FUNCTIONAL PROPERTIES OF PROTEIN AND CHITIN FROM COMMERCIAL CRICKET FLOUR

by

Andrew J. Hirsch

A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Food Science

West Lafayette, Indiana

December 2018

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Owen Jones, Co-Chair

Department of Food Science

Dr. Brad Kim, Co-Chair

Department of Animal Science

Dr. Osvaldo Campanella

Department of Agricultural and Biological Engineering

Approved by:

Dr. Arun Bhunia

Head of the Graduate Program

I dedicate this to my parents, for without them, I would have never made it this far.

ACKNOWLEDGMENTS

I would like to thank my professor, Dr. Owen Jones, for taking a chance on me and allowing me to join his lab. His help and guidance over the past couple of years has really helped me grow as a researcher and person. My fellow lab members for their continued help throughout my time at Purdue. And fellow grad students for their comradery through the journey that is grad school.

I would also like to thank my family. Their continued interest and encouragement really helped me get through the tough times when my experiments weren't working out or I just felt like I just had no motivation. And my friends for always being willing to listen to me complain about my research when things were going bad and always being there to celebrate with me when things were going well.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Author: Hirsch, Andrew, J. MS
Institution: Purdue University
Degree Received: December 2018
Title: Functional Properties of Protein and Chitin from Commercial Cricket Flour
Committee Chair: Dr. Owen Jones and Dr. Brad Kim

The House Cricket (*Acheta domesticus*) is a promising alternative to traditional protein sources, as these insects produce over 12 times the mass of protein for a given mass of food/water when compared to cattle, while also producing lower amounts of greenhouse gases and NH_3 emissions (Kim et al. 2017, Hanboonsong, Jamjanya and Durst 2013, Van Huis 2013). Additionally, previous studies have demonstrated significant emulsification and gelling properties of insect flours, such as from cricket, which has been attributed to the functional properties of the protein (Kim et al. 2017). Ground cricket flours contain significant quantities of both protein and fibrous polysaccharides, particularly chitin. Since chitin particles are also capable of preparing emulsions as a Pickering stabilizer, there remains a question on the relative role of the protein and chitin components in crickets for stabilizing emulsion products. Relative contributions of each component was identified by first isolating the water-soluble protein and water-insoluble chitin fractions from ground cricket flour and then determining their interfacial properties and stability of prepared oil-in-water emulsions. Dynamic interfacial tension measurements indicated significant surface activity of the protein fraction, while there was minimal evidence of significant surface pressure development in the presence of 5-10 μm chitin particles. 10 % (w/w) canola oil-in-water emulsions were prepared with 0.5-2% (w/w) of the water-soluble protein fraction and 5.29% (w/w) canola oil-in-water emulsions were prepared with 0.688% of the chitin fraction. Stability of the emulsions against creaming was between 75% and 90% for emulsions stabilized

by the protein fraction over three weeks of storage and between 93% and 96% for emulsions stabilized by chitin over 24 hours of storage. Significant fractions of precipitate- and oil-layers found in chitin-stabilized dispersions was attributed to the presence of large chitin particles (79 μm volume weighted mean diameter) and inefficient adsorption to droplet interfaces during homogenization, respectively. Volume-weighted mean diameter of emulsified oil droplets remained at 17-24 μm among protein-stabilized (>1.5 wt%) emulsions over three weeks of storage but only 60 μm over 24 hours among chitin-stabilized emulsions. Light micrographs of emulsion droplets showed successful adsorption of chitin fractions to oil droplets in the emulsion layer, verifying their potential as Pickering stabilizers. These findings demonstrated that both water-soluble protein and chitin particles obtained from ground cricket flours are legitimate emulsion stabilizers, yet the chitin fraction is much less effective without a more intensive approach to reduce their particle size.

CHAPTER 1. INTRODUCTION AND OBJECTIVES

1.1 Introduction

Edible insects are a major source of nutrition throughout the world, with over 1900 species of insects being consumed as human food. When compared to traditional livestock, edible insects contain a larger amount of protein per kilogram of body weight. Additionally, the protein of most edible insect species is a complete protein, meaning that they contain all of the essential amino acids. The use of edible insects as a source of ingredients for food products would have beneficial impacts in both sustainability and in the environment. With a projected global population of 9.5 billion by 2050 and increased demand for animal-derived protein, alternate sources of protein are heavily sought after. The use of edible insects as this source of protein would decrease the greenhouse gas emissions of the agricultural industry as well as slow the destruction of undeveloped land. Insects also contain a significant amount of the polysaccharide-based structural material, chitin, which typically ends up within protein-rich ground insect products. The use of edible insect proteins, along with this chitin, is a logical choice for a stabilizer to be used in other food products.

Emulsions are a colloid system where the continuous and dispersed phases are immiscible, meaning they are inherently unstable. The use of a stabilizer to prevent aggregation and creaming increases the products quality. Cricket protein can be used to stabilize emulsions by absorbing onto the interface of the system, creating a barrier, and lowering the interfacial tension. The chitin from crickets can be used as a Pickering stabilizer, where it absorbs onto the interface and creates a barrier to protect the dispersed droplets from flocculation and coalescence. Cricket protein and chitin are readily available materials that are being underutilized in the food industry. They have the capability of being a natural food ingredient that can stabilize food emulsions while

maintaining the clean food label that is demanded by consumers. Current research shows promising results for the emulsifying properties of insect proteins and whole insect powder, but there is a lack of information on the relative contributions of the fractions that make up the insects. Knowledge of stabilizing properties of the fractions, as well as the requirements of purification, is limited and must be further researched to determine usefulness of insect fractions as stabilizers in the food industry. Filling in these gaps, while not necessarily providing enough evidence to justify immediate use in food products, will provide useful information for furthering the research on the effectiveness of insect fractions.

1.2 Objectives

The main objective of this research was to determine the relative contribution of isolated protein and chitin from whole cricket flour to ingredient-related functional attributes with potential utility in emulsified food products. The specific goals of this research were to i) determine the relative surface activity of the isolated protein and chitin, ii) assess if this surface activity translated into being an effective emulsifier, and iii) determine the long-term stability of emulsions made with cricket protein and chitin. It is hypothesized that the cricket protein will significantly increase the surface pressure of a model heptane-water interface, while both cricket protein and chitin will show positive emulsion stabilizing abilities.

.

CHAPTER 2. BACKGROUND

2.1 Edible Insects

2.1.1 Insects as an Alternative Protein-Rich Food Ingredient

Much of the world's population consumes insects as part of their normal diet. Especially in developing countries, approximately 1900 species of insects are consumed as human food (Van Huis 2013). This practice is not due to lack of other suitable food sources, but to the availability of insects and their exceptional nutritive properties. In developed countries, it is the visual features of the insects that keep most people from including insects in their diets, so called "yuck factor" (Megido et al. 2016). But if this fear were conquered it would be beneficial. Most insects commonly consumed as part of a diet are shown to contain all of the essential amino acids, specifically lysine, threonine, and methionine, the common limiting amino acids (Van Huis 2013). Additionally, they contain favorable proportions of saturated to unsaturated fats, on a level comparable with poultry and fish (DeFoliart 1991). Furthermore, it is estimated that insects commonly contain a higher amount of the micronutrients iron, zinc, and calcium when compared to the rest of the diet of developing countries (Christensen et al. 2006, Oliveira et al. 1976).

In addition to the increased population mentioned previously, the global demand for animal-derived protein is expected to double by 2050 due to the increased income that much of the world is experiencing (Henchion et al. 2017). As incomes rise, individuals replace lower quality foods with more expensive sources of nutrition, such as meat, eggs, and dairy products (Drewnowski and Popkin 1997). A rising demand for animal-proteins and a slowing trend in productivity points towards an estimated increase of 30-50% in the price of meat (Van Huis 2013). Moreover, this increase in demand raises concerns for sustainability as agricultural production, specifically the raising of livestock, is one of the largest contributors to greenhouse gas emissions

in the world. Beef production has the highest single environmental impact with 14.8 kg in CO_2 equivalents produced for every 1 kg of beef (Van Huis 2013). Furthermore, the need for increased production of animal protein means there will be an increased production for animal feed, resulting in overutilized soil and expansion into current natural lands (Henchion et al. 2017). These natural lands are currently combatting the massive CO_2 emissions caused by livestock production. Converting these lands to fields for feed production would have negative ecological and environmental impacts (van Zanten et al. 2016).

As a response to this impending future where there is a shortage of animal protein, increased prices for meat, and a rapidly deteriorating environment, commercially reared edible insects are starting to be considered as a partial dietary substitute for traditional livestock meat. Besides many species being complete proteins, edible insects offer many advantages over traditional livestock. Insects produce lower amounts of both greenhouse gases and ammonia per kilogram compared to cattle, pigs, and chickens (Van Huis 2013). This will help alleviate some of the damage done by the greenhouse effect by lowering overall input. Edible insects also require less total space for production. They require less space for feed as crickets have a feed to liveweight ratio of 1.7, while cattle's ratio is 10. But when this ratio is changed to feed to edible weight, the ratios change to 2.1 and 25, respectively (Van Huis 2013). Due to the overall smaller size of the insects compared to traditional livestock and a smaller amount of feed required, edible insects are able to provide 100 people with animal protein with only 40 m^3 of space (Van Huis 2013).

On major way that insects could be utilized in the food industry is through incorporation into products as a food ingredient. Current research has shown that insect proteins and their hydrolysates have the potential of usefulness, especially in the area of emulsion stability. Tropical banded cricket protein hydrolysates have been proven to have some effectiveness in emulsions

stability (Hall et al. 2017). Analysis of whole giant African cricket has also given evidence of the usefulness of insect protein as an emulsifier, but it did have low stability during storage (Adebowale, Adebowale and Oguntokun 2005). Additionally, moth larva and silkworm have shown to have effective emulsification properties and stability (Omotoso 2015). These studies have all shown a potential usefulness for insect proteins as emulsifiers, but there is very little fundamental evidence of how and why these insect proteins will provide stable emulsions, therefore the functionality of the proteins must be further investigated to determine the true potential of insects and insect proteins as food ingredients.

2.1.2 Insect Biology and Major Components

Much like traditional livestock differ in composition when compared across species, so do edible insects. Their major components, as well as their fractions of protein, fat, and carbohydrates, differ wildly. Edible insects can be broken down into four groups, differentiated by their order: Lepidoptera (butterflies, moths, caterpillars), Coleoptera (beetles), Orthoptera (grasshoppers, locusts, crickets), and Isoptera (termites). There are general trends that exist for percentages of fractions within orders, but exceptions exist. The order Lepidoptera is characterized by containing a larger amount of protein than other orders, with percentages well over 50% (Amadi and Kiin-Kabari 2016). Coleoptera is characterized by having a larger moisture content (Amadi and Kiin-Kabari 2016). While Orthoptera and Isoptera have a relatively balanced distribution across all of their fractions. Specifically, when looking at the house cricket (*Acheta domesticus)* of the order Orthoptera, values for its fractions show a large amount of crude protein (62%) which qualifies as a complete protein as it contains all of the essential amino acids. It also contains a balanced caparison for fats (7.5%), ash (4.6%), crude fiber (7.0), and water (5.2%) (Nakagaki, Sunde and DeFoliart 1987), These values differ greatly when compared to the results for the order Orthoptera

in the previous study. The house cricket also contains high levels of zinc and iron, both deficient minerals in developing countries (Christensen et al. 2006).

How these fractions are utilized in the insect also determines its effectiveness as an edible insect. Much of the crude fiber of the house cricket is in the form of chitin. This makes up the cuticle of the insect. Within this cuticle is also unavailable protein. These proteins are utilized by the insect to harden the cuticle by linking the chitin fibers together (Jonas-Levi and Martinez 2017). The rest of the protein is available for digestion by the human body. The lipid profile of *Acheta domesticus* is most made up mostly of palmitic acid, stearic acid, oleic acid, and linoleic acid. A large majority of the lipids are unsaturated fats with only oleic and linoleic acids comprising over 60% of all lipids for every stage of life for both male and female house crickets (Grapes, Whiting and Dinan 1989).

2.2 Insect Fractions as Emulsion Stabilizer

2.2.1 Emulsion Theory

An emulsion is a mixture of two immiscible liquids, usually oil and water, with one of the liquids being dispersed as small droplets within the other (McClements 2015). The emulsion is defined by which liquid is the dispersed phase and which is the continuous phase. An emulsion with dispersed oil droplets in a water continuous phase would be an oil-in-water emulsion, and dispersed water droplets in oil would be a water-in-oil emulsion. Due to the fact that emulsions consist of immiscible liquids, they are inherently unstable. This causes a constant battle between emulsifiers and the laws of thermodynamics as an emulsion always wants to destabilize to minimize interfacial area. The most common mechanisms by which an emulsion destabilizes are creaming, flocculation, and coalescence. Creaming is a gravitation separation defined by the upward movement of droplets due to the difference in density of oil and water. Flocculation and

coalescence are both forms of droplet aggregation but differ in that during flocculation the aggregated droplets retain their integrity while during coalescence the droplets merge to form a larger droplet.

To stabilize an emulsion, small-molecular surfactants and commonly used to reduce the interfacial tension between the dispersed and continuous phases. Additionally, particulate matter with intermediate hydrophobicity between the phases can also be used (Pickering 1907). The particles absorb onto the interface and create a barrier that increases stability to coalescence. In the case of both approaches, the continuous phase of the emulsion is usually determined by the relative wettability of the stabilizer (Finkle, Draper and Hildebrand 1923). A particle stabilizer typically does not have any effect on the surface pressure of the interface, where a traditional surfactant reduces the interfacial tension by an appreciable amount (McClements 2015).

2.2.2 Emulsions in Foods

Emulsions are a fundamental component of foods that are consumed by billions of people across the world. Many natural and processed food are or contain emulsions. Milk, salad dressings, mayonnaise, and butter are just a few examples of everyday products that many people do not realize are emulsions. The appeal of emulsions for food products lies in their versatility. Emulsions allow you to alter the texture of a product by controlling the oil or water in a product to modify characteristics, such as creaminess or mouthfeel (Benjamins et al. 2009). They also can be used as delivery systems. They can transport flavors, colors, nutraceuticals, vitamins, and many other useful proponents (McClements 2015). The goal of emulsion based delivery systems is to encapsulate, protect, and release these ingredients to serve a purpose, such as improving the product or targeted delivery (McClements 2015).

Many products that naturally contain both water and oils undergo homogenization to maintain a more uniform appearance as well as to increase the stability of the product. For example, milk traditionally undergoes homogenization to minimize the size of the oil droplets to create a uniform taste of the product, as well as to extend the shelf life (Yamada et al. 1997, Pereda et al. 2007). Without this homogenization, the oil droplets would likely aggregate and rise to the surface of the product, and this physical destabilization defines the predicted shelf life of pasteurized milk rather than the microbiological safety.

One common feature that is found in all emulsion food products is the inclusion of some form of stabilizer, whether it is natural or added. In foods these stabilizers are generally surfactants, which stabilize by lowering the interfacial tension, and thickening agents, which stabilize through increased viscosity (McClements 2004, Dickinson 2009). Hydrocolloids are traditionally used as thickening agents in food products but must have higher concentrations as low concentrations tend to destabilize the emulsions. Hydrocolloids are used in products such as chocolate milk, carbonated soft drinks, and salad dressings (Dickinson 2009). They increase the viscosity of the system and control the kinetics of phase separation by the creation of a gel-like network of interconnected emulsion droplets (Dickinson 2009). Surfactants found stabilizing food emulsions range from fats to proteins, such as lecithin and sodium caseinate. Sauces and cream liqueurs are examples of products stabilized by surfactants (Kralova and Sjöblom 2009). These emulsions are generally stabilized by reducing the interfacial tension between the dispersed and continuous phases at the interface (Kralova and Sjöblom 2009).

2.2.3 Proteins' Use as an Emulsifier

Proteins used as emulsifiers stabilize by adsorbing to a surface, lowering the interfacial tension, and forming a protective coat (McClements 2015). The proteins diffuse through the

continuous phase and adsorb to the interface of the immiscible fluids. By adsorbing, the protein lowers the interfacial tension at the interface and improves stabilization creating a protective membrane around the oil droplets that hinders interaction between separate droplets. The greater the binding of protein to the interface, the greater the overall stabilization of the emulsion (McClements 2004).

Protein concentration is extremely important when it comes to emulsions stabilization because it affects so many different components. If there is insufficient protein, it increases the likelihood that there will be dispersed droplets surrounded by membranes that contain gaps (McClements 2004). The gaps cause the droplet to be susceptible to coalescence if it comes into contact with a gap of another droplet. Moreover, it could also limit the effectiveness of homogenization by limiting the ability of oil droplets to decrease to a size necessary for emulsions stability (Pandolfe 1995). Regardless of the capabilities of the homogenizer, if the protein concentration is too low, not enough protein will adsorb onto the interface and effective membranes will not develop (McClements 2004). But when a protein is in excess, the droplet size then becomes more dependent on the capabilities of the homogenizer rather than the concentration of the surfactant (Pandolfe 1995). Some proteins are capable of forming multiple layers when in excess and thus provide a stronger barrier to prevent aggregation of the dispersed droplets.

Influences of aqueous phase composition also have an effect on stabilized interfaces. Proteins used as surfactants are usually thin and electrically charged, which aids in preventing flocculation due to electrostatic repulsion (McClements 2004).This, however, also makes them very sensitive to changes in pH and ionic strength. As the pH of the system nears the isoelectric point of the protein, the emulsions tend to flocculate. High ionic strengths also increase flocculation due to lowering the electrostatic repulsion between the droplets which no longer

allows them to resist attractive interactions, such as van der Waals or hydrophobic (McClements 2004).

2.2.4 Chitins' Use as an Emulsifier

Aside from traditional surfactants, another way to keep an emulsion stable is through particle stabilization. Commonly, this form of stability is referred to as Pickering stabilization, named after a pioneer of particle stabilization, S.U. Pickering, for his work published in 1907 (Pickering 1907). The basic principle of Pickering stabilization is that a solid non-dissolved particle that demonstrates some surface activity is aiding in the stabilization by absorbing onto the interface of the immiscible liquids and acting as a barrier to prevent coalescence of the dispersed droplets (Pickering 1907). The level of protection from destabilization depends on the density of packing and the level of energy required to remove the particles from the interface (Dickinson 2012). The particles themselves can also have an effect of the type of emulsion. Just as traditional surfactants can be considered hydrophilic or hydrophobic, so can particles used for stabilization. They can determine if an emulsion will be oil-in-water or water-in-oil (Binks 2002). Other factors that will affect the efficiency of the barrier properties of the particles are the size of the particles and by which phase the particles are wetted. It is desired for the particles to be wetted by the continuous phase of the emulsion, in which case the particles tend to stay on the outer surface of the dispersed droplets. Wetting by the dispersed phase causes the particles to migrate into the droplets where they are not creating an effective barrier to coalescence (Dickinson 2012, Leal-Calderon and Schmitt 2008). Particle material plays a large role in the effectiveness of stabilization because it greatly effects the contact angle of absorption onto the interface of the droplets. The energy of attachment, which dictates the level of effectiveness by which a material can create a barrier, is related to both contact angle as well as the tension of the interface. If the contact angle

is altered away from the ideal 90°, the level of attachment decreases which allows for easier destabilization of the absorbed barrier (Binks 2002).

Chitin and chitosan, both in bulk and in the form of nanoparticles/nanocrystals, exhibit properties that give reason to believe they maintain some functionality in emulsion stability. Bulk chitin exhibits both hydrophobic and hydrophilic moieties which gives indication that they would be able to absorb at the oil-water interface found in emulsions (Magdassi and Neiroukh 1990). To increase the functionality of chitin, it is generally converted to its acid soluble derivative – chitosan. This is achieved by a deacetylation reaction (Zhang et al. 2015). Chitin nanocrystals on the other hand contain positive charges at their surface due to the protonation of the amino groups that occurs during the acid-hydrolysis that takes place to process them (Tzoumaki et al. 2011). This increases their interaction with the water phase of the emulsion due to the polarity of water. Also, the non-spherical shape of chitin nanocrystals have shown an ability to stabilize emulsions that were unable to be stabilized by spherical particles due to the differing aspect ratio (Tzoumaki et al. 2011).
Bulk chitin has shown the ability to stabilize 10% paraffin oil emulsions, with exceptional stability of the emulsions as low as 1% chitin showing only ~1% oil separation over the course of 30 days (Magdassi and Neiroukh 1990). Chitin nanocrystals exhibited lower stability showing small signs of destabilization at 1% chitin nanocrystals in an emulsion prepared with 10% corn oil (Tzoumaki et al. 2011). Chitin has been shown to exhibit evidence of oil sorption properties. It is benefitted by the addition of acid and follows a pseudo-secord-order and intra-particle diffusion kinetic model (Elanchezhiyan, Sivasurian and Meenakshi 2014).

2.3 Objectives and Hypothesis

The overall objective of this research is to determine the relative contributions of cricket protein and chitin extracted from whole cricket powder to ingredient-related functional attributes

with potential utility in food systems, such as emulsion stability. It is hypothesized that the protein will show evidence of altering surface pressure at the water-oil interface, as well as provide substantial stabilization to an emulsion in storage. The chitin is expected to show limited effect on surface pressure of the water-oil interface but provide evidence of some ability to bind onto the interface and provide some stabilization to the emulsion by creating a barrier at the interface. The experimental design will test the ability of these materials to alter surface pressure, giving evidence of absorption onto the interface, as well as monitor their ability to stabilize emulsions during storage, giving a direct insight into their utility as an emulsifier. If the hypothesis is correct, further investigation into the emulsification properties of insect powders could soon lead into their utilization in the food industry.

CHAPTER 3. ANALYTICAL METHODS

3.1 Interfacial Tension and Surface Pressure

Droplet Shape Analysis (DSA) is useful technique when the effects of materials and substances on the interface are being investigated. DSA is most commonly used for measurements of surface tension, interfacial tension, and contact angles (Chen et al. 1998). It provides an advantage over other techniques as it can be used for both liquid-gas and liquid-liquid interfacial tension measurements. Additionally, DSA requires a small sample size when compared to other techniques. Droplet shape analysis uses the measurable shape of the drop and the known force of gravity to calculate the surface tension (Chen et al. 1998).

The different ways to measure using DSA is with a pendant drop and a sessile drop. Sessile drop analysis is performed by placing a drop of a liquid onto a surface. The three-phase contact angle formed by the droplet phase, surrounding phase, and surface is measured and used to determine the surface energies of the surface and/or droplet phase (Murphy 2017). Pendant drop analysis is used to determine the interfacial tension between a droplet and the continuous phase.

Components of pendant drop analyzers include a syringe with a known end diameter, a camera, a light source, one liquid for liquid-gas measurements and two liquids for liquid-liquid measurements (Krüss 1997). The light source is used to illuminate the sample and contrast the droplet. The camera sends the acquired images to an attached computer where necessary calculations are made. The syringe must have a known end diameter because this allows the computer to relate pixels to real-world distances (Krüss 1997). It is important to know the density of both of the continuous and droplet phases, whether they be liquid or gas. If the droplet phase is denser than the surrounding phase, a conventional needle is used (Krüss 1997). If the droplet phase is less dense than the surrounding phase, then a J-hook needle is used, which forces the use of an

inverted pendant drop test (Krüss 1997). Additionally, the difference in density is considered when the measurements are made. This difference, acting as gravity or buoyancy, causes the drops to elongate. Interfacial tension acts on the droplet to keep the shape spherical, therefore minimizing the surface area of the droplet. If the difference in density and the dimensions of the droplet are known, it is possible to determine the surface tension of the droplet by use of the Young-Laplace equation (Berry et al. 2015):

$$\Delta P \equiv \gamma\left(\frac{1}{R_1} + \frac{1}{R_2}\right) \quad (2.1)$$

Where ΔP is the Laplace pressure, γ is the interfacial tension and R_1 and R_2 are the principle radii of curvature of the droplet (Noordmans and Busscher 1991). The Laplace pressure is defined by the difference in pressure between the inside and outside of the droplet which is caused by the presence of a curved interface (Murphy 2017). The Laplace pressure can also be defined as (Berry et al. 2015):

$$\Delta P \equiv \Delta P_0 - \Delta pgz \quad (2.2)$$

Where ΔP_0 is the reference pressure at the drop apex, Δp is the density difference between the droplet phase and the continuous phase, g is the acceleration due to gravity, and z is the vertical distance from the reference point. Due to the axisymmetric nature of the droplet, the Laplace Young equation can use cylindrical coordinates r (horizontal) and z (vertical, the tangent angle φ, and the arc length from the droplet apex s. The observed droplet shape profile can be used to determine the Bond number B_0, the ratio of the gravitational and interfacial forces (Berry et al. 2015):

$$B^0 \equiv \frac{\Delta p g R_0^2}{\gamma} \quad (2.3)$$

Where R_0 is the radius at the drop apex. Droplet area (A_d) and volume (V_d) can also be determined by the following equations (Berry et al. 2015, Murphy 2017):

$A_d = 2\pi \int \bar{r} \, d\bar{s}$ (2.4)

$V_d = \pi \int \bar{r}^2 sin\varphi \, d\bar{s}$ (2.5)

3.1.1 Interfacial Tension Measurements

3.1.1.1 Dynamic Interfacial Tension

As a surfactant is introduced to an interface it binds itself and alters the interfacial tension of the system. The measurement and characterization of this binding over time is referred to as dynamic interfacial tension. At initial moments in the measurement, there are little or no surface-active agents present at the interface. Results at such early measurements times should be identical to a system with no surface-active agents present at all. As surface-active agents migrate and adsorb to the interface from the surrounding phase, a significant fraction of the interfacial area is covered, lowering the interfacial tension. Surface Pressure is generally used to define this change in interfacial tension and is calculated by:

$\Pi = \gamma - \gamma_0$ (2.6)

Where γ_0 is the initial interfacial tension. It is important to determine the surface pressure of a system to determine the potential effectiveness of a material as a surfactant.

3.1.1.2 Determination of Protein Saturation at the Interface

Certain destabilization mechanisms are dependent upon the interfacial tension of the emulsified droplets, while homogenization to prepare stable emulsions is assisted by the ability of surfactants to reduce the tension. When a surfactant is introduced to the interface, it adsorbs and lowers the interfacial tension, assisting in the creating of stable emulsions (McClements 2015). But assuming that there is no multi-layer packing occurring, the measure of how much the interfacial tension can be lowered is finite (McClements 2004). This point is referred to as saturation. Once saturation is reached, excess protein in the continuous phase will not contribute

to a further reduction in the interfacial tension. The Langmuir adsorption model is a well-known mathematical description of small molecules binding to an available surface. It operates on a few assumptions: i) the surface of the binding site is homogenous, ii) the surfactant adsorbs into an immobile state, iii) all binding sites are equal, iv) binding sites can bind only one unit of surfactant, and v) there are no interactions between units of surfactant (Masel 1996). The Langmuir equation allows for the prediction of the equilibrium surface pressure at any given concentration when the equilibrium surface pressure of a small number of concentrations is known:

$$\Gamma = \frac{BC_2}{a+C_2} \quad (2.9)$$

Where Γ is the surface pressure, B and a are fitting parameters describing the saturation and binding properties, and C_2 is the concentration of surfactant in molar-basis. When a and B are known this equation relates surface pressure with concentration.

3.2 Measurements of Emulsion Stability

The efficiency of a surfactant or Pickering stabilizer is ultimately determined by the stability it offers to the emulsion, specifically, the ability of the material to keep the dispersed phase from creaming, flocculating, or coalescing. Some examples of techniques to characterize stability of emulsions over time are the creaming index, the emulsifying capacity, the emulsion stability index, and scattering-based determination of the size distribution among the dispersed phase droplets.

Creaming index is a simple method used to determine the level of creaming that takes place in an emulsion over time. As the emulsion destabilizes, the dispersed phase starts to physically separate from the continuous liquid phase of the emulsion due to their difference in density. If the dispersed phase is relatively less dense, it will migrate upwards and float at the top of the emulsion. This is a precursor to coalescence, where the dispersed droplets merge into larger droplets,

eventually progressing towards the full separation of a discrete upper liquid. The creaming index is the measure of the height of the emulsified layer compared to the total height of the emulsified and creamed layer (Yasumatsu et al. 1972).

$$\frac{emulsified\ layer\ height}{total\ height\ of\ emulsified\ layer\ and\ creamed\ layer} \times 100 \quad (2.10)$$

Emulsifying capacity of a water-soluble emulsion, such as cricket protein, is defined as the maximum amount of oil that can be dispersed in an aqueous solution containing a specific amount of emulsifier without the emulsion breaking down or inverting into a water-in-oil emulsion (Sherman 1995). It is measured by titrating oil into a continuously agitated emulsifier solution. The measurement ends when the emulsion destabilizes or inverts (McClements 2007). Emulsifying capacity relates to creaming index, emulsion stability index, and the particle size determination method for emulsion stability by giving an indication of the minimum concentration that can be used to accurately measure destabilization of an emulsion over time.

Emulsion stability index (ESI) is a measure of the change in particle size of an emulsion after storied for a specified length of time (McClements 2007). The procedure includes measuring the mean droplet diameter over time. These measurements are then used to determine the ESI by use of an equation:

$$ESI = \frac{d(0)t}{d(t)-d(0)} \quad (2.11)$$

Where d(0) is the initial mean droplet diameter and d(t) is the mean droplet diameter at time t (McClements 2007). The ESI over time of an emulsion gives numerical evidence of the stability of the emulsion. This method is comparable to the creaming index because both methods show a numerical trend of the stabilization of the sample. It also is comparable to the particle size determination method because the ESI is calculated by determination of mean droplet diameter. Both methods utilize particle sizing to determine stability.

Measuring the size of the dispersed phase droplets over time is another approach to determine efficiency of an emulsifier. Over time, the emulsion will start to destabilize, and the droplet size of the dispersed phase will disproportionate and possibly coalesce. This causes the average size of the droplets to increase proportionally with the rate of emulsion destabilization. A unimodal size distribution of the droplets that increases over time would give reason that the surfactant indicates an unstable emulsion susceptible to flocculation or coalescence. A bimodal size distribution of the droplets is most likely caused by insufficient emulsifier content. In this outcome, only a fraction of the droplets were relatively destabilized. If there is an increasing difference in the peaks of the curves with stabilized smaller droplets, it supports that only the droplets that are more stable had full absorption onto the interface and the destabilized droplets had gaps which allowed aggregation to take place. If the larger droplets are increasing and size and the smaller droplets are decreasing, this would be due to disproportionation.

3.3 Confocal Laser Scanning Microscopy (CLSM)

CLSM is a light microscopy technique that can be used to clearly view a thin optical section of a three-dimensional fluorescent sample, as well as reconstruct the sample to view internal characteristics and properties (Fjne 2005). Light from the fluoresced point passes through an aperture and is detected; light from out of focus planes are blocked. This level of precision is due to the inclusion of an optical pinhole that sits in front of the detector and blocks any light that is not focused. This allows for an image that is a very clear cross-section of the sample (Fjne 2005). Other advantages of confocal microscopy is that it improves resolution and decreases noise, but these come at the cost of longer exposure times and the need for more advance photodetectors due to the reduced intensity that results from the block light (Inoué 2006). The high level of precision and clarity that come with confocal microscopy requires great care to preserve the sample; over-

exposure to light can lead to photobleaching, which reduces fluorescence and optical contract. The level of clarity can also be reduced by motion which can be overcome by use of fixatives or by increasing the viscosity of the suspended phase (Inoué 2006). If a sample contains multiple components, such as lipids and polysaccharides, confocal imaging makes it possible to differentiate between the components by use of fluorescent dyes without compromising the sample.

3.4 Protein Determination Methods

3.4.1 Nitrogen Combustion

Traditionally, Kjeldahl's method proposed in 1883 is used to determine the nitrogen content of a sample (Hansen 1989). This is a go-to method when nitrogen content is sought, usually to ultimately determine crude protein content. But the catalysts commonly used are toxic and offer waste problems that make the method cumbersome and difficult. Alternatively, combustion of the sample can be used to determine nitrogen content. The sample is destroyed in pure oxygen at 950-1000°C which yields products of CO_2, H_2O, O_2, NO_x, and N_2 which are collected in a gas ballast volume and mixed. To achieve accurate results of nitrogen content, it is important to remove all other products. A portion of the mixture is passed through a series of filters which leaves just the helium carrier and N_2 to be measured by a thermal conductivity cell. The H_2O is absorbed by two filters, one containing iron chips and the other magnesium chloride. The CO_2 is removed by a filter containing sodium hydroxide. The NO_x is reduced by a filter with copper turnings and N-catalyst. The results are corrected by a blank value and the instrument is calibrated with nicotinic acid. The N_2 that remains is measured by a thermal conductivity cell. This measurement can then be calculated into percent nitrogen which can be used to determine crude protein content based on ratios of the nitrogen content of protein (Hansen 1989). Commonly 6.25 is used but recent studies

have shown that different species of animals typically require the use of a different ratio to determine true protein content (Jonas-Levi and Martinez 2017, Janssen et al. 2017).

3.4.1.1 Effect of Chitin and other Fractions on Percent Nitrogen Content

Most reported protein contents of edible insects are overestimation due to the common practice of using the percent nitrogen to protein content ratio of 6.25. This ratio is based on the idea that proteins are composed of about 16% nitrogen (Jonas-Levi and Martinez 2017). When used for traditional livestock, it gives a relatively accurate protein content. The fundamental error in the practice of using this ratio for edible insects is that it overlooks the obvious, insects contain chitin. The presence of chitin is important because of the incluision of nitrogen in its makeup. The exoskelative of insects, known as the cuticle, is composed mainly of chitin fibres which contain glucosamines and N-acetylglucosamine, both of which contain nitrogen atoms (Jonas-Levi and Martinez 2017). Additionally, not all proteins appear to be digestible due to the interaction with the cuticle. Some proteins link the chitin fibers and thus disclude themselves from being available to to animals by reacting with quinones in the chitin (Andersen, Hojrup and Roepstorff 1995). This could explain why protein yield in isolation procedures is generally reported in literature as lower than expected. Furthermore, when an insect sample is then combusted to determine percent nitrogen, this nitrogen is recorded and allows for the protein content to be overestimated. New ratios have been determined based on the amino acid profile, chitin content, and calculated non-protein nitrogen (Janssen et al. 2017). These results give a more accurate ratio of 4.76 ± 0.09 for whole insect powders as they contain a larger amount of chitin and non-protein nitrogen. After purification and extraction, this ratio increase to 5.60 ± 0.24 (Janssen et al. 2017).

3.4.2 Protein Determination by Ultra-Violet Absorption Methods

A quick method of determining protein concentration is by measuring the absorbance of the sample at 280nm. The downside of this method is that it is susceptible to interference or false positives. Proteins strongly absorb light at ultra-violet 280nm because of tryptophan and tyrosine residues in the proteins (Chang 2010). This absorption is specifically due to the aromatic rings present in these amino acids (Murphy 2017). The levels of tryptophan and tyrosine are relatively constant within a particular source, this allows the absorption to be used to determine protein content. The accuracy of this measurement though, relies on having a known extinction coefficient for the exact protein that is being measured or being able to accurately measure the coefficient if a clean sample with a known concentration is available. An extinction coefficient is a measure of how strong a substance absorbs light at a particular wavelength. Using an extinction coefficient of a different protein can result in over-or-under-estimation of the protein content.

Another method developed on the basis of absorption is the Bicinchoninic Acid (BCA) Protein Assay. Traditionally, Bovine Serum Albumin (BSA) is used as the protein source to create the standard curve. A pure BSA source is diluted in a serious of serial dilutions and is reacted with the BCA working reagent. After the reaction has had ample time to occur, the absorbance of the solutions is read at 562nm. This creates the standard curve which is used to determine the unknown concentration of a sample. The unknown sample is reacted with the BCA agent in the same fashion as the BSA. The absorbance at 562nm is also ready and compared against the standard curve. The concentration of protein can then be compared to the amount of sample used to determine purity (Krieg et al. 2005).

3.5 Determination of Ash Content by Dry Ashing Method

Determination of ash content is necessary to determine the relative amounts of inorganic matter in a sample. Dry ashing requires the use of a muffle furnace at temperatures between 500°C and 600°C (Marshall 2010). The principles behind the method is the vaporization of water and volatiles and the burning of organic substances. The organic substances are turned into CO_2 and oxides of N_2 upon incineration (Marshall 2010). These gases along with the vaporized substances are vented away from the sample leaving only inorganic materials which is referred to as ash.

CHAPTER 4. MATERIALS AND METHODS

4.1 Materials

Griopro® 100% Cricket Powder was purchased from All Things Bugs LLC. (Midwest City, OK). This is the only source of starting material and was bought in bulk so as to avoid any deviation in results due to different growing conditions or other factors that could change the properties of a single batch. The powder was then dried overnight in an oven to remove any moisture before further steps were taken. Petroleum ether was purchased from Fisher Chemical (Lot 176076). Concentrated HCl was purchased from Sigma-Aldrich (St. Louis, MO) and diluted to the desired concentration. NaOH was also purchased from Sigma-Aldrich (St. Louis, MO) in anhydrous form and was hydrated to desired concentration. The $Na_2CO_3 \cdot H_20$ was purchased from Fischer Scientific (Chicago, IL) and was hydrated to desired concentration. Pure canola oil used for emulsions was cleaned using Florisil purchased from Fischer Scientific (Chicago, Illinois). Ultrapure water ($\sigma \geq 18$ mΩ-cm) used for all solutions was obtained from a water filtration system (Barnstead E-pure, Thermo Scientific, Waltham, MA).

4.1.1 Defatted Whole cricket Flour

The method developed to defat the cricket powder was developed from the American Oil Chemists' Society Procedure for Rapid Determination of Oil/Fat Utilizing High-Temperature Solvent Extraction (Am 2009). The modified method was less labor intensive and produced better results. The whole cricket powder was stirred in petroleum ether for 8 hours. The ratio of cricket powder to petroleum ether was set at 1g:20mL. This was based off the ratio used for the Soxhlet method. After 8 hours, the solution was vacuum filtered and left to dry overnight in a vacuum oven set to 77ºC. It was then sealed and stored in a desiccator at 22°C.

4.1.2 Extracted Protein

Protein was extracted from defatted whole cricket powder based on an established procedure (Zhang et al. 2000) with some modification. In brief, Ultrapure water was added to the defatted cricket powder at a ratio of 1g powder:20mL water. The pH was adjusted to 9.5 using 1.0 N NaOH. It was sealed and stirred for 18hrs to allow the protein to fully solubilize according to previous studies that have used this cricket powder (Kim et al. 2017). This slurry was then centrifuged at 9,000 RPM (14,334 x G) on a Beckman Avanti J-25I centrifuge for 30 minutes. The supernatant was collected and centrifuged again at 16,000 RPM (35,267 x G) for 1 hour, while the pellet was sealed and stored for later use at -40°C. The new supernatant was then collected and filtered using 0.45µm disc filters to remove any remaining non-solubilized proteins or contaminants. The filtered solution was frozen and lyophilized on a Labconco Freezone6 (Labconco Corporation; Kansas City, MO). The lyophilized protein was sealed and stored in a desiccator at 22°C.

4.1.3 Isolated Chitin

The stored pellet from the protein isolation was then subjected to a series of three refluxes (Figure 4.1) in different chemicals based upon an existing method used to isolate chitin from insects (Zhang et al. 1999). First, the pellet was stirred with 1 N HCl (Sigma-Aldrich) and then refluxed for 20 minutes at 100ºC. This was to remove the catechols that were present in the pellet. After 20 minutes, this was centrifuged, and the supernatant was poured off and the pellet was neutralized with ultrapure water. This was then centrifuged, and the pellet was used for the next step while the supernatant was discarded. This pellet was mixed with 1 N NaOH (Sigma-Aldrich) and refluxed for 28 hours at 85ºC, this was done to remove any polysaccharides and colors that were present. This was then centrifuged and neutralized like the previous step. Finally, the pellet

after neutralization was mixed with 0.4% Na_2CO_3 for 20 hours at 115ºC. This ultimate step was used to completely remove any proteins that had remained in the sample until this point. This was then centrifuged and neutralized. The final pellet after neutralization was then dried down overnight under vacuum at 77ºC, then sealed and stored in a desiccator at 22°C to be used in the future.

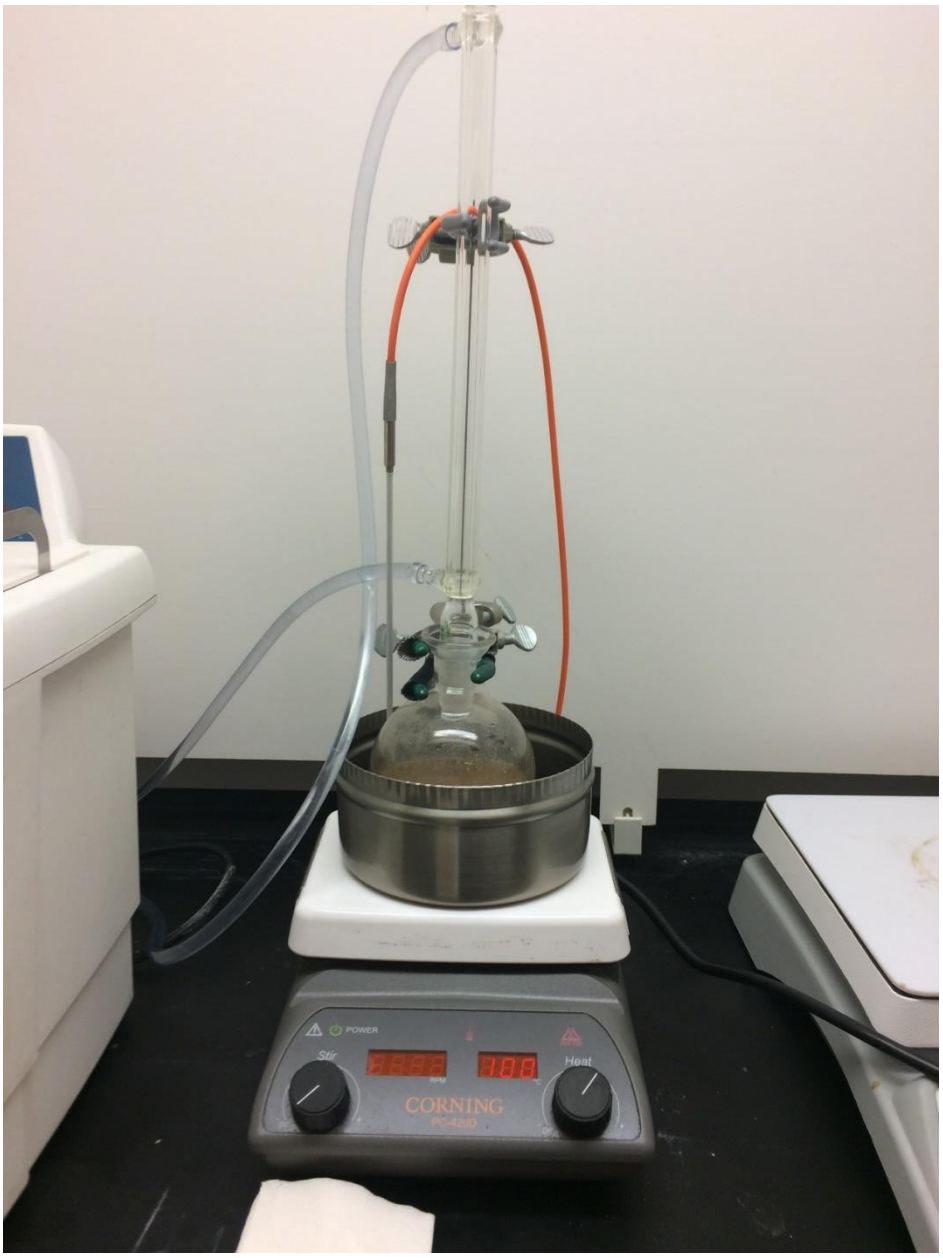

Figure 4.1: Setup for isolation of chitin (oil bath with a round bottom flask connected to a distilling column).

In order to produce a powder of homogenous and small particle size, the isolated chitin was milled initially with a Cyclotec^{TM} 1093 Mill (Foss; Hillerød, Denmark). The ground chitin was then further processed with a Retsch ball mill (Retsch; Haan, Germany) using multiple quarter-inch diameter stainless steel spheres to further reduce the particle size to a desired 1-5

micrometers. Particle size of the chitin was determined by a Morphologi G3-ID (Malvern, Worcestershire, England). This is a particle sizer with a dry dispersion unit that uses visual imaging to determine particle size by converting number mean to volume weighted mean. There is a margin of error introduced due to assumption of spherical particles.

4.2 Preparation of Samples

4.2.1 Preparation of Emulsions

Emulsions were prepared using Pure Canola Oil, ultrapure water, and either cricket protein or chitin. For the cricket protein emulsions (Figure 4.2), isolated protein powder was dispersed in ultrapure water and stirred until fully dissolved to reach final concentrations of 0.5% (wt/wt), 1% (wt/wt), 1.5% (wt/wt), and 2% (wt/wt). Oil (20g) was added to these solutions to reach a final mixture of 200g with a 10% oil content. Mixtures were stirred with a stir bar for 15 minutes to allow for initial mixing and then homogenized using an Ultra-Turrax Tissue Tearor (Sigma-Aldrich; St. Louis, MO) for 60s at 24,000 rpm to create an initial emulsion. Initial emulsions were further homogenized using a GEA Panda homogenizer (GEA; Columbia MD) operating at 10,000 PSI. These emulsions were then sealed and stored at 22°C for further testing.

Figure 4.2: Emulsions Prepared Using 0.5%, 1%, 1.5%, and 2% Cricket Protein and 10% Oil.

For chitin emulsions (Figure 4.3), chitin was added in excess (1-2%) and the emulsions were prepared similarly to the protein emulsions but were not homogenized with the GEA Panda homogenizer to avoid damaging the machinery with the insoluble chitin particles. Prepared emulsions were allowed to settle for an hour before a top oil layer was manually removed with a glass pipette. The remaining emulsified layer was transferred to a new container to be sealed and stored at 22°C for future testing.

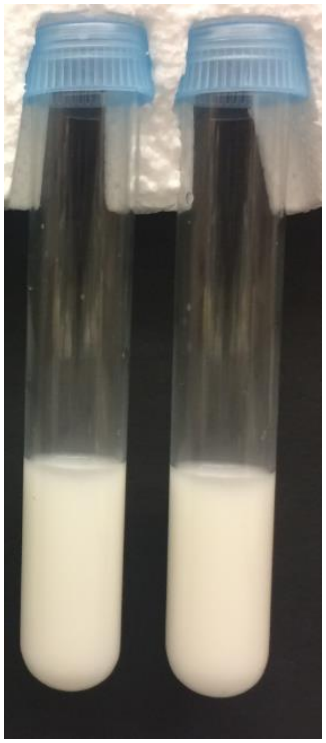

Figure 4.3: Emulsions Prepared Containing 1.56% Cricket Chitin and 5.29% Oil.

4.3 Analysis

4.3.1 Determining the Interfacial Tension of Chitin

Measurements to determine the interfacial tension of the isolated and ground chitin were performed on a Krüss Droplet Shape Analyzer (DSA30b; Krüss GMbH; Hamburg, Germany). Interfacial tension is measured to assess the potential of a material to absorb onto the interface of two immiscible liquids and alter the surface pressure of that interface. Knowing the change in interfacial tension gives a good indication on the effective of the material as an emulsifier. Droplets of ultrapure water were dispensed into air at room temperature from a stainless-steel needle (Krüss; Hamburg, Germany) with a measured diameter of 0.503 mm. The interfacial tension of the protein was measured at four different concentrations (0.0% wt/wt, 0.01% wt/wt, 0.05% wt/wt, 0.1% wt/wt). Samples were prepared by adding the appropriate amount of chitin to 25mL of ultrapure water. These solutions were allowed to stir until visual homogeneity was reached. The tests were

run for at least 300 minutes to determine the interfacial tension and allow for equilibrium, with measurements being taken at 1 second intervals. Surface pressure was calculated from interfacial tension by:

$$\Pi = \gamma - \gamma_0 \quad (4.1)$$

Where γ is the interfacial tension at a given time and γ_0 is the measured interfacial tension of ultrapure water in air.

4.3.2 Determining the Interfacial Tension of Protein

Measurements to determine the interfacial tension of the isolated cricket protein were performed on a Krüss Droplet Shape Analyzer (DSA30b; Krüss GMbH; Hamburg, Germany)(Figure 4.4). Interfacial tension is measured to assess the potential of a material to absorb onto the interface of two immiscible liquids and alter the surface pressure of that interface. Knowing the change in interfacial tension gives a good indication on the effective of the material as an emulsifier. Droplets of n-heptane were dispensed into 25 mL of aqueous phase using a J-shaped stainless-steel needle (Krüss; Hamburg, Germany) with a measured diameter of 0.910 mm. The interfacial tension of the protein was measured at six different concentrations of protein previously dispersed within the aqueous phase (0.0% wt/wt, 0.1% wt/wt, 0.2% wt/wt, 0.4% wt/wt, 0.6% wt/wt, 0.8% wt/wt). Tests were run for at least 60 minutes to determine the interfacial tension and allow for equilibrium, with measurements being taken at 1 second intervals. Surface pressure was calculated from interfacial tension by:

$$\Pi = \gamma - \gamma 0 \quad (4.1)$$

Where γ is the interfacial tension at a given time and γ_0 is the interfacial tension of the 0.0% protein solution at the same given time.

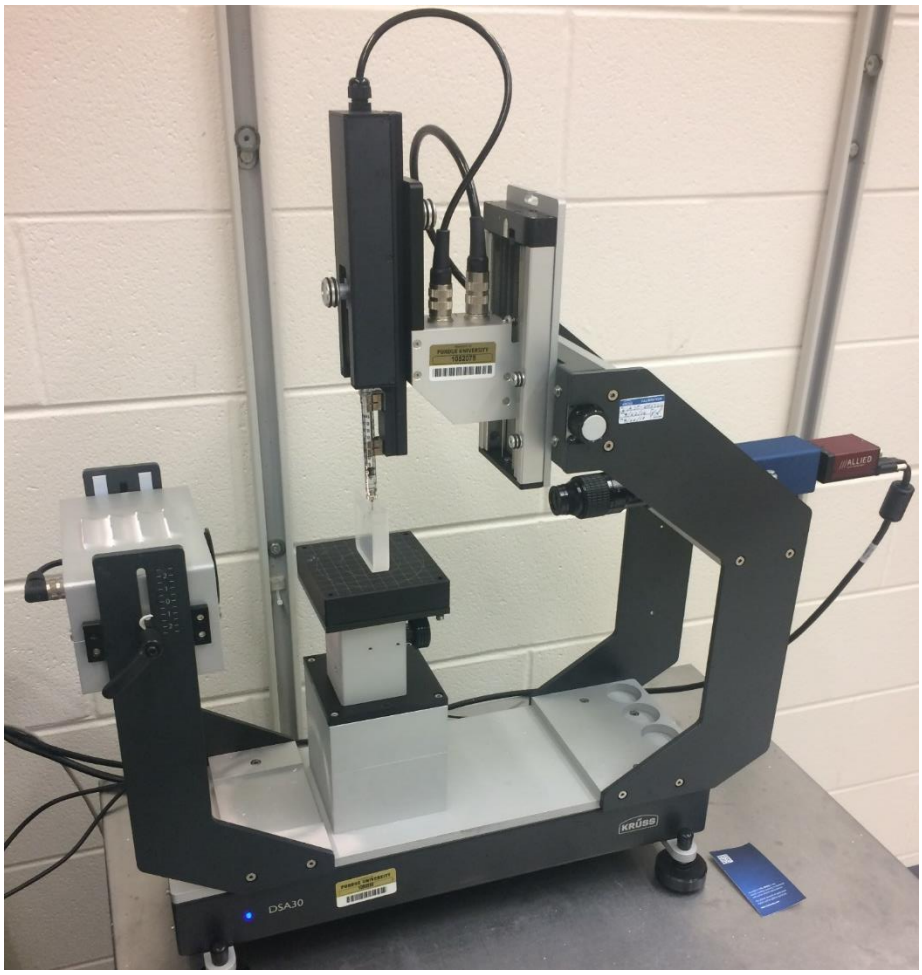

Figure 4.4: DSA30 (Droplet Shape Analyzer with syringe, J-hook needle, and glass cuvette).

4.3.2.1 Determination of Protein Saturation at the Interface

Equilibrium surface pressures were measured from pendant droplets after 60 minutes in the presence of 0.01%-0.08% cricket protein solutions. Equilibrium surface pressures were used to determine the fitting parameters *B* and *a*, describing sorption, from the Langmuir equation:

$$\Gamma = \frac{BC_2}{a+C_2} \quad (4.2)$$

This equation was discussed in Section 3.1.1.2. It was used to determine surface pressures are various concentrations. The predicted saturation point can be determined by:

$$\Gamma_{saturation} = BRT \quad (4.3)$$

Where $\Gamma_{saturation}$ is the surface pressure at the point of saturation, B is the inverse of the intercept of the plot for Langmuir fitting parameters, R is the gas constant, and T is temperature. All units are standardized. This was used to determine the predicted saturation point where additional cricket protein would have no effect on the surface pressure.

4.3.3 Oil Droplet Size Determination

Oil droplet sizes were determined among prepared emulsions during storage using multi-angle static light scattering (Malvern Mastersizer 2000; Malvern, Worcestershire, England)(Figure 4.5). Oil droplet size during storage was measured as it is a direct measurement on the instability of an emulsion. It is a clear indication on the effectiveness of a material as an emulsion stabilizer. Directly prior to analysis, emulsion samples were vortexed for 25 seconds to ensure homogeneity. Sample aliquots were added to a continuously agitated liquid cell until the obscuration of the laser reached a level of 12-13%. Measurements were taken every day until emulsions displayed significant instability. Average droplet size was presented as either the Sauter Mean Diameter or the Volume Weighted Mean. The lens was cleaned periodically to remove any oil residue that could distort accurate measurements of obscuration and size.

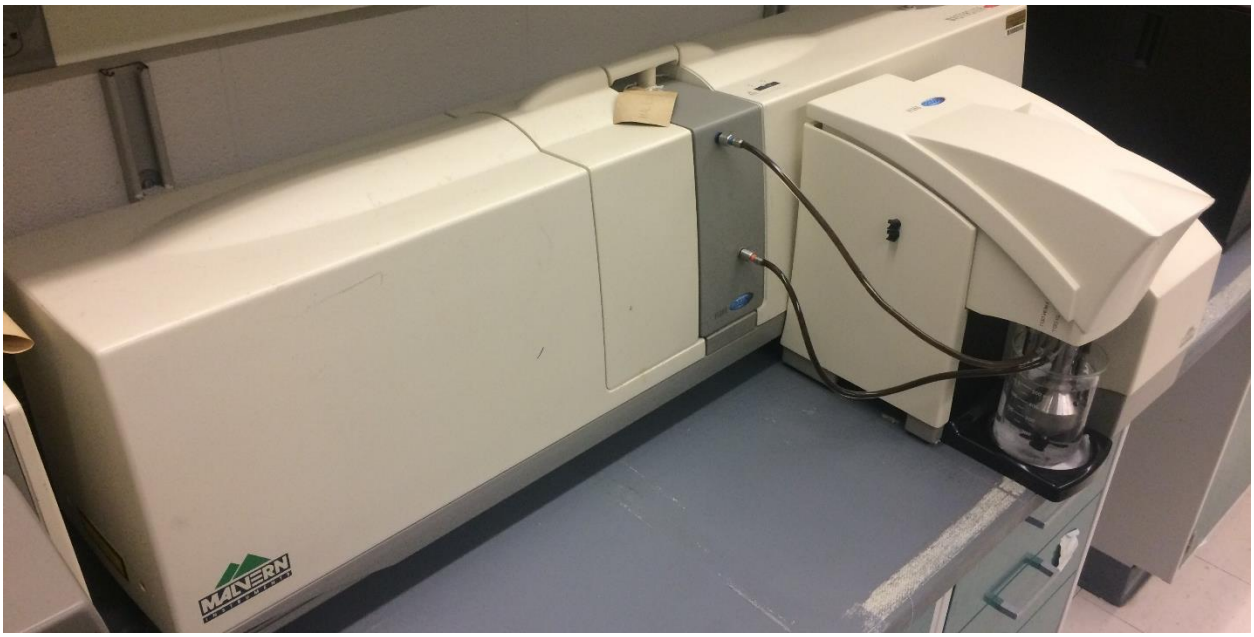

Figure 4.5: Malvern Mastersizer

4.3.4 Creaming Index

Stability of the emulsions was also determined through measuring the creaming index of the emulsions. 12mL of the emulsion samples were pipetted into test tubes and sealed. The tubes were suspended in a custom-made apparatus (Figure 4.6) and imaged with a digital camera.

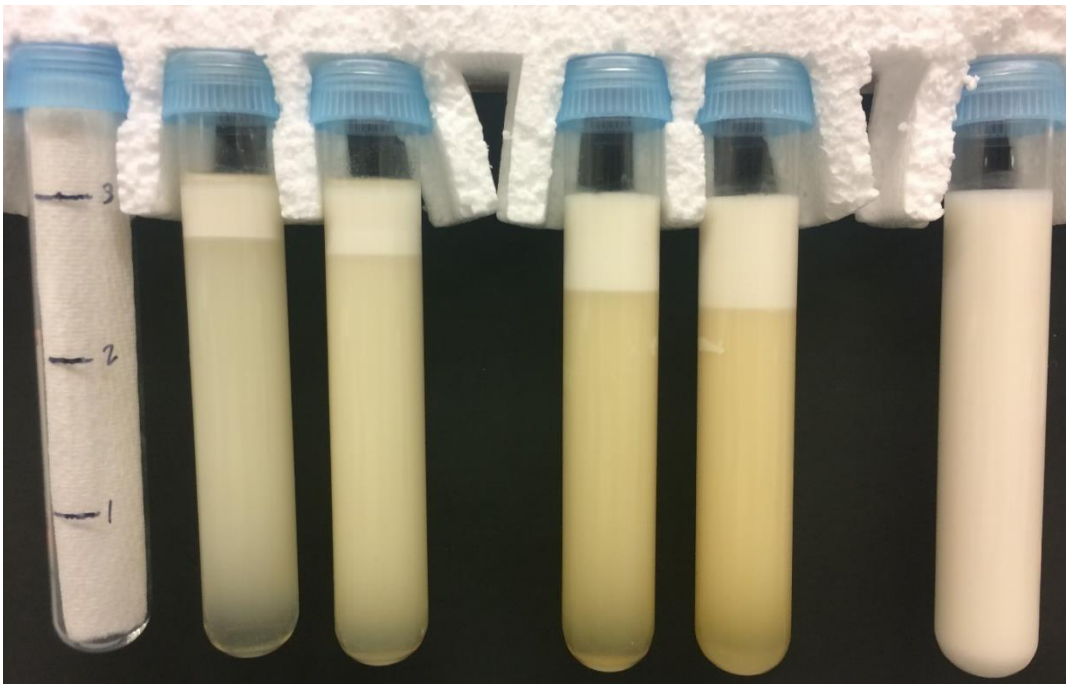

Figure 4.6: Apparatus used to hold tubes for creaming index measurements

Obtained images were analyzed with ImageJ (National Institutes of Health; Bathesda, Maryland) to accurately measure the emulsion layer height and total height of the samples. There was an incomplete separation of the serum layer, so creaming index was determined by:

$$\frac{emulsified\ layer\ height}{total\ height\ of\ emulsified\ layer\ and\ creamed\ layer} \times 100 \quad (4.4)$$

By this definition, 100% was a fully emulsified sample with no creaming. When used with an oil droplet size determination test, creaming index gives a fairly direct indication on the ability of a material to stabilize an emulsion long term.

4.3.5 Confocal Microscopy

Confocal Microscopy was used to identify the location of chitin in prepared emulsions. A Nikon A1-Rsi confocal microscope (Nikon Instruments Inc.; Melville, NY) was used to capture the images of the chitin stabilized oil droplets. 15µL of 0.1% Nile Red dye (Sigma-Aldrich; St.

Louis, MO), to visualize lipids, and 15µL of 0.4% Trypan Blue dye (Sigma-Aldrich; St. Louis, MO), to visualize chitin, was added to 50µL of emulsion sample. This was mixed and allowed to sit at 22°C for 60 minutes to allow for proper association between the dyes and their respective components. 15µL of this mixture was then pipetted onto a glass slide and covered with a #1.5 glass cover slip. Nile Red was excited using a 561nm laser and Trypan Blue was excited using a 640nm laser and imaged using a 60x oil immersion objective lens.

4.3.6 Ash Content Determination

To determine the effectiveness of the protein isolation procedure and to identify the present fractions in the samples, the ash content of the whole cricket powder and of the protein extract were measured. Porcelain crucibles and lids were dried in a furnace over at 550°C to remove any moisture. The mass was measured and recorded, and the crucibles and lids were stored in a desiccator. Triplicates of both the whole cricket powder and protein extract were weighed to approximately 2.5000g into a tared crucible and the mass was recorded. They were placed in a desiccator to transfer to the muffle furnace. The crucibles with lids and samples were placed in a muffle furnace set to 550°C for 15 hours to fully incinerate the samples. After 15 hours the muffle furnace was turned off and the samples were allowed to cool to workable temperature for six hours. After six hours they were removed from the oven and placed in a desiccator to be transferred to the scale. The mass of the crucible + lid + ashed sample was recorded in whole for all samples. Calculations were made to determine the weight of the remaining ash:

$$Mass_{ash} = mass_{total} - mass_{crucible} - mass_{lid} \quad (4.5)$$

This was used to determine the percent ash content in both the whole cricket powder as well as the protein extract:

$$Percent\ ash\ content = \left(\frac{mass_{ash}}{mass_{sample}}\right) \times 100 \quad (4.6)$$

4.3.7 Nitrogen Combustion

To determine the crude protein content of the samples, the percent nitrogen content of whole cricket powder, protein extract, and defatted protein extract were measured. Approximately 0.2500 grams of sample were measured in duplicate into clay boats. The mass was recorded, and the samples were loaded onto a LECO nitrogen analyzer (LECO Corporation, St. Joseph, Michigan, USA). After analysis of the samples percent nitrogen content was determined and used for determination of crude protein content.

4.3.8 Determination of Crude Protein Content

In order to determine the true protein content of the whole cricket powder and the protein extract, it was necessary to calculate the crude protein content within the samples. Results from the nitrogen combustion measurements were used to perform the calculations. Two values were calculated for each sample, one using the traditional ratio of 6.25 and the second using newer more accurate ratios, 4.78 for whole cricket powder and 5.60 for extracts, that help give a more truthful value (Janssen et al. 2017).

$$mass_{protein} = percent\ nitrogen\ \times ratio \quad (4.7)$$

4.4 Statistical Analysis

All experiments were performed in triplicate and reported as the mean and standard deviation, unless otherwise stated. Significance testing was conducted using single or multiple comparison ANOVA and Tukey's method to determine significant differences.

CHAPTER 5. RESULTS AND DISCUSSION

5.1 Isolation of Water-soluble Protein and Chitin from Whole Cricket Powder

In order to test the functionality of the cricket protein and chitin, it was necessary to obtain extracted samples of these fractions. To do this, the lipid fraction was removed by petroleum ether, which accounted for 16.81% ± 0.408 of the total weight. This extracted lipid was less than reported values of 22% (w/w) fat that was described on the nutritional label for the purchased whole cricket powder but much greater than the reported 3.6% ± 0.4 (w/w) fat content from a previous study (Yi et al. 2013).

Protein was extracted from the defatted powder with a content of 20.729% ± 1.35 (w/w) in relation to the defatted powder. The sample contained all of the water-soluble proteins, but none of the bound proteins in the cuticle. A water-soluble extract is more reasonable as opposed to the full system of protein due the inability to extract the bound chitin without denaturing the protein. The means by which to remove the protein from the cuticle would put the protein under conditions which could portentially alter their ability to function as an emulsifier. This would make the effort and time necessary to extract the proteins useless. Again, this content was lower than the reported protein content on the nutritional label, which stated 67g of protein per 100 g of whole cricket powder. However, the measured percentage was very near to the reported isolated protein fraction of 21.5% ± 0.5 from a previous study (Yi et al. 2013). Chitin was isolated from the remaining precipitate with a content of 5.137% ± 0.299 (w/w) in relation to the defatted cricket powder. No comparable data in the literature provided an estimate of the expected amount of chitin within *Acheta domesticus* or associated powder products. Reports for other insects, including other cricket species, found as much as 15.3 ± 3.6% (Barker, Fitzpatrick and Dierenfeld 1998) or as little as 0.014% chitin contents (Rumpold and Schlüter 2013).

5.2 Analysis of Cricket Protein Extract

		Nitrogen Combustion Results			
	Ash Content (%)	Nitrogen Content (%)	Protein Content (%)[1]	Protein Content (%)[2]	Protein Content (%)[3]
Whole Cricket Powder	4.3670 ± 0.0166	10.9535 ± 0.2906	68.46 ± 1.82	52.14 ± 1.38	N/A
Isolate	25.7096 ± 0.4847	8.9464 ± 0.3181	55.92 ± 1.99	50.10 ± 1.78	88.425 ± 0.036
Defatted Isolate	N/A	9.6095 ± 0.0134	60.06 ± 0.08	53.81 ± 0.08	N/A

Table 5.1: Summary of Ash and Protein Content Determination Results for Whole Cricket Powder, Protein Extract, and Defatted Protein Extract.

1: Converted from Nitrogen Content using the ratio 6.25

2: Converted from Nitrogen Content using the ratios 4.78 (whole cricket powders) and 5.60 (extract, defatted extract)

3: Determined by direct absorption measurements at 280nm using the extinction coefficient for Bovine Serum Albumin

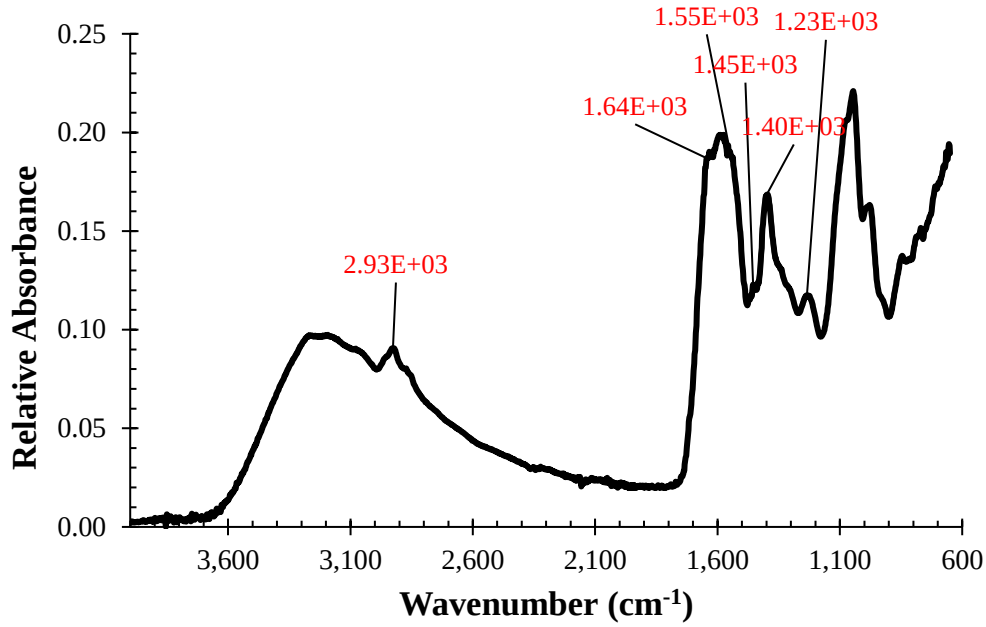

Figure 5.1: Infrared Spectra of Protein Isolate

Infrared spectroscopy analysis (Figure 5.1) of the extract found peaks in the Amide I, Amide II, and Amide III regions, at 1650cm^{-1}, 1600cm^{-1}, and 1400cm^{-1}, respectively. Existence of C=O bonds (1650cm^{-1}), CN bonds (1550cm^{-1}), CH stretching (1400cm^{-1}), and PO_2 stretching (1240cm^{-1}) strongly suggested the identity protein in the sample. Peaks were also found in the C-H methylene region at 2924 cm^{-1} that typically suggest the inclusion of lipids in the sample.

Protein concentration of the whole cricket powder and protein extract was determined by nitrogen combustion and spectrophotometry of prepared solutions. Nitrogen contents of the whole cricket powder and extract were found to be 10.9535 ± 0.2906% and 8.9464 ± 0.3181%, respectively (Table 5.1). To verify that lipids were not present in significant concentrations, the protein extract was rinsed several additional times with petroleum ether; the nitrogen content did not significantly change. Using revised estimates for the correct nitrogen-to-protein ratio of insect ingredients (Janssen et al. 2017), the determined protein contents of whole cricket flour,

the protein extract, and the further defatted protein extract were 52.14 ± 1.38%, 50.10 ± 1.78%, and 53.81 ± 0.08%. Protein content of the protein extract by direct absorption of UV light at 280nm was 88.425 ± 0.036%. However, this value was likely inaccurate because there was no literature value for the extinction coefficient of the insect protein and the value for Bovine Serum Albumin was utilized.

Ash content of the whole cricket powder was found to be 4.3670 ± 0.0166%, while ash content of the protein extract was significantly increased to 25.7096 ± 0.4847% (Table 5.1). This is most likely due to fractionation taking place during the extraction procedure. This also can explain the lower than expected protein content of the extract. During the extraction procedure, the protein fractions with low solubility most likely did not fully solubilize and were separated into the pellet during centrifugation. This removed them from the collected portion that was measured to determine protein content. But the majority of the ash from the whole cricket powder solubilized and remained in the supernatent fluid after centrifugation and was not filtered out before lyophilzation. By removing a portion of the protein and all other insoluble fractions, the percent of ash increased from less than 5% to over a quarter of the sample.

5.3 Analysis of Stabilizing Ability

5.3.1 Effects of Concentration of Protein on Surface Pressure

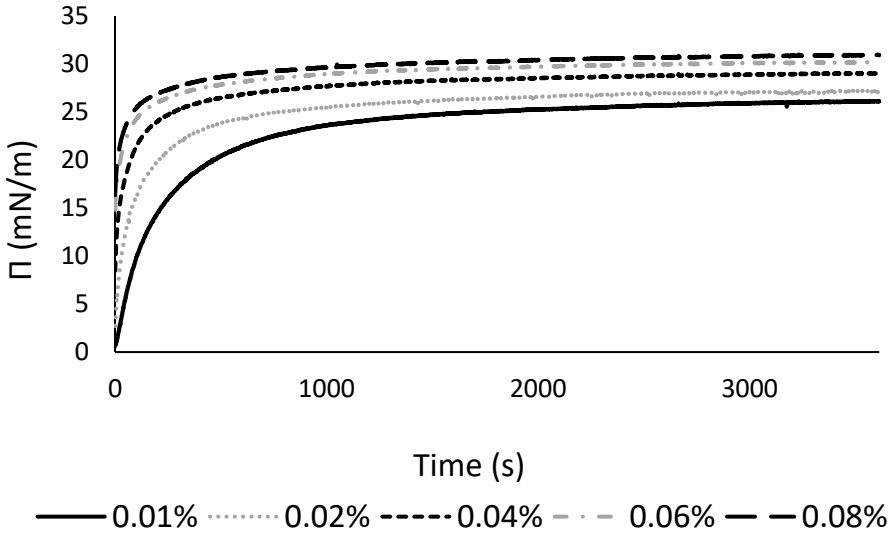

Figure 5.2: Dynamic Surface Pressure (Π) of Hexane Droplets in Water with Increasing Content of Water-Soluble Cricket Proteins.

In order to determine the potential effectiveness of the cricket protein, the surface activity of the protein was determined between concentrations of 0.01% to 0.08% (Figure 5.2). Higher concentrations of cricket protein showed a higher overall surface pressure at equilibrium. Moreover, the time taken to approach the equilibrium surface pressure decreased with concentration. This showed that as the concentration of the surfactant in the continuous phase increased, the absorption onto the interface occurred more readily allowing the surface pressure to reach equilibrium more quickly. When compared with whey protein isolates (WPI), cricket protein has a much greater increase in surface pressure when it absorbs onto the interface. WPI yields an increase in surface pressure of <5 mN/m even at concentrations of 10% protein (Davis and Foegeding 2007). Cricket proteins at concentrations as low as 0.08% have yielded increases in

surface activities ~29 mN/m. This higher surface activity gives reason to believe that cricket protein has the potential to be a more effective emulsion stabilizer than WPI.

5.3.2 Determination of Protein Saturation at the Interface

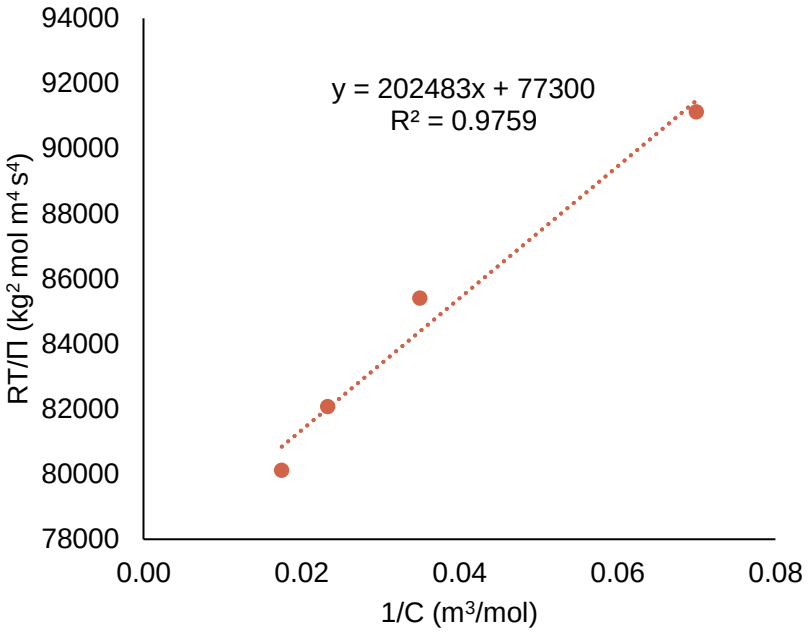

Figure 5.3: Reciprocal Plot of the Equilibrium Surface Pressure of Cricket Protein Solutions as a Function of Concentration to Obtain Langmuir Sorption Model Fitting Parameters.

In order to determine the saturation surface load of the cricket protein on a model emulsion droplet, the concentration dependent equilibrium surface pressure , obtained from the data of Figure 5.2, was fit with the Langmuir sorption model (Figure 5.3). A linear correlation was found for the equilibrium surface pressures at added concentrations of 0.02-0.08 wt%. Using equations 3.2 and 3.3, the predicted surface pressure at full interfacial saturation would be 32.05 mN/m, corresponding to a concentration of the isolated cricket protein fraction slightly in excess of 1% (w/w). It should be stated that the reliability of the parameters is quite low given the few data points measured and the measurement of concentrations well below the 1% (w/w) predicted for

interfacial saturation. Measuring a wider range of concentrations, especially higher concentrations, would greatly increase the accuracy of the results.

5.3.3 Stability of Cricket Protein and 10% Oil Emulsions

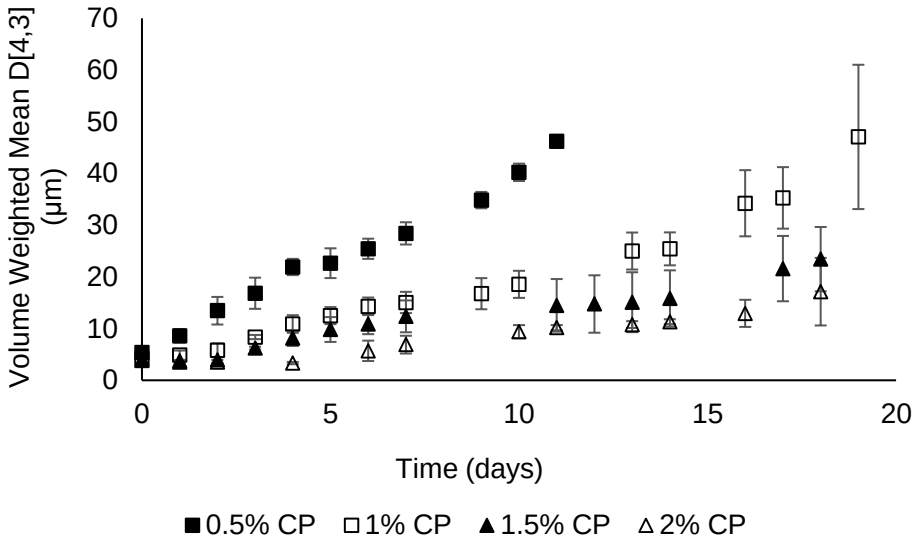

Figure 5.4: Comparison of Oil Droplet Size Over Time in Emulsions Using 10% Oil and Different Concentrations of Cricket Protein - D[4,3]

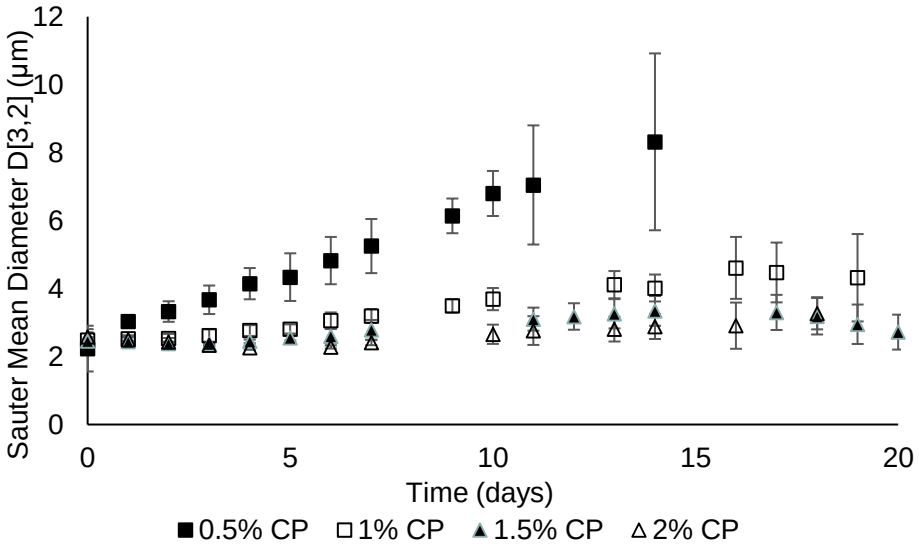

Figure 5.5: Comparison of Oil Droplet Size Over Time in Emulsions Using 10% Oil and Different Concentration of Cricket Protein - D[3,2]

In order to assess the effectiveness of the cricket protein as an emulsifier, emulsions were created using the cricket protein at concentrations between 0.5% and 2%. These emulsions were then measured over time to determine the size of the oil droplets within the emulsion. The size of the oil droplets was displayed as Volume Weighted Mean D[4,3] (Figure 5.4) and Sauter Mean Diameter D[3,2] (Figure 5.5). All concentrations showed a near linear increase in size over time when displayed as D[4,3]. Lower concentrations showed a greater rate of droplet growth during storage, and resistance to this droplet growth increased with greater concentration of cricket protein. There was less difference in the average sizes of oil droplets at higher protein concentrations, giving evidence that the higher concentrations studied here were sufficient to stabilize the emulsions. Though the Sauter Mean Diameter (D[3,2]) results appeared to show greater stability over time when compared to the results displayed as Volume Weighted Mean D[4,3], this can be credited to how the results are weighted. D[3,2] relies more heavily on the surface area of the droplets and D[4,3] is weighted more towards the volume of the droplets. As the size of the droplets increased, they increased in both volume and surface area, but the ratio of surface area to volume is 3/r where r is the radius. This explains why the D[4,3] results had a greater increase in size over time when compared to the D[3,2], because as sphere size increased, volume increased at a greater rate than surface area. When compared with whey-protein isolate (WPI) stabilized emulsions, initial particle size was much larger. With an average particle size of 2.58 μm (d[3,2]), cricket protein stabilized emulsions had initial oil droplets over 4 times larger than WPI stabilized emulsions (d[3,2] ≈ 0.6 μm) (Demetriades, Coupland and McClements 1997).

5.3.4 Physical Separation of Cricket Protein Emulsions during Storage

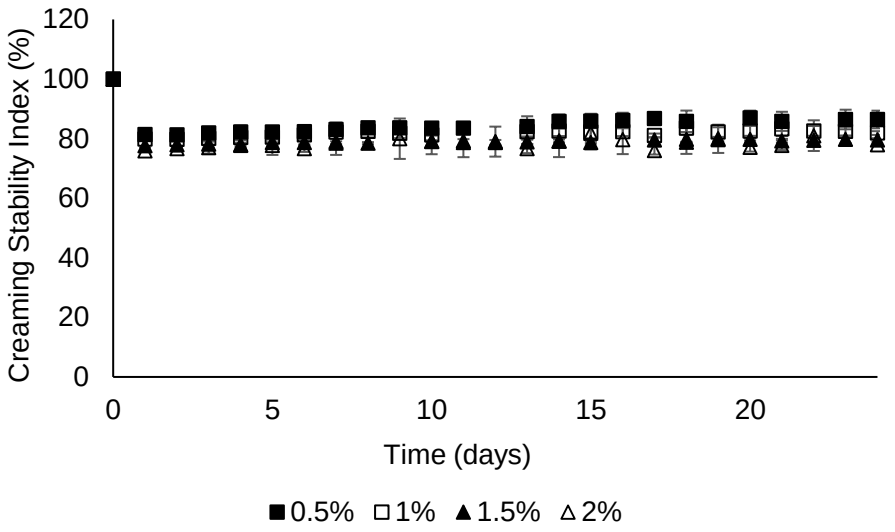

Figure 5.6: Creaming Stability Index of Emulsions (10% oil content) stabilized with Different Concentrations of Cricket Protein

Creaming stability index is a widely accepted way to measure the stability of an emulsion, which ultimately helps determine the effectiveness of an emulsifier. Over the course of 24 days, creaming stability index of emulsions stabilized by 0.5%, 1.5%, or 2% cricket protein showed no significant difference after the initial measurement at day 0, with days 1 through 24 being significantly similar, while the 1% cricket protein emulsions showed significant differences over time as creaming increased. No noticeable difference in the creaming stability index was noted during the observed period (Figure 5.6). This observation was most likely caused by insufficient absorption of protein onto the interface of the oil and water. The oil droplets that were not fully saturated aggregated and creamed out. This left only the fully stabilized oil droplets in the emulsified layer and resulted in the observed stabilization over the course of days 1 through 24. When compared to WPI stabilized emulsions, the stability of the cricket protein stabilized emulsions was much greater. After 24 hours of storage WPI stabilized emulsions reported a

creaming stability index of 0 (Demetriades et al. 1997), where cricket protein stabilized emulsions reported a creaming stability index of ~75. This gives some evidence that cricket protein has a higher capacity for stability than WPI. But to fully determine the level of stability and initial destabilization, the level of creaming should be measured more thoroughly over the course of the first twenty-four hours.

5.3.5 Effect of Concentration of Cricket Protein on Solution Viscosity

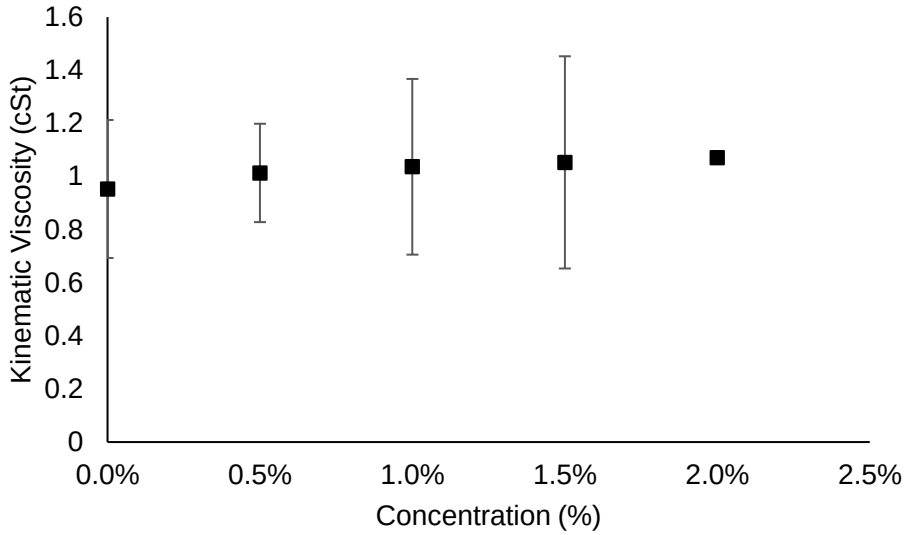

Figure 5.7: Kinematic Viscosity of Cricket Protein Solutions at Increasing Concentrations

To be classified as a true traditional surfactant, a material must adsorb to a surface, form a protective coat, and lower the interfacial tension (McClements 2015). This altered tension increases the surface pressure and increases the stability of an immersed droplet in a solution. It had been proven that the cricket protein possessed this property and was corroborated by the determination of oil droplet sizes over time, but it was necessary to determine the effect that increased viscosity will have on the stability of the emulsions. In order to rule out that increased concentrations of cricket protein increased the viscosity to the point that it added stabilization to

an emulsion outside of the interaction at the interface, the kinematic viscosity of cricket protein solutions were measured at the concentrations used for the emulsions, from 0% to 2% (Figure 5.7). There was no statistical difference at $\alpha=0.05$ in viscosity over the entire range of concentrations and the measured viscosity is essentially that of water. The protein was not contributing anything to the viscosity profile, which provides further evidence that the increased stability in emulsions is due to the cricket proteins ability to decrease pressure at the interface of the immiscible liquids of the emulsion.

5.3.6 Stability of Cricket Chitin and 5.29% Oil Emulsions

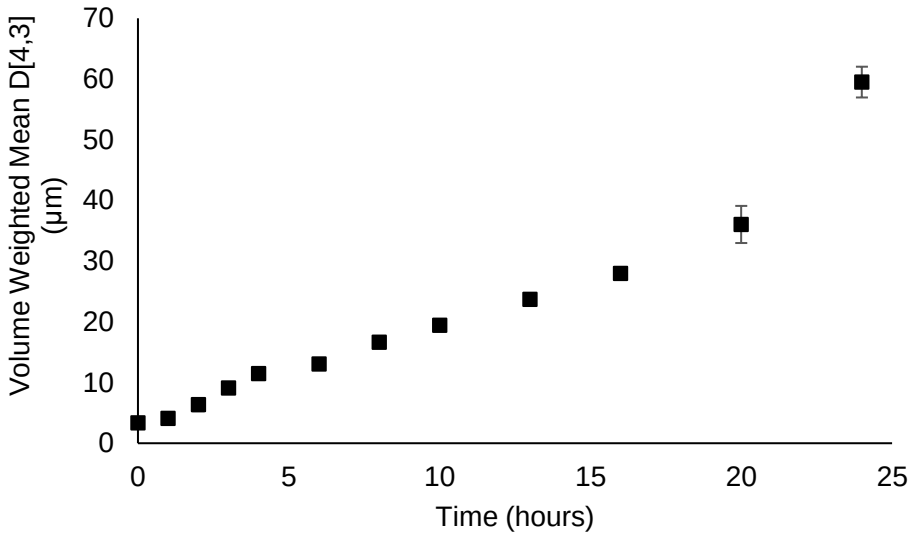

Figure 5.8: Comparison of Oil Droplet Size Over Time in Emulsions Containing 5.29% Oil and Cricket Chitin - D[4,3]

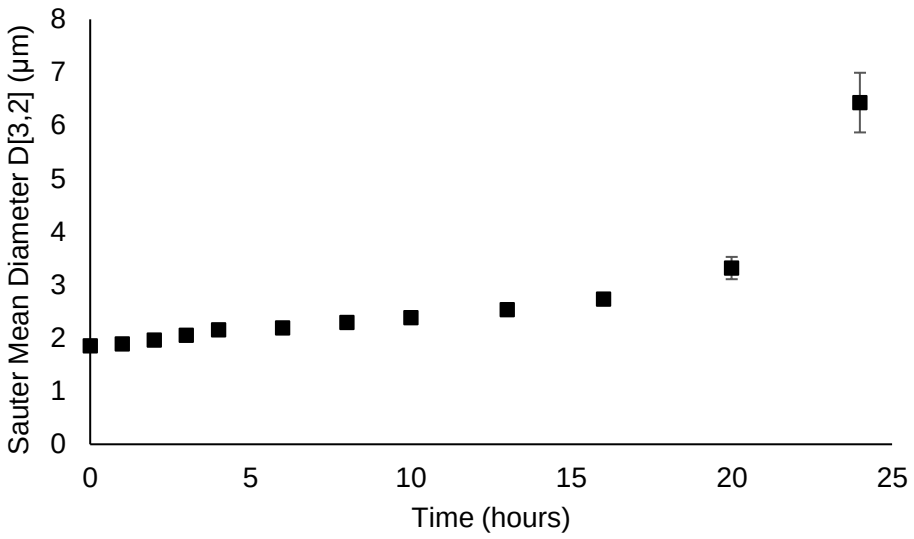

Figure 5.9: Comparison of Oil Droplet Size Over Time in Emulsions Using 5.29% Oil and Cricket Chitin - D[3,2]

In order to assess the effectiveness of the chitin fraction of crickets as a Pickering stabilizer, emulsions were created using excess concentrations of cricket chitin. The size of formed oil droplets was displayed as Volume Weighted Mean D[4,3] (Figure 5.8) and Sauter Mean Diameter D[3,2] (Figure 5.9). D[4,3] of the emulsions increased linearly over time except for the 25th hour, which showed a greater increase in size than was expected. Similarly, D[3,2] of the emulsions showed a linear increase over time until the final measurement at 24 hours where there was an increase in size uncharacteristic of the trend. As was identified with the cricket protein emulsions, the results displayed as D[3,2] appeared to show a greater degree of stability when compared to those displayed as D[4,3], but this is simply due to how the measurements are weighted and the relationship between surface area and volume. When compared to soy protein isolate nanoparticles (SPINP), another particle stabilizer, chitin stabilized much smaller oil droplets. SPINP emulsions had an initial average oil droplet size of 60-70 μm (Liu and Tang 2013), where the chitin stabilized

emulsions had an initial average oil droplet size of 3.33 μm. This provides some evidence that chitin is a practical Pickering stabilizer with promising functionality.

5.3.7 Physical Separation of Chitin Chitin Emulsions During Storage

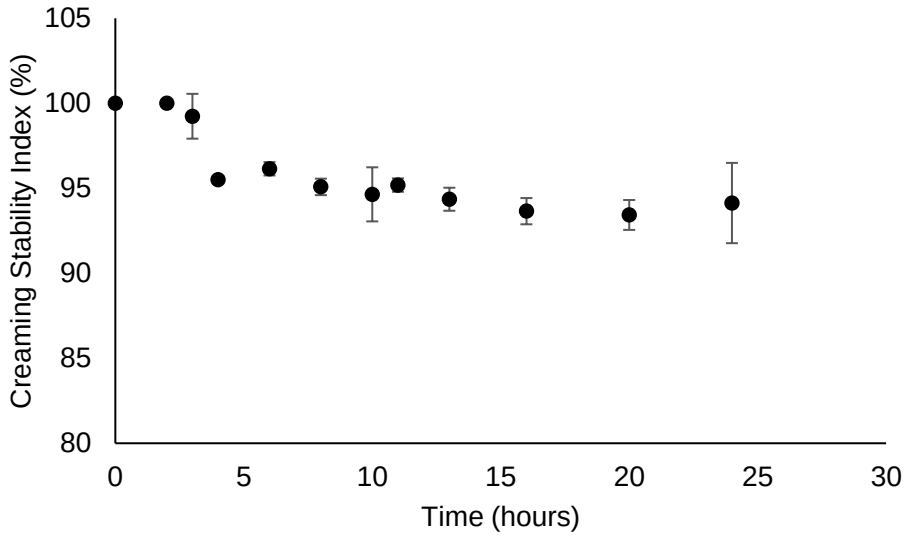

Figure 5.10: Creaming index of emulsions (5.29% oil content) stabilized by Cricket Chitin during storage

To assess the emulsification abilities of the cricket chitin, the creaming index of emulsions prepared with isolated cricket chitin was measured over time from hour 0 through hour 24 (Figure 5.10). Upon settling after homogenization, 10g of oil separated and the oil content decreased from 10% to 5.29%. Settling also showed a decrease in chitin content from 2% to 1.56%, as 0.7916g of the original 3.60g of chitin settled of the emulsion. Over the course of the time measured, the cricket chitin emulsion showed a significant difference at $\alpha=0.05$ as the creaming stability index decreased over time. Unlike as was observed with the cricket protein emulsions, there was no rapid decrease in creaming stability index after the initial 24 hours. The creaming stability index of the cricket chitin stabilized emulsions showed a slow but steady decrease over time, indicating a high capacity for emulsion stability as very little creaming took place, but a constant destabilization.

5.4 Microscopic Analysis

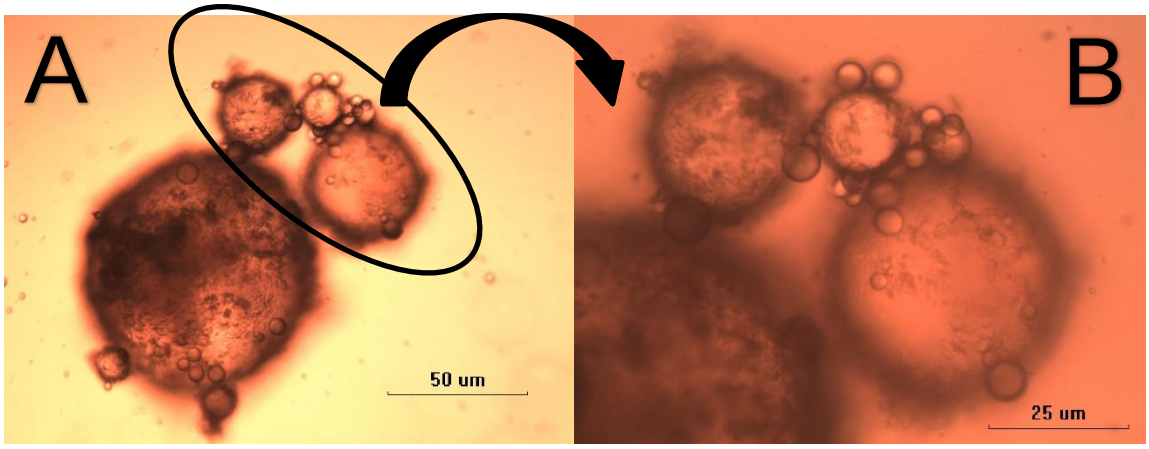

Figure 5.11: Chitin Stabilized Oil Droplets under Light Microscope

Due to the nature of Pickering stabilization, the material creating the barrier at the interface is solid and therefore visible if viewed with a microscopy technique capable of visualizing those solid particles (Pickering 1907). In order to verify that the emulsions prepared with cricket chitin as the sole stabilizer were being stabilized by chitin, oil droplets were isolated from the separated emulsion and evaluated under a light microscope (Figure 5.11). From the images it was clearly seen that the oil droplets were surrounded and stabilized by chitin fragments. The droplets were able to be identified as oil and not just a coagulation of chitin due to the diffusion of light through the droplet, if it were just a grouping of chitin, the light would be blocked and not transmitted through the sphere. The main question that needed to be answered was if the chitin was capable of being broken down enough to stabilize oil droplets in a fully stable emulsion. Figure 5.11b provides evidence of chitin stabilized oil droplets that fit within the range of the size of the oil droplets when the emulsion was still considered stable (<50μm).

5.4.1 Confocal Microscope Analysis of Chitin Emulsion Oil Droplets

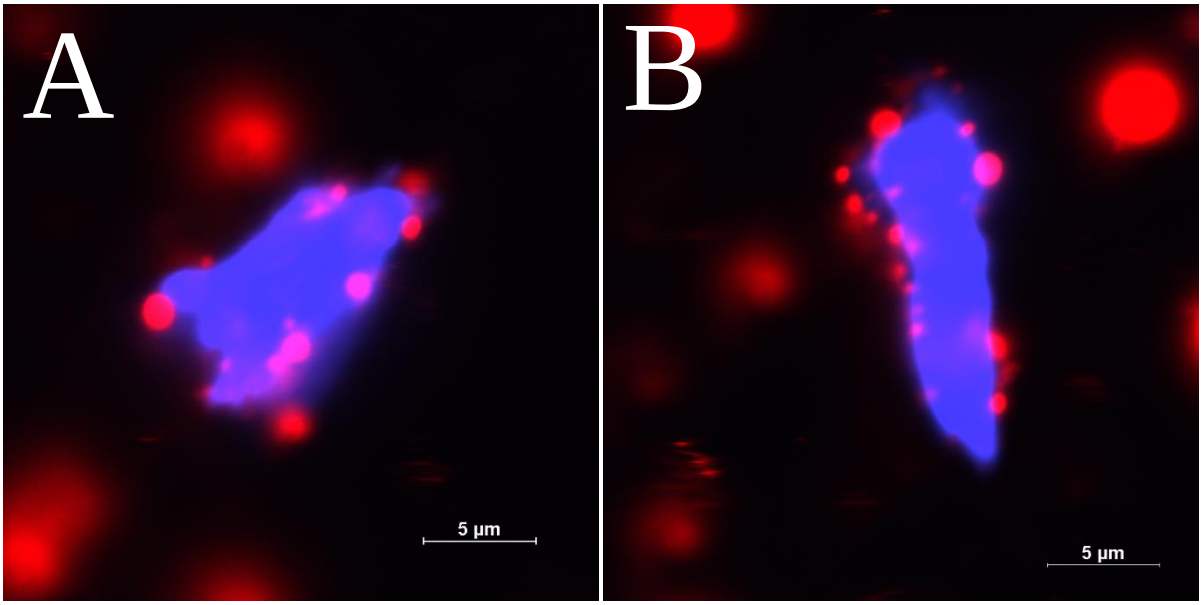

Figure 5.12: Confocal Laser Scanning Micrographs of Oil Droplets (Stained with Nile Red) and Two Chitin Fragments (Stained with Trypan Blue) Obtained from the Same Stabilized Emulsions of 1.56% Cricket Chitin and 5.29% Oil.

When confocal imaging was used to find evidence of chitin stabilized oil droplets, the expectation was to find an oil droplet surrounded by fragments of chitin. When in fact, the opposite was discovered. The images show chitin fragments stabilizing oil droplets by giving them a surface to adhere and stay immobile (Figure 5.12). This provides evidence that the chitin particles have affinity with the oil phase. This affinity with the oil phase implies a potential surface activity of the chitin particles and functionality for emulsification, which agreed with the observed stability of emulsions prepared with chitin particles (Figure 5.10 and 5.11).

CHAPTER 6. CONCLUSIONS

Crickets are a reliable source for isolation of protein and chitin. The isolation of a pure sample, although challenging, is possible and reproducible under the right conditions. Percent yields for protein fractions are lower than expected but most likely due to protein binding the chitin particles and becoming unavailable. This causes an overestimation when the traditional ratio of percent nitrogen content to protein content is used. Ultraviolet-visible spectroscopy methods also overestimate the protein due to an unknown extinction coefficient for *Acheta domesticus*.

Cricket protein displayed an ability to increase the surface pressure of a system at low concentration. This was corroborated by its ability to stabilize emulsions during storage. The protein acted as a traditional surfactant, binding to the interface, creating a barrier and decreasing the interfacial tension. The saturation point of the protein was found to be within usable limits for food products, making it a reasonable option as a food ingredient. There was no increase in viscosity in solution as concentration increased that was aiding in the stabilization of the emulsion. Further purification of the protein extract will only increase the emulsifying properties of the protein, most likely increasing long term stability of sample emulsions. This would also allow for smaller portions of cricket protein to be used for full stabilization of an emulsion, increasing its usefulness as an added ingredient without altering other qualities of the product.

Cricket chitin presented evidence of Pickering Stabilization by adsorbing to the interface of oil droplets in an emulsion and creating a barrier. The chitin displayed an ability to emulsify a fraction of the oil phase with good stability over a 24 hour period, although its emulsification and stabilizing capabilities were much less when compared to the water-soluble cricket protein. This difference in capacity for stabilization can partially be explained by the significant fraction of large chitin particles present in the samples; such large chitin particles would have been less effective

as emulsion stabilizers due to their long adsorption times and inability to stabilize droplets with large interfacial curvature. If particle size of the chitin was further reduced, emulsification properties should increase because more of the chitin would participate in stabilizing the emulsion droplets. More research will need to be conducted to fully understand the practicality of the chitin fraction as an emulsifying component.

Overall, crickets are a viable source for natural and food-grade emulsifiers. Both cricket protein and chitin showed evidence of being able to stabilize emulsions over a period of time without rapid or drastic destabilization. They prevented major flocculation, coalescence, and creaming over the course of the storage. The protein displayed an ability for long term stabilization of a product without agitation to promote break up of aggregation. These results were also supported by its ability to decrease the interfacial tension of an emulsion system. The chitin gave promising evidence of its ability to stabilize an emulsion over time. Further research is needed to fully understand the necessary processing needed to utilize the chitin to its maximal stabilizing potential.

REFERENCES

Adebowale, Y. A., K. O. Adebowale & M. O. Oguntokun (2005) Evaluation of nutritive properties of the large African cricket (Gryllidae sp). *Pakistan Journal of Scientific and Industrial Research,* 48**,** 274.

Am, A. A. P. (2009) Rapid Determination of Oil/Fat Utilizing High Temperature Solvent Extraction.

Amadi, E. N. & D. B. Kiin-Kabari (2016) Nutritional composition and microbiology of some edible insects commonly eaten in Africa, hurdles and future prospects: A critical review. *Journal of Food: Microbiology, Safety & Hygiene,* 1**,** 35-42.

Andersen, S. O., P. Hojrup & P. Roepstorff (1995) Insect cuticular proteins. *Insect biochemistry and molecular biology,* 25**,** 153-176.

Barker, D., M. P. Fitzpatrick & E. S. Dierenfeld (1998) Nutrient composition of selected whole invertebrates. *Zoo Biology: Published in affiliation with the American Zoo and Aquarium Association,* 17**,** 123-134.

Benjamins, J., M. H. Vingerhoeds, F. D. Zoet, E. H. A. De Hoog & G. A. Van Aken (2009) Partial coalescence as a tool to control sensory perception of emulsions. *Food Hydrocolloids,* 23**,** 102-115.

Berry, J. D., M. J. Neeson, R. R. Dagastine, D. Y. C. Chan & R. F. Tabor (2015) Measurement of surface and interfacial tension using pendant drop tensiometry. *Journal of colloid and interface science,* 454**,** 226-237.

Binks, B. P. (2002) Particles as surfactants—similarities and differences. *Current opinion in colloid & interface science,* 7**,** 21-41.

Chang, S. K. C. (2010) Protein Analysis. *Food Analysis***,** 133-146.

Chen, P., D. Y. Kwok, R. M. Prokop, O. I. Del Rio, S. S. Susnar & A. W. Neumann. 1998. Axisymmetric drop shape analysis (ADSA) and its applications. In *Studies in Interface Science,* 61-138. Elsevier.

Christensen, D. L., F. O. Orech, M. N. Mungai, T. Larsen, H. Friis & J. Aagaard-Hansen (2006) Entomophagy among the Luo of Kenya: a potential mineral source? *International Journal of Food Sciences and Nutrition,* 57**,** 198-203.

Davis, J. P. & E. A. Foegeding (2007) Comparisons of the foaming and interfacial properties of whey protein isolate and egg white proteins. *Colloids and Surfaces B: Biointerfaces,* 54**,** 200-210.

DeFoliart, G. R. (1991) Insect fatty acids: similar to those of poultry and fish in their degree of unsaturation, but higher in the polyunsaturates. *The Food Insects Newsletter,* 4**,** 1-4.

Demetriades, K., J. N. Coupland & D. J. McClements (1997) Physical properties of whey protein stabilized emulsions as related to pH and NaCl. *Journal of Food Science,* 62**,** 342-347.

Dickinson, E. (2009) Hydrocolloids as emulsifiers and emulsion stabilizers. *Food hydrocolloids,* 23**,** 1473-1482.

--- (2012) Use of nanoparticles and microparticles in the formation and stabilization of food emulsions. *Trends in Food Science & Technology,* 24**,** 4-12.

Drewnowski, A. & B. M. Popkin (1997) The nutrition transition: new trends in the global diet. *Nutrition reviews,* 55**,** 31-43.

Elanchezhiyan, S. S., N. Sivasurian & S. Meenakshi (2014) Recovery of oil from oil-in-water emulsion using biopolymers by adsorptive method. *International journal of biological macromolecules,* 70**,** 399-407.

Finkle, P., H. D. Draper & J. H. Hildebrand (1923) The theory of emulsification1. *Journal of the American Chemical Society,* 45**,** 2780-2788.

Fjne, A. (2005) Confocal Microscopy: Principles. *Imaging in Neuroscience and Development: A Laboratory Manual***,** 43.

Grapes, M., P. Whiting & L. Dinan (1989) Fatty acid and lipid analysis of the house cricket, Acheta domesticus. *Insect biochemistry,* 19**,** 767-774.

Hall, F. G., O. G. Jones, M. E. O'Haire & A. M. Liceaga (2017) Functional properties of tropical banded cricket (Gryllodes sigillatus) protein hydrolysates. *Food Chemistry,* 224**,** 414-422.

Hanboonsong, Y., T. Jamjanya & P. B. Durst. 2013. *Six-legged livestock: edible insect farming, collection and marketing in Thailand*.

Hansen, B. (1989) Determination of nitrogen as elementary N, an alternative to Kjeldahl. *Acta Agriculturae Scandinavica,* 39**,** 113-118.

Henchion, M., M. Hayes, A. M. Mullen, M. Fenelon & B. Tiwari (2017) Future protein supply and demand: strategies and factors influencing a sustainable equilibrium. *Foods,* 6**,** 53.

Inoué, S. 2006. Foundations of confocal scanned imaging in light microscopy. In *Handbook of biological confocal microscopy,* 1-19. Springer.

Janssen, R. H., J.-P. Vincken, L. A. M. van den Broek, V. Fogliano & C. M. M. Lakemond (2017) Nitrogen-to-protein conversion factors for three edible insects: Tenebrio molitor, Alphitobius diaperinus, and Hermetia illucens. *Journal of agricultural and food chemistry,* 65**,** 2275-2278.

Jonas-Levi, A. & J.-J. I. Martinez (2017) The high level of protein content reported in insects for food and feed is overestimated. *Journal of Food Composition and Analysis,* 62**,** 184-188.

Kim, H. W., D. Setyabrata, Y. Lee, O. G. Jones & Y. H. B. Kim (2017) Effect of House Cricket (Acheta domesticus) Flour Addition on Physicochemical and Textural Properties of Meat Emulsion Under Various Formulations. *Journal of food science,* 82**,** 2787-2793.

Kralova, I. & J. Sjöblom (2009) Surfactants used in food industry: a review. *Journal of Dispersion Science and Technology,* 30**,** 1363-1383.

Krieg, R. C., Y. Dong, K. Schwamborn & R. Knuechel (2005) Protein quantification and its tolerance for different interfering reagents using the BCA-method with regard to 2D SDS PAGE. *Journal of biochemical and biophysical methods,* 65**,** 13-19.

Krüss, D. S. A. (1997) Drop Shape Analysis. *Manual, Krüss GmbH, Hamburg*.

Leal-Calderon, F. & V. Schmitt (2008) Solid-stabilized emulsions. *Current Opinion in Colloid & Interface Science,* 13**,** 217-227.

Liu, F. & C.-H. Tang (2013) Soy protein nanoparticle aggregates as Pickering stabilizers for oil-in-water emulsions. *Journal of agricultural and food chemistry,* 61**,** 8888-8898.

Magdassi, S. & Z. Neiroukh (1990) Chitin particles as O/W emulsion stabilizers. *JOURNAL OF DISPERSION SCIENCE ANDTECHNOLOGY,* 11**,** 69-74.

Marshall, M. R. 2010. Ash analysis. In *Food analysis*, 105-115. Springer.

Masel, R. I. 1996. *Principles of adsorption and reaction on solid surfaces*. John Wiley & Sons.

McClements, D. J. (2004) Protein-stabilized emulsions. *Current opinion in colloid & interface science,* 9**,** 305-313.

--- (2007) Critical review of techniques and methodologies for characterization of emulsion stability. *Critical reviews in food science and nutrition,* 47**,** 611-649.

---. 2015. *Food emulsions: principles, practices, and techniques*. CRC press.

Megido, R. C., C. Gierts, C. Blecker, Y. Brostaux, É. Haubruge, T. Alabi & F. Francis (2016) Consumer acceptance of insect-based alternative meat products in Western countries. *Food quality and preference,* 52**,** 237-243.

Murphy, R. (2017) Production, Interfacial Behavior, Modification and Functionality of Whey Protein Microgels.

Nakagaki, B. J., M. L. Sunde & G. R. DeFoliart (1987) Protein quality of the house cricket, Acheta domesticus, when fed to broiler chicks. *Poultry Science,* 66**,** 1367-1371.

Noordmans, J. & H. J. Busscher (1991) The influence of droplet volume and contact angle on liquid surface tension measurements by axisymmetric drop shape analysis-profile (ADSA-P). *Colloids and surfaces,* 58**,** 239-249.

Oliveira, J. F. S., J. P. De Carvalho, R. F. X. B. De Sousa & M. M. Simao (1976) The nutritional value of four species of insects consumed in Angola. *Ecology of Food and Nutrition,* 5**,** 91-97.

Omotoso, O. T. (2015) An Evaluation of the Nutrients and Some Anti-nutrients in Silkworm, Bombyxmori L.(Bombycidae: Lepidoptera). *Jordan Journal of Biological Sciences,* 8.

Pandolfe, W. D. (1995) Effect of premix condition, surfactant concentration, and oil level on the formation of oil-in-water emulsions by homogenization. *Journal of dispersion science and technology,* 16**,** 633-650.

Pereda, J., V. Ferragut, J. M. Quevedo, B. Guamis & A. J. Trujillo (2007) Effects of ultra-high pressure homogenization on microbial and physicochemical shelf life of milk. *Journal of dairy science,* 90**,** 1081-1093.

Pickering, S. U. (1907) Cxcvi.—emulsions. *Journal of the Chemical Society, Transactions,* 91**,** 2001-2021.

Rumpold, B. A. & O. K. Schlüter (2013) Nutritional composition and safety aspects of edible insects. *Molecular nutrition & food research,* 57**,** 802-823.

Sherman, P. (1995) Critique of some methods proposed for evaluating the emulsifying capacity and emulsion stabilizing performance of vegetable proteins. *Italian journal of food science: IJFS= Rivista italiana di scienza degli alimenti*.

Tzoumaki, M. V., T. Moschakis, V. Kiosseoglou & C. G. Biliaderis (2011) Oil-in-water emulsions stabilized by chitin nanocrystal particles. *Food hydrocolloids,* 25**,** 1521-1529.

Van Huis, A. (2013) Potential of insects as food and feed in assuring food security. *Annual Review of Entomology,* 58**,** 563-583.

van Zanten, H. H. E., H. Mollenhorst, C. W. Klootwijk, C. E. van Middelaar & I. J. M. de Boer (2016) Global food supply: land use efficiency of livestock systems. *The International Journal of Life Cycle Assessment,* 21**,** 747-758.

Yamada, H., Y. Mizota, K. Toko & T. Doi (1997) Highly sensitive discrimination of taste of milk with homogenization treatment using a taste sensor. *Materials Science and Engineering: C,* 5**,** 41-45.

Yasumatsu, K., K. Sawada, S. Moritaka, M. Misaki, J. Toda, T. Wada & K. Ishii (1972) Whipping and emulsifying properties of soybean products. *Agricultural and Biological Chemistry,* 36**,** 719-727.

Yi, L., C. M. M. Lakemond, L. M. C. Sagis, V. Eisner-Schadler, A. van Huis & M. A. J. S. van Boekel (2013) Extraction and characterisation of protein fractions from five insect species. *Food chemistry,* 141**,** 3341-3348.

Zhang, M., A. Haga, H. Sekiguchi & S. Hirano (2000) Structure of insect chitin isolated from beetle larva cuticle and silkworm (Bombyx mori) pupa exuvia. *International Journal of Biological Macromolecules,* 27**,** 99-105.

Zhang, Y., Z. Chen, W. Bian, L. Feng, Z. Wu, P. Wang, X. Zeng & T. Wu (2015) Stabilizing oil-in-water emulsions with regenerated chitin nanofibers. *Food chemistry,* 183**,** 115-121.