MICROENCAPSULATION OF OMEGA-3 FATTY ACIDS FROM FLAXSEED OIL IN FLAXSEED PROTEIN AND FLAXSEED GUM BASED MATRIX

By

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ABSTRACT

The overarching goal of this research was to develop microencapsulated flaxseed oil as a plant based source of omega-3 fatty acids. To accomplish this, flaxseed oil was microencapsulated in a novel matrix composed of flaxseed protein isolate (FPI) and flaxseed gum (FG) and converted into a powder by freeze drying and spray drying. The primary objectives were: a) To evaluate the physicochemical and functional properties of FPI and FG; b) to optimise the process of complex coacervation between FPI and FG to maximise the yield of complex coacervates; c) to characterise the flaxseed oil microcapsules obtained through complex coacervation followed by freeze drying or spray drying.

FPI and FG were extracted from whole flaxseeds at optimised temperatures to obtain 90% purity. The physicochemical and functional properties of FPI were found superior to most of the commonly used proteins. Lower extraction temperatures (30, 50 °C) of FG yielded higher levels of neutral monosaccharides and lower levels of acidic monosaccharides. The functional properties of FG, such as EAI and WAC, were negatively affected by the rise in extraction temperature. Electrostatic complexation studies between these two biopolymers showed that the optimum FPI-to-FG ratio is 3:1 and the optimum pH is 3.1. The complex coacervates of FPI-FG were used to microencapsulate flaxseed oil at different core to wall ratios (1:2, 1:3 and 1:4), and converted to powder through spray drying and freeze drying. The spray dried solid microcapsules had higher oil microencapsulation efficiency, lower surface oil content and higher oxidation stability compared to the freeze dried microcapsules. The oxidation stability obtained from spray dried microcapsules at core-to-wall ratio of 1:4 was nearly double to that of the unencapsulated flaxseed oil. This study affirms the potential of a solely plant based encapsulating matrix that returns superior nutritional outcomes to other commonly used wall materials.

STATEMENT OF AUTHORSHIP

Except where explicit reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis by which I have qualified for or been awarded another degree or diploma. No other person's work has been relied upon or used without due acknowledgement in the main text and bibliography of the thesis.

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Dedication

I dedicate this thesis to my husband, Vikas Kaushik who remained a continuous source of support, inspiration and motivation throughout my PhD journey.

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Chapter 1

Introduction

1.1 BACKGOUND

Omega-3 fatty acids have been identified as a shortfall nutrient by various health organisations such as U.S. Department of Agriculture (USDA), the Pan American Health Organization/World Health Organization (PAHO/WHO) and the Food and Agriculture Organization of United Nations (FAO) (Simopoulos, Leaf, & Salem, 1999). From the last three decades continuous emphasis is made to obtain adequate amounts of omega-3 fatty acids by including plant based sources (Kris-Etherton, Harris, Appel, & AHA Nutrition Committee, 2003; Swanson, Block, & Mousa, 2012).

Flaxseed oil is the richest plant sources of the essential omega-3 fatty acid ALA (α–linolenic acid), one of the two essential fatty acids which are not synthesized by the human body (Frasure-Smith et al., 2004). However, it plays an important role in many physicological functions in human body (Carneiro, et al, 2012). Other dietary sources of ALA include vegetable oils from canola and soybean; seeds and nuts such as chia and walnuts; and some green leafy vegetables. ALA is a short chain omega-3 fatty acid, which is converted into long chain omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahaxaneoic acid (DHA) by the enzymes elongase and desaturase present in the human body.

Health benefits of omega-3 fatty acids are well substantiated through both epidemiologic and clinical trials (Delgado-Lista et al., 2012; Mozaffarian et al., 2012). Omega-fatty acids are considered essential not only for normal growth and development of the human body but also for their positive effects on heart, brain, eyes, joints, skin, mood and behaviour (Connor, 2000; Simopoulos, 1991). Higher intakes of omega-3 fatty acids aids in the prevention of coronary artery disease, hypertension, diabetes, arthritis, inflammatory and autoimmune

disorders, and cancer (Tur et al., 2012). Many studies have supported the significance of the adequate intake of omega-3 fatty acids by women during pregnancy and lactation, specifically to support the healthy development of retina and brain in fetus (Connor, 2000; Koletzko et al., 2001). Consequently, there has been increased interest by the food industry to incorporate larger amounts of omega-3 oils into human diets.

The recommended daily intakes of omega-3 fatty acids varies with age, gender and personal needs such as pregnancy, lactating period and any sickness pertaining to heart, brain or nervous system. Dose recommendations issued by countries such as Canada, Sweden, United Kingdom, Australia, Japan and various health organizations such as WHO and North Atlantic Treaty Organisation vary from 0.8 to 1.1 g/d of alpha linolenic acid and 0.3 to 0.5 g/d of EPA and DHA (Kris-Etherton, 2000). In contrast, the reported current daily intake of omega-3 fatty acids is quite low, with average reaching only 65% of the recommended amounts in some countries including Australia (Ervin et al., 2004; Gregory et al., 1988; Meyer et al., 2003).

A number of factors may contribute to the lower intake of omega -3 fatty acids. The decreased consumption of fish, which is a good source of omega-3 fatty acids is noted in many communities. The consumption of fish has decreased in recent years due to concerns such as pollution in environment, decreasing availability, low sensory acceptability owing to its characteristic odour etc. (Harris et al., 2003). Further, the availability of fatty fish coupled with rising prices of fish oils has greatly decreased their affordability and consumption (Turchini et al., 2009). Although, plant based sources of omega-3 fatty acids are comparatively inexpensive and renewable; their consumption is limited due to the unstable nature of omega-3 fatty acids. Omega-3 fatty acids are highly unsaturated and are very sensitive to light and heat and; hence, are easily oxidised. The oxidation products (hydroperoxides, aldehydes and ketones) are deemed undesirable by consumers due to their

characteristic off flavour and off odour (Liu et al., 2010). This limits their broader application as potential food ingredients.

To overcome this problem, microencapsulation has been used by many researchers (Carneiro et al, 2012; Heinzelmann et al., 2000; Liu et al., 2010). Microencapsulation is the process of entrapping any active ingredient within a coating substance. Encapsulated materials are generally referred as core material, active ingredient, or payload (Versic, 1988). The outer continuous and protective material around core is called the capsule, encapsulant, wall material, membrane, carrier, shell, or encapsulation matrix (Shahidi and Han, 1993). In addition to stabilizing the oils and increasing their shelf-life, it also masks the characteristic unpleasant odour and taste, converts it into easy to handle form and enhances the bioavailability. All these benefits enable and promote the production of omega-3 fortified foods (Heinzelmann et al., 2000). Microencapsulation of omega-3 fatty acids with different encapsulation and drying techniques using numerous wall materials are reported and include spray drying, spray cooling or chilling, freeze drying and complex coacervation. Complex coacervation gives the highest pay load (Barrow et al., 2007). The higher payload is advantageous in meeting the daily dietary recommendations of omega-3 fatty acids. This is because less powder is required to deliver the recommended amount of omega-3 fatty acid when the payload is higher (Kralovec et al., 2012).

Microencapsulation of oils is usually carried out in two steps. In the first step, an emulsion is made with oil and the desired wall material/s and in the second step; the emulsion is dried to powder form. Complex coacervation followed by spray drying is reported as the most promising technology for stabilization of omega-3 fatty acids by microencapsulation while delivering the highest pay load (40-60%) (Barrow et al., 2007; Liu et al., 2010). However, most of the microencapsulated products have gelatin (from animal origin) as wall material, which is unacceptable to the vegetarian population (Kralovec et al., 2012; Liu et al., 2010).

There is also a safety concern associated with the use of gelatin due to the emergence of prion diseases (Morrison et al., 1999). Hence, it is highly desirable to find alternatives to gelatin as a shell material for microencapsulation.

In this context, the complex coacervates of plant-based proteins such as soy protein (Trubiano and Makarious, 2005), pea protein and cereal protein (Ducel et al., 2004), flaxseed protein (Wang et al., 2010) with gum arabic has been explored for different active ingredients. Specifically for microencapsulation of flaxseed oil, the different wall materials reported include whey protein isolate (WPI) (Partanen et al. 2008), zein protein (Quispe-Condori et al., 2011), gum arabic (Tonon et al., 2011), and different combinations of maltodextrin (MD), gum Arabic (GA), whey protein concentrate (WPC) and modified starch (Carneiro et al, 2012; Omar et al., 2009). The application of some plant protein particularly wheat protein and soy protein in the coacervation process is not expected to receive wide spread application due to possible allergen issues (Ducel et al., 2004). In another study, zein the prolamin fraction of corn protein was also examined to assess its ability in encapsulating flaxseed oil (Quispe-Condori et al., 2011). Results from this study indicated that zein as a wall material provided good microencapsulation efficiency but the powder properties of the final product were poor in terms of flowability.

Based on the nutritional amino acid profile and associated health benefits, flaxseed protein is emerging as potential ingredient for the food industry (Oomah & Mazza, 1993). To date, there are no published clinical studies indicating that flaxseed protein triggers allergic responses when consumed as part of a normal diet (Muir, & Westcott, 2003). Flaxseed protein can be used for malnutritioned populations with milk protein allergies (Weisdorf, 1998). In addition, flaxseed protein possesses promising functional properties, such as high emulsifying activity and foaming capacity (Dev & Quensel, 1988). Another flaxseed component, the soluble dietary fiber or flaxseed gum (FG) is reported to have favourable

functional properties and can be used as a substitute for gum arabic in food formulations (Mazza and Biliaderis, 1989). It has also been reported as a potential emulsifier for oil in water emulsions (Wang et al., 2010). None of the published studies on the microencapsulation of flaxseed oil have used the flax seed protein isolate (FPI) and flaxseed gum (FG) together as wall materials. Hence, FPI and FG which are emerging as potential emulsifiers should be considered for their efficacy in terms of payload, preventing oxidation as well as structural strength of the coacervates.

1.2 RESEARCH QUESTION

There is clearly a gap between the recommended and average dietary intake of omega-3 fatty acids. For much of the human population, it is imperative that this gap be addressed. The research questions for this study are:

1. How can the flaxseed oil (as source of ALA) be best encapsulated to be used as an food ingredient?
2. How flaxseed protein and flaxseed gum can be combined (as complex coacervates) to form a coating material for microencapsulation of flaxseed oil?

1.3 OBJECTIVES

The overall aim of this project is to develop and produce flaxseed oil microcapsules by using complex coacervation technology followed by spray drying. Such capsules will deliver these essential micronutrients to the vegetarian population in particular but also to a wider un-met market of concerned consumers. The specific objectives of this research are to:

1. Preparation of flaxseed protein isolate (FPI) from flaxseeds and characterization of its functional properties.
2. Extraction of flaxseed gum (FG) from flaxseeds and characterization of its functional properties.

3. Optimization of complex coacervation process between FPI and FG (in terms of FPI to FG ratio, pH, oil to water ratio, surface charge and the amount of crosslinker) in order to use FPI and FG as wall materials and flaxseed oil as core, to maximise the payload with minimum surface oil.
4. Characterization and comparison of microcapsules obtained through complex coacervation followed by freeze drying or spray drying.

1.4 OUTLINE OF THESIS

This work follows a thesis with publications model and is divided into seven chapters as shown in Figure 1.1. The publication details of each chapter (from chapter 2-6) is listed in Table 1.1.

Chapter 1. This chapter provides the introductory context for the research work which includes background, existing knowledge gaps and the significance of the study.

Chapter 2. This chapter critically reviews the various microencapsulation techniques used for omega-3 rich oils. This literature review also covers the advantages and disadvantages associated with each technique, characterization of omega-3 fatty acid rich microcapsules and their stability and controlled release.

Chapter 3. This chapter documents the extraction, characterization and physicochemical properties of FPI. The functional properties of FPI such as emulsifying activity index, emulsion stability index, water holding capacity, fat absorption capacity, emulsion zetapotential and emulsion droplet size were compared with other commonly used animal and plant proteins.

Chapter 4. This chapter documents the effect of extraction temperature on yield, composition structure, physicochemical properties and functional properties of FG. The relationship

between changes in structure and composition of FG as function of extraction temperature were related to the variation in its functional properties.

Chapter 5. In this chapter optimisation of complex coacervation between FPI and FG is documented in detail. The electrostatic complexation between two polymers as function of different FPI to FG ratios and pH were studied using turbidometric and zetapotential measurements. The changes in the secondary structure of FPI as a function of pH were also documented in this chapter.

Chapter 6. This chapter documents the detailed protocol for the microencapsulation of flaxseed oil in a matrix composed of FPI-FG complex coacervates. Two drying processes freeze drying and spray drying were used to produce powdered microcapsules. Microcapsules formed though two processes were compared for yield, microencapsulation efficiency, pay load and oxidative stability.

Chapter 7. In this chapter, key conclusions drawn from current study are summarised. This chapter details the contributions made by this study to work done in the fields of: functional properties of flaxseed protein and flaxseed gum, complex coacervation between plant protein and polysaccharides, microencapsulation of omega-3 fatty acids from flaxseed oil. This chapter also provides future research directions based on the knowledge obtained in this study.

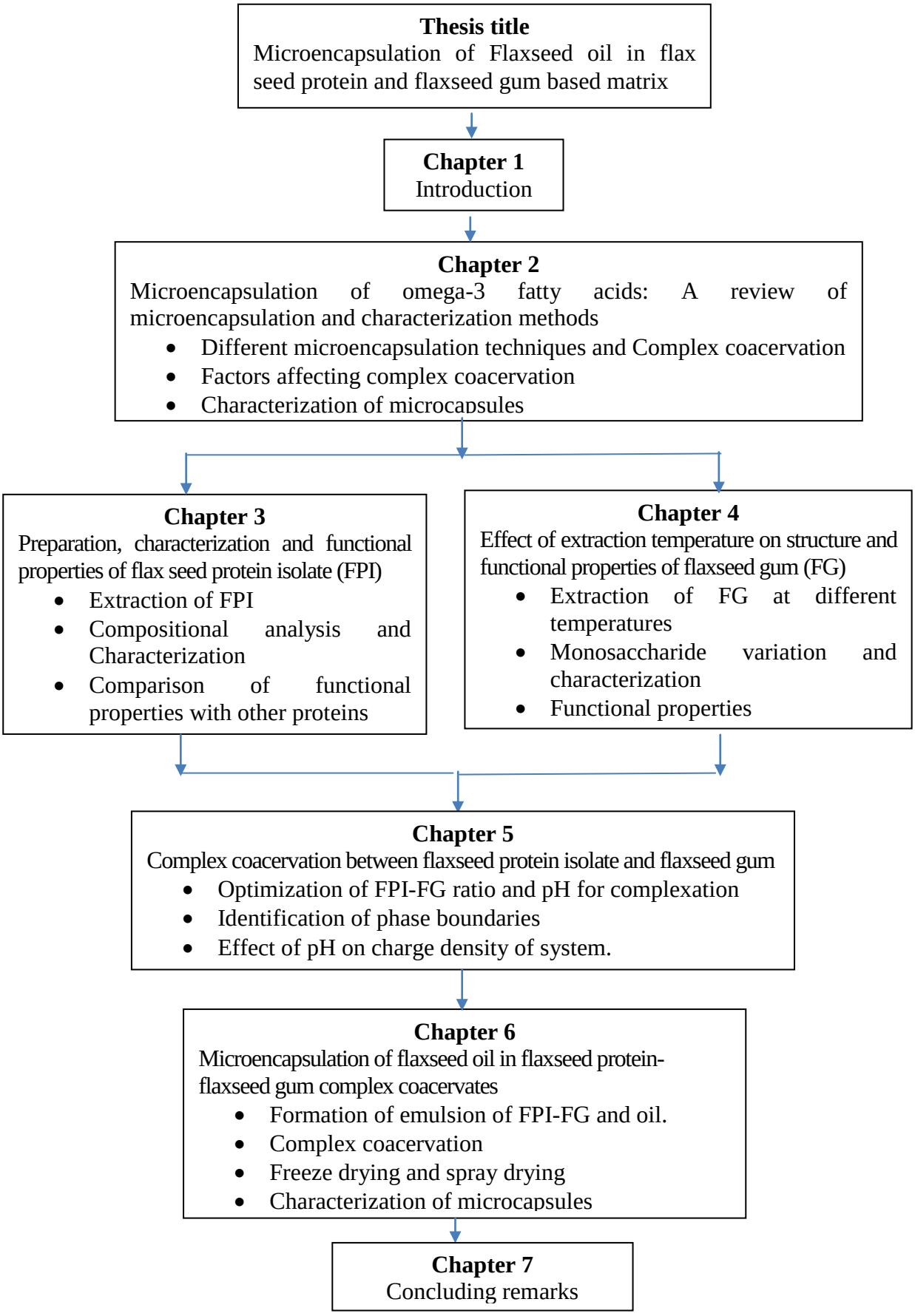

Figure 1.1 Structural flow of thesis

Table 1.1 Publication summary of Chapters

Chapter no.	Title of the paper	Journal	Status	Contribution of candidate (%)
2	Microencapsulation of omega-3 fatty acids: A review of microencapsulation and characterization methods	Journal of Functional foods	Published	80%
3	Preparation, characterization and functional properties of flax seed protein isolate	Food Chemistry	Published	80%
4	Effect of extraction temperature on composition, structure and functional properties of flaxseed gum	Food Chemistry	Published	80%
5	Complex coacervation between flaxseed protein isolate and flaxseed gum	Food Research International	Published	85%
6	Microencapsulation of flaxseed oil in flaxseed protein-flaxseed gum complex coacervates consolidated by maltodextrin	Food Research International	Published	80%

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Chapter 2

Microencapsulation of omega-3 fatty acids: A review of microencapsulation and characterization methods

ABSTRACT

To improve consumption of omega-3 fatty acids, foods can be enriched with omega-3 rich oils. Microencapsulation of omega-3 oils minimises oxidative deterioration and allows their use in stable and easy-to-handle form. Microencapsulation of omega-3 fatty acids can be achieved by using a variety of methods, with the two most commonly used commercial processes being complex coacervation and spray dried emulsions. A variety of other methods are in development including spray chilling, extrusion coating and liposome entrapment. The key parameter in any of these processes is the selection of wall material. For spray dried emulsions and complex coacervates protein or polysaccharides are primarily used as shell material, although complex coacervation is currently commercially limited to gelatin. Here we review the need for microencapsulation of omega-3 oils, methods of microencapsulation and analysis, and the selection of shell material components. In particular, we discuss the method of complex coacervation, including its benefits and limitations. This review highlights the need for research on the fundamentals of interfacial and complexation behaviour of various proteins, gums and polyphenols to encapsulate and deliver omega-3 fatty acids, particularly with regard to broadening the range of shell materials that can be used in complex coacervation of omega-3 rich oils.

Key words: Alapha linolenic acid**,** Microencapsulation, Complex coacervation, Biopolymers, Oxidative stability.

2.1. BACKGROUND

The health benefits of omega-3 fatty acids are substantiated through extensive and rigorous *in vivo* studies (Connor, 2000; Frank et al., 2002). A wide range of literature indicates that omega-3 fatty acids are essential not only for normal growth and development but also for their positive effects on heart, brain, eyes, joints, skin, mood and behaviour (Connor, 2000; Simopoulos, 1991). Omega-3 fatty acids are also implicated in the prevention of coronary artery disease, hypertension, diabetes, arthritis, other inflammatory and autoimmune disorders, and cancer (Tur et al., 2012). Many studies encourage the adequate intake of omega-3 fatty acids by pregnant and lactating women to support overall health of foetal and healthy development of retina and brain in foetus (Connor, 2000; Koletzko et al., 2001). Some studies argue that the claimed health benefits of omega-3 fatty acids are inconclusive, particularly with regard to cardiovascular events, cancer, cognitive health and slowing down the age-related macular degeneration (AMD). For example, Hooper et al (2006) reported that the beneficial effect of omega-3 fatty acids on combined cardiovascular events and cancer prevention is not conclusive. Sydenham, Dangour and Lim (2012) suggested that the positive effect of omega-3 on cognitive decline and dementia are also not conclusive. Lawrenson and Evans (2012) opined that there is currently no evidence to support that an explicit increase in the level of omega 3 in the diet could slow down the progression of AMD. However, the majority of studies indicate that omega-3 fatty acids bring about substantial benefits to human health, and some benefits, such as the lowering of serum tryglyceride levels in blood, are conclusively established.

Omega-3 fatty acids belong to family of polyunsaturated fatty acids (PUFAs). As the name suggests, these have multiple double bonds with the first double bond placed at the third carbon starting from the methyl end. The first member of the omega-3 family is alpha–linolenic acid (ALA, 18:3n-3), which is not synthesized by the human body. However, ALA

plays an important role in several physicological functions in human body and hence is recognized as essential in the diet. The other nutritionally important omega-3 fatty acids are the longer chain metabolites of ALA, eicosapentaenoic acid (EPA, 20:5n-3) and docosahaxaneoic acid (DHA, 22:6n-3). Major dietary sources of ALA, EPA and DHA are shown in Table 2.1. EPA and DHA are long chain PUFA (LCFUFA) obtained in our diet principally from marine sources.

Table 2.1 Omega-3 fatty acids and their natural sources

Omega-3 fatty acid	**Sources**
Alpha –Linolenic acid (ALA)	Dark green leafy vegetables, certain nuts, seeds and their oils flaxseed, hempseed and walnut, canola, perilla, chia, kiwifruit
Eicosapentaenoic acid (EPA)	Fatty fish such as herring and mackerel, liver of lean white fish such as cod and halibut, blubber of marine mammals such as whales and seals and algal species
Docosahaxaneoic acid (DHA)	

Desired levels of omega-3 fatty acids in diets can be achieved by including various foods enriched with omega-3 PUFA. Although a variety of food products enriched with omega-3 fatty acids are available in the market, there are technical challenges in their production, transportation, storage, bioavailability and sensory acceptability (Kolanowski, 1999). The physical and chemical characteristics of omega-3 oils limit their application as a potential food ingredient. Due to the highly unsaturated nature of omega-3 fatty acids, these are susceptible to oxidation and readily produce hydroperoxides, off flavours and odours, which are deemed undesirable by consumers.

To overcome the above mentioned problems, the use of microencapsulation technology has been explored by various researchers (Klinkesorn et al., 2005). Omega-3 fatty acids have been microencapsulated using different encapsulation techniques. So far, spray drying, complex coacervation and extrusion are the most commonly used commercial techniques for microencapsulation of omega-3 fatty acids. Spray drying offers many advantages over other drying methods such as freeze drying, including low operational cost, ability to handle heat-sensitive materials, readily available machinery and reliable operation and ability to control the mean particle size of the powders for spray dried emulsions. However, only limited numbers of wall materials are compatible with this technology (Desai & Park, 2005). Hence, there is a need for new wall materials to be developed that can be used in high temperature and high evaporation flux conditions which prevail in the spray drying environment. Moreover, the amount of oil that can be encapsulated with conventional methods is low when compared to the amount of omega-3 PUFA required to meet recommended daily dietary allowances for humans (Garg et al., 2006).

Therefore there is a need to explore different wall materials which can microencapsulate increased amounts of omega-3 oils and can improve the protective efficacy and subsequent bioavailability of omega-3 fatty acids. This is particularly true for complex coacervation, where the particles are formed during the coacervation process rather than in the dryer. Proteins other than gelatin have been shown to form poor coacervates compared with gelatin, particularly with regard to outer shell formation in agglomerated multicore coacervates (Zhang et al. 2009). Given the recognition of dietary deficiency of omega-3 fatty acids globally and particularly in western diets (Ervin et al., 2004; Gregory et al., 1988; Meyer et al., 2003) (Fig. 2.1) the need to look for alternative sources is an important issue.

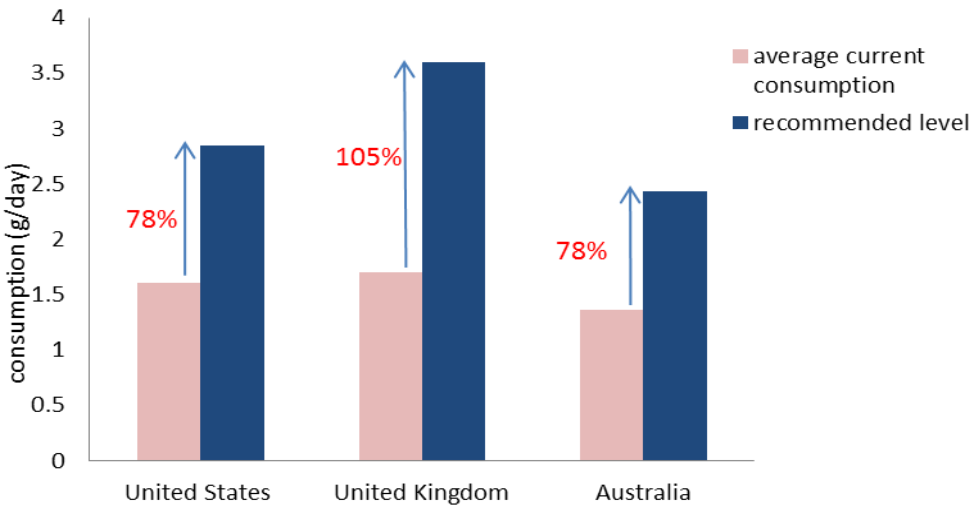

Figure 2.1 Current consumption verses recommended levels of omega -3 fatty acids

In this context this review was undertaken with the objectives 1) To suggest some means to cover the gap found between the recommended and current dietary intake of omega-3 fatty acids among people of different countries; 2) To critically review the research work done so far on the microencapsulation of omega-3 fatty acids using different techniques and shell materials with, particular emphasis on complex coacervation of gelatin and its alternatives.

2.2. MICROENCAPSULATION

Increased consumption of omega-3 oils can be achieved by fortifying staple foods such as bread, milk and yogurt with omega-3 fatty acids. However, incorporation of omega-3 fatty acids into foods is restricted by their oxidative instability and the formation of oxidized products carrying off-flavours. Microencapsulation technology can partially prevent oxidation and extends the shelf-life of omega-3 fatty acids, offering practical solutions for stabilization and improved delivery of omega-3 fatty acids in food products (Kolanowski, 1999). Microencapsulation is the process of entrapping any active ingredient within another coating substance. Encapsulated materials are generally referred to as core material, active

ingredient, fill, internal phase or payload (Versic, 1988). The outer continuous and protective material around the core is called the capsule, encapsulant, wall material, membrane, carrier, shell, or encapsulation matrix (Shahidi & Han, 1993). The wall materials of the microcapsules protect the core substance against environmental effects (oxygen, light, humidity etc.), thus improving its stability, handling conditions and overall acceptability (Garg et al., 2006). It also extends the shelf-life of products, improves functionality of additives and expands the application range of nutritionally important food ingredients including omega-3 fatty acids.

2.3. METHODS USED FOR MICROENCAPSULATION OF OMEGA -3 OILS

2.3.1 Spray dried emulsions

Spray drying of emulsions is one of the most commonly used microencapsulation and drying technologies in food and pharmaceutical industries because the process is flexible, economical, efficient, easy to scale-up, uses easily available equipment and produces good quality powder (Ashady, 1993). It has been extensively used in the encapsulation of fats, oils, flavours and oil soluble ingredients. The general process of spray drying involves dispersion of a core material into a polymer solution, forming an emulsion or dispersion, pumping of the feed solution/emulsion and atomization of the mixture and dehydration of the atomized droplets to produce microcapsules (Fig. 2.2(a)) (Risch, 1995). Depending upon the solids concentration of starting feed solution and operating conditions, the size of the microcapsules produced by spray drying can vary from 10-50μm at the fine end to 2-3mm at the large end (Gharsallaoui et al., 2007; Nedovic et al., 2011).

The production of microencapsulated omega-3 oil involves the selection of core material (source of omega-3 oil) and wall materials, design of the formulation (ratio of core to wall) and selection of the appropriate encapsulation technology. A successful microencapsulation

system is evaluated on the basis of encapsulation efficiency and storage stability of omega-3 oil microcapsules, which mainly depends on the type and composition of wall material used. A number of wall materials (as shown in Table 2.2) have been used to produce omega-3 microcapsules by spray drying of emulsions. These include proteins, carbohydrates, lipids and gums used either alone or in combination to achieve desirable characteristics. For example, Drusch et al. (2006) observed that low molecular weight carbohydrates get crystallized at a temperature above their glass transition temperature and thus lose the protection given to the core material. But this crystallization may be retarded by combining sugars with polymers or with divalent cations (Buera et al., 2005).

In a recent study Helena et al. (2013) evaluated the potential of maltodextrin combination with different wall materials (starch, whey protein concentrate and gum Arabic) for microencapsulation of flaxseed oil through spray drying. Results indicated that maltodextrin (MD) in combination with modified starches gave the best encapsulation efficiency in comparison to a gum Arabic and whey protein concentrate (WPC) combination. Whereas the best emulsion stability and oxidation protection was observed in MD-WPC combination (Helena et al., 2013).

In addition to wall materials, processing methods used in the emulsification process also impact the encapsulation efficiency. It has been observed that emulsion droplet size has a pronounced effect on the encapsulation efficiency of oils during spray drying (Soottitantawat et al., 2003). Small oil droplets get enclosed and embedded more efficiently within the wall matrix of the microcapsules.

Table 2.2 Technologies and wall materials used for microencapsulation of omega 3 fatty acids

Technologies	Wall materials	References
Spray drying (Fish oil)	Gelatin, caseinate and maltodextrin	Lin et al., 1995
Spray-drying(Fish oil)	Casein and lactose	Keogh et al., 2001
Spray-drying (Fish oil)	Sodium caseinate and dextrose equivalence	Hogan et al ., 2003
Spray-drying (Fish oil)	Highly branched cyclic dextrin and sodium caseinate	Kagami et al. (2003)
Spray-drying (Fish oil)	Methylcellulose Hydroxypropyl methylcellulose	Kolanowski, et al., 2004
Spray-drying (Fish oil)	Sodium caseinate, glucose, glucose syrup	Augustin, et al., 2006
Spray-drying(Fish oil)	n-octenylsuccinate Derivatized starch/glucose syrup or terhalose	Drusch, et al., 2006
Spray-drying (Fish oil)	Methylcellulose Hydroxypropyl methylcellulose	Kolanowski et al., 2006
Spray-drying (Fish oil)	Sugar beet pectin and glucose syrup	Drusch, 2007
Spray-drying (Fish oil)	Gum arabic	Fang et al., 2005
Spray-drying (Fish oil)	Corn syrup solids	Shaw et al., 2007
Spray drying	Starch/whey protein concentrates	Jafari et al.,2008
Spray drying (Flaxseed)	Whey protein isolate	Partanen et al.,2008
Spray drying (flaxseed)	Guma arabic and lecithin	Omar et al., 2009
Spray drying (flaxseed oil)	Maltodextrin, whey protein concentrate, gum arabic and two chemically modified starches: Tapioca starch and waxy maize	Helena et al., (2012)
Freeze-drying (Fish oil)	Sodium caseinate, carbohydrate	Heinzelmann et al., 2000
Spray and freeze drying (Fish oil)	Egg white powder	Taguchi et al.,1992
Spray and freeze-drying	Gum arabic	Minemoto et al.,

Technologies	Wall materials	References
(Fish oil)		1997
Spray and freeze-drying (Fish oil)	Lactose and maltodextrin	Heinzelmann and Franke, 1999
Simple coacervation and Spray-drying (Fish oil)	Hydroxypropyl methylcellulose	Wu et al., 2005
Complex coacervation and freeze drying (flax seed oil)	Gelatin and gum arabic	Liu et al., 2010
Complex coacervation (micoalgal oil)	Gelatin-gum Arabic with transglutaminase (TG) as cross-linking agent	Zhang et al., 2012
Double emulsification and subsequent enzymatic gelation (Fish oil)	soy protein, whey protein, wheat protein sodium caseinate, Transglutaminase	Cho et al., 2006
Electrostatic layer by layer (mutli layer) deposition and Spray drying (Fish oil)	Lecithin and chitosan	Klinkesorn et al., 2006
Ultrasonic atomization and freeze drying (Fish oil)	Chitosan	Klaypradit and Huang, 2008
Electrospraying (Fish oil)	Zein prolamine (Corn protein)	Sergio et al., 2010
Spray Granulation and Fluid Bed Film Coating (Fish oil)	SSPS and maltodextrin, hydroxypropyl betacyclodextrin (HPBCD)	Anwar et al., 2010
Comparison of spray granulation, spray drying, and freeze drying (Fish oil)	Soybean soluble polysachharide (SSPS) with maltodextrin, hydroxypropyl betacyclodextrin and octenyl succinic anhydride	Anwar et al., 2011

Hence, the methods that produce small emulsion droplets encapsulate higher amounts of oil and allow less oil exposed to the microcapsule particle surface. Based on this theory, Jafari et al. (2008) used a microfluidizer to reduce the droplet size during emulsion preparation and observed that a higher encapsulation efficiency and lower surface oil was obtained using this method, compared to other methods of preparing emulsions, such as ultrasonication. In a similar study, Tonon et al. (2011) reported that higher oil concentration and lower solid content resulted in emulsions with lower viscosity, larger droplet size, poor oil loading and high lipid oxidation.

Although spray drying is the most commonly used technology for microencapsulation of omega-3 fatty acids, some studies have also pointed out drawbacks of this technology (Fang et al., 2005; Wu et al., 2005). These authors reported that the use of air as the drying medium at very high temperature produces particles with a porous structure. Hence, the spray dried powder particles can readily undergo oxidation, which decreases their shelf life. Kolanowski et al. (2006) reported that spray dried fish oil powders were more prone to oxidation compared to the fish oil upon storage. Industry has strong interest in manufacturing stable fish oil powders through spray drying and a current research focus is to overcome the oxygen sensitivity of these powders. Use of cross-linked wall materials for example, carbohydrates and proteins via Maillard reaction, has been suggested as a potential solution (Augustin et al., 2006; Luff, 2007). It has also been reported that the application of complex coacervation methods by incorporating maltodextrin into an emulsion of fish oil and hydroxypropyl methyl cellulose (HPMC) also improved the oxidation stability of fish oil (Ke-Gang et al., 2005). Hence, shells formed by coacervating and further crosslinking of polymers can help in protecting the omega-3 oils against post spray drying oxidation.

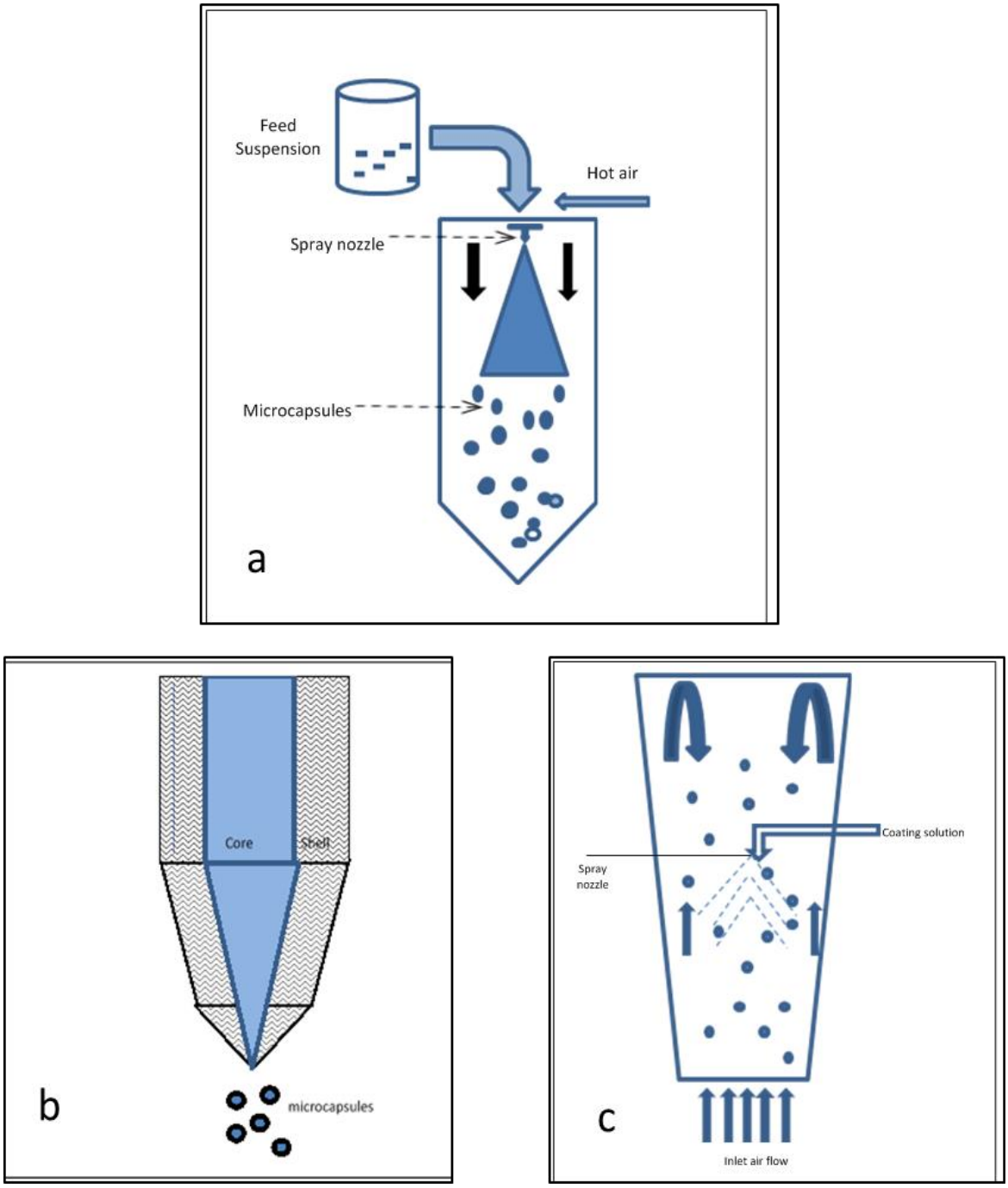

Figure 2.2 Different Techniques for Microencapsulation: a) Spray Drying, b) Extrusion and c) Fluidized Bed Coating

2.3.2 Freeze dried emulsions

For freeze drying the emulsion is frozen at temperature between -90 and -40º C and then dried by sublimation under low pressure. The main advantage of this technology is the removal of oxygen and the application of low temperatures, which help minimize product oxidation. These advantages of freeze drying make this technology suitable for the microencapsulation of highly sensitive ingredients, such as probiotics and PUFAs.

Few studies have explored the microencapsulation of omega-3 fatty acids using freeze drying. Heinzelmann et al. (2000) reported that freeze drying of microencapsulated fish oil gives powders with highly porous structures and a short shelf-life. In similar studies, Harayani et al. (2011) compared the characteristics of microencapsulated fish oil products using spray drying, spray granulation and freeze drying and found that porous, irregular, and flake-like structure of the freeze dried powder accelerates the oxidation due to the easy access of oxygen to the interior of such powders. In addition, freeze drying is 30 to 50 times more costly than spray-drying (Gouin, 2004). Hence, freeze drying of emulsions is an expensive technology for commercial production of microencapsulated omega-3 products and requires technical advancement to overcome the porosity issues in the structure of the final powder.

2.3.3 Fluidized bed drying

Microencapsulation using fluidized bed coating is a technique in which a coating is applied onto powder particles in a batch process or a continuous set-up (Rumpler & Jacob, 1998). The powder particles are suspended in an air stream maintained at a specific temperature and sprayed with a coating material as shown in Fig. 2.2(b). Generally powdered omega-3 oils can be coated with a lipid layer to prevent oxidation upon storage. Melted fats and waxes are preferred as shell materials over aqueous-based formulations as no evaporation step is required in lipid based coatings, resulting in savings of energy and time. Skelbaek and

Andersen (1994) patented a process of double encapsulation of fish oil. First, the fish oil was emulsified by using caseinate as an emulsifier and this emulsion was spray dried. This powder was then coated by using corn starch as a spraying agent. In another patent, Ponginebbi and Publisi (2008), produced spray dried fish oil powders and subsequently coated by spraying molten 30% (w/w) hydrogenated palm wax to these powders to increase the oxidation stability.

However, this method has only been used to provide additional coating on top of already microencapsulated fish oil but never for direct microencapsulation of omega-3 PUFAs. Therefore, this method is a secondary method useful for improving microencapsulation stability or other properties with a tailored coating, rather than a primary microencapsulation method for producing omega-3 powders.

2.3.4 Extrusion

Extrusion is another potential technology for microencapsulation of omega-3 oils. This technology can be used to produce high density encapsulated products. It involves mixing of molten carriers with omega-3 oils, allowing the emulsion to pass through a die or nozzle at high pressure (Fig. 2.2(c)). The fundamental approach of this technology has been patented by Saleeb and Arora (1999). According to these authors, extrusion is preferred over spray drying because the extruded products are less porous (Serfert et al., 2009). However, it has also been observed that cost of extrusion is almost twice that of spray drying and the use of screw extruders at high pressure generating high shear forces which are detrimental to the stability of sensitive core material such as omega-3 oil (Gouin, 2004).

Co-extrusion or centrifugal extrusion is a type of extrusion technology which is quite commonly used in microencapsulation processes. In this technology, nozzles consisting of concentric orifices are used. Heated aqueous polymer solution flows through outer tube and the oil to be encapsulated flows through the inner tube and finally both the fluids are

discharged into moving stream of carrier fluid. The particle size range of microcapsules obtained by co-extrusion is 500-1000 μm (Gouin, 2004). The particle size is too high for useful inclusion in many foods, since particles above about 100 μm impact mouth feel.

2.3.5 Melt Injection

Melt injection processes begin with mixing of omega-3 oil in a matrix containing starch, anti-oxidants, sugars, emulsifiers and water at about 130º C (Valentinotti et al., 2006). The mixture is then extruded through a filter and collected in a bath filled with cold organic solvent (Isopropanol or liquid nitrogen) which solidifies the sugar matrix and transforms it into a glassy state material which is then washed with a terpene (eg. limonene) to remove the surface oil. Oil microencapsulated using this method was found to be very stable when stored with $a_w \leq 0.3$. Moreover, this process makes it possible to avoid the ubiquitous use of gelatin or other proteins in encapsulation of omega-3 oils. However, particle size is large, limiting the utility of this method for food ingredient preparation.

2.3.6 Complex Coacervation

Coacervation is generally defined as the separation of two liquid phases in a colloidal solution. Out of the two phases one is rich in polymer and called coacervate phase and another devoid of it is called equilibrium solution. In case of simple coacervation there is only one polymer whereas complex coacervation involves the interaction of two oppositely charged colloids (Fig. 2.3) (Ke-Gang et al., 2005). In microencapsulation of omega-3 oils by simple coacervation the oil component is usually dispersed in gelatin solution and then a pH adjustment causes the gelatin to coacervate and form a coating over oil droplets. The subsequent cooling step hardens the coating and encapsulates the oil.

Complex coacervation uses two oppositely charged polymers and is one of the most promising technologies for stabilization of omega-3 oils by microencapsulation while

delivering highest pay load (40-60%) (Barrow et al., 2007; Liu et al., 2010). The two typical hydrocolloids used in the complex coacervation of omega-3 oils are gelatin or whey proteins and oppositely charged gum arabic, sodium polyphosphate or carboxy methyl cellulose. The formation of an external coating by these oppositely charged moieties is induced by adjusting the pH. Solidification of the shell formed is then carried out either by thermally denaturing the protein or by cross linking the protein chains by glutaraldehyde or transglutaminase (Barrow et al., 2007; Strauss & Gibson, 2004).

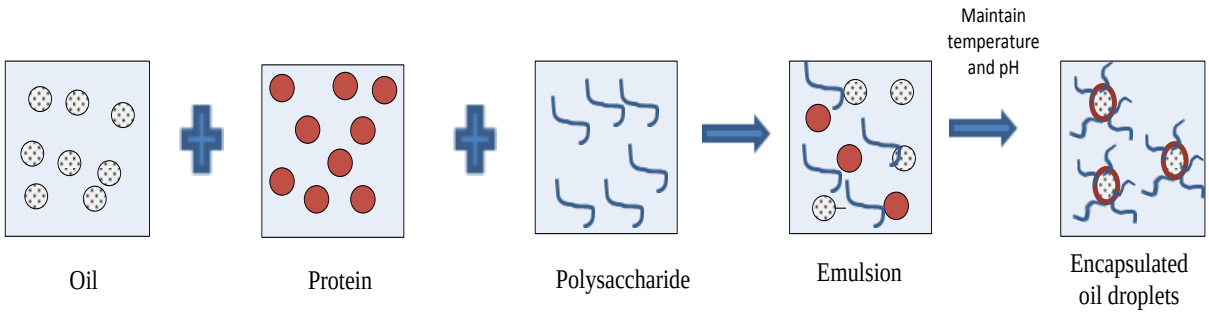

Figure 2.3 Flow diagram of microencapsulation by complex coacervation

Morphology of microcapsules obtained using this technology is either mono-nucleated (single core - single oil droplet surrounded by shell) or poly-nucleated (multi core - multi oil droplets surrounded by a common shell). But the size and shape of cells depend on both the method of emulsion formation and the material-based parameters, such as type of polymers, their molecular weight, charge density, concentration and their ratio. Similarly the size and the shape of the cells also depend on process-based parameters such as pH, temperature, cooling and solidification rates. Due to the interdependency of all these factors, optimization of the process is challenging.

The main advantage of complex coacervation is the production of microcapsules with smaller particle size that ranges from about 1 to 1000 µm. Moreover, as compared to other

microencapsulation processes complex coacervation gives unusually higher payload of up to 90% for single core and 60% for multi core. This process also successfully prevents the oil migrating to the particle surface and the concentration of the surface oil is normally low compared with other methods, particularly in the multi core process. A low surface oil concentration is conducive in maintaining the sensory properties of the complex coacervate powders during storage of omega-3 fortified food products. The higher payload is required to address the need for increasing daily dietary intake of omega-3 fatty acids and for lowering cost by decreasing the amount of shell material components required for gram of omega-3 (Kralovec et al., 2012).

The complex coacervation technology also comes with some disadvantages. Current technology works as a batch process which is time consuming and can offset the savings associated with using less shell material. In addition, this process requires an extra step to enable crosslinking of protein. Moreover, the coacervates formed with this technique are stable on a very narrow range of pH and ionic strength and so reaching the correct endpoint before cross-linking requires careful monitoring even at production levels (Stainsby, 1980; Zhang et al. 2009). Current processes primarily use gelatin as the positively charged polymer, but animal derived gelatin is not acceptable to the vegetarian population (Kralovec et al., 2012). Fish gelatin is also in use in place of the animal-based gelatin. However, the raw material for production of fish gelatin is limited, particularly if high bloom gelatin is required (Schmid et al., 2001; Zhang et al. 2009). In addition, the cost of fish gelatin is 4-5 times higher than the pork gelatin (Choi et al., 2010). The above mentioned disadvantages associated with the pork and fish gelatins suggest that there is a need to source a variety of coacervating agents which are of plant origin and can give a high payload, structural strength and oxidative stability.

2.3.7. Inclusion complexation

This technology normally uses cylodextrins as encapsulants. Cyclodextrins are cyclic oligosaccharides of six to eight D-glucose units, which are enzymatically linked through alpha 1-4 linkages to form a ring. Cyclodextrins form complexes with LCPUFAs, and provide some protection against oxidation. In a patent assigned to Schmid et al. (2001), fish oil was mixed with gamma cyclodextrin (containing eight glucose molecules) to form a complex. This inclusion complex was formed by continuously stirring of the fish oil-gamma cyclodextrin mixture in the presence of nitrogen at 45^{o}C for 24 h. The resultant complexed product contained the fish oil payload of 15-40% and was stable when stored under air at 100^{o}C for 24 h.

Choi et al. (2010) encapsulated fish oil in beta cylodextrin and reported an encapsulation efficiency of 84.1% and payload of 62.7%. However, the surface oil after freeze-drying was 11.0%, which is quite high compared to the surface oil in powders obtained from using complex coacervation.

2.3.8. Liposome entrapment

A liposome is a lipid vesicle composed of lipid bilayers that enclose a number of aqueous compartments. Phospholipids are primarily used as the encapsulating agents in liposome based microencapsulation processes (Kim et al., 1991). Liposomes have been used to encapsulate omega -3 fatty acids by dissolving the source oil in phospholipid before addition of water. This mixture comprising phospholipid, omega-3 oil and water is sonicated to form encapsulated product and finally dried into powder. The oil encapsulated in liposomes is quite stable against oxidation (Kubo et al., 2003). The main advantage of this technology is that encapsulated ingredients such as enzymes can be targeted to specific locations within food products, such as the curd component of milk in cheese ripening (Gouin, 2004).

However, the high cost and sometimes low stability limit the application of this technology for microencapsulating omega-3 fatty acids.

2.3.9. Emerging microencapsulation methods of omega-3 oils

2.3.9.1. Electrospraying for ultrathin coating

Sergio et al. (2010) used electrospraying to microencapsulate DHA in Ultrathin films of zein prolamine. Nanometer sized capsules were formed using electrospraying and induction period for encapsulated oil was increased, showing improved oxidative stability due to the ultrathin coating of zein prolamin. The presence of this coating had minimal impact on the textural characteristics of the product, indicating that this is a promising technology for food industry.

2.3.9.2. Spray granulation and fluid bed film coating

To enhance the stability of fish oil, spray granulation and fluid bed coating were used in combination for microencapsulation (Anwar et al., 2010). In this technology, the fish oil microcapsules were produced in two successive steps. Firstly, fish oil was emulsified with soybean polysaccharides and maltodextrin and sprays dried to produce granules. Secondly, the granules were coated using hydroxypropyl betacyclodextrin. However, the authors found the coating was not effective in preventing the lipid oxidation and so this method is not useful for omega-3 stabilisation without further development.

2.3.9.3. Encapsulation using ultrasonic atomizer

Conventional pressure spray nozzles produce a wide size distribution of droplets as they are incapable of controlling droplet size (Bittner & Kissel, 1999). Ultrasonic atomizers employ ultrasound energy to atomize fluids and provide smaller droplets with relatively narrow size distribution (Topp and Eisenklam, 1972).

Klaypradit and Huang (2008) encapsulated fish oil using an ultrasonic atomizer. They obtained small particle size and good emulsion stability in a combined wall material matrix of

chitosan and maltodextrin. These freeze dried microcapsules contained 240 mg/g of EPA and DHA. Ultrasonic atomizer-based atomization technology has some potential for the microencapsulation of omega-3 oils, but payload is relatively low.

In a similar study, Legako and Dunford (2010) used three-fluid pressure nozzle and two-fluid ultrasonic nozzle. In these nozzles oil and the aqueous solution containing wall material flow in separate channels and do not mix until they meet at the tip of the nozzle. The application of these nozzles eliminates the need for the emulsion preparation step prior to drying. The elimination of the emulsion preparation step eliminates oxidation that can occur during emulsification. Although, results from these studies demonstrated improved oxidative stability during processing, microencapsulation using three-fluid nozzle and ultrasound nozzles resulting in relatively low encapsulation efficiency. Hence, further research is required to better assess the benefit of using a three-fluid nozzle in producing microcapsules from fish oil.

2.4. METHODS AND PROPERTIES FOR THE MICROENCAPSULATION OF OMEGA-3 OILS USING COMPLEX COACERVATION

Complex coacervation is one of the commercially successful methods for microencapsulation of flavour oils and the oils rich in omega-3 fatty acids (Gouin, 2004; Barrow, 2010). In this section we discuss the impact of polymer characteristics on the process and outcome for the microencapsulation of omega-3 using complex coacervation.

2.4.1. Processes for the microencapsulation of omega-3 oils using complex coacervation

Complex coacervates formed by proteins and polysaccharides have been widely used as vehicles for encapsulation of omega-3 oils. Wu et al. (2005) encapsulated fish oil by complex coacervation of hydroxypropyl methylcellulose with maltodextrin and spray drying of the resultant coacervates. It was found that better stability of fish oil against oxidation was

achieved by replacing 40% maltodextrin with gum acacia. Microencapsulation of fish oil by complex coacervation with gum arabic and gelatin has also been reported. The limitation of this method was that the coacervates obtained had high water content after spray-drying (Lamprecht et al., 2001).

Barrow et al. (2007) developed an improved coacervation technology which involved controlled agglomeration and formation of an outer shell that surrounds multiple agglomerates. This technology has been scaled up to industrial level and is used commercially to stabilise and deliver omega-3 oils into foods and beverages. Drusch (2007) microencapsulated fish oil rich in long-chain polyunsaturated fatty acids in a combined matrix of sugar beet pectin and glucose syrup. Results of this study indicated that sugar beet pectin could be an alternative, cost effective wall material like gum Arabic; however, the maximum oil load obtained with sugar beet pectin in this study was not more than 50%.

Zhang et al. (2009) used whey protein and gum Arabic to encapsulate fish oil using a combination of complex coacervation and thermal crosslinking processes. Free flowing powders were produced through spray drying the coacervates. Instead of applying chemical or enzymatic crosslinking of proteins, authors used a thermal crosslinking process to solidify the microcapsules and prevent their dissolution. The microcapsules obtained with whey protein isolate were more stable in high thermal treatments than those obtained by using gelatin.

To preserve and protect omega-3 fatty acid in flaxseed oil, this oil was microencapsulated by complex coacervation using gelatin-gum Arabic which yielded free flowing powders. The production of primary and secondary oxidative products was inhibited in these powders when stored at ambient temperature for 25 days (Liu et al., 2010). A survey of literature indicates that gelatin is the material of the choice for use in complex coacervation processes due to its unique properties as described in the next section. However, as most of the available gelatin

is of animal origin, and gelatin has its own sensory issues, the use of gelatin has some limitations in food and beverage applications. Therefore, it is necessary to find alternatives to gelatin for complex coacervation. Plant-based materials, such as flaxseed and chia proteins, provide vegetarian alternatives and should be studied for their efficacy in terms of payload, preventing oxidation as well as structural strength of the coacervates. Plant polyphenols have been used as cross-linking agents and in the microencapsulation of omega-3 oils (Montero et al., 2005). Polyphenolics have also been used as antioxidants to increase the shelf-life of microencapsulated flaxseed oil (Rubilar et al., 2012).

2.4.2. Properties of coacervates

2.4.2.1. Structural properties

For a protein to be suitable in a coacervation process, it should have a particular amino acid composition and charge density profile (Ducel et al., 2004). Gelatin is the most commonly used protein in complex coacervation process partly because its amino acid profile and charge density is suitable for the coacervation process. The amino acid composition of gelatin is characterized by repeating sequence of Glycine-X-Y triplets, where X is mostly proline and Y is mostly hydroxyproline (Eastoe & Leach, 1997). The coiled structure of gelatin maintains sufficient charge on its chains to avoid precipitation irrespective of the anionic compound used. Similarly gum Arabic also has a coiled polysaccharide configuration, which preserves the charges as well as allows a considerable quantity of water to be occluded between the chains to avoid precipitation (Ducel et al., 2004). The random coil configuration of two polymers is favourable for the formation of coacervates as this configuration better preserves the charges and increases the interaction between the polymers (Schmitt et al., 2010).

2.4.2.2. Rheological properties

Rheological properties of the coacervates formed by the interactions of proteins and polysaccharides are very important for production of microcapsules. Burgess (1994) reported that a high viscosity of the coacervates can yield more stable capsules. Mixing conditions leading to complete neutralization of charges on both the polymers gives the maximum viscosity.

Rheological behaviour of coacervates is influenced by many factors, including molecular weight, molecular structure and charge. In addition, protein–polysaccharide ratio, pH, temperature and ionic strength also affect the rheology of coacervates. Viscosities of both the coacervate phase and the coacervation medium also influence the size of the resulting microspheres/microcapsules.

2.4.3. Factors affecting complex coacervation

The process of coacervation occurs as a consequence of associative interactions between the biopolymers present in the solution. These interactions are weak, attractive and nonspecific, including electrostatic, van der Waals, hydrophobic and hydrogen bonding. The most common interaction between proteins and polysaccharides is electrostatic (McClements, 2006), which depend on the pH and ionic strength of the system. Hence, both the pH and ionic strength significantly impact the coacervation process. It has been observed that coacervation starts only when the two polymers involved carry net opposite charge (Burgess & Carless, 1985). In addition to charge and pH, there are some other factors that influence outcomes, such as type, ratio, molecular weight and total concentration of the polymers used, extent of turbulence (agitation) in the system, emulsion size and temperature (Turgeon et al., 2007).

Wang et al., (2000) observed that molecular weights of polymers have considerable effect on the process of coacervation, and that the size of coacervates formed can be controlled by

adjusting the molecular weight. Large molecular weight polymers appear to form large coacervates with lower solubility (Wang et al., 1996). Semenova (1996) observed that coacervation between soy protein and dextran increased with increasing dextran weight.

The pH of the medium impacts the charge density of polymers and in turn the strength of coacervation. For a variety of polymers a narrow pH range gives the best coacervation results (Turgeon et al., 2003). This effective pH range depends on the molecular weight and the distribution of the reactive groups of the polymers. For example, coacervation of soybean protein isolate and chitosan occurred within a pH range of 5.5-6.5 (Huang et al., 2012). Similarly the effective pH range in the case of α-lactalbumin-chitosan pair was found to be 5.5-7.0 (Lee & Hong, 2009).

The mixing ratio of biopolymers affects the extent of coacervation by changing the available amount of each polymer for electrostatic interaction. Burgess and Carless (1985) reported that maximum coacervation is obtained when an equal ratio of polymers by weight are mixed at electrical equivalence pH. That is, a pH at which the charges on both the polymers are equal and opposite (Burgess & Carless, 1985). All these process conditions are interrelated and influence the process of coacervation collectively. Microencapsulation of fish oil by complex coacervation using maltodextrin and hydroxypropyl methyl cellulose (HPMC) illustrates how sets of parameters control the process. Coacervation was observed only when the dextrose equivalence of maltodextrin, concentration of HPMC solution and the percentage of fish oil in microcapsules were no more than 20, 5 and 40%, respectively (Ke-Gang et al., 2005).

2.5. STABILITY AND CONTROLLED RELEASE OF ENCAPSULATED OMEGA-3 FATTY ACIDS

As described in Section 3 and 4 above, microcapsules of omega-3 rich oils can be made in different ways, including by trapping in glassy matrices, stabilizing in emulsion systems with

low molecular weight surfactants or film forming biopolymers, or encapsulating in liposomes or cyclodextrins. But most techniques involve emulsification as a key step. A stable emulsion containing small oil droplets dispersed in aqueous solutions of polymers result into successful microencapsulation irrespective of the method used for drying. Different types of emulsion based systems used to encapsulate various lipophilic ingredients in the food industry include single layered oil in water emulsion, multiple emulsions and multilayered oil in water emulsion.

Protection against oxidative deterioration is the main purpose behind the microencapsulation of oils containing omega-3 fatty acids. In order to achieve the best level of emulsion stability, different emulsion formulations have been tried using a variety of ingredients. Klinkesorn et al. (2005) produced spray dried tuna oil microcapsule powders which were emulsified using a mixture of lecithin and chitosan. This multilayer coating produced emulsion droplets with interfacial membranes that are cationic and thick, providing better protection against the lipid oxidation. This study indicates that multi layered emulsions provide better stability for omega-3 oils than single layered emulsions. However, the technology of producing multi-layered or double emulsions involves more than one wall materials and a complex double emulsion system.

Encapsulation using double emulsification and an enzymatic gelation method using microbial transglutaminase cross-linked proteins is also used to prepare protein based microcapsules containing fish oils. This process using two emulsions in succession provides better protection to active ingredients. Emulsions can be either water-oil-water (w/o/w) or oil-water-oil (o/w/o). For encapsulation of omega-3 oils from marine origin the o/w/o emulsion is commonly used. Cho et al. (2006) microencapsulated fish oil in double emulsions made by enzymatic gelation technique. Firstly, an oil-in-water emulsion was produced by dispersing fish oil in soy protein isolate. This emulsion was subsequently

dispersed in corn oil to form O/W/O double emulsion. Transglutaminase enzyme was used to carry out the gelation. The capsules thus obtained were spherical with having narrow size distribution with a mean diameter of 23 µm. Overall this method improves the stability of the ingredients and allows controlled release.

The mechanism of release of omega-3 is one of the most important aspects of encapsulation technologies. It was observed that the process of hardening of coacervated capsules affects the release of oil. Zhang et al. (2012) reported that the release of oil from gelatin-gum Arabic coacervated microcapsules cross-linked using transglutaminase (TG) could be regulated by changing the cross-linking parameters such as hardening time, pH, temperature and the concentration of TG.

2.6. CHARACTERIZATION OF MICROENCAPSULATED OMEGA-3 OILS

Microencapsulated omega-3 oils can be produced by different microencapsulation techniques using a number of suitable shell materials. The processing parameters and the material characteristics determine the final characteristics of the microcapsules (Zhang et al., 2009). The characteristic of the microcapsules are important for both bioavailability and success in the targeted application. Microencapsulated oil can be characterized using physical, chemical or physicochemical properties. These characteristics generally include determination of encapsulation efficiency, surface oil and payload, particle size and shape of microcapsules, oxidation stability and sensory performance.

2.6.1 Encapsulation efficiency

This can be defined as the ratio of the mass of the core material which is encapsulated in the wall material to the mass of the core material used in the formulation. For higher encapsulation efficiency, the mass of the free oil should be as low as possible and the mass of fully encapsulated oil should be as high as possible. Free oil refers to the non-encapsulated oil

and is also known as surface oil. Measurement of surface oil is important as this oil can oxidise extremely rapidly, so that high surface oil tends to correlate with off-flavor of microcapsules and poor food application stability. Surface oil can be measured using a standard method of fat determination involving extraction of fat by using a soxhlet apparatus with an organic solvent like hexane, ethyl acetate or methanol, and measuring the extracted fat either gravimetrically or by using quantitative spectroscopic methods like UV-VIS or IR spectroscopy. Ideally for safe storage of microcapsules the surface oil content should be below 0.1% (w/w), although many emulsion products are significantly higher than that.

2.6.2 Payload

This is the percentage of the oil or active ingredient per gram of the powder. When an encapsulation process achieves a higher load, the production of the microcapsules become economical and the process becomes more economically feasible. High payload means that a lower amount of powder is required per serving of the food. Payload is calculated by taking the ratio of mass of encapsulated oil (or omega-3) to total mass of powder. The mass of encapsulated oil can be determined gravimetrically by quantitative extraction of oil from a known quantity of microcapsules, followed by weighing of extracted oil. This is a destructive method and is time consuming and expensive for quality control purposes. A Fourier transform Infrared (FTIR) spectroscopy based non-destructive method has been recently developed to determine payload (Vongsvivut et al., 2012).

2.6.3 Particle size

The size of microcapsules for food applications should be below 100μm to avoid impacting the mouth feel of the food product. The distribution of the particle size should also be as narrow as possible in order to maintain product consistency. The size of the microcapsules can be measured using techniques such as laser scattering or particle size imaging using

microscopy. High resolution imaging using electron microscopy or confocal laser scanning microscope (CSLM) is useful for studying the detailed morphology of microcapsules. CSLM can be combined with staining techniques to gain better insights into the characteristics and distribution of the hydrophobic core and the hydrophilic shell.

2.6.4 Stability

The primary purpose of microencapsulation of omega-3 rich oils is to protect the sensitive omega-3 fatty acids against oxidation by providing an oxygen barrier in the form of wall materials. Different types of wall materials offer oxidative stability to different extent, primarily depending upon their ability to inhibit the transfer of oxygen. Oxidative stability of oil in microcapsules is measured by storing the microcapsules under a set of temperature and relative humidity for a defined period and measuring the oxidative products formed. Conjugated dienes, aldehydes, trans fats and peroxides are the oxidation products of omega-3 fatty acids and can be quantitatively measured in oil samples (Table 3). Propanal distinctive for omega-3 oils and can be used as an indicator of oxidation of omega-3 fatty acids (Frankel et al., 2002). The extent of oxidation can also be quantified by measuring the oxygen head space concentration or by accelerated analysis using a rancimat or oxypres (Kulas and Ackman, 2001). The advantages and limitation of these methods are listed in Table 2.3.

Association of off-flavours with oxidation enables assessment of the extent of oxidation, particularly at relatively low oxidation levels where these volatile aldehydes responsible for off-flavours first form. Sensory evaluation can also provide qualitative assessment of degree of oxidation of the omega-3 microencapsulated products. A trained sensory panel is one of the fastest ways of qualitatively detecting the initial oxidative deterioration of omega-3 in encapsulated products.

Table 2.2 Different methods for measurement of oxidation of omega-3 fatty acids

Method	Principle	Advantages	Disadvantages
Peroxide value (AOCS methods Cd 8-53 and Cd 8b-90)	Idiometric titration	Established method, most commonly used	Time consuming, Measures unstable and intermediate products (Kolanowski et al., 2007)
Conjugated dienes (AOCS method Ti 1a-64)	Spectrophotometric (232-234 nm)	Fast simple and less sample size required	Depends on sample fatty acid composition (Shahidi and Wanasundara, 1996)
Propanal measurement	Static headspace gas chromatography	Specific marker (Shen et al., 2010)	Time consuming for large number of samples (Boyd et al., 1992; Shaw et al., 2007)
Thiobarbituric acid reactive substances (TBARS)	Spectrophotometric (532-535 nm)		Not a good indicator at lower oxidation levels (Kolanowski et al., 2007)
Rancimat	Measures Induction period	Rapid with multiple sample at one time, good reproducibility	High cost of apparatus
Sensory Panel	Sensory characteristics	Quick results	Qualitative only

2.7. BIOAVAILABILITY OF MICROENCAPSULATED OMEGA-3 FATTY ACIDS

The health benefits associated with microencapsulated omega-3 fatty acids depend on their bioavailability. Shell materials used for microencapsulation are primarily protein and carbohydrates that are known to be digestible and susceptible to stomach acid and intestinal enzymes, so bioavailability would be anticipated. Specific studies to assess the bioavailability of omega-3 in food materials enriched with microencapsulated omega-3 fatty acids, have shown bioavailability that is equivalent to fish oil supplements provided as capsules (Higgins et al., 1999; Wallace et al., 2000). Barrow et al. (2009) showed that omega-3 rich fish oil that was microencapsulated using complex coacervation had a similar triacylglycerol lowering effect in blood to that of fish oil supplements. It has also been reported that the application of whey protein as wall material increased the bioavailability of microencapsulated omega-3 fatty acids (Richelle et al., 2002). New technologies for microencapsulating fish oil need to be assessed for bioavailability. However, where protein is the major shell material, bioavailability would be expected. If materials with known low digestibility, such as chitosan or cellulose, are added to the shell material then testing for bioavailability becomes particularly important.

2.8. FUTURE TRENDS

There is considerable scientific evidence that the consumption omega-3 fatty acids not only reduces the risk of a number of diseases but also promotes physical and mental wellbeing. Many countries have issued recommendations for increasing the daily consumption of these healthy fats. As a consequence, the demand of omega-3 fatty acids is increasing in the functional food market. Microencapsulated omega-3 fatty acids are being incorporated into wide range of products such as breads, milk, fruit juices, tortillas, chocolate, yoghurt drinks, spreads, peanut butter, eggs and meat. Comparing the commercially available

microencapsulated products of omega-3s Barrow et al., 2007, found that complex coacervation is a successful technique for the microencapsulation of LCPUFA. This technology besides giving a high payload also yields products with lowest surface oil and cost per dose of omega-3s. The biggest disadvantage of this technology is limited availability of shell materials. So far, gelatin is the only protein which is successful commercially.

A number of studied have reported that the plant proteins are capable of forming coacervates in the presence of polysaccharides. This means that plant proteins can be used instead of animal proteins in complex coacervation process. Ducel et al (2004) used alpha gliadin (cereals) and pea globulin (legume) in complex coacervation process. These authors found that both these proteins form excellent complex coacervates with the gum Arabic. However, the application of alpha gliadin in the coacervation process will not achieve wide spread acceptance as this protein is an allergen in some instances (Ducel et al., 2004). So, there is a need to test other plant polysaccharides for their potential for the safe delivery of active ingredients. Recently whole buttermilk (Agustin et al., 2014) was used to successfully microencapsulate fish oil. In a different strategy, omega-3 lipids were used as effective carriers for lutein delivery (Lacatusu, et al 2013), indicating that co-delivery of lipid ingredients is an important future strategy.

Emulsifying properties of flaxseed protein concentrate (FPC) were evaluated by Wang et al. (2010). It was found that the emulsions stabilized by FPC at neutral pH and in the absence of salt had a smaller droplet size and higher surface charge which makes them good candidates to be used in coacervation process. The FPC-stabilized emulsions were more stable against the effect of salt concentration. The FPC can be effective stabilizing emulsions where droplet size and zeta-potential are major factors influencing the emulsion stability. Flaxseed gum is also found to possess good potential in stabilizing the protein-based emulsions (Wang et al., 2011). The electrostatic complexes formed by these two polymers can be potential shell

materials for microencapsulation of active ingredients by complex coacervation process. But currently there is no study available on the complexation behaviour of these two polymers. Hence, further research is needed to standardize the process of coacervation

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Chapter 3

Preparation, characterization and functional properties of flax seed protein isolate

ABSTRACT

Flaxseed Protein Isolate (FPI) was extracted from flaxseeds and its amino acid composition and functional properties (solubility, thermal stability, emulsifying properties and electrostatic charge density, water holding and fat absorption capacities) were determined. The highest purity of FPI (90.6%) was achieved by extraction at 60 °C. FPI had a low lysine to arginine ratio of 0.25 which is desired in heart-healthy foods and infant formulas. The denaturation temperature of FPI was 105 °C. FPI had highest emulsion activity index (375.51 m^2/g), highest emulsion stability index (179.5 h) and zeta potential (-67.4 mV) compared to those of other commonly used proteins such as sodium caseinate (SC), whey protein isolate (WPI), gelatin (Gel) and soy protein isolate (SPI). The average emulsion droplet size of emulsions stabilized by these proteins was in the order SC<FPI<WPI<Gel<SPI. Water holding and fat absorption capacities of FPI were similar to those of the above mentioned proteins.

Key words: Flaxseed protein isolate; Solubility; Zeta potential; Emulsion activity index; Droplet size

3.1. INTRODUCTION

Flaxseed is an important oilseed crop that provides healthy food components including oil, protein, dietary fibre and lignans (Jenkins et al., 1999). Flaxseed protein is a major component comprising 18-22% of seed weight depending upon variety and geographical origin (Oomah & Mazza, 1993). Flaxseed protein is composed of two protein fractions: a salt soluble, high molecular weight (11S-12S) fraction; and a water soluble, low molecular weight (1.6S-2S) fraction. Flaxseed protein is rich in aspartic acid, glutamic acid, leucine and arginine (Oomah & Mazza, 1993). Due to its amino acid profile, flaxseed protein offers potential health benefits to malnutrioned populations and those with milk protein allergies (Weisdorf, 1998). Allergen studies reported for flaxseed either relate to flaxseed oil used as a laxative, or other seeds present in food (Alonso et al., 1996; Leon, Rodriguez, & Cuevas, 2002). There is no published clinical study indicating that flaxseed protein triggers allergy when consumed as part of a normal diet (Muir, & Westcott, 2003).

In addition, flaxseed protein possesses promising functional properties, such as high emulsifying activity and foaming capacity (Dev & Quensel, 1988), making it an attractive ingredient for the food industry. Moreover, there is an increasing trend of replacing animal proteins with plant proteins in food formulations, resulting in plant proteins such as soybean protein (Molina, Papadopoulou, & Ledward, 2001), lentil protein (Joshi et al., 2012), pea, chick pea (Boye et al., 2012) being extensively studied for their physicochemical and functional properties. Flaxseed protein products containing mucilage were also reported to possess favourable functional properties (Dev & Quensel, 1988) and recent studies have reported favourable functional properties for flaxseed protein concentrate (Kuhn, Netto, & Cunha, 2014; Wang, Li, Wang, & Ozkan, 2010).

Application of flaxseed in the food industry is primarily limited to its oil and the whole seeds. The interference of flaxseed mucilage in protein isolation, and the lack of effective extraction methods, have prevented the broader utilization of flaxseed protein isolate (FPI) (Oomah & Mazza, 1993). Studies have identified that the presence of polysaccharides in the seed coat of flaxseed hinder the separation of proteins, due to their swelling in aqueous media (Oomah & Mazza, 1993; Chung, Lei, & Li-Chan, 2005). The removal of these mucilage type polysaccharides prior to protein extraction greatly enhances the recovery and purity of protein (Wanasundara & Shahidi, 1997; Li-Chan, & Ma, 2002). Polysaccharide degrading enzymes were used by Wanasundara and Shahidi (1997), to minimise the interference of mucilage in the extracted protein. Although advances have been made in the extraction and isolation of proteins in the last 20 years, the extraction of flaxseed protein from flaxseed is still challenging. The water-soluble mucilage that is rich in the hull can increase the viscosity of the mixture and interfere with subsequent protein extraction. In 2009, Zhang et al. (2009) developed a wet process for extraction and fractionation of mucilage from flaxseed, which is continuous and easy to scale up. However, this process was time consuming, required substantial amount of water and resulted in low protein recovery.

Studies carried out to determine or assess the functional properties of flaxseed protein have either used flaxseed protein concentrate (Wang et al., 2010) or enzymatically modified or fractionated protein (Mueller et al., 2010; Wanasundara & Shahidi, 1997) rather than native FPI. In addition, there is no systematic study of emulsifying properties of FPI and these properties have not been compared with those of other commonly used food proteins such as gelatin (Gel), whey protein isolate (WPI), sodium caseinate (SC), soy protein isolate (SPI).

In the above context, the objectives of this study were to: 1) Optimize the extraction of FPI and maximize its yield without compromising protein purity. That is, to minimise the interference of flaxseed mucilage in protein extraction; 2) characterize the physicochemical

properties (thermal stability, solubility, and electrostatic charge density) of the extracted FPI; and 3) compare the functional properties (emulsion activity index, emulsion stability index, water holding and fat absorption capacity emulsion droplet size and charge density) of FPI with those of commonly used proteins such as Gel, WPI, SC and SPI.

3.2. MATERIALS AND METHODS

3.2.1. Materials

The golden flaxseeds (*Linum usitatissimum*) were received from Stoney Creek Oil Product Pty. Ltd (Talbot, VIC, Australia). Gelatin (Type A) was purchased from Sigma-Aldrich Corporation (Castle Hill, NSW, Australia). Whey protein isolate (WPI 895^{TM}) was donated by Fonterra Dairy Company (Christchurch, Canterbury, New Zealand). Sodium caseinate (SC, MC2972) and soy protein isolate (SPI, PRO-FAM 974) were provided by Murray Goulburn Cooperative Limited (Southbank, VIC, Australia) and Archer Daniels Midland Company (Decatur, IL, USA), respectively. Sunflower oil was purchased from a local supermarket and was used to prepare oil-in-water emulsions. All other chemicals used in this study were purchased from Sigma-Aldrich Australia (Sydney, New South Wales, Australia) and were used as received.

3.2.2. Preparation of FPI

FPI was extracted from whole raw flaxseed using a method described by Oomah, Mazza and Cui (1994), with some minor modifications, as shown schematically in Fig. 3.1. Briefly, the flaxseeds were demucilaged three times by soaking in Milli-Q water at a flaxseed to water ratio of 1:18 at 30-60 ^{o}C for 3 h. The demucilaged seeds obtained in this way were dried in a hot air oven at 50 ^{o}C for 24 h and were pulverized using a coffee grinder (EM0415, Sunbeam Corporation Ltd. NSW, Australia). The hull was separated from the meal by screening the

tailings using a 0.15mm sieve to further reduce the interference of the mucilage during protein extraction.

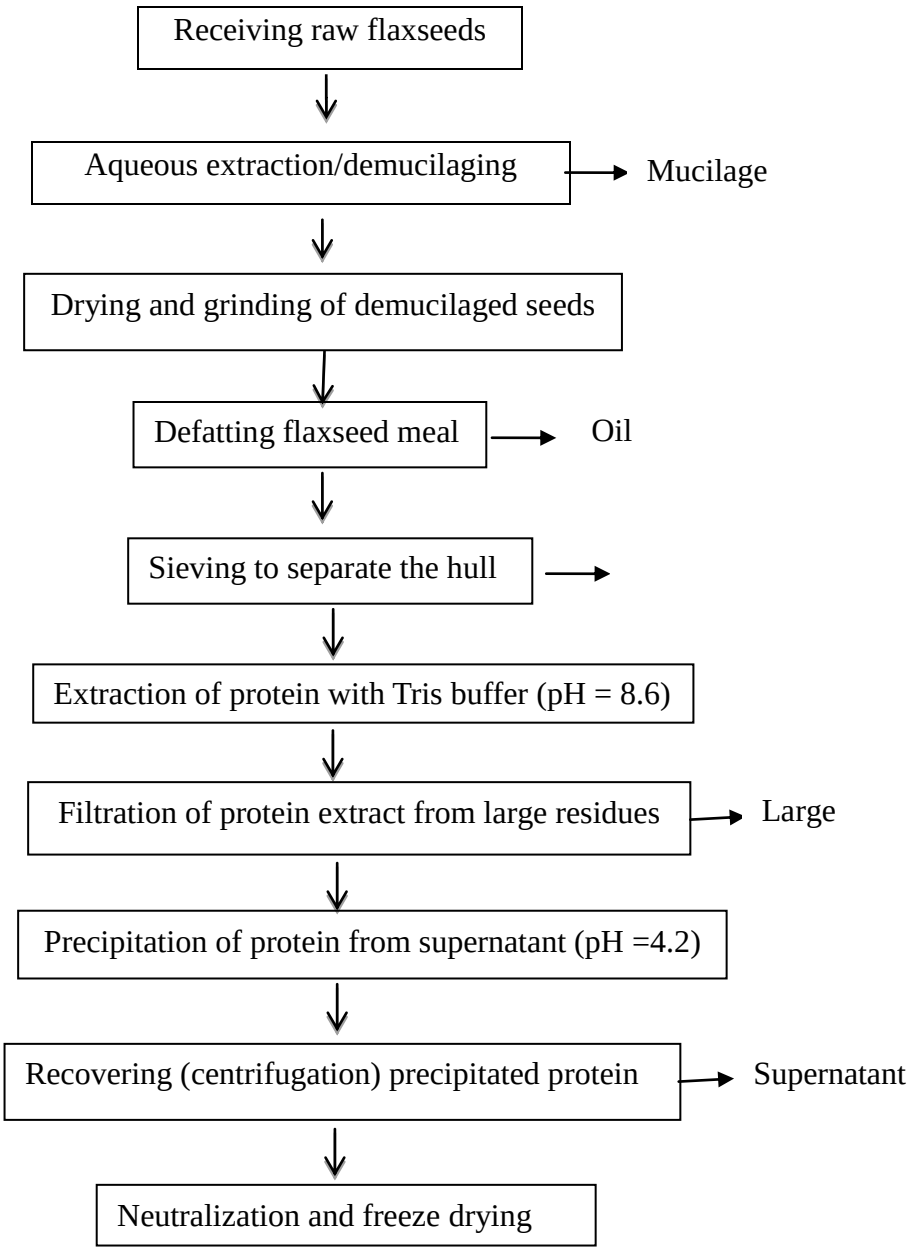

Figure 3.1 Schematic process flow diagram used to extract flaxseed protein isolate (FPI) from whole flaxseeds

The crushed flaxseed meal was further defatted for 3 h using hexane at a meal to hexane ratio of 1:6. This powder was subsequently soaked in 0.1 M tris buffer (pH 8.6) at a seed to buffer ratio of 1:16 for 24 h. The large residues were then separated using double layered cheesecloth from the protein extract. Subsequently, the extract was centrifuged at 9,600×g for 20 min using an ultracentrifuge (Sorvall Instruments, Wilmington, DE). The supernatant was collected and its pH was adjusted to 4.2 using 0.1 M HCl to precipitate the flaxseed protein. The extracted protein was then stored at 4 ^{o}C for 16 h to allow complete protein precipitation. This precipitated protein was subsequently centrifuged at 17,000×g for 20 min, redispersed in Milli-Q water, and then neutralized using 0.1 M NaOH. Finally, the flaxseed protein isolate (FPI) was obtained by freeze drying the sample at -45 °C compressor temperature and 66.66 Pa vacuum pressure using a freeze drier (DYNAVAC, Dynavac Engineering, Australia). The freeze-dried FPI was ground and vacuum sealed and stored at 4 °C till further use.

3.2.3. Determining the effect of demucilaging temperature on yield and purity

To minimise the interference or amount of residual mucilage in the FPI extract, the demucilaging process was carried out at four different temperatures, (30, 40, 50 and 60 °C). The effect of temperature on yield and purity of the extracted FPI was investigated. Yield of extracted protein was calculated follows:

$$Yield(\%) = (P/S) \times 100 \quad (1)$$

Where '*P*' is the weight of protein powder in grams obtained after extraction and freeze drying from whole flaxseeds and '*S*' is the weight of seeds in grams taken for extraction.

Purity of protein extract was reported as the protein content obtained from the Kjeldhal protein estimation method (AOAC Method No. 920.87). Briefly, 1 g of the sample was digested with 98% (w/w) sulphuric acid at sample/acid ratio of 1/10 to convert the organic nitrogen to ammonium sulphate. Subsequently, 40ml NaOH (30%, w/v) was used to

neutralise the digest and the liberated ammonia was distilled into boric acid. Finally, the nitrogen content in the sample (N) was estimated by the amount of (0.1M) hydrochloric acid needed to titrate the formed ammonium borate and the total protein content in the sample was further calculated using 6.25×N.Percentage protein recovery was calculated by the equation given below:

$$Recovery\ (\%) = (P_p / S_p) \times Yield\ (\%) \quad (2)$$

Where '*Pp*' is the protein content (%) of extracted protein and '*Sp*' is the protein content (%) of the seed used for extraction of protein.

3.2.4. Determining the moisture, protein and ash contents

The moisture, protein (% N×6.25) and ash contents of the FPI samples were analysed using the standard AOAC (2005) method numbers of 925.1, 920.87 and 923.03, respectively. All the measurements were made in triplicate and the mean values are reported.

3.2.5. Determination of amino acid profile

The FPI sample was hydrolysed in order to determine the amino acid profile. The hydrolysis was carried out using 6 M HCl at 110°C for 24 h. For cysteine analysis, the sample was oxidised using performic acid followed by gas phase hydrolysis at 110 ^{o}C for 24 h. For tryptophan analysis, 5 M NaOH was used for liquid hydrolysis of the sample at 110 ^{o}C for 24 h. After hydrolysis all amino acids were determined by an amino acid analyser (Waters Acquity UPLC) using an AccQTag Ultra chemistry column. Samples were analysed in duplicate and results are reported as an average.

3.2.6 Physicochemical Properties of FPI

3.2.6.1. Measurement of X-ray diffraction

X-ray diffractograms were obtained using a Siemens diffractometer (model D501, Siemens AG, Karlsruhe, Germany) with Co Kα < 1 radiation. The samples were scanned digitally with

a scan rate of 1° per min over the (2θ) angle range of 5° to 55° (2θ) range at an increment of 0.05°.

3.2.6.2. Measurement of zeta-potential

The zeta-potential of FPI was determined as a function of pH using an electrophoresis instrument (Zetasizer ZS-90, Malvern instruments, Worcestershire, UK). The FPI powder was dispersed at 0.1 M tris buffer (pH 8.6) at a concentration of 0.5% (w/v), followed by filtration using Wattmann No.5 filter paper. The filtered protein dispersion was transferred into seven glass cuvettes and subsequently diluted 100 times with MilliQ water to avoid multiple scattering effects. The pH of these diluted samples was adjusted in the range of 2.0-8.0 using 0.1 M HCl or 0.1 M NaOH.

3.2.6.3. Thermal analysis of FPI

Thermal characteristics of FPI were determined using a differential scanning calorimeter (Q2000, TA instruments, New Castle, DE, USA). Seven milligrams of FPI sample was accurately weighed in an aluminium pan. The pan was hermetically sealed and heated from 30 to 160 °C at a heating rate of 10 °C/min. An empty aluminium pan was used as the reference. The onset (T_m) and peak (T_d) denaturation temperatures and enthalpy of denaturation (ΔH) was determined from the thermograms using the associated software (TA Universal Analysis 2000).

3.2.6.4. Determination of solubility

The solubility of FPI was determined as a function of pH using the method of Morr et al. (1985). The FPI powder was dissolved in 0.1 M tris buffer at 1% (w/v) and the pH was adjusted to achieve specific values within the range of 2.0 to 10.0 using 0.1 N HCl or 0.1 N NaOH. These FPI dispersions were agitated at ambient temperature (20 ± 2 °C) for 60 min and then centrifuged (Sorvall SS-34, Sorvall Instruments, Wilmington, DE) at 17,500×g for 30 min at ambient temperature. Subsequently, the protein content in the supernatant was

determined using the Kjeldahl method (% N×6.25). The protein solubility (%) was determined by dividing the protein content in the supernatant by the total protein content in the powder used. Experiments were performed in triplicate and average values are reported.

3.2.6.5. Determination of water holding and fat absorption capacities.

The water holding capacity (WHC) and fat absorption capacity (FAC) were determined using the method of Tomotake et al. (2002) with minor modifications. One gram of protein was weighed into pre-weighed centrifuge tube and 10 ml of Milli-Q water was added. The mixture was vortexed for 1 min and allowed to stand for 30 min at ambient temperature and then centrifuged at 5,000×g for 30 min. The supernatant was decanted carefully and the tube with sediment was weighed again. WHC was calculated as the amount of water held by one gram of FPI sample. The FAC was determined using similar procedure using 0.5 g of FPI and 5 ml sunflower oil. The FAC was expressed as the amount of oil per gram of FPI sample.

3.2.7. Preparation and characterisation of emulsions

The oil-in-water emulsion was prepared by dispersing 10% sunflower seed oil (v/v) in the water phase containing 1% protein (w/v), followed by homogenization using an ultra Turax (RW 20, IKA GmbH Co., Bitterfeld-Wolfen, Germany) at 10,000 rpm for 1 min. The prepared emulsions were stored at room temperature until needed.

3.2.7.1. Emulsifying activity index and emulsion stability index.

The emulsifying activity index (EAI) of FPI and the emulsion stability index (ESI) of protein emulsions were determined by using a turbidimetric method (Pearce & Kinsella, 1978). Freshly prepared emulsion was diluted 100 times with 0.1% (w/v) sodium dodecyl sulphate (SDS) and mixed for one minute using a vortex mixer. The absorbance of this diluted emulsion was measured at 500 nm wavelength using a UV spectrophotometer (UV-1800,

Shimadzu, Kyoto, Japan). The turbidity of emulsion was calculated using Eq. (3) given below.

$$T = 2.303 \times \frac{A}{L} \times D \quad (3)$$

where T is the turbidity of emulsion in m^{-1}, A is the absorbance (dimensionless), D is the dilution factor (dimensionless) and L is the light path length in m.

The emulsifying activity index (EAI, expressed in m^2/g) of FPI and emulsion stability index (ESI, expressed in h) were calculated using Eq. (4) and (5), respectively.

$$EAI = \frac{2 \times T_0}{\phi \times C \times 1000} \quad (4)$$

$$ESI = \frac{T_0}{T_0 - T} \times t \quad (5)$$

where, T_0 is the turbidity of fresh emulsion in m^{-1}, ϕ is the oil volume fraction (dimensionless), C is the concentration of protein in the dispersion (mg/ml), t is the time interval (24 h) and T is the turbidity of the emulsion after 24 h storage.

3.2.7.2. Determination of droplet size and zeta-potential of FPI stabilised emulsions

The Z-average diameter and zeta-potential of droplets in a FPI-stabilized oil-in-water emulsion were determined using a Zetasizer (ZS-90, Malvern instruments Ltd, Worcestershire, UK), within 24 h of emulsion preparation. To avoid multiple scattering effects, the emulsions were diluted 100 times with Milli-Q water prior to these measurements.

3.2.8 Statistical Analyses

ANOVA was used to compare the means. The means were obtained from triplicate determinations for all the above mentioned variables. The EAI and ESI were measured on

three different days, producing each day two different emulsions of the same sample, and taking two aliquots (replicates) of each emulsion. The results are given as the mean±standard deviation. The significant difference between two means was calculated using the Tukey method at 95% confidence level ($p< 0.05$).

3.3. RESULTS AND DISCUSSION

3.3.1 Effect of demucilaging temperature on the yield and purity of FPI

The extraction yield and purity of the extracted FPI at different demucilaging temperatures are shown in Table 3.1. The yield of extracted protein was not significantly affected by the temperature ($p > 0.05$). However, the purity of the FPI dramatically increased with increasing temperature within a temperature range of 25-50 ^{o}C ($p < 0.05$). This could be due to the more efficient removal of mucilage at higher temperature, which consequently decreases the interference of mucilage in subsequent extraction of protein.

The increase of temperature in the range of 25 to 50 ^{o}C also resulted in a significant increase in the recovery of protein. At the demucilaging temperature of 60 ^{o}C, FPI with high purity (90.60 ± 1.31%) was extracted. Madhusudhan and Singh (1985) reported that heat treatment of linseed meal not only reduced its solubility as compared to untreated meal but also diminishes its functional properties. Hence, in the present study demucilaging of seeds was only carried out up to 60 ^{o}C in order to preserve the functionality of flaxseed protein.

Many different methods have been used to extract flaxseed protein from whole flaxseeds or defatted flaxseed meal and different levels of purity and yield have been reported. Madhusudhan and Singh (1985) extracted the protein from degummed, defatted and dehulled flaxseed meal with 1M NaCl at pH 7 and reported the yield of protein extracted as 85%. Wanasundara and Shahidi (1994) reported that removal of polar compounds with a two phase solvent extraction process prior to protein extraction enhanced the protein content of flaxseed

meal from 42.9% to 55.29%. Recently, Kuhn et al. (2014) extracted the FPI from flaxseed meal without demucilaging resulting in a lower level of extracted FPI purity (68.53±0.33%) and a higher dietary fibre (mucilage) content (16.20±0.29%), however, the yield of the extracted protein was not noted. In contrast, Karaca, Low, and Nickerson (2011) demucilaged flaxseed in alkali prior to protein extraction and obtained a higher purity of extracted protein (89.25%), which is in good agreement with the present study. Hence, demucilaging the whole flaxseeds, prior to fat and protein extraction can enhance the purity of protein. However, more work is required to increase the yield of the FPI extracted by this method. Similar limitations were reported by Zhang et al. (2009), who reported that aqueous extraction of flaxseed mucilage resulted in significant loss in protein recovery.

Table 3.1 Effect of temperature on yield and purity and recovery of FPI

Temperature(°C)	Protein extracted Yield (%)	Protein purity (%)	Protein Recovery (%)
25	$4.50^{a} \pm 0.74$	$51.64^{a} \pm 0.51$	$12.10^{a} \pm 1.16$
40	$5.00^{a} \pm 0.62$	$58.03^{b} \pm 0.39$	$15.11^{b} \pm 1.07$
50	$5.36^{a} \pm 0.90$	$68.21^{c} \pm 1.82$	$19.04^{c} \pm 1.94$
60	$4.30^{a} \pm 0.88$	$90.60^{d} \pm 1.31$	$20.29^{c} \pm 1.57$

The values carrying different letters as superscript are significantly different ($p<0.05$).

3.3.2 Proximate Composition and amino acid profile of FPI

The proximate composition of the extracted FPI was as follows: Protein 90.6 ± 1.31% (w/w); moisture 4.2 ± 0.3% (w/w) and ash 2.8 ± 0.24% (w/w). The purity of protein obtained in this study was similar to that obtained by Karaca, Low, and Nickerson (2011) (89.25% pure) using similar methodology with an additional step of dialyzing the extracted protein against water. The amino acid profile of FPI extracted in this study is shown in Table 3.2. The amino

acid compositions of sodium caseinate (SC), whey protein isolate (WPI) and soybean protein isolate (SPI) are also compiled in this table for comparison.

Table 3.2 Amino acid profile of flaxseed protein isolate (FPI). SC = sodium caseinate, WPI= whey protein isolate, SPI= soy protein isolate

Amino acid name	Amino acid content (mg/g)			
	FPI[a]	SC[b]	WPI[c]	SPI[d]
Histidine	21.8	27	21.1	29.0
Serine	47.0	46	41.7	54.8
Arginine	108.0	33	24.9	75.7
Glycine	48.2	16	17.2	38.6
Aspartic acid	101.8	63	112.6	118
Glutamic acid	185.1	190	171.8	212.9
Threonine	33.9	37	49.9	41.0
alanine	43.6	27	57.1	38.3
Proline	37.7	nr	44	52.9
Lysine	27.5	71	134.2	53.9
Tyrosine	25.6	55	36.6	37.1
Methionine	18.6	26	nr	9.3
Valine	55.2	60	35.6	44.1
Isoleucine	45.4	49	39.7	44.8
Leucine	54.9	84	125.1	70.0
Phenylalanine	53.1	45	31.3	53.0
Tryptophan	20.4	nr	nr	nr
Cysteine	10.7	0.4	nr	0.6

[a] Data from present study, [b] Wang et al., (1999), [c] Pena-Ramos, Xiong, and Arteaga (2004), [d]Tang, Ten, Wang and Yang (2006)

The essential amino acid profile of FPI was similar to those observed in other proteins in this study. However, FPI had higher arginine and cysteine content than that of other proteins ($p < 0.05$). High level of these amino acids is useful for some nutritional supplements, such as

those used to improve the health of low birth weight infants (Wang et al., 1999). Hence, FPI may be a useful protein for use in some infant foods.

The lysine to arginine ratio is used as a measure of cholesterolaemic and atherogenic effects of a protein (Czarnecki & Kritchevsky, 1992). A protein with a lower ratio of lysine to arginine has lesser lipidemic and atherogenic effects. In the present study FPI exhibited a lysine to arginine ratio of 0.25, which is similar (0.22) to that reported by Marambe, Shand, and Wanasundara (2008). The lower lysine to arginine ratio (0.25) for FPI, compared with SPI (0.71), SC (2.15) and WPI (5.38), indicates that FPI may be a better protein for cardiac health. FPI may therefore be a useful protein ingredient for addition to both infant formula and formulations intended to improve cardiac health.

3.3.3 Physicochemical properties of FPI

3.3.3.1 Amorphous nature

X-ray diffractograms of FPI extracted from the flaxseed meal demucilaged at different temperatures are shown in Fig. 3.2. All the FPI samples exhibited a single peak around 19^{o} with no significant difference in peak intensities. Low intensities of this peak clearly indicates a lack of crystallinity or orderly arrangement in the structure of extracted FPI. Wang et al. (2006) reported three peaks at 8.5^{o}, 19.5^{o} and 24.5^{o} for soy protein isolate. In the present study demucilaging temperature has no effect on the amorphicity of the extracted FPI. Although, DSC results in section 3.3.4 showed a glass transition at 53 °C, FPI obtained by demucilaging at 60 °C lacked crystallinity. While demucilaging at 60 °C was carried out the seed was intact, and the protein, which is in the inner layer of the seed, may experience a lower temperature, providing an explanation for the lack of conformational change observed.

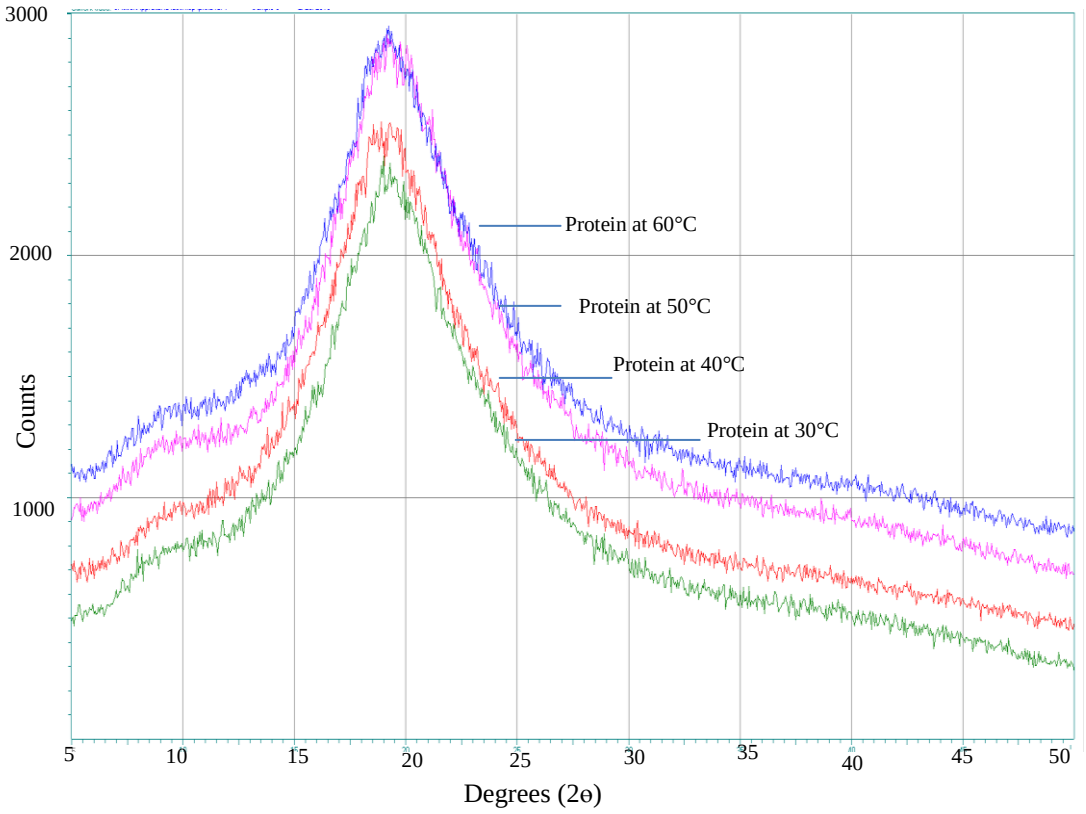

Figure 3.2 The X-ray diffractograms of FPI extracted from flaxseed meal demucilaged at different temperatures (30, 40, 50 and 60 °C).

3.3.3.2. Surface charge

The variation in surface charge (zeta potential) of FPI in the pH range of 2.0-8.0 is shown in the Fig. 3.3(a). The maximum negative charge was observed at pH 8.0, due to increased exposure of anionic groups on the protein surface. The magnitude, or the absolute value, of the surface charge of FPI decreased as pH decreased and became neutral at pH 4.2, which is the isoelectric point of FPI (Oomah & Mazza, 1993).

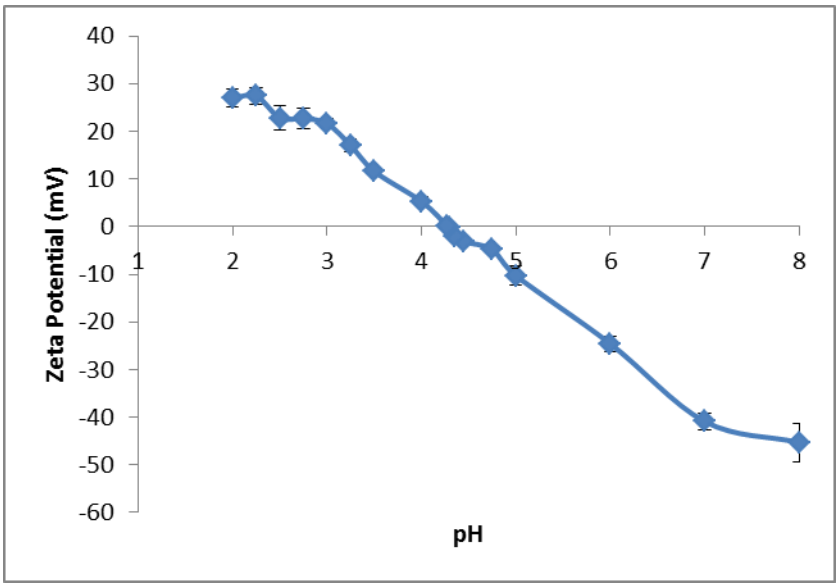

Figure 3.0.3(a) Zeta potential of 0.5% (w/v) flaxseed protein isolate dispersion at different pH values.

3.3.3.3. Thermal Characteristics

The ability of a protein to resist aggregation upon heating is a measure of its thermal stability (Horax, Hettiarachchy, Chen, & Jalaluddin, 2004). The thermal stability of a protein is indicated by 'T_d', the temperature at which denaturation occurs and ΔH, the amount of heat required to induce denaturation.

The overall thermogram of FPI indicated two transitions within the temperature scanning range of 30 to 160 ^{o}C, as shown in Fig. 3.3(b). The first transition observed at 53.72 ^{o}C indicates the glass transition temperature of freeze dried FPI and the second one at 105.05 ^{o}C indicates its denaturation, before final decomposition at 150 ^{o}C. Rahman et al., (2008) reported a similar thermogram for a commercial gelatin sample and observed a glass transition temperature at 60 ^{o}C, denaturation at 118 ^{o}C and deterioration near 280 ^{o}C.

The onset denaturation temperature(T_m), peak denaturation temperature (T_d) and enthalpy of transition (ΔH) determined from the thermogram of FPI were 83.4 °C, 105 °C and 8.25 J/g, respectively. These results are consistent with the T_d and ΔH values of 106.4 °C and 14.16

J/g, respectively, reported by Green, Milanova, and Logie (2005). The broad nature of the denaturation peak of FPI observed in this study may be due to the merging of denaturation bands of 2S and 11S components of FPI, as the former denatures at about 83^{o}C (Koppelman et al., 2005) while the latter denatures near 100^{o}C (Molina, Papadopoulou & Ledward, 2001).

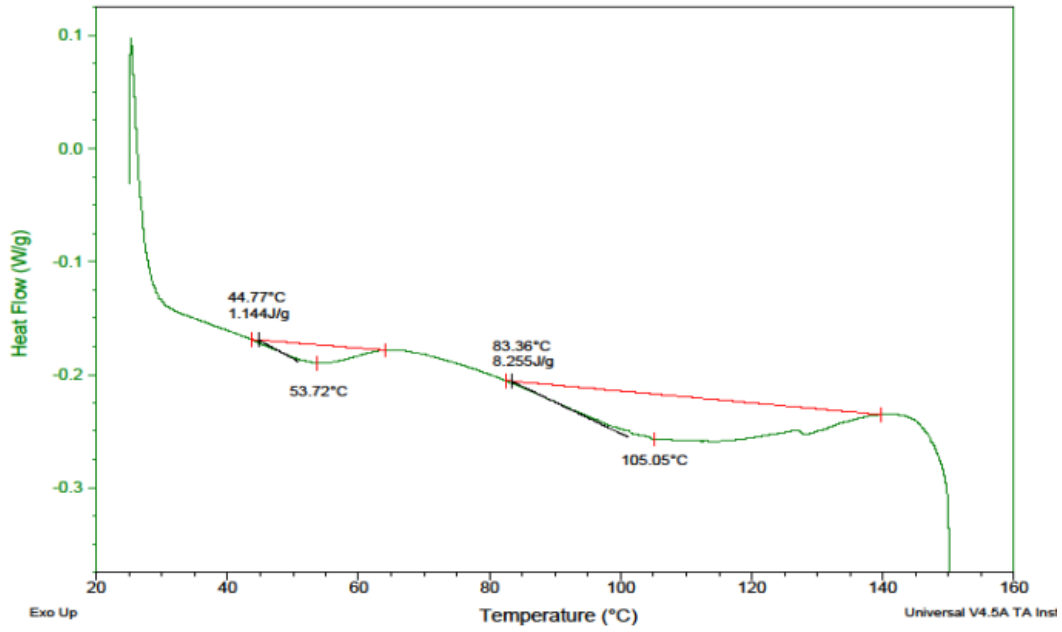

Figure 3.3(b) Thermogram of flaxseed protein isolate obtained from the differential scanning calorimeter.

In the current study, extracted FPI exhibited a peak denaturation temperature of 105 °C, indicating that 11S (globulin) is the predominant fraction, while 2S (albumin) is a minor fraction in this isolate. Unlike soy protein, which contains 5S and 7S globulins with a denaturation temperature at 75 and 95 ^{o}C, respectively, the FPI in this study exhibited only one typical denaturation temperature above 100 ^{o}C, which is in agreement with Li-Chan and Ma (2002). The denaturation temperature of flaxseed is higher than that of other plant and animal proteins such as soy protein (96 °C, Horax et al., 2004), hemp seed protein (95 °C, Tang, Ten, Wang, & Yang, 2006) and whey protein (65 °C, Haque, Aldred, Chen, Barrow, &

Adhikari, 2013), but lower than that of gelatin (118 °C, Rahman, Al-Saidi, & Guizani, 2008). These differences in the denaturation temperature for different proteins can be attributed to the varying extraction methods, purity, moisture content and the presence of non-protein components such as salt (Murray, Arntfield, & Ismond, 1985).

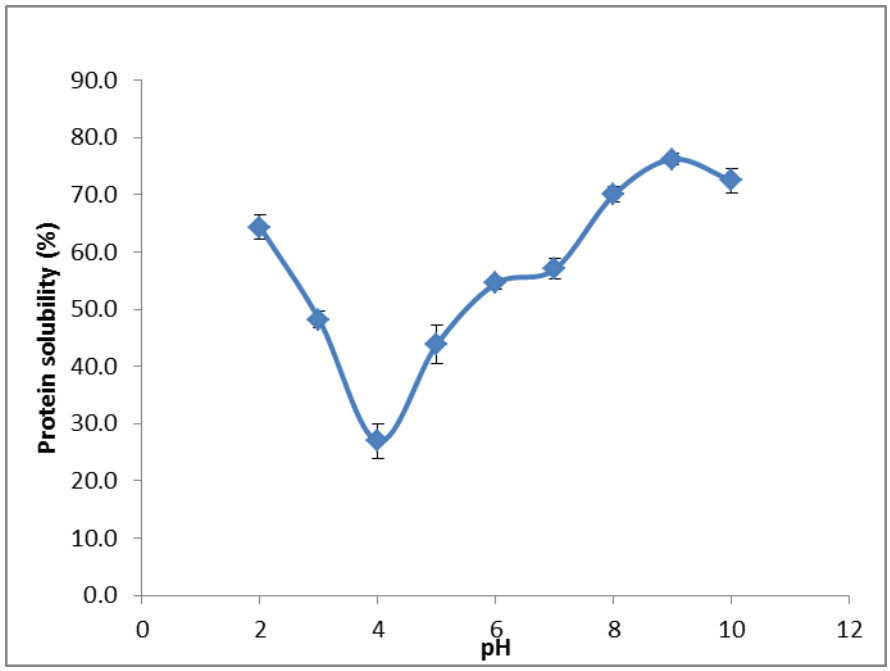

Figure 3.3(c) Solubility of flaxseed protein isolate as a function of pH.

3.3.3.4. Solubility

The solubility of FPI in the pH range of 2.0-10.0 is shown in Fig. 3.3(c). The minimum solubility occurred at pH 4.0, which is close to the isoelectric point of FPI. Being alkali soluble, the solubility of FPI increased at a slow rate at pH values > 5.0. The solubility of FPI was 57% at pH 7.0 and 76% at pH 9.0. This trend of increasing solubility with increasing pH was observed previously for other oilseed proteins, including hemp seed protein (Tang et al., 2006). However, the maximum solubility obtained for FPI is less (76%) than that of observed

for hemp seed protein (90%). Similar results on solubility of FPI were obtained by Dev and Quensel (1988) containing differing levels of mucilage.

3.3.3.5. Water holding and fat absorption capacities

The water holding capacity (WHC) and the fat absorption capacity (FAC) are described as the ability of a protein to hold an amount of added water or oil. As can be observed from Fig. 3.4(a), the WHC of FPI was significantly higher than that of SC, WPI and gelatin, but lower than that of SPI, which is in agreement with a previous study (Tang et al., 2006). The FAC of the studied proteins was found to be in the following order: WPI>FPI>SC>Gel>SPI. The lower FAC for SPI, compared with the other proteins, indicates its hydrophilic nature. Similarly, WPI had the lowest WHC but the highest FAC, indicating the presence of more lipophilic sites present in its structure than in the other proteins.

The WHC and FAC of FPI were higher than those of most of the proteins investigated here. The higher FAC of FPI can be attributed to its greater lipophilic character, when compared to that of SPI (Oomah & Mazza, 1993) and the other proteins. Kinsella (1976) reported that a higher proportion of the non-polar amino acids in proteins results in more lipophilic characteristics. The hydrophilic characteristics of FPI can be attributed to the presence of mucilage and the porous structure of the freeze dried FPI. Wanasundara and Shahidi (1997) reported similar FAC but higher WHC of flaxseed meals, which can be attributed to the higher proportion of the residual mucilage.

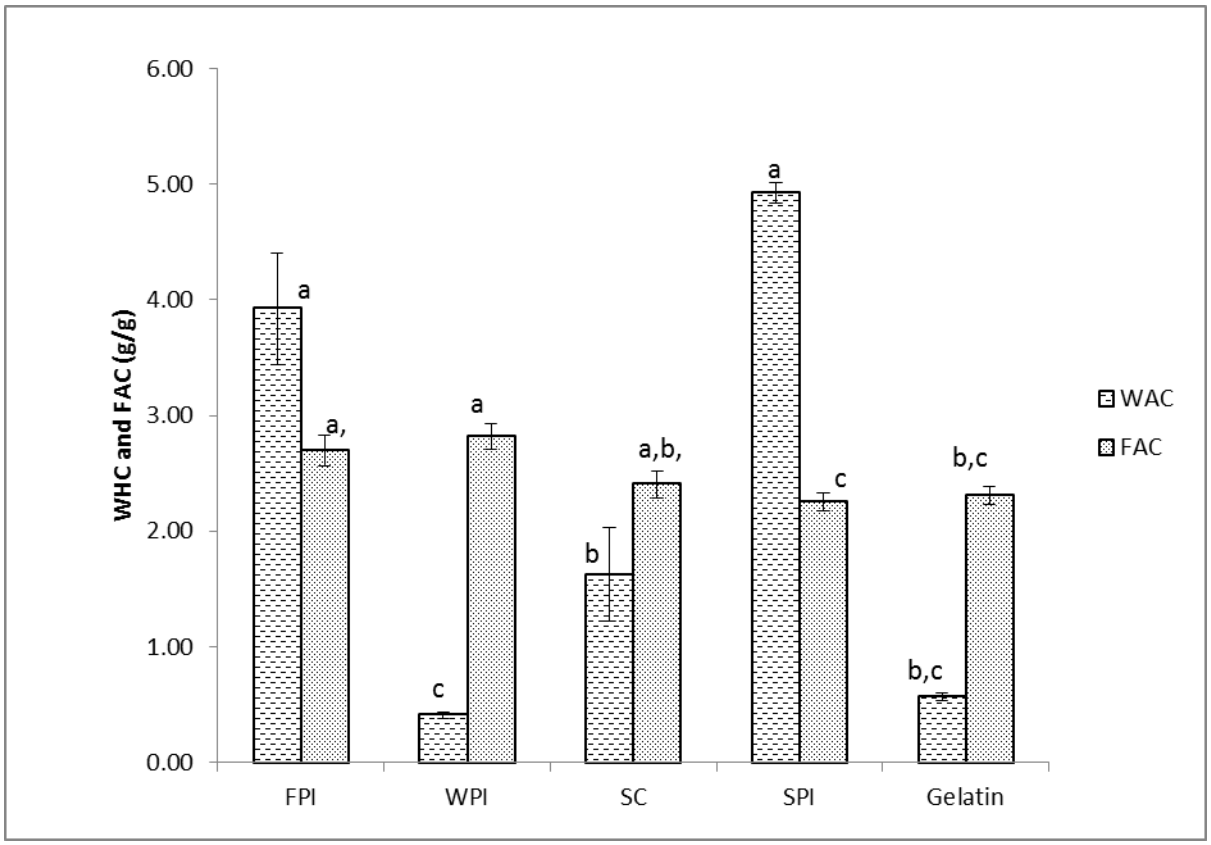

Figure 3.4(a) Comparison of water absorption capacity (WHC) and fat absorption capacity (FAC) of FPI with different proteins. The bars with different letters are significantly ($p<0.05$) different.

3.3.4 Emulsifying properties of the FPI

3.3.4.1. Emulsifying activity and emulsion stability indices

The ability of a protein to form an emulsion and to stabilize it over time is measured by using the emulsifying activity index (EAI) and the emulsion stability index (ESI), respectively. EAI is measured as the interfacial area stabilized per unit mass of protein based on the turbidity of an emulsion (Pearce & Kinsella, 1978). The change in turbidity of that diluted emulsion measures the ESI of protein in units of time. The EAI of FPI and some selected proteins and ESI of emulsions stabilised by these proteins are presented in Fig. 3.4(b). EAI of these proteins ranged from 187 to 375 m^2/g, whereas the ESI results ranged from 56 to 180 h. FPI

exhibited the highest EAI, and the ESI of FPI stabilized emulsion was the highest among the protein stabilized emulsions. These results, especially the very high ESI of FPI, indicate that it can be preferably used as emulsifier in oil-in-water emulsions.

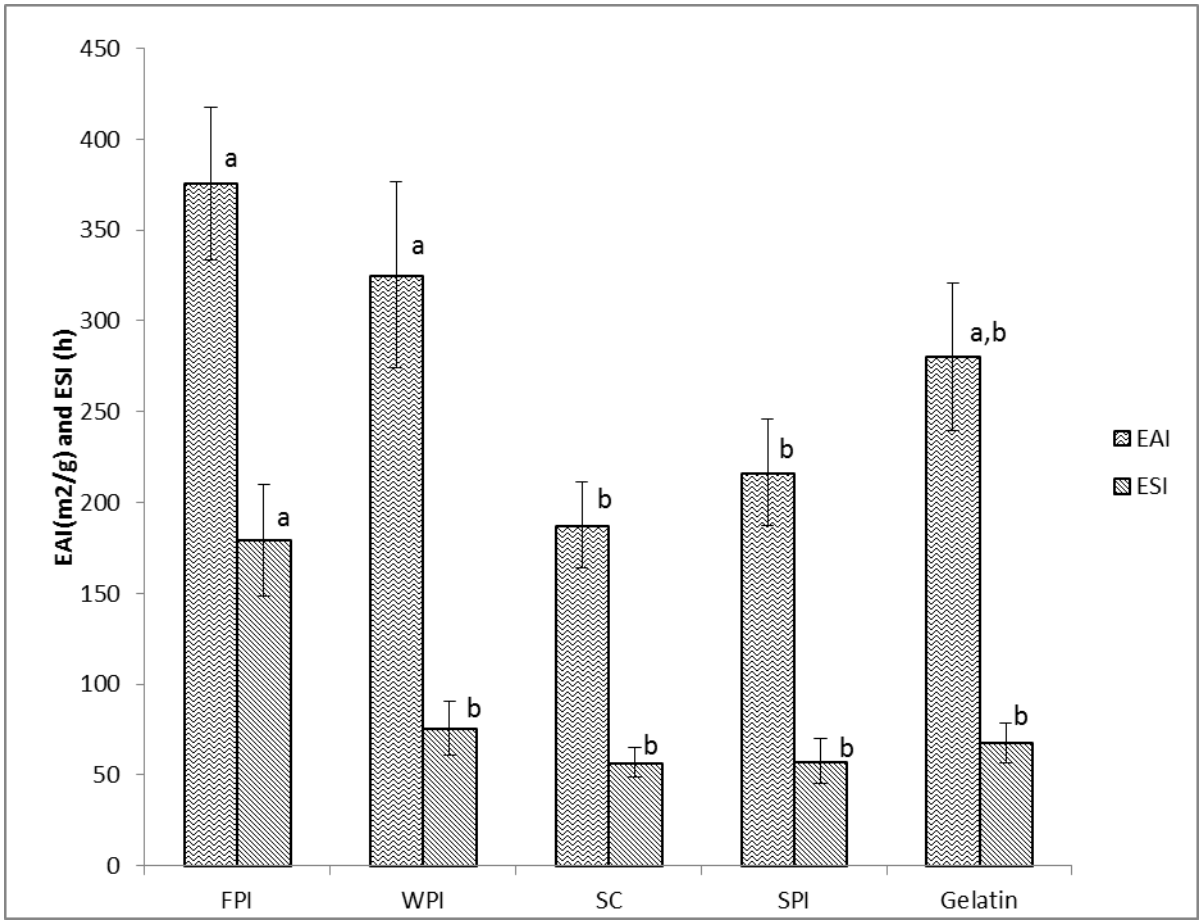

Figure 3.4 (b) Comparison of emulsion activity index (EAI) and emulsion stability index (ESI) of FPI with different proteins. The bars with different letters are significantly ($p<0.05$) different.

Dev and Quensel (1988) reported that flaxseed protein exhibited better emulsion activity and emulsion stability than did soy protein. The EAI of FPI obtained in this study was 375 m^2/g, which is significantly higher than that obtained by Krause, Schultz, and Dudek (2002) (220 m^2/g), which may be attributed to the difference in protein extraction conditions, final protein content and the pH at which emulsions are made in the respective studies, since these factors

strongly influence the functional properties of proteins (Krause, Schultz, & Dudek 2002). The emulsion stability for FPI obtained in this study (86%) is similar to that obtained by Dev and Quensel (1988) (84%) for their low mucilage protein isolate.

3.3.4.2. Emulsion droplet size and Zeta potential

Creaming is one of the key manifestations of instability of emulsions. This is attributed to the tendency of oil droplets in the emulsion to move upwards and form a layer at the top (McClements, 2007). In this context, the size and zeta potential of the oil droplets are critical parameters influencing the stability of an emulsion (Joshi et al., 2012). The Z-average diameter of FPI stabilised-emulsion droplets was 647.5 nm, which is smaller than the droplet size of emulsions stabilized by WPI, Gel and SPI [Fig. 3.4(c)], but larger than that in SC-stabilized emulsions. The droplet size for all the emulsion was below 1µm, except in the case of the soy protein emulsion (1.344 µm). Larger droplet size in soy emulsions is consistent with its lower EAI and ESI, compared to those of other proteins. However, for SC the droplet size was the smallest (246.8 nm) among the proteins studied, which contrasts with the ESI results. This result indicates that droplet size is not the only factor affecting stability of an emulsion over time. Other possible parameters that may contribute to the instability of emulsions are the density difference between the dispersed phase and continuous phase and the viscosity of the continuous phase (McClements, 2007). In the present study the viscosity of SC was the lowest amongst the protein dispersions (data not shown), which may be the reason for the high creaming and low ESI of SC dispersions, even though the droplet size was small.

Proteins generally stabilize emulsions by forming a coating over oil droplets and thus preventing agglomeration. The electrostatic force of repulsion between protein coated oil droplets depends on the amount of charge they carry, and the magnitude of this charge is measured using zeta-potential (ZP).

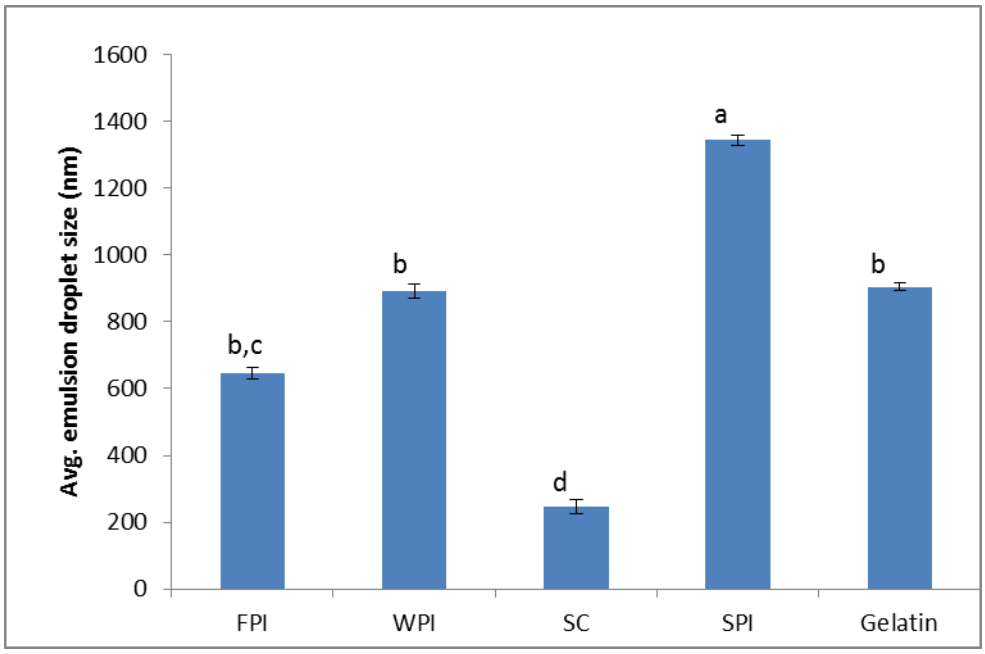

Figure 3.4(C) Average droplet size (diameter, nm) of oil in emulsion stabilised by different proteins. The bars with different letters are significantly ($p<0.05$) different.

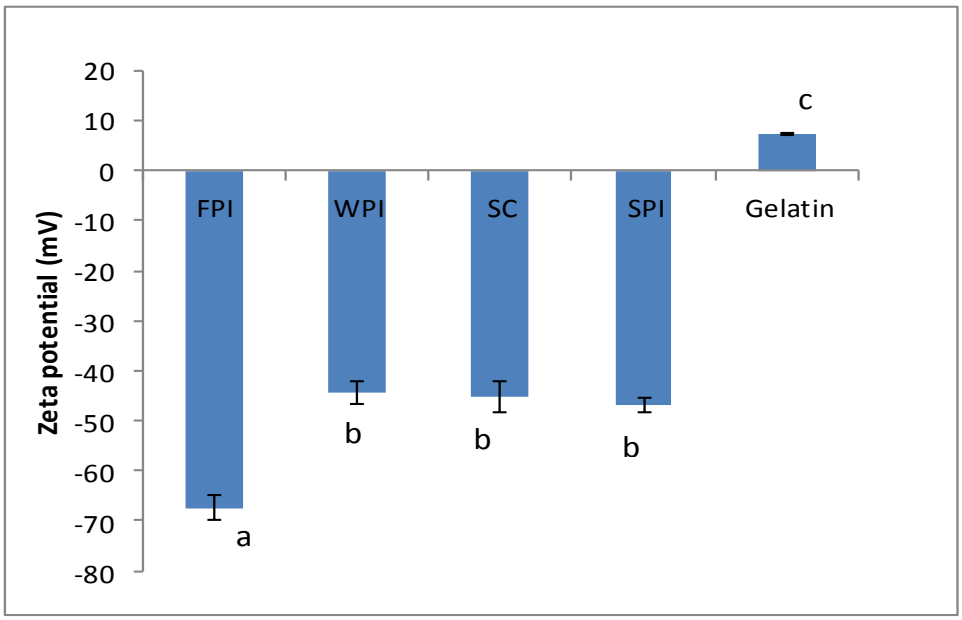

Figure 3.4(d) Zeta potential of oil-in-water emulsions of FPI and some selected proteins. The bars with different letters are significantly ($p<0.05$) different.

For an electrostatically stabilized emulsion the minimum zetapotential is ±30 mV (Wu, Eskin, Cui, & Pokharel, 2015). As shown in Fig. 3.4(d), the emulsions of FPI and other tested proteins, except gelatin, were negatively charged. The positive charge of gelatin (Type A) indicates that it had a higher isolectric point (pH= 6.5-8.0, Digenis, Gold, & Shah, 1994) than the pH values at which these emulsions were prepared. Using the same reasoning, the negative charge in the emulsions of FPI and other remaining proteins indicates that these proteins had isolelecric point lower than the pH at which these emulsions were prepared. The gel-stabilised emulsion had the lowest magnitude of zeta-potential (7.5 mV). The zeta potential of the FPI stabilised-emulsion is much higher (-67.4 mV) than those of SPI, SC, WPI and Gel-stabilised emulsions. Similar results for flaxseed protein concentrate (approx -63 mV) and soy protein concentrate (-45 mV) were reported previously (Wang et al., 2010). The results obtained in the current study suggest that FPI-stabilized emulsions can be more electrostatically stable at lower pH values than those stabilised with other proteins. This indicates that FPI-stabilised emulsions will be useful for the controlled/targeted delivery of oils soluble nutrients in the intestine, due to their higher stability in high acid environments.

3.4 CONCLUSIONS

Flaxseed protein isolate with nearly 90% protein content was extracted from flaxseeds by demucilaging at 60 ^{o}C. The lysine to arginine ratio of FPI was found to be 0.25, which is lower than that of other commonly used proteins (SPI, SC, WPI and Gel), indicating that FPI may be a preferable ingredient in some infant formulae and also formulations intended to improve cardiac health. The solubility of FPI in water was relatively low and was 57% at pH 7.0 and 76% at pH 9.0. The onset and peak denaturation temperatures of FPI were 83.4 °C and 105 °C, suggesting that it is a relatively thermally stable protein. The FPI-stabilised emulsions were more stable (ESI= 375.51 m^2/g, zeta potential = -67.4mV) than the emulsions stabilised with SPI, WPI, SC or Gel. The emulsions stabilised by FPI were relatively more

stable at low pH values than the emulsions stabilised by SPI, WPI, SC or Gel, and hence, may be preferable for use in controlled/targeted delivery in high acid environments such as in the human digestive system.

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Chapter 4

Effect of extraction temperature on composition, structure and functional properties of flaxseed gum

ABSTRACT

Flaxseed gum (FG) was extracted at four different temperatures (30, 50, 70 and 90 °C). Chemical composition and structural features of FG extracted at different temperatures were investigated to determine the effect of temperature. Content of acidic monosaccharides and denatured protein increased with increasing FG extraction temperature. The ratio of neutral to acidic monosaccharides decreased from 6.7 to 5.7 as the extraction temperature was increased from 30 to 90 °C. Physicochemical and functional properties, including zeta-potential, surface morphology, emulsifying activity index (EAI) and emulsion stability index (ESI), water absorption capacity (WAC) and fat absorption capacity (FAC) of FG samples, were also investigated as a function of extraction temperature. EAI and WAC of FG samples reduced significantly with rise in extraction temperature. Our study suggests that FG extracted at different temperatures may be specifically targeted for different applications such as for emulsification or gel formation in food systems.

Key words: Neutral monosaccharides, Acidic monosaccharides, Xylose, Emulsion activity index, Zeta potential

4.1. INTRODUCTION

Flaxseed is an important functional food with the potential to deliver diverse health benefits. The nutritionally important components of flaxseed include alpha lenolenic acid, protein, lignin and dietary fibre (Jenkins et al., 1999). The average composition of flaxseed is fat - 41%, protein - 20%, total dietary fiber - 28%, moisture-7.7%, and ash- 3.4% (Shim, Gui, Arnison, Wang, & Reaney, 2014). The dietary fiber of flaxseed is composed of soluble dietary fiber (9%) and insoluble dietary fiber (20%) (Cui 2000). The soluble fiber is a viscous seed coat gum composed of neutral (75%) and acidic (25%) monosaccharides (Warrand et al., 2005). This soluble fraction of FG is of particular interest for its nutritional and functional properties. Different studies indicate that inclusion of FG in the diet helps reduce the risk of diabetes and coronary heart diseases, and aids in preventing colon and rectal cancer (Cunnane et al., 1993; Jenkins et al., 1999; Thakur, Mitra, Pal, & Rousseau, 2009). Moreover, flaxseed gum possesses good gelling, foaming and emulsifying capacity, and can potentially replace gum Arabic in food emulsions (Chen, Xu, & Wang, 2006; Mazza & Biliaderis, 1989; Wang, Li, Wang, Li, & Adhikari, 2010).

In the last 20 years a number of studies have been carried out on the extraction, characterization, chemical composition and physicochemical properties of the soluble fraction of FG. Cui, Mazza and Biliaderis, (1994) optimised the extraction process in terms of temperature, pH and seed to water ratio. In a subsequent study they found that chemical composition and physical properties of FG varied significantly with the cultivar (Cui & Mazza, 1996). Fedeniuk and Biliaderis, (1994) fractionated the aqueously extracted FG from flaxseed meal into acidic and neutral components and reported significant differences in the rheological properties of each fraction. Structural investigations of FG revealed that it is an arabinoxylan with a major neutral fraction composed of l-arabinose, d-xylose and d-galactose (3.5:6.2:1), and a minor acidic fraction composed of l-rhamnose, l-fucose, l-galactose, and d-

galacturonic acid (2.6:1:1.4:1.7) (Muralikrishna, Salimath, & Tharanathan, 1987; Warrand et al., 2005).

Understanding the properties of plant polymers is important in optimising their use as stabilizing, foaming and emulsifying agents. The functional properties of FG and its constituting fractions (acidic, neutral and without protein) are reported in literature. Qian, Cui, Wu, and Goff, (2012) found that an acidic fraction with protein content of 8% w/w exhibited better emulsification properties and higher surface activity than a neutral fraction with protein removed. In contrast, Ding et al., (2014) reported that a non-protein FG fraction exhibited better surface activity than FG fractions containing protein. In a more recent study, conlinin, the 2S storage protein, was shown to be the major protein associated with FG. This study also concluded that the protein significantly contributes to the favourable emulsifying properties exhibited by FG (Liu, Shim, Poth, & Reaney 2016). Cui et al. (1994) studied the effect of extraction temperature on the yield, composition and rheology of the extracted gum from flaxseeds. Although the effect of extraction temperature on the functional properties of FG is clear, the structure-function relationship for the temperature effect is not yet adequately understood.

In order to determine the structural factors which link temperature to FG functional properties, we present: 1) Aqueous extraction of FG from whole flaxseed at four different temperatures 2) Characterization of the extracted FG samples in terms of monosaccharide composition and physicochemical properties (electrostatic charge density, surface morphology and crystallinity) 3) Comparison of functional properties (emulsion activity index, emulsion stability index, water absorption and fat absorption capacity) of FG samples extracted at different temperatures.

4.2. MATERIALS AND METHODS

4.2.1. Materials

The golden flaxseeds (*Linum usitatissimum*) were received from Stoney Creek Oil Products Pty. Ltd (Talbot, VIC, Australia). All other chemicals used in this study were purchased from Sigma-Aldrich Australia (Sydney, New South Wales, Australia).

4.2.2. Extraction of FG

FG was extracted from whole raw flaxseed using the method of Cui et al. (1994) with slight modification. Briefly, the flaxseed was soaked in Milli-Q water at a flaxseed-to-water ratio of 1:18 (w/w) at four different temperatures (30, 50, 70, 90 °C) with continuous and gentle stirring over a magnetic plate for 2h for each of two consecutive cycles of extraction. The soaked seeds were filtered and the water containing the dissolved gum was treated with three volumes of 95% ethanol to precipitate the gum. The precipitated gum was collected by centrifuging at 4,000 x g for 10 min. The precipitated gum was vacuum dried at 50 °C and stored at 4 °C.

4.2.3. Effect of extraction temperature on yield and composition and structure of FG

The FG samples extracted at different temperatures (30, 50, 70, 90 °C) were compared for yield and composition.

4.2.3.1 Determining the yield

Yield of extracted gum was calculated follows:

$$Yield(\%) = (G/S) \times 100 \quad (1)$$

Where '*G*' is the mass (wet basis) of gum powder in grams obtained after extraction and vacuum drying from whole flaxseeds and '*S*' is the mass (wet basis) of seeds in grams taken for extraction.

4.2.3.2 Determining the moisture, protein and ash contents

The moisture, protein (% N×6.25) and ash content of the FG samples were analysed using the standard AOAC (2005) method numbers of 925.1, 920.87 and 923.03, respectively. All the measurements were made in triplicate and the average values are reported.

4.2.3.3 Determination of monosaccharide composition

To determine monosaccharide composition 0.1% (w/v) FG solution was prepared in MilliQ water. The FG solution was subjected to strong acid conditions to promote acid hydrolysis. Subsequently, the solution was dried under vacuum and the residue was reconstituted using an internal standard (for neutral and amino monosaccharide analysis: 100 µl of 2-deoxy-D-glucose solution (100 µM); for acidic monosaccharide analysis: 100 µl of ketodeoxynonulosonic acid (100 µM)). The monosaccharide analysis was carried out on a high-performance anion-exchange chromatograph system with pulsed amperometric detection (HPAEC-PAD), fitted with a BioLC amino trap guard column (3 x 50 mm) connected to a CarboPac PA10 column (4 x 250 mm) held at 25 °C. The sample was injected into the HPEAC-PAD and analysed using basic solvents, at a flow rate of 0.5 ml/min. The analytes detected were quantified with internal calibration. The sample was analysed in triplicate and average results are reported.

4.2.3.4 Acquiring Nuclear Magnetic Resonance (NMR) spectra

To identify the glycosidic bonds and type of side chains, NMR spectroscopy was employed. ^{1}H NMR and ^{13}C NMR spectra of the FG samples were obtained with a Bruker Av500 spectrometer, operating at a ^{13}C frequency of 125.7 MHz. Sample was dissolved high quality D_2O (99.96%) and the final concentration of FG in the solution was 20 mg mL^{-1}. The spectra were obtained at 45 C using a relaxation delay of 1 s and a 30 pulse. ^{13}C NMR spectra were recorded for 96 h at 45^{o}C to ensure good signal to noise and sharp resonance signals.

4.2.4 Physicochemical Properties of FG

4.2.4.1. Measurement of X-ray diffraction

X-ray diffractograms were obtained using a Siemens diffractometer (model D501, Siemens AG, Karlsruhe, Germany) with Co Kα < 1 radiation. The samples were scanned digitally with a scan rate of 1° per min over the (2θ) angle range of 5° to 55° (2θ) range at an increment of 0.05°.

4.2.4.2 Acquiring scanning electron micrograph

A Scanning Electron Microscopy (JEOL JSM6300 SEM, Tokyo, Japan) was used to acquire the morphology of gum samples extracted at different temperatures. Samples were lightly gold sputter coated (Sputter coater, Agar Aids, England) for 45 seconds and imaged under scanning electron microscope operated at 7kV and low beam current.

4.2.4.3. Measurement of zeta-potential

The zeta-potential of FG was determined as a function of pH using an electrophoresis instrument (Zetasizer ZS-90, Malvern instruments, Worcestershire, UK). The FG powder was dispersed in MilliQ water at a concentration of 0.1% (w/v), followed by filtration using Wattmann No.5 filter paper. The filtered gum dispersion was transferred into seven glass cuvettes and subsequently diluted 100 times with MilliQ water to avoid multiple scattering effects. The pH of these diluted samples was adjusted in the range of 2.0-8.0 using 0.1 M HCl or 0.1 M NaOH.

4.2.5 Determination of functional properties of FG

4.2.5.1 Water holding and fat absorption capacities.

The water holding capacity (WHC) and fat absorption capacity (FAC) were determined using the method of Tomotake et al. (2002) with minor modifications. One gram of gum was weighed into a pre-weighed centrifuge tube and 10 ml of Milli-Q water was added. The

mixture was vortexed for 1 min and allowed to stand for 30 min at ambient temperature and then centrifuged at 5,000×g for 30 min. The supernatant was decanted carefully and the tube with sediment was weighed again. WHC was calculated as the amount of water held by one gram of FG sample. The FAC was determined using similar procedure using 0.5 g of FG and 5 ml sunflower oil. The FAC was expressed as the amount of oil per gram of FG sample.

4.2.5.2 Preparation of emulsions

The oil-in-water emulsion was prepared by dispersing 10% flaxseed oil (v/v) in the water phase containing 0.5% gum (w/v), followed by homogenization using an ultra Turax (RW 20, IKA GmbH Co., Bitterfeld-Wolfen, Germany) at 10,000 rpm for 1 min.

4.2.5.3 Emulsifying activity index and emulsion stability index.

The emulsifying activity index (EAI) of FG and the emulsion stability index (ESI) of protein emulsions were determined by using a turbidimetric method (Pearce & Kinsella, 1978). Freshly prepared emulsion was diluted 100 times with 0.1% (w/v) sodium dodecyl sulphate (SDS) and mixed for one minute using a vortex mixer. The absorbance of this diluted emulsion was measured at 500 nm wavelength using a UV spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The turbidity of emulsion was calculated using Eq. (3) given below.

$$T = 2.303 \times \frac{A}{L} \times D \qquad (3)$$

where T is the turbidity of emulsion in m^{-1}, A is the absorbance (dimensionless), D is the dilution factor (dimensionless) and L is the light path length in m.

The emulsifying activity index (EAI, expressed in m^2/g) of FG and emulsion stability index (ESI, expressed in h) were calculated using Eq. (4) and (5), respectively.

$$EAI = \frac{2 \times T_0}{\phi \times C \times 1000} \quad (4)$$

$$ESI = \frac{T_0}{T_0 - T} \times t \quad (5)$$

where, T_0 is the turbidity of fresh emulsion in m^{-1}, ϕ is the oil volume fraction (dimensionless), C is the concentration of protein in the dispersion (mg/ml), t is the time interval (24 h) and T is the turbidity of the emulsion after 24 h storage.

4.2.6 Statistical Analyses

ANOVA was used to compare the means. The means were obtained from triplicate determinations for all the above mentioned variables. The EAI and ESI were measured on three different days, producing each day two different emulsions of the same sample, and taking two aliquots (replicates) of each emulsion. The results are given as the mean ± standard deviation. The significant difference between two means was calculated using the Tukey method at 95% confidence level ($p < 0.05$).

4.3. RESULTS AND DISCUSSION

4.3.1 Effect of extraction temperature on the yield and composition of FG

FG samples extracted at different temperatures (30, 50, 70, 90 °C) are shown in Fig 1. These FG samples differed only in extraction temperature, all other treatments applied were same. As shown in Fig.1 the visual appearance of FG samples varied in terms of colour and texture. The FG samples extracted at lower temperatures 30 °C and 50 °C are more transparent and light in colour as compare to the FG extracted at higher temperatures. Ideally, for food applications the gum should be transparent in colour to avoid any colour alterations (Wang et al., 2010).

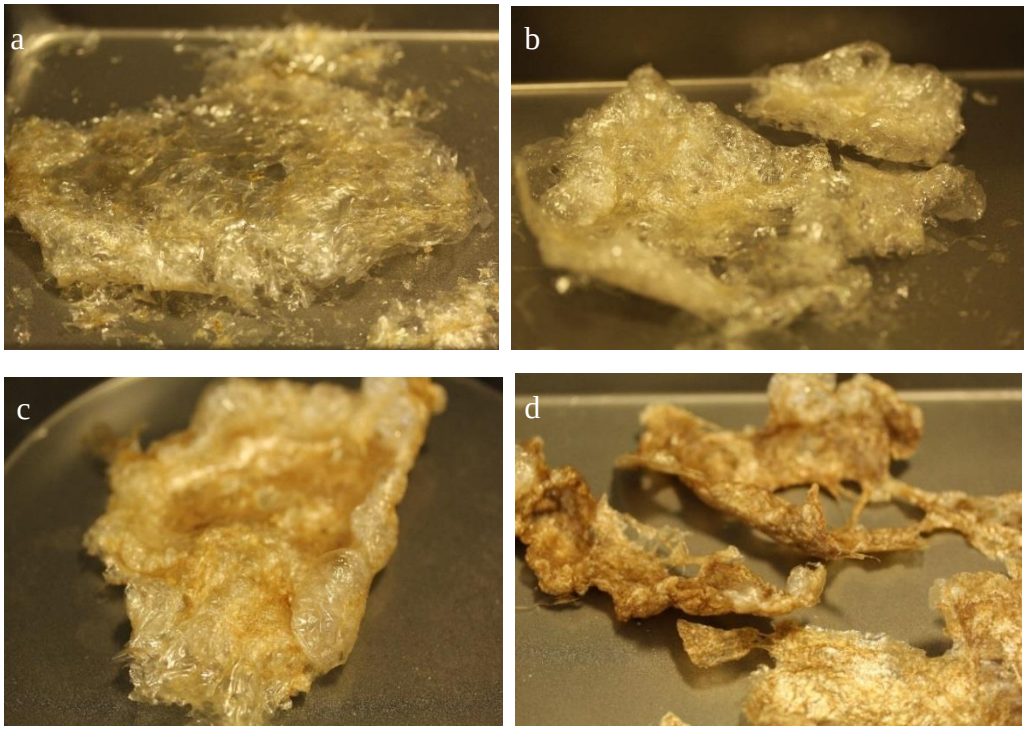

Figure 4.1 Vacuum dried flaxseed gum extracted from whole flaxseed by aqueous extraction at (a) 30 °C (b) 50 °C (c) 70 °C (d) 90 °C

The extraction yield and composition of the extracted FG at different extraction temperatures are shown in Table 1. The yield, purity (carbohydrate content) and protein content of the extracted gum was significantly affected by the extraction temperature ($p < 0.05$). The moisture content of the FG extracted at 30 ^{o}C was found significantly higher than FG extracted at 90 ^{o}C, which is probably due to more compact nature of gum extracted at high temperature which retained less free water (Table 1 and Fig 1). The yield of FG increased from 2.1 to 8.4 % (of seed weight) with increasing extraction temperature from 30-90 ^{o}C ($p < 0.05$). However, the purity of the gum, as indicated by carbohydrate content, decreased with increasing temperature of extraction ($p < 0.05$). This is because, the extraction yield of water soluble protein is greater at higher temperatures, which consequently increased the yield but decreased the purity of extracted gum. The protein content of FG samples increased significantly ($p<0.05$) from 4.4% to 15.1% as the extraction temperature increased from 30-

90 ^{o}C. A similar trend for protein content was reported by others in flaxseed gum extracted at 25 ^{o}C and 100 ^{o}C (Barbary, Al-Sohaimy, El-Saadani, & Zeitoun, 2009). However the yield of flaxseed gum extracted at room temperature, reported in the current study (2.1 ± 0.4%), was lower than that reported (3.0-5.2%) by Barbary et al. (2009). This may be attributed to longer extraction times (8h) used by Barbary et al. as compared to 2h in the current study. Moreover, recently Kaewmanee et al. (2014) concluded that gum yield can vary with cultivar type, climate and crop age.

Table 4.1 Yield and proximate composition of flaxseed gum extracted at four different temperatures

Numbers in a row with different superscript are significantly different.

Constituents (%)	FG extracted at 30 °C	FG extracted at 50 °C	FG extracted at 70 °C	FG extracted at 90 °C
Yield	2.1 ± 0.3^{a}	3.7 ± 0.8^{b}	5.7 ± 1.0^{c}	8.4 ±1.0^{d}
moisture	4.1 ± 0.4^{a}	3.8 ± 0.3^{a,b}	3.7 ± 0.3^{a,b}	3.4 ± 0.3^{b}
Protein	4.4 ± 0.7^{a}	4.7 ± 0.7^{a}	8.4 ± 0.7^{b}	15.1 ± 0.9^{c}
Ash	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}	0.7 ± 0.1^{a}	0.8 ± 0.1^{b}
Fat	0.5 ± 0.1^{a}	0.6 ± 0.2 ^{a}	0.6 ± 0.2^{a}	0.7 ± 0.2^{a}
Carbohydrate	90.4 ± 1.1^{a}	90.3 ± 0.9^{a}	86.6 ± 1.3^{b}	80.0 ± 1.4^{c}

Carbohydrate content of FG extracted at different temperatures was further investigated to determine the influence of extraction temperature on monosaccharide composition (Table 4.2). Monosaccharide analysis showed that FG is a heteropolysaccharide consisting of neutral and acidic monosaccharides. Xylose, arabinose and galactose constituted the neutral monosaccharides of FG and contributed more than 85% of total sugar, while rhamnose, glucose and fucose constituted the acidic monosaccharides and account for less than 15% of

total sugar. The level of galacturonic acid in FG is used as an index for the amount of acidic monosaccharides, while the level of xylose indicates the amount of neutral monosaccharides (Cui, et al., 1994).

Table 4.2 Monosaccharide composition of flaxseed gum extracted at four different temperatures

Monosaccharides	FG extracted at 30 °C	FG extracted at 50 °C	FG extracted at 70 °C	FG extracted at 90 °C
Xylose	47.6 ± 0.7^{b}	47.4 ± 0.7^{b}	46.4 ± 0.7^{b}	42.9 ± 0.6^{a}
Arabinose	26.2 ± 0.7^{a}	25.4 ± 0.6^{a}	25.4 ± 0.7^{a}	28.0 ± 0.7^{b}
Galactose	13.0 ± 0.5^{a}	13.3 ± 0.4^{a}	13.2 ± 0.2^{a}	14.0 ± 0.4^{a}
Rhamnose	7.0 ± 0.5^{a}	7.8 ± 0.5^{a}	8.2 ± 0.6^{a}	8.2 ± 0.5^{a}
Glucose	3.1 ± 0.2^{b}	2.3 ± 0.2^{a}	3.2 ± 0.2^{b}	2.8 ± 0.3^{b}
Fucose	2.9 ± 0.3^{a}	3.6 ± 0.3^{a}	3.3 ± 0.3^{a}	3.8 ± 0.5^{a}
Galacturonic acid	n.d	n.d	n.d	n.d
NM : AM	6.7 ± 0.4^{c}	$6.2 \pm 0.4^{b,c}$	$5.7 \pm 0.4^{a,b}$	$5.7 \pm 0.3^{a,b}$

Numbers in a row with different superscript are significantly different.
NP= neutral monosaccharide, AP= acidic monosaccharide, n.d = not detected

Accordingly, in the current study the absence of galacturonic in all the FG samples and the presence of high levels of xylose (47.6-42.9%) confirms the high content of neutral monosaccharides present in the extracted FG. The content of both neutral monosaccharides and acidic monosaccharides decreased with the rise in extraction temperature of gum ($p<0.05$). The ratio of the neutral to acidic fraction also decreased with the increasing temperature from 30-90 °C, indicating higher temperatures more efficiently extracted acidic monosaccharides. According to Barbary et al. (2009) the reason for decreased monosaccharide content at higher temperature is degradation and interference of starch and protein in the extraction of gum.

The major monosaccharides present in FG are arabinose (26 %) and xylose (47 %), which is consistent with FG being a arabinoxylan type polysaccharide (Warrand et al., 2005). The ratio of arabinose to xylose ranged from 0.6 to 0.7 and did not vary with extraction temperature. This ratio is an indicator of a moderately branched structure of FG. A branched structure of a polymer helps in the formation of stable solutions with high viscosity even at low concentration. Arabinoxylans exist structurally as random coil in aqueous solutions.

4.3.2 Effect of extraction temperature on the structure of FG

Composition profiles of FG extracted at four different temperatures 30 ^{o}C, 50 ^{o}C, 70 ^{o}C and 90 ^{o}C were compared using ^{1}H and ^{13}C spectra, with spectra shown in Fig. 4.2(a) and Fig. 4.2(b). The ^{1}H NMR spectrum of FG (Fig. 4.2(a)) were consistent with the presence of glucosyl residues.

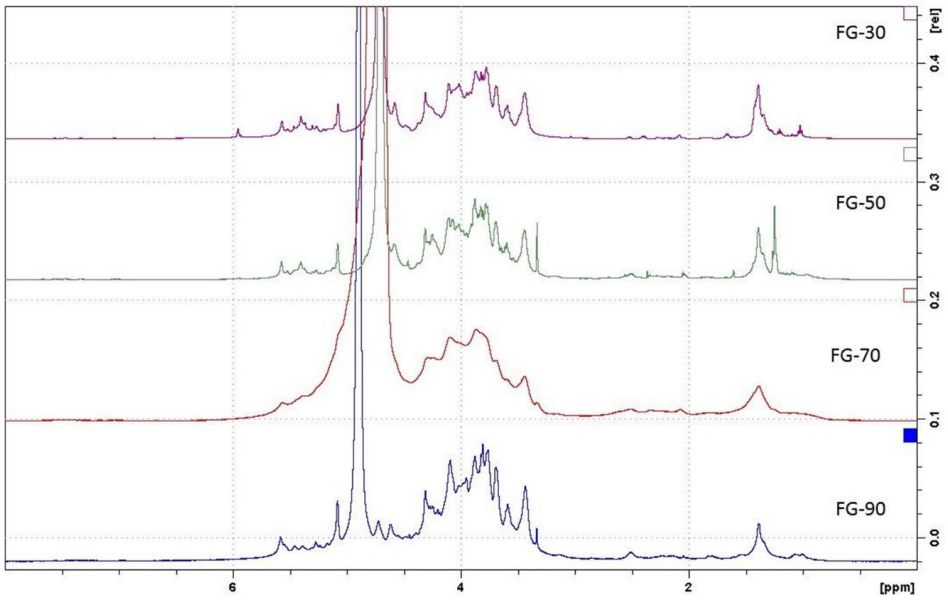

Figure 4.2(a) Comparative 1H spectra of flaxseed gum extracted at 30, 50, 70 and 90 °C

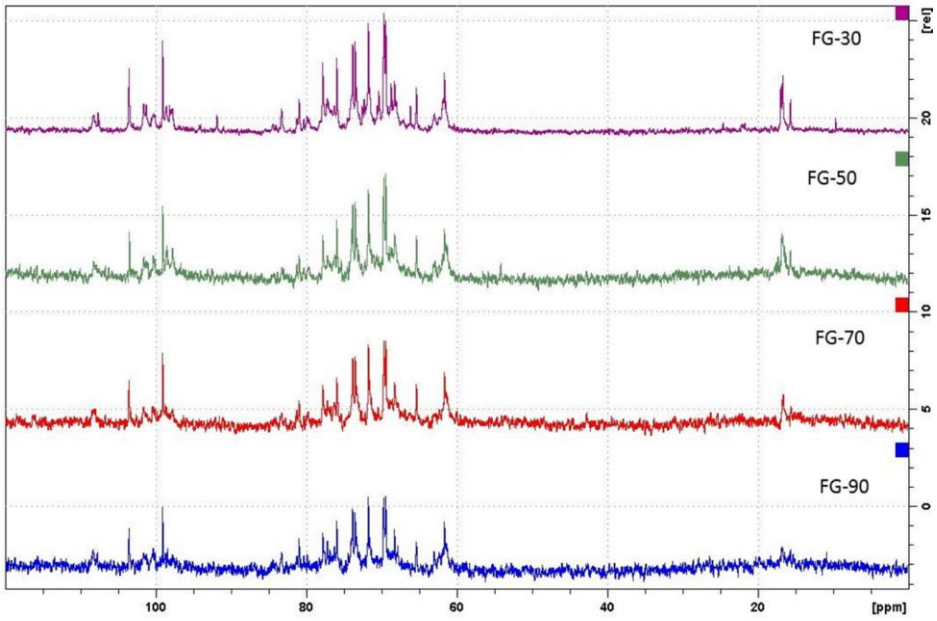

Figure 4.2(b) Comparative 13C spectra of flaxseed gum extracted at 30, 50, 70 and 90 °C

The anomeric protons resonate in the downfield region of the spectra, usually between 4.3 and 6.0 ppm, while all other ring protons from monosaccharides resonate between 3.1 and 4.3 ppm (Cui et al., 1995). ^{1}H NMR showed resonances of anomeric protons well separated and in agreement with previously reported spectra (Warrand et al., 2005). The FG-50 sample showed a well resolved peak at 1.24 ppm, indicative of fucose methyl protons. In general, samples extracted at higher temperature (70 ºC and 90 ºC) showed less resolved resonances for both ring and anomeric protons, probably due to denatured protein impurities contributing to the reduced solubility of samples in the solvent at these higher temperatures.

^{13}C NMR spectra of samples extracted at different temperature are shown in Fig. 4.2 (b), the spectra are similar to those previously reported (Cui et al., 1995; Warrand et al., 2005). The signal at 16.7 ppm (Hanniffy et al., 1999) is characteristic of the C6 carbon of L-fucose and decreased in intensity with the increasing extraction temperature. Signal at 97.4 ppm (C1) and 77.4 ppm (C4) indicated a ß-anomeric conformation of the 1,4-linked D-xylopyranose backbone (Kardosova et al, 1998). The absence of a resonance near 176.9 ppm indicates no galacturonic acid is present in the FG sample, which is consistent with ^{1}H NMR and GC

analysis. All other assigned anomeric configurations of the residues in the polysaccharide were in close agreement with those reported by Warrand et al. (2005).

4.3.3 Effect of extraction temperature on the physicochemical properties of FG

4.3.3.1 Surface morphology a nd amorphicity

The surface morphology of extracted FG was studied using SEM. The SEM micrograph Fig. 4.3(a) showed that the flaxseed gum powder particles exhibited an irregular shape and flaky structure that is typically observed in vacuum dried powders.

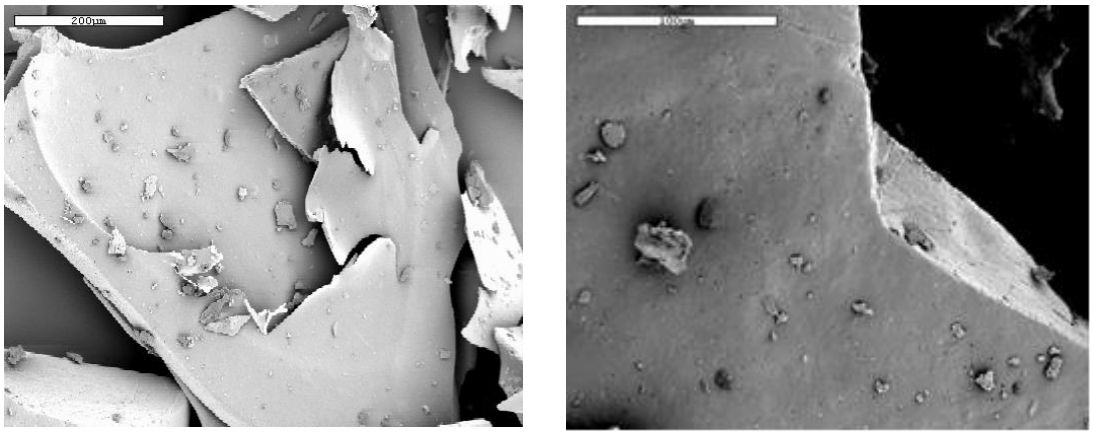

Figure 4.3(a) SEM images of vacuum dried flaxseed gum

X-ray diffractograms of FG extracted from whole flaxseed at different temperatures are shown in Fig. 3(b). All FG samples exhibited a single peak around 22^{o} with no significant difference in peak intensities. Low intensities of this peak indicates a lack of crystallinity or orderly arrangement in the structure of extracted FG. Moreover, the diffractograms show that extraction temperature had no effect on the amorphicity of the extracted FG.

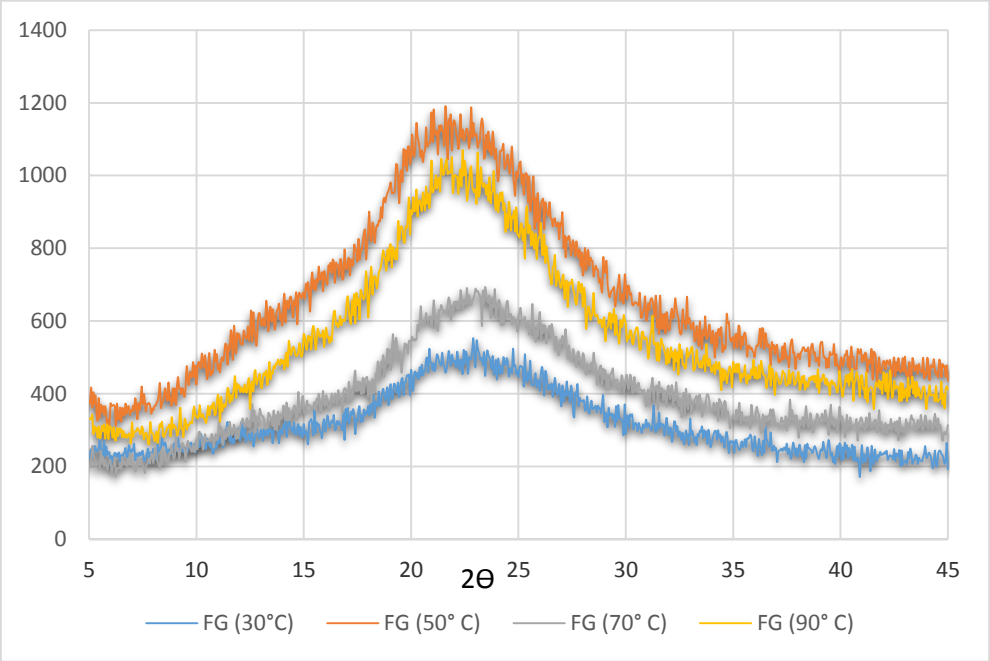

Figure 4.3(b) The X-ray diffractograms of flaxseed gum extracted from whole flaxseed at different temperatures (30, 50, 70 and 90 °C). The diffractrograms were shifted vertically for more clarity.

4.3.3.2 Zeta potential

The variation in zeta potential of FG in the pH range of 1.5-7.0 is shown in Fig. 4.4. The maximum negative charge for all FG samples extracted at different temperatures was observed at pH 7.0. Among all the FG samples the maximum value of zeta potential (-54.3 mV) was observed for gum extracted at 90 ^{o}C, followed by 47.0 mV for 70 ^{o}C gum, 43.3mV for 50 ^{o}C gum and 38.4mV for 30 ^{o}C gum. The lower Zeta potential at lower temperature is consistent with the increasing anionic character of FG with the higher extraction temperature, as described in section 3.1. This is due to the reduced ratio of neutral monosaccharide to acidic monosaccharide from 6.7 to 5.7.

The higher amounts of protein present in gum extracted at higher temperatures also contribute to the change in observed zeta potential. As reported in our previous study, the

isoelectric point (PI) of flaxseed protein isolate is pH 4.2 (Kaushik et al., 2015). Hence, at pH 7.0 the protein is negatively charged, contributing to an overall higher negative charge.

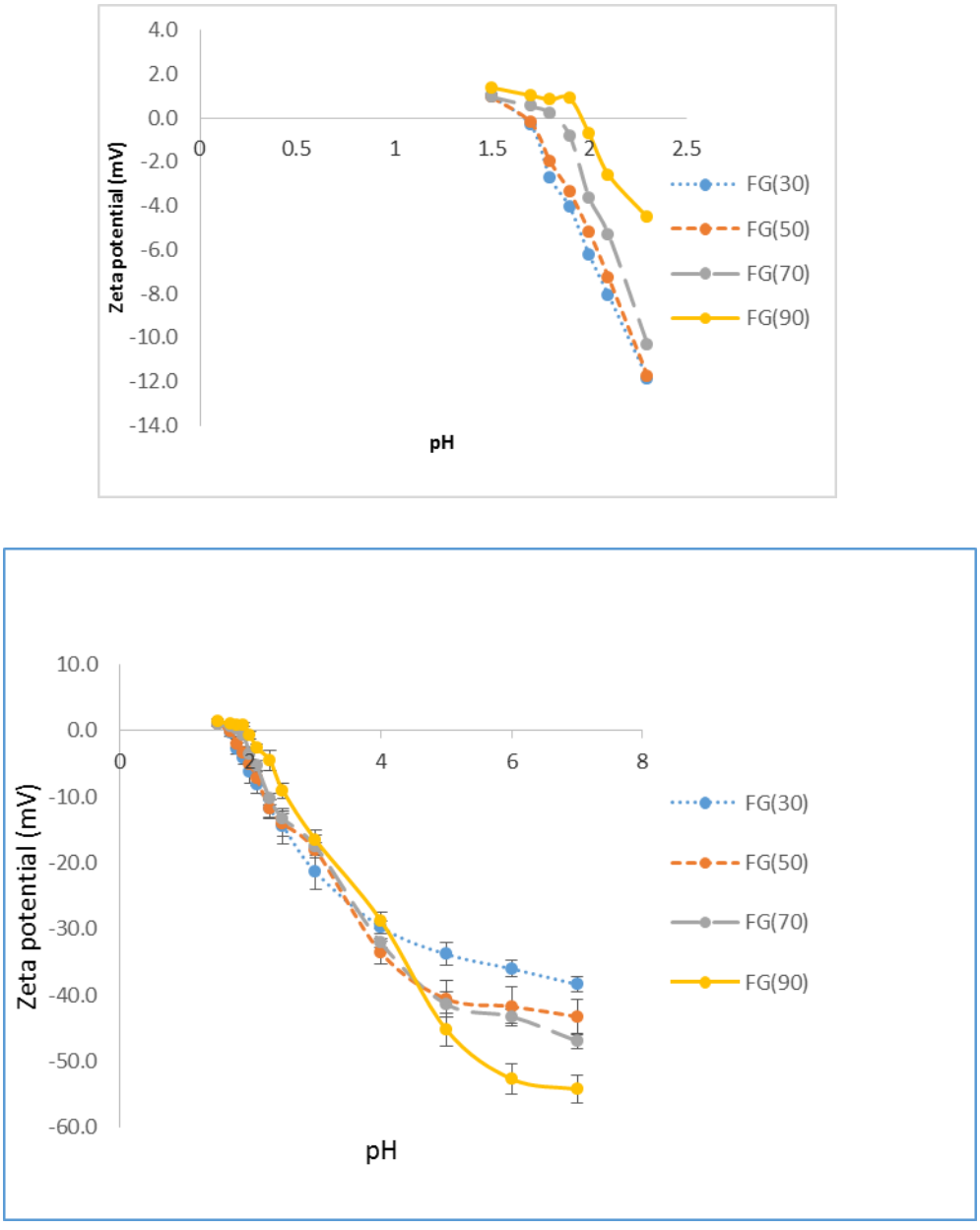

Figure 4.4 Zeta potential of 0.5% (w/v) flaxseed gum extracted from whole flaxseed at different temperatures (30, 50, 70 and 90 °C) as a function of pH. Inset figure shows the difference in the isoelectric points of FG extracted at different temperatures

The magnitude of the zeta potential for FG samples decreased as pH decreased, and became zero near pH 2.0. The Figure 4 inset graph shows that the PI of extracted gums shifts from pH

1.7 to 2.0 with the rise in extraction temperature from 30 to 90 °C. This shift in PI of the gum samples is a result of more protein being extracted at higher temperatures. As the pH of the gum solution is lowered below the PI of flaxseed protein (pH=4.2), the positively charged protein combines and neutralises a proportion of negatively charged gum. Therefore, the neutralisation point for gum samples containing higher amount of protein is achieved at higher pH.

4.3.4 Effect of extraction temperature on functional properties

4.3.4.1 Emulsifying activity and emulsion stability indices

The EAI and ESI of FG extracted at different temperatures is presented in Fig 4.5(a). EAI of these samples ranged from 61.4 to 151.9 m^2/g, indicating that the extraction temperature of FG has a significant effect on the emulsifying activity of FG. Initially, with the rise in extraction temperature from 30 to 70 ^oC the EAI increased from 133.8 to 151.9 m^2/g ($p < 0.05$), but subsequent temperature increase from 70 to 90 °C, resulted in a drop to 61.4 m^2/g. This indicates that the protein content of gum plays an important role in the emulsification properties of the gum. The underlying mechanism for higher EAI of FG with higher protein is related to the electrostatic interaction between charged protein and polysaccharide groups. Another contributing mechanism is the presence of hydrophobic groups in the protein structure that can adsorb on the oil phase of emulsions (Garti & Leser, 2001). In the current study, the protein content is highest for the gum extracted at 90 ^oC, but the EAI is lowest. This can be attributed to the decreased solubility of protein above its denaturation temperature, as the denaturation temperature of one of the flaxseed protein fractions is 83 ^oC (Koppelman et al., 2005).

However, the extraction temperature of FG has no significant effect on the stabilisation of emulsions formed by different FG samples. As shown in Fig. 4.5(a), ESI of FG samples ranged from 69.1 to 63.3 h as the temperature of extraction of FG increased from 30 to 90 ^oC.

This may be due to the higher viscosity of FG, which can prevent creaming of the emulsion over time. Mathur (2012) reported that polysaccharides act as emulsion stabilisers by increasing the viscosity of the emulsion.

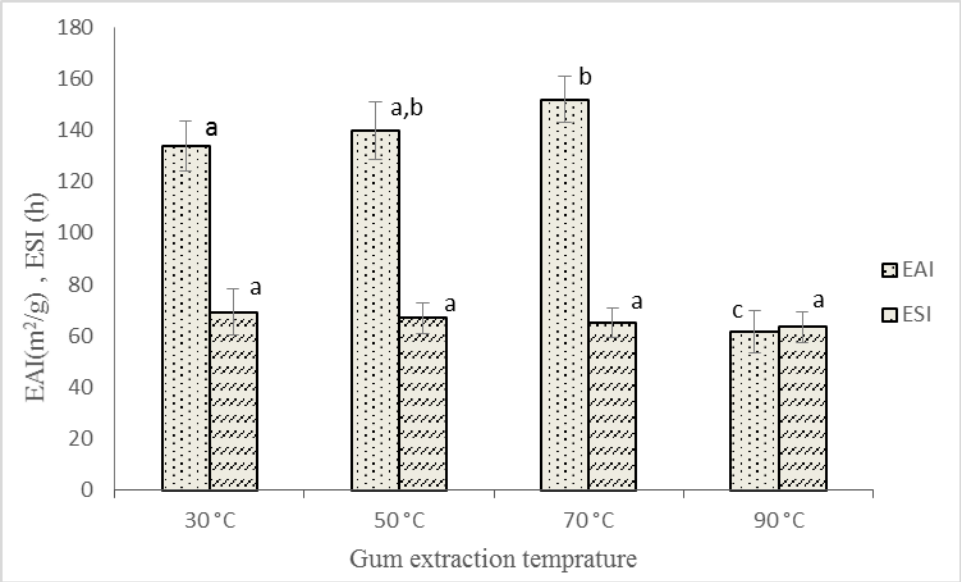

Figure 4.5(a) Comparison of emulsion activity index (EAI) and emulsion stability index (ESI) of flaxseed gum extracted at different temperatures (30, 50, 70 and 90 °C). The bars with different letters are significantly ($p<0.05$) different.

4.3.4.2 Water absorption and fat absorption capacities

The water absorption capacity (WAC) of FG samples extracted at different temperatures Fig. 4.5(b) varied significantly in the temperature range 50-90 ^{o}C ($p < 0.05$). The maximum water absorption capacity of 25.89 g/g of FG was observed for gum extracted at 30 ^{o}C, which is comparable to that of guar gum (22 g/g) and in good agreement with previous results (16-33 g/g) reported by Fedeniuk and Biliaderis (1994). However, with the rise in extraction temperature the WAC of FG decreased significantly and dropped to nearly half of its maximum value, that is 12.13 g/g at 90 ^{o}C. This reduction in FG is due to the decreased dietary fibre/carbohydrate content in the FG sample extracted at elevated temperatures. Moreover, as the maximum reduction in WAC was observed for FG extracted at 90 ^{o}C, it is

possible that the denaturation of the protein fraction present in gum has contributed to the result (Amid & Mirhosseini, 2012). The WAC data of FG extracted at different temperatures suggests that food applications where high WAC is required, FG extracted at temperatures below 50 ^{o}C should be employed.

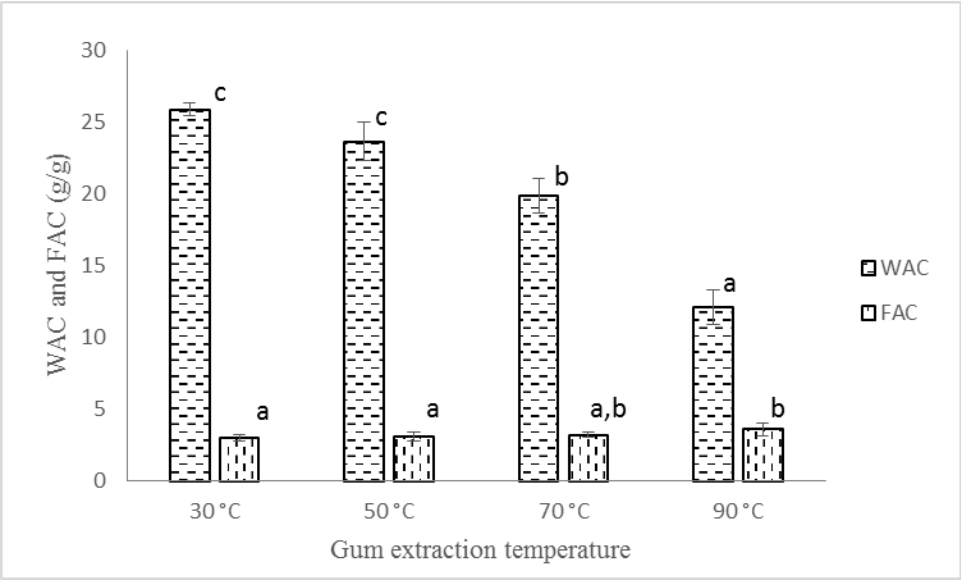

Figure 4.5(b) Comparison of water absorption capacity (WAC) and fat absorption capacity (FAC) of flaxseed gum extracted at different temperatures (30, 50, 70 and 90 °C). The bars with different letters are significantly ($p<0.05$) different.

FAC of the FG samples did not vary with the temperature of extraction. As shown in Fig. 4.5(b), the FAC of all FG samples, except the one extracted at 90 ^{o}C, have no significant difference ($p>0.05$). In the current study FAC ranged from 3.03-3.60g/g. The higher FAC of FG extracted at 90 °C is due to higher protein content. Proteins are more lipophilic in character than carbohydrates, due to the presence of non-polar amino acids and hence they bind fat more effectively than carbohydrates. Therefore, FG extracted at higher temperatures such as 90 ^{o}C is probably more effective for fat/oil absorption applications than those extracted at lower temperatures.

4.4 CONCLUSIONS

Flaxseed gum extracted at four different temperatures 30, 50, 70, 90 °C varied significantly in chemical composition with regard to type and content of monosaccharides and protein. Lower extraction temperatures (30, 50 °C) yielded FG samples with higher levels of neutral monosaccharides and lower levels of acidic monosaccharides. In addition, the protein contamination of FG samples increased with the rise in extraction temperature. NMR spectra of FG sample extracted at different temperatures indicated that all samples contained similar types of monosaccharides. The functional properties of FG, such as EAI and WAC, were negatively affected by the rise in extraction temperature. The FAC of the FG extracted at higher temperatures (70 or 90 °C) was higher than that of the sample extracted at lower temperatures (30 or 50 °C), probably due to the increased amount of protein extracted at higher temperatures. The results indicate the importance of selecting the appropriate extraction temperature to match the FG properties of the specific application. FG extracted at lower temperatures is better for emulsifying applications, while FG extracted at higher temperatures is more suitable for food systems where high oil absorption capacity is required.

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Chapter 5

Complex coacervation between flaxseed protein isolate and flaxseed gum

ABSTRACT

Flaxseed protein isolate (FPI) and flaxseed gum (FG) were extracted and the electrostatic complexation between these two biopolymers was studied as a function of pH and FPI-to-FG ratio using turbidimetric and electrophoretic mobility (zeta potential) tests. The zeta potential values of FPI, FG and their mixtures at the FPI-to-FG ratios of 1:1, 3:1, 5:1, 10:1, 15:1 were measured over a pH range 8.0-1.5. Alteration of the secondary structure of FPI as a function of pH was studied using circular dichroism. The proportion of α-helical structure decreased, whereas the both β-sheet structure and random coil structure increased with the lowering of pH from 8.0 to 3.0. The acidic pH affected the secondary structure of FPI and the unfolding of helix conformation facilitated the complexation of FPI with FG. The optimum FPI-to-FG ratio for complex coacervation was found to be 3:1. The critical pH values associated with the formation of soluble (pHc) and insoluble ($pH_{\phi 1}$) complexes at the optimum FPI-to-FG ratio were found to be 6.0 and 4.5, respectively. The optimum pH (pH_{opt}) for the optimum complex coacervation was 3.1. The instability and dissolution of FPI-FG complex coacervates started ($pH\phi_2$) at pH 2.1. These findings contribute to the development of FPI-FG complex coacervates as delivery vehicles for unstable albeit valuable nutrients such as omega-3 fatty acids.

Key word: Flaxseed protein isolate; Flaxseed gum; Secondary structure; Complex coacervation; Zeta potential; Turbidity

5.1 INTRODUCTION

The process of complex coacervation or associative phase separation in protein polysaccharide mixtures occurs due to electrostatic attraction of oppositely charged molecules, eventually leading to a solvent-rich and a biopolymer-rich phase (Schmitt & Turgeon, 2011; Tolstoguzov, 1991). Other factors influencing the complex coacervation are charge density, relative ratio and total concentration of biopolymers, pH, and temperature of the solvent (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Protein-polysaccharide mixtures form electrostatic complexes in a narrow pH range. Proteins are positively charged below their isoelectric point (pI) and can undergo complexation with negatively charged polysaccharides, resulting into soluble complex coacervates at pH_c, where pH_c is defined as the pH at which noncovalent interaction between protein and polysaccharide initiates (Aryee & Nickerson, 2012). Further reduction in mixture pH results into the formation of insoluble complexes at $pH_{\phi 1}$, where $pH_{\phi 1}$ is defined as the pH at which interaction between protein and polysaccharide is strong enough to cause macroscopic phase separation (Turgeon, Beaulieu, Schmitt, & Sanchez, 2003). The yield of complex coacervates is highest at pH_{opt}, where the net charge on the system is zero. The dissolution of complex coacervates back to solution state due to the protonation of polysaccharide occurs at $pH\phi_2$, where $pH\phi_2$ is defined as the pH beyond which interaction between protein and polysaccharide starts decreasing. (Elmer, Karaca, Low, & Nickerson, 2011). Determination of these important pH values (pH_c, $pH_{\phi 1}$, pH_{opt} and $pH_{\phi 2}$) for any protein-polysaccharide combinations provide better understanding of complexation behaviour as a function of pH. Kruif, Weinbreck, and Vries (2004) suggested that pH induced changes in the conformation of protein also influence the complexation of polymers with proteins.

A number of studies have reported that plant proteins are capable of forming complex coacervates in the presence of polysaccharides. Pea protein is the most widely studied protein

for complex coacervation (Ducel, Richard, Saulnier, Popineau, & Boury, 2004; Elmer et al., 2011; Klemmer, Waldner, Stone, Low, & Nickerson, 2012; Liu, Low, & Nickerson, 2009; Liu, Elmer, Low, & Nickerson, 2010). Other plant proteins considered appropriate for coacervation include soy protein (Jaramillo, Robert, & Coupland, 2011), canola protein (Klassen, Elmer, & Nickerson, 2011) and corn protein (Quispe-Condori, Saldana, & Temelli, 2011).

There are plant proteins that are theoretically known to possess favourable characteristics for coacervation but are unexplored practically. Dickinson, (2003) reported that charge density and droplet size are two important characteristics required for the stabilization of an emulsion. Wang, Li, Wang, Adhikari, and Shi, (2010) observed that flaxseed protein concentrate when compared to soy protein concentrate, possessed higher surface charge and smaller emulsion droplet size. Recently, Kuhn, Silva, Netto, and Cunha, (2014) found that flaxseed protein isolate (FPI) based emulsion are more stable than mixed FPI-whey protein isolate stabilised emulsions. In addition, the amino acid profile of flaxseed protein is nutritionally desirable and it is considered nutritionally similar to other oil seed proteins such as soybean (Oomah, 2001). However, the complexation behaviour of flaxseed protein with its own polysaccharide or with other polysaccharides has not been studied.

Flaxseed gum (FG) is another plant polymer identified as a good emulsifier (Cui, Ikeda, & Eskin, 2007). FG is a heteropolysaccharide composed of xylose, arabinose, glucose, galactose, galacturonic acid, rhamnose and fucose (Cui, Mazza, Oomah, & Biliaderis, 1994). Functional properties of flaxseed gum are comparable to those of gum Arabic and hence it can be used to replace gum Arabic in emulsions (Mazza & Biliaderis, 1989). Moreover, consumption of flaxseed gum as dietary fibre is reported to reduce the blood glucose level thereby reducing the risk of coronary artery disease (Oomah & Mazza, 2000).

The important nutritional characteristics of flaxseed protein and gum mean that they can be economical source of functional foods (Oomah, 2001). A thorough study on the complexation behaviour of these two biopolymers would help produce novel FPI-FG complex coacervates which can be preferentially used to microencapsulate active bio-ingredients such as omega-3 oils. This study determines the optimum pH range, FPI-to-FG ratio and total biopolymer concentration required for the formation of soluble and insoluble complexes between FPI and FG. In order to gain greater insight into the formation of these complex coacervates, the underlying structural change of flaxseed protein as a function of pH was also investigated. Except for this work, the complexation behaviour of flaxseed protein and flaxseed gum has not, so far, been reported.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Golden flaxseeds (*Linum usitatissimum*) were received from Stoney Creek Oil Product Pty. Ltd (Talbot, VIC, Australia). FG and flaxseed protein isolate (FPI) were extracted in the laboratory at Federation University, Australia. All other chemicals used in this study were purchased from Sigma-Aldrich Australia (Sydney, New South Wales, Australia) and were analytical grade.

5.2.2 Methods

5.2.2.1. Extraction of flaxseed gum

FG was extracted from whole raw flaxseed using the method of Cui et al. (1994) with slight modification (Fig. 5.1). Briefly, the flaxseed was soaked in Milli-Q water at a flaxseed-to-water ratio of 1:18 at 50 °C with continuous and gentle stirring for 2h for each of two consecutive cycles of extraction. The soaked seeds were filtered and the water containing the dissolved gum was treated with three volumes of 95% ethanol to precipitate the gum. The

precipitated gum was collected by centrifuge at 4,000g for 10 min. The precipitated gum was vacuum dried at 50 °C and stored at 4 °C.

5.2.2.2. Extraction of flaxseed protein isolate

Flaxseed protein was extracted from whole raw flaxseed following method of Oomah, Mazza and Cui (1994) with minor modifications. Firstly, the flaxseeds were demucilaged as described in section 2.2.1. The demucilaged seeds were dried in a hot air oven at 50 °C for 24h and pulverized using a coffee grinder (EM0415, Sunbeam Corporation Ltd. NSW, Australia). The crushed meal was defatted for 3 hours using hexane at a flaxseed-to-hexane ratio of 1:6. The hull was separated from the kernel by screening the tailings using a 0.15mm sieve to further reduce the interference of the mucilage during protein extraction. This fat extracted powder was subsequently soaked in 0.1M tris buffer (pH 8.6 with 0.1M NaCl) for 6h at a powder-to-buffer ratio of 1:16. The large residues were then separated from the protein extract using double layered cheesecloth. This filtered sample was centrifuged at 9,000g for 20 min using an ultracentrifuge (Sorvall Instruments, Wilmington, DE). The supernatant was collected and the pH was adjusted to 4.2 using 0.1 M HCl to precipitate the flaxseed protein. Once the pH was adjusted the sample was stored at 4 °C for 16h in order to provide sufficient time for protein to precipitate completely. The precipitated protein was recovered by centrifuging at 12,000g for 20min. The recovered solid mass was redispersed in Milli-Q water and was neutralized using 0.1 M NaOH. Finally, the FPI was obtained by freeze drying the sample at -45 °C compressor temperature and 0.5mm vacuum pressure using a freeze drier (DYNAVAC, Dynavac Engineering, Australia). The freeze-dried FPI was ground, vacuum sealed and stored at 4 °C.

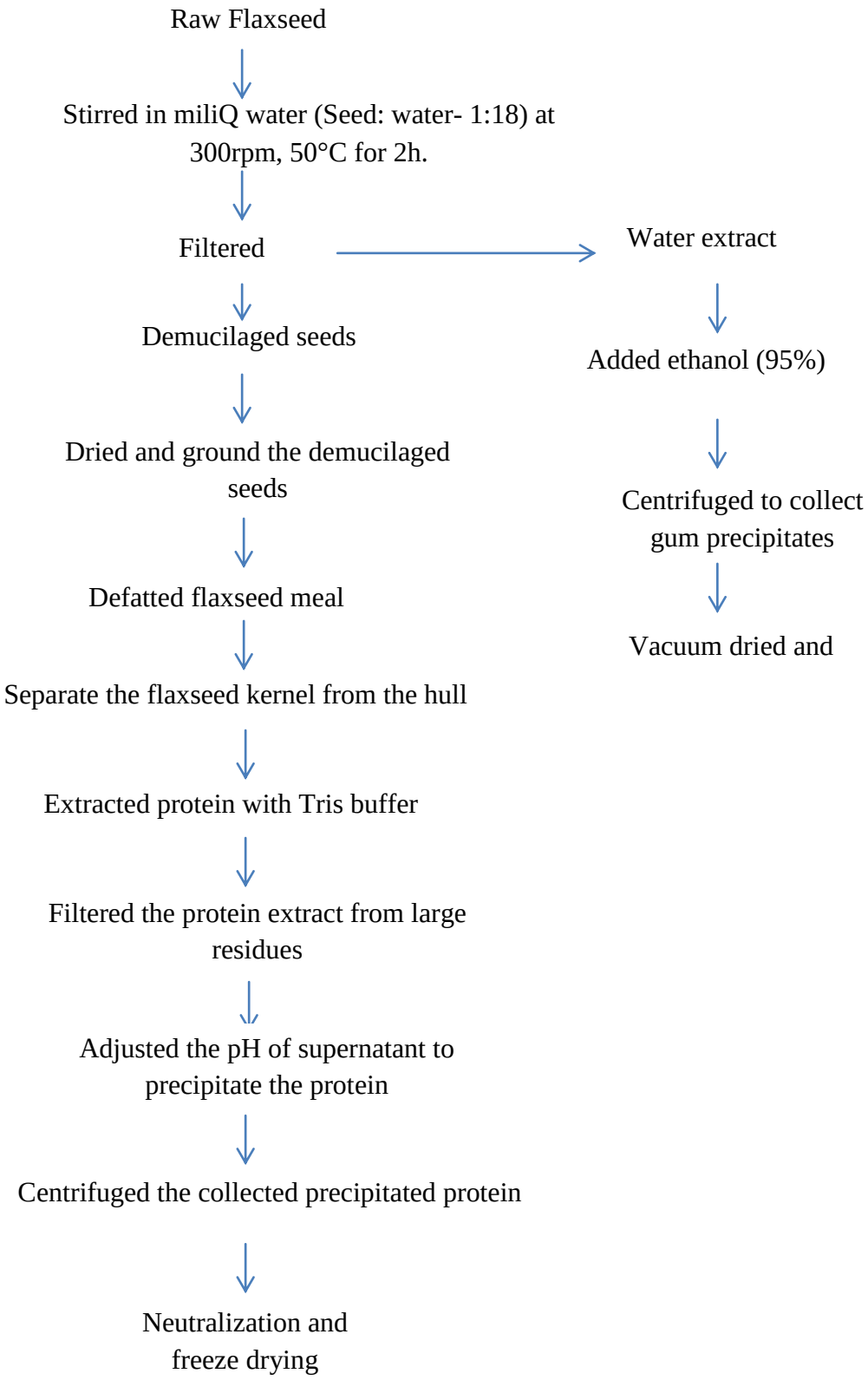

Figure 5.1 The protocol of extraction of flaxseed gum (FG) and flaxseed protein isolate (FPI) from whole flaxseeds

5.2.3. Chemical analysis of FPI and FG

Chemical analyses on all materials were performed according to AOAC Methods 925.10 (moisture), 923.03 (ash), 920.87 (crude protein) and 920.85 (lipid) (AOAC, 2003). Carbohydrate content was calculated on percent differential from 100%.

5.2.4. Identifying pHc, $pH_{\phi 1}$, pH_{opt} and $pH_{\phi 2}$ by turbidimetric analysis

FPI (1% w/w; pH 8.4) and FG stock solutions (0.3%, w/w; pH 7.0) were prepared by dispersing FPI and FG powders in tris buffer(0.1M pH 8.4) and Milli-Q water, respectively, followed by stirring at 500 rpm for 16 h at room temperature (21–22 °C), and 1 h at 40 °C to dissolve the protein. Tris buffer at pH 8.4 was used to prepare FPI stock solution to get better solubility. The FG concentration was fixed based on the preliminary experiments as higher concentrations (< 0.3%) showed high turbidity and viscosity. Moreover, past studies have indicated that FG dissolves completely at concentration within 0.2%-0.3% (w/w) (BeMiller, Whistler, Barkalow, & Chen, 1993). Therefore stock of 0.3% FG was made and used further in mixtures of FPI and FG at different ratios. Turbidity measurements were performed on individual FPI and FG systems and mixed systems at FPI-to-FG mass ratios of 1:1, 1.5:1, 3:1, 5:1, 10:1 and 15:1. The total solid concentration (FPI+FG) was maintained at 0.5% (w/w). The pH range in these tests was 8.00–1.50. In order to measure the turbidity in terms of light absorption, a UV spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) at 700 nm was used. The plastic cuvettes provided a path length of 1 cm. The mixture was acidified by adding 0.3M HCl dropwise to bring the pH to 6.0. Further decrease in pH to 2.0 was achieved by adding 0.1M HCl dropwise. To reach pH 1.5, 0.3M HCl was used. A different concentration of HCl was used to go to pH 1.5 so as to minimize the effect of dilution. Structure-forming transitions (pHc, $pH_{\phi 1}$ and $pH_{\phi 2}$) were determined graphically from the curve according to Weinbreck, Nieuwenhuijse, Robijn, & Kruif (2004), whereas pH_{opt}

corresponds to the pH value at which the highest optical density at 700 nm was observed. All these measurements were carried out in triplicate.

5.2.5 Measurement of zeta potential

A Zetasizer (ZS-90, Malvern instruments Ltd, UK) was used to measure the zeta potential values of individual (FPI, FG) solutions and mixed FPI-FG formulations as a function of pH within the range 7.0-2.0. The apparatus measures the direction and the velocity of the charged particles by applying an electric field and calculates their zeta potential using Smoluchowski model (Kirby & Hasselbrink, 2004). In these tests, the individual FPI (0.5%) and FG (0.2%) solutions were diluted by a factor of 100 using MilliQ water. Mixed FPI-FG formulations at different FPI-to-FG ratios (1:1, 3:1, 5:1, 10:1, 15:1) were prepared maintaining total solid (FPI+FG) concentration of 0.5% (w/w) then diluted by a factor of 100 using MilliQ water. Triplicate measurements were made for each sample and average values were reported.

5.2.6. Circular Dichroism

Circular dichroism (CD) spectral tests of FPI were carried out under nitrogen atmosphere at room temperature using a Jasco J-815 CD spectrophotometer (Jasco Corporation, Japan) using a quartz cell of 0.1 cm path length (Starna Pty. Ltd., Atascadero, CA, USA). Protein solution was prepared at a concentration of 2 mg/ml in deionized water (pH=8.4) and then injected in the required amounts into previously prepared citric acid buffer solutions. The concentration and pH of the final test samples were maintained at 1 mg/ml and 3.0-8.0, respectively. Absorption spectra of the above samples were recorded between 190 and 250 nm using the following instrumental parameters: bandwidth = 1.0 nm, time constant =1.0s and scanning rate=20 nm/min. Each acquired spectrum represented an average of three consecutive scans. The composition (%) of α-helix, β-sheet, β-turn and unordered structures

in the test specimen were calculated as suggested by Raussens, Ruysschaert, and Goormaghtigh (2003).

5.2.7. Statistical analysis

Results were expressed as the mean value ± standard deviation of three replicate experiments. The analysis of variance (ANOVA) was conducted using Minitab statistical software package (Minitab Inc., ver. 17, 2014). The significant difference between two mean values were calculated using the Tukey method at 95% confidence level ($P<0.05$).

5.3. RESULTS AND DISCUSSION

5.3.1. Chemical analysis of FPI and FG

Chemical analyses of the extracted FG showed: 4.42±0.47% moisture, 9.35±0.84% protein (%N×6.25), 1.75±.022% lipid, 3.17±0.43% ash and 81.31% carbohydrate.

Analysis of extracted FPI showed: 90.60±1.31% protein, 4.2±0.3% moisture, 2.20±0.24% ash, and 1.06±0.18% lipid and 1.94±0.37% carbohydrate. All of the above results are on weight (w/w) basis.

5.3.2. Effect of pH on the secondary structure of flaxseed protein

Fundamental understanding of pH induced conformational changes in protein structure provides insight on complexing behaviour of the protein. Mapping of the protein conformation changes aids in identifying the optimum conditions its functionality is preseserved before and after its conjugation with other polymers (Shang, Wang, Jiang, & Dong, 2007).

As shown in Table 5.1, the secondary structure of flaxseed protein is clearly affected by the variation of pH within the tested range. The lowering of the pH 8.0-3.0 promoted the formation of mostly β-sheets and random coils to an extent at the expense of α-helix. Both

α-helix and β-sheet are regular form of secondary structure, held together with amide back-bone of hydrogen bonds. However, in a α-helix they are formed within a single strand with an average length of 1.5A whereas in a β-sheet, they are formed between different strands with an average length of 3.5A (Walsh, 2012). Hence providing β-sheet structure, some extra flexibility and stability than α-helix. In the current study as the solvent conditions of FPI were changed from low alkaline (pH 8.0) to low acid (pH 6.0) a significant proportion of helices (50% of the original content) were dissociated. In contrast, the proportion of the β-sheets increased by 50 % from the original content observed at pH 8.0. With further lowering of the pH, the same trend continued for α-helical and β-sheet forms. With respect to the random coil content, there is a gradual but statistically insignificant ($P>0.05$) increase from pH 8.0-3.5. However, on lowering the pH from 3.5 to 3.0, the random coil content increased significantly ($P<0.05$), indicating considerable disorder in the structure of FPI in association with the helical unwinding. The amount of β-turns is essentially unchanged at 12.1-12.5% ($P>0.05$) across the pH range.

Table 5.1 The composition (%) of secondary structural features of FPI in a pH range of 8.0-3.0

Structure (%)	pH						
	8	7	6	5	4.2	3.5	3
α-Helix	23.6^{a}	19.0^{b}	15.0^{c}	13.5^{c}	13.0^{c}	12.0^{c}	7.5^{d}
β-sheet	13.9^{c}	29.4^{b}	30.2^{b}	37.9^{a}	32.9^{b}	35.9^{a}	31.6^{b}
Turn	12.1^{a}	12.3^{a}	12.3^{a}	12.2^{a}	12.2^{a}	12.1^{a}	12.5^{a}
Random coil	31.8^{b}	34.7^{b}	35.4^{b}	34.0^{b}	35.5^{b}	34.9^{b}	39.9^{a}

Numbers in a row with different superscript are significantly different.

Similar changes in the secondary structure of the wheat protein α-gliadin due to change in pH were reported by Chourpa, Ducel, Richard, Dubois, and Boury (2006). However, the authors attributed this change (more of alpha helices and random coil above pH 3.0 and more of β-sheets below pH 3) to the complexation of gum Arabic with α-gliadin. In the present study we confirmed that the changes in the conformation of FPI are a result of pH change and not complexation. The observed unfolding of flaxseed protein may facilitate its complexation with other polymers.

5.3.3. Complex coacervation of FPI and FG

5.3.3.1 Effect of pH

Changes in turbidity (measured as optical density (O.D.)) during an acid titration were measured for individual FPI (0.5%) and FG (0.3%) solutions (Fig. 5.2(a)), as well as for mixtures of FPI and FG (Fig. 2(b)) at FPI-to-FG ratios of 1:1, 1.5:1, 3:1, 5:1, 10:1, 15:1. The total solid content (FPI+FG) in these tests was maintained at 0.5% (w/w).

For FPI alone, O.D. started increasing near pH 6.0 (Fig. 5.2(a)) and the highest O.D. was observed at pH 3.4, after which it decreased with further lowering of pH down to 1.5. The increase in O.D. can be attributed to the decrease in solubility of FPI in that pH range. In separate experiments it was observed that the pI of FPI was 4.2; thus, the highest value of O.D. was observed below the pI of FPI. This may be due to the fact that some low molecular weight fractions of FPI have their pI at 3.5 (Oomah et al., 1994). Moreover, the presence of carbohydrates (approx. 2%) in FPI as mentioned in section 3.1 also might have contributed to this observation. These carbohydrates are basically the acidic fraction of flaxseed gum, composed of rhamnose and glacturonic acid (Cui, Kenaschuk, & Mazza, 1996).

In the case of FG, the O.D. started increasing near pH 6.0 (Fig. 5.2(a)); however, the increase was slow and the highest O.D. value (0.267) was quite low when compared to that for FPI

(0.024-0.529). The initial increase of O.D. in FG takes the form of a plateau between pHs ~3.75 and 3.00 (corresponding to a maximum O.D. value of 0.267), before starting to decrease in magnitude from pH 2.9, with a minimum of 0.177 at pH 1.5. Unlike other polysaccharides such as gum Arabic (Aryee & Nickerson, 2012), FG showed an increase in O.D. with decrease in pH most probably due to the presence of protein (10%).

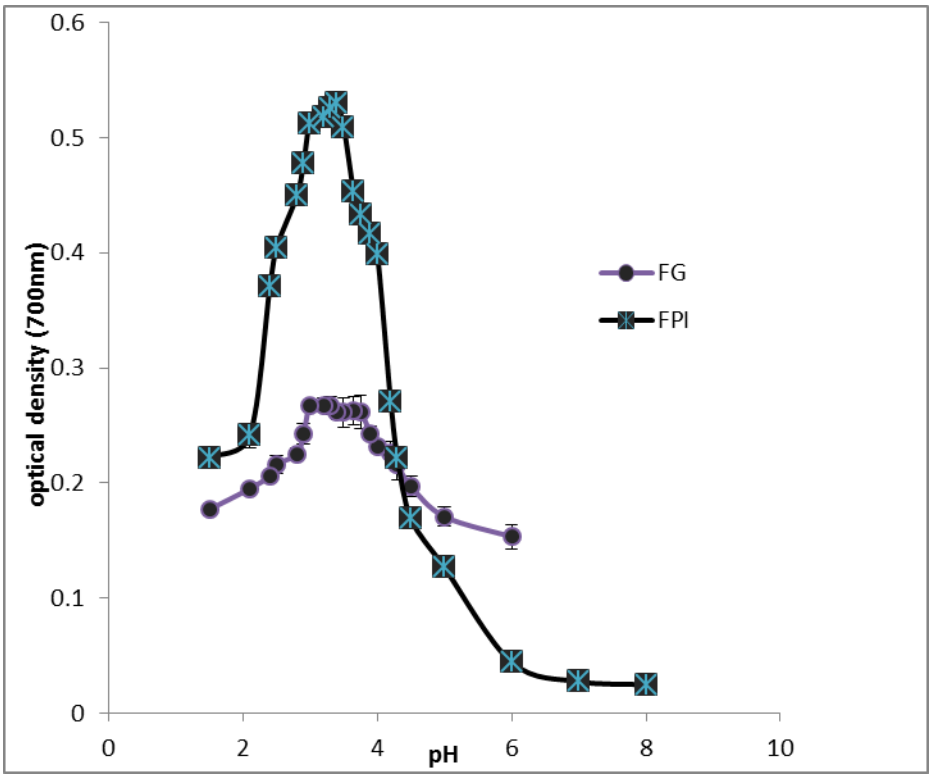

Figure 5.2(a) The turbidity values (optical density) as a function of pH for FPI (0.5% w/w) and FG (0.3% w/w). Data represent mean ± standard deviation (n=3).

In the case of mixtures of FPI and FG, the trend of variation of O.D. with pH was similar to that of FPI, except for the 1:1 mixture, in which the O.D. value of the mixture was lower than that of the pure protein. However, the highest O.D. values of the mixtures were significantly higher than that of FPI and FG alone (Fig. 5.2(b)). This can be attributed to the formation of complexes between these two polymers. Interestingly, the process of complexation started at the relatively high pH value 6.0, albeit the process was very slow. Though this pH is higher than the pI of FPI (4.2) and FPI is still negatively charged at this pH, the reason for this

interaction can be attributed to electrostatic attraction between anionic FG and cationic patches of FPI. Similar findings have been observed in whey protein-gum Arabic systems (Weinbreck, Vries, Schrooyen, & Kruif, 2003). The FPI-FG mixtures became slightly turbid due to this interaction; however, as no phase separation occurred the complex coacervates formed in this condition were believed to be soluble. Further acidification of this mixture, adjusted the pH towards the pI of FPI (4.2) and hence resulted in FPI having a net positive charge. The electrostatic attraction between the positively charged FPI and negatively charged FG resulted in the formation of insoluble complexes (at pH =4.5) and the O.D. of the mixture continuing to increase. The highest O.D. (0.913) was observed in the case sample with FPI-to-FG ratio of 15:1 at pH 3.3. When the pH was lowered further, slow dissociation of complex coacervates began to occur due to the protonation of reactive sites in the anionic polysaccharide.

5.3.3.2 Effect of FPI-to-FG ratio

Protein to polysaccharide ratio is another important factor affecting the process of complex coacervation. Different mixing ratios influence the charge balance between protein and polysaccharide, ultimately affecting the intensity of interaction and complexation (Liu et al., 2009).

The formation of complex coacervate at different FPI-to-FG ratios was investigated as a function of pH by varying the ratio from 1:1 to 15:1. The data presented in Fig. 5.2(b) illustrates that the O.D. of the mixtures increased with the increase in the proportion of FPI. The variation of O.D. of the mixtures as a function of FPI-to-FG ratio in terms of the highest O.D. values is presented in Fig. 5.3(a). The peak O.D. values for FPI-to-FG ratios of 3:1, 5:1, 10:1 and 15:1 were 0.831, 0.869, 0.875 and 0.913, respectively are not significantly different ($P>0.05$). This observation indicates that the formation of complex coacervates does not increase above the FPI-to-FG ratio of 3:1 as all of the negative sites available with the FG

have complexed with the positive sites of FP and the remaining positive sites of FP are in excess in the mixture.

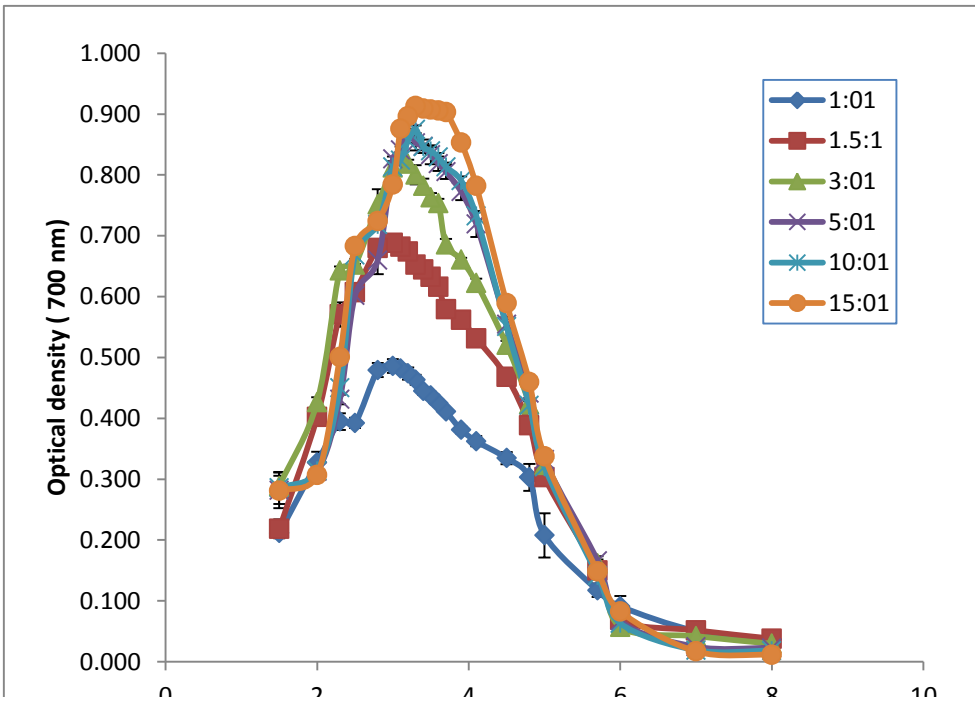

Figure 5.2(b) The turbidity values (optical density) as a function of pH for FPI-FG mixed systems at different FPI-to-FG ratios. Data represent mean ± standard deviation (n=3).

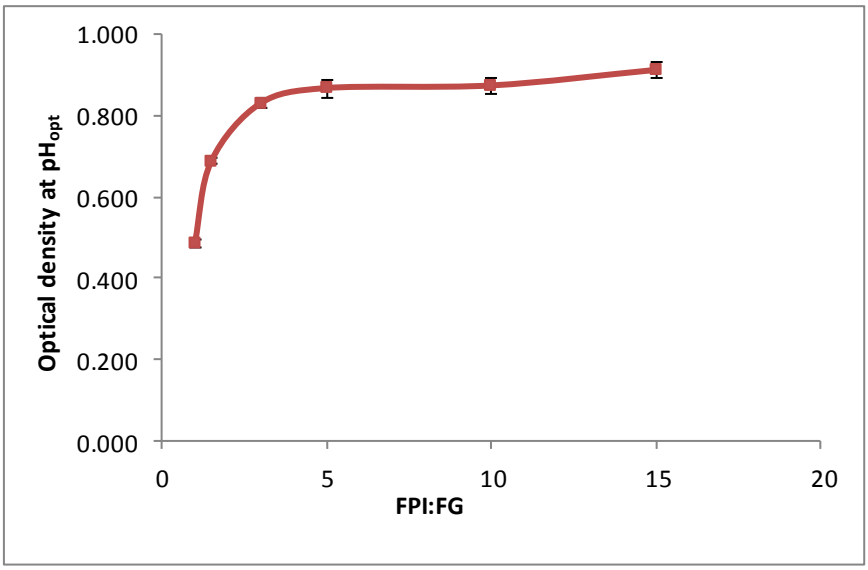

Figure 5.3(a) The highest optical density obtained at pHopt for different FPI-to-FG mixtures. Data represent mean ± standard deviation (n=3).

Hence, optimum complexation between FPI and FG was achieved at the FPI-to-FG ratio of 3:1. Similar observations were made by Elmer et al. (2011) using a base titration, for cationic polysaccharide chitosan and pea protein isolate.

5.3.3.3 Identification of pHc, $pH_{\phi 1}$, pH_{opt} and pH $_{\phi 2}$

As described in Section 1, identification of the phase boundaries pHc, $pH_{\phi 1}$, pH_{opt} and $pH_{\phi 2}$ is important for determining the critical pH values associated with structure forming events observed in the complex coacervation process. In the present study we selected the optimum FPI-to-FG ratio (3:1) to identify the critical pH values for complexation of FPI and FG. The process of complexation follows two structure-forming events associated with the formation of soluble and insoluble electrostatic complexes (Aryee & Nickerson, 2012). As shown in Fig. 5.3(b), the soluble FPI-FG complex coacervates were formed at pH_c = 6.0, as indicated by the slight increase in the O.D. due to increase in acidity. As the pH is lowered further, formation of insoluble complex coacervates started at pH 4.5. Both FPI and FG are negatively charged at this $pH_{\phi 1}$ = 4.5. However, as explained in Section 3.3.1, these interactions were assumed to be between negative sites of FG and the positive patches present in the molecular structure of FPI. As the pH approaches the pI of FPI (4.2), the process of complexation between oppositely charged polymers accelerated and the O.D. reaches its highest value at pH_{opt} (3.1). When the pH of the mixture was reduced further, it resulted in a decline in O.D. indicating the dissolution of complexes due to the protonation of negatively charged sites of FG. The dissolution of electrostatic complexes started near pH 2.1 as indicated by a steep decrease in the O.D. and continued until pH 1.5 (pHϕ2) to give an O.D. value of 0.289. This O.D. at pH 1.5 is still higher than the starting O.D., because at pH 1.5 solubility of FPI and FG is not 100% as indicated by their individual turbidity plots in Fig. 5.2(a) in the previous section.

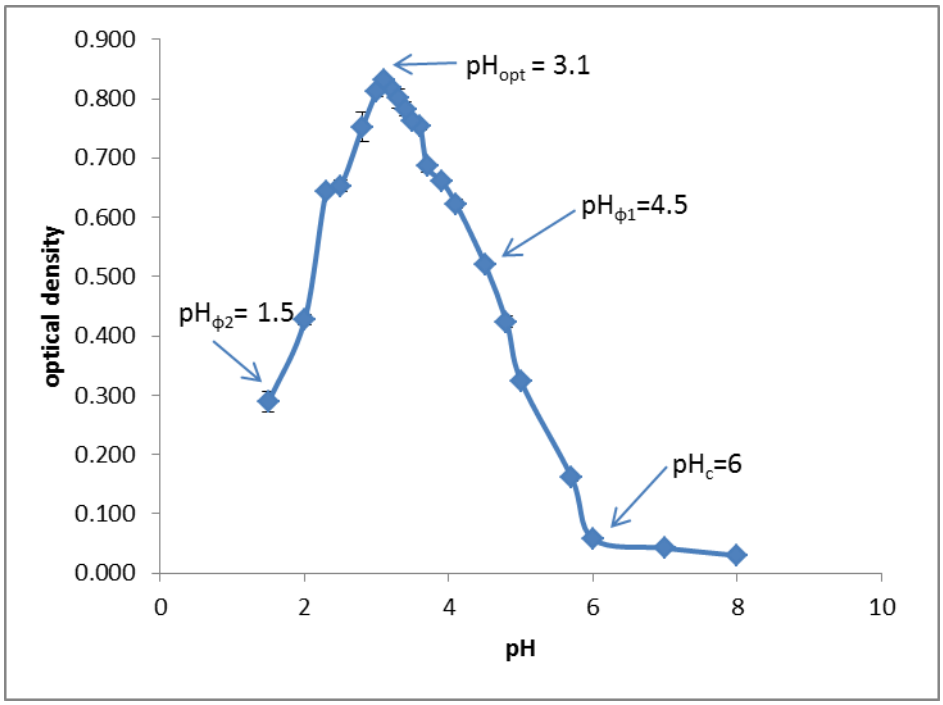

Figure 5.3(b) Optical density as function of pH for a formulation containing FPI-to-FG ratio of 3:1, demonstrating the various structure forming events associated with formation of soluble (pHc) and insoluble (pHϕ1) complex coacervates, the highest optical density (pH

Phase diagram (Fig. 5.3(c)) of FPI-FG system is used to demonstrate the effect of pH and FPI-to-FG ratios on the complex coacervation. Among these phase boundaries (pH_c, $pH_{\phi1}$, pH_{opt}, and $pH_{\phi2}$) pHc and $pH_{\phi2}$ were found to be independent of FPI-to-FG mixing ratios ($P>0.05$). In contrast, $pH_{\phi1}$ and pH_{opt} were influenced by the change in the mixing ratios. Specifically, $pH_{\phi1}$ values shifted to lower pHs as mixing ratios (FPI-to-FG) increased from 1:1 to 3:1; however, $pH_{\phi1}$ became independent when mixing ratio increased further ($p>0.05$).

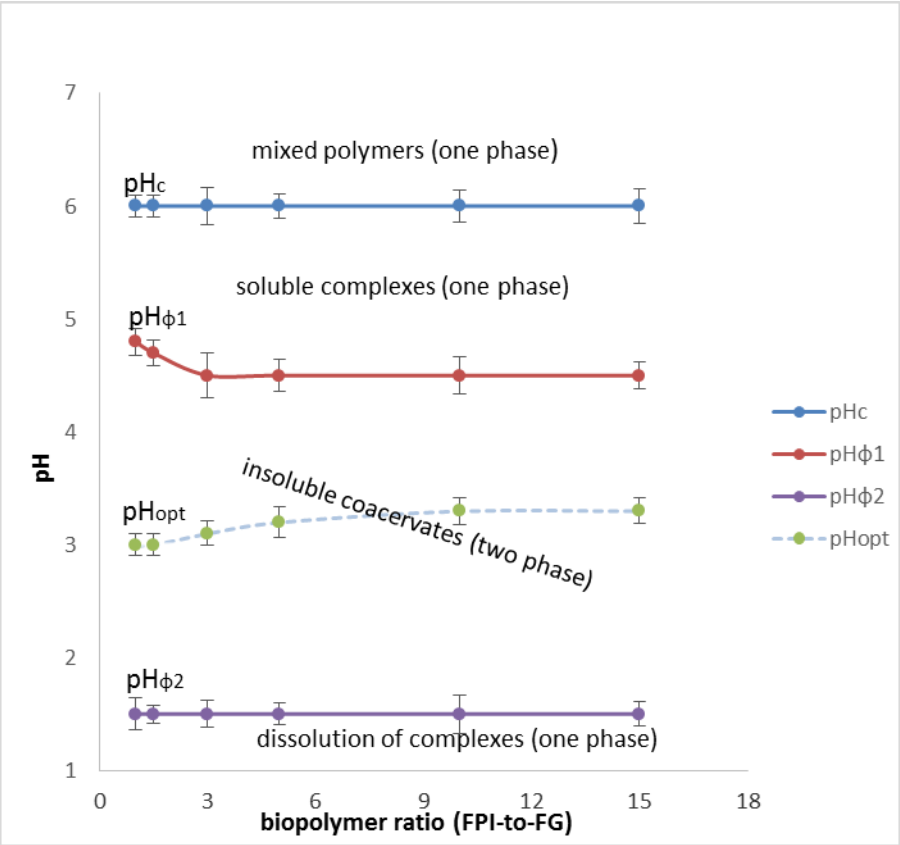

Figure 5.3(c) Phase diagram of FPI-FG system demonstrating the effect of pH and biopolymer ratio on complex coacervation Data represent mean ± standard deviation (n=3).

The pH_{opt} values shifted to higher pHs for the mixing ratios ranging from 1:1 to10:1 and was stable when the rations were increased further. Similar trends for pH_c and $pH_{\phi 2}$ were reported by Mattison, Brittain, and Dubin (1995) in BSA-polydimethyldiallylammonium chloride system and by Aryee and Nickerson (2012) in lentil protein-gum Arabic system, respectively. In other studies some differing trends have also been reported regarding the dependence of phase boundaries with the biopolymer ratio. For example, Weinbreck et al. (2003) observed that in the case of whey protein isolate-gum Arabic system, out of all the phase boundaries only pH_c was independent of biopolymer ratio. In contrast, Liu et al. (2009) found that pH_c was dependent on the biopolymer ratio in pea protein isolate-gum Arabic mixtures. The

differences in the above findings can be attributed to the different characteristics of the protein and polysaccharides mixed systems (Weinbreck et al., 2003).

5.3.4. Effect of pH on the charge density

The charge density of individual FPI, FG solutions and their admixtures at FPI-to-FG ratios of 1:1, 3:1, 5:1, 10:1, 15:1 were determined as a function of pH in order to confirm the pH_{opt} for each individual ratio of FPI and FG mixed systems. Net surface charge density of FPI and FG as a function of pH (8.0-2.0) is shown in Fig. 5.4(a).

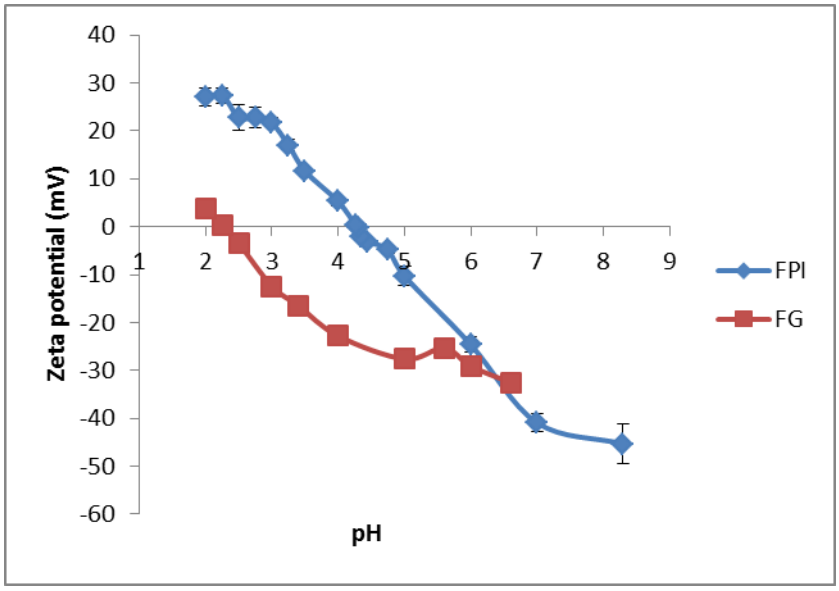

Figure 5.4(a) Zeta-potential values as a function of pH for the individual FPI and FG. Data represent mean ± standard deviation (n=3).

Electrostatic complexes are formed under solvent conditions when the participating polymers have opposite charges. As shown in Fig. 5.4(a), the net charge of FPI and FG solutions is zero at pH 4.20 and 2.25, respectively. This means effective formation of complex coacervates between FPI and FG can occur between pH values of 4.20 and 2.25.

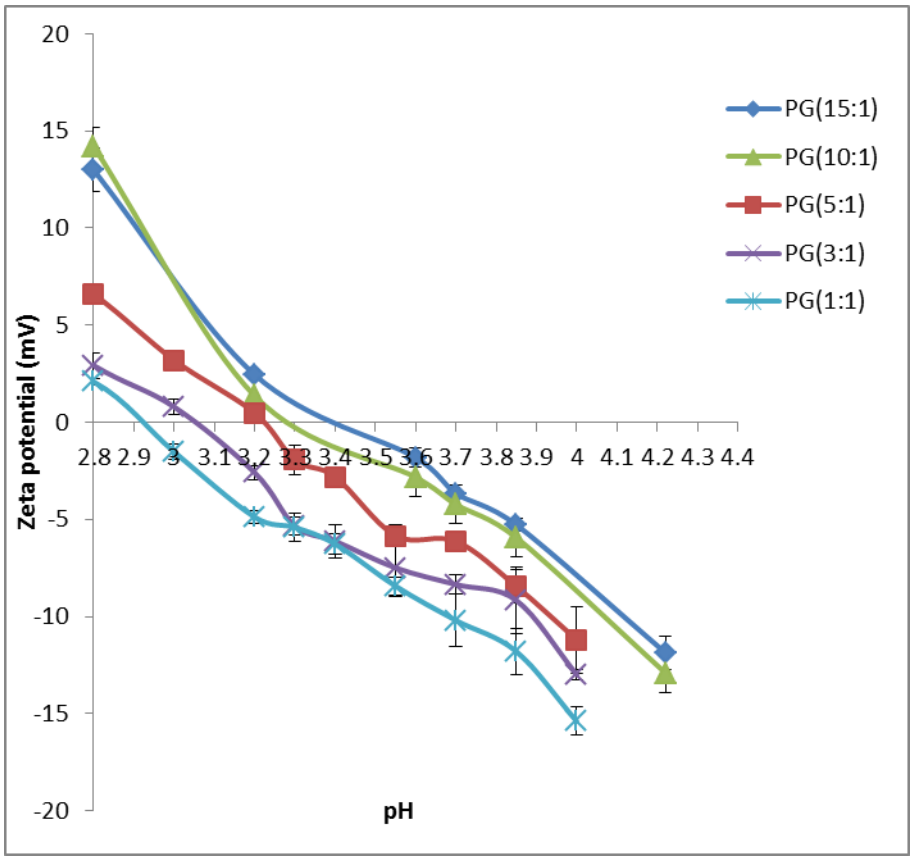

Figure 5.4 (b) Zeta-potential values as a function of pH for the mixed systems at different FPI-to-FG ratios. Data represent mean ± standard deviation (n=3).

The points of electrical equivalence (a pH value at which the net charge of the oppositely charged moieties is zero) of different FPI-to-FG ratios are shown in Fig. 5.4(b). As shown, the net neutrality (zeta potential = 0 mV) of FPI-FG mixed systems at FPI-to-FG ratios 1:1 to 15:1 occurred within the pH range of 2.9 and 3.4. It can be observed from Fig. 5.4(b) that when the proportion of FPI in FPI-to-FG ratio increased, the point of neutrality or the point of electrical equivalence shifted to higher pH values. These data sets provide important insights to determine the optimal protein-to-polysaccharide ratio in order to optimize the complex coacervation process. Fig. 5.4(b) also shows that the pH of neutrality for all the FPI-to-FG ratios except 1:1 was same as pH_{opt} determined through optical density tests, showing that both these methods are capable of determining the optimum pH and optimum FPI-to-FG ratio for maximum yield of complex coacervates.

5.4. CONCLUSIONS

This study demonstrated that flaxseed protein isolate (FPI) and flaxseed gum (FG) can be successfully complexed at optimized conditions. The variation of pH affected the secondary structure configuration of FPI and the unfolding of helix conformation facilitated the complexation of FPI with FG at lower pH values. The optimum FPI-to-FG ratio and pH value for complexation between FPI and FG were 3:1 and 3.1, respectively and the resultant complex coacervates were found to be stable at low pH values up to 2.1.

These FPI-FG complex coacervates can be preferentially used as novel, economic and nutritionally valuable delivery vehicles for active and unstable food ingredients. Further research is needed to study the practical applications of this delivery matrix and the ability to microencapsulate and protect sensitive ingredients such as omega-3 oils, vitamins or probiotics.

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Chapter 6

Microencapsulation of flaxseed oil in flaxseed protein-flaxseed gum complex coacervates

ABSTRACT

Flaxseed oil, a rich source of omega-3 fatty acids was microencapsulated in a novel matrix formed by complex coacervation between flaxseed protein isolate (FPI) and flaxseed gum (FG). This matrix was consolidated by crosslinking with glutaraldehyde and adding matodextrin. Liquid microcapsules with three core (oil)-to-wall ratios (1:2, 1:3 and 1:4) were prepared and spray dried and freeze dried to produce powders. The microencapsulation efficiency, surface oil, morphology and oxidative stability of these solid microcapsules were determined. The spray dried solid microcapsules had higher oil microencapsulation efficiency, lower surface oil content, better morphology and higher oxidation stability compared to the freeze dried microcapsules. The highest microencapsulation efficiency obtained in spray dried microcapsules was 87% with a surface oil of 2.78% at an oil load of 20%. The oxidation stability obtained from spray dried microcapsules at core-to-wall ratio of 1:4 was nearly double to that of the unencapsulated flaxseed oil.

Keywords: Omega-3 fatty acids, Microencapsulation, Complex Coacervation, Flaxseed protein isolate, Flaxseed gum

6.1 INTRODUCTION

Flaxseed oil (FO) is the richest source of ALA (57% of total fatty acids) (Carneiro et al., 2013). However, like fish oil, its unsaturated nature renders it very prone to oxidation with consequent loss of biological functionality. Microencapsulation is used by many researchers (Carneiro et al., 2013; Heinzelmann et al., 2000; Liu et al., 2010) to address this issue. Process of complex coacervation followed by spray drying has been recognized as one of the most promising technologies for stabilization of omega-3 oils by microencapsulation while delivering the highest pay load (40-60%) (Barrow, Nolan & Jin 2007; Liu et al., 2010).

However, most of the microencapsulated products have gelatin included in the wall material (Liu et al., 2010) rendering it unacceptable to the vegetarian population (Kralovec et al., 2012). There are also safety concerns associated with gelatin due to the recognition of prion diseases (Morrison et al., 1999). Moreover, there is increasing interest in industry to find plant-based ingredients as encapsulating shell material due to their healthy image. Hence, it is essential to find alternatives to gelatin as a shell material for microencapsulation.

In this context, the complex coacervates of plant-based proteins such as soy protein (Jun-xia et al., 2011), pea protein and cereal protein (Ducel et al., 2004), flaxseed protein (Wang et al., 2011) with gum arabic has been explored for different active ingredients. Specifically for microencapsulation of FO, different wall materials used to date include whey protein isolate (Partanen et al., 2008), zein protein (Quispe-Condori et al., 2011), gum Arabic (Tonon et al., 2011), and different combinations of maltodextrin, whey protein concentrate and modified starch (Carneiro et al., 2013; Omar et al., 2009). None of the published studies on the microencapsulation of FO have used the flaxseed protein isolate (FPI) or flaxseed gum (FG) as wall materials and such an association should be considered natural. FPI and FG which are

emerging as potential emulsifiers (Oomah, 2001) should be considered for their efficacy in terms of payload, preventing oxidation as well as the structural strength of the coacervates.

Hence, this study had three key objectives: Firstly, to encapsulate FO in crosslinked FPI–FG complex coacervates consolidated by maltodextrin at varying core-to-wall ratios. Secondly, to spray and freeze dry and spray dry the liquid microcapsules and finally, characterize the dried microcapsules in terms of oxidative stability, microencapsulation efficiency, surface oil content and morphology.

6.2 MATERIALS AND METHODS

6.2.1 Materials

The golden flaxseeds (Linum usitatissimum) and flaxseed oil (FO) were received from Stoney Creek Oil Products Pty Ltd (Talbot, VIC, Australia). Flaxseed protein isolate (FPI) and flaxseed gum (FG) were extracted in the laboratory as described in the section 2.2. All other chemicals were purchased from Sigma–Aldrich Australia (New South Wales, Australia) and were of analytical grade.

6.2.2 Proximate analysis

Proximate composition analyses for extracted FPI and FG were conducted according to AOAC official Methods 925.10 (moisture), 923.03 (ash), 920.87 (crude protein, by using %N × 6.25) and 920.85 (lipid) (AOAC, 2003). Carbohydrate content was determined on the basis of percent difference from 100%.

6.2.3 Extraction of FPI and FG

Extraction of FPI and FG were carried out by the method described in our previous work (Kaushik et al., 2015). Briefly, flaxseed was soaked in Milli-Q water at a flaxseed-to-water ratio of 1:18 at 50 ^{o}C with continuous gentle stirring for 2 h. Subsequently, the seeds were

filtered and the water containing the dissolved gum was treated with three volumes of 95% ethanol to precipitate the gum. The precipitated gum was collected by centrifugation at 4000×g for 10 min. The precipitated gum was vacuum dried at 50 °C and stored at 4 °C till further use.

Flaxseed protein was extracted from demucilaged flaxseed. The demucilaged seeds were dried in a hot air oven at 50 °C for 24 h and ground using a coffee grinder (EM0415, Sunbeam Corporation Ltd. NSW, Australia). The crushed meal was defatted for 3 hours using hexane at a ratio of 1:6. The hull was separated from the kernel by screening the tailings using a 0.15mm sieve to further reduce the interference of the mucilage during protein extraction. This defatted powder was subsequently soaked in 0.1M tris buffer (pH 8.6 with 0.1M NaCl) at a seed-to-buffer ratio of 1:16 for 24 h. The large residues were then separated from the protein extract using double layered cheesecloth. The filtered sample was centrifuged at 9000×g for 20 min using an ultracentrifuge (Sorvall Instruments, Wilmington, DE). The supernatant was collected and the pH was adjusted to 4.2 using 0.1 M HCl to precipitate the flaxseed protein. The extract was then stored at 4 °C for 16h in order to provide sufficient time for protein to precipitate completely. The precipitated protein was recovered by centrifuging at 12,000×g for 20min. The recovered solid mass was redispersed in Milli-Q water and was neutralized using 0.1 M NaOH. Finally, the FPI was obtained by freeze drying the sample, freeze-dried FPI was ground, vacuum sealed and stored at 4 °C.

6.2.4 Complex coacervation of FPI and FG and microencapsulation of FO

Complex coacervation between FPI and FG was optimized at pH 3.1 and FPI-to-FG ratio of 3:1 (Kaushik et al., 2015). Firstly, 250 g FPI solution (6%, w/w) was prepared by dissolving the FPI in distilled water at 50 °C for first two hours and then allowed to hydrate overnight at ambient temperature using a magnetic stirrer. Next day different amount of FO (5.0 g, 3.3 g,

and 2.5 g) was dispersed in the protein to maintain the core-to- wall ratios at 1:2, 1:3, and 1:4. Subsequently, first homogenization was carried out using an ultra Turax (RW 20, IKA GmbH Co., Bitterfeld-Wolfen, Germany) at 12,000 rpm for 5 min to produce an O/W emulsion. After that 250 g FG solution (2%, w/w) was added drop wise into this O/W emulsion with continuous stirring. A second homogenization was carried out at 18,000 rpm for 15min. The pH of this emulsion was adjusted to 3.1 by adding 0.1M HCl drop wise to induce interaction between the FPI and FG. A microscope (Eclipse 80 i, Nikon, Japan) was used to obtain optical images of the coacervate microcapsules. The coacervation procedure was carried out at 50 ^{o}C for 1 h, followed by crosslinking step. Crosslinking of the wall materials was carried out by addition of 4g of 70% (w/w) glutaraldehyde solution (Devi & Maji, 2011). The liquid microcapsules thus formed were cooled to 5 ^{o}C at a slow rate of 5 ^{o}C/h using a programmable water bath (PolyScience, Niles, Illinois, USA). After maintaining the sample at 5 ^{o}C for 4 h, 7.5g maltodextrin (10 DE) was added to the liquid microcapsules before drying. Maltodextrin with its high solubility and low viscosity aids in the spray drying of the samples (Adhikari et al., 2004; Gharsallaoui et al., 2007). Finally, the microcapsules were dried (section 2.5) to produce solid microcapsules.

6.2.5 Drying of microencapsulated flaxseed oil

6.2.5.1 Spray drying

Microencapsulated FO produced as per section 2.4 was spray dried (Mini spray dryer B-290, BUCHI Labortechnik, Switzerland) using inlet and outlet temperatures of 190 °C and 95±2 °C, respectively. These spray dried microcapsules were collected and stored at 4°C for further characterization.

6.2.5.2 Freeze drying

The freeze drying of the liquid microcapsules was carried out by first freezing them at -80 °C for overnight. Frozen samples were then freeze dried (Christ Alpha 2-4LD, Osterode, Germany). The temperature of the ice condenser was set at -50 °C and the vacuum pressure was set to 0.04 mbar. The frozen samples were dried for approximately 36 hrs and the dried product was collected and stored at 4 °C for further characterization.

6.2.6 Physicochemical properties

6.2.6.1 Solid yield

The solid yield was calculated as the ratio of the powder mass collected after every drying experiment to the initial amount of solids in the dispersion volume before drying.

6.2.6.2. Moisture content and water activity

The moisture content of powdered microcapsules was determined by drying the sample in a hot air oven at 105 °C until constant weight was obtained. The water activity of the powdered microcapsules was determined using a water activity meter (Novasina, Switzerland). The temperature during the water activity tests was maintained at 24±0.5°C.

6.2.6.3 Microencapsulation efficiency

Microencapsulation efficiency (ME) was calculated by measuring the surface oil (SO) and total oil (TO) of the microcapsules. Surface oil was determined by the washing method described by Liu et al. (2010) with slight modification. Briefly, three grams of dried microcapsule sample was dispersed in 30 ml of hexane followed by vigorous shaking for 60 s. The solvent was then filtered through filter paper (Whatman, 5µm) into a 50 ml beaker and the beaker was placed in the fume hood overnight to allow evaporation of the solvent. The beaker was then heated at 100 °C for 1h to remove any residual solvent and SO content was determined gravimetrically.

The TO content in the dried microcapsules was determined by an acid digestion method as described by Eratte et al (2015) with minor modifications. Briefly, 3.0 g of powdered microcapsule sample was dispersed in 30 ml of 4N HCl and shaken at 225 rpm for 15 minutes on an orbital shaker (Stuart SSL-1, Carl Roth, Karlsruhe, Germany) in order to dissolve the shell material. To extract the oil, 15 ml of hexane was added to this mixture and then shaken for 4 h at ambient temperature. The mixture was centrifuged at 24,471×g at 20 ^{o}C for 30 minutes. The hexane phase containing the dissolved oil was collected and dried under nitrogen in a fume hood. The sample was further dried at 100 ^{o}C in an oven to remove the residual solvent. The TO content was then determined gravimetrically. The percent ME was calculated using equation given below

$$ME\ (\%) = [(TO\text{-}SO)/TO] \times 100 \quad (1)$$

where TO and SO are the mass values (g) of total oil and surface oil of the microcapsules, respectively.

6.2.6.4 Surface morphology of solid microcapsules

A Scanning Electron Microscopy (JEOL JSM6300 SEM, Tokyo, Japan) was used to acquire the morphology of spray dried and freeze dried microcapsules. Samples were coated with thin layer of gold for 2 min, using a gold sputter (Sputter coater, Agar Aids, England). The sample was examined at an accelerating voltage of 7 kV.

6.2.7 Oxidative stability test

Peroxide value and rancimat analysis were carried out for all the microencapsulated samples in order to determine the effect of core-to-wall ratio and drying methods on the oxidative stability.

6.2.7.1 Peroxide value (PV)

Oxidative stability of original FO and microencapsulated oil was measured by storing the microcapsules at 4 °C for 30 days and measuring the primary oxidative product (peroxides) formed. The peroxide values of the samples were measured for 30 days at an interval of 10 days as described by Karaca et al. (2013). Approximately 0.2 g of extracted FO was weighed into a 100 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 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 0.001 N sodium thiosulfate ($Na_2S_2O_3$) until the purple colour disappeared. The required PV was calculated as given by equation (2).

$$PV = (S\text{-}B) \times N \times 1000/W \quad (2)$$

where, S is the volume of $Na_2S_2O_3$ used by sample, B is the volume of $Na_2S_2O_3$ used by blank, N is the normality of $Na_2S_2O_3$ solution, and W is the sample mass (g).

6.2.7.2 Rancimat analysis

Oxidation stability of the liquid oil and microencapsulated FO produced by freeze drying and spray drying was determined using a Rancimat (model 743, Metrohm, Herisau, Switzerland) following Farhoosh (2007). Accelerated oxidation was carried out with 4 ml of FO or 1.5 g dried microcapsule powder by heating the samples at 90^{o}C under purified air (flow rate of 20L/h). The induction time of the test sample was recorded and used as the oxidative stability index (OSI). Analyses were performed in triplicates.

6.2.8. Interaction between FPI and FG

To study the interaction between FPI and FG as they complex to form coacervates the infrared spectra of wall materials (FPI and FG), core (FO) and dried microcapsules were

recorded on a Nicolet 6700-FTIR (Thermo Scientific, Waltham, MA, USA). FTIR spectrometer was equipped with a thermoelectrically cooled deuterated triglycine sulphate (DTGS) detector and an attenuated total reflectance (ATR) sampling accessory. Each sample was applied on to the face of diamond crystals of the sample accessory. IR spectra were collected in the range of 4000–625 cm^{-1}at a resolution of 4 cm^{-1}. A background spectrum was individually acquired on a clean surface of the ATR crystal prior to acquiring the IR spectra of each sample. For each sample, 16 scans were carried out and averaged.

6.2.9 Statistical Analysis

All measurements were performed in triplicate and the results are reported as mean ± standard deviation. The SPSS statistical package (Version 21, Lead Technologies, USA) was used for the analysis of variance (ANOVA) to determine whether or not significant difference existed between two mean values. The confidence level of 95% ($p<0.05$) was used.

6.3. RESULTS AND DISCUSSION

6.3.1 Morphology of microcapsules

6.3.1.1 Liquid microcapsules

Optical microscopy was used to observe the formation of microcapsules having flaxseed oil as core and flaxseed protein and flaxseed gum as outer shell. As per the protocol described in section 2.4 after mixing all the core and wall materials; as the pH reduced from 6.5 to 3.1, coacervation occurred in the oil in water emulsion. These coacervate particles migrated to the surface of oil droplets, forming a primary coating around them (Wang et al., 2014). In general, microcapsules can be single core or multicore depending upon the various process conditions (Prata & Grosso, 2015). In the current study we observed that the microcapsules formed were multicore type (Fig 6.1(a)). It is assumed that the two stage homogenisation of the emulsion (as described in section 2.4) carried out before the coacervation step, reduced

the size of the oil droplets to <2µm (calculated based on 100 µm scale bar). However, after coacervation and the primary coating of oil droplets, they aggregated and formed multicore capsules. Similar formation of multicore capsules was reported by Yeo et al. (2005) under a high degree of homogenization. Multicore capsules are suggested to have better core protection and release properties (Barrow et al., 2007; Yeo et al., 2005).

Burgess and Ponsart (1998) suggested that coacervated micro-particles without crosslinking do not sustain spray drying process. Hence, we used glutaraldehyde as a crosslinker to strengthen the wall material. The crosslinking step was carried out after the coacervation step, at 50 ^{o}C for proper mixing of the glutaraldehyde in the dispersion. This was followed by slow cooling of the microcapsules to 5 ^{o}C. These two steps (crosslinking and cooling) resulted in the binding and absorption of the free coacervate on the surface of aggregated droplets. Thus an outer wall is formed around the already microencapsulated and aggregated droplets, ensuring these stay intact and are more robust in structure. The overall morphology of microcapsules (Fig 6.1(a)) indicates that these were of irregular shape with a wide size range of 90-130microns (measured based on 100 microcapsules). However, after crosslinking (Fig 6.1(b)) the liquid microcapsules attained an oval like shape. Similar crosslinking effects on the morphology of liquid microcapsules were reported by Wang et al., (2014).

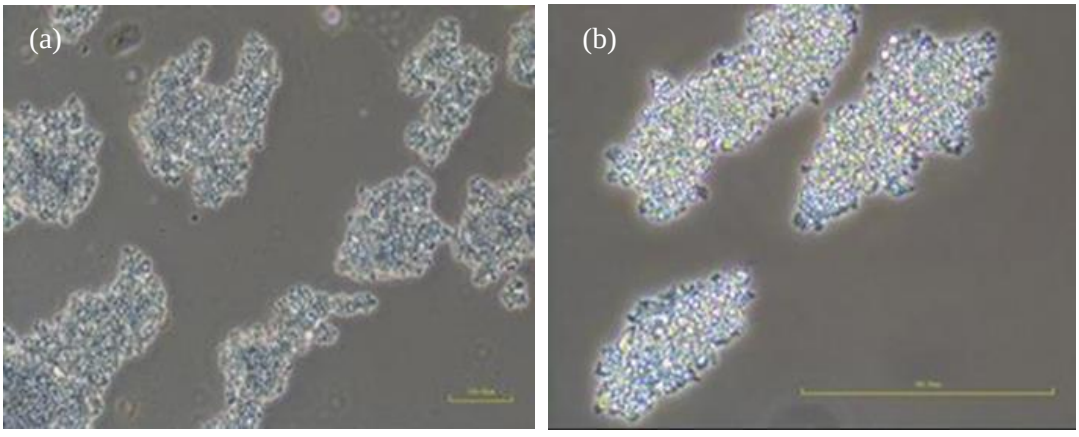

Figure 6.1 Optical microscopic images of liquid microcapsules (a) before cross linking (b) after cross linking at scale bar of 100µm

6.3.1.2 Solid microcapsules

The SEM micrographs of both SDM and FDM are shown in Fig 6.2(a) and 2(b), respectively. A fair comparison can be drawn about the structure of microcapsules produced by these two drying methods.

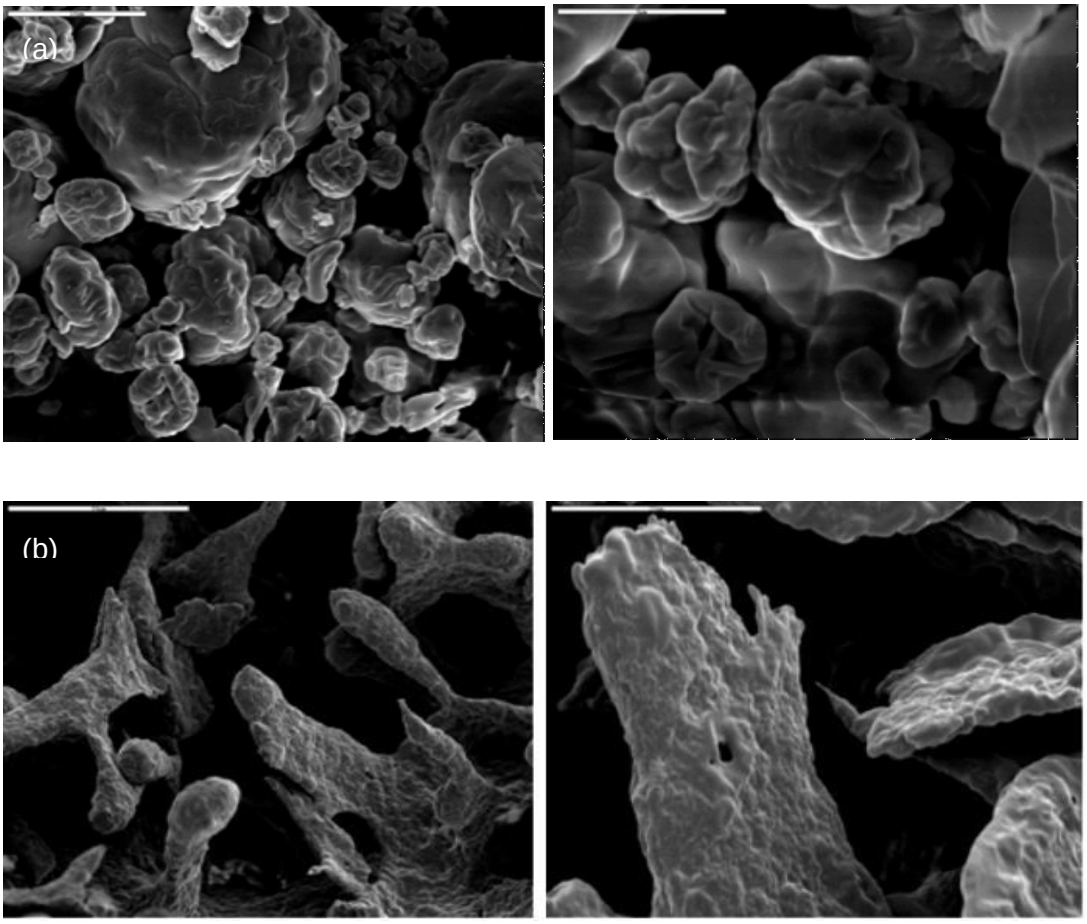

Figure 6.2 SEM images of (a) Spray dried and (b) freeze dried flaxseed oil microcapsules. Scale bar 10 µm and 5 µm.

The freeze dried microcapsules appeared to have a lumpy structure with pores or cavities on the surface, while the spray dried microcapsules showed more or less spherical shape in various sizes with wrinkles on the surface. A porous microcapsule morphology has also been observed by Karaca et al. (2013) for freeze-dried microcapsules of FO with chick pea protein and lentil protein as wall material. Eratte et al. (2015) attributed the formation of wrinkles on

the surface of spray dried microcapsules to the uneven shrinkage of the particles during the drying process associated with rapid evaporation of water.

6.3.2 Complexation between FPI and FG

The infra-red spectra of the individual wall materials (FPI and FG), core material (original FO) and the microencapsulated product are shown in Fig 6.3. The spectra of microcapsules produced at different core-to-wall formulations were found to be similar, therefore only one spectrum (at core-to-wall ratio of 1:2) is presented.

In case of FPI the peak appearing at approximately 3300 cm^{-1} is the characteristic of the amine groups (N-H stretch) which are positively charged at a pH below isoelectric point of protein (Comunian, et al., 2013). In the spectrum of flaxseed gum, the high intensity peak that appeared at approximately 3200 cm^{-1} is actually the emergence of overlapping of O-H stretching (3500-2900 cm^{-1}) and C-H vibrations (2900-2950 cm^{-1}) arising from the negatively charged carboxylic groups present (Cui et al., 2007). During complex coacervation, the amino groups of FPI interact with the carboxyl groups of FG to form a complex containing an amide bond. The characteristic peaks of amides in the spectrum of microcapsules can be visualised at 1632 cm^{-1} (Amide I) and 1552 cm^{-1} (Amide II) confirming the formation of coacervates. Additionally, a significant reduction in the intensity of the peak corresponding to O-H bond (3500-2900 cm^{-1}) and complete elimination of characteristic peak for the amine group (at 3300 cm^{-1}) in the microcapsules spectrum, suggests that the available functional groups on the encapsulating agents had become involved in bonding. Many recent studies on the complex coacervation between protein and polysaccharide have reported similar observations through FTIR (Comunian et al., 2013; Santos et al., 2015). However, the observation of characteristic galacturonic acid peak (1038 cm^{-1}) (though in reduced intensity) in the spectrum of microcapsules, suggested that some of the galacturonic

acid present in FG did not participated in complex formation. Similar observation was reported by Rocha-Selmi et al. (2013) for gum Arabic while studying complex coacervation between gelatine and gum Arabic.

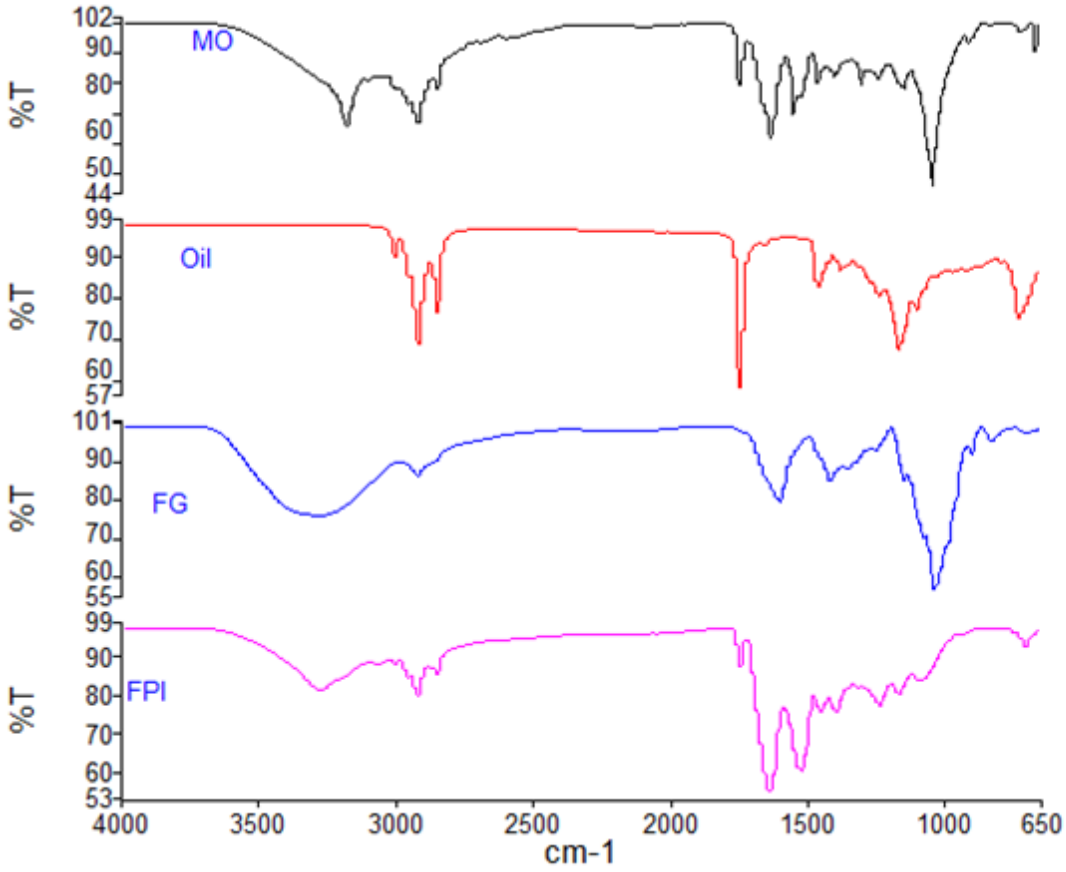

Figure 6.3 FTIR spectra of main ingredients and microcapsules. MO = Microencapsulated oil, FG = Flaxseed gum, FPI = Flaxseed protein isolate

6.3.3 Effect of core-to-wall ratio and drying methods on the physicochemical characteristics of the microcapsules

The FO microcapsules produced by complex coacervation followed by freeze drying or spray drying were characterized in terms of solid yield, moisture content, water activity, SO, TO and ME (Table 1). Solid yield for freeze dried microcapsules (93.05% - 94.26%) were significantly higher ($p<0.05$) than that of spray dried microcapsules (35.46% - 52.60%). The low recovery of powder in spray drying operation was attributed the loss of 'fines' which

were not collected in the cyclone. A portion of the microcapsule powders was lost due to wall deposition as the drying chamber was small (inner diameter = 165 mm). In the case of spray dried microcapsules (SDM), the yield of powder increased with the decrease in the oil content as core to wall ratio changed from 1:2 to 1:4. Similar trends were observed by Tan et al. (2005) for spray drying of fish oil microcapsules.

Table 6.1 Physicochemical properties of freeze dried and spray dried microcapsules at different core to wall ratio.

	Freeze dried			Spray dried		
(%)	1:2	1:3	1:4	1:2	1:3	1:4
Moisture	4.47 ± 0.20^{c}	4.37 ± 0.09^{c}	4.18 ± 0.15^{c}	3.70 ± 0.13^{b}	3.34 ± 0.10^{a}	3.20 ± 0.08^{a}
Water activity	0.323±0.003	0.313±0.018	0.286±0.008	0.272±0.004	0.251±0.008	0.241±0.008
Yield	93.05 ± 2.87^{d}	94.26 ± 3.51^{d}	93.57 ± 3.14^{d}	35.46 ± 3.68^{a}	45.26 ± 3.06^{b}	52.60 ± 3.24^{c}
Surface oil	19.40 ± 1.26^{d}	14.26 ± 1.07^{c}	8.26 ± 0.95^{b}	6.78 ± 0.56^{b}	4.64 ± 0.82^{a}	2.76 ± 0.87^{a}
Total oil	46.74 ± 2.14^{c}	30.45 ± 3.07^{b}	$24.47\pm2.63^{a,b}$	$25.52\pm1.13^{a,b}$	23.85 ± 1.11^{a}	22.27 ± 1.83^{a}
Encapsulation efficiency	58.49 ± 3.36^{a}	66.3 ± 3.48^{b}	67.06 ± 2.72^{b}	$73.43\pm3.26^{b,c}$	80.54 ± 2.68^{c}	87.6 ± 3.06^{c}

Note: The numbers in a row with different superscripts are significantly ($p<0.05$) different.

The moisture content varied from 3.20% to 3.70% and 4.07% to 4.19% (w/w) for SDM and FDM respectively and water activities ranged from 0.241 to 0.323. Most of these results were within the maximum moisture specification for dried powders in the food industry which targets 3-4% (Klinkesorn et al., 2006).

Microencapsulation efficiency is the actual amount of oil which is encapsulated inside the matrix. As shown by equation (1), ME was calculated based on SO and TO contents. Surface oil content represents the unencapsulated oil found on the surface of the microcapsules, which is more prone to oxidation than encapsulated oil. In the current study microcapsules produced by spray drying had lower SO content and higher ME than those produced by freeze drying irrespective of the core to wall ratio. Similar comparisons of drying methods were reported by Anwar and Kunz (2011) and Quispe-Condori et al. (2011). The maximum microencapsulation efficiency obtained in spray drying (87.60%) was significantly higher ($p<0.05$) than that obtained in freeze drying (67.06%). This may be attributed to the porous structure in freeze dried solid microcapsules which will be explained in the next section.

The variation in the concentration of oil or wall material also influenced the SO and TO contents and efficiency of encapsulation. The oil on the surface of SDM was lowest (2.76%) at a low core-to-wall ratio (1:4). When the amount of wall material decreased and the core-to-wall ratio (1:3 and 1:2) increased, the SO content increased up to 6.78% indicating there was insufficient wall material to encapsulate the oil. Similar trends were observed in the FDM where the SO varied from 8.26% to 19.4% with when the ratio increased from 1:4 to1:2. The TO content also followed the same trend in both SDM and FDM samples. In contrast, the ME followed the opposite trend as it is inversely proportional to the SO content (equation 1). Moreover, as suggested by Gharsallaoui et al. (2007), ME increases with the increase in the wall material content due to better coating of oil droplets.

6.3.4 Oxidation stability of microcapsules

6.3.4.1 Peroxide value

The oxidation patterns of original and encapsulated FO stored for a period of 30 days are illustrated in Fig 6.4. The first measurement of PV was made on the 5^{th} day after the

production of microcapsules because of the time needed to extract oil. The PV of original flaxseed oil, on the 5th day, was 3.18 meq active O_2/kg while that of FDM ranged from 3.25 - 3.33 meq active O_2/kg and that of SDM ranged from 2.85 to 3.31 meq active O_2/kg; with no significant difference ($p>0.05$) observed between different core-to-wall formulations or drying methods. This observation indicates that the emulsification, homogenisation and encapsulation processes did not negatively impact oil stability which is in agreement with the reports of Karaca et al. (2013) and Martinez et al. (2015).

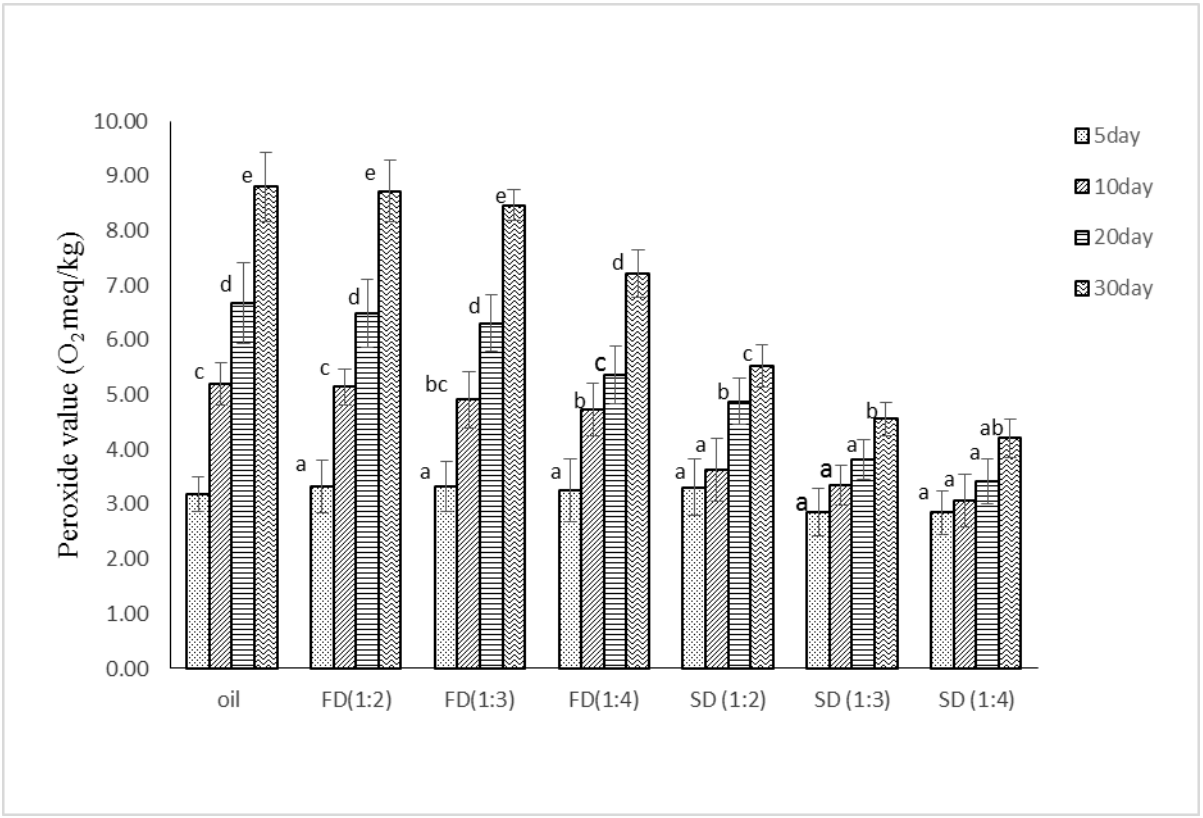

Figure 6.4 Changes in peroxide value (PV) of freeze dried and spray dried microcapsules at different core-to-wall ratios. FD = freeze dried, SD= spray dried. Data represented means ± standard deviation (n=3).

In the succeeding storage stage (10-30day), freeze dried and spray dried microcapsules having different core-to-wall ratios followed a similar oxidation pattern. However, the rate of

oxidation and extent of oxidation in FDM was significantly ($p<0.05$) higher than that of SDM irrespective of the oil load. Additionally, the overall storage data of 30 days showed that PV for original FO varied from 3.18 - 8.80, for FDM varied from 3.25-8.72 and for SDM varied from 2.85-5.52. These data points indicated that freeze drying was unable to provide better oxidative in microcapsules compared to that in the original FO (based on 8.80 and 8.72, $p>0.01$). Conversely, oxidation stability of FO was significantly ($p<0.05$) improved when the liquid microcapsules were spray dried. Eratte et al. (2014) also reported that fish oil microcapsules produced by spray drying had better oxidation stability than those produced by freeze drying. In contrast, Liu et al. (2010) encapsulated FO in gelatin–gum Arabic matrix and after freeze drying and demonstrated a significant reduction in oil oxidation, based on PV and p-anisidine values when compared to the free oil. Similarily, Grattard et al. (2002) reported that freeze-drying of FO encapsulated in a maltodextrin–lecithin–xanthan gum matrix, provided good protection to the entrapped oil against oxidation. The poor stability of FDM observed in the current study is related to the higher SO content and lower encapsulation efficiencies of FDM when compared to SDM. As explained above, surface oil triggers the oxidation and forms the primary oxidation products in the microcapsules.

The variation of oil load in microcapsules having different core-to-wall ratio also affected the oxidative stability of the encapsulated oil. This effect was more pronounced in FDM than in SDM. This is clearly demonstrated by the steady rise ($p<0.05$) of PV value in FDM after 5 days of storage microcapsules with increasing oil content and core-to-wall ratio (1:4 to 1:2). In the case of SDM, the increase in PV was not significant ($p>0.05$) for the first 10 days of storage in all the core-to-wall ratios. However, after 10 days of storage the PV value of formulation with the ratio of 1:2 increased significantly $p<0.05$); however, the PV values of formulations with the ratios of 1:3 and 1:4 remained stable up to 30 days. The data suggests that stability of microcapsules is greatly influenced by the amount of oil load in the

microcapsules. Tonon et al. (2011) reported earlier that higher oil concentration in microcapsules leads to higher lipid oxidation due to lower encapsulation efficiency.

6.3.3.2 Oxidation stability index

The oxidation stability index (OSI) values of both freeze dried and spray dried solid microcapsules with different core to wall ratios are presented in Fig 6.5. The original FO was used as a control for comparison. All the spray dried FO microcapsules had higher stability against oxidation (higher OSI values) than freeze dried FO microcapsules.

Two out of three spray dried formulations (core-to-wall ratios of 1:3 and 1:4) were found to be significantly stable ($p < 0.05$) against oxidation compared to the original FO and freeze dried microcapsules. This can be attributed to the less porous skin-like surface of spray dried microcapsules as compare to the freeze dried microcapsules as explained in section 3.1.2. Moreover, the lower SO content of spray dried microcapsules aids to their higher stability. Specifically, in spray died microcapsules the formulation with core-to-wall ratio of 1:4 exhibited the highest stability against oxidation followed by the formulation with the ratio of 1:3. Both of these formulations had significantly higher ($p<0.05$) stability than the original FO. Interestingly, the spray dried formulation with high core-to-wall ratio (1:2) showed oxidative stability poorer than that of the oil. These results clearly indicate that there is a significant decrease in the oxidative stability of microcapsules with the increase in the oil load core-to-wall ratio. In this particular case, it appeared that when the core-to-wall ratio was 1:2 the amount of wall material was insufficient to encapsulate the high oil content (33.34 % of total solids). Similar observations were reported earlier by Gallardo et al. (2013) when lower fish oil encapsulation efficiency was observed for oil load higher than 25%. It can be observed that all the microcapsule formulations with a SO content higher than 6.78% (Table 1) exhibited poorer oxidative stability when compared to the unencapsulated flaxseed oil irrespective of the method of drying. This observation is also corroborated by the PV values.

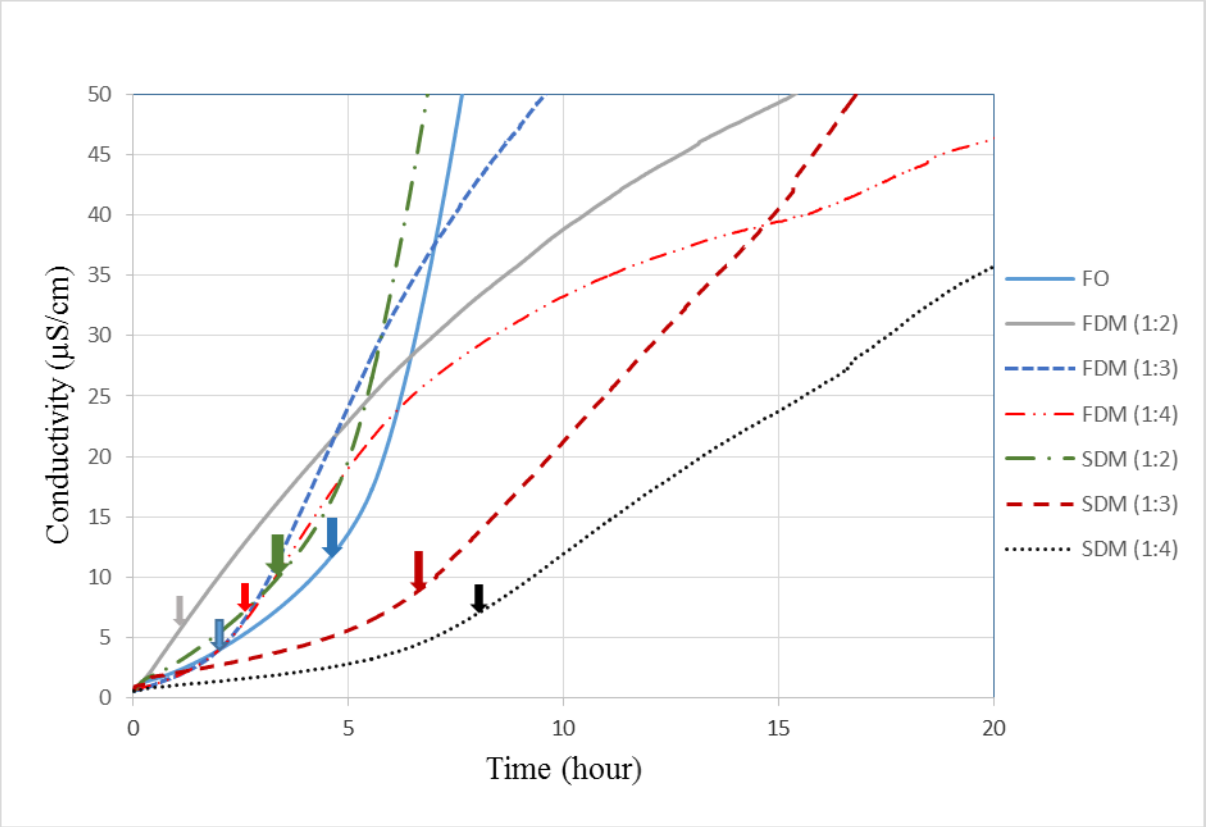

Figure 6.5 Oxidative stability index (OSI) of freeze dried and spray dried microcapsules as determined by RancimatTM . FO = Flaxseed oil, FDM = Freeze dried microcapsules, SDM= Spray dried microcapsules

Hence, the oxidation study of microencapsulated and original FO, indicated that FO microcapsules produced by freeze drying due to their porous structure and higher surface oil content exhibited poor oxidation stability during storage irrespective of the oil load. While, the FO microcapsules produced by spray drying in which the oil load was less than or equal to 25% (based on total solids) were stable against oxidation for 30 days.

6.4. CONCLUSIONS

Flaxseed oil was microencapsulated in flaxseed protein isolate (FPI)-flaxseed gum (FG) complex coacervate matrix consolidated by maltodextrin and glutaraldehyde. Three different formulations of core-to-wall ratios (1:2, 1:3 and 1:4) were studied. Liquid microcapsules of

flaxseed oil were dried through spray drying and freeze drying. The flaxseed oil microcapsules prepared by spray drying (SDM) and freeze drying (FDM) exhibited significantly different physicochemical, morphological, structural and storage properties. The SDM had higher microencapsulation efficiency and lower surface oil content compared to that of FDM. FDM was not protected by the wall material against oxidation due to the porous structure of the microcapsules. SDM were found to be protected against oxidation for up to 30 days for oil load up to 25%. This study concludes that the crosslinked FPI-FG complex coacervates consolidated by maltodextrin can effectively microencapsulate flaxseed oil, and the solid microcapsules produced using spray drying can give a low surface oil (<3%), high encapsulation efficiency (~87%) for an oil load of 20%. These findings suggest that application of flaxseed oil can be increased in food formulations by encapsulating it in crosslinked FPI-FG complex coacervates consolidated by maltodextrin.

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Chapter 7

Concluding remarks

The overarching goal of this research was to microencapsulate flaxseed oil (FO) in a flaxseed protein isolate (FPI) and flaxseed gum (FG) based matrix formed by complex coacervation. For this purpose, FPI and FG were extracted from flaxseeds, characterized and evaluated for their emulsification and functional properties. Subsequently, both flaxseed protein and gum were combined at optimized conditions to form complex coacervates and FO was microencapsulated in the composed matrix. Finally, the characteristics of microencapsulated FO were determined.

7.1 Extraction of FPI and investigation of its functional properties

Flaxseed protein with its unique amino acid profile has gained prominence as a potential ingredient for the food industry. Flaxseed protein contributes 18-22% of seed weight, but this significant protein contribution is adversely affected by the interference of mucilage during extraction in terms of both the recovery and purity of protein. To assess and minimise the interference of mucilage and increase the recovery, extraction of flaxseed protein was carried out at four different temperatures (30, 40, 50, 60 ^{o}C). The recovery of extracted protein was not significantly affected by the temperature change. However, the purity of the FPI dramatically increased with the rise in temperature, and 90.6 % pure FPI was extracted at 60 ^{o}C.

Physicochemical characteristics, amino acid profile and functional properties of FPI were evaluated by comparison with commonly used proteins such as sodium caseinate (SC), whey protein isolate (WPI), gelatin (Gel) and soy protein isolate (SPI). The essential amino acid profile of FPI was similar to those observed in the above mentioned proteins. The higher arginine and cysteine content of FPI when compared to many other proteins commonly used in the food industry increases its applicability for nutritional supplements. The maximum

solubility of FPI at room temperature and pH 9.0 was found as 75%, which is lower than most of the plant proteins such as soy protein, pea protein and hemp seed protein. The onset and peak denaturation temperatures of FPI were found as 83.4 °C and 105 °C, suggesting that it is a relatively thermally stable protein.

In terms of emulsifying properties, FPI had the highest emulsion activity index (375.51 m^2/g), emulsion stability index (179.5 h) and zeta potential (-67.4 mV) compared to those of other commonly used proteins such as sodium caseinate (SC), whey protein isolate (WPI), gelatin (Gel) and soy protein isolate (SPI). The average emulsion droplet size of emulsions stabilized by these proteins was in the order SC<FPI<WPI<Gel<SPI. The functional properties of FPI such as WHC and FAC were found to be higher than those of the proteins mentioned above. The higher WHC of FPI was attributed to the mucilaginous impurities of FPI and higher FAC was attributed to the presence of a higher proportion of non-polar amino acids as compared to other proteins. The results obtained in this study suggest that FPI-stabilized emulsions can be more electrostatically stable at lower pH than those stabilised with other proteins. This indicates that FPI-stabilised emulsions will be useful for the controlled/targeted delivery of oils soluble nutrients in the intestine, due to their higher stability in high acid environments.

These results fulfilled the first objective of the research work and provided useful information about extraction conditions and functional characteristics of FPI. Similar information about the extraction conditions and functional characteristics of FG are required before carrying out complexation between FPI and FG.

7.2 Extraction of Flaxseed Gum (FG) and investigation of its functional properties

Gum from flaxseed is another nutritionally important component and its functional properties are reported as comparable with gum Arabic. Hence, flaxseed gum can be used in place of gum Arabic for the encapsulation of active ingredients. In this study, it was found that

extraction temperature plays an important role in the separation of FPI and FG as individual components. Hence, before practical application of FG as a wall material in combination with FPI it is important to study the effect of extraction temperature on its composition and functional properties.

Analysis showed that yield, carbohydrate content and protein content of the extracted gum was significantly affected by the temperature ($p < 0.05$). The yield of FG dramatically increased from 2.1 to 8.4 % (of seed weight) with increasing extraction temperature from 30-90 ^{o}C ($p < 0.05$). In contrast, the purity of the gum decreased with the rise in extraction temperature. A possible explanation for this result is the increased contamination of gum with water soluble protein extracted at higher temperatures, which consequently increased the yield but decreased the purity of extracted gum. It was further noted that the content of acidic monosaccharides and denatured protein increased in the FG that was extracted at higher temperatures. Also, the ratio of neutral to acidic monosaccharides increased from 2.3 to 2.6 as the extraction temperature was increased from 30 to 90 °C.

Physicochemical and functional properties such as zeta-potential, surface morphology, emulsifying activity index (EAI) and emulsion stability index (ESI), water absorption capacity (WAC) and fat absorption capacity (FAC) of FG samples, as a function of extraction temperature, were also considered. The isoelectric point of FG samples increased from a pH 1.7 to pH 2.0 as the temperature of extraction increased from 30 to 90 °C. Some of the functional properties of FG such as EAI and WAC were also negatively affected with the rise in extraction temperature. Interestingly, the FAC of FG sample extracted at higher temperatures (70, 90 °C) was found to be higher than the sample extracted at lower temperatures (30, 50 °C). The significance of this finding is that FG extraction temperature can be tailored for targeted applications. FG extracted at lower temperatures is better for

emulsification and water holding (to prevent synersis) applications, whereas FG extracted at higher temperature can be applied to food systems where high oil absorption capacity is required.

Findings from this portion of the study fulfilled the second objective of the research work. The fundamental knowledge gained on FPI and FG extraction and characterization, aids in the design of studies to enable successful complexation to form coacervates. It also establishes the versatility of the process to customise out for specific industry needs.

7.3 Complex Coacervation between FPI and FG

After extraction, characterization and investigation of functional properties of both FPI and FG, the logical next step involves combination of both polymers to form a matrix for encapsulation of FO. Hence, electrostatic complexation between these two biopolymers was studied as a function of pH and FPI-to-FG ratio using turbidimetric and electrophoretic mobility (zeta potential) tests. Changes in turbidity (measured as optical density (O.D.) during an acid titration were recorded for individual FPI and FG solutions and their mixtures at the FPI-to-FG ratios of 1:1, 3:1, 5:1, 10:1, 15:1 over a pH range 8.0-1.5. According to the turbidity tests, the optimum FPI-to-FG ratio and pH value for complexation between FPI and FG were 3:1 and 3.1, respectively. These results were further confirmed by the electrophoretic mobility measurements of FPI and FG solutions and their mixtures at the same ratios as mentioned above.

The structural mechanism behind the complexation of FPI and FG was found by mapping the conformational changes in protein secondary structure as a function of pH. It was found that with the lowering of pH from 8.0 to 3.0, the proportion of α-helical structure decreased, whereas the amount of both β-sheet structure and random coil increased. The acidic pH

affected the secondary structure of FPI and the unfolding of helix facilitated the complexation of FPI with FG.

The critical pH associated with the formation of soluble (pHc) and insoluble ($pH_{\phi 1}$) complexes at the optimum FPI-to-FG ratio were found to be 6.0 and 4.5, respectively. The optimum pH (pH_{opt}) for optimal complex coacervation was 3.1. The instability and dissolution of FPI-FG complex coacervates started ($pH\phi_2$) at pH 2.1. These findings contribute to the development of FPI-FG complex coacervates as a delivery vehicle for unstable, albeit essential, nutrients such as omega-3 fatty acids and fulfilled the third objective of this research study. The next step involved the evaluation of application of FPI-FG coacervates for encapsulation of FO.

7.4 Microencapsulation of FO in complex coacervates of FPI and FG

After identifying the optimum conditions for carrying out the complex coacervation of FPI and FG, oil from flaxseed was microencapsulated in the novel biopolymers matrix. This matrix was consolidated by crosslinking with glutaraldehyde and adding maltodextrin. Liquid microcapsules with three core (oil)-to-wall ratios (1:2, 1:3 and 1:4) were prepared and spray dried or freeze dried to produce powders. Different characteristics of solid microcapsules such as microencapsulation efficiency, surface oil, surface morphology and oxidative stability were determined. Determination of these properties is essential for further application of microencapsulated FO in the food industry.

The powder yields of liquid microcapsules obtained from the two drying processes were significantly different. The freeze drying process produced more than a 90% yield in all formulations. Whereas, in the case of spray drying, the maximum yield obtained was 52% and it decreased significantly with the rise in oil load. The reason for this difference was the different operational parameters (temperature and pressure) and different equipment used in

the two processes. In terms of surface morphology, the freeze dried microcapsules appeared to have a lumpy and uneven surface structure with pores or cavities on the surface, while the spray dried microcapsules showed more or less spherical shape in various sizes with folds on the surface.

The spray dried solid microcapsules had higher oil microencapsulation efficiency and lower surface oil content than those produced by freeze drying irrespective of the core to wall ratio and hence had higher stability against oxidation. The maximum microencapsulation efficiency obtained in spray drying (87.60%) was significantly higher ($p<0.05$) than that obtained in freeze drying (67.06%). This may be attributed to the porous structure in freeze dried solid microcapsules. It was also observed that in both drying processes, an increase in the oil concentration resulted in a concomitant increase in microcapsule surface oil content and a decrease in oil encapsulation efficiency.

The main objective of microencapsulation of FO is to provide protection against oxidation. To evaluate the oxidation stability of the designed matrix of FPI and FG, the solid microcapsules were subjected to a variety of oxidation studies including Rancimat and storage oxidation using the recording of peroxide (PV) development in samples over 30 days. The storage data of PV suggested that the rate and extent of oxidation in freeze dried microcapsules (FDM) was significantly ($p<0.05$) higher than that of spray dried microcapsules (SDM), irrespective of the oil load. Additionally, the overall storage data of 30 days showed that PV for original FO varied from 3.18 - 8.80, for FDM varied from 3.25-8.72 and for SDM varied from 2.85-5.52. These data points indicate that FPI-FG matrix was unable to provide any oxidative stability to FDM. Conversely, oxidation stability of FO was significantly ($p<0.05$) improved when the liquid microcapsules were spray dried. The oxidation stability obtained from spray dried microcapsules at a core-to-wall ratio of 1:4 was

nearly double that of the unencapsulated FO. This study concludes that the crosslinked FPI-FG complex coacervates consolidated by maltodextrin can effectively microencapsulate FO, and the solid microcapsules produced using spray drying can give a low surface oil (<3%) and a high encapsulation efficiency (~87%) for an oil load of 20%. These findings suggest that the development of an effective plant based encapsulating matrix is possible and this could lead to increased utilization of FO and other bioactive ingredients in food.

7.5 Contributions and advances made in this study

This research work has made a significant contribution in food component product design to address a global dietary shortage of omega-3 fatty acids by stabilising FO, a richest plant based source of omega 3 fatty acids and provide an alternative to fish oil. Microencapsulation of FO can help increase the dietary intake of omega-3 fatty acids among various population groups which do not consume fish for a variety of reasons such as allergies, mercury related risks, ethical grounds, availability and economical conditions. In addition, the wall material matrix designed here from FPI and FG can be preferentially used as novel, vegetarian, economic and nutritionally valuable delivery vehicles for active and unstable food ingredients.

The overarching contributions made by this research are in two different domains, one in the work done on plant polymers and their utilization as encapsulates and other in the encapsulation of bioactive ingredients such as FO. This study started with temperature optimisation of extraction protocol of both flaxseed protein and flaxseed gum, followed by physicochemical characterization and finally evaluating their functional properties. This study also demonstrated that both FPI and FG have potentiality to be used as functional ingredients. Yet another contribution of this thesis is the interaction between these two biopolymers to form complex coacervates which can be used as strong wall materials after

crosslinking. This thesis has identified the phase boundaries required for the formation of soluble and insoluble complexes, optimum pH, ratio and concentration of biopolymers for maximum yield of complex coacervates. To provide a better understanding of the formation of electrostatic complexes between FPI and FG, various changes occurred in the structure of flaxseed protein as functions of pH were also explained in this study. The understanding and knowledge of these protocols and parameters will help in developing FPI-FG coacervates as potential wall materials for microencapsulation in the food industry. In addition, this study also demonstrated the practical application of FPI- FG coacervates for microencapsulation of FO through spray drying and freeze drying. Characterization of solid microcapsules done in this study helped to show which drying technique works better for plant polymers when used as wall materials. It also showed that if FO is microencapsulated in FPI-FG based matrix and spray dried at a particular oil load, its oxidative stability is at least double that of the unencapsulated oil.

In summary, findings of this study suggest that the developed plant-based microencapsulation system could lead to increased utilization of FO and flaxseed protein and gum in food and bioproduct formulations and applications.

7.6 Recommendations for further study

The food processing industry is increasingly interested in developing microencapsulated bioactive products. FO, being one of the richest plant based source of omega-3 fatty acids is in high demand and is a current focus of development. Although, FO microencapsulated in gelatin is already commercially available, there is great interest in plant based wall materials due to their nutritional benefits, the consistency of using plant based products and economical desire to use all components of the flaxseed. In this context, this study extracted the pure protein and gum from flaxseed, combined and crosslinked them and successfully used them

to microencapsulate FO. Findings of current study suggested, spray dried microencapsulated FO is stable against oxidation. Building from the current study, further research on the process of spray drying to produce powders complying with industrial specifications is required. As in process of spray drying, the resultant drying powder yield and physical and chemical properties such as bulk density, particle size, flowability, moisture content and surface morphology can be modified by changing the processing conditions.

In terms of wall material developed in this study, flaxseed protein and gum were successfully complexed and crosslinked to produce a wall material to protect FO. However, the crosslinker used in this study has no nutritional benefits. There is an increasing interest in application of plant polyphenols as crosslinker due to their unique health benefits. Hence, further work using phenols as crosslinking agent would add value to the research. Furthermore, the glass transition temperature studies of FPI-FG matrix can be used to improve their performance in spray drying as well as in providing protection against oxygen. Production of an amorphous glassy matrix is generally recommended for slowing down oxygen diffusion. Hence, high glass transition temperature for FPI- FG matrix could be used as a decision criterion for choosing the optimum formulation. A further recommendation could be the use of confocal and/or SEM, TEM microscopy to get a deeper insight in to the distribution pattern of protein and gum in outer wall formed around the oil droplets.

And finally, the performance of microencapsulated FO powder food matrices needs to be evaluated in terms of bioavailability before moving the technology to industry.