UTILIZATION OF NATURAL EMULSIFIERS AND THEIR DERIVATIVES TO FORMULATE EMULSION-BASED DELIVERY SYSTEMS FOR HYDROPHOBIC NUTRACEUTICALS

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https://doi.org/10.7275/9950696.0 https://scholarworks.umass.edu/dissertations_2/1007

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UTILIZATION OF NATURAL EMULSIFIERS AND THEIR DERIVATIVES TO FORMULATE EMULSION-BASED DELIVERY SYSTEMS FOR HYDROPHOBIC NUTRACEUTICALS

A Dissertation Presented

by

CANSU EKIN GUMUS

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2017

Food Science
UTILIZATION OF NATURAL EMULSIFIERS AND THEIR DERIVATIVES TO
FORMULATE EMULSION-BASED DELIVERY SYSTEMS FOR
HYDROPHOBIC NUTRACEUTICALS

A Dissertation Presented

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In memory of my mother- Suheyla Unver…

To my beloved family and friends for always supporting, and helping me.

“Listen to the mustn`ts child, listen to the don`ts.
Listen to the shouldn`ts, the impossibles, the won`ts.
Listen to the never haves, then listen close to me.
Anything can happen, child. Anything can be.”

Shel Silverstein
ACKNOWLEDGMENTS

First of all, I would like to thank the Ministry of National Education of Turkey for the International Graduate Fellowship provided for my doctoral degree and my former professor Dr. Aziz Tekin at Ankara University (Turkey) for encouraging me to accept this scholarship to pursue my doctoral degree abroad. Furthermore, I am really thankful to Dr. Decker and Dr. McClements for accepting me to their research groups and supporting me throughout my entire doctoral studies. I want to thank my other committee members, Dr. Corradini and Dr. Liu, for guiding me at all stages of my dissertation.

I would also like to thank the current and past lab mates, especially Jen Komaiko for her precious friendship, for always taking the time to proofread my works and listen to my practice presentations, Gabriel Davidov for being a great mentor and sharing his expertise with me, Sandra Ebert for her support and friendship whenever I needed, Ruojie and Zipei Zhang for always being there to help me. My special thanks go to Jean Alamed for the guidance, support, and help she provided to me and other lab members; without her I wouldn’t be able to finish my research this smoothly and as planned. Furthermore, I will always appreciate the support of our department secretary Fran Kostek, who always helped me with troubles I had outside my research.

I want to thank my loving family, and friends, who encouraged me to study abroad and have supported me over the past five years. I would like to thank my father- Alaettin Gumus- for raising me as an independent and strong character and for always believing in me, and my step-mother Sevgi Gumus for listening to me and
calming me down at times I needed to vent. Most importantly, I am extremely thankful to my little sister Ela Gumus, who was two years old when I left home to start my degree. She has been very patient and mature in the period of my studies despite her age. Her presence has been sometimes the only thing that has kept me sane and hopeful. It has not been easy to overcome the stress of a PhD life being away from home and family, so I also want to thank Emrah Ilker Ozay and Noam Goldstein for being my family away from home. Last but not least, I would like to thank Ceyda Hafizoglu, Yagmur Kocak, and Nesrin Senbil for always being there to talk and listen to me when I needed despite the time differences.

The research that is made for my doctoral dissertation is based upon work supported by the Cooperative State Research, Extension, Education Service, Massachusetts Agricultural Experiment Station (Project No. 831), and by the United States Department of Agriculture, NRI Grants (2011-03539, 2013-03795, 2011-67021, and 2014-67021), as well as by Agriculture and Food Research Initiative Grant no. 2014-67017-21635 from the USDA National Institute of Food and Agriculture. It was also partly supported from funding provided by DSM.
ABSTRACT

UTILIZATION OF NATURAL EMULSIFIERS AND THEIR DERIVATIVES TO FORMULATE EMULSION-BASED DELIVERY SYSTEMS FOR HYDROPHOBIC NUTRACEUTICALS

MAY 2017

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Directed by: Distinguished Professor David J. McClements

There is increasing consumer demand for food products that are more natural, sustainable, and environmentally friendly. Industry has responded by trying to identify natural alternatives to synthetic functional ingredients within these products. In this study, the ability of Maillard conjugation products, and several legume proteins were investigated to act as nature-derived or natural emulsifiers in oil-in-water emulsions fortified with hydrophobic nutraceuticals.

Casein-coated oil droplets enriched with lutein were highly unstable to flocculation near their isoelectric point due to the reduction in electrostatic repulsion. However, casein-dextran-coated droplets were stable, which was attributed to strong steric repulsion by the dextran moiety. The casein-coated droplets were unstable to aggregation in the gastric phase of a simulated gastrointestinal tract (GIT), whereas the casein-dextran-coated ones were still stable, which was again attributed to
increased steric repulsion. Emulsifier type did not strongly influence lutein bioaccessibility.

Pea, lentil, and faba bean protein concentrates all proved to be effective emulsifiers for forming and stabilizing 10 wt% oil-in-water emulsions produced by high-pressure homogenization. The droplet size decreased with increasing emulsifier concentration, and relatively small oil droplets ($d < 0.3$ mm) could be formed. Lentil protein-coated droplets were the most stable to environmental stresses such as pH, ionic strength and temperature changes. Our results showed that there were no significant differences in the free fatty acid release in the small intestine phase among these systems and a whey protein-stabilized emulsion, with the emulsified lipids being rapidly and fully digested in all cases. Overall the emulsions formed using whey protein, that had smaller particle sizes than the others, were slightly more stable to lipid oxidation during the period of storage. Blocking the free sulfhydryl groups of proteins did not affect their ability to inhibit lipid oxidation in emulsion systems.

These results have important implications for the production of functional foods and beverages from natural plant-based ingredients and Maillard conjugates that can improve the stability of emulsions without adversely affecting the bioaccessibility of the bioactive agent.
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CHAPTER 1

NATURAL EMULSIFIERS – BIOSURFACTANTS, PHOSPHOLIPIDS, BIOPOLYMERS, AND COLLOIDAL PARTICLES: MOLECULAR AND PHYSICOCHEMICAL BASIS OF FUNCTIONAL PERFORMANCE

1.1 Introduction

Oil-in-water emulsions are an integral part of many commercial products used in the food, supplements, personal care, cosmetic, detergent, and pharmaceutical industries [1-3]. The lipid droplets in these emulsion-based products strongly contribute to their desirable physicochemical and sensory attributes, such as appearance, texture, stability, and interactions with the human body [4]. For example, the addition of lipid droplets to an aqueous solution increases its turbidity and viscosity. The lipid droplets in emulsions may also be utilized as delivery systems to encapsulate, protect, and release non-polar active ingredients, such as hydrophobic colors, flavors, vitamins, nutrients, nutraceuticals, pharmaceuticals, antimicrobials, and antioxidants [3, 5-7]. Oil-in-water emulsions are thermodynamically unstable systems that consist of small lipid droplets dispersed within an aqueous medium. To produce commercial products with sufficiently long shelf lives and with resistances to the environmental stresses they may encounter during their utilization it is necessary to incorporate stabilizers, such as emulsifiers, thickening agents, gelling agents, weighting agents, or ripening inhibitors [4]. Emulsifiers are particularly important functional ingredients for forming stable emulsions with appropriate shelf lives and functional attributes. Many of the emulsifiers currently used industrially to stabilize oil-in-water emulsions are synthetic surfactants [8-10]. However, there has been increasing consumer demand for more natural, environmentally friendly, and
sustainable commercial products [11-13], and so many manufacturers have been reformulating their products to replace synthetic surfactants with more label-friendly natural alternatives [14]. In particular, manufacturers would often like to create new products entirely from natural ingredients so that they can make “all-natural” claims on their labels.

This chapter reviews the physicochemical basis for the ability of emulsifiers to form and stabilize oil-in-water emulsions, because this information is critical for understanding the requirements of any natural emulsifier that will be used as an alternative to a synthetic one. It then outlines a series of standardized tests that can be used to test and compare emulsifiers, which is useful for establishing the suitability of a particular emulsifier for different applications, and for comparing the relative performance of natural and synthetic emulsifiers. Finally, a review of the different kinds of natural emulsifiers available for use in foods is given (i.e., proteins, polysaccharides, phospholipids, biosurfactants, and bioparticles), and their advantages and disadvantages are highlighted. This chapter mainly focuses on the development of natural emulsifiers that can be used in food emulsions, but a great deal of the material discussed is also pertinent to other types of commercial emulsion-based products. It should also be stressed that the utilization of emulsifiers in the food industry is of great economic importance, with the market for these ingredients being estimated to be around $2.1 billion in 2012 and predicted to rise to around $2.9 billion by 2018 [15]. Consequently, the identification of natural alternatives to synthetic emulsifiers has considerable economic implications.
1.2 Physicochemical Principles of Emulsifier Performance

Emulsifiers play two key roles in the creation of successful emulsion-based products (Figure 1.1): (i) they facilitate the initial formation of fine lipid droplets during homogenization; (ii) they enhance the stability of the lipid droplets once they have been formed [4]. A brief review of the physicochemical basis for the ability of emulsifiers to form and stabilize emulsions is given in this section, with special consideration being given to the performance of natural emulsifiers in these roles.

Figure 1.1: Emulsifiers play two key roles in the production of commercial emulsion-based products: (i) they facilitate emulsion formation and (ii) they promote emulsion stability.

1.2.1 Emulsion Formation

1.2.1.1 Principles of Homogenization

Oil-in-water emulsions may be formed using either high- or low-energy approaches [16, 17]. High-energy approaches can be characterized by the utilization of specially designed mechanical devices (known as “homogenizers”) that create powerful disruptive forces that disrupt and intermingle the oil and water phases...
leading to the production of fine lipid droplets [18, 19]. The most commonly utilized mechanical devices in the food industry are high shear mixers, colloid mills, high-pressure valve homogenizers, microfluidizers, and sonicators [18-21]. Most natural emulsifiers can be utilized with most types of mechanical homogenizers; however, there are some examples where one must be careful. Polysaccharides or proteins may be depolymerized or denatured within sonicators due to the high local temperature and pressure gradients generated, which can adversely affect their functional performance [22]. Globular proteins may also be denatured and aggregate within high-pressure homogenizers or microfluidizers, which again alters their functional performance [23]. Low-energy homogenization approaches can be characterized by the spontaneous formation of emulsions when the composition or environment of an emulsifier-oil-water mixture is changed in a particular way [24, 25]. The most commonly used low-energy approaches for producing emulsions are the phase inversion temperature (PIT), spontaneous emulsification (SE), and emulsion inversion point (EIP) methods [26, 27]. Commercially, high-energy approaches are much more commonly utilized by the food industry to prepare emulsions than low-energy approaches, and only high-energy approaches are suitable for creating emulsions containing small lipid droplets for most natural emulsifiers. For these reasons, this section will mainly focus on the role of emulsifiers during homogenization using high-energy methods.

1.2.1.2 Role of Emulsifier

The role of the emulsifier in emulsion formation can be understood by examining the major physicochemical events that occur within a homogenizer (Figure 1.2). For the sake of clarity, only a high-pressure valve homogenizer will be considered here since it is the most commonly used mechanical device to form small lipid droplets
industrially (Figure 1.3). Nevertheless, fairly similar physicochemical processes occur within other types of homogenizers [17, 20]. Initially, the emulsifier is dissolved within the aqueous phase (although this is not always the case), and then the oil and aqueous phases are combined and intermingled using a high-shear mixer, which leads to the formation of a coarse emulsion. This coarse emulsion contains relatively large droplets (typically \( d > 1 \mu m \)) that are coated by emulsifier, with the remaining emulsifier molecules being dispersed within the aqueous phase. The coarse emulsion is then pumped through a small valve in the homogenizer at high pressure, which produces powerful disruptive forces (cavitation, turbulence, and shear) that break up the larger droplets into smaller ones [28]. The dimensions of the droplets initially produced inside the homogenizer depend on the relative magnitude of the disruptive forces and the interfacial restoring forces [29, 30].

Figure 1.2: Representation of the major physicochemical processes occurring within a homogenizer during the formation of an emulsion: droplet disruption; droplet coalescence; emulsifier adsorption; and droplet stabilization. Small droplets tend to be formed when the emulsifier adsorbs more rapidly than droplet collisions occur.
Figure 1.3: Schematic diagram of the typical two-step procedure used to produce oil-in-water emulsions using a high-energy method (i) a coarse emulsion is formed using a high-shear mixer; (ii) a fine emulsion is formed by passing the coarse emulsion through a high-pressure valve homogenizer.

Facilitation of Droplet Fragmentation: The interfacial restoring forces are related to the tendency for the droplets to adopt a spherical shape because this minimizes the thermodynamically unfavorable contact area between the oil and water phases as described by the Laplace Pressure ($\Delta P_L$):

$$\Delta P_L = \frac{4\gamma}{d}$$

(1)

Here, $\gamma$ is the oil-water interfacial tension and $d$ is the droplet diameter [4]. Large droplets are typically fragmented into smaller droplets when the disruptive forces produced inside a homogenizer are appreciably higher than the Laplace pressure [29, 30]. Thus, the intensity of the disruptive forces required to break down droplets tends to increase as $\gamma$ increases or $d$ decreases. As a consequence, smaller droplets will be produced during homogenization at fixed energy intensity (e.g., operating pressure) as the interfacial tension decreases.

An emulsifier can therefore expedite the production of fine droplets inside a homogenizer by rapidly adsorbing to the droplet surfaces and depressing the interfacial tension. The greater the ability of an emulsifier to reduce $\gamma$, the smaller will
be the droplets that can be generated using fixed homogenization conditions, such as pressure and number of passes [29, 31]. However, the emulsifier adsorption rate must be faster than the droplet fragmentation rate, otherwise the droplets will not be fully coated with emulsifier before a droplet break up event occurs [18, 19, 32]. There are major differences between the ability of natural emulsifiers to rapidly adsorb to lipid droplet surfaces during homogenization and therefore in their ability to rapidly decrease the interfacial tension during homogenization, which leads to considerable differences in the size of the droplets that can be generated within a homogenizer (see later). In addition, some biopolymers are not as efficient at screening the thermodynamically unfavorable contact between the oil and water phases as small molecule surfactants, and therefore lead to higher interfacial tensions and larger droplets during homogenization [33, 34].

Inhibition of Droplet Coalescence: Once the large droplets have been broken down into smaller ones it is important to prevent their coalescence within the homogenizer (Figure 1.2). Immediately after a large droplet has been broken down into two or more smaller ones the new droplet surfaces formed are not completely covered with emulsifier due to the increase in oil-water interfacial area [29, 35]. The stability of lipid droplets to coalescence inside a homogenization chamber depends on the degree of surface coverage [36]. If the surfaces can be completely covered by the amount of available emulsifier, and the emulsifier is effective at generating sufficiently strong repulsive forces (e.g., steric or electrostatic), then relatively stable droplets can be produced. However, if the droplets can only be partially covered by the available emulsifier, then they are liable to coalesce when they collide, which leads to larger droplets exiting the homogenizer [36]. Consequently, it is important that the lipid droplet surfaces are saturated with emulsifier molecules before they
collide with their neighbors [35, 37, 38]. Another important feature of an emulsifier is therefore its adsorption rate relative to the droplet collision rate. Emulsifiers that rapidly adsorb to the surfaces of the lipid droplets tend to be more effective at inhibiting droplet coalescence inside a homogenizer [39]. This is one of the reasons that synthetic or natural small molecule surfactants are so effective at forming emulsions containing small droplets since they are able to rapidly adsorb to the droplet surfaces during homogenization, thereby rapidly lowering the interfacial tension and forming a protective coating [18, 40, 41]. On the other hand, some natural emulsifiers (such as polysaccharides) are relatively large molecules that adsorb to lipid droplet surfaces relatively slowly and are therefore less efficient at creating fine droplets [42, 43].

To form small droplets and to optimize energy efficiency, it is important that there is adequate emulsifier present to completely cover the surfaces of the lipid droplets formed inside the homogenizer [36]. A certain amount of emulsifier can only cover a certain amount of oil-water surface area, which depends on oil content, droplet size, and the packing of emulsifier molecules at the droplet surfaces [36]. The smallest mean droplet diameter ($d_{\text{min}}$) that can theoretically be achieved during homogenization is given by the following equation [20]:

$$d_{\text{min}} = \frac{6 \cdot \Gamma_{\text{sat}} \cdot \phi}{c_S}$$  \hspace{1cm} (2)

Here, $d_{\text{min}}$ is the surface-weighted mean diameter ($d_{32}$), $\Gamma_{\text{sat}}$ is the emulsifier surface load at saturation (in kg m$^{-2}$), $\phi$ is the disperse phase volume fraction (unitless), and $c_S$ is total emulsifier concentration in the emulsion (in kg m$^{-3}$). This equation assumes that stable droplets can only be formed when they are fully coated.
with emulsifier, that droplet diameter is not limited by the strength of the disruptive forces produced by the homogenizer, and that all the emulsifier adsorbs to the lipid droplet surfaces. An estimation of the dependence of the mean droplet diameter on emulsifier concentration for emulsifiers with different surface loads is shown in Figure 1.4. This estimation shows that the droplet diameter decreases with increasing emulsifier concentration, and that the minimum droplet size that can be produced at a given emulsifier concentration increases with increasing surface load. Typically, the surface load of natural emulsifiers follows the order: small molecule surfactants (such as saponins) < globular proteins (such as whey protein) < flexible proteins (such as caseinate) < polysaccharides (such as gum arabic) [44, 45]. Consequently, one would expect saponins to form much smaller droplets than gum arabic when used at the same concentration. Experimental measurements of the mean droplet diameter versus emulsifier concentration support these theoretical estimations (Figure 1.5). In practice, it is often not possible to reach the theoretically estimated minimum droplet size because the emulsifiers do not adsorb rapidly enough, some of the emulsifier remains in the water phase, some droplet coalescence occurs, or the homogenizer is unable to generate sufficiently strong disruptive forces.
**Figure 1.4:** The droplet size typically increases with increasing emulsifier concentration under fixed homogenization conditions (pressure and number of passes), provided the homogenizer can generate small droplets. The effectiveness of different emulsifiers can be compared by plotting mean particle diameter ($d_{32}$) versus emulsifier concentration.

**Figure 1.5:** The effectiveness of different emulsifiers can be compared by plotting mean particle diameter ($d_{32}$) versus emulsifier concentration. Data from Ozturk et al (2015).

**Figure 1.6:** The droplet size typically decreases with increasing homogenization pressure, provided there is sufficient emulsifier present to cover the surfaces of the entire droplet formed. In some situations, the droplet size increases at high homogenization pressures (“over processing”), *e.g.*, due to heating effects.
Another important factor to consider during emulsion formation is the dependence of the droplet size on homogenization pressure [18, 46]. Typically, the mean droplet diameter decreases with increasing pressure, but the dependence of this relationship depends on emulsifier type and concentration [20]. A number of possible situations are highlighted in Figure 1.6:

(i) **Excess Emulsifier:** If there is an excess of emulsifier present, then the droplet diameter will continue to decrease with increasing homogenization pressure. Eventually, the upper limit for droplet disruption by the homogenizer will be reached, and the droplet size will not decrease any further. In this case, droplet size is determined by homogenization pressure and there is typically a linear log-log relationship between them. Droplet size also depends on the ease of droplet disruption. In food-grade oil-in-water emulsions the ease of droplet disruption tends to increase with diminishing interfacial tension and dispersed-to-continuous phase viscosity ratio [46, 47]. Thus, natural emulsifiers that are better at decreasing the interfacial tension tend to lead to smaller droplets [44, 45].

(ii) **Limited Emulsifier:** If there is only a limited amount of emulsifier present, then the droplet size decreases with increasing homogenization pressure until a certain droplet size is reached [36]. At this point, all of the emulsifier initially added to the system is adsorbed to the droplet surfaces, and so the droplet size cannot be reduced any further since there is not enough emulsifier available to cover any more droplets. As a result, any smaller droplets formed within the homogenizer will not be fully covered with emulsifier, and so they will tend to coalesce with each other. In this
case, the minimum droplet size that can be produced is mainly determined by the initial emulsifier concentration added as discussed earlier.

(iii) Over-processing: In some situations, the droplet size may initially decrease with increasing homogenization pressure, but then increase, which is often referred to as “over-processing” [39]. There is often a considerable increase in the temperature of a sample during homogenization at high pressures due to frictional losses. High pressures and temperatures sometimes cause an increase in droplet diameter due to a reduction in functionality of the emulsifiers, e.g., due to depolymerization or unfolding of biopolymer chains or due to dehydration of surfactant head-groups. These effects are likely to be highly system specific. As mentioned earlier, some proteins and polysaccharides are susceptible to depolymerization or unfolding in certain types of homogenizers, and therefore this effect has to be taken into account when deciding the most appropriate homogenization method.

1.2.2 Emulsion Stability

Once the droplets in an oil-in-water emulsion have been formed during homogenization it is important to keep them stable throughout the expected lifetime of the product [4, 48, 49]. Emulsions may become unstable through numerous physicochemical processes, including gravitational separation (creaming and sedimentation), aggregation (floculation, coalescence, and partial coalescence), Ostwald ripening, phase inversion, and chemical degradation (Figure 1.7). The vulnerability of a particular type of emulsion to these instability mechanisms depends on its precise composition, microstructure, and thermal-mechanical history. Each product must be carefully formulated to resist the range of conditions that it may be
exposed to throughout its lifetime, e.g., changes in pH, ionic strength, dilution, ingredient interactions, temperature, mechanical forces, and water activity. The choice of the most appropriate emulsifier is one of the most important decisions that scientists must make when formulating commercial emulsion-based product, since the interfacial layer has a marked impact on many of these instability mechanisms. Some of the most important ways that emulsifiers can influence emulsion stability are outlined below, again with special emphasis on the behavior of natural emulsifiers.

**Figure 1.7:** Oil-in-water emulsions may become physically unstable through numerous physicochemical processes, including gravitation separation, flocculation, coalescence, and phase separation.

### 1.2.2.1 Gravitational Separation

Gravitational separation is the upward (“creaming”) or downward (“sedimentation”) movement of droplets due to a density difference between them and the surrounding medium (**Figure 1.7**). To a first approximation, in dilute emulsions the creaming velocity ($v$) is given by Stokes’ Law [4]:

$$v_{Stokes} = -\frac{g(\rho_2 - \rho_i)d^2}{18\eta_l}$$  \hspace{1cm} (3)
Here, \( g \) is the gravitational field, \( d \) is the droplet diameter, \( \rho \) is density, \( \eta \) is shear viscosity, and the subscripts 1 and 2 refer to the continuous and dispersed phases, respectively. The sign of the creaming velocity is an indication of whether the droplets cream (+) or sediment (-).

Emulsifiers may influence gravitational separation in emulsions through both direct and indirect means. First, the mean diameter of the droplets in an emulsion is influenced by the effectiveness of an emulsifier at rapidly adsorbing to the droplet surfaces during homogenization thereby facilitating droplet fragmentation and inhibiting droplet coalescence (Section 1.2.1). Emulsifiers vary considerably in their ability to produce fine droplets inside of an homogenizer [41, 50], which will therefore influence their subsequent creaming stability. Second, emulsifiers may alter the effective density of the droplets by forming a dense interfacial coating around them [16, 26]. Typically, emulsifiers have a higher density than water, whereas oil has a lower density. Consequently, the presence of an emulsifier layer tends to reduce the difference in density between the droplets and surrounding medium, thereby reducing the creaming velocity (Equation 3). However, this effect is only really significant in emulsions that contain relatively small droplets and thick interfacial layers [51].

The droplets in an oil-in-water emulsion may aggregate through numerous mechanisms (Figure 1.7), with the most common being flocculation, coalescence, and partial coalescence [20, 52]. Flocculation involves the association of two or more droplets into a clump, with each individual droplet retaining its original dimensions [48]. Coalescence is the process whereby two or more droplets merge together to form a single larger droplet [36]. Eventually, this process may lead to phase separation (“oiling-off”), which is the formation of a separate oil layer on top of an emulsion. Partial coalescence is the process whereby two or more partially crystalline
lipid droplets form a clump, which is often initiated by protrusion of fat crystals inside one droplet into the fluid region of neighboring droplets [49]. In this case, the droplets do not fully merge together because of the mechanical strength of the three-dimensional fat crystal network inside them [49, 53].

The nature of the emulsifiers present in an emulsion may influence droplet aggregation in numerous ways. First, the type of emulsifier adsorbed to the droplet surfaces plays a major role in determining the attractive and repulsive colloidal interactions operating in an emulsion [54]. The droplets in an emulsion tend to aggregate when the attractive interactions dominate, but be stable when the repulsive interactions dominate [4]. Typically, emulsifiers inhibit droplet aggregation by generating strong electrostatic and/or steric repulsive interactions (Figure 1.8). However, in some cases they may promote droplet aggregation by generating attractive interactions between the droplets, such as hydrophobic attraction when they have exposed non-polar regions [55] or depletion attraction when there are high levels of non-adsorbed emulsifier [56].

![Steric and Electrostatic Interactions](image)

**Figure 1.8:** Natural emulsifiers typically stabilize lipid droplets against aggregation through steric and/or electrostatic interactions. The relative magnitude of these colloidal interactions depends on the thickness, chemistry, and charge of the emulsifier molecules.

A brief summary of some of the most important properties that may influence the colloidal interactions between oil droplets coated by natural emulsifiers is given below:
**Electrostatic interactions:** The electrostatic repulsive interactions acting between lipid droplets suspended in water depends on the surface charge density, as well as on solution conditions, such as ionic strength and solvent type [4, 54]. Typically, the higher the surface charge density and the lower the ionic strength the stronger and longer range is the electrostatic interaction. The nature of the emulsifier molecules surrounding the lipid droplets in an emulsion strongly influences the surface charge density, as well as its pH-dependence. For example, the magnitude of the electrical charge (ζ-potential) on globular protein-coated droplets goes from highly positive at low pH, to zero at intermediate pH, to highly negative at high pH (Figure 1.9). For instance, legume proteins are constituted of around 70% globulin and 30% albumin [57-59]. The isoelectric point for globulins is around pH 4.5, whereas it is around pH 6 for albumins, and so the net isoelectric point for the overall system is around pH 4.9 [60]. Consequently, protein-based emulsifiers are typically only useful for preventing droplet aggregation through electrostatic repulsion at low pH and high pH, but not at intermediate pH values close to their isoelectric point [61].

![Figure 1.9: Change in droplet charge (ζ-potential) and mean particle diameter with pH for different kinds of natural emulsifiers: phospholipids (lecithin); quillaja saponins (Q-Naturale); gum arabic (GA); and, whey protein isolate (WPI).](image-url)
• **Steric repulsion:** The magnitude and range of the steric repulsion operating between oil droplets is largely determined by the thickness and packing of the emulsifier molecules at the droplet surfaces [4, 54]. Typically, the denser the packing and the thicker the interface, the stronger and longer range is the steric repulsion. Emulsifiers differ considerably in their molecular organization at oil-water interfaces, which influences their ability to generate steric repulsion between droplets. For example, polysaccharides that form thick interfacial layers (such as gum arabic) are highly effective at inhibiting droplet aggregation through steric interactions [42, 43]. Conversely, globular proteins (such as whey proteins) that form thin interfacial layers are not effective at preventing droplet aggregation through steric repulsion alone because the range of the van der Waals attraction exceeds the range of the steric repulsion. In this case, droplet aggregation may be inhibited by ensuring the globular proteins have a strong electrical charge (next section) or by covalently attaching hydrophilic chains that increase the effective thickness of the interface [48, 62]. Interfacial thickness, and therefore steric interactions, can be tailored by choosing natural emulsifiers with different surface properties or by using the layer-by-layer electrostatic deposition method to form multilayered interfaces [52, 63, 64]. The presence of a thick interfacial layer may also inhibit partial coalescence by preventing fat crystals penetrating from one droplet to another droplet [65].

• **Hydrophobic interactions:** After adsorption to the surfaces of lipid droplets, certain types of emulsifiers have non-polar regions that remain exposed to the surrounding water, which generates a hydrophobic attraction between the droplets that can promote aggregation [4, 54]. Amphiphilic proteins have both polar and non-polar groups along the polypeptide backbone, and after they adsorb to lipid
droplet surfaces the non-polar groups tend to protrude into the lipid phase, whereas the polar groups tend to protrude into the water phase. Nevertheless, some of the non-polar groups on the surfaces of the adsorbed proteins may still be directed towards the water phase, and therefore cause the droplet surfaces to have some hydrophobic character. In addition, globular proteins (such as whey, soy, and pea proteins) typically undergo conformational changes after adsorption to oil droplet surfaces (“surface denaturation”) or after an emulsion is heated (“thermal denaturation”), which leads to an increase in the number of hydrophobic groups exposed to the surrounding aqueous phase [55, 66-68]. As a result of this surface hydrophobicity, a strong hydrophobic attraction is often generated between protein-coated droplets that can promote droplet flocculation (Figure 1.10).

**Figure 1.10:** Droplet aggregation may occur in globular-protein stabilized emulsions when they are heated above their thermal denaturation temperature due to an increase in surface hydrophobicity. β-lactoglobulin stabilized emulsions containing 150 mM NaCl (added before heating).

Hydrophobic interactions are typically less important for lipid droplets coated by non-ionic surfactants or phospholipids, provided that all of the droplet surfaces are saturated with emulsifier so none of the underlying lipid phase is exposed. There may
be some contribution to the overall colloidal interactions from hydrophobic interactions for polysaccharides that have exposed non-polar groups, but this is likely to be highly dependent on the nature of the polysaccharide used, and there have been few studies in this area.

- **Covalent interactions:** Some food emulsifiers have chemically reactive functional groups capable of forming covalent bonds with other emulsifiers on the same or on different lipid droplets depending on solution and environmental conditions. One of the commonest examples of this phenomenon are globular proteins (such as whey, soy, and egg proteins) that have free sulfhydryl groups (-SH) or disulfide bonds (-S-S-) that can react with each other [69, 70]. If covalent bonds are formed amongst proteins adsorbed to the same droplet surfaces, then they can improve the aggregation stability of emulsions [55, 66]. Conversely, if the covalent bonds are formed between proteins adsorbed onto different droplets, then they can lead to aggregation with the droplets being held together by strong covalent bonds [70]. In general, covalent interactions are relatively strong short-range interactions, and therefore they can only form when the reactive groups are in close proximity. Consequently, they may work in concert with other physical interactions, such as van der Waals, electrostatic, hydrophobic, or hydrogen bonding interactions. For example, protein-coated droplets may come into close contact due to a reduction in electrostatic repulsion or an increase in hydrophobic attraction, and then the covalent bonds form between the adsorbed layers on the different droplets [55, 66]. The formation of covalent bonds depends on the presence of chemically reactive functional groups, as well as the precise solution and environmental conditions of the system. This type of interaction therefore
tends to be less important for many natural surfactants, phospholipids, and polysaccharides because they have less chemically reactive functional groups.

- **Overall interactions:** Individual colloidal interactions (such as van der Waals, electrostatic, hydrophobic, and steric interactions) can be classified by three major attributes: sign (highly positive to highly negative), range (short to long), and magnitude (weak to strong) (Figure 1.11). This means that the overall interaction between emulsifier-coated lipid droplets may be relatively complex due to the contribution of a number of different colloidal interactions with different attributes [20]. The type of colloidal interactions to include in this type of analysis depends on the nature of the emulsifiers used to stabilize the systems. Typically, there is always a van der Waals attraction between lipid droplets that will favor their aggregation, which may be supplemented by other types of attractive interaction such as hydrophobic or depletion attraction. Consequently, the emulsifier layer must generate some kind of repulsive force that is strong enough to overcome these attractive interactions. Emulsifiers that can generate repulsive interactions that are stronger and longer range than the attractive interactions can completely inhibit droplet aggregation by preventing them from coming close together [4, 48]. On the other hand, droplet aggregation may occur in emulsions containing emulsifiers that are unable to generate sufficiently strong or long-range repulsive interactions. In this case, weak flocculation, strong flocculation, or coalescence may occur depending on the nature of the emulsifier layer and its resistance to disruption.
Overall colloidal interactions depend on the range, magnitude and sign of the attractive and repulsive forces. $h$ is the surface-to-surface droplet separation, $r$ is the radius of the droplet, and $\delta$ is the interfacial layer thickness.

Understanding the major types of colloidal interactions that operate in a particular emulsifier-stabilized system is particularly important for understanding the major factors that will influence its aggregation stability. Emulsifier-coated lipid droplets that are primarily stabilized by electrostatic repulsion tend to be highly sensitive to pH and ionic strength, e.g., proteins, phospholipids, and ionic surfactants [48]. Conversely, those primarily stabilized by steric repulsion are much less sensitive to changes in environmental conditions [50]. In addition, emulsifiers that tend to undergo conformational changes upon heating (such as globular proteins) that lead to exposure of non-polar groups may be susceptible to droplet aggregation driven by hydrophobic attraction [66].

- **Impact on partial coalescence:** Some types of food-grade emulsifiers are able to impact the tendency for partial coalescence to occur in emulsions containing partly crystalline droplets [49]. Firstly, some small molecule surfactants are able to alter the nucleation and crystallization of emulsified lipids by acting as templates,
thereby altering the number, size, and location of the fat crystals present at the oil-water interface [71]. Secondly, some emulsifiers are able to form thick interfacial coatings around lipid droplets that can prevent a crystal from one droplet penetrating into the liquid portion of another droplet, e.g., caseinate can form thick interfacial layers that inhibit partial coalescence [65, 72]. As a result, emulsifier type may have a strong influence on the stability of emulsions to partial coalescence. This is important knowledge for controlling partial coalescence. In some cases, partial coalescence leads to emulsion instability and should therefore be inhibited by using natural emulsifiers that form thick interfacial layers that prevent fat crystal penetration. In other cases, partial coalescence is an important stage in the production of food products, such as margarine, butter, ice cream, and whipped cream. In this case it may be important to use a natural emulsifier that forms a thin interfacial layer that is easy to penetrate, such as a biosurfactant or phospholipid.

1.2.2.2 Ostwald Ripening

Ostwald ripening (OR) causes instability in those oil-in-water emulsions where the oil phase has some solubility in the water phase, which is the case for flavor oils, essential oils, and short chain triglycerides [47, 73, 74]. OR leads to a progressive increase in the mean droplet size over time as a result of diffusion of oil molecules from the small droplets (high curvature) to large droplets (low curvature) through the intervening water phase [75]. The thermodynamic driving force for this process is the greater solubility of the oil phase in the immediate vicinity of small droplets than in the immediate vicinity of large droplets. This effect occurs because the water-solubility of an oil phase increases as the curvature of the oil-water interface increases, i.e., the droplet size decreases. The higher concentration of oil molecules
around the small oil droplets compared to the large ones leads to a concentration gradient that “pumps” oil molecules from small to large droplets. The rate of droplet growth due to OR can be described by the following equation [76]:

$$d(t)^3 = d_0^3 + \left( \frac{64\gamma V_m^2}{9RT} \right) S(\infty)Dt$$

(4)

Here \(S(\infty)\) is the equilibrium water-solubility of the oil phase for a droplet with infinite curvature (a planar oil-water interface), \(d(t)\) is the droplet diameter at time \(t\), \(d_0\) is the initial droplet diameter, \(V_m\) is the molar volume of the oil molecules, and \(\gamma\) is the oil-water interfacial tension. This equation indicates the OR rate is strongly influenced by the water-solubility of the oil phase, but it also depends on some emulsifier properties.

Emulsifiers may influence the OR rate in oil-in-water emulsions through various mechanisms. First, the rate of OR is proportional to the oil-water interfacial tension (Equation 4), and so the more effective an emulsifier is at decreasing the interfacial the more effective it should be at inhibiting droplet growth through this mechanism [75]. Small molecule surfactants tend to be better at reducing the interfacial tension that proteins or polysaccharides, and may therefore be more effective at inhibiting OR through this mechanism. Second, some emulsifiers can form rigid shells around oil droplets that can inhibit Ostwald ripening by mechanically retarding droplet shrinkage or growth [74, 77]. Third, some emulsifiers are capable for forming colloidal structures (such as micelles) that can increase the solubility of the oil phase in the aqueous phase, thereby increasing the OR rate [78]. The type of natural emulsifier used may therefore have an influence on the tendency for OR to occur in emulsions. Having said this, the most effective way at inhibiting OR is usually to add highly
hydrophobic lipids (such as long-chain triacylglycerols or ester gums) into the oil phase because this generates a thermodynamic driving force that opposes OR due to an entropy of mixing effect [47, 76, 79].

1.2.2.3 Lipid Oxidation

Lipid oxidation is an important factor causing loss of product quality and nutrients in many foods [80, 81]. Moreover, potentially toxic reaction products, such as carcinogenic or inflammation-promoting substances, may be formed in foods as a result of lipid oxidation [81, 82]. Lipid oxidation in oil-in-water emulsions is a particular problem when the oil phase contains appreciable levels of polyunsaturated lipids, such as ω-3 oils or carotenoids [83-85]. Lipid oxidation typically involves an interaction between an unsaturated lipid and oxygen leading to the formation of hydroperoxides and their breakdown products [86]. The lipid oxidation reaction can be divided into four major steps: initiation, propagation, decomposition, and termination [86]. This reaction may be initiated by autooxidation, photosensitizer-induced oxidation, or enzyme-induced oxidation depending on system composition and environmental conditions. Controlling the rate of lipid oxidation in emulsions has proved to be a major challenge, and many different strategies have been developed, including controlling environmental conditions (such as oxygen, light, and temperature), controlling ingredient quality, adding antioxidants, adding chelating agents, and engineering the droplet interface [80, 81, 83, 87]. The interfacial layer formed by emulsifiers around lipid droplets has a major impact on the stability of emulsions to lipid oxidation [88, 89]. Some emulsifiers have been shown to inhibit lipid oxidation, whereas others have been shown to accelerate it. For example, proteins can inhibit lipid oxidation by scavenging free radicals, chelating pro-
oxidative transition metals, or physically forming a barrier to separate lipids from other reactive species [90]. The metal-catalyzed decomposition of lipid hydroperoxides is a major oxidation pathway in emulsions [81]. Lipid hydroperoxides are surface-active molecules that migrate to droplet surfaces after formation, where they decompose by a metal-catalyzed pathway. Proteins can inhibit lipid oxidation in emulsions by hindering the access of metals to the interface by electrostatic repulsion or by creating a steric barrier due to their thickness and denseness [90, 91]. Some proteins are able to bind transition metals and thereby alter their ability to promote lipid oxidation [92, 93]. If the proteins are present within the aqueous phase, then they will keep the transition metals away from the lipid substrate and inhibit oxidation. However, if the proteins are adsorbed to the droplet surfaces, they may bring the transition metals into close proximity to the droplet surfaces and thus promote oxidation. Proteins that can inhibit lipid oxidation by binding transition metals include casein, whey protein, soy protein, bovine serum albumin, zein, and potato protein [90]. Also, animal proteins such as egg protein and gelatin have been reported to inhibit lipid oxidation [90]. In addition, saponins (commercially available as Q-Naturale) and certain types of phospholipids may also be effective at inhibiting lipid oxidation in emulsions because of their natural free radical scavenging capacity [94-96]. In addition, colloidal particles used to stabilize Pickering emulsions have also been reported to inhibit lipid oxidation by forming thick interfacial layers and physically separating the pro-oxidant compounds in the continuous phase from the lipid hydroperoxides located at the droplet interface [97].

Environmental and solution conditions are known to affect the anti- or pro-oxidative properties of emulsifiers. For instance, lipid oxidation is inhibited by adsorbed proteins at pH values below their isoelectric due to their ability to
electrostatically repel transition metals, but may be promoted above their isoelectric point due to their ability to electrostatically attract transition metals [98, 99]. Conversely, the opposite may be true for non-adsorbed proteins since they can pull transition metals away from the droplet surfaces when they bind them. Thus, the ratio of free-to-adsorbed emulsifier may have to be controlled, as well as solution and environmental conditions, for emulsions prone to lipid oxidation.

In summary, some natural emulsifiers may promote lipid oxidation whereas others may inhibit it depending on their molecular properties, location, and environmental conditions. Consequently, the selection and application of an appropriate emulsifier is particularly important in commercial products that are prone to lipid oxidation.

1.2.3 Gastrointestinal Fate

Emulsions are often used as delivery systems to encapsulate and protect lipophilic bioactive components within commercial products [5, 100, 101]. However, it is important that any delivery system is able to release the bioactive component at the appropriate site of action after the product has been ingested. In some cases, a lipid may be encapsulated so well, that it is not released within the gastrointestinal tract (GIT) and therefore does not have its potential beneficial effects. The nature of the emulsifier used can have a pronounced influence on the GIT fate of emulsions, and selection of an appropriate natural emulsifier may therefore be important for commercial products that are intended for oral delivery of bioactive components.

In order to select an appropriate emulsifier it is useful to have an understanding of the behavior of emulsions within the GIT after ingestion. Initially, an emulsion-based product will enter the oral cavity where it will spend a few seconds or so depending on the nature of the product [16, 102, 103]. On entering the mouth, an
emulsion is mixed with saliva and may experience changes in pH, ionic strength, shearing, and temperature, as well as being exposed to mucin and the surfaces of the tongue, palate, and cheeks. After swallowing, the bolus travels through the esophagus and into the gastric cavity, where it encounters highly acidic gastric fluids that contain minerals and digestive enzymes (such as pepsin and lipase) [104]. In addition, the lipid droplets may be exposed to complex fluid flows and forces due to the motility of the stomach [105]. Typically, an emulsion may spend from a few minutes to a few hours in the stomach depending on its composition and physicochemical properties, as well as those of the surrounding matrix.

After a food has been sufficiently disrupted within the gastric cavity, the resulting chyme passes through the pylorus sphincter (a biological valve) and into the small intestine, where the pH increases due to the secretion of pancreatic fluids containing bicarbonate [106, 107]. The pancreatic fluids also contain digestive enzymes (such as lipase, amylase, and protease) that hydrolyze the lipids, starches, and proteins remaining in the chyme. In addition, phospholipids and bile salts are mixed with the chyme, which serve to displace some of the existing emulsifiers from the droplet surfaces, and to solubilize the free fatty acids formed during lipolysis [108]. The changes in the environment of the lipid droplets as they pass through the GIT cause alterations in their composition, size, and aggregation state [107]. Droplet composition may be changed due to displacement of some of the original emulsifiers from the droplet surfaces, or due to hydrolysis of the lipids or emulsifiers. Droplet size may be changed due to lipid hydrolysis, coalescence, or fragmentation processes. Droplet aggregation state may be altered due to flocculation induced by bridging, depletion, or electrostatic screening mechanisms. Many of these processes depend on the nature of the emulsifier used to stabilize the original lipid droplets, and can
therefore be modulated by selection of an appropriate natural emulsifier. Consequently, it may be possible to design food emulsions with improved nutritional aspects, such as increased bioavailability, targeted release, or enhanced satiety response.

The rate and extent of lipid digestion within the small intestine is one of the most important factors affecting the release, solubilization, and transport of encapsulated bioactive components [5]. Oil type has a major impact on the potential gastrointestinal fate of emulsions [109-111], but will not be considered further because it is not directly related to emulsifier properties. Droplet size has also been shown to influence the rate of lipid digestion, with smaller droplets (bigger surface area) being digested more rapidly [112, 113]. Consequently, natural emulsifiers that produce emulsions containing smaller lipid droplets are more effective at ensuring rapid lipid digestion and bioactive release within the GIT [114, 115]. Studies have also shown that lipid digestion may be directly influenced by the nature of the emulsifier used to stabilize the droplets. Lipid digestion may be inhibited when an emulsifier coating restricts the adsorption of lipase to the oil droplet surfaces, thereby preventing it from coming into close contact with the lipids [116-119]. For example, the initial rate of lipid digestion was much slower for caseinate-coated oil droplets than for lactoferrin- or Tween-coated ones (Figure 1.12) because the caseinate-coated droplets were highly flocculated when they entered the small intestine, which restricted the ability of the lipase to reach the lipid phase [120]. Other studies have also shown that emulsions that are highly aggregated when they enter the small intestine have slower lipid digestion rates [121, 122]. As mentioned earlier, caseinate-stabilized emulsions are highly susceptible to flocculation within the stomach, which can influence their aggregation state and digestion in the small
intestine [123]. On the other hand, saponins-stabilized emulsions are more stable to droplet aggregation in the stomach, and therefore have a higher surface area and faster digestion rate in the small intestine [124].

Figure 1.12: Influence of emulsifier type on the release of free fatty acids released from oil-in-water emulsions under simulated small intestine conditions (Zhang et al 2015).

As well as acting on the lipid phase within oil droplets, digestive enzymes may also act upon the emulsifier molecules that coat the droplets. For example, proteases within the stomach (pepsin) or small intestine (trypsin and chymotrypsin) may hydrolyze the layer of protein molecules adsorbed to lipid droplet surfaces, thereby affecting their susceptibility to lipid digestion [125-127]. Studies have also shown that the type of natural emulsifier coating the lipid droplets in an emulsion may influence the extent of lipid digestion and the type of lipid digestion products produced, i.e., the ratio of monoacylglycerols, diacylglycerols and triacylglycerols [128]. In this study, the extent of lipid digestion was greater for gum arabic stabilized emulsions than for whey protein stabilized ones, which was attributed to the ability of the whey protein molecules to partly inhibit the adsorption of the lipase molecules.
A number of other studies have compared the ability of different natural emulsifiers to influence the lipid digestion process under simulated GIT conditions. The free fatty acid release was reported to be faster when oil-in-water emulsions were stabilized by proteins than by lecithin [114], and when emulsions were stabilized by saponins than by Tween 20 [129]. There have been a number of recent studies on the potential GIT fate of oil-in-water emulsions stabilized by natural colloidal particles (“Pickering emulsions”). For instance, the rate of lipid digestion was found to be slower for oil droplets coated by chitin nanoparticles than for droplets coated by whey protein or caseinate [130]. On the other hand, coating oil droplets with lactoferrin nanoparticles appeared to have little influence on their rate of lipid digestion [131]. These differences may be because chitin nanoparticles are indigestible, whereas lactoferrin nanoparticles are digested by proteases. Overall, these studies suggest that it may be possible to alter the GIT fate of emulsions by choosing appropriate natural emulsifiers to coat the lipid droplets.

1.2.4 Summary of Role of Natural Emulsifiers

In summary, a natural emulsifier must be a surface-active molecule or colloidal particle that can rapidly adsorb to the surfaces of the oil droplets produced during homogenization. After adsorption, the emulsifier should rapidly depress the interfacial tension so as to facilitate droplet disruption and the generation of fine droplets, and it should form a coating that protects the droplets from aggregation. In addition, the emulsifier may have to be selected to provide protection against the chemical degradation of encapsulated lipids (such as the oxidation of polyunsaturated lipids), as well as guaranteeing that the lipids are completely digested and absorbed within the GIT. The level of emulsifier needed to form an emulsion containing
droplets with a particular size is largely determined by its surface load ($\Gamma$), which may vary appreciably for natural emulsifiers.

1.3 Experimental Methods for Comparing Performance of Natural Emulsifiers

If a manufacturer would like to select the most appropriate natural emulsifier to use in a particular commercial product, they need to have some standardized analytical tests that can be used to compare different emulsifier types. In this section, some practically viable analytical tests for characterizing and comparing the performance of natural emulsifiers according to their capability to form and stabilize emulsions is given.

1.3.1 Emulsion Formation

Practically, two of the most important attributes of an emulsifier related to emulsion formation are: (i) the minimum amount of emulsifier needed to form an emulsion with a given droplet size; and, (ii) the smallest droplet size achievable under a specified set of homogenization conditions. Information related to these attributes can be obtained using fundamental and/or empirical methods depending on the needs of the investigator.

1.3.1.1 Fundamental Methods

Fundamental information about emulsifier properties can be obtained by measuring their effectiveness at reducing the tension of an oil-water interface [20]. Typically, the interfacial tension is measured as a function of increasing emulsifier level, and then the surface pressure versus emulsifier concentration profile is calculated (Figure 1.13). The surface pressure ($\Pi$) is defined as the difference in interfacial tension between a clean interface and an interface in the presence of
emulsifier: $\Pi = \gamma_0 - \gamma$. In general, the surface pressure rises from zero in the absence of emulsifier to $\Pi_{\text{sat}}$ when the interface is saturated with emulsifier.

![Graph](image)

**Figure 1.13:** The interfacial properties of an emulsifier can be characterized by measuring the interfacial tension versus concentration profile, and then converting into surface pressure data.

A number of valuable pieces of information can be obtained from a plot of $\Pi$ versus emulsifier concentration:

- **Saturation Surface Pressure:** The value of $\Pi_{\text{sat}}$ gives an indication of how effectively an emulsifier can reduce the interfacial tension after it adsorbs to the droplet surfaces, which is related to how easily droplets are fragmented within a homogenizer. The greater $\Pi_{\text{sat}}$, the smaller the size of the droplets generated under fixed homogenization conditions (assuming there is enough emulsifier available and that it adsorbs rapidly enough).

- **Surface Activity:** Practically, the surface activity (SA) of an emulsifier can be taken to be the reciprocal of the emulsifier concentration at which the surface pressure reaches 50% of the saturation value: $\text{SA} = 1/C_{50\%}$. The thermodynamic affinity of an emulsifier for an oil-water interface increases
as its surface activity increases. At a molecular level, the surface activity depends on how effectively the emulsifier shields the thermodynamically unfavorable oil-water interactions that occur at the interface, which depends on interfacial packing efficiency.

- **Surface Load:** The surface load of an emulsifier can be calculated from the gradient of an interfacial tension *versus* logarithm of emulsifier concentration plot (Figure 1.13). As mentioned earlier, the surface load is related to the level of emulsifier needed to stabilize a given amount of interfacial area.

As discussed in Section 1.2, the dimensions of the droplets leaving a homogenizer depend on the speed at which emulsifier molecules are able to adsorb to the droplet surfaces during homogenization. Information about the kinetics of emulsifier adsorption (under quiescent conditions) can be obtained by acquiring interfacial tension *versus* time profiles [20, 132]. Nevertheless, the time scales that can be accessed in conventional interfacial tension meters is not usually fast enough to accurately mimic the highly dynamic events occurring within a homogenizer. The stability of emulsifier-coated droplets within a homogenizer depends on interfacial properties such as thickness and charge, which can be measured using a variety of analytical tools, such as dynamic light scattering and particle electrophoresis [20].

### 1.3.1.2 Empirical Methods

Fundamental methods are useful for providing quantitative information about the interfacial properties of natural emulsifiers that can be related to their molecular characteristics and that can be compared between different laboratories. However, they usually provide little insight into how a particular emulsifier functions in practice under commercial manufacturing homogenization conditions. Consequently,
empirical methods based on test conditions that more closely mimic the way an emulsifier is actually used in practice [20]. For example, if a manufacturer were preparing a commercial emulsion-based product using a particular homogenizer, then standardized laboratory conditions could be established to mimic this process. In this case, a coarse oil-in-water emulsion could be prepared with a composition similar to the commercial product (e.g., oil content, oil type, aqueous phase composition, pH, and ionic strength). This coarse emulsion would then be passed through a homogenizer operated under standardized conditions that mimic the industrial process (e.g., homogenizer type, operating pressure, and number of passes), and the mean droplet diameter ($d_{32}$) would be measured. This procedure is repeated for emulsions containing a range of emulsifier levels, and then the data are plotted as mean droplet diameter $versus$ emulsifier concentration (Figure 1.4). This kind of plot is particularly useful for characterizing and comparing the properties of different natural emulsifiers (Figure 1.5). For example, it can be used to identify the amount of emulsifier required to produce droplets of a particular size. Since the droplet size ($d_{32}$) and disperse phase volume fraction ($\phi$) of emulsions are typically known, and then the effective surface load ($\Gamma$) of an emulsifier can be estimated by fitting the equation 2 to the experimental data. Indeed, plotting $d_{32}$ versus $1/C$ should result in a linear line that goes through the origin. The slope of this line should be $6\Gamma\phi$, and therefore the surface load is given by: $\Gamma = \text{slope}/6\phi$. This approach assumes that the droplet size is limited by the amount of emulsifier present, rather than by the disruptive forces that can be generated by the homogenizer, and therefore only the data at relatively low emulsifier concentrations should be used in the analysis. In addition, it assumes that the interfacial composition and structure does not change with increasing emulsifier concentration, e.g., due to multilayer formation. Despite these limitations this approach is a useful means of
comparing emulsifiers under similar conditions that mimic commercial processes. For example, based on the data shown in Figure 1.5 the surface load of quillaja saponins, whey protein, and gum arabic are 0.001, 1, and 25 mg m$^{-2}$, respectively. Hence, a much lower concentration of the quillaja saponins is required to form an emulsion than for the other emulsifiers.

1.3.2 Emulsion Stability

Emulsions are thermodynamically unstable colloidal dispersions that may breakdown through numerous instability pathways, including creaming, sedimentation, flocculation, coalescence, and Ostwald ripening [20, 36, 49, 52, 75]. The type of natural emulsifier used to stabilize an oil-in-water emulsion has a major influence of type of instability mechanisms that the droplets are most susceptible to [12]. Analytical tools and experimental protocols are therefore needed to characterize and compare the stability of emulsions stabilized by different kinds of natural emulsifiers [133].

1.3.2.1 Analytical methods for measuring emulsion stability

Numerous analytical tools exist for measuring the stability of emulsions, which have been reviewed in detail elsewhere [4, 133]. For this reason, only a concise overview of the major methods is given here. A particularly important factor that influences the stability of many emulsions is the size and aggregation state of the droplets they contain. Particle size is usually measured using specialized analytical instruments, such as those based on light scattering, particle counting, or microscopy. Typically, an emulsion sample is diluted (if required) and then placed within the measurement chamber of the instrument. The instrument then analyzes the sample and
provides information about the particle size distribution and mean particle diameter (often within a few minutes).

The electrical properties of the interfaces formed by natural emulsifiers have a major impact on emulsion stability and performance. There are several methods available to measure the electrical characteristics of emulsion droplets, but the simplest and most widely used method is based on micro-electrophoresis [20]. Instruments based on this principle measure the direction and velocity of colloidal particles in a well-defined electrical field, and then use this information to calculate the sign and magnitude of the \( \zeta \)-potential. The thickness of the interfacial layer formed by a natural emulsifier plays an important role in determining the steric repulsion between droplets, as well as their protective and release characteristics. X-ray and neutron scattering or reflection techniques can be utilized to determine the thickness of the interfacial layer, but they require specialized instrumentation that is often not widely available. Interfacial thickness can sometimes be determined using dynamic light scattering instruments by determining the difference in particle diameter between naked and emulsifier-coated latex beads [134].

Information about the aggregation state of the droplets in emulsions is usually obtained using microscopy methods, such as optical or electron microscopy [20]. This kind of structural information is particularly useful for distinguishing between droplet flocculation, coalescence, and Ostwald ripening. The susceptibility of an emulsion to gravitational separation can be established by simple visual observation, or using specialized instruments that scan the droplet concentration as a function of sample height (e.g., using a laser).
1.3.2.2 Emulsion Testing Protocols

An important criteria to consider when choosing a natural emulsifier for a particular application is to determine whether it will form emulsions that remain stable under the solution conditions found in commercial products (e.g., pH, ionic strength, and ingredient profile), as well as under the various environmental changes that a product experiences throughout its lifetime (e.g., temperature variations, water activity, mechanical forces) [133]. It is therefore useful to develop standardized testing protocols to identify the solution and environmental conditions that an emulsion containing droplets coated by a particular natural emulsifier will remain stable. Initially, a stock emulsion is produced using the emulsifier to be tested using conditions where the system is known to be stable (e.g., pH, ionic strength, temperature, etc.). This stock emulsion is then used to prepare samples that are exposed to a range of solution conditions and environmental stresses:

- **pH:** The stock emulsion is used to prepare samples with pH values spanning the range that might be encountered within commercial products or within the gastrointestinal tract (e.g., 2 to 8).

- **Ionic strength:** The stock emulsion is used to prepare samples with a range of ionic strengths by adding different quantities of salts (e.g., 0 to 500 mM NaCl; 0 to 50 mM CaCl₂). The type and levels of salts chosen should represent those that an emulsion may experience within a typical commercial product or during passage through the gastrointestinal tract.

- **Thermal processing:** The stock emulsion is adjusted to a certain pH and ionic strength (chosen to mimic the values of the commercial product it may be used in), and then a series of samples are prepared that are exposed to different temperatures (e.g., 0 to 90 ºC) for a specific time (e.g., 20 minutes), or that or exposed to a
certain temperature (e.g., 90 °C) for varying times (e.g., 0 to 30 minutes). Alternatively, thermal processing conditions that mimic an industrial process such as pasteurization, sterilization, or cooking can be used.

- **Freeze-thaw stability:** The stock emulsion is adjusted to a certain pH and ionic strength, and then samples are exposed to freezing (e.g., -20 °C for 24 hours) and thawing (e.g., +20 °C for 24 hours). This procedure may be repeated numerous times to simulate thermal fluctuations that might be experienced by a commercial product. The holding temperatures chosen are important because the water and fat phases may crystallize at different temperatures.

- **Mechanical stress:** The stock emulsion is adjusted to a certain pH, ionic strength and temperature, and then samples are exposed to standardized mechanical stress conditions e.g., shearing at a constant rate (e.g., 500 s⁻¹) for a fixed time (e.g., 20 minutes); exposing samples to a series of fixed shear rates (e.g., 0 to 500 s⁻¹) for a fixed time at each shear rate (e.g., 5 minutes); or shearing at a constant rate (e.g., 500 s⁻¹) for increasing times (e.g., 0 to 60 minutes).

- **Light stability:** The stock emulsion is adjusted to a certain pH, ionic strength and temperature, and then samples are exposed to standardized ultraviolet or visible radiation of a known intensity versus wavelength profile.

After exposure to these environmental stresses, changes in the particle size, aggregation state, and creaming stability can be measured, as well as other relevant characteristics, such as rheology, optical properties, flavor profile, or chemical degradation.

### 1.4 Natural Emulsifiers

In the context of oil-in-water emulsions, the term “emulsifier” refers to
amphiphilic substances that have the ability to adsorb to oil droplet surfaces, reduce the interfacial tension, and protect them from aggregation [20]. The most frequently utilized food-grade emulsifiers are proteins, polysaccharides, phospholipids, and small molecule surfactants [8-10]. Nevertheless, recently there has been great interest in identifying food-grade colloidal particles to stabilize food emulsions through a Pickering mechanism [135-138]. Food emulsifiers vary considerably in their abilities to form and stabilize oil-in-water emulsions depending on their unique chemical and structural properties [4]. An ideal emulsifier needs to rapidly adsorb to the oil droplet surfaces generated during homogenization, appreciably decrease the oil-water interfacial tension (to facilitate droplet fragmentation), and generate a protective coating (to inhibit droplet coalescence within the homogenizer) (Section 1.2.1). Moreover, the emulsifier coating should keep the lipid droplets stable under the conditions that a commercial product might confront during its production, transport, storage, and utilization (Sections 1.2.2 and 1.3.2). In this section, natural emulsifiers that are already used in commercial food products are reviewed, as well as some that are currently being investigated for their potential application. In addition, the major factors that affect the functionality of different food emulsifiers are discussed so that their potential range of application can be established.

1.4.1 Phospholipids

1.4.1.1 Molecular and Physicochemical Characteristics

Phospholipids are polar lipids naturally found in animal, plant, and microorganism cell walls [139]. In nature, phospholipids form semi-permeable membranes that play important roles in the separation, protection, and transportation of cellular constituents, as well in cellular integrity and signaling [140]. Phospholipids
consist of a glycerol backbone with two fatty acids and a phosphoric acid moiety attached [139]. The fatty acid chains make up the non-polar lipophilic tail of the emulsifier, whereas the phosphoric acid moiety and any attached groups form the polar hydrophilic head. Because phospholipids have appreciable non-polar and polar regions within the same molecule they are amphiphilic molecules that can adsorb to oil-water interfaces and stabilize lipid droplets [141, 142]. When a phospholipid adsorbs to an oil-water interface the non-polar fatty acid tails protrude into the oil phase, whereas the polar hydrophilic head-groups protrude into the surrounding aqueous phase (Figure 1.14). In some circumstances, phospholipids form monolayers around oil droplets, but in other circumstances they may form multiple bilayers (with the molecules lined up head to head), which may impact the stability and properties of emulsions [141, 143].

**Figure 1.14:** Some natural surfactants that can be used to stabilize food emulsions, with some examples given in brackets.

The phospholipid-based functional ingredients used as emulsifiers in commercial products are usually called *lecithins* [9, 142]. Lecithins can be isolated from numerous biological sources, with the most common being soybeans, eggs, milk, rapeseed, canola seed, cottonseed, and sunflower [144]. Commercial lecithins typically contain a combination of various phospholipids and other lipophilic...
materials (such as triglycerides, glycolipids, and sterols), but they can be fractionated
to create more refined ingredients [145]. The most common phospholipids found in
commercial lecithin ingredients are: phosphatidylcholine (PC),
phosphotidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA)
[139]. The hydrophilic head-groups of phospholipids are typically either anionic (PI
and PA) or zwitterionic (PC and PE), with the charge depending strongly on pH. The
non-polar tail groups of phospholipids usually have two fatty acids, which can vary in
the number of carbon atoms and double bonds they contain. In some commercial
lecithin ingredients (“lysolecithins”), one of the fatty acid tails is removed to alter
their functional characteristics [146].

1.4.1.2 Factors Affecting Emulsion Formation and Stability

Unlike most other natural emulsifiers, phospholipids may be dispersed in the
oil or the aqueous phase prior to homogenization. The most appropriate phase to
disperse the phospholipids is governed by the food application, and depends on the
nature of the phospholipids, oil, and aqueous phase and would have to be determined
empirically.

Oil-in-water emulsions have been formed using sunflower lecithins, but the
dimensions of the oil droplets created was reported to be relatively large (30 to 160
µm), which may be because only a high-shear mixer was used to homogenize them
[145]. Another study using sunflower lecithins to form oil-in-water emulsions showed
that most of the phospholipids were adsorbed to the droplet surfaces when used at low
concentrations, but that phospholipid vesicles were formed in the aqueous phase at
higher concentrations [147]. The presence of these vesicles could influence emulsion
appearance, rheology, and stability. The oil droplets created in this study were again
relatively large (40 to 100 µm) due to the fact that only a high shear mixer was used to prepare the emulsions. Nevertheless, recent studies in our laboratory have shown that sunflower lecithins can be used to form emulsions containing small droplets ($d_{32} < 200$ nm) when a high-pressure homogenizer (microfluidizer) is used to fabricate them (Figure 1.15).

![Figure 1.15](image)

**Figure 1.15:** Influence of phospholipid-to-oil ration on the ability of a sunflower lecithin to produce fish oil-in-water emulsions. Data supplied by Jennifer Komaiko.

Another recent study showed that oil-in-water emulsions containing relatively small droplets ($d_{32} < 400$ nm) could be fabricated by microfluidization using soy lecithin as an emulsifier [148]. Under neutral pH conditions, the lecithin-coated droplets were highly negatively charged, which led to good aggregation stability because of the strong electrostatic repulsion between them. However, under highly acidic conditions (pH 1.6), the droplets were not stable to aggregation because the phospholipid head-groups lost their negative charge ($\text{pK}_a = 1.5$). Soy lecithin has also been used to create vitamin E-enriched emulsions containing small droplets ($d < 200$ nm) using microfluidization [44]. Without salt addition, the lecithin-coated lipid droplets were stable to aggregation from pH 8 to 3, but became highly flocculated at
pH 2. Again, this phenomenon can be attributed to the fact that the phospholipid headgroups lost much of their negative charge at these low pH values thereby reducing the electrostatic repulsion between them. At neutral pH, the emulsions underwent appreciable droplet aggregation when the salt concentration exceeded about 100 mM NaCl, presumably due to electrostatic screening of the anionic phospholipid headgroups by cationic sodium ions. Without salt addition, these emulsions were stable to heat treatment (30 to 90 °C, 30 min), which can again be attributed to the strong electrostatic repulsion between the strongly anionic droplets at pH 7.

A number of other studies have also examined the emulsifying properties of lecithins. The mean droplet diameter has been reported to decrease with increasing lecithin concentration during homogenization; with the droplet size produced depending on homogenization method and operating conditions [145, 147, 149, 150]. Emulsion stability has also been related to the molecular composition of the phospholipids used, e.g., the ratio of PC to PE [145]. Phospholipid ingredients with high levels of PC were reported to produce smaller oil droplets [149]. Formulation-composition maps have been developed to predict the optimum lecithin-oil-water ratios needed to produce stable emulsions [151]. Certain types of phospholipids may also be effective at retarding the oxidation of emulsified lipids because of their natural free radical scavenging capacity [94, 95].

Some commercial lecithin ingredients are not particularly good at stabilizing oil-in-water emulsions when used in isolation because they have low or intermediate hydrophilic-lipophilic-balance numbers (HLB= 2 to 8). Nevertheless, these ingredients can be combined with other natural emulsifiers to form stable emulsions. For example, lecithin has been combined with caseins to form antimicrobial emulsions [152], with caseins to form fish oil emulsions [153], with whey proteins to
form lutein-loaded emulsions [154], and with monoacylglycerols to form infant formula emulsions [155]. The functionality of lecithin may also be improved by utilizing cosolvents, such as ethanol, which alter the properties of the surfactant monolayer (such as optimum curvature) thereby facilitating emulsion formation and stability [156]. Alternatively, natural lecithins can be modified by chemically or enzymatically cleaving one of the fatty acid tails from the glycerol backbone to create more polar surfactants (“lysolecithins”) that are suitable for forming and stabilizing emulsions, especially when used in combination with other emulsifiers [146, 157]. The physical and chemical stability of lecithin-coated lipid droplets can also be improved by coating them with oppositely charged biopolymers to form multilayer emulsions, e.g., cationic chitosan has been used to coat anionic lecithin-coated droplets [158-160]. The same approach can be used to alter the potential gastrointestinal fate of lecithin-coated lipid droplets [161].

1.4.2 Biosurfactants

1.4.2.1 Molecular and Physicochemical Characteristics

Saponins are natural small molecule surfactants that are isolated from the bark of a tree (Quillaja saponaria). These biosurfactants typically contain a complex mixture of different amphiphilic constituents that have been shown to form micelles when dispersed in water, and that can facilitate the formation and stability oil-in-water emulsions [162-166]. The dominant amphiphilic components identified within the natural extracts from the Quillaja saponaria tree are saponins [166-168]. The saponins are amphiphilic because they have regions that are hydrophilic (e.g., sugar groups) and regions that are hydrophobic (e.g., phenolic groups) distributed within a single molecule [164, 169]. An emulsifier derived from the quillaja saponin extract
(Q-Naturale®, Ingredion, Bridgewater, NJ) is available commercially for application within the food industry. This ingredient is typically provided in either a powdered form or dissolved within an aqueous solution. It has been reported that the critical micelle concentration (CMC) of quillaja saponins is around 0.025 wt%, and that each molecule occupies about 1 nm$^2$ at the interface [166], which corresponds to a surface load of about 2.8 mg m$^{-2}$. The same study reported that the surface tension at saturation was around 40 mN m$^{-1}$, and that adsorption of the surfactant molecules to interfaces was much slower than predicted by simple diffusion, which suggested that there was a large energy barrier to adsorption. This study also reported that adsorbed saponins form relatively strong elastic interfaces with a surface dilatational elasticity around 280 mN/m and a surface shear elasticity around 26 mN/m. Finally, it has been shown that the interfacial rheology of saponin layers depends on the nature of the oil phase, with the interfacial elasticity increasing with increasing hydrophobicity [170].

1.4.2.2 Factors Affecting Emulsion Formation and Stability

Numerous studies have reported that quillaja saponin is a particularly efficacious emulsifier for forming and stabilizing oil-in-water emulsions. This biosurfactant can form emulsions containing small oil droplets ($d < 200$ nm) that are stable to aggregation over a range of conditions (pH, ionic strength, and temperature) that make it suitable for application in a wide variety of foods [44, 165, 171]. For instance, it has been shown that quillaja saponin can form vitamin E-enriched nanoemulsions ($d < 200$ nm) (Figure 1.5), that may be used as delivery systems to fortify foods and other products with oil-soluble vitamins [44]. In the absence of salt, saponin-coated oil droplets had high aggregation stability from pH 8 to 3, but flocculated at pH 2. At the higher pH values, the droplets were prevented from aggregating because of the high negative charge on them, but once the pH fell below a
certain value the oil droplets became less negatively charged and so became flocculated (Figure 1.9). At neutral pH, the droplets were highly unstable to flocculation at elevated salt levels ($\geq$ 400 mM NaCl, pH 7) due to the reduction in electrostatic repulsion caused by electrostatic screening. The saponin-coated oil droplets also had good heat stability (30 to 90 °C, 30 min, no salt, pH 7) due to the strong steric and electrostatic repulsion between them. Quillaja saponins have also been shown to protect oil droplets from aggregation when the lipid phase crystallizes, which is important for preventing partial coalescence and for the production of solid lipid nanoparticles (SLN) or nanostructured lipid carriers (NLC) [172]. Part of the ability of saponins to form stable emulsions may be due to the fact that they form interfacial layers with a high dilatational elasticity [166], which may inhibit droplet deformation and coalescence. A study of the ability of different kinds of emulsifiers to produce nanoemulsions and emulsions by low energy methods (emulsion phase inversion) reported that quillaja saponins were ineffective because they could not be dissolved in the oil phase [173], which is important for this type of emulsion formation method. Moreover, simulated GIT studies have shown that lipid droplets stabilized by saponins are still rapidly digested [129]. Finally, saponin-stabilized oil-in-water emulsions showed better lipid oxidation stability than those stabilized by synthetic emulsifiers, which was attributed to their free radical scavenging capacity [96].

1.4.3 Proteins

1.4.3.1 Molecular and Physicochemical Characteristics

Proteins are biopolymers consisting of strings of amino acid units covalent linked by peptide bonds [86, 174, 175]. The type, number, and position of amino
acids in the polypeptide chain determine the molecular, physicochemical, and functional properties of food proteins. Most proteins contain a mixture of polar and non-polar amino acids and are therefore amphiphilic molecules that can attach to oil-water interfaces and stabilize lipid droplets in emulsions [12]. The relative balance of polar and non-polar groups exposed on their surfaces governs the surface activity of proteins. If the surface hydrophobicity is too low, then the driving force for protein adsorption is not strong enough to overcome the loss of entropy associated with adsorption. Conversely, if the surface hydrophobicity is too high, then the proteins tend to aggregate, become water-insoluble, and lose their surface activity. Consequently, an optimum level of surface hydrophobicity is typically required for a protein to be a good emulsifier.

Most proteins also have a mixture of anionic, neutral, and cationic amino acids along their polypeptide chains, which determines their electrical characteristics under different pH conditions [175]. The electrical characteristics of a protein have a major influence on its functional properties in emulsions. In particular, electrostatic repulsion plays a critical role in preventing protein-coated oil droplets from aggregating [12, 20, 48]. In addition, electrostatic interactions have an impact on the stability of emulsions to lipid oxidation, since anionic droplet surfaces may attract cationic transition metals that catalyze the oxidation of lipids within the droplets [176, 177]. The distribution of the charges on the surfaces of proteins is also important since this influences the adsorption of other charged species, e.g., charged biopolymers can adsorb to the surfaces of similarly charged droplets if they have sufficiently large patches of opposite charge [178, 179].

Proteins may adopt various conformations in aqueous solutions and at oil-water interfaces depending on the balance of van der Waals forces, hydrophobic
interactions, electrostatic interactions, hydrogen bonding, covalent bonds, steric effects, and entropy effects [12, 68, 180]. This balance is determined by solution and environmental conditions, such as pH, ionic strength, dielectric constant, and temperature. Consequently, the conformation of a protein in solution or at an interface may change when these conditions are altered. The two most common conformations of surface-active proteins used as emulsifiers in the food industry are globular and random coil [52]. Globular proteins have fairly compact spheroid structures where the majority of non-polar groups are located within the interior, and the majority of polar groups are present at the exterior [181]. Nevertheless, many globular proteins still have surface activity because some of the non-polar groups remain exposed at their surfaces, which gives a driving force for adsorption to oil-water interfaces [182]. There are a wide variety of surface-active globular proteins that can be used as emulsifiers, including whey, soy, egg, and plant proteins (Table 1.1). Random coil proteins have a more open flexible structure, although there may still be some regions that have local order such as helical or sheet structures. The most common random coil proteins used as emulsifiers in foods are casein and gelatin (Table 1.1). The structure of proteins often changes after they adsorb to oil-water interfaces because the resulting change in their environment alters the delicate balance between the different molecular interactions and entropy effects [182]. For example, globular proteins may unfold after they adsorb to droplet surfaces and expose groups normally located in their interiors, such as non-polar and sulfhydryl groups [68, 69, 183, 184]. As a result, the proteins may react with other proteins adsorbed to the same or different lipid droplets through hydrophobic or disulfide bonds, which may influence the stability of the droplets to coalescence and flocculation. After adsorption to oil droplet surfaces protein molecules tend to adopt a configuration where many of
the hydrophilic groups protrude into the water phase, whereas many of the hydrophobic groups protrude into the oil phase (Figure 1.14).

The most common proteins used as food emulsifiers are whey proteins and caseins from bovine milk [185]. In addition, other proteins derived from animal sources are also widely used in some food products, such as gelatin and egg proteins [12]. Nevertheless, there is a major push towards identifying, isolating and characterizing alternative types of proteins that can be used as emulsifiers in foods, particularly those from plant sources, such as soy, pea, lentil, chickpea, bean and canola proteins [11, 12]. The various kinds of proteins that may be utilized as emulsifiers are summarized in Table 1.1.

1.4.3.2 Factors Affecting Emulsion Formation and Stability

Proteins differ considerably in their abilities to form and stabilize oil-in-water emulsions, with some proteins being highly effective at producing stable emulsions containing small droplets, and others being highly ineffective [11, 12, 20]. These differences in performance are due to differences in the molecular and physicochemical characteristics of proteins from diverse sources. These characteristics depend on their biological origin, as well as the isolation, processing, and storage conditions used. If a protein is too hydrophilic, then it will not have an appreciable surface activity, e.g., certain types of gelatin [186]. Conversely, if a protein is too hydrophobic, then it may be insoluble in water and form aggregates that have poor surface activity, e.g., zein [187]. Proteins that are water-soluble and that do have sufficient surface activity still differ in their effectiveness at forming and stabilizing emulsions due to differences in their adsorption rates, surface loads, saturation surface pressures, interfacial thickness, surface hydrophobicity, and electrical characteristics [184, 188]. For example, β-lactoglobulin can form smaller
Table 1.1: Comparison of properties of emulsifiers isolated from various natural sources that may be utilized within the food industry. The information in the table was taken from a variety of sources [86, 100, 175].

<table>
<thead>
<tr>
<th>Emulsifier</th>
<th>Molecular Properties</th>
<th>Emulsion properties</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Small molecule surfactants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Quillija saponis</em></td>
<td>Surface active because they contain both hydrophilic (e.g., sugars) and hydrophobic (e.g., phenolics) regions</td>
<td>Can form small droplets at low levels using high-pressure homogenization. Emulsions unstable at highly acidic conditions (pH &lt; 3), and at high ionic strengths. Stable to heating.</td>
</tr>
<tr>
<td><strong>Phospholipids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lecithin</em></td>
<td>Surface active because of polar head-group (phosphate moiety) and non-polar (two fatty acids) tail group</td>
<td>Can forms fairly small droplets at low levels using high pressure homogenization. Unstable under acidic conditions (pH &lt; 3), and at high ionic strength. May breakdown at high temperatures.</td>
</tr>
<tr>
<td><strong>Lysolecithin</strong></td>
<td>Surface active because of polar head-group (phosphate moiety) and non-polar (one fatty acid) tail group</td>
<td>Can forms fairly small droplets at low levels using high-pressure homogenization. Unstable under acidic conditions (pH &lt; 3), and at high ionic strength. May breakdown at high temperatures.</td>
</tr>
<tr>
<td><strong>Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Whey protein</em></td>
<td>Mixtures of globular proteins from milk. MW = 18 kDa; pl = 5, Tm = 80 °C</td>
<td>Unstable at pH near pl, at high ionic strength, and at temperatures &gt; Tm.</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>Globular protein from whey protein. MW = 18.4 kDa; pl = 4.4; Tm = 83 °C</td>
<td>Unstable at pH near pl, at high ionic strength, and at temperatures &gt; Tm.</td>
</tr>
<tr>
<td>α-lactalbumin</td>
<td>Globular protein from whey protein. MW = 14.2 kDa; pl = 4.4; Tm = 83 °C</td>
<td>Unstable at pH near pl, at high ionic strength, and at temperatures &gt; Tm.</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>Globular protein from whey protein. MW = 66.3 kDa; pl = 5.1; Tm = 75 °C</td>
<td>Unstable at pH near pl, at high ionic strength, and at temperatures &gt; Tm.</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Globular glyco-protein from whey protein. MW = 80 kDa; pl = 8; Tm = 60 and 85 °C</td>
<td>Unstable at pH near pl, at high ionic strength, and at temperatures &gt; Tm.</td>
</tr>
<tr>
<td>Caseinates</td>
<td>Mixtures of flexible proteins from milk. MW = 24 kDa; pl = 5</td>
<td>Unstable at pH near pl, and at high ionic strength. Stable to heating.</td>
</tr>
<tr>
<td>αs-casein</td>
<td>Flexible protein from milk. MW = 23.6 kDa; pl = 4.1</td>
<td>Unstable at pH near pl, and at high ionic strength. Stable to heating.</td>
</tr>
<tr>
<td>β-casein</td>
<td>Flexible protein from milk. MW = 24.0 kDa; pl = 5.5</td>
<td>Unstable at pH near pl, and at high ionic strength. Stable to heating.</td>
</tr>
<tr>
<td>Egg proteins</td>
<td>Mixtures of globular proteins from egg white or yolk.</td>
<td>Unstable at pH near pl, at high ionic strength, and at temperatures &gt; Tm.</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>Globular protein from egg white. MW = 45 kDa; pl = 4.5; Tm = 80 °C</td>
<td>Unstable at pH near pl, at high ionic strength, and at temperatures &gt; Tm.</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Globular protein from egg white. MW = 14.3 kDa; pl = 11.3; Tm = 72 °C</td>
<td>Unstable at pH near pl, at high ionic strength, and at temperatures &gt; Tm.</td>
</tr>
<tr>
<td>Legume proteins (Soy, pea, lentil, chickpea, faba bean etc.)</td>
<td>Mixtures of globular proteins from legumes with variable molecular weights. pl = 4.3-5.0; Tm = 82-90 °C</td>
<td>Unstable at pH near pl, at high ionic strength, and at temperatures &gt; Tm.</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Fairly hydrophilic: flexible proteins from animal sources (collagen). Variable molecular weight depending on processing conditions. pl = 5 (Type B) or 8 (Type A); Tm = 10-30 °C</td>
<td>Often not very surface active due to high hydrophilic character.</td>
</tr>
<tr>
<td><strong>Polysaccharides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gum arabic</td>
<td>Branched glycoprotein. MW = 1,000 kDa; pKa ≈ 3.5</td>
<td>Requires a high surfactant-to-oil ratio, but forms droplets stable to a wide range of pH, ionic strength, and temperature.</td>
</tr>
<tr>
<td>Beet Pectin</td>
<td>Branched anionic hydrophilic polysaccharide with hydrophobic ferulic acid groups.</td>
<td>Requires a high surfactant-to-oil ratio, but forms droplets stable to a wide range of pH, ionic strength, and temperature.</td>
</tr>
<tr>
<td>Citrus Pectin</td>
<td>Branched anionic hydrophilic polysaccharide with hydrophobic protein groups attached.</td>
<td>Requires a high surfactant-to-oil ratio, but forms droplets stable to a wide range of pH, ionic strength, and temperature.</td>
</tr>
</tbody>
</table>
droplets than lactoferrin under similar conditions (emulsifier concentration, homogenization pressure, and number of passes) [189], which may be attributed to its smaller surface load, faster adsorption kinetics, and/or higher surface pressure.

For food proteins, the surface tension values are typically between about 22 to 42 mN m\(^{-1}\) and the interfacial tension values are typically between about 8 and 22 mN m\(^{-1}\) depending on oil type [190]. Surface loads for food proteins are usually around 2 to 4 mg m\(^{-2}\) depending on protein type and concentration and system conditions, such as pH, ionic strength, and temperature [190]. Many globular proteins form viscoelastic gel-like interfaces after they adsorb to surfaces due to intermolecular cross-linking with their neighbors, e.g., it has been reported that \(\beta\)-lactoglobulin forms an interface with a surface dilatational modulus around 150 mN m\(^{-1}\) [191]. After adsorption to droplet surfaces globular proteins may undertake conformational changes in response to their new molecular environment, which leads to exposure of hydrophobic groups and sulfhydryl groups. As a result, neighboring protein molecules may form hydrophobic or disulfide bonds with each other [192]. On the other hand, more flexible proteins tend to form layers that are more viscous than elastic, such as casein [192].

Adsorbed proteins usually form interfacial layers that are rather thin (< 10 nm) compared to those formed by adsorbed polysaccharides (> 10 nm), which means that steric repulsion alone is often not sufficiently long-range to inhibit droplet aggregation [12, 48, 52, 61]. Instead, protein-coated droplets are often stabilized against aggregation by having a high electrical charge, which may generate a strong and long-range electrostatic repulsion under appropriate solution conditions, i.e., sufficiently low ionic strength. Hence, protein-coated droplets are highly susceptible to flocculation under conditions where their surface charge is reduced, such as high
salt levels or pH values close to the isoelectric point. On the other hand, they may still be stable to coalescence due to the strong short-range steric repulsion generated by the adsorbed protein layer. Globular proteins tend to expose non-polar groups when they are held at temperatures above their thermal denaturation temperature, which can increase the surface hydrophobicity of the droplets. As a result, the hydrophobic attraction between the droplets becomes stronger, and can lead to aggregation if any repulsive forces (such as electrostatic repulsion) operating in the system are not strong (Figure 1.10). In addition, sulfhydryl groups may be exposed when a globular protein unfolds, which results in the formation of covalent linkages between other proteins adsorbed on the same or different droplets [55, 66].

Proteins adsorbed to oil droplets surfaces have been shown to protect the underlying oil phase from lipid oxidation [193-195]. This may occur due to a number of physicochemical mechanisms associated with the adsorbed protein layer, including free radical scavenging, chelation, steric hindrance, and electrostatic repulsion [87]. Whey proteins, soy proteins and caseinate have been shown to inhibit lipid oxidation in oil-in-water emulsions [90]. Chickpea and lentil proteins have also been shown to inhibit lipid oxidation in emulsions [196, 197]. The metal-catalyzed decomposition of lipid hydroperoxides is the dominant oxidation pathway in emulsions [81]. Copper and iron are pro-oxidative transition metals that are widely found in foods. Some proteins can form complexes with transition metals and thus influence the fate of the lipid oxidation in foods [90].

In the food industry, the most widely utilized protein emulsifiers are whey proteins and caseins isolated from bovine milk [12, 185]. Whey proteins consist of a mixture of globular proteins, whereas caseins consist of a mixture of flexible proteins.
Both types of protein have good water-solubility, high surface-activity, and the ability to stabilize oil-in-water emulsions over a range of conditions. Both the yolk and white of eggs also contain a mixture of surface-active globular proteins that are able to form and stabilize emulsions [12, 198]. Other animal-based proteins, such as gelatin, have also been shown to be effective emulsifiers under certain circumstances [186, 199, 200]. Nevertheless, there has been interest in finding plant-based alternatives to these animal-based proteins for labeling, economic, allergenicity, and functionality reasons [12]. Consequently, researchers have examined various types of plant-based proteins, including those isolated from soy, peas, lentils, beans, chickpeas, and corn [12]. Some of these proteins have been shown to have potential as emulsifiers, although in many cases the proteins have to be physically, chemically, or enzymatically modified before they are effective. In certain cases, the modification method used means that the resulting emulsifiers can no longer be considered to be natural. In addition, the performance and economic viability of any new protein-based emulsifiers needs to be established under the demanding conditions experienced within many food products. Protein-based emulsifiers are available as fairly crude extracts (such as whey protein concentrates) or as more purified extracts (such as β-lactoglobulin or α-lactalbumin). Typically, the more pure the extract, the more expensive is the ingredient. The properties of different protein-based emulsifiers are summarized in Table 1.1.

Consumers are changing their dietary preferences and are leaning more towards clean labels [201]. In particular, there is a shift towards plant-based proteins rather than animal-based ones [202] because of their wide availability, low-cost, consumer desirability, and nutritional benefits [203, 204]. In addition, whey proteins and caseins have been reported to be food allergens [127], while some plant proteins are not. Therefore, there is an increase in studies on sources of novel protein sources,
such as faba bean, lentil, pea, and chickpeas [11, 196, 197, 202, 205]. Legume proteins are globular proteins that can stabilize emulsions by forming relatively thick and charged layers around oils droplets that generate strong steric and electrostatic repulsion [206]. Soybean proteins have been widely used as food emulsifiers because of their high solubility and good surface activity [207]; however, there is a high risk of allergic reactions combined to soy. Chickpea, pea, lentil and faba bean proteins have particularly strong potential as food emulsifiers because of their non-genetically modified production style, high nutritional value, and low risk of allergic reactions [59, 60, 202, 205, 206, 208-211].

As mentioned earlier, some proteins have been shown to be particularly effective at improving the stability of emulsions to lipid oxidation [212]. Lipid oxidation is typically inhibited by the proteins at pH values below the pI of the protein due to electrostatic repulsion of the cationic transition metals by the cationic droplet surfaces [98]. The pI of legume proteins usually ranges from around pH 4.3 to 5.0, so at neutral pH the net charge on the legume proteins is negative. As a consequence, they may be less effective as antioxidants because there is an electrostatic attraction between the cationic transition metals and anionic droplets, which brings these pro-oxidants into close proximity to the lipids.

Legume proteins typically have lower digestibility than proteins from other sources, which could affect the bioavailability of any encapsulated lipids [213]. The hydrolysis of vegetable proteins has been reported to lead to the formation of larger peptides than those formed by animal proteins [214]. Conversely, pea proteins were reported to be completely digested in in vivo studies [215]. The digestibility of lentil and faba bean proteins was reported to be more extensive to that of chickpeas [216].
It should be noted that the functional properties of proteins may vary considerably depending on their native structures, but also on the way they are isolated, purified, stored, and processed, since these steps may alter their molecular conformation, aggregation state, and functional properties. Indeed, this is often an important consideration when developing new protein-based ingredients: producing a final ingredient with well-defined and consistent properties from batch-to-batch.

1.4.4 Polysaccharides

1.4.4.1 Molecular and Physicochemical Characteristics

Polysaccharides are natural polymers consisting of one or more types of monosaccharide linked together by glycosidic bonds [86, 174, 175]. Polysaccharide molecules vary considerably in their molar masses, degree of branching, electrical charge, hydrophobicity, and polarity, which alter their physicochemical attributes and functional performance. Some polysaccharides have polypeptides (glyco-proteins) or lipids (glyco-lipids) covalently attached to them, which often influences their ability to act as emulsifiers. Many polysaccharides are not good emulsifiers because they are mainly comprised of hydrophilic monosaccharides and are therefore not particularly surface active [52]. Nevertheless, some polysaccharides do contain a balanced appropriate mixture of non-polar and polar groups and are therefore amphiphilic molecules that can adsorb to oil droplet surfaces and thereby stabilize emulsions. The non-polar groups may be part of the carbohydrate molecule (e.g., methylated groups) or they may be non-carbohydrate moieties (e.g., lipids or proteins) that are covalently or physically attached to the carbohydrate molecules.

By far the most widely used natural polysaccharide emulsifier in the food industry is gum arabic [217-219]. Gum arabic is amphiphilic because it has a non-
polar polypeptide backbone with a number of polar polysaccharide chains attached. After adsorption to oil droplet surfaces, the polypeptide chain protrudes into the oil phase, whereas the polysaccharide chains dangle into the water (Figure 1.14). This leads to the formation of a relatively thick hydrophilic coating around oil droplets, which gives them good stability against aggregation due to strong steric repulsion (Figure 1.8). A new form of gum arabic, based on a controlled heating and humidity process, has been shown to have improved emulsification properties [220]. Two other polysaccharide-based emulsifiers used in the food industry are modified starch and modified cellulose, which have non-polar hydrocarbon chains covalently attached to polysaccharide chains [221]. However, these emulsifiers are not natural since their synthesis involves the chemical modification of starch or cellulose molecules, and so they will not be considered further here.

A number of researchers have focused on the identification of new sources of amphiphilic polysaccharides suitable for use as emulsifiers. Pectin fractions isolated from various sources (beet, citrus, apple, and okra) have been shown to have surface activity and the ability to stabilize oil-in-water emulsions [222-224]. Pectin fractions with higher levels of protein were reported to be more effective at forming small droplets during homogenization, which can be attributed to the fact that the proteins have non-polar groups that help anchor the molecules to the oil phase. Corn fiber gum can be used to fabricate oil-in-water emulsions containing relatively small stable droplets [225, 226]. This polysaccharide contains some non-polar hydrophobic groups (possibly polypeptide and/or phenolic groups) attached to a polar polysaccharide backbone. Another polysaccharide that appears to be a highly effective emulsifier is water-soluble yellow mustard mucilage, which has been shown to form stable emulsions at much lower levels than gum arabic [227]. Chitosan, a cationic
polysaccharide typically derived from crustacean shells, has also been shown to be capable for facilitating emulsion formation and stability [228]. Other sources of polysaccharide that have been shown to be effective as emulsifiers include those isolated from soybeans [229], basil seeds [230], gum tragacanth [231], and olives [232]. Further work is needed to thoroughly test these emulsifiers under standardized conditions, and to establish their potential commercial applications, economic feasibility, batch-to-batch consistency, and reliability of source.

1.4.4.2 Factors Affecting Emulsion Formation and Stability

Many amphiphilic polysaccharides have relatively large molecular weights and dimensions, and therefore have high surface loads (Γ). As a result, relatively high amounts are required to produce small droplets during homogenization (Figure 1.5). For example, typically a 1:1 mass ratio of emulsifier-to-oil is required to form small droplets using gum arabic (Γ = 26 mg m⁻²) [220] compared to less than 1:10 for whey proteins (Γ = 2 mg m⁻²). A similar challenge is likely to exist for other types of amphiphilic polysaccharides that have high molecular weights, although it has been reported that some of them can be used at appreciably lower amounts than gum arabic [227].

The relatively thick and hydrophilic biopolymer layers formed by polysaccharide-based emulsifiers often means that they are mainly stabilized by steric repulsion [20, 52]. Nevertheless, many polysaccharides do have an appreciable electrical charge, which can impact their ability to act as emulsifiers, e.g., by influencing their interactions with charged mineral ions, surfactants, proteins, or other polysaccharides. Indeed, the electrical charge on polysaccharides is critical for the
assembly of many types of structured emulsions, such as filled hydrogels, coacervates, or multilayer emulsions (Figure 1.16) [1].

Figure 1.16: Emulsion droplets can be stabilized by small colloidal particles that adsorb to the droplet surfaces, which is referred to as Pickering stabilization.

The fact that polysaccharide-coated lipid droplets are primarily stabilized by steric repulsion means that the emulsions tend to be much less affected by changes in pH and ionic strength than protein-coated droplets [20, 52]. For example, gum arabic-coated droplets are stable to droplet flocculation over a range of pH values (3 to 9), salt conditions (0 to 500 mM NaCl and 0 to 25 mM CaCl₂), and temperatures (30 to 90 °C) [42, 43, 45, 50]. The high stability of these systems to environmental stresses can again be attributed to the strong steric repulsion between them, and is one of their major advantages over other types of natural emulsifiers.

1.4.5 Natural Colloidal Particles

A considerable research effort has recently been directed to the identification of food-grade colloidal particles that can be used to stabilize oil-in-water emulsions through a Pickering stabilization mechanism [135, 233]. This type of colloidal particle tends to become strongly attached to oil-water interfaces because their surfaces are partially wetted by both oil and water phases (Figure 1.16). When the colloidal particles are wetted better by the aqueous phase than the oil phase they tend to
protrude into the water and can therefore stabilize oil-in-water emulsions (Figure 1.16).

Some examples of nanoparticles and microparticles derived from natural sources that have potential to stabilize oil-in-water emulsions through a Pickering mechanism include chitin [234, 235], cellulose [236], starch [237], zein [238], pea protein [239], soy protein [240], kafirin [241] and cocoa [242] particles. A comprehensive overview of different kinds of food-grade colloidal particles that have been investigated is given elsewhere [243]. A major advantage of using colloidal particles to stabilize emulsions is that they can lead to systems that are very stable to droplet coalescence. On the other hand, a major drawback is that they can typically only be used to form emulsions containing relatively large oil droplets ($d > 2 \mu m$). This means that the droplets do not have very good stability against gravitational separation. In addition, colloidal particles used to stabilize Pickering emulsions may inhibit lipid oxidation by forming thick interfacial layers and physically separating the prooxidant compounds in the continuous phase from the lipid hydroperoxides located at the droplet interface [97]. Consequently, there is currently great interest in identifying alternative sources of natural food-grade colloidal particles that can be used to stabilize emulsions with small droplets [243]. Ideally, these should be ultrafine particles that rapidly adsorb to the droplet surfaces during homogenization, and form small oil droplets coated by a layer of colloidal particles that protrude into the aqueous phase.

The GIT fate of Pickering emulsions stabilized by natural colloidal particles has not been widely studied. One in vitro study showed that lipid digestion was retarded in emulsions containing oil droplets coated by chitin nanocrystals [244]. Another study showed that the rate of lipid digestion in emulsions containing oil
droplets coated by kafirin nanoparticles was between that of bulk oil emulsions containing oil droplets coated by a synthetic surfactant [241]. This effect was attributed to the fact that the protein nanoparticles were digested by proteases in the simulated GIT, which led to droplet coalescence and therefore a decrease in droplet surface area. These studies show that the potential gastrointestinal fate of Pickering emulsions depends on the nature of the colloidal particles used, which highlights the need for further studies in this area.

1.4.6 Emulsifier Complexes

The ability of some natural emulsifiers to form and stabilize emulsions can be improved by using them in combination with other emulsifiers, e.g., proteins-polysaccharides, surfactants-proteins, or surfactants-polysaccharides.

![Figure 1.17: Schematic representation of different kinds of mixed interfacial layer that can be formed at oil droplet surfaces to stabilize emulsions](image)

Emulsifiers can be used in combination using different approaches (Figure 1.17):

- **Co-adsorption**: In this case, the two emulsifiers are both adsorbed to the lipid droplet surfaces as individual molecules [64, 245]. The resulting interface may consist of a homogeneous mixture of the two different emulsifiers, or it may have regions rich in one emulsifier and depleted in another. The emulsifiers
may be both incorporated into the system prior to homogenization by dispersing them in the oil and/or water phases. Alternatively, one emulsifier may be added before homogenization, and the other emulsifier added after homogenization. The overall composition of the interface will depend on the relative affinity of the two emulsifiers for the oil-water interface (their surface activities), as well as their relative concentrations.

- **Complexation:** In this case, the two components (which may be two emulsifiers or an emulsifier and another molecule) form a complex through physical or non-physical interactions, such as electrostatic, hydrogen bonding, hydrophobic forces, or covalent bonding [64]. The complexes may be formed before or after homogenization. In the first case, the two components are mixed together in the aqueous phase to form a complex, and then the aqueous phase is homogenized with an oil phase. In the second case, one of the components (an emulsifier) is used to form an emulsion containing emulsifier-coated lipid droplets, and then the other component is added to form a complex.

- **Layer-by-layer deposition:** Initially, an emulsion is fabricated by homogenizing oil, water, and emulsifier together [246]. The emulsifier used should have some ionizable groups, so that the emulsifier-coated droplets have an electrical charge. This emulsion is then mixed with a solution containing polymers or particles that have an opposite charge to the emulsifier-coated droplets, which causes them to be adsorbed onto the droplet surfaces through electrostatic attraction. The resulting “multilayer” emulsion typically has an opposite charge to the original emulsion. The electrostatic deposition process can be repeated a number of times to form a series of layers around the
droplets, which may improve their stability and functional performance. Nevertheless, the system composition and structure must be carefully controlled during the electrostatic deposition process to avoid droplet aggregation [246].

There are appreciable differences between the emulsifying abilities of individual natural emulsifiers. For instance, when used at low levels, protein emulsifiers are often more effective at generating fine oil droplets during homogenization than polysaccharide emulsifiers. Conversely, polysaccharide emulsifiers are usually more effective at generating oil droplets that are stable to a broader range of environmental conditions, such pH, ionic strength, temperature, and freezing. Some of the approaches mentioned above may therefore be used to form emulsifier combinations that can overcome the challenges using individual emulsifiers. Indeed, it has been reported that protein-polysaccharide complexes are better emulsifiers than either of the biopolymers used on its own [52, 64, 246]. It has been shown that considerable improvements in the stability of oil-in-water emulsions to pH changes, salt addition, heating, freezing, and drying [246]. As an example, depositing an anionic polysaccharide (pectin) onto the surfaces of protein-coated lipid droplets improves the pH stability of the emulsions (Figure 1.18). In this example, the pectin molecules form a coating around the droplets that increases the steric and electrostatic repulsion between the droplets, and therefore helps prevent the droplets from aggregation. The complexes formed by proteins and polysaccharides may be held together by physical or covalent bonds, and they may be created prior to, during, or after the homogenization process. Commercial emulsifiers based on protein-polysaccharide complexes will have to meet regulatory requirements, be economically feasible, and provide enhanced functionality before they are used in the food industry.
Figure 1.18: Example of ability of multilayer formation through layer-by-layer deposition to improve the pH stability of protein-coated lipid droplets. An anionic polysaccharide (pectin) was deposited onto the surfaces of β-lactoglobulin coated lipid droplets. As a result less droplet aggregation occurs around the isoelectric point of the protein in the presence of the polysaccharide.

1.5 Conclusions

There is a strong demand from consumers for “all-natural” foods and beverages, which has driven researchers in the food industry to identify natural alternatives to many synthetic ingredients currently utilized in foods. This chapter has focused on recent progress in the identification and characterization of natural emulsifiers, such as biosurfactants, phospholipids, proteins, polysaccharides, and colloidal particles. Many of these natural emulsifiers are capable of forming oil-in-water emulsions containing relatively small droplets that are stable over a range of environmental conditions, and may therefore be suitable for utilization within commercial food products. Nevertheless, there are still challenges to overcome for many types of natural emulsifiers. Proteins are capable of forming small droplets at low usage levels, but the droplets formed are often highly susceptible to aggregation.
at certain pH values, high ionic strengths, or after thermal processing. Conversely, high levels of polysaccharides are typically needed to form emulsions containing small droplets, but the droplets formed have excellent stability to environmental stresses, such as pH, ionic strength, and temperature changes. Biosurfactants, such as saponins, are capable of forming small droplets at low levels that are stable to a wide range of environmental conditions, and may therefore be particularly suitable for food applications.

For certain applications in the food industry it would also be useful to identify natural emulsifiers that have enhanced functional performance, such as stability to freezing/thawing, protection of encapsulated components against chemical degradation, or controlled release properties. Consequently, there is still a need for researchers to search the natural world for new sources of emulsifiers. Based on our current understanding of the structure-function relationships of emulsifiers, these molecules should have a number of characteristics: they should be water-dispersible and amphiphilic; they should be relatively small so that they can rapidly adsorb to droplet surfaces during homogenization; and, they should form thick hydrophilic layers to give good steric stabilization. Each newly identified natural emulsifier should be carefully characterized in terms of its ability to form and stabilize emulsions.
CHAPTER 2

LUTEIN-ENRICHED EMULSION-BASED DELIVERY SYSTEMS: INFLUENCE OF PH AND TEMPERATURE ON PHYSICAL AND CHEMICAL STABILITY

2.1 Introduction

An important trend in the modern food industry is for products that are manufactured "without artificial additives" as preservatives, flavorings, and colorings [247]. In addition, consumers are tending to purchase more functional food products that claim to provide additional health benefits beyond their normal nutritional effects [247]. Lutein is a natural pigment that has been shown to exhibit a range of potentially beneficial biological effects, and it is therefore an interesting food ingredient for replacing artificial dyes and for creating functional foods. Indeed, it has recently been reported that lutein, which is mainly extracted from Marigold flowers (Tagetes erecta), has the fastest growing market among the carotenoids with a market value of around US$233 million in 2010, projected to grow to US$309 million by 2018 [248].

Like other carotenoids, lutein is one of the major pigments in fruits and vegetables that lead to their characteristic yellow, red and orange colors. These carotenoids are found in appreciable levels in green leafy vegetables such as kale, spinach, lettuce, broccoli, peas, Brussel sprouts, and parsley, as well as in egg yolks, tomatoes, corn, and marigold flowers [249-252]. Lutein belongs to the xanthophyll class of carotenoids, which are oxygenated carotenes [252].

Lutein, as well as other xanthophylls, may decrease the risk of age-related macular degeneration and cataracts [249, 250, 252]. Xanthophylls accumulate in the
pigmented region of the human eye, which is called the macula, and since they have high absorptivity within a specific wavelength range, they absorb the blue light that reaches the eye. Moreover, they can act as antioxidants by scavenging free radicals or quenching singlet oxygen [251-253], thus decreasing oxidative stress in the retina. Since carotenoids, including lutein, cannot be synthesized in the human body, it is essential that they be consumed as part of the daily diet [252-254]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that the acceptable daily intake (ADI) for lutein and zeaxanthin is 0-2 mg/kg body weight [255]. In addition, 10 mg/day was found to be an effective dose to provide protection against diseases such as age-related macular degeneration and cataracts [154]. Average lutein and zeaxanthin intake of a US consumer is around 2 mg per day [256]. Dosages of up to 40 mg/day in humans showed no adverse effects after eye examinations. The presence of lutein crystals that could cause retinal damage was also not found. The only adverse effect was carotenedermia, which is a reversible and harmless cutaneous hyperpigmentation [257]. Eggs are one of the major natural sources for carotenoids and also contain them in a very bioavailable form. However, there are some concerns about the consumption of eggs leading to increased serum cholesterol levels.

Another concern is that lutein is sensitive to the thermal processing and storage process and thus can degrade in foods that are naturally rich or enhanced with lutein [258]. Carotenoid oxidation can be enhanced by photodegradation, thermal degradation, acid exposure, autoxidation, and singlet oxygen; these different pathways can cause bioactivity and quality (color loss and rancidity) loss in food products fortified with carotenoids [250, 252]. Therefore, it is crucial to understand the
degradation process of lutein in order to develop better protection systems for them in foods [250].

One of the major challenges to utilizing lutein as a functional food ingredient is its relatively low and variable oral bioavailability [252-254]. The poor bioavailability profile of lutein can be attributed to its low water-solubility, high melting-point, and poor chemical stability [154, 259]. As a result of these challenges, carotenoids cannot usually be directly incorporated into aqueous-based foods. Instead, a colloidal delivery system, such as an oil-in-water emulsion, is often required to overcome these limitations [250]. An oil-in-water emulsion consists of small lipid droplets (containing the lipophilic bioactive) suspended in an aqueous medium. This type of emulsion-based delivery system provides a suitable means of dispersing a lipophilic bioactive into the aqueous environments found in many commercial food products. In addition, the lipid phase breaks down within the human gastrointestinal tract to form colloidal structures (mixed micelles) that are capable of solubilizing and transporting the bioactive agents, thereby increasing their bioavailability [253]. Furthermore, emulsion-based delivery systems may also be designed to inhibit the rate of carotenoid degradation [250].

For commercial applications, it is important that any delivery system should remain physically and chemically stable when exposed to the different pH and temperature environments during its processing, storage, and transportation [260]. The aim of this work was therefore to study the effect of temperature and pH on the physical and chemical stability of lutein-enriched emulsions. A natural protein-based emulsifier (caseinate) was used to stabilize the emulsions, and a source of long chain triacylglycerols (corn oil) was used as the lipid phase since this type of lipid has previously been shown to increase the bioaccessibility of carotenoids [113, 261].
2.2 Materials and Methods

2.2.1 Materials

Lutein (MariLut Lutein Oil 20% in corn oil) was kindly donated by PIVEG (San Diego, CA). Mazola corn oil was purchased from a local store. Spray dried sodium caseinate was purchased from the American Casein Company (Burlington, NJ). A lutein standard for chromatography analysis was purchased from Extrasynthese (France). Sodium azide and mono- and dibasic sodium phosphate were purchased from Sigma-Aldrich (St. Louis MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Waltham, MA, USA).

2.2.2 Emulsion Preparation

An organic phase was prepared by diluting 2.5% (w/w) of the commercial lutein in corn oil. An aqueous phase was prepared by dispersing 1% (w/w) powdered sodium caseinate into aqueous buffer solution (5 mM phosphate, pH 7.0). A coarse oil-in-water emulsion was prepared by mixing the organic phase (10%, w/w) and the aqueous phase (90%, w/w) using a high-speed mixer M133/1281-0 (Biospec Products, Inc. Bartlesville, OK, USA) for 2 min at 10,000 rpm. The resulting coarse emulsion was then passed through a high-pressure microfluidizer M-110L for five passes at 12,000 psi (Microfluidics, Newton, MA, USA). The fine emulsion produced was then diluted (1:1, v/v) with buffer solution containing an antimicrobial agent (5 mM phosphate buffer, pH 7.0, 0.04% (w/v) sodium azide). The final diluted emulsion that were used for the stability studies contained 5% (w/w) oil phase and 250 mg/L lutein.
2.2.3 Stability Study

The emulsions were stored for 7 and 14 days at different temperatures (5, 20, 37, 55 and 70 ºC) and different pH values (2-8), respectively. The pH values were adjusted to the desired values using 0.1 and 1.0 N of hydrochloric acid and/or sodium hydroxide solutions. The emulsions with pH values of 2, 3, 4, 5, 6, 7 and 8 and were stored at 20 ºC, while samples at pH 7 were stored at 5, 20, 37, 55 and 70 ºC.

2.2.3.1 Chemical Stability

The chemical stability of lutein was assessed by measuring the change in color and lutein concentration in the emulsions during storage. The color was monitored using a colorimeter ColorFlex EZ (HunterLab Reston, VA, USA). For the color analysis, 10 mL of emulsion was pipetted onto a plastic petri dish and the readings were performed against a black background. The concentration of lutein was determined from absorbance measurements (460 nm) made using a UV–visible spectrophotometer Cary 100 UV-VIS (Agilent Technologies, Santa Clara, Ca, USA). To prepare the samples for the spectrophotometric measurements, the emulsions were diluted 100 times in DMSO (50 µL of emulsion was diluted in 4.95 mL of DMSO). The emulsion without lutein was used as blank. A calibration curve was made by dissolving the lutein standard in DMSO in a range from 0.5 to 5 mg/L ($r^2=0.9992$).

2.2.3.2 Physical Stability

The physical stability of the emulsions was assessed by measuring the change in mean droplet diameter and ζ-potential of the emulsions after 7 or 14 days of storage. The mean droplet diameters, particle size distributions, and ζ-potential were measured using a dynamic light scattering/micro-electrophoresis instrument Zetasizer Nano ZS (Malvern Instruments, Malvern, England). Samples were diluted 100 times
in buffer solution (5 mM phosphate) at the proper pH to avoid multiple scattering and measurements were made at 25 °C.

2.2.4 Data Analysis

All experiments were performed in triplicate and the results are given as mean values ± standard deviation. Differences among the treatments were determined using an analysis of variance (ANOVA) and a post-hoc Tukey test with a confidence level of 95%. The analyses were made using SPSS software (IBM Corporation, Armonk, NY, USA).

2.3 Results and Discussion

2.3.1 Emulsion Preparation

Emulsion-based delivery systems were prepared by homogenizing the organic and aqueous phases together to create a 10% w/w oil-in-water emulsion that contained 476 ± 22 mg of lutein per liter of emulsion. If this emulsion were diluted ten times to create a low-fat dairy-like beverage, then the amount of lutein per serving (240 mL) would be approximately 11.5 mg, which is higher than the recommended daily intake of lutein (10 mg) necessary to exert a beneficial effect on human health [154]. Emulsions prepared with and without lutein had monomodal distributions and mean droplet diameters of 231.8 ± 1.6 and 220.2 ± 0.5 nm, with polydispersity indexes of 0.155± 0.015 and 0.144± 0.05, respectively (Figure 2.1). The slight increase in droplet size in the presence of lutein may have been because it affected droplet fragmentation within the homogenizer due to an increase in viscosity of the oil phase. It is known that an increase in oil phase viscosity increases the particle diameter of emulsions produced by homogenization, and that lutein increases the viscosity of the oil phase [46, 262, 263].
Figure 2.1: Droplet size distribution of corn oil and corn oil-lutein enriched emulsions stabilized by sodium caseinate (0.25% lutein, 5% corn oil, 0.5% sodium caseinate in 5 mM phosphate buffer at pH 7).

2.3.2 Impact of Storage Conditions on Chemical Stability of Lutein-enriched Emulsions

2.3.2.1 Impact of Temperature

Changes in the color of lutein-enriched emulsions were measured throughout storage at different incubation temperatures (Figure 2.2). The color was assessed using a colorimeter to obtain the Commission Internationale de l’Eclairage (CIE L*a*b*) color coordinates. There was strong evidence of color fading in many of the emulsions during storage, as evidenced by a decrease in color intensity (particularly a*-value) and increase in lightness (L*-value) [264]. The rate of color fading clearly increased as the storage temperature increased. For example, the red color of the emulsions (a*-value) remained relatively constant at the lowest incubation temperature (5 ºC), but it fell increasingly rapidly as the incubation temperature was increased. The reduction in the red color of these emulsions can be explained by the
increased degradation of the lutein at higher temperatures. Similarly, the lightness of the emulsions remained relatively stable at the lowest incubation temperatures, but grew increasingly as the storage temperature was increased (Figure 2.2A). This increase in lightness can be attributed to the fact that less light was absorbed by lutein at elevated temperatures, and therefore more light was scattered by the emulsion droplets [264, 265].

(A)  

(B)  

(C)  

(D)
Figure 2.2: Effect of storage temperature on the (A) lightness (L-value); (B) green-red axis (a*-value); (C) blue-yellow axis (b*-value); (D) total color change (ΔE*); (E) appearance of lutein-enriched nanoemulsions stabilized by sodium caseinate. Emulsion composition: 0.25% lutein, 5% corn oil, 0.5% sodium caseinate in 5 mM phosphate buffer at pH 7.

The concentration of lutein remaining in the emulsions during storage followed a similar trend as for the redness (a*-values) of the emulsions (Figure 2.3A). Carotenoids may degrade through various mechanisms, such as oxidation and isomerization, depending on their composition and storage conditions [250]. Nevertheless, the overall degradation rate for these reactions usually increases with a rise in temperature. Heating in the presence of oxygen and metals can lead to the formation of various radical species that are transformed into peroxyl radicals, which can undergo propagation reactions with carotenoids [250, 266]. Moreover, it was reported that the degradation rate of lutein was higher when it was emulsified compared to when it is dispersed in a bulk oil phase [254], which may be because the carotenoid is more exposed to pro-oxidants in the aqueous phase of an emulsion.
Figure 2.3: Effect of storage temperature on the (A) concentration of lutein and (B) color and lutein degradation rate in lutein-enriched nanoemulsions stabilized by sodium caseinate. Emulsion composition: 0.25% lutein, 5% corn oil, 0.5% sodium caseinate in 5 mM phosphate buffer at pH 7.

Interestingly, there was only an appreciable decrease in the intensity of the yellowness of the emulsions (b*-values) when they were stored at 70 ºC. In addition, there was actually a slight increase in the yellowness of the emulsions stored at 37 and 55 ºC. As with other xanthophylls, lutein exhibits a red color at high concentrations, but an intense yellow color at lower concentrations due to the light that is absorbed by the double bonds on the backbone of the molecule [252]. The observed changes in the a*- and b*-values of the emulsions can therefore be related to their overall color at the different incubation temperatures. At relatively low temperatures (5 and 20 ºC), the emulsions maintained an orange color, at intermediate temperatures (37 and 55 ºC) they had a slightly less intense orange color, and at high temperatures (70 ºC) they only had a slight yellowish color.

The total color difference was calculated so as to compare the differences in color intensity of the emulsions using a single value [267]:
\[ \Delta E^* = \sqrt{(L^* - L_i^*)^2 + (a^* - a_i^*)^2 + (b^* - b_i^*)^2} \]  

(1)

Where, \( L^*, a^*, b^* \) are the measurements of the CIE L*a*b* space at time \( t \), and \( L_i^*, a_i^*, b_i^* \) are the initial measurements immediately after emulsion preparation. The total change in color followed a fairly linear trend during the initial stages of storage (Figure 2.2D). The overall color of the emulsions stored at 5 and 20 °C remained relatively stable, while it changed increasingly rapidly with increasing storage temperature. To be able to clearly compare the fading of the color of the enriched emulsions and the reduction in the lutein concentration at the studied temperatures, the daily rate of the changes was calculated as the slope of the linear regression of \( \Delta E^* \) versus time in the linear region of the plots. The rate of lutein loss clearly increased with temperature (Figure 2.3B). There was a very strong correlation between the rate of color fading and the rate of lutein loss (Pearson correlation coefficient = 0.9996). The activation energy associated with lutein loss during storage was calculated using the Arrhenius equation:

\[ \ln(k) = \ln(A) - \frac{E_a}{RT} \]  

(2)

Where, \( k \) is the color fading or concentration reduction rate calculated as the slope of the linear regression of \( \Delta E^* \) versus time \( A \) is a pre-exponential factor; \( E_a \) is activation energy; \( R \) is the universal gas constant, and \( T \) is the absolute temperature in degrees Kelvin. The activation energy of the color fading was calculated using equation 2 and resulted in 37.98 kJ/mol (\( R^2=0.95 \)), which is similar to the activation energy of the lutein concentration reduction 38.43 kJ/mol (\( R^2=0.98 \)), again highlighting the close relation between lutein degradation and color fading in the emulsions. The activation energy obtained in this work was lower than the one obtained by Lim, Griffin [266] who encapsulated lutein in whey protein isolate single layer and layer-by-layer freeze-
dried emulsions (58.9 and 45.9 kJ/mol, respectively). This difference may be due to differences in the nature of the emulsifiers used (casein versus whey protein), as well as differences in the physical state (liquid versus solid) of the emulsions used in the two studies. The activation energy for lutein degradation in model emulsions and in orange juice were reported to be 60.1 and 65 kJ/mol, respectively [265], which are again higher than the values found in the current study. Again, these differences may be due to the different compositions and structures of the delivery systems being tested [81]. A common method used to estimate the impact of temperature in the loss of quality of a food product is the $Q_{10}$ value which gives the increase in the rate of color fading when the temperature is increased by 10 °C. The following equation is used to calculate the $Q_{10}$ value [268]:

$$Q_{10} = \exp\left(\frac{10E_a}{RT(T+10)}\right)$$  \hspace{1cm} (3)

For color fading, the $Q_{10}$ value determined was 1.73 which means that the loss of color nearly doubles when increasing the temperature by 10 °C.

### 2.3.2.2 Impact of pH

Food products and beverages present a wide range of pH from acidic beverages such as soft drinks or juices to neutral beverages such as milk. Therefore, the influence of pH on color and lutein loss was studied (Figure 2.4). The study was carried out at room temperature and extended to 14 days due to the relatively high stability of lutein at this incubation temperature. The samples at pH 4 and 5 were completely unstable to droplet aggregation and creaming (Figure 2.4D), and therefore color and lutein measurements were not carried out for these treatments. For the physically stable emulsions, there was little impact of storage pH on the rate of color fading or lutein loss, with the exception of the samples stored at pH 8 that appeared to
be slightly more stable. The change in lightness and color intensity of the samples followed similar trends as the ones discussed in section 3.2.1, i.e., there was a slight increase in lightness and yellowness and a slight decrease in redness (data not shown). Nevertheless, the overall visual appearance of the samples did not change noticeably at any of the pH values studied, except for the physically unstable emulsions at pH 4 and 5 (Figure 2.4D). It is known that carotenoids undergo protonation of the carbon atoms of the conjugated systems in acidic environments that results in accelerated degradation and isomerization of these compounds [250, 269]. The total color change of the lutein emulsions at pH 3 was around 10 times lower compared to the total color change of β-carotene encapsulated in orange oil emulsified with β-lactoglobulin [270]. The higher stability of lutein could be related to the presence of hydroxyl groups, which alter the protonation of the carotenoids [269]. In addition, Khalil, Raila [254] found that lutein esters are more stable when encapsulated in emulsions made with medium chain triglyceride oils than in orange oil.

![Graph A](image)

![Graph B](image)
2.3.3 Impact of Storage Conditions on Physical Stability of Lutein-enriched Emulsions

2.3.3.1 Impact of Temperature

The physical stability of emulsion-based delivery systems during manufacture, transport, storage, and utilization is a critical aspect for their practical application. Changes in temperature can affect the stability of emulsions through numerous mechanisms. Heating leads to an increase in the droplet-droplet collision frequency, which can promote aggregation under conditions where there is not a strong repulsion between the droplets [260]. Heating can also cause conformational changes of any emulsifier molecules adsorbed to the droplet surfaces, which can alter their ability to stabilize the droplets against aggregation. For example, thermal denaturation of adsorbed globular proteins can promote droplet aggregation through increased
hydrophobic interactions and/or disulfide bond formation when the proteins unfold [271]. However, this effect is less important for flexible proteins such as the casein.

In this study, we found that emulsions containing casein-coated lipid droplets were relatively stable to thermal processing. Different treatments presented statistical differences (p<0.05) but with no observed increase in mean particle size (Figure 2.5). Caseins are relatively flexible and disordered proteins that lack cysteine groups, and therefore they are less likely to promote droplet aggregation through hydrophobic attraction or disulfide bonds [272, 273].

![Figure 2.5: Effect of storage temperature on the droplet diameter of lutein-enriched nanoemulsions stabilized by sodium caseinate after 7 days of storage. Emulsion composition: 0.25% lutein, 5% corn oil, 0.5% sodium caseinate in 5 mM phosphate. Different letters are significantly different for each storage condition s (p < 0.05)](image)

2.3.3.2 Impact of pH

It is known that emulsions containing protein-coated lipid droplets are mainly stabilized by electrostatic repulsion, and are highly susceptible to changes in solution pH [48, 61]. For this reason, the influence of pH on the mean particle diameter of the lutein-enriched emulsions was measured after 14 days storage (Figure 2.6A). At pH 4
and 5, the mean particle diameter increased steeply to values > 1000 nm and the emulsions underwent visible phase separation, with a white cream layer on top of a clear serum layer (Figure 2.4D). These pH values are close to the isoelectric point of sodium caseinate (pH 4.6) causing a reduction in the ζ-potential of the system (Figure 2.6B). As a result, the electrostatic repulsion between the droplets was not sufficiently large to prevent droplet aggregation. It has been estimated that a minimum ζ-potential of ± 30 mV is required to generate an electrostatic repulsion strong enough to overcome the attractive interactions (van der Waals and hydrophobic) [274]. This is supported by the particle size measurements shown in Figure 2.6, which indicate that the protein-coated lipid droplets were fairly stable to aggregation with no statistical differences (p>0.05) at pH values far below or far above their isoelectric point because the magnitude of the ζ-potential was near or above 30 mV.

Figure 2.6: Effect of pH on the (A) droplet diameter and (B) ζ-potential of lutein-enriched nanoemulsions stabilized by sodium caseinate after 14 days of storage. Emulsion composition: 0.25% lutein, 5% corn oil, 0.5% sodium caseinate in 5 mM phosphate stored at 20 ºC. Different letters are significantly different for each storage conditions (p < 0.05).
2.4 Conclusions

This study has shown that it is possible to encapsulate lutein in emulsion-based delivery systems fabricated from all-natural ingredients (lutein, corn oil, and milk protein). These lutein-enriched emulsions can be used to create natural colorants or to fortify functional foods at a level that may be beneficial to human health. Elevated temperatures promoted rapid chemical degradation of lutein leading to color fading, and so it is important to avoid exposing the delivery systems to high temperatures during their manufacture, storage, transport, and utilization. However, lutein stability was not strongly influenced by the storage pH. Conversely, pH did have a major impact on the physical stability of the emulsions, with extensive droplet flocculation occurring near the isoelectric point of the adsorbed caseinate molecules. As would be expected, the rate of color degradation positively correlated with the rate of lutein degradation. This work provides important information for the design of stable emulsion-based delivery systems for utilization as natural colorants or nutraceutical ingredients.
3.1 Introduction

Lutein is a natural colorant found in a variety of biological materials, such as yellow corn, egg yolk and marigold flowers. Lutein belongs to the xanthophyll class of carotenoids, which are oxygenated carotenes [252]. As with other xanthophylls, lutein has an intense yellow color when present at low concentrations but a reddish color when present at high concentrations. Its characteristic color is due to selective absorption of electromagnetic radiation in the visible region by conjugated double bonds in its backbone [252]. Lutein is known to accumulate in the pigmented region of the human eye, which is called the macula. The accumulation of lutein in the macula has been associated with a decrease in the risk of age-related macular degeneration and cataracts [249, 252, 275]. One of the proposed mechanisms for the protection of the macula by carotenoids is the absorbance of damaging light waves [251]. Moreover, they can act as antioxidants by scavenging free radicals or quenching singlet oxygen, which protects cells including the ones in the macula from oxidative stress. Lutein cannot be synthetized by the human body and must therefore be ingested through the diet [252-254]. The acceptable daily intake for lutein approved by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) is 0 to 2 mg/kg body weight [255]. Moreover, the effective dose of lutein to provide protection against diseases such as age-related macular degeneration and cataracts has been reported to be about 10 mg/day [154].
Due to the beneficial effects of lutein on human health it can be considered to be a nutraceutical ingredient to create functional foods and beverages. Moreover, its yellow-red color and its hydrophobicity make lutein a natural lipid-soluble colorant that can be used to replace artificial ones. An important trend in the modern food industry is towards products that are manufactured "without artificial additives" such as preservatives, flavorings, and colorings [247]. In addition, consumers are tending to purchase more functional food products that claim to provide additional health benefits beyond their normal nutritional effects [247]. Lutein is a promising ingredient to fulfill these market trends, indeed it has recently been reported that lutein, which is mainly extracted from Marigold flowers (Tagetes erecta), has the fastest growing market among the carotenoids with a market value of around US$233 million in 2010, projected to grow to US$309 million by 2018 [276].

Nevertheless, the use of lutein in the food industry presents challenges related to its poor chemical stability, water-solubility, and bioaccessibility characteristics. In common with other carotenoids, lutein is sensitive to heat and acidic environments, which in the presence of oxygen enhance its degradation through autoxidation [275]. The degradation of lutein causes a reduction in its bioactivity, as well as a change in its desirable quality attributes due to color fading and formation of rancid off-flavors [252, 275]. The poor oral bioaccessibility of lutein can be attributed to its low water-solubility, high melting point, and poor chemical stability [154, 259]. As with other lipophilic compounds, lutein has to be solubilized within the mixed micelle phase formed in the small intestine before it can be absorbed by the epithelial cells, packaged into lipoproteins, and transported to the blood stream [249]. The efficacy of solubilization in the mixed micelle phase therefore plays a major role in determining the overall bioavailability of lutein [253].
Oil in water (O/W) nanoemulsions are a promising platform for creating delivery systems to incorporate lipophilic compounds into food products and increase their bioavailability [277]. O/W nanoemulsions are thermodynamically unstable colloidal systems in which oil is dispersed in water in the form of small spheres ($r < 100$ nm) [16, 278]. The functional performance of nanoemulsions can be tailored to specific applications by controlling their compositions or structures. A particularly promising approach to improving nanoemulsion performance is to use novel emulsifiers formed by covalently linking proteins and polysaccharides together using the Maillard reaction [279-281]. The protein part helps the emulsifiers rapidly adsorb to oil droplet surfaces, whereas the polysaccharide part helps prevent the oil droplets from aggregating by generating a strong steric repulsive interaction. For example, studies have shown that protein-polysaccharide emulsifiers formed by the Maillard reaction can improve the physical stability of emulsions, and alter their gastrointestinal fate [281, 282].

The Maillard reaction is a non-enzymatic reaction that involves the condensation of the carbonyl group of a reducing carbohydrate with a free amino group of a protein (such as a lysine or an arginine residue or an N-terminal amino group). In the initial stages of the reaction an aldimine (Schiff base) is formed. The Schiff base subsequently undergoes an Amadori rearrangement when aldoses are involved or a Heyns rearrangement in the case of ketoses [283, 284]. It is often important to prevent the later stages of the Maillard reaction from occurring when preparing protein-polysaccharide conjugates since they lead to the degradation of the Amadori products and the formation of a wide variety of undesirable reaction products [285].
The aim of this work was to establish the impact of casein-dextran Maillard conjugates on the physicochemical stability and gastrointestinal fate of lutein-enriched nanoemulsions. In particular, this study examined if these conjugates could improve the stability of the nanoemulsions, without adversely affecting the bioaccessibility of lutein. A source of long chain triacylglycerols (corn oil) was used as the lipid phase since this type of lipid has previously been shown to increase the bioaccessibility of carotenoids [113, 261].

3.2 Materials and Methods

3.2.1 Materials

MariLut (20% lutein in corn oil) was kindly donated by PIVEG (San Diego, CA). Mazola corn oil was purchased from a local store. Spray dried sodium caseinate was purchased from the American Casein Company (Burlington, NJ). A lutein standard for HPLC analysis was purchased from Extrasynthese (Genay, France). Sodium azide, calcium chloride, sodium phosphate mono- and dibasic, dextran 37 kDa, porcine bile extract, pepsin and lipase were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO), potassium persulfate, ethanol, and 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Fisher Scientific (Waltham, MA).

3.2.2 Maillard Conjugates Formation

Sodium caseinate (2.00 w/v%) and dextran (3.5 w/v%) were individually solubilized overnight at 5 °C in water. The completely solubilized and hydrated samples were subsequently mixed in a one-to-one ratio, which led to final sodium caseinate and dextran concentrations of 1.00 and 1.75 w/v%, respectively. The mixture was spray-dried using a mini spray drier (Buchi B-290, Switzerland) with an
inlet temperature of 150 ºC, a feed rate of 7.5 mL/minute, a compressed air pressure of 600 kPa, and an air flow rate of 35 m³/h [286]. Maillard conjugation reactions were performed by incubating the spray-dried mixture at 76% relative humidity (using a saturated KBr solution in a desiccator) and 60 ºC (in an incubator) for 48 hours [279, 287]. After conjugation, the samples were allowed to cool to room temperature and ground using a mortar and pestle. The samples were subsequently stored in a desiccator prior to use.

3.2.3 Maillard Conjugates Characterization

3.2.3.1 Conjugation Efficiency

The conjugation efficiency was determined by measuring the reduction in free amino groups using the OPA assay (Pan et al., 2006). The OPA reagent was prepared according to Pan, Mu [287]. In short, 40 mg OPA (dissolved in 1.0 mL 95% ethanol), 25 mL 0.100 M sodium tetraborate buffer (pH 9.5), 2.5 mL 20% SDS solution, and 0.10 mL 2-mercaptoethanol were mixed together and brought to a final volume of 50 mL. The OPA reagent was prepared freshly before use. After dispersing the conjugates, 0.10 mL of the dispersion was mixed with 2.70 mL of OPA reagent and incubated for 1 minute at room temperature, the absorbance at 340 nm was measured immediately using an UV–visible spectrophotometer Ultrospec 3000 pro (Biochrom Ltd., Cambridge, England). A calibration curve was constructed using L-leucine (0.2-5 mM) as a standard amino group-containing compound [279, 287]. The conjugation efficiency was defined as follows:

\[
\text{Conjugation efficiency (\%)} = \left(1 - \frac{\text{amine groups after conjugation (M)}}{\text{amine groups before conjugation (M)}}\right) \times 100
\]
3.2.3.2 Conjugation Yield

The quantification of the non-complexed protein remaining in the system was assessed based on the methodology described by Markman and Livney [279]. The samples were dissolved in double distilled water at concentrations of 10 mg/mL, and subsequently acidified to pH 4.6 with HCl. The sample suspensions were centrifuged at 1000 g for 10 minutes and then filtrated through P5 filter paper (Fisher Scientific, Pittsburgh, PA). The pH of the supernatant was readjusted to 7.0 with NaOH. The protein content of the suspension (after pH adjustment and before centrifugation) and supernatant was measured using an UV–visible spectrophotometer (Ultrospec 3000 pro, Biochrom Ltd., Cambridge, England) at 278 nm. The amount of protein was subsequently calculated using a calibration curve prepared using sodium caseinate concentrations ranging from 0.2 to 1.0 mg/mL. The conjugation yield was defined as follows:

\[ \text{Conjugation yield (\%)} = \left( \frac{\text{protein in supernatant (mg/L)}}{\text{protein in the suspension (mg/L)}} \right) \times 100 \]

3.2.3.3 Antioxidant Activity Measurement

The antioxidant activity of protein and Maillard conjugate solutions were calculated using an ABTS assay [288]. For this assay, 7 mM ABTS and 2.45 mM potassium persulfate were allowed to interact overnight at room temperature in the absence of light to produce the ABTS radical. The ABTS radical solution was then diluted in ethanol to give an absorbance of 0.7 cm\(^{-1}\) at 734 nm. 10 µL aliquots of samples to be analyzed were mixed with 1 mL of the diluted radical solution and absorbances were read at 734 nm after 1 minute. The percent reduction in absorbance
at 734 nm and the antioxidant capacities were calculated using a Trolox standard curve and the results are reported as Trolox equivalents (TE)

3.2.4 Emulsion Formation

An organic phase was prepared by diluting the lutein in corn oil to a final concentration of 2.5% lutein (w/w). An aqueous phase was prepared by dispersing 1% (w/w) powdered sodium caseinate or 1% (w/w) caseinate-dextran complexes based on the protein weight of the Maillard conjugates into aqueous buffer solution (5 mM phosphate, pH 7.0). A coarse oil-in-water emulsion was prepared by mixing the organic phase (10% w/w) and the aqueous phase (90% w/w) using a high-speed mixer M133/1281-0 (Biospec Products, Inc. Bartlesville, OK, USA) for 2 min at 10,000 rpm. The resulting coarse emulsion was then passed through a high-pressure microfluidizer for three passes at 20,000 psi (Purenano, Microfluidics, Newton, MA). The fine emulsion produced was then diluted (1:1) with buffer solution containing an antimicrobial agent (5 mM phosphate buffer, pH 7.0, 0.04% (w/v) sodium azide). The final emulsion therefore contained 5% (w/w) oil phase and 250 mg/L lutein.

3.2.5 Stability Study

Selected emulsions were stored for 7 days at different temperatures (5, 20, 37, 55 and 70 ºC) or at different pH values (pH 3-8). The pH values were adjusted to the desired values using 0.1 and 1.0 N of hydrochloric acid and/or sodium hydroxide solutions. The emulsions with pH values of 3, 4, 5, 6, 7 and 8 were stored at 37 ºC to accelerate their destabilization, while samples at pH 7 were stored at 5, 20, 37, 55 and 70 ºC.
3.2.5.1 Chemical Stability

The chemical stability of lutein was assessed by measuring the change in color in the emulsions during storage. The color was monitored using a colorimeter (ColorFlex EZ, HunterLab Reston, VA, USA). For the color analysis, 10 mL of emulsion was pipetted onto a plastic petri dish and the readings were performed against a black background.

3.2.5.2 Physical Stability

The physical stability of the emulsions was assessed by measuring changes in particle size and charge after 7 days of storage. The mean droplet diameters, particle size distributions, and ζ-potentials were measured using a dynamic light scattering/micro-electrophoresis instrument (Zetasizer Nano ZS, Malvern Instruments, Malvern, England) and a laser diffraction particle size analyzer (LS 13 320, Beckman Coulter, Brea, Ca, USA). Samples were diluted in buffer solution (5 mM phosphate) at the proper pH to avoid multiple scattering and measurements were made at 25 °C.

3.2.6 In vitro Digestion Model

A dynamic in vitro gastrointestinal model was used to study the influence of the emulsion interface on the bioaccessibility of lutein. The gastrointestinal model was based on the work by Salvia-Trujillo, Qian [113] with some slight modifications. The mouth phase was not included in this work since most liquids do not require an oral phase, mainly due to the very short residence times in the oral cavity [289].

3.2.6.1 Gastric Phase

Simulated gastric fluid stock solution (SGFSS) was prepared by dissolving 2 g of NaCl and 7 mL of HCl (37%) in 1 L of double distilled water. Simulated gastric fluid work solution (SGFWS) was prepared by mixing 20 mL of SGFSS and 0.064 g
of pepsin (amounts are per sample) 45 minutes before running the gastric phase. The emulsion was diluted to 1 % (w/w) oil and then mixed with SGFWS at a 50:50 volume ratio so that the final mixture contained 0.5% (w/w) oil. The pH of the sample was adjusted to 2.5 using NaOH and incubated at 37 °C for 2 h with continuous agitation at 100 rpm in an incubator shaker (Innova 4080, New Brunswick Scientific, Enfield, CT).

### 3.2.6.2 Small Intestinal Phase

An instrumental automatic titration (pH-stat) device (835 Titrando, Metrohm USA Inc., Riverview, FL) was used to simulate the conditions in the small intestinal phase of the gastrointestinal tract. An aliquot of 30 mL of the gastric chyme was placed in a water bath at 37°C and the pH was set to 7.0 using NaOH solution. Then, 1.5 mL of calcium chloride (37 mg/mL) and sodium chloride (219 mg/mL) and 3.5 mL of bile extract (53.5 mg/mL) dissolved in 5 mM phosphate buffer solutions were added to the sample and the pH was re-adjusted to 7.0. Afterwards, 2.5 mL of freshly prepared lipase suspension (24 mg/mL) dissolved in 5 mM phosphate buffer was incorporated into the mixture. The pH of the mixture was monitored and the volume of 0.1 M NaOH (mL) necessary to neutralize the free fatty acids (FFA) released from the lipid digestion (i.e., to keep pH at 7.0) was recorded during 2 h. The amount of free fatty acids released was calculated using the following equation:

\[
\%\text{FFA} = 100 \times \frac{V_{\text{NaOH}} \times m_{\text{NaOH}} \times m_{\text{lipid}}}{w_{\text{lipid}} \times 2}
\]

Where, \(V_{\text{NaOH}}\) is the volume of titrant in liters, \(m_{\text{NaOH}}\) is the molarity of the sodium hydroxide, \(m_{\text{lipid}}\) is the molecular weight of corn oil (872 g/mol), \(w_{\text{lipid}}\) is the weight of oil in the digestion system in grams.
3.2.7 Bioaccessibility and transformation

Bioavailability of a compound depends on three factors: bioaccessibility, transformation, and adsorption [290]. The bioaccessibility and transformation of lutein was evaluated after the samples had passed through the simulated small intestine phase of the gastrointestinal model. Aliquots of 10 mL of the samples were centrifuged (4000 rpm for 40 min at room temperature) using a bench top centrifuge (Sorvall ST8, Thermo Scientific, Tewksbury, MA). After centrifugation, the samples separated into a sediment phase at the bottom and a clear micelle phase at the top. The intestinal phase and the micelle phase were dissolved in dimethyl sulfoxide and read at 460 nm. The bioaccessibility (B) and transformation (T) were determined using the following equation:

\[
B(\%) = \frac{C_M}{C_R} \times 100
\]

\[
T(\%) = \frac{C_R}{C_I} \times 100
\]

where, \(C_M\) is the lutein concentration in the micelle phase, \(C_R\) is the concentration of lutein in the entire intestinal phase, which is considered the raw digesta, and \(C_I\) is the concentration of lutein initially [291]. The emulsions without lutein were used as blanks. The concentration of lutein was determined from absorbance measurements (460 nm) made using a UV–visible spectrophotometer (Cary 100 UV-VIS, Agilent Technologies, Santa Clara, CA, USA). A calibration curve was prepared by dissolving lutein (standard) in DMSO in a range from 0.5 to 5 mg/L (\(r^2=0.9992\)).
3.2.8 Data Analysis

All experiments were performed in triplicate and the results are given as mean values ± standard deviation. Differences among the treatments were determined using an analysis of variance (ANOVA) and a post-hoc Tukey test with a confidence level of 95 %. The analyses were made using SPSS software (IBM Corporation, Armonk, NY, USA).

3.3 Results and Discussion

3.3.1 Maillard Conjugates Formation and Characterization

Sodium caseinate and dextran mixtures were spray dried and stored at 60 °C and 76% relative humidity for 48 h to produce Maillard conjugates. Dry conditions were used to minimize the presence of post-Amadori reactions [292]. After 48 hours, we monitored the extent of conjugation using the OPA test, which measures the unreacted amino groups of the protein, and the conjugation efficiency was found to be 23.8 ± 0.4%. It has been reported that the number of amino groups per caseinate molecule available to react with polysaccharides is 13.6 [279, 287, 293]. This means that the number of dextran molecules attached to each of the caseinate molecules in our system was around 3.3. The percentage of protein participating in the Maillard reaction was measured by quantifying the protein that did not precipitate at pH 4.6 (isoelectric point of casein). This percentage is referred as the conjugation yield. For this study the conjugation yield was determined to be 93.7 ± 3.2%, which verifies that the majority of the caseinate was conjugated to dextran molecules. Formation of Maillard conjugates was also verified using SDS-PAGE (data not shown).
3.3.2 Emulsion Formation

Oil-in-water emulsions were made using 10% corn oil as the dispersed phase and 90% aqueous solution as the continuous phase (phosphate-buffered saline at pH 7). The continuous phase contained either caseinate or caseinate-dextran conjugates as emulsifiers, with the caseinate concentration being the same (1%) in both cases. Commercial lutein (20% in corn oil) was dispersed at a concentration of 2.5% in the oil phase to reach a lutein concentration of 500 mg/L in the final emulsion. The lutein concentration used in this study was based on the amount required to have a biologically significant effect on macular degeneration, i.e., 10 mg of lutein for a 200 mL serving size of a 1% fat beverage. Initially, emulsions containing relatively small droplets (d < 300 nm) that were stable to phase separation could be produced using both emulsifiers.

3.3.3 Influence of pH on Emulsion Stability and Properties

Emulsion-based delivery systems may be utilized in food products that have different pH values, and they are exposed to changes in pH as they pass through the different regions of the gastrointestinal tract. For this reason, the influence of pH on the physical and chemical stability of lutein-enriched emulsions stabilized by either caseinate or caseinate-dextran conjugates was measured. The pH-stability of the emulsions was compared by adjusting them to different pH values (pH 3 to 8), and then storing them at 37 °C for one week.

The physical stability of the emulsions was determined by measuring their particle size, particle charge, and overall appearance at the end of the storage period. The emulsions stabilized by caseinate alone underwent extensive droplet aggregation at pH 3, 4 and 5, as seen by a large increase in particle size (Figure 3.1A) and visible
phase separation (Figure 3.1C). This effect can be attributed to a reduction in the magnitude of the electrical charge on the caseinate-coated oil droplets around their isoelectric point (Figure 3.1B), which reduces the electrostatic repulsion between them [16]. Presumably, the casein layer around the droplets was not sufficiently thick to prevent aggregation through steric repulsion [48, 294]. Probably, the emulsions exhibited droplet aggregation at pH 3 (Figure 3.1A), despite the fact that the droplets had a relatively high positive charge at this pH (Figure 3.1B), because they were initially prepared at neutral pH and then adjusted to the final acidic pH. Consequently, they had to pass through the isoelectric point, which may have resulted in some irreversible droplet flocculation. The change in ζ-potential with pH for this system was typical of that observed for protein-coated droplets, changing from highly negative at pH values well above the isoelectric point to highly positive at pH values well below the isoelectric point. The point of zero charge (between pH 4 and 5) for the protein-coated emulsions was consistent with the published isoelectric point of caseins (around pH 4.6).
Figure 3.1: (A) Mean droplet diameter (B) \( \zeta \)-potential (C) pictures of lutein-enriched emulsions stabilized by non-conjugated sodium caseinate and Maillard conjugates adjusted to pH 3-8 and stored at 37 °C, values were taken at the end of 7 days of storage. Different lower case letters are significantly different for non-conjugated samples (p<0.05). Different upper case letters are significantly different for conjugated samples (p<0.05).

The emulsions containing droplets coated with caseinate-dextran conjugates were stable to droplet aggregation across the entire pH range studied, with no evidence of an increase in particle size or visible phase separation (Figures 3.1A and 3.1C). The high stability of these systems can be attributed to the ability of the hydrophilic dextran molecules to generate a steric repulsion that is strong enough to overcome any attractive interactions (such as van der Waals) between the droplets [295]. The \( \zeta \)-potential versus pH profile of the emulsions containing conjugated caseinate followed a similar general trend to those containing non-conjugated caseinate, i.e., the droplet charge went from negative at high pH to positive at low pH, with a point of zero charge between pH 4 and 5 (Figure 3.1B). However, the magnitude of the \( \zeta \)-potential values was appreciably lower for the caseinate-dextran coated droplets than the caseinate-coated droplets at the same pH. This effect can be attributed to the influence of the dextran molecules on the distance from the droplet surfaces where the effective electrical properties are measured, i.e., the shear plane.
In the presence of dextran, the electrical properties are measured at a distance that is further from the droplet surfaces, and so there has been a greater decay in the electrical potential. These results show that conjugation of caseinate with dextran leads to an appreciable increase in the pH-stability of emulsions.

Lipid oxidation reactions produce reactive species that can further interact with other oxidizable compounds, such as the lutein in our samples. The interfacial characteristics of the emulsion will affect the susceptibility of lutein to degradation. Proteins located at the oil-water interface can inhibit oxidation at pH levels below their isoelectric points due to electrostatic repulsion of transition metals since they are both positively charged [90, 98, 296]. On the other hand, proteins in the continuous phase act as pro-oxidants above their isoelectric points due to their iron-binding properties since they are oppositely charged [87, 93]. Previous work has shown that carotenoid oxidation (including lutein) leads to color fading of the emulsion and that the Pearson correlation values between these two phenomena was close to >0.9 [297]. Therefore, the influence of pH on the chemical degradation of lutein in the emulsions was monitored by measuring changes in the L, a, b values and then calculating the total color change (ΔE). The observed total color change (Figures 3.2A and 3.2B) was primarily the result of a reduction in positive b-values (decreased yellowness), a reduction in positive a-values (decreased redness), and an increase in lightness (color fading) during storage, which is indicative of lutein degradation (data not shown).
Figure 3.2: Changes in ΔE values of lutein-enriched emulsions prepared with (A) sodium caseinate and (B) Maillard conjugates adjusted to different pH values (pH 3-8) (C) Effect of storage at different pH values on lutein color degradation rate. All samples were kept at 37 ºC for 7 days.

For the emulsions containing caseinate alone, reliable color measurements could not be made during storage at pH 4 and 5 because the samples rapidly phase separated (Figure 3.1C). For the remainder of the samples it was clear that the rate of color fading increased with decreasing pH (Figure 3.2). Previous studies have reported that carotenoids undergo chemical degradation under acidic conditions due to protonation of carbon atoms [269, 275]. Color measurements could be made across
the entire pH range for the emulsions containing droplets coated by caseinate-dextran conjugates because they were physically stable (Figure 3.1). In these systems, the rate of color fading clearly increased with decreasing pH (Figures 3.2A and 3.2C), which is in agreement with previous studies with other carotenoids [269, 275]. Conjugation did not appear to have a major impact on the rate of color degradation (Figure 3.2C); however, color fading did appear to occur somewhat more rapidly for the emulsions stabilized by the caseinate-dextran complexes at pH 3, but more slowly for the same systems from pH 6 to 8.

Overall, these results indicate that it may be better to store lutein-enriched emulsions under neutral conditions to inhibit color fading, and that these systems may be susceptible to some degradation under the highly acidic conditions of the stomach.

3.3.4 Influence of Temperature on Emulsion Stability

Food and beverage products are often exposed to a range of temperatures during their production, storage, transport, and utilization, and therefore it is useful to examine the influence of temperature on the physical and chemical stability of the lutein delivery systems. Emulsion thermal-stability was established by storing them at different temperatures (5 to 70 °C) for 7 days at pH 7.

A significant increase in droplet diameters was only observed at 70°C when the emulsions were stabilized with protein alone. However, significant differences in droplet size were observed at storage temperatures 37, 50 and 70°C when the conjugates were used to stabilize the emulsions (p<0.05) (Figure 3.3A). Figure 3.3C shows that the polydispersity index values of the treatments follow the same trend as the droplet size values. There was also an appreciable change in the electrical charge
at elevated temperatures, particularly of the emulsions stabilized by the non-conjugated protein (Figure 3.3B).

**Figure 3.3:** (A) Mean droplet diameter and (B) ζ-potential (C) polydispersity index of lutein-enriched emulsions stabilized by Maillard conjugates adjusted to pH 7 and stored at different temperatures (5-70 ºC), values were taken at the end of 7 days of storage. Different lower case letters are significantly different for non-conjugated samples (p<0.05). Different upper case letters are significantly different for conjugated samples (p<0.05).

The temperature-dependence of the chemical stability of lutein was determined by monitoring the changes in emulsion color during storage (Figure 3.4). For both emulsion systems, higher temperatures led to faster color fading. Previous
studies have also reported that exposure of carotenoids to elevated temperatures leads to more rapid chemical degradation and formation of colorless products [298, 299]. The use of casein-dextran conjugates did not have a significant effect on the rate of color change in the emulsions. Some studies have reported that the Maillard reaction between proteins and low molecular weight sugars resulted in an increased antioxidant activity of the conjugates [300-303]. In the case of this study, the antioxidant activity of the Maillard conjugates and the casein alone measured by ABTS (909 ± 29 mM TE and 879 ± 25 mM of TE, respectively) were not significantly different (p>0.05). This lack of difference in antioxidant activity of the emulsifiers may account for the similar trends of color fading observed in both systems (Figure 3.4). Similar results were found by Drusch, Berg [304]. In their work, the conjugation of caseinate with dextran did not prevent the oxidation of fish oil, while conjugation of casein with glucose showed a protection against lipid oxidation.
Figure 3.4: Changes in ΔE values of lutein-enriched emulsions prepared with (A) sodium caseinate and (B) Maillard conjugates exposed to different temperatures (5-70°C) (C) Effect of storage at different temperatures on lutein color degradation rate. All samples were adjusted to pH 7 and kept for 7 days.

After the storage period at temperatures above 37°C the ζ-potential of the enriched emulsions stabilized by the Maillard conjugates presented a significant decrease (p<0.05) and the droplet diameter of the samples stored at 70°C had the same increase as the samples stabilized with sodium caseinate alone. The decrease in the ζ-potential and the increase in droplet diameter could be explained by the possible breakdown/degradation of conjugates between the sodium caseinate and dextran when they are exposed to intermediate products of the carotenoids autooxidation reactions. The degradation of Amadori compounds can be initiated by lipid oxidation products and continue as a chainlike reaction [305]. As mentioned before, the exposure of carotenoids to temperatures of 37 °C and above will cause their oxidation and produce highly reactive radicals [275]. The degradation of Maillard products due to the continuous heating of emulsions in the presence of casein and long chain carbohydrates has also been attributed to the progression of the Maillard reaction and the presence of post-Amadori products [306]. To verify this possibility, a solution of
the conjugates was heated using the same conditions as the emulsions and the increase in absorbance was measured at 420 nm (an indicator of the formation of post-Amadori products). The observed lack of browning in the samples supported the hypothesis that the degradation of the Maillard products was probably initiated by lipid and lutein oxidation.

3.3.5 Digestion and Bioaccessibility

The digestion rate (FFA release) depends on several factors including droplet size and interfacial structure [259]. The effect of using Maillard conjugates as emulsifiers on the fate of the digestion of the enriched emulsions was examined. The pH-stat method was used to compare the rate and extent of lipid digestion among the emulsions stabilized with protein only and protein-polysaccharide conjugates [113]. The digestion model utilized for these samples do not include a mouth phase since the liquids do not spend long enough time in mouth to cause a significant change. Also, it is not necessary for liquid foods that does not contain a significant amount of starch [289]. Figure 3.5 shows the changes in ζ-potential, particle size distribution and average particle diameter of the emulsions along the in vitro digestion model. The ζ-potential of the emulsions at pH 7 prior starting the digestion model was -38.73 ± 0.76 mV for the emulsions with non-conjugated emulsifier. The ζ-potential of the emulsions stabilized by Maillard conjugates was significantly lower (p<0.05) with a value of -7.75 ± 0.67 mV. Both samples presented a negative charge because the pH of the emulsion was higher than the isoelectric point (pI) of sodium caseinate. The lower charge of the droplets in the emulsions stabilized by the Maillard conjugates has been explained earlier in the emulsion formation and stability section. In the stomach phase the pH drops to 2.5 and this affects the electric charge of the droplets
which became positively charged 9.15 ± 1.38 mV and 2.52 ± 1.09 mV for samples with non-conjugated and conjugated sodium caseinate, respectively. Positive charge is expected since the pH is lower than the pI. Another reason for the lower charge in the stomach phase can be that the pepsin present in the gastric juice hydrolyzes sodium caseinate, especially at around pH 2 [307] and the resulting peptides yield a lower ζ-potential. The emulsion stabilized with the Maillard conjugates is still stable at the gastric phase (will be explained further below). That is why the change in the ζ-potential is less drastic than the emulsion stabilized with protein alone, which is not stable at this step anymore (Figure 3.5A). During the intestinal phase the pH is 7 and this yields to negatively charged droplets in both samples. The average charge became strongly negative and was -40.88 ± 2.30 mV for the emulsions stabilized by sodium caseinate alone and -37.75 ± 0.91 mV for the Maillard conjugates. The ζ-potentials in this phase are similar and high not only because sodium caseinate has strong negative charge in neutral pH but also because the bile salts and phospholipids in the intestinal fluid are negatively charged and may contribute to the charge load. Furthermore, the FFAs produced can also yield to negative charges [308].
Figure 3.5: Changes in (A) ζ-potential (B) average particle diameter and (C) particle size distribution and of lutein-enriched emulsions stabilized by non-conjugated sodium caseinate and Maillard conjugates during the in vitro digestion model. Different lower case letters indicate significant difference between non-conjugated and conjugated samples at the same digestion stage (p<0.05). Different upper case letters indicate significant difference between different digestion stages for each of the samples (p<0.05).

All samples showed monomodal particle size distribution (Figure 3.5C) in the initial stage. The average initial droplet diameter of the emulsion formed by using sodium caseinate was 144 ± 0.82 nm, whereas it was 138.25 ± 0.5 nm for the emulsions stabilized by Maillard conjugates, showing that Maillard conjugates reduced the droplet size significantly (p<0.05) (Figure 3.5B). Markman and Livney (2012) claim that the Maillard conjugation changes the packing characteristics of the surface-active materials and increases the curvature of the emulsion droplets that stabilize smaller droplet sizes. Also, the microstructures of the emulsions after each stage are presented in Figure 3.6. It can be seen that the droplets were evenly distributed and no coalescence or flocculation were observed in the initial stage. The emulsions with Maillard conjugates stayed stable during the gastric phase with an average droplet diameter of 136 ± 0.82 nm (p>0.05). On the other hand, the emulsions
stabilized by sodium caseinate yielded to 3.19 ± 0.48 µm in average droplet diameter (Figure 3.5B). The emulsion stabilized with caseinate flocculated and showed coalescence as shown by the increase in average droplet diameter and the appearance of multimodal particle size distributions. This was further confirmed by microscopic observations (Figure 3.6). The sodium caseinate emulsions destabilization might have occurred because of the loss of charge due to the pH changes or proteolysis by pepsin present in this phase [282, 293, 309]. The stability of the emulsions made with the Maillard conjugates in the gastric phase could be due to the steric repulsion caused by the dextran moieties on the surface of the droplets. The steric hindrance prevents the pepsin from reaching the surface of the droplet and therefore impedes the proteolysis of the caseinate molecules [279, 293, 310]. In the intestinal phase both emulsions showed a much higher average droplet size; 11.27 ± 0.90 µm for the emulsions with sodium caseinate alone and 13.6 ± 1.54 µm for the samples with the Maillard conjugates (Figure 3.5B). Both treatments showed flocculation and coalescence (Figure 3.6) leading to bigger droplet sizes after the intestinal phase, probably due to the replacement of sodium caseinate or Maillard conjugates from the droplet surface by the bile salts and free fatty acids present in the intestinal fluid [113, 293, 309, 311]. It has been shown that the bile salts in the gastric fluids strongly replace the emulsifiers around the droplets, even in multilayer emulsions stabilize by enzymatic cross-linking [311]. The replacement by the bile salts and FFA can be further confirm by the similarity between the samples stabilized with sodium casein alone and Maillard conjugates after the intestinal phase (Figure 3.5A).
The effect of Maillard conjugates on the digestion rate and extent of the corn oil was also examined. The FFA release is calculated by determining the amount of sodium hydroxide used during the titration at intestinal phase. Figure 3.7 shows the FFA release during the intestinal phase of the in vitro digestion model. The steep increase in the FFA release in the beginning of the digestion shows that lipase quickly hydrolyzed the triglycerides (Figure 3.7). However, the increase in the FFA release during the first 5 minutes of digestion is slower for the samples stabilized with caseinate alone. This effect could be due to the lower droplet diameter of the emulsions stabilized with Maillard conjugates after the gastric phase that yielded a higher surface area at the beginning of the intestinal step. These findings are in accordance with other studies [281, 311, 312]. Zeeb, Lopez-Pena [311] explained that
the interfacial properties do not play an important role in the fate of the digestion, but the stability and particle size tend to be more determinative. On the other hand, there is no obvious difference between the extents of FFA releases of the two emulsions. This may be due to exchange of surface-active materials during the digestion.

**Figure 3.7:** Free fatty acids (FFA) release during the intestinal stage of the *in vitro* digestion model lutein-enriched emulsions stabilized with non-conjugated sodium caseinate and Maillard conjugates. Insert: FFA release during the first five minutes.

Finally, the bioaccessibility and transformation of the lutein in the enriched emulsions was monitored by measuring the lutein present in the mixed micelle phase after the digestion [313]. Bioavailability is calculated as the multiplication of Bioaccessibility, Adsorption, and Transformation (BA=B*A*T) [314]. We only focused on bioaccessibility and transformation, and have not found a negative effect on the bioaccessibility and transformation of lutein when the caseinate was replaced by the conjugates. The bioaccessibility of lutein was 8.20±0.73% when the emulsion was stabilized by caseinate, and 7.55±0.66% when it was stabilized by the conjugates. The transformation of lutein was 13.40±1.38% when the emulsion was stabilized by caseinate, and 14.54±1.07% when it was stabilized by the conjugates. The multiplication of these two factors (Bioaccessibility x Transformation) was equal to 1.1% for both systems and there was no significant difference between the two
(p>0.05). The similar size and charge of the samples after the small intestine phase can be a reason for the similar bioaccessibility. The fact that the bioaccessibility was lower than 10% could be explained by the high amount of lutein in the original emulsions and the maximum capacity of the micelles to incorporate it. Further research with higher and lower lutein concentrations should be done to prove this.

3.4 Conclusions

Lutein can be used as a natural colorant in foods, as well as a functional ingredient in functional foods to improve eye health. Caseinate alone yields to unstable lutein-enriched emulsion at acidic pH values (pH 3-5), whereas the conjugation with dextran through Maillard reaction results in a surface-active complex that provides stability to the emulsions in the same conditions. The oxidation of lutein at high temperatures had an impact on the stability of the Amadori compounds of the Maillard conjugates, which resulted in a slight increase in the particle size of those emulsions stored at high temperatures. Finally, the Maillard conjugates did not affect the fate of digestion and the bioaccessibility of lutein. The results of this study should facilitate the rational design and fabrication of nanoemulsion-based delivery systems for utilization in functional foods and beverages.
CHAPTER 4

FORMATION AND STABILITY OF OMEGA-3 OIL EMULSION-BASED DELIVERY SYSTEMS USING PLANT PROTEINS AS EMULSIFIERS: LENTIL, PEA AND FABA BEAN PROTEINS

4.1 Introduction

Emulsifiers are surface active molecules that adsorb to the surfaces of oil droplets and form protective coatings around them [203]. Many proteins act as natural emulsifiers because they have an appropriate balance of hydrophilic and hydrophobic amino-acid groups, and adopt three-dimensional conformations where some of the hydrophobic groups are exposed at their surfaces [12]. Proteins therefore have the tendency to adsorb to oil droplet surfaces during homogenization and reduce the interfacial tension, which facilitates further droplet fragmentation by decreasing the Laplace pressure [124]. After adsorption, they may undergo conformational changes that increase the number of protein hydrophobic groups in contact with the oil phase, which may also lead to interfacial cross-linking of the proteins [55]. The protein coating formed around oil droplets also helps protect them against aggregation by generating electrostatic and steric repulsive forces [315]. Finally, the protein layer may also provide protection against chemical degradation by acting as a physical barrier, chelating agent, or antioxidant [296]. Compared to animal-based proteins, such as those from milk, eggs, meat or fish, the emulsification properties of plant-based proteins are much less well understood [203].

Proteins from legumes are of particular interest for use in the food industry due to their widespread abundance, low-cost, sustainability, low allergenicity, nutritional benefits, positive consumer perception, and good functional attributes.
Pulses, which are the edible seeds of legumes, have been shown to contain amphiphilic proteins that form relatively thick layers around oil droplets, thereby enhancing emulsion formation and stability [11]. Legume proteins contain around 70% globulins and 10-20% albumins [57, 59, 316]. The main globulins found in legume proteins are vicilin and legumin [317]. Vicilin was found to be a better surface-active material than legumin, due to its low molecular weight and more flexible tertiary structure [60].

In this study, we investigated the ability of legume protein concentrates to act as plant-based emulsifiers in the development of emulsions fortified with omega-3 polyunsaturated fatty acids (PUFAs) from a non-animal source (algae). Two of the most biologically active sources of PUFAs currently utilized in the food industry are eicosapentaenoic acid (22:5 omega-3, EPA) and docosahexaenoic acid (22:6 omega-3, DHA). A diet rich in these omega-3 PUFAs may help prevent a variety of health problems, including cardiovascular disease, inflammation, diabetes, cancer, asthma, schizophrenia and depression [318-320]. Individuals can obtain nutritionally beneficial levels of these bioactive lipids by consuming sufficient quantities of fatty fish or isolated fish oils [318]. However, many individuals do not consume enough of these oil sources, including vegans, vegetarians, pregnant women (avoiding mercury), or people who dislike the taste of fish [318]. Consequently, there is considerable interest in identifying alternative sources of these long-chain omega-3 PUFAs, such as algae oil. In this case, it is necessary to incorporate the algae oil into a functional food or beverage product that consumers find desirable. There are a number of challenges associated with fortifying foods with omega-3 PUFAs due to their poor water solubility and high susceptibility to lipid oxidation [208]. These challenges can often be overcome by using well-designed emulsion-based delivery systems that can be
used to conveniently incorporate these beneficial lipids into functional food and beverage products [83, 321].

The aim of the present study was to provide an understanding of the relative advantages and disadvantages of three different kinds of legume protein concentrate (pea, lentil, and faba bean) as natural emulsifiers for formulating emulsions enriched with omega-3 PUFAs. The influence of protein type, protein concentration, and processing conditions on emulsion formation and stability was investigated. This is the first part of a more comprehensive study using the same proteins, which also includes studies of their ability to inhibit lipid oxidation and their potential gastrointestinal fate to be published later. The information obtained from these studies may benefit the food industry by demonstrating the potential benefits of pulse protein concentrates as natural emulsifiers for formulating functional foods and beverages.

4.2 Materials and Methods

4.2.1 Materials

Pea, lentil, and faba bean protein concentrates (Vitessence Pulse 1550, 2550 and 3600, respectively) were kindly donated from Ingredion Inc. (Bridgewater, NJ). The composition of the legume protein concentrates is shown in Table 4.1. Glutamine and asparagine were the most abundant amino acids in the legume protein concentrates, making up to 17.8-19.5% and 11.6-12.8% of total amino acids, respectively. Whey protein isolate with 94% protein content (BiPro JE 011-4-420) was provided by Davisco Foods International Inc. (Le Sueur, MN). Algae oil (O55-O100 life`OMEGA 60) was provided by DSM Nutritional products LLC (Parsippany, NJ). This product was reported to have a total omega-3 content of at
least 550 mg/g, with 300 mg/g coming from DHA and 150 mg/g from EPA. All other
chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Table 4.1:** Compositional information of the pea, lentil and faba bean protein
concentrates used in this study (provided by the supplier).

<table>
<thead>
<tr>
<th></th>
<th>Vitessence Pulse 1550 (Pea)</th>
<th>Vitessence Pulse 2550 (Lentil)</th>
<th>Vitessence Pulse 3600 (Faba bean)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Color</strong></td>
<td>Pale yellow</td>
<td>Pale yellow</td>
<td>Pale cream, gray</td>
</tr>
<tr>
<td><strong>Protein (%)</strong></td>
<td>55</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td><strong>Fat (%)</strong></td>
<td>3.1</td>
<td>3.0</td>
<td>3.1</td>
</tr>
<tr>
<td><strong>Ash (%)</strong></td>
<td>5.0</td>
<td>4.9</td>
<td>5.0</td>
</tr>
<tr>
<td><strong>Moisture (%)</strong></td>
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</tr>
<tr>
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<tr>
<td><strong>Dietary fiber (%)</strong></td>
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<td>11</td>
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<tr>
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<td>3.2</td>
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</tr>
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<td><strong>Other Carbohydrates (%)</strong></td>
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<td>17</td>
<td>13</td>
</tr>
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<td>8.9</td>
</tr>
<tr>
<td></td>
<td>Histidine</td>
<td>1.2</td>
<td>2.8</td>
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<tr>
<td></td>
<td>Isoleucine</td>
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</tr>
<tr>
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<td>Leucine</td>
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<td>Lysine</td>
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<td>Methionine</td>
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<td></td>
<td>Phenylalanine</td>
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<td>Threonine</td>
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<td></td>
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<td>Asparagaine</td>
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<td>Cysteine</td>
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<td></td>
<td>Glycine</td>
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<tr>
<td><strong>Non-protein nitrogen</strong></td>
<td>8.7</td>
<td>N/A</td>
<td>9.3</td>
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</table>
4.2.2 Methods

4.2.2.1 Protein content of plant protein concentrates

Information about the protein content of the plant protein concentrates was obtained using a modification of the method described by Joshi et al (2011) [322]. Protein concentrates were dissolved in distilled water (20% w/w) and the mixture was adjusted to pH 8 using NaOH solutions. The dispersions were then stirred for an hour at room temperature. Any insoluble materials were then removed by centrifugation (Sorvall Lynx 4000 Centrifuge, Thermo Scientific, Agawam, MA) at 15,000 g for half an hour. The supernatants were collected and the solutions were adjusted to pH 4.5 using hydrochloric acid (HCl) solutions. The solutions were centrifuged as described above and the protein concentrates were collected in petri dishes and frozen by placing them into a -80°C freezer. The frozen protein concentrates were then freeze-dried overnight using a Virtis Freeze-dryer (Virtis Company, Gardiner, NY, USA). The dried protein concentrates were ground using a mortar and pestle.

The Lowry assay was used to quantify the protein content of the legume protein concentrate solutions [323]. The amount of protein present was calculated using a calibration curve prepared using bovine serum albumin as a standard.

The protein contents of the freeze-dried faba bean, lentil and pea protein concentrates were 74.5, 76.1 and 76.2%, respectively. This suggests that there must have been other components within the powders that co-precipitated with the proteins at pH 4.5, such as lipids, carbohydrates, or minerals.
4.2.2.2 Protein solubility assays

Protein solubility was determined following a modification of the method used by Aluko and Yada (1997) [324]. Protein concentrates were dispersed in sodium phosphate buffer (0.05% w/v) with sodium azide (0.02% w/v). For the pH-solubility assay, the pH values were then adjusted to pH 2-9 using 1 N NaOH or HCl solutions. The solutions were then stirred overnight at room temperature and the pH values were re-adjusted to the appropriate values. To determine the total soluble protein content, a control group was carried by mixing the same concentration of the protein concentrates in 0.1 N NaOH solution. The protein solutions were centrifuged at 10,000 g for 20 minutes using a benchtop centrifuge (Sorvall ST8, Thermo Scientific, Tewksbury, MA, USA) and the protein concentrations of the supernatants were determined by the Lowry method [323]. Protein solubility (PS%) was calculated as:

\[ PS(\%) = 100 \times \frac{\text{protein content of sample}}{\text{protein content of control}} \]

4.2.2.3 Emulsion Formation

Emulsions were prepared using algae oil as the dispersed phase and a buffered protein solution as the continuous phase (10 mM sodium phosphate buffer, pH 7). These conditions were selected to simulate food and beverage products that have pH values around neutral. The powdered plant-protein concentrates were initially dissolved in sodium phosphate buffer overnight at 4°C at concentrations ranging from 0.25 to 5% (w/w) based on the mass of the powder used. The pH values of the protein solutions were adjusted back to pH 7 after they were dissolved using sodium hydroxide (NaOH) solution. The manufacturer reported that these commercial ingredients contained 55-60% of protein, and therefore the actual protein contents were quantified using the Lowry method (Table 1). After dispersion in sodium
phosphate buffer the protein concentrates tended to form cloudy dispersions. For this reason, the protein concentrate solutions were centrifuged (Sorvall Lynx 4000 Centrifuge, Thermo Scientific, Agawam, MA) at 15,000 g for 15 minutes to remove any insoluble particular matter. Emulsions were prepared using both centrifuged and non-centrifuged protein solutions, to determine the influence of this process on their functionality.

Coarse oil-in-water emulsions were prepared by blending the oil (10% w/w) and aqueous (90% w/w) phases together using a high-shear mixer (M133/1281-0, Biospec Products Inc., Bartlesville, OK) for 2 min at 10,000 rpm. This coarse emulsion was then passed three times through a high-pressure homogenizer (PureNano microfluidizer, Microfluidics, Newton, MA) operating at 10,000 psi. This system includes a Y- and a Z-type chamber to decrease the droplet size. The temperature of the emulsions was kept low (<15°C) during homogenization by using an ice bath to cool the interaction chamber. This was done to prevent excessive heating of the emulsions at the high pressures used.

4.2.2.4 Emulsion Stability

A series of tests was carried out to establish the impact of environmental stresses on the stability of protein-coated oil droplets. These environmental stresses were selected to cover a range of representative conditions that emulsions may experience in commercial food products:

- **pH**: Emulsions were prepared at pH 7 and then diluted 10-fold using 10 mM sodium phosphate buffer. A series of emulsions with different pH values (2 to 9) was then obtained by adjusting the diluted emulsions to the specified pH values using NaOH and HCl solutions.
• **Ionic strength:** Emulsions were prepared at pH 7 and then they were diluted 10-fold using pH 7 sodium phosphate buffer containing a range of salt levels to achieve the final values of 0 to 500 mM sodium chloride (NaCl).

• **Temperature:** Emulsions prepared at pH 7 were diluted 10-fold using pH 7 sodium phosphate buffer. They were then placed in glass test tubes (10 mL each), incubated in water baths set at different temperatures (20 to 90 °C) for 30 minutes, and then cooled down to room temperature.

After preparation, all of the emulsions were stored in the dark for 24 hours at room temperature prior to analysis for particle size, particle charge, and emulsion appearance using the methods described in the following sections.

**4.2.2.5 Droplet Characteristics**

Droplet sizes were measured using a static light scattering instrument (MasterSizer 2000, Malvern Instruments, Westborough, MA). The resulting data is presented as particle size distributions or surface-weighted mean diameters ($d_{32}$). Droplet surface potentials ($\zeta$-potentials) were measured by particle electrophoresis (Zetasizer Nano ZS Series, Malvern Instruments). Samples were diluted 1:100 with sodium phosphate buffer having the same pH and salt concentration as the sample prior to analysis to avoid multiple scattering effects.

**4.2.2.6 Data Analysis**

All data shown represents the mean values ± standard deviation of two repeated measurements from two replicates. Data results were analyzed by analysis of variance (ANOVA) using IBM SPSS Statistics 20 package program.
4.3 Results and Discussion

4.3.1 Emulsion Formation

The protein contents of the protein solutions were measured before and after centrifugation and are reported in Table 4.2. The percentages of proteins present were calculated from the ratio of the measured value to the amount of protein concentrate added to the solution. For the non-centrifuged samples, the protein contents were around 51.0%, 53.2%, and 63.5% for the pea, lentil, and faba bean proteins, respectively, which is in good agreement with the manufacturer’s specifications. For the centrifuged samples, the protein contents were around 43.1%, 43.3%, and 58.3% for the pea, lentil, and faba bean proteins, respectively. These results suggest that an appreciable amount of insoluble proteins were removed by centrifugation. Whey protein isolate was also used to compare the plant-based proteins with a widely-used animal-sourced protein for their potential to fabricate omega-3 emulsions.

The particle size distributions of emulsions stabilized using the centrifuged and non-centrifuged protein solutions were measured to determine the influence of this processing step on protein functionality (Figure 4.1). The emulsions prepared from the non-centrifuged protein concentrates had a bimodal distribution, with a population of relatively small particles (peak around 0.2 μm) and another population of relatively large particles (peak around 20 μm). It was confirmed that the bimodal behavior was due to the insoluble particles, but not bigger oil droplets, by microscopic imaging and size measurements of centrifuged and non-centrifuged protein solutions. The microscopic images showed similar distributions among samples prepared with centrifuged and non-centrifuged protein solutions (data not shown). Also, the protein solutions had micron sizes before centrifugation, while it dropped to nano sizes.
afterwards (data not shown). Conversely, the emulsions prepared from the centrifuged protein concentrates had a monomodal distribution, with a single peak around 0.5 μm. These results suggest that the non-centrifuged samples contained some relatively large insoluble particles that contributed to the light scattering signal measured by the instrument used to measure the particle size distribution. Interestingly, the population of small particles in the emulsions prepared from non-centrifuged solutions was smaller than those in the emulsions prepared from centrifuged solutions (Figure 4.1). This suggests that there may have been some small particles that were also removed by centrifugation, or that there were some highly effective emulsifiers in the non-centrifuged samples that produced small lipid droplets during homogenization, but that were removed by centrifugation. Further work is clearly needed to determine the physicochemical origin of this interesting effect. For the remainder of this study, the centrifuged protein samples were utilized because they produced emulsions with a monomodal particle size distribution, which would be more suitable for most commercial applications. Our results suggest that it may be advantageous for the manufacturers of the plant protein-based emulsifiers to include additional steps to remove any large insoluble aggregates from ingredients intended for applications in emulsions.
**Table 4.2:** Protein concentrations of the protein powders measured using the Lowry method before and after centrifugation.

<table>
<thead>
<tr>
<th></th>
<th>2.5</th>
<th>3.4</th>
<th>5</th>
<th>7.5</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pea Protein</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-centrifuged</td>
<td>1.29±0.02</td>
<td>1.61±0.03</td>
<td>2.54±0.04</td>
<td>3.77±0.08</td>
<td>5.30±0.25</td>
<td>11.0±0.7</td>
<td>15.4±0.1</td>
<td>21.4±0.8</td>
<td>25.5±0.6</td>
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<td>Centrifuged</td>
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<td>1.98±0.02</td>
<td>2.94±0.05</td>
<td>4.25±0.08</td>
<td>8.40±0.13</td>
<td>12.9±0.2</td>
<td>19.2±0.6</td>
<td>21.6±1.2</td>
</tr>
<tr>
<td><strong>Lentil Protein</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-centrifuged</td>
<td>1.25±0.02</td>
<td>1.62±0.03</td>
<td>2.47±0.02</td>
<td>3.65±0.06</td>
<td>5.34±0.10</td>
<td>10.1±0.3</td>
<td>15.9±0.3</td>
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<td>26.6±0.5</td>
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<tr>
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<td>1.77±0.02</td>
<td>2.60±0.02</td>
<td>3.50±0.03</td>
<td>7.31±0.13</td>
<td>10.8±0.4</td>
<td>15.4±0.3</td>
<td>21.7±0.2</td>
</tr>
<tr>
<td><strong>Faba bean</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-centrifuged</td>
<td>1.35±0.03</td>
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<td>2.80±0.04</td>
<td>3.65±0.31</td>
<td>5.81±0.19</td>
<td>10.9±0.6</td>
<td>16.5±0.7</td>
<td>22.9±0.1</td>
<td>31.8±1.0</td>
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<tr>
<td>Centrifuged</td>
<td>1.11±0.02</td>
<td>1.43±0.02</td>
<td>2.4±0.3</td>
<td>3.33±0.03</td>
<td>4.62±0.02</td>
<td>9.66±0.05</td>
<td>14.4±0.4</td>
<td>19.1±0.4</td>
<td>29.2±0.4</td>
</tr>
</tbody>
</table>
Figure 4.1: Particle size distributions of algae oil-in-water emulsions formed using either non-centrifuged or centrifuged faba bean protein concentrate, lentil protein concentrate or pea protein concentrate solutions. The protein-to-oil mass ratio used was 0.27:1.

The influence of protein type and concentration on the mean droplet diameter ($d_{32}$) of the emulsions was also investigated (Figure 4.2). All the plant proteins used in this study led to a similar trend of decreasing mean particle size with increasing protein concentration. Figure 4.2a shows the emulsion samples stabilized using non-centrifuged protein solutions, where smaller droplet sizes could be achieved with less amounts of protein concentrates. On the other hand, when the protein solutions were centrifuged, it required using more protein concentrates to achieve smaller droplets. This trend has been attributed to the fact that the droplet size that can be produced in a homogenizer is limited by the amount of emulsifier available to cover the surfaces of the droplets formed [325]. Commercially, it is important that fortified emulsions have relatively small droplet sizes ($d < 0.5 \mu m$) to reduce creaming and increase bioavailability [326]. In most previous studies using pea, bean, lentil, and chickpea protein concentrates to form oil-in-water emulsions it was reported that it was difficult to produce oil droplets with diameters below about 1 $\mu m$ [211, 327, 328]. However, one study was able to produce smaller droplets by continuously passing pea protein stabilized emulsions
through a high pressure homogenizer [317]. In the present study, we were able to fabricate emulsions with mean particle diameters below 0.35 \( \mu m \) for all plant protein concentrates. For example, for pea, lentil, and faba bean protein concentrates (5% w/w) the mean droplet diameters were 0.35, 0.35, and 0.28 \( \mu m \) for the centrifuged samples, respectively. The smaller size of the droplets produced in the current study may be due to the fact that a microfluidizer was used to produce the emulsions, which is known to be more efficient than high-pressure valve homogenizers at producing small droplets. It is also possible that the nature of the proteins used in this study was different from that used in previous studies due to differences in origin or isolation. Overall, our results suggest that the faba bean protein concentrates were able to produce the smallest droplets under standardized homogenization conditions.

![Graphs](A) (B)

**Figure 4.2:** Effect of protein type and concentration on the mean particle diameter \((d_{32})\) of algae oil-in-water emulsions produced using (A) non-centrifuged or (B) centrifuged plant protein solutions.
4.3.2 Surface Load Calculations

The saturation surface load ($\Gamma_{\text{sat}}$) is the mass of emulsifier adsorbed per unit surface area when an interface is completely covered with emulsifier, and it therefore provides a useful indication of the minimum amount of emulsifier needed to prepare an emulsion [20]. The saturation surface load can be calculated from the following expression:

$$\Gamma = \frac{C_S d_{32}}{6 \phi}$$

where $C_S$ is the emulsifier concentration in the emulsion, $d_{32}$ is the surface-weighted mean droplet diameter, and $\phi$ is the disperse phase volume fraction [20]. In our study, the emulsions were prepared with a disperse phase mass fraction of 0.1 (10% w/w), and so it is necessary to convert this value into a volume fraction [20]:

$$\phi = \frac{\phi_m \rho_1}{\rho_1 \phi_m + \rho_2 (1 - \phi_m)}$$

Here $\rho_1$ and $\rho_2$ are the densities of the continuous and dispersed phases, respectively. This equation was used to calculate the disperse phase volume fraction ($\phi = 0.104$) from the densities of the two phases and the mass fraction ($\phi_m = 0.100$).

As seen in figure 4.2a, whey protein-stabilized samples provided the smallest sizes with lowest emulsifier concentration; therefore, it would be expected for whey protein to have the smallest surface load, i.e., to be the most effective emulsifier among all in this study. Equation 1 suggests that a plot of $d_{32}$ against $1/C_S$ should be a linear line with a slope equal to $6\Gamma \phi$, which was observed in practice (Figure 4.3). This approach was therefore used to estimate the saturation surface loads of the different emulsifiers (Table 4.3). As expected from the particle size data, faba bean protein concentrate proved to be the most efficient plant-based emulsifier, i.e., it had the lowest surface load. Relatively small globular proteins (such as bovine serum albumin, $\alpha$-lactalbumin, and $\beta$-lactoglobulin) typically have surface loads
around 1 to 3 mg m$^{-2}$ [329-334]. On the other hand, relatively large globular proteins (such as soy proteins) and some flexible proteins (such as casein) have been reported to have surface loads around 4 to 11 mg m$^{-2}$ [335-337]. The higher values for these proteins may be due to their high molecular weights or their ability to form multilayers around oil droplets [334, 336]. It should be noted that the method used to calculate the surface load in our study is based on the assumptions that all of the proteins adsorb to the droplet surfaces, and that the surface load does not depend on initial protein concentration. In practice, these assumptions may not be valid, which would lead to some errors in the estimated values. Nevertheless, this approach does provide some valuable information about the effective surface load of emulsifiers under conditions that simulate those that would be used commercially to fabricate emulsions.

Table 4.3: Calculated surface loads of the various plant proteins used. The plant proteins were centrifuged before making the emulsions to remove insoluble matter. The results for whey protein isolate are shown as a comparison.

<table>
<thead>
<tr>
<th>Protein source</th>
<th>Surface load (mg/m$^2$)</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey</td>
<td>1.68</td>
<td>0.946</td>
</tr>
<tr>
<td>Pea</td>
<td>5.94</td>
<td>0.912</td>
</tr>
<tr>
<td>Lentil</td>
<td>10.6</td>
<td>0.799</td>
</tr>
<tr>
<td>Faba bean</td>
<td>4.97</td>
<td>0.888</td>
</tr>
</tbody>
</table>

4.3.3 Effect of Environmental Stresses on Emulsion Stability

For the stability studies, 10% (w/w) oil-in-water emulsions containing 3% (w/w) protein (centrifuged to remove insoluble matter) were prepared, because the droplet size appeared to reach a plateau region between 2 to 3% protein (Figure 4.2B). The properties of the emulsions were measured after they had been exposed to the different pH, salt, and temperature conditions described earlier (Section 4.2.2.3).
Figure 4.3: Example of method used to determine the surface load of the protein emulsifiers from the particle diameter versus protein concentration data. In this case the emulsions were prepared using centrifuged lentil protein concentrate and Cs is the protein concentration measured by the Lowry method.

4.3.3.1 Effect of pH

For commercial applications, it is often important that the emulsifier-coated oil droplets stay stable over the range of pH values typically found in emulsion-based food and beverage products. The aim of this study was therefore to evaluate the impact of storage pH on the properties of emulsions stabilized by the different legume protein concentrates. The electrical properties of the oil droplets were characterized by measuring their pH versus ζ-potential profiles (Figure 4.4A). All of the emulsions exhibited a fairly similar behavior, with the ζ-potential moving from positive at low pH values to negative at high pH values, with an isoelectric point (pI) around pH 5. Legumes typically consist of a major fraction of globulins and a minor fraction of albumins [57, 59, 316]. The pI for globulins is around pH 4.5, whereas the pI of albumins is around pH 6 [60], and so the pH where the droplets have net zero charge would be expected to be between these values. Other researchers have also reported that pea, lentil, and soy protein isolates have a low net charge around pH 5 [34].
(A) Graph showing the change in zeta potential (mV) with pH for Faba bean, Lentil, and Pea.

(B) Graph showing the change in d32 (nm) with pH for Faba bean, Lentil, and Pea.

(C) Images of Faba bean, Lentil, and Pea at different pH levels (2 to 9).
Figure 4.4: Influence of pH on (A) droplet charge, (B) particle size, and (C) physical appearance of algae oil-in-water emulsions stabilized by different plant proteins. The protein-to-oil mass ratio in the emulsions was 0.27:1. The influence of pH on (D) the solubility of the protein in buffer solutions is also shown.

The emulsions stabilized by lentil protein concentrate were the most stable to pH changes with the mean particle diameter being relatively low at all pH values except pH 5 (Figure 4.4B), which is close to the pH where the droplets carry no charge. However, extensive phase separation due to creaming was observed from pH 4 to 6 in this system (Figure 4.4C). The most likely reason for this observation is that the flocs were only held together by relatively weak attractive forces and so they were easily disrupted when the samples were diluted for the light scattering measurements. For the emulsions stabilized by pea protein concentrate, extensive droplet aggregation and creaming occurred in the range from pH 3 to 6. The emulsions stabilized by faba bean protein concentrate were the least stable to pH changes, with extensive aggregation and creaming occurring from pH 2 to 6. These results can be attributed to changes in the magnitude of the electrostatic repulsion between the droplets with pH [61, 315]. The protein-coated droplets have a high net charge at pH values well above or below their isoelectric point, which generates a strong electrostatic
repulsion between them. Conversely, they have a low net charge at pH values around the pI, and so the electrostatic repulsion is not strong enough to overcome the van der Waals attraction, thereby leading to flocculation. Also, even though the electrical charge around the droplets was very similar in all samples (Figure 4.4A), lentil protein-stabilized droplets were more stable under extreme pH values (Figure 4.4C). This suggests that electrostatic interactions were not the only factor responsible for the different behaviors observed in the stability of emulsions. On the other hand, differences in steric interactions can also account for this effect and probably, lentil proteins create thicker coatings around droplets that increase emulsion stability.

It is interesting to compare the flocculation stability of the protein-coated emulsion droplets to the solubility of the protein molecules in solution, since both of these phenomena depend on electrostatic interactions, i.e. either between protein molecules or between protein-coated lipid droplets. For this reason, we measured the protein solubility as a function of pH (Figure 4.4D). All of the plant proteins have a relatively high solubility at relatively high or low pH values because of the strong electrostatic repulsion between them, but they all aggregate around the isoelectric point because of the reduction in electrostatic repulsion. Thus, the solubility behavior of the protein molecules in solution follows a similar trend as the aggregation stability of the protein-coated droplets in emulsions.

4.3.3.2 Effect of Salt

Foods and beverages contain different levels of mineral ions and so it is important to understand the impact of salts on the properties of protein-coated oil droplets. For this reason, we examined the impact of NaCl on the stability of legume protein concentrate-stabilized emulsions at pH 7.
The influence of salt concentration on the particle size, particle charge, and physical appearance of the emulsions was measured (Figure 4.5). For all the protein concentrates, there was a decrease in the magnitude of the ζ-potential with increasing salt concentration (Figure 4.5A), which can be attributed to electrostatic screening, *i.e.*, the preferential accumulation of counter-ions (Na⁺) around the negatively charged droplet surfaces [20, 315]. Nevertheless, there were large differences in the stability of emulsions with different salt concentrations depending on the nature of the protein used. No increase in mean particle diameter or visible creaming was observed in the lentil protein-emulsions for all salt levels studied (Figures 4.5B and 4.5C), which suggested that this protein concentrate produced droplets that were highly resistant to salt addition. Conversely, an increase in mean particle diameter and extensive creaming was observed in the faba bean-emulsions at 100 and 200 mM NaCl, and in the pea protein-emulsions at 100 mM (Figures 4.5B and 4.5C). Surprisingly, these emulsions became stable to aggregation again at higher salt levels. Thus, it appeared that these emulsions were unstable to salt at intermediate ionic strengths. This effect may be due to the ability of salt to alter various types of electrostatic interactions in the emulsions, both attractive and repulsive. The addition of salt screens the electrostatic interactions between the droplets, which should decrease the electrostatic repulsion between the droplets and lead to flocculation [315]. On the other hand, addition of salt may also alter the conformation of the adsorbed protein molecules leading to a thicker interfacial layer that increases the steric repulsion between the droplets. Alternatively, adsorption of salt ions to charged groups on droplet surfaces can increase the hydration repulsion between droplets due to the water of hydration associated with the salt ions [338, 339].
Figure 4.5: Influence of salt (NaCl) concentration on (A) droplet charge, (B) particle size, and (C) physical appearance of algae oil-in-water emulsions stabilized by different plant proteins. The protein-to-oil mass ratio in the emulsions was 0.27:1.
Other studies have also reported that emulsions stabilized by certain types of plant proteins are stable to aggregation at elevated salt levels, such as coconut or tomato seed proteins [340-342]. This effect can be partly attributed to the fact that the solubility of some proteins increases with increasing salt content due to the ability of the salts to weaken the attractive interactions between protein molecules (also known as the `salting in` effect) [343, 344].

4.3.3.3 Effect of Temperature

Foods and beverages may be exposed to elevated temperatures during their processing, transportation, storage, and handling, and so it is useful to study the impact of thermal processing on the stability of protein-coated oil droplets. For this reason, the influence of temperature on the stability of emulsions prepared using different plant protein concentrates was examined.

In the absence of added salt, the ζ-potential on the droplets changed appreciably after heat treatment depending on the holding temperature (Figure 4.6A). The magnitude of the negative charge on the droplets was much higher at temperatures below 60°C, than at higher temperatures. This result suggests that there was some change in the electrical characteristics of the droplets induced by heating. This change may have been brought about by a conformational change of the adsorbed proteins above their thermal denaturation temperature, which altered the exposure of charged groups or altered the number of counter-ions bound. The thermal denaturation temperatures of a number of plant proteins have been reported previously: faba bean protein, 88°C [345]; pea protein, 80-86 °C [346, 347]; red bean globulin, 90°C [348]; legume proteins, 84°C [349]; and lentil proteins, 80 °C [350]. Typically, a protein will start to unfold at temperatures considerably below its thermal denaturation temperature, and so it is possible that protein conformational changes may explain the
observed effects on droplet charge with temperature (Figure 4.6A). Alternatively, the solubility of any mineral ions in the system (such as calcium) may have changed with temperature, which could have altered their interactions with the adsorbed proteins, thereby modifying the surface potential.

![Figure 4.6: Influence of incubation temperature on the (A) droplet charge and (B) particle size of algae oil-in-water emulsions stabilized by different plant proteins in the absence of added salt.](image)

Despite the observed decrease in droplet charge with heating in the absence of salt, the emulsions were still relatively stable to droplet aggregation (Figure 4.6B) and creaming (data not shown). Presumably, the electrostatic and steric repulsions between the droplets were still strongly enough to prevent flocculation at low ionic strengths, as it was in other studies [351].

In the presence of salt (150 mM NaCl), the $\zeta$-potential on all the coated droplets remained fairly constant (-9 to -16 mV) regardless of the temperature the emulsions were held at (Figure 4.7A). This result suggests that there was little change in protein conformation or ion binding effects with temperature in the presence of relatively high salt levels. It has been reported that the denaturation temperature of oat globulin [352], faba bean protein [345], red bean globulin [348] and pea proteins [347] increase with NaCl addition. Consequently, it is
possible that the unfolding of the proteins did not occur at the higher temperatures studied in the presence of salt. However, this effect is unlikely, because we did observe extensive droplet aggregation (Figure 4.7B) and creaming (Figure 4.7C) in some of the emulsions after they were exposed to the higher temperatures. This phenomenon may have occurred due to an increase in the hydrophobic attraction between the oil droplets when the protein molecules unfold and expose non-polar amino acids normally buried in their hydrophobic interiors [66]. Also, the salt present in the samples can screen the charge around the droplets and therefore, the repulsive electrostatic forces might not be enough to overcome the attractive forces and cause to droplet aggregation during heating [66]. Interestingly, the lentil protein-coated droplets appeared to be relatively stable to aggregation across the entire temperature range studied, since we observed little change in their mean particle diameter (Figure 4.7B) or creaming stability (Figure 4.7C) with storage temperature.
The origin of the stability of the lentil protein emulsions is currently unknown, but may be due to differences in the surface hydrophobicity or thickness of the adsorbed protein layer. The fact that the electrical characteristics of all the plant protein-coated droplets was similar (Figure 4.7A), suggests that differences in hydrophobic or steric interactions are more likely to account for this effect than differences in electrostatic interactions.

### 4.4 Conclusions

This study has shown that omega-3 fortified emulsions can be produced using plant proteins as emulsifiers. These emulsions are completely free of synthetic or animal-based ingredients, and may therefore be suitable for consumers with particular dietary requirements, such as vegans and vegetarians. The initial droplet size decreased with increasing protein...
concentration, and relatively small droplets \( d < 0.3 \, \mu m \) could be produced using all plant protein types. The lentil protein-stabilized emulsions had better stability to environmental stresses (pH, salt, and temperature) than the faba bean- and pea protein-stabilized ones. The origin of the higher physical stability of the lentil protein-stabilized emulsions is currently unknown. However, the fact that the electrical characteristics of all the protein-coated droplets were very similar suggests that the higher stability of lentil protein-stabilized emulsions is due to differences in surface hydrophobicity or interfacial thickness. In summary, the results generated through this study may provide practical strategies for the food industry to formulate clean-label fortified foods and beverages, as well as other commercial emulsion-based products, such as personal care or cosmetic products.
CHAPTER 5

GASTRONINTESTINAL FATE OF EMULSION-BASED Ω-3 OIL DELIVERY SYSTEMS STABILIZED BY PLANT PROTEINS: LENTIL, PEA, AND FABA BEAN PROTEINS

5.1 Introduction

Eicosapentaenoic acid (22:5 omega-3, EPA) and docosahexaenoic acid (22:6 omega-3, DHA) are omega-3 polyunsaturated fatty acids (PUFAs) that are abundant in fish and algae oils [353, 354]. A diet rich in omega-3 PUFAs may promote cardiovascular health and reduce inflammation, diabetes, cancer, asthma, schizophrenia, and depression [353, 355-357]. Due to their high susceptibility to lipid oxidation and their low water-solubility, oil sources rich in omega-3 PUFAs are usually incorporated into colloidal delivery systems to protect them during processing, storage, and transport [83, 89, 208]. Emulsion-based delivery systems, which consist of emulsifier-coated lipid droplets dispersed within an aqueous medium, have been shown to be particularly suitable for this purpose because of their ease of preparation and flexibility of design [1, 358]. Many of the emulsifiers currently used in the food industry to stabilize emulsions are either synthetic (often esters of fatty acids) or animal-based (such as milk, egg, or meat proteins) [9]. There is increasing demand from consumers for “clean label” products that are formulated from plant-based ingredients, and therefore there is interest in replacing synthetic or animal-based emulsifiers with plant-based ones in food emulsions [12, 359].

Pulses are a particularly good source of edible proteins because of their relative abundance, sustainable supply, and low cost [58]. The Food and Agriculture Organization (FAO) of the United Nations describes pulses as the edible and dry seeds of legumes that are members of the Fabaceae or Leguminosae families which includes chickpeas, peas, lentils,
beans, and lupins [360]. Pulses have a good nutritional profile due to their low sodium, fat and cholesterol content, low glycemic index, and high protein, iron, folate, potassium and fiber content [361]. Plant proteins can be isolated from pulses and converted into functional ingredients using commercially viable extraction and purification methods [362]. The major protein fractions in pulses are globulins (such as 7S and 11S) and albumins, while the minor protein fractions are prolamins and glutelins [363].

A potential disadvantage of pulse proteins is their lower digestibility in the gastrointestinal tract (GIT) compared to proteins from animal sources [213]. If pulse proteins are going to be utilized as emulsifiers in emulsion-based delivery systems, then it is important that they will release the encapsulated bioactive lipids within the GIT [277]. Typically, the ingested triacylglycerols (TAGs) must be hydrolyzed by gastric and pancreatic lipases within the stomach and small intestine, which leads to the formation of free fatty acids (FFAs) and monacylglycerols (MAGs) [364, 365]. These lipid digestion products then interact with bile salts and phospholipids from the small intestinal secretions to form mixed micelles, which transport the FFAs and MAGs to the epithelium cells where they are absorbed [366, 367]. The bioavailability of bioactive lipids could therefore be reduced if pulse protein-coated lipid droplets are not fully digested within the GIT. There have been relatively few previous studies on the potential gastrointestinal fate of lipid droplets coated by pulse-proteins. A recent study on the utilization of pea and soy proteins to coat conjugated linoleic acid (CLA) droplets indicated that only about 22-25% of the FFAs were released in a simulated GIT model [368]. This result suggests that plant-proteins may suppress lipid digestion, which would be a major disadvantage for their application as delivery systems. An alternative explanation for the relatively low level of lipid digestion reported in this study is that the concentrations of gastrointestinal components used in the GIT model (e.g., digestive enzymes, bile salts, and calcium ions) did not adequately reflect human gastrointestinal conditions.
Another recent study reported that high concentrations of pea proteins in oil-in-water emulsions retarded the rate of β-carotene release under simulated GIT conditions, but this may have been because these high protein levels promoted extensive droplet flocculation [370].

The main objective of this chapter was therefore to determine the impact of three pulse protein emulsifiers (isolated from lentil, pea, and faba bean) on the digestibility of fish oil-in-water emulsions using a simulated GIT model. In addition, the impact of these plant-based proteins on lipid digestion was compared to that of a widely used animal-based protein (whey). The results from this study should provide valuable information about the potential utilization of pulse proteins to create emulsion-based delivery systems for bioactive lipids.

5.2 Materials and Methods

5.2.1 Materials

Pea, lentil, and faba bean concentrates (Vitessence Pulse 1550, 2550 and 3600) were provided by Ingredion, Inc. (Bridgewater, NJ). Fish oil was provided by DSM, Inc. (Columbia, MD). Whey protein isolate (BiPro JE 011-4-420) was provided by Davisco Foods International, Inc. (Le Sueur, MN). An aqueous sodium phosphate buffer (10 mM, pH 7.0) was used to prepare all protein solutions and emulsions. All other chemicals and reagents used in this study were analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

5.2.2 Protein purification

The pulse protein concentrates provided by the manufacturer contained about 55-60% protein, and so a purification step was carried out before producing the emulsions. The protein purification protocol used was based on a method described previously with some modifications [322]. The protein concentrates were dispersed in buffer solutions by stirring for 2 hours at room temperature at pH 7.5 at a concentration of 20% (w/w). Then the solutions
were centrifuged (Sorvall Lynx 4000 Centrifuge, Thermo Scientific, Agawam, MA) at 15,000 g for 30 min at 10 °C to remove any starch, fiber, and insoluble matter, including insoluble proteins. The supernatant was then collected and centrifuged again using the same conditions. The resulting supernatant was then collected in another beaker and the pH was adjusted to 4.5 using hydrochloric acid (HCl) solution to promote isoelectric precipitation of the proteins. Solutions were then centrifuged again using the same conditions as described previously to precipitate and collect the proteins. The supernatant was removed and replaced with fresh buffer solution (pH 7.0). The precipitate was dispersed in buffer solution at room temperature for 60 minutes and the mixture was adjusted back to pH 7.0 using sodium hydroxide (NaOH) solution. To ensure complete dispersion, the protein solutions were stirred overnight at 5 °C. The resulting solutions were brought to room temperature by stirring for 30 minutes, and then adjusted to pH 7.0 and centrifuged again. The protein contents of the resulting solutions were determined using the Lowry method [323], and calculated using a standard curve prepared with a bovine serum albumin standard (R=0.992). The protein solutions were diluted to 20 mg/mL protein content using buffer solutions prior to emulsion formation.

5.2.3 Emulsion formation

Emulsions were prepared by homogenization of 10% (w/w) oil phase (fish oil) and 90% (w/w) aqueous phase (protein solution, pH 7.0) at ambient temperature. A high-shear mixer (M133/1281-0, Biospec Products Inc., Bartlesville, OK) was used to blend the two phases for 2 minutes at 10,000 rpm to produce coarse oil-in-water (O/W) emulsions. These emulsions were then passed through a high-pressure microfluidizer (PureNano, Microfluidics, Newton, MA) 3 times at 10,000 psi. This device contained a series of X- and Y-interaction chambers to breakdown the droplets, which were cooled throughout homogenization using an ice bath to prevent a rise in emulsion temperature.
5.2.4 In vitro digestion

The gastrointestinal fate of the emulsions was monitored using a static simulated GIT that has been widely utilized in our laboratory [369]. Initially, the samples were diluted with buffer solution (1:5) to obtain a fat content of 2%, since this level of fat is usually appropriate to give full digestion under the simulated GIT conditions used.

• **Mouth phase:** 20 mL of the emulsions were mixed with 20 mL of artificial saliva solution containing 0.6 g mucin that was prepared according to previous studies [124, 371, 372]. The mixture was then adjusted to pH 6.8 and incubated at 37°C for 10 minutes with continuous agitation at 100 rpm (Innova Incubator Shaker, Model 4080, New Brunswick Scientific, New Jersey, USA).

• **Stomach phase:** Simulated gastric fluid stock solution (SGFSS) was prepared by dissolving 2 g of NaCl and 7 mL of 12 N HCl in 1 L of double distilled water. 20 mL of the bolus sample taken from the mouth phase was mixed with 20 mL of artificial gastric fluid that was prepared by mixing 20 mL of SGFSS with 0.064 g of pepsin. The mixture was then adjusted to pH 2.5 and incubated at 37°C for 2 hours with continuous agitation at 100 rpm.

• **Small intestine phase:** 30 mL of the chyme sample taken from the stomach phase was placed in a water bath at 37°C in a beaker and the adjusted to pH 7.0. Then, 1.5 mL of calcium chloride (36.7 mg/mL) and sodium chloride (219.1 mg/mL) solution was added. Next, 3.5 mL of bile extract (53.6 mg/mL) dissolved in buffer solution was added to the sample and the pH was re-adjusted to 7.0. Lastly, 2.5 mL of lipase (24 mg/mL) dissolved in buffer solution was incorporated into the mixture. An automatic titration (pH-stat) device (835 Titrando, Metrohm USA Inc., Riverview, FL) was then used to determine the volume of NaOH solution required to maintain the system at pH 7.0 throughout the
incubation period. The amount of free fatty acids released at the small intestine phase was calculated using the following equation:

\[
\%\text{FFA} = 100 \times \frac{V_{\text{NaOH}} \times m_{\text{NaOH}} \times m_{\text{lipid}}}{w_{\text{lipid}} \times 2}
\]

where, \(V_{\text{NaOH}}\) is the volume of titrant consumed in liters, \(m_{\text{NaOH}}\) is the molarity of the NaOH solution used (0.1 N), \(m_{\text{lipid}}\) is the molecular weight of fish oil (868 g/mol), \(w_{\text{lipid}}\) is the weight of oil in the digestion system in grams (0.15 g).

### 5.2.5 Droplet characterization

The mean particle diameters of the emulsions were measured using a static light scattering instrument (Mastersizer 2000, Malvern Instruments, Westborough, MA). The results are reported as surface-weighted mean diameters (\(d_{32}\)) or volume-weighted mean diameters (\(d_{43}\)). The electrical surface potentials (\(\zeta\)-potentials) of the droplets were measured using a particle electrophoresis instrument (Zetasizer Nano ZS Series, Malvern Instruments, Westborough, MA). The emulsions were diluted (1:100) using buffers of the same pH. A confocal microscope (C1 Digital Eclipse, Nikon, Tokyo, Japan) was used to monitor the microstructures of the samples after each phase with a 60\(\times\) oil immersion objective. Nile red and fluorescein isothiocyanate (FITC) were used as dyes to stain the lipid and protein parts of the samples, respectively.

### 5.2.6 Data analysis

All experiments were carried out in triplicate, with two repeated measurements per sample. Means and standard deviations were then calculated from these values, and then analysis of variance (ANOVA) was carried out using a statistical software package (SPSS Statistics 20, IBM). The Tukey test was employed to determine significant differences amongst samples at a 5% significance level (p<0.05).
5.3 Results and Discussion

5.3.1 Influence of protein type on particle stability under simulated GIT conditions

Initially, the impact of the type of protein used to coat the lipid droplets on the gastrointestinal fate of the emulsions under simulated GIT conditions was examined. The mean particle diameter, particle size distribution (PSD), and microstructure of the emulsions was measured after each GIT stage. For the sake of brevity, only the PSD measurements for the whey and pea proteins are shown because the other pulse proteins showed similar results as the pea protein.

- **Initial systems:** Emulsions were prepared using an animal protein (whey) and three plant proteins (pea, lentil, and faba bean). The surface-weighted mean droplet diameters ($d_{32}$) were determined after each stage of digestion (Figure 5.1). The whey protein-stabilized emulsions contained appreciably smaller droplets ($d_{32} = 129$ nm) than the pulse protein-stabilized ones ($d_{32} = 392$ to $485$ nm). All of the emulsions initially had monomodal PSDs (Figure 5.2) with relatively small lipid droplets (stained red) evenly dispersed throughout the aqueous phase (Figure 5.3). The fact that the aqueous phase had a greenish color when observed by confocal fluorescence microscopy (Figure 5.3), indicated that there were non-adsorbed proteins (stained green) in the aqueous phase.
Figure 5.1: Influence of gastrointestinal tract (GIT) stage and emulsifier type on the (A) surface-weighted ($d_{32}$) (B) volume-weighted ($d_{43}$) mean particle diameter of fish oil-in-water emulsions. Different lower case letters represent significant differences ($p < 0.05$) between different samples in a given in vitro digestion stage (initial, mouth, stomach or small intestine).

Figure 5.2: Particle size distribution (PSD) of fish oil-in-water emulsions stabilized by pea protein (white symbols) or whey protein (black symbols) after exposure to different stages of the simulated GIT. The other pulse protein-stabilized emulsions had similar PSD profiles, and therefore only one sample is shown as a representative.
Figure 5.3: Influence of emulsifier type and GIT stage on the microstructure of fish oil-in-water emulsions exposed to different GIT stages determined using confocal fluorescence microscopy. The proteins are stained green, while the lipid phase is stained red.

The effectiveness of emulsifiers at producing small droplets during homogenization depends on a number of factors: (i) the speed they adsorb to the droplet surfaces; (ii) their saturation surface loads; (iii) their ability to lower the interfacial tension; and, (iv) their ability to generate strong repulsive forces [39, 359, 373]. The minimum droplet diameter that can be produced by a particular emulsifier can be calculated as [20]:

\[ d_{\text{min}} = \frac{6\times\Gamma \times \phi}{C_s} = \frac{6\times\Gamma \times \phi}{(1 - \phi)C_s} \]
where, $\Gamma$ is the surface load (kg m$^{-2}$), $\phi$ is the disperse phase volume fraction, $C_s$ is the emulsifier concentration in the emulsion (kg m$^{-3}$), and $C'_s$ is the emulsifier concentration in the continuous phase (kg m$^{-3}$). The surface load of the same proteins was determined in our previous study to be around 1.7 mg m$^{-2}$ for whey protein, 5.0 mg m$^{-2}$ for faba bean protein, 5.9 mg m$^{-2}$ for pea protein, and 10.6 mg m$^{-2}$ for lentil protein (unpublished results). The emulsions used in this study initially contained 10% (w/w) oil droplets ($\phi$$\approx$0.1) and contained 20 mg/mL protein in the aqueous phase ($C'_s = 20$ kg m$^{-3}$). Therefore, the predicted minimum droplet diameters for these emulsions should be around 57, 167, 197, and 353 nm for whey, faba bean, pea and lentil proteins, respectively. The actual mean droplet diameters (129, 455, 392 and 485 nm) were considerably higher than these theoretical values, which suggests that other factors limited the particle size. For example, the homogenizer pressure used may have been insufficient to break the droplets down to the theoretical limit, the emulsifiers may not have adsorbed fast enough to the droplet surfaces to prevent coalescence, some of the protein molecules may not have adsorbed to the droplet surfaces, or some droplet flocculation may have occurred after homogenization [39, 359].

- **Mouth phase:** Interestingly, there was a slight decrease in the surface-weighted mean particle diameter ($d_{32}$) of the three emulsions stabilized by the pulse proteins when they were exposed to simulated mouth conditions, but a substantial increase for the emulsions stabilized by the whey proteins (Figure 5.1B). However, there was an increase in the volume-weighted mean particle diameter ($d_{43}$) of all the emulsions after exposure to the simulated mouth conditions (Figure 5.1B). The $d_{43}$ value is more sensitive to the presence of large particles than the $d_{32}$ values, which may account for this effect [20]. Indeed, the confocal microscopy images of the different emulsions indicated that they were all highly aggregated in the mouth phase (Figure 5.3). Droplet aggregation may have occurred due to the presence of mucin (an anionic biopolymer) in the simulated saliva, which has
previously been reported to induce depletion and/or bridging flocculation in the mouth phase of GIT models [372, 374, 375]. Bridging flocculation occurs when mucin molecules bind to the surfaces of two or more droplets [52, 365], whereas depletion flocculation occurs when the concentration of non-adsorbed mucin molecules in the aqueous phase is high enough to generate a strong osmotic attraction between the droplets [376, 377]. Droplet flocculation may also have been partially the result of electrostatic screening effects, i.e., the accumulation of positively charged counter-ions around the negatively charged lipid droplet surfaces [124, 315]. The emulsions had bimodal PSDs in the mouth stage (Figure 5.2), which suggests that only a fraction of the droplets aggregated in the simulated saliva. The fact that the emulsions had a bimodal distribution accounts for the different behaviors of the $d_{32}$ and $d_{43}$ values of the emulsions [20].

- **Stomach phase:** After exposure to the stomach phase, the mean particle diameter increased appreciably (Figure 5.1), and the presence of large aggregates was observed in the particle size distributions (Figure 5.2) and in the microstructure images (Figure 5.3) for all emulsions. The observed increase in particle aggregation can be attributed to a number of phenomena. First, the anionic mucin molecules originating from the simulated saliva may have promoted bridging flocculation of the cationic protein-coated droplets in the acidic gastric fluids [378, 379]. Second, the pepsin present in the gastric fluids may have partially hydrolyzed the protein coating around the lipid droplets, which would have altered the thickness and charge of the interfacial layer, and therefore reduced the steric and electrostatic repulsion between the droplets [380, 381]. Third, the relatively high ionic strength of the simulated gastric fluids may have reduced the strength of the electrostatic repulsion between the droplets [378, 379].

- **Small intestine phase:** After exposure to the small intestine phase, the mean particle diameter remained relatively high (Figure 5.1), and there was still evidence of large
aggregates in the particle size distributions (Figure 5.2) and microscopy images (Figure 5.3) for all emulsions. However, the size of these aggregates was significantly lower (p<0.05) than those observed in the stomach phase for all systems. The composition and structure of the digested materials present in the small intestine phase after lipid digestion is typically highly complex [382]. Various constituents arising from the emulsions or from the GIT fluids may be present in the digesta, including free fatty acids, monoacylglycerols, peptides, bile salts, phospholipids, enzymes, mineral ions, and undigested lipids and proteins. These constituents can assemble into different types of colloidal particles with different dimensions, morphologies, and aggregation states, including micelles, vesicles, protein aggregates, insoluble calcium salts, and undigested lipid droplets. Consequently, it is difficult to conclusively establish the nature of the particles present in the small intestine fluids after digestion from the light scattering and microscopy measurements.

5.3.2 Influence of protein type on particle charge under simulated GIT conditions

Changes in the surface potential of the particles in different regions of the GIT were carried out because this provides some valuable information about changes in interfacial composition (Figure 5.4).
Figure 5.4: Influence of gastrointestinal tract (GIT) stage and emulsifier type on the particle charge ($\zeta$-potential) of fish oil-in-water emulsions. Different lower case letters represent significant differences ($p < 0.05$) between different samples in a given in vitro digestion phase (initial, mouth, stomach or small intestine).

- **Initial systems:** Initially, faba bean, pea, and lentil protein-coated droplets had moderately high negative surface potentials (-20, -20, and -25 mV, respectively), while whey protein-coated droplets had considerably more negative values (around -41 mV). For electrostatically-stabilized colloidal systems, the magnitude of the $\zeta$-potential on the particles should be greater than about 30 mV to generate repulsive electrostatic forces that are strong enough to overcome attractive van der Waals forces, and thereby prevent particle aggregation over extended periods [383]. Our results suggest that the emulsions containing plant protein-coated droplets may be more susceptible to droplet aggregation than those containing whey protein-coated ones. However, other types of repulsive force may also determine the overall aggregation stability of protein-coated droplets, such as steric repulsion [20]. The surface potentials measured in our study are in accordance with those determined for protein-coated lipid droplets in other studies at the same pH [42, 202, 384].

- **Mouth phase:** After exposure to the mouth phase, there was an appreciable decrease in the magnitude of the surface potential for the whey protein-coated droplets, while the surface potential of the plant protein-coated droplets remained relatively constant (Figure 5.4). In general, changes in the $\zeta$-potential of particles are due to alterations in either the surface charge density and/or the ionic strength of the surrounding aqueous phase [20]. Our results therefore suggest that there was a difference in the changes in interfacial composition of the whey and plant protein-coated droplets after exposure to the simulated mouth phase. For both systems, there will have been some electrostatic screening caused by the presence of mineral ions in the simulated saliva [20], but in the case of the whey protein-coated droplets there may have been more mucin molecules adsorbed to their surfaces [375, 385, 386]. This may have occurred because the whey protein molecules had more exposed
cationic groups that could attract anionic groups on the mucin molecules, but further research is needed to confirm this hypothesis.

- **Stomach phase:** After exposure to the stomach phase, the surface potentials of all the emulsions were fairly similar (+1.1 to +1.5 mV), with no statistical difference (p>0.05) amongst them (Figure 5.4). The simulated stomach phase is highly acidic (pH 2.5), and therefore one would have expected the droplets to have a much higher positive ζ-potential that actually observed because this pH is well below their isoelectric point [34]. The fact that the measured ζ-potential under simulated stomach conditions was actually close to neutral can be attributed to the adsorption of anionic mucin molecules to the surfaces of the cationic protein-coated droplets, leading to charge neutralization [378, 387]. The relatively low charge on the lipid droplets under simulated stomach conditions would account for the high degree of droplet aggregation observed (Figures 5.1 to 5.3); the electrostatic repulsion was insufficient to overcome the van der Waals attraction [315]. In addition, the relatively high ionic strength of the simulated gastric fluids would have led to electrostatic screening effects, which would decrease the magnitude of the surface potential on the droplets [20].

- **Small intestine phase:** After exposure to the small intestine phase, all of the samples contained particles with a strongly negative surface potential (Figure 5.4). Under the neutral conditions of the simulated intestinal fluids, any proteins remaining should have a strong negative charge because this pH is well above their isoelectric point. Moreover, the intestinal fluids will contain various other types of anionic species, including free fatty acids, bile salts, and phospholipids that can form anionic colloidal particles, such as micelles and vesicles [364, 365]. The presence of anionic lipid digestion products in all of the samples may account for the fact that they all had fairly similar surface potentials at the end of the small intestine phase. Nevertheless, the ζ-potential of the particles in the digesta resulting from the whey protein emulsions was significantly more negative than that for the
plant protein emulsions (p < 0.05), which may have been because the whey proteins were initially more negatively charged at neutral pH (Figure 5.4).

5.3.3 Influence of protein type on *in vitro* digestion

If a plant protein is going to be used to stabilize the lipid droplets in emulsion-based delivery systems, then it is important that it does not inhibit the release of the bioactive agents. For this reason, the impact of protein type on the rate and extent of free fatty acid (FFA) release from the different emulsions in the small intestine phase was monitored using a pH-stat method. There was a rapid increase in FFA release during the first 15 minutes of the small intestine phase, with around 85 to 92% of the lipids being digested in this initial period (Figure 5.5). From 15 to 120 minutes, there was a further slow increase in the amount of FFAs released from the lipid droplets, with complete lipid digestion occurring by the end of the small intestine phase for all of the samples. These results suggest that there were no major differences in the ability of lipase to hydrolyze the emulsified fish oil in the emulsions stabilized by the different kinds of proteins. This result is markedly different from that of a recent study on the digestion of CLA droplets coated by pea or soy proteins, where it was reported that only about 22-25% of the FFAs were released by the end of the small intestine phase [368]. The most likely reason for this apparent discrepancy is that the levels of enzymes and bile salts used in this latter study were insufficient to digest all of the lipids and solubilize all the free fatty acids released in the emulsions used [369]. The rate of lipid digestion in emulsions is known to increase with decreasing droplet size, because this increases the surface area of the lipid phase exposed to the lipase [113, 121]. The whey protein emulsions initially had smaller mean droplet diameters ($d_{32}$) than the plant protein emulsions (Figure 5.1A), and might therefore have been expected to be digested more rapidly. However, it is the size of the lipid droplets reaching the small intestine, rather than the initial size, that determines rate of
lipid digestion, which may account for the fact that the whey protein emulsions were not digested more rapidly than the plant protein emulsions.

**Figure 5.5:** Release of free fatty acids (FFA) from fish oil-in-water emulsions stabilized by different emulsifier types during exposure to simulated small intestine conditions.

The digestion of pulse proteins in foods is often inhibited due to the presence of anti-nutritional factors (ANFs) present in pulses, such as protease inhibitors, lectins, tannins, saponins, and phytates [58, 388]. Indeed, it has been reported that the in vitro digestibility of pulse proteins varies between about 60 to 80% depending on pulse type and processing methods [389]. Despite this phenomenon, the lipid droplets coated by all three types of pulse-proteins used in our study were fully digested under simulated GIT conditions (**Figure 5.5**). There are a number of possible reasons that may account for this observation. First, anti-nutritional factors are usually eliminated during the isolation procedures used to extract and purify protein ingredients [213]. Second, the proteins may have been displaced from the lipid droplet surfaces by bile salts and lipase, and therefore they did not need to be fully digested before the lipids were digested.
5.4 Conclusions

This study has shown that plant protein isolates (from lentils, peas, and faba beans) could be used to successfully fabricate oil-in-water emulsions containing relatively small fish oil droplets ($d_{32} < 500$ nm). These emulsions could therefore be used as delivery systems for omega-3 polyunsaturated fatty acids, allowing them to be incorporated into aqueous functional foods and beverages. The delivery systems stabilized by plant proteins were shown to behave similarly in a simulated gastrointestinal tract as delivery systems stabilized by a commonly used animal protein (whey protein isolate). Moreover, the lipid droplets coated by the plant proteins were completely digested under simulated GIT conditions, and would therefore be expected to fully release the encapsulated omega-3 polyunsaturated fatty acids. These plant proteins may therefore be suitable for the formation of delivery systems for bioactive lipids, although further research is needed to establish their stability under commercial food product conditions, and to determine their sensory attributes.
CHAPTER 6

IMPACT OF LEGUME PROTEIN TYPE AND LOCATION ON LIPID OXIDATION IN FISH OIL-IN-WATER EMULSIONS: LENTIL, PEA, AND FABA BEAN PROTEINS

6.1 Introduction

Lipid oxidation is an important factor causing loss of product quality and nutrients in foods [83, 390]. The primary products of lipid oxidation, such as lipid hydroperoxides, are odorless and tasteless, whereas the secondary products, such as hexanal, change the flavor of the product considerably [81, 90]. Moreover, potentially toxic reaction products, such as carcinogenic or inflammation-promoting substances, may be formed as a result of lipid oxidation in foods [82, 391, 392]. The oxidation rate of lipids increases with an increase in number of conjugated double bonds, because conjugation increases the ease of hydrogen abstraction and there are more sites available for attack. Due to their high level of unsaturation, omega-3 polyunsaturated fatty acids (PUFAs) are subject to rapid oxidation when exposed to air, light and high temperature [83, 390]. Consequently, food manufacturers must develop effective strategies to manage lipid oxidation in functional food and beverage products enriched with these bioactive lipids.

A variety of approaches have been developed to manage lipid oxidation, including addition of antioxidants, utilization of chelating agents, control of oxygen levels, engineering of interfacial levels, and control of storage conditions (such as light exposure, temperature, and water activity) [81]. Antioxidant addition is one of the most widely used approaches because of its effectiveness, versatility, and simplicity [393]. There are a number of highly effective synthetic antioxidants that can be used in foods, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and propyl gallate (PG), but their utilization is
declining because of consumer interest in more clean-label products [82]. Consequently, there is a great interest in the identification and evaluation of natural antioxidants, especially from plant sources, to inhibit lipid oxidation in foods [82, 394, 395].

A number of food proteins have been shown to be effective at inhibiting lipid oxidation in foods, and can therefore be used as natural antioxidants [90, 396]. Proteins are typically oxidized faster than unsaturated fatty acids, thereby delaying lipid oxidation and rancidity [90]. The preferential oxidation of proteins occurs if they are more susceptible to oxidation than the fatty acids in the system, or if they are physically located closer to the free radicals or reactive oxygen species (ROS), where they will scavenge them before they get closer to the lipids [90, 397]. Proteins inhibit lipid oxidation by scavenging free radicals, inactivating ROS, chelating pro-oxidative transition metals (such as iron or copper), reducing hydroperoxide formation, and by altering the interfacial properties of foods so as to physically separate reactive species [90]. Consequently, proteins can act as multifunctional antioxidants capable of inhibiting lipid oxidation through different mechanisms [90, 398].

Emulsified food products, such as beverages, creamers, desserts, dressings, and sauces, are an important category of functional foods that may be fortified with polyunsaturated fatty acids [81, 83, 399]. Transition metal-catalyzed decomposition of lipid hydroperoxides is the dominant oxidation pathway in emulsions [81, 400]. Lipid hydroperoxides are surface active molecules and therefore tend to migrate to the lipid droplet surfaces after they are formed, where they are then decomposed by a metal-catalyzed pathway. Proteins can influence lipid oxidation in emulsions through a number of mechanisms: (i) non-adsorbed proteins may bind metal ions and prevent them from reaching the lipid droplet surfaces; (ii) adsorbed proteins may bind metal ions and bring them into close proximity with the droplet surfaces; (iii) adsorbed proteins with a positive charge may electrostatically repel cationic metal ions; (iv) adsorbed proteins may form a physical barrier that sterically hinders the ability of metal ions
to interact with peroxides; and, (v) proteins have antioxidant side groups that can scavenge free radicals [89, 90, 399]. The relative importance of these different mechanisms will depend on the type, concentration, and location of the proteins present in an emulsion.

There is a growing interest by consumers in products containing plant-based natural ingredients, rather than those of animal origin (such as milk, egg, fish, or meat proteins), and so the food industry is looking for effective plant-based protein emulsifiers [401]. Legumes are gaining popularity for this purpose due to their high natural abundance, sustainability, low cost, and functional attributes [202]. As well as being effective emulsifiers, many legume proteins are also effective antioxidants [402-404]. Previous studies have shown that incorporation of chickpea or lentil proteins into flaxseed oil-in-water emulsions inhibits lipid oxidation of the powdered product during storage [196, 197].

The objective of the current study was to compare the efficacy of a number of legume-based proteins (pea, lentil, and faba bean) at forming and stabilizing oil-in-water emulsions, with particular emphasis on their ability to inhibit lipid oxidation during storage. The results obtained for the legume proteins were compared to those obtained for whey protein isolate, since this animal-based protein is widely used as an emulsifier in the food industry. We hypothesized that there would be appreciable differences in the ability of the legume proteins to act as antioxidants depending on their type and location within the system (adsorbed versus non-adsorbed). The results of this study would provide valuable information that could be used to form plant-based functional foods and beverages fortified with polyunsaturated fatty acids.
6.2 Materials and Methods

6.2.1 Materials

Pea, lentil, and faba bean protein concentrates (Vitessence Pulse 1550, 2550 and 3600, respectively) were donated by Ingredion, Inc. (Bridgewater, NJ). Whey protein isolate (BiPro JE 011-4-420) was donated by Davisco Foods International, Inc. (Le Sueur, MN). Fish oil was donated by DSM, Inc. (Columbia, MD). All other chemicals were purchased from the Sigma-Aldrich Co. (St. Louis, MO, USA). Double distilled water (DDW) was used for all experiments.

6.2.2 Protein Purification

The pulse protein concentrates provided by the ingredient suppliers only contained around 55 to 60% of protein by weight. Therefore, a protein isolation and purification procedure was carried out as described previously [405], with some slight modifications. Briefly, the pulse protein concentrates were dispersed in sodium phosphate buffer (10 mM, pH 7) for one hour using a magnetic stirrer followed by centrifugation (Sorvall Lynx 4000 Centrifuge, Thermo Scientific, Agawam, MA) for 30 minutes at 15,000 g at 10 °C. The supernatant was collected and centrifuged again using the same conditions to remove any starch, fiber, and insoluble compounds. The protein extract was then adjusted to pH 4.5 using hydrochloric acid (HCl) solution to precipitate the protein. The protein precipitate was recovered by centrifugation, washed with distilled water and dispersed in pH 7.0 sodium phosphate buffer by stirring at room temperature for an hour. The solution was then readjusted to pH 7.0 using sodium hydroxide (NaOH) solution and the system was stirred overnight at 5°C to ensure complete protein dispersion. The protein solution was brought to room temperature with continuous stirring for 30 minutes and then centrifuged to remove any insoluble protein. The protein content of the resulting supernatant were determined by the
Lowry method [323], using a standard curve prepared with bovine serum albumin (R=0.992). The protein solutions were diluted to 20 mg/mL protein content using buffer solutions prior to utilization.

6.2.3 Blocking of Protein Sulfhydryl Groups

The importance of sulfhydryl groups for the antioxidant activity of the proteins was determined by using N-ethylmaleimide (NEM), which is a chemical known to block sulfhydryl groups [406, 407]. Protein solutions (20 mg/mL) were stirred with NEM (3.45 mmol/g protein) in a water bath at 25°C for 15 minutes. The excess NEM was then removed by dialysis at 5°C for 24-hours with continuous stirring using a 3.5 kDa molecular cutoff dialysis tube (Spectra/Por 3, Spectrum Laboratories, Inc., Rancho Dominguez, CA). The protein solutions were then diluted by adding 100 parts of sodium phosphate buffer (pH7, 10 mM) to one part of protein solution. The buffer was replaced after 3, 6, and 12 hours. The protein content within the dialysis tube was calculated using the Lowry method as described in the previous section.

6.2.4 Iron Nitrilotriacetate-Protein-Binding Experiments

The iron binding capacity of the different proteins was determined using a method described previously [408], with some slight modifications. Briefly, 10 mL of protein solution (10 mg/mL) was dialyzed against 1 L of HEPES buffer (0.05 M in DDW) solution to which 4 mL of nitrilotriacetate (NTA) solution and 2 mL of iron chloride (FeCl₃) solution were added. The NTA solution was prepared by dissolving NTA in double distilled water (0.5 M). FeCl₃ solution was prepared by dissolving FeCl₃ in 0.05 M HCl (0.5 M). The dialysis was performed for 24 hours at 5°C with continuous stirring. The protein content within the dialysis tube was calculated using the Lowry method as described previously.
A protein precipitation solution was prepared by dissolving hydroxylamine hydrochloride (0.72 M) and trichloroacetic acid (0.61 M) in 1.2 N HCl. Then 2 mL of the protein solution from the dialysis tube was mixed with 1 mL of the protein precipitation solution in a test tube and incubated overnight at room temperature. The tube was centrifuged (Sorvall ST8 Centrifuge, Thermo Scientific, Agawam, MA) at 1750 g for 10 min at room temperature. 1 mL of supernatant was mixed with 2 mL of ammonium acetate buffer (10% w/v) and 0.5 mL of Ferrozine reagent (9 mM), prepared in DDW. The absorbance values were measured after 1 hour at a wavelength of 562 nm using a UV-visible spectrophotometer (Ultraspec 3000 pro, Biochrom Ltd., Cambridge, UK).

6.2.5 Emulsion formation

Coarse oil-in-water emulsions were prepared by blending 10% oil phase with 90% aqueous phase (w/w) using a high-shear mixer (M133/1281-0, Biospec Products Inc., Bartlesville, OK) for 2 minutes at 10,000 rpm. Fish oil was used as the oil phase and buffered protein solutions (20 mg/mL) were used as the aqueous phase. The coarse emulsions were passed through a dual channel high-pressure microfluidizer (PureNano, Microfluidics, Newton, MA) for 3 times at 10,000 psi to further breakdown the droplets. The interaction chamber (which consisted of consecutive X and Y channels) was soaked in ice water to avoid a rise in temperature during homogenization.

Tests were carried out on 4 different types of emulsions prepared from the initial ones: unwashed, washed with added buffer, washed with added protein solution, and washed with added NEM-treated protein solution. Emulsion washing was carried out using a method described previously [409]. Around 30 grams of emulsion was weighed into a centrifuge tube and centrifuged at 36,000 g at 10°C for 60 minutes. The supernatant was then replaced by the same amount of fresh buffer solution, and centrifugation was repeated twice more. After the
third time, supernatants were replaced by the same amount of fresh buffer solution, protein solution, or NEM-treated protein solution and were vortexed for 5 minutes. The supernatants from the first and third centrifugation step were collected for protein content analysis by the Lowry method after an additional centrifugation step.

6.2.6 Particle characterization

The surface potential (ζ-potential) of the protein-coated lipid droplets was measured using a particle electrophoresis instrument (Zetasizer Nano ZS Series, Malvern Instruments, Westborough, MA). The emulsions were diluted 100-times using sodium phosphate buffer (pH 7.0) prior to analysis to avoid multiple scattering effects. The particle size distribution and surface-weighted mean droplet diameter (d_{32}) of diluted emulsions were measured using a static light scattering (Mastersizer 2000, Malvern Instruments, Westborough, MA). The microstructure of the emulsions was measured using confocal microscopy (C1 Digital Eclipse, Nikon, Tokyo, Japan) with a 60× oil immersion objective. Nile red was added to the emulsions to highlight the lipid regions in the confocal images.

6.2.7 Lipid oxidation measurements

Emulsions were diluted 10-fold using sodium phosphate buffer (pH 7.0) containing sodium azide (0.03% w/v), and then stored in dark brown bottles (to avoid light exposure) at 37°C. For iron-accelerated studies, 100 μM iron sulfate was added to the samples.

6.2.7.1 Primary oxidation products

The peroxide value (PV) was measured using a method based on that described earlier [410]. Briefly, 0.3 mL of sample was mixed with 1.5 mL isooctane: 2-propanol (3:1 v/v) mixture in a test tube and then vortexed 3 times for 10 seconds each. Then the test tubes were centrifuged (Sorvall ST8 Centrifuge, Thermo Scientific, Agawam, MA) at 1000 g for 1 minute to ensure phase separation. 0.2 mL of the top layer was transferred into a new test tube
and 2.8 mL of methanol: 1-butanol mixture (2:1 v/v) was added. Iron sulfate solution (0.144 M) was prepared daily in DDW and 1 mL of this solution was mixed with 1 mL of barium chloride stock solution (0.132 M in 0.4 N HCl). The resulting cloudy mixture was then separated by centrifugation at 3000 g for 3 minutes. 1 mL of the supernatant was mixed with 1 mL of ammonium thiocyanate stock solution (3.94 M) prepared in double distilled water. 30 µL of this pink solution was added to the test tubes content and they were then incubated for 20 minutes at room temperature. The absorbance values were then measured at a wavelength of 510 nm using a UV-visible spectrophotometer (Ultraspec 3000 pro, Biochrom Ltd., Cambridge, UK). Hydroperoxides were calculated using a standard curve (R=0.995) prepared with different concentrations of cumene hydroperoxide (0-0.4 mM).

6.2.7.2 Secondary oxidation products

Thiobarbituric acid reactive substances (TBARS) analysis was followed according to a method described previously [411]. TBARS stock reagent was prepared by mixing 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, and HCl (0.25 M) in double distilled water and mixing this with 2% (w/v) BHT in ethanol at a 100: 3 (v/v) ratio. 1 mL of the emulsion samples were transferred into test tubes and 2 ml of the TBARS reagent were added. The mixture was incubated in a water bath at 90°C for 15 minutes, cooled in a water bath at room temperature for 10 minutes, and then centrifuged at 15,000 g for 15 minutes. The supernatants were pipetted into the cuvettes and absorbance values were measured at 532 nm using a UV-visible spectrophotometer (Ultraspec 3000 pro, Biochrom Ltd., Cambridge, UK). The concentrations were calculated using a standard curve (R=0.999) that was prepared using different concentrations of 1,1,3,3-tetraethoxypropane (0-20 µM).
6.2.8 Data analysis

The results were calculated using two measurements from each triplicate and are reported as the mean ± standard deviation. Statistical analysis was performed using a statistical software program (IBM SPSS Statistics 20). Significant differences were calculated using one-way analysis of variance (ANOVA) using the Tukey test with a significance level of 5% (p<0.05).

6.3 Results and Discussion

Particle size, particle charge, microstructure, and lipid oxidation were monitored throughout emulsion storage. Oil-in-water emulsions containing 10% fish oil were prepared using the three plant-based proteins (pea, lentil, faba bean proteins) and a widely used animal-based protein (whey protein). These emulsions were then diluted to 1% w/w oil content and stored at 37°C. The initial experiments were carried out using emulsions that were formed directly by homogenization (“unwashed emulsions”), while the remainder of the experiments was carried out using emulsions that had been washed so as to establish the impact of protein location (adsorbed versus non-adsorbed).

6.3.1 Properties of Unwashed Emulsions

6.3.1.1 Droplet Characteristics and Physical Stability

Preliminary experiments showed that all the unwashed emulsions were relatively stable to lipid oxidation when stored in the absence of added pro-oxidants (data not shown). For this reason, iron was added to the emulsions to accelerate the rate of lipid oxidation so that the experiments could be carried out over a reasonable timeframe.

There were no appreciable differences among the initial mean particle diameters of the unwashed emulsions stabilized by the different plant proteins, with the D[3,2] values being
around 376, 407, and 409 nm for faba bean, lentil, and pea proteins, respectively (Figure 6.1A). On the other hand, the mean particle diameter of the emulsions produced using the whey protein isolate was appreciably smaller (130 nm). This effect can be attributed to the fact that whey proteins have a smaller surface load than legume proteins (i.e., mass of protein adsorbed per unit surface area at saturation), and therefore produce smaller droplets when used at the same level [412]. There was little change (< 5%) in the mean particle diameter of the legume-protein stabilized emulsions after 21-days storage, and only a small increase (≈15%) for the whey protein-stabilized emulsions (Figure 6.1A). These results suggest that all of the unwashed emulsions were relatively stable to droplet aggregation under the experimental storage conditions used.

![Figure 6.1](image.png)

**Figure 6.1:** Change in (A) mean particle diameter and (B) droplet surface potential of emulsions stabilized by different proteins and held at 37°C with added iron. All emulsions were diluted to 1 wt% total oil phase. Different lower case letters represent significant differences (p < 0.05) between the different samples at the same day. Different upper case letters represent significant differences (p < 0.05) between same samples at different days.

All of the protein-coated lipid droplets were negatively charged, but the magnitude of the initial surface potential (ζ-potential) depended on protein type: whey > lentil > faba bean > pea (Figure 6.1B). The electrical characteristics of emulsions droplets are important because they determine the strength of the electrostatic repulsion between them [20], as well as the
interaction of the droplets with other charge species in the system, such as cationic transition metals [413]. There was a significant increase in the magnitude of the negative charge on the protein-coated lipid droplets in all of the emulsions after 21-days storage (p>0.05) (Figure 6.1B). This change suggests that there was some alteration in the interfacial composition during storage. The observed increase in the negative charge of the droplets after storage may have been due to the accumulation of anionic reaction products at the droplet surfaces. Lipid oxidation may lead to the formation of surface-active organic acids [414, 415], whereas protein oxidation may alter the electrical characteristics of amino acids [90, 416].

6.3.1.2 Chemical Stability

In emulsion-based systems, lipid oxidation typically occurs at the surface of the droplets where the unsaturated fatty acids and hydroperoxides from the oil phase can interact with transition metals from the aqueous phase [399, 417]. Consequently, we anticipated that the rate of lipid oxidation would depend on the nature of the emulsifier at the droplet surfaces.

In a preliminary experiment, it was found that all of the unwashed emulsions were relatively stable to lipid oxidation when stored at 37°C for 33 days (data not shown). For this reason, an accelerated storage study was carried out by adding a pro-oxidant (100 μM iron sulfate) to the emulsions at the beginning of the experiment to accelerate the oxidation rate. The formation of primary (hydroperoxides) and secondary (TBARS) reaction products was then measured throughout storage (Figure 6.2). There was a steady increase in the level of hydroperoxides generated during the first 15 days, and then a slight decrease at longer storage times (Figure 6.2A). This effect can be attributed to the fact that the breakdown of primary reaction products was faster than their formation at longer storage times. There was a steady rise in the level of TBARS throughout the incubation period (Figure 6.2B). Interestingly, no lag-period was observed for any of the emulsions, which suggests that the transition metals
rapidly adsorbed to the lipid droplet surfaces and promoted oxidation. All of the emulsions appeared to oxidize at a fairly similar rate, e.g., the TBARS values increased by about 4.6 to 5.7 µM per day for all of the samples.

Figure 6.2: (A) Hydroperoxide and (B) TBARS values for emulsions stabilized by different proteins and held at 37°C with added iron for 21 days. All emulsions were diluted to 1 wt% total oil phase.

6.3.2 Properties of Washed Emulsions

In this series of experiments, the original emulsions were centrifuged and washed to remove any non-adsorbed proteins. In some experiments, additional protein (with or without NEM treatment) was added back to the aqueous phase of the emulsions so that the final protein level was the same as in the original emulsions. The physical and chemical stability of the washed emulsions was then measured and compared to that of the unwashed emulsions. For these experiments, no additional iron was added to the emulsions as a pro-oxidant, since the rate of lipid oxidation in the washed emulsions was already relatively fast. For the sake of concision, the full results are only shown for the emulsions stabilized by the lentil proteins, but the other types of plant protein behaved qualitatively similarly. For this reason, only the TBARS data are used to compare the impact of protein type on oxidation.
6.3.2.1 Droplet Characteristics and Physical Stability

The unwashed emulsions initially contained relatively small droplets (Figure 6.3A) and had a monomodal particle size distribution (Figure 6.3B). Interestingly, there was evidence of a population of relatively large particles in all of the washed emulsions, as seen in the particle size distribution (Figure 6.3B) and microstructure (Figure 6.4) measurements. There are a number of possible reasons for this phenomenon. Firstly, the centrifugation process used to wash the emulsions may have promoted droplet aggregation as the lipid droplets were forced together by the centrifugal forces [418]. Secondly, the decrease in protein concentration at the droplet surfaces due to washing may have increased their surface hydrophobicity, thereby promoting aggregation due to the increase in hydrophobic attraction between the droplets [419]. Despite the evidence of large aggregates in the emulsions, the majority of the droplets were still relatively small, which led to a relatively low mean droplet diameter, \( d_{3,2} \) = 340 to 413 nm (Figure 6.3A). Nevertheless, the confocal microscopy images indicated that there were some large individual droplets present (Figure 6.4), which indicated that some droplet coalescence had occurred in the emulsions during the washing process. After storage for 33 days, there was a noticeable increase in the mean particle diameter of most of the samples, with the exception of the washed emulsion containing added protein. This suggested that a limited amount of further droplet aggregation occurred during storage. It should be noted that the particle size distribution of the emulsions stabilized with whey protein isolate did not change after centrifugation and washing (data not shown), which is in agreement with previous studies [409]. It therefore seems that the whey protein-coated droplets are more stable to centrifugation/washing than the legume-coated ones.
All of the unwashed and washed lentil-stabilized emulsions initially had a moderately strong negative charge (-24 to -25 mV), which can be attributed to the fact that the pH was appreciably above the isoelectric point of the proteins. Moreover, the fact that the charge was fairly similar in all of the systems suggests that the interfacial composition was fairly similar.

There was a pronounced increase in the magnitude of the negative charge in all of the
emulsions after 33-days storage, which may be indicative of changes in the chemistry of the interfacial lipid or protein molecules resulting from oxidation, as discussed earlier.

**Figure 6.4:** Microstructure of unwashed and washed emulsions stabilized by lentil proteins at day 0 and 33 of storage determined using confocal fluorescence microscopy.
6.3.2.2 Chemical Stability

There were distinct differences in the oxidative stability of the lentil-stabilized fish oil-in-water emulsions depending on whether they were washed or unwashed, and on the level of protein they contained in the aqueous phase. Both the primary (Figure 6.5) and secondary (Figure 6.6A) reaction products demonstrated similar trends. The fastest rate of lipid oxidation occurred in the washed emulsions containing no additional protein in the aqueous phase (“Washed+buffer”). The rate of lipid oxidation was considerably less for the unwashed emulsion, which can be attributed to the presence of free protein in the aqueous phase. The lowest rate of lipid oxidation was observed in the washed emulsions to which additional protein was added after homogenization.

Figure 6.5: Hydroperoxide values for unwashed and washed emulsions stabilized by lentil proteins and held at 37°C for 33 days. All emulsions were diluted to 1 wt% total oil phase.
Figure 6.6: TBARS values for unwashed and washed emulsions stabilized by (A) lentil (B) pea (C) faba bean (D) whey proteins and held at 37°C for 33 days. All emulsions were diluted to 1 wt% total oil phase.

The same general trends were observed for the other types of proteins studied (faba bean, pea, and whey proteins), as seen in the TBARS results (Figures 6.6B – 6.6D). For all protein types, the rate and extent of lipid oxidation was appreciably higher in the washed emulsions containing buffer, than in the unwashed emulsions or the washed emulsions containing added protein. Having said this, the progress of lipid oxidation also appeared to
depend on protein type. A “lag-period” was defined as the time at which the TBARS levels first increased steeply. After this time, the emulsions would be perceived as being unacceptable to consumers due to the formation of appreciable levels of volatile secondary reaction products that would make the product rancid. The susceptibility of the washed emulsions to lipid oxidation increased in the following order as determined by their lag-periods: pea and faba bean proteins (14 days) < whey and lentil proteins (5 days). As mentioned earlier, the lag-period for the unwashed emulsions was much greater than for the washed emulsions, and was difficult to determine for some of the systems due to the relatively low levels of TBARS produced. Nevertheless, it is clear that the rate of TBARS formation was considerably higher for the emulsions stabilized by lentil proteins than for those stabilized by the other proteins.

In the following section, we examine a number of factors that may account for the observed differences in the oxidative stability of different samples.

6.3.3 Potential factors affecting oxidation rates

6.3.3.1 Droplet size effects

The different types of proteins produced emulsions containing lipid droplets with different particle sizes (Figure 6.1A). Moreover, the centrifugation and washing procedure also led to differences in the particle size (Figure 6.3A). In principle, the rate of lipid oxidation should increase with decreasing droplet size because there would be a greater surface area of lipid exposed to the aqueous phase [420-422]. However, the whey protein-stabilized emulsions contained the smallest droplet sizes (Figure 6.1A), but they oxidized slightly slower than the legume-protein stabilized emulsions (Figure 6.2). In addition, there did not appear to be a correlation between the oxidative stability (Figure 6.5) and the particle
size (Figure 3a) of the washed and unwashed emulsions. Thus, it seems that particle size was not a major factor affecting the rate of lipid oxidation in the emulsions used in this study.

**6.3.3.2 Droplet charge effects**

It has been reported that the rate of lipid oxidation in oil-in-water emulsions depends on the electrical potential of the lipid droplets [413]. Positively charged interfacial layers electrostatically repel cationic metal ions (such as Fe$^{2+}$ or Fe$^{3+}$), thereby preventing these potent pro-oxidants from coming into close contact with the lipids close to the droplet surfaces. Conversely, negatively charged interfacial layers electrostatically attract cationic metal ions, and may therefore bring them into close proximity to the lipids. In this study, all of the emulsion droplets were negatively charged, and therefore we would have expected that cationic metal ions would be adsorbed to the droplet surfaces. However, there was no correlation between the magnitude of the negative charge and the rate of lipid oxidation. For instance, the whey protein-coated lipid droplets had the highest negative charge (Figure 6.1A), but they oxidized slightly more slowly than the legume-protein coated ones (Figure 6.2). Moreover, the washed and unwashed emulsions had very similar initial surface potentials (Figure 6.3C), but the rates of lipid oxidation were very different (Figure 6.5). These results suggest that the surface potential of the lipid droplets is not a good indication of their oxidative stability.

**6.3.3.3 Iron binding effects**

Another possible explanation for the observed differences in the lipid oxidation rates of the emulsions is due to differences in the ability of the proteins to bind iron. As mentioned earlier, iron ions are highly potent pro-oxidants that can accelerate lipid oxidation [81]. Consequently, if a protein can bind iron ions strongly it may be able to either promote or inhibit lipid oxidation depending on its location (adsorbed or non-adsorbed). Therefore, we
compared the iron-binding capacities of the various proteins used. An iron nitriloacetate-protein binding assay was used to determine the iron binding capacities of the different proteins [408]. The iron binding capacities of lentil, pea, faba bean, and whey proteins were 278.4±4.5, 273.9±3.3, 270.0±6.0, and 236.7±6.6 μmoles of bound iron per % protein, respectively. The iron binding capacity of whey protein determined in this study was in good agreement with that reported in an earlier study [409], while the authors could not find any reported iron binding capacities for pea, lentil and faba bean proteins. There were no significant differences among the plant proteins’ iron binding capacities (p>0.05). However, whey protein’s iron binding capacity was significantly less (12.5-15% less) than the plant proteins (p<0.05). These results suggest that all of the proteins were able to bind iron.

6.3.3.4 Sulfhydryl group effects

Another factor that may account for differences in the antioxidant properties of proteins is the number of free sulfhydryl groups they contain [407, 423]. However, there are contradictory results on the effect of free sulfhydryl groups on lipid oxidation. Some studies suggest that the existence of free sulfhydryl groups retards lipid oxidation [407, 409, 424], whereas others suggest they have little effect [91, 425]. In the current study, we therefore used NEM-treatment of the proteins to provide some insight into the potential role of free sulfhydryl groups, since NEM blocks free sulfhydryl groups [406]. Either protein or NEM-treated protein was added to washed emulsions to study the effect of free sulfhydryl groups on the oxidation rate.

The rate of lipid oxidation in the emulsions to which NEM-treated protein was added was fairly similar to that observed in the emulsions to which untreated protein was added (Figure 6.5). This suggests that free sulfhydryl groups did not make a major contribution to the antioxidant mechanism of the proteins.
6.3.3.5 Protein location effects

Finally, we examined the impact of the location of the proteins in the emulsions (adsorbed versus non-adsorbed) on their stability to lipid oxidation. Our results clearly show that the location of the proteins was the most important factor impacting lipid oxidation (Figures 6.5 and 6.6). In all of the systems studied, the lipid oxidation rate was faster for the washed emulsions than for the unwashed emulsions. Moreover, the addition of protein back into the aqueous phase of the washed emulsions improved their oxidative stability. These results indicate that the non-adsorbed protein acts as an effective antioxidant in the emulsions.

The most likely physicochemical origin of this effect is the ability of the proteins to change the location of the pro-oxidant metal ions in the system. Anionic proteins may bind cationic iron ions through electrostatic interactions, or through other types of interaction, as highlighted by the iron binding results discussed earlier. When the proteins are adsorbed to the droplet surfaces they will bring the iron ions into close proximity to the lipid substrate and thereby promote oxidation, but when the proteins are dispersed in the aqueous phase they will pull the iron ions away from the droplet surfaces and thereby retard oxidation [426]. A number of other studies have highlighted the importance of other types of non-adsorbed proteins at inhibiting lipid oxidation in emulsions, e.g., α-lactalbumin [427], caseinate [428], soy protein isolate [409]. This effect seems to be a fairly generic one, and should be taken into account when formulating emulsion-based delivery systems for unstable lipids.

Information about the protein concentrations in the aqueous phase of the unwashed and washed emulsions is shown in Table 1. As expected, the washed emulsions had much lower non-adsorbed protein levels than the unwashed emulsions (a >95% reduction). There were no significance differences among the protein levels in the aqueous phase of the emulsions stabilized by plant proteins (p>0.05), but the amount of whey protein in the aqueous phase was significantly higher than for the plant proteins (p<0.05). Interestingly, the
washed emulsions formed using lentil proteins had the lowest non-adsorbed protein concentration, and were the most unstable to lipid oxidation (Figure 6.6). This again highlights the potential importance of non-adsorbed protein in the aqueous phase at inhibiting lipid oxidation.

Table 6.1: Protein concentration in the continuous phase of oil-in-water emulsions after 1st (unwashed) and 3rd (washed) centrifugation stages (mg/ mL)

<table>
<thead>
<tr>
<th>Protein type</th>
<th>Unwashed</th>
<th>Washed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lentil</td>
<td>11.91±0.86</td>
<td>0.12±0.03</td>
</tr>
<tr>
<td>Pea</td>
<td>11.94±0.49</td>
<td>0.47±0.06</td>
</tr>
<tr>
<td>Faba bean</td>
<td>11.13±0.70</td>
<td>0.25±0.04</td>
</tr>
<tr>
<td>Whey</td>
<td>13.83±0.35</td>
<td>0.77±0.04</td>
</tr>
</tbody>
</table>

6.4 Conclusions

The physical and chemical stability of fish oil-in-water emulsions produce using pea, faba bean or lentil proteins as emulsifiers were compared to those produced using whey proteins. Emulsions produced using whey protein had appreciably smaller droplet diameters and slightly better stability to lipid oxidation than those produced using the legume proteins. In all systems studied, the presence of non-adsorbed proteins in the aqueous phase appeared to be the most important factor affecting the rate of lipid oxidation. The removal of non-adsorbed proteins from the emulsions by washing led to faster lipid oxidation, which was attributed to binding of transition metals to the adsorbed proteins, thereby bringing them into close proximity to the emulsified lipids. Overall, this study shows that oil-in-water emulsions can be produced using legume proteins as emulsifiers that have an oxidative stability fairly similar to emulsions produced using a commonly utilized animal protein (whey protein isolate). The main advantage of using the legume proteins would be the positive consumer perception, lower cost, and better sustainability. However, the sensory aspects of commercial food and beverage products created using legume proteins still need to be investigated.
CHAPTER 7

CONCLUSIONS

There is a demand from consumers for “all-natural” foods and beverages, which has driven researchers in the food industry to identify natural alternatives to synthetic ingredients utilized in foods. This thesis has focused on the identification and characterization of some natural emulsifiers that can be capable of forming oil-in-water emulsions containing relatively small droplets that are stable over a range of environmental conditions, and may therefore be suitable for utilization within commercial food products. Nevertheless, there are still challenges to overcome for many natural emulsifiers. Proteins are capable of forming small droplets at low usage levels, but the droplets formed are often highly susceptible to aggregation at certain pH values, high ionic strengths, or after thermal processing. This study has shown that it is possible to encapsulate hydrophobic nutraceuticals in emulsion-based delivery systems fabricated from all-natural or plant-based ingredients. These emulsions can be used to create natural colorants or to fortify functional foods at a level that may be beneficial to human health. Overall, these studies show that oil-in-water emulsions can be produced using legume proteins or Maillard conjugates of a milk protein as emulsifiers. The definition of a ʻnatural ingredientʻ is not well established and since Maillard conjugates include a reaction before their use in the food systems, it might be open to discussion for its ʻnaturalʻ or ʻminimally processedʻ situation. The main advantage of using the legume proteins would be the positive consumer perception, lower cost, and better sustainability. However, the sensory aspects of commercial food and beverage products created using legume proteins still need to be investigated. In summary, the results generated through this study may provide practical strategies for the food industry to formulate clean-label fortified foods and beverages.
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