Utilization of mildly fractionated pea proteins in the development of thermally stable beverage emulsions

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By

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ABSTRACT

Mild fractionation is a non-invasive protein extraction technique that can be used to retain protein fractions of high purity and functionality. The overall aim of this research was to develop beverage emulsions using mild-fractionated soluble pea proteins with improved stability against thermal processing. Initially, soluble pea proteins were retained from a pea protein concentrate dispersion via mild fractionation using simple aqueous centrifugation. Later, high-pressure homogenized (20,000 psi for 6 cycles) 5 wt% oil-in-water emulsions were made using different concentrations of soluble proteins as the aqueous phase. After various emulsion characterization tests were carried out, 2.5 wt% of protein in the aqueous phase was concluded to be the ideal concentration of protein for optimum emulsion stability. All the emulsions for further characterization were made using 2.5 wt% of mildly fractionated soluble proteins in the aqueous phase.

It was important to test the stability of pea protein-stabilized emulsions against various environmental stresses: heat treatment at 90°C for 30 minutes, addition of 0.0 M-1 M salt, and the effect of two different pH (2 and 7). Heating caused extensive emulsion destabilization due to droplet and protein aggregation at both the pH values. The emulsions at pH 2 showed destabilization even without heating. The problem of aggregation could be due to the denaturation of proteins during heat-treatment which caused the exposure of hydrophobic groups, in turn causing emulsion destabilization.

To overcome the problem, it was hypothesized that partial denaturation of the soluble proteins by pre-heating and thereafter making the emulsions with the heated protein solutions could solve the problem of protein aggregation in emulsions after heat treatment. Therefore, emulsions were prepared at heated conditions using heat-treated (75°C) partially denatured soluble pea proteins. From the characterization tests, it was found that the heat-treated protein-stabilized emulsions at pH 7 had superior stability at all salt concentrations without any sign of extensive droplet and protein aggregation even after heating the emulsion to 90°C for 30 minutes. A similar improvement in stability was, however, not observed for the pH 2 emulsions prepared under comparable conditions.

The effect of protein pre-heat treatment on the emulsion lipid digestibility was also determined by *in-vitro* digestion tests, which showed that the unheated protein unheated emulsion showed the maximum lipid digestion of 97.51%, followed by 73.47% and 56.06% lipid digestibility for heat-treated protein heated emulsion and heat-treated protein unheated emulsion, respectively. Overall, this research showed that the pre-treatment of mildly fractionated soluble proteins could significantly improve the stability of beverage emulsions and influence the protein structure to reduce the lipid digestibility of the emulsions.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celsius
PP55	Pea protein concentrate 55
µm	Micro metre
µl	Micro litre
g	Gravitational force (9.81 m/s^2)
M	Molarity
PUFA	Poly unsaturated fatty acids
mW	Milli watt
O/W	Oil-in-water
W/O	Water-in-oil
mPa	Milli Pascal
mN/m	Milli-Newton per metre
wt %	Weight percentage
G′	Storage moduli
G″	Loss moduli
mM	Milli molar
rpm	Rotations per minute
wt%	Weight percentage
psi	Pound force per square inch
d_{32}	Droplet surface area mean
nm	Nano metre
ml	Milli litre
mg	Milli gram
J/g	Joule per gram

1. INTRODUCTION

1.1 Overview

In recent years, there has been an increase in the consumption of plant-based products due to their health benefits as well as their sustainability. Plant proteins are one of the chief ingredients in most of these novel plant-based products. They have been utilized by the food industry to develop various products, such as plant-based meats, spreadables, salad dressings and beverages, due to their functional properties such as water holding capacity, solubility, foaming ability, emulsifying properties *etc*. (Shevkani *et al*., 2019). Pulses, such as peas, lentils, and faba beans, are an abundant source of plant proteins that are consumed all over the world (Hughes *et al.*, 2011). However, before pulse proteins can be utilized as a food ingredient, they must be extracted from the pulses. There are many ways for extraction, dry fractionation (Pelgrom *et al*., 2013), wet extraction, and alkaline extraction (Kornet *et al*., 2020) being some of the most common methods. Dry fractionation helps to retain protein functionality, but the purity of the proteins is relatively low (Pelgrom *et al*., 2015). Whereas wet fractionation and alkaline extractions offer to retain highly pure protein fractions, but the functionality of the proteins is disturbed due to the harsh conditions during the extraction (Pelgrom *et al*., 2013). Therefore, it is important to develop a non-invasive mild fractionation method to retain the purity and functionality of pea protein fractions.

The overall objective of this research was to utilize mildly fractionated soluble pea proteins to develop thermally stable beverage emulsions. Initially, pea proteins were extracted through mild fractionation by centrifugation method. The soluble protein fraction obtained directly from centrifugation served as the aqueous phase of the oil-in-water beverage emulsions without any further processing of the soluble protein. The emulsions were made via high-pressure homogenization at 20,000 psi for 6 cycles. Various concentrations (0.5 to 2.5 wt%) of the soluble proteins were tested for emulsion stability. Emulsion stability was characterized by various tests, including droplet size, zeta potential, creaming velocity and degree of flocculation. These tests led

to the conclusion that 2.5 wt% of protein in the aqueous phase was ideal for emulsion stability, which was then utilized in further emulsion studies to understand stability against various environmental stresses such as heat treatment (90°C for 30 minutes), salt addition (0.0 M to 1 M) and change in pH (2 and 7). Heat treatment led to the destabilization of emulsions which was a major issue to be resolved. It was hypothesized that, pre-heating the soluble proteins before making the emulsions might prevent aggregation. Hence, the soluble proteins were heated to 75°C before making the emulsions. The emulsions were later characterized for average droplet size, zeta potential, creaming velocity, viscosity and confocal microscopy. Finally, *in-vitro* digestion was done to understand whether protein heat pre-heat treatment had any influence on the lipid digestibility of the emulsions.

This thesis study illustrated the possibility of utilizing mild fractionation as an effective method to extract soluble proteins that have the characteristics required for the stabilization of beverage emulsions. It was also found out that the soluble proteins extracted via mild fractionation could be directly used with minimal heat treatment for enhanced emulsion stability. The soluble protein does not have to be extensively processed via freeze-drying or spray-drying before making the emulsion. Therefore, effective utilization of the pea protein for the development of beverage emulsions has been demonstrated in this research.

1.2 Objectives

To achieve the overall research goal four objectives were developed.

1. To extract functional pea proteins by centrifugal separation-based mild fractionation of pea protein concentrate.
2. To characterize the mildly fractionated soluble proteins to understand the protein structure and functionality, which are prerequisites for their effective utilization.
3. To develop highly stable oil-in-water emulsions using the soluble proteins extracted via mild fractionation and assess the stability of these emulsions under various environmental stresses.
4. To improve emulsion stability under heat-treatment by pre-heat treating the soluble proteins to partially denature and making the emulsions at heated condition.

1.3 Hypothesis

The following hypothesis will be tested to support the above objectives.

1. Mild fractionation would be an ideal extraction method to extract highly functional proteins as the extraction takes place in non-invasive conditions. As mild fractionation is carried out at native pH of the concentrate and does not involve any harsh chemicals, it helps to preserve the functionality of the protein.
2. The proteins extracted via this method could be directly used to develop highly stable oil-in-water emulsions due to the enrichment of functional soluble proteins than the original pea protein concentrate.
3. Heat treatment of the emulsions will lead to structural changes in the protein, causing the emulsion to destabilize.
4. Partial denaturation of the soluble protein will help in improving the emulsion stability and prevent the damage to the emulsion structure caused due to heat treatment. When the protein is partially denatured, it exposes the hydrophobic patches which are better attracted to lipid droplets when compared to native proteins and offer better stabilization to emulsions than unheated proteins.

2. LITERATURE REVIEW

2.1 Pulses – overview and composition

2.1.1 Introduction to pulses

Pulses, such as pea, lentil and faba bean, play an important role in fulfilling our nutritional requirements due to the starch and protein components present in them. Soybeans and peanuts are not considered as pulses because they contain higher amount of fat and generally regarded as oil yielding crops (Singh, 2017). They have been consumed for centuries as a staple food in many countries such as India, China, and many other eastern countries. Pulses can be consumed to fulfil protein requirements in vegan and vegetarian diets and serve as an alternative to animal proteins (Hall *et al*., 2017).

The earliest evidence of humans growing pulses dates to the 7th century BC when lentils and chickpeas were grown in Fertile Crescent, a region in the Middle East (Kislev *et al.* 1988). Today, pulses are grown in almost all parts of the world. The main producers of pulses include India, Canada, Myanmar, China, Brazil, Australia, USA, Russia, and Tanzania (Akibode & Maredia, 2012). The most common pulses, including pea, lentil, faba bean, and chickpea, are grown in Canada. In 2011, the pulse seeded areas were 2.2 million hectares in Canada (Bekkering, 2014). Canada is the top exporter of lentils and chickpeas in the world trade (Maphosa & Jideani, 2017). In 2011, Saskatchewan was the largest pulse-growing region in the country with 1.7 million hectares dedicated to pulse crop production (Bekkering, 2014).

One of the most widely used pulses is pea. Peas are spherical seeds which present in the fruit (pod) of *Pisum sativum*, each pod contains about 5-7 seeds which are either yellow or green in color. The pods are considered as fruits because they develop from the ovary of the flower and contain seeds (Nene, 2006). Lentil (*Lens esculenta*) is an annually grown plant known for its

lens-shaped seeds. Usually, one seed is present per pod which is developed from aerial flowers. It is consumed all over the world and referred by several names such as mercimek, daal, paripappu, hiramame etc. in different countries (Nene, 2006). Fababean (*Vicia faba*) has many different varieties, including field bean and tick bean which are also fed to horses and other animals. They are also seeds which are present in the pods. They are grown as cover crops because they fix atmospheric nitrogen making the soil more fertile (Doughty 1982). Chickpeas (*Cicer arietinum*) are mostly consumed in Middle East regions (Nene, 2006). There is usually a single seed present in a pod which develops from the stem of the plant. The chickpeas are used in different dishes such as chana masala in India and hummus in Middle Eastern region. It is also ground into flour to make falafel.

2.1.2 Composition and nutritional quality of pulses

Generally, pulses contain about 45%-55% starch, 20%-30% protein, 20%-25% dietary fiber and 2%-7% lipids. They are a rich source of protein, high in fiber and low in fat. Most of the pulse protein consist of water-soluble albumin (10-20%) and salt-soluble globulin fractions (about 70%) (Schaafsma, 2000). It can be observed that pulse composition mainly consists of carbohydrates followed by protein, making pulses one of the richest sources of plant protein. The compositions given in Table 1 are general values and vary depending on different cultivars of the plants.

Table 1: Dry composition of different pulses (Adopted without modification from Asif *et al*., 2013)

Name	Crude Protein (%)	Lipid (%)	Carbohydrate (%)	Crude Fibre (%)	Minerals (%)
Garden beans	24.1	1.8	65.2	4.5	4.4
Chickpeas	22.7	5.0	66.3	3.0	3.0
Lentils	28.6	0.8	67.3	0.8	2.4
Peas	25.7	1.6	68.6	1.6	3.0

The quality of the protein is determined by PDCAAS method (protein digestibility- corrected amino acid score), which evaluates the protein's ability to provide adequate essential amino acids for human needs (Hughes *et al*., 2011). PDCAAS has been adopted by FAO/WHO as the preferred method for the measurement of the protein value in human nutrition. From such an evaluation, it appears that dairy proteins are superior to plant proteins (Schaafsma, 2000). The protein with maximum quality has a PDCAAS of one. Dairy proteins casein, egg white proteins, and isolated soy proteins have a PDCAAS value of one. Whereas the PDCAAS of pulse proteins approximately lies from 0.5 to 0.6. Some of the PDCAAS values of different pulse proteins are given in Table 2.

Table 2: PDCAAS of black beans, chickpeas, lentils, and peas (adopted and modified from Hughes *et al*., 2011).

Pulse Names	PDCAAS
Black Beans	0.53
Chickpeas	0.52
Lentils	0.63
Peas	0.64

Pulses are an excellent source of good quality protein that is generally rich in many essential amino acids. It is also a good source of lysine which is a limiting amino acid in cereals. However, pulse proteins have methionine, cysteine and cystine as well as tryptophan in limiting quantities making them an incomplete source of protein (Rebello *et al*., 2014). Pulses and cereals complement each other in terms of protein quality, which can be significantly improved when pulses are blended with cereals. For better nutritional balance, pulses and cereals are recommended to be consumed in a ratio 35:65 (Maphosa & Jideani, 2017). According to a recent study, the addition of pulses to either wheat or rice increases the overall PDCAAS values to 0.71 (wheat + pulses) and 0.75 (rice + pulses) in the blends.

Table 3: Amino acid composition (g/16g of nitrogen) of pulses (tyrosine and phenylalanine reported together as phenylalanine, cysteine and methionine reported together as methionine) (adopted and modified from Hall *et al*., 2017).

Amino Acid	Black Beans	Lentils	Chickpeas	Peas
Alanine	NR	4.2	4.4	4.5
Arginine	6.7	7.2	10.3	7.9
Aspartic Acid	NR	11.3	11.4	11.9
Glutamic Acid	NR	15.1	17.3	16.5
Glycine	NR	3.9	4.1	4.5
Histidine	2.9	2.8	3.4	2.8
Isoleucine	4.1	4.6	4.1	4.9
Leucine	7.7	7.2	7.0	7.5
Lysine	6.7	6.8	7.7	7.7
Methionine	2.0	2.9	1.6	3.3
Phenylalanine	5.1	7.8	5.9	8.1
Proline	NR	3.8	4.6	4.2
Serine	NR	4.3	4.9	4.1
Threonine	3.1	3.6	3.6	3.8
Tryptophan	NR	0.7	1.1	0.9
Valine	4.9	5.0	3.6	5.2

NR: Not reported

2.2 Pulse protein extraction

Pulse proteins are usually separated and enriched from the original pulse which are utilized for further development of many food products. Pulse proteins are a popular food ingredient and generally used in the development of a variety of food products including plant-based meat alternatives, plant-based whipped creams, plant-based yogurts and most vegan alternatives. This makes pulse protein extraction a critical process. Different protein extraction methods have been developed based on the desired quality and purity of the proteins and the requirements of the end product. Protein extraction is important before utilizing them in food products because of the other components present in the pulse flour, such as starch and lipids, which can negatively influence the structure of the end product. Generally, protein extraction from pulses involves two different methods, dry fractionation, and wet fractionation (Pelgrom *et al.*, 2013). Less invasive methods, such as mild fractionation, have been recently used to preserve the functional properties of the extracted protein, which might have a better application in emulsion-based food product development.

2.2.1 Dry fractionation

Dry fractionation is a density-based separation method which separates the protein and starch-based on density differences. It is a non-invasive method that helps to preserve the functionality of the retained protein fraction (Boye *et al*., 2010). An impact mill or a jet mill can be used for dry fractionation of pulses (Pelgrom *et al*., 2013). In impact milling, the size reduction of pulses is due to the collisions between the powder particles and the mill wall. Whereas, in jet milling, the size reduction is due to inter-particle collisions (Létang *et al.,* 2001). The speed of the mill generally determines the time of milling and the energy consumed. By impact milling and jet milling at various classifier speeds, pulses can be ground into flour with different particle size distribution. Subsequently, air classification is applied to separate the lighter protein-rich fraction from the heavier starch-rich fraction to obtain protein concentrates. The product of dry fractionation is generally called protein concentrate which usually have a protein concentration of 51-55 wt% (Pelgrom *et al*., 2013). This method is found to be suitable for legume crops low in fat, such as field pea and common bean (Schutyser & van der Goot, 2011). Flours are first fractionated into starch-rich and protein-rich concentrates. The starch-rich fraction is re-milled

to separate the remaining protein from the starch-rich fraction. Usually, dry fractionation results in protein fractions of less purity and more functionality, whereas wet fractionation is utilized for retaining high purity protein fractions.

2.2.2 Wet fractionation

In wet fractionation, pulse flours are dissolved in an aqueous phase with appropriate ionic strength and pH before the proteins are extracted. It usually involves the following steps (Schuster-Gajzágó, 2011): 1) preparing a flour suspension, 2) extraction by solubilizing the proteins under alkaline conditions, 3) isoelectric precipitation, 4) re-dispersion, and 5) spray drying. Wet fractionation has the advantage of extracting isolates that are relatively pure in proteins (>90 %). The disadvantages are the enormous amounts of water and energy associated with the extraction of the pure isolates. Another disadvantage is that the harsh conditions adopted during the extraction process are detrimental to the functionality of the final product (Schuster-Gajzágó, 2011). A typical procedure for wet fractionation process is described in Figure 1 which involves the centrifugation process, isoelectric precipitation and re-dispersion to obtain the isolate.

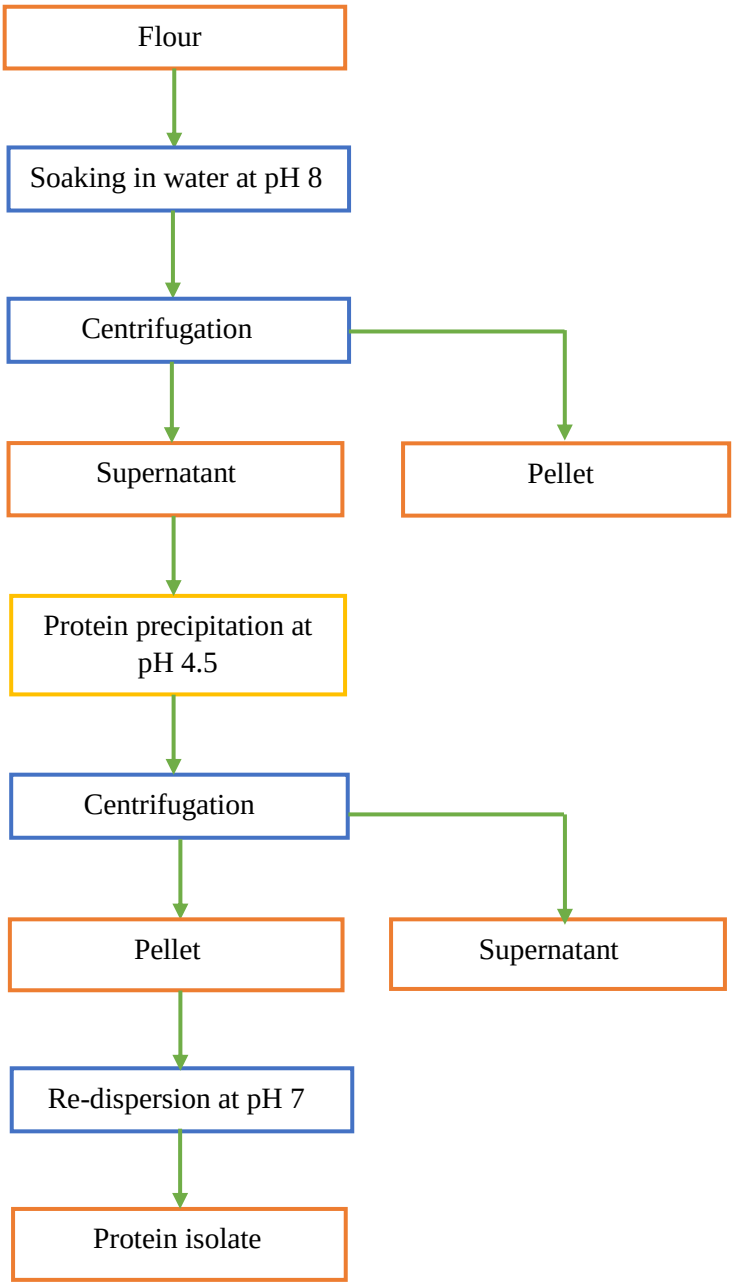

Figure 1: Schematic flow diagram explaining the procedure of wet fractionation of pulses for the extraction of protein isolate. The figure has been modified from Kornet *et al.* (2020).

2.2.3 Mild fractionation

Conventional fractionation methods focus on improving purity of pulse protein ingredients. Dry fractionation yields somewhat lower contents of proteins whereas wet fractionation yields a high purity of proteins as discussed above. The difference in the fractionation also results in different functional quality. The dry fractionation does not influence the functionality, whereas wet fractionation generally degrades the functionality through its harsh fractionation conditions. Loss of functionality is a major cause of concern which gives scope to create fractionation methods which are mild and preserve the quality and functionality of proteins. Pelgrom *et al*. (2015) described a fractionation method for yellow peas, which followed mild processing steps with limited usage of chemicals. Here, a yellow pea flour was obtained by milling the flour which was then mildly fractionated by centrifugation. Upon centrifugation, the top layer was enriched with protein and the bottom was rich in starch. This process did not involve harsh physical and chemical conditions, the purity levels of protein via mild fractionation did not match the purity levels of those obtained via conventional methods but it helped preserve the functionality of the proteins. Geerts *et al*. (2017) observed that emulsions made with mildly fractionated protein had similar droplet sizes when compared to the emulsions made with commercial protein isolate. Moreover, the emulsions made with mildly fractionated proteins had an added advantage of being stable upon freeze-thaw treatment which was not observed in the emulsions made with commercial protein isolate. Preserving functional properties is an important aspect to be considered while creating emulsions. Therefore, it is important to understand and consider the effect of extraction methods on the functional properties of the proteins before creating emulsions.

2.3 Pulse proteins as a value-added food ingredient

2.3.1 Functional properties of pulse proteins

In recent years, the development and application of plant proteins have drawn increasing scientific and industrial interests. Pulse protein components are generally considered hypoallergenic, and many studies have highlighted the health benefits associated with their consumption. Apart from pulse protein's nutritional benefits, they also have many functional properties such as solubility, foaming ability, oil absorption capacity, gelation, and emulsifying ability. These properties of different proteins determine their demand and usage in the food

industry, which in turn control the physical, chemical and organoleptic properties of foods and beverages. The functional properties of the proteins may change according to different processing conditions. It is important to remember that the functional properties of the protein are important in many cases than the purity of the protein. When we aim for obtaining pure forms of protein, the purifying procedures may involve extreme temperature or extreme pH conditions, which might deteriorate the functionality of protein (Geerts *et al*., 2017). In the sections below, the significance of a few functional properties of pulse proteins are discussed.

Solubility: Solubility is the amount of protein in a sample that dissolves in a solution under specific conditions. Protein recommended food additives can be partly or completely soluble or completely insoluble (Zayas 1997). The solubility of the protein varies at different pH conditions and temperatures. Several studies show that pea vicilin showed higher emulsifying properties than pea legumin. This was attributed due to higher solubility and surface hydrophobicity of vicilin proteins. Solubility of vicilin proteins depend both on the source and their protein concentration (Singhal *et al*., 2016). In one of the studies done by Keivaninahr *et al*. (2021) it was observed that pea protein isolates had lower solubility when compared to pea protein concentrates. These effects are attributed to the differences in processing conditions. The protein isolates were prepared with acid and alkali treatments using wet extraction followed by spray drying which led to denaturation and subsequent aggregation, while the concentrates were obtained by simple dry fractionation.

Foaming ability: The property of proteins to form stable foams is important in the production of a variety of foods. Under mechanical whipping, proteins rapidly adsorb at the interface and form a stabilizing film around air bubbles which help in foam formation. The basic function of proteins in foams is to decrease interfacial tension, to increase viscosity of the liquid phase and to form strong films around the air bubbles. Foaming properties determine the applications of protein in food products where air is to be incorporated in the food products. Chiffon cakes, fudges, whipped cream/toppings, mousses, ice-cream mixes are some of the examples of the products. Foaming properties of pulse proteins are measured in terms of foaming capacity (FC) and foam stability (FS) (Shevkani *et al*., 2019). FC is an indicator of increase in volume after

whipping, it depends on the ability of the proteins to diffuse at the interface, change its orientation, and form a viscous film. FS is the ability of the protein to maintain the foam. The lower foaming of globulins is due to their reduced ability to unfold/reorient at the interface, which limits stabilization of air bubbles, whereas albumins show increased foaming abilities due to enhanced protein unfolding. The foaming capacity and foaming stability for albumins from lentils was 76.7% and 66.7% respectively, which were much higher than that from globulins (16.7% and 8.9%, respectively) (Ghumman *et al*., 2016).

Water and fat absorption capacity: Water and fat/oil absorption of proteins are related to texture, mouth feel and flavour retention in foods. Water absorption capacity is an important property of proteins in viscous foods such as soup, dough, custard and baked foods which are supposed to imbibe water without the dissolution of proteins. Water absorption capacity is defined as the ability of proteins to physically hold water against gravity (Kinsella, 1976), and it is expressed as the mass of water absorbed by a known weight of protein. The water absorption capacity of pulse proteins is mainly due to the hydrophilic parts, the polar and charged side chains of proteins as well as carbohydrates present in the sample which have an affinity for water molecules. Proteins from kidney bean, field pea, and cowpea varieties showed a varying water absorption capacity between 1.6 and 4.8 g/g (Shevkani *et al.*, 2015). Fat or oil absorption capacity is defined as the amount of fat or oil that can be absorbed by the protein. In general, proteins interact with lipids through the binding of nonpolar side chains of amino acids with aliphatic chains of oils and fats. Different pulse proteins have different fat absorption capacities. For example, fat absorption capacity of protein isolates from kidney bean, field pea and cowpea varied between 4.7-6.9 g/g, 5.5-7.2 g/g and 1.4-2.0 g/g, respectively (Shevkani *et al*., 2015).

Gelation ability: Gelation is an important functional property of proteins in viscous foods such as puddings, soups, gels, curds, heated-minced meats, *etc.* The protein gels can be formed by application of heat, pressure and changing ionic strength, though heat-induced gelation is the most commonly used method of gelation for pulse proteins (Shevkani *et al*., 2019). Gelation temperature is generally the minimum temperature for gel formation and is generally detected as the crossover temperature between G′ and G″ during the temperature sweep rheological experiments (Mession

et al., 2015). Heat-induced gelation depends on several factors including protein structure, composition, and pH of the medium. The gelation temperature of proteins is generally dependent on the thermal stability of the proteins and is higher than the denaturation temperature (Shevkani *et al*., 2019). Gelation temperatures range from 87.4 to 94.5°C for protein isolates of kidney beans of different varieties and from 84.0 to 93.1°C for protein isolates of different lines of field peas (Shevkani *et al.,* 2015).

Emulsifying properties: Emulsifying properties of protein play an important role in its applications as a food ingredient. A protein with superior emulsifying properties could be used to develop stable oil-in-water emulsion-based food products such as coffee creamer, mayonnaise, ice cream, beverages etc. Emulsifying capacity, emulsifying ability, emulsifying activity index, emulsifying stability index and creaming stability are some of the quality indexes commonly used to evaluate the emulsifying properties of a protein (Boye *et al*., 2010). For pulse proteins, different researchers used various indexes and units to describe their emulsifying properties, which makes it difficult to compare the results (Ge *et al*., 2020). Overall, researchers found that the method of extraction plays an important role in determining the emulsifying ability of the proteins. Even with the same extraction methods, different cultivars or genotypes of pulses can have a significantly different emulsifying properties (Barac *et al*., 2010; A. C. Y. Lam *et al*., 2017).

2.4 Proteins-based emulsifiers for food and beverage application

2.4.1 Introduction to emulsifier

An emulsion consists of two immiscible liquids, usually oil and water, with one of them being dispersed as small spherical droplets in the other (McClements, 2007). The material that makes up the droplets is referred to as the dispersed phase, whereas the surrounding liquid is referred to as the continuous phase. The preparation of emulsions that are kinetically stable over a period requires the incorporation of substances known as emulsifiers. Emulsifiers are amphiphilic surface-active molecules that are used to facilitate the formation of droplets during homogenization and provide stability to the resulting emulsion. Usually, low molecular weight emulsifiers tend to be more surface active than large molecular weight proteins. Emulsifiers,

being amphiphilic, i.e., consisting of both the hydrophilic and hydrophobic parts, help to stabilize the oil droplets in the aqueous phase of an oil-in-water emulsion.

During emulsification, emulsifiers migrate from the continuous phase to the droplet surface and realign and absorb at the oil-water interface to lower the interfacial tension and form a protective layer around the droplet surface (Piorkowski & McClements, 2014). The emulsifier should be in enough concentration to coat the droplets completely. They should adsorb on the surface of the droplets faster than the droplets coalescing with each other during homogenization (Piorkowski & McClements, 2014). Generally, there are two types of emulsifiers depending on their molecular structure: small molecule emulsifiers, which include lecithin, polysorbates, monoglyceride and polymeric emulsifiers, which include dairy proteins such as sodium caseinate, whey proteins, and pulse proteins such as pea proteins etc.

Based on the charge of the hydrophilic moiety, small molecule emulsifiers are categorized into non-ionic, ionic and zwitterionic. Non-ionic emulsifiers do not possess any charge. Non-ionic emulsifiers have been widely used to form nanoemulsions because of their low toxicity and capacity to form nanoemulsions by both high-energy and low-energy approaches (Rao & McClements, 2011). Some of them include polyoxyethylene ether (POE), and ethoxylated sorbitan esters (Rao & McClements, 2011). Ionic emulsifiers impose either positive or negative charges to emulsion droplets. Most of the food-grade ionic emulsifiers are negatively charged such as citric and fatty acid esters of glycerol (CITREM), diacetyl tartaric acid ester of mono- and diglycerides (DATEM) and sodium lauryl sulphate (SLS). One of the positively charged emulsifiers that is available for food applications is lauric arginate (Rao & McClements, 2011). Zwitterionic or amphoteric emulsifiers have two or more oppositely charged ionizable groups on the same molecule. They can have a positive charge or negative charge or neutral charge depending on the pH of the solution. Phospholipids, such as lecithin is a common zwitterionic emulsifiers used in the food industry (Chowdhury *et al*., 2019).

2.4.2 Proteins as emulsifiers

Protein-based emulsifiers are generally used in the food and beverage industry in fruit-based

drinks, plant-based milk, coffee creamer, salad dressings etc. Proteins can adsorb at the oil-water interface and decrease the interfacial tension, thereby reducing the energy required to make an emulsion. A balanced hydrophobicity and hydrophilicity of proteins are required for good absorption to the interface between the aqueous and oil phases. The adsorption of proteins to the interface usually occurs in two phases. Due to its hydrophilic nature, proteins initially migrate to the interface in a solubility-dependent manner (Shevkani *et al*., 2015). After the migration of proteins to the interface, its hydrophobic patches promote adsorption. In the second stage, a structural rearrangement of proteins occurs such that proteins may partially denature and realign themselves to make the hydrophilic portions face the aqueous phase while the hydrophobic portions reside in the oil phase (Lam & Nickerson, 2013). It also provides a steric barrier at the oil droplet surface, which helps prevent droplet coalescence (Shevkani *et al*., 2019). Proteins also contribute to emulsion stability by increasing the viscosity of the continuous phase, which decreases the rate of movement of oil droplets in the continuous phase. Of the commonly used proteins, whey protein and casein proteins have been used for a long time (Dickinson *et al*., 1988). The difference in these two dairy proteins is mainly caused by structural differences in the molecules since casein has a flexible coil structure while whey protein is globular. There is now growing demand for plant proteins as alternatives to animal proteins (Silva *et al*., 2019). Various plant proteins are being investigated as an emulsifier, including proteins from soy, pea, cowpea, wheat gluten etc. (Burger & Zhang, 2019). These can be broadly categorized into globular proteins and flexible random coil proteins.

2.4.2.1 Flexible random coil protein-based emulsifiers

The main sources of flexible proteins are casein from bovine milk and gelatin from fish (McClemments, 2005). Gelatin is prepared by disrupting the native structure of collagen and by boiling it in the presence of acid (Type A gelatin) or base (Type B gelatin). Gelatin is a random coil molecule at high temperatures but undergoes coil to helix transition upon cooling from 10°C to 25°C (Dickinson *et al*., 1988). Gelatin, however, form relatively large droplets when used in isolation. So, they are generally used along with other ingredients to improve their stabilization capacity. There are many casein-based emulsifiers that can be purchased commercially with different functions, such as sodium caseinate and calcium caseinate (Piorkowski & McClements,

2014). Caseins are highly surface active and have been widely used in developing oil-in-water emulsions for various food applications (Wusigale *et al*., 2020). Casein protein-stabilized emulsions generally have smaller droplets with higher stability against aggregation under heat treatment and salt addition compared to other globular protein-stabilized emulsions (Patel, 2017).

2.4.2.2 Globular protein-based emulsifiers

Native globular proteins tend to have fairly compact spheroid structures with non-polar amino acids present within their hydrophobic interiors and polar amino acids located in the exteriors. Whey, soy, egg, pea and other pulse proteins are some of the major sources of globular protein (Kim *et al*., 2020). Globular proteins tend to form a layer around oil droplets and stabilize the emulsion through electrostatic and steric repulsion. Globular proteins are particularly sensitive to droplet flocculation near isoelectric points. Emulsions stabilized by globular proteins are sensitive to temperature changes as they tend to unfold at high temperatures exposing the hydrophobic and sulphydryl groups, which lead to the attraction between the oil droplets and cause emulsion destabilization (Kim *et al*., 2020). The isoelectric point of most globular proteins, such as whey proteins, is in between pH 4 to 6, and the thermal denaturation temperature is around 70-90°C (Geerts et al., 2017). The isoelectric point of pea proteins is in the range of pH 4.8 to 5.5, and the denaturation temperature lies between 65-85°C (Kim *et al*., 2020; Mession *et al*., 2013). Globular proteins have been widely used as emulsifiers in stabilizing various food emulsions (Kim *et al*., 2020). Pulse proteins are one of the most commonly available globular proteins which can effectively be used for the development of beverages with or without modifications based on the requirements.

2.4.3 Pulse proteins as emulsifiers

Pulse proteins are garnering attention across the globe due to their wide range of applications in the food industry. In recent years, they have been considered an alternative to conventional animal proteins due to their low cost, lower allergenicity and wider acceptability (Carbonaro *et al*., 2015). Most pulse proteins are composed of albumin and globulin fractions; different

cultivars have different ratios of these fractions. Besides low cost and low allergenicity, pulse proteins also have various functional properties, including solubility, water and fat holding capacities, foaming abilities and emulsifying property (Burger & Zhang, 2019). They are amphiphilic molecules that offer stability to emulsions, their emulsifying capacity depends on various factors, including the method of extraction of the protein. They tend to lose some of their functional properties when extracted under harsh alkaline or acidic conditions (Geerts *et al*., 2017). Apart from the internal factors such as functional properties as emulsifiers, their concentration, the amount of dispersed and continuous phases are also important to determine the stability of an emulsion (McClements, 2007). It is also important to understand how the emulsion behaves under different environmental stresses to ensure a stable emulsion over a long period of time in different conditions of foods.

2.4.4 Effect of heat, salt, and pH on the emulsion stability:

Emulsifiers used in the food industry must be capable of functioning under a range of different environmental conditions, which are highly dependent on the type of food product. Different products may have appreciable variations in pH, ionic strength, and temperature during manufacturing and storage. Therefore, it's important to understand the range of the conditions where the emulsifier can successfully operate (McClements, 2007). Emulsions under observation are usually subjected to thermal processes mimicking sterilization, cooking or pasteurization. The stability of such emulsions can be tested by placing them in a controlled water bath at high temperatures for a defined period (e.g., 30 to 90°C for 30 minutes), followed by cooling to ambient temperature and performing the characterization tests (McClements, 2007). The resistance of protein-stabilized emulsions to pH and ionic concentrations is tested by adjusting them to different pH values (from pH 2-8) and mineral concentrations (from 0.1 M to 1 M NaCl or $CaCl_2$) and then characterizing them after storing for a fixed time period (McClements, 2007). Protein-coated droplets have a high net charge at pH far from their isoelectric point (pI), which generates strong repulsion between them. Whereas, they have a low net charge at pH values close to the pI and so the electrostatic repulsion is not strong enough to overcome the van der Waals attraction, leading to flocculation (Gumus *et al*., 2017). Usually, there is a decrease in zeta potential with the increase in salt concentration, which is due to electrostatic screening, i.e., the accumulation of counter-ions

(Na^+) around the negatively charged droplet surfaces (Ozturk *et al*., 2015). It was observed that emulsions flocculate at low salt concentrations (100 mM to 400 mM NaCl) but are stable to aggregation at high salt concentrations (above 500 mM NaCl). This may be due to the ability of salt to decrease the electrostatic repulsion between the droplets at lower concentrations leading to flocculation (Dickinson, 2011). On the other hand, high salt concentrations may also alter the conformation of adsorbed protein molecules leading to a thicker interfacial layer that increases the steric repulsion between the droplets making the emulsion stable (Parsons & Salis, 2015). Alternatively, this effect might be because the solubility of some proteins increases with an increase in salt concentration due to the ability of salts to weaken the attractive interactions between the protein molecules, also known as the 'salting in' effect (Aluko & Yada, 1995). Furthermore, emulsions stabilized by globular proteins, such as pulse proteins, are particularly sensitive to thermal treatments as there is a possibility of the proteins to unfold and expose hydrophobic groups originally located within their hydrophobic domains leading to attraction and, in turn, causing aggregation of the protein-coated droplets, destabilizing the emulsion (Peng *et al*., 2016)

In one of the studies carried out by Sarkar *et al*. (2016) on tomato seed protein isolate-stabilized emulsions, the effect of pH (2–9), salt (0–250 mM NaCl) and heat (30–90 °C, 30 minutes) was studied. Emulsions were stable to droplet flocculation at all pH ranges except pH 2–4, due to the proximity to the isoelectric point. Emulsions showed stability to high NaCl concentrations (250 mM) at pH 6–8. Droplet aggregation was observed when the emulsions were heated above 80°C due to the denaturation of the globular protein fractions adsorbed at the surface. In another study by Keivaninahr *et al.* (2021), the effect of salt (1% NaCl), pH (2 and 7), and thermal treatment (90°C for 30 minutes) was examined for emulsions made with pea protein isolate and concentrate. At pH 2, the aggregate size of the emulsions was significantly higher when compared to that of the emulsions made at pH 7. Heat treatment caused phase separation in pH 2 emulsions, leaving a clear aqueous phase at the bottom of the glass vials. Whereas, at pH 7, the emulsions did not show any phase separation upon heating, indicating improved stability. Partial destabilization of heated emulsions at pH 2 was attributed to the denaturation of protein at acidic conditions causing aggregation of proteins, and in turn causing emulsion destabilization. At pH 2, adding salt led to visual phase separation in emulsions. At pH 7, the emulsions were stable at high salt concentrations

above 400 mM NaCl, which was ascribed to the salting-in effect of the proteins. These studies could help us to understand the conditions that are ideal for a protein to act as an emulsifier to stabilize an emulsion.

2.5 Beverage emulsions

Emulsions are extensively used in various food products such as whipping cream, margarine, butter, coffee creamer, plant-based milk, and beverages. Emulsion-based beverages include fruit drinks, flavoured drinks, juices, sports drinks and many more. Beverages are usually O/W emulsions, where the oil droplets are dispersed in the aqueous phase with the help of emulsifiers (Molet-Rodríguez *et al*., 2018). Usually, the aqueous phase mainly consists of water and water-soluble components such as vitamins, minerals, sugars, organic acids, proteins or polysaccharides. The oil phase acts as a carrier for oil-soluble components such as carotenes, fat-soluble vitamins, plant essential oils, and flavour components, among others. Finally, the emulsifiers and stabilizers help in decreasing the interfacial tension and help in stabilizing the emulsions (Molet-Rodríguez *et al*., 2018). It is not necessary that all beverages should contain all these components. One of the common exceptions is club sodas otherwise known as sparkling water, which is nothing but carbonated water but, are still considered as beverages. Based on the desired appearance, texture and mouth feel properties, beverage emulsions can be tailored in many ways. For example, if the requirements include transparent or translucent emulsions, then the droplet size should be nanoscale and can be achieved by homogenizing the emulsion in a high-pressure homogenizer (Chanamai & McClements, 2002). Nanoscale droplets scatter light poorly and the emulsion tends to be more transparent. For opaque beverages, the droplet size could be larger to scatter more light which helps in giving an opaque appearance (Chanamai & McClements, 2002). If a creamy mouth feel is required, the viscosity of the emulsion can be altered by adding thickening agents or by increasing the oil concentration (McClements, 2007). Based on their function, beverage emulsions can either be used to impart flavour to the beverage or behave as clouding agents. Flavour emulsions are developed with non-polar flavour oils as the dispersed phase, which is responsible for imparting flavour to the beverage. As clouding agents, the main function of the dispersed oil phase is to offer turbidity to the emulsion, causing an opaque appearance (McClements, 2007).

2.5.1 Flavour emulsion components

The main ingredient of the oil phase in flavour emulsions includes flavoured essential oils from various sources such as orange, lemon, and peppermint (Misharina *et al*., 2010). The flavour oils differ in their composition based on their origin due to the different folds of the oil. Oils which have been extracted by cold pressing are usually referred to as single-fold (1×) flavour oils, whereas the oils which have undergone further processing are known to have higher folds such as, 3×, 5×, and 10× (Gamarra *et al*., 2006). Generally, higher fold oils tend to have more intense flavours. The oil droplets in an emulsion containing oil of lower fold tend to be more unstable and grow in size during the storage period, due to Ostwald Ripening (Rao & McClements, 2012). It is a process in which the droplet size grows due to the diffusion of oil molecules from small droplets to large droplets, this occurs when the oils exhibit partial water-solubility. An effective means of preventing Ostwald Ripening is to add ripening inhibitors which are hydrophobic components with very low water solubility (Kabal'nov *et al*., 1987). A ripening inhibitor commonly used in beverages is corn oil which has long chain triglycerides that are hydrophobic and water insoluble (Rao & McClements, 2012). Nevertheless, insoluble components inherently present in the flavour oils of higher folds (10×) behave as ripening inhibitors and help prevent Ostwald Ripening (Rao & McClements, 2012). Another challenge with beverage emulsion stability is the large difference in densities of flavour oils compared to the beverage aqueous phases, leading to faster separation of oil droplets due to gravity-induced creaming (McClemments, 2005). The creaming rate in the beverage emulsion can be reduced by decreasing the density differences between the aqueous phase and the dispersed phase. The most common method of increasing the density of the oil phase is by adding weighting agents, which are hydrophobic components with a density considerably greater than flavour oil. Brominated vegetable oil, dammar gum, ester gum and sucrose acetate are some of the common weighting agents that are used to increase the density of the aqueous phase and prevent creaming (Chanamai & McClements, 2002). The rate of degradation of the oils also depends on many different environmental factors, such as temperature, exposure to light and oxygen and the presence of antioxidants (Dickinson, 2011).

2.5.2 Cloud emulsions components

The oil phase in cloud emulsions is mainly made up of non-flavour oil such as triacylglycerols or terpene oils (McClemments *et al*., 2009). These oils have very low water solubility and tend to be stable against Ostwald ripening. The main purpose of cloud emulsions is to scatter light strongly to give an opaque, turbid, or cloudy visual appearance (Piorkowski & McClements, 2014). They do not help in contributing to the flavour of the beverage but might lead to the development of off-flavour when they are degraded in unsuitable conditions. Polyunsaturated oils, oil-soluble antioxidants and vitamins are also some of the important components which are introduced into the beverages because of the nutritional benefits they offer (Pool *et al*., 2013). Some components such as carotenoids and oil-soluble colourants are also added to impart a desired colour to the beverage.

2.5.3 Emulsifiers for beverage emulsions

The most common emulsifiers used for beverages are modified starch, gum Arabic and proteins. The emulsification behaviour of proteins was reviewed in detail in section 2.3.1 and 2.4.1. Here emulsification behaviour of modified starch and gum Arabic are discussed.

2.5.3.1 Modified starch as an emulsifier for beverage emulsions

Starch contains hydrophilic components which are responsible for its solubility in aqueous solutions. Starch is modified by adding hydrophobic side chains making it an amphiphilic molecule, suitable for emulsifying applications. These side groups anchor the molecule to the oil droplet surface, while the hydrophilic groups protrude into the aqueous phase to protect droplets from aggregation. One of the most commonly used modified starches is the octenyl succinate derivative of waxy maize starch (Piorkowski & McClements, 2014). Cheng *et al*. (2021) developed 1–5 wt% octenyl succinic anhydride (OSA) modified pea starch, normal corn starch and waxy corn starch and compared their application in beverage emulsion formation and stabilization. The OSA-modified waxy corn starch showed the highest emulsifying properties and created the most stable beverage emulsions when compared to the other modified starches

(Cheng *et al*., 2020).

2.5.3.2 Gum Arabic as an emulsifier for beverage emulsions

Gum Arabic is one of the most widely used emulsifiers in the beverage industry (Given, 2009). It is a natural exudate harvested from *acacia* trees in sub-Saharan Africa, especially Sudan (Buffo *et al*., 2002). It consists of arabinogalactan blocks attached to a polypeptide backbone. The hydrophobic polypeptide chain anchors the molecules to the droplet surface, while the carbohydrate block extends into the aqueous phase. The stability offered by gum Arabic is mainly provided by steric repulsion and partially by electrostatic repulsion (Piorkowski et al. 2014). It has been shown that emulsions stabilized using gum Arabic are stable to flocculation when exposed to a wide range of environmental conditions, for example, pH (3–9), ionic strength (0–500 mM NaCl) and thermal treatment (90°C) (Charoen *et al*., 2011). Cheng *et al*. (2020) showed that gum Arabic-stabilized beverage emulsions showed long-term stability over a period of 28 days and were comparable to or even better than the emulsions prepared with OSA-modified pea and normal corn starches.

2.5.4 Beverage emulsion manufacture

Beverage emulsions are generally produced in a two-step process. The first step usually involves producing beverage concentrates via high-energy methods. The concentrated emulsified systems were designed to carry and protect those water-insoluble ingredients to be incorporated into the final beverages (Molet-Rodríguez *et al*., 2018). The concentrates have a longer shelf life can be efficiently transported to various places to produce the required beverages. In the second step, the concentrates are diluted according to the required concentration of the final beverage product. Pasteurization or sterilization is carried out to prevent the spoilage of the products (Guzey & McClements, 2007).

2.5.4.1 Beverage emulsion concentrates

In the production of beverage emulsion concentrates, the aqueous phase and the oil phase are

prepared separately. The aqueous phase is heated and mechanically agitated to facilitate the dissolution of various components including thickening agents, emulsifiers, minerals, and other water-soluble components (Tan, 2004). The oil phase is also mechanically agitated in a similar way to solubilize some components such as antioxidants, ripening inhibitors, weighting agents, and other oil-soluble pigments. Once the aqueous phase and the oil phase are prepared, they are blended by high-shear mixer to form a coarse emulsion, which is then homogenized to prepare a fine emulsion (Acosta, 2009; Tadros *et al*., 2004). High energy methods are widely used in the industry because they can be utilized with a large variety of oils and emulsifiers. High energy approaches include the usage of high-pressure valve homogenizers, microfluidizers and ultra-sonic homogenizers. After, the concentrate is prepared, it is pasteurized, or heat treated to decrease the microbial load in the product and later transported to the place where it will be used to make the final beverage.

2.5.4.2 The final beverage processing

The finished product is created by diluting the beverage concentrate with water or any other appropriate aqueous solution containing other ingredients such as flavours, colours, preservatives, sugars and pH regulators. The concentrate is generally diluted 500 to 1000 times to form the final product and usually contains less than 20 mg of oil per 1 litre of beverage (Given, 2009). The final product can be homogenized again to make sure the colourants and flavours are distributed homogenously. The shelf life of the final product depends on the concentration of emulsifiers, the presence of other additives and the environmental factors. The preservatives and pH regulators, which are added during dilution, help to maintain the shelf life and to obtain the desired sensory characteristics of the final beverage product. One of the major concerns in the beverage industry is product degradation, which is caused by many reasons, microbial infestation being the one of the primary concerns. Usually, beverages are subjected to thermal treatment, pasteurization (72°C for 16 seconds) or sterilization (30 minutes at 110°C) depending on the requirement to prevent the growth of microorganisms and spores (McClements, 2007). Beverage emulsions are also susceptible to chemical degradation, such as changes in flavour or colour fading due to the degradation of the colourants. These challenges can be overcome by using appropriate packaging materials or proper formulation. Apart from

the chemical and microbial degradation, beverage emulsions can also undergo physical changes leading to destabilization. Different mechanisms of emulsion destabilization is discussed in the following section.

2.5.5 Destabilization in beverage emulsions

When the amount of emulsifier added is insufficient or too much, or due the effect of environmental stresses such as pH and temperature differences, an emulsion may undergo droplet aggregation leading to phase separation and ultimately emulsion destabilization (Dickinson, 2011). Different kinds of emulsion destabilization mechanisms are illustrated in Figure 2.

Creaming: In O/W emulsions, gravitational separation is one of the most common forms of instability. Oils are less dense compared to water, so the oil droplets tend to rise to the top of the emulsion, form a cream layer and destabilize the emulsion (Figure 2a). The most common phenomenon in beverages would be a ring formation at the top, which is the accumulation of a visible ring of oil droplets on the top part of the beverage container. The velocity that an oil droplet moves upwards in a dilute emulsion due to gravity (g) is given by Stokes's law (Piorkowski & McClements, 2014),

$$v = \frac{2gr^2(d_1 - d_2)}{9\mu} \tag{2.1}$$

Where v is the velocity of creaming, r is the droplet radius, d_1 is the density of the dispersed phase, d_2 is the density of the continuous phase and μ is the viscosity of the continuous phase. This equation shows that the rate of droplet creaming should decrease as the droplet size decreases, the density difference decreases, or the aqueous phase viscosity increases. One of the methods to prevent creaming would be to match the density of the oil phase with that of the aqueous phase by adding weighting agents such as sucrose acetate isobutyrate, dammar gum, ester gum etc. (Lim *et al*., 2011). Creaming can also be inhibited by reducing the size of the oil droplets, or by increasing the viscosity of the aqueous phase by adding suitable thickening agents (Piorkowski *et al*. 2014).

Flocculation: A flocculation is a reversible form of aggregation; the droplets are aggregated but can be easily separated upon gentle mixing (Figure 2b). Flocculation is a process which accelerates gravitational separation and reduces the shelf-life of a beverage. Droplets in a colloidal system are in continuous motion. This motion is due to the thermal energy, mechanical agitation or gravitational forces leading to collision between the droplets. Normally, the droplets stay apart due to steric or electrostatic repulsion, but they aggregate if the repulsive barrier is not enough. Flocculation can be due to reduced electrostatic repulsion, where the droplets flocculate when the electrostatic repulsion between them is reduced. Depletion attraction, induced by the osmotic pressure differences from the excess soluble biopolymer in an emulsion, is often a leading cause of droplet flocculation in emulsions (McClemments, 2005). Increased hydrophobic interaction also causes flocculation, one example would be the effect of thermal treatment on oil-in-water emulsions stabilized with globular proteins. The globular proteins unfold when they are heated above 70°C and cause the exposure of interior hydrophobic patches leading to the attraction between the hydrophobic groups and in turn cause flocculation (Demetriades et al., 1997). The formation of biopolymer bridges among two or more droplets also causes flocculation, for example, a positively charged biopolymer such as chitosan might adsorb to the surface of two negatively charged droplets causing them to flocculate, or a negatively charged polymer such as carrageenan might adsorb to the surface of two positively charged droplets causing them to flocculate (McClements et al., 2007). Flocculation can generally be prevented if the repulsive forces dominate the attractive forces.

Coalescence: Coalescence is the fusion between multiple droplets due to rupture of their interfacial layers leading to the formation of larger droplets (Figure 2c). Coalescence increases the rate of gravitational separation due to the increased size of the droplets. Coalescence usually leads to the formation of oil layer on top of some unstable beverages which is generally termed as oiling off (McClemments, 2005). Generally, the occurrence of coalescence is due to certain forces such as gravitational, colloidal, hydrodynamic, and mechanical forces acting on the system which causes interfacial layer to rupture (McClemments, 2005). This can usually be prevented with colloidal complexes with partial wettability which are able to accumulate at the interface to prevent coalescence. In one of the studies done by Doost *et al*. (2019), almond gum and whey protein complexes were used to stabilize tricaprylin oil-in-water emulsions. It was

observed that the complexes prevented the coalescence of oil droplets and offer more stability when compared to the emulsions made with native whey protein over a time period of 40 days.

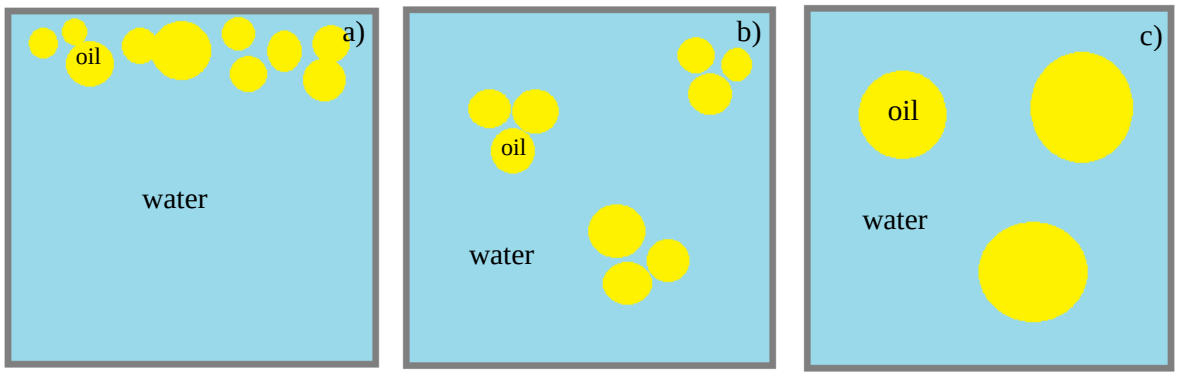

Figure 2: Schematic illustration of emulsion destabilization, (a) creaming in O/W emulsions because of gravitational separation due to the difference in density between the aqueous and lipid phases, (b) flocculation due to the dominant attractive interaction between droplets compared to the repulsive forces, and (c) coalescence between the droplets due to the rupture of inter-droplet film.

Ostwald ripening: Vegetable oils are hydrophobic and tend to remain insoluble in the aqueous phase. But there are some flavour oils (such as lemon oils), which are partially hydrophilic and soluble in the aqueous phase (Rao & McClements, 2012). Due to the higher solubility of oil molecules from smaller droplets compared to larger droplets, partially soluble oil molecules from smaller droplets dissolve in the aqueous phase and diffuse through the intervening phase towards the large droplets (Kabal'nov *et al*., 1987). This leads to the continuous increase in the size of the larger droplets at the expense of smaller droplets (Figure 3). This form of destabilization is known as Ostwald ripening (Wooster *et al*., 2008). To prevent Ostwald ripening, small amounts of insoluble vegetable oils such as corn, sunflower, or canola oils are mixed with the flavour oil phase of a beverage emulsion (Piorkowski & McClements, 2014). Wooster *et al*. (2008) determined the effect of the oil molecular weight on the rate of Ostwald ripening. The authors prepared one set of emulsions with *n*-alkanes and another set of emulsions with peanut oil under the same conditions. For alkanes emulsion, the rate of Ostwald ripening decreased with an increase in the molecular weight. Moreover, the emulsions made with peanut

oils showed higher stability against Ostwald ripening when compared to *n*-alkanes due to the high molar volume of the former when compared to *n*-alkanes. The molar volume of oil has a direct relationship with its solubility in water. The insolubility of the triglycerol peanut oil in water acted as a kinetic barrier to Ostwald ripening, making the triglyceride emulsions highly stable compared to the alkane emulsions.

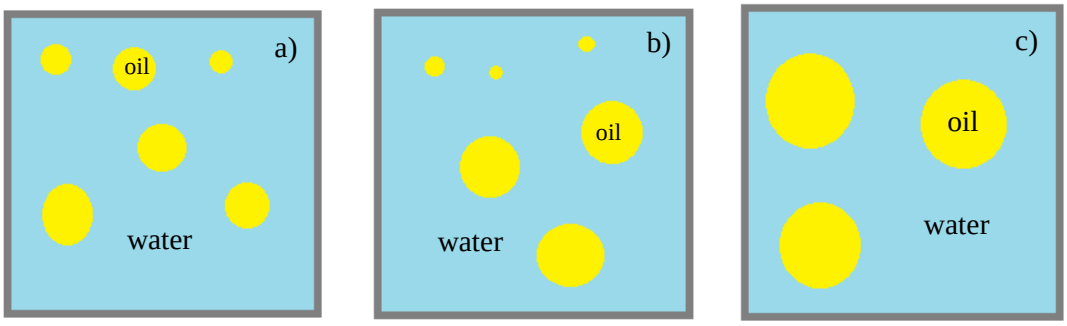

Figure 3: Stages of Ostwald ripening, (a) droplets of different sizes, (b) smaller droplets dissolving and increasing the size of larger droplets (c) small droplets completely dissolved and form larger droplets which destabilize the emulsion.

2.6 Delivery of lipophilic bioactives via beverage emulsions

The dispersed oil droplets of beverage emulsions can be utilized for the delivery of lipophilic bioactive compounds. There are many lipophilic bioactives such as flavonoids, carotenoids, polyunsaturated fatty acids etc. that can be delivered via the dispersed oil droplets of beverage emulsions. The following section describes a few different kinds of lipophilic bioactives, and their delivery, release, and bioavailability from emulsions.

2.6.1 Lipophilic bioactives and their importance

It has been known for many centuries that consuming fresh fruits and vegetables enhances human health. Epidemiological and prospective studies on fruits, vegetables and nuts revealed that they contain compounds to reduce the risk of cancers and cardiovascular diseases (Patil *et al*., 2009). Evidence from *in vitro* and *in vivo* digestion experiments showed convincing results

regarding the health benefits of bioactives (Patil *et al*., 2009). There are many different classes of bioactive compounds important for food application and human health. Among these bioactives, there are a few lipophilic bioactives which are more commonly used in emulsions, such as flavonoids, carotenoids and omega-3 fatty acids.

Flavonoids: During the 1800s, the red color of wine and the blue pigment of cornflower were ascribed to the presence of flavonoids (Svirbely1 & Szent-Gyorgyi, 1932). As of today, more than 5000 flavonoids have been identified, of which some of them are extensively used for clinical purposes (A. Y. Sun *et al*., 2002). It has been proven that flavonoids have antioxidant activity and are helpful for the prevention of cardiovascular diseases, cancer, hemorrhoidal and Alzheimer's disease (Engelhart *et al*., 2002). Some the flavonoids which are recognized as strong antioxidants include quercetin (from onion), myricetin (from peppers) and tangeritin (from citrus fruits) (Patil *et al*., 2009).

Carotenoids: Carotenoids are fat soluble natural compounds found in plants, algae, and photosynthetic bacteria. With the discovery of Vitamin-A, it was established that carotenes and vitamin-A had close correlation. In 1930, it was reported that β-carotene can be converted into Vitamin-A and may show a pro-vitamin A activity (Goodwin, 1961). Most of the studies have shown that β-carotene has anti-cancer properties and radical scavenging properties. This property is useful for inhibiting the proliferation of cancer cells and anti-inflammatory properties (Patil *et al*., 2009). Apart from β-carotene, lycopene is also a carotenoid which is said to have anti-cancer properties like inhibiting cell invasion, metastasis and help to induce apoptosis (Sidhu *et al*., 1998). These carotenoids can be found in carrots, beet roots, pomegranates, and many other plant sources.

Polyunsaturated Fatty acids: Polyunsaturated fatty acids (PUFA) are required for normal development and functioning of brain and heart, and for all the tissues and organs to be at equilibrium (Sokoła-Wysoczańska *et al*., 2018). Omega-6 and omega-3 fatty acids are some of the common polyunsaturated fatty acids that have an important role in fulfilling nutritional deficiencies. The first double bond in omega-3 family occurs at the third carbon from the methyl end of the carbon chain (hence the name omega-3) and in the case of omega-6 family, the first

double bond occurs at the sixth carbon from the methyl end of the carbon chain (Sokoła-Wysoczańska *et al*., 2018). They are found in vegetable oils such as flaxseed, canola, hemp seed, nuts such as walnuts as well as chia seeds (Meyer et al., 2003). Alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid are the prominent representatives of the omega-3 family (Lee *et al*., 2009). Consumption of omega-6 linoleic acid is important to prevent fatty liver, skin lesions and reproductive failure (Sokoła-Wysoczańska *et al*., 2018). Whereas deficiency of omega-3 fatty acids leads to reduced vision. According to the current guidelines of the European Society of Cardiology, treatment with omega-3 PUFAs may be considered a new option to treat acute myocardial infarction, the treatment with omega-3 PUFAs has demonstrated a 20% reduction in the mortality rates of affected patients (Marchioli & Levantesi, 2013). Studies on rodents have revealed that deficiency of omega-3 PUFA affects learning, memory, and cognitive behaviour (Fedorova *et al*., 2009). It is important to maintain the right ratio of the intake of polyunsaturated fatty acids since excess consumption of omega-6 may lead to low-grade chronic systemic inflammation leading to civilizational diseases (Pallebage-Gamarallage *et al*., 2012).

Apart from the bioactives mentioned above, there are many others which offer benefit to human health. Regarding health concerns, general immunity against the common flu and cough has become one of the main concerns for consumers (Molet-Rodríguez *et al*., 2018). This led to a marked rise in the sales of beverages based on probiotics, green tea, and antioxidant super fruits like acai, acerola, noni and mangosteen (Given, 2009). The food and beverage industry have taken advantage of this trend to manufacture beverages which are being fortified with vitamins, minerals and many other health benefitting bioactives (Gunathilake *et al*., 2013). Another trend in consumers is their distrust of artificial flavours and colourants. These two trends have led to the development of beverages which are fortified with health-benefitting bioactives with natural flavours and colourants.

2.6.2 Delivery of bioactives using emulsion-based systems

The majority of the bioactives are hydrophobic and their delivery in an aqueous human GI tract system is a challenge. In this respect, an oil-in-water emulsion-based delivery system could be

suitable by dissolving the bioactives in the oil phase prior to making the emulsion. A number of different emulsion-based delivery systems have been used to deliver bioactives, including conventional emulsions, multiple emulsions, multilayer emulsions, solid lipid particles and filled hydrogel particles (McClements *et al*., 2007). There are some important factors to consider for an ideal emulsion-based delivery system, for example, the delivery system should be food grade, economically viable, should protect the bioactive from chemical degradation, should be able to encapsulate a relatively large amount of bioactives and should protect the bioactives till it is delivered. The delivery of the bioactive should also be site-specific and the delivery system should enhance the bioavailability of the encapsulated components (McClements *et al*., 2007). Plant oils such as sunflower, safflower, corn, flaxseed oils and flavour oils such as lemon and orange oils are some of the common oils which were used to construct bioactive delivery systems (Pool *et al*., 2013). Conventional emulsions for bioactive delivery are made by dispersing the bioactive component in the oil phase, which is later homogenized with the aqueous phase in the presence of a water-soluble emulsifier (Walstra, 2003). If the bioactive lipid was crystalline (phytosterols or carotenoids) then the bioactives are dissolved in the oil phase by heating the oil in the presence of the bioactive before making the emulsion (Mcclements & Decker, 2000). If the bioactive is susceptible to chemical degradation such as omega-3 fatty acids, then it is important to control the homogenization and emulsion storage conditions that may cause degradation, such as high temperatures, exposure to oxygen and light or the presence of transition metals. Oil-in-water emulsions have been used to encapsulate omega-3 fatty acids-rich oils so that they can be incorporated into food products such as milk, yogurt, ice cream and meat patties (McClements *et al.*, 2007). The bioavailability of ingested hydrophobic bioactive depends on their behaviour as they pass through the different regions in the gut (Tan *et al*., 2022). *In-vitro* digestion models were therefore designed to mimic the conditions of the human gut, and to understand the bioavailability of these bioactives and their release from the delivery systems at the targeted regions of the digestive system (Li *et al*., 2012).

2.6.3 Emulsion *in-vitro* digestion to determine lipid digestibility and bioavailability of bioactives

Digestion is a complex process that helps us to absorb and assimilate the nutrients that we

consume in the form of food. It comprises of many chemicals and enzymes, which help in the breakdown and hydrolysis of various components of food, which in turn facilitates better absorption of nutrients. Compared to *in vivo* studies using live animals, *in vitro* studies are easier to conduct due to no ethical issues involved (Wang *et al*., 2018). Various methods have been standardized to carry out *in vitro* digestion analysis; however, recent global consensus method proposed by Minekus *et al*. (2014) and by Brodkorb et al. (2019) is now widely accepted. A typical *in vitro* digestion set-up consists of three phases, oral, gastric and intestine. During the oral phase of digestion, α-amylase plays a major role in the digestion of starch. It should be considered as an important phase for the digestion of emulsions containing starch fractions (Brodkorb et al., 2019). The gastric phase of digestion involves several enzymes and chemicals, HCl being a major factor controlling the pH in the range of 2–3. Most of the protein digestion takes place in the gastric phase due to the presence of pepsin. In the case of emulsions stabilized by proteins, pepsin helps to digest most of the protein, coating the oil droplets, causing the lipid to be exposed, thereby promoting lipid digestion in the intestine phase (Guevara-Zambrano *et al*., 2022). The intestinal phase mostly comprises of lipid digestion, although protein digestion can also be observed due to the presence of proteolytic enzymes. The intestinal phase involves many salts and enzymes, including bile, trypsin, chymotrypsin, co-lipase and lipase. Bile helps solubilize fats and fat-soluble bioactives, whereas chymotrypsin and trypsin are involved in protein digestion (Brodkorb *et al*., 2019). Co-lipase is responsible for activating the lipase, which in turn promotes lipid hydrolysis (Tan *et al*., 2022). Simulating a complex digestion process *in-vitro* makes it more flexible for researchers to understand how numerous factors and emulsion composition could affect lipid and protein digestibility and bioaccessibility of encapsulated bioactives. Bioaccessibility is a measure of release of lipophilic bioactives in the simulated small intestine, which can be correlated to their bioavailability, which is a measure of uptake of the bioactives from the intestine into the blood circulation system (McClements and Rao, 2011).

In the recent times, many studies have investigated the effect of emulsion structure, type of emulsifier and droplet size on the lipid digestibility of an emulsion (Tan *et al*., 2022). The lipid phase content plays a major role in determining the maximum amount of bioactive substance that can be incorporated into the delivery system, as well as the rate and extent of digestion of lipid for

bioaccessibility of the bioactives (Zhang *et al*., 2006). Emulsifiers may also influence lipid digestion and bioaccessibility due to their ability to interact with components present in gastrointestinal fluids such as enzymes, calcium ions or bile salts (McClemments *et al*., 2007). The results from many studies suggest that the bioaccessibility of hydrophobic bioactives can be enhanced by using an emulsifier that produces small oil droplets during homogenization and digested easily and that are also resistant to extensive aggregation within the gastrointestinal tract (Tan *et al*., 2022; McClemments *et al*., 2007).

3. MATERIALS AND METHODS

3.1 Materials

Pea protein concentrates, PP55 and PP55P30 were kindly donated by AGT Foods and Ingredients (Saskatoon, SK, Canada). Canola oil was obtained from a local grocery (Saskatoon, SK, Canada). Sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate, sodium hydroxide and hydrochloric acid were purchased from Fisher Scientific (Whitby, Ontario, Canada). Sodium azide and glacial acetic acid were purchased from Sigma-Aldrich (Milawaukee, WI, USA). Sodium chloride was purchased from VWR International (Edmonton, AB, Canada). The water used in this study was Milli-Q^{TM} (EMD Millipore, Billerica, MA, USA). For *in vitro* digestion, all the enzymes and chemicals such as porcine pepsin (P7012), pancreatin lipase (L3126), bile extract (B8631), porcine pancreatin extract (8× USP specifications, P7545) and calcium chloride purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were analytical grade and purchased from Sigma-Aldrich.

3.2 Methods

3.2.1 Protein extraction

Initially 7 wt% PPC powder dispersions were made and left overnight for stirring at 220 rpm at room temperature (25 ± 2 ºC), the pH of the solution being 6.4 followed by centrifugation on the next day. Initially, two step centrifugation was tested (step-1: 4,000×*g* for 1 min, step-2: 16,000×*g* for 30 minutes), this procedure was followed according to Geerts *et al*. (2017). Soluble protein fraction, starch-rich fraction and insoluble protein fractions were resulted after the 2-step centrifugation process (Figure 4). Protein quantification of the three fractions were done using the modified Lowry's method. Two step centrifugation process resulted in insignificant amount of insoluble protein after the second centrifugation and did not have much difference when compared to one step centrifugation process. Hence, one step centrifugation was opted which helped to save

time yielding similar quantity of protein in the soluble protein fraction. The protein quantities of starch-rich fraction and the insoluble protein fraction were obtained by drying the samples overnight at 60°C and later solubilizing them with 0.1 M NaOH as they are insoluble in water. The protein yields in the various fractions are mentioned in Table 5.

$$Protein\ yield\ = \frac{Protein\ concentration\ in\ soluble\ fraction\ wt\%}{Protein\ concentration\ in\ protein\ concentrate\ wt\%} \times 100 \qquad (3.1)$$

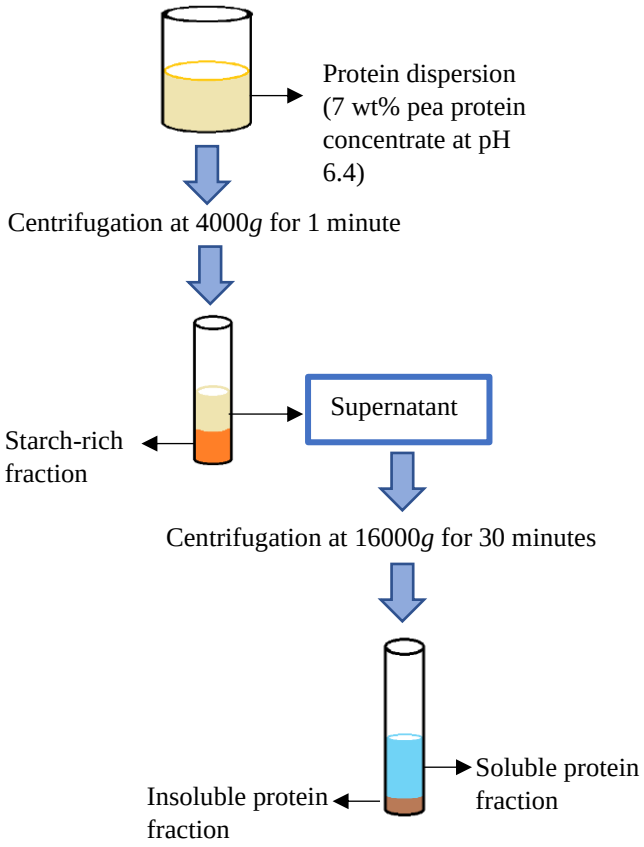

Figure 4: Flowchart describing the two-step centrifugation process to retain soluble protein fraction.

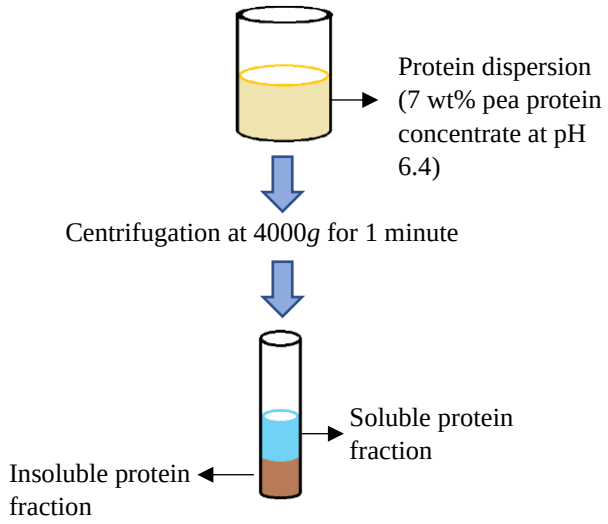

Figure 5: Flowchart describing the single-step centrifugation process to retain soluble protein fraction.

3.2.2 Emulsion preparation

After centrifugation, the supernatant was separated, and protein quantification was done for the wet fraction without drying. Once, the protein quantity in the soluble fraction was determined, the

concentration of the soluble protein for emulsion preparation was adjusted by adding water. O/W emulsions were prepared by mixing 5 wt% oil phase with 95 wt% aqueous phase. The emulsions were coarsely homogenized using a rotor-stator mixer (Polytron, Brinkmann instruments, Ontario, Canada) for 1 minute at 10,000 rpm, followed by high-pressure homogenization (EmulsiFlex-C3, Avestin Inc., Ottawa, ON, Canada) at a pressure of 20,000 psi for 6 cycles. Five different aqueous phase protein concentrations were used: 0.5, 1, 1.5, 2 and 2.5 wt%. The emulsions were stored in 50 ml glass vials for further analysis and visual observation.

3.2.3 Emulsion Characterization

3.2.3.1 Droplet Size

Droplet sizes distribution of all the emulsions were measured in fresh condition using a static laser diffraction particle size analyzer (Mastersizer 3000, Malvern Instruments, Montreal, QC, Canada) with a relative refractive index of the dispersed phase versus continuous phase as 1.465. For the emulsion with 2.5 wt% protein concentration, droplet size measurements were done for fresh emulsions as well as weeks old emulsions. The average droplet size of the emulsions was characterized by surface area mean diameter d(3,2). The dispersed phase during the measuring of droplet size was at the same pH and salt concentration of the respective emulsion.

3.2.3.2 Degree of flocculation

To test if there was any flocculation present, sodium dodecyl sulphate (SDS) was added to the emulsions and the droplet sizes were measured. SDS being a small ionic emulsifier can replace the protein at the interface and help in lowering interfacial tension, breaking any flocculation if present. Degree of flocculation (D of F) was calculated using the formula:

$$D\ of\ F\% = (d_{(3,2)}\ in\ water - d_{(3,2)}\ in\ SDS) / (d_{(3,2)}\ in\ water) \quad (3.2)$$

3.2.3.3 Zeta potential

Surface charge of the emulsions were measured using a Zetasizer Nano ZS 90 (Malvern Instrument, Westborough, MA, USA). The emulsion was diluted (200 µl of emulsion added to 100 ml of deionized water and the pH adjusted to the pH of the emulsion) to prevent multiple scattering peaks. The diluted emulsion was loaded into the cuvette to measure the electrophoretic mobility (U_E) of the droplets when a potential difference is applied. The procedure carried out by Primozic et al., 2018 was followed to measure the zeta potential. By using the Henry's equation, zeta potential is calculated using the electrophoretic mobility (U_E) of the samples:

$$U_E = 2\varepsilon \times \zeta \times f\,(k\,\alpha)3\eta \tag{3.3}$$

Where U_E is the rate of migration of charged particle per unit electric field strength (m/s), ε is the permittivity, $f\,(k\alpha)$, known as Smolulchowki approximation is a function associated with the ratio of the particle radius (α) to the Debye length (k), and η is the viscosity (mPa·s) of the dispersing medium (water, 1mPa·s). The Smoluchowki approximation $f\,(k\alpha)$ for this study was taken as 1.5.

3.2.3.4 Accelerated gravitational separation

The accelerated gravitational separation was carried out by using a photocentrifuge dispersion analyzer (LUMisizer, LUM Americas, Boulder, CO, USA). The emulsions were loaded into 8 mm × 2 mm cuvettes and centrifuged at 3,000 rpm (1,056×g) for 165 minutes. During centrifugation, the transmission of laser at 865 nm through the tube were collected every 10 seconds. The transmission profile reflected the droplet movement under the centrifugal force. Data analysis and determining creaming velocity were done with SEPview software, v 4.1 (LUM, GmbH, Berlin, Germany).

3.2.3.5 Change in the environmental conditions: addition of salt, changing pH conditions and heat treatment

To examine the stability of 2.5 wt% protein-stabilized O/W emulsions under different pH conditions the pH of one set emulsions was changed to pH 2 by adding 1 N HCl and pH of the other set of emulsions was changed to 7 by adding 1 N NaOH. Under each pH condition three

ionic strengths, 0.0 M, 0.1 M, and 0.5 M were tested by adding appropriate amounts of NaCl. The emulsions were also examined under heat treatments at 90°C for 30 minutes and later cooled down to room temperature. The droplet size, zeta potential and accelerated gravitational separation was measured for each sample with different pH, ionic strength and heat treatment. Droplet size and zeta potential were also examined for 1-week aged emulsions.

3.2.3.6 Viscosity of heat-treated protein emulsions

The viscosity of freshly prepared heat-treated protein emulsions was determined by a rheometer (AR G2, TA instruments, Montreal, QC, Canada) equipped with a 40 mm cross-hatched parallel plate geometry. The samples were loaded on the bottom Peltier plate using a spatula. The viscosity was measured by rotational shear between two parallel plates at 25°C with a gap of 1,000 µm as a function of increasing shear rate from 0.01 s^{-1} to 100 s^{-1}. The viscosity of the samples was recorded with the TRIOS software version 4.5.0.42498 (TA Instruments, Montreal, QC, Canada).

3.2.3.7 Microstructure of emulsions

Confocal microscope was used to study the microstructure of the pH 7 and pH 2, untreated and heat-treated emulsions without salt. For the confocal microscope, the emulsions were observed with a Nikon C2 microscope (Nikon Inc., Mississauga, ON, Canada) with a combination of 543 nm and 633 nm lasers, a 60× Plan APO VC (numerical aperture 1.4) oil immersion objective lens and 2.5 times digital zoom. For confocal microscopy, emulsions were prepared by adding 0.01 wt% Nile red dye (excitation by 543 nm laser, emission collected in 573-613 nm range) to the oil phase prior to the homogenization. Fast green (0.01 wt%) (excitation by 633 nm laser, emission collected using a 650 nm long-pass filter) was then added to the final emulsion sample to stain the proteins.

3.2.3.8 Lipid and protein digestibility

A static *in-vitro* digestion model with simulated gastric and intestinal conditions as proposed by (Minekus et al., 2014) was used to study the lipid and protein digestion of untreated protein

unheated emulsion, heated-treated protein unheated emulsion, heat-treated protein heated emulsion.

Preparation of stock solutions

The stock solutions of simulated gastric (SGF) and intestinal fluid (SIF) were made up with a mixture of various sodium, potassium and magnesium and ammonium salts according to Table. For each of the simulated fluid (gastric or intestine) the corresponding volumes of stock solutions were mixed and diluted up to 400 ml using deionized water. The volumes of stock solution stated in Table was calculated by (Minekus *et al*., 2014) to give a correct final electrolyte concentration of SGF and SIF at 500 ml, after the addition of emulsions, enzymes, bile extract, calcium salt solution and water. The $CaCl_2$ $(H2O)_2$ solution was added directly in the simulated digestion mixture to prevent Ca^{2+} precipitation.

Gastric phase

Untreated unheated, heat-treated unheated, heat-treated heated emulsions were homogenized at 20,000 psi at 2.5 wt% protein concentration in the aqueous phase. They were digested by simulated gastric fluid (SGF) in stomach simulating conditions. The SGF was prepared from the electrolyte stock solution according to the table. Simulated gastric digestion was carried out by adding SGF, pepsin with enzyme activity 3,200–4,500 U/ml (Sigma Aldrich, St. Louis, MO), $CaCl_2 \cdot 2(H_2O)$ and water according to the mentioned sequence. The pH of the mixture was maintained at pH 3 by adding 1N HCl to mimic the conditions in the stomach. The emulsion-SGF mixture (gastric chyme) was then incubated at 37°C in swirling motion in a dynamic water bath.

Intestinal phase

After 2 hours of incubation at pH 3 in the gastric phase, the chyme is ready to enter the intestinal phase. The pH is adjusted to pH 7 by adding 1N NaOH to create the ideal conditions of intestinal phase. The SIF (simulated intestinal fluid) is prepared by adding the required electrolytes mentioned in the table. SIF was followed by bile extract (B8631, Sigma Aldrich, St. Louis MO), porcine pancreatin enzyme (PPE) and porcine pancreatic lipase (PPL). $CaCl_2 \cdot 2(H_2O)$ was added prior to adding the PPE and PPL enzymes to activate the enzymes. PPE was added before PPL

as PPE contains co-lipase enzyme which is required for the activation of PPL (lipase) to carry out lipid hydrolysis. The percentage of free fatty acid release was calculated from the number of moles of NaOH needed for the neutralization of free fatty acid.

3.2.4 Protein characterization

3.2.4.1 Differential scanning calorimetry

Thermal properties of the protein samples were measured using the differential scanning calorimetry (DSC 8000, Perkin Elmer, Woodbridge, ON, Canada). The samples were weighed to 9–10 mg and thoroughly wetted with three volumes of distilled water in stainless-steel pan. After being hermetically sealed, the sample was equilibrated at room temperature for at least 2 hours. The sample was being heated from 10 to 150°C at a rate of 10°C/minute, with an empty pan being used as the reference. The thermal parameters were determined using Pyris software (Perkin Elmer, Woodbridge, ON, Canada).

3.2.4.2 Interfacial tension

Interfacial tension of the protein solutions against canola oil was measured using Wilhelmy plate method via a K20 tensiometer (Kruss, Germany), operated at room temperature (25 ± 2 °C). A Wilhelmy plate was immersed 3 mm into 25 ml the 2 wt% aqueous dispersion after initial surface detection. Then canola oil (40 ml) was pipetted gently into the cup on top of the aqueous phase, and the plate was raised 3 mm back to the original position. IT was calculated using the following equation.

$$\sigma = F/(L \times cos\theta) \quad (3.4)$$

Where σ is the IT, F is the force detected by the force sensor, L is the wetted length of the plate, which is 40.2 mm, and θ is the contact angle between the aqueous phase and the plate. Since the plate was made of roughened platinum and it is optimally wetted, the contact angle would be 0. IT was recorded every minute for 30 minutes to obtain the values at equilibrium.

3.2.4.3 Intrinsic fluorescence

A spectrofluorometer (FluroMax-4, Horiba Jobin Yvon Inc., Edison, N.J., USA) was used to determine the intrinsic fluorescence for 0.01 wt% protein solutions made from freeze dried untreated and heat-treated protein solutions. A constant excitation wavelength of 295 nm (slit width 2.5 nm) and an emission range between 300 and 450 nm (slit width 5.0 nm, increment of 0.5 nm) was used to determine the selective fluorescence spectra of the aromatic amino acid tryptophan.

3.2.4.4 SDS PAGE (Gel electrophoresis)

Freeze-dried untreated and heat-treated proteins were subjected to electrophoresis using NuPAGETM BisTris Mini Gels. In brief, 30 μL (7.5 mg/mL) of the sample was mixed with 7.5 μL of NuPAGETM LDS Sample buffer (4×), 3 μL of NuPAGETM reducing agent (10×), 19.5 μL of deionized water, and the total volume was 60 μL. The sample was then heated at 70 °C for 10 minutes. The markers (Thermo Scientific PageRulerTM Plus Prestained Protein Ladder) were prepared with the same proportion, except the final volume was 40 μL. Next, 15 μL of sample and marker was loaded to the gel placed in an InvitrogenTM Mini Gel Tank with diluted (1×) 1 L NuPAGETM MES SDS Running buffer (20×). The SDS PAGE tank was then connected to a power supply (VWRTM Power Source TM 300 V, constant 200 V, 400 mA) until the electrophoresis was complete. After that, the gel was placed in a solution of 50 wt% ethanol and 10 wt% acetic acid and heated in the microwave for up to 35 s. This process was used to fix the proteins on the gel and remove the buffer components (mostly SDS) that might interfere with the staining process. The gel was then cooled down and transferred to a solution containing 400 μL Coomassie blue, 5 wt% ethanol and 7.5 wt% acetic acid for staining and was heated again in the microwave for up to 35 s and cooled down for 5 to 10 min. Finally, the gel was rinsed with distilled water a few times to remove the non-protein bound stain until the bands were clearly observed.

3.3 Statistical analysis

All experiments were conducted in triplicates. The results were reported as mean ± standard deviation. The results were analyzed by using independent t-test and analysis of variance (ANOVA) with a 95% confidence level where $p < 0.05$ indicates a significant difference. The statistical analysis was done by using Microsoft Excel software.

4. RESULTS

4.1 Centrifugal separation of various protein fractions

Table 4 shows the dry weight and protein content of dry matter of the three fractions after the two-step centrifugation process. The dry weights of the fractions were determined by measuring the weight of each fraction before and after drying overnight at 60°C. The soluble fraction had the highest protein content when compared to the other two fractions. PP55 had higher protein content (83.3%) in the dry matter of the soluble fraction compared to PP55P30 (82.2%) ($p < 0.05$). The insoluble fractions of both PP55 and PP55P30 was very minimal and contained insignificant amounts of protein in it.

Table 4: Distribution of various fractions after two-step centrifugation process for the 7 wt% aqueous dispersion of PP55P30 and PP55

	PP55		PP55P30	
	Dry wt%	Protein content of dry matter (wt%)	Dry wt%	Protein content of dry matter (wt%)
Soluble fraction	42.8 ± 2.3	83.3 ± 1.6	43.4 ± 1.9	82.2 ± 2.1
Insoluble starch-rich fraction	50.1 ± 0.56	30.2 ± 1.2	49.4 ± 0.32	29.47 ± 0.8
Insoluble fraction	7.1 ± 0.02	0.004	7.17 ± 0.02	0.004
Total	100%	-	100%	-

From Table 4 it was concluded that the insoluble protein fraction had an insignificant amount of protein; hence two-step separation process would not be necessary. In the next step, one-step centrifugation was done in which only the starch fraction and the soluble protein fractions were obtained. Analysis was carried out to check whether protein retention was similar in the single-step centrifugation when compared to the two-step centrifugation process. The dry weight of various fractions and their protein content from one-step centrifugal separation is shown in Table 5. By comparing Tables 4 and 5, it was observed that single-step centrifugation yielded similar amounts of protein in the dry matter of the two fractions as in the two-step process. Therefore, single-step centrifugation was opted for mild fractionation of proteins to recover the soluble fraction for emulsification purpose.

Table 5: Distribution of various fractions after single-step centrifugation process for PP55P30 and PP55

	PP55		PP55P30	
	Dry wt%	Protein content of dry matter (wt%)	Dry wt%	Protein content of dry matter (wt%)
Soluble fraction	43.1 ± 3.1	83.3 ± 2.6	43.57 ± 2.8	82.2 ± 0.9
Insoluble starch-rich fraction	56.9 ± 1.4	30.2 ± 0.9	56.43 ± 1.6	29.47 ± 1.3
Total	100%	-	100%	-

Protein yield is a measure of the protein concentration of the soluble protein fraction retained from the pea protein concentrate after centrifugation. The protein yield of the soluble protein fraction of the two pea protein concentrates (PP55P30 and PP55) after the two-step and one-step centrifugal

separation process is shown in Table 6. One-step and two-step centrifugation led to similar protein yields in both the PP55 and PP55P30 samples. The soluble protein from PP55 concentrate had a slightly higher protein yield (71.1%) when compared to PP55P30 (70.2%), although the values are not statistically different ($p > 0.05$). Also, the sample PP55P30 was specially processed by AGT Foods and Ingredients after dry fractionation, while the sample PP55 was just dry fractionated. For these reasons, further studies on emulsion development were carried out using the soluble proteins retained from PP55 concentrate. A similar protein yield of 71.0% was observed in the soluble protein fraction when pea flour was subjected to mild fractionation at pH 6.8 in a study done by Moller et al. (2022).

Table 6: Protein yield of the soluble fractions after two-step vs. one-step centrifugal separation process

Protein concentrate	Two-step	One-step
PP55P30	70.21 ± 3.4	70.24 ± 3.7
PP55	71.1 ± 3.8	71.1 ± 4.1

4.2 Emulsion preparation and characterization

After the retention of the soluble protein fraction from PP55 concentrate, it was directly used as the aqueous phase for emulsion without any processing. The protein concentration of the soluble protein fraction was 2.5 wt%. This solution was further diluted at various degrees with deionized water to 0.5, 1.0. 1.5, 2.0 and 2.5 wt%. Emulsions were made with these five soluble protein preparations to determine the effect of protein concentration on emulsion stability.

4.2.1 Droplet size measurement

Initially, the emulsions were made with 0.5 and 1 wt% protein concentrations in the aqueous phase at pH 7. However, at these concentrations, the emulsions showed extensive creaming likely due to the insufficient amounts of proteins to cover all the droplet surfaces. Such conditions will lead to

sharing of proteins among the droplets, which is known as bridging flocculation (McClemments, 2004), leading to the creaming phenomena. Figure 6 shows the surface average droplet size d(3,2) for all the emulsions with different protein concentrations in their aqueous phase. The droplet size was measured with and without the presence of SDS to see if there was any flocculation. Small molecular weight surfactants such as SDS are known to displace proteins from the interface and separate the flocculated droplets due to their ability to lower interfacial tension to a greater extent (Gunning *et al*., 1999). The droplet size decreased with an increase in protein concentration of the aqueous phase. The highest droplet size was observed for emulsions containing 0.5% protein in the aqueous phase. Upon addition of SDS, the droplet size of the 0.5% protein emulsion decreased from 1.43 to 0.66 μm indicating there was extensive flocculation. With an increase in protein concentration, the difference between the average droplet size with and without SDS became smaller even though they had statistical difference ($p < 0.05$) for the emulsions with 1.5% and higher protein concentrations in the aqueous phase. There was very minimal droplet flocculation at these higher protein concentrations. Nevertheless, the most stable emulsions in terms of average droplet size were obtained at 2.0% and 2.5% protein concentrations in the aqueous phase compared to the emulsions with lower protein concentrations. The average d(3,2) for the emulsion with 2.0% and 2.5% protein was 0.372 μm and 0.344 μm, respectively, without adding SDS. A similar decrease in emulsion droplet size with the increase in protein concentration was also observed before (Patel *et al*., 2019; Yerramilli *et al*., 2017). It was ascribed to providing enough protein to fully cover the oil-water interface in the emulsion. When the interface becomes saturated, no further decrease in droplet size could be observed at a specific oil-protein ratio. From the present work, it is apparent that the droplet surface became close to saturation at 2.5% proteins in the aqueous phase. Due to the limitation of the maximum amount of soluble proteins recovered from the mild fractionation process, it was not possible to further increase the protein concentration in the aqueous phase of the emulsions.

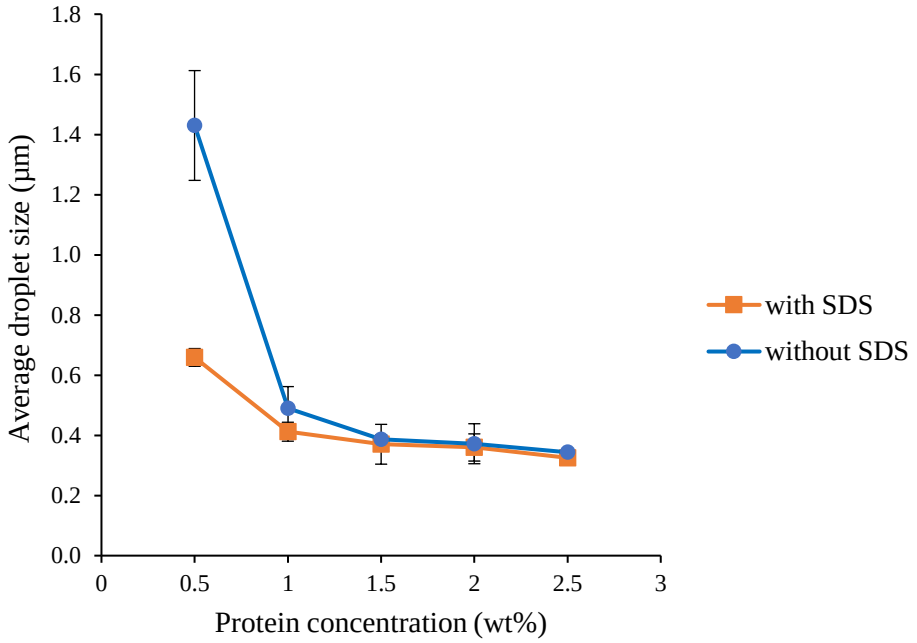

Figure 6: Average droplet size d(3,2) of emulsions with and without SDS as a function of soluble pea protein concentrations from 0.5 to 2.5 wt% in the aqueous phase at pH 7.

4.2.2 Emulsion creaming velocity under accelerated gravitation

The creaming velocity was calculated from the photo-centrifuge transmission profiles collected at 3,000 rpm (1,056×*g*) for a period of 165 minutes. The creaming velocity of the emulsions decreased gradually as the concentration of the protein in the aqueous phase increased from 0.5 wt% to 2.5 wt% (Figure 7). The highest creaming velocity (17.35 µm/s) was observed for the 0.5 % protein emulsion. In contrast, the lowest creaming velocity (2.74 µm/s) was observed for 2.5 wt% protein emulsion, although the creaming velocity at 2 wt% proteins was also not significantly different ($p > 0.05$) from that at 2.5 wt% proteins. It indicates that 0.5 wt% protein took the least time to show creaming, and the time required for creaming increased as the protein concentration increased. This shows that the stability against accelerated gravitation increased with an increase in protein concentration and a decrease in droplet size. Due to the lowest creaming velocity and no indication of flocculation for the emulsions prepared with 2.5 wt% proteins, it was selected for further analysis in Section 4.3.

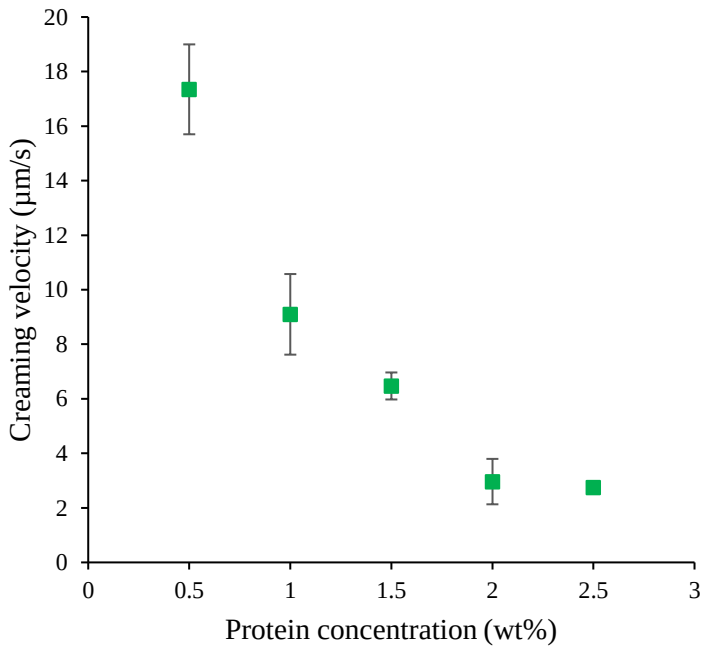

Figure 7: Creaming velocity at a centrifugal speed of 1050 g for emulsions as a function of aqueous phase protein concentrations from 0.5 to 2.5 wt% at pH 7 (obtained via photocentrifuge LUMiSizer).

4.2.3 Emulsion zeta potential

Zeta potential is the droplet charge and influences various properties of an emulsion, such as repulsive interactions among the droplets and, ultimately, emulsion long-term stability (Dickinson, 2010). It is mainly influenced by the pH and ionic strength of the emulsion. The zeta potentials of the emulsions prepared with different soluble protein concentrations in the aqueous phase at pH 7 are presented in Figure 8. A high absolute value of zeta potential is desired for emulsion stability. It was observed that the zeta potential of the emulsions remained similar at all protein concentrations ($p > 0.05$). The average zeta potential of the emulsions prepared with 0.5 to 2.5 wt% soluble proteins ranged from -31.17 to -48.95 mV. The emulsion containing 2.5 wt% protein in the aqueous phase had a zeta potential of -41.18 mV. Since pH 7 is higher than the pea protein isoelectric point, it is expected that the protein would have a negative zeta potential. When the proteins are adsorbed on the oil droplet surface, the droplets would also get a similar charge as the proteins. Since the absolute value of the zeta potential of emulsion droplets is more than 30 mV,

it is expected that the emulsions would be stable. It was observed in some research studies that emulsions made using pea protein concentrates and pea protein isolates had zeta potentials around -60 to -50 mV (Keivaninahr *et al*., 2021). The difference in zeta potential could be attributed to the difference in the composition of emulsions. Keivaninahr *et al*. (2021) made their coarse emulsions using a high-speed blender by dispersing 40 wt% canola oil in an aqueous phase containing 2 wt% protein, and 0.25 wt% xanthan gum. In contrast, the emulsions made in the current research were made using a high-pressure homogenizer and contained only 5 wt% oil and 2.5 wt% soluble proteins in the aqueous phase. Removal of the insoluble components in the pea protein concentrate in the current study might have attributed to a lower value of zeta potential in the current study compared to Keivaninahr *et al*. (2021). Also, a much lower protein-to-oil ratio in the previous study might have resulted in more opening of the protein molecule on the oil droplet surface, leading to a larger exposure of charged groups and an increase in emulsion zeta potential. Zhi *et al*. (2022) have also observed that pea protein-stabilized emulsions at pH 7 have zeta potential around -38 mV. Their emulsions (average droplet size 0.58 μm) were made with 15 wt% oil with an aqueous phase containing 1.5 wt% commercial pea protein isolate using a microfluidizer at a high pressure. Whereas, in the current research the emulsions were made with the soluble pea proteins extracted from pea protein concentrate using a high-pressure homogenizer. Similar processing conditions (high-pressure homogenization) of the emulsions might have contributed to the similarities in the zeta potential reported in the current research and the study done by Zhi *et al*. (2022). Differences in processing conditions of the pea protein, environmental conditions during the preparation of the emulsion, different pea cultivars could contribute to the differences or similarities in zeta potentials of pea protein emulsions.

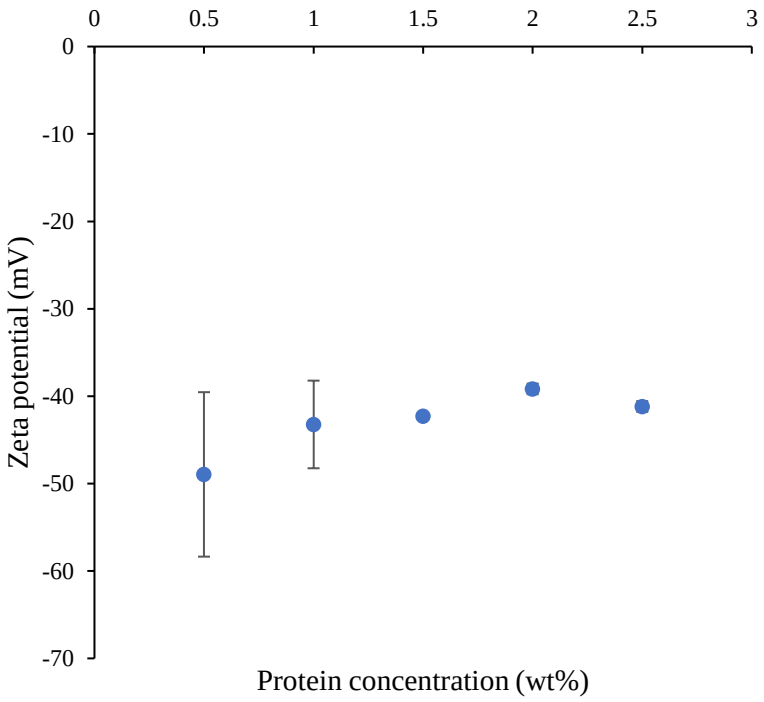

Figure 8: Zeta potential of the emulsions as a function of protein concentrations from 0.5 to 2.5 wt% in their aqueous phase at pH 7.

4.2.4 Degree of flocculation

The degree of flocculation was calculated to check which emulsions showed the maximum flocculation and thereby determine which emulsion would more stable to creaming and phase separation under long-term storage. The lower the degree of flocculation, the greater the stability of the emulsion. The emulsions prepared with 0.5 wt% soluble proteins had the highest degree of flocculation (53.34%) (Figure 9), which could be due to the extensive bridging flocculation at a very low protein concentration as the proteins tried to cover multiple droplets. The degree of flocculation decreased as the protein concentration in the aqueous phase of the emulsions increased. The emulsions containing 1.5, 2 and 2.5 wt% of protein showed a lower degree of flocculation, 3.35 ± 2.16%, 2.48 ± 1.28% and 5.34 ± 2.98%, respectively ($p < 0.05$). Due to relatively low degree of flocculation, lower average droplet size and creaming velocity, along with higher zeta potential for the emulsion prepared with 2.5 wt% protein, it was considered for further analysis of the effect of various environmental conditions on emulsion stability (Section 4.3).

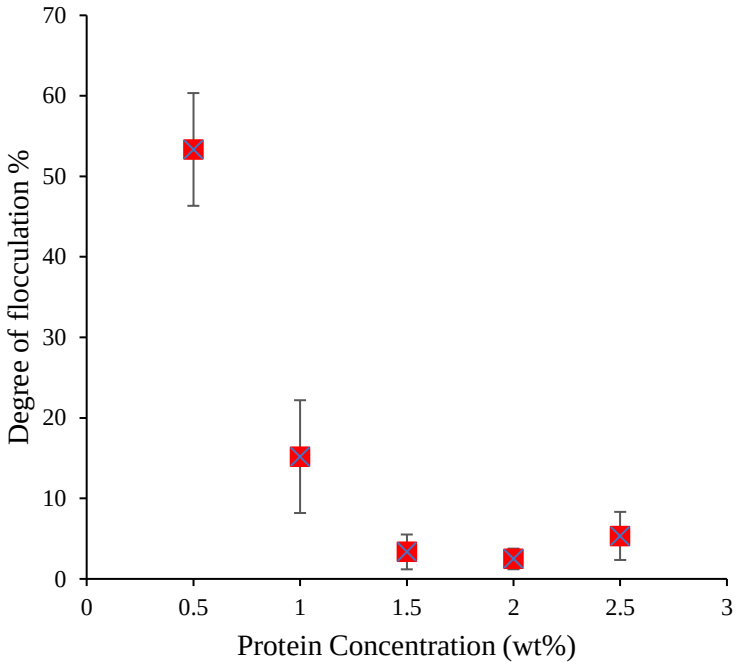

Figure 9: Degree of flocculation % of the various emulsions as a function of protein concentrations from 0.5 to 2.5 wt% in their aqueous phase at pH 7.

4.3 Effect of different environmental conditions (pH, ionic strength, and heat treatment) on emulsion stability

4.3.1 Effect of different environmental conditions on emulsion droplet size

Emulsions prepared with 2.5 wt% proteins were examined under different ionic conditions at two pH levels (pH 2 and 7) and under heat treatment to check their stability. All emulsions showed aggregation when heated to 90°C for 30 minutes and cooled to room temperature. For example, at pH 7, after heat treatment, the surface average droplet size was increased to 0.472 µm, 0.567 µm, 0.945 µm and 1.175 µm at ionic strengths of 0.0 M, 0.1 M, 0.5 M, and 1.0 M, respectively, compared to the emulsion without heat treatment (0.326 µm, 0.344 µm, 0.348 µm, and 0.330 µm at ionic strengths 0.0 M, 0.1 M , 0.5 M, and 1.0 M, respectively) (Figure 10a). It is known that a high salt concentration (above 0.1 M of NaCl) would lead to extensive droplet aggregations due to ionic bindings and charge screening effect (Dickinson, 2010). However, in the present case, the soluble pea protein-stabilized unheated emulsions were surprisingly stable without any sign of droplet aggregation (Figure 10 a). It was also observed in the work done by Keivaninahr *et al*.

(2021) that droplet sizes remained similar at pH 7 at various salt concentrations. The addition of salt generally induces electrostatic charge screening, leading to droplet aggregation. However, addition of salt may also alter the conformation of the adsorbed protein molecules leading to thicker interfacial layer that increases the steric repulsion between the droplets making the emulsion stable (Parsons & Salis, 2015). Alternatively, some plant proteins are stable to aggregation to salt, this can be due to the increase in protein solubility with increase in salt content due to the ability of salts to weaken the attractive interactions between protein molecules (salting in effect) (Aluko & Yada, 1995).

Interestingly, heating led to a increase in droplet size, and the effect of salt on the heat-treated emulsions was far worse than the unheated emulsions. For example, without any salt, upon heat treatment, droplet size increased from 0.326 µm to 0.472 µm, while at 1.0 M salt, upon heat treatment, the droplet size increased from 0.330 µm to 1.175 µm. Heat unfolds globular pea proteins on the surface of the droplets, followed by inter-droplet attraction due to hydrophobic interaction among the denatured proteins in heated emulsions (Mession *et al*., 2013). A similar effect of heat-induced droplet aggregation in pea protein-stabilized emulsions was also observed by Keivaninahr *et al*. (2021) and Gummus *et al*. (2017). These studies observed that there was extensive droplet aggregation after heating them to high temperatures (90°C for 30 minutes).

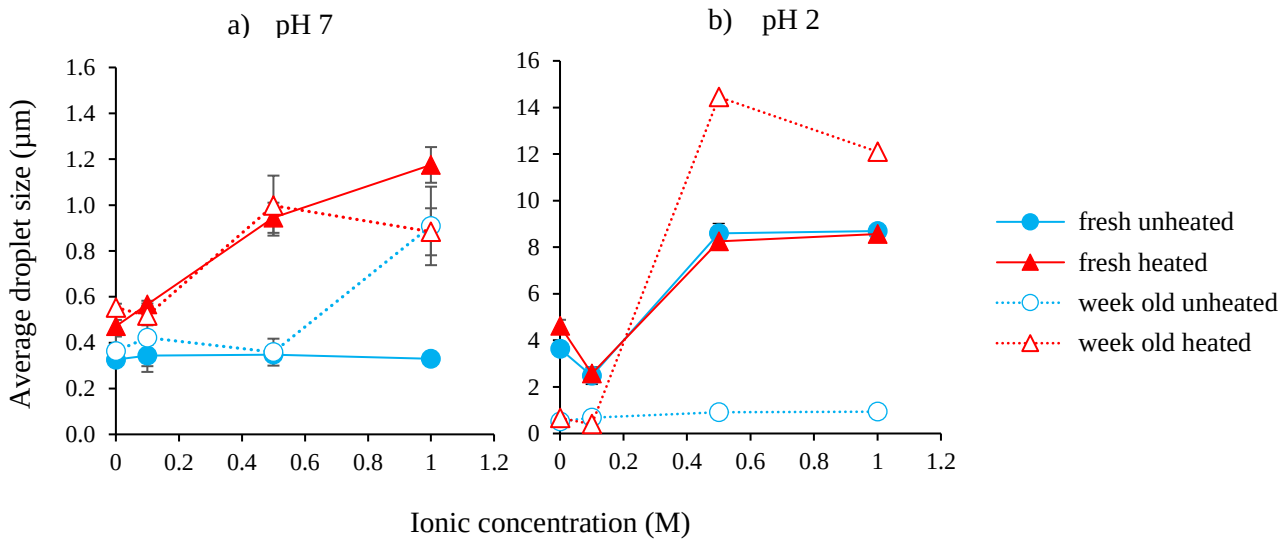

Figure 10: Average droplet size of emulsion as a function of different ionic concentrations (0–1 M) with and without heat treatment. Data for fresh and one week old emulsions are shown. (a) pH 7, (b) pH 2. Note that the Y-axis is different for Figure (a) and (b).

Changing the pH of the emulsions to pH 2 showed extensive aggregation even without any heating and without the addition of any salt (Figure 10 b). The average aggregate size reached 8.69 µm at pH 2 at 0.0 M ionic strength which was nearly 10-times higher than the corresponding value at pH 7 During lowering the pH of the emulsion, they pass through the isoelectric point at which the proteins lose their charge and extensively aggregate, (Patel *et al*., 2019) which could still remain even after reaching pH 2 leading to droplet aggregation. To minimize this aggregation, the pH was lowered very quickly but, there was still some droplet aggregation at pH 2. Such droplet aggregation under acidic pH was also observed by others when pea proteins were used to stabilize emulsions (Keivaninahr *et al*., 2021; Castellani *et al*., 1998). Castellani *et al*. (1998) have suggested a close relationship with the stability of amaranth globulin emulsion and its pH. They observed that protein was stable at pH 5 to 9, but by decreasing the pH to 3 the protein rapidly unfolded, which led to the aggregation of protein coated droplets. At acidic pH values, globulin proteins (11s globulins) undergo denaturation with dissociation into their sub-unit polypeptides. The dissociation of protein subunits at acidic pH might contribute to the higher hydrophobicity compared to higher pH values (Chang *et al*., 2015). At acidic pH, the protein loses stability due

to partial unfolding which causes the exposure of hydrophobic components leading to protein aggregation (Castellani *et al*., 1998). In the present case, it is possible that globulin proteins underwent complete dissociation and aggregation at acidic pH conditions. This contributed to the greater hydrophobicity and aggregation of the proteins causing emulsion destabilization. At pH 2, droplet aggregate size also increased to a large extent with the addition of 0.5 M and 1.0 M salt (from 3.63 µm and 4.62 µm, without salt to 8.59 µm and 8.25 µm, with 0.5 M salt and 8.69 µm and 8.56 µm, with 1.0 M salt) (Figure 10b) at unheated and heated conditions respectively. Such effect of ionic strength at acidic pH was also observed by (Keivaninahr *et al*., 2021). When the pH was changed from 7 to 2, the aggregate size increased by around 10-times, which was similar to the results observed in the current research. Keivaninahr *et al*. (2021) observed a slight increase in the droplet aggregate size with the addition of salt, whereas in this research we observed significant increase in aggregate size after the salt addition. This might be due to the high concentrations of salt being added (0.5 M and 1 M) in the present research compared to the previous work (0.16 M).

The droplet sizes were also measured for all the emulsions after a week to observe the effect of time. For the unheated and heated pH 7 emulsions, the droplet size seemed similar for the fresh and one week old emulsions (Figure 10 a; $p > 0.05$). It was observed that the droplet aggregate size for 0.0 M and 0.1 M heated emulsions decreased after 1 week. For 0.5 M and 1 M ionic strength emulsion at pH 2 the droplet size increased significantly from 8.25 µm to 14.44 µm and from 8.56 µm to 12.10 µm respectively after 1 week for the heated emulsion (Figure 10 b). The unheated pH 2 emulsions at 0.0 M and 0.1 M ionic concentration had the similar droplet size as that of the heated emulsion after 1 week. But at higher salt conditions (0.5 M and 1 M), the formation of larger aggregates can be observed due to salting out effect of emulsions. At acidic pH and high salt concentrations (0.5 M and above), pea proteins exhibit salting out effect which might be responsible for the large aggregate size at (Sun, 1975). The aggregate size of the 0.5 M and 1 M concentration heated emulsion at pH 2 after also showed similar aggregate sizes as that of the unheated emulsions at respective salt concentrations when fresh but they showed increased aggregate sizes after one week. Aggregates broke down with time at unheated conditions as the

interactions were weaker than the heated emulsions. Heating caused larger aggregate formation at 0.5 M and 1 M salt concentrations after one week when compared to fresh emulsions.

4.3.2 Effect of different environmental conditions on emulsion zeta potential

Emulsion zeta potential values as a function of pH, salt and heat treatment are shown in Figure 11. Pea protein has an isoelectric point at pH 4.5 where their charge would be neutral (Gumus *et al*., 2017). Therefore, at pH 2.0, below the isoelectric point, all emulsions showed positive zeta potential, while at pH 7, above the isoelectric point, it was negative. It was observed that at both pH values, the absolute values of zeta potential decreased as the ionic strength of the emulsions increased. The highest zeta potential (-34.65 mV) was observed for the emulsion that did not contain salt (0.0 M), which was decreased to -5 mV at 1.0 M salt. After a week of storage, the emulsion without salt showed an increase in zeta potential from -34.65 mV to -44.90 mV. Protein adsorption at the oil droplet surface has been reported and attributed to the surface denaturation of proteins leading to more exposure of the ionic groups towards the aqueous phase (Keivaninahr *et al*., 2021), the adsorption might have taken place over time leading to the increase in zeta potential after one week. Upon addition of salt, however, the lower values of zeta potential remained unchanged after one week of storage. Heated emulsions showed higher zeta potential at 0.0 and 0.1 M salt than emulsions without heat treatment. However, at a higher ionic strength, no significant difference in zeta potential was observed after heat treatment ($p > 0.05$).

For all the emulsions at pH 2, a similar trend in zeta potential of the emulsions was observed, which decreased as the ionic strength of the emulsions increased (Figure 11 b). For example, the zeta potential of unheated emulsions decreased from 28.17 mV without salt to 7.25 mV at 1.0 M ionic strength. Upon heat treatment at pH 2, no significant change in emulsion zeta potential was observed at any of the ionic strengths studied ($p > 0.05$). Similar to most samples at pH 7.0, after 1 week of storage, zeta potential at pH 2 also did not change significantly ($p > 0.05$).

The absolute value of the zeta potential of the emulsion without any salt was slightly higher at pH 7 than at pH 2. Upon addition of salt, the emulsion zeta potential decreased at a much faster rate

at pH 7 than that at pH 2, and at 1.0 M salt, zeta potential of unheated emulsions at pH 7 was -4.3 mV, while that at pH 2 was 7.25 mV ($p < 0.05$).

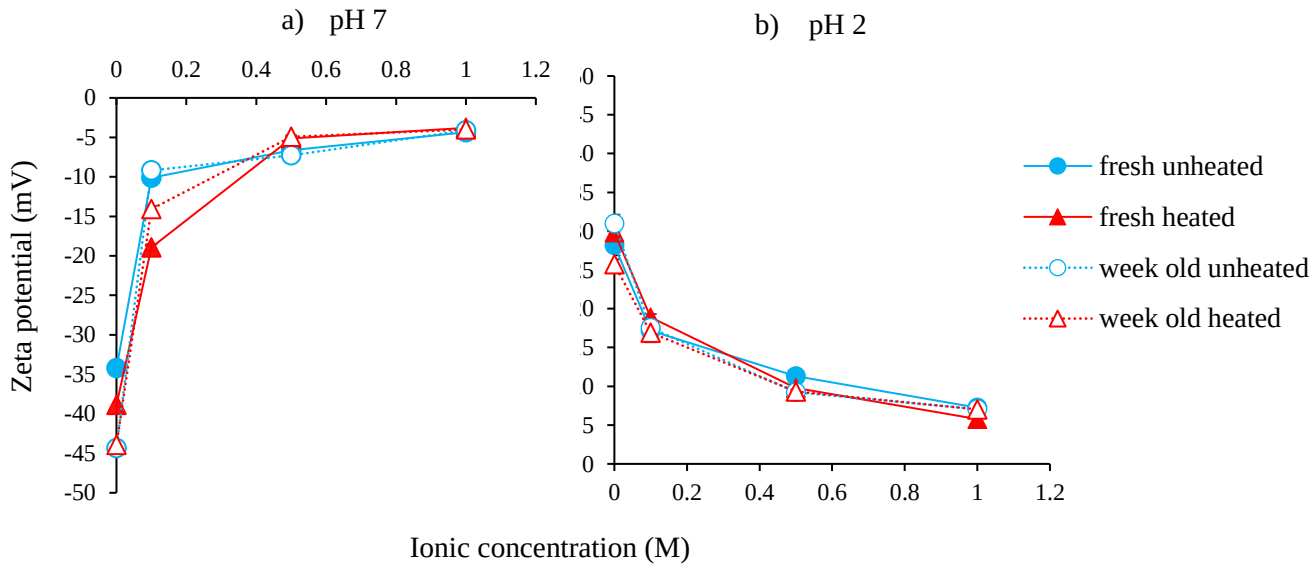

Figure 11: Zeta potential of emulsions as a function of aqueous phase with different ionic concentrations (0.0 M, 0.1 M, 0.5 M, 1 M) with and without heat for fresh and one week old emulsions. (a) pH 7, (b) pH 2

4.3.3 Effect of different environmental conditions on emulsion visual observation

The visual observations of fresh emulsions are presented in Figure 12. The non-heated emulsions were stable at both pH 7 and pH 2 when they were fresh (data not shown). Upon heating at 90°C and cooling to room temperature, it was observed that the pH 7 emulsions showed extensive droplet aggregation leading to weak gelling and emulsion phase separation (Figure 12 a, b, c, d). The droplet aggregation and emulsion destabilization increased upon increase in salt concentration, the strongest aggregate was observed for emulsions containing 1 M NaCl. Interestingly, at pH 2, the emulsions did not show any visual indication of droplet aggregation upon heat treatment, and the emulsions flowed like a liquid when the glass vials were laid horizontally (Figure 12 e, f, g, h). With 1 M salt at pH 2, some indication of aqueous phase separation could be seen in Figure 12h. All emulsions at pH 2, however showed a large increase in aggregate size upon addition of salt and heat treatment (Figure 10 b). Such discrepancy between aggregate size and visual observation at pH 7 and pH 2 could be due to the strength of inter-droplet

interactions and size of the clusters of droplet aggregate. It is possible that at pH 7 droplet aggregates formed a larger network which could form a gel-like structure, but the aggregates broke down during mixing in the droplet size analyzer, leading to a smaller size compared to that at pH 2. At pH 2, on the contrary, the aggregates were stronger and could withstand the mixing and pumping through the droplet size analyzer, however, the aggregates did not form a space-spanning network, hence the emulsion could flow as viscous liquid and appeared stable.

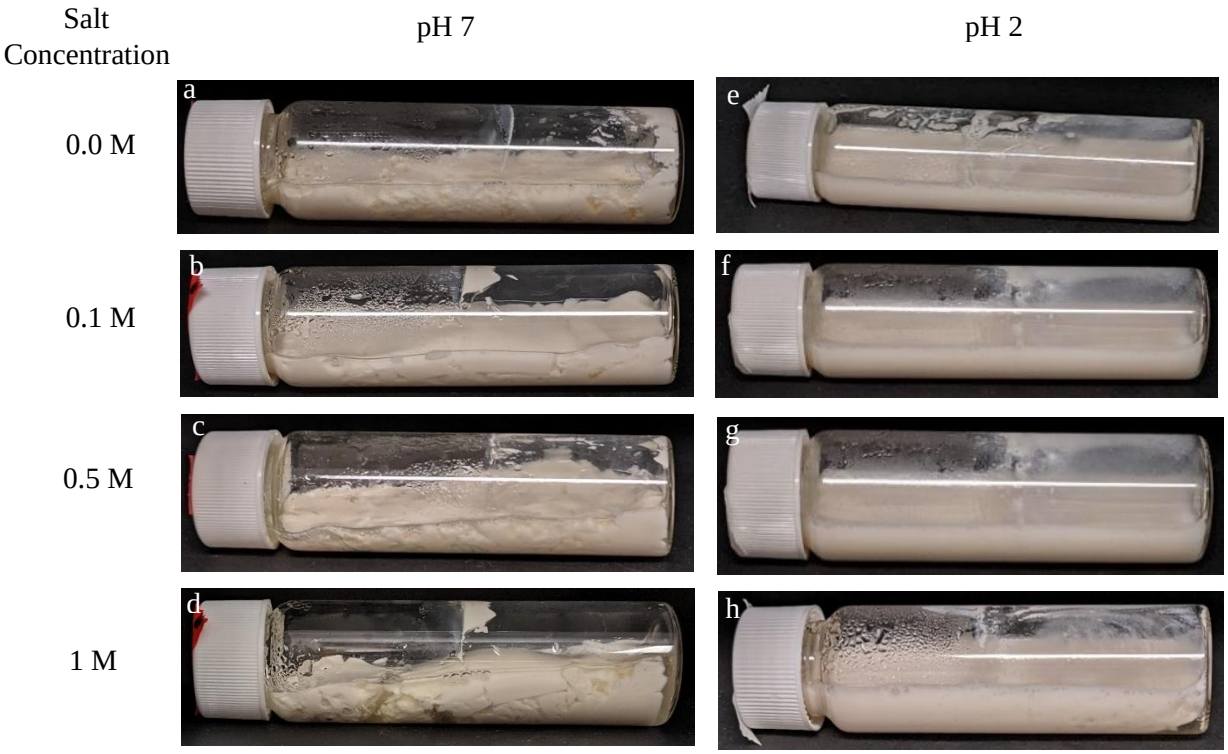

Figure 12: Visual observation emulsion flow behaviour after heating at 90ºC for 30 min as a function of different salt concentration. Salt concentrations and emulsion pH are shown on the figure. Emulsions in the glass vials were placed horizontally and photos were taken within 5 seconds.

The visual observations of one week old emulsions at pH 7 and pH 2 are presented in Figure 13 and 14 respectively. For the non-heated emulsions at pH 7, it was observed that the emulsion without salt showed aqueous phase separation after 1 week (Figure 13 a). However, with salt, after

1 week, the emulsions were stable without any phase separation (Figure 13 b, c, d). Enhanced emulsion stability due to salt is explained in section 4.3.1. For the heated emulsions at pH 7, however, after 1 week of storage, extensive droplet aggregation and separation of aqueous phase from the aggregated emulsions were observed, indicating syneresis of the aggregated droplets with time (Figure 13 e, f, g compared to Figure 12 b, c, d).

At pH 2, after 1 week, all non-heated emulsions showed aqueous phase separation irrespective of salt concentration, although the emulsion remain flowable, indicating creaming of flocculated droplets (Figure 14 a, b, c, d). For the heated emulsions at pH 2, after 1 week, the droplet showed signs of extensive aggregation and separation of aqueous phase, which increased with increase in ionic strength (Figure 14 e, f, g, h). Comparing pH 7 *vs.* pH 2, upon heat treatment, and after 1 week, both emulsions showed extensive droplet aggregation-induced destabilization, although visually, the emulsion at pH 7 appeared more affected by heat treatment compared to that at pH 2.

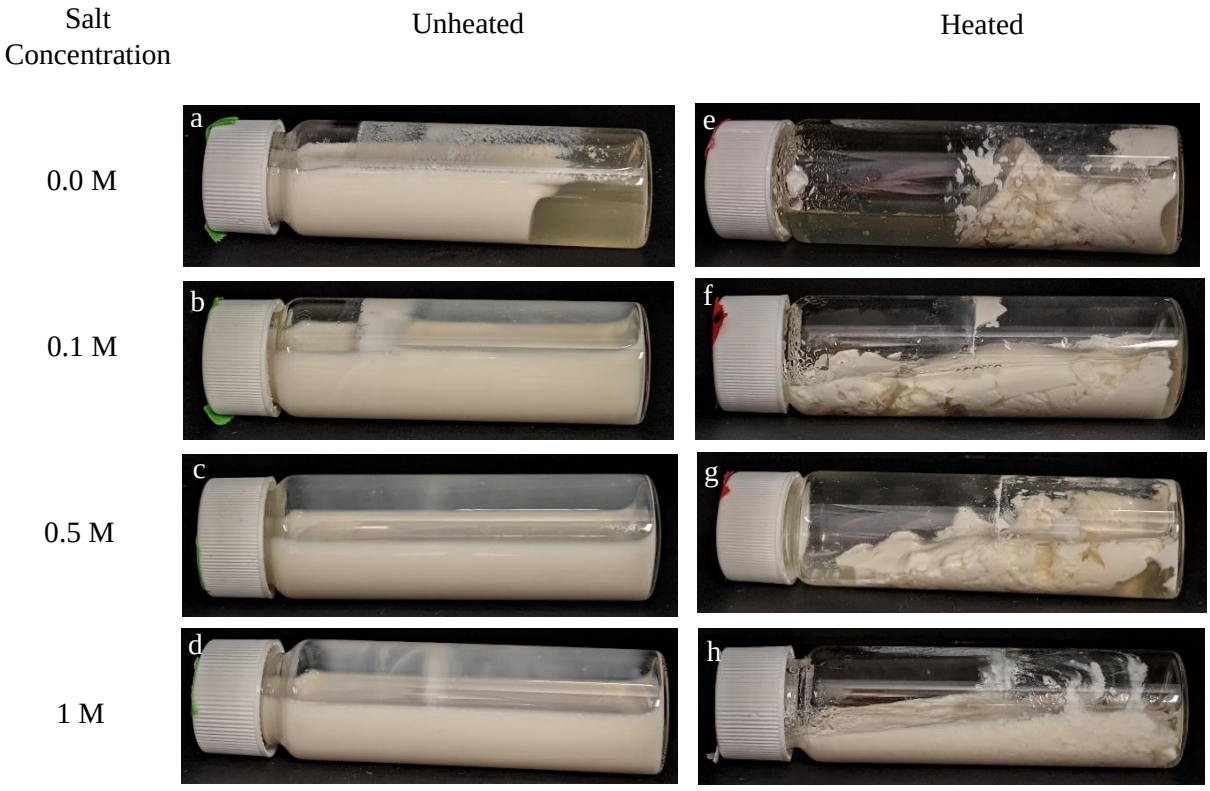

Figure 13: Visual observation of pH 7 emulsions after one week. The figure includes emulsions which were not heated and those which were heated for 30 minutes at 90°C and then cooled down to room temperature as a function of different salt concentration. Salt concentrations are shown in the figure. The photos were taken within 5 seconds after placing the vials horizontally.

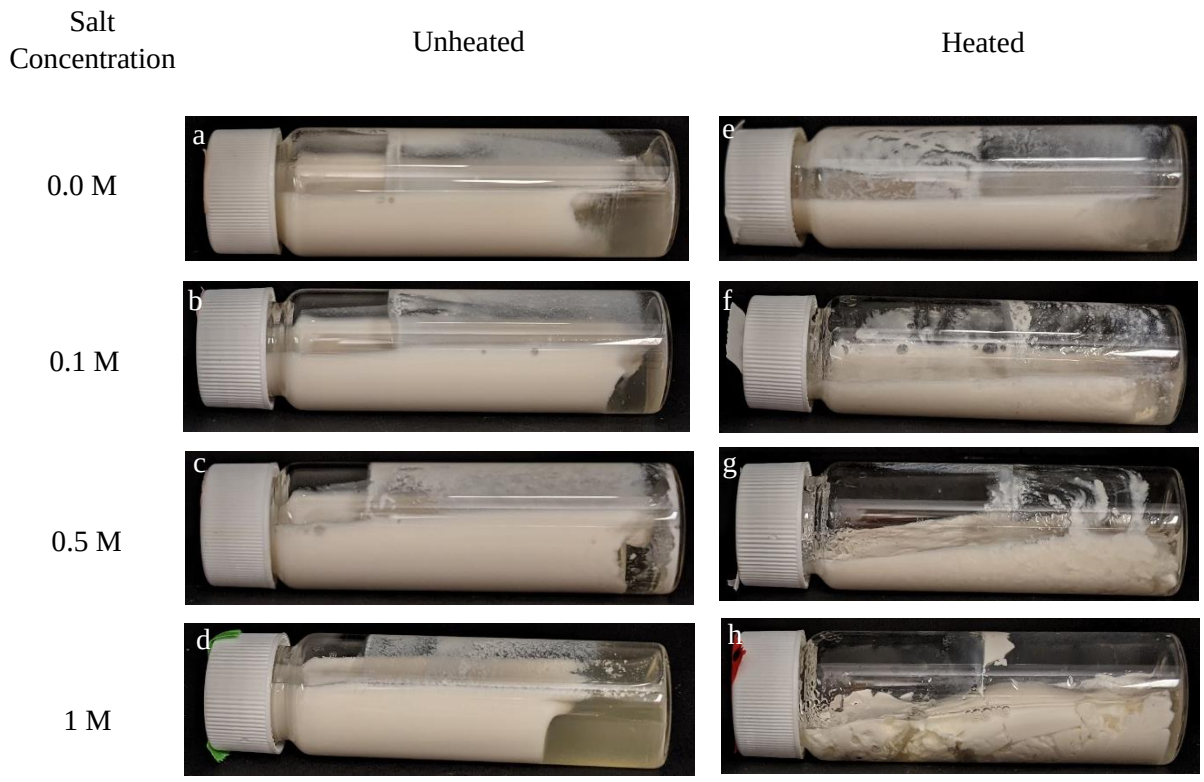

Figure 14: Visual observation of pH 2 emulsions after one week. This figure includes heated and non-heated emulsions as a function of salt concentration. The heating conditions are same as that of pH 7 emulsions. The salt concentrations are mentioned in the figure. The photos were taken 5 seconds after placing the vials horizontally.

4.3.4 Effect of different environmental condition on emulsion stability under accelerated gravitation

The creaming velocities of the non-heated emulsions under accelerated gravitation were lower than the heated emulsions for both the pH conditions indicating higher stability for the former (Figure 15). At pH 7, the creaming velocity of the unheated emulsions was 2.40 µm/s without salt, which decreased with an increase in ionic strength and reached 1.38 µm/s at 1 M salt. This matches with the visual observation that the addition of salt improved emulsion stability at pH 7 for the unheated emulsions. For the heated emulsions at pH 7, the creaming velocities increased from 6.00 µm/s without salt to 31.09 µm/s with 1M salt. At pH 2, without any salt, the creaming velocity of unheated emulsions was similar to the pH 7 unheated emulsions. However, the creaming velocity

rapidly increased with salt at pH 2 compared to pH 7 and reached a maximum value of 95.88 µm/s at 1 M salt. Such increase in creaming velocity at pH 2 matches with the aggregate size and visual observation of phase separation reported in Figures 10 b and 14 a, b, c, d, respectively. For the heated emulsions at pH 2, creaming velocity was similar to the unheated emulsions, which was much higher than pH 7 and increased with an increase in salt concentration. The aggregate size of the heated and unheated emulsions at pH 2 as a function of salt concentration was also very similar and increased to a large value with an increase in salt concentration (Figure 10 b), which led to their similar and high creaming velocities. It is possible that the proteins were already denatured at pH 2, before it was heated, therefore, it did not have much effect on the creaming velocity as the aggregates formed are pretty much similar in heated and unheated emulsions at pH 2. Overall, heat treatment had more detrimental effect on creaming velocities at pH 7 compared to pH 2, however the creaming velocities at pH 2 for both with and without heat treatment, and with salt were much higher than at pH 7. Larger aggregate size at pH 2 compared to pH 7 was certainly responsible for their higher creaming velocity. Unheated pH 7 emulsions were quite stable with salt leading to their lower creaming velocity. The gel-like structure observed in the pH 7 heated emulsion could also prevent their faster creaming as in the pH 2 emulsions, however, as observed in Figures 12 and 13, the pH 7 heated emulsions were extensively aggerated and the emulsion destabilized. Overall, it can be said that the soluble pea protein stabilized emulsions prepared in this work could not withstand the applied heat treatment due to heat-induced protein denaturation at the droplet interface and subsequent aggregation.

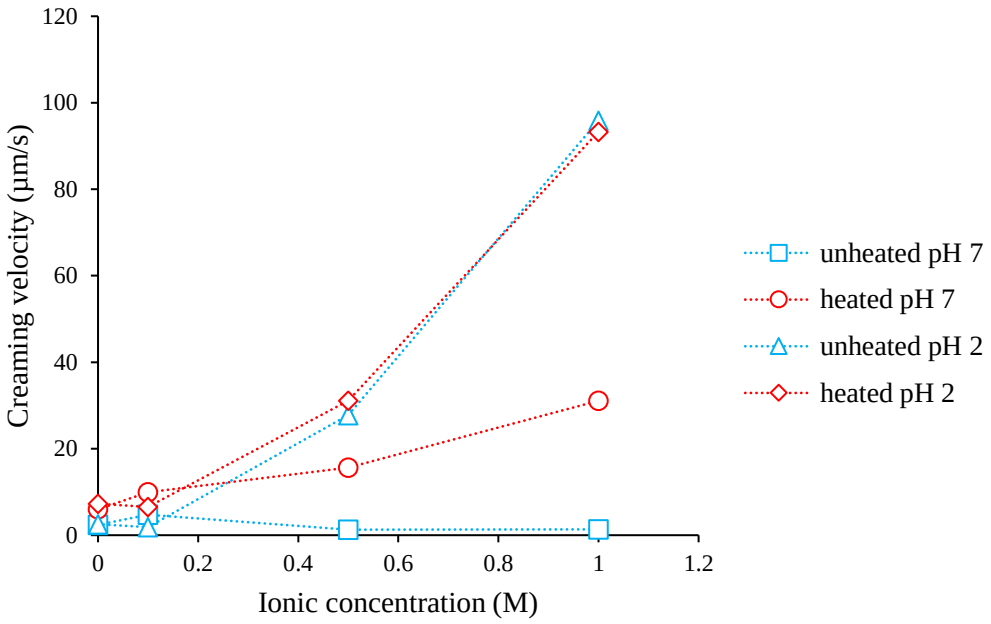

Figure 15: Calculated creaming velocities at a centrifugal force of 1050*g* as a function of ionic strength and emulsion pH (pH 7 unheated: square, pH 7 heated: circle, pH 2 unheated: triangle and pH 2 heated: diamond). Data for both heated (filled symbols) and unheated emulsions (open symbols) are shown. The creaming velocities were obtained using a LUMiSizer dispersion analyzer.

4.4 Heat-treatment of soluble protein to form heat-treated protein emulsions

After the application of different environmental stresses, it was observed that there was extensive destabilization of emulsions after the heat treatment at 90°C for 30 minutes. The emulsions became strongly aggregated resembling a weak gel. This heat treatment can be compared to pasteurization treatment given to products in the industry, hence it is a vital step in beverage processing, and cannot be omitted. Therefore, it was important to overcome this problem. To find an appropriate solution, it was hypothesized that if the emulsion is made with partially denatured proteins it would adsorb on the oil droplets with those partially opened hydrophobic patches, which might prevent their heat-induced aggregation and stop further emulsion destabilization. To test this hypothesis, the soluble protein solution was heated to 75°C, and the hot protein solutions were used to prepare the emulsions under similar heated conditions. The protein solutions were not allowed to cool down after the partial denaturation until the emulsions were made. It was known that complete denaturation of pea proteins happens at 88–99 °C and hence 75°C was chosen for partial

denaturation (Arntfield & Murray, 1981). In a research study by Chao & Aluko (2018), pea protein isolates were heated at different temperatures ranging from 50–100 °C, followed by cooling to room temperature and freeze drying. The authors then prepared emulsions with 1:5 canola oil and partially-denatured protein mixtures at pH 7 using a high-pressure homogenizer to obtain heat-treated protein-stabilized emulsions. Their research concluded that, even though the emulsions made with heat-treated proteins showed smaller droplet sizes at pH 7 and pH 3 when compared to emulsions made with untreated protein, heat-treatment did not improve emulsion stability. In the current research, the emulsions were made at the ratio of 95% aqueous phase (containing 2.5 wt% proteins) and 5% oil phase without allowing the proteins to cool down after heat treatment using high-pressure homogenization. The emulsions were later cooled to room temperatures. The emulsions prepared with heat-treated proteins were characterized similar to those of the untreated protein-stabilized emulsions including, droplet size, zeta potential and creaming velocity at different environmental stresses (salt, pH and heat treatment of emulsions). Apart from these characterization tests, viscosity of the heat-treated protein emulsions was also determined to understand the effect of protein processing and emulsion heat treatment on the emulsion flow behaviour. Since, the emulsion were made at the heated conditions, it is hypothesized that the hydrophobic patches would be exposed and not aggregated (Peng *et al*., 2016), and the exposed hydrophobic groups would help in better stabilization of the lipid droplets in the emulsion.

4.4.1 Droplet size of heat-treated emulsions

For the heat-treated protein-stabilized fresh emulsions at pH 7, the droplet sizes were similar for all the emulsions at different ionic concentrations (Figure 16 a). The effect of salt on emulsion stability at pH 7 is explained in section 4.3.1. The droplet size changed from 0.313 µm without any salt to 0.305 µm at 1.0 M ionic strengths ($p > 0.05$), which was similar to those prepared with untreated proteins (Figure 10 a) ($p > 0.05$). These emulsions were also stable and did not show aggregation over a period of one week. For the week-old pH 7 heat-treated protein-stabilized emulsions, the droplet sizes changed from 0.329 µm without any salt to 0.430 µm at 1.0 M ionic strength (Figure 16 a) ($p > 0.05$). Overall, there wasn't too much change observed in the droplet size of pH 7 heat-treated protein-stabilized emulsions over a period of one week, except for the one with 1.0 M salt. The most striking improvement in emulsion stability was observed when the

heat-treated protein-stabilized emulsions were heated at 90ºC for 30 min, which did not show any significant change from the unheated emulsions at pH 7 without salt (Figure 16 a). Even with 1.0 M salt, the average droplet size increased from 0.305 µm to only 0.381 µm ($p > 0.05$). The corresponding increase in droplet size for untreated protein-stabilized emulsion was nearly 3-times, from 0.472 to 1.175 µm (Figure 10 a) after the emulsions were heated. Even after one week of storage, no significant change in emulsion average droplet size was observed for the heat-treated emulsions. This shows that our hypothesis on improving emulsion stability against heat treatment by utilizing partially denatured proteins to prepare the emulsions was correct. Such an approach could be a valuable addition to the value-added utilization of pea proteins in developing stable beverage emulsions that could withstand the heat treatment used in food processing.

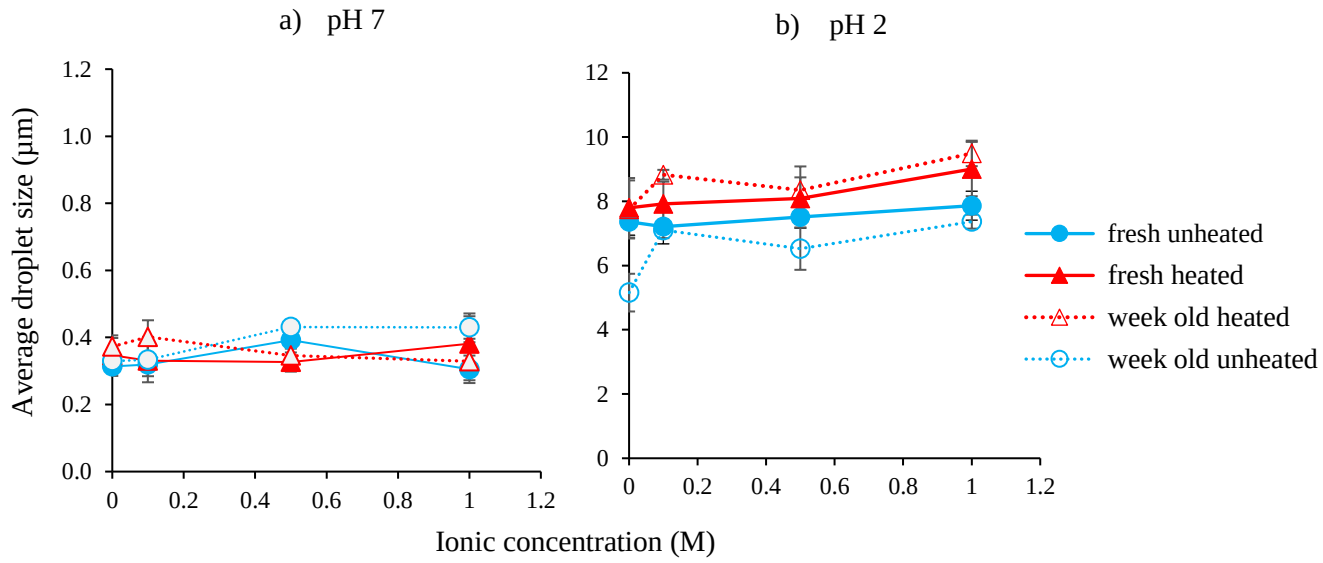

Figure 16: Average droplet size of heat-treated protein-stabilized emulsions as a function of ionic concentrations (0–1 M) with and without heat treatment. Data for fresh and one week old emulsions are shown. (a) pH 7, (b) pH 2. Note that Y-axis is different for Fig (a) and (b).

The situation was slightly different for the heat-treated protein-stabilized emulsions at pH 2. The average droplet sizes of pH 2 emulsions were much greater than pH 7 emulsions. Without any salt, the average droplet size increased from 0.313 µm at pH 7 to 7.36 µm at pH 2 (Figure 16 b), which was even higher than pH 2 emulsions prepared with untreated proteins (3.63 µm from Figure 10

b). However, with an increase in ionic strength, the droplet size of pH 2 emulsions prepared with heat-treated proteins did not change significantly (7.86 μm at 1.0 M salt, $p > 0.05$). After one week, the pH 2 heat-treated protein-stabilized emulsions showed relatively smaller droplet sizes than the fresh emulsions due to the breakdown of the aggregates with time. When the pH 2 heat-treated protein-stabilized emulsions were heated at 90ºC for 30 min, the average droplet size became slightly higher (statistically difference is insignificant) when compared to the corresponding unheated emulsions ($p > 0.05$). After one week, the droplet aggregate sizes of these emulsions remained unchanged at all ionic strength, which is in contrast with the emulsions prepared with untreated proteins (Figure 10 b), where a large increase in average size was observed at higher salt concentrations. Overall, the droplet and aggregate sizes of pH 2 emulsions were similar for unheated and heated emulsions under fresh and one-week-old conditions and with the range of ionic strength studied. It can be said that extensive aggregation was found in pH 2 emulsions irrespective of pre-heating the soluble protein before making the emulsions; however, the effect of salt was less severe for the former.

4.4.2 Zeta potential of heat-treated emulsions

At pH 7, zeta potential of both the heated and unheated emulsions prepared with heat-treated proteins was significantly different from each other ($p < 0.05$) (Figure 17 a). Zeta potential decreased with the increase in salt concentration, attributed to the charge screening effect. There was a significant decrease in the zeta potential when the salt concentration of the emulsion increased from 0.0 M to 0.1 M from -35.8 mV to -9.83 mV. After that, the zeta potential did not decrease much with increase in salt concentration, became -5 mV at 1 M. The zeta potential of the heat-treated pH 7 emulsions did not change significantly compared to the unheated emulsions at all ionic strength ($p > 0.05$).

At pH 2, the heat-treated protein-stabilized emulsions' zeta potentials decreased from 17.29 mV to 6.29 mV with an increase in ionic strength from 0.0 to 1.0 M (Figure 17 b). For the heated pH 2 emulsions, the zeta potential decreased from 20.76 mV to 6.93 mV with an increase in ionic strength from 0.0 to 1.0 M, which was not significantly different than the unheated emulsions (p

> 0.05). The zeta potential values of the heat-treated protein-stabilized emulsions, at pH 2 without salt, were significantly lower than the zeta potential of the corresponding emulsions prepared with untreated proteins (Figure 11 b). This could be due to the partial denaturation of the proteins, which changed the interfacial orientation of the proteins under acidic pH leading to lowering of surface charge, which was also observed in the study done by Keivaninahr *et al*. (2021).

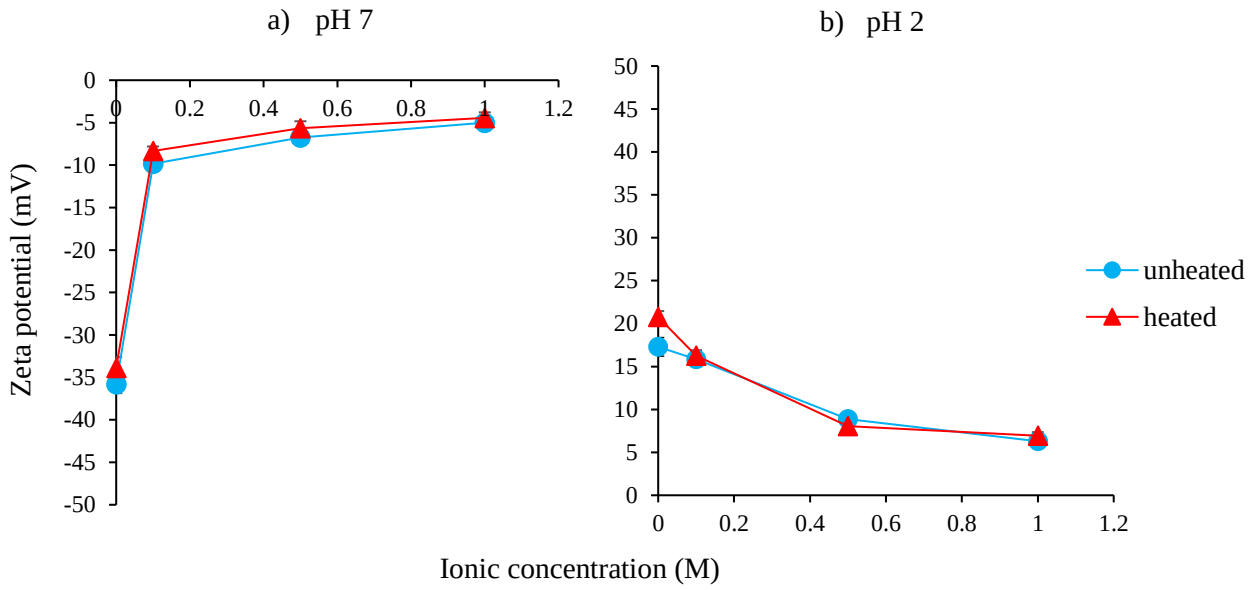

Figure 17: Zeta potential of heat-treated protein-stabilized emulsions as a function of ionic concentrations (0–1 M) with and without heat treatment. (a) pH 7, (b) pH 2.

4.4.3 Effect of different environmental conditions on heat-treated protein emulsion visual observation

The visual observations of fresh pH 7 and pH 2 heat-treated protein-stabilized emulsions are presented in Figure 18. These emulsions were thermally treated at 90°C for 30 minutes by placing them in a water bath followed by cooling to room temperature. Even after the heat-treatment we can observe that both the pH 7 emulsions and the pH 2 emulsions were flowing and showed the no signs of aggregation. The heated pH 7 emulsions show similar properties at all salt concentrations. These emulsions, after heat treatment, did not show extensive aggregation or

stabilized emulsions which were heated (Figure 12). This indicates that preheating the proteins and making emulsions under hot condition, without allowing the partially denatured proteins to cool down, significantly improved emulsion stability against heat treatment

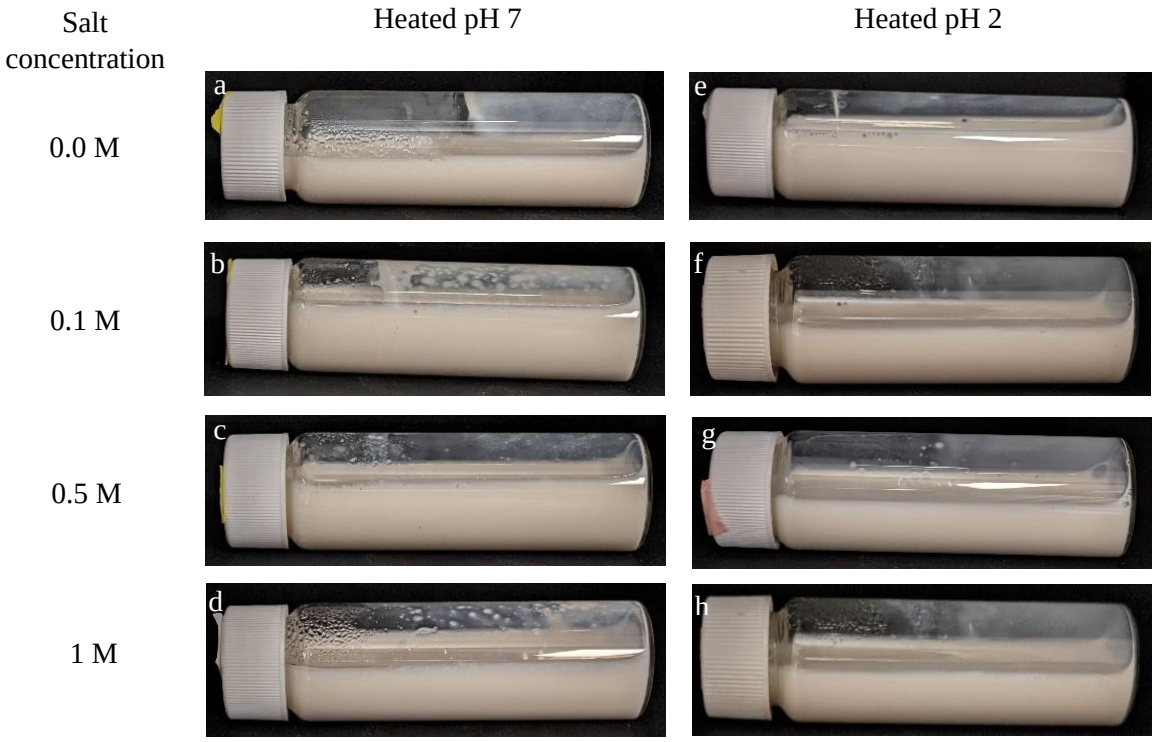

Figure 18: Visual observation of heat-treated protein-stabilized emulsions' flow behaviour after heating at 90ºC for 30 min and then cooled down to room temperature at different salt concentrations. Salt concentrations and emulsion pH is shown on the figure. Emulsions in the glass vials were placed horizontally and photos were taken within 5 seconds.

The visual observations for week-old pH 7 and pH 2 heat-treated protein-stabilized emulsions are presented in Figure 19 and Figure 20, respectively. For the pH 7 unheated emulsions (Figure 19 a, b, c, d), there was not any sign of aggregation, and the emulsions did not show any signs of creaming or destabilization after one week of storage. The corresponding untreated protein-stabilized emulsions without heating at pH 7 showed some phase separation at 0.0 M ionic strength, which disappeared, and the emulsions became stable with increasing ionic strength

emulsions (Figure 13 a, b, c, d). The increasing salt concentrations did not have much effect on the stability of the pH 7 unheated emulsions, they seemed to have similar flow behaviour when observed visually. The visual observation is also similar to the droplet size results, where untreated and heated emulsions have similar droplet sizes at all salt concentrations even after one week. This in in complete contrast with the untreated protein-stabilized emulsions after heating, where complete emulsion destabilization due to extensive droplet and protein aggregation was observed. The enhanced stability in heat-treated soluble proteins emulsions at heated conditions is explained in section 4.4.1.

The heat-treated protein-stabilized, pH 2 emulsions at unheated conditions after one week showed a slight sign of aggregation (apparently from the emulsion sticking to the top of the glass surface) but still had flowing behaviour and did not show any creaming sign of aqueous phase separation (Figure 20 a, b, c, d). This is also an improvement compared to the aqueous phase separation as that observed in Figure 14 a, b, c, d for the corresponding untreated protein-stabilized emulsions. Finally, the increasing salt concentration did not have much effect on the heat-treated protein emulsions at pH 2 after one week. The heated pH 2 emulsions prepared with heat-treated proteins showed some sign of aggregation, which increased with ionic strength (Figure 20 e, f, g, h), but not as much as the aggregation observed in the corresponding untreated protein-stabilized emulsions at untreated pH 2 emulsions at heated conditions (Figure 14 e, f, g, h).

Overall, making emulsions with heat-treated proteins in their partially denatured conditions improved the stability of the emulsions to a great extent when compared to untreated protein-stabilized emulsions. The damaging effects at pH 2 in the untreated protein-stabilized unheated emulsions are also decreased to a certain extent by making the emulsions with partially denaturated soluble proteins.

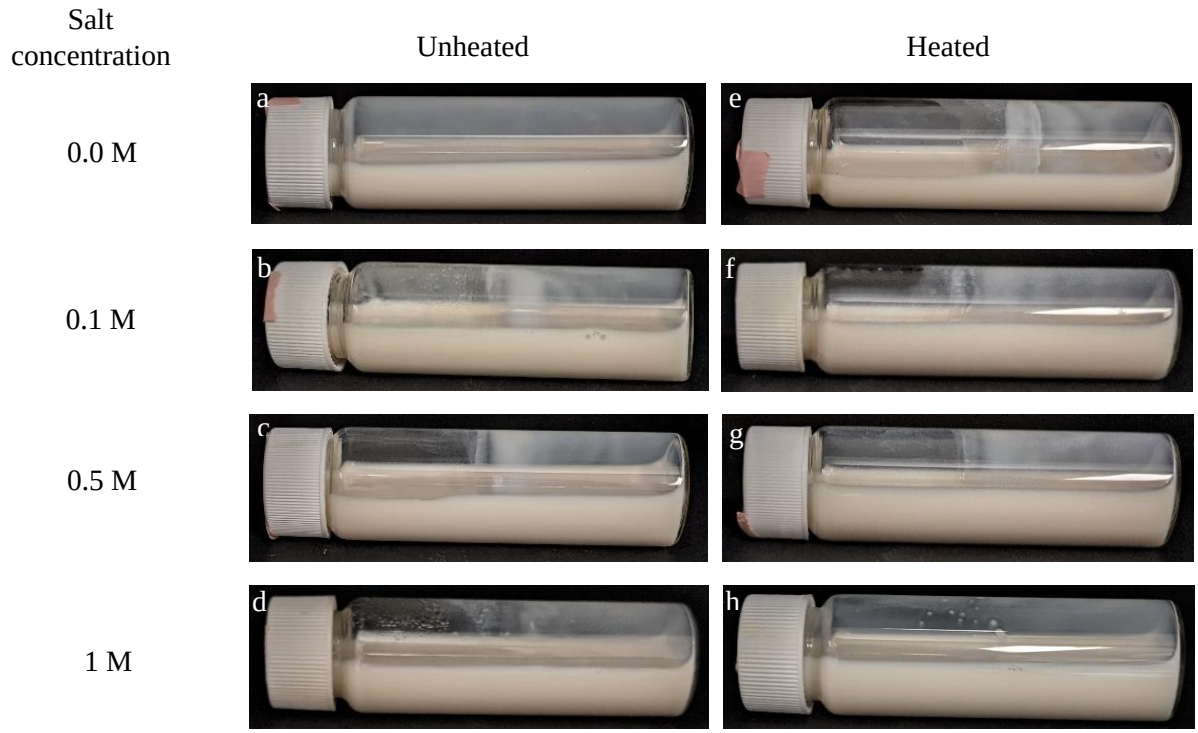

Figure 19: Visual observation of pH 7 heat-treated protein-stabilized emulsions after one week. The figure includes emulsions which were not heated and those which were heated for 30 minutes at 90°C and then cooled down to room temperature at different salt concentrations. Salt concentrations are shown in the figure. Emulsions were placed horizontally, and the photos were taken within 5 seconds.

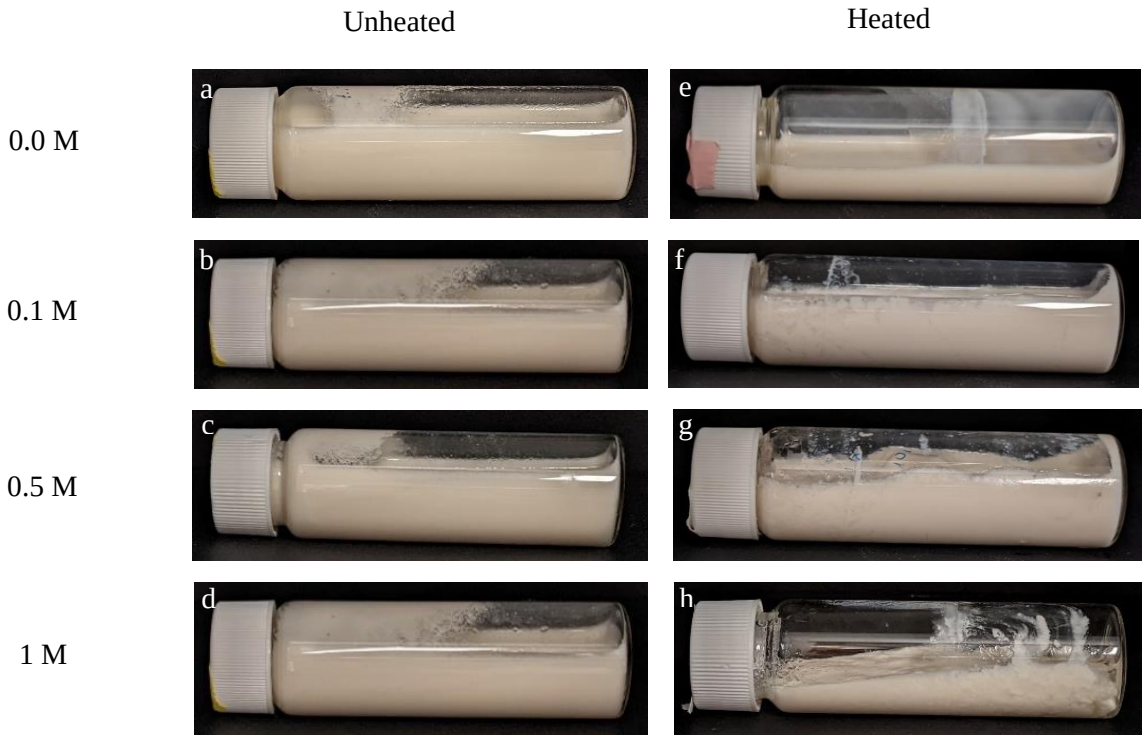

Figure 20: Visual observation of pH 2 heat-treated protein-stabilized emulsions after one week. This figure includes heated and unheated emulsions at different salt concentrations. The heating conditions are same as that of pH 7 emulsions. The salt concentrations are mentioned in the figure. The photos were taken 5 seconds after placing the vials horizontally.

4.4.4 Accelerated creaming velocity of heat-treated emulsions

For the heat-treated protein-stabilized emulsions at pH 7, accelerated creaming velocity was less than 2 μm/s, which did not change significantly with an increase in ionic strength ($p > 0.05$). Similar behaviour was also observed for the un-treated protein-stabilzed emulsions (Figure 15). However, a significant improvement in emulsion creaming stability was observed when the heat-treated protein-stabilized emulsions were heated at 90ºC for 30 min (Figure 21) compared to the un-treated protein-stabilzied emulsions under the similar condition (Figure 15). In Figure 15, we have seen that the creaming velocities increased from 6.00 μm/s to 31.09 μm/s when ionic strength increased from 0.0 to 1.0 M salt after heat treatment of the emulsions. However, when the emulsion was prepared with heat-treated partially denatured proteins, the creaming velocity did not change

at all and remain less than 2 μm/s at all ionic strengths (Figure 21). This shows the advantage of partially denaturing the proteins prior to making the emulsions, which led to an emulsion stable to further heat treatment.

For pH 2 heat-treated emulsions, the creaming velocities were very high when compared to the creaming velocities of pH 7 heat-treated emulsions (Figure 21). The creaming velocities of emulsions having 0.0 M and 0.1 M salt showed lower creaming velocities, but the creaming velocities increased as the salt concentration increased to 0.5 M and 1 M, this was also observed in un-treated protein stabilized emulsions (Figure 15). Heated pH 2 emulsions showed higher creaming velocities at 0.5 M and 1 M than unheated emulsions. This shows that heat treatment of emulsions affects the creaming velcity. It can also be observed from Figure 16 that the droplet size of pH 7 emulsions was quite low when compared to pH 2 emulsions, and hence the creaming velocity of pH 7 emulsions was very low. Whereas, the droplet size of pH 2 emulsions was very high, which led to a very high creaming velocity at pH 2.

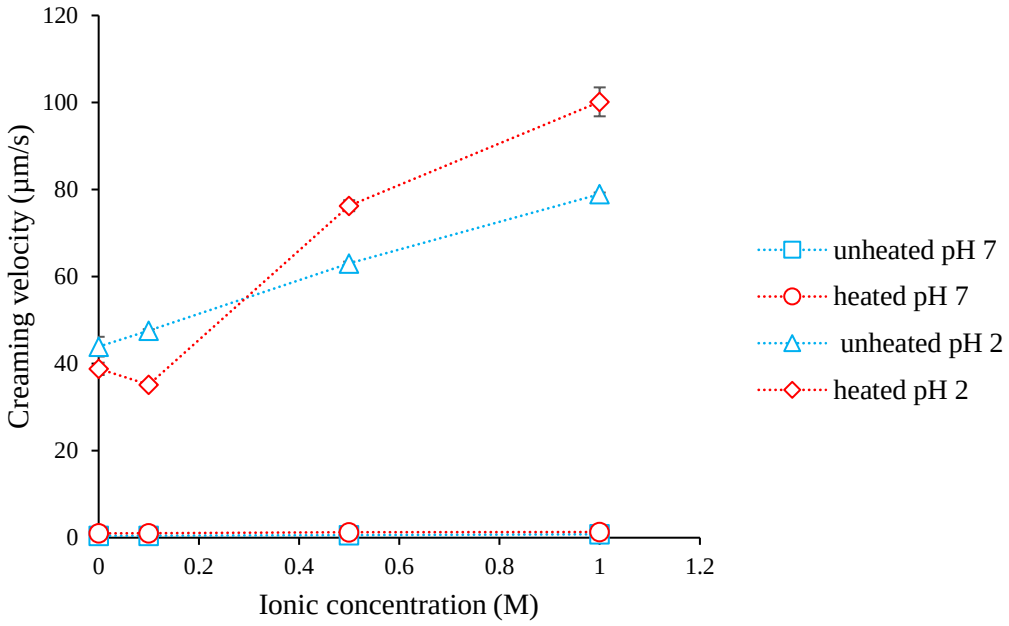

Figure 21: Calculated creaming velocities of the emulsions prepared with heat-treated proteins at a centrifugal force of 1050*g* as a function of ionic strength and emulsion pH (pH 7 unheated: square pH 7 heated: circle, pH 2 unheated: triangle and pH 2 heated: diamond). The creaming velocities were obtained using LUMiSizer dispersion analyzer.

4.4.5 Microstructure of the emulsions prepared with untreated and heat-treated proteins

4.4.5.1 Emulsion microstructure at pH 7

Emulsion microstructure was captured using a confocal microscope. Only the emulsions without any added salt were used for microscopy. Images with both 2.5× and 5.0× magnification are shown in Figure 22 to better understand the effect of protein pre-treatment and emulsion heat treatment on microstructure. It can be observed in Figure 22 a, b that oil droplets and proteins are dispersed in the emulsion. After the heat treatment, not much change in emulsion microstructure was observed (Figure 22 c, d), although some droplet flocculation and protein network formation could be visible. Perhaps, the weak protein and droplet network was responsible for the weak gel-like structure observed visually (Figure 12 pH 7). Interestingly, the microstructure of the emulsions

made with heat-treated proteins was quite different than the untreated protein-stabilized emulsions. In Figure 22 e, f it can be observed that the protein is aggregated into clumps, and the lipid droplets are present without any sign of aggregation. Most of the lipid droplets are present inside the protein clumps, and a few of the lipid droplets are free in the continuous phase. However, proteins clumps were not forming a space-spanning network as in Figure 21 c, d, rather they were well dispersed in the continuous aqueous phase. This could be the reason behind their flowable nature observed in Figure 12. The similar size of aggregate and droplets (Figure 10 a) and similar creaming velocities (Figure 15) of the two unheated emulsions, one prepared with un-treated proteins and the other prepared with heated proteins, also indicate the protein clumps observed in Figure 22 e and f were loosely bound and did not affect emulsion stability against phase separation. When the heat-treated protein-stabilized emulsions were heated at 90ºC for 30 min, it appeared that the heating led to partial breakdown of the protein clumps and the proteins and oil droplets are more dispersed in the emulsions (Figure 22 g, h), leading to similar droplet size (Figure 16a), creaming velocity (Figure 21), and visual observation (Figure 18) as with the emulsion before heat treatment. It appears that the emulsions prepared with heat-treated proteins formed loosely bound protein clumps, which disintegrated into a more dispersed state after the emulsion heat treatment, leading to a significant improvement in emulsion stability compared to the emulsions prepared with un-treated proteins. It can also be observed visually in Figure 18 how the heat-treated protein stabilized heated emulsions are stable and free flowing even though there are small clumps of protein present in the emulsion which is observed under the microscope. Heat-treated emulsions which are made at heated conditions show better stability, when they are cooled to room temperature after making the emulsions. The protein surrounding the oil droplets show some aggregation (due to the exposure of the hydrophobic patches), but because majority of the exposed hydrophobic groups contribute to the stabilization of the lipid droplets, there is no extensive protein clump formation. The protein clumps which are present are small and even with their presence, emulsion is still stable. Similar aggregation was observed in lentil protein emulsions that were heat-treated in a study done by Nawaz *et al*. (2021). They observed formation of bridges between droplets as well as the presence of large protein aggregates on the surface of emulsions.

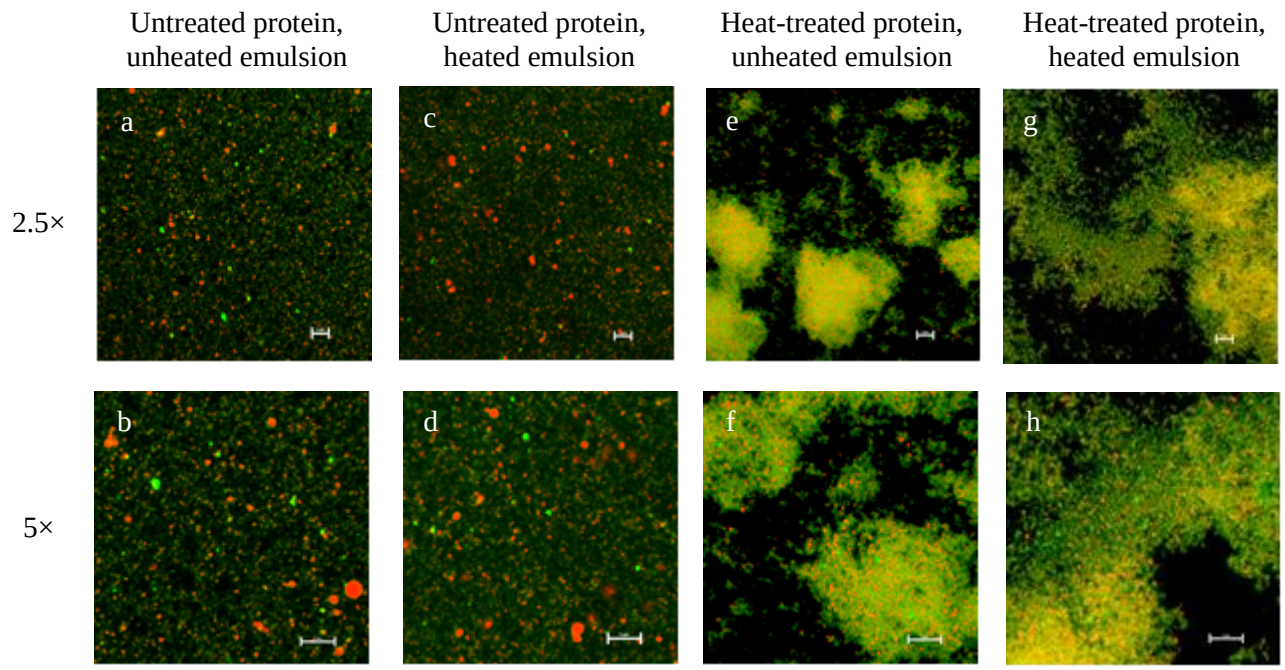

Figure 22: Confocal micrograph of untreated and heat-treated protein-stabilized emulsions at pH 7 without any added salt. Both emulsions were either heated at 90ºC for 30 min or not. Nile-red stained oil droplets are shown in red and fast-green stained protein is shown in green. Scale bar is 5 µm.

4.4.5.2 Emulsion microstructure at pH 2

When the untreated protein-stabilized emulsions were adjusted to pH 2, a drastic change in emulsion microstructure was observed (Figure 23 a, b). The emulsions formed stronger aggerates than that at pH 7. Castellani *et al*. (1998) showed that their amaranth globulin protein was stable at pH 5 to 9, but aggregation was observed when the pH decreased to 3, the protein rapidly unfolded leading to aggregation of the protein coated droplets. Similar aggregates can also be seen in Figure 23 a, b which appeared as large droplets (nearly 10-times larger) as reported in Figure 10. When the pH 2 emulsions were heated at 90ºC for 30 min, the aggregates formed a space-spanning network holding the oil droplets in the protein aggregates (Figure 23 c, d). This led to a certain extent of emulsion destabilization more than the unheated emulsions. For the heat-treated protein-stabilized emulsions, large aggregates of protein and oil droplets were observed (Figure

23 e, f). Unlike pH 7, no free oil droplets can be seen in these emulsions. These aggregates appeared to be larger than the corresponding pH 7 emulsions (Figure 22 e, f) and also in the untreated protein-stabilized emulsions (Figure 23 a, b). Heating the pH 2 emulsions prepared with heat-treated proteins led to re-dispersion of the large aggregates (Figure 23 g, h), which was similar to the pH 7 heat-treated protein-stabilized heated emulsions (Figure 22 g, h). Overall, more extensive protein and droplet aggregation was observed in pH 2 emulsions compared to the pH 7 emulsions. Pea proteins unfold at pH below 5, which exposed the hydrophobic patches causing attraction between them, in turn causing the protein aggregation (Castellani *et al*., 1998; Gumus *et al.*, 2017).

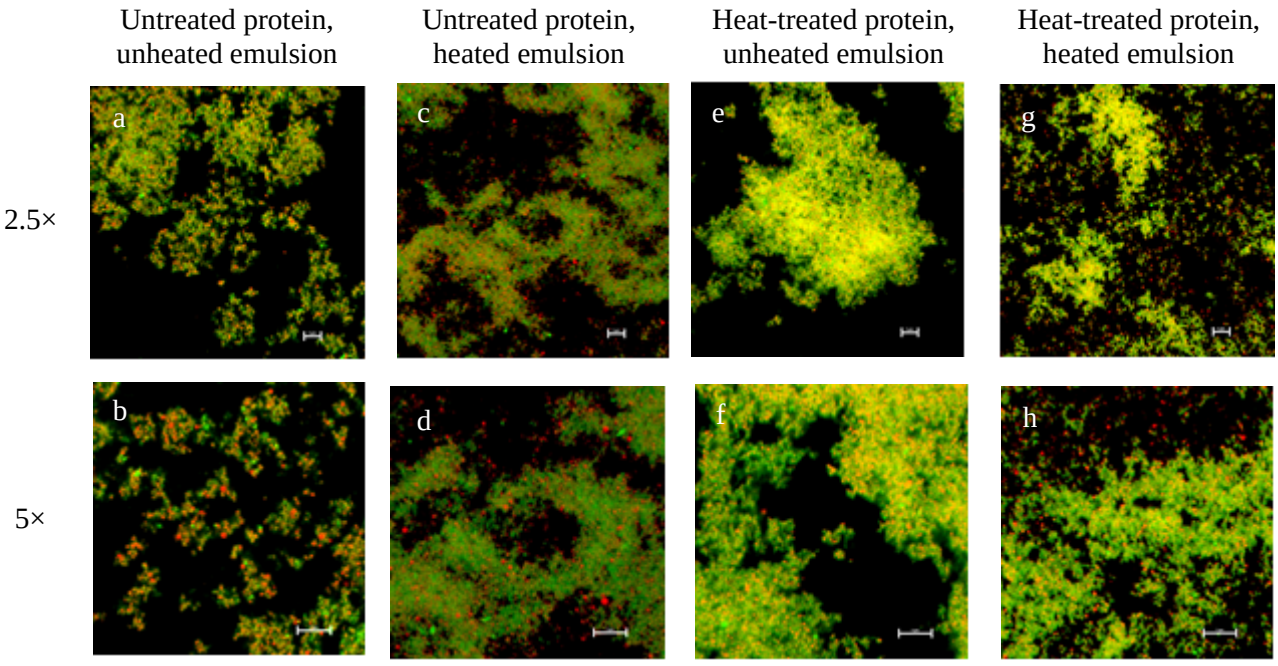

Figure 23: Confocal micrograph of untreated and heat-treated protein-stabilized emulsions at pH 2 without any added salt. Both emulsions were either heated at 90ºC for 30 min or not. Nile-red stained oil droplets are shown in red and fast-green stained protein is shown in green. Scale bar is 5 µm.

4.4.6 Viscosity of heat-treated emulsion

Viscosity was measured for the heat-treated protein stabilized emulsions at pH 7 and pH 2 before and after heating the emulsions. All emulsions showed a decrease in viscosity with an increase in shear rate, indicating their shear thinning behavior (Figure 24). It was observed that in the unheated emulsion at pH 7, the viscosity decreased with the addition of salt (Figure 24 a), whereas the heated emulsions had similar viscosity at different salt concentrations (Figure 24 b). Overall, the heated pH 7 emulsions showed higher viscosity when compared to unheated emulsions (Figure 24 a and b).-The heating of the emulsion also caused aggregation, leading to an elevated viscosity when compared to unheated emulsions. The unheated emulsions at pH 2 also showed a decreasing trend in the viscosity with increase in salt concentration. The heated pH 2 emulsions showed similar viscosities at all salt concentrations. Overall, pH 2 emulsions show higher viscosity when compared to pH 7 emulsions, which could be related to the larger aggregate size observed in pH 2 emulsion compared to pH 7 emulsions (Figure 16). To compare the apparent viscosities of various emulsions prepared with heat-treated proteins, their viscosities at a low shear rate of 0.04 s^{-1} were compared (Figure 25). Highest viscosity was observed for pH 2 heated emulsion (9.92 mPa·s at 0.5 M salt and the lowest viscosity was observed for pH 7 emulsion (0.136 mPa·s) at 0.5 M salt at unheated conditions. Unheated and heated emulsions at pH 7 showed similar viscosities at 0.04 s^{-1} shear rate ($p > 0.05$). Unheated and heated emulsions at pH 2 also have significantly different viscosities at that particular shear rate ($p < 0.05$). Unheated emulsions at pH 7 and pH 2 also have significantly different viscosities ($p < 0.05$). Heated emulsions at pH 7 and pH 2 had significantly different viscosities ($p < 0.05$) at 0.04 s^{-1} shear rate, viscosity of pH 2 emulsions being significantly higher. Formation of large aggregates might be responsible for the high viscosity of pH 2 emulsions. This can also be confirmed visually in Figure 19 and 20, there is slight aggregate formation observed in pH 2 emulsions whereas, at pH the emulsions appear to be free flowing without any large aggregate formation. Larger aggregate size of pH 2 heated emulsions compared to pH 7 heated emulsions can also be observed from their average particle size data (Figure 16).

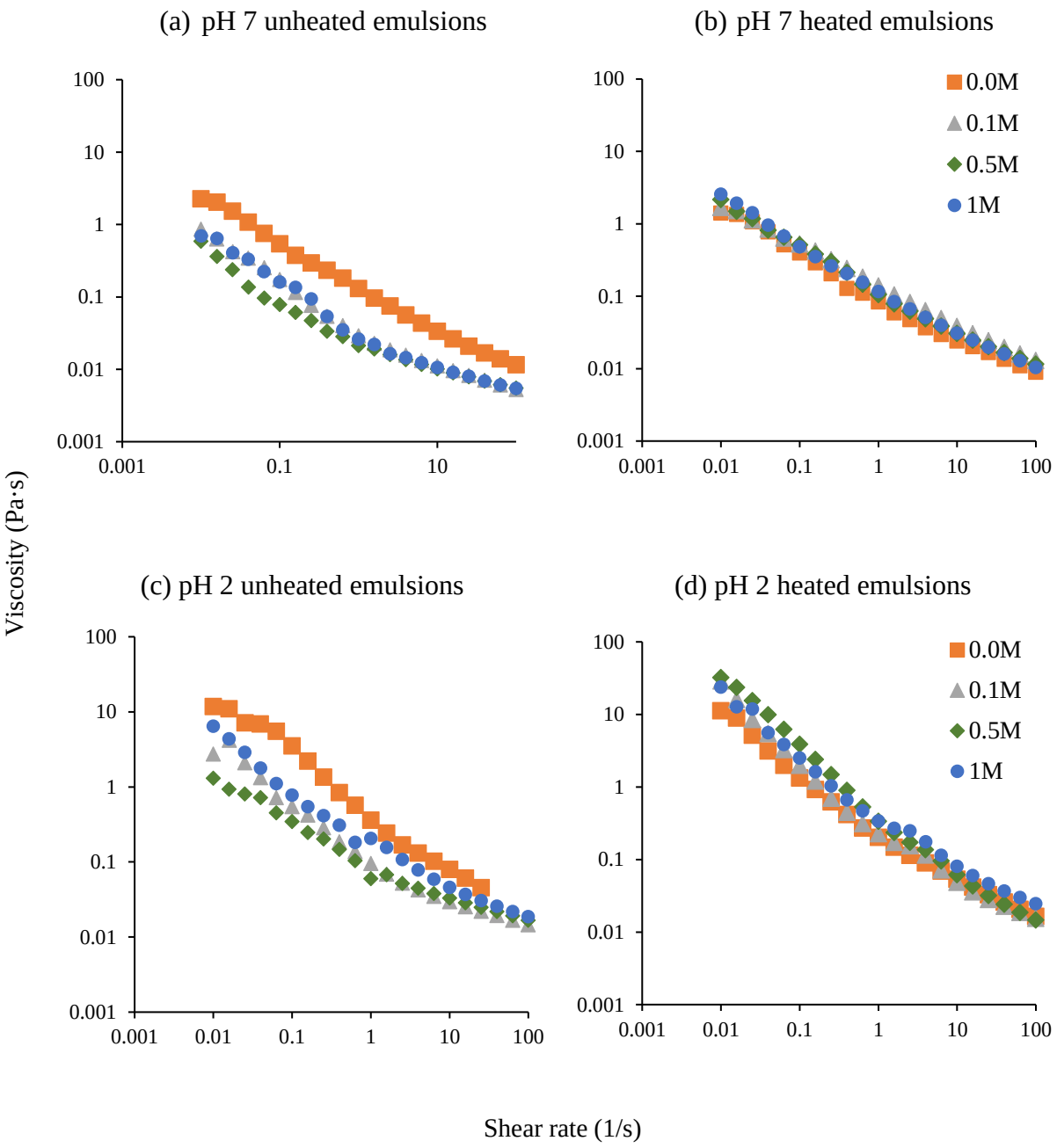

Figure 24: Viscosities of emulsions prepared with heat-treated protein as a function of shear rate for different ionic concentrations. Data for (a) pH 7 unheated and (b) at pH 7 heated emulsions are shown along with (c) pH 2 unheated and (d) pH 2 heated emulsions.

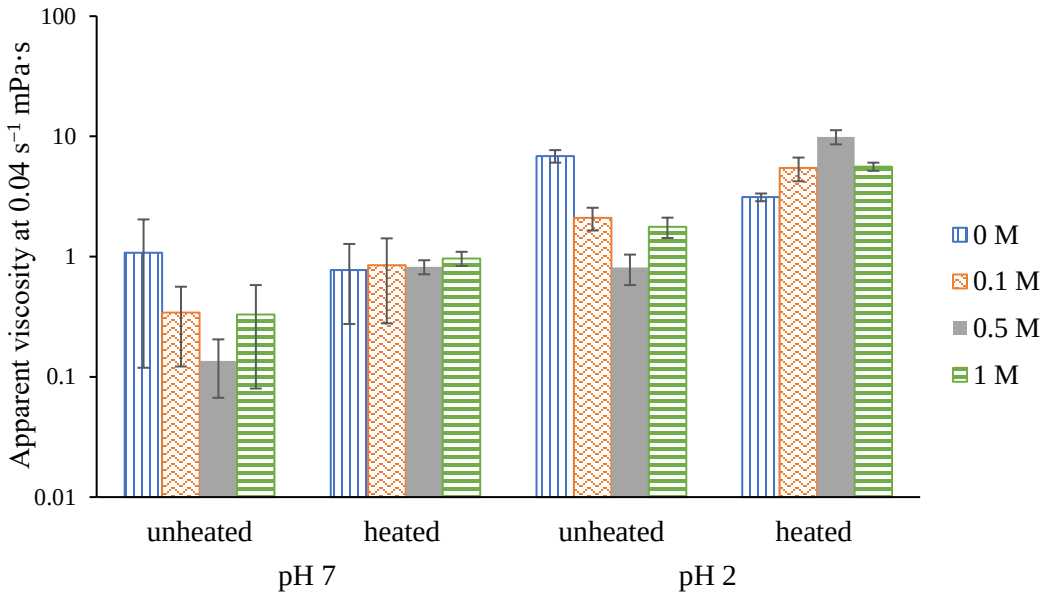

Figure 25: Apparent viscosity of heat-treated protein emulsions at 0.04 s^{-1} shear rate as a function of pH and emulsion heat treatment at various ionic concentrations.

4.5 Influence of emulsion and protein heat treatments on the *in-vitro* lipid digestibility

Emulsion technology is one of the most widely used systems for bioactive delivery. There are many factors that influence bioactive delivery, including their molecular weight, structure, functional group, polarities, charge, etc. (McClements *et al*., 2007). These factors are important to design an emulsion system such that the bioactive delivery is done efficiently. Apart from the characteristics of the bioactive, the structure of the emulsion and the droplet interfacial structure also play a major role in the lipid digestibility and releasing the bioactives dissolved in the lipid droplets and thereby determining their bioavailability (McClemments *et al*., 2007). Interfacial structure and digestibility of the emulsifier during digestion determine how much exposure the lipid droplets have and thereby influence lipid digestibility (Li *et al*., 2019). In this research, it was found that heat treatment modified the interfacial structure of the protein and the emulsion droplet aggregation. Hence *in vitro* lipid digestibility of the emulsions was determined to understand how

the interfacial structure of the protein and droplet aggregation influence the digestibility of the lipids.

The *in vitro* digestion was carried out on the emulsions made with heat-treated proteins before and after heating the emulsions to 90ºC for 30 min (Figure 26). As a control, unheated emulsion made with untreated proteins was also used. Lipid digestibility is observed only in the intestinal phase and hence, only the intestinal phase (which started at 120 minutes, after the gastric phase) has been reported in Figure 26. For the first ten minutes of intestinal phase, the rate of lipid digestion is similar for all three emulsions, but the rate of lipid digestion varied after that point. Lipid digestion for the first 10 minutes of intestinal phase (130^{th} minute of digestion) was rapid for all the three emulsions (Figure 26). After that, the rate of lipid digestion slowed down significantly for the heat-treated protein-stabilized unheated emulsion. After about 136 minutes of digestion, the heat-treated protein-stabilized heated emulsion showed a shift in the rate of digestion to a lower value. Lipid digestion rate of the untreated protein-stabilized unheated emulsions slowed down after 140 minutes of digestion. The shift in the rate of lipid digestion after the inititial higher rate might be due to the saturation of bile salts at the oil-water interface (Pilosof, 2017). The total percentage of free fatty acid release from the *in vitro* digestion was determined after 120 minutes of intestinal phase, as the typical intestinal phase lasts for 2 hours.

At the end of 2 hour of gastric and 2 hours of intestinal digestion, it was observed that for untreated protein-stabilized unheated emulsions, the cumulative release of free fatty acids was 97.51 ± 1.1%, indicating that the lipids were almost completely digested in the emulsions without any heat treatment. However, the total lipid digestibility in heat-treated protein-stabilized unheated emulsions showed a large drop to 56.06 ± 0.1% (Figure 26). Such differences in final lipid digestibility must be due to the differences in protein and droplet interfacial structure in various emulsions which was influenced by the heat treatment of the proteins and heating of emulsions or their absence thereof. Sarkar *et al*. (2016) also observed about 9% decreased lipid digestibility in emulsions stabilized by heat-treated whey proteins (46% lipid digestion) compared to the emulsions stabilized by whey proteins without any thermal treatment (42% of lipid digestion). It was also observed by Ruiz *et al*. (2016), that the heat treatment of quinoa proteins led to a

decreased lipid digestion in emulsions compared to untreated quinoa protein-stabilized emulsions. The authors also reported that as the heating temperature increased from 90°C to 120°C, lipid digestibility decreased from 12.5% to 11.5% (Ruiz *et al*., 2016). Due to the heat treatment of the proteins in the heat-treated protein-stabilized unheated emulsions, the proteins encapsulating the lipid droplets were aggregated at the oil droplet surface, thereby increasing the interfacial strength, and preventing easy accessibility of bile salts and lipase towards the internal lipids. Recently, Guevara-Zambrano *et al*. (2022) proposed that the structure of interfacial proteins played an important role in determining the extent of lipid hydrolysis in emulsions. The authors stated that the aggregated globulins present in pea proteins hindered the accessibility of gastric lipase to the oil droplets, thereby inhibiting complete lipolysis.

Interestingly the lipid digestibility of heat-treated protein-stabilized heated emulsions increased to 73.47 ± 0.1%, compared to 56.06 ± 0.1% for the corresponding unheated emulsions (Figure 26). The microstructure of the emulsions (Figure 22) showed that heating the emulsions, prepared with heat-treated proteins, led to a better dispersion the droplet and protein aggregates. Possibly, this led to an increased exposure of the lipid droplets to the external surroundings thereby providing more accessibility to the bile salts and lipase to access the lipids and aid in better digestion than unheated emulsions. Interfacial composition and strength also play a major role in determining the extent of lipid digestion. Sarkar, Murray, *et al*. (2016) have observed that heat-treated whey-protein microgels protected the lipids against digestion when compared to unheated whey protein microgels. It was observed by Pilosof (2017) that the composition and crosslinking of protein itself do not have a role in determining the extent of lipolysis. But the characteristics such as crosslinking and aggregation at the interface are attributed to their low resistance to proteases action or to displace bile salts that facilitate the anchoring of lipase/colipase complex that determine the rate of lipid digestion (Pilosof, 2017). In the current research, differences in lipid digestibility among the emulsions can be attributed to the differences in protein structure due to various heat-treatments of the emulsions. It is possible that the effect of the interfacial protein structures on bile salts to facilitate the anchoring of lipase enzyme complex contributed to the differences in lipid digestibility of the emulsions. Guevara-Zambrano *et al*. (2022) also observed that, protein aggregation had slowed down lipid digestibility in emulsions. They reported that even though the

emulsions stabilized by native pea proteins and heat-treated pea proteins showed similar lipid digestibility, heat-treated protein emulsions took longer time for lipid digestion (Guevara-Zambrano *et al*., 2022). In the current research, heat-treated protein emulsions had lower rate of digestion when compared to untreated protein emulsion. It is possible that if the digestion was carried out for a longer period of time, the heat-treated protein emulsions might also show similar free fatty acid release as the untreated protein emulsions.

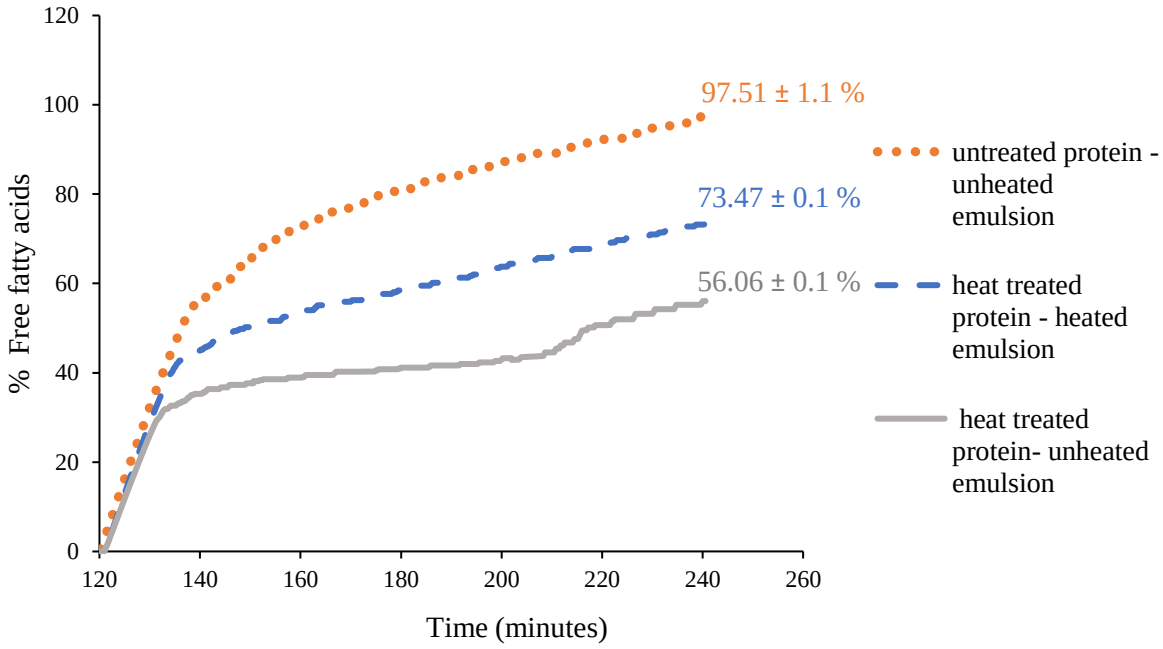

Figure 26: Percentage of free fatty acids released during intestinal digestion as a function of time in untreated-unheated, heat treated-unheated and heat treated-heated emulsion. Intestinal phase takes place 2 hours after of the start of gastric phase of digestion; hence the X-axis starts from 120 min.

4.6 Characterization of the effects of heat treatment on protein structure and functional properties

To explain why heat treatment of the protein and subsequently making the emulsion under hot condition led to a such a significant improvement in thermal stability, it would be critical to understand how heat treatment of the protein influenced its structure and functional properties. However, it should be noted that the proteins had to be cooled down to room temperature before

they were characterized, as opposed to their emulsification behaviour under hot condition without allowing the proteins to re-aggregate upon cooling. Nevertheless, even if the proteins were aggregated after they were denatured by heat treatment, the change in their structure and functional properties should be detectable, which might help explain the improved thermal stability of the emulsions.

4.6.1 Interfacial tension

Interfacial tension is a force that minimizes the contact area between two immiscible phases. To disperse an oil phase into an aqueous phase, or to increase the area of contact between the oil and aqueous phases, surface active molecules are introduced which accumulate at the oil-water interface and lower the interfacial tension (Shevkani *et al*., 2015). This minimizes the energy required to make an emulsion and creates smaller droplets with higher stability. In the present case, the soluble pea proteins were the surface-active molecules which decreased the interfacial tension and aid in emulsification. To better understand how partial denaturation of the proteins due to heat treatment influenced their emulsification ability, the oil-water interfacial tension of the untreated and heat-treated proteins at pH 7 was determined as a function of time. As proteins are large molecules, they need time to diffuse towards the interface from the aqueous phase (Shevkani *et al*., 2015). Even after reaching the interface, protein molecules undergo surface denaturation by unravelling their internal structure and the exposure of the hydrophobic group leading to adsorption to the interface (Lam & Nickerson, 2013). During this time, the interfacial tension decreases slowly, and only when the proteins complete their conformational adjustment the interfacial tension reaches an equilibrium. From Figure 27, it can be seen that the untreated proteins and heat-treated proteins have decreased the interfacial tension to a different rate. The heat-treated proteins reached equilibrium after 15 mins, much quicker than the untreated proteins, which reached equilibrium after 27 minutes. Heat treatment of soluble proteins at 75ºC led to breakdown of protein particle aggregates and the partial exposure of hydrophobic patches. Smaller protein aggregates diffused faster towards the interface. The partially exposed hydrophobic patches improved the adsorption of the proteins to the oil droplets compared to the untreated proteins, which had to undergo conformational change before adsorption. Even if the proteins were cooled down after heat treatment, the partially exposed hydrophobic patches remained available to adsorb

on the oil droplets. This led to further lowering of the interfacial tension for the heat-treated proteins compared to the untreated proteins. Both the heat-treated and untreated proteins showed different interfacial tensions from the beginning, the initial interfacial tension of untreated protein was 8.1 mN/m whereas it was 6.5 mN/m for heat-treated protein. The rate of decrease in the interfacial tension was also different for both the untreated protein and the heated treated proteins. The equilibrium interfacial tension of the heat-treated proteins was 0.8 mN/m, whereas for the untreated proteins, it was significantly higher, 1.2 mN/m ($p < 0.05$). This small but significant decrease in interfacial tension, however, did not significantly change the oil droplet size of the emulsions (pH 7) made with these two proteins (Figure 10 a vs 16 a). Nevertheless, the emulsions made with heat-treated proteins were significantly more stable to thermal treatment than those made with untreated proteins, which could be ascribed to the more suitable adsorption of the proteins at the oil droplet surface in the former. It is possible that as the proteins were already partially denatured, their adsorption at the interface happened with those already exposed hydrophobic patches; therefore, heating the emulsion did not further affect the interfacial proteins to such an extent that they would as strongly aggregate as seen in the untreated protein-stabilized emulsions. It was also reported by Peng *et al*. (2016) that a heat-treated (95 °C for 30 minutes) 3 wt% pea protein solution showed a lower interfacial tension (25.02 ± 0.2 mN/m) when compared to a similar but untreated protein solutions. Heat-treatment of soluble proteins caused the exposure of hydrophobic groups which improved the hydrophobicity of the protein. Shen *et al*. (2022) observed in their study that heat-treated soy proteins had lower interfacial tension when compared untreated soy proteins. They mentioned that increased hydrophobicity in heat-treated soy proteins could influence interfacial properties. The exposed hydrophobicity of each protein would decrease the energy barrier for the adsorption to the interface, which could increase the interfacial adsorption rate. Similarly, in the current research higher interfacial activity (lower interfacial tension) was observed for heat-treated pea proteins when compared to untreated pea proteins.

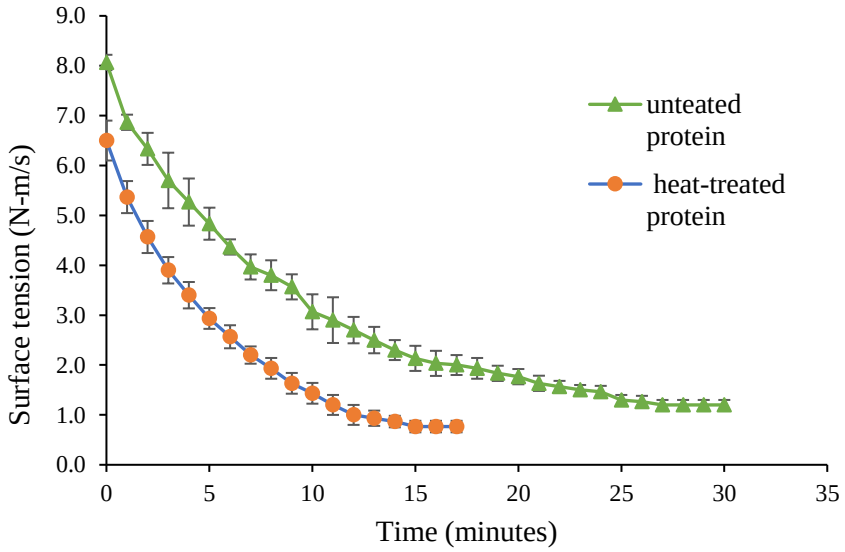

Figure 27: Oil-water interfacial tension of untreated and heat-treated protein as a function of time. The interfacial tension was measured till the system reached equilibrium.

4.6.2 Intrinsic fluorescence

If the hydrophobic amino acids of the proteins are exposed due to heat-treatment, their fluorescence spectra might also be affected, which was tested by measuring the intrinsic fluorescence of the protein solutions. In proteins, the fluorescence emitted by tryptophan, phenylalanine, and tyrosine can be used to determine the structural changes in proteins. Of the three, tryptophan is the most dominant fluorophore. Tryptophan's fluorescence emission is affected by changes in protein's tertiary and quaternary structure, binding to a substrate, and the polarity of the solvent (Yerramilli *et al.*, 2017). Figure 28 shows the intrinsic fluorescence of tryptophan residues from the heat-treated and untreated soluble pea protein solutions. A significant decrease in fluorescence intensity was observed for the heat-treated proteins compared to the untreated proteins. Fluorescence intensity of proteins can be decreased by many different processes, however, one of the most reasons behind such a decrease in intensity is known as quenching. Collisional quenching occurs when excited-state fluorophore is deactivated when it comes in contact with some other molecule in solution, which is usually the quencher (Lakowicz, 2010). Collisional quenching can be used to determine the extent of tryptophan exposure to the aqueous phase. If the tryptophan residue is buried inside the protein, then, quenching is not expected to occur, whereas if the tryptophan residue is exposed, then quenching is expected

(Peyrano *et al*., 2016). In this case, the tryptophan residues are expected to be buried inside the untreated soluble pea proteins which prevents collisional quenching and results in high fluorescence emission when compared to heat-treated protein. Whereas, when soluble protein was heat-treated, it led to the partial exposure of hydrophobic patches that may contain the tryptophan residues (Peng *et al*., 2016). This consequently leads to collisional quenching resulting in the decrease of fluorescence emission from the from the heat-treated proteins. Oxygen molecule is one of the well-known quenchers that participate in collisional quenching (Lakowicz, 2010). Apart from quenching, the fluorescence emission of the heat-treated protein had a red shift. Typically, the excitation of the fluorophores occurs at 280–295 nm and the emission are observed at 340 nm when the fluorophores are buried within the native protein. But a red shift is observed, i.e., the emission occurs at a longer wavelength when the protein is unfolded. In Figure 28, we can observe that the maximum emission is observed at 340 nm for untreated protein whereas, for the heat-treated protein, the maximum emission occurs at slightly higher wavelengths, around 348 nm. Hence, red shift is observed under such conditions when the protein is heated. It indicates that the chromophores such as tyrosine and tryptophan residues shifted to the exterior part of the protein which clearly indicates the unfolding of proteins at heated conditions (Wu *et al*., 2020).

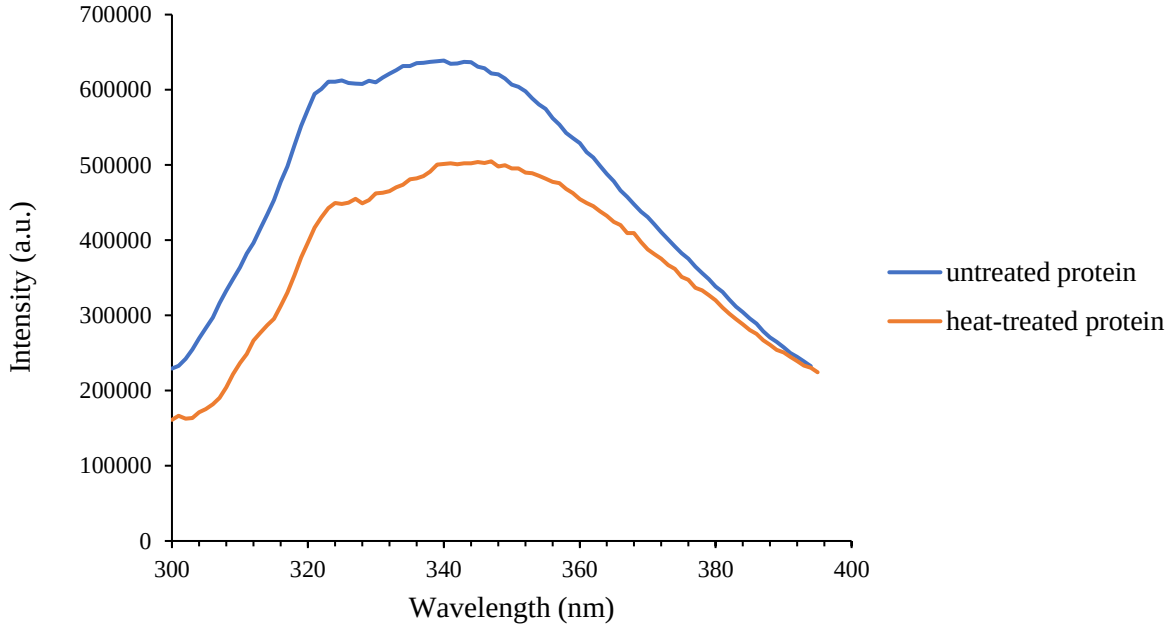

Figure 28: Tryptophan fluorescence spectra of pea protein solutions carried out at a constant excitation wavelength of 295 nm. Spectra for untreated and heat-treated soluble pea protein solutions are shown.

4.6.3 SDS PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

Gel electrophoresis was run for the untreated and heat-treated proteins under non-reducing and reducing conditions. Under non-reducing condition, untreated proteins showed distinct bands for various protein fractions, such as vicilin, convicilin, legumin α, legumin β and albumin fractions (Figure 29 a). Fainter bands were observed for heat-treated proteins under non-reducing condition when compared to untreated proteins (Figure 29 b). Partial denaturation of the heat-treated proteins could induce aggregation due to attraction among the hydrophobic patches, which could make the proteins too large to enter the gel. Hence fainter bands were observed for the heat-treated proteins, as only the unaggregated proteins can enter the gel. Similar results showing heat-induced aggregation and formation of large protein molecules that did not enter the gel have also been reported for pea protein isolates (Chao & Aluko, 2018). The addition of β-mercaptoethanol and doing the gel electrophoresis under reducing condition confirms that the heat-treatment did not lead to any changes in the polypeptide composition and all protein fraction bands were clearly visible. Previous works have also shown that heat-treatment of cow pea proteins (Peyrano *et al.*,

2016) and whey protein (Patel *et al*., 2004) did not produce any changes in the polypeptide patterns after SDS PAGE run under reducing condition.

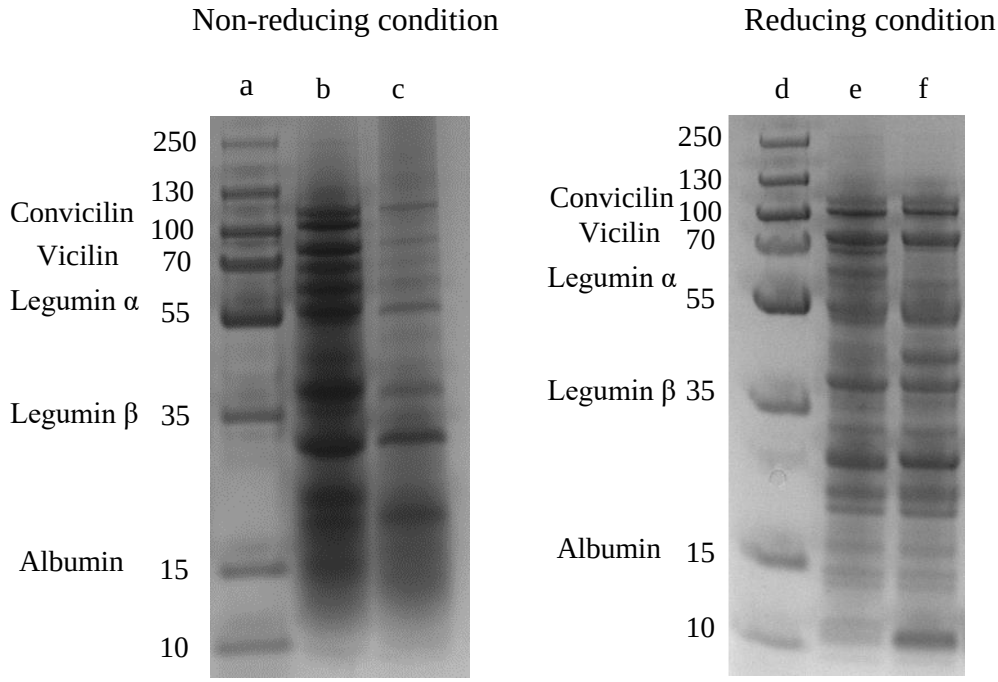

Figure 29: Gel electrophoresis under non-reducing condition for (b) untreated and (c) heat-treated proteins; and under reducing condition for (e) untreated and (f) heat-treated protein. Bands in lane (a) and (d) indicate the molecular weight of marker ranging from 10 to 250 kDa. Convicilin, vicilin, legumin α, legumin β and albumin are various protein fractions marked according to their molecular weights.

4.6.4 Differential Scanning Calorimetry (DSC)

To understand the denaturation temperatures and peak enthalpy changes of untreated and heat-treated soluble proteins, differential scanning calorimetry (DSC) analysis was done on the samples. As a control, the original pea protein concentrate sample (PP55) was also analyzed. In Figure 28, we can observe that there are multiple peaks for PP55 sample, one at 73.9°C and another peak at 93.1°C. The peak at 73.9°C indicates starch gelatinization, which was absent in heat-treated and unheated protein samples. It shows that the majority of the starch fractions were absent in unheated and heat-treated protein fractions due to mild fractionation. The curves of untreated and heat-treated proteins were relatively flat when compared to the PP55 curve. In the unheated proteins,

peaks were observed at 90.0°C and 99.6°C, whereas for the heat-treated proteins, there was only one peak observed at 91.4°C. This indicates that heat treatment of soluble proteins had a profound effect on their denaturation temperature. Thermal denaturation of pea proteins usually ranges from 88–100°C depending on the pea cultivar, processing conditions and rate of heating (Arntfield & Murray, 1981; Sun & Arntfield, 2011).

The enthalpy change of protein denaturation for PP55 concentrate was 1.82 J/g of protein. For the untreated soluble proteins, it was 2.88 J/g of protein, whereas for the heat-treated sample it was 0.24 J/g of protein. The enthalpy change is given for per gram of protein present in the respective samples as the protein concentrations are different in soluble proteins and PP55 samples. PP55 sample contains 55 wt% of proteins, whereas soluble proteins have 82.8 wt% of proteins, which explains the higher enthalpy change of untreated proteins when compared to PP55 concentrate sample. There were two major peaks observed for untreated proteins, whereas there was only one major peak observed for heat-treated proteins. Thermal transition peak is missing in heat-treated protein indicating that heat-treated protein was denatured due to heat-treatment. Ma *et al*., (2022) also observed much reduced enthalpy change for commercial pea protein isolate (0.033 J/g of protein) when compared to laboratory-produced pea protein isolate (15.8–17.8 J/g of protein), indicating that commercial pea protein isolate was further denatured. They observed that the transition peak was missing when commercial pea protein isolate was heated above 86°C, meaning that most protein isolates were already denatured during the process. Similar trends are observed in the current research. The decrease in enthalpy change of heat-treated proteins can be attributed to the partially denatured state of the protein prior to the DSC experiment. Since, in heat-treated protein, the protein is already exposed to heat and partial denaturation has occurred, it needs a much lower amount of heat for the denaturation of the rest of the native proteins compared to the untreated protein. From DSC analysis it was understood that heat-treatment of soluble proteins effected the structure of the proteins, which resulted in decreased denaturation temperature and enthalpy change of protein denaturation.

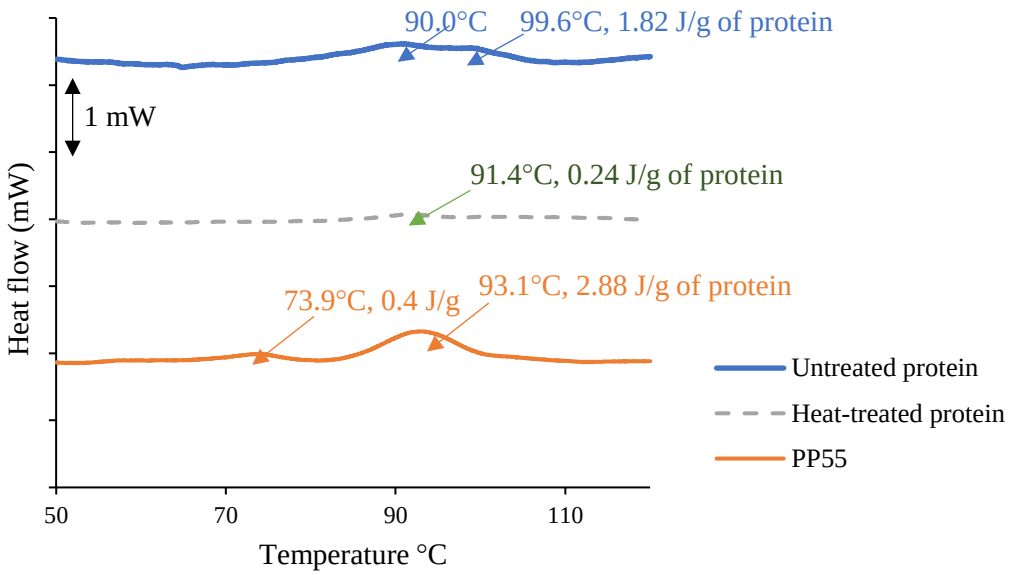

Figure 30: Heat flow of PP55 concentrate, untreated protein, and heat-treated protein in temperature range of 50°C to 120°C, at heating rate of 10°C/min. The denaturation temperatures and peak enthalpy changes are mentioned for the respective samples. The first peak in PP55 sample indicates starch gelatinization.

These characterizations of the soluble proteins helped to understand why the heat-treated proteins improved the emulsion thermal stability better than the untreated proteins. From the interfacial tension experiment, it was understood that the heat-treated proteins helped in lowering the interfacial tension better than the untreated proteins. The intrinsic fluorescence showed a shift of aromatic fluorophores from the interior parts of the protein to the exterior, which indicates that heating led to the unfolding of the proteins, thereby increasing their hydrophobicity. SDS PAGE indicated that heating caused aggregation of the protein, but the structural components of the untreated protein and heat-treated protein did not differ. Thermal analysis of the protein samples showed that the denaturation peak enthalpy change of the heat-treated soluble proteins was significantly lower than the untreated soluble proteins indicating that the heat-treatment affected the secondary structure of the soluble proteins. From all the characterization tests, we understood that heat-treatment caused unfolding in the proteins that had a positive impact on the stability of the emulsions, but it is important to consider that these protein characterization tests were done

after the heat-treated proteins were either cooled to room temperature or freeze-dried. Whereas the heat-treated protein-stabilized emulsions were made under hot conditions, where the proteins were not allowed to cool down till the emulsions were made. During the formation of the emulsions, because the proteins were at heated conditions, the hydrophobic patches unfolded but did not aggregate. Since these patches were unfolded, they helped the proteins adsorbing on the lipid droplets in a better way when compared to the untreated proteins. Hence, the heat-treated protein-stabilized emulsions offered superior stability at pH 7 in terms of prevention of extensive droplet and proteins aggregation compared to the emulsions made with the untreated proteins.

5. GENERAL DISCUSSION

The objective of this research was to seek the possibility of using mildly fractionated soluble protein fraction of pea protein concentrate as an emulsifier for the development of canola oil-based beverage emulsions which could enhance the value-added utilization of pea proteins. Two pea protein concentrate samples (PP55 and PP55P30, containing 55 and 53 wt% proteins, respectively) were used to extract the soluble-protein fractions. Initially, a two-step centrifugation process was opted, which led to the separation of three fractions: starch-rich fraction, soluble protein-rich fraction, and insoluble protein fraction. The soluble-protein fraction from PP55P30 and PP55 retained 70.21% and 71.1% of the total proteins, respectively. It was observed that the insoluble fraction was very minimal and contained an insignificant amount of protein. Therefore, single-step centrifugation was opted for as it would be more economically viable. Single-step centrifugation gave rise to two fractions: a soluble protein-rich fraction and an insoluble fraction. The soluble protein fraction also retained about 70–71% of the total proteins for both PP55P30 and PP55, which was not significantly different from the two-step centrifugation process. Hence, single-step centrifugation was chosen to extract the soluble proteins in the development of beverage emulsions. The lower concentration of proteins in the soluble protein-rich fraction of PP55P30 is attributed to its coarse granular structure when compared to PP55 which is in powder form. The structure of the sample played an important role in the extraction of the soluble proteins. PP55 pea protein concentrate was chosen for the further study.

After extracting the soluble proteins via centrifugation, next, the optimum concentration required for O/W emulsion stabilization was determined. Emulsions were made with 5 wt% oil and 0.5 to 2.5 wt% soluble proteins in the aqueous phase using a high-pressure homogenizer. From the various emulsion characterization tests, including droplet size, degree of flocculation, and accelerated creaming velocity it was observed that the emulsion with 2.5 wt% of protein in the

aqueous phase had the highest emulsion stability. Hence, further research on emulsion stability under various environmental stresses was done with 2.5 wt% proteins in the aqueous phase.

Various environmental stresses were applied to the emulsions to understand their stability in the presence of salt, in acidic pH 2 and after heat treatment at 90ºC for 30 min. These treatments were selected, such as to mimic the conditions of a beverage in the industry, heat treatment being similar to pasteurization, salt to maintain the ionic concentration and preservation and pH change to understand how the beverage would stabilize in acidic pH. At unheated conditions, pH 7 emulsions were stable at different salt conditions, whereas when the emulsions were heated, they showed aggregation due to protein denaturation. At pH 2, all unheated emulsions showed aggregation, however, upon heat treatment, the pH 2 emulsions showed flowable nature despite their large aggregate size at various salt concentrations. After a week of storage, heated emulsions at pH 7 showed strong aggregation, whereas the unheated emulsions were stable, which was attributed to the salting-in effect of the proteins (Keivaninahr *et al*., 2021). But unheated pH 2 emulsions after one week showed creaming and serum layer separation, which was ascribed to the unfolding of globulin proteins under acidic pH, leading to aggregation of protein-coated droplets (Keivaninahr *et al*., 2021). Aggregation was also found in heated pH 2 emulsions after one week, but they were not as strong as the aggregates found in heated pH 7 emulsions. Overall heat had a prominent effect on both the emulsions, with pH 7 emulsions being more affected compared to pH 2 emulsions.

After the emulsions were heated at 90ºC for 30 min, there was extensive droplet and protein aggregation leading to a weak gelation and phase separation which made the emulsions unsuitable for beverages. Hence, it was a critical problem that had to be resolved if the emulsions were to be used in beverages. It was understood that heating the emulsions to a high temperature (90°C) caused the interfacially adsorbed proteins to denature, which exposed the hydrophobic patches on the oil droplet surface, thereby aggregating the droplets and proteins and destabilizing the emulsion. To solve this problem, it was hypothesized that by partially denaturing the proteins by pre-heating them before making the emulsions with the heated partially denatured proteins might solve the problem of aggregation that occurred due to emulsion heating. The partially denatured proteins with exposed hydrophobic patches would adsorb better on the lipid droplets and would

not re-aggregate after making the emulsions, even if the emulsion was heated at 90°C. Chao *et al*. (2018) also observed that heat treatment of pea protein isolates helped to obtain emulsions with lower droplet sizes when compared to the emulsions made with untreated pea protein isolates. Based on this hypothesis, the soluble protein fractions were heated to 75°C causing partial denaturation, and the emulsions were also made at that same heated condition. Proving the hypothesis, exposure of the hydrophobic patches due to partial denaturation of the proteins during emulsion formation helped in better stabilization of the lipid droplets. The emulsions made with heat-treated proteins at pH 7 remained stable even after heating emulsions to 90°C, even though the emulsions at pH 2 showed aggregation. The average droplet size of all pH 7 emulsions made with heat-treated protein ranged from 0.3 µm to 0.4 µm irrespective of emulsion heat treatment and ionic concentration. Due to their smaller droplet sizes, creaming velocities of pH 7 emulsions were also relatively low (less than 2 µm/s) when compared to pH 2 emulsions (40–110 µm/s). Smaller droplet size, lower creaming velocities and no sign of aggregation even after one week indicated that pH 7 emulsions made with heat-treated protein were highly stable. Acidic pH plays an important role in denaturing the protein structure which causes aggregation and cannot be prevented by pre-treating the protein via heating. At pH 2, the average droplet size of emulsions was much higher (4–9 µm) ue to extensive aggregation, despite the heat treatment of the soluble protein. The pH 2 emulsions showed stronger aggregation with increase in salt concentration after one week. Overall, heat treatment of soluble proteins did not have a major effect on pH 2 emulsions.

Lipid digestibility of the beverage emulsions were done to understand the extent of release of free fatty acids from canola oil in the simulated digestive system and assess its bioaccessibility. Canola oil is a good source of polyunsaturated fatty acids which are important to maintain heart and brain health, hence a higher lipid digestibility of the beverage emulsions would improve their health-promoting functional properties. It was also important to understand how the structure of the heat-treated interfacial proteins around the oil droplets impacted lipid digestibility in pH 7 emulsions. In untreated protein-stabilized unheated emulsions, 97.51 ± 1.1% lipid digestion was observed. Such a high value of lipid digestion was possible as the lipase enzyme was able to access the lipid droplets without protein interference in the untreated protein-stabilized unheated emulsions. The

protein was in its native form without any aggregation which was also observed in confocal microscopy. In contrast, only 56.06 ± 0.1% lipid digestion was observed in the heat-treated protein-stabilized unheated emulsion. The confocal image of this emulsion suggested that there was extensive aggregation in the protein due to the heat-treatment of the emulsion which serves as a barrier for the digestion of lipid. Interfacial protein aggregation plays a major role in lipid digestion. The presence of strong protein aggregates at the oil-water interface resulted in a decreased lipid digestion. Interestingly, the heat-treated protein-stabilized heated emulsion showed 73.47 ± 0.1% lipid digestion, lower than the original untreated protein-stabilized emulsions, but still much higher than the heat-treated protein-stabilized unheated emulsions. These emulsions were made with the heat-treated protein under heated conditions, followed by further heat treatment of the emulsions, which broke down the aggregates leading to the formation of smaller proteins and droplet aggregates as observed in the confocal microscopy. Smaller aggregates improved lipid digestion; however, it was still lower than the emulsions without any aggregates. Therefore, protein structure, especially at the oil-water interface of lipid droplets plays an important role in determining the degree of lipid digestion in emulsions.

It was important to understand how partial denaturation of proteins helped improve the stability of emulsions. It is well known that the structure and functionality of proteins play a significant role in emulsion stability. To know the structural changes before and after heat-treatment of proteins and how it impacted the emulsification behaviour, proteins were characterized by SDS PAGE, intrinsic fluorescence, DSC and interfacial tension. From the SDS PAGE analysis it was found that heat-treatment caused soluble protein aggregation. Bands indicating the untreated soluble protein were distinct whereas, the bands indicating heat-treated protein were faint. Heat-treatment caused aggregation of soluble proteins which prevented it from entering the wells of the gel as the proteins became too large to enter after aggregation. Interfacial tension analysis showed that heat treatment led to a rapid equilibrium of the proteins at the oil-water interface compared to the untreated proteins, which was ascribed to the partial protein denaturation causing partial exposure of the hydrophobic patches leading to lower interfacial tension and higher surface activity of the heat-treated proteins and better emulsion stability. From DSC analysis, the thermal enthalpy change of the pea protein concentrate, heat-treated protein and untreated protein was understood.

The absence of starch gelatinization peaks in untreated soluble protein and the heat-treated soluble protein samples indicated that the starch was absent in the soluble protein fractions due to mild fractionation. The lower enthalpy change of heat-treated proteins when compared to untreated proteins might be due to the partial denaturation of the heat-treated protein which was carried out before the DSC experiment. This suggests that partial denaturation of pea proteins resulted in structural changes of the protein. Heat-treated protein also showed lower fluorescence intensity when compared to untreated protein, which indicated exposure of hydrophobic residues to the exterior parts of the proteins due to heat-treatment of protein. The exposed hydrophobic amino acids showed fluorescence quenching leading to a lowering of their intrinsic fluorescence. From, these characterizations we can understand that partial denaturation caused the exposure of the hydrophobic patches that helped to enhance the stability of emulsions towards further heat treatment. The pH 7 emulsions made with heat-treated soluble proteins showed lower creaming velocity, smaller droplet size and flowable consistency even after the heat-treatment of the emulsions at 90°C for 30 min, indicating superior emulsion stability when compared to emulsions made with untreated soluble proteins.

6. CONCLUSION

The overall objective of this research was to create canola oil beverage emulsions that would be stabilized by mildly fractionated pea proteins. Initially, beverage emulsions were made using pea protein concentrate, to check if it was capable of stabilizing the emulsion without any further processing. Significant thickening and phase separation was observed in the emulsions made with the concentrate, which was not ideal for beverage emulsions. Starch and other insoluble components of the concentrate were believed to cause thickening in the emulsion. Hence, it was concluded that mild fractionation was necessary to separate the soluble proteins which would offer superior stability to the emulsions when compared to the concentrate. It was observed that, of the two-pea protein concentrate samples, PP55 yielded higher quantities of soluble proteins compared to PP55P30, and hence PP55 was used for further analysis. Both two-step and one-step centrifugation processes yielded similar quantities of proteins in the soluble fraction. The soluble protein fraction had 2.5 wt% of protein in the aqueous phase after centrifugation, which was directly used to develop beverage emulsion. When compared to the other dilutions of proteins in the aqueous phase, emulsions containing 2.5 wt% proteins in the aqueous phase had the highest stability considering various factors such as droplet size, zeta potential and creaming velocity. Emulsion stability was tested at various environmental stresses (heat, salt and pH). It was observed that the stability of emulsions increased upon addition of salt at pH 7 due to the salting in effect. Whereas emulsions at pH 2 weren't stable due to extensive protein and droplet aggregation. The acidic pH denatured the proteins causing the emulsions to aggregate, thereby destabilizing the emulsion. After a week of storage, phase separation was observed at low salt concentrations in pH 7 emulsions and in all pH 2 emulsions with or without salt.

When the fresh pH 7 and pH 2 emulsions were subjected to heat (90°C for 30 minutes), the heated emulsions at both the pH conditions showed extensive protein and droplet aggregation due to the heat-induced unfolding of proteins on the oil droplet surface leading to hydrophobic attraction

causing aggregation. The strength of the aggregate increased with an increase in salt concentration at pH 2. The aggregation of heated pH 2 emulsions at 1 M ionic concentration was stronger than the aggregation of emulsions at all other conditions. The aggregate size of this particular emulsion was also the highest when compared to all other emulsions, indicating that 1 M heated pH 2 emulsion was the least stable one.

Since it was observed that heating the emulsion showed extensive aggregation, the next step was to resolve the problem. The soluble proteins were heat-treated at 75°C to induce partial denaturation, which helped expose the hydrophobic patches. These hydrophobic patches subsequently helped in conferring better stability to the lipid droplets in the emulsion. The emulsions made with this heat-treated soluble protein were further characterized to understand how stable they are under various environmental stresses. It was observed that heat-treated soluble protein-stabilized emulsions at pH 7, were stable at different salt concentrations compared to all other emulsions, even after heating the emulsion to 90°C for 30 min. However, the pH 2 emulsions made with heat-treated soluble proteins were not stable and suffered extensive protein and droplet aggregation. *In vitro* lipid digestibility was determined for the untreated protein-unheated emulsion, heat-treated protein unheated and heated emulsions. It was observed that untreated protein-unheated emulsion had 97.51% lipid digestion, whereas the heat-treated protein unheated emulsion and heat-treated protein heated emulsion had 56.06% and 73.47% of lipid digestion, respectively. The differences in lipid digestibility were attributed to the differences in the protein structure of the various emulsions corresponding to the heat treatments.

Finally, to understand how the structure of the heat-treated proteins influenced emulsion stability, the proteins were characterized using SDS-PAGE, intrinsic fluorescence, DSC analysis and interfacial tension. From the various characterization tests, it was concluded that the partially denaturing the proteins exposed their hydrophobic patches, which made them adsorbed better on the oil droplet surface. Upon further heating the emulsions, the already denatured proteins adsorbed at the oil droplet surface at pH 7 were protected from further aggregation leading to a much-improved emulsion stability compared to the untreated proteins.

The results presented in this thesis were in accordance with the four hypotheses proposed at the beginning of the thesis. First, mild centrifugation was carried which helped in retaining the functional proteins. Protein enrichment was done by separating soluble protein fractions from the protein concentrate. Second, the soluble proteins extracted via this method were directly used to prepare canola oil beverage emulsions without any further processing of the protein. Third, as expected, heat treatment led to structural changes in the soluble proteins which destabilized the emulsions. Finally, partial denaturation of soluble proteins helped in the development of stable emulsions and prevented the damage caused by heat treatment of emulsions at pH 7.

7. FUTURE STUDIES

This study involves the extraction of soluble pea proteins via simple centrifugation for the utilization of stabilizing canola oil-in-water beverage emulsions. During mild fractionation, centrifugation of a 7 wt% pea protein concentrate dispersion was done at 4000×g for 1 minute to separate a soluble protein fraction containing 2.5 wt% proteins from the insoluble fraction. Various concentrations of the pea protein concentrate can also be tested to see if it has an impact on soluble protein retention. Different centrifugation speeds can also be tested to check how it impacts the separation of the protein fraction and the insoluble fraction. The effect of different pH of the dispersion during centrifugation can also be examined. It was observed that 2.5 wt% of soluble proteins in the aqueous phase help in stabilizing the emulsions containing 5 wt% oil. Future studies can be done to check how higher protein concentrations in the aqueous phase impact the stability of the emulsions. Higher concentrations could not be tested in the current research as 2.5 wt% of proteins was the maximum protein retained in the soluble protein fraction after centrifugation of the 7 wt% pea protein concentrate dispersion. Since the aim of this research was to utilize the mildly extracted pea proteins, the soluble protein was not further spray dried or freeze dried; instead, soluble protein fraction from centrifugation was directly used to stabilize the emulsions. Canola oil was used in the present research in the development of beverage emulsions. In future, research can focus on the development of flavour oil-based beverage emulsion. The emulsion stability and characterization can be done on these flavour oil emulsions and can be compared with the emulsions made from canola oil. It would be interesting to know how flavour oils such as citrus oils, orange oil and lemon grass oil would impact emulsion stability as Ostwald ripening could be a problem with utilizing these flavour oils due to their partial water solubility. This can, however, be prevented by adding ripening inhibitors to promote emulsion stability.

Emulsions are ideal delivery systems for hydrophobic bioactives, such as vitamin A, vitamin D, curcumin, resveratrol, etc. Future studies can focus on incorporating such bioactives into the beverage emulsion, which would enhance their nutraceutical value and create functional foods for

improving health benefits. In the current research, lipid digestibility tests were performed to understand how protein pre-heat treatment and emulsion heat treatment would affect emulsion digestion. However, protein digestibility tests were not performed in the current research, understanding how various environmental factors, such as heat treatment, would affect protein digestibility alone would be important to know. In the current research, it was proposed that interfacial aggregation of heat-denatured protein was responsible for their lower lipid digestibility. To test that hypothesis, interfacial rheology of the heat-treated and unheated proteins could also be performed. It would also be interesting to know if the addition of salt and changing into acidic pH would have any impact on the lipid digestibility of the emulsions. Protein digestibility and its impact on lipid digestibility would be something important to know.

Protein characterization is very important to understand the structure and functionality of the proteins and its impact on emulsion stabilization. It would be important to determine the secondary structure of soluble proteins extracted via mild fractionation using FTIR spectroscopy. The changes in the protein secondary structure after heat treatment would also be something important to know to effectively utilize the proteins. Finally, one of the major problems in this research was protein aggregation in the acidic pH 2. Many industrial beverages are acidic in nature; therefore, it would be important for the emulsion to be stable at acidic pH. Further research needs to be done to modify the structure of the pea proteins or to modify the mild fractionation process such that the protein withstands the damaging effects of pH 2 and promotes emulsion stability at acidic pH as well.

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