JUNE 2020

M.Sc. in Food Engineering

HATİCE TUBA KÖKLÜ

REPUBLIC OF TURKEY

GAZİANTEP UNIVERSITY

GRADUATE SCHOOL OF NATURAL & APPLIED SCIENCES

RASPBERRY SEED OIL ENCAPSULATION BY COMPLEX COACERVATION OF HETEROPROTEIN

M.Sc. THESIS

IN

FOOD ENGINEERING

BY

HATİCE TUBA KÖKLÜ

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Supervisor

Prof. Dr. Şenol İBANOĞLU

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FOOD ENGINEERING

Name of the Thesis: Raspberry Seed Oil Encapsulation by Complex Coacervation of Heteroprotein

Name of the Student: Hatice Tuba KÖKLÜ

Exam Date: 04.06.2020

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This is to certify that we have read this thesis and that in our consensus opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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Hatice Tuba KÖKLÜ

ABSTRACT

RASPBERRY SEED OIL ENCAPSULATION BY COMPLEX COACERVATION OF HETEROPROTEİN

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M.Sc. in Food Engineering
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June 2020
85 pages

In this study, the encapsulation of raspberry seed oil (RSO) which has a high amount of Omega 3 and 6 fatty acids by heteroprotein complexes between the pea protein isolate (PPI) and Lactoferrin (LF) was investigated. In the first part of the thesis, main goal was increasing the solubility of the pea protein isolate (PPI). The solubility of the pea protein isolate was increased by enzyme hydrolysis. For this purpose, different amounts of enzymes and different hydrolysis times were used for the hydrolysis. Hydrolysis times were chosen for 5,10,15,30 and 60 minutes. The enzyme/substrate ratios were chosen as 1:250 ($HPPI_{250}$), 1:1000 ($HPPI_{1000}$), 1:2000 ($HPPI_{2000}$), 1:3000 ($HPPI_{3000}$) and 1:4000 ($HPPI_{4000}$). In the second part of the thesis, the formation of complex coacervate was examined between two oppositely charged globular proteins, lactoferrin and hydrolyzed pea protein isolate. The effect of biopolymer ratio, pH, ionic strength and temperature on complex formation was investigated. Optimum emulsion model was prepared with 10% oil (wt%) and 1% protein (wt%). In the third part of this thesis, emulsions were freeze dried to evaluate encapsulation efficiency, moisture content and color properties. The results revealed that the hydrolyzed of pea protein isolate with enzyme cause a significant increase effect on solubility. It was decided that the different amounts of enzymes and different hydrolysis times were used for the hydrolysis significantly affect the degree of hydrolysis. Capsules produced at optimized conditions with RSO was evaluated for their properties and it was found that heteroprotein coacervates can be used to encapsulate omega-rich oil.

Key Words: Lactoferrin, Pea Protein Isolate, Raspberry Seed Oil, Complex Coacervation, Encapsulation

ÖZET

FRAMBUAZ ÇEKİRDEĞİ YAĞININ PROTEİN-PROTEİN KOMPLEKSLERİ İLE KAPSÜLLENMESİ

KÖKLÜ, Hatice Tuba
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Haziran 2020
85 sayfa

Bu çalışmada, bezelye proteini izolatı (PPI) ve Lactoferrin (LF) arasındaki heteroprotein kompleksleri tarafından yüksek miktarda Omega 3 ve 6 yağ asidine sahip frambuaz çekirdeği yağı (FÇY) kapsüllenmesi araştırılmıştır. Tezin ilk bölümünde, bezelye proteini izolatının çözünürlüğünün arttırılması amaçlanmıştır. Bezelye proteini izolatının çözünürlüğü enzim hidrolizi ile artırılmıştır. Hidroliz için farklı miktarlarda enzimler ve farklı hidroliz süreleri kullanılmıştır. Hidrolize zamanları 5,10,15,30 ve 60 dakika olarak seçilmiştir. Enzim/substrat oranları 1:250 ($HPPI_{250}$), 1:1000 ($HPPI_{1000}$), 1:2000 ($HPPI_{2000}$), 1:3000 ($HPPI_{3000}$) ve 1:4000 ($HPPI_{4000}$) olarak seçilmiştir. Tezin ikinci bölümünde, birbirine zıt yüklü iki küresel protein, laktoferrin (LF) ve hidrolize bezelye proteini izolatı (HPPI) arasında kompleks koaservat oluşumu incelenmiştir. Biyopolimer oranı, pH, iyonik güç ve sıcaklığın kompleks oluşumu üzerindeki etkisi araştırılmıştır. Optimum emülsiyon modeli, %10 yağ ve %10 protein (% ağırlıkça) ile hazırlanmıştır. Bu tezin üçüncü bölümünde, kapsülleme verimliliği, nem içeriği ve renk karakteristiklerini değerlendirmek için emülsiyonlar dondurularak kurutulmuştur. Sonuçlar, bezelye proteini izolatının enzim ile hidrolize edilmesinin, çözünürlük üzerinde önemli bir artış etkisine neden olduğunu göstermiştir. Hidroliz için farklı miktarlarda enzimlerin ve farklı hidroliz sürelerinin kullanılmasına, hidroliz derecesini önemli ölçüde etkilediğine karar verilmiştir. FÇY ile optimize edilmiş koşullarda üretilen kapsüller özellikleri açısından değerlendirilmiş ve heteroprotein koaservatların omega açısından zengin yağı kapsüllemek için kullanılabileceği anlaşılmıştır.

Anahtar Kelimeler: Laktoferrin, Bezelye Proteini İzolatı, Frambuaz Çekirdeği Yağı, Kompleks Koaservasyon, Enkapsülasyon

"I would like to dedicate this thesis, to women who don't have the opportunity for education. "

ACKNOWLEDGEMENTS

I would like to express my gratitude to Prof. Dr. Şenol İBANOĞLU, a valuable consultant who will give me a different perspective on the field of food sciences, I am proud to be a student and will guide me through my academic career. I would like to express that I consider myself lucky to work under his guidance.

I would like to express my deepest gratitude to Prof. Dr. Esra İBANOĞLU for her guidance, advice, criticism, encouragements and insight throughout this study.

I would like to thank to my dear advisor, Dr. Eda ADAL who contributed to my academic career, excellent advice to enable me to progress through my study.

I also express my gratitude to TUBITAK (Project No: 1180297) for the scholarship from 1001- Supporting Scientific and Technological Research Projects.

I would like to acknowledge the staff of the Food Engineering Department, the University of Gaziantep for technical and administrative support and special thanks to Food Engineer Nazan ÇAĞLAR and Ph.D. Student Zeynep Tuğba ÖZASLAN for their help about studies and life.

I would like to special thanks to my parents, my brothers, my sisters for their encouragement, patience, and trust during my whole life and also especially thanks to beloved Ahmet BAŞ who encouraged me to pursue my dreams and finish my dissertation.

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CHAPTER I

INTRODUCTION

Sensitivity and consciousness about healthy eating habits have increased significantly. The demographic changes and socio-economic change of the populations in the world and in Turkey show the need for healthy and easy to prepare food. In line with all these changes, scientific research has shifted to the search and development of foods that are defined as " functional foods " that contain natural bioactive components. The studies on functional foods that are rich in phytochemicals and antioxidants and contain certain minerals, vitamins, fatty acids and dietary fibers have started.

Essential fatty acids are fatty acids that are necessary for human life, which can not be synthesized in the body and must be taken by diet. There are two types of essential fatty acids; Omega 3 (alpha-linolenic acid) and Omega 6 (linoleic acid) fatty acids, which are known for their beneficial and protective properties for human health. Today, oils containing Omega 3 and 6 fatty acids are directly involved in food. It is stated that raspberry seed oils are beneficial for health due to the omega-3 (ω-3), omega-6 (ω-6) and beneficial sterols (Yang et al., 2011). According to researches, omega-3 and 6 polyunsaturated fatty acids have been shown to be useful in reducing heart disease, preventing cancer, strengthening the immune system and treating many neurological diseases such as Alzheimer's (Abeywardena and Head, 2001; Riediger et al., 2009; Weitz et al., 2010; Wendel and Heller, 2009). Omega fatty acids are not synthesized in the human body and must be taken from outside. The food and agriculture sectors produce large quantities of by-products around the world. This waste economy creates serious problems in agriculture dependent countries. When fruit juice, jam, marmalade, jelly, sugar, cake are produced from raspberry-like fruits, fruit seeds generally remain as waste. Researches indicate that these kernels contain omega fatty acids and bioactive substances that are beneficial for health (Bada et al., 2014; Yılmaz & Gökmen, 2013). Bada et al. (2014) found the raspberry seed oil was found to contain 54.27% linoleic (omega-6) 26.68% α-linolenic (omega-3) fatty acids. In addition, it is stated raspberry seed oil contains beneficial sterols (Van Hoed et al.,

2009). Raspberry seed oils which are sensitive to light, heat, and moisture undergo oxidation during storage and processing of food products. Encapsulation is one of the most common methods used to partially prevent this problem of oils, to give a new form (i.e. powder) and to increase its functionality for use in food products. Encapsulation is a method of protecting the bioactive substances by forming a barrier between the encapsulated substance and the environment. Since the oils rich in omega fatty acids used in this study are sensitive to environmental factors (heat, temperature, oxygen, etc.) they may be damaged during other methods than coacervation. In addition, according to researches, higher products and more encapsulated capsules provide more durable capsules. For this reason, it is stated that the coacervation process is more effective than other methods in the encapsulation of oils rich in omega fatty acids (Kaushik et al., 2015; Bakry et al., 2016).

According to the literature review, spray drying, freeze-drying, extrusion, fluidized bed, co-crystallization and coacervation are the most common methods used to encapsulate food ingredients. Coacervation is a physical method used in the encapsulation of bioactive substances such as omega fatty acids. Coacervation is a term used in colloidal chemistry to indicate the unifying phase separation process induced by modification of the medium (i.e. pH, ionic strength, temperature, solubility) under controlled conditions. In this process, while the colloid-rich phase is known as the coacervate phase, those containing a very small amount of colloids are known as the balance phase (Nairm, 1995). Depending on the polymer systems involved in the reaction and the phase separation mechanism, namely two types of coacervation processes: these are simple and complex coacervations. In a simple coacervation process, a single polymer forms a coacervate. It is caused by dehydration or "lack of water" mechanism caused by the addition of a salt or desolvation fluid or inducing agent. On the other hand, in complex coacervation processes, two or more opposite-charged polymers lead to the coacervate formation and phase separation by ionic interactions (Newton, 1991; Bachtsi et al., 1996). The main factor for complex coacervation is the electrostatic interaction between charged macromolecules found in the reaction medium. However, van der Waals intermolecular force, hydrophobic interaction in dipolar proteins and nucleic acid conformational changes due to intermolecular hydrogen bonding also play an important role in the complex coacervation mechanism (Schmitt et al., 2011; Turgeon

et al., 2007; Xiao et al., 2014). Complex coacervation is a simple, low-cost method that can provide very high payload and provide high encapsulation efficiency (Gosh, 2006; Specos, et al., 2010; Drusch et al., 2012). Biopolymer type and size, mixing rates, total biopolymer concentration, electrical charge density and solvent conditions (i.e. pH, ionic strength, temperature, mixing and pressure) are the factors affecting coacervation. Complexes are aggregated to reduce the free energy of the system until its dimensions and surface properties lead to degradation and liquid-liquid phase separation (Schmitt and Turgeon 2011). The coacervate formation is determined by numerous non-covalent and H-binding interactions.

Gelatin, whey proteins, casein, and caseinates are generally preferred in the encapsulation process as a coating material. Gelatin, emulsion and the film-forming feature is a good coating material for microencapsulation by spray drying method due to its high water solubility and edible properties. The properties and morphologies of gelatin microparticles can be improved by the addition of sugar alcohol mannitol (Bruschi et al., 2003). It has been preferred as gelatin-coating material in the microencapsulation of lemon (Kaushik and Roos, 2006), phospholipids (Caiyuan et al., 2007), lycopene (Chiu et al., 2007) and fish oil (Curtis et al., 2008). The use of whey proteins as a coating material in encapsulation dates back to the 1990s (Moreau and Rosenberg, 1993; Young et al., 1993). Whey proteins were preferred as a coating material in the encapsulation of milk fat and provided an encapsulation yield of approximately 90% (Young et al., 1993). The first study on complex coacervation in the literature is Bungenberg de Jong et al. (1929) by the gelatin and gum systems. Generally, complex coacervation studies have been among protein polysaccharides: β-lactoglobulin-acacia gum (Schmitt et al., 1999), whey powder protein-Arabic gum (Weinbreck et al., 2003), vegetable protein-Arabic gum (Ducel et al., 2005), gelatin-sodium montmorillonite (Qazvini et al., 2012), gelatin-κ carrageenan (Fang et al., 2006), gum arabic-cytosine (Espinosa-Andrews et al., 2007). In addition, mixtures of protein-polyelectrolyte (Wang et al., 2000) and polyelectrolyte-micelle (Leisner and Imae, 2003) were used. However, protein-protein complex coacervation, which is more functional and biologically effective, has just been explored and very limited information is available on this subject (Yan et al., 2013; Flanagan et al., 2015; Tavares et al., 2015; Anema and Kruif, 2016). Complex coacervation usually occurs at low ionic strength and within the pH range that corresponds to the isoelectric pH of proteins

(Croguennec et al., 2017; Desfougères et al., 2010). Encapsulation with complex coacervation usually occurs in 4 stages. These stages are emulsion formation, complexing of polymers, coating formation, and encapsulated formation. This method has been used to encapsulate omega-3-rich oils (Kaushik et al., 2015; Wang et al., 2014) and essential oils (Jun Xia et al., 2011; Xiao et al., 2014). As a result of the preliminary studies, it has been observed that coacervate forms between lactoferrin and pea protein isolate, and with the following, the main factors affecting coacervation (i.e.biopolymer ratio, pH, ionic strength, temperature) (Adal et al., 2017).

Lactoferrin (LF) is a metal-binding glycoprotein with a molar mass of 89 kDa with an isoelectric point (pI) of ~ 8.7. One of its most important features is that it is positively charged in a wide pH range. LF has been commercially isolated from cow milk in recent years as a multi-functional additive due to its antibiotic, anticancer and immune system strengthening properties. It is also reported that it can inhibit pathogenic bacteria including *Escherichia coli*, *Staphylococcus pneumonia, Salmonella typhimurium, Shigella dysenteriae* and even some viruses (Bokkhim et al., 2015; Bengoechea et al., 2011). Lactoferrin which is also found in the mother's milk is a glycoprotein known to have antimicrobial property. Emulsifying properties of LF can be improved by forming conjugates or complexes with proteins, polysaccharides or polyphenols (Yang et al., 2017; Lesmes et al., 2010). Cationic LF and β-lactoglobulin (Flanagan et al, 2015; Kizilay et al, 2014; Yan et al, 2013) and anionic proteins such as casein (Anema and Kruif, 2012), pea protein isolate (Adal et al., 2017) and osteopontin (Yamniuk et al., 2009) heteroprotein complex coacervation among these proteins includes recent studies.

While the need for protein has been met mostly from animal protein, the need for vegetable proteins has increased in recent years due to the increase in obesity, diseases of animal origin and animals fed with antibiotics (Aiking, 2011). Apart from containing high quality protein, animal protein sources contain high levels of components such as cholesterol and saturated fatty acids that cause diseases such as heart and vascular diseases, cancer, when consumed too often. In addition, the fact that vegetable proteins preferred by private consumer groups such as vegan and vegetarian are cheaper and have a wide variety of resources, making it an alternative source of protein for vegetable proteins to be used in food applications (Asgar et al., 2010). Vegetable proteins are produced as protei isolates (protein content 90% and above) or

concentrate (protein content 48─70%) for use in food applications (Sari et al., 2015; Moure et al., 2006). Legumes (peas, chickpeas, broad beans, lentils) have a protein content richer than cereals with 20─30% protein content. However, legume proteins display a balanced amino acid profile with high amounts of lysine, leucine, aspartic acid, glutamic acid and arginine (Miñarro et al., 2012). The most commonly used vegetable protein in legumes is pea protein. Peas are rich in protein and carbohydrates, but contain a relatively high concentration of insoluble dietary fiber and low levels of fat.

Pea (*Pisum sativum Leguminosae*) proteins have been very popular in recent years due to its nutritional and functional properties at low cost. According to the American Food and Drug Administration (FDA), it does not contain any allergens, so it can be easily used instead of allergic vegetable and animal-derived proteins (Bajaj et al. 2017). The pea protein consists of two main proteins; legumin and vicilin. Legumin has a molecular weight of 320 kDa while vicilin has a molecular weight of 170 kDa. Pea protein isolate was used directly and its solubility was found to be low (Adal et al., 2017). The isoelectric point of the pea protein isolate is ~ 5.4 (Liang and Tang, 2013). In previous studies, pea protein isolate was used directly and its solubility was found to be low (Adal et al., 2017). There are studies showing that hydrolysis of pea protein with enzyme increases solubility (Tavares et al., 2015; Barac et al., 2011) Research shows that emulsions prepared with hydrolyzed pea protein isolate yield more stable and high-yield encapsulation products (Bajaj et al., 2017).

The first part of this thesis deals with increasing the solubility of the pea protein isolate (PPI). The pea protein was hydrolyzed with trypsin enzyme and used in coacervation to increase this solubility. The low solubility of the pea protein isolate leads to the low efficiency of the emulsions. This hydrolysis process will provide high efficiency of emulsions made using PPI. The fact that the pea protein, which has increased solubility after emulsion, will be used in the formation of coacervation with lactoferrin and increasing the encapsulation efficiency. Required conditions (i.e. pH, biopolymer ratio, ionic strength, temperature) for coacervation formation of lactoferrin (LF) and hydrolyzed pea protein isolate (HPPI) are examined according to particle size, zeta potential, and coacervation efficiency. These parameters are the basic parameters used in coacervation characterization (Weinbreck et al., 2003; Liu et al., 2010; Anema and Kruif, 2016). Most of the previous coacervation studies are between proteins and

polysaccharides. This increases the unique value of this study and sheds light on the work to be done in this field.The lactoferrin-pea protein isolate hydrolyzate (HPPI) complex coacervation encapsulation is of the first study in literature. There is no study that coacervate obtained using HPPI and lactoferrin is used as a wall in the encapsulation of raspberry seed oils. Optimum coacervation conditions obtained from these data were used in emulsion studies.

The purpose of this thesis is to stabilize the emulsions obtained by looking at the droplet size, zeta potential and cream index. There is limited information in the literature about encapsulating of raspberry seed oil by protein-protein coacervation to be applied in our study. Therefore, we believe that results obtained in this thesis will lead to a clearer understanding of the encapsulation of fruit seed oils by protein–protein coacervation.

CHAPTER II

LITERATURE REVIEW

2.1 Encapsulation

Encapsulation is the process of coating bioactive molecules and living cells with another wall material for preserving. Therefore, encapsulation is the process of covering an core material with the wall material. This technique is very diverse in terms of applications. It is widely used in food, chemical, pharmaceutical and cosmetic industries. The first use of capsules dates back to the 1950s. Encapsulated dyes were firstly produced by coacervation of gum arabic and gelatin. According to Barac et al. (2010); Boye et al. (2010) encapsulation is also used in the dairy and pharmaceutical industries.

Capsules are classified size and morphology. Microcapsule morphology is divided into 3 groups; monocored, polycored and matrix species (Dubey et al., 2009). As shown in Figure 2.1, microcapsules have single-core, with single cavity, multicore consists of multiple cavities within the shell and matrix-type capsules contain more than one matrix structure component within the shell. The morphology of the capsules may vary depending on the morphology of the inner structure, the material of the shell and the method of encapsulation.

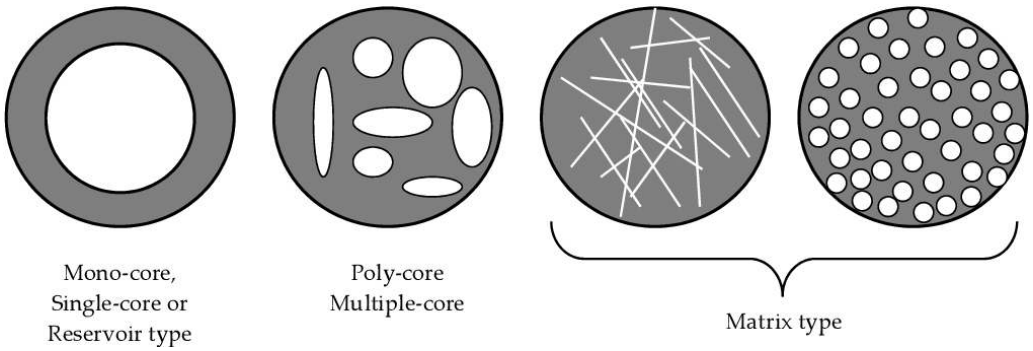


Figure 2.1 Microcapsules with different morphological

Considering the reasons for using the encapsulation technology can protect the sensitive bioactive substances from the environment, that is, from external influences and can also be used to mask the unpleasant taste and smell of bioactive substances. The main purpose of encapsulating foodstuffs is used to stabilize the food ingredients with increased bioavailability. Another reason for the encapsulation of the active ingredients is to protect the products so that they are not affected by any external influences during processing. In addition, the encapsulation of foods containing volatile compounds or aroma compounds prevents evaporation and aroma contents are preserved. Also, the encapsulation process is used to prevent feelings such as unpleasant bitter taste during meals. Another purpose of using encapsulation is to prevent active compound from reacting with these compounds in oxygen and water sensitive food products. Encapsulation also ensures that cells or enzymes remain stable in fermentation or metabolite-producing processes. Due to these benefits, the capsulation process is a system that provides high productivity by improving the quality of food products and there is an increasing demand for studies and solutions in this regard (Nedovic et al., 2011).

In the encapsulation process, the material to be encapsulated must be coated, dissolvable and/or dispersible, the material in solid, liquid phase. Core materials used for food products may be additives, stabilizers, oxidants, high nutritional values preservatives, colourants, cross-linking and setting agents, agents with undesirable flavours and odours, essential oils, amino acids, vitamins, minerals , flavouring agents oils, spices, seasonings and sweeteners. The coating material should be compatible with the core material, capable of holding the core material, well stabilized, not interact with the active substances and should emit in a controlled manner when certain conditions are met. Depending on the material to be physically encapsulated, coating material can be flexible, hard or thin. In addition, easy availability and being cheap are among the criteria of the selected coating material. The coating material can be gums, starch derivatives, dextrin, maltodextrins and alginates and also be protein-based materials such as polypeptone, soy protein, milk-derivatives and gelatin derivatives (Jyothi and Seethadevi et al., 2012).

2.1.1 Encapsulation Methods

The classification of encapsulation techniques depends on the core material and the shell material that will surround that material. The type of treatment that determines the encapsulation techniques is selected according to the material to be encapsulated and the properties of the coating material (de Vos et al., 2010).

2.1.1.1 Spray Drying

Spray drying method is a well known method used in different industries. It is often used to easily convert concentrated liquids into powder. Spray drying has important role in the making of microcapsules, especially bioactive food products and live probiotics. Spray dryer is often used for encapsulation of proteins as such sodium caseinate, whey proteins, gelatin, and gums such as gum arabic and guar gum. Gum arabic is one of the most common material used in encapsulation. Spray drying is also fast and inexpensive compared to other methods. It provides low cost high production rate in a short time (Dordevic et al., 2015). In general, the success of encapsulation depend on the minimum level of oil on the surface of the capsules. The spray drying main aim is to dissolve the inner material in dispersion. Then it is to atomize the dissolved and solvent-containing medium in heated air (160–220ºC). Atomizing increases the separation between solvent (i.e water) and the medium, allowing it to be removed rapidly (Figure 2.2). This process takes a very short time, which ensures that there is no significant loss in heat-sensitive products (de Vos et al., 2010).

Besides the very common use of the spray dryer, it also has some disadvantages. Equal adjustment of the particle size of nonhomogeneous samples is difficult in this method. Also, the difficulty of using the equipment in this technique is one of the reasons. Another main disadvantage is that very high temperature is required to remove water in the system. This situation restricts its use for heat-sensitive materials (J.Risch., 1995).

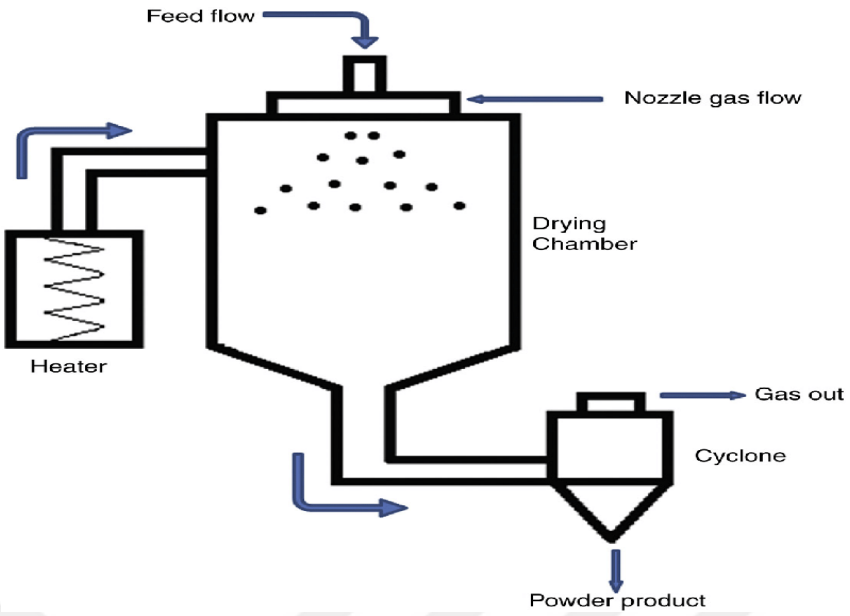


Figure 2.2 Schematic presentation of spray drying

2.1.1.2 Spray Chilling and Spray Cooling

In spray cooling, the core material is distributed with a coating material as in spray drying. In this method, however, the heated mixture is atomized with cooled air. Spray-cooling or spray-chilling are technologies used to produce lipid-based components. The difference between the melting points of lipids determines the difference between spray cooling and spray chilling techniques. In the spray cooling the temperature is in the range of 34-42 °C whatever in the spray cooling temperature rate is higher. Spray cooling is a continuous technique with high efficiency and product capacities.

Spray cooling is mainly used for vitamin-mineral encapsulation. Frozen liquids, especially heat sensitive materials, and materials insoluble in the desired solvent can be encapsulated with this method. In spray cooling, the coating material generally consists of vegetable oils. Some food ingredients that use spray cooling are: bakery products, dry powder soup mixtures and foods with high fat content. These techniques are quite common for use in aroma compounds as well as in dry foods. (F. Gibbs et al., 1999; Dordevic et al., 2015) .

2.1.1.3 Fluidized Bed Coating

Fluidised bed coating method is a powerful tool for coating solid particles with air suspension. In the fluidized bed coating the particles are sprayed with a stream of air. The core material to be coated is placed in a section with high-speed air and circulated there. As it circulates, the coating material is atomized to the core material and is deposited on its surface. Determination of the appropriate coating amount is controlled by the time the particles spent in the chamber (J. Risch 1995). Factors such as the speed of spraying, amount of water, airflow, humidity and temperature of the point where air enters are important for optimization. One of the main problems in this process is the uncontrolled and nonhomogeneous collection of particles. This method allows larger coating materials to be used compared to spray drying. In this method, attention is paid to have the current viscosity, thermal properties and film-forming properties of the coating material. A fluidized bed coating method has been used to encapsulate many minerals and vitamins including iron salts and B and C vitamins (Dordevic et al., 2015).

2.1.1.4 Freeze Drying

Freeze-drying is known as one of the method to achieve a high-quality products. Rodríguez et al. (2016) determined that the freeze drying is more suitable to prevent oxidation than spray drying. The method consists of sublimation in three steps; freezing, primary and secondary drying. The most important goal is the active compound is frozen before dried under a high vacuum. This method provides more protection of products with parameters such as lower oxidation and less chemical interaction due to the use of vacuum and low temperature. This state makes drying methods a sufficient method for drying the heat-sensitive products. Table 2.2 shows the materials used for the freeze drying method (Rodríguez et al., 2016). The main disadvantage of freeze-drying is required high energy and long drying time.

2.1.1.5 Extrusion

The extrusion method is the process of encapsulation of the core material in a carbohydrate melt. The carbohydrate substance is mixed with the core material at a temperature above 100 °C. The mixture is exposed to a dehydration fluid to better capture and coat the core material. The main purpose is that the coating agent hardens

when it comes into contact with the dehydration fluid so that the core material is encapsulated. The advantage of extrusion is that the core material is almost completely covered with the coating material (J. Risch 1995).

The most common material used to make the dehydration and coating more sheltered and tough is isopropyl alcohol. The hardened structure is cut into small pieces and dried. Extrudates can consist of gums, starches, sugars, maltodextrins, proteins, modified starches, emulsifiers and lipids. It is widely used for vitamin C and color compounds. Also, since the encapsulated material is water-soluble, it is highly used in food applications, such as drinks, cakes, gelatin and dessert mixtures (Zuidam and Nedović, 2009).

2.1.1.6 Emulsion

This method consists of two main stages. In the first stage, emulsion is formed primarily with the polymer solution and the aqueous phase. The polymer solution is suspended by mixing it with a non-solvent oil. In the second stage, the solvents are removed and the particles remain. The initial polymer precipitates in the form of a nanocapsule, and the oil remains in the polymer matrix. This method assumed that the encapsulation of both fat and water-soluble food materials. With this method, bioactive compounds such as fish oil, vitamins, fatty acids, omega-3 polyunsaturated fatty acids, phenolics, anthocyanins are encapsulated (Rodríguez et al., 2016).

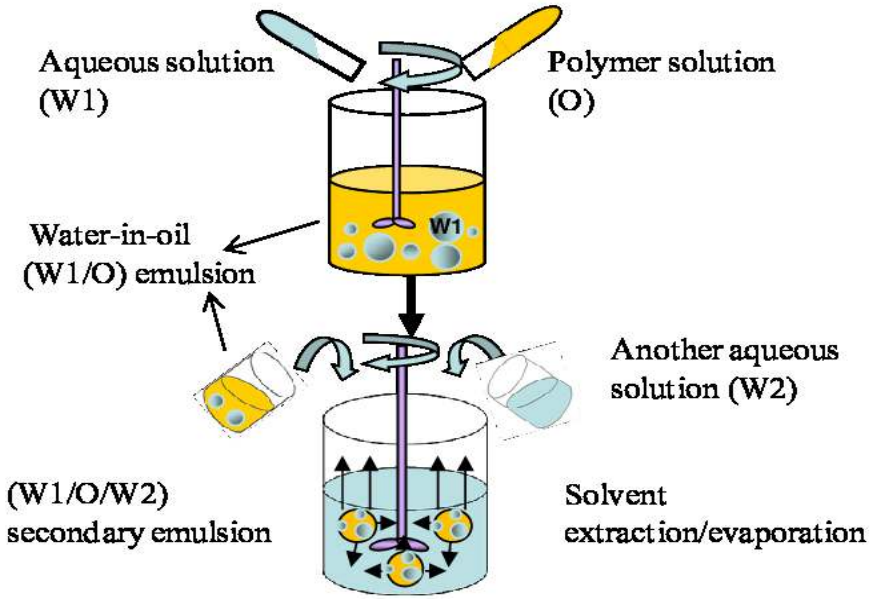


Figure 2.3 Schematic representation of emulsion

2.1.1.7 Coacervation

Coacervation is a phase separation process. There are two types of coacervation namely simple and complex coacervation. In simple coacervation, only one polymer is used, whereas in complex coacervation, two opposed polymers are used. For example, in complex coacervation it is possible to use gelatin (positive charge) or gum arabic (negative charge). The logic is basically the same in both types of coacervation, which is the complete dissolution of the particle in the polymer structure. Firstly, the polymer is dissolved in water and coating material (oil) is dispersed on the prepared suspension. The coating combines with the core material and is stabilized to form microcapsules. The capsules produced with this method are very important for their good release properties and heat resistant properties (Rodríguez et al., 2016).

Coacervation is usually consist of protein and polysaccharide compounds. Protein-protein complexes have been recently studied (Adal et al. 2017; Santos et al. 2018; Zheng et al. 2020). Coacervation is environmentally friendly and it is a very useful method for heat-sensitive materials. The main advantage of coacervation methods encapsulation efficiency can be very high under optimized conditions (Dordevic et al., 2015).

Table 2.1 Encapsulation methods in the food industry (Desai and Jin Park, 2005)

Encapsulation Method	Encapsulation material	Applications
Spray drying	Gum arabic, maltodextrin, modified starch, polysaccharides, proteins	Bioactive food products and live probiotics, flavor compounds, polyphenols and vitamins
Spray chilling and spray cooling	Frozen liquids and especially materials sensitive to heat, vegetable oils.	Flavor compounds, minerals, vitamins and probiotics.
Fluidized bed coating	Gelatin, carbohydrates, lipids	Minerals and iron salts, especially B and C vitamins, color and flavor in meat product, dry sausage products, bakery products
Freeze drying	Sodium caseinate, carboxymethulcelullose maltodextrin, lecithin .	Flavor compounds, fatty acids, probiotics
Extrusion	Carbohydrate matrix with a glassy structure.	Flovor compounds (citrus oil, mint oils, garlic oils, onion oils) vitamins (β-Karoten) and food ingredients (lactic acid, acetic acid)
Emulsion		Bioactive compounds including vitamins, fatty acids, anthocyanins, bioactive extracts, phenolic compounds, oils.
Coacervation	Chitosan, k-carrageenan , gum arabic, gelatin	Fatty acids, flavonoids

2.1.2 Encapsulation Materials

Various materials can be used to encapsulate solids, gases and liquids with different properties. Coating materials is generally biocompatible, food-grade, free of toxic substances providing protection by creating a barrier at the interface of the core material and coating material. The most important step of encapsulation is to choose a suitable wall material that must protect the core material. The wall material should have a protective mechanism against the material to be encapsulated and should cover all the particles of the core material. Coating material is determined by looking at the physical and chemical properties of microcapsules. The coating material; (i) should be compatible with the material to be encapsulated and should form a film to show good encapsulation efficiency; (ii) coating material should not chemically interact with the core material; (iii) the coating material should provide in-demand coating properties such as stability, strength, optical properties, impermeability. It should be paid attention that the cost of the selected material is appropriate, its properties such as taste and smell should not be sharp and should be stable while preserving its properties during storage. Wall materials and encapsulating materials used in food applications are given in (Table 2.2). Maltodextrins, proteins and gum arabic are the most commonly used wall materials. Maltodextrins is generally used in spray drying. Maltodextrin is preferred for its low cost and good encapsulation efficiency. Maltodextrin is used as an alternative wall material enriched by mixing with gum arabic (Vila et al., 2015).

There are functional foods used as the core material in microencapsulation. These include eliminating incompatibility, increasing bioavailability, removing unwanted flavors, etc. Among the foods that are highly encapsulated in the food industry; polyphenols, carotenoids, omega fatty acids, vitamin, protein hydrolysates and peptides (Mohan et al., 2015). Encapsulation is applied for flavoring agents, sweeteners, colorants, acids, bases, preservatives, antioxidants. For example, the encapsulation method is widely used for aspartame and flavors in chewing gum. New research is being done on these foods and new products are being developed and taking their place in the markets due to reasons for reduced cost (Gibbs et al., 2017).

Table 2.2 Commonly used materials and their category of food ingredients

Encapsulation Materials	Category of Food Ingredients	Advantages and Disadvantages
	Polysaccharides	
Gum Arabic Modified Starches Hydrolyzed Starches (Maltodextrins) Alginates Pectin Carrageenan Corn Syrups		Higly stable , plenty of accessibility and cheap costs.High energy to obtain small size capsules
	Proteins	
Gelatin Wheat Protein Casein , Milk Serum Soy Protein Gluten Whey Proteins Other Vegetable Proteins		Nutritional benefits and good emülsification and gelation properties but Core material and shell material can be similarlly.
	Fats	
Hydrogenated Vegetable Oils , Natural Fats, Waxes Lecithin, Liposomes, Static Acid Tristearic Acid, Mono-And Triglycerides		Appropriate for peptides with distinct properties. But easy to lipid oxidation and thermal stability
Chitosan, Cyclodextrins	**Other carbohydrates**	

2.2 Coacervation

The interest in encapsulation techniques in the agriculture, food, cosmetics, pharmacy and printing industries has been increasing. The meaning of the word coacervate is derived from the word “acervus”, which means mass or cluster in Latin. The prefix "co" in front of the word means the colloidal parts merge. In general terms, the term "coacervate" means a stable suspension of ion-rich particles that represent the liquid-liquid phase, electrostatically condensing. The more concentrated part in this phase is the coacervate phase and the other phase is the balanced solution. Another definition of coacervate is the name of the spherical particles formed by the colloidal droplets in the suspension by connecting them with hydrophobic bonds (Devi et al., 2017). Coacervates are a system of colloids, such as oppositely charged proteins and surfactants in an aqueous solution. Tiebackx (1911) first used the term coacervation, but Jong and Kruyt (1929) first investigated the protein/polysaccharide complex coacervation systems. Overbeek and Voorn (1957) improved the model of complex coacervation. Coacervates in coacervation systems are formed by electrostatic forces although they are miscible in the solvent (Eghbal and Choudhary, 2018).

Generally, coacervation processes consist of severel stages and require continuous stirring: (i) formation of chemical phase that does not mix with each other; (ii) collection of coating material; (iii) formation of thick coating. Firstly, a separation technique is applied directly or *in situ* to a three-phase system consisting of a liquid phase, core and coating material. In the direct addition technique, insoluble solutions, such as waxes are added to the coating material, provided that it does not mix with the other phase. In the *in situ* separation technique, the monomer dissolves in the liquid carrier and polymerizes at the interface. The deposition of the liquid polymer coating around the core material, while the coating phase is liquid, the physical mixing of the liquid core material in a controlled manner is a prerequisite for effective coating. Finally, the liquid coating material solidifies and forms microcapsules. The microcapsules formed are taken from the medium by filtration or centrifugation and are dried by appropriate drying techniques, such as spray, fluid bed and freeze-drying (Desai & Jin Park et al., 2005) .In the coacervation method, the core material should not be water-soluble and reactive with the wall material. However, the wall materials gelatin and gum acacia, which are well known as coating materials and whose

structures are well researched, are widely used. Recently, modified coacervation processes have been developed to overcome some problems that may occur with typical gelatin-gum acacia coacervates. For example, encapsulation of heat sensitive essential oils can be performed at room temperature (Desai & Jin Park et al., 2005). Advantages of the coacervation method can be summarized as follow; it is possible to make capsules with a controlled particle size with this system. Also, the shape of the capsules created is quite spherical. The vast majority of the capsules created to provide good protection against external influences. The active material can be released by pressure, hot water or chemical means (Balassa et al., 1971). The disadvantage of the coacervation method is that it is expensive and rather complicated. In addition, glutaraldehyde, which is used to bind wall materials, should be used carefully according to the national legislation. However, this problem can be solved by using enzymatic binders instead of using chemical binders. For example, in a study, the transglutaminase enzyme was used to bind proteins in the shell material. The enzyme was brought to a certain temperature and added to the microencapsulation medium adjusted to pH 7 to form a hardened coacervate shell around the oil droplets (Desai & Jin Park et al., 2005). Schematic demonstration of coacervation is given in (Figure 2.4).

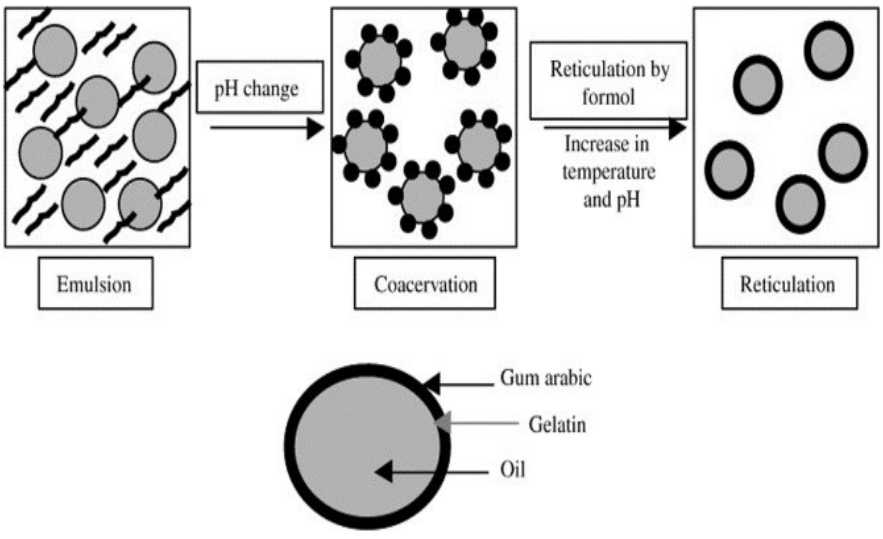


Figure 2.4 Principle of coacervation formation (Eghbal and Choudhary, 2018)

2.2.1. Simple Coacervation

Unlike the complex coacervation, only one polymer is used in simple coacervation, such as gelatin or cellulose. The steps of the simple coacervation method are as follows; (i) the core material is dispersed in one polymer solution; (ii) assembling around the nucleus with the help of hydrophilic structures. (iii) forming gel structure and microcapsule hardening. Simple coacervation is a lower cost and flexible work compared to complex coacervation. However, phase separation the use of only inorganic salts restricts in the simple coacervation. Complex coacervation is more useful in terms of a since phase separation can be made even at low pH (Wu and Xiao, 2005).

2.2.2 Complex Coacervation

Complex coacervation refers to the involvement of the complex between two opposed charged polymers. The basic principle of encapsulation with a complex coacervation method is that the cationic and anionic polymers become water-soluble and form a phase in water. The polymer-rich part covers the particles of the core. Polymers which coating the core materials are selected according to the negative or positive charge state. Negatively charged polymers are polysaccharides such as acacia, pectin, alginate and alginate derivatives, carboxymethyl cellulose and carrageenan. Positive polymers are typically used with proteins such as gelatin, whey proteins and chitosan. The steps for microcapsules produced using the complex coacervation method are given in (Figure 2.5) (Jayanudin et al., 2016).

The most important factors of the complex coacervation process are the pH of the mixture and emulsion size, temperature and the ratio of the two polymers used and the ionic strength between them. The importance of pH is enormous in complex coacervation since pH, affects the ionization degree of the carboxyl group of the carbohydrate and the amino groups of proteins (Jayanudin et al., 2016).

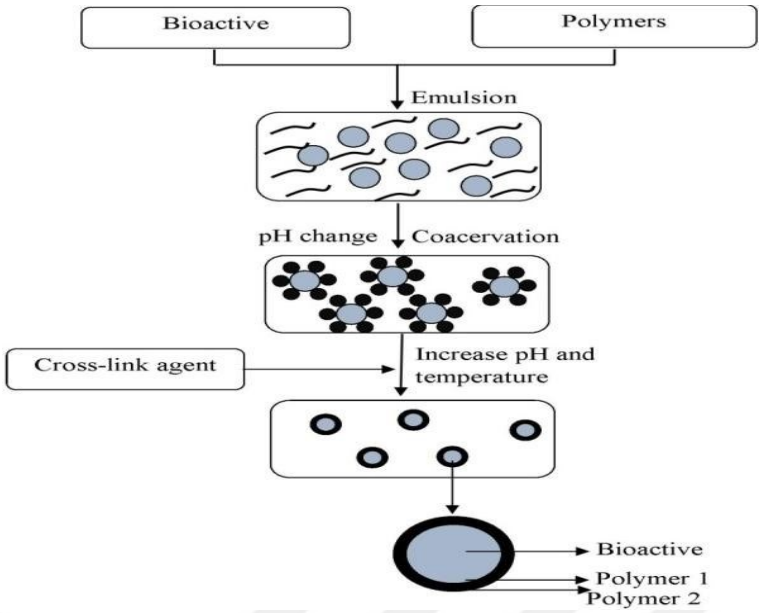


Figure 2.5 Steps of complex coacervation (Jayanudin et al., 2016)

2.2.3 Complex Coacervation of Proteins

Proteins are natural biopolymers that can interact with hydrophobic, hydrophilic or charged polymers due to their chemical properties. Aggregates, hydrogels and complex coacervates are the products of this nano and microstructure. Complex coacervation is the liquid phase separation by electrostatic interactions between two mutually charged macromolecules. Numerous studies on this subject are related to complex coacervation including proteins and polysaccharides. However, very few studies have been reported in food science in terms of heteroprotein complex coacervation, especially systems containing spherical proteins. It is known that the role of pH is very important for heteroprotein liquid-liquid phase separation (Croguennec and Tavares , 2016). Heteroprotein complex coacervates obtained by opposite-charged proteins indicates the balance of interaction between the proteins (Figure 2.6). Literally heteroprotein complex coacervation is the balance of attractive forces. However, if the balance between the proteins cannot be achieved, the proteins precipitate or remain in solution. Electrostatic interactions have a dominant role in the formation of coacervates, changes in pH and ionic strength. Factor affecting the formation of coacervation is given in (Figure 2.7).

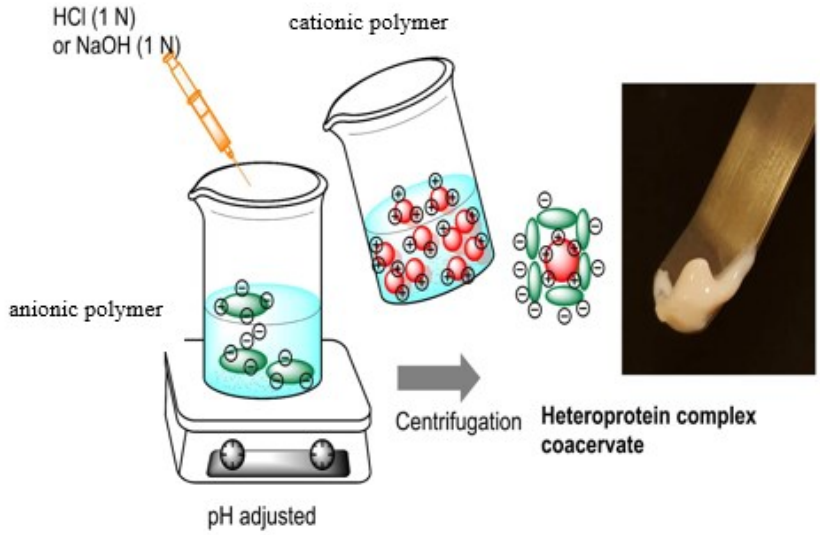


Figure 2.6 Preparation of heteroprotein by complex coacervation method (Adal et al., 2017)

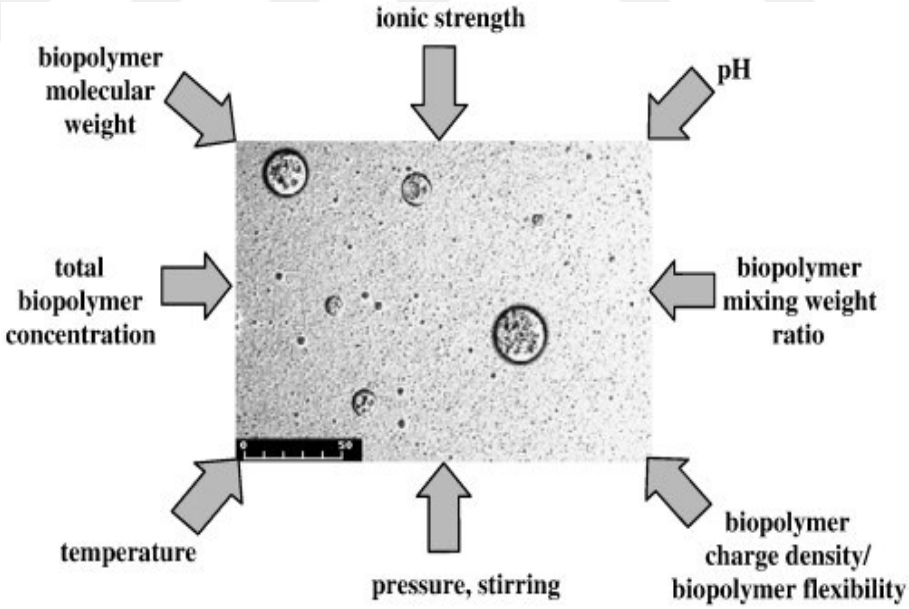


Figure 2.7 Parameters affecting the formation of coacervates (Schmitt et al., 2011)

2.3 Encapsulation of Omega Rich Oils by Complex Coacervation in Food Industry

Fatty acids (FAs), the primary component of lipids, have an aliphatic chain and a carboxyl group. These fatty acids are divided into saturated and unsaturated fatty acids, and the main difference between them is that the unsaturated fatty acids have an unstable structure due to the double bonds in their structure. They have chemical structure consisting of unsaturated fatty acids methylene groups and having two or more double bonds separated from the carbon atom by methylene. The structure of unsaturated fatty acids has a *cis* configuration due to double bonds. This causes unsaturated fatty acids to be unstable against light, oxygen, metallic interactions and environmental factors.

Omega-3 and omega-6 fatty acids belong to the unsaturated fatty acids family. The most commen omega-3 fatty acids (ω-3) are known for their positive effects on diseases such as reducing the risk of coronary heart disease, anti-inflammatory mediators, allergies, diabetes and neurological diseases such as Alzheimer's. Also, since the human body cannot adequately synthesize unsaturated fatty acids (PUFAs), these omega fatty acids are known as essential fatty acids (EFA) acids. Essential fatty acids should be taken to the body as a dietary supplement. Besides the food industry, these fatty acids are widely used also cosmetics and pharmaceutical industries. Omega -3 is available from a variety of foods. These are sources such as animal ones (fish oil) and vegetable ones (flax seed oil, chia oil). In addition, there are many food products enriched with these fatty acids such as dairy products, juices, eggs, baby food and meat products. Functionally, the use of these omega oils in the food industry has a wide range. However, since it is easily affected by environmental factors due to the double bonds in the structure of unsaturated fatty acids, the oxidative stabilization of fatty acids is only achieved by encapsulation. Some factors may oxidize the lipids in their structure resulting in undesirable compounds in the environment. Minimizing these unwanted compounds can be prevented by encapsulation. In the encapsulation process, antioxidants can be added to improve the structure of foods that are prone to oxidation. Several authors have studied the production of functional bread enriched with encapsulated omega -3 fatty acids. According to these authors, encapsulation has

reduced lipid oxidation in breads during cooking (Comunian and Trindade, 2016; Gouin, 2004). During the encapsulation process, the bioactive substance, which is generally rich in fatty acids, is trapped in a coating material. The coating material protects the core material especially against light, oxygen, and metals in order to prevent external effects. At the same time, the coating is applied for reasons such as improving the flavor and sensory properties of the core material. Coating materials can be proteins, polysaccharides and mixtures thereof. There are several methods that have been used for encapsulation of omega oils, such as complex coacervation, spray drying, freeze-drying and ultrasonic atomizer (Comunian and Trindade., 2016).

In a research that was performed by Klaypradit and Huang (2008) tuna oil was encapsulated using emulsion technique and chitosan, maltodextrin, and whey protein were used a wall material. These authors obtained good quality microcapsules with improving flavor and odor structures. They suggested that the coating materials used in different combinations will increase the stability of tuna and other oils.

Eratte et al. (2015) applied an encapsulation system by complex coacervation method with omega-3 fatty acids and probiotic bacteria, being whey protein isolate and gum arabic as a coating material. They observed that while the probiotic bacteria remained alive with encapsulation the oxidative stability of the tuna oil used for omega -3 fatty acid was significantly increased. The flaxseed oil with a high amount of omega-3 fatty acid was encapsulated with gelatin-gum Arabic complex coacervation method and its effects were investigated by keeping at room temperature for 25 days. The main effects of encapsulation, on the oxidation of oil and against the oxidative products have been reported (Liu et al., 2010).

2.4 Pea Protein Isolate

Peas (Pisum sativum L.) has been useful nutrients due to their functional content and rich protein source and is used in the food industry for improving the texture of food products. It has no allergenic properties and is preferred in the food formulation as it has rich content when examined in terms of nutritional values of pea seeds (Barac et al., 2010). Considering peas, other properties, fiber content is considerably high and it is also rich in lysine, tryptophan, minerals, carbohydrates, folate, vitamin B, in calcium, iron, and potassium.

In addition to high nutritional value of peas, it is considered important as an agricultural product. Most uses in legumes varieties of pea grains, which are used as a direct food as, in the production of canned food in recent years. Therefore, it is widely used in pea protein concentrates, pea flour soups. The content of pea protein isolate (PPI) is given in Table 2.3. As can be seen from the table, the proportion of protein in peas is considerably higher than other components. Dried peas contain lysine-rich proteins. Pea proteins are available easily and it has a low cost. The protein groups called albumin and globulin are predominantly found in the structure of pea protein. Of these, albumin is a water-soluble protein that makes up about 20% of the total protein in pea protein. The proportion of globulin in pea protein is greater than albumin. The globulins are in salt-soluble format and contain some of the highly essential amino acids. When pea proteins are examined, the most prominent proteins are legumin (11S) and vicilin (7S) and convicilin globular proteins (Lam et al., 2016). The molecular forms of the three main proteins contained in the protein are shown in (Figure 2.8).

Figure 2.8 Molecular forms of legumin, vicilin and convicilin (Barac et al., 2010)

In addition, pea protein contributes to the structure also of the food surface. The storage proteins in the protein structure of peas, which are in the group of globulins, are 7S (vicilin) and 11S (legumin). These proteins can provide a barrier between oil and water emulsions and are known to reduce stress and improve stabilization by forming a durable membrane at their interface. Pea protein are associated with good gelling properties and good oil-water absorption (Taherian et al., 2010). Differences between the structures vicilin and legumin in the composition appear in functional properties and nutrition. Legumin contains more sulfur amino acids in its structure than vicilin. However, there are some studies examining the different functional properties of these protein units. According to Bora et al. (1994) it was stated that vicillin gels in the presence of temperature but legumin does not. However, O'Kane et al., found that both the vicilin and legumin forms of pea protein can be gelled. These studies have shown that the gelling properties of legumin and vicilin also differ and that the vicilin has better emulsifying properties.

Table 2.3 Content of pea protein isolate (PPI) (S. Tömösközi et al., 2001).

	Pea Protein Isolate (%)
Protein	89,6
Lipid	1,6
Ash	2,6
Moisture	5,3

The pea protein isolate has been reported many times for achieving the desired food functions such as foaming, emulsification and water absorption (Chao et al., 2018). Changes in the workability of PPI have been studied by chemical modification. It has been functionalized to provide more benefits than proteins that are now popularly used in the food processing industry. One of the methods applied to increase the solubility of vegetable proteins is treatment with enzymes. Pea protein is cheaper and available than other expensive proteins on the market such as soy protein or egg white protein.

Figure 2.9 Powder form of green pea

Several methods to hydrolyze protein which are called chemical and enzymatic hydrolysis. Enzymatic hydrolysis is more preferred because when hydrolyzing it should also not damage the structure of the protein but also improve the nutritional quality. Enzymatic hydrolysis is important because protease enzymes that break down protein are more controlled and more natural. Chemical hydrolysis is less preferred than enzymatic hydrolysis because chemical processes are performed at elevated temperatures using acids or bases, which is necessary to break the bonds of the protein. The chemical hydrolysis method is easy to apply, but undesirable results may occur. These are the disappearance of some essential amino acids that we need, and the formation of toxic products by some undesirable reactions (Humiski and Aluko, 2017).

2.5 Lactoferrin

Lactoferrin (LF) is an iron-binding glycoprotein with a molecular weight of 80 kDa. It is found in mammalian milk and in many secretions such as saliva, tears, semen and mucosal making it the second most abundant protein in milk after caseins (González et al., 2009). Lactoferrin was first isolated from cow's milk and purified on industrial scale from skimmed milk and whey powder. Five to 30% of a saturated lactoferrin molecule consists of iron ions. The iron binding stability of lactoferrin has a broad pH limit and is not adversely affected by gastric acidity. Lactoferrin can bind more iron ions than transferrin, form iron chelates even under very acidic conditions such as pH 3.0 and is more resistant to oxidative change than transferrin. Iron ions, calcium, magnesium, zinc, sodium, potassium, copper manganese, cobalt ions and DNA and RNA also has the ability to bind. In addition, lactoferrin has been shown to be highly resistant to proteolytic degradation. Although there are differences in the structural and biochemical properties of lactoferrin in cow and human milk, bioactivities have been shown to be quite similar in animal studies (Alkın, 2008). It includes N and C, two symmetric lobes in the polypeptide chain (Figure 2.10). These two lobes (N-lobes and C-lobes) are connected to each other by α-helix. In both lobes there are two areas called N1, N2 and C1 and C2, and there is a deep iron binding zone between them (Baker, 2005).

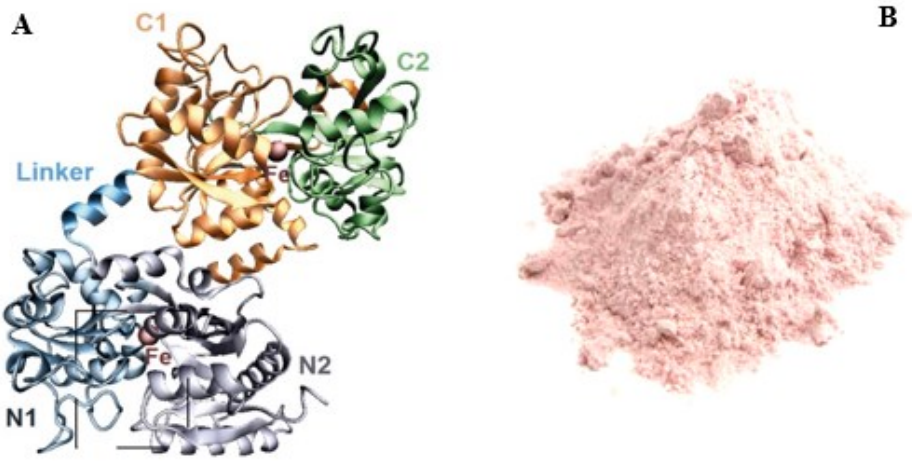


Figure 2.10 Structure of Lactoferrin (A) , powder form of Lactoferrin (B)

Lactoferrin is thought to play an important role in preventing infections by binding iron in existing pathogens as an antimicrobial function. The effect of gram-positive and gram-negative bacteria and some acid and alcohol resistant bacteria have been proven in many studies.The metals that it binds are the Fe_2^+ or Fe_3^+ ions, but it has also been observed to bind Cu_2^+, Zn_2^+ and Mn_2^+ ions. Lactoferrin has recently been commercially isolated from cow's milk and applied to foodstuffs due to its antimicrobial and probiotic properties as a functional additive.The isoelectric point of lactoferrin has a high value. When the isoelectric points for cattle and humans are compared, it is 8.0 and 5.5, respectively. This difference may be due to the method used and changes in the N-terminus of the molecule, which is rich in arginine (Steijns and Hooijdonk , 2000). According to many studies, lactoferrin is biologically well defined active proteins. Although lactoferrin obtained in vitro or from animals differs structurally and biochemically, their bioactivity is similar. Because of these properties, lactoferrin is actively used as iron dispersant in commercial products. In addition, lactoferrin is an alternative to breast milk and is resistant to infections and is highly preferred for enhancing the development of the defense mechanism. It is an important protein in terms of finding a natural antioxidant mechanism and cleaning iron. Many other uses are given in (Table 2.4).

Table 2.4 Current commercial application of lactoferrin

Availabilitiy	**Functionality**
Milk-Based Products (Infant Formulas)	Close to breast milk, resistance to pathogens
Health Supplements	Aid in the absorption of iron in the body, e.g. immune enhancer in pregnant women
Functional Food Drinks	Increases iron solubility and absorption
Cosmetics	Antioxidant
Oral Care Products, Chewing Gums	Improved Oral Hygiene
Feed Supplements	Anti-Feline Virus In Cats

2.6 Raspberry Seed Oil (RSO)

Berry fruits, which are rich in phenolic compounds such as phenolic acid, flavonoid, and anthocyanin, are known to have anti-cancer, anti-inflammatory and antineurodegenerative effects owing to their antioxidants. It is known that raspberry contains a significant amount of phenolic compounds, ascorbic acid and antioxidants. Blackberry kernel extracts were examined and the result was reported to that blackberries containing polyphenolic compound had a positive effect on human DNA. In some studies, they suggested that black raspberry seeds can be used as a functional food due to the presence of omega-3 fatty acids and antioxidants (Dimić et al., 2012). In a study, peroxide values were evaluated after storing raspberry seed oil (RSO) at high temperatures and it was reported that the oil was oxidized very slowly even at a temperature of 60 ˚C. The oil obtained from raspberry seeds has a potential use in foods due to excellent reasons such as being very good in fatty acid compositions, containing omega fatty acids and high nutritional value, high tocopherol content, increasing the shelf life and also protecting against oxidative deterioration. The raspberry seed oil has many beneficially properties such as being used as a dietary supplement owing to its rich functional ingredients and micro components, highlighting the use of an edible source in the market and also improving the environment by adding value to agricultural products. Raspberry seed oil has been patented in nonfood industries such as, cosmetic and pharmaceutical products for preventive effects on gingivitis, eczema, rash and other skin diseases. It is known that raspberry seed oil has better anti-damaging activity than other oils (i.e. avocado oil, grape seed oil, hazelnut, and wheat germ oil) (Oomah et al., 2000).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Pea protein isolate (PPI) was obtained from Roquette (France) and had 85% protein content, 7% moisture content and 4% ash content. Lactoferrin (LF) was purchased from Ingredia (France) and it is known to be purified from cattle milk and has 96% protein, 0.5% ash, and iron saturation of 10-20%. In the experiments, Milli-Q water (Milli-Q device, Millipore Corp., Bedford, MA, USA) was used as solvent. Trypsin enzyme (Type IX-S) used in the hydrolysis stage was purchased from Sigma-Aldrich. Raspberry seed oil (RSO) was obtained from Apres-V (Prosser, WA). Sodium azide and all other chemicals were obtained from the Sigma - Aldrich chemical company (St. Louis, MO, USA).

3.2 Methods

3.2.1 Hydrolysis of Pea Protein Isolate

Pea protein isolate (2g/100mL) was added to distilled water and mixed for 30 minutes on a magnetic stirrer. The temperature was adjusted to 37°C and the pH was adjusted to 8.0 for the determination of optimum enzyme hydrolysis conditions (L.M.Humiski et al., 2007). Trypsin enzyme was then added to pea protein isolate solutions at the following enzyme/substrate (E/S[w/w]) ratios: 1:250 ($HPPI_{250}$), 1:1000 ($HPPI_{1000}$), 1:2000 ($HPPI_{2000}$), 1:3000 ($HPPI_{3000}$) and 1:4000 ($HPPI_{4000}$). The enzyme reaction times were 5, 10 ,15, 30 and 60 minutes. The activity of the enzyme was stopped for at 75 ° C and the solution was rapidly cooled to 25 °C with a cooling bath. The resulting mixture was centrifuged at 5000 rpm (revolutions per minute) for 10 minutes. The supernatant taken from the above of solution was freeze-dried (CHRIST Alpha 1-4 LDPlus, Martin Christ, Germany). Powder form of hydrolyzed pea protein isolate (HPPI) were kept tightly in glass tubes at + 4 ° C until analysis. A table showing the design for optimization is given in (Table 3.1).

Table 3.1 Design for optimization of the degree of hydrolysis with different hydrolyzation time and different E/S ratios

Enzyme/Substrate Ratios (w/w)	Degree of Hydrolysis
$HPPI_{250}$	
$HPPI_{1000}$	
$HPPI_{2000}$	
$HPPI_{3000}$	
$HPPI_{4000}$	

Hydrolyzation Time (min.)	Degree of Hydrolysis
5	
10	
15	
30	
60	

where, $HPPI_{250}$: E/S[w/w] ratio: 1:250, $HPPI_{1000}$: E/S[w/w] ratio: 1:1000, $HPPI_{2000}$; E/S[w/w] ratio: 1:2000, $HPPI_{3000}$: E/S[w/w] ratio: 1:3000 and $HPPI_{4000}$: E/S[w/w] ratio: 1:4000

3.2.1.1 Solubility Measurement of Pea Protein Hydrolyzate

The solubility value determination was described in Jiang et al. (2010). Protein dispersion of 0.5% (w/v) was prepared with hydrolysed protein samples (250 mg). The dispersions were stirred on a magnetic stirrer for 1 hour at room temperature and centrifuged at 2700 rpm for 15 minutes. Supernatants taken after centrifugation were measured by Biuret method (AACC, 2000). For the reliability of the samples, analyzes were performed 3 times. Solubility values were calculated using the equation (Eq.(3.1)) below (Bajaj et al., 2017).

$$\text{Solubility (\%)} = \frac{\text{protein in supernatant } (\frac{mg}{ml})}{\text{total protein in solution } (\frac{\text{mg}}{ml})} \times 100 \qquad (3.1)$$

3.2.1.2 Determination of Degree of Hydrolysis

The degree of hydrolysis (DH) was determined the method described by Kim et al. (1990). The degree of hydrolysis was calculated by measuring the amount of nitrogen soluble in 10% trichloroacetic acid using the following equation (Eq. (3.2)) ;

$$\text{DH}(\%) = \frac{\text{soluble nitrogen in 10\%TCA}}{\text{total nitrogen}} \times 100 \qquad (3.2)$$

10 mL of hydrolyzate was mixed with 10 mL of 20 % trichloroacetic acid (TCA) and centrifuged at 10000 rpm for 15 minutes. The amount of dissolved nitrogen in the supernatant was determined by the Kjeldahl method (AOAC, 1997). The total amount of nitrogen was found using 10 mL suspension prepared without enzyme. Nitrogen content was determined by the Kjeldahl method.

3.2.1.3 Differential Scanning Calorimeter (DSC) Analysis

Differential Scanning Calorimeter (DSC) device (Perkin Elmer, Pyris DSC-6) was used for the determination of the thermal properties of hydrolyzed and non-hydrolyzed pea protein isolates modified by Bongoechea (2011). 0.2 g of powder samples were weighed and mixed for 2 hours in 2 mL of distilled water. 10 mg of sample was placed in the cylinder pans and the pans were hermetically sealed. An empty aluminum pan container was used as a reference. Denaturation was examined in the temperature range of 30 to 180 °C a heating rate of 10 °C/min. The denaturation temperature (T_d), enthalpy of denaturation (ΔH) were computed from the thermograms by the Universal Analysis Program, Version 1.9 D (TA Instruments).

3.2.2 Heteroprotein Complex Coacervation Formation

Coacervations was performed according to method described by Tamm et al. (2016). Coacervations were prepared from the hydrolyzates obtained by 1:3000 E/S ratio ($HPPI_{3000}$) and 15 minutes enzyme application. Lactoferrin (0.4%) and pea protein isolate hydrolysates (0.4%) were added separately to 100mL distilled water and mixed for 2 hours. Their pH was adjusted to 7. Then, LF and HPPI solutions taken in equal volume were mixed in a magnetic stirrer for 30 minutes.

3.2.2.1 Determination of Optimum Biopolymer Ratios in Protein-Protein Complex Coacervation Formation

The hydrolyzate of pea protein isolate and lactoferrin was mixed in distilled water for 2 hours. Lactoferrin was kept constant at 0.4% (w/w) and different pea protein hydrolyzes concentrations were studied (0.005, 0.01, 0.03 and 0.04 wt%). HPPI and LF volume was chosen 1:1 and the mixture was stirred 30 minutes on the stirrer.

3.2.2.2 Determination of Optimum pH in Protein-Protein Complex Coacervation Formation

After the determination of optimum biopolymer ratio between the HPPI and LF. The pH was adjusted using 0.1 N standard HCl or NaOH from 2-9 to the target pH while on the stirrer. The LF stock solution were mixed into an equally freshly prepared PPI solution. Stirring was continued at about 300 rpm on the stirrer at 30 minutes. After stirring the pH of the solution was adjusted between pH 2-9. The optimum biopolymer ratio and optimum pH was determined based on the lowest zeta potential, highest particle size, and the highest turbidity value.

3.2.2.3 Determination of the Effect of Ionic Strengths on Protein-Protein Complex Coacervation Formation

By keeping fixed the optimum biopolymer ratio and pH for complex formation, the ionic strength of the complex solutions was changed and the effect of ionic strength on the complex formation was investigated. For this purpose, zeta potentials and turbidity of solutions at different NaCl concentrations (i.e. 20mM, 50mM, 100mM, 300mM) were investigated.

3.2.2.4 Analyzes for Protein-Protein Complex Coacervation

I. Zeta Potential and Hydrodynamic Diameter Determination

Measurements for hydrolyzed pea protein isolates, lactoferrin, complex coacervates were done with the Zetasizer device (Zetasizer Nano ZS instrument, Malvern Instruments, Worcestershire, U.K). The zeta potential values were measured using a laser Doppler velocity measurement, phase analysis light scattering (M3-PALS0) and disposable electrophoretic cells (DTS 1060). Z potential was calculated according to Henry's equation (Eq.(3.3)):

$$UE= 2\varepsilon zf(\kappa a)/3\eta \quad (3.3)$$

where UE is electrophoretic mobility, ε dielectric constant, z zeta potential, f(κa) Henry's function and η dispersion viscosity. F(κa) relates to the particle radius (a), and κ is the Debye length, which is called equal to 1.5 is referred Smoluchowski approximation in this study . After the solutions were prepared, approximately 2 mL were placed in the cuvettes and 3 readings were made and the values were given on average. The cuvettes have two conductive electrodes which are to make the external voltage come into contact with the liquid sample inside. Hydrodynamic diameter values were measured with dynamic light scattering (DLS) at 633 nm. The dimensions of the diameter were calculated by scattering at an angle of 173 ° C with an interval of 10 seconds using the Stokes-Einstein equation (Eq.(3.4)) ;

$$Rh = kBT/(6\pi\eta DT) \quad (3.4)$$

where, T is temperature, η is solution viscosity, kB is Boltzman constant, D is diffusion constant and d is hydrodynamic diameter. Samples for emulsions were measurement by diluting with water due to their high concentration (Anema and de Kruif, 2014; Can Karaca et al., 2011).

II. Turbidity Determination

The turbidity of pure protein solutions and their complex/ coacervate were measured by a nano spectrophotometer Optima SP-3000 (Slovakia) using a 1 cm disposable plastic cuvette at 600 nm (Flanagan, S. E. et al., 2015). Milli-Q water was used as blank reference resulting in 100% transmittance. The turbidity (OD600) was calculated using equation below; (Eq.(3.5))

$$OD_{600} = -\ln (I / I_{0)} \quad (3.5)$$

where, I is the intensity of light reaching the detector from the sample and I_0 is the intensity of distilled water.

III. Determination of Isoelectric Points of Proteins

Isoelectric points of lactoferrin, hydrolyzed and non-hydrolyzed pea protein isolates were determined. Samples (0.2 wt%) were mixed with pure water for 2 hours. The pH was adjusted to 7.0 for the stock solutions. The pH of the solutions was changed in the range of 2-9 and then zeta potential and particle size values were determined at each pH value. The lowest zeta potential and the highest particle size value were considered as the isoelectric point.

3.2.3 Preparation of Emulsion

The protein solutions were prepared by dispersing either LF powder or HPPI powder into Milli-Q water and then stirring for 2 h at room temperature to provide complete dispersion. The pH of the protein solutions was adjusted to 7.0 using 0.1 M NaOH or 0.1 M HCl. Raspberry Seed Oil (RSO) (10 wt%) made use of to prepare emulsions. LF solution was mixed with raspberry seed oil at a certain concentration. The mixture was pre-homogenized by using a lab-scale homogenizer (Art-Micra D-8, Germany) at 12,000 rpm for 2 minutes (Figure 3.1-A) and then ultrasound at 10% amplitude was applied for 1 minute to each emulsion (Soniprep 150 Ultrasonic Disintegrator, UK) (Figure 3.1-B). The temperature rise that may occur during ultrasonication was controlled using water at 15 ºC. Then, the pea protein isolate hydrolyzate ($HPPI_{3000}$) at pH 7.0 was added to the LF-covered solution. The mixture was stirred for 30 minutes on a magnetic stirrer and the final pH of the emulsion was adjusted to 6.90 by using 1 M NaOH or 0.1 M HCl (Figure 3.1-C). The final protein solution was 1 wt% while oil was 10 %. The method was modified from the study of Kermasha et al. (2018).

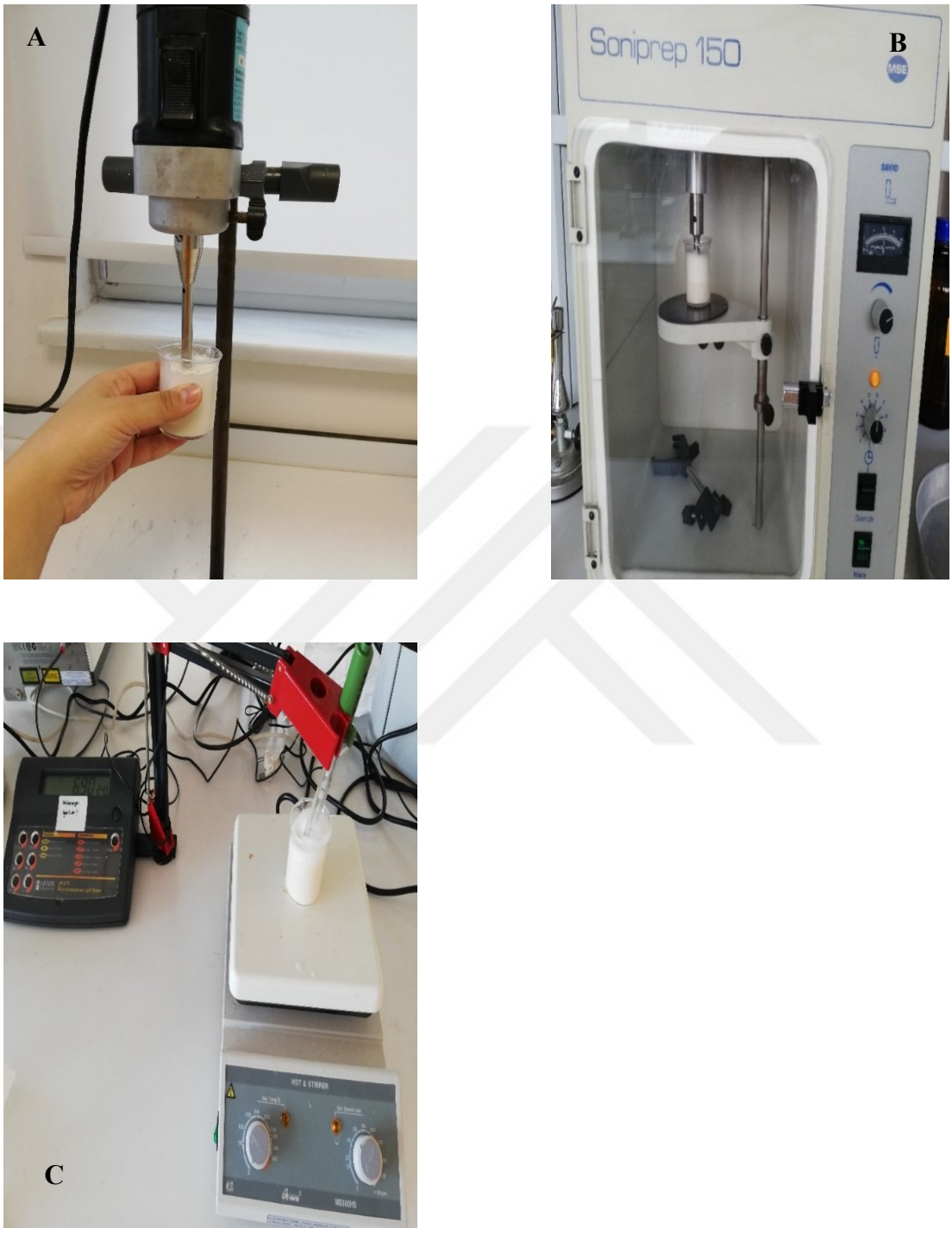


Figure 3.1 Preparation of Emulsion with RSO by $HPPI_{3000}$-LF complexes **A)** Image of homogenizer application **B)** Image of Ultrasound application **C)** Final emulsion pH adjusted to 6.90

3.2.3.1 Analyser for Determination of Emulsion Properties

I. Droplet Size Analysis

Emulsion droplet size distribution was measured using a light scattering instrument (Horiba particle size analyzer (LA-950, Irvine, CA, Amerika). This measuremet is an angular measurement of the intensity of light scattered from the emulsion droplet. Refractive indices of water and oil of raspberry seed were entered as 1.330 and 1.480, respectively (Can Karaca et al., 2011). The average droplet size is given as the volume mean diameter (d_{43}) as follows equation (Eq. (3.6));

$$d_{a,b}=\sum d_i n_i^a / \sum d_i n_i^b \quad (3.6)$$

In this equation, d_i is the diameter of oil droplets and n_i is the number of droplets.

II. Creaming Index

The emulsions were placed in sealed plastic caps as 5 mL each and then stored at room temperature. After seven days of storage, the extent of separation was examined and photographs of emulsion samples were taken over a period of 7 days using a digital camera to record the separation (Sarkar et al., 2016). Creaming index was calculated according to the formula below (Eq.(3.7)) ;

$$\text{Creaming index} = 100 \times (L/L_0) \quad (3.7)$$

where, L is cream layer length, L_0 is total emulsion length.

III. Optical Microscopy

Emulsion was viewed using a camera (20 x magnification) (Pixera PVC 100C, USA) using an Olympus BX 51 microscope (Tokyo, Japan). A small drop of emulsion was put into the microscope slide and covered with a coverslip (Erçelebi and Ibanoğlu, 2009).

3.2.4 Encapsulation Optimization of Raspberry Seed Oil

The samples prepared according to Section 3.2.3 were chosen (10% oil and 1% protein wt%) and the samples were taken into aluminum containers and kept in the freezer overnight. Then frozen emulsions were freeze-dried (CHRIST Alpha 1-4 LDPlus, Martin Christ, Germany). After freeze-drying the powder capsules were storred the +4 ˚C (Kermasha et al., 2018).

3.2.4.1 Moisture Content Determination of Powders

The moisture content of the capsules that were dried and powdered by the freeze-drying method was determined according to the (AOAC, 1995). One g dried powder capsule was placed in a petri dish and oven-dried at 105 ºC for 24 hours. After weighing, the samples were taken to the desiccator, the percentage of moisture was calculated with the formula below (Eq.(3.8));

$$\% \text{ Moisture content} = ((m_1-m_2) / m_1) * 100 \quad (3.8)$$

where m_1 is wet weight of the sample and m_2 is weight of the sample after drying.

3.2.4.2 Color Measurement

Color measurements of RSO and encapsulated RSO were measurement according to the CIELAB system with HunterLab Colorflex (A-60-1010-615 Model Colorimeter, HunterLab, Reston, VA.) device. This system uses three values L *, a *, b * to define the location of color in the three-dimensional visible color space. The L*, a*, b* color space was used to express the color changes. Color values are expressed as L* (whiteness, brightness / blackness), a* (redness / greenness) and b* (yellowness / blueness) and yellowness index (YI). Three measurements were taken for each sample. The instrument was calibrated against the standard reference white tiles (L*= 93.41, a* =-1.12, b* =1.07) (Timilsena et al., 2016).

3.2.4.3 Encapsulation Efficiency

The total oil content was determined according to the method described by Shi et al. (2018) with some modifications. One g of raspberry seed oil capsule was mixed with 15 mL HCl and 15 mL of hexane and shaken for 2 min to extract the free oil. Then, the dispersion was passed through Whatman filter paper No:4. The filter was washed three times with 20 ml of hexane. The filtrate was collected and the solvent was removed using a rotary evaporator (Heidolph Laborota 4000 Efficient, Germany) at 60°C.

For the calculation of surface oil, the method described by Frascareli et al. (2012) was modified. One g sample was mixed with 6 mL petroleum ether for 2 minutes with vortex. The sample was then filtered. The sample on the paper was washed 2 times with 2mL petroleum ether. The solvent and oil mixture were taken to fixed weighed petri dishes and removed from the solvent in the oven at 60 ℃ for 2 hours. The encapsulation efficiency was calculated with the formula below (Eq.(3.9)); (Quispe-Condori et al., 2011).

$$EE\ (\%) = ((\text{Total oil} - \text{Surface oil}) / \text{Total oil}) \times 100 \qquad (3.9)$$

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Hydrolysis of Pea Protein Isolate with Enzyme Treatment

4.1.1 Solubility Measurement on Pea Protein Isolate Hydrolysis

Pea protein isolate was hydrolyzed using trypsin enzyme according to section 3.2.1 method. The amount of protein used in solubility calculation (Biuret method) was calculated according to the calibration curve indicated in Figure 4.1. The solubility of hydrolyzed pea protein isolate (HPPI) prepared using 2% (w / w) pea protein isolate (PPI) was found to be 87%. Therefore, the goal of increasing the solubility of pea protein isolate by hydrolysis was achieved by increasing 80%. Klost and Drusch (2019) hydrolyzed the pea protein using trypsin enzyme and found its solubility at pH 7 at 90%. In another study, it was observed that the solubility level of the flax seed, whose solubility value was analyzed at pH 7.0 and above, increased by 85% by proteolysis with trypsin (Wei Yin et al., 2008). According to Sijtsma et al. (1998); Bajaj et al. (2017) and Barac et al. (2012) enzymatic hydrolysis can significantly alter the functional and solubility properties of pea protein a compared to native ones.

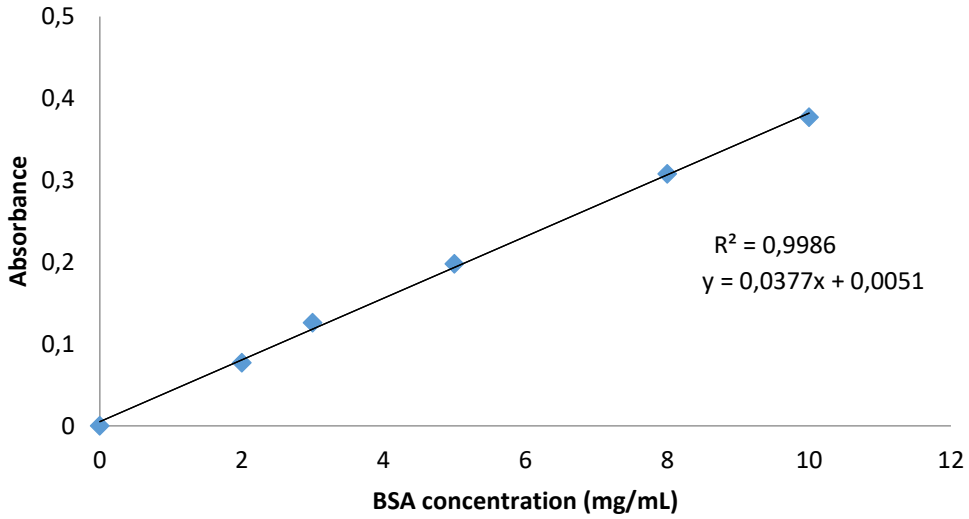


Figure 4.1 Protein calibration curve by Biuret methods

4.1.2 Determination of Degree of Hydrolysis

The degree of hydrolysis (DH) was found that an enzymatic hydrolysis results in a larger interface area compared to the unmodified protein (Tamm et al., 2016). Barac et al. (2011) studied HPPI with chymosin enzyme for 60 minutes and they reported DH from 3.9 to 4.7%. The low degree of hydrolysis is important for emulsions in terms of functional properties (1-10%) (O'regan & Mulvihill, 2010). Wani et al. (2014) found that protein hydrolysis increases the functional properties of the original protein, but these properties are lost above a certain degree of hydrolysis. The reason for this is that peptides break down into very small pieces and lose their function and cause nutritional loss. For this reason, in our study, the hydrolysis time and enzyme/substrate ratios were changed and the degree of hydrolysis was targeted at 10%. The hydrolysis of proteins was considered optimal and they showed better functional properties by varying their hydrolysis levels depending on the hydrolysis time or the ratio of the substrate to the enzyme (Avramenko et al., 2013).

4.1.3 Effects of Different Hydrolysis Time on the HPPI

The hydrolysis times were changed to 5, 10, 15, 30, 60 minutes and the effect of the time on the degree of hydrolysis was examined. The change in the degree of hydrolysis depending on time is given in Figure 4.2. As seen in the figure, the degree of hydrolysis has changed in enzyme applications up to 15 minutes, but no major change has been observed after 15 minutes. For this reason, the hydrolysis time was determined as 15 minute in this study.

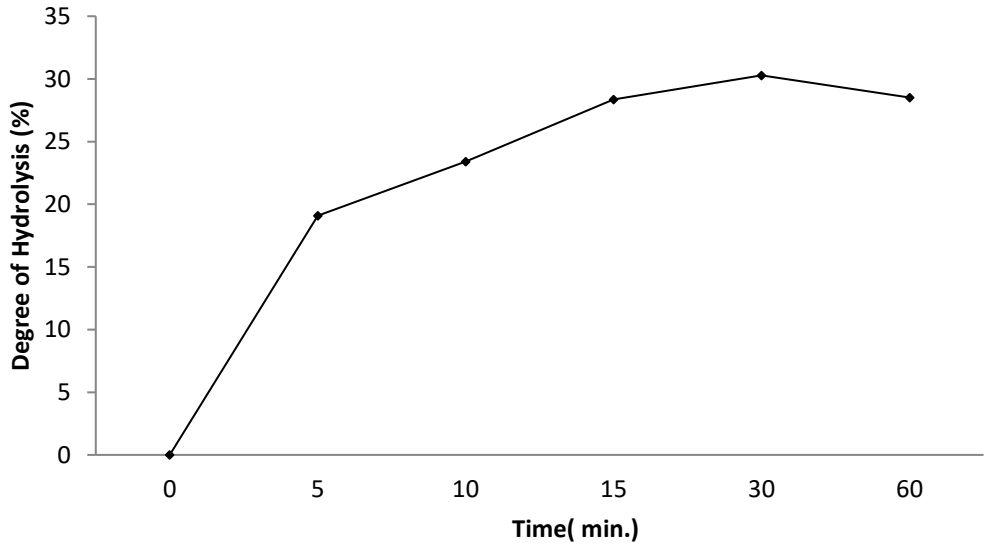


Figure 4.2 Degree of hydrolysis with time

Barac et al. (2011) stated that the hydrolysis curves in the hydrolysis of the pea protein hydrolyzate with chymosin enzyme were rapidly hydrolysis in the first 15 minutes at the beginning of the reaction and that they underwent less intensive hydrolysis in the following minutes. More hydrolysis (60 minutes) caused small fractions to almost completely disintegrate.

4.1.4 Effects of Different Enzyme/Substrate (E/S) Ratio on HPPI

Different trypsin enzyme/substrate (E/S) ratios were studied to examine the change of degree of hydrolysis. Figure 4.3 shows the different levels of enzyme/substrate for the degree of hydrolysis. As can be seen from the graph, the degree of hydrolysis decreases as the concentration of enzymes decreases. The DH% value for different E/S ratio was 28.36% for 1:250 ($HPPI_{250}$), 22.87% for 1:1000 ($HPPI_{1000}$), 15.6% for 1:2000 ($HPPI_{2000}$), 9.92% for 1:3000 ($HPPI_{3000}$) and 7.8% for 1:4000 ($HPPI_{4000}$).

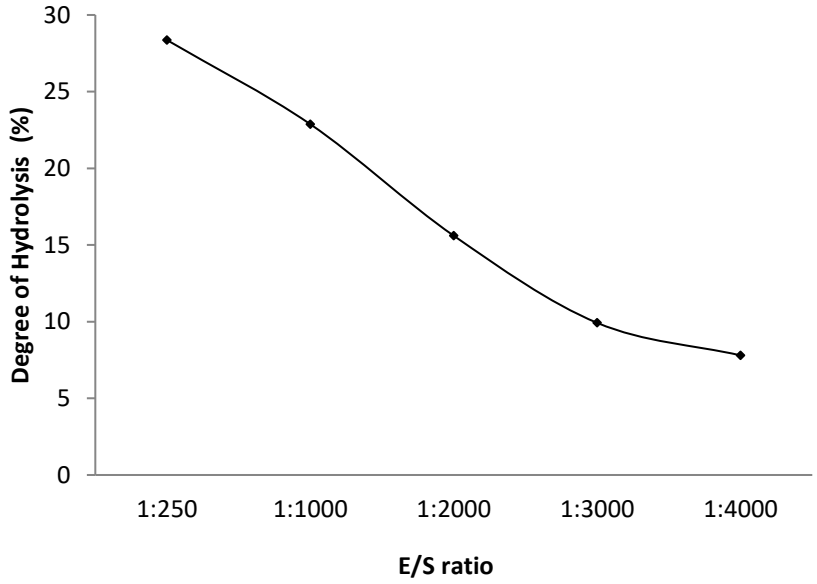


Figure 4.3 The degree of hydrolysis relative to the enzyme/substrate ratio (E/S)

In a similar study, hydrolysis of lentil protein isolate with trypsin at different E/S ratios was discussed. In this study, E/S ratios were selected between 1:100 and 1:1000 and degree of hydrolysis were compared. After the 40 minute hydrolysis of lentil protein isolate, the degrees of hydrolysis was determined as ~42% for 1: 100, ~18% for 1: 250, ~17% for 1: 500, and ~4% for 1: 1000. They also observed that the degree of hydrolysis decreases as the amount of enzymes decreases. In addition, the researchers assumed that the selected DH% range will positively affect emulsion formation and stability when lower than 10% (Boye et al., 2010).

The zeta potential and hydrodynamic diameter values of HPPI at different enzyme/substrate (E/S) ratios were investigated. The effect of the change of E/S ratio on zeta potentials was not seen significantly (Figure 4.4). The zeta potential value for $HPPI_{250}$, $HPPI_{1000}$, $HPPI_{2000}$, $HPPI_{3000}$ and $HPPI_{4000}$ we found as -23.3, -23.56, -24.73, -22.90,-23.63mV, respectively.

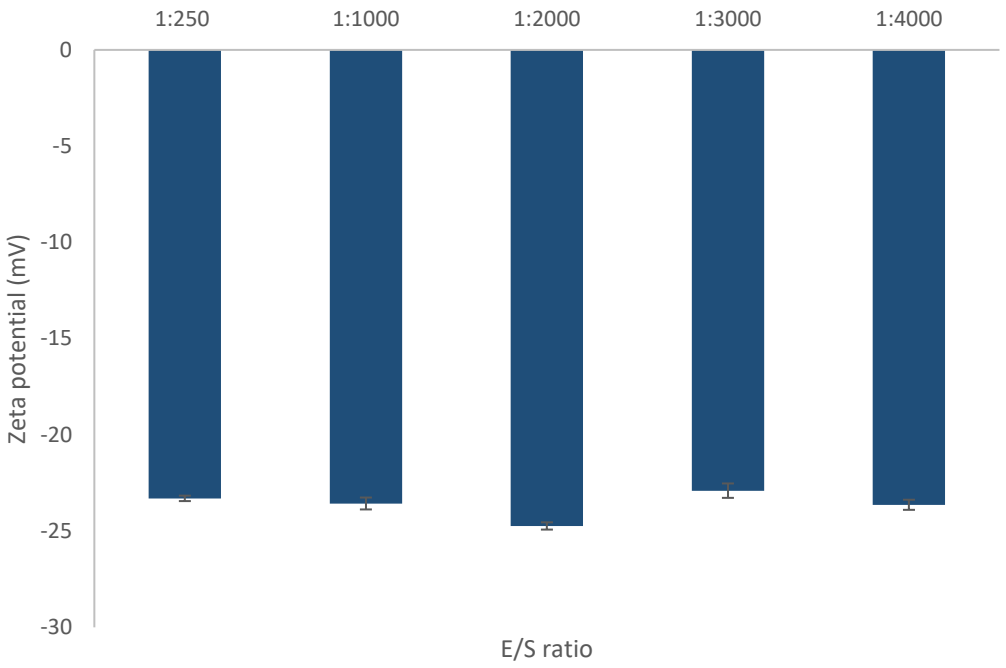


Figure 4.4 Zeta potential values of HPPI at different E/S ratios

It has been observed that $HPPI_{250}$ gives the maximum hydrodynamic diameter (282.5 µm) (Figure 4.5). The decrease in the amount of enzyme caused a decrease in the hydrodynamic diameter. Based on these results, the E/S ratio was chosen as 1:3000 ($HPPI_{3000}$), and the coacervation and emulsion studies continued with $HPPI_{3000}$. However, in characterization studies of the hydrolyzed protein, the results were given for $HPPI_{250}$ and $HPPI_{3000}$.

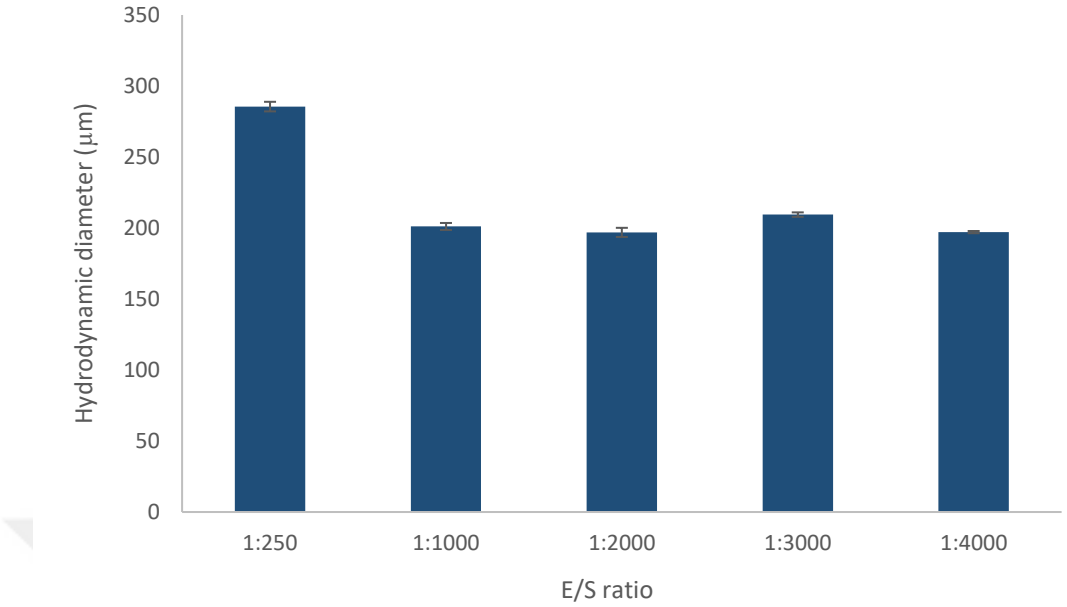


Figure 4.5 Change of hydrodynamic diameter according to E / S ratio

4.1.5 Determination of Differential Scanning Calorimeter (DSC)

The effect of hydrolysis on the thermal stability of PPI and non-hydrolyzed PPI were determined by DSC (Figure 4.6). Thermal denaturation temperature (Td) and denaturation enthalpy of proteins ΔH (J/g) are given in (Table 4.1). The hydrolysis process decreased both the Td and ΔH values. $HPPI_{250}$ has lower thermal stabilities compared to the other samples because it has lower denaturation temperature and ΔH (Table 4.1). Lower denaturation and ΔH indicate a lower secondary structure content (Wani et al., 2014). Lopez et al. (1998) studied soy protein isolates with enzymatic hydrolysis and they reported an increased protein denaturation and lower thermal stability. A similar decrease observed by Wani et al. (2009) in the hydrolysis of lentil protein isolates.

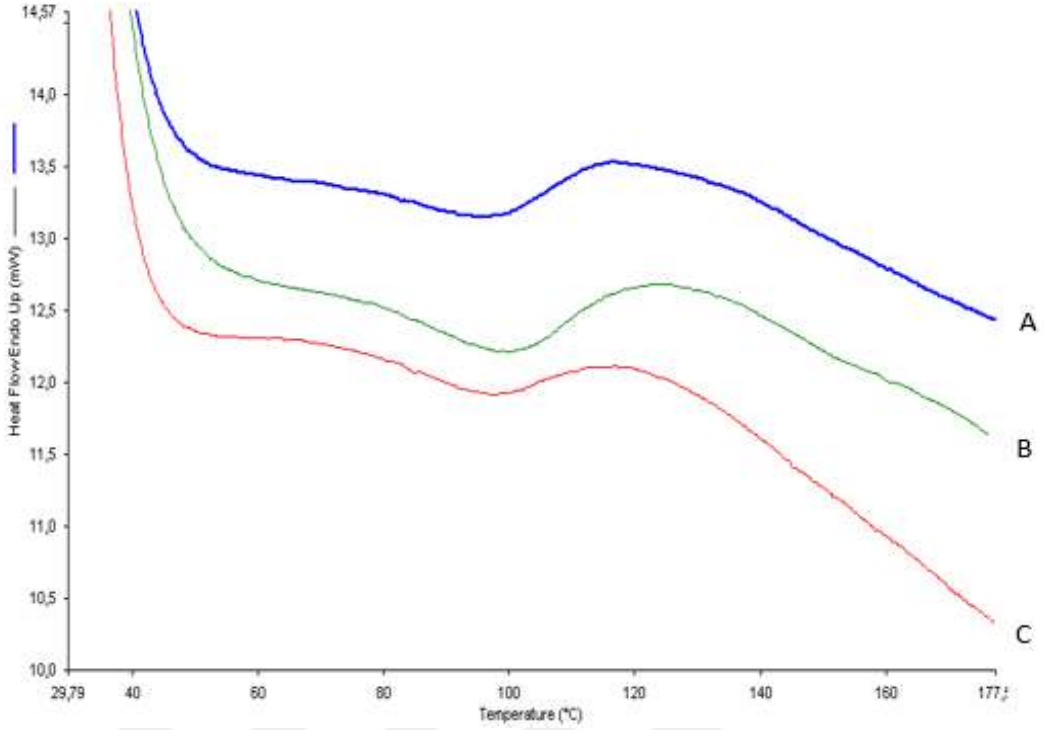


Figure 4.6 DSC thermograms of hydrolyzed pea protein isolates (HPPI) at different E / S ratios A: $HPPI_{3000}$, B: PPI, C: $HPPI_{250}$ and non-hydrolyzed (PPI)

Enzyme hydrolysis causes some bonds to break, it decreases the thermal stability of the protein, causing the protein to denature at lower temperatures. Similarly, the low ΔH value means that the energy required for thermal denaturation is lower. Yin et al. (2008) hydrolyzed the hemp seed isolate with trypsin and showed a decrease in ΔH.

Table 4.1 T_d and ΔH values for PPI, $HPPI_{3000}$ and $HPPI_{250}$

	T_D (°C)	Δ*H*(j/g)
PPI	100.09	0.6526
$HPPI_{3000}$	96.76	0.3913
$HPPI_{250}$	96.25	0.2942

4.2 Formation of Heteroprotein Complex Coacervation Between the Hydrolyzed Pea Protein Isolate (HPPI) and Lactoferrin (LF)

The isoelectric points of HPPI and non-hydrolyzed PPI were determined for coacervation formation. Samples were prepared as $HPPI_{250}$, $HPPI_{3000}$ and pea protein isolate (PPI) for a better comparison. The pH is defined as the isoelectric point where the zeta potential is closest to 0, hydrodynamic diameter and turbidity measurement is closest to the highest value (Anema and de Kruif, 2014; Schmitt, 2011; Kayitmazer, 2013). The isoelectric point of the PPI was also determined as pH=4.6, as shown in Figure 4.7. According to Lan et al. (2018), the isoelectric point of PPI varies between pH = 4-5. In Figure 4.8, isoelectric point of $HPPI_{3000}$ is determined as pH=4.5. In Figure 4.9, the isoelectric point of $HPPI_{250}$ is determined as pH=3.8. According to this information, the increase in the amount enzyme caused the isoelectric point to decrease. Ochiai et al. (1982) saw the same reduction in the hydrolysis of soy protein with trypsin. The complex occurs between the isoelectric point (pI) of the proteins (Santos et al., 2018). In this study, evaluated with the effect of different ratios HPPI-LF (w/w) on complex formation at pH=7.

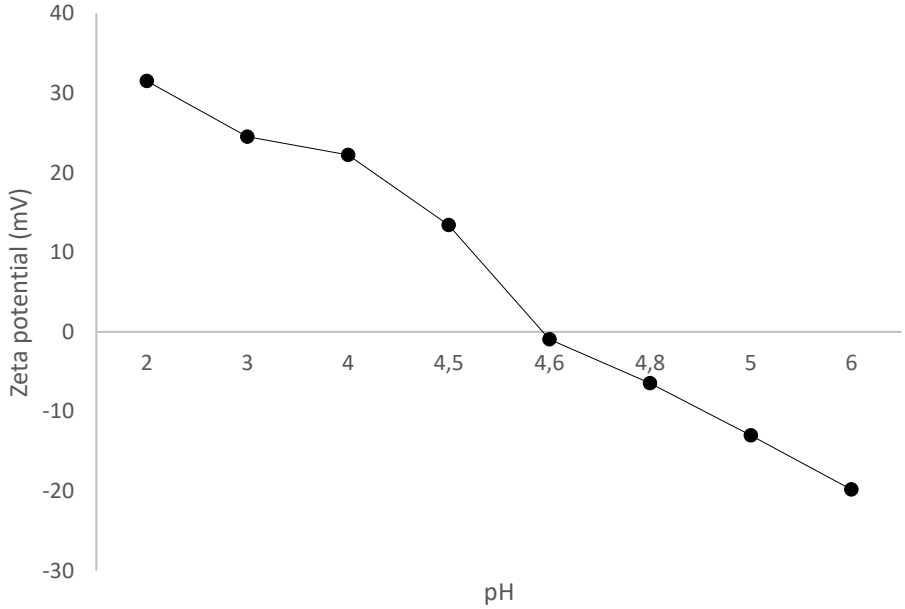


Figure 4.7 Zeta potential of PPI with pH

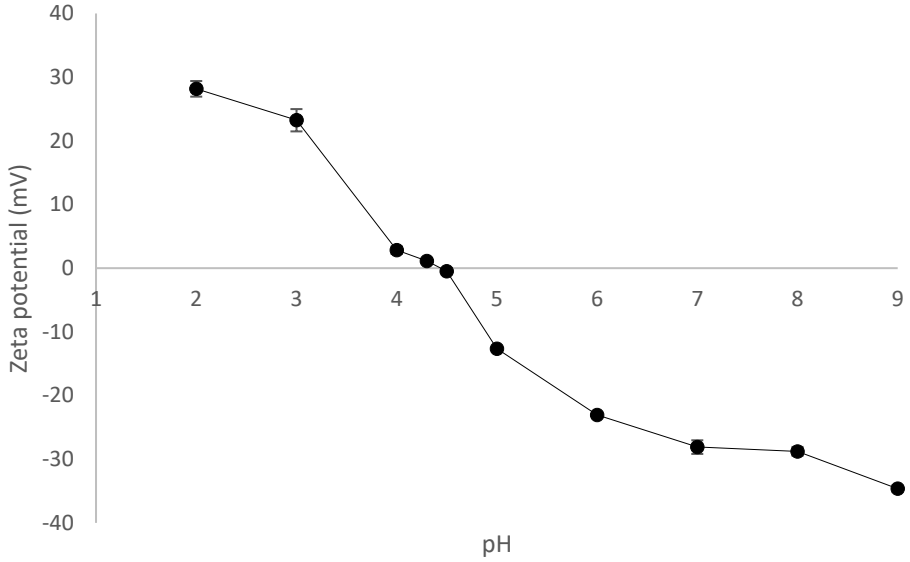


Figure 4.8 Zeta potential of $HPPI_{3000}$ with pH

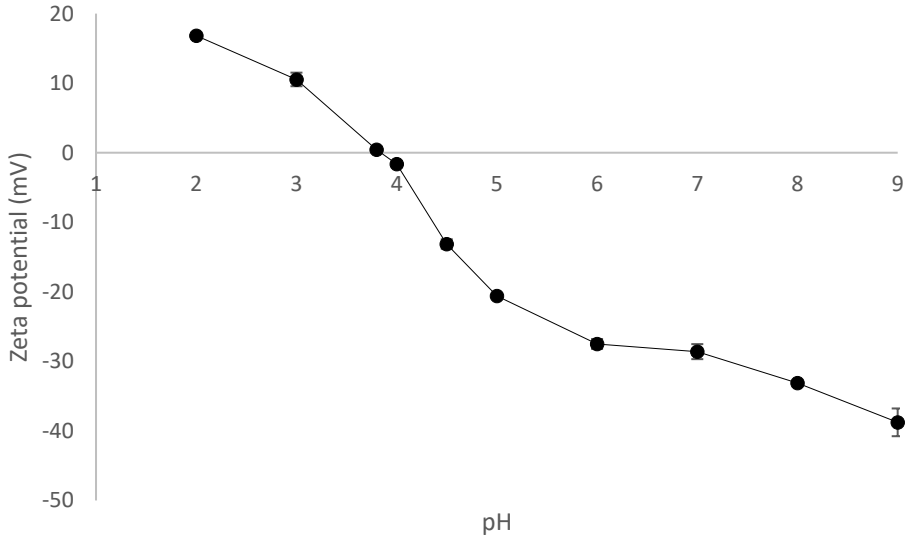


Figure 4.9 Zeta potential of $HPPI_{250}$ with pH

It was observed that the hydrodynamic diameter increased between pH= 2-3 for all three samples and reached the maximum value at the isoelectric point of proteins (Figures 4.10, 4.11 and 4.12). Then the hydrodynamic diameter dropped between pH 5-6 and no major changes between pH 6-7 were observed. Since the proteins are insoluble at the isoelectric point, the hydrodynamic diameter is expected to be large (Tiwari et al., 2009; Peixoto et al., 2016).

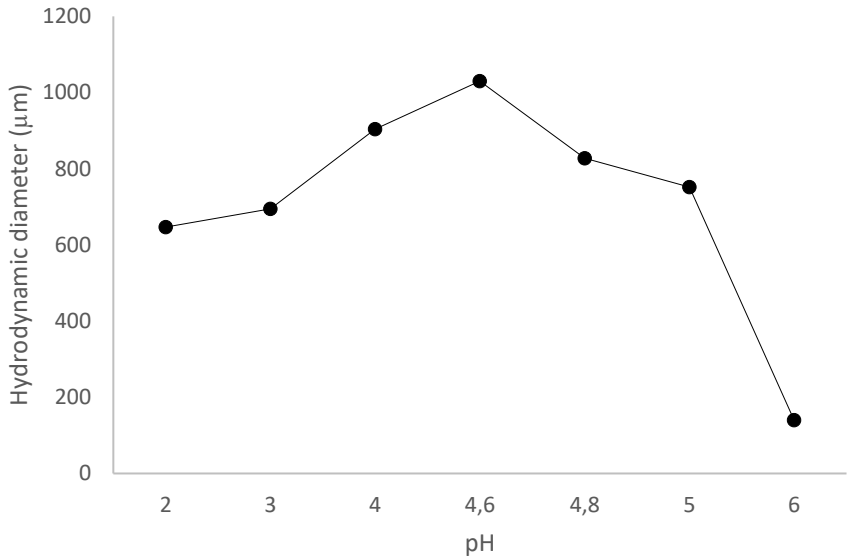


Figure 4.10 Hydrodynamic diameter of PPI with pH

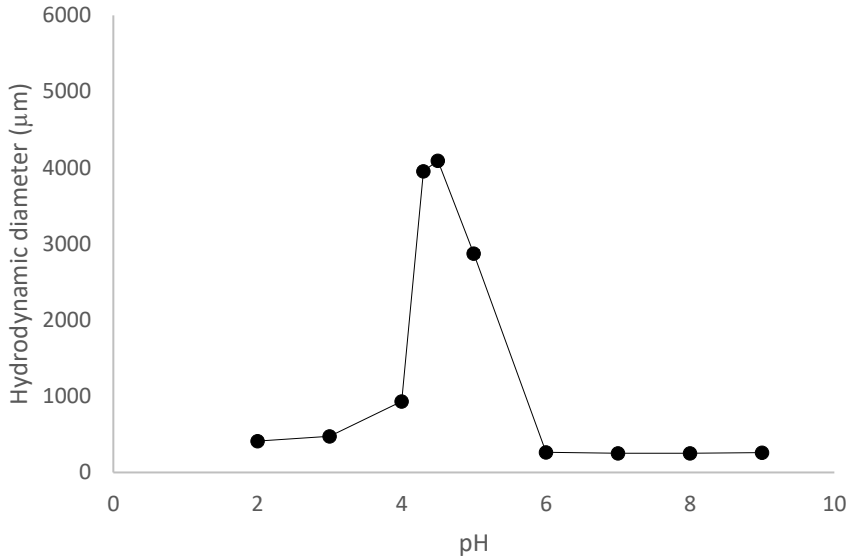


Figure 4.11 Hydrodynamic diameter of $HPPI_{3000}$ with pH

As understood from Figures 4.10, 4.11 and 4.12 the maximum hydrodynamic diameter was observed at pHs with isoelectric points of proteins. The hydrodynamic diameter of the non-hydrolyzed pea protein isolate (PPI) at pH 4.6 is 1030 µm (Figure 4.10). A maximum diameter of 4093 µm was observed in the pH 4.3-4.5 range for $HPPI_{3000}$ (Figure 4.11) and the hydrodynamic diameter in the pH 3.8-4.0 range for $HPPI_{250}$ was observed as 5669 µm (Figure 4.12).

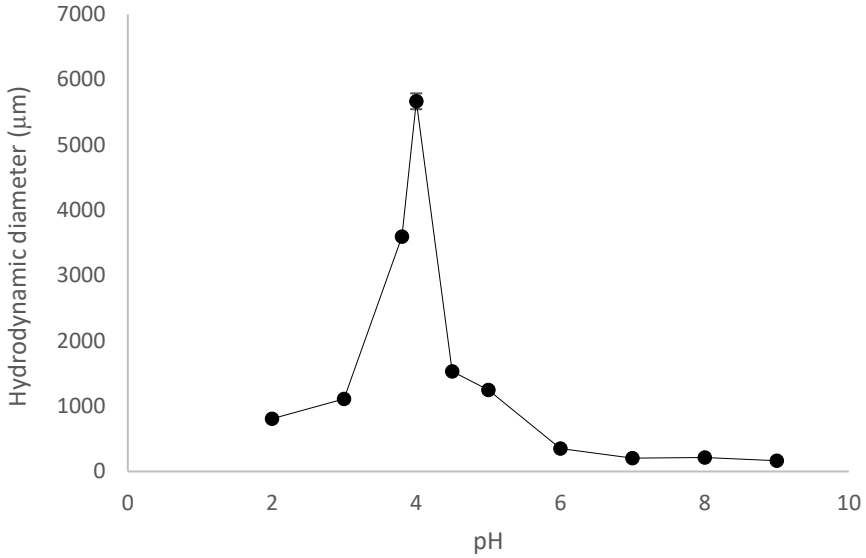


Figure 4.12 Hydrodynamic diameter of $HPPI_{250}$ with pH

In Figure 4.13, the isoelectric point of lactoferrin to be used in complex coacervation was determined as pH=8.7. Gulao et al. (2014), Anema and de Kruif (2012) and Bokkhim et al. (2015) found similar results. The hydrodynamic diameter graph of lactoferrin (Figure 4.14) also shows a large diameter (257.7 µm) value at pHs close to the isoelectric point.

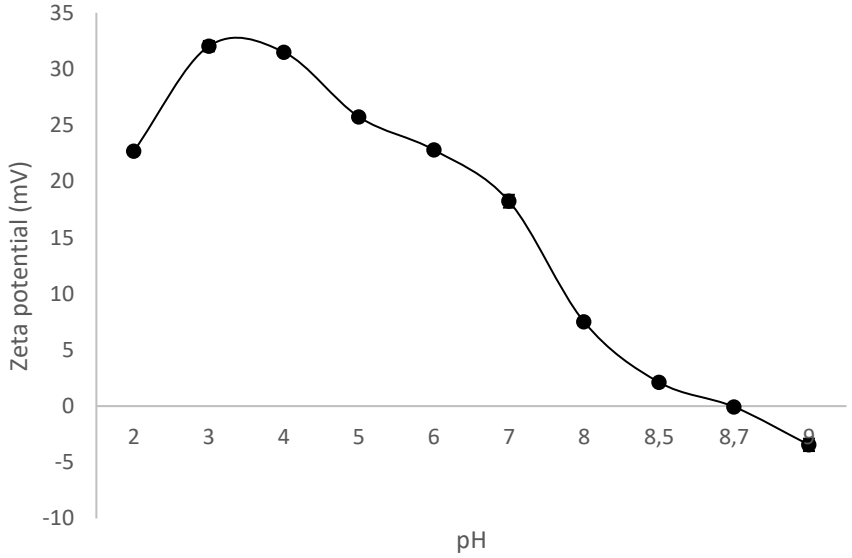


Figure 4.13 Zeta potential of Lactoferrin with pH

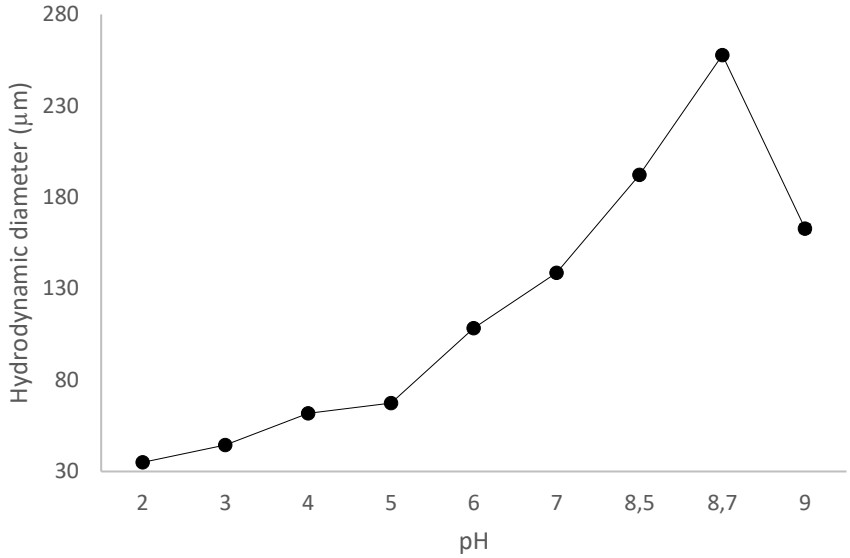


Figure 4.14 Hydrodynamic diameter of LF with pH

4.2.1 Effect of Biopolymer Ratio of Between the HPPI –LF on the Complex Formation

The LF ratio was kept constant at 0.4% by weight in the complex and the pea protein hydrolysates (HPPI) were changed with different biopolymer ratios by weight. The pea protein hydrolysates samples were determined by two different E/S ratios of 1:250 ($HPPI_{250}$) and 1:3000 ($HPPI_{3000}$). In the examples, zeta potential and hydrodynamic diameter analyzes were performed depending on the complex ratio. The biopolymer ratio has been determined as the optimum ratio to create a complex, that is the zeta potential is closest to 0, the hydrodynamic diameter and the turbidity value gives the highest value. As seen in Figure 4.15, 4.16 and 4.17, the rate of biopolymer for $HPPI_{250}$ was determined by weight as 0.2% LF and 0.04% $HPPI_{250}$. The increase of turbidity indicates that the resolution decreases after complex formation. Therefore the highest turbidity indicating the formation of insoluble complexes (Santos et al., 2018).

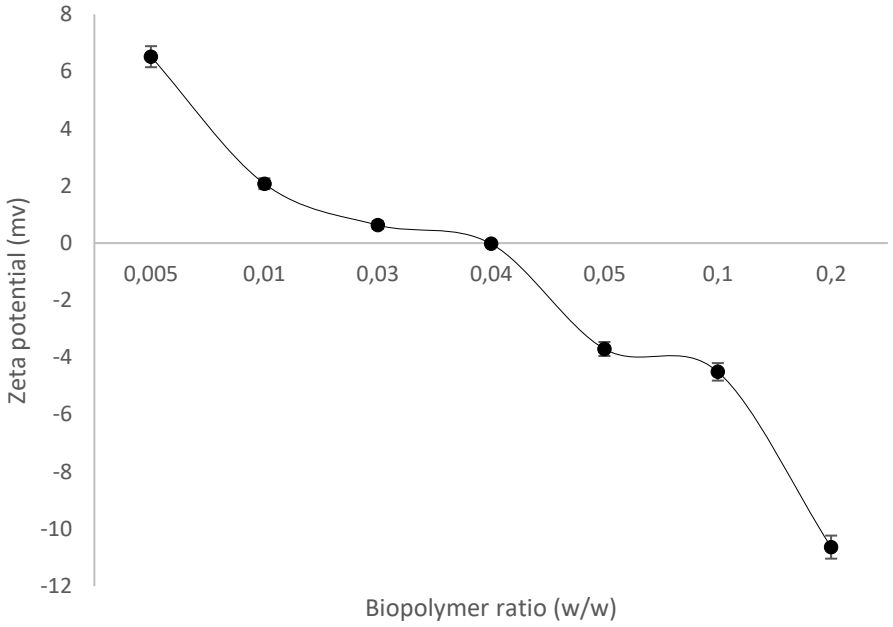


Figure 4.15 Zeta potential with biopolymer ratio in the complex formation of HPPI250– LF

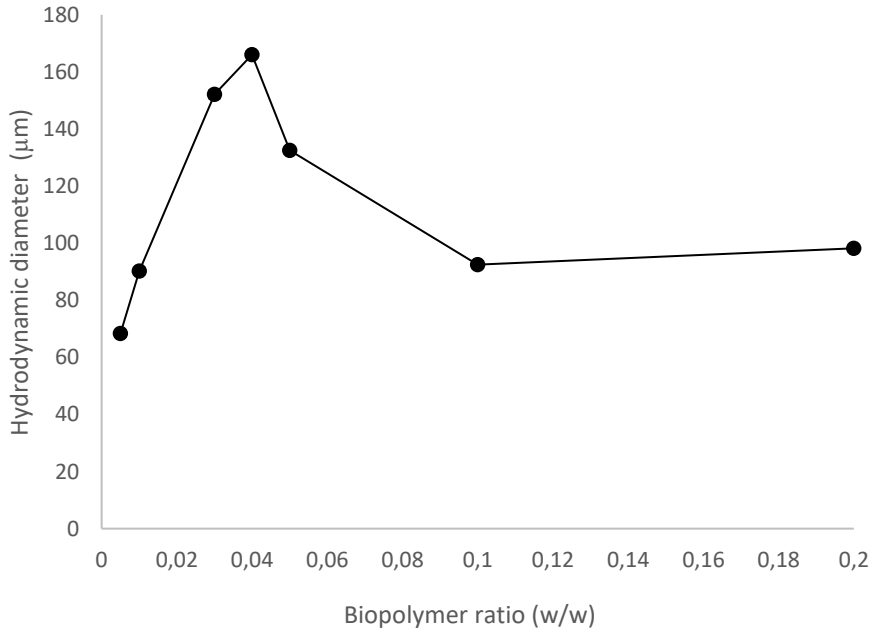


Figure 4.16 Hydrodynamic diameter with biopolymer ratio in the complex formation of $HPPI_{250}$ - LF

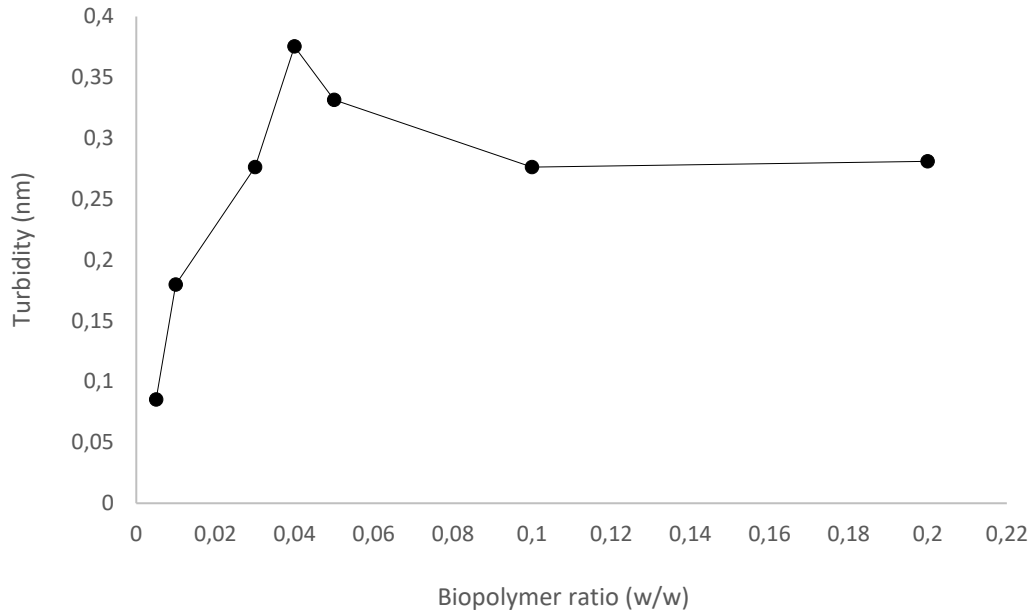


Figure 4.17 Turbidity with biopolymer ratio in the complex formation of $HPPI_{250}$ – LF

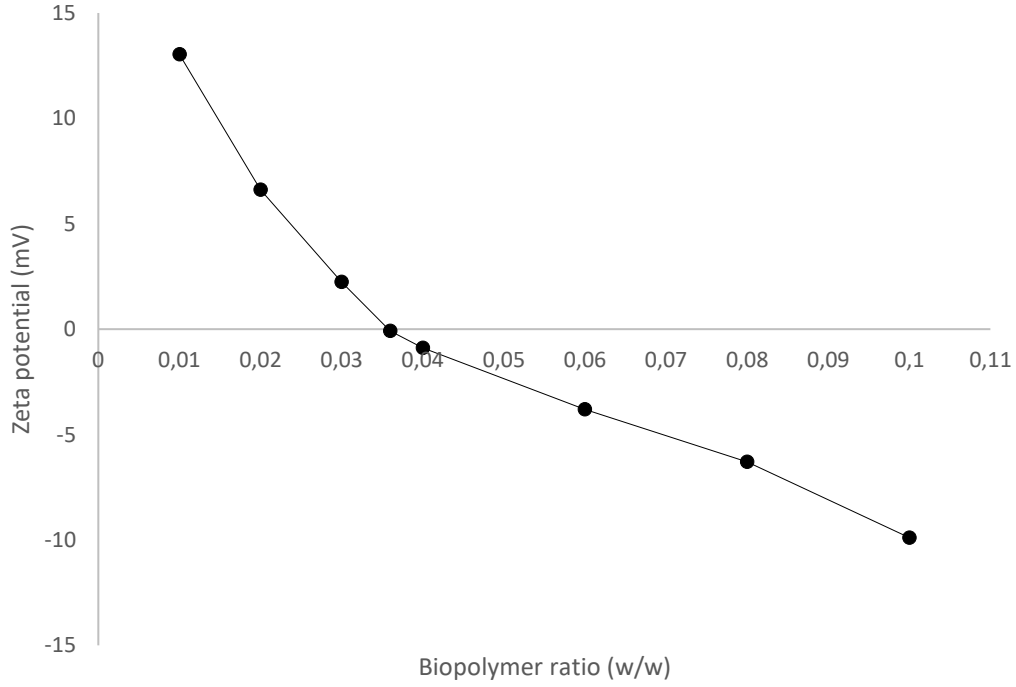


Figure 4.18 Zeta potential with biopolymer ratio in complex formation of $HPPI_{3000}$-LF

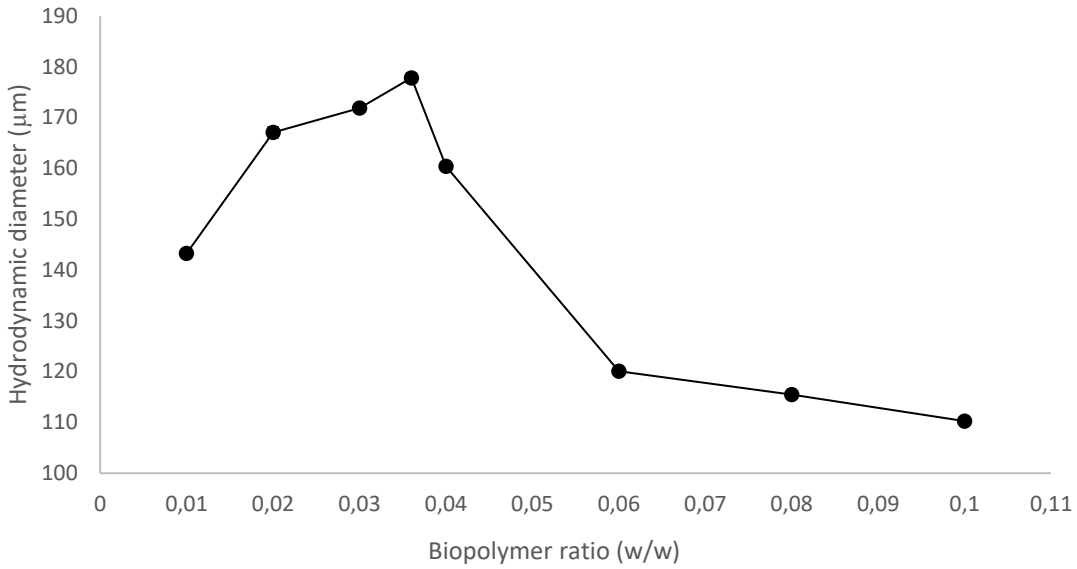
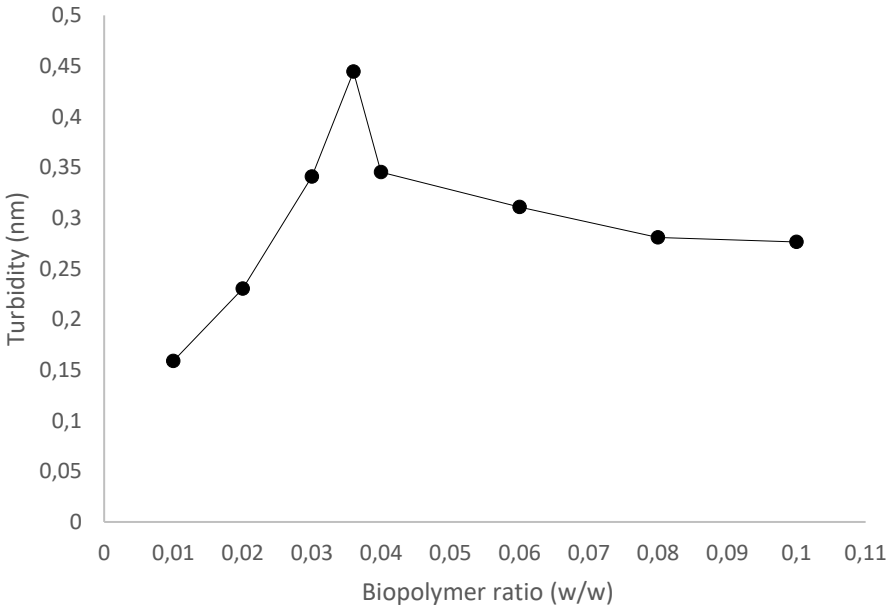


Figure 4.19 Hydrodynamic diameter with biopolymer ratio in complex formation of $HPPI_{3000}$ – LF

According to Figures 4.18, 4.19 and 4.20, the biopolymer ratio of LF and $HPPI_{3000}$ were determined by as 0.2% and 0.036% by weight, respectively. When we look at the complexes formed with hydrolyzed pea protein isolates, there was no significant change in hydrodynamic diameter between $HPPI_{250}$ and $HPPI_{3000}$. However, it was observed that the electrical charge of complexes formed with $HPPI_{3000}$ is higher than the $HPPI_{250}$ (Figure 4.18). Mahmoud et al. (1992), observed that in the enzymatic hydrolysis of casein, as the degree of hydrolysis increases, the electrical interaction of small-sized particles with each other becomes easier and this causes the net charge to decrease. While the degree of hydrolysis is high, although some bonds have disappeared, the interaction of opposite groups with each other is low due to the density of molecular groups, since the protein's globular structure is still preserved. In other words, the increase in the degree of hydrolysis causes the zeta potential to decrease. In addition, the increase in zeta potential provides stability against agglomeration in later emulsion studies (Tamm et al., 2016). On the basis of all these, since the enzyme ratio is higher than $HPPI_{3000}$ in complexes made with $HPPI_{250}$, it can be interpreted in such a way that proteins will be further broken down and the complex formation will be negatively affected. In addition, using less enzymes is economically important (Avramenko et al., 2013).



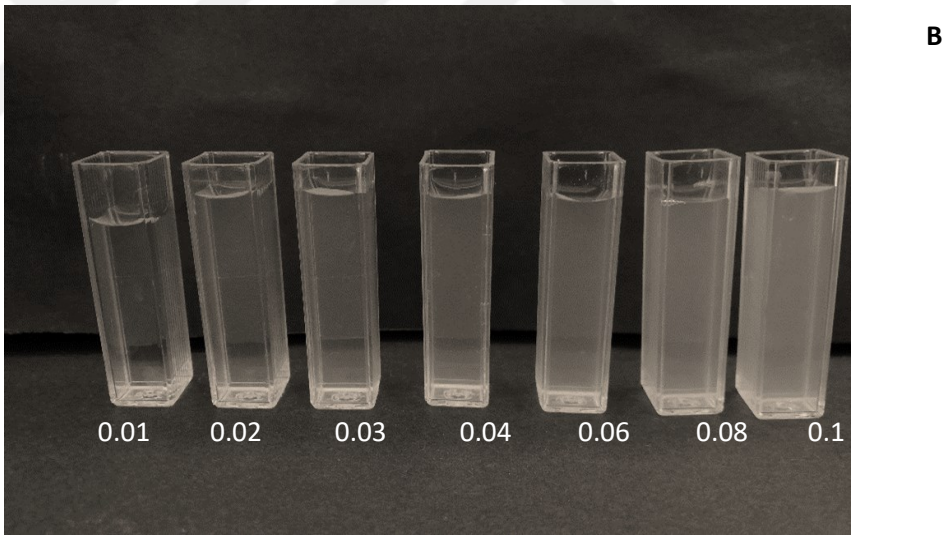


Figure 4.20 A) Turbidity with biopolymer ratio **B)** images of the complex formation with biopolymer ratios $HPPI_{3000}$ – LF

4.2.2 Effect of pH on The Formation of Heteroprotein Complexes

The zeta potential, hydrodynamic diameters and turbidity of the samples at different pHs were investigated to determine the optimum pH for protein-protein complex formation with biopolymer ratios determined. In complexes prepared with $HPPI_{250}$, the electrical charge up to the isoelectric point has a positive value, while it has become negative after this point. In other words, zeta potential value of complexes formed with $HPPI_{250}$ has decreased from + 36mV to -15.6 mV between pH 3-9 and the minimum value has been reached at pH 6.60 (Figure 4.21). This point is determined as the pH where the complex has the lowest solubility, that is, the maximum coacervation (Anema and de Kruif, 2016). The results were also confirmed by hydrodynamic diameter and turbidity measurement. At pH, where the zeta potential is closest to 0, the maximum hydrodynamic diameter was observed as 229.16 µm (Figure 4.22). The maximum turbidity value was 0.207nm and it is shown in (Figure 4.23) that pH is 6.60, where the turbidity is maximum.

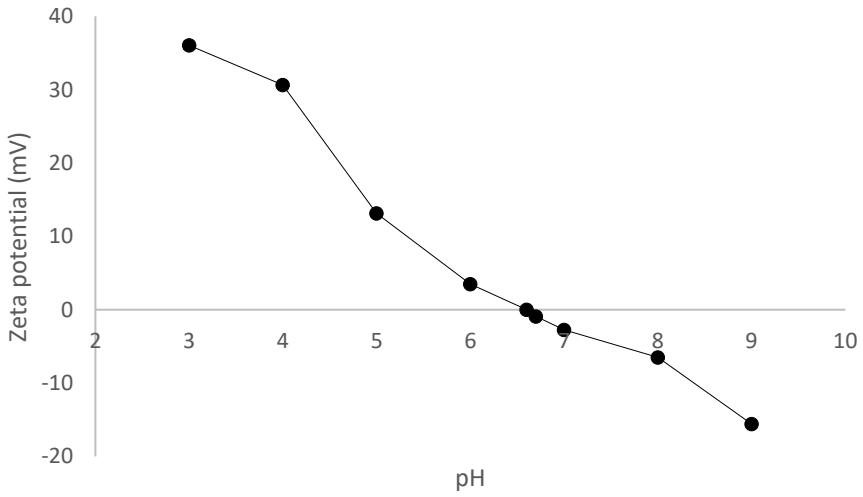


Figure 4.21 Zeta potential in the complex formation of $HPPI_{250}$ – LF with pH

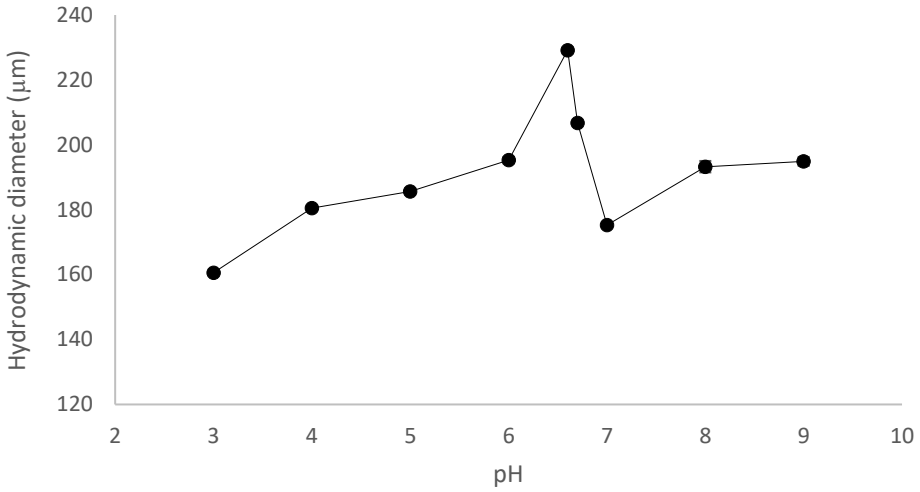


Figure 4.22 Hydrodynamic diameter in complex formation of $HPPI_{250}$ – LF with pH

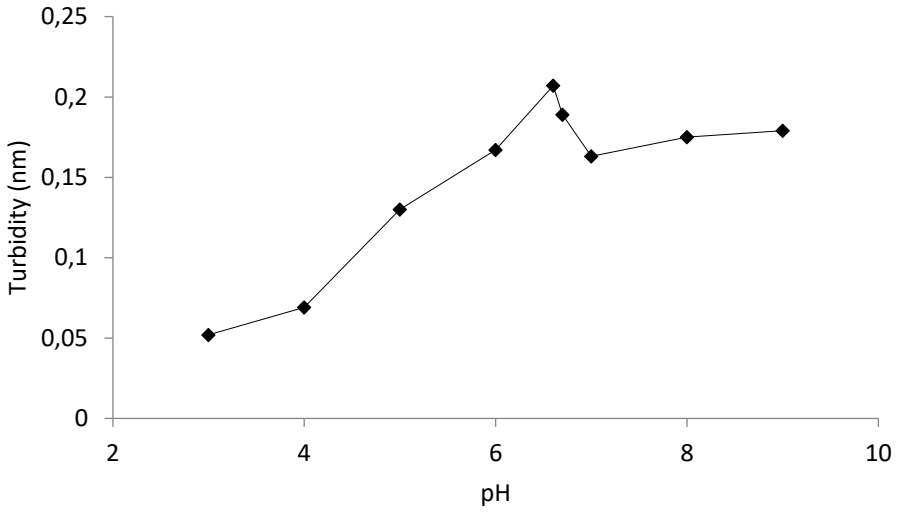


Figure 4.23 Turbidity chart in complex formation of $HPPI_{250}$ -LF with pH

For the complexes made with $HPPI_{3000}$, the zeta potential value has decreased from + 35.3 mV to to -9.18mV between pH 3-9 and the maximum value has been reached at pH 6.90 (Figure 4.24). For the $HPPI_{3000}$-LF complex, this pH was the pH where the solubility was the lowest. The maximum size of the hydrodynamic diameter (168.9µm) was observed at pH 6.90 (Figure 4.25). Similarly maximum turbidity value (0.380 nm) was observed (Figure 4.26) at pH 6.90. Complex formation using $HPPI_{3000}$ and $HPPI_{250}$ have been studied depending on the biopolymer ratio and pH. It was seen that there was not much difference in electrical charge in both examples. When hydrodynamic diameters are compared, it is observed that the complexes formed with $HPPI_{250}$ have larger diameters. It is thought that this was due to the fact that relatively small peptides that appear as the degree of hydrolysis increases, causing the hydrodynamic diameter to be larger (Tamm et al., 2016). The results also correlate with the turbidity obtained.

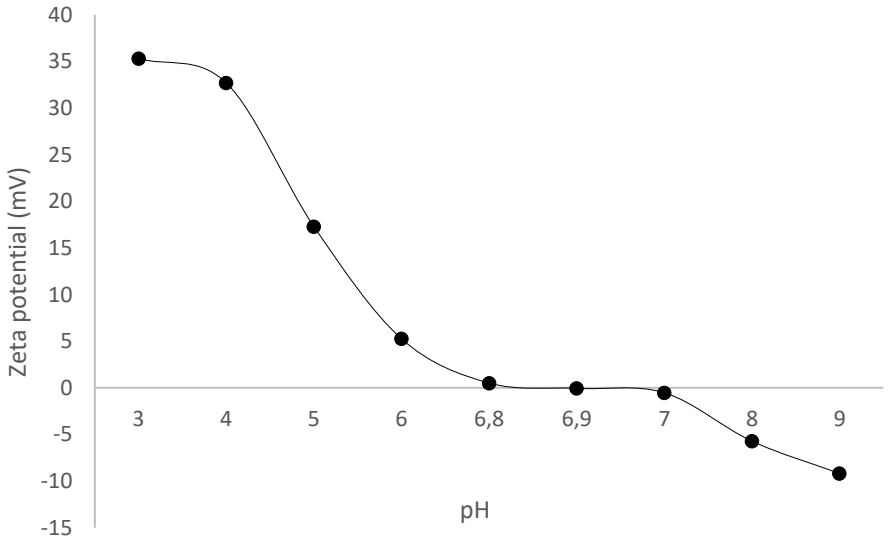


Figure 4.24 Zeta potential in the complex formation of $HPPI_{3000}$ – LF with pH

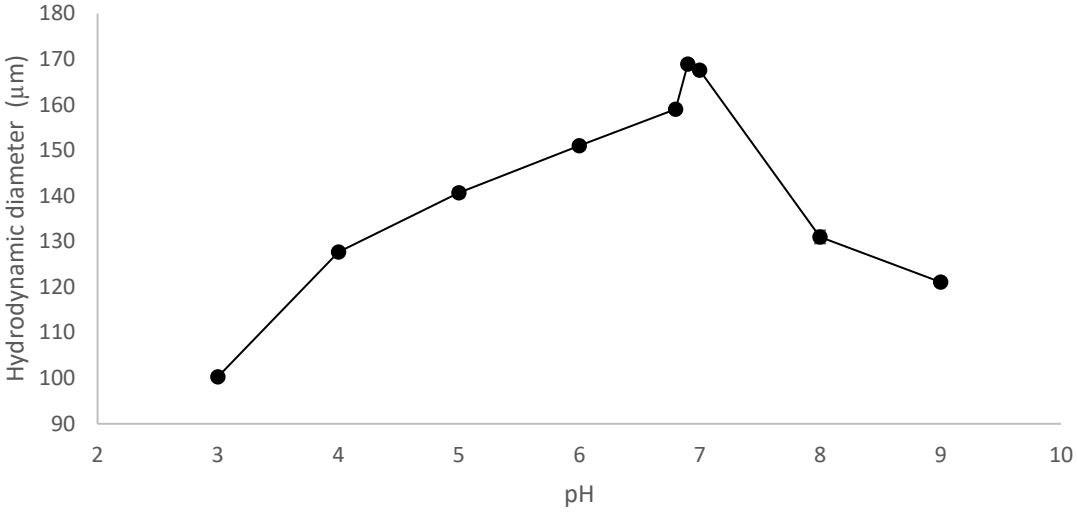


Figure 4.25 Hydrodynamic diameter in complex formation of $HPPI_{3000}$ – LF with pH

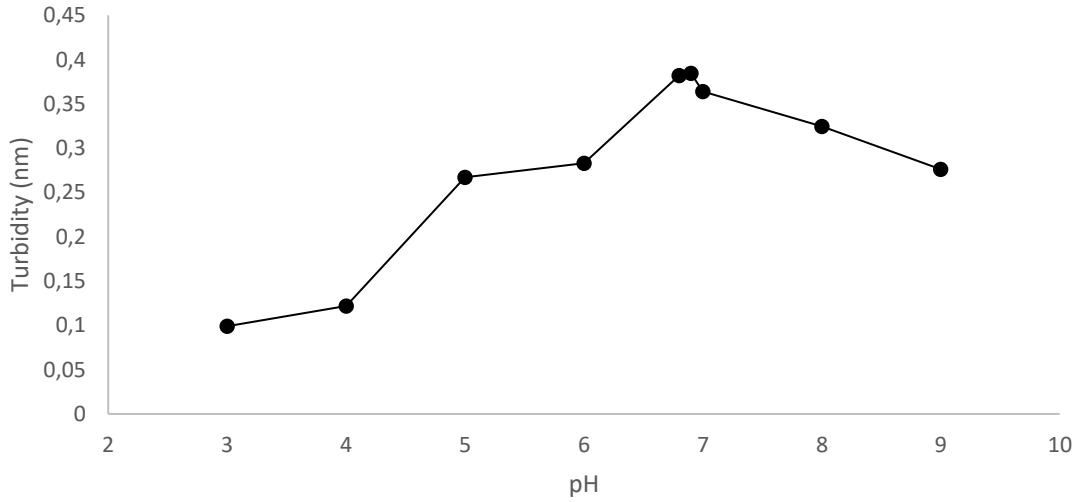


Figure 4.26 Turbidity in complex formation of $HPPI_{3000}$ – LF with pH

4.2.3 The Effect of Ionic Strength on the Protein-Protein Complex Coacervation Formation

The formation of the complex among biopolymers depends on the electrical charge density and the gravity of the polymers. Therefore, pH and ionic strength play a major role in complex formation (de Kruif et al., 2004). For this purpose, the effect of ionic strength on complex formation was examined by keeping the optimum biopolymer ratio and pH fixed for complex formation while changing the ionic intensity of the complex solutions. For this purpose, $HPPI_{3000}$ was selected and the zeta potentials and turbidity of the solutions in different NaCl concentrations (20mM, 50mM, 100mM, 300mM) of between pH 3-12 were examined (Figure 4.27 and Figure 4.28). As seen in Figure 4.27, increasing NaCl concentration decreased the pH value obtained with maximum coacervation. The optimum pH previously determined for the formation of the $HPPI_{3000}$-LF complex was 6.90. The pH was decreased to 6.0 for 20mM ionic strength while for 50-100 and 300 mM ionic strength pH was decreased to 4-5 (Figure 4.27).

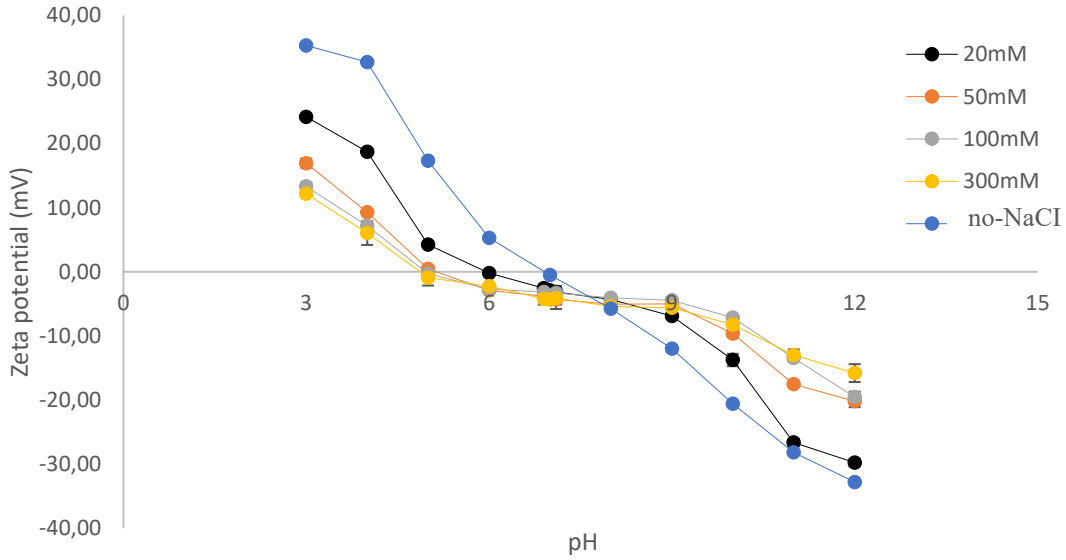


Figure 4.27 Effect of ionic strength on complex formation of $HPPI_{3000}$- LF with pH

According to these results, it was observed that NaCI added even at low concentrations negatively affected the formation of coacervate, and the same result was observed in the turbidity measurements obtained (Figure 4.28). According to Figure 4.28, a low concentration which is the 50mM has the highest turbidity value. This result confirms low concentration ionic strength can affect negatively. Santos et al. (2018a) was investigated the effect of ionic strength on bovine serum albumin and lysosome interaction with 10-50 mM NaCl concentration. It was observed that salt concentration had a negative effect. As a result, pure water was used to prepare solutions in our ongoing study.

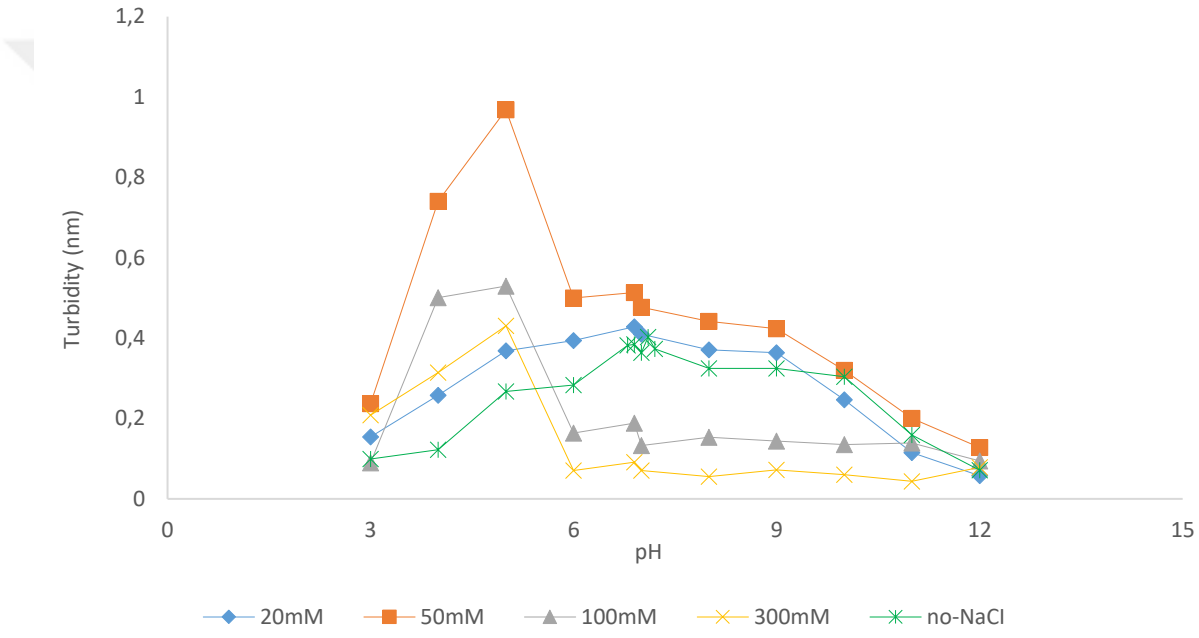


Figure 4.28 Turbidity measurement of different ionic strength on complex formation of $HPPI_{3000}$ - LF

4.3 Emulsion Optimization with Raspberry Seed Oil (RSO)

In our emulsion study, primarily stabilized with lactoferrin, $HPPI_{3000}$ solution was added to the emulsion. In general, pea protein isolate is known to have a lower emulsification property than lactoferrin. Therefore, in this study, the raspberry seed oil (RSO) is first emulsified with LF and added after the $HPPI_{3000}$ solution and the pH was adjusted 6.90. This method is the mostly used method for complex coacervation (Dong et al., 2011, Mendenha et al., 2009). Hydrodynamic diameters and zeta potential of prepared emulsions were determined. Zeta potential was found -1.46±0.45 mV, hydrodynamic diameter was 263,3 ±0,94 nm. Before the emulsion, the complex between LF and $HPPI_{3000}$ had a zeta potential of 0.064 mV and a hydrodynamic diameter of 168.9 nm. Although proteins form complexes, LF was primarily involved in the interface, $HPPI_{3000}$ molecules may be located on the outer surface of the droplet. This has caused the electrical charge to be slightly negative. This change has not been significantly evaluated. It was observed that the protein-protein complex was stable and the electric charge did not change much by emulsifying with raspberry seed oil (RSO). Although the emulsion formed does not change greatly in terms of zeta potential, there has been an increase in the hydrodynamic diameter, which is interpreted as the core material being surrounded and the diameter is larger. The large hydrodynamic diameter indicates that the complex forming biopolymers are interfacially to form emulsions due to their large structure.

Following the zeta potential and hydrodynamic diameters, the droplet size of the emulsion was determined with time in terms of d_{43} (volume diameter to medium) values to examine the emulsion stability. The droplet sizes of the emulsions stabilized with the LF-$HPPI_{3000}$ complex against time were given in Figure 4.29. It was observed that the emulsions obtained were stable and small in size. Optical microscope image also confirmed this result (Figure 4.30). The creaming index was 20% (Figure 4.31).

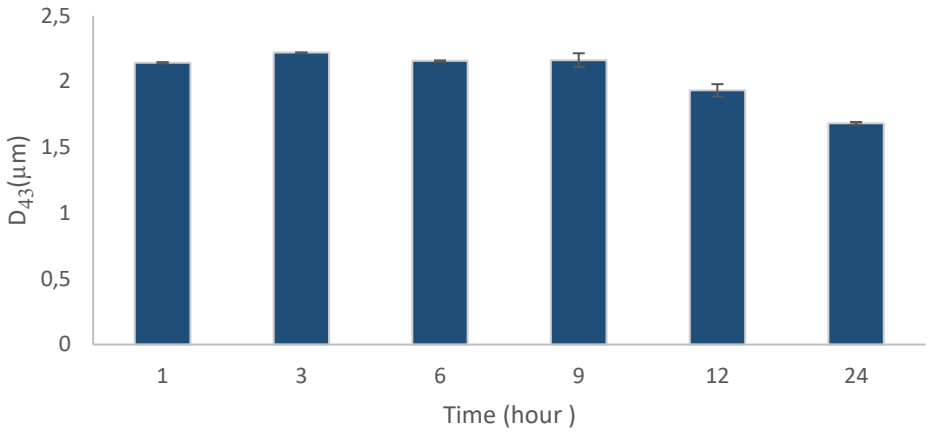


Figure 4.29 Droplet size of the emulsion by the LF - $HPPI_{3000}$ with time

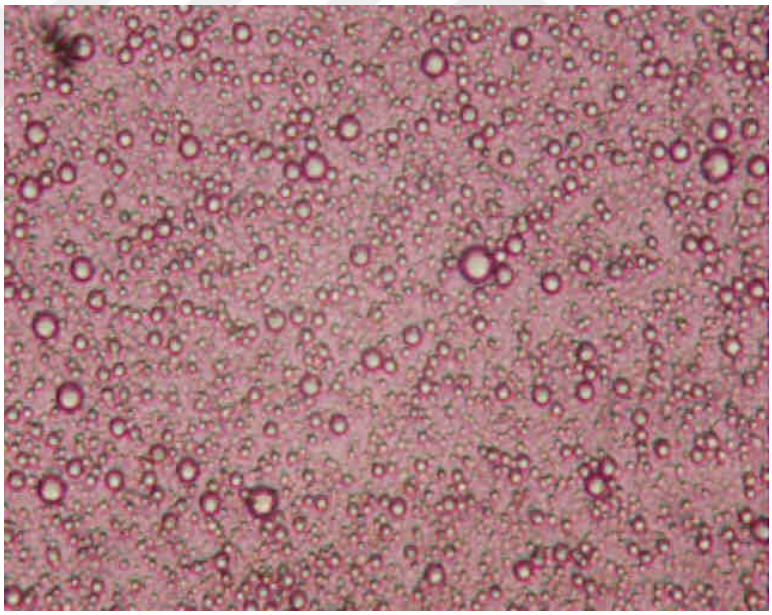


Figure 4.30 Optical microscopic images of the emulsion prepared with RSO by the LF- $HPPI_{3000}$ complex (scale: 75 µm)

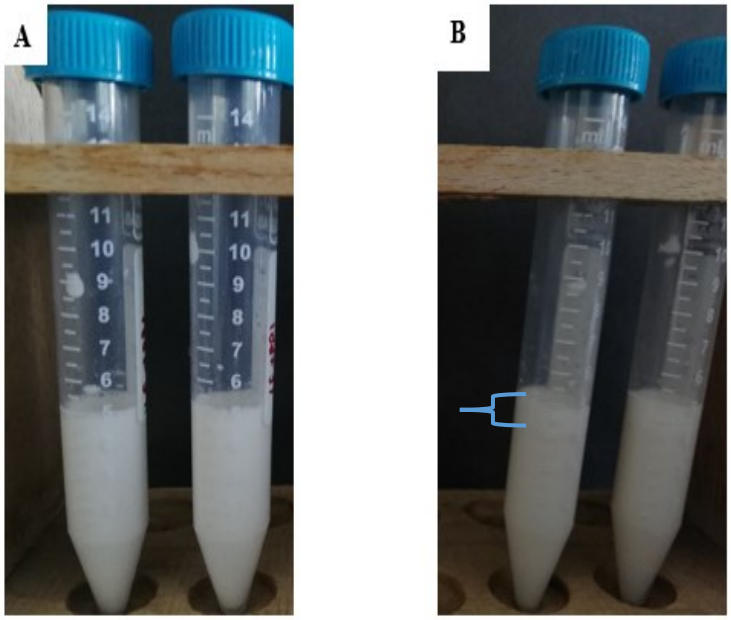


Figure 4.31 Creaming index of the emulsions prepared with RSO by the LF-HPPI$_{3000}$. A) Day 1 and B) Day 7

4.4 Formation of Encapsulated Raspberry Seed Oil

Freeze drying of RSO with complexes LF-HPPI$_{3000}$ was carried out according to the method described by Kermasha et al. (2018). Emulsions prepared in Section 3.2.3 at %10 oil ratio was put into round aluminum plates and placed in a freezer at -22°C for 24 h. Emulsion samples were freeze-dried for 72 h. (Figure 4.32-A). After freeze drying (Figure 4.32-B), the encapsules were immediately put into tightly sealed a glass cups (Figure 4.32-C) and stored at +4 ℃ temperature.

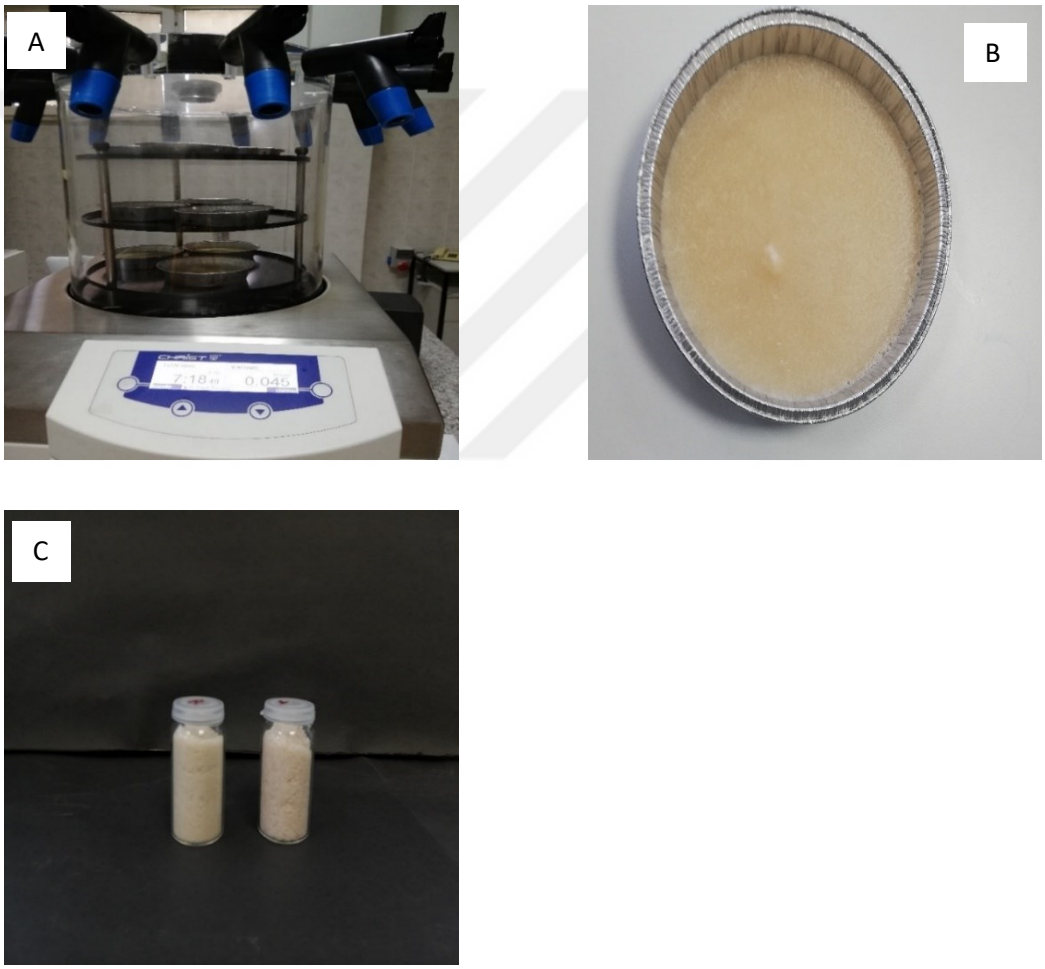


Figure 4.32 A) Image of freeze drying of samples **B)** Emulsion sample after freeze drying **C)** Image of stored glass cups

4.4.1 Moisture Content Determination of Encapsulated RSO

In the encapsulation of oils, the amount of moisture is important. Moisture content is a significant property of products because of that they were relative to stability. (Labuza et al., 1972). During the freeze-drying of the emulsions, water loss, occurred. Since the free water in the structure will accelerate the breakdown of the oil, the moisture content values and water activity values of the powder products are important.

At the latest stages of this thesis, RSO encapsules coated with complexes LF and $HPPI_{3000}$ have been produced. The moisture content of freeze dried powders was as 2.39% (%, d.b.). Similar results were in found with previous studies (Kermasha et al., 2018; Quispe-Condori et al., 2011). Gharsallaoui et al. (2007) found that between 3-4 % the moisture content of the encapsulated formation with lactoferrin. In a study by Domian and Wąsak (2008), they reported that the moisture values of microecapsulated powder products were between 2.5% and 4.8%. They also said that the maximum moisture level in powder products in the food industry is 3% to 4%.

4.4.2 Color Characteristic of Encapsulated RSO

Color analysis of control oil samples and capsules produced from those oils were analyzed. Color values are expressed as L*(lightness/ whiteness), a* (redness/ greenness) and b* (yellowness / blueness). These values of capsulated RSO and RSO samples are given in Table 4.3. Encapsulated RSO has a* value higher than RSO. The a* value originated from the pink color of the lactoferrin powder. Negative a* values indicate the green color of the oils and positive b* values indicate the yellow color of the oils. The b* value approaches the yellow color and this might be due to the yellow color of the RSO (Figure 4.33). Encapsulated RSO was lighter than RSO as indicated in L* value.

Table 4.2 Color values of encapsulated RSO and RSO

Values	Encapsulated Rso	Rso
L*	79.99±0.0081	1.45 ±0.09
a*	3.65±0.0125	-0.8±0.12
b*	18.93±0.0424	1.25 ±0.07
YI	40.35±0.0543	35.23 ± 1.79

Figure 4.33 Image of freeze-dried encapsulated RSO

4.4.3 Encapsulation Efficiency

Encapsulation efficiency (EE) is one of the major parameters for demonstrating the success of encapsulated powder after freeze drying. The drying method can affect the encapsulation efficiency according to studying by Wilkowska et al. (2016). In their study, they used two different drying methods (spray and freeze-drying) and they found that the spray-dried method had less efficiency. Quispe-Condori et al. (2011) found higher efficiency (70-78%) for zein encapsulated flaxseed oil by freeze-drying.

EE is determined by dividing the mass of the core material encapsulated in the wall material by the core material utilized in the formulation. The mass of the free oil (non-encapsulated or surface oil) ought to be low for higher efficiency values of encapsulation to be attained. On the other hand, the mass of the completely encapsulated oil needs to have high values. It is important to determine the surface oil because it can happen to oxidize fast and this means that high surface oil is related to weak stability and flavor of encapsules (Kaushik et al., 2015). Low surface oil content is of great importance in terms of shelf life due to the oxidative stability of encapsulated products. The encapsulation efficiency was calculated by analyzing the surface oil and total oil of the obtained powders. Encapsulation efficiency (EE%) of RSO with LF-HPPI$_{3000}$ complex coacervation encapsulated was 53.7% for samples prepared at optimum conditions.

CHAPTER V

CONCLUSION

In the first part of the study, the pea protein isolate has been hydrolyzed and its solubility has been increased from about 30% to 87%. Then, hydrolyzed pea protein with trypsin enzyme was examined in terms of functionality and the most suitable hydrolysis parameters were determined. Firstly, the hydrolysis times were changed to 5, 10, 15, 30, 60 minutes and the effect of the time on the degree of hydrolysis was examined. The degree of hydrolysis has changed in enzyme applications up to 15 minutes, but no major change has been observed after 15 minutes. For this reason, the hydrolysis time was determined as 15 minute in this study. Different trypsin enzyme/substrate (E/S) ratios were studied to examine the change of degree of hydrolysis. Enzyme/Substrate ratios were changed to 1:250 ($HPPI_{250}$), 1:1000 ($HPPI_{1000}$), 1:2000 ($HPPI_{2000}$), 1:3000 ($HPPI_{3000}$), 1:4000 ($HPPI_{4000}$). The most suitable hydrolyzates were chosen as $HPPI_{250}$ and $HPPI_{3000}$ for the complex formation to a better comparison.

In the second part of the study, formation of the complex with cationic lactoferrin (LF) and anionic hydrolyzed pea protein isolate (HPPI). The most suitable biopolymer ratio and pH were determined for complex and coacervate formation and also the effect of ionic strength and temperature on complex formation was investigated. The results were determined based on the particle size, zeta potential and turbidity. The optimum coacervation conditions obtained from these data were used in emulsion studies. Based on these results, the E/S ratio was chosen as 1:3000 ($HPPI_{3000}$), and the coacervation and emulsion studies continued with $HPPI_{3000}$. The most appropriate biopolymer ratio for the coacervate formation was chosen as 0.036% $HPPI_{3000}$ and 0.2% lactoferrin by weight of and the most suitable pH was determinated 6.90.

In the third part of the study, the oil is first emulsified with LF and added after the $HPPI_{3000}$ solution and the pH was adjusted 6.90. The droplet size, zeta potential, hydrodynamic diameter and stability of the emulsions were examined. Zeta potential was found -1.46±0.45 mV, hydrodynamic diameter was 263,3 ±0,94 nm. Before the emulsion, the complex between LF and $HPPI_{3000}$ had a zeta potential of 0.064 mV. This means that the protein-protein complex was stable and the electric charge did not change much by emulsifying with raspberry seed oil (RSO). The results revealed that the emulsions obtained were stable and small in size. The optical microscope image also confirmed this result.

In the last part of the study, the amount of oil that for the 1 wt% of the protein was determined 10 wt% which followed by freeze-drying of the emulsions. After the freeze-drying, moisture content, color properties, encapsulation efficiency of encapsules produced with RSO were evaluated. Encapsulation efficiency (EE%) of RSO with LF-$HPPI_{3000}$ complex coacervation encapsulated determined was 53.7%.
The results confirmed that encapsulate formation was achieved by hetero protein complexes and coacervates and of oils has successfully coating.

All results confirmed that lactoferrin and hydrolyzed pea protein isolate can formation complexes and coacervates. Furthermore, the appropriate emulsion and electrostatic interaction based method for the formation of LF-HPPI coated omega-rich oils increasing their usage in the food industry.

REFERENCES

Abeywardena, M. Y., and Head, R. J. 2001. Longchain n− 3 Polyunsaturated Fatty Acids and Blood Vessel Function. *Cardiovascular research*, **523,** 361-371.

Adal, E., Sadeghpour, A., Connell, S., Rappolt, M., Ibanoglu, E., & Sarkar, A. (2017). Heteroprotein Complex Formation Of Bovine Lactoferrin And Pea Protein Isolate: A Multiscale Structural Analysis. *Biomacromolecules*, ***18*(2)**, 625-635.

Aiking, H., 2011. Future Protein Supply. *Trends In Food Science And Technology*, **22**: 112120.

Alkin, E. Laktoferrin ve Gidalarda Kullanimi. Gıda ve Yem Bilimi Teknolojisi Dergisi, **(10)**.

Anema, S. G., De Kruif, C. K. (2016). Phase Separation And Composition Of Coacervates Of Lactoferrin And Caseins. *Food Hydrocolloids*, ***52***, 670-677.

Anema, S. G., de Kruif, C. G. (2011). Interaction of Lactoferrin and Lysozyme with Casein Micelles. *Biomacromolecules*, **12(11)**, 3970-3976.

Anema, S. G., And De Kruif, C. G. (2012). Coacervates Of Lactoferrin and Caseins. *Soft Matter*, **8(16)**, 4471-4478.

Anema, S. G., De Kruif, C. G. (2014). Complex Coacervates of Lactotransferrin and Beta-Lactoglobulin. *Journal Of Colloid And Interface Science*, **430**, 214-220.

AOAC. (1995). Official methods of analysis of Association of Official Analytical Chemists Washington, DC, USA.

Asgar, M. A., Fazilah, A., Huda, N., Bhat, R., Karim, A. A. (2010). Nonmeat Protein Alternatives as Meat Extenders and Meat Analogs. *Comprehensive Reviews in Food Science and Food Safet*y, **9(5**), 513-529.

Avramenko, N. A., Low, N. H., Nickerson, M. T. (2013). The Effects Of Limited Enzymatic Hydrolysis on the Physicochemical and Emulsifying Properties of A Lentil Protein Isolate. *Food Research International*, **51(1)**, 162-169.

Bachtsi, A. R., & Kiparissides, C. (1996). Synthesis and Release Studies Of Oil-Containing Poly (Vinyl Alcohol) Microcapsules Prepared by Coacervation. *Journal of Controlled Release*, ***38*(1)**, 49-58.

Bada, J. C., Leon-Camacho, M., Copovi, P., and Alonso, L. (2014). Characterization of berry and currant seed oils from asturias, spain. *International journal of food properties*, ***17*(1),** 77-85.

Bajaj, P. R., Bhunia, K., Kleiner, L., Joyner, H. S., Smith, D., Ganjyal, G., and Sablani, S. S. (2017). Improving functional properties of pea protein isolate for microencapsulation of flaxseed oil. *Journal of microencapsulation*, ***34*(2)**, 218-230.

Bakry, A. M., Abbas, S., Ali, B., Majeed, H., Abouelwafa, M. Y., Mousa, A., and Liang, L. (2016). Microencapsulation of oils: A comprehensive review of benefits, techniques, and applications. *Comprehensive reviews in food science and food safety*, ***15*(1)**, 143-182.

Balassa, B. (1977). 'Revealed'comparative advantage revisited: An analysis of relative export shares of the industrial countries, 1953–1971. *The Manchester School*, **45(4),** 327-344.

Barac, M., Cabrilo, S., Pesic, M., Stanojevic, S., Zilic, S., Macej, O., and Ristic, N. (2010). Profile and functional properties of seed proteins from six pea (Pisum sativum) genotypes. *International Journal of Molecular Sciences*, **11(12)**, 4973-4990.

Barac, M., Pesic, M., Stanojevic, S., Kostic, A., and Cabrilo, S. (2015). Technofunctional properties of pea (Pisum sativum) protein isolates: A review. *Acta Periodica Technologica* **(46)**, 1-18.

Bengoechea, C., Jones, O. G., Guerrero, A., and McClements, D. J. (2011). Formation and characterization of lactoferrin/pectin electrostatic complexes: Impact of composition, pH and thermal treatment. *Food Hydrocolloids*, **25(5)**, 1227-1232.

Bokkhim, H., Bansal, N., Grøndahl, L., and Bhandari, B. (2015). Interactions between different forms of bovine lactoferrin and sodium alginate affect the properties of their mixtures. *Food Hydrocolloids*, ***48*****,** 38-46.

Bokkhim, H., Bansal, N., Grøndahl, L., and Bhandari, B. (2016). In-vitro digestion of different forms of bovine lactoferrin encapsulated in alginate micro-gel particles. *Food Hydrocolloids*, ***52***, 231-242.

BORA, PUSHKAR S.; BREKKE, CLARK J.; POWERS, JOSEPH R. Heat induced gelation of pea (Pisum sativum) mixed globulins, vicilin and legumin. *Journal of Food Science*, 1994, **59(3)**, 594-596.

Boye, J., Zare, F., and Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International*, **43(2)**, 414-431.

Bruschi, M. L., Cardoso, M. L. C., Lucchesi, M. B., and Gremião, M. P. D. (2003). Gelatin microparticles containing propolis obtained by spray-drying technique: preparation and characterization. *International journal of pharmaceutics*, ***264*****(1-2)**, 45-55.

Yu, C., Wang, W., Yao, H., and Liu, H. (2007). Preparation of phospholipid microcapsule by spray drying. *Drying Technology*, ***25*****(4),** 695-702.

Can Karaca, A., Nickerson, M. T., and Low, N. H. (2011). Lentil and chickpea protein-stabilized emulsions: Optimization of emulsion formulation. *Journal of agricultural and food chemistry*, ***59*****(24),** 13203-13211.

Chao, D., Jung, S., and Aluko, R. E. (2018). Physicochemical and functional properties of high pressure-treated isolated pea protein. *Innovative Food Science & Emerging Technologies,* **45**, 179-185.

Chiu, Y. T., Chiu, C. P., Chien, J. T., Ho, G. H., Yang, J., and Chen, B. H. (2007). Encapsulation of lycopene extract from tomato pulp waste with gelatin and poly (γ-glutamic acid) as carrier. *Journal of agricultural and food chemistry*, ***55*****(13)**, 5123-5130.

Comunian, T. A., and Favaro-Trindade, C. S. (2016). Microencapsulation using biopolymers as an alternative to produce food enhanced with phytosterols and omega-3 fatty acids: A review. *Food hydrocolloids*, **61**, 442-457.

Croguennec, T., Li, N., Phelebon, L., Garnier-Lambrouin, F., and Gésan-Guiziou, G. (2012). Interaction between lactoferrin and casein micelles in skimmed milk. *International dairy journal*, ***27*****(1-2),** 34-39.

Croguennec, T., Tavares, G. M., and Bouhallab, S. (2017). Heteroprotein complex coacervation: A generic process. *Advances in colloid and interface science*, ***239***, 115-126.

Curtis, J. M., Berrigan, N., and Dauphinee, P. (2008). The determination of n-3 fatty acid levels in food products containing microencapsulated fish oil using the one-step extraction method. Part 1: Measurement in the raw ingredient and in dry powdered foods. *Journal of the American Oil Chemists' Society*, ***85*****(4),** 297-305.

de Jong, B. (1929). Coacervation. In *Proc. Royal Acad. Amsterdam* (Vol. 32, pp. 849-856).

de Kruif, C. G., Weinbreck, F., and de Vries, R. (2004). Complex coacervation of proteins and anionic polysaccharides. *Current Opinion in Colloid & Interface Science*, **9(5)**, 340-349.

de Vos, P., Faas, M. M., Spasojevic, M., and Sikkema, J. (2010). Encapsulation for preservation of functionality and targeted delivery of bioactive food components. *International Dairy Journal*, **20(4**), 292-302.

Desai, K. G. H., and Jin Park, H. (2005). Recent Developments in Microencapsulation of Food Ingredients. *Drying Technology*, **23(7),** 1361-1394.

Desfougeres, Y., Croguennec, T., Lechevalier, V., Bouhallab, S., and Nau, F. (2010). Charge and size drive spontaneous self-assembly of oppositely charged globular proteins into microspheres. *The Journal of Physical Chemistry B*, ***114*****(12)**, 4138-4144.

Devi, N., Sarmah, M., Khatun, B., and Maji, T. K. (2017). Encapsulation of active ingredients in polysaccharide–protein complex coacervates. *Advances in colloid and interface science*, **239**, 136-145.

Dimic, E., Vujasinovic, V., Radocaj, O., and Pastor, O. (2012). Characteristics of blackberry and raspberry seeds and oils. *Acta periodica technologica* **(43)**, 1-9.

Domian, E., and Wasak, I. (2008). Microencapsulation of rapeseed oil based on the spray drying method. *Polish journal of food and nutrition sciences*, **58(4)**.

Dong, Z., Ma, Y., Hayat, K., Jia, C., Xia, S., and Zhang, X. 2011. Morphology and release profile of microcapsules encapsulating peppermint oil by complex coacervation. *Journal of Food Engineering*, **1043**, 455-460.

Dordevic, V., Balanc, B., Belscak-Cvitanovic, A., Levic, S., Trifkovic, K., Kalusevic, A., Kostic, I., Komes, D., Bugarski, B., and Nedovic, V. (2015). Trends in Encapsulation Technologies for Delivery of Food Bioactive Compounds. *Food Engineering Reviews*, **7(4**), 452-490.

Drusch, S., Regier, M., and Bruhn, M. (2012). Recent advances in the microencapsulation of oils high in polyunsaturated fatty acids. In *Novel Technologies in Food Science* (pp. 159-181). Springer, New York, NY.

Drusch, S., Serfert, Y., Van Den Heuvel, A., and Schwarz, K. (2006). Physicochemical characterization and oxidative stability of fish oil encapsulated in an amorphous matrix containing trehalose. *Food Research International*, ***39*(7),** 807-815.

Dubey, R., Shami, T. C., Rao, K. B., Yoon, H., and Varadan, V. K. (2009). Synthesis of polyamide microcapsules and effect of critical point drying on physical aspect. *Smart materials and structures*, **18(2)**, 025021.

Ducel, V., Richard, J., Popineau, Y., and Boury, F. (2005). Rheological interfacial properties of plant protein− arabic gum coacervates at the oil−water interface. *Biomacromolecules*, ***6*(2)**, 790-796.

Eghbal, N., and Choudhary, R. (2018). Complex coacervation: Encapsulation and controlled release of active agents in food systems. *Lwt*, **90**, 254-264.

Eratte, D., McKnight, S., Gengenbach, T. R., Dowling, K., Barrow, C. J., and Adhikari, B. P. (2015). Co-encapsulation and characterisation of omega-3 fatty acids and probiotic bacteria in whey protein isolate–gum Arabic complex coacervates. *Journal of functional foods*, **19**, 882-892.

Ercelebi, E. A., and Ibanoğlu, E. (2009). Characterization of phase separation behavior, emulsion stability, rheology, and microstructure of egg white–polysaccharide mixtures. *Journal of food science*, ***74*(6)**, C506-C512.

Espinosa-Andrews, H., Báez-González, J. G., Cruz-Sosa, F., and Vernon-Carter, E. J. (2007).Gumarabic−chitosan,complex,coacervation. *Biomacromolecules*, ***8*(4),** 1313-1318.

F. Gibbs, Selim Kermasha, Inteaz Alli, Catherine N.Mulligan, B.(1999). Encapsulation in the food industry: a review. *International journal of food sciences and nutrition*, ***50*(3)**, 213-224.

Fang, Y., Li, L., Inoue, C., Lundin, L., and Appelqvist, I. (2006). Associative and segregative phase separations of gelatin/κ-carrageenan aqueous mixtures. *Langmuir*, ***22*(23),** 9532-9537.

Flanagan, S. E., Malanowski, A. J., Kizilay, E., Seeman, D., Dubin, P. L., Donato-Capel, L., Bovetto, L., and Schmitt, C. (2015). Complex Equilibria, Speciation, and Heteroprotein Coacervation of Lactoferrin and beta-Lactoglobulin. *Langmuir*, **31(5)**, 1776-1783.

Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A., and Saurel, R. (2007). Applications of spray-drying in microencapsulation of food ingredients: An overview. *Food research international*, **40(9)**, 1107-1121.

González-Chávez, S. A., Arévalo-Gallegos, S., and Rascón-Cruz, Q. (2009). Lactoferrin: structure, function and applications. *International journal of antimicrobial agents*, **33(4)**, 301e1.

Gosh, S. K. (2006). Functional coatings by polymer microencapsulation.

Gouin, S. (2004). Microencapsulation: industrial appraisal of existing technologies and trends. *Trends in food science & technology*, **15(7-8),** 330-347.

Gulão, E. D. S., de Souza, C. J., da Silva, F. A., Coimbra, J. S., and Garcia-Rojas, E. E. 2014. Complex coacervates obtained from lactoferrin and gum arabic: Formation and characterization. *Food research international*, **65**, 367-374.

Humiski, L. M., and Aluko, R. E. (2007). Physicochemical and bitterness properties of enzymatic pea protein hydrolysates. *Journal of Food Science*, **72(8)**, S605-S611.

Jayanudin, J., Rochmadi, R., Renaldi, M. K., and Pangihutan, P. The Influence Of Coating Material Difference Against Encapsulation Efficiency Of Red Ginger Oleoresin. *Alchemy Jurnal Penelitian Kimia*, **13(2),** 274-286.

Jiang, J., Xiong, Y. L., and Chen, J. 2010. pH shifting alters solubility characteristics and thermal stability of soy protein isolate and its globulin fractions in different pH, salt concentration, and temperature conditions. *Journal of agricultural and food chemistry,* **5813**, 8035-8042.

Jun-xia, X., Hai-yan, Y., and Jian, Y. (2011). Microencapsulation of sweet orange oil by complex coacervation with soybean protein isolate/gum Arabic. *Food Chemistry*, ***125*(4)**, 1267-1272.

Jyothi, S. S., Seethadevi, A., Prabha, K. S., Muthuprasanna, P., and Pavitra, P. (2012). Microencapsulation: a review. *International Journal Of Pharmacy And Biological Sciences,* **3**, 509-531.

Kaushik, V., and Roos, Y. H. (2007). Limonene encapsulation in freeze-drying of gum Arabic–sucrose–gelatin systems. *LWT-Food Science and Technology*, ***40*(8),** 1381-1391.

Kayitmazer, A. B., Seeman, D., Minsky, B. B., Dubin, P. L., and Xu, Y. 2013. Protein–polyelectrolyte interactions. *Soft Matter*, **99**, 2553-2583.

Kermasha, S., Aziz, S., Gill, J., and Neufeld, R. (2018). Microencapsulation of esterified krill oil, using complex coacervation. *Journal of microencapsulation*, ***35*(1),** 36-48.

Kim, S.Y., Park, P.S.W. and Rhee, K.C. 1990. Functional properties of proteolytic enzyme modified soy protein isolate. *Journal of Agricultural and Food Chemistry*, **38**, 651–656.

Kizilay, E., Kayitmazer, A. B., and Dubin, P. L. (2011). Complexation and coacervation of polyelectrolytes with oppositely charged colloids. *Advances in Colloid and Interface Science*, **167(1-2)**, 24-37.

Klaypradit, W., and Huang, Y. W. (2008). Fish oil encapsulation with chitosan using ultrasonic atomizer. *LWT-Food Science and Technology*, **41(6)**, 1133-1139.

Klost, M., and Drusch, S. (2019). Functionalisation of pea protein by tryptic hydrolysis–Characterisation of interfacial and functional properties. *Food hydrocolloids*, 86, 134-140.

Labuza, TP, McNally, L., Gallagher, D., Hawkes, J, and Hurtado, F. (1972). Stability of intermediate moisture foods. 1. Lipid oxidation. *Journal of Food Science*, **37(1),** 154-159.

Lan, Y., Chen, B., and Rao, J. (2018). Pea protein isolate–high methoxyl pectin soluble complexes for improving pea protein functionality: Effect of pH, biopolymer ratio and concentrations. *Food hydrocolloids*, **80**, 245-253.

Leisner, D., and Imae, T. (2003). Interpolyelectrolyte complex and coacervate formation of poly (glutamic acid) with a dendrimer studied by light scattering and SAXS. *The Journal of Physical Chemistry B*, ***107*(32)**, 8078-8087.

Lesmes, U., and McClements, D. J. (2009). Structure-function relationships to guide rational design and fabrication of particulate food delivery systems. *Trends in Food Science & Technology*, **20(10)**, 448-457.

Liang, H. N., and Tang, C. H. (2013). pH-dependent emulsifying properties of pea [Pisum sativum (L.)] proteins. *Food Hydrocolloids*, **33(2)**, 309-319.

Liu, C., Yang, X.-Q., Lin, M.-G., Zhao, R.-Y., Tang, C.-H., Luo, L., et al. (2011). Complex coacervation of chitosan and soy globulins in aqueous solution: A electrophoretic mobility and light scattering study. *International Journal of Food Science & Technology,* **46,** 1363e1369.

Liu, S., Cao, Y. L., Ghosh, S., Rousseau, D., Low, N. H., and Nickerson, M. T. (2010). Intermolecular interactions during complex coacervation of pea protein isolate and gum Arabic. *Journal of agricultural and food chemistry*, **58(1)**, 552-556.

Lopez, L. M. I., Brullo, A., Natalucci, C. L., Caffini, N. O., Sorgentini, D. A., and Wagner, J. R. (1998). Thermal behavior, solubility and structural properties of soy concentrate hydrolyzed by new plant proteases. *Journal of Food Biochemistry*, **22(2)**, 125–141.

Mahmoud, M. I., MALONE, W. T., and Cordle, C. T. 1992. Enzymatic hydrolysis of casein: effect of degree of hydrolysis on antigenicity and physical properties. *Journal of Food Science*, **575**, 1223-1229.

Manojlović, V., Nedović, V. A., Kailasapathy, K., and Zuidam, N. J. (2010). Encapsulation of probiotics for use in food products. In Encapsulation technologies for active food ingredients and food processing (pp. 269-302). Springer, New York, NY.

Mendanha, D. V., Ortiz, S. E. M., Favaro-Trindade, C. S., Mauri, A., Monterrey-Quintero, E. S., and Thomazini, M. 2009. Microencapsulation of casein hydrolysate by complex coacervation with SPI/pectin. *Food Research International*, **428**, 1099-1104.

Miñarro, B., Albanell, E., Aguilar, N., Guamis, B., Capellas, M. 2012. Effect of legume flours on baking characteristics of glutenfree bread. *Journal of Cereal Science*, **56**: 476-481

Mohan, A., Rajendran, S. R., He, Q. S., Bazinet, L., and Udenigwe, C. C. (2015). Encapsulation of food protein hydrolysates and peptides: a review. *Rsc Advances*, **5(97)**, 79270-79278.

Moreland, N., Ashton, R., Baker, H. M., Ivanovic, I., Patterson, S., Arcus, V. L.,and Lott, J. S. (2005). A flexible and economical medium-throughput strategy for protein production and crystallization. Acta Crystallographica Section D: *Biological Crystallography*, **61(10)**, 1378-1385.

Moure, A., Sineiro, J., Domínguez, H., and Parajó, J. C. (2006). Functionality of oilseed protein products: a review. *Food research international*, **39(9)**, 945-963.

Nairm, J. G. (1995). 3 Coacervation-phase separation technology. In *Advances in pharmaceutical sciences* (Vol. 7, pp. 93-219). Academic Press.

Nedovic, V., Kalusevic, A., Manojlovic, V., Levic, S., and Bugarski, B. (2011). An overview of encapsulation technologies for food applications. 11th International Congress on Engineering and Food (Icef11), **1**, 1806-1815.

Newton, D. W. (1991). Coacervation: Principles and applications. *Polymers for Controlled Drug Delivery*, 67-81.

O'Regan, J., and Mulvihill, D. M. (2010). Sodium caseinate–maltodextrin conjugate stabilized double emulsions: Encapsulation and stability. *Food Research International*, **43(1)**, 224-231.

Ochiai, K., Kamata, Y., and Shibasaki, K. 1982. Effect of tryptic digestion on emulsifying properties of soy protein. *Agricultural and Biological Chemistry*, **461**, 91-96.

O'Kane, F. E., Happe, R. P., Vereijken, J. M., Gruppen, H., and van Boekel, M. A. (2004). Heat-induced gelation of pea legumin: comparison with soybean glycinin. *Journal of Agricultural and Food Chemistry*, **52(16)**, 5071-5078.

Oomah, B. D., Ladet, S., Godfrey, D. V., Liang, J., and Girard, B. (2000). Characteristics of raspberry (Rubus idaeus L.) seed oil. *Food Chemistry*, **69(2)**, 187-193.

Overbeek, J. T. G., and Voorn, M. J. (1957). Phase separation in polyelectrolyte solutions. Theory of complex coacervation. *Journal of Cellular and Comparative Physiology*, **49(S1),** 7-26.

Peixoto, P. D., Tavares, G. M., Croguennec, T., Nicolas, A., Hamon, P., Roiland, C., and Bouhallab, S. 2016. Structure and dynamics of heteroprotein coacervates. Langmuir, 3231, 7821-7828.

Qazvini Firouz, A., and Torabi, F. (2012, January). Feasibility study of solvent-based huff-n-puff method (cyclic solvent injection) to enhance heavy oil recovery. In *SPE Heavy Oil Conference Canada*. Society of Petroleum Engineers.

Quispe-Condori, S., Saldaña, M. D. A., and Temelli, F. (2011). Microencapsulation of flax oil with zein using spray and freeze drying. *LWT - Food Science and Technology,* **44(9)**, 1880-1887.

Riediger, N. D., Othman, R. A., Suh, M., and Moghadasian, M. H. (2009). A systemic review of the roles of n-3 fatty acids in health and disease. *Journal of the American Dietetic Association*, ***109*****(4),** 668-679.

Risch, S. J., and Reineccius, G. A. (1995). Encapsulation and controlled release of food ingredients. ACS Symposium Series 590.

Rodríguez, J., Martín, M. J., Ruiz, M. A., and Clares, B. (2016). Current encapsulation strategies for bioactive oils: From alimentary to pharmaceutical perspectives. *Food Research International*, **83**, 41-59.

Santos, M. B., da Costa, A. R., and Garcia-Rojas, E. E. (2018). Heteroprotein complex coacervates of ovalbumin and lysozyme: Formation and thermodynamic characterization. *International Journal of Biological Macromolecules*, **106**, 1323-1329.

Santos, M. G., Bozza, F. T., Thomazini, M., and Favaro-Trindade, C. S. (2015). Microencapsulation of xylitol by double emulsion followed by complex coacervation. *Food Chemistry*, ***171***, 32-39.

Sari, Y. W., Mulder, W. J., Sanders, J. P., and Bruins, M. E. (2015). Towards plant protein refinery: review on protein extraction using alkali and potential enzymatic assistance. *Biotechnology journal*, **10(8)**, 1138-1157.

Schmitt, C., and Turgeon, S. L. (2011). Protein/polysaccharide complexes and coacervates in food systems. *Advances in Colloid and Interface Science*, ***167*****(1-2),** 63-70.

Shi, L., Beamer, S. K., Yang, H., and Jaczynski, J. (2018). Microemulsification/ encapsulation of krill oil by complex coacervation with krill protein isolated using isoelectric solubilization/precipitation. *Food Chemistry*, **244**, 284-291.

Sijtsma, L., Tezera, D., Hustinx, J., and Vereijken, J. M. (1998). Improvement of pea protein quality by enzymatic modification. *Food/Nahrung*, **42(03-04)**, 215-216.

Specos, M. M., García, J. J., Tornesello, J., Marino, P., Vecchia, M. D., Tesoriero, M. D., and Hermida, L. G. (2010). Microencapsulated citronella oil for mosquito

repellent finishing of cotton textiles. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, ***104*****(10),** 653-658.

Steijns, J. M., and Van Hooijdonk, A. C. M. (2000). Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. *British Journal of Nutrition*, **84(S1)**, 11-17.

Taherian, A. R., Mondor, M., Labranche, J., Drolet, H., Ippersiel, D., and Lamarche, F. (2011). Comparative study of functional properties of commercial and membrane processed yellow pea protein isolates. *Food Research International*, **44(8)**, 2505-2514.

Tamm, F., Herbst, S., Brodkorb, A., and Drusch, S. 2016. Functional properties of pea protein hydrolysates in emulsions and spray-dried microcapsules. *Food Hydrocolloids*, **58**, 204-214.

Tavares, Guilherme M., Croguennec, Thomas, Hamon, Pascaline, Carvalho, Antonio F., and Bouhallab, S. (2015). Selective coacervation between lactoferrin and the two isoforms of β-lactoglobulin. *Food Hydrocolloids*, **48**, 238-247.

Timilsena, Y. P., Adhikari, R., Barrow, C. J., and Adhikari, B. (2016). Microencapsulation of chia seed oil using chia seed protein isolate-chia seed gum complex coacervates. *International Journal of Biological Macromolecules*, **91**, 347-357.

Tiwari, A., Bindal, S., and Bohidar, H. B. 2008. Kinetics of protein− protein complex coacervation and biphasic release of salbutamol sulfate from coacervate matrix. *Biomacromolecules*, **101**, 184-189.

Tömösközi, S., Lásztity, R., Haraszi, R., and Baticz, O. (2001). Isolation and study of the functional properties of pea proteins. *Food/Nahrung*, **45(6)**, 399-401.

Turgeon, S. L., Schmitt, C., and Sanchez, C. (2007). Protein–polysaccharide complexes and coacervates. *Current Opinion in Colloid & Interface Science*, ***12*****(4-5),** 166-178.

Van Hoed, V., De Clercq, N., Echim, C., Andjelkovic, M., Leber, E., Dewettinck, K., and Verhé, R. (2009). Berry seeds: a source of specialty oils with high content of bioactives and nutritional value. *Journal of Food Lipids*, ***16*****(1)**, 33-49.

Vila, M. M., Chaud, M. V., and Balcão, V. M. (2015). Microencapsulation of natural anti-oxidant pigments. In Microencapsulation and Microspheres for Food Applications (pp. 369-389). Academic Press.

Wang, B., Adhikari, B., and Barrow, C. J. (2014). Optimisation of the microencapsulation of tuna oil in gelatin–sodium hexametaphosphate using complex coacervation. *Food Chemistry*, ***158***, 358-365.

Wang, Y., Kimura, K., Dubin, P. L., and Jaeger, W. (2000). Polyelectrolyte− micelle coacervation: Effects of micelle surface charge density, polymer molecular weight, and polymer/surfactant ratio. *Macromolecules*, ***33*****(9)**, 3324-3331.

Wani, I. A., Sogi, D. S., Shivhare, U. S., and Gill, B. S. (2015). Physico-chemical and functional properties of native and hydrolyzed kidney bean (Phaseolus vulgaris L.) protein isolates. *Food Research International*, **76**, 11-18.

Weinbreck, F., De Vries, R., Schrooyen, P., and De Kruif, C. G. (2003). Complex coacervation of whey proteins and gum arabic. *Biomacromolecules*, **4(2)**, 293-303.

Weitz, D., Weintraub, H., Fisher, E., and Schwartzbard, A. Z. (2010). Fish oil for the treatment of cardiovascular disease. *Cardiology in Review*, ***18*****(5)**, 258.

Wendel, M., and Heller, A. R. (2009). Anticancer actions of omega-3 fatty acids-current state and future perspectives. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, ***9*****(4),** 457-470.

Wilkowska, A., Ambroziak, W., Czyżowska, A., and Adamiec, J. (2016). Effect of microencapsulation by spray-drying and freeze-drying technique on the antioxidant properties of blueberry (Vaccinium myrtillus) juice polyphenolic compounds. *Polish Journal of Food and Nutrition Sciences*, ***66*****(1)**, 11-16.

Wu, K. G., and Xiao, Q. (2005). Microencapsulation of fish oil by simple coacervation of hydroxypropyl methylcellulose. *Chinese Journal of Chemistry*, **23(11),** 1569-1572.

Xiao, Z., Liu, W., Zhu, G., Zhou, R., and Niu, Y. (2014). A review of the preparation and application of flavour and essential oils microcapsules based on complex

coacervation technology. *Journal of the Science of Food and Agriculture*, ***94*(8)**, 1482-1494.

Yamniuk, A. P., Burling, H., and Vogel, H. J. (2009). Thermodynamic characterization of the interactions between the immunoregulatory proteins osteopontin and lactoferrin. *Molecular immunology*, **46(11-12)**, 2395-2402.

Yan, Y. F., Kizilay, E., Seeman, D., Flanagan, S., Dubin, P. L., Bovetto, L., Donato, L., and Schmitt, C. (2013). Heteroprotein Complex Coacervation: Bovine beta- Lactoglobulin and Lactoferrin. *Langmuir*, **29(50)**, 15614-15623.

Yang, J., Mao, L., Yang, W., Sun, C., Dai, L., and Gao, Y. (2018). Evaluation of non-covalent ternary aggregates of lactoferrin, high methylated pectin, EGCG in stabilizing β-carotene emulsions. *Food chemistry*, ***240*,** 1063-1071.

Yılmaz, C., and Gökmen, V. (2013). Compositional characteristics of sour cherry kernel and its oil as influenced by different extraction and roasting conditions. *Industrial Crops and Products*, ***49***, 130-135.

Yin, S. W., Tang, C. H., Cao, J. S., Hu, E. K., Wen, Q. B., and Yang, X. Q. (2008). Effects of limited enzymatic hydrolysis with trypsin on the functional properties of hemp (Cannabis sativa L.) protein isolate. *Food chemistry*, **106(3),** 1004-1013.

Young, S. L., Sarda, X., and Rosenberg, M. (1993). Microencapsulating properties of whey proteins. 1. Microencapsulation of anhydrous milk fat. *Journal of Dairy Science*, ***76*(10)**, 2868-2877.

Zhang, W., Yan, C., May, J., and Barrow, C. (2009). Whey protein and gum Arabic encapsulated Omega-3 lipids. The effect of material properties on coacervation. *Agro food industry hi-tech,* **20(4)**, 20-23.

Zhao, J. J., Wei, T., Wei, Z. H., Yuan, F., and Gao, Y. X. (2015). Influence of soybean soluble polysaccharides and beet pectin on the physicochemical properties of lactoferrin-coated orange oil emulsion. *Food Hydrocolloids*, **44**, 443-452.

Zheng, J., Gao, Q., Tang, C. H., Ge, G., Zhao, M., and Sun, W. (2020). Heteroprotein complex formation of soy protein isolate and lactoferrin: Thermodynamic formation mechanism and morphologic structure. *Food Hydrocolloids*, **100**, 105415.

Zuidam, N. J., and Shimoni, E. (2010). Overview of Microencapsulates for Use in Food Products or Processes and Methods to Make Them. In N. J. Zuidam andViktor Nedovic (Eds.), *Encapsulation Technologies for Active Food Ingredients and Food Processing* (pp. 3-29). New York, NY: Springer New York.