ENCAPSULATION OF FLAXSEED OIL USING PLANT PROTEINS

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ABSTRACT

The overall goal of this research was to develop a plant protein-based microcapsule capable of carrying, protecting and delivering flaxseed oil within the food and gastrointestinal environment. Specifically, the research aimed to: a) screen a variety of plant proteins and pre-treatment conditions based on their emulsifying properties for use as a wall material; b) develop and optimize encapsulation protocols for entrapping flaxseed oil; and c) study the oxidative stability and delivery of entrapped oils from capsules under different environmental and simulated gastrointestinal conditions.

In Chapter 3 and 4, the emulsifying and physicochemical properties of legume and oilseed protein isolates, respectively produced from isoelectric precipitation and salt extraction were investigated. Findings in Chapter 3 indicated that both the legume source and method of production showed significant effects on the emulsifying and physicochemical properties of chickpea (ChPI), faba bean (FbPI), lentil (LPI), pea (PPI), and soy (SPI) protein isolates. The emulsion capacity (EC) values ranged between 476-542 g oil/g protein with LPI showing the highest capacity. Isoelectric-precipitated ChPI and LPI displayed higher emulsion activity index (EAI) (~46.2 m^2/g), (emulsion stability index) ESI (~84.9 min) and (creaming stability) CS (98.6%), which were comparable to those of SPI. In Chapter 4, findings indicated that both protein source and method of production had significant effects on the physicochemical and emulsifying properties of canola (CaPI) and flaxseed protein isolates (FlPI). CaPI showed significantly higher EC (~515.6 g oil/g protein) than FlPI (~498.9 g oil/g protein). EAI for FlPI was found to be higher (~40.1 m^2/g) than CaPI (~25.1 m^2/g) however, ESI values of CaPI and FlPI were similar. Creaming stability of emulsions stabilized by CaPI and FlPI ranged between 86.1 and 96.6%. CaPI and FlPI were shown to have emulsion forming properties; however their stability was low.

In Chapter 5, ChPI and LPI-stabilized emulsions were optimized based on pH, protein concentration and oil content for their ability to form and stabilize oil-in-water emulsions using response surface methodology. Droplet charge was shown to be only affected by pH, while droplet size and creaming index were affected by protein concentration, oil content and pH. Optimum conditions for minimal creaming (no serum separation after 24 h), small droplet size (<2 μm), and high net droplet charge (absolute zeta potential (ZP) value >40 mV) were identified as: 4.1% protein, 40.0% oil, and pH 3.0 or 8.0, regardless of the plant protein used for emulsion preparation.

Flaxseed oil was microencapsulated by freeze (Chapter 6) or spray (Chapter 7) drying employing ChPI or LPI and maltodextrin. Effects of emulsion formulation (oil, protein and maltodextrin levels) and protein source (ChPI vs. LPI) on the physicochemical characteristics, oxidative stability, and release properties of the resulting capsules were investigated. Optimized capsule designs were found to have high encapsulation efficiencies, low surface oil, and afforded protection against oxidation over a 25 d room temperature storage study relative to free oil. Microcapsules were also able to deliver 84.2% of the encapsulated oil in the simulated gastrointestinal environments.

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LIST OF SYMBOLS AND ABBREVIATIONS

ALA	α-Linolenic acid
ANOVA	Analysis of variance
ANS	8-Anilino-1-naphthalenesulfonic acid
A_w	Water activity
BHT	Butylated hydroxytoluene
BSE	Bovine spongiform encephalopathy
CaPI	Canola protein isolate
ChPI	Chickpea protein isolate
CS	Creaming stability
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
EAI	Emulsion activity index
EC	Emulsion capacity
EE	Encapsulation efficiency
EDTA	Ethylenediamine tetraacetic acid
EPA	Eicosapentaenoic acid
ESI	Emulsion stability index
FbPI	Faba bean protein isolate
FI	Fluorescent intensity
FlPI	Flaxseed protein isolate
H_0	Surface hydrophobicity
LPI	Lentil protein isolate
MDA	Malondialdehyde
pI	Isoelectric point
PV	Peroxide value
PPI	Pea protein isolate
PUFA	Polyunsaturated fatty acid
RH	Relative humidity
RSM	Response surface methodology
S	Svedberg Unit
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy

SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SPI	Soy protein isolate
SSPS	Soybean soluble polysaccharide
TBARS	2-Thiobarbituric acid reactive substances
U_E	Electrophoretic mobility
UF	Ultra-filtration
WPI	Whey protein isolate
$d_{3,2}$	Volume-surface mean diameter
$d_{4,3}$	Volume-length mean diameter
ε	Permittivity
γ	Interfacial tension
κ	Debye length
η	Viscosity
ζ	Zeta potential

1. INTRODUCTION

1.1 Summary

Flaxseed oil is rich in essential fatty acids (e.g., α-linolenic acid) known to possess a variety of health benefits, including reducing the risk of coronary heart diseases (Li et al., 2003) and the prevention of certain types of cancer (Bougnoux and Chajès, 2003). Despite its health promoting properties, flaxseed oil remains underutilized by the food industry due to its susceptibility to oxidation because of its high polyunsaturated fatty acid content, and its lack of miscibility in aqueous food systems. However, with the use of encapsulation technologies these hurdles can be circumvented so as to afford protection against lipid oxidation due to the harsh environmental conditions experienced during processing and storage, and to improve flaxseed oil miscibility in foods. Encapsulation is defined as a process whereby an active ingredient becomes enclosed or packaged within micron-sized carrier matrices, which in turn segregates and protects the inner core from the surrounding environment (Gibbs et al., 1999). Depending on the active ingredient and application, there are various physical and chemical methods for producing these capsules, along with a variety of wall materials to choose from. Gelatin is one of the most widely used encapsulating materials; however due to several perceived safety concerns (e.g., prion disease), religious and dietary restrictions, alternative plant-based materials are being sought. The focus of this research project is to develop microencapsulation technologies employing plant protein-based wall materials for the encapsulation of flaxseed oil. Based on these discoveries, novel methods for the encapsulation of a variety of bioactive compounds and their targeted delivery in human and animal systems should be realized.

1.2 Objectives

The overall goal of this research was to develop a plant protein-based microcapsule capable of carrying, protecting and delivering flaxseed oil within the food and gastrointestinal environments. Specific objectives were as follows: a) to screen a variety of plant proteins and pre-treatment conditions based on their emulsifying/interfacial properties for use as a wall material; b) to develop and optimize encapsulation protocols for entrapping flaxseed oil

within the identified wall material by simple coacervation; and c) to study the oxidative stability and delivery of entrapped oils from capsules under different environmental and simulated gastrointestinal conditions.

1.2 Hypotheses

The following hypotheses were tested: a) legume proteins will provide better emulsifying properties compared to oilseed proteins; b) proteins away from their isoelectric point will have better emulsifying properties than those near; c) isolates produced by isoelectric precipitation method will have improved emulsifying properties compared to those produced by salt extraction; d) encapsulation efficiency will decrease with increasing core to wall ratio; and e) oxidative stability of microencapsulated flaxseed oil will be higher than free oil.

2. LITERATURE REVIEW

2.1 Overview of microencapsulation

Microencapsulation has been used by the food industry for decades for coating food ingredients such as flavours, antioxidants, colours, acidulants, probiotics, polyunsaturated fatty acids (PUFAs), enzymes, vitamins, etc (Pegg and Shahidi, 2007). The application of microencapsulation allows protection of the core material from environmental factors such as temperature, oxygen, moisture, pH, etc., controlled release of the ingredient under specific conditions, masking of unpleasant odours and tastes, dilution and uniform dispersion of the active ingredient and easier handling (Desai and Park, 2005). Microencapsulation is defined as 'the technique by which solid, liquid or gaseous materials are packaged in miniature, sealed capsules or entrapped within a matrix that can release their contents at controlled rates under specific conditions' (Desai and Park, 2005; Madene et al., 2006; Champagne and Fustier, 2007; Pegg and Shahidi, 2007; Augustin and Hemar, 2009; Kailasapathy, 2009). Generally, the encapsulated material is referred to as the core, active ingredient or fill whereas the coating material is called the matrix, wall, carrier, or shell.

Microcapsules can be classified into different groups according to their size (>100 nm to 1000 microns) and morphologies (Finch and Bodmeier, 2000; Augustin and Hemar, 2009; Kailasapathy, 2009). Capsules tend to be either mono- or multinuclear in nature depending on the preparation method/conditions and the wall materials employed. In mononuclear capsules, the core ingredient is concentrated at the center and surrounded by the wall material(s), whereas within multinuclear systems, the core material is dispersed as small droplets throughout the wall material resembling that of an aggregated cluster of mononuclear capsules (Gouin, 2004; Dong et al., 2007). Mono, and multinuclear capsules tend to display rapid burst or prolonged release of their core ingredient, respectively (Gouin, 2004; Dong et al., 2007). Coatings can also be added for improved protection, more tailored release profiles (i.e., combination of burst, prolonged or delayed release) or release of multiple core materials, however pay loads tend to be lowered when compared to uncoated capsules (Gouin, 2004). Currently, there are several preparation techniques and wall materials used for the microencapsulation of lipophilic cores using proteins, which are summarized in Table 2.1. The selection of the appropriate encapsulation method depends on various factors such as: the

physical and chemical properties of the core and wall materials, and the final product; the desired core release profile; estimated production costs; and processing conditions involved in the manufacturing of the final product (Finch and Bodmeier, 2000; Desai and Park, 2005; Madene et al., 2006; Martins et al., 2008).

The entrapment of lipophilic materials (e.g., antioxidants, colourants, essential oils, flavours, lipid-soluble vitamins, and polyunsaturated fatty acids) within protein-based microcapsules have been numerous over the past decade, with the main purpose of inhibiting oxidation to prolong shelf life (Table 2.1). However, the entrapment of oils containing ω-3 polyunsaturated fatty acids (PUFAs), such as α-linolenic acid (ALA, 18:3), eicosapentaenoicacid (EPA, 20:5), and docosahexaenoic acid (DHA, 22:6) have received the most attention due to their purported health promoting properties including but not limited to, decreasing the risks of cardiovascular diseases, protection against inflammation, and their positive roles in infant development (McClements et al., 2007; Subirade and Chen, 2008; Augustin and Hemar, 2009)). Despite these purported health promoting properties, their use in foods has been hindered due to their high susceptibility to oxidation, distinct flavour, and lack of miscibility in aqueous products. Encapsulation provides a means to circumvent these challenges.

2.2 Proteins, as a wall material

Food proteins (e.g., gelatin, sodium caseinate, soy protein and whey protein), have been widely used as encapsulating agents due to their amphiphilic nature, ability to stabilize oil-in-water emulsions and film forming abilities (Augustin and Hemar, 2009). Proteins are also advantageous due to their potential for controlled release applications, as the capsule wall materials can be formulated to be sensitive to pH through complex coacervation (i.e. electrostatic attraction between a positively charged protein and negatively charged polysaccharide), temperature (e.g., thermal properties of gelatin) or enzymes (e.g., proteases) (Chen et al., 2006). As such, environmental triggers could be a means to induce site specific degradation of the capsule wall coupled with active ingredient release; such as in the gastrointestinal tract. Proteins that exhibit high solubility, low viscosities at high concentrations and gel forming capabilities are also key characteristics as encapsulating agents (Madene et al., 2006; Lee and Ying, 2008; Augustin and Hemar, 2009). An ideal capsule wall for oil entrapment should be entirely food grade, be able to emulsify the active core ingredient to form a stable oil-in-water emulsion, provide core ingredient protection against oxidation and mechanical stress, possess a high load capacity and have a low surface

oil content (Desai and Park, 2005; McClements et al., 2007; Pegg and Shahidi, 2007). In addition, capsules should be miscible in the food product, be able to withstand processing, have controlled release profiles and retain the bioavailability of its core ingredient (Desai and Park, 2005; McClements et al., 2007; Pegg and Shahidi, 2007).

Table 2.1 Examples of studies involving the entrapment of lipophilic core materials using proteins as a wall material component.

Active ingredient	**Wall material(s)**	**Microencapsulation method**	**Reference**
Microalgal oil	Sodium caseinate	Spray drying	Bao et al., 2011
Sunflower oil	Dextrin and milk protein	Spray drying	Ahn et al., 2012
Olive oil	Sodium caseinate, gelatin, gum Arabic gum, starch, lactose, and maltodextrin	Spray drying	Calvo et al., 2010
Flaxseed oil	Gelatin and gum Arabic	Complex coacervation	Liu et al., 2010
Alpha-tocopherol	Pea protein, carboxymethylcellulose and maltodextrin	Spray drying	Pierucci et al., 2007
Paprika oleoresin	Gum Arabic and soy protein isolate	Spray drying	Rascón et al., 2011
Orange oil	Soybean protein isolate and gum Arabic	Complex coacervation	Jun-Xia et al., 2011
Limonene	Gum Arabic, sucrose and gelatin	Freeze drying	Kaushik and Roos, 2007
Coffee oil	Whey protein isolate	Spray drying	Frascareli et al., 2012
Miglyol 812	Pea protein and pectin	Spray drying	Gharsallaoui et al., 2010
Fish oil	Wheat gluten	Double emulsification and heat polymerization	Liao et al., 2012
Fish oil	Barley protein	Spray drying	Wang et al., 2011

Food proteins derived from animal sources are the most commonly used wall materials for encapsulation, either alone or in combination with polysaccharides. The most common animal proteins used are gelatin, whey and caseinate based on their cost, highly solubility and excellent emulsifying properties. The majority of studies in literature have focused on both wall formulation and processing to achieve the most stable capsules for oil entrapment. As an example, Heinzelmann et al. (2000) microencapsulated fish oil using sodium caseinate in combination with lactose or maltodextrin as the wall materials, followed by freeze drying. The authors also examined the effects of antioxidant addition, use of carbohydrates, homogenisation/freezing conditions and grinding on the oxidative stability of the entrapped fish oil. They reported, that the addition of antioxidants was necessary to obtain adequate shelf life of their dried product. Pegg and Shahidi (2007) reported that as the amount of surface oil increased so did the rates of oxidation for oil-containing capsules, leading to increased rancidity in the final product. As such, antioxidants (e.g., rosemary) or chelating compounds (e.g., ethylenediamine tetraacetic acid, citrates or phosphates) are often used in capsule formations (Hu et al., 2004; Drusch and Mannino, 2009, Ahn et al., 2012). Partanen et al. (2008) entrapped flaxseed oil within a whey protein isolate matrix coupled with spray drying, and investigated the effect of relative humidity on powder characteristics and oxidative stability. The wall material afforded oxidative protection relative to free oil, however oxidation rates were found to increase at low (0%) and high (91%) humidity, with the lowest occurring at 75%. Liu et al. (2010) entrapped flaxseed oil using complex coacervation involving gelatin-gum Arabic mixtures at a 1:1 biopolymer mixing ratio and 1:1 core-to-wall ratio. The authors reported high entrapment efficiencies (84%) and showed a protective effect against the production of primary and secondary oxidative products versus non-encapsulated oil over a 25 d room temperature storage study. The authors reported that depending on the homogenization rates employed during emulsification, capsule morphology transitioned from a spherical mononuclear to irregular-shaped multinuclear capsule.

Despite the wide use of proteins as encapsulating agents, consumer concerns relating to the use of animal-derived products (e.g., bovine spongiform encephalophathy), and dietary restrictions due to religious or moral beliefs are on the rise, leaving industry searching for plant-based protein alternatives. Recent studies involving plant proteins as encapsulating agents have included: wheat gliadin (Ezpeleta et al., 1996; Mauguet et al., 2002), soy glycinin (Lazko et al., 2004), and soy protein (Rascón et al., 2011). Ducel et al. (2004) studied the potential of α-gliadin and pea globulin as wall materials in the microencapsulation of a model oil (Miglyol 812N) using a complex coacervation process involving a range of anionic

polysaccharides (gum Arabic, sodium alginate and carboxymethylcellulose). The authors reported that mixtures of α-gliadin and gum Arabic at a protein:polysaccharide mixing ratio of 30:70 and pH 2.8, and pea globulin and gum Arabic at protein:polysaccharide mixing ratio of 50:50 and pH 3.0, were best suited for encapsulation. Coacervate coated oil droplets were observed under both sets of conditions. In another study, Rascón et al. (2011) investigated the performance of soy protein isolate (SPI) on the microencapsulation of paprika oleoresin by spray drying. The authors reported that oleoresin retention in the microcapsules increased as inlet air temperature was increased from 160 to 200°C. Microcapsules with the highest oleoresin retention were stored at 35°C for 35 d under different water activities (0.108, 0.318, 0.515 and 0.743) and maximum stability for oleoresin oxidation was found at a water activity of 0.743. Jun-Xia et al. (2011) used SPI-gum Arabic (GA) coacervates for the microencapsulation of sweet orange oil. Effects of pH, ionic strength, SPI:GA ratio, core material load and addition of sucrose and maltodextrin on complex coacervation and microencapsulation efficiency were investigated. The optimum conditions for high coacervate yield and microencapsulation efficiency were determined as, pH 4.0, 0 ionic strength), 1:1 SPI:GA ratio and 10% core material load. The authors also reported that the addition of sucrose at a 1:1 ratio with SPI increased the microencapsulation yield from 65 to 78%.

2.3 Effect of protein characteristics on emulsification

A prerequisite for protein encapsulation of oils is their ability to adsorb at the oil-water interface (Damodaran, 2005; Dickinson, 2010). Proteins act to decrease the interfacial tension and prevent coalescence of lipid droplets in an emulsion by forming a physical barrier at the oil-water interface (Jiang et al., 2009). The protein film around the oil droplets decreases the rate of droplet aggregation by means of its electrostatic charge and steric hindrance (McClements, 2005a). Proteins have a net charge when the pH is above or below their isoelectric point. Oil droplets covered by charged protein layers repel each other. Steric stability is also an important factor in the prevention of droplet flocculation and coalescence. Segments of the protein that remain suspended into the aqueous phase prevent oil droplets from coming into contact with each other (Damodaran, 2005). Furthermore, proteins increase the viscosity of the continuous phase, further contributing to the stability of formed emulsions (Sikorski, 2001). The emulsifying properties of proteins depend on their surface hydrophobicity, molecular size, solubility, flexibility, and method of preparation (Moure et al., 2006; Bueno et al., 2009; Papalamprou et al., 2010). Also, environmental factors such as pH, ionic strength, and the presence of other components such as small molecule emulsifiers or

polysaccharides are also crucial factors affecting the emulsifying properties of proteins (Luyten et al., 2004; Makri and Doxastakis, 2006).

A brief summary relating emulsifying properties to protein characteristics is as follows:

(a) Surface hydrophobicity relates to the relative percentage of hydrophobic groups exposed on the surface of the protein, where higher amounts of these groups allows for greater absorption to the oil phase at the oil-water interface. Often proteins are partially denatured to expose buried hydrophobic sites in order to increase their emulsification capacity (Sikorski, 2001).

(b) Protein flexibility relates to the ability for the protein to re-align itself once absorbed to the oil-water interface, in order to position the majority of hydrophilic groups towards the aqueous phase and hydrophobic groups towards the oil phase; decreasing interfacial tension in the process (Damodaran, 2005). Depending on the protein composition, hydrophilic strings of amino acids may extend out from the oil-water interface into the aqueous phase to give steric hindrance (Damodaran, 2005).

(c) Molecular size of the proteins can impact their migration to the oil-water interface during emulsion formation, and the gelling or film-forming abilities of the protein once there. Small proteins tend to have higher diffusion rates to the interface than larger ones (Luyten et al., 2004), whereas the latter have more effective film forming abilities (Sikorski, 2001).

(d) High protein solubility is desired during encapsulation in order to have greater migration to the oil-water interface and increased continuous phase viscosity (Sikorski, 2001).

(e) *Solvent pH and ionic strength* influences protein solubility as well as attraction or repulsive forces between neighboring droplets leading to either emulsion instability or stability, respectively. In order to enhance emulsion stability (and increase entrapment efficiencies), charge repulsion is desired within the system, occurring at a solvent pH away from the protein's isoelectric point or under low ionic conditions (McClements, 2004).

(f) Preparation method of the protein ingredients also plays an important role, where depending on the method, different protein compositions and levels of degradation may occur. Several researchers have reported that preparation method of protein isolates has an appreciable impact on the functional properties of the protein (Papalamprou et al., 2010).

2.4 Effects of emulsification of the entrapment of lipophilic core materials

Emulsions are defined as dispersions of two immiscible liquids in which one of the liquids is dispersed in the other as small droplets (0.1-100 μm) (McClements, 2005b). Physicochemical properties of emulsions play an important role in the formation, structure

and oxidative stability of microcapsules containing oils (Lee and Ying, 2008). Good emulsion stability is a prerequisite for maximizing oil encapsulation efficiency and oxidative stability. Emulsion stability is the ability of an emulsion to resist changes in its nature over time. These changes can be either physical or chemical processes that result in an alteration in the distribution or organization of droplets such as creaming, flocculation and coalescence, and/or in chemical reactions such as hydrolysis and oxidation. Controlling droplet size, use of stabilizers (emulsifiers or texture modifiers), and environmental conditions such as temperature, pH, and ionic strength are the key factors in emulsion stability (McClements, 2005a). The stability of an emulsion is highly dependent on its droplet size and distribution (McClements, 2007). Emulsions with smaller droplets tend to have greater stability and surface coverage from proteins at the oil-water interface than larger ones; with the latter leading to reduced surface oil content on the dried encapsulated product (McClements, 2005a; Lee and Ying, 2008). Droplet size can be controlled by modifying the homogenizing conditions such as shear rate, or concentration and type of the emulsifier used (e.g., protein) (McClements, 2007). In general, increased oil concentrations in the emulsion result in a decrease in encapsulation efficiency as a result of having an insufficient amount of wall material for complete coverage of the emulsified oil droplets (Rusli et al., 2006; Polavarapu et al., 2011).

2.5 Improving the performance of plant protein-based systems as wall materials

Plant proteins have relatively lower solubility in aqueous systems than do animal-derived proteins (Ezpeleta et al., 1996; Lazko et al., 2004; Can Karaca et al., 2011a); therefore modifications are usually required to improve their solubility, and subsequently emulsifying properties and entrapping abilities. For instance, Jiang et al. (2009) modified the native structure of soy protein by pre-treating in acid (pH 1.5-3.5) and alkaline (pH 10.0-12.0) solutions for various times (0 to 4 h). The authors reported that the pH pre-treatments resulted in an increase in surface hydrophobicity because the protein adopted a molten globule-type conformation, resulting in a significant improvement to its emulsifying properties. Augustin et al. (2006) investigated the effect of heat treatments on mixtures of proteins (sodium caseinate, whey protein isolate and soy protein) and carbohydrates (glucose, dried glucose syrup, oligosaccharide) on the encapsulation of fish oil; observing higher entrapment efficiencies in heated versus unheated controls. They also reported that the oxidative stability of fish oil microcapsules was improved by increasing the temperature-time treatment of protein-carbohydrate mixtures before emulsification.

Paraman et al. (2007) modified rice endosperm protein by controlled glycosylation, deamidation and enzymatic hydrolysis methods so as to improve their emulsion activity and stability relative to unmodified protein. The authors found that alkali-deamidation was the most effective method at improving the emulsifying properties of rice endosperm protein, presumably due to denaturation, which increased the hydration and net charge of the protein. Wong et al. (2011) prepared deamidated wheat protein-dextran Maillard conjugates and investigated the effect of size and location of conjugated polysaccharide on the steric stabilization of emulsions at acidic pH. Experimental results showed that the number of dextrans conjugated and the location of conjugation was dependent on the size of the dextran. The conjugated wheat protein-dextran complexes produced formed a thicker interfacial layer and provided more effective steric stabilization than adsorbed protein alone. Tang et al. (2011) investigated the effect of glycosylation with glucose on the physicochemical and conformational properties of kidney bean vicilin (phaseolin). The authors reported that phaseolin underwent a tertiary conformation unfolding and subsequent rearrangement process, whereas the quaternary conformational flexibility progressively increased upon increasing degree of glycosylation which played a major role in the enhanced emulsifying properties of glycosylated vicilins.

Additionally, the presence of polysaccharides in combination with proteins in an emulsion can enhance overall stability (Jourdain et al., 2008). Polysaccharides are added to protein-stabilized oil-in-water emulsions to increase the stability of the interfacial film separating droplets (Ghoush et al., 2008) and to reduce the rate at which droplets move by increasing the viscosity of the continuous phase or by forming a gel (McClements, 2005a). In microencapsulation formulations, maltodextrins are commonly used as a secondary wall material (filler) to improve capsule drying properties (Kagami et al., 2003; Gharsallaoui et al., 2007).

2.6 Choice of plant protein materials

In the present study, the emulsifying properties of protein isolates produced from legume (chickpea, lentil, pea, faba bean and soy) and oilseed (canola and flaxseed) crops were investigated. Legume proteins appear to be a promising source to animal proteins as functional food ingredients because of their nutritional value, functional properties, low cost, availability, and possible beneficial health effects (Duranti, 2006; Boye et al., 2010a). Also, canola and flaxseeds are economically important oilseed crops and although their protein rich

meals are used as animal feeds, the economic prospects for their proteins remain underutilized by the food industry (Krause and Schwenke, 2001; Tan et al., 2011).

Globulins and albumins are the main storage proteins in legume seeds. Globulins represent ~70% of the protein found in legume seeds and are soluble in salt-water solutions (Roy et al., 2010). They are classified as either 11S (legumins) or 7S (vicilins) proteins according to their sedimentation (S; Svedberg Unit) coefficients. Legumin is a hexameric protein with an overall molecular mass of 300-400 kDa whereas vicilin is a trimeric protein with a molecular mass between 150-180 kDa (Derbyshire et al., 1976). The α- and β-chains of legumin are linked by disulfide bridges, and hydrophilic α-chains are located on the surface of the molecule while hydrophobic sections are buried in the interior, minimizing their contact with water. Globulins dissociate into their subunits at extreme pH values and ionic strength (Henning et al., 1997). Water soluble albumins constitute 10–20% of the protein in legume seeds and can have variable molecular masses (16-483 kDa) (Papalamprou et al., 2010). Boye et al. (2010b) compared the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration (UF) and isoelectric precipitation (IEP) techniques and reported that the emulsifying activity indices for these legume protein concentrates ranged between 4.6 m^2/g for yellow pea-UF to 5.7 m^2/g for the desi and kabuli chickpea-IEP. With respect to their emulsion stability index, the lowest value of 17.8 min was observed for green lentil-IEP and the highest of 19.7 min was found for the kabuli chickpea-UF protein concentrate. Kimura et al. (2008) compared the functional properties of 7S and 11S globulins from pea, faba bean, cowpea, and French bean proteins with those of soybean. The authors reported that oil-in-water emulsions stabilized with 7S globulin of French bean had smaller droplet size and exhibited excellent emulsion stability when compared to other proteins studied.

The main storage proteins in canola seeds are 12S globulin (cruciferin) and 2S albumin (napin). Cruciferin is a hexameric protein with an overall molecular mass of ~300 kDa. Each subunit consists of α- (30 kDa) and β- (20 kDa) chains linked by intramolecular disulfide bridges (Lampart-Szczapa, 2001). Napins have a low molecular mass (12.5-14.5 kDa) and are composed of two polypeptide chains of 4.5 kDa and 10 kDa held together by disulfide bonds (Bérot et al., 2005). Flaxseed proteins are also composed of 11-12S globulins and 1.6-2S albumins, which are referred to as linin and conlinin, respectively (Vassel & Nesbitt, 1945). Flaxseed globulin has an overall molecular mass of ~320 kDa, a pI of ~4.75 (Wanasundara & Shahidi, 2003), and is comprised of at least five subunits having molecular masses ranging from 11 to 61 kDa held together by disulfide linkages (Oomah & Mazza,

1993). In contrast, flaxseed albumin is a basic protein containing a single polypeptide chain that has a molecular mass between 16–18 kDa (Wanasundara & Shahidi, 2003; Chung et al., 2005). Wu and Muir (2008) compared the emulsifying properties of cruciferin and napin isolated from defatted canola meal and found that emulsions prepared by cruciferin had significantly higher specific surface area and lower droplet size than that of napin. Wang et al. (2010a) compared the ability of flaxseed protein concentrate to stabilize oil-in-water emulsions with that of soybean protein concentrate and found that the flaxseed protein-stabilized emulsions had smaller droplet sizes and higher surface charges but poorer stability when compared to soybean protein-stabilized emulsions.

2.7 Production of protein isolates

Legumes and oilseeds contain a range of proteins, including albumins, globulins, prolamins, and glutelins which differ in size, molecular mass and solubility within various solvents (Xu and Diosady, 2003). For instance, globulins are salt soluble, albumins are water soluble, prolamins are alcohol soluble, and glutelins are soluble in dilute acid or alkali (Xu and Diosady, 2003; Boye et al., 2010a; Roy et al., 2010). Depending on the protein source and method of extraction, protein profiles within the final isolate may differ; ultimately influencing the physicochemical properties of the protein isolate.

The following are the four major methods employed for protein isolate production. In brief:

(a) Isoelectric precipitation is one of the most frequently used methods for the production of plant protein isolates. In general, ground and defatted flour is dispersed in water, and then pH adjusted to alkaline conditions (pH 8-11) and allowed to stir for a specific time period so as to facilitate protein solubility. The suspension is subsequently centrifuged and filtered to remove any insoluble material. The supernatant is then adjusted to the isoelectric point of the protein to promote precipitation, which is collected after centrifugation and freeze dried to yield a protein rich powder. The isoelectric precipitation method mainly precipitates globulins (Papalamprou et al., 2010).

(b) *Salt precipitation* is based on dissolving the proteins of interest in an aqueous solution in the presence of salts (e.g. 0.1-1.0 M NaCl). After a specific time of stirring, insoluble material is removed by centrifugation and filtration. The supernatant is subsequently dialyzed to remove the salt so as to induce precipitation of the proteins. The precipitate is collected by centrifugation and freeze dried to yield a protein rich powder. Products of salt extraction method typically comprise of a mixture of globulins and albumins (Liu et al., 2008).

(c) *Ultra-filtration* (UF) is based on alkaline or acid raw material extraction followed by membrane separation. Membrane type, molecular weight cut-off, volume concentration ratio and diafiltration conditions are important factors impacting protein separation by UF (Boye et al., 2010a). The process involves dissolving a flour in pH adjusted (~7-8) aqueous solution, followed by the removal of insoluble material by UF and collection of the proteins within the filtrate, which is then freeze dried. Depending on the material, enzymes may be added to help remove carbohydrates present. Protein extracts obtained by UF method are a mixture of globulins and albumins, with the globulin fraction dominant (Papalamprou et al., 2010).

(d) *Micellization* process is based on the ability for proteins to self-associate into a thermodynamically stable micelle configuration (Ismond et al., 1991). In this method, defatted flour is dispersed in a salt solution (e.g. 0.5-0.8 M NaCl) at pH 7.0 and stirred for a specific time period. The extract is centrifuged and the supernatant is concentrated by UF. The concentrated protein solution is then diluted (1:4-1:12) with cold distilled water. After allowing to stand for a specific time, the protein is recovered by centrifugation and freeze dried to yield a protein rich powder. Proteins precipitated in the form of micelles are reported to undergo a smaller degree of denaturation compared to isolates produced by isoelectric precipitation (Cordero-de-los-Santos et al., 2005). The micellization technique precipitates mainly the globulin fraction of the raw material (Rodriguez-Ambriz et al., 2005).

3. EMULSIFYING PROPERTIES OF CHICKPEA, FABA BEAN, LENTIL AND PEA PROTEINS PRODUCED BY ISOELECTRIC PRECIPITATION AND SALT EXTRACTION[1]

3.1 Abstract

The emulsifying (emulsion capacity, EC; emulsion activity/stability indices, EAI-ESI and creaming stability, CS) and physicochemical properties (surface charge/hydrophobicity, protein solubility, interfacial tension, and droplet size) of chickpea (ChPI), faba bean (FbPI), lentil (LPI), and pea (PPI) protein isolates produced by isoelectric precipitation and salt extraction were investigated relative to each other and a soy protein isolate (SPI). Both the legume source and method of isolate production showed significant effects on the emulsifying and physicochemical properties of the proteins tested. All legume proteins carried a net negative charge at neutral pH, and had surface hydrophobicity values ranging between 53.0 and 84.8 (H_0-ANS), with PPI showing the highest value. Isoelectric precipitation resulted in isolates with higher surface charge and solubility compared to those produced via salt extraction. The EC values ranged between 476-542 g oil/g protein with LPI showing the highest capacity. Isoelectric-precipitated ChPI and LPI had relatively high surface charges (~−22.3 mV) and formed emulsions with smaller droplet sizes (~1.6 μm), they also displayed high EAI (~46.2 m^2/g), ESI (~84.9 min) and CS (98.6%) results, which were comparable to the SPI.

3.2 Introduction

The ability of food proteins to form and stabilize emulsions is critical to their role as food ingredients in a wide range of applications. However, the role of plant-derived proteins as emulsifiers is less understood. With the exception of soy and gluten, plant proteins remain largely underutilized by the food industry in part due to insufficient structure-function information relating to their performance. Emulsion stability describes an emulsion's ability to resist change from a thermodynamically stable state to one that is unstable (i.e., separation

[1]Reproduced with permission. Can Karaca, A., Low, N. and Nickerson, M. 2011. Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction. Food Research International, 44, 2742–2750. Copyright (2011) Elseiver Ltd.

into oil and water layers) (Dickinson, 2003; McClements, 2007). Protein-stabilized emulsions are dependent on: protein characteristics (e.g., protein source, concentration, size, surface hydrophilic-hydrophobic properties and solubility); processing (e.g., level and duration of shear); environmental conditions (e.g., temperature, pH and ionic strength); mixing ratio; emulsion droplet properties (e.g., size and size distribution, level of coalescence and spatial arrangement of droplets); emulsion viscosity; and time (Dickinson, 2003; McClements, 2004). Droplet size, environmental conditions such as temperature, pH, and ionic strength and the use of emulsifiers are key factors used by the food industry to control emulsion stability (McClements, 2005b).

Proteins are widely used as emulsifiers, due to their ability to adsorb at the oil-water interface and form stabilizing layers around oil droplets. During emulsion formation, individual proteins or aggregates become adsorbed at the surface of newly formed oil droplets in the form of a densely packed layer (Dickinson, 2010). Proteins then act to decrease interfacial tension and prevent coalescence by forming a physical barrier at the oil-water interface (Jiang et al., 2009). The protein film surrounding the lipid droplets decreases the rate of droplet aggregation by electrostatic repulsion if the pH is away from the isoelectric point (pI) of the protein (McClements, 2005a) and by steric hindrance if segments of the protein extend outwards into the aqueous medium to physically restrict neighbouring droplets from coalescing (Damodaran, 2005). Proteins also increase the viscosity of the continuous phase, which contributes to emulsion stability by decreasing the rate of droplet movement (Sikorski, 2001). For a protein to be an effective emulsifier, it should be able to readily adsorb to the oil-water interface, unfold at the interface, and be able to form a cohesive film around oil droplets through intermolecular interactions (Damodaran, 2005).

There has been a growing interest by the food industry towards utilizing plant proteins as substitutes for animal-based proteins in new product formulations. Legume proteins are of special interest because of their nutritional value, availability, low cost and beneficial health effects (Duranti, 2006). Proteins from several legumes such as beans (Tsoukala et al., 2006), chickpea (Papalamprou et al., 2010), faba bean (Galazka et al., 1999), lentil (Bora, 2002; Boye et al., 2010b), pea (Ducel et al., 2004), cowpea (Kimura et al., 2008), and lupine (Jayasena et al., 2010) have been investigated for their emulsifying properties. However a comprehensive study focused at understanding the modes of action of legume protein-stabilized emulsions, prepared under controlled and consistent preparation and testing conditions is lacking in literature.

The main storage proteins in legume seeds are globulins and albumins. Globulins are soluble in salt-water solutions and represent ~70% of the protein found in legume seeds (Roy et al., 2010). They are classified as either 11S (legumins; S - Svedberg Unit) or 7S (vicilins) proteins according to their sedimentation coefficients. Legumin is a hexameric protein with an overall molecular weight of 300-400 kDa whereas vicilin is a trimeric protein with a molecular weight between 150-180 kDa (Derbyshire et al., 1976). The α- and β-chains of legumin are linked by disulfide bridges, and hydrophilic α-chains are located at the surface of the molecule while hydrophobic sections are buried at the interior, minimizing their contact with water. Globulins dissociate into their subunits at extreme pH values and ionic strength (Henning et al., 1997). Water soluble albumins constitute 10–20% of the protein in legume seeds and can have variable molecular weights (16-483 kDa) (Papalamprou et al., 2010). The ratio between globulins and albumins and/or legumins and vicilins in isolates may show differences due to the species and/or their method of production which could influence their physicochemical properties (Swanson, 1990).

Aqueous alkaline extraction followed by isoelectric precipitation and salt extraction processes are widely used techniques for producing legume protein isolates. It has been reported that the isolate production method has a significant effect on protein functionality in emulsion systems, since it may influence both the globulin/albumin or legumin/vicilin ratio and the physicochemical characteristics of the protein (Papalamprou et al., 2010). The overarching goal of this study was to compare the emulsifying and physicochemical properties of protein isolates from chickpea, faba bean, lentil and pea produced by isoelectric precipitation and salt extraction relative to a similarly produced soy protein isolate.

3.3 Material and Methods

3.3.1 Materials

Chickpea (CDC Frontier, Kabuli), faba bean (CDC SSNS), lentil (CDC Grandora), and pea (CDC Leroy) were provided by the Crop Development Centre at the University of Saskatchewan (Saskatoon, SK, Canada). Whole legume seeds were ground into a fine flour using a coffee grinder for 1 min, and then defatted using hexane (1:3 (w/v) flour:hexane ratio) for 40 min. The mixture was then filtered through a 110 mm Whatman #1 filter paper (Whatman International Ltd., Maidstone, United Kingdom), and air-dried in a fume hood. The defatting procedure was repeated twice for each flour. Defatted soy flour and flaxseed oil were kindly donated by Cargill Inc. (Cedar Rapids, IA, Prolia 200/20, Lot #: 071909G) and Bioriginal Food & Science Corp. (Saskatoon, SK, Canada), respectively. Proximate

composition of all flours and protein isolates were performed according to AOAC Official Methods 925.10 (moisture), 923.03 (ash), 920.85 (lipid), and 920.87 (crude protein using %N × 6.25 for chickpea, faba bean, lentil and pea; %N × 5.71 for soy; according to Kolakowski, 2001) (AOAC, 2003). Carbohydrate content was determined on the basis of percent differential from 100%. All protein isolates and flours were stored at 4°C. All chemicals used were of reagent grade and purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.3.2 Preparation of protein isolates by isoelectric precipitation and salt extraction

Chickpea protein isolates (ChPI):

Isoelectric-precipitated ChPI was prepared according to the method of Papalamprou et al. (2010). In brief, defatted flour (100 g) was mixed with Milli-Q^{TM} water (Millipore Corporation, MA, USA) at a 1:10 ratio (w/v), adjusted to pH 9.0 using 0.1 M NaOH and stirred at 500 rpm for 45 min at room temperature (20-22°C). The suspension was then centrifuged at 4,500 × *g* for 20 min at 4°C using a Sorvall RC-6 Plus centrifuge (Thermo Scientific, Asheville, NC, USA) to collect the supernatant. The resulting pellet was re-suspended in Milli-Q^{TM} water at a ratio of 1:5 (w/v), adjusted to pH 9.0, stirred for an additional 45 min, followed by centrifugation (4,500 × *g*, 20 min, 4°C). Both supernatants were pooled and adjusted to pH 4.6 using 0.1 M HCl to precipitate the protein. The protein was recovered by centrifugation, collected and stored at −30°C until freeze-dried.

The salt-extracted ChPI was produced according to the method of Bhatty (1982) with minor modifications. Briefly, defatted flour (100 g) was mixed with 5% potassium sulphate aqueous solution at 1:10 ratio (w/v), adjusted to pH 7.0 with 0.1 M NaOH and stirred at 500 rpm for 1 h at room temperature. The slurry was centrifuged at 17,700 × *g* for 20 min at 4°C. The resulting supernatant was collected and dialyzed at 4°C for 72 h (6-8 kDa cut off; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) against Milli-Q^{TM} water refreshing several times until the conductivity of the dialysis water reached 2.0-2.5 mS/cm. Following dialysis the supernatant was stored at −30°C until freeze-dried.

Freeze-drying was performed using a Labconco FreeZone 6 freeze drier (Labconco Corp., Kansas City, MO, USA) to yield the final isolate powder.

Faba bean protein isolates (FbPI):

Isoelectric-precipitated FbPI was produced according to the method of Makri et al. (2006). Briefly, defatted flour (100 g) was dispersed in Milli-Q^{TM} water at 1:10 ratio (w/v), adjusted to pH 9.5 using 1.0 M NaOH, and stirred at 500 rpm for 40 min at room temperature.

The alkali extract was centrifuged at 1,600 × *g* for 20 min at 4°C and the supernatant was collected. The pellet was subjected to an additional extraction (1:5 w/w pellet:water ratio) followed by centrifugation at 1,600 × *g*, 20 min, 4°C. Supernatants were pooled and adjusted to pH 4.5 using 0.1 M HCl. The precipitated protein was isolated by centrifugation, collected and stored at −30°C until freeze-dried.

The salt-extracted FbPI was produced in a similar manner as previously described for ChPI.

Lentil protein isolates (LPI):

Isoelectric-precipitated LPI was produced using the combined methods of Bamdad et al. (2006) and Lee et al. (2007). Briefly, defatted flour (100 g) was mixed with Milli-Q^{TM} water at 1:10 ratio (w/v), adjusted to pH 9.5 with 1.0 M NaOH, and stirred at 500 rpm for 1 h at room temperature. The mixture was kept static at 4°C overnight to allow for non-protein constituent sedimentation. Following centrifugation at 1600 × *g* for 30 min at 4°C, the supernatant was collected and the pH was adjusted to 4.5 with 0.1 M HCl. The precipitated protein was collected by centrifugation (1,600 × *g*, 30 min, 4°C) and stored at −30°C until freeze-dried.

The salt-extracted LPI was produced according to the method of Bora (2002) with minor modifications. Briefly, defatted flour was dispersed in 50 mM potassium phosphate buffer (pH 7.2) containing 0.5 M NaCl at a 1:5 ratio (w/v). The dispersion was stirred at 500 rpm for 1 h at room temperature before centrifugation at 17,700 × *g* for 20 min at 4°C to remove insoluble residues. The supernatant was collected and dialyzed (6-8 kDa cut off) against Milli-Q^{TM} water at 4°C for 72 h and stored at −30°C until freeze-dried.

Pea protein isolates (PPI)

Isoelectric-precipitated PPI was produced according to the method of Boye et al (2010b) with minor modifications. Briefly, defatted flour (100 g) was dispersed in Milli-Q^{TM} water at 1:15 ratio (w/v), adjusted to pH 9.5 with 1.0 M NaOH, and stirred at 500 rpm for 1 h at room temperature. The mixture was then centrifuged at 4,500 × *g* for 20 min at 4°C. The supernatant was collected and its pH was adjusted to 4.5 using 0.1 M HCl. The precipitate was collected by centrifugation (4,500 × *g*, 20 min, 4°C) and stored at −30°C until freeze-dried.

The salt-extracted PPI was prepared according to the method of Liu et al. (2009). Briefly, defatted flour (100 g) was mixed with 0.1 M phosphate buffer (pH 8.0) containing

6.4% KCl at 1:10 ratio (w/v). The mixture was stirred at 500 rpm for 24 h at room temperature and centrifuged at 4,500 × *g* for 20 min at 4°C. The supernatant was collected and dialyzed (6-8 kDa cut off) against Milli-Q^{TM} water at 4°C for 72 h and stored at −30°C until freeze-dried.

Soy protein isolates (SPI)

Isoelectric-precipitated SPI was produced according to the method of Jiang et al. (2009) with minor modifications. Briefly, defatted flour (100 g) was dispersed in Milli-Q^{TM} water at 1:10 ratio (w/v), adjusted to pH 8.0 with 0.1 M NaOH, and then stirred at 500 rpm for 2 h at room temperature. The suspension was then centrifuged at 17,700 × *g* for 30 min at 4°C. The supernatant was collected and adjusted to pH 4.5 using 1 M HCl. The precipitated protein was collected by centrifugation (17,700 × *g*, 30 min, 4°C) and stored at −30°C until freeze-dried.

The salt-extracted SPI was produced according to the method of Oomah et al. (1994) with modifications. Briefly, defatted flour was (100 g) was mixed with 50 mM sodium phosphate buffer (pH 8.0) containing 0.8 M NaCl at a 1:10 ratio (w/v). This dispersion was stirred at 500 rpm for 30 min at room temperature before centrifugation at 17,700 × *g* for 30 min at 4°C. The supernatant was collected and dialyzed (6-8 kDa cut off) against Milli-Q^{TM} water at 4°C for 72 h and stored at −30°C until freeze-dried.

3.3.3 Physicochemical properties

For all physicochemical tests, protein solutions were prepared by dispersing the isolates (corrected on a weight basis for protein content) in 10 mM sodium phosphate buffer (pH 7.0) and adjusted to pH 7.0 with either 0.1 M NaOH or 0.1 M HCl followed by stirring at 500 rpm overnight (~16 h) at 4°C.

Surface charge (Zeta potential):

The overall surface charge of the protein isolates was determined by measuring the electrophoretic mobility *(U_E)* of protein solutions (0.05%, w/w) at pH 7.0 using a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA). U_E was used to calculate the zeta potential *(ζ)* by applying Henry's equation:

$$U_E = \frac{2\varepsilon \cdot \zeta \cdot f(\kappa\alpha)}{3\eta} \qquad \text{[eq. 3.1]}$$

where, ε is the permittivity, $f(\kappa\alpha)$ is a function related to the ratio of particle radius (α) and the Debye length (κ), and η is the dispersion viscosity. For this study, the Smoluchowski approximation $f(\kappa\alpha)$ equalled 1.5.

Average surface hydrophobicity (H_0):

Average surface hydrophobicity was determined using the fluorescent probe, 8-anilino-1-naphthalenesulfonic acid (ANS) (Kato and Nakai, 1980) with slight modifications described by Wang et al. (2005). All fluorescence measurements were made using a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) with the excitation and emission wavelengths at 390 and 470 nm, respectively. The excitation and emission slit widths were each set at 1 nm. Protein solutions (0.01%, w/w) prepared in 10 mM sodium phosphate buffer (pH 7.0) were diluted in the same buffer to obtain concentrations of 0.002%, 0.004%, 0.006%, 0.008% and 0.01% (w/w). To 4 mL of protein solution, 20 μL of 8 mM ANS solution in 10 mM sodium phosphate buffer (pH 7.0) was added and mixed well by vortexing for 10 s. After keeping each sample for 15 min in the dark, the fluorescent intensity (FI) was measured. FI values for the ANS blank and diluted protein blanks (without the ANS probe) were measured and subtracted from the FI of the protein solutions with ANS. The initial slope of the plot of FI (corrected) versus protein concentration was calculated by linear regression analysis and used as an index of protein surface hydrophobicity.

Percent protein solubility:

Percent protein solubility was determined using the method of Morr et al. (1985). Briefly, protein solutions were prepared by dispersing 0.2 g of sample in 19.8 mL (1.0%, w/v) of 10 mM sodium phosphate buffer (pH 7.0) with pH adjustment to 7.0 with either 0.1 M NaOH or 0.1 M HCl, followed by stirring at 500 rpm overnight at 4°C. Solutions were centrifuged at 9,100 × *g* for 10 min at room temperature. The nitrogen content of the supernatant was determined using a micro-Kjeldahl digestion and distillation unit (Labconco Corp., Kansas City, MO, USA). Percent protein solubility was calculated by dividing the nitrogen content of the supernatant by the total nitrogen in the sample (×100%).

Interfacial tension:

Interfacial tension between protein solutions (0.25%, w/w) and flaxseed oil was determined according to the Du Noüy ring method using a semi-automatic tensiometer (Lauda

TD2, Lauda Dr. R. Wobser GmbH & Co., Lauda-Königshofen, Germany); and was compared to the interfacial tension between Milli-Q^{TM} water and flaxseed oil (without protein). Interfacial tension was calculated from the maximum force *(F_{max})* using the following equation:

$$\gamma = \frac{F_{\max}}{4\pi R\beta} \qquad \text{[eq. 3.2]}$$

where, γ is the interfacial tension, R is the radius of the ring, β is a correction factor that depends on the dimensions of the ring and the density of the liquid involved.

3.3.4 Emulsifying properties

Emulsion capacity (EC):

A series of emulsions were prepared by homogenizing 3 g of a 0.25% (w/w) protein solution with differing amounts (3-5 g) of flaxseed oil in 50 mL plastic centrifuge tubes using an Omni Macro Homogenizer (Omni International, Marietta, GA, USA) with a 20 mm saw tooth generating probe at speed 4 (~7,200 rpm) for 5 min. The conductivity of the resulting emulsions was measured immediately after homogenization using an Orion 3-Star bench top conductivity meter (Thermo Scientific, Waltham, MA, USA) with a 4-electrode conductivity cell. Emulsion capacity was determined at the inversion point where an oil-in-water emulsion turns into a water-in-oil emulsion as indicated by a sudden drop in conductivity. Emulsion capacity was expressed as g of oil homogenized per g of protein before the inversion was observed.

Emulsifying activity (EAI) and stability (ESI) indices:

Emulsifying activity and stability indices of protein samples were determined by the method described by Pearce and Kinsella (1978). Briefly 5 g of 0.5% (w/w) protein solution and 5 g of flaxseed oil were homogenized as described above. A 50 μL emulsion sample was immediately taken from the bottom of the tube and diluted in 7.5 mL of 10 mM sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulphate (SDS) and this solution was vortexed for 10 s. An aliquot of this suspension was taken at 10 min, and the absorbance of the diluted emulsion was measured at 500 nm using a Genesys 10 UV-visible spectrophotometer (Thermo Scientific, Madison, WI, USA) using plastic cuvettes (1 cm path length). *EAI* and *ESI* were calculated by using the following equations:

$$EAI(m^2/g) = \frac{2 \cdot 2.303 \cdot A_0 \cdot N}{c \cdot \varphi \cdot 10000} \quad \text{[eq. 3.3]}$$

$$ESI(\text{min}) = \frac{A_0}{\Delta A} \cdot t \quad \text{[eq. 3.4]}$$

where, A_0 is the absorbance of the diluted emulsion immediately after homogenization, N is the dilution factor (×150), c is the weight of protein per volume (g/mL), φ is the oil volume fraction of the emulsion, ΔA is the change in absorbance between 0 and 10 min $(A_0 - A_{10})$ and t is the time interval, 10 min.

Creaming stability:

Oil-in-water emulsions (20 mL) were prepared by homogenizing (as previously described) 16 mL of 1.0% (w/w) protein solutions, 4 mL of flaxseed oil and ~5 mg of Oil Blue N dye (a lipid-soluble dye, added to improve visualization during creaming). Emulsions (10 mL) were then transferred into 10 mL sealed graduated glass cylinders (inner diameter = 10.5 mm; height = 160 mm; as measured by a digital calliper) immediately after preparation. The stability of the emulsions was monitored by observing the separation of a cream layer after 1 h of storage at room temperature. At this point, emulsions had separated into an optically opaque darker blue cream layer (top), and a turbid layer at the bottom with a similar appearance to the original emulsion. Creaming stability *(CS)* was expressed as:

$$CS(\%) = \frac{H_T}{H_E} \cdot 100 \quad \text{[eq. 3.5]}$$

where, H_T is the height of the turbid layer and H_E is the total height of the emulsion.

Droplet size:

Emulsion droplet size distribution was measured using a Mastersizer 2000 laser light scattering instrument (Malvern Instruments Ltd., Worcestershire, United Kingdom) equipped with a Hydro 2000S sample handling unit (containing 10 mM sodium phosphate buffer at pH 7.0). Emulsions were prepared as outlined in the creaming study, with samples being taken from the bottom of the tube immediately after homogenization for analysis. The sample was stirred continuously within the sample cell to ensure homogeneity at room temperature, and all measurements were performed at ~14% obscuration by buffer addition. Droplet size distributions were calculated by the instrument according to the Mie Theory which uses the refractive index difference between the droplets and the dispersing medium to predict scattered light intensity. The ratio of the refractive index of flaxseed oil (1.479) to that of the

dispersion medium (1.330) was 1.112. Droplet size measurements were reported as volume-surface mean diameters *($d_{3,2}$)*, which is expressed as:

$$d_{3,2} = \frac{\sum_{i=1} n_i \cdot d_i^3}{\sum_{i=1} n_i \cdot d_i^2} \qquad \text{[eq. 3.6]}$$

where, n_i is the number of droplets of diameter (d_i) (McClements, 2005c).

3.3.5 Statistical analyses

All experiments were performed in triplicate and reported as the mean ± one standard deviation. A two-way analysis of variance (ANOVA) with a Scheffe post-hoc test was used to measure statistical differences in emulsifying and physicochemical properties as a function of legume source and production method. Simultaneous multiple regression analysis was conducted to examine the relationship between surface charge, surface hydrophobicity, protein solubility, interfacial tension and droplet size (for CS only) on the emulsifying properties (e.g., EAI, ESI, EC and CS). Pearson correlation coefficients (r) were also calculated to describe the relationship between the emulsifying and physicochemical properties. All statistical analyses were performed with SPSS version 17.0 software (SPSS Inc., 2008, Chicago, IL, USA).

3.4 Results and Discussion

3.4.1 Proximate composition of flours and protein isolates

The proximate composition of all raw materials (flours) and protein isolates are shown in Table 3.1. Protein levels found for all raw materials were comparable to those reported in literature (Boye et al., 2010; Comai et al., 2007); with concentrations in chickpea, faba bean, lentil and pea flours that ranged between 16.7% and 23.9%. In contrast, commercial soy flour contained significantly higher levels of protein (45.4%). An analysis of variance indicated that the raw material and method of extraction, along with their interaction affected protein levels in many of the isolates ($p<0.001$). Overall, isolates produced by isoelectric precipitation yielded higher levels of protein (~85.6%) than those by salt extraction (~78.4%, $p<0.001$). Isolates produced by isoelectric precipitation gave the highest levels of protein for PPI and SPI (~88.2%), followed by ChPI and FbPI (~84.8%), and LPI (81.9%). In contrast, those produced by salt extraction gave isolates with similar levels for FbPI, ChPI, and PPI (~81.6%), followed by LPI (74.7%) and SPI (72.6%). For this study, all prepared materials were labelled as an ‘isolate’, despite some having protein levels <80%. Currently,

there is no universal classification separating a protein concentrate from an isolate for all legumes. In the case of soy, Pearson (1983) developed criteria requiring a minimum protein content of 85% on a dry weight basis (6.25 nitrogen conversion factor) to be classified as an isolate. Converting protein levels from a wet to dry basis in the present study, coupled with the 6.25 conversion factor, protein levels in the soy products were calculated to range from 88.4 to 100.4%. In the isoelectric precipitation method, proteins were extracted under dilute alkali (pH 8.0-10.0) conditions due to their high solubility at high pH, and were precipitated at pH conditions close to their isoelectric point (4.5-5.0). For the salt extraction method, various salt concentrations were used to solubilise the legume proteins. Removal of salt during the dialysis step resulted in protein precipitation, as hydration layers surrounding the protein's surface was disrupted (Aluko, 2004). In the present study, isoelectric precipitation was found to be more effective at isolating legume proteins, yielding higher protein concentrations in the isolates.

The lipid content for all raw materials were generally low (<1%) with the exception of chickpea (3.8%). It has been shown (Leyva-Lopez et al., 1995) that defatting prior to isolate preparation leads to improved protein extraction as protein-lipid interactions are significantly reduced. For all isolate materials, fat content was reduced to <1.0% (Table 3.1). Overall, the ash content in ChPI, FbPI, LPI, and PPI was higher than that observed in the raw material, whereas ash in the commercial soy flour was reduced in the isolate (Table 3.1). Sosulski and McCurdy (1987) indicated that strong alkali or acid used in isoelectric precipitation methods may result in salt formation and a subsequent higher ash level in the protein isolate relative to the flour. Similarly, salts remaining after dialysis would contribute to higher ash contents in the isolates compared to starting materials.

3.4.2 Surface characteristics

The physicochemical characteristics of the legume proteins such as molecular size, surface hydrophobicity, net charge, steric hindrance, and molecular flexibility have been found to greatly influence their emulsifying properties (Sikorski, 2001). Among these factors, surface hydrophobicity and net surface charge were proposed to be the most important molecular features that influence their functional attributes (Schwenke, 2001). The amphiphilic nature of proteins allows them to simultaneously remain in the aqueous phase and adsorb at the surface of oil droplets, where they generate stabilizing electrostatic forces and steric interactions (Claesson et al., 2004). A major requirement for protein adsorption at the oil-water interface is the presence of hydrophobic patches on its surface. Additionally, the

net charge on the protein must be large enough to overcome various attractive forces (e.g., van der Waals, hydrophobic or depletion) and lead to significant stabilizing electrostatic repulsive forces between oil droplets (McClements, 2004).

Table 3.1 Proximate composition of raw materials (flours) and protein isolates prepared by isoelectric precipitation and salt extraction (as is basis). Data represent the mean ± one standard deviation (n = 3).

Material	Protein (%)	Moisture (%)	Lipid (%)	Ash (%)	Carbohydrate[1] (%)
(a) Raw materials (flours)					
Chickpea	16.71 ± 0.91^{a}	5.74 ± 0.10^{a}	3.77 ± 0.59^{a}	2.72 ± 0.03^{a}	71.06
Faba bean	23.94 ± 0.27^{b}	6.37 ± 0.10^{b}	0.73 ± 0.03^{b}	2.89 ± 0.01^{b}	66.07
Lentil	18.43 ± 0.30^{c}	6.22 ± 0.09^{b}	0.73 ± 0.05^{b}	2.56 ± 0.03^{a}	72.06
Pea	18.76 ± 0.28^{c}	6.77 ± 0.18^{c}	0.89 ± 0.20^{b}	2.73 ± 0.03^{a,b}	70.85
Soy	45.41 ±0 .67^{d}	6.23 ± 0.14^{b}	0.59 ± 0.07^{b}	6.27 ± 0.11^{c}	41.50
(b) Protein isolates prepared by isoelectric precipitation					
ChPI	85.40 ± 0.45^{a}	6.52 ± 0.27^{a}	0.92 ± 0.21^{a}	3.05 ± 0.33^{a}	4.11
FbPI	84.14 ± 0.45a	6.46 ± 0.12^{a}	0.39 ± 0.22^{b}	4.03 ± 0.41^{b}	4.98
LPI	81.90 ± 0.87^{b}	5.04 ± 0.16^{b}	0.43 ± 0.02b	3.63 ± 0.09^{a,b}	9.00
PPI	88.76 ± 0.04^{c}	5.08 ± 0.08^{b}	0.55 ± 0.08^{a,b}	5.59 ± 0.34^{c}	0.02
SPI	87.59 ± 0.37^{c}	4.47 ± 0.08^{c}	0.62 ± 0.03^{a,b}	2.09 ± 0.15^{d}	5.23
(c) Protein isolates prepared by salt extraction					
ChPI	81.63 ± 0.54^{a}	6.95 ± 0.04^{a}	0.56 ± 0.08^{a}	3.65 ± 0.33^{a,b}	7.21
FbPI	81.98 ± 0.65^{a}	7.16 ± 0.02^{a}	0.34 ± 0.08^{b}	3.57 ± 0.37^{a,b}	6.95
LPI	74.71 ± 0.29^{b}	6.87 ± 0.35^{a}	0.45 ± 0.05^{a,b}	4.60 ± 0.75^{b,c}	13.37
PPI	81.09 ± 0.88^{a}	9.55 ± 0.06^{b}	0.58 ± 0.09^{a}	5.33 ± 0.33^{c}	3.45
SPI	72.64 ± 0.31^{c}	10.08 ± 0.38^{b}	0.27 ± 0.05^{b}	3.27 ± 0.15^{a}	13.74

[1] Calculated by percent differential from 100%.
Means in each column followed by different letters were significantly different ($p<0.05$).

Surface charge (zeta potential) and surface hydrophobicity (H_0-ANS) of all protein isolates at pH 7.0 are shown in Table 3.2. In all cases, proteins carried a net negative charge at neutral pH as they were above their pI values (pI ~4.5 for globulins; ~6.0 for albumins) (Swanson, 1990). Surface charge values measured were comparable to those reported in literature for soy (Lam et al., 2008) and pea protein isolates (Liu et al., 2009). An analysis of variance showed that the legume source and method of extraction, plus their interaction were significant ($p<0.001$). Overall, protein isolates produced by isoelectric precipitation had slightly higher surface charge (mean of ~−22.3 mV) compared to salt extraction (mean of ~−20.2 mV, $p<0.001$). However, effect of legume source followed a different trend depending on the method of extraction. For proteins produced by isoelectric precipitation, ChPI, LPI, SPI, and FbPI had similar net charges at ~−22.6 mV ($p>0.05$), while PPI was found to carry a significantly lower net charge (−21 mV, $p<0.05$). Among salt-extracted proteins, surface charge was found to be the lowest for FbPI at −18.3 mV, and then increased for ChPI ≈ LPI ≈ PPI (~-20.4 mV), and again for SPI (−21.7 mV).

In terms of surface hydrophobicity, legume source and method of extraction, along with their interaction was also found to be significant ($p<0.05$, Table 3.2). In case of proteins produced by isoelectric precipitation, H_0-ANS ranged between 55.2 and 84.8; increasing in the following order: FbPI ≈ SPI < LPI < ChPI ≈ PPI. On the other hand, H_0-ANS was found to be the lowest for SPI and LPI (~54.6), and then increased for ChPI ≈ FbPI, and again for PPI (77.8) for salt-extracted proteins. Isoelectric-precipitated proteins showed greater H_0-ANS relative to the salt-extracted proteins in the case of ChPI, LPI, and PPI ($p<0.05$). Differences in H_0-ANS between the two extraction methods could be caused by variances in their composition. Isoelectric precipitation mainly precipitates globulins (Papalamprou et al., 2010), whereas products of salt extraction method typically comprise of a mixture of globulins and albumins (Liu et al., 2008). Surface hydrophobicity for globulins were reported to be higher than albumins (Papalamprou et al., 2009); therefore isolates produced by isoelectric precipitation method would be expected to be richer in globulins; showing relatively higher H_0-ANS than salt extractted proteins. Besides, conditions used for alkaline extraction of globulin proteins (e.g., legumin (11S, hexamer) or vicilin (7S, trimer)-type) may disassociate into their subunits; resulting in increased H_0-ANS due to the exposure of the originally buried hydrophobic side-chain groups. Protein disassociation in legume proteins have been previously reported for chickpea (Papalamprou et al., 2009) and pea protein isolates (Gueguen, 1989).

Table 3.2 Physicochemical properties of legume protein isolates (pH 7.0) prepared by isoelectric precipitation and salt extraction. Data represent the mean ± one standard deviation (n = 3).

Material	Surface Charge (mV)	Surface Hydrophobicity (H_0-ANS)	Percent Solubility	Interfacial Tension (mN/m)
(a) Protein isolates prepared by isoelectric precipitation				
ChPI	−22.0 ± 0.67^{a,b}	80.36 ± 1.94^{a}	91.20 ± 0.37^{a}	42.00 ± 0.30^{a}
FbPI	−23.0 ± 0.70^{a}	55.23 ± 2.23^{b}	89.65 ± 0.24^{b}	42.16 ± 0.28^{a}
LPI	−22.6 ± 0.55^{a}	64.67 ± 1.69^{c}	90.73 ± 0.35^{a,b}	42.48 ± 0.11^{a}
PPI	−21.0 ± 0.26^{b}	84.76 ± 1.16^{a}	61.42 ± 0.77^{c}	41.91 ± 0.13^{a}
SPI	−22.7 ± 0.06^{a}	55.32 ± 0.58^{b}	96.53 ± 0.04^{d}	42.68 ± 0.26^{a}
(b) Protein isolates prepared by salt extraction				
ChPI	−19.9 ± 0.57^{a}	67.71 ± 2.45^{a}	30.16 ± 0.22^{a}	42.48 ± 0.31^{a}
FbPI	−18.3 ± 0.35^{c}	73.52 ± 0.45^{a,b}	52.54 ± 0.25^{b}	42.71 ± 0.32^{a}
LPI	−20.4 ± 0.15^{a}	58.60 ± 2.33^{c}	89.88 ± 0.22^{c}	41.91 ± 0.24^{a}
PPI	−20.9 ± 0.44^{a,b}	77.83 ± 0.69^{b}	38.12 ± 0.08^{d}	42.63 ± 0.42^{a}
SPI	−21.7 ± 0.32^{b}	50.62 ± 5.22^{c}	96.79 ± 0.24^{e}	41.94 ± 0.33^{a}

Means in each column (for each production method) followed by different letters were significantly different ($p<0.05$).

3.4.3 Solubility and interfacial properties

Proteins when well dispersed and in solution, generally have good emulsifying properties as they can readily migrate to the oil/water interface (Sikorski, 2001). Protein solubility is related to the balance of protein-protein and protein-solvent interactions. The former is facilitated through hydrophobic interactions and leads to precipitation, whereas the latter promotes protein hydration and solubility (Damodaran, 1996). Protein-solvent interactions are also influenced by environmental factors (e.g., pH, ionic strength and temperature), solvent-type (McClements, 2009), and by processing (e.g., extraction or post-extraction treatments) (Kinsella, 1979). In the present study, the solubility of all protein isolates was investigated at neutral pH (Table 3.2). An analysis of variance indicated that legume source and method of production, along with their interaction were highly significant ($p<0.001$). Overall, solubility was significantly higher for isolates produced by isoelectric precipitation (~85.9%) relative to those produced by salt extraction (~61.5%, $p<0.05$). In the

case of isoelectric-precipitated isolates, their solubility was lowest at 61.4% for PPI, and was >90% for FbPI, LPI, and ChPI, and was highest at 96.5% for SPI. For salt-extracted isolates, their solubility values showed much greater variation (30.2-96.8%); increasing in the following order: ChPI < PPI < FbPI < LPI < SPI. The solubilities of all legume protein isolates (with the exception of ChPI) were found to be positively correlated with their surface charge ($r = 0.664$, $p<0.01$) and negatively correlated with their surface hydrophobicity ($r = -0.556$, $p<0.01$, Table 3.3). The high solubility of FbPI, LPI and SPI (~89.7-96.5%; prepared by isoelectric precipitation) is attributed to their relatively low surface hydrophobicity and high surface charge ($p<0.05$, Table 3.2). In contrast, the high surface hydrophobicity and low surface charge of PPI ($p<0.05$, Table 3.2) results in its lower solubility (61.4%). In the case of ChPI, solubility remained high (91.2%) for isolates prepared by isoelectric precipitation despite its high relative surface hydrophobicity, whereas low solubility (30.2%) was found for salt-extracted isolates, despite having more intermediate hydrophobicity values relative to the other legume proteins tested (Table 3.2). Variations in solubility have been reported for different legume proteins by Tsoukala et al. (2006) and Kimura et al. (2008) and isolates produced by different extraction methods (Boye et al., 2010b). In the present study, the differences observed between the two extractions methods are thought to reflect differences in protein composition, which influence total protein surface characteristics (i.e., number of exposed hydrophilic and hydrophobic groups, folding and aggregation), impacting their interactions with water.

Table 3.3 Pearson correlation coefficients (r) for physicochemical and emulsifying properties of legume protein isolates.

Parameter	SC	SH	SOL	IT	EC	EAI	ESI	CS
SC	1							
SH	−0.367*	1						
SOL	0.664**	−0.556**	1					
IT	−0.173	0.071	−0.351	1				
EC	0.284	−0.570**	0.669**	−0.311	1			
EAI	0.763**	0.038	0.633**	−0.200	0.161	1		
ESI	0.773**	−0.242	0.670**	0.073	0.232	0.699**	1	
CS	0.597**	0.105	0.754**	−0.311	0.366	0.771**	0.445*	1
DS	−0.650**	0.081	−0.670**	0.339	–	–	–	−0.830**

SC, Surface charge; SH, surface hydrophobicity; SOL, solubility; IT, Interfacial tension; EC, emulsion capacity; EAI, emulsifying activity index; ESI, emulsion stability index; CS, creaming stability; DS, droplet size.

* Correlation is significant at the 0.05 level.

** Correlation is significant at the 0.01 level.

Once in solution, the migration and adsorption of proteins to the oil-water interface occurs first during emulsion formation (Damodaran, 1996). Proteins with high molecular flexibility, mobility, and surface hydrophobicity tend to show the greatest surface activity (Kinsella, 1979). The ability of all protein isolates prepared in this study (0.25%, w/w; pH 7.0) to lower the interfacial tension between an aqueous and a flaxseed oil phase was investigated (Table 3.2). All isolates were able to decrease interfacial tension (~42.3 mN/m) relative to water (48.4 mN/m), however no significant differences were observed for either legume source or isolate production method ($p>0.05$). Ducel et al. (2004) determined the interfacial tension between water and vaseline oil to be 51.7 mN/m using the pendant drop method. A pea globulin solution of 0.4% (pH 2.6) was reported to reduce the interfacial tension to ~34 mN/m.

3.4.4 Emulsion formation and stability

In the present study, the emulsifying properties (EC, EAI and ESI) for all isolates (0.25%, w/w; pH 7.0) with flaxseed oil were investigated (Table 3.4). Emulsion capacity defines the amount of oil that can be emulsified by a standard amount protein under a specific set of conditions (Pearce and Kinsella, 1978). An analysis of variance revealed only legume

source to be significant ($p<0.001$). EC values ranged between ~481 to ~513 g oil/g protein and increased as follows, PPI ≈ ChPI < FbPI < SPI ≈ LPI (Table 3.4). The EC of isolates were found to be positively correlated with solubility ($r = 0.669$, $p<0.01$) and negatively correlated with surface hydrophobicity ($r = -0.570$, $p<0.01$, Table 3.3). A predictive multiple regression model of EC supported the inclusion of the following factors: surface charge, surface hydrophobicity, and solubility and interaction between solubility and surface charge. The model was able to predict 63.1% of data variability ($F = 10.668$; $p<0.001$), which suggested that other factors were affecting EC (Table 3.5). In general, EC increased as the isolate surface charge and solubility increased, and surface hydrophobicity decreased. Akintayo et al. (1998) determined the EC of pigeon pea, lima bean and African yam bean protein isolates produced by isoelectric precipitation. The authors found that the EC values varied between 146-1673 mL oil/g of protein and were dependent upon emulsifying speed, rate of oil addition, and protein concentration. Typically, EC testing is performed by the addition of oil to the protein solution at a constant rate and the EC value is the point (i.e., volume of oil added) at which a significant drop in conductivity occurs, or by direct visual inspection of phase changes as the material transitions from a water-in-oil to an oil-in-water emulsion (McClements, 2007). The latter method is complicated by the rate of oil addition and inconsistent homogenization duration. In the method devised for this study, the method was improved by preparing a series of emulsions at different oil concentration, followed by homogenization of all samples at constant homogenization duration.

Emulsifying activity (EAI) describes the ability of a protein to form an emulsion (Hill, 1996), with the EAI providing an estimation of the interfacial area stabilized per unit weight of protein based on the turbidity of a diluted emulsion (Pearce and Kinsella, 1978). Also, the ESI provides a measure of the stability of the same diluted emulsion over a defined time period. An analysis of variance found both the legume source and method of isolate preparation, along with their associated interaction to be significant ($p<0.001$, Table 3.4). Overall, the EAI values were found to be lower for isolates produced by salt extraction, suggesting that proteins were less effective at forming the emulsion. For isolates prepared by isoelectric precipitation, EAI values for PPI, SPI, FbPI and LPI were similar in magnitude ranging between 42.9 to 44.5 m^2/g of isolate ($p>0.05$), whereas the values for ChPI were significantly higher (47.9 m^2/g of isolate, $p<0.05$). For isolates prepared by salt extraction, EAI values were found to be the lowest for ChPI at 33.8 m^2/g of isolate, and then increased for FbPI ≈ LPI (37.1 m^2/g of isolate), and again for SPI ≈ PPI (~43.0 m^2/g of isolate). The

EAIs were found to be positively correlated with isolate surface charge ($r = 0.763$, $p<0.01$) and solubility ($r = 0.633$, $p<0.01$, Table 3.3).

Table 3.4 Emulsifying properties of legume protein isolates (0.25% w/w; pH 7.0) prepared by isoelectric precipitation and salt extraction. Data represent the mean ± one standard deviation (n = 3).

Material	Emulsion Capacity (g oil/g protein)	Emulsifying Activity Index (m^2/g)	Emulsion Stability Index (min)	Creaming Stability (%)	Mean Droplet Diameter (d_{32})
(a) Protein isolates prepared by isoelectric precipitation					
ChPI	504.43 ± 10.20^{a,b}	47.90 ± 1.88^{a}	82.94 ± 3.18^{a}	98.63 ± 0.55^{a}	1.69 ± 0.04^{a}
FbPI	513.33 ± 0.00^{b}	44.29 ± 0.55^{b}	69.39 ± 3.71^{b}	98.74 ± 0.25^{a}	1.41 ± 0.32^{a}
LPI	484.44 ± 7.70^{a,c}	44.51 ± 1.06^{a,b}	86.79 ± 4.14^{a}	98.52 ± 0.63^{a}	1.59 ± 0.18^{a}
PPI	477.78 ± 3.85^{c}	42.87 ± 0.80^{b}	12.40 ± 0.04^{c}	98.91 ± 0.03^{a}	1.85 ± 0.09^{a}
SPI	520.00 ± 13.33^{b}	44.20 ± 0.92^{b}	85.97 ± 5.33^{a}	95.76 ± 1.05^{b}	1.51 ± 0.10^{a}
(b) Protein isolates prepared by salt extraction					
ChPI	475.55 ± 3.85^{a}	33.83 ± 0.25^{a}	10.92 ± 0.03^{a}	82.02 ± 0.70^{a}	10.13 ± 0.49^{a,b}
FbPI	486.67 ± 0.00^{a}	37.11 ± 0.98^{b}	10.97 ± 0.08^{a}	ND	10.75 ± 0.31^{b}
LPI	542.22 ± 7.70^{b}	37.17 ± 0.31^{b}	11.02 ± 0.09^{a}	97.39 ± 0.54^{b}	1.02 ± 0.06^{c}
PPI	484.45 ± 3.85^{a}	42.73 ± 0.15^{c}	10.89 ± 0.03^{a}	ND	8.90 ± 0.28^{d}
SPI	504.45 ± 3.85^{c}	43.35 ± 0.12^{c}	25.04 ± 0.62^{b}	94.06 ± 0.41^{c}	9.24 ± 0.25^{a,d}

Means in each column (for each production method) followed by different letters were significantly different ($p<0.05$).

ND, not detected; characteristic creaming behaviour was not observed in emulsions prepared with FbPI and PPI produced by salt extraction.

A multiple regression predictive model for EAI is presented in Table 3.5, which identified significant isolate factors including surface charge and hydrophobicity, solubility, and the interaction between solubility and surface hydrophobicity. The model accounted for 85.6% of the variation found in the data ($F = 37.296$; $p<0.001$; Table 3.5). Protein isolates produced by isoelectric precipitation had significantly higher surface charge ($p<0.001$) and solubility ($p<0.05$), which contributed to their higher EAI when compared to salt-extracted

isolates. The individual effects of solubility and surface hydrophobicity were positive while their interaction had a negative effect on EAI, which emphasized the important balance between hydrophilic and hydrophobic interactions on emulsifying properties.

Table 3.5 Multiple regression predictive models for estimating the emulsifying properties (EC, EAI, ESI and CS) from the physicochemical properties of legume protein isolates.

Dependent variable	Independent variable	Coefficient	p-value	Model fit	
(a) Emulsion capacity					
	SC	16.705	0.083	R^2 =	0.631
	SH	−0.758	0.014	SE[a] =	14.053
	SOL	6.598	0.017	p	<0.001
	SOL×SC	−0.290	0.028		
	Constant	169.491	0.362		
(b) Emulsion activity index					
	SC	1.633	0.000	R^2 =	0.856
	SH	0.562	0.002	SE =	1.685
	SOL	0.401	0.009	p	<0.001
	SOL×SH	−0.004	0.036		
	Constant	−38.997	0.005		
(c) Emulsion stability index					
	SC	63.702	0.003	R^2 =	0.835
	SH	34.039	0.000	SE =	15.333
	SOL	0.819	0.000	p	<0.001
	SC×SH	−0.729	0.018		
	SH^2	−.0129	0.000		
	Constant	−2023.177	0.000		
(d) Creaming stability					
	SC	0.557	0.003	R^2 =	0.989
	SOL	1.283	0.000	SE =	0.641
	SOL^2	−0.009	0.000	p	<0.001
	DS	−0.191	0.006		
	Constant	42.167	0.000		

SC, Surface charge; SH, surface hydrophobicity; SOL, solubility; DS, droplet size.

[a] Standard error of the estimate

In terms of emulsion stability (ESI), an analysis of variance showed that the legume source and method of isolate preparation, plus their interaction to be significant ($p<0.001$, Table 3.4). Overall, ESI was greatest for isolates produced by isoelectric precipitation. ESI was lowest for PPI (12.5 min) and steadily increased to 69.3 min for FbPI and to ~84.0 min for ChPI ≈ SPI ≈ LPI for isoelectric-precipitated isolates. In contrast, ESI values for salt-extracted isolates were found to be similar for PPI, ChPI, FbPI and LPI (~11 min), and was 25.0 min for SPI. ESI values were found to be positively correlated with isolate surface charge ($r = 0.773$, $p<0.01$) and solubility ($r = 0.670$, $p<0.01$, Table 3.3). A multiple regression predictive model was devised, identifying significant factors such as surface charge, solubility, surface hydrophobicity, along with the square of surface hydrophobicity, and interaction between surface charge and surface hydrophobicity; explaining 83.5% of the variability in the data ($F = 24.328$; $p<0.001$; Table 3.5). In general, isolates with higher surface charge and solubility showed higher ESI values. Among isolates produced by isoelectric precipitation, PPI showed the lowest surface charge and solubility, and the lowest ESI. For salt-extracted isolates, the high ESI value of SPI can be explained by its high solubility and surface charge ($p<0.05$). Also, the interaction between isolate surface charge and hydrophobicity had a negative effect on ESI, indicating that both hydrophobic and hydrophilic interactions were important in these protein-stabilized emulsion systems. In general, high ESI values of isoelectric-precipitated proteins could be attributed to their higher surface charge, higher surface hydrophobicity (with the exception of FbPI), and higher solubility compared to salt-extracted proteins. Differences in EC, EAI and ESI values in the present study are thought to reflect differences in protein composition and physicochemical properties induced by the different extraction methods.

3.4.5 Creaming stability and droplet size

One of the most common mechanisms for emulsion instability is creaming, which occurs as a result of the density difference between the oil and water phases. Oil droplets have a lower density compared to the water phase, so they tend to move upwards and accumulate at the top of the emulsion (McClements, 2009). The ability of an emulsion to resist creaming is highly dependent on the droplet size, density difference between the dispersed and continuous phases, and the viscosity of the continuous phase. Emulsions with smaller droplets, a lower density contrast between phases, and higher viscosity are more stable to creaming (McClements, 2007). The creaming stability of emulsions containing 1.0% protein isolate

(w/w) and 20% flaxseed oil (w/w) are presented in Table 3.4. Legume source and method of isolate preparation, as well as their associated interaction were found to be significant ($p<0.001$). Overall, the creaming stability values of emulsions stabilized by isolates produced by isoelectric precipitation of ~98.1% was significantly higher than those produced by salt extraction of ~91.2% ($p<0.05$). For isolates prepared by isoelectric precipitation, their creaming stabilities were found to be similar for LPI, ChPI, FbPI, and PPI (~98.5%, $p>0.05$) which was significantly higher than that observed for SPI (95.8%, $p<0.05$). For isolates produced by salt extraction, characteristic creaming behaviour was not observed in emulsions prepared with FbPI and PPI as a rapid separation of an aqueous layer was observed. For the remaining samples, creaming stability values ranged from 82.0 to 97.4%; increasing in the following order: ChPI < SPI < LPI. It has been shown (Lucassen-Reynders, 1996) that rapid separation occurs in emulsions where the emulsifier fails to cover the oil-water interface thoroughly, resulting in a completely mobile continuous phase. Rapid separation into two phases has been observed in emulsions prepared with cowpea protein isolates (Kimura et al., 2008). Creaming stability was found to be positively correlated with isolate surface charge ($r = 0.597$, $p<0.01$) and solubility ($r = 0.754$, $p<0.01$) and negatively correlated with oil droplet size ($r = -0.830$, $p<0.01$, Table 3.3). A multiple regression predictive model for creaming stability identified the following significant isolate factors: surface charge, oil droplet size, solubility, and solubility^2, which was able to explain 98.9% of data variability ($F = 416.061$; $p<0.001$; Table 3.5). A quadratic effect of solubility indicated that creaming stability increased rapidly at low levels of isolate solubility, and was slower as isolate solubility increased. In general, isolates prepared by isoelectric precipitation had relatively higher surface charge and solubility compared to those produced by salt extraction, and were able to form emulsions with smaller droplets which were stable to creaming. Among salt-extracted isolates, ChPI had a lower creaming stability (82.0%) than SPI (94.1%) and LPI (97.4%), which could be explained by its relatively lower surface charge and solubility ($p<0.05$, Table 3.2). Also, isolates which had high EAI and ESI values also showed high creaming stability.

The mean droplet diameter of emulsions used for creaming is shown in Table 3.4. An analysis of variance indicated that legume source and method of isolate preparation, along with their interaction were significant ($p<0.001$). Overall, protein isolates produced by isoelectric precipitation formed emulsions with significantly smaller droplets (~1.6 μm) compared to emulsions formed with salt-extracted isolates (~8.0 μm, $p<0.001$). This finding is in accordance with the high creaming stability of emulsions stabilized with these samples. Isolates produced by isoelectric precipitation formed emulsions with similar droplet sizes

ranging between 1.4-1.9 μm ($p>0.05$). For salt-extracted isolates, LPI formed emulsions with the smallest droplets (1.0 μm). Droplet size for the other salt-extracted proteins ranged from 8.9 to 10.7 μm; increasing in the following order: PPI ≈ SPI < ChPI < FbPI. Droplet size was found to be negatively correlated with isolate surface charge ($r = -0.650$, $p<0.01$) and solubility ($r = -0.670$, $p<0.01$, Table 3.3) which suggested that proteins that are highly soluble in the continuous phase are better emulsifiers as they can easily migrate to the oil/water interface during emulsification and can lead to formation of smaller droplets. All emulsions were polydisperse, with mono- or multimodal droplet size distributions (Figure 3.1). Similar multimodal size distributions were previously reported for emulsions stabilized with pea (Aluko et al., 2009), broad bean and lupine protein isolates (Tsoukala et al., 2006).

3.5 Conclusions

Both legume source and isolate production method had significant impacts on the physicochemical and emulsifying properties of legumes studied. The EC, EAI and ESI values of protein isolates were all affected by surface charge and hydrophobicity and solubility; whereas their creaming stabilities were related to surface charge, solubility and droplet size. In general, isolates produced by isoelectric precipitation had higher surface charge and solubility compared to those produced by salt extraction. For all legume isolates studied, PPI had the lowest emulsion capacity and stability, which was attributed to its high surface hydrophobicity, low surface charge and low solubility. The ChPI and LPI produced by isoelectric precipitation had the highest surface charge and solubility, formed emulsions with smaller droplet size and showed high emulsifying activity and stability that were comparable to the SPI. These findings suggest that chickpea and lentil protein isolates have the potential to serve as an alternative to soy protein isolates, for stabilizing oil-in-water emulsions.

3.6 Linkage

Findings from this study described the effects of protein source and method of isolate production on the physicochemical and emulsifying and properties of the legume proteins tested. Isoelectric precipitated ChPI and LPI had relatively high surface charges and formed emulsions with smaller droplet sizes, and displayed high EAI, ESI and CS results, which were comparable to those of SPI. The focus of the next study was to investigate the emulsifying properties of canola and flaxseed protein isolates produced by isoelectric precipitation and salt extraction relative to a commercial whey protein isolate.

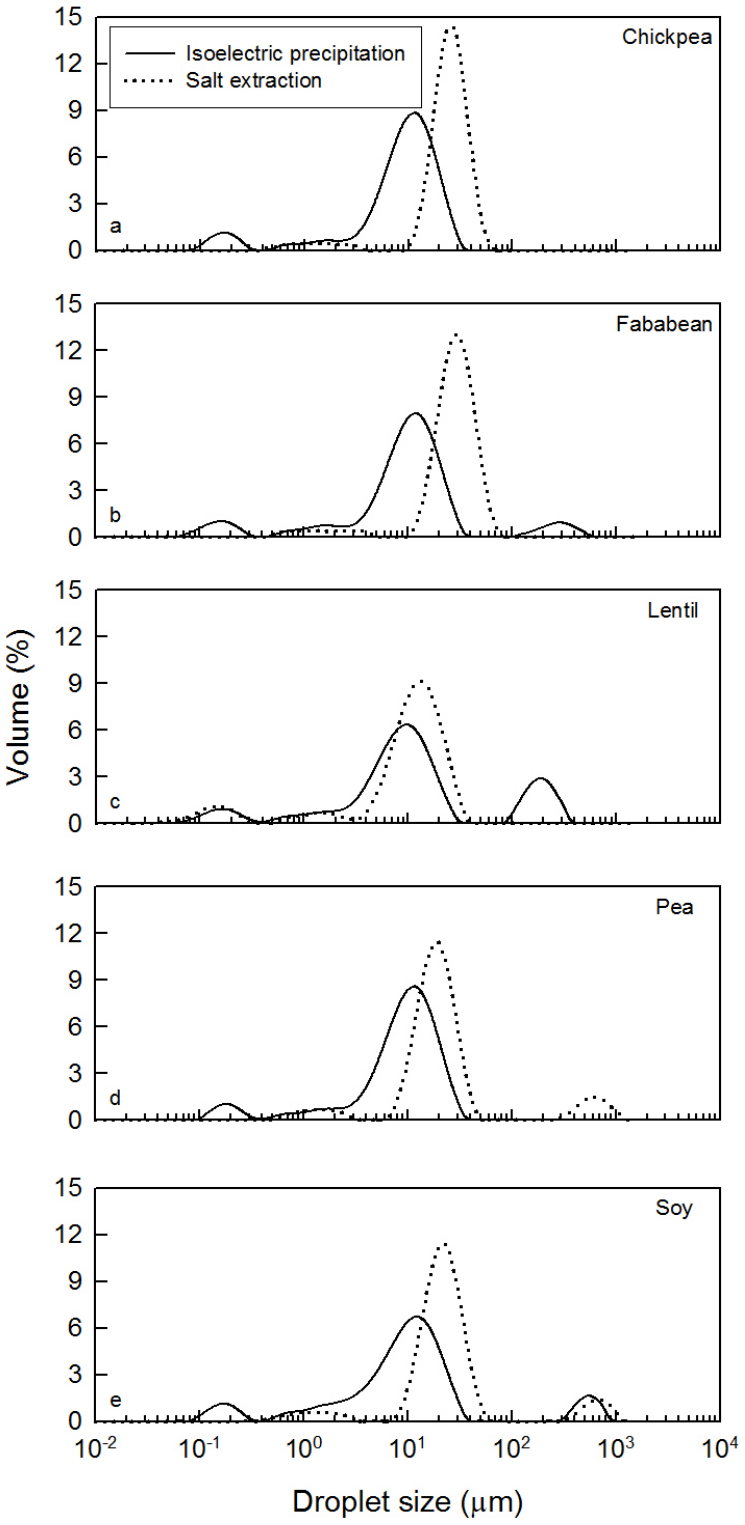

Figure 3.1 Droplet size distribution of legume-protein stabilized emulsions prepared at a 20:80 (w/w) oil-to-water ratio with flaxseed oil, using protein isolates (1.00%, w/w) prepared by isoelectric precipitation and salt extraction.

4. EMULSIFYING PROPERTIES OF CANOLA AND FLAXSEED PROTEIN ISOLATES PRODUCED BY ISOELECTRIC PRECIPITATION AND SALT EXTRACTION[2]

4.1 Abstract

The emulsifying (emulsion capacity (EC), emulsion activity/stability indices (EAI-ESI) and creaming stability (CS)) and physicochemical (surface charge/hydrophobicity, protein solubility, interfacial tension, and droplet size) properties of canola (CaPI) and flax (FlPI) protein isolates produced by isoelectric precipitation and salt extraction were investigated relative to whey protein isolate (WPI). Both protein source and method of production were found to have significant effects on the physicochemical and emulsifying properties of both protein isolates. All proteins carried a net negative charge at neutral pH, whereas surface hydrophobicity for CaPI and FlPI (~120.6) was found to be significantly higher than that of WPI (~61.9). CaPI and FlPI produced by salt extraction showed higher solubility and interfacial activity compared to those produced by isoelectric precipitation. CaPI showed significantly higher EC (~515.6 g oil/g protein) than FlPI (~498.9 g oil/g protein) which was comparable to WPI (520.0 g oil/g protein). However, EAI and ESI values for CaPI and FlPI were significantly lower than that of WPI. The mean EAI value for FlPI was higher (~40.1 m^2/g) than CaPI (~25.1 m^2/g) however, ESI values of CaPI and FlPI were similar. Creaming stability of emulsions stabilized by CaPI and FlPI ranged between 86.1 and 96.6%, which was comparable to WPI-stabilized emulsions (90.8%). The mean droplet diameter for FlPI-stabilized emulsions (~11.7 μm) was smaller than that of CaPI-stabilized emulsions (~14.8 μm). The EC of CaPI and FlPI was related to their solubility, surface characteristics and ability to reduce interfacial tension, while emulsion stability was a function of solubility, surface characteristics and droplet size. These results suggest that CaPI and FlPI have emulsion forming properties; however their stability is low when compared to WPI.

[2] Reproduced with permission. Can Karaca, A., Low, N. and Nickerson, M. 2011. Emulsifying properties of canola and flaxseed protein isolates produced by isoelectric precipitation and salt extraction. Food Research International, 44, 2991–2998. Copyright (2011) Elseiver Ltd.

4.2 Introduction

Canola and flaxseed are economically important oilseed crops grown in Western Canada primarily for their oil content and fatty acid composition, leaving protein-rich meals as an underutilized by-product. Within the food protein ingredient market, industry is pushing towards finding plant-based alternatives to animal-derived ingredients based on consumer perceived fears (e.g., prion disease), religious inhibitions, and dietary and moral preferences associated with consuming animal by-products. In order to increase the value and market integration to plant protein usage (e.g. canola and flaxseed consumption), a greater understanding of their structure-function relationships is needed. Krause and Schwenke (2001) and Tan et al. (2011) suggested that protein-rich plant meals are suitable raw materials for producing highly functional ingredients (e.g., emulsifiers). Canola (Paulson and Tung, 1988; Aluko and McIntosh, 2001; Wu and Muir, 2008) and flaxseed proteins (Wanasundara and Shahidi, 1997; Krause et al., 2002; Wang et al., 2010a-b) have been investigated for their emulsifying properties with mixed results. Processing methods used for extracting oilseed proteins have been reported to influence both their composition and functionality (Aluko and McIntosh, 2001; Krause et al., 2002). To date, there is a general lack of knowledge relating emulsification and physicochemical properties of canola and flaxseed protein isolates under the same testing conditions. The aim of this study was to investigate the emulsifying properties of canola and flaxseed protein isolates produced by isoelectric precipitation and salt extraction relative to a commercial whey protein isolate (WPI), and to relate the emulsifying properties of these proteins to their physicochemical attributes. WPI was used as a protein control for comparative purposes as it is a widely used emulsifier in food systems because of its lowcost, high solubility and excellent emulsifying properties.

Canola seeds contain two predominant classes of storage proteins: 12S (S - Svedberg Unit) salt-soluble globulin (cruciferin) and 2S water-soluble albumin (napin). Cruciferin is a hexameric protein with an overall molecular mass of ~300 kDa. Each subunit consists of α- (30 kDa) and β- (20 kDa) chains linked by intramolecular disulfide bridges (Lampart-Szczapa, 2001). Cruciferin differs from other oilseed globulins with its neutral isoelectric point (pI ~7.20) (Krause and Schwenke, 2001) and high level of glycosylation (~13% carbohydrate) (Lampart-Szczapa, 2001). Cruciferin may also dissociate into subunits at extreme pH and in the presence of urea (Schwenke, 1994; Bérot et al., 2005). Napins have a low molecular mass (12.5-14.5 kDa), high pI (10.0-11.0), and they are composed of two polypeptide chains of 4.5 kDa and 10 kDa held together by disulfide bonds (Bérot et al., 2005). Flaxseed proteins are also composed of salt-soluble 11-12S globulins and water-

soluble 1.6-2S albumins, which are referred to as linin and conlinin, respectively (Vassel and Nesbitt, 1945). Flaxseed globulin has an overall molecular mass of ~320 kDa, a pI of ~4.75 (Wanasundara and Shahidi, 2003), and is comprised of at least five subunits having molecular masses ranging from 11 to 61 kDa held together by disulfide linkages (Oomah and Mazza, 1993). In contrast, flaxseed albumin is a basic protein containing a single polypeptide chain that has a molecular mass between 16–18 kDa (Wanasundara and Shahidi, 2003; Chung et al., 2005).

4.3 Material and Methods

4.3.1 Materials

Canola seeds (SP Desirable *Brassica napus*, Lot#: 168-8-129810), flaxseeds (CDC Sorrel, *Linum usitatissimum*) and commercial whey protein isolate (BiPro JE061-7-440) were provided by Viterra (Saskatoon, SK, Canada), the Crop Development Centre at the University of Saskatchewan (Saskatoon, SK, Canada), and Davisco Foods International Inc. (Le Sueur, MN, USA), respectively. Flaxseed oil was kindly donated by Bioriginal Food & Science Corp. (Saskatoon, SK, Canada). Proximate composition of all raw materials and protein isolates were performed according to AOAC (2003) Official Methods 925.10 (moisture), 923.03 (ash), 920.85 (lipid), and 920.87 (protein; nitrogen conversion factors of 5.70, 6.25 and 6.38 were used for canola, flaxseed and WPI, respectively; Schwenke et al., 1998). Carbohydrate content was determined on the basis of percent differential from 100%. All flours and protein isolates were stored at 4°C. All chemicals used were of reagent grade and purchased from Sigma-Aldrich (Oakville, ON, Canada). The water used in this research was product from a Millipore Milli-Q^{TM} water system (Millipore Corp., Milford, MA, USA).

4.3.2 Preparation of protein isolates by isoelectric precipitation and salt extraction

Canola protein isolates (CaPI)

Defatted canola meal was prepared by pressing canola seeds using a continuous screw expeller (Komet Type CA59 C, IBG Monforts Oekotec GmbH & Co., Monchegladbach, Germany), followed by hexane extraction at a 1:1 meal to hexane ratio (w/v) for 16 h. The meal was then air-dried in a fume hood for 8 h at room temperature (20-22°C), followed by a second hexane extraction and drying step.

Isoelectric-precipitated CaPI was prepared according to the method of Aluko and McIntosh (2001) with minor modifications. Briefly, defatted canola meal (100 g) was mixed with a 0.1M NaOH solution at 1:10 ratio (w/v) and stirred at 500 rpm for 20 min at room

temperature. The suspension was then centrifuged at 10,000 × *g* for 30 min at 8°C (Sorvall RC-6 Plus, Thermo Scientific, Asheville, NC, USA) to collect the supernatant which was then filtered through a 110 mm Whatman #1 filter paper (Whatman International Ltd., Maidstone, UK). The filtrate was then adjusted to pH 4.0 with 0.1 M HCl. The precipitate was recovered by centrifugation (10,000 × *g*, 30 min, 8°C), dispersed in water at 1:1 ratio (w/v) and dialyzed at 4°C for 24 h using Spectro/Por tubing with a 6-8 kDa cutoff (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) against water refreshing several times until the conductivity of the dialysis water reached 2.0-2.5 mS/cm. The protein was collected by centrifugation (10,000 × *g*, 30 min, 8°C) and stored at −30°C until freeze-dried, which was performed using a Labconco FreeZone 6 freeze drier (Labconco Corp., Kansas City, MO, USA) to yield a free flowing powder.

Salt-extracted CaPI was produced according to the method of Folawiyo and Apenten (1996) with minor modifications. Briefly, defatted canola meal (100 g) was mixed with 0.05 M Tris-HCl buffer (pH 7.0) containing 0.1 M NaCl at 1:10 ratio (w/v) and stirred at 500 rpm for 2 h at room temperature. The suspension was then centrifuged at 18,600 × *g* for 1 h at 4°C and the supernatant was recovered. A second centrifuge step for 30 min was used to further clarify the supernatant of insoluble residues, followed by dialysis (6-8 kDa cut off) against water at 4°C for 72 h. Precipitated salt soluble proteins were collected by centrifugation (18,600 × *g*, 1 h, 4°C) and stored at −30°C until freeze-dried.

Flaxseed protein isolates (FlPI)

In order to remove mucilage in the seed coat, flaxseeds were mixed with a 0.5 M $NaHCO_3$ solution at a 1:8 ratio (w/v) and stirred at 500 rpm for 18 h at room temperature (Marambe et al., 2008). Seeds were recovered by filtration, manually rubbed against an aluminum wire mesh and washed thoroughly with Milli-Q^{TM} water. The extraction and washing procedures were repeated twice. Seeds were collected and air-dried in a fume hood overnight. Demucilaged flaxseeds were ground into a fine flour using a coffee grinder for 1 min, and then defatted using hexane at a 1:3 (w/v) flour to hexane ratio for 6 h. The mixture was then filtered through a 110 mm Whatman #1 filter paper and the defatting procedure was repeated twice. Defatted flour was collected by filtration and air-dried in a fume hood overnight.

Isoelectric-precipitated FlPI was produced according to the method of Marambe et al. (2008). Briefly, demucilaged, defatted flaxseed flour (100 g) was dispersed in water at 1:10 ratio (w/v), adjusted to pH 8.5 using 1.0 M NaOH, and stirred at 500 rpm for 1 h at room

temperature. The suspension was then centrifuged at 8,820 × *g* for 20 min at 4°C to collect the supernatant. The resulting pellet was re-suspended in water at a ratio of 1:10 (w/v), adjusted to pH 8.5, and was stirred for 1 h, followed by centrifugation (8,820 × *g*, 20 min, 4°C). Supernatants were pooled and adjusted to pH 3.8 with 0.1 M HCl to precipitate the protein. Protein was recovered by centrifugation (8,820 × *g*, 20 min, 4°C), dispersed in water at 1:1 ratio (w/v) and dialyzed (6-8 kDa cut off) at 4°C for 24 h. The protein slurry was stored at −30°C until freeze-dried.

Salt-extracted FlPI was produced according to the method of Oomah et al., (1994). Briefly, demucilaged, defatted flaxseed flour (100 g) was mixed with 50 mM Na_3PO_4 buffer (pH 8.0) containing 0.8 M NaCl at 1:10 ratio (w/v). The mixture was stirred at 500 rpm for 30 min at room temperature and centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatant was collected and dialyzed (6-8 kDa cut off) at 4°C for 72 h as described above and stored at −30°C until freeze-dried.

4.3.3 Physicochemical properties

For all physicochemical tests, protein solutions were prepared by dispersing the isolates (corrected on a weight basis for protein content) in 10 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) and adjusted to pH 7.0 with either 0.1 M NaOH or 0.1 M HCl followed by stirring at 500 rpm overnight at 4°C. All experiments were conducted in triplicate.

Surface charge (Zeta potential):

The overall surface charge of the protein isolates was determined by measuring the electrophoretic mobility (U_E) of protein solutions (0.05%, w/w) at pH 7.0 using a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA). U_E was used to calculate the zeta potential (ζ) by applying the Henry's equation:

$$U_E = \frac{2\varepsilon \cdot \zeta \cdot f(\kappa\alpha)}{3\eta} \qquad \text{[eq. 4.1]}$$

where ε is the permittivity, $f(\kappa\alpha)$ is a function related to the ratio of particle radius (α) and the Debye length (κ), and η is the dispersion viscosity. For this study, the Smoluchowski approximation $f(\kappa\alpha)$ equalled 1.5.

Average surface hydrophobicity (H_0):

Average surface hydrophobicity was determined using the fluorescent probe, 8-anilino-1-naphthalenesulfonic acid (ANS) according to the method of Kato and Nakai (1980) with slight modifications described by Wang et al. (2005). All fluorescence measurements were made using a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) with the excitation and emission wavelengths at 390 and 470 nm, respectively. The excitation and emission slit widths were each set at 1 nm. Protein solutions (0.01%, w/w) prepared in 10 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) were diluted in the same buffer to obtain concentrations of 0.002%, 0.004%, 0.006%, 0.008% and 0.01% (w/w). To 4.0 mL of the protein solution, 20 μL of 8 mM ANS solution in 10 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) was added and mixed well by vortexing for 10 s. After keeping each sample in the dark for 15 min, the fluorescence intensity (FI) was measured. FI values of the ANS blank and diluted protein blanks (without the ANS probe) were also measured and subtracted from the FI of the ANS-protein solutions. The initial slope of the plot of the FI (corrected) versus protein concentration was calculated by linear regression analysis and used as an index of protein surface hydrophobicity.

Percent protein solubility:

Percent protein solubility was determined using the method of Morr et al. (1985). Protein solutions were prepared by dispersing 0.2 g of sample in 19.8 mL (1.0%, w/v) of 10 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) and adjusting the pH to 7.0 with either 0.1 M NaOH or 0.1 M HCl followed by stirring at 500 rpm overnight at 4°C. Solutions were centrifuged at 9,100 × *g* for 10 min at room temperature to remove insoluble residues. Nitrogen content in the supernatant was determined using a micro-Kjeldahl digestion and distillation unit (Labconco Corp., Kansas City, MO, USA). Percent protein solubility was calculated by dividing the nitrogen content in the supernatant by the total nitrogen in the sample (×100%).

Interfacial tension:

Interfacial tension between protein solutions (0.25%, w/w) and flaxseed oil was determined according to the Du Noüy ring method using a semi-automatic tensiometer (Lauda TD2, Lauda Dr. R. Wobser GmbH & Co., Lauda-Königshofen, Germany) and compared with the interfacial tension between water and flaxseed oil (without protein). Interfacial tension was calculated from the maximum force (F_{max}) by the following equation:

$$\gamma = \frac{F_{\max}}{4\pi R\beta} \qquad \text{[eq. 4.2]}$$

where γ is the interfacial tension, R is the radius of the ring, and β is a correction factor that depends on the dimensions of the ring and the density of the liquid involved.

4.3.4 Emulsifying properties

Emulsion capacity (EC):

A series of emulsions were prepared by homogenizing 3.0 g of a 0.25% (w/w) protein solution with differing amounts (3-5 g) of flaxseed oil in 50 mL plastic centrifuge tubes by using an Omni Macro Homogenizer (Omni International, Marietta, GA, USA) with a 20 mm saw tooth generating probe at speed 4 (~7,200 rpm) for 5 min. Emulsion conductivity was measured immediately after homogenization using an Orion 3-Star bench top conductivity meter (Thermo Scientific, Waltham, MA, USA) with a 4-electrode conductivity cell. Emulsion capacity was determined at the inversion point where an oil-in-water emulsion turns into a water-in-oil emulsion as indicated by a sudden drop in conductivity. Emulsion capacity was expressed as g of oil homogenized per g of protein before the inversion was observed.

Emulsifying activity (EAI) and stability (ESI) indices:

Emulsifying activity and stability indices of protein samples were determined as described by Pearce and Kinsella (1978). Five grams of a 0.5% (w/w) protein solution and 5.0 g of flaxseed oil were homogenized as described above. A 50 µL emulsion sample was immediately taken from the bottom of the tube and diluted in 7.5 mL of 10 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) containing 0.1% sodium dodecyl sulphate (SDS) and then vortexed for 10 s. An aliquot of this mixture was taken after 10 min of static storage at room temperature. Sample absorbance was measured at 500 nm using a Genesys 10 UV-visible spectrophotometer (Thermo Scientific, Madison, WI, USA) using plastic cuvettes (1 cm path length). EAI and ESI values were calculated using the following equations:

$$EAI(m^2/g) = \frac{2 \cdot 2.303 \cdot A_0 \cdot N}{c \cdot \varphi \cdot 10000} \qquad \text{[eq. 4.3]}$$

$$ESI(\text{min}) = \frac{A_0}{\Delta A} \cdot t \qquad \text{[eq. 4.4]}$$

where A_0 is the absorbance of the diluted emulsion immediately after homogenization, N is the dilution factor (×150), c is the weight of protein per volume (g/mL), φ is the oil volume fraction of the emulsion, ΔA is the change in absorbance between 0 and 10 min $(A_0 - A_{10})$ and t is the time interval (10 min).

Creaming stability:

Oil-in-water emulsions (20 mL) were prepared by homogenizing 16.0 g of a 1.25% (w/w) protein solution, 4.0 g of flaxseed oil and ~5 mg of Oil Blue N dye (a lipid-soluble dye, added to improve visualization during creaming). Emulsions (10 mL) were then transferred into 10 mL sealed graduated glass cylinders (inner diameter = 10.5 mm; height = 160 mm; as measured by a digital calliper) immediately after preparation. Creaming stability was determined by observing the separation of a 'cream' layer after 1 h of storage at room temperature. At this time interval, emulsions had separated into an optically opaque, darker blue cream layer (top) and a turbid layer (bottom) with a similar appearance to the original emulsion. Creaming stability (CS) was expressed as:

$$CS(\%) = \frac{H_T}{H_E} \cdot 100 \qquad \text{[eq. 4.5]}$$

where H_T is the height of the turbid layer and H_E is the total height of the emulsion.

Droplet size:

Emulsion droplet size distribution was measured using a Mastersizer 2000 laser light scattering instrument (Malvern Instruments Ltd., Worcestershire, United Kingdom) equipped with a Hydro 2000S sample handling unit (containing 10 mM Na_2HPO_4-NaH_2PO_4 buffer at pH 7.0). Emulsions were prepared as per the creaming study, with samples being taken from the bottom of the tube immediately after homogenization. The sample was stirred continuously within the sample cell at room temperature to ensure homogeneity. Obscuration in all measurements was kept at ~14% by buffer addition. Droplet size distributions were instrument calculated according to the Mie Theory which uses the refractive index difference between the droplets and the dispersing medium to predict the intensity of the scattered light. The ratio of the refractive index of flaxseed oil (1.479) to that of the dispersion medium (1.330) was 1.112. Droplet size measurements were reported as volume-surface mean diameters ($d_{3,2}$), which is expressed as:

$$d_{3,2} = \frac{\sum_{i=1} n_i \cdot d_i^3}{\sum_{i=1} n_i \cdot d_i^2} \qquad \text{[eq. 4.6]}$$

where n_i is the number of droplets of diameter (d_i) (McClements, 2005c).

4.3.5 Statistical analyses

All experiments were performed in triplicate and reported as the mean ± one standard deviation. Student's t-test for independent samples was applied to determine statistical significance of differences in the proximate composition of raw materials and protein isolates, and creaming stability data. An individual degree of freedom (orthogonal) contrast analysis using the general linear model (Li, 1964) was employed to measure statistical differences in physicochemical and emulsifying (except creaming stability) properties as a function of protein source and production method. The following individual degree of freedom contrasts were tested: oilseed protein source (CaPI vs. FlPI); oilseed proteins vs. WPI; production method (isoelectric precipitation vs. salt extraction method for oilseed proteins only), and the interaction between oilseed protein source and production method. Simultaneous multiple regression analysis was conducted to examine the relationship between surface charge, surface hydrophobicity, protein solubility, interfacial tension and droplet size (for CS only) on the emulsifying properties (e.g., EAI, ESI, EC and CS). Statistical analyses were performed with Systat (SPSS Inc., Ver. 10, 2000, Chicago, IL) and SPSS software (SPSS Inc., Ver. 17, 2008, Chicago, IL).

4.4 Results and Discussion

4.4.1 Composition of defatted meals and protein isolates

The proximate composition of all raw materials and protein isolates are given in Table 4.1. Mean protein levels for defatted canola and flaxseed meals were 25.41% and 31.93%, respectively, which were comparable to those reported by Klockeman et al. (1997) and Jhala and Hall (2010). Depending on the extraction method, protein levels in produced isolates differed. Overall, salt extraction resulted in higher protein levels than those produced by isoelectric precipitation for CaPI. Reduced levels in the latter may reflect the heterogeneous nature of canola proteins, which are known to have differing isoelectric points that complicate their precipitation (Wu and Muir, 2008). Gillberg and Törnell (1976) reported that acid precipitation of rapeseed protein extracts obtained by alkali extraction resulted in poorer yields. In contrast, protein levels for FlPI prepared by both extraction methods gave

high protein levels (>87%). Oomah (2003) reported protein levels of 90–95% in isolates prepared from mucilage-free flaxseed meal by isoelectric precipitation. In the present study, the lipid, moisture and ash levels for all isolates were at or below ~1.9%, ~8.3% and ~4.6%, respectively (Table 4.1).

Table 4.1 Proximate composition (as is basis) of raw materials (defatted meals) and protein isolates of canola (CaPI) and flaxseed (FlPI) prepared by isoelectric precipitation or salt extraction and whey protein isolate (WPI). Data represent the mean ± one standard deviation (n = 3).

Material	Protein (%)	Moisture (%)	Lipid (%)	Ash (%)	Carbohydrate[1] (%)
(a) Raw materials (defatted meals)					
Canola	31.93 ± 0.55	5.22 ± 0.13	1.98 ± 0.13	7.31 ± 0.02	53.56
Flaxseed	25.41 ± 0.85	5.98 ± 0.04	0.69 ± 0.06	5.12 ± 0.03	62.80
(b) Protein isolates prepared by isoelectric precipitation					
CaPI	75.31 ± 0.25	5.57 ± 0.12	1.72 ± 0.35	1.50 ± 0.07	15.36
FlPI	89.25 ± 0.78	8.28 ± 0.21	0.37 ± 0.02	2.08 ± 0.27	0.02
(c) Protein isolates prepared by salt extraction					
CaPI	93.10 ± 0.92	4.43 ± 0.53	1.90 ± 0.14	0.56 ± 0.17	0.01
FlPI	87.39 ± 0.26	7.58 ± 0.61	0.40 ± 0.05	4.58 ± 0.20	0.05
(d) Control					
WPI	89.78 ± 0.41	4.92 ± 0.04	0.10 ± 0.01	2.06 ± 0.04	3.14

[1] Calculated by percent differential from 100%.

4.4.2 Surface characteristics

For a protein to display acceptable surface activity, it should possess hydrophobic patches on its surface and have a good solubility in the aqueous phase (Dickinson, 2003). The net protein charge should also be large enough to afford electrostatic repulsion between oil droplets to prevent aggregation (McClements, 2007). Surface charge (zeta potential, mV) and

surface hydrophobicity (H_0-ANS) of all protein isolates at pH 7.0 are shown in Table 4.2. In all cases, proteins carried a net negative charge at this pH value. An individual degree of freedom (orthogonal) contrast analysis found that all main effects: oilseed proteins vs. WPI; oilseed protein source; isolate production method; the interaction between oilseed protein source and isolate production method were significant ($p<0.001$). The presence of a significant interaction between oilseed protein source and isolate production method indicates that effect of oilseed protein source (ChPI vs. FlPI) was dependent on isolate production method. Overall, isoelectric precipitation resulted in isolates with significantly higher surface charge (~−28.1 mV) compared to those produced by salt extraction (~−14.7 mV, $p<0.001$). Also, FlPI showed a higher surface charge relative to CaPI ($p<0.001$) for both isolate production methods, and oilseed proteins (~−21.3 mV) gave significantly higher surface charge when compared to WPI (~−17.9 mV) ($p<0.001$).

Table 4.2 Surface charge (zeta potential, mV) and average surface hydrophobicity (Ho-ANS) of protein isolates (pH 7.0) of canola (CaPI) and flaxseed (FlPI) prepared by isoelectric precipitation or salt extraction and whey protein isolate (WPI). Data represent the mean ± one standard deviation (n = 3).

Material	Surface Charge (Zeta Potential, mV)	Surface Hydrophobicity (H_0-ANS)
(a) Protein isolates prepared by isoelectric precipitation		
CaPI	−22.7 ± 0.8	148.4 ± 4.9
FlPI	−33.4 ± 0.3	65.5 ± 0.4
(b) Protein isolates prepared by salt extraction		
CaPI	−11.3 ± 0.1	111.0 ± 2.7
FlPI	−18.1 ± 0.7	157.3 ± 4.1
(c) Control		
WPI	−17.9 ± 0.3	61.9 ± 1.8

In terms of average surface hydrophobicity (H_0-ANS), all main contrasts and the interaction between oilseed protein source and isolate production method were found to be

significant ($p<0.001$). The relationship between protein source and isolate surface hydrophobicity differed depending on their preparation method. For isoelectric-precipitated isolates, CaPI showed significantly higher H_0-ANS compared to FlPI, whereas the opposite was found for salt–extracted isolates ($p<0.001$, Table 4.2). Overall, the surface hydrophobicities of oilseed protein isolates (~120.6) were found to be significantly greater than that of WPI (~61.9, $p<0.001$, Table 4.2). The method of isolate production has been reported to affect protein composition and physicochemical properties for legume protein isolates (Papalamprou et al., 2009). However, published scientific results relating the surface hydrophobicity of oilseed protein isolates produced by different methods is lacking. Apenten and Folawiyo (1996) studied the effect of acid and alkali treatments on canola globulin (cruciferin) binding to ANS and indicated that acid treatment (pH 2.0) resulted in the unfolding of the native structure of cruciferin which in turn led to exposure of previously buried hydrophobic groups for ANS binding. In contrast, an alkali treatment (pH 10.0) did not show a significant effect on surface hydrophobicity for cruciferin. Paulson and Tung (1987) investigated the effect of succinylation, pH and ionic strength on some physicochemical properties of canola protein isolates prepared by an isoelectric precipitation method. Surface hydrophobicity (H_0-ANS) of the unmodified canola protein isolate was reported to be significantly higher at pH 3.5 when compared to pH 6.5. Succinylation resulted in a decrease in H_0-ANS while the effect of NaCl varied with pH. For the unmodified isolate, at pH 3.5 and 5.0, NaCl decreased hydrophobicity while the opposite effect was observed at higher pH values (Paulson and Tung, 1987).

4.4.3 Solubility and interfacial properties

High protein solubility is required for rapid migration to and adsorption at the oil-water interface (Damodaran, 2005). Percent solubility of protein isolates at neutral pH is presented in Figure 4.1. An individual degree of freedom contrast analysis indicated that all main effects and interaction between protein source and isolate production method were significant ($p<0.001$). Overall, protein isolates from oilseeds showed significantly lower solubility when compared to WPI ($p<0.001$). The salt-extracted isolates were found to have significantly higher solubilities relative to those prepared by isoelectric precipitation ($p<0.001$), whereas for both production methods, FlPI showed greater solubility than CaPI ($p<0.001$). Aluko and McIntosh (2001) reported low solubility values at pH 7.0 (<6%) for acid-precipitated CaPI, and higher solubility (~78.8%) for the calcium-precipitated isolate (1 M $CaCl_2$). Yoshie-Stark et al. (2008) indicated that a rapeseed protein isolate produced by

isoelectric precipitation showed significantly lower solubility at pH 7.0 (~25%) compared to the isolate obtained with ultrafiltration (~90%). Krause et al. (2002) compared the composition and functional properties of FlPI prepared with micellization (extraction with 0.5 M NaCl solution followed by ultrafiltration) and by isoelectric precipitation. The authors reported that both isolates contained the same major 11S globulin fraction identified by chromatography and electrophoresis. The FlPI produced by isoelectric precipitation was found to have a higher content of phytic acid and pentosans, and a distinctly lower solubility at pH 7.0 (~40%) compared to the isolate produced by micellization (~90-95%). The authors suggested that protein denaturation during acid precipitation occurred, and interactions between protein and non-protein components accounted for the lower solubility observed (Krause et al., 2002). Pedroche et al. (2004) also proposed that the low solubility of an isoelectric-precipitated *Brassica carinata* (Ethiopian mustard) protein isolate at acidic and neutral pH could be due to the insoluble phytic acid-protein complexes that form between pH 3.0-7.0.

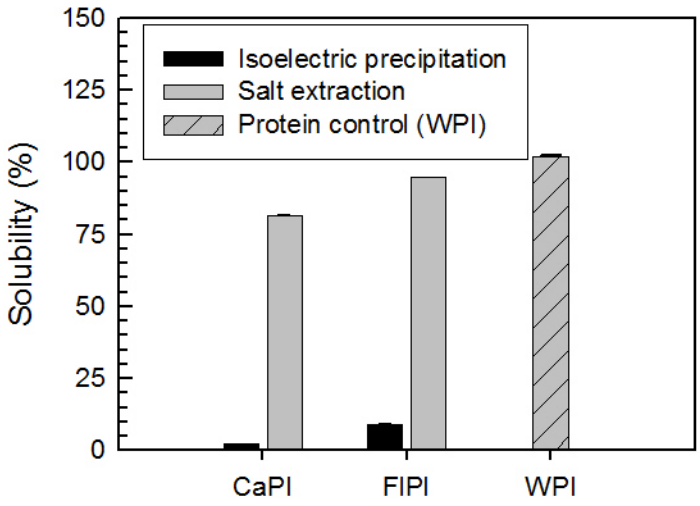

Figure 4.1 Percent solubility of protein isolates (1.0%, w/w; pH 7.0) of canola (CaPI) and flaxseed (FlPI) prepared by isoelectric precipitation or salt extraction and whey protein isolate (WPI). Data represent the mean ± one standard deviation (n = 3).

Proteins, as an emulsifier should have the ability to orient their hydrophobic residues to the oil phase and hydrophilic residues to the aqueous phase in order to reduce the interfacial tension (Dickinson, 2003). The ability for all isolates (0.25%, w/w; pH 7.0) to

lower interfacial tension between an aqueous and a flaxseed oil phase is shown in Figure 4.2. An individual degree of freedom contrast analysis revealed that only two of the main contrasts were significant: oilseed protein source vs. WPI ($p<0.001$) and production method ($p<0.05$). Interfacial tension between water and flaxseed oil was measured as 48.4 mN/m. Oilseed proteins were slightly more effective in reducing the interfacial tension (~43.1 mN/m) compared to WPI (~44.5 mN/m). However, there was no significant difference found between CaPI and FlPI ($p>0.05$). The ability of salt-extracted oilseed protein isolates to reduce interfacial tension (~42.4 mN/m) was slightly higher than that of isoelectric-precipitated isolates (~43.8 mN/m, $p<0.05$).

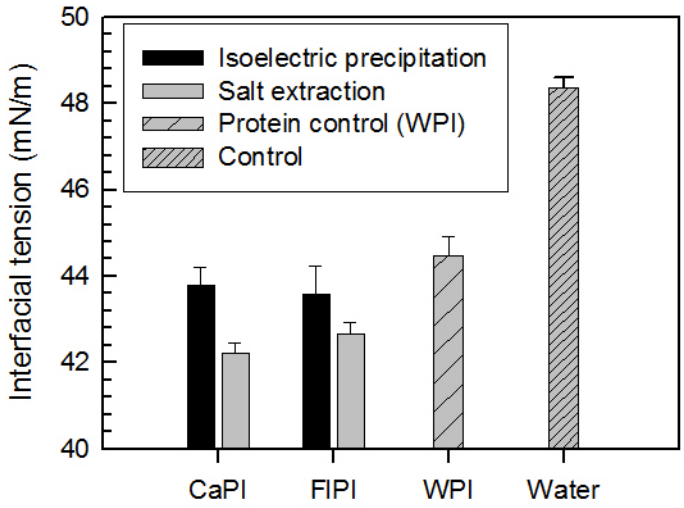

Figure 4.2 Interfacial tension (mN/m) at the interface between flaxseed oil and protein isolates (0.25%, w/w; pH 7.0) of canola (CaPI) and flaxseed (FlPI) prepared by isoelectric precipitation or salt extraction and whey protein isolate (WPI). Data represent the mean ± one standard deviation (n = 3).

4.4.4 Emulsion formation and stability

The emulsifying properties of protein isolates (emulsion capacity, emulsion activity and stability indices) are presented in Figure 4.3. Emulsion capacity (EC) is defined as the amount of oil that can be emulsified by a standard amount of protein under specific conditions (Pearce and Kinsella, 1978). An individual degree of freedom contrast analysis found that all main contrasts and interaction between protein source and production method were significant ($p<0.05$). Overall, WPI had a higher EC (520.0 g oil/g protein) compared to protein isolates

from oilseeds (~507.2 g oil/g protein, $p<0.05$), and CaPI showed significantly higher EC (~515.6 g oil/g protein) than that of FlPI (~498.9 g oil/g protein, $p<0.05$). The magnitude of EC values for CaPI was dependent upon the extraction method used. The EC for CaPI produced by salt extraction (528.9 g oil/g protein) was found to be greater than the isoelectric-precipitated isolate (502.2 g oil/g protein). In the case of FlPI, EC values were similar in magnitude regardless of the method of isolate production (497.8-500.0 g oil/g protein). A multiple regression predictive model for EC is presented in Table 4.3, which identified significant factors such as, solubility, interfacial tension, and the interaction between solubility and surface charge. The model accounted for 92.7% of the variation found in the data ($F = 33.660$; $p<0.001$; Table 4.3). In the present study, EC increased with increasing isolate solubility and decreasing interfacial tension. The negative interaction term (solubility × surface charge) suggests that EC is inversely related to charge (i.e., the higher the charge, the lower the EC). Decreased protein-protein interactions as a result of increased electrostatic repulsion between these molecules would prevent formation of a stable film around oil droplets during emulsion formation (Wanasundara and Shahidi, 1997). Yoshie-Stark et al. (2008) measured the EC of rapeseed protein isolates by titrating oil into a 1% protein solution until the emulsion collapsed. The authors reported that a rapeseed protein isolate produced by ultrafiltration method had significantly higher EC (693 mL oil/g protein) compared to an isoelectric-precipitated isolate (400 mL oil/g protein), which was attributed to better protein solubility in the former. Thompson et al. (1982) determined the EC of a rapeseed protein concentrate using a similar oil titration method, and reported it to have a lower EC (108.0 ml oil/g protein) than a soy protein isolate (191.3 mL oil/g protein). Martinez-Flores et al. (2006) measured the EC of a flaxseed protein concentrate produced by isoelectric precipitation as ~65% (percentage of oil emulsified) at pH 8.0 by measuring the height of the emulsion layer after centrifugation.

Table 4.3 Multiple regression predictive models for estimating the emulsifying properties from the physicochemical properties of protein isolates from canola and flaxseed.

Dependent variable	Independent variable	Coefficient	p-value	Model fit	
(a) Emulsion capacity					
	SOL	0.531	0.004	R^2 =	0.927
	IT	-11.665	0.012	SE[a] =	4.558
	SOL×SC	-0.039	0.000	F =	33.660
	Constant	1013.360	0.000	p	<0.001
(b) Emulsion activity index					
	SOL	0.339	0.000	R^2 =	0.987
	SC	1.036	0.000	SE =	1.481
	SH	-0.134	0.000	F =	194.129
	Constant	10.682	0.018	p	<0.001
(c) Emulsion stability index					
	SOL	0.064	0.000	R^2 =	0.983
	SC	0.215	0.000	SE =	0.299
	SH	0.019	0.000	F =	158.460
	Constant	2.672	0.006	p	<0.001
(d) Creaming stability					
	SOL	-0.126	0.001	R^2 =	0.986
	SC	-0.461	0.033	SE =	0.724
	DS	-0.581	0.188	F =	119.433
	Constant	112.302	0.000	p	<0.001

Abbreviations: SOL, solubility; IT, interfacial tension; SC, surface charge; SH, surface hydrophobicity; DS, droplet size.

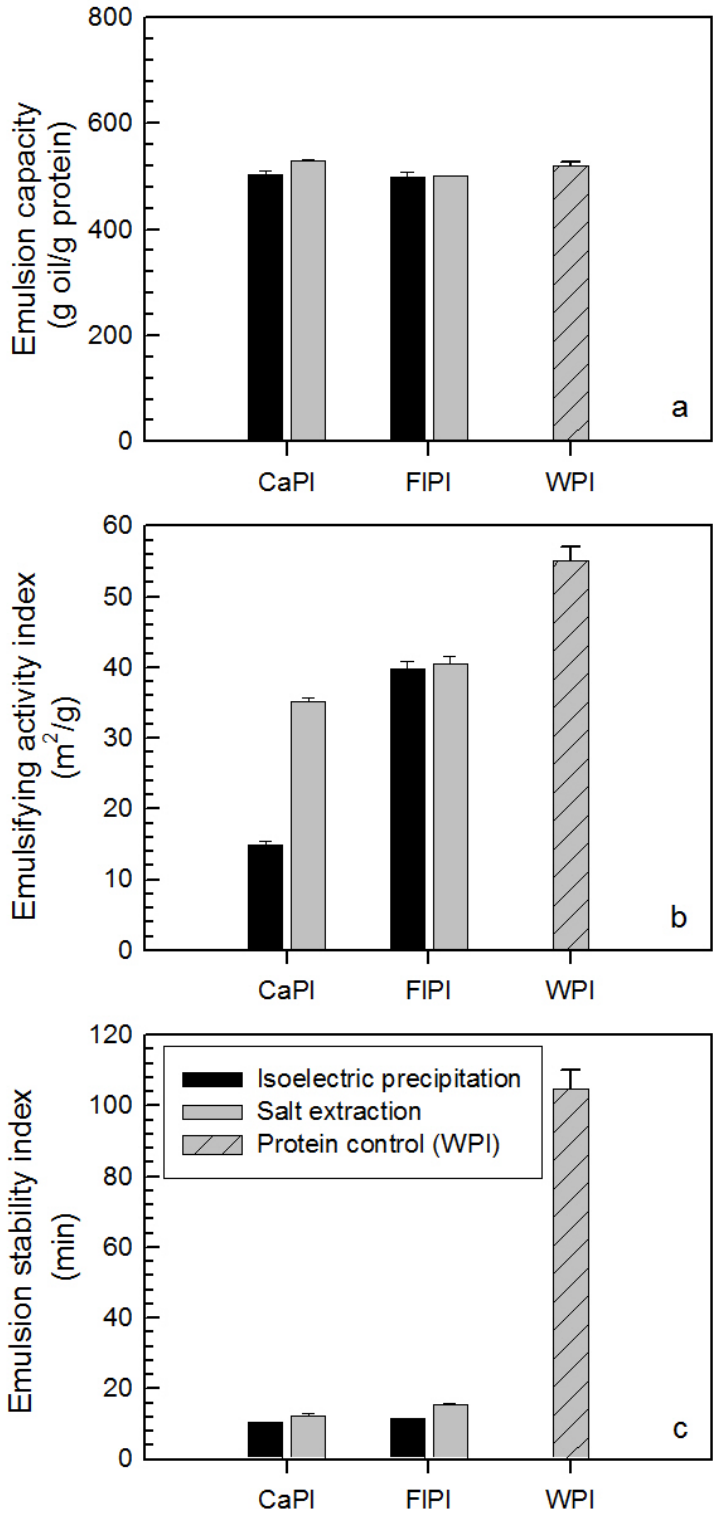

Figure 4.3 Emulsifying properties of protein isolates (0.25%, w/w; pH 7.0) of canola (CaPI) and flaxseed (FlPI) prepared by isoelectric precipitation or salt extraction and whey protein isolate (WPI): (a) emulsion capacity (g oil/g protein), (b) emulsifying activity index (m^2/g), and (c) emulsion stability index (min). Data represent the mean ± one standard deviation (n = 3).

The emulsifying activity index (EAI) is a measure of available interfacial area that can be stabilized per unit amount of protein and is estimated from the turbidity of a diluted emulsion (Pearce and Kinsella, 1978). Emulsion stability index (ESI) provides a measure of the stability of the same diluted emulsion over a defined time period (Yust et al., 2010). An individual degree of freedom contrast analysis indicated that all the main contrasts, and interaction between oilseed protein source and isolate production method were highly significant ($p<0.001$). Overall, WPI showed significantly higher EAI (55.0 m^2/g) compared to oilseed protein isolates (~32.6 m^2/g, $p<0.001$), and EAI values for FlPI were significantly higher (~40.1 m^2/g) than CaPI (~25.1 m^2/g, $p<0.001$). However, the magnitude of EAI values for CaPI and FlPI were dependent upon the extraction method used. EAI values for CaPI produced by salt extraction (35.1 m^2/g) were higher than those produced by isoelectric precipitation (15.0 m^2/g), however for FlPI, the mean EAI value was similar regardless of the method of isolate production (39.7-40.5 m^2/g). Aluko and McIntosh (2001) found that EAI of calcium-precipitated CaPI (81.9 m^2/g) was significantly higher than that of an acid-precipitated CaPI (25.1 m^2/g). Krause et al. (2002) also reported higher EAI values for FlPI prepared by micellization compared to those produced by isoelectric precipitation. A predictive multiple regression model of EAI in the present study indicated inclusion of the following factors: surface charge, surface hydrophobicity and solubility (Table 4.3). The model was able to explain 98.7% of the variation found in the data ($F = 194.129$; $p<0.001$; Table 4.3). In general, EAI increased with increasing surface charge, solubility and decreasing surface hydrophobicity. The high EAI values observed for FlPI compared to CaPI could be explained by its relatively higher surface charge, solubility, and lower surface hydrophobicity ($p<0.001$). The model identifies the importance of the balance between hydrophilic and hydrophobic interactions for good emulsifying properties.

In terms of emulsion stability (ESI), an individual degree of freedom contrast analysis revealed that only one of the main contrasts was significant: oilseed protein source vs. WPI ($p<0.001$). ESI values measured for oilseed protein isolates (~10.5-15.5 min) were significantly lower than that of WPI (104.7 min) suggesting that although emulsion formation was similar to WPI; stability was much less under the conditions tested (e.g., 0.25% w/w protein; 50% w/w oil in the emulsion). A multiple regression predictive model was devised, identifying significant factors such as solubility, surface charge and surface hydrophobicity; explaining 98.3% of the variability in the data ($F = 158.460$; $p<0.001$; Table 4.3). Wang et al. (2010a) compared the emulsifying properties of flaxseed protein concentrate with soy protein concentrate and reported that flaxseed protein showed lower EAI and ESI than soy protein.

4.4.5 Creaming stability and droplet size

Creaming is one of the most common instability mechanisms in emulsions that lead to macroscopic phase separation into cream and serum layers (Dickonson, 1997). The creaming stability of emulsions containing 1.0% protein (w/w) and 20% flaxseed oil (w/w) are presented in Figure 4.4. The creaming stability of emulsions stabilized by FlPI produced with either method, and isoelectric-precipitated CaPI ranged between 86.1 to 96.6%, which was comparable to WPI-stabilized emulsions (90.8%, $p>0.05$). Emulsion stability is thought to be related to the formation of a viscoelastic film around the oil droplets, as well as the continuous phase viscosity. Rapid separation of an aqueous layer was observed in emulsions stabilized by salt-extracted CaPI preventing determination of a CS value. Lucassen-Reynders (1996) found that fast drainage occured in emulsions when the emulsifier failed to cover the oil-water interface thoroughly, resulting in a completely mobile continuous phase that drains through the oil droplets. A multiple regression predictive model for creaming stability including solubility, surface charge and droplet size accounted for 98.6% of the data variability ($F = 119.433$; $p<0.001$; Table 4.3). The mean droplet diameter of emulsions used for creaming is shown in Figure 4.5. An individual degree of freedom contrast analysis found that all main contrasts and interaction between oilseed protein source and production method were highly significant ($p<0.001$). Overall, WPI formed emulsions with significantly smaller droplets (1.6 μm) compared to emulsions formed with protein isolates from oilseeds (~13.2 μm, $p<0.001$). Furthermore, mean droplet diameter for FlPI-stabilized emulsions (~11.7 μm) were overall smaller than that of CaPI-stabilized emulsions (~14.8 μm, $p<0.001$). For salt-extracted isolates, CaPI formed larger droplets than FlPI, whereas the reverse trend was apparent for isoelectric-precipitated isolates (Figure 4.5). All emulsions were polydisperse, with a bimodal droplet size distribution (Figures 4.6 & 4.7). Similar bimodal size distributions were previously reported for emulsions stabilized with flaxseed (Wang et al., 2010b) and canola protein isolates (Wu and Muir, 2008).

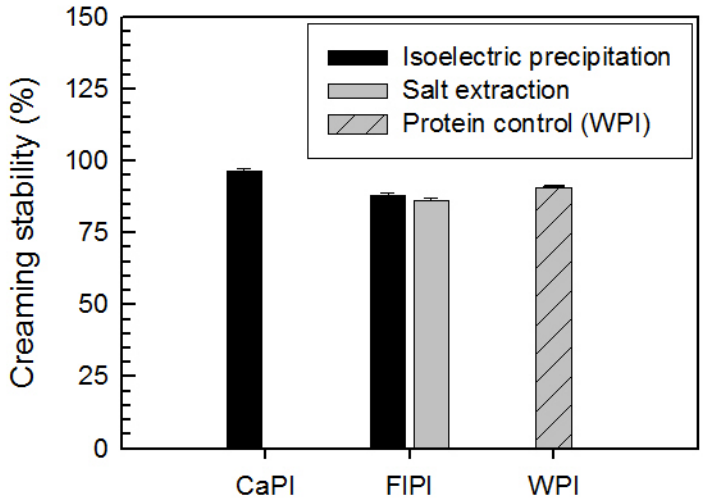

Figure 4.4 Creaming stability (%) of protein stabilized emulsions prepared at a 20:80 oil-to-water ratio with flaxseed oil, using protein isolates (1.00%, w/w) of canola (CaPI) and flaxseed (FlPI) prepared by isoelectric precipitation or salt extraction and whey protein isolate (WPI). Data represent the mean ± one standard deviation (n = 3).

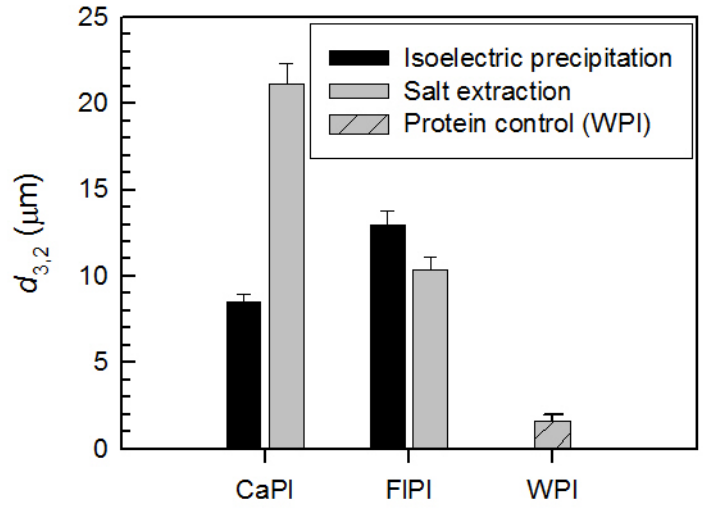

Figure 4.5 Mean droplet diameter (d_{32}) of protein stabilized emulsions prepared at a 20:80 oil-to-water ratio with flaxseed oil, using protein isolates (1.00%, w/w) of canola (CaPI) and flaxseed (FlPI) prepared by isoelectric precipitation or salt extraction and whey protein isolate (WPI). Data represent the mean ± one standard deviation (n = 3).

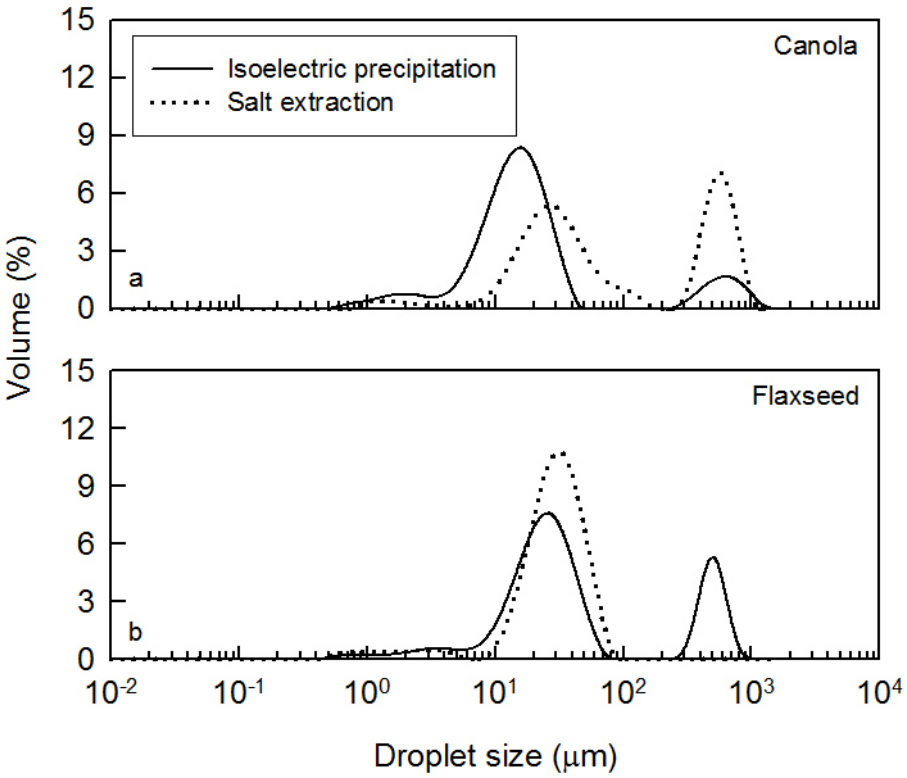

Figure 4.6 Droplet size distribution of protein stabilized emulsions prepared at a 20:80 oil-to-water ratio with flaxseed oil, using protein isolates (1.00%, w/w) of (a) canola (CaPI) and (b) flaxseed (FlPI) prepared by isoelectric precipitation or salt extraction. Data represent the mean ± one standard deviation (n = 3).

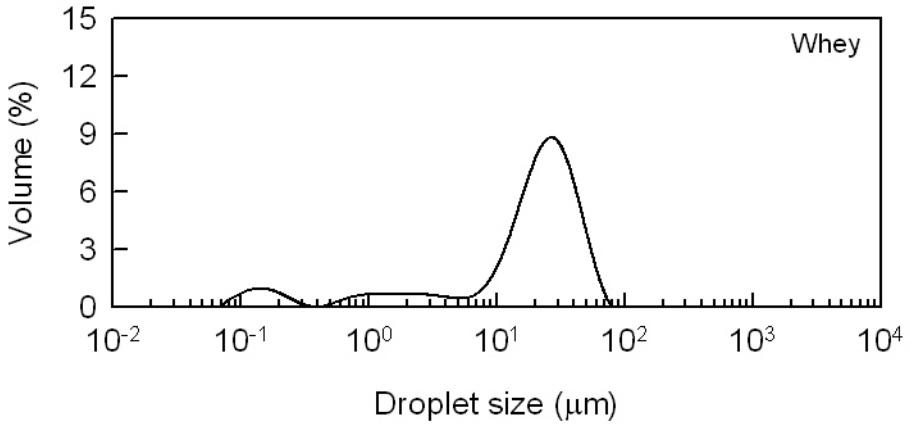

Figure 4.7 Droplet size distribution of whey protein isolate (WPI)-stabilized (1.00%, w/w) emulsions prepared at a 20:80 oil-to-water ratio with flaxseed oil. Data represent the mean ± one standard deviation (n = 3).

4.5 Conclusions

The method of production influenced both the physicochemical and emulsifying properties of canola and flaxseed protein isolates. Salt-extracted isolates were found to have higher solubility and interfacial activity compared to those produced by isoelectric precipitation. Multiple regression analyses indicated that emulsion forming ability (EC and EAI) was related to isolate solubility, surface characteristics and ability to decrease interfacial tension, while emulsion stability was a function of solubility, surface characteristics and droplet size.

4.6 Linkage

Both protein source and method of production were found to have significant effects on the physicochemical and emulsifying properties of the produced canola and flaxseed protein isolates. The findings of this study suggested that CaPI and FlPI have oil-in-water emulsion forming properties; however their stability was low when compared to WPI. Based the poor emulsion stabilizing properties of oilseed proteins (Chapter 4) and the good emulsifying properties of chickpea and lentil protein isolates produced by isoelectric precipitation (Chapter 3), only the latter was moved forward in this research in terms of optimization of the emulsion formulation and for encapsulation.

5. LENTIL AND CHICKPEA PROTEIN-STABILIZED EMULSIONS: OPTIMIZATION OF EMULSION FORMULATION[3]

5.1 Abstract

Chickpea and lentil protein-stabilized emulsions were optimized based on pH (3.0-8.0), protein concentration (1.1-4.1% w/w) and oil content (20-40%) for their ability to form and stabilize oil-in-water emulsions using response surface methodology. Specifically, creaming stability, droplet size and droplet charge were accessed. Optimum conditions for minimal creaming (no serum separation after 24 h), small droplet size (<2 μm), and high net droplet charge (absolute value of ZP >40 mV) were identified as: 4.1% protein, 40% oil, and pH 3.0 or 8.0, regardless of the plant protein used for emulsion preparation.

5.2 Introduction

The emulsifying properties of food proteins play an important role in the food industry for controlling food quality and texture. An emulsion is defined as a dispersion of two immiscible liquids in which one is dispersed in the other as small droplets (0.1-100 μm) (McClements, 2005c). Emulsion stability is the ability of an emulsion to resist changes in its nature over time. These changes can be either physical or chemical in nature, such that an alteration in the distribution or organization of molecules occurs (i.e. creaming, flocculation, coalescence). Key factors in controlling emulsion stability include, droplet size, use of stabilizers, and environmental conditions such as temperature, pH, and ionic strength (McClements, 2005b). The amphiphilic structure of proteins (i.e. possessing both hydrophobic and hydrophilic components) allows them to be used as emulsifiers. The role of plant proteins as emulsifiers in food and bioproduct systems is less well understood compared to the more widely used animal proteins. Chickpea and lentil proteins appear to be a promising source for producing substitutes for animal-based proteins in new product formulations because of their nutritional value, continuum of production, low cost and possible beneficial health effects (Duranti, 2006; Boye et al., 2010b).

[3] Reproduced with permission. Can Karaca, A., Nickerson, M. T. and Low, N. H. 2011. Lentil and chickpea protein-stabilized emulsions: Optimization of emulsion formulation. Journal of Agricultural and Food Chemistry, 59, 13203–13211. Copyright (2011) American Chemical Society.

Response surface methodology (RSM) consists of a group of mathematical and statistical techniques used in the modeling and analysis of situations in which a response is affected by several variables, alone or in combination (Fomuso et al., 2011). One of the main advantages of RSM is enabling the prediction of behaviour of different parameters under a given set of conditions with a reduced number of experiments. Granato et al. (2010) used RSM to optimize the sensory attributes of a soy protein-pink guava juice dessert over a wide range of juice (22-32%) and protein (1-3%) concentrations. Liu and Yang (2011) optimized the formulation of an emulsion containing evening primrose oil employing gum Arabic, maltodextrin and sodium caseinate. To our knowledge, studies using RSM to determine the optimum conditions for non soy-based plant protein-stabilized emulsions are lacking. The main goal of this study was to determine the optimum formulation for chickpea and lentil protein-stabilized oil-in-water emulsions and to better understand mechanisms of instability as emulsion formulations change.

5.3 Material and Methods

5.3.1 Materials

Chickpea (CDC Frontier, Kabuli) and lentil (CDC Grandora) were provided by the Crop Development Centre at the University of Saskatchewan (Saskatoon, SK, Canada). Flaxseed oil was also kindly donated by Bioriginal Food & Science Corp. (Saskatoon, SK, Canada, 2010). All chemicals used were of reagent grade and purchased from Sigma-Aldrich (Oakville, ON, Canada). The water used in this research was product from a Millipore Milli-Q^{TM} water system (Millipore Corp., Milford, MA, USA).

5.3.2 Proximate analysis

Whole chickpea and lentil seeds were ground into a fine flour using a coffee grinder for 1 min, and then defatted using hexane (1:3 [w/v] flour:hexane ratio) for 40 min. The mixture was then filtered employing Whatman Gr. 1 paper (110 mm; Whatman International Ltd., Maidstone, United Kingdom), and air-dried in a fume hood. This defatting procedure was repeated twice for each flour. Proximate composition analyses were performed on the produced defatted material according to AOAC Official Methods 925.10 (moisture), 923.03 (ash), 920.85 (lipid), and 920.87 (crude protein by using %N × 6.25) (AOAC, 2003). Carbohydrate content was determined on the basis of percent differential from 100%.

5.3.3 Preparation of protein isolates

Chickpea protein isolate (ChPI) was prepared according to the method of Papalamprou et al. (2010). In brief, defatted flour (100 g) was mixed with water at 1:10 ratio (w/v), adjusted to pH 9.0 using 1.0 M NaOH and stirred at 500 rpm for 45 min at room temperature (20-22°C). The suspension was then centrifuged at 4,500 × *g* for 20 min at 4°C using a Sorvall RC-6 Plus centrifuge (Thermo Scientific, Asheville, NC, USA) to collect the supernatant. The resulting pellet was re-suspended in water at a ratio of 1:5 (w/v), adjusted to pH 9.0, stirred for an additional 45 min, followed by centrifugation (4,500 × *g*, 20 min, 4°C). Supernatants were pooled and adjusted to pH 4.6 using 0.1 M HCl to precipitate the protein. The protein was recovered by centrifugation as above, collected and stored at −30°C until freeze-drying which was performed using a Labconco FreeZone 6 freeze drier (Labconco Corp., Kansas City, MO, USA) to yield a free flowing powder. Proximate analysis of ChPI showed a composition of, 85.40% protein, 6.52% moisture, 3.05% ash, 4.11% carbohydrate and 0.92% lipid.

Lentil protein isolate (LPI) was produced employing the combined methods of Bamdad et al. (2006) and Lee et al. (2007). Defatted flour (100 g) was mixed with water at 1:10 ratio (w/v), adjusted to pH 9.5 with 1.0 M NaOH, and stirred at 500 rpm for 1 h at room temperature. The mixture was kept static at 4°C overnight to allow for non-protein sedimentation. After centrifugation at 1,600 × *g* for 30 min at 4°C, the supernatant was collected; and the pH was adjusted to 4.5 with 0.1 M HCl. The precipitated protein was collected by centrifugation (1,600 × *g*, 30 min, 4°C) and stored at −30°C until freeze-drying. Proximate analysis of LPI showed a composition of, 81.90% protein, 5.04% moisture, 3.63% ash, 9.00% carbohydrate and 0.43% lipid.

5.3.4 Percent protein solubility

Percent protein solubility was determined using the method of Morr et al. (1985). Protein solutions were prepared by dispersing 0.2 g of sample in 19.8 mL (1.0%, w/v) of water and were adjusted to the following pHs, 1.3, 3.0, 5.5, 8.0 and 9.7 with either 0.1 M NaOH or 0.1 M HCl followed by stirring at 500 rpm overnight at 4°C. Solutions were centrifuged at 9,100 × *g* for 10 min at room temperature to remove insoluble residues. Nitrogen content in the supernatant was determined using a micro-Kjeldahl digestion and distillation unit (Labconco Corp., Kansas City, MO, USA). Percent protein solubility was calculated by dividing the nitrogen content in the supernatant by the total nitrogen in the sample (×100%).

5.3.5 Preparation of emulsions

Prior to the homogenization, the pH of the protein solutions was adjusted to the following pHs, 1.3, 3.0, 5.5, 8.0 and 9.7 as described above. Oil-in-water emulsions were prepared by homogenizing varying amounts (4.25-6.95 g) of 0.11-7.32% (w/w) protein solutions with differing amounts (1.05-3.75 g) of flaxseed oil in 15 mL plastic centrifuge tubes employing a Polytron PT 2100 Homogenizer (Kinematica AG, Lucerne, Switzerland) with a 12 mm PT-DA 2112/2EC generating probe at 13,000 rpm for 3 min. The ranges for protein and oil concentration were 1.1-4.1% and 20.0-40.0% on a w/w basis, respectively (Table 5.1).

Table 5.1 Central composite rotatable design arrangement with coded and decoded levels of factors.

Standard	***Protein %***		***Oil %***		***pH***	
order	*Coded*	*Decoded*	*Coded*	*Decoded*	*Coded*	*Decoded*
1	−1	1.10	−1	20.0	−1	3.0
2	+1	4.10	−1	20.0	−1	3.0
3	−1	1.10	+1	40.0	−1	3.0
4	+1	4.10	+1	40.0	−1	3.0
5	−1	1.10	−1	20.0	+1	8.0
6	+1	4.10	−1	20.0	+1	8.0
7	−1	1.10	+1	40.0	+1	8.0
8	+1	4.10	+1	40.0	+1	8.0
9	−1.682	0.08	0	30.0	0	5.5
10	+1.682	5.12	0	30.0	0	5.5
11	0	2.60	−1.682	13.2	0	5.5
12	0	2.60	+1.682	46.8	0	5.5
13	0	2.60	0	30.0	−1.682	1.3
14	0	2.60	0	30.0	+1.682	9.7
15	0	2.60	0	30.0	0	5.5
16	0	2.60	0	30.0	0	5.5
17	0	2.60	0	30.0	0	5.5

5.3.6 Electrophoretic mobility

The average surface charge of oil droplets or protein isolates was determined by measuring electrophoretic mobility (U_E) of droplets using a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA). U_E was used to calculate the zeta potential *(ζ)* employing the Henry's equation:

$$U_E = \frac{2\varepsilon \cdot \zeta \cdot f(\kappa\alpha)}{3\eta} \qquad \text{[eq. 5.1]}$$

where ε is the permittivity, *f(κα)* is a function related to the ratio of particle radius (α) and the Debye length (κ), and η is the dispersion viscosity. For this study, the Smoluchowski approximation *f(κα)* equalled 1.5. In all cases, emulsions were diluted to a droplet concentration of 0.005% oil (v/v) using water, and then pH adjusted to the specific pH.

5.3.7 Creaming index

Emulsion samples (8 mL) were transferred into 10 mL sealed graduated glass cylinders (inner diameter = 10.5 mm; height = 160 mm; as measured by a digital calliper), and then stored for 24 h at room temperature. During storage appreciable emulsion separation into an optically opaque cream layer at the top and a turbid serum layer at the bottom could be viewed visually, and by measuring the total height of the emulsion (H_E) and the height of the serum layer (H_S). Creaming index (CI) was expressed as:

$$CI(\%) = \frac{H_S}{H_E} \cdot 100 \qquad \text{[eq. 5.2]}$$

where H_S is the height of the serum layer and H_E is the total height of the emulsion (McClements, 2007).

5.3.8 Droplet size

Emulsion droplet size distribution was measured using a Mastersizer 2000 laser light scattering instrument (Malvern Instruments Ltd., Worcestershire, United Kingdom) equipped with a Hydro 2000S sample handling unit (containing water). Emulsion samples were taken immediately after homogenization from the bottom of the tube. This sample was stirred continuously within the sample cell to ensure homogeneity at room temperature. Obscuration in all the measurements was kept at ~14% by water addition. Droplet size distributions were calculated by the instrument according to the Mie Theory which uses the refractive index difference between the droplets and the dispersing medium to predict the intensity of the

scattered light. The ratio of refractive index of flax seed oil (1.479) to that of the dispersion medium (1.330) was 1.112. Droplet size measurements were reported as volume-surface mean diameters ($d_{3,2}$), which is expressed as:

$$d_{3,2} = \frac{\sum_{i=1} n_i \cdot d_i^3}{\sum_{i=1} n_i \cdot d_i^2} \qquad \text{[eq. 5.3]}$$

where n_i is the number of droplets of diameter (d_i) (McClements, 2005c).

5.3.9 Emulsion morphology

A Nikon Eclipse E400 light microscope equipped with a Nikon DS-FiL color camera and a long working distance 10× len and condenser (Nikon Instruments Inc., Melville, NY, USA) were used to acquire bright field micrographs. The image resolution was 2560 by 1920 pixels.

5.3.10 Experimental design

A central composite rotatable design was used for the RSM studies, and 17 experimental settings were generated with three factors (Montgomery, 1997). The ranges of settings for the variable factors were chosen based on preliminary experiments and similar studies from literature (Akintayo et al., 1998; Papalamprou et al., 2005; Gharsallaoui et al., 2009; Aluko et al., 2009) and were as follows: 1.1-4.1% for protein concentration, 20-40% for oil concentration, and 3.0-8.0 for pH. Conditions for each experimental setting, coded and decoded, are shown in Table 5.1. Experiments were carried out in a randomized order in triplicate and reported as the mean ± one standard deviation. Data were fitted to a second-order polynomial model:

$$Y = \beta_0 + \sum_{i=1}^{a} \beta_i \cdot X_i + \sum_{i=1}^{a} \beta_{ii} \cdot X_i^2 + \sum_{i,j=1(i \neq j)}^{a} \beta_{ij} \cdot X_i \cdot X_j \qquad \text{[eq. 5.4]}$$

where Y is the response value predicted by the model (mean droplet charge, creaming index or mean droplet diameter), β_0 is the constant coefficient, β_i is the coefficient of the linear effect, β_{ii} is the coefficient of the quadratic effect, β_{ij} is the coefficient of the interaction effect, and X_i and X_j are the independent variables i and j, respectively. Experimental design, data analysis and contour plots were performed with Statistica 9 software (StatSoft, Inc., Tulsa, OK, USA). Optimization of the emulsion formulation in terms of pH, protein and oil concentration was achieved by an evaluation of the contour plots. Student's t-test for independent samples was

applied to determine the statistical significance of differences in the solubility of protein isolates using SPSS software (SPSS Inc., Ver. 17, 2008, Chicago, IL).

5.4 Results and Discussion

5.4.1 Protein solubility

High solubility is required in order for a protein to be an effective emulsifier. This important physical property is required as the protein must be able to readily migrate to the oil/water interface (Sikorski, 2001). Percent solubility of ChPI and LPI at the pH values used in the emulsion preparations in this study are shown in Figure 5.1. The typical U-shaped protein solubility profile was observed for both proteins and ChPI and LPI were found to be highly soluble (>80%) at acidic (pH<3.0) and basic pH values (pH>8.0). These results are supported by the pI values for these proteins of 4.49 (ChPI) and 4.56 (LPI) (Figure 5.2), where a net charge is generated as the pH of the medium moves away from these pH values. With the introduction of a net charge on the protein, there is a) a concomitant increase in protein-water interactions due to the hydration of charged moieties on the protein surface; and b) an increase in electrostatic repulsive forces between neighboring proteins in solution. Each of these factors will promote protein solubility in an aqueous environment. Boye et al. (2010b) reported high (80-90%) solubility values for chickpea and lentil protein isolates at pHs ranging from 1.0 to 3.0 and 7.0 to 10.0. Among the pH values tested in this study, the lowest protein solubility (4.22%) was observed at pH 5.5 for both ChPI and LPI. The maximum solubilities for ChPI (97.92%) and LPI (99.93%) were found at pH values of 8.0 and 9.7, respectively. In general, protein solubilities increased as pH values moved away from their pIs. Experimental results showed that ChPI solubility was significantly ($p<0.05$) higher than that observed for LPI at basic pH, whereas LPI solubility was significantly ($p<0.05$) higher than ChPI at acidic pH (Figure 5.1). It is postulated that these solubility differences as influenced by pH are due to the exposed surface amino acid composition of these protein isolates with higher levels of carboxyl groups in LPI, and amino groups in ChPI.

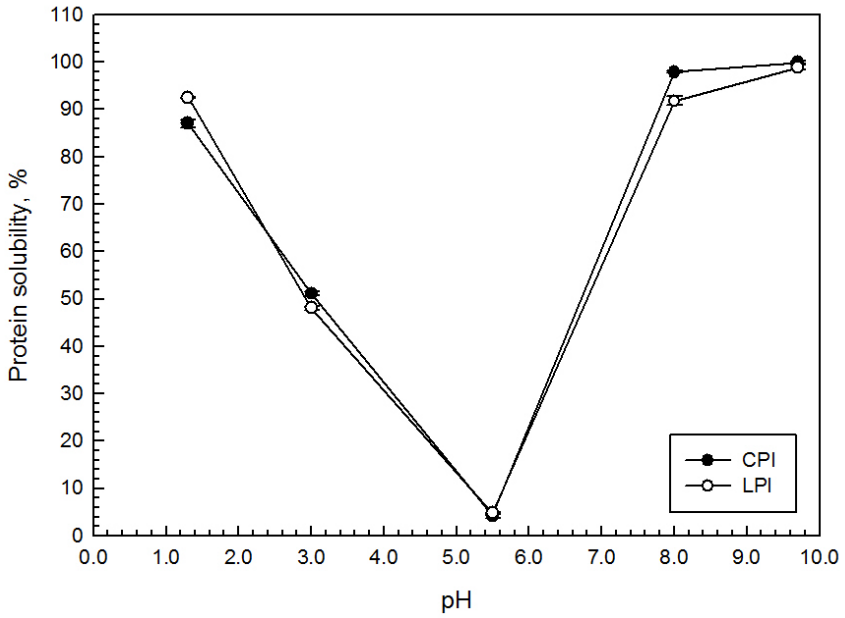

Figure 5.1 Percent protein solubility as a function of pH for ChPI and LPI (data represent mean values (n = 3) ± one standard deviation).

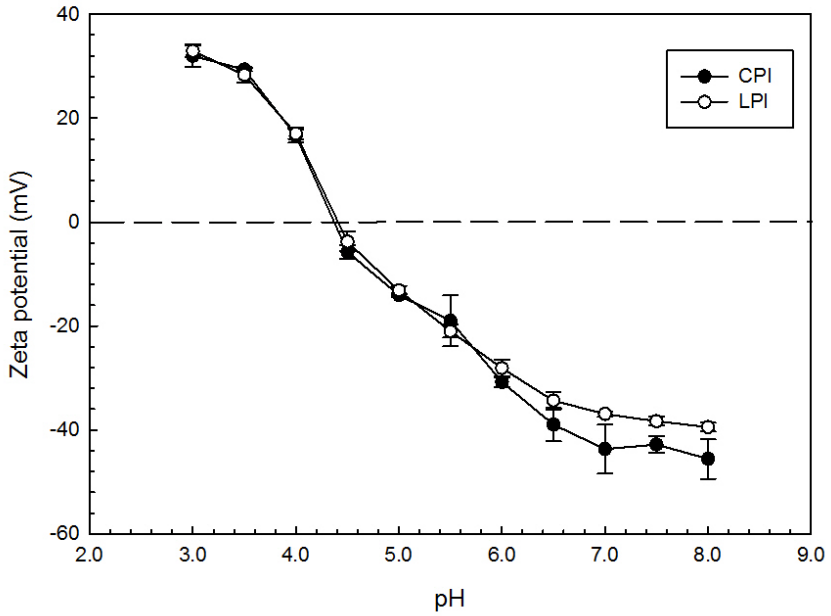

Figure 5.2 Zeta potential (mV) values for ChPI and LPI as a function of pH (data represent mean values (n = 3) ± one standard deviation).

5.4.2 Droplet charge

In an emulsion, the interfacial membranes formed by proteins induce a droplet surface charge which inhibits droplet aggregation through electrostatic repulsive forces. The mean electrical charge (zeta potential, mV) on droplets of ChPI- and LPI-stabilized emulsions are presented in Table 5.2. Predictive models for estimating droplet charge identified pH as the only significant factor ($p<0.05$, Tables 5.3 & 5.4). These models accounted for 88.3% and 85.0% of the variation found in the data for ChPI- and LPI-stabilized emulsions, respectively. Contour plots for mean oil droplet charge are shown in Figure 5.3. The zeta potential of the droplets was positive at pH 3.0 (>+30 mV), became less positive with increasing pH until it reached zero (pH 4.5-5.0), and then became increasingly negative as the pH increased; reaching a maximum of −53.3 mV at pH 9.7. Gharsallaoui et al. (2009) found that the zeta potential of droplets in a pea protein-stabilized emulsion was highly positive at pH 2.4 (+30.4 mV), reached zero at around pH 4.3 and reached a maximum value of −59.3 mV at pH 8.0. The zeta potential-pH dependence of ChPI- and LPI-stabilized emulsions is attributed to the electrical characteristics of the adsorbed chickpea and lentil protein molecules, which have isoelectric points of 4.49 and 4.56, respectively (Wagner and Gueguen, 1999).

Table 5.2 Measured responses for each run for chickpea and lentil protein-stabilized emulsions. Data represent the mean ± one standard deviation (n = 3). See Table 5.1 for formulations corresponding to standard order numbering.

Std.	***Creaming Index (%)***		***Droplet Size (μm)***		***Droplet Charge (mV)***	
order	*Chickpea*	*Lentil*	*Chickpea*	*Lentil*	*Chickpea*	*Lentil*
1	69.08 ± 0.97	69.01 ± 0.14	1.9 ± 0.5	1.7 ± 0.3	39.7 ± 0.9	40.4 ± 2.5
2	22.48 ± 1.68	64.42 ± 1.83	1.7 ± 0.8	1.8 ± 0.2	42.1 ± 0.9	42.1 ± 1.0
3	41.28 ± 0.44	41.14 ± 0.55	8.9 ± 0.2	9.2 ± 0.4	40.6 ± 1.5	42.2 ± 2.6
4	0.00 ± 0.00	0.00 ± 0.00	8.1 ± 0.1	9.5 ± 0.2	41.8 ± 0.3	41.8 ± 1.0
5	68.66 ± 0.58	72.47 ± 2.50	1.5 ± 0.4	1.8 ± 0.3	−51.4 ± 0.8	−50.4 ± 1.0
6	69.18 ± 0.84	70.92 ± 0.46	1.7 ± 0.1	1.2 ± 0.2	−50.6 ± 0.9	−49.3 ± 2.3
7	39.91 ± 0.40	44.24 ± 1.09	7.6 ± 0. 8	8.2 ± 0.2	−47.7 ± 1.1	−48.1 ± 0.8
8	0.00 ± 0.00	2.86 ± 0.04	1.4 ± 0.5	1.2 ± 0.2	−49.2 ± 1.3	−49.4 ± 0.8
9	-[1]	-	-	-	-	-
10	19.20 ± 3.18	17.19 ± 2.06	1.8 ± 0.0	1.3 ± 0.2	−29.3 ± 1.5	−29.5 ± 0.8
11	51.70 ± 1.50	63.43 ± 0.44	9.6 ± 0.5	11.9 ± 0.2	−26.9 ± 0.5	−30.6 ± 2.0
12	1.32 ± 2.28	15.13 ± 1.21	24.1 ± 0.5	21.7 ± 1.2	−28.0 ± 0.8	−29.4 ± 1.9
13	35.78 ± 2.50	47.83 ± 0.74	9.2 ± 0.2	9.1 ± 0.3	23.9 ± 0.3	19.0 ± 1.4
14	61.05 ± 3.04	62.90 ± 1.81	1.6 ± 0.1	1.4 ± 0.4	−56.8 ± 2.0	−49.7 ± 1.8
15	24.75 ± 1.59	24.27 ± 1.59	11.7 ± 2.3	11.6 ± 0.4	−24.3 ± 1.4	−26.2 ± 0.4
16	25.43 ± 0.37	22.79 ± 0.43	12.1 ± 0.7	9.3 ± 0.3	−23.7 ± 0.6	−24.8 ± 0.3
17	24.88 ± 1.12	20.39 ± 1.13	11.7 ± 2.9	10.2 ± 1.1	−24.9 ± 0.7	−26.1 ± 1.1

[1] No measurements could be made for the 9th run as the sample separated into two phases immediately after homogenization.

Table 5.3 Predictive models for estimating creaming index, droplet size and droplet charge for chickpea protein-stabilized emulsions.

Dependent variable	Independent variable	Coefficient	p-value	Model fit
(a) Creaming Index				
	Protein	−25.2230	p<0.001	R^2 = 0.963
	Protein^2	2.8439	NS	F = 17.105
	Oil	0.2879	p<0.001	p = 0.001
	Oil^2	0.0013	NS	
	pH	−8.3506	p<0.05	
	pH^2	1.2596	p<0.05	
	Protein × Oil	−0.2926	NS	
	Protein × pH	1.6162	NS	
	Oil × pH	−0.2383	NS	
	Constant	108.5375	p<0.05	
(b) Droplet Size				
	Protein	13.6530	NS	R^2 = 0.891
	Protein^2	−2.1281	p<0.05	F = 5.428
	Oil	0.0379	p<0.05	p = 0.026
	Oil^2	0.0107	NS	
	pH	6.1959	NS	
	pH^2	−0.4774	p<0.05	
	Protein × Oil	−0.0588	NS	
	Protein × pH	−0.1659	NS	
	Oil × pH	−0.0379	NS	
	Constant	−26.2415	NS	
(c) Droplet Charge				
	Protein	−15.5722	NS	R^2 = 0.883
	Protein^2	2.8971	NS	F = 5.051
	Oil	−1.3625	NS	p = 0.031
	Oil^2	0.0224	NS	
	pH	−25.6915	p<0.001	
	pH^2	0.9811	NS	
	Protein × Oil	−0.0286	NS	
	Protein × pH	−0.1411	NS	
	Oil × pH	0.0218	NS	
	Constant	128.5941	NS	

NS, not significant (p>0.05).

Table 5.4 Predictive models for estimating creaming index, droplet size and droplet charge for lentil protein-stabilized emulsions.

Dependent variable	Independent variable	Coefficient	p-value	Model fit
(a) Creaming Index				
	Protein	−0.6638	$p<0.05$	R^2 = 0.968
	Protein^2	2.2601	NS	F = 19.890
	Oil	−3.8595	$p<0.001$	p = 0.0008
	Oil^2	0.0608	$p<0.05$	
	pH	−19.1446	NS	
	pH^2	1.8827	$p<0.05$	
	Protein × Oil	−0.6365	$p<0.05$	
	Protein × pH	0.0934	NS	
	Oil × pH	−0.0200	NS	
	Constant	169.8918	$p<0.05$	
(b) Droplet Size				
	Protein	12.8059	NS	R^2 = 0.907
	Protein^2	−1.9043	$p<0.05$	F = 6.513
	Oil	−0.3268	$p<0.05$	p = 0.017
	Oil^2	0.0164	NS	
	pH	5.6414	NS	
	pH^2	−0.3920	$p<0.05$	
	Protein × Oil	−0.0530	NS	
	Protein × pH	−0.2649	NS	
	Oil × pH	−0.0435	NS	
	Constant	−18.8599	NS	
(c) Droplet Charge				
	Protein	−18.1951	NS	R^2 = 0.850
	Protein^2	3.3026	NS	F = 3.770
	Oil	−1.0674	NS	p = 0.060
	Oil^2	0.0197	NS	
	pH	−26.6280	$p<0.05$	
	pH^2	1.1445	NS	
	Protein × Oil	−0.0369	NS	
	Protein × pH	−0.0478	NS	
	Oil × pH	0.0042	NS	
	Constant	127.2986	NS	

NS, not significant ($p>0.05$).

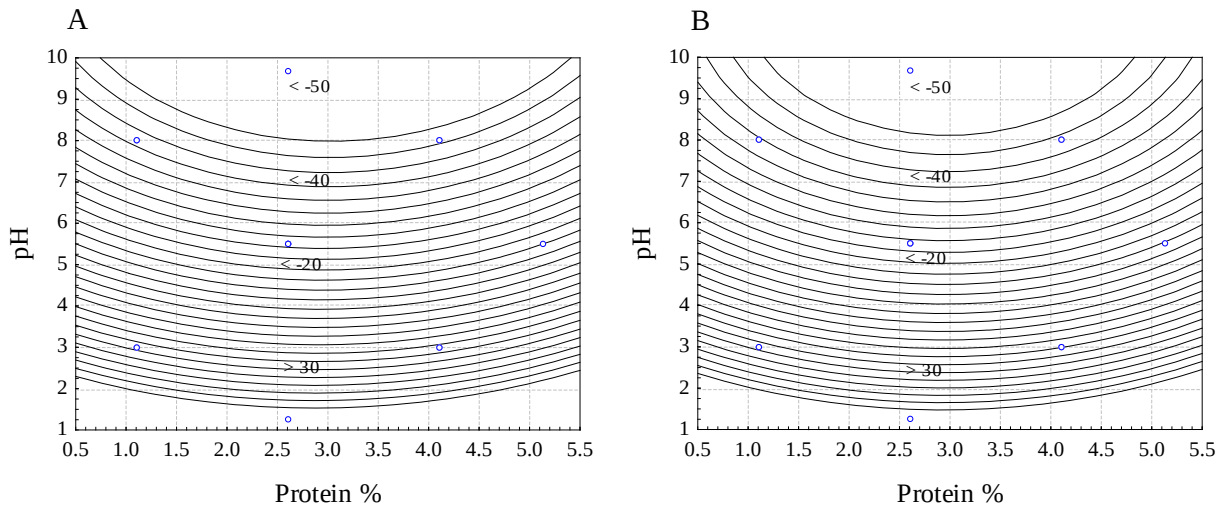

Figure 5.3 Contour plots for mean droplet charge (zeta-potential, mV) for chickpea (A) and lentil (B) protein-stabilized emulsions at 30% oil.

5.4.3 Creaming

A common emulsion instability mechanism is creaming, which leads to macroscopic phase separation into both cream and serum layers. For ChPI-stabilized emulsions, a predictive model for estimating its creaming index supported the inclusion of the following factors: protein concentration, oil content, pH and pH^2 (Table 5.3). This model was able to predict 96.3% of data variability. For LPI- stabilized emulsions, the predictive model identified the following significant factors: protein concentration, oil content and oil content^2, pH^2, and the interaction(s) between protein concentration and oil content, which predicted 96.8% of data variability (Table 5.4). Figure 5.4 shows the effect of varying protein concentration and oil content for each isolate on creaming index at pH 3.0, 5.5 and 8.0. Low creaming index values of 0-5% are indicative of low serum separation and higher emulsion stability. Experimental results showed that as the protein concentration and oil content increased to >3.5% and >35%, respectively, the degree of creaming stability of the resulting emulsion increased. Serum separation was found to increase as the protein and/or oil concentration/content decreased to <3.0% and <30%, respectively for both ChPI and LPI-stabilized emulsions at all pH values. According to Stokes' Law, emulsions with smaller droplet sizes, a lower density contrast between phases, and higher phase viscosities are more stable to creaming. By increasing the protein concentration at the oil-water interface it is possible to decrease the creaming rate as the density difference between the oil and water phases decreases (Sun and Gunasekaran, 2009). According to Dickinson and Golding (1997), as the oil content of an emulsion is increased, a concomitant increase in oil droplet packing

occurs, which increases emulsion viscosity and lowers the creaming rate. Also, emulsion stability improves as a function of increased protein concentration as the rigidity of the film surrounding the oil droplets increases (Dickinson and Golding, 1997). Finally, as discussed in the droplet size section below, as the protein concentration increased to 4.1%, the mean oil droplet size decreased, resulting in lower creaming indices (0-5%). The higher creaming index values (>25%) observed in emulsions containing lower protein concentrations (<3.0%) can be attributed to an insufficient content of emulsifier so as to cover the oil droplets, which promotes droplet flocculation/coalescence (McClements, 2005b). Makri and Doxastakis (2006) reported that creaming indices of emulsions stabilized with common bean and scarlet runner bean proteins decreased from 12-17% to 5-7% when the protein concentration was increased from 1% to 3%.

5.4.4 Droplet size

Emulsion stability is highly dependent on its droplet characteristics. An emulsion with small droplets usually has a longer shelf life than one containing larger droplets (McClements, 2005b). The volume-surface mean diameters ($d_{3,2}$) of emulsions stabilized by ChPI and LPI ranged between 1.4-24.1 μm and 1.2-21.7 μm, respectively (Table 5.2). Predictive models for estimating droplet size of both ChPI- and LPI-stabilized emulsions revealed that following factors were significant: square of protein concentration, oil content and square of pH (Tables 5.3 & 5.4). These predictive models accounted for 89.1% and 90.7% of the variation found in the data for ChPI- and LPI-stabilized emulsions, respectively. Figure 5.5 shows the effect of protein concentration and oil content on mean droplet diameter at pH 3.0, 5.5 and 8.0. Interactions between these two components appeared as saddle surfaces, where mean droplet size decreased as the protein concentration increased or decreased from around the midpoint of 2.5-3.0%, regardless of pH.

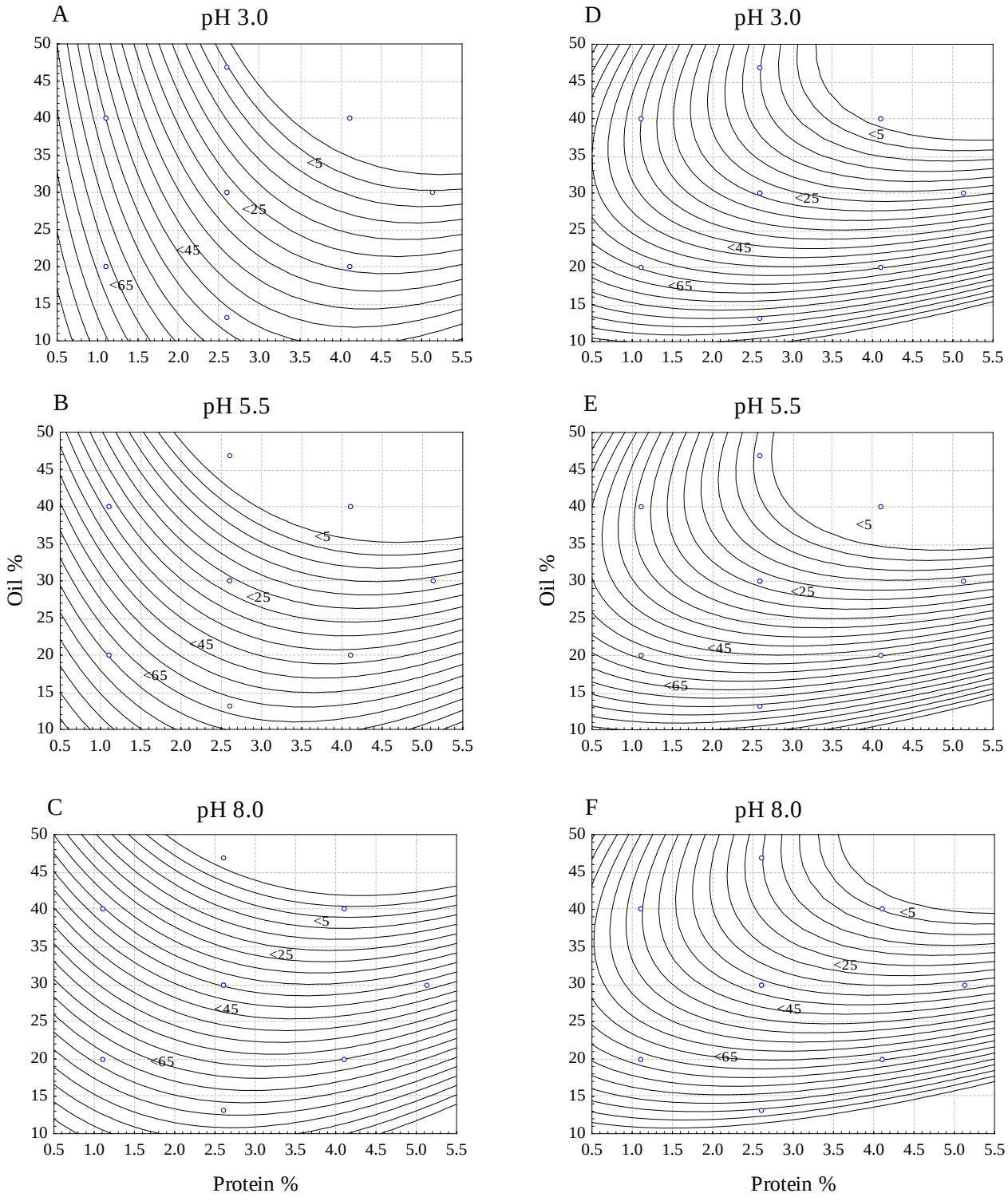

Figure 5.4 Contour plots for creaming index (%) for chickpea (A, B, and C) and lentil (D, E, and F) protein-stabilized emulsions at different pH values.

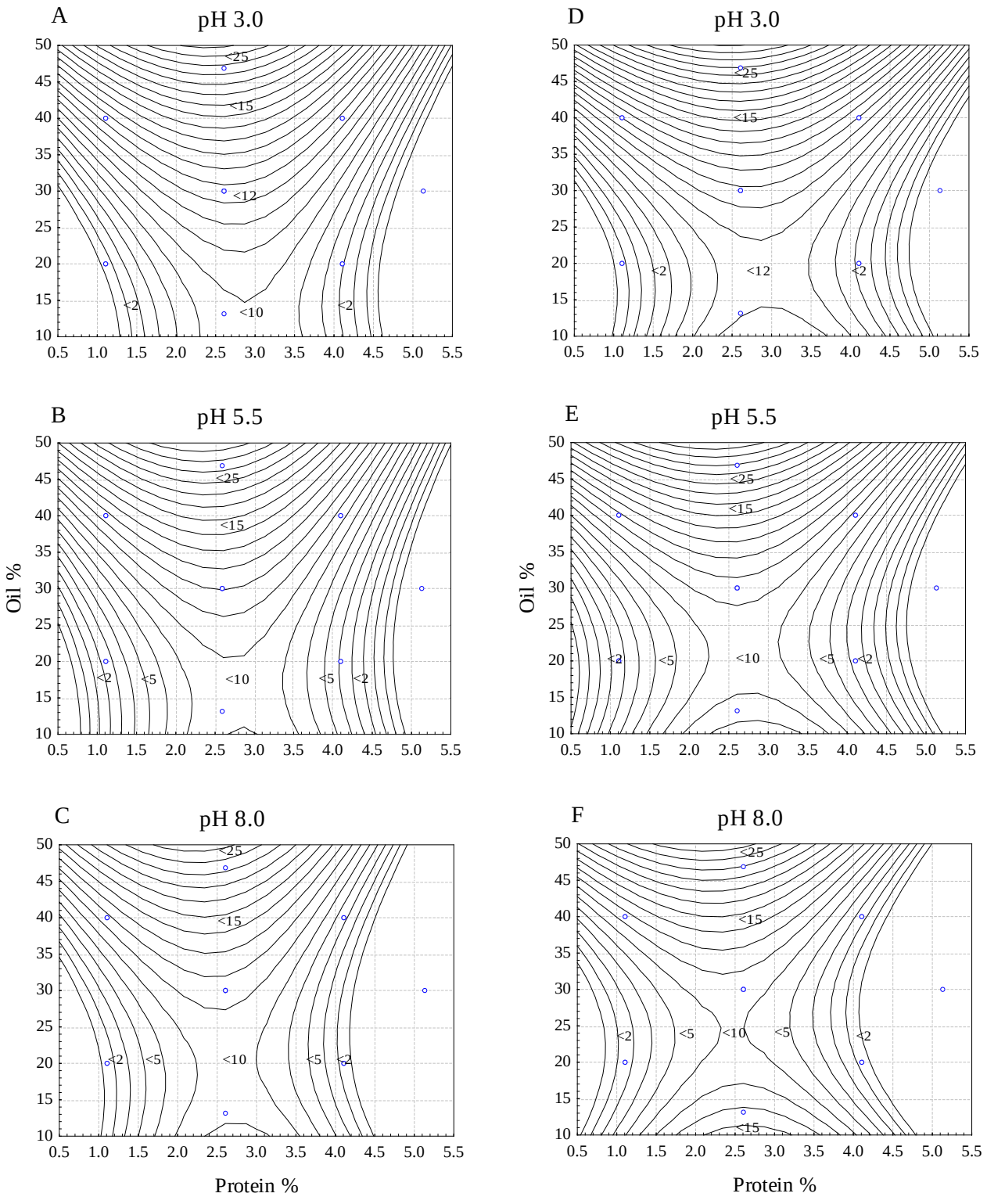

Figure 5.5 Contour plots for mean droplet diameter ($d_{3,2}$) for chickpea (A, B, and C) and lentil (D, E, and F) protein-stabilized emulsions at different pH values.

Experimental results showed that the particle size decreased (from ~25.0 μm to ~2.0 μm) as the oil content decreased from 50% to 35%, until the saddle point of the response surface (2.5-3.0% for protein concentration and 15-20% for oil content) was reached. It was observed that varying the protein concentration and oil content around this midpoint had no effect on mean droplet size. It has been reported that as the emulsifier concentration in a protein-stabilized emulsion increases, protein absorption increases on the surface of oil droplets, which prevents droplets from aggregating and results in the formation of smaller droplets (Wang et al., 2010a). At protein concentrations below or above the observed midpoint, the mean droplet size decreased with decreasing (<30%) oil concentration. Similar trends of decreased mean droplet diameter with increased protein concentration were reported by Makri and Doxastakis (2006) for emulsions stabilized with protein isolates from common bean and scarlet runner bean and by Wang et al. (2010a) for emulsions stabilized with a soybean protein concentrate. Overall, smaller droplet size distributions of 1.2-9.5 μm were observed at pH 3.0 and 8.0 compared to 1.3-24.1 μm for pH 5.5 for both ChPI- and LPI-stabilized emulsions. These findings suggests that ChPI and LPI are more effective at producing small droplets during the homogenization step of emulsion formation at pH 3.0 and 8.0 than at pH 5.5 as they possess a net charge and become more soluble at pH values significantly removed from their isoelectric points. Emulsion droplets tend to flocculate immediately after homogenization at pH values close to the pI of the protein due to the lack of significant electrostatic repulsion between the absorbed proteins on the interfacial film (Dickinson et al., 1988). Zhang et al. (2009) also found that an oil-in-water emulsion produced with a chickpea protein isolate produced smaller droplet sizes at pH 3.0, 7.0 and 9.0 compared to pH 5.0.

5.4.5 Optimization

A numerical optimization procedure was carried out to determine the optimum set of independent variables leading to the following desired emulsion characteristics: 0% creaming (no serum separation after 24 h), low droplet size (<2 μm), and high net droplet charge (absolute value of ZP >40 mV). From the conducted RSM experiments, the overall optimum region was achieved by a combined level of 4.1% protein, 40% oil, and pH 3.0 or 8.0 for both ChPI- and LPI-stabilized emulsions. However, the optimum values obtained by RSM are case sensitive and may not apply if the oil droplet size distribution is much smaller; i.e., if the emulsions are homogenized using a high pressure homogenizer. Microscopy images of the

emulsions produced using these optimum conditions are shown in Figure 5.6, which revealed that the emulsions contained small, evenly distributed and closely packed oil droplets.

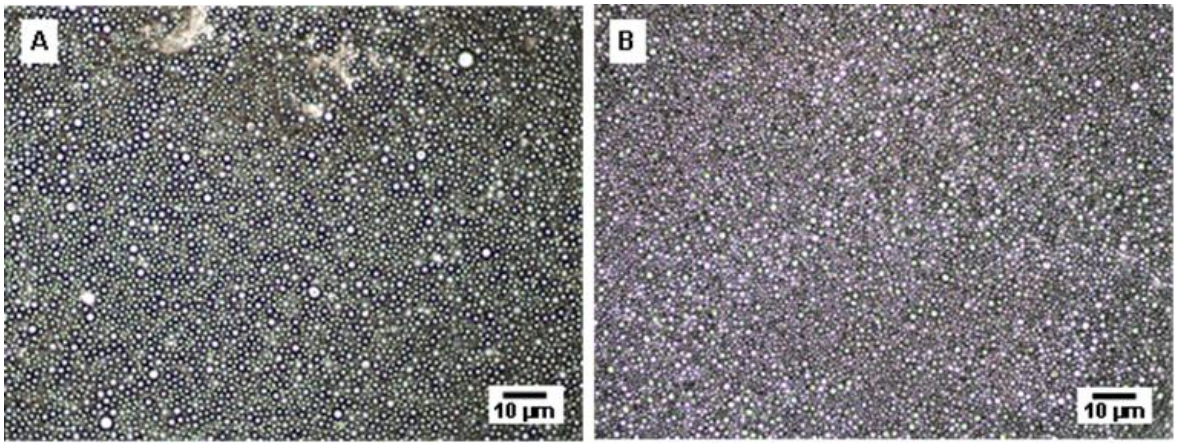

Figure 5.6 The microscopy images (at 10× magnification) of emulsions containing 4.1% protein and 40% oil stabilized by chickpea (A) and lentil (B) protein isolates at pH 8.0.

5.5 Conclusions

In conclusion, RSM was effectively used to identify three important emulsion parameters, protein concentration, oil content and pH as they related to mean droplet characteristics and the overall stability of a protein-stabilized oil-in-water emulsion. All three variables were found to have a significant effect on final emulsion characteristics. Droplet charge was shown to be only affected by pH, while droplet size and creaming index were affected by protein concentration, oil content and pH. The modeling of the obtained experimental data afforded the generation of useful equations for predicting the behaviour of the system under a variety of experimental conditions. Stable emulsions with small mean droplet sizes and high net droplet charge can be obtained using the optimized formulations.

5.6 Linkage

Chickpea and lentil protein-stabilized oil-in-water emulsions were optimized based on pH, protein concentration and oil content. The stable emulsion systems obtained could be used for the microencapsulation of flaxseed oil in legume protein-based matrices. The next study was designed to investigate the role of oil concentration, protein source and maltodextrin type and concentration on both the physicochemical characteristics and

microstructure of the microcapsules produced by freeze drying. In addition, the oxidative stability and release characteristics of the microcapsules produced were also determined.

6. MICROCAPSULE PRODUCTION EMPLOYING CHICKPEA OR LENTIL PROTEIN ISOLATES AND MALTODEXTRIN: PHYSICOCHEMICAL PROPERTIES AND OXIDATIVE PROTECTION OF ENCAPSULATED FLAXSEED OIL

6.1 Abstract

Flaxseed oil was microencapsulated employing a wall material matrix of either chickpea (ChPI) or lentil protein isolate (LPI) and maltodextrin, followed by freeze-drying. Effects of oil concentration (5.3-21.0%), protein source (ChPI vs. LPI) and maltodextrin type (DE 9 and 18) and concentration (25.0-40.7%) on both the physicochemical characteristics and microstructure of the microcapsules were investigated. It was found that an increase in emulsion oil concentration resulted in a concomitant increase in oil droplet diameter and microcapsule surface oil content, and a decrease in oil encapsulation efficiency. Optimum flaxseed oil encapsulation efficiency (~83.5%), minimum surface oil content (~2.8%) and acceptable mean droplet diameter (3.0 μm) was afforded with 35.5% maltodextrin-DE 9 and 10.5% oil. Microcapsules formed employing these experimental conditions showed a protective effect against oxidation versus free oil over a storage period of 25 d at room temperature.

6.2 Introduction

Flaxseed oil is rich in essential fatty acids (e.g., α-linolenic acid) which are purported to induce a variety of health benefits upon consumption. These health benefits include, reducing the risk of coronary heart diseases (Li et al., 2003) and the prevention of breast and prostate cancers (Bougnoux and Chajès, 2003). Despite these purported health promoting properties, flaxseed oil remains underutilized by the food industry due to its susceptibility to oxidation because of its high polyunsaturated fatty acid (PUFA) content, and due to its lack of miscibility in aqueous food systems (Łukaszewicz et al., 2004; Bozan and Temelli, 2008). However, through the use of encapsulation technologies these limitations can be circumvented so as to offer PUFA protection to the harsh environmental conditions experienced during food processing and storage, and improve flaxseed oil miscibility in foods.

Encapsulation is defined as a process whereby an active ingredient becomes enclosed or packaged within micron-sized carrier matrices, which in turn segregates and protects the inner core from the surrounding environment (Gibbs et al., 1999). Depending on the active ingredient and food matrix, a selection of physical and chemical methods for capsule production are available (Gouin, 2004; Madene et al., 2006). Although gelatin is one of the most widely used encapsulating materials it suffers from a number of perceived safety concerns (e.g., prion disease), and religious and dietary restrictions. Therefore, the development of plant protein based encapsulation systems as an alternative to animal proteins is of considerable interest and importance. Legume proteins can serve as a potential source for this purpose because of their high nutritional value, low cost and purported beneficial health benefits including but not limited to, reducing the risk of cardiovascular disease, as an aid in glycemic control in diabetic individuals, and in the prevention of digestive tract diseases (Boye et al., 2010; Duranti, 2006). Literature reports of the use of legume proteins as wall materials for lipid encapsulation are few however, flaxseed oil has been previously entrapped within other non-legume protein matrices with some success.

Grattard et al. (2002) encapsulated flaxseed oil into a matrix composed of maltodextrin, lecithin and xanthan gum via freeze-drying. They indicated that the resulting microcapsules efficiently protected flaxseed oil from oxidation. Liu et al. (2010) optimized the encapsulation of flaxseed oil within a gelatin-gum Arabic matrix via complex coacervation followed by freeze-drying. Optimized microcapsules with an oil encapsulation efficiency of 84% showed a protective effect against oxidation. Quispe-Condori et al. (2011) microencapsulated flaxseed oil by spray drying and freeze-drying methods using zein as the coating material and investigated the effects of zein and flaxseed oil concentration on microcapsule efficiency. They reported significantly higher encapsulation efficiency for spray drying (93.3%) when compared to freeze-drying (59.6%). The objectives of this study were to study flaxseed oil microencapsulation potential of chickpea and lentil protein isolates and maltodextrin as wall materials, and to investigate the physicochemical properties, surface microstructure, and flaxseed oil oxidative protection of the produced microcapsules.

6.3 Material and Methods

6.3.1 Materials

Chickpea (CDC Frontier, Kabuli) and lentil (CDC Grandora) seeds were provided by the Crop Development Centre at the University of Saskatchewan (Saskatoon, SK, Canada). Maltodextrin samples (DE 9, Dry MD^{TM} 01918 and DE 18, Dry MD^{TM} 01909-Z) were

donated by Cargill Inc. (Cargill Texturizing Solutions, Cedar Rapids, IA, USA). Flaxseed oil was kindly donated by Bioriginal Food & Science Corp. (Saskatoon, SK, Canada). All chemicals used were of reagent grade and purchased from Sigma-Aldrich (Oakville, ON, Canada). The water used in this research was produced from a Millipore Milli-Q^{TM} water system (Millipore Corp., Milford, MA, USA).

6.3.2 Proximate analysis

Proximate composition analyses for protein isolates and maltodextrin-DE samples were conducted according to AOAC Official Methods 925.10 (moisture), 923.03 (ash), 920.85 (lipid), and 920.87 (crude protein by using %N × 6.25) (AOAC, 2003). Carbohydrate content was determined on the basis of percent differential from 100%.

Proximate analysis of maltodextrin samples

The chemical composition of maltodextrin-DE 9 was determined to be: 4.6% moisture, 0.0% protein, 0.0% lipid, 95.0% carbohydrate and 0.4% ash. For maltodextrin-DE 18 the results were: 4.7% moisture, 0.0% protein, 0.0% lipid, 95.0% carbohydrate and 0.3% ash.

6.3.3 Protein isolate preparation

Whole chickpea and lentil seeds were ground into a fine flour using an IKA A11 basic analytical mill (IKA Works Inc., Wilmington, NC, USA) for 1 min, and then defatted using hexane (1:3 [w/v] flour:hexane ratio) for 40 min. The mixture was then filtered employing Whatman Gr. 1 paper (110 mm; Whatman International Ltd., Maidstone, United Kingdom), and air-dried in a fume hood. This defatting procedure was repeated twice for each flour.

Chickpea protein isolate (ChPI) was prepared according to the method of Papalamprou et al. (2010). In brief, defatted flour (100 g) was mixed with water at a 1:10 ratio (w/v), adjusted to pH 9.0 using 1.0 M NaOH and stirred at 500 rpm for 45 min at room temperature (21-23°C). The suspension was then centrifuged at 4,500 × *g* for 20 min at 4°C using a Sorvall RC-6 Plus centrifuge (Thermo Scientific, Asheville, NC, USA) to collect the supernatant. The resulting pellet was re-suspended in water at a ratio of 1:5 (w/v), adjusted to pH 9.0, stirred for an additional 45 min, followed by centrifugation (4,500 × *g*, 20 min, 4°C). Supernatants were pooled and adjusted to pH 4.6 using 0.1 M HCl to precipitate the protein. The protein was recovered by centrifugation as above, collected and stored at −30°C until freeze-drying which was performed using a Labconco FreeZone 6 freeze drier (Labconco Corp., Kansas City, MO, USA) to yield a free flowing powder. Proximate analysis of ChPI

showed a chemical composition of, 85.40% protein, 6.52% moisture, 3.05% ash, 4.11% carbohydrate and 0.92% lipid.

Lentil protein isolate (LPI) was produced employing the combined methods of Bamdad et al. (2006) and Lee et al. (2007). Defatted flour (100 g) was mixed with water at a 1:10 ratio (w/v), adjusted to pH 9.5 with 1.0 M NaOH, and stirred at 500 rpm for 1 h at room temperature. The mixture was kept static at 4°C overnight to allow for non-protein sedimentation. After centrifugation at 1,600 × *g* for 30 min at 4°C, the supernatant was collected, and pH was adjusted to 4.5 with 0.1 M HCl. The precipitated protein was collected by centrifugation (1,600 × *g*, 30 min, 4°C) and stored at −30°C until freeze-drying. Proximate analysis of LPI showed a chemical composition of, 81.90% protein, 5.04% moisture, 3.63% ash, 9.00% carbohydrate and 0.43% lipid.

6.3.4 Emulsion preparation

Protein solutions (ChPI or LPI, 4.0%) were prepared by dispersing the isolates (corrected on a weight basis for protein content) in water followed by adjustment to pH 3.0 with 0.1 M HCl. The resulting mixtures were stirred at 500 rpm overnight at 4°C to ensure complete dispersion. Maltodextrin solutions were prepared by dispersing either DE 9 or 18 in water followed by stirring at 300 rpm overnight at 4°C. Prior to sample homogenization, the pH of the protein solutions was re-adjusted to 3.0 as described above. Twenty-eight oil-in-water emulsions were prepared (Table 6.1) by homogenizing (Polyton PT2100, Kinematica AG, Lucerne, Switzerland) varying amounts of protein isolate, maltodextrin solutions and flaxseed oil in 15 mL plastic centrifuge tubes employing a 12 mm PT-DA 2112/2EC generating probe at 13,000 rpm for 3 min.

Additional ChPI and LPI emulsions were prepared at pH 7.0, 10.5% oil and 35.5% maltodextrin (DE 9 only) for encapsulation purposes based on the optimization of emulsion formulation at pH 3.0 (see Section 6.5).

Table 6.1 Formulations of ChPI- and LPI-stabilized emulsions prior to freeze drying.

Protein (ChPI or LPI, %)	Maltodextrin (DE 9 or 18, %)	Oil (%)	Water (%)
4.0	40.7	5.3	50.0
4.0	38.1	7.9	50.0
4.0	35.5	10.5	50.0
4.0	32.8	13.2	50.0
4.0	30.2	15.8	50.0
4.0	27.6	18.4	50.0
4.0	25.0	21.0	50.0

6.3.5 Droplet size measurements

Droplet size distributions of initial and reconstituted emulsions were measured using a Mastersizer 2000 laser light scattering instrument (Malvern Instruments Ltd., Worcestershire, United Kingdom) equipped with a Hydro 2000S sample handling unit (containing water). Emulsion samples were taken from the bottom of the tube immediately after homogenization for analysis. This sample was stirred continuously within the sample cell to ensure homogeneity at room temperature. Obscuration in all the measurements was kept at ~14% by water addition. Droplet size distributions were calculated by the instrument according to the Mie Theory which uses the refractive index difference between the droplets and the dispersing medium to predict the intensity of the scattered light. The ratio of the refractive index of flaxseed oil (1.479) to that of the dispersion medium (1.330) was 1.112. Droplet size measurements were reported as volume-length mean diameters ($d_{4,3}$) which is expressed as:

$$d_{4,3} = \frac{\sum_{i=1} n_i \cdot d_i^{\,4}}{\sum_{i=1} n_i \cdot d_i^{\,3}} \qquad \text{[eq. 6.1]}$$

where n_i is the number of droplets of diameter (d_i) (McClements, 2005c).

Emulsion reconstitution

Freeze-dried microcapsule samples of 0.5 g were dispersed in 4 mL of water and stirred at 500 rpm for 5 min. Samples were withdrawn for particle size distribution with measurements performed as described above.

6.3.6 Freeze-drying

The emulsion preparation (section 2.3) samples were placed in aluminum pans (diameter = 70 mm; approximate layer thickness = 5 mm) and frozen at −40°C for 24 h. Freeze dried emulsions were prepared as previously described with the ice condenser set at −50°C, and the vacuum pressure was approximately 0.120 mbar; the freeze drying time was 72 h. Following freeze drying the samples were manually ground to obtain a fine powder.

6.3.7 Microcapsule characterization

Moisture content and water activity:

The moisture content of freeze dried microcapsules was determined gravimetrically, following drying in a forced-air oven at 105°C for ~12 h. Microcapsule water activity was determined using an AquaLab CX-2 water activity meter (Decagon Devices, Inc., Pullman, WA, USA).

Colour measurements:

The tristimulus colour values of freeze dried microcapsules were measured using a Hunter colourimeter (ColorFlex EZ 45/0, Hunter Associates Laboratory, Inc., Reston, VA, USA), which was standardized using a white reference tile. The results were expresses as *L* (lightness), *a* (redness), and *b* (yellowness) tristimulus values.

Microcapsule surface and total oil content:

Microcapsule surface oil was determined according to the method of Liu et al. (2010). Briefly, 1 g of microcapsules was dispersed in 30 mL of hexane followed by vigorous shaking for 30 s. The solvent was filtered (Whatman Gr. 1 paper) into a 40 mL beaker, and the beaker plus solvent was placed in a fume hood overnight to afford solvent evaporation. Microcapsule surface oil was then determined gravimetrically, after heating the beaker at 105°C for 30 min to remove any residual solvent.

Total oil content of the microcapsules was determined using the method described by Klinkesorn et al. (2006) with some modifications. Briefly, 4 mL of water was added to 1 g of microcapsules followed by mixing at 300 rpm for 2 min. The resulting solution was then mixed with 25 mL hexane/isopropanol (3:1 v/v), stirred at 300 rpm for 15 min and centrifuged at 1500 × *g* for 2 min. The clear organic phase was collected and the aqueous phase was re-extracted with the aforementioned solvent mixture. The organic phases were

pooled and filtered through anhydrous Na_2SO_4, and the solvent was allowed to evaporate overnight in a fume hood. Total oil content was determined gravimetrically, after heating at 105°C for 30 min.

Flaxseed oil encapsulation efficiency:

The encapsulation efficiency (EE) was calculated from the quantitative determinations as follows (Anwar and Kunz, 2011):

$$EE = (\text{Total Oil} - \text{Surface Oil}) / \text{Total Oil} \times 100\% \qquad \text{[eq. 6.2]}$$

Scanning electron microscopy (SEM):

Microcapsule samples were mounted onto aluminum stubs with double-sided tape and gold coated with a sputter coater. The coated samples were then viewed with a Philips SEM 505 (Eindhoven, The Netherlands) operating at an accelerating voltage of 27 kV with 6× and 1000× magnification.

6.3.8 Oxidative stability

Oxidative stability of free (i.e. control) and encapsulated flaxseed oil was characterized during storage at room temperature over a 25 d period employing both the peroxide value and 2-thiobarbituric acid reactive substances tests. Microcapsules (3-4 g/bottle) or free oil (~2 mL) were stored in individually sealed nitrogen-flushed 10 mL amber glass bottles for storage stability studies. Oxidative testing was carried out every 5 d over the 25 d testing period, using a new set of unopened samples. Flaxseed oil extraction from the microcapsules followed the same procedure as that outlined previously for total oil determination, except the extraction solvent was dried under a stream of nitrogen.

Peroxide value (PV):

In brief, ~0.2 g of extracted flaxseed oil was weighed into a 250 mL Erlenmeyer flask, followed by the addition of 30 mL of 3:2 acetic acid/chloroform (v/v) solution and 0.5 mL of saturated potassium iodide (KI). After vigorous shaking for exactly 1 min, 30 mL of water was added to this mixture. A 0.5 mL aliquot of 1% (w/v) starch indicator was then added to the mixture, and the resulting solution was titrated using 0.001N sodium thiosulfate ($Na_2S_2O_3$) until the purple colour disappeared. Sample PV was calculated as:

$$PV = (S - B) \times N \times 1000 / W \qquad \text{[eq. 6.3]}$$

where *S* is the volume of $Na_2S_2O_3$ added to the sample, *B* is the volume of $Na_2S_2O_3$ of the blank, *N* is the normality of $Na_2S_2O_3$ solution, and *W* is the sample weight (g). PV was expressed as meq active O_2 (peroxide milliequivalent) per kg sample (Pegg, 2005).

2-thiobarbituric acid reactive substances (TBARS):

In brief, ~40 mg of extracted flaxseed oil was weighed into a 10 mL volumetric flask and was dissolved and brought to volume with n-butanol. To a 2.0 mL Eppendorf tube was added, 50 µL of 8.1% (w/v) SDS, 375 µL of 20% acetic acid, 375 µL of 0.8% (w/v) TBA, 8.25 µL of 0.02% (w/v) BHT (in DMSO) and 200 µL of the oil-butanol mixture. A standard curve was prepared using malondialdehyde (MDA) (1.25-50 µM) under the same experimental conditions. Samples and standards were then heated at 95°C for 1 h. After cooling in cold water, 0.9 mL of n-butanol/pyridine (15:1, v/v) was added, followed by vigorous shaking for 30 s. Samples and standards were centrifuged at 4000 × *g* for 10 min, and the upper organic layer was transferred to a 1.5 mL cuvette and the absorbance at 532 nm was measured against a butanol blank. TBA values were expressed as mg MDA eq/mg oil, which equates to the reactive aldehyde content (nmol)/sample oil weight (mg) (modified from Pegg, 2005 and Akhlaghi and Bandy, 2010).

6.3.9 Release characteristics

Release behaviour of flaxseed oil from the microcapsules triggered by pH and ionic strength was determined by the combined methods of Zhong and Jin (2009) and Choi et al. (2010). In brief, microcapsule samples of 1 g were individually dispersed in 10 mL aqueous NaCl solutions (0, 50, 100, 150 and 200 mM) that were pH adjusted (0.1 M HCl or NaOH) to produce values of 3.0, 5.0, 7.0 or 9.0 followed by stirring at 500 rpm for 1 h. The amount of released oil was determined by gravimetric analysis after two 30 mL hexane extractions.

In-vitro release behaviour of microencapsulated flaxseed oil was also investigated using a simulated gastrointestinal model according to the method of Burgar et al. (2009). Simulated gastric fluid (SGF) was prepared by dissolving 2.0 g of NaCl and 7.0 mL 36% HCl in 900 mL of water. After the addition of 3.2 g pepsin, the solution pH was adjusted to 1.2 with 0.1 M HCl and the final volume was made up to 1000 mL with water. Simulated intestinal fluid (SIF) was prepared by dissolving 6.8 g K_2HPO_4 in 800 mL of water. To this solution was added 77 mL of 0.2 M NaOH and 100.0 g of pancreatin and the solution was

stirred overnight at 4°C. Solution pH was adjusted to 6.8 with 1 M NaOH or 1 M HCl and the final volume was made up to 1000 mL with water.

A 2 g microcapsule sample was mixed with 20 mL of SGF and incubated for 2 h at 37°C and 100 rpm in a water bath. Released oil was extracted using hexane and then determined gravimetrically. For exposure to SGF and SIF in sequence, a 2 g microcapsule sample was mixed with 20 mL of SGF and incubated under same conditions for 2 h. Sample pH was adjusted to 6.8 using 1 M NaOH, followed by addition of 20 mL of SIF and the sample was incubated under the same conditions for 3 h. The amount of flaxseed oil released from the microcapsules was determined by gravimetric analysis as outlined above.

6.3.10 Statistical analyses

All experiments were performed in triplicate and reported as the mean ± one standard deviation. A one-way analysis of variance (ANOVA) with a Scheffe post-hoc test was used to measure statistical differences in microcapsule characteristics among different formulations. A general linear model was employed to measure statistical differences in: (1) the physicochemical characteristics of the microcapsules as a function of protein source (ChPI vs. LPI), maltodextrin type (DE 9 vs. DE 18) and oil concentration; and (2) microencapsulated flaxseed oil release properties as a function of protein source, pH of the protein solution used for preparing the microcapsules, and pH or ionic strength of the release medium. All statistical analyses were performed with SPSS version 17.0 software (SPSS Inc., 2008, Chicago, IL, USA).

6.4 Results and Discussion

6.4.1 Physicochemical characteristics of ChPI- and LPI-based microcapsules produced at pH 3.0 and 7.0

The moisture content and water activity of freeze dried flaxseed oil microcapsules containing ChPI and LPI produced at pH 3.0 are shown in Tables 6.2 and 6.3, respectively. The moisture content of the microcapsules varied from 2.26 to 4.18% while their water activities ranged between 0.07-0.19. The majority of these results were within the maximum moisture specification for dried powders in the food industry which is between 3-4% (Klinkesorn et al., 2006). Changes in oil concentration, protein source (ChPI vs. LPI) and maltodextrin type (DE 9 vs. DE 18) did not have a significant effect on microcapsule moisture content and water activity ($p>0.05$).

Table 6.2 Moisture content of freeze-dried ChPI- and LPI-based microcapsules produced at pH 3.0. Data represent the mean ± one standard deviation (n = 3).

Oil (%)	ChPI		LPI	
	DE 9	DE 18	DE 9	DE 18
5.3	2.44 ± 0.45	2.77 ± 0.66	2.76 ± 0.13	3.40 ± 0.11
7.9	3.25 ± 0.20	3.10 ± 0.18	2.84 ± 0.13	3.24 ± 0.19
10.5	3.89 ± 0.06	3.95 ± 0.14	4.18 ± 0.57	2.48 ± 0.10
13.2	3.28 ± 0.28	3.54 ± 0.14	3.25 ± 0.18	2.90 ± 0.12
15.8	2.77 ± 0.03	2.70 ± 0.10	2.44 ± 0.15	2.63 ± 0.17
18.4	2.68 ± 0.32	3.20 ± 0.19	3.13 ± 0.24	3.17 ± 0.14
21.0	2.94 ± 0.12	2.63 ± 0.24	2.78 ± 0.05	2.26 ± 0.06

Table 6.3 Water activity of freeze-dried ChPI- and LPI-based microcapsules produced at pH 3.0. Data represent the mean ± one standard deviation (n = 3).

Oil (%)	ChPI		LPI	
	DE 9	DE 18	DE 9	DE 18
5.3	0.12 ± 0.02	0.17 ± 0.01	0.19 ± 0.01	0.17 ± 0.00
7.9	0.14 ± 0.01	0.12 ± 0.01	0.16 ± 0.01	0.16 ± 0.01
10.5	0.15 ± 0.00	0.16 ± 0.01	0.15 ± 0.01	0.07 ± 0.00
13.2	0.14 ± 0.01	0.16 ± 0.00	0.14 ± 0.00	0.15 ± 0.01
15.8	0.15 ± 0.00	0.14 ± 0.02	0.13 ± 0.01	0.14 ± 0.00
18.4	0.13 ± 0.02	0.14 ± 0.00	0.13 ± 0.01	0.15 ± 0.02
21.0	0.13 ± 0.01	0.14 ± 0.02	0.14 ± 0.01	0.09 ± 0.02

The *L* (lightness), *a* (redness), and *b* (yellowness) tristimulus colour values of freeze dried microcapsules containing flaxseed oil produced at pH 3.0 are presented in Table 6.4. Microcapsules containing ChPI were slightly yellow in colour, which was illustrated by *L* values ranging from 87.3 to 90.6, *a* values from −0.5 to 0.3, and *b* values from 11.2 to 20.3. On the other hand, microcapsules containing LPI were beige in colour with *L* values ranging from 77.9 to 84.4, *a* values from 2.1 to 2.9, and *b* values from 14.1 to 20.1. As microcapsule oil content increased, the *L* value decreased and the *b* value increased for both ChPI- and LPI-containing microcapsules ($p<0.05$) which indicated that the microcapsules became more yellow as the amount of flaxseed oil increased. Maltodextrin type (DE 9 vs. DE 18) did not have a significant effect on microcapsule colour ($p>0.05$). Overall, the ChPI-microcapsules

had significantly higher *L* values (~88.9) compared to LPI-microcapsules (~82.5, $p<0.05$), whereas *a* values of LPI-microcapsules (~2.3) were significantly higher than those of ChPI-microcapsules (~0.0). The darker colour of the LPI-microcapsules is most likely due to the presence of hull pigments which were extracted by the alkaline solvents used in isolate preparation (Bamdad et al., 2006).

Table 6.4 The Hunter colour values of freeze dried ChPI- and LPI-based microcapsules produced at pH 3.0. Data represent the mean ± one standard deviation (n = 3).

Oil (%)	**ChPI**		**LPI**	
	DE 9	DE 18	DE 9	DE 18
L *(Lightness; L = 0 indicates black; L = 100 indicates white)*				
5.3	90.5 ± 0.4	90.6 ± 0.5	83.1 ± 0.4	84.0 ± 0.9
7.9	90.3 ± 0.3	90.3 ± 0.3	83.5 ± 0.9	84.4 ± 0.6
10.5	89.1 ± 0.5	88.7 ± 0.9	83.5 ± 0.7	82.2 ± 0.5
13.2	89.7 ± 0.1	88.9 ± 0.6	83.7 ± 0.4	83.3 ± 0.6
15.8	88.6 ± 0.6	88.0 ± 0.2	82.1 ± 0.3	82.2 ± 0.1
18.4	88.1 ± 0.3	87.5 ± 0.3	82.2 ± 1.0	81.6 ± 0.4
21.0	87.7 ± 0.4	87.3 ± 0.4	81.3 ± 0.2	77.9 ± 0.5
a *(negative 'a' values indicate green; positive 'a' values indicate red/magenta)*				
5.3	-0.3 ± 0.1	-0.5 ± 0.2	2.3 ± 0.1	2.1 ± 0.1
7.9	-0.3 ± 0.1	-0.5 ± 0.3	2.3 ± 0.2	2.1 ± 0.1
10.5	0.1 ± 0.1	0.0 ± 0.1	2.1 ± 0.2	2.3 ± 0.1
13.2	0.1 ± 0.1	0.0 ± 0.1	2.0 ± 0.1	2.1 ± 0.1
15.8	0.1 ± 0.1	0.1 ± 0.1	2.3 ± 0.1	2.1 ± 0.0
18.4	0.1 ± 0.1	0.3 ± 0.1	2.4 ± 0.2	2.4 ± 0.1
21.0	0.1 ± 0.3	0.3 ± 0.2	2.7 ± 0.1	2.9 ± 0.1
b *(negative 'b' values indicate blue; positive 'b' values indicate yellow)*				
5.3	11.9 ± 0.2	11.2 ± 0.1	14.3 ± 0.1	14.1 ± 0.4
7.9	13.7 ± 0.2	12.7 ± 0.9	14.8 ± 0.5	14.1 ± 0.5
10.5	14.6 ± 0.4	14.9 ± 0.6	14.8 ± 0.4	15.5 ± 0.2
13.2	14.5 ± 0.3	15.5 ± 0.4	14.7 ± 0.4	15.2 ± 0.4
15.8	16.2 ± 0.7	17.0 ± 0.1	16.3 ± 0.4	16.8 ± 0.1
18.4	17.6 ± 0.3	18.6 ± 0.5	17.6 ± 0.5	17.7 ± 0.4
21.0	19.0 ± 0.6	20.3 ± 0.6	18.3 ± 0.2	20.1 ± 0.1

Surface oil represents the portion of oil present on the surface of the microcapsule (Bao et al., 2011). Minimizing the amount of surface oil is crucial in lipid microencapsulation as this material can oxidize at more rapid rates than the encapsulated oil, causing rancidity and reducing the shelf life of the finished product (Pegg and Shahidi, 2007). The effect of emulsion formulation on surface oil content is presented in Figure 6.1. It was noted that the

surface oil content of the microcapsules produced at pH 3.0 ranged from 0.7-19.8% depending on the formulation. An analysis of variance revealed the following as significant factors: oil concentration ($p<0.001$), maltodextrin type ($p<0.001$) and interactions between protein source × maltodextrin type ($p<0.05$), protein source × oil concentration ($p<0.001$) and maltodextrin type × oil concentration ($p<0.001$). Overall, the surface oil in the microcapsules increased from 1.0 to 16.9% as the oil concentration in the initial emulsions increased from 5.3 to 21.1%. Kagami et al. (2003) encapsulated fish oil using a blend of dextrin/maltodextrin and sodium caseinate. An increase in surface oil was reported as the oil load of the microcapsules increased, regardless of the wall material used. In the present study, two types of maltodextrin (DE 9 and 18) were used as a secondary wall material (i.e., filler) to improve microcapsule drying properties (Kagami et al., 2003). Maltodextrins are widely used as wall materials for capsule formation as they exhibit good solubility and low viscosities at high solids contents (Gharsallaoui et al., 2007). Overall, microcapsules prepared with maltodextrin-DE 9 had lower surface oil contents (6.5%) when compared to microcapsules prepared with maltodextrin-DE 18 (8.3%). Maltodextrin-DE 18 is a more hydrolyzed starch product with a higher concentration of lower molecular weight glucose polymers, which are responsible for its higher water solubility compared to maltodextrin-DE 9. The lower surface oil content observed in microcapsules prepared with DE 9 is most likely due to its higher hydrophobicity when compared to DE 18 due to the presence of higher molecular weight glucose polymers in this material.

The effect of emulsion formulation on flaxseed oil encapsulation efficiency is shown in Figure 6.2. The encapsulation efficiency of flaxseed oil ranged from 46.2 to 92.1% in the microcapsules depending on the formulation. An analysis of variance showed that oil concentration ($p<0.001$), maltodextrin type ($p<0.001$) and interactions between protein source × maltodextrin type ($p<0.05$), protein source × oil concentration ($p<0.001$) and maltodextrin type × oil concentration ($p<0.001$) were significant. Overall, as the concentration of flaxseed oil increased in the emulsion from 5.3 to 21.0%, encapsulation efficiency values decreased from ~89 to ~53%, respectively, which agreed with the trend observed in surface oil content. Polavarapu et al. (2011) reported lower encapsulation efficiencies at higher oil concentrations for fish oil and extra virgin olive oil in a sugar beet-pectin matrix. The authors attributed these results to a capsule wall material content that was unable to form a dense, tightly packed matrix around the dispersed oil droplets.

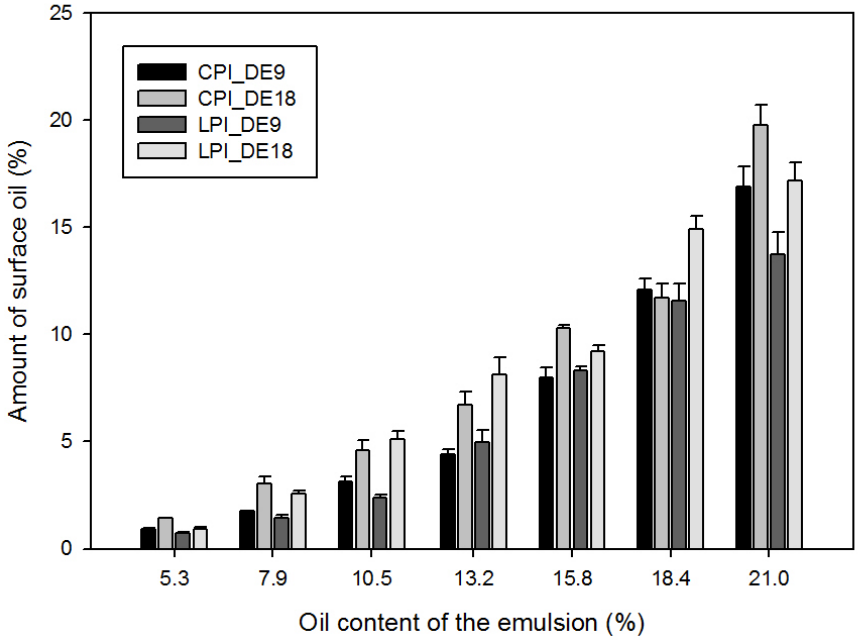

Figure 6.1 Changes in surface oil content as a function of emulsion formulation at pH 3.0. Data represent the mean ± one standard deviation (n = 3).

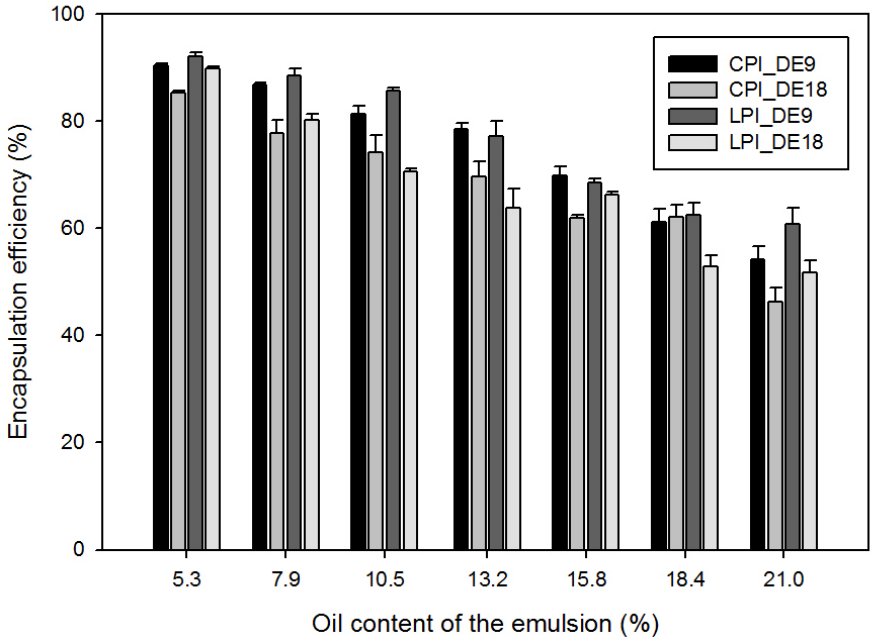

Figure 6.2 Changes in flaxseed oil encapsulation efficiency as a function of emulsion formulation at pH 3.0. Data represent the mean ± one standard deviation (n = 3).

In this study, higher flaxseed oil encapsulation efficiencies coupled with lower surface oil contents were achieved in ChPI- and LPI-based microcapsules containing maltodextrin-DE 9 (Figure 6.2). Experimental results also showed that flaxseed oil encapsulation efficiencies in ChPI- and LPI-maltodextrin systems were dependent upon multiple factors.

The particle size of the active ingredient (e.g. flaxseed oil) dispersed within the aqueous phase of the emulsion has been shown to be a significant factor for retention within microcapsules, where the smaller the particle size the greater the retention (Rish and Reineccius, 1988). Smaller oil droplet sizes have also be shown to minimize microcapsule surface oil, increase oil encapsulation efficiency and decrease lipid oxidation rates (Lee and Ying, 2008). The mean droplet diameter of ChPI- and LPI-stabilized emulsions before freeze-drying and after reconstitution are shown in Figure 6.3.

The volume-weighted mean droplet diameter range ($d_{4,3}$) of flaxseed oil-in-water emulsions stabilized by ChPI and LPI were 2.4-4.8 μm and 2.7-4.5 μm, respectively, depending on the formulation. An analysis of variance revealed that protein source ($p<0.01$), maltodextrin-type ($p<0.001$), flaxseed oil concentration ($p<0.001$), the interactions between protein source × maltodextrin-type ($p<0.05$), protein source × oil concentration ($p<0.001$) and maltodextrin-type × oil concentration ($p<0.01$) were all significant. Overall, despite having a significant protein-type main effect, the $d_{4,3}$ values of 3.7 and 3.9 μm for LPI and ChPI stabilized emulsions, respectively, were similar. Whereas emulsions containing maltodextrin DE 9 and DE 18 showed quite different overall $d_{4,3}$ values of 3.6 and 4.0 μm, respectively. The lower droplet diameters observed for emulsions prepared with the less hydrolyzed maltodextrin (DE 9) may be due to a higher continuous phase viscosity imparted by the larger concentration of high molecular weight glucose polymers in this material when compared to DE 18. Dokic et al. (2004) reported a similar phenomenon while investigating the effects of various maltodextrin products (DE 5 – 20) on the droplet size of sunflower oil-in-water emulsions. Also, as the concentration of flaxseed oil in the emulsion increased from 7.9% to >10%, the $d_{4,3}$ values increased from ~3.1 μm to ~4.1 μm. The larger oil droplets observed at oil concentrations >10% could be attributed to the limited availability of protein to cover the oil surface to sufficiently form a dense adsorption layer so as to prevent coalescence. Sun and Gunasekaran (2009) and Achouri et al. (2011) reported a similar trend in whey protein isolate-xanthan gum and soy protein isolate stabilized oil-in-water emulsions, respectively.

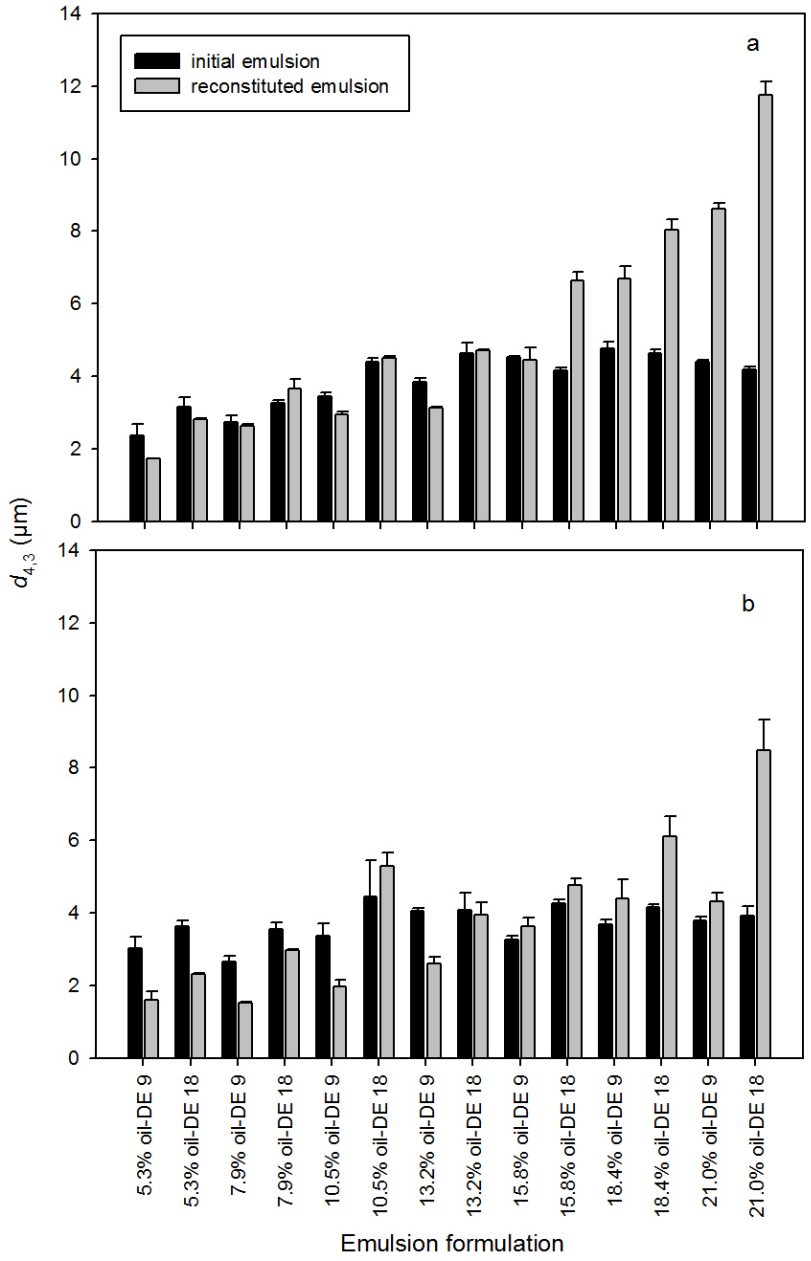

Figure 6.3 Effect of freeze-drying on mean droplet diameter for a) ChPI- and b) LPI-stabilized emulsions at pH 3.0. Data represent the mean ± one standard deviation (n = 3).

The droplet size of water-redispersed freeze dried microcapsules containing flaxseed oil showed that this drying process resulted in a significant increase in mean droplet diameter ($p<0.05$) at oil concentrations of 18.4% and 21.0% regardless of the protein source and maltodextrin type. The observed increase in droplet size in reconstituted emulsions with

higher amounts of oil could be attributed to coalescence of the surface oil after drying and/or upon reconstitution (Polavarapu et al., 2011; Shaw et al., 2007).

The goal of the next section of this study was to produce microcapsules containing flaxseed oil that would be suitable for use in food commodities with a more neutral pH such as dairy products. The physicochemical characteristics of microcapsules produced at pH 7.0 are presented in Table 6.5. Microcapsules produced with ChPI at pH 7.0 had lower moisture content and water activity compared to pH 3.0 ($p<0.05$). In addition, lower *L* (lightness) and higher *a* (redness) and *b* (yellowness) values were observed in microcapsules produced with ChPI at pH 7.0 compared to pH 3.0 ($p<0.05$), resulting in a slightly darker yellowish colour. No significant differences in surface oil content and flaxseed oil encapsulation efficiency were found between microcapsules produced at pH 7.0 and 3.0 ($p>0.05$). Microcapsules produced with LPI at pH 7.0 maintained their original droplet diameter after water-redispsersion of freeze-dried material while this process resulted in an increase in oil droplet diameter in microcapsules produced with ChPI at pH 7.0. Microcapsules produced with LPI at pH 7.0 had similar moisture content ($p>0.05$) but lower water activity ($p<0.05$) than those produced at pH 3.0. A lower *L* (lightness) value was found in microcapsules produced with LPI at pH 7.0 compared to pH 3.0 ($p<0.05$). Microcapsules produced with LPI at pH 7.0 and 3.0 had similar surface oil content, encapsulation efficiency and maintained their original droplet diameter after water-redispersion following freeze-drying ($p>0.05$).

When comparing microcapsule formation at pH 7.0; LPI-based materials had a lower moisture content and darker colour than ChPI-based ones ($p<0.05$), and no significant difference was observed in surface oil content and encapsulation efficiency between these protein isolates ($p>0.05$). Water-redispersion of the freeze-dried microcapsules resulted in an increase in droplet diameter for ChPI-based materials while droplet diameter remained unchanged in LPI-based ones (Table 6.5).

Table 6.5 Physicochemical characteristics of freeze dried ChPI- and LPI-based (4.0%) microcapsules produced at pH 7.0 containing 10.5% oil and 35.5% maltodextrin (DE 9). Data represent the mean ± one standard deviation (n = 3).

Sample	Physicochemical characteristics				
a) Moisture, A_w and colour					
	Moisture	**A_w**	**Color**		
			L	***a***	***b***
ChPI-based microcapsules	2.80 ± 0.17[a]	0.14 ± 0.01[a]	88.3 ± 0.2[a]	0.5 ± 0.1[a]	15.7 ± 0.5[a]
LPI-based microcapsules	3.23 ± 0.20[b]	0.13 ± 0.00[a]	78.4 ± 0.4[b]	2.2 ± 0.1[b]	13.2 ± 0.2[b]
b) Surface oil and encapsulation efficiency					
	Surface oil (%)	**Encapsulation efficiency (%)**			
ChPI-based microcapsules	2.68 ± 0.36[a]	83.40 ± 2.65[a]			
LPI-based microcapsules	2.66 ± 0.41[a]	83.17 ± 1.75[a]			
c) Droplet diameter, $d_{4,3}$ (μm)					
	Before drying	**After drying**			
ChPI-based microcapsules	2.15 ± 0.03[a]	4.33 ± 0.70[a]			
LPI-based microcapsules	2.45 ± 0.05[b]	2.36 ± 0.08[b]			

Means in each column followed by different letters were significantly different ($p<0.05$).

6.4.2 Surface morphology of ChPI- and LPI-based microcapsules containing flaxseed oil

SEM images of freeze-dried ChPI- and LPI-based microcapsules containing 10.5% flaxseed oil are shown in Figure 6.4. All four samples (microcapsules produced at both pH 3.0 and 7.0) had similar surface morphology which were highly porous. A porous microcapsule morphology has also been observed by other research groups for freeze-dried oil-in water emulsions (Anwar and Kunz 2011; Heinzelmann et al., 2000).

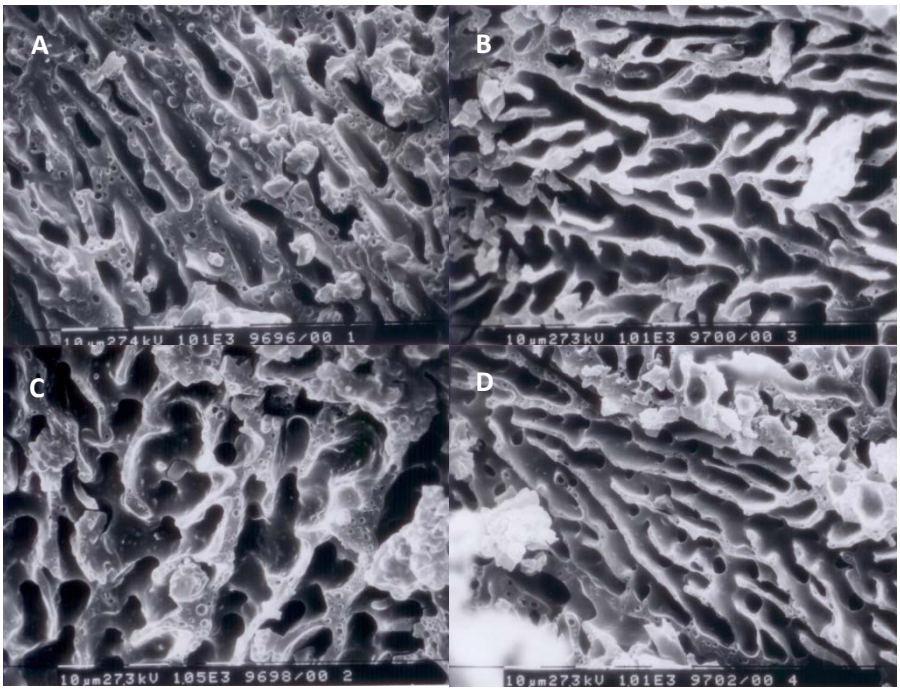

Figure 6.4 SEM images of freeze-dried ChPI- and LPI-based microcapsules produced at optimum conditions of 10.5% flaxseed oil and 35.5% maltodextrin-DE 9: a) ChPI at pH 3.0; b) LPI at pH 3.0; c) ChPI at pH 7.0; d) LPI at pH 7.0.

6.4.3 Oxidative stability of microencapsulated flaxseed oil

As flaxseed oil is rich in polyunsaturated fatty acids (PUFAs) it is highly susceptible to oxidation resulting in the onset of rancidity. Łukaszewicz et al. (2004) investigated the oxidative stability of flaxseed oil produced from nine different cultivars by measuring both conjugated dienes and 2-thiobarbituric acid-reactive substances (TBARS) formation following sample storage in air at 140°C for 40 min. Results from these experiments showed that the concentration of conjugated dienes reached 50-200 mol/kg, whereas TBARS reached 0.1-0.5 mol/kg. Based on these results, the authors concluded that flaxseed oil isolated from all nine cultivars was easily oxidized. One of the main goals in this study was to investigate the ability of ChPI- and LPI-based microcapsules to delay flaxseed oil oxidation.

Based on previous results, a ChPI- and LPI-based microcapsule formulation was chosen consisting of 4.0% protein, 35.5% maltodextrin-DE 9, and 10.5% oil. The reasons for this choice included high flaxseed oil encapsulation efficiency (~83.5%), minimum surface oil content (~2.8%) and acceptable mean droplet diameter (3.0 μm). The oxidative stability of free and the ChPI- and LPI-based microencapsulated flaxseed oil stored under nitrogen and held at room temperature was monitored over a 25 d period, with sample peroxide value (PV)

and TBARS results determined at five-day intervals for all samples (Figure 6.5). The PV of free flaxseed oil at time zero was 5.88 ± 010 meq active O_2/kg while that of ChPI- or LPI-based microencapsulated flaxseed oil at immediately following freeze drying and extraction (time zero) ranged from 5.76-6.40 meq active O_2/kg; with no significant difference observed between protein source (ChPI vs. LPI) and pH (3.0 vs. 7.0). Because the PV of time zero microencapsulated flaxseed oil was found to be similar to that of the free oil, the emulsification and encapsulation processes did not negatively impact oil stability. The PV results for ChPI and LPI-based microencapsulated flaxseed oil remained unchanged over the 25 d storage period ($p>0.05$), whereas that of the free oil steadily increased to 9.08 meq active O_2/kg at day 15, and to 11.43 meq active O_2/kg at day 20, and to 13.57 meq active O_2/kg at day 25 ($p<0.05$). The primary oxidative products (hydroperoxides) measured by the PV test are odourless and colourless but can readily participate in the autoxidation process producing a variety of secondary oxidation products, such as aliphatic aldehydes, alcohols, ketones, cyclic compounds, and hydrocarbons which can have an adverse effect on the sensory attributes of the oil/product (Pegg, 2005). In the present study, secondary oxidation products were measured using the TBARS test. The TBARS value of ChPI- and LPI-based microencapsulated flaxseed oil remained unchanged (~1.85 nmol/mg oil at time zero and ~2.15 nmol/mg oil at day 25) over the 25 d storage period ($p>0.05$), with no significant differences observed between protein isolate and pH. The TBARS values for free oil remained unchanged during the first 15 days (2.12-2.51 nmol/mg oil) but increased to 3.22 nmol/mg oil at day 20 and to 3.98 nmol/mg oil at day 25 ($p<0.05$). These results indicate that the increased formation of secondary oxidation products over time was significant when compared to the microencapsulated flaxseed oil ($p<0.05$). Experimental results clearly show that the ChPI- and LPI-based microcapsules provided oxidative protection to the encapsulated flaxseed oil over the 25 d storage period.

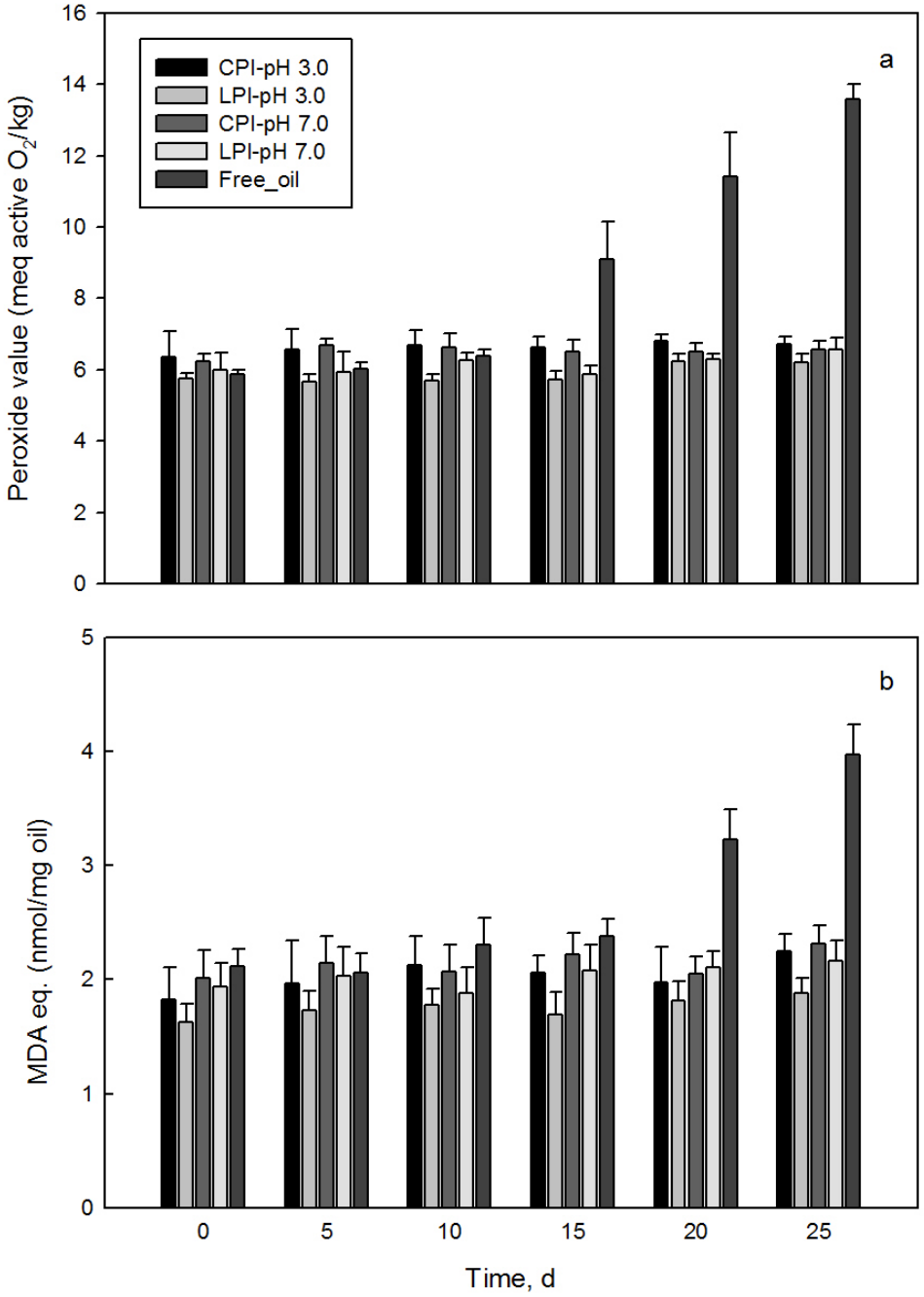

Figure 6.5 Changes in a) peroxide value (PV) and b) thiobarbituric acid-reactive substances (TBARS) for free and microencapsulated flaxseed oil. Data represent the mean ± one standard deviation (n=3). ChPI- and LPI-based microcapsules were produced at optimum conditions of 10.5% flaxseed oil and 35.5% maltodextrin-DE 9 for both ChPI and LPI at pH's 3.0 and 7.0.

To best of our knowledge, the microencapsulation of flaxseed oil in legume protein-based matrices has not been reported in literature. However, Grattard et al. (2002) encapsulated flaxseed oil in a maltodextrin-lecithin-xanthan gum matrix followed by freeze-drying and this material was reported to protect the entrapped oil against oxidation. Partanen et al. (2008) found that the oxidation of flaxseed oil encapsulated in a spray-dried whey protein isolate matrix was retarded when compared to that of free oil. The authors reported that the comparison of oxidation rate of free versus encapsulated oil is not straightforward because the surface area of dispersed oil is much higher and oxygen concentration in the oil-solid interface and the oil-gas interface are different. Liu et al. (2010) encapsulated flaxseed oil within a gelatin-gum Arabic matrix and reported a significant reduction in oil oxidation based on PV and p-anisidine values when compared to the free oil. Pu et al. (2011) reported improved oxidative stability of flaxseed oil microencapsulated in a sodium caseinate-lactose matrix when the pigment astaxanthin (from shrimp) was added to the oil prior to microencapsulation.

6.4.4 Release characteristics of microencapsulated flaxseed oil

The relationships between flaxseed oil release from ChPI- and LPI-based microcapsules as a function of a selection of NaCl (i.e. ionic strength) concentrations, and the pH of the microcapsule preparation protocol (3.0 and 7.0) and release medium (3.0, 5.0, 7.0 and 9.0) are shown in Table 6.6. With respect to pH-triggered release, an analysis of variance showed that the protein source, pH of the protein solution used for microcapsule preparation, pH of the release medium, and the interaction of the last two factors were significant ($p<0.001$). In general, the amount of flaxseed oil released from the microcapsules was found to be the lowest (2.6-4.5%) for pH 5.0 and increased in conjunction with pH to 7.2-9.2% at pH 9.0. The observed increase in released flaxseed oil from the protein-based microcapsules at acidic (pH 3.0) and basic pH values (pH 9.0) is most likely due to the increased solubility of ChPI and LPI, resulting in the loss of capsule integrity/structure with concomitant flaxseed oil release. For salt-triggered release, an analysis of variance of the results showed that the protein source, ionic strength (50, 100, 150 and 200 mM NaCl), and pH of the protein solution used for microcapsule preparation were significant ($p<0.001$). The amount of flaxseed oil released from the microcapsules was found to be the lowest (4.1-5.1%) at 0 mM NaCl and steadily increased to 9.1-10.0% at 200 mM NaCl. The observed increase in flaxseed oil release from the protein-based microcapsules as a function of ionic strength may be explained by the increased solubility of ChPI and LPI at elevated NaCl concentrations (i.e.

200 mM) resulting in the loss of capsule integrity/structure with concomitant flaxseed oil release.

The *in-vitro* release behaviour of ChPI- and LPI-based microencapsulated flaxseed oil was investigated employing both simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) systems. In general, microencapsulated flaxseed oil release under SGF conditions ranged from 36.6-43.4% with the highest value observed for the ChPI capsules prepared at pH 7.0, whereas the lowest value was observed for the LPI capsules prepared at pH 7.0. Microencapsulated flaxseed oil release under the combined SGF-SIF conditions was significantly higher (84.5-92.6%) than that observed for SGF only. This result is most likely explained by the presence of the proteolytic enzyme pepsin in SGF which catalyses protein hydrolysis resulting in a change in capsule structure (e.g. large pore formation) with concomitant oil release.

6.5 Conclusions

Experimental results showed that ChPI- and LPI-based microcapsule formation was efficient for the entrapment and gastrointestinal delivery of flaxseed oil. Microcapsules prepared employing these two plant proteins in conjunction with maltodextrin exhibited a protective effect against oxidation over a 25 d storage period at room temperature as indicated by a lack of significant change in initial PV and TBARS results. The optimum microcapsule formulation of those studied included ChPI or LPI, maltodextrin-DE 9 and 10.5% flaxseed oil followed by freeze drying. These microcapsules had a low surface flaxseed oil content (<3%), a high flaxseed oil encapsulation efficiency (~83%), and showed high (>84%) targeted release properties under simulated gastrointestinal conditions. These findings suggest that the developed plant-based microencapsulation system could lead to increased utilization of flaxseed oil and legume proteins in food and bioproduct formulations and applications.

6.6 Linkage

Flaxseed oil was microencapsulated by freeze drying using a ChPI-maltodextrin or LPI-maltodextrin matrix as wall materials. Microcapsules formed employing the optimum formulation had a high flaxseed oil encapsulation efficiency, showed a protective effect against oxidation versus free oil over a storage period of 25 d at room temperature and high targeted release characteristics under simulated gastrointestinal conditions. Based on these findings the goal of the next study was to encapsulate flaxseed oil employing spray drying

and investigate the effects of emulsion formulation and protein source on the physicochemical characteristics, oxidative stability, and release properties of the resulting capsules.

Table 6.6 Release behavior of freeze dried ChPI- and LPI-based microcapsules triggered by pH, ionic strength and gastrointestinal environments. Values represent the percentage of encapsulated oil released. Data represent the mean ± one standard deviation (n = 3).

Capsule	**Release conditions**				
a) Effect of pH					
	3.0	5.0	7.0	9.0	
ChPI, pH 3.0	$6.2 \pm 0.1^{a,b}$	$3.4 \pm 0.3^{a,b}$	7.4 ± 0.4^{a}	7.6 ± 0.3^{a}	
LPI, pH 3.0	5.6 ± 0.2^{a}	2.6 ± 0.6^{a}	7.6 ± 0.5^{a}	7.2 ± 0.2^{a}	
ChPI, pH 7.0	6.4 ± 0.2^{b}	4.5 ± 0.5^{b}	9.2 ± 0.2^{b}	9.2 ± 0.6^{b}	
LPI, pH 7.0	5.6 ± 0.4^{a}	$3.6 \pm 0.5^{a,b}$	$8.5 \pm 0.6^{a,b}$	$8.6 \pm 0.8^{a,b}$	
b) Effect of ionic strength (mM NaCl)					
	0	50	100	150	200
ChPI, pH 3.0	4.2 ± 0.1^{a}	6.9 ± 0.5^{a}	$7.8 \pm 0.3^{a,b}$	8.7 ± 0.5^{a}	9.2 ± 0.4^{a}
LPI, pH 3.0	4.1 ± 0.4^{a}	6.2 ± 0.8^{a}	6.9 ± 0.5^{a}	9.3 ± 0.4^{a}	9.1 ± 0.4^{a}
ChPI, pH 7.0	5.1 ± 0.5^{a}	7.4 ± 0.2^{a}	8.6 ± 0.5^{b}	9.4 ± 0.2^{a}	10.0 ± 0.8^{a}
LPI, pH 7.0	4.6 ± 0.6^{a}	7.2 ± 0.7^{a}	$8.2 \pm 0.8^{a,b}$	9.1 ± 0.1^{a}	9.2 ± 0.3^{a}
c) Effect of simulated gastrointestinal conditions					
	SGF	SGF+SIF			
ChPI, pH 3.0	$37.5 \pm 0.5^{a,b}$	85.6 ± 3.8^{a}			
LPI, pH 3.0	$38.7 \pm 2.4^{a,b}$	92.6 ± 4.1^{a}			
ChPI, pH 7.0	43.4 ± 3.3^{b}	90.0 ± 4.7^{a}			
LPI, pH 7.0	36.6 ± 2.2^{a}	84.5 ± 3.0^{a}			

Means in each column followed by different letters were significantly different ($p<0.05$).
Abbreviations: SGF, simulated gastric fluid; SIF, simulated intestinal fluid.

7. ENCAPSULATION OF FLAXSEED OIL BY SPRAY DRYING WITHIN LEGUME PROTEIN-MALTODEXTRIN MICROCAPSULES

7.1 Abstract

Flaxseed oil was microencapsulated employing a wall material matrix of either chickpea (CPI) or lentil protein isolate (LPI) and maltodextrin followed by spray drying. Effects of emulsion formulation (oil and maltodextrin levels) and protein source (CPI vs. LPI; 4.0%) on the physicochemical characteristics, oxidative stability, and release properties of the resulting capsules were investigated. Microcapsule formulation containing higher oil levels (20%) were found to have higher surface oil and lower encapsulation efficiencies. Overall, LPI-maltodextrin capsules gave higher flaxseed oil encapsulation efficiencies (~88.0%) relative to CPI-maltodextrin matrices (~86.3%). However, both designs were found to provide encapsulated flaxseed oil protection against oxidation over a 25 d room temperature storage study relative to free oil. Flaxseed oil release from the microcapsules was found to be triggered by both pH (3.0-9.0) and ionic strength (0-200 mM NaCl) in a similar manner, regardless of the protein isolate source. Percent oil released from the microcapsules was found to be the lowest for pH 5.0 at ~2.7%, and increased to a maximum of ~6.8% at pH 9.0. In the presence of NaCl, flaxseed oil release was found to increase (from ~3.6% to ~8.8%) with ionic strength over the range studied (0-200 mM). Overall, ~37.6% of encapsulated flaxseed oil was released after 2 h under simulated gastric fluid (pH 1.2 + 0.32% pepsin), followed by the release of an additional ~46.6% over a 3 h period under simulated intestinal fluid (pH 6.8 + 10.0% pancreatin) conditions.

7.2 Introduction

Canada is the largest producer and exporter of flaxseed (*Linum usitatissimum*), with Saskatchewan accounting for approximately 70% of Canada's total production (Anon., 2011). Flaxseed oil represents a rich source of polyunsaturated fatty acids (PUFAs) (e.g., α-linolenic acid), which have been positively correlated with a variety of human health benefits, such as reducing the risk of coronary heart diseases (Li et al., 2003), protection against inflammation (Bloedon et al., 2008) and prevention of certain types of cancer (Bougnoux and Chajès, 2003). However, its use in foods has been hindered due to its lack of miscibility in aqueous systems,

susceptibility to oxidation, and distinct flavour. Microencapsulation technology offers a means to circumvent these problems by protecting flaxseed oil PUFAs against oxidation, improving their aqueous miscibility and masking its taste. Similar technology has been examined for PUFAs protection in fish oils (Hogan et al., 2003, Kagami et al., 2003; Klinkesorn et al., 2005; Drusch et al., 2006; Kolanowski et al., 2006; Jafari et al., 2008; Anwar and Kunz, 2011; Pop, 2011; Wan et al., 2011).

Spray drying is one of the most common steps used in the production of microencapsulated food ingredients (Pegg and Shahidi, 2007). Spray-drying involves the atomization of an emulsion into a wall material (e.g., whey protein isolate, gum Arabic, maltodextrin, etc.) under a hot air current, resulting in rapid water evaporation and instantaneous entrapment of the core material (Gharsallaoui et al., 2007). Flaxseed oil has been previously encapsulated by spray drying within a variety of wall materials, such as whey protein isolate (Partanen et al., 2008), gum Arabic (Tonon et al., 2011), zein (Quspe-Condori et al., 2011) and sodium caseinate/lactose (Pu et al., 2011). Partanen et al. (2008) employed spray drying during flaxseed oil encapsulation with a whey protein isolate, and investigated powder characteristics and oxidative stability at relative humidities between 0% and 91% at 37°C. The authors concluded that the oxidation of microencapsulated flaxseed oil within whey protein isolate was reduced when compared to free oil; but followed the same oxidation pattern as free oil with respect to relative humidity (RH). A high rate of oxidation was found for both low and high humidity conditions whereas the lowest rate of oxidation was found at a RH of 75%. Tonon et al. (2011) studied the effect of emulsion composition/properties and spray dryer inlet air temperature on the oxidative stability and entrapment efficiency of microencapsulated (gum Arabic) flaxseed oil. The authors reported that oil encapsulation efficiency increased (>84%) with high wall material content (26-30% total solids) and low oil concentrations (10-14% oil with respect to total solids). The authors noted that lipid oxidation was lower (0.047 meq peroxide/kg oil) at high emulsion viscosities (~0.1 Pa.s) and low droplet sizes (~2.27 μm). In addition, flaxseed oil oxidation increased concomitantly with increased (170 to 200°C) inlet air temperature of the spray drying process. Quispe-Condori et al. (2011) encapsulated flaxseed oil using zein as the wall material and spray and freeze drying. The authors optimized the microencapsulation process with respect to zein and flaxseed oil concentrations. Encapsulation efficiency (~93.3%) of the spray drying process was found to be higher than that observed for freeze drying (~59.6%). The bulk density of spray dried flaxseed oil was found to decrease with an increase in zein concentration at the same flaxseed oil concentration. Pu et al. (2011) spray dried flaxseed oil containing shrimp

astaxanthin using sodium caseinate and lactose as wall materials. The authors found that the oxidation of microencapsulated flaxseed oil containing astaxanthin was lower than that of microencapsulated flaxseed oil at 5, 25, and 40°C during 26 days of storage.

To best of our knowledge, the microencapsulation of flaxseed oil using legume proteins as wall materials has not been reported in literature. Chickpea and lentil proteins appear to be promising alternatives to animal proteins in encapsulation systems due to their nutritional value, low cost and possible beneficial health effects (e.g., reducing the risk of cardiovascular diseases, diabetes, digestive tract diseases, and obesity) (Duranti, 2006; Boye et al., 2010b). The major storage proteins in legume seeds are globulins and albumins. Globulins represent ~70% of the protein found in legume seeds and are classified as either 11S (legumins; S - Svedberg Unit) or 7S (vicilins) based on their sedimentation coefficients (Roy et al., 2010). Legumin is a hexameric protein with an overall molecular weight of 300-400 kDa whereas vicilin is a trimeric protein with a molecular weight between 150-180 kDa (Derbyshire et al., 1976). Albumins constitute 10–20% of the protein in legume seeds and can have variable molecular weights (16-483 kDa) (Papalamprou et al., 2010). In the present study, maltodextrin-DE 9 was used as a secondary wall material (i.e., filler) to improve microcapsule drying properties (Kagami et al., 2003). Maltodextrins are widely used as wall materials for capsule formation as they exhibit good solubility and low viscosities at high solids contents (Gharsallaoui et al., 2007). Our group has investigated the emulsifying (emulsion capacity, emulsion activity/stability indices, and creaming stability) and physicochemical properties (surface charge/hydrophobicity, protein solubility, interfacial tension, and droplet size) of chickpea (ChPI), faba bean (FPI), lentil (LPI), soy (SPI) and pea (PPI) protein isolates produced by isoelectric precipitation and salt extraction (Can Karaca et al., 2011b). We found that the ChPI and LPI produced by isoelectric precipitation had the highest surface charge and solubility, formed emulsions with smaller droplet sizes and showed high emulsifying activity and stability that were comparable to SPI. Based on these results, ChPI and LPI were selected as wall materials for this study. Maltodextrin was used as a secondary wall material (i.e., filler) so as to improve the drying properties of sprayed droplets by enhancing the formation of a dry crust around drying droplets (Bae and Lee, 2008).

The objectives of this study were to investigate the effects of oil concentration and wall material type on the physicochemical properties of microcapsules containing flaxseed oil as produced by spray drying. In addition the oxidative stability and release properties of microencapsulated flaxseed oil were assessed.

7.3 Material and Methods

7.3.1 Materials

Chickpea (CDC Frontier, Kabuli) and lentil (CDC Grandora) seeds were provided by the Crop Development Centre at the University of Saskatchewan (Saskatoon, SK, Canada). Maltodextrin (DE 9; Dry MD^{TM} 01918) was donated by Cargill Inc. (Cargill Texturizing Solutions, Cedar Rapids, IA, USA). Flaxseed oil was kindly donated by Bioriginal Food & Science Corp. (Saskatoon, SK, Canada). All chemicals used were of reagent grade and purchased from Sigma-Aldrich (Oakville, ON, Canada). The water used in this research was produced from a Millipore Milli-QTM water system (Millipore Corp., Milford, MA, USA).

7.3.2 Proximate analysis

Proximate composition analyses for protein isolates and maltodextrin-DE 9 were conducted according to AOAC Official Methods 925.10 (moisture), 923.03 (ash), 920.85 (lipid), and 920.87 (crude protein by using %N × 6.25) (AOAC, 2003). Carbohydrate content was determined on the basis of percent differential from 100%.

Proximate composition of maltodextrin

The chemical composition of maltodextrin-DE 9 was determined to be: 4.6% moisture, 0.0% protein, 0.0% lipid, 95.0% carbohydrate and 0.4% ash.

7.3.3 Protein isolate preparation

Whole chickpea and lentil seeds were ground into fine flour using an IKA A11 basic analytical mill (IKA Works Inc., Wilmington, NC, USA) for 1 min, and then defatted using hexane (1:3 [w/v] flour: hexane ratio) for 40 min. The mixture was then filtered through a 110 mm Whatman Gr. 1 filter (Whatman International Ltd., Maidstone, United Kingdom), and air-dried in a fume hood. This defatting procedure was repeated twice for each flour.

Chickpea protein isolate (ChPI) was prepared according to the method of Papalamprou et al. (2010). In brief, defatted flour (100 g) was mixed with water at 1:10 ratio (w/v), adjusted to pH 9.0 using 1.0 M NaOH and stirred at 500 rpm for 45 min at room temperature (21-23°C). The suspension was then centrifuged at 4,500 × *g* for 20 min at 4°C using a Sorvall RC-6 Plus centrifuge (Thermo Scientific, Asheville, NC, USA) to collect the supernatant. The resulting pellet was re-suspended in water at a ratio of 1:5 (w/v), adjusted to pH 9.0, stirred for an additional 45 min, followed by centrifugation (4,500 × *g*, 20 min, 4°C). Supernatants were pooled and adjusted to pH 4.6 using 0.1 M HCl to precipitate the protein.

The protein was recovered by centrifugation as above, collected and stored at −30°C until freeze-drying which was performed using a Labconco FreeZone 6 freeze drier (Labconco Corp., Kansas City, MO, USA) to yield a free flowing powder. Proximate analysis of ChPI showed a composition of, 85.40% protein, 6.52% moisture, 3.05% ash, 4.11% carbohydrate and 0.92% lipid.

Lentil protein isolate (LPI) was produced with a combined method of Bamdad et al. (2006) and Lee et al. (2007). In brief, defatted flour (100 g) was mixed with water at 1:10 ratio (w/v), adjusted to pH 9.5 with 1.0 M NaOH, and stirred at 500 rpm for 1 h at room temperature. The mixture was kept static at 4°C overnight to allow for the sedimentation of non-protein constituents. After centrifugation at 1,600 × *g* for 30 min at 4°C, the supernatant was collected; and pH was adjusted to 4.5 with 0.1 M HCl. The precipitated protein was collected by centrifugation (1,600 × *g*, 30 min, 4°C) and stored at −30°C until freeze-drying. Proximate analysis of LPI showed a composition of, 81.90% protein, 5.04% moisture, 3.63% ash, 9.00% carbohydrate and 0.43% lipid.

7.3.4 Emulsion preparation

Protein solutions were prepared by dispersing the isolates (corrected on a weight basis for protein content) in water followed by adjustment to pH 3.0 with 0.1 M HCl. The resulting mixtures were stirred at 500 rpm overnight at 4°C to ensure complete dispersion. Maltodextrin solutions were prepared by dispersing the samples in water followed by stirring at 300 rpm overnight at 4°C. Prior to the homogenization, pH of the protein solutions was re-adjusted to 3.0. Oil-in-water emulsions were prepared by homogenizing protein solutions, maltodextrin solution and flaxseed oil (Table 7.1) in a 500 mL container by using Omni Macro Homogenizer (Omni International, Marietta, GA, USA) with a 20 mm saw tooth generating probe at speed 4 (~7,200 rpm) for 10 min. Results from the corresponding formulations will be denoted by their oil content in the final powder (10, 15 and 20%) for discussion purposes.

Table 7.1 Formulations of ChPI- and LPI-stabilized emulsions before and after spray drying.

a) In initial emulsion

% Oil	% Protein	% Maltodextrin	% Total Solids	Core	Wall
2	4	14	20	1	9
3	4	13	20	1	5.7
4	4	12	20	1	4

b) In spray-dried powder

% Oil	% Protein	% Maltodextrin	% Total Solids
10	20	70	100
15	20	65	100
20	20	60	100

7.3.5 Droplet size measurements

Droplet size distributions of initial and reconstituted emulsions were measured using a Mastersizer 2000 laser light scattering instrument (Malvern Instruments Ltd., Worcestershire, United Kingdom) equipped with a Hydro 2000S sample handling unit (containing water). Emulsion samples were taken from the bottom of the container immediately after homogenization for analysis. The sample was stirred continuously within the sample cell to ensure homogeneity at room temperature. Obscuration in all the measurements was kept at ~14% by adding distilled water. Droplet size distributions were calculated by the instrument according to the Mie Theory which uses the refractive index difference between the droplets and the dispersing medium to predict the intensity of the scattered light. The ratio of refractive index of flaxseed oil (1.479) to that of the dispersion medium (1.330) was 1.112. Droplet size measurements were reported as volume-length mean diameters ($d_{4,3}$), which is expressed as:

$$d_{4,3} = \frac{\sum_{i=1} n_i \cdot d_i^{\,4}}{\sum_{i=1} n_i \cdot d_i^{\,3}} \qquad \text{[eq. 7.1]}$$

where n_i is the number of droplets of diameter (d_i) (McClements, 2005c).

Emulsion reconstitution

Spray-dried microcapsule samples of 0.5 g were dispersed in 4 mL of water and stirred at 500 rpm for 5 min. Samples were withdrawn for particle size distribution measurements performed as described above.

7.3.6 Spray drying

The emulsion samples were spray-dried by a mini spray drier B-290 (Büchi Labortechnik AG, Flawil, Switzerland) with an atomizer nozzle of 700 μm diameter. The dryer had an evaporation rate of 1 L/h and a chamber with diameter of 70 cm. The inlet air temperature was adjusted to 180°C, and the outlet temperature was kept at 90 ± 3°C by controlling the flow rate. In order to maintain homogeneity and to prevent coalescence of oil droplets, the emulsions were gently stirred using a magnetic stirrer while fed into the spray dryer. The spray-dried microcapsules were collected in the cyclone collection vessel.

7.3.7 Microcapsule characterization

Moisture content and water activity:

Moisture content of spray-dried microcapsules was determined gravimetrically, after drying the capsules in a forced-air oven at 105°C for ~12 h, whereas the water activity was determined using an AquaLab CX-2 water activity meter (Decagon Devices, Inc., Pullman, WA, USA).

Colour measurements:

The colour values of spray-dried microcapsules were measured using a Hunter colourimeter (ColorFlex EZ 45/0, Hunter Associates Laboratory, Inc., Reston, VA, USA), which was standardized using a reference white tile. The results were expresses as L (lightness), a (redness), and b (yellowness) tristimulus values.

Microcapsule surface and total oil content:

Surface oil of the microcapsules was determined according to the method of Liu et al. (2010). Briefly, 2 g of microcapsules was dispersed in 30 mL of hexane followed by vigorous shaking for 30 s. The solvent was filtered through a Whatman Gr. 1 paper into a 40 mL beaker, and the beaker plus solvent was placed in a fume hood overnight to afford solvent evaporation. Microcapsule surface oil was then determined gravimetrically, after heating the beaker at 105°C for 30 min to remove any residual solvent. Total oil content of the microcapsules was determined using a method described by Klinkesorn et al. (2006) with some modifications. Briefly, 8 mL of water was added to 2 g of microcapsules followed by mixing at 300 rpm for 2 min. The resulting solution was then mixed with 40 mL hexane/isopropanol (3:1 v/v), stirred at 300 rpm for 15 min and centrifuged at 1500 × *g* for 2

min. The clear organic phase was collected and the aqueous phase was re-extracted with the solvent mixture. The organic phases were pooled and filtered through anhydrous Na_2SO_4, and then the solvent was allowed to evaporate overnight in a fume hood. The amount of total oil was determined gravimetrically, after heating the beaker at 105°C for 30 min. The flaxseed oil encapsulation efficiency (EE) was calculated from the quantitative determinations as follows (Anwar and Kunz, 2011):

$$EE = \frac{Total\ oil - Surface\ oil}{Total\ oil} \times 100\% \qquad \text{[eq. 7.2]}$$

7.3.8 Oxidative stability

Oxidative stability of free (i.e. control) and encapsulated flaxseed oil was characterized during storage at room temperature over a 25 d period using the peroxide value and 2-thiobarbituric acid reactive substances tests. Microcapsules (~5 g/bottle) or bulk oil (~3 mL) were stored within individually sealed nitrogen-flushed 10 mL amber glass bottles for storage stability studies. Oxidative testing was carried out every 5 d over the 25 d testing period, using a separate unopened bottle of microcapsules and oil. Extraction of flaxseed oil from the microcapsules followed the same procedure as that described previously for total oil determination, except the solvent was dried under a stream of nitrogen.

Peroxide value (PV):

In brief, ~0.2 g of sample oil was weigh into a 250 mL Erlenmeyer flask, followed by the addition of 30 mL of 3:2 acetic acid/chloroform (v/v) solution and 0.5 mL of saturated potassium iodide (KI). After vigorous shaking for exactly 1 min, 30 mL of water was added to this mixture. Half a millilitre aliquot of 1% (w/v) starch indicator was then added to the mixture, and the resulting solution was titrated using 0.001 N sodium thiosulfate (Na2S2O3) until the purple colour disappeared. PV was calculated as:

$$PV = \frac{(S - B) \times N \times 1000}{W} \qquad \text{[eq. 7.3]}$$

where S is the volume of $Na_2S_2O_3$ added to the sample, B is the volume of $Na_2S_2O_3$ of the blank, N is the normality of $Na_2S_2O_3$ solution, and W is the sample weight (g). PV was expressed as meq active O2 (peroxide milliequivalent) per kg sample (Pegg, 2005).

2-thiobarbituric acid reactive substances (TBARS):

In brief, ~40 mg of extracted flaxseed oil was weighed into a 10 mL volumetric flask and was dissolved and brought to volume with n-butanol. To a 2.0 mL Eppendorf tube was added, 50 μL of 8.1% (w/v) SDS, 375 μL of 20% acetic acid, 375 μL of 0.8% (w/v) TBA, 8.25 μL of 0.02% (w/v) BHT (in DMSO) and 200 μL of the oil-butanol mixture. A standard curve was prepared using malondialdehyde (MDA) (1.25-50 μM) under the same experimental conditions. Samples and standards were then heated at 95°C for 1 h. After cooling in cold water, 0.9 mL of n-butanol/pyridine (15:1, v/v) was added, followed by vigorous shaking for 30 s. Samples and standards were centrifuged at 4000 × *g* for 10 min, and the upper organic layer was transferred to a 1.5 mL cuvette and the absorbance at 532 nm was measured against a butanol blank. TBA values were expressed as mg MDA eq/mg oil, which equates to the reactive aldehyde content (nmol)/sample oil weight (mg) (modified from Pegg, 2005 and Akhlaghi and Bandy, 2010).

7.3.9 Release characteristics

Release behaviour of the flaxseed oil from the microcapsules triggered by pH and ionic strength was determined by a combined method of Zhong and Jin (2009) and Choi et al. (2010). In brief, microcapsule samples of 5 g were dispersed in 50 mL of aqueous NaCl solutions (0, 50, 100, 150 and 200 mM) or water (pH adjusted to 3.0, 5.0, 7.0 or 9.0 with 0.1 M HCl or NaOH) followed by stirring at 500 rpm for 1 h. The amount of released oil was determined by gravimetric analysis after two 30 mL hexane extractions.

In-vitro release behaviour of microencapsulated flaxseed oil was also investigated by using a simulated gastrointestinal model according to the method of Burgar et al. (2009). Simulated gastric fluid (SGF) was prepared by dissolving 2.0 g NaCl and 7.0 mL 36% HCl in 900 mL of water. After the addition of 3.2 g pepsin to this solution, pH was adjusted to 1.2 with 0.1 M HCl and the final volume was made up to 1000 mL with water. Simulated intestinal fluid (SIF) was prepared by dissolving 6.8 g K_2HPO_4 in 800 mL of water. After addition of 77 mL 0.2 M NaOH and 100.0 g pancreatin, the solution was left stirring overnight at 4°C. The pH was adjusted to 6.8 with 1 M NaOH or 1 M HCl and the final volume was made up to 1000 mL with water.

Microencapsulated flaxseed oil sample of 5 g was mixed with 50 mL of SGF and incubated for 2 h at 37°C and 100 rpm in a water bath. Released oil was extracted using hexane and then determined gravimetrically. For exposure to SGF and SIF in sequence, 5 g of microcapsule sample was mixed with 50 mL of SGF and incubated under same conditions for

2 h. The pH was adjusted to 6.8 using 1 M NaOH, followed by addition of 50 mL of SIF, and the sample was incubated under the same conditions for another 3 h. The amount of flaxseed oil released from the microcapsules was determined at the end of exposure to SGF and SIF. The amount of released oil was determined by gravimetric analysis as outlined above.

7.3.10 Statistical analyses

Three replicates were measured on duplicate batches of capsules. All experiments were reported as the mean ± one standard deviation. A two-way analysis of variance (ANOVA) with a Scheffe post-hoc test was used to measure statistical differences in microcapsule characteristics and oxidative stability as a function of protein source and oil concentration. A general linear model was employed to determine statistical differences in release profile of the microcapsules as a function of protein source, oil concentration, pH or ionic strength of the release medium. All statistical analyses were performed with SPSS version 17.0 software (SPSS Inc., 2008, Chicago, IL, USA).

7.4 Results and Discussion

7.4.1 Physicochemical characteristics of microcapsules

The moisture contents and water activities of spray dried produced ChPI and LPI microcapsules containing flaxseed oil are shown in Table 7.2. The moisture content of the microcapsules ranged between 3.65 and 4.12% and their water activity varied from 0.05 to 0.08. These results meet both the maximum moisture and water activity specifications for dried powders in the food industry which are 3-4% and ~0.3, respectively (*38*). The Hunter *L* (lightness), *a* (redness), and *b* (yellowness) tristimulus colour values of spray-dried microcapsules containing flaxseed oil differed significantly for ChPI and LPI as shown in Table 7.3 ($p<0.05$). ChPI-microcapsules were creamy in surface colour, which was demonstrated by a mean *L, a, b* values of 91.3, -0.3, and 9.1, respectively. Whereas, LPI-microcapsules were darker (beige) in colour with mean *L, a, b* values of 87.2, 1.6, and 11.4, respectively. The observed darker colour of the LPI-microcapsules containing may be explained by the hull colour of the lentil proteins used in isolate production (Bamdad et al., 2006).

Table 7.2 Moisture content (%) and water activity of spray-dried flaxseed oil microcapsules. Data represent the mean ± one standard deviation (n = 6).

Oil (%)	Moisture Content (%)		Water Activity	
	ChPI	*LPI*	*ChPI*	*LPI*
10	3.66 ± 0.32	4.12 ± 0.31	0.08 ± 0.01	0.08 ± 0.01
15	4.07 ± 0.31	3.89 ± 0.23	0.08 ± 0.00	0.05 ± 0.01
20	3.71 ± 0.46	3.65 ± 0.10	0.06 ± 0.00	0.06 ± 0.01

Table 7.3 The Hunter colour values of spray-dried microcapsules. Data represent the mean ± one standard deviation (n = 6).

Oil (%)	ChPI Microcapsules			LPI Microcapsules		
	L	*a*	*b*	*L*	*a*	*b*
10	89.2 ± 0.0	-0.5 ± 0.0	10.4 ± 0.0	87.5 ± 0.0	1.6 ± 0.0	11.4 ± 0.0
15	92.9 ± 0.0	-0.1 ± 0.0	8.5 ± 0.0	87.8 ± 0.0	1.4 ± 0.0	11.0 ± 0.0
20	91.8 ±0.0	-0.4 ± 0.0	8.4 ± 0.0	86.3 ± 0.0	1.6 ± 0.0	11.8 ± 0.0

The presence of oil on the microcapsule surface has been shown to have an adverse effect on several characteristics of spray-dried powders such as flow, dispersion and oxidative stability (Bae and Lee, 2008). The effect of emulsion formulation on both surface oil content and encapsulation efficiency is presented in Table 7.4. The lowest surface oil and highest encapsulation efficiency for flaxseed oil with either plant protein wall material was observed at an initial oil concentration of 10%; with values of 1.13 and 1.08% for surface oil and 88.72 and 90.42% for encapsulation efficiency for ChPI and LPI, respectively. An analysis of variance ($p<0.05$) indicated that as the amount of flaxseed oil used in the emulsion formulation increased (from 10 to 20%), surface oil increased whereas the encapsulation efficiency decreased for both ChPI and LPI. The observed increase in surface oil as a function of oil content in the emulsion formulation was in accordance with the findings of Rusli et al. (2006) and Polavarapu et al. (2011), both of whom reported lower encapsulation efficiencies

at higher oil concentrations. The authors postulated that this was a result of having an insufficient amount of wall material for complete coverage of the emulsified oil droplets.

Table 7.4 Changes in surface oil and encapsulation efficiency as a function of emulsion formulation. Data represent the mean ± one standard deviation (n = 6).

Oil (%)	Surface Oil (%)		Encapsulation Efficiency	
	ChPI	*LPI*	*ChPI*	*LPI*
10	1.13 ± 0.07^{a}	1.05 ± 0.08^{a}	88.72 ± 0.69^{a}	90.42 ± 0.64^{a}
15	1.49 ± 0.11^{b}	1.45 ± 0.12^{b}	86.69 ± 0.95^{b}	87.89 ± 0.96^{b}
20	2.64 ± 0.04^{c}	2.49 ± 0.07^{c}	83.62 ± 0.24^{c}	85.61 ± 0.40^{c}

Means in each column followed by different letters were significantly different ($p<0.05$).

The mean droplet diameter of CPI- and LPI-stabilized emulsions before spray-drying and after reconstitution are shown in Figure 7.1. Experimental results showed that the *volume*-weighted mean oil droplet diameters ($d_{4,3}$) of flaxseed oil-in-water emulsions stabilized by CPI and LPI ranged between 16.3-24.0 and 21.0-26.1 μm, respectively. An analysis of variance of droplet size indicated that the main effects of total oil concentration ($p<0.001$) and sample conditions (i.e., those found in fresh vs. reconstituted emulsions) ($p<0.01$) were found to be significant, whereas protein-type (CPI vs. LPI) ($p>0.05$) was not. Furthermore, all 2-way interaction terms were found to be significant ($p<0.001$). For clarity, only the main effects will be discussed. As the total oil content increased within the sample, size of the droplets increased significantly from ~15.8 μm at the 2% level within the emulsion (10% in the reconstituted capsules) to 24.6 μm at the 3- 4% level within the emulsion (15-20% in the reconstituted capsules) ($p<0.001$). Droplet size was similar at the two higher oil concentrations ($p>0.05$). Overall, droplet size was found to be reduced from ~22.4 μm in the fresh emulsion to ~21.0 μm in the reconstituted emulsion ($p>0.01$).

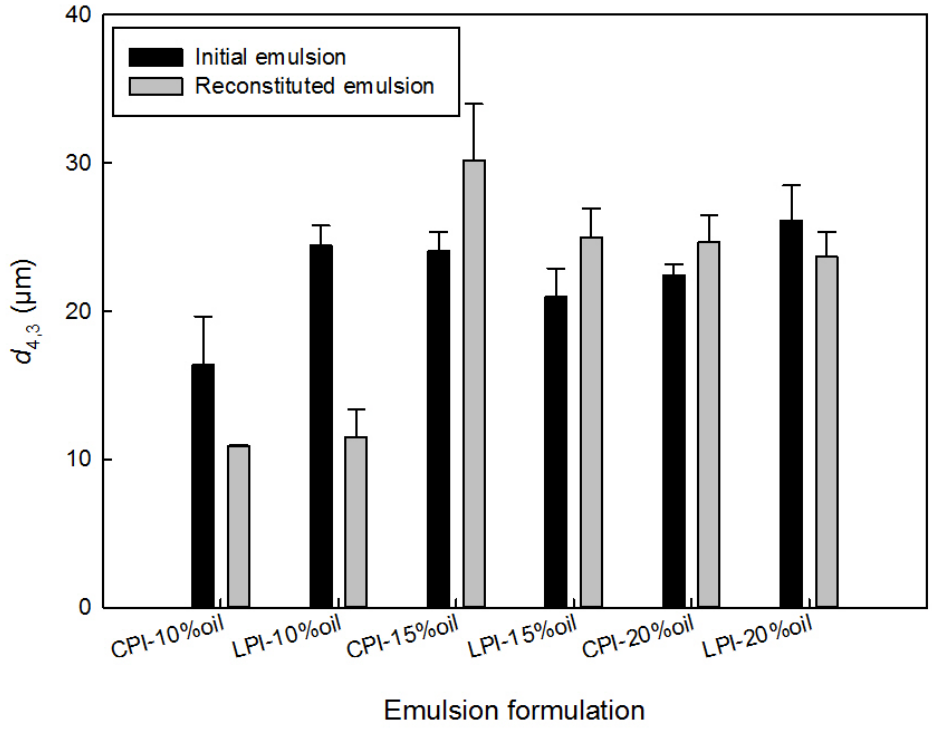

Figure 7.1 Effect of spray drying on mean droplet diameter for ChPI- and LPI-stabilized emulsions. Data represent the mean ± one standard deviation (n = 6).

7.4.2 Oxidative stability of encapsulated flaxseed oil

The peroxide value (PV) and TBARS results for free and CPI and LPI encapsulated flaxseed oil maintained at room temperature over a 25 d period are presented in Table 5. Primary oxidation products, mainly peroxides, are highly reactive and readily break down to free radicals, which propagate oxidation reactions. They also participate in the autoxidation process producing a variety of secondary oxidation products, such as aldehydes and ketones (Pegg, 2005). The PV of flaxseed oil before microencapsulation was 5.73 ± 0.30 meq active O_2/kg while that of the microencapsulated oil immediately after spray drying (Day 0) ranged from 6.31-6.80 meq active O_2/kg. This increase in PV value for flaxseed oil during the microencapsulation process can be attributed to oxygen contact with oil during the emulsification and spray drying processes. Similar results have been reported for the encapsulation of fish oil in modified cellulose (Kolanowski et al., 2006), and a blend of n-octenylsuccinate-derivatized starch and glucose syrup (Pop, 2011). The PV results for both CPI and LPI microencapsulated flaxseed oil versus that of free oil were significantly different at storage days 15 to 25 ($p<0.05$) (Table 5). This is illustrated by the PV values for free, CPI and LPI microencapsulated flaxseed oil values at storage day 25 of, 7.31 ± 0.56, 6.86 ± 0.40

and 12.91 ± 0.40 meq active O_2/kg, respectively. These results clearly show that the plant protein microencapsulation process employed in this study provides significant protection to flaxseed oil oxidation during a 25 d storage period at room temperature. Modest increases in PV values for microencapsulated flaxseed oil were observed for both CPI and LPI, however these changes were not found to be significant. In addition, no significant differences in PV values in microencapsulated flaxseed oil was found between the two plant protein sources. On the other hand, the PV of bulk oil started to increase from 6.12 to 9.38 meq active O_2/kg at day 15 and kept increasing to 11.28 meq active O_2/kg at day 20 and finally to 12.91 meq active O_2/kg at day 25 ($p<0.05$, Table 5).

In this study, the TBARS test was employed to measure the secondary oxidation products of free and CPI and LPI microencapsulated flaxseed oil. The TBARS value of flaxseed oil before microencapsulation was 2.21 ± 0.15 MDA eq/mg oil while that of the microencapsulated oil immediately following spray drying (Day 0) ranged from 1.99-2.14 MDA eq/mg oil. These results were not significantly different showing that the microencapsulation process had no effect on the formation of secondary oxidation products in flaxseed oil. The TBARS value of microencapsulated flaxseed oil in both CPI- and LPI-containing microcapsules was between 1.90-2.47 MDA eq/mg oil and did not change over the 25 day storage period ($p>0.05$, Table 5). In contrast, TBARS value of bulk oil started to increase from 2.29 to 3.15 nmol MDA eq/mg oil at day 20 and kept increasing to 3.95 MDA eq/mg oil at day 25 ($p<0.05$, Table 5); indicating an increase in secondary oxidative products such as aliphatic aldehydes, alcohols, ketones, cyclic compounds, and hydrocarbons (Table 5). These results clearly show that the legume protein-maltodextrin matrices tested improved the oxidative stability of flaxseed oil when compared to bulk oil as indicated by both primary and secondary oxidative products during a 25 d storage period at room temperature. To our knowledge, spray drying of flaxseed oil using legume proteins as wall materials has not been reported yet. Partanen et al. (2008) spray dried flaxseed oil using whey protein isolate matrix and found that oxidation of microencapsulated flaxseed oil was retarded compared to that of bulk oil. Pu et al. (2011) reported that oxidative stability of flaxseed oil microencapsulated in a sodium caseinate and lactose matrix was further improved by addition of shrimp astaxanthin into flaxseed oil prior to microencapsulation.

Table 7.5 Changes in a) peroxide value (PV) and b) thiobarbituric acid-reactive substances (TBARS) for free and microencapsulated flaxseed oil. Data represent the mean ± one standard deviation (n = 6).

	Protein source in the microcapsule	Oil (%)[1]	Day 0	Day 5	Day 10	Day 15	Day 20	Day 25
PV (meq active O_2/kg)	**ChPI**	10	6.33 ± 0.10[a]	6.23 ± 0.14[a]	6.45 ± 0.23[a]	6.62 ± 0.21[a]	6.48 ± 0.32[a]	6.68 ± 0.36[a]
		15	6.43 ± 0.22[a]	6.34 ± 0.37[a]	6.51 ± 0.33[a]	6.80 ± 0.36[a]	6.69 ± 0.35[a]	6.71 ± 0.55[a]
		20	6.80 ± 0.21[a]	7.08 ± 0.23[a]	7.18 ± 0.26[a]	7.36 ± 0.31[a]	7.27 ± 0.26[a]	7.31 ± 0.56[a]
	LPI	10	6.31 ± 0.23[a]	6.29 ± 0.21[a]	6.42 ± 0.33[a]	6.57 ± 0.24[a]	6.54 ± 0.21[a]	6.62 ± 0.40[a]
		15	6.47 ± 0.25[a]	6.34 ± 0.22[a]	6.52 ± 0.30[a]	6.75 ± 0.38[a]	6.62 ± 0.26[a]	6.82 ± 0.31[a]
		20	6.73 ± 0.24[a]	6.84 ± 0.21[a]	6.74 ± 0.24[a]	6.89 ± 0.32[a]	6.99 ± 0.35[a]	6.86 ± 0.46[a]
	Free oil	—	5.73 ± 0.30[a]	6.25 ± 0.16[a]	6.37 ± 0.14[a]	9.38 ± 0.75[b]	11.28 ± 0.36[c]	12.91 ± 0.40[d]
TBARS (nmol MDA eq./mg oil)	**ChPI**	10	2.04 ± 0.27[a]	1.90 ± 0.22[a]	2.20 ± 0.15[a]	2.24 ± 0.18[a]	1.92 ± 0.25[a]	2.13 ± 0.24[a]
		15	2.00 ± 0.27[a]	2.07 ± 0.23[a]	2.29 ± 0.18[a]	2.18 ± 0.24[a]	2.10 ± 0.26[a]	2.22 ± 0.24[a]
		20	2.14 ± 0.21[a]	2.22 ± 0.18[a]	2.34 ± 0.14[a]	2.33 ± 0.16[a]	2.24 ± 0.24[a]	2.47 ± 0.18[a]
	LPI	10	2.03 ± 0.15[a]	2.03 ± 0.19[a]	2.12 ± 0.26[a]	1.91 ± 0.24[a]	2.21 ± 0.19[a]	2.13 ± 0.25[a]
		15	1.99 ± 0.18[a]	1.99 ± 0.20[a]	2.16 ± 0.35[a]	2.09 ± 0.27[a]	2.14 ± 0.22[a]	2.22 ± 0.24[a]
		20	2.10 ± 0.22[a]	2.02 ± 0.26[a]	2.37 ± 0.24[a]	2.22 ± 0.17[a]	2.31 ± 0.21[a]	2.40 ± 0.27[a]
	Free oil	—	2.21 ± 0.15[a]	2.13 ± 0.20[a]	2.42 ± 0.16[a]	2.47 ± 0.09[a]	3.15 ± 0.27[b]	3.94 ± 0.30[c]

[1] Concentration of oil in the microcapsule.

Means in each row followed by different letters were significantly different ($p<0.05$).

7.4.3 Release characteristics of spray-dried flaxseed oil

The relationship between flaxseed oil release from CPI- and LPI-microcapsules as a function of pH, ionic strength and simulated gastrointestinal environments are shown in Figures 7.2, 7.3 and 7.4, respectively. For pH-triggered release, an analysis of variance showed that protein source, oil concentration, pH of the release medium and interactions between these factors were highly significant ($p<0.001$). Percentage of oil released was found to be the lowest for pH 5.0 at ~2.7%, and increased in conjunction with pH to 6.8% at pH 9.0. Lowest amount of oil released at pH 5.0 could be arising from the low solubility of CPI and LPI at pH values close to their isoelectric point whereas increased amounts of released oil from the microcapsules at lower (pH 3.0) and higher pH values (pH 7.0-9.0) could be attributed to increased solubility of CPI and LPI at these regions. In case of salt-triggered release, an analysis of variance indicated that protein source, oil concentration, ionic strength of the release medium plus the interactions between protein source-oil concentration and protein source-ionic strength were significant ($p<0.05$). Percentage of oil released was found to be the lowest at 0 mM NaCl and increased with increasing ionic strength ($p<0.05$), as it was assumed the addition of NaCl promoted protein solubility through increasing ordering of water molecules around the capsule's surface (i.e., salting-in effect) (Arakawa and Timasheff, 1984). Increased amounts of oil in the microcapsules resulted in increased amounts of released oil ($p<0.05$) regardless of the protein source used in the microcapsules and ionic strength of the release medium.

The in-vitro release behaviour of CPI- and LPI-microencapsulated flaxseed oil was investigated employing both simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) models. Overall, CPI and LPI microcapsules released ~37% of encapsulated flaxseed oil after 2 h in pepsin-containing simulated gastric fluid (pH 1.2), with a further ~47% release after 3 h in pancreatin-containing simulated intestinal fluid (pH 6.8). The differences in the amount of oil released are thought to the different susceptibilities of the matrix components to digestion by different enzymes. For the CPI and LPI microcapsules, no significant differences ($p>0.05$) in the amount of oil released under these experimental condition was observed with respect to the oil concentration (10-20%) of the microcapsules.

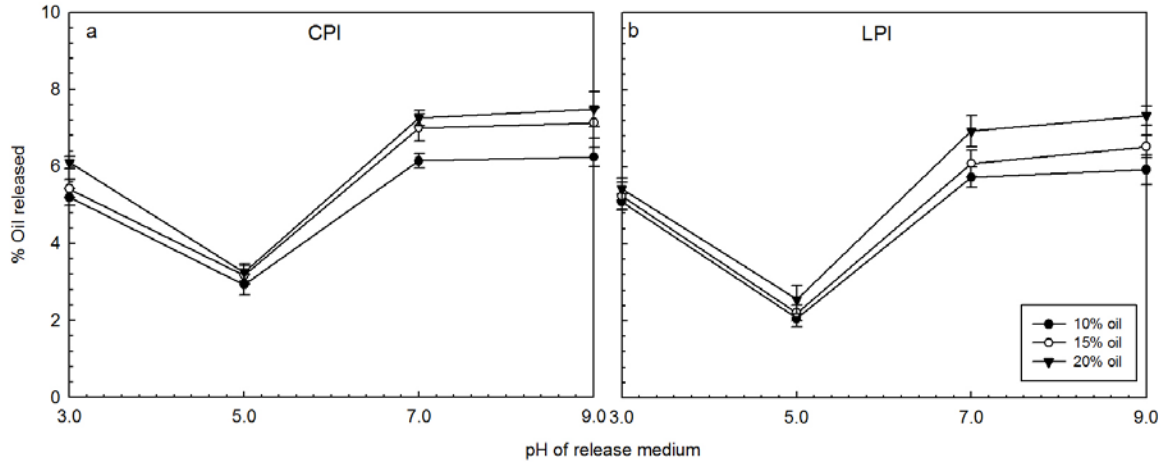

Figure 7.2 Release behavior of flaxseed oil microcapsules containing a) ChPI; b) LPI, triggered by pH. Data represent the mean ± one standard deviation (n = 6).

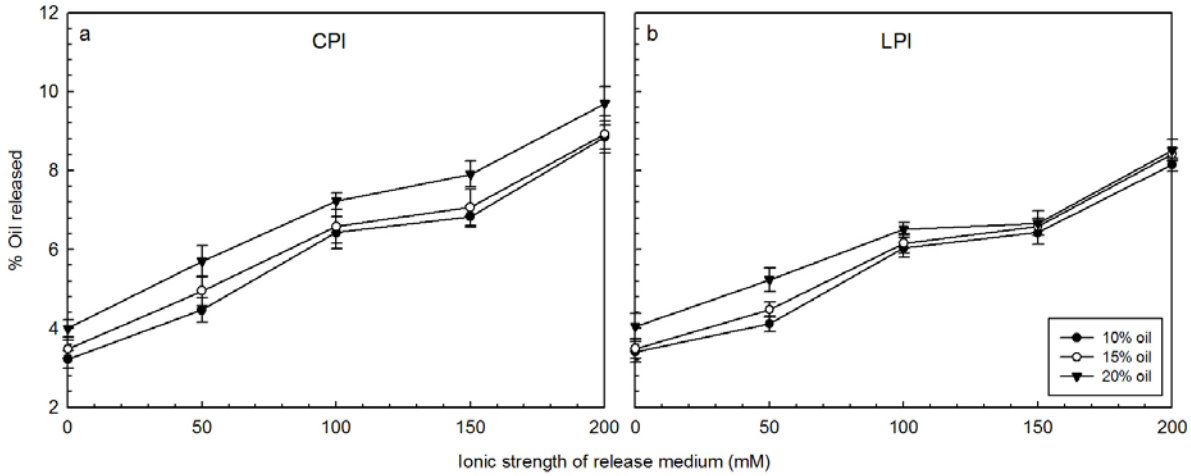

Figure 7.3 Release behavior of flaxseed oil microcapsules containing a) ChPI; b) LPI, triggered by ionic strength. Data represent the mean ± one standard deviation (n = 6).

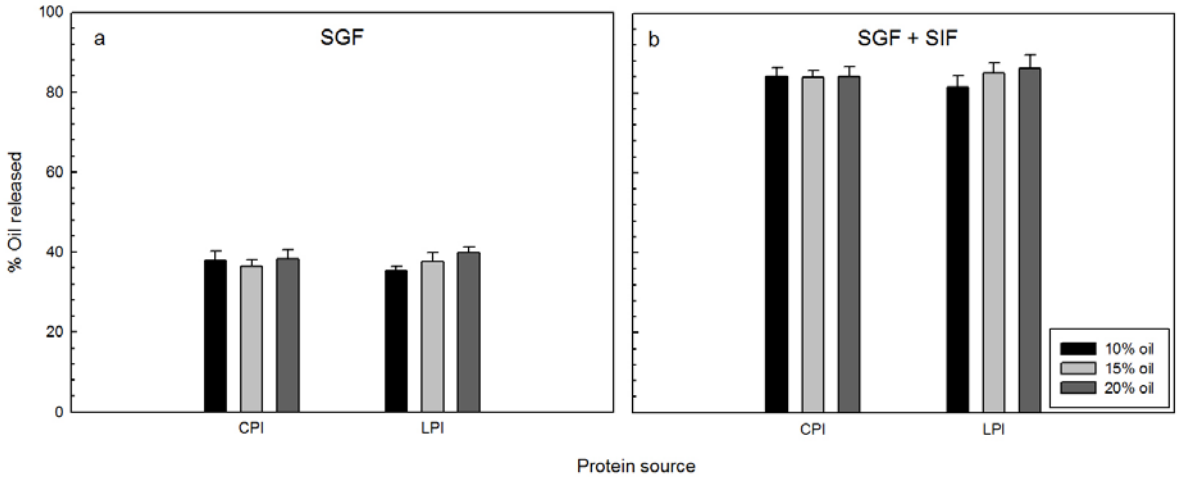

Figure 7.4 Release behavior of flaxseed oil microcapsules containing ChPI and LPI in a) simulated gastric fluid (SGF); b) sequential exposure to simulated gastric and intestinal fluids (SGF + SIF). Data represent the mean ± one standard deviation (n = 6).

7.5 Conclusions

In conclusion, findings of the present study indicated that CPI- and LPI-based microcapsule formation was efficient for the entrapment and gastrointestinal delivery of flaxseed oil. Oil concentration and protein source had significant effects on physicochemical characteristics, encapsulation efficiency and release characteristics of microcapsules prepared by spray drying flaxseed oil using CPI or LPI and maltodextrin as wall materials. Encapsulation matrices tested showed a protective effect against oxidation over a 25 d period of room temperature storage. Microcapsules were able to deliver 84.2% of the encapsulated oil within the gastrointestinal environments. The findings suggest that the legume protein-based microcapsule systems tested were capable of carrying, protecting and delivering flaxseed oil. This study also identified opportunities for increasing the utilization of flaxseed oil and legume proteins in food and bioproduct applications.

8. GENERAL DISCUSSIONS

The overarching goal of this research was to develop a plant protein-based microcapsule capable of carrying, protecting and delivering flaxseed oil within the food and gastrointestinal environments. For this purpose, a variety of plant proteins including legume proteins such as chickpea, faba bean, lentil, and pea, and oilseed proteins such as canola and flaxseed were screened for their ability to form and stabilize flaxseed oil-in-water emulsions.

The physicochemical characteristics of the legume proteins, especially surface hydrophobicity and net surface charge have been found to greatly influence their emulsifying properties (Schwenke, 2001; Sikorski, 2001). It has been shown that the isolate production method has a significant effect on protein functionality, since it may influence the globulin/albumin ratio and the physicochemical characteristics of the protein (Papalamprou et al., 2010). In our study, both the protein source and method of isolate production showed significant effects on the emulsifying and physicochemical properties of the proteins tested.

In case of legume proteins tested, isoelectric-precipitated proteins showed greater surface hydrophobicity relative to the salt-extracted proteins in the case of ChPI, LPI, and PPI. Differences in surface hydrophobicity between the two extraction methods were attributed to the variances in their composition, which influence total protein surface characteristics (i.e., number of exposed hydrophilic and hydrophobic groups, folding and aggregation). Isoelectric precipitation also resulted in isolates with higher surface charge and solubility compared to those produced via salt extraction. Variations in solubility have been previously reported for isolates produced by different extraction methods (Boye et al., 2010). The EC, EAI and ESI values of protein isolates were all affected by surface charge and hydrophobicity and solubility; whereas their creaming stabilities were related to surface charge, solubility and droplet size. Differences in emulsifying properties in the present study are thought to reflect differences in protein composition and physicochemical properties induced by the different extraction methods.

For the oilseed proteins tested, canola and flaxseed protein isolates showed significantly higher surface hydrophobicity and lower solubility when compared to WPI ($p<0.001$). The salt-extracted isolates were found to have significantly higher solubilities relative to those prepared by isoelectric precipitation. Aluko and McIntosh (2001) also

reported low solubility values for acid-precipitated CaPI, and higher solubility for the calcium-precipitated isolate. Krause et al. (2002) suggested that protein denaturation during acid precipitation occurred, and interactions between protein and non-protein components accounted for the lower solubility observed in isoelectric-precipited flaxseed protein isolate. CaPI showed significantly higher EC than FlPI which was comparable to WPI. However, EAI and ESI values for CaPI and FlPI were significantly lower than that of WPI. CaPI and FlPI had emulsion forming properties; however their stability was low when compared to WPI.

Overall, EC values of plant proteins tested ranged between 476-542 g oil/g protein, with salt-extracted LPI showing the highest value whereas EC of WPI and isoelectric-precipiated SPI was ~520 g oil/g protein. EAI and ESI values of the plant proteins studied ranged between 15.0-47.9 m^2/g and 10.5-86.8 min, respectively. Isoelectric-precipiated SPI had an EAI value of ~44.2 m^2/g and an ESI of ~86.0 min whereas those values for WPI were ~55.0 m^2/g and ~104.7 min, respectively. Mean droplet diameter of the emulsions stabilized with plant proteins tested ranged between 1.4-21.1 μm with isoelectric-precipiated FbPI showing the lowest value. Isoelectric-precipiated SPI formed emulsions with a mean droplet diameter of ~1.5 μm while WPI-stabilized emulsions had a mean droplet diameter of 1.6 μm.

Chickpea and lentil protein-stabilized emulsions were optimized based on pH, protein concentration and oil content for their ability to form and stabilize oil-in-water emulsions using response surface methodology. As the protein concentration and oil content increased to >3.5% and >35%, respectively, the degree of creaming stability of the resulting emulsion increased. Lower creaming stability observed in emulsions containing lower protein concentrations can be attributed to an insufficient content of emulsifier so as to cover the oil droplets, which promotes droplet flocculation/coalescence. Findings were comparable to those reported by Makri and Doxastakis (2006) for emulsions stabilized with common bean and scarlet runner bean proteins. A decrease in particle size (from ~25.0 μm to ~2.0 μm) was negatively correlated with protein concentration and oil content, until the midpoint of the response surface (2.5-3.0% for protein concentration and 15-20% for oil content) was reached. Similar trends of decreased mean droplet diameter with increased protein concentration were reported by Wang et al. (2010a) for emulsions stabilized with a soybean protein concentrate. ChPI and LPI were found to be more effective at producing small droplets during the homogenization step of emulsion formation at pH 3.0 and 8.0 than at pH 5.5 as they possess a net charge and become more soluble at pH values significantly removed from their isoelectric points. Optimum conditions for minimal creaming, small droplet size, and high net droplet

charge were identified as: 4.1% protein, 40% oil, and pH 3.0 or 8.0, regardless of the plant protein used for emulsion preparation.

After identifying the optimum formulation for ChPI and LPI-stabilized emulsions, flaxseed oil was microencapsulated employing a wall material matrix of either ChPI or LPI and maltodextrin, followed by freeze-drying. An increase in emulsion oil concentration resulted in a concomitant increase in oil droplet diameter and microcapsule surface oil content, and a decrease in oil encapsulation efficiency. These findings are in accordance with those previously reported by Kagami et al. (2003) who encapsulated fish oil using a blend of dextrin/maltodextrin and sodium caseinate and by Polavarapu et al. (2011) for encapsulation of fish oil and extra virgin olive oil in a sugar beet-pectin matrix. Emulsions containing maltodextrin DE 9 had smaller particle size compared to those containing maltodextrin DE 18 which was attributed to to a higher continuous phase viscosity imparted by the larger concentration of high molecular weight glucose polymers. Dokic et al. (2004) reported a similar phenomenon for sunflower oil-in-water emulsions. The ability of ChPI- and LPI-based microcapsules to delay flaxseed oil oxidation was investigated. The peroxide and TBARS values of ChPI and LPI-based microencapsulated flaxseed oil remained unchanged over the 25 d storage period, whereas those of the free oil increased significantly.

Flaxseed oil was also microencapsulated employing a wall material matrix of either chickpea (ChPI) or lentil protein isolate (LPI) and maltodextrin followed by spray drying. The lowest surface oil and highest encapsulation efficiency for flaxseed oil with either plant protein wall material was observed at an initial oil concentration of 10%. As the amount of flaxseed oil used in the emulsion formulation increased from 10 to 20%, surface oil increased whereas the encapsulation efficiency decreased which is in accordance with the findings of Rusli et al. (2006) and Polavarapu et al. (2011). The peroxide value of spray dried flaxseed oil increased slightly as a result of oxygen contact with oil during the emulsification and spray drying processes. Similar results were also en reported for the encapsulation of fish oil in modified cellulose (Kolanowski et al., 2006), and a blend of modified starch and glucose syrup (Pop, 2011). Flaxseed oil released from the microcapsules was found to be the lowest for pH 5.0 at ~2.7%, and increased to a maximum of ~6.8% at pH 9.0. In the presence of NaCl, flaxseed oil release was found to increase (from ~3.6% to ~8.8%) with ionic strength over the range studied (0-200 mM). Microencapsulation systems studied were found to provide flaxseed oil protection against oxidation over a 25 d room temperature storage study relative to free oil as no significant differences in PV and TBARS values in microencapsulated flaxseed oil were found between the two plant protein wall materials. Oil

concentration was the only factor affecting PV of the spray dried flaxseed oil throughout the whole storage period. PV of microcapsules containing 20% oil was significantly higher than that of microcapsules containing 10-15% oil. Rusli et al. (2006) and Tonon et al. (2011) also reported that lower solid content and higher oil concentration led to higher peroxide values.

9. GENERAL CONCLUSIONS

Overall, both protein source and isolate production method had significant impacts on the physicochemical and emulsifying properties of the plant proteins studied. Surface charge and hydrophobicity, and solubility affected the EC, EAI and ESI values of legume protein isolates; whereas their creaming stabilities were related to only their surface charge, solubility and droplet size. Overall, legume protein isolates produced by isoelectric precipitation had higher surface charge and solubility compared to those produced by salt extraction. Among the legume proteins tested, PPI had the lowest emulsion capacity and stability as a result of its its high surface hydrophobicity, low surface charge and low solubility. The isoelectric-precipitated ChPI and LPI had the highest surface charge and solubility, formed emulsions with smaller droplet size and showed high emulsifying activity and stability. The performance of ChPI and LPI were comparable to SPI, and as such have the potential to serve as an alternative for stabilizing oil-in-water emulsions.

Salt-extracted oilseed protein isolates showed higher solubility and interfacial activity compared to those produced by isoelectric precipitation. Their EC and EAI values were related to isolate solubility, surface characteristics and ability to decrease interfacial tension, while emulsion stability was affected by solubility, surface characteristics and droplet size. Salt extraction method resulted in higher solubility and interfacial activity compared to isoelectric precipitation in oilseed protein isolates studied. CaPI showed high EC which was comparable to WPI; however, EAI and ESI values for CaPI and FlPI were significantly lower than that of WPI. It was shown that CaPI and FlPI had emulsion forming properties; however their stability was low when compared to WPI.

Protein concentration, oil content and pH were found to have a significant effect on mean droplet characteristics and the overall stability of ChPI and LPI-stabilized oil-in-water emulsions. Optimum conditions for minimal creaming (no serum separation after 24 h), small droplet size (<2 μm), and high net droplet charge (absolute value of ZP >40 mV) were identified as: 4.1% protein, 40% oil, and pH 3.0 or 8.0, regardless of the plant protein used for emulsion preparation.

Freeze-dried microcapsules prepared using ChPI and LPI proteins in combination with maltodextrin showed a protective effect against oxidation over a 25 d storage period at room temperature. An increase in emulsion oil concentration resulted in an increase in oil droplet diameter and microcapsule surface oil content, and a decrease in oil encapsulation efficiency. The optimum microcapsule formulation was identified as 4.0% ChPI or LPI, 35.5% maltodextrin-DE 9 and 10.5% flaxseed oil followed by freeze drying. This formulation resulted in a low surface flaxseed oil content (<3%), a high flaxseed oil encapsulation efficiency (~83%), and showed high (>84%) targeted release properties under simulated gastrointestinal conditions.

Oil concentration and protein source significantly affected physicochemical characteristics, encapsulation efficiency and release characteristics of microcapsules prepared by spray drying flaxseed oil using CPI or LPI and maltodextrin as wall materials. Spray-dried microcapsules containing higher oil levels were found to have higher surface oil and lower encapsulation efficiencies. Overall, LPI-maltodextrin capsules gave higher flaxseed oil encapsulation efficiencies (~88.0%) relative to CPI-maltodextrin matrices (~86.3%). However, both designs showed a protective effect against oxidation over a 25 d period of room temperature storage. Microcapsules were able to deliver 84.2% of the encapsulated oil within the simulated gastrointestinal environments.

Findings of this study suggest that the developed plant-based microencapsulation system could lead to increased utilization of flaxseed oil and legume proteins in food and bioproduct formulations and applications.

10. FUTURE STUDIES

The development of plant protein based food ingredients as an alternative to animal proteins is of considerable interest and importance. Legume proteins appear to be a promising source for this purpose because of their nutritional value, low cost and possible beneficial health effects. However, they still remain largely underutilized by the food industry partly due to insufficient structure-function information relating to their performance. In the present study, the significance of physicochemical properties of proteins in forming and stabilizing emulsions was revealed and legume protein-based encapsulation systems capable of carrying, protecting and delivering flaxseed oil were developed.

Spray drying is one of the most common techniques used in the food industry for production of microencapsulated food ingredients. In order to increase the utilization of flaxseed oil further, the oil load in the spray dried microcapsules could be increased without compromising the quality of the microcapsule in terms of encapsulation efficiency, surface oil, oxidative stability, etc.

Furthermore, studies on the glass transition properties of encapsulation matrices tested may provide useful information on their performance in spray drying as an amorphous glassy matrix is generally recommended for slowing down oxygen diffusion and hence improving oxidative stability of the product. Along with high emulsification capacity and stability; high glass transition temperature could be used as a decision criterion for choosing the optimum matrix formulation.

Flaxseed oil microcapsules can be incorporated in a variety of food products such as bakery products, beverages, dairy products, baby foods, nutrition bars, soups and salad dressings. Microencapsulated flaxseed oil can also find application as a feed ingredient to develop α-linolenic acid acid-rich animal-based food products. The performance of the encapsulated oil in a food product could be investigated to reveal whether it would interact with other ingredients in the product or affect the sensory properties and shelf life of the end product.

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