studies found in the literature. The height of the emulsions and the separated serum layers were measured for three months. The first measurement was taken three days after the preparation of the emulsions, and one measurement was taken every week.

During storage, a darker-colored layer was observed over the emulsions combined with the separation of the outer aqueous phase composed of the pea flour solutions at the bottom of the tubes. In samples prepared using 15% pea flour, the separation of the outer phase from the emulsions at the bottom started as early as the first measurement (3rd day), while in samples with 20% pea flour, it occurred around 2-3 weeks.

Storage conditions such as temperature have a strong effect on emulsions stability (Can Karaca, 2020). It was observed that emulsions which were stored in 4°C had higher stability compared to the emulsions stored in 20°C (Table 3.3).

Table 3.3 Final stability (%) of emulsions after 12 weeks of storage at 4°C and 20°C

Homogenization Method	Flour Concentration (%)	Stability (%) 4°C	Stability (%) 20°C
HSH	15	70.74 ± 0.27^{c}	57.44 ± 1.53^{d}
	20	74.78 ± 2.53^{bc}	73.79 ± 1.17^{b}
	25	86.72 ± 2.45^{a}	86.22 ± 0.08^{a}
US	15	66.90 ± 1.52^{c}	56.81 ± 0.19^{d}
	20	75.33 ± 2.76^{abc}	66.00 ± 1.00^{c}
	25	85.25 ± 1.80^{ab}	71.14 ± 1.14^{bc}

*Different letters in the same column represents significant difference ($p \leq 0.05$)

For emulsions stored at 4°C, stability of emulsions after 12 weeks of storage varied between 66.9% and 86.7%, while for the emulsions kept at 20°C, it was between 56.6% and 86.22%. Kocaman et al. (2020) also reported that when the release behavior was compared at 4, 20, and 37 °C, the fastest release was found at the highest temperature due to the increased solubility in the oil phase and the faster diffusion rate at this temperature.

An increase in viscosity due to the gelatinization of starch molecules during the heating treatment contributes to the stability of the emulsions. However, as retrogradation takes place during the long-term storage of emulsions, a change in the properties of the outer phase might occur. These changes, as a result, might add to the instability. The rate of retrogradation depends on the temperature and thus is affected by the storage conditions of the samples.

The effect of storage temperature was found to be more prominent in 15% emulsions due to the significantly lower viscosity of the outer aqueous phase, which contributes to the higher diffusion rate between the inner and outer phases. The stability of emulsions with 15% flour and HSH decreased from 92.80% to 57.44% when stored at 20°C (Figure 3.9). For emulsions with US, on the other hand, stability values changed from 85.37% to 56.63% at the same storage conditions (Figure 3.10).

As graphs for the long-term stability (Figure 3.9, Figure 3.10, Figure 3.11, Figure 3.12) suggest, the highest decrease in stability was between 3rd and 4th weeks, which can be seen more clearly in storage at 20°C.

Emulsions with high apparent viscosity and lower mean particle size are observed to have higher stability values. According to Stoke's law, a droplet's speed is directly proportional to the square of the radius of the droplet and drops as fluid viscosity increases. As a result, it may be said that droplets with lower particle sizes move slowly due to gravitational forces, and thus decreasing particle size slows down gravitational separation (Yildirim et al., 2017). Likewise, Assadpour et al. (2016) emphasized that higher surfactant concentrations could prevent emulsion droplets from coalescing, aid in the breakdown of large droplets and the production of smaller, microscopic droplets that are very stable through decreasing surface tension rapidly which can provide a higher stability.

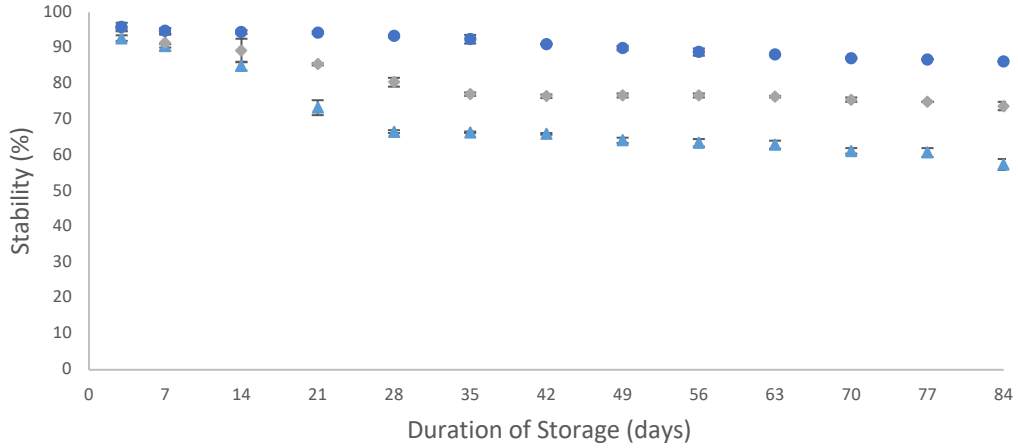

Figure 3.9 Long-term stability of double emulsions prepared with different concentrations of pea flour and high-speed homogenization during 12 weeks storage at 20°C (▲):15%, (♦):20%, (●):25%

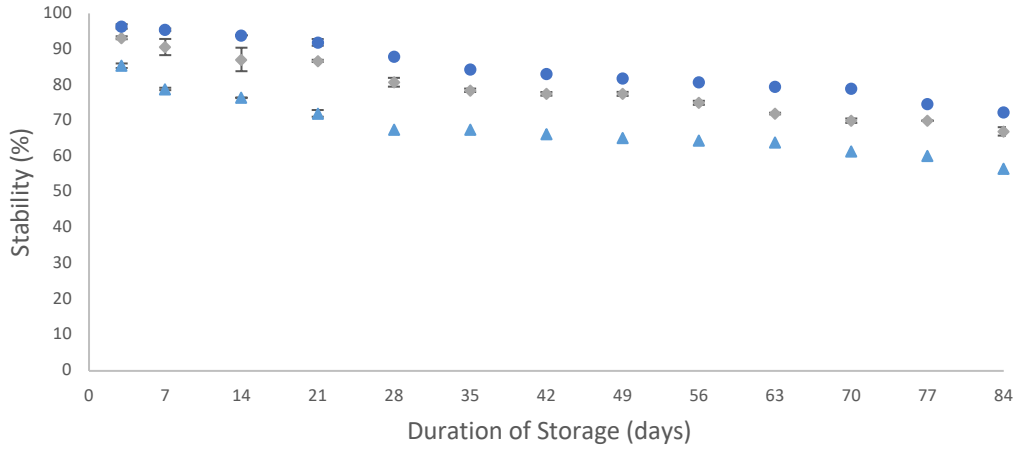

Figure 3.10 Long-term stability of double emulsions prepared with different concentrations of pea flour and ultrasonication during 12 weeks storage at 20°C (▲):15%, (♦):20%, (●):25%

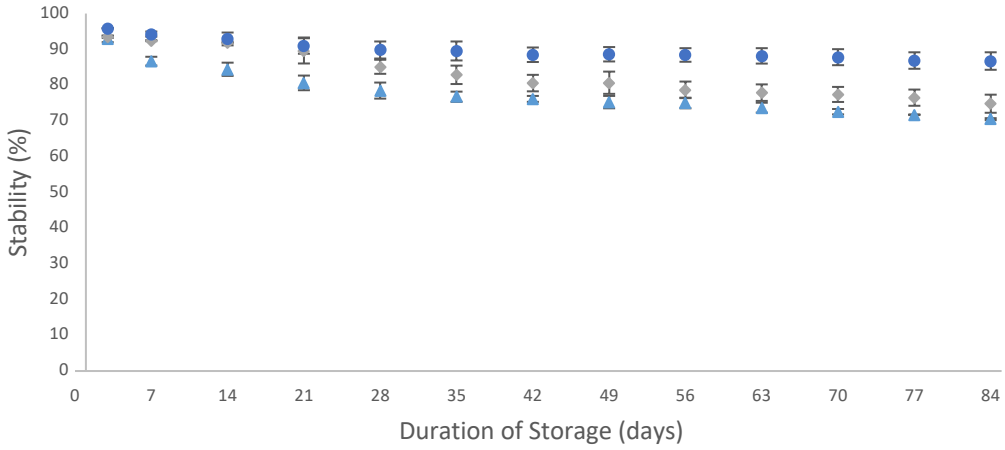

Figure 3.11 Long-term stability of double emulsions prepared with different concentrations of pea flour and high-speed homogenization during 12 weeks storage at 4°C (▲):15%, (♦):20%, (●):25%

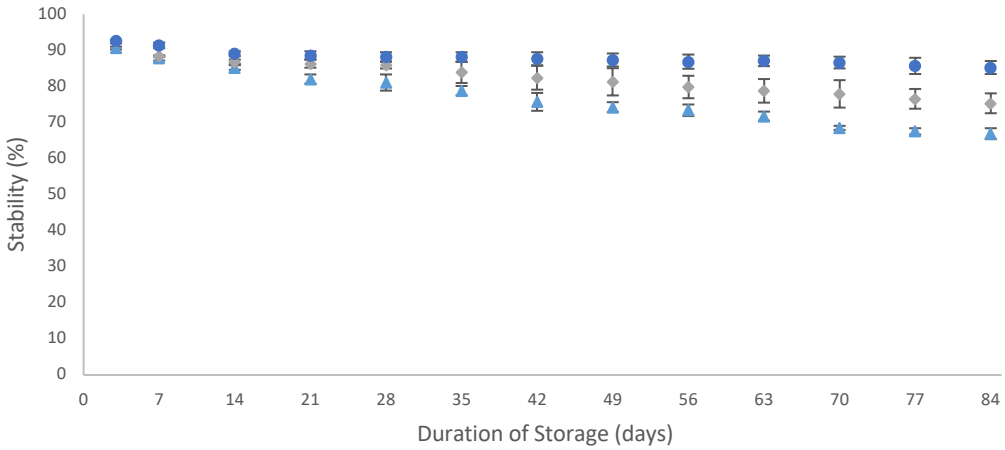

Figure 3.12 Long-term stability of double emulsions prepared with different concentrations of pea flour and ultrasonication during 12 weeks storage at 4°C (▲):15%, (♦):20%, (●):25%

After storage of emulsions in two different environments for 12 weeks, it was observed that emulsions with higher flour concentration had higher stability. At 4°C, the stability of samples prepared with 25% flour was found as 86.72% and 85.25% for HSH and US, respectively (Table 3.3). On the other hand, in emulsions stabilized with 15% flour, the concentration of biopolymers to stabilize the interface was lower, and thus oil particles could not be covered sufficiently, which led to lower stability values ($p<0.05$).

The concentration of biopolymers (maltodextrin, gum Arabic, tamarind kernel powder) was found to affect the creaming stability of emulsions. A two-fold increase in Tamarind kernel powder concentration (from 1.35 to 2.70 wt%) resulted in an increase in viscosity (42.15 to 68.45 mPa.s) and a decrease in creaming rate (52.50 to 33.99%).

Moreover, increasing the other biopolymers in the solution also had a significant effect on the stability of the double emulsions. The lower creaming stability was commented to be related to the increasing viscosity as it retarded the flocculation of droplets (Pitchaon et al., 2013). Assadpour et al. (2016) investigated the preparation of whey protein-maltodextrin emulsion systems through spontaneous emulsification. After experimenting on properties such as viscosity and encapsulation efficiency, researchers concluded that surfactant concentration was the parameter that had the highest effect on the qualities of the final product.

In our study, the long-term stability of double emulsions prepared with different methods and flour concentrations were correlated with consistency index, apparent viscosity, and particle size diameter with the correlation coefficient of 0.950 ($p=0.004$), 0.984 ($p=0.000$) and -0.902 ($p=0.014$), respectively.

Furthermore, in preliminary tests, it was acknowledged that an antimicrobial agent was needed for long-term stability tests. As pea flour is rich in nutrients, the outer phase of the emulsions, which is not entirely isolated from the environment, is prone to microbial growth. Emulsions stored at 4°C did not exhibit any microbial growth, whereas gas formation and turbidity were observed in emulsions stored at

20°C due to the growth of microorganisms. Previously, it was hypothesized that the antimicrobial properties of OLE would inhibit the deterioration of emulsions. However, the concentration of phenolic compounds in the outer phase proved to be insufficient to inhibit the growth of microorganisms. Deterioration was more evident in emulsions prepared with US since air was incorporated in the emulsions during homogenization. Thus, sodium azide, an antimicrobial agent, was added to the inner phase.

As previously mentioned, NaCl was also included in the aqueous phases of emulsions. In double emulsions, the difference in solute concentration between the internal and external phases exerts a force on the surface of the inner and outer droplets, referred as osmotic pressure (Khadem & Sheibat-Othman, 2019). Thus, NaCl is also added as an osmotic regulator. When added to double emulsions, electrolytes can cause a reduction of interfacial tension and enhance the adsorption of the emulsifiers.

When incorporation of different salt concentrations to the double emulsions was investigated, it was experimented that salt increased the efficiency of emulsifiers and had an effect on Laplace pressure. Researchers also commented that a synergistic effect between NaCl and PGPR in the preparation of W/O primary emulsions was critical for the overall success of the double emulsions (Sapei et al., 2012). Similarly, Kwak et al. (2022) compared the performance of different electrolytes as osmotic regulators in double emulsions. They concluded that the addition of electrolytes such as $MgCl_2$, NaCl, and KCl was effective to achieve a higher encapsulation efficiency and stability. Kabakci (2022) also demonstrated that emulsions with NaCl showed significantly better stability both after preparation and during storage.

Nevertheless, in some cases, the incorporation of osmotic pressure regulators such as electrolytes or polysaccharides may not be sufficient to control the expulsion of W1 droplets since some emulsifiers may facilitate or accelerate transport

phenomena (Garti & Aserin, 1996). Therefore, it is essential to choose the concentration and type of such osmotic regulators deliberately.

3.5 Optical Imaging

An inverted light microscope was used to examine the morphological properties of the emulsions. The particle size of the emulsions was in the suitable range, and the particles were visible when lenses with 10× and 40× magnification were used.

However, dilution of the samples was needed to observe the particles clearly. Distilled water was considered as the appropriate solvent as the outer phase of the double emulsions was water. Obtaining a thin layer of emulsion on the glass slide was also found to be crucial. When samples were diluted, it was easier to obtain a clear image by properly focusing due to overlapping particles of the emulsion.

The left image in Figure 3.13 shows the structure of the hydrated pea flour. The particles in the figure can also be seen in some of the double emulsion images. On the other hand, the right image shows the particles of W/O primary emulsion prepared with high-speed homogenization. Due to the small particle size of the primary emulsion, it was hard to obtain a clear image, even when a 40× objective was used.

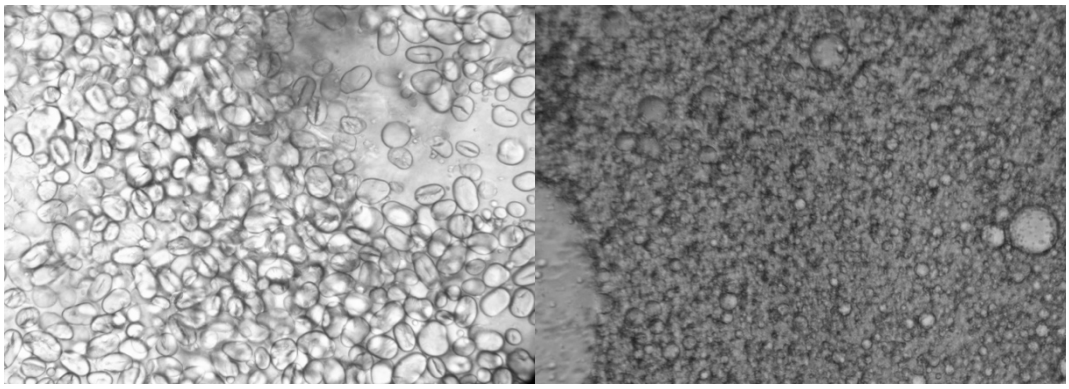

Figure 3.13 Optical images of hydrated pea flour (left image) and primary emulsion (right image) without dilution

In Figure 3.14, taken during the preliminary studies, the multiple-phase structure of double emulsions can be seen. The contrast of the image was increased to observe the oil droplets and the inner water droplets more clearly.

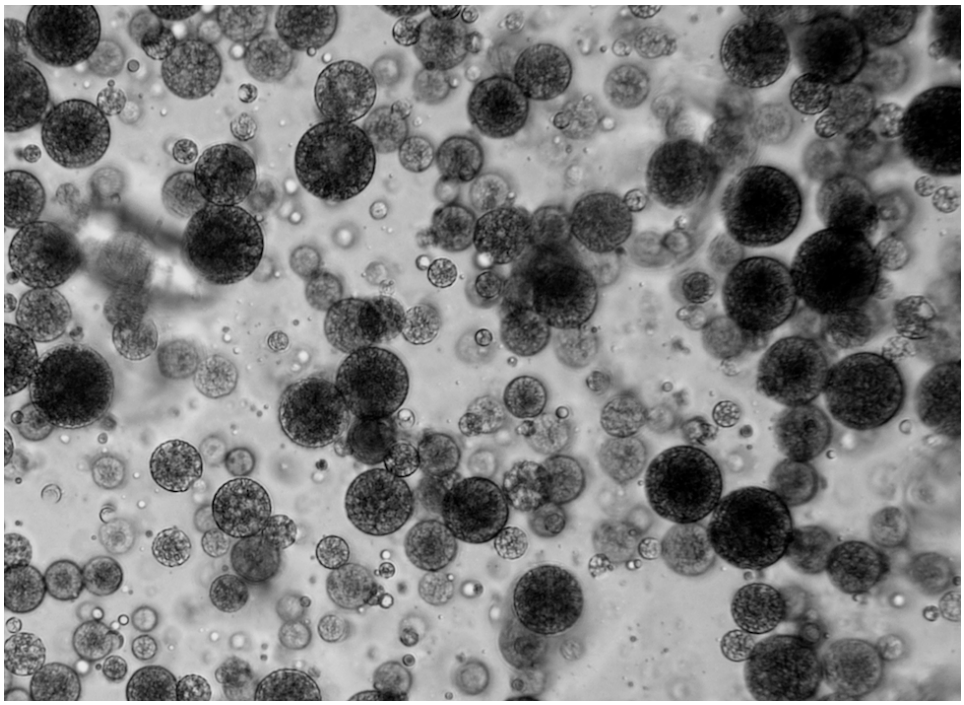

Figure 3.14 Optical image of double emulsion with 20% pea flour prepared with high-speed homogenization in the preliminary studies (40× magnification)

Owing to the superior emulsifying ability of pea proteins, which was experimented in many studies (Can Karaca, 2020; Liang & Tang, 2013), emulsions with spherical droplets are obtained. In our research, droplets of both primary and double emulsions for all concentrations of pea flour (15%, 20%, 25%) and homogenization methods (HSH, US) were identified as spherically shaped. Flour addition enables the formation of a matrix around dispersed droplets, and the oil phase is trapped inside the W2 phase.

Moreover, Kabakci et al. (2021) observed that the homogenization method might affect the morphology of the double emulsions considerably. When ultrasonication was applied, the shape and the diameters of emulsion droplets changed. In their study, primary emulsions were treated with ultrasonication equipment for 10 min at 160 W power, 20 kHz frequency, and 50% pulse as opposed to the lower power level and higher duration employed in this study. They commented that the formation of droplets with irregular shapes resulted from the sonication device's high energy application.

The type of emulsifier can also lead to differences in the shape of the particles. Non-spherical droplets were obtained when lecithin was used as the only surfactant for stabilization of the oil phase of W/O/W emulsions loaded with mango peel extract (Velderrain-Rodríguez et al., 2019). Combining lecithin with other surfactants such as PGPR can aid in the formation of more homogenously shaped droplets which was observed in this study. Moreover, this shows that the surfactant used in the primary emulsion is also effective in the microstructure of the double emulsions and hence should be carefully selected when the shape of the droplets is considered.

Optical images of double emulsions prepared with 15%, 20% and 25% pea flour are given in Figure 3.15, Figure 3.16 and Figure 3.17, respectively. Multiple phased structure of double emulsions is confirmed with images taken with the light microscope. Images taken with 10× magnification are given in Appendix B.

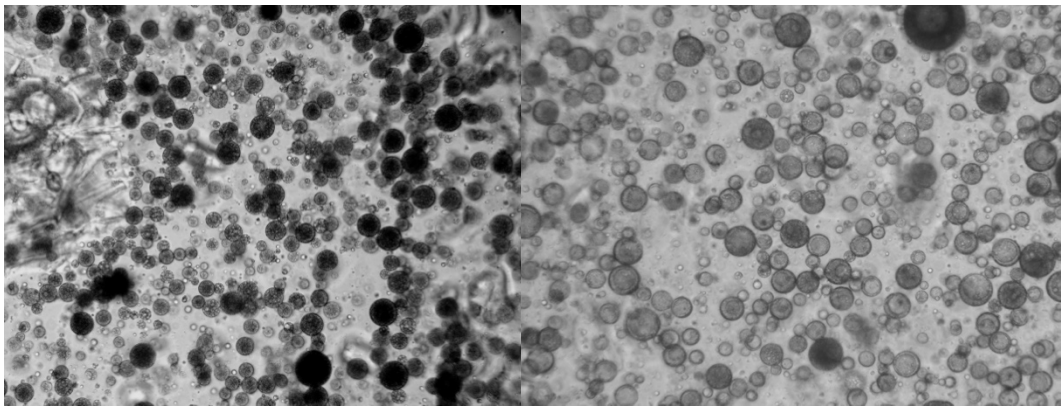

Figure 3.15 Optical images of double emulsions prepared with 15% pea flour using high speed homogenization (left) and ultrasonic homogenization (right) with 40× magnification

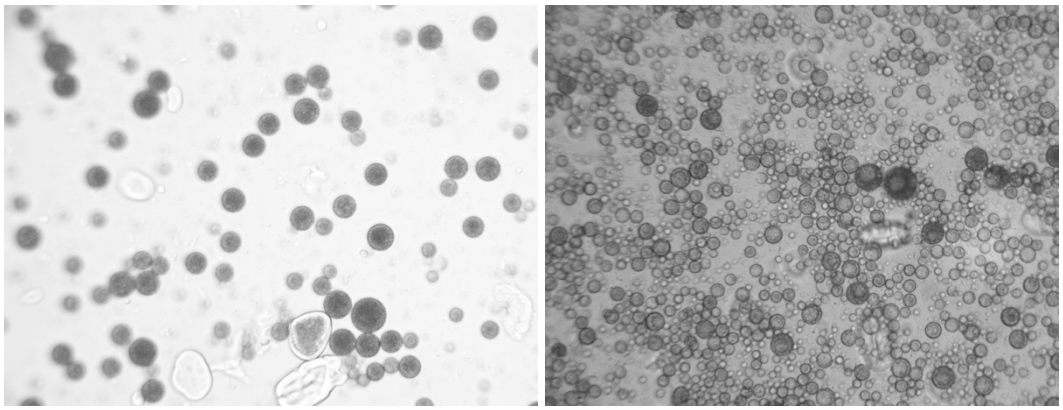

Figure 3.16 Optical images of double emulsions prepared with 20% pea flour using high speed homogenization (left) and ultrasonic homogenization (right) with 40× magnification

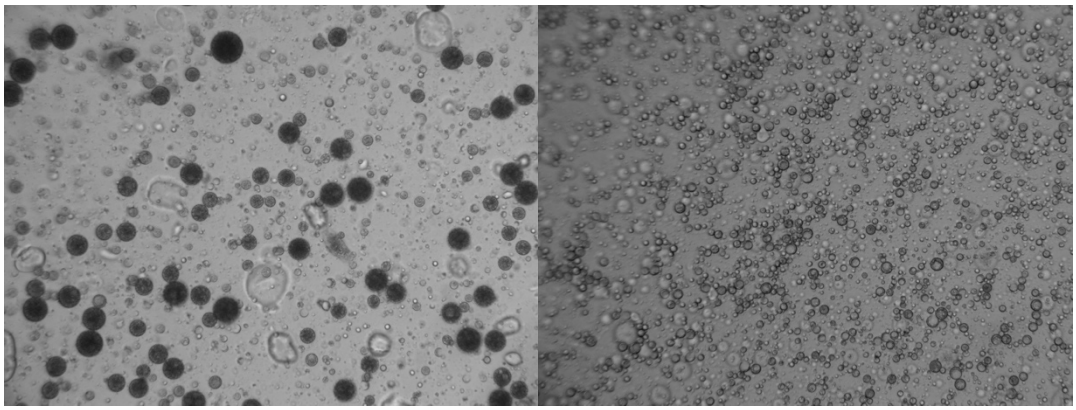

Figure 3.17 Optical images of double emulsions prepared with 25% pea flour using high speed homogenization (left) and ultrasonic homogenization (right) with 40× magnification

Although it is not possible to determine the exact particle sizes through the microscope since only a small portion of the samples can be observed, optical images obtained from the microscope were in agreement with the results from the particle size analyzer. Smaller particle size was observed as the concentration of the flour was increased.

3.6 Encapsulation Efficiency of Emulsions and Release (%) of OLE

The effectiveness with which the bioactive ingredient is retained in the inner phase of the emulsion determines the quality of the emulsions (Mudrić et al., 2019). In double emulsions, encapsulation efficiency is referred to as the amount of the core material that is entrapped within the primary emulsion after the second homogenization process. The length and complexity of the analytical procedure render it difficult to quantify the efficiency, even though it is one of the most crucial characteristics for the quality of a double emulsion.

Our study evaluated the encapsulation efficiency of emulsions after 4 hours of storage. For the calculation of efficiency, the W2 phase of the emulsions was recovered through centrifugation and filtration. The phenolic content found in the W2 phase was then subtracted from the phenolic content of the extract added during the preparation of the primary emulsion. The total phenolic content of the samples was determined through the Folin-Ciacalteou method.

The total phenolic content of the liquid olive leaf extract was found as 17.82 mg GAE/g liquid extract. The phenolic content of extracts varies significantly on the variety of the olive tree and the parameters used in the extraction of the phenolic compounds.

As pea flour was also rich in phenolic compounds, another factor that was necessary to be considered when calculating the efficiency was the phenolic content of the outer phase of the emulsion. Thus, emulsions without the addition of extract were prepared. The application of high temperatures may increase the phenolic content of pulses (Vasilean et al., 2018). For this reason, the contribution of pea flour to the total phenolic content results was evaluated through the preparation of emulsions without the core material.

The phenolic content of emulsions without the OLE was referred to as $TPC_{W2,0}$ and was subtracted from the TPC of the outer phase to find the corrected phenolic content of the W2 phase ($TPC_{W2,corrected}$ or $TPC_{W2,c}$). The phenolic content of pea flour used in the experiments was 2.95 mg GAE/g flour.

Phenolic content of pea flour changes substantially with respect to the variety. According to Turkmen et al. (2005) phenolic content of peas grown in Turkey was 1.83 mg GAE/g dry weight. Ladjal Ettoumi & Chibane (2015) found the TPC of pea flour as 2.36 mg GAE/g for a common variety of peas in Algeria, while for peas grown in Mexico, the phenolic content was quantified as 5.84 mg GAE/g (Borges-Martínez et al., 2022). Marathe et al. (2011), on the other hand, reported the TPC for pea flours obtained from India between 0.93 and 1.04 mg GAE/g.

Samples prepared with US were found to have lower EE compared to the double emulsions prepared with HSH ($p<0.05$) (Figure 3.18). The lower apparent viscosity of the emulsions observed in 15% and 20% coupled with the US treatment may have had a detrimental effect on the encapsulation of the core compound. Even though the optical images did not reveal changes in the morphological properties of droplets, high shear and temperature caused by the US treatment can disrupt the integrity of droplets, also explained by over-processing phenomena.

The encapsulation efficiency of the emulsions was also related to the particle size of the emulsions. Emulsions with lower particle size typically exhibit higher steric stability (Huang et al., 2019). However, the small size and thus, the large specific surface area of the droplets in emulsions may encourage the chemical breakdown of the core material (Niknam et al., 2020). As smaller particle size was obtained when a higher concentration of pea flour was used, the concentration of the flour significantly affected the encapsulation efficiency ($p<0.05$). For HSH, EE values were found as 89.73%, 88.52%, and 86.40% for emulsions prepared with 15%, 20%, and 25%, respectively.

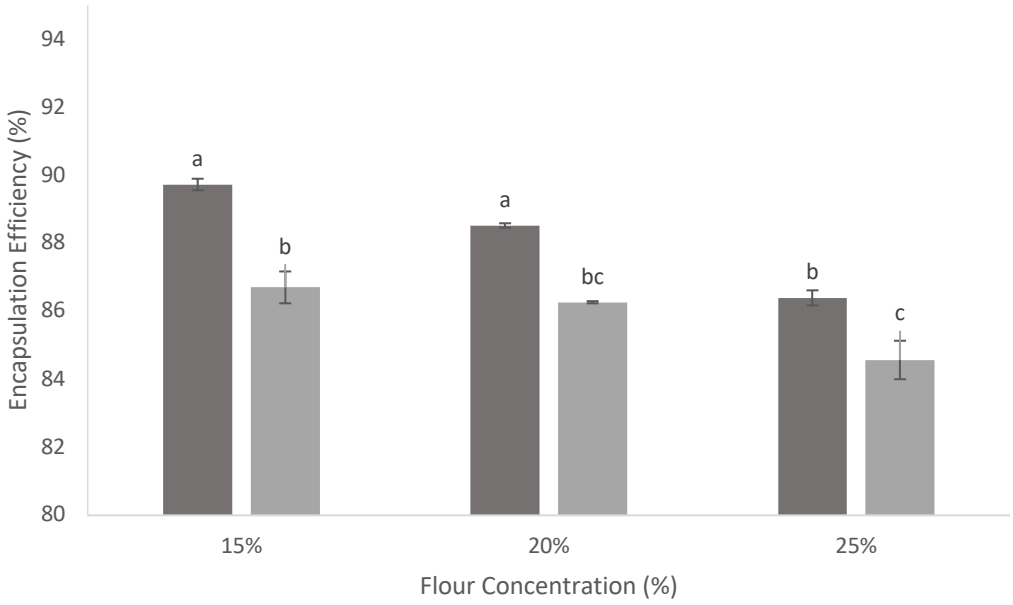

Figure 3.18 Encapsulation efficiency (%) of double emulsions prepared with different pea flour concentrations (15%, 20%, 25%) and homogenization methods (HSH: ■, US:■) *Different letters in the bars represent a significant difference ($p \leq 0.05$)

Yildirim et al. (2017) and Kabakci et al. (2021) also observed a strong correlation between larger emulsion droplets and higher efficiency values. As the particle size of oil particles decreased, a larger overall area of the O/W2 interface was formed. Compared to bigger particle emulsions with the same amount of oil phase, the total area of contact and transfer increase as particle size decreases due to the increase in the total area of oil droplets. As a result, in comparison to larger oil droplets, the possibility of inner aqueous phase (W2) interaction with the oil-water interface is higher for smaller oil droplets (Schuch et al., 2014). A higher amount of water is encapsulated inside the droplets of emulsions when the size of droplets is larger. Thus, a higher encapsulation efficiency is achieved (Schuch et al., 2015).

Encapsulation efficiency for mango seed kernel powder and methyl gallate W/O/W emulsions increased with increasing biopolymer concentrations. The type of the polysaccharide was also effective, which is mainly explained by the change in the viscosity of the system (Pitchaon et al., 2013). Mansuroglu et al. (2020) observed a similar trend for encapsulation of black pepper seed oil using chickpea protein and maltodextrin by spray drying. The efficiency increased from 74.1 to 99.3 as the protein concentration was increased from 1% to 4%. Researchers also observed a significant decrease in surface oil which was identified as an important parameter for the success of encapsulation.

Pea flour is mainly composed of proteins and polysaccharides. The approximate protein content of yellow pea flour was found as 24.5 g, while the polysaccharide content was 53.5 g per 100 g of flour (Han & Baik, 2008). High protein and carbohydrate amount of the pea flour were effective in keeping the phenolic compounds in the olive leaf extract contained inside the emulsion. Pea proteins contributed to the encapsulation efficiency of emulsions mainly due to their amphiphilic character, which was emphasized by many studies (Hadidi et al., 2021; Ladjal Ettoumi & Chibane, 2015).

Polysaccharides, on the other hand, was more effective in the manipulation of the outer phase viscosity and slowing down the transport of the encapsulated material as well as keeping the material inside the primary emulsion. However, in some cases, the osmotic balance-driven leakage of water from the inner-water phase to the outer-water phase might dilute the outer-phase concentration of polysaccharides, making them unstable and having a lower encapsulation efficiency after storage (Pitchaon et al., 2013).

Another characteristic that affects the encapsulation efficiency was gelling of the outer phase. Gel formation was effective to increase encapsulation efficiency and to decrease the release of caffeine from its capsule to the outer aqueous phase (Dickinson, 2011). Yildirim et al. (2017) likewise concluded that the gelling ability of xanthan gum enhanced the encapsulation efficiency of the double emulsion.

Similarly, when thermal treatment was applied, starch molecules in the flour solution could undergo gelatinization and thus increase the viscosity. Viscosity increase due to gelatinization of starch molecules was demonstrated by Yang et al. (2021). Since pea flour is abundant in starch, increasing the concentration of flour might slow down the coalescence of emulsions through increasing viscosity and decreasing the movement of particles. Gelatinization of pea starch happens around 70-75°C (Sun et al., 2021). Thus, samples were kept in the water bath until the solution temperature reached to 75°C.

Encapsulation efficiency of oleuropein, the main phenolic compound in olive leaf extract, was found as 34% when it was encapsulated using soy phosphatidylcholine liposomes. Prepared liposomes were incorporated into acidic model drinks and were capable of protecting the phenolic-rich extract from degradation (González-Ortega et al., 2021). Furthermore, for W/O/W emulsions containing whey protein concentrate and whey protein-pectin, encapsulation efficiency for olive leaf phenolics was 93.34% and 96.64%, respectively. On the 20th storage day, these values were reduced to 72.73% and 88.81%. It could be seen that emulsions prepared with only whey protein as the emulsifier showed higher release rates (Mohammadi, Jafari, Esfanjani, et al., 2016).

In addition, single and double emulsions encapsulating OLE was prepared by Jolayemi et al. (2021). The effect of encapsulated and unencapsulated OLE on the oxidation of the salad dressings was investigated. Encapsulation of the extract provided a slower and more controlled release of the core compound. The physical instability of dressings was also addressed.

The storage test regarding the release of OLE revealed that the double emulsions were effective in the controlled release. We did not observe a sudden release over the duration of the storage (Figure 3.19).

Dickinson (2011) commented that a double emulsion system can be thought to have satisfactory stability if the initial yield is around 95% and the yield after a few weeks of storage remains in the region of 70-80%. In our study, EE efficiency remained over 70% after a storage period of 13 days at 37°C.

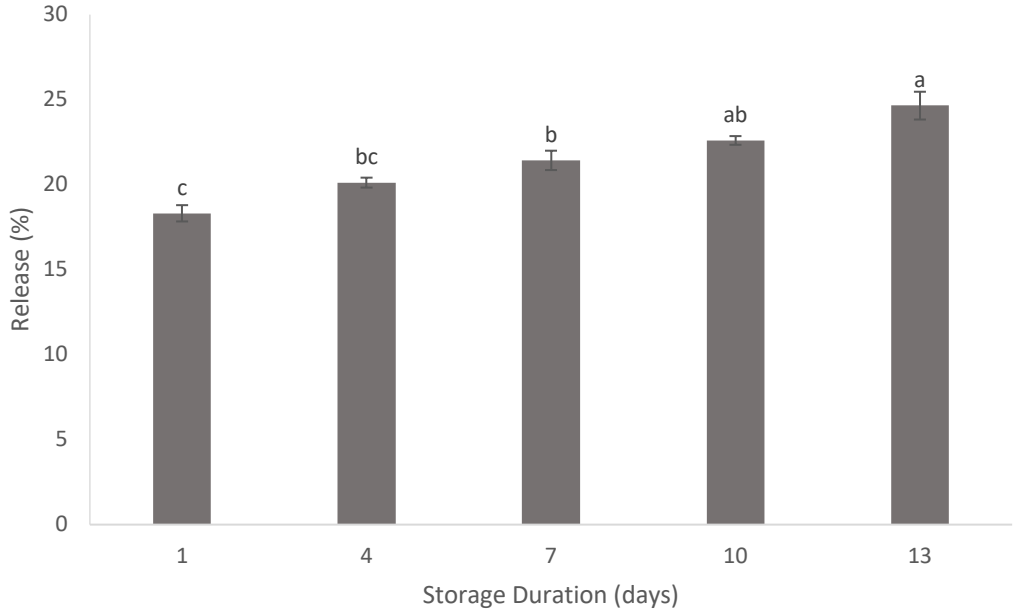

Figure 3.19 Release (%) of OLE from emulsions prepared using HSH and 25% pea flour during 13 days of storage at 37°C *Different letters in the bars represent a significant difference ($p \leq 0.05$)

Furthermore, linear regression was performed to gain insight into the release of the core compound to the outer phase. According to Çilek Tatar (2018), first-order kinetics can be used to represent the release on OLE. Indeed when the natural logaritm of the data was fitted with respect to storage duration, a high coefficient of determination value (R^2=0.991) was obtained.

The kinetic constant concerning the phenolic content of the outer phase was found as 0.0237 d^{-1} for emulsions prepared with 25% pea flour (Figure 3.20). The contribution of pea flour to the phenolic content of the phases was again taken into account when calculating the TPC. The positive sign of the kinetic constant indicates that the amount of olive leaf extract released through W2 during storage had an increasing trend.

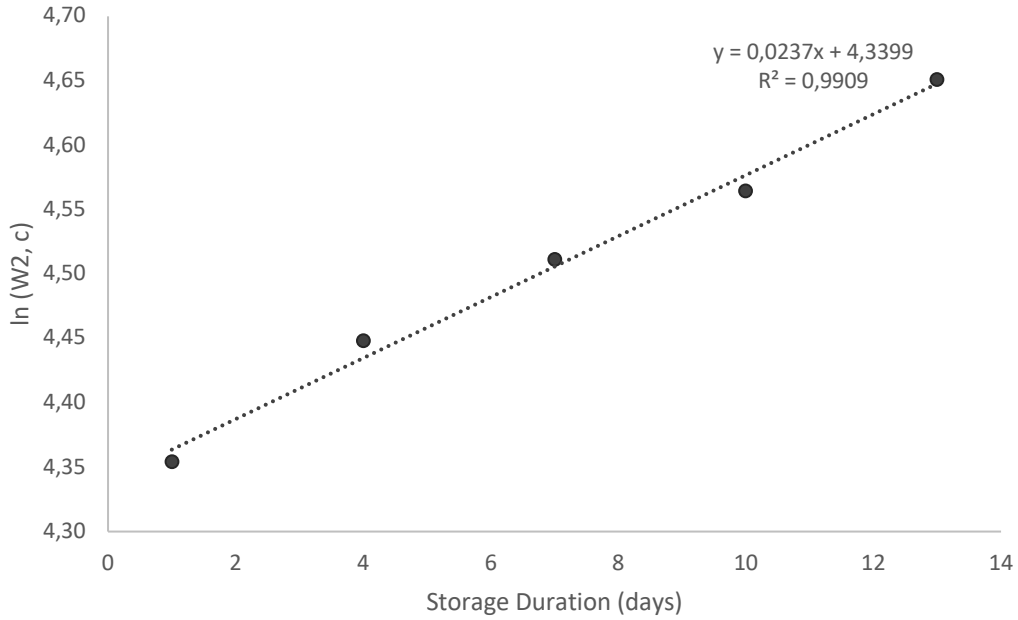

Figure 3.20 Fittings of the first-order kinetics of the corrected W2 results of double emulsions prepared using HSH and 25% pea flour during 13 days of storage at 37°C

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

The main objective of this study was the use of pea flour, which is a natural and inexpensive emulsifier, for the encapsulation of olive leaf extract without purification. The double emulsion systems were hypothesized to increase the stability of the phenolic compounds extracted from a by-product of olive production. The effect of different pea flour concentrations (15%, 20%, 25%) and different homogenization methods (HSH, US) were investigated.

For the characterization of the samples; particle size, rheology, encapsulation efficiency, stability, and optical images of the emulsions were evaluated. Emulsions prepared with both methods and all concentrations demonstrated shear thinning behavior and multi-modal particle size distribution. Furthermore, both the long-term and instant stability of emulsions were correlated to the viscosity and particle size of the emulsions. As a result of high biopolymer concentration and thus, emulsifying ability, pea flour was found to be a successful encapsulating agent. It was observed that the emulsions prepared with 25% flour had the highest stability due to their higher viscosity and smaller particle size. Ultrasonication treatment was not effective in reducing the average particle size of the emulsions. Lower values for D_{90} was obtained; however, this decrease did not contribute to the stability of the emulsions. It was concluded that HSH is a more effective method for obtaining higher encapsulation efficiencies.

In addition, storage temperature also affected the stability of emulsions as emulsions stored at 20°C showed faster degradation as compared to 4°C. Moreover, due to the lower viscosity, this effect was more prominent in emulsions in which 15% pea flour was employed. Average encapsulation efficiency values for double emulsions prepared with HSH and US were found as 88.3% and 85.9%,

respectively. In addition, double emulsions were effective in controlled release of OLE, double emulsion method can therefore be used for successful and efficient encapsulation of olive leaf extract.

Future studies on this subject can include the incorporation of the prepared double emulsions in a real food matrix which will be used for the fortification of food which can contribute to the development of functional foods with proposed health benefits. Further in-depth research is needed to understand how each emulsifier interacts with the interface and the core compound, OLE. In vitro tests could be used to evaluate the protection capability of emulsions against unfavorable environmental conditions. Furthermore, in-vivo tests might also be conducted to gain insight into the bioavailability of phenolic compounds. Lastly, the industrial applicability of our method for the preparation of pea flour emulsions can also be investigated and optimized. As OLE is obtained from a by-product of olive and olive oil production, its encapsulation can support sustainable agriculture.

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APPENDICES

A. Calibration Curves

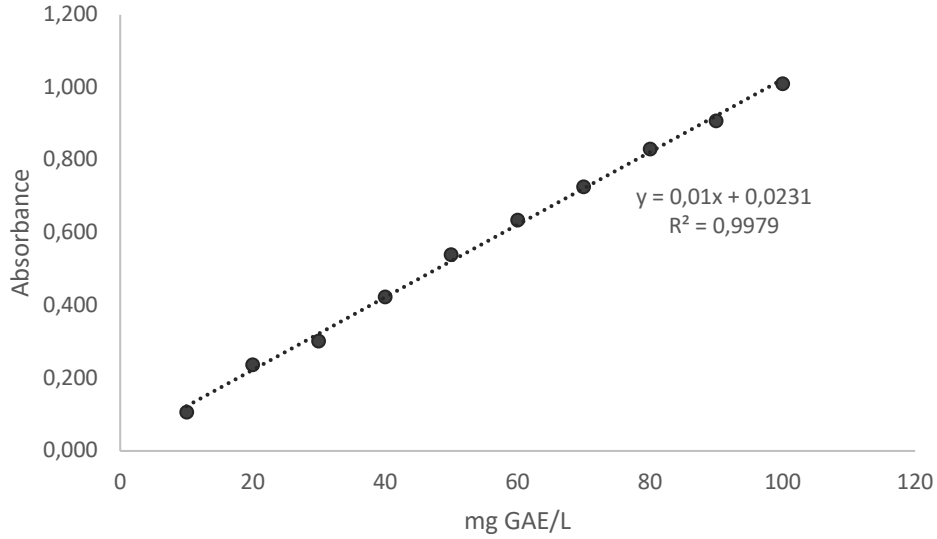

Figure 6.1 Calibration curve prepared by gallic acid in ethanol:water mixture (50:50 v/v) for determination of total phenolic contents

$$Absorbance\ (760\ nm) = 0.01 * \left(mg\frac{GAE}{L}\right) + 0{,}0231$$

$$R^2 = 0.9979$$

B. Optical Images of Double Emulsions

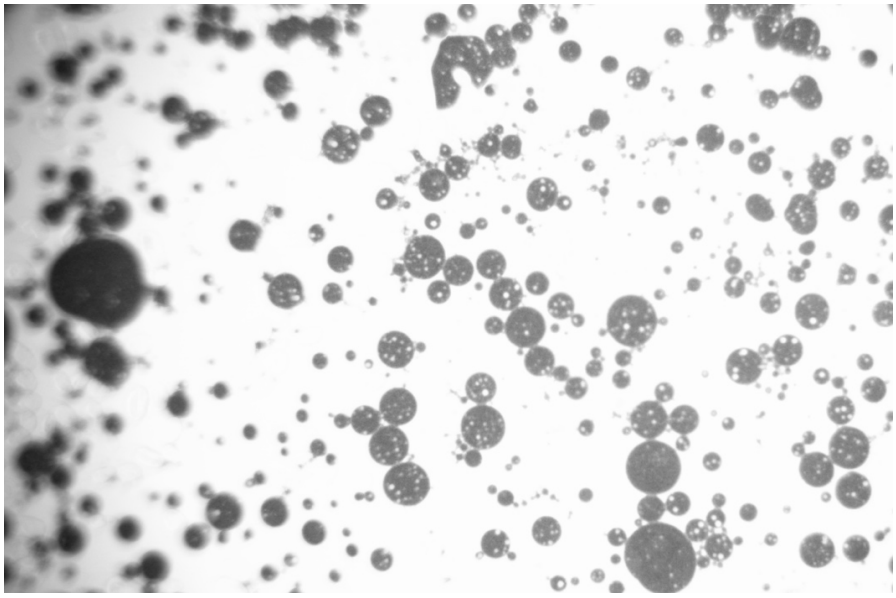

Figure 6.2 Optical image of double emulsion prepared using HSH and with 15% flour (taken with 10x lens)

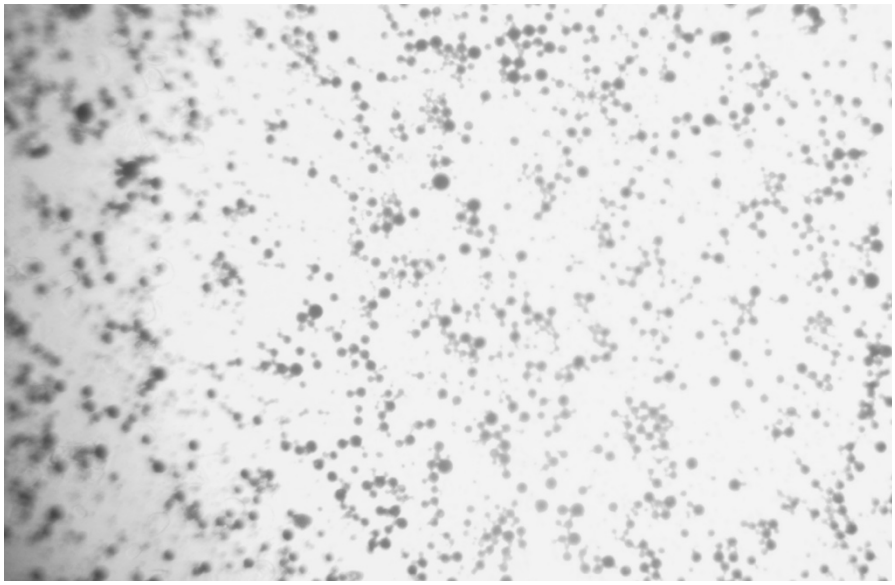

Figure 6.3 Optical image of double emulsion prepared using HSH and with 20% flour (taken with 10x lens)

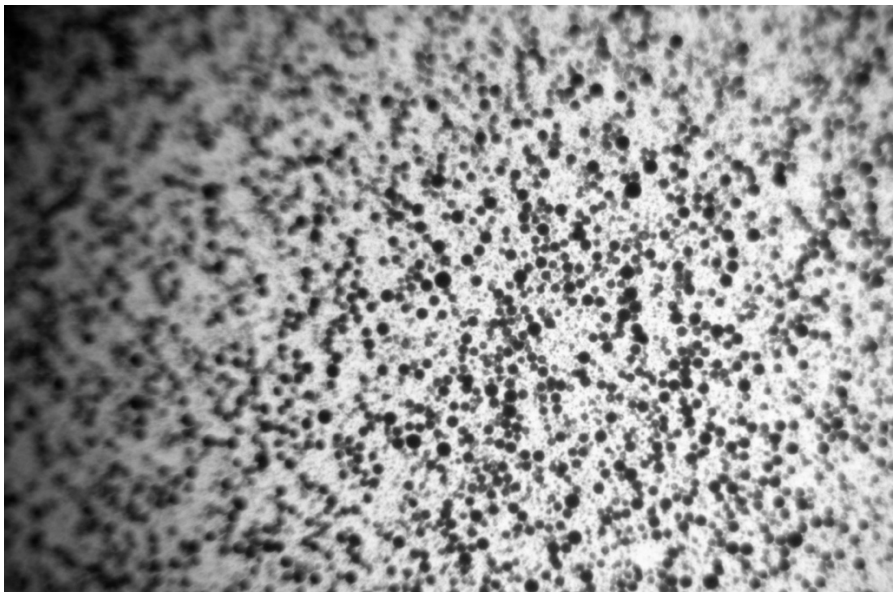

Figure 6.4 Optical image of double emulsion prepared using HSH and with 25% flour (taken with 10x lens)

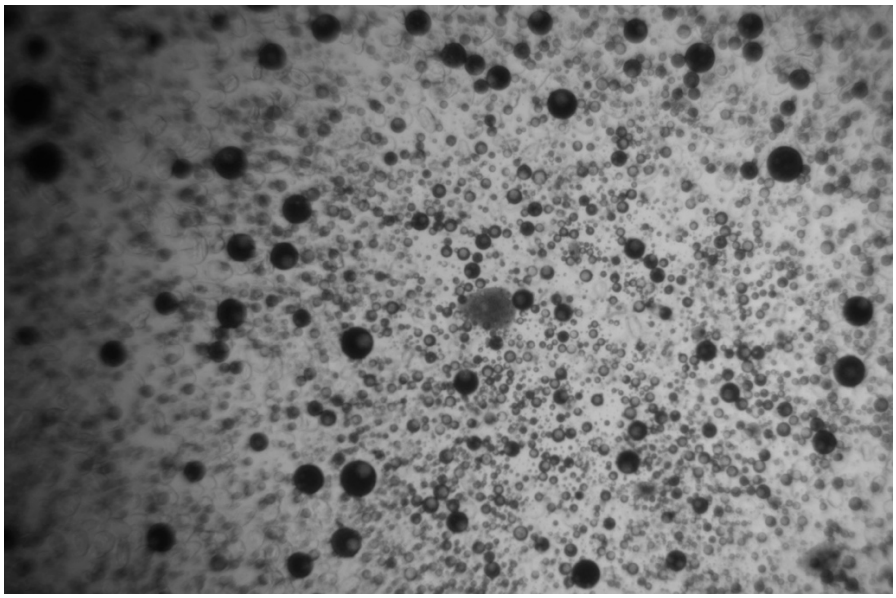

Figure 6.5 Optical image of double emulsion prepared using US and with 15% flour (taken with 10x lens)

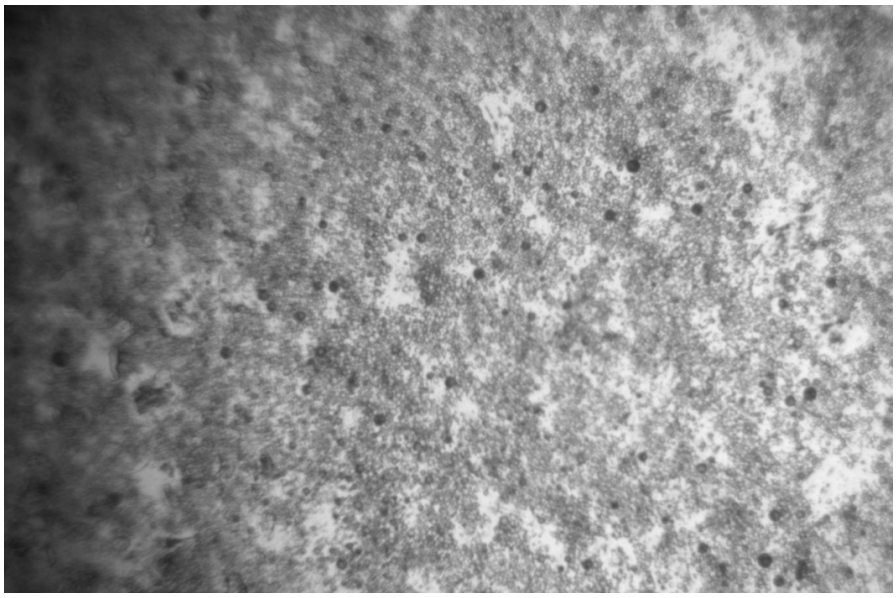

Figure 6.6 Optical image of double emulsion prepared using US and with 20% flour (taken with 10x lens)

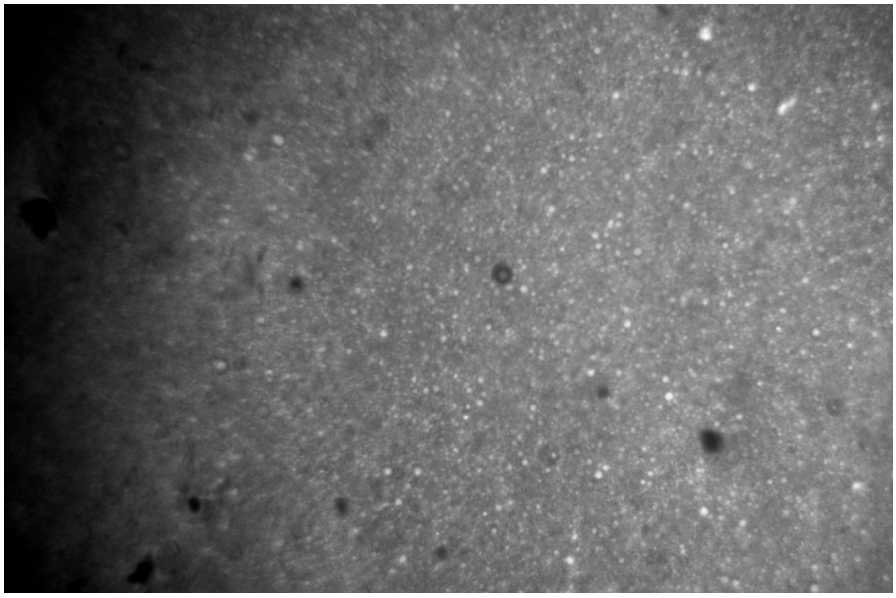

Figure 6.7 Optical image of double emulsion prepared using US and with 25% flour (taken with 10x lens)

C. Statistical Analysis

Table 6.1 Two-way ANOVA and Tukey's comparison test for surface mean diameter (D3,2) of double emulsions prepared by HSH and UH; for 15%, 20% and 25% pea flour concentrations

General Linear Model: D3,2 versus method, concentration

Factor Information

Factor	Type	Levels	Values
method	Fixed	2	HSH, US
concentration	Fixed	3	15, 20, 25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
method	1	2.283	2.2834	10.40	0.018
concentration	2	71.971	35.9856	163.97	0.000
method*concentration	2	1.884	0.9420	4.29	0.070
Error	6	1.317	0.2195		
Total	11	77.455			

Comparisons for D3,2

Tukey Pairwise Comparisons: method

method	N	Mean	Grouping
HSH	6	9.47576	A
US	6	8.60333	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: concentration

concentration	N	Mean	Grouping	
15	4	12.4506	A	
20	4	7.8534	B	
25	4	6.8146		C

Means that do not share a letter are significantly different.

Table 6.1 (Continued)

Tukey Pairwise Comparisons: method*concentration

method*concentration	N	Mean	Grouping		
HSH 15	2	12.7563	A		
US 15	2	12.1450	A		
HSH 20	2	8.8269		B	
US 20	2	6.8800			C
HSH 25	2	6.8442			C
US 25	2	6.7850			C

Means that do not share a letter are significantly different.

Table 6.2 Two-way ANOVA and Tukey's comparison test for volume mean diameter (D4,3) of double emulsions prepared by HSH and UH; for 15%, 20% and 25% pea flour concentrations

General Linear Model: D4,3 versus method, concentration

Factor Information

Factor	Type	Levels	Values
method	Fixed	2	HSH, US
concentration	Fixed	3	15, 20, 25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
method	1	79.93	79.928	7.68	0.032
concentration	2	837.10	418.549	40.21	0.000
method*concentration	2	18.83	9.413	0.90	0.454
Error	6	62.46	10.410		
Total	11	998.31			

Table 6.2 (Continued)

Comparisons for D4,3

Tukey Pairwise Comparisons: method

method	N	Mean	Grouping
HSH	6	59.3050	A
US	6	54.1433	B

Tukey Pairwise Comparisons: concentration

concentration	N	Mean	Grouping		
15	4	67.5750	A		
20	4	55.3400		B	
25	4	47.2575			C

Tukey Pairwise Comparisons: method*concentration

method*concentration	N	Mean	Grouping		
HSH 15	2	68.855	A		
US 15	2	66.295	A		
HSH 20	2	57.530	A	B	
US 20	2	53.150		B	C
HSH 25	2	51.530		B	C
US 25	2	42.985			C

Means that do not share a letter are significantly different.

Table 6.3 Two-way ANOVA and Tukey's comparison test for D_{50} value of double emulsions prepared by HSH and UH; for 15%, 20% and 25% pea flour concentrations

General Linear Model: D50 versus method, concentration

Factor Information

Factor	Type	Levels	Values
method	Fixed	2	HSH, US
concentration	Fixed	3	15, 20, 25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
method	1	129.90	129.90	6.22	0.047
concentration	2	645.36	322.68	15.44	0.004
method*concentration	2	39.81	19.91	0.95	0.437
Error	6	125.37	20.90		
Total	11	940.45			

Comparisons for D50

Tukey Pairwise Comparisons: method

method	N	Mean	Grouping
HSH	6	33.1440	A
US	6	26.5637	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: concentration

concentration	N	Mean	Grouping
15	4	40.1650	A
20	4	25.6630	B
25	4	23.7335	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: method*concentration

method*concentration	N	Mean	Grouping	
HSH 15	2	43.7450	A	
US 15	2	36.5850	A	B
HSH 20	2	31.0250	A	B
HSH 25	2	24.6621		B
US 25	2	22.8050		B
US 20	2	20.3010		B

Means that do not share a letter are significantly different.

Table 6.4 One-way ANOVA and Tukey's comparison test for D_{90} value of double emulsions prepared by HSH and UH; for 15%, 20% and 25% pea flour concentrations

One-way ANOVA: D90 versus method

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
method	2	HSH, US

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
method	1	6366	6366.0	14.99	0.003
Error	10	4246	424.6		
Total	11	10612			

Table 6.4 (Continued)

Tukey Pairwise Comparisons

method	N	Mean	Grouping
HSH	6	142.7	A
US	6	96.60	B

Means that do not share a letter are significantly different.

Table 6.5 Two-way ANOVA and Tukey's comparison test for flow behaviour index of double emulsions prepared by HSH and UH; for 15%, 20% and 30% pea flour concentrations

General Linear Model: n versus method, concentration

Factor	Type	Levels	Values
method	Fixed	2	HSH, US
concentration	Fixed	3	15, 20, 25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
method	1	0.004028	0.004028	10.34	0.018
concentration	2	0.108868	0.054434	139.73	0.000
method*concentration	2	0.006429	0.003214	8.25	0.019
Error	6	0.002337	0.000390		
Total	11	0.121663			

Comparisons for n

Tukey Pairwise Comparisons: method

method	N	Mean	Grouping
US	6	0.657150	A
HSH	6	0.620506	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: concentration

concentration	N	Mean	Grouping		
15	4	0.745225	A		
20	4	0.657171		B	
25	4	0.514088			C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: method*concentration

method*concentration	N	Mean	Grouping		
US 15	2	0.768600	A		
HSH 15	2	0.721850	A		
US 20	2	0.700975	A		
HSH 20	2	0.613367		B	
HSH 25	2	0.526300			C
US 25	2	0.501875			C

Means that do not share a letter are significantly different.

Table 6.6 Two-way ANOVA and Tukey's comparison test for consistency index of double emulsions prepared by HSH and UH; for 15%, 20% and 30% pea flour concentrations

General Linear Model: k versus method, concentration

Factor	Type	Levels	Values
method	Fixed	2	HSH, US
concentration	Fixed	3	15, 20, 25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
method	1	0.136	0.136	0.53	0.494
concentration	2	256.098	128.049	499.57	0.000
method*concentration	2	5.815	2.907	11.34	0.009
Error	6	1.538	0.256		
Total	11	263.586			

Comparisons for k

Tukey Pairwise Comparisons: method

method	N	Mean	Grouping
HSH	6	4.85752	A
US	6	4.64465	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: concentration

concentration	N	Mean	Grouping	
25	4	11.2085	A	
20	4	2.3816	B	
15	4	0.6631		C

Means that do not share a letter are significantly different.

Table 6.6 (Continued)

Tukey Pairwise Comparisons: method*concentration

method*concentration	N	Mean	Grouping		
US 25	2	12.0279	A		
HSH 25	2	10.3891	A		
HSH 20	2	3.2407		B	
US 20	2	1.5225		B	C
HSH 15	2	0.9427			C
US 15	2	0.3835			C

Means that do not share a letter are significantly different.

Table 6.7 Two-way ANOVA and Tukey's comparison test for apparent viscosity of double emulsions prepared by HSH and UH; for 15%, 20% and 30% pea flour concentrations

General Linear Model: Apparent Viscosity versus Method, Concentration

Factor	Type	Levels	Values
Method	Fixed	2	HSH, US
Concentration	Fixed	3	15, 20, 25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Method	1	0.02939	0.029387	6.46	0.044
Concentration	2	1.49632	0.748162	164.36	0.000
Method*Concentration	2	0.00393	0.001967	0.43	0.668
Error	6	0.02731	0.004552		
Total	11	1.55695			

Table 6.7 (Continued)

Comparisons for Apparent Viscosity

Tukey Pairwise Comparisons: Method

Method	N	Mean	Grouping
HSH	6	0.576858	A
US	6	0.477886	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration

Concentration	N	Mean	Grouping
25	4	1.00937	A
20	4	0.39949	B
15	4	0.17325	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Method*Concentration

Method*Concentration	N	Mean	Grouping	
HSH 25	2	1.03390	A	
US 25	2	0.98485	A	
HSH 20	2	0.46640	B	
US 20	2	0.33258	B	C
HSH 15	2	0.23028	B	C
US 15	2	0.11623		C

Means that do not share a letter are significantly different

Table 6.8 Correlation coefficients and p values of instant stability versus consistency index (k) and average particle diameter ($D_{3,2}$)

Pairwise Pearson Correlations

Sample 1	Sample 2	Correlation	95% CI for ρ	P-Value
k	Instant stability	0.995	(0.955, 1.000)	0.000

Sample 1	Sample 2	Correlation	95% CI for ρ	P-Value
D3,2	Instant stability	-0.823	(-0.980, -0.033)	0.044

Table 6.9 Correlation coefficients and p values of instant stability versus long-term stability at 4°C and 20°C

Pairwise Pearson Correlations

Sample 1	Sample 2	Correlation	95% CI for ρ	P-Value
storage stability (4°)	Instant Stability	0.945	(0.812, 0.985)	0.000

Sample 1	Sample 2	Correlation	95% CI for ρ	P-Value
storage stability (20°)	Instant Stability	0.767	(0.344, 0.931)	0.004

Table 6.10 Two-way ANOVA and Tukey's comparison test for instant stability of double emulsions prepared by HSH and UH; for 15%, 20% and 30% pea flour concentrations

General Linear Model: Instant Stability vs Method, Concentration

Factor Information

Factor	Type	Levels	Values
Method	Fixed	2	HSH, US
Concentration	Fixed	3	15, 20, 25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Method	1	0.16	0.156	0.10	0.759
Concentration	2	1687.81	843.904	555.44	0.000
Method*Concentration	2	7.73	3.867	2.54	0.158
Error	6	9.12	1.519		
Total	11	1704.81			

Comparisons for Instant Stability

Tukey Pairwise Comparisons: Method

Method	N	Mean	Grouping
HSH	6	51.9333	A
US	6	51.7052	A

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration

Concentration	N	Mean	Grouping
25	4	68.3453	A
20	4	46.0350	B
15	4	41.0775	C

Means that do not share a letter are significantly different.

Table 6.10 (Continued)

Tukey Pairwise Comparisons: Method*Concentration

Method*Concentration	N	Mean	Grouping	
US 25	2	69.3555	A	
HSH 25	2	67.3350	A	
HSH 20	2	46.5750	B	
US 20	2	45.4950	B	
HSH 15	2	41.8900	B	C
US 15	2	40.2650		C

Means that do not share a letter are significantly different.

Table 6.11 Correlation coefficients and p values of stability versus consistency index (k), apparent viscosity and average particle diameter ($D_{3,2}$)

Pairwise Pearson Correlations

Sample 1	Sample 2	Correlation	95% CI for ρ	P-Value
k	storage stability (4°)	0.950	(0.602, 0.995)	0.004

Sample 1	Sample 2	Correlation	95% CI for ρ	P-Value
apparent viscosity	storage stability (4°)	0.984	(0.859, 0.998)	0.000

Sample 1	Sample 2	Correlation	95% CI for ρ	P-Value
D3,2	storage stability (4°)	-0.902	(-0.989, -0.336)	0.014

Table 6.12 Two-way ANOVA and Tukey's comparison test for encapsulation efficiency (EE%) of double emulsions prepared by HSH and UH; for 15%, 20% and 25% pea flour concentrations

General Linear Model: EE (%) versus Method; Concentration

Factor Information

Factor	Type	Levels	Values
Method	Fixed	2	HSH; US
Concentration	Fixed	3	15; 20; 25

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Method	1	16,8270	16,8270	80,67	0,000
Concentration	2	15,7598	7,8799	37,78	0,000
Method*Concentration	2	0,7393	0,3696	1,77	0,248
Error	6	1,2516	0,2086		
Total	11	34,5776			

Comparisons for EE (%)

Tukey Pairwise Comparisons: Method

Method	N	Mean	Grouping
HSH	6	88,2167	A
US	6	85,8483	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Concentration

Concentration	N	Mean	Grouping
15	4	88,2175	A
20	4	87,3975	A
25	4	85,4825	B

Means that do not share a letter are significantly different.

Table 6.12 (Continued)

Tukey Pairwise Comparisons: Method*Concentration

Method*Concentration	N	Mean	Grouping		
HSH 15	2	89,730	A		
HSH 20	2	88,525	A		
US 15	2	86,705		B	
HSH 25	2	86,395		B	
US 20	2	86,270		B	C
US 25	2	84,570			C

Means that do not share a letter are significantly different.

Table 6.13 Two-way ANOVA and Tukey's comparison test for release (%) of double emulsions prepared by HSH and 25% flour

One-way ANOVA: RR (%) versus Days

Factor Information

Factor	Levels	Values
Days	5	1, 4, 7, 10, 13

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Days	4	46.313	11.5782	21.10	0.002
Error	5	2.743	0.5486		
Total	9	49.056			

Tukey Pairwise Comparisons

Days	N	Mean	Grouping		
13	2	24.645	A		
10	2	22.595	A	B	
7	2	21.430		B	
4	2	20.115		B	C
1	2	18.310			C

Means that do not share a letter are significantly different.