Insect proteins as emulsifiers in oil-in-water emulsions

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Abstract

Insect protein was extracted using an alkaline extraction method with an ethanol defatting step from six species and two life stages of commercially available insect species. The protein extracts and whole insect powders were analysed for composition and protein structure, oil-in-water emulsions were produced from the protein extracts at 0.44 % protein with a sunflower oil dispersed phase.

The first study compared protein extracts from Acheta domesticus (house cricket), Gryllodes sigillatus (banded crickets), Gryllodes bimaculatus (black crickets) and Gryllus assimilis (silent crickets) by composition, emulsion formation and the emulsion stability over a 40-day period. Little difference was found in the protein secondary structure in either the protein extracts or the whole insect powders. The amino acid composition of the four cricket protein extracts was found to be similar. House cricket protein extract produced emulsions with a marginal but significantly lower droplet diameter. Emulsions produced from all four cricket protein extracts were stable to coalescence over the 40-day period. These results suggest that protein from an insect species within the order *Orthoptera* can be used interchangeably to stabilise oil-in-water emulsions.

The second study compared the composition from protein extracts form *Tenebrio molitor* (mealworm) larvae and adult beetles, *Achroia grisella* (waxworm) larvae and banded crickets and emulsions the proteins formed. No difference was found in between droplet diameter of emulsions at pH 7 when heat-treated emulsions or non-heat-treated, with or without non-ionic surfactants. Heat-treatment at pH 5 increased the droplet diameter of emulsions from all insect protein types, but not in the presence of a non-ionic surfactant. Banded cricket protein emulsions adjusted to pH 5 with a non-ionic surfactant showed an increase in droplet diameter, mealworm beetle protein emulsions did not, and the larval protein emulsion droplet diameter increased marginally. These results suggest that there are important differences between proteins extracted from different insect order when used in emulsions.

The third study investigates the reasons behind the difference in the emulsions from the banded cricket and mealworm beetle protein extracts. Differences in interfacial protein concentration, droplet charge and interfacial activity were found.

This research has shown that insect proteins emulsions are stable in a range of processing conditions and have potential to be used in food products.

1 Introduction

Insects have been a food source for humans since at least 800 BCE and have continued to be eaten in parts of Asia, Africa, South America and Mexico up to the present day (Van Huis et al., 2013). That is to say that Europe and populations of European descent are the anomalies in not consuming insects. In fact, eating insects is often associated with disgust in European populations (Ruby et al., 2015, Russell and Knott, 2021). It is not within the scope of this study to try to understand why the peoples of Europe have come to view insects with this attitude but that insects are beginning to be legalised and sold for human consumption provide the opportunity for attitudes to shift. Moreover, the large number of people who already eat insects, and in some cases, consider specific species a delicacy should not be ignored.

Research into if and how to utilise insects in food has been increasing in volume since around 2013 when Van Huis wrote the Food and Agriculture Organization (FAO) paper *Edible insects: future prospects for food and feed security.* Research into the nutritional composition of insects had been performed as early as the 1980s usually with regards to use in animal feeds (Defoliart et al., 1982, Delvalle et al., 1982, Redford and Dorea, 1984). In the past decade, research has increased in both volume and depth of study. Beginning with the (Yi et al., 2013) showing insect protein from five species had a range of functional properties. A specific journal was created in 2015 at Wageningen University *Journal of Insects as food and feed* demonstrating the volume of work that has been done.

Food systems today involve the mass production of food products which are given specific organoleptic and nutritional properties by food ingredients. To this end, research has diversified to include the technical function of whole insect powders and insect fractions to assess the potential for use in food products. The high protein content of some insect species and the multiple functional properties of protein in food structure: gelling, oil binding, water binding, foams emulsions has led to a focus on protein utilisation. However, to take insect proteins from a potential ingredient, replacing the current suite of proteins such as whey, casein and soy, to a viable commercial option a deeper understanding of how proteins from specific insects behave in the conditions of a food product are needed. Insect protein functionality with regards to pH, ionic strength, heating, cooling, moisture content and presence of other molecules which may interact with the protein are all essential.

Emulsified oil droplets are present in traditional foods such as soups, sauces, dressings and stews; and in more recent innovations such as many types of soft drinks, milkshakes and meal drinks. When producing these products at scale proteins which behave in known and predictable way are essential because mistakes and inconsistencies can lead to waste on a mass-produced scale.

In countries where whole insects are already consumed, insect protein is a potential ingredient to be used in emulsion type food products. Furthermore, as countries grow economically and industrialise then demand for processed food products increases and the demand for ingredients such as proteins which can be used in these products.

It has been suggested that insects used as an ingredient as a food ingredients where the consumer does not see whole insects that the people studied were more willing to try and eat foods containing insects in western countries. While this shows a potential mechanism for increasing acceptance caution must be taken. Adding insects to foods which previously did not contain them then simply writing it on the ingredients list without any other signalling could lead to people feeling mislead and therefore discourage future attempts to use insects.

There are considerable benefits to be had in the introduction of insect proteins in processed food products and animal feeds. Before insect proteins can be utilised in foods the insect protein concentration, yield and functionality need to be understood. This last point functionality as it pertains to oil-in-water emulsions is the focus of this doctoral thesis.

The principle aim of this thesis was to further understand which proteins from insects are a viable emulsifier in oil-in-water food emulsions and under which physicochemical conditions. There are over a million known species of insects so the question arises as to protein from which species can be used as an emulsifier and at which level of taxonomic classification the differentiation occurs between emulsion formation and no emulsion formation.

The first objective was to test if emulsions within a taxonomic order of insects have a similar protein extract composition, protein structure and capacity to form emulsions. The order of Orthoptera was tested because of the commercial availability of the cricket species from this order.

The second objective was to determine if insects from different orders and life-stages have similar protein extract composition, protein structure and form emulsions with the same properties at different pH levels, heat treatments, in the presence of a surfactant. Chapter 6 brings the model emulsions to closer to a food product with the introduction of processing steps which begin to reflect those of a food product such as pH shifts, temperature processing and the addition of interfacially active ingredients. Furthermore, the investigation into the level of taxonomic classification at which the insect proteins produce emulsions with different properties by investigating three different taxonomic orders. The introduction of life stage as a factor allowed for the difference within the life of a single species to be investigated.

The third objective was to determine the underlying reason for the differences in found in Chapter 6, taking two of the insect protein extracts and investigating the properties of the emulsions produced more deeply.

This thesis is organised into literature review chapter, techniques chapter, three self-contained chapters of experimental results and discussions, a chapter of overall conclusions from all three results chapters together with proposed further work. Each of the results chapters is structured in the manner of an academic paper with an introduction giving the rational for the work contained in the chapter in the context of the current literature. Each introduction contains different a broader argument as to why it is worthwhile expending resources to investigate. The techniques chapter gives an overview of the techniques used; the details of the methods are given in each results

chapter with specific parameters because the details for some techniques differ between chapters.

2 Literature Review

2.1 Protein Stabilised Emulsions in Foods

Emulsions are kinetically stable colloidal dispersions of two immiscible phases. A thermodynamically lower energy state would be achieved by complete separation of the two phases and a minimum interfacial area (McClements, 2016, Lam and Nickerson, 2013). However, the presence of an amphiphilic molecule at the interface stabilises a larger interfacial area by the lowering of interfacial tension and creating a kinetic barrier to phase separation. Separate oil and aqueous phases have a lower Gibbs free energy than emulsion where one of the phases is dispersed droplets. In order for the phases of the emulsion to separate the kinetic barrier provided by the emulsifier at the interphase must be overcome. Emulsions involving aqueous and oil phases can be oil-in-water (o/w), water-in-oil (w/o) or double emulsions such as oil-in-water-in-oil (o/w/o) and water-in-oil-water (w/o/w) . At the time of writing, published literature of insect protein stabilised emulsions has focused on o/w emulsions and is limited in scope. As a result, this review will include the current literature on proteins used commercially in o/w emulsions: milk proteins, meat proteins, soy protein isolate and insect protein stabilised emulsions. Milk proteins are established emulsifiers and provide insight into the interfacial properties through the contrasting structures of casein and whey proteins. Insect myofibrillar proteins

are similar in structure to vertebrate myofibrillar proteins and so meat proteins are of relevance to the interfacial properties of insect protein.

2.1.1 Emulsion Formation

Emulsions are generally considered to have droplet diameters in the region of 0.1 – 100 µm and nanoemulsions have droplet diameters of less than 100 nm (Lam and Nickerson, 2013). To form an emulsion the two immiscible phases are dispersed through the application of shear forces in a homogenisation process. Homogenisation is usually a single stage process but can involve multiple stages. In a multistage process, the first phase is usually a dispersal with a high shear mixer to produce an initial emulsion with a larger droplet diameter than requires then a second homogenisation often with an alternative method to produce a smaller droplet diameter of a given distribution (McClements, 2016).

The Laplace pressure (ΔP_L) explains the forces governing the spherical shape of an emulsion droplet and the forces required to disrupt a larger emulsion droplet into smaller droplets.

Equation 1 The equation for the Laplace pressure of a droplet where ϒ is the interfacial tension, d is the droplet diameter

$$\Delta P_L = \frac{4\gamma}{d}$$

Where γ is the interfacial tension and *d* is the diameter of the droplet. The force required to disrupt a droplet increases as the diameter of the droplet decreases and as the interfacial tension increases. The disruptive forces and energy input differ by homogenisation process and consequently the diameter of emulsion droplet produced by each process differs. High speed mixers can produce

emulsions with minimum droplet diameter of around 2 µm, colloid mills 1 µm; whereas high-pressure homogenisation, micro-fluidisation and ultrasonic techniques which involve cavitational forces as well as turbulent and laminar flow can produce emulsions of 0.1 µm or lower in some cases. Highly efficient membrane processes can produce emulsions with droplets of 0.3 µm (McClements, 2016).

Proteins and surfactants lower interfacial tension by adsorbing to the oil-water interface. Protein adsorption takes place in three phases: diffusion to the interface, adsorption to the interface and reorganisation and conformation changes at the interface (Graham and Phillips, 1979). In protein stabilised o/w emulsions, the protein is dispersed in the aqueous phase. The rate at which a protein molecule diffuses to the interface plays a role in the rate at which an emulsion droplet can be stabilised and therefore the diameter of the emulsion droplet which can be produced by a given protein. Generally, a smaller more stable emulsion droplet is produced from proteins with lower molecular weight, higher surface hydrophobicity, higher molecular flexibility, higher surface charge and consequently aqueous solubility (Sharif et al., 2018). However, surface hydrophobicity and surface charge have a degree of interdependence resulting in negative interaction effect because they affect the droplet diameter through different mechanisms (Karaca et al., 2011b). Proteins diffuse more slowly to the oil-water interface than smaller surfactant molecules (McClements, 2016, Lam and Nickerson, 2013). A higher protein surface charge increases solubility of the protein in the aqueous phase and subsequently increases the rate of diffusion to the interface and has a

significant effect on emulsion droplet diameter (Karaca et al., 2011b). The emulsifying capacity of a protein is dependent on the surface hydrophobicity of the protein molecule (Kato and Nakai, 1980, Keshavarz and Nakai, 1979). The higher surface hydrophobicity allows for more efficient adsorbance to the oil-water interface up the point where it affects solubility in the continuous aqueous phase whereby diffusion rate may be reduced. Protein flexibility and conformation influence emulsifying properties, more flexible proteins can orient with hydrophobic regions in the oil phase and charged and polar regions in the aqueous phase reducing the interfacial tension at a faster rate than more rigid and globular proteins (Freer et al., 2004a, Freer et al., 2004b, Bos and van Vliet, 2001). Emulsifier concentration in the initial solution before emulsification can impact the droplet diameter in emulsions. A higher emulsifier concentration allows smaller droplets to be formed in an emulsion because more molecules are available and a larger interfacial area can be covered for the same volume of continuous phase.

2.1.2 Emulsion Stability

The processes which to lead to the degradation of the emulsion structure are creaming, flocculation, coalescence and Ostwald ripening (McClements, 2016, Tcholakova et al., 2006). Ostwald ripening is the diffusion of the dispersed phase from smaller diameter droplets to larger diameter droplets via the continuous phase (Taylor, 1998). The higher Laplace pressure in smaller droplets, shown in Equation 1, provide the driving force for the diffusion to larger droplets. The solubility of the dispersed phase in the continuous phase is the principle factor in the rate of Ostwald ripening but the composition of the

interfacial layer is still a lesser factor (Dickinson et al., 1999b). Ostwald ripening in protein emulsions occurs at a low rate because the mechanical strength of the interfacial layer which is a result of the protein interactions and cross links at the interfacial layer (Trevino et al., 1993, Taylor, 1998, Dickinson et al., 1999b, McClements, 2016) So due to both low solubility in the aqueous continuous phase and the interfacial layer Ostwald ripening is insignificant in comparison to coalescence as a mode of microstructure degradation in protein stabilised emulsions (Lam and Nickerson, 2013).

Coalescence is the fusing of a least two droplets of the dispersed phase into one larger droplet with a thermodynamically favourable reduction in interfacial area. In order to coalesce droplets must first come into physical contact through creaming and flocculation. Creaming is the propensity for droplets to separate on a basis of density. In the case of o/w emulsions the lower density dispersed oil phase forms a cream layer at the top of the emulsions allowing for closer associations between droplets which can act as a precursor to coalescence (McClements, 2016). The creaming rate is affected by the droplet radius, the density differential and viscosity of the continuous phase and is explained by the stokes law equation. v_{stokes} is the terminal velocity of a emulsion droplet in an emulsion and is calculated from the following stokes law equation:

Equation 2 The Stoke's law equation which calculates the velocity of an emulsion droplet

$$v_{Stokes} = -\frac{2gr^2(\rho_2 - \rho_1)}{9\eta_1}$$

Where ρ_1 is the density of the continuous phase, ρ_2 is the density of the dispersed phase, η_1 is the viscosity of the continuous phase and when v_{Stokes} is positive droplets move up which indicates creaming and negative droplets move down indicating sedimentation in the unit ms^{-1} (McClements, 2016). Creaming or sedimentation increase as the droplet radius increases, the difference in density of the dispersed and continuous phase and the viscosity of the continuous phase decreases. Therefore, creaming in the case of an oil-in-water emulsion can be controlled by reducing the droplet radius or increasing the viscosity of the dispersed phase by adding a hydrocolloid, for example. In addition, creaming can be controlled by increasing the volume fraction of the dispersed oil phase above a critical value whereby a stable microstructure is produced which spans the entire vertical space, this is dependent on droplet distribution and can be lower when the droplets are strongly flocculated (Dickinson, 2010, Dickinson and Golding, 1997).

Flocculation is the association of two or more droplets into an aggregate where the viscoelastic interfacial film of each individual droplet remains intact (Dickinson, 2010). It has been reported that in β-lactoglobulin stabilised emulsions with no excess of protein in the aqueous phase are less stable to flocculation from heat compared to emulsions with an excess of protein in the continuous aqueous phase, particularly if the excess protein is added after homogenisation (Kim et al., 2005b).

A lower aqueous phase concentration of protein produces a thinner monolayer of protein at the oil-water interface because the protein can alter conformation

and cover a larger area, as protein concentration is increased the density of the molecules at the interface can increase and the properties of the interface change (Bos and van Vliet, 2001). Proteins form stable viscoelastic films at the interface stabilising the oil droplet against phase seperation and coalescence. The principle factors maintaining the stability of an emulsion against flocculation are electrostatic repulsion between droplets, steric hindrance provided by the molecules composing the interfacial layer and the viscosity of the continuous phase inhibiting droplet collisions.

The charge groups on the protein are oriented towards the aqueous phase, so when the pH of the continuous aqueous phase is above the isoelectric point of the protein the net charge on the droplets is negative consequently there is a repulsive force between the emulsion droplets and equally when the pH is below the isoelectric point the charge is positive and repulsive (Dickinson, 2010, Tcholakova et al., 2006). At the isoelectric point of the protein the net charge is neutral and maximal droplet aggregation can occur.

The differing conformation at the interface has been confirmed in both differential scanning calorimetry in soy protein isolate (Keerati-u-rai and Corredig, 2009) and Fourier transform infrared spectroscopy in β-lactoglobulin (Fang and Dalgleish, 1997). Proteins which can form long protrusions from the interface into the continuous phase composed of majority hydrophilic amino acids providing a steric barrier against coalescence of oil droplets (Tcholakova et al., 2006).

2.1.3 Protein emulsifiers by type

2.1.3.1 Milk Proteins

Milk proteins can be divided into two main fractions: whey proteins and caseins. Whey protein would have been the soluble fraction which remained after the enzyme precipitation with rennet of the caseins for cheese making. Caseins are mostly commonly used in the form of sodium caseinate in the food industry as an emulsifier (Phillips and Williams, 2011b).

2.1.3.1.1 Caseins

Casein fraction of milk is composed of α_{S1}-casein 40 %, α_{S2}-casein 10 %, β-casein 40 % and κ-casein 10 % (Phillips and Williams, 2011b). β-casein is 24 kDa amphiphilic flexible random coil protein. β-casein has a hydrophobic head and a hydrophilic tail which protrudes into aqueous phase producing steric hinderance stabilised by 5 phosphoserine residues, producing a compressed inner layer at , < 2 nm, of protein at the interface and reducing to 1 % at 10 nm and a total thickness of 10 -15 nm (Dickinson, 2001, Atkinson et al., 1995). α_{S1}-casein has a slightly lower molecular weight than β-casein and the hydrophobic residues are distributed along the entire amino acid chain producing higher charged dispersed oil droplets with protein loops into the aqueous layer producing a further steric hinderance between drops (Dickinson, 2001).

Caseinates are the casein fraction of milk without further purification into individual proteins; this can be in the form of sodium caseinate, calcium caseinate or manufactured combinations of caseins to form a caseinate. Caseinate emulsions at 2 % wt, 20 % soybean oil, 20 mM buffer salt were heat

stable to 90 °C and 121 °C for 30 min at pH 7 but not at pH 3 (Hunt and Dalgleish, 1995). Caseinate can stabilise emulsions at lower minimum surface concentration than whey protein at 1.00 mg/m^2 and 1.50 mg/m^2 respectively which was attributed to the more flexible conformation of the caseinate protein molecules (Hunt and Dalgleish, 1994). Yet, the interfacial saturation concentration in the bulk aqueous phase for both caseinate and whey protein isolate is 2.25 % wt (Hunt and Dalgleish, 1994). Caseinate absorbs preferentially to the oil water interface over whey protein isolate when both proteins are in excess due to the more rapid orientation at the interface (Hunt and Dalgleish, 1994).

2.1.3.1.2 Whey

Whey proteins are a group of globular highly soluble proteins composed of by mass 60 % β-lactoglobulin, 20 % α-lactoglobulin, 10 % immunoglobulin G, 3 % bovine serum albumin and < 1 % lactoferrin (Farrell et al., 2004). β-lactoglobulin is a 162 amino acid residue, 18.363 kDa in size, isoelectric point of pH 5.35 with two variants A and B (Farrell et al., 2004). β-lactoglobulin is rich in hydrophobic amino acids with 22 Leucine residues, 10 Isoleucine and 9 or 10 Valine residues depending on the variant (Phillips and Williams, 2011b).

Whey protein isolate is the form commonly utilised in food products. The high solubility combined with a large proportion of hydrophobic amino acids allows diffusion to the oil-water interface and amphipathic properties allow for the stabilisation of the oil water interface. Whey protein isolate: 2 % wt, at pH 7 and pH 3, 20.0 mM buffer salts in an emulsion with 20 % wt soybean oil

produced stable emulsions with no change in droplet diameter ($d_{4,3}$) when heated to 90 °C and 121 °C for 30 mins (Hunt and Dalgleish, 1995). Whey protein isolate did not form an emulsion at pH 4 due to poor solubility from proximity to the isoelectric point (Hunt and Dalgleish, 1995). KCl concentrations of less than 25 mM had no effect on emulsion droplet diameter, at KCl concentrations up to 200 mM emulsion droplet diameter ($d_{4,3}$) increased particularly at pH 3 (Hunt and Dalgleish, 1995). Above 1 % wt continuous phase protein concentration a smaller emulsion droplet diameter was not achieved showing that protein concentration was not the limiting factor (Hunt and Dalgleish, 1994). The interfacial concentration of whey protein isolate showed a plateau where increasing continuous phase concentration from 1 % to 1.75 % did not increase interfacial concentration, above 1.75 % a sharp increase in interfacial concentration was observed suggesting a multilayer interface (Hunt and Dalgleish, 1994). β-lactoglobulin produces a more compact interface than the casein proteins of 2-3 nm (Atkinson et al., 1995) in air-water which develops from a single layer of protein particles (Boerboom et al., 1996) into a viscoelastic gel layer through the unfolding and inter linking of the protein molecules at the interface through covalent and electrostatic bonds (Wijmans and Dickinson, 1999, Wijmans and Dickinson, 1998, Petkov et al., 2000).

2.1.3.2 Meat Proteins

Meat batters are oil-in-water emulsions stabilised by myofibrillar proteins and are a production stage in many emulsion type meat products such as sausages (Chen et al., 2018). Amiri et al. (2018) estimates that myofibrillar proteins account for more than 75 % and possibly as high as 90 % of the emulsifying

capacity of meat. And of the myofibrillar proteins actin and myosin are the primary interfacially active proteins (Smith, 1988). Meat proteins and particularly myofibrillar proteins have not been used to the same extent as milk and soy proteins because of low water solubility (Chen et al., 2017) and aggregation to form gel networks under heating which can disrupt the oil-water interface (Li et al., 2018). Although emulsion gels where the continuous phase is gelled can prevent coalescence.

Emulsions produced with salt extracted myofibrillar chicken protein, 8 mg/ml and soybean oil 20 % in a high shear mixer had a monomodal particle distribution with $d_{4,3}$ = 2082.67 ± 45.76 nm (Li et al., 2020). An increased particle diameter with increased protein concentration was attributed to increased viscosity of the continuous phase, protein aggregation and increased interfacial protein concentration (Li et al., 2020). Through isolation of the proteins at the oil-water interface and SDS-PAGE it was shown that the myosin heavy chain 220 kDa and actin 43 kDa were the main proteins present with lesser amounts of tropomyosin relative to non-absorbed protein with C-protein and a-actin also present at the interface in smaller amounts due to lower proportions in the myofibrillar proteins (Li et al., 2020). The protein secondary structure of the myofibrillar protein is 41.9 % ± 2.64 α-helix, 19.95 % ± 2.71 β-sheet, 22.73 % ± 1.65 β-turn and 15.43 % ± 2.70 random coil (Li et al., 2020).

Alkaline extracted myofibrillar proteins can produce stable o/w emulsions. Alkaline extracted isoelectric point precipitation protein from less functional pale, soft exudative chicken breast produced emulsions by high shear mixing

with 20 % soybean oil produced monomodal droplet diameter distribution with $d_{4,3}$ of 89 µm at 50 mg/ml, 306 µm at 100 mg/ml and 546 µm at 150 mg/ml stable up to 72 h, the maximum storage period measured (Zhao et al., 2019). Zeta potentials ranged from -19.5 mg/ml at 50 mg/ml protein concentration to -36.7 mg/ml at 150 mg/ml protein (Zhao et al., 2019).

Myofibrillar proteins emulsions have shown to form aggregates when heated in alkaline conditions. A 75 °C heat treatment for 30 min at pH 9.0 of chicken myosin produced emulsions with no significant difference in interfacial concentration compared to native myosin in an o/w emulsion (Li et al., 2018). The $d_{4,3}$ of the pH 9 heat treated emulsion was slightly higher than native myosin, yet the rate of reduction in interfacial tension was higher (Li et al., 2018). A faster rate of interfacial tension reduction would be expected to decrease the emulsion $d_{4,3}$ suggesting flocculation may be higher in the heat and alkaline treated myosin emulsions.

2.1.3.3 Soy Proteins Isolate

Soy protein isolate can produce o/w emulsions with smaller droplet diameter than whey protein and sodium caseinate, and has been used in dairy based yoghurts, ice-creams and other emulsion food products (Damodaran, 2005, Yada, 2017).

Soy protein isolate is composed of storage proteins β-conglycinin (7S globulin) and glycinin (11S globulin). Glycinin has a molecular mass 300 – 380 kDa, denatures at 85 °C (Keerati-u-rai and Corredig, 2009) , a hexamer with five main subunits: Group I A_{1a} B_{1b} of 53.6 kDa and A_2B_{1a} of 52.4 kDa; Group II $A_{1b}B_2$ of

52.2 kDa, $A_5A_4B_3$ of 61.2 kDa, and A_3B_4 of 55.4 kDa (Nielsen, 1985, Nielsen et al., 1989, Thanh and Shibasaki, 1979) . The subunits are composed of two polypeptides one basic and one acidic of around 20 kDa and 35 kDa respectively joined by a disulphide bridge (Staswick et al., 1984). β-conglycinin has a molecular mass of 150 – 220 kDa, denatures at 68 °C (Keerati-u-rai and Corredig, 2009), and is composed of three principle subunits α' of 72 kDa, α of 68 kDa and β of 52 kDa and a lesser γ subunit (Thanh and Shibasaki, 1979). The protein is heterogenous in the specific combinations of the subunits.

The β-conglycinin is more interfacially active than glycinin and of the β-conglycinin subunits have interfacial activity from highest to lowest α', α then β (Puppo et al., 2011). β-conglycinin secondary structure as measured by x-ray diffraction is composed of 10 % α-helix, 33 % β-structure and 57 % disordered or random coil (Utsumi et al., 1997). Glycinin secondary structure 8 % α-helix, 36 % β-structure and 56 % disordered or random coil (Phillips and Williams, 2011b, Utsumi et al., 1997).

The emulsion stability and emulsion capacity of soy flour, soy protein concentrate and soy protein isolate increased with an increase of pH level from pH 6.0 – pH 8.0 (Wang and Zayas, 1992). Emulsions stabilised with 1 % soy protein isolate, 10 % oil and homogenised in a Microfluidizer produced monomodal emulsions with a $d_{4,3}$ of 1 µm (Keerati-u-rai and Corredig, 2009). A 75 °C or 95 °C heat treatment after emulsification caused a shift to a bimodal distribution but greater stability in droplet diameter overtime, likely through aggregation of droplets. Whereas a heat treatment of the protein dispersion

before emulsification of 75 °C produced a bimodal distribution with one group of droplet diameter < 0.1 µm and a second between 1 µm and 10 µm and at 95 °C produced a monomodal distribution of $d_{4,3}$ of 10 µm. The smaller droplet diameter species at 75 °C were explained by dissociation of the subunits of β-conglycinin giving faster adsorption to the interface and partial denaturation of glycinin, the larger droplet diameter was attributes to denaturation and aggregation of both β-conglycinin and glycinin both these conclusion were supported by SDS-PAGE analysis of absorbed protein composition (Keerati-u-rai and Corredig, 2009). A third thermal transition was observed in protein recovered from the o/w interface at over 90 °C, suggesting conformational changes at the interface.

2.2 Insect Composition

2.2.1 Insect Taxonomy

Insect taxonomy is the classification of organisms based on the morphology or exhibited phenotype (Barnard, 2011). Taxonomy of insects was occurring at least as early as the 16th century with the Italian Ulisse Aldrovandi, and pre-dates the concept of evolution which was proposed by Charles Darwin in the 19th century (Kjer et al., 2016). Since the understanding of DNA structure and genetics was yet to be discovered taxonomy was entirely based on morphology initially, then as the understanding of evolutionary theory developed morphology with reference to the evolutionary origins which is now known as phylogeny (Kjer et al., 2016). Although the first phylogenetic tree was published in 1904, Willi Hennig systematised the creation of phylogenetic trees in the 20th

century by determining monophyletic groups with a single common ancestor and a formalising some of the commonly used methodology.

Modern molecular phylogenetics, where phylogenetic trees are constructed based on genetic evidence started in the 1980s and were based on RNA data. More recently DNA barcoding has been used to distinguish between species and form phylogenetic trees using mitochondrial genetic markers in 16S r RNA, 12S r RNA, ND (1-6 genes) (De Mandal et al., 2014). The advent of large but incomplete datasets of genetic data, increasing computing power and improved modelling techniques has led to improved and ever evolving phylogenetic trees (Chesters, 2017, Gullan, 2014). However, the genetic and morphological approaches are not independent. DNA barcoding can aid in the differentiation of cryptic species, where a single identified species may in actuality be two or more species (Bickford et al., 2007). The differentiation through DNA barcoding then leads to the identification of subtle morphological differences of these species in one or more of the insect life stages (Burns et al., 2008).

Gullan (2014) defines the class *Insecta* morphologically as possessing three principle segments: head, thorax and abdomen. Legs of six segments are attached to the thorax and the abdomen has 11 segments with the gonopore on segment 8 in females and 9 in the male. The body segments stay consistent through larval or nymph stage but are often less developed. The Insecta possess simple eyes (ocelli) and compound eyes, cerci protrude from the abdomen and O_2 and CO_2 transfer occurs via trachea. The mouthparts are external which can

be a key differentiating factor from other Hexapoda. While almost all insects species lay eggs, life stages vary, with larval and pupal, or nymph stages possible in addition to an adult life stage (Halloran et al., 2018).

By species Insecta is the largest class of organisms in the subphyla Hexapoda which is in turn accounts for the largest number of organisms in the phylum Arthropoda. Arthropoda account for 80 % of the 1 552 319 named species in the Kingdom Animalia (Zhang, 2011). The Arthropoda contains by number of named species in each subphyla 67 000 Crustacea, 12 000 Myriapoda, 112 000 Chelicerata and 1 024 000 Hexapoda (Stork, 2018). While there are around one million known species of insect there are an estimated seven million species in total (Stork, 2018). Within the class Insecta the six orders with the largest number of species account for 90 % of the named species as shown in Table 1, and 88 % of the consumed species. In the United Kingdom there are 24 000 species of insect due to the temperate climate with the tropics having disproportionate diversity of species, and the Hymenoptera are the most numerous order in the United Kingdom (Barnard, 2011, Stork, 2018).

Table 1 Number of named species in the six larges orders in the subphyla Insecta, adapted from Stork (2018) data from Zhang (2011) and Van Huis et al. (2013)

Order	Common Names	Number of Named Species	Proportion of Total Consumption by Species
Coleoptera	Beetles	387 000	31 %
Lepidoptera	Caterpillars, Butterflies, Moths	157 000	18 %
Diptera	True Flies	155 000	2 %
Hymenoptera	Bees, Wasps, Ants	117 000	14 %
Hemiptera	True bugs	104 000	10 %
Orthoptera	Crickets, Grasshoppers	24 000	13 %

There are disagreements about the exact number of insect orders with classifications anywhere between 25 to 30 listed but this changed little over time (Gullan, 2014). However, the phylogenetic classification is under constant flux in line with new data and DNA analysis results (Gullan, 2014). According to Zhang (2011) there are 30 orders of insect and 9 extinct orders. The Isoptera (termites) have been added the Blattodea and the orders Phthiraptera (parasitic lice) combined with the Psocoptera giving the order Psocodea leaving 28 orders (Gullan, 2014).

2.2.2 Insect Composition

The proportions of fat, proteins, carbohydrates, micronutrients, vitamins and minerals vary by species, life stage, growth environment and food source. The major components of insects are protein, fat and fibre in the form of chitin (Van Huis et al., 2013).

Chitin is insoluble long chains of N-acetyl glucosamine, a nitrogen containing derivative of glucose which is indigestible for humans. Chitin forms an important structural component of the cuticle in insects and the content varies between 11.6 mg/kg to 137.2 mg/kg on a dry mass basis (Finke, 2007). The nitrogen content of chitin may lead to an overestimate of protein content, when protein is calculated based on nitrogen content then converted to protein using a 6.25 nitrogen factor (Finke, 2007). A nitrogen factor of 4.76 has been suggested for larvae of *Tenebrio molitor*, *Alphitobius diaperinus*, and *Hermetia illucens* (Janssen et al., 2017) and 5.00 for cricket species *Acheta domesticus* and *Gryllus bimaculatus* (Ritvanen et al., 2020). Although Finke (2007) found that chitin nitrogen accounts for a small proportion of total nitrogen in a number of Coleoptera larvae including *Tenebrio molitor* and cricket nymphs.

The fat content of insects can range from as low as 9.12 % for the grasshopper *Zonocerus variegates* and 67.25 % for the cricket *Homorocoryphus nitidulus* on a dry mass basis and is higher in the larval life stage (Womeni et al., 2009, Xiaoming et al., 2010). The range of fat content in a number of insect orders are listed in Table 2. Of the fats contained in insects a large proportion is composed

of triacylglycerides rich in polyunsaturated and monounsaturated fatty acids but this also varies by species (Van Huis et al., 2013, Womeni et al., 2009).

In an evaluation of the protein content Xiaoming et al. (2010) collated ranges of protein content on a dry matter basis including the two largest orders Coleoptera and Lepidoptera, partly summarised in Table 2. The lower and upper bounds of the protein content were species both in the order Hymenoptera at 13 – 77 %, but all of the reported orders showed a large variation in protein content, the smallest variation was the homoptera at 45 – 57 %. In a systematic review of nutrients composition of twelve insect species Payne et al. (2016) noted standard deviations of 50 % of the mean in the protein content of insects of the same species. Adult insects tend to have higher protein contents than the earlier life stages (Ademolu et al., 2010).

Table 2 The range of protein and fat content on a dry mass basis for insect orders adapted from (Van Huis et al., 2013) and (Xiaoming et al., 2010)

Insect Order	**Stage**	**Protein (% range)**	**Fat (% range)**
Coleoptera	Adults and larvae	23 – 66	14 – 36
Lepidoptera	Pupae larvae	14 – 68	5 – 49
Hymenoptera	Adults, pupae, larvae and eggs	13 – 77	8 – 55
Orthoptera	Adults and nymph	23 – 65	-
Hemiptera	Adults and larvae	42 – 74	10 – 44
Homoptera	Adults, larvae and eggs	45 – 57	25 – 31
Odonata	Adults and naiad	46 – 65	14 – 41

2.2.3 Insect Proteins

The main sources of protein in insects by mass are the integument, muscles, the fat body and to a lesser extent the haemolymph (Lamsal et al., 2019). Other groups of proteins present can vary between orders and species but include fibroins which form silk and other filaments, enzymes, hormones and in some cases bioluminescence proteins (Rockstein, 1978). This review will attempt to summarise the principle proteins present in insects their basic function and characteristics of these proteins.

2.2.3.1 Insect Muscle

2.2.3.1.1 Function

Insects contain striated muscle similar to vertebrates but do not contain the structurally different smooth muscle or cardiac muscle (Nation, 2016). Insect muscle can be divided broadly into two or three groups determined by the location of the muscle: skeletal, visceral and wing or cardiac muscles and may be placed in a discrete groups depending on classification (Nation, 2016, Chapman, 2013). Flight muscle can account for up to 60 % of mass in some insects (Marden, 1989). The difference in function of insect muscle is produced by physiology and the number, position and operation of neurons on the muscles not by the structure of the proteins within the muscle (Chapman, 2013, Nation, 2016). Vertebrae striated muscle is synchronous in that there is one contraction per nerve input. Some insects have asynchronous flight or sound producing muscles which contract multiple times for a single neuron input allowing for rapid beating of the wings. Asynchronous muscle is also known as

fibrillar muscle owing to the loose structure of the loose association of the muscle fibres. Visceral muscle can be classified into heart and dorsal vessels, alimentary canal, Malpighian tubules, accessory pulsatile organs, diaphragm and glandular structures.

Muscles are composed of fibres which are constructed of myofibrils which are in turn constructed of segments called sarcomeres. The sarcomeres contain filaments composed of the protein actin and separate filaments of the protein myosin. The sarcomeres are divided by z-bands which are also bound to the actin filaments which protrude across the sarcomere but not to the subsequent z-bands. Between the ≈5 nm diameter actin filaments are ≈20 nm diameter myosin filaments (Ashhurst and Doreen, 1967). The ratio of actin to myosin filaments varies by muscle type with 3:1 ratio common in wing muscles but 6:1 found in intersegmental muscles (Smith 1966). A large number of mitochondria are present in the muscles between the myofibrils, in particular wing muscles, and tracheoles protrude into the muscle to provide oxygen (Nation, 2016). A sarcoplasmic reticulum is located along the surface of the muscle fibres and present to a greater extent in fast synchronous muscle than in slow moving synchronous muscle and fibrillar asynchronous muscles.

2.2.3.1.2 Muscle Proteins

The proteins present in insect muscle are largely the same as in the striated muscle of other animals although some differences in molecular weight and subunit structure have been noted (Nation, 2016, Kominz et al., 1962). Myosin filaments have a tail of three coiled α-helices and large globular double heads

the action of which rely on calcium and ATPase as well as sites for binding to actin. Actin is composed of globular subunits called G-actin which make up filaments of F-actin, with one filament composed of an α-helix of two F-actin chains. There is an active site for myosin attachment on each G-actin subunit. Two α-helical entwined chains of tropomyosin fit into the grooves of the F-actin α-helix and cover the active site for binding to myosin. Troponin, composed of tropomyosin binding, calcium binding and actin binding subunits is globular and activated by nerve impulses whereby it detaches tropomyosin from the myosin binding sites on the F-actin. In mammals as glycogen and ATP decrease, the actin filaments irreversibly crosslink to the myosin filaments forming actomyosin complex and causing rigor after death (Lawrie and Ledward, 2006). When insects are killed by freezing, the insect go into dormancy or diapause before death, consequently the state of the muscle proteins is unclear.

Bullard et al. (1973) studied the flight muscles of *Lethocerus cordofanus*, *L. maximus* (water bugs) and *Heliocorpis japetus* (dung beetle) in comparison to minced rabbit muscle. The myosin of all three insect species was found to be composed of a heavy sub-unit of molecular weight around 200 kDa and two light subunits, the dung beetle subunits were lighter than the *Lethocerus* subunits at 15 kDa and 27 kDa compared to 17 kDa and 30 kDa, the rabbit myosin had three light subunits of 16 kDa, 19 kDa and 25 kDa. The actin in all three species had molecular weight 45 kDa. Tropomyosin and troponin of subunit molecular weight 18 kDa, 27 kDa and 30 kDa were extracted.

A further muscle protein called arthrin was isolated from *Drosophila* and identified as ubiquinated actin; it was suggested there may be one arthrin unit per actin-tropomyosin-troponin unit (Ball et al., 1987). High molecular weight polypeptide doublets with molecular weights 700 kDa for (Hmp I) and 600 kDa (Hmp II) were extracted from *Apis mellifera* (Bees) flight and leg muscle and *Drosophila melanogaster*, and both extracted and studied in *Locusta migratoria* (migratory Locusts) leg muscles (Nave and Weber, 1990). Similar but larger polypeptide doublets are found in vertebrates sarcomeric muscle called titin I and tintin II. Both are connective proteins however, Hmp I/Hmp II is thought to connect I band to the A band whereas vertebrate titin I/titin II connects the Z band to the M band in the myofibrils (Nave and Weber, 1990).

2.2.3.2 Insect Storage Proteins

Proteins which are stored and synthesised in the fat body are known as storage proteins. These storage proteins are at their highest concentration in the late larval stage of many species of insect called the last instar or the period before pupal formation (Nation, 2016). Storage proteins serve as a reservoir of amino acids, in particular aromatic amino acids, for biosynthesis over proceeding growth stages as the insect develops into the adult phase (Nation, 2016, Rockstein, 1978, Haunerland, 1996). Initially discovered in *Calliphora erythrocephala* (a type of blow fly), and named *Calliphorins*, this protein accounts for 60 % of the soluble proteins in the last instar of the larval stage and is a hexamer rich in tyrosine, phenylalanine and methionine (Munn et al., 1971, Munn and Greville, 1969, Munn et al., 1969). Munn and Greville (1969) identified analogous proteins in the orders *diptera* and *lepidoptera*. The

proteins with a greater than 15 % tyrosine and phenylalanine content have been referred to as arylphorins, but often the term hexamerin is used which includes other hexameric storage proteins with different amino acid composition (Haunerland, 1996, Nation, 2016). Hexamerins are composed of six subunits and have been found in other species and orders including bees (Shipman et al., 1987), ants (Wheeler and Martinez, 1995), beetles (Dekort and Koopmanschap, 1994) and locusts (Dekort and Koopmanschap, 1987). Insect storage protein mass and composition fluctuates depending not just on life stage but on the specific instar of the life stage. Storage proteins may account for a greater proportion of extracted proteins if the insect is harvested during the final instar of a pre-adult life stage.

2.2.3.3 Insect Integument Proteins

The cuticle forms the exoskeleton of an insects and together with the epidermal cells from which the cuticle is secreted make up the integument, which is shown in Figure 1. The cuticle has multiple functions in insects including protection against bacteria, fungi, environmental chemicals, predators and parasites; and muscle attachment. The integument as whole has a myriad of functions some common across all species and others specific. These functions include breathing, water control and osmoregulation, feeding and excretion. Due to this wide range of purposes for the integument the chemical makeup and structure can vary (Vincent and Wegst, 2004).

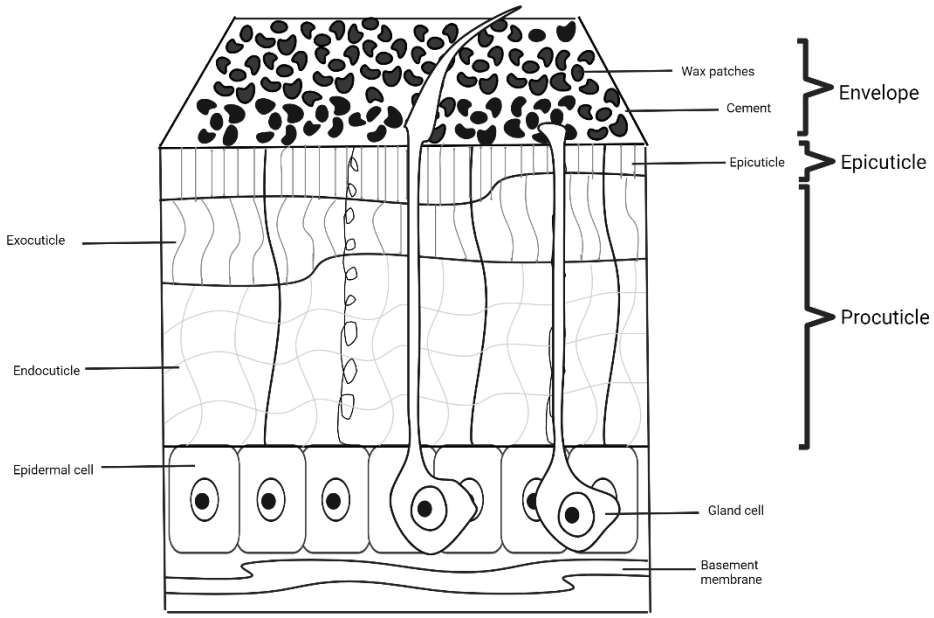

Figure 1 A diagram of the insect integument structure reproduced from Nation (2016) diagram created with BioRender.com

Locke (2001) divided the insect cuticle into three sections: cuticulin envelope, the epicuticle and the procuticle. The cuticulin envelope is poorly understood and is only 10 - 30 nm thick (Locke, 2001).

The epicuticle is 1 – 4 µm thick and is composed of sclerotised proteins, lipoproteins, waxes and cement as well as minerals. The epicuticle contains no chitin and derives its hardness and strength from protein sclerotised by covalent bonds with phenols and quinones. The procuticle is composed of both chitin and protein, the portion termed the exocuticle can be highly sclerotised depending on species and life stage. In many larvae the exocuticle is almost absent giving the cuticle more flexible properties. Whereas in harder adult insects the exocuticle is much harder and rigid. The endocuticle located below the exocuticle is less sclerotised and contains chitin and protein.

2.2.3.3.1 Proteins of the Cuticle

Insect cuticle proteins are extremely diverse with over 1 % of the insect genome codes for cuticle protein (Pan et al., 2018). The material in the insect cuticle excluding chitin is mostly protein and accounts for between 25 % and 37 % of dry weight (Hackman, 1953). A fraction of the protein in insect cuticle is water-soluble. The extractable water-soluble proteins are known not to coagulate in hot water, be soluble in hot not cold aqueous trichloroacetic acid; precipitated by ethanol solutions greater than 45 %, by one-third saturated ammonium sulphate and by saturated sodium chloride and readily redissolves after precipitation (Hackman, 1953).

Proteins are the matrix in which other components such as the chitin are constrained. The proteins are cross-linked and sclerotized to a greater extent in harder insects but still to some degree in softer insects which include many larvae. It has not been possible to assess the degree of crosslinking between chitin and protein because once the protein is sclerotized the extraction processes required to extract the protein breaks any crosslinks between the protein and chitin (Nation, 2016). In Locusts about 70 % of the dry weight of the insect cuticle is protein, before sclerotization 90 % of proteins are extractable but whereas after few are extractable (Nation, 2016). In *Locusta migratoria,* the mass of cuticle increases up to day 13 of the adult life stage, as a proportion of total protein cuticular protein fluctuates but does not increase over the same time period (Phillips and Loughton, 1981). Unsclerotized proteins are dominated by proteins from 10 kDa and 40 kDa in molecular weight in larvae and pupae in *Tenebrio molitor* (mealworms) (Andersen, 2002).

There is evidence that a common amino acid pattern in the primary structure of cuticle protein known as the R & R consensus, due to being first discovered by Rebers and Riddiford (Rebers and Riddiford, 1988, Rebers and Willis, 2001). The R & R consensus may be capable of binding to chitin and forming a β-sheet structure (Iconomidou et al., 2005). The CPR family of cuticle proteins accounts for a large proportion of the cuticle protein genes across numerous genera and forms an amphipathic β-sheet, β-turns and loops and an absence of α-helix (Willis et al., 2012). It has been proposed that insect cuticle proteins have an abundance of intrinsically disordered regions (Andersen, 2011).

The cuticle proteins can be different between the sexes, life stages, body part and species. Cuticle proteins can differ by life stage, in mealworms, it was shown that a water soluble 18 kDa protein was present in larvae that was not present in pupae or adults (Lemoine and Delachambre, 1986).

2.2.3.4 Interstitial Structural Proteins

While the integument provides the structural support for the insect body, other structural proteins perform important structural roles within the insect. Principal among these is collagen, which is common to many other animals. Collagen has 28 different types but some key structural features common to all types. Collagen is a trimer composed of three chain which combine in a triple helix. The amino acid composition of around one third glycine in a [gly-X-Y] repeating amino acid motif where positions X and Y are often proline or hydroxyproline (Phillips and Williams, 2011b, Bella, 2016, Ramachandran and Kartha, 1954, Ricard-Blum, 2011). Each of the three chains is offset by one

amino acid residue to the other, which combined with every third amino acid residue being glycine allows for the tight packing of the chain and each glycine residue is positioned at the helical axis (Bella, 2016). Collagen is insoluble so is not useful for use at interfaces, but when partially hydrolysed into gelatin, in acid or alkaline conditions, gains gelation and interfacial properties (Mad-Ali et al., 2016, Balti et al., 2011). Acid treated Type A gelatin has an isoelectric point of pH 6 – 9 so is positively charged in most food products, alkali treated Type B gelatin, which is less commonly used in food, has an isoelectric point of pH 5.0 so may be positively or negatively charged (Yada, 2004). Type A gelatin is produced from less cross-linked collagen sources such as pig, poultry and fish (Yada, 2004). Gelatin is a polydisperse groups of proteins of varying molecular weight which can be grouped into α- chains 90 – 110 kDa, β-chains 180 – 220 kDa and γ-chains 270 – 300 kDa lower temperature extractions produce longer chain gelatins (Phillips and Williams, 2011b).

As of 1968 collagen had been found in most insect orders (Ashhurst, 1968). Locust and cockroach collagen is organised into fibrils and fibres, composed of 1000 amino acids, and contains 12 % proline and 10 % hydroxyproline (Ashhurst and Bailey, 1980, Sutherland et al., 2013). Only 0.1 % of the protein extracted by trichloroacetic acid from cockroach carcasses was collagen (Rockstein, 1978).

The majority of the structural support in insects is provided by chitin, however collagen containing basement membranes provide additional structural support holding organ and other internal elements in place (Ashhurst, 1968,

Whitten, 1962, Whitten, 1964). The organs in many insects are enclosed in a layer of connective tissue and collagen forms part of this as well as the stroma associated with haemolymph and nerves (Rockstein, 1978). Basement membranes are an extracellular matrix which in some cases is continuous between organs and internal components, sheaths the nerves and anchors Malpighian tubules to other structures such as the gut and exoskeleton (Ashhurst, 1968). Collagen in the neural lamella, an extracellular basement membrane, of *Locusta migratoria* was shown to be collagen in mucopolysaccharide matrix (Ashhurst and Richards, 1964, Ashhurst, 1968).

Type (IV) collagen similar to vertebrate type (IV) collagen has been identified in basement membranes of Drosophila (Yasothornsrikul et al., 1997). Cockroach collagen precipitates at 4.0 M NaCl, contains 8.6 % hexose, the collagen converts to gelatin at 38.5 °C as opposed to 36 °C for calf skin gelatine (Francois et al., 1980). Collagen from the ejaculatory duct of adult male Locusts was analogous to α-1 (I) collagen chains in mammals but with elevated levels of hydroxylysine (Ashhurst and Bailey, 1980).

2.2.3.5 Dumpy

Dumpy is a 2.5 MDa protein first identified in Drosophila with similar proteins identified in numerous other insect orders (Willis, 2010, Wilkin et al., 2000). The dumpy protein is classified as a cuticular protein as it anchors muscles to the cuticle, but also stabilises the tissue around the trachea (Willis, 2010).

2.2.3.6 Proteins of the Haemolymph

Many of the proteins produced in the fat body are released into the haemolymph and reabsorbed into the fat body. Mealworms contain a "moderately abundant" odorant binding protein, dubbed THP12, around 12 kDa in size which has hydrophobic cavities for transporting odorant molecules (Graham et al., 2001). The odorant binding protein comprises about 5% of the proteins in the haemolymph and are abundant in the final instar of the larval stage and in later adult stages of the mealworm life cycle and similar proteins are found in other insect species (Graham et al., 2001). THP12 was previously mistaken as an antifreeze protein. There are a family of anti-freeze proteins found in *Tenebrio molitor* (mealworms) having a range of isoforms of varying molecular weight:8.4 kDa to 10.8 kDa, charge, hydrophobicity and give variable results in SDS-PAGE (Liou et al., 1999).

2.3 Protein Structure and Physicochemical Properties

2.3.1 Protein Structure

The emulsifying and interfacial properties of a protein are determined by the molecular weight, conformation, flexibility, polarity, charge and hydrophobicity. The molecular characteristics are ultimately determined by the type and order of the amino acids. The tertiary structure in reference to the globular or more open random coil nature of the protein has been linked to emulsion droplet diameter and viscoelasticity of the oil-water interface in emulsions (Lam and Nickerson, 2013). The "conformational flexibility " has been linked to the emulsifying properties of proteins as well as the secondary structure with both the secondary and tertiary structure adapting at the interface (Tang, 2017, Tang and Shen, 2013, Zhai et al., 2013). The relationship between protein structure and interfacial properties will be covered in more detail in Section 2.5 Insect Protein Emulsions, p 87.

2.3.1.1 Primary Structure

The sequence of amino acid known as the primary structure of proteins is the exact sequence and combination of the 21 possible amino acids. Each amino acid is joined by a peptide bonds to the preceding and subsequent amino acids. The bond between the α-carbon containing the carbonyl group and the amide nitrogen in the preceding amino acid is non-rotational due to the electron distribution of the carbonyl double bond being across the C-N bond. The C-N and C-C bonds either side of the central chiral carbon where the R- side chain is bonded, are fully rotational but in practice take a limited number of defined

bond angles. Proline and hydroxyproline are exceptions, because the C-N is on pyrrolidone ring so is non-rotational. The R- groups on the amino acids are usually in a *trans* configuration with the exception of proline and hydroxyproline which can take a *cis* configuration but are still more commonly found in a *trans* configuration.

2.3.1.2 Secondary Structure

The secondary structure is formation of stable ordered structures from the primary structure. The predominant forms of primary structure are right-handed α-helix, parallel and anti-parallel β-sheet, β-turn and absence of ordered structure known as random coil. Secondary structures are stabilised by hydrogen bonds. Globular proteins have multiple secondary structure elements whereas fibrous proteins such as collagen are often characterised by a single secondary structure element. Four of the main secondary structure arrangements are covered below but others exist such as the 3_{10}-helix and the Poly(Pro) II-helix.

2.3.1.2.1 α-Helix

An α-helix is formed by hydrogen bonds between the oxygen of the carbonyl groups with a N-H separated by four residues on the peptide chain. The hydrogen bonds are parallel to the axis of the α-helix. The α-helix has on average 3.6 residues and a length of 0.54 nm per turn and 0.15 nm rise per residue. α-helices can be disrupted by electrostatic repulsion of charged residue, steric repulsion of residues or hydrogen and ionic bonds between side chains. Right-handed α-helices are more stable than left-handed α-helices due

to the steric hinderance of the carbonyl groups and can be intertwined to form super secondary structures. These α-helices are rich in glycine, proline and hydroxyproline with the glycine residues α-helices are amphipathic with polar amino acids forming an area on one side of the helix and polar amino acids on the other (Sharadadevi et al., 2005, Creighton, 2010). A higher hydrophobic moment across the α-helix can produce emulsions of smaller droplet diameter (Poon et al., 2001).

2.3.1.2.2 β-sheet

β-sheet or β-pleated sheet are adjacent chains of amino acids from either the same peptide or different peptides with the chains either running in the same direction known as parallel or opposite directions know as antiparallel. In parallel formation all peptides run from N-terminus to C-terminus in the same direction in anti-parallel the chain alternate direction. Combinations of parallel and antiparallel β-sheet can also occur (Creighton, 2010). The structure is stabilised by hydrogen bonds between the oxygen on the carbonyl groups on the peptide bonds and the hydrogen on the amide N-H groups. Glycine and alanine preferentially form β-sheet secondary structures.

Parallel β-sheet are least common β-sheets and anti-parallel β-sheets the most common. Parallel β-sheets involve a minimum of four strands, anti-parallel can consist of as little as two strands, whereas mixed sheets can contain 3 – 15 strands (Creighton, 2010). β-barrel are formed where entirely anti-parallel or parallel β-sheets curves into a barrel, this can be combined with an α-helix around the outside of the barrel connecting two β-sheets (Creighton, 2010).

2.3.1.2.3 β-turn

β-turns are a structure where the peptide chain folds back on itself involving four amino acid residues. The structure is stabilised by a hydrogen bond from the oxygen of the first carbonyl to the amide on the fourth residue. β-turns allow proteins to form a more compact globular structure.

2.3.1.2.4 Disordered or Random Coil

When a peptide does not take a given secondary structure with a regular pattern it is known as random coil. A given sequence has only one conformation with a minimum energy state and only a few conformations which will be kinetically stable. Therefore, the conformation is not truly random in nature.

2.3.1.3 Tertiary Structure

The tertiary structure is how the secondary structure folds to form a specific shape which plays a determining role in functionality. The secondary structures are folded into domains in proteins above around 200 residues with specific functional properties and a final three-dimensional structure is formed through disulphide bridges, salt bridges, non-covalent bonds and solvent interactions which determines the position of each amino acid residue in space. The surface of the protein is uneven and irregular.

Water soluble protein molecules have Aspartic acid, Glutamic acid, Lysine and Arginine make up on average 27 % of the surface residues but 4 % of the interior residues (Creighton, 2010). Charged groups are often found in close proximity to opposing charges on the surface but do not form salt bridges. The interior amino acids are predominantly hydrophobic, around 63 % Valine, Leucine,

Isoleucine, Phenylalanine, Alanine and Glycine (Creighton, 2010). Larger globular proteins and membrane proteins tend to have a higher proportion of hydrophobic amino acid residues.

Protein folding is based on the enthalpy of the bonds formed compensating for the decrease in entropy from a more constrained structure. The excess in free energy at 25 °C is around 20 – 100 kJ/mol for proteins without disulphide bridges meaning that the stability of the tertiary structure is minimal (Yada, 2017, Trivedi et al., 2009).

The quaternary structure is two or more protein subunits joined to form a larger functional protein molecule as in the case of myosin. Single subunit protein have molecular weights 10 kDa – 100 kDa, whereas multiple subunit proteins can be many times larger such as the Dumpy protein in insect which is 2.5 MDa (Wilkin et al., 2000, Yada, 2017).

The internal hydrophobic interactions between molecules involve transient dipoles-dipole bonds on hydrophobic residues and interactions with the π electrons on the aromatic rings of Phenylalanine, Tyrosine and Tryptophan residues. The electron clouds above and below the aromatic rings on the amino acid residues, oxygen or sulphur interact with the positively charged hydrogens atoms at the edge of the aromatic rings or positively charged residues.

2.3.2 Solubility

Protein solubility is determined by the amino acid residues which are present at the protein surface and the protein dispersion medium (Cutler, 2004). The exact combination and proportions of polar, charged or hydrophobic amino

acids at the surface of the protein will determine the range of solvents, salt concentration and pH levels at which the protein is soluble (Cutler, 2004). Soluble globular proteins have large areas of charged and polar amino acid residues with some patches of hydrophobic amino acids although these are mostly in the interior of the protein as discussed in the previous section (Creighton, 2010). For solubility in water the surface Glutamic acid, Aspartic acid and Serine residues contribute to solubility to a greater extent than other hydrophilic amino acids (Trevino et al., 2007).

The net charge at a given pH will be determined by the quantity and location of the charged amino acid residues: lysine, arginine, histidine, aspartic acid and glutamic acid (Creighton, 2010). The external conditions affect the solubility of protein molecules. Proteins are most soluble in aqueous solution when the highest charge exists across the protein and least soluble at the protein isoelectric point where the net charge is neutral (Creighton, 2010). In a medium with pH below the isoelectric point of the protein the charge on the protein is positive and above the isoelectric point the charge is negative (Boye et al., 2010). The isoelectric point of a large proportion proteins in many materials such as soya beans, lentils and cereals is pH 4 – 7 (Zhao et al., 2018, Jarpa-Parra et al., 2014, Jarpa-Parra, 2018, Ciborowski and Silberring, 2016). Soya protein was shown to be most soluble below pH 3.5 and above pH 7 (Lawhon et al., 1981b). In a number of insect species solubility minima have been reported at pH 4 to pH 5: *Tenebrio molitor* larvae (Azagoh et al., 2016), *Schistocerca gregariai* and *Apis mellifera* (Mishyna et al., 2019), *Locusta migratoria* (Purschke et al., 2018b) and *Hermetia illucens* (Bussler et al., 2016).

2.3.3 Denaturation

Protein denaturation is the alteration of the quaternary, tertiary or the secondary structure or the protein. Denaturation may be reversible and the protein can refold into the tertiary structure based on the primary structure, many food proteins, egg white protein for example, denature irreversibly (Yada, 2017). Small globular protein denaturation does not involve stable intermediate states whereas large proteins have stable intermediate states due to the number of domains.

Both increasing and decreasing temperature beyond a threshold specific to each protein can cause denaturation from the native structure. Alterations to the tertiary structure can alter the function of a protein by exposing hydrophobic groups causing decreased solubility and aggregation. The state transition involved in denaturation exhibits an increased heat absorption and so can be measured by calorimetric methods (Creighton, 2010). Cold temperatures can unfold proteins in a manner that is thermodynamically the same as heat denaturation, through a decrease in the hydrophobic effect. The disruption of salt bridges ionization of amino acid residues in the core of the protein molecule can cause the unfolding of the native structure.

The shearing forces during processing and mixing may denature the tertiary structure but this may be dependent on the flow conditions. It was concluded that in laminar flow condition would require a flow rate of $> 10^{-7}\ s^{-1}$ to denature small globular proteins (Jaspe and Hagen, 2006). Although higher molecular

weight proteins are more susceptible to shear than small globular proteins (Siedlecki et al., 1996).

2.4 Protein Extraction Methods

Protein extraction in wet processes from solid or semi-solid starting materials involves comminution, extraction of the protein in a liquid medium, recovery from the liquid medium and a drying step. In dry processes, drying may be the first step if required, followed by comminution and separation of the protein from polysaccharide or lipid materials. Lipid extraction or defatting can be performed before the protein extraction if required. The material to extraction medium ratio, temperature, stirring speed, time and filtration particle size limit or centrifugation time and speed can all have an effect on the proteins extracted and the extent of any denaturation or hydrolysis (Dunford, 2012, Sari et al., 2015). Since the publication of the FAO report by Van Huis et al. (2013) publications on extracting insect proteins have accelerated, yet still remains limited in scope. Therefore, this review will cover protein extraction protocols used in food production and on other food materials such as soybeans, pulses and meat. Section: Insect Protein Extraction 2.4.7 p79 will cover the current literature on insect protein extraction for food purposes specifically.

2.4.1 Defatting

Defatting increases the proportion of protein through the removal of lipids by mechanical processes, aqueous or organic solvents, this is particularly true of high lipid materials such as soybeans and rapeseeds (Rosenthal et al., 1996, Rosenthal et al., 1998, Ghodsvali et al., 2005, Sari et al., 2013). In addition, the

removal of lipids can help prevent emulsions forming during the protein extraction, which can prevent the protein from fully solubilising (Yada, 2004). Insects vary from 9.12 % to 67.25 % as stated in section Insect Composition 2.2.2 p39 indicating a defatting step would be beneficial before a protein extraction in insects (Womeni et al., 2009, Xiaoming et al., 2010).

Common solvents for defatting include ethanol, petroleum ether and hexane (Yada, 2004). Hexane extraction is reported to recover almost 95 % (Rosenthal et al., 1996) and 99 % (Russin et al., 2011) of oil from oilseeds. Soybean hexane oil extraction process involves grinding (comminution), incubation in the solvent and recovery of the lipid containing solvent (Russin et al., 2011). More oil can be extracted from a flour than a flake in a given time period in soybeans (Nieh and Snyder, 1991).

In soybeans flakes, hexane has a higher rate of lipid extraction than heptane or ethanol (Gandhi et al., 2003). However, hexane can have negative consequences if released into the environment, so alternative solvents for defatting have been investigated. A 30 s ethanol-water extraction from soybeans in ratios varying from 9:1 to 1:1 after a hexane extraction extracted more phospholipids and free fatty acid from soybeans compared to a hexane extraction only with the proportion of phospholipids increasing with increasing water content (Nieh and Snyder, 1991). The increased extraction of phospholipids is due to the polar nature of water molecules.

As an alternative to non-polar solvents, aqueous lipid extraction methods have been developed which solubilise ground oil seed material remove the insoluble material, then separate emulsified oil phase from the protein rich aqueous phase (Lawhon et al., 1981b, Lawhon et al., 1981c, Lawhon et al., 1981a, Russin et al., 2011). The oil recovery is only 65 %, but the oil content of final protein isolates is low < 10 % suggesting the non-recovered oil remains in the insoluble material (Rosenthal et al., 1998, Lawhon et al., 1981b, Lawhon et al., 1981c, Lawhon et al., 1981a).

Supercritical extraction of lipids is an alternative extraction method to hexane which operates from the properties of CO_2 above the critical temperature and pressure. Supercritical CO_2 has the density of a liquid and the diffusivity of a gas allowing for the penetration of materials and solubilisation of lipids (Friedrich et al., 1982). While supercritical extraction yields can be comparable to hexane in soybeans (Friedrich and List, 1982, Friedrich et al., 1982, Friedrich and Pryde, 1984) and other materials such as freeze dried mackerel (Ikushima et al., 1986), the equipment required requires considerable investment (Hruschka and Frische, 1998).

2.4.1.1 Defatting Insect Protein

Insects have a different structure and composition to oil seeds and so most efficient solvents and the material extracted will differ. Zhao et al. (2016) found that ethanol extraction in mealworms gave a fat yield of 33.1 % after 1 hour and 34.8 % after 24 hours, compared to 31.6 % for 3:2 hexane:isopropanol but with no significant difference. The differing solvents in the fat extraction had

little effect of crude protein yield. Ethanol, as previously noted, extracts more phospholipids and free fatty acids so the excess yield may not be accounted for with triacylglycerides. In milled crickets, genus *Gryllidae* species not given, ethanol yielded a significantly higher fat compared to hexane reducing the fat content from 20.86 % to 9.27 % and 11.98 % respectively (Amarender et al., 2020). Supercritical CO_2 extraction was compared to three phase partitioning and Soxhlet extraction with four different solvents including hexane and ethanol in *Acheta domesticus* (house crickets) and mealworms (Laroche et al., 2019). Ethanol Soxhlet extraction gave a significantly higher fat yield than the other extraction methods for house crickets and gave the highest extraction yield in mealworms but no significant difference was found compared to the other methods. However, the standard deviation in fat yield was higher for the ethanol Soxhlet than other methods at ± 2.9 for mealworms and ± 5.9 for house crickets with next highest being ± 2.4 for mealworms and ± 2.0 for house cricket from the three-phase partitioning extraction. Furthermore, ethanol was shown to reduce the fat content of *Hermetia illucens* (black soldier fly) from 32.09 % to 1.05 %. Ethanol appears to be comparable hexane in fat extractions in species from orders Coleoptera and Orthoptera, and is effective in one species in the Diptera order. Hexane fat extraction reduced the fat content in mealworms, *Gryllus bimaculatus* (field cricket) and *B. mori* (silkworm) pupae to less than 1.5 % fat after a 24 hour process increasing the protein yield from a subsequent protein extraction process (Choi et al., 2017).

Overall, defatting in insects has been shown to increase protein yield in protein extraction (Mintah et al., 2020). Ethanol appears at minimum to be comparable

hexane in fat extractions in species from orders Coleoptera and Orthoptera, and is effective in one species in the Diptera order. Therefore, ethanol would be preferable over to hexane for a defatting step in terms of toxicity and as a result environmental impact.

2.4.2 Protein Extraction Methods

Protein extraction methods can be used to produce protein concentrate and isolates for increasing the protein contents of food stuffs or for functional purposes such as emulsification. Protein extraction methods can be divided broadly into two types: wet methods which involve dispersion of the original material in water or another solvent or dry methods which separate the material either in it's original form or after drying. In wet methods the quantity and type of solvent combined with the time and conditions will determine the efficiency of the extraction and purity of the extracts given the same initial material.

2.4.3 Alkaline Extraction

A basic alkaline extraction involves dispersing a mass of material in an alkali at pH 8 – 11 in a ratio of anywhere from 1:5 to 1:20, held at a temperature of up to 65 °C and stirred or agitated at the given temperature for 30 – 180 mins then filtered or centrifuged to remove any insoluble material (Dunford, 2012). Alkaline extraction (AE) is often combined with an isoelectric point precipitation (IEP) to separate the proteins from other alkaline soluble components such as sugars, polysaccharides and salts. In the IEP process, the pH level of the alkaline protein extract solution is adjusted to pH 4 – 7 where a large proportion of the

proteins have a solubility minimum allowing for collection of the protein precipitate by filtration or centrifugation. If the isoelectric point of a protein or group of proteins is known then they may be precipitated selectively (Cutler, 2004).

Alkaline extraction has been optimised for many common protein sources and is widely used in industry. Alkaline protein extraction has been used on food materials such as soya beans (Lawhon et al., 1981b, Lawhon et al., 1981c), rapeseeds (Tan et al., 2011, Aider and Barbana, 2011), microalgae (Schwenzfeier et al., 2011) cotton seeds (Zhang et al., 2009), rice bran (Phongthai et al., 2017), lentils (Jarpa-Parra et al., 2014) and tea pulp (Shen et al., 2008).

In soya beans, the ratio of extraction solution to raw material was found to affect the protein recovery more than the pH of the extraction solution (Lawhon et al., 1981b). A solvent to soy flour ratio of 30:1 at pH 8 and pH 6.65 extracted 94.2 % and 91.4 % of the nitrogen respectively, whereas at pH 9 a 25:1 ratio and a 12:1 ratio extracted 89.3 % and 80.4 % of the nitrogen respectively (Lawhon et al., 1981b). Since the nitrogen extracted is predominantly protein in terms of yield, solvent ratio appears to be a more important than the pH of the extraction solvent. It was found in tea pulp that an optimum material to solvent ratio was 1:40, there was a 20 % increase in recovery from 1:20 to 1:40 with higher volumes of solvent increasing extraction rate from 35 % to 37 – 38 % (Shen et al., 2008). The increased solvent to material ratio maintains a higher protein concentration gradient as protein

diffuses from the material to the solvent gives a larger driving force and increasing yield. These data suggest that a higher solvent to material ration would give higher protein yield in insect protein extractions. Further analysis of the tea pulp protein extraction showed the extraction time to be the most important parameter for protein recovery, followed by alkali concentration and the least important was solvent ratio (Shen et al., 2008). The effect of solvent to material ratio on tea pulp was further confirmed (Zhang et al., 2014) which has been attributed to the buffering capacity of the lignin and other biomass components (Sari et al., 2015, Jasaitis et al., 1987). Indeed, a key difference between the plant-based protein extractions and insects as a starting material is the presence of a cell wall. The cell wall and intracellular fibres such as lignins, celluloses and hemi-celluloses provide a barrier to extraction in a manner not present only present perhaps in the integument of insects. Subsequently, if lignin and other fibres are acting as a buffer, this effect may not be present in insect protein extractions.

While touched on previously the pH level of the extraction medium has an effect on the extraction of proteins. There are two main mechanisms for the pH effect: the proximity to the isoelectric point reduces solubility reducing extraction yields and a highly acidic or alkali medium can degrade the material reducing barriers to protein extraction. At pH 2.5 and pH 3 rapeseed meal, soybean meal, and microalgae meal gave protein yields of 15 %, 17 % and 16 % respectively, with the low yields attributed to minimal cell wall degradation and proximity to the isoelectric point (Sari et al., 2013). At pH 9.5 the protein yield was around 15 % for rapeseed meal, 80 % for soybean meal and 40 % for

microalgae meal with little difference at pH 10 or pH 11 (Sari et al., 2013). Even within different plant materials extraction yields differ by a factor of five, suggesting that in an animal material such as insects the optimum conditions are likely to be both different to plants and specific due to the structural differences.

Moving from pH 4 to pH 9 for 1 hour at a 1:5 ratio, temperature not given, increased protein yield from 3 mg/g to 80 mg/g in corn germ (Gu and Glatz, 2007), the increase in yield was explained by moving away from the isoelectric point between pH 4 – 7 (Nielsen et al., 1973) and increased glutelin extraction at pH 7-9 (Menkhaus et al., 2004). One optimisation of alkaline extraction with isoelectric point precipitation method for peas and lentils produced the following parameters: pH 9.5, 1:5 material to extraction medium ratio at 35 °C for desi and Kabuli chickpeas, and yellow peas; pH 9.0 with 1:10 material to extraction medium ratio at 25 °C for red and green lentils (Boye et al., 2010). The protein concentrations of the protein extracts produced from the alkaline extraction were 81.7 % in yellow peas, 79.1 % in green lentils, 78.2 % in red lentils, 73.6 % in desi chickpeas and 63.9 % in kabuli chickpeas (Boye et al., 2010). Similarly, a pilot scale protein extraction of peas in a 1:4 ratio at pH 9, 40 °C for 60 mins followed by an isoelectric point precipitation at pH 4.5 produced 77.1 % protein extract of mostly albumins and globulins which showed little difference in molecular weight by SDS-PAGE or solubility compared to salt extracted proteins (Tian et al., 1999). In a study of proteins extracted from chickpea, faba bean, lentil, pea and soy alkaline extraction at pH 8.00 – 10.00 with isoelectric point pH 4.50 – 5.00 precipitations were found to produce

significantly higher protein content protein extracts at 85.6 % to 78.4 % (Karaca et al., 2011b).

Using an extraction method similar to Boye et al. (2010), defatted pea protein was extracted from a variety of cultivars producing protein extracts at 83.3 – 86.9 % protein and 62.6 – 76.7 % yield (Stone et al., 2015). Salt extraction 0.1 M sodium phosphate buffer with 6.4 % KCl at 1:10 ratio, room temperature for 24 h then centrifuged, dialysed with 6 – 8 kDa cut-off produced 71.5 - 79.3 % concentrates (Stone et al., 2015). Minor difference in amino acid composition were found by extraction protocol AE IEP, salt extraction and reverse micellular extraction in peas (Stone et al., 2015).

Both acid at pH 2.5 – 3.5, and alkaline at pH 10.8 – 11.5, extraction can be utilised to extract meat proteins followed by a centrifugation to extract lipids and insoluble material then an isoelectric point precipitation at pH 5.5 (Matak et al., 2015). Acid solubilisation giving a slightly higher yield than alkaline extractions in meat proteins (Nolsoe and Undeland, 2009). The similarity of mammalian and avian myofibrillar proteins to insect muscle proteins would indicate that similar extraction conditions may be appropriate in both cases. Although there remains a higher proportion of non-muscle protein in insects which could lead to only the muscle proteins being extracted.

It was shown that peanut proteins recovered by alkaline extraction and isoelectric point precipitation from peanuts were significantly less soluble at pH 3 - 9, partially unfolded (denatured), lowered surface sulfhydryl groups and disulphide bonds, had lower surface hydrophobicity yet significantly lower

emulsifying activity index (Liu et al., 2011). When proteins are subjected to alkaline conditions and heat a number of negative chemical changes can occur. Racemisation is the alteration between L and D enantiomers in the presence of heat and alkali. Most amino acids show a linear relationship of increasing temperature increasing racemisation at temperatures under 55 °C (Schwass and Finley, 1984). Aspartic acid/asparagine, phenylalanine and serine racemise to a greater degree at temperature between 22 – 55 °C with serine completely racemised by 60 min at 55 °C (Schwass and Finley, 1984). In terms of pH levels at 85 °C, serine begins racemising at pH 7.0, whereas other amino acids do not increase above basal levels until pH 10 (Schwass and Finley, 1984). There are differences in serine racemisation between proteins which is suggested to be because of the difference in denaturation at a given pH level exposing the amino acids (Schwass and Finley, 1984).

In general, higher pH level, greater solvent to material ratios higher temperatures and increased extraction times increase extraction yields but beyond a level specific for each material increased yields are minimal. Increasing the pH level and the temperature can increase protein denaturation, racemisation and hydrolysis. The different structures of plant materials with cell walls and fibres mean that insects are likely to differ in some respects to plants in terms of protein extraction. While the different protein composition and the presence of the integument mean that protein extraction protocols for mammalian of avian meats may preferentially extract insect muscle proteins.

2.4.4 Acid Extraction

Acid extraction, similarly to alkaline extraction, operates by the principle of increased solubility of proteins at a pH level away from the isoelectric point (Dunford, 2012). At pH levels lower than 4 the solubility of some proteins is increased (Dunford, 2012). Alkali extraction is more commonly used than acid extractions because it often gives higher yields than acid extraction as the isoelectric point of many proteins is pH 4 – 7 so acid extractions at pH 1 – 4 are closer to the isoelectric point of the protein (Dunford, 2012). Acid pretreatments are commonly used in the production of type A gelatin, where an initial collagen rich material, often pigskin, is treated with acid (pH 1.5 – 3.0) to partially hydrolyse the collagen and increase solubility (Phillips and Williams, 2011a). Insects do contain collagen, but not at levels comparable to pigskin.

Aqueous water extraction of buckwheat groats followed by isoelectric point precipitation at pH 4.3 produced a 71.6 % protein extract with a recovery rate of 63.9 % and yield 12.4 % (Zheng et al., 1997). A 1:6 solvent ratio, at pH 7 gave a soy protein yield of 55.5 %, pH 4 an extraction yield of 31.4 % and 46.0 % at pH 5.0 showing lower yields at the protein isoelectric point (Jung et al., 2006).

Protein extracted at pH 2.5 and pH 3.5 with a subsequent isoelectric point precipitation at pH 5.2 of mechanically recovered turkey meat has a lower emulsifying activity index and lower surface hydrophobicity (Hrynets et al., 2010). Acid treatment of cod proteins at pH 2.5 increased the emulsifying activity index of the proteins but not as much as alkali pretreatment, and had a significantly higher interfacial tension than non-acid treated proteins

(Kristinsson and Hultin, 2003). These data are mixed on the applicability of acid extractions for extracting proteins to be used in oil-in-water emulsions. With any increases in emulsifying index being a potential benefit but the higher interfacial tension potentially inhibiting the formation of lower diameter emulsion droplets depending on the initial rate of interfacial tension reduction.

2.4.5 Salt Extraction

Proteins have varying solubilities in salt solutions. At low salt concentrations of 0.5 – 1.0 M there is a "salting in" effect where the salt ions promote solubility by reducing the electrostatic interactions between charged and ionised residues within protein molecules and promoting salt ion-protein group interactions (Dunford, 2012). The hydration of the ions and charges which were previously within and between protein molecules increases solubility (Dunford, 2012). At higher concentrations of ions above 1 M, protein "salting out" occurs where the water interacts preferentially with the salt ions precipitating the protein (Dunford, 2012, Cutler, 2004).

Salt extractions involve dispersing a material in a salt solution of 0.5 M to 1 M at a specific temperature and pH level and for a specific time (Yada, 2004, Dunford, 2012). Commonly, salt extraction is followed by dialysis against a buffer or water to remove some or all of the salt before drying and storage (Stone et al., 2015, Yada, 2004). Removal of the salt can precipitate the globulin fraction from the water-soluble albumin fraction, allowing for further fractionation. Myofibrillar proteins are a protein of note which are soluble in high ionic strength solutions. In order to completely solubilise meat proteins, a

minimum of 0.3 M sodium chloride or potassium chloride is required, whereas only half of the proteins are soluble in water and low ionic strength solutions (Krishnamurthy et al., 1996).

Vertebrae muscle proteins can be grouped by solubility, the water soluble or low ionic strength proteins are the sarcoplasmic proteins, myofibrillar proteins are soluble above 0.3 M salt solution, and connective proteins which are insoluble at high ionic strength (Lee et al., 2010, Ito et al., 2003). Myofibrillar proteins are now commonly extracted at 0.6 M KCl and pH 6.0 (Chen et al., 2017). Subsequent separation of proteins can be achieved by adjusting to specific pH levels and ionic strengths (Chen et al., 2017).

In comparisons of salt protein extractions to AE IEPP extractions in legume and oil seed proteins the properties of the extracted proteins do not show a consistent pattern.

In proteins extracted from chickpea, faba bean, lentil, pea and soy AE IPP were found to produce significantly higher protein content, higher solubility, but formed emulsions with significantly smaller mean droplet diameter and similar interfacial tensions (Karaca et al., 2011b). Similar higher soubility was found in the flax and canola oil seed proteins extracted by AE IPP compared to salt extraction with proteins from both methods produced stable oil-in-water emulsions with similar droplet diameter (Karaca et al., 2011a).

Salt extracted proteins had higher solubility, oil holding capacity, foaming capacity and lower water holding capacity than alkaline extraction isoelectric point precipitation and micellular extracted proteins (Stone et al., 2015).

Yet the amino acid composition of pea protein was similar for protein extracted by alkaline extraction isoelectric point precipitation, salt extraction with dialysis and micellular extraction suggesting similar proteins were extracted meaning the difference in properties was conformational (Stone et al., 2015). Pea protein produced through salt extraction showed little difference in solubility and molecular weights compared to AE IEPP (Tian et al., 1999).

In summary, salt extraction may be beneficial in extracting insect sarcoplasmic and myofibrillar proteins. For non-muscle proteins there is no clear evidence that the surface charge or hydrophobicity and as a result the solubility or emulsions formed are different between proteins from AE IPP and salt extraction. So, the properties of insect proteins extracted by the two methods would need to be compared.

2.4.6 Alternative protein extraction methods

Numerous alternative protein extraction methods exist to either maximise extraction efficiency, extract specific protein fractions or to minimise the use of water, energy or environmentally damaging chemicals. A brief summary of some of these alternative protein methods is provided here. However, a more extensive review of literature is out with the scope of this work.

2.4.6.1 Reverse micellular extraction

Reverse micellular extraction involves the separation of proteins by creating a two-phase system where the proteins are dispersed in an aqueous phase and the organic solvent phase with monodisperse aggregates of surface-active molecules which range in size from 1 nm to 10 nm in diameter (Dunford, 2012,

Grandison and Lewis, 1996). Alternatively, the protein can be added in a solid state and added to the organic phase directly (Zhao et al., 2011, Dunford, 2012). On agitation the proteins become encapsulated with a small amount of water in the reverse micelles in the organic phase, this step if often termed the forward extraction (Dunford, 2012, Grandison and Lewis, 1996). The protein is then extracted from the reverse micelle by agitation of the reverse micelle containing solvent with a high salt solution which can disrupt the reverse micelles and the protein disperses in the high salt solution, this step is termed the backwards extraction (Dunford, 2012, Grandison and Lewis, 1996). The reverse micelle prevents denaturation by the apolar solvent and can be tuned to select specific proteins by optimising the water content, type and concentration of surfactant, salt type and concentration and pH (Grandison and Lewis, 1996). Furthermore, the solvent and surfactants can be recovered and reused from the process decreasing cost and the process scaled up so is a viable commercial method (Sun and Bandara, 2019). The ability to select for specific proteins would be advantageous in investigating specific insect fractions for emulsifying properties.

2.4.6.2 High-pressure processing

High pressure processing for food products is a term which covers techniques which use 100 – 800 MPa of pressure and can also be known as high hydrostatic pressure or ultrahigh pressure processing (Balasubramaniam et al., 2015). Temperature and pressure cannot be separated, since for a set volume of a given mass of a material there is a temperature and pressure (Balasubramaniam et al., 2015). Changes in pressure and temperature are

assumed to be instantaneous according to the isostatic principle so differ considerably from other methods of heating such as conduction, radiation and convection which take a longer period of time to reach a given temperature (Balasubramaniam et al., 2015). High pressure processing is used primarily as food preservation method because it can disrupt cells without many of the degradative properties of prolonged heating consistent with other heating methods.

High pressure treatments reduce the distance between molecules so can alter reaction rates both positively and negatively; impact density, solubility and other physicochemical properties, as well as biasing chemical equilibria towards the alleviation of higher-pressure states (Balasubramaniam et al., 2015). The anti-microbial properties of high pressure treatments are both disruption of vegetative cells (Sauer et al., 1989) and by altering cell function to change the internal pH (Molina-Gutierrez et al., 2002). Cell lysing could be an advantage in releasing proteins such as muscle proteins which are contained in cells but the extent to which protein could be released from the sclerotised protein matrix of the insect integument is an open question.

2.4.6.3 High-pressure homogenisation

High-pressure homogenisation and similar brand name equipment called Microfluidizers, involve liquid cavitation, similar to ultrasound, and by extension shearing forces which act in combination with high hydrostatic pressure (Sauer et al., 1989, Smelt, 1998). High pressure homogenisation has been used to modify protein properties. The covalent bonds in proteins have

been shown to be stable up to 1500 MPa so any modifications are at the secondary, tertiary and quaternary structure level (Mozhaev et al., 1994). The solubility of chicken myofibrillar proteins in water was increased from 2 % to 69 % by the use of 103 MPa high pressure homogenisation by disrupting the structure of the myofibril (Chen et al., 2016). Microfluidizer treatment decreased α-helix and β-turn structure, and correspondingly increased β-sheet and random coil structure as measured by Fourier Transform Infrared Spectroscopy and circular dichroism (Hu et al., 2011).

2.4.6.4 Ultrasound

Ultrasound in food processing is the transferal of high frequency waves through a liquid medium. Low-intensity ultrasound uses high frequencies of > 1 MHz and low amplitudes which do not rupture materials (Kentish and Feng, 2014). High-intensity or power ultrasound uses larger amplitude waves at lower frequencies, with power ultrasound in the range 20 kHz to 100 kHz often used to alter the physicochemical properties of food materials (Kentish and Feng, 2014). As the ultrasonic waves pass through the material, areas of high and low pressure are created with cavitations occurring at the bubbles created in the low-pressure areas (Kentish and Feng, 2014, Tiwari, 2015). Through formation and collapse of the bubbles shearing forces occur and a mixing of the fluid (Kentish and Feng, 2014). Ultrasonication of materials for protein extraction is not an independent extraction method, but a method for disruption and solvent permeation of the material. The pressure and shearing forces caused by the cavitations disrupt cells, cause surface microfissures, increase the porosity of the material and increase mass transfer of protein into the solvent

(Tiwari, 2015). Ultrasound can increase extraction efficiency and the protein content of isolates (Karki et al., 2009, Preece et al., 2017). Ultrasound can potentially denature proteins and induce covalent linkages between proteins but has been shown not to increase protein hydrolysis (Pojic et al., 2018, Yang et al., 2017). Ultrasound has the potential to disrupt the structure of insect increasing extraction efficiency with minimal denaturation or hydrolysis of the protein.

2.4.6.5 Enzyme assisted extraction

Enzymes in protein extraction can be used to break down the structures which contain the protein, such as cell wall in oilseed crops and cell membranes as well as proteolysis of proteins which can aid solubility and diffusion into the extraction medium (Sari et al., 2015, Dunford, 2012, Russin et al., 2011). Enzymes can be used individually or in mixed enzyme systems and in concert with acidic or alkaline conditions depending on the optimum pH level for functioning of the enzymes. In oilseeds and other material of plant origins proteases are more effective than carbohydrases at increasing protein extraction yields (Sari et al., 2015, Sari et al., 2013). In insects, specific enzymes for disrupting cell membranes, sclerotised protein and breaking down chitin could be investigated. Disruption of the insect structures could allow for the release and extraction of greater levels of protein.

2.4.6.6 Dry fractionation

Dry fractionation has been utilised for protein extraction on soybean hulls (Wolf et al., 2002), yellow pea (Pelgrom et al., 2015a, Pelgrom et al., 2015b), lupine

(Pelgrom et al., 2015c) and barley endosperm (Silventoinen et al., 2018). The dry fractionation process has two stages: the grinding or particle reduction process and the particle separation or classification process. The starch granules, fibrous material and sometimes lipids can be separated from the protein. Protein can be extracted in a native state if the temperature of the grinding process is controlled sufficiently because no denaturing solvents are being used such as the alkaline solutions used in alkaline extractions.

Grinding can be performed by numerous methods which have differing physiochemical properties to the particles. Types of mills are hammer mills which are rotating impact hammers, pin mills which break particles with a rotating pins, attrition milling which reduces particle using a shearing force and jet milling which utilises a jet of gas to cause particle collisions and collisions with the interior walls of the jet mill to reduce particle size (Assatory et al., 2019). The classification of particles can be performed based on particle density in the case of air classification, or particle charge as in the case of electrostatic separation (Assatory et al., 2019). The lower energy input compared to wet methods make dry-fractionation a likely method for producing insect protein extracts commercially.

2.4.6.7 Ultrafiltration

Ultrafiltration is a protein purification and concentration method which operates by passing a protein solution through a selectively permeable membrane with the application of pressure (Burgess et al., 2009). The pore size and mechanical strength of the membrane are selected on the basis of the

impurities or solvent required to pass through the membrane and the size of the protein molecules to be retained (Burgess et al., 2009). Ultrafiltration membranes can be utilised to concentrate proteins of molecular mass 0.3 – 3 000 kDa and of size 0.02 – 10 µm and in combination with a drying method can be used as an alternative to isoelectric point precipitation to obtain a protein concentrate or isolate (Dunford, 2012). Ultrafiltration is used commercially as a method of producing whey protein isolates from milk (Akpinar-Bayizit et al., 2009). Diafiltration is a similar mechanism but involves the addition of water or buffer to the retained protein which is refiltered to lower the proportion of soluble impurities. Ultrafiltration and diafiltration open the opportunity to select protein fractions based on mass. Different molecular weight insect protein fractions could be analysed for interfacial activity and emulsifying properties.

2.4.6.8 Ammonium sulphate precipitation

Ammonium sulphate is used as a method of protein recovery from a protein solution through salting out. The protein solution is saturated with the highly soluble ammonium sulphate salt which preferentially forms electrostatic bonds with the water molecules causing the proteins to precipitate (Yada, 2004). Salt precipitation methods can be used to fractionate albumins from globulins based on the relative salt solubilities (Yada, 2004).

2.4.7 Insect Protein Extraction

2.4.7.1 Alkali Extraction with Isoelectric Point Precipitation

Alkali extraction with an isoelectric point precipitation is one of the most widely used protein extraction methods. Some of the first work on the bulk extraction of insect proteins utilised this method. Protein was extracted from *Anastrepha ludens* (Mexican fruit fly) at pH 10 and precipitated at minimum solubility of pH 5 (Delvalle et al., 1982). An alkali extraction at pH 10.5 with sodium hydroxide was also used to produce a 64.2 % protein concentrate from dried honey bees at 52.0 % protein with minimal change in amino acid composition (Ozimek et al., 1985). It was shown that the protein solubility was over 50 % at above pH 11 compared to around 20 % at pH 7 for dried ground *Cirina forda* (pallid emperor moth) further validating this approach (Omotoso, 2006). Zhao et al. (2016) optimised the alkaline extraction process for *Tenebrio molitor* (mealworms) showing that an ethanol fat extraction is as effective as a hexane-isopropanol mixture and that 1 hour at 40 °C is as effective as overnight at extracting fat and that 0.25 M sodium hydroxide at 40 °C for 60 min was optimum for the alkali extraction. The optimised extraction protocol increased the protein concentration of the mealworm powder from 43 % to 75 % in the protein concentrate with a 70 % extraction rate (Zhao et al., 2016). Similarly, protein was extracted from mealworms and *Hermetia illucens* (black soldier fly) at pH 10, increasing the protein content from 53.8 % in the larval extract to 63.8 % for the mealworms and 31.7 % to 44.9 % for the black soldier fly (Bussler et al., 2016). On the relationship between pH and solubility, extracted mealworm protein and heat treated mealworm meal have a minimum solubility at pH 3 to

pH 4 and pH 3 to pH 5 respectively, suggesting an optimum pH for isoelectric point precipitation (Azagoh et al., 2016).

In *Locusta migratoria* (migratory locust), a hexane fat extraction was used followed by an alkali extraction at pH 10 leaving a residual alkali insoluble fraction, and an isoelectric point precipitation at pH 4 giving an acid soluble supernatant fraction and an alkali soluble-acid insoluble fraction (Clarkson et al., 2018). The protein content of the three fractions were insoluble fraction 81.29 %, acid soluble fraction 69.13 % and alkali soluble fraction 73.64 % and the protein recovery 39.85 %, 9.64 % and 52.17 % respectively (Clarkson et al., 2018). It is possible that a large proportion of the protein is sclerotised in the adult migratory locust, and so is resistant to extraction at pH 10 and that the nitrogen in the chitin is also increasing the apparent protein content in the alkali insoluble fraction. By an alkali extraction process at pH 9, migratory locusts of 65.87 % protein produced an 82.26 % protein extract (Purschke et al., 2018b). From this protein extraction the proteins showed a lower solubility at pH 10 than the maximum at pH 9 which was attributed to partial denaturation (Purschke et al., 2018b). In black soldier fly prepupae an alkali extraction with 1 M sodium hydroxide was found to extract 96 % of the total protein compared to <85 % for the stepwise Osbourne fractionation method, however the alkali extracted protein showed a much greater degree of hydrolysis (Caligiani et al., 2018). Depending on the functional properties of hydrolysed and denatured proteins compared to native proteins, there may be an optimum pH for extraction for a given protein function.

2.4.7.2 Acid Extraction Methods

In an 9.46 mM ascorbic acid aqueous extraction of mealworms, *Acheta domesticus* (house crickets), *Blaptica dubia* (Dubia roach), *Zophobas morio* (Superworm), *Alphitobius diaperinus* (lesser mealworm) the soluble supernatant fractions were between 50 % and 61 % protein and rich in low molecular weight proteins (Yi et al., 2013). Using a similar extraction protocol on *Patanga succincta* (Bombay locust) and *Chondracris roseapbrunner* (Spur-throated grasshopper) an abundance of water soluble proteins with molecular weight below 100 kDa but with some bands in the 100-150 kDa region on SDS-PAGE were found (Chatsuwan et al., 2018). The same extraction protocol from Yi et al. (2013) was repeated on house crickets but the supernatant fraction was not retained or analysed making preventing comparisons of the soluble protein fraction (Ndiritu et al., 2017). The aqueous extraction protocol used by Yi et al. (2013) was found to extract a higher proportion of protein than a salt extraction in mealworms, *Allomyrina dichotoma* and *Protaetica brevitaris,* the maximum protein content achieved was the 5.58 % protein solution for aqueous extracted *Allomyrina dichotoma* (Kim et al., 2019). There was no drying step in this study so the purity that could be achieved is not directly comparable to other studies but does give a proof of concept in these species. An acid extraction of house crickets using 0.5 M ascorbic acid compared favourably to a 0.5 M sodium hydroxide alkali extraction with protein concentrations of 69.69 % and 61.75 % respectively (Amarender et al., 2020).

2.4.7.3 Alternative Extraction Methods

Sonication and fat extraction were shown to increase protein yield from mealworms, *Bombyx mori* (silkworms) and *Gryllus bimaculatus* (black cricket) with sonication particularly effective in silkworm pupae increasing yield from around 20 % to 89 % after 5 min (Choi et al., 2017). For alkali extractions at pH 10 with a prior hexane lipid extraction of *A. millifera* (honey bees) and *Schistocerca gregaria* (edible grasshopper) sonication was found to increase protein yield and improve functional properties in both insect species (Mishyna et al., 2019). Enzyme extraction of black soldier fly prepupae gave an extraction rate of 60 % with Bacillus licheniformis protease (Caligiani et al., 2018).

A dry fractionation method involving lipid extraction with supercritical carbon dioxide followed by air separation to course and fine fractions was used on house crickets and mealworms (Sipponen et al., 2018). Protein was concentrated from 48.6 % to 59.5 % in house crickets and 39.6 % to 56.8 % in the course fraction and slightly lower in the fine fraction using a 4.76 nitrogen factor (Sipponen et al., 2018).

2.4.8 Compare & Contrast

The alkaline extraction with isoelectric point precipitation method consistently produces high yield, high protein concentration protein concentrates or isolates across numerous materials: pea (Stone et al., 2015), lentils and pulses (Boye et al., 2010, Jarpa-Parra et al., 2014), soy (Sari et al., 2013). Alkaline extraction has already been proven in multiple studies to produce protein

extracts of > 80 % in multiple insect orders (Purschke et al., 2018b, Mintah et al., 2020, Kim et al., 2020).

Acid extraction may produce higher protein yields in meat proteins (Matak et al., 2015) although the evidence is mixed (Nolsoe and Undeland, 2009) and at a lower surface hydrophobicity and emulsifying activity index (Hrynets et al., 2010).

The isoelectric point for recovery of the proteins from the extraction medium is often selected for maximum yield. The specific pH of precipitation however selects proteins with that isoelectric point, so may be used to select proteins which would have maximum solubility in food products of a specific pH level.

Acid extraction often produces lower purity protein concentrates, > 70 % protein, relative to alkaline extraction, > 90 % protein, due to acidic isoelectric point for the majority of soybean proteins being pH 4 – 5. Acid pre-treatments as with collagen conversion to gelatin are time consuming, in the region of months and a large proportion of insect protein is in muscles and the integument as discussed in Section 2.2.3.1.2 Muscle Proteins p 42 and Section 2.2.3.3 Insect Integument Proteins p 45 respectively, so undertaking this process is questionable in terms of overall yield.

Salt extractions have been shown to produce high protein content extracts in multiple plant materials and is particularly effective in myofibrillar proteins. Salt extraction has a high potential for extracting the insect myofibrillar proteins because of the similarity between insect muscles proteins and vertebrate striated muscle protein. Vertebrate myofibrillar proteins stabilise oil-water

interfaces in emulsion types meat products which is covered in more detail in Section 2.1.3.2. Meat Proteins p 31 It seems a reasonable hypothesis that salt extracted insect proteins may contain elevated level of myofibrillar proteins. Salt extracted proteins have reduced solubility at lower salt content mediums reducing emulsifying properties.

High pressure processing, ultrasound and enzymic extraction are all additions to an extraction in an aqueous medium with a given pH and salt type and concentration. All three of the extraction conditions can disrupt the cell structure and intracellular structure in the starting materials and can modify the structure and functionality of proteins independent of extraction yield. Sclerotisation of the protein in the cuticle is a factor which limits protein extraction yield. The extents to which any process can release the highly variable protein from the sclerotised matrix has potential to increase yield.

Enzyme assisted extraction can either hydrolyse the protein of breakdown the material in which the protein is bound. Proteases are more effective in increasing protein yield than carbohydrases in oil seeds. Proteases hydrolyse the proteins with similar results on the interfacial properties as hydrolysis by alkaline conditions. Each enzyme has an optimum pH which in addition to the enzymic hydrolysis may have a denaturing effect of the protein. A second enzyme would be required break the bond in the chitin chains and any chitin-protein bonds, but this would introduce chitin into the protein extract. Cleaving the sclerotised bonds and any chitin-protein bonds selectively but since

protein-chitin bonds are poorly understood it would be difficult to find an enzyme.

High pressure processing should be considered as pure hydrostatic pressure processes; and high-pressure homogenisation processes and Microfluidization processes which produce cavitation and consequently high shear forces. Therefore, a high-pressure cavitation process could greatly disrupt the structure of a material to a greater degree including muscle cells and insect cuticle, for example, releasing more protein breaking protein aggregates and denaturing the protein, hydrolysis is unlikely unless combined with acidic or alkali extraction medium and heat.

High intensity ultrasound provides both mixing and cavitation forces. The effects are both time and amplitude dependant, the majority of studies have used frequencies in the region 20 – 30 kHz. The correct balance of amplitude and time is fundamental to extracting the maximum yield with minimal denaturation. Ultrasound needs to be powerful enough to disrupt the cell structure and protein matrix in the cuticle, but be low enough energy to minimise protein denaturation and hydrolysis.

High pressure processing, enzymic extraction and ultrasound all alter the protein structure and by extension the functional properties. High pressure processing alters secondary structure and breaks up aggregates, enzymic extraction hydrolyses proteins and ultrasound alters tertiary structure at minimum.

Dry fractionation can extract protein in near native form with high degrees for solubility and has been shown to produce insect protein extracts. There is an issue with heat produced in the grinding process denaturing proteins and reducing solubility. The protein concentration in the extract has a trade off with yield and concentrations are lower than in salt and alkaline extraction.

Reverse micellular protein does appear to have some effect on protein secondary structure but can produce highly soluble proteins with similar functional properties to alkaline extracted proteins. The process can be optimised for yield or specific proteins through selection of surfactants and apolar solvents.

2.5 Insect Protein Emulsions

The potential for the use of insect protein to stabilise food emulsions has been established since 2006 at least with Omotoso (2006) showing emulsifying capacity in *Cirina forda* (westwood). Although some of the earliest work such as Delvalle et al. (1982) suggested no emulsifying capacity was present in *Anastrepha ludens* (Mexican fruit fly) larvae, a number of factors suggested that further work was required to establish the applicability of these findings to insects more generally. The similarity of insect muscle proteins to striated vertebrate muscle suggested that a subcategory of insect proteins could stabilise emulsions. In addition, the number and diversity of insect species discussed in Chapter 2 2.2.1 Insect Taxonomy, p 35, combined with a greater understanding of proteins of the integument indicated there is much yet to be understood.

It was shown that both salt concentration and pH have a significant effect on solubility and have an interaction with a solubility maximum at 3 % sodium chloride (the maximum tested) and pH 9 (Purschke et al., 2018b).

The initial work on insect protein emulsions was focused on measures of emulsion stability over time, the volume of oil emulsified per mass of emulsifier or turbidity. Some of the measures used were emulsifying capacity (Bussler et al., 2016), emulsion stability (Hall et al., 2017), emulsion activity (Purschke et al., 2018b, Zielinska et al., 2018, Udomsil et al., 2019) or emulsion activity index (Hall et al., 2017). The purpose of these studies was to determine if insect protein emulsions had potential to be used in oil-in-water emulsions and under

what conditions. In both mealworms and migratory locust the maximal ability to form an emulsion was found at pH 5 close to the isoelectric point of the protein (Bussler et al., 2016, Purschke et al., 2018b). Usually, the lower solubility at closer proximity to the isoelectric point results in reduced emulsification. In a comparison of alkali extractions of protein from multiple insect species, it was found that *Gryllodes sigillatus* (banded cricket) had a higher emulsion activity compared to *Schistocerca gregaria* (desert locust), and mealworms but banded crickets showed a lower emulsion stability (Zielinska et al., 2018). In a study of the effect of defatting and comminution methods, whole spray dried mealworm powder had a significantly higher emulsifying capacity than all other powders but lower stability that hexane defatted mealworms ground in a cutter mill and pressure defatted mealworms ground in a jet mill (Son et al., 2019). So, some of the non-polar components may play a role in stabilising the oil-water interface.

No significant difference in emulsifying capacity on a wet mass basis was found between the emulsifying capacity of whole insect powder, defatted powder and acid-hydrolysed mealworm larvae and silkworm larvae despite the higher protein content of the acid-hydrolysed powder (Kim et al., 2016). However, whole Edible grasshopper powders and defatted powders had a significantly lower emulsifying capacity per gram of protein, than alkaline extracted proteins, which was comparable to whey protein indicating the advantage of using a protein extraction protocol (Mishyna et al., 2019). Therefore, there may be a difference in emulsifying properties based on the pH of the extraction or pretreatment.

In a 20 % oil model emulsion study, mealworm protein was shown to stabilise emulsions with a similar droplet diameter to whey protein emulsions but at a lower protein concentration by rapidly decreasing interfacial tension (Gould and Wolf, 2018). Using a pH 9.5 alkali extraction, lower than that of 0.25 M sodium hydroxide used by Gould and Wolf (2018), it was shown that house crickets protein lowered interfacial tension rapidly and produced stable emulsions, whereas the house cricket chitin produced unstable emulsions (Hirsch et al., 2019b). Using an extraction protocol similar to Zhao et al. (2016), the emulsion activity and stability of mealworms decreased as processing temperature increased and as processing time at a given temperature increased (Lee et al., 2019).

Salt extracted and water soluble proteins form mealworms, *Allomyrina dichotoma* (Japanese rhinoceros beetle) and *Protaetia brevitarsis* (a beetle native to Asia) all formed oil-in-water emulsions (Kim et al., 2019) Salted extracted *Protaetia brevitarsis* had a significantly higher emulsifying capacity on a per gram of protein basis, an abundance of hydrophobic amino acid residue and proteins of molecular weight 180 kDa and 75 kDa compared to the other extracts (Kim et al., 2019). The higher emulsifying capacity indicates an increased interfacial activity in the *Protaetia brevitarsis* proteins over the proteins from the other insect species. Further evidence that the extraction protocol and insect species may affect emulsifying properties. Interestingly, the three insect species are from the same taxonomic order *Coleoptera*, suggesting there may be some differences in emulsifying properties within an order.

There has been a number of studies which do not directly analyse o/w emulsions stabilised by insect proteins, but nonetheless give insights into the emulsifying properties of insect proteins. Three insect species: *Alphitobius diaperinus* (lesser mealworm beetle), mealworms and *Zophobas morio* (superworms), were shown to produce batters similar to meat batters but with requiring more heating to reach similar viscoelastic properties to meat batters (Scholliers et al., 2019). Oil extracted from Black soldier fly was shown to form stable nano emulsions with Hydrogenated lecithin and Tocopheryl polyethylene glycol 1000 succinate stabilising the interface (Chou et al., 2020).

The extraction protocol parameters: pH, temperature, time, lipid extraction solvent and additional processing methods have been shown to affect protein yield, solubility, emulsifying properties and degree of hydrolysis. The majority of insect proteins studied to date show a maximum solubility at pH 9 to pH 11 and minimum solubility at pH 3 to pH 5 and many have emulsifying properties. There remains scope to optimise extraction protocols further, and find insect species which produce proteins with specific emulsifying properties. In addition, many protein extraction methods have yet to be trialled on insects or have been in a limited fashion.

3 Techniques

3.1 Freeze drying

Freeze drying relies on the removing water by freezing the water present in the material and then lowering the pressure below the triple point of water and heating to cause sublimation of the water. The freeze drying was performed on a solvent capture unit with an oil sealed rotary vane vacuum pump (E1M18, Edwards, West Sussex, United Kingdom.) Freeze drying was performed until a constant weight was achieved.

3.2 Amino acid analysis

Amino acid compositional analysis of insect proteins and powders was performed by the following procedure. A known mass of the sample was treated with formic acid/hydrogen peroxide solution to oxidise the sulphur containing amino acids cysteine and methionine to acid stable derivatives cysteic acid and methionine sulfone respectively. Without this step the sulphur containing amino acids would be partially or fully oxidised in the acid hydrolysis and become unmeasurable. The sample is then hydrolysed with concentrated hydrochloric acid to produce free amino acids for measurements. The solutions were adjusted to pH 2.9 centrifuged and passed through a 0.22 µm filter before dilution to 1 – 10 µg/L of nitrogen. During the hydrolysis the asparagine and glutamine are deaminated to aspartic acid and glutamic acid and tryptophan is broken down so cannot be measured.

Amino acids were quantified by high performance liquid chromatography (Biochrom 30, Biochrom, Cambridge, United Kingdom), or liquid

chromatography with tandem mass spectrometry (LC-MS/MS). The LC-MS/MS system was an ultra-high performance liquid chromatography system (Thermo-Fisher Vanquish, Thermo Fisher Scientific, Massachusetts, USA) fitted with an a Thermo Scientific Acclaim Trinity P1 mixed mode column (150 mm x 2.1 mm, 3 µM) coupled to a Triple Quadrupole Mass spectrometer (MS/MS) (Altis, Thermo Fisher Scientific, Massachusetts, USA) with heated electrospray ionization (H-ESI) system.

Amino acids were quantified in comparison to a standard and two replicates were performed for each sample. The exact methodology used for amino acid composition differs between Chapter 5.2.2.1, p 112 and Chapter 7.2.2.5 Amino Acid Analysis p 194.

3.3 Protein content

Protein content was determined through the Dumas method with an Elemental Analyser (Flash EA1112, Thermo Scientific, Massachusetts, USA). The Dumas method involves combusting a 5 – 6 mg of the material weighed precisely in a tin capsule at 1000 °C in pure oxygen to achieve complete combustion. The nitrogen oxides are then reduced in a copper column at 600 °C. Gas chromatography is used to measure total nitrogen with helium as the mobile phase and a thermal conductivity detector. The total nitrogen includes both organic and inorganic nitrogen, the organic nitrogen includes nitrogen from not only protein but other nitrogen containing compounds such as chitin in the case of whole insects.

A nitrogen conversion factor is used to estimate the protein content from the total nitrogen. A global nitrogen factor of 6.25 is used when no other data is

available. The nitrogen factor is calculated from a total protein as measured by full amino acid analysis divided by total nitrogen. Specific nitrogen factors have been calculated for whole insects: 4.76 ± 0.09 (Janssen et al., 2017), 5.0 (Ritvanen et al., 2020), 5.33 (Boulos et al., 2020); and 5.6 for insect protein isolates (Boulos et al., 2020). The lower nitrogen factors for the whole insect powders reflect both the presence of non-protein nitrogen in the form of chitin and a lower proportion of amino acid residues with nitrogen containing side chains. A protein isolate would contain little chitin so the lower nitrogen factor is due to the amino acid residues alone. However, in this study the 6.25 conversion factor was used for three reasons. At the time of the initial work in this study only the Janssen et al. (2017) paper was published so there was not sufficient evidence to support a lower nitrogen factor. Secondly, to allow comparison through the three chapters the nitrogen factors were kept consistent. Thirdly, there is still only limited evidence for the nitrogen factor on insect protein isolates and this may vary between insect species and extraction methods. The protein content estimates in this study are likely to be overestimates, but could be recalculated from the data given if new information of nitrogen factors is produced.

Equation 3 Calculation of protein from nitrogen

$$Total\ protein = total\ nitrogen * nitrogen\ conversion\ factor$$

3.4 Thermogravimetric Analysis

Thermogravimetric analysis (TGA) is the measuring of mass changes of a material while heating under a controlled atmosphere. TGA measures loss of volatile compounds including water and the thermal stability in a given

atmosphere under a controlled heating regime. The Mettler-Toledo TGA/DSC 3+ (Mettler-Toledo, Switzerland) used for TGA operates by heating a precisely weighed mass of sample of around 3 – 6 mg in an appropriate pan type, usually aluminium, stainless steel or ceramic. The pans are open topped to allow gases to escape and the controlled atmosphere to enter. The purge gas can be an inert gas commonly nitrogen but helium or argon may be used or a gas which facilitates combustion or other reactions such as oxygen or hydrogen. The sample and pan are heated in a furnace at a specific rate between 0.02 Kmin-1 and 150 $Kmin^{-1}$ up to a maximum temperature of 1600 K on a balance accurate to and the mass change measured over the temperature change. An empty pad is heated and the mass measured to account for the change in density of the gas around the sample affecting the buoyancy, this allows for a differential scanning calorimetry (DSC) heat measurement to be made. The initial loss of mass around 100 K are due to the loss of volatiles such as water, solvents and monomers. At higher temperatures the decomposition of larger molecules accounts for the change in mass but this is specific to each sample.

3.5 High-shear Emulsification

Emulsification of an oil-in-water emulsions involves using mechanical energy to disperse the oil phase in the aqueous phase allowing the emulsifier to adsorb to the oil-water interface forming oil droplets dispersed in the aqueous phase. A distribution of droplet diameters are produced and as the emulsion is processed further the larger droplet are disrupted and dispersed into smaller droplets through mechanical shear forces and the emulsifier adsorbing to these

smaller emulsion droplets. Emulsions where produced in a high-shear mixer (L5M Series, Silverson, Chesham, UK) 8000 rpm fitted with an emulsor screen for 2 min.

3.6 Laser diffraction particle sizing

Laser diffraction particle sizing uses the unique scattering of light from the surface of a particle when the particle has a different refractive index from the continuous phase in which it is dispersed. The laser diffraction particle sizer (LS 13 320, Beckman Coulter, High Wycombe, UK) contains a 750 nm, 5mW laser diode, in the visible light range to maximise scattering rather than absorption. The light scattering patterns of spherical particle can be described by Mie theory if the wavelength polarisation of the light and refractive index are known. The light scatters from the surface of the spherical particle at maxima and minima of intensity symmetrical from the angle of the angle of the incident light. The exact angles are dependent on the size of the particle with large particle scattering mostly at small angle due to diffraction effects at the particle limits. Therefore, the distribution of particle sizes can be produced through calculating the intensity of scattered light from the laser. The calculation of particle sizes is dependent on the concentration of particles in the particle sizer being sufficient to produce a scattered light intensity which can be detected but below the detector saturation. The measurements were performed in in this research were performed with an aqueous module using reverse osmosis water continuous phase. A customised model based on MIE theory was used with refractive indices 1.333 for the water phase and 1.464 for the dispersed

sunflower oil phase to improve accuracy over a more general model. Three independent repeat measurements were taken for each sample to achieve a representative measurement.

3.7 Light microscopy

Brightfield microscopy relies on a light being transmitted through and diffracted by a sample to an objective lens and eye-piece lens magnifying the image against a bright background. The brightfield microscopy was performed on an inverted microscope where the light source is above sample and a camera below to display the image on a screen (EVOS f1, AMG, Washington, USA) and an upright microscope (Nikon Eclipse, Nikon, Amstelveen, The Netherlands) where the light source and the eyepiece and camera positions are reversed.

3.8 Fourier transform infrared spectroscopy

Mid-infrared spectroscopy uses the absorption of electromagnetic radiation in the infrared range by covalent bonds to elucidate the nature and environments of these bonds. The Fourier transform infrared spectrometer (FTIR) (Tensor 27, Bruker Optics Inc., Billerica, MA, USA) has a KBr beam splitter and was fitted with an attenuated total reflectance attachment (Golden Gate, SPECAC Ltd, Orpington, Kent, UK) which had a diamond crystal and was internally purged with dry air. The FTIR operates by the beam from an infrared source being directed through an interferometer. The interferometer splits the beam on to a fixed mirror in one direct and a moving mirror at a right angle. The moving mirror travels at a known velocity and a known distance or more accurately a known difference in distance travelled by the infrared beams going to the two

mirrors, know as optical path difference. The beam is recombined the in the interferometer creating an interference pattern when recorded becomes an interferogram of the light intensity vs optical path length. The combined beam is passed through the sample and the resulting interferogram is converted to a sample absorbance spectrum through a Fourier transform.

The attenuated total reflectance method allows the analysis of materials in their current state without further grinding and dispersion in material such as potassium bromide, which dons not absorb infrared radiation in the analysis range. Therefore, any structural alterations from the sample reparation process are avoided and the procedure required less time so more measurements can be made in a given time. The attenuated total reflectance module operates by the creating a contact between the sample and the diamond crystal. The infrared beam from the interferogram is passed through the crystal and the internal reflectance if the beam in the crystal creates an evanescent wave which is attenuated by the sample and passed to the detector. Measurements were taken at 4000 – 500 cm^{-1}, 128 scans and a 4 cm^{-1} resolution with at least 5 replicate measurements.

3.9 Force tensiometry

A force tensiometer (Sigma 700, Biolin Scientific UK, Manchester, UK) was used for interfacial tensions measurements. A force tensiometer operates by measuring the forces exerted by the interface on an object of known geometry and properties and calculating the interfacial tension from this information. It this research a platinum microroughened Wilhelmy plate was used of length

19.52 mm and thickness 0.1 mm with a 0° contact angle with water. The buoyancy of the Wilhelmy plate was first measured in the light phase (sunflower oil). To take a measurement, the Wilhelmy plate is lowered into the heavy phase (aqueous phase), the light phase carefully poured on to the heavy phase and the Wilhelmy plate moved to the interface ($F_{external}$) of the two phases. By measuring the force required to keep the Wilhelmy plate at the interface the interfacial tension can be calculated using the following equation.

Equation 4 Wilhelmy plate interfacial tension forces relationship

$$F_{external} + F_{bouyancy} + 2(l + w)\gamma - F_{gravity} = 0$$

Where $F_{external}$ is the force exerted by the Wilhelmy plate in Newtons, $F_{bouyancy}$ is the buoyancy force acting on the plate in N, l is the plate length in m, w the plate thickness in m, γ the interfacial tension in Nm^{-1} and $F_{gravity}$ is the force of gravity on the plate in N. A plot of interfacial tension against time was produced with measurements taken every 1 s.

3.10 ζ-potential

ζ-potential was measured using photon correlation spectroscopy equipment (Delsa Nano, Beckman Coulter, High Wycombe, UK). The ζ-potential is a measure of the electric potential of the double layer on a droplet in the case of an emulsion. The higher the electrical potential on the droplet in either the positive or negative direction the greater the repulsive forces between droplets. As the repulsive forces decrease the tendency for the emulsion droplets to flocculate so the emulsion is less stable to creaming and possibly coalescence depending on the structure of the interfacial layer. A charged

particle in solution attracts and binds a layer of opposingly charged particle ions called the Stern layer, beyond this is a diffuse layer of ions creating a double layer. When the droplets move in a liquid the point where the attached ion remain with the droplet and ions beyond this point remain in solutions is called the slipping plane and this is point at which the electrical potential is measured for a ζ-potential measurement. When an electrical potential is placed across a emulsions, by measuring the velocity of the droplet by electrophoretic light scattering the electrical potential at the slipping plain can be calculated using Henry's equation:

Equation 5 Henry's Equation

$$U_E = \frac{2\varepsilon\zeta F(\kappa a)}{3\eta}$$

Where U_E is electrophoretic mobility, ε is dielectric constant, ζ is ζ-potential, $f(\kappa a)$ is Henry's function, η is the viscosity of the liquid (Kaszuba et al., 2010).

Before measurements, emulsions were diluted 1:10 in distilled water and adjusted the pH with 0.1 M hydrochloric acid or 0.1 M sodium hydroxide as required. Measurements were performed at 20 °C and carried out by taking three independent measurements for each sample.

4 Methods

This chapter details the methods which are common across all three results chapters. Each results chapter contains a methods section which is specific to the experimental work within that chapter.

4.1 Protein extraction

The insect proteins were extracted using the protocol proposed by Zhao et al. (2016) and adapted by Gould and Wolf (2018) with minor changes. Insects were killed by freezing at -80 °C. After freezing at -80 °C, the insects were freeze dried to a constant weight then ground for 30 s pulsing to avoid heat accumulation in a coffee grinder (De'Longhi KG49, Havant, UK). The dried insect powder was defatted by incubating 30 g in 200 ml of ethanol at 40 °C for 1 hour, followed by filtration through Whatman Grade 1 filter paper (Whatman GF/F, Kent, UK) pore size 11 µm, the lipid extraction was performed twice. The residual ethanol was removed by vacuum drying at 40 °C overnight (Weiss Gallenkamp, Loughborough, UK). The defatted powder was dispersed in 400 g of 0.25 M NaOH for 1 hour at 40 °C with gentle agitation, centrifuged at 3500 g for 20 min and 4 °C (J2-21M Induction Drive Centrifuge, Beckman, High Wycombe, UK), the resulting supernatant and gel were collected, and the alkaline extraction process repeated on the pellet. The protein was precipitated from the supernatant by adjusting to pH 4.3 – 4.5 with 2 M HCl. The protein suspension was centrifuged at 2500 g for 15 min and 4 °C (J2-21M Induction Drive Centrifuge, Beckman, High Wycombe, UK), then washed with double distilled water and centrifuged at 2500 g for 10 min, the washing step and centrifugation

were repeated. The protein pellet was dispersed in double distilled water and adjusted to pH 7 with 0.25 M NaOH to select for the proteins with emulsifying properties at a neutral pH (McClements, 2016). The protein dispersion was agitated overnight before adjusting to pH 7 again if required and a further centrifugation at 3500 g for 20 min (J2-21M Induction Drive Centrifuge, Beckman, High Wycombe, UK) from which the supernatant and gel were retained frozen at -80 °C and freeze-dried until a constant weight was reached.

4.2 Nitrogen analysis

Nitrogen content of the insect powders and the protein concentrates were measured with an Elemental Analyser (Flash EA1112, Thermo Scientific, Massachusetts, USA) and the protein content calculated using a 6.25 conversion factor. Lower nitrogen conversion factors for whole insects have been proposed to account for the nitrogen in the chitin , 4.5 by Mishyna et al. (2019) for *Schsitocerca gregaria* (desert locust) and Janssen et al. (2017) for non-*Orthoptera*. Due to the limited evidence and reduction in chitin in the resultant extract the 6.25 global factor was used in this study for all samples. Three replicates were performed for each sample.

Moisture content for the calculation of dry mass was determined by thermogravimetric analysis Mettler-Toledo TGA/DSC 3+ (Mettler-Toledo, Switzerland) by weighing out precisely around 5 mg heated from 20 °C to 550 °C with a 5 °C/min in an aluminium pan with a nitrogen atmosphere. The moisture content was estimated by determining the mass loss between 100 °C and 200 °C.

4.3 Compositional analysis

4.3.1 Midinfrared absorbance spectra

The midinfrared absorbance spectra were collected for the whole insect powder and protein extracts to assess the composition. The spectra were collected using a Fourier transform infrared spectrometer (Tensor 27, Bruker Optics Inc., Billerica, MA, USA) in the 4000-500 cm^{-1} spectral range fitted with a KBr beam splitter and a diamond crystal Golden Gate Attenuated Total Reflectance attachment (SPECAC Ltd, Orpington, Kent, UK). For each measurement, 128 scans at a 4 cm^{-1} resolution were performed and the background subtracted. The spectra were vector normalised in the OPUS software (Version 7.2.1.39.1294., Bruker Optics, Inc., Billerica, MA, USA).

4.4 Protein Solutions

Solutions were produced of the four cricket species at 0.44 % w/w protein content, the actual mass of protein concentrate added was adjusted to account for variations in the protein content, and 0.02 % sodium azide added to prevent microbial growth in either double distilled water or in a pH 7 50 mM sodium phosphate buffer.

4.5 Sunflower oil treatment

Where sunflower oil was treated to remove surface active components the following process was used. Treated sunflower oil was produced by washing sunflower oil with 4 % Florisil for 30 min at 600 rpm, then centrifuged at 2900 g for 30 min at 20 °C (Jouan CR3i multifunction Centrifuge, ThermoFisher

Scientific, Massachusetts, USA), the process was repeated to remove all surface-active impurities as measured by interfacial tension.

4.6 Emulsion Preparation

Emulsions were produced with a 4:1 ratio of protein solution to sunflower oil in a high-speed mixer (L5M Series, Silverson, Chesham, UK) fitted with an emulsor screen at 8000 rpm for 2 min. The emulsions were stored at 20 °C in 100 ml glass vials for 24 hours before first analysis, which was taken as Day 1. The emulsions were not analysed after initial formation because the aim was to measure the stability over a longer time period and to allow the emulsions to stabilise before initial analysis.

4.7 Particle Size Analysis

The diameter of the emulsion droplets was measured by laser diffraction (LS 13 320, Beckman Coulter, High Wycombe, UK) by taking the mean of three independent samples from each emulsion replicate. The droplet diameter was calculated using Mie theory in the particle sizer software with the refractive indices of the continuous water phase of 1.333 and the dispersed sunflower oil phase of 1.464 appropriate for the 780 nm laser, the absorption was set to zero. The volume based mean diameter ($d_{4,3}$) was calculated from the droplet diameter distribution.

4.8 Interfacial Saturation Concentration

To measure the interfacial tension at equilibrium, 35 ml of protein solution at a range of concentrations were measured into a 70 mm circular glass cuvette. The degassed protein solution was placed in the force tensiometer (Sigma 700,

Biolin Scientific UK, Manchester, UK) and the Wilhelmy plate submerged in the protein solution. 50 ml of purified sunflower oil was poured over the protein solution and the Wilhelmy plate moved to the interface. The interface was allowed to equilibrate for 24 hours, in previous pendant drop tensiometer measurements the interface was found to be at equilibrium after 20 hours, data not shown. Measurements were then taken every 1 s and the mean value of the 15 min after 24 hours taken as the equilibrium interfacial tension. The results were plotted protein concentration against interfacial tension and curve-fit performed using OriginPro software, Version 2023 (OriginLab Corporation, Northampton, MA, USA) with a single exponential decay function and Levenberg-Marquardt iteration function to estimate the interfacial tension when the interface is saturated.

5 The Emulsifying and Interfacial Properties of Cricket Proteins

5.1 Introduction

The world population is predicted to grow from 7.8 billion in 2020 to 9.7 billion in 2050 with a 0.34 billion 95 % prediction interval (United Nations, 2019a). With increased population comes increased demand for dietary energy and protein. Protein requirements to fulfil demand could drop by 13 % if each person met the protein demand for a sedentary adult, but an increase in requirement of 33 % is considered "highly likely" (Henchion et al., 2017). As incomes increase, consumption of carbohydrates decreases in favour of animal proteins and vegetables (FAO, 2018). In the FAO "Business-As-Usual" scenario in the FAO Pathways to 2050 report, the mean daily protein intake will decrease from 83.68 grams per person per day to 83.25 per person per day (FAO, 2018). However, with the projected increase population this still amounts to a substantial increase in gross protein demand. Moreover, as the population in many countries age, increased protein consumption maybe desirable to prevent sarcopenia (Deer and Volpi, 2015, Coelho et al., 2022) which prevents falling and the associated injuries in the elderly (Landi et al., 2012). In England and Wales over the period 2018-2020 the crude death rate caused by falls was 11.29 per 1 000 persons for 65-74 year olds and 97.30 per 1 000 persons in over 75 year olds as compared to 2.45 per 1 000 persons for 35-64 year olds(NHS Digital, 2022).

The environmental impact of proteins is higher for the currently utilised animal proteins relative to plant proteins. Global consumption of protein in 2018 was 33.07 g/capita/day from animal sources and 49.84 g/capita/day from plant sources (FAO, 2021). Mammalian and avian animal proteins have been found to produce more greenhouse gas emissions per kilogram of food produced than to plant-based protein sources, with mean emission for beef at 50 kg CO_2eq/100g protein, poultry at 5.7 kg CO_2eq/100g protein compared to 2.0 kg CO_2eq/100g protein for tofu and 2.7 kg CO_2eq/100g protein for grains (Xu et al., 2021, Poore and Nemecek, 2018). The quality of the protein in animal and plant sources differs. Protein quality is judged on the essential amino acid, digestibility, absorption and utilisation. Generally, animal based proteins are higher quality protein relative to plants due to higher digestibility (Tome, 2013) fewer anti-nutritional factors compared to legumes and cereals (Gilani et al., 2012), and an essential amino acid profile which more closely matches human needs (Gorissen and Witard, 2018). Although some legume isolates have similar digestibility (Santos-Hernandez et al., 2020).

Insect proteins have been proposed as an alternative source of animal protein with interest growing in the past 10 year due to high nitrogen conversion ratios when corrected for edible portions (Oonincx et al., 2015), suitable essential amino acid profiles and digestibility (Zielinska et al., 2015, Hawkey et al., 2021). In the European Union insect consumption has been given further impetus with the approval for novel food status of *Acheta domesticus* (house crickets) (SLU et al., 2018), *Tenebrio molitor* (mealworm) (Turck et al., 2021b) and *Locust migratoria* (migratory locust) (Turck et al., 2021a).

A major impediment to the widespread use of insect proteins as a food source in many countries is acceptance by the population due to factors such as disgust, a lack of familiarity, food safety concerns and religion (Natalia et al., 2021). European countries are more averse to eating insects that many Asian and African countries (Hartmann et al., 2015, Van Huis et al., 2013), however within Europe Northern European countries show a higher acceptance than central European countries (Piha et al., 2018). Acceptance of many foods has altered with increasing familiarity and wider consumption by early adopters. Sushi and raw fish consumption in the United States of America is a particular example (Edwards, 2012). In the wider world, insects are an accepted and valuable food source for many countries. Eight countries are projected to count for 50 % of the increased population between now and 2050: Democratic Republic of Congo, Egypt, Ethiopia, India, Nigeria, Pakistan, the Philippines and the United Republic of Tanzania (Nations, 2022). Of these eight countries, insects are consumed in all but Ethiopia and Pakistan (Bernard, 2017).

The insect order *Orthoptera* contains around 24 000 of the one million known species of insects and accounts for 13 % of the consumed species of insects (Stork, 2018, Van Huis et al., 2013, Zhang, 2011). Two of the species approved for novel food status in the European Union, migratory locust and house crickets, belong to the order Orthoptera. The mean protein content of insect species varies greatly by order with *Isoptera* found to have a mean protein content by dry mass of 35 % whereas *Orthoptera* 61 % mean protein content (Rumpold and Schluter, 2013). For the order *Orthoptera,* the essential amino acids per kg of protein meets human requirements (Rumpold and Schluter,

2013) and has high digestibility at 80 – 90 % (Rodríguez-Rodríguez et al., 2022). Similarly, to legume proteins, insect protein isolates have been shown to be more digestible than whole insects (Sere et al., 2021). The mean amino acid contents of all nine essential amino acids of 17 studies of *Orthoptera* were above the mg of amino acid per gram of protein required for humans (Rumpold and Schluter, 2013). However, five of the studies were for *Acheta domesticus* and all five showed house crickets to be slightly deficient in lysine and tryptophan (Rumpold and Schluter, 2013). Although ultimately it was concluded that house crickets have a similar essential amino acid profile to soya (Hawkey et al., 2021). The combination of a stable legal status in many countries for some species, known consumption as a food, high protein content and adequate proportion of essential amino acids make the order *Orthoptera* a suitable candidate for wider use in food products.

Proteins have a wide range of functions in foods such as a primary macronutrient, oil binder, gelling agent, and emulsifying agent. Initially research for utilising insect proteins in foods focused on protein extraction and basic functional properties of insect protein (Yi et al., 2013, Purschke et al., 2018b, Zielinska et al., 2018) with some work on hydrolysates (Purschke et al., 2018a, Hall et al., 2017). Other work has investigated the potential of using whole insects and protein extracts in food products (Kim et al., 2016, Gonzalez et al., 2019).

Some studies began to focus more on specific use cases. Gould and Wolf (2018) showed that *Tenebrio molitor* (mealworms) protein extract stabilise emulsions

comparably to whey protein in terms of droplet diameter and stability of droplet diameter over time at lower protein concentrations. Using an extraction protocol similar to Gould and Wolf (2018) which originated from Zhao et al. (2016), the emulsion activity and stability of mealworms decreased as processing temperature increased and as processing time at a given temperature increased (Lee et al., 2019).

The *Orthoptera* order of insects, which include crickets, locusts and grasshoppers, have the potential to be economically viable and exhibit interfacial functionality. Protein extracted from the *Orthoptera* order of insects have some emulsifying capacity: *Schsitocerca gregaria* (Mishyna et al., 2019); *Gryllodes sigillatus* and *Schistocerca gregaria* (Zielinska et al., 2018); *Gryllodes sigillatus* hydrolysates (Hall et al., 2017) and *Locusta migratoria* (Purschke et al., 2018b). In addition, *Acheta domesticus* (house crickets) protein lowered interfacial tension rapidly and produced stable emulsions, whereas the house cricket chitin produced unstable emulsions showing that it is the protein component which stabilises the oil-water interface (Hirsch et al., 2019b). Pulsed electric field treatment of house crickets when extracting the protein was shown to increase the emulsifying capacity (Psarianos et al., 2022). *Acheta domesticus* (house crickets) are already produced commercially at a large scale for reptile and other animal feed and consumed as a food in many Asian countries and to a lesser extent in European countries (Weissman et al., 2012).

Although house crickets have been approved as a novel food, a singular source of protein can be susceptible to supply disruption. Indeed, house crickets

production was greatly disrupted by the Acheta domesticus Densovirus (AdDNV) leading to the suggestion of the more resilient *Gryllus bimaculatus* (black cricket), *Gryllus assimilis* (silent crickets) and *Gryllodes sigillatus* (banded crickets) as replacements (Maciel-Vergara and Ros, 2017, Szelei et al., 2011, Weissman et al., 2012). The replacement protein must behave similarly at the oil-water interface of an emulsion to provide a viable alternative in a food product. Therefore, as the house cricket become an accepted food ingredient it is important to investigate alternative species as protein sources for use in oil-in-water emulsions. Other insect species within the taxonomic order *Orthoptera* within the family *Gryllidae* are the best candidates for the substitution of house crickets being the most similar physiologically.

The aim of this study is to test the use of proteins extracted from four cricket species: house cricket, banded cricket, silent cricket and black cricket as emulsifier and to gain insights into the cricket protein structure. Understanding this future protein source would be a significant step towards the utilisation as a functional ingredient in commercial food products.

5.2 Materials & Methods

5.2.1 Materials

Acheta domesticus (house crickets) 15 – 18 mm; *Gryllus bimaculatus* (black cricket) 18 – 25 mm; *Gryllus assimilis* (silent crickets) 18 – 25 mm; and *Gryllodes sigillatus* (banded crickets) 15 – 18 mm were purchased live from Monkfield Nutrition (Royston, UK). For the protein extraction and emulsion preparation, sodium azide (NaN_3), Florisil < 200 mesh were purchased from Sigma-Aldrich

(Dorset, UK). Hydrochloric acid (HCl), ethanol and sodium hydroxide (NaOH), were purchased from Fisher Scientific (Loughborough, UK), sunflower oil was purchased from a retailer (Sainsbury's, London, UK).

For the amino acid analysis, hydrogen peroxide, formic acid 98 – 100 % analytical reagent, phenol, sodium metabisulphite analytical reagent, hydrochloric acid, ammonium formate, acetonitrile LC/MS grade, isopropanol LC/MS grade were purchased from Fischer Scientific (Loughborough, Leicestershire, UK). Cell free amino acid mixture ^{13}C,^{15}N, amino acid standard, cysteic acid, methionine sulfone, alanine ^{15}N, phenylalanine ^{15}N were purchased from Sigma-Aldrich (Merck Life Science UK Limited, Gillingham, Dorset, UK). Phenylalanine $^{13}C_6$ was purchased from Cambridge Isotopes Limited (Cambridge, MA).

5.2.2 Methods

The cricket protein extracts were produced by the method in 4.1 Protein extraction p100. The protein contents of both the whole cricket powders and protein extracts were estimated by the method in 4.2 Nitrogen analysis p101. Compositional analysis was performed using midinfrared absorbance spectroscopy by the method in 4.3 Compositional analysis p102 with five replicates. Protein solutions were produced with double distilled water by the method in 4.4 Protein Solutions p102. Four replicates of each emulsion were produced with 80 g of protein solution and 20 g of untreated sunflower oil with the method given in 4.6 Emulsion Preparation p103. To determine if the surface-active components of the sunflower oil were impacting emulsion

structure, banded cricket protein emulsions were produced by the same process with sunflower oil treated to remove surface active impurities. Particle size analysis was performed using the method detailed in 4.7 Particle Size Analysis p103. Interfacial saturation concentration was determined by the method given in 4.8 Interfacial Saturation Concentration p103 with protein dialysed by the process given below.

5.2.2.1 Amino Acid Analysis

The amino acid content of the four cricket protein extracts and banded cricket powder was analysed by first oxidising approximately 5 mg of material in hydrogen peroxide formic acid phenol solution for 16 hours. The excess oxidation solution was decomposed with 0.42 g of sodium metabisulphite. 25 ml of HCl was added and the samples were heated for 30 min at 150 °C in a microwave (Multiwave pro, Anton Paar, Graz, Austria). After cooling the samples were adjusted to pH 2.20 using 7.5 N and 1 N NaOH and made up to 100 ml with 2 ml of norleucine standard and pH 2.20 tri-sodium citrate buffer. The hydrolysates were centrifuged at 3000 rpm for 2 mins and the supernatant passed through a 0.22 µm filter and analysed with an amino acid analyser (Biochrom 30, Biochrom, Cambridge, United Kingdom).

5.2.2.2 Microscopy

Micrographs were recorded on an optical microscope (EVOS f1, AMG, Washington, USA) using brightfield microscopy to assess the microstructure and support the particle size measurements. A drop of emulsion was placed on a microscope slide and a coverslip placed over. Micrographs were taken at Day 1, Day 7, Day 20 and Day 40.

5.2.2.3 Protein Dialysis

To remove any impurities such as residual salts which could impact interfacial tension measurements the banded cricket protein was dialysed. For the interfacial measurements fresh protein extractions were performed for banded crickets and the pH 7 soluble fractions were dialysed for three days using 1 kDa dialysis tubing (Spectrum Spectra/Por Dialysis Membrane, Repligen, Waltham, Massachusetts, USA) to remove any impurities such as salts which could change the interfacial tension. The protein was dialysed against reverse osmosis water at 4 °C changing the water three times the first day, and twice a day for two further days.

5.2.2.4 Statistical Analyses

One-way ANOVAs was performed using VSN International (2020). Genstat for Windows 21st Edition. (VSN International, Hemel Hempstead, UK. Web page: Genstat.co.uk). A post-hoc Tukey test was applied with a $P < 0.05$ threshold for significance to show difference between treatments of the mean volume-based diameter ($d_{4,3}$) of the emulsion samples and the interfacial tension values for protein concentration.

5.3 Results and Discussion

The difference in cricket protein properties was assessed by the amino acid composition. The functional properties were assessed by forming oil-in-water emulsions and measuring interfacial tension.

5.3.1 Protein Content

The protein content of the freeze-dried insect powders and the protein extracts from the four cricket species were determined to evaluate the efficacy of the extraction process in concentrating the cricket proteins. Furthermore, the protein content was used equalise the mass of protein in the protein stabilised emulsions for each cricket species.

The protein content of the four cricket species and freeze-dried powders is shown in Table 3. The protein content of the freeze-dried powders from the four cricket species was similar ranging for 70 (± 1) % for house crickets to 61 (± 2) % for black crickets. Protein content can be affected by diet as previously

Table 3 The protein content measured by the Dumas method calculated with a 6.25 nitrogen factor on a dry mass basis for the four cricket powders and protein extracts, n=3, standard deviation in parentheses

Cricket species	Protein content dried Powder (%)	Lipid (%)	Protein content of dried protein extract (%)	Protein Extraction Efficiency (%)
House Cricket	70 (± 1)	29 (± 2)	93 (± 1)	12 (± 3)
Black Crickets	61 (± 2)	37 (± 1)	94 (± 1)	15 (± 3)
Silent Crickets	64 (± 2)	26 (± 0.8)	89 (± 1)	14 (± 3)
Banded Crickets	69 (± 4)	27 (± 0.2)	96 (± 1)	14 (± 2)

noted (Oonincx and van der Poel, 2011, Collavo et al., 2005, Oonincx et al., 2015), sex (Kulma et al., 2019), age, life stage (Ademolu et al., 2010) and growing conditions (Gligorescu et al., 2018).

House crickets have the most published data regarding protein content of the four species because they have been approved for human and animal consumption in the European Union and other countries. The house crickets used in this study have a higher protein content than previously recorded for house crickets, although differing analysis methods and nitrogen conversion factors make direct comparisons difficult. House cricket protein content was shown to vary between 56.2 % and 60.0 % on a dry mass basis with a 6.25 nitrogen conversion factor, depending on diet (Collavo et al., 2005). Using amino acid analysis, the protein content of house crickets has been measured at 54.9 (± 0.8) %, considerably lower than the value stated in Table 3, and the nitrogen factor calculated to be 5.25 (± 0.12) % this would account for 11 percentage points of the discrepancy (Boulos et al., 2020). The true protein content of house crickets has been measured to be lower still at 46 % and 41 % on a wet weight basis, from two different samples giving nitrogen factors of 4.53 and 4.80 respectively (Belghit et al., 2019). Therefore, the 70 % protein content for house crickets is likely an overestimate, however the range of reported values shows the variability in protein content of house crickets. Specific nitrogen factors were not available at the time the work was performed for cricket protein extracts by this extraction method so the protein content of both the cricket protein extracts and whole cricket powders were estimated using the global 6.25 nitrogen factor to allow a direct comparison.

Whole oven dried banded cricket meal was shown to have a protein content of 67.76 % on a dry mass basis similar to the 69 % reported in this study (Kilburn et al., 2020) , whereas cricket nymphs were shown to have a protein content of

56% (Jozefiak et al., 2016) supporting the conclusion that life stage impacts nutrient composition.

For the black crickets the 61 (± 1) % on a dry mass basis protein content is similar to the hot air dried black crickets at 59.88 g/100 g (Seong and Kim, 2021) and 58.5 % dried at 45 °C (Dobermann et al., 2019) on a wet weight basis, and 64.9 % (Jeong et al., 2021b) on a dry weight basis. The black cricket protein content is within the bounds of the reported values from the literature; however, the same caveats apply regarding the nitrogen factor as for the house crickets. The lower protein content in black crickets may be accounted for by a higher lipid content at 37 (± 1) % in black crickets compared to 26 (± 0.8) % for silent crickets 27 (± 0.2) % for banded cricket and 29 (± 2) % for house crickets, shown in Table 3. A protein content of 62.76 (± 1.12) % has been reported for silent crickets (Machado and Thys, 2019) similar to the 64 (± 2) % level reported in Table 6, what is more 0.75 % non-protein nitrogen was found to be present which would have been counted as protein by the methodology used in this study. However, in a study where silent crickets were collected around a village in Benue State Nigeria and sun-dried, rather than farmed commercially the protein content was 73.6 % on dry weight basis using a 6.25 nitrogen conversion factor (Oibiokpa, 2017). Furthermore, the lipid content from the wild caught insects by Soxhlet was 7.3 % considerably lower than the lipid content of silent crickets at 27 (± 0.2) % and of silent crickets previously reported 20.96 (± 0.28) % on a dry mass basis (Machado and Thys, 2019). The differing lipid contents are in line with the evidence on feed composition, life stage and environmental conditions affecting macronutrient composition. Wild

silent crickets could be expected to have vastly different diets and more changeable growing conditions than farmed silent crickets. A secondary factor is that ethanol was used as a solvent here whereas the non-polar petroleum ether was utilised in the other two studies. Ethanol extracts more polar lipids such as phospholipids and glycolipids compared to the non-polar petroleum ether (Xie et al., 2017, Jeong et al., 2021a).

The aim of this study was to investigate the potential for cricket protein to be used in food emulsions, to this end a protein extraction was used to minimise other components such as chitin which could potentially adsorb to the oil-water interface. The protein content of the protein extracts from Table 3 by dry mass protein extracts were 93 (± 1) % for house crickets, 94 (± 1) % for black crickets, 89 (± 1) % for silent crickets and 96 (± 1) % for banded crickets. When a Soxhlet lipid extraction was followed by a similar alkaline protein extraction on house crickets the protein concentration of the extract was 58.3 (± 0.5) % with no prior defatting, and 74.3 – 78.5 % depending on the solvent used with a prior Soxhlet extraction with no significant difference for the value for hexane extraction and ethanol extraction (Laroche et al., 2019). Hirsch et al. (2019b) produced a protein extract of 50.1 (± 1.8) % from house crickets through an alkaline extraction at pH 9.5 adjusted to pH 7.5 and retaining the supernatant. The lower protein concentration in this extract without an isoelectric point precipitation step suggest further impurities are being excluded through this step. Hall et al. (2017) produced protein extracts of up to 70.8 (± 0.01) % using an enzymic hydrolysis compared to a 55.8 (± 0.01) % control extracted at pH 8.0. The protein concentrations of the protein extracts shown in Table 3, are

high compared to extracts produced by other extraction protocols, even when considering a higher nitrogen factor. The higher protein content is due to the higher pH of the 0.25 M NaOH extraction medium relative to other extraction processes, the isoelectric point precipitation step and the selection of pH 7 soluble proteins.

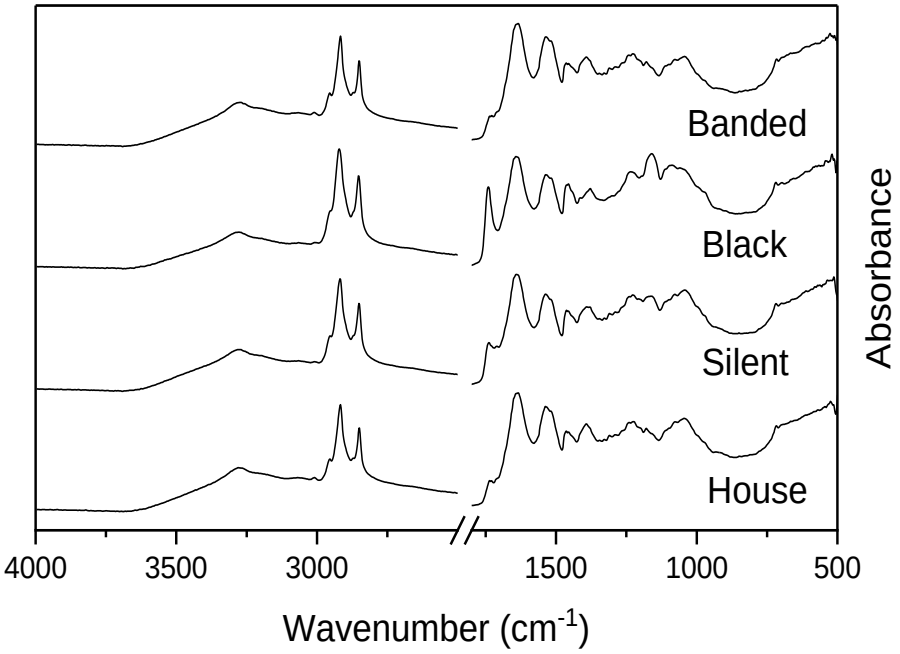

Figure 2 Mid-infrared spectra of whole cricket powders

The infrared absorbance spectra were qualitatively analysed to confirm if the defatting and protein extraction process effectively removed a large proportion of the lipids, reduced the chitin content and increased the protein content. The C-H bonds in the aliphatic fatty acid chains show absorbance at 2936 – 2915

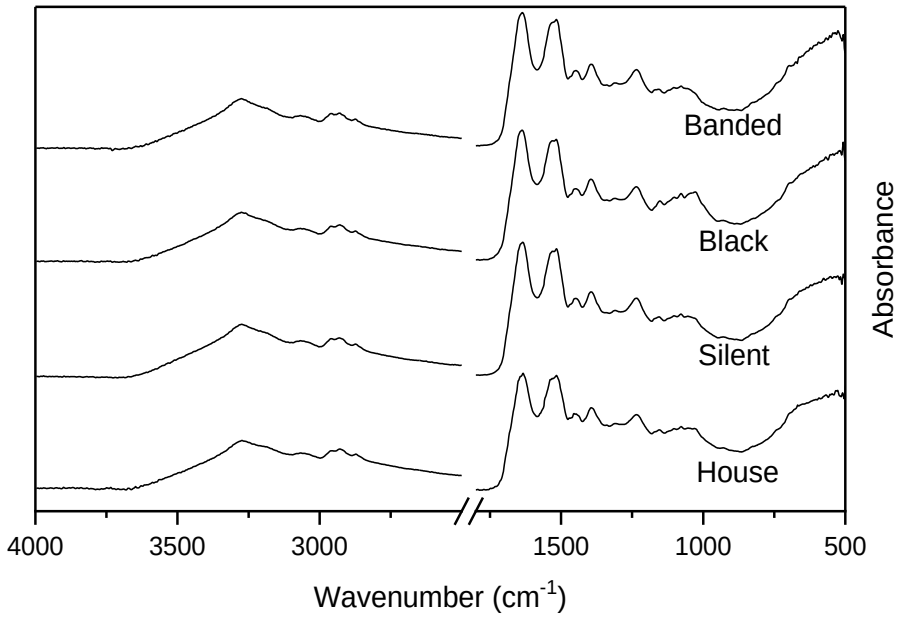

Figure 3 Mid-infrared spectra of cricket protein extracts

cm^{-1} and 2865 – 2833 cm^{-1} wavenumbers causing an asymmetric stretch in; a C=O stretch in the 1750 – 1735 cm^{-1} region is characteristic on the ester bond in lipids (Larkin, 2017). The asymmetric C-H stretch peaks at 2916 – 2921 cm^{-1} and 2849 – 2955 cm^{-1} in the whole insect powders in Figure 2 are almost absent in the protein extracts shown in Figure 3. Furthermore, all four cricket species show C=O stretch at 1736 cm^{-1} in banded, 1738 cm^{-1}, 1741 cm^{-1} and three replicates of house cricket powder shown in Figure 2, but is not present in the extracts in Figure 3. The black cricket powder which contained the highest proportion of lipids had the largest C=O stretch peak. The reduction of the C=O peak and the C-H peak supports the lipid extraction results in Table 3 to show a reduction in lipids from the whole powder to protein extracts.

The C=O stretch from the amide bond in proteins produces a band around 1650 cm^{-1} and is known as the amide I region (Pelton and McLean, 2000). The amide

I peak is present at 1634 cm^{-1} for banded crickets, 1636 cm^{-1} for black crickets, 1638 cm^{-1} for silent crickets and 1635 cm^{-1} for house cricket powders from Figure 2. The amide I peak remains present in the protein extracts, in Figure 3, at 1635 cm^{-1} for banded crickets, 1634 cm^{-1} for black crickets, 1636 cm^{-1} for silent crickets, 1634 cm^{-1} for house crickets, supporting the nitrogen analysis data that the protein is retained through the extraction process.

A reduction in the absorbance peaks due to chitin in the protein extracts is present relative to the whole insect powders. Chitin shows peaks at ≈1160 cm^{-1} for the ether bond between the N-acetylglucosamine monomer units and ≈1030 cm^{-1} and ≈1070 cm^{-1} for the ether bond within the N-acetylglucosamine (Johnson and Naiker, 2020). Banded cricket powder shows peaks 1045 cm^{-1}, 1076 cm^{-1}, and 1179 cm^{-1}, which are absent in the protein extract, indicating the reduction of chitin in the final protein extract relative to the whole insect powder.

5.3.2 Amino Acid Analysis

The amino acid composition of the protein extracts and the powders were assessed to ascertain if the cricket species would be expected to produce emulsions with differing properties or differing emulsifying capacities.

Table 4 shows the amino acid composition of the protein extract of the banded cricket, black cricket, house cricket and silent crickets and one example of the insect powders from the banded cricket. The amino acid composition is broadly consistent across all four cricket protein concentrates. The protein concentrates are relatively high in Glutamic acid at between 15 and 16 g/100g

protein, and Aspartic acid at between 11 and 12 g/100 g protein. The pK_a of the carboxylic acid group on the side chains of pH 4.25 for Glutamic acid and aspartic acid is pH 3.65 which is close to the pH 4.3 - 4.5 isoelectric point precipitation. Therefore, proteins rich in these amino acids may have been preferentially isolated during the extraction. Both Glutamic acid and Aspartic acid showed an increase from the banded cricket powder to the banded cricket protein concentrate, further suggesting concentration through the extraction process. As previously reported for house crickets both the protein concentrates and banded cricket powder were low in the sulphur containing amino acids cysteine and methionine (Sipponen et al., 2018). There have only been comparison at the time of writing between whole insect powders and extracted proteins for house crickets. The amino acid composition found for house crickets proteins differs in some key aspects to that reported for whole house cricket powders, higher proportions of aspartic acid and glutamic acid, and either lower phenylalanine or tyrosine (Yi et al., 2013, Sipponen et al., 2018). The increase aspartic and glutamic acid reported in the literature agree with the results for banded crickets here. However only tyrosine fell in the banded cricket extracts with phenylalanine increasing slightly in proportion.

Amino acids with aliphatic or aromatic side chain which could be considered hydrophobic are Tyrosine, Tryptophan, Alanine, Isoleucine, Leucine, Valine, Phenylalanine, Methionine and Cysteine (Creighton, 2010). As a proportion of total measured amino acids, the hydrophobic amino acids excluding tryptophan which was not measured, account for 38 % in banded crickets, 37 % in black crickets, 37 % in house crickets and 36 % in silent crickets, this is

comparable to the 38 % calculated from Gorissen et al. (2018) for whey proteins.

All four protein extracts have similar proportions of hydrophobic amino acids, so it could be hypothesised that the four protein extracts will have similar emulsifying properties.

Table 4 The amino acid composition and standard deviation of freeze-dried banded cricket powder and the protein concentrate of the four cricket species, the colouring represents relative concentration of amino acids in (green higher, red lower) n=2.

Amino acid	Banded cricket powder (g/100 g protein)		Banded cricket protein (g/100 g protein)		Black powder protein (g/100 g protein)		House cricket protein (g/100 g protein)		Silent cricket protein (g/100 g protein)	
Cystine	1.8	± 0.2	1.1	± 0.2	1.0	± 0.1	1.0	± 0.1	1.0	± 0.0
Aspartic acid	9.5	± 0.7	12.0	± 1.4	11.7	± 1.5	12.1	± 0.3	12.4	± 0.8
Methionine	2.1	± 0.4	2.2	± 0.1	1.8	± 0.0	2.2	± 0.0	1.7	± 0.2
Threonine	4.1	± 0.7	3.8	± 0.6	3.7	± 0.8	4.0	± 0.3	3.9	± 0.9
Serine	5.0	± 0.3	4.7	± 1.2	5.0	± 0.8	5.2	± 0.1	5.2	± 0.4
Glutamic acid	13.6	± 0.1	15.8	± 0.6	14.9	± 0.4	15.4	± 0.1	15.8	± 0.2
Glycine	5.9	± 0.7	5.4	± 1.0	5.9	± 1.2	5.5	± 0.3	5.8	± 1.0
Alanine	12.0	± 2.5	7.8	± 1.3	9.0	± 1.2	8.1	± 0.3	9.1	± 1.1
Valine	4.7	± 0.9	5.2	± 1.2	4.8	± 1.5	4.0	± 0.2	4.6	± 0.7
Isoleucine	3.1	± 0.8	3.8	± 1.7	3.2	± 1.6	3.0	± 0.4	3.1	± 0.8
Leucine	7.4	± 0.1	8.5	± 0.7	8.4	± 0.9	8.6	± 0.2	8.4	± 0.4
Tyrosine	4.9	± 0.1	4.0	± 0.3	4.5	± 0.4	5.1	± 0.2	3.2	± 0.3
Phenylalanine	3.8	± 0.1	4.5	± 0.4	4.5	± 0.2	4.6	± 0.2	4.4	± 0.3
Lysine	6.1	± 0.3	7.2	± 0.6	6.8	± 0.5	6.9	± 0.1	6.7	± 0.4
Histidine	2.7	± 0.4	2.3	± 0.1	2.3	± 0.4	2.1	± 0.1	2.3	± 0.1
Arginine	7.6	± 0.8	6.7	± 0.2	6.8	± 0.2	6.6	± 0.0	6.7	± 0.0
Proline	5.7	± 0.3	5.0	± 0.0	5.6	± 0.6	5.4	± 0.3	5.9	± 0.2

5.3.3 Cricket Protein Stabilised Oil-in-Water Emulsions

The emulsifying capacity and stability of the cricket protein extracts was assessed over a 40-day period. Oil-in-water emulsions were produced from the 0.44 % protein solutions of all four cricket species which was confirmed by brightfield microscopy on Day 1. Microscopy image shown for day 7 in Figure 4, day 20 in Figure 5 and day 40 in Figure 6 confirm that the emulsions maintained intact microstructure over the 40-day period.

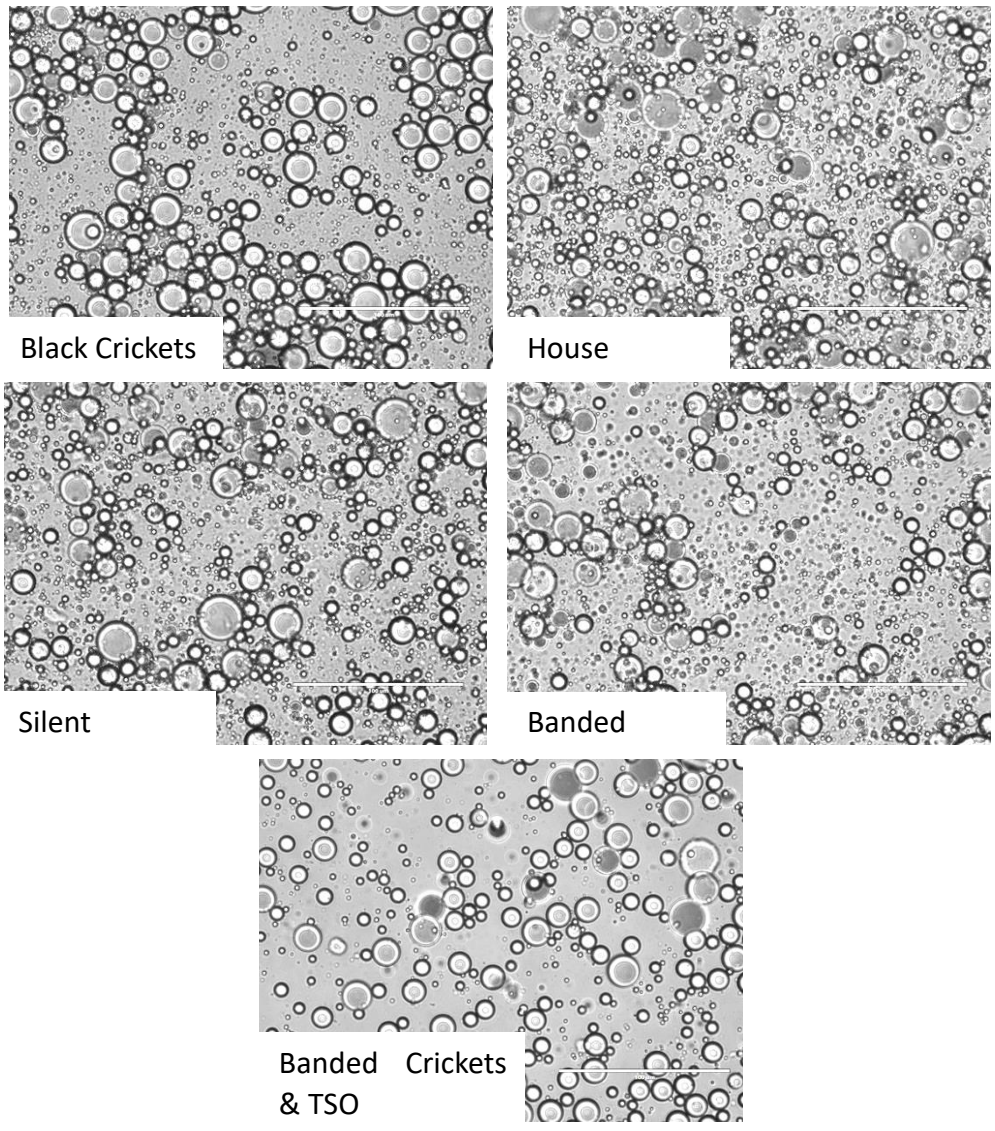

Figure 4 Day 7 micrographs of the emulsions stabilised with protein from the four cricket species,, treated sunflower oil (TSO), scale bars 100 µm

The volume-based droplet diameter distribution, shown in Chapter 9.1 Appendix I p230, for the emulsions produced from the house cricket, silent cricket and black cricket protein and untreated sunflower oil were monomodal over the measured time. Whereas the volume-based droplet diameter distribution for the emulsions produced from the banded cricket protein and untreated sunflower oil were bimodal with a second minor peak at 4.5 µm. The micrographs confirmed droplet diameter results from the laser diffraction particle sizer, showing a range of droplet diameters with numerous droplets 10 µm or lower and fewer larger droplets of 20 µm or larger.

The mean $d_{4,3}$ of the emulsion oil droplets for the four cricket species on Day 1 from Figure 6, were house crickets (11.7(± 0.3) µm, black cricket (13.7 (± 0.5) µm, banded cricket 13.4 (± 0.3) µm and silent cricket 14.0 (± 0.2) µm. The mean $d_{4,3}$ of the house cricket emulsion droplets was significantly lower ($F_{(14,45)}$ = 15.85, $p < 0.05$) than the other three cricket species although the absolute difference is minimal at less than 3 µm. The $d_{4,3}$ for the four cricket protein emulsions produced with untreated sunflower oil and the $d_{4,3}$ for the banded cricket emulsions produced with treated sunflower oil were not significantly different between Day 1 and Day 40 evidenced by the presence in the same groups from the Tukey's post hoc test in Figure 6. The house crickets for example showing a small increase of 1.5 µm to (13.2 ± 0.5) µm. The lack of

significant difference between the mean $d_{4,3}$ of the untreated sunflower oil cricket emulsions indicates no more coalescence at Day 40 than Day 1.

The banded cricket with treated sunflower oil emulsions showed a slightly larger mean $d_{4,3}$ at 16.7 (± 1.4) µm decreasing to 15.1 (± 1) µm after 40 days, although the lack of significant difference suggests this is measurement or sampling error. The significant difference between the treated sunflower oil and untreated sunflower oil indicates that surface-active components of the untreated sunflower influence enable the formation of smaller emulsion droplets. But the stable emulsion formed with the treated sunflower oil indicates the cricket proteins form stable emulsion droplets independent of any interfacially active components in the untreated sunflower oil. Untreated sunflower oil contains lecithins, 0.1 – 0.2 % depending on extraction method (Carelli et al., 1997), which can lower interfacial tension at an oil-water interface and form oil-in-water emulsions at levels of 0.1 % (Pan et al., 2002). In protein stabilised emulsions, lecithins can improve emulsions properties and stability by allowing for smaller droplet diameters and lower interfacial tension

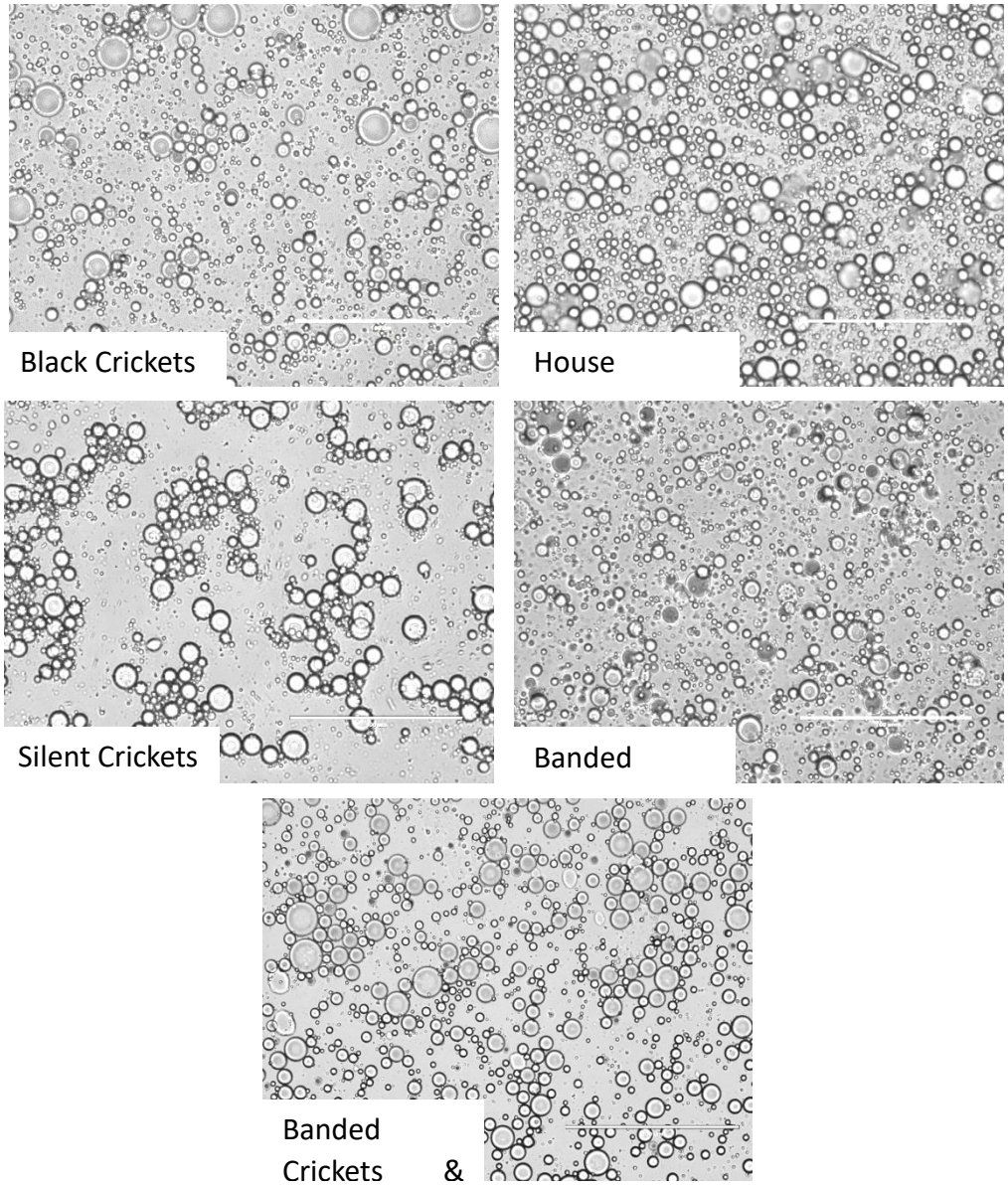

Figure 5 Day 40 micrographs of the emulsions stabilised with protein from the four cricket species, scale bars 100 µm

or be detrimental by increasing droplet diameters depending on the concentrations and complexations formed (van Nieuwenhuyzen and Szuhaj, 1998, Sunder et al., 2001). For example, inclusion of a low level of soy lecithin 0.25 – 0.75 % has been associated with a reduction in droplet diameter of whey protein isolate stabilised oil-in-water emulsions through complex formation with the proteins at the interface, however inclusion at 1 – 2 % concentration

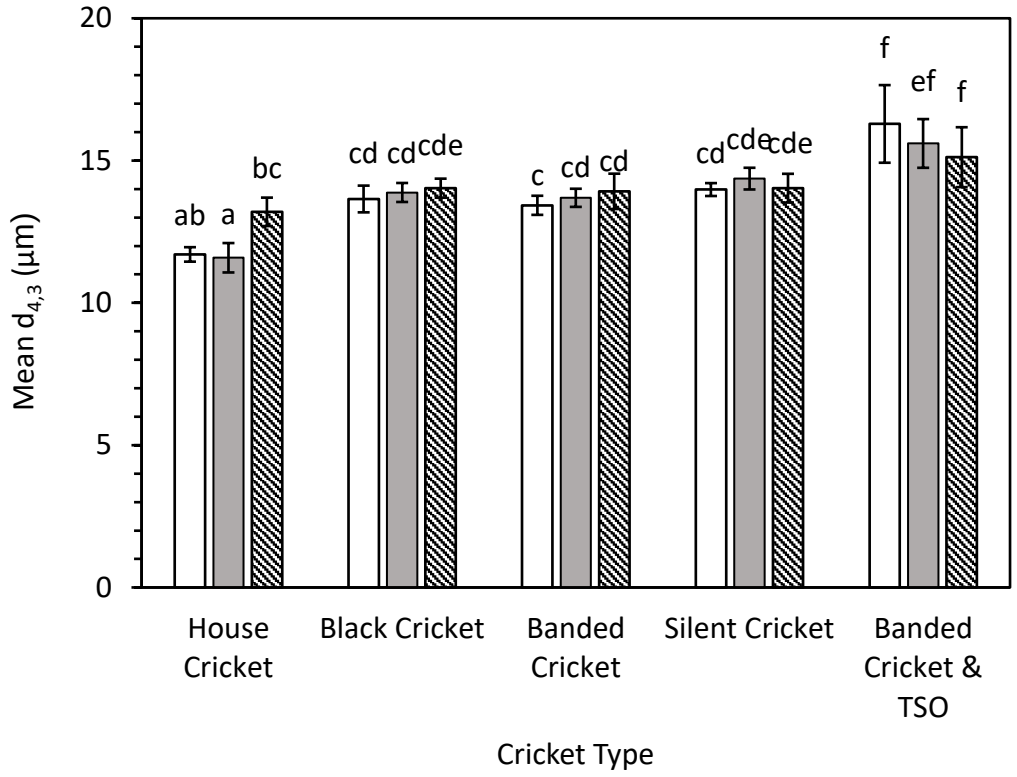

Figure 6 Mean droplet diameter of emulsions stabilised with cricket proteins as measured by laser diffraction particle sizing, white bars are at day 1, grey bars day 20, striped bars day 40, error bars show standard deviation, letters denote significant, difference $p < 0.05$, n=4 for all cricket types and banded cricket with treated sunflower oil (TSO)

enables the lecithins to displace the proteins at the interface increasing droplet diameter (Wang et al., 2017).

The mean $d_{4,3}$ of the banded cricket-treated sunflower oil emulsions decreased over the 40-day period but with a larger standard deviation and only the day 40 emulsion and not statistically different. There was no visible oil separation on any of the emulsions suggesting no phase separation of larger droplet for the banded cricket-purified sunflower oil emulsions. The reduction in droplet diameter may be partially explicable by the higher standard deviation of the mean $d_{4,3}$ for the banded cricket purified sunflower oil emulsion or incomplete homogenisation before analysis in earlier measurement as the larger diameter emulsion droplets would cream most rapidly and be at the top of the emulsion.

The results show that all four cricket species can form oil-in-water emulsions which are stable to coalescence over a 40-day storage period, in the presence other surface-active components from the untreated sunflower oil. The minimal difference between mean $d_{4,3}$ of the untreated cricket species and the stable banded cricket-purified sunflower oil emulsions suggest all four species would form stable emulsions in purified sunflower oil.

Findings presented are in agreement with previous studies that showed house crickets can form stable emulsions in purified canola oil and that house cricket protein extracts produced emulsions stable over 20 days with a minimal increase $d_{4,3}$ at 2.5 % protein w/w (Hirsch et al., 2019b). The mean $d_{4,3}$ of oil-in-water emulsions stabilised by mealworm proteins from a similar extraction protocol produced with 20 % sunflower oil-in-water emulsions with a similar droplet diameter of around 12 µm and stability over a two-month period (Gould and Wolf, 2018).

There is a minimum droplet surface coverage for a protein to produce stable emulsions and a maximum coverage where no more protein can be adsorbed at the interface (Fang and Dalgleish, 1993). The stability of the emulsions over 40 days suggests the 0.44 % for this specific protein extraction protocol is above the maximum interfacial coverage for these emulsion parameters, Gould and Wolf (2018) tested mealworm protein from the same protocol at concentrations up to 3 % protein with no further decrease in $d_{4,3}$ further supporting this hypothesis.

5.3.4 Interfacial Tension

The principal properties required of a molecule to form a stable emulsion are to lower interfacial tension and prevent coalescence of droplets over the life of the emulsion (Damodaran, 2005, McClements, 2016). Protein stabilised interfaces typically exhibit a rapid reduction in interfacial tension with an equilibrium surface pressure of between 15 mN/m and 20 mN/m (Damodaran, 2005). The interfacial tension was measured at a range of concentrations to confirm the mechanism for droplet stabilisation and establish the concentration at which the oil-water interface becomes saturated with protein molecules.

Protein absorption takes place in three phases: diffusion to the interface, adsorption to the interface and reorganisation and conformational changes at the interface (Graham and Phillips, 1979). In protein stabilised oil/water emulsions, the protein is dispersed in the aqueous phase. The rate at which a protein molecule diffuses to the interface plays a role in the rate at which an emulsion droplet can be stabilised and therefore the diameter of the emulsion droplet which can be produced from proteins with lower molecular weight (Lam and Nickerson, 2013), higher surface hydrophobicity (Kato and Nakai, 1980), higher molecular flexibility (Tang, 2017), higher surface charge and consequently aqueous solubility (Sharif et al., 2018, Karaca et al., 2011b).

The interfacial tension of the water-treated sunflower oil interface was 28.8 (± 0.7) mN/m. The protein solutions at all concentrations showed a steep reduction in interfacial tension in the first 25 min, the 0.01 % protein solution

decreased by 11.4 mN/m or within 32 % of the equilibrium value. The lowest protein concentration at 0.001 % tested reached within 10 % of the equilibrium interfacial tension by 32 000 s or less than 9 h, with all other protein concentrations requiring less time.

The interfacial tension of the protein solution-treated sunflower oil interface shows a sharp reduction from 0.001 % protein reaching an equilibrium value of 12.8 mN/m at 0.01 % shown in Figure 7. The exponential decay function was fit to estimate the equilibrium value of interfacial tension and the rate of reduction but does not accurately model the protein adsorption kinetics. The interface could be considered to have saturated monolayer at a protein solution concentration of 0.01 %, this concentration is similar to β-casein 0.01 % but lower than bovine serum albumin at 0.1 % (Lucassen-Reynders et al.,

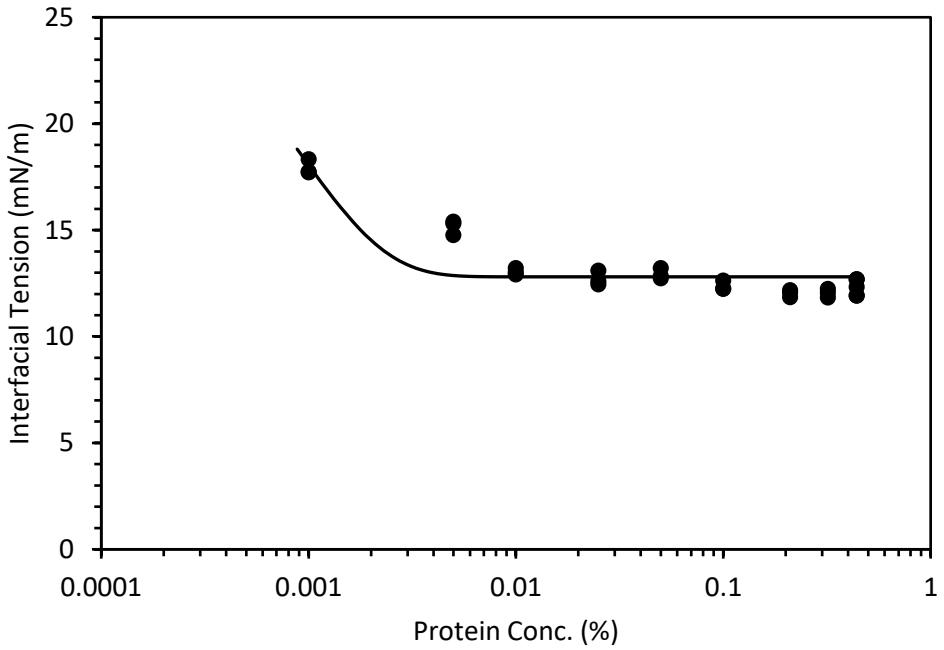

Figure 7 The interfacial tension of banded cricket protein solution against treated sunflower oil, line shows an exponential decay function curve fit

2010), considerably lower than 2 % for faba beans (Felix et al., 2019b) and 3 % for pH 7.5 for chickpea protein (Felix et al., 2019a).

The interfacial pressure (Π) at 0.01 % protein is 15.7 mN/m, comparable to the values reported for 0.1 % bovine serum albumin (BSA) and 0.01 % β-Casein against purified sunflower oil at both 16 mN/m (Lucassen-Reynders et al., 2010, Benjamins et al., 2006). Protein flexibility and conformation influence emulsifying properties, more flexible proteins can orient with hydrophobic regions in the oil phase and charged and polar regions in the aqueous phase reducing the interfacial tension at a faster rate than more rigid and globular proteins (Freer et al., 2004a, Freer et al., 2004b, Bos and van Vliet, 2001). The low protein concentration at which the monolayer is formed suggests a more flexible protein able to orient as such to cover the interface at a low concentration (Razumovsky and Damodaran, 1999).

5.4 Conclusion

The four cricket species produced protein extracts with little difference in protein concentration and amino acid content. Showing that overall, the alkaline extraction process separates proteins with similar amino acid compositions for all of the cricket species. The high protein concentration of the protein extract shows that insect protein isolates of greater than 90 % protein are possible. Insect protein isolates would allow for the addition of insect protein as an ingredient with minimal other macronutrient components.

All four cricket species produce stable oil-in-water emulsions over a 40-day period through lowering of interfacial tension and the formation of an

interfacial layer. Therefore, there is considerable evidence that the four cricket species may be used interchangeably to stabilise oi-in-water emulsions. This builds on previous studies indicating cricket proteins and insects more generally extracted by well-established processes may be a viable alternative to more established animal protein to stabilise oil droplets in emulsion systems.

This study raises the question as to whether other orders or life stages of insects would produce stable oil-in-water emulsions. Food emulsions are complex multicomponent systems subject to heating processes, pH changes and addition of other interfacially active species. Cricket proteins have been shown to stabilise a model system with sunflower lecithins present, however understanding the effect of production processes and other ingredients would be necessary before utilisation in a consumer food product to ensure stability and quality.

6 The effect of insect order and life stage on insect protein stabilised emulsion microstructure as relates to food processing conditions

6.1 Introduction

During the Sars-Cov-2 pandemic numerous supply chains have been shown to come under increasing pressure and shortage of many materials occurred (Bakalis et al., 2020). The shortages have led to large price fluctuations which have priced many manufacturers out the market leading to a lack of supply of goods to consumers. Geopolitical instability has compounded the problems of supply particularly in wheat and sunflower seeds at present (Dongyu, 2022). In the food manufacturing sector, pandemic and geopolitical issues come in addition to the ever-present problems of weather, pestilence and disease which affect the yields, quality and availability of materials for food production (Tendall et al., 2015). A greater emphasis on flexibility in both sources of materials and the types of materials is required. With regards to the use of insects as functional food ingredients, there is an opportunity to develop ingredients which can be extracted from multiple insect species. This flexibility in species would allow production of insects in different locations and the substitution of one species for another in the case of disease of a lack of feed input. The risk of disease in insects is not theoretical, with the *Acheta domesticus* (house cricket) adenovirus decimating cricket farms in the 2010s and other more resilient species being farmed as replacements (Maciel-Vergara and Ros, 2017, Szelei et al., 2011, Weissman et al., 2012).

Numerous studies have shown the viability of insect proteins as emulsifiers in oil-in-water emulsions (Wang et al., 2021, Gould and Wolf, 2018, Hirsch et al., 2019a). In Chapter 5, protein from four different species of cricket were shown to form stable oil-in-water emulsions, showing that at least within the order *Orthoptera* the emulsifying properties of the proteins may be similar. Much work has focused on the *Tenebrio molitor* (mealworm) larvae due to the EU commission approving mealworms as a novel food (Turck et al., 2021b) and the existing production infrastructure for reptile feed, bird feed and fishing bait. Indeed, Gould and Wolf (2018) showed the potential for the use of protein extracted from mealworm larvae to be utilised as an emulsifying agent. Both *Acheta domesticus* (house crickets) (Fernandez-Cassi et al., 2019) and *Locusta migratoria* (migratory locust) (Turck et al., 2021a) have received similar approval by the EU commission showing the order *Orthoptera* is a source of insect protein which must be considered. The ability to utilise both mealworms and orthoptera interchangeable as a protein source would be highly advantageous.

Insects of one species, unlike mammals, often exhibit more than one morphology during a lifetime. The two main modes of insect metamorphosis which are holometabolous involving a complete alteration in morphology and hemimetabolous which undergo a partial change in morphology (Hall and Martin-Vega, 2019). *Coleoptera* and *Lepidoptera* are two examples of insect orders which are holometabolous having larval, pupal and adult life stages; the many composite anatomical components are the same between life stage but vary greatly in size and shape as discussed in Chapter 2 Section 2.2.1. Insect

Taxonomy, p 35. Whereas the order *Orthoptera* is hemimetabolous with life stages: pronymph, nymph and adult which are similar anatomically. Numerous changes occur in the insect proteins during the metamorphosis from larvae to pupa to adult in holometabolous insects: the quantity of total protein, proportion of different kinds of proteins (Rockstein, 1978). Larval stages tend to have a higher lipid content than adult insects because energy stores are required for metamorphosis because pupae cannot consume food (Ademolu et al., 2010). The higher lipid content means proportionally lower protein contents in the larvae. However, the cuticle of the larvae contains protein which is less sclerotised relative to adult insects so may be more available for extraction in larvae (Nation, 2016). Larvae contain abundant storage proteins which act as precursors in biochemical processes (Nation, 2016, Rockstein, 1978, Haunerland, 1996). But perhaps the principal consideration is that a main component of insect protein is muscle protein. Insect muscle structure and proteins present are comparable to animal striated muscle and does not change much between life stages (Kominz et al., 1962, Nation, 2016). Therefore, it is hypothesised that the muscle proteins extracted would be similar between species and life stages.

While the protein extracts produced in Chapter 5 were high in protein, approaching that of isolates, other minor non-protein components remain in the extract. In order for there to be differences between the emulsifying properties of the protein extracts, one of three differences in extract composition would need to occur. Either the extract needs to have a different proportion of non-protein material in the extract, a different composition of

non-protein material, or a different protein composition. A different proportion of non-protein material gives a reduction in protein concentration and consequently could reduce protein oil-water interface coverage but this can compensated for by adjusting for protein content.

A different composition of the protein material with different structures or the same proteins in different proportions can lead to different emulsifying activity. Generally, a smaller more stable emulsion droplet is produced from proteins with lower molecular weight, higher surface hydrophobicity, higher molecular flexibility and higher surface charge (Sharif et al., 2018). If different proteins are extracted this could lead to a reduction in emulsion droplet diameter if the proteins are lower molecular weight and diffuse more rapidly to the interface or adsorbs more readily. In addition, the stability to flocculation and coalescence could be affected by the charge on the protein, the sensitivity to depletion flocculation, steric hinderance between proteins between droplets. The thickness and viscoelasticity of the interfacial layer depends on the structure of the protein at the interface, and the strength of the interactions between the proteins. If proteins can form disulfide bridges for example, then the viscoelastic interfacial layer will be more resistant to tearing and coalescence as has been noted in whey protein emulsions (Dickinson, 1986).

Emulsions in food products may undergo further physicochemical transformations during processes applied to increase shelf life, alter sensory properties, or progress production to a final product. To this end, an emulsion will be subject to temperature processing such as freeze-thaw processing,

cooking, pasteurisation or storage, changes in pH, changes in ionic strength, the addition of other surface-active ingredients, surfactants or wetting agents for example (McClements, 2016). The interfacial composition of the protein and any non-protein components will affect the emulsion stability when the emulsion is subjected to the afore mentioned processing steps.

Oil-in-water emulsions are present in soups, sauces, dairy desserts and increasingly plant-based meat analogues (Degner et al., 2014, McClements, 2016). Protein is the predominant emulsifying agent in many of these emulsions. The dairy derived proteins whey and sodium caseinate are used for stabilising many food emulsions and are present in ice cream.

In the production of oil-in-water emulsion under high-shear the protein must first diffuse to the interface, followed by adsorption to the interface and then a further rearrangement of protein orientation and structure to minimise interfacial tension. Proteins undergo surface denaturation at the interface to achieve the lowest energy conformation with hydrophobic amino acid residues orienting towards the oil phase and the charged and other hydrophilic residues orienting towards the water phase (Lam and Nickerson, 2013, Fang and Dalgleish, 1997).

One of the key strengths of insects as a food source is the diversity of species estimated to be around 5 million (Stork, 2018). The diversity of species could be an advantage in diversity of supply for protein which produces emulsions of the same properties. Or it could be the diversity of species and life stages gives a diversity of materials leading to an increase in the variety of emulsion

properties which can be achieved. The implication of the transition from the larval to the adult stage for the emulsifying properties of the protein extracts has not been found in the published literature at the time of writing. Furthermore, the specific interactions with functional ingredients, pH changes and temperature treatments need to be understood to enable insects to be utilised commercially in food products. This chapter will focus on the properties of oil-in-water emulsions formulated with insect proteins extracted from three different species in three different taxonomic orders and two different life stages, larval and adult. Further, the effect of the reduction to pH 5, heat treatment, freeze-thaw treatment, the addition of a non-ionic surfactant and combination of these treatments on emulsion microstructure is evaluated. This work will aid in the progression of insects being deployed as a food ingredient in future.

6.2 Materials and Methods

6.2.1 Materials

Tenebrio molitor (mealworm) 18 – 26 mm larvae from the order *Coleoptera*; *Gryllodes sigillatus* (banded crickets) 15 – 18 mm adult insect from the order *Orthoptera;* and *Achroia grisella* (waxworm) 15 – 20 mm larvae from the order *Lepidoptera;* were purchased from Monkfield Nutrition (Royston, UK). Mealworm beetles adult insect from the order *Coleoptera* were purchased from Norfolk Pet Supplies (Kings Lynn, UK). For the protein extraction and emulsion preparation, sodium azide (NaN_3), Florisil < 200 mesh, Polyoxyethylene (20) sorbitan monolaurate (polysorbate 20 or Tween 20),

sodium phosphate dibasic and monobasic were purchased from Sigma-Aldrich (Dorset, UK), hydrochloric acid (HCl), ethanol and sodium hydroxide (NaOH), were purchased from Fisher Scientific (Loughborough, UK), sunflower oil was purchased from a retailer (Sainsbury's, London, UK). All water used was double distilled.

6.2.2 Methods

The four insect protein extracts were produced by the method in 4.1 Protein extraction p100. The protein contents of both the whole insect powders and protein extracts were estimated by the method in 4.2 Nitrogen analysis p101. Compositional analysis was performed using midinfrared absorbance spectroscopy by the method in 4.3 Compositional analysis p102 with fifteen replicates. Protein solutions were produced with a pH 7 50 mM sodium phosphate buffer by the method in 4.4 Protein Solutions p102. Particle size analysis was performed using the method detailed in 4.7 Particle Size Analysis p103.

6.2.2.1 Emulsion Processing

Four replicates of each emulsion were produced with 120 g of protein solution and 30 g of treated sunflower oil with the method given in 4.6 Emulsion Preparation p103. To model food processing conditions further surfactant addition, pH adjustment and temperature treatments were performed as detailed in Figure 8.The process is described here briefly, 0 %, 0.5 % or 1 % Tween 20 was added to each emulsion and the equivalent mass of doubly distilled water to keep the mass equal between treatment levels and the

emulsion stirred for 30 min at 300 rpm. The emulsion was divided into two aliquots, one aliquot was reduced to pH 5 with 2 M HCl, the second aliquot was

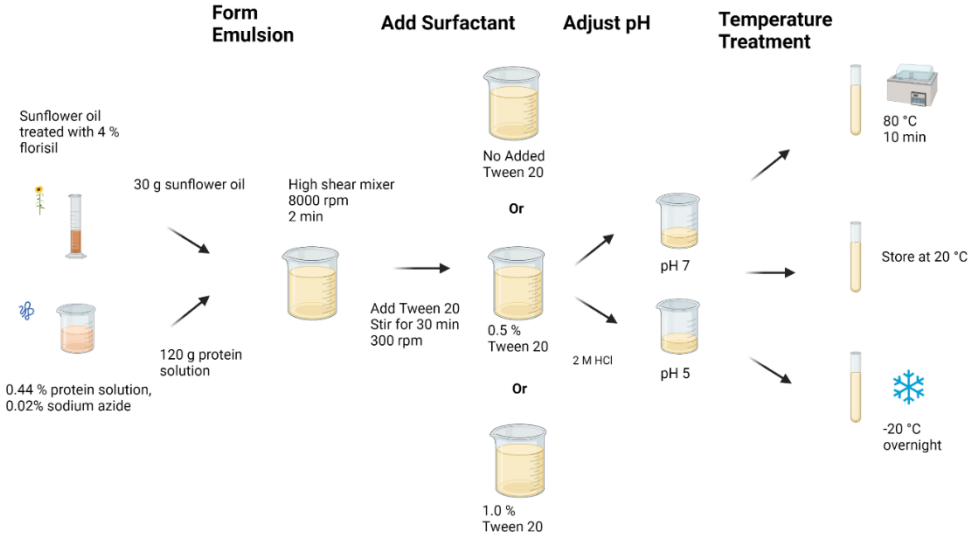

Figure 8 Insect protein extract emulsion production method diagram created with BioRender.com

equalised in mass with doubly distilled water (pH 7.0). Each aliquot was subdivided into three more aliquots, the first was placed at -20 °C overnight (20 hours), the second maintained at 20 °C until analysis and the third was heat-treated in a water bath at 80 °C for 10 min. The heat-treated emulsions were cooled at room temperature and stored at 20 °C. The day after production the frozen emulsion samples were defrosted for 2 hours at room temperature and all of the samples were analysed.

6.2.2.2 Microscopy

Micrographs were recorded on an optical microscope (Nikon Eclipse, Nikon, Amstelveen, The Netherlands) using brightfield microscopy to assess the microstructure. A drop of emulsion was placed on a microscope slide and a coverslip placed over.

6.2.2.3 *Creaming Index*

The creaming index was measured by placing 10.00 g of emulsion in 16 mm diameter and 150 mm long test tube and storing at 20 °C for 24 hours. The height of the total emulsion sample and the height of the serum phase measured and the creaming index was calculated as follows:

Equation 6 Creaming index

$$Creaming\ Index = \frac{Serum\ height}{Emulsion\ height} * 100$$

6.2.3 Statistics

To test for statistical differences in emulsion mean $d_{4,3}$ ANOVA analyses were performed (IBM Corp. Released 2021. IBM SPSS Statistics for Windows, Version 28.0. Armonk, NY: IBM Corp). The level of statistical significance was set at p = 0.05 and a Tukey's pairwise comparison of means was carried out forthe ANOVA test.

6.3 Results & Discussion

6.3.1 Composition

Table 5 shows the protein content of the freeze-dried ground powders of the four insect species and life stages. The freeze-dried insect powders had protein contents of: mealworm larvae 64 (± 3.6) %, mealworm beetle 72 (± 8.6) %, waxworms 46 (± 5.8) % and banded crickets 66 (± 1.3) %, comparable to figures reported in the literature. Mealworm larvae 60 % (Finke, 2015), 53 % (Jones et al., 1972), 43.75 % (Pennino et al., 1991), 49 % (Finke, 2002), 52 % (Zielinska et

al., 2015); Waxworms 40 % (Finke, 2015), 34 % (Finke, 2002) ; Banded cricket 70 % (Zielinska et al., 2015); and Mealworm beetle 65 % (Finke, 2002) .

A standard 6.25 nitrogen factor was used to calculate the protein contents of the insect powders and proteins which is a probable overestimate of protein content. Insects contain varying proportions of chitin composed of β(1,4)-N-acetylglucosamine monomer units in addition to other non-protein nitrogen such as nucleic acids, phospholipids and ammonia destined for excretion (Mariotti et al., 2008, Weihrauch et al., 2012, Janssen et al., 2017). In the larval form of *Tenebrio molitor* (Mealworm), *Alphitobius diapernius* (Lesser mealworm) and *Hermetia illucens* (Black soldier fly), Janssen et al. (2017) calculated a nitrogen factor of 4.76 and non-protein nitrogen of between 11 % and 26 % for the whole insect powder with 3.0 – 6.8 % of total nitrogen accounted for by chitin. While Belghit et al. (2019) proposed a 4.21 – 5.0 nitrogen factor for the same three insect larvae. Another study suggested nitrogen factors of 5.41 for mealworm larvae, 5.25 for house crickets and 5.33

Table 5 Insect powder and protein composition on a dry mass basis, n=3 standard deviation in parentheses. Lipid indicates the mass fraction extracted through the ethanol extraction, protein extract indicates the mass fraction isolated through the alkaline extraction process.

Insect species	Whole Insect powder protein content (%)	Lipid extract (%)	Protein extract (%)	Protein content of protein extract (%)	Protein extraction efficiency (%)
Mealworm beetle	72 (± 8.6)	27 (± 2.0)	12 (± 1.0)	90 (± 1.3)	16 (± 1.3)
Mealworm larvae	64 (± 3.6)	32 (± 1.5)	27 (± 0.8)	97 (± 1.2)	40 (± 1.2)
Waxworm larvae	46 (± 5.8)	57 (± 1.0)	4.3 (± 0.6)	89 (± 3.4)	8 (± 1.2)

for migratory locusts with a mean of 5.33 suggested for standard use across all species (Boulos et al., 2020). A nitrogen factor of 5.6 has also been suggested for protein extracts (Boulos et al., 2020). In protein extracted in 0.1 M citric acid and 0.2 M citric acid buffer the nitrogen factor was calculated as 5.60 (Janssen et al., 2017). The current balance of evidence shows the 6.25 factor is an overestimation. However, in light of the limited number studies on insect nitrogen factors generally and the lack of specific nitrogen factors for all of the insect species and life stages, 6.25 was utilised in this study. Furthermore, the common 6.25 nitrogen factor will facilitate comparison with previous studies.

Defatting is the removal of non-polar lipids with a solvent leaving a material with a higher protein content. A defatting treatment can increase yield from a protein extraction process (Choi et al., 2017, Azagoh et al., 2016). No significant difference has been found between defatting with ethanol as compared to numerous organic solvents such as hexane and isopropanol at a 3:2 ratio for mealworm larvae (Zhao et al., 2016). Furthermore, while ethanol might be expected to extract some proteins (prolamins) no difference was found in protein species by SDS-PAGE in the final extract compared to hexane defatting (Kim et al., 2022). The lipid content of the waxworms at 57 (± 1.0) g/100 is considerably higher than the other three insect types, with the mealworm larvae, for example at 32 (± 1.5) g/100, shown in Table 5, with a corresponding difference in protein content at 46 (± 5.8) %, and 64 (± 3.6) % respectively. The fat contents as measured by ethanol extract found in Table 5 were in the middle range of the reported values for both mealworms:, 45 % (Pennino et al., 1991), 35.42 % (Jones et al., 1972) 35 % (Finke, 2002), 25 % (Zielinska et al., 2015); and

waxworms 56 % (Pennino et al., 1991), 60 % (Finke, 2002), 51.4 % (Barker et al., 1998), . The fat contents reported previously for banded cricket 18 % (Zielinska et al., 2015) and mealworm beetle 15 % (Finke, 2002) are lower than the 26 (± 2.0) % and 25 (± 0.9) % respectively measured in this study. In addition to differences in extraction solvents for fat content, factors such as feed type, environmental growth conditions and exact life stage can affect the macronutrient makeup of the whole insects which could account for the variation in reported results (Hawkey et al., 2021). The lower fat content of the mealworm beetles relative to mealworm larvae is consistent with previous findings that larval life stages have higher fat contents than adult insects (Barker et al., 1998).

The extraction process was optimised for mealworm larvae by Zhao et al. (2016), which is represented by the higher efficiency of the extraction process at 40 (± 1.2) % more than double that of the other three species and life stages. While all four extractions were of a high purity near or above 90 % so all except for the waxworm larvae extract could be considered isolates, the protein estimation by way of nitrogen content is likely an overestimate (Koshy et al., 2015). During the final centrifugation step for separating pH 7 soluble fraction some supernatant was discarded with the pellet to ensure no pH 7 insoluble protein was present in the protein extracts. The purity of the pH 7 soluble protein was prioritised over the efficiency because the aim of this study was to assess the emulsifying properties of the proteins not to optimise the extraction process. Nevertheless, the extraction efficiency was calculated and included to show the potential optimisations which may be possible, as Zhao et al. (2016)

showed with mealworms. The extraction protocol was least efficient for the waxworm larvae which combined with the lower protein content of the waxworm powder gave an overall lower yield. Further optimisation of the protocol would be required for larger scale trials or commercialisation. There are two possibilities for the difference in extraction efficiency between mealworm larvae and the other three insect types. In the adult insects, mealworm beetle and banded crickets, the cuticle proteins are more sclerotised and therefore not soluble as discussed in the Literature Review Chapter 2 section 2.2.3.3 Insect Integument Proteins, p 45. The proportion of total proteins with the isoelectric point in the range pH 4.3 – 4.5 is lower for the banded crickets, mealworm beetles and waxworm larvae than the mealworm larvae. Or the proportion of protein soluble at pH 7 was higher for the mealworm larvae than the three other insect types. The minimum solubility for banded crickets and mealworms was shown to be at pH 5, but banded cricket had a solubility of 57 % at pH 4 compared to just below 20 % for mealworm larvae and similar solubility at pH 7 of around 30 % (Zielinska et al., 2018).

6.3.1.1 Compositional Analyses

Infrared spectroscopy compositional analysis Infrared spectroscopy was performed to confirm the crude composition data. If there were any chitin or chitosan in the protein extract, then the estimation of protein by nitrogen would be an over estimation, as discussed in the previous Section 6.3.1 Composition section. Although chitosan is not present in the whole insects it is produced by the alkaline deacetylation of the β(1,4)-N-acetylglucosamine chitin polymer which has increased solubility. As a result, it was necessary to confirm

the exclusion of chitin and chitosan from the extract by a secondary method to provide some secondary validation for the nitrogen content measurements.

Qualitative infrared spectroscopy is also necessary to assess the effectiveness of the lipid extraction step. Ethanol is a polar solvent, and while has lower toxicity than hexane (which is used for lipid extraction) is less effective generally at removing non-polar lipids. Consequently, the infrared spectra can give useful insights into the reduction in non-polar lipids with reference specifically to the C-H asymmetric stretch peaks at 2936 – 2915 cm^{-1} and 2865 – 2833 cm^{-1} the C=O stretch peak at 1750 – 1735 cm^{-1} from the fatty acid glycerol ester bond (Larkin, 2017).

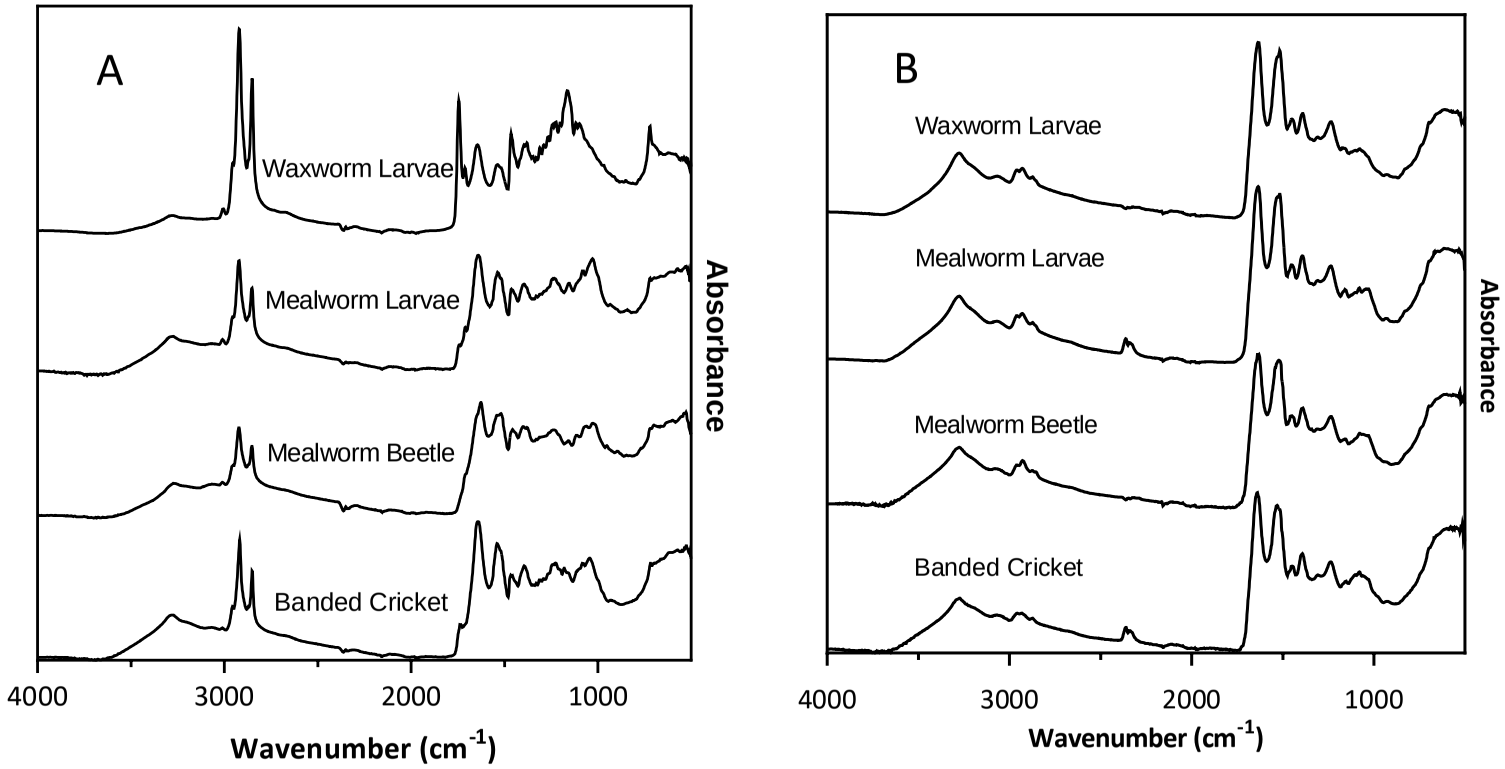

Figure 9 Mid-infrared spectra of whole insect powders (A), and insect protein extracts (B), normalised and baseline extracted

It might be hypothesised that some degree of protein denaturation would occur due to the alkaline extraction environment.

6.3.1.1.1 Qualitative Composition

All four insect extracts show a reduction in the -CH_2 stretch peak at 2916 – 2920 cm^{-1}, as shown in Figure 9 (B), relative to the powder where the peak is shifted at 2930 – 2932 cm^{-1}, suggesting a reduction in the presence of lipids (Chalmers and Griffiths, 2002, Forfang et al., 2017). The waxworm larvae powder spectra shows a distinct peak at 1744 cm^{-1} for the C=O stretch from the ester bond in glycerides which is consistent with the higher ethanol extract of 57 % from Table 5 containing lipids specifically glycerides (Forfang et al., 2017). The same C=O stretch is present in both mealworm larvae powder and banded cricket powder but reduced to a shoulder of the Amide I peak in the mealworm beetle. Chitin exhibits absorbance in the region 1000 – 1200 cm^{-1} typically, with specific peaks at ≈1160 cm^{-1} for the ether bond between the N-acetylglucosamine monomer units and ≈1030 cm^{-1} and ≈1070 cm^{-1} for the ether bond within the N-acetylglucosamine (Johnson and Naiker, 2020, Khoshmanesh et al., 2017). Banded cricket, mealworm larvae and mealworm beetle powders have chitin peaks at 1045 cm^{-1} and 1080 cm^{-1}, 1028 cm^{-1} and 1080 cm^{-1}, 1026 cm^{-1} and 1063 cm^{-1} respectively which are reduced in the protein extracts, comparing the (A) figure of the powder with the (B) figure of the protein extracts in Figure 9. The waxworm powder spectra show an additional peak at 1161 cm^{-1} from the C-O-C bond of the ester bond in the lipids which would occur with the same

frequency as the C=O bond. The infrared spectra show the reduction of lipids and chitin consistent with the Table 5 composition results.

6.3.2 Insect protein emulsions at pH 7

6.3.2.1 Non-heat-treated

Emulsions were produced with 0.44 % protein solutions, 20 % treated sunflower oil with the protein extracts from all four insect types in a pH 7 sodium phosphate buffer. Micrographs and laser diffraction particle sizing were used to assess the microstructure of the emulsions in terms of droplet diameter and flocculation. The micrographs in Figure 10 show that all four insect protein extracts produced stable oil-in-water emulsions before any further treatment. Emulsions produced had mean $d_{4,3}$ values of 16.1 (± 1.1) µm for banded cricket, mealworm beetle 14.9 (± 2.1) µm, mealworm larvae 13.0 (± 3.6) µm and waxworms 15.7 (± 1.3) µm, shown in Figure 11 (A). There was no significant difference between the $d_{4,3}$ of emulsions produced from different insect protein types, ANOVA ($F_{(15,32)}$ = 11.84, p < 0.05), indicated by the letter denotations in Figure 11 (A). This finding that protein from all insect species and life stages produced stable oil-in-water emulsions is in agreement with the hypothesis that alkaline extracted protein from all species of insects and life stages can produce stable oil-in-water emulsions. Micrographs for the emulsion, formed from all four insect extracts showed little evidence of flocculation. The results for banded crickets are in agreement with the results for banded cricket protein emulsions in Section 5.3.3 p 124, and the results for

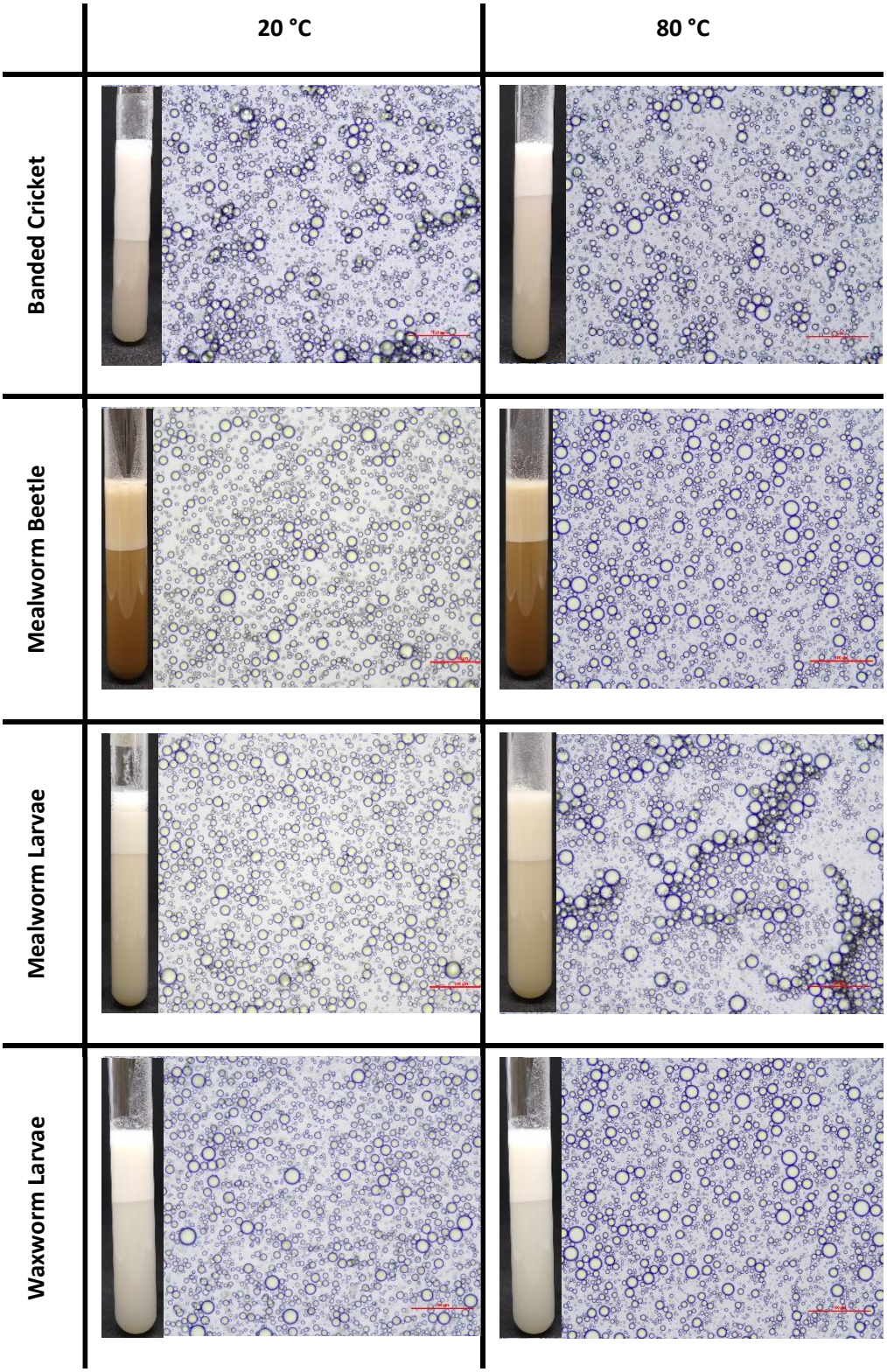

Figure 10 Micrographs and emulsions stabilised by all four insect protein extracts maintained at pH 7 without added surfactant, before and after heat-treatment at 80 °C for 10 mins Samples imaged after 24 hours storage at 20 °C. Scale bars represent 100 µm

mealworms in agreement with previously published data (Gould and Wolf, 2018).

After the initial droplets are formed the further reduction in droplet diameter depends on the disruption of coarse droplets into smaller droplets during homogenisation. Emulsion droplet diameter is limited by the diffusion rate of the emulsifier to the droplet interface, reduction of interfacial tension on adsorption to the interface and the maximum energy of the emulsion production technique (Freer et al., 2004a, Noskov, 2014, Kotsmar et al., 2009) Since the droplets from all of the insect protein stabilised emulsions, before further stresses are applied, are of similar dimensions it can be inferred that the proteins are similar in diffusion and adsorbance characteristics, and initial reduction in interfacial tension (McClements, 2016).

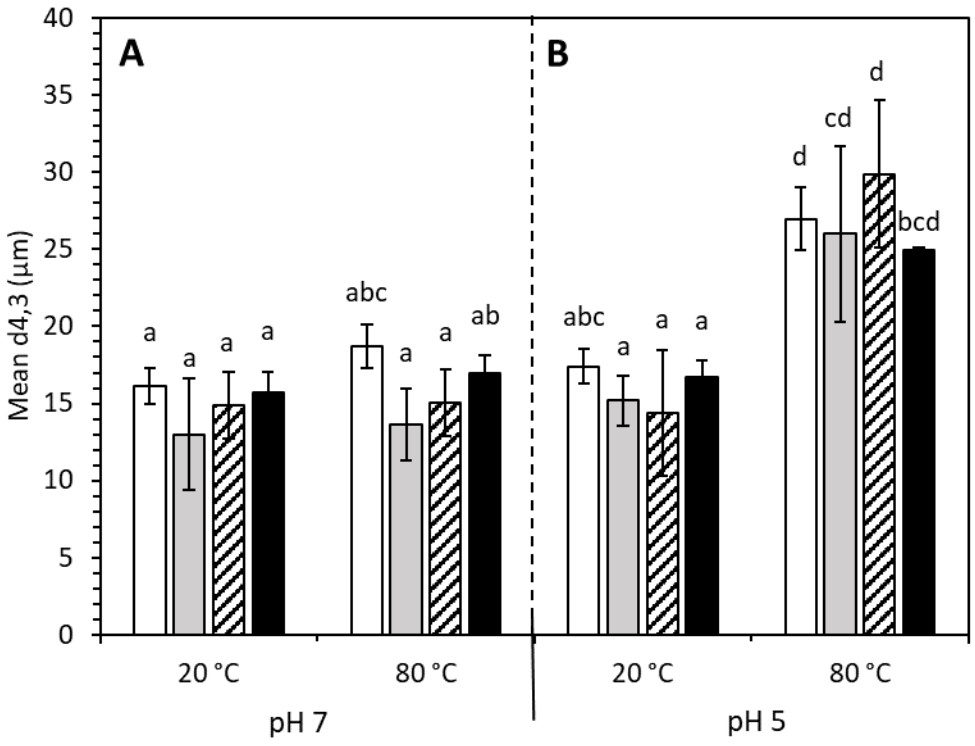

Figure 11 Mean $d_{4,3}$ of insect protein extract stabilised emulsions (A) maintained at pH 7, 20 °C (non-heat-treatment) and 80 °C for 10 mins (heat-treated); (B) adjusted to pH 5, 20 °C (non-heat-treatment) and 80 °C for 10 mins (heat-treated) white bars are banded cricket, grey bars are mealworm larvae, striped bars are mealworm beetle and black bars are waxworm larvae, letters denote significant difference from post-hoc Tukey test from one-way ANOVA ($F_{(15,32)} = 11.84$, $p < 0.05$) , n=3 error bars show standard deviation.

All four insect extract emulsions showed creaming after 24 hours, as seen in the images in Figure 12 (A). Creaming rate in emulsions maintained at pH 7 showed no significant difference, ANOVA ($F_{(15,32)}$ = 20.90, p < 0.05) between insect species shown by the same grouping in a post-hoc Tukey's test in Figure 12 (A). Creaming rate increases with droplet radius in an oil-in-water emulsion because of the lower density of the dispersed phase in accordance with Stoke's Law (McClements, 2016). So the creaming index results support the lack of difference found in the $d_{4,3}$ results. A lower droplet diameter which would lower the creaming rate could be achieved by a higher energy emulsification technique such as high-pressure homogenisation (Desrumaux and Marcand,

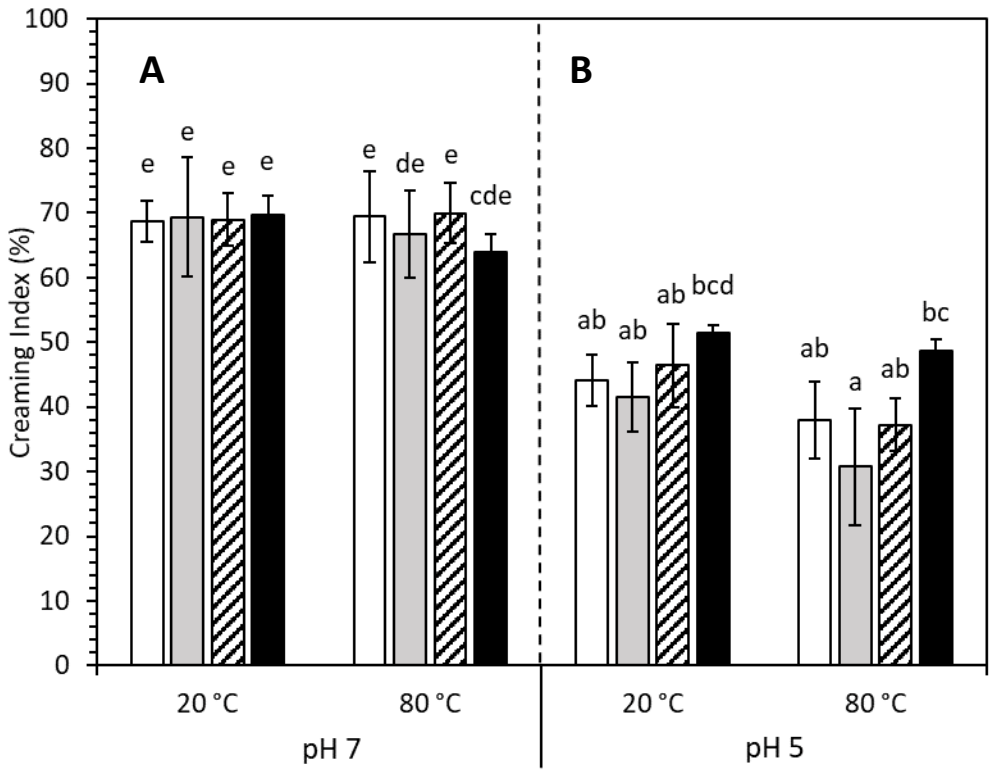

Figure 12 Mean creaming index of insect protein extract stabilised emulsions (A) maintained at pH 7, 20 °C (non-heat-treatment) and 80 °C for 10 mins (heat-treated); (B) adjusted to pH 5, 20 °C (non-heat-treatment) and 80 °C for 10 mins (heat-treated) white bars are banded cricket, grey bars are mealworm larvae, striped bars are mealworm beetle and black bars are waxworm larvae, letters denote significant difference from post-hoc Tukey test from one-way ANOVA ($F_{(15,32)}$ = 20.90, p < 0.05) , n=3 error bars show standard deviation.

2002) or a lower energy technique such as the dynamic membranes of tuneable pore size used by Wang et al. (2021).

6.3.2.2 Heat-treated

A heat processing step of 10 min at 80 °C was applied to assess the impact on droplet stability and microstructure at pH 7. Heating is an important step in many food production processes. Pasteurisation to reduce or eliminate vegetative bacterial cells in sauces is essential to ensure the safety and stability of emulsion-based products.

The heat-treatment had no observable effect on degree of flocculation or coalescence when assessed by microscopy, images shown in Figure 10. Emulsion droplet diameter, shown in Figure 11 (A), showed no significant difference in $d_{4,3}$ compared to the emulsions stored at 20 °C or between insect protein types, ANOVA ($F_{(15,32)} = 11.84$, $p < 0.05$), with a $d_{4,3}$ of 18.7 (± 1.4) µm for banded cricket, mealworm beetle 15.1 (± 2.1) µm, mealworm larvae 13.6 (± 2.3) µm and waxworm 16.9 (± 1.2) µm. Similarly, in mealworm protein stabilised emulsions heat treated for 30 mins, droplet diameter increased marginally from 11 µm to around 14 µm (Gould and Wolf, 2018). The volume-based particle distributions, data not shown, for the banded cricket protein and mealworm beetle protein stabilised emulsions were monomodal for the emulsions at both heat-treated and non-heat-treated at pH 7. Whereas the mealworm larvae stabilised emulsions showed evidence of a second mode between 3 µm and 5 µm at between 1 % and 4 % volume. In the emulsions stabilised by waxworm larvae proteins the volume-based droplet distribution is

bimodal with minor peak of 1 % at 4 -5 µm. This change in emulsion structure may be too minor to be observed in the micrographs in Figure 10.

There was no significant difference in creaming index between the heat-treated emulsions and non-heat-treated emulsions at pH 7 for any of the insect protein extracts, ANOVA ($F_{(15,32)} = 20.90$, $p < 0.05$), shown in Figure 12 (A). The creaming index results support the lack of difference in emulsion microstructure observed in the micrographs and $d_{4,3}$ measurements. These data suggest insect protein stabilised emulsions may be suitable for heat processing.

Proteins can undergo further denaturation to any which has occurred during the extraction process, with the application of heat which can increase flocculation between emulsion droplets and aggregation of the free protein through increased hydrophobic amino acid residues on the surface of the droplets creating protein bridges between droplets (Demetriades et al., 1997, Sliwinski et al., 2003, Keerati-u-rai and Corredig, 2009, Tang, 2017). Moreover, if sufficient protein exists in the serum this can aggregate with the emulsions droplets to form a gel, this is protein specific however (Hunt and Dalgleish, 1995). Heat-treating the protein before emulsification was not trialled in this study so it cannot be said conclusively if aggregation did or did not occur. In the case of the insect protein extracts any further denaturation caused by heat treatment used in this study was not sufficient to increase flocculation, and subsequently coalescence, at the temperature and time period analysed.

6.3.3 Insect protein emulsions adjusted to pH 5

6.3.3.1 Non-heat-treated (20 °C)

In addition to temperature changes, the physiochemical properties of emulsions are affected by pH level. The pH of a food product may be lowered for flavour, as a barrier to microbial growth or as a processing step to induce change structure. The insect protein stabilised emulsions were adjusted from pH 7 to pH 5 to assess the stability of the emulsions to flocculation and coalescence under acidic conditions at a pH level close to the isoelectric point of the insect protein extracts.

The micrographs in Figure 13 show an increase in flocculation when the emulsions are adjusted to pH 5 emulsions produced with all insect protein types. No increase in coalescence was observable, by assessing droplet diameters in the emulsion micrographs nor any coalescence events. Furthermore, the decrease in opacity of the serum layer observed in the emulsion images in Figure 13 suggests the flocculation.

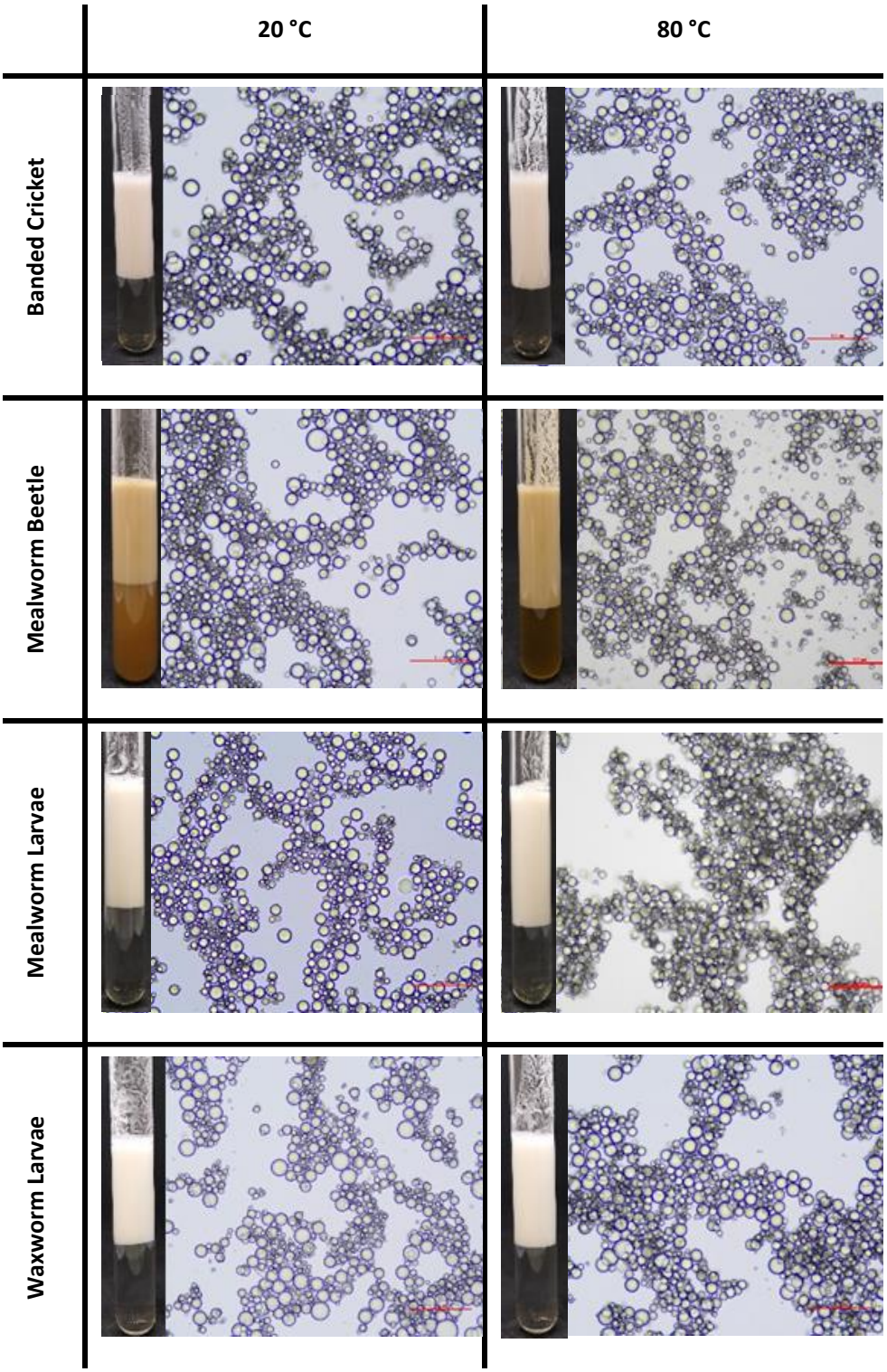

Figure 13 Micrographs and emulsions stabilised by all four insect protein extracts adjusted to pH 5 without added surfactant, with and without heat-treatment at 80 °C for 10 mins Samples imaged after 24 hours storage at 20 °C. Scale bars represent 100 µm

Emulsions stabilised by all four insect protein extracts showed no significant change in $d_{4,3}$, ($F_{(15,32)} = 11.84$, $p > 0.05$) after lowering from pH 7 to pH 5 shown in Figure 11 (B). The lack of difference in the droplet diameter data is likely to be due to the forces exerted on the emulsion from the pump in the laser diffraction particle sizer disrupting the flocs. If the emulsion droplet flocs are indeed disrupted the $d_{4,3}$ data reflects the volume-based droplet diameter of individual emulsion droplets. Therefore, the $d_{4,3}$ data confirm the observations in the micrographs that flocculation was presence but not coalescence.

The creaming index of the emulsions was significantly lower for all insect protein types, ($F_{(15,32)} = 20.90$, $p < 0.05$) , 64 – 70 % at pH 7 to 42 – 51 % at pH 5, shown in Figure 12. The lower creaming index indicates an increase in creaming at pH 5 relative to pH 7. An increase in the attractive forces between emulsion droplets can increase both creaming rate and decrease the density of the cream layer which increases the volume occupied by the cream layer leading to a decrease in creaming index. An increase in droplet-droplet attraction causes flocs to form in the emulsion which cream at a faster rate than individual droplets due to the combined buoyancy of all of the droplet in the floc, by Stokes's law. The flocs and therefore cream layer decrease in density because the increased droplet-droplet forces prevent rearrangement of the droplets within a floc leading to a more open floc structure.

The primary interactions acting on the emulsion droplets are electrostatic, steric and van der Waals forces. In protein stabilised emulsions electrostatic repulsion tends to be the predominant repulsive force between emulsion

droplets. When the pH of the serum is not near to the isoelectric point of the protein, then the net charge of the protein is negative above the isoelectric point and positive below it resulting in repulsive forces of the same charge between droplets preventing flocculation. At the isoelectric point of the protein, flocculation is increased due to the net neutral charge on the protein reducing electromagnetic repulsion. The proteins were precipitated at pH 4.4 during the extraction process which would be assumed to be the isoelectric point of the proteins. The solubility minima of mealworm larvae and banded crickets were shown to be pH 5, so the pH adjustment used may be even closer to the isoelectric point than assumed (Zielinska et al., 2018). The proximity to the isoelectric point reduces the net charge on the proteins reducing the electrostatic repulsive forces which accounts for the increased flocculation suggested by the decrease in creaming index and observed in the micrographs (Dickinson, 2010).

6.3.3.2 Heat-treated

The insect protein stabilised emulsions formed at pH 7 were heat-treated for 10 min at 80 °C after adjustment to pH 5 to assess heat stability in acidic conditions. The heat-treated emulsions showed similar levels of flocculation in the micrographs in Figure 13 to the non-heat-treated emulsions. It is not possible to assess from the Figure 13 micrographs any coalescence has occurred.

The heat treatment, after the change to more acidic conditions, resulted in a significant increase in $d_{4,3}$ for all insect species from the non-heat-treated pH 7

emulsions to between 25 µm and 30 µm shown in Figure 11 ANOVA ($F_{(15,32)}$ = 11.84, $p < 0.05$).

The creaming index for the pH 5 adjusted heat-treated emulsion, showed a significant decrease relative to the non-heat-treated pH 7 emulsions for all four insect protein extracts, ANOVA ($F_{(15,32)} = 20.90$, $p < 0.05$), shown by the differing groups in the post-how Tukey test in Figure 12. Yet when the emulsions adjusted to pH 5 are considered, the heat-treated emulsions show no significant difference from the non-heat-treated shown by presence in the same groups of the post-hoc Tukey's test in Figure 12. The lack of difference in creaming index suggests that the density of the cream layers are similar and therefore the structure flocs which make up the cream layer. It is hypothesised that the increase in $d_{4,3}$ from the pH 5 non-heat-treated in the heat-treated emulsion is due to flocs which are not broken in the laser diffraction particle sizer, not from droplet coalescence.

The reduction from pH 7 to pH 5 decreased the repulsive forces between emulsion droplets as discussed in the previous section; the subsequent heat-treatment increased the attractive forces between droplets. The increased temperature from the heat-treatment may be sufficient to denature the proteins both at the oil-water interface in the emulsions and any remaining free protein in the serum. The protein denaturation exposes hydrophobic amino acid residues from the interior of the protein molecule through the alteration of the tertiary structure, as discussed in section 2.3.3 p 58 of the literature review chapter. The increased surface hydrophobic amino acid residue residues

increase the hydrophobic interactions between the protein molecules leading to stronger droplet-droplet attractive forces and flocculation. The increase in attractive forces from the heat-treatment combined with the decrease in repulsive forces from the lower pH is leading to flocs which are strong enough to persist in the laser diffraction particle size analysis, resulting in the increase in $d_{4,3}$ for the emulsions adjusted to pH 5 and heat treated. This could be confirmed by measuring the viscosity in relation to shear stress of the emulsions or the shear modulus in the linear viscoelastic region. Greater attractive forces between droplets leads to a larger shear stress required to reach an equilibrium viscosity (Quemada and Berli, 2002).

6.3.4 Insect protein emulsions at pH 7 with added non-ionic surfactant

6.3.4.1 Non-heat-treated

To model the presence surfactants in food products, a non-ionic low molecular weight surfactant was added at 0.5 % and 1 % of total mass to the 0.44 % insect protein emulsions after emulsification at pH 7. A maximum surfactant level of 1% was selected because this is the legal maximum quantity in a food product according to EU law at the time of writing (European Commision, 2011).

The overall microstructure of the oil-in-water emulsions remained intact after the addition of Tween 20 show in Figure 14. The micrographs in Figure 14 show no observable increase in flocculation due to the addition of Tween 20 at either 0.5 % or 1 %. There does appear to have been some coalescence of emulsion droplets in the waxworm larvae and mealworm larvae protein emulsions as a result of the addition of Tween 20. Although, there was no significant difference

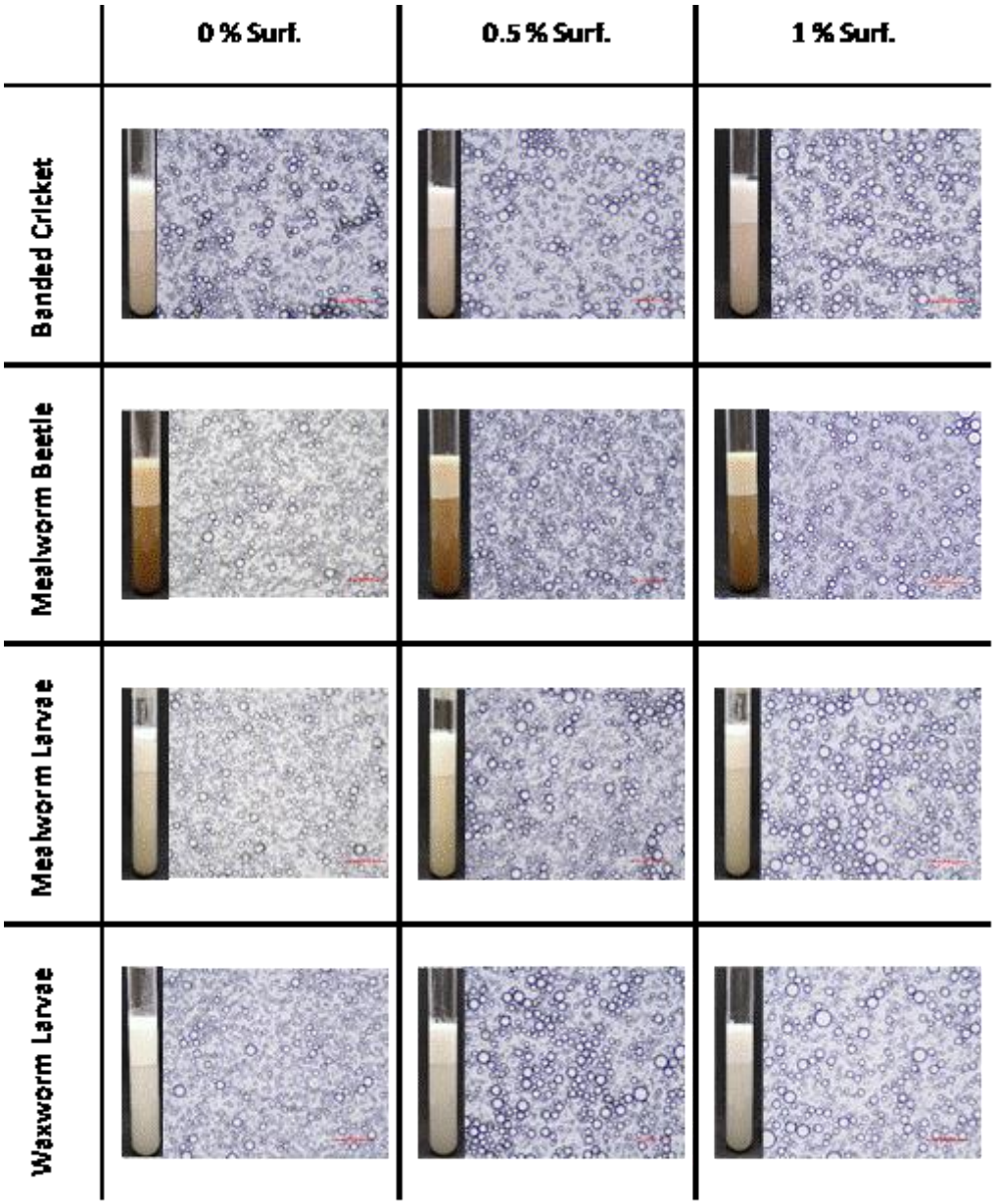

Figure 14 Micrographs and emulsions stabilised by insect protein extracts at pH 7 with 0 %, 0.5 % and 1 %, added surfactant, no heat-treatment, after 24 hours, scale bars 100 µm

in $d_{4,3}$ ANOVA ($F_{(23,48)}$ = 1.94, p = 0.27), shown in Figure 15 (A), between the emulsions with added Tween 20 and the emulsions without at 20 °C. The lack of difference in $d_{4,3}$ suggests the observed coalescence in the micrograph was a sampling artefact. The creaming index results in Figure 16 (A) supported the $d_{4,3}$ results with no significant difference between the three levels of added Tween 20, ANOVA ($F_{(23,48)}$ = 1.09, p = 0.39), indicate no increase or decrease in

the strength of droplet-droplet interactions altering the density of the cream layer nor an increased creaming rate.

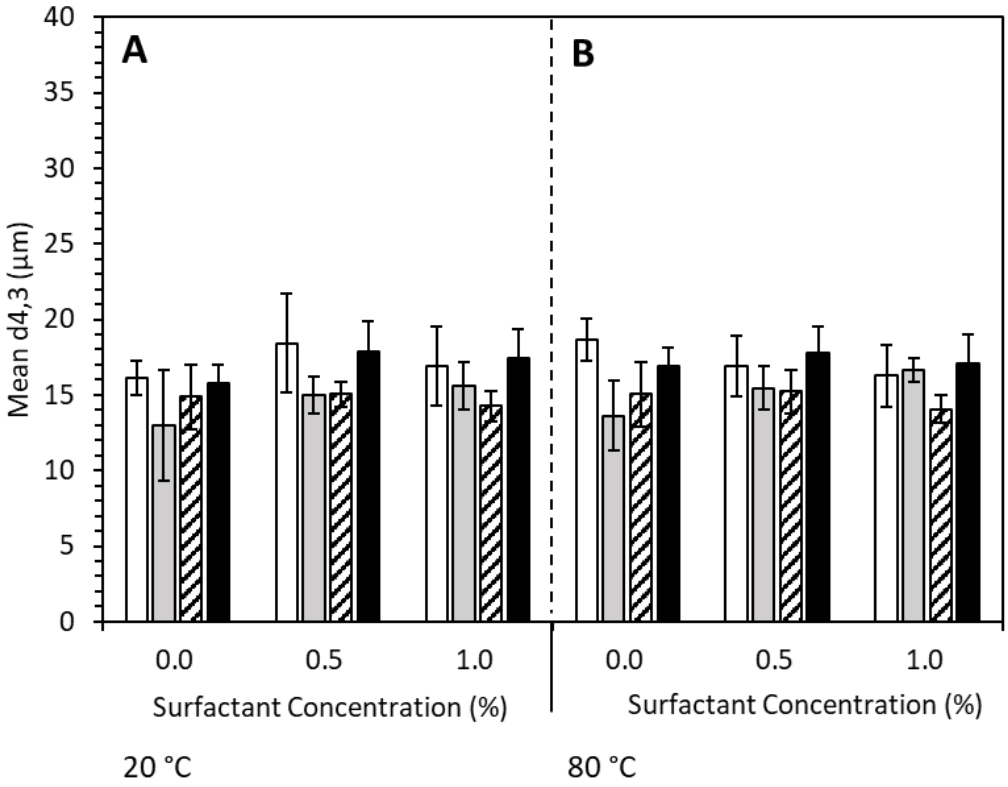

Figure 15 Mean $d_{4,3}$ of insect protein extract stabilised emulsions maintained at pH 7 with 0 %, 0.5 % and 1 %, added surfactant, heat-treated at 80 °C for 10 mins and without heat-treatment, white bars are banded cricket, grey bars are mealworm larvae, striped bars are mealworm beetle and black bars are waxworm larvae, n=3 error bars show standard deviation.

6.3.4.2 Heat-treated

To assess the stability of the insect protein emulsions to heat treatment with three levels of added Tween 20, 0 %, 0.5 % and 1 %, the emulsions were heated at 80 °C for 10 min.

The micrographs in Figure 17 for the emulsions heat-treated at 80 °C showed increased flocculation when surfactant was added at 0.5 % Tween 20 in the case of mealworm beetle protein and waxworm larvae protein emulsions and for all four insect protein emulsions at 1 % Tween 20.

However, the addition of Tween 20 to the insect protein stabilised emulsions caused no significant increase in mean $d_{4,3}$ ($F_{(23,48)}$=1.94, p=0.27) from Figure 15 (B) in either the heat-treated despite increased flocculation, indicating the flocs observed in the micrographs were broken and dispersed in the laser diffraction particle sizer and that no measurable no coalescence had occurred. Furthermore, the creaming index of four insect protein extract emulsions showed no significant difference on addition of Tween 20 at 0.5 % nor 1 % when heat-treated shown in Figure 16, ANOVA ($F_{(23,48)}$ = 1.09, p = 0.39),in line with the $d_{4.3}$ results.

While the $d_{4,3}$ and creaming index results appear to contradict the observations from the Figure 14 micrographs, two potential effects of the addition of Tween 20 protein emulsions may explain the results. Firstly, non-ionic surfactants can adsorb to the oil-water interface and dislodge the protein molecules in a process known as orogenic displacement. The low molecular weight surfactants are able to adsorb to the oil-water interface between the protein molecules, rapidly reducing interfacial tension more than the proteins, which compresses the area of the interface which the protein occupies eventually dislodging the protein (Mackie et al., 2000, Woodward et al., 2009). Non-ionic surfactants at the oil-water interface of an emulsion prevent flocculation by steric repulsion rather than the electrostatic and steric repulsion for protein stabilised

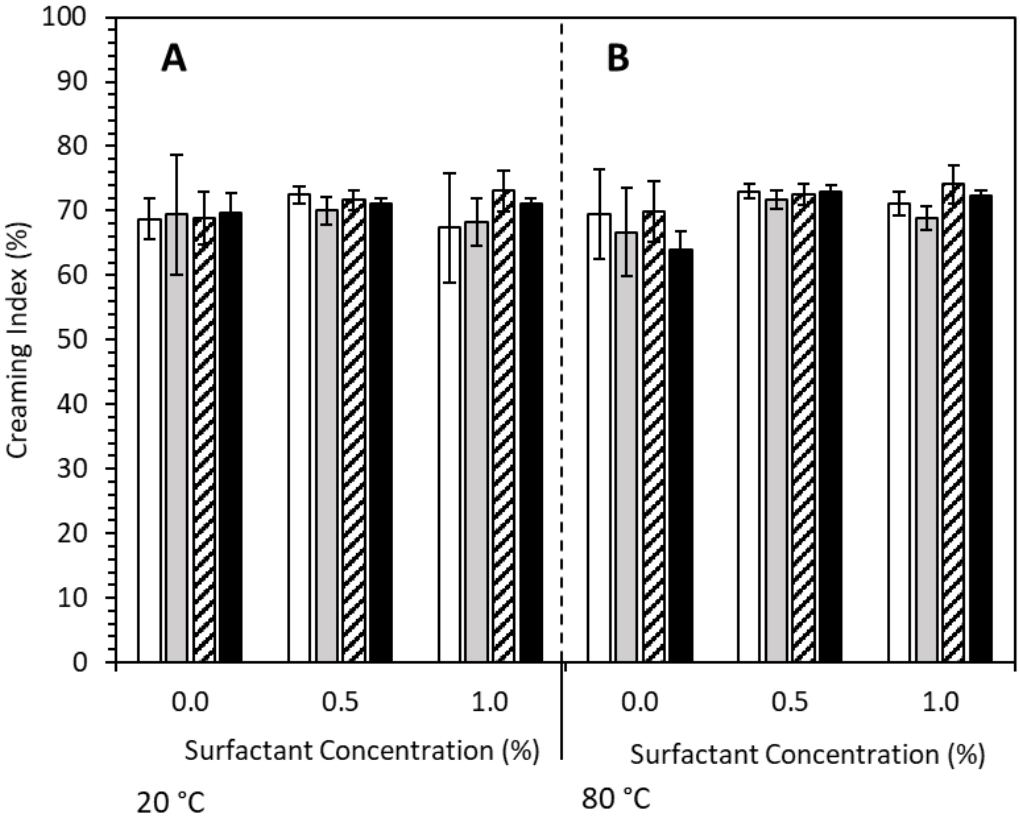

Figure 16 Mean creaming index of insect protein stabilised emulsions with surfactant added at 0 %, 0.5 % and 1 %, adjusted to pH 7, heat-treated at 80 °C for 10 mins and without heat-treatment, n=3 error bars show standard deviation

emulsions discussed in Insect protein emulsions adjusted to pH 5, 6.3.3, p 156. The effect of Tween 20 addition on whey protein emulsions has been shown to differ from the flocculation induced in the insect protein emulsions. Adding Tween 20 to whey protein emulsions caused less flocculation due to heat treatment at 78 °C for 30 mins showing no change in viscosity, droplet diameter or creaming stability relative to non-heat-treated emulsion compared to whey protein emulsions which showed considerable flocculation (Demetriades and McClements, 1998).

Secondly, at higher concentrations of unabsorbed protein or surfactant, depletion flocculation of oil-in-water emulsions can occur. Depletion flocculation results from the exclusions of the surfactant micelles or protein molecules from between the emulsion droplets due to the concentration of dispersed droplets causing the water to diffuse from the high water concentration region between the droplets to the wider serum inducing flocculation (Dickinson, 2019, Dickinson, 2010, Liang et al., 2014). Therefore, the depletion flocculation must be a result of changes to the protein component, either the heat treatment causing greater displacement of proteins from the oil/water interface inducing depletion flocculation or the denaturation of the remaining protein at the interface in combination with depletion flocculation increasing flocculation. The laser diffraction particle sizer measures droplet diameter of the emulsion diluted in water, therefore the depletion flocculation effects would not be present because of the lower concentration of surfactant and protein molecules. The addition of Tween 20 to sodium caseinate emulsions has been shown to increase depletion

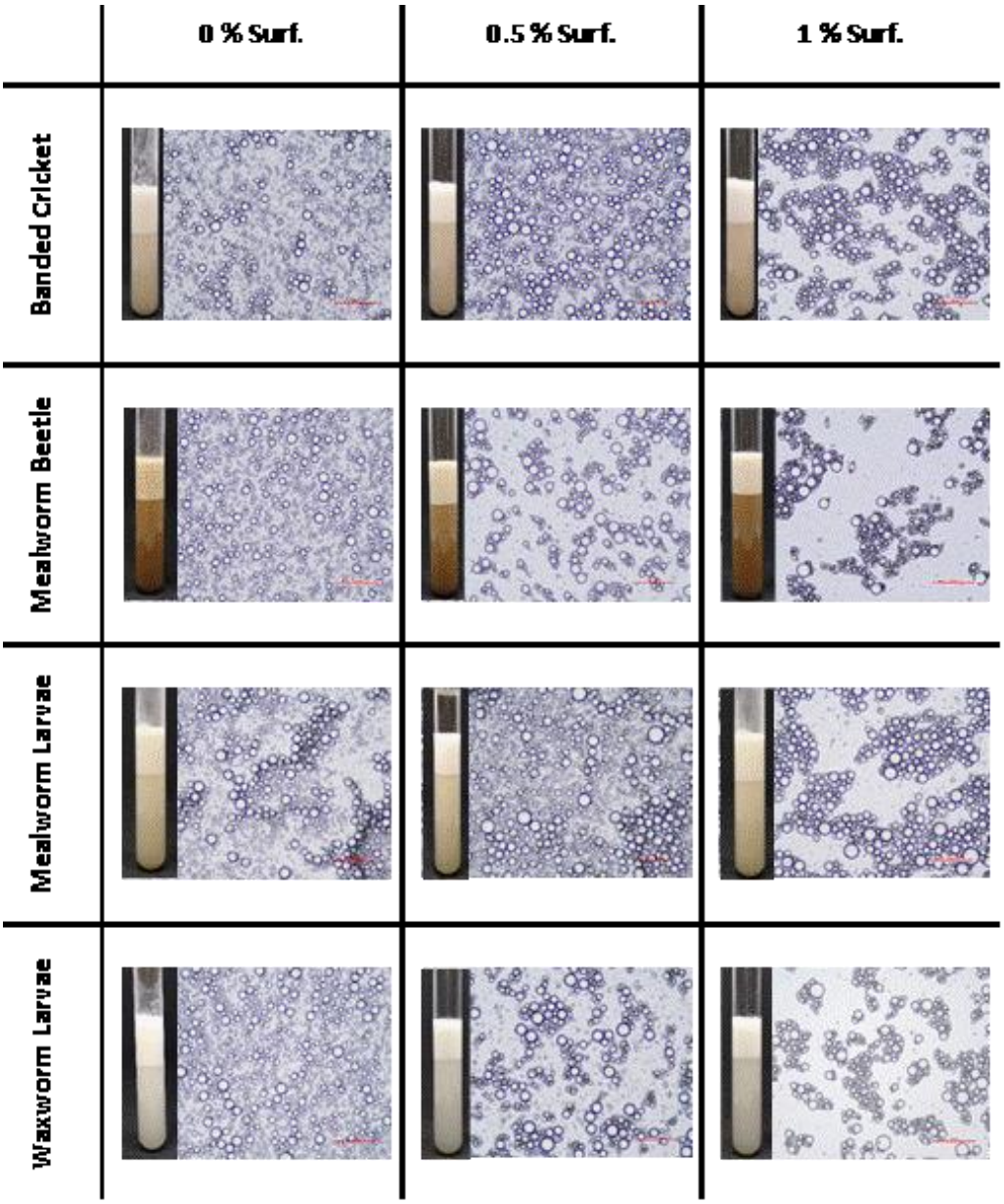

Figure 17 Micrographs and emulsions stabilised by insect protein extracts at pH 7 with 0 %, 0.5 % and 1 %, added surfactant, 80 °C, after 24 hours, scale bars 100 µm

flocculation but decrease it in the presence of calcium due to aggregation of protein molecules (Dickinson et al., 2003, Dickinson et al., 1999a).

Neither life stage nor insect species appears to greatly affect the tendency for the emulsions to flocculate when heated after the addition of surfactant. The limited flocculation observed, which can be mitigated in products with stabilisers, and the absence of coalescence suggest all four insect protein

extracts may be suitable for use in a food product with added surfactant at these conditions.

6.3.5 Insect protein emulsions at pH 5 with added non-ionic surfactant

6.3.5.1 Non-heat-treated

The emulsions produced with 0.44 % insect protein for the four insect types were adjusted from pH 7 to pH 5 after the addition of either 0 %, 0.5 % or 1 % Tween 20 to assess the impact of the addition of a non-ionic surfactant on the emulsion stability during pH adjustment. Emulsion behaviour under acidic conditions in the presence of surface-active species is important since low pH is a common method of reducing microbial growth and altering flavour in foods.

At 0 % added surfactant the emulsions from all four insect protein types appear to be comparably flocculated in Figure 18. With added surfactant, the micrographs of the banded cricket stabilised protein in Figure 18 show the most flocculation and mealworm beetle protein stabilised emulsions little or no flocculation. The mealworm larvae and waxworm larvae protein emulsions exhibit flocculation at 0.5 % and 1 % added Tween 20 at pH 5 in Figure 18 but to lesser degree than the banded cricket protein emulsions.

For the emulsions where the addition of Tween 20 is followed by the addition of hydrochloric acid to lower to pH 5 there are significant differences in emulsion microstructure between both orders of insect and life stages. Figure 19 shows the addition of 0.5 % Tween 20 before the lowering to pH 5, induces a significant increase in mean $d_{4,3}$ ($F_{(11,24)} = 16.70$, $p < 0.05$) for banded cricket

protein emulsions from 17 (± 1.1) µm to 31 (± 1.6) µm, with no further increase in $d_{4,3}$ on the addition of 1 % Tween 20. Whereas, mealworm beetle protein emulsions showed no significant increase in $d_{4,3}$ when surfactant is added prior to lowering to pH 5. The mealworm larvae and waxworm larvae showed an intermediate level of increase from 15 (± 1.6) µm and 17 (± 1.1) µm to 22 (± 3.4) µm and 25 (± 3.2) µm respectively, with only waxworms showing a significant

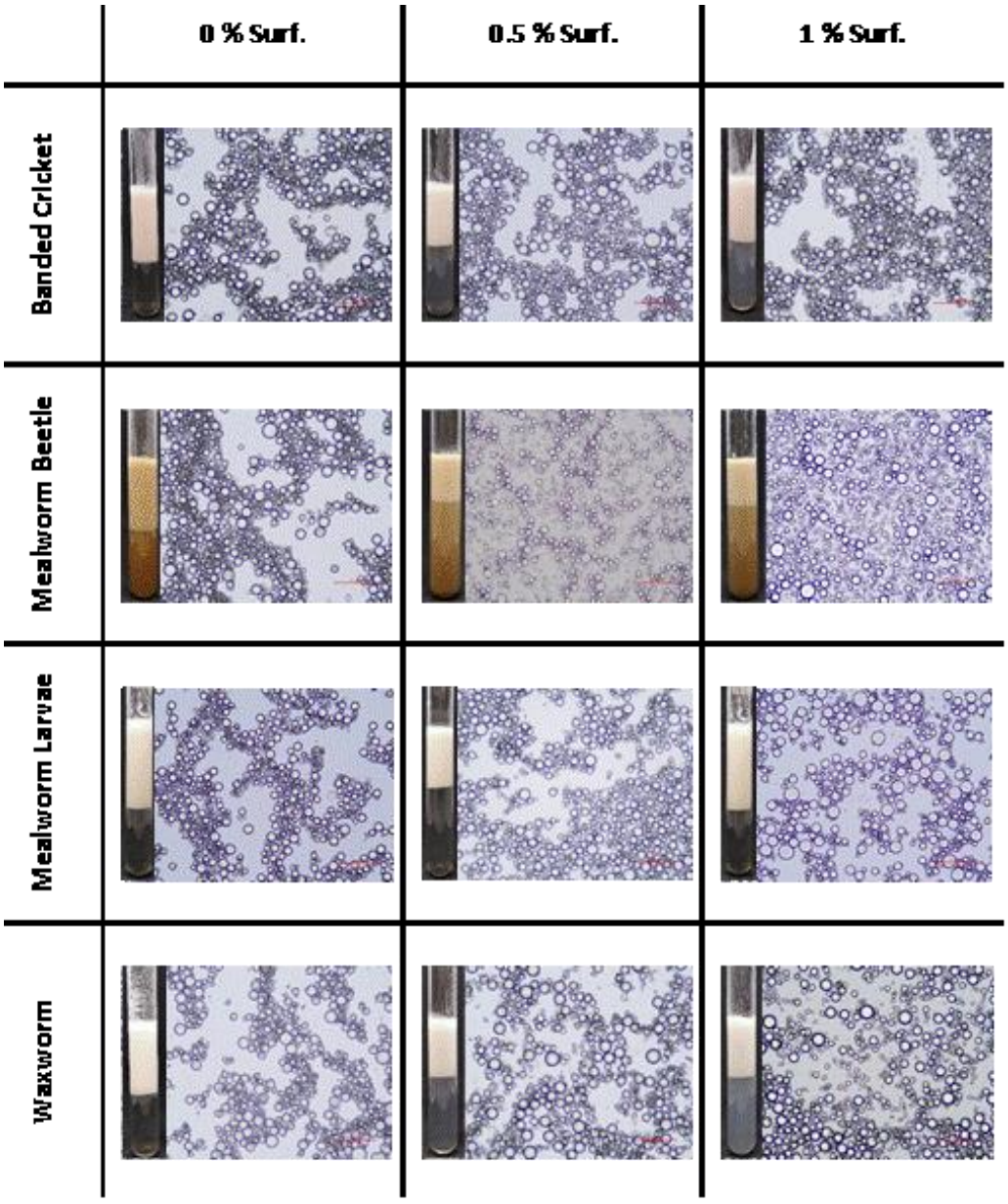

Figure 18 Micrographs and emulsions stabilised by insect protein extracts adjusted to pH 5 with 0 %, 0.5 % and 1 %, added surfactant, without heat treatment after 24 hours, scale bars 100 µm

difference. Differences in in mean $d_{4,3}$ when adjusted to pH 5 indicate a difference in degree of displacement of protein by the surfactant at the interface, surfactant protein interaction or differences in depletion flocculation in presence of surfactant. The creaming index, in Figure 20, for the pH 5 emulsions in the presence of surfactants showed no significant difference between levels of surfactant or insect protein type ($F_{(11,24)} = 0.43$, $p > 0.05$).

In emulsions stabilised by non-ionic surfactants, steric forces prevent the flocculation of droplets (McClements and Jafari, 2018, Demetriades and McClements, 1998). The steric forces are less sensitive to changes in pH and ionic strength than electromagnetic forces between proteins since it relies on the unfavourable entropy, not charge (McClements, 2016, McClements and

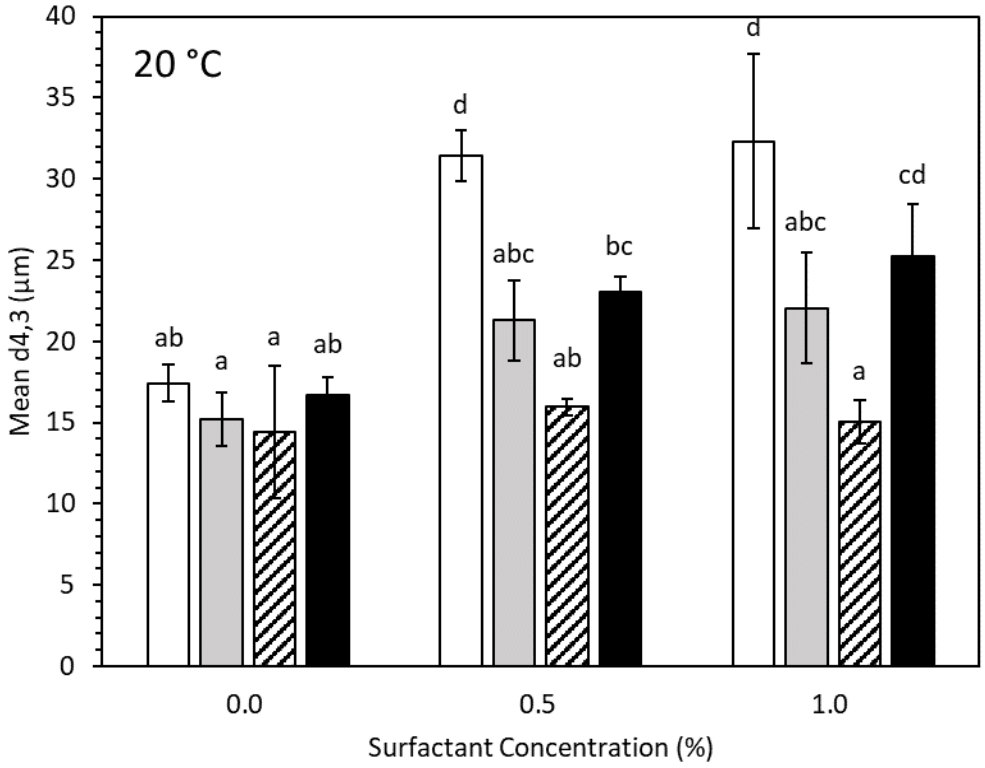

Figure 19 Mean $d_{4,3}$ of insect protein extract stabilised emulsions adjusted to pH 5 with 0 %, 0.5 % and 1 %, added surfactant, without heat-treatment, white bars are banded cricket, grey bars are mealworm larvae, striped bars are mealworm beetle and black bars are waxworm larvae, n=3 error bars show standard deviation.

Jafari, 2018) . Therefore, it is hypothesised that the degree of protein displacement from the interface is lesser in the banded cricket protein stabilised emulsion as these emulsions were altered to a greater degree by the change in pH. The heterogeneous surfactant-protein interfacial composition found in the banded cricket emulsions would be increasing the coalescence relative to the more surfactant laden interface in mealworm beetle emulsions. When surfactants are added to a preformed protein stabilised emulsion the surfactant molecules can: not interact with the protein and only interact with the other surfactant molecules forming micelles; interact with free protein molecules; interact with protein molecules at the interface; or displace protein molecules at the interface (Kotsmar et al., 2009, Aguirre-Ramirez et al., 2021, Fainerman et al., 2016, Mackie and Wilde, 2005, Wilde et al., 2004).

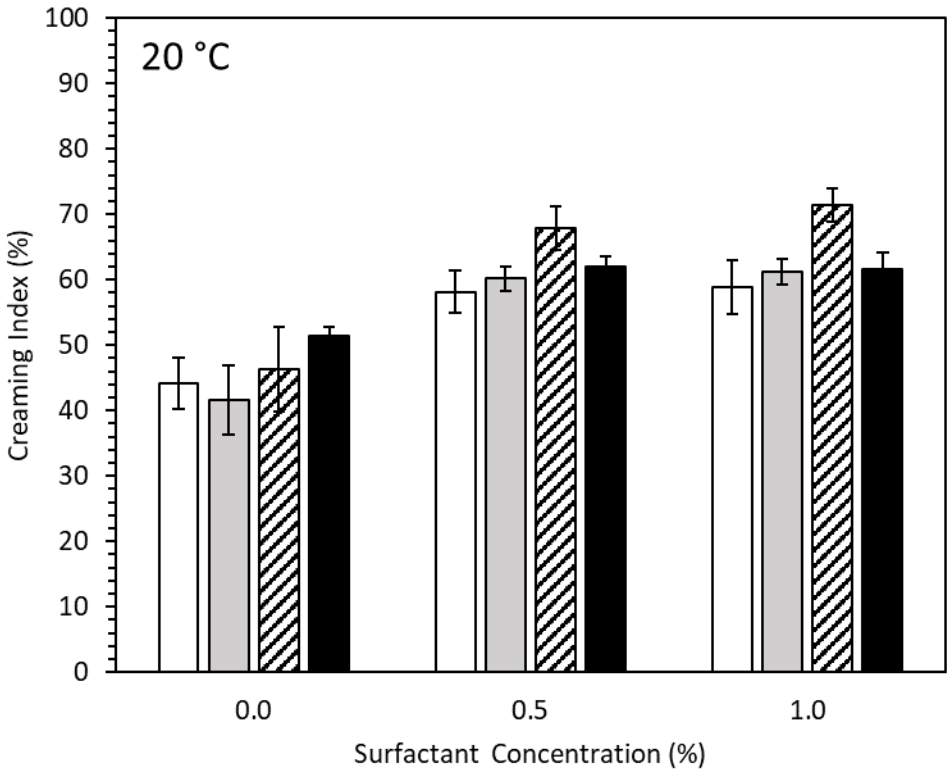

Figure 20 Mean creaming index of insect protein stabilised emulsions with surfactant added at 0 %, 0.5 % and 1 %, adjusted to pH 5, non-heat-treated, n=3 error bars show standard deviation

Displacement can be either be direct replacement or a via a complex formed between the protein and surfactant. Tween 20 has been shown to displace whey protein (Demetriades and McClements, 1998), sodium caseinate (Dickinson et al., 1999a), soy protein isolate (Zhang et al., 2021a) from an oil-in-water emulsions. In whey protein stabilised emulsions, adding Tween 20 reduced flocculation in response to pH changes (Demetriades and McClements, 1998), in agreement with results presented for the mealworm beetle sample.

6.3.5.2 Heat-treated

The oil-in-water insect protein emulsions after emulsification had surfactant added at 0 %, 0.5 % or 1 %, were adjusted to pH 5 then heat-treated at 80 °C for 10 min to determine the effect of these processing conditions on emulsion microstructure and any differences in stability of the emulsions produced from different insect proteins. Combinations of heat pasteurisation and acidification are used in food processing to prevent microbial growth so understanding the impact on microstructure of emulsion formed from insect proteins will help elucidate if commercial usage may be viable.

The micrographs in Figure 21 showed flocculation in the case of all four insect protein emulsions for 0 %, 0.5 % and 1 % added Tween 20 when heat-treated. In both cases of added surfactant, 0.5 % and 1 %, there appears to be dense flocs of emulsion droplets and perhaps protein and surfactant. However, the opaque serum at 0.5 % and 1 % Tween 20 as compared to 0 % Tween 20 indicates the presence of emulsion droplets and so less flocculation. The limited reduction in flocculation is further confirmed by the increase in creaming index

in Figure 22 Therefore, the reduction in flocculation seen with the addition of Tween 20 in the mealworm beetle proteins emulsion adjusted to pH 5 in Figure 18, is present but to a lesser extent, when the emulsion is heat treated.

The addition of surfactant, reduction to pH 5 then heating the emulsions to 80 °C resulted in similar behaviour between both insect orders and life stages. All four insect protein extracts showed a smaller increase in $d_{4,3}$ compared to the absence of surfactant after heating from Figure 23, resulting in a significantly lower $d_{4,3}$,($F_{(11,24)} = 4.14$, $p < 0.05$) for mealworm beetles and waxworms. The presence of Tween 20 in the formulation is to some degree protecting the sample from droplet diameter increases caused by heating, shown in Figure 23.

The lower $d_{4,3}$ indicates that the displacement of the protein by surfactants reduces flocculation and possibly coalescence during heating. Temperature increases affect surfactants and proteins differently on the droplet interface. In the case of polysorbate surfactants such as Tween 20 the heat dehydrates the polysorbate head increasing interactions between the surface of droplets during collisions (McClements, 2016). Moreover, the increased kinetic energy can allow surfactant molecules to break free of the droplet surface disrupting droplets and increasing coalescence. However, at 80 °C for 10 min appears to be insufficient for the dehydration or liberation of the surfactant molecules in the insect protein emulsions. Tween 20 has been shown to prevent flocculation

in whey protein emulsions when heat processed through displacement (Demetriades and McClements, 1998). Moreover, due to the non-ionic nature of Tween 20 the interface is less affected by acidic conditions. The flocculation observed in the micrographs does not persist in the laser diffraction particle sizer in the presence of added Tween 20, suggesting the displacement of the protein from the oil-water interface by the Tween 20 reduces the attractive forces between droplets sufficiently to allow the disruption of flocs in the

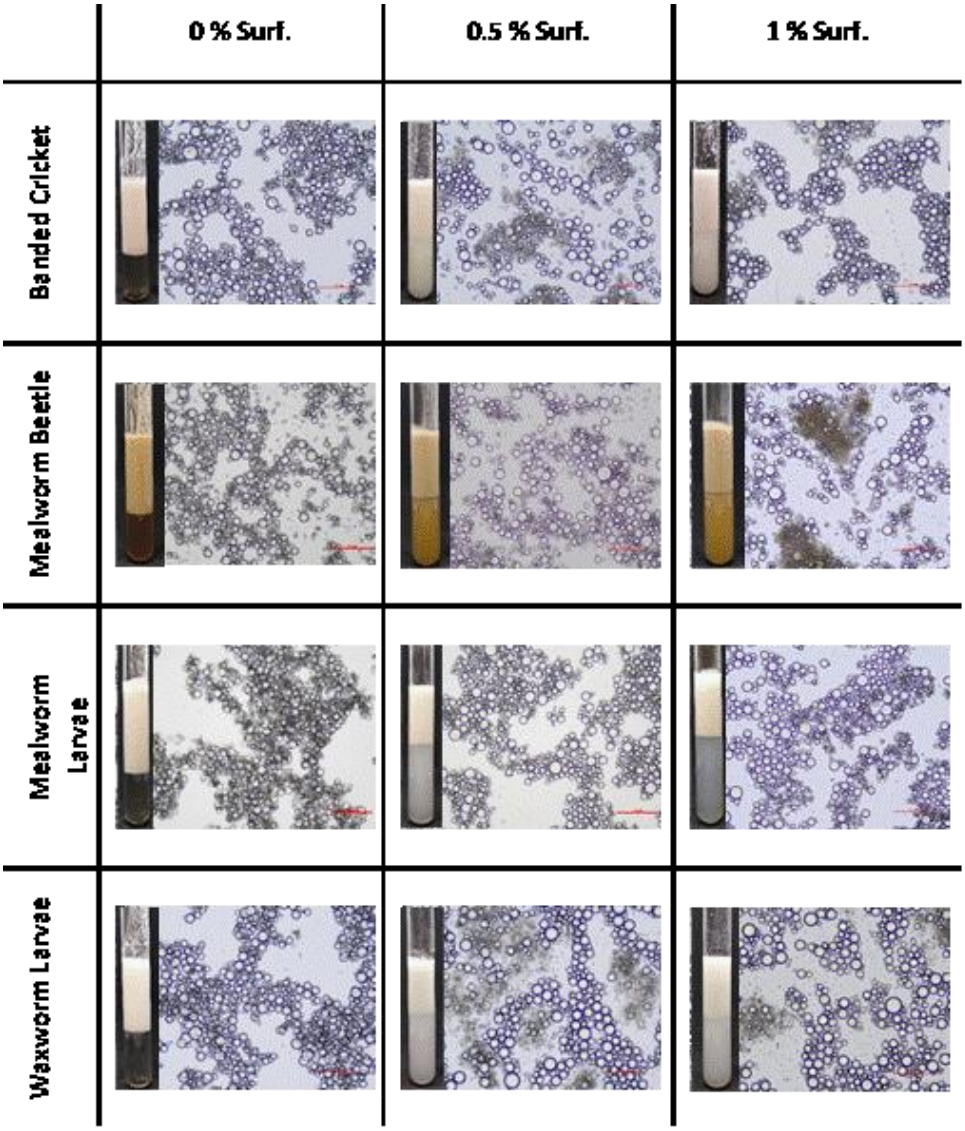

Figure 21 Micrographs and emulsions stabilised by insect protein extracts adjusted to pH 5 with 0 %, 0.5 % and 1 %, added surfactant, heat-treated at 80 °C for 10 min after 24 hours, scale bars 100 µm

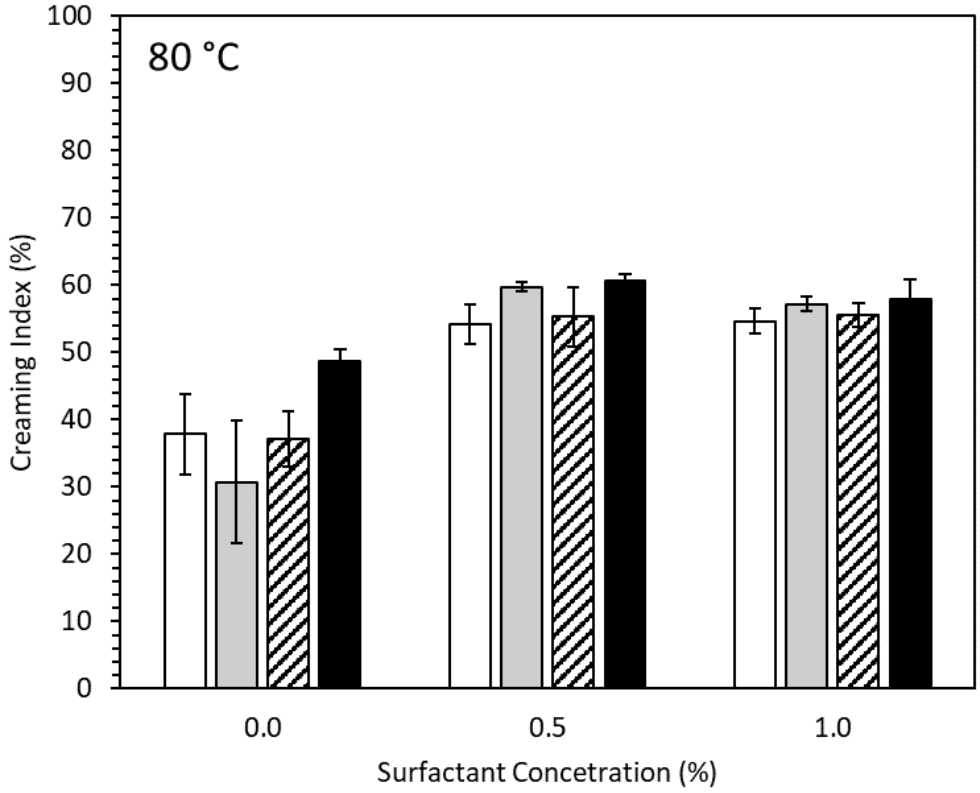

Figure 22 Mean creaming index of insect protein stabilised emulsions with surfactant added at 0 %, 0.5 % and 1 %, adjusted to pH 5, heat-treated, n=3 error bars show standard deviation

particle sizer. In the section: Heat-treated 6.3.5.2 p 172, it was suggested the increase in $d_{4,3}$ was due to an increase in hydrophobic droplet-droplet interaction due to denaturation of protein at the oil-water interface. Displacement of some of the protein at the oil-water interface by Tween 20

would decrease these hydrophobic interactions and allow the flocs to be disrupted in the laser diffraction particle sizer.

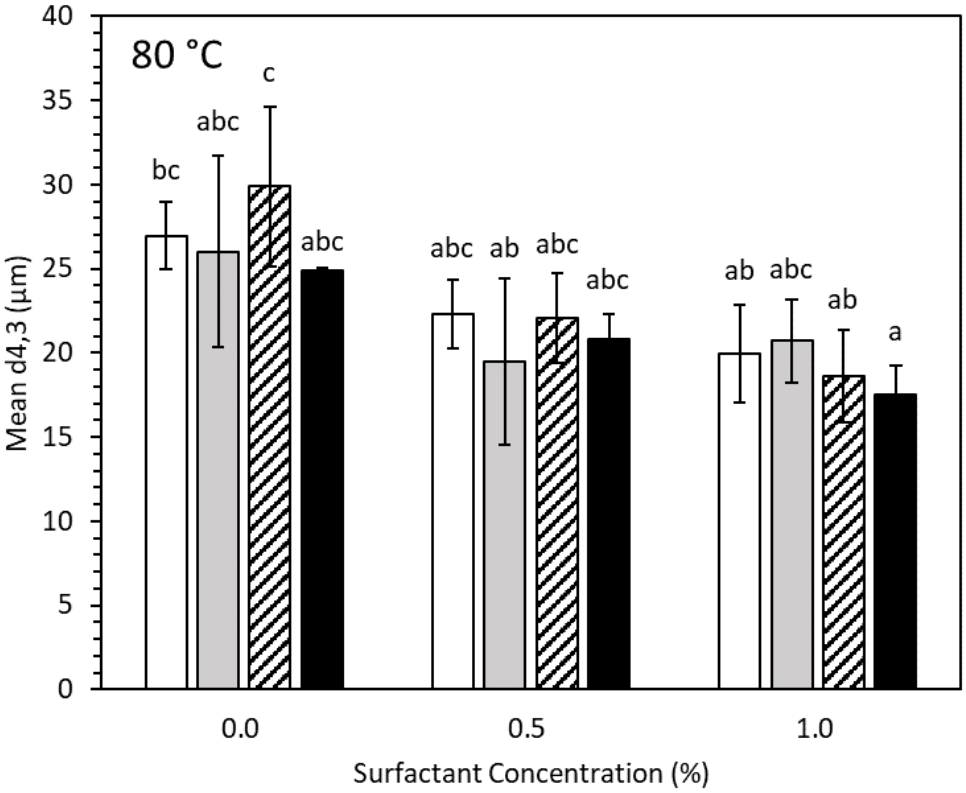

Figure 23 Mean $d_{4,3}$ of insect protein extract stabilised emulsions adjusted to pH 5 with 0 %, 0.5 % and 1 %, added surfactant, heat-treated at 80 °C for 10 mins, white bars are banded cricket, grey bars are mealworm larvae, striped bars are mealworm beetle and black bars are waxworm larvae, n=3 error bars show standard deviation.

6.3.6 Freeze-Thaw Emulsion Microstructure and Stability

The 0.44 % insect protein emulsions with Tween 20 added at 0 %, 0.5 % and 1 %, at pH 7 and adjusted to pH 5 were tested for freeze-thaw stability at -20 °C. Freezing is a common food preservation method which prevents microbial growth by the removal of liquid water from a food system and lowering reaction rates of spoilage processes. Accordingly, it is necessary to understand the freeze-thaw stability of emulsions to be used in food products. The freezing process involves the emulsion serum becoming supercooled and as a result supersaturated, nucleation of water and fat crystals, crystal growth and

reformation of the crystals as molecules gain enough energy to become part of the remaining serum or oil phase then recrystalise on other crystals (Hartel, 2002, Degner et al., 2014). The emulsifier, fat phase composition, ionic strength and pH of the serum phase all play a role in the determining the freeze-thaw stability (Degner et al., 2014).

6.3.6.1 Freeze-Thaw Emulsion Microstructure and Stability at pH 7

In the emulsions stored at -20 °C pH 7 there was visible oil separation in all emulsions with some residual emulsions droplets as seen in Figure 24. The micrographs in Figure 24 show that a portion of the emulsion droplets remained stable through the freeze thaw process. The $d_{4,3}$ for all of the emulsions, shown in Figure 25 in the absence of Tween 20 have a high standard deviation ranging from 97 µm for mealworm larvae to 27 µm for waxworm larvae, reflecting the large-scale flocculation and coalescence of the emulsions. The lower $d_{4,3}$ and related standard deviation for emulsions stabilised by proteins with added non-ionic surfactant from all species reflects that the Tween 20 displacing the insect protein at the emulsion oil-water interface provides some protection against disruption by the freeze-thaw process. The droplet volume-based distribution is highly multimodal with differences between replicates demonstrating the interference by phase separated sunflower oil. Tween 20 stabilised emulsions have been shown to be less stable to coalescence and oiling off when frozen relative to casein and whey, so displacement is unlikely to be protective to phase separation which appears to contradict the protective effect seen in Figure 25 (Thanasukarn et al., 2004b).

The freezing process increases the ionic strength of the serum phase of the emulsions through the removal of water to ice crystals, the emulsion droplets are simultaneously concentrated into a smaller volume of serum. The increase in ionic strength alters the charges on the proteins at the droplet interface causing coalescence on freezing or thawing. The oil phase will crystallise, and the crystals can breach the interfacial layer then aggregate with similar fat

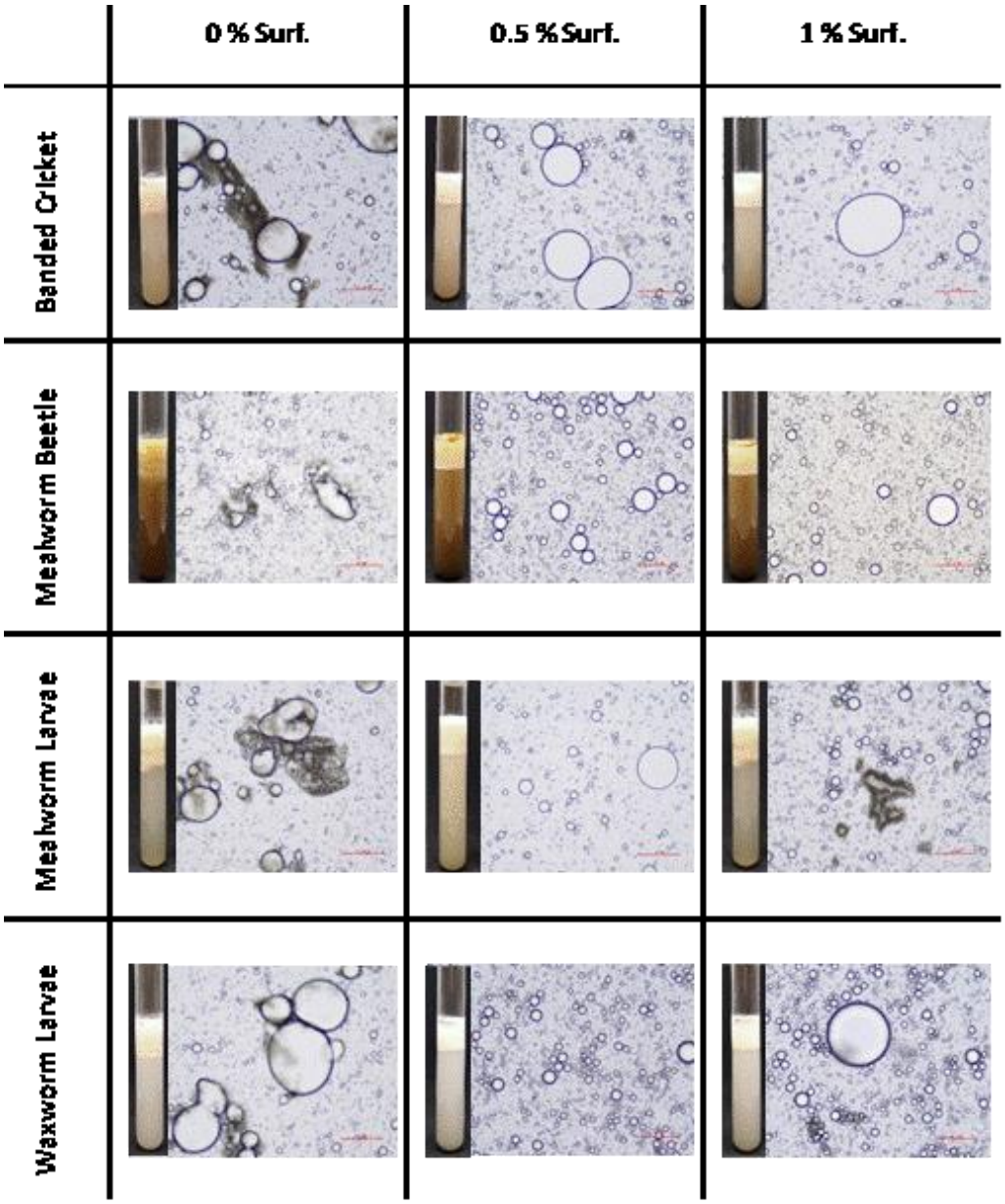

Figure 24 Micrographs and emulsions stabilised by insect protein extracts maintained at pH 7 with 0 %, 0.5 % and 1 %, added surfactant, frozen at -20 °C for 20 h and thawed

crystals on other droplets promoting coalescence (Cramp et al., 2004, Coupland, 2002). The formation of ice crystals in the continuous aqueous phase of an oil-in-water emulsion can lead to disruption the microstructure. Dehydration of the protein molecules in addition to the potential freeze

denaturation are the final mechanisms by which the droplet disruption and coalescence occurs.

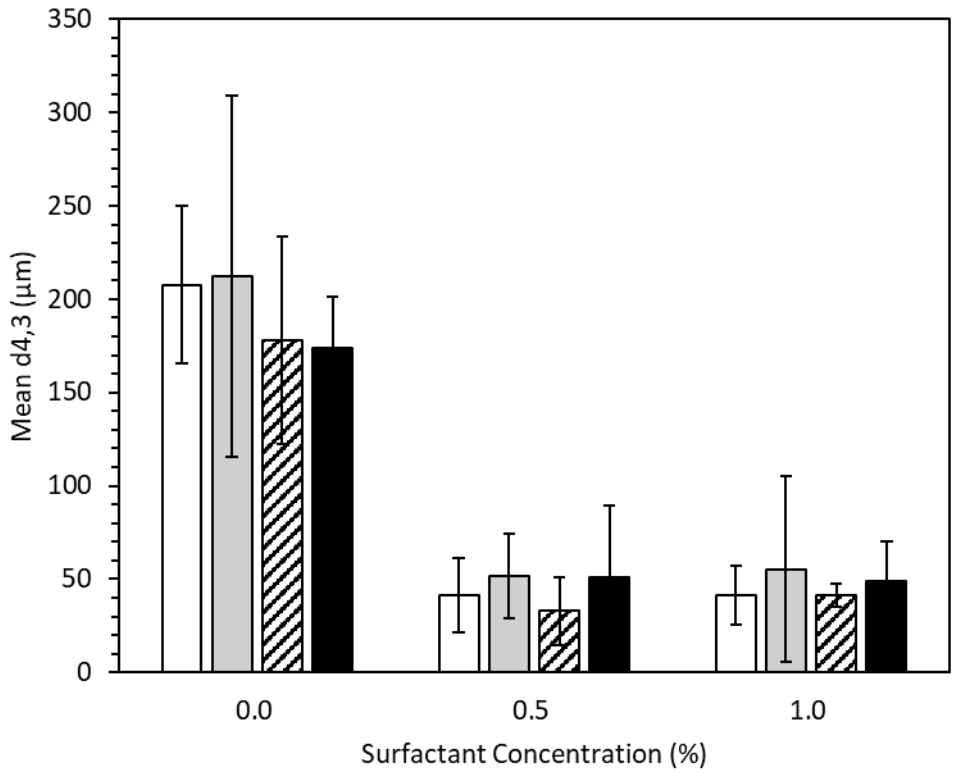

Figure 25 Mean $d_{4,3}$ of insect protein extract stabilised emulsions maintained at pH 7 with 0 %, 0.5 % and 1 %, added surfactant, frozen at -20 °C for 20 h, white bars are banded cricket, grey bars are mealworm larvae, striped bars are mealworm beetle and black bars are waxworm larvae, n=3 error bars show standard deviation.

6.3.6.2 Freeze-Thaw Emulsion Microstructure and Stability at pH 5

The lowering of the emulsions to pH 5 visibly reduces the volume of oil in the separated oil layer showing a reduction in the disruption to the emulsion structure. The proximity to the emulsion isoelectric point allows protein in solution to aggregate at the interface as demonstrated by the reduction in opacity of the serum in the emulsions adjusted to pH 5 in Figure 26. The

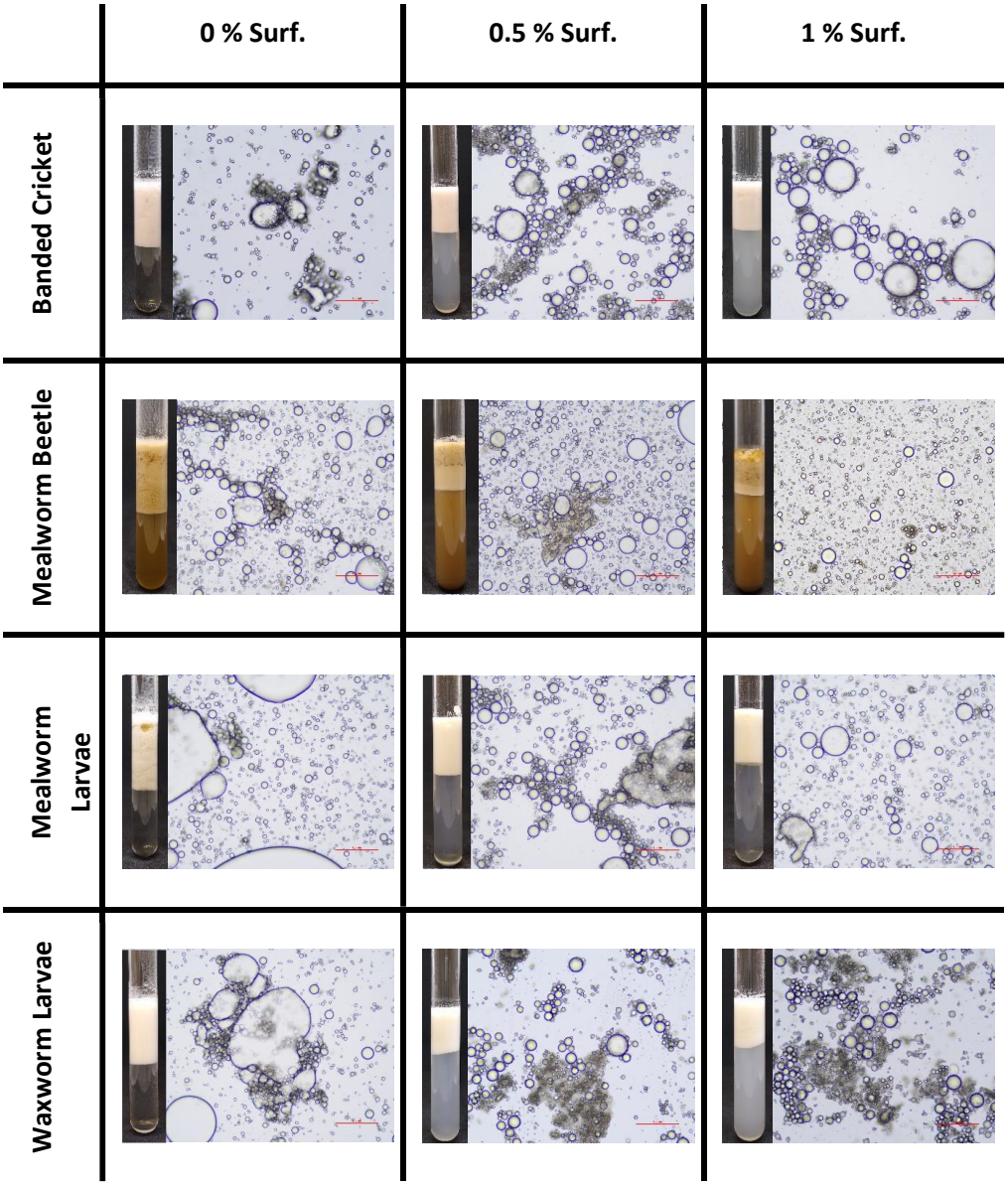

Figure 26 Micrographs and emulsions stabilised by insect protein extracts adjusted to pH 5 with 0 %, 0.5 % and 1 %, added surfactant, frozen at -20 °C for 20 h and thawed

increased thickness of the interfacial layer provides protection through greater structure to resist film rupture from ice crystals and fat crystals in addition to the prevention of film thinning and rupture, which would lead to oil separation. Increased thickness of the interfacial layer has been attributed to increased freeze-thaw stability on whey protein stabilised emulsions (Thanasukarn et al., 2004b, Cornacchia and Roos, 2011). The $d_{4,3}$ of the reaming intact insect protein emulsion structures show similar disruption to microstructure as was observed at pH 7 in the pH 5 adjusted emulsions shown in Figure 27.

The emulsions lowered to pH 5 would also have an increased ionic strength from the addition of hydrochloric acid to overcome the buffer. Increased sodium chloride has been found to disrupt the emulsion structure when one or both of the phases are crystallised in whey protein stabilised emulsions, this was explained by charge screening of the droplets allowing more droplet-droplet interactions (Thanasukarn et al., 2004a). However, one emulsion system cannot be generalised to another (Degner et al., 2014). The increased ionic strength will affect both the charge screening on the emulsion droplets and the crystallisation of water in the serum phase. Previous work with mealworm larvae from a similar extraction protocol, and the same emulsion production method found that freezing to -20 °C for 24 h gave a reduction in mean droplet diameter and no mention of phase separation (Gould and Wolf, 2018). Emulsions were produced using 1.75 % whey protein solutions, which showed similar freeze-thaw instability to the four insect protein extracts by the same method (data not shown). The key differences between the emulsions in

this study and that of Gould and Wolf (2018) is the lower strength of the buffer in the emulsions in this study and additionally the lower salt content in the final mealworm extract contributing to an overall lower ionic strength in this study. Furthermore, the holding time at -20 °C lower in this study at 18 hours compared to 24 hours in (Gould and Wolf, 2018). Ultimately, higher ionic strength reduces dispersed phase crystallisation potentially improving freeze-thaw stability, but droplet charge screening may reduce stability. While the increased interfacial load may provide a robust viscoelastic layer around the droplets increasing freeze-thaw stability. The results here suggest that for emulsions from all four insect proteins show increased stability at pH 5 so the

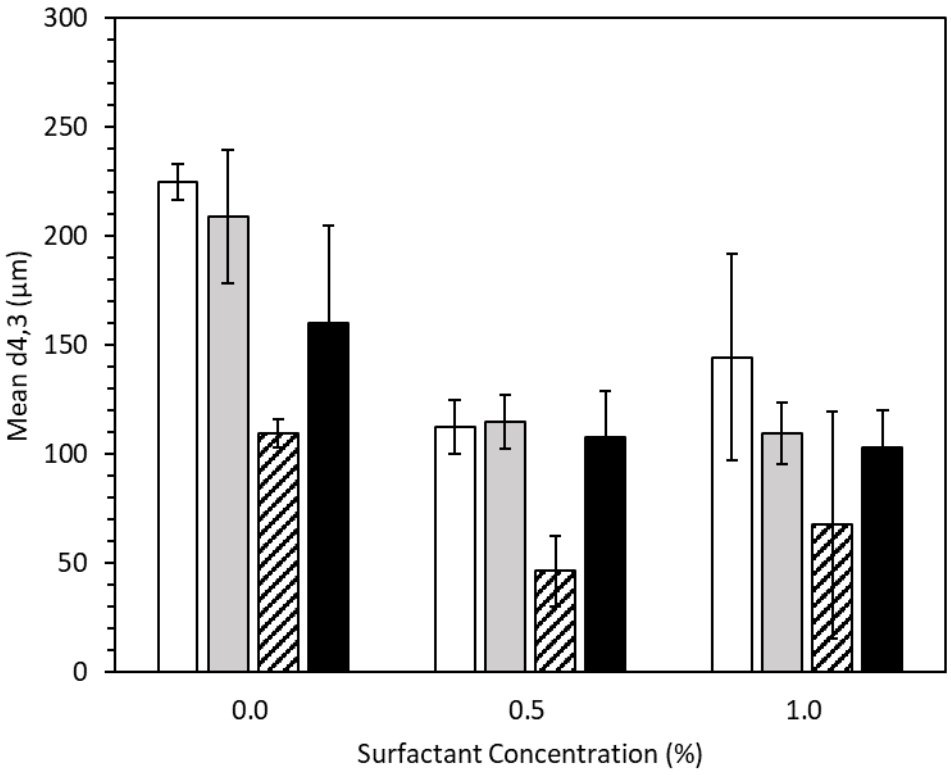

Figure 27 Mean $d_{4,3}$ of insect protein extract stabilised emulsions adjusted to pH 5 with 0 %, 0.5 % and 1 %, added surfactant, frozen at -20 °C for 20 h, white bars are banded cricket, grey bars are mealworm larvae, striped bars are mealworm beetle and black bars are waxworm larvae, n=3 error bars show standard deviation

increased interfacial load helps prevent any coalescence from increased droplet-droplet interactions due to charge screening.

None of the insect protein extracts formed an emulsion stable to a basic freeze-thaw treatment at -20 °C, showing no effect of insect of order or life stage. A more detailed study of freezing rates and temperatures would be valuable to understand if insect protein emulsions might be stable under other conditions.

6.4 Conclusion

The whole freeze-dried insect powders from banded crickets, mealworm beetles, mealworm larvae and waxworm larvae showed differing protein contents and fat composition. With The mealworm beetle powder having the highest protein content and waxworm protein content was the lowest reflecting the higher fat content of the waxworm larvae. So, the higher lipid contents of the mealworm larvae relative to the mealworm beetles and the waxworms larvae to the other insect types shows there may be increased lipids and in the insect larval stage. Furthermore, there are differences in insect composition between the orders of adult insects and larval insects.

Protein extracted from the all four insect types: banded crickets, mealworm beetles, mealworm larvae and waxworm larvae produced stable oil-in-water emulsions at pH 7 with similar microstructures. When the emulsions were adjusted to pH 5, flocculation visibly increased, creaming index was reduced but no change in droplet diameter. If the insect protein emulsions were heated after adjustment to pH 5 the droplet diameter increased, which was hypothesised to be due to droplet flocculation. No differences were observed

between emulsions formed from insect protein if different life stage or taxonomic order.

While the addition of surfactant had little effect at pH7, when the four insect protein emulsions were adjusted to pH 5 after the addition of surfactants differences between the insect protein types became apparent. The banded cricket protein emulsions showed an increase in droplet diameter and flocculation whereas mealworm beetle protein emulsions showed no increase in droplet diameter and no observable flocculation.

These results suggest under specific conditions in a food environment, insect protein extracts may be used interchangeably. However, if a non-ionic surfactant is present, protein from a specific insect order may be required, depending on if disruption or maintenance of the emulsion structure is the aim. No conclusive difference was found between emulsions formed from adult compared larval protein extracts. Before insect proteins are utilised in emulsion food products, a greater understanding of whether protein displacement is occurring in the presence of Tween 20 and to what degree would be beneficial.

7 The Interfacial Composition, Properties and displacement of *Tenebrio molitor* beetles and *Gryllodes Sigillatus* protein

7.1 Introduction

The world population is urbanising, with 4.2 billion people living in urban areas in 2018, which projected to grow to 6.9 billion by 2050. The majority of this increase is projected to come from Asia and Africa increasing from 0.5 billion to 1.5 billion in Africa and 2.3 billion to 3.5 billion in Asia (United Nations, 2019b). Increasing urbanisation leads to reduction in the proportion of the population working in agriculture and an increase in processed food consumption (Tschirley et al., 2015, Jayne et al., 2018, de Bruin et al., 2021). The majority of countries which have a tradition of insect consumption are found in Africa and Asia, indeed China, India and Nigeria are expected to make up 37 % of the increase in urbanisation and a subset of these populations already consume insects (Van Huis et al., 2013, United Nations, 2019b). Therefore, there is value in understanding the functional behaviour of insects and insect derivatives such as protein extracts in processed foods to meet the needs of future urban dwellers.

Attitudes and the legal position of insect for human consumption in Europe, USA, Canada and Australia suggest any increase in consumption will be slow. In the European Union and the United Kingdom insects are being legalised on a species-by-species basis as safety data is produced and submitted to the

relevant food safety authorities (Turck et al., 2021a, Turck et al., 2021b). In addition, there remains an aversion to among many western countries to eating insects due to disgust and a lack of familiarity (McDade and Collins, 2019). In view of this aversion to insect consumption, the cautious legal approach is indeed sensible as a major food safety incident would only serve to further increase aversion. However, these two factors mean the adoption of insect products in processed foods in Europe and North America excluding Mexico, is likely to be lower than that of Africa, Asia and South America in the near term.

Emulsions are an important structural component in processed foods, as covered in Chapter 2 2.1 Protein Stabilised Emulsions in Foods, p 22, emulsions are two immiscible liquids one dispersed within the other in small droplets, in separate phases. Emulsions are found in milkshakes and dairy drinks, grain and nut drinks, soups, sauces, dressings, marinades, vegetable-based purees and soft drinks (Piorkowski and McClements, 2014), vegan cheese alternatives, vegan meat alternatives and the increasingly popular meal drinks (McClements, 2016, Lam and Nickerson, 2013). Furthermore, emulsifiers play an important role in products such as ice cream to displace interfacially active proteins from the surface of fat particles (Goff and Jordan, 1989). The emulsifier utilised in any given emulsion containing food is dependant the specific function of the emulsifier on the processing conditions: ionic strength, pH level, heating, cooling, freezing, mixing; the conditions in the final product, the storage conditions and use case of the product. Understanding the behaviour of an emulsifier before utilisation in a food product allows for matching to the correct processing conditions for the desired end result.

Proteins are a commonly used emulsifier for oil-in-water emulsions because some are soluble in aqueous media, adsorb readily to phase interfaces, add to macronutrient content of foods, proteins are acceptable to consumers and produce differing interfacial properties and structure depending on structure. In western countries, the most common protein type used as an emulsifier in foods is caseinates followed by whey proteins and increasingly plant proteins such as soy isolate (Phillips and Williams, 2011b).

In oil-in-water emulsions smaller molecular weight proteins diffuse and adsorb to interface more rapidly than larger molecular weight proteins (Sharif et al., 2018). Proteins with a more open flexible structure adsorb to the interface and reduce interfacial tension more rapidly than proteins with a more globular structure (Freer et al., 2004a, Tang and Shen, 2015, Freer et al., 2004b). The mass of protein required to stabilise and saturate the oil-water interface is dependent on protein structure.

Whey protein is a commonly used emulsifier in foods, it has a heterogeneous composition but is composed of approximately 60 % β-lactoglobulin and 20 % α-lactoglobulins (Farrell et al., 2004). Whey protein is highly soluble, diffuses and adsorbs to the interface sufficiently to form an emulsion, and takes many hours to reach an equilibrium interfacial tension as the protein rearranges into a lower energy state. Casein protein is also a heterogeneous protein mixture but is mostly composed of flexible proteins, such as β-casein 40 % and α_{S1}-casein 40 % (Phillips and Williams, 2011b). Caseins diffuse and adsorb to the oil-water interface more rapidly than whey proteins and reach an equilibrium

interfacial concentration more rapidly than whey protein (Zhou et al., 2022). Insect proteins have been shown to produce stable oil-in-water emulsions and may serve as a viable emulsifier instead of milk proteins in emulsion type foods in countries which already consume insect (Wang et al., 2021, Gould and Wolf, 2018, Hirsch et al., 2019a).

Food products are complex mixtures containing a number of interfacially active compounds. Polysorbate also known as Tweens are used both as emulsifiers and to displace proteins from lipid globules in ice-cream promoting agglomeration and adsorbing to lipid interface in liquid non-aeriated creams to prevent agglomeration during high-pressure homogenisation and preventing fat bloom in chocolate coatings (Norn, 2015, Goff, 2013). Polysorbate based emulsifiers are non-ionic low molecular weight surfactants which are highly soluble in aqueous media. Tweens are a hydrophilic polyoxyethylene head group of varying average units bonded to a specific hydrophobic fatty acid tail group. Non-ionic low molecular weight surfactants can displace proteins from the interface by adsorbing in the gaps between the larger protein molecules and lowering the interfacial tension to a greater degree, compressing the protein layer and eventually displacing the proteins completely (McClements and Jafari, 2018). Moreover, surfactants can form hydrophobic interactions with both proteins at the interface or free proteins in solution. Although the rate and degree of displacement and any direct protein-surfactant interactions are dependent on the structure of both the surfactant and the protein (Aguirre-Ramirez et al., 2021, Li and Lee, 2019). To this end understanding the

interaction between polysorbate surfactants and insect proteins which could potentially be used in food products is highly important

Chapter 5 showed insects from within one order could all form oil-in-water emulsions with similar properties. Chapter 6 demonstrated that the proteins from different life stages and taxonomic orders produced similar emulsions at a neutral pH level, when the same emulsions were acidified to pH 5 in the presence of a non-ionic surfactant differences in the volume-based droplet diameter became apparent most clearly between Gryllodes sigillatus (banded cricket) proteins and *Tenebrio molitor* (mealworm) beetle proteins. It was hypothesised that mealworm beetle proteins were more readily displaced than banded cricket proteins by Tween 20 surfactant, the Tween 20 surfactant being non-ionic is less susceptible to disruption by pH shifts (Demetriades and McClements, 1998). Understanding the differences in emulsion properties in the presence of food-grade surfactants under conditions which may be expected in a food product provides valuable information for the use of insect protein as a food ingredient. To this end, Chapter 6 will focus on investigating the similarities and differences in interfacial composition between emulsions formed from mealworm beetle protein and banded cricket protein, and the degree of displacement by Tween 20 surfactant.

7.2 Materials and Methods

7.2.1 Materials

Gryllodes sigillatus (banded crickets) 15 -18 mm adult insects from the order *Orthoptera* were purchased from Monkfield Nutrition (Royston, UK).

Mealworm beetles adult insects from the order *Coleoptera* were purchased from Norfolk Pet Supplies (Kings Lynn, UK). For the protein extraction and emulsion preparation, sodium azide (NaN_3), Florisil < 200 mesh, Polyoxyethylene (20) sorbitan monolaurate (polysorbate 20 or Tween 20), sodium phosphate dibasic and monobasic were purchased from Sigma-Aldrich (Dorset, UK), hydrochloric acid (HCl), ethanol and sodium hydroxide (NaOH), were purchased from Fisher Scientific (Loughborough, UK), sunflower oil was purchased from a retailer (Sainsbury's, London, UK). All water used was double distilled. The rhodamine B for microscopy was purchased from Sigma-Aldrich (Dorset, UK).

For the amino acid analysis, hydrogen peroxide, formic acid 98 – 100 % analytical reagent, phenol, sodium metabisulphite analytical reagent, hydrochloric acid, ammonium formate, acetonitrile LC/MS grade, isopropanol LC/MS grade were purchased from Fischer Scientific (Loughborough, Leicestershire, UK). Cell free amino acid mixture $^{13}C,^{15}N$, amino acid standard, cysteic acid, methionine sulfone, alanine ^{15}N, phenylalanine ^{15}N were purchased from Sigma-Aldrich (Merck Life Science UK Limited, Gillingham, Dorset, UK). Phenylalanine $^{13}C_6$ was purchased from Cambridge Isotopes Limited (Cambridge, MA).

7.2.2 Methods

The two insect protein extracts were produced by the method in 4.1 Protein extraction p100. The protein contents of both the whole insect powders and protein extracts were estimated by the method in 4.2 Nitrogen analysis p101.

Protein solutions were produced with a pH 7 50 mM sodium phosphate buffer by the method in 4.4 Protein Solutions p102.

7.2.2.1 Emulsion Preparation and Processing

Emulsions were produced with 120 g of protein solution and 30 g with the method given in 4.6 Emulsion Preparation p103. Three emulsion replicates for each insect protein extract were produced. The emulsions were allowed to rest for 30 min, then either 1 % Tween 20 or the same mass of ultrapure water and the emulsion stirred at 300rpm for 30 min. The emulsions were subdivided in two at a ratio of 1:2 by mass. The lower mass aliquot was stored at 20 °C. The larger aliquot was adjusted to pH 5 with 2 M HCl and further subdivided into two aliquots. One aliquot was heat-treated in a 80 °C water bath for 10 min cooled at room temperature and stored at 20 °C. All emulsion samples were stored overnight at 20 °C before analysis.

7.2.2.2 Emulsion Imaging

7.2.2.2.1 Protein Staining

Rhodamine B solution 1 g/L was produced using ultrapure water. Emulsions were combined at 50:1 ratio with the 1 g/L rhodamine B solution in a 1.5 ml Eppendorf tube, gently agitated and allowed to stand at 20 °C for 30 min before analysis.

7.2.2.2.2 Confocal Microscopy

To identify the presence of proteins at the oil-water interface the rhodamine B stained emulsions were imaged using a confocal laser scanning microscope (LM 880, Carl Zeiss Ltd, Cambridge, United Kingdom) with Zen blue software version

3.4.91.00000, (Carl Zeiss Ltd, Cambridge, United Kingdom), 20x and 40x oil objective lens, using 561 nm excitation and 634 nm emission wavelength channel and a T-PMT channel. Images were recorded and overlayed.

7.2.2.3 Droplet Surface Charge

The ζ-potential of the emulsion droplets was measured using photon correlation spectroscopy (Delsa Nano, Beckman Coulter, High Wycombe, UK). Three independent replicates were prepared from each emulsion by diluting the emulsion 1:10 on a volume basis. The pH was adjusted to pH 5 or pH 7 ± 0.05 with 0.1 M hydrochloric acid and 0.1 M sodium hydroxide. Three measurements of each replicate were recorded.

7.2.2.4 Interfacial Protein Concentration

Aqueous and creamed phases of emulsions were fractioned for the interfacial protein concentration analysis. Emulsions were poured into separation funnels after production and allowed to cream overnight. The serum layer was drawn into glass vials. The serum was freeze dried by pipetting 7 – 10 ml accurately and recording the mass, then freezing to -80 °C then freeze drying to a constant mass. The protein content was estimated by the Dumas method with a 6.25 nitrogen-to-protein conversion factor after subtraction of the nitrogen due to sodium azide.

7.2.2.5 Amino Acid Analysis

The amino acid composition of the banded cricket and mealworm beetle proteins and powders, emulsion serums at pH 7 and for mealworm beetle only emulsion serum adjusted to pH 5 with no heat-treatment, as well as a soy flour

standard. Formic acid-phenol working solution was prepared with 735 ml of formic acid, 111 ml ultrapure water and 4.73 g of phenol. Oxidation solution was prepared from the formic acid-phenol solution with hydrogen peroxide added to a final concentration of 3 %, this was prepared fresh before oxidising samples. Hydrolysis reagent was produced by diluting hydrochloric acid to 5 M.

Approximately 30 mg of material was weighed accurately into glass tubes and chilled. Oxidation solution was prepared from the formic acid-phenol solution with hydrogen peroxide added to a final concentration of 3 %, this was prepared fresh before oxidising samples. To each tube 2.5 ml of chilled oxidation solution was added, the samples were stored at 4 °C for 17 hours. To decompose excess oxidation reagent 0.42 g of sodium metabisulphite was added to each tube.

Asparagine and glutamine are deaminated to aspartic acid and glutamic acid, and tryptophan is completely broken down (Fountoulakis and Lahm, 1998). Hydrolysis reagent, 2.5 ml of 12 M HCl and 0.5 ml of 6 M HCl and 1 % phenol were added to each tube which were then allowed to cool. The tubes were sealed and placed in a 110 °C oven for 24 hours. The hydrolysed material was quantitatively transferred to 50 ml centrifuge tubes washing with ammonium formate pH 2.8, 20 mM. The hydrolysed material was partly neutralised with 16 ml of 4 M ammonium formate, keeping below 40 °C. The pH was adjusted to pH 2.8 with 4 M, 100 mM and formic acid then made up to 50 ml with pH 2.8, 20 mM ammonium formate buffer. The tubes were centrifuged at 3000 rpm for 10 min, the supernatant was passed through a 0.22 µm filter into glass

vials. The amino acid solutions were diluted to 1 -10 µg/l of nitrogen and stored at 4 °C until analysis.

Each amino acid was quantified against a standard using an LC-MS/MS system. The LC-MS/MS system was an ultra-high performance liquid chromatography system (Thermo-Fisher Vanquish, Thermo Fisher Scientific, Massachusetts, USA) fitted with an a Thermo Scientific Acclaim Trinity P1 mixed mode column (150 mm x 2.1 mm, 3 µM) coupled to a Triple Quadrupole Mass spectrometer (MS/MS) (Altis, Thermo Fisher Scientific, Massachusetts, USA) with heated electrospray ionization (H-ESI) system. The data was analysis software is TraceFinder Version4.1. Two replicates were produced and analysed.

7.2.2.6 Interfacial Tension

7.2.2.7 Force Tensiometry

The banded cricket protein measurements were not performed since these were completed in Chapter 5.3.4.

7.2.2.7.1 Drop Profile Tensiometry

The surface tension of the banded cricket and mealworm beetle protein solutions in air was measured using a drop profile tensiometer (PAT1, SINTERFACE technologies, Berlin, Germany). The surface tension was measured for protein concentration 0.44 %, the same as the concentration in the emulsions, and 0.001 % to assess the interfacial tension at t < 60 s after the air water interface is formed. A 2 mm capillary was used at drop area 28 mm^2 for 86 400 s at 20 °C, recording measurements at 1 s intervals. A new droplet was formed at the start of the measurement period. The initial interfacial tension

was taken as the first recorded measurement point after the drop area had stabilised. The equilibrium interfacial tension value was taken as the mean of the final 1200 s of the 86 400 s period. Measurements were performed in triplicate.

7.3 Result and Discussion

Emulsions were produced at 0.44 % banded cricket protein and mealworm beetle protein with 20 % treated sunflower oil to investigate presence and concentration of protein at the oil-water interface and the properties of the interfacial layer.

Extraction and composition have been covered for banded crickets in Chapter 5.3.1 Protein Content, p 114, and both banded cricket and mealworm beetle in Chapter 6.3.1 Composition, p 142, so consequently will not be covered here.

7.3.1 Emulsion Microstructure

To confirm visually if protein was present at the oil-water interface in the insect protein stabilised emulsions, staining laser scanning confocal microscopy was performed. Rhodamine B fluorescent stain was used to visualise the protein

7.3.1.1 At pH 7

The images in Figure 28 show that both mealworm beetle protein and banded cricket protein produced stable oil-in-water emulsions at pH 7 with protein stained red at the oil-water interface. Some free protein particulate is also present in the serum phase which was not visible in the brightfield microscopy in Section 5.3.3 Cricket Protein Stabilised Oil-in-Water Emulsions, p 124, or in older emulsion samples. Both the mealworm beetle protein and banded cricket show evidence of protein present in the solution at pH 7.

In the presence of 1 % Tween 20 the stained protein ring around the emulsion droplet is not present and the stained material is present in the emulsion serum as evidenced in Figure 28. In fact, when surfactant is added there is no evidence

of protein at the interface in any the confocal laser scanning microscopy images, even at higher magnification in the mealworm beetle proteins stabilised emulsion in Figure 29. At pH 7 with 1 % added Tween 20 there is no observable difference in the interfacial composition between the two insect protein stabilised emulsions.

Both emulsion types, containing either mealworm beetle protein or banded cricket protein, exhibit some limited flocculation at pH 7 in the presence of 1 % surfactant but in the banded cricket stabilised emulsions there is evidence of some aggregated protein between the emulsion droplets in Figure 28. In Chapter 6.3.4.1 Section Non-heat-treated, p 161, there was no evidence of flocculation at pH 7 with 1 % Tween 20 in either mealworm beetles nor banded cricket proteins, so it is possible that the flocculation is induced by the

Rhodamine B stain, although no evidence was found of this in the literature, or it is an artefact due to the microscope slide coverslip.

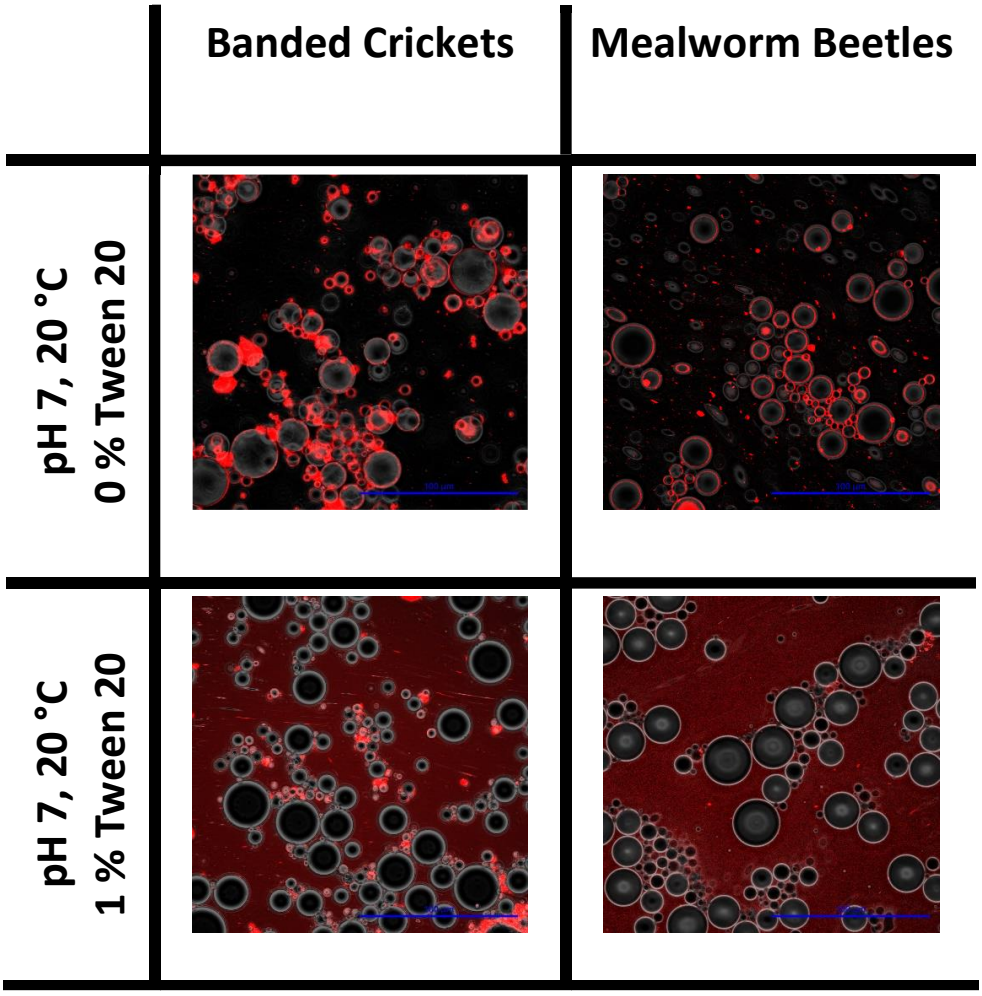

Figure 28 Image of oil-in-water emulsions stained with rhodamine B produced using confocal microscopy, scale bars show 100 µm

7.3.1.2 At pH 5

When lowered to pH 5 the quantity of the protein at the interface increased as shown by the increased staining around the droplets in Figure 30, for mealworm beetle emulsions.

Protein oil-in-water emulsions are maintained in an unflocculated state by electrostatic repulsion and steric hinderance (Dickinson, 1999, Dalgleish, 1996).

When the mealworm beetle protein stabilised emulsions were adjusted to pH 5 flocculation increased considerably as the pH of the serum phase moved closer to the isoelectric point of the protein at the oil-water interface reducing the droplet charge and electrostatic repulsions between droplets. In addition, the closer proximity to the isoelectric point of the protein caused the aggregation of the free protein in the dispersion with the protein at the interface.

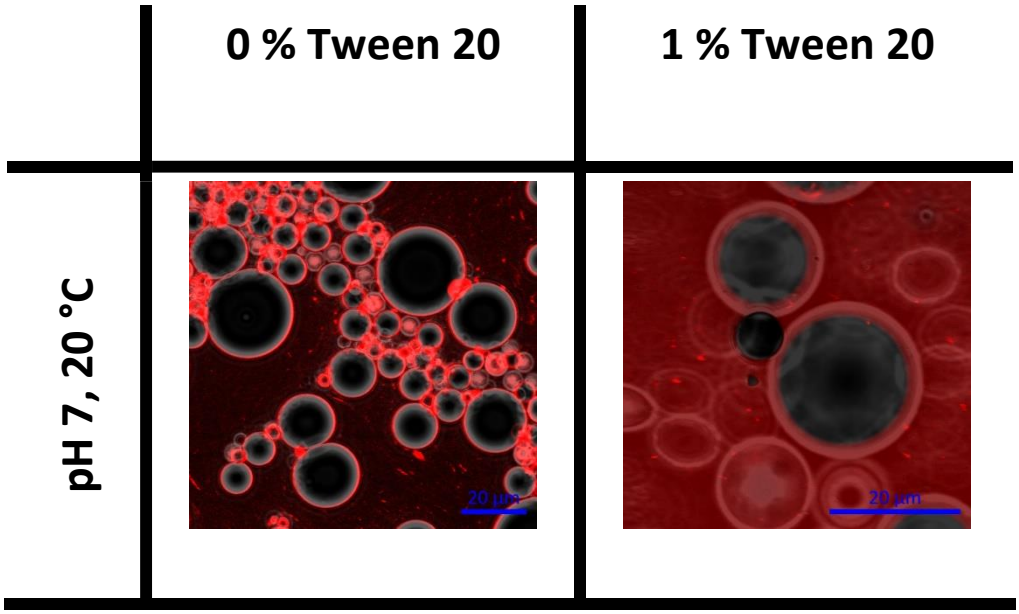

Figure 29 Images of mealworm beetle protein oil-in-water emulsions stained with rhodamine B produced using confocal microscopy, scale bars show 100 µm

When the mealworm beetles are reduced to pH 5 in the presence of 1 % Tween 20 as shown in Figure 30 similarly to sample at pH 7, there is an absence of stained protein at the interface, some protein remained aggregated between the droplets and increased staining in the solution was observed. Visual observations in Figure 30 from show less flocculation of emulsion droplets in the emulsions adjusted to pH 5 with added Tween 20 compared to without Tween 20 surfactant in mealworm beetle protein emulsions.

Tween 20 is non-ionic and therefore when it is at the oil-water interface in emulsions the repulsive forces between droplets are dominated by steric forces rather than electrostatic repulsion so changes in pH alter flocculation to a lesser degree than in proteins (Dimitrova and Leal-Calderon, 1999).

The aggregated protein between emulsion droplets suggests either surfactant-protein interactions or residual protein at the interface. The likelihood of the two afore mentioned scenarios depends on the charge of the protein molecules and emulsions droplets at pH 5.

It was hypothesised in Chapter 6 that there was greater protein displacement by the Tween 20 in the mealworm beetle emulsions than in the banded cricket protein which led to an increase in emulsion droplet diameter in banded cricket protein emulsions in the presence of 1 % surfactant. From the laser scanning confocal microscopy image produced here in appears that both the banded cricket protein and mealworm beetle protein emulsions had protein present at the oil-water interface in the absence of Tween 20. With added Tween 20 at pH 7, the insect protein emulsions showed no evidence of

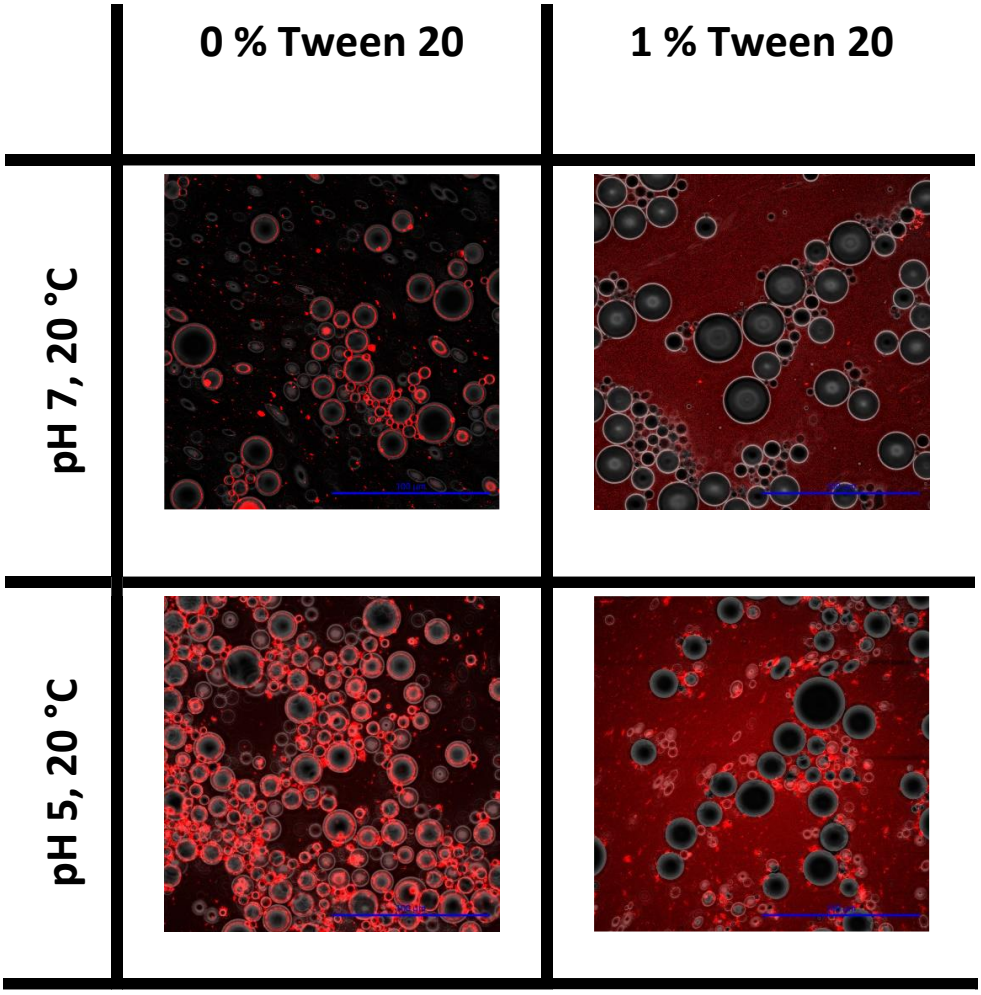

Figure 30 Images of mealworm beetle oil-in-water emulsions stained with rhodamine B, produced by confocal microscopy, scale bars show 100 µm

protein at the oil-water interface in either emulsion. At pH 5 in mealworm beetle protein emulsions there was little evidence of protein at the oil-water when 1 % Tween 20 is added. Banded cricket protein emulsions with 1 % Tween 20 were at pH 5 were not imaged.

7.3.2 Droplet Charge

To assess the charge on the droplet at differing pH levels and to understand if the protein has been displaced at the interface the ζ-potential of the droplets was measured. ζ-potential is a measure of electrical potential at the point of

slip between the charged bi-layer around a droplet or particle and the aqueous medium (Cano-Sarmiento et al., 2018).

Emulsions were measured at pH 7 without prior heat-treatment (20 °C), pH 5 with (80 °C) and without heat-treatment (20 °C), with and without added Tween 20 at 1 %, from both banded cricket and mealworm beetle protein. These analyses were performed to confirm or refute the complete displacement of protein by Tween 20 shown in confocal laser scanning microscopy images.

At pH 7 the ζ-potential of the emulsion droplets was -34.1 (± 0.9) mV for banded crickets and -34.6 (± 1.4) mV for mealworm beetle shown in Figure 31. These ζ-potentials are beyond the ± 30 mV generally regarded as required for an

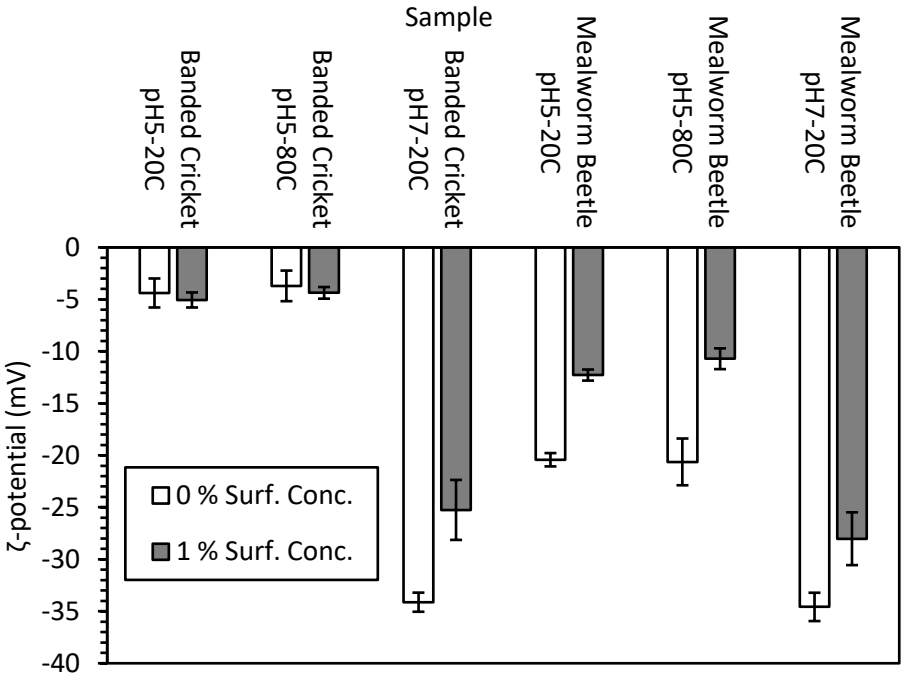

Figure 31 ζ-potential of insect protein oil-in-water emulsions, error bars show standard deviation, no added Tween 20 (white bars), 1 % added surfactant (grey bars)

emulsion to remain stable to flocculation and consequently, coalescence for the emulsions in the absence of surfactant (Grumezescu, 2016). This would be consistent with the presence of protein at the interface being negatively charged and above the isoelectric point of the protein. In the extraction process the protein was precipitated at pH 4.4 indicating this would be proximal to the isoelectric point for any proteins precipitated as the overall neutral charge decreases solubility and results in precipitation. Proteins are charged molecules due to the nitrogen on the amine groups on arginine and lysine, acting as a proton acceptor and carboxylic acid charged amino acid side chains. The ζ-potential of proteins is negative above the isoelectric point of the protein as protons are donated from the acidic amino side chains, such as aspartic and glutamic acid and released form positively charged protein acceptors. Below the isoelectric point the ζ-potential is positive as the concentration of H^+ ions increases the proton acceptors on the protein to become positively charged. The ζ-potential in the absence of surfactant at pH 7 is similar to that of whey protein emulsions at -38 mV (Demetriades and McClements, 1998) and higher than the -23 mV reported for soy protein isolate emulsions (Zhang et al., 2021b).

On the addition of 1 % Tween 20 the ζ-potential at pH 7 of the emulsion droplets became less negative at -25.3 (± 2.9) mV for banded cricket protein and -28.0 (± 2.5) for mealworm beetle protein, shown in Figure 32. The ζ-potential results in Figure 32 suggest partial displacement of protein at pH 7 since droplets stabilised with the non-ionic Tween 20 exhibits a low ζ-potential as the polar head group has minimal charge (Cheng et al., 2010, Kaltsa et al., 2014) yet the

ζ-potential of the droplets remains negative. A ζ-potential of -36 mV for caseinate emulsions was reduced on the addition of 1 % wt Tween 60 to between -21 mV and -15 mV depending on caseinate concentration, a larger reduction than for the insect proteins suggesting greater displacement (Dalgleish et al., 1995). In whey protein emulsions a Tween 20 molar ratio of 10 produced a reduction of the ζ-potential to less than -20 mV (Demetriades and McClements, 1998).

Non-ionic low molecular weight surfactants adsorb to the interface in the gaps in the heterogeneous protein layer lowering the surface free energy more than the protein, however any intramolecular interactions between the protein must be overcome and specific surface pressure reached before the surfactant can completely displace the protein (Morris and Gunning, 2008). Random-coil structured proteins such as β-casein with weaker intramolecular interactions are displaced more at lower surface pressure than globular β-lactoglobulin which creates a more elastic film (Mackie et al., 1999). The ζ-potential of the surfactant containing emulsions could be due to either the remaining undisplaced insect proteins at the interface, free protein and protein aggregates. The negative ζ-potential at pH 7 in the presence of 1 % Tween 20 implies but does not confirm protein present at the oil-water interface which is not visible in the confocal laser scanning microscopy images in Figure 28 and Figure 29.

The banded cricket emulsions adjusted to pH 5 exhibited ζ-potentials of -4.4 (± 1.4) mV at pH 5 for non-heat-treated and -3.7 (± 1.5) mV, from Figure 32, for

heat-treated is evidence of the surface proteins approaching the isoelectric of the protein.

In the mealworm beetle protein emulsion adjusted to pH 5 the ζ-potential reduced to -20.4 (± 0.64) mV for non-heat-treated and -20.6 (± 2.3) mV for heat-treated. Despite this large difference in ζ-potential between banded cricket protein emulsions and mealworm beetle emulsions at pH 5, in Chapter 6.3.3.1 Non-heat-treated (20 °C), p 156, showed little difference in microstructure shown in the micrograph in Figure 13 and $d_{4,3}$ in Figure 11. The more negative ζ-potential for mealworm beetles shows the mealworm beetle proteins may have a lower isoelectric point, but a broader range where the proteins are insoluble allowing precipitation at pH 4.4 despite the difference in isoelectric point. It is possible that the buffer region of the mealworm beetle protein is elongated meaning a greater volume of acid is required to adjust to a given pH and therefore a larger charge on the protein.

Mealworm beetle emulsion samples with 1% Tween had ζ-potentials of -12.3 (± 0.5) mV for non-heat-treated emulsions and -10.7 (± 1.0) mV for heat treated, from Figure 31, a similar reduction in ζ-potential in absolute terms as for the pH 7 emulsions.

In the banded cricket protein emulsions, the ζ-potential at pH 5 with 1 % Tween 20 added was -5.1 (± 0.7) mV in non-heat-treated emulsions and -4.4 (± 0.6) mV in heat-treated emulsions, no different to emulsions without added surfactant. The ζ-potential measurements show there is a difference in charge of the

protein at the interface when the emulsion is adjusted to pH 5 between mealworm beetle protein and banded cricket protein.

There is clear evidence of protein displacement by the addition of Tween 20 at pH 7 for both insect protein emulsion types and for mealworm beetle protein emulsions at pH 5. However, the low droplet charge on the banded cricket protein emulsion at pH 5 does not allow conclusions about displacement to be made. These results could potentially mean a difference in the protein displacement by the Tween 20, but this needs to be determined by an alternative method.

7.3.3 Interfacial Protein Concentration

The concentration of protein in the emulsion serum, separated from the cream layer by gravity, was measured by nitrogen to infer the protein concentration at the oil-water interface and elucidate the reasons for the ζ-potential measurements and the resolve contradictions with the confocal laser microscopy images. The interfacial protein concentration will determine whether in the presence of surfactant the staining microscopy, which showed no evidence of protein at the oil-water interface, or ζ-potential, which showed evidence of protein at the oil-water interface, is more accurate.

The 0.44 % protein solutions had slightly lower protein when back calculated to the protein content of the solution at 0.42 (± 0.04) % for banded crickets and 0.38 (± 0.05) % for mealworm beetle, shown in Figure 32. In the banded cricket protein emulsions (pH 7, no added surfactant), the serum protein concentration at 0.14 (± 0.01) % is lower than that of the protein solution

showing a proportion of the protein is present in the cream layer and as shown in the microscopy images at the emulsion droplet interface. The protein serum concentration supports that the negative ζ-potential in the banded cricket protein emulsion at pH 7 from Figure 31 is due to the charge of the protein adsorbed at the interface.

For the mealworm beetle protein emulsions there was 0.30 (± 0.02) % protein in the pH 7 emulsion serum implying a lower interfacial protein concentration than the banded cricket emulsion. The similar protein concentration in the serum to the protein solution (0.38%) implies that there is little protein at the oil-water interface, indicating why the stained protein rings around the droplets in the staining microscopy are thinner in the case of the mealworm beetle protein emulsions in Figure 28. Although, an alternate explanation may be a larger proportion of nitrogen containing non-protein material in the serum, which could also account for the brown colour in the mealworm beetle protein.

The addition of surfactant to the mealworm beetle emulsion did not substantially alter the high quantity of protein in the serum phase, changing from 0.31 (± 0.02) % to 0.30 (± 0.01) %. In the presence of surfactant (1% Tween 20), the protein concentration in the banded crickets serum phase increased from 0.14 (± 0.01) % to 0.26 (± 0.02) % showing a decrease in protein in the cream layer and consequently the oil-water interface, suggesting partial displacement of the proteins from the interface. The interfacial protein results for banded cricket protein emulsions appear to agree with the ζ-potential results from Figure 31 showing a partial displacement of protein from the oil-

water interface by the addition of Tween 20. The mealworm beetle protein emulsions exhibit no displacement of protein from the interface when measured by serum protein concentration, contrary to the partial displacement which would be hypothesised from the ζ-potential data.

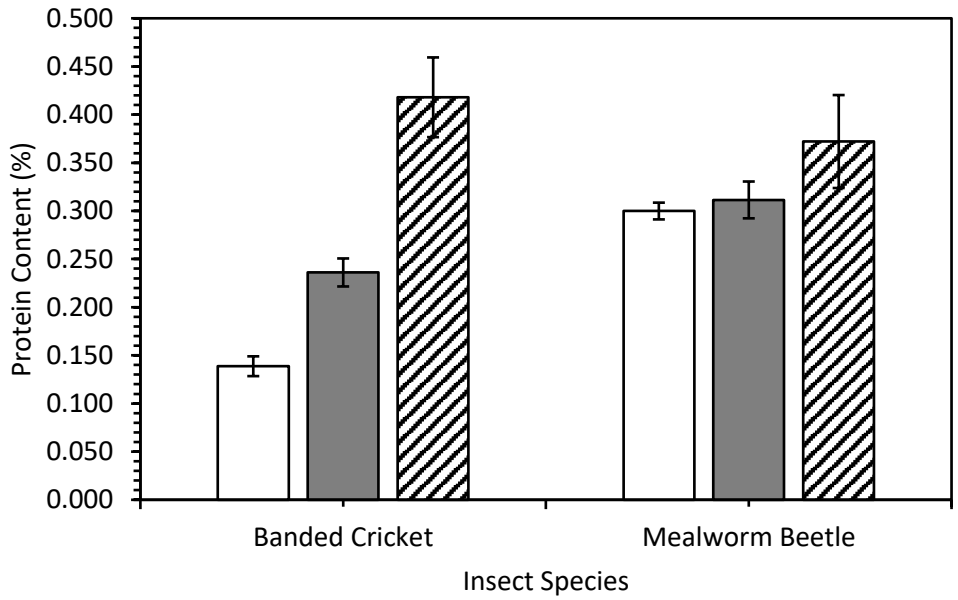

Figure 32 Protein concnetration of protein solutions and emulsions serums of emulsions at pH 7, 0.44 % solution (striped bar), no added Tween 20 (white bars), 1 % added surfactant (grey bars)

The serum protein concentration after the reduction to pH 5 decreases in both banded cricket and mealworm beetle to -0.02 (± 0.02) % and 0.00 (± 0.03) % respectively shown in Figure 33. As the serum approached the isoelectric point of the protein the neutral charge causes the serum protein to aggregate to the protein at the interface. Heat-treatment makes no measurable difference in the serum protein concentration.

When reduced to pH 5 in the presence of 1 % Tween 20 there is no evidence of increased serum protein concentration in banded cricket protein emulsions with protein content without surfactant 0.00 (± 0.03) %, and with surfactant

0.00 (± 0.01) % from Figure 33. The banded cricket serum concentration suggests that if there is displacement in the banded cricket protein emulsions the protein remains in the cream layer, either aggregating with the Tween 20 or with any remaining protein at the interface. It is, however, unlikely that the protein aggregated with Tween 20 at the interface because hydrophobic interactions predominate between surfactants and proteins (Aguirre-Ramirez et al., 2021). There was no notable difference in protein displacement with heat-treatment for banded cricket protein stabilised emulsions at -0.01 (± 0.03) % protein in the serum in the absence of Tween 20 and 0.00 (± 0.01) % with 1 % added Tween 20 from Figure 33. These banded cricket serum protein concentration result in conjunction with the slightly negative ζ-potential results from Figure 31 suggest that there is a protein laden oil-water interface in a serum phase close to the isoelectric point of the protein.

In the mealworm beetle protein emulsions at pH 5 in the presence of surfactant leads to an increase in serum concentration from 0.00 (± 0.03) to 0.23 (± 0.02) % a level similar to that in the banded cricket emulsions serum at pH 7 in the presence of surfactant inFigure 33. The increase in serum concentration is

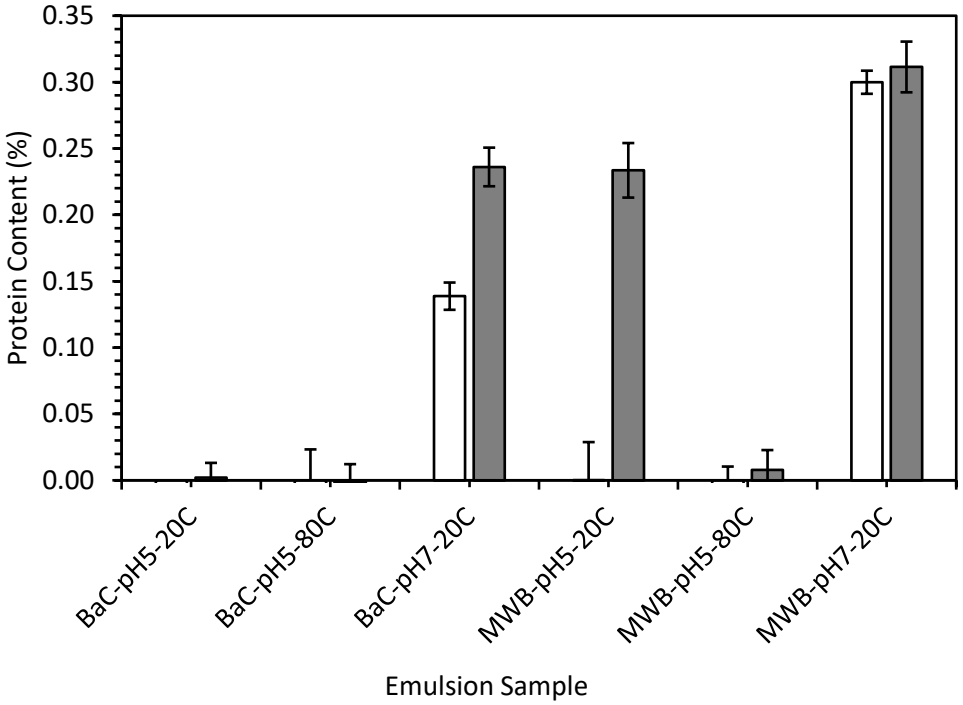

Figure 33 Serum protein concentration of insect protein oil-in-water, nitrogen conversion factor 6.25, n=3, error bars show standard deviation, no added Tween 20 (white bars), 1 % added surfactant (grey bars)

evidence of protein displacement from the interface by the Tween 20. Yet when heat-treated the in the presence of surfactant the serum protein concentration stays constant at -0.01 (± 0.02) %. The lack of change in the serum protein concentration implies a change in the protein when heated at 80 °C which prevents displacement, or agglomeration to residual interfacial protein so remain in the cream layer as expected with increased hydrophobic protein-protein interactions from denaturation.

7.3.4 Amino Acid Composition

The amino acid composition of the insect powder and protein extracts was analysed to compare to the amino acid composition might explain higher interfacial protein concentration for banded cricket protein emulsions and the lower protein displacement at pH 5 by Tween 20 relative to mealworm beetle protein emulsions. To this end the, the amino acid composition of the emulsion serums to establish if there is a change in the protein composition at the oil-water interface relative to the whole protein composition.

The protein content by nitrogen content for the whole insect powders and protein extracts is higher than the protein content by amino acid analysis in Table 6. The difference in protein contents between the two methods is partly due to tryptophan not being measured in the amino acid data, although the degree of oxidative degradation of Tryptophan during the alkaline extraction process is unknown. An additional factor affecting the difference in protein contents is the presence of chitin in the whole insect powders. The difference in protein content by the two methods reverses in the banded cricket protein extract with protein by amino acid analysis at 94.2 (± 0.1) % and by nitrogen 90.3 (± 2.4) %. However, for the mealworm beetle protein the protein by amino acid analysis is 82.3 (± 2.4) % and by nitrogen content is 90.0 (± 6.6) %. The protein content results for the mealworm beetle by nitrogen content has a high standard deviation at 6.6 %, which may account for some of the difference.

Of the eight amino acids with nitrogen containing side chains arginine, histidine, lysine and proline are measured independently, asparagine and

glutamine are oxidised to aspartate and glutamate respectively and tryptophan is oxidised during the acid digestion process. A higher proportion of nitrogen containing amino acids in the mealworm beetle protein would lead to a higher estimated protein content by the Dumas method using the same 6.25 nitrogen factor. The mealworm beetle protein contains a slightly lower proportion of arginine and lysine, slightly higher proportion of histidine and no difference in proline as compared to banded cricket protein from Table 6. The proportion of aspartate is slightly higher in mealworm beetle protein but the proportion of glutamate is higher in banded cricket protein. The effect on total nitrogen of the proportions of glutamate and aspartate would depend on the sub proportion accounted for by the oxidised glutamine and asparagine. The higher the proportion of the amine forms of these amino acids in the protein extract the higher the nitrogen content will be, in addition to any differences in tryptophan.

The experimental errors and the differences in proportions of amino acids could account for the differences in protein by nitrogen content and amino acid content. While much literature has been published on *Tenebrio molitor* larvae (Poelaert et al., 2018, Janssen et al., 2017, Jensen et al., 2019, Tran et al., 2023, Kim et al., 2020), there was no published literature found on whole adult beetle *Tenebrio molitor* amino acid composition at the time of writing. For banded cricket protein, the free amino acid composition reported by Hall et al. (2017) although reported on a /100 g of banded cricket protein extract but is of limited use in comparing to total amino acid composition.

Table 6 Protein concentration of insect proteins, whole insect powder and freeze-dried emulsion serums, errors show standard deviation, n=3 for nitrogen analysis, n=2 for amino acid analysis

	Banded Cricket Protein (%)	Banded Cricket Powder (%)	Mealworm Beetle Protein (%)	Mealworm Beetle Powder (%)	Banded Cricket, Surf. 0 %, pH 7, 20 °C (%)	Mealworm Beetle, Surf. 0 %, pH 7, 20 °C (%)	Mealworm Beetle, Surf. 0 %, pH 5, 20 °C (%)
Protein by Total AA	94.2 (± 0.1)	57.3 (± 1.4)	82.3 (± 2.4)	49.3 (± 0.5)	16.9 (± 3.6)	20.3 (± 2.3)	7.9 (± 1.4)
Protein by Nitrogen	90.3 (± 2.4)	65.1 (± 1.3)	90.0 (± 6.6)	68.8 (± 8.2)	10.5 (± 1.0)	15.9 (± 2.1)	0.69 (± 4.0)

By comparing the amino acid composition of the serum protein with the whole protein in Table 7, the composition of the protein at the oil-water interface can be inferred. The banded cricket protein shows a lower proportion of glutamic acid/glutamine but an increased proportion of the aliphatic valine, threonine, leucine and isoleucine at the interface. Whereas mealworm beetle protein at the interface showed a slight increase in proportion of glutamic acid/glutamine and lysine, but a much larger reduction in glycine and an increase in isoleucine and leucine.

Generally, proteins with higher solubility, surface charge, surface hydrophobicity, molecular flexibility and lower molecular weight have superior emulsifying properties (Sharif et al., 2018). So, in practice a balance between hydrophobicity to absorb to the oil-water interface and hydrophilicity to give solubility for diffusion of the protein to the interface is optimal(Karaca et al., 2011b, Karaca et al., 2011a).

The lower proportion of charged amino acids and higher proportion aliphatic residues would allow for increased hydrophobic interactions protein-oil but also between protein molecules increasing the elasticity of the interfacial layer.

As the net effect of charge decreases at pH 5, the hydrophobic interactions dominate, reducing displacement because the hydrophobic protein-protein interactions must be overcome to displace the protein (Morris and Gunning, 2008)., increasing interfacial protein-protein interactions and preventing displacement. Heat treating soy protein isolates (Zhang et al., 2021a) or β-lactoglobulin (Dickinson and Hong, 1994) has been shown to reduce displacement by Tween 20. Although this is different than heating the already formed emulsion these studies indicate that protein denaturation can reduce Tween 20 displacement in principle.

The lack of displacement in the mealworm beetle at pH 5 when heat-treated would concur with this hypothesis if further hydrophobic residues were exposed through denaturation as in whey protein (Kim et al., 2005a). However, increased hydrophobic residues would conflict with the more negative ζ-potential in the mealworm beetle protein emulsions with added Tween 20.

It is evident from Figure 33 that the proportion of the protein in the cream layer increased as the emulsions were adjusted form pH 7 to pH 5 in both the banded cricket emulsions and the mealworm beetle emulsions. In the case of the mealworm beetle emulsions the proportion of glutamic acid/glutamine decreased as much as the glycine while the tyrosine increased substantially, leucine increased further along with phenylalanine. There were further small decreases in the proportion of alanine, proline and lysine.

Table 7 Amino acid composition of in insect proteins g/100g of protein, whole insect powder and emulsion serums, errors show standard deviation, n=2, colours represent relative quantity of amino acid (green higher and red lower)

Amino Acid	Molecular Mass (g/mol)	Banded Cricket Protein	Banded cricket Powder	Mealworm Beetle Protein	Mealworm Beetle Powder	Banded Cricket, Surf. 0 %, pH 7, 20 °C	Mealworm Beetle, Surf. 0 %, pH 7, 20 °C	Mealworm Beetle, Surf. 0 %, pH 5, 20 °C	Soy Flour
Alanine	**89.1**	6.7 (± 0.1)	8.3 (± 0.4)	5.7 (± 0.1)	9.5 (± 0.3)	7.4 (± 0.3)	5.6 (± 0.0)	6.1 (± 0.2)	4.3 (± 0.1)
Arginine	**174.2**	7.0 (± 0.1)	7.8 (± 0.3)	5.6 (± 0.3)	4.6 (± 0.0)	6.7 (± 0.2)	5.8 (± 0.0)	5.9 (± 0.2)	7.3 (± 0.0)
Aspartate	**133.1**	9.1 (± 0.1)	7.1 (± 0.1)	9.9 (± 0.5)	6.6 (± 0.4)	9.5 (± 0.2)	10.4 (± 1.0)	10.4 (± 0.4)	10.1 (± 0.1)
Cysteine	**121.16**	0.2 (± 0.0)	0.4 (± 0.1)	0.2 (± 0.0)	0.3 (± 0.0)	0.1 (± 0.0)	0.2 (± 0.0)	0.2 (± 0.1)	0.5 (± 0.0)
Glutamate	**147.13**	18.6 (± 0.8)	19.1 (± 0.0)	17.1 (± 0.3)	15.2 (± 1.4)	21.0 (± 0.7)	16.2 (± 2.8)	21.4 (± 0.3)	25.1 (± 0.1)
Glycine	**75.07**	6.1 (± 0.3)	8.4 (± 0.4)	6.7 (± 0.1)	13.6 (± 0.4)	6.4 (± 0.1)	11.1 (± 0.6)	10.6 (± 1.1)	5.3 (± 0.1)
Histidine	**155.15**	2.6 (± 0.0)	2.9 (± 0.0)	3.0 (± 0.2)	3.7 (± 0.2)	2.4 (± 0.1)	3.2 (± 0.1)	3.1 (± 0.0)	2.8 (± 0.0)
Isoleucine	**131.17**	4.9 (± 0.2)	4.5 (± 0.3)	6.2 (± 0.4)	5.9 (± 0.2)	4.5 (± 0.6)	4.9 (± 1.0)	4.9 (± 0.2)	4.4 (± 0.0)
Leucine	**131.17**	6.8 (± 0.2)	5.7 (± 0.2)	7.7 (± 0.1)	7.1 (± 0.7)	6.2 (± 0.3)	6.3 (± 0.7)	5.6 (± 0.0)	5.9 (± 0.4)
Lysine	**146.19**	7.6 (± 0.5)	6.8 (± 0.1)	6.3 (± 0.1)	5.2 (± 0.5)	7.8 (± 0.7)	5.7 (± 0.1)	6.6 (± 0.5)	6.6 (± 0.1)
Methionine	**149.21**	1.9 (± 0.1)	1.6 (± 0.0)	1.9 (± 0.1)	1.1 (± 0.0)	1.6 (± 0.1)	1.8 (± 0.0)	1.3 (± 0.0)	1.3 (± 0.0)
Phenylalanine	**165.19**	5.0 (± 0.1)	4.0 (± 0.2)	5.9 (± 0.0)	3.3 (± 0.1)	4.8 (± 0.1)	5.5 (± 0.2)	4.4 (± 0.1)	5.4 (± 0.0)
Proline	**115.13**	4.7 (± 0.0)	6.0 (± 0.1)	4.7 (± 0.1)	6.7 (± 0.1)	4.9 (± 0.0)	4.6 (± 0.2)	5.2 (± 0.1)	5.2 (± 0.1)
Serine	**105.09**	4.0 (± 0.0)	3.8 (± 0.2)	4.1 (± 0.2)	3.6 (± 0.5)	3.8 (± 0.3)	3.7 (± 0.0)	4.0 (± 0.1)	4.3 (± 0.2)
Threonine	**119.12**	4.9 (± 0.1)	4.3 (± 0.1)	5.2 (± 0.1)	4.4 (± 0.1)	4.2 (± 0.1)	4.8 (± 0.4)	4.8 (± 0.1)	4.3 (± 0.2)
Tyrosine	**181.19**	4.1 (± 0.3)	4.5 (± 0.0)	4.3 (± 0.1)	2.8 (± 0.0)	4.0 (± 0.1)	4.3 (± 0.2)	0.7 (± 0.1)	2.8 (± 0.0)
Valine	**117.15**	5.7 (± 0.1)	4.7 (± 0.0)	5.4 (± 0.2)	6.4 (± 0.2)	4.7 (± 0.3)	5.8 (± 0.7)	4.9 (± 0.2)	4.4 (± 0.2)

7.3.5 Interfacial Tension

The relationship between interfacial tension and protein concentration was investigated to determine if the mealworm beetle protein was more interfacially active than banded cricket protein. Data for the banded cricket protein was collected in Chapter 5.3.4 Interfacial Tension, p 130 and is presented along with the mealworm beetle data in Figure 34.

The interfacial tension response to mealworm beetle protein solution concentration was fit to an exponential decay function ($p < 0.01$, $F_{(2,33)} = 307.9$, $R^2 = 0.95$) shown in Figure 34 to give insights into the relationship between the protein concentration and the interfacial tension. Both the banded cricket and the mealworm beetles follow a similar relationship between the protein concentration and interfacial tension. The mealworm beetle protein reaches an equilibrium by 0.0025 % protein concentration half that of the banded cricket

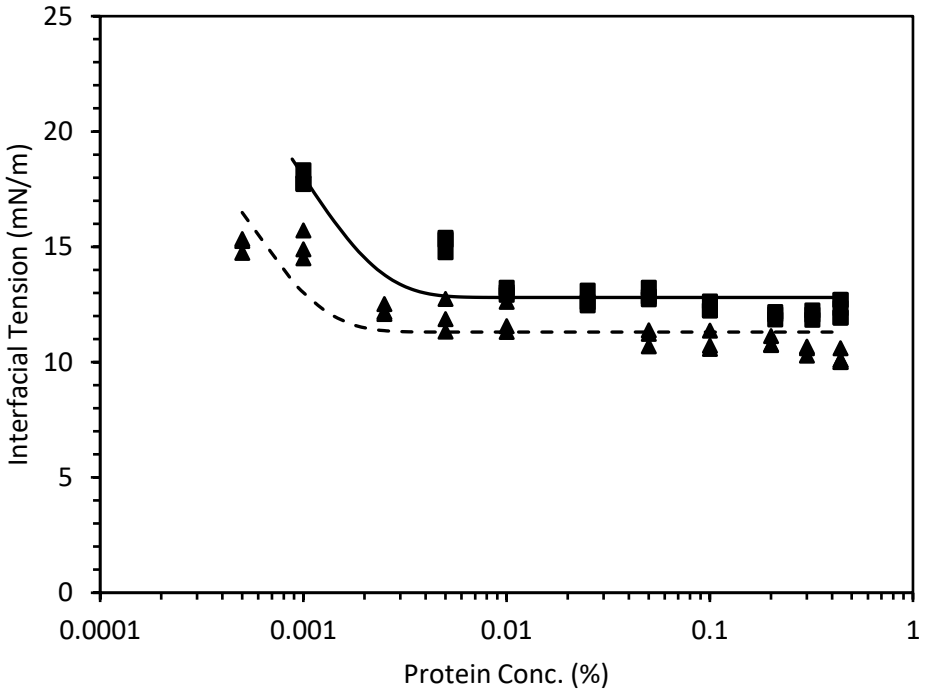

Figure 34 Interfacial tension of the treated sunflower oil-protein solution interface by protein concentration, banded cricket protein data from chapter 5 (solid line, squares), mealworm beetle protein (dashed line, triangles)

protein at 0.005 %. The difference in equilibrium concentration could be due to either differences between the proteins or in non-protein components which are surface active. With regards to proteins, the saturation concentration is partly determined by protein conformation, with more flexible proteins able to produce lower interfacial tension for a given concentration more rapidly (Freer et al., 2004a). Secondly, the distribution of hydrophilic and hydrophobic amino through protein molecule determines is the protein molecule can orient and alter conformation to allow the hydrophobic residues to reside in the oil phase and hydrophilic residues in the aqueous phase reducing the interfacial tension. Other surface active components in the protein extract would have the effect of a greater reduction in interfacial tension for a given concentration of protein. So it is possible that the mealworm beetle protein extract contains a higher proportion of surface active non-protein components. The mealworm beetle protein extract and emulsions are visibly darker in colour than the banded cricket proteins suggesting an abundance of pigment molecules. Molecules such as anthraquinones, aphins, pterins, ommochromes, tetrapyroles, melanins, papiliochromes, carotenoids, anthocyanins and flavones provide pigmentation in insects (Shamim et al., 2014). The presence some of these non-protein components could both interact with the protein altering the interfacial activity or influence the interfacial tension more directly.

The equilibrium interfacial tension of an oil-water interface measured by force tensiometry at the 0.44 % shows a similarly small discrepancy between the mealworm and banded cricket proteins, at 11.3 m/Nm and 12.8 mN/m taken from the fitted curve in Figure 34. The equilibrium interfacial tension is higher

than the 6.85 mN/m reported for mealworm larvae protein by isoelectric point precipitation, although the oil used in this study was not treated to remove surface active ingredients (Gkinali et al., 2022) and 3.4 mN/m for *Hermetia illucens* (Wang et al., 2021). *Clanis Bilineata Tingtauica Mell* was found to have an equilibrium interfacial tension of 11.22 (± 0.03) mN/m (Wang et al., 2022), and 11.1 mN/m for mealworm larvae (Gould and Wolf, 2018) which is more in line with the results in Figure 34.

Drop profile tensiometry was performed in pendant drop configuration with a protein solution internal phase drop and air as the external phase. The measurements were not measured oil-water due to equipment constraints. While oil-water interfaces differ from oil-water interfaces, air-water interfacial tension data can be used in preliminary manner to give insights into the initial adsorption of protein to the interface.

The drop profile tensiometer allows for the measurement of interfacial tension at 1 s after the air-water interface is formed, giving an insight into the rate of diffusion and adsorption of the protein to the interface. From Figure 35, at 0.001 % the protein adsorption is diffusion rate limited causing the initial plateau in interfacial tension measurements from 0 s to 400 – 600 s (Graham

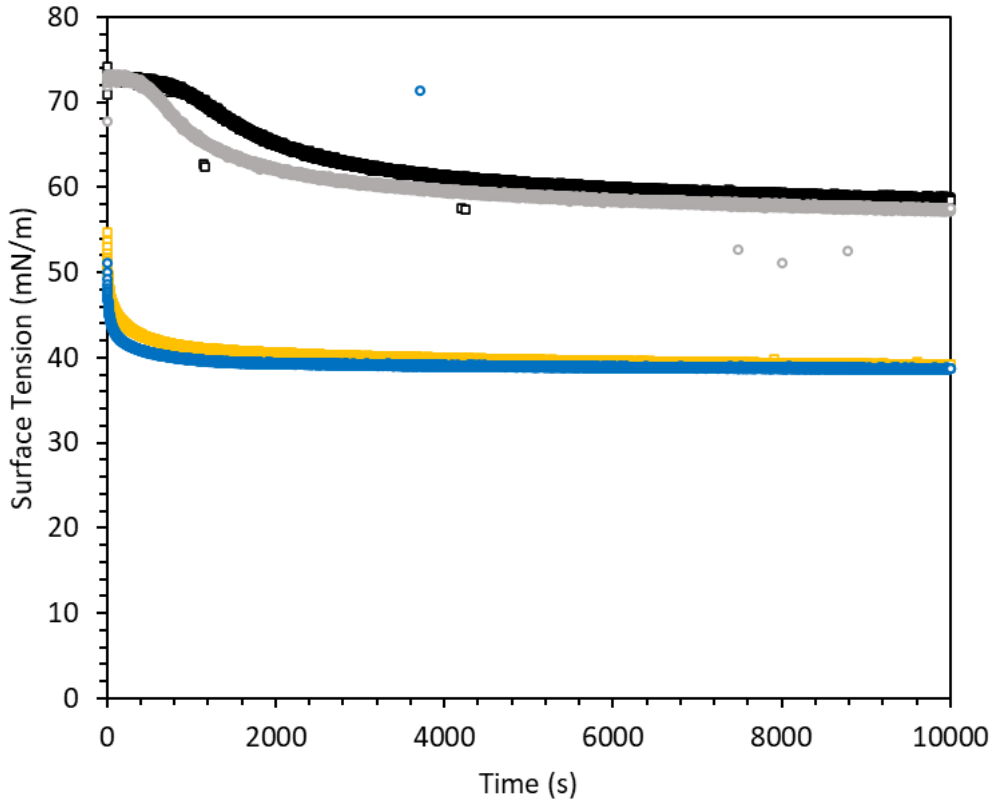

Figure 35 Surface tensions of protein solution-air interface at 0.001 % protein banded cricket protein (black), mealworm beetle protein (grey); and 0.44 % banded cricket protein (yellow), mealworm beetle protein (blue)

and Phillips, 1979, Freer et al., 2004a). The mealworm beetle protein has a shorter initial induction plateau period compared to the banded cricket protein as seen in Figure 35. The shorter induction period suggests, similarly to the interfacial tension discussions, either a differing protein composition or a variation in non-protein components rapidly diffusing and adsorbing to the air-water interface.

At 0.44 % protein the initial plateau induction period is not present, with the interfacial tension reducing rapidly from t = 1 s because the protein adsorption not the diffusion is the rate limiting step (Graham and Phillips, 1979). The initial interfacial tension at t = 1 s is slightly lower, shown in Table 8 for mealworm beetle protein at 50.2 (± 0.9) mN/m, compared to 53.4 (± 0.4) mN/m for banded cricket protein suggesting a slightly faster adsorbtionin the mealworm beetle proteins. The mean interfacial tensions at equilibrium, after 24 h, was 37.7 (± 0.7) mN/m for mealworm beetle and 37.1 (± 1.6) mN/m for banded cricket suggesting little difference in an air-water interface allowed to further rearrange. The interfacial tension data shows that the mealworm beetle protein is able to produce a similar interfacial tension with a lower protein

Table 8 Mean surface tensions of air-protein solution interface, banded cricket protein, mealworm beetle protein, at t=1s and 24 h,

Sample	Mean at t = 1 s	Mean at 24 h
0.001 % Banded Cricket	71.5 (± 0.7)	53.4 (± 0.1)
0.001 % Mealworm Beetle	72.5 (± 0.4)	49.0 (± 3.1)
0.44 % Banded Cricket	53.4 (± 0.4)	37.1 (± 1.6)
0.44 % Mealworm Beetle	50.2 (± 0.9)	37.7 (± 0.7)

concentration, due to either differing protein composition and conformations or the presence of non-protein components. These results may explain why the in mealworm beetle protein emulsions and the banded cricket emulsions at pH

7 produced emulsions with similar microstructure in Chapter 6.3.2 Insect protein emulsions at pH 7, p 150 despite the lower interfacial load.

7.4 Conclusion

Mealworm beetle protein extract has a lower interfacial load in terms of protein than banded cricket protein extract at pH 7. But mealworm beetle protein is displaced by Tween 20 more effectively when adjusted to pH 5 than banded cricket protein. The amino acid composition of the interfacial layer in the mealworm beetle protein emulsions has increased glutamine/glutamic acid, reduced glycine and aliphatic amino acids relative to banded cricket protein emulsion at pH 7. It is hypothesised that the increased aliphatic amino acids in the banded cricket protein emulsion interfacial layer leads to increased hydrophobic interactions between the protein molecules resisting orogenic displacement by Tween 20. Heat-treating the mealworm beetle emulsion appeared to reduce protein displacement in the mealworm beetle emulsions by serum protein concentration but not in terms of ζ-potential. However, both protein extracts are heterogeneous mixtures potentially containing interfacially active non-protein components. The mealworm beetle protein visually appears to contain more pigment, which could be an alternate explanation for the differences in interfacial properties. The non-protein component of the protein extracts would need to be established before firm conclusions were drawn.

In an emulsion-based food product mealworm beetle protein extract may be more suitable where displacement is required but for banded cricket protein extract the opposite is true. Further investigation is required to ascertain the composition of both the protein extracts and the composition of the oil-water interface. It can be concluded that there are difference in the behaviour of

protein extracts from different insect species in emulsion systems, but differences in pure proteins remain to be established.

8 Conclusions

A total of six insect species and two life stages of one of the species giving seven insect protein extract types produced from the same protein extraction process were investigated for emulsifying functionality in this research.

All seven of the insect protein types produced oil-in-water emulsions at 0.44 % protein pH 7, with similar $d_{4,3}$ with little difference between the emulsions produced with treated sunflower oil and the emulsions produced with untreated sunflower oil. The seven insect types spanned three taxonomic orders, with the four species of cricket within a single taxonomic order suggesting the proteins extracted from each species by the specific alkaline extraction technique used in this study are sufficiently similar to be interchangeable at pH 7 with or without surface active sunflower oil components present.

The similarity in the behaviour of proteins from the four cricket species in the Chapter 5 emulsions from whole untreated sunflower oil, with the surface-active components, suggests that the other cricket proteins may well behave similarly to banded crickets from Chapter 6 in the presence of non-ionic surfactants. Therefore, at pH 7 it is likely to be the case that all seven of the insect types would not be destablised by 1 % non-ionic surfactant. The lack of difference in amino acid profile for all four of the cricket species and the amino acid profile for banded crickets and mealworm beetle whole powder and protein are similar indicating difference between species may be minimal.

The emulsions produced with banded cricket proteins showed no significant difference in emulsion structure if reduced to pH 5 although some increase in flocculation was observed. Taking into account the similarities found in the cricket protein in Chapter 5 then the four cricket species could reasonably be used interchangeable at a lower pH range. It is likely that one of the species from the taxonomic order *Orthoptera* would produce protein most efficiently and that economies of scale would lead to one species being used predominantly. However, given the previous occurrence of viruses which limited house cricket production and the problems of supply chains during the SARS-Cov-2 pandemic, having the option of using an alternative insect from the same order to rapidly alleviate supply problems would be advantageous.

Differences between taxonomic orders were exhibited most clearly in the case of mealworm beetles and banded crickets with regards to protein displacement at pH 5. The banded crickets showed an increase in droplet diameter on adjustment to pH 5 with added Tween 20 and mealworm beetles showed no difference in droplet diameter. The surface load of the mealworm beetle protein was considerably lower than that of the banded cricket protein and the less negative ζ-potential banded cricket emulsion implies a difference in protein structure. The waxworm larvae and the mealworm larvae appeared to occupy an intermediate region between the banded crickets and mealworm beetles where in the presence of a surfactant adjusted to pH 5 the $d_{4,3}$ increased significantly in the case of the waxworm and not in the case of the mealworm larvae. If it were to be investigated a reasonable hypothesis may be that the interfacial load and the ζ-potential are between that of the banded cricket and

mealworm beetle and the degree of displacement. Given the difference in desired protein displacement required for different food products either a specific species and life stage could be selected or an appropriate blend of two or more proteins.

Fundamentally, this research has shown that proteins extracted from within a taxonomic order such as *Orthoptera* can be used interchangeably to produce oil-in-water emulsions at pH 7. Secondly, that insects from different orders and life stages: larval or adult, can stabilise oil-in-water emulsions at pH 7 but adjusted to pH 5 in the presence of a non-ionic surfactant produce differing microstructures. Thirdly, that the differing microstructures of emulsion with added surfactant at pH 5 are due to different interfacial protein loads, from differing protein properties. This research helps move insects protein extracts towards commercial use in emulsion type foods.

8.1 Further Work

This thesis aimed to investigate the effect of insect species, order and life stage on the emulsion microstructure and stability produced with the extracted protein. It was impossible to answer all of the questions and resulting hypotheses which arose from the literature and experiments covered herein due to either time constraints, resource constraints, equipment accessibility or being without the scope of the thesis. To this end a summary of the most relevant future work is included forthwith.

A further characterisation of the proteins extracted through the alkaline extraction method would be useful to confirm the infrared spectroscopy results

and expand understanding to include molecular mass. An understanding of molecular mass through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) which could then be used to guide ultrafiltration of the protein extracts into fraction. It could then be established which fractions stabilise oil-in-water emulsions. To investigate the infrared spectroscopy secondary structure elements circular dichroism would be valuable and infrared spectroscopy in solution to provide two complementary data streams giving secondary structure data.

Interfacial rheology would complement the protein structure measurements by simultaneously giving insights into the nature of the intramolecular bonds and the viscoelastic properties of the interfacial layer. By comparison to proteins with known structures such as the more disordered β-casein and the globular β-lactoglobulin the overall structure of the insect proteins at the interface could be inferred.

Alkaline extraction has a high extraction efficiency yet may denature the proteins, other protein extraction methods may extract proteins with differing emulsifying properties. Other extraction protocols such as salt extractions, reverse micellular, high-pressure, pulsed electric field and enzymic could be investigated.

Interactions between insect proteins and other ingredients in emulsion-based food products would be of value to bring products to market. Starches and other polysaccharides are used as emulsion stabilisers which increase the viscosity of the continuous phase to decrease the number of droplet-droplet

interactions and prevent creaming. Understanding which stabilisers do not disrupt the emulsion structure and in what concentration would be invaluable for designing food products.

9 Appendices

9.1 Appendix I

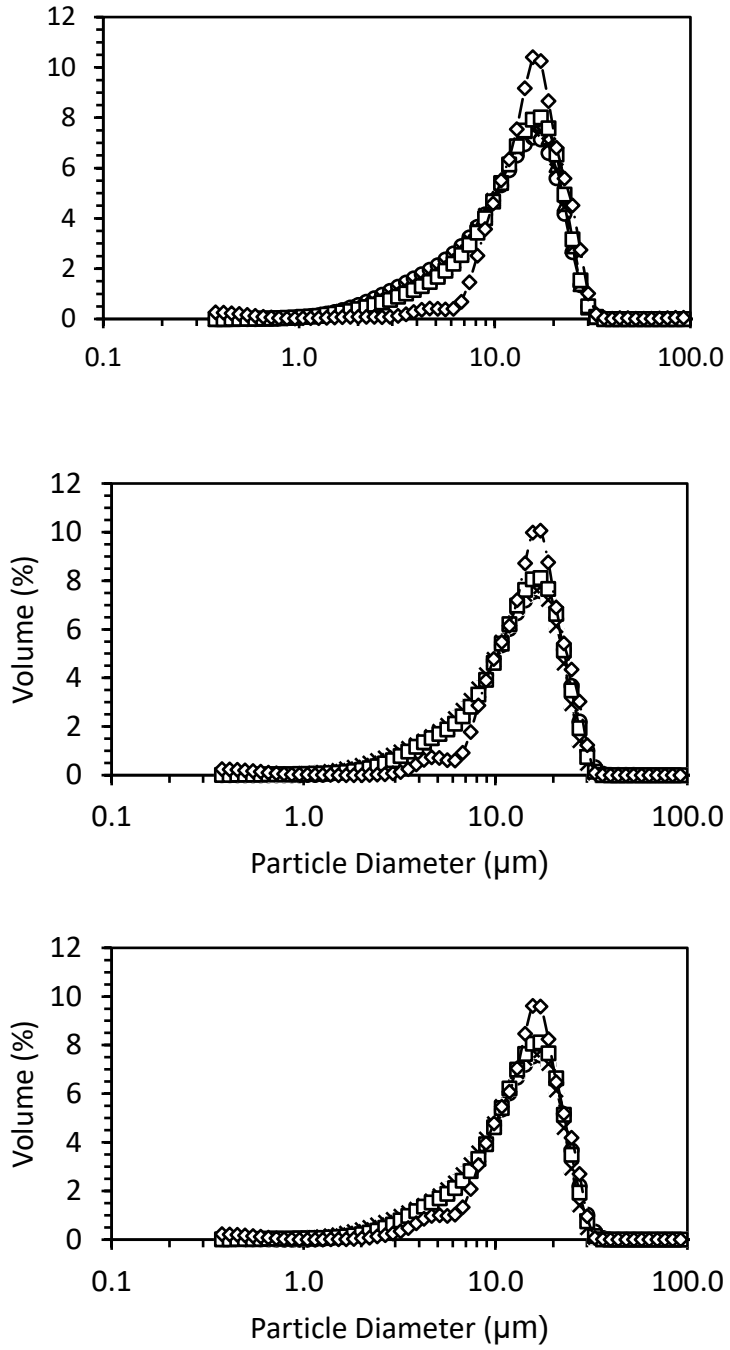

Figure 36 The emulsion droplet volume-based diameter distribution from Chapter 5, house cricket is circles with solid line, black cricket protein is triangles with dotted line, banded cricket protein is crosses with medium dashed line, silent cricket protein is squares with long dashed line, banded cricket protein with treated sunflower oil is diamonds with variably dashed line

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