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Kinetic And Oxidative Stability Of Oil-In-Water Emulsions Prepared With Denatured Soy Whey Protiens And Soy Soluble Polysaccharides

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KINETIC AND OXIDATIVE STABILITY OF OIL-IN-WATER EMULSIONS PREPARED WITH DENATURED SOY WHEY PROTEINS AND SOY SOLUBLE POLYSACCHARIDES

by

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Kinetic and oxidative stability of oil-in-water emulsions prepared with denatured soy whey proteins and soy soluble polysaccharides

Abstract

With increasing consumer awareness and growing demand for healthier processed food options, there is an ever-present push for the incorporation of nourishing ingredients into foods. Many health-promoting ingredients, for example Omega-3 fats, are prone to rancidity and are insoluble in water. A current challenge facing the Ontario agri-food sector is the addition of such ingredients that can normally be added to fatty foods, but not into water-based foods such as many store-bought beverages. Furthermore, oils such as flaxseed oil are also very sensitive to oxidation when in the presence of light, heat or air, resulting in the formation of undesirable odours and flavours as well as loss in nutritional properties. The use of food emulsions is considered an attractive approach to preserve their healthfulness while minimizing rancidity.

The overall goal of the present thesis was to incorporate soybean or flaxseed oil as micron-sized droplets within water-continuous emulsions using biopolymers derived from soy industrial processing waste to help 'protect' the oil from visual phase separation and coalescence as well as oxidative rancidity. To meet the goal, an extraction protocol to purify and concentrate the soy whey proteins (SWP) was initially developed. This was followed by establishing a method to increase the surface activity of the SWP via denaturation (dSWP). Subsequently, emulsions consisting of soybean oil or flaxseed oil prepared with dSWP and commercially-available soy soluble polysaccharides (SSPS) were analyzed for their kinetic and oxidative stability.

Results clearly showed that the combination of dSWP and SSPS could: i) kinetically stabilize model oil-in-water emulsions against coalescence and phase separation more so than dSWP or SSPS alone and ii) effectively protect emulsions containing flaxseed oil from oxidative rancidity to a greater extent than a commonly-used emulsifier (polysorbate 20).

Overall, this thesis yielded a novel method to emulsify and protect polyunsaturated oils using soy-based proteins and polysaccharides. The outcomes of this study offer the attractive potential of using soy-based ingredients from industrial waste in value-added food products such as beverage-type emulsions. Findings from this study may be applied to non-food products where there is a need for the development and stabilization of emulsions (e.g., pharmaceutical, cosmetics).

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Acronyms and abbreviations

(in alphabetical order)

CD spectroscopy	Circular dichroism spectroscopy
DLVO theory	Derjaguin, Landau, Verwey, and Overbeek theory
DSD	Droplet size distribution
dSWP	Denatured soy whey protein
IEP	Isoelectric point
KTI	Kunitz trypsin inhibitor
L	Lectin
MDA	Malondialdehyde
nSWP	Native soy whey protein
O/W	Oil-in-water
PSD	Particle size distribution
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSPS	Soy soluble polysaccharide
TBA	Thiobarbituric acid

List of symbols

(in alphabetical order)

b	Path length through a sample
c	Sample concentration
$D_{4,3}$	Volume-weighted average particle or droplet size
dx/dt	Terminal velocity of a particle undergoing sedimentation or creaming
ΔG	Gibbs' Free energy
$F_{suspension}$	Percent of suspension layer
g	Gravitational constant
I	Intensity of scattered light
I_0	Intensity of incident light
I_d	Intensity of light at a distance d from a scattering particle
I_t	Intensity of transmitted light
k	Boltzmann Constant
κ^{-1}	Debye screening length
ℓ	Path length through a sample
N_A	Avogadro number
p	Probability statistic

r	Particle radius
T_r	Transmission of light through a sample
V_A	London-van der Waals attractive interaction energy
V_{DL}	Electrical double layer repulsive interaction energy
V_{max}	Primary energy barrier for particle/droplet interaction
V_R	Electrostatic repulsive interaction energy
V_T	Total interaction potential
x	Distance of scattered light from a particle
y	Ratio of the refractive index of a dispersed particle or droplet relative to the medium
ΔBS	Difference in backscattering
ε	Dielectric constant of a medium
ε_λ	Molar absorptivity
ζ	Zeta potential
η	Viscosity
λ	Wavelength of light
μ_e	Electrophoretic mobility
ρ	Density
τ	Turbidity

Chapter 1: Introduction

1.0 General Introduction

A considerable amount of research has been published on the stabilization of oil-in-water (O/W) emulsions by proteins, polysaccharides or protein-polysaccharide complexes. The uniqueness of the present research was to use soy whey protein-polysaccharide interactions to promote the kinetic and oxidative stability of oil dispersed within O/W emulsions. A certain number of publications have been published on the stabilization of emulsions with soy soluble polysaccharides (SSPS) alone or in combination with other proteins, but denatured soy whey proteins (dSWP) have not extensively been utilized, given the difficulties associated with their extraction. This chapter briefly summarizes some of the key points of colloidal stability and their importance in emulsion kinetic stability.

1.1 Emulsion stabilization

In general, an emulsion consists of two immiscible liquids, normally oil and water, where one forms dispersed or internal phase and the other is the continuous or external phase [1]. The contact between these two phases is thermodynamically unfavourable and as a result, the interface must be kinetically stabilized by surfactants such as low molecular weight emulsifiers, proteins, polysaccharides, protein-polysaccharide complexes or other surface-active species. The presence and adsorption of such components results in an interfacial layer around dispersed droplets that help retard droplet flocculation and coalescence. Kinetic stability against coalescence and phase separation can also be achieved by reducing the rate of creaming/sedimentation through a reduction in droplet size, increasing continuous phase viscosity and/or minimizing the density difference between the dispersed and continuous phase [2].

1.2 Fundamentals of colloidal systems

Forces acting between particles in suspensions and emulsions significantly influence the stability of aqueous dispersions, colloidal particles as well as O/W emulsions. These forces are now discussed.

1.2.1 DLVO theory

The interactions and forces involved with electrically-charged colloidal particles during flocculation can be described by the DLVO theory, named after Derjaguin, Landau, Verwey, and Overbeek [3]. The stability of a colloidal system depends on the total interaction potential (V_T) at the colloidal surface. V_T is the sum of attractive long range van der Waals forces (V_A) and electrostatic repulsive forces (V_R) (Equation 1.1).

$$V_T = V_R + V_A \qquad \text{(Eqn. 1.1)}$$

When two particles approach each other, the change in total energy can be estimated from DLVO theory (Figure 1.1).

If repulsive forces dominate, the total energy of the system increases and the system is stable. Electrostatic repulsive forces increase exponentially when inter-particle distances (H_0) decrease. Accordingly, when attractive forces dominate, total energy decreases and the system is destabilized.

The DLVO theory only considers the long range repulsion as a consequence of interacting electrical double layers and attractive forces due to van der Waals interactions. It cannot explain short range interactions between particles a few nanometres from each other. Such attractive and repulsive forces may modulate the stability of colloidal dispersions and emulsions. These include non-DLVO forces such as hydrophobic interactions, hydration effects and steric repulsion, that can at times be more influential than DLVO forces [3]. As later discussed, steric effects were central to the emulsions' stabilization herein studied.

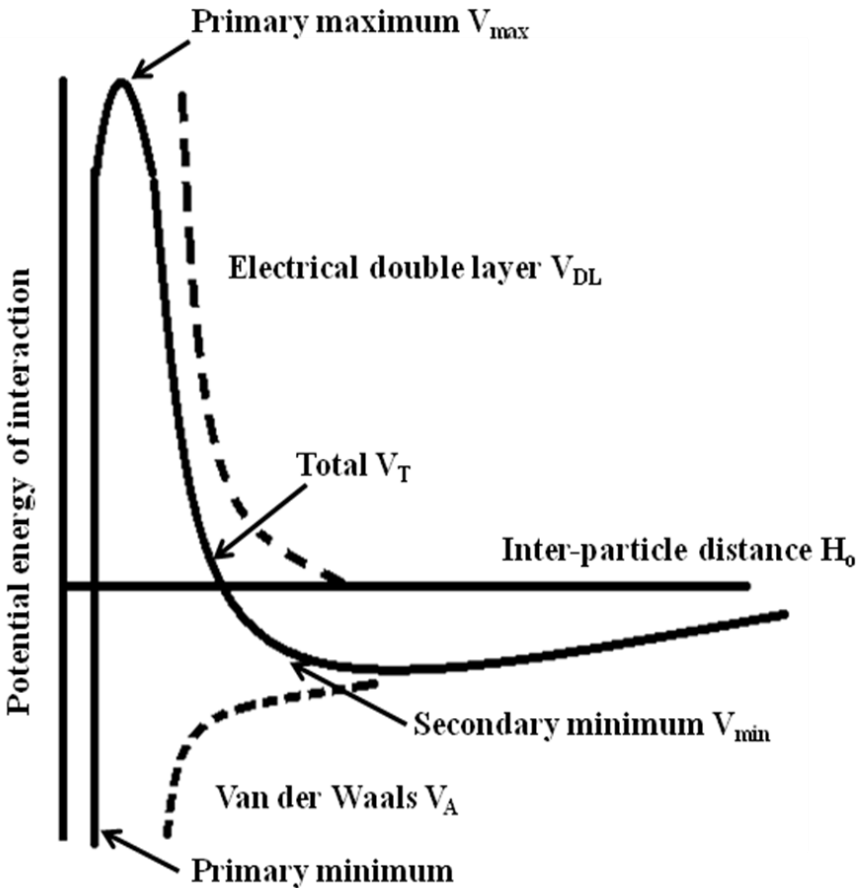

Figure 1.1: Schematic representation of the DLVO theory showing the overall potential energy of the system as a function of inter-particle distance (H_0). The electrical double layers (repulsive interaction energy) (V_{DL}), van der Waals dispersion forces (attractive interaction energy) (V_A) and the total interaction potential V_T are shown (adapted from [1]).

1.2.2 Non-DLVO forces

Hydrophobic forces are attractive by nature and also play a major role in determining the behaviour of many colloidal systems, for example food emulsions, lipids and proteins [2]. These strong forces act between nonpolar groups separated by water. Normally, water molecules hydrogen bond with their nearest neighbours whereas nonpolar molecules only form van der Waals bonds. When a nonpolar molecule is introduced into liquid water, water molecules in its immediate vicinity rearrange themselves by disrupting the hydrogen bonding structure with neighbouring water and/or polar groups. This minimizes the water-nonpolar group contact [1]. As a result, this leads to hydrophobic bonding between hydrophobic surfaces to minimize contact

with water molecules. Such an effect is dominant in water-continuous food emulsions as, in the absence of any surfactant; the dispersed oil droplets will flocculate and coalesce to minimize oil-water surface area.

Hydration interactions originate from the structuring of water molecules around polar and ionic groups. Hydration interactions can produce repulsive forces between colloidal particles and/or emulsion droplets by disrupting the bonding between polar groups and the water molecules in their immediate vicinity. This effect can increase repulsive interactions between two approaching particles because they must lose the water of hydration before interacting with each other. With higher ionic strengths ($\geq$ 1 mM), the hydration effect is significant [2]. The magnitude and range of hydration interactions depend on the number and strength of the bonds formed between the polar groups and the water molecules. Hydration interactions are important in regards to the thermodynamics of folding and unfolding of proteins present in an aqueous phase, as they play a significant role in their native structure before interfacial adsorption during emulsion preparation [2]. This effect thus plays a significant role in emulsion stability, particularly at higher ionic strengths when charged molecules may adsorb at the interface [1].

Steric repulsion is generated when the electron clouds of two or more neighbouring atoms or molecules overlap. Correspondingly, when a considerable amount of interfacially-adsorbed material extends outwards from the droplet/particle surface, a large steric repulsive force is produced and may therefore prevent emulsion droplets/particles from aggregating [3]. When a particle or emulsion droplet surface is covered with a highly-branched biopolymer, the hydrophilic branches may cause structuring of aqueous phase during the close approach of two droplets/particles, resulting in short-range, volume-limited repulsive forces that lead to steric stabilization. The overall magnitude, sign and range of steric interactions are strongly dependent on the characteristics of the interfacial layers, e.g., thickness, packing, emulsifier type and molecular interactions [2]. Densely-packed thick interfacial layers can prevent droplet flocculation and coalescence as well as improve emulsion stability in higher ionic strength environments [1]. Steric barriers may also act as obstacles against aqueous phase pro-oxidants, (e.g., transition metals or radicals), thereby limiting their ability to permeate through the interface.

1.3 Instability of emulsion systems

The kinetic stability as well as the taste, texture or mouth-feel of emulsions strongly depend on their composition, local environmental conditions (e.g., pH, ionic strength) and processing conditions (e.g., homogenization, thermal treatment, storage condition). All of these factors will impact the key modes of destabilization that may occur in food emulsions which are discussed here, namely creaming/sedimentation, flocculation and coalescence (Figure 1.2).

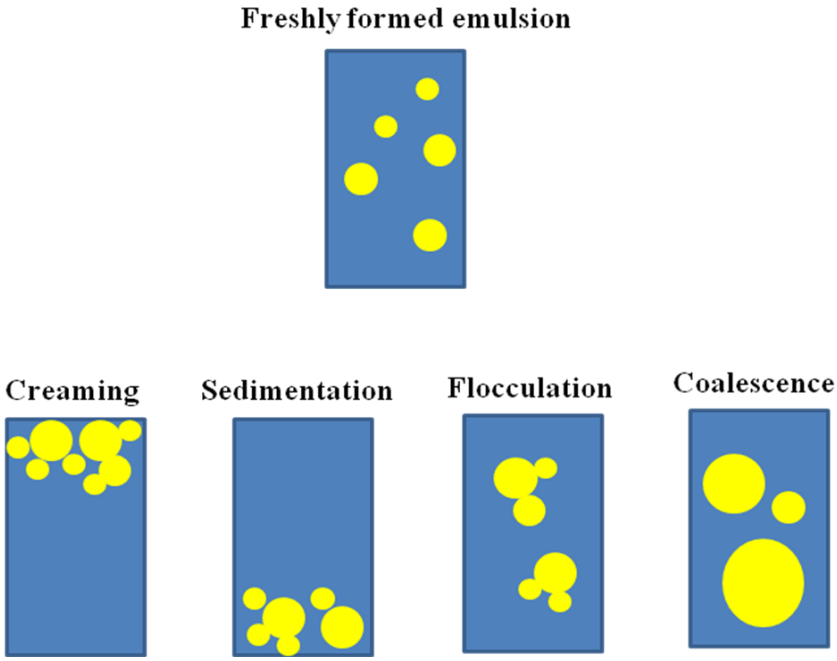

Figure 1.2: Common mechanisms of destabilization for typical emulsions.

1.3.1 Flocculation and aggregation

As a result of Brownian motion, colloidal particles and droplets below a critical size move randomly and may collide with neighbouring particles depending on the relative magnitude of the attractive and repulsive forces present. If the total attraction energy between droplets/particles is higher than the total repulsion energy, they will adhere to one another (Figure 1.2). During flocculation, droplets/particles cluster together but do not merge [2]. Flocculation is a reversible phenomenon where it is possible to re-disperse the clustered entities

by gentle agitation. However, if the droplets/particles collide with a sufficiently high energy to overcome the primary energy maximum, they permanently cluster in a process known as aggregation [3].

Due to random collisions between dispersed droplets/particles, there are two possible types of flocculation that have direct relevance to food emulsions: bridging or depletion flocculation. In the former, large surface-active biopolymers (e.g., proteins or polysaccharides) have multiple sites to interact with other molecules, which can increase the possibilities of interacting ('bridging') between more than two entities. Normally, if the amount of surface-active biopolymer is less than the saturation concentration required to fully cover the droplet surface, bridging flocculation occurs [2]. Depletion flocculation arises from local changes in osmotic pressure in emulsions containing unadsorbed polymers in the continuous phase. Typically, given their large hydrodynamic radius, biopolymer molecules tend to be excluded from the gap between two approaching droplets/particles. The region between droplets is thus depleted of biopolymers compared to the bulk aqueous phase [3]. As a consequence, the solvent between the droplets/particles is reduced due to osmotic pressure gradient, and this facilitates the depletion flocculation.

1.3.2 Coalescence

Coalescence occurs when two or more droplets irreversibly merge into a large daughter droplet. This process can only occur when droplets are in close proximity and the thin film of continuous phase separating them is ruptured [2]. The rate of coalescence is highly dependent on numerous factors, namely the composition of the adsorbed surfactant layers as well as the local environmental conditions (e.g., ionic strength, pH, temperature). Contrary to flocculation, this process is irreversible and leads to emulsion breakdown.

If droplets are attracted to and/or pushed into one another by an external force, a thin solvent layer is formed between them [4]. Its thickness is controlled by the balance between attractive and repulsive forces between droplets as well as the interfacial layer composition. For example, an interfacial layer at the surface of a droplet consisting of highly-branched hydrophilic biopolymers will increase the solvent film thickness by encompassing an appreciable amount of solvent. Thus, film thickness will decrease as two or more particles/droplets approach each other

more closely. Change in ionic strength can also modulate film thickness by binding counter ions to the adsorbed charged interfacial polymeric layers, which partially neutralizes surface charge causing a reduction in the effective range of the electrostatic double layers present [4]. Finally, near the isoelectric point (IEP) of adsorbed proteins (i.e., the pH at which the global surface charge of the protein is nil), electrostatic repulsion between two droplets may be reduced thereby decreasing solvent layer thickness thus increasing the propensity towards coalescence.

1.3.3 Creaming and sedimentation

Depending on the density difference between the dispersed and continuous phases, flocculated or aggregated droplets may either settle to the bottom or rise to the top of a sample [2]. When higher density droplets settle to the bottom of a vessel, this is known as sedimentation whereas lower density droplets migrate to the top in a process known as creaming (Figure 1.2).

Whether a particle or droplet undergoes sedimentation or creaming depends on its size and density as well as the density and viscosity of the continuous phase. Stoke's law provides an estimate of the rate of sedimentation and creaming:

$$\frac{dx}{dt} = \frac{2r^2(\rho_2 - \rho_1)g}{9\eta} \qquad \text{(Eqn. 1.2)}$$

where dx/dt is the terminal velocity of the particle/droplet, r is the particle/droplet radius, ρ_1 is the density of the continuous phase, ρ_2 is the density of the particle/droplet, g is the gravitational constant and η is the viscosity of the continuous phase [3]. Sedimentation/creaming rates increase with the square of the particle radius, clearly identifying particle size as a dominant factor in this equation. However, particle size may consist of either individual or clustered droplets. Hence, prevention of flocculation is an important route to retarding phase separation. Gravity also plays an important role in creaming and sedimentation (Equation 1.2) as aggregated droplets will cream/sediment more quickly. If the bulk phase viscosity is reduced due to the absence of any thickening agent, the friction forces acting on the dispersed droplets will be smaller, thereby accelerating phase separation [2]. Finally, increasing the density difference between the dispersed and continuous phases leads to faster breakdown.

1.4 Improving emulsion stability

Emulsion stability refers to the resistance of dispersed droplets in an emulsion to resist physical degradation processes such as flocculation, coalescence, creaming and sedimentation. Given the presence of a significant interfacial tension between oil and water, the contact between two immiscible liquid phases, e.g., between the dispersed and continuous phase of a freshly-prepared emulsion, is thermodynamically unfavourable. Hence, given enough time, any emulsion will break down. To retard such destabilization, emulsifiers and/or surfactants are used to reduce interfacial tension. As well, the presence of electrostatic and/or steric repulsion at the interface (i.e., by adsorbed proteins or surface-active polysaccharides) can further retard droplet-droplet contact thereby limiting emulsion destabilization.

Emulsion stability depends on a number of factors, including the:

(a) Physical nature of the interfacial film

Improved emulsion stability is achieved with densely-packed surfactants at the oil-water interface that confer strong lateral intermolecular forces and thus higher film elasticity [5, 6].

(b) Presence of an electrical and/or steric barrier on droplets

Interfacially-adsorbed emulsifiers with highly-branched hydrophilic charged groups oriented towards aqueous phase improve the stability of O/W emulsions via electrostatic and steric repulsion between neighbouring droplets [2].

(c) Droplet size distribution

Smaller-sized droplets result in more kinetically-stable emulsions. As well, an emulsion with a fairly uniform narrow size distribution is more stable than one with the same average particle size but a wider size distribution [5, 6].

(d) Phase volume ratio

Surfactants are most effective at emulsion stabilization when present at their critical micelle concentration (CMC), which results in the monolayer coverage of all droplets in an emulsion.

For a given dispersed phase volume and surfactant concentration at the CMC, an increase in surface-to-volume ratio will result in the re-distribution of surfactants onto more droplets, thereby yielding a surfactant concentration below that of the CMC. In this scenario, the resulting emulsion is less stable as the droplets are not fully covered by surfactant, thereby exposing bald patches more prone to flocculation and coalescence [7, 8].

(e) Viscosity

Addition of thickening agents to the continuous phase can increase the continuous phase viscosity and promote the kinetic stability of an emulsion.

(f) Temperature

An increase in temperature increases the kinetic energy of an emulsion, which results in a number of destabilization phenomena including a reduction in the concentration of interfacially-adsorbed surfactant, denaturation (i.e., of proteins), a reduction in continuous phase viscosity and perhaps surfactant solubility [5, 6].

1.5 Soy-based ingredients

From a food industry standpoint, high-value food products such as functional foods (e.g., sports beverages) are valuable revenue generators as they are less price-sensitive than 'standard' grocery items. For example, in 2005, global consumption of low-pH protein beverages was $27B US [9]. Furthermore, there is a continued (and growing) demand from many food manufacturers for ingredients that provide functional benefits to their products at a reduced cost.

Ontario produces 80% of Canadian soybeans (2.5MT valued at $727M in 2006), and is the province's most valued field crop. Most Ontario soybeans are crushed for low-value soymeal used for animal feed and other under-valued applications. When current worldwide sales of nutraceuticals at ~$80B US [9] and an annual growth rate of 15-20%, are factored in, the use of under-valued soy-based ingredients may offer advantages for use in novel value-added food applications.

Significant industrial waste results from the production of both tofu and soymilk. Waste effluent from the former contains an appreciable amount of proteins [soy whey proteins (SWPs)] whereas the latter contains soy soluble polysaccharides (SSPS). Both of these biopolymers were shown to successfully form and stabilize O/W emulsions. Given its success, the outcomes of this research may result in significant value addition to agricultural waste associated with Ontario-grown soybeans.

1.6 Hypotheses

The research hypotheses explored within this thesis were that:

i) The combination of SSPS and dSWP would provide improved O/W emulsion stability compared to SSPS or dSWP alone;

ii) Synergy between SSPS and dSWP would be most effective at acidic pH, and;

iii) Combined use of dSWP and SSPS would improve the kinetic and oxidative stability of flaxseed oil-based emulsions.

1.7 Thesis objectives

The overall objectives of the present thesis were to investigate the synergistic ability of dSWP and SSPS to:

i) kinetically stabilize O/W emulsions against physical breakdown, and;

ii) prevent the oxidative rancidity of a polyunsaturated oil dispersed within the emulsion as droplets.

The specific objectives were as follows:

i) Extract soy whey proteins from soy flour and characterize its composition and molecular weight;

ii) Investigate the ability of combined dSWP and SSPS to stabilize O/W emulsions against physical and oxidative breakdown, and;

iii) Study the effects of local environment (pH, ionic strength and temperature) on the kinetic and oxidative stability of emulsions stabilized with dSWP and/or SSPS.

1.8 Thesis organization

The thesis is divided into eight chapters organized as follows:

Chapter 1: Introduction

This chapter describes fundamental aspects associated with the stability of emulsions and interfaces. It also includes a brief overview of soy-based ingredients as well as the thesis hypotheses and objectives.

Chapter 2: Literature review

Chapter 2 presents a brief review of recent work on emulsions stabilized by proteins and/or polysaccharides with a focus on their kinetic and oxidative stability.

Chapter 3: Material and experimental methods

The chapter elaborates on the materials and experimental techniques used in this thesis, and provides a brief discussion of the concepts behind the experimental techniques used.

Chapter 4: Stabilization of soybean oil-in-water emulsions using denatured soy whey proteins and soy soluble polysaccharides interactions

This chapter covers the isolation, purification, denaturation and characterization of SWP as well as the formulation, characterization and kinetic stability of dSWP+SSPS-stabilized emulsions. This work was published in *Food Research International* [10].

Chapter 5: Physical and oxidative stability of flaxseed oil-in-water emulsions stabilized with soy whey proteins and polysaccharides

The chapter focuses on the formulation, characterization and oxidative stability of dSWP+SSPS-stabilized flaxseed oil-based emulsions as a function of pH and storage temperature. It was written in a manuscript format.

Chapter 6: Overall conclusion and prospects

Chapter 6 summarizes the results presented in the thesis and discusses potential applications of the emulsions herein developed.

Chapter 7: Future studies

This chapter highlights areas for possible future activities on emulsions stabilized by proteins and polysaccharides.

Chapter 8: References

This chapter consists of the references used in this thesis.

Chapter 2:

Literature review – food emulsions

2.0 Introduction

Emulsions are dispersions of one liquid within another, where the two liquids are immiscible. Emulsions are encountered in many fields, including foods, pharmaceuticals, cosmetics and crude oil [4]. In food systems, both water-in-oil (W/O) (e.g., butter, margarine) and oil-in-water (O/W) emulsions (e.g., salad dressings, soups) are encountered. These systems are inherently thermodynamically unstable. Hence, the energy required to create emulsions involves generating a large increase in the interfacial area between the two immiscible phases.

As previously mentioned, emulsions may be destabilized by a number of mechanisms. Creaming (or settling) is the separation of the two phases induced by differences in densities between the two phases. Flocculation involves the attraction of droplets to nearby neighbours by weak colloidal interactions. When droplets flocculate, they maintain their structural integrity [11, 12], and in some cases can even be re-dispersed. Coalescence is the complete merger of droplets, associated with inter-droplet film thinning and rupture. Phase inversion may occur where droplets of the continuous phase partially coalesce and as a result entrap the once-continuous phase within it. This is the mechanism by which butter (a W/O emulsion) is formed from milk (an O/W emulsion) during churning. Ostwald ripening, which is a lesser factor in most food emulsions, involves the growth of larger droplets at the expense of smaller droplets and is driven by solubility gradients created by differences in droplet Laplace pressures. The Laplace pressure inside a droplet is the pressure increase due to contraction of the droplet surface and the resulting decrease in volume. The pressure inside the droplet, p_{in}, is related to the pressure outside the droplet, p_{out}, by the surface tension of the droplet interface, γ, and the radius of the droplet, r [12, 13].

$$p_{in} = p_{out} + \frac{2\gamma}{r} \qquad \text{(Eqn. 2.1)}$$

Smaller droplets will have a greater internal pressure than larger droplets, thus exhibiting accelerated rates of Oswald ripening.

The kinetic stability of emulsions can be improved through a number of methods. One method is to decrease the average droplet size thereby reducing the rate of creaming or settling, as is done in the homogenization of milk [14]. Surfactants may be added to lower the interfacial tension between the two fluids. Surfactants also act to stabilize emulsions by forming a cohesive film around the droplets that resists coalescence [15]. Especially in aqueous systems, the addition of surface-active proteins can stabilize emulsions by increasing the interfacial elasticity, thereby slowing the rate of film drainage, and thus coalescence. Adsorbed biopolymers will resist droplet coalescence through steric effects but, as mentioned above, may contribute to flocculation through depletion interactions [16]. Conventional emulsions possess limited strength against environmental stresses such as pH, ionic, and thermal changes, and they have a limited capability to modulate functional performance such as ingredient protection and encapsulation [2, 15].

This chapter focuses on emulsion formation as well as the use of proteins and polysaccharides as emulsion stabilizers. Specifics on the structural and physical aspects of soy whey proteins and soy polysaccharides are also discussed. Finally, factors influencing emulsion stability are explored.

2.1 Thermodynamic perspective

Emulsions are not thermodynamically stable systems. This is because the most energetically-favourable state for the oil and water phases is when they are completely separated. Oil and water do not spontaneously form an emulsion - the application of some external action such as mixing is required for their formation. Once emulsified, the components tend to move towards regaining thermodynamic stability [2, 15, 17]. Emulsions that are said to be “stable” are in fact those for which the destabilization rate has been reduced enough so that the visual manifestation of emulsion breakdown will not begin within a practical timeframe (weeks, months or years), depending on the desired shelf-life [4].

Consider the energy of molecules contained in an emulsion system. At any given point, there are two possible states, a low-energy state, $E_{low,}$ and a high-energy state, E_{high}. The state that is more favourable for molecules is one that has the lowest free energy. The Boltzmann distribution given in Equation 2.2 describes how the two states are distributed when the system is at thermodynamic equilibrium.

$$\frac{\phi_{high}}{\phi_{low}} = \exp\left[-\frac{(E_{low} - E_{high})}{kT}\right] \qquad \text{(Eqn. 2.2)}$$

where ϕ is the fraction of molecules that occupies the energy level E, k is Boltzmann's constant (k = 1.38 x 10^{-23} J K^{-1}), and T is the absolute temperature. The product of kT represents the thermal energy of the system. When the difference between the high and low energy states is large compared to the thermal energy of the system, the fraction of molecules in the lower energy state is also high.

Before a system can reach equilibrium, it must overcome an energy barrier (ΔE^*) present between the high and low-energy states. For the system to move into the low-energy state, the system must acquire an amount of energy that is greater than this barrier. The lower the height of the energy barrier, the easier it is for the emulsion to become thermodynamically stable, hence the faster the rate of transformation from a high to low energy state will be. Although in a state of thermodynamic instability, an emulsion may remain at a high-energy state for a long period of time if the energy barrier is sufficiently high. At this point, the emulsion is considered to have kinetic stability or be in a metastable state.

2.2 Food emulsifiers

Emulsifiers (or surfactants) are amphiphilic molecules that consist of hydrophilic and hydrophobic moieties. During emulsion preparation, they adsorb to the surface of dispersed droplets formed during homogenization. Surfactant adsorption onto the droplet surface occurs when the hydrophilic head interacts with the aqueous phase and the hydrophobic tail interacts with the non-aqueous phase [2, 15, 18]. The result is a reduction in the interfacial tension between oil and water, which results in lower energy required for breaking up emulsion droplets

during homogenization. Once emulsified, surfactants may provide a repulsive force between droplets, hence reducing flocculation. For example, the addition of charged surfactants produces the same electric charge on all droplets leading to electrostatic repulsion. Short-range repulsive forces such as steric, hydration, and thermal fluctuation interactions are usually produced by non-ionic surfactants, and also aid in preventing droplet aggregation. Other surfactants are capable of reducing droplet aggregation by forming multilayers around the emulsion droplets rather than a monolayer [15, 18].

There are two broad classes of emulsifying agents used in food industry: small-molecule surfactants (e.g., monoglycerides, polysorbates, lecithins and fatty acid salts) and macromolecular emulsifiers (e.g., proteins and polysaccharides). There are four categories of small-molecule surfactants: ionic, non-ionic, zwitter ionic and cationic. Given that the focus of this thesis was on macromolecular species, these four categories are not further discussed. Attention is given to proteins and polysaccharides below.

There are three main characteristics that enable surfactants to enhance the formation and stability of emulsions: (1) rapid adsorption of surfactant molecules onto the droplets during homogenization; (2) monolayer coverage and reduction of the interfacial tension, and (3) reduction of destabilization phenomena [15, 18]. Small-molecule surfactants are usually very efficient at reducing interfacial tension compared to macromolecular species. Larger emulsifiers such as proteins and surface-active polysaccharides form interfacial layers that increase the ability of the oil-water interface to resist tangential stresses during unit operations that involve shear and changes in temperature [15, 18].

2.2.1 Proteins

Proteins are naturally-occurring biopolymers that consist of covalently-linked monomers of amino acids. Each amino acid contain at least one primary amino ($-NH_2$), one carboxyl (-COOH) group and R-group that strongly influences a protein's capacity to stabilize an emulsion. As a result of their amphiphilic property, proteins are often the emulsifier of choice for food processors. From a fundamental perspective, the relationship between protein structure and emulsifying activity depends on a combination of factors such as molecular flexibility, molecular

size, surface hydrophobicity, net charge, and amino acid composition. The molecular weight of proteins ranges from ~5000 to upwards of 500000 Da (e.g., myosin) [19].

Proteins are a popular choice to help incorporate bioactive lipids in emulsions due to their high digestibility, their ability to increase the bioavailability of incorporated hydrophobic bioactives as well as their generally-recognized-as-safe (GRAS) status. In addition, they can retard droplet coalescence by forming protective layers around droplets that help to stabilize dispersed droplets via electrostatic or steric repulsion as well as improving the mechanical strength of the interface [20].

The effectiveness of a protein to stabilize a dispersed droplet may also depend on pre-treatments, e.g., exposure of proteins to heat, high shear, high pressure and pH extremes – all conditions that may cause protein denaturation. Denaturation may expose previously unexposed hydrophobic groups, thereby potentially improving the emulsifying ability of the protein by enhancing its capacity to sit at the oil-water interface [21-24]. However, if the exposed hydrophobic groups increase inter-protein interactions, this may reduce overall emulsifying activity. Different chemical and/or enzymatic treatments, e.g., acylation, alkylation, oxidation, phosphorylation, deamidation, cross-linking with transglutaminase and digestion with enzymes such as proteases, have also been applied to modify the emulsifying ability of proteins [25-27].

Proteins may undergo surface denaturation after interfacial adsorption to maximize the number of favourable interactions with the oil and aqueous phases. Such adsorption and surface-induced denaturation depends on the molecular flexibility of the protein, with greater flexibility inducing more reorientation along the protein backbone chain. More rigid proteins, such as globular proteins, e.g. β-lactoglobulin, take longer to unwind and are less sensitive to surface denaturation whereas relatively flexible proteins, e.g., caseins, undergo rapid surface denaturation. In general, adsorption is often irreversible and is associated with a negative change in free energy [18].

2.2.1.1 Soy whey proteins

Soybean-based proteins are seeing increased interest due to their nutritional properties, functionality (e.g., emulsification, gelation, foaming, etc.) and increasing availability to food

processors. There are two families of soybean proteins obtained via aqueous extraction - storage globulins and whey proteins [28]. During the isoelectric precipitation of storage globulins, a residual liquid called 'soy whey' is generated. The main component of soy whey proteins (~ 10 % total extractable proteins) are lectin (L) and the Kunitz trypsin inhibitor (KTI) with molecular masses of 120 kDa and 20-22 kDa, respectively [28-30].

Lectin or soybean hemaglutinin is a tetramer composed of identical 30 kDa subunits (Figure 2.1). It consists of two carbohydrate binding sites, thus creating the possibility of complex formation with sugars [31]. KTI is a monomeric, non-glycosylated protein made up of 181 residues. The overall structure is composed of a sphere ~ 3-5 nm in diameter, consisting of 12 criss-crossing antiparallel β-strands stabilized by hydrophobic side chains (Figure 2.2). KTI has two disulfide bridges - Cys 39-Cys 86 and Cys 138-Cys 145, which are solvent-exposed and responsible for its inhibitory nature against trypsin enzyme [32-34] and resistance to thermal and chemical denaturation. It is also classified as a β-II or 'disordered' protein [35, 36]. Lectin and KTI are stable against pH-induced denaturation over wide pH ranges (3-10 and 2.2-10.8, respectively) [30, 37].

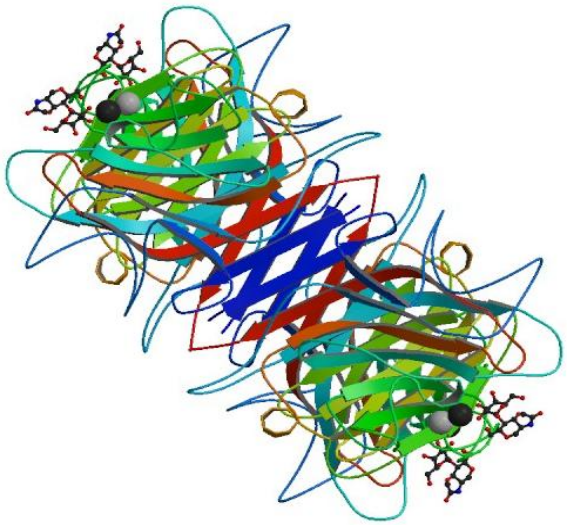

Figure 2.1: X-ray crystallographic structure of lectin (obtained from RSCB database [38]).

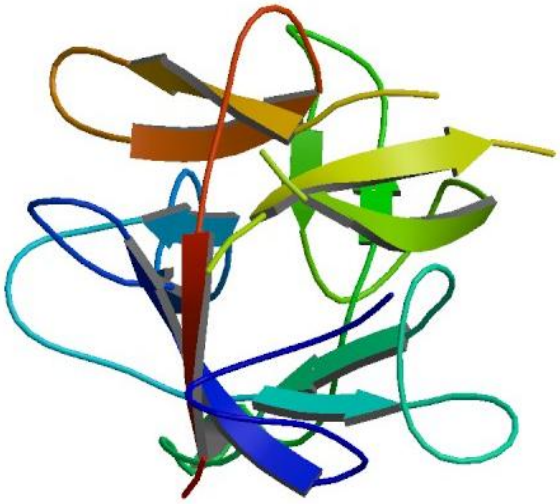

Figure 2.2: X-ray crystallographic structure of KTI (obtained from RSCB database [39]).

The limited scientific literature on the emulsifying ability of soy whey proteins (SWPs) has shown that dSWPs demonstrate better emulsifying activity than native SWPs [40, 41]. Further details on dSWPs are discussed in Chapter 4.

2.2.2 Polysaccharides

Most thickening agents are long-chain, straight or branched polysaccharide-based biopolymers that contain hydroxyl groups capable of hydrogen bonding. Polysaccharides are polymers of monosaccharides and the degree of polymerization is typically between 1,000 to 10,000 [2, 15]. Starches and cellulose are examples of homopolymers as they are made up of *n* repeating units of the same monomers. The sugar monomers can contain linked side units, or substituent groups, such as sulfates, methyl ethers, esters and acetals. They can be neutral or anionic.

Polysaccharides are used in numerous processed foods for emulsification, thickening, stabilization and gelation. The effectiveness of surface-active polysaccharides (e.g., derivatives of cellulose such as methylcellulose, carboxymethylcellulose, and hydroxypropylcellulose) as emulsifiers is based on their speed of adsorption at an interface, so that droplet aggregation can be inhibited. Following their interfacial adsorption, short-range steric repulsive forces help to prevent droplet flocculation. If the interface is electrically-charged, electrostatic repulsion may

help to further prevent flocculation. Ionic strength and pH clearly affect the magnitude of the electrostatic repulsions between droplets stabilized by charged polysaccharides.

Increasing the viscosity of the continuous aqueous phase of an emulsion via thickening or gelling improves the emulsion's kinetic stability [15]. Examples of thickeners include starches, celluloses, guar gum, etc. The ability of a biopolymer to increase the viscosity of an emulsion is based on its molecular weight, conformation, degree of branching, and flexibility. The biopolymers selected for this purpose are generally highly-hydrated molecules having extended structures, or they may be aggregates of molecules. Many polysaccharides (e.g. carrageenan, cellulose, pectin) have the ability to cause the aqueous phase in emulsions to gel. During gel formation, the biopolymers entrap water within their three-dimensional networks, forming a structure that has properties similar to those of a weak solid [2]. Gel properties depend largely on the biopolymer molecules themselves (type, structure and interactions). Gels may be hard, soft, brittle or rubbery, have homogenous or heterogeneous composition, and may also appear opaque.

2.2.2.1 Soluble soybean polysaccharides (SSPS)

A number of studies have documented that certain polysaccharides such as gum arabic and SSPS contain a protein moiety representing 2-4 % of their total molecular weight [42-44]. Such species exhibit surface/interfacial activity, which may be highly-beneficial for food emulsion formation. As well, their high water solubility, low bulk viscosity, good emulsifying ability and capacity to create a strong protective film around dispersed oil droplets have received considerable interest [42-44].

In this regard, SSPS is a promising choice for the stabilization of low pH food emulsions [43]. It is an acidic polysaccharide extracted from the carbohydrate by-product of soy protein isolate production composed of a main rhamnogalacturonan backbone branched by β-1,4- galactan and α-1,3- or α-1,5- arabinan chains and homogalacturonan (Figure 2.3) [42, 43, 45-47]. SSPS contains a hydrophilic carbohydrate portion covalently bound to a protein moiety of ~ 50 kDa that allows it to bind to oil-water interfaces while the carbohydrate portions form a steric barrier (~17 nm thick, though this is system-dependent) [47]. SSPS has demonstrated high water

solubility (up to 30 wt%), low bulk viscosity, good emulsifying properties, and ability to form strong interfacial films that play a major role in its emulsifying and stabilizing abilities.

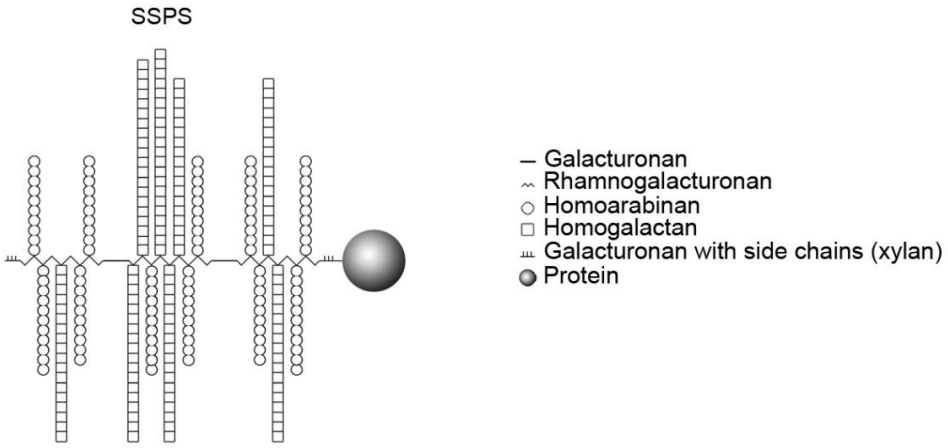

Figure 2.3: The structure of SSPS (adapted with permission from [48]).

Emulsions prepared with SSPS have been shown to remain stable at pH 3-7 and in ionic strengths of 5-25 mM [43]. SSPS is also resistant to heat denaturation up to 70˚C [49]. Literature data also indicates that SSPS can offer superior functional properties compared to gum arabic, modified starch, and pectin [31-36].

2.3 Protein-polysaccharide interactions upon mixing

Mixing of a protein and polysaccharide in a dispersion may give rise to three different outcomes depending on the biopolymer composition, their concentrations, pH, ionic strength, conformation, flexibility and charge density: 1) co-solubility, 2) incompatibility, or 3) complex coacervation (Figure 2.4).

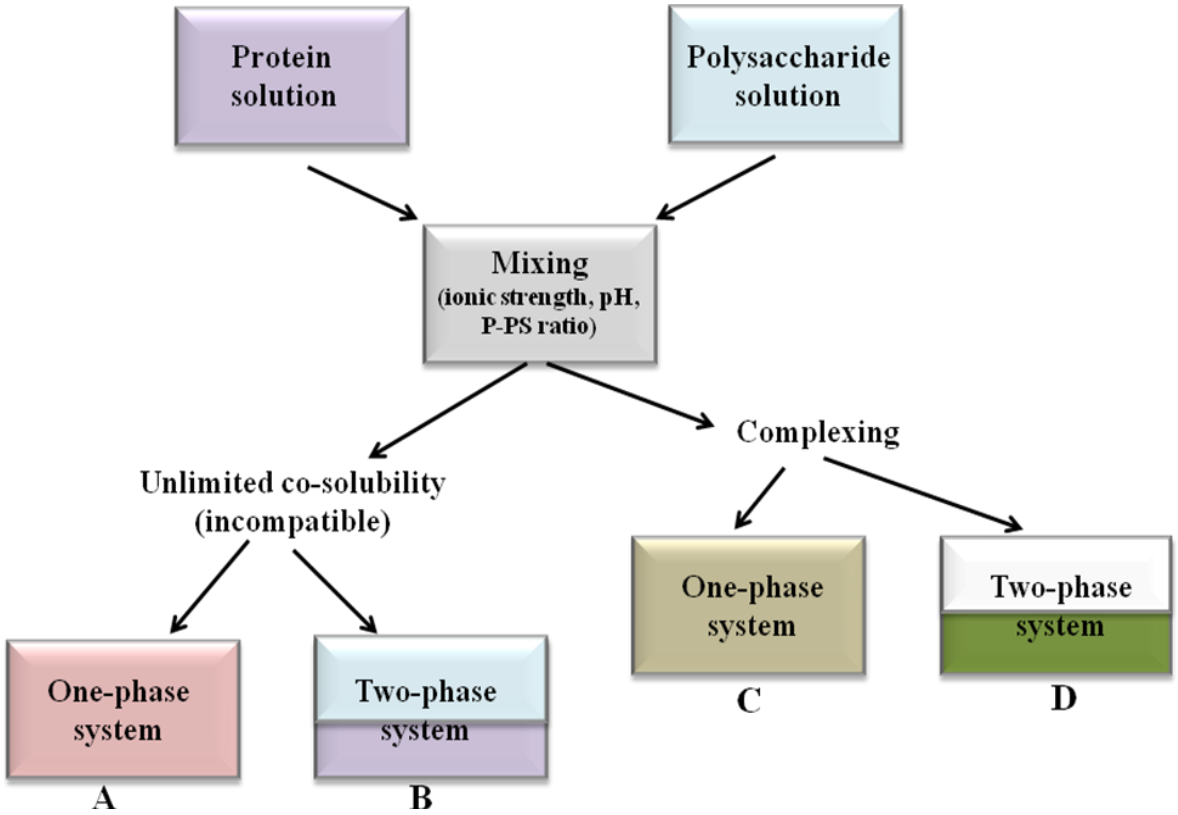

Figure 2.4: Possible interactions between proteins and polysaccharides in aqueous suspension: co-solubility (A), phase separation (B), soluble complexes (C), and insoluble complexes (D) (adapted from [50]).

For very dilute dispersions, the mixing entropy dominates protein-polysaccharide mixed dispersions, and both biopolymers will be co-soluble. Below the co-solubility threshold concentration, protein and polysaccharides are miscible and exist as a homogeneous suspension (Figure 2.4A). Above a critical concentration, they are incompatible, resulting in a two-phase separation, i.e., with one phase rich in protein while the other is polysaccharide-rich (Figure 2.4B).

Attractive interactions between proteins and polysaccharides can result in soluble and/or insoluble complexes (Figure 2.4C, D). Insoluble complexation leads to coacervate formation or associative phase separation [51]. Associative interactions produce primarily soluble complexes that interact to form electrically-neutralized aggregates that ultimately sediment to form a coacervate phase containing both biopolymers. Thus, two co-existing phases are

generated: 1) a solvent-rich phase with very small amount of biopolymers, and; 2) a biopolymer-rich phase containing the complexed biopolymers (Figure 2.4D).

In aqueous suspensions, protein-polysaccharide association is of physical origin, arising from hydrophobic, ionic interactions or hydrogen bonding. Electrostatic interactions are predominant when two oppositely-charged biopolymers are mixed at a pH below the IEP of the protein but above that of the polysaccharide (if charged), thus forming strong electrostatic complexes. Weaker reversible complexes are formed between anionic polysaccharides and proteins that are (nearly) neutral (pH ≈ IEP) and/or with a net negative charge (pH > IEP) [50]. On adjusting the pH and/or ionic strength of the aqueous phase, protein-polysaccharide interactions may be modulated, from net attractive to net repulsive or vice versa [52]. However, the presence of unfavourable repulsive interactions between segments on different biopolymers can lead to a high probability of mutual exclusion of each polymer from the local vicinity of the other. At sufficiently high polymer concentrations, such repulsion spontaneously produces two distinct phases due to their thermodynamic incompatibility [52].

2.3.1 Molecular interactions

Several important molecular interactions and entropy effects influence the conformation of biopolymers in aqueous solutions: hydrophobic and electrostatic interactions, hydrogen bonding and configurational entropy. Some of these are now discussed.

2.3.1.1 Electrostatic interactions

The molecular structure and aggregation of biopolymers are strongly influenced by electrostatic interactions. Proteins may contain amino acids that can form positively charged ions (such as arginine, lysine, and proline) or negatively charged ions (such as glutamic and aspartic acids). Polysaccharides may also have ionizable groups along their backbone structures (such as sulfate or phosphate groups). Electrostatic interactions may be affected by the pH of the surrounding aqueous phase and the pKa of the ionizable groups [9]. The effects due to electrostatic screening, dependent on the concentration and type of counter ions that may be present in the aqueous phase, may also be important. The structure of biopolymers is largely dependent on the type of charged groups it contains. If the groups have similar charges, then the molecule tends to stretch

out in order to produce more space between them and hence reduce electrostatic repulsions. If the groups are oppositely-charged, the molecule will take on a globular structure, since folding up would maximize electrostatic attractions.

Aggregation of biopolymer molecules in solution is also dependent on electrostatic interactions. Biopolymers with similar charges tend to remain as individual molecules due to repulsion, whereas aggregation occurs between molecules that are oppositely charged. Electrostatic interactions are also responsible for the binding of low-molecular-weight ions, such as Na^+ and Ca^+ to biopolymers. This may be important since many formulations often include various metal salts or pro-oxidant metals as discussed later.

2.3.1.2 Hydrogen bonding

Hydrogen bonding is a relatively weak bond that exists between molecules, but is strong overall if present in large numbers. Systems attempt to maximize the number and strength of these bonds within their structures. Proteins and polysaccharides both contain monomers that exhibit this type of bonding, which consequently affects their structures. Some may form very ordered structures (such as helices), while others may favour less ordered structures and form hydrogen bonds with the surrounding water molecules. The different forms are either entropically favourable or unfavourable. In general, the more highly ordered the conformation is, the less entropically favourable is the structure. Therefore, extensive intramolecular hydrogen bonding is less entropically favourable than extensive intermolecular hydrogen bonding (random-coil conformation).

Environmental conditions also play a key role in a biopolymer's structure formation since the strength of the hydrogen bond is relatively weak compared to the other forces of interactions such as hydrophobic and electrostatic, and configurational entropy that may be involved.

2.3.1.3 Hydrophobic interactions

The mixing between water and apolar substances such as many fatty acids and alcohols is thermodynamically unfavourable ($\Delta G > 0$). The antagonistic behaviour between water and these substances drives them to restructure into a conformation that reduces the contact of their hydrophobic domains with water. Therefore, in an aqueous environment apolar groups will

associate with each other rather than with the aqueous phase. A reduction in the interfacial surface area between the water and the apolar groups will result in an increase in the overall thermodynamic favourability of the system ($\Delta G < 0$).

Proteins generally contain a fairly high proportion of non-polar groups along their polypeptide backbones as ~ 40% of amino acids have non-polar side chains (e.g., methyl group in alanine, benzyl group in phenylalanine, isopropyl group in valine and mercaptomethyl group in cysteine). By contrast, polysaccharides are mainly hydrophilic, with few exceptions (e.g., gum arabic). The effect of hydrophobic interactions on their structures is therefore not as pronounced as in proteins.

2.3.1.4 Configurational entropy

The configurational entropy of a structure is the amount of free energy within its structure that results from its conformation. The conformation and aggregation of biopolymers depends largely on their configurational entropy, of which there are local and non-local contributions. The number of conformations that can be formed by individual monomers in a chain is referred to as local entropy whereas non-local entropy refers to the concerted number of conformations that the biopolymer chain can adopt. Random coil structures are highly flexible and can take on many conformations - therefore they have a high configurational entropy. More rigid structures such as compact globular proteins have much lower configurational entropies.

2.4 Protein-polysaccharide interfacial layers

Associative (i.e., net attractive) interactions in protein-polysaccharide mixtures can result in stable interfacial layers at the oil-water interface. Such protein-polysaccharide complexes may possess high surface activity, the ability to increase the bulk viscosity as well as the capacity to create gel-like charged and thick interfacially-adsorbed layers [53]. Normally, adsorption of neutral complexes leads to comparatively more densely-packed layers than adsorption of charged complexes (Figure 2.5) [54, 55]. As a result, protein-polysaccharide interactions can lead to the formation of food emulsions with a highly kinetically-stable dispersed phase.

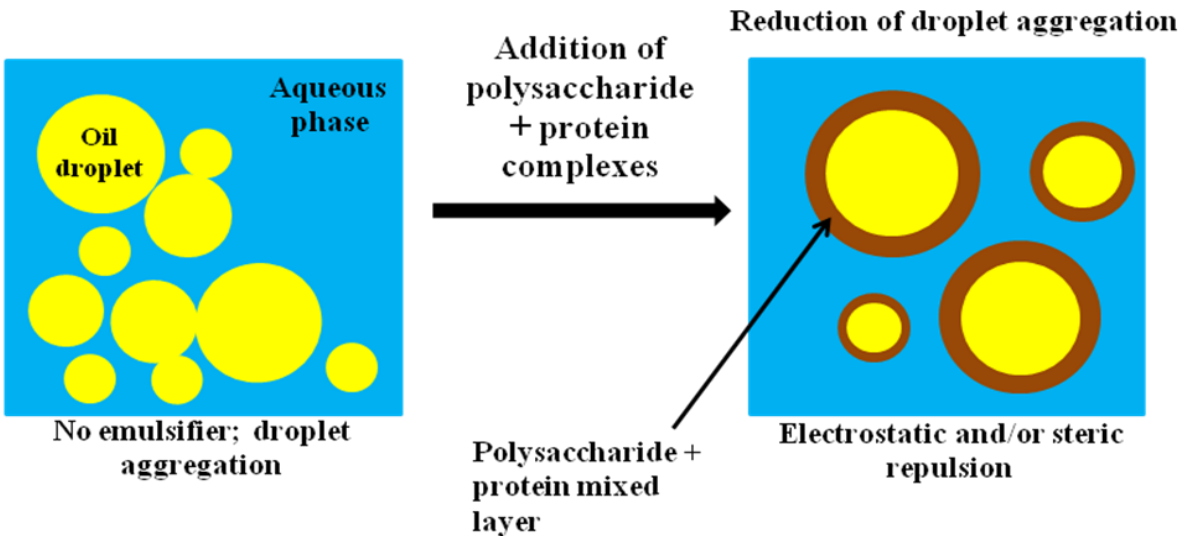

Figure 2.5: Putative mixed protein-polysaccharide layer formation at the oil-water interface.

2.5 Protein-polysaccharide interfacial layer formation

Multi-layered emulsions consist of two or more layers of proteins and polysaccharides that envelop dispersed oil droplets, in a process called 'layer-by-layer' deposition (Figure 2.6). They are based on the electrostatic deposition of charged biopolymers onto oppositely-charged sub-layers. An O/W emulsion is obtained by homogenizing the oil and aqueous phases in the presence of an emulsifier (usually a protein) [56, 57]. An oppositely-charged biopolymer is then added to this "primary emulsion", which leads to the electrostatic deposition of the second biopolymer onto the droplet surface. This creates a "secondary emulsion" that contains oil droplets coated by a double biopolymer layer [58]. By adding the next oppositely-charged biopolymer, a multi-laminated interface with three layers is formed, and a "tertiary emulsion" is obtained. Thick and highly-charged interfacial layers usually increase inter-droplet repulsion, thereby reducing flocculation and coalescence [59].

The mixed layer approach consists of the direct adsorption of protein-polysaccharide complexes formed before, during or after homogenization. This technique involves the preparation of bulk aqueous suspension of protein-polysaccharide complexes which are then used as the emulsifying agent during homogenization (Figure 2.6).

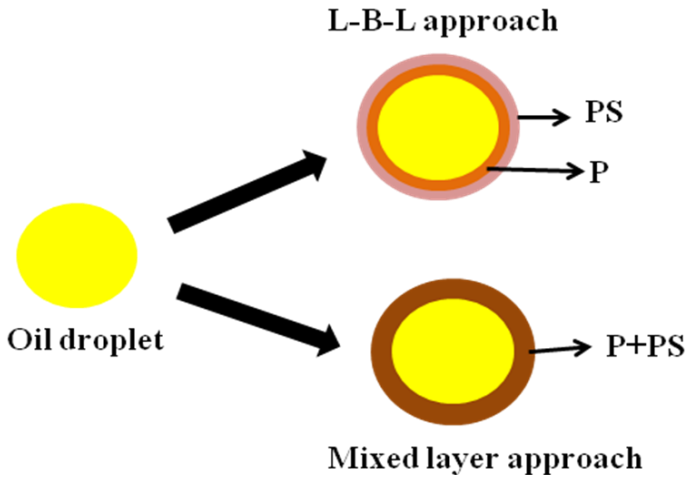

Figure 2.6: Schematic representation of layer-by-layer (L-B-L) vs. mixed layer interfacial deposition, showing the protein layer (P), polysaccharide layer (PS) and mixed protein-polysaccharide layer (P+PS).

In comparing both techniques, the layer-by-layer approach has a tendency to lead to extensively-flocculated emulsions during processing. When oil droplet collisions occur faster than polysaccharide adsorption onto the protein-coated primary emulsion surface, this enhances bridging flocculation. Sometimes, the polysaccharide concentration is much higher than a critical concentration value, which increases the possibility of depletion flocculation. With the mixed layer approach, protein-polysaccharide complexation in the bulk phase may slow the adsorption of the formed complexes towards the interface.

2.6 Factors influencing the stability of interfacial layers and emulsions

The properties of protein-polysaccharide interfacial layers can be controlled in different ways, notably pH, ionic strength and temperature.

2.6.1 Protein-polysaccharide complex properties

The formation of a stable protein-polysaccharide interfacial layer depends on the types and properties of the biopolymers used (e.g., concentration, charge status, molecular weight and architecture, etc.). Even when used at low concentration, proteins are good emulsifiers that can result in micron-sized droplets using high-pressure valve homogenization whereas

polysaccharides are able to stabilize a wide range of emulsion systems under different environmental conditions. It is thus advantageous to combine these two types of biopolymers as their synergistic effects can significantly improve an emulsion's shelf life [60-64].

2.6.1.1 Effect of pH

In emulsions with complexed proteins and polysaccharides, the electrical charge on the emulsion droplets will depend on pH, which can alter both intermolecular and intra-molecular electrostatic interactions involving the adsorption and/or desorption of biopolymer molecules [2].

Electrostatic interactions between biopolymers will depend on the sign, type, number, and charge distribution along the biopolymer chains. For example, at pH 7 anionic β-lactoglobulin-coated droplets do not complex with anionic pectin due to large electrostatic repulsion between the pectin and protein. At pH 3, however, the anionic pectin readily adsorbs onto positively-charged β-lactoglobulin-coated droplets [58]. Under certain circumstances, two biopolymers of the same net charge can form an electrostatic complex, particularly when both biopolymers contain oppositely-charged patches. One supporting example of this phenomenon is the adsorption of carrageenan onto the anionic β-lactoglobulin surface at pH 6, which demonstrates the fact that some positive patches are present on the adsorbed β-lactoglobulin [65].

2.6.1.2 Salt and ionic strength

Electrostatic interactions between charged biopolymers are strongly influenced by changes in ionic strength. The addition of salts such as NaCl or KBr results in electrostatic charge screening between oppositely-charged biopolymers, which decreases the attraction between oppositely-charged species and the repulsion between similarly-charged species [2]. Charge screening is strengthened by an increase in the concentration and valency of the counter ions in the solution. The range of the electrostatic charge screening effect is characterized by Debye screening length (κ^{-1}) that varies with the inverse square root of the ionic strength [66]:

$$\kappa^{-1} = \sqrt{\frac{(\epsilon_0 \epsilon_R kT)}{(2\, N_A e^2\, I)}} \qquad \text{(Eqn. 2.3)}$$

where ε_0 is the permittivity of free space, ε_R is the dielectric constant of the medium, k is Boltzmann constant, T is temperature (in Kelvin), N_A is Avogadro number, e is the elementary charge and I is the ionic strength of the electrolyte (mole/m^3).

It has also been reported that the efficiency and effectiveness of ionic surfactant adsorption onto similarly-charged species can be improved by increasing the ionic strength of the aqueous phase [67, 68]. In particular, multivalent countercations (e.g., Fe^{2+}, Fe^{3+}, Ca^{2+}) are more effective in electrostatic charge screening than monovalent counterions (e.g., Na^+, K^+, Cl^-) [2, 17, 66]. This is presumably due to the adsorption of multivalent countercations, which yields positively-charged sites that facilitate negatively-charged biopolymer adsorption [66].

The amount of biopolymer adsorbed on the droplet surface also tends to be higher in the presence of salts [69]. Generally, biopolymers form a thin layer at the surface in the absence of salts, which can be attributed to the highly-extended conformation of biopolymer molecules in solution. Thus, during adsorption onto droplet surfaces, biopolymers will form a thicker layer in the presence of salt due to the more compact chain conformation in the solution and the weaker electrostatic attraction between charged biopolymer and surface groups [11, 69-72].

2.6.2 Temperature

In typical food applications, emulsions undergo thermal processing such as pasteurization or sterilization [73]. In the case of conventional protein-stabilized emulsions, protein unfolding at higher temperatures increases the likelihood of emulsion destabilization due to droplet flocculation or coalescence. However, the thick protective layers provided by protein-polysaccharide complexes around lipid droplets confer stability to emulsions during such treatments [5, 74].

Literature review – lipid oxidation

2.7 Introduction

Oils and fats begin to decompose from the moment they are isolated from their natural environment. For food applications, it is obviously undesirable, yet unavoidable. In fact, only a small proportion of the oil needs to be oxidized before it results in objectionable smells and tastes and becomes unacceptable from a sensory perspective.

Lipid oxidation often leads to deterioration in processed food quality, including changes in flavour, colour, and nutritional value, with the end result being a reduced shelf life [75-80]. Improvements in product shelf life have generally been achieved either by reducing the polyunsaturated fatty acid content through genetic engineering, hydrogenation or the outright replacement of an oil with less unsaturated counterparts. Other approaches such as oxygen exclusion from product during manufacture, addition of antioxidants, reduction of metal and/or methods to control production of free radicals have also been used [75, 81-83].

2.8 Properties of flaxseed oil

Flaxseed oil is extracted from the seeds of the flax plant (*Linum usitatissimum, L.*). The oil consists of 73 % polyunsaturated fatty acids (PUFA), 18 % monounsaturated fatty acids (MUFA) and 9 % saturated fatty acids [84]. It is the richest source of the essential fatty acid omega (ω)-3, ALA (α-linolenic acid), which represents about 55 % of its total fatty acids [85]. The percentage of ALA in flaxseed is 5.5 x higher than the next highest sources (canola oil and walnuts).

There are a significant number of research publications that have reported on the beneficial health properties of flaxseed oil [86]. For example, Caughey et al. showed that the incorporation of ALA from flaxseed oil into a human diet for 4 weeks reduced the production of tumor necrosis factors in healthy male subjects by ~30 % [87]. As well, Allman et al. demonstrated that the daily consumption of 40 g of flaxseed oil over 23 days decreased platelet aggregation in

healthy young men [88]. The use of flaxseed oil in our diets in place of more common oils (e.g., canola or corn oil) may thus improve one's overall health [86].

2.9 Lipid oxidation mechanisms

Lipid oxidation proceeds via different pathways: autoxidation, photooxidation, thermal oxidation and/or due to the presence of enzymes, notably lipoxygenase. Each pathway depends on the presence of free radicals, although initiation differs in each mechanism [83, 89]. Enzymatic breakdown is much more common in processed foods and is not addressed here.

Mechanisms: Autooxidation of unsaturated fatty acids occurs via a free radical mechanism, and involves 3 steps – I) initiation, propagation and termination.

Initiation: In the presence of an initiator such as hydroxyl radical (•OH), a hydrogen atom from a methylene group (-CH_2-) is abstracted leaving behind an unpaired electron on the carbon (-CH-) or free lipid radical (L•). The radical can form a conjugated diene, which can combine with oxygen to form a peroxy radical (LOO• or LO_2•).

$$LH \rightarrow L\bullet + H\bullet$$

Propagation: The peroxy radical can abstract hydrogen from another unsaturated fatty acid (LH) to form hydroperoxides (LOOH) as well as a new free lipid radical (L•), leading to an autocatalytic chain reaction via which lipid oxidation continues [75]. It is important to note that hydrogen abstraction can occur at different points on the acyl chain and several isomeric hydroperoxides are formed. The radical also reacts with molecular oxygen to form a new peroxy radical (LOO•):

$$LO_2\bullet + LH \rightarrow LOOH + L\bullet$$

$$L\bullet + O_2 \rightarrow LOO\bullet$$

Hydroperoxides are the primary products of lipid oxidation. While not directly responsible for off-flavours and aromas, hydroperoxides decompose into secondary oxidation products, including aldehydes, ketones, carbonyls and smaller amounts of epoxides and alcohols, with

aldehydes being the most important source of undesirable organoleptic properties [90]. Propagation is accelerated by elevation in temperature, pressure and oxygen concentration, prior oxidation, presence of metal ions, lipoxygenases, removal of antioxidants (naturally-occurring or otherwise), time, and light (both visible and UV).

Termination: The termination of the free radical oxidative reactions occurs when two radical species react with each other to form a non-radical product. Reaction with an antioxidant may also terminate this reaction.

$$LOO\bullet + LOO\bullet \rightarrow LOOL + O_2$$

$$L\bullet + L\bullet \rightarrow LL$$

$$LOO\bullet + L\bullet \rightarrow LOOL$$

Numerous detailed reports on unsaturated fatty acid oxidation are available in the literature, namely Frankel [75].

Photooxidation also involves the formation of hydroperoxides in a direct reaction of singlet oxygen and an unsaturated fatty acid without the formation of a free radical, Singlet oxygen is extremely reactive, being ~1500 x more reactive than its molecular counterpart. Light, and in particular UV radiation, may be involved in the initiation of this pathway [83, 89].

Oxidation is normally a slow reaction that only occurs to a limited degree. The rate of oxidation depends primarily on the fatty acid composition and less on the stereospecific distribution within the triacylglycerols. Oil oxidation will be roughly proportional to the degree of unsaturation. The most important components that are capable of reacting with oxygen are polyunsaturated fatty acids. Thus, linolenic acid (18:3) is more susceptible than linoleic acid (18:2), which in turn is more susceptible than oleic acid (18:1). Unfortunately, the oxidative stability of flaxseed oil greatly limits its applicability in foods. Given its high polyunsaturated fatty acid content, it is highly susceptible to oxidation.

2.9.1 Flaxseed oil oxidation

A number of studies have highlighted the susceptibility of flaxseed oil to oxidation. Kuhn et al. studied the influence of emulsion composition and homogenization pressure on the incorporation

of flaxseed oil in emulsion, demonstrating that an increase in the number of homogenization cycles increased flaxseed oil degradation [91]. Tonon et al. compared the effect of oil load (10-40 wt%) and biopolymer type (e.g., gum arabic, modified starch or whey protein concentrate) on flaxseed oil encapsulation via spray-drying [92]. They found that an increase in oil load resulted in a lower encapsulation efficiency and higher level of lipid oxidation. In a separate study, Carneiro et al. found that the combination of maltodextrin and whey protein concentrate or maltodextrin and modified starch resulted in efficient flaxseed oil encapsulation with good oxidative stability [93].

2.9.2 Kinetics of lipid oxidation

Lipid oxidation follows two distinct phases. A lag phase first produces low and undetectable levels of oxidation products. This is followed by an exponential phase where the concentration of oxidation products increases dramatically [94]. Such products lead to characteristic rancid odours and flavours in processed foods. The extent of lipid oxidation can be monitored by measuring primary and secondary oxidation products, where lipid hydroperoxides indicate primary oxidation [94]. As oxidation progresses, they decompose into secondary oxidation products (e.g., aldehydes, ketones, epoxides and alcohols).

2.10 Control of lipid oxidation in O/W emulsions

Lipid oxidation has been widely studied in bulk fats and oils, and there is now a clear understanding of the factors that affect oxidation in such systems [83, 89]. However, oils are often used in complex, heterogeneous foods such as emulsions, where many different molecular species chemically and physically interact with one another (e.g., margarine, salad dressings and mayonnaise). The oxidation of emulsified vegetable oils differs from that of bulk lipids, predominantly because of the presence of the emulsion droplet interface, and the partitioning of ingredients between the oil, aqueous and interfacial regions [83].

As mentioned above, hydroperoxides are the primary products of lipid oxidation. As they are more polar than unoxidized oil, they may migrate towards the droplet interface. Studies have suggested that the interaction between interfacially-bound lipid hydroperoxide and pro-oxidants

such as transition metals in the aqueous phase promotes lipid oxidation in O/W emulsions [95-97].

2.11 Factors influencing lipid oxidation in emulsions

The presence and properties of the oil-water interface are determining factors responsible for the unique oxidation behaviour observed in food emulsions. Though the presence of the interface generally promotes oxidation of emulsified oil, the judicious selection of an emulsifier may significantly counter this effect [94]. Numerous studies have shown that the interface (and the corresponding increase in surface-to-volume ratio) increases the interactions between lipid hydroperoxides and aqueous phase pro-oxidants (Figure 2.7).

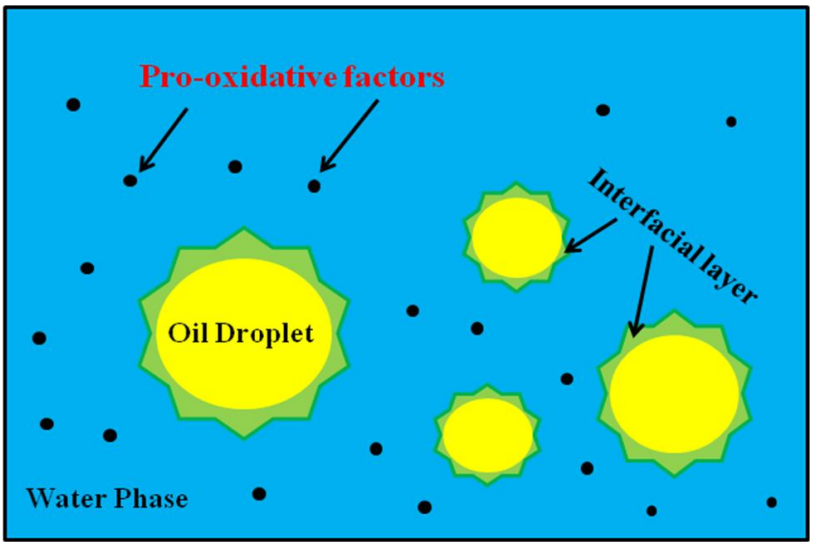

Figure 2.7: Schematic representation of aqueous phase pro-oxidative factors and interfacial barrier against oxidation. [98-100].

2.11.1 Aqueous phase components

By definition, food emulsions are compositionally complex. The components potentially present in the aqueous phase include polysaccharides, sugars, proteins, surfactants, salts, acids, bases and buffer, all of which may influence lipid oxidation either as pro- or anti-oxidants depending on their chemical nature and the local environmental conditions. It is therefore important to determine the role of common ingredients used in food emulsions on lipid oxidation.

2.11.1.1 Polysaccharides and sugars

Polysaccharides are usually added to emulsions to increase continuous phase viscosity, which can improve emulsion stability by slowing droplet-droplet interactions. However, it has also been reported that polysaccharides can inhibit lipid oxidation in O/W emulsions via free radical scavenging, transition metal binding and hydrogen donation [101, 102]. Common polysaccharides such as xanthan gum, low methoxyl pectin, high methoxyl pectin, sodium alginate, methyl cellulose and α–carrageenan have been reported to chelate metals and inhibit lipid oxidation [101, 103, 104]. Gum tragacanth has been shown to act as a radical chain breaker by donating hydrogen [103]. Glycoproteins, e.g., gum arabic and SSPS, have also been shown to retard lipid oxidation via free radical scavenging by the protein fraction [102, 105, 106]. Other studies have reported that sugars, e.g., pentose, hexose and reducing disaccharides, can act as strong pro-oxidants. Specifically, reducing sugars promote lipid oxidation whereas non-reducing sugars, e.g., sucrose, inhibit lipid oxidation [107-109].

2.11.1.2 Proteins

Besides providing stability against droplet flocculation or coalescence, proteins have also been shown to inhibit lipid oxidation via diverse mechanisms, including free radical scavenging, metal chelation, inactivation of reactive oxygen species and hydroperoxide reduction [110]. The antioxidant ability of a protein inherently depends on its chemical properties and the solvent accessibility of its amino acid residues [111, 112]. Biologically-derived amino acids containing either nucleophilic sulfur-containing side chains (e.g., cysteine, methionine) or aromatic side chains (e.g., tryptophan, tyrosine, phenylalanine) have been shown to be effective in preventing lipid oxidation [110]. For example, the antioxidant activity of whey proteins is thought to be due to the presence of sulfhydryl and nonsulfhydryl functional groups [113-115].

A number of studies have reported that proteins may act as pro-oxidants under certain conditions whereas others such as heme proteins and lipoxygenases are pro-oxidants by nature. For example, Donnelly et al. found that oxidation of polysorbate 20-stabilized menhaden O/W emulsions increased with the addition of whey protein isolate. The authors suspected that the presence of various pro-oxidant impurities (e.g., hydroperoxides or transition metals) in the protein sample helped to promote lipid oxidation [116].

2.11.1.3 Surfactants

Small-molecule surfactants readily form a physical barrier at the oil-water interface in emulsions. When the interface is saturated (i.e, at the CMC), excess surfactant molecules will partition into the continuous phase and form surfactant micelles that may increase oxidative stability [2, 15]. For example, hemoglobin-catalyzed oxidation of emulsified sunflower oil was inhibited due to the presence of excess anionic surfactant in the continuous aqueous phase [107]. The authors surmised that the presence of negatively-charged micelles interacted with the transition metals thereby decreasing pro-oxidant levels at droplet surface [117-119].

2.11.1.4 Salts

As previously mentioned, salts can have a significant impact on the kinetic stability of O/W emulsions. In regards to oil oxidation, salt may also screen electrostatic interactions transition metals with the oil-water interface, thereby potentially increasing or decreasing their attraction with the interface. For example, addition of NaCl to negatively-charged SDS (sodium dodecyl sulfate)-stabilized corn oil emulsions showed a slight reduction in lipid oxidation rate in the absence of added iron whereas in the presence of iron, the lipid oxidation rate slightly increased [95, 96, 120, 121]. Different studies have suggested that depending on the system, salt could act as pro-oxidant or antioxidant [120, 121].

2.11.2 Oil phase

The chemical structure and composition of lipids are significant factors dictating the susceptibility of an oil to oxidation. As previously mentioned, saturated lipids are significantly more stable than unsaturated lipids against oxidation due to the absence of reactive sites [83].

Differences in the molecular structure of unsaturated lipids in aqueous colloidal dispersions can show surprising results. For example, Miyashita et al. reported that unsaturated fatty acids solubilized in non-ionic surfactant micelles (polysorbate 20) showed increased oxidative stability with an increasing degree of unsaturation [122, 123]. In another study, Miyashita et al. demonstrated that when double bonds were closer to methyl end, fatty acids were more susceptible to oxidation [124]. Coupland et al. showed that lipid oxidation was faster when triacylglycerols were located at the droplet surface rather than within the interior [125, 126].

Therefore, the polarity and surface activity of lipid molecules present within an emulsion droplet will strongly impact whether they will be located within the emulsion droplet or at its surface.

2.11.3 Emulsion droplet properties

The oxidation of emulsified lipids will vary depending on the droplet size, concentration, surface charge and physical state of the dispersed phase.

2.11.3.1 Emulsion droplet size

The typical diameter of emulsion droplets is 0.2-10 μm. The presence of a larger surface area (resulting from a smaller average droplet size) may lead to greater oxidation as the emulsified is potentially exposed to more aqueous phase pro-oxidants [127]. However, other studies have reported that emulsion droplet size has a minimal impact on oxidation rate. Gohtani et al. demonstrated that the oxidation rate of emulsified docosahexaenoic acid did not increase with a decrease in droplet size [127]. This was likely due to the lack of reactive species present at the droplet interface [128, 129].

2.11.3.2 Emulsion droplet interfacial thickness

The thickness of a biopolymer interfacial layer can have a significant impact on the susceptibility of emulsified oil to oxidation. Layer thickness invariably depends on the molecular weights and dimensions of the biopolymer used. It has been reported that a casein-based interfacial layer (~ 10 nm thick) conferred enhanced stability against emulsified oil oxidation compared to a whey protein-based interfacial layer (1-2 nm) [130].

2.11.3.3 Emulsion droplet surface charge

Lipid oxidation depends on the droplet charge status, with the type of emulsifier and/or pH of the medium impacting interfacial charge [105, 131-133]. A number of studies have demonstrated that emulsions stabilized with anionic emulsifiers oxidize more rapidly than those stabilized with positively-charged emulsifiers [95, 96, 130, 134, 135]. In the case of protein-stabilized emulsions, lipid oxidation has been shown to be faster at pHs above the protein's IEP [95, 99, 100, 116, 135, 136]. However, droplet charge does not necessarily equate with susceptibility to oxidation. For example, Silvestre et al. demonstrated that casein-stabilized emulsions showed

improved oxidative stability compared to whey proteins, though the latter had a higher droplet surface charge (+ 56 vs. 30 mV) [130]. Other factors (e.g., amino acid profile, free radical scavenging ability, etc.) may also play a role in inhibiting lipid oxidation.

2.11.4 Effect of available oxygen

There is always sufficient oxygen present in oil phase to initiate lipid oxidation as oxygen solubility in oils is three times higher than in water [137]. As a result, an effective option to retard lipid oxidation is to reduce available oxygen concentration, for example by packaging the lipid-containing products under nitrogen or vacuum. Food processing unit operations introduce oxygen during grinding, mixing and/or homogenization, which can increase the oxidative stress on the final product. It has been observed that at low oxygen concentrations, the rate-limiting step for the oxidation of emulsified oil was dependent on oxygen diffusion through the aqueous phase [138, 139].

2.11.5 Influence of antioxidants

One of the most effective ways to retard lipid oxidation is to introduce antioxidants within processed foods. The ability of an antioxidant to retard lipid oxidation depends on its concentration, polarity, physical location and environment (e.g., temperature, pH and ionic strength) [75, 80]. Different studies have demonstrated that both non-polar and surface-active antioxidants (e.g. TBHQ, α-tocopherol, δ-tocopherol and propyl gallate) effectively inhibit lipid oxidation in O/W emulsions [140-142]. Antioxidants inactivate different types of pro-oxidants (e.g., reactive oxygen species, metals and enzymes) and/or scavenge free radicals generated at different stages of oxidation [75, 82, 143, 144].

Though synthetic antioxidants are highly-effective (e.g., BHA, BHT, TBHQ), there is growing consumer demand for more naturally-occurring ingredients in processed foods. This has motivated the food industry to search for more 'label-friendly' alternatives such as certain biopolymers (e.g., proteins or polysaccharides), carotenoids, tocopherols and fruit/plant extracts as natural antioxidants.

Chapter 3: Materials and experimental methods

3.0 Introduction

This chapter is focused on the materials and experimental methods used to formulate and characterize the dispersions and O/W emulsions herein studied. Specific parameters and experimental details are discussed within the experimental sections of the subsequent chapters. All experiments were performed in triplicate, as were their respective measurements.

3.1 Materials

Defatted solvent-free soy flour (7B Soy Flour: min. 53% protein, max. 9% moisture, 32% carbohydrates, max. 3% fat, 18% total dietary fibre) was obtained from Archer Daniels Midland Company (Decatur, IL, USA). Commercial-grade SSPS (SOYAFIBE-S-CA200: moisture: 5.8 %, crude protein: 7.8 %, crude ash, 7.8 %. Saccharide composition (%): Rhamnose: 5.0, Fucose: 3.2, Arabinose: 22.6, Xylose: 3.7, Galactose: 46.1, Glucose: 1.2, Galacturonic acid: 18.2) was provided by Fuji Oil Ltd (Osaka, Japan). Denatured soy whey proteins (dSWPs) were extracted from defatted solvent-free soy flour (discussed in Chapter 4). Soybean oil (acid value < 0.2) and flaxseed oil (acid value ≈ 1.78 mg KOH/g of oil) were purchased from a local grocery store. All chemicals used were reagent-grade. Deionized water was used throughout all experiments. Unless noted otherwise, all reagents were used without further purification.

3.2 Protein-polysaccharide suspension preparation

Stock dispersions of dSWP and SSPS were prepared independently by adding appropriate amounts of dSWP and/or SSPS to deionized water to desired concentrations. The final stock suspensions were mixed together, and the final pH was adjusted to 3. The mixture was stirred for 3 h. The final suspension mixture was mixed thoroughly with a bench top homogenizer (Polytron PT 10-35, Kinematica GmbH, Switzerland). This unit is a rotor/stator that consists of a rotating rotor rod surrounded by a fixed stator cylinder (Figure 3.1).

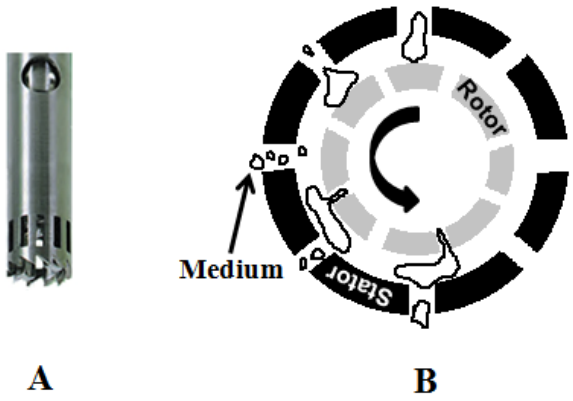

Figure 3.1: Image of the rotor-stator of a Polytron PT 10-35 bench top homogenizer (A) and a cross-section schematic of a rotor-stator (B) (Adapted from [145]).

The spinning rotor forces the liquid medium through a narrow gap between the rotor and stator, thus generating shear. This shear force breaks the bulk liquid into smaller droplets resulting in a coarse emulsion.

3.3 Emulsion preparation

A two-step process was used to prepare emulsions stabilized with dSWP, SSPS alone or mixed dSWP and SSPS. First, a coarse emulsion was formed by mixing the oil and its respective aqueous phase with a bench top rotor/stator. The coarse emulsion was then passed through a high pressure valve-homogenizer (APV 1000, APV, Albertslund, Denmark). Figure 3.2 depicts high pressure valve homogenization. Emulsion preparation is discussed in Chapters 4 and 5.

At high pressure and very low velocity, the coarse emulsion (E) enters the adjustable clearance area between the valve (A) and the valve seat (B). The applied pressure is inversely proportional to the volume of the adjusted clearance area. The coarse emulsion flows through the small gaps between the valve (A) and the valve seat (B). At that point, the inside pressure drops and rapidly increases the velocity of the fluid. This generates turbulent flow that disrupts the bigger size droplets at the discharge gap (D). The homogenized fluid impinges on the impact ring (C) and then flows out to be collected (F) at low pressure and high velocity [146]. Repeated passes through the homogenizer further decrease droplet size until it reaches a minimum size that is controlled by the pressure used [1, 147, 148].

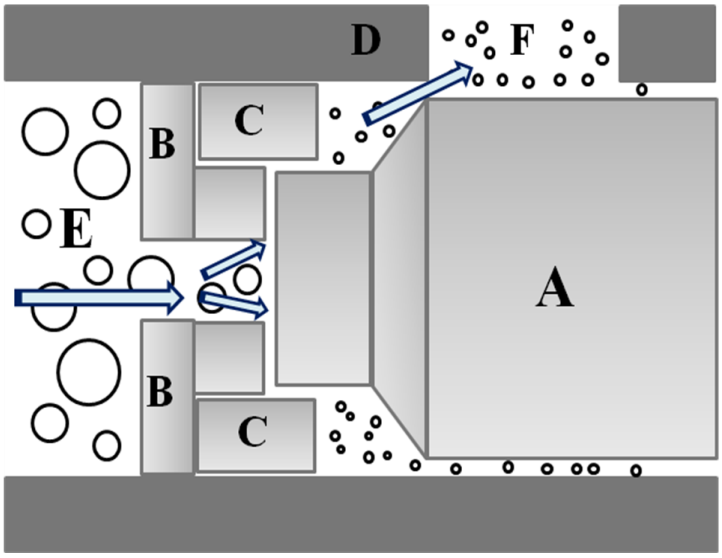

Figure 3.2: A schematic representation of the high pressure emulsification process in an APV 1000 valve homogenizer (adapted from [147]).

3.4 Determination of particle/droplet size distribution

The particle sizes of protein, polysaccharide and/or protein-polysaccharide mixed dispersions were measured via dynamic light scattering (Brookhaven 90Plus/BI-MAS DLS Particle Size Analyzer, Brookhaven Instruments Corporation, Holtsville, NY, USA). Laser light diffraction (LD) (Malvern Mastersizer 2000S, Malvern Instruments, Worcestershire, UK) was used to determine droplet size distributions of dispersed droplets.

3.4.1 Dynamic light scattering (DLS)

DLS is used to determine droplet or particle size and polydispersity typically in sub-micron dispersions and emulsions. When a coherent light beam interacts with colloidal particles undergoing Brownian motion, the particles scatter light. The original measurement is a time correlation function of the scattered intensity of the particles within the dispersion/emulsion. The decrease of this correlation function with time (lag time) is used to extract the diffusion coefficient of a particle or droplet in solution [149]. The measured diffusion coefficient can be used to calculate a hydrodynamic radius (R_h) of the droplet or particle using the Stokes-Einstein equation:

$$R_h = kT / 6\pi\eta D \qquad \text{(Eqn. 3.1)}$$

where k is the Boltzmann constant, T is the absolute temperature in Kelvins; η is the viscosity (cP) of the continuous phase, and D (cm^2/s) is the diffusion coefficient [150]. For proper interpretation of results, it is very important to establish whether the R_h values are based on unimodal or multimodal distributions.

A particle size analyzer (Brookhaven 90 Plus with BI-MAS-multi angle particle sizing option, Brookhaven Instruments Corporation, New York, USA) was used to measure sample mean particle diameter, particle size distribution and conductance as well as polydispersity. Measurements were carried out at a 90° scattering angle, at 25 °C.

3.4.2 Laser diffraction

Laser diffraction is used to measure particle/droplet sizes from ~ 50 nm to ~ 2-3 mm in diameter in polydispersed samples. This technique is based on the determination of the angular diffraction of light scattered from a dispersion/emulsion when irradiated with a laser beam. The intensity of scattered light is inversely proportional to the particle/droplet radius, *i.e.,* smaller particles/droplets show more intense scattering at higher angles compared to larger ones. Particle/droplet size distributions are calculated based on comparisons of the angular information with a scattering model (the Mie theory) that applies to absorbing or non-absorbing spherical particles/droplets. Calculations using the Mie theory require an input of the illumination wavelength and polarization state, the refractive index (both real and imaginary) of both the particles/droplets and the medium, the diameter of the particles/droplets and the angle of observation relative to the incident illumination (also called the scattering angle) [151]. The instrument used was a Malvern Mastersizer 2000S (Malvern Instruments, Worcestershire, UK). The instrument calculates a droplet/particle size distribution based around volume terms independent of the number of particles in the sample. It measures the volume or mass moment mean diameter $D_{4,3}$ also called the De Brouckere mean diameter, and assuming that particle density is constant, generates a volume distribution data equal to the weight distribution directly from the initial measurements [146].

3.5 Droplet surface charge measurement

Particles or droplets dispersed in a liquid medium may carry a surface charge that is represented as an electric double layer. The double layer consists of the Stern layer (inner region) and diffuse layer (outer layer). Counter ions are strongly associated with the droplet/particle surface but are loosely attached to diffuse layer (Figure 3.3).

The surface potential of particles in the Stern layer cannot be measured directly [152]. Within the diffuse layer, an imaginary boundary exists where charges are assumed to be stable. The electric potential at the surface of this boundary (or surface of hydrodynamic shear) is called the zeta (ζ) or electrokinetic potential [152] and can be measured by laser Doppler electrophoresis.

An instrument (as discussed below) measures the electrophoretic mobility U_e of the sample and converts it into the ζ potential by Henry's equation [152]:

$$U_e = \frac{2E\zeta f(\kappa a)}{3\eta} \qquad \text{(Eqn. 3.2)}$$

where U_e is the electrophoretic mobility, E is the dielectric constant of the dispersion medium, ζ is the zeta potential (mV), $f(\kappa a)$ is Henry's function (most often the Hückel and Smoluchowski approximations of 1 and 1.5, respectively, are used) and η is the viscosity of the solvent medium [1, 146, 153].

The ζ potential is an important parameter to determine electrostatic colloidal dispersion stability. The instrument used to measure the ζ potential of the protein, polysaccharide and protein-polysaccharide particles as well as that of the biopolymer-coated emulsion droplets was a Brookhaven 90Plus PALS Zeta Potential Analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA).

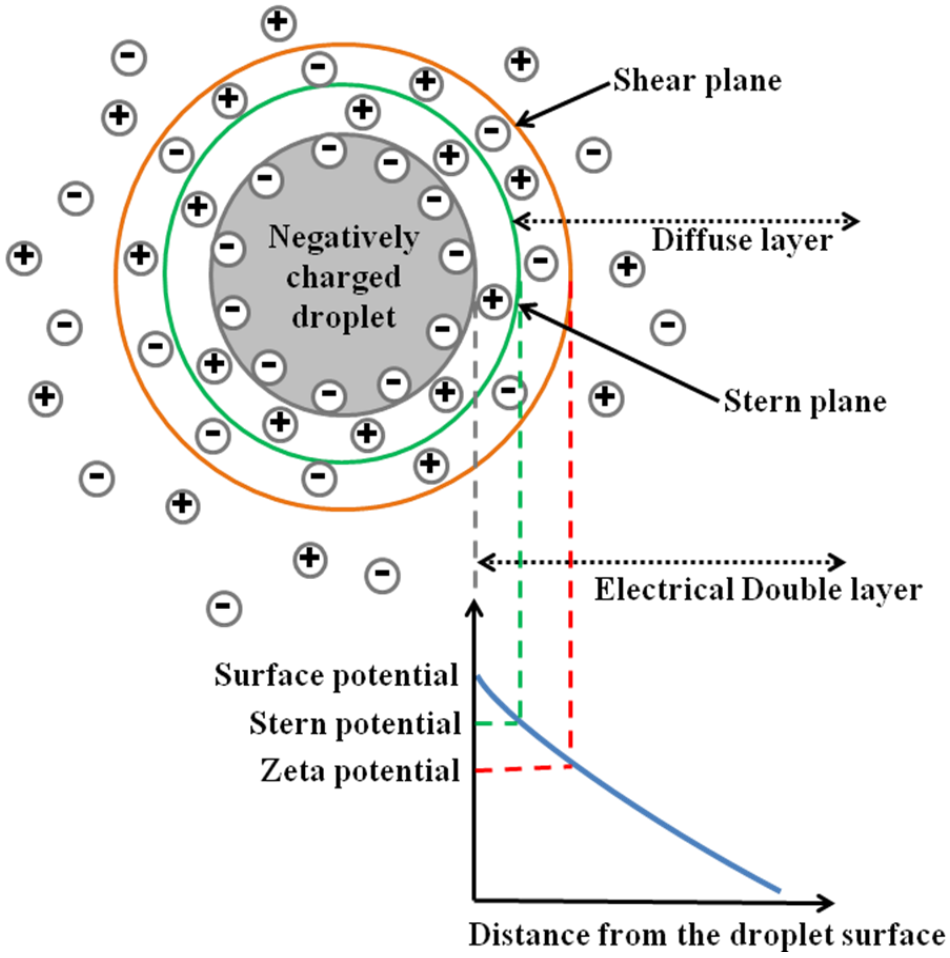

Figure 3.3: Schematic of the electrical double layer and other potential gradients on a charged droplet/particle surface (adapted from [1, 146, 153]).

3.6 Turbidity analysis

Turbidity measurements can be used to study the stability profile of an emulsion as a function of time. Typically, in an unstable emulsion system, particle size will increase due to particle coagulation and coalescence, eventually leading to bulk phase separation.

When a light source sends a beam of light (λ = 880 nm, in our case) through the sample cell of the instrument, the amount of backscattered light is a measure of turbidity, with the transmission of light through a sample calculated using:

$$T_r = \frac{I_t}{I_0} = \exp(-\tau \ell) \qquad \text{(Eqn. 3.3)}$$

where τ is the turbidity, I_t and I_0 are the intensities of the transmitted and incident light, respectively, T_r is the transmission, and l is the path length through the sample [154]. The scattered light intensity depends on the shape and size of the particles along with the difference in refractive index between the dispersed particles and surrounding medium. The average particle/droplet size in a suspension or emulsion can be estimated via turbidity measurement, but the size distribution cannot be measured [154]. A vertical scan turbidity analyzer (Turbiscan LAb, Formulaction, L'Union, France) was used to measure the turbidity of dSWP, SSPS, dSWP-SSPS mixed dispersions and biopolymer-stabilized O/W emulsions.

3.7 Inverted light microscopy

In an inverted light microscope, the light source and condenser are located above the stage and point down while the objectives and turret are located below, pointing up towards the stage [2]. Emulsion droplets were observed for droplet/particle size and shape using an inverted light microscope.

Emulsion sample images were observed using a Zeiss Axiovert 200M inverted light microscope (Zeiss Canada, Toronto, ON, Canada). Images captured on CCD camera were analysed with Northern Eclipse software, Version 7.0. (Empix Imaging, Inc., Mississauga, ON, Canada).

3.8 UV-Vis spectroscopy

Ultraviolet-visible spectroscopy (UV-Vis) refers to absorption spectroscopy in the ultraviolet-visible spectral region. UV/Vis spectroscopy is routinely used in the quantitative determination of solutions of biological macromolecules and lipid oxidation products. The concentration of an analyte in solution can be calculated by measuring the absorption at a particular wavelength and applying the Beer-Lambert law. The amount of light, I, transmitted through a transparent solution of an absorbing compound can be related to:

$$-\log\left(\frac{I}{I_o}\right) = A = \varepsilon_\lambda bc \qquad \text{(Eqn. 3.4)}$$

where I_o is the incident light intensity, A is the absorbance (also referred to as the optical density), b is the cell path length in cm, c is the solution concentration in moles/litre, and ε_λ is the molar absorptivity or the molar extinction coefficient, which has units of litre/mole/cm.

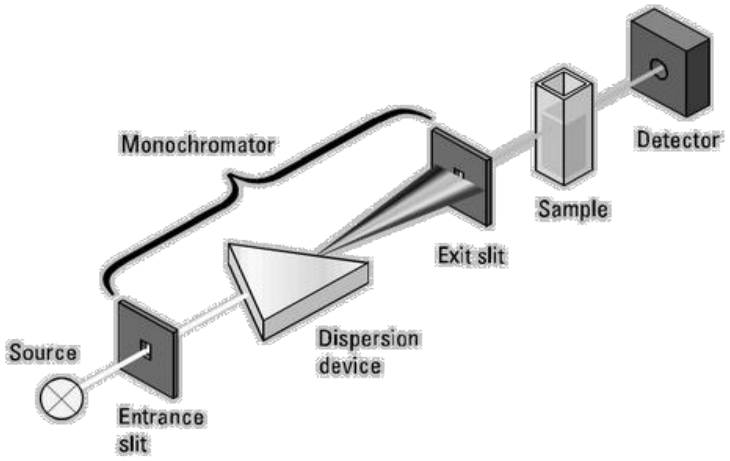

Figure 3.4: Schematic representation of a UV-Vis spectrometer operating principle (adapted from [155]).

The basic parts of a spectrophotometer are a light source, a sample holder, a diffraction grating (e.g., a monochromator or prism) to separate the different wavelengths of light and a detector. The radiation source is often a hydrogen or deuterium arc lamp for UV measurements and a tungsten filament lamp for visible light measurements [155].

A double beam UV-Visible absorption spectrometer (PerkinElmer, lambda 2000, Woodbridge, ON, Canada) was used to measure the concentration of oxidized lipid products (discussed in Chapter 5).

3.9 Data analysis

All reported experiments in this thesis were carried out at least in triplicate, and the results are expressed as means ± standard deviation. Statistical analysis was performed using SigmaStat 4 integrated in SigmaPlot 11 (Systat Software, Chicago, IL, USA). Student's *t*-test for single pair comparisons or one-way ANOVA with single-step Tukey's multiple comparison *post-hoc* test for multiple groups were used.

Chapter 4

Stabilization of soybean oil-in-water emulsions using denatured soy whey protein and soy soluble polysaccharide interactions

4.0 Abstract

Mixtures of denatured soy whey proteins (dSWP) and soluble soybean polysaccharides (SSPS) were used to stabilize 5 wt% soybean oil-in-water (O/W) emulsions against coalescence and phase separation. Emulsions prepared with either dSWP or SSPS at low concentrations demonstrated limited stability. At an optimal SSPS:dSWP ratio of 1.5:1.0 (corresponding to 2.5 wt% total biopolymer in the aqueous phase), emulsions did not phase separate for > 60 days at pH 3 and 21 days at pH 8. Irrespective of the protein-polysaccharide ratio, emulsions prepared at lower pH (3-4) showed better long-term stability versus pH 5-8. The negligible surface charge (-2 mV) at low pH suggested the presence of dSWP-SSPS complexes that promoted emulsion stability *via* steric hindrance. The higher surface charge at pH 7-8 (near -20 mV) prevented mixed dSWP-SSPS layer formation around the dispersed oil droplets resulting in limited emulsion stability. Overall, this study showed that the presence of dSWP-SSPS interfacial layer promoted the capacity of O/W emulsions to resist oil droplet coalescence and phase separation.

Keywords: emulsion, soy whey protein, soluble soybean polysaccharide, electrostatic interaction, steric stabilization**.**

4.1 Introduction

Mixed protein-polysaccharide systems are well-recognized macromolecular assemblies capable of stabilizing O/W food emulsions [15, 156-162]. The presence of polysaccharides can significantly influence protein functionality (*e.g.*, surface activity, solubility, emulsifying property, gel forming ability, foaming property, conformational stability, *etc*.) [163, 164] though this depends on the extent of their interactions and mass ratio, local environmental conditions and external inputs such as temperature and shear [50, 158, 159, 165, 166].

In the preparation and stabilization of O/W emulsions, oil-water interfacial adsorption of protein-polysaccharide complexes is significantly dependent on how they are complexed. Complexation prior to, or during, emulsification may alter the resulting emulsification efficacy of the protein-polysaccharide amphiphiles, particularly if the accompanying polysaccharide is surface-active [54, 55]. As an example, a comparative study using a sodium caseinate-dextran sulfate complex to stabilize *n*-tetradecane-water emulsion suggested that improvements in interfacial film shear visco-elasticity with the pre-emulsified complex led to emulsions with an improved shelf life [57].

Both mixed film and layer-by-layer deposition of proteins and polysaccharides can create a thick interfacial film that greatly minimizes inter-droplet contact [167-170]. As well, compared to protein films alone, protein-polysaccharide films can provide superior resistance against environmental stresses such as large changes in pH or ionic strength and typical unit operations such as thermal processing or freezing [58, 70, 171-173]. For example, O/W emulsions stabilized with β-lactoglobulin-pectin multilayers showed better resistance against salt-induced droplet flocculation compared to β-lactoglobulin-coated emulsions [174].

However, the soluble fractions of soy protein aqueous extracts, *i.e.*, soy whey proteins, have seen comparatively little effort devoted to their structure-functionality relationship. Native soy whey proteins (nSWPs) are a waste by-product following the isoelectric precipitation of soy protein isolate. Their main components are lectin and the Kunitz trypsin inhibitor (KTI) with molecular weights of 120 kDa and 20-22 kDa, respectively [28-30]. The limited literature available on

SWPs has revealed that denatured soy whey proteins (dSWP) show somewhat better emulsifying activity compared to nSWP, but that they are incapable of preventing droplet coalescence over extended periods [40, 41]. Previous studies have demonstrated that raw soybean proteins contain antinutritional components that may inhibit vertebrate pancreatic serine proteinases, resulting in a range of deleterious physiological effects [175]. Though soybean trypsin inhibitor does not appear to be hazardous in humans, soymeal for use in animal feed must be heat-treated to minimize any potential health risks [176].

Soy soluble polysaccharides (SSPS) are pectin-like acidic biopolymers also extracted from the residual carbohydrate by-product of soy protein isolate production. They consist of a main rhamnogalacturonan backbone branched with β-1,4-galactan and α-1,3 or α-1,5-arabinan chains, and homogalacturonan covalently bound to a ~50 kDa protein moiety [47]. SSPS is effective in stabilizing emulsions at pH 3-7 and ionic strengths up to 25 mM NaCl or $CaCl_2$ [45].

The characterization and utilization of SSPS and SWP from soymilk and tofu preparation may offer new avenues for the use of these undervalued agricultural resources. The goal of this study was to develop O/W emulsions stabilized with mixed dSWP and SSPS to impart improved stability compared to protein-based systems. dSWP and SSPS dispersions were first analyzed and the resulting findings were extended to the stabilization of O/W emulsions.

4.2 Material and methods

The different materials and characterization methods used to study the kinetic stability of the O/W emulsions herein developed are described below.

4.2.1 Materials

Defatted solvent-free soy flour (7B Soy Flour: min. 53% protein, max. 9% moisture, 32% carbohydrates, max. 3% fat, 18% total dietary fibre) was obtained from Archer Daniels Midland Company (Decatur, IL, USA). Commercial-grade SSPS (SOYAFIBE-S-CA200: moisture: 5.8 %, crude protein: 7.8 %, crude ash, 7.8 %. Saccharide composition (%): Rhamnose: 5.0, Fucose: 3.2, Arabinose: 22.6, Xylose: 3.7, Galactose: 46.1, Glucose: 1.2, Galacturonic acid: 18.2) was

provided by Fuji Oil Ltd (Osaka, Japan). Soybean oil (acid value < 0.2) [177] was purchased from a local grocery store. All chemicals used were reagent grades. Deionized water was used throughout all experiments. Unless noted otherwise, all reagents were used without further purification.

4.2.2 Extraction of denatured soy whey proteins (dSWP)

The method of Sorgentini and Wagner (1999) was followed to extract the nSWP. After centrifugation (at 10^4 x *g*) of a 10 wt% suspension of defatted solvent-free soy flour in water, the supernatant was brought to pH 4.5 using 0.1 M HCl and held 1 h at room temperature [28]. The resulting suspension was again centrifuged at 10^4 x *g*. The collected supernatant was filtered through a defatted cotton sheet and brought to pH 8 with 0.1 M NaOH, then stirred for 1 h at room temperature and centrifuged (SORVALL-RC 5C Plus Superspeed Centrifuge-Thermo-Scientific, Ottawa, ON, Canada) at 10^4 x *g* for 30 min at 4 °C. The resulting clear supernatant was dialyzed for 24 h at 10 °C against a 0.02 wt% sodium azide aqueous solution, resulting in a nSWP dispersion with a concentration of 0.1 wt%. The nSWP dispersion was denatured at 100 ^{o}C for 45 min followed by freeze-drying for 4 d at - 52 °C to yield a dSWP free-flowing powder (residual moisture content ~ 1.5 wt%). Protein concentration was assayed spectroscopically (4284D READER-Thermo Labsystems Multiskan Ascent, Haverhill, MA, USA).

4.2.3 Preparation of stock dSWP and SSPS dispersions

Stock dispersions of dSWP (2 wt%) and SSPS (3 wt%) in deionized water were independently prepared, adjusted to pH 3 and stirred for 3 h. Sodium azide (0.02 wt%) was added to prevent microbial growth. At this low concentration, sodium azide did not have any effect on protein-polysaccharide interactions in bulk or in emulsions.

4.2.4 Preparation of O/W emulsions

Emulsion consisted of 5 wt% oil, 1 wt% dSWP and 0.15-1.5 wt% SSPS in deionized water. Volumes of stock dSWP and SSPS dispersions at pH 3 were mixed at appropriate ratios and stirred for 30 min. The emulsions were prepared by mixing 5 wt% soybean oil with the dSWP-SSPS dispersion to produce 5% O/W emulsions. Coarse emulsions were prepared using a rotor/stator (Polytron PT-10-35 with PCU-2 control, Kinematica GmbH, Switzerland) at 27,000

RPM for 1 min followed by high-pressure valve homogenization (3 passes at 60 MPa) (APV, model 1000, Albertslund, Denmark) at room temperature (~25 °C). Emulsion pH was adjusted from pH 3 to 8 with 0.1 M NaOH or 0.1 M HCl. Emulsion samples were kept sealed at 4 °C until analysis.

4.2.5 Protein SDS-PAGE

Volumes (15 µL) of nSWP and molecular weight standards were resolved on a linear 8 % acrylamide SDS-PAGE gel using a Bio-Rad mini electrophoresis system (Bio-Rad Laboratories, Mississauga, ON, Canada) in tricine electrophoresis buffer, following the method of Schägger & von Jagow [178]. The gel was run for 30 min at 30 V and then at 125 V until the tracking dyes reached the end of the gel. The gel was released from glass plates and the protein bands were stained with a Coomassie brilliant blue R-250 staining solution (Bio-Rad Laboratories, Mississauga, ON, Canada). Images of the gels were captured with a digital camera (Canon, Toronto, ON, Canada).

4.2.6 Circular dichroism (CD) spectroscopy

CD spectroscopy was performed on Jasco J-810 CD spectrophotometer (Jasco Inc, Easton, MD, USA). The secondary structures of nSWP and dSWP were determined by scanning 300 µL protein samples (1 wt%) at pH 7 using a 0.1 cm path length cuvette. Samples were scanned from 250 nm to 190 nm with 1 s averaging time for each wavelength.

4.2.7 Droplet/particle size distribution (DSD/PSD)

Laser light scattering experiments were conducted on prepared emulsions with a Malvern Mastersizer 2000 with a Hydro 2000S wet cell attachment (Malvern Instruments, Worcestershire, UK) weekly, for two months. Homogeneous samples were gently mixed *via* inversion to ensure representative sampling and then dispersed into the instrument's sample cell until an obscuration level of 5-10 % was reached. The pH of the dispersant water was adjusted to match the pH of the samples. The background and sample integration times were 12 s and 10 s, respectively. The optical parameters used were: a refractive index of 1.47 and an absorption index of 0.001 for the soybean oil and a refractive index of 1.33 for the water. Results were analyzed with the Malvern Mastersizer 2000 software v.5.54. Volume-weighted average droplet

sizes ($D_{4,3}$) values are reported. The DSDs shown are representative distributions of the emulsion prior to visible emulsion sedimentation.

4.2.8 Zeta potential

Variations in emulsion zeta potential (ζ) were monitored weekly using a Brookhaven Zeta PALS system mated to a 90 Plus dynamic light scattering unit (Brookhaven Instruments Corporation, Holtsville, NY, USA) for two months. The samples were prepared by diluting 20 µL of the emulsions with 3.0 mL of pH-matched deionized water. Samples were analyzed at 25 °C and the instrument parameters used were: water as the dispersant with a viscosity of 0.890 cP, a refractive index of 1.330, and a dielectric constant of 78.54.

4.2.9 Particle size measurement

The particle size of the dSWP and dSWP-SSPS dispersions at different pH values was measured with a DLS at 25 °C using a Multi Angle Brookhaven 90 Plus Particle Size Analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA). The samples were prepared by diluting 150 µL of the dispersions in 3.0 mL of pH-matched deionized water in a 1 cm transparent polystyrene cell.

4.2.10 Surface tension

The du Noüy ring method was used to determine the apparent interfacial tension of soybean oil-aqueous phase (dSWP , SSPS or dSWP-SSPS mixed dispersions) at 25 °C using a Fisher Surface Tensiomat, model 21 (Fisher Scientific, Nepean, ON, Canada). A platinum-iridium ring was immersed inside the dense aqueous phase, and the breaking point of the distended film formed at the oil-aqueous phase interface is the measure of apparent interfacial tension. The breaking point is indexed through circular scale.

4.2.11 Emulsion phase separation

Emulsion creaming/sedimentation was determined using a vertical scan analyzer (Turbiscan, Formulaction, Toulouse, France). The backscattering profiles of 3 cm high samples stored in cylindrical glass tubes (d=20 mm) were monitored on days 0, 1, 7, 14, 21, 28 and 60 with the resulting profiles analyzed using the instrument's built-in software.

4.2.12 Emulsion microstructure

Samples were observed using an inverted light microscope (Zeiss Axiovert 200M, Zeiss Canada, Toronto, ON, Canada) with a 20x objective and 10x oculars at 25 °C (magnification 200 x). Images were captured with a Q-Imaging CCD camera and analyzed with Northern Eclipse software v.7.0 (Empix Imaging, Inc., Mississauga, ON, Canada).

4.2.13 Data analysis

All reported experiments were carried out in triplicate, and the results are expressed as mean ± standard deviation. Statistical analysis was performed using SigmaStat 4 integrated in SigmaPlot 11 (Systat Software, Chicago, IL, USA). The Student's *t*-test for single pair comparisons or one-way ANOVA with Tukey's multiple comparison test for multiple groups was used. Differences were considered statistically significant at $p \leq 0.05$.

4.3 Results and discussion

The isolation of dSWP, properties of the dSWP-SSPS dispersions and the kinetic stability of model O/W emulsions prepared with either dSWP, SSPS or mixed dSWP and SSPS are discussed in following the segments.

4.3.1 SWP composition and conformation

The extraction and characterization of dSWPs are highlighted in the next two sections.

4.3.1.1 nSWP SDS-PAGE

SDS-PAGE analysis of nSWP expectedly showed the presence of the KTI and lectin at 21 kDa and 120 kDa, respectively (Figure 4.1) [28]. The band at 40 kDa was due to the hydrolysis of lectin. Mass spectrometry was used to verify the molecular weight and representative protein moiety of the three bands seen in the SDS-PAGE analysis (results not shown).

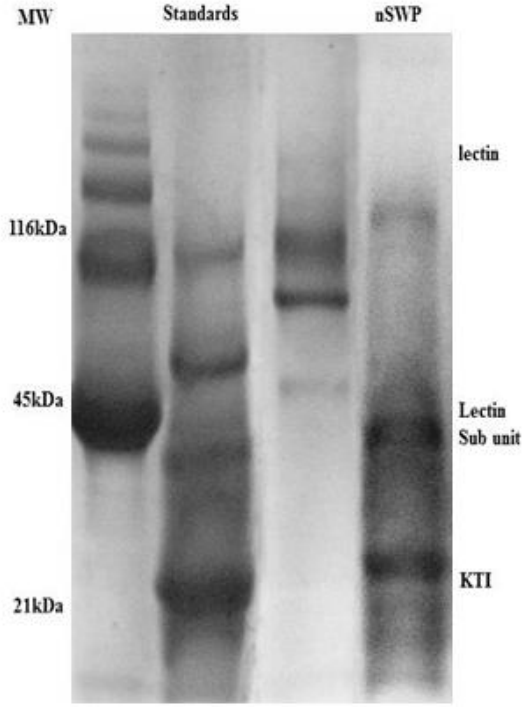

Figure 4.1: SDS-PAGE of nSWP and molecular weight standards.

4.3.1.2 CD spectroscopy

CD spectroscopy was performed to determine the secondary structure of nSWP and dSWP at pH 7 (Figure 4.2). In both spectra, the peak near ~ 222 nm was interpreted as an α–helix [179] whereas the peak at ~ 200 nm was indicative of a β-sheet conformation [36]. Finally, the shoulder at ~ 195 nm in the dSWP spectrum was characteristic of β-II proteins, and provided evidence of changes in secondary structure with some solvent exposure [35, 36]. However, based on the similarities between both spectra, there was little difference in the secondary structure of the nSWP and dSWP, implying probable disruption of tertiary structure.

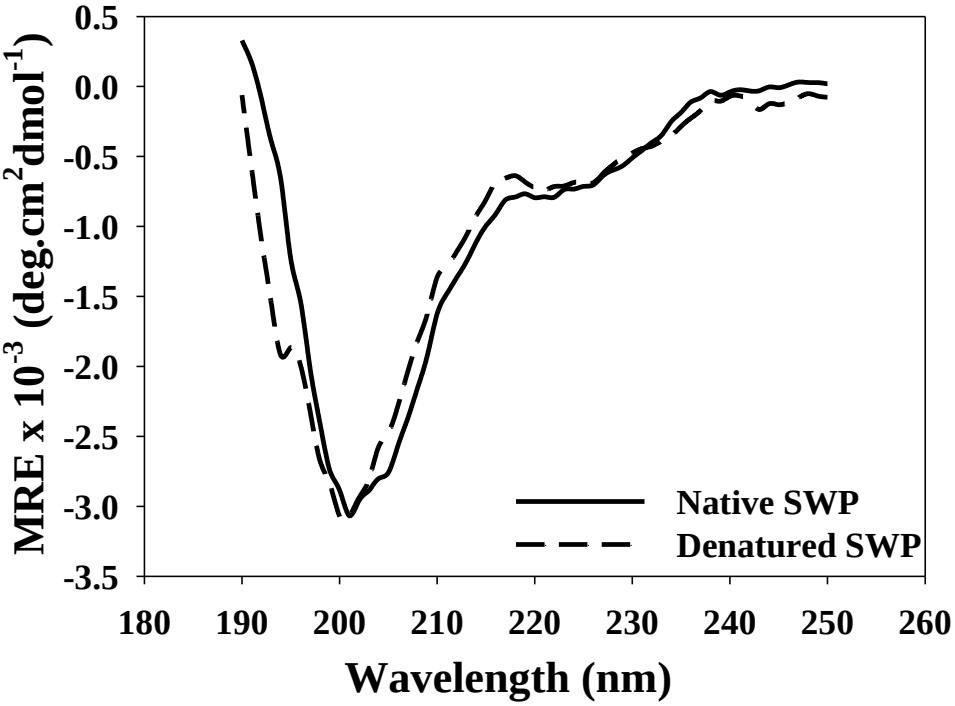

Figure 4.2: CD spectra of native and denatured SWP.

4.3.2 Protein-polysaccharide dispersions

The particle size and zeta potential of aqueous dispersion of dSWP and dSWP-SSPS mixed systems at different pHs is explored in the next segment.

4.3.2.1 Particle size

To gain insight into their role on emulsion formation and stability, the pH-dependent interactions of mixed dSWP (1 wt%) + SSPS (up to 1.5 wt%) aqueous dispersions were determined (Table 4.1). On decreasing pH from pH 8 to 5, the average particle size of dSWP-only dispersions dipped from 316 ± 2 nm to 309 ± 9 nm ($p \leq 0.05$), only to increase at pH 4 (428 ± 8 nm) and pH 3 (462 ± 10 nm) ($p \leq 0.05$). At pH 1 and 2, average particle sizes were 248 ± 9 nm and 395 ± 7 nm, respectively ($p \leq 0.05$). Hence, there was a significant pH-dependent effect on dSWP particle size with strong evidence of aggregation at lower pH. Addition of SSPS to the 1 wt% dSWP dispersion strongly hindered protein aggregation at lower pH only, *e.g.*, at pH 3, presence of 1.5 wt% SSPS yielded a 46 % reduction in dSWP particle size (249 ± 4 nm) ($p \leq 0.05$)

whereas the admixture of just 0.15 wt% SSPS led to a 36 % reduction in average particle size (297 ± 8 nm) ($p \leq 0.05$). At pH 4-8, presence of 0.25 - 1.5 wt% SSPS also yielded smaller dSWP particle sizes compared to the protein alone ($p \leq 0.05$). With only 0.15 wt% SSPS, particle size rose from 309 ± 9 nm to 321 ± 6 nm at pH 5 and from 316 ± 2 nm to 405 ± 8 nm at pH 8 respectively ($p \leq 0.05$), indicating that low levels of SSPS could not prevent protein aggregation.

Table 4.1: pH dependent variation of average particle size and zeta potential of a 1.0 wt% dSWP dispersion and 1.0 wt% dSWP + 1.5 wt% SSPS mixed dispersion (n=3).

pHs	**Average particle size (nm)**		**Zeta potential (mV)**	
	1.0 wt% dSWP	**SSPS:dSWP (1.5)**	**1.0 wt% dSWP**	**SSPS:dSWP (1.5)**
3	462 ± 10	249 ± 4	-1 ± 1	-2 ± 1
4	428 ± 8	247 ± 4	-3 ± 0.5	-4 ± 0.8
5	309 ± 9	253 ± 3	-4 ± 0.3	-6 ± 0.7
6	311 ± 8	254 ± 2	-7 ± 0.4	-8 ± 0.3
7	315 ± 5	256 ± 5	-10 ± 0.4	-11 ± 0.2
8	316 ± 2	256 ± 4	-12 ± 1	-11 ± 0.4

4.3.2.2 Zeta potential

The dSWP-only dispersion showed a gradually less negative surface charge upon decreasing pH from 8 to 3 (-12 ± 1 mV to -1 ± 1 mV) ($p \leq 0.05$) and a slightly positive charge at pH 1 and 2 (2 mV) ($p > 0.05$). Between pH 5 and 8, all mixed SSPS+dSWP dispersions had ζ-potential values < -11.0 mV (Table 4.1). Near pH 3, ζ-potential values were still negative but small (-4 mV) due to protein-polysaccharide complexation mediated by negatively and positively-charged patches on the SSPS and dSWP, respectively [180].

Broadly speaking, there were no differences in ζ-potential between the only protein and protein-polysaccharide mixed (1:1.5) dispersion (Table 4.1). When protein-polysaccharide combined

with the clear differences in particle size, this suggested weak dSWP-SSPS complexation that improved protein solubility by electrostatic repulsion and/or steric effects [181].

4.3.3 dSWP + SSPS stabilized O/W emulsions

The kinetic stability of emulsions prepared with either dSWP, SSPS or mixed dSWP and SSPS at pH 3-8 is explored below.

4.3.3.1 Droplet size distribution (DSD) and visual sedimentation

dSWP-based emulsions were highly-unstable and rapidly phase-separated following preparation, e.g., the DSDs of fresh 1 wt% dSWP emulsion showed primarily bimodal distributions dominated by a mode at 90 µm regardless of pH (Figure 4.3A). Addition of SSPS greatly diminished the emulsions' initial DSD, *e.g.,* at pH 3 (Figure 4.3B).

At all dSWP:SSPS ratios, emulsion DSDs shifted to lower droplet sizes, particularly at higher SSPS loads and lower pH (Figure 4.4A). Bimodal DSDs (~ 0.2 µm - 180.0 µm) were observed at protein-polysaccharide ratios ≤ 0.25 (Figure 4.4D) whereas a nearly unimodal DSD (~ 0.1 - 2.0 µm range) appeared for ratios ≥ 0.5 at pH 3 and 4 (Figure 4.4A and C). The $D_{4,3}$ value of 1 wt% dSWP + 1.5 wt% SSPS emulsion at pH 3 increased from 0.7 ± 0.1 µm on day 0 to ~ 1.7 ± 0.2 µm after 28 d (Fig. 4.4A,B).

However, at pH 8, the $D_{4,3}$ of the same formulation increased from 1.5 ± 0.1 to 3.5 ± 0.5 µm from day 0 to 21, indicating system instability. The pH 8 emulsions at dSWP:SSPS ratios of 0.25 and 0.15 visually phase-separated within 12 h, hence no DSDs were measured. These results were in support of the visual sedimentation data (Tables 4.2 and 4.3), which clearly showed that all emulsions experienced significantly improved shelf life with mixed dSWP and SSPS compared to dSWP or SSPS only. When mixed with 0.15-1.5 wt% SSPS, there was no phase separation at any SSPS:dSWP ratio at low pH, suggesting formation of a liquid coacervate or the steric stabilization of the dSWP by the SSPS [182, 183]. There was a very little variation in the measured viscosity of all dSWP-SSPS combinations used in the emulsions (average viscosity ~ 0.003 Pa·s at 25 °C regardless of pH), implying no effect of viscosity on complex formation and emulsion stability (results not shown) ($p > 0.05$). Overall, these results provided insight on the

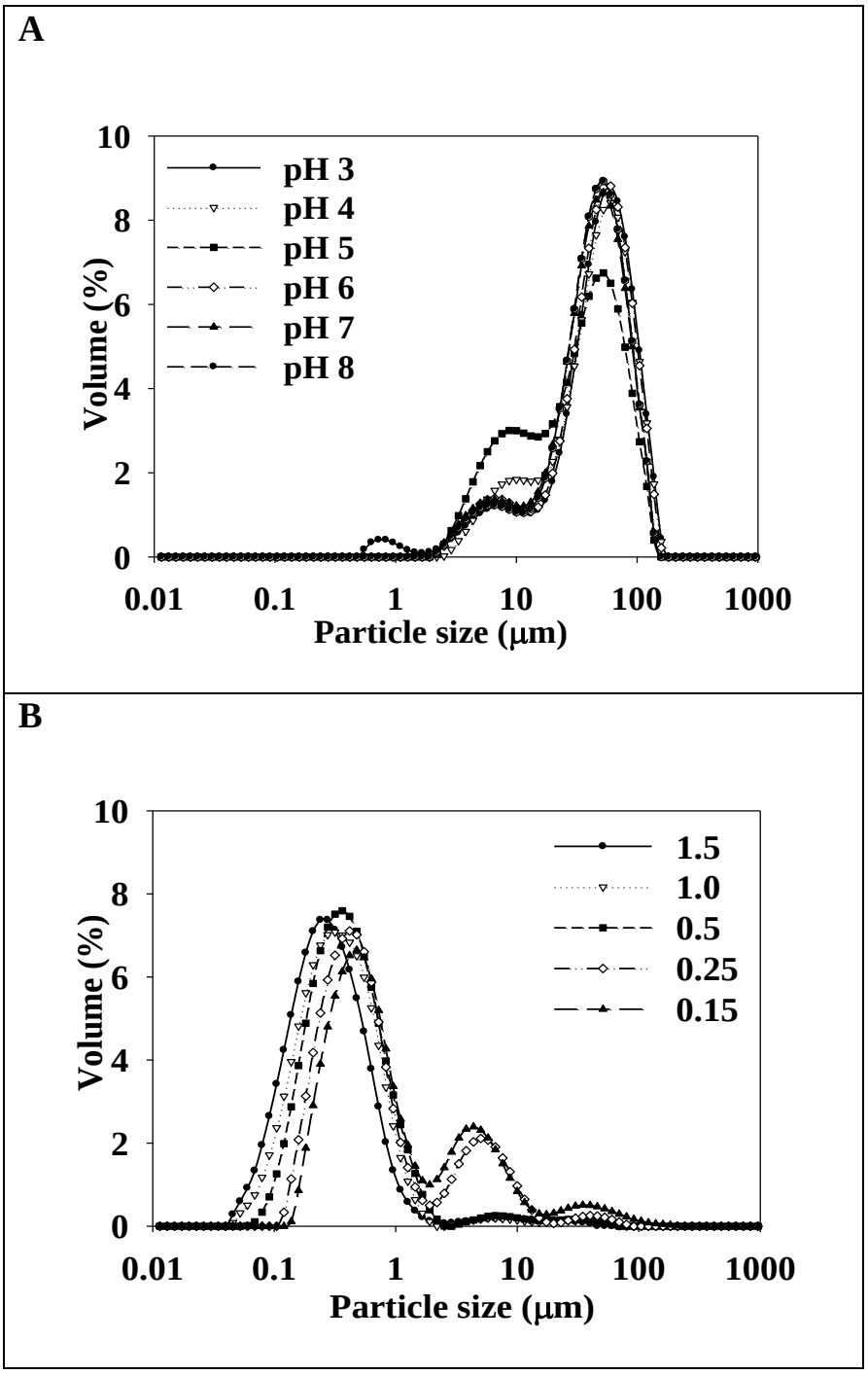

Figure 4.3: (A) pH-dependent droplet size distribution of freshly-prepared 1 wt% dSWP emulsions; (B) The effect of added SSPS (wt%) on the droplet size distribution of freshly-prepared dSWP emulsions at pH 3.

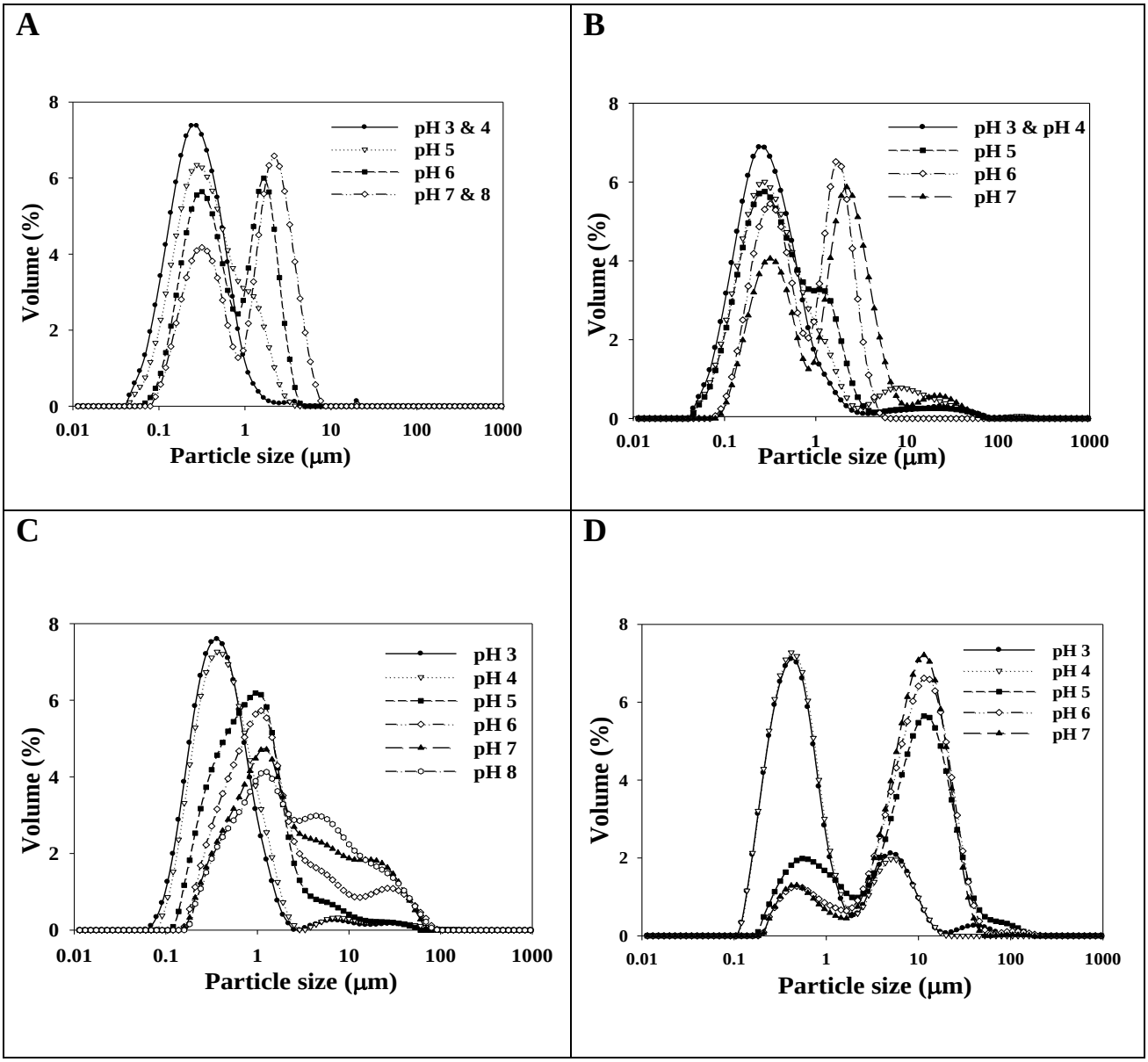

Figure 4.4: The effect of pH, SSPS:dSWP ratio (1.5, 0.5 or 0.25) and storage time on the droplet size distributions of 5% O/W emulsions: (A) ratio of 1.5 on day 0; (B) ratio of 1.5 on day 28; (C) ratio of 0.5 on day 0; (D) ratio of 0.25 on day 0. Note: pH 8 emulsions not shown in B and D due to phase separation.

Table 4.2: Onset of visual phase separation for emulsions stabilized with 1.0 wt% dSWP and 0.15-1.5 wt% SSPS at various pH values. 'Ratio' corresponds to SSPS:dSWP ratio used in the emulsion (n=3).

Ratio	**pH 3**	**pH 4**	**pH 5**	**pH 6**	**pH 7**	**pH 8**
1.5:1.0	> 60 d	60 d	60 d	28 d	28 d	21 d
1.0:1.0	> 28 d	28 d	28 d	14 d	7 d	7 d
0.5:1.0	28 d	28 d	21 d	7 d	7 d	7 d
0.25:1.0	14 d	14 d	48 hr	< 24 hr	< 24 hr	< 12 hr
0.15:1.0	14 d	14 d	48 hr	< 24 hr	< 24 hr	< 12 hr

Table 4.3: Onset of visual phase separation for a 5% O/W emulsion stabilized with 0.15-1.5 wt% SSPS only at various pHs (n=3).

wt% SSPS	**pH 3**	**pH 4**	**pH 5**	**pH 6**	**pH 7**	**pH 8**
1.5	14 d	14 d	7 d	7 d	< 7 d	< 7 d
1.0	7 d	7 d	< 7 d	< 7 d	< 7 d	< 7 d
0.5	< 7 d	< 7 d	< 7 d	< 7 d	< 7 d	< 7 d
0.25	1 d	1 d	< 48 hr	< 24 hr	< 24 hr	< 12 hr
0.15	< 24 hr	< 24 hr	< 24 hr	< 24 hr	< 24 hr	< 12 hr

role of pH-dependent charge interactions between the dSWP and SSPS on emulsion stability. At pH 6-8, the greater negative charges on both dSWP and SSPS created weaker, more negatively-charged dSWP-SSPS complexes that perhaps formed more diffuse interfacial layers due to electrostatic repulsion between neighbouring complexes. The resulting diffuse layer structure could not impede droplet-droplet flocculation and emulsion destabilization as effectively. Such charge repulsion likely allowed the dSWP-SSPS complexes to bind to more than one droplet thereby promoting bridging flocculation [17, 166]. The emulsions' initial $D_{4,3}$ values at SSPS:dSWP ratios $\geq$ 1.0 at these pHs a greater penchant to flocculation and phase separation

(Table 4.2). This likely resulted from surface charge-mediated depletion flocculation given the increasing amount of dSWP-SSPS complexes present in the bulk phase at SSPS:dSWP ratios ≥ 1.0 [184], *i.e.*, the bulk protein concentration at pH 8 was ~8.2 % vs 4.1 % at pH 3.

At pH 3 to 5, nearly neutral complexes provided an effective steric barrier that reduced inter-droplet contact [185]. The significant electrostatic interactions and resulting strong dSWP-SSPS complexes (at all protein-polysaccharide ratios) generated stable emulsions, *e.g.*, at pH 3-4 and an SSPS:dSWP ratio of 1.5 (Figure 4.4A), the $D_{4,3}$ was ~0.7 µm. Time-dependent changes in droplet size highlighted the positive effect of dSWP:SSPS ratio on emulsion stability. For example, at a ratio of 1.5, the $D_{4,3}$ of 28 d emulsions rose to ~ 2 µm (Figure 4.4B), though no visual phase separation was evident ≥ 60 days. Lower SSPS:dSWP ratios (≤ 0.5) produced greater droplet aggregation (Figure 4.4C and D), with longer storage (≤ 28 d) leading to extensive droplet flocculation as a result of a sub-optimal protein-polysaccharide ratio and/or insufficient material to englobe the oil droplets.

The optimum dSWP-SSPS mixed system (ratio = 1.5) yielded a higher apparent interfacial tension at pH 3 (41.0 ± 1.4 mN/m) compared to pH 8 (36 ± 1.2 mN/m) ($p \leq 0.05$). The 1.5 wt% dSWP-SSPS (1:0.5) combination aqueous solution-oil interface at pH 3 demonstrated a significantly lower apparent interfacial tension (~32 ± 0.0 mN/m) than the optimum dSWP-SSPS combination ($p < 0.05$) (Table 4.4). Based on Tables 4.2 and 4.3, the most stable emulsion was formed at pH 3 with 1 wt% dSWP + 1.5 wt% SSPS. As this emulsion had a higher interfacial tension, this strongly suggested that emulsion stability resulted from the formation of a structured elastic interfacial film that resisted emulsion droplet coalescence [186].

The combination of 0.5 wt% SSPS and 1.0 wt% dSWP reduced the $D_{4,3}$ to 0.6 µm, shifted the DSD towards lower values (0.03-2.10 µm) and retarded the onset of visible phase separation to 28 d from < 12 h (1 wt% dSWP), < 7 d (0.5 wt% SSPS) or 14 d (1.5 wt% SSPS) (Table 4.2 and 4.3). Table 4.2 and 4.3 further indicate that the combination of dSWP + SSPS noticeably increased the time point at which visual phase separation occurred compared to SSPS-stabilized emulsions.

Table 4.4: Variation of apparent interfacial viscosity (unit mN/m) for soybean oil-aqueous phase of dSWP, SSPS or dSWP-SSPS mix dispersion at pH 3 and 8 (n=3).

pH	1.0 wt% dSWP	2.5 wt% dSWP	1.5 wt% SSPS	2.5 wt% SSPS	1.5 wt% (dSWP-SSPS)	2.5 wt% (dSWP-SSPS)
3	31.5 ± 0.7	31.5 ± 0.7	34.5 ± 0.7	36.0 ± 1.6	32.0 ± 0.0	41.0 ± 1.4
8	32.0 ± 0.0	--	32 ± 0.4	--	--	36.0 ± 1.2

4.3.3.2 Total biopolymer content

An increase in total biopolymer content and dSWP-SSPS complexation significantly improved emulsion stability compared to the dSWP (1 wt%) or SSPS (0.15-1.5 wt%) alone. Fig. 4.5A shows that at pH 3, the 1 wt% dSWP emulsion produced a multimodal DSD with a range of 1 µm to 158 µm ($D_{4,3}$ ~ 49 µm), the 0.5 wt% SSPS yielded a narrower unimodal DSD with a range of ~ 1 - 8 µm ($D_{4,3}$ ~ 3 µm) and the 1.5 wt% SSPS emulsion was even more narrowly-distributed (0.1-3.3 µm; $D_{4,3}$ ~ 0.8 µm) (Figure 4.5B).

4.3.3.3 Zeta potential

Generally speaking, ζ-potential values of ≤ -30 mV or ≥ +30 mV are required for particles to be considered stable against electrostatic aggregation [187]. No ζ-potentials for the dSWP-only emulsions (average particle size > 30 µm) were measurable due to instrument limitations. Blank emulsions solely prepared with SSPS were slightly more negatively-charged compared to those with SSPS-dSWP complexes, *i.e.*, on day 0, ζ-potentials of -13 ± 1 mV at pH 3 and -41 ± 0 mV at pH 8 were observed for the emulsion with 1.5 wt% SSPS whereas the combination of 1.0 wt% dSWP and 1.5 wt% SSPS showed ζ-potentials of -11 ± 0 mV at pH 3 and -37 ± 0 mV at pH 8 (Figure 4.6).

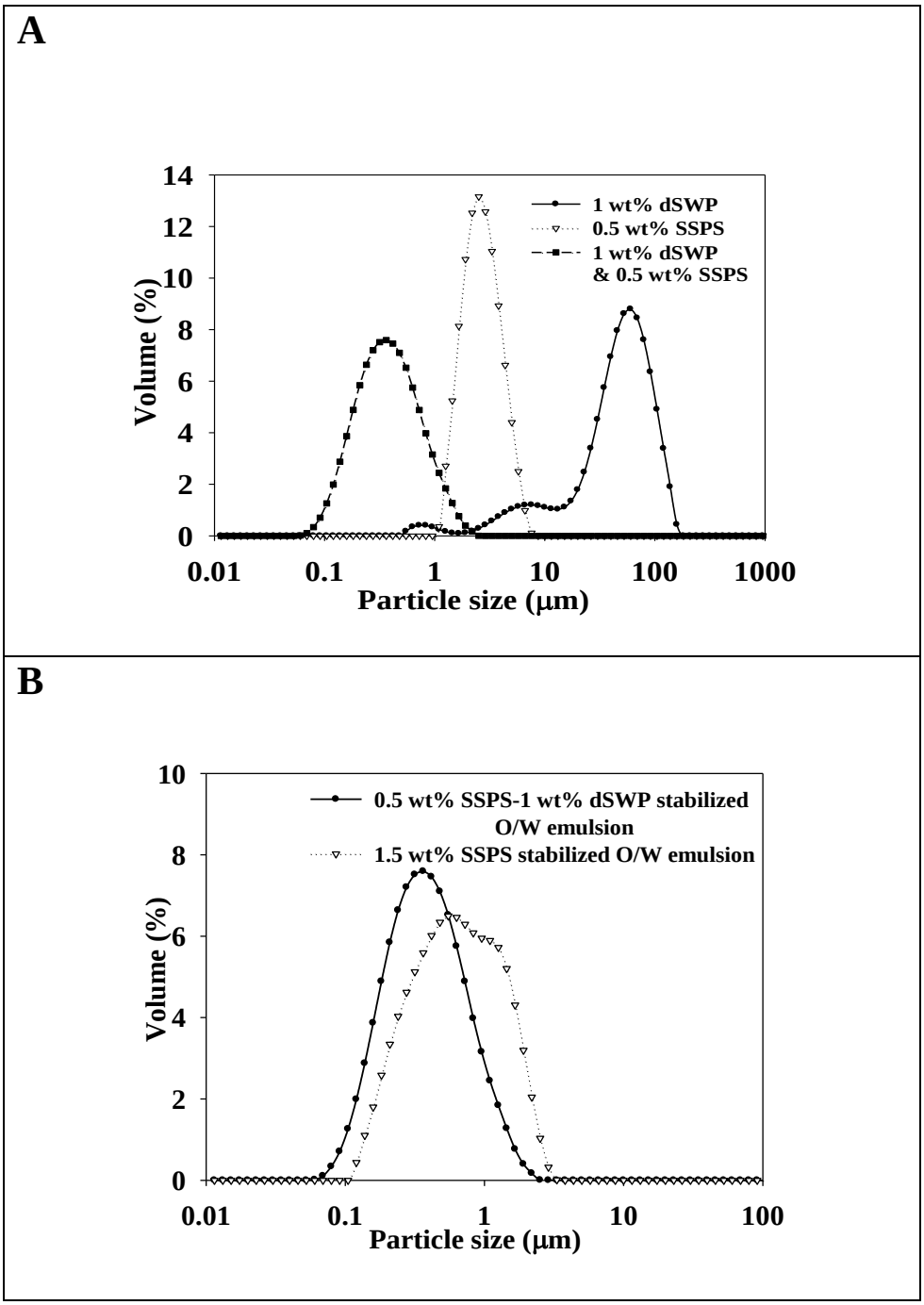

Figure 4.5: Total biopolymer combination effect on the initial droplet size distribution of freshly-prepared emulsions at pH 3: (A) 1.0 wt% dSWP, 0.5 wt% SSPS and 1.0 wt% dSWP + 0.5 wt% SSPS emulsions; (B) 1.5 wt% SSPS *vs.* 1.0 wt% dSWP + 0.5 wt% SSPS emulsions.

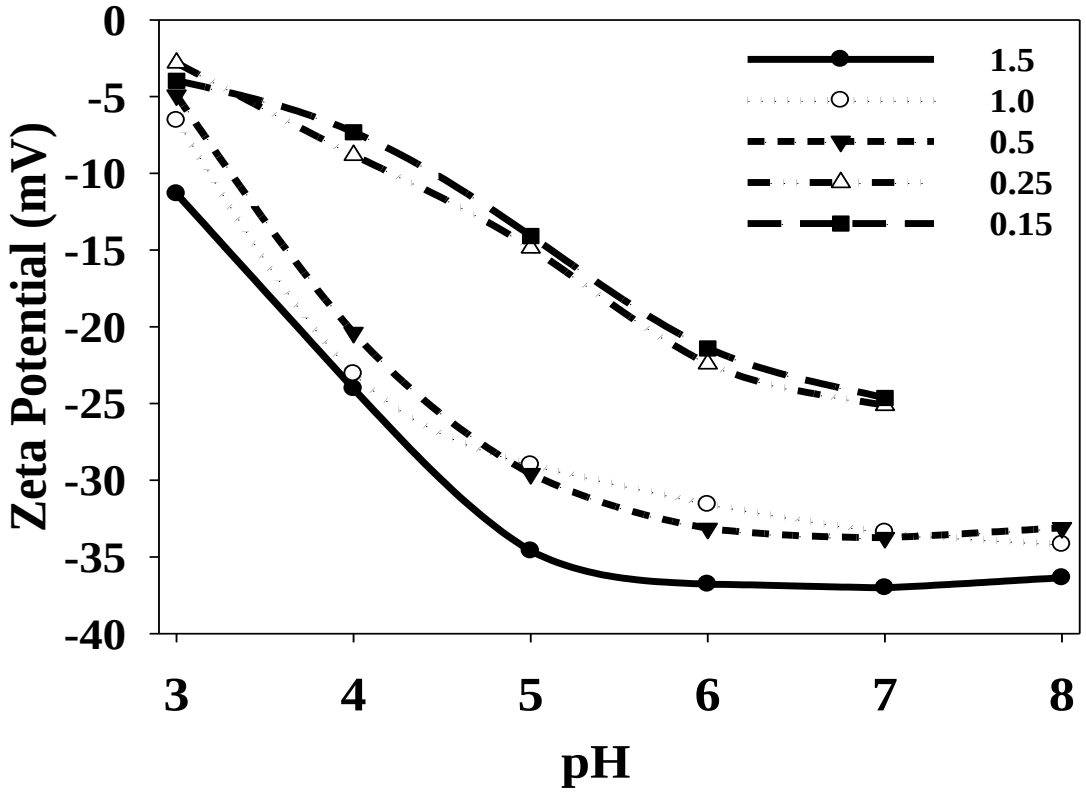

Figure 4.6: Zeta potential of freshly-prepared dSWP + SSPS emulsions as a function of pH. Emulsions consisted of 1 wt% dSWP and 0.15, 0.25, 0.5, 1.0 or 1.5 wt% added SSPS. Error bars removed for clarity (n=3).

This decrease in ζ-potential was attributed to charge neutralization and electrostatic complexation between the SSPS and dSWP. A further possibility was the adsorption of more dSWP-SSPS complexes at the droplet surface. Moschakis et al., who studied the influence of chitosan and gum arabic mixtures on emulsion stability at pH 3, concluded that enhanced adsorption of these mixtures on droplet surfaces caused an overall charge density reduction [182]. A similar mechanism was perhaps observed with the present emulsions as at pH 3, the ζ-potential values were -4 to -12 mV irrespective of SSPS:dSWP ratio. Though harbouring less negative ζ-potential values when compared to pH 5-8, emulsion stability was enhanced at lower pH (3 & 4), strongly suggesting that steric effects were the dominant mode of emulsion stabilization.

4.3.3.4 Phase separation

Emulsions prepared solely with dSWP phase-separated within < 12 h of preparation whereas in control emulsions with SSPS, separation occurred ≤ 14 d. Combining 0.15 wt% or 1.5 wt% SSPS with 1.0 wt% dSWP increased sedimentation stability at pH 3 to ≥ 14 or > 60 d, respectively (Figure 4.7A). Ganzevles et al. [188] showed that a neutral protein-polysaccharide complex favoured the formation of a dense, thick and compact interfacial layer with a higher interfacial shear modulus than with only protein. Conversely, highly negatively-charged complexes led to a more diffuse layer at the interface. In our case, at low pH the dSWP+SSPS complexes had less negative surface charges compared to pH 5-8 and adsorbed more effectively to the oil-water interface thereby leading to a more densely-packed interface. As a result, emulsion phase separation was retarded at low pH compared to emulsions at pH 5-8 (Table 4.2).

The height-dependent backscattering profile of emulsions containing 1.5 wt% SSPS + 1.0 wt% dSWP were evaluated as a function of time at pH 3 (Figure 4.7A) and pH 8 (Figure 4.7B). Such profiles may be used to delineate changes in emulsion breakdown prior to the onset of visual phase separation. Zone I indicates gravity-induced oil-water creaming and zone II shows accumulation of oil droplets near the emulsion surface [41, 189].

The change in zone I and II backscattering profiles as a function of time was markedly more rapid at pH 8 showing the tendency of this emulsion to break down more rapidly. These results confirmed the trend in droplet size evolution whereby at pH 8, $D_{4,3}$ increased more rapidly than at pH 3. Larger droplets are known to accelerate emulsion breakdown.

4.3.3.5 Emulsion morphology

Emulsion morphology also revealed a significant pH-dependent trend (Figure 4.8). At pH 3-4, no significant size variation was apparent at SSPS:dSWP = 1.5 after 1 month whereas slight changes in morphology were apparent after one month at pH 5.

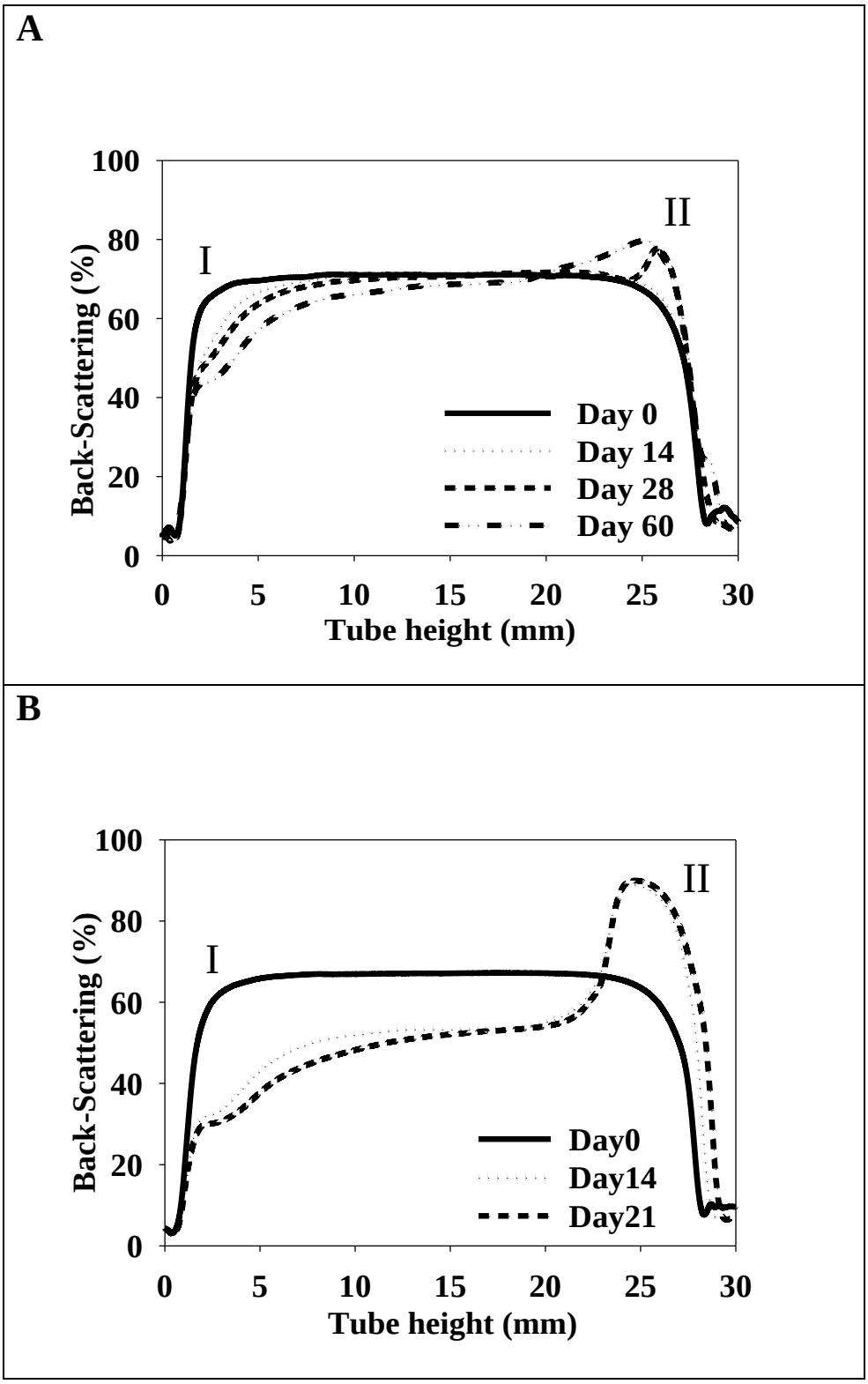

Figure 4.7: Creaming profile of a 1.0 wt% dSWP + 1.5 wt% SSPS emulsion as a function of storage time at pH 3 (A) or pH 8 (B). Zone I indicates gravity-induced oil-water creaming and zone II shows accumulation of oil droplets near the emulsion surface.

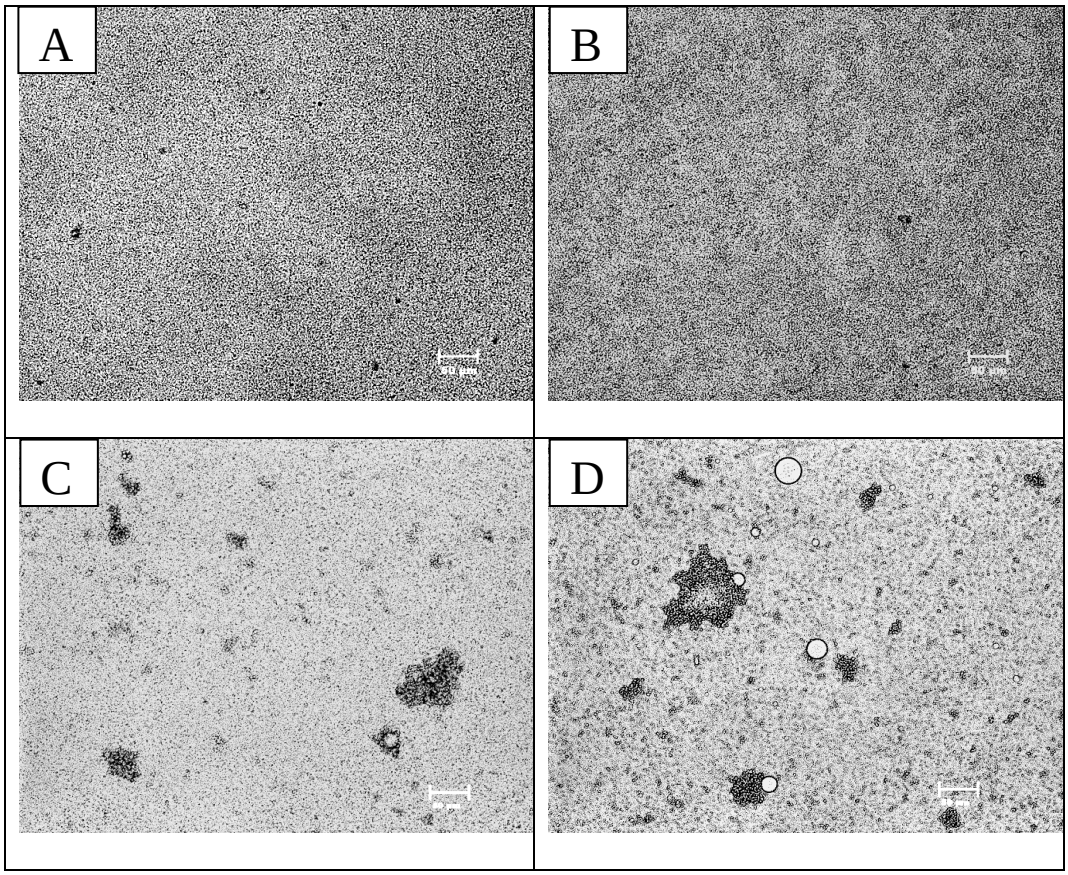

Figure 4.8: Microstructure of a 1.0 wt% dSWP + 1.5 wt% SSPS emulsion as a function of pH after 14 days of storage. A) pH 3-4; B) pH 5; C) pH 6; D) pH 7-8. Size bar = 50 µm.

A significant amount of droplet flocculation was evident at pH 6-8 from day 1 onwards, gradually leading to the presence of large flocs. Presumably, the adsorbed interfacial complexes were diffuse at this pH and incapable of hindering droplet flocculation and coalescence [188].

4.3.3.6 Presence of salt vs. emulsion stability

The presence of NaCl at 1.0 M greatly influenced emulsion morphology (Figure 4.9), ζ-potential (Figure 4.10) and droplet size evolution (Figure 4.11) of emulsions stabilized with 1.0 wt% dSWP + 1.5 wt% SSPS at pH 3-8. With post-emulsification addition of salt, as the dSWP and SSPS had already complexed, the observed flocculation and creaming were due to aggregation of droplets with pre-formed dSWP-SSPS complexes (Figure 4.9A). Though the overall surface charge was small at all pHs (between -2 and -13 mV) (Figure 4.10), larger flocs only appeared at

pH 5-8. This was likely due to the presence of greater electrostatic interactions between the protein and polysaccharide at lower pH which resulted in more salt-resistant flocs that sterically hindered flocculation. Droplet size results concurred with the microscopy images whereby there was an increase in the population of larger droplets with increasing pH (Figure 4.11A). At pH 5-8, the salt worked to integrate the local biopolymer environment thus causing electrostatic screening [173]. As protein-polysaccharide complexes may dissociate upon addition of electrolytes [52], this would result in the displacement of SSPS from the surface of the dSWP, thus allowing for hydrophobic interactions between charge-reduced patches on the protein.

With NaCl added prior to dSWP-SSPS complexation and emulsification, all emulsions destabilized within 48 h. As per above, large flocculated droplets appeared at pH 5-8 with extensive aggregation also present at pH 3-4 (Figure 4.9). There were tri-modal DSDs at all pHs with the population of larger droplets noticeably increasing at lower pH (Figure 4.11B). The ζ-potential trend with addition of salt prior to protein-polysaccharide complexation mimicked the 'salt-free' pH-dependent trend with a gradual charge reduction < pH 5. If the Na^+ and Cl^- ions indeed screened the dSWP and SSPS in bulk thus partly countering their complexation [50, 158, 190], then the measured surface charge was not only that of the complexed biopolymers but also that of the individual proteins and polysaccharides. There was support for this possibility based on the similar DSDs of the 1 wt% dSWP emulsion at pH 3 (Figure 4.5A) and the present pH 3 emulsion which indicated that under these conditions, the dSWP and/or weakly-associated dSWP and SSPS adsorbed to the emulsion surface, thereby resulting in a poor emulsion.

By contrast, there was a lesser effect on emulsion DSD at higher pH, implying that more protein-polysaccharide complexation took place. The more highly negatively-charged dSWP and SSPS may have repelled the Cl^- ions thereby limiting its ability to screen the positive patches on the dSWP. As a result, some dSWP-SSPS complexation occurred leading to better emulsification and a shift towards smaller droplet sizes compared to pH 3-4.

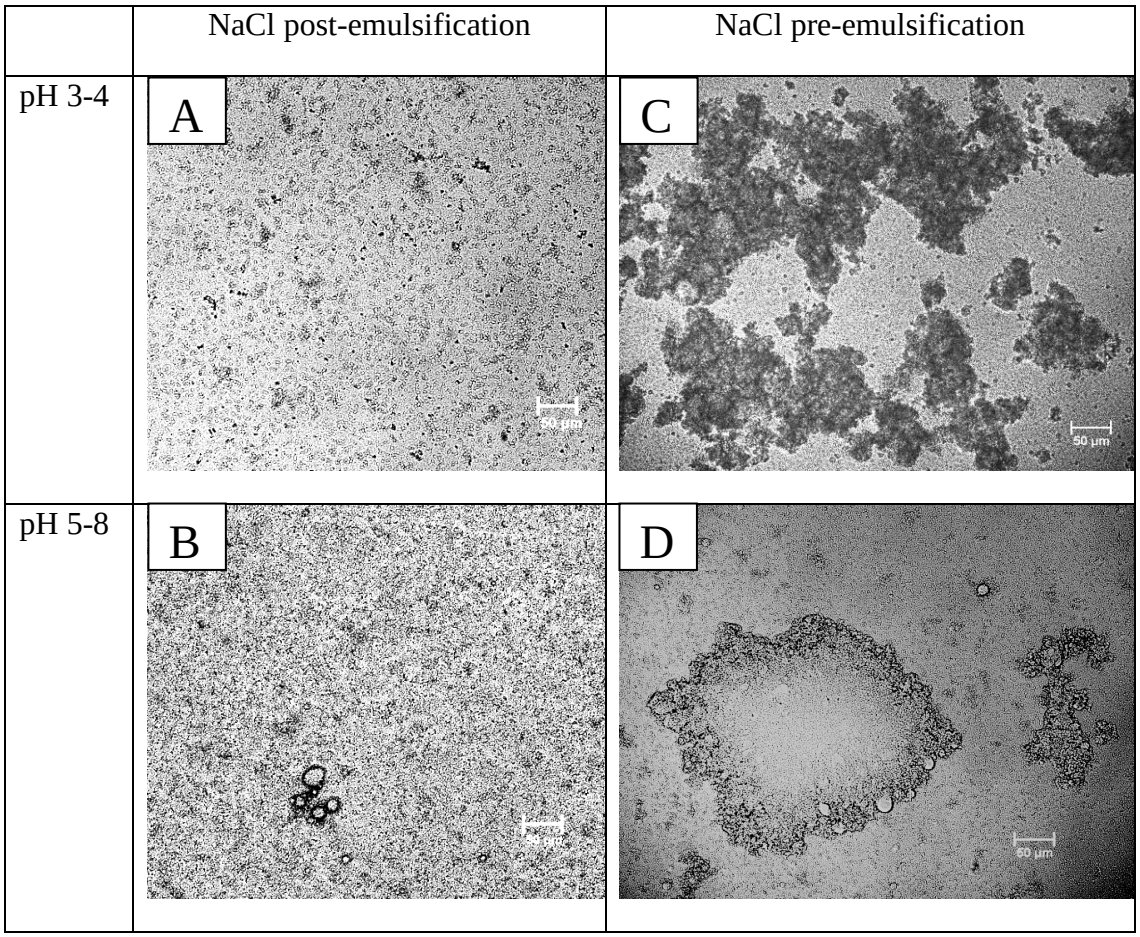

Figure 4.9: Microstructure after 24 hrs of a 1.0 wt% dSWP + 1.5 wt% SSPS emulsion containing 1M NaCl added either after emulsion preparation. Representative morphologies for pH 3-4 and pH 5-8 are shown. Size bar = 50 µm.

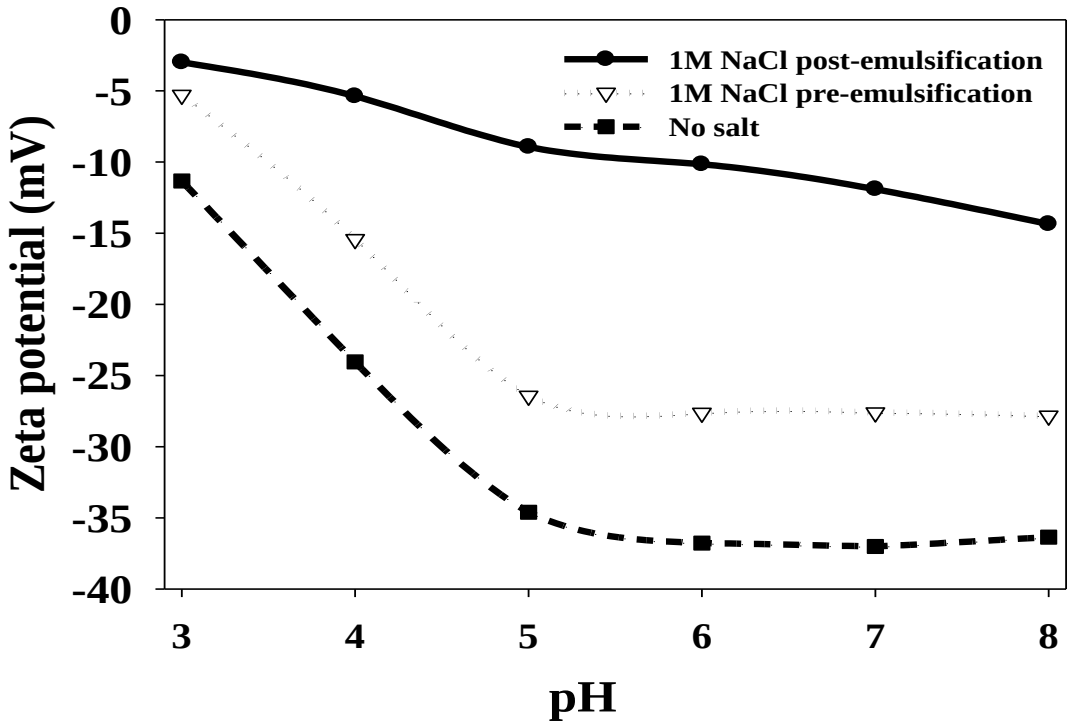

Figure 4.10: pH-dependent zeta potential of a 1.0 wt% dSWP + 1.5 wt% SSPS emulsion containing 1M NaCl added either before or after emulsion preparation. Error bars removed for clarity.

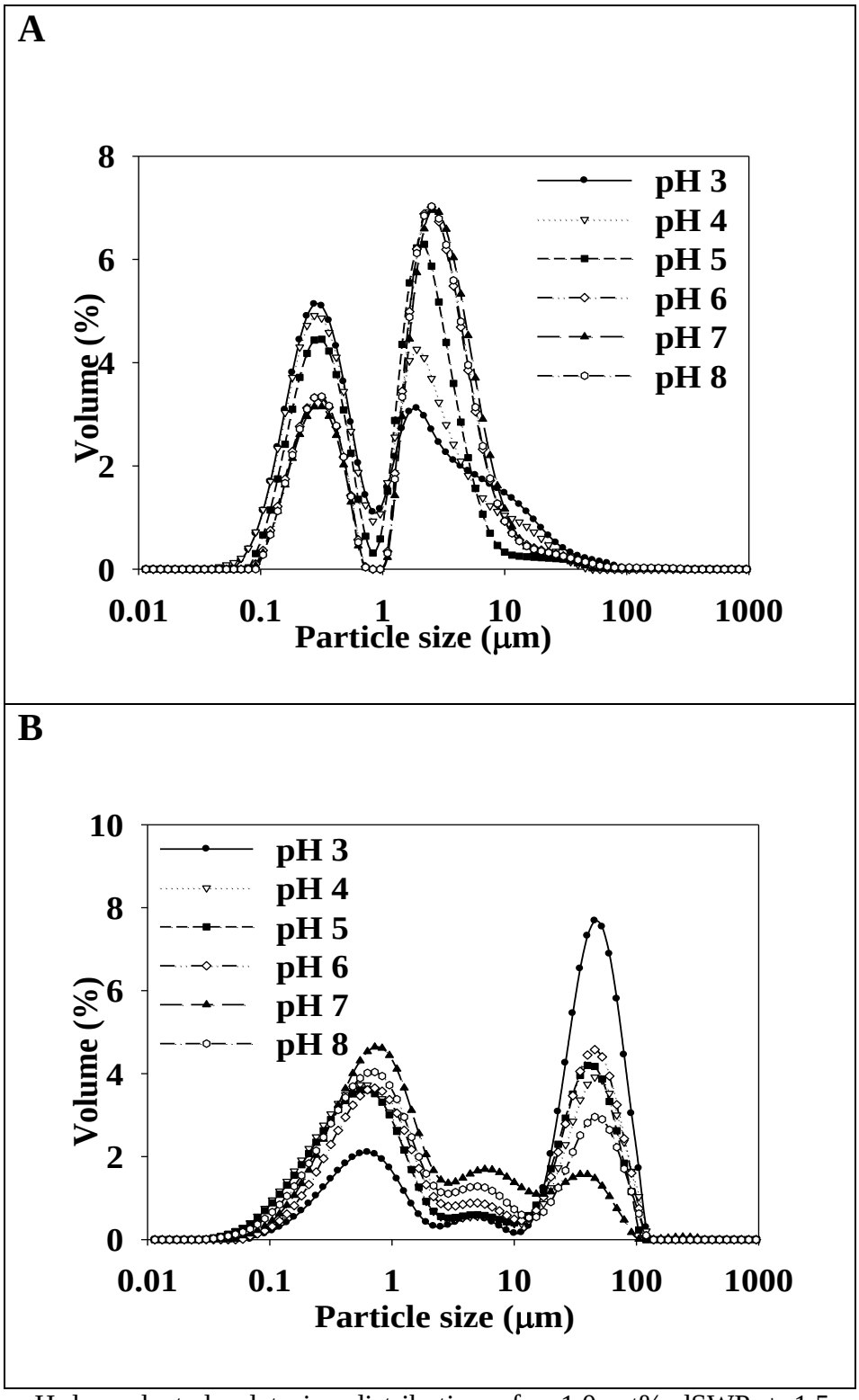

Figure 4.11: pH-dependent droplet size distribution of a 1.0 wt% dSWP + 1.5 wt% SSPS emulsion containing 1M NaCl added either after (A) or before (B) emulsion preparation.

4.3.3.7 Mechanistic considerations

Fundamentally, post-emulsification, the oil-water interface was covered by either dSWP, SSPS or dSWP-SSPS complexes, with the latter offering the greatest improvement in emulsion stability. The two main factors that determined the packing density and layer thickness of the dSWP-SSPS mixed layer were the net charge density and molecular architecture of the dSWP + SSPS complexes. During emulsion formation, adsorption of the dSWP-SSPS complexes to the interface was presumed to form a uniform mixed layer with the overall charge and dSWP-SSPS complex stoichiometry determining interfacial adsorption and layer thickness. Chen and Soucie [180] stated that a decrease in ζ-potential could occur due to increasing electrostatic binding of oppositely-charged molecules or ions. As at pH 3 and 4, the net negative charge of the dSWP + SSPS complexes was low, it could be postulated that these pH conditions facilitated denser packing of the dSWP + SSPS complexes around the oil droplets. Such increased layer thickness and density would provide greater steric stabilization and an enhanced emulsion shelf life. Conversely, at pH 5-8, the more negatively-charged dSWP + SSPS complexes prevented formation of a more compact layer at the O/W interface. The more negatively-charged dSWP + SSPS complexes would also be more apt to electrostatically repel neighbouring complexes thereby either promoting their desorption from the interface and/or reducing the possibility of adsorption of new dSWP + SSPS complexes present in the bulk. With a lower concentration of adsorbed complexes that were spatially sparser, there would be the greater likelihood of direct droplet-droplet contact, flocculation and emulsion coalescence.

Generally speaking, there was increased emulsion stability at higher SSPS loads, however this was significantly dependent on pH as at higher pH there was evidence of depletion flocculation. As well, NaCl addition after emulsification induced surface charge screening of the oil droplets whereas added salt before emulsification provided hindered electrostatic interactions between dSWP and SSPS, resulting in more extensive emulsion instability. These results clearly demonstrated that by modulating SSPS and dSWP concentrations, pH and ionic strength, optimal conditions were obtained that resulted in emulsions with long-term stability.

4.4 Conclusion

This study has shown that mixtures of dSWP and SSPS may be used to stabilize dilute O/W emulsions against coalescence and phase separation. Compared with emulsions solely prepared with either dSWP or SSPS, emulsions at an optimal SSPS:dSWP ratio of 1.5:1.0 (corresponding to 2.5 wt% biopolymer in the aqueous phase) did not phase separate for > 60 days at pH 3. Based on our findings, dSWP-SSPS complexes promoted emulsion stability *via* steric hindrance. However, should such an approach be used, pitfalls associated with either depletion or bridging flocculation would need to be taken into account. As well, a fundamental question that remains is whether SSPS preferentially interacts with KTI or lectin in dSWP, which may provide guidance regarding the dynamics of dSWP activity. Overall, however, the long-term stability achieved by complexing dSWP with SSPS at low pH ranges may be used as a stepping stone towards the use of soy processing by-products in value-added processed foods.

Chapter 5

Physical and oxidative stability of flaxseed oil-in-water emulsions stabilized with soy whey proteins and polysaccharides

5.0 Abstract

Electrostatic interactions between denatured soy whey proteins (dSWP) and soluble soybean polysaccharides (SSPS) were used to stabilize dilute flaxseed oil-in-water emulsions against physical breakdown and lipid oxidation. Using MDA measurements to evaluate latter stages of lipid oxidation, interfacially-adsorbed dSWP-SSPS complexes were shown to effectively slow the oxidation of encapsulated flaxseed oil at pH 3-8 and different storage temperatures (4, 25 and 50 °C). Emulsions prepared with only SSPS (1.5 wt%) more effectively inhibited oxidation vs. dSWP (1.0 wt%) at low pH, with higher temperatures expectedly accelerating oil oxidation irrespective of protein-polysaccharide composition (4 < 25 < 50 °C). The composition leading to the highest physical stability (1.5 wt% SSPS + 1.0 wt% dSWP) also most effectively retarded flaxseed oil oxidation, with no evidence of oxidation at pH 3 and 4 °C for 21 days. Compared to pH 3, pH 7-8 MDA concentrations in day-old emulsions were higher by 30 % at 4 °C, 67 % at 25 °C and 91 % at 50 °C, due to the lack of protein-polysaccharide complexation at higher pH. Overall, the study showed that the presence of combined dSWP and SSPS retarded flaxseed oil oxidation, though this was highly dependent on pH.

Keywords: emulsion; soy whey protein; soluble soybean polysaccharides; flaxseed oil; lipid oxidation; coalescence.

5.1 Introduction

The consumption of foods rich in polyunsaturated (PUFA) fatty acids can reduce the risk of chronic diseases such as heart disease, arthritis, and some types of cancers [191-193]. Unfortunately, PUFAs are highly susceptible to oxidative deterioration that results in objectionable off-flavours (e.g., metallic, fishy, and rancid aromas) and loss of nutritional value [194]. Reduction of oxygen levels (e.g., with packaging), low-temperature storage and/or addition of metal chelators or antioxidants retards lipid oxidation in PUFA-rich foods [83, 89]. The dispersion of oil within oil-in-water (O/W) emulsions is also considered a viable tool to protect labile oils such as flaxseed, fish and safflower oils from oxidation [83, 195].

The oil-water interface has been shown to act as a physical barrier that retards lipid oxidation of emulsified oil phase from pro-oxidants present in the neighbouring continuous aqueous phase [115, 196]. As well, a number of studies have suggested that the rate of lipid oxidation in O/W emulsions can be controlled by changes in the properties of the interfacial layer surrounding dispersed droplets, including composition, thickness as well as charge status and density [83, 89, 100, 110, 197, 198]. For example, Silvestre et al. compared the oxidation rate in salmon oil O/W emulsions stabilized with either Brij 76 (polyoxyethylene (2) stearyl ether) or Brij 700 (polyoxyethylene (100) stearyl ether), with the latter retarding oxidation more extensively given its thicker interfacial layer [130].

Certain proteins inhibit lipid oxidation in food emulsions, including caseins, lactoferrin and dairy whey proteins [83, 89, 100, 110, 197, 198]. Mechanisms associated with their antioxidant properties include: inactivation of reactive oxygen species, free radical scavenging, metal chelation, hydroperoxide reduction, enzymatic removal of oxidants and creation of a physical barrier between reactants [110]. Of relevance to the present study, Taylor and Richardson found that thermal denaturation improved the antioxidant activity of milk whey proteins [199].

Certain polysaccharides (e.g., xanthan gum, low methoxy pectin, high methoxy pectin, sodium alginate, methyl cellulose and α–carrageenan) have also shown the capacity to retard lipid oxidation in O/W emulsions, via mechanisms such as free radical scavenging, transition metal

chelation and viscosity enhancement [25, 102-104]. Certain glycoproteins such as gum arabic and SSPS may also retard lipid oxidation via free radical scavenging via their attached protein moiety [102, 105, 106].

Flaxseed oil is a polyunsaturated oil extracted from the flax plant (*Linum usitatissimim*) that is rich in α-linolenic acid, an ω-3 fatty acid that represents ~ 57 % of its total fatty acids [200]. Though recognized as having beneficial health effects [201], it is highly susceptible to oxidative deterioration and the production of undesirable flavours [202]. Different proteins and polysaccharides have been used to encapsulate flaxseed oil, with the goal of improving its oxidative stability. These include whey protein isolate [91] as well as mixtures of gelatin and gum arabic [203], barley and beta glucan [204] as well as maltodextrin, whey protein concentrate and modified starch [93]. Generally, these studies have shown that higher homogenization pressures and oil volume fractions increase its oxidation.

As we have previously shown, mixtures of denatured soy whey proteins (dSWP) and soluble soybean polysaccharides (SSPS) can successfully be used to kinetically stabilize dilute O/W emulsions against coalescence and phase separation [10]. The present study hypothesized that the oxidative stability of emulsified flaxseed oil would increase in the presence of a mixed dSWP and SSPS as emulsion stabilizers, with the role of protein-polysaccharide complexation and continuous phase pH playing key roles in efficacy. To this end, the oxidative stability of flaxseed oil droplets coated by dSWP and/or SSPS at pH 3-8 and different temperatures was tested.

5.2 Material and methods

The different materials and characterization methods used to study the kinetic and oxidative stability of the O/W emulsions herein developed are described below.

5.2.1 Materials

Defatted solvent-free soy flour (7B Soy Flour: min. 53 % protein, max. 9 % moisture, 32 % carbohydrates, max. 3 % fat, 18% total dietary fibre) was obtained from Archer Daniels Midland (Decatur, IL, USA). Commercial-grade SSPS (SOYAFIBE-S-CA200: moisture: 5.8 %, crude

protein: 7.8 %, crude ash, 7.8 %. Saccharide composition (%): Rhammnose: 5.0, Fucose: 3.2, Arabinose: 22.6, Xylose: 3.7, Galactose: 46.1, Glucose: 1.2, Galacturonic acid: 18.2) was provided by Fuji Oil Ltd (Osaka, Japan). Flaxseed oil (Royal Harvest Flax oil, Shape Foods Inc, Brandon, MB, Canada) was purchased from a local store (acid value ≈ 1.78 mg KOH/g of oil). Polysorbate 20 was purchased from Fisher Scientific (Nepean, ON, Canada). All chemicals used were reagent grade. Deionized water was used throughout all experiments. Unless noted otherwise, all reagents were used without further purification.

5.2.2 Preparation of stock dispersions

dSWP was extracted and prepared as previously published [10]. Dispersions of dSWP (2 wt%) and SSPS (3 wt%) in deionized water were independently prepared, adjusted to pH 3 and stirred for 3 h. Sodium azide (0.02 wt%) was added to prevent microbial growth. At this low concentration, sodium azide did not have any effect on protein-polysaccharide interactions in bulk or in emulsions.

5.2.3 Preparation of O/W emulsions

Emulsions consisted of 5 wt% flaxseed oil, 1 wt% dSWP and/or 1.5 wt% SSPS in deionized water. This dSWP-SSPS combination was deemed optimal based on earlier work [10]. Coarse emulsions were prepared using a rotor/stator (Polytron PT-10-35 with PCU-2 control, Kinematica GmbH, Switzerland) operated at 27,000 RPM for 1 min followed by high-pressure valve homogenization (3 passes at 60 MPa) (APV, model 1000, Albertslund, Denmark) at room temperature (~25 °C). Emulsion pH was adjusted from pH 3 to 8 with 0.1 M NaOH or 0.1 M HCl. Emulsion samples were kept sealed at three different storage temperatures (4, 25 or 50 °C) until analysis. Most physical and oxidative stability results herein reported are based on the optimal emulsion formulation consisting of 1 wt% dSWP + 1.5 wt% SSPS (1:1.5). For comparison, 5 wt% flaxseed O/W emulsions with no emulsifier or with 0.25 wt% polysorbate 20 were also prepared using the same protocols as above.

5.2.4 Droplet/particle size distribution

Laser light scattering experiments were conducted on prepared emulsions with a Malvern Mastersizer 2000 with a Hydro 2000S wet cell attachment (Malvern Instruments,

Worcestershire, UK) weekly for 3 weeks. Homogeneous samples were gently mixed *via* inversion to ensure representative sampling and then dispersed into the instrument's sample cell until an obscuration level of 5-10 % was reached. The pH of the dispersant water was adjusted to match the pH of the samples. The background and sample integration times were 12 s and 10 s, respectively. The optical parameters used were: a refractive index of 1.47 and an absorption index of 0.001 for the flaxseed oil and a refractive index of 1.33 for the water. Results were analyzed with the Malvern Mastersizer 2000 software v.5.54. Volume-weighted average droplet sizes ($D_{4,3}$) values are reported. Representative distributions of the emulsion prior to visible emulsion sedimentation are reported.

5.2.5 Zeta potential

Variations in emulsion zeta potential (ζ) were monitored weekly using a Brookhaven Zeta PALS system mated to a 90 Plus dynamic light scattering unit (Brookhaven Instruments Corporation, Holtsville, NY, USA) for 3 weeks. The samples were prepared by diluting 20 µL of the emulsions with 3.0 mL of pH-matched deionized water. Samples were analyzed at 25 °C and the instrument parameters used were: water as the dispersant with a viscosity of 0.890 cP, a refractive index of 1.330, and a dielectric constant of 78.54.

5.2.6 Emulsion phase separation

Emulsion creaming/sedimentation was determined using a vertical scan analyzer (Turbiscan, Formulaction, Toulouse, France). The backscattering profiles of 3 cm high samples stored in cylindrical glass tubes (d = 20 mm) were monitored on days 0, 1, 7, 14 and 21with the resulting profiles analyzed using the instrument's built-in software.

5.2.7 Emulsion microstructure

Samples were observed using an inverted light microscope (Zeiss Axiovert 200M, Zeiss Canada, Toronto, ON, Canada) with a 20x objective and 10x oculars at 25 °C (magnification 200 x). Images were captured with a Q-Imaging CCD camera and analyzed with Northern Eclipse software v.7.0 (Empix Imaging, Inc., Mississauga, ON, Canada).

5.2.8 Lipid oxidation

Thiobarbituric acid (TBA) assay values, which were used to quantify the generated secondary oxidation product malondialdehyde (MDA) present in the emulsified flaxseed oil, were measured as per Mei et al. [95]. A TBA solution was prepared by mixing 15 g of trichoroacetic acid, 0.375 g of TBA, 1.76 mL of 12 N HCl, and 82.9 mL of H_2O yielding a 18.16 wt% TBA solution. One hundred mL of TBA solution was mixed with 3 mL of 2 wt% butylated hydroxytoluene in ethanol, and 2 mL of this solution was mixed with 1 mL of emulsion diluted 30 times in water. The mixture was vortexed and heated in a boiling water bath for 15 min, cooled to room temperature, and centrifuged at 3000 x *g* for 15 min. Absorbance was measured at 532 nm using a UV-vis spectrophotometer (PerkinElmer, Lambda 2000, Woodbridge, ON, Canada). The extent of secondary lipid oxidation was expressed by quantifying malondialdehyde (MDA) concentration based on its molar absorptivity ($1.56 \times 10^5\ M^{-1}cm^{-1}$). To monitor lipid oxidation during storage, emulsions (10 mL) were placed in sealed 15mL Falcon tubes and stored at 4, 25 or 50 °C in the dark. Variations in TBA values were monitored weekly at 4 and 25 °C samples and hourly for samples stored at 50 °C given emulsion breakdown.

5.2.9 Data analysis

All reported experiments were carried out at least in triplicate, and the results are expressed as mean ± standard deviation. Statistical analysis was performed using SigmaStat 4 integrated in SigmaPlot 11 (Systat Software, Chicago, IL, USA). The Student's *t*-test for single pair comparisons or one-way ANOVA with Tukey's multiple comparison test for multiple groups was used. Differences were considered statistically significant at $p \leq 0.05$.

5.3 Results and discussion

The kinetic and oxidative stability of flaxseed-based O/W emulsions prepared with either dSWP, SSPS or mixed dSWP and SSPS are discussed in the following segments.

5.3.1 dSWP + SSPS stabilized O/W emulsions

The following section explores the kinetic stability of flaxseed oil dispersed as droplets in O/W emulsions stabilized with either dSWP, SSPS, mixed dSWP and SSPS or polysorbate 20 as a function of pH and temperature.

5.3.1.1 Phase separation

Flaxseed O/W emulsions at pH 3-8 prepared with no emulsifier phase-separated within 24 h whereas with dSWP alone, they broke down within 1 h of preparation, signifying that dSWP was completely ineffective as an emulsifier (Table 5.1). With 1.5 wt% SSPS, visual phase separation took $\leq$ 7 d at both 4 and 25 °C (Table 5.1). Stability of the mixed dSWP-SSPS emulsions was highest at low pH. Within 24 h, all emulsion stored at 50 °C completely phase-separated, even at lower pH (results not shown).

To ascertain the pre-visual emulsion phase separation, the changes in height-dependent backscattering profiles of the mixed dSWP-SSPS emulsions were monitored as a function of time at pH 3 (Figure 5.1A) and pH 8 (Figure 5.1B). Zone I exhibits gravity-induced oil-water creaming and zone II shows accumulation of oil droplets near the emulsion surface [41, 189]. The change in zone I and II backscattering profiles as a function of time were markedly more rapid at pH 8 showing the tendency of this emulsion to break down more rapidly. Presence of larger droplets and/or droplet aggregation facilitated destabilization at pH 8 vs. pH 3. These results further confirmed that pH had a significant impact on the phase separation behaviour of these emulsions.

Table 5.1: Onset of visual phase separation for emulsions stabilized with 1.5 wt% SSPS and/or 1.0 wt% dSWP at pH 3-8 and 4 or 25 °C. Results for surfactant-free emulsion are also shown. Emulsions with dSWP alone phase-separated within 1 h of preparation.

Emulsion	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
No emulsifier	<12 hr	<12 hr	<24 hr	<24 hr	<24 hr	<24 hr
1.5 SSPS (4 °C)	7 d	7 d	7 d	5 d	5 d	5 d
1.5 SSPS (25 °C)	7 d	7 d	7 d	5 d	5 d	5 d
1.5 SSPS + 1.0 dSWP (4 °C)	> 21 d	> 21 d	21 d	7 d	7 d	7 d
1.5 SSPS + 1.0 dSWP (25 °C)	21 d	21 d	14 d	7 d	7 d	7 d

5.3.1.2 Emulsion droplet size evolution

Emulsions solely prepared with dSWP were highly unstable and phase-separated very quickly after preparation. Freshly-prepared emulsions consisting of 1.0 wt% dSWP demonstrated bimodal distributions with a dominant mode at ~ 92 µm regardless of pH (result not shown). Presence of 1.5 wt% SSPS resulted in bimodal distributions at 4 and 25 °C, with their breaths dependent on pH (Figure 5.2 A,B). At pH 3, two dominant DSD modes at 0.4 and 1.5 µm were observed whereas at pH 8, these were higher at 1.2 and 3.3 µm ($p < 0.05$).

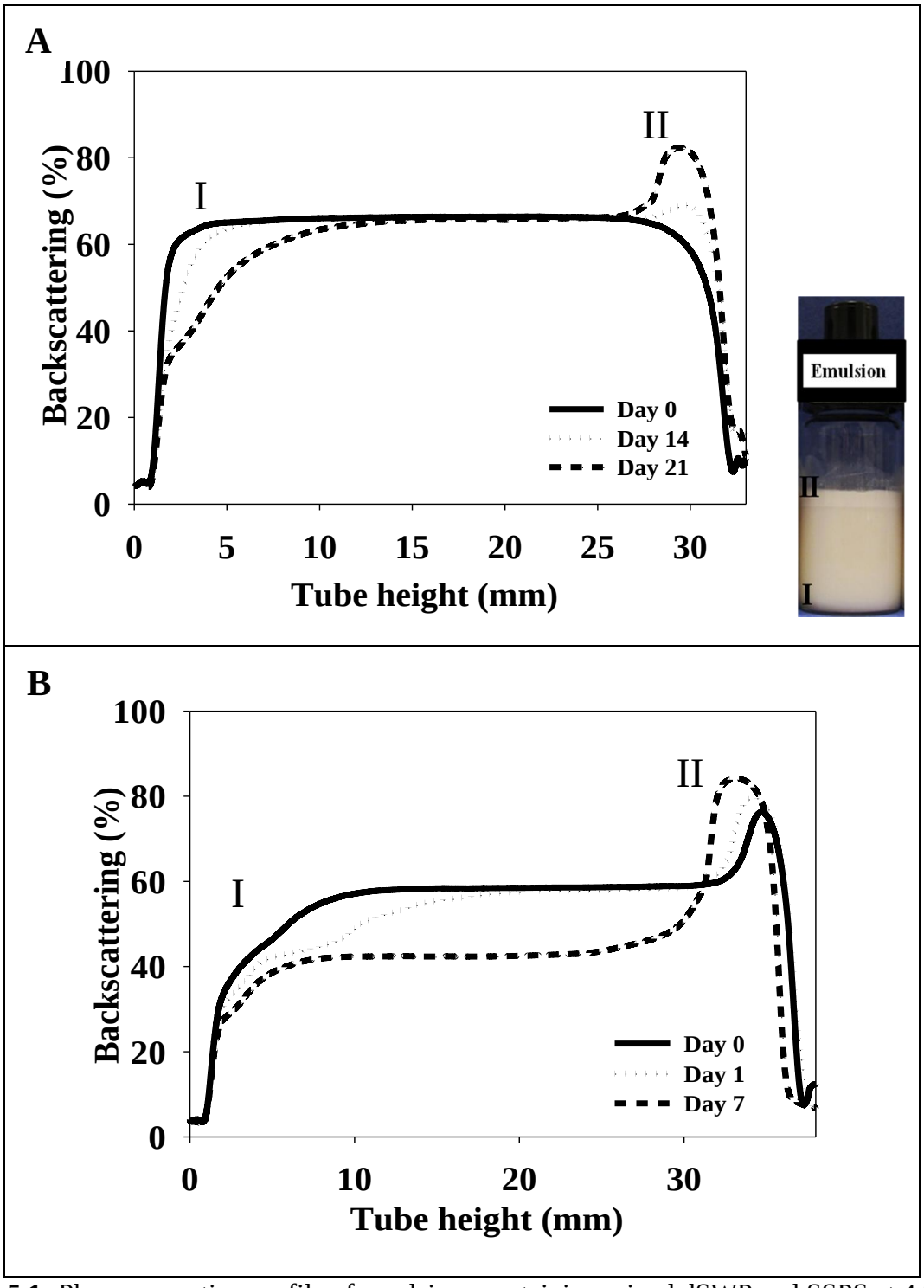

Figure 5.1: Phase separation profile of emulsions containing mixed dSWP and SSPS at 4 °C as a function of storage time and pH; (A) pH 3 and (B) pH 8. Zone I indicates gravity-induced oil-water settling and zone II shows accumulation of oil droplets near the emulsion surface, as per side image in A. Representative profiles are shown (n=3).

The $D_{4,3}$ values of these emulsions was somewhat pH-dependent rising from ~0.7 at pH 3-5 to ~2.5 µm at pH 8 (at both 4 and 25 °C) ($p > 0.05$) (Figure 5.3). By comparison, the $D_{4,3}$ of the emulsion with no added emulsifier was constant at 1.1-1.3 µm irrespective of pH ($p > 0.05$) whereas that of a polysorbate 20 control emulsion remained unchanged at ~0.5 µm. Combining 1.0 wt% dSWP and 1.5 wt% SSPS resulted in nearly unimodal distributions at 4 and 25 °C at low pH and bimodal DSDs at high pH (Figure 5.2 A,B). At both of these temperatures, the dominant DSD mode at pH 3 was at 0.4 µm with two modes at ~ 1 and ~3 µm at pH 8. Importantly, the $D_{4,3}$ values of the dSWP-SSPS emulsions were significantly pH-dependent demonstrating significant increases at ≥ pH 6 (at both 4 and 25 °C) (Figure 5.3). Time-dependent changes in the droplet size distribution of the emulsions containing mixed dSWP and SSPS were more prominent at pH 8 than pH 3 (Figure 5.4). There was no significant change in the breadth of the droplet size distribution at pH 3 over 21 days whereas at pH 8, droplet size growth was evident after day 7 ($p<0.05$). These results confirmed that protein-polysaccharide complexation at low pH retarded emulsions coalescence and phase separation more effectively than either SSPS or dSWP alone.

Figure 5.5 shows the pH-dependent droplet size distributions of the combined dSWP and SSPS emulsions at 4 and 25 °C. Distributions were nearly unimodal at pH 3-5, with the key mode centred at 0.2-0.3 µm. A small mode was also visible at ~ 3 µm. At pH 6, the distribution was still nearly unimodal, but the primary mode had increased to ~0.4 µm, indicating a somewhat less stable emulsion. Finally at pH 7 and 8, bi- or multimodal emulsions were apparent, with a growing portion of the emulsion's population consisting of droplets ≥ 10 µm in diameter (Figure 5.5). These results supported the visual sedimentation data (Table 5.1), which showed that all emulsions experienced significantly improved shelf life when dSWP and SSPS were mixed.

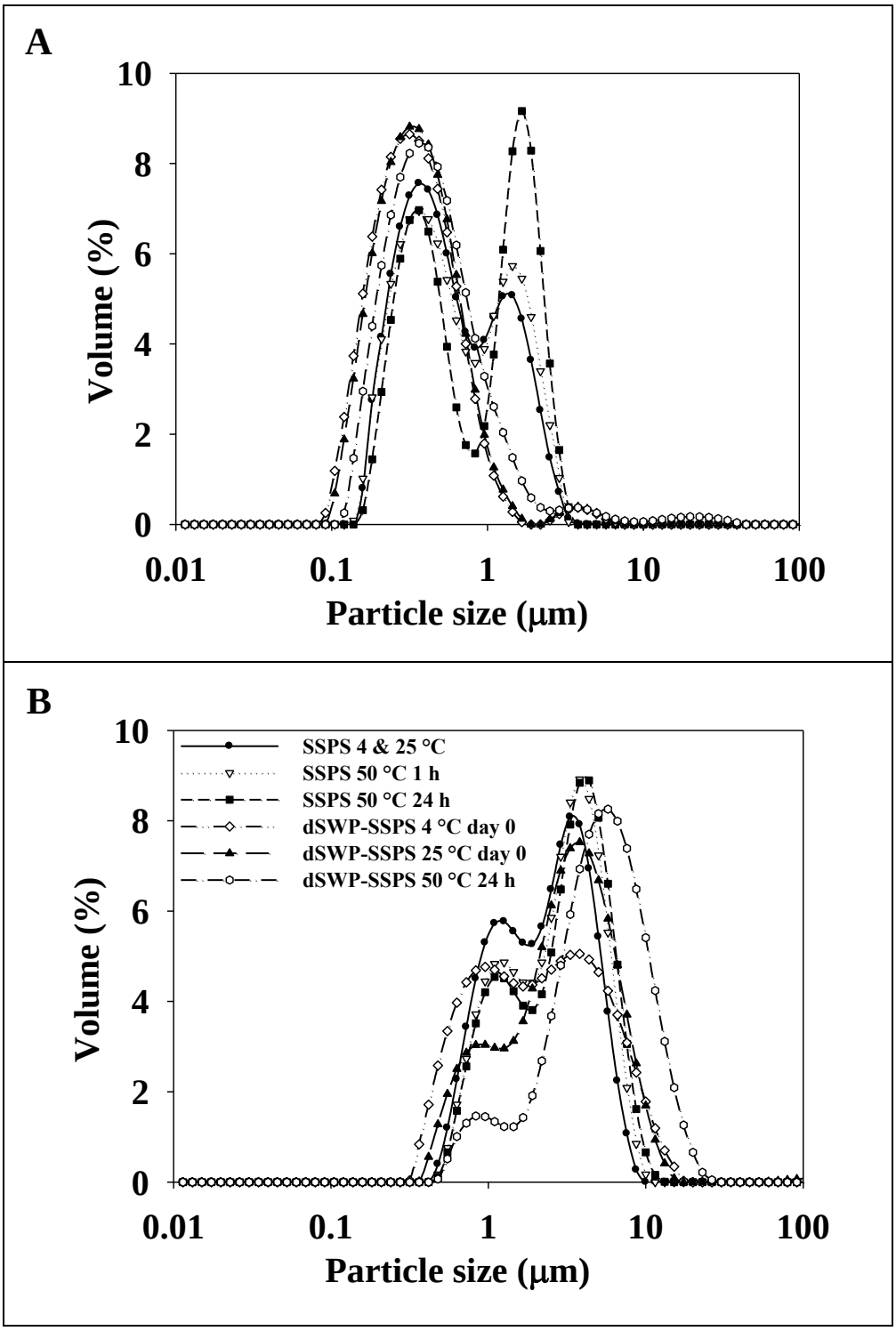

Figure 5.2: Temperature-dependent droplet size distributions of freshly-prepared flaxseed O/W emulsions at (A) pH 3 and (B) pH 8. Representative distributions are shown (n=3). Legend applies to both (A) and (B).

Mechanistically, the significant electrostatic interactions and resulting strong dSWP-SSPS complexes led to more kinetically-stable emulsions at pH 3-5 as the nearly neutral complexes provided an effective steric barrier that reduced inter-droplet contact probability [185]. At higher pH 6-8, the greater negative charges on both dSWP and SSPS produced more negatively-charged, weak dSWP-SSPS complexes that formed more diffuse interfacial layers due to electrostatic repulsion between charged neighbouring complexes, thus facilitating bridging flocculation [17, 166].

Storage at 50 °C accelerated emulsion breakdown, particularly at pH 6-8. All emulsion stored at 50 °C completely broke down within 24 h, even at lower pH where they were expected to remain somewhat kinetically stable. Nevertheless, there were notable pH-dependent differences in the size distributions of these emulsions as per those stored at 4 and 25 °C. Thus, at pH 3-5, primarily unimodal distributions were present with key a mode at ~0.5 µm (Figure 5.6). By contrast, at pH 6-8, bimodal distributions with a shift toward larger droplets were observed. Euston et al. (2000) and Sliwinski et al. (2003) suggested that the extent of droplet aggregation in whey protein-stabilized emulsions was markedly enhanced due to the thermal treatment of the emulsion [205, 206]. Both groups also proposed that the presence of non-adsorbed proteins in the bulk promoted droplet bridging. This was also likely observed with the present results, where the higher temperature reduced biopolymer interfacial adsorption. The non-adsorbed dSWP now present in the bulk thus promoted droplet bridging.

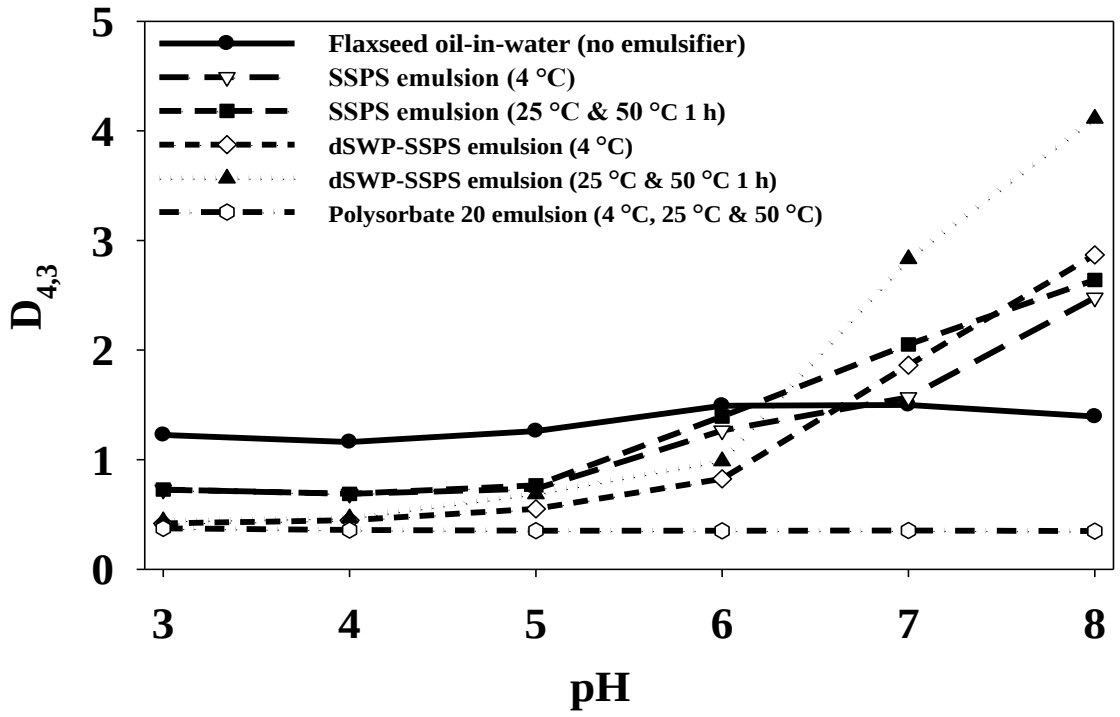

Figure 5.3: $D_{4,3}$ as a function of pH for freshly-prepared flaxseed O/W emulsions with either no emulsifier, SSPS alone or mixed dSWP and SSPS at 4 or 25 °C (n=3). Error bars removed for clarity.

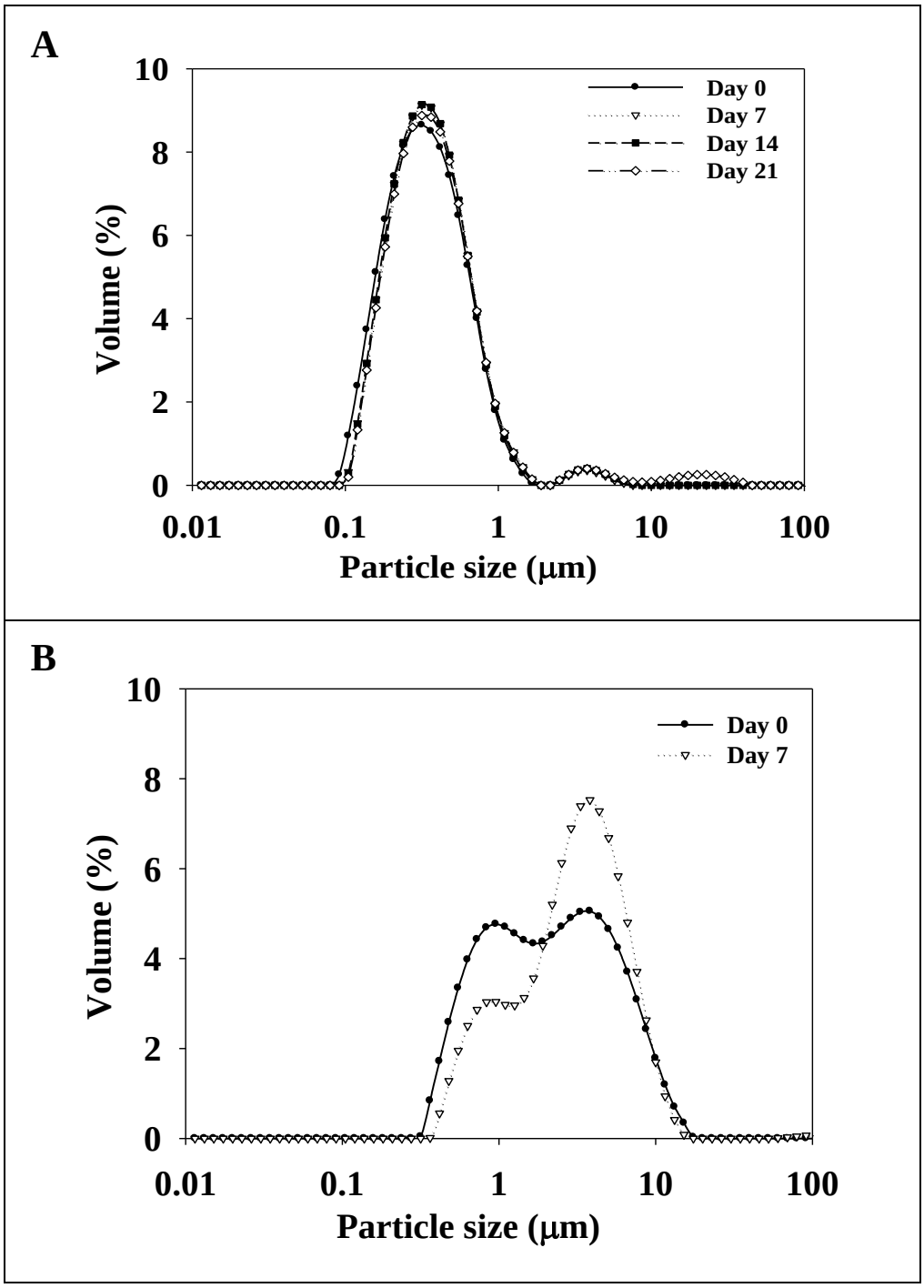

Figure 5.4: The effect of storage time on the droplet size distributions of flaxseed O/W emulsions at 4 °C as a function of pH : (A) pH 3, (B) pH 8.

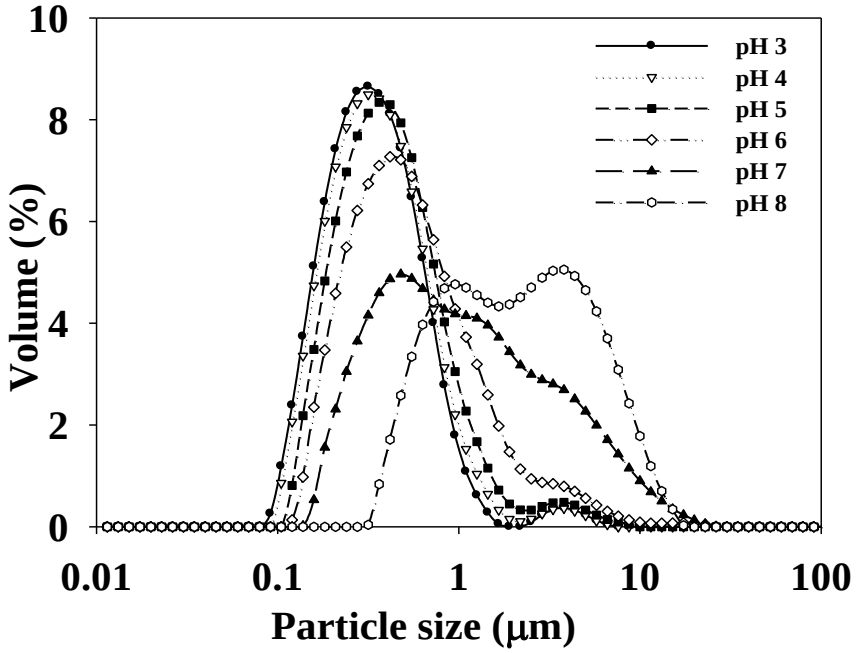

Figure 5.5: Temperature and pH-dependent droplet size distributions of freshly-prepared flaxseed O/W emulsions containing mixed dSWP and SSPS on day 0 at 4 °C. Representative distributions are shown (n=3).

5.3.2 Zeta potential

Charge density is a significant factor responsible for emulsion droplet resistance against aggregation and/or interactions with other charged species [131, 132, 207, 208]. Emulsions prepared with no emulsifier were highly negatively-charged with ζ-potential values of ca. -31 mV at pH 3 and ca. -90 mV at pH 8 emulsions (Figure 5.7). Addition of SSPS or the dSWP-SSPS complex resulted in substantially less negative ζ-potential values, with values of about -10 mV at pH 3 and -30 to -35 mV at pH 4 ($p < 0.05$). There were no differences in ζ-potential between these systems at pH 5-8, with values of -35 to -40 mV ($p > 0.05$). Figure 5.7A and B show that with time the ζ-potential of the emulsions stabilized with both dSWP and SSPS became slightly less negative from day 0 to 21 at both 4 and 25 °C. At both temperatures, emulsion ζ-potential at pH 3 evolved from ca. -10 mV on day 0 to -8 mV on day 21. At pH 5, the values rose from -42 mV (day 0) to -30 mV (day 21). Emulsions at pH 7-8 emulsions demonstrated nearly identical ζ -potential values on days 0 and 7. At 50 °C, the initial ζ-potential values of dSWP-SSPS emulsions ranged from -9 mV at pH 3 to -39 mV at pH 8.

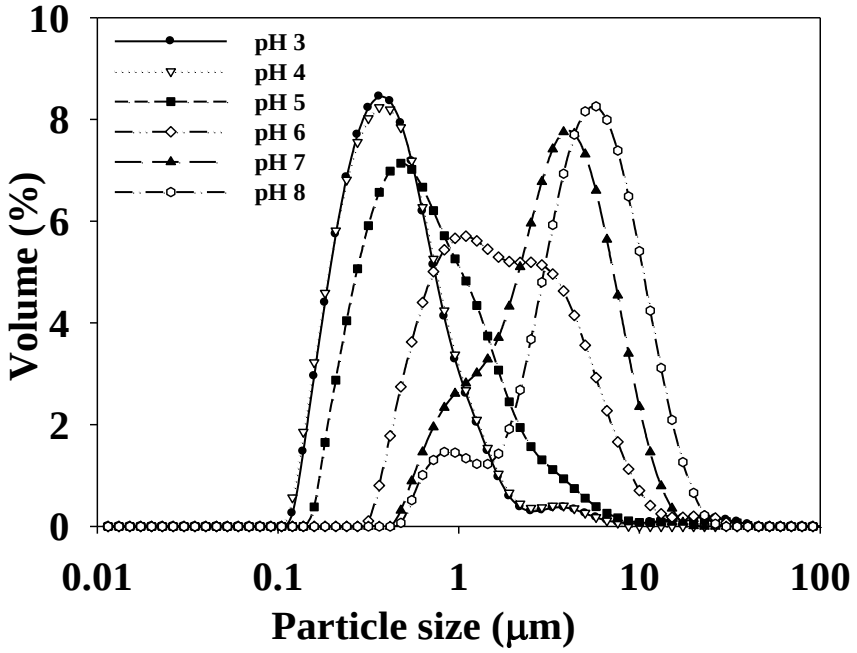

Figure 5.6: pH-dependent droplet size distributions of freshly-prepared flaxseed O/W emulsions containing mixed dSWP and SSPS at 50 °C after 24 h. Representative distributions are shown (n=3).

The pH-dependent ζ-potential pattern indicated at lower pH, steric stabilization by SSPS or the mixed dSWP and SSPS was dominant [209]. At pH 6-8, both the SSPS and dSWP-SSPS stabilized emulsions exhibited more negatively-charged droplets than at lower pH, which was likely indicative of a weaker dSWP-SSPS complex and/or reduced interfacial deposition due to the higher electrostatic repulsion between neighbouring dSWP-SSPS complexes [54, 55, 188].

However, the ζ-potential patterns only partly explained the observed changes in emulsion stability, particularly in light of the fact that the SSPS and mixed dSWP-SSPS emulsions had nearly identical ζ-potential values at all pH values, yet notable differences in sedimentation existed. Such results implied that steric effects dominated emulsion stabilization. For example, at 4 °C, at pH 3, though harbouring identical ζ-potential values (-10 mV), the SSPS-stabilized emulsion demonstrated visible phase separation after 7 days compared to the mixed dSWP-SSPS emulsion which stayed visually homogeneous 21 days (3 times longer). Ganzevles et al. (2008) reported that neutral protein-polysaccharide complexes favoured the formation of dense, thick

and compact interfacial layers with a higher interfacial shear modulus than with only protein [188]. In our case, at low pH the dSWP + SSPS complexes had less negative surface charges compared to pH 6-8 implying greater accumulation of such complexes at the interface at low pH.

5.3.3 Emulsion morphology

There were clear composition, temperature and pH-dependent differences in emulsion droplet size and aggregation behaviour. In emulsions solely prepared with dSWP, there was significant droplet flocculation (result not shown). Emulsions with only SSPS demonstrated more droplet aggregation at pH 6-8 compared to pH 3-5, which agreed with the visual phase separation results.

At pH 3-8, no significant morphological differences was observed between dSWP-SSPS emulsions stored at 4 or 25 °C (Figure 5.8). At pH 3-5, there was no droplet aggregation presumably due to steric repulsion between the droplets. At pH 6, somewhat larger droplets were present. Slight droplet aggregation was detected at pH 7-8. Changes in morphology became apparent after 7 days at pH 6-8 and after 21 days at pH 3-5.

When stored at 50 °C, the changes in emulsion morphology mirrored the changes in droplet size distribution. Hence, more significant changes were seen at pH 6-8 than at pH 3-5 (Appendix A.1). After 24 h, all emulsions had bulk phase-separated.

5.3.4 Flaxseed oil oxidation

The extent of lipid oxidation was monitored by measuring MDA concentration in the oil phase of 5 wt % flaxseed O/W emulsions and crude flaxseed oil. These emulsions were stabilized with either SSPS, dSWP or the dSWP-SSPS complex. The roles of both pH (3-8) and temperature (4, 25 and 50 °C) were ascertained. For comparison, the oxidative stability of emulsions prepared with no added emulsifier or with polysorbate 20 was also evaluated. MDA is a secondary degradation product in lipid oxidation that results in the development of objectionable off-flavours and odours. It is thus a commonly-used indicator of the latter stages of lipid oxidation.

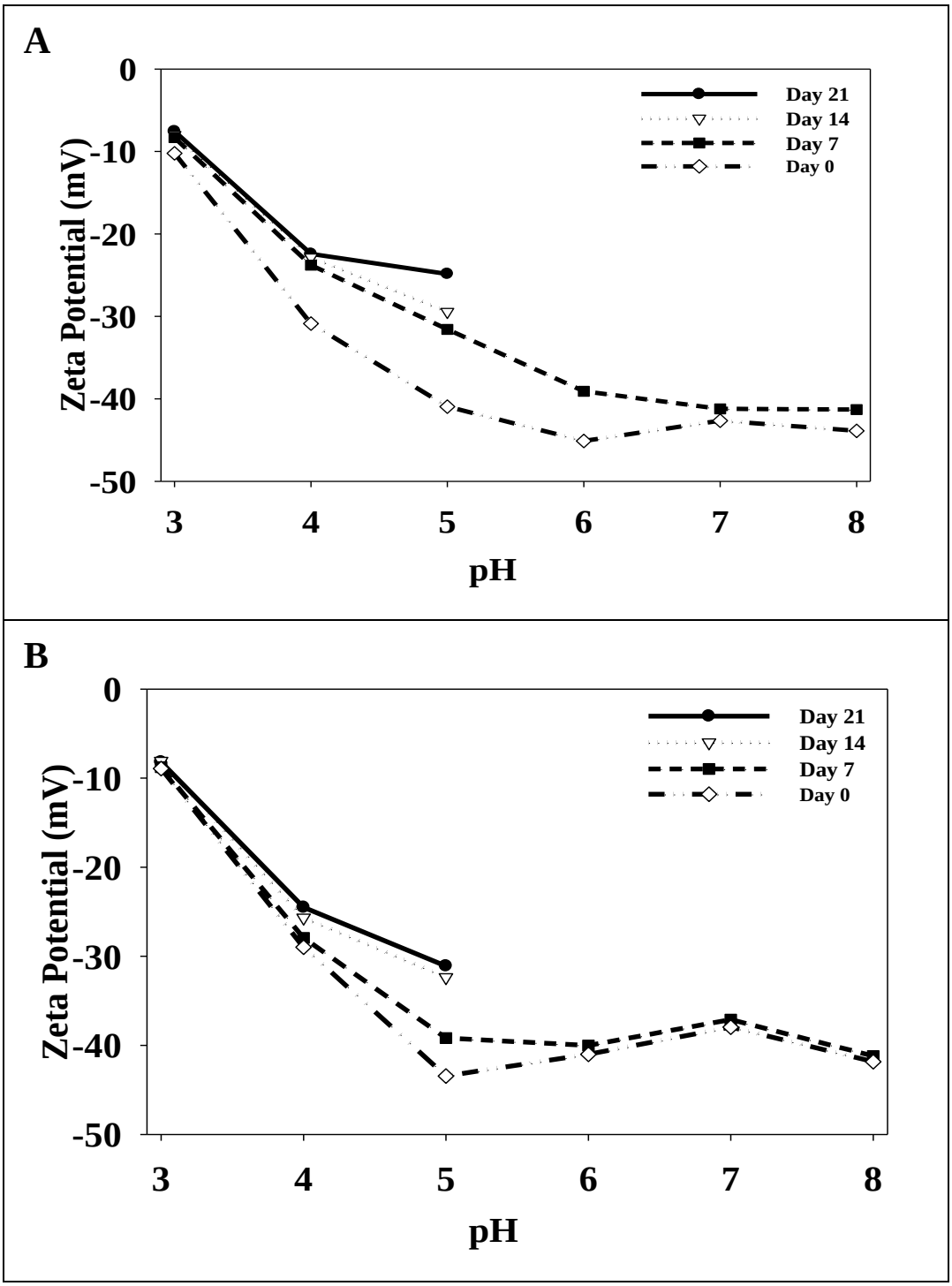

Figure 5.7: Zeta potential of mixed dSWP and SSPS containing flaxseed O/W emulsions as a function of pH, storage time and temperature; (A) 4 °C and (B) 25 °C (n=3). Error bars removed for clarity.

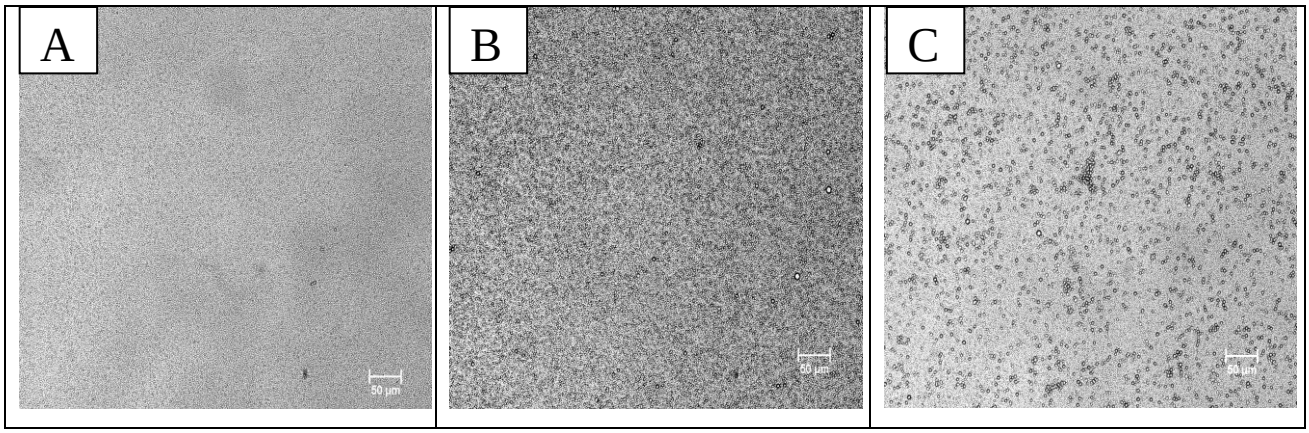

Figure 5.8: Microstructure of emulsions with mixed dSWP and SSPS as a function of pH after 24 h of storage. (A) pH 3-5 (B) pH 6 and (C) pH 7-8. Similar results were obtained at both 4 and 25 °C. Size bar = 50 µm.

Overall, the combination of dSWP and SSPS conferred the best oxidative stability both at pH 3 and 8 by retarding MDA generation more extensively than any other emulsifier (Figure 5.9). For example, compared to the polysorbate 20-stabilized emulsion at 4 °C, the presence of the dSWP-SSPS complex hindered oxidation by a factor of 2 at pH 3 and 43 at pH 8. For the control with no emulsifier, MDA formation was pH-dependent, with values of 1.07 ± 0.3 µM at pH 3 and 0.85 ± 0.1 µM at pH 8 ($p < 0.05$), values 2-3 times higher than in emulsions stabilized with mixed dSWP and SSPS. The observed oxidative stability trend for freshly-prepared emulsions was as follows: dSWP-SSPS (~ 199 %) > SSPS (~ 63 %) > dSWP (~ 29 %) > no emulsifier ($p \leq 0.05$) at 4 °C and pH 3. A different trend was present at pH 8: dSWP-SSPS (~ 133 %) > dSWP (~ 26 %) > no emulsifier ($p \leq 0.05$). This clearly indicated that at lower pH, the presence of stabilizing biopolymer(s) conferred greater oxidative stability than at higher pH.

When comparing the concentration of MDA in crude flaxseed oil vs. the emulsions, only the mixed dSWP and SSPS yielded similar MDA values (bars 2 and 6 in Figure 5.9 A and B) ($p > 0.05$). Use of all other emulsifiers resulted in higher MDA values ($p \leq 0.05$), signifying that they there were not as effective as the combined biopolymers in suppressing the initial oxidation of the flaxseed oil.

At pH 3, the nearly neutral dSWP-SSPS complexes that were accumulated at the interface more effectively suppressed the interaction of the aqueous phase pro-oxidant and dispersed flaxseed

oil compared to pH 8. This was conceptually similar to the results in Chapter 4 where it was shown that the combination of dSWP and SSPS was more effective than SSPS or dSWP alone in kinetically stabilizing dilute O/W emulsions. Hence, generating an emulsion more stable against flocculation and coalescence conferred a greater resistance to lipid oxidation.

Additionally, both SSPS and dSWP have been shown to have the inherent ability to retard lipid oxidation. SSPS possesses significant radical scavenging ability given the presence of galacturonic acid [102, 105, 106]. It has also been reported that the protein moiety of SSPS can inhibit lipid oxidation by preventing the formation of secondary lipid oxidation products, though this is pH-dependent [102, 105, 106]. As well, the presence of specific solvent-accessible amino acids residues (namely Cys, Met, Trp, Tyr, Phe and His) in both dSWP proteins (KTI and lectin) can reduce lipid oxidation via rapid free radical scavenging, inactivation of reactive oxygen species and/or reduction of hydroperoxides [110, 210]. Therefore, beyond their emulsion stabilization capacity shown in Chapter 4, complexing dSWP and SSPS also resulted in a synergistic effect where the antioxidant ability of the protein and polysaccharide were combined.

Storage temperature also influenced the oxidative stability profile of dSWP-SSPS stabilized emulsions, though similar trends were observed at both 4 and 25 °C (Figures 5.10A and B). In general, an increase in pH and storage time resulted in an increase in MDA. The greatest MDA content was seen at pH 8 and longer storage times whereas the smallest MDA concentration in the fresh emulsions at pH 3.

At 50 °C, MDA content increased from 0.60 ± 0.02 µM (after 1 h) to 2.30 ± 0.1 µM (after 24 h) at pH 8 ($p < 0.05$), and from 0.4 ± 0.01 µM (after 1 h) to 0.9 ± 0.03 µM (after 24 h) at pH 3 ($p < 0.05$) (Appendix A.2). Strikingly, MDA values rose by 95 % at pH 3 and 200 % at pH 8 over 24 h vis-à-vis the values at 4 °C. These results clearly showed that storage at higher temperatures accelerated lipid oxidation regardless of pH.

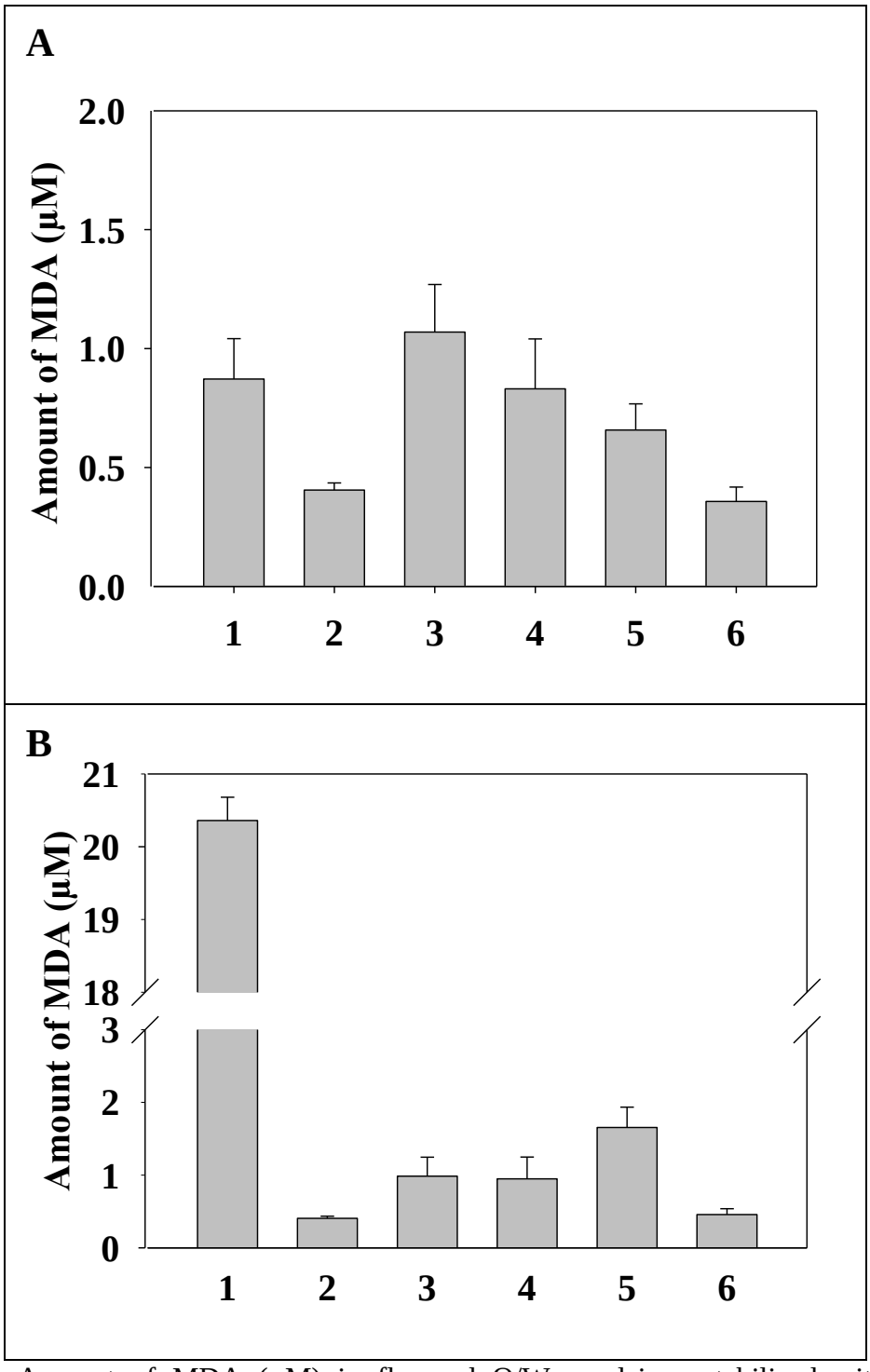

Figure 5.9: Amount of MDA (µM) in flaxseed O/W emulsions stabilized with either no emulsifier, SSPS alone or mixed dSWP and SSPS at 4 °C. (A) pH 3; (B) pH 8. Profiles: (1) Polysorbate 20 emulsion; (2) Crude flaxseed oil; (3) flaxseed O/W emulsion (no emulsifier); (4) dSWP-only emulsion; (5) SSPS-only emulsion; (6) emulsion with mixed SSPS and dSWP (ratio=1.5). Note different y-axis scales in (A) and (B) (n=3).

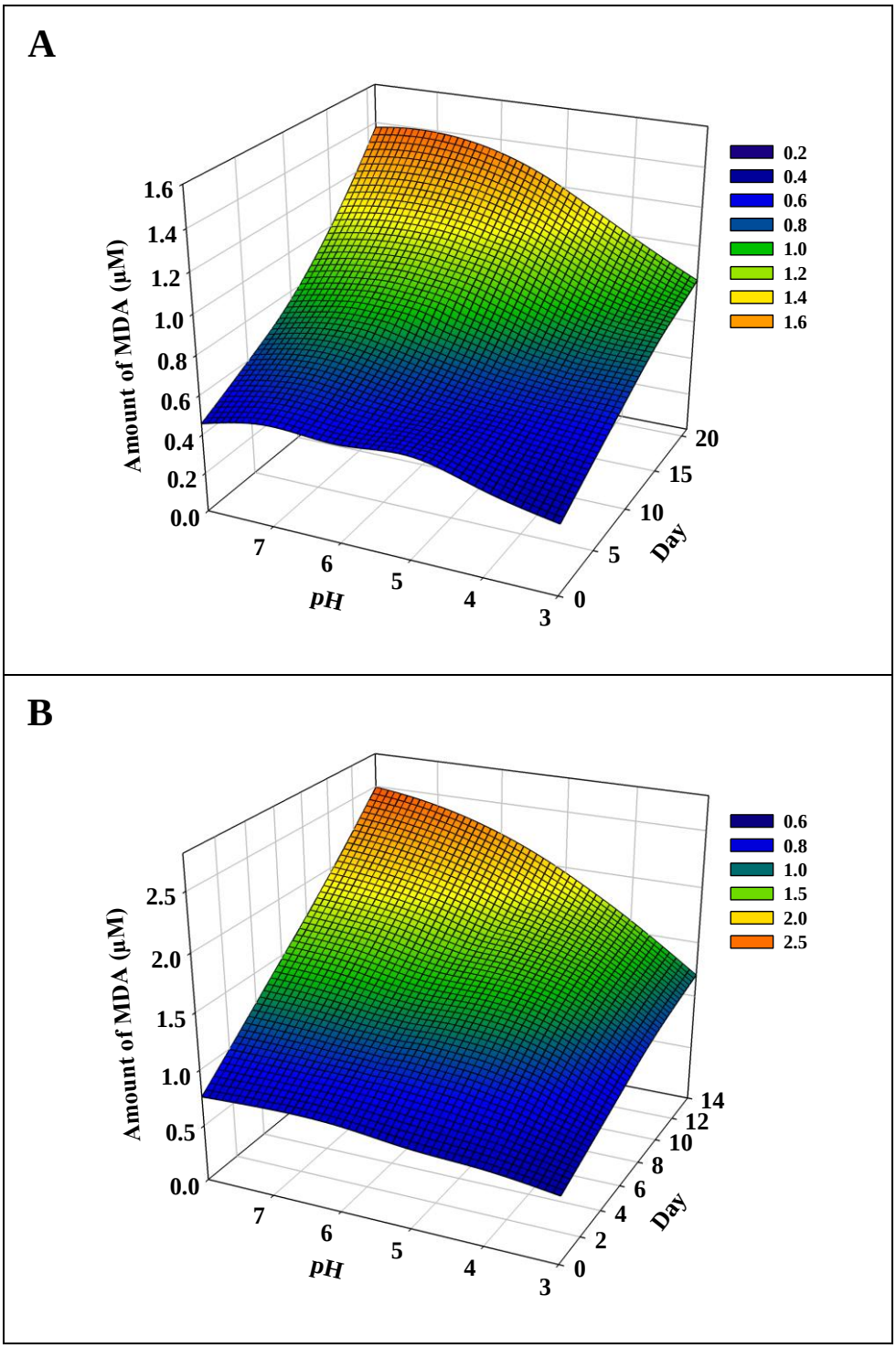

Figure 5.10: Amount of MDA (µM) in flaxseed O/W emulsions containing mixed dSWP and SSPS as a function of pH and storage time at (A) 4 °C and (B) 25 °C (n=3). Note different z-axis scales in (A) and (B) (n=3). Legends corresponds to amount of MDA (µM).

The dSWP and SSPS, being larger-sized biopolymers, formed better interfacial barriers than the polysorbate 20 thus hindering interactions between the emulsified flaxseed oil and pro-oxidants in the aqueous phase more effectively [83, 211]. As well, regardless of pH, SSPS suppressed oxidation more effectively than polysorbate 20, possibly due to SSPS' inherent radical scavenging ability [102, 105, 106], larger size, and efficient barrier-forming ability, which likely prevented the diffusion of oxidation initiators into the oil droplets. Furthermore, the polysorbate 20 may have been a possible source of hydroperoxides itself, thereby biasing the MDA results, particularly at pH 8 [211].

A possible concern was the role of the somewhat different starting average droplet sizes and distributions on susceptibility to oxidation. As previously mentioned, pro-oxidants have a tendency to be polar and may adsorb and accumulate at the oil-water interface [83, 211]. It was surmised that as smaller droplet sizes have a higher surface-to-volume ratio, this may have resulted in the emulsified flaxseed oil being more 'accessible' to oxidation [83]. However, this was not the case as no relationship was found between $D_{4,3}$ value and MDA content in any emulsion, i.e., the level of oxidation did not increase with a decrease in droplet size. Both Roozen et al. [128] as well as Min et al. [212] reported a similar lack of relationship in various O/W emulsions.

5.4 Conclusion

The physical and oxidative stability of flaxseed oil-based emulsions were influenced by the presence of dSWP, SSPS or dSWP-SSPS complexes at the oil-water interface, with the latter offering the greatest improvements. During emulsion formation, at pH 3-5, the dSWP-SSPS complexes at the interface formed a mixed layer whose low negative charge implied that steric repulsion dominated emulsion stabilization. Conversely, at pH 6-8 the more negatively-charged dSWP-SSPS complexes prevented the effective formation of interfacial layers, which reduced emulsion stability thereby promoting oxidation compared to the corresponding pH 3-4 emulsions. A similar effect was observed at all measurement temperature (4, 25 and 50 °C). The conditions most effectively retarding oil oxidation were obtained at lower pH 3-4 and lower storage temperature (4 °C) in the presence of the mixed protein-polysaccharide system. Other than the likely relationship between kinetic stability and oxidative stability, it is possible that

both soy biopolymers aided in radical scavenging and possibly the inactivation of active oxygen species. From a practical standpoint, emulsion stabilization with mixed dSWP and SSPS offers an attractive avenue for the stabilization of polyunsaturated oils over commonly-used food based surfactants such as polysorbate 20 as well as the utilization of soy processing by-products in commercial food and beverage products.

Chapter 6: Overall conclusion and prospects

There were two key deliverables from this thesis:

i) Mixtures of proteins and polysaccharides extracted from soymilk processing by-products were used to prepare and kinetically stabilize dilute O/W food emulsions, and;

ii) Soy protein-polysaccharide complexation and use thereof to coat dispersed emulsified flaxseed oil effectively suppressed oil oxidation compared to a commonly-used emulsifier.

The long-term emulsion stability achieved by combining soy-based proteins and polysaccharides, particularly at low pH, may thus be used as a stepping stone to the use of soy processing by-products in value-added beverage-type processed foods.

The key beneficiaries of this research will be the segments of the food industry where there is a need to disperse oil in water-based foods, such as the sports nutrition industry, which is valued at $21B in the US [9]. Many sports beverages are acidic. Often, when trying to add proteins to such beverages, they phase-separate as the proteins precipitate. The combination of soy proteins and polysaccharides can be used in such products as the polysaccharides protect the proteins from undesired precipitation.

Ultimately, this research has resulted in a novel method of utilizing soy industrial by-products to emulsify and stabilize polyunsaturated oils in food emulsions. Such an approach may be used by food processors looking to incorporate healthy oils into acidic beverages and potentially other commercial food products. For example, if a food company is interested in developing a soy-based beverage that contains ‘healthy’ (e.g., Omega-3) oils, then the approach developed in this research project should be considered. The results of this research could also be used in acidic beverages that do not contain any oil (e.g., shelf stable soy-based shakes) as the SSPS also prevents phase separation of dSWP dispersions under acidic conditions.

Chapter 7: Future Studies

Potential areas of future work include the study of:

i) Protein-polysaccharide interfacial adsorption and packing from a more fundamental perspective, for example by measuring the dilatational rheology and interfacial shear rheology of SSPS:dSWP mixtures as a function of pH. Such rheological studies could provide further insight on the sensitivity of protein-polysaccharide interactions and structural cohesion at the oil-water interface.

ii) The destabilization profile of enzyme-digested SSPS and dSWP-stabilized emulsions to help understand the extent of the steric stabilization effect that SSPS offers, in particular at low pH where the surface charge is almost zero.

iii) Other polysaccharides for their synergistic behaviour with dSWP, namely non-surface active polysaccharides such as xanthan gum, carrageenan, etc. and neutral polysaccharides such as guar gum. Such studies would highlight how effective the use of dSWP as a functional food ingredient may be.

iv) The antioxidant mechanisms of dSWP and SSPS as a function of different environmental conditions (pH, temperature, ionic strength, etc.).

v) The stability and breakdown of dSWP-SSPS emulsions under gastric conditions, with an eye to commercially-relevant applications.

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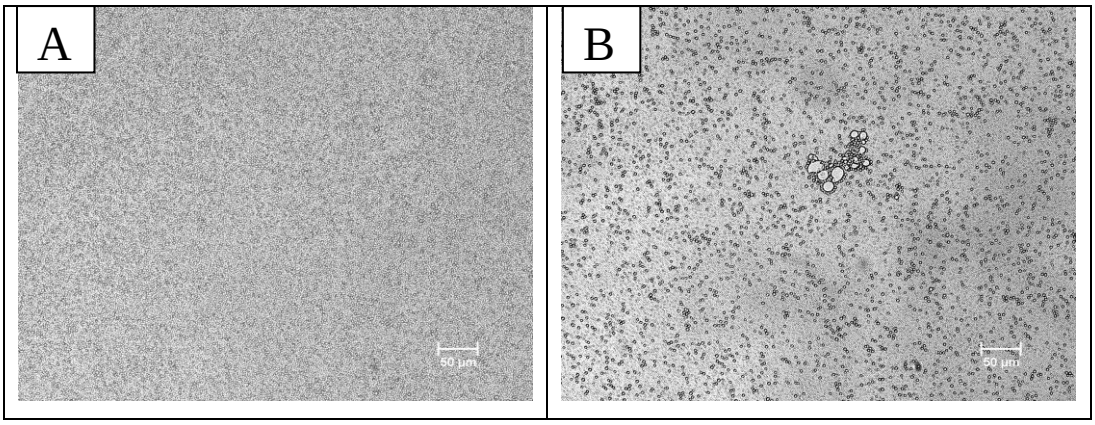
Appendix A

A.1 Emulsion morphology at 50 °C

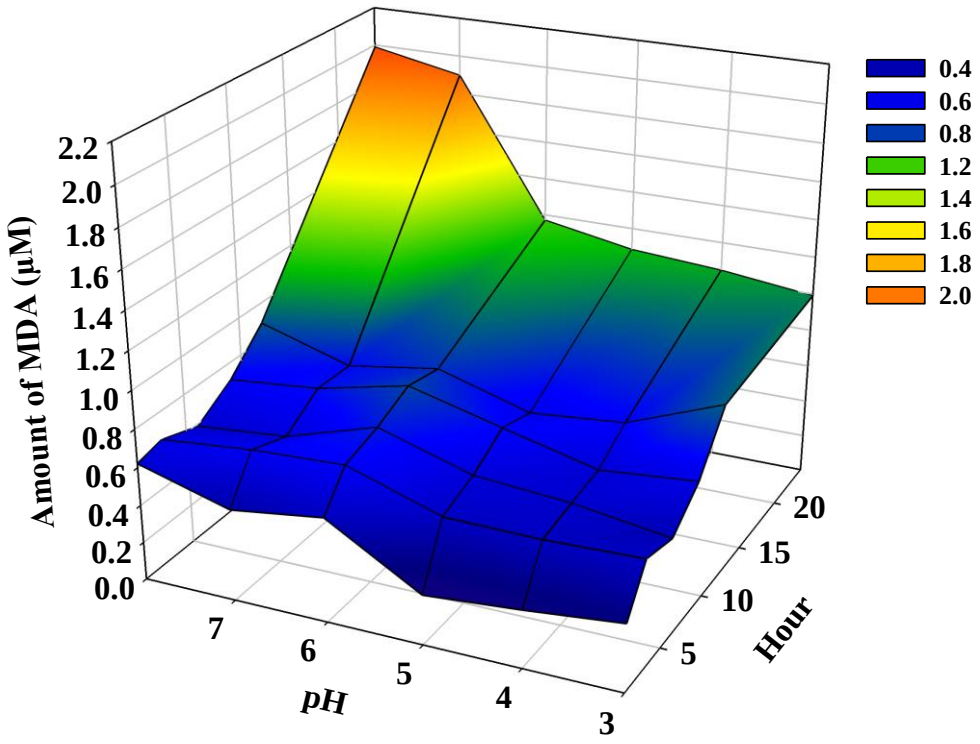
Flaxseed oil emulsions stabilized with mixed dSWP and SSPS stored at 50 °C demonstrated significant changes in morphology as a function of pH. The observed changes were more prominent at pH 6-8 than at pH 3-5.



Appendix A.1: pH-dependent microstructure of 1-day old flaxseed O/W emulsions containing mixed dSWP and SSPS at 50 °C. (A) pH 3-5 and (B) pH 6-8. Size bar = 50 µm.

A.2 Emulsified flaxseed oil oxidation at 50 °C

Emulsion storage at high temperature (50 °C) accelerated MDA production in emulsions stabilized with dSWP and SSPS at pH 3-8. Results clearly showed that accumulation of MDA was greater at higher than lower pH.



Appendix A.2: Amount of MDA (µM) in flaxseed O/W emulsions containing 1.0 wt% dSWP and 1.5 wt% SSPS at 50 °C as a function of pH and storage time (up to 24 h) (n=3).