DELAYING LIPID OXIDATION IN EMULSIONS BY LOCALIZING LENTIL PROTEIN-POLYPHENOL CONJUGATES AT THE OIL-WATER INTERFACE

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ABSTRACT

Polyphenols are naturally derived antioxidants. In the present study, the ability of polyphenols to prevent lipid oxidation was used in synergism with the surface activity of lentil proteins to fabricate flaxseed oil-in-water emulsions. The conjugation of lentil protein isolate with polyphenols (quercetin, rutin and ellagic acid) was achieved by a simple grafting technique involving high alkaline pH of 9.0. The resulting lentil proteins-polyphenol conjugates were characterized by their structural and functional properties. The extent of covalent binding in lentil protein was 21.0%, 11.7% and 4.4% for quercetin, ellagic acid and rutin molecules, respectively. Results from Fourier transform infrared (FTIR) spectroscopy revealed an increase in random coils and a subsequent decrease in the β-sheet for all conjugates. Surface hydrophobicity of the conjugates was lower than control lentil proteins suggesting possible involvement of protein hydrophobic groups towards covalent bonding with polyphenols. The antioxidant activity of lentil proteins-polyphenol conjugates was 0.30 to 1.75-fold higher for reducing power assay and 0.05 to 0.08-fold higher for free radical scavenging than the control lentil proteins. Further, the prepared conjugates were also successful in lowering the interfacial tension of oil-water interface and therefore, were employed for stabilizing oil-in-water emulsions.

Lentil protein isolate-quercetin and lentil protein isolate-ellagic acid conjugates were selected for emulsifying flaxseed oil and to retard its oxidation in a location-dependent manner. The emulsions stabilized using the conjugates showed superior oxidative stability compared to those stabilized using the original lentil protein isolate or control lentil proteins with an equivalent amount of quercetin or ellagic acid in their respective aqueous phases, thereby demonstrating the advantage of placing the polyphenols at the oil droplet surface. However, emulsions prepared using conjugates showed limited stability towards changes in environmental factors such as pH, ionic strength, and heat. Results from accelerated gravitational separation analysis showed emulsions made using conjugates had similar instability indices as those made using lentil proteins only. The distribution of the conjugates at the surface of the oil droplets was observed using confocal laser scanning microscopy. Overall, the results from this study demonstrated the advantage of localizing antioxidants at the oil-water interface of an emulsion in better protecting the lipid core against oxidation.

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I would like to thank my loving parents, and my brother who have supported and encouraged me throughout my life. Finally, respect and special thanks to my grandma for her love and support in all the years she has been with me and without.

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LIST OF ABBREVIATIONS AND SYMBOLS

ArOH	Phenol
ArȮ	Phenoxyl radical
BDE	Bond dissociation enthalpy
BHA	Butylated hydroxyanisole
BSA	Bovine serum albumin
CITREM	Citric acid ester of monoglyceride
ED-group	Electron donating group
EW-group	Electron withdrawing group
d_{32}	Volume surface mean droplet diameter
d_{43}	Surface average droplet diameter
E	Ellagic acid
g	Acceleration due to gravity
ΔH_{HB}	Hydrogen bond enthalpy
L̇	Alkyl radical
LH	Fatty acid
LȮ	Alkoxyl radical
LOȮ	Peroxyl radical
LOOH	Lipid hydroperoxide
LbL	Layer-by-layer
LPI	Lentil protein isolate
LPI-D	Lentil protein isolate dialyzed
LPI-E	Lentil protein isolate and ellagic acid conjugate
LPI-R	Lentil protein isolate and rutin conjugate
LPI-UD	Lentil protein isolate un-dialyzed
LPI-Q	Lentil protein isolate and quercetin conjugate
MDA	Malondialdehyde
–OMe	Methoxy group
O/W	Oil-in-water emulsion
O/W/O	Oil-in-water-in-oil emulsion

ΔP	Laplace pressure
p-AV	*para*-Anisidine value
pI	Isoelectric point
PV	Peroxide value
Q	Quercetin
r	Droplet radius
R	Rutin
TAG	Triacylglycerol
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBHQ	tert-Butylhydroquinone
W/O	Water-in-oil emulsions
W/O/W	Water-in-oil-in-water emulsion
γ	Interfacial tension
η	Viscosity (Pa.s)
ρ	Density

1. OVERVIEW

1.1. Summary

Emulsions constitute a predominant part of the food products we consume. A majority of the foods are either wholly or partly emulsions or go through an emulsified stage sometime during their processing (Dickinson & Stainsby, 1982; Friberg et al., 2003; McClements, 2004b). For example, milk is a natural oil-in-water emulsion whereas mayonnaise is manufactured as an oil-in-water emulsion. Ice cream undergoes from being a simple oil-in-water emulsion to a complex one with the incorporation of air. This diverse physicochemical and sensory characteristic exhibited by an emulsion system can be attributed to the ingredients and processing conditions used and exploited by many food manufacturers (McClements, 2004b).

An emulsion can be defined as a mixture of two immiscible liquids (usually oil and water), with one phase (called the dispersed phase) suspended in the other (called the continuous phase) as small droplets (McClements, 2004b). In an oil-in-water emulsion, the oil droplets are dispersed in the continuous aqueous phase as in milk, mayonnaise, salad dressings, and soups. Some examples of water-in-oil emulsions include margarine and butter. The possibility of having both the oil and aqueous phases in the same system poses a thermodynamic limitation in which the droplets tend to coalesce upon collision leading to complete phase separation. For this reason, emulsifiers are significant because of their amphiphilic behavior.

The health benefit of a diet rich in polyunsaturated fats is well established (Lomova et al., 2010). But the high susceptibility of polyunsaturated fatty acids (PUFAs) to oxidation and demand for fewer additives makes it imperative that an alternate approach to stabilizing PUFA is made. Numerous researches have suggested that lipid oxidation in an oil-in-water emulsion can be better controlled by localizing the antioxidants at the interface of the dispersed droplets (Berton-Carabin et al., 2014; McClements & Decker, 2000). To improve and add to these findings, the present research will explore the possibility of improving the effectiveness of natural polyphenol antioxidants by spatially locating them at the oil-water interface by conjugating with a plant protein acting as an emulsifier rather than having them dispersed in the bulk of the continuous phase.

1.2. Objectives

To reach the overall goal of the proposed research which is to achieve an emulsion stable towards lipid oxidation, the following objectives are summarized:

1. To synthesize protein-polyphenol complexes by selectively conjugating lentil protein isolate with polyphenols (antioxidants) using a simple grafting technique.
2. Develop emulsions with the protein-polyphenol complex as an emulsifier and investigate its stability.
3. To compare the efficiency of a protein-polyphenol conjugate stabilized emulsion in preventing lipid oxidation compared to an emulsion with an equal amount of polyphenol dispersed in its bulk aqueous phase.

1.3. Hypotheses

To address the overall goal of this thesis, the following hypotheses will be tested:

1. Simple grafting at an alkaline pH is a well-known method used to improve the functionality of natural and synthetic polymers. Complexation of proteins with polyphenols to form conjugates will have synergistic effects on the antioxidative and emulsifying properties of the substrates and on inhibiting lipid oxidation in oil-in-water emulsions.

2. The creation of a large interfacial area between the aqueous and lipid phases is assumed to promote the contact between the unsaturated fat and dissolved pro-oxidants in its surroundings. Therefore, polyphenols located at the interface will provide better oxidative stability to an emulsion than direct addition to the bulk phases of the emulsion.

2.1. Lipid oxidation in food systems

Edible oil and fat-containing foods may develop undesirable off flavors and odors and become 'rancid' over long-term storage. This volatile rancid flavor is due to the generation of low molecular weight and potentially toxic saturated, and unsaturated carbonyls and organic acids through a myriad of lipid degradation reactions (Frankel, 1998a; Huang et al., 1996). Rancidity affects the nutritional, organoleptic and potential safety of the food product. It is one of the principal causes of food spoilage and is a challenge for food manufacturers. Oxidative degradation of lipid in foods is known to follow "auto-oxidation", where molecular oxygen reacts with lipids through a self-catalytic mechanism initiated by free radicals and promoted by certain metal ions. A thorough knowledge of the mechanism is important to better prevent lipid oxidation and deliver shelf-stable food products to consumers.

2.1.1. Mechanism of lipid oxidation

Lipid oxidation occurs in a cascade of steps, often cited as initiation, propagation and termination:

2.1.1.1. Initiation

In the initiation step, a fatty acid radical, also called alkyl radical ($\dot{L}$) is formed with the abstraction of a hydrogen atom at positions α to the fatty acid (*LH*) double bond (Coupland & McClements, 1996). The reaction can also begin with the decomposition of lipid hydroperoxides (*LOOH*) into highly reactive peroxyl ($L\dot{O}O$) and alkoxyl ($L\dot{O}$) radicals in the presence of an initiator. The reaction is catalyzed by a range of means important to a food system including exposure to light, transition metal ions, and certain enzymes and leads to the generation of lipid free radicals (Coupland & McClements, 1996).

$$LH + Initiator \xrightarrow{catalyst} free\ radicals\ \left(\dot{L}, L\dot{O}, LO\dot{O}\right) \quad (2.1)$$

2.1.1.2. Propagation

In the first step of propagation, the lipid radical ($\dot{L}$) rapidly reacts with oxygen to form a peroxyl radical $(L\dot{O}O)$ which has higher energy than an alkyl radical. The peroxyl radical therefore, abstracts hydrogen from the methylene group of another lipid molecule (LH) to yield hydroperoxides (*LOOH*) and a new free radical ($\dot{L}$) is generated. This propagates a complex chain of reactions.

$$\dot{L} + O_2 \rightarrow L\dot{O}O \tag{2.2}$$

$$L\dot{O}O + LH \rightarrow LOOH + \dot{L} \tag{2.3}$$

2.1.1.3. Termination

A high concentration of free radicals in the food system allows for a greater probability of radical-radical collision to form a non-radical species, and this terminates the oxidation sequence.

$$\dot{L} + \dot{L} \rightarrow L_2 \tag{2.4}$$

$$\dot{L} + L\dot{O}O \rightarrow (LO)_2 \tag{2.5}$$

$$L\dot{O}O + L\dot{O}O \rightarrow LOOL + O_2 \tag{2.6}$$

This step is not as important as initiation or propagation since the food is already rancid before significant termination reactions take place (Chaiyasit et al., 2007).

2.1.2. Lipid oxidation decomposition products

The food becomes rancid when the unsaturated fatty acids decompose into volatile compounds. These oxidation products are produced from the decomposition of fatty acid hydroperoxides (Frankel, 1998b). The homolytic cleavage of hydroperoxides yields an alkoxyl $(\dot{L}O)$ and a hydroxyl radical $(\dot{O}H)$ and is the most likely hydroperoxide decomposition pathway (Min & Boff, 2002). The alkoxyl radical being more energetic than alkyl ($\dot{L}$) or peroxyl radical $(L\dot{O}O)$ then enters into a number of other pathways (Figure 2.1).

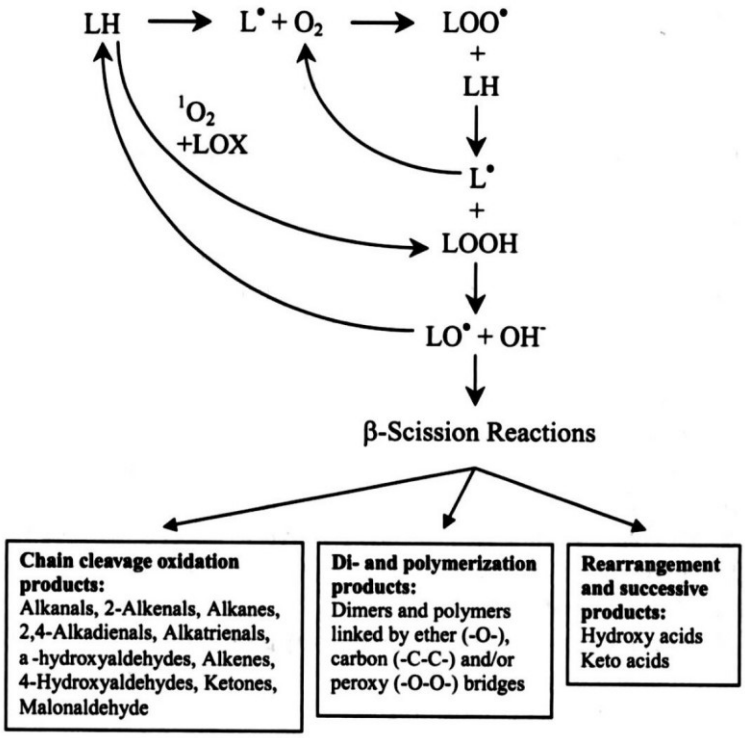


Figure 2.1 Schematic of lipid oxidation (adopted without modification from Chaiyasit et al. (2007). Used with permission from Taylor & Francis Informa Ltd., United Kingdom of Great Britain and Northern Ireland.

The alkoxyl radical may attack another unsaturated fatty acid, a pentadiene group within the same fatty acid or the covalent bonds adjacent to the alkoxyl radical (Chaiyasit et al., 2007). When the alkoxyl radical attacks the covalent bonds adjacent to it, a new array of reactions begin, called as β-scission reactions, which are critical from the food quality perspective as it leads to the generation of a wide variety of different molecules, including aldehydes, ketones, alcohols, and hydrocarbons, responsible for the characteristic physicochemical and sensory properties of oxidized lipids (Frankel, 1985).

2.1.3. Measurement of lipid oxidation

It is challenging to evaluate lipid oxidation in a system due to a number of reasons such as the variety of compounds formed as a function of time, extent of oxidation and mechanism(s) involved (Barriuso et al., 2013). Furthermore, it is not only the lipid as a substrate which influences

oxidation, but also the type and concentration of proteins, antioxidants and prooxidants present in the food system as well as its physicochemical parameters that adds to the complexity (Eymard et al., 2009). However, there are a number of analytical techniques available for measuring primary and secondary lipid oxidation products in food systems as described below:

2.1.3.1. Primary oxidation products

Lipid hydroperoxides are the primary products of lipid oxidation. The redox properties of hydroperoxides form the key basis in their quantification. Many simple inorganic ions, such as iodide and ferrous ions are oxidized in the presence of hydroperoxides. These methods however, requires the formation of coordination complexes to build on the sensitivity of the substrate under consideration.

Volumetric method

Iodometry is the most widespread and conventional method used for the analysis of hydroperoxides because of its experimental simplicity. It involves the reaction of hydroperoxides and other peroxides with the iodide ion to generate iodine under acidic conditions. The iodine is then titrated against a sodium thiosulfate solution in the presence of starch. Peroxide value is considered to represent the quantity of active oxygen (in meq) contained in 1 kg of lipid that could oxidize potassium iodide in the reaction mixture (Barriuso et al., 2013).

Ferrous oxidation method

The ferrous oxidation spectrophotometric method is used for the determination of peroxide content in a sample. It consists of Fe(II) to Fe(III) oxidation, mediated by hydroperoxide reduction in the presence of either thiocyanate or xylenol orange in acidic conditions (Barriuso et al., 2013). These two compounds provide the spectrophotometric properties by forming complexes with ferric ion, giving maximum absorbance peaks at 500 and 560 nm, respectively, which are measured using a spectrophotometer in the visible range.

2.1.3.2. Secondary oxidation products

Lipid primary oxidation products yield secondary oxidation products if further exposed to oxidation conditions. These secondary oxidation products include aldehydes, ketones, epoxides, hydroxy compounds, oligomers and polymers (Barriuso et al., 2013). These compounds differ mostly in their voaltility, polarity and molecular weight.

Measurement of malondialdehyde

One of the most abundant aldehydes generated during secondary lipid oxidation and therefore, a commonly used secondary oxidation marker is malondialdehyde (MDA). Spectrophotometric determination of the red fluorescent MDA-thiobarbituric acid (MDA-TBA) complex is the most employed method for MDA determination. The chromophore complex has its absorbance maxima at 532 nm. The reaction depends on the concentration of TBA solution used, the temperature conditions and the pH (Fernández et al., 1997).

A number of other aldehydes are generated apart from MDA during lipid secondary oxidation. Another spectroscopic method uses *p*-anisidine to provide useful information on the formation of carbonyl compounds, especially non-volatile α-unsaturated aldehydes (such as 2-alkenals and 2,4-dienals). The reaction is based on the reactivity of the aldehyde carbonyl bond on the *p*-anisidine amine group, forming a Schiff's base with an absorption maxima at 350 nm. The *p*-anisidine value is defined as 100 times the absorbance of a solution containing 1 g of fat in 100 mL of solvent (Barriuso et al., 2013).

2.2. Antioxidants

Antioxidants are compounds which delay or prevent the oxidation of auto-oxidizable substrates. These are added in low concentrations to food formulations rich in unsaturated lipids which are prone to oxidation (Halliwell et al., 1995). Hundreds of compounds have been identified which exhibit antioxidant properties. These may be synthetic, for example, tert-butylhydroquinone (TBHQ) and butylated hydroxyanisole (BHA) or extracted from natural sources, such as catechin and epicatechin from tea leaves and cacao beans (Prior et al., 2001).

2.2.1. Mechanism of action

According to Howard and Ingold (1968), antioxidants are classified into two broad groups based on their mechanism of action such as primary and secondary antioxidants. Primary antioxidants or chain-breaking antioxidants (*ArOH*) are molecules which act as hydrogen donors or free radical acceptors and help to form stable products (*LOOH*) on reaction with lipid free radicals ($LO\dot{O}$) (Nawar, 1985).

$$LO\dot{O} + ArOH \rightarrow LOOH + Ar\dot{O} \tag{2.7}$$

However, *ArOH* acting as an antioxidant here closely competes with the *LH* of the chain propagating step (Equation 2.3) in lipid peroxidation for the lipid peroxyl radicals. The efficiency of a primary antioxidant molecule, therefore, depends on which of the above two reactions occur first and faster. This in turn is a function of bond dissociation enthalpy (BDE) of $ArOH$ where the effectiveness of an antioxidant increases with its decreasing bond strength and, decreasing steric crowding around the –OH group. For instance, the presence of an electron donating group (methoxy group, –OMe) lowers the BDE of O-H by stabilizing the phenoxyl radical $Ar\dot{O}$ produced after H-atom removal (Figure 2.2) (Amorati et al., 2003). However, an electron donating group at the ortho position on a phenolic ring could participate in intramolecular H-bonding and stabilize the parent phenol rather than contributing to lowering the BDE of O-H with respect to the parent phenol (Figure 2.2) (Amorati et al., 2012).

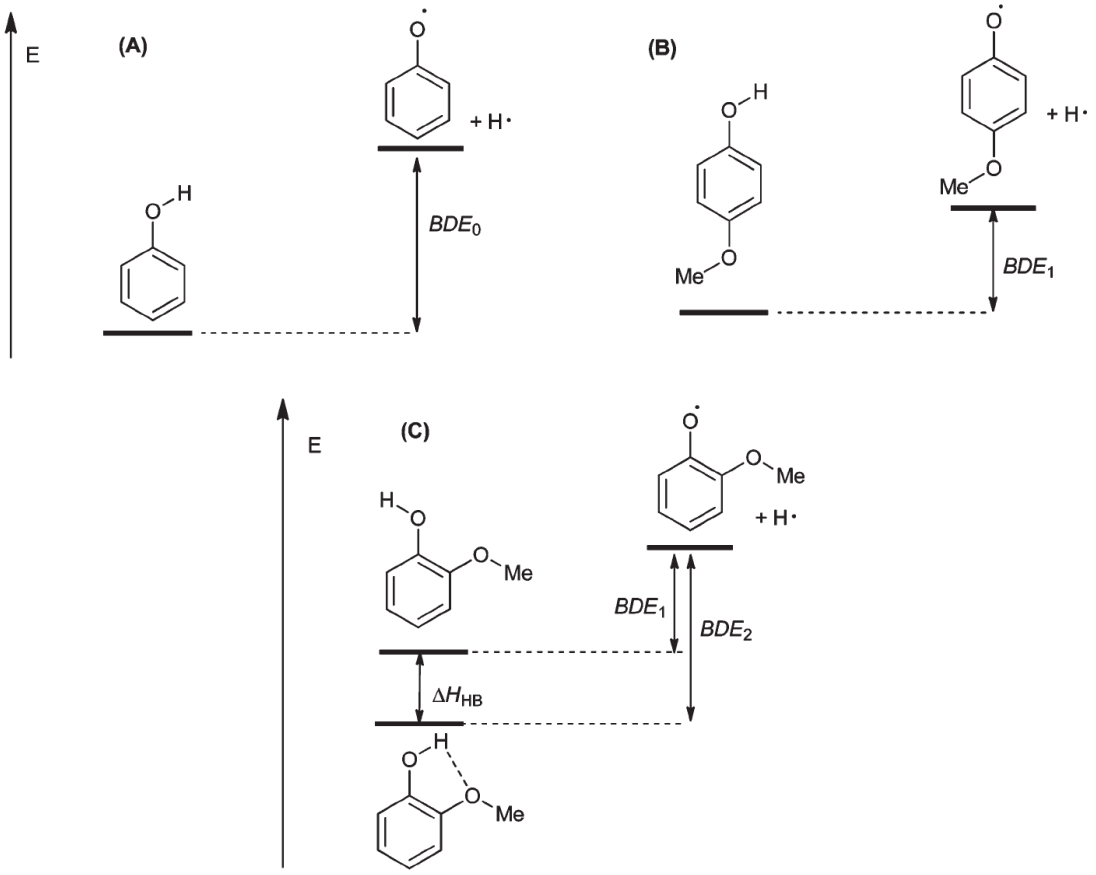


Figure 2.2 Energy diagram for O–H bond dissociation in phenols with ortho or para –OMe and –OH groups, showing the effect of intramolecular H-bonding. (A) Unsubstituted phenol. (B) A para methoxy (or hydroxyl) group substituted phenol. (C) An ortho methoxy group (Armorati et at., 2012). Used with permission from Royal Society of Chemistry, Great Britain.

Secondary or preventive antioxidants such as citric acid, phytic acid and phosphites retard the rate of auto-oxidation of lipids by interrupting the chain initiation step (Equation 2.1). They do this in a variety of ways; e.g., metal chelation, oxygen scavenging, decomposing hydroperoxides to non-reactive species, absorbing UV radiation to counteract the peroxidation chain reaction, and deactivating free radical intitiators like singlet oxygen species to name a few (Gordon, 1990). However, the ability of secondary antioxidants to act as chelators or sequestrants usually takes place in the presence of primary antioxidants (Núñez-Delicado et al., 1997). For example, a very widely used primary antioxidant in the food industry is ascorbic acid, which functions to protect

the double bonds of fatty acids and scavenge singlet oxygen. But its property as a reducing agent also helps to convert oxidized antioxidants (for example, tocopherol) back to its reduced form (Gordon, 1990). This synergism allows tocopherol to be added in small concentrations as a secondary antioxidant along with ascorbic acid. Other examples include cyclodextrins as secondary antioxidants in synergism with ascorbic acid (Núñez-Delicado et al., 1997).

2.2.2. Polyphenols as antioxidants

Because of the growing demand for the use of natural products in place of synthetic ingredients, antioxidants from various plant materials have been extensively explored (Murphy et al., 1992). Most dietary antioxidants from fruits and fresh vegetables, cereals and beverages belong to a large class of compounds called polyphenols (Amorati & Valgimigli, 2012). Polyphenols account for more than 8,000 compounds found in plants (Pandey & Rizvi, 2009). They are grouped into different classes of compounds as shown below (Figure 2.3).

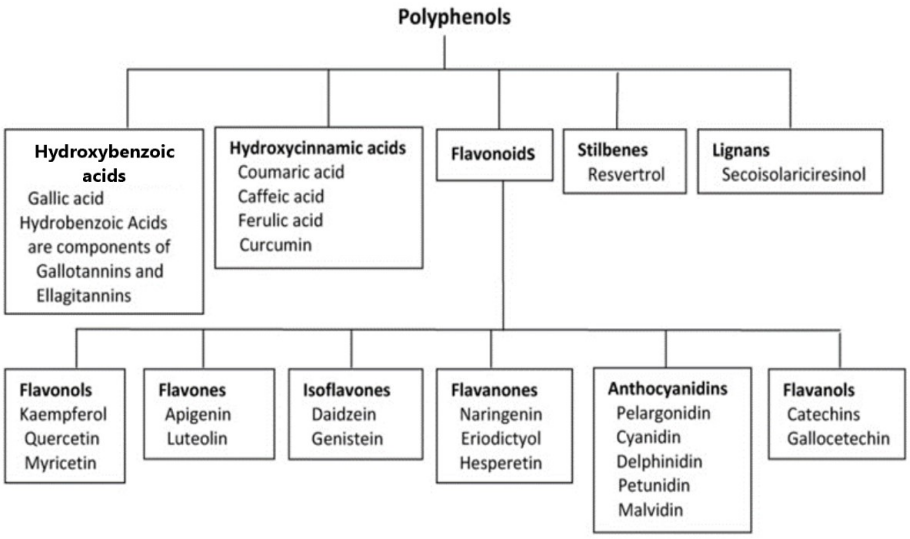


Figure 2.3 Polyphenol classifications with examples (adopted with modification from Hardman, 2014).

Polyphenols are secondary metabolites, present as active compounds in plants assisting in modulating the activity of a wide range of enzymes and cell receptors (Middleton et al., 2000).

Moreover, they are also synthesized in plants in response to generated stress which could be a physical injury, exposure to ultraviolet radiation or pathogenic insect attack, to name a few (Beckman, 2000). Chemically, all polyphenols are known to possess one or more than one aromatic ring with multiple hydroxyl groups and bearing one or more hydroxy substituents with an array of methyl, methoxyl, amino or glycosyl residues (Tsao, 2010). Polyphenols are classified into two main groups, namely, flavonoids and non-flavonoids. All flavonoids share the same basic structure comprised of 15 carbons, with two aromatic rings linked together by a three carbon bridge making a C6-C3-C6 configuration (Figure 2.4) (Williams, 1995). Flavonoids can be further divided into several structural classes (Figure 2.5). In addition to their antioxidative effect, researchers have also associated plant polyphenols with several health benefits. There is evidence for their active role in preventing diseases such as cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Arts & Hollman, 2005; Graf et al., 2005; Salah et al., 1995).

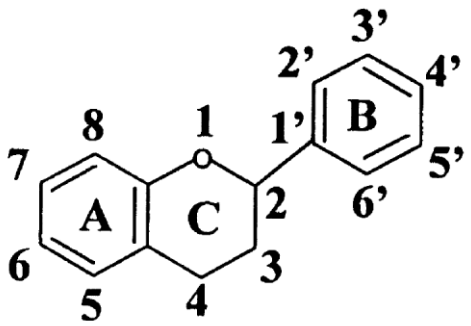


Figure 2.4 Basic flavonoid structure (Cook et al., 1996). Used with permission from Elsevier Inc., Amsterdam, Netherlands.

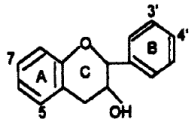
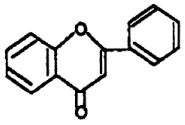
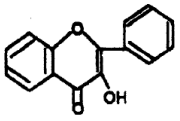
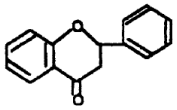
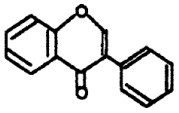
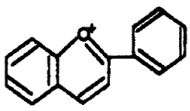
Class	General structure	Flavonoid	Substitution Pattern
Flavanol		(+)-catechin	3,5,7,3′,4′-OH
		(-)-epicatechin	3,5,7,3′,4′-OH
		Epigallocatechin gallate	3,5,7,3′,4′,5′-OH,3-gallate
Flavone		chrysin	5,7-OH
		apigenin	5,7,4′-OH
		rutin	5,7,3′,4′-OH, 3-rutinose
		luteolin	5,7,3′,4′-OH
		luteolin glucosides	5,7,3′-OH, 4′-glucose
			5,4′-OH, 4′,7-glucose
Flavonol		kaempferol	3,5,7,4′-OH
		quercetin	3,5,7,3′,4′-OH
		myricetin	3,5,7,3′,4′,5′-OH
		tamarixetin	3,5,7,3′-OH,4′-OMe
Flavanone (dihydroflavon		naringin	5,4′-OH,7-rhamnoglucose
		naringenin	5,7,4′-OH
		taxifolin	3,5,7,3′,4′-OH
		eriodictyol	5,7,3′,4′-OH
		hesperidin	3,5,3′-OH,4′-OMe, 7-rutinose
Isoflavone		genistin	5,4′-OH, 7-glucose
		genistein	5,7,4′-OH
		daidzin	4′-OH, 7-glucose
		daidzein	7,4′-OH
Anthocyanidin		apigenidin	5,7,4′-OH
		cyanidin	3,5,7,4′-OH,3,5-OMe

Figure 2.5 Structure of flavones, flavonols, flavanones, isoflavones and anthocyanins (adopted without modification from Rice-evans et al., 1995. Used with permission from Taylor & Francis Informa, United Kingdom of Great Britain and Northern Ireland.

The antioxidative capacity of polyphenols comes from their excellent hydrogen or electron donating capacity (Nawar, 1985). The resonance delocalization of the π electrons (Figure 2.6) and

limited positions for nucleophilic attack by molecular oxygen on the radical intermediates of phenols (ArO˙) make them relatively more stable than alkyl radicals (Ṙ) (Nawar, 1985). For this reason, the transfer of phenolic hydrogen to lipid peroxyl radicals occurs much faster compared to the hydrogen transfer from an alkyl molecule to a lipid peroxyl radical during chain propagation in lipid peroxidation (Section 2.1.1.2., Equations 2 & 3) (Amorati & Valgimigli, 2012). The great abundance of polyphenolic compounds in our diet, their potential role in participating against diseases related to oxidative stress (Scalbert et al., 2005; Tsao, 2010), and efficient antioxidative properties has led to their incorporation into foods as natural additives in many novel ways. For instance, they have been integrated in active packaging films (de Dicastillo et al., 2016; Wang et al., 2013), added directly to bulk vegetable oils or rendered animal fat (Satyanarayana et al., 2000; Teguh et al., 2014), or encapsulated in delivery systems for example, cocoa polyphenols in beverage emulsions (Ferrazzano et al., 2009). Irrespective of their method of inclusion, food systems offer a very complex environment to the functionality of these polyphenols, where, their antioxidative properties, are viewed as a dynamic function of their chemical and physicochemical environment (Scalbert et al., 2005).

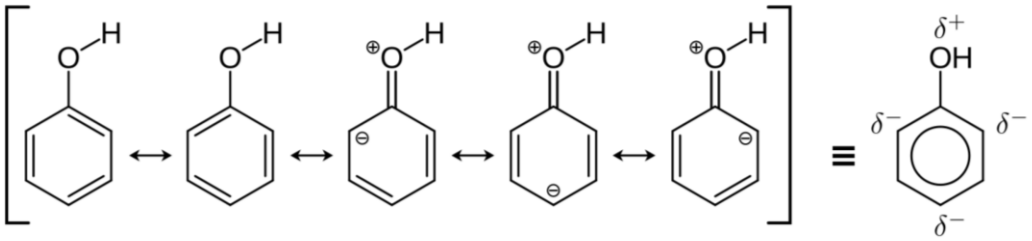


Figure 2.6 Resonance delocalization of π-electrons in a phenol molecule. Adopted without modification from Finar (1956).

2.2.3. Protein-polyphenol interactions and their antioxidant activities

The oldest documentation of protein-polyphenol interaction dates to the early 1940s. Barnell and Barnell (1945), in their method of tracking changes in ripening bananas, reported that the astringency of tannins was a result and function of their binding with proteins. Concerns about the isolation of plant enzymes due to polyphenol interference was also expressed by Loomis and

Battaile (1966), and in fact, they were the first to state that such interactions are more prevalent and complex than realized. McManus et al. (1985) examined complexation of simple phenols and inter-related esters of gallic acid with bovine serum albumin (BSA). Results from these findings showed that pH, molecular size, protein conformational flexibility and mobility in polyphenol containing substrates strongly determined the extent of their interaction with proteins. Plant polyphenols are significantly small molecules (180-700 Da) compared to macromolecular proteins (14,000-350,000 Da). Therefore, it is logical to assume that more than one polyphenol molecule may bind to one protein molecule. Furthermore, McManus et al. (1985) also revealed that at higher protein concentrations polyphenols displayed the ability to form cross-links with adjacent protein molecules rather than complexing with just one.

Polyphenol binding to protein can be reversible or irreversible depending on the type of interaction between the two species. The combination is reversible if non-covalent linkages, such as hydrogen bonding, π-bonding, hydrophobic effects and ionic pairing exist (McManus et al., 1985). Non-covalent bonding was exclusively studied by Rawel et al. (2005), who calculated the binding constants and number of binding sites for a range of phenolics (chlorogenic, ferulic, and gallic acids, quercetin, rutin and isoquercetin) to different proteins (human serum albumin, bovine serum albumin, soy glycinin, and lysozyme). The free enthalpy change for binding constants were negative which underlines the fact that the binding affinity of a polyphenol to a protein is, in fact, high and energetically favourable. At the same time, they also studied the influence of increasing temperature and ionic strength as well as decreasing pH on reducing polyphenol binding. It was also suggested that protein-polyphenol binding could play a protective role against oxidative damage due to the presence of high phenol dosage in plant-based food supplements.

In the second category of polyphenol-protein interactions, the combination is irreversible if covalent linkages are formed (Loomis & Battaile, 1966). The phenols are transformed into quinones which may react with nucleophilic groups on the protein molecule (Beart et al., 1985) (Figure 2.7). Theses complexes can be found or made. Ali et al. (2012) demonstrated the existence of covalent bonds between caffeoylquinic acid and coffee bean storage proteins. On the other hand, Liu et al. (2015) used a multistep, non-toxic, and high efficiency process called free-radical grafting, to form these polyphenol-protein (lactoferrin from bovine whey) conjugates. This procedure involves the oxidation of ascorbic acid by H_2O_2 at room temperature with the generation

of hydroxyl radicals to initiate the reaction (Kitagawa & Tokiwa, 2006). Good antioxidant activities were displayed by the functionalized material prepared. $ABTS^{\bullet+}$ scavenging power of lactoferrin–polyphenol conjugates showed a 0.23- to 2.10-fold increase compared to the control lactoferrin.

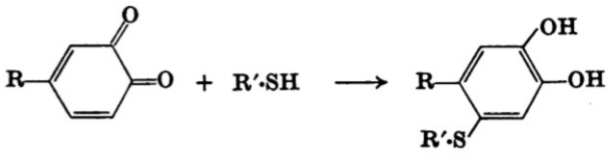


Figure 2.7 The reaction of amino acids (here cysteine) with chlorogenoquinone (Pierpoint, 1969). Used with permission from Biochemistry Society of London.

Covalently bound polyphenol-protein conjugates have shown compelling evidence of synergism with improved functional properties of the protein (antioxidant activities, solubility, and emulsifying properties), depending on the phenolic compound employed (Liu et al., 2015). Such conjugation provides a novel and efficient way to combine the advantages of using a biodegradable, biocompatible macromolecule such as protein and the antioxidant properties of polyphenolic compounds.

2.3. Emulsion: definition and food applications

Emulsions constitute principle delivery systems for industries including but not limited to: pharmaceuticals, cosmetics, foods, paint, industrial chemicals, and agriculture. Out of these, the food industry applies emulsion technology to create the most diverse range of products. The formulation of emulsion-based systems allows the selection of ingredients (e.g., water, oil, emulsifiers, thickeners, minerals, acids, bases, flavors, etc.) and processing conditions (e.g., mixing, homogenization, pasteurization, sterilization, etc.) which are convenient to adjust, to the manufacturer's and further, to the consumer's benefit (McClements, 2004a).

Emulsions may be defined as a suspension of two immiscible phases whereby the suspended phase (dispersed phase) is in the form of small droplets in the continuous phase with an interfacial layer keeping the two phases separate (Dalgleish, 2006). Three principle types of emulsions encountered

are: (1) O/W emulsions: These are oil droplets suspended in the aqueous phase, for example mayonnaise, cream liqueurs, creamers, ice cream mixes. These emulsion types have wide applicability due to an easier control of the surfactants and aqueous phase components (McClements, 2004a). (2) W/O emulsions: This system has water droplets dispersed in a fat phase, for example, margarine and, butter. Properties of these emulsions can be influenced by controlling the fat phase and surfactants used (Dalgleish, 2006). (3) Multiple emulsions of O/W/O or W/O/W: Described as "emulsions of emulsions," these systems have two dispersed phases, one dispersed as droplets in the other, which is in turn dispersed in another continuous phase (Muschiolik, 2007). Double emulsions have a great potential for applicability in the food processing industry for better encapsulation and protection of sensitive compounds from oxidation, controlled release of flavor compounds and in the production of reduced fat foods (Daisuke et al., 2004; Gaonkar, 1994; Okonogi et al., 1994)

2.4. Emulsion components and their functions

2.4.1. Oil phase

The oil phase of an O/W emulsion influences the nutritional, organoleptic, and physicochemical properties of the final emulsion (Dalgleish, 2006; McClements, 2004a). The fat globules aid in partial coalescence through crystallization and in total coalescence that leads to emulsion instability. The oil phase may also act as carriers for other important food components like vitamins, antioxidants, preservatives and essential oils. The opaque nature of most food emulsions is due to the light scattering by the oil droplets. Fats and oils are also a major source of energy and nutrients in an emulsion. Additionally, there are also apprehensions regarding to lipid oxidation in emulsions following improper processing and packaging of these products.

2.4.2. Aqueous phase

The aqueous phase of food emulsions dissolves an array of water-soluble or dispersible constituents like acids and bases (co-solvents), proteins, polysaccharides, sugars (sweeteners), vitamins and minerals, preservatives, antioxidants and colorants. The interaction between these components and water contributes to the structure of food emulsion (Dalgleish, 2004). For instance, the presence of polysaccharides in food emulsions may lead to three consequences. First, the thermodynamic limitation of the macromolecule along with the emulsion droplet may lead to

phase separation (Dickinson et al., 1995). Second, gel formation may occur, and this provides stability against aggregation (Dalgleish, 2004). Third, these polysaccharides may interact with the surface of the emulsion droplets. This interaction may be due to opposite charges on the polysaccharide and emulsifier at a given pH, for example in casein-stabilized emulsions with pectin where casein and pectin are negatively and positively charged, respectively (Dalgleish & Hollocou, 1997).

2.4.3. Interfacial layer

The interfacial layer is defined as the thin boundary between the oil and water phases usually spanning over a few nanometers. For small droplets of diameter 0.1 μm, the interfacial layer accounts for a sizable portion of the entire droplet volume (Berton-Carabin et al., 2014). The contribution of the water-lipid interface towards emulsion stability/instability cannot be underestimated. For example, a positive surface charge on emulsion droplets repels positively charged metal ions present in the continuous phase and helps to retard lipid oxidation by free metal initiators. The principal component present in the interfacial layer of an emulsion is the surface-active emulsifier, whose polar and non-polar groups align themselves in the aqueous and oil phases, respectively according to their polarity, thereby lowering the interfacial tension and reducing the amount of energy required to form oil droplets during emulsification.

2.4.4. Emulsifier

Emulsions are thermodynamically unstable systems and require stabilizing agents to avoid spontaneous separation of the phases. Stabilizing agents include a texture modifier (which increases the viscosity of the continuous phase and reduces droplet movement and hence collision) and emulsifier (which reduces surface tension) (Berton-Carabin et al., 2014). Common emulsifiers used in the food industry are solid particles, small amphiphilic molecules and amphiphilic biopolymers. Solid particles ranging from a few nanometers up to several micrometers may participate in protecting the emulsion droplet against coalescence. These colloidal systems are known as Pickering emulsions. Chemically modified octenyl succinic starch introduces sufficient hydrophobic character to the starch granules to stabilize coarse O/W Pickering emulsions (Dickinson, 2013). However, these particles are much less effective in stabilizing emulsions

compared to small-molecule surfactants or proteins owing to their low diffusive mobility and a reduced capacity to decrease the interfacial tension (Dickinson, 2013).

Small-molecule emulsifiers include relatively low-molecular weight surface active species consisting of a hydrophilic head and a hydrophobic hydrocarbon chain for its “tail”, which can be linear, branched or aromatic. Based on the charge on the head group, small-molecule emulsifiers are classified as anionic (e.g., citric acid ester of monoglyceride, CITREM), cationic (e.g., lauric arginate), zwitterionic (e.g., phospholipids) and non-ionic (e.g., sorbitan esters of fatty acids, polysorbates). The long-chain fatty acid residues they contain interacts with the lipid phase of the oil-water interface and results in their adsorption.

Amphiphilic polymers like proteins and certain polysaccharides possess the ability to stabilize emulsions and act as emulsifiers. When added to an emulsion, the hydrophobic regions of proteins lie on or even partially dissolve in the oil phase. Depending on protein structure and ionic strength of the aqueous phase, stabilization by these macromolecules is influenced by their steric or/and electrostatic interactions (Semenova & Dickinson, 2010; Dickinson, 1992). Casein from dairy milk, for example, acts as both electrostatic and steric stabilizer (Dickinson, 1999). After adsorption on the droplet surface, the strong repulsive barrier from the long hydrophilic chain of casein as well as their electrostatic charge at pHs away from their isoelectric point prevents the neighboring casein-coated droplets to come together and coalesce. Other protein emulsifiers include whey, gelatin, and proteins extracted from varius plant soucres, such as, soy and pulses (pea, lentil).

Polysaccharides stabilize emulsions by means of viscosity enhancement and their interfacial action. Because of their predominant hydrophilic character, they are used extensively in the food industry for thickening and gelation. Some polysaccharides including gum arabic, chitosan, some types of pectin, modified starch/cellulose also act as surface active agent. Their surface activity arise from either (1) modifying the starch/cellulose hydrophilic backbone to include a non-polar chemical group or, (2) the presence of a protein moiety linked covalently to the polysaccharide polymers such as gum arabic (Dickinson, 2013).

2.5. Plant proteins

Proteins are one of the vital macronutrients included in the human diet providing energy, normal body growth and function (Sharif et al., 2018). With the world population on an increase, the demand for dietary proteins are also increasing. A higher income has led to consumers' demand for an improved food experience, both from an organoleptic and nutritive aspect. Therefore, we see shifting trends towards dietary choices based on health (e.g., allergies), ethical (e.g., genetic modifications, vegan), and/or religious inclinations (e.g., vegetarian) and environmentally sustainable options (Toews & Wang, 2013). For the same reason, legume proteins have become popular as they provide a potential alternative to animal-based proteins. They display a fair nutritional value, availability, low cost, and health benefits (Duranti, 2006). But most importantly, their production demands the utilization of less resources such as land and water.

2.5.1. Sources

Legumes are the third-largest land plant family with about 751 genera and some 19,000-known species (Ratnayake et al., 2001). Of these 60 have been domesticated and include soybeans, mung bean, chickpea, and lentils (Sharif et al., 2018). The word legume is derived from the word "legumin" which is Latin for harvesting of grains present inside a pod (Sharif et al., 2018). Legumes are plants belonging to the family Fabaceae or Leguminosae or the seed of such a plant called a pulse (Sharif et al., 2018). Legumes are cultivated all over the world primarily for human and animal consumption, and to produce oils for industrial uses.

2.5.2. Structure

Legumes are a cheap dietary source of good quality protein with protein contents higher than other plant foods. The proteins in legumes are classified into albumins, globulins and glutelin. Globulins constitute 70% of the total protein fraction. They are made up of multi-subunits of two main fractions-vicilin which is a trimeric protein with molecular weights between 175-180 kDa and legumin which is hexameric with overall molecular weight of 300-370 kDa (Derbshyre et al., 1976). Vicilin and legumins have sedimentation coefficients of 7 and 11 (S value, S—Svedberg unit), respectively (Derbshyre et al., 1976). The α- and β-chains of legumins are linked by disulfide bridges where the hydrophilic α-chains are on the surface and hydrophobic chains are buried deep within the protein structure (Karaca et al., 2011). Globulins dissociate into their subunits at extreme

pH and salt concentrations (Henning et al., 1997). Albumins are the water soluble protein fraction of legumes with concentration between 10-20% and molecular weight ranging from 16-483 kDa (Papalamprou et al., 2010). Prolamins and glutelins are minor components of legume proteins with molecular weights ranging from 1-100 kDa and 45-150 kDa, respectively (Shewry & Halford, 2002).

2.5.3. Functionality

Protein functionality varies based on their method of extraction, isolation and purification, which in turn dictates compositional and physicochemical characteristics (Farooq & Boye, 2011). Studies on protein functionality generally analyze their gelling, emulsifying, foaming, solubility and water- and oil-absorption capacity. Protein solubility is an important attribute as most others depend on it (Joshi et al., 2012). It, therefore, serves as a useful performance indicator in food systems. As an example, when compared to other proteins at pH 9.5, lentil protein had a water soluble protein content (WSPC) approximately 1.3 times higher than chickpea proteins, and was significantly higher than soy protein, and was comparable to whey protein isolate (Aydemir & Yemenicioğlu, 2013). In another study, lentil protein presented minimum solubility in the pH range 4.0-6.0 with solubility increasing in the regions both above and below this range irrespective of the extraction process (Boye et al., 2010). Solubility of the protein affects how quickly it diffuses onto a fluid-fluid interface. Because of the surface-active nature of proteins, due to the presence of both hydrophilic and lipophilic units, they have been used to aid in the emulsification of oil and water. Studies have shown great variability depending on extraction and testing conditions, however, in general, the emulsifying capacity of various pulse proteins are found to be similar to each other (Jarpa-Parra, 2018).

2.6. Emulsion formation

In the process of mixing oil and water together in a container, the unfavorable thermodynamic driving forces causes the oil and water to rearrange into a configuration which allows for a minimum area of contact between the two phases (McClements, 2004a). As a result, owing to hydrophobic interactions and gravity, two separate layers form, oil on top of an aqueous layer. To construct an emulsion, it is necessary to provide an energy input, usually as mechanical agitation in a homogenizer, to disrupt and blend the oil and aqueous phases together (McClements, 2004a).

Emulsion formation may be accomplished in a single step or a series of steps (McClements, 2004a). Primary homogenization involves the formation of coarse emulsions with fairly large droplets of the suspended phase in the continuous phase. This is achieved using equipment such as a colloid mill or a high-speed blender. The droplet size can be further reduced during secondary homogenization using a high-pressure homogenizer, subjecting the primary emulsion to pressures ranging from 34,000-206,842 kPa (5,000-30,000 psi) (Dalgleish, 2004; McClements, 2004a). The high presssure forces the liquid to pass through a narrrow valve at a high speed. This generates a turbulence and cavitation energy which forces the oil droplets to split into finer droplets (Pandolfe, 1981, 1983). Ususally, multiple passes are made through a homogenizer to reduce the polydispersity of the emulsion droplets obtained at the end of each cycle.

One of the key roles of secondary homogenization is to control the droplet size of the final emulsion. The droplet diameter in an emulsion has a direct impact on products' stability, texture, appearance, and taste (McClements, 2004a). A food manufacturer looks to create an emulsion which has a droplet size distribution in the range desired for the optimum functionality of their product. Achieveing acceptable droplet size from a homogenizer depends on a balance between two competing forces of droplet disruption and droplet coalescence (McClements, 2004a). A droplet breaks up if the disruptive forces generated inside the homogenizer overpower the interfacial forces responsible for keeping the droplet in a spherical shape (McClements, 2004a). Laplace pressure (ΔP_L), which characterizes the interfacial force, acts across the oil-water interface and towards the center of the droplet, is a function of interfacial tension (γ) and droplet diameter (d) (Walstra, 1996, 1983). It holds the droplet in its spherical form and is given by:

$$\Delta P_L = \frac{4\gamma}{d} \tag{2.8}$$

From the above equation it can be inferred that as the droplet radius decreases, it becomes progressively difficult to break the droplets and therefore, greater energy is required to form them. Disruptive forces, which pull the droplet apart is a function of the flow profile (laminar, turbulent, or cavitational) generated in the homogenizer and hence depends on the type of homogenizer used (Walstra & Smulders, 1998). In the presence of emulsifiers, the droplet interfacial tension and hence the Laplace pressure is significantly reduced. However, their influence on the rheology of the interfacial membrane makes the droplet resistant to disruptive tangential stresses (McClements,

2004a). At the same time, insufficient coverage of the droplet with an emulsifier immediately after its formation makes coalescence of the newly formed droplets highly likely. Emulsions are a highly dynamic system, with the droplets in continuous motion and perpetually colliding with each other. The rate at which an emulsifier adsorbs to the droplet surface and forms a protective layer ultimately prevents droplet coalescence and is a measure of its effectiveness towards droplet disruption and against droplet coalescence (Walstra, 1993).

2.7. Emulsion Characterization

Research and development of high-quality food emulsions requires a detailed understanding of its molecular, colloidal, physicochemical and sensory properties. To monitor the food quality during the manufacturing process and to verify whether it meets the set standards, appropriate analytical techniques and methodologies need to be followed (McClements, 2004a). Some of the common parameters employed to characterize an emulsion system are discussed in the next section of the thesis.

2.7.1. Droplet size distribution

Food emulsions are polydisperse systems and contain a wide distribution of droplet sizes, much more complicated than an ideal monodisperse emulsion containing droplets of a singular size (McClements, 2004a). In most cases, knowledge of the average droplet size and the range of the distribution are sufficient to characterize the emulsion (Lyklema, 1991). Average droplet size can be denoted in several ways of which surface area average diameter (d_{32}) and volume average diameter (d_{43}) are most commonly used (McClements, 2004a; Walstra, 2003). Droplet size distribution described for a typically encountered food emulsion may change from monomodal to bimodal in nature with the passage of time (McClements, 2004a), where the second peak results from excessive droplet flocculation. Laser diffraction used to analyze droplet size distribution works on the principle that a beam of light directed through an emulsion is scattered by its droplets in a well-defined manner (Lyklema, 1991). The extent of scattering is further used to estimate the droplet size distribution using Mie Theory (Farinato & Rowell, 1983).

2.7.2. Droplet charge

Droplets in an emulsion are, most often, associated with a surface charge. The absence of charge could be a result of using non-ionic surfactants for example, polysorbates (Tweens) or sorbitan esters (Spans). The existence of charge, however, is due to the adsorption of ionic or ionizable emulsifiers (e.g., proteins, polysaccharides, and surfactants) at the interface. The magnitude and sign of the charge is a function of the type of emulsifier adsorbed, the concentration of it at the surface and the existing environmental conditions, which include pH, temperature, and ionic strength of the continuous phase (McClements, 2004a). The same sign of the charge (negative/positive) on the droplets creates electrostatic repulsions and therefore, contributes in stabilizing the emulsion. Zeta-potential or the measure of surface charge is estimated using electrophoresis which works on the principle that when a static electric field is applied across an emulsion sample, via a pair of electrodes, the charge on the droplets will cause them to move towards the oppositely charged electrode (Hunter, 1993; Lyklema, 1991). The direction in which the charged droplets move, determines the sign of their charge. Other intruments using the principles of electroacoustics are also used to measure droplet charge.

2.7.3. Microstructure

Unaided human eyes can perceive objects that are greater than about 0.1 mm (100 μm) (Aguilera, 1990). Particles encountered in an emulsion are much smaller than this limit and hence we require the help of various microscopic techniques to study their structure and mutual interactions (Dickinson, 1992). Optical microscopy, atomic force microscopy, and electron microscopy are a few techniques used to investigate the structure of emulsions (McClements, 2005). These microscopy techniques work on different physicochemical principles to examine and generate "images" of different levels and types of structural organizations (Kirby et al., 1995; McClements, 2004a). Confocal laser scanning microscopy is another novel method to examine distinct components of the dispersed droplets by staining the respective phases of an emulsion with lipophilic or hydrophilic fluorescent dyes (Blonk & van Aalst, 1993). It allows the generation of higher clarity, three-dimentional images of structures without the need to physically section the sample (McClements, 2004a).

2.7.4. Appearance

Attributes like opacity, color, and homogeneity influence the overall appearance of a food emulsion and are characteristics of the interactions between light waves and the emulsion particles (McClements, 2004a). Emulsions being colloidal systems exhibit Tyndall effect; that is, the scattering of light by the colloid particles dispersed in an otherwise-light-transmitting medium. For an emulsion to display this effect the individual particles need to fall in the range of, 40 and 900 nm, which includes the wavelength of visible light (400 to 750 nm). Milk is an example of an O/W emulsion which appears white due to light-scattering by the fat globules and casein micelles. A variety of techniques to measure appearance includes spectrophotometry, light scattering techniques, and colorimetry.

2.8. Emulsion stability

An emulsion is labelled ‘stable’ if it can resist changes in its properties with alterations made to its environment or to itself (McClements, 2004a). A perfectly stable colloidal system is one in which there is no decrease in the total number of particles with time (Lawrence & Mills, 1954). However, food emulsions are thermodynamically unstable systems and will eventually break up. Although, kinetic stability of an emulsion dictates the rate at which this breakdown happens and the overall property exhibited by them (McClements, 2004a). The different mechanisms by which an emulsion destabilizes are gravitational separation, flocculation, coalescence, Ostwald ripening, and phase inversion (Figure 2.8).

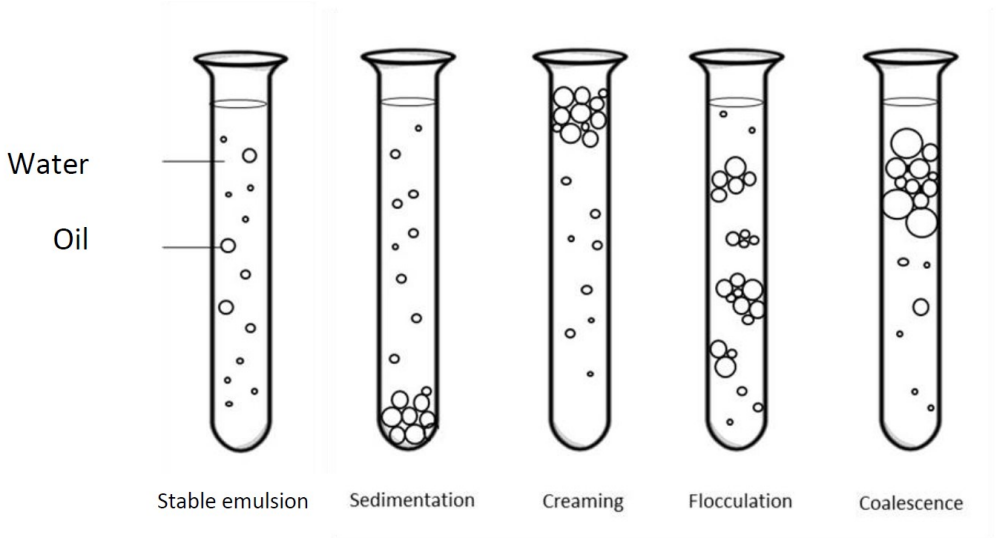


Figure 2.8 Schematic diagrams of different forms of oil-in-water emulsion destabilization.

2.8.1. Gravitational Separation

Gravitational separation in an emulsion is a consequence of density difference between the dispersed phase and the surrounding aqueous phase (Figure 2.8) (Dickinson, 1992). Edible oils have densities lower than that of water which leads to sedimentation of water droplets to the bottom of a W/O emulsion, and creaming of oil droplets to the surface of an O/W emulsion (McClements, 2004a). Phase separation of the droplets results in the loss of homogeneity. This does not only affect the intrinsic textural attribute of the product but also its holistic appeal to the consumer. Stability against creaming/sedimentation can be estimated using Stoke's Law as follows:

$$v_{Stokes} = -\frac{2gr^2(\rho_2-\rho_1)}{9\eta} \tag{2.9}$$

where v is the creaming velocity, r is the particle radius, g is acceleration due to gravity, ρ_1 and ρ_2 refer to densities of the continuous and dispersed phases, respectively, and η is the shear viscosity. v_{Stokes} quantifies the rate at which an isolated droplet in an ideal liquid creams or sediments depending on the sign calculated. Theoretically, an oil droplet of radius 1 μm suspended in water

would cream at a rate of 17 mm/day. This does not represent a very stable emulsion system. Generally, a creaming rate of less than about 1 mm/day results in a stable emulsion. However, large deviations are encountered while predicting creaming velocity using Stoke's law and those from experimental measurements due to the assumptions taken into account.

2.8.2. Flocculation

Droplets in any colloidal system are in a state of continuous motion. This motion is associated to the thermal energy of the system, mechanical agitation or gravitational forces and results in collision between the droplets (Lips et al., 1991). Two outcomes can result from this collision. First, the droplets move apart and are stable to aggregation due to the strong electrostatic and/or steric repulsions between them or second, they aggregate if the repulsive energy barrier between them is not large enough for them to stay apart. Flocculation in food emulsions is characterized by droplets coming together to form flocs, however, they retain their original shape and size (Figure 2.8) (McClements, 2004a; Walstra, 1993). Biopolymers may stimulate flocculation in some cases through bridging between two or more droplets (Dickinson, 2003; Lips et al., 1991). This usually tends to happen when an insufficient quantity of biopolymer, used as an emulsifier, is present to completely coat the oil-water interface. Another incidence of depletion interaction ensues when an excess of nonadsorbing biopolymer or surfactant micelles occupy the continuous phase of an emulsion. A moment of depletion of these entities from a narrow region between two approaching droplets generates an osmotic pressure gradient and pulls the neighboring droplets together and into depletion flocculation. The reversibility of flocculation depends on the size and concentration of polyelectrolyte molecules and the emulsion droplets, as well as the system conditions such as ionic strength, temperature, pH, and stirring. It is sometimes possible to disrupt the formed flocs by including an additional mechanical agitation step (Guzey & McClements, 2006).

2.8.3. Coalescence

The second major type of droplet destabilization is coalescence where a rupture of the interfacial layer between two or more droplets causes them to merge and form a single larger droplet (McClements, 2004a; Saether et al., 2004). The interfacial layer plays a significant role towards coalescence, and hence it becomes important to control the characteristics of the emulsifier employed at the oil-water interface and not only its existing surrounding conditions (McClements,

2004a). Increasing the thickness of the interfacial layer limits coalescence occurrence in emulsions to some extent. A thin interfacial membrane is more likely to deform. When thermal fluctuations are large enough, a hole develops in the film which extends from one droplet to another and causes its rupture. Coalescence stability can be improved by increasing inter-droplet repulsions. This can be achieved by varying the emulsifier type, pH, ionic strength, or temperature of the system. Coalescence decreases the contact area between the continuous and dispersed phases and is a principle mechanism by which emulsions move to their lowest free energy state (McClements, 2003).

2.8.4. Phase Inversion

Phase inversion is characterized by a changeover from an oil-in-water emulsion to a water-in-oil emulsion or vice versa (McClements, 2004a). This becomes an important phenomenon in manufacturing butter but is undesirable in most other food products for it leads to an alteration in the product's aesthetic quality and stability. If the dispersed phase volume fraction of an emulsion is increased keeping other system parameters like, emulsifier type, emulsifier concentration, temperature and shear rate, constant, then beyond a certain critical volume fraction the system either undergoes a spontaneous phase inversion or a breakdown with the dispersed phase forming a layer on the top. The point at which phase inversion takes place is called the "balance point" and involves a system with a non-uniform blend of regions having O/W emulsion, W/O emulsion, multiple emulsion, and bi-continuous phases (McClements, 2003). All phase inversions are accompanied by a notable change in emulsion viscosity (McClements, 2003).

2.8.5. Ostwald Ripening

Ostwald ripening (OR) is the process by which larger droplets grow at the expense of smaller ones. The driving force behind this instability arises from increased solubility of dispersed phase molecules in the continuous phase from small droplets compared to the larger ones (Ratke & Voorhees, 2013). Therefore, the molecules around the smaller droplet move through the continuous phase and re-dissolve into the larger ones. Therefore, OR occurs by dissolution and diffusion of the dispersed phase molecules and is more prominent in systems having appreciable mutual solubility between the lipid and water phases (McClements, 2004a; Princen, 1983). As triacylglycerols (TAGs) are insoluble in water, it is less of a concern in food emulsions.

Nevertheless, it appears in O/W beverage emulsions containing flavor oils (e.g., orange oil, lemon oil, etc.) due to their partial miscibility in water. To inhibit OR in such systems, high molecular weight hydrophobic components, such as an ester gum or even TAGs are mixed with the flavor oils (Tan, 2004). Some other ways to retard OR involve the use of less polar-long chain TAGs as the oil phase, create a narrow droplet size distribution, increase the thickness of the interfacial membrane and use an oil phase containing a mixture of lipids with different water solubility.

2.8.6. Influence of environmental factors on emulsion stability

Development of stable emulsion system with enhanced physicochemical properties requires a thorough knowledge on how its stability relates to the variety of solution conditions (pH, ionic strength) and processing conditions (thermal, mechanical, freeze-thaw) it goes through (McClements, 2004a). Emulsion instability and breakdown manifests itself as, for example, oil on the surface of a defrosted cheese sauce, coagulation of proteins in milk, or formation of cream layer at the top of a liquid beverage.

2.8.6.1. Effect of pH on emulsion stability

One of the most important factors influencing the formation and properties of multilayer colloids is solution pH. It dictates the ionization of surface groups and therefore the final surface charge density of the droplets (Israelachvili & Ninham, 1977). The magnitude and sign of the droplet charge due to the presence of ionized species (e.g., ionic emulsifiers, polyelectrolytes, or mineral ions) adsorbed on them can vary with pH (McClements, 2005). For example, many polyelectrolytes such as proteins and polysaccharides used in the food industry have ionizable groups. For anionic polyelectrolytes, the negative charge mostly comes from the sulphate, phosphate, or carbonate groups with pK_a values in the acidic range. While the cationic polyelectrolytes carry positive charges from amino or imino groups with pK_b in the basic range (Ai et al., 2003). It is, therefore, possible to control the degree of polyelectrolyte adsorption to the particle surface by manipulating solution pH.

2.8.6.2. Effect of salt concentration on emulsion stability

Electrolytes influence the interactions between emulsion droplets in several ways. Most influential being the electrostatic charge screening by salt where a ‘cloud’ of counterions accumulate around

the charged surface forming an electrical double layer (McClements, 2005). These ions reduce the strength of the electrical field around a charged group and this alters the range of intermolecular electrostatic interactions keeping the droplets from coming together (McClements, 2015). The range of this effect is characterized by the thickness of the electrical double layer and is called Debye screening length (κ^{-1}) which is inversely proportional to the square root of ionic strength (McClements, 2015). Other types of interactions include salt bridge formation by multivalent ions and biopolymer re-orientation which also affects steric repulsions and depletion interactions (McClements, 2005).

2.8.6.3. Effect of temperature on emulsion stability

Temperature plays a vital role in inducing phase inversions in emulsions. Crystallization-induced phase inversion occurs on cooling an O/W emulsion to partly crystallize the oil fraction, followed by shearing. Surfactant-stabilized phase inversion takes place upon heating the emulsion over/near the phase inversion temperature where the emulsifier loses its emulsifying properties (McClements, 2005). Additionally, short range hydration forces play an important role in providing flocculation stability in sterically stabilized emulsions (Evans & Wennerström, 1999). When these emulsions are heated, the emulsifier head groups are progressively dehydrated which leads to emulsion destabilization (Aveyard et al., 1990). Apart from these, temperature also affects the competitive adsorption of surfactants at the interface, and droplet size due to its influence on viscosity. In general, temperature affects the interactions between emulsion droplets and surrounding colloidal entities, and hence influences emulsion stability (McClements, 2005).

2.9. Emulsion as a delivery system for lipid-soluble bioactives

The inclusion of bioactives and nutrients such as vitamins, probiotics, minerals, polyphenols, omega-3-fatty acids, and phytosterols has become a recent food formulation trend. Although these compounds add potential health benefits, their stability against oxygen, light, heat and water become critical parameters to control (Đorđević et al., 2015). Encapsulation of these sensitive bioactives in O/W emulsions is one way to enhance their solubility, bioavailability, and stability. However, these emulsions are subjected to a broad range of environmental stresses, starting from their manufacture, to transportation, storage and their final deconstruction in the gastrointestinal tract. Under such conditions, some chemically active components including the lipid used as a

carrier may become oxidized leading to a loss of components of alimentary interest and limiting the shelf-life of the product (Berton-Carabin et al., 2014; McClements et al., 2007). High susceptibility of polyunsaturated lipids to oxidation, for example, has restricted their application in simple emulsions in many food systems despite their many health benefits (McClements & Decker, 2000). A way to tackle this problem lies in the advantages of multilayer emulsions. The increased thickness of the interfacial layer of a multilayer emulsion provides better protection to its encapsulated bioactives in addition to enhanced stability against environmental stresses (Maswal & Dar, 2014; Sagalowicz & Leser, 2010). The novel technique of fabricating multilayer emulsons by layer-by-layer (LbL) deposition of functional biopolymers has been explored by many researchers (Klinkesorn et al., 2005; Ogawa et al., 2003a). Some have also included antioxidant molecules that selectively partition at the oil-water interface forming a layer of its own (Frankel et al., 1996; Huang et al., 1996). Other conventional methods involve dispersing the antioxidant in the aqueous or oil phase of the emulsion where the location would depend on the antioxidant's polarity. Both of these approaches will be discussed further in this research study.

2.9.1. Lipid oxidation in O/W emulsions

Physical properties of a food system play an important role in the chemistry of lipid oxidation (McClements & Decker, 2000; Halliwell et al., 1995). Many studies have claimed that there is a significant difference between lipid oxidation taking place in bulk oils as opposed to that in O/W emulsions, where it is seen to occur faster in the latter (Lomova et al., 2010; Berton-Carabin et al., 2014; Coupland & McClements, 1996). In a study to analyze the oxidative stability of phytosterols in bulk and O/W emulsions, it was shown that the extent of both lipid hydroperoxides and hexanal formation was faster in emulsified oils than its bulk counterpart (Cercaci et al., 2006). The reason for this observation was postulated to be the large surface area to volume ratio of contact between the oil and water phases presented by the oil droplet interface. A larger surface area to volume ratio promotes contact between unsaturated lipids and prooxidant compounds, such as transition metal ions, and even molecular oxygen, dissolved in the aqueous phase of the emulsion(Berton-Carabin et al., 2014). In another study, Mao et al. (2009) reported the process of emulsification itself to accelerate lipid degradation in O/W emulsion. Two factors promoting this could be: first, the large surface area of the oil droplets as a result of size reduction to micro/nanometer range and second, the possible formation of free radicals during the high-pressure

homogenization process. However, out of all the parameters explored, the contribution of the oil-water interface to lipid oxidation has been studied most extensively (Berton-Carabin et al., 2013; Genot et al., 2003; McClements & Decker, 2000; Waraho et al., 2011). These studies have unanimously validated the involvement of the interfacial layer as critical in the development of lipid oxidation in an O/W emulsion system. In the present research the efficacy of the presence of antioxidants at the oil/water interafce in retarding emulsified lipid oxidation will be investigated.

2.10. Encapsulation to prevent lipid oxidation in emulsions

The problem of lipid oxidation has initiated a growing interest among food technologists, to develop protective delivery systems appropriate for the incorporation of plant oils rich in polyunsaturated fatty acids (PUFAs), and the lipophilic components such as oil-soluble vitamins, carotenoids, and flavonoids they carry, into the human diet (Kennedy et al., 2012). Microencapsulation, whereby tiny particles or droplets containing food ingredients, cells or enzymes are coated on a microscale, and the addition of antioxidants have shown some effectiveness against lipid oxidation and thus, widely applied to preserve PUFA and other bioactives (Sun-Waterhouse et al., 2011; Taherian et al., 2011). It has been a constant endeavor to fabricate the microcapsules in a way which fulfills and optimizes its purpose, whether it is to protect, deliver or aid in controlled release of the payload. For example, charged or sterically branched microcapsule wall materials have been developed to improve long-term stability and prevent droplet aggregation in colloidal systems (Grigoriev et al., 2007; Shchukin et al., 2005; Wheatley & Singhal, 1995). Whereas, mechanically robust and permeable shells made of gelatin or sodium alginate, for example, are employed for delivery and controlled release functions (Crespy et al., 2007; Suslick et al., 1994). Emulsion-based systems, such as hydrogel beads, liposomes or multi-layered droplets (using protein and polysaccharide layers) have been widely used for the microencapsulation, protection, and release of lipophilic bioactives (McClements, 2012). These systems provide ample flexibility to modulate and optimize their interfacial characteristics by tailoring their chemical and microscale structure. Additionally, these delivery systems, are also best exploited owing to their compatibility with water-based food and beverage systems (McClements & Rao, 2011). Two types of microencapsulation systems for lipid oxidation will be discussed here based on their ability to prevent lipid oxidation and deliver the payload in a controlled and efficient manner.

2.10.1. Encapsulation by layer-by-layer deposition

This strategy is based on the deposition of multiple layers of proteins and polysaccharides using a layer-by-layer (LbL) electrostatic deposition technique (Figure 2.9) (Pan et al., 2015; Ogawa et al., 2003a). The technique requires the synthesis of a "primary" emulsion with an emulsifier that rapidly adsorbs on the droplet surface. In the next step, a "secondary" emulsion is obtained when an oppositely charged polyelectrolyte is added to the "primary" emulsion. This "secondary" emulsion is a system with a two-layered interface (Guzey & McClements, 2007). Literature shows that a two-layered system improves the oxidative stability of O/W emulsions (Djordjevic et al., 2004; Ogawa et al., 2003a; Taherian et al., 2011). This improvement is attributed to a number of reasons.

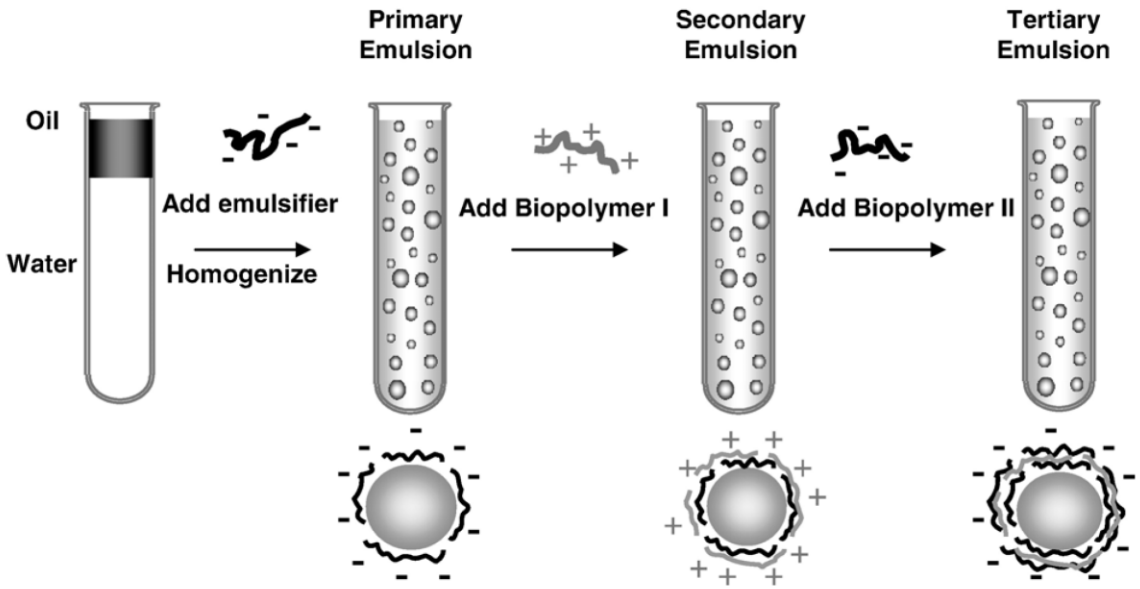


Figure 2.9 Schematic representation of LbL technique in preparing multilayer O/W emulsions Adopted without modification from Ogawa et al. (2004). Used with permission from American Chemical Society, USA.

The most apparent reason is the increased thickness of the interfacial multilayer which acts as an effective barrier against oxidation initiators (Kiokias et al., 2006). The oil-water interfacial thickness in an emulsion stabilized with an ionic emulsifier is only a few nanometers, and for relatively smaller emulsion droplets, this interface makes up for a significant fraction of the droplet

volume (McClements & Decker, 2000). Using macromolecules like proteins and polysaccharides to stabilize the droplet can increase the interfacial layer thickness to 10-15 nm (Atkinson et al., 1995; Fang & Dalgleish, 1993). This accounts for an increase of 140% in thickness compared to that imparted by ionic and other small molecule emulsifiers, and the value may still increase if larger proteins are used. For example, casein stabilized droplets produce a thicker interfacial layer (~10 nm) compared to others, such as whey proteins (~2 nm) (Atkinson et al., 1995; Patel, 2017). In a study by Hu et al., (2003), casein stabilized emulsions gave better oxidative stability than whey protein isolate (WPI) or soy protein isolate (SPI) stabilized emulsions. Increased thickness of the interfacial layer reduces the permeability of pro-oxidative species such as singlet oxygen, present in the aqueous phase into the lipid core. Tikekar et al. (2011) found that a chitosan-SDS stabilized multilayer emulsion showed a decreased oxygen transport rate into the oil-core compared to an emulsion stabilized only with an SDS layer. Slower movement of oxygen across the thicker boundary is, therefore, another reason stated for the observed improvement in oxidative stability of O/W multilayer emulsions. Other studies by Klinkesorn et al. (2005) and Ogawa et al. (2004) have shown positive results towards prevention of lipid oxidation in O/W emulsions prepared by LbL technique using polysaccharides and small molecule emulsifier. Klinkesorn et al. (2005) prepared primary emulsions with 15 wt% tuna oil and lecithin as the emulsifier. Secondary emulsions were prepared by depositing chitosan on the primary emulsions. After storing the emulsions for 35 days in the dark, there was a nine-fold reduction in hydroperoxide and a five-fold reduction in TBARS (thiobarbituric acid reactive substances, markers of secondary oxidation) recorded for the secondary emulsions when compared to the primary emulsions.

Inherent antioxidative properties of interfacial proteins may also contribute to the improvement in the prevention of lipid oxidation in emulsions. Research by Hu et al. (2003) reported that some amino acid residues, for example, tyrosine and methionine, have inherent antioxidant properties. Therefore, due to the higher percentage of tyrosine and methionine, caseinate-stabilized emulsions exhibited better oxidative stability in most cases, when compared to WPI and SPI stabilized emulsions (Berton-Carabin et al., 2013). Hasni et al. (2011) conducted binding and docking studies on the interaction of tea polyphenols with α-casein and β-casein in solution. They reported the order of binding to increase as the number of –OH group on the polyphenol increased which could affect the electron donating capacity of the polyphenols as it decreases the number of hydroxyl groups available in the solution.

Different polysaccharides are being used in conjugation with proteins in the interfacial engineering of O/W emulsions. Proteins as emulsifiers face some limitations including complex molecular conformation, susceptibility to aggregation, and alterations in functional properties upon handling and storage (Mun et al., 2016). A protein/polysaccharide bilayer as a composite emulsifier combines the exceptional emulsifying ability of the hydrophobic proteins adsorbed on the oil-water interface, with the stabilizing capacity of the hydrophilic polysaccharide solvated by the continuous aqueous phase (Akhtar & Dickinson, 2003). Although, past literature has shown promising results towards the stability of polyunsaturated fatty acids in O/W LbL emulsions, an effective way to boost the functionality of the protein/polysaccharide composites at the oil-water interface against lipid oxidation could involve the inclusion of polyphenols with antioxidative properties into the shell matrix.

Lomova et al. (2010) studied lipid peroxidation in micron size O/W emulsion droplets coated with multiple layers of polyelectrolytes/tannic acid compared to an antioxidant-free biodegradable multilayer coating shell assembly with 10,000 ppm of mixed tocopherols added to the oil core. The ζ-potential measured at the droplet surface shifted polarity after the deposition of each component layer during the preparation of the multilayer assembly with the final droplet surface charge positive. It is a common practice to keep the surface charge of the final composite at the oil-water interface positive for the effective electrostatic repulsion of the prooxidant transition metal ions and therefore, extended oxidative stability (Lomova et al., 2010; Ogawa et al., 2003b). The group used 10% linseed oil to prepare these emulsions and were able to show better oxidative stability for emulsions with tannic acid at the oil/water interface than emulsions encapsulated with the antioxidant-free shell with mixed tocopherols in the oil core. Additionally, O/W emulsions with a multilayer shell comprising tannic acid did not oxidize over 15 days of storage at 37 °C and remained stable in a solution of prooxidant Fe^{2+} added at a concentration 10 times greater than its physiological concentration in human blood serum.

2.10.2. Encapsulation by protein-polyphenol conjugates at the interface

The idea behind the encapsulation of oil droplets by protein-polyphenol conjugates lies in the possibility to selectively locate the antioxidant molecules at the oil-water interface (Figure 2.10). Protein-polyphenol complexation can be achieved either by covalent bonding or non-covalent interactions (electrostatic, H-bonding, hydrophobic, van der Waals) (Liu et al., 2016a). However,

it has been well established that complexes held together by covalent bonds offer a greater improvement to protein functionality, including solubility, thermal processing, emulsifying characteristics, and foaming ability (Zou et al., 2015; Evans et al., 2013; Liu et al., 2012a; Xu et al., 2012) than those held together by the weaker non-covalent bonds (Kim et al., 2013). To illustrate a better oxidative stability imparted by localized antioxidants at the oil-water interface, Pan et al. (2015) engineered the antioxidant gallic acid at the interface in conjugation with ε-polylysine and dextran sulfate as an added coating and showed that after 48 hours of storage, approximately 95%, 77%, 43% and 56% of encapsulated retinol was degraded in SDS, SDS-EPL, SDS-EPL-GA emulsions and SDS emulsion with equivalent amount of gallic acid added to the bulk aqueous phase, respectively. With the lowest retinol degradation reported for emulsions with GA at the interface (43%), the authors were able to retain the encapsulated bioactive in oil-in-water emulsion.

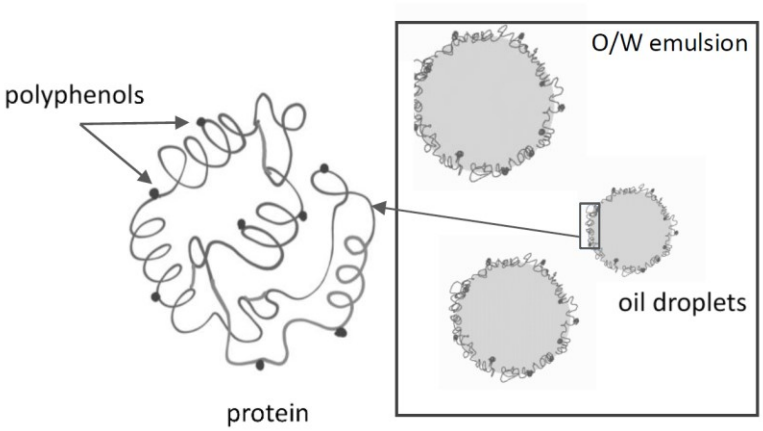


Figure 2.10 Schematic diagram depicting polyphenols at the interface of an oil-in-water emulsion stabilized using protein-polyphenol conjugates.

Antioxidants participating in conjugation with proteins can be chosen such that their physicochemical properties lead to their maximum binding with the protein and favorable partitioning at the oil-water interface (Berton-Carabin et al., 2014). Prigent et al. (2008) studied the covalent interactions between quinones derived from caffeoylquinic acid and amino acids.

Their study revealed that lysine and tyrosine were more reactive to their binding with quinones than histidine and tryptophan. Many studies have used lactoferrin as their model protein (Liu et al., 2017b; Liu et al., 2016d). Lactoferrin is a globular protein with a single-chain glycoprotein and a well-defined structure (Orsi, 2004; Ward et al., 2002). Liu et al. (2015) analyzed the reactivity of different antioxidants including epigallocatechin gallate (EGCG), chlorogenic acid (CA), and gallic acid (GA) with lactoferrin (LF). The degree of conjugation followed the order: LF-EGCG > LF-CA > LF-GA. It was demonstrated that reactivity with free tyrosine groups of LF ranked in the order: EGCG > CA > GA which followed the same order as the percent conjugation of the polyphenol to LF and also, the number of hydroxyl groups on the phenolic moiety.

Notwithstanding, there have also been concerns by many groups regarding the decrease in antioxidant activity of polyphenols chemically bound to protein emulsifiers. This decrease was in response to the exhaustion of the hydroxyl groups participating in the hydrogen-donating activity of the antioxidant now being involved in forming hydrogen bonds with active protein sites (Khan et al., 2011). Figure 2.11 shows the proposed hydrogen bond interaction between the quercetin molecule and binding sites in human serum albumin and the consequent blocking of –OH groups on quercetin molecule.

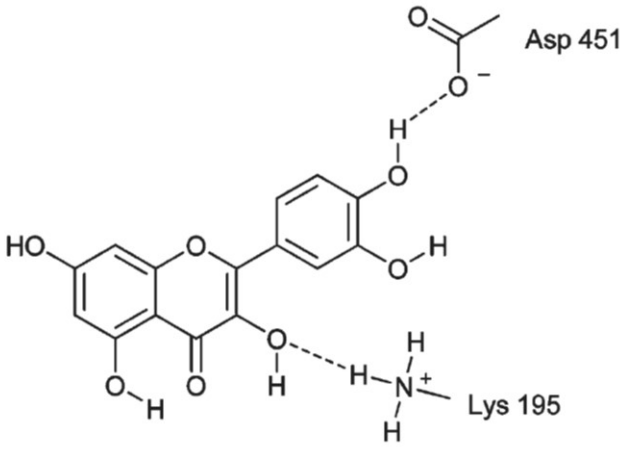


Figure 2.11 Proposed binding of quercetin to aspartate and lysine residues of human serum albumin (adopted without modification from Khan et al., 2011). Used with permission from Royal Society of Chemistry, UK.

However, selecting an appropriate polyphenol with abundant hydroxyl groups per aromatic ring placed at strategic locations on the ring should be sufficient in counteracting the limitation stated above. There are many pieces of evidence from literature which suggest an improved lipid oxidative stability using protein-polyphenol conjugates and polysaccharide-coated multilayered O/W emulsions (Wei et al., 2015; Tokle et al., 2010). Liu et al. (2016d) investigated the stability of β-carotene O/W emulsions coated with LF-polyphenol (EGCG or CA) and polysaccharide (soybean soluble polysaccharides (SSPS) and beet pectin (BP)) composites prepared using the LbL technique. The results obtained showed better physical stability for secondary emulsions (LF-CA/EGCG-BP/SSPS) over primary (LF or LF-CA/EGCG) emulsions. The secondary emulsions were stable over a wide pH range (3.0-9.0) which was attributed to the relatively thick emulsifier composite surrounding the oil droplet. Secondary emulsions from both SSPS and BP were successful in inhibiting β-carotene degradation compared to primary emulsions. Recently, protein-antioxidant-polysaccharide ternary conjugates have also been developed by the same group. In contrast to the LbL technique where different layers are deposited individually at the oil-water interface, the ternary conjugates were developed via mild Maillard reactions before making the emulsions (Liu et al., 2016a). The ternary conjugates of chlorogenic acid-lactoferrin-polydextrose were reported to exhibit strong emulsifying properties and chemical and physical stability for the encapsulated β-carotene moiety against freeze-thaw treatment and UV exposure compared to when lactoferrin, chlorogenic acid-lactoferrin, and chlorogenic acid-lactoferrin-polydextrose physical mixtures were used as emulsifiers.

The studies from this group, however, did not analyze the oxidative stability of the dispersed oil phase of the emulsions which is the main aim of the present research. Assessment of lipid free radicals and other lipid oxidation products in emulsion is important and directly influences the stability of the bioactive substances in the lipid phase. However, these studies were successful in formulating physically stable O/W emulsions for the effective carriage of lipid biosensitive compounds, ß-carotene. It was demonstrated that emulsion systems with composite emulsifiers prepared using LbL technique or mild Maillard reactions provide superior and long-term protection to the encapsulate compared to a conventional emulsion system. The different components of the composite are responsible for the enhanced performance of the emulsion system as a whole. The protein contributes to the surface activity of the conjugates and helps in its adsorption at the lipid droplet interface; the polysaccharide contributes to droplet stability by

providing steric repulsion between the droplets, and the polyphenols improve the oxidative stability of the system by acting as interfacial antioxidants (Liu et al., 2017b). Such delivery systems have tremendous potential in inhibiting lipid oxidation in O/W emulsion-based systems and are yet to be explored to their full extent. In the present research, conjugates of lentil proteins with polyphenols will be used to prepare flaxseed oil-in-water emulsions to test their ability to retard lipid oxidation.

2.11. Choice of materials

2.11.1. Emulsifier

There is a clear trend of consumer interest in alternative proteins sources. Where at one time, plant-based proteins remained relatively underutilized as food ingredients they are now fast replacing proteins sourced from animals. Canada is one of the major contributors to global pulse production. Utilizing pulse proteins would not only promote innovative food solutions and sustainability but also add as a valuable commodity to the nation's agriculture. For the same reason, lentil protein isolate, extracted using isoelectric precipitation at the POS Biosciences, Canada was utilized as an emulsifier for this study.

2.11.2. Oil phase

There are many studies enumerating the health benefits of consuming ω-3 fatty acids, especially those relating to cardiovascular disease prevention (Astrup et al., 2011). A good vegetable source of ω-3 fatty acids is flaxseed oil, also sometimes known as linseed oil when used for industrial appliction, which contains more than 50% α-linolenic acid (Bozan & Temelli, 2008). However, ω-3 polyunsaturated fatty acids are easily oxidized due to their high degree of unsaturation. Flaxseed oil was chosen as a preferential oil source for its beneficial health effects as well as its liability to oxidize easily.

2.11.3. Polyphenols

Three polyphenols were chosen for this study based on the differences pertaining to their polarity and/or phenolic classification:

Quercetin (Q) (5,7,3′,4′-flavon-3-ol) and rutin (R) (quercetin-3-O-rutinoside) belong to the flavonoid group of polyphenols. Q is widely found in vegetables, fruits and plants such as onions, teas, kale, and apples (Meyer et al., 1998). Q displays limited water solubility and can only well dissolve in nonpolar solvents, e.g., acetic ether, chloroform, acetone (Xu et al., 2006). R (quercetin-3-O-rutinoside), on the other hand, is the flavonol quercetin with a disaccharide rutinose combined to it. The sugar group in rutin makes it more polar than quercetin. R is a citrus flavonoid and is found abundant in citrus fruits. Luo et al. (2011) in their study measured the partition coefficient of quercetin and rutin between water and a nonpolar solvent (octanol) to be 144.54 and 0.134 respectively, and further tested the stability of these polyphenols in emulsifying n-tetradecan emulsions droplets where R formed "good" with no observable oiling off after 1 week storage and Q formed "no" emulsions with clear oil and water separating within 1 h.

Ellagic acid I (4,4′,5,5′6,6′-hexahydroxydiphenic acid 2,6,2′6′-dilactone), a phenolic acid, is a dimeric derivative of gallic acid (Losso et al., 2004). It is found in plants such as woody plants, berries, grapes and nuts (Talcott & Lee, 2002). E is an amphiphatic molecule. The four phenolic rings and two lactone groups represent the hydrophilic part whereas, the two phenyl rings represent the hydrophobic part of the molecule (Losso et al., 2004). Ellagic acid is only sparingly soluble in aqueous media and therefore, possesses low polarity but showed potential to be used for an emulsion system.

3. STRUCTURE-FUNCTIONALITY OF LENTIL PROTEIN-POLYPHENOL CONJUGATE

3.1. Abstract

The purpose of the present study was to estimate how lentil protein's interaction with plant polyphenols (quercetin (Q), rutin (R), ellagic acid (E)) affects the physicochemical, structural, and functional characteristics of the conjugates when compared with the lentil protein isolate (LPI) control. The interaction between polyphenols and protein was achieved by a simple grafting method where the subtrates were made to react in alkaline conditions (pH 9.0) in the presence of free oxygen. The resulting protein-polyphenol conjugates were characterized using spectroscopic and thermal techniques. Structural analysis was made using Fourier transform infrared (FTIR) spectroscopy, surface hydrophobicity and intrinsic fluorescence. This study was conducted to get a better understanding of the functionality of the protein-polyphenol conjugates. Effect of different polyphenols on the solubility and interfacial property of LPI was investigated. Finally, the antioxidant capacity of the prepared conjugates was estimated using the $DPPH^{\cdot}$ free radical scavenging and Ferric Reducing Antioxidant Power (FRAP) assays. The degree of conjugation determined using proximate analysis of the samples was in the order: LPI-Q > LPI-E > LPI-R. The results show changes in the secondary structure of the covalently conjugated protein with increased random coil configuration in addition to giving compelling evidence of synergism – with improved antioxidant activities, depending on the phenolic compound employed. However, there was no significant change in the interfacial tension and thermal properties of the LPI-polyphenol conjugates. Such conjugation provides a novel and efficient way to combine the advantages of using plant protein and polyphenols in developing a healthier ingredient for the food industry.

3.2. Introduction

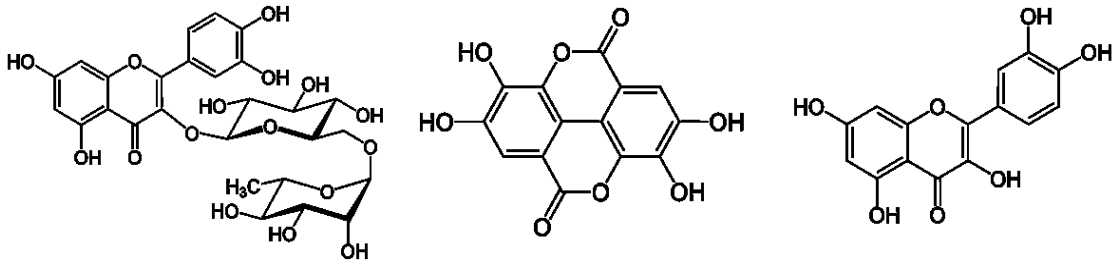
Polyphenols are one of the most widely distributed compounds available in various vegetables, cereals, cocoa, and dry legumes, including fruits and plant-derived beverages such as fruit juices, tea, coffee and red wine (Scalbert et al., 2005). There are more than 8,000 of these compounds

reported in various food sources, making the total dietary intake of polyphenols to about 1 g per day (Fraga et al., 2010; Scalbert et al., 2005). Chemically, all polyphenols are known to possess more than one aromatic ring with multiple phenolic hydroxyl groups (Tsao, 2010). Their excellent electron donating capacity makes them popular as natural antioxidants serving to delay or prevent oxidation by reactive oxygen species (ROS) or pro-oxidant metal ions (Nawar, 1985).

Interactions between proteins and polyphenols have been well discussed in literature. The oldest documentation of protein-polyphenol interaction dates to the early 1940s when Barnell et al. (1945) in their method of tracking changes in ripening bananas, reported that the astringency of tannins was a result and function of their binding with proteins. There are also reports supporting the role of polyphenols in foam stabilisation and gelatin gel-network strengthening by phenolic dimers inducing protein cross-linking (Strauss & Gibson, 2004; Sarker et al., 1995). Polyphenol binding to protein can be reversible or irreversible depending on the type of interaction between the two species. The combination is reversible if non-covalent linkages, such as hydrogen bonding, π-bonding, hydrophobic interactions and ionic pairing exist (McManus et al., 1985). Non-covalent bonding was exclusively studied by Rawel et al. (2005), who calculated the binding constants and number of binding sites for a range of phenolics (chlorogenic, ferulic, and gallic acids, quercetin, rutin and isoquercetin) to different proteins (human serum albumin, bovine serum albumin, soy glycinin, and lysozyme). The free enthalpy change for binding revealed that the binding affinity of a polyphenol to a protein is, in fact, high and energetically stable.

For irreversible polyphenol-protein interactions, covalent linkages are formed (Haslam, 1996; Loomis & Battaile, 1966). During alkaline hydrolysis, the polyphenols are oxidized to their corresponding quinones (Hurrell & Finot, 1984). Quinones, being powerful electrophilic intermediates, are then attacked by nucleophilic amino acid moieties such as lysine, methionine, cysteine, and tryptophan on the protein chain leading to the formation of protein-polyphenol conjugates (Hurrell & Finot, 1984). Covalent interactions have been studied by many research groups for animal proteins, such as lactoferrin with EGCG, chlorogenic acid and gallic acid (Liu et al., 2015), myoglobin with ferulic and gallic acids (Kroll & Rawel, 2001), and bovine serum albumin with chlorogenic acid (Rawel et al., 2002b). However, to the best of our knowledge, there is no study documenting the behavior of lentil proteins with polyphenols, specifically their antioxidant activity.

Conjugation of proteins and polyphenols, in general, provides a novel and an efficient way to combine the advantages of biodegradable, biocompatible macromolecules and the antioxidant properties of the polyphenolic compounds (Liu et al., 2015). Therefore, the objective of the present study was to synthesize and characterize plant protein – polyphenol complexes by conjugating lentil proteins with natural polyphenols. There is a growing impetus to replace animal-based proteins in our foods with the plant-derived counterpart. The later confers advantages such as nutritional value, low cost, availability, environmental sustainability and beneficial health effects (Gumus et al., 2017). Proteins from various legume sources such as lentil, faba bean, chickpea, pea, cowpea, and lupine are being intensively investigated for their use in new product formulations. In this work, we have used lentil protein isolate (LPI) as a source of plant protein. Three different polyphenols – quercetin (Q), rutin (R), and ellagic acid (E) were used for this purpose differing in polarities and/or polyphenolic classification. While Q belongs to the sub-class flavonoids, E is a phenolic acid. Furthermore, R is a glycoside of Q with a disaccharide rutinose attched to it making it more polar than Q. Structures for Q, R and E are presented in Figure 3.1. Following conjugation, these new entities were characterized in terms of their structural (Fourier transform infrared (FTIR) spectroscopy, fluorescence spectroscopy, surface hydrophobicity), antioxidant (DPPH and ferric ion reducing power (FRAP)) and functional (interfacial properties and turbidity) make-up in comparison to the plant protein alone.



Rutin (R) Ellagic acid (E) Quercetin (Q)

Figure 3.1 Chemical structure of the polyphenols used for binding with lentil protein isolate (LPI).

3.3. Materials and methods

3.3.1. Materials

Lentil protein isolate (LPI) was kindly provided by POS Bio-sciences (Saskatoon, SK, Canada), after being produced by alkaline extraction and isoelectric precipitation according to the method of Karaca et al. (2011). It contained 3.8% moisture, and 75.2% protein on a dry basis as determined by POS Bio-scienes (Section 3.3.3). Canola oil used in this study was purchased from a local supermarket (Saskatoon, SK, Canada). Quercetin (Q, purity ≥95%) was obtained from Millipore-Sigma (Oakville, Ontario, Canada). (+)-Rutin trihydrate (R, purity ≥97%) and Ellagic acid (E, purity ≥97%) were obtained from VWR International (Edmonton, AB, Canada). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade.

3.3.2. Preparation of protein-polyphenol conjugates

The protein-polyphenol conjugates were prepared following the method described by Liu et al. (2015). Briefly, 5 g protein was dissolved in 400 mL deionized water at pH 9.0 and stirred overnight at room temperature (23 ± 2 ºC). The following day, 1.25 g polyphenol (quercetin, rutin, and ellagic acid) was dissolved in 50 mL distilled water (0.25 wt%) and its pH brought to 9.0 using 0.1 N NaOH. The polyphenol solution was stirred for 1 hour following which it was mixed with the protein solution and the total volume was made to 500 mL. The reaction mixture was kept under constant stirring for 24 h at room temperature with free air contact to allow complexation. The reaction mixture was then dialyzed (Spectra-Por cellulose ester dialysis bags, molecular weight cut off 8-10 kDa, Spectrum Labs, Rancho Dominguez, CA, USA) to remove any unreacted polyphenol. The dialysate water was changed multiple times and the water was tested for traces of polyphenols using UV spectra in the wavelength range of 350-450 nm until the aborbance of the dialysate was less than 0.002 and no polyphenols were detected. The reaction mixture in the dialysis tubes was then freeze-dried to obtain lyophilized protein-polyphenol conjugates. All conjugates were prepared in replicates, n=3.

3.3.3. Chemical characterization of the protein-polyphenol conjugates

Proximate analysis and the amino acid profiles of the proteins and the protein-polyphenol conjugates were performed by POS BioSciences, Saskatoon, SK. Moisture was analyzed using AOCS Ba 2a-38, protein using AOCS Ba 4e-93, ash using AOCS Bc 5-49, oil using Troeng, S.(1955). Carbohydrate was estimated by difference (i.e. 100 – moisture, protein, ash, oil). Amino acids were analyzed using the Waters Pico-Tag Amino Acid Analysis System, 1984. The references for these methods are: AOAC method 994.12 for 16 amino acids; AOAC Method 988.15 for Tryptophan and Method 982.30 for the sulfur containing amino acids – methionine and cysteine.

3.3.4. Conformational and surface hydrophobicity analysis

3.3.4.1. FTIR spectroscopy

FTIR was used to assess any difference in the secondary structure between LPI and its conjugates. All freeze-dried samples were further ground into a powder, pressed on the observation slides, and analyzed using a Renishaw Invia Reflex Raman Microscope (Renishaw Group, Gloucestershire, UK) fitted with a IlluminatIR II FTIR microscope accessory (Smith's Detection, Danbury, CT, USA), in the mid-infrared region (650-4000 cm-1). Absorbance from the polyphenol was subtracted from the conjugate spectra using a WIRE 3.3 (Renishaw Group, Gloucestershire, UK) software inbuilt function. The protein's FTIR spectra was self-deconvoluted in the amide I band (1600-1700 cm-1), and the major peaks for protein secondary structure were resolved by curve-fitting using the WIRE 3.3 software. The area under the individual component bands: α-helix (1,648–1,660 cm-1), β-sheet (1,612–1,641 cm-1), β-turn (1,662–1,684 cm-1), and random coil (1,640–1,650 cm-1) was measured with the Gaussian function, added up and divided by the total area to give percent area under each band. All measurements were performed at room temperature.

3.3.4.2. Fluorescence spectroscopy

Fluorescence measurements were performed using a spectrofluorometer (FluroMax-4, Horiba Jobin Yvon Inc., Edison, NJ, USA) at room temperature. Samples (LPI and its conjugates) were taken at a concentration of 1 mg/mL. A constant excitation wavelength of 285 nm (slit width 2.5 nm) was used to selectively excite the aromatic amino acid residues and emission collected

between 300 and 400 nm (slit width of 5 nm and increment of 0.5 nm). Absorbance from pure polyphenol was subtracted from the conjugate sample reading. Each spectrum presented is an average of three replicates.

3.3.4.3. Surface hydrophobicity

Surface hydrophobicity was determined according to a modified method of Kato and Nakai (1980) using a fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS). LPI and its conjugates (0.025 % w/w) were dissolved in 10 mM sodium phosphate buffer (pH 7.0) and stirred overnight at 4°C. Several dilutions of the solution (0.005, 0.010, 0.015 and 0.020%) were made in 10 mM sodium phosphate buffer (pH 7.0). 20µL of 8 mM ANS solution (dissolved in 10 mM sodium phosphate buffer at pH 7.0) was added to 1.6 mL of each dilutions and vortexed for 10 s and kept in the dark for 5 min. Fluorescence intensity was measured using a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) with excitation and emission wavelengths of 390 and 470 nm, respectively, and slit widths of 1 nm. Fluorescence intensity values for the ANS and protein blanks were subtracted from the fluorescence intensity of the sample solutions containing ANS. The relative fluorescent intensity was determined using the equation provided by Chaudhuri et al. (1993):

$$RFI = \frac{F_S - F_0}{F_0} \tag{3.1}$$

where F_s and F_0 are fluorescence intensity of the protein-ANS conjugate and ANS alone, respectively. Surface hydrophobicity was expressed as the initial slope of the plot of RFI versus protein concentration (mg/mL).

3.3.4.4. DSC measurement

Calorimetric breakdown of LPI and its conjugates was determined using a diferential scanning calorimeter (DSC) (model Q-2000, TA Instruments, New Castle, DE, USA). A 3 mg sample of powder was placed in a Tzero aluminium hermatic pan and sealed tightly with a Tzero aluminum lid using a crimper. An empty aluminum pan was used as a reference. The pans were heated from 25 to 120 ºC at a constant rate of 5 ºC min-1 and a constant purge of dry nitrogen gas at 50 mL min-1. The peak temperature of denaturation was recorded from the thermal curve using the Universal Analysis Software (Ver. 4.5A, TA Instruments, New Castle, DE, USA).

3.3.5. Functional properties of the protein-polyphenol conjugates

3.3.5.1. Turbidity

Turbidity of LPI and its conjugates at two different pH values (2.0 and 7.0) was measured as an indirect method of solubility. Samples were dissolved in distilled water at a concentration of 1 mg/mL and adjusted to pH 2.0 or 7.0 with 0.1 N HCl or NaOH. A UV-vis spectrophotometer (Beckman DU 530) at wavelength 600 nm was used to measure the absorbance.

3.3.5.2. Interfacial tension

Interfacial tension was determined between the protein or protein-polyphenol conjugate solutions (2.0%, w/w) and canola oil at pH 7.0 using a semi-automatic interfacial Tensiometer (Lauda TD2, GmbH & Co., Lauda-Konigshofen, Germany) with a Du Noüy ring (20 mm diameter). In brief, the protein solution was added into the glass sample cup, and then the Du Noüy ring was lowered into the protein solution, followed by the addition of canola oil. The maximum force measured while pulling the ring upwards without breaking the oil-protein interface was recorded. Successive measurements were taken until the standard deviation was lower than 0.10 mN/m.

3.3.5.3. DPPH˙ scavenging activity

The free radical scavenging properties of LPI and its conjugates was evaluated by their reaction with a stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH˙), according to the method of Gong et al. (2012). 500 µM DPPH˙ was freshly prepared in 70% (v/v) aqueous methanol. 2 mL of diluted sample (1 mg/mL) was mixed with 2 mL DPPH solution. This mixture was stored in the dark for 1 hr and the residual DPPH concentration was determined using using a UV- vis spectrophotometer (Beckman DU 530) at 517 nm. Finally, free radical scavenging activity of the samples was calculated as the decrease in DPPH˙ absorbance, and expressed as percent inhibition of DPPH˙ radicals as:

$$\text{Inhibition } \% = \frac{A_0 - A_1}{A_0} \times 100 \quad (3.2)$$

Where A_0 is the absorbance of the control solution containing only DPPH˙, and A_1 is the absorbance of the protein or conjugate samples. A Trolox caliberation curve was formulated from

concentrations 7.5-75 µg of Trolox/mL solvent and the %DPPH inhibition of the samples were compared to %DPPH inhibition of Trolox and finally expressed as Trolox equivalents.

3.3.5.4. Ferric reducing antioxidant power (FRAP)

The method described by Yıldırım, Mavi, and Kara (2001) was used to determine the iron (III) reducing ability of the conjugates. Briefly, 1 mL of samples (1 mg/mL in MeOH) was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6). 2.5 mL of 1% potassium ferricyanide was added to initiate the reaction. The reaction mixture was then incubated at 50ºC for 20 min. Following this, 2.5 mL of 10% (w/v) trichloroacetic acid was added and the mixture was subjected to centrifugal force at 3000 rpm for 10 min. 2.5 mL of the supernatant was then separated and mixed with 2.5 mL distilled water and 0.5 mL FeCl3 (0.1%, w/v). The absorbance was measured at 700 nm using a UV-vis spectrophotometer (Beckman DU 530) where a higher absorbance indicated a higher reducing power. A Trolox calibration curve was formulated in the concentration rage 0.05-0.80 mg/mL following the same method as described above. Final values are expressed as µmol Trolox equivalents per g sample.

3.3.6. Statistics

All assays were measured in triplicates for each replicates (n=3) of the conjugates and reported as the mean ± standard deviation. Statistical significance was determined using a two way analysis of variance (ANOVA) available in Microsoft Excel (Microsoft Canada Co, Mississauga, ON, Canada) with a 95% confidence interval where $p < 0.05$ was considered as statistically significant.

3.4. Results and discussion

3.4.1. Chemical profile of the protein-polyphenol conjugates

3.4.1.1. Polyphenol content of the conjugates

Table 3.1 presents the protein and polyphenol content (dry basis) of the various samples. After dialysis, protein content of the LPI increased from 79.9 to 80.6 %, due to the removal of some non-protein materials such as fibres and carbohydrates. For the conjugates, different amounts of polyphenols were bound to the protein molecules, which was highest for Q (21 %) and lowest for R (4.4 %). Polyphenol bound for each conjugate was determined by subtracting the ‘other

materials' (in Table 3.1) from its protein content assuming it had the same content of 'other materials' as the dialyzed LPI (19.4%). The extent of binding depended on the polyphenol type where for Q and E binding increased with increasing phenolic hydroxyl groups on the polyphenol molecule. A similar reporting of the influence of phenolic hydroxyl group on protein-polyphenol binding was also observed by Afanas'ev et al. (1989). (+)-Rutin trihydrate, also known as quercetin-3-O-rutinoside, a more water-soluble analog of Q with a rutinose moiety attached to it, possibly posed a steric hinderance to its complexation with LPI leading to a lower level of conjugation. Also, quercetin with an additional active phenolic hydroxyl, is a slightly stronger electrophile than rutin. Protein content of the conjugates also varied depending on the amount of polyphenol bound to them. For example, for LPI-Q, the highest polyphenol binding led to lowest protein content (59.6 % by weight) of all the conjugates. Similarly, LPI-R contained the highest amount of protein (76.2 %) as the polyphenol content of the conjugate was lowest.

Table 3.1 Proximate analysis of the protein and protein-polyphenol conjugates.

Sample	Protein content (%, dry basis)	Other materials (%, dry basis)	Polyphenol content (%, dry basis)
LPI-UD	79.9	20.1	-
LPI-D	80.6	19.4	-
LPI-Q	59.6	19.4	21.0
LPI-R	76.2	19.4	4.4
LPI-E	68.9	19.4	11.7

Note: Other materials includes: percentage of ash + lipid + CHO. The polyphenol concentration was estimated based on the protein level minus the 'other materials', assuming it had the same content as the dialyzed sample (19.4%).

3.4.1.2. Amino acid profile of the protein and protein-polyphenol conjugates

The amino acid profile of LPI and LPI-polyphenol conjugates is given in Table 3.2. As a control, LPI-UD and LPI-D were also analyzed. After dialysis, a drop in glutamic acid, serine and arginine levels was observed, whereas levels of alanine, proline, tyrosine, valine, isoleucine, leucine and phenylalanine increased. Minor alternations in amino acid levels may be the result of losses in some protein fractions in the dialysis water. After conjugation, a minor drop in aspartic acid,

glutamic acid concentration was observed for LPI-Q, and LPI-R compared to the LPI-D, while for most of the other amino acids no change was observed after conjugation (Table 3.2), suggesting that grafting of the polyphenol to the conjugate had little effect on the overall ratio of AA with values differing by <1%.

Table 3.2 Amino acid (AA) profiles of the protein and protein-polyphenol conjugates (normalized to 100% for comparative purposes). Analysis was made with one replicate.

	Percent weight of total amino acids				
AA	LPI-UD	LPI-D	LPI-Q	LPI-R	LPI-E
Aspartic Acid	13.5	13.0	12.0	11.8	12.9
Glutamic Acid	20.2	18.6	17.6	17.2	18.6
Serine	7.4	6.3	8.7	6.4	6.5
Glycine	3.8	4.0	3.9	3.6	3.5
Histidine	2.8	2.6	2.7	2.5	2.7
Arginine	9.1	8.0	8.3	7.6	8.8
Threonine	3.1	3.1	3.3	3.1	3.0
Alanine	4.1	4.5	4.8	4.3	4.1
Proline	3.8	4.1	4.1	4.1	4.0
Tyrosine	3.3	3.5	3.3	3.4	3.3
Valine	4.4	5.0	5.0	5.0	4.8
Methionine	0.4	0.7	0.5	0.7	0.6
Cystine	0.7	0.8	0.6	0.8	0.8
Isoleucine	4.1	4.6	4.5	5.0	4.8
Leucine	7.1	8.2	7.8	8.8	8.3
Phenylalanine	4.8	5.5	5.3	6.3	5.7
Lysine	6.7	6.7	7.1	8.1	6.8
Tryptophan	0.9	1.1	0.8	1.1	1.1
Total AA	100	100	100	100	100

3.4.2. Structural characterization of the protein-polyphenol conjugates

3.4.2.1. FTIR analysis

FTIR spectroscopy provided an insight into the changes in the secondary structure of protein and was used to characterize the effect of LPI-polyphenol complexation. Information from stretching or bending of the peptide chains as determined by the amide bands I, II, and III is useful for decoding changes of secondary structures such as: α-helix, β-turns, β-sheet and random coils of the protein (Liu et al., 2015). Figure 3.2 presents the deconvolution and Gaussian curve-fitting for

the control LPI-UD over the spectral range of 1600-1700 cm^{-1} (amide I region, representative of C–O stretching/hydrogen bonding coupled with COO–). The curve is deconvoluted to accommodate contributions from α-helix, β-turns, β-sheet and random coils. The area percent from each of these curves is tabulated in Table 3.3. The IR frequencies in the amide I region (mainly C=O stretch) are segmented as follows: strong bands around 1,654 cm^{-1} are observed for α-helical conformed proteins, bands around 1,625 cm^{-1} are reported for β-sheets, while, β-turns and random coils are generally assigned the bands around 1,673 and 1,645 cm^{-1} respectively (Pelton & McLean, 2000; Torii et al., 1996; Goormaghtigh et al., 1994).

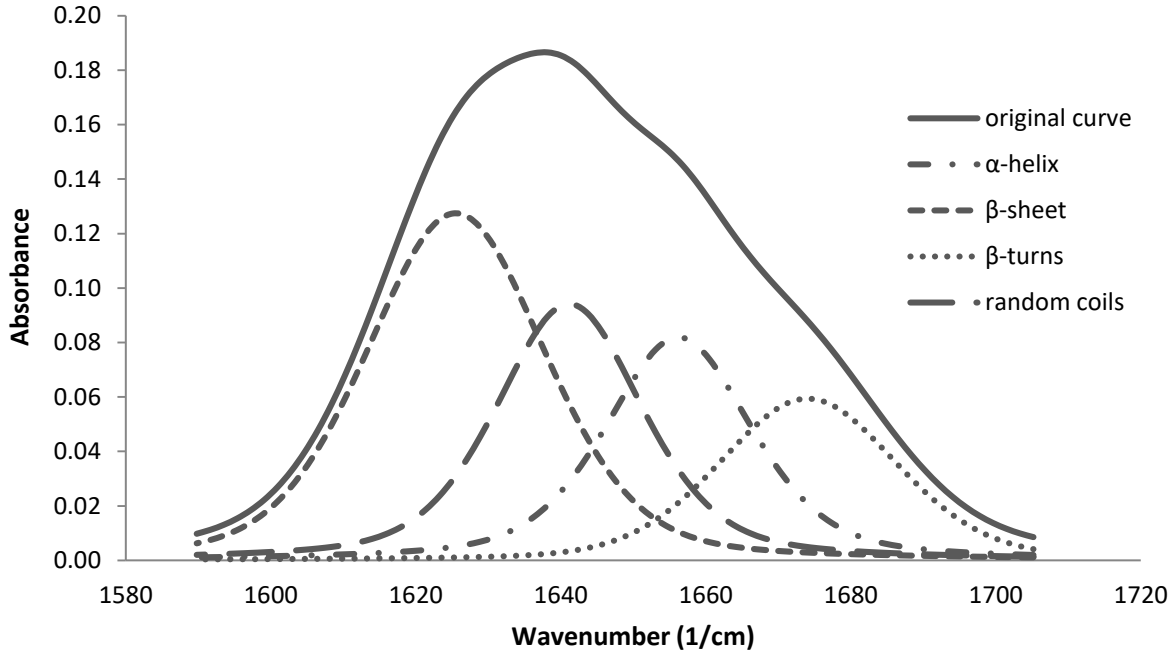


Figure 3.2 FTIR spectra of the amide I region of control LPI-UD deconvoluted to show the contribution from α-helix, β-turns, β-sheet and random coils.

The fractions of α-helix, β-sheet, β-turns and random coils were estimated using WiRE 3.3 software (Renishaw Canada Limited, Mississauga, ON) and are given in Figure 3.3. Complexation of protein with different polyphenols causes a full or partial unfolding or denaturation of the protein chain and thus alters their secondary and tertiary structures. There was no significant change in LPI due to dialysis as the content of all secondary structures between LPI-UD and LPI-

D remained unchanged. No significant change in α-helix and β-turn content was observed for the conjugates compared to the LPI-D. However, stabilization of the α-helix came at a cost of subsequent loss in the β-sheet content for all LPI conjugates compared to the LPI control. Roy et al. (2012) reported similar changes for the interactions of (+)-catechin and (+)-epicatechin with human serum albumin using FTIR. Additionally, the fraction of random coil structure for all conjugates significantly increased compared to the LPI control, suggesting possible uncoiling and exposure of hydrophobic sites to facilitate conjugation, leading to a more unordered protein structure.

Table 3.3 Percent area contributed by the secondary structure elements in the control LPI-UD sample.

Secondary structure	Peak area	Area (%)
β-turns	2.0	17.0
α-helix	2.5	21.2
random coils	2.9	24.3
β-sheet	4.4	37.6

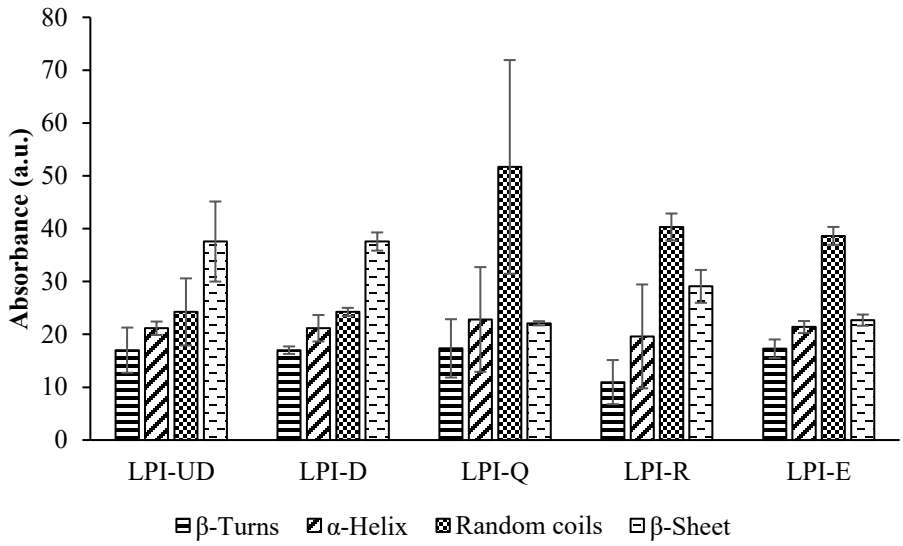


Figure 3.3 Secondary structure fractions of LPI-D and LPI-UD, LPI-Q and LPI-R, and LPI-E from the deconvolution of the amide I region of the FTIR spectra. Data represent the mean ± one standard deviation (n =3).

3.4.2.2. Fluorescence spectroscopy

Conformational changes in LPI were evaluated by measuring the intrinsic fluorescence intensity of the aromatic side chains of phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) residues on the protein. Changes in the emission spectra of these amino acids are common in the presence of an external stimuli and is often considered in the analysis of protein folding and ligand associations (Labieniec & Gabryelak, 2006). The effect of polyphenol conjugation on the LPI fluorescence intensity is shown in Figure 3.4 in the wavelength range of 320-350 nm. We also observed an increase in the fluorescence intensity of LPI-D from LPI-UD. Dialysis could have removed some shorter chain peptides from LPI and led to the exposure of buried aromatic amino residues and therefore higher fluorescence intensity. In addition, conjugation with Q, R and E resulted in an appreciable decrease in the fluorescence intensity of LPI-UD ($p < 0.05$). Drop in fluorescence intensity is normally seen due to the quenching of the aromatic amino acids by polar or charged species (e.g., polyphenols). It is postulated that the polyphenols, that were unable to penetrate into the hydrophobic core of the protein, were bound to the aromatic amino acid residues

on the protein surface causing a depression in its fluorescence intensity (Skrt et al., 2012; Tian et al., 2004). Therefore, to infer from our results, LPI-E showed the maximum (hence lowest fluorescence intensity) and LPI-Q showed the least (hence highest fluorescence intensity) preference towards binding with these aromatic side chains on LPI. Moreover, the peak emission wavelength for LPI-UD was obtained at 333 nm with no parallel red shift of the spectral maxima with subsequent conjugation. This suggests that the aromatic amino residues of the protein were not exposed to the aqueous phase due to dialysis or conjugation with polyphenols (Lakowicz, 2004). These observations were consistent with the result of Rawel et al. (2002a), who studied the interactions of different phenolic acids and flavonoids with soy proteins and also reported a decrease in fluorescence intensity upon conjugation with no wavelength shift.

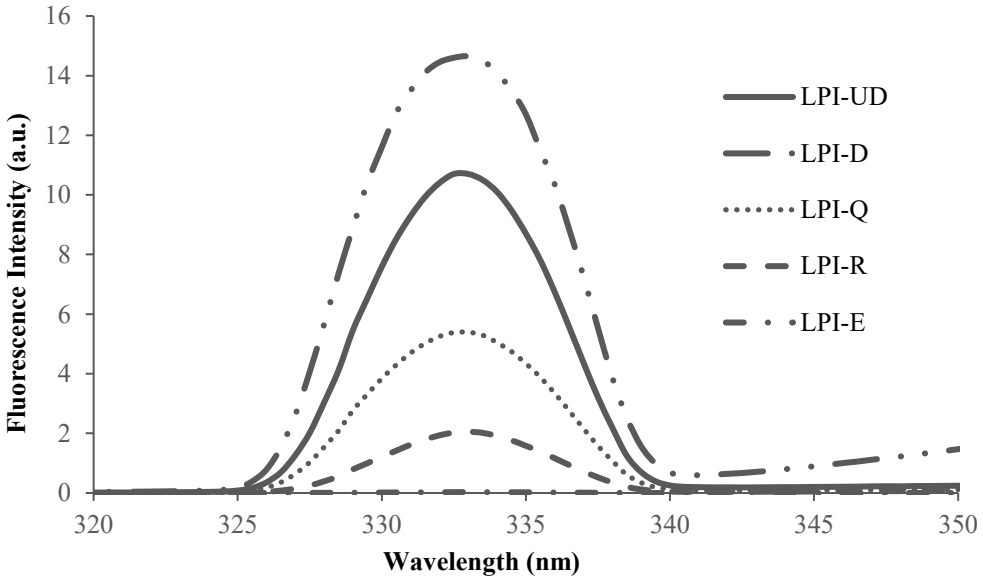


Figure 3.4 Fluorescence intensity of LPI-UD, LPI-D and conjugates of LPI-Q and LPI-R, and LPI-E at 332 nm wavelength. Data represent the mean of three curves.

3.4.2.3. Surface hydrophobicity

The surface hydrophobicity values of LPI-UD, LPI-D and its conjugates are compared in Figure 3.5. Among all the samples tested, LPI had the highest surface hydrophobicity (403.57 ± 4.7) which decreased significantly for the conjugates. The surface hydrophobicity value for LPI is comparable with the report by Joshi et al. (2012). Surface hydrophobicity for the conjugates

followed the order LPI-E > LPI-R > LPI-Q although not significantly different from each other. Surface hydrophobicity is a measure of ANS binding to the aromatic amino acid side chains of phenylalanine, tryptophan and tyrosine (Nakai and Li-Chan, 1988). Therefore, lower surface hydrophobicity for the conjugates suggests that the complexation of the polyphenols to these exposed amino acid side chains on the protein limits ANS binding to these sites, giving lower readings compared to the LPI control. It could also give information about the specificity of binding of the polyphenols to these aromatic amino acids. For example, lowest surface hydrophobicity of LPI-Q implies that Q is slightly more preferentially conjugated to the aromatic amino acids than E or R. High percentage of Q binding to the LPI (Table 3.1) may also be another reason for its lowest signal from ANS probe binding. Surface hydrophobicity has been correlated with emulsifying and foaming properties of food proteins (Kato & Nakai, 1980) as it influences intermolecular interactions taking place between protein-protein and protein-lipid and subsequent adsorption on the water-air/lipid interface (Beverung et al., 1999). The presence of free hydrophobic amino acids on the protein-polyphenol conjugates indicate their potential for surface activity and ability to stabilize emulsions and foams.

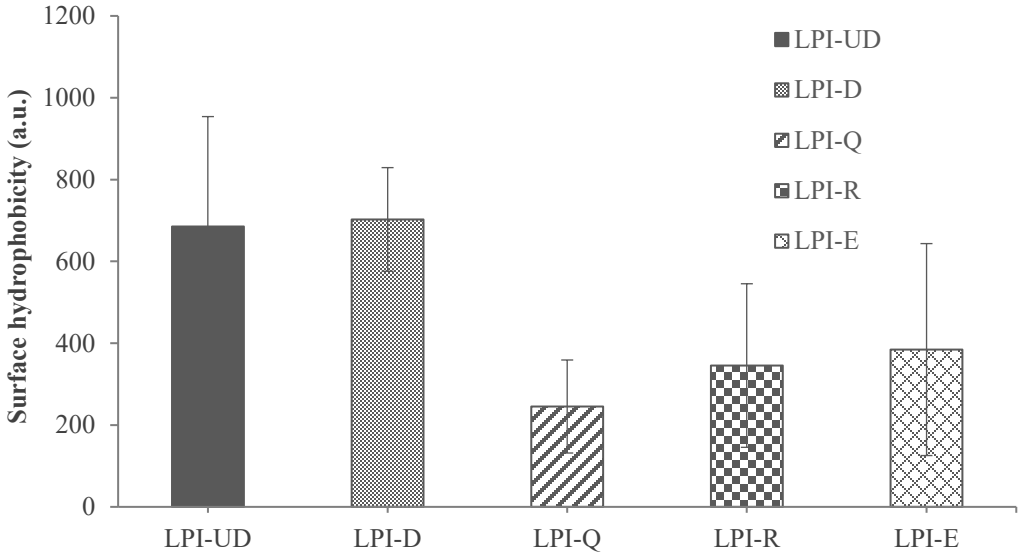


Figure 3.5 Surface hydrophobicity of LPI-UD, LPI-D and its conjugates LPI-Q, LPI-R, and LPI-E. Data represent the mean ± one standard deviation (n = 3).

3.4.2.4. Thermal behaviour of the protein and the conjugates

Heat treatment affects the structural and functional properties of globular proteins as it promotes thermal denaturation (Liu et al., 2012b). Thermal denaturation involves the transition of the protein from its folded to unfolded state (Privalov & Khechinashvili, 1974) which can be estimated using a differential scanning calorimeter (DSC). The temperature of protein denaturation gives information on its conformational stability. Peak denaturation temperatures (T_p) for LPI and its polyphenolic conjugates were analysed using a DSC and are listed in Table 3.4. The DSC profiles for all samples showed a single exothermic transition peak (data not shown) around 100-115°C, indicative of protein denaturation. T_p of LPI-UD and LPI-D were similar ($p > 0.05$), indicating that the alkaline treatment and dialysis did not alter the thermal stability and molecular conformation of LPI, consistent with the FTIR and surface hydrophobicity data (Figures 3.3 and 3.5, respectively). Joshi et al. (2011) reported average T_p for freeze-dried, spray-dried and vacuum-dried LPI ranging from 118 to 123 ºC. These differences were attributed to different cultivars, preparation method or concentrations of protein used for the analysis (Joshi et al., 2011). In the present case, T_p for all protein-polyphenol conjugates and the control LPIs were not significantly different ($p > 0.05$). Similar observations have been made by Liu et al. (2017a) when grafting quercetagetin and chlorogenic acid to zein protein. The T_p values of these conjugates were similar to the control zein. It was explained that, chlorogenic acid did not interact strongly with zein and further, the structural changes in zein induced by quercetagetin were not enough to promote modifications in its thermal stability, resulting in no appreciable change in the T_p value, similar to what we observed in the present study. Other studies (Kim & Cavaco-Paulo, 2012; Liu et al., 2016c; Rawel et al., 2002a) have, however, shown an increase in the T_p value of proteins upon its conjugation. According to Brandts and Lin (1990) if the ligand (polyphenol, in this case) binds to the native state of the protein an increase in the T_p would be observed indicating stability of the protein conformation; however, if the ligand interacts with the denatured protein, the T_p would decrease. Thermal stability of a protein in the existence of a ligand therefore, depends on the molecular state the protein is in.

Table 3.4 Thermal denaturation temperatures of LPI-D, LPI-UD and conjugates LPI-Q and LPI-R, LPI-E. Data represent the mean ± one standard deviation (n = 3).

Sample	Peak denaturation temperature (ºC)
LPI-UD	106.1 ± 0.0
LPI-D	106.1 ± 0.1
LPI-Q	105.8 ± 0.1
LPI-R	105.9 ± 0.1
LPI-E	106.1 ± 0.0

3.4.3. Functional properties of the protein-polyphenol conjugates

3.4.3.1. Turbidity

Turbidity of a dispersion holds an indirect measurement of the solute's solubility. For a protein, solubility becomes an important index of its functionality. Many researchers showed that higher solubility of proteins led to higher emulsification and foaming capabilities (Nick Pace et al., 2004; Sathe et al., 2018; Smith Jr et al., 1959). Proteins isolated from legumes frequently show low solubility and therefore, find limited commercial use (Karaca et al., 2011). The turbidity of LPI and its conjugates at two different pH values (2.0 and 7.0) is given in the Figure 3.6. Turbidity is minimum for the control LPI at both pH values, indicating highest solubility. After conjugation, however, the turbidity increased significantly ($p < 0.05$) for all LPI-polyphenol samples, which suggested a drop in the protein solubility due to the presence of polyphenols in their structure. For LPI-Q and LPI-E, turbidity decreased from pH 2.0 to 7.0 ($p < 0.05$), while for LPI-R the value at pH 2.0 was nearly half of that at pH 7.0. Of all the conjugates, LPI-R had lowest turbidity suggesting maximum solubility, which could be due to the presence of the disaccharide rutinose in the R molecule (Figure 3.1). Solubility in general requires a balance of protein-protein and protein-solvent interactions. While the former is enabled by hydrophobic interactions and promotes precipitation, the latter helps in protein hydration and solubilization (Damodaran, 1996). The presence of polyphenols could reduce protein-solvent interaction, leading to a drop in the solubility. Clearly, conjugation has led to a change in the intensity of the protein-protein vs. protein-solvent interactions which is why there are turbidity/solubility differences among

conjugates and controls. We tried to explain the turbidity results using zeta potential measurements of the protein and its conjugates at pH values 2.0 and 7.0 (Figure 3.7). At pH 7.0 (pH higher than LPI isoelectric point), all samples had a negative zeta potential, whereas at pH 2.0 (lower pH than the protein isoelectric point) they all displayed a positive zeta potential. A significant drop in zeta potential was observed at both pH values when the LPI was dialyzed, which could be due to the removal of charged protein fragments during dialysis. However, the values of zeta potential of the conjugates did not always correspond with their turbidity. For example, at pH 7.0 the zeta potential of LPI-R was similar to LPI-D ($p>0.05$), while its turbidity was much higher, hence solubility was poorer than LPI-D. Similarly, at pH 2.0, zeta potential of LPI-UD was similar to LPI-Q and LPI-R ($p>0.05$), while the turbidity of the latter two samples were much higher compared to LPI-UD, indicating poor solubility. Moreover, the increased turbidity for conjugates do not correlate well with their decreased surface hydrophobicity (Figure 3.5) which can only be explained by the existence of cross-linked proteins due to presence of polyphenols. Different authors reported a wide range of solubility of the protein-polyphenol conjugates compared to the protein. For example, Liu et al. (2015) found that solubility of lactoferrin at pH 7.0 improved upon conjugation with epigallocatechin gallate, chlorogenic acid and gallic acid, while Rawel et al. (2001) found a significant drop in solubility at the same pH when lysozyme was conjugated with gallic acid, ferulic acid and dihydroxybenzene. In contrast to what we obtained, Rawel et al. (2002a) having worked with plant proteins, reported that when soy glycinin was conjugated with quercetin the solubility of the conjugate improved at both pH 2.0 and 7.0 compared to the protein but not when it was conjugated to soy trypsin inhibitor. The difference, the group wrote, underlined the role of their structure and molecular parameters in reactions with quercetin. For the present study, the differences in the protein and polyphenol contents of the conjugates clearly influenced their turbidity. LPI-Q and LPI-E with lower protein but higher polyphenol content showed higher turbidity than LPI-R with a much higher protein and a much lower polyphenol content.

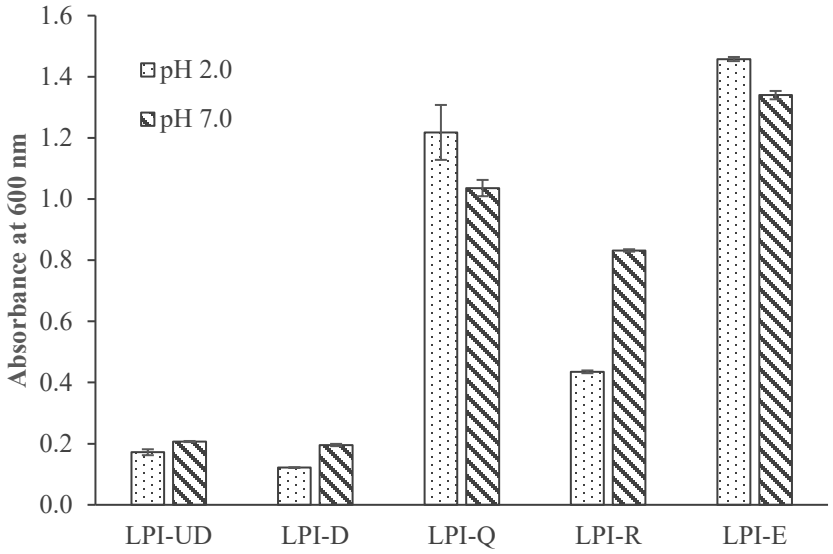


Figure 3.6 Turbidity of dispersions at pH 2.0 and pH 7.0 measured via absorbance at 600 nm. Data for LPI-UD, LPI-D and conjugates LPI-Q, LPI-R, and LPI-E are shown. Data represent the mean ± one standard deviation (n = 3).

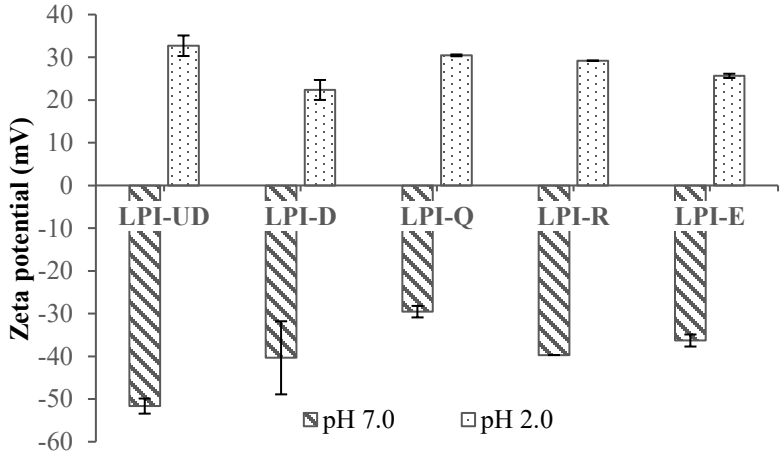


Figure 3.7 Zeta potential of protein and protein-polyphenol conjugates at pH 2.0 and 7.0.

3.4.3.2. Interfacial tension

Interfacial tension is a measure of a compound's surface activity and its ability to stabilize oil-water or air-water interfaces. The lower the interfacial tension, the higher its surface activity and

the better the ability to stabilize oil-water interface. In the present case, canola oil-water interfacial tension of LPI was about 15.2 ± 0.1 mN/m, which was lower than the pure oil-water interfacial tension (22.0 ± 0.3 mN/m), indicating good surface activity of LPI (Figure 3.8). When LPI was conjugated with polyphenols, its surface activity decreased, as can be seen from the increase in interfacial tension values of the LPI-polyphenol conjugates in Figure 3.8. For all conjugated samples, interfacial tension significantly increased from LPI ($p < 0.05$), ranging from 17.5 to 18.3 mN/m. No significant difference in interfacial tension was observed among the different conjugates ($p > 0.05$). The increased interfacial tension might be due to the decreased surface hydrophobicity of the modified protein (Figure 3.5). The interfacial activity was unchanged after modification of gelatin with tannic acid and oxidized ferulic acid and was also reported to have increased after complexing with oxidized caffeic acid (Aewsiri et al., 2009). From our results, however, even though the surface activity of conjugates decreased, their interfacial tension was still lower than the pure oil-water interface, indicating their potential for use in emulsion stabilization.

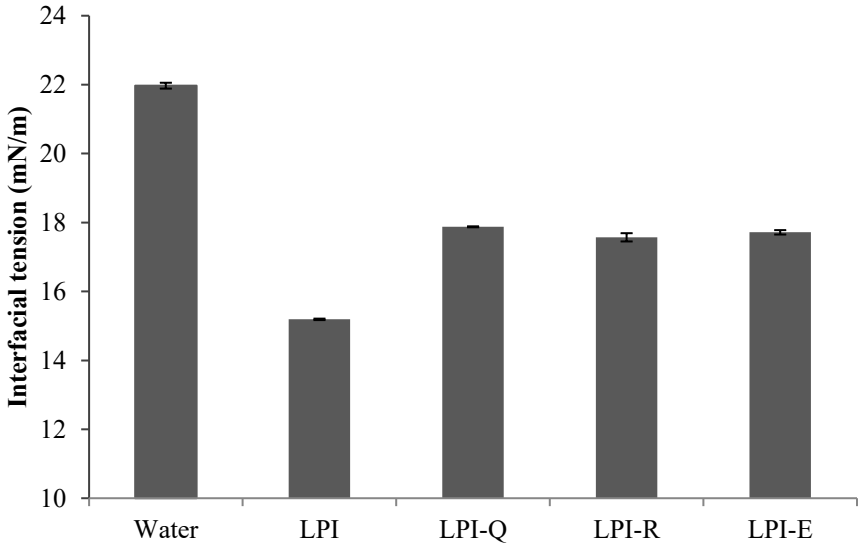


Figure 3.8 Canola oil-water interfacial tension of of LPI (control) and conjugates of LPI-Q, LPI-R, and LPI-E. For comparison, pure oil-water interfacial tension is also shown. Data represent the mean ± one standard deviation (n = 3).

3.4.3.3. Ferric reducing antioxidant power (FRAP)

Reducing power of a compound provides a measure of its potential antioxidant activity (Yıldırım et al., 2001). As the name suggests, the ferric reducing antioxidant potential (FRAP) assay measures the conversion of Fe^{3+} to Fe^{2+} by an antioxidant and was reported here as its Trolox equivalent (μmole/g sample). Figure 3.9 presents the FRAP values of LPI versus its conjugates and pure polyphenols. Reducing power among the conjugates were observed in the following order: LPI-Q conjugate > LPI-E conjugate > LPI-R conjugate. The activities of these conjugates were 5 to 35-fold greater than LPI-UD or LPI-D which demonstrated negligible reducing potential. Pure polyphenols carried a significantly higher reducing power than their corresponding conjugates with LPI. The FRAP values for LPI-Q, LPI-R and LPI-E were 46%, 13% and 43% of their corresponding pure polyphenol. However, considering the extent of conjugation was only 21%, 4.4% and 11.7% for Q, R and E (Table 3.1), respectively, these findings demonstrated that binding polyphenols to the protein synergistically improved their antioxidant capacity. The performance of polyphenols as antioxidants depends on the bond dissociation enthalpy (BDE) of phenolic O–H bond, and the steric crowding around the group (Amorati & Valgimigli, 2012). It is known that electron-donating (ED) groups lower the BDE, while electron-withdrawing (EW) groups increase the BDE. If the polyphenol is covalently attached to an ED group on the protein (such as glutamic acid), it could lead to a greater stability of the phenoxyl radical formed after the H-atom abstraction, which would explain, for example 43% higher reducing ability of LPI-E, at just 11.7% conjugation. Therefore, the type of amino acid residues the polyphenol shows preference to during conjugation, could have an influence on its antioxidant potential. These results show adequate potency of the conjugates to donate electrons to the Fe3+ ions, reducing them to Fe2+. Conjugating polyphenols to the LPI could be a novel way to impart reducing ability to the proteins.

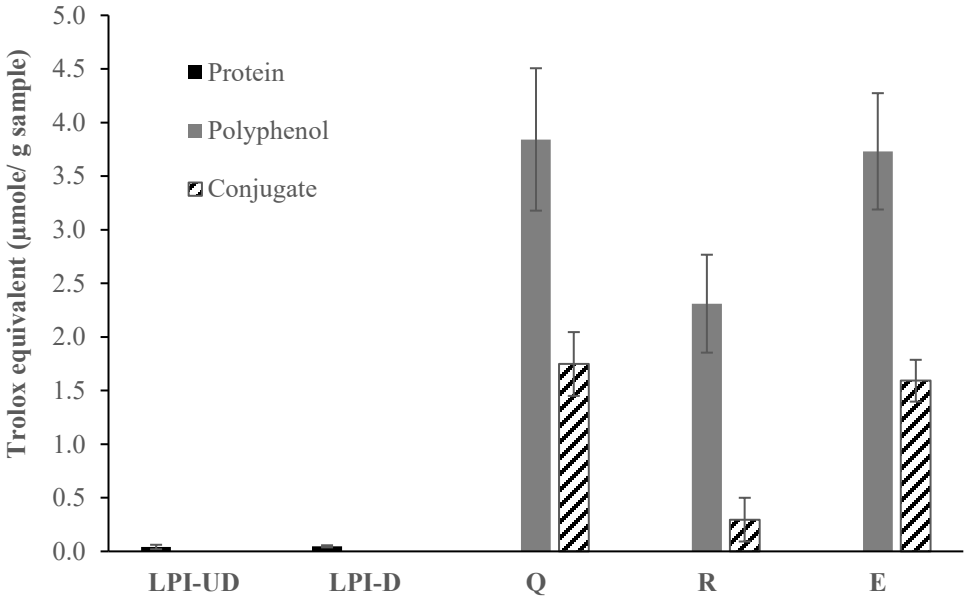


Figure 3.9 FRAP antioxidant activity expressed as µmoles of trolox per g of sample of LPI, its conjugates, and pure polyphenols. Data represent the mean ± one standard deviation (n = 3).

3.4.3.4. DPPH˙ scavenging activity

Antioxidant capacities of the conjugates were tested in vitro using DPPH˙ (2,2-diphenyl-1-picrylhydrazyl) radical. DPPH˙ is a stable free-radical which gains its diamagnetic properties (paired electrons) upon neutralization (Sharma & Bhat, 2009). The radical has an intense violet color with strong absorbance centred around 520 nm. DPPH˙ turns colorless or pale yellow when neutralized and the disappearance of color is directly correlated to the antioxidant capacity of the sample being tested. Figure 3.10 reports the DPPH˙ radical scavenging ability of the conjugates compared to pure polyphenols expressed as Trolox equivalent (µmole Trolox/g sample). Control LPI (both before and after dialysis) gave negligible DPPH˙ radical scavenging even at high concentrations (1-5 mg/mL) and therefore was not included in Figure 3.10. Of the three pure polyphenols tested, DPPH˙ scavenging power was obtained highest for Q, consistent with the findings from Kumari et al. (2010) and Apak et al. (2007). However, unlike others (Fukumoto & Mazza, 2000), we found DPPH˙ radical powers similar for R and E. Furthermore, the protein-polyphenol conjugates displayed a lower scavenging ability than their corresponding pure

polyphenols. Values for LPI-Q, LPI-R and LPI-E were however, 66, 82 and 61% of their polyphenol counterparts when the degree of conjugation was 21%, 4.4% and 11.7% for LPI-Q, LPI-R and LPI-E, respectively (Table 3.1). These results suggest that when these polyphenols were conjugated to LPI, the overall antioxidant activity of the protein was evidently increased. For example, it is interesting to note that the DPPH˙ scavenging powers of LPI-R was significantly higher than LPI-E, while the latter had nearly three times more polyphenol conjugated to the protein. This indicates that the ability of R to scavenge free radicals improved after conjugation to a greater extent compared to both Q and E. It is previously stated in literature that compounds having a free catechol group on the B-ring (like Q and R) are shown to be the most potent towards free radical scavenging activity, although the blockage of hydroxyl group at the C7 position has a slightly negative effect on R when compared to Q (Kessler et al., 2003) (Figure 2.4). This is reflected well in Figure 3.10. However, an antioxidant may exhibit different behavior in scavenging radicals in different solvent environment (Shen et al., 2005). All the samples tested under the DPPH˙ assay were dissolved in 50% methanol, keeping in mind the solubility of protein and polyphenol in the conjugates. With the polarity of the polyphenols following order R > Q > E, the partial solubility of LPI-Q and LPI-E in 50% methanol could have interfered with the radical scavenging property. Pulido et al. (2000) evaluated the antioxidant dose required to cause a 50% inhibition under FRAP and DPPH˙ assays in 100% methanol or 100% distilled water to show that the antioxidants they used (quercetin, gallic acid, catechin, caffeic acid, resveratrol, Trolox, ascorbic acid) performed better in the former than the latter. The variability observed between the DPPH and FRAP assays could also be due to the differences in target compounds being reduced/quenched since iron is used in the FRAP and DPPH in the other.

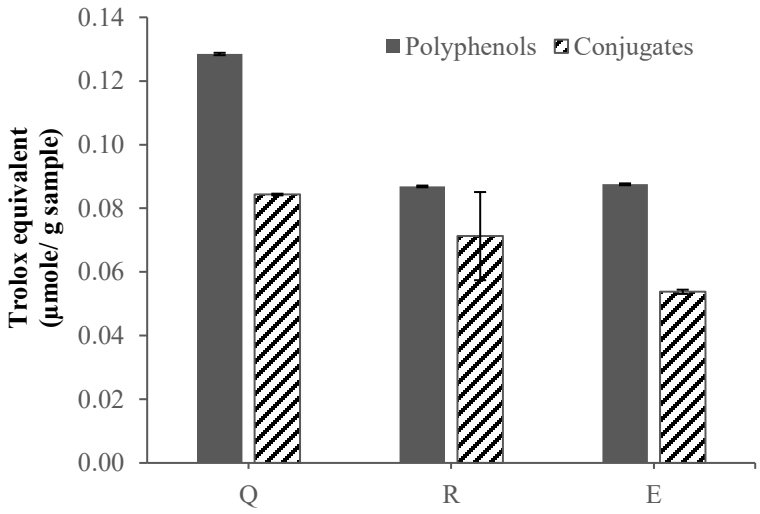


Figure 3.10 Antioxidant activity of pure polyphenols and LPI-polyphenol conjugates expressed as Trolox equivalent (µmole Trolox/g sample). Data represent the mean ± one standard deviation (n = 3).

3.5. Conclusion

To conclude, it was possible to form conjugates of polyphenol with LPI using a relatively simple alkaline grafting method. The resulting complexation was able to alter and, in some cases, improve the functional performance of LPI. Highest conjugation was obtained for Q, followed by E and R. FTIR analysis of protein secondary structure showed no significant change in α-helix and β-turn content, however, a significant loss in β-sheet content and increase in random coil structure was observed upon conjugation, which suggests possible uncoiling and exposure of hydrophobic sites to facilitate conjugation, thus leading to a more unordered protein structure. Fluorescence intensity of the conjugates dropped significantly from the pure LPI, which was attributed to the quenching of the aromatic amino acid signals by the bound polyphenols. Surface hydrophobicity of the conjugates, measured via ANS probe binding to the free aromatic amino acid, also decreased upon conjugation as the polyphenols limited the ANS binding sites on the protein molecules. However, no change in the thermal behaviour of the conjugates was observed compared to the pure protein. Functional properties of the protein-polyphenol conjugates were also significantly changed compared to the pure proteins. Both solubility and surface activity of conjugates decreased compared to the pure LPI, however surface activity of the conjugates were still better than pure

oil-water interface, which could facilitate their ability to form stable emulsions. Finally, significant improvement in antioxidative properties to LPI was observed upon conjugation with the polyphenols. DPPH and FRAP assays also seemed to show better results for the prepared conjugates when compared to their pure polyphenol counterpart and the degree of substitution they hold, which would be important if the protein-polyphenol conjugates are to be further applied in the encapsulation and delivery of sensitive bioactive lipids.

3.6. Connection to the next study

In the present chapter, LPI-polyphenol conjugates were prepared and characterized. The conjugates differed in their structural and functional properties, which was chiefly due to the type and concentration of polyphenols, amount of protein and their mutual interactions. It was observed that the conjugates lowered the interfacial tension between oil and water to a fair extent and therefore, were used in stabilizing oil-in-water emulsions. Improved antioxidative properties of the conjugates was also a significant result from the present chapter. For the same reason, the second chapter also investigated the prevention of lipid oxidation by localizing lentil-portein polyphenol conjugates at the oil-water interface in flaxseed oil-in-water emulsions.

4. PHYSICAL AND OXIDATIVE STABILITY OF FLAXSEED OIL-IN-WATER EMULSIONS STABILIZED WITH LENTIL PROTEIN-POLYPHENOL CONJUGATES

4.1. Abstract

The present work was aimed at evaluating the potential of lentil protein isolate (LPI) and polyphenol conjugates as emulsifiers in inhibiting lipid oxidation in 5 wt% flaxseed oil-in-water emulsions. Quercetin (Q) and ellagic acid (E) were chosen to selectively complex with LPI. The emulsions stabilized using LPI-Q and LPI-E conjugates show superior oxidative stability compared to those stabilized using LPI-undialyzed or LPI-dialysed emulsifiers and compared to those with an equivalent amount of Q or E in their respective aqueous phases. All emulsions were stable with an average droplet size less than 3 μm, while upon 4-week storage some increase in droplet was observed. Visually emulsions were stable to phase separation when stored at 5 ºC, while at 25 ºC they showed extensive phase separation. Emulsions prepared using the conjugates showed limited stability towards changes in environmental factors such as ionic strength, and heat treatment, while their stability against change in pH was better. Confocal laser scanning microscopy allowed us to observe the distribution of the conjugates around the oil droplets. These findings show that LPI-Q and LPI-E can act as efficient emulsifier to protect functional oil cores from oxidative degradation in emulsion because of their higher antioxidant activity and interfacial accumulation of polyphenols.

4.2. Introduction

Antioxidants present in our food help to limit oxidative damage to cells, DNA, lipids and so forth (Scalbert et al., 2005). In recent years, there has been a pressing incentive to shift to naturally sourced antioxidants due to speculated carcinogenicity of synthetic ones (Iverson, 1995) or to simply keep up with the trend of avoiding or minimizing the use of artificial additives (Frankel, 1999). Plant-derived polyphenols make for powerful natural antioxidants. The phenolic groups in polyphenols readily donate a hydrogen atom or an electron to the free radicals, neutralizing them, while forming a relatively stable phenoxy radical and terminate the chain propagation step of the oxidation mechanism (Kehrer, 1994). The initial concentration of polyphenols in plant-based foods

is near its optimum value and any fortification with polyphenols has led to only minor changes (Pokorný et al., 2001). However, it may be useful to add polyphenols to products where a rapid dissipation of antioxidants is observed, for example in plant oils rich in polyunsaturated fatty acids (flaxseed oil, soybean oil), that are widely used in food formulations for direct human consumption (Waterhouse et al., 2014). These oils are susceptible to oxidative deterioration, especially when present in an emulsion and exposed to oxygen, heat, light, moisture and transition metal ions.

Lipid oxidation in foods results in the loss of nutrients and flavor, and the production of deleterious products such as reactive oxidation species (ROS). Protecting the oil quality therefore becomes imperative and critical for the food industry. Oil encapsulation and addition of antioxidants have been used to develop protective delivery systems appropriate for the incorporation of polyunsaturated oils into the human diet (Waterhouse et al., 2014). The present study explores plant protein-polyphenol conjugates as emulsifiers to develop flaxseed oil-in-water emulsions and investigate their potential role in reducing the rate of oxidation of the lipid core. There are evidences in literature that suggest that locating antioxidants at the oil-water interface can help to control lipid oxidation in emulsions (Berton et al., 2011). For example, emulsions prepared using zein/chitosan particles loaded with curcumin showed improved stability against lipid oxidation compared to those prepared with zein/chitosan particles with curcumin dispersed in the oil phase, suggesting an interfacial role of curcumin in controlling lipid oxidation (Wang et al., 2015a).

Recently, plant proteins extracted from legumes have attracted the interest of the food industry because they are an effective and cheaper alternative to animal proteins with beneficial health effects and nutritional value (Duranti, 2006). Furthermore, to derive functional benefits, researchers have investigated emulsifying properties of legume proteins (Bora, 2002; Boye et al., 2010). Chang et al. (2015) investigated emulsifying and physicochemical properties of lentil, pea and canola protein isolates and found that LPI produced the most stable emulsions and therefore was accepted for the present study.

Flavonoids and phenolic acids are abundant bioactive compounds in plants and are making their way into commercial foods and beverages (Ho, 1992). Flavonoids carry a core structure with two aromatic rings joined in a chroman structure by a three-carbon unit: C6-C3-C6 (Macheix et al., 1990). Quercetin (Q), the flavonoid of interest in the present study, is widely distributed in plant-based foods such as onions, tea, apples and exhibits poor water and oil solubility (Di Mattia et al.,

2010). Ellagic acid (E), a phenolic acid also used in this study, is a dimeric derivative of gallic acid. It is found in woody plants, berries, grapes, and nuts and exhibits better water solubility than Q while its oil-solubility is quite poor (Talcott & Lee, 2002).

The objective of the present work was to evaluate the physical and oxidative stability of flaxseed oil-in-water emulsions stabilized using lentil protein isolate (LPI) and polyphenol conjugates in comparison with emulsions prepared with unconjugated LPI. Flaxseed oil was chosen because of the higher degree of unsaturation and therefore, its faster oxidation. All emulsions were characterized for their physicochemical properties, stability against environmental stresses and finally by measuring lipid oxidation where the effect of the presence of protein-polyphenols conjugates at the oil droplet surface was compared with emulsions stabilized by non-conjugated proteins containing an equivalent quantity of the polyphenol in the bulk aqueous phase.

4.3. Materials and methods

4.3.1. Materials

Lentil protein isolate (protein content 75.2% w/w, w.b.) was kindly provided by POS Bio-sciences (Saskatoon, SK, Canada), produced using isoelectric precipitation in a pilot-scale facility. Flaxseed oil (Willow Creek Organic Grain Co., Watson, SK, Canada) used in this study was cold pressed organic and purchased from a local supermarket (Federated Co-operatives Limited, Saskatoon, SK, Canada). The oil had no added antioxidants as declared by the supplier. Quercetin (Q, purity ≥ 95%) was obtained from Millipore-Sigma (Oakville, Ontario, Canada). Ellagic acid (E, purity ≥ 97%) was obtained from VWR International (Edmonton, Alberta, Canada). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade.

4.3.2. Preparation of protein-polyphenol conjugates

The protein-polyphenol conjugates were prepared following the method described in section 3.3.2.

4.3.3. Preparation of flaxseed oil-in-water emulsions

Undialysed (LPI-UD) and dialysed LPI (LPI-D), LPI-Q and LPI-E conjugates were dispersed in deionized water at a protein concentration of 1 wt% and pH 7.0. To prevent microbial growth sodium azide (0.02% w/v) was added and the protein solutions were stirred overnight to ensure

complete dissolution. Emulsions were prepared by adding 5 wt% flaxseed oil to 95 wt% protein solutions, mixing with a rotor-stator blender (Polytron, Brinkman, ON, Canada) at power setting of 5 for 2 min to form a coarse emulsion, followed by homogenizing using a high-pressure homogenizer (Emulsiflex C3, Avestin Inc., Ottawa, ON, Canada) at 138 MPa (20,000 psi) for 8 cycles. The temperature of the emulsions during homogenization was kept below 20 ºC using a cooling coil submerged in ice water bath. The emulsions were transferred to 120 mL glass bottles (VWR International, Edmonton, AB, Canada), wrapped in foil to prevent any exposure to direct light, and stored in a refrigerator (~5 ºC) for further analysis. All emulsions were prepared in triplicate.

4.3.4. Particle characterization

4.3.4.1. Droplet size and size distribution

Volume average droplet size (d_{43}) and size distribution of the emulsions were determined using a laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments, Montreal, QC, Canada). The relative refractive index of the dispersed versus continuous phases was used as 1.479. The emulsions were gently shaken before the experiment. Drops of emulsion was added to the sample dispersion unit of the instrument until the obscuration index reached about 15% before starting the measurement.

4.3.4.2. Zeta potential

A zeta potential analyzer (Zetasizer 90, Malvern Instruments, Montreal, QC, Canada) was used to measure the magnitude of electrostatic charge on the emulsion droplets. The emulsions were diluted (1:500) with pH adjusted water (7.0) and injected into the cuvette containing the electrodes, transferred into the instrument and equilibrated for 120 s before collecting the droplet charge data. The zeta potentials (mV) were obtained by measuring the direction and velocity of the droplet movement in a well-defined electric field.

4.3.4.3. Confocal Laser Scanning Microscopy

All samples were prepared by adding 0.01 wt% fast green (excitation by 633 nm laser, emission collected using a 650 nm long pass filter) to the final emulsion to stain the proteins within the

continuous phase and droplet interface. Microstructure of the emulsions were examined using a Nikon C2 microscope (Nikon Inc., Mississauga, ON, Canada) using 633 nm laser, a 60× Plan Apo VC (numerical aperture 1.4) oil immersion objective lens and 2.5 times digital zoom.

4.3.5. Creaming stability

4.3.5.1. Visual observation

Visual observation for the extent of creaming of the emulsions in 20 mL glass vials was recorded with a digital camera after every week of storage for 4 weeks for the samples stored at room temperature (25 ± 1 ºC) and in a refrigerator at 5 ºC.

4.3.5.2. Accelerated gravitational separation

Physical stability of the emulsions was also analyzed using a photocentrifuge dispersion analyzer (LUMiSizer, LUM Americas, Boulder, CO, USA) to determine the instability index and creaming rate of freshly prepared emulsions at an accelerated gravitation. In brief, 380 µL of freshly prepared nanoemulsions were transferred into 8 mm x 2 mm rectangular polycarbonate cuvettes and centrifuged at 2000 × g for 16 h and temperature 25 ºC. Data analysis and calculation of separation or instability index was done using the SEPView software v 4.1 (LUM GmbH, Berlin, Germany).

4.3.6. Effect of different environmental conditions on emulsion stability

4.3.6.1. Effect of change in pH

The pH stability of the emulsions was evaluated by measuring the zeta potential and droplet size distribution at different pH values (pH 2.0 - 9.0). Hydrochloric acid (0.1 N) and sodium hydroxide (1.0 N) solutions were used to adjust the pH of the emulsions to the desired value and de-ionized water with pre-adjusted pH (same as the emulsion) was used to avoid multiple scattering effects during zeta potential analysis. All measurements were made at 25 ºC.

4.3.6.2. Effect of change in ionic strength

Emulsions were mixed with the same volume of NaCl solution to get final concentration of 0.1, 0.5 and 1 N NaCl. The mixture was stirred for 30 min at 120 rpm followed by incubation at 25 ºC

overnight. The stability of the emulsions was evaluated using the zeta potential and droplet size distribution analysis. For zeta potential analysis, emulsions were diluted with de-ionized water with pre-adjusted ionic strength (same as the emulsion). All measurements were made at 25 ºC.

4.3.6.3. Effect of heat treatment

To evaluate the thermal stability, emulsions in glass vials were incubated in a hot water bath at 90 ºC for 10 min, and then cooled and stored at 25 ºC for 24 h. Emulsion stability was evaluated using droplet size and zeta potential analysis. For the latter, the emulsions were diluted with de-ionized water with pre-adjusted pH (same as the emulsion) prior to analysis. All measurements were made at 25 ºC.

4.3.7. Oxidative stability of flaxseed oil in emulsions

Oxidative stability (peroxide value and *p*-anisidine value) of the oil phase of the emulsions (at pH 7.0) was determined at room temperature and at 5 ºC during four weeks of storage in 40 mL screw-capped clear glass vials (VWR International, Edmonton, AB, Canada) (unless stated otherwise) to allow exposure to light, an initiator of peroxidation.

4.3.7.1. Peroxide value (PV) determination

PV (meq. cumene hydroperoxide /kg oil) was determined according to the method described by Sun et al. (2007). A certain quantity (0.3 g) of emulsion was added to 2.8 mL isooctane/isopropanol (3:2, v/v) solution. This mixture was vortexed 3 times for 10 s each followed by centrifugation for 2 min at 10,000 rpm. A small amount (0.2 mL) of the clear upper solvent layer was collected and mixed with methanol/1-butanol (2:1, v/v) and thiocyanate/Fe^{2+} solution made by combining one part 3.94 M thiocyanate solution with one part 0.072 M Fe^{2+} solution (obtained from the supernatant of a freshly prepared mixture of one part 0.144 M FeSO4 and one part 0.132 M BaCl2 in 0.4 M HCl). The final mixture was left to incubate for 20 min at room temperature and absorbance was measured using a UV/visible spectrophotometer (Beckman DU 530, Beckman Coulter, Brea, CA, USA) at 510 nm. Lipid hydroperoxide content was determined using a cumene hydroperoxide standard curve where 0.3 g of cumene hydroperoxide was used instead of the emulsion following the same procedure as stated above (Richards et al., 2002).

4.3.7.2. p-Anisidine value (*p*-AV) determination

p-Anisidine value was determined according to the modifications set by British Standard Method (Hamilton & Rossell, 1986) and as described by Sun et al. (2007). About 1 g of the emulsion was added to a 25 mL volumetric flask and the flask was filled to volume by adding iso-octane. The contents of the flask were transferred to 50 ml clear polypropylene centrifuge tubes (VWR international, AB, Canada). The tubes were vortexed 2 times for 10 s each. This mixture was centrifuged for 10 min at 5000 rpm (International Equipment Co., Needham, Massachusetts, USA) and the supernatant was analyzed spectrophotometrically at 350 nm against pure isooctane and the absorbance was recorded as A1. Aliquots (5 mL) of the sample or isooctane (as blank) was then transferred to 10-mL test tubes and 1 mL para-anisidine solution (0.25% w/v solution in glacial acetic acid) was added to it. This mixture was centrifuged for 10 min at 5000 rpm and the supernatant was analyzed spectrophotometrically at 350 nm against isooctane containing para-anisidine (as blank) and the absorbance was recorded as A2. The *p*-AV value was calculated using the following equation, where sample mass refers to the amount of oil in the emulsion:

$$AV = \frac{25 \times (1.2 \times (A_2 - A_1))}{Sample\ mass} \quad (4.1)$$

4.3.7.3. Schaal oven test

The Schaal oven test is a tradictional method to measure the oxidative stability of oils especially in baked products (Moslavac et al., 2015). Samples were placed into 40 mL amber, airtight bottles (VWR International, Edmonton, AB, Canada) with screw caps and subjected to accelerated lipid oxidation in an hot air oven at 60 ± 2 °C for 24 h. The oxidative deterioration level of the emulsions was measured using PV and p-AV as described in section 4.3.7.1, and 4.3.7.2.

4.3.8. Statistics

All experiments were conducted in triplicate and reported as the mean ± one standard deviation. Statistical significance was determined using a one-way analysis of variance (ANOVA) available in Microsoft Excel (Microsoft Canada Co, Mississauga, ON, Canada) with a 95% confidence interval where $p < 0.05$ was considered as statistically significant.

4.4. Results and Discussion

4.4.1. Droplet size and size distribution

Figures 4.1a and 4.1b present the droplet size distribution for all emulsions prepared fresh and after 4 weeks of storage at room temperature, respectively. The initial pH values of the emulsions ranged from 6.7 to 7.0. For fresh emulsions, LPI-UD showed a monomodal distribution with a peak <1 μm, indicating the LPI concentration used for the emulsion preparation was sufficient for the droplet coverage under the given conditions (Wang et al., 2015b). A similar distribution for LPI-stabilized emulsion at 1 wt% protein was also reported by Primozic et al. (2017). Emulsions made using dialyzed LPI (LPI-D) presented a bimodal distribution same as the emulsions made using conjugated proteins, with a second smaller peak at >1 μm suggesting aggregated oil droplets and proteins in the continuous phase. Appearance of a second peak in the emulsion stabilized with dialyzed proteins could be due to the removal of proteins that are essential for emulsion stability during dialysis. After 4 weeks, the droplet size distribution for LPI-UD remained monomodal but shifted to the right (larger droplet size) with peak at >10 μm. LPI-D and LPI-E retained its bimodal distribution where the peak at smaller droplet size reduced in height, while the larger droplet size peak increased. Droplet size distribution of LPI- Q stabilized emulsion shifted right, where the peak <1 μm become smaller and the one >1 μm grew into a large peak over the duration of 4 weeks. With time, droplets flocculated and extensive protein network formed in the continuous phase entrapping the oil droplets and giving rise to the increased second peak in the distribution (Diftis et al., 2005; McClements, 2004b). Better long-term stability of emulsion stabilized with dialyzed protein compared to undialyzed could be due to the removal of components such as fibre from LPI that were responsible for protein aggregation. Holm and Eriksen (1980) evaluated the emulsifying capacity of undialysed and dialyzed potato protein concentrate in soybean oil-in-water emulsions and also found that dialysis removed low molecular weight material and therefore, had a high positive effect on the emulsification capacity (50% increase). A larger second peak for LPI-Q in contrast with LPI-E after 4 weeks of storage could be due to more protein and oil droplet aggregation with the former.

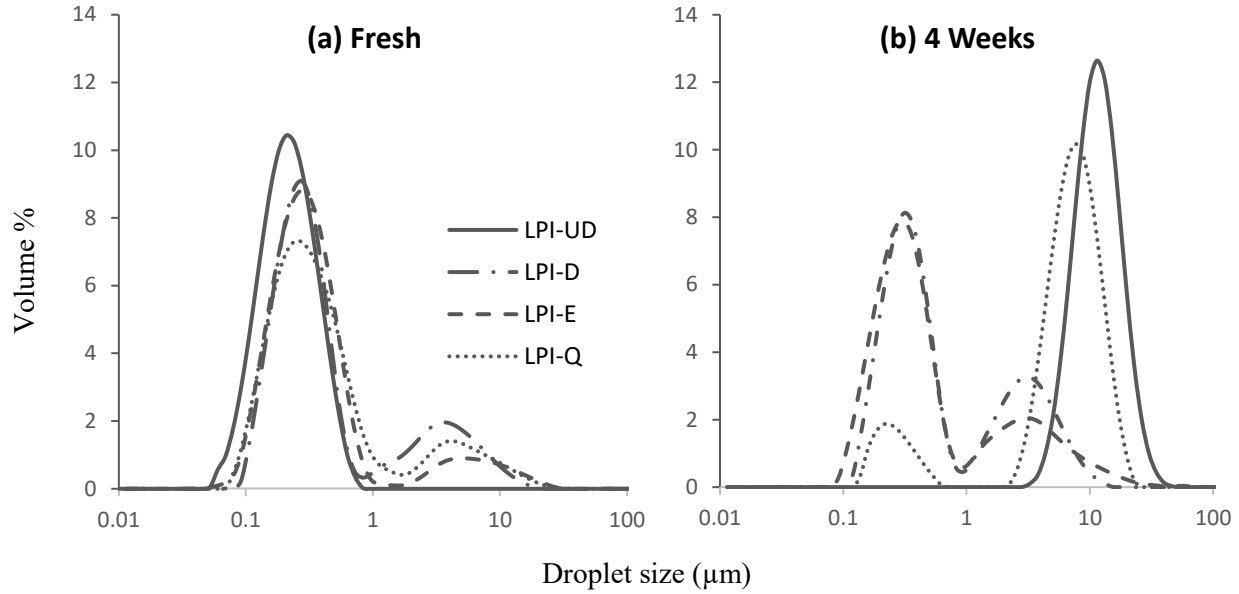


Figure 4.1 Droplet size distribution of 5 wt% flaxseed oil-in-water emulsions stabilized with 1 wt% LPI-UD, LPI-D, and the two LPI conjugates LPI-Q and LPI-E prepared (a) fresh and, (b) after 4 weeks.

Furthermore, to better assess the average droplet size of the emulsions, the d_{43} values were plotted in Figure 4.2. For the fresh emulsions, LPI-UD has an average droplet size of 0.2 ± 0.0 μm. However, average sizes for all other fresh emulsions were >1 μm and not statistically different from each other ($p > 0.05$). The wide difference between droplet sizes of LPI-UD and other emulsions suggests that dialysis followed by freeze-drying could have removed surface active proteins and influenced the surface activity of the remaining proteins. However, after 4 weeks of storage, droplet size for control LPI-UD stabilized emulsions increased to 19.3 ± 9.5 μm and was the largest among all emulsions. Although LPI-D, LPI-Q and LPI-E started with larger droplet sizes, after 4-weeks storage their droplet sizes followed the order: LPI-E (1.3 ± 0.4 μm) < LPI-D (2.8 ± 1.9 μm) < LPI-Q (6.6 ± 3.0 μm). Conjugating protein with polyphenol improved emulsion storage stability in case of LPI-E. A similar trend was reported by Feng et al. (2018) where the control emulsions made using dialyzed ovalbumin more than doubled over a period of 14 days storage compared to the emulsions made using ovalbumin and catechin complexes.

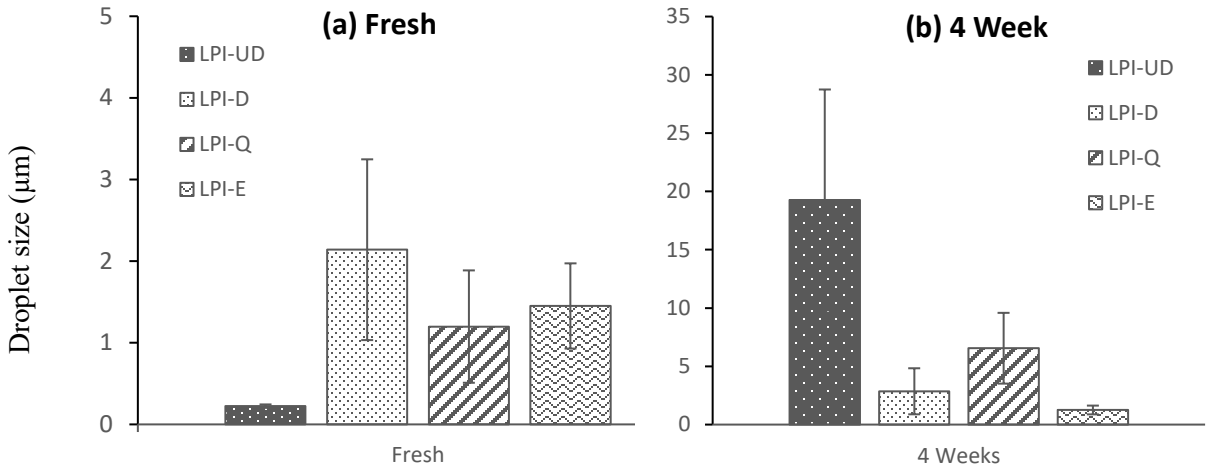


Figure 4.2 Volume average droplet diameter (d_{43}) of of 5 wt% flaxseed oil-in-water emulsions stabilized with 1 wt% LPI-UD, LPI-D, and the two LPI conjugates LPI-Q and LPI-E prepared (a) fresh and, (b) after 4 weeks. Values are means ± SD (n = 3).

4.4.2. Emulsion stability under accelerated gravitation

A photocentrifuge (LUMiSizer®) employs a centrifugal force to accelerate instability and subsequent phase separation (Xu et al., 2013). At the same time, a near-infrared light illuminates the entire sample cell and the instrument measures the intensity of this transmitted light as a function of time and position over the entire sample length. The integrated transmission profiles display the percent light transmittence per unit time, from which the creaming rate and instability index of the emulsions can be calculated (Liu et al., 2016b). The transmission profiles of the samples as a function of time and sample height in cuvettes is plotted in Figure 4.3. For all samples, the initial transmission of 80% dropped to almost zero when the laser reached the top surface of the emulsion in the cuvette. The red color initial transmission line remains close to zero, meaning no light was passing through the cuvette as the oil droplets and proteins were homogeneously distributed throughout the emulsion. Lerche (2002) observed similar small changes in the initial transmission profile at the end of the cuvette (at 127 mm) and called it the 'cuvette wall effect'. Centrifuging over time, the oil droplets moved toward the top forming a cream layer, leading to clarification of the bottom end of the cuvette. This progressive movement in the transmission profile is indicated by an arrow pointing towards the left of the graph. The more change in

transmission profile with centrifugation, the less stable an emulsion would be. In general, all emulsions displayed very similar profiles.

To achieve a better comparison of the emulsion stability under accelerated gravitation, the transmission profiles were quantified, and instability index (Figure 4.4a) and creaming velocities (Figure 4.4b) were calculated. Instability index is a dimensionless number between 0 and 1, where 0 indicates no change in transmission (highest stability) and 1 represents a complete separation of phases (lowest stability) under an applied force field. It was calculated from the ratio of clarification that was achieved due to phase separation at the end of the experiment to the maximum possible clarification (Detloff et al., 2013). Instability indices for all emulsions were > 0.85 and there were no significant differences among the different emulsion ($p > 0.05$).

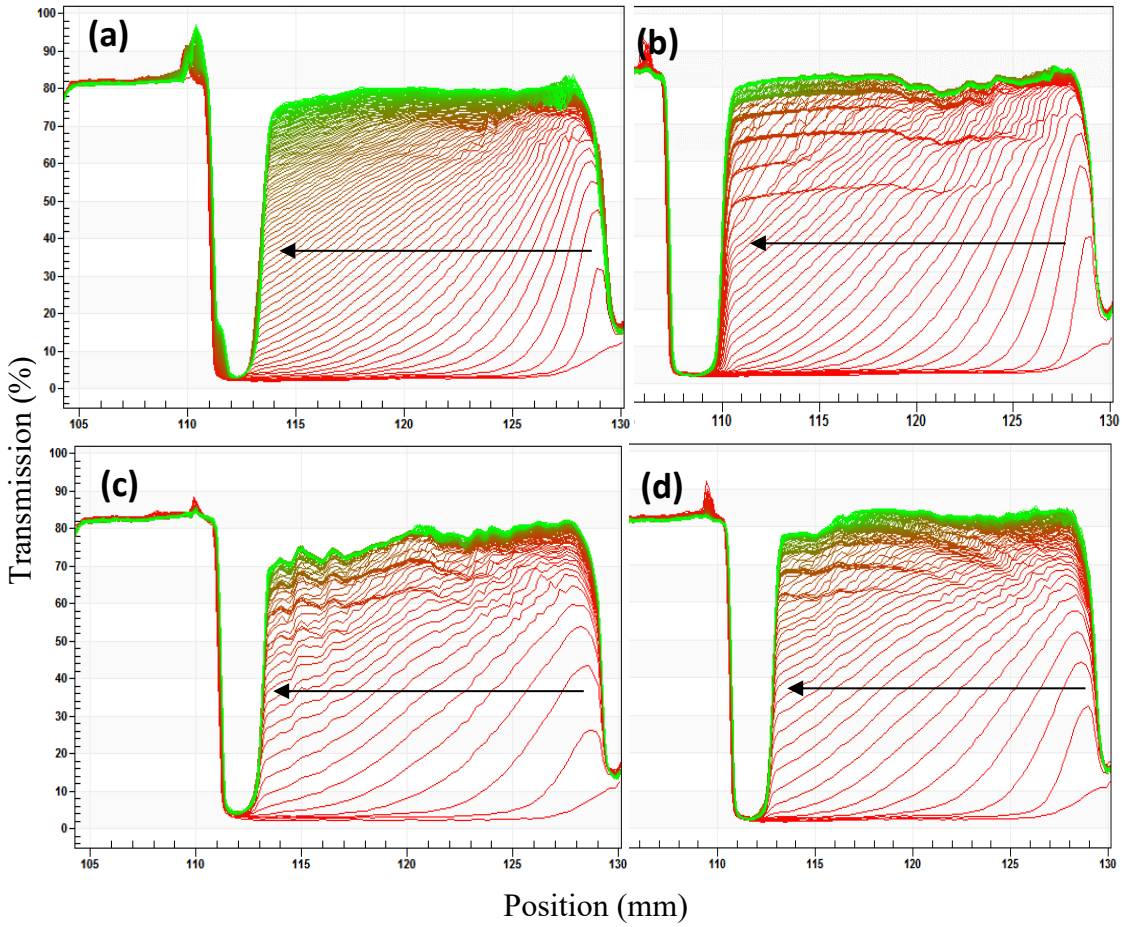


Figure 4.3 Photocentrifuge transmission profiles as a function of sample length in cuvettes for the emulsions made with (a) LPI-UD, (b) LPI-D, (c) LPI-Q, and (d) LPI-E. The color of the transmission profiles changed from red to green as the time of centrifugation progressed from 0 to 16 h. Arrows indicate movement of the transmission profiles as a function of time.

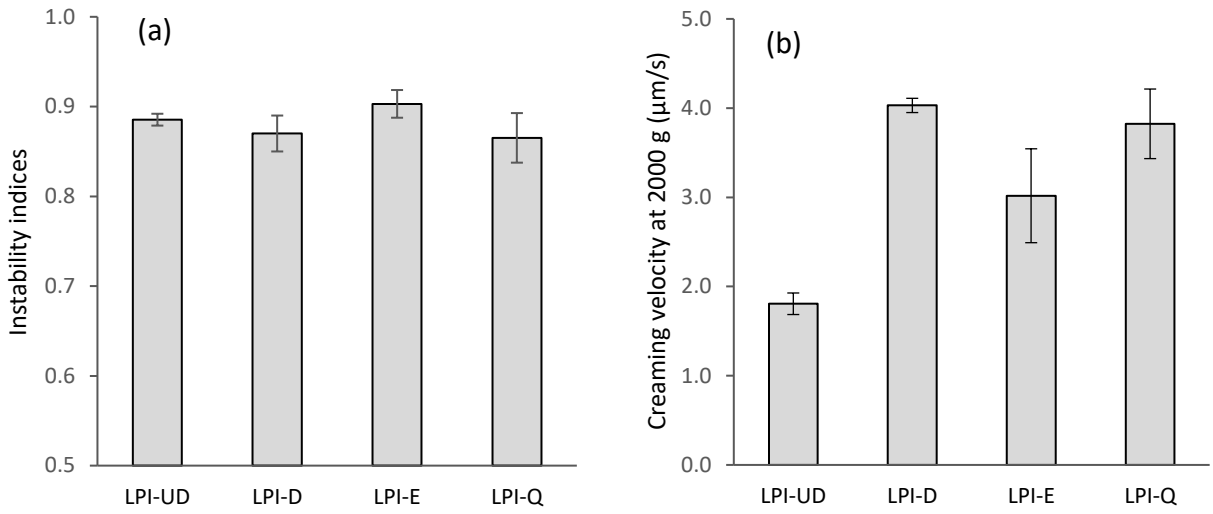


Figure 4.4 (a) Instability indices of freshly-made emulsions calculated from the photocentrifuge transmission profiles, and (b) creaming velocity at 2000×g for emulsions made using LPI (UD and D), LPI-Q and LPI-E conjugates. Values are means ± SD (n = 3).

Creaming rate was calculated from the slope of the plot of integrated transmission profile against time (Petzold et al., 2009). As shown in Figure 4.4b, creaming velocity was lowest for emulsions made using LPI-UD (1.8 ± 0.1 μm/s) indicating highest stability against gravitation (2000 × g). Dialysis of LPI (LPI-D) seemed to have increased the creaming rate to more than double the value (4.0 ± 0.1 μm/s). In fact, creaming velocities of emulsions made using conjugated dialyzed proteins were also greater than the emulsions made using LPI-UD. The creaming velocity under accelerated gravitation matched the droplet size of the freshly-prepared emulsions (Figure 2a), where the LPI-D emulsion with monodispersed distribution and lowest droplet size gave lowest creaming velocity, and LPI-D with highest droplet size gave highest creaming velocity. Additionally, emulsions made using LPI-E gave a lower creaming velocity indicating higher stability, which conflicts with our findings from its instability index. Yerramilli and Ghosh (2017), upon similar sightings claimed it to be a limitation of the centrifugal separation method where an oversaturation of the instrument detector in the presence of excess proteins in the emulsion continuous phase prevented clear detection of droplet movement. Liu et al. (2016c) reported a decrease in the emulsion creaming rate at earth gravitation following conjugation of lactoferrin with polyphenols. In the present case, compared to the LPI-D, a decrease in creaming velocity was observed for LPI-

E ($p < 0.05$), while for LPI-Q, no significant change was observed under accelerated gravitation ($p > 0.05$). Higher creaming rate for LPI-Q compared to LPI-E could be related to the presence of aggregated structure in the former as shown by microscopy (section 4.4.4).

4.4.3. Visual observation of emulsions stability

Visual observations were made to understand the extent of overall creaming and emulsion destabilization at normal gravitation. Pictures of the samples collected over the duration of the experiment - fresh (a) and after 4 weeks of storage (b) at 25 ºC are shown in Figure 4.5. The emulsions displayed different colors characteristic of the proteins and polyphenols used for their preparation. Emulsions made using LPI-Q and LPI-E conjugates were colored because of the polyphenols in them - Q and E were bright yellow and tan colored amorphous powders, respectively.

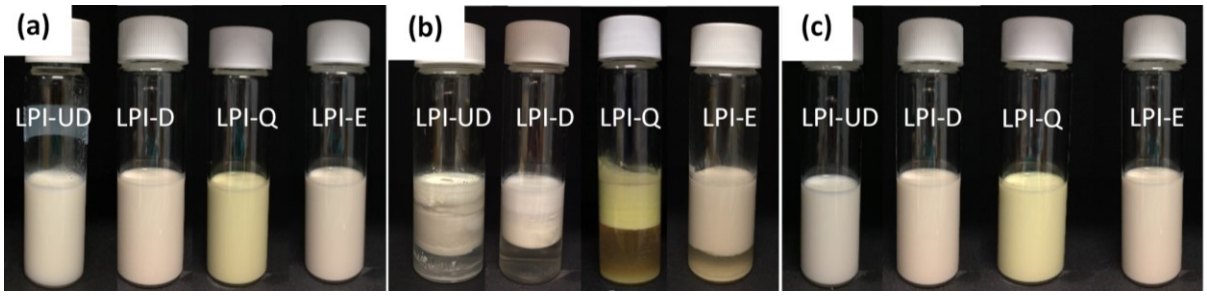


Figure 4.5 Visual observation of the emulsions in clear glass vials (a) fresh, (b) after 4 weeks of storage at room temperature (~25 ℃), (c) after 4 weeks of storage at ~5 ℃.

After 4 weeks of storage, all emulsions were separated into a cream layer leaving a near clear serum layer at the bottom (Figure 4.5b). However, the oil droplets were weakly flocculated and dispersible upon gentle shaking. Extensive droplet flocculation in emulsion (as evident from Figure 4.2b) could lead to a stable oil droplet network which could undergo compaction and restructuring, expelling the aqueous serum layer to the bottom (Yerramilli & Ghosh, 2017; Sun et al., 2007). Figure 5c presents emulsions in glass vials left undisturbed for 4 weeks at 5 ºC. The emulsions were stable to creaming for the entire duration of the test. This can be explained by no significant change in average droplet size at this temperature (data not shown) and minimum lipid peroxidation (shown in section 4.4.6), since both these parameters contribute to emulsion

destabilization. Lipid oxidation is known to occur at the oil-water interface, where water soluble prooxidants meet the polyunsaturated fatty acids in the oil (Yi et al., 2019). Lipid oxidation products, such as free radicals, lipid hydroperoxides, and secondary oxidation products, react with proteins at the interface and promote protein oxidation. Lipid-protein co-oxidation products have been shown to damage proteins in emulsions and cause destabilization (Yi et al., 2019; Schaich, 2008). Since increasing temperature increases the rate of oxidation, we observed little or no change in emulsions at 5 ºC, but extensive creaming at 25 ºC.

4.4.4. Confocal laser scanning microscopy

Confocal laser scanning micrographs of the emulsions are shown in Figure 4.6, where only the proteins were tagged with fast green and visible. In all micrographs, oil droplets were observed with a layer of proteins around them (some were marked with a white circle for identification). In case of emulsions stabilized using LPI-UD (Figure 4.6a), it can be observed that particle aggregation was minimal. This agrees with its droplet size distribution with a single peak below 1 µm (Figure 4.1a) and is also consistent with the results from Primozic et al. (2017) working with 1 wt% LPI-stabilized nanoemulsions. Emulsions made using LPI-D displayed larger aggregates and flocculated droplets, in comparison, as was evident from its droplet size distribution (Figure 4.1a). The process of dialysis must have removed some soluble protein fractions in addition to carbohydrates that contribute to droplet stabilization. The arrow in Figure 6b, indicates a droplet surrounded by an aggregate of LPI-D protein molecules. Structures like these were present throughout the sample slide and suggested flocculation via bridging or hydrophobic interactions between different protein residues. For emulsions stabilized using the conjugates LPI-Q (Figure 4.6d) showed more protein aggregation compared to LPI-E emulsion (Figure 4.6c). Similar observations were made by Karefyllakis et al. (2017), who studied oil-in-water emulsions stabilized with 1 wt% soy protein isolates and soy protein isolate-chlorogenic acid conjugates under light microscopy. Aggregates were absent in case of emulsions stabilized with soy proteins alone but were very apparent when complexes were used as emulsifiers. Indeed, polyphenol crosslinking at the oil/water interface and in the continuous phase could have influenced emulsion microstructure and participated in the visible clustering seen here.

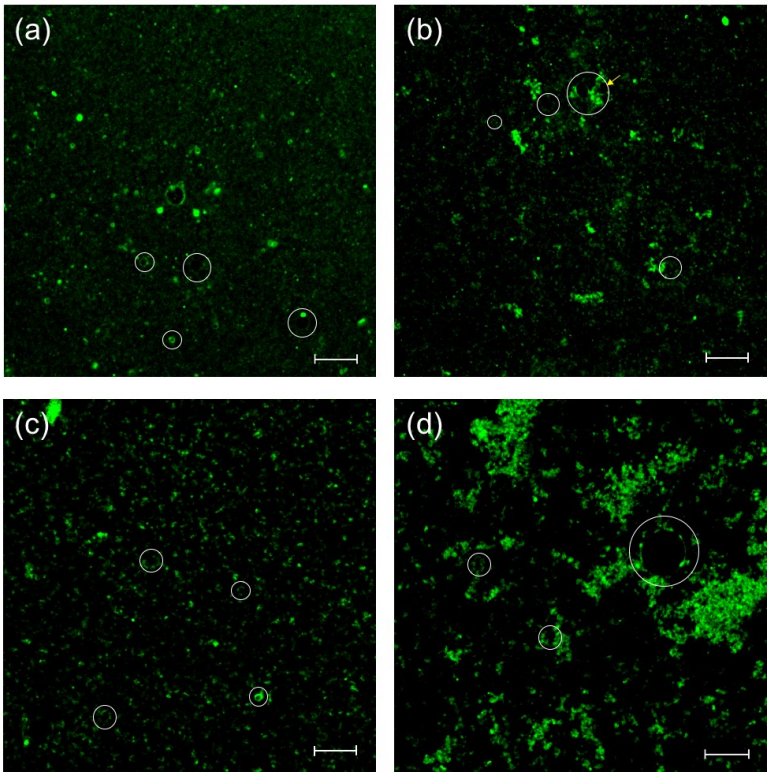


Figure 4.6 Confocal laser scanning micrographs of 5 wt% flaxseed oil-in-water emulsions stabilized by 1 wt% (a) LPI-UD, (b) LPI-D, (c) LPI-E and (d) LPI-Q. All images were captured at a working magnification of 60× with a 2.5× times digital zoom. Only the proteins were stained with fast green. Scale bars represent 10 µm. Some oil droplets are marked with circles around them for the ease of their visualization.

4.4.5. Effect of different environmental conditions on emulsion stability

An emulsion goes through appreciable changes when the surrounding environment is altered. For example, during its transportation, or processing. The aqueous phase for certain food emulsions are tailored from acidic (soft drinks) to mildly basic (nutritional beverages) or different degrees of saltiness (meats, mayonnaise, salad dressings, milk). For this reason, it is beneficial to know the impact of changing pH, ionic concentrations and heat on the emulsion stability. We have examined the droplet size distribution, mean droplet size and zeta potential under different conditions to understand how changing environments affect emulsion stability.

4.4.5.1. Effect of change in pH

Figure 4.7 and Figure 4.8a show the droplet size distribution and average droplet sizes for all emulsions at different pH values, respectively. All emulsions except the control LPI-UD gave a bimodal distribution at pH 7.0 (Figure 4.7f), similar to Figure 4.1a. At pH 7.0 all emulsions showed smallest droplet sizes (Figure 4.8a). From the zeta potential values recorded in Figure 8b, it can be inferred that the high negative charge (from -40 to -55 mV) of the droplets at pH 7.0 resulted in strong electrostatic repulsion among the droplets, leading to a smaller droplet size and stable emulsions at pH 7.0. Joshi et al. (2012) reported zeta potential values of -43.3 ± 0.6 mV for LPI-stabilized emulsions at pH 7.0 comparable to -44.9 ±1.0 mV in the present study. It is important to mention that there was no significant difference in the droplet size and zeta potential values between the emulsions stabilized with LPI-Q and LPI-E across all pH values (Figure 4.8b). As the pH decreased from 7.0 to 5.0, droplet size increased for all emulsions (Figure 4.8a), which could be attributed to the decrease in zeta potential (Figure 4.8b) At pH 5.0, a sharp increase in the mean droplet size for LPI-UD was observed which is close to the isoelectric point for LPI (4.5-5.0). For dialyzed LPI (LPI-D) the droplet size also reached maximum at pH 5.0, corresponding to the lowest zeta potential (-9.1 ± 1.7). Interestingly, emulsions stabilized by the conjugates maintained the lowest droplet size (8.6 ± 1.0 μm for LPI-Q and 8.3 ± 0.4 μm for LPI-E) when compared to emulsions stabilized with LPI-UD and LPI-D (16.8 ± 4.0 μm for LPI-UD and 12.6 ± 2.9 μm for LPI-D) at pH 5.0. There was a stark difference in the pH values at which LPI-E stabilized emulsions reached their isoelectric potential (~4.5) versus other emulsions (~5.0). This could have been due to the structure of E possessing four phenolic hydroxyl groups (Priyadarsini et al., 2002) leading to pKa values ranging from 4.5 to 12. Therefore, at pH 5, deprotonation of one para-phenolic hydroxyl group would make E negatively charged, which could be why the zeta potential for LPI-E emulsions remained high compared to other emulsions. At pH 5.0 the droplet size distribution for all emulsions were monomodal, although shifted towards 10 μm (Figure 4.7d). The impact of changes in protein charge at pH 5 could cause some buried hydrophobic residues to expose themselves (Karaca et al., 2011) that may react preferentially with the polyphenols forming supramolecular structures. A study by Rawel et al. (2005) had reported observing a higher binding affinity for bovine serum albumin to ferulic acid and chlorogenic acid close to the protein's isoelectric pH. At pH values lower than 5.0, the zeta potential values were large enough (more than +30 mV, except for LPI-E at pH 4) to restore electrostatic repulsion between the droplets

leading to a drop in average droplet size and increase in emulsion stability. For the emulsion stabilized with LPI-UD, droplet size decreased as the pH decreased from 4.0 to 2.0 ($p < 0.05$). For LPI-D emulsion, a large decrease in droplet size was observed at pH 4.0, which further decreased at pH 3.0 followed by an increase at pH 2.0 ($p > 0.05$) (Figure 4.8a). The droplet size distribution of LPI-D also followed similar shift where the second peak at larger droplet size increased at pH 2.0 (Figure 4.7a). For the emulsion stabilized by the conjugates (LPI-E and LPI-Q), a slight increase in droplet size was observed at pH 4.0 , followed by a decrease at pH 3.0 and 2.0 , similar to LPI-UD. Overall, the emulsions stabilized by the conjugates showed higher resistance against emulsion destabilization near the protein isoelectric point.

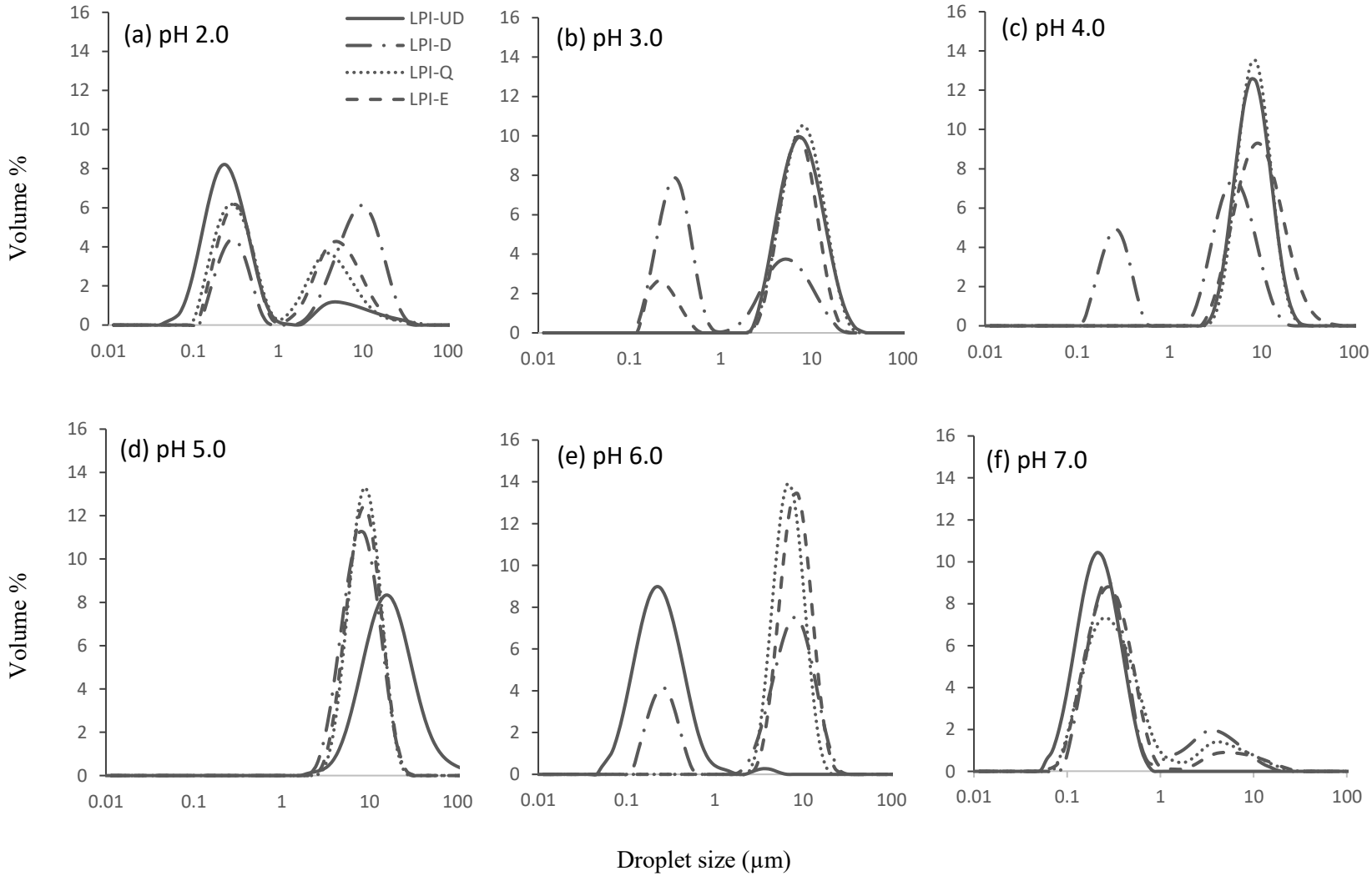


Figure 4.7 Droplet size distribution for emulsions stabilized by LPI (UD and D), LPI-Q and LPI-E conjugates at different pH values from 2.0 to 7.0.

4.4.5.2. Effect of change in emulsion ionic strength

The salt content or ionic strength of the medium which carries the oil droplets changes considerably depending on the type of food product. In the present case, the physical stability of emulsions was determined at 0.1-1.0 M NaCl concentrations at pH 7.0 at room temperature. With the addition of salt, the droplet size distribution of the emulsions shifted towards larger size (Figure 4.9). For LPI-UD, the peak below 1 µm reduced and the and the peak above 1 µm increased. For all other emulsions, salt addition resulted in larger droplet diameters (Figure 4.9b, c, d). It can be said that the emulsion stabilized with LPI-UD was better able to prevent salt-induced droplet aggregation compared to all other proteins.

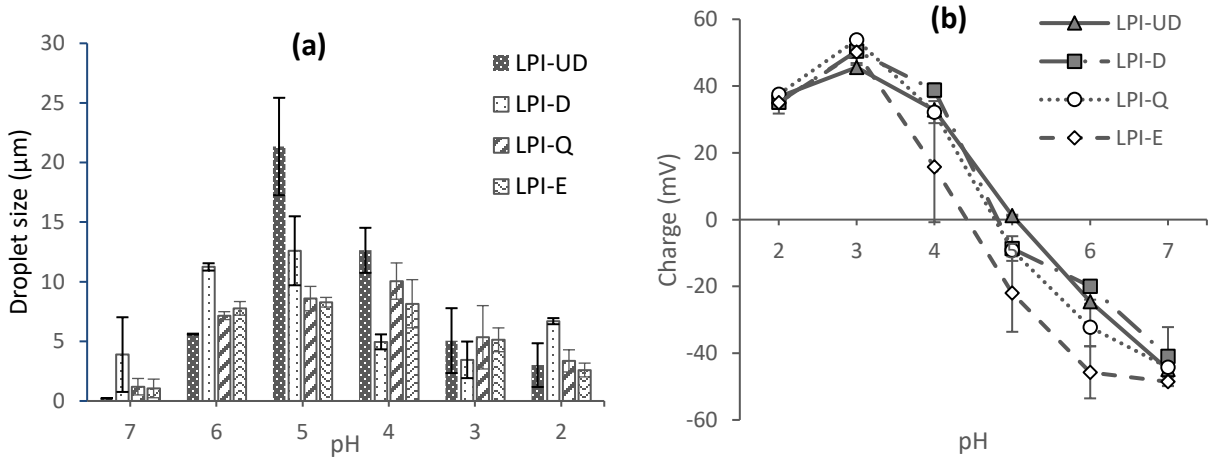


Figure 4.8 (a) Volume average droplet diameter (d_{43}) and (b) zeta potential of emulsions stabilized by LPI (UD and D), LPI-Q and LPI-E conjugates at different pH values from 2.0 to 7.0. Values are means ± SD (n = 3)..

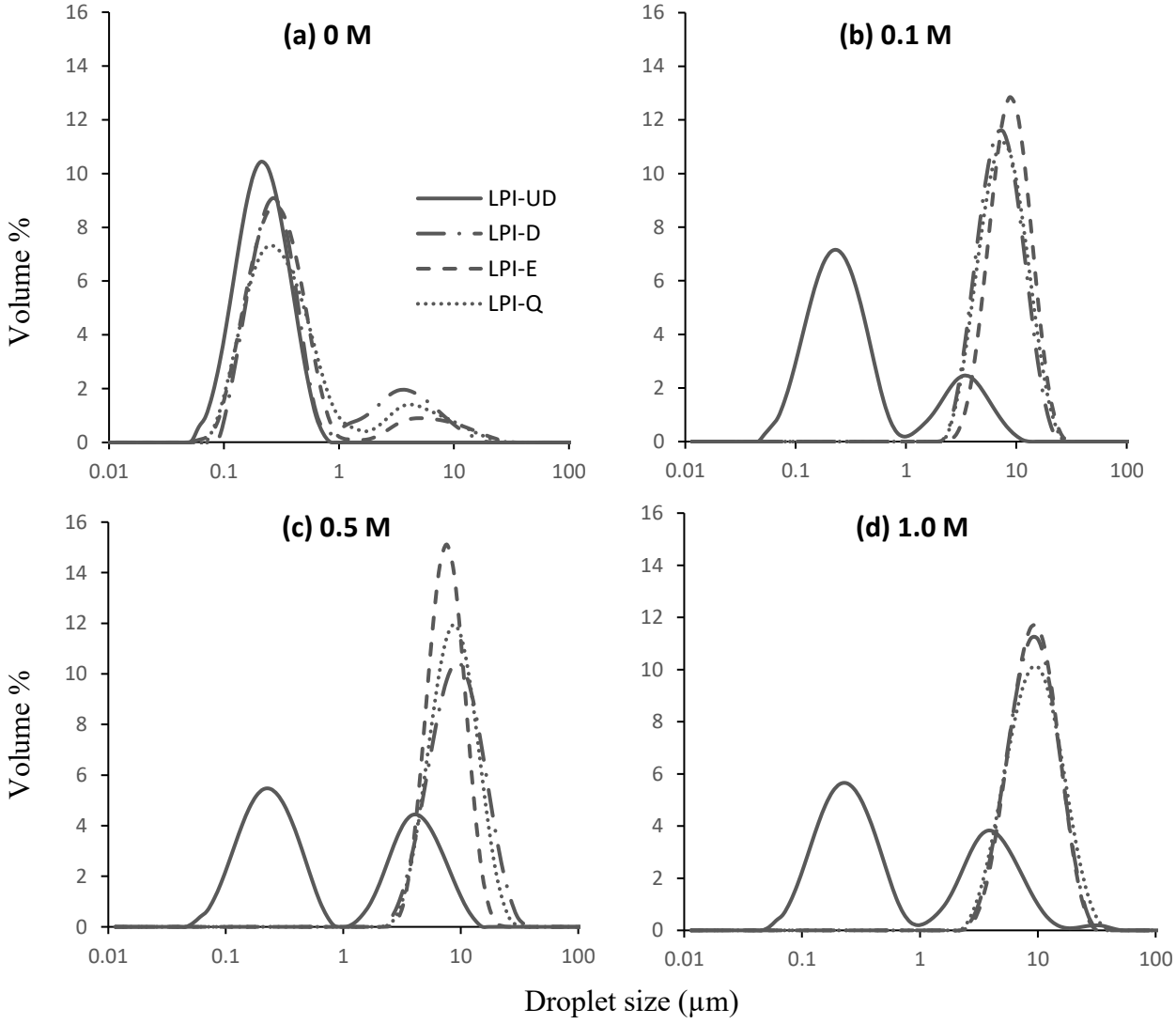


Figure 4.9 Droplet size distributions of emulsions stabilized by LPI (UD and D), LPI-Q and LPI-E conjugates at different salt concentrations (a) 0, (b) 0.1, (c) 0.5, and (d) 1.0 M.

Average droplet sizes (Figure 4.10a) for the emulsion stabilized with LPI-UD were the smallest at all salt concentrations. With salt, the droplet size for the emulsion made using LPI-UD increased to a maximum of 2.5 ± 0.7 μm. However, for emulsions made using dialyzed proteins (LPI-D, LPI-Q and LPI-E) the droplet size increase was to a far greater extent (ranged from 6.5 to 10.5 μm) and not significantly different from each other ($p > 0.05$). Similar observations have also been reported upon addition of NaCl to LPI stabilized emulsions in other studies (Ettoumi et al., 2016; Joshi et al., 2012). This destabilization of the emulsions upon salt addition could be attributed to the screening of droplet surface charge on the protein coated oil droplets by Na+ and Cl- ions (McClements, 2015). This is evident from the zeta potential data shown in Figure 4.10b. Magnitude of zeta potential decreased as concentration of salt increased. For concentrations > 0.1 M, the surface charge on the oil droplet dropped to about 10 mV in magnitude. The zeta potential of charged surfaces can reduce asymptotically to zero in the presence of Na+ and Cl- ions (Hunter, 2013). Clearly, the charge was not adequate to overcome the van der Waals and hydrophobic

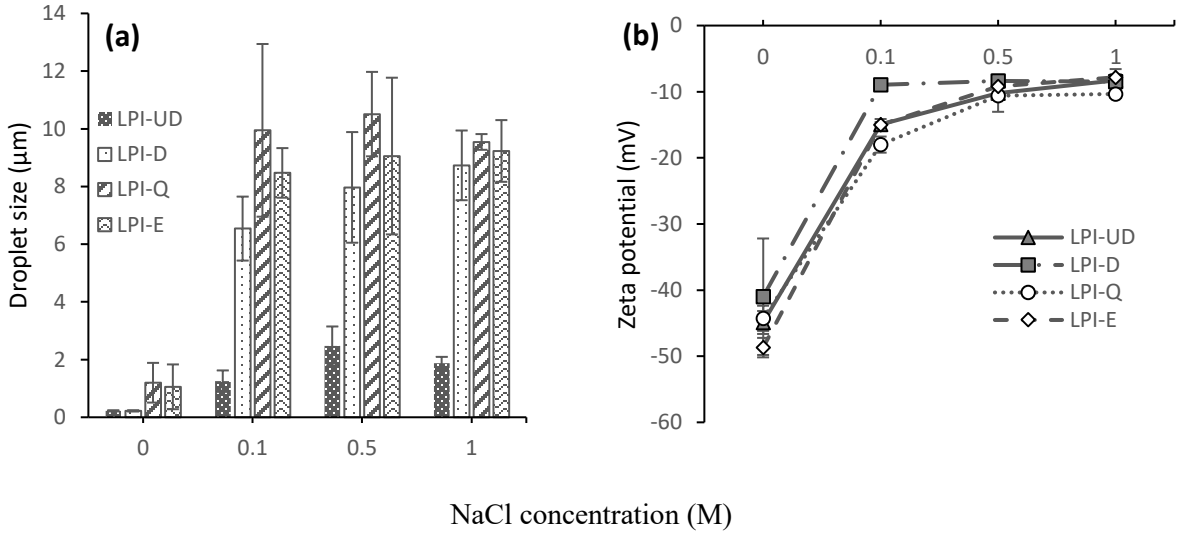


Figure 4.10 (a) Volume average droplet diameter (d_{43}) (b) zeta potential of emulsions stabilized by LPI (UD and D), LPI-Q and LPI-E conjugates at different salt concentrations (0 to 1.0 M). Values are means ± SD (n = 3).

attractions between the protein groups and therefore at concentrations at or above 0.1 M NaCl the attractive forces dominated leading to droplet aggregation. Nevertheless, the emulsions stabilized

by LPI-UD were significantly better at preventing droplet aggregation compared to any other emulsions, which could be attributed to the presence of certain protein fractions that could provide steric repulsion among the droplets. Similar stability against salt-induced charge screening was also observed by many researchers for sodium caseinate and has been related to the high steric barrier posed by the long protein molecules (Hu et al., 2003; Hunt & Dalgleish, 1995; Srinivasan et al., 2002). The process of dialysis removed those proteins leading to destabilization with the addition of salt for LPI-D, LPI-Q and LPI-E emulsions.

4.4.5.3. Effect of heat treatment

The effects of heat treatment (90 ºC, 10 min) on LPI stabilized emulsions (at pH 7.0) are presented in Figures 4.11 and 4.12. From the droplet size distributions in Figure 11, the emulsions prepared using LPI-UD and LPI-D did not change upon heat treatment. Both of these emulsions were seen to be stable to the heat treatment; the average droplet size increasing to a maximum value of 4.3 ± 3.6 μm in case of LPI-D and 0.22 ± 0.02 μm in case of LPI-UD stabilized emulsions (Figure 4.12a). The behavior of emulsions made using the conjugates, LPI-Q and LPI-E were comparable to each other but different from the emulsions stabilized with LPI-UD and LPI-D. After heating, their droplet size distribution peak shifted from < 1 μm to > 10 μm. Emulsions stabilized using the conjugates were unstable to the heat treatment: the average size increased to 25.5 ± 1.6 μm in case of LPI-Q and 15.8 ± 0.6 μm in case of LPI-E stabilized emulsions from ~2.0 μm before heat treatment. Zeta potential of LPI-UD and LPI-D stabilized emulsions were decreased to nearly half with the heat treatment, however, the values for LPI-D and LPI-UD were similar to each other ($p > 0.05$) (Figure 4.12b). For protein-stabilized emulsions, heating leads to the formation of covalent disulfide bonds between the adsorbed molecules, which could lead to loss of surface charge, while reinforcing the adsorption layers and contributes strongly to emulsion stability (Denkov et al., 2006).

Conjugation of the LPI with polyphenols on the other hand, reduced the emulsion stability when heated to 90 ºC. Zeta potential values for the emulsions made using conjugates were higher than -40 mV and did not change significantly with heating, both between and within the groups ($p > 0.05$) (Figure 4.12b). Therefore, it was expected that ample electrostatic repulsions between the protein residues would keep the droplets from flocculating/coalescing. However, the increase in droplet size for LPI-Q and LPI-E could be due to an increase in the hydrophobic attraction between

lipid droplets. At high temperatures, hydrophobic groups buried inside the native protein conformation are exposed (Kim et al., 2002). This ensues protein-protein interaction and aggregation. At the same time, there could also be a decrease in the disulfide bond formation due to thiols now occupied by polyphenols leading to a weaker configuration of the adsorbed layer. For Joshi et al. (2012), heating LPI-stabilized emulsions to 60 ºC did not change the initial average droplet size of 0.5 μm significantly. However, the value increased to > 3.5 μm when the emulsion was subjected to 70 ºC.

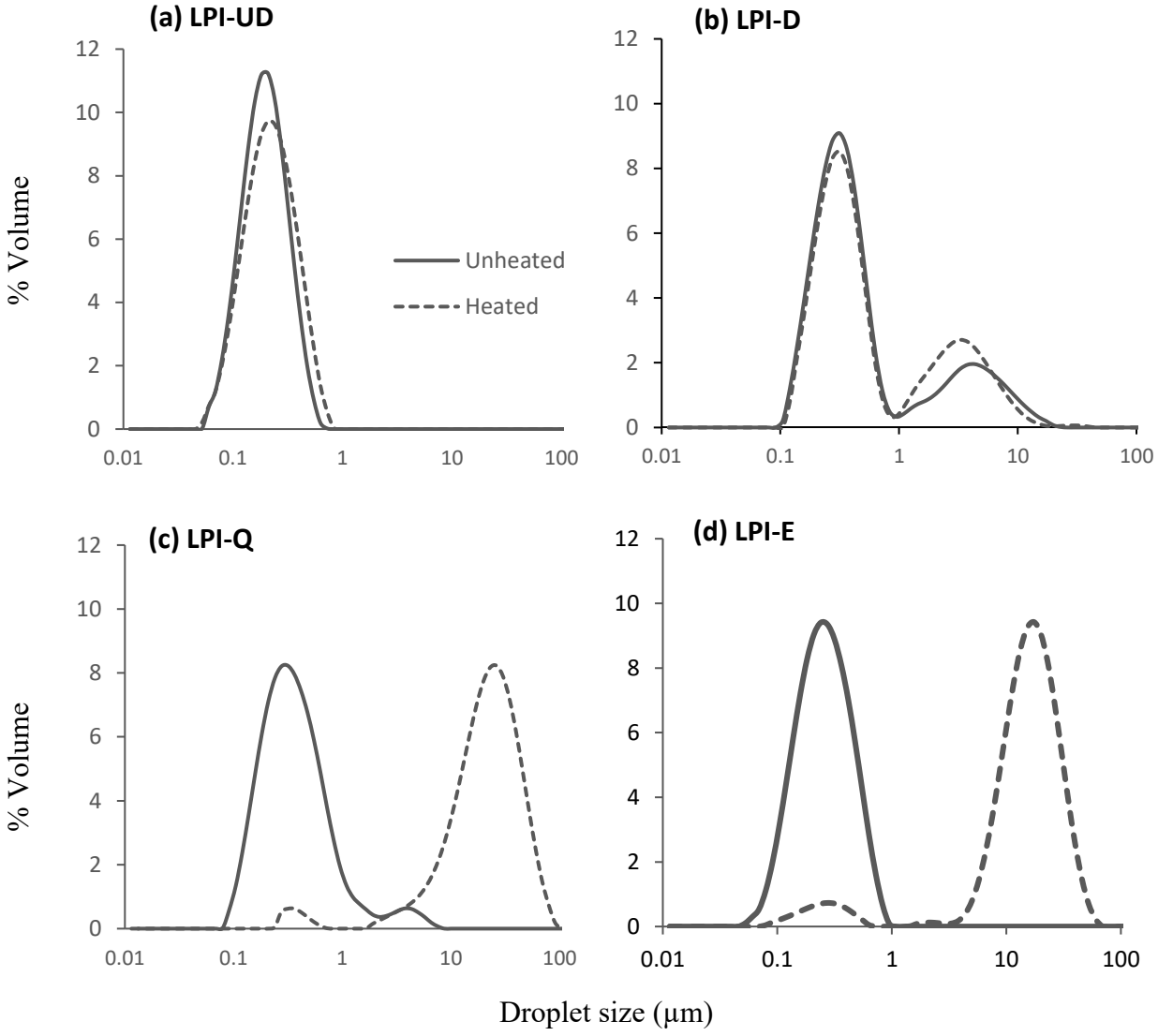


Figure 4.11 Droplet size distributions of emulsions stabilized by (a) LPI-UD, (b) LPI-D, (c) LPI-Q and (d) LPI-E before (solid line) and after heat treatment (dashed line) at 90 ℃ for 10 minutes.

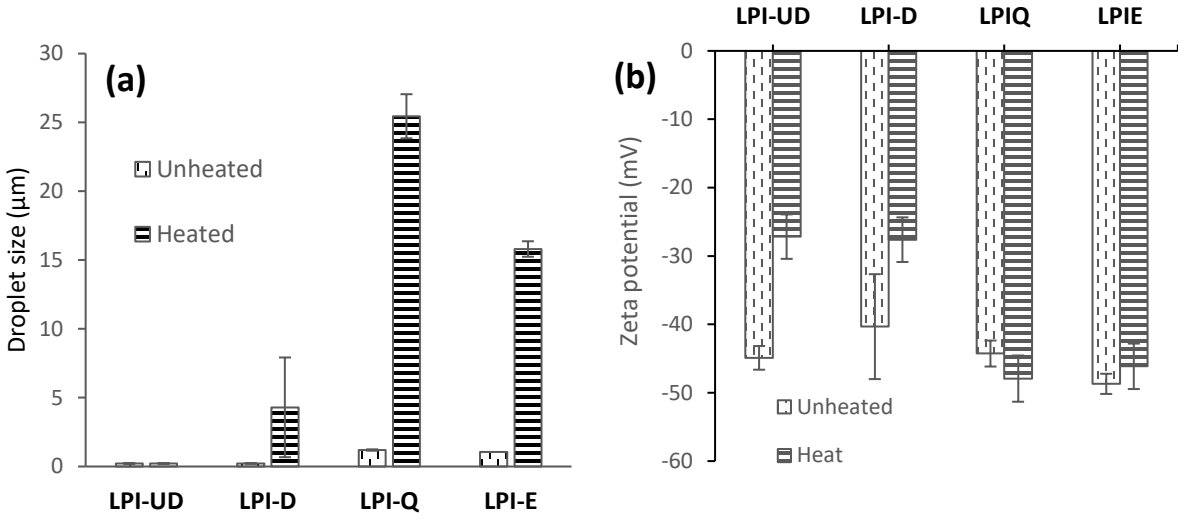


Figure 4.12 (a) Volume average droplet diameter (d43) (b) zeta potential of emulsions stabilized by LPI, LPI-Q and LPI-E conjugates before and after heat treatment at 90℃ for 10 minutes. Values are means ± SD (n = 3).

4.4.6. Oxidative stability of flaxseed oil in emulsions

Flaxseed oil naturally contains high levels of ω-3 polyunsaturated fatty acids (PUFAs) (linolenic acid) which makes the oil very prone to oxidation. Lipid hydroperoxides, generated from the reaction between oxygen and the PUFAs, in addition to the secondary oxidation products are indicators of lipid oxidation. The overall goal of this experiment was to compare the efficacy of localizing antioxidants at the oil droplet interface (by using protein-polyphenol conjugates to make emulsions) against radical permeation in an oil-in-water emulsion. LPI-UD emulsion was also compared with LPI-D emulsion to understand the effect of protein dialysis on lipid oxidation. To demonstrate the benefit of localizing the polyphenols at the oil droplets' surface, oxidative stability of LPI-D emulsions were also investigated with equivalent amount of polyphenols (Q or E) added to the emulsion aqueous phase. Emulsions were investigated at two different temperatures (5 and 25 ºC) every week for four consecutive weeks and the final PV and AV values are reported in Figure 4.13. Samples were also analyzed after 24 h of storage at 60 ºC (Schaal oven test).

4.4.6.1. Peroxide Value

Peroxide value (PV) was measured to assess the oxidation status of fats and oils, mainly to account for the primary oxidation products. In general, the PVs for all emulsions increased as storage proceeded (data not shown). After 4 weeks, the values were greater at 25 ºC (Figure 13c) compared to 5 ºC (Figure 4.13a), however remained below 18 meq/kg oil, indicating acceptable oil quality (Sun-Waterhouse et al., 2011). For emulsions stored at 5 and 25 ºC, those stabilized using LPI-UD and LPI-D (without any polyphenol in them) generated the highest PV, suggesting that these were oxidized fast. However, the values were statistically no different from PVs for emulsions with polyphenols in the bulk aqueous phase (Figure 4.413a, c). For some polyphenols, a relatively high concentration of their radical form have been reported to be pro-oxidant in their activity and seen to enhance hydroxyl radical generation by producing superoxide radical and hydrogen peroxide upon auto-oxidation (Miura et al., 1998; Cao et al., 1997; Laughton et al., 1989). Researchers, such as Huang and Frankel (1997) and Di Mattia et al. (2009), have shown pro-oxidant activities for gallic acid/catechin and quercetin dispersed in the aqueous phase of oil-in-water emulsions, with PVs, in some cases, even higher than their controls.

Having said that, placing polyphenols at the droplet interface offered greater protection against lipid oxidation, especially at 25 ºC, since lower PVs were obtained for emulsions made using both the conjugates than with the polyphenols in the bulk phase of the emulsion (Figure 4.13c). Also, PVs for emulsions made using LPI-Q were lower than LPI-E at both temperatures. This not only points to a higher percent of conjugation in LPI-Q (21%) than LPI-E (11%), but also a greater ability of Q to interrupt free-radical propagation compared to E. Furthermore, from the results, we don't see the differences in their interfacial antioxidant potentials translate into the bulk aqueous phase, which further indicates the ability of Q and E to provide a more targeted activity when placed at the interface than otherwise. Pan and Nitin (2015) has worked towards a similar hypothesis but used a slightly different approach at interfacial engineering in emulsions. By means of layer-by-layer coating they deposited a chemical conjugate of gallic acid and an electropositive polymer ε-polylysine on an anionic sodium dodecyl sulphate stabilized droplet in an oil-in-water emulsion. With the antioxidant at the droplet interface, their results were positive against inhibiting oxidation of an encapsulated bioactive.

Accelerated stability tests using elevated temperatures provide a prediction of the oil's oxidative fate during storage (Warner & Eskin, 1995). LPI and LPI-conjugate-stabilized emulsions were therefore exposed to the Schaal oven test. PVs reported in Figure 4.13e were statistically significant between the two emulsions with polyphenols (at the interface vs. in the bulk phase). The values obtained are also similar to those reported previously in literature (Huang et al., 1994; Mohanan et al., 2018; Moslavac et al., 2015). Lowest PVs were obtained for emulsions made using LPI-UD and LPI-D (0.8 ± 0.3 meq/kg oil), while, diminished antioxidant activity and higher PVs (ranging from 0.91 to 1.96 mM/g oil) were observed for emulsions containing polyphenols in them. From these results we can stipulate that the antioxidants could remained effective over a period, but their effectiveness decreased as oxidation accelerated (Landrault et al., 2001). PVs for emulsions with polyphenols at the interface (for the conjugates) however, remained lower than when they were added in the bulk aqueous phase. A possible explanation for the observed lower PVs in case of LPI-UD and LPI-D emulsions could be associated with globular proteins, modifying their secondary structure upon heating above 60 ºC. In this "molten globule" state, internal hydrophobic functional groups are exposed and may interact with other neighboring groups or molecules leading to a more compact interface in case of LPI-UD/LPI-D stabilized emulsions (Nicolai et al., 2011). However, such interactions may be not be feasible or reduced for the conjugates with hydrophobic functional groups now employed towards bonds with the polyphenols. For the same reason, there could have been a weaker interface for LPI-Q and LPI-E emulsions where the oil droplets are more susceptible to pro-oxidant attack and lipid oxidation.

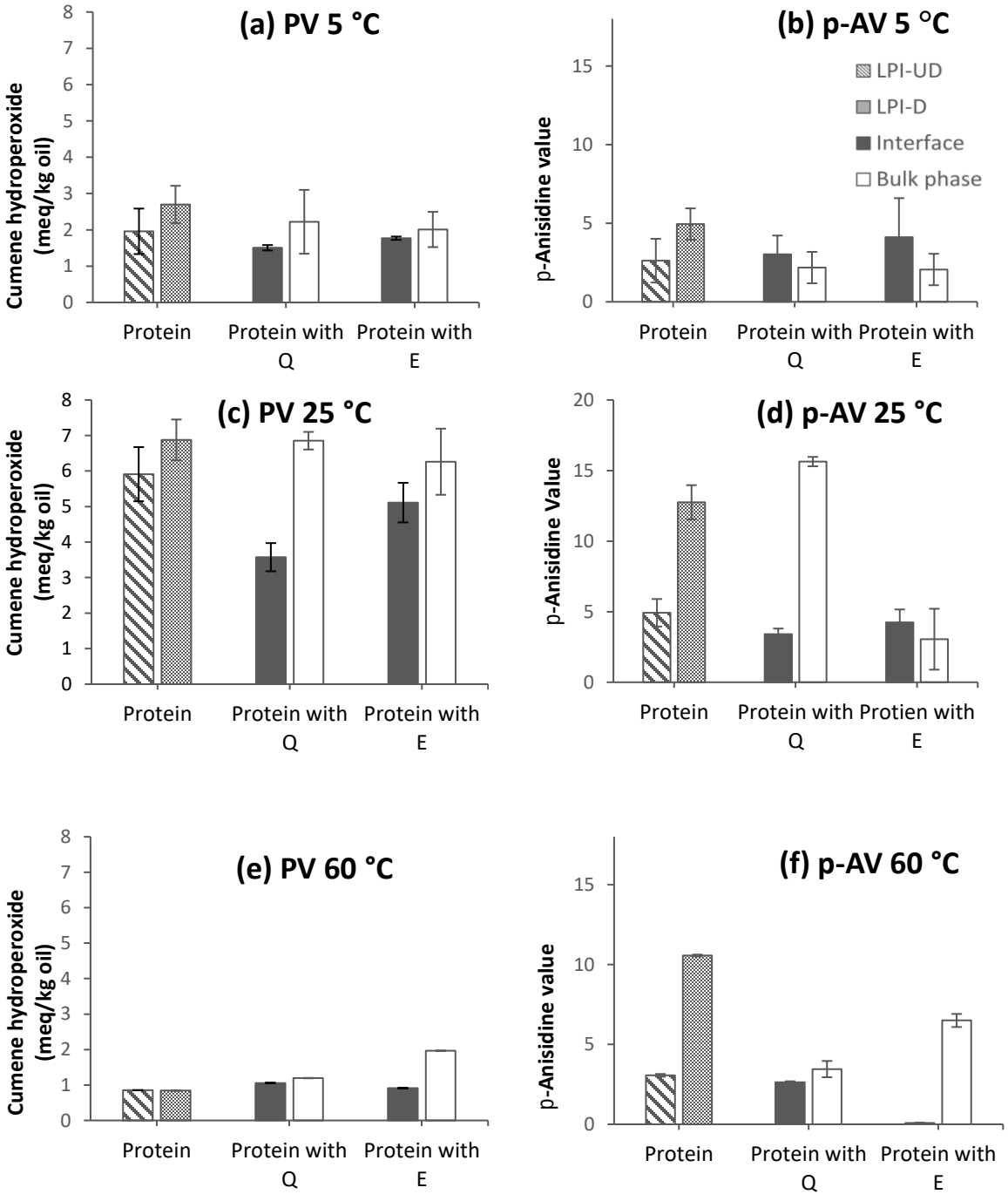


Figure 4.13 Oxidative stability tests on emulsions stabilized with LPI (UD (striped bar) and D (gray bar)), LPI-Q and LPI-E. Peroxide values (PV) (a, c, e) and *p*-anisidine values (AV) (b, d, f) were measured after four weeks of storage at 5 ºC (a, b), 25 ºC (c, d) and one day of storage at 60 ºC (e, f). The Q and E polyphenols were either located at the oil droplet surface while conjugated with LPI (dark bars) or freely suspended in the bulk aqueous phase (white bars) of the LPI-D-stabilized emulsion. Values are means ± SD (n = 3).

4.4.6.2. *p*-Anisidine value

para-Anisidine value (*p*-AV) quantifies secondary oxidation in oils and fats. Non-volatile carbonyl compounds generated in the advanced stages of lipid oxidation are responsible for the off-flavors due to their low sensory threshold value (Allen & Hamilton, 1994). The level of these reaction products in the system can be measured using *p*-anisidine which condenses readily with aldehydes and ketones to form Schiff bases and absorb at 350 nm (Mitchell & Waring, 2000). It is one of the oldest methods for evaluating lipid oxidation with a value of less than 2 indicating a good quality oil (Subramanian et al., 2000). Predictably, PVs initially increase and then begin to decrease as they are decomposed in the secondary stages of oxidation at which point *p*-AVs begin to increase (McClements & Decker, 2000). Results from Figure 4.13 (b, d, f) indicate that secondary oxidation had commenced in all emulsions. The extent of secondary oxidation increased with the increase in temperature. For example, *p*-AVs obtained for samples at 5 ºC (Figure 13b) following 4 weeks of storage was lower than that stored at 25 ºC (Figure 13d). In general, the extent of secondary oxidation was more for LPI-D emulsions than LPI-UD. For example, at 25 ºC, *p*-AV for LPI-D (12.8 ± 1.2) was double of that measured for LPI-UD (4.9 ± 1.0), while at 60 ºC it was nearly three times as high. Further from our analysis, emulsions stored at 5 ºC (Figure 4.13b) showed substantial variability with polyphenols at the interface yielding a slightly higher average *p*-AV than when placed in the bulk. The values were, however, not statistically significant ($p > 0.05$), both between and within the groups.

For the emulsions stored at 25 ºC (Figure 13d), those stabilized with the conjugates generated a significantly lower *p*-AV compared to LPI-D and LPI-UD emulsions. In keeping with our stated hypothesis, emulsion made with LPI-Q conjugate had a lower *p*-AV at 25 ºC than emulsions with Q in the bulk aqueous phase. However, the observation did not hold true for emulsions containing E in them, where, for the duration of this storage study, no significant differences in *p*-AV were obtained between the conjugates and the emulsions with E in the bulk aqueous phase (Figure 4.13d). Such differences in the antioxidant activities for Q and E towards lipid oxidation can be ascribed to factors such as differences in solubilities, concentration, and partitioning behaviour between aqueous and lipid phases in emulsions (Meyer et al., 1998).

Samples stored at 60 ºC (Figure 4.13f) for 24 hr were able to furnish lower *p*-AVs for emulsions made using the conjugates than otherwise. Similar to 5 and 25 ºC storage, LPI-UD emulsions

showed lower *p*-AV compared to the LPI-D emulsions, indicating removal of certain components of LPI during dialysis lowered its antioxidative properties. Emulsions made using LPI-D generated the highest *p*-AV at 60 ºC, followed by those with E in the bulk phase. Interestingly, compared to the latter, emulsions with conjugated E showed negligible *p*-AV, suggesting little or no secondary oxidation but only primary oxidation when E was present at the oil droplet surface. Di Mattia et al. (2009), working with gallic acid, catechin and quercetin in the aqueous phase of 20% (w/w) olive oil-in-water emulsions at concentration of 250, 350 and 500 µM, observed an enhancement of primary oxidation but an inhibition of secondary oxidation when catechin was used in the media, directing to rapid inactivation of peroxyl radicals that moved to the interface from the lipid core along with the radicals formed due to hydroperoxide degradation. The effects of quercetin and gallic acid when compared to catechin were limited and intermediate, respectively. For the present study, the presence of Q, at 60 ºC, both at the interface and bulk was unable to repress secondary oxidation and the effect was lower compared to when E was present at the interface. However, the value at the interface (2.6 ± 0.1) were significantly lower than when Q was placed in the bulk aqueous phase (3.5 ± 0.5).

4.5. Conclusions

LPI-polyphenol conjugates were used to prepare flaxseed oil-in-water emulsions for physical stability and lipid oxidation studies. Two different polyphenols, Q and E, were used to develop the conjugates. As controls, emulsions were also prepared with non-conjugated, but similarly processed proteins (LPI-D) and native LPI without any processing (LPI-UD). The emulsions made using LPI-polyphenol conjugates displayed similar droplet size compared to the non-conjugated proteins (1.2 to 2.1 µm), while the one prepared with LPI-UD was the smallest (0.2 µm). After four weeks of storage at room temperature all emulsion showed extensive creaming and the separation of clear aqueous phase, however, they were redispersible. In contrast, when stored at 5 ºC, all emulsions were stable without any visible separation. The largest increase in droplet size after four weeks was observed for LPI-UD, while the smallest change was recorded for LPI-E. Increment in droplet size around the isoelectric pH of LPI was minimal in emulsions stabilized using conjugates, however, they were not stable to salt, and heat treatments. In addition, accelerated gravitational separation using a photocentrifuge to calculate instability indices were similar for all emulsions prepared in the study. The rate of creaming, was higher for emulsions

using dialysed emulsifiers. Lastly, from the oxidation studies, it is hypothesized that LPI-polyphenol conjugated emulsions provided better protective effects and slower degradation of flaxseed oil compared to LPI-UD and LPI-D emulsions stored at room temperature. In doing so, the conjugates conferred the advantage of holding the polyphenols at the oil-water interface to delay lipid oxidation in a location dependent manner. In many incidences, we saw emulsions made using LPI-conjugates were more efficient in retarding oxidation than emulsions made with LPI-D with an equivalent amount of polyphenol added to their bulk aqueous phase. However, differences in the efficiencies of these conjugates in preventing lipid oxidation in emulsions were observed. When stored at room temperature, the oil in LPI-Q was less oxidized compared to LPI-E, which was attributed to more conjugation of polyphenols in case of Q and the ability of Q to interrupt free-radical propagation more efficiently than E. The inhibition of oxidation in flaxseed oil-in-water emulsions by localizing antioxidants at the oil-water interface could take us to a step closer in the design and formulation of efficient delivery systems for healthy lipids.

5. GENERAL DISCUSSION

To counteract lipid oxidation in foods rich in fats and oils, manufactures have added antioxidants in low concentrations since the early 1900s. Due to some toxicological concerns regarding the nature of synthetic antioxidants, antioxidants from natural sources are being investigated extensively (Madhavi et al., 1995). Polyphenols, derived from fruits, vegetables, cereals and beverages, have been shown to exhibit antioxidative properties due to their excellent hydrogen or electron donating capacities and are progressively replacing synthetic ones in food systems (Amorati & Valgimigli, 2012; Nawar, 1985). It is therefore, isn't just the nature of antioxidants used but also the physical properties of food systems that play an important role in the chemistry and scope of lipid oxidation (Halliwell et al., 1995; McClements & Decker, 2000). Many studies have shown that there is a significant difference between lipid oxidation taking place in bulk oils and in O/W emulsions, where it is seen to occur faster in the latter (Berton-Carabin et al., 2014; Lomova et al., 2010). A closer look at lipid oxidation in oil-in-water emulsions has revealed that the droplet interface plays a crucial role in the initiation of oxidation of the oil core and has therefore been studied most elaborately (Berton et al., 2011; Waraho et al., 2011). The present research builds on the previous works and strives to develop a flaxseed oil-in-water emulsion stabilized using protein-polyphenol complexes to strategically locate the polyphenols at the oil droplet interface. It further compares the oxidative stability of the engineered emulsions with the ones made using the protein with an equivalent amount of polyphenol dispersed in the aqueous phase.

To localize polyphenols at the interface, they were conjugated to proteins. Proteins are natural emulsifiers due to their amphiphilic nature and therefore, are situated at the droplet interface when used to stabilize an emulsion. Recently, pulse proteins are gaining wide popularity as substitutes for animal-based proteins. In the present work, LPI extracted using isoelectric precipitation was used as the protein for emulsification. The structure of lentil protein presents binding sites for polyphenols to complex with, allowing the later to protect or modulate their activity. The polyphenols used in this study were Q, R and E. Q belongs to the polyphenol sub-class flavonoids with a basic C6-C3-C6 backbone. They are abundant in onion, tea and apple. R, also called

quercetin-3-O-rutinoside, is a glycoside combining flavonoid Q and the disaccharide rutinose at the C3 position. It is widely distributed in plants including citrus fruits. Lastly, E is a phenolic acid. It is a dimeric condensation product derived from hexahydroxydiphenic acid and found in walnuts, pecans, peaches, and pomegranate. These polyphenols were conjugated to LPI at an alkaline pH of 9.0 using a simple grafting method, dialyzed to remove the unreacted polyphenols, lyophilized to obtain dry powders of protein-polyphenol conjugates which were then characterized in the first objective of this study.

Upon experimentation, it was observed that the highest conjugation was obtained for Q, followed by E and R. FTIR analysis of protein secondary structure showed no significant change in α-helix and β-turn content upon conjugation, however, a significant loss in β-sheet content and increase in random coil structure was observed, which suggested possible uncoiling and exposure of hydrophobic sites to facilitate conjugation and thus, leading to a more disordered protein structure. Fluorescence intensity of the conjugates dropped significantly from the pure LPI, attributing to the quenching of the aromatic amino acid signals by the bound polyphenols. Surface hydrophobicity of the conjugates, measured via ANS probe binding to the free aromatic amino acid, also decreased upon conjugation as the polyphenols occupied the ANS binding sites on the protein molecules. However, no change in the thermal behaviour of the conjugates was observed compared to the pure protein. There was significant improvement in antioxidative properties of LPI upon conjugation with the polyphenols, which suggests some potential to apply protein-polyphenol conjugates in the encapsulation and delivery of sensitive bioactive lipids. From the DPPH$^{\bullet}$ radical scavenging test it was found that the free radical scavenging ability of conjugates decreased in the order: LPI-Q > LPI-R > LPI-E. Polyphenols Q and R with a free catechol group in the B ring is the most potent towards free radical scavenging and the effect was translated well into the conjugates. Here, solubility in the solvent used for the assay is important to understand the antioxidative power of polyphenols and conjugates used in the study. Since 50% methanol was used as the solvent for the analysis, radical scavenging of LPI-E suffered while that of LPI-R benefitted. Reducing power (FRAP) of the conjugates followed a different trend with LPI-Q > LPI-E > LPI-R which reflected the order for the pure polyphenols Q > E >R. In general for both DPPH$^{\bullet}$ and FRAP assays, the conjugates performed better than their corresponding pure polyphenols when taking the percent conjugation into account. Lastly, functional properties of the protein-polyphenol conjugates were also significantly changed compared to the pure proteins.

Turbidity increased significantly for all conjugates and therefore solubility decreased. Also, interfacial tension of conjugates increased indicating loss of surface activity compared to LPI against canola oil, however surface activity of the conjugates was still better than pure canola oil-water interface which, was hypothesized, could facilitate their ability to form stable emulsions.

LPI-Q and LPI-E conjugates were selected for the second part of this study based on their higher degrees of conjugation. 1.0 wt% LPI-Q or LPI-E stabilized 5 wt.% flaxseed oil-in-water emulsions were developed using a high pressure homogenizer. Flaxseed oil was selected to act as a model functional oil core due to its high content of polyunsaturated fatty acids prone to lipid oxidation. The emulsions stabilized with LPI-Q and LPI-E (LPI-polyphenol conjugates) and dialysed LPI (LPI-D) displayed an avaergae droplet size of 1.2 ± 0.6, 1.5 ± 0.5 μm and 2.1 ± 1.1 μm, respectively not significantly different from each other but significantly larger than the droplets in undialysed LPI (LPI-UD) stabilized emulsions (0.2 ± 0.0 μm). The droplet sizes of the LPI-Q, LPI-E and LPI-D, however were smaller at the end of 4-week storage compared to emulsions stabilized with LPI-UD. This may be due to better oxidative stability of the emulsions prepared using conjugates, keeping the interface in place with minimum protein displacement by the generated surface-active lipid oxidation products. Kato and Nakai (1980) have previously demonstrated a strong correlation between surface hydrophobicity, interfacial tension and emulsification properties of proteins. Because proteins with a structurally flexible random coil configuration tend to be more surface-active, conjugated LPI should have produced a better stable emulsion with lower droplet sizes based on the increase in random coil from FTIR analysis (Section 3.4.2.1., Figure 3.3). The result, however, is compromised due to the reduced surface hydrophobicity of LPI conjugates (Section 3.4.2.3., Figure 3.5) allowing reduced hydrophobic sites for emulsion stabilization now complexed to polyphenols. The creaming velocity analyzed using the photocentrifuge, was higher for emulsions using dialysed emulsifiers owing to the larger droplet sizes in them. Extended storage stability of the prepared emulsions at room temperature showed that all emulsions destabilized after 4 weeks, however, were stable at refrigeration temperature with minimum lipid oxidation in them. Confocal scanning microscopy allowed to examine the distribution of the protein and conjugates around the oil droplets in the emulsion. There were protein aggregation present in the system, chiefly for emulsions prepared using the conjugates which can be explained from their increased turbidity at pH 7.0 (Section 3.4.3.1., Figure 3.6). Varying conditions of pH, salt and temperature led us to a few important conclusions. Increment in the droplet size around the

isoelectric pH (pI) (4.0 to 5.0) of LPI was minimal in emulsions stabilized using the conjugates compared to emulsions with LPI alone. Again, we saw evidence of the conjugate interface staying intact in an environment of changing protein conformations as a function of pH. However, the conjugate stabilized emulsions were not as stable to salt, and heat treatments compared to the non conjugated protein.

Lastly, from the oxidation studies performed at temperatures 5 ℃, 25 ℃ and 60 ℃, the results indicated that LPI-polyphenol conjugated emulsions provided better protective effects and slower degradation of lipids compared to LPI-UD and LPI-D emulsions with or without the polyphenols. For example, after 4 weeks of storage at 25 ℃, PV (Section 4.4.6.1., Figure 4.13) for LPI-Q emulsions (3.6 ± 0.4 meq/kg oil) were nearly half of that obtained with Q added to the bulk of the LPI-D emulsion (6.8 ± 0.2 meq/kg oil). For emulsions at 5 ℃, the values were 1.5 ± 0.1 meq/kg oil and 2.2 ± 0.9 meq/kg oil for Q at interface and bulk, respectively. For emulsions at 60 ℃, the values were 1.0 ± 0.1 meq/kg oil and 1.2 ± 0.6 meq/kg oil for Q at interface and bulk, respectively. In preparing the emulsions using LPI-Q and LPI-E, the conjugates conferred the advantage of holding the polyphenols at the oil-water interface to delay lipid oxidation in a location dependent manner. For example, to draw from the observations made in the first objective, Q was reported as a powerful antioxidant both in the FRAP and DPPH assays done (Sections 3.4.3.3 and 3.4.3.4., Figures 3.9 and 3.10, respectively). For the same reason, Q was more successful at the interface than E. The inhibition of oxidation in flaxseed oil-in-water emulsions localizing antioxidants at the oil-water interface is a significant result from this research and a step closer to facilitating the design and formulation of delivery systems for healthy lipids.

6. OVERALL CONCLUSION

The overall objective of the present work was to develop LPI and plant polyphenol conjugates, to determine their physicochemical, structural, and functional characteristics and further, to use LPI and polyphenol conjugates as emulsifiers in inhibiting lipid oxidation in flaxseed oil-in-water emulsions. It was shown that polyphenols are powerful antioxidants, and when complexed to proteins on the oil droplet surface, could contribute to an enhanced stability in oil-in-water emulsions. The degree of conjugation was revealed to be higher for conjugates formed using Q and E. Antioxidant capacities, estimated using the DPPH free radical scavenging and ferric reducing antioxidant power (FRAP) assays, were also highest for LPI-Q and LPI-E conjugates. The results from FTIR showed changes in the secondary structure of the conjugated protein, particularly an increase in the random coil configuration and a decrease in its β-sheet. From the second objective, emulsions stabilized using LPI-Q and LPI-E conjugates showed superior oxidative stability compared to those stabilized using LPI-UD or LPI-D emulsifiers and compared to those with an equivalent amount of Q or E in their aqueous phases respectively. Results from accelerated gravitation using LUMiSizer describe emulsions made using conjugates to have similar instability indices as those made using LPI-UD and LPI-D as emulsifiers. Creaming velocity, on the other hand, was higher for the emulsions made using conjugates than those made using LPI-D (4.03 ± 0.08 μm/s) compared to LPI-UD emulsions (1.81 ± 0.12 μm/s). Emulsions prepared using conjugates also showed greater stability was observed towards changes in environmental factors such as pH, although, limited stability towards changes in ionic strength, and heat treatment.

The present study was able to answer the stated hypotheses. In brief, as a result of conjugation we saw the surface hydrophobicity and intrinsic fluorescence of the LPI decrease suggesting the number of hydrophobic and aromatic groups on the proteins also decreased. Conjugation enhanced the antioxidant activity of the polyphenols and emulsifying capacity of the proteins, leading to a somewhat symbiotic arrangement. Furthermore, it was feasible to prepare O/W emulsions with the protein-polyphenol conjugates synthesized in the objective one of this study. To summarize, these findings show that LPI-Q and LPI-E can act as efficient encapsulating agent to retard oxidative

deterioration of functional oil cores because of their higher antioxidant activity and the interfacial accumulation of polyphenols.

7. FUTURE STUDIES

The present study can be made better in the future to further our understanding of the functional advantages of using protein-polyphenol conjugates. First and foremost, the protein-polyphenol binding can be examined at the molecular level. Groups who have studied interactions between protein and polyphenols have applied numerous other techniques such as, nuclear magnetic resonance (NMR) (Baxter et al., 1997; Murray et al., 1994), small-angle X-ray scattering (SAXS) (Jöbstl et al., 2004), dynamic light scattering (DLS) (Poncet-Legrand et al., 2006), and more recently, isothermal titration microcalorimetry (ITC) (Frazier et al., 2006; Karefyllakis et al., 2017) to investigate the complexation from the molecular perspective. For example, Karefyllakis et al. (2017) who studied physical binding of sunflower proteins with polyphenols were able to analyze the thermodynamics of the binding reaction based on the heat evolved during the molecular association of sunflower proteins and increasing amounts of chlorogenic acid. In another study by Baxter et al. (1997), polyphenol's affinity to proline rich peptides was investigated by titrating a series of polyphenols onto a synthetic polypeptide fragment. The results confirmed the predominant mode of association between the two entities to be hydrophobic in nature. Another study (Luo et al., 2011) employed microscopy to study localization in Pickering emulsions stabilized with rutin particles. The group used a confocal laser scanning microscope in fluorescence mode to show the flavonoid particles neatly arranged at the oil-water interface. Talking of location, University of Saskatchewan is situated near the Canadian Light Source. The facility offers a range of light scattering techniques which could be explored in future for a better proof of the presence of polyphenol at the interface.

The conjugates of LPI and polyphenols could also be made using an alternate multistep, non-toxic, and higher efficiency process called free-radical grafting. This procedure involves the oxidation of ascorbic acid by H2O2 at room temperature with the generation of hydroxyl radicals to initiate the reaction (Kitagawa & Tokiwa, 2006). Following this method of covalent modification, Liu et al. (2015) were able to conduct structural characterization and functional evaluation of protein (lactoferrin)-polyphenol conjugates.

In addition to looking at different ways to conjugate proteins to polyphenols and using those to prepare emulsions, interfacial engineering itself could be achieved differently from what was seen here. The strategy includes depositing multiple layers of proteins and polysaccharides using a layer-by-layer (LBL) electrostatic deposition technique (Ahmad et al., 2011). Literature shows that a two-layered system improves the oxidative stability of O/W emulsions due to the increased thickness of interfacial multilayer which acts as an effective barrier against pro-oxidant initiators (Taherian et al., 2011). For Pan and Nitin (2015), adding a layer of tannic acid to the multilayer emulsion provided better stability against degradation of linseed oil compared to free dispersed tocopherols in the oil phase of the same multilayered emulsion. The possibility of placing an additional layer of polyphenol to a multilayered droplet interface could be another extension of the present work.

Plant oils rich in unsaturated fatty acids, such as those derived from canola and flaxseed, are widely used in food formulations and direct human consumption (Waterhouse et al., 2014). However, these oils are chemically unstable and highly susceptible to oxidative deterioration, especially when exposed to oxygen, heat, light, moisture and transition metal ions. Microencapsulation, whereby tiny particles or droplets containing food ingredients, cells or enzymes are coated on a micro-scale are shown to be effective and thus, widely applied to preserve PUFA and other bioactive compounds. These microcapsules or dry emulsions are obtained by removing the water from an oil-in-water emulsion using rotary evaporation (Myers & Shively, 1992), freeze drying (Heinzelmann & Franke, 1999) or spray drying (Cui et al., 2007). While temperature remains controlled in the first two processes, during spray drying the outlet temperature can reach 60-120°C (Maas et al., 2011). Therefore, having polyphenols conjugated to the protein and used as an encapsulating agent at the oil droplet interface could be helpful during spray drying rather than have the polyphenol simply dispersed in the bulk aqueous phase. Hence an appropriate future study of the present research could be to spray dry the emulsions and test the conjugates' ability to protect the internal core oil against degradation.

Finally, coming to the application aspect of the present study, all emulsions prepared had the potential to be developed further into being marketed as a beverage. Conventionally, beverage emulsions are made with low oil concentrations carrying necessary flavor compounds which are later diluted to a greater extent to obtain the final beverage (Piorkowski & McClements, 2014).

Firstly, since the emulsions in the study were formulated with just 5 wt.% oil, they displayed beverage-like characteristics. Secondly, although the emulsions completely separated at 25 ℃ after 4 weeks of storage, at a temperature of 5 ℃ they exhibited no visible creaming and were stable for up to 1.5 months. Moreover, The polyphenols incorporated in the conjugates imparted a characteristic color to the product apart from unique antioxidative properties. This, in combination with certain flavor oils (e.g., citrus oil, orange oil) incorporated into the lipid phase, can be utilized in the development of a novel and efficient way to deliver the benfits of plant proteins, polyphenols and other nutraceuticals towards healthier foods for the market.

REFERENCES

Aewsiri, T., Benjakul, S., & Visessanguan, W. (2009). Functional properties of gelatin from cuttlefish (Sepia pharaonis) skin as affected by bleaching using hydrogen peroxide. *Food Chemistry, 115*(1), 243-249.

Afanas'ev, I. B., Dcrozhko, A. I., Brodskii, A. V., Kostyuk, V. A., & Potapovitch, A. I. (1989). Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochemical Pharmacology, 38*(11), 1763-1769.

Aguilera, J. & Stanley, D. (1999). *Microstructural Principles of Food Processing & Engineering.* (2nd Edition). Gaithersberg, MA: Aspen Publishers, Inc.

Ahmad, A., Kusumastuti, A., Derek, C., & Ooi, B. (2011). Emulsion liquid membrane for heavy metal removal: An overview on emulsion stabilization and destabilization. *Chemical Engineering Journal, 171*(3), 870-882.

Ai, H., Jones, S. A., & Lvov, Y. M. (2003). Biomedical applications of electrostatic layer-by-layer nano-assembly of polymers, enzymes, and nanoparticles. *Cell Biochemistry and Biophysics, 39*(1), 23.

Akhtar, M., & Dickinson, E. (2003). Emulsifying properties of whey protein–dextran conjugates at low pH and different salt concentrations. *Colloids and Surfaces B: Biointerfaces, 31*(1), 125-132.

Ali, M., Homann, T., Kreisel, J., Khalil, M., Puhlmann, R., Kruse, H.-P., & Rawel, H. (2012). Characterization and modeling of the interactions between coffee storage proteins and phenolic compounds. *Journal of Agricultural and Food Chemistry, 60*(46), 11601-11608.

Allen, J., & Hamilton, R. (1994). *Rancidity in Foods*. (3rd Edition). Blackie Academic and Professional: Chapman & Hall, London.

Amorati, R., Lucarini, M., Mugnaini, V., Pedulli, G. F., Minisci, F., Recupero, F., Fontana, F., Astolfi, P., & Greci, L. (2003). Hydroxylamines as oxidation catalysts: thermochemical and kinetic studies. *Journal of Organic Chemistry, 68*(5), 1747-1754.

Amorati, R., & Valgimigli, L. (2012). Modulation of the antioxidant activity of phenols by non-covalent interactions. *Organic & Biomolecular Chemistry, 10*(21), 4147-4158.

Apak, R., Güçlü, K., Demirata, B., Özyürek, M., Çelik, S., Bektaşoğlu, B., Berker, K., & Özyurt, D. (2007). Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules, 12*(7), 1496-1547.

Arts, I. C., & Hollman, P. C. (2005). Polyphenols and disease risk in epidemiologic studies. *The American Journal of Clinical Nutrition, 81*(1), 317S-325S.

Astrup, A., Dyerberg, J., Elwood, P., Hermansen, K., Hu, F. B., Jakobsen, M. U., Kok, F. J., Krauss, R. M., Lecerf, J. M., & LeGrand, P. (2011). The role of reducing intakes of saturated fat in the prevention of cardiovascular disease: where does the evidence stand in 2010? *The American Journal of Clinical Nutrition, 93*(4), 684-688.

Atkinson, P. J., Dickinson, E., Horne, D. S., & Richardson, R. M. (1995). Neutron reflectivity of adsorbed β-casein and β-lactoglobulin at the air/water interface. *Journal of the Chemical Society, Faraday Transactions, 91*(17), 2847-2854.

Aveyard, R., Binks, B. P., Clark, S., & Fletcher, P. D. (1990). Cloud points, solubilisation and interfacial tensions in systems containing nonionic surfactants. *Journal of Chemical Technology & Biotechnology, 48*(2), 161-171.

Aydemir, L. Y., & Yemenicioğlu, A. (2013). Potential of Turkish Kabuli type chickpea and green and red lentil cultivars as source of soy and animal origin functional protein alternatives. *LWT-Food Science and Technology, 50*(2), 686-694.

Barnell, H., & Barnell, E. (1945). Studies in Tropical Fruits: XVI. The distribution of tannins within the banana and the changes in their condition and amount during ripening. *Annals of Botany, 9*(33), 77-99.

Barriuso, B., Astiasarán, I., & Ansorena, D. (2013). A review of analytical methods measuring lipid oxidation status in foods: a challenging task. *European Food Research and Technology, 236*(1), 1-15.

Baxter, N. J., Lilley, T. H., Haslam, E., & Williamson, M. P. (1997). Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry, 36*(18), 5566-5577.

Beart, J. E., Lilley, T. H., & Haslam, E. (1985). Polyphenol interactions. Part 2. Covalent binding of procyanidins to proteins during acid-catalysed decomposition; observations on some polymeric proanthocyanidins. *Journal of the Chemical Society, Perkin Transactions 2*(9), 1439-1443.

Beckman, C. H. (2000). Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? *Physiological and Molecular Plant Pathology, 57*(3), 101-110.

Berton-Carabin, C., Genot, C., Gaillard, C., Guibert, D., & Ropers, M. (2013). Design of interfacial films to control lipid oxidation in oil-in-water emulsions. *Food Hydrocolloids, 33*(1), 99-105.

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

Berton, C., Ropers, M.-H., Viau, M., & Genot, C. (2011). Contribution of the interfacial layer to the protection of emulsified lipids against oxidation. *Journal of Agricultural and Food Chemistry, 59*(9), 5052-5061.

Beverung, C., Radke, C., & Blanch, H. (1999). Protein adsorption at the oil/water interface: characterization of adsorption kinetics by dynamic interfacial tension measurements. *Biophysical Chemistry, 81*(1), 59-80.

Blonk, J. C. G., & van Aalst, H. (1993). Confocal scanning light microscopy in food research. *Food Research International, 26*(4), 297-311.

Bora, P. S. (2002). Functional properties of native and succinylated lentil (Lens culinaris) globulins. *Food Chemistry, 77*(2), 171-176.

Boye, J., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E., & Rajamohamed, S. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International, 43*(2), 537-546.

Bozan, B., & Temelli, F. (2008). Chemical composition and oxidative stability of flax, safflower and poppy seed and seed oils. *Bioresource Technology, 99*(14), 6354-6359.

Brandts, J. F., & Lin, L. N. (1990). Study of strong to ultratight protein interactions using differential scanning calorimetry. *Biochemistry, 29*(29), 6927-6940.

Cao, G., Sofic, E., & Prior, R. L. (1997). Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radical Biology and Medicine, 22*(5), 749-760.

Cercaci, L., Conchillo, A., Rodriguez-Estrada, M. T., Ansorena, D., Astiasarán, I., & Lercker, G. (2006). Preliminary study on health-related lipid components of bakery products. *Journal of Food Protection, 69*(6), 1393-1401.

Chaiyasit, W., Elias, R. J., McClements, D. J., & Decker, E. A. (2007). Role of physical structures in bulk oils on lipid oxidation. *Critical Reviews in Food Science and Nutrition, 47*(3), 299-317.

Chang, C., Tu, S., Ghosh, S., & Nickerson, M. (2015). Effect of pH on the inter-relationships between the physicochemical, interfacial and emulsifying properties for pea, soy, lentil and canola protein isolates. *Food Research International, 77*, 360-367.

Chaudhuri, T. K., Das, K. P., & Sinha, N. K. (1993). Surface hydrophobicity of a low molecular weight basic trypsin subtilisin inhibitor from marine turtle eggwhite. *Journal of Biochemistry, 113*(6), 729-733.

Cook, N. C., & Samman, S. (1996). Flavonoids—chemistry, metabolism, cardioprotective effects, and dietary sources. *The Journal of Nutritional Biochemistry*, *7*(2), 66-76.

Coupland, J. N., & McClements, D. J. (1996). Lipid oxidation in food emulsions. *Trends in Food Science & Technology, 7*(3), 83-91.

Crespy, D., Stark, M., Hoffmann-Richter, C., Ziener, U., & Landfester, K. (2007). Polymeric nanoreactors for hydrophilic reagents synthesized by interfacial polycondensation on miniemulsion droplets. *Macromolecules, 40*(9), 3122-3135.

Cui, F., Wang, Y., Wang, J., Feng, L., & Ning, K. (2007). Preparation of an enteric-soluble solid-state emulsion using oily drugs. *International Journal of Pharmaceutics, 338*(1-2), 152-156.

Daisuke, W., Noriko, I., Akira, U., & Takuya, S. (2004). Fat-soluble vitamins-containing food and drink product, and method for stabilizing fat-soluble vitamins. Japan patent *JP 2,00,40,97,113 A*.

Dalgleish, D. G. (2004). Food emulsions: their structures and properties. *Food Emulsions*, *4,* 1-44.

Dalgleish, D. G. (2006). Food emulsions—their structures and structure-forming properties. *Food Hydrocolloids, 20*(4), 415-422.

Dalgleish, D. G., & Hollocou, A. -L. (1997). Stabilization of protein-based emulsions by means of interacting polysaccharides. In E. Dickinson & B. Bergenståhl (Ed.), *Food Colloids: Proteins, Lipids and Polysaccharides*, (pp. 236-244). Cambridge UK: Royal Society of Chemistry.

Damodaran, S. (1996). Functional properties. In S. Nakai & H. W. Modler (Ed.), *Food Proteins: Properties and Characterization* (pp. 167-234). New York, USA: VCH Publishers.

de Dicastillo, C. L., Rodríguez, F., Guarda, A., & Galotto, M. J. (2016). Antioxidant films based on cross-linked methyl cellulose and native Chilean berry for food packaging applications. *Carbohydrate Polymers, 136*, 1052-1060.

Denkov, N. D., Tcholakova, S., & Ivanov, I. B. (2006). Globular proteins as emulsion stabilizers-similarities and differences with surfactants and solid particles. Paper presented at the The Proceedings of the 4th World Congress on Emulsions.

Derbshyre, E., Wright, D., & Boutler, D. (1976). Legumin and vicilin storage proteins of legume seed. *Phytochemistry, 15*, 3-24.

Detloff, T., Sobisch, T., & Lerche, D. (2013). Instability index. *Dispersion Letters Technical, T4*, 1-4.

Di Mattia, C. D., Sacchetti, G., Mastrocola, D., & Pittia, P. (2009). Effect of phenolic antioxidants on the dispersion state and chemical stability of olive oil O/W emulsions. *Food Research International, 42*(8), 1163-1170.

Dickinson, E. (1992). *Introduction to Food Colloids*. Oxford University Press.

Dickinson, E. (1999). Caseins in emulsions: interfacial properties and interactions. *International Dairy Journal, 9*(3), 305-312.

Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids, 17*(1), 25-39.

Dickinson, E. (2013). Stabilising emulsion-based colloidal structures with mixed food ingredients. *Journal of the Science of Food and Agriculture, 93*(4), 710-721.

Dickinson, E., Goller, M. I., & Wedlock, D. J. (1995). Osmotic pressure, creaming, and rheology of emulsions containing nonionic polysaccharide. *Journal of Colloid and Interface Science, 172*(1), 192-202.

Dickinson, E., & Stainsby, G. (1982). *Colloids in Food*. Barking, Essex, UK: Applied Science Publishers.

Diftis, N., Biliaderis, C., & Kiosseoglou, V. (2005). Rheological properties and stability of model salad dressing emulsions prepared with a dry-heated soybean protein isolate–dextran mixture. *Food Hydrocolloids, 19*(6), 1025-1031.

Djordjevic, D., Kim, H. J., McClements, D. J., & Decker, E. A. (2004). Physical stability of whey protein-stabilized oil-in-water emulsions at pH 3: potential ω-3 fatty acid delivery systems (Part A). *Journal of Food Science, 69*(5), C351-C355.

Đorđević, V., Balanč, B., Belščak-Cvitanović, A., Lević, S., Trifković, K., Kalušević, A., Kostić, I., Komes, D., Bugarski, B., & Nedović, V. (2015). Trends in encapsulation technologies for delivery of food bioactive compounds. *Food Engineering Reviews, 7*(4), 452-490.

Duranti, M. (2006). Grain legume proteins and nutraceutical properties. *Fitoterapia, 77*(2), 67-82.

Ettoumi, Y. L., Chibane, M., & Romero, A. (2016). Emulsifying properties of legume proteins at acidic conditions: Effect of protein concentration and ionic strength. *LWT-Food Science and Technology, 66*, 260-266.

Evans, D. F., & Wennerström, H. (1999). *The Colloidal Domain, where Physics, Chemistry, Biology, and Technology Mee*t. New York, NY: VCH Publishers.

Evans, M., Ratcliffe, I., & Williams, P. A. (2013). Emulsion stabilisation using polysaccharide–protein complexes. *Current Opinion in Colloid & Interface Science, 18*(4), 272-282.

Eymard, S., Baron, C. P., & Jacobsen, C. (2009). Oxidation of lipid and protein in horse mackerel (Trachurus trachurus) mince and washed minces during processing and storage. *Food Chemistry, 114*(1), 57-65.

Fang, Y., & Dalgleish, D. G. (1993). Dimensions of the adsorbed layers in oil-in-water emulsions stabilized by caseins. *Journal of Colloid and Interface Science, 156*(2), 329-334.

Farinato, R., & Rowell, R. (1983). Optical properties of emulsions. *Encyclopedia of Emulsion Technology, 1*, 439-480.

Farooq, Z., & Boye, J. (2011). Novel food and industrial applications of pulse flours and fractions. In Tiwari, B. K., Gowen, A., McKenna, B., (Eds.), *Pulse Foods: Processing, Quality and Nutraceutical Applications*, (pp. 283-323). Amsterdam, Netherlands: Elsevier.

Feng, J., Cai, H., Wang, H., Li, C., & Liu, S. (2018). Improved oxidative stability of fish oil emulsion by grafted ovalbumin-catechin conjugates. *Food Chemistry, 241*(Supplement C), 60-69.

Fernández, J., Pérez-Álvarez, J. A., & Fernández-López, J. A. (1997). Thiobarbituric acid test for monitoring lipid oxidation in meat. *Food Chemistry, 59*(3), 345-353.

Ferrazzano, G. F., Amato, I., Ingenito, A., De Natale, A., & Pollio, A. (2009). Anti-cariogenic effects of polyphenols from plant stimulant beverages (cocoa, coffee, tea). *Fitoterapia, 80*(5), 255-262.

Fraga, C. G., Galleano, M., Verstraeten, S. V., & Oteiza, P. I. (2010). Basic biochemical mechanisms behind the health benefits of polyphenols. *Molecular Aspects of Medicine, 31*(6), 435-445.

Frankel, E. (1998a). Antioxidants. *Lipid Oxidation*, (pp. 209-258). (2nd Edition). Cambridge, UK: Woodhead Publishing Ltd.

Frankel, E. (1998b). Methods to determine extent of oxidation. *Lipid oxidation*, (pp. 79-98). (2nd Edition). Cambridge, UK: Woodhead Publishing Ltd.

Frankel, E. N. (1985). Chemistry of autoxidation: mechanism, products and flavor significance. In Min, D.B., Smouse, T.H., (Eds.), *Flavor Chemistry of Fats and Oils*, (pp. 1-37). Champaign, Ill.: American Oil Chemists' Society.

Frankel, E. N. (1999). Food antioxidants and phytochemicals: present and future perspectives. *Lipid/Fett, 101*(12), 450-455.

Frankel, E. N., Huang, S.-W., Aeschbach, R., & Prior, E. (1996). Antioxidant activity of a rosemary extract and its constituents, carnosic acid, carnosol, and rosmarinic acid, in bulk oil and oil-in-water emulsion. *Journal of Agricultural and Food Chemistry, 44*(1), 131-135.

Frazier, R. A., Papadopoulou, A., & Green, R. J. (2006). Isothermal titration calorimetry study of epicatechin binding to serum albumin. *Journal of Pharmaceutical and Biomedical Analysis, 41*(5), 1602-1605.

Friberg, S., Larsson, K., & Sjoblom, J. (2003). *Food Emulsions.* (4th Edition). New York, NY: Marcel Dekker Inc.

Fukumoto, L., & Mazza, G. (2000). Assessing antioxidant and prooxidant activities of phenolic compounds. *Journal of Agricultural and Food Chemistry, 48*(8), 3597-3604.

Gaonkar, A. G. (1994). Stable multiple emulsions comprising interfacial gelatinous layer, flavor-encapsulating multiple emulsions and low/no-fat food products comprising the same. U.S. Patent No. 5,332,595. Washington, DC: U.S. Patent and Trademark Office.

Genot, C., Meynier, A., & Riaublanc, A. (2003). Lipid oxidation in emulsions. *Lipid Oxidation Pathways* (pp. 190-244). (1st Edition). New York, NY: AOCS Publishing.

Gong, Y., Liu, X., He, W.-H., Xu, H.-G., Yuan, F., & Gao, Y.-X. (2012). Investigation into the antioxidant activity and chemical composition of alcoholic extracts from defatted marigold (Tagetes erecta L.) residue. *Fitoterapia, 83*(3), 481-489.

Goormaghtigh, E., Cabiaux, V., & Ruysschaert, J.-M. (1994). Determination of soluble and membrane protein structure by Fourier transform infrared spectroscopy. *Subcellular Biochemistry*, 23, 363-404.

Gordon, M. (1990). The mechanism of antioxidant action in vitro. *Food Antioxidants* (pp. 1-18). (1st Edition). Elsevier Applied Science Publishers.

Graf, B. A., Milbury, P. E., & Blumberg, J. B. (2005). Flavonols, flavones, flavanones, and human health: epidemiological evidence. *Journal of Medicinal Food, 8*(3), 281-290.

Grigoriev, D., Miller, R., Shchukin, D., & Möhwald, H. (2007). Interfacial assembly of partially hydrophobic silica nanoparticles induced by ultrasonic treatment. *Small, 3*(4), 665-671.

Gumus, C. E., Decker, E. A., & McClements, D. J. (2017). Formation and stability of ω-3 oil emulsion-based delivery systems using plant proteins as emulsifiers: lentil, pea, and faba bean proteins. *Food Biophysics, 12*(2), 186-197.

Guzey, D., & McClements, D. J. (2006). Formation, stability and properties of multilayer emulsions for application in the food industry. *Advances in Colloid and Interface Science, 128*, 227-248.

Guzey, D., & McClements, D. J. (2007). Impact of electrostatic interactions on formation and stability of emulsions containing oil droplets coated by β-lactoglobulin− pectin complexes. *Journal of Agricultural and Food Chemistry, 55*(2), 475-485.

Halliwell, B., Aeschbach, R., Löliger, J., & Aruoma, O. (1995). The characterization of antioxidants. *Food and Chemical Toxicology, 33*(7), 601-617.

Hamilton, R. J., & Rossell, J. B. (1986). *Analysis of Oils and Fats* (pp. 1-90). New York, NY: Elsevier Applied Science Publishers.

Hardman, W. E. (2014). Diet components can suppress inflammation and reduce cancer risk. *Nutrition Research and Practice*, *8*(3), 233-240.

Haslam, E. (1996). Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *Journal of Natural Products, 59*(2), 205-215.

Hasni, I., Bourassa, P., Hamdani, S., Samson, G., Carpentier, R., & Tajmir-Riahi, H.-A. (2011). Interaction of milk α-and β-caseins with tea polyphenols. *Food Chemistry, 126*(2), 630-639.

Heinzelmann, K., & Franke, K. (1999). Using freezing and drying techniques of emulsions for the microencapsulation of fish oil to improve oxidation stability. *Colloids and Surfaces B: Biointerfaces, 12*(3-6), 223-229.

Henning, T., Mothes, R., Dudek, S., Krause, J., & Schwenke, K. (1997). Structural and functional changes of faba bean legumin during super-limited tryptic hydrolysis. *Food/Nahrung, 41*(2), 81-86.

Ho, C. T. (1992). Phenolic compounds in food. In Ho, C., Lee, CY. and Huang, M, (Eds.), *Phenolic Compounds in Food and their Effects on Health Volume I: Analysis, Occurrence and Chemistry* (pp. 1-7). New York, NY: AOCS Publishing.

Holm, F., & Eriksen, S. (1980). Emulsifying properties of undenatured potato protein concentrate. *International Journal of Food Science & Technology, 15*(1), 71-83.

Howard, J., & Ingold, K. (1968). Self-reaction of sec-butylperoxy radicals. Confirmation of the Russell mechanism. *Journal of the American Chemical Society, 90*(4), 1056-1058.

Hu, M., McClements, D. J., & Decker, E. A. (2003). Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey protein isolate, and soy protein isolate. *Journal of Agricultural and Food Chemistry, 51*(6), 1696-1700.

Huang, S.-W., Frankel, E. N., Schwarz, K., Aeschbach, R., & German, J. B. (1996). Antioxidant activity of carnosic acid and methyl carnosate in bulk oils and oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 44*(10), 2951-2956.

Huang, S. W., & Frankel, E. N. (1997). Antioxidant activity of tea catechins in different lipid systems. *Journal of Agricultural and Food Chemistry, 45*(8), 3033-3038.

Huang, S. W., Frankel, E. N., & German, J. B. (1994). Antioxidant activity of. alpha.-and. gamma.-tocopherols in bulk oils and in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 42*(10), 2108-2114.

Hunt, J. A., & Dalgleish, D. G. (1995). Heat stability of oil-in-water emulsions containing milk proteins: effect of ionic strength and pH. *Journal of Food Science, 60*(5), 1120-1123.

Hunter, R. J. (1993). *Introduction to Modern Colloid Science.* Oxford, UK: Oxford University Press.

Hunter, R. J. (2013). *Zeta Potential in Colloid Science: Principles and Applications* (Vol. 2). Waltham, MA: Academic press.

Hurrell, R. F., & Finot, P.-A. (1984). Nutritional consequences of the reactions between proteins and oxidized polyphenolic acids. *Nutritional and Toxicological Aspects of Food Safety* (pp. 423-435). New York, NY: Plenum Press.

Israelachvili, J., & Ninham, B. (1977). Intermolecular forces—the long and short of it. *Journal of Colloid and Interface Science, 58*(1), 14-25.

Iverson, F. (1995). Phenolic antioxidants: health protection branch studies on butylated hydroxyanisole. *Cancer Letters, 93*(1), 49-54.

Jarpa-Parra, M. (2018). Lentil protein: a review of functional properties and food application. An overview of lentil protein functionality. *International Journal of Food Science & Technology, 53*(4), 892-903.

Jöbstl, E., O'Connell, J., Fairclough, J. P. A., & Williamson, M. P. (2004). Molecular model for astringency produced by polyphenol/protein interactions. *Biomacromolecules, 5*(3), 942-949.

Joshi, M., Adhikari, B., Aldred, P., Panozzo, J., & Kasapis, S. (2011). Physicochemical and functional properties of lentil protein isolates prepared by different drying methods. *Food Chemistry, 129*(4), 1513-1522.

Joshi, M., Adhikari, B., Aldred, P., Panozzo, J., Kasapis, S., & Barrow, C. (2012). Interfacial and emulsifying properties of lentil protein isolate. *Food Chemistry, 134*(3), 1343-1353.

Karaca, A. C., Low, N., & Nickerson, M. (2011). Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction. *Food Research International, 44*(9), 2742-2750.

Karefyllakis, D., Altunkaya, S., Berton-Carabin, C. C., van der Goot, A. J., & Nikiforidis, C. V. (2017). Physical bonding between sunflower proteins and phenols: Impact on interfacial properties. *Food Hydrocolloids, 73*, 326-334.

Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta (BBA)-Protein structure, 624*(1), 13-20.

Kehrer, J. P. (1994). Free radicals in biology: sources, reactivities, and roles in the etiology of human diseases. *Natural Antioxidants in Human Health and Disease*, 25-62.

Kennedy, E. T., Luo, H., & Ausman, L. M. (2012). Cost implications of alternative sources of (n-3) fatty acid consumption in the United States. *The Journal of Nutrition, 142*(3), 605S-609S.

Kessler, M., Ubeaud, G., & Jung, L. (2003). Anti-and pro-oxidant activity of rutin and quercetin derivatives. *Journal of Pharmacy and Pharmacology, 55*(1), 131-142.

Khan, M. K., Rakotomanomana, N., Dufour, C., & Dangles, O. (2011). Binding of citrus flavanones and their glucuronides and chalcones to human serum albumin. *Food & Function, 2*(10), 617-626.

Kim, H.-J., Decker, E., & McClements, D. (2002). Role of postadsorption conformation changes of β-lactoglobulin on its ability to stabilize oil droplets against flocculation during heating at neutral pH. *Langmuir, 18*(20), 7577-7583.

Kim, S.-H., Nam, J., Kim, J. W., Kim, D.-H., Han, S.-H., & Weitz, D. A. (2013). Formation of polymersomes with double bilayers templated by quadruple emulsions. *Lab on a Chip, 13*(7), 1351-1356.

Kim, S., & Cavaco-Paulo, A. (2012). Laccase-catalysed protein–flavonoid conjugates for flax fibre modification. *Applied Microbiology and Biotechnology, 93*(2), 585-600.

Kiokias, S. N., Dimakou, C. P., Tsaprouni, I. V., & Oreopoulou, V. (2006). Effect of compositional factors against the thermal oxidative deterioration of novel food emulsions. *Food Biophysics, 1*(3), 115.

Kirby, A. R., Gunning, A. P., & Morris, V. J. (1995). Imaging xanthan gum by atomic force microscopy. *Carbohydrate Research, 267*(1), 161-166.

Kitagawa, M., & Tokiwa, Y. (2006). Polymerization of vinyl sugar ester using ascorbic acid and hydrogen peroxide as a redox reagent. *Carbohydrate Polymers, 64*(2), 218-223.

Klinkesorn, U., Sophanodora, P., Chinachoti, P., McClements, D. J., & Decker, E. A. (2005). Increasing the oxidative stability of liquid and dried tuna oil-in-water emulsions with electrostatic layer-by-layer deposition technology. *Journal of Agricultural and Food Chemistry, 53*(11), 4561-4566.

Kroll, J., & Rawel, H. M. (2001). Reactions of plant phenols with myoglobin: influence of chemical structure of the phenolic compounds. *Journal of Food Science, 66*(1), 48-58.

Kumari, A., Yadav, S. K., Pakade, Y. B., Singh, B., & Yadav, S. C. (2010). Development of biodegradable nanoparticles for delivery of quercetin. *Colloids and Surfaces B: Biointerfaces, 80*(2), 184-192.

Labieniec, M., & Gabryelak, T. (2006). Interactions of tannic acid and its derivatives (ellagic and gallic acid) with calf thymus DNA and bovine serum albumin using spectroscopic method. *Journal of Photochemistry and Photobiology B: Biology, 82*(1), 72-78.

Lakowicz, J. R. (2004). Principles of Fluorescence Spectroscopy. (3rd Edition). New York, NY: Kluwer Academic/Plenum Publishers.

Landrault, N., Poucheret, P., Ravel, P., Gasc, F., Cros, G., & Teissedre, P.-L. (2001). Antioxidant capacities and phenolics levels of French wines from different varieties and vintages. *Journal of Agricultural and Food Chemistry, 49*(7), 3341-3348.

Laughton, M. J., Halliwell, B., Evans, P. J., Robin, J., & Hoult, S. (1989). Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin: effects on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. *Biochemical Pharmacology, 38*(17), 2859-2865.

Lawrence, A. S. C., & Mills, O. S. (1954). Kinetics of the coagulation of emulsions. *Discussions of the Faraday Society, 18*, 98-104.

Lerche, D. (2002). Dispersion stability and particle characterization by sedimentation kinetics in a centrifugal field. *Journal of Dispersion Science and Technology, 23*(5), 699-709.

Lips, A., Campbell, I., & Pelan, E. (1991). Aggregation mechanisms *in* food colloids and the role of biopolymers. *Food Polymers, Gels and Colloids* (pp. 1-15). Cambridge, UK: Royal Society of Chemistry.

Liu, F., Ma, C., McClements, D. J., & Gao, Y. (2016a). Development of polyphenol-protein-polysaccharide ternary complexes as emulsifiers for nutraceutical emulsions: Impact on formation, stability, and bioaccessibility of β-carotene emulsions. *Food Hydrocolloids, 61*, 578-588.

Liu, F., Ma, C., McClements, D. J., & Gao, Y. (2017a). A comparative study of covalent and non-covalent interactions between zein and polyphenols in ethanol-water solution. *Food Hydrocolloids, 63*, 625-634.

Liu, F., Ma, C., Zhang, R., Gao, Y., & McClements, D. J. (2017b). Controlling the potential gastrointestinal fate of β-carotene emulsions using interfacial engineering: Impact of coating lipid droplets with polyphenol-protein-carbohydrate conjugate. *Food Chemistry, 221*, 395-403.

Liu, F., Sun, C., Yang, W., Yuan, F., & Gao, Y. (2015). Structural characterization and functional evaluation of lactoferrin–polyphenol conjugates formed by free-radical graft copolymerization. *RSC Advances, 5*(20), 15641-15651.

Liu, F., Wang, D., Sun, C., & Gao, Y. (2016b). Influence of polysaccharides on the physicochemical properties of lactoferrin–polyphenol conjugates coated β-carotene emulsions. *Food Hydrocolloids, 52*, 661-669.

Liu, F., Wang, D., Sun, C., McClements, D. J., & Gao, Y. (2016c). Utilization of interfacial engineering to improve physicochemical stability of β-carotene emulsions: Multilayer coatings formed using protein and protein–polyphenol conjugates. *Food Chemistry, 205*, 129-139.

Liu, F., Wang, D., Xu, H., Sun, C., & Gao, Y. (2016d). Physicochemical properties of β-carotene emulsions stabilized by chlorogenic acid–lactoferrin–glucose/polydextrose conjugates. *Food Chemistry, 196*, 338-346.

Liu, H., Wang, C., Zou, S., Wei, Z., & Tong, Z. (2012a). Simple, reversible emulsion system switched by pH on the basis of chitosan without any hydrophobic modification. *Langmuir, 28*(30), 11017-11024.

Liu, Y., Zhao, G., Zhao, M., Ren, J., & Yang, B. (2012b). Improvement of functional properties of peanut protein isolate by conjugation with dextran through Maillard reaction. *Food Chemistry, 131*(3), 901-906.

Lomova, M. V., Sukhorukov, G. B., & Antipina, M. N. (2010). Antioxidant coating of micronsize droplets for prevention of lipid peroxidation in oil-in-water emulsion. *ACS Applied Materials & Interfaces, 2*(12), 3669-3676.

Loomis, W. D., & Battaile, J. (1966). Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry, 5*(3), 423-438.

Losso, J. N., Bansode, R. R., Trappey II, A., Bawadi, H. A., & Truax, R. (2004). In vitro anti-proliferative activities of ellagic acid. *The Journal of Nutritional Biochemistry, 15*(11), 672-678.

Luo, Z., Murray, B. S., Yusoff, A., Morgan, M. R., Povey, M. J., & Day, A. J. (2011). Particle-stabilizing effects of flavonoids at the oil− water interface. *Journal of Agricultural and Food Chemistry, 59*(6), 2636-2645.

Lyklema, J. (1991). Foundations of colloid science, vol. II. *Journal of Colloid and Interface Science, 141, 594-595*.

Maas, S. G., Schaldach, G., Littringer, E. M., Mescher, A., Griesser, U. J., Braun, D. E., Walzel, P. E., & Urbanetz, N. A. (2011). The impact of spray drying outlet temperature on the particle morphology of mannitol. *Powder Technology, 213*(1-3), 27-35.

Macheix, J., Fleuriet, A., & Billot, J. (1990). Phenolic compounds in fruit processing. *Fruit Phenolics, 1*, 295-358.

Madhavi, D. L., Deshpande, S., & Salunkhe, D. K. (1995). *Food Antioxidants: Technological: Toxicological and Health Perspectives.* Boca Raton: CRC Press.

Mao, L., Xu, D., Yang, J., Yuan, F., Gao, Y., & Zhao, J. (2009). Effects of small and large molecule emulsifiers on the characteristics of b-carotene nanoemulsions prepared by high pressure homogenization. *Food Technology and Biotechnology, 47*(3), 336-342.

Maswal, M., & Dar, A. A. (2014). Formulation challenges in encapsulation and delivery of citral for improved food quality. *Food Hydrocolloids, 37*, 182-195.

McClements, D. (2003). Analysis of food products. *Food Science, 581*, 1-57.

McClements, D., & Decker, E. (2000). Lipid oxidation in oil-in-water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. *Journal of Food Science, 65*(8), 1270-1282.

McClements, D. J. (2004a). *Food Emulsions: Principles, Practices, and Techniques.* Boca Raton: CRC Press.

McClements, D. J. (2004b). Protein-stabilized emulsions. *Current Opinion in Colloid & Interface Science, 9*(5), 305-313.

McClements, D. J. (2005). Theoretical analysis of factors affecting the formation and stability of multilayered colloidal dispersions. *Langmuir, 21*(21), 9777-9785.

McClements, D. J. (2012). Advances in fabrication of emulsions with enhanced functionality using structural design principles. *Current Opinion in Colloid & Interface Science, 17*(5), 235-245.

McClements, D. J. (2015). *Food Emulsions: Principles, Practices, and Techniques.* Boca Raton: CRC press.

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

McClements, D. J., & Rao, J. (2011). Food-grade nanoemulsions: formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Critical Reviews in Food Science and Nutrition, 51*(4), 285-330.

McManus, J. P., Davis, K. G., Beart, J. E., Gaffney, S. H., Lilley, T. H., & Haslam, E. (1985). Polyphenol interactions. Part 1. Introduction; some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *Journal of the Chemical Society, Perkin Transactions 2*(9), 1429-1438.

Meyer, A. S., Heinonen, M., & Frankel, E. N. (1998). Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chemistry, 61*(1-2), 71-75.

Middleton, E., Kandaswami, C., & Theoharides, T. C. (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Reviews, 52*(4), 673-751.

Min, D. B., & Boff, J. M. (2002). Lipid oxidation of edible oil. In Akoh, C. C., Min, D. B. (Eds.), *Food Lipids* (pp. 335-364). (2nd Edition). New York, NY: Marcel Dekker, Inc.

Mitchell, S., & Waring, R. (2000). *Ullmanns Encyclopedia of Industrial Chemistry*. Verlag, Weinheim, Germany: John Wiley & Sons.

Miura, Y. H., Tomita, I., Watanabe, T., Hirayama, T., & Fukui, S. (1998). Active oxygens generation by flavonoids. *Biological and Pharmaceutical Bulletin, 21*(2), 93-96.

Mohanan, A., Nickerson, M. T., & Ghosh, S. (2018). Oxidative stability of flaxseed oil: effect of hydrophilic, hydrophobic and intermediate polarity antioxidants. *Food Chemistry*, *266*, 524-533.

Moslavac, T., Jokić, S., Šubarić, D., Aladić, K., & Dominković, O. (2015). The influence of antioxidants on oxidative stability of sunflower and olive oil. *Technologica Acta, 8*(2), 25-30.

Mun, S., Shin, M., & Kim, Y.-R. (2016). Emulsifying properties of proteins isolated from various rice cultivars. *Food and Bioprocess Technology, 9*(5), 813-821.

Murphy, P., Myers, D., Davies, M., Webster, N., & Jones, J. (1992). The antioxidant potential of propofol (2, 6-diisopropylphenol). *British Journal of Anaesthesia, 68*(6), 613-618.

Murray, N. J., Williamson, M. P., Lilley, T. H., & Haslam, E. (1994). Study of the interaction between salivary proline-rich proteins and a polyphenol by 1H-NMR spectroscopy. *European Journal of Biochemistry, 219*(3), 923-935.

Muschiolik, G. (2007). Multiple emulsions for food use. *Current opinion in colloid & interface science, 12*(4), 213-220.

Myers, S. L., & Shively, M. L. (1992). Preparation and characterization of emulsifiable glasses: Oil-in-water and water-in-oil-in-water emulsions. *Journal of colloid and interface science, 149*(1), 271-278.

Nakai, S., & Li-Chan, E. (1988). Hydrophobicity-functionality relationship of food proteins. *Hydrophobic Interactions in Food Systems, 2,* 47-48.

Nawar, W. W. (1985). Chemistry of thermal oxidation of lipids. *Flavor Chemistry of Fats and Oils*, 39-60.

Nick Pace, C., Trevino, S., Prabhakaran, E., & Martin Scholtz, J. (2004). Protein structure, stability and solubility in water and other solvents. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 359*(1448), 1225-1235.

Nicolai, T., Britten, M., & Schmitt, C. (2011). β-Lactoglobulin and WPI aggregates: formation, structure and applications. *Food Hydrocolloids, 25*(8), 1945-1962.

Núñez-Delicado, E., Sánchez-Ferrer, A., & García-Carmona, F. (1997). Cyclodextrins as secondary antioxidants: synergism with ascorbic acid. *Journal of Agricultural and Food Chemistry, 45*(8), 2830-2835.

Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC International) 16th Ed., 1995: 940.33 (Chapter 45).

Ogawa, S., Decker, E. A., & McClements, D. J. (2003a). Influence of environmental conditions on the stability of oil in water emulsions containing droplets stabilized by lecithin− chitosan membranes. *Journal of Agricultural and Food Chemistry, 51*(18), 5522-5527.

Ogawa, S., Decker, E. A., & McClements, D. J. (2003b). Production and characterization of O/W emulsions containing cationic droplets stabilized by lecithin−chitosan membranes. *Journal of Agricultural and Food Chemistry, 51*(9), 2806-2812.

Ogawa, S., Decker, E. A., & McClements, D. J. (2004). Production and characterization of o/w emulsions containing droplets stabilized by lecithin−chitosan−pectin mutilayered membranes. *Journal of Agricultural and Food Chemistry, 52*(11), 3595-3600.

Okonogi, S., Kato, R., Asano, Y., Yuguchi, H., Kumazawa, R., Sotoyama, K., Takahashi, K., & Fujimoto, M. (1994). Methods for producing emulsions, low-fat spread and oil-in-water-in-oil type spread. U.S. Patent No. 5,279,847. Washington, DC: U.S. Patent and Trademark Office.

Orsi, N. (2004). The antimicrobial activity of lactoferrin: current status and perspectives. *Biometals, 17*(3), 189-196.

Pan, Y., & Nitin, N. (2015). Effect of layer-by-layer coatings and localization of antioxidant on oxidative stability of a model encapsulated bioactive compound in oil-in-water emulsions. *Colloids and Surfaces B: Biointerfaces, 135*, 472-480.

Pan, Y., Tikekar, R. V., Wang, M. S., Avena-Bustillos, R. J., & Nitin, N. (2015). Effect of barrier properties of zein colloidal particles and oil-in-water emulsions on oxidative stability of encapsulated bioactive compounds. *Food Hydrocolloids, 43*, 82-90.

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity, 2*(5), 270-278.

Pandolfe, W. D. (1981). Effect of dispersed and continuous phase viscosity on droplet size of emulsions generated by homogenization. *Journal of Dispersion Science and Technology, 2*(4), 459-474.

Pandolfe, W. D. (1983). Homogenizing apparatus and method. U.S. Patent No. 4,383,769. Washington, DC: U.S. Patent and Trademark Office.

Papalamprou, E. M., Doxastakis, G. I., & Kiosseoglou, V. (2010). Chickpea protein isolates obtained by wet extraction as emulsifying agents. *Journal of the Science of Food and Agriculture, 90*(2), 304-313.

Patel, A. D. (2017). *Stability, rheology and the effect of various environmental factors on the formation of food protein-stabilized nanogels from nanoemulsions*. (Unpublished master's thesis). University of Saskatchewan, Saskatoon, Canada.

Pelton, J. T., & McLean, L. R. (2000). Spectroscopic methods for analysis of protein secondary structure. *Analytical Biochemistry, 277*(2), 167-176.

Petzold, G., Goltzsche, C., Mende, M., Schwarz, S., & Jaeger, W. (2009). Monitoring the stability of nanosized silica dispersions in presence of polycations by a novel centrifugal sedimentation method. *Journal of Applied Polymer Science, 114*(2), 696-704.

Pierpoint, W. S. (1969). o-Quinones formed in plant extracts. Their reactions with amino acids and peptides. *Biochemical Journal*, *112*(5), 609-616.

Piorkowski, D. T., & McClements, D. J. (2014). Beverage emulsions: Recent developments in formulation, production, and applications. *Food Hydrocolloids, 42*, 5-41.

Pokorný, J., Trojáková, L., & Takácsová, M. (2001). The use of natural antioxidants in food products of plant origin. In *Antioxidants in Food* (pp. 355-372). Cambridge, UK: Woodhead Publishing.

Poncet-Legrand, C., Edelmann, A., Putaux, J.-L., Cartalade, D., Sarni-Manchado, P., & Vernhet, A. (2006). Poly (L-proline) interactions with flavan-3-ols units: Influence of the molecular structure and the polyphenol/protein ratio. *Food Hydrocolloids, 20*(5), 687-697.

Prigent, S. V., Voragen, A. G., Li, F., Visser, A. J., van Koningsveld, G. A., & Gruppen, H. (2008). Covalent interactions between amino acid side chains and oxidation products of

caffeoylquinic acid (chlorogenic acid). *Journal of the Science of Food and Agriculture, 88*(10), 1748-1754.

Primozic, M., Duchek, A., Nickerson, M., & Ghosh, S. (2017). Effect of lentil proteins isolate concentration on the formation, stability and rheological behavior of oil-in-water nanoemulsions. *Food Chemistry, 237*, 65-74.

Princen, H. M. (1983). Rheology of foams and highly concentrated emulsions. I. Elastic properties and yield stress of a cylindrical model system. *Journal of Colloid and Interface Science, 91*(1), 160-175.

Prior, R. L., Lazarus, S. A., Cao, G., Muccitelli, H., & Hammerstone, J. F. (2001). Identification of procyanidins and anthocyanins in blueberries and cranberries (Vaccinium spp.) using high-performance liquid chromatography/mass spectrometry. *Journal of Agricultural and Food Chemistry, 49*(3), 1270-1276.

Privalov, P., & Khechinashvili, N. (1974). A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. *Journal of Molecular Biology, 86*(3), 665-684.

Priyadarsini, K. I., Khopde, S. M., Kumar, S. S., & Mohan, H. (2002). Free radical studies of ellagic acid, a natural phenolic antioxidant. *Journal of Agricultural and Food Chemistry, 50*(7), 2200-2206.

Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of Agricultural and Food Chemistry, 48*(8), 3396-3402.

Ratke, L., & Voorhees, P. W. (2013). *Growth and Coarsening: Ostwald Ripening in Material Processing.* Berlin, Germany: Springer Science & Business Media.

Ratnayake, W., Hoover, R., Shahidi, F., Perera, C., & Jane, J. (2001). Composition, molecular structure, and physicochemical properties of starches from four field pea (Pisum sativum L.) cultivars. *Food Chemistry, 74*(2), 189-202.

Rawel, H. M., Czajka, D., Rohn, S., & Kroll, J. (2002a). Interactions of different phenolic acids and flavonoids with soy proteins. *International Journal of Biological Macromolecules, 30*(3-4), 137-150.

Rawel, H. M., Kroll, J., & Rohn, S. (2001). Reactions of phenolic substances with lysozyme—physicochemical characterisation and proteolytic digestion of the derivatives. *Food Chemistry, 72*(1), 59-71.

Rawel, H. M., Meidtner, K., & Kroll, J. (2005). Binding of selected phenolic compounds to proteins. *Journal of Agricultural and Food Chemistry, 53*(10), 4228-4235.

Rawel, H. M., Rohn, S., Kruse, H.-P., & Kroll, J. (2002b). Structural changes induced in bovine serum albumin by covalent attachment of chlorogenic acid. *Food Chemistry, 78*(4), 443-455.

Rice-evans, C. A., Miller, N. J., Bolwell, P. G., Bramley, P. M., & Pridham, J. B. (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research*, *22*(4), 375-383.

Roy, D., Dutta, S., Maity, S. S., Ghosh, S., Roy, A. S., Ghosh, K. S., & Dasgupta, S. (2012). Spectroscopic and docking studies of the binding of two stereoisomeric antioxidant catechins to serum albumins. *Journal of Luminescence, 132*(6), 1364-1375.

Saether, O., Sjoblom, J., & Dukhin, S. (2004). Droplet flocculation and coalescence in dilute oil-in-water emulsions. *Food Emulsions* (pp. 175-220). (*4th Edition)*. NEw York, NY: Marcel Dekker, Inc.

Sagalowicz, L., & Leser, M. E. (2010). Delivery systems for liquid food products. *Current Opinion in Colloid & Interface Science, 15*(1), 61-72.

Salah, N., Miller, N. J., Paganga, G., Tijburg, L., Bolwell, G. P., & Riceevans, C. (1995). Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Archives of Biochemistry and Biophysics, 322*(2), 339-346.

Sarker, D. K., Wilde, P. J., & Clark, D. C. (1995). Control of surfactant-induced destabilization of foams through polyphenol-mediated protein-protein interactions. *Journal of Agricultural and Food Chemistry, 43*(2), 295-300.

Sathe, M., Muthukrishnan, G., Rae, J., Disanza, A., Thattai, M., Scita, G., Parton, R. G., & Mayor, S. (2018). Small GTPases and BAR domain proteins regulate branched actin polymerisation for clathrin and dynamin-independent endocytosis. *Nature Communications, 9*(1), 1835.

Satyanarayana, A., Giridhar, N., Joshi, G., & Rao, D. (2000). Ascorbyl palmitate as an antioxidant for deep fat frying of potato chips in peanut oil. *Journal of Food Lipids, 7*(1), 1-10.

Scalbert, A., Manach, C., Morand, C., Rémésy, C., & Jiménez, L. (2005). Dietary polyphenols and the prevention of diseases. *Critical Reviews in Food Science and Nutrition, 45*(4), 287-306.

Schaich, K. M. (2008). Co-oxidation of proteins by oxidizing lipids. *Lipid oxidation pathways*, *2*, 181-272.

Semenova, M. G., & Dickinson, E. (2010). *Biopolymers in food colloids: Thermodynamics and molecular interactions.* Boca Raton: CRC Press.

Sharif, H. R., Williams, P. A., Sharif, M. K., Abbas, S., Majeed, H., Masamba, K. G., Safdar, W., & Zhong, F. (2018). Current progress in the utilization of native and modified legume proteins as emulsifiers and encapsulants–A review. *Food Hydrocolloids, 76*, 2-16.

Sharma, O. P., & Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food Chemistry, 113*(4), 1202-1205.

Shchukin, D. G., Köhler, K., Möhwald, H., & Sukhorukov, G. B. (2005). Gas-filled polyelectrolyte capsules. *Angewandte Chemie International Edition, 44*(21), 3310-3314.

Shen, L., Zhang, H.-Y., & Ji, H.-F. (2005). Successful application of TD-DFT in transient absorption spectra assignment. *Organic Letters, 7*(2), 243-246.

Shewry, P. R., & Halford, N. G. (2002). Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental Botany, 53*(370), 947-958.

Skrt, M., Benedik, E., Podlipnik, Č., & Ulrih, N. P. (2012). Interactions of different polyphenols with bovine serum albumin using fluorescence quenching and molecular docking. *Food Chemistry, 135*(4), 2418-2424.

Smith Jr, C., Earle, F., Wolff, I., & Jones, Q. (1959). Seed protein solubility, comparison of solubility characteristics of selected seed proteins. *Journal of Agricultural and Food Chemistry, 7*(2), 133-136.

Srinivasan, M., Singh, H., & Munro, P. (2002). Formation and stability of sodium caseinate emulsions: influence of retorting (121 C for 15 min) before or after emulsification. *Food Hydrocolloids, 16*(2), 153-160.

Stevenson, E. M., Horne, D. S., & Leaver, J. (1997). Displacement of native and thiolated β-casein from oil—water interfaces—effect of heating, ageing and oil phase. *Food Hydrocolloids, 11*(1), 3-6.

Strauss, G., & Gibson, S. M. (2004). Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. *Food Hydrocolloids, 18*(1), 81-89.

Subramanian, R., Nandini, K., Sheila, P., Gopalakrishna, A., Raghavarao, K., Nakajima, M., Kimura, T., & Maekawa, T. (2000). Membrane processing of used frying oils. *Journal of the American Oil Chemists' Society, 77*(3), 323.

Sun-Waterhouse, D., Zhou, J., Miskelly, G., Wibisono, R., & Wadhwa, S. (2011). Stability of encapsulated olive oil in the presence of caffeic acid. *Food Chemistry, 126*(3), 1049-1056.

Sun, C., Gunasekaran, S., & Richards, M. P. (2007). Effect of xanthan gum on physicochemical properties of whey protein isolate stabilized oil-in-water emulsions. *Food Hydrocolloids, 21*(4), 555-564.

Suslick, K., Grinstaff, M., Kolbeck, K., & Wong, M. (1994). Characterization of sonochemically prepared proteinaceous microspheres. *Ultrasonics Sonochemistry, 1*(1), S65-S68.

Taherian, A. R., Britten, M., Sabik, H., & Fustier, P. (2011). Ability of whey protein isolate and/or fish gelatin to inhibit physical separation and lipid oxidation in fish oil-in-water beverage emulsion. *Food Hydrocolloids, 25*(5), 868-878.

Talcott, S. T., & Lee, J.-H. (2002). Ellagic acid and flavonoid antioxidant content of muscadine wine and juice. *Journal of Agricultural and Food Chemistry, 50*(11), 3186-3192.

Tan, C.-T. (2004). Beverage emulsions. In Friberg, S.E. (Ed.), *Food emulsions* (pp. 491-524). (3rd Edition). New York, NY: Marcel Dekker.

Teguh, W., Eka, N., & Noor, A. (2014). The utilization of cocoa polyphenol extract to improve the shelf life of bulk frying oil used in open and deep frying. *International Food Research Journal, 21*(1), 111.

Tian, J., Liu, J., He, W., Hu, Z., Yao, X., & Chen, X. (2004). Probing the binding of scutellarin to human serum albumin by circular dichroism, fluorescence spectroscopy, FTIR, and molecular modeling method. *Biomacromolecules, 5*(5), 1956-1961.

Tikekar, R. V., Johnson, A., & Nitin, N. (2011). Real-time measurement of oxygen transport across an oil–water emulsion interface. *Journal of Food Engineering, 103*(1), 14-20.

Toews, R., & Wang, N. (2013). Physicochemical and functional properties of protein concentrates from pulses. *Food Research International, 52*(2), 445-451.

Tokle, T., Lesmes, U., & McClements, D. J. (2010). Impact of electrostatic deposition of anionic polysaccharides on the stability of oil droplets coated by lactoferrin. *Journal of Agricultural and Food Chemistry, 58*(17), 9825-9832.

Torii, H., Tasumi, M., Mantsch, H., & Chapman, D. (1996). Infrared Spectroscopy of Biomolecules. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1767*(9), 1073-1101.

Troëng, S. (1955). Oil determination of oilseed. Gravimetric routine method. *Journal of the American Oil Chemists' Society*, *32*(3), 124-126.

Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. *Nutrients, 2*(12), 1231-1246.

Walstra, P. (1983). Formation of emulsions. *Encyclopedia of emulsion technology*, *1*, 58-127.

Walstra, P. (1993). Principles of emulsion formation. *Chemical Engineering Science, 48*(2), 333-349.

Walstra, P. (1996). Dispersed systems: basic considerations. In Fennema, O. R. (Ed.), *Food Chemistry* (pp. 95-156). (3rd Edition). New York, NY: Marcel Dekker, Inc.

Walstra, P. (2003). Studying food colloids: past, present and future. *Special Publication-Royal Society of Chemistry, 284*, 391-400.

Walstra, P., & Smulders, P. E. (1998). Emulsion formation. *Modern Aspects of Emulsion Science* (pp. 56-99). Cambridge, UK: The Royal Society of Chemistry.

Wang, L.-J., Hu, Y.-Q., Yin, S.-W., Yang, X.-Q., Lai, F.-R., & Wang, S.-Q. (2015a). Fabrication and characterization of antioxidant pickering emulsions stabilized by zein/chitosan complex particles (ZCPs). *Journal of Agricultural and Food Chemistry, 63*(9), 2514-2524.

Wang, L., Dong, Y., Men, H., Tong, J., & Zhou, J. (2013). Preparation and characterization of active films based on chitosan incorporated tea polyphenols. *Food Hydrocolloids, 32*(1), 35-41.

Wang, X., Liu, F., Liu, L., Wei, Z., Yuan, F., & Gao, Y. (2015b). Physicochemical characterisation of β-carotene emulsion stabilised by covalent complexes of α-lactalbumin with (−)-epigallocatechin gallate or chlorogenic acid. *Food Chemistry, 173*, 564-568.

Waraho, T., McClements, D. J., & Decker, E. A. (2011). Mechanisms of lipid oxidation in food dispersions. *Trends in Food Science & Technology, 22*(1), 3-13.

Ward, P. P., Uribe-Luna, S., & Conneely, O. M. (2002). Lactoferrin and host defense. *Biochemistry and Cell Biology, 80*(1), 95-102.

Warner, K., & Eskin, M. (1995). *Methods to Access Quality and Stability of Oils and Fat-Containing Foods.* Champaign, Ill: AOCS Publishing.

Waterhouse, G. I., Wang, W., & Sun-Waterhouse, D. (2014). Stability of canola oil encapsulated by co-extrusion technology: Effect of quercetin addition to alginate shell or oil core. *Food Chemistry, 142*, 27-38.

Wei, Z., Yang, W., Fan, R., Yuan, F., & Gao, Y. (2015). Evaluation of structural and functional properties of protein–EGCG complexes and their ability of stabilizing a model β-carotene emulsion. *Food Hydrocolloids, 45*, 337-350.

Wheatley, M., & Singhal, S. (1995). Structural studies on stabilized microbubbles: development of a novel contrast agent for diagnostic ultrasound. *Reactive Polymers, 25*(2-3), 157-166.

Williams, C. (1995). Healthy eating: clarifying advice about fruit and vegetables. *The Bmj, 310*(6992), 1453-1455.

Xu, D., Wang, X., Jiang, J., Yuan, F., Decker, E. A., & Gao, Y. (2013). Influence of pH, EDTA, α-tocopherol, and WPI oxidation on the degradation of β-carotene in WPI-stabilized oil-in-water emulsions. *LWT-Food Science and Technology, 54*(1), 236-241.

Xu, D., Wang, X., Jiang, J., Yuan, F., & Gao, Y. (2012). Impact of whey protein–Beet pectin conjugation on the physicochemical stability of β-carotene emulsions. *Food Hydrocolloids, 28*(2), 258-266.

Xu, M.-S., Luo, M.-F., Xing, X.-H., & Chen, H.-Z. (2006). Characteristics of quercetin transglycosidation catalysed by *Penicillium decumbens* glycosidase. *Food and Bioproducts Processing, 84*(3), 237-241.

Yerramilli, M., & Ghosh, S. (2017). Long-term stability of sodium caseinate-stabilized nanoemulsions. *Journal of Food Science and Technology, 54*(1), 82-92.

Yi, J., Ning, J., Zhu, Z., Cui, L., Decker, E. A., & McClements, D. J. (2019). Impact of interfacial composition on co-oxidation of lipids and proteins in oil-in-water emulsions: Competitive displacement of casein by surfactants. *Food Hydrocolloids, 87*, 20-28.

Yıldırım, A., Mavi, A., & Kara, A. A. (2001). Determination of antioxidant and antimicrobial activities of Rumex crispus L. extracts. *Journal of Agricultural and Food Chemistry, 49*(8), 4083-4089.

Zou, Y., Guo, J., Yin, S.-W., Wang, J.-M., & Yang, X.-Q. (2015). Pickering emulsion gels prepared by hydrogen-bonded zein/tannic acid complex colloidal particles. *Journal of Agricultural and Food Chemistry, 63*(33), 7405-7414.

APPENDIX

Critical reviews in food science & nutrition

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Diet components can suppress inflammation and reduce cancer risk

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Abstract

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